BRCA1-PALB2 INTERACTION AND ITS ROLES IN MAINTENANCE OF GENOME STABILITY AND SUPPRESSION OF CANCER DEVELOPMENT

by

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ABSTRACT OF THE DISSERTATION

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DDR is often considered as a critical barrier during tumor initiation with most pre-malignant cells accumulate endogenous DNA damage before acquiring additional genetic alterations that provides a survival advantage. The work in this thesis focused on delineating the biological roles of BRCA1-PALB2 coiled-coil domain interaction, particularly on utilizing patient-derived VUS as means to identify novel BRCA1 function and possibility therapeutic targets in BRCA-associated cancers. No known structural information of the BRCA1-PALB2 interaction is available to date. The BRCA1-PALB2 coiled-coil domain is still potentially targetable due to the different post-translational modifications flanking this domain; protein modifications such as phosphorylation and ubiquitination can be explored further to modulate the BRCA1-PALB2 interaction. The BRCA1-PALB2 interaction is important for HR and suppression of error prone repair pathways. This protein-protein interaction is also required for efficient cell cycle checkpoint to ensure optimal repair timing when DNA damage occurs. We first investigated several PALB2 N-terminal coiled-coil domain mutants to not only determine the critical residues involved in PALB2 function but also to assess if varied repair ability would translate equally to chemotherapy sensitivity. Although PALB2 c.104T>C, p.L35P behaves as expectedly as a bona fide pathogenic missense variant, our results on other hypomorphic mutations such as c.83A>G , p.Y28C

raised an interesting question on the complexity of VUS. Hypomorphic mutations can significantly reduce HR activity and increase risk of tumor development. The HR ability of cells often correlate well with drug resistance as genotoxic stress are often alleviated by HR. However, reduction of PALB2 HR activity might not necessarily confer drug sensitivity as retained HR activity, albeit lower, is sufficient for resistance against chemotherapy. Unlike pathogenic mutations that often resulted from frameshift mutations, hypomorphic PALB2 missense mutation that still retained HR activity above a poorly understood threshold is a challenge for personalized treatment of cancer patients. We also examine the direct relationship between known protein kinases such as ATM/ATR, CDK and PLK1 on BRCA1 function. BRCA1, a critical DDR protein, can function in various component of DDR network of responses. Several S/TQ sites on BRCA1 were widely reported based on either in vitro biochemical reactions or mass spectrometric analysis. Interestingly, the BRCA1-PALB2 binding coiled-coil domain is positioned within this S/TQ rich region. However, our experimental results focusing on the HR and SSA suppression activity of widely reported SQ BRCA1 mutants suggest that most of these phosphorylation events may not be as critical in DDR as previously reported. S1387, T1394 and S1423 are phosphorylatable residues immediately flanking the PALB2 binding coiled-coil domain. Here, we report that abolishing phosphorylation of both S1387 and S1423 leads to partial sensitization of cells to both cisplatin and olaparib despite similar to wt HR activity. T1394 phosphorylation, although undetectable in most mass spectrometry studies to date, appears to be a critical event as single amino acid alteration on this site is sufficient to partially reduce HR activity, increase the more erroneous SSA activity and completely sensitized cells towards cisplatin and olaparib. Both artificial and patient-derived VUS affecting T1394 phosphorylation do not affect PALB2 binding. Phosphospecific antibody against pT1394 confirms that this residue is potentially a ATM/ATR target that can be induced by DNA damage. Results and observations of this study can contribute to

improvement in our knowledge of DDR by targeting BRCA1 function, while assessing the potential of ATM/ATR inhibition for cancer therapy. In conclusion, this thesis offers new insights in understanding the biological relevance of the BRCA1-PALB2 coiled-coil domain interaction, particularly, as therapeutic targets in *BRCA*-associated cancers. My observations established that BRCA1-PALB2 interaction is critical for maintenance of genome stability and suppression of cancer development. Moreover, our data offers support for better risk assessment and clinical decision making for carriers of BRCA1 and PALB2 mutations affecting the BRCA1-PALB2 interaction.

Acknowledgement and dedication

This thesis is dedicated to my late father, Foo See Kuan, who was the constant source of support and encouragement throughout my life. As a single father, he was the rock that me and my sister rely on. This thesis is also for the friends and family who have always been there for me.

Although the following thesis is an individual work, I could never have reached this stage without the support and guidance of a lot of people. I would like to first acknowledge my mentor, Dr. Bing Xia, for instilling in me the qualities of being a good scientist. His strong dedication to the research that we perform and his leadership by example are inspirational. I would also like to thank my committee members, Dr. Nancy Walworth, Dr. Shridar Ganesan and Dr. Samuel Bunting for their advices and support throughout the years. I would also acknowledge current and former members of the Xia lab for a conducive research environment. I would like to specifically thank two important lab members: Dr. Srilatha Simhadri for her infectious smiles and thought provoking discussions; Dr. Gabriele Vincelli for his patience in working with me and the late nights we sometimes spend talking about science.

Table of Contents	**Pages**
Abstract of dissertation	ii-iv
Acknowledgements	v
Table of Contents	vi-ix
List of Tables	x
List of Figures	xi-xiii
List of Appendices	xiii
List of Abbreviations	xiv-xv

List of tables

List of Figures

List of appendices

List of abbreviations

BRCA1- breast and ovarian cancer type 1 susceptibility protein

BRCA2 -breast and ovarian cancer type 2 susceptibility protein

BARD1- BRCA1-associated RING domain protein 1

PALB2- Partner and localizer of BRCA2

RING domain – “Really Interesting New Gene” domain

BRCT domain- BRCA1 C-terminal domain

VUS-Variants of uncertain significance

ATM-ataxia telangiectasia mutated serine/threonine kinase

ATR- ataxia telangiectasia and Rad3-related protein

DNAPK- DNA-dependent protein kinase

CDK- cyclin-dependent protein kinase

PLK1- Polo-like kinase 1

IR-ionizing radiation

IRIF- IR-induced foci formation

PARP-poly(ADP-ribosyl) polymerase

PARPi- PARP inhibitor

RPA-Replication Protein A

DDR-DNA Damage Response

ssDNA- Single strand DNA

DSB- Double stranded breask

SSB- single-strand breaks

NER- Nucleotide excision repair

ICLR- Interstrand crosslink repair

HR- Homologous recombination

NHEJ- Non-homologous end joining

SSA- Single strand annealing

ROS-reactive oxygen species

FA-Fanconi anemia (FA)

PDACs-pancreatic ductal adenocarcinomas

CHK1-checkpoint kinase 1

CHK2-checkpoint kinase 2

KAP1- KRAB-associated protein-1 / Tripartite motif-containing 28 (TRIM28)

S/TQ- Serine/Threonine Glutamate motif

siRNA- Small interfering RNA

KD- knockdown

IP-immunoprecipitation

IF-immunofluorescence

MRN -CtIP/MRE11-RAD50-NBS1

53BP1- TP53-Binding protein-1

MMC- Mitomycin C

HU- Hydroxyurea

CtIP -CtBP-interacting protein

ER-Estrogen receptor

PR- Progesterone receptor

HER2- Human epidermal growth factor receptor 2/neu

TNBC-Triple-negative breast cancers

LOH- Loss of heterozygosity

CRISPR- Clustered Regularly Interspaced Short Palindromic Repeats

KEAP1- Kelch-like ECH-associated protein 1

Chapter 1: Introduction

1.1 Different forms of DNA damage and modes of DNA repair

The DNA damage response(DDR) is a complex biological process that cells react in response to both endogenous and exogenous stress that threatens the integrity of genetic materials critical for cell viability. Rapidly proliferating cells will often undergo replication stress, together with production of cellular metabolites. Therefore, cells require an efficient way to sense DNA damage, signals the necessary repair machinery and further propagating the repair signals for downstream effector pathways. DDR is tightly regulated by crosstalks of various pro-survival machinery such as cell cycle checkpoints, cell death signaling pathways, inhibition of transcription or replication etc. It was estimated that an average human cell experience ~70,000 lesions daily, mostly single-strand DNA (ssDNA) breaks, when not repaired effectively can be converted into the more deleterious DNA double-stranded breaks (DSBs). Table 1.1 summarized the different types of endogenous and exogenous DNA damage that a cell might undergo daily before damaged DNA lesions are effectively repaired.

Endogenous DNA Damage	DNA Lesions Generated	Number Lesions/Cell/Day	
Depurination	AP site	10000[a]	
Cytosine deamination	Base transition	100–500[a]	
SAM-induced methylation	3meA	600[a]	
	7meG	4000[a]	
	O^6meG	10–30[b]	
Oxidation	8oxoG	400–1500[c]	
Exogenous DNA Damage	Dose Exposure (mSv)	DNA Lesions Generated	Estimated Number Lesions/Cell
Peak hr sunlight	—	Pyrimidine dimers, (6–4) photoproducts	100,000/day[d]
Cigarette smoke	—	aromatic DNA adducts	45–1029[e]
Chest X-rays	0.02[f,g,h]	DSBs	0.0008[i]
Dental X-rays	0.005[f,g,h]	DSBs	0.0002[i]
Mammography	0.4[f,g,h]	DSBs	0.016[i]
Body CT	7[f]	DSBs	0.28[i]
Head CT	2[f,g]	DSBs	0.08[i]
Coronary angioplasty	22[h]	DSBs	0.88[i]
Tumor PET scan (^{18}F)	10[h]	DSBs	0.4[i]
^{131}I treatment	70–150[h]	DSBs	2.8–6[i]
External beam therapy	1800–2000[j]	DSBs	72–80
Airline travel	0.005/hr[f]	DSBs	0.0002/hr[i]
Space mission (60 days)	50[k]	DSBs	2[i]
Chernobyl accident	300[l]	DSBs	12[i]
Hiroshima and Nagasaki atomic bombs	5–4000[k]	DSBs	0.2–160[i]

Type and number of DNA lesions are indicated. The number of lesions/cell has been estimated as described.

Table 1.1: Endogenous and exogenous forms of DNA damage that a normal cell may experience daily.

Table adapted from [64]

1.2.1 Different types of DNA damages require specific modes of repair

As dividing cells requires a robust DNA repair system, alterations in the functions of DNA repair genes will inevitably leads to genomic instability that subsequently increases the chances of cells accumulate additional mutations at genomic site for cellular transformation. Depending on the source of DNA damage, cells would need one or more types of repair pathways. For example, exposure to ionizing radiation would lead to generation of oxygen radicals that may result in depurination and single-strand breaks that can be repaired by Base Excision Repair (BER), while exposure to higher dose of radiation will inevitably leads to DSBs that requires an even more complex modes of repair[64]. Exposure to chemical agents such as crosslinking agents will result in either inter or intra-strand crosslinking that may lead to the distortion of the DNA double strand helix. Repair pathways such as nucleotide excision repair (NER) and interstrand crosslink repair (ICLR) are required for removal such a bulky adducts before DSBs is generated. As actively replicating cells would inevitably generate errors in the newly synthesized nucleotide codes, mismatch repair (MMR) is instead critical to recognize the mismatched bases and effectively remove it before a potentially mutagenic sequence is transcribed. DSBs are the most lethal form of DNA damage and are often repaired by two distinct pathways called homologous recombination (HR) and non-homologous end-joining (NHEJ). Mutations in critical components of DDR genes often resulted in impaired repair of a specific type of DNA damage, ultimately leading to developmental abnormalities and even cancer as cells acquire pro-survival mutations to compensate for the loss of DNA repair.

Repair pathway	Base excision repair	Nucleotide excision repair	Mismatch repair	Interstrand crosslink repair	Double strand repair		
					Homologous recombination (HR)	Non homologous end joining (NHEJ)	Single Strand Annealing (SSA)
Source of DNA damage	Ionizing radiation Oxygen Radicals Alkylating agents Spontaneous deamination	Ultra-violet DNA crosslinkers Platinum drugs	Replication errors DNA polymerase proofreading error	Alkylating agents	Ionizing radition Interstrand cross link Genotoxic agents (Topoisomerase inhibitor) Replication stress		
Types of DNA damage	Uracil Abasic site 8-oxoguanine Altered base Single strand break	Bulky adducts Intrastrand crosslink (6-4) photoproduct Pyrimidine dimers	Insertions Deletions Base mismatches	O6-alkyl-guanine Pyrimidine dimer	Interstrand cross linking Double stranded breaks		
Sensor proteins	DNA glycosylase APE1 PARP	XPC CSA DDB2 PARP	MSH2/6 MLH1	FA core complex	MRN complex PARP	Ku70/ku80	MRN complex
Example of critical protein	XRCC1 FEN1 TOP1 LIG1 LIG3A	XPA XPF RPA XPG ERCC1 POLE POLD1 LIG1/3	EXO1 PCNA POLD LIG1	Shares with HR and NER Structure-specific nucleases Translesion DNA polymerases BLM	ATM/ATR CtIP RPA BRCA1 PALB2 BRCA2 RAD51	DNAPK XRCC4 XLF ARTEMIS LIG4 PAXX WRN	CtIP RPA RAD52
Associated disease	Neurodegeneration Immunodeficiency	Cancer Hypogonadism Microcephaly Neurodegeneration Growth defects	Hereditary nonpolyposis colon cancer (HNPCC)	Fanconi anemia Cancer Microcepahly Neurodegeneration Growth defects	Fanconi anemia Cancer Microcepahly Neurodegeneration Growth defects	Anemia Microcephaly Immunodefciency Developmental abnormality	Not reported

Table1.2: summary of different DNA repair pathways in response to both intrinsic and extrinsic DNA damage [31, 64, 81, 120]

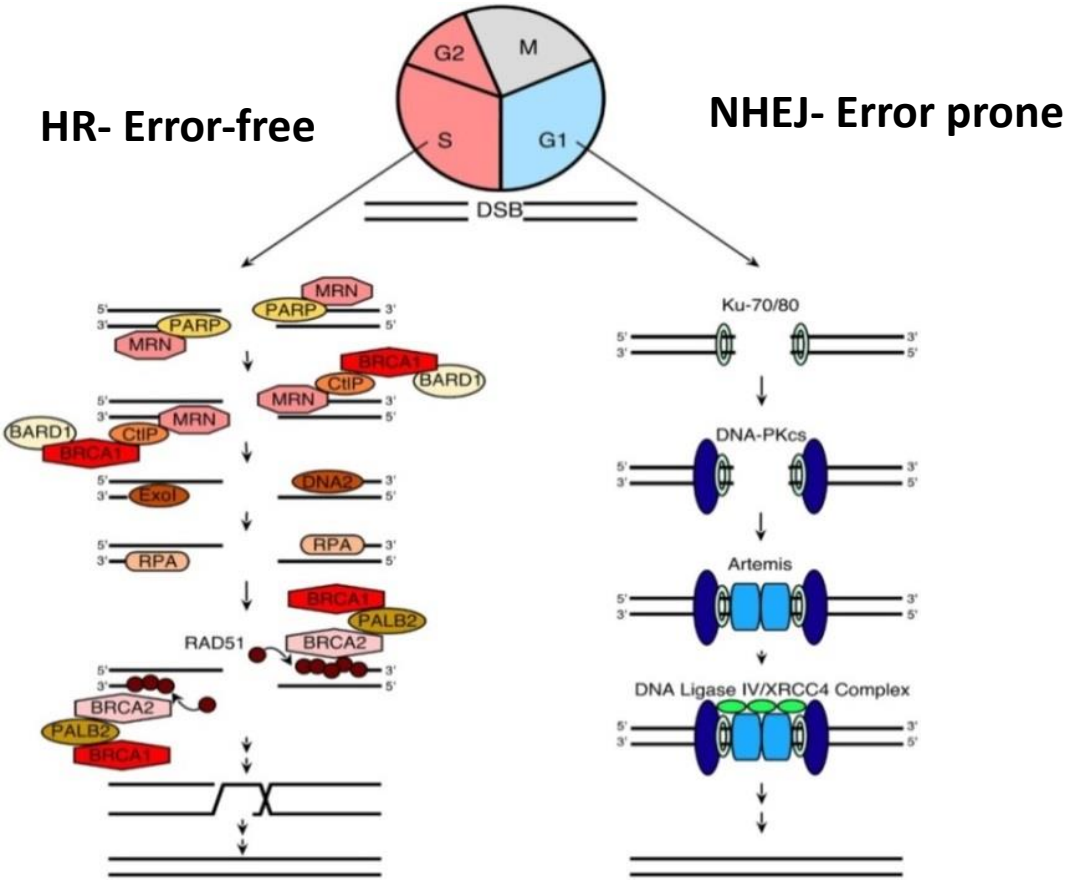

Figure 1.1 : NHEJ and HR pathway are used for repair of DSBs ; Figure adapted from [80]

DSB repair is often considered as a cell-cycle dependent process as the modes of repair is dependent upon the cell cycle state in which DNA damage is introduced. NHEJ is mostly localized at G0/G1 in which it relies on stabilization of broken DNA ends by Ku70/Ku80 proteins and DNA-PKcs before DNA end processing by exonucleases such as Artemis and subsequently DNA ligase IV mediated blunt-end ligation[80, 333]. Despite its fast kinetics in repair DSBs, NHEJ is often considered as an error-prone process that resulted in insertions or deletions at break point irrespective of sequence homology.

Alternatively, DSB can be repaired by homologous recombination (HR) with higher fidelity using a sister or homologous chromatid as template for repair, predominantly during mid-S to mid-G2 cell cycle phase when DNA replication and sister template is

present[54, 236]. Repair choices between NHEJ and HR are often proposed to be dependent on cyclin-dependent protein kinases (CDKs) control of DSB resection by resection factors such as CtIP/MRE11-RAD50-NBS1 (MRN) for short range resection (20bp in mammalian cells or 100-300bp in yeast) and EXO1-DNA2 for generation of longer ssDNA tracts [15, 25, 54, 149, 155, 333]. NHEJ is favored in G1 due to reduced CDK activity and is inhibited by end resection with the generated single-stranded DNA (ssDNA) 3'-overhang that needs to be protected by single-strand DNA binding protein Replication Protein A (RPA) [54, 141, 333, 385]. The RAD51 recombinase is then must be recruited efficiently to form RAD51 nucleofilament to displace RPA from the ssDNA followed by homology search and ultimately strand invasion into the homologous sister chromatid. The intermediate complex Holliday junction formed will later be dissolved by helicases or resolved by endonucleases[236]. If RAD51 function is impaired or lost, mutagenic repair pathways using the resected ends such as Single Strand Annealing (SSA) with depend on RAD52, a RAD51 paralog, to mediate end joining between interspersed nucleotide repeats (< 100bp) in the genome and involves deletions of intervening sequence between the repeats.

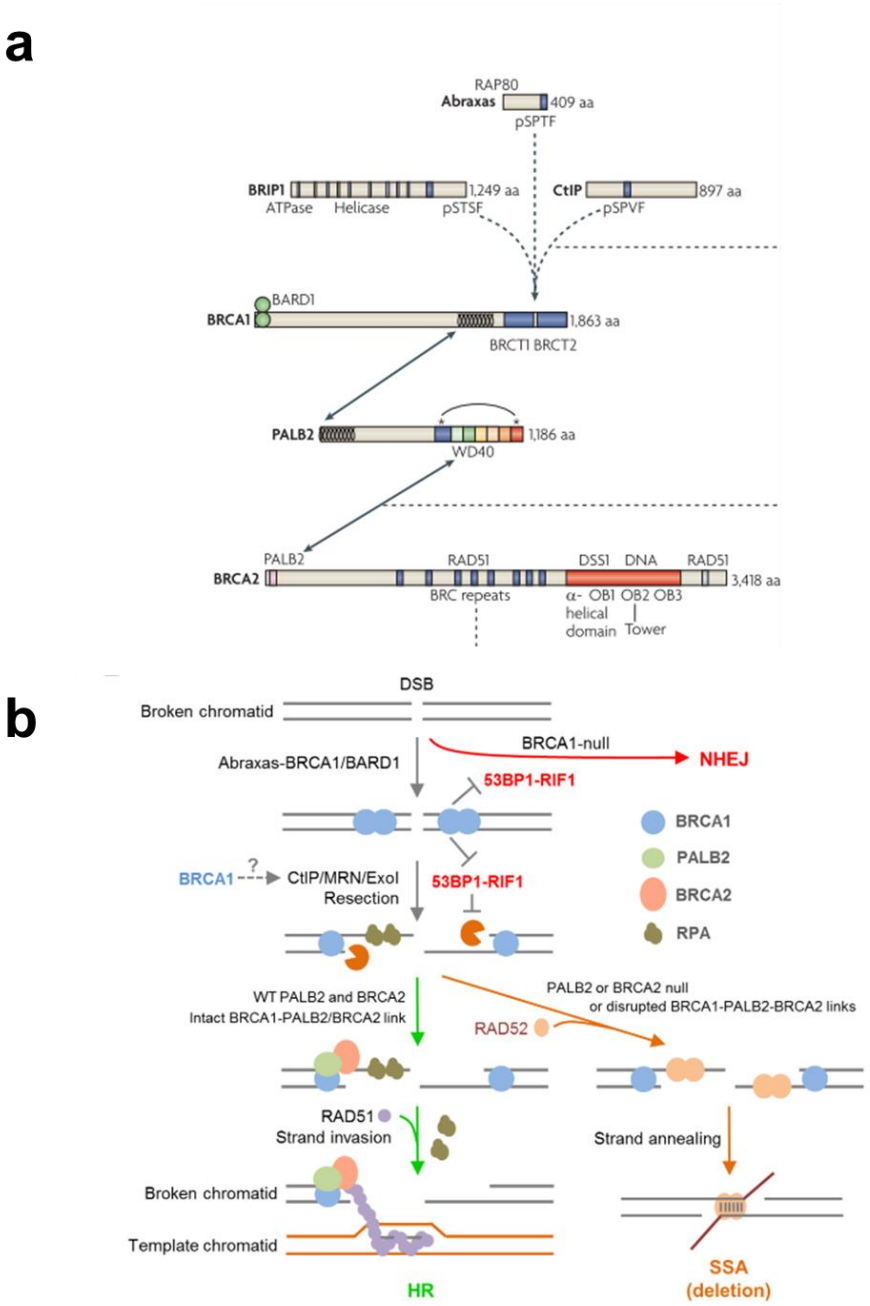

Figure 1.2: BRCA1/PALB2/BRCA2 complex formation is required for effective recruitment of RAD51 for HR

Figure adapted and modified from[11, 236]

a) Key protein-protein interaction domains in BRCA1 allows for its diverse roles in maintaining genomic stability through formation of various BRCA1 containing complexes.

b) Timely recruitment of BRCA1-PALB2-BRCA2 complex is important for RAD51-mediated strand invasion

1.2.2 Roles of BRCA1-PALB2-BRCA2 complex in tumor suppression

Figure 1.2a summarizes some of the key protein interacting domains involved in ensuring proper function of the BRCA protein complex. Stable interaction between breast and ovarian cancer type 1 susceptibility protein (BRCA1), partner and localizer of BRCA2 (PALB2) and BRCA2 are critical for RAD51-mediated homologous recombination. Depicted in the figure, BRCA1 has 3 key protein interacting domain, namely it's N-terminal RING domain, coiled-coil domain and its C-terminal tandem BRCT domain. BRCA1 binds to either BRIP1[65], Abraxas[377] or CtIP[355] in a mutually exclusive manner via recognition of phosphorylated SerXXPhe (pSerXXPhe) by its tandem BRCT domain[393]. It's N-terminal RING domain is important for binding to another RING domain containing protein called BRCA1 associated RING domain protein (BARD1), forming a heterodimer that is important for BRCA1 E3 ligase function[34, 35]. Interaction between BRCA1 and PALB2 is dependent upon a hydrophobic interaction between N-terminal coiled-coil domain of PALB2 and coiled-coil domain of BRCA1.PALB2 can also self-dimerize in vitro via its coiled-coil domain. The C-terminal WD40 domain of PALB2 is important for binding to a short N-terminal region of BRCA2 for proper recruitment of BRCA2-RAD51 complex and BRCA2 stability [247, 378]. BRCA2 can bind at least 6 RAD51 molecules at a time via its conserved BRC repeats that not only serves to recruit RAD51 for displacement of RPA-coated ssDNA but also to stabilize RAD51 nucleofilament formation[48, 60, 79, 162, 205, 341, 373]. The oligonucleotide–oligosaccharide-binding (OB) fold in BRCA2 was not only identified to function as its DNA binding domain for RAD51 loading ssDNA–dsDNA junctions but also as a PAR binding domain[236, 399]. Proteins involved in *BRCA*-mediated DNA repair such as BRCA1, BRCA2 and RAD51 can have expanded roles in maintaining genomic integrity independent of homologous recombination such moderating replication stress via protection of nascent replication fork degradation and fork restart [40, 133, 258, 262, 300, 358].

Following DSB formation, the broken chromatid will first be recognized by PARP1 which is then auto-parylated to promote recruitment of chromatin remodeler to alter the chromatin landscape adjacent to site of damage to increased accessibility of DNA ends for either KU70/80 binding for NHEJ or resection that direct the choice towards HR[333].The BRCA1/BARD1 complex was recruited to DNA damage sites by Abraxas and perhaps another yet to be defined factor(s) (Figure 1.2b). TP53-Binding protein-1 (53BP1) is a large protein that acts as a barrier towards resection and needs to be displaced by BRCA1 containing complex[39, 57]. The presence of BRCA1 at damage sites promotes resection in a mechanism that remains unclear and inhibits non-homologous end joining (NHEJ), at least in part, by counteracting the resection-inhibiting activity of the 53BP1-RIF1 complex(Figure 1.2b)[45, 58, 85, 102, 405].

Following DNA resection, BRCA1 is critical for recruitment the PALB2/BRCA2 complex, which then displaces RPA from resected ssDNA ends and loads RAD51 to initiate HR. Through the use of laser microirradiation coupled with immunofluorescence microscopy, Bekker-Jensen et al (2006) proposed that DSBs induces complex sub-compartmentalization of DDR repair factors with chromatin marked by gamma-H2AX occupied by ataxia telangiectasia-mutated (ATM) kinase, MRN complex, Mdc1, BRCA1, and 53BP1 ; BRCA1/2, Rad51, RAD52, ATR and ATRIP instead focused mostly in sub-chromatin micro-compartments with single-stranded DNA (ssDNA)[21]. The presence of BRCA1 in both chromatin compartments strongly supports its roles in priming the early stages of HR via stimulation of resection before careful positioning of the PALB2/BRCA2/RAD51 complex at RPA-coated ssDNA for recombination to occur.

Interestingly, deletion of *Trp53bp1* in mice model that were reported to rescue BRCA1 deficiency [27, 39, 160] , failed to do so in PALB2/BRCA2 deficient background[30]. Such an observation is consistent with the antagonistic relationship between 53BP1 and BRCA1 in modulating end processing at DNA breakpoints but not when the ends are already

resected and requires the recruitment of BRCA2/RAD51 for HR. When PALB2 or BRCA2 is lost or when the direct interactions in the BRCA1-PALB2-BRCA2 axis are disrupted, RAD52 binds to resected ends and mediates SSA when homologous sequences are available, leading to genomic deletions (Figure 1.2b). Patient-derived point mutations on BRCA1 RING and BRCT domain were observed to result in reduced HR and SSA due to presumably impaired BRCA1-mediated resection, as well as, impaired BRCA1 recruitment. Hence, disrupted BRCA1-PALB2 or PALB2-BRCA2 interaction instead drastically reduced HR but leads to the de-repression of SSA. It is unclear whether PALB2 or BRCA2 can directly suppress the binding of RAD52 to ssDNA or inhibiting its strand annealing activity.

1.2.3 BRCA-associated tumor spectrum

BRCA associated breast cancer and ovarian cancer. Although hereditary breast cancer only accounts for ~15% of all reported breast cancer cases [10], mutations in breast cancer suspectibility genes such as *BRCA1*, *PALB2* and *BRCA2* have been highly implicated in a majority of familial breast cancers **(Figure 1.3)**. As high as 70% of *BRCA1*-related breast cancers are higher grade, estrogen receptor-negative, progesterone receptor-negative and *HER-2*-negative, or “triple negative” , while 16-23% of BRCA2 breast tumors are triple-negative [111, 325]. Triple-negative breast cancers (TNBC) patients often show early recurrence of disease and poor prognosis. Mutations in tumor suppressor genes such as *TP53* and *PTEN* occur at higher frequencies in *BRCA1*-mutated breast cancer than in sporadic cases [95].

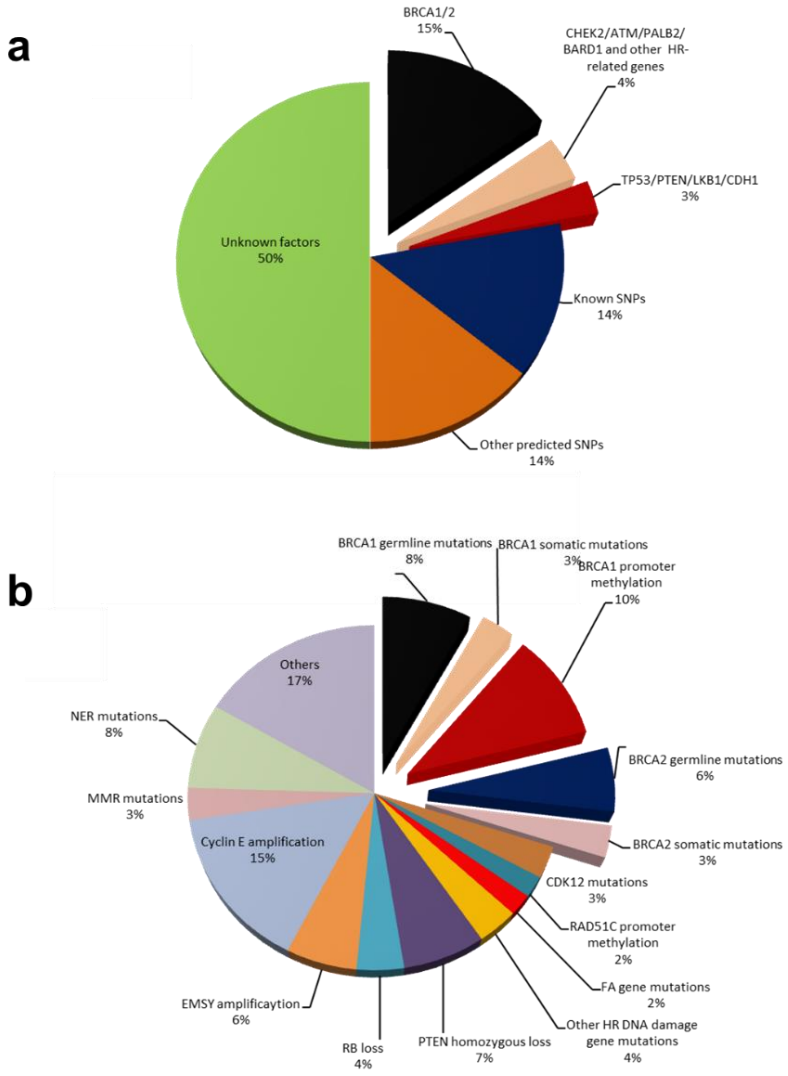

Figure 1.3: Genetic alterations that may contribute to predisposition for hereditary breast or ovarian cancer

a) Pie chart showing contribution of DDR related genes (~20%) and other possible SNPs involved in hereditary breast cancer [71]
b) Pie chart showing contribution of DDR related genes (~50% with BRCA1/2 loss of function contributes ~30% o) in high-grade serous epithelial ovarian cancer[185]

PALB2 mutation carriers have greater risk of developing breast cancer and ovarian cancer in comparison with the general population [13, 51, 74, 245]. Interestingly, breast cancer risk associated with *PALB2* mutation carriers overlaps with *BRCA2* mutation carriers [13].

High grade, triple negative tumors were first observed in tumors associated with the Finnish *PALB2* c.1592delT founder mutation [99, 136] and subsequent discovery of deleterious mutations in a large cohort of patients with TNBC unselected for family history of breast or ovarian cancer also supported the correlation between *PALB2* mutations and aggressiveness of the cancer [72]. While most *BRCA1*-associated tumors are triple-negative, only ~30% of *PALB2*-associated breast cancers are triple negative [13, 74] with most PALB2-deficient tumors exhibit a mixed ER/PR status similar to *BRCA2* tumors [301, 338, 339]. Although *PALB2* loss of heterozygosity (LOH) was observed in several cases of breast cancer patients to date [51, 115, 132], it is interesting to note that LOH remains to be inconsistent in *PALB2*-associated tumors [132, 343]. A similar inconsistency was also observed in previously reported ovarian cancer cases with deleterious *PALB2* mutations [75, 171, 345, 357] while up to 100% and ~75% LOH was instead observed for BRCA1 and *BRCA2* associated ovarian cancers[171] . It is possible that partial loss of PALB2 function is sufficient to drive tumor development instead.

Mutations in *BRCA1/2* and *PALB2* have also been associated with increased risk of male breast cancer (MBC) that is rare male cancer with a mean age at diagnosis of ~65 years [186, 266]. Pathology analysis of MBC revealed that tumors are significantly enriched for mutations affecting DNA repair–related genes [266] with majority of MBC cancers are ER+ and/or PR+ and HER2- [186, 266, 315]. Compared to *BRCA1*, *BRCA2* was found to be more frequently mutated in MBC cohorts [70, 117, 272, 315]. Moreover, young male BRCA2 mutation carriers were recently reported to be more susceptible to aggressive forms of MBCs [315]. Despite the rarity of MBCs, deleterious *PALB2* mutations have also been identified in breast cancer families with MBCs [22, 88, 272, 316].

BRCA genes and Fanconi Anemia. Fanconi anemia (FA) is an autosomal recessive pediatric disease that resulted in developmental abnormalities such as bone marrow failure, a variety of physical and organ malformations, with patients often have greater risk of developing cancers such as acute myeloid leukemia (AML) or solid tumors[135]. To date, 19 genes have been identified to encode for FA complementation group proteins implicated in the pathogenesis of FA[91, 96]. Cells derived from FA patients showed hypersensitivity to DNA inter-strand crosslinking agents and genomic instability signature. The complexity and heterogeneity of FA are suggestive of the intricate cellular networks and crosstalk involved in responding to and suppressing genomic instability. In fact, deleterious mutations in several genes involved in the FA pathway also predispose patients to greater incidence of gynecological cancers such as breast and ovarian cancers, further confirming the critical functions of FA proteins as tumor suppressors.

Although a majority of FA patients harbors mutations in *FANCA* (~60%), both *BRCA2* and *PALB2* homozygous mutation carriers accounts for ~3% and <1% of FA patients respectively [135]. There also appears to be a direct correlation between the severity of the typical FA phenotype as recent report on a family with two PALB2 mutations (one truncating mutation in one allele and an in-frame deletion Thr839_Lys862del on the other allele) proposed that hypomorphic *PALB2* allele can lead to mild form of FA with the patients did not experience FA-associated developmental disabilities and developed B cell lymphomas rather than FA solid tumors [41]. Interestingly, only 2 individuals with biallelic deleterious mutations of *BRCA1* were identified to date with the two unrelated patients carries a truncating mutation in one allele and a hypomorphic pathogenic missense variant in the BRCT domain of the second allele [89, 299]. Although neither patients exhibit any bone marrow failures commonly observed in FA patients, they do exhibit both cellular and clinical phenotypes consistent with FA-like features such as cellular sensitivity towards crosslinking agents, predisposition towards either ovarian cancer or breast cancer,

developmental defects such as short stature, developmental delay, microcephaly, supporting the inclusion of *BRCA1* as a new FA gene (*FANCS*) [299]. Solid tumors such as medulloblastoma in FA patients have so far been described only in FA patients with biallelic inactivation either *BRCA2/FANCD1* or *PALB2 (FANCN)* [84, 139, 231, 286]. Wilms tumor or nephroblastoma were also identified in FA patients with *PALB2* mutations [1, 144, 286, 304].

***BRCA1/2* and other cancer types.** Over 90% of pancreatic ductal adenocarcinomas (PDACs) contains *KRAS* mutation with pathogenic mutations in *TP53* detected in approximately 75% of PDACs[180, 356]. DNA repair and maintenance gene mutations are also associated with these highly aggressive tumors with BRCA-related mutations often resulted in tumors with unstable chromosomal structures [356]. Deleterious mutations in *PALB2* have been observed in cases of pancreatic cancer even though the reported percentage so far is lower (0.6%-2.1%) than *BRCA1/BRCA*2 mutations (*BRCA1*, 1-2.65% ; *BRCA2*, 3.6-8.6%) depending on the study cohort [109, 143, 294, 402].

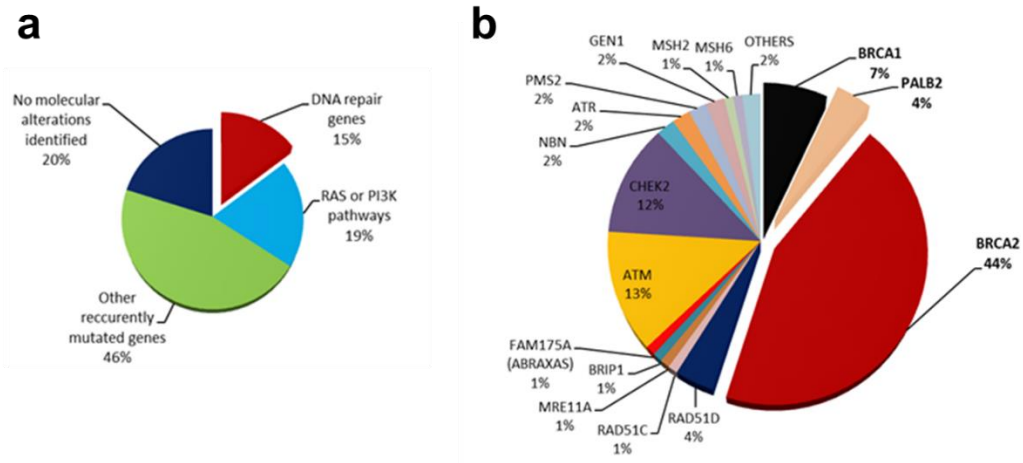

Figure 1.4: Association between DDR gene defects and prostate cancer.

a) DDR repair genes contributes to ~15% of primary prostate cancer[240]
b) Distribution of DDR gene mutations identified by Pritchard et al (2016) in ~12% cases of metastatic prostate cancer patients (692 cases) [271]

Prostate cancer is the most common cancer in men and the second leading cause of cancer related deaths in men. Approximately 15-20% cases of prostate cancer exhibit alterations in the DNA repair pathway [240, 271, 287]. Male mutation carriers of *BRCA1/2* and have an increased risk of PCa with relative risk of PCa by 65 yr is estimated at 1.8-fold to 4.5-fold for *BRCA1* carriers and at 2.5-fold to 8.6-fold for BRCA2 carriers [18, 98, 199]. Two recent studies reported that germinline *BRCA2* mutations were detected in 5.01-5.3% of unselected metastatic prostate cancers, with *BRCA1* (0.3-0.9%) and *PALB2* (0.4-0.6%) combined accounts for less than 1.5% of the cases observed [12, 271]. Glass et al, using samples from 936 localized and metastatic prostate cancers, proposed that the contribution of DDR gene defects on prostate cancers may even be higher (24.4%) than previously reported with frequently mutated genes are *BRCA2* (11.43%), *ATM* (5.77%), *MSH6* (2.46%), *MSH2* (2.14%), *ATR* (1.60%), *MLH1* (1.28%), and *BRCA1* (1.18%)[121].

BRCA1 overexpression have been proposed to be associated with poor prognosis in lung cancer [3, 246] while a rare variant in *BRCA2* have also been implicated in lung cancer [361]. In addition, BRCA1 may also play a role in hereditary and sporadic colon cancer due to *BARD1* mutations that affect BRCA1 stability, localization and BRCA1/BARD1 ligase function [400]. Moreover, recent reports have raised the possibility of altered BRCA genes such as *BRCA1/2, PALB2* and *RAD51* to confer susceptibility in colorectal cancer [6, 260, 396], stomach cancer [213] and gastric cancer [293]. Strangely, a recent case report also identified familial multiple subcutaneous lipomatosis (FML) or Dercum disease, a rare autosomal dominant disorder, in a family carrying the *PALB2* c.2716delT gene deletion (p.Trp906GlyfsTer17) with the 41 year old male proband also developed TNBC [284]. Although the genetic basis of FML is poorly understood and most families with deleterious *PALB2* mutations so far did not report signs of FML, it is still possible that *PALB2* mutations in combination with other environmental and genetic factors may contribute towards the abnormal growth of fatty tissues in the body.

Whole genome sequencing on tumor sample to determine mutational landscape and identifying mutational signatures as potential biomarker of HR defect

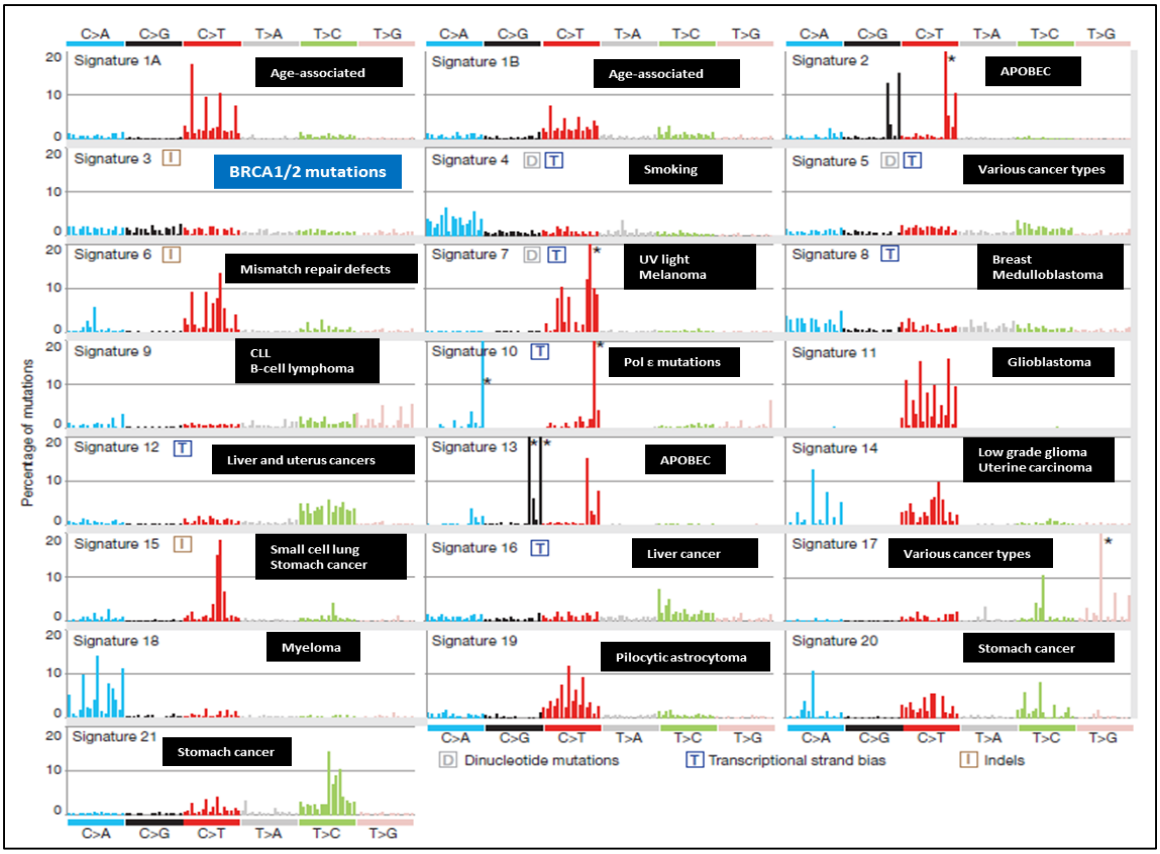

Figure 1.5: 96 substitution classification system proposed by Alexandrov et al (2013) allows for complex analysis of specific tumor mutational signatures

Since there are 6 classes of base substitutions, C>A, C>G,C>T, T>A,T>C, T>G, sequence information on bases immediately 5' and 3' to each base substitution can also be assessed. 96 substitution classification system proposed by Alexandrov et al (2013) focused on distinguishing mutational signatures that cause the same substitutions but in different sequence context. Distinct validated mutational signature can then be applied to further delineate the cancer types or genetic mutations (black boxes) associated with specific signatures such as **Signature 3 (blue box)** may only be limited to BRCA or HR deficient tumor. **Figure adapted and modified from** [7]

Growing body of evidences from next generation sequencing of cancer cells have revealed distinct mutational signatures caused by specific defective repair pathways. However, it remains to be determined why organ specificity arises for genes such as BRCA1/2 are more frequently identified in hereditary breast, pancreatic, prostate and

gynecological cancers when the genes are ubiquitously expressed in all cell types due to their critical functions. One possibility in variations of cancer risks could be originated from tissue specific vulnerabilities towards DNA damage, resulting in distinct mutational signatures [352].

Although the TNBC features of *Brca1*^{null}/*p53*^{null} and *Brca2*^{null}/*p53*^{null} mice breast tumors are similar to that of a *Palb2*-null tumor, it is interesting to note that Bowman-Colin et al (2013) also observed a distinct genomic profile within *Palb2*-null tumor that separates them from the *Brca1/2* tumors samples[30]. Such an observation is interesting from the perspective of *BRCA*-cancer genetics as *BRCA*-tumors are classically perceived to have genomic instability; advancement in whole genome sequencing technology has enabled researchers to delineate the forms of genomic alterations that maybe present in *BRCA*-tumors and if specific subtypes exist even within these HR-deficient tumors. Analysis of cancer genome mutations can be informative in revealing distinct patterns of single nucleotide substitution, cause of the patterns such as carcinogen, replicative stress or specific gene alterations during tumor development[7, 8].

Tumors harboring pathogenic mutations in *BRCA1/2, BARD1* and *PALB2* share a common mutational Signature 3, a distinct somatic mutation signature based off a 96-mutation classification that includes the six substitution types together with the bases immediately 5′ and 3′ to the mutated base , often identified in the genome of *BRCA1/2* associated breast, ovarian and pancreatic tumors [7, 110, 267, 356]. Interestingly, tumors harboring deleterious mutations in other breast cancer susceptibility genes such as *ATM* and *CHEK2* do not showed elevated signature 3, confirming that it may be specific to the functions of the BRCA complex [267, 364]. Interestingly, signature 3 was also identified in *BRCA1/2* WT type chemoresistant ovarian tumors with no discernible evidence of HR defect, suggesting that revertant tumors likely retained high levels of signature 3 inherited during tumor initiation even though HR was restored[257, 267].Signature 3 is arguably

beneficial as an independent marker to predict HR due to its sensitivity in detecting germline or somatic mutations in *BRCA1/2*, as well as, identifying not readily detectable, rare mutations of HR genes that may generates a mutational landscape similar to a HR defective tumor [7, 9, 78, 257, 267].

Prior study on the mutational signatures of breast cancers reported that *BRCA1* and *BRCA2* mutant cancers exhibited more and larger indels (>3bp) despite carrying a similar base substitution signature[244]. *BRCA*-associated tumors were also observed to exhibit tandem duplications <10kb (rearrangement signature 3), which is mostly concentrated in *BRCA1* mutant tumor but not *BRCA2*, even though most HR-deficient tumors do exhibit tandem duplications >100kb (rearrangement signature 1) or deletions <10kb (rearrangement signature 5) [78, 242, 243]. It was later observed that this ~10kb microhomology-mediated tandem duplications signature of *BRCA1*-associated breast cancer as a product of a replication restart-bypass mechanism terminated by end joining or by microhomology-mediated template switching[371]. Although, BRCA1, BRCA2 and PALB2 all participate within the same complex for homologous recombination, it is possible that PALB2 may have alternative roles independent of BRCA1/2 mediated DSB repair. Unfortunately, the mutational signature profiles for *PALB2*-mutant cancer in comparison to *BRCA1/BRCA2* mutant tumors are currently understudied due to limited amounts of *PALB2*-associated tumors sequenced. Nevertheless, a better understanding of the clonal heterogeneity, epigenomic variability and functional complexity of TNBC cancer genomes can be a powerful tool to be integrated into rational cancer therapeutic strategies[118, 388].

1.2.4 BRCA1 RING domain function and interaction with another RING domain protein BARD1

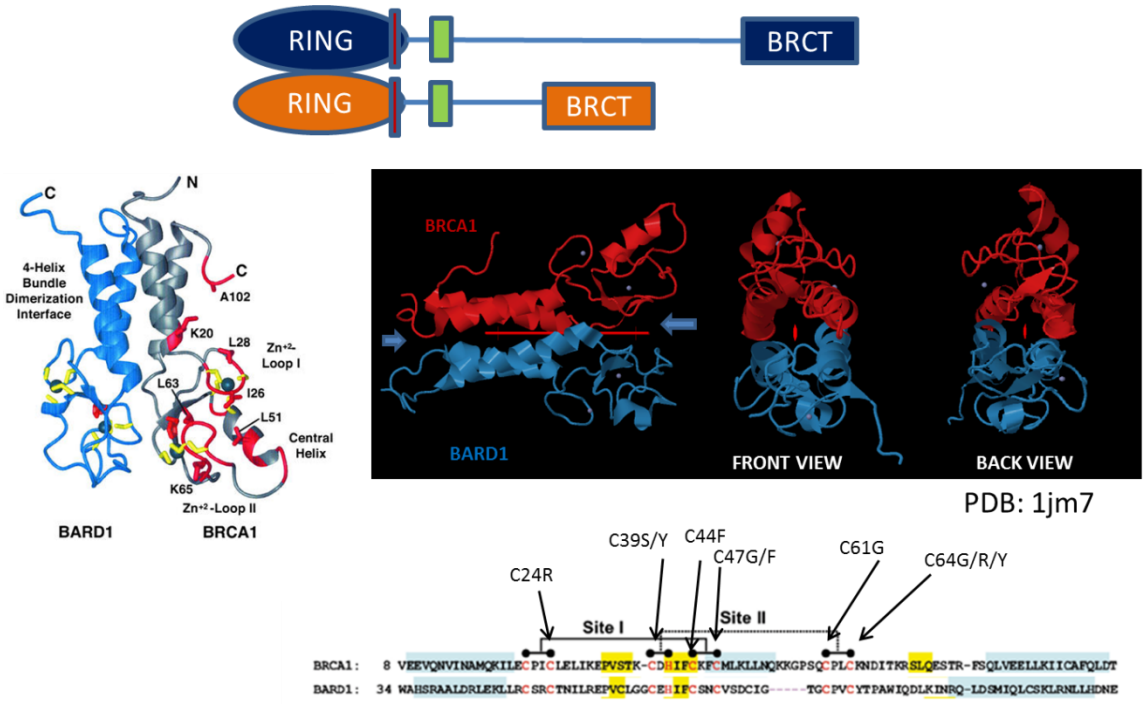

Figure 1.6: The BRCA1-BARD1 interaction is dependent upon a four helix bundle dimerization that can be affected by patient-derived mutations affecting the zinc ion coordination.

Both BRCA1 and BARD1 share 2 structurally similar domains, namely the N-terminal RING domain and the C-terminal BRCT domain. The BRCA1-BARD1 4-helix dimerization is important to mask their respective nuclear export sequence, as well as protein stability. Generally, the E3 ligase activity of this heterodimer complex is dependent on BRCA1 as BARD1 lacks a substrate coordinating central helix that is present in BRCA1.Mutations within the zinc coordinating loop or the zinc coordinating cysteine residues in either BRCA1 or BARD1 resulted in destabilization of the protein structure, leading to a misfolded protein that affects the various BRCA1/BARD1 functions that will be elaborated further.

Figure adapted and modified from[34, 35] ; PDB# 1jm7

Forming a heterodimer with BRCA1-associated RING domain protein 1 (BARD1) at its N-terminal RING domain [34, 35, 134], BRCA1 interacts with at least 8 different E2 ubiquitin-conjugating enzymes *in vitro* to either mono-or polyubiquitylate substrate proteins[35, 62, 220, 380] such as RNA polymerase II, estrogen receptor-α [97, 106], γ-Tubulin [323], histone H2A/H2AX [170] and Nucleophosmin [156]. It also was reported that the BRCT domain

of BARD1, but not BRCA1's, is able to recognize poly (ADP-ribose) (PAR) during early phase of DNA damage for rapid recruitment of BRCA1 that later transition into the more stable γH2AX-RAP80-BRCA1 complex [201]. Although deletions of the RING, Ankyrin repeat and BRCT motif of BARD1 greatly decreases HDR efficiency, BRCT point mutants of BARD1 later discovered to be unable to recognize PAR still retained HDR function similar to BARD1-WT [192]. It was also observed that BARD1 binding is necessary for BRCA1's DNA binding ability[319].Moreover, functional dependency of the two proteins is further complicated by the observation that BRCA1 nuclear retention and localization is dependent upon its interaction with BARD1 [11, 104, 288].

Through in vitro ubiquitination assays and structural information, the BRCA1-BARD1 complex was reported to mediate the ubiquitination of nucleosomal histones, particularly H2A , via a conserved positively charged nucleosome binding loop at BRCA1 and a possibly BARD1[83, 170, 220, 228, 326]. Densham et al proposed that the BRCA1–BARD1 Ub ligase activity is critical for chromatin modification (H2A-Ub) for promotion of resection, as well as repositioning of 53BP1, a known barrier of resection [83]. Interestingly, recent in vitro assay performed by Zhao et al suggest that the BRCA1-BARD1 interaction is important to prime the assembly of RAD51 synaptic complex apart from BRCA1–BARD1's role in facilitating resection of DNA ends to generate a single-stranded template for the recruitment of the BRCA2–PALB2-RAD51 complex[401]. The group determined that four or five human RAD51 molecules (but not RAD51 of other species) are bound by BRCA1–BARD1 independent of their DNA binding ability. Through the use of separation of function mutations in BARD1 that retained BRCA1 binding but lost RAD51-binding (^{133}FDA^{136} to ^{133}AAE^{136}), Zhao et al also established that loss of this presynaptic complex formation leads to deficiency in HR even though RAD51 focus formation is not affected[401]. Interestingly, Lin et al reported that 53BP1 may be a potential E3 ligase substrate of

BRCA1/BARD1 based on in vitro ubiquitination assay; this potential BRCA1-53BP1 interaction maybe critical to regulate 53BP1 function in S/G2 as PTIP, a 53BP1 downstream effector PTIP, formed more foci readily in BRCA1 deficient cells reconstituted with BRCA1-C61G and BRCA1-I26A than WT expressing cells[108].

Since most cancer predisposing missense mutations affecting BRCA1-BARD1 interaction at either the BRCA1 or BARD1 RING domain leads to misfolding or destabilization of the RING structure [11, 34, 35, 195, 326], it remains to be understood which of the aforementioned functions of BRCA1-BARD1 interaction is crucial for BRCA1's tumor suppressor functions. As most cancer predisposing mutations in the BRCA1 RING domain affects its heterodimerization with BARD1 and ultimately cause the loss of its E3 ligase function, Brzovic et al (2003) have made significant contributions in better understanding of the BRCA1/BARD1 ligase function through the generation of BRCA1-I26A synthetic mutation that do not severely affect heterodimerization with BARD1 but abolished the ability of BRCA1 to interact with the E2 ligase UbcH5c, ultimately affecting only the E3 ligase function of BRCA1/BARD1 [35]. Interestingly I26A knock-in mice embryonic stem cells are viable with HDR activity similar to wild type mice, while whole body knock in BRCA1-I26A mice are viable and born at Mendelian ratio with homozygous male mice were reported to be sterile[285, 307].Interestingly, we and another group have observed reduced sensitivity towards olaparib in cells expressing BRCA1-I26A [11, 83].

1.2.5 BRCA1 tandem BRCT domain mediated phospho-protein interaction

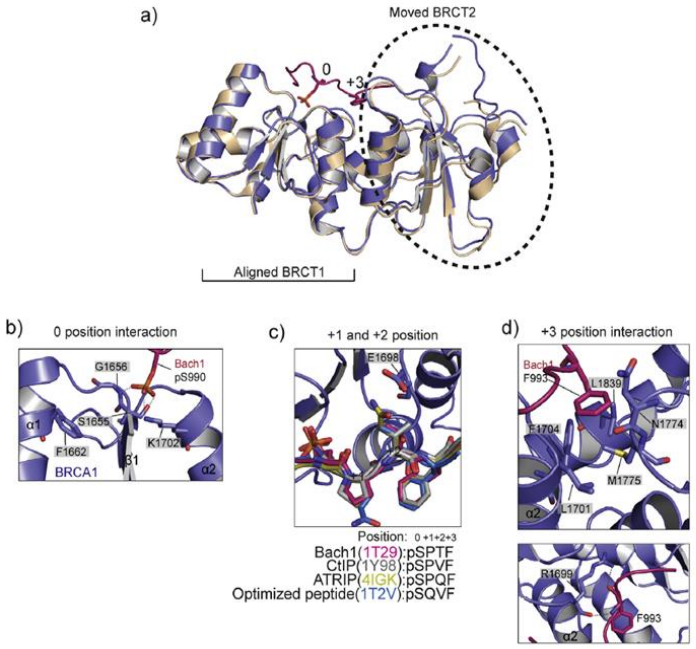

Figure 1.7: The BRCA1 C-terminal tandem BRCT domain interacts with its phosphopeptide partners via a conserved pSXXF motif

a) Alignment of BRCA1 BRCT domain alone (milky white; PDB: 1JNX) with BACH1-bound BRCT domain (purple; PDB: 1T29, BACH1 peptide in pink) suggest that the second BRCT domain (BRCT2) will undergo a slight movement to accommodate the binding of the phosphopeptide.

b) The phosphate group of he phosphorylated serine residue of BACH1 interacts with the side chains of several amino acids within the first BRCT. For example, the BRCA1 S1665F patient derived mutation will affect the binding of BRCA1 BRCT to its phosphopeptide partners.

c) Structural alignments of BRCA1 BRCT in complex with various phosphopetides reveals a conserved pSXXF motif for BRCA1 binding.

d) The bulky phenylalanine (F) at position +3 is buried within a hydrophobic pocket between BRCT1 and BRCT2

Figure adapted from [376]

The C terminal domain of BRCA1 is a tandem BRCT domain [374, 393] known to contribute significantly to BRCA1's interaction with known phosphorylated binding proteins such as ABRAXAS [177, 359, 376, 377], BACH1 [47, 65, 204], and CtIP [355, 391, 392, 394, 395]. Mutually exclusive protein-protein interaction with the aforementioned phosphoproteins via the BRCT domain lead to different BRCA1 containing complexes involved in various modes

of DNA damage response (DDR) such as DNA resection, cell cycle checkpoints, chromatin localization, interstand crosslink repair and double stranded break repair [203]. Pathogenic BRCA1 BRCT mutations often resulted in misfolded and mislocalized protein that can also be unstable[197, 288, 367, 368]. Exogenously expressed BARD1 that can only minimally rescue the HR defects of BRCT points suggest that deleterious missense mutations of BRCA1 BRCT can have multiple dire consequences that are difficult to separate from one another[11].

ABRAXAS, BACH1 and CtIP all share a conserved CDK phosphorylation motif pSPXF or pSXXF (X is any amino acid) that is recognized by the BRCA1 BRCT domain. This tandem BRCT domain of BRCA1 adopts a head to-tail configuration with a large hydrophobic interface positioned between BRCT1 and BRCT2. Binding of the phosphopeptide to the BRCT domain to this hydrophobic cleft is mediated by a “two-anchor” interaction, in which the phosphorylated Ser (position0) and the Phe (position þ3) interact with the BRCT1 and BRCT2 respectively. BRCT2 was also observed to move closer to BRCT1 when a phosphopeptide is bound. The only salt bridge across the interface occurs between Arg 1699 (R1699), immediately N-terminal to Ala 1708 (A1708) and Met 1775 M1775). Therefore, it is not too surprising that cancer predisposing mutations in residues such as R1699, A1708, M1775 and other residues positioned within this tandem BRCT interface have been identified to destabilize the BRCT structure, abolish BRCA1 binding to its interacting partners, resulting in defective HR and sensitivity to DNA damaging agents.

1.2.6 BRCA1-PALB2 interaction is mediated via their respective coiled-coil domain

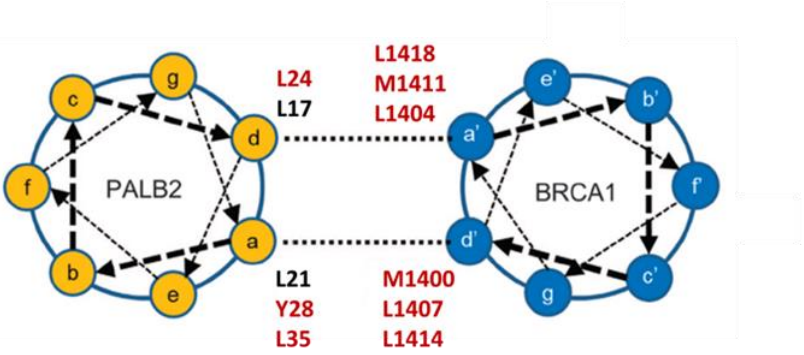

Figure 1.8: The BRCA1-PALB2 coiled-coil domain interaction is hydrophobic.

Predicted model of the interaction between the coiled-coil motifs of PALB2 and BRCA1. Hydrophobic residues at the interface are shown. Residues affected by VUSs that may potentially affect the binding of the interaction are shown in red. Potential ionic salt bridges stabilizing the two coiled-coil domain interaction is not shown.

Upstream of the tandem BRCT domain, the coil-coiled domain of BRCA1 is critical for binding of Partners and Localizer of BRCA2 (PALB2), another breast cancer susceptibility protein, to bridge the DNA damage repair signal triggered by BRCA1 to BRCA2-RAD51 for homology-directed repair (HDR) of DNA lesions [36, 378]. PALB2's interaction to BRCA1 is proposed to be dependent on a hydrophobic interaction between its N-terminal coiled-coil domain (aa 9-42) with the coiled-coil domain of BRCA1 (aa 1397–1424)[331, 398]. Structural information of PALB2-BRCA1 complex remains poorly understood due to the present lack of crystal structure or NMR spectroscopy.

Interestingly, in vitro studies have independently showed that the N-terminal coiled-coil motif of PALB2 is also required for its self-oligomerization [37, 317, 332] . The three-dimensional structure of the PALB2 homodimer, resembling a classical anti-parallel coiled-coil leucine zipper, was reported recently[322]. However, the biological functions of PALB2 self-oligomerization remains poorly understood. Biochemically, PALB2 has been reported to bind to DNA, preferably the D-loop structure, followed by ssDNA and splayed arms [36].

In fact, through its physical interaction with RAD51 at aa101–184 and to a lesser degree aa 372–561, PALB2 was reported to stimulate RAD51-mediated D-loop formation [36, 92]. Overexpression of PALB2 lacking its N-terminal coiled-coil motif for BRCA1-mediated recruitment affects loading of the PALB2-BRCA2-RAD51 complex to DNA damage sites, while overexpression of the coiled-coil domain alone is sufficient to severely affects RAD51 loading by sequestering the PALB2-BRCA1 interaction [37, 332]. Such observations suggest that self-oligomerized PALB2 can act to suppress proper BRCA1-PALB2-BRCA2 complex formation in mechanism that remains to be studied. However, whether an overexpression model is suitable to delineate the PALB2 self-oligomerization and the BRCA1-PALB2 interaction is debatable. It is also possible that there is indeed a defined threshold between the PALB2-PALB2 oligomerization complex and the PALB2-BRCA1 complex at the cellular level that can later be shifted to favor the loading of RAD51 for HR.

1.2.7 PALB2-KEAP1 interaction is mediated via a conserved ETGE -motif downstream of the BRCA1 binding coiled-coil domain

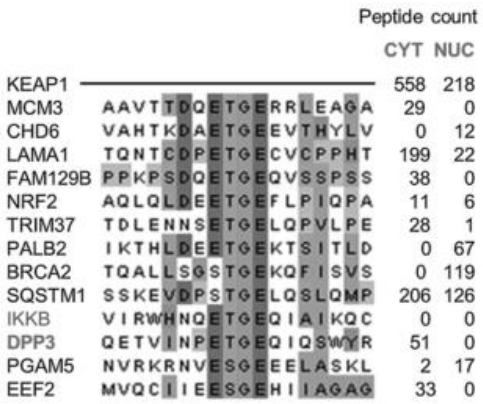

Figure 1.9: Tandem affinity purification of cytoplasmic and nuclear containing KEAP1 complexes

Alignment of amino acid sequences of the "ETGE" or "ETGE"-like motifs and their immediate surrounding regions in proteins identified in the KEAP1 complexes purified.

The PALB2 ^{91}ETGE^{94} motif is a highly conserved KEAP1 binding motif

CYT, cytosol; NUC, nucleus.

Table adapted from [214]

Aside from its primary roles in DNA damage response, BRCA1 may play a broader role in genome protection and transcriptional regulation in response to diverse regulatory signals. Earlier genome-wide expression profiling and ChIP-chip analysis have suggested

that even though specific consensus sequence for BRCA1 DNA binding does not exist, BRCA1 can still be present on defined promoters poised to mount a differential response to genotoxic stress [124]. In fact, chromatin immunopercipitation sequencing (CHIP-seq) and RNA-seq analysis in breast epithelial cells (MCF-10A) discovered strong occupancy of both BRCA1 and PALB2 at transcription start sites (TSS) of modulators of inflammation and stress[116].

Through its N-terminal ETGE-type KEAP1 binding motif (Table 1.3), our lab identified that PALB2 directly interact with KEAP1 (KELCH-like ECH-associated protein 1), an oxidative stress sensor involved in regulation of the master antioxidant transcription factor NRF2 [214, 218]. Overexpressed or free PALB2 can function as a regulator of cellular redox by sequestering NRF2 from KEAP1-mediated protein turn over and ultimately lead to the accumulation of NRF2 to lower cellular ROS levels[218]. More importantly, this ROS modulating function of PALB2 is independent of its BRCA1/2 binding as overexpression of PALB2-Y28A (BRCA1 binding hypomorph) and PALB2-A1025R (BRCA2 binding mutant) are still able to promote NRF2 activity similar to WT-PALB2. Interestingly, BRCA2 also shares an “ETGE-like” motif similar to another *bona fide* KEAP1 interacting partner p62/sequestosome 1 (SQSTM1) and can be identified to be within the same complex as nuclear KEAP1 (Table 1.3). It remains undetermined if KEAP1 can also directly bind to BRCA2 independent of PALB2 and if the greater BRCA2 peptide counts observed was simply a result of the sheer size of BRCA2 that is almost twice the size of PALB2. Nonetheless, defective DNA repair is often associated with increased ROS level in cells. A HR-independent role of PALB2 in modulating physiological ROS levels, provides insights in the various modes of tumor suppression mediated by PALB2.

1.2.8 PALB2-BRCA2 interaction is mediated by an insertion of BRCA2 N-terminal helix into the hydrophobic pocket between blades 4 and 5 of PALB2 C-terminal WD40

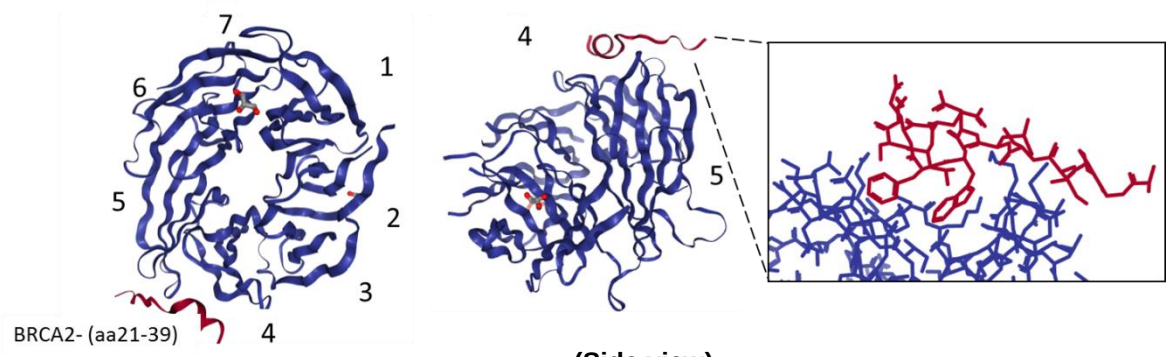

Figure 1.10: Structure of PALB2-BRCA2 interaction.

The PALB2-BRCA2 interaction is mediated by BRCA2 W31, F32 AND L35 (red color peptide) inserting into the hydrophobic pocket at the crossover between blades of 4 and 5 of the PALB2-β-propeller (PDB # 3EU7).

PALB2 interacts with the N-terminal aa 21-39 of BRCA2 via its C-terminal WD40-type 7-bladed β-propeller [247]. We and others have shown that PALB2 is essential for nuclear recruitment of the BRCA2-RAD51 assembly for effective DNA repair[378].Patients derived mutations in BRCA2 such as G25R, W31C and W31R has been reported to have impaired PALB2 binding as reflected in reduced HR repair ability [131, 378].BRCA2 is critical for assisting the loading of RAD51 recombinase at DSBs via its C-terminal DNA binding domain that binds to exposed ssDNA after resection and its BRC repeats which binds to RAD51 molecules for assembly onto ssDNA [48, 77, 101, 305, 341].

Previous reports on BRCA2 function have established that a certain degree of redundancy in DNA binding is present within the PALB2-BRCA2 complex in recruitment of RAD51 recombinase to DNA. BRCA2 fusion peptide lacking DNA binding domain requires its interaction with PALB2 to perform HR, while BRCA2 fusion peptide unable to

bind to PALB2 is instead dependent on its DNA binding for effective HR [313]. Moreover, generation of PALB2 deficient DT40 cells revealed a possible dependency of PALB2-related phenotypes on BRCA2 function as PALB2-/- cells was observed to have a milder phenotype in cellular sensitivity towards various DNA-damaging agents compared to BRCA2$^{-/-}$ cells [4]. In addition, PALB2 and BRCA2 were previously reported in a RNA interference screens to function as a regulator for Ionizing radiation-induced G2 check point maintenance [230].

The function of WD40 domain in binding BRCA2 is further confounded by a recent report on RNF168-PALB2 interaction which mapped a direct protein-protein interaction between the chromatin associated ubiquitin ligase RNF168 to the PALB2 WD40 domain[217].Such an interaction was reported to be critical for proper recruitment of PALB2 to DNA lesions. Whether there is a competition between BRCA2 and RNF168 for binding at the WD40 domain remains unclear, but it is reasonable to surmise that RNF168-PALB2 acts to further stabilize the recruitment of PALB2 to DNA lesions before initiating HR through protein interaction with BRCA2-RAD51.

Patients derived WD40 domain mutations of PALB2 such as L939W (c.2816T>G) and L1143P were reported as hypomorphic mutations that exhibit partial disruption of the PALB2-BRCA2 complex with decreased HR ability and increased sensitivity towards increasing dose of IR [254]. Yet, subsequent studies challenged the pathogenicity of mutations such as L939W that was also identified in control population and showed almost similar to wild type HR activity [53]. Another patient-derived WD40 mutation, T1030I (c.3089C>T) was observed to decrease protein stability and modestly decreased binding with RAD51C and RAD51 compared to wildtype in vitro[254]. Whether or not the T1030I mutation still retains any BRCA2 binding was not reported. Pathogenic PALB2 mutations such Y1183X (c.3549C>G), a premature stop codon introduce before the last 3 codon of protein, have been identified in multiple individuals with fanconi anemia, female breast

cancer, ovarian cancer, pancreatic cancer, and male breast cancer[13, 88, 142, 275, 276, 286]. Interestingly, the absence of PALB2 protein due to this mutation is most likely not the result of nonsense-mediated decay[286], but rather a misfolded WD40 domain that is unable to close properly and subsequently destabilizes the protein[247]. It is possible that missense substitution of PALB2 within the WD40 domain may disrupt proper folding of the domain and subsequently affect protein stability, resulting in impaired DNA repair function. Nevertheless, there have yet to be a bona fide pathogenic WD40 domain missense mutation of PALB2 reported so far. **Chapter 3 of this thesis will also expand on collaborative efforts that we undertook in studying the importance of PALB2-BRCA1 interaction by identifying PALB2 N-terminal VUSs and also identifying PALB2 c.104T>C (L35P) the first known pathogenic missense mutation of PALB2 known to date.**

1.3 BRCA1 post-translational modifications and their effects on BRCA1 functions

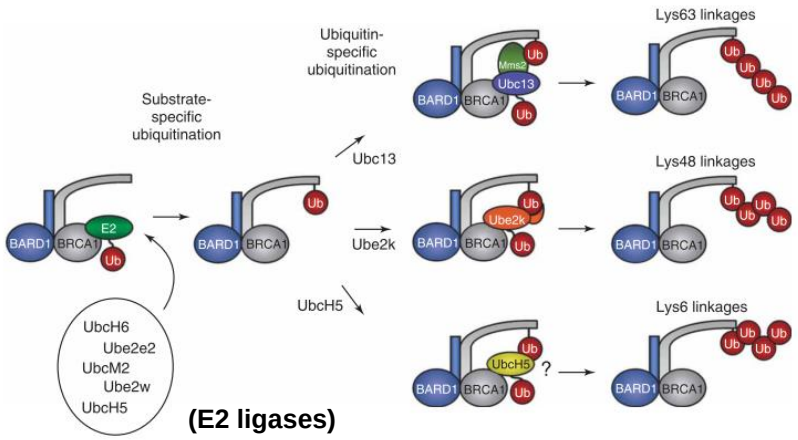

Figure 1.11: BRCA1-BARD1 autoubiquitination is dependent on its interaction with specific E2 ligases

Through the use of both yeast-two-hybrid and NMR, Christensen, et al. (2007) identified several E2 ligases that can interact with the BRCA1/BARD1 complex, leading to a variety of possibilities for BRCA1 to target different substrates for different fates (proteolytic vs non-proteolytic). BRCA1 was proposed to first be monoubiquitinated by Ube2w, followed by polyubiquitination by other E2 ligases.

Figure adapted from [62]

Ubiquitin is typically known for targeting protein for 26S proteasome mediated degradation. While ubiquitin itself has seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) that can be further conjugated, K11/48 linked polyubiquitination often resulted in proteasomal degradation[61, 154, 158].. Due to the dynamic nature of ubiquitin conjugation, polyubiquitination through other lysine residues such as K63-linked ubiquitination can have non-proteolytic functions such as DDR signaling [61, 154, 158]. The BRCA1-BARD1 hetero-dimerization has been reported to be critical for BRCA1 K6-,K48- and K63-linked poly-ubiquitination on its substrate proteins[62, 380]. Moreover, BRCA1 auto-ubiquitination was reported to increase its E3 ligase activity[35, 134, 220, 380]. Although the auto-ubiquitination of BRCA1 was widely reported, the specific lysine residue to be ubiquitination remains poorly understood.

BRCA1 sumoylation at residue lysine 119 (K119) were reported to be important for the E3 ligase activity of the BRCA1/BARD1 complex[235] . In addition, BRCA1 SUMO interaction motifs (SIM) were also reported previously[255, 381] . However, depletion of UBC9, the critical SUMO E2 conjugating enzyme, was reported to have no effects on HR nor SSA[148]. We reported that BRCA1-K119R had no effect on BRCA1 HR or SSA activity, nor did it affect cisplatin resistance, in agreement with the notion that the E3 ligase activity does not play a significant role in HR[11]. However, colony formation in the presence of olaparib was reduced by ~30%, suggesting an HR-independent role of sumoylation for PARPi resistance[11].

BRCA1 was also reported to be poly-ADP-ribosylated (PARsylated) at its DNA binding domain (^{630}NLSPPNCTELQIDSCSSSEEIKK^{652}) with this modification functions to reduce BRCA1 DNA-binding and ultimately prevents hyper-recombination and chromosomal instability[146]. It was also recently reported that acetylation of BRCA1 at K830 (further confirmed by mass spectrometry) is required for BRCA1-mediated intra-S checkpoint after DNA damage[191]. BRCA1 can be acetylated by the acetyltransferases P300/CBP-associated factor (pCAF), GCN5, and p300 with SIRT1 is the specific deacetylase of BRCA1[191]. Generation of BRCA1-K830R embryonic stem (ES) cells using a transcription activator-like effector nuclease (TALEN) system showed greater viability, impaired intra-S checkpoint and reduced checkpoint kinase 1 (CHK1) phosphorylation in K830R cells as compared with WT cells after UV exposure[191].

Phosphorylation of BRCA1 by different kinases has been implicated in a variety of its tumor suppressor functions such as cell cycle checkpoint regulation, BRCA1-mediated transcription regulation, recruitment of downstream DNA repair effector proteins etc. BRCA1 was initially observed to be phosphorylated mostly in S-phase upon DNA damage[303]. Interestingly, the PALB2-BRCA1 binding domain of BRCA1 is positioned

within “SQ cluster region” (SQR) that contains over a dozen “SQ” sites that are targets for ATM/ATR-mediated phosphorylation. Among these phosphorylatable sites, S1387, S1423, S1457 and S1524 have been subjected to considerable amount of study [20, 68, 226, 383]. Although additional ATM/ATR phosphorylation sites flanking the coiled-coil domain such as S1239, S1245,S1330,S1336, S1342, S1466 and S1542 have also been found to be phosphorylated, prior vitro kinase assay suggested that key ATR phosphorylation sites at this region are only limited to S1387, T1394, S1423 and S1457 [342]. Interestingly, the coil-coil domain of BRCA1 where PALB2 binds is positioned within the S/TQ cluster domain, which contains ~14 potential phosphorylation sites by ATM, ATR, CDK and DNA-PKs. **The functional relevance of these phosphorylation sites will be elaborated in detail in Chapter 4 of this thesis.**

1.4 PALB2 post-translational modification and their effects on cellular function

The identification of post-translational modifications and protein-protein interaction at the PALB2 N-terminal are strong evidence that an intricate regulation of BRCA1/PALB2 mediated tumor suppression might exist. A recent report provided exciting observations that the interaction of BRCA1 with the PALB2-BRCA2 complex is cell cycle dependent, in which G1-specific ubiquitylation of PALB2 by the KEAP1-CUL3-RBX1 complex at specific lysine residues (K20, K25, K30) surrounding its BRCA1 interacting domain is inhibitory for BRCA1-PALB2-BRCA2 complex formation [250]. As cells progress through S-phase, the ubiquitin marks on PALB2 can then be removed by the deubiquitylase USP11, which is itself under cell cycle control. Whether or not the ubiquitination of PALB2 also equally affects PALB2 dimerization in a cell cycle specific manner or conversely promotes its oligomerization remains to be studied. However, we and others do note a slight increase in BRCA1-PALB2 binding detected via immunoprecipitation when the PALB2 ETGE-type KEAP1 binding motif is either mutated or deleted[38, 218]. Importantly, KEAP1-mediated

ubiquitination of PALB2 do not seem to greatly affect overall protein stability or subcellular localization, consistent with what was observed in the case of the DNA helicase minichromosome maintenance 3 (MCM3), another ETGE-containing nuclear protein that is also targeted by the KEAP1-CUL3-RBX1 complex[38, 218, 237].

We and others have also observed phosphorylation of PALB2 at its N-terminal by ATM/ATR in response to ionizing radiation[2, 38, 128, 226]. Loss of BRCA1 was observed to result in reduced PALB2 phosphorylation at Ser157 and Ser376, leading to sustained activation of DDR which should be resolved as cells recover from IR-induced DNA damage[128].Whether or not confirmation changes that occurs between BRCA1-PALB2 allows for more accessible phosphorylation by kinases such as ATM/ATR remains unknown. Triple serine to alanine mutant S59A/S157A/S376A showed reduced RAD51 binding compared to phosphomimetic (S59D/S157D,S376D) mutant or WT-PALB2, without affecting BRCA2 binding[2]. However, PALB2-RAD51 and PALB2-BRCA2 binding were reported to be unaffected after chemical inhibition if ATR[38]. Nevertheless, Ahlskog et al (2016) also noted that the checkpoint activation role of PALB2 following IR was not affected in the triple alanine or triple aspartate mutants, but the checkpoint recovery function was impaired in the triple alanine mutant presumably due to impaired RAD51 foci formation leading to more time necessary to repair damaged lesions[2].

Apart from its N-terminal SQ motifs, CDK phosphorylation at residue S64 downstream of the PALB2 coiled-coil domain further complicate our understanding of the fine balance between BRCA1-PALB2 post-translational modifications and the co-ordination of BRCA-PALB2 binding for HR. Recently, it was proposed that BRCA1-PALB2 interaction can be stimulated by ATR mediated phosphorylation of residues such as S59 and hypo-phosphorylation of the CDK site S64[38]. Buisson et al raised an interesting observation that although phosphorylation of S64 by CDK do not affect ATR phosphorylation at the upstream site S59, persistent pS59 favors BRCA1 binding while

pS64 is not. PALB2-S59A/S64E were observed to have lower HR activity(~30% reduction), consistent with its weak BRCA1 binding following IR[38] .Whether or not this slight reduction in BRCA1-PALB2 binding is deleterious for cell viability remains undetermined as ~30% reduction in HR activity may still be well tolerated provided that damaged lesions can still be repaired in a timely manner. Although reduced HR activity will lead to increased time required for the BRCA1-PALB2-BRCA2 to repair damaged lesion, it is possible that the longer checkpoint arrest coupled with a specific threshold of repair capacity is sufficient to maintain cell viability.

1.5 Knockin mouse models as means to understand BRCA1, PALB2 and BRCA2 functions

Mouse models are useful in providing insightful observation for the study of the BRCA complex in tumor suppression and other biological functions in vivo. Whole body knock out mice have been instrumental in establishing the critical role of BRCA complex in embryonic development and possible genetic interactors that can rescue the embryonic lethality [67, 86, 130, 215, 277]. Tissue specific deletions of BRCA genes have also widely used to determine the biological functions of BRCA proteins in either tumor suppression or cell survival[103, 112, 216, 253]. Yet, gene deletion strategies still have their limitations; it is difficult to determine the specific functional domain of BRCA proteins that is critical for embryonic and post-natal development. Hence, knockin mice using either artificial or patients derived mutations are also powerful tools to investigate the importance of a specific protein-protein interaction within the BRCA complex.

Early works on BRCA1 RING mutants have noted radiation hypersensitivity in cells lacking a functional BRCA1 E3 ligase activity [291]. Similar to *Brca1*-null mice, *Bard1*-null mice is embryonic lethal with observed genome instability and such a phenotype is unable to be rescued even with a *Trp53* -null background [227]. Conditional knock out of either *Brca1*

or *Bard1* in murine mammary epithelial cells was observed to generate breast carcinomas that are indistinguishable from each other in terms of tumor formation frequency, latency, histopathology and cytogenetic features [306]. The BRCA1 E3-ligase defective I26A knockin mice embryonic stem cells are viable with HDR activity similar to wild type mice albiet modestly reduced gene targeting efficiencies and increased chromosomal aberrations in response to MMC treatment [285]. The *in vivo* tumor model of BRCA1 E3 ligase mutant (I26A) observed that deleterious nature of RING domain mutations in tumor development is possibly attributed to the BARD1-BRCA1 heterodimer formation and not its E3 ligase activity [307]. Interestingly, homozygous *BRCA1*$^{I26A/I26A}$ male mice exhibits impaired spermatogenesis with smaller testes and seminiferous tubules lack elongated spermatids and spermatozoa[307]. *BRCA1*$^{I26A/I26A}$ mouse embryonic fibroblasts (MEFs) revealed that the I26A mutation had no measurable effect on cellular proliferation, chromosomal stability, senescence induction, centrosome number, spindle formation, resistance to genotoxic stress in comparison to other hypomorphic *Brca1* deletion mouse models. However, this does not fully address the biological relevance of BRCA1/BARD1 ligase activity since E3 ligase activity of both BRCA1/BARD1 heterodimer are greater compared to the individual proteins alone [134]. Observations made by Drost et al (2011) on a *Brca1-C61G* conditional mice breast tumor model (*KB1C61GP*; *K14cre*;*Brca1*$^{F/C61G}$;*Trp53*$^{F/F}$) revealed that loss of BARD1 binding by the C61G might not necessarily greatly destabilize the BRCA1 protein with the tumors can still elicit partial resistance towards cisplatin and olaparib than *Brca1* null tumors[93]. RAD51 IRIFs and fewer γH2AX in these tumor cells suggest that C61G is a hypomorphic mutation that can still retain a partially intact DNA damage response in *KB1C61GP* tumor cells[93]. Yet, the group also made the confounding observation that BRCA1-deficient ES cells expressing BRCA1-C61G do not show functional HR activity and are still sensitive to cisplatin and olaparib[93]. Subsequent work by 2 independent groups using mouse models expressing RING-less BRCA1, products of novel translation

start sites downstream of a frame shift or exon deletion, showed that despite loss of BARD1 binding and genomic instability, RING-less BRCA1 can still promote therapy resistance with detectable RAD51 foci formation after DNA damage[94, 202].

Brca1$^{S1598F/S1598F}$ knockin mice provided interesting findings on the effects of BRCT point mutations on BRCA1-mediated tumor suppression[307]. Mice expressing BRCA1-S1598F (S1655F in human) mutation were highly tumor-prone with mammary and pancreatic tumor formation at rates similar to mice with conditional Brca1 inactivation. Unable to form BRCA1 and RAD51 IRIF, the *Brca1*$^{S1598F/S1598F}$ MEFs show reduced proliferation, spontaneous chromosomal aberrations, and centrosome amplification [307]. Yet, phenotypes in BRCT point mutant mice model are not fully recapitulated in its interacting partners that had their binding abolished. Mouse model of CTIP-S326A that is unable to bind to BRCA1 has confirmed that the BRCA1-CtIP interaction is not required for viability, DNA resection for HDR-mediated DSB repair or tumor suppression [268, 282].It is also interesting to note that while mammary-specific biallelic CtIP deletion in mice breast cancer model did not elicit breast tumors in a manner similar to of BRCA1 loss[283]. Unlike in the case of CtIP loss, mice deficient in Abraxas, another BRCT domain interacting protein, are viable, with heterozygous and homozygous whole body knock-out mice develop more B-cell lymphoma than WT mice. *Abraxas*$^{-/-}$ mice are also more sensitive to ionizing radiation (7.5Gy) with MEF cells derived from this mice exhibit are more genomically unstable with persistent DNA damage[52]. So far, there has not been any reported mice model harboring Serine to Alanine mutation that abolished interaction with BRCA1-BRCT in either Abraxas or BACH1/BRIP1.

Similar to previously reported systemic *BRCA*-knock out mice models, *Palb2*$^{-/-}$ mice exhibits embryonic lethality at E9.5 at the latest [277], further strengthening the importance of PALB2 as the critical mediator protein between BRCA1 and BRCA2. Interestingly, mice harboring a hypomorphic *Palb2* allele expressing mutant PALB2

protein with abolished BRCA1 binding (^{24}LKK^{26} to ^{24}AAA^{26} or *Palb2*^{CC6}) are viable despite impaired HR activity and increased genomic instability; male mice harboring this knockin allele showed reduced fertility with impaired male meiosis [317]. Indeed, both immunohistochemistry (IHC) and immunofluorescence (IF) also demonstrated that different tissues of the mutant mice have higher levels of endogenous DSBs (γH2AX foci) and slower DSB repair kinetics after ionizing radiation (IR). Yet, mutant cells were more resistant to cell death despite higher levels of higher levels of DNA breaks, stronger induction of p53/p21 and elevated levels of reactive oxygen species (ROS) following IR. The *Palb2*^{CC6} mice exhibited constitutive activation of NFκB, an anti-apoptotic transcription factor inducible by both DNA damage and oxidative, leading to the aforementioned radioresistance and tumor development[219].

Following ionizing radiation, *Palb2*^{CC6} B-cells showed greatly increased chromosomal abnormalities due to combined defects in HR and checkpoint control [318]. We also observed that these homozygous knockin mice with a p53 deficient background accelerates *Trp53*-associated thymic lymphoma and osteosarcoma (Mahdi et al, manuscript in prep), suggesting impaired PALB2 function as a driving factor behind tumor development. Such an observation is consistent with increased tumor susceptibility observed in a prior knockin mouse models harboring *Brca2*^{G25R}, a *BRCA2* patient derived mutation that resulted in weak binding to PALB2 and subsequently impairs HR function[131, 378]. *Brca2*$^{G25R/G25R}$ homozygous mice are viable with increased B-cell lymphoma formation compared to wt or heterozygous mice[131]. Interestingly, a previous case study of two B cell non-Hodgkin lymphoma patients from a single family, carrying biallelic *PALB2* mutations (one truncating with the second a hypomorphic allele with in-frame deletion of exon 6) did not observe severe developmental defects often associated with *PALB2* FA patients[41]. Yet, patient derived fibroblasts from these patients exhibit impaired RAD51 foci formation and sensitivity towards radiation and mitomycin C (MMC)[41]. Hence, genomic instability

induced in cells harboring hypomorphic alleles of BRCA proteins may not be sufficient to mount a strong apoptotic response but it may be significant enough to promote cellular transformation and subsequently tumor development[110, 131, 317].

1.6 Roles of protein kinases in modulating DDR to allow for timely repair of damaged DNA.

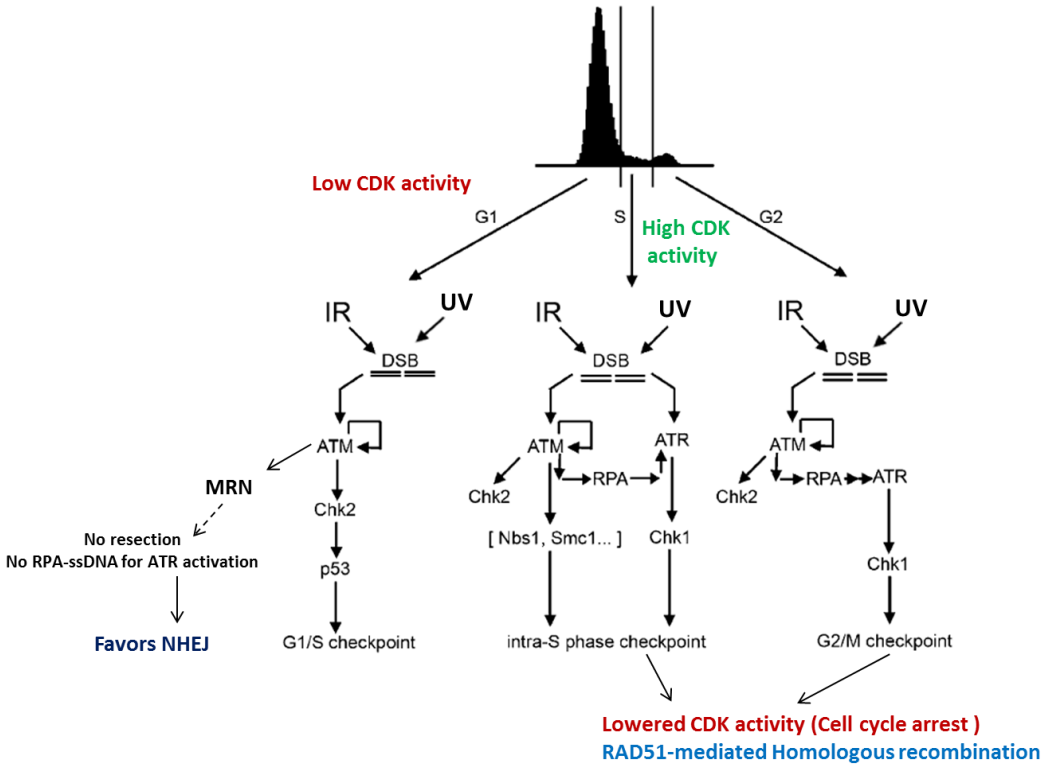

Figure 1.12: Interactions between ATM, ATR and checkpoint kinases Chk1 and Chk2 in the DNA damage response

This figure briefly illustrates the activation of ATM and ATR protein kinases to control DDR at different stages of cell cycle in response to either IR or HU. While ATR and Chk1 activity, occurs mostly in S and G2 phase, ATM activity is required through the cell cycle via phosphorylation of ATM targets. There exist two stages of CDK activity that needs to be tightly regulated by ATM/ATR to allow for sufficient DNA resection in favor of HR and a gradual decrease of CDK activity while repair is ongoing.

Figure adapted and modified from [73, 161]

Members of phosphatidylinositol-3-kinase-like kinase family (PIKKs) such as ATM, ATR and DNA-PKcs (DNA-dependent protein kinase) are key players in transmitting DDR signals for efficient repair of DNA lesions. When DNA damage occurs, the recruitment and signaling activation of these DDR regulators to damaged lesions are mediated via their interaction with Nbs1 (ATM), ATRIP (ATR) and Ku80 (DNAPK) proteins respectively[105]. ATM and ATR can further amplify the phosphorylation signals through activation of cell cycle checkpoint kinases such as CHK1, CHK2, and MK2 to ensure sufficient time is provided to repair damaged DNA [221]. ATM has been linked to the error prone NHEJ occurring throughout the cell cycle while HDR occurring mostly during S phase at DNA replication forks or after replication during G2 is more dependent on CDK and ATR. While ATM is mostly activated by double stranded breaks, ATR can be activated by spectrum of DNA damage such as DSB and replication-related stress.

Depending on the cell cycle state in which DNA damage occurs, cells can undergo various cell cycle checkpoints to cope with damaged lesions and provide additional time for repair to occur. If DSB occurs during G1, activation of ATM will lead to activation of checkpoint kinase 2 (Chk2) which in return activate the tumor suppressor p53 for activation of the G1/S checkpoint in a p21 dependent manner[138, 224, 225]. As most cancer cells carry a loss-of-function mutation or its downstream effectors, the G1/S checkpoint is disrupted with the damaged lesion carried into the subsequent stage of the cell cycle S/G2.HR occurs primarily in S and G2 phase with the presence of homologous chromatid as template for repair. During S-phase, ATM can still be activated at earlier time points after damage, leading MRN complex activation to generate RPA-coated ssDNA that is needed for ATR recruitment, auto-phosphorylation and subsequently Chk1 activation[161, 206, 207, 406, 407]. During this state, CDK1/2 activity is also necessary for activation of key DNA resection factors to generate resection DNA protected by the single-strand binding protein RPA[155, 350]. CDK1 is critical for phosphorylation of BRCA1 interacting partners such as

CtIP at residue S326 for the BRCA1-CTIP interaction via the BRCA1 C-terminal BRCT domain, as well as T847 for CtIP's nuclease activity [150, 187]. BRCT point mutations of BRCA1 are deleterious and are associated with loss of tumor suppression [307]. Nevertheless, mouse model of CTIP-S326A that is unable to bind to BRCA1 has confirmed that the BRCA1-CtIP interaction is not required for viability, DNA resection for HDR-mediated DSB repair or tumor suppression [268, 282]. Still, CtIP T87 phosphorylation is critical for efficient DNA resection after which ATR-Chk1 mediated checkpoint maintenance [187, 296]. Abolishing CDK phosphorylation at four C-terminal CDK sites S639, T732, S815 and T824) at EXO1 (another key resection factor involved in long range resection) also resulted in impaired DNA resection and ultimately HR[350].

As DNA ends are progressively resected, the activation of ATM is gradually reduced with decreased in DSB ends in favor of ssDNA signifies the transition between ATM to ATR activation[221]. Extensive RPA accumulation will subsequently activate ATR which can then activate the checkpoint kinase Chk1 for the intra-S phase checkpoint and subsequently the G2/M checkpoint if the damage signal persists even longer[206, 207, 312, 406]. Buisson et al proposed that CDK-mediated phosphorylation can have both a positive and negative effect on HR with elevated CDK activity leads to gradual activation of the ATR pathway that later inhibits CDK to limit resection and eventually promote repair using the resected ends[38]. As cells slowly progressed into the G2/M phase, cyclin B-CDK1 activity first appears at the G2 to M phase transition, peaks in mid-M phase, and declines rapidly by the end of M phase[141]. In fact, CDK-mediated phosphorylation at BRCA2-S3291 on BRCA2 was also observed to be inhibitory for HR as the phosphorylation is inhibitory for RAD51 filament formation on single-stranded DNA cells slowly approaches M-phase, together with a concomitant increase in pS3291[79, 100, 188].

1.7 Therapeutic strategies in treatment of *BRCA*-associated tumors

Platinum salts or other forms of crosslinking drugs are commonly considered as effective in the treatment of tumors with clear DNA repair defects as in the case of BRCA-associated tumors[42, 261].As a major DNA crosslinker and strong inducer of ROS, cisplatin mostly form 1,2-intrastrand crosslinking (~90%) with activated platinum complexes can react with nucleophilic centers of purine bases generating intrastrand dGpG cross-link that leads to strong distortion in the DNA double helix [76] [159]. Standard treatment of ovarian cancers is often with platinum salts in combination with taxane such as Paclitaxel that can bind preferentially to microtubule leading to aberrant mitosis and mitotic catastrophe [76]. Although *BRCA1/2* defective cells generally exhibit genomic instability and impaired HDR abilities that can be exploited by chemotherapy, it is important to note that the molecular basis behind *BRCA*-gene mediated tumor suppression may still vary greatly [314] as *BRCA1*, for example, when lost either by epigenetic silencing or gene deletions not only confer sensitivity towards DNA damaging agents but also confer resistance to microtubule toxins[274, 328]. Similar to most Fanconi anemia genes, *PALB2* deficient cells are also hypersensitive to cross linking agents such as Mitomycin C (MMC) and can be rescued through complementation of an ectopically expressed wild type protein or gene reversion [286, 379]. Apart from crosslinking agents, HR-deficient BRCA-associated tumors can also be effectively targeted by topoisomerase inhibitors such as anthracyclines/doxorubicin[125, 292, 309].

Development of targeted therapy such as poly(ADP-ribosyl) polymerase (PARP) inhibitors to synthetically kill DNA repair defective tumors have been encouraging with FDA approval of PARP inhibitors (PARPi) such as olaparib[14, 174, 176, 353], rucaparib[17, 66, 251, 330] and niraparib[234, 295] in treatment of *BRCA1/2* related advanced breast or ovarian cancers [33, 107, 210, 212] **(Figure 1.12)**. Application of PARPi are also observed in the treatment of prostate cancers showing impaired or reduced HR ability [18, 223, 271].

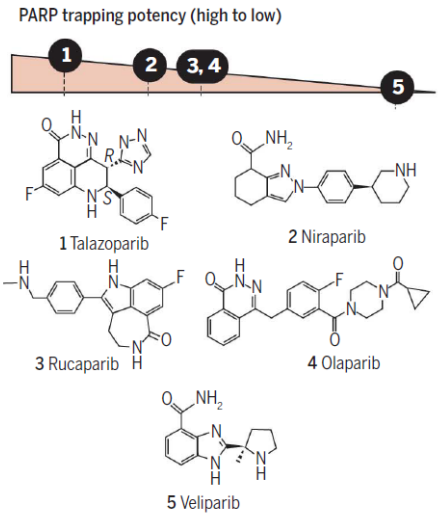

Figure 1.13: Different PARPi has varying degrees of PARP trapping potentials

Development of clinical PARPi such as talazoparib (Lead/Biomarin/Medivation/Pfizer), niraparib (Merck/Tesaro), rucaparib (Pfizer/Clovis), olaparib (KuDOS/AstraZeneca), and veliparib (Abbvie) led to the exciting discovery that the different PARPi has varying degree of PARP-trapping. Although PARPi catalytically inhibits the PARylation functions of PARP1/2, the PARP trapping potency of each individual PARPi vary. Ideally, the PARP trapping potential should not be too strong to avoid extreme cytotoxicity with moderate PARP-trapping a favored option.

Figure adapted from [212]

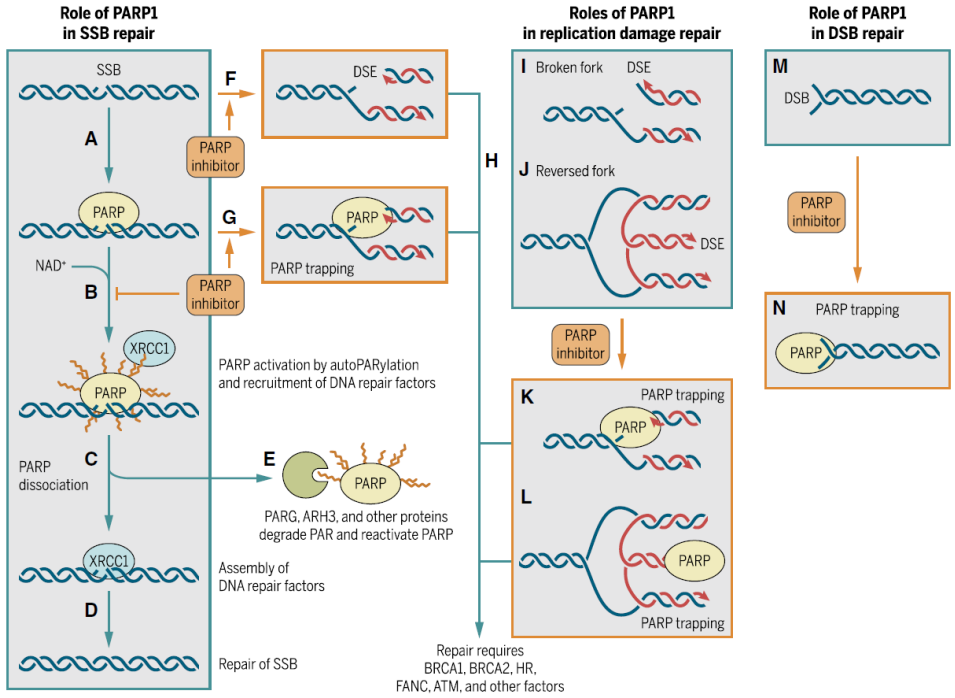

Figure 1.14: The mechanism PARPi- mediated killing of HR-deficient cells
Synthetic lethality with PARP inhibition in HR –deficient cells can be achieved by direct inhibition of PARP1 enzymatic activity and trapping of the PARP polymerase at site of DNA damage. The trapped PARP will result in a bulky adduct that needs to be cleared before collision with either the replication or transcriptional machinery, resulting in DSBs. **Figured adapted from** [269]

When DNA single-strand breaks (SSB) occurs, recruitment of PARP1 to break sites is an important first step in base excision repair (BER)[212, 269, 280] **(Figure 1.13 A-D).** Generation of PAR polymers by PARP either for PARP auto-PARylation or PARylation on neighboring chromatin proteins is a NAD+ dependent process [212, 269, 280] **(B).** PARylation is required for the recruitment of repair factors in a wide variety of repair machinery. Most PARP inhibitors (PARPi) targets the NAD+ binding catalytic domain of PARP1/2, which prevents PARylation and ultimately inhibition of SSB repair[80, 212, 269]. Persistent SSB that is not repaired will eventually be converted into DSBs **(F).** It was also observed that PARPi binding can also prevents the dissociation of PARP from damaged site **(E)** or better known as PARP-trapping **(G)[238, 327].** Since PARP is also important in replication fork repair **(I,J,M)** and HR **(M)**, PARP–trapping **(G,K,L,N)** pose a challenge for cells as PARP needs to be dissociate from DNA lesions in order to provide sufficient access to downstream repair proteins. DSB generated from PARPi needs to be repaired efficiently via either HR, Fanconi and replication bypass pathways before cells accumulates too much damaged DNA and induce apoptosis[80, 212, 269]. Interestingly, NHEJ-mediated cell death can also be achieved in HR deficient tumors as NHEJ becomes the only pathway available for repair during PARPi, leading to elevated genomic instability[80, 114].Therefore, selective killing of cancer cells deficient in HR or manipulation of other related repair pathways to induce BRCAness is a potential approach in personalized cancer treatments.

Acquired resistance towards PARPi can still occur within HR defective tumors either via enhanced drug metabolism or altered drug transporters [28, 210, 289]. Resistance can also be achieved by restoration of a functional HR activity either through genetic reversion or even secondary mutations that instead rewires the defective DDR pathway to restore function[27, 94, 184, 362, 363]. Therefore, it is not too surprising that combinational therapy are currently explored together with PARPi with heighten efforts such as liquid

biopsy or analysis of circulating cell-free DNA to identify potential biomarkers for monitoring resistance or efficacy of a treatment[123, 273, 363].

1.8 Interpretation of Variants of Uncertain Significance and its challenges

Genetic testing for breast cancer (BRCA) susceptibility genes BRCA1 and BRCA2 have been instrumental in promoting integration of genomic information in screening, prevention and treatment strategies for breast cancer. Genetic alterations of BRCA1 (intronic alterations, frameshifts, insertions, deletions, missense, nonsense, and neutral substitutions) have been widely reported in breast cancer databases. While single nucleotide substitutions or missense variants accounts for a majority of Variants of uncertain significance (VUS) identified, interpretation of VUS is further complicated by other genetic alterations such as small in frame deletions or insertions (no frame shifts), synonymous nucleotide substitutions, truncating mutations in the last exons of genes that does not result in gross destabilization of proteins, alterations in noncoding sequences or in untranslated regions. The rarity of a VUS compared to the general population leads to uncertainty when a VUS cannot be categorized as potentially disease causing or otherwise because the meaning of this type of genetic change is not yet known. Moreover, functionally characterizing mutations or polymorphisms on large proteins such as BRCA1/2 is undoubtedly painstaking even with the aid of quantitative cell based assays to establish their biological relevance [69].

Nevertheless, extensive studies on familial cases of breast cancer and cell based assays have narrowed down the diverse roles of BRCA1 to be attributed to 2 frequently mutated regions namely the N terminal RING domain, and the C terminal BRCT domain. *BRCA1* exon 11 which accounts for > 60% of the protein is also frequently mutated due to the sheer size of the exon itself. For other genes such as *BRCA2, BARD1* and *PALB2*, functional characterization of VUS remains limited but is slowly gaining attention in the

field of DNA repair[126, 127, 195, 254, 326, 401]. In fact, VUS analysis can be challenging as in silico predictions are only useful in assessing if a certain amino acid substitution is located at a conserved genomic region or if the substitution is conserved enough; designation of a potentially deleterious VUS requires careful deliberation and a series of stringent criteria to be met cautiously. The following table addresses some possible factors to be taken into consideration when a potentially pathogenic VUS is assessed:

Questions to consider when addressing a potentially pathogenic VUS	Yes	No
Does the VUS track with cancer in the family?	Possibly deleterious	Unlikely deleterious
Has the VUS been reported in the general population, ie is there a difference in population frequency between reported cases and control?	Possible	Unlikely
Has the VUS been observed together with deleterious mutations in other known genes?	Very unlikely	Possible
Is the amino acid substitution at a conserved residue across species? Is the substitution a conserved one (structurally similar amino acid)?	Possible Unlikely	Unlikely Possible
Is loss of heterozygosity observed in the tumor specimen?	Possible	Unlikely
Have there been functional assays conducted to confirm the deleterious effects of this specific missense variant on protein function?	Probable	Unlikely

Table 1.3: Criteria that can be applied in determining potential pathogenicity of a VUS. Table adapted from [90] **.**

Chapter 2: Materials and Methods

2.1 Plasmids and siRNAs

Exogenously expressed PALB2 or BRCA1 proteins in Chapter 3 and 4 were expressed using either the pOZ-FH-C1-PALB2 retroviral vector or pcDNA-3xMyc-BRCA1 with all mutations were generated through site-directed mutagenesis as previously described[11, 317]. Site-directed mutagenesis was performed according to the QuikChange protocol (Agilent Technologies). DNA transfections were carried out using X-tremeGENE 9 or X-tremeGENE HP (Roche).

2.1.1 Plasmids used in Chapter 3 and chapter 3 appendix	**Notes**
pOZ-FH-C1-PALB2-WT	Wild type cDNA sequence
pOZ-FH-C1-PALB2-K18R	c.53A>G
pOZ-FH-C1-PALB2-K20R	Artificial mutation ; Appendix (3)
pOZ-FH-C1-PALB2-L24F	c.72G>C; Appendix (2)
pOZ-FH-C1-PALB2-L24S	c.71T>C; Appendix (2)
pOZ-FH-C1-PALB2-K25R	Artificial mutation ; Appendix (3)
pOZ-FH-C1-PALB2-Y28C	c.83A>G
pOZ-FH-C1-PALB2-Y28N	c.82T>A; Appendix (2)
pOZ-FH-C1-PALB2-K30R	Artificial mutation ; Appendix (3)
pOZ-FH-C1-PALB2-K30N	c.90G>T
pOZ-FH-C1-PALB2-L35P	c.104 T>C
pOZ-FH-C1-PALB2-R37H	c.110G>A
pOZ-FH-C1-PALB2- ΔTG	Artificial mutation ; Appendix (3)
pOZ-FH-C1-PALB2-ΔETGE	Artificial mutation ; Appendix (3)
pOZ-FH-C1-PALB2-E91A/E94A	Artificial mutation ; Appendix (3)
pOZ-FH-C1-I944N	c.2831T>A; Appendix (2)
pOZ-FH-C1-T1030I	c.3089C>T ; Appendix (2)
pOZ-FH-C1-L1070P	c.3209T>C ; Appendix (2)
2.1.2 Plasmids used in Chapter 4	
pcDNA-3xMyc-BRCA1-WT	Wild type cDNA sequence
BRCA1- S1164I	c3491G>T

BRCA1- S1189A/S1191A	Artificial mutation
BRCA1-S1497A	Artificial mutation
BRCA1- S1189A/S1191A/S1497A	Artificial mutation
BRCA1-S1387A	Artificial mutation
BRCA1-S1387E	Artificial mutation
BRCA1-S1423A	Artificial mutation
BRCA1-S1524A	Artificial mutation
BRCA1- S1387A /S1423A	Artificial mutation
BRCA1- S1387E /S1423E	Artificial mutation
BRCA1- S1387A /S1423A/S1457A	Artificial mutation
BRCA1- S1387A /S1423A/S1524A	Artificial mutation
BRCA1- S1423A/S1457A/S1524A	Artificial mutation
BRCA1- S1387A /S1423A/S1457A/S1524A	Artificial mutation
BRCA1-T1394A	Artificial mutation
BRCA1-T1394E	Artificial mutation
BRCA1-T1394I	c.4181C>T
BRCA1-Q1395R	c4184A>G
BRCA1-Q1395H	c.4185G>C
BRCA1- S1387A /T1394A/S1423A	Artificial mutation
BRCA1- S1387E /T1394E/S1423E	Artificial mutation
BRCA1-M1400V	c.4198A>G
BRCA1-L1407P	c.4220T>C
BRCA1-M1411T	c.4232T>C

2.1.3 List of siRNA used in this thesis

NSC1 UUCGAACGUGUCACGUCAAdTdT
Sigma 1 control siRNA-Mission siRNA UNIVERSAL Negative control #1
Sigma 2 control siRNA-Mission siRNA UNIVERSAL Negative control #2
BRCA1 -296 unpublished sequence
BRCA1-719 unpublished sequence
BRCA1-6252 GGAUCGAUUAUGUGACUUAdTdT
PALB2-718 GGAAAAGACUAAAGGAACAdTdT
PALB2-1099 unpublished sequence
PALB2-1493 UCAUUUGGAUGUCAAGAAAdTdT

PALB2-1981 unpublished sequence
PALB2-2210 unpublished sequence
PALB2-2693 GCAUAAACAUUCCGUCGAAdTdT
BRCA2-1949 GAAGAAUGCAGGUUUAAUAdTdT
BRCA2-2618 GCUCAAAGGUAACAAUUAUdTdT
BRCA2-4915 GGGCAAAGACCCUAAAGUAdTdT
Abraxas-772 unpublished sequence
Abraxas-1158 unpublished sequence
Abraxas-1330 unpublished sequence

2.2 Cell lines and cultures

U2OS/DR-GFP HR or SA-GFP reporter cells, 293T and SV40-transformed EUFA1341 fibroblasts were previously described.[11, 378, 379] EUFA1341 cell lines reconstituted with wt or variant PALB2 proteins were generated as previously described.[379] These and 293T cells were all cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1X Penicillin-Streptomycin (Pen-Strep), at 37^{o}C in a humidified incubator with 5% CO_2.

MDA-MD-436 cells was cultured in DMEM/F12 (1:1) supplemented with 10% FBS and 1x Penicillin-Streptomycin. All cells were cultured in a humidified chamber with 5% CO2 at 37˚C. Mycoplasma was not tested, but the cells have been cultured in the presence of Plasmocin (ant-mpt, InvivoGen) to eliminate potential mycoplasma contamination.

No commonly misidentified cell lines were used.

siRNA knockdown of U2OS/DR-GFP cell.

siRNAs were transfected using Lipofectamine RNAiMax (Invitrogen) following manufacturer's instructions. For Western blotting, DRU2OS cells were plated at a 200,000 cells/well in 6-well plates. The final concentration of siRNAs was 10 nM. For the gene conversion assays, cells were instead seeded on a 10cm plate at 1,000,000 cells/plate as previously described. For BRCA1 pS1423/Ps1524 phosphorylation experiments, cells were harvested or treated with 16hr 2mM HU prior to cell lysate collection at 72hr. At least

three control siRNA sequences were used and three individual sequences for each gene studied were selected and pooled for knockdown analysis.

2.3 antibodies and chemicals

Antibodies used for the different chapters are as follows:

Myc (9E10, Covance), PALB2 antibody used (M11, against aa 601-880) was described before[317, 378], BARD1 (H300, Santa Cruz), Abraxas (ab139191, AbCam), HA (HA-7; H3663 Sigma) , polyclonal BRCA1 antibody (07-434, EMD Milipore), BRCA1 N-terminal monoclonal antibody (Abcam MS110), BRCA2 (OP95, EMD Milipore), RAD51 (H-92, Santa Cruz) , GAPDH (FL-335, Santa Cruz), B-Actin (AC-15, Santa Cruz), phospho-RPA (S4/S8) (A300-245A , Bethyl Laboratories), phospho-KAP1 (S824) (A300-767A, Bethyl Laboratories), RPA-32 kDa subunit (9H8, Santa Cruz), Chk1-pS317 (2344S, Cell Signalling), Total Chk1 (2344S ,Cell signaling), PLK (F-8, Santa Cruz) and P53 (DO-1 Santa Cruz). BRCA1 phosphospecific antibodies used include Rabbit monoclonal against pT1394 (described in Chapter 4), BRCA1-pS1423 (SC-101647, Santa Cruz) BRCA1-pS1524 (#9009;Cell Signalling). The secondary antibodies used were Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (NA931V, GE Healthcare) and donkey anti-rabbit IgG (NA9340V, GE Healthcare).

Chemical reagents used are as follows:

DMSO (D2650, SIGMA); Olaparib (S1060, Selleckchem); Bleomycin Sulfate (S1214, Selleckchem); Hydroxyurea (H8627, Sigma); Cisplatin (S1166, Selleckchem); Mitomycin C (M4287, Sigma); Camptothecin (S1288, Selleckchem); MG132 **(**M8699, Sigma); Cycloheximide (C7698, Sigma); ATM inhibitor (KU-55933) (S1092, Selleckchem); ATR

inhibitor (VE-821) (S8007 Selleckchem); DNA-PK inhibitor (NU7441) (S2638 Selleckchem)

2.4 Immunofluorescence

Cells were seeded onto coverslips in 12-well plates at a density of 150,000 cells per well the day before analysis. For MDA-MB 436 stable cell lines and 293T cells, coverslips were pre-treated with 0.01% Poly-L-lysine solution for 20min followed by one time PBS wash before cell seeding. Following 10 Gy of IR and 6 hr recovery, cells were washed with PBS and fixed with 3% paraformaldehyde/PBS for 6 min at room temperature (RT). For staining, cells were permeabilized with 0.5% triton X-100 for 5 min on ice and then incubated sequentially with primary and secondary antibodies for 30 min each at 37°C, with 3 PBS washes in between. The primary antibodies used were PALB2 (M11), RAD51 (H-92) and BRCA1 (D9, Santa Cruz), and the secondary antibodies were FITC-conjugated goat anti-rabbit and Rhodamine-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch). Coverslips were mounted onto glass slides with VECTASHIELD Mounting Medium with DAPI (Vector Labs). Images were captured using a Nikon Eclipse TE-2000-U microscope. Images of the same group were captured with identical exposure time using NIS-Elements Basic Research software. Amount of IR induced foci were determined using the NIS-Elements Basic Research software.

2.5 Immunoprecipitation (IP) and western blotting

2.5.1 Immunoprecipitation (IP)

The whole procedure was performed essentially as previously described [11, 317]. In brief, 0.5-2 ug of cDNA expression constructs per well were transfected into 293T cells in 6-well plates ($5x10^5$cells/well) using X-tremeGENE HP. Cell lysates were prepared ~30 hr

post-transfection using a NETNG-250 lysis buffer (250 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, 0.5% Nonidet P-40, and 10% glycerol) containing Complete® protease inhibitor mixture (Roche). The FLAG-HA-tagged PALB2 were IPed with anti-FLAG M2-agarose beads (Sigma). The 3xMyc-agged BRCA1 proteins were IPed for 3–4 hr with Pierce™ Anti-c-Myc Magnetic Beads (Thermo Fisher Scientific). Proteins were resolved on 4–12% Tris-Glycine SDS gels, transferred onto nitrocellulose membranes and analyzed by immunoblotting. Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used to develop the blots.

For co-expression experiments to determine PALB2-PALB2 self-oligomerization or BRCA1-PALB2 heterodimerization, the plasmids were mixed at a 1:1 ratio to a maximum DNA amount of 2ug transfected into 293T cells. For PALB2 self-oligomerization, Myc/GFP tagged PALB2 were co-transfected with FLAG-HA-tagged PALB2 containing the variants. For BRCA1-PALB2 heterodimerization, Myc-tagged BRCA1 were co-transfected instead. Anti-FLAG M2 agarose beads were used for immunoprecipitation in both experiments.

2.5.2 Gel filtration

PALB2 proteins tagged with FLAG-HA epitopes at the C terminus were overexpressed in 293T cells by transient transfection. Cells were collected 30 hr after transfection and lysed in NETNG250 (250 mM NaCl, 1mM EDTA, 20mM Tris-HCl, 0.5% Non-Idet P-40, 10% glycerol) with 5 mM NaF. Insoluble material was removed by high speed centrifugation (16,000 rpm for 30 min) at 4°C. 2 mg of each extract was analyzed on an FPLC AKTA Purifier (GE Healthcare) with a Superpose 6, 10/300 GL Tricorn column pre-equilibrated with NETNG250 (with 0.2% Non-Idet P-40) buffer containing 5mM NaF. 0.6 ml fractions were collected, and the proteins in the fractions were analyzed by western blotting using anti-PALB2.

2.5.3 Cycloheximide chase and MG132 treatment in chapter 4

At least 30hr following BRCA1 siRNA knockdown, U2OS cells were plated into 6-well plates at 250,000 cells per well after BRCA1 #6252 siRNA knockdown for at least 30hr. 48hr following siRNA knockdown, cells were transfected with 1ug of BRCA1 cDNA constructs for another 48hr. Individual wells were treated with 40ug/ml of cycloheximide. DMSO (1 mL per well) was added to control wells. Cells were trypsinized at 0, 3 and 6 hours after cycloheximide treatment and lysed in NETNG250; proteins were analyzed by Western blotting. To also determine the involvement of proteosomal degradation in BRCA1 mutant plasmids that expressed lower, cells were treated with 1uM of MG132 for 6hr before whole cell lysates were collected.

2.6. Gene Conversion assays

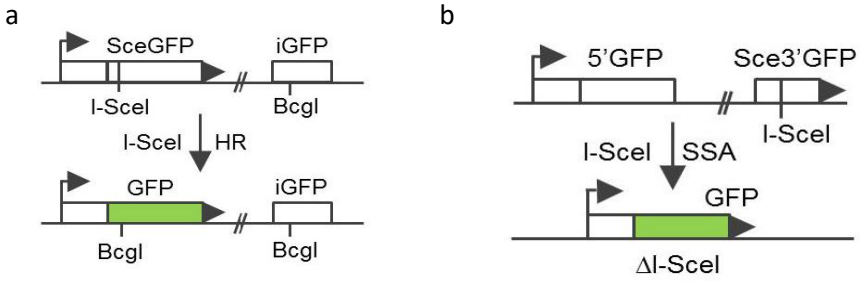

Figure 2: Schematic diagram of the HR and SSA reporter assays

a) DR-GFP HR reporter assay
b) SA-GFP reporter assay

Figure adapted from[11]

2.6.1 DR-GFP HR assay

To quantitatively assess the activity of BRCA1 or PALB2 variants in HR, we utilized our previously described HR assay based on a 'protein replacement' strategy[11] U2OS DR-GFP (HR) reporter cell line (Figure 2a) [239].The stably expressed DR-GFP reporter is

composed of two differentially mutated GFP genes oriented as direct repeats (DR) in which a 18bp I-SceI (yeast-specific endonuclease) site is inserted within the upstream copy of the GFP gene (SceGFP; Figure 2.1a) [263]. The upstream GFP fragement are defective due to a I-Sce1 restriction site (that also supply two in-frame stop codons) rendering the gene out of frame. Downstream of the 5' fragment is an 812-bp internal GFP fragment (iGFP) lacking the 3' end[263]. Both of these fragments are 3.7kb apart.

We first knockdown either endogenous BRCA1 or PALB2 using siRNA at least 48hr before plasmid reconstitution. For the PALB2 constructs, silent mutations were introduced to make the PALB2 cDNA resistant against PALB2 #1493 siRNA (5'-UCAUUUGGAUGUCAAGAAAdTdT-3'), while siRNA targeting the 3'-UTR of the BRCA1 was used (the BRCA1 cDNAs lacks the 3'-UTR targeted by the BRCA1 #6252 siRNA). To ensure uniformity of the knockdowns, siRNA transfections were performed in 10 cm plates and the cells were harvested, mixed and reseeded into six-well plates (200-250k cells/well) before the second transfection. 500ng of siRNA resistant pOZ-FH-C1-PALB2 or 1ug pcDNA-3xMyc-BRCA1-WT can then be co-transfected with 1.5ug pCBASceI plasmid (I-SceI endonuclease) into the knockdown-ed cells. After transient expression of the I-SceI restriction enzyme for a minimum of 48hr, site specific DSBs introduced by exogenous expressed I-Sce1 endonuclease will need to be repaired by homologous recombination or more specifically gene conversion utilizing the downstream GFP fragment as a template, in which the correct GFP reading frame is then restored (the I-Sce1 site is lost and instead replaced with BcgI site)[263, 324]. The GFP signals generated represents repair efficiency of the cells and can be measured via FACS. We would expect that DDR proteins defective would be unable to repair DNA lesion effectively, thereby generating lower GFP reads that may even be similar to vector control cells. As additional controls, expression of both overexpressed constructs and the HA-tagged I-Sce1 protein

needs to be assed via western blot to better interpret if the impaired activity is a result of reduced protein expression or I-Sce1 levels.

2.5.2 DR-GFP SSA assay

To complement our observations from the U2OS DR-GFP (HR) reporter, we also used a similar protein replacement strategy with another U2OS-based reporter cell line containing an integrated SA-GFP reporter [324] (Figure 2b). Single Stand Annealing (SSA) is an error-prone repair pathway that also utilizes resected DNA ends but instead of RAD51-mediated homologous, relies on RAD52 to search for homologous shorts sequences, anneals the short homology region with one copy of the homologous repeat and intervening sequence between the homologous repeated deleted[54]. Similar to the DR-GFP reporter, the SA-GFP reporter consist of direct repeats of the GFP gene. However, the I-Sce1 restriction site is instead positioned at the SceGFP3′ fragment, which has 266 bp homology with the upstream 5′GFP fragment (lacks the 3' end)[324]. Induction of I-SceI-generated DSB in SceGFP3′ can have two choices of repair, either HR or SSA. If the DSBs are repaired by HR utilizing the short homology between the fragments (estimated to be at least 30-fold less frequent), a non-functional GFP gene is instead generated. Hence, the SA-GFP assay is specific for assessment of SSA activity with a DNA strand from SceGFP3′ is annealed to the complementary strand of 5′GFP, followed by DNA-processing to generate a 2.7-kb deletion[324]. The restored GFP reading frame lacks the I-Sce1 site and the resulting GFP signal can later be measured using FACS.

2.7 Drug sensitivity assays

2.7.1 CellTiter Glo assay

EUFA1341 cells were seeded at 2,000 cells per well in 96-well plates. MDA-MB 436 stables were seeded at 4,000 cells per well. The day after seeding, drug compounds were first diluted in the medium and then added to the wells to achieve the desired final

concentrations. Cells were incubated with the drugs for 96 hr and survival rates were measured using CellTiterGlo Cell Viability Assay (Promega) according to manufacturer's instructions.

2.7.2 Clonogenic survival assay and MDA-MB 436 stable cell line generation

MDA-MB-436 cells were seeded into six-well plates at a density of 1X 106 cells per well the day before transfection. Cells were transfected with 1 ug of empty vector or 1 ug of BRCA1 expression vectors using 6 ml of X-tremeGENE 9 (Roche). Cells were trypsinized 36 hr after transfection and reseeded into 10 cm plates at 100,000 cells per plate in a volume of 10 ml. Another 16 hr later, 1 ml of 10X G418 (6 mg/ml dissolved in the same culture medium) or 10X G418 containing 2.2 mM cisplatin or olaparib was added to each plate. The final concentrations of G418, cisplatin and olaparib were 550 ug/ml, 200 nM and 200 nM, respectively. All experiments were performed in duplicate plates for each construct. For cells selected with G418 alone, 15 days after selection, one plate was trypsinized and cells counted with a Vi-CELL Cell Counter (Beckman Coulter) to determine viable cell number, and the other plate was stained with Crystal Violet (0.5% w/v in 95% ethanol, 5% acetic acid) to count the number of colonies. For cells selected with both G418 and cisplatin, both plates were stained 21 days after selection. For cells subjected to G418 and olaparib double selection, both plates were stained 28 days after selection. Colonies with approximately 50 cells or more were counted, and the numbers were normalized against the number of viable cells on corresponding plates with G418 alone. The vast majority of constructs were measured by three or more independent experiments (each with duplicate sets of plates), with the only exceptions being when the first two experiments produced nearly identical results or were carried out using two independent plasmids.

For generation of the MDA-MB-436 stable cell lines, transfected cells were instead seeded at 20,000 cells into 10cm plates with the same G418 selection concentration used.

At least 21 days after selection, individual colonies were trypsinized and first grew in regular DMEM/F12 (1:1) supplemented with 10% FBS and 1x Penicillin-Streptomycin for a week before being screened using immunofluorescence microscopy to identify of BRCA1 positive cells. BRCA1 expressing clones were subjected to a final round of G418 selection at 250ug/ml for a week before growing in regular media.

2.8 Align-GVD grade assessment

Align-GVGD is a freely available, web-based program that was used to predict if a specific missense substitution in genes of interest fall in a spectrum of grades of clinical significance based on the biophysical characteristics of amino acids and protein multiple sequence alignments[336]. Align-GVD grade assessment on each individual BRCA1 VUS were determined using the Huntsman Cancer Institute (University of Utah) HCI database of prior probabilities of pathogenicity for single nucleotide substitutions (http://priors.hci.utah.edu/PRIORS/BRCA/indexBRCA1.php). The Align-GVD grade assessments are constructed under the assumption that each given missense substitution is either high-risk (in the same sense as a truncating mutation) or neutral with respect to risk. Grade C65 has the highest relative odds of a high-risk variant and grade C0 having the lowest odds. Align-GVGD grade for C0 to C65 denote increasing likelihood of a variant to cause damage (to protein function).

2.9 Statistical analysis

Statistical significance was analyzed by either Student's t-test or one way ANOVA using GraphPad Prism 5.0 (GraphPad Software). P values of less than 0.05 were considered statistically significant.

Chapter 3

Effects of compromised PALB2-BRCA1 and PALB2-BRCA2 interactions on breast cancer risk

3.1 Compromised BRCA1-PALB2 interaction is associated with breast cancer risk

Work in this section was contributed to the following publication

Foo, T. K., et al. (2017). "Compromised BRCA1-PALB2 interaction is associated with breast cancer risk." Oncogene 36(29): 4161-4170.

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Running title: PALB2-BRCA1 interaction and breast cancer risk

Abstract

The major breast cancer suppressor proteins BRCA1 and BRCA2 play essential roles in homologous recombination (HR)-mediated DNA repair, which is thought to be critical for tumor suppression. The two BRCA proteins are linked by a third tumor suppressor, PALB2, in the HR pathway. While truncating mutations in these genes are generally pathogenic, interpretations of missense variants remains a challenge. To date, patient-derived missense variants that disrupt PALB2 binding have been identified in BRCA1 and BRCA2; however, there has not been sufficient evidence to prove their pathogenicity in humans, and no variants in PALB2 that disrupt either its BRCA1 or BRCA2 binding have been reported. Here, we report on the identification of a novel *PALB2* variant, c.104T>C [p.L35P], that segregated in a family with a strong history of breast cancer. Functional analyses showed that L35P abrogates the PALB2-BRCA1 interaction and completely disables its abilities to promote HR and confer resistance to platinum salts and PARP inhibitors. Whole-exome sequencing of a breast cancer from a c.104T>C carrier revealed a second, somatic, truncating mutation affecting *PALB2*, and the tumor displays hallmark genomic features of tumors with *BRCA* mutations and HR defects, cementing the pathogenicity of L35P. Parallel analyses of other germline variants in the PALB2 N-terminal BRCA1-binding domain identified multiple variants that affect HR function to varying degrees, suggesting their possible contribution to cancer development. Our findings establish L35P as the first pathogenic missense mutation in *PALB2* and directly demonstrate the requirement of the PALB2-BRCA1 interaction for breast cancer suppression.

Key words: BRCA1, PALB2, homologous recombination, breast cancer

Introduction

The BRCA1 and BRCA2 tumor suppressor proteins play critical roles in the repair of DNA double strand breaks (DSBs) by homologous recombination (HR), which is generally believed to be essential for their tumor suppressive function. The two BRCA proteins are linked in the HR pathway by a third tumor suppressor protein, PALB2, via direct protein-protein interactions.[331, 378, 397] Similar to *BRCA1* and *BRCA2*, mono-allelic mutations in *PALB2* increase the risk of breast, ovarian and pancreatic cancers,[344, 357] whereas bi-allelic mutations cause Fanconi anemia (FA).[344] In a way akin to the risk conferred by *BRCA2* germline mutations, in women under 40 years of age, the risk of breast cancer development conferred by *PALB2* mutations is 8-9 times that of controls and in women older than 60, the risk is 5 times that of controls.[13]

PALB2 and BRCA2 interact with each other via a WD40-repeat domain at the C-terminus of PALB2, which forms a 7-bladed β-propeller structure, and a highly conserved motif in the N-terminus of BRCA2 (aa 21-39) that forms an α-helix.[247] The PALB2-BRCA1 interaction, on the other hand, is mediated by what appears to be a hydrophobic interaction between a conserved coiled-coil motif at the N-terminus of PALB2 (aa 9-42) and a similar motif in BRCA1 (aa 1393–1424)[331, 397, 398]. Interestingly, the N-terminus of PALB2 has also been reported to mediate its own dimerization or oligomerization,[37, 332] suggesting a possible competition between the PALB2-PALB2 self-interaction and the PALB2-BRCA1 complex formation.

Numerous sequence alterations in *PALB2* have been identified in germline genetic testing of familial breast and pancreatic cancers and in tumor DNA sequencing. Based on available data as of 2014, the frequency of *PALB2* truncating mutations is estimated to be ~2.4% in patients with family history of breast cancer worldwide.[13] In the United States, a study found the rate of truncating mutations to be 3.4% in 972 families without *BRCA1*/*2* mutations but unselected for ancestry.[51] To date, at least 339 unique sequence variants

in *PALB2* have been found in diverse populations (http://databases.lovd.nl/shared/variants/PALB2/unique), with ~100 being protein-truncating and the rest being mostly missense variants of unknown significance (VUSs). The crystal structure of the PALB2 C-terminal WD domain, combined with results from FA patient-derived cells, has shown that deletion of just 4 amino acids from the C-terminus of PALB2 would result in a collapse of the β-propeller structure and degradation of the protein.[247, 286] Also, premature termination of translation often leads to mRNA degradation by nonsense-mediated decay (NMD). As such, practically all *PALB2* truncating mutations can be considered deleterious and pathogenic. The interpretation of VUSs, however, requires detailed functional and genetic analyses. In this regard, the vast majority of *PALB2* VUSs have not been characterized at all and the associated risks remain undetermined for all *PALB2* VUSs.

Results

A breast cancer family carrying the c.104T>C [p.L35P] variant in *PALB2*

The unaffected proband reported a maternal family history of breast cancer (Figure 1a). Her mother was diagnosed with invasive breast cancer of the right breast at the age of 33; she was treated by a total mastectomy and chemotherapy and later died of an unrelated cause at 41 years. In the previous generation (II), the maternal grandmother was diagnosed cancer of the left breast at 70 and non-Hodgkin's Lymphoma at 71. The breast cancer was found to be estrogen receptor (ER) positive, progesterone receptor (PR) negative and HER2 negative. The patient underwent total mastectomy and showed excellent response to subsequent letrozole treatment. The patient is presently 78 years old. Her mother was reportedly diagnosed with bilateral breast cancer, at the ages of 62 and 68. Clinical testing of germline samples from the proband revealed a VUS in the *PALB2* gene: c.104T>C [p.L35P] (confirmed by Sanger sequencing, Figure 1b, upper

trace). The same variant was discovered in germline and somatic (tumor) samples from the grandmother, confirming that the proband's mother is an obligate heterozygote for this mutation. No tissue was available for the mother. To determine whether the tumor of the maternal grandmother of the proband, diagnosed with invasive ductal breast cancer at age 70 years, had undergone loss of heterozygosity (LOH) at *PALB2*, DNA was extracted from macrodissected tumor and subjected to PCR-sequencing analysis. Both wild-type (wt) and mutant allele were present in the tumor cells and the ratio appears to be 1:1, similar to that in the germline/blood DNA (Figure 1b, lower trace), indicating a lack of LOH.

Whole exome sequencing of germline and tumor DNA from a c.104T>C *PALB2* carrier

To gain a better understanding of the pathogenicity and somatic mutation pattern associated with the L35P variant, blood and tumor DNA from the proband's maternal grandmother were analyzed by whole-exome sequencing (WES) (see supplementary methods and tables S1 and S2). L35P was found to be present in 45% and 48% of DNA from normal (blood) and tumor tissues, respectively (Figure 1c, supplementary table S2), confirming the lack of LOH. A clonal somatic nonsense mutation affecting the *PALB2* gene (Q61*) was identified, providing a mechanism for *PALB2* bi-allelic inactivation, and suggesting that PALB2 is likely dysfunctional in this cancer. Additionally, WES identified 230 somatic mutations in the tumor, of which 175 were non-synonymous mutations, some of which affected known cancer genes including *ARID1A* (G794E), *CDK12* (P670S) and *BCOR* (T581N) (Figure 1d-e). The tumor displayed a complex genome, with numerous low-level copy number gains and losses, and a focal amplification on 8q22. Consistent with the notion that *PALB2* is inactivated and that this tumor would lack competent HR DNA repair, WES analysis revealed a high score of 27 for large-scale state transitions[270] (LST, Figure 1d and Table S1) and a mutational Signature 3[242] (Figure 1f), both hallmark

features of tumors with *BRCA1* or *BRCA2* mutations and HR deficiency (HRD).[270] Both features have been further confirmed by our re-analysis of i i) breast cancers harboring BRCA1 germline mutations regardless of ER and HER2 status, ii) breast cancers harboring BRCA2 germline mutations regardless of ER and HER2 status, and iii) ER-positive (ER+) and HER2-negative (HER2-) breast cancers lacking BRCA1 or BRCA2 germline mutations from The Cancer Genome Atlas (TCGA) database (Supplementary Figure 1). The L35P tumor showed positive nuclear staining for BRCA1 as analyzed by immunohistochemistry (IHC) (Supplementary Figure 2), ruling out the possibility of its HRD phenotype being due to silencing of BRCA1 expression.

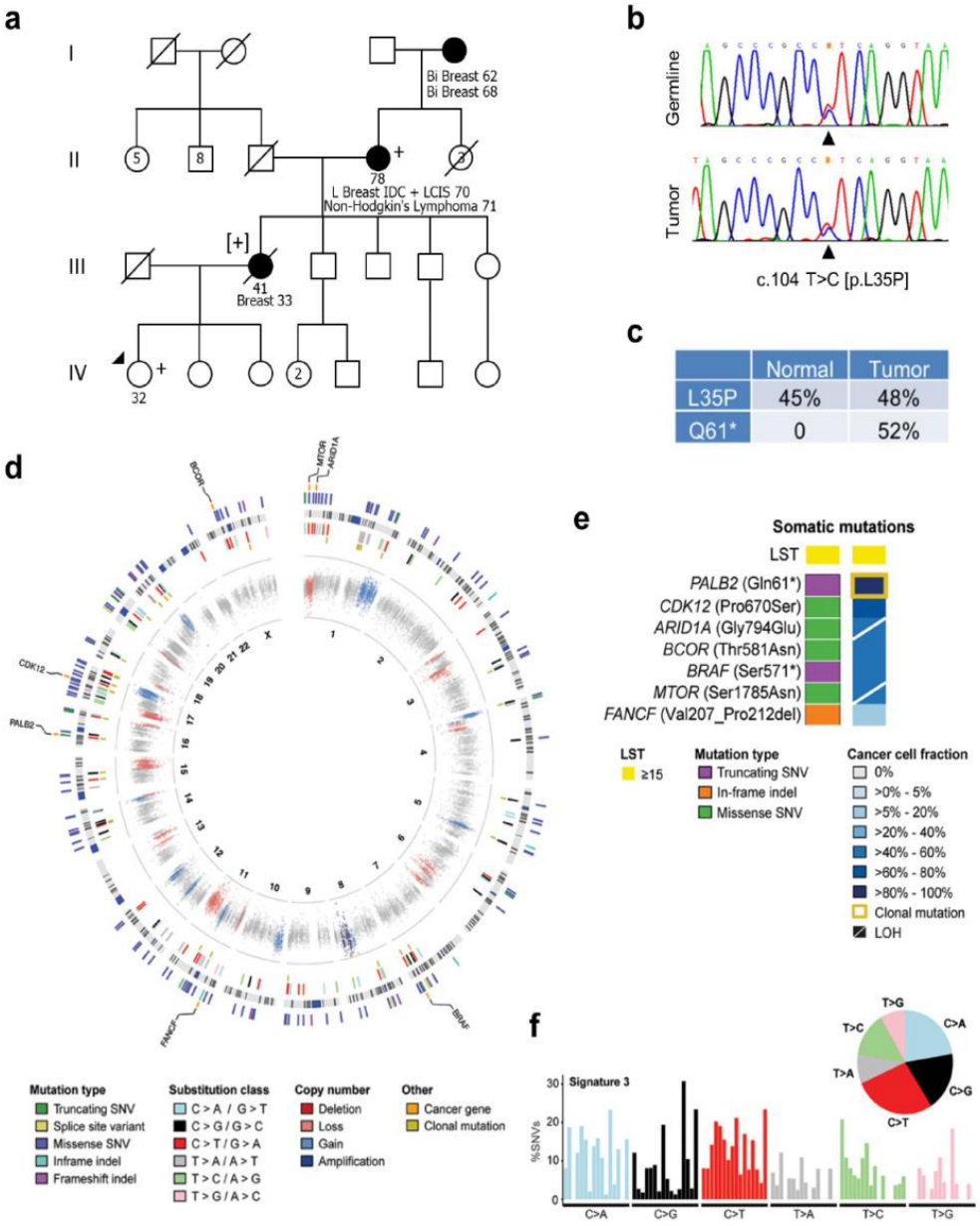
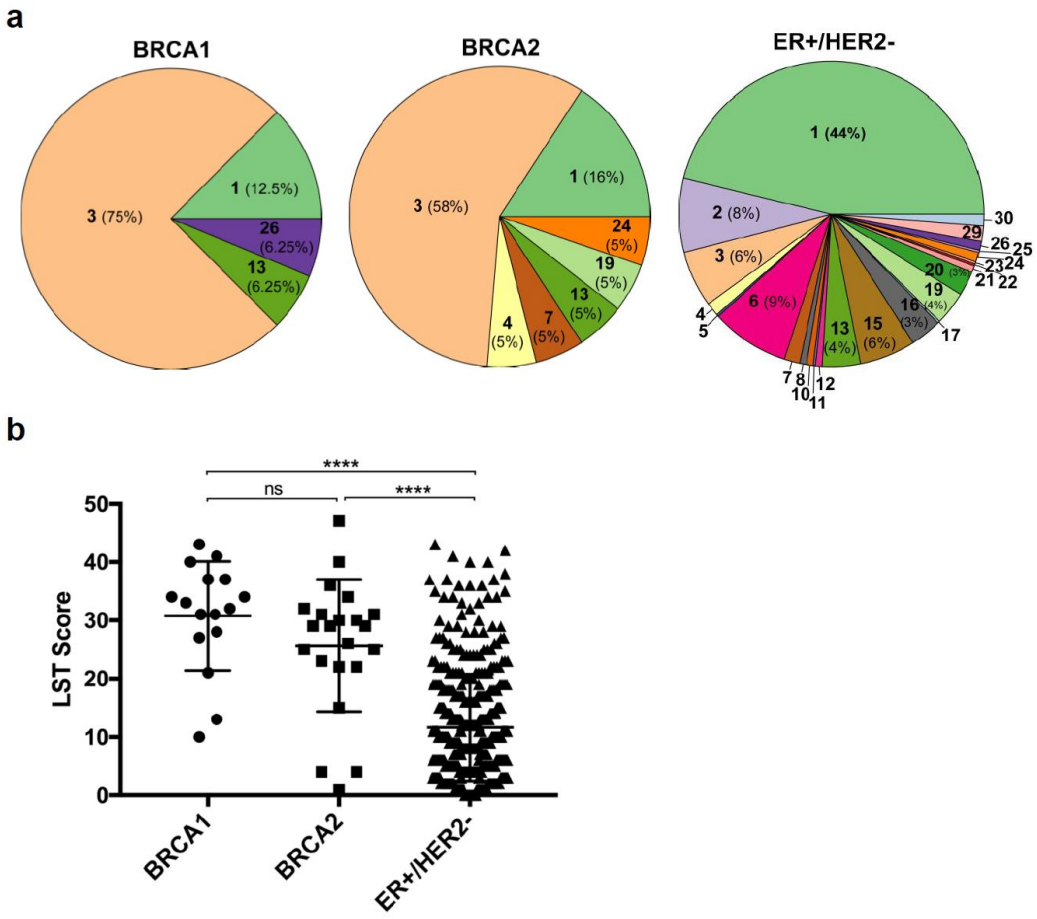


Figure 1. Characterization of a breast cancer from a family carrying the *PALB2* c.104T>C [p.L35P] variant. **(a)** Pedigree of the family. The proband is marked by a filled triangle. Confirmed mutations carriers are indicated by a "+" sign. Obligate carriers are indicated by a [+] sign. Mutation status in all other person is unknown. **(b)** Presence of the L35P mutation in the germline DNA and tumor DNA of the affected grandmother. **(c)** Presence of the L35P and Q61* mutations in normal (blood) and tumor DNAs of the affected

grandmother. (**d**) Circos plot depicting the mutations and copy number alterations across the genome. Mutations are shown along the outside, including annotations on cancer gene status and mutation type (color-coded according to the legend), with the chromosomal position arranged along the middle ring. The 96 substitution classifications defined by the substitution classes are shown, and clonal mutations are indicated with a golden mark. Copy number alterations are depicted along the center ring color-coded according to the legend. **(e)** Diagram depicting the somatic mutations identified affecting cancer genes (see Supplemental Methods). The LST status (top), mutation types (left) and cancer cell fractions (right) are shown, color-coded according to the legend. (**f**) Mutational signature of all somatic synonymous and non-synonymous SNVs identified. Variants are displayed according to the 96 substitution classification and the 5' and 3' sequence context, normalized to the trinucleotide frequency of the human genome. Indel, small insertion and deletion; LOH, loss of heterozygosity; LST, large-scale state transition; SNV, single nucleotide variant.



Supplementary Figure 1. Mutational signatures and large-scale state transitions (LSTs) in different subgroups of breast cancer. Re-analysis of i) breast cancers harboring *BRCA1* germline mutations, ii) breast cancers harboring *BRCA2* germline mutations, and iii) ER-positive (ER+) and HER2-negative (HER2-) breast cancers lacking *BRCA1* or *BRCA2* germline mutations from The Cancer Genome Atlas (see Methods). (**a**) Pie charts depicting the percentage of mutational signatures identified in breast cancers harboring *BRCA1* germline mutations (n=16; left), breast cancers harboring *BRCA2* germline mutations (n=19; middle), and ER+/HER2- breast cancers lacking *BRCA1* or *BRCA2* germline mutations (n=420; right). Numbers refer to a given mutational signature. (**b**), LST scores identified in breast cancers harboring *BRCA1* germline mutations (n=16), in breast cancers harboring *BRCA2* germline mutations (n=22), and in ER+/HER2- breast cancers lacking *BRCA1* or *BRCA2* germline mutations (n=448). Bars show mean ± s.d. ****, $p<0.0001$; n.s., not significant; Mann-Whitney *U* test. The *PALB2* c.104T>C breast cancer described in this manuscript displays a mutational signature 3 and a LST score of 27 (see Results and Figure 1).

cDNA mutation	Amino acid change	POLYPHEN/SIFT prediction	Description	Reference
c.53A>G	K18R	Probably damaging/ tolerated mutation	Reported 19 times in 5 independent studies **1.** Identified in 1 out of 95 in probands from families with cases of early onset prostate cancer (age at diagnosis <55 years) enrolled at University of Michigan Prostate Cancer Genetics Project (PCGP). **2.** Identified in 3 out of 139 early-onset African-American breast cancer cases recruited at Parkland Hospital, affiliated with the University of Texas Southwestern Medical Center. **3.** Identified in 3 out of 158 German patients with bilateral breast cancers. **4.** Identified in 11 out of 1240 successfully sequenced probands enrolled through USA familial cancer clinics by the Breast Cancer Family Registry. **5.** Identified in 1 out of 279 African American women with breast cancer recruited at the Cancer Risk Clinic at the University of Chicago.	24, 87, 241, 347, 403
c.83A>G	Y28C	Probably damaging/alters protein function	Single patient identified from study on 115 male breast cancer patients, with the patient diagnosed with breast cancer at age 46 and having one first degree and two second-degree female relatives diagnosed with breast cancer.	88
c.90G>T	K30N	Probably damaging/alters protein function	Identified in 1 out of 747 youngest women from multiple-case breast cancer families not known to carry a mutation in BRCA1 or BRCA2.	338
c.104 T>C	L35P	Probably damaging/alters protein function	Identified in a family of Western European descent with strong evidence of familial breast cancer.	This study.
c.110G>A	R37H	Probably damaging/alters protein function	Reported in 2 independent studies **1.** Identified in 1 out of 132 non-BRCA1/BRCA2 Spanish breast/ovarian cancer families with at least one pancreatic cancer case. **2.** Identified in 1 out of 565 unilateral breast cancer patient recruited from WECARE study involving breast cancer patients from USA and Denmark diagnosed before age of 55.	23, 346

Table 1: Overview of the 5 *PALB2* N-terminal VUSs examined in this study. POLYPHEN/SIFT predictions were made at the time of original reports. See *PALB2* mutation database (http://databases.lovd.nl/shared/variants/PALB2/unique) for further details.

VUSs in the coiled-coil motif of PALB2

Based on the results of the WES analysis, we posited that the *PALB2* c.104T>C, p.L35P mutation would result in a dysfunctional PALB2 protein unable to elicit HR and DNA repair. Interestingly, the affected residue is located in PALB2's N-terminal coiled-coil motif, the binding site for BRCA1.[331, 397, 398] Notably, in the coiled-coil motif there are at least 4 other VUSs (K18R, Y28C, K30N and R37H) previously identified in the germline samples of patients with familial breast cancer (Figure 2a and Table 1). Among them, Y28C was originally discovered in a family with both female and male breast cancers.[88] K18R is also notable as it has been found 18 times in 5 different studies (Table 1). All affected residues are highly conserved in vertebrates. Based on a previously proposed PALB2-BRCA1 interaction model,[331] Y28 and L35 are both located at the predicted hydrophobic interaction interface, whereas K18, K30 and R37H are placed away from the interface (Figure 2b). As such, only Y28C and L35P are expected to affect BRCA1 binding. It should be noted, however, that the model remains speculative due to the lack of crystal or nuclear magnetic resonance (NMR) structural information.

Effect of the PALB2 variants on BRCA1 binding and HR function

To determine the functional impact of the above VUSs, we first tested their abilities to interact with BRCA1 by immunoprecipitation (IP). Y28C and L35P both abrogated the co-IP of the endogenous BRCA1 with the ectopically expressed PALB2 proteins in 293T cells, whereas K18R, K30N and R37H did not significantly affect the complex formation (Figure 2c and data not shown). Interactions of the PALB2 variants with BRCA2 and RAD51 were all unaffected, consistent with the fact that BRCA2 binds to the C terminus of PALB2.[247, 332] We next asked if any of these variants would disrupt PALB2 HR function, by testing their ability to rescue HR following the depletion of the endogenous PALB2 in U2OS/DR-GFP reporter cells.[378] Surprisingly, all of the variants but K30N caused varying

degrees of reduction in HR ability (Figure 2d). Consistent with the genomics observations made in the WES analysis of the L35P *PALB2* mutant breast cancer, the L35P mutation completely abrogated the HR activity of PALB2. Although PALB2-Y28C behaved similarly to PALB2-L35P in the co-IP assay, it retained ~35% of HR activity of the wt protein. These results suggest that the variants may affect the integrity of the coiled-coil motif and that even a modest distortion of the structure could result in reduced HR activity, even if the binding of BRCA1 is not affected.

Effects of the variants on PALB2 dimer/oligomerization

In addition to binding BRCA1, the N-terminus of PALB2 also mediates its own dimerization or oligomerization.[37, 332] Deletion of the N-terminus results in dissociation of PALB2 dimer/oligomers, higher DNA binding affinity and increased activity in promoting RAD51 nucleoprotein filament formation and strand invasion in vitro.[37] Nonetheless, due to the fact that deletion of the N terminus also abolishes BRCA1 binding, which is critical for PALB2 recruitment to DNA damage sites, the in vivo relevance of PALB2 dimer/oligomer formation has been difficult to assess. To determine the effect of the variants on dimer/oligomer formation, we introduced the sequence alterations in a "mini-PALB2" that lacks the sequence encoded by exon 4.[379] Due to the smaller size of the mini-PALB2 (PALB2Δ4) proteins, they can be clearly separated from the endogenous PALB2 on a western blot. When the proteins were transiently expressed in 293T cells and IPed, a small amount of endogenous PALB2 was co-IPed with all of them (Figure 2e and data not shown). Y28C and L35P moderately but reproducibly reduced the amount of endogenous PALB2 co-IPed, whereas K18R showed no significant effect (Figure 2e). To more directly measure dimer/oligomer formation, we overexpressed the full-length variant proteins in 293T cells and subjected the lysates to gel filtration. L35P showed no discernible effect on the elusion profile of the overexpressed PALB2 (largely free of

binding proteins due to overexpression), while Y28C caused a very slight shift of the PALB2 peak to the right (smaller molecular weight) (Fig. 2f). As a positive control, deletion of the coiled-coil motif caused a clear shift to the right, indicative of a compromised self-interaction. Taken together, these data suggest that Y28C and L35P may weaken but do not disrupt PALB2 self-interaction. Note that PALB2 appears to form both dimers and oligomers, but the exact mode and in vivo function of PALB2 self-interaction remains unknown.

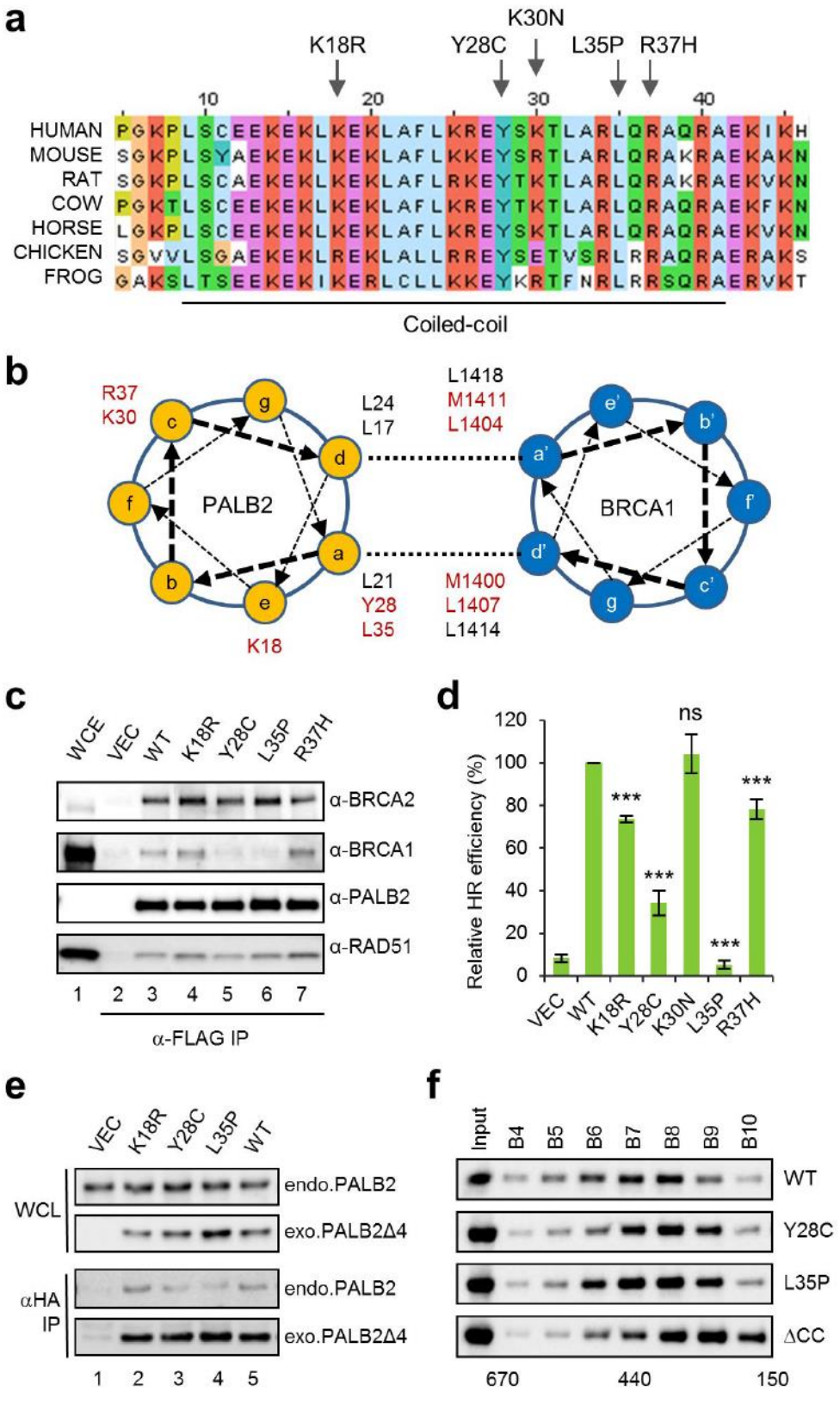

a
K18R
Y28C
K30N
L35P
R37H
10
20
30
40
HUMAN PGKPLSCEEKEKLKEKLAFLKREYSKTLARLQRAQRAEKIKH
MOUSE SGKPLSYAEKEKLKEKLAFLKKEYSRTLARLQRAKRAEKAKN
RAT SGKPLSCAEKEKLKEKLAFLRKEYTKTLARLQRAKRAEKVKN
COW PGKTLSCEEKEKLKEKLAFLKREYTKTLARLQRAQRAEKFKN
HORSE LGKPLSCEEKEKLKEKLAFLKREYSKTLARLQRAQRAEKVKN
CHICKEN SGVVLSGAEKEKLREKLALLRREYSETVSRLRRAQRAERAKS
FROG GAKSLTSEEKEKIKERLCLLKKEYKRTFNRLRRSQRAERVKT
Coiled-coil
b
R37
K30
L24
L17
L1418
M1411
L1404
PALB2
BRCA1
L21
Y28
L35
M1400
L1407
L1414
K18
c
WCE
VEC
WT
K18R
Y28C
L35P
R37H
α-BRCA2
α-BRCA1
α-PALB2
α-RAD51
1 2 3 4 5 6 7
α-FLAG IP
d
Relative HR efficiency (%)
ns
***
VEC
WT
K18R
Y28C
K30N
L35P
R37H
e
VEC
K18R
Y28C
L35P
WT
WCL
endo.PALB2
exo.PALB2Δ4
αHA IP
endo.PALB2
exo.PALB2Δ4
1 2 3 4 5
f
Input
B4
B5
B6
B7
B8
B9
B10
WT
Y28C
L35P
ΔCC
670
440
150

Figure 2. Effects of PALB2 N-terminal VUSs on BRCA1 binding, HR activity and PALB2 self-interaction. **(a)** Amino acid sequence alignment of the PALB2 N terminus. The alignment as generated by ClustalW. The VUSs studied are marked on top. **(b)** Predicted model of the interaction between the coiled-coil motifs of PALB2 and BRCA1. Hydrophobic residues at the interface are shown. Residues affected by VUSs are shown in red. **(c)** Effects of the PALB2 variants on BRCA1 and BRCA2 binding. The proteins were transiently expressed in 293T cells and IPed with anti-FLAG M2 beads. The amounts of relevant proteins in the input whole cell lysate (WCL) and IPed materials were determined by western blotting. **(d)** Effects of the variants on the HR activity of PALB2. U2OS/DR-GFP cells were first depleted of the endogenous PALB2 by an siRNA and then rescued with the wt and variant PALB2 proteins by transient transfection. Error bars represent standard deviations (SDs) from at least three independent experiments. Statistical significance was analyzed by one way ANOVA and Newman-Keuls post hoc analysis using GraphPad Prism 5.0 (GraphPad Software). ***, $p<0.001$; ns, not significant. **(e-f)** Effects of the variants on PALB2 self-interaction. The variants were introduced into PALB2Δ4 and the binding between these shortened PALB2 proteins and the endogenous PALB2 were assessed by IP-western following transient transfection into 293T cells (**e**). FLAG-HA-tagged full-length PALB2 variant proteins were transiently overexpressed in 293T cells and analyzed by gel filtration followed by western blotting using anti-PALB2. Fraction numbers are marked above the blots and the approximate corresponding molecular weights of the fractions below.

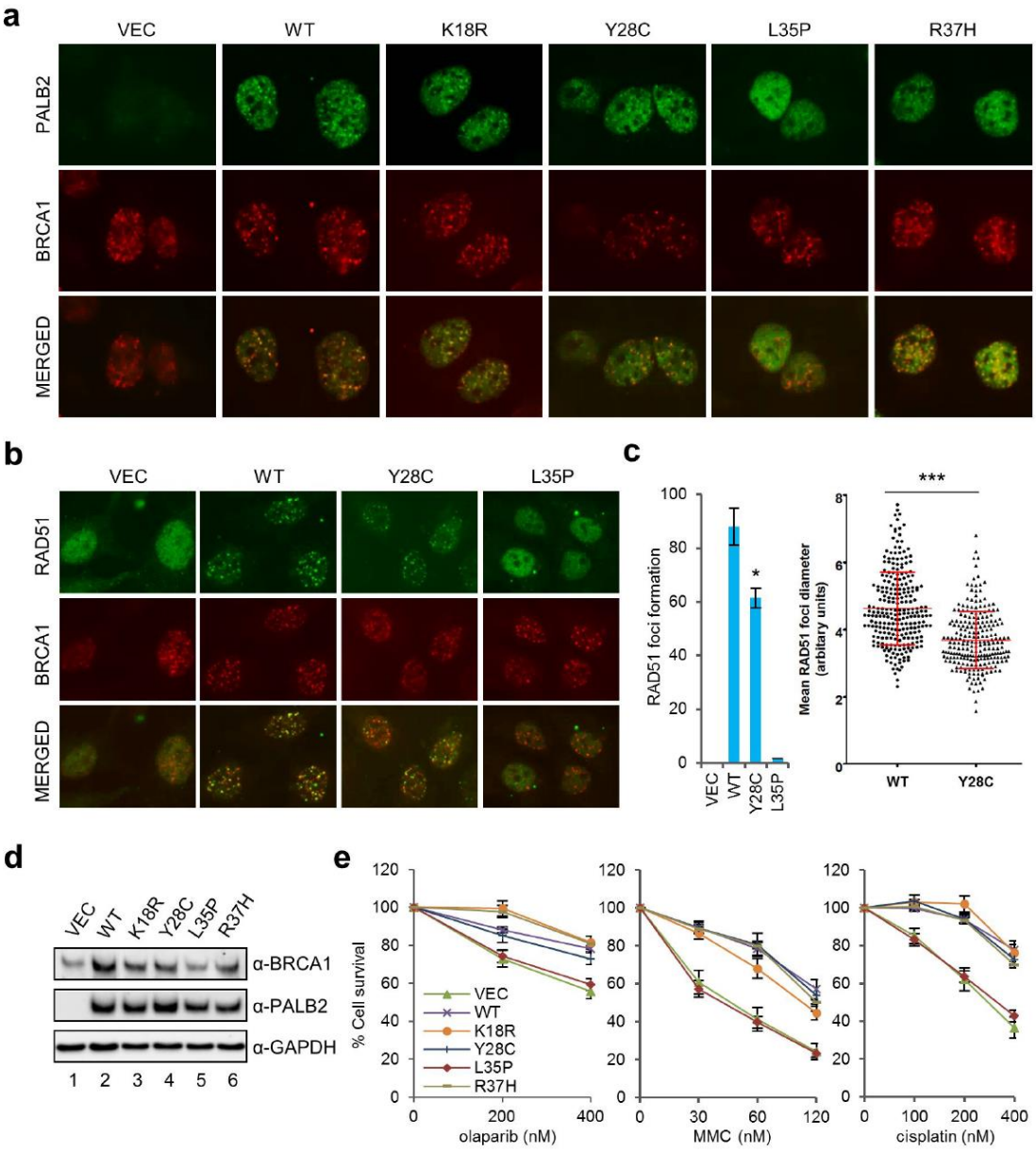


Figure 3. Effects of PALB2 N-terminal VUSs on PALB2 and RAD51 foci formation and cellular sensitivity to DNA damaging agents. **(a-b)** PALB2 and RAD51 IRIF formation in

EUFA1341 stable cell lines. Cells were treated with 10 Gy of IR, fixed after 6 hr recovery and analyzed by immunofluorescence using PALB2 (**a**) and RAD51 (**b**) antibodies, respectively. BRCA1 was co-stained with each of PALB2 and RDA51 to indicate sites of DNA damage and level of co-localization. **(c)** Efficiency and size of RAD51 foci formation in the above stable cell lines. Left, the percentage of RAD51 foci positive cells among BRCA1 foci positive (S and G2 phase) cells; right, sizes of the RAD51 foci in EUFA1341 cells expressing wt PALB2 and PALB2-Y28C. Error bars represent standard deviations (SDs) from at least three independent experiments. Statistical significance was calculated with Student's t-test. *, $p<0.05$; ****, $p<0.001$. **(d)** Representative western blots showing the levels of PALB2 and BRCA1 in the EUFA1341 stable cell lines. GAPDH was used a loading control. **(e)** Sensitivities of the EUFA1341 stable cell lines to cisplatin, olaparib and MMC. Cells were incubated with the drugs for 96 hr. Data presented are the averages of at least three independent experiments. Error bars represent standard deviations.

L35P abrogates PALB2 and RAD51 recruitment to DNA damage sites

We next sought to characterize in greater detail the basis of the HR repair deficiency caused by the VUSs, except PALB2-K30N, which was completely functional in HR (Figure 2d). EUFA1341 cell lines stably expressing each of these variant PALB2 proteins were generated. EUFA1341 is a SV40-transformed skin fibroblast cell line derived from an FA-N patient with biallelic mutations in *PALB2*, a nonsense mutation (c.1802T>A, p.Y551*) on one allele and a loss of the other allele due to a genomic deletion, which result in the expression of a truncated PALB2 protein lacking the ability to bind BRCA2 and recruit BRCA2-RAD51 following DNA damage.[379] As we and others have shown that BRCA1 promotes the recruitment of PALB2 to DSBs,[331, 397, 398] the ability of the PALB2 variants to form ionizing radiation-induced foci (IRIF) was determined. As depicted in Figure 3a, wt PALB2 forms IRIF that largely co-localize with those of BRCA1, and PALB2-

K18R and R37H behaved similarly to the wt protein. The Y28C variant was also able to form IRIF, but the foci were fewer, suggesting that its recruitment is partially impaired. In contrast, PALB2-L35P failed to form any foci, indicative of a completely abrogated recruitment. Consistent with our previous observations,[379] EUFA1341 cells were completely defective in RAD51 IRIF formation, and the defect was fully restored upon re-expression of wt PALB2 (Figure 3b). Again, PALB2-L35P was completely unable to support RAD51 foci formation, and Y28C appeared to be hypomorphic as the protein was able to support RAD51 foci formation but the foci were evidently smaller and also modestly fewer in number (Fig. 3c). Normal RAD51 foci formation was observed in cells expressing PALB2-K18R and R37H (data not shown). These observations demonstrate the important role of BRCA1 for PALB2 recruitment and the requirement of PALB2 for RAD51 foci formation. The expression levels of wt and variant proteins were largely comparable (Figure 3d).

PALB2-L35P is unable to confer resistance to DNA damaging agents

Defects in HR-mediated repair have been shown be predictive of clinical response to commonly used platinum drugs among breast and ovarian cancer patients[261, 337]. Similarly, we have shown that *PALB2*-deficient cells are hypersensitive to mitomycin C (MMC) and the poly (ADP-ribose) polymerase (PARP) inhibitor olaparib.[152, 379] Therefore, we assessed the sensitivity of EUFA1341 cells expressing the different variants to these DNA damaging agents. As expected, while re-expression of wt PALB2 in EUFA1341 cells conferred resistance to all three types of drugs, cells expressing PALB2-L35P were indistinguishable from vector-harboring cells (Figure 3e). Cells expressing other variant proteins showed resistance to all 3 types of drugs, although there were modest differences in their sensitivities to olaparib and MMC. Surprisingly, despite its substantially reduced HR activity as measured by the DR-GFP reporter assay (Figure 2d), PALB2-Y28C

conferred a wild-type level of resistance to both cisplatin and MMC and nearly wild-type level of resistance to olaparib (Figure 3e), suggesting that the residual HR activity was sufficient to confer resistance to the drugs.

Discussion

VUSs are commonly found during clinical genetics tests but their clinical and biological significance is often difficult to define, and this uncertainty poses significant challenges for clinicians and patients. Although our understanding of how cancers develop following the loss of BRCA1/2 and PALB2 remains far from complete, it is generally accepted that the resulting DNA repair defect and ensuing genome instability are a root cause. Moreover, the HR defect of *BRCA*/*PALB2* mutant tumor cells is now being rationally targeted by DNA damaging agents that generate lesions that require HR for repair, such as platinum salts and PARP inhibitors.[209, 211] Therefore, functional assessment of DNA repair properties of VUSs is required for the understanding of their pathogenicity, and this information is germane to treatment decision-making, risk prediction and management of both patients and family members.

PALB2 directly interacts with both BRCA1 and BRCA2 and acts as essential linker between the two proteins in the HR pathway.[331, 397, 398] While patient-derived missense mutations that disrupt PALB2 binding have been identified in both BRCA1 and BRCA2,[331, 378] there has been limited evidence that confirm their pathogenicity in humans, and no such mutations in PALB2 have been reported to date. Here, we identify a novel missense variant, L35P, in the coiled-coil motif of PALB2 that mediates BRCA1 binding. We establish that L35P is a *bona fide* null mutation that disrupts BRCA1 binding and completely abrogates the HR activity of PALB2 and its ability to confer resistance to DNA damaging agents. Whole-exome sequencing analysis of a breast cancer from a L35P germline mutation carrier provides direct evidence of bi-allelic inactivation of *PALB2*

through a second, somatic, truncating mutation in the gene. Moreover, the tumor displays genomic features of breast cancers with HR DNA repair defects, including complex patterns of copy number alterations, the Signature 3[242] and a high large-scale state transitions score.[270] Thus, L35P is a pathogenic mutation, and tumors from L35P mutation carriers are likely to respond to agents that target HR DNA repair defects, such as olarparib, cisplatin and MMC, provided that the wt allele of *PALB2* is deleted, mutated or epigenetically silenced.

Interestingly, the HR function of PALB2 is also affected by multiple other VUSs in the coiled-coil motif, particularly Y28C, which causes a 65% reduction of HR activity (Figure 2d). Although Y28C affects the PALB2-BRCA1 co-IP to a similar extent to L35P (Figure 2c), it can still be recruited to BRCA1-containing foci when stably expressed in EUFA1341 cells, albeit with moderately reduced efficiency (Figure 3a). This suggests that Y28C may in fact reduce the stability of the PALB2-BRCA1 complex to a point where the complex can no longer withstand the cell lysis or IP conditions used, rather than disrupting the complex formation altogether. The substantially reduced HR activity of PALB2-Y28C suggests that the foci are less productive, potentially due to imprecise location or suboptimal configuration. This scenario is supported by the observation that the RAD51 foci in EUFA1341 cells expressing PALB2-Y28C are noticeably smaller (Figure 3c,d). Yet, these cells are fully resistant to cisplatin and MMC with only a slight sensitivity to olaparib (Figure 3e), indicating that the residual HR activity is largely sufficient to confer drug resistance under the setting used. To our surprise, K18R and R37H also impair the HR activity of PALB2 even though they do not appear to reduce the PALB2-BRCA1 interaction (Figure 3b). One potential explanation is that they may affect higher order structures of the PALB2-BRCA1 and PALB2-PALB2 complexes, which could fine-tune HR activity. Similar to Y28C, these variants do not cause significant changes in drug sensitivity (Figure 3d). Thus, partial HR impairment may not translate into meaningful sensitivity to genotoxic

therapies. However, variants with intermediate HR defects may well increase cancer risk, as recently demonstrated in a mouse model for the *BRCA2* G25R variant, which weakens its binding to PALB2.[131, 378]

We have previously reported a *Palb2* knockin mouse strain with a mutation in the coiled-coil motif (CC6, ^{24}LKR^{26} to ^{24}AAA^{26}).[317] The CC6 mutation appears to abrogate the BRCA1-PALB2 interaction as assessed by co-IP and the HR activity of PALB2 as measured with U2OS/DR-GFP cells. The homozygous mutant mice are viable but show a moderate defect in spermatogenesis. B cells isolated from the mutant mice are able to form detectable RAD51 foci following MMC treatment, but the foci are smaller and dimmer, and the cells are hypersensitive to the drug. Human PALB2-Y28C is similar to mouse PALB2-CC6 in that it also affects the interaction with BRCA1 and can only support formation of smaller RAD51 foci; however, RAD51 foci in EUFA1341 cells expressing the Y28C protein are brighter and more distinct than those in the above-mentioned mouse B cells. Moreover, PALB2-Y28C retains significant HR activity and, at least when overexpressed, is fully capable of conferring resistance to DNA damaging agents. L35P, on the other hand, is a null mutation that completely abrogates the ability of PALB2 to support RAD51 foci formation and its HR function. Overall, these results together demonstrate the importance of the PALB2-BRCA1 interaction for RAD51 foci assembly. At the same time, the results also point to the existence of a possible threshold in the size and structure of RAD51 foci for the determination of HR activity and drug resistance, as well as a possible difference in the degree of dependence of RAD51 foci assembly on the PALB2-BRCA1 interaction in mouse and human cells.

It has been well established that *BRCA1* tumors are predominantly triple negative and *BRCA2* tumors are mostly ER positive. The underlying mechanisms that cause the dramatic difference remain poorly understood, although there have been reports that BRCA1 regulates ER transcription.[360] As for PALB2 tumors, recent consensus is that

around 70% are ER+ and 30% are triple negative.[13] Thus, the PALB2 phenotype sits between BRCA1 and BRCA2 but is much closer to BRCA2, consistent with the fact that it functions in a stable and high stoichiometry complex with BRCA2 while its interaction to BRCA1 is, though critical for HR, of much lower stoichiometry or and/stability. In others words, PALB2 has considerably more in common with BRCA2 than BRCA1. Moreover, as mentioned above, BRCA1 has been reported to play significant roles in transcriptional regulation, which could be the key for its triple negative cancer phenotype but completely independent of its role in recruiting PALB2 (and therefore BRCA2 and RAD51) to DNA damage sites. These could explain the ER+ phenotype of the L35P tumor studies here. Although patient-derived BRCA1 missense variants that disrupt PALB2 binding have been identified[331], there have not been any reports on the phenotypes of those BRCA1 tumors. It would be interesting to know if the tumors will be triple negative like most *BRCA1* null tumors or instead ER+ similar to what was observed in the L35P tumor.

In summary, we have now identified a novel *PALB2* variant, c.104T>C [p.L35P], which segregates in a family with a strong history of breast cancer. Our results from WES and functional analyses established L35P as the first *bona fide* null and pathogenic missense mutation in PALB2. These results for the first time directly demonstrate the requirement of the BRCA1-PALB2 interaction for breast cancer suppression. Our expanded analyses of other germline VUSs in the coiled-coil motif showed a certain VUS, such as Y28C, can significantly affect HR activity but have little or no effect on drug resistance, suggesting that a mutation may increase cancer risk but may not predict therapy response. The present study and our above-noted mouse study corroborate with each other to demonstrate the importance of the PALB2-BRCA1 interaction in RAD51 foci formation and drug resistance, as well as in male fertility and suppression of cancer development.

Materials and methods

Patients and genotyping

The proband attended the Hereditary Cancer Clinic at the Jewish General Hospital, Montreal, QC, Canada, on account of her strong family history of breast cancer. Chart notes confirmed her mother's diagnosis, and pathology reports and personal report confirmed her maternal grandmother's diagnosis. Blood was sent from the proband to Invitae (San Francisco, CA) where massively parallel sequencing of *BRCA1*, *BRCA2*, *CHEK2*, *PALB2* and *TP53* was performed. c.104T>C in *PALB2* was the only variant called by Invitae, and it was categorized as a VUS. We obtained both saliva and formalin-fixed, paraffin-embedded (FFPE) breast tumor tissue from the maternal grandmother. Sequencing of the lymphocyte and tumor-derived DNA, was carried out as previously described.[343] The study was approved by the Institutional Review Board of the Faculty of Medicine of McGill University, Montreal, QC, Canada, no. A12-M117–11A.

Whole-exome sequencing

Extracted DNA samples from the microdissected tumor and the matched germline DNA extracted from peripheral blood were subjected to whole exome capture using the SureSelect Human All Exon v4 (Agilent) capture system and to massively parallel sequencing on an HiSeq 2000 at the Memorial Sloan Kettering Cancer Center Integrated Genomics Operation (IGO) following validated protocols.[140, 182] Whole-exome sequencing analysis was performed as described previously[265]. The coverage was 202.21x for the tumor sample and 77.52x for the normal sample.

Paired-end reads in FASTQ format were aligned to the reference human genome GRCh37 using Burrows-Wheeler Aligner (BWA, v0.7.5a) [200]. Local realignment and base quality adjustment was performed using the Genome Analysis Toolkit (GATK, v2.7.4) [229]. Somatic single nucleotide variants (SNVs) were identified using MuTect (v1.0) [63] Small insertions

and deletions (indels) were detected using Strelka (v2.0.15)[297] and VarScan 2 (v2.3.7).[181] Mutations were filtered as previously described [265]. Copy number alterations (CNAs) were identified using FACETS [310], which performs a joint segmentation of the total and allelic copy ratio and infers allele-specific copy number states, as previously described.[266] The cancer cell fraction (CCF) of each mutation was inferred using the number of reads supporting the reference and the alternate alleles and the segmented Log_2 ratio from MPS as input for ABSOLUTE (v1.0.6).[50] Solutions from ABSOLUTE were manually reviewed as recommended [50, 193]. A mutation was classified as clonal if its clonal probability, as defined by ABSOLUTE, was >50%[193] or if the lower bound of the 95% confidence interval of its CCF was >90%. Mutations that did not meet the above criteria were considered subclonal. Mutations were annotated using a combination of driver prediction methods, Mutation Taster,[302] CHASM (breast)[49] and FATHMM,[311] and presence in three cancer gene lists[113, 172, 194] to define the potential functional effect of each non-synonymous SNV.

Circos Plot

A circos plot for case IDC53 was produced by binning all variants of high confidence (curation methodology described previously), into one of four categories “frameshift in-del,” “truncating SNV,” “missense SNV,” “inframe in-del,” “splice site variant,” “upstream, start/stop, or *de novo* modification,” or “silent” according to the functional salience of each aberrant locus, after which silent variants were discarded. Remaining variants were subsequently annotated if present in one of three reference sets diagnostic of potential pathogenicity as described above. Copy number assignments made using the FACETS algorithm [310] and post-processed (as described above) to determine per-gene allelic status as either “deleted,” “lost,” “neutral,” “gained,” or “amplified,” were then mapped to correspondent segmentation data. The annotated variant and copy number information

were then displayed using the OmicCircos software package [147] with respect to genomic position using the hg19 reference.

Mutational signatures

To define mutational signatures in the breast tumor, we measured the mutational context of all synonymous and non-synonymous somatic SNVs within the target regions. For each tumor component, we extracted the 5' and 3' sequence context of each mutation from the GRCh37 reference genome and the SNVs were categorized into C>A, C>G, C>T, T>A, T>C and T>G bins according to the type of substitution and subcategorized them according to the nucleotides preceding (5') and succeeding (3') the mutated base. The number of SNVs for each of the 96 sub-bins representing the tri-nucleotides [A|C|G|T] [C>A|C>G|C>T|T>A|T>C|T>G] [A|C|G|T] were counted.

The proportion of mutations belonging to the 96 sub-bins were normalized using the observed trinucleotide frequency in the target regions of the respective sequencing platforms to that in the human genome as previously described.[7, 8] The normalized mutational patterns were compared to the mutation signatures using non-negative least squares, such that a linear combination of the mutation signatures that is equal to the mutation pattern was found. The mutation signature of the tumor analyzed here was defined as the mutation signature with the highest coefficient.

Large-scale state transitions (LSTs)

Using previously established classification guidelines,[270] LSTs were defined as chromosomal breaks (i.e., changes in copy number of major allele counts) between adjacent regions of at least 10Mb. LSTs were quantified after smoothing and filtering small-scale copy number variations (<3Mb). The tumor had an LST score of ≥15, which

was categorized as high (i.e. associated with HR DNA repair defects, according to the original report describing LSTs.[270]

Re-analysis of The Cancer Genome Atlas (TCGA) breast cancer samples

To define the LST scores and mutational signatures of i) breast cancers harboring BRCA1 germline mutations regardless of ER and HER2 status, ii) breast cancers harboring BRCA2 germline mutations regardless of ER and HER2 status, and iii) ER-positive (ER+) and HER2-negative (HER2-) breast cancers lacking BRCA1 or BRCA2 germline mutations, we retrieved the MAF file of the breast cancers analyzed by TCGA[46] from the Broad Firehose portal (http://gdac.broadinstitute.org/), and the Affymetrix SNP6 array data for tumor and normal samples from the TCGA Data portal (https://tcga-data.nci.nih.gov/tcga/) on 1/28/15. Affymetrix SNP6 array data were used to determine LST scores for each TCGA sample as described above. Whole-exome sequencing data were employed to define the mutational signatures as described above.

All other materials and methods used in this chapter has been summarized in Chapter 2.

Acknowledgements

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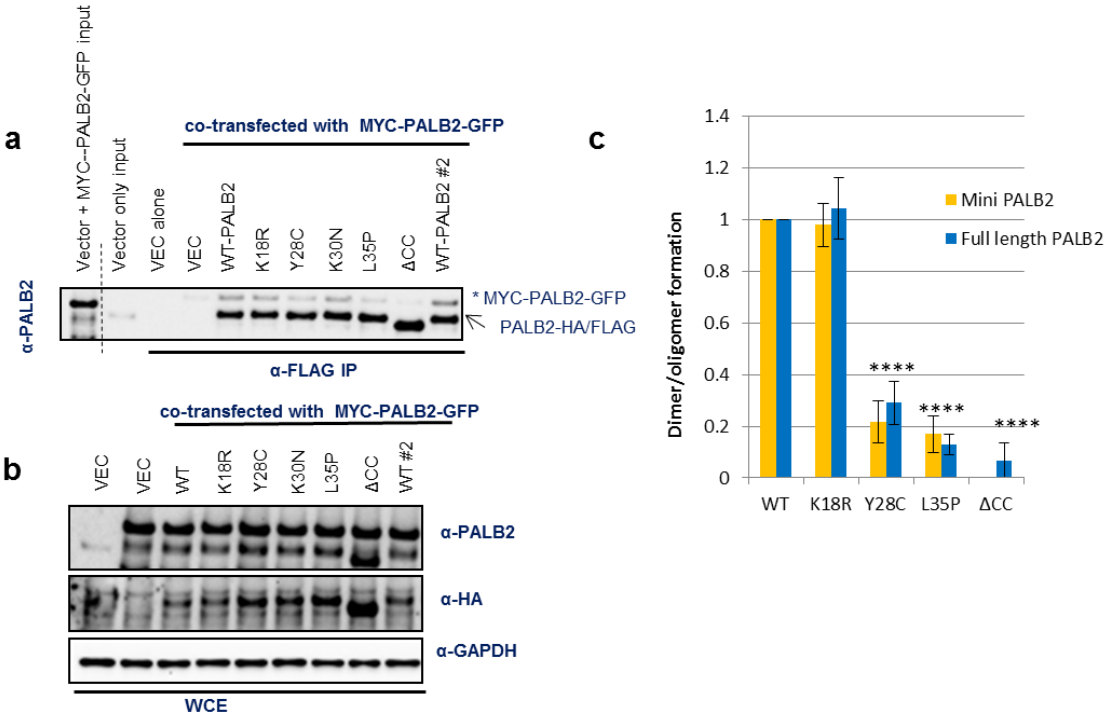

Appendix (1) Reduced PALB2 self-oligomerization in N-terminal CC domain mutants
(a)Full length PALB2 N-terminal variants were transiently co-transfected with Myc/GFP-tagged PALB2 similar to (2E).
(b) whole cell lysate of (a)
(c) Binding between either full length PALB2 or the ΔExon 4 "mini PALB2" harboring K18R, Y28C and L35P variants with MYC-PALB2-GFP were quantified and the ratio was calculated and plotted

3.2 BRCA1-PALB2 and PALB2-BRCA2 patient VUS lead to different effects on PALB2 mediated HR

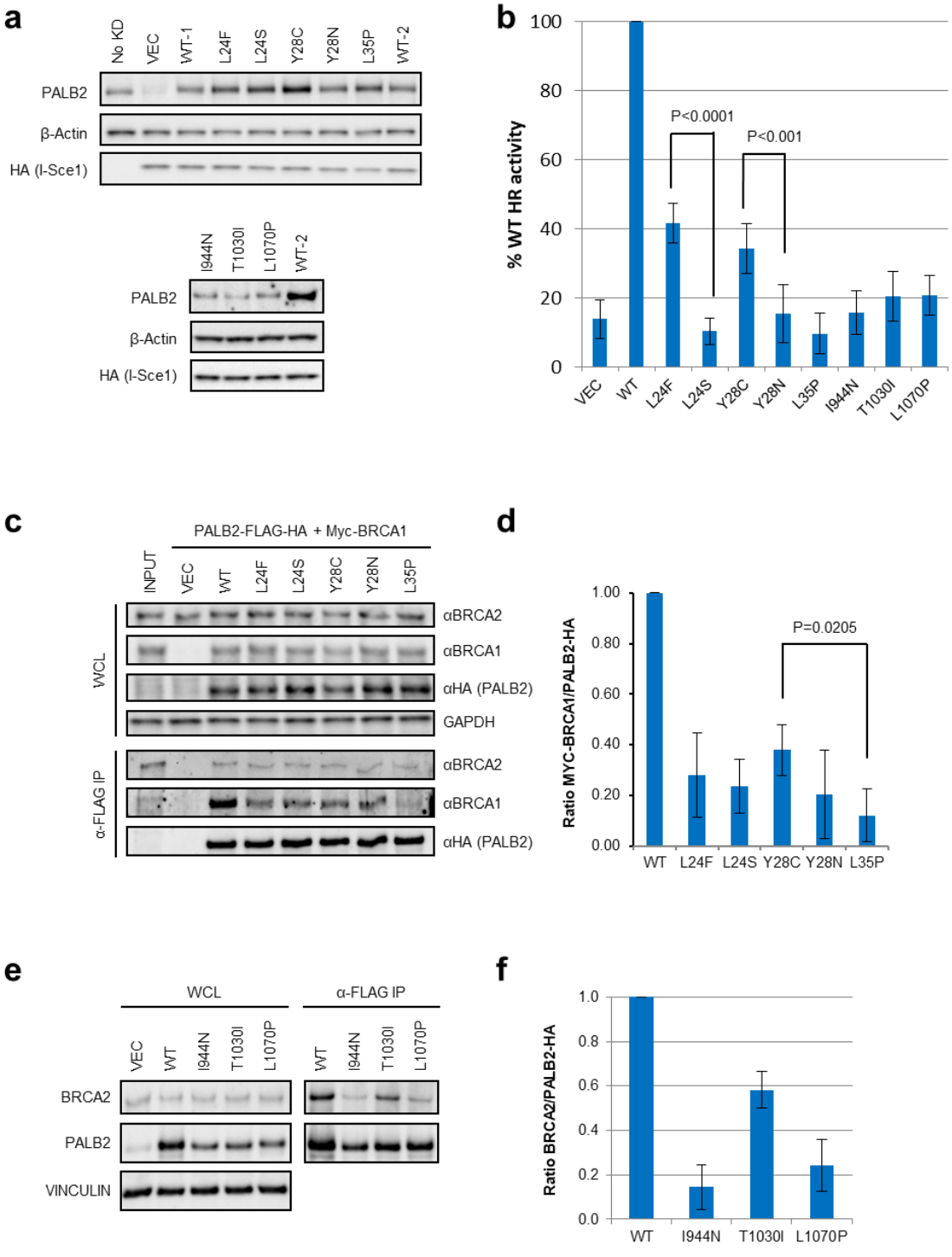

Figure 3.4: Effects of the PALB2 N and C-terminal variants on the HR activity of PALB2.

a) U2OS/DR-GFP cells were first depleted of the endogenous PALB2 by an siRNA and then rescued with the wt and variant PALB2 proteins by transient transfection. Whole cell lysates were collected with western blot of the respective PALB2 variant expression and I-Sce1 shown (b) Effects of PALB2 variants on its HR activity when measured in U2OS/DR-GFP cells. (c) Full length PALB2 N-terminal variants were transiently co-transfected with Myc-tagged BRCA1 with binding between either full length PALB2 or with MYC-BRCA1 were quantified and the ratio was calculated and plotted in (d) (e) Full length PALB2 C-terminal variants were transiently transfected into 293T cells with binding of PALB2 with endogenous BRCA2 were quantified and the respective ratios plotted in (f)

As we previously observed RAD51 foci is still retained in the case of PALB2-Y28C expressing EUFA1341 stable cell lines with ~35% HR activity, we wanted to ask if Y28C is truly a hypomorphic mutant of BRCA1 binding. To have a clearer comparison, we also tested in parallel 3 other N-terminal PALB2 coiled-coil domain VUS that potentially affects the PALB2-BRCA1 interaction (L24F, L24S,Y28N) identified from the ClinVar database that hosted sequencing reports of germline testing in patients with history of hereditary cancer. L24F, L24S, Y28N all showed reduced HR activity compared to WT-PALB2 even though their expressions are similar to or slightly higher than WT protein **(Figure 3.4 a)**. Interestingly, we observed a significant difference between L24F and L24S HR activity, as well as, between Y28C and Y28N **(Figure 3.4b)**. It appears that substitution of L24 and Y28 to more polar residues have a stronger effect on impairment of the HR function of PALB2 (~30-40% vs ~15-20%). Because we previously observed almost similar loss of endogenous BRCA1 binding in the case of Y28C and L35P, we decided to co-express both FLAG/HA tagged PALB2 together with Myc-tagged BRCA1 in 293T. When we look at the binding of these N-terminal variants to exogenously expressed BRCA1, we observed that all of the VUSs, with the exception of L35P, still retain some degree of BRCA1 binding (~20-30% of WT binding) **(Figure 3.4 c and d)**. These results suggest that the BRCA1-PALB2 interaction is hydrophobic with substitution of critical residues within this interface with a polarly charged residue will lead to an even greater effect on PALB2-mediated HR function.

Six amino acid residues (Leu17, Leu21, Leu24, Tyr28, Thr31 and Leu35) were reported to be critical for the PALB2 coiled-coil homodimer interface[322]. While homodimeric PALB2cc (aa1-60) has a dissociation constant (Kd) of ~ 80 µM, which is weaker than the corresponding PALB2cc:BRCA1cc heterodimer (~ 6 µM), it is unclear if the same preference for BRCA1-PALB2 heterodimer formation is recapitulated in the context of full-length proteins and *in vivo*[322]. In fact, L24A synthetic mutation on PALB2cc was observed to affect both homodimer and heterodimer formation with a stronger effect on homodimer formation[322]. Whether the homo or oligomerization of PALB2 is biologically relevant remains poorly understood.

We also looked at the effects of PALB2 WD40 domain VUS on its BRCA2 binding and HR activity. PALB2-T1030I was previously reported to be unstable, while its BRCA2 binding remains unknown[254]. All three PALB2 WD40 domain VUS, I944N, T1030I and L1070P, were observed to express much weaker than WT-PALB2 with almost similar to vector level of HR activity **(Figure 3.4a and b)**. Immunoprecipitation of over-expressed plasmids harboring these mutations revealed that these mutant proteins can still weakly bind to BRCA2 although to different degrees **(Figure 3.4 e and f)**. This result suggests that the three WD40 domain VUS are introducing amino acid alterations detrimental to the WD40 domain folding, leading to impaired BRCA2 binding. Unlike the N-terminal VUS that mostly affects the BRCA1-PALB2 interaction without affecting protein expression **(Figure 3.4a)**, the three WD40 domain VUS that binds weakly to BRCA2 are also unstable and subsequently destabilized.

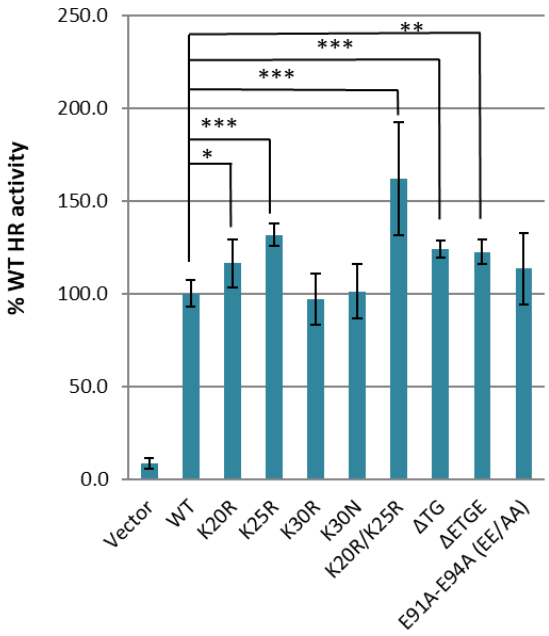

Figure 3.5: Effects of the PALB2 N-terminal coiled-coil domain putative ubiquitylation on HR activity

U2OS/DR-GFP cells were first depleted of the endogenous PALB2 by an siRNA and then rescued with the wt and variant PALB2 proteins by transient transfection. Standard deviations were shown. Unpaired T-test were used to calculate statistical significance.

Orthwein et al (2015) proposed that KEAP1-mediated ubiquitylation of PALB2 N-terminal Lysine residues (K20, K25 and K30) is critical for regulation of the PALB2-BRCA1 interaction in a cell cycle dependent manner. Mutations of K20/25/K30 together to arginine (R) lead to BRCA1–PALB2–BRCA2 complex assembly in G1 cells[250]. Increased BRCA1-PALB2 interaction were observed when the ETGE KEAP1-binding motif of PALB2 were mutated or deleted [38, 318]. Although the sidechain of K25 forms an intrachain salt bridge with Glu27 (E27), none of the aforementioned ubiquitylation sites are important for PALB2 homodimerization[322]. We observed modest but statistically significant increase in HR activity for PALB2 harboring a mutation in K20R, K30R or small deletions in the PALB2 ETGE motif responsible for its KEAP1 interaction **(Figure 3.5)**. Contrary to published results by Orthwein et al, K30 does not appear to be a critical residue with K30R artificial mutation and K30N patient-derived VUS showing similar to WT HR activity. K20R/K25R double mutation greatly elevated HR activity, further confirming the importance of these residues in PALB2-mediated HR. The effects of these Lysine to Arginine modifications on PALB2-BRCA1 interaction and cellular drug resistance remains to be determined.

Chapter 4

Roles of BRCA1 phosphorylation on its tumor suppressor function

4.1 Abstract

DNA double stand breaks (DSBs) are the most hazardous form of DNA damage which, if unrepaired or misrepaired, can lead to cell death or genome instability that drives tumorigenesis. The breast cancer tumor suppressors BRCA1 and BRCA2 play essential roles in cellular response to DSBs by enabling homologous recombination (HR)-mediated DSB repair and cell cycle checkpoints. The two BRCA proteins are physically and functionally linked by a third tumor suppressor protein, PALB2 (partner and localizer of BRCA2). Interestingly, PALB2/BRCA2 binds to a region in BRCA1 that contains 14 serine/threonine residues that can be phosphorylated by ATM (Ataxia telangiectasia mutated) and/or ATR (Ataxia telangiectasia and Rad3 related). ATM and ATR are master kinases pivotal in orchestrating DNA repair and cell cycle checkpoints after DNA damage and DNA replication stress. However, functional importance of previously identified ATM/ATR phosphorylation sites in BRCA1 (S1387, S1423, S1457, and S1524) remains unclear. Here, we report that most BRCA1 phosphorylation sites are not critical for its function in supporting HR, suppression of single strand annealing (SSA) and conferring resistance to either cisplatin or olaparib when mutated individually. Combined mutation of S1387 and S1423 (S1387A/S1423A) led to moderately reduced resistance to cisplatin and olaparib without disruption of BRCA1 HR function and PALB2 binding. Mutation of either S1457 or S1524 in combination with S1387A/S1423A partially restored cellular resistance to cisplatin and olaparib but not when mutated together (S1387A/S1423A/S1457A/S1524A). Importantly, artificial and patient-derived mutations of T1394, a largely uncharacterized site immediately upstream of the PALB2 binding motif, significantly impaired BRCA1 function in supporting HR, suppressing SSA and conferring drug resistance. Yet, these T1394 mutations do not affect PALB2 binding. A phosphospecific antibody generated against pT1394 confirmed that T1394 is indeed phosphorylated upon DNA damage in a ATM/ATR dependent manner. Further studies are

necessary to establish if BRCA1 T1394 phosphorylation can affect repair protein dynamics in the event of DNA damage. Overall, insights from this study can be used to better understand the complex relationship between BRCA1 phosphorylation and maintenance of genome stability.

4.2 Introduction

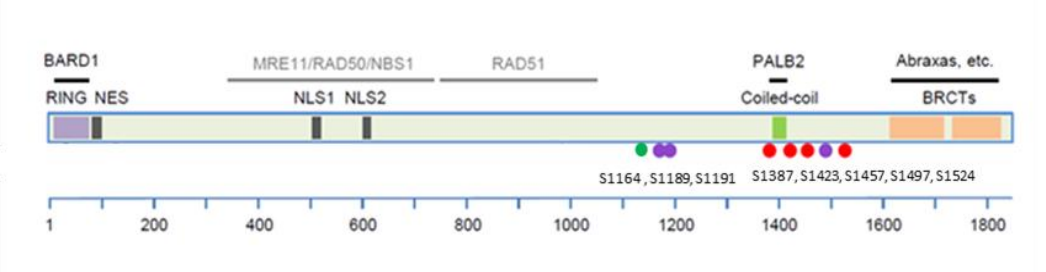

Figure 4. 1: General overview of BRCA1 phosphorylation within the Serine Cluster Domain.

PLK1 phosphorylation site (S1164) is represented as green circle.
CDK phosphorylation sites (S1189, S1191 & S1497) are represented as purple circle.
ATM/ATR phosphorylation sites (S1387,S1423,S1457,S1524) are represented as red circle.
Biological functions of these phosphorylation sites are summarized in **Table 4.1.**

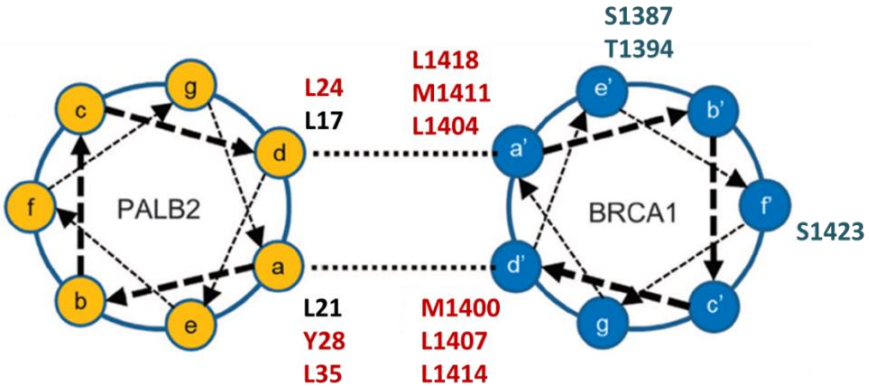

Figure 4.2: The BRCA1-PALB2 coiled-coil domain interaction is hydrophobic

Predicted model of the interaction between the coiled-coil motifs of PALB2 and BRCA1. Hydrophobic residues at the interface are shown. Residues affected by VUSs that may potentially affect the binding of the interaction are not shown. The two proteins are shown in red. Polarly charged residues such as S1387, T1394 and S1423 are positioned away from the hydrophobic interface. Potential ionic salt bridges stabilizing the two coiled-coil domain interaction is not shown.

Phosphorylation of BRCA1 by different kinases has been implicated in a variety of its tumor suppressor functions such as cell cycle checkpoint regulation, BRCA1-mediated transcription regulation, recruitment of downstream DNA repair effector proteins etc. BRCA1 was initially observed to be phosphorylated mostly in S-phase upon DNA damage[303]. Interestingly, the PALB2-BRCA1 binding domain of BRCA1 is positioned within “SQ cluster region” (SQR) that contains over a dozen “SQ” sites that are targets for ATM/ATR-mediated phosphorylation **(Figure 4.1- 4.3)**. Among these sites, S1387, S1423, S1457 and S1524 have been subjected to considerable amount of study [20, 68, 226, 383]. Although additional ATM/ATR phosphorylation sites flanking the coiled-coil domain such as S1239, S1245,S1330,S1336, S1342, S1466 and S1542 have also been found to be phosphorylated, an in vitro kinase assay suggested that the key phosphorylation sites at this region are limited to S1387,T1394,S1423 and S1457 only [342].

Similar to BRCA1, several 53BP1 phosphorylation sites have been detected via either mass spectrometry or use of 53BP1 phosphospecific antibodies[168]. Site-directed mutagenesis of 53BP1 at a combination of at least 15 phosphosites (PI3-like kinase; S/TPQ motifs) in the N terminus of 53BP1 were observed to disrupt its interaction with RIF1 and affect 53BP1 function[45, 58, 85, 102, 405] . However, mutating all putative phosphorylation sites in a single protein may disrupt protein folding. Structural information on large proteins such as BRCA1 and 53BP1 is limited only to specific functional domains which is not where the phosphorylation sites are located [26, 34, 65, 153, 166, 355, 369, 370, 372, 377]. It is possible that phosphorylation of BRCA1 S/TQ sites can differentially regulate the binding of BRCA1 to its interacting partners and ultimately places BRCA1 as a core signaling adaptor in regulating DDR. Characterization of this S/TQ cluster may therefore provide us with a clear understanding of how BRCA1 function is tightly regulated by protein kinases in the event of DNA damage. For example, it was reported that phosphoserine

1423 (pS1423) is important for protein-protein interactions between BRCA1 and BCL2-associated transcription factor1 (BCLAF1) for pre-mRNA splicing of DDR genes [298].Phosphorylation of S1423 and S1524 were also reported to be important for binding of X-linked inhibitor of apoptosis protein (XIAP) to promote Caspase3 activation and subsequently apoptosis in cells experiencing extensive DNA damage [222].

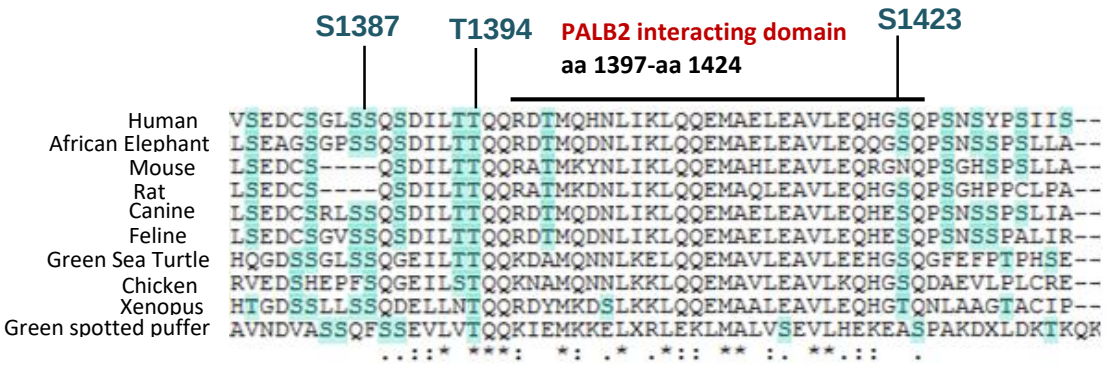

Figure 4.3: S1387 and T1394 are positioned upstream of the coiled-coil domain, whereas S1423 are positioned within the coiled-coil domain.

Among the three phosphorylation sites, T1394 is the most conserved residue and exist in lower organisms such as xenopus and pufferfish (no BRCA1 orthologs have been identified in the zebrafish genome despite the presence of PALB2 and BRCA2 gene [145]) with some degrees of flexibility in the relative position of S1387 and S1423 phosphorylation site.

Loss of BRCA1 in cells was observed to result in a dysregulated S-phase checkpoint, the G2/M checkpoint, the spindle checkpoint and centrosome duplication[82]. However, loss of BRCA1 function also leads to elevated DNA damage that would inevitably activate cell cycle checkpoints to regulate cell proliferation and induce apoptosis when damaged lesions are beyond repair. Phosphorylation of specific residues such as S1423 was discovered to be critical in maintaining the G2/M checkpoint but not the ionizing irradiation-induced S-phase checkpoint of BRCA1 [382].Phosphorylation of S1387, on the other hand, was reported to be important for radiation induced S-phase arrest but not the G2/M checkpoint [383]. It was proposed that effective recruitment of BRCA1 to a chromatin

region flanking DNA double stranded breaks (DSB) is important for S1387 and S1423 phosphorylation [122]. Interestingly, both S1387 and S1423 are conserved SQ sites flanking the PALB2-binding coiled-coil domain with a previously uncharacterized T1394 site positioned even closer to the PALB2 binding region.

In addition to the SQR, it has been reported that CDK1 mediated phosphorylation of BRCA1 at S1189, S1191 and S1497 promotes BRCA1 focus formation and DNA repair activity, consistent with G1/S-specific increase in BRCA1 phosphorylation [163, 290]. Interestingly, Kim et al reported delayed mammary gland development, aging-like phenotype and increased hypersensitivity to high dose of γ-irradiation in a mouse model of *Brca1* $^{S1152A/S1152A}$ (corresponding to human S1189) [179]. Moreover, homozygous *Brca1* $^{S1152A/S1152A}$ mice also exhibited significantly increased incidence of tumor formation following sublethal dose of γ-irradiation. Tumors developed by the mice were either differentiated mammary adenocarcinomas cyclin D1+/ ER-α+/ *Trp53*- or ER-α- less differentiated adenocarcinomas [179]. MDA-MB-436 cells reconstituted with a triple CDK phosphorylation mutant (S1189A/S1191A/S1497A) were reported to be more sensitive towards both cisplatin and rucaparib due to reduced BRCA1 foci formation upon DNA damage [163, 164]. CDK1 is also critical for phosphorylation of BRCA1 interacting partners such as CtIP at residue S326 for the BRCA1-CTIP interaction via the BRCA1 C-terminal BRCT domain, as well as T847 for CtIP's nuclease activity [150].BRCT point mutations of BRCA1 is deleterious and is associated with loss of tumor suppression [307]. Nevertheless, mouse model of CTIP-S326A that is unable to bind to BRCA1 has confirmed that the BRCA1-CtIP interaction is not required for viability, DNA resection for HDR-mediated DSB repair or tumor suppression [268, 282]. As RPA accumulation was not affected in S1189A/S1191A/S1497A expressing cells [163], it would be important to question if the previously observed reduced BRCA1 foci corresponds to HR function of BRCA1.

PLK1-mediated phosphorylation of at S1164 BRCA1 was also recently reported to be critical for formation of BRCA1 foci after DNA damage [55]. Mutation of S1164 on BRCA1 impairs formation of BRCA1 foci after DNA damage, mimicking the phenotype observed with PLK1 inhibition or depletion. Such an observation is intriguing as activation of PLK1 is required for proper entry into mitosis, which does not favor DSB repair, as it might result in increased sister telomere fusions, resulting in aneuploidy [249]. Loss of PALB2 and BRCA2 was observed to result in activated PLK1 and premature entry into mitosis [230] . Work on BRCA1's function during mitosis is poorly understood but microscopy imaging of mitotic chromosomes alluded to the possible functions of BRCA1 and its interacting partner CtIP in promoting the joining of de-protected telomeres via alternative NHEJ [16],a process that is undesirable as it will lead to genome instability.

Phase I/II clinical trials on DDR-related kinase inhibitors, particularly ATM/ATR inhibitors, as potential chemo- and radio- sensitizers in a variety of cancer are ongoing **(table 4.2)**, and there have been exciting reports of synergistic killing of cancer cells with DNA damaging chemotherapy [167, 190, 264, 281, 340].Chemical inhibition of DDR–related kinases such as ATM/ATR has also been insightful in understanding potentially novel methods to further target BRCA-related tumors that are HR deficient. Although ATM is generally required for the early stages of HR, it is interesting to note multiple ATM-defective mouse models suggests that HR activity is not severely impaired in the absence of ATM function[173, 278]. Yet, ATM loss or inhibition is synthetically lethal with the *Brca1*^{S1598F} knockin mouse model with severely reduced HDR that can still be rescued with *53bp1* deletion[59]. Huntoon et al (2013) observed that ATR inhibition via VE-821 in BRCA1-deficient ovarian cancer cells can further sensitize these cells to DNA damaging agents such as cisplatin, topotecan, and veliparib beyond the already potent sensitization caused by defective HR[151]. HR-defective tumors accumulate greater amounts of genomic instability that subsequently leads to stronger dependency on replication checkpoint signaling, that can

then be targeted via ATR inhibition[189]. Moreover, chemical inhibition of ATR activity not only affects RAD51 loading to DSBs but also caused stalled replication forks[389].

Because S/TQ rich sites are positioned flanking the PALB2 binding domain of BRCA1**(Figure 4.3)**, we are interested in examining the effects of BRCA1 phosphorylation on PALB2 binding and whether mutations on these phosphorylation sites would impair PALB2 binding and subsequently BRCA1 function. It is also noteworthy that some of these phosphorylation sites such as S1387, S1423 and S1524 have been shown to be significant in the context of cell cycle progression in the event of DNA insults. Whether single or combination of phosphorylation events at these residues can impact BRCA1 functions remains poorly understood. Phosphorylation of the S/TQ cluster may play a significant role in balancing the different cellular signals for DNA repair, cell cycle checkpoint arrest and ultimately cell survivability. Therefore, it will be interesting to determine the effects of PALB2 binding on the phosphorylation status of specific S/TQ residues and ask if they are essential for PALB2-BRCA1 interaction during DNA damage. Since phosphorylated BRCA1 is important for proper cell cycle control, we expect that multiple phosphorylation events may be necessary for BRCA1 to both transduce and maintain checkpoint activation signals especially during DNA damage. It is also crucial for us to better understand the effects of PALB2 binding on BRCA1 function as the large PALB2-BRCA2 complex may mask the S/TQ sites of BRCA1, thereby limiting accessibility of kinases such as ATM/ATR. Hence, a clear understanding of BRCA1 phosphorylation status and the PALB2-BRCA1 complex assembly dynamics is greatly insightful in the context of DDR and its implications during tumorigenesis. Despite the extensive study of BRCA1 phosphorylation, functional roles of BRCA1 SQR in the context of HR activity, SSA suppression and drug sensitivity remain poorly understood.

Residue	Kinases involved	Detectable via Mass spectrometry (MS) analysis	Biological Significance if applicable	Reference
S1164	PLK	248, 308	PLK1 phosphorylates BRCA1 mainly on S1164 with mutation of PLK1 sites on BRCA1 impairs formation of BRCA1 foci after DNA damage	55
S1189, S1191	CDK1	226, 248, 308	Together with S1497 phosphorylation resulted S-phase DNA Damage checkpoint and formation of BRCA1-containing foci	68, 163
S1387	ATR ATM	N/A	ionizing irradiation-induced S-phase arrest	68, 119, 342, 383
T1394	ATR ATM	N/A	None reported	342
S1423	ATR ATM	68	G2-M checkpoint Phosphorylation required for XIAP for anti-apoptosis BCLAF1 binding for mRNA splicing	68, 119, 222, 298, 342, 383
S1457	ATR ATM	68	Not reported but identified in multiple reports of ATM-mediated BRCA1 phosphorylation	68, 119
S1497	CDK1 CDK2	175, 308	Together with S1189/S1191 phosphorylation resulted S-phase DNA Damage checkpoint and formation of BRCA1-containing foci	163, 226, 290
S1524	ATM	68, 226, 248, 308	Phosphorylation in combination with S1423 is required for XIAP binding	

Table 4.1: A brief summary of BRCA1 SQR phosphorylation sites and their reported biological roles

Clinical trial #	Inhibitor	Kinase	Sponsor	Phase	Combination therapy	Conditions
NCT02588105	**AZD0156**	**ATM**	AstraZeneca	I	Olaparib	Advanced Solid Tumours
NCT02157792	**VX-970**	**ATR**	EMD Serono Research & Development Institute	I	Gemcitabine/Cisplatin/ Etoposide/Carboplatin	Advanced Solid Tumor
NCT02589522	**VX-970**	**ATR**	NCI	I	Whole Brain Radiation Therapy	Brain Metastases from Non-small Cell Lung Cancer, Small Cell Lung Cancer, or Neuroendocrine Tumors
NCT02595931	**VX-970**	**ATR**	NCI	I	Irinotecan	Metastatic/Unresectable solid tumors
NCT02723864	**VX-970**	**ATR**	NCI	I	Cisplatin + veliparib	Advanced refractory solid tumors
NCT02567409	**VX-970**	**ATR**	NCI	I/II	Cisplatin and Gemcitabine	Recurrent and Metastatic Ovarian, Primary Peritoneal, or Fallopian Tube Cancer
NCT02487095	**VX-970**	**ATR**	NCI	I/II	Topotecan	Small / Non-Small -Cell Lung Carcinoma Ovarian Neoplasms ; Uterine Cervical Neoplasms Carcinoma, Neuroendocrine
NCT02627443	**VX-970**	**ATR**	NCI	I/II	Carboplatin and Gemcitabine	Recurrent and Metastatic Ovarian, Primary Peritoneal, or Fallopian Tube Cancer
NCT02595892	**VX-970**	**ATR**	NCI	II	Gemcitabine Hydrochloride	Recurrent Ovarian, Primary Peritoneal, or Fallopian Tube Cancer
NCT02567409	**VX-970**	**ATR**	NCI	II	Cisplatin and Gemcitabine	Metastatic Urothelial Cancer
NCT02567422	**VX-970**	**ATR**	NCI	I	Cisplatin and radiation therapy	Locally Advanced HPV-Negative Head and Neck Squamous Cell Carcinoma
NCT03309150	**VX-970**	**ATR**	Merck KGaA	I	Carboplatin /Paclitaxel	Advanced Stage Solid Tumors
NCT03328273	**AZD6738**	**ATR**	Acerta Pharma BV	I/II	Acalabrutinib	Chronic Lymphocytic Leukemia
NCT02223923	**AZD6738**	**ATR**	Royal Marsden NHS Foundation Trust	I	Palliative radiotherapy	Solid Tumour Refractory to Conventional Treatment

NCT03330847	**AZD6738**	**ATR**	AstraZeneca	II	Olaparib	Metastatic Triple Negative Breast Cancer
NCT02264678	**AZD6738**	**ATR**	AstraZeneca	I/II	Carboplatin /olaparib / MEDI4736 (Durvalumab)	Adv Solid Malig - H&N SCC, ATM Pro / Def NSCLC, Gastric & Breast Cancer
NCT03334617	**AZD6738**	**ATR**	AstraZeneca	II	Durvalumab	anti-PD-1/PD-L1 progressed Metastatic non-small cell lung cancer (NSCLC)
NCT01955668	**AZD6738**	**ATR**	Samsung Medical Center	I	Monotherapy	Relapsed/refractory CLL, PLL or B cell lymphoma.
NCT02630199	**AZD6738**	**ATR**	AstraZeneca	I	Paclitaxel	Refractory Cancer
NCT03022409	**AZD6738**	**ATR**	AstraZeneca	I	Olaparib	Head and Neck Squamous Cell Carcinoma
NCT03188965	**BAY1895344**	**ATR**	Bayer	I	N/A; radium-223 dichloride	Advanced solid tumors and lymphomas; castration-resistant prostate cancer (CRPC)

Table 4.2: Current clinical trials on ATM/ATR inhibitors are focused on chemical inhibition of ATM/ATR in combination with DNA damaging agents for treatment of advanced tumors (https://clinicaltrials.gov) ; Accessed 8.22.2018

This chapter focuses on the following aims

1. To determine if the loss of PALB2 affects BRCA1 SQR phosphorylation
2. To understand the functional roles of protein kinases (ATM/ATR, CDK & PLK1) in regulating BRCA1 function by introducing serine to alanine in previously reported BRCA1 phosphorylation sites (Table 4. 1).
3. To investigate the biological significance of BRCA1 phosphorylation in HR, SSA suppression and drug sensitivity
4. To determine the biological relevance of a previously uncharacterized putative phosphorylation site of BRCA1 (T1394)
5. To understand the clinical relevance of patient-derived mutations that may potentially affect BRCA1 phosphorylation and its functions.

Results

4.3.1 Phosphorylation of S1423 and S1524 is induced upon DNA damage

BRCA1 is constitutively phosphorylated *in vivo* even in the absence of DNA damage and the phosphorylation can be removed by addition of λ-phosphatases **(Figure 4.4a lane 1, 2)**. In the presence of DNA damage such as 10Gy IR, we can observe greater induction of phospho-BRCA1 **(Figure 4.4a, lane 3)**. We first focused specifically on the effects of S1423 (pS1423) and S1524 phosphorylation (pS1524) following various DNA damaging insults. S1423 phosphorylation is of interest to us as opposed to pS1387 because pS1387 is implicated in the intra-S checkpoint while pS1423 was reported to be functionally important for the G2/M checkpoint. PALB2 has been implicated in regulating G2/M checkpoint with loss of PALB2 leading to impaired G2/M checkpoint activation and maintenance [230]. Phosphorylation of S1524 has consistently been detected via mass spectrometry analysis although it is not known if this phosphorylation is constitutively present in the cell and if the phosphorylation has any significant biological consequences. We first compared S1423 and S1524 phosphorylation of endogenous BRCA1 in the presence of various DNA damaging chemotherapeutic drugs. We observed that phosphorylation of both S1423 and S1524 are significantly elevated after treatment with commonly used DNA damaging agents such as Hydroxyurea (HU), Cisplatin, Mitomycin C (MMC) and Bleomycin. HU treatment showed the strongest induction of phosphorylation at both sites in a concentration- and time-dependent manner **(Figure 4.4 b,c)**. We also tested the induction of phosphorylation at these sites following chronic DNA damage (16hr treatment of DR-U2OS with either 2mM HU or 1μM MMC) in the presence of ATM/ATR inhibition, to test if there is a compensatory relationship between ATM or ATR in modulating these residues.

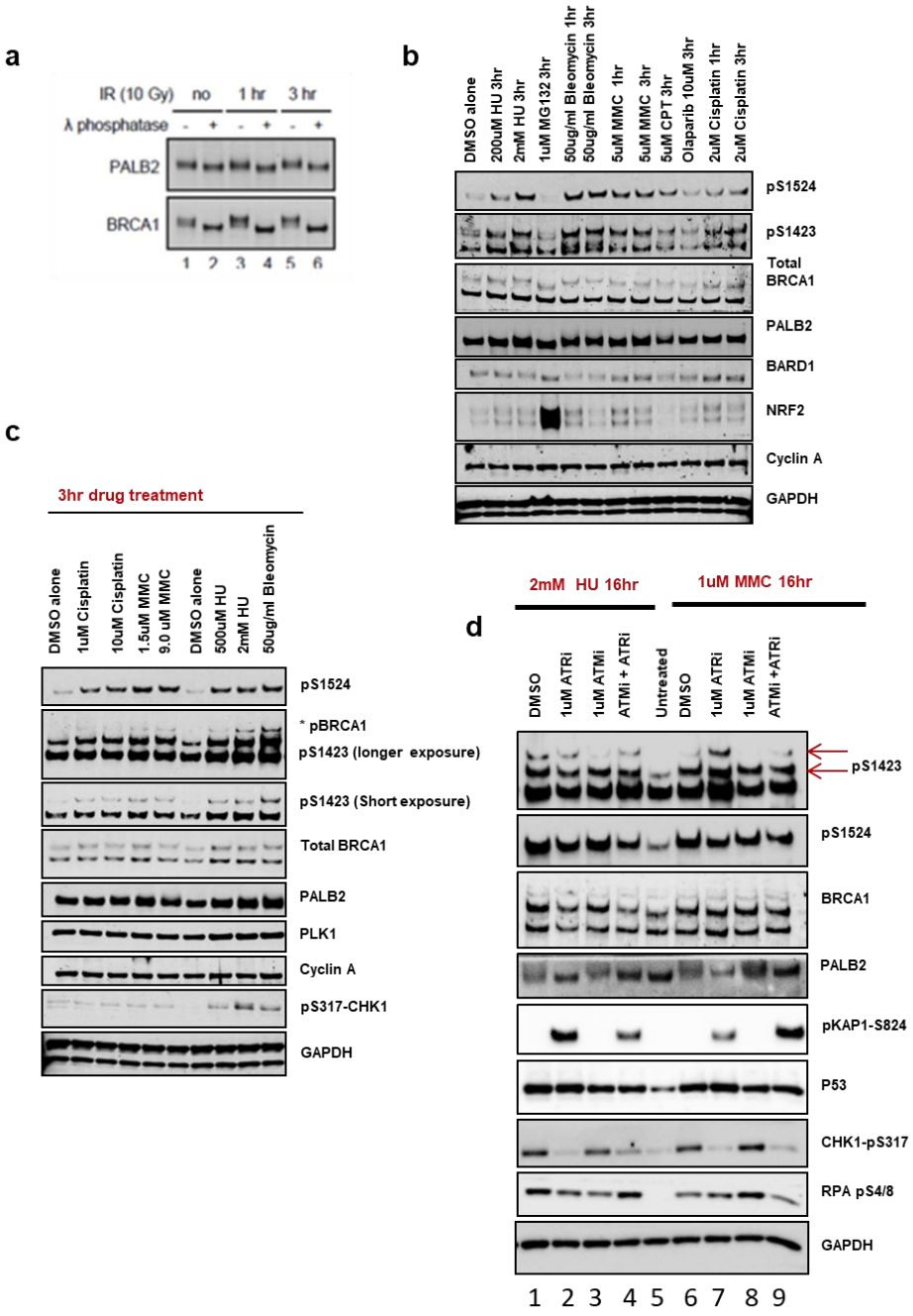

Figure 4.4: BRCA1 pS1423 and pS1524 are induced following DNA damage in a PIKK kinase dependent manner

a) BRCA1 is constitutively phosphorylated even in the absence of DNA damage with a further induction of BRCA1 phosphorylation following 10Gy IR. B-c) DR-U2OS treated with different DNA damaging agents.BRCA1 pS1423 and pS1524 are induced by DNA damaging agents (cisplatin, MMC, HU and Bleomycin). c) DR-U2OS cells treated with either 2mM HU or 1uM MMC overnight (16hr) in the presence of DMSO control, ATM inhibitor (1uM KU-55933), ATR inhibitor (1uM VE-821) or both. Inhibitors are added 15minutes before addition of HU or MMC.

Hydroxyurea (HU) promotes DNA damage as it inhibits ribonucleotide reductase that catalyzes the rate-limiting step in the biosynthesis of all four precursors for DNA replication, thereby resulting in depletion of the dNTP pool and subsequently replication fork arrest in actively replicating cells[320, 386]. Overnight treatment of 2mM HU induced a strong activation of pS1423 (top and bottom arrow) and pS1524 together with hyper-phosphorylated PALB2, induced CHK1-pS317 and RPA-pS4/S8 **(Figure 4.4d, compare lane 1 with lane 5)**. Inhibition of ATR activity (1μM of VE-821 treatment overnight), however, slightly reduces hyper-phosphorylated BRCA1-pS1423 (red arrows) with reduced PALB2 phosphorylation, CHK1-pS317 and RPA-pS4/S8 **(Figure 4.4d lane 2)**. KAP1 is a heterochromatin protein that is involved in the relaxation of the chromatin surrounding DNA damage region and is phosphorylated mostly by ATM at S824 for its activity[157, 365, 366]. Increased pKAP1 after ATR inhibition is consistent with previous observations that in the absence of proper ATR signaling when cells are challenged with replication stress, ATM activation becomes more predominant due to extensive DSBs being generated [348]. Treatment of the cells with ATM inhibitor, however, reduces hyper-phosphorylated BRCA1-pS1423, reduced RPA-S4/S8 but do not affect BRCA1-pS1524, PALB2 and CHK1-S317 phosphorylation **(lane 3)**. The drastically-reduced PALB2 phosphorylation in the absence of ATR activity but not ATM, suggests that PALB2 is mostly phosphorylated by ATR consistent with previous mass spectrometric analysis[226]. Interestingly, inhibition of both ATM and ATR only slightly reduces hyper-phosphorylated BRCA1-pS1423 **(lane 4)**. BRCA1-pS1524 appears to be lower in both cases when cells were treated with ATRi, whether this is due to a reduced BRCA1 amount remains uncertain. Reduced PALB2 phosphorylation and CHK1-pS317 with a slightly increased pKAP1, indicates that the ATR inhibition indeed was accomplished in the combination. KAP1 phosphorylation can still be mediated by other members of the PIKK family such as DNA-PK in the absence of ATM/ATR phosphorylation[44, 366]. P53 induction, a known target

of ATM is slightly impaired in cells treated with ATM inhibitor. Since RPA-pS4/S8 is similar to DMSO only control, it is possible that the inhibition of both ATM and ATR leads to a compensatory activation of DNA-PK in phosphorylating BRCA1-pS1423 but not BRCA1-pS1524.

Mitomycin C (MMC) can alkylate DNA, leading to its role as a known DNA interstrand crosslinker that also have other cytotoxic modes of actions such as redox cycling and inhibition of rRNA[81, 259, 349]. Overnight treatment of cells with 1μM MMC, on the other hand, produced results different from HU treatment. Treatment with MMC alone produced a similar result as HU, with hyper-phosphorylated BRCA1-pS1423/pS1524, phospho-PALB2, induced CHK1-pS317 and RPA-pS4/S8 **(Figure 4.4 lane 7)**. Inhibition of ATR activity, however, reduces hyper-phosphorylated PALB2 and BRCA1-pS1524, CHK1-pS317. Yet, unlike HU treatment, overnight treatment of MMC in combination with ATRi induced even more reduces hyper-phosphorylated BRCA1-pS1423, with a slight induction of pKAP1 **(lane 7)**.Inhibition of ATM, on the other hand, reduces hyper-phosphorylated BRCA1-pS1423 and p53 induction with a surprising increase in CHK1-pS317 and RPA-pS4/S8.Combined inhibition of ATM/ATR in the presence of MMC restored hyper-phosphorylated BRCA1-pS1423 and increased pKAP1 with a concomitant reduction in CHK1-pS317 and RPA-pS4/S8.Such an observation remains puzzling as we would expect a compensatory role by DNA-PK when both ATM/ATR activities are inhibited. Based on the results mentioned above, it is likely that BRCA1-pS1423 can be phosphorylated by all three members of the PIKK family of kinases with a stronger preference for ATM; BRCA1-pS1524 and PALB2 phosphorylation appears to be mostly ATR driven. Induction of pS1189/pS1191/pS1497, pS1164, pS1387 and pS1457 were not determined in this study due to limited access to reliable phosphospecific antibodies.

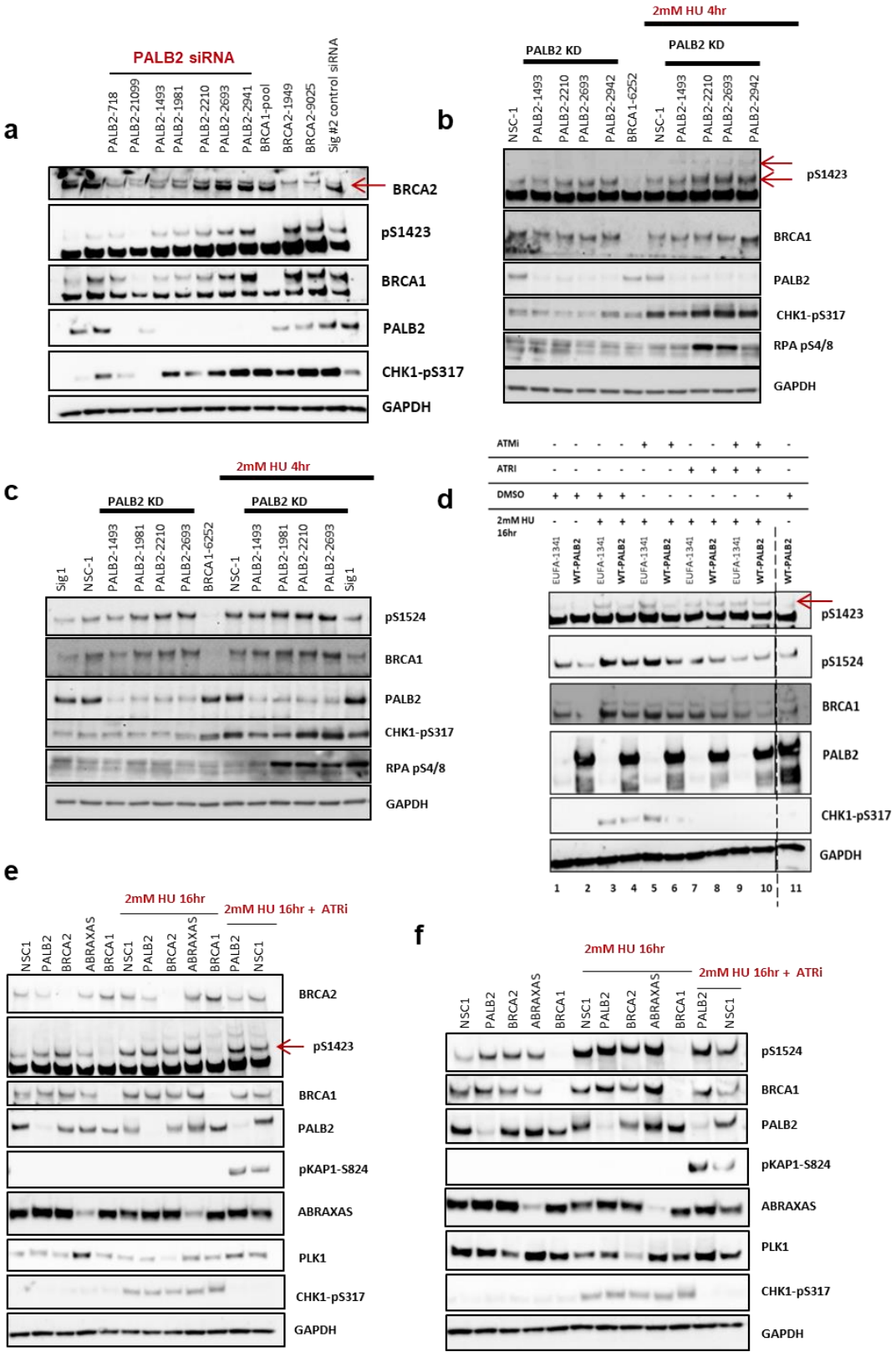
a
PALB2 siRNA
PALB2-718
PALB2-21099
PALB2-1493
PALB2-1981
PALB2-2210
PALB2-2693
PALB2-2941
BRCA1-pool
BRCA2-1949
BRCA2-9025
Sig #2 control siRNA
BRCA2
pS1423
BRCA1
PALB2
CHK1-pS317
GAPDH
b
2mM HU 4hr
PALB2 KD
PALB2 KD
NSC-1
PALB2-1493
PALB2-2210
PALB2-2693
PALB2-2942
BRCA1-6252
NSC-1
PALB2-1493
PALB2-2210
PALB2-2693
PALB2-2942
pS1423
BRCA1
PALB2
CHK1-pS317
RPA pS4/8
GAPDH
c
2mM HU 4hr
PALB2 KD
PALB2 KD
Sig1
NSC-1
PALB2-1493
PALB2-1981
PALB2-2210
PALB2-2693
BRCA1-6252
NSC-1
PALB2-1493
PALB2-1981
PALB2-2210
PALB2-2693
Sig1
pS1524
BRCA1
PALB2
CHK1-pS317
RPA pS4/8
GAPDH
d
ATMi
ATRi
DMSO
2mM HU 16hr
EUFA-1341
WT-PALB2
pS1423
pS1524
BRCA1
PALB2
CHK1-pS317
GAPDH
1 2 3 4 5 6 7 8 9 10 11
e
2mM HU 16hr
2mM HU 16hr + ATRi
NSC1
PALB2
BRCA2
ABRAXAS
BRCA1
BRCA2
pS1423
BRCA1
PALB2
pKAP1-S824
ABRAXAS
PLK1
CHK1-pS317
GAPDH
f
2mM HU 16hr
2mM HU 16hr + ATRi
NSC1
PALB2
BRCA2
ABRAXAS
BRCA1
pS1524
BRCA1
PALB2
pKAP1-S824
ABRAXAS
PLK1
CHK1-pS317
GAPDH

Figure 4.5: pS1423 and pS1524 are induced following siRNA mediated knockdown of PALB2/BRCA2.

(a)Individual siRNA knockdown of PALB2 and BRCA2 in DRU2OS cells. Sig1, Sig2 and NSC-1 were used as control siRNA with pooled BRCA1 siRNA used as negative control for BRCA1.
(b) Individual siRNA knockdown of PALB2 in DRU2OS cells with or without 2mM HU treatment for 4hr leads to slight induction of pS1423 and (c) pS1524
(d) EUFA1341 cells lacking a functional PALB2 showed elevated pS1423 and pS1524 than cells reconstituted with WT PALB2 following overnight treatment of 2Mm HU
(e) pooled siRNA knockdown of PALB2, BRCA2 and ABRAXAS treated with or without overnight treatment of 2mM HU. An additional set of NSC1 and PALB2 siRNA knockdown were also treated with 1uM ATRi before HU treatment.

4.3.2 pS1423 and pS1524 are induced following loss of PALB2

We and others have previously observed that in the absence of BRCA1, phosphorylation of specific residues surrounding the PALB2 coiled-coil domain is impaired following DNA damage [38, 128]. Considering that pS1423 is inducible following DNA damage, it is critical for us to investigate if the binding of PALB2 to BRCA1 is suppressing phosphorylation of any of these residues surrounding the coiled-coil domain. We also question if the BRCA1-PALB2 interaction is disrupted in the event of S/TQ phosphorylation upon different forms of DNA damage (IR, and replication stress) and whether the specific phosphorylation events are regulated by PALB2 binding. If PALB2 is truly limiting the access of kinases involved in S1423 and S1524 phosphorylation, we would expect to see increased S1423 and S1524 phosphorylation in the absence of PALB2.

We first tested if loss of PALB2 will either promote or impair the induction of BRCA1 phosphorylation of any of these residues before and after DNA damage **(Figure 4.5a)**. Interestingly, individual siRNA knockdown of PALB2 resulted in a slight increase of both pS1423 and pS1524. 5 out of the 7 PALB2 siRNA (PALB2 siRNA #2941 not considered due to increased BRCA1 expression) tested showed increased pS1423. Although the PALB2 siRNA knockdown efficiency is relatively good, only 4 out of 7 siPALB2 oligos used in this experiment resulted in reduced levels of cellular BRCA2. Evidently, siRNA

knockdown of BRCA2 that also resulted in slightly reduced PALB2 expression can also mildly induce pS1423. Elevated CHK1-pS317 levels were detected, indicating the activation of ATR signaling following the loss of key HR players such as BRCA1/PALB2/BRCA2. Taken together, we observed that individual siRNA knockdown of PALB2 or BRCA2 leads to increased pS1423 in a PALB2-dependent manner. A synergistic effect was also observed when the siRNA knockdown cells were treated with 2mM HU for 4hours; knock down of PALB2 can still further elevated both pS1423 and pS1524 even in the presence of HU-induced replication stress **(Figure 4.5b,c)**.

Because our siRNA knockdown of both PALB2 and BRCA2 caused slight induction in BRCA1 phosphorylation, we tested if the observed increase in BRCA1-pS1423/pS1524 is a consequence of increased accessibility of ATM/ATR to phosphorylate BRCA1 at the region where PALB2 binds, or is instead caused by a direct effect of elevated DNA damage caused by impaired DDR. To address this, we tried to confirm our observations in the PALB2-deficient EUFA1341 cell line, a SV40-transformed skin fibroblast cell line derived from a Fanconi Anemia patient with biallelic mutations in *PALB2*. This cell line has a nonsense mutation (c.1802T>A, p.Y551*) on one allele and a loss of the other allele due to a genomic deletion, resulting in the expression of a truncated, unstable PALB2 protein[379] When exposed to chronic replication stress (2mM HU 16hr), it is apparent that EUFA1341 cells lacking PALB2 have a slightly stronger induction of BRCA1-pS1423 and pS1524 compared to cells reconstituted with wild type protein **(Figure 4.5d lane 3, 4)**. Inhibition of ATM in the presence of HU slightly increased BRCA1 pS1423 while pS1524 remains the same; inhibition of ATR in the presence of overnight HU treatment leads to overall reduced BRCA1 pS1423 and pS1524 **(Figure 4.5d Lane 7-10)**.

Our observation supports a model in which the BRCA1-PALB2 interaction to some extent limits BRCA1 phosphorylation. However, we cannot rule out the possibility that loss of PALB2 leads to elevated DNA damage, which in return activates ATM/ATR to further

phosphorylate BRCA1. A previous report using the ChIP assay to measure BRCA1 recruitment after IR suggest that RAP80-dependent recruitment of BRCA1 to chromatin flanking DNA breaks is required for BRCA1 phosphorylation at Serine 1387 and 1423 by ATM after 10Gy IR[122]. Since RAP80-ABRXAS in complex with BRCA1 is important for proper localization of the BRCA1-A complex to sites of damage, we also tested the effects of pooled siRNA knockdown of *Abraxas* on BRCA1-pS1423 **(Figure 4.5e)**. Interestingly, reduced ABRAXAS expression alone did not lead to induction of BRCA1-pS1423 as observed in the case of *Palb2* or *Brca2* knockdown. However, in the presence of HU-mediated DNA damage, *Abraxas* knockdown cells showed the greatest induction of BRCA1-pS1423 **(Figure 4.5e)**. This result suggests that impaired recruitment of BRCA1 to DSBs when ABRAXAS is depleted can lead to elevated DNA damage and further ATM/ATR mediated BRCA1 phosphorylation. Strangely, PLK1 levels were observed to increase in the Abraxas knockdown set. PLK1 levels are generally lower after HU treatment, most prominently in BRCA2 knockdown cells, suggesting the persistent HU-mediated DNA damage leads to increased G2/M checkpoint where PLK1 protein levels are reduced following severe DNA damage[19, 32]. Upon ATR inhibition in the presence of HU, we also noted a marked increase of BRCA1-pS1423 in PALB2 knockdown set, due to persistent DSB that stimulates ATM activity. In the case of BRCA1-pS1524, PALB2, BRCA2 and ABRAXAS knockdown is sufficient to induce this specific phosphorylation with further induction upon overnight treatment of HU **(Figure 4.5f)**. Overall, these observations validate the importance of the BRCA1-PALB2 interaction in regulating BRCA1 pS1423 and pS1524 that can be further induced in response to elevated DNA damage caused by impaired DNA repair.

4.3.3 Previously reported ATM/ATR-mediated BRCA1 SQR phosphorylation does not severely affect HDR or SSA suppression activity.

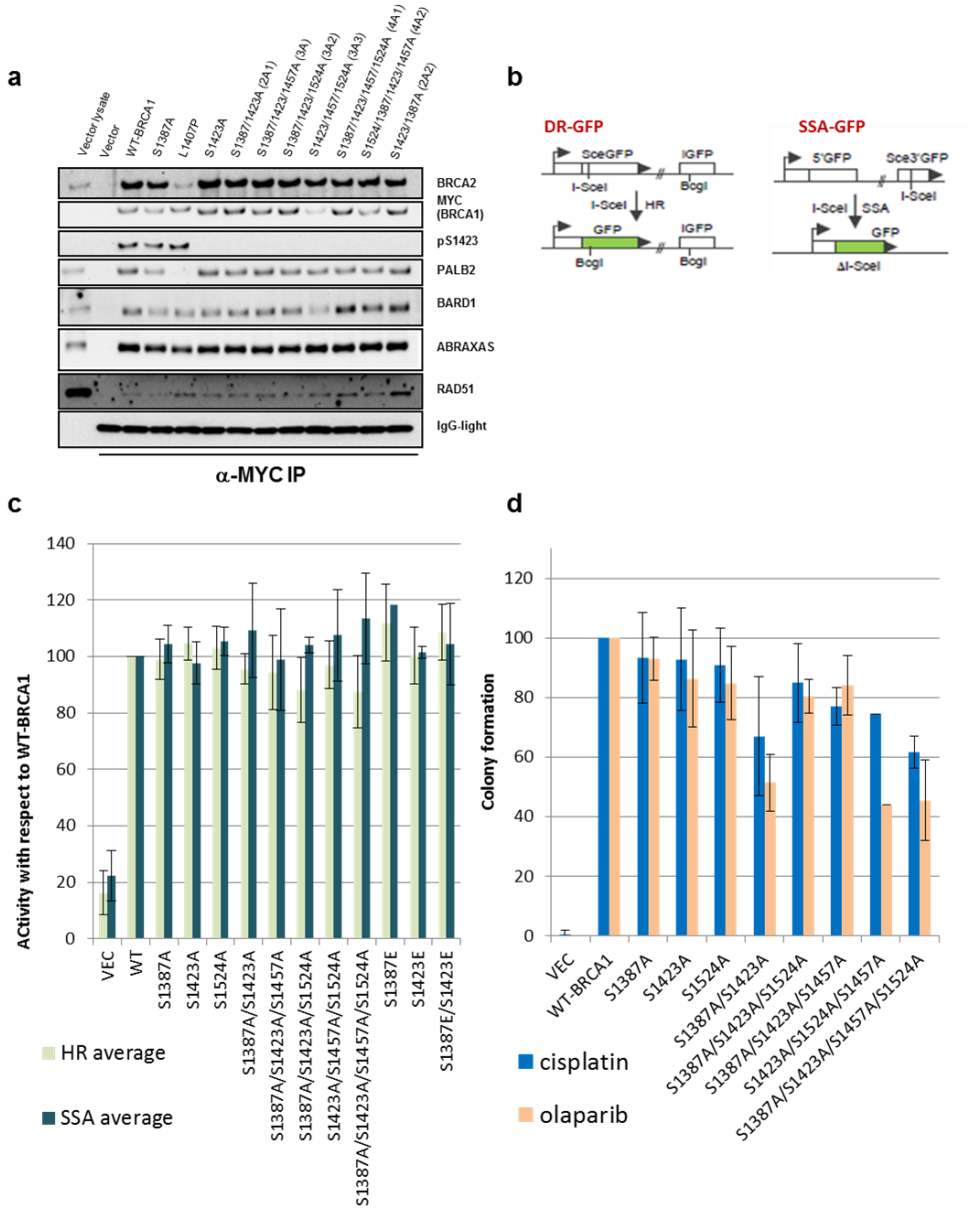

Figure 4. 6: BRCA1 phosphorylation at SQ sites S1387, S1423, S1457 and S1524 is dispensable for HR and SSA suppression, but may potentially affect sensitivity towards cisplatin and olaparib

(a)Plasmids harboring single and different combinations of BRCA1 SQR phosphomutants were transfected into 293T cells with the whole cell lysates later collected for immunopercpitation . (b) schematic diagram of the HR and SSA reporter assay used to assess BRCA1 phosphomutant functions (c) HR and SSA activity of BRCA1 SQR phosphomutant (d) Colony formation of MDA-MB-436 cells transfected with BRCA1 plasmids harboring the different phosphomutants and treated with either cisplatin or olaparib.

Due to BRCA1's crucial roles in DDR, targeting wild type BRCA1 function via use of kinase inhibitors may be potentially useful in sensitizing cancer cells towards DNA damage. Chemical inhibition of protein kinases such as ATM or ATR may introduce uncontrollable variables due to the broad spectrum of substrates that can be targeted by the PIKK kinase family, single amino acid substitution of S/TQ sites on BRCA1 will be a more direct and effective way of understanding the genetic interaction between ATM/ATR and BRCA1.

Previously, we observed that single Serine to Alanine (S to A) mutations at S1387, S1423 or S1524, which abolish phosphorylation of the specific residues, do not affect the BRCA1-PALB2 interaction[398]. Considering the dynamic nature of BRCA1 phosphorylation, it is important to determine if specific phosphorylation events contributes to BRCA1 function. Since both S1387 and S1423 are positioned flanking the coiled-coil domain, we first tested if simultaneously mutating both residues has any discernible effect on the BRCA1-PALB2 interaction. We also generated several additional combination mutations to test if differential phosphorylation of the two SQ residues in combination with constitutive identified phosphorylation sites such as S1457 or S1524 can affect PALB2 binding. Immunoprecipitation of exogenously expressed BRCA1 phosphomutants in 293T cells instead confirmed that abolishing currently known BRCA1 SQR phosphorylation sites does not affect its PALB2-binding **(Figure 4.6a)**. This observation is consistent with the nature of the BRCA1-PALB2 interaction that is mostly hydrophobic and confirms that most of the widely reported BRCA1 phosphorylation sites are not required for PALB2 binding. However, we cannot rule out if binding of PALB2 to phosphorylated BRCA1 can be affected in the case of phosphomimetic D/E mutants.

We and others have reported that patients derived mutations that impaired the PALB2-BRCA1 interaction resulted in loss of HR activity that is critical for cisplatin and olaparib resistance [11, 29, 331]. It has been reported that loss of BRCA2 increases SSA in

mouse cells [324]. We previously observed that BRCA1 depletion reduced the efficiency of both HR and SSA, whereas depletion of PALB2/BRCA2 led to severe loss of HR, but increased SSA[11]. Through the use of different patient derived mutations affecting the RING, coiled-coil and BRCT domain of BRCA1, we further established that BRCA1 interactions with BARD1 and BRCT-binding partners are both required for its function in the resection step of the DSB repair, whereas the BRCA1-PALB2 interaction functions downstream to guide ssDNA towards HR and reduce SSA[11].

Loss of PALB2-BRCA1 interaction also resulted in loss of Single-strand Annealing (SSA) suppression[11]. We did not observe much difference between WT-BRCA1 and the phosphoalanine mutations in the context of HR and SSA suppression activity **(Figure 4.6c)**. Our results are not in full agreement with a prior study that observed drastically reduced HR efficiency in ovarian cancer cells expressing S1387A/S1423A double mutant and BRCA1 S1387A/S1423A/S1457A/S1524A quadruple mutant (4SQA) [20]. Beckta et al (2015) also proposed that S1387A/S1423A is sufficient to redirect the preferred modes of DNA repair rom the “error-free” HDR pathway to the more error-prone NHEJ pathway. Hence, we looked at the activity of the BRCA1 phosphomutants in terms of their ability to suppress SSA, a deletion-causing and therefore mutagenic process that also utilizes resected DNA ends. Although, SSA suppression in S1387A/S1423A was observed to be weaker than wild type, such a difference is not significant. We also generated single S1387E, S1423E and a combination of both phosphomimetic (S1387E/S1423E) but did not observed any significant difference in HR and SSA suppression, indicative that the PALB2-BRCA1 interactions remains intact context of HR and SSA suppression activity **(Figure 4.6c)**.

4.3.4 Differential BRCA1 SQR phosphorylation may have implications on cellular sensitivity towards either cisplatin or olaparib

We next assessed the combinatorial effects of the different SQR phosphorylation on BRCA1-mediated resistance towards DNA damaging drugs such as cisplatin and olaparib. Since mutation of most single sites to alanine produced little to no effect on HR activity **(Figure 4.6c)**, the effects of single serine mutations on cisplatin and olaparib resistance was also modest **(Figure 4.6d)**. This result is consistent with previous reports by Xu et al (2002) that BRCA1 deficient HCC1937 cells expressing S1387A, S1423A, or S1423/1524A did not exhibit any significant difference in radiation sensitivity (0-6Gy) despite reported cell cycle checkpoint abrogation in these phosphomutant cells[382, 383]. Yet, Chang et al (2009) reported that S1423A/S1524A is still able to support *Brca1*-null mouse ES cell viability despite sensitivity to ionizing radiation[56].

In this study, combined mutations of S1387 and S1423 had little effect on HR activity but moderately reduced colony formation in the presence of either cisplatin or olaparib **(Figure 4.6d)**. Further mutation of S1457 or S1524 in the S1387A/S1423A double mutant caused very slight reductions in HR activity but, surprisingly, partially restored drug resistance. Interestingly, S1423A/S1457A/S1524A triple mutant that does not show much difference in HR and SSA suppression leads to a partial defect in drug sensitivity. When all 4 serine residues were mutated, the HR activity of the quadruple mutant remained similar to the triple mutants; however, its ability to support colony formation was reduced again to a level similar to that of the S1387A/S1423A double mutant **(Figure 4.6d)**. These results suggest that either S1457 or S1524 phosphorylation alone is sufficient to modestly restore drug resistance in the absence of S1387 and S1423 phosphorylation. Furthermore, as shown in the context of the S1387A/S1423A/S1457A/S1524A quadruple phosphomutant (4SQA), S1457 and S1524 may act redundantly to one each in an exclusive manner.

4.3.5 Cyclin Dependent Kinase (CDK)- and Polo-Like Kinase 1 (PLK1)-mediated BRCA1 phosphorylation events are dispensable for BRCA1 HR/SSA function

In addition to the SQR, it has been reported that CDK phosphorylation of BRCA1 at S1189, S1191 and S1497 promotes BRCA1 focus formation and DNA repair activity [163]. In fact, abolishing CDK phosphorylation at these sites was reported to result in a partial sensitization of cancer cells towards cisplatin and rucaparib [163, 164]. Therefore, mutations of these sites were also generated and tested in parallel with sites in the SQR. Given the proximity of S1189 and S1191, these two sites were mutated together. The S1189A/S1191A double mutant showed an 8% reduction in HR, and S1497A displayed a 10% increase **(Fig. 4.7b)**, both considered within the margin of experimental error. S1497A, independently studied in two separate mouse ES cell-based systems [29, 56], was observed to confer resistance towards cisplatin. Drug resistance overall was not significantly affected by either mutation, although S1497A appeared to cause a slight sensitivity to olaparib. S1189A/S1191A /S1497A triple mutant also did not have any significant difference with wild type in the context of SSA suppression. Combined mutation of S1189, S1191 and S1497 led to a triple mutant with 100% HR activity but reduced ability to support cisplatin and olaparib resistant colony formation by ~20%**(Fig. 4.7c)**. Thus, CDK phosphorylation of BRCA1 on these sites does not affect HR but has a modest role in promoting cell survival after DNA damage. We also examined the possible effect of PLK1 mediated phosphorylation of BRCA1 by mutating S1164 to a patient-derived S1164I VUS. Align-GVGD grade assessment on S1164I (C0) suggest that it is most likely a neutral variant **(Table 4.3)** even though prior yeast-based HR assay and stable cell lines suggest that the S1164I mutation may severely affects HR [43, 55]. When tested, we observed no significant difference of S1164I in terms of HR/SSA activity and drug sensitivity.

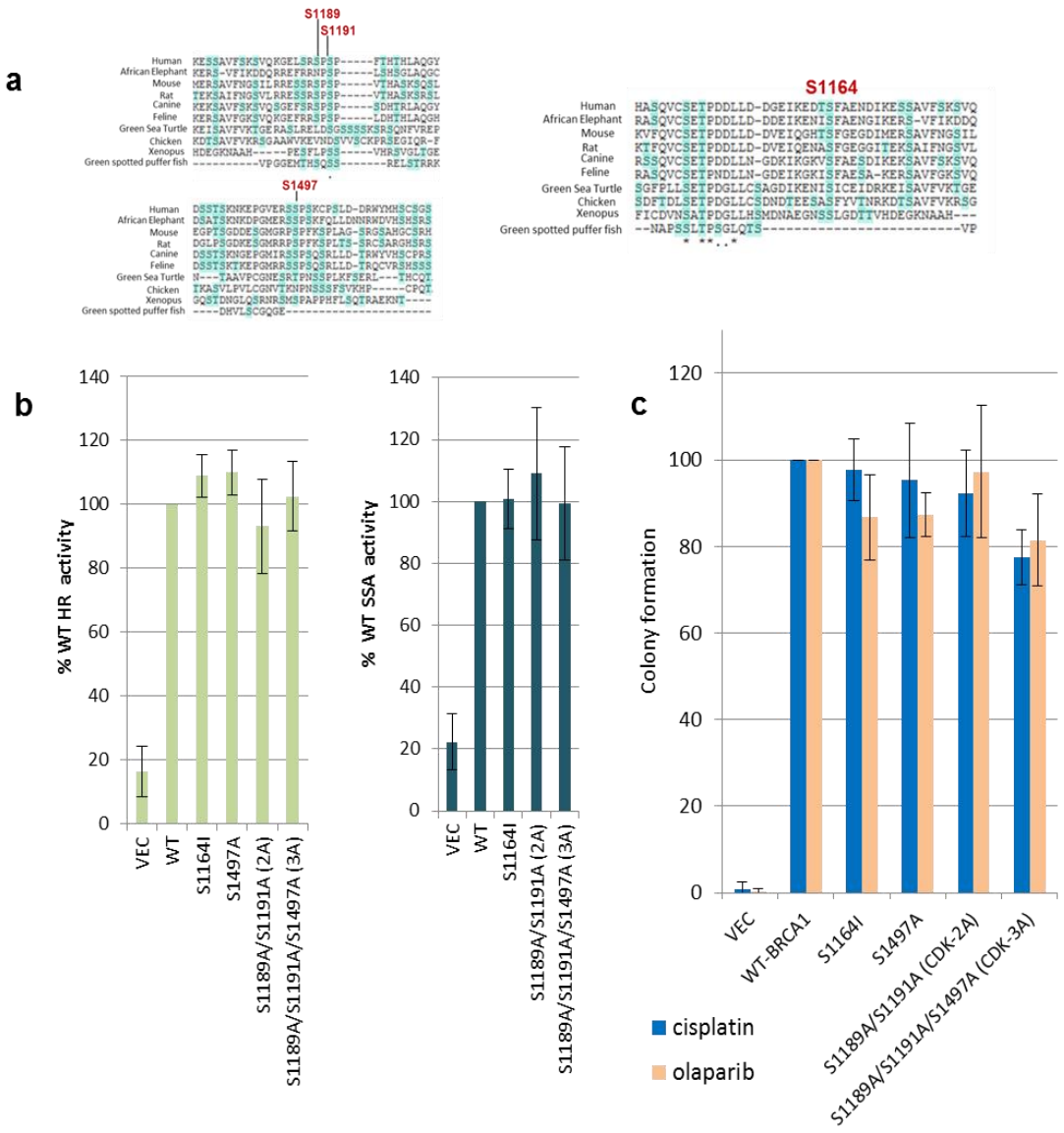

Figure 4.7: Cyclin Dependent Kinase (CDK)- and Polo-Like Kinase 1 (PLK1)-mediated BRCA1 phosphorylation events are dispensable for BRCA1 HR/SSA function

(a) Sequence alignment of conserved CDK sites (S1189/S1191/S1497) and PLK1 site S1164 across different species

(b) HR and SSA activity of CDK and PLK phosphomutants.

(c) Drug resistance of CDK and PLK phosphomutants.

4.3.6 Identification of T1394 as a critical residue that can affect BRCA1 functions independent of PALB2 binding

Previous observations made by Tibbet et al (2000) proposed that S1143, S1280, S1387, T1394, S1423, S1457 are key ATR phosphorylation sites in BRCA1 following incubation with ATR. T1394 is of particular importance to us as it is one of the three conserved S/TQ motifs flanking the PALB2 binding coiled-coil domain. As there has not been further characterization of T1394 other than the possibility of it can be phosphorylated by ATR in vitro, we first tested if BRCA1-T1394A can support cell viability following cisplatin or olaparib treatment. Interestingly, T1394A single mutant itself showed a defect in conferring drug resistance while addition of T1394A onto the partially defective S1387A/S1423A drastically reduces drug resistance **(Figure 4.8a).**

Since T1394 is positioned closely to the PALB2-binding coiled-coil domain, it is feasible that T1394 is critical in providing structural support for the coiled-coil. To rule out such a possibility, we compared the PALB2 binding ability of T1394A, T1394E and Q1395R, a patient-derived VUS that presumably abolished the phosphorylation of T1394 [129, 169], with previously reported PALB2 binding mutants M1400V, M1411T and L1407P. M1400V is a hypomorphic mutation that binds weakly to PALB2 ; L1407P is a pathogenic mutation that introduces a proline into the helical structure that disrupts the structure of the coiled-coil ,abolishing PALB2 binding [11, 331]. The Q1395R VUS was previously reported in African American patients receiving known breast cancer diagnosis at a young age (age <50 years at diagnosis)[129, 252]. Binding of T1394A, T1394E and Q1395R to PALB2 is similar to WT-BRCA1**(Figure 4.8b)**. In fact, the amount of BRCA2-bound PALB2 IP-ed by either T1394A or T139E are comparably more than the known PALB2 binding mutants (M1400V, M1411T, L1407P). Moreover, triple alanine mutants S1387A/T1394A/S1423A (T1394-3A) and triple glutamate mutant S1387E/T1394E/S1457E (T1394-3E) also do not greatly affect BRCA1-PALB2 interaction **(Figure 4.8c).**

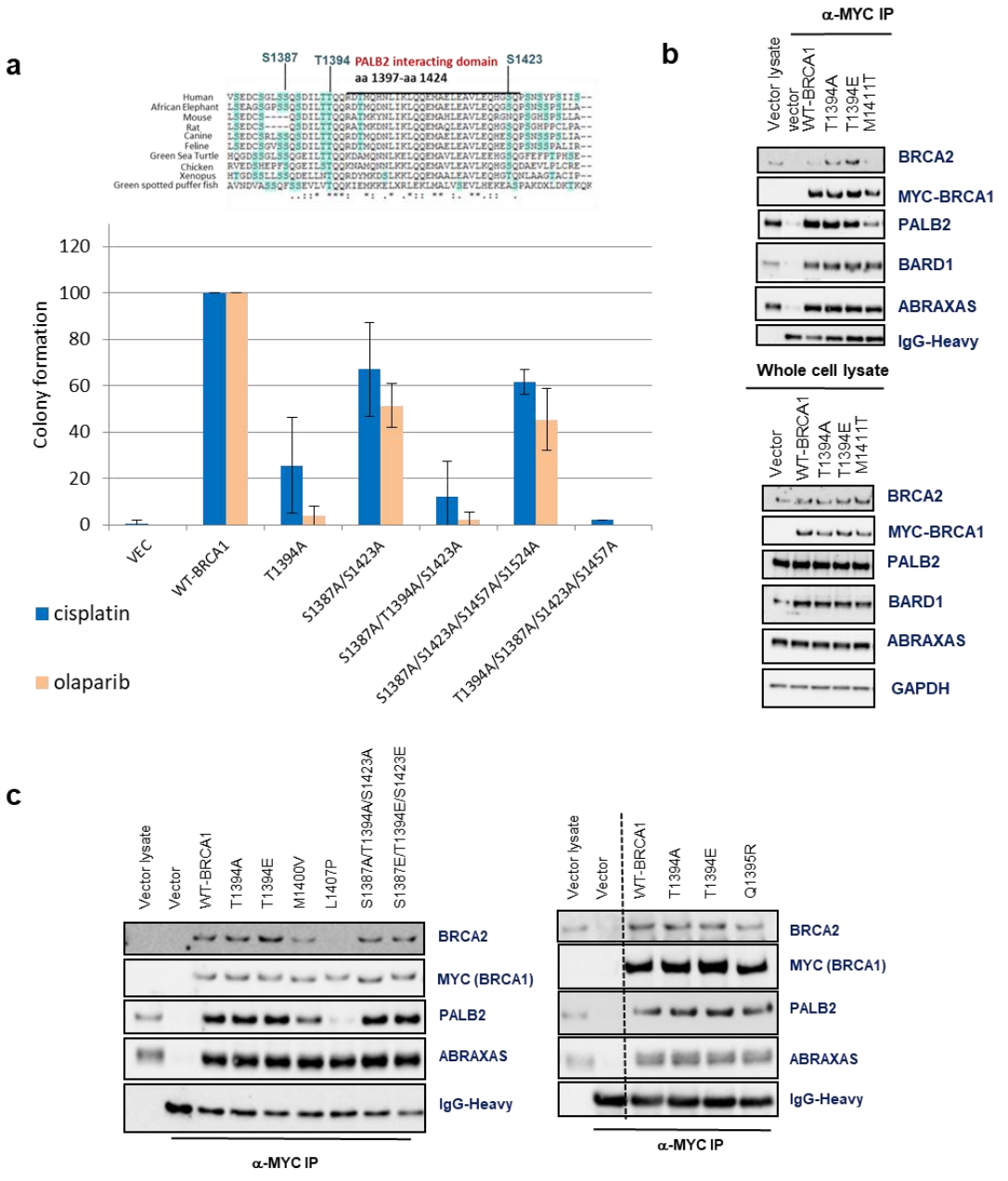

Figure 4.8: BRCA1 residue T1394 is critical for drug sensitivity even though mutations on T1394 or its adjacent Q1395 do not affect PALB2 binding

- **a)** T1394 is a highly conserved residue upstream of the BRCA1-PALB2 interaction motif with T1394A mutations greatly sensitized cells to cisplatin and olaparib.
- **b)** Immunoprecipitation of BRCA1-T1394A and T1394E revealed retained PALB2 binding
- **c)** BRCA1-T1394A, T1394E, Q1395R and T1394 triple mutants (S1387A/TR1394A/S1423A & S1387E/T1394E/S1423E) still retains PALB2 similar to WT and is more than previously reported BRCA1 hypomorph M1400V and pathogenic mutant L1407P.

Patients derived mutations identified on T1394 and its adjacent Q1395.

Detailed examination of the phosphorylation status of BRCA1 based on high throughput methods and software predictions can be useful for better characterization of VUS that may affect patterns of BRCA1 phosphorylation [351]. A simple search into the ClinVar and CbioPortal databases revealed that there has been reported single amino acid alterations that can potentially affect some of the phosphorylation sites mentioned in this study **(Table 3).** Mutations of phosphorylation sites can either be a non-conserved substitution of the Serine (S) or Threonine (T) residues or direct alterations of the kinase consensus site, rendering the phosphorylation site inactive. Due to lack of functional studies, most of these patient-derived missense variants are often designated as variants of uncertain significance (VUS) or conflicting interpretation may arise due to limited reports of a certain VUS from the general population. Although *in silico* approaches for prediction of missense changes based on protein structure and sequence alignment maybe informative, it remains insufficient to determine if an altered BRCA1 phosphorylation site may potentially drive tumor formation or development. We identified several patient derived VUS in phosphorylation sites for S1189/1191, S1387, S1423, S1497 and S1524.

Although most of the Align-GVD assessments **(Chapter 2.8)** on these variants are C0 or likely to be neutral, we cannot rule out the possibility that the non-conserved substitution will have any impact on the structural integrity of the BRCA1 protein. Still, we would expect that our single S/T to alanine substitution result could be informative in determining the likelihood of BRCA1 function in these patient derived VUS. In the case of T1394, we are intrigued to observe a total of 13 reported cases with patient derived VUS either affecting T1394 itself (T1394I, T1394P) or the adjacent Q1395 (Q1395R, Q1395H, Q1395E) **(Table 3)**.Similar to Q1395R, T1394I (c.4181C>T, initially misreported as 4300C>T) was reported in a black woman affected by invasive breast carcinoma < 50 years old [252]. Moreover, a metastatic specimen of invasive breast carcinoma of a 68-year-

old black, female T1394I patient was found within the AACR Project Genomics Evidence Neoplasia Information Exchange (GENIE); GENIE is a multi-phase, multi-year, national and international project that focused on a regulatory-grade registry of clinical-grade cancer genomic data with clinical outcomes of cancer patients treated at participating institutions. Interestingly, the T1394I breast cancer metastases exhibit amplification of CCDN1, KRAS, as well as potentially oncogenic PI3KCA (H1047L) mutation. Since Align-GVGD grade for T1394 associated VUS suggest that the non-conserved substitution may alter protein structure and function, we decided to also functionally characterize T1394I, Q1395R and Q1395H in parallel with T1394A/E **(Table 4.3).**

Residue	Kinases	Previously reported VUS that may affect phosphorylation		Times reported in ClinVar	Times reported in CBioPortal	ClinVar or CBioportal assessment	Align-GVGD grade
		HGVS cDNA	ClinVar Designation				
S1164	PLK	c3491G>T	S1164I	1	-	not provided	C0
S1189, S1191	CDK1	c.3569C>T	P1190L	2	1	Uncertain significance	C0
		c.3569C>A	P1190H	-	1	Unknown clinical implications	C0
		c3572G>A	S1191N	2	-	Uncertain significance	C0
S1377	N/A	c.4131C>A	S1377R	2	-	Uncertain significance	C0
S1387	ATR ATM	c4159T>C	S1387P	2	-	Uncertain significance	C0
		c.4160C>T	S1387F	-	1	Unknown clinical implications	C0
		c.4162C>G	Q1388E	-	1	Unknown clinical implications	C0
		c.4164G>C	Q1388H	-	2	Unknown clinical implications	C0
T1394	**ATR ATM**	**c.4180A>C**	**T1394P**	**1**	**-**	**Uncertain significance**	**C35**
		c.4181C>T	**T1394I**	**4**	**1**	**Likely benign (1); Uncertain significance(3)**	**C65**
		c4184A>G	**Q1395R**	**5**	**-**	**Likely benign (1); Uncertain significance (4)**	**C35**
		c.4185G>C	**Q1395H**	**1**	**-**	**Uncertain significance**	**C15**
		c.4183C>G	**Q1395E**	**-**	**1**	**Unknown clinical implications**	**C25**
Q1396		c.4186C>A c.4187A>G c.4188G>T	Q1396K Q1396R Q1396H	2 41 -	- - 1	Uncertain significance Benign Unknown clinical implications	C45 C35 C15
S1423	ATR ATM	c4268G>T	S1423I	1	-	Uncertain significance	C0
		c4272G>C	S1423R	-	2	Unknown clinical implications	C0
		c.4272G>T	Q1424H	1	-	Uncertain significance	C0
S1497	CDK1/2	c.4489T>C	S1497P	-	1	Likely Neutral	C0
S1524	ATM	c4574A>G	Q1525R	1	-	Uncertain significance	C0
		c.4575A>C	Q1525H	-	1	Unknown clinical implications	C0

Table 4.3 : Variants of uncertain significance may directly affect BRCA1 phosphorylation by either affecting S/T residue or adjacent residue to alter kinase consensus motif.

4.3.7 T1394 mutant BRCA1 proteins are unable to suppress SSA with lower HR.

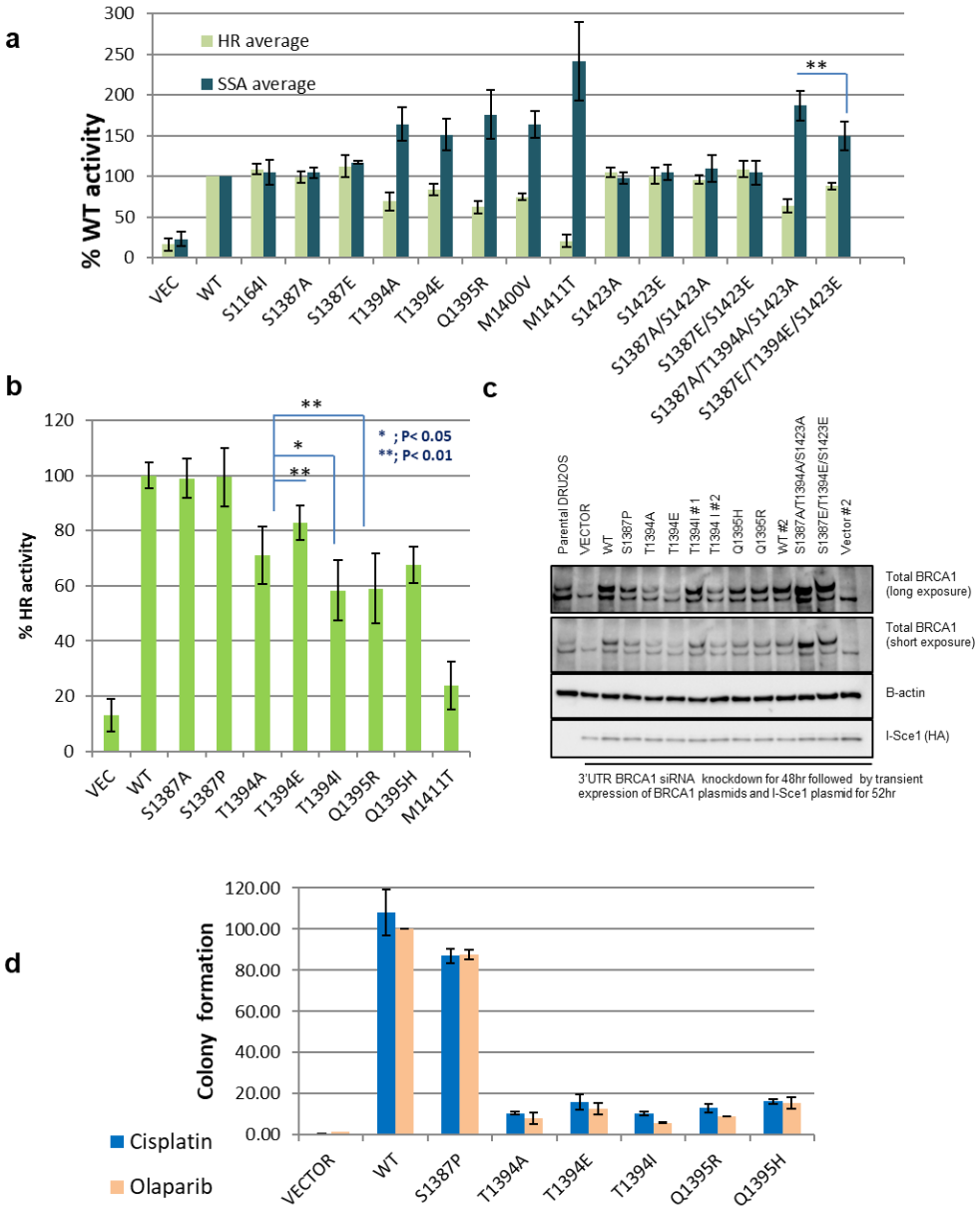

Figure 4.9: T1394 phosphomutants have reduced HR activity, impaired SSA suppression and sensitized cells towards DNA damaging agents

a) HR and SSA activity of BRCA1 T1394 phosphomutants in comparison with S1387 and S1423 phosphomutants

b) HR activity of BRCA1 T1394 patient derived VUS in comparison with the artificial T139A/E mutations

c) Levels of BRCA1 knockdown and re-expression of BRCA1 T1394 phosphomutants re-expressed in U2OS/DR-GFP cells. Cells untreated with any siRNA were used as a control for the endogenous protein abundance.

d) Abilities of the BRCA1-T1394 mutants to confer resistance to cisplatin and olaparib

When assessed for their respective HR activity, T1394A, T1394I, Q1395R and Q1395H showed reduced HR activity at ~55-70% WT activity **(Figure 4.9a,b)**. Interestingly, T1394E phosphomimetic mutant also showed reduced HR efficiency (~85%) but is consistently higher than T1394A or any of the patient derived VUSs despite significantly lower protein expression **(Figure 4.9b, c)**. Consistent with the T1394A and Q1395R single mutants, T1394-3A triple mutant also showed similar reduction of HR (~60%); phosphomimetic mutants T1394E and T1394-3E has HR activity of ~80%. Such an observation is interesting as the triple A and triple E mutants are both slightly higher in expression than the single phosphomutants and WT alone. PALB2 binding hypomorph M1400V, on the other hand, retained ~85% activity while M1411T due to a more severe PALB2 binding defect is more similar to vector control (~15%).

We tested if BRCA1 phosphorylation at T1394 can also affect SSA as we previously reported PALB2 binding mutants showed reduced HR with a concomitant increase in SSA[11]. Interestingly, T1394A, T1394E and Q1395R behave similarly to the PALB2 binding hypomorph M1400V in their inabilities to suppress SSA despite lower HR activity with better PALB2 binding **(Figures 4.8c and 4.9a)**. A previously reported mammalian two hybrid system suggest that Q1395R may not be able to bind to PALB2 effectively even though the study did not detect any significant disruption of the coiled-coil domain formation [375]. However, our immunoprecipitation experiment clearly demonstrated that these mutants bind to similar amounts of PALB2 as WT-BRCA1 and much more PALB2 binding than M1400V, L1407P and M1411T **(Figure 4.8c)**. We also noted that introduction of T1394A in S1387A/S1423A double mutant slightly elevated the SSA activity than T1394A alone. It is possible that the phosphorylation of S1387 and S1423 can act to partially suppress the elevated SSA activity following abolishment of T1394 phosphorylation. In fact, T1394-3E triple glutamate mutant do not exhibit a further increase

in SSA activity than T1394E alone and is lower in SSA activity than the T1394-3A triple alanine mutant **(Figure 4.9 a)**.

Similar what was already observed in S1387A, the S1387P VUS do not exhibit much difference in terms of HR activity with SSA suppression highly unlikely to be affected **(Figure 4.9d)** with an expected resistance to cisplatin and olaparib. Moreover, T1394A, T1394I, Q1395R and Q1395H mutations that presumably abolish T1394 phosphorylation are unable to support HR activity with cell sensitive to both cisplatin and olaparib **(Figure 4.9b, d)**. Interestingly, although T1394E has significantly higher HR activity than T1394A, MDA-MB 436 cells reconstituted with T1394E are also unable to generate an expected resistance towards either cisplatin or olaparib.

Interestingly, the expression of BRCA1-T1394A was lower than wild type protein **(Figure 4.9c)**, raising the possibility that abolishing this phosphorylation site would result in structural alterations, which in return destabilize the protein. However, the patient-derived mutations T1394I expressed similar to BRCA1-WT, confirming a direct relationship between BRCA1 function and T1394 phosphorylation. Structurally, an isoleucine (I) is much similar to the branched configuration of threonine (T) as opposed to alanine. A cycloheximide chase experiment at 40µg/ml concentration was performed on BRCA1-WT, T1394A and T1394I reconstituted in BRCA1 knockdown U2OS to rule out if the lower expression of the proteins is indeed the result of destabilized proteins cleared via proteosomal degradation or a matter of plasmid mRNA expression. Indeed, BRCA1-T1394A, expressed at lower levels than WT protein **(Figure 4.10a, c)** are much more unstable than BRCA1-WT and BRCA1-T1394I **(Figure 4.10b, c)** and can be further stabilized by inhibition of the 26S proteasome through treatment of 1uM MG132. The degradation kinetics of BRCA1-T1394I is similar with wild type protein, while BRCA1-T1394A is indeed relatively unstable **(Figure 4.10 d)**. These observations confirm that the

reduced HR activity, de-repression of SSA and drug sensitivity of the T1394-derived mutations are mostly dependent on phosphorylation at this site.

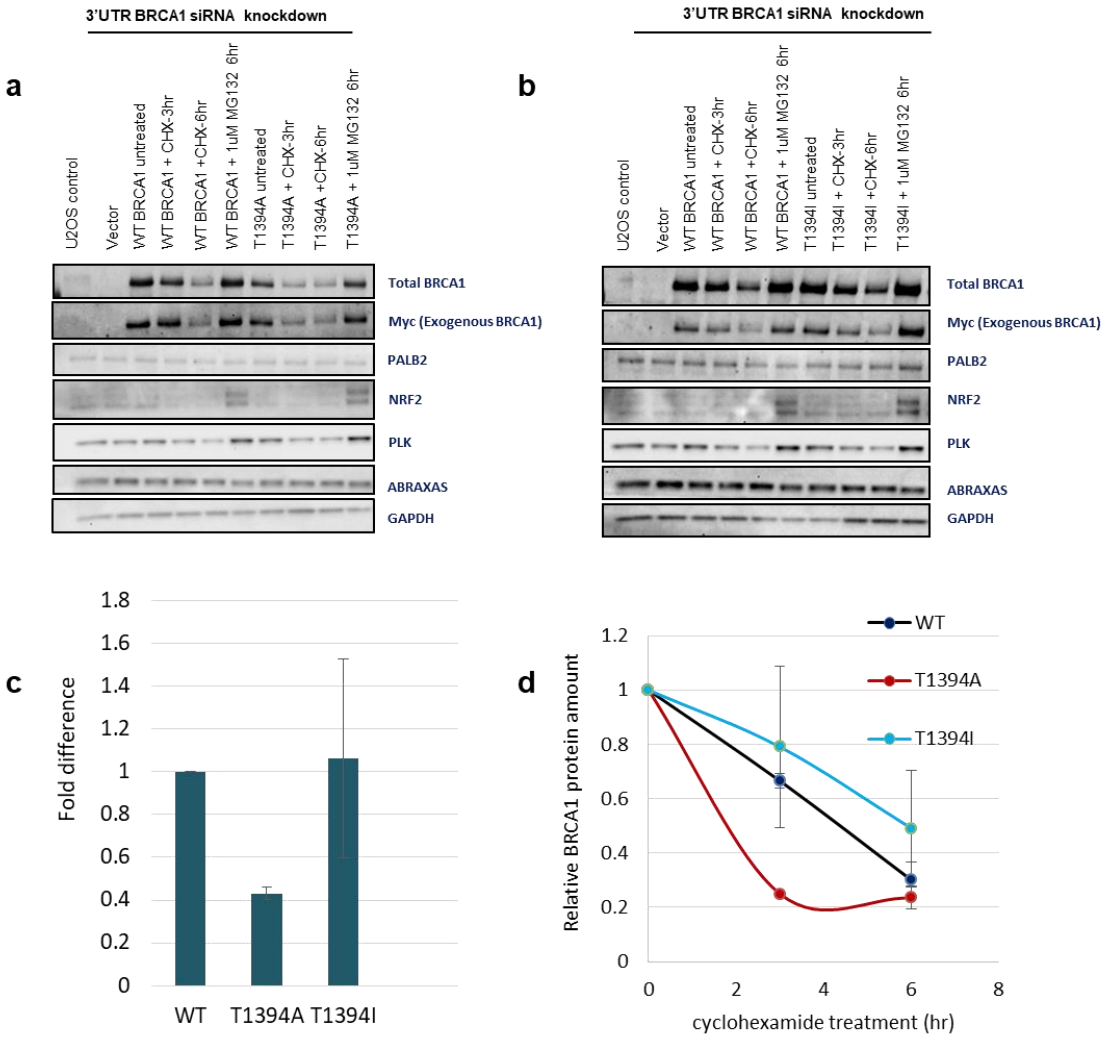

Figure 4.10: BRCA1-T1394A expressed less and is more unstable compared to WT-BRCA1 and BRCA1-T1394I

a) Cycloheximide treatment of BRCA1-T1394A / b) BRCA1-T1394I reconstituted in U2OS cells depleted of endogenous BRCA1 by siRNA Cells were either untreated or treated with 40ug/mL of cycloheximide for 3 and 6 hours, and the proteins were analyzed by Western blotting

c) Comparison of BRCA1-T1394A and T1394I protein levels with respect to WT BRCA1

Nevertheless, our results based of on T1394 phosphomutants underline the possible complexity of T1394 phosphorylation and its effects on BRCA1 function in coordinating DNA repair choice between HR and SSA. In comparison with S1387 and S1423 that also flanked the two ends of the PALB2 binding domain, single amino acid alteration on this TQ site is sufficient to impair HR activity, SSA suppression and drastically sensitized cells to DNA damage. It is also feasible that alteration of the phosphorylation dynamics of BRCA1, particularly on S1387, T1394 and S1423 can lead to a structural change in the BRCA1-PALB2 interaction that is not able to be fully determined via immunoprecipitation.

4.3.8 Detection of BRCA1 T1394 phosphorylation by phospho-specific antibody

Because T1394 is part of a highly conserved TTQQ motif, it is important to determine if the drug sensitivity of cells expressing BRCA1-T1394 mutations is a direct consequence of abolished phosphorylation or simply a matter of structural distortion of BRCA1 coiled-coil domain that is not detectable via immunoprecipitation. Putative BRCA1 phosphorylation sites such as S1387, T1394 and S1423 have been reported to be phosphorylatable in vitro [342]. However, previous mass-spectrometry-based approaches were unable to detect the phosphorylation events of these residues in vivo unlike pS1524 that was frequently detected [68, 226, 248, 308]. Interestingly, pS1423 has only been identified from BRCA1 isolated from ATM in vitro kinase assays but not in vivo despite we and others who observed a clear induction of pS1423 following DNA damage[68]. As shown **Figure 4.11a**, it is possible that peptide fragments containing any of these 3 residues were too long following trypsin digestion, thereby limiting the detection of any phosphorylation event close to the PALB2-BRCA1 binding motif. In vivo phosphorylation of S1164, S1189, S1191, S1497 and S1524 are also detectable in mitotic cells [226, 248, 308].

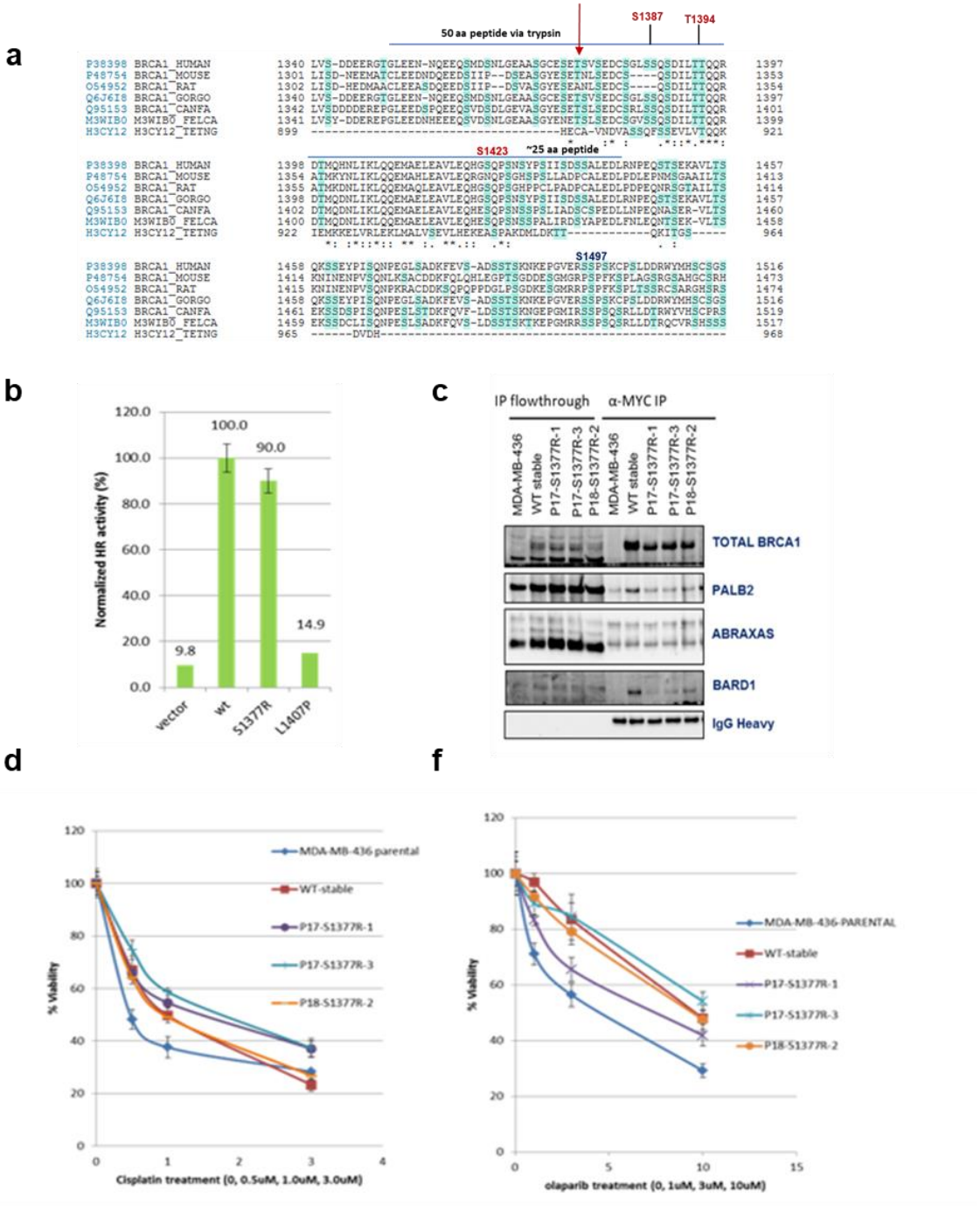

Figure 4.11: Generation of MDA-MB 436-S1377R stable cell line

a) Strategy to generate a cleavable BRCA1 peptide for mass spectrometric analysis of phosphorylation sites flanking the BRCA1 coiled-coil domain (aa 1397-1424), red arrow denotes S1377 that can potentially be mutated to S1377R

b) HR activity of BRCA1 S1377R patient derived VUS in comparison with WT and BRCA1-L1407P

c) Levels of BRCA1 immunoprecipitated from 3 independent MDA-MB 436-S1377R stable cell line

d) MDA-MB 436-S1377R stable cells confer resistance to cisplatin and olaparib (e)

A careful look at trypsin digestible fragments surrounding the PALB2-BRCA1 binding motif (aa 1397-1424) indeed confirmed the technical difficulty in identifying these phosphorylation sites. Both S1387 and T1394 are positioned at a possibly 50 amino acid peptide that may be too large for mass-spectrometry detection that usually utilized 7-35 amino acid residues[329] **(Figure 4.11a)**. Although S1423 is positioned at a possibly 25 amino acid fragment, it is also often undetectable via mass-spectrometry analysis performed by various labs except the original report in which it was first identified[68]. Interestingly, a follow up study by Matsuoka et al that utilized a BRCA1-pS1423 phosphospecific antibody for mass spectrometric analysis of ATM/ATR phosphorylation substrates could identify a wide range of S/TQ substrates but not BRCA1 pS1423 that the antibody was initially designed for[226]. Serine residues downstream of this domain such as S1457 and S1524 have consistently been identified to be phosphorylated following DNA damage via mass-spec analysis, in agreement with in vitro observations [68, 226].

To address this technical challenge, we first BRCA1-S1377R, a patient derived VUS, that was reported in the Breast Cancer Database (bic.gov) to be a neutral variant. BRCA1-S1377R appears to be comparable in terms of HR activity to BRCA1-WT **(Figure 4.11b)**. We next generated MDA-MB-436 stable cell lines expressing this VUS and tested their ability to confer similar to WT resistance of cisplatin and olaparib **(Figure 4.11d, e)**. In order to determine pT394 phosphorylation following varying DNA damaging conditions via mass spectrometric analysis, we first attempted to immunoprecipitate myc-tagged BRCA1 expressed in these S1377R stable cell lines. Unfortunately, we could only immunoprecipitate a fraction of the BRCA1 expressed in the stable cell line and there is still BRCA1 remained in the IP flow through **(Figure 4.11c)**. We also observed that the amount pulled down is relatively less than the WT stable with also lower PALB2 and

BARD1 binding detected. Further work is necessary to optimize the conditions necessary for mass spec analysis**.**

T1394 phosphorylation is not required for effective BRCA1-PALB2 complex formation following DNA damage

We generated a novel phosphospecific antibody (GeneScript) against pT1394 using phosphopeptide ranging from amino acid S1390-D1398 (^{1390}DILT**[pT]**QQRD^{1398}C with the non-phosphorylated peptide ^{1390}DILTTQQRD^{1398}C). The resulting Rabbit anti-human affinity-purified phosphospecific pT1394 antibody was first tested in 293T cells over-expressing either vector plasmid or WT-BRCA1 6-hour post 10Gy IR. Following ionizing radiation, the pT1394 antibody is able to detect phosphorylated BRCA1 in vector control cells and more in the WT-BRCA1 expressing cells **(Figure 4.12a)** over time **(Figure 4.12b)**. However, the signal detected for pT1394 is not proportional to the amount of exogenous BRCA1 detected in the whole cell lysate. Nevertheless, the induction of pT1394 (upper band of two bands) in WT-BRCA1 can be clearly demonstrated when the myc-tagged BRCA1 was immunoprecipitated **(Figure 4.12a, b)**. As ATM-mediated phosphorylation often occurs immediately after DNA damage with a slow transition into ATR-mediated cell signaling, we expected that pT1394 induction after 6hr to be mostly a ATR-driven event. When cells were treated with ATRi, we observed reduced pT1394 (upper band) but the overall signal for pT1394 are still not completely reduced **(Figure 4.12b)** as in the case of T1394I or S1387A/T1394A/S1423A **(Figure 4.12 d,e)**. Such an observation suggests that other members of the PIKK family such as ATM or DNA-PK can still phosphorylate pT1394 in the absence of ATR kinase activity. Since T1394 is a TQ motif that can be phosphorylatable by ATM, ATR or DNA-PK, we treated the cells with the respective kinase inhibitors to determine if all of these kinases are required for pT1394.Inhibition of either ATM (KU-55933) or ATR (VE-821) is able to reduce

phosphorylated T1394 after 10Gy IR while inhibition of DNA-PK (NU7441) do not affect pT1394 **(Figure 4.12c)**. Treatment of IR-irradiated cells with both ATM and ATR inhibitors are sufficient to completely impair T1394 phosphorylation **(Figure 4.12c)**.

We next tested if PALB2 binding to T1394 mutants such as T1394I are affected following IR-mediated DNA damage. We first treated 293T cells overexpressing BRCA1 plasmid with IR (10Gy) followed by 6-hour recovery before collection for immunoprecipitation. We observed binding of hyper-phosphorylated PALB2 (as indicated by a shift in PALB2 migration and diffusive migration pattern) following IR was not affected in any of the mutants tested **(Figure 4.12b)**. As expected, patient-derived mutations such as T1394I **(Figure 4.12d)** and Q1395R **(Figure 4.12e)** do not affect PALB2 binding before and after ionizing radiation.

We also investigated if phosphorylation at T1394 is dependent upon the phosphorylation of other phosphosites that we previously assessed to have minimal effect on BRCA1 function **(Figure 4.12e)**. It is possible that abolishing the phosphorylation of a specific SQ site may lead to compensatory phosphorylation at other neighboring residues. We compared pT1394 signal between WT-BRCA1, S1387E/T1394E/S1423E (T1394-3E) and the quadruple 4SQA. As negative controls, Q1395R and T1394-3A mutants do not have detectable pT1394 and PALB2 binding remains unaffected similar to WT. Interestingly, we observed a detectable pT1394 signal in the T1394-3E mutant, further confirming the specificity of the antibody. More importantly, we observed the BRCA1-4SQA is unable to migrate the same level as WT or T1394-3E, but can still exhibit pT1394 signal. Whether or not any of the 4SQ sites are critical for induction of pT1394 remains to be determined. However, the presence of pT1394 signal in the 4SQA mutant do calls into question if the partial drug resistance observed in the BRCA1-2-SQA and 4SQA mutants is compensated by pT1394.

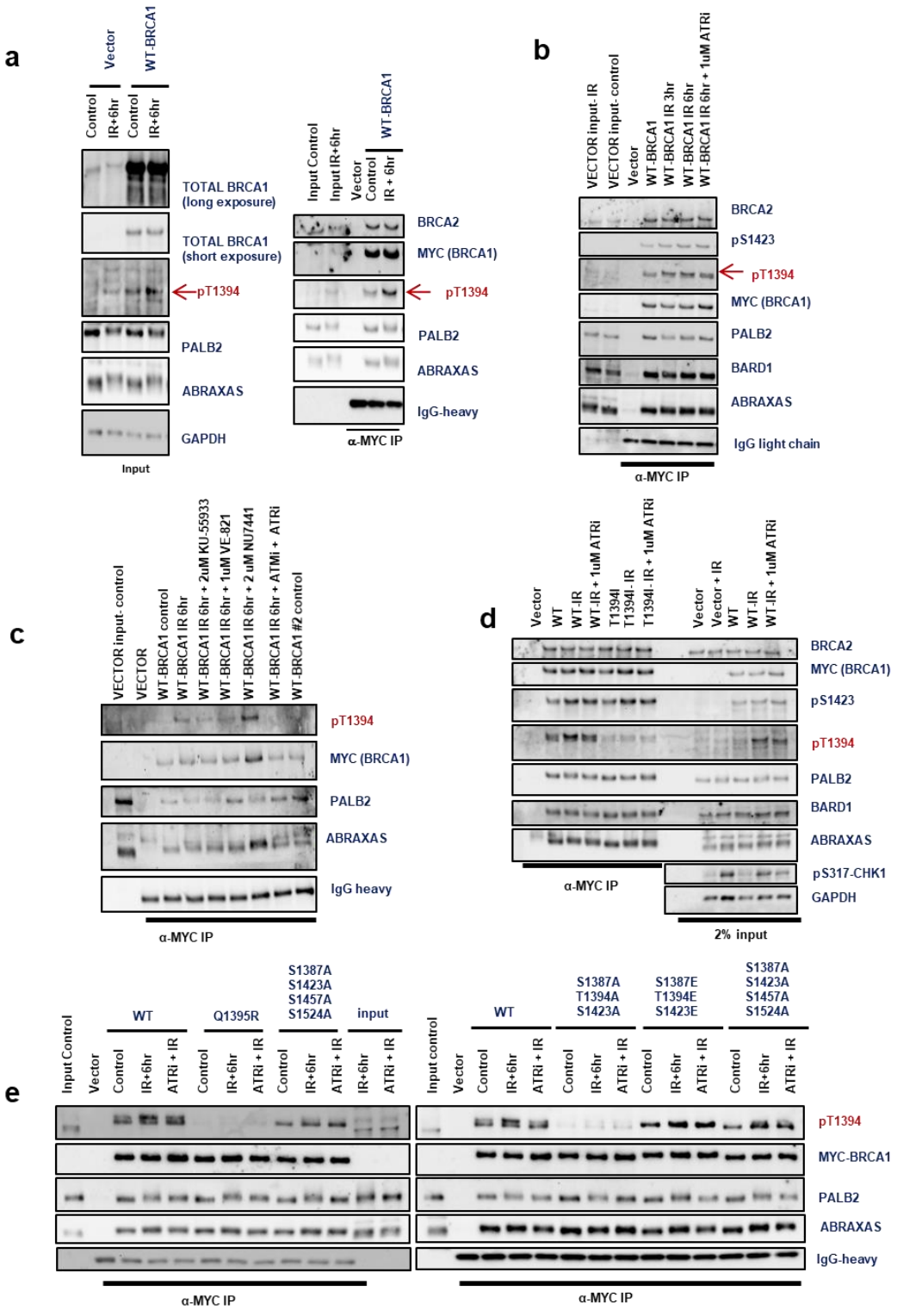
a
Vector
WT-BRCA1
Control
IR+6hr
Control
IR+6hr
TOTAL BRCA1 (long exposure)
TOTAL BRCA1 (short exposure)
pT1394
PALB2
ABRAXAS
GAPDH
Input
Input Control
Input IR+6hr
Vector Control
WT-BRCA1 IR + 6hr
BRCA2
MYC (BRCA1)
pT1394
PALB2
ABRAXAS
IgG-heavy
α-MYC IP
b
VECTOR input-IR
VECTOR input- control
Vector
WT-BRCA1
WT-BRCA1 IR 3hr
WT-BRCA1 IR 6hr
WT-BRCA1 IR 6hr + 1uM ATRi
BRCA2
pS1423
pT1394
MYC (BRCA1)
PALB2
BARD1
ABRAXAS
IgG light chain
α-MYC IP
c
VECTOR input- control
VECTOR
WT-BRCA1 control
WT-BRCA1 IR 6hr
WT-BRCA1 IR 6hr + 2uM KU-55933
WT-BRCA1 IR 6hr + 1uM VE-821
WT-BRCA1 IR 6hr + 2 uM NU7441
WT-BRCA1 IR 6hr + ATMi + ATRi
WT-BRCA1 #2 control
pT1394
MYC (BRCA1)
PALB2
ABRAXAS
IgG heavy
α-MYC IP
d
Vector
WT
WT-IR
WT-IR + 1uM ATRi
T1394I
T1394I- IR
T1394I- IR + 1uM ATRi
Vector
Vector + IR
WT
WT-IR
WT-IR + 1uM ATRi
BRCA2
MYC (BRCA1)
pS1423
pT1394
PALB2
BARD1
ABRAXAS
pS317-CHK1
GAPDH
α-MYC IP
2% input
e
Input Control
Vector
WT
Q1395R
S1387A S1423A S1457A S1524A
input
Control
IR+6hr
ATRi + IR
WT
S1387A T1394A S1423A
S1387E T1394E S1423E
S1387A S1423A S1457A S1524A
pT1394
MYC-BRCA1
PALB2
ABRAXAS
IgG-heavy
α-MYC IP
α-MYC IP

Figure 4.12: BRCA1 T1394 can be phosphorylated following DNA damage in an ATM/R dependent manner

a) Detection of phosphorylated pT1394 on overexpressed WT-BRCA1 in 293T before and after 10Gy ionizing radiation. 6hr following IR treatment, whole cell lysate was collected and myc-tagged BRCA1 was immunoprecipitated using anti-myc-magnetic beads
b) Detection of phosphorylated pT1394 on overexpressed WT-BRCA1 in a time dependent manner following 10Gy IR.
c) Treatment of PIKK inhibitors identify both ATM and ATR as key protein kinase involved in phosphorylation of T1394. Inhibitors were added at least 15min before 10Gy IR. Whole cell lysates were collected 6hr later for immunoprecipitation.
d) T1394I patient derived mutation do not affect PALB2 binding, before and after IR
e) Mutations on widely reported BRCA1 SQ sites do not significantly impair the induction of pT1394 following IR.

4.3.9 BRCA1 T1394A mutation does not affect BRCA1 recruitment to DNA lesions

Previous work on BRCA1 CDK and PLK phosphorylation proposed that alterations on specific BRCA1 phosphorylation sites affects the recruitment of BRCA1 to damaged DNA lesions, subsequently impairing HR[55, 163]. It is possible that phosphorylation of T1394 affects BRCA1 recruitment to DNA lesions. To address such a possibility, we generated BRCA1 T1394A mutation on BRCA1-GFP construct (experiment performed by Yu lab, City of Hope CA). Following UV laser strip experiment, the recruitment of BRCA-T1394A is not affected within the first 15min after DNA lesions are introduced and showed similar recruitment kinetics as WT-BRCA1 or a possibly neutral variant R1397M. Such a result is as expected as the recruitment of BRCA1 to damaged lesion is mostly dependent on its nuclear localization sequence, its interaction with BARD1 and BRCT-mediated nuclear localization **(Figure 4.13 a,b)**. We also looked at foci formation ability of transiently expressed BRCA1-GFP plasmids following chronic DNA damage such as 10μM cisplatin for 24hr. Interestingly, preliminary results of foci formation in BRCA1-T1394A transfected cells appears to be significantly higher in both MDA-MB-436 cells and 293T cells.

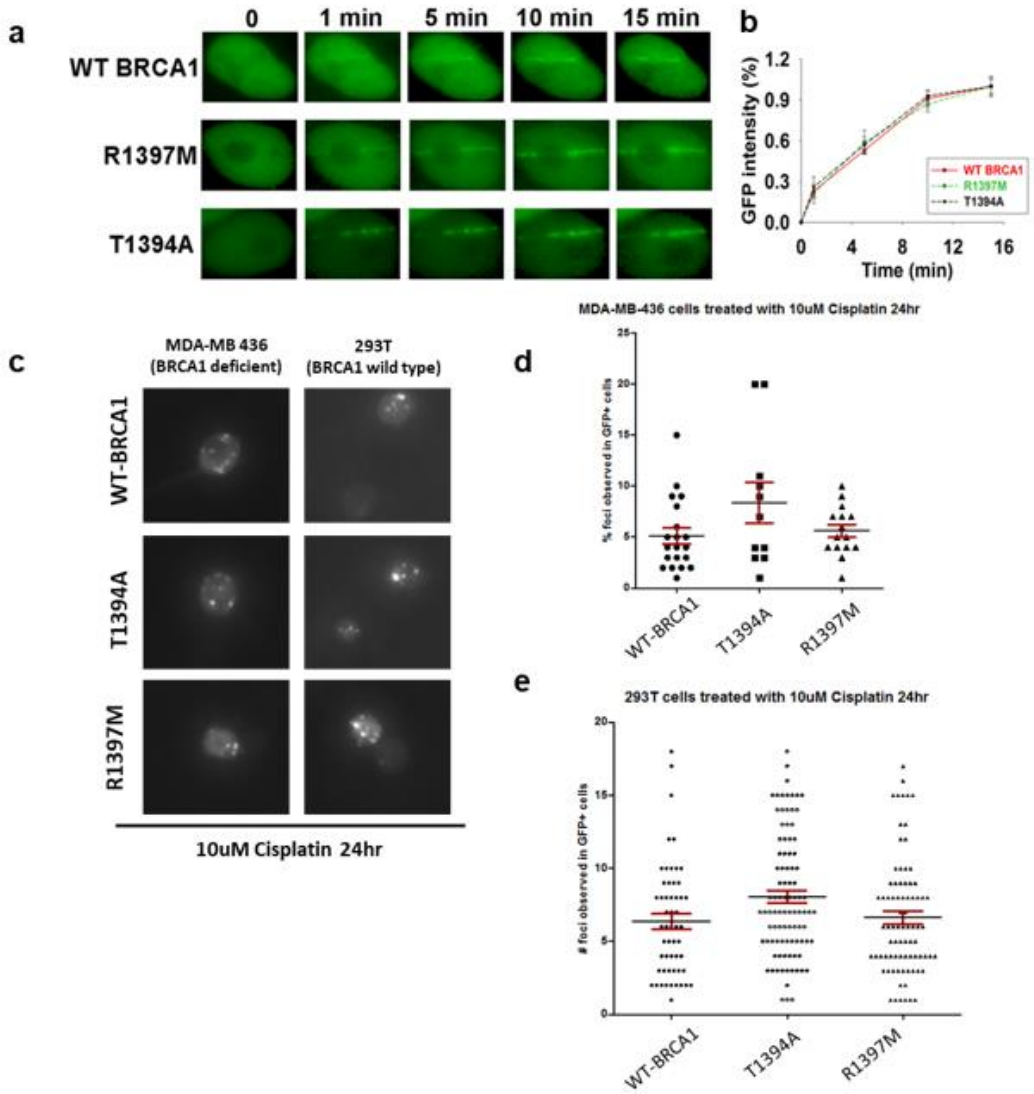

Figure 4.13: Recruitment of transiently expressed GFP-tagged BRCA1-T1394A is not affected following DNA damage

a) 293T cells transfected with the indicated plasmids were laser microirradiated using setting as previously reported[201] GFP fluorescence at the laser line was converted into a numerical value (relative fluorescence intensity) using Axiovision software (version 4.5).
b) Normalized fluorescent curves from 20 cells from three independent experiments were averaged. The error bars represent the SD
c) MDA-MB 436 and 293T cells transiently transfected with GFP-tagged BRCA1 plasmids were treated with 10uM Cisplatin with 24hr. Numbers of nuclear foci observed in each cells were counted and plotted in d) for MDA-MB 436 and c) for 293T cells; error bars represent SD

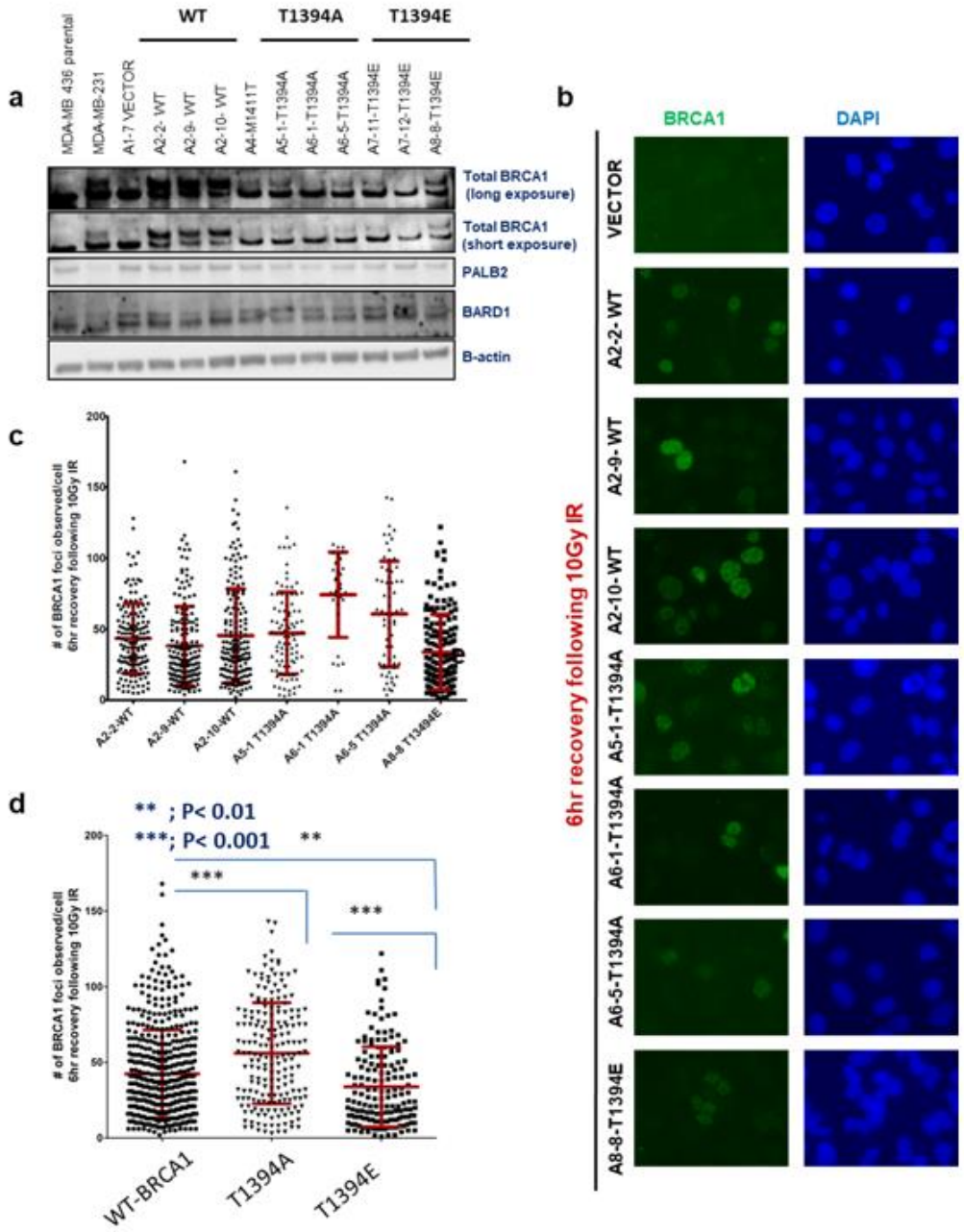

Figure 4.14: BRCA1 T1394A stable cell lines expressed weaker T1394 but still do not affect BRCA1-IRIF

a) Expression of BRCA1 proteins in G418-selected MDA-MB-436 clones. MDA-MB 231 cell lysate were used as a positive control for BRCA1 positive cells.

b) MDA-MB 436 stable cell lines were exposed to 10Gy ionizing radiation with irradiated cells fixed 6hr post IR. Nuclear BRCA1 were stained with BRCA-D9 antibody (Santa Cruz).

c) Numbers of nuclear foci observed in each individual stable cells in (b) were counted and plotted. The error bars represent the SD. d) Summary of foci counts in WT, T1394A and T1394E stable cell lines in (c). The error bars represent the SD.

As transient expression of BRCA1 may not fully reflect the recruitment dynamics of T1394 phosphomutants, we tried generating MDA-MB-436 stable cell lines harboring T1394A and T1394E mutations to first understand the effects of pT1394 in BRCA1 recruitment. MDA-MB-436 cells transfected with cDNA constructs expressing wt BRCA1 or the T1394 variants were subjected to selection with either Geneticin (G418) alone with colonies were allowed to grow for a period of 3–4weeks before being screened for BRCA1 expression and BRCA1 IR-induced foci formation (IRIF). Interestingly, BRCA1-T1394A/E stable cell lines expressed the exogenous BRCA1 plasmid at levels lower than WT-BRCA1, consistent with what was observed in the DR/GFP U2OS cell lysates **(Figure 4.14a)**. Moreover, the disparity between protein levels renders subsequent analysis such as cell cycle profile, cell sensitivity and DNA fiber analysis to be very difficult. Nevertheless, we are still able to observe BRCA1-IRIF and count the number of BRCA1 foci formed for each individual stable cell lines **(Figure 4.14b)**. To our surprise, T1394A stable cell lines showed significantly more foci formation that WT-stables, while the T1394E stable cells have relatively lower number of foci formed **(Figure 4.14c, d)**. Overall our results using the stable cell lines are consistent with our previously mentioned transient experiment with GFP-BRCA1. We also recently generated T1394I stable cell lines and are currently characterizing the foci forming abilities of these cells. Since the BRCA1 foci forming abilities are largely unaffected, a future plan is to determine if recruitment of downstream factors such as RPA, PALB2 and RAD51 are also affected in these stable cell lines.

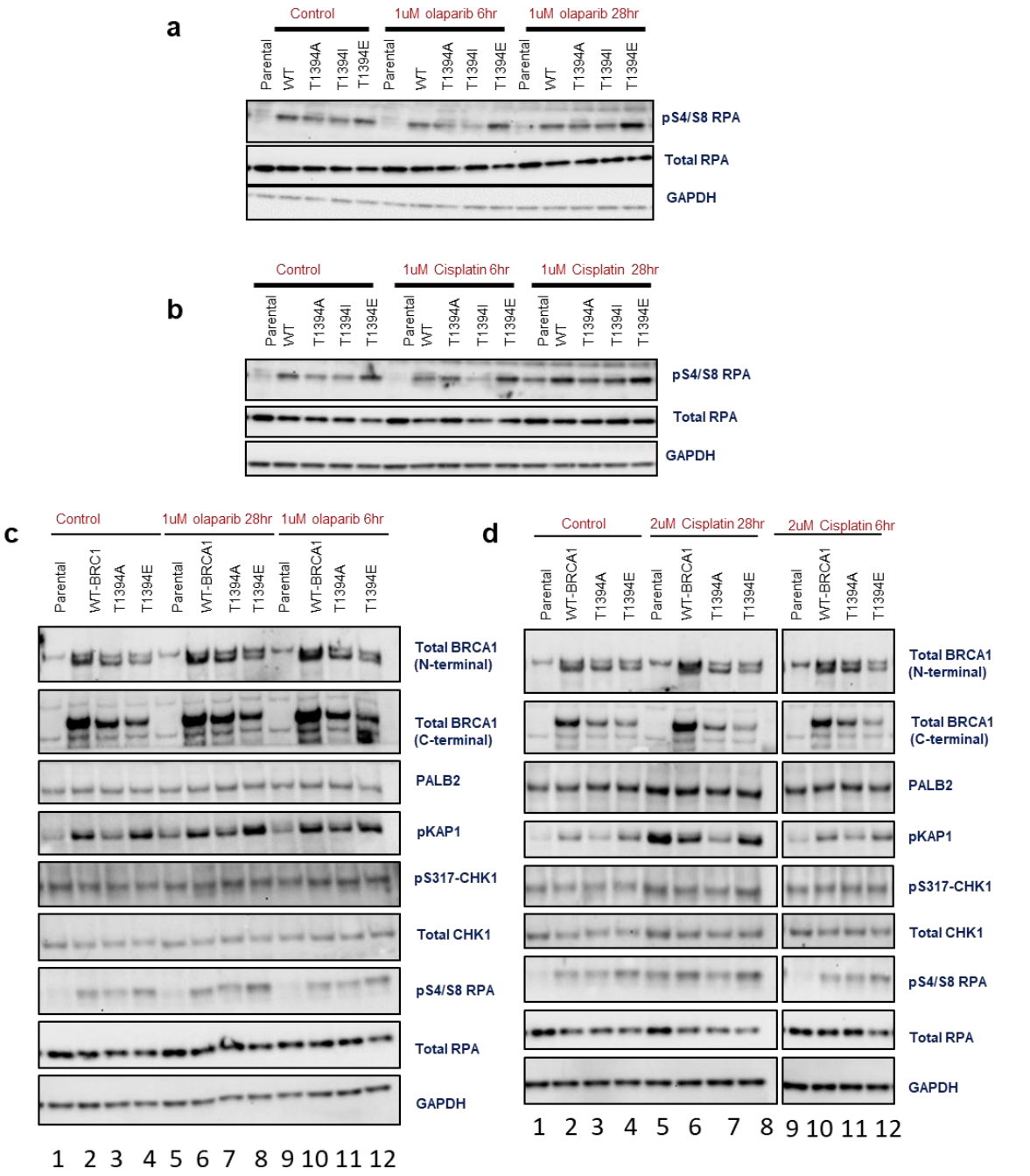

Figure 4.15: Transient expression of BRCA1-T1394A protein leads to altered DDR signaling.

a) State of hyperphosphorylated RPA (S4/S8) in MDA-MB 436 cells transiently expressing BRCA1 T1394 phosphomutant before and after treatment with 1uM Olaparib and b) 1Um cisplatin

c) Whole cell lysate of MDA-MB-436 transiently expressing BRCA1 WT, T1394A and T1394E before and after treatment with olaparib and (d) cisplatin.

4.3.10 Possible regulation of ATM signaling by T1394 phosphorylation

So far, our experimental results points to the inability of T1394 mutant BRCA1 to support HR activity and suppress SSA with defective DNA repair for drug survival. However, we observed retained BRCA1-PALB2 interaction with the foci forming abilities of T1394 mutants largely unaffected or is even more than WT proteins. It is possible that the increased foci formation of T1394A mutant cells is an indirect consequence of defective DNA repair as more of the hypomorphic BRCA1 proteins are recruited to repair damaged DNA. To address such a possibility, we first investigated the effects of BRCA1 T1394 mutants on potential markers of DNA damage such as RPA S4/S8 in MDA-MB 436 cells. If damaged DNA is indeed effectively repaired, we would expect to see reduced phosphorylation of RPA S4/S8 indicative of reduced single stranded resected ends for ATR signaling. Transient expression of wild type BRCA1 in BRCA1-deficient MDA-MB 436 cells leads to increased pRPA S4/S8 in control cells with increasing time of treatment with olaparib and cisplatin; transient expression of T1394A and T1394I instead showed weaker pRPA S4/S8 **(Figure 4.15a, b)**. Intriguingly, T1394E, appears to have a similar to wild type induction of pRPA S4/S8 that is clearly stronger than both T1394A and T1394I. As MDA-MB-436 parental cells are BRCA1-null, it is possible that transient expression of exogenous BRCA1 may lead to excessive resected ends with increased pRPA S4/S8. It is also possible that out of cell cycle overexpression of BRCA1 can have negative outcomes in terms of overactivation of the DDR signaling pathway.

As pRPA S4/S8 are found in the most hyperphosphorylated form of RPA and can be subjected to multiple different regulations by members of the PIKKs targeting other phosphorylated residues on RPA[208], we next investigated other indicators of PIKKs activity such as pKAP1(ATM) and pCHK1 (ATR) to determine if transiently expressed T1394A and T1394E would also differ in terms of their effects on DDR **(Figure 4.15c, d)**. We observed that in control cells without DNA damage, transient expression of wild type

BRCA1 and T1394E led to increased pKAP1, suggesting elevated ATM activity that does not appear to be induced in parental cell and T1394A expressing cells **(Figure 4.15c lanes 1-4; Figure 4.15d lanes 1-4)**. Upon treatment with either olaparib or cisplatin, pKAP1 induction can be observed in parental MDA-MB 436 cells as the cells lacks the necessary HR machinery for survival **(Figure 4.15c lanes 5 and 9; 4.15d lanes 5 and 9)**. Even though protein expression of T1394E is lower than WT and T1394A, it is interesting to note that T1394E has the strongest induction of pRPA S4/S8 before and after treatment with 6hr or 28hr of 1μM olaparib **(Figure 4.15c lanes 4, 12 and 8)**. A similar trend was also observed in the cisplatin treatment set (Figure 4.14d lane 4,8,12). T1394A appears to not trigger a strong DDR response as WT or BRCA1-T1394E protein, with lower pKAP1 and pRPA S4/S8 before and after olaparib or cisplatin treatment. No discernible difference was observed for CHK1-S317 phosphorylation. Considering the distinct pKAP1 and pRPA levels observed between WT, T1394A and T1394E transiently expressed cells, it is important for us to also confirm this phenotype in the MDA-MB 436 BRCA-T1394 stable cell lines that we have generated.

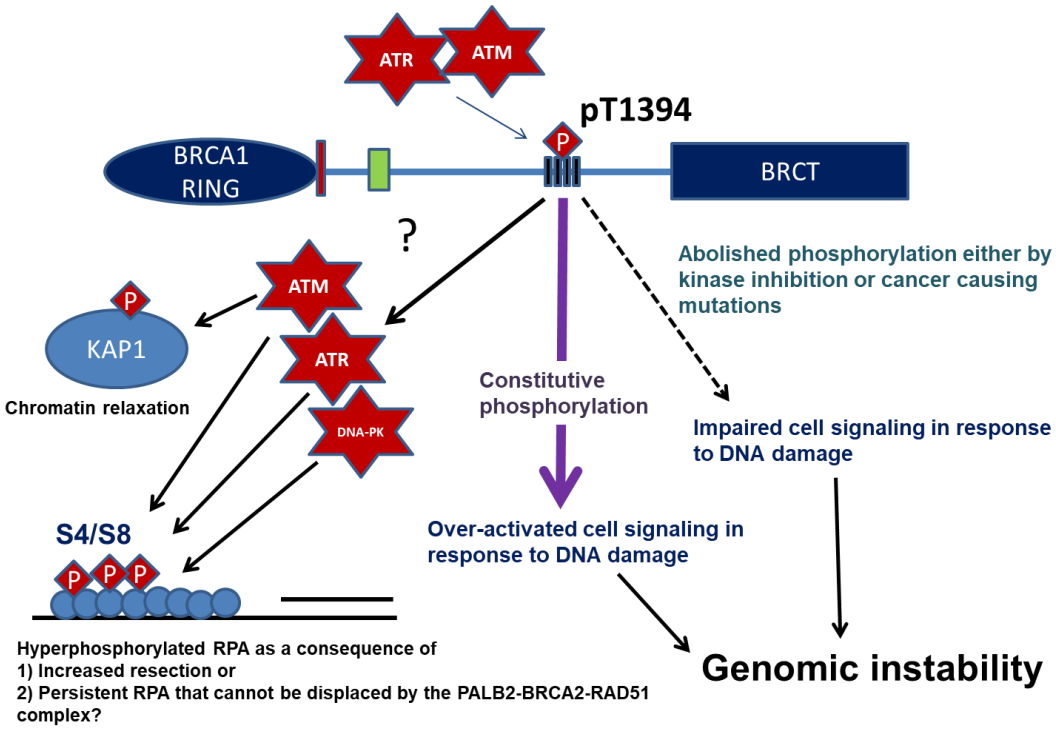

Figure 4.16: ATM/ATR-mediated phosphorylation of T1394 is a critical molecular switch in DDR

Phosphorylation of T1394 by ATM/ATR occurs in a DNA damage inducible manner. Cancer causing mutations affecting this phosphosite will lead to impaired DNA damage repair and cell signaling, resulting in elevated genomic instability. This phosphorylation event, however, also requires tight regulation as constitutive phosphorylation may lead to over-activated cell signaling pathways in response to DNA damage. Cells that adapted to constitutive pT1394 may instead acquire additional mutations to cope with the genomic instability. It remains unclear if the hyperphosphorylated RPA observed in the case of T1394E is a consequence of increased resection activity or inability of RAD51-mediated displacement of RPA.

Based on our observations, we first established that phosphorylation of BRCA1 at T1394 by ATM/ATR is critical step in DDR. Patient-derived VUSs that abolished the potential phosphorylation of this residue or chemical inhibition of ATM/ATR leads to reduced HR activity with a concomitant increase of SSA activity, an error-prone homology directed repair that also utilized resected DNA ends. Since HR and SSA activity are not drastically reduced as previously observed in RING and BRCT point mutants of BRCA1, it is likely that DNA resection is not impaired in T1394-associated mutations. However, we

cannot rule out if the resected DNA ends are utilized for HR in a timely manner. Impaired DDR signaling in response to DNA damage by T1394-related mutations as shown in the reduced HR activity, reduced RPA phosphorylation and also potentially pKAP1 are proposed to contribute towards genomic instability that is a key driver of tumorigenesis **(Figure 4.16)**. However, T1394E, mimicking constitutively phosphorylated T1394, showed greater HR activity that may point to increased resected ends for HR to utilize. Although it is possible that T1394E is instead a phosphomutant unable to be phosphorylated, we consistently observed significantly higher HR activity for T1394E or T1394-3E than all other T1394 phosphomutant; this supports a clear separation of function within T1394-mutations with T1394A and all other patients-derived mutations lead to reduced HR (~60—70%), while T1394E can still retain ~80% HR activity. However, such a constitutive state of DNA resection in the case of T1394E may pose additional stress on the cell as the PALB2-BRCA2-RAD51 complex may not be recruited efficiently for displacement of RPA. Such an exhaustive use of RPA, may lead to massive DNA breakage, genomic instability and, ultimately, cell death if the T1394 phosphorylation is not effectively removed.

4.4 Discussion

The DNA damage response (DDR) is a critical barrier to tumor initiation with most pre-malignant cells showing increased endogenous DNA damage and activation of the DDR before acquiring additional genetic alterations that lead to malignant transformation. The major breast cancer susceptibility protein BRCA1 plays critical roles in multiple aspects of the DDR, particularly HR-mediated repair of DNA double strand breaks (DSBs) and cell cycle checkpoint control, thereby maintaining genome integrity and suppressing cancer development. BRCA1 functions as a differential mediator of chemotherapy-induced apoptosis, whereby it sensitizes cells towards anti-microtubule agents (paclitaxel

and vinorelbine) but also confer resistance towards DNA damaging agents (cisplatin, 5-fluorouracil etoposide or bleomycin) ; G2/M arrest was observed in response to both type of chemotherapeutic drugs[274]. Restored or upregulated expression of BRCA1 in chemotherapy resistant cancer cells is a great hurdle that needs to be overcome for effective treatment. A comprehensive understanding of the effects of BRCA1 phosphorylation opens the possibility of utilizing kinase inhibitors as a possible target for BRCA1 tumor suppressor function. In this study, we sought to provide a detailed look at the effects of BRCA1 phosphorylation on its tumor suppressor function, specifically Homologous recombination (HR), suppression of Single Strand Annealing (SSA) and drug sensitivity. We generated mutations of 8 different BRCA1 phosphorylation sites (5 ATM/ATR, 3 CDK and 1 PLK1 sites) **(Table 4.1)** that have been reported to either be phosphorylated or has an effect on the tumor suppressor function of BRCA1.

To understand the precise role of ATM/ATR-mediated BRCA1 phosphorylation, we first generated single amino acid substitutions of the widely cited S/Q sites in BRCA1. We have previously reported that single serine to alanine (S to A) mutations at S1387, S1423 or S1524 do not affect the BRCA1-PALB2 interaction [398]. These three sites, together with S1457, have been widely assumed to be the most important ATM/ATR sites in BRCA1. By immunoprecipitation (IP) of exogenously expressed BRCA1 proteins, we confirmed that abolishing these SQ sites not only individually but also in combination (4A) does not affect PALB2-binding. We further tested the functions of these SQ residues using the well-established U2OS/DR-GFP HR and U2OS/SA-GFP SSA reporter cells to quantitatively assess HR and SSA activities. I did not observe any significant difference between wt and mutant BRCA1 other than a small reduction of HR and small increase of SSA when all 4 sites were mutated to alanine. Then, I used our recently developed colony formation assay [11] to assess the roles of these phosphorylation sites in drug sensitivity. When mutated individually, none of these widely cited SQ sites were found to reduce

colony formation; double mutation of S1387 and S1423 or quadruple mutation of all 4 sites indeed reduced, but only moderately, colony formation in the presence of the two agents.

Our results also raise an intriguing question in the context of understanding BRCA1 phosphorylation at the SQR domain. The two closest phosphorylation sites flanking the PALB2 binding domain of BRCA1 are T1394 and S1423 respectively. The true *in vivo* function of S1423 is harder to determine in mice, as mice lack a S1423 site. The mice equivalent of this residue is an Asparagine (N1379) which instead carries a positive charge **(Figure 4.3)**. Considering that phosphorylated Serines are often negatively charged, such a non-conserved substitution (Serine to Asparagine) in mice suggest that S1423 phosphorylation in mouse cells may not be essential compared to other organisms. Since the three phosphorylatable residues (S1387, T1394, S1423) are positioned closely to one another, it possible that S1387/T1394 phosphorylation compensates for the loss of S1423 phosphorylation. We also observed that phosphorylation of S1423 are induced following DNA damage, as well as, loss of PALB2. This observation supports the complex roles of BRCA1-PALB2 interaction in potentially modulating the phosphorylation states of BRCA1 and PALB2. We and others observed that PALB2 phosphorylation is dependent on the presence of BRCA1 with the BRCA1-PALB2 interaction potentially reconfigure the coiled-coil domain structures into a state that is more accessible for protein kinases to phosphorylate PALB2. In the case of BRCA1 SQR phosphorylation, however, an opposite effect was observed. Increased phosphorylation of BRCA1 pS1423 upon siRNA-mediated knockdown of *PALB2* or PALB2-deficient cell lines suggest the role of BRCA1-PALB2 interaction in possibly limiting the accessibility of protein kinases to phosphorylate BRCA1.In HeLa cells, dynamic PALB2 N-terminal phosphorylation by CDK and ATR was reported to be critical in regulating the BRCA1-PALB2 interaction during the cell cycle[38]. Increased BRCA1 interaction was observed in PALB2 phosphorylated at S59 (an ATR site), with a hypo-phosphorylated S64 (a CDK site)[38]. In the case of BRCA1

phosphorylation surrounding the PALB2-binding domain, we did not observe any significant difference in the PALB2 interaction by BRCA1 4SQA or even the T1394-3A mutants that covers of most of the SQ residues closest the coiled-coil domain. With the exception of T1394E, we did not observe any significant difference in the HR/SSA activity of S1387E, S1423E and S1387E/S1423E phospho-mimetic mutants **(Figure 4.6c)**, indirectly excluding the potential effects of S1387 and S1423 phosphorylation on BRCA1-PALB2 binding.

In vivo ATM dependent phosphorylation of BRCA1 S1387, S1423, S1457, S1524 and S1542 in response to IR had been previously reported in accordance with rapid ATM kinase activity after IR [119]. However, delayed BRCA1 phosphorylation still occurs in cells lacking ATM activity supporting the roles of other kinases such as ATR in BRCA1 phosphorylation[68, 119]. Moreover, the exact functional significance of these phosphorylation events remains poorly understood in the context of DNA damage repair. Using a (DR)-GFP reporter-based mouse model to study HDR in primary cell types derived from diverse lineages, Kass et al showed that ATM is dispensable for homology directed repair in primary somatic cells (fibroblasts, glial, and mammary epithelial cells) even though chemical inhibition of ATM does affect HDR and MMC sensitivity[173]. Such an observation agrees with previous findings that low dose of UV, MMC and HU treatment leads to S-phase phosphorylation of BRCA1 in an ATM, DNA-PK independent manner [303]. In fact, ATR has been observed to phosphorylate BRCA1 on 6 Ser/Thr residues (S1143, S1280, S1387, T1394, S1423 and S1457) within the SQR domain [342].Since ATM is dispensable in the case of HDR, it would be important to us to re-examine if other kinases such as ATR is acting redundantly to phosphorylate BRCA1 for HDR and whether or not such a phosphorylation event can affect PALB2 binding to BRCA1. It is also possible that phosphorylation of BRCA1 SQ sites have no effect on HR but instead on other cellular processes associated with BRCA1 function that can be manipulated pharmacologically.

Phosphorylation of S1387 was reported to be critical for the intra-S checkpoint, while S1423 phosphorylation was important for the G2/M checkpoint following IR[382, 383]. Although Serine to Alanine mutation for most of the SQR phosphorylation do not affect HR and SSA activity, combination of S1387A/S1423A leads to partial sensitization to cisplatin and olaparib. BRCA1-deficient HCC1937 cells stably expressing S1387A, S1423A and S1423A/S1524A were observed to be radioresistant[383]; therefore, the relative contributions of cell cycle checkpoint functions of these phosphorylation in drug sensitivity remains poorly understood. Nonetheless, single phosphomutants of S1387A, S1423A and S1524A indeed do not affect HR, SSA activity and drug resistance. The partial drug sensitivity observed in cells expressing S1387A/S1423A is consistent with a previous report on UWB1.289 (BRCA1-null human ovarian cancer cell line) that showed partial sensitization to MMC when S1387A/S1423A was re-expressed[20].

S1387A/S1423A/S1524A were previously reported to be unable to support mouse ES cell viability as it is not expressible[56], while we could see a slight rescue of cisplatin and olaparib resistance with ~90% HR activity. S1423A/S1524A, on the other hand, was observed to support mouse ES cell viability. Yet, the radiation sensitivity of cells expressing S1423A/S1524A is contradictory as a previous mouse ES cell system proposed that it sensitized cells to IR, while another study using BRCA-null HC1397 re-expressing this phosphomutants reported no significant difference in terms of its radiosensitivity. Moreover, combinations of different BRCA1 SQR mutations leads to varied drug sensitivity despite similar to wild type HR and SSA activity. Our colony formation assay results also suggest that most BRCA1 phosphorylation events may be redundant but can still be mutually exclusive as shown in the context of drug resistance conferred by different combinations of S1387A/S1423A such as S1387A/S1423A/S1457A (3A1), S1387A/S1423A/S1524A (3A2), S1423A/S1457A/S1524A (3A3) and S1387A/S1423A/S1457A/S1524A (4SQA) **(Figure 4.6d)**. In the case of 3A1, 3A2, it is

possible that the constitutive phosphorylation of S1457 and S1524 are redundant of each other with either phosphorylation being critical for cell survival only after the other phosphorylation is abolished. Interestingly, S1423A/S1457A/S1524A (3A3) leads to a partial resistance towards olaparib, similar to S1387A/S1423A or 4SQA.Whether or not S1387 phosphorylation itself is sufficient to compensate for both S1457 and S1524 phosphorylation remains uncertain. Overall, these data suggest that ATM/ATR phosphorylation of BRCA1 in widely reported SQ sites has limited impact on HR and suppression of SSA; however, phosphorylation on multiple sites can collectively contribute to cellular resistance to DNA damaging agents, possibly through modulating DNA damage signaling.

Recent studies have reported sensitization of myeloma, tamoxifen resistant breast cancer cell lines and TNBC cells to either cisplatin or PARP1/2 inhibitors in combination with dinaciclib, a CDK 1, 2, 5, and 9 inhibitor, with notably reduced expression of HR repair genes, BRCA1 S1497 phosphorylation and BRCA1/RAD51 foci formation following damage [5, 165, 256, 404]. Although the application of CDK inhibitors to directly target BRCA1 phosphorylation as a mode of altering the efficiency of HR is indeed an interesting concept, it is more likely that the effects of CDK inhibition may have a more global effect on multiple cellular pathways independent of BRCA1 function. In fact, treatment of dinaciclib not only inhibit CDK-mediated phosphorylation of BRCA1 but also reduces the transcription of BRCA1 mRNA[5, 165, 404]. Surprisingly, in our study, we did not observe a drastic reduction of cellular sensitivity by the BRCA1 triple CDK mutants to either cisplatin or olaparib as previously reported[163, 164]. It is possible that the MDA-MB-436 stable cell lines generated and used in the two studies have accumulated unknown adaptations that affects its DNA repair abilities as Johnson et al (2009) observed drastically reduced BRCA1 foci formation following DNA damage in BRCA1 CDK mutants[163]. Additionally, two independent mouse ES cell systems provide some confounding results on the true nature

of BRCA1 CDK phosphorylation with ES cells stably expressing BRCA1-S1497A was reported to be viable, sensitive towards ionizing radiation by one group [56] , but resistant to cisplatin in another separate study [29]. We previously reported that mis-localization or impaired recruitment of BRCA1 by deletion of its nuclear localization signal (NLS) will lead to impaired HR activity together with a reduced SSA activity[11]. The CDK mutants in our study do not exhibit reduced HR and retained similar to WT levels of SSA suppression, indirectly confirming that the recruitment of BRCA1 CDK mutants are largely unaffected.

Polo-like kinase 1 (PLK1) is widely studied for its function during mitosis. When G2-phase cells are exposed to DNA damage, the activity of PLK1 is actively downregulated to prevent entry into mitosis and is required to restart the cell cycle, a process called checkpoint recovery[321, 354]. While several studies have proposed the roles of PLK1 phosphorylation in regulating the functions of HR proteins such as BRCA1[55, 175], BRCA2[196, 335] and RAD51[387], the intricate regulation of PLK1 activity and its substrate HR function during DDR remains poorly understood. Using a patient-derived VUS, S1164I, we established that PLK1-mediated phosphorylation at this specific site is also dispensable for the BRCA1 functions tested.

To search for potential phosphorylation sites that may indeed play a key role in regulating BRCA1 DNA repair function, we shifted our attention to T1394. This highly conserved threonine is followed by a glutamine, with the two constituting a TQ site that has been predicted to be a target of ATR phosphorylation [342]. However, it has never been detected by mass spec, due presumably to a lack of lysine or arginine residues in its near vicinity to yield detectable peptides upon trypsin digestion, and has never been functionally characterized **(Figure 4.11a)**. Like S1387 and S1423, T1394 is located very close to the BRCA1 coiled-coil motif (aa1397-1424), where PALB2 binds. Remarkably, single substitution of T1394 with an alanine (T1394A) significantly impaired BRCA1 function in supporting HR, suppressing SSA and conferring drug resistance. Note that the impact of

this single mutation was more profound than that of the quadruple mutation of all 4 commonly cited sites **(Figure 4.8-9)**.

To confirm that T1394 is indeed phosphorylated by ATR/ATM, we generated a phosphospecific antibody for this site, and my results confirmed that T1394 is indeed a target of ATM/ATR following DNA damage **(Figure 4.12).** Given that reduced HR activity with a concomitant increase in SSA activity have only been observed in PALB2 binding mutants of BRCA1 [11], our results suggest that phosphorylation of T1394 may affect PALB2 binding. Yet, the T1394A mutation does not appear to affect PALB2 binding as assessed by co-IP experiment **(Figure 4.8)**. Moreover, the T1394A mutation does not affect the early recruitment of BRCA1 to UV laser-induced DNA damage **(Figure 4.13)**, raising the possibility that the kinetics of BRCA1 or PALB2 recruitment, particularly at later time points may be affected or the composition or dynamics of BRCA1-containing protein complexes may be altered.

To assess the possible role of T1394 in BRCA1 tumor suppression function, I also searched the ClinVar and cBioPortal databases for patient-derived mutations or variants of this and the following Q1395 residues **(Table 4.3)**. Given the challenge to assign clinical relevance to VUS in cancer genes, their functional characterization is important for better clinical management. A total of 5 variants of these 2 residues are listed in the databases with at least 13 cancer patients identified. One of these variants, T1394I, has been found in at least 4 different breast cancer patients but is still classified as a VUS. Interestingly, BRCA1-T1394I showed an HR activity even lower than the T1394A mutant **(Figure 4.9b)** with its PALB2 binding seemingly unaffected before and after ionizing radiation **(Figure 4.12d)**. Moreover, transient transfection of BRCA1-T1394I and other Q1395 variants, such as Q1395H and Q1395R, also failed to rescue the sensitivity of MDA-MB-436 cells to either cisplatin or olaparib in our colony formation assay **(Figure 4.9d)**. Together, my

preliminary results suggest a critical regulation of BRCA1 function by ATR/ATM through phosphorylation of T1394, with the exact mechanism still unclear.

A previous Transcription Activation (TA) luciferase-based reporter assay, based on a GAL4 DBD:BRCA1 fusion protein and the firefly luciferase gene under the control of a GAL4 responsive promoter as a reporter, did not detect any significant difference for most BRCA1 coiled-coil mutations with the exception of known deleterious VUSs such as L1407P and M1411T[375]. Although the TA assay is a classical approach for assessing BRCT domain mutations due to the highly acidic nature of the BRCT domain[233], it may not be suitable to discern the effects of coiled-coil domain mutations that was not known to have any DNA binding abilities. In fact, the aforementioned TA reporter study did not observe any significant difference in terms of the effects of T1394I and Q1395R on BRCA1 transcriptional activation activity. Here, we are able to determine the potential pathogenic nature of patient-derived mutations (T1394I, Q1395R, Q1395H) affecting T1394 with reduced HR activity with concomitant de-repression of SSA. The T1394-VUSs also consistently showed sensitivity towards cisplatin and olaparib similar to T1394A mutants. Identification of T1394 phosphorylation as a potential target provides additional evidence in the context of potentially sensitizing cancer cells to chemotherapy via direct inhibition of BRCA1 function using PIKK inhibitors.

Since the foci forming ability of T1394-derived mutant BRCA1 proteins are unaffected, it is possible that these mutations affect PALB2 binding through subtle but deleterious alterations on protein folding. Compared to well established PALB2 binding hypomorphs such as M1400V, L1407P or M1411T, T1394 mutant BRCA1 retained similar to WT level of PALB2 binding. It is possible that positioning of the BRCA1-PALB2 complex is affected by T1394, as evinced in the reduced HR activity with a concomitant increase of SSA derepression. Prediction modeling of Q1395R suggest that the mutation do not affect the stability of the coiled-coil in comparison with pathogenic mutations such as

L1404P or L1407P[375]. Unfortunately, there is currently no known PALB2-BRCA1 structure available to date to determine the possible outcome of T1394 phosphorylation. As the T1394 residue is most likely positioned within a loop region or positioned at beginning of the coiled-coil domain helix, it is possible that phosphorylation of this specific residue maybe critical in modulating a timely transition between the different BRCA1-containing complexes to the BRCA1-PALB2-BRCA2 homologous repair machinery.

A limitation of our study is the use of an overexpression system for most of the assays performed to investigate the effects of specific BRCA1 phosphorylation on its functions. It is possible that abolished phosphorylation at specific residues may impact the HR and SSA suppression activity at levels that are not fully captured by the HR and SSA reporter assays. We previously defined a HR activity of ~55% to represent the cut off threshold that captures the specificity (the ability to detect variants showing loss of BRCA1 function) and sensitivity (the ability to detect variants retaining wt activity of BRCA1) of the HR assay at 100%, respectively[11]. The colony formation assay used in this study is a more direct and unbiased approach in identifying the effects of BRCA1 phosphorylation because we focused on examining any potential effects of phosphorylation on HR over a longer period of time than what is accomplished for the gene conversion assays.

4.5 Future directions

It is important to acknowledge that obtaining patient-derived cells harboring mutations that can possibly affecting BRCA1 phosphorylation is a challenging task due to the rarity of VUS itself. Overexpressed system may be informative in terms of confirming the deleterious nature of impaired BRCA1 phosphorylation, as shown in the case of BRCA1-T1394 mutations, but it remains insufficient, especially in terms of understanding the impact of these mutations on other components of DDR such as cell cycle. Therefore,

the best way to investigate the physiological roles of BRCA1 phosphorylation on DDR would be to utilize knockin mutations generated on the endogenous BRCA1 allele.

The 1394-TQ-1395 motif is conserved in the mouse, with T1350 being the mouse equivalent of human T1394. In order to define the role of T1394 phosphorylation *in vivo*, we attempted to generate a *Brca1*^{T1350I} knockin mouse strain using the CRISPR-Cas9 technology The effort came to fruition very recently, and we in fact obtained several homozygous *Brca1*^{T1350I} mice among the founders. These mice are viable and so far do not appear to have any major developmental defects or overt phenotypes by 8 weeks of age. Given the partial HR defect of the human BRCA1-pT1394, the homozygous mice being viable is not surprising. Importantly, this allows us to thoroughly analyze the impact of this mutation on the DNA damage response *in vivo* and on BRCA1-mediated tumor suppression. In fact, partial loss of HR activity may well lead to tumor development, as is the case of *Brca2* mutant mice with a mutation that partially affect PALB2 binding[131]; partial loss of HR activity may instead strike a balance between genome instability and cell fitness allowing affected cells to both accumulate mutations and proliferate without too much hardship. Splenic B cells and mouse embryonic fibroblast (MEFs) from the *Brca1*^{T1350I} knockin mice can be isolated to perform IF, neutral comet, metaphase spread and drug sensitivity assays to assess the extent of HR and other potential DNA repair defects. IR- and drug-induced RAD51 foci formation will be informative to determine if T1350 is critical for the recruitment of the PALB2/BRCA2 complex. Neutral comet assay and metaphase spread analysis will determine the presence of double stranded breaks and the types of chromosomal aberrations that may arise due to loss of T1350 phosphorylation.

ATM and ATR are central regulators of DNA damage-induced cell cycle checkpoints. Several studies have implicated BRCA1 in both the intra-S and the G2/M checkpoints [178, 202, 384]. In particular, phosphorylation of (human) BRCA1 at S1387 and

S1423 has been implicated in inta-S and G2/M checkpoints, respectively [382, 383] Very recently, we demonstrated that cells derived from mice with a knockin mutation in PALB2 that disengages its interaction with BRCA1 (*Palb2*^{CC6}) [317] show significant defects in both the activation and maintenance of the G2/M checkpoint[318] To study the role of BRCA1 T1350 phosphorylation in regulating cell cycle checkpoints, I will treat B cells and MEFs from the wt and *Brca1*^{T1350I} mice with IR to determine the mitotic population and DNA synthesis ATM is often considered as the critical kinase involved in G2/M checkpoint activation, while ATR is more associated with the maintenance of the checkpoint. Depending on the types of G2/M checkpoint defects observed, it may be a direct evidence of ATM/ATR phosphorylation promoting BRCA1-mediated checkpoint regulation.

In addition to genome instability, HR-defective tumors show increased replication stress that subsequently leads to stronger dependency on ATR-mediated replication checkpoint signaling, which can then be targeted by ATR inhibition [189]. A better understanding of how ATM/ATR and BRCA1 function cooperatively to promote DSB repair and replication fork stability has significant implications in cancer therapy. Independent from its HR function, BRCA1 is also important for mitigating replication stress. It is well established that BRCA1/2 is critical in protecting nascent DNA at the replication fork from MRE11-mediated degradation via stabilization of RAD51 filament formation during HU-induced replication stress [133, 183, 198, 279, 300, 334, 390]. ATM and ATR also play key roles in replication stress through modifications of its target proteins such as replisome complex proteins and HR factors. However, little is known about the roles of these master kinases in regulating the BRCA1-PALB2-BRCA2 axis in the context of replication stress.

Using B cells isolated from the *Brca1*^{T1350I} mice, I will also study the effects of abolished T1350 phosphorylation on DNA replication. I will label the cells with IdU and CldU and perform DNA combing/fiber analysis to examine different aspects of DNA

replication such as origin firing, fork progression, stalling, protection, and restart before and after DNA damage. We previously reported that the BRCA1-PALB2 interaction is critical for the suppression of RAD52-mediated SSA [11]. Recent studies have also suggested that loss of RAD52 may alleviate the degradation of stalled replication forks in BRCA2-deficient cells [137, 232]. However, whether there is any interplay between the BRCA1/PALB2/BRCA2 complex and RAD52 during replication stress remains unknown. Since the SSA suppression activity of BRCA1 T1394 mutants was weakened, I would expect to observe a mis-localization of RAD52, resulting in increased fork degradation during stalled replication fork. This can be tested by BRCA1/RAD52 double staining on IF.

Despite strong HR defects observed *in vitro*, several whole-body knockin mutant mice in BRCA1, PALB2 or BRCA2 have been reported to be viable, with homozygous male mice exhibited reduced fertility due to germ cell loss [86, 131, 285, 307, 317]. So far, only two BRCA1 phosphorylation mutant mice have been reported, specifically S971A (CHK2 site) and S1164A (CDK/ATM site). *Brca1*$^{S971A/S971A}$ mice developed normally but are susceptible to spontaneous tumor formation, with increased incidence of IR-induced lymphoma [178]. The *Brca1*$^{S1164A/S1164A}$ mice also developed normally but 40% of them exhibited aging-like phenotypes including growth retardation, skin abnormalities, and delayed mammary gland morphogenesis. Similar to the *Brca1*$^{S971A/S971A}$ mice, *Brca1*$^{S1164A/S1164A}$ mice are also prone to IR-induced lymphoma [179]. It is important to note, however, that I have tested the HR and SSA activities and drug sensitivities of cells expressing BRCA1 mutants of both of these sites and neither showed any effect (data not shown). This fact makes the potential effect of the T1350I mutation all the more interesting to study.

It would be important to determine if the *Brca1*^{T1394I} mice are also susceptible to radiation-induced tumors such as but not limited to lymphoma, osteosarcoma, breast and lung tumors. For the *Brca1*^{T1350I} knockin mouse that we have generated, I can set up

cohorts of mutant mice (and wt controls) and monitor both spontaneous and radiation-induced tumor development. I will treat the mice with 3 doses of 3 Gy of IR , one week apart, starting from 8 weeks of age, as we did before[219]. Considering the hypomorphic nature of human T1394I, we would expect *Brca1*^{T1350I} knockin mouse to be susceptible to radiation-induced tumors.

Chapter 5: Overall summary

The work presented in this thesis focused on delineating the biological roles of BRCA1-PALB2 coiled-coil domain interaction, particularly on utilizing patient-derived VUS as means to identify novel BRCA1 function and possibility therapeutic targets in *BRCA*-associated cancers. No known structural information of the BRCA1-PALB2 interaction is available to date. The BRCA1-PALB2 coiled-coil domain is still potentially targetable due to the different post-translational modifications (PTMs) flanking this domain. PTMs such as phosphorylation and ubiquitination can be explored further as means to modulate the interaction between BRCA1 and PALB2. The BRCA1-PALB2 interaction is not only important for HR and suppression of error prone repair pathways, but also for efficient cell cycle checkpoint to ensure optimal repair timing when DNA damage occurs.

Interpretation of patient VUS is clinically challenging due to limited functional information of a specific VUS. In chapter 3, we investigated several N-terminal coiled-coil domain mutants to not only determine the critical residues involved in PALB2 function but also to assess if varied repair ability would translate equally to chemotherapy sensitivity. Although *PALB2* c.104T>C, p.L35P behaves as expectedly as a bona fide pathogenic missense variant, our results on other hypomorphic mutations such as c.83A>G , p.Y28C raised an interesting question on the complexity of VUS. The HR ability of cells often correlate well with drug resistance as genotoxic stress are often alleviated by HR. Unlike pathogenic mutations that often resulted from frameshift mutations, hypomorphic PALB2 missense mutation that still retained HR activity above a poorly understood threshold is a challenge for personalized treatment of cancer patients. While defective HR activity can increase the risk of tumor development, the exact threshold necessary to confer chemosensitivity remains unclear. As shown in the case of Y28C, hypomorphic missense mutations maybe defective in comparison to wt or other neutral VUS but cells expressing

this type of variant are still resistant to DNA damaging chemotherapy. It is clear that not all missense mutations on a single specific amino acid behaves similarly; one mutation may confer ~30% of WT PALB2 activity while another variant that is not well tolerated will behave similar to a completely pathogenic mutation (L24F vs L24S or Y28C vs Y28N). These observations also confirm the hydrophobic nature of coiled-coil domain interaction with hydrophilic or polar substitutions have a more severe effect on PALB2 function.

DDR is often considered as a critical barrier during tumor initiation with most pre-malignant cells accumulate endogenous DNA damage before acquiring additional genetic alterations that provides a survival advantage. Recent, ongoing clinical trials on altering DDR focused on targeting the ATR-CHK1 network as tumors with defects in the G1 checkpoint (or *P53* mutated) are believed to rely more on ATR-CHK1 activity, and consequently more susceptible to ATR/CHK1 inhibition. Chapter 4 explores potential direct relationship between known protein kinases such as ATM/ATR, CDK and PLK1 on BRCA1 function. BRCA1, a critical DDR protein, can function in various component of DDR network of responses. Hence, understanding the effects of PIKK-mediated phosphorylation on BRCA1 will lead to better understanding of the direct relationship between PIKK and BRCA1 function. Several S/TQ sites on BRCA1 were widely reported based on either in vitro biochemical reactions or mass spectrometric analysis. Moreover, the BRCA1-PALB2 binding domain is positioned within this S/T rich region, raising the possibility that BRCA1-PALB2 interaction can either be affected by specific phosphorylation events or vice versa. Our experimental results focusing on the HR and SSA suppression activity of widely reported SQ BRCA1 mutants suggest that most of these phosphorylation events may not be as critical as previously proposed in the literature. However, we observed that the drug resistance of cells expressing distinct serine to alanine combination phosphomutants are partially reduced despite minimal effects on HR activity.

S1387, T1394 and S1423 are phosphorylatable residues immediately flanking the PALB2 binding coiled-coil domain. Here, we report that abolishing phosphorylation of both S1387 and S1423 leads to partial sensitization of cells to both cisplatin and olaparib despite similar to wt HR activity. T1394 phosphorylation, although undetectable in most mass spectrometry studies to date, appears to be a critical event as single amino acid alteration (artificial and patients derived VUS) on this site is sufficient to partially reduce HR activity and completely sensitized cells towards cisplatin and olaparib. Interestingly, the effects of modulating these S/TQ phosphosites appears to be independent of PALB2 binding. It is important to note that cells expressing T1394 mutants showed reduced HR and increased SSA activity that is often observed in PALB2 binding hypomorphs of BRCA1. Phosphospecific antibody against pT1394 confirms that this residue is potentially a ATM/ATR target. Understanding how T1394 phosphorylation affects DSB repair pathway choice following resection will have at least two implications: first, it will provide new insights on the dynamic nature of BRCA1 phosphorylation near the PALB2 binding domain to better understand if any previously unknown intermediaries dictating HR/SSA choice exists; second, it will help to identify possible molecular targets that may specifically bind only to phosphorylated BRCA1.Results and observations of this study will hopefully contribute to improvement in our knowledge of DDR by targeting BRCA1 function, while assessing the potential of ATM/ATR inhibition for cancer therapy.

In conclusion, this thesis offers new insights in understanding the biological relevance of the BRCA1-PALB2 coiled-coil domain interaction, particularly, as therapeutic targets in *BRCA*-associated cancers. My observations established that BRCA1-PALB2 interaction is critical for maintenance of genome stability and suppression of cancer development. Moreover, our data offers support for better risk assessment and clinical decision making for carriers of BRCA1 and PALB2 mutations affecting the BRCA1-PALB2 interaction.

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