UNIVERSITY OF BIRMINGHAM

INTERPLAY BETWEEN REGULATION OF PROMOTER PROXIMAL PAUSING, BRCA1 AND PARP INHIBITOR, AND THEIR EFFECTS ON G-MIDS

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Content

Abbreviation	Full name	
APH	aphidicolin	
BER	Base Excision repair	
BRCA1	Breast Cancer Type 1 Susceptibility Protein	
BRCA2	Breast Cancer Type 2 Susceptibility Protein	
BrdU	Bromodeoxyuridine	
CDK	cyclin-dependent kinase	
CFS	Common Fragile Site	
COBRA1	Cofactor Of BRCA1	(Also known as NELF-B)
CPSF	cleavage and polyadenylation specificity factor	
CSTF	cleavage stimulation factor	
CTD	C-terminal domain	
DDR	DNA Damage Response	
DMEM	Dulbecco's Modified Eagle Medium	
dNTP	deoxyribonucleotides	
DSB	Double Strand Break	
DSIF	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) sensitivity inducing factor	
EdU	5-ethynyl-2′-deoxyuridine	
ERα	Estrogen Receptor α	
FA	Fanconi Anaemia	
FBS	Fetal bovine serum	
G-MiDS	G2/M DNA synthesis	
HR	Homologous Recombination	
HSF	heat shock factor	
hsp	heat shock protein	
ICL	Interstrand Crosslink	
IEG	Immediate Early Gene	
IR	Ionizing radiation	
KOW motif	Kyprides, Ouzounis, Woese motif	
MCM	Minichromosome Maintenance Complex Component 7	
MiDAS	Mitotic DNA Synthesis	
MMEJ	Microhomology-Mediated End Joining	
MMR	Mismatch Repair	
NELF	Negative Elongation Factor	
NER	Nucleotide Excision Repair	
NHEJ	Nonhomologous End Joining	
OB folds	oligonucleotide/oligosaccharide binding fold	
PAF1	Pol II-associated factor 1	
PALB2	Partner And Localizer Of BRCA2	

PAR	Poly-ADP-Ribosylation	(Also known as parylation)
PARP	Poly-ADP ribose polymerase	
PARPi	PARP inhibitor	
PIC	pre-initiation complex	
PPP	Promoter Proximal Pausing	
P-TEFb	positive transcription elongation factor b	
RD motif	Arg-Asp dipeptide repeats	
RNA Pol II	RNA Polymerase II	
rNTP	ribonucleotides	
ROS	Reactive Oxygen Species	
RPA	Replication Protein A	
RRM	RNA recognition motif	
RTC	Replication-Transcription Collision	
Ser	serine	
Ser-2P	serine-2 phosphorylation	
Ser-5P	serine-5 phosphorylation	
SETX	Senataxin	
SUPT6H/SPT6	Suppressor Of Ty 6	
SSB	single strand break	
SUPT4H1/SPT4	Suppressor of Ty4	
SUPT5H/SPT5	Suppressor of Ty5	
TFIID	TATA-binding protein (TBP)-containing transcription factor IID	
Thr	threonine	
TSS	Transcription Start Site	
Tyr	tyrosine	
UTR	3'-untranslated region	
UV	ultraviolet	
WHSC2	Wolf-Hirschhorn Syndrome Candidate 2 Protein	(Also known as NELF-A)

Abstract

Previously in the lab we found that RNA Pol II persists around TSS region and blocks replication progression throughout S phase, it subsequently leads to under-replication of TSS region. It has been shown that cells complete duplication of this region using G2/M DNA synthesis after RNA Pol II removal. Promoter proximal pausing is a key step to regulate transcription, and it is regulated by DSIF and NELF complexes. By modulating factors (SUPT5H and NELF-A) involved in regulating promoter proximal pausing, we can control the removal of RNA Pol II from TSS region and alter the level of G2/M DNA synthesis. Additionally, PARPi has been shown to increase mitotic DNA synthesis, and NELF is a known target for PARPi. Here we show that PARPi can also increase G2/M DNA synthesis. Our studies in U2OS cells showed that depletion of SUPT5H and NELF-A leads to no difference in PARPi sensitivity comparing to control cells but failed to rescue PARPi hypersensitivity in BRCA1 knockdown cells. Importantly, we discovered functional links between BRCA1 and BRCA2 with PPP factors. Knockdown of SUPT5H, BRCA1 and BRCA2 exhibit similar pattern of transcriptional defects, and co-depletion of either SUPT5H or NELF-A with BRCA1 can induce synthetic lethality. This may have an impact on the survival/relapse of BRCA1/BRCA2 mutated patients, by analysing BRCA1 and BRCA2 mutant ovarian and breast cancer patients' clinical data, we found some associations between the expression level of DSIF and NELF complex subunits and patients' overall survival and relapse rate.

(245 words)

Introduction

DNA damage response and tumorigenesis

Several hallmarks of cancer cells have been described over the past 30 years. These include avoiding apoptosis, replicative immortality, uncontrolled proliferation, and the ability to escape from growth suppressors and the immune system, invading surrounding tissues and metastasis [1,2]. One of the most important mechanism underlying these features of cancer cells is genome instability. DNA damages generated by either endogenous (E.g., replication) or exogenous (E.g., radiation) sources are the main threat to maintaining genome stability. Accumulation of genome instability due to replication stress (E.g., defective replication forks progression), DNA damage response (DDR) defects and mutations in genes involved in DDR and cell cycle checkpoints, has been proved to disrupt genome stability and genome integrity [2,3,4]. These are classified as internal triggers for tumorigenesis. Especially replication stress caused by oncogene activation (E.g., oncogene activation can disrupt replication initiation in various ways, such as origin over-usage, under-usage or re-usage [3-6]) and stalled replication forks are key drivers for such mutagenesis in cells [3,7-10]. To prevent aforementioned mutagenesis, cells employ tight cell cycle progression control and DDR. Depending on cell cycle stage and type of lesion, different repair pathways are used to protect genome stability and integrity.

During cancer development, tumour cells need to avoid cell death triggered by programmed cell death, immune cell detection and antitumor drug treatment. Therefore, cancer cells acquire certain levels of genomic changes helping them to

bypass cell cycle checkpoints, avoid cell apoptosis, adapt to the environmental selection pressure, and build up resistance to certain treatment, e.g., chemotherapy [11]. Therefore, an accumulation of nucleotides variations, deletion, insertion, and chromosomal abnormalities (e.g. chromosomal exchange, insertion, and deletion) would gradually build up within cancer cells, which can be potentially the underlying mechanism of drug resistance [11].

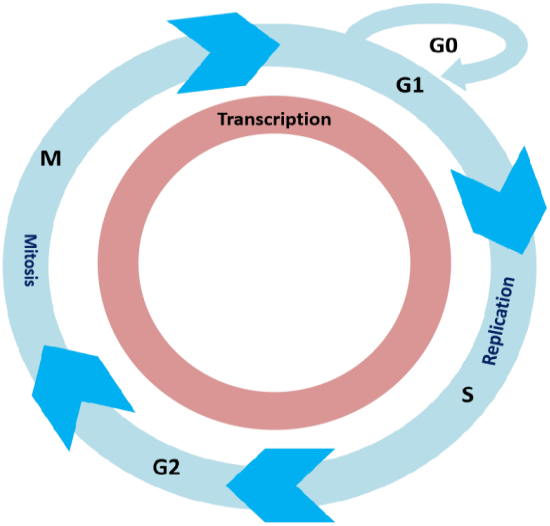


Figure.1 Cell cycle and checkpoints. Quiescent or resting cell in a metabolically active state without undergoing DNA synthesis and cell division is set as G0 phase. The cell cycle is divided into four major phases: G1, S, G2 and M phase. Quiescent cells enter G1 phase to prepare themselves for DNA replication in S phase, subsequently, G2 phase is for cells to get ready for cell mitotic division in M phase. Two daughter cells either enter G1 phase to replicate again or exit cell cycle and become quiescent cells.

Cell cycle progression

Within the cell, all the cellular processes are tightly controlled at different stages through-out the cell cycle. Quiescent cells (G0) enter G1 phase when they want to proliferate, in order to prepare the right cellular environment for the further steps in the process (i.e., cell needs to increase its size and produce proteins required for DNA synthesis). When the cell passes the first cell cycle checkpoint, it enters the synthesis (S) phase to duplicate its genome. During S phase, replication machinery is used to duplicate the whole genome, and towards the end of S phase, if the cell

successfully duplicates the whole genome and passes the intra-S checkpoint, it then moves to G2 phase. Same as in G1 phase, G2 gives the cell a chance to increase its size and prepare proteins required for mitotic (M) phase. When the cell passes the G2/M checkpoint, then it can enter M phase for cell division. During M phase, the spindle checkpoint is used to check if the chromosomes are properly attached to the spindle and ready for division. After cell split into two daughter cells, they either become quiescent cells or re-enter the cell cycle for cell proliferation (Fig.1).

Therefore, different cell cycle checkpoints are employed throughout cell cycle to make sure the cell can be divided into two healthy daughter cells. Indeed, if a cell fails to pass any of these checkpoints, due to unfinished DNA replication, unrepaired DNA damage, and/or chromosomal abnormalities, the cell cycle checkpoint is activated [2,4]. Consequently, cell cycle progression pauses until the problem is solved, or cell apoptosis is activated if the problem cannot be fixed. However, even under these tight controls, cancer cells can survive when things go wrong.

DNA damages and damage repair pathways

As mentioned above, cells face various endogenous and exogenous threats constantly during development, these risk factors can lead to different types of DNA damage (Fig.2), which may be a threat to genome stability and integrity [12-15]. For example, endogenous damage can arise from ribonucleotides (rNTP) misincorporation into DNA strand during replication. Due to the differences between rNTPs and deoxyribonucleotides (dNTPs), rNTP is prone to hydrolysis,

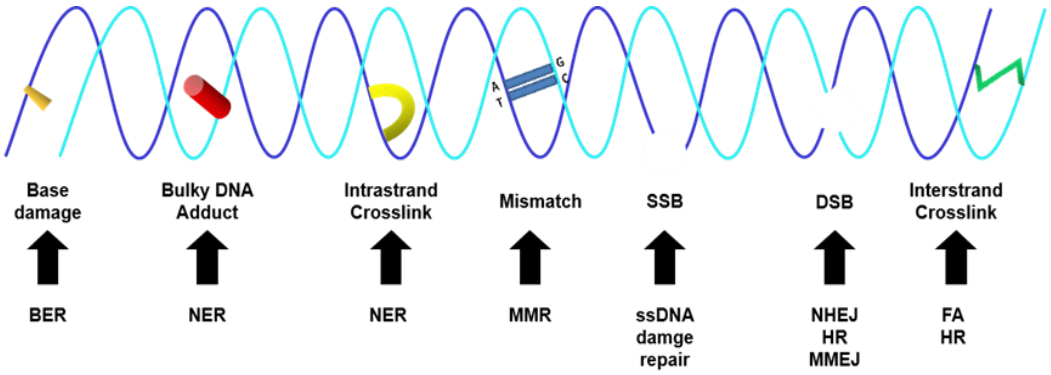


Figure.2 Different types of DNA lesions require different repair pathways. Lesions caused by either exogenous or endogenous sources occur commonly in the genome, it varies from minor to major modification of DNA structure, gaps, SSBs and DSBs. DNA base lesions are chemical modifications of the base of a nucleotide, and it is the most common DNA damage. There are four major causes of base damage: oxidation, deamination, alkylation, and hydrolysis. Base excision repair (BER) is the main repair pathway that cells use to repair base damages in both nuclei and mitochondria. Bulky DNA adducts can be generated from UV light, certain types of chemotherapeutic drugs, and many classes of environmental mutagens such as polycyclic aromatic hydrocarbons (PAHs). This type of lesion causes DNA helix distortions and is repaired by nucleotide excision repair (NER) in mammalian cells. Intrastrand crosslinks generated by chemotherapeutic drugs such as Cisplatin, is also classified as bulky DNA lesion and resolved by NER. DNA base mismatches occur naturally during replication, whereas polymerization errors are rare as DNA polymerase and exonuclease can resolve the problem during replication. This type of lesion can be single base mismatch, deletion mismatch or addition mismatch, mismatch repair (MMR) is responsible for the removal of the errors from nascent DNA strands and restore the template sequence. SSBs are generated either directly by disintegration of DNA sugar back bone, or indirectly during enzymatic removal of damaged base by BER, and Topoisomerase I (Topo-1) when unwind the DNA double helix. PARP1 binds to the SSBs and synthesizes poly (ADP-ribose), which recruits other required proteins to the damage site and activates SSB repair pathway. Unrepaired SSBs can develop into DSBs, which is the most lethal threat to genomic stability. DSBs require either non-homologous end joining (NHEJ) or homologous recombination (HR) to repair, in some cases, microhomology-mediated end joining (MMEJ) is used as well. Interstrand crosslink (ICL) is generated from exposure to environmental mutagens and ICL-based chemotherapeutic treatment. FANCM recognizes the lesion, recruits associated FA core complex to repair such damage. Depending on cell cycle stage, HR or other repair pathways are combined with FA pathway to finish the repair of ICL at a later stage.

hence, this can lead to DNA single base lesions [16]. Other endogenous risks such as incorporation of wrong dNTP during replication, and reactive oxygen species (ROS) generated by mitochondrial respiration [15], which subsequently leads to DNA single strand breaks (SSBs). Exogenous risks for DNA could be ultraviolet (UV) light, ionizing radiation (IR), as well as certain chemicals that are commonly used in chemotherapy. These exogenous factors cause either SSBs or double strand breaks (DSBs) depending on their effects [12, 13, 15]. Therefore, proper detection

and repair of DNA damages is crucial for cell survival and prevent the possible carcinogenesis. Based on the cell cycle stage and types of lesion caused by various risk factors, cells have multiple different repair pathways to protect genome stability and integrity [12, 13, 15].

Cells are equipped with different repair pathways to repair distinct damage in various cell cycle stages, namely mismatch repair (MMR), base excision repair (BER), and nucleotide excision repair (NER) for SSBs [12], nonhomologous end joining (NHEJ), and homologous recombination (HR) for DSBs [12,13], Fanconi anaemia (FA) pathway for interstrand crosslink (ICL) (Fig.2) [15]. Evidence has shown that microhomology-mediated end joining (MMEJ) is also used to repair DSBs, and this pathway is important for cell survival in HR-deficient cells [12]. Among all these repair mechanisms, HR is well studied in the past decades, because it was thought to be the accurate repair pathway to protect genome stability and integrity. In parallel, HR is found to be less error-prone in comparison with NHEJ in repairing DSBs. On the other hand, these two repair pathways are used in different cell cycle stage, NHEJ (Fig.3A) can be used at any stages of the cell cycle, but HR (Fig.3B) can only be used in S and G2 phase when an undamaged sister chromatid is available for use as a homology template for repair.

Replication-transcription collisions are an endogenous threat for genome stability and integrity

Unlike replication, which normally occurs within S phase, transcription occurs throughout the whole cell cycle without restrictions (Fig.1A). Hence, when

transcription and replication both happen during S phase, the same DNA template may be required for both machineries. On such occasion, replication-transcription collision (RTC) could occur when the two processes get close together. Since replication machinery cannot bypass the transcription machinery as it is an equally big complex, this will lead to a stalled replication fork, which subsequently increases replication stress in cells [4,8,9,10]. Unresolved replication stress can develop into DNA SSBs or DSBs [8,10], which activates the DDR and delays replication completion within S phase (Fig.4).

Hence, RTCs are a key internal source of replication stress, which may subsequently induce genome instability and genome integrity, and if not handled properly, can eventually lead to tumorigenesis [4,8,9]. From this perspective, it is important to understand the mechanisms underlying such events (i.e., how to avoid and resolve RTCs during S phase without compromising genome stability and integrity), as it may be able to provide a new target for cancer therapy. However, although the implications for genome stability from RTCs are clear, how these two processes are regulated and coordinated during S phase to reduce RTC occurrence are less well studied. To answer the above question and gain a better understanding of RTCs, we focused on regulation of transcription throughout the cell cycle.

Transcription process

The central dogma of molecular biology is that in order to produce proteins, the genetic information encoded in the DNA template needs to be transcribed into RNA by a specific RNA polymerase, and then this RNA is translated into protein peptides

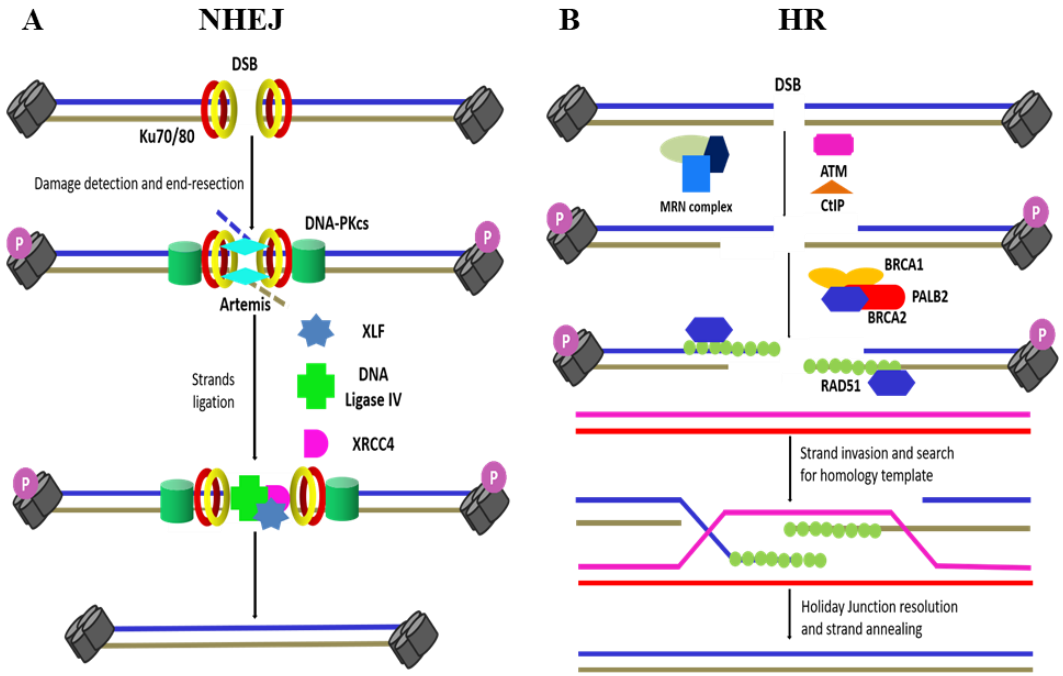


Figure.3 NHEJ and HR are used to repair DSBs. When endogenous or exogenous source causes dsDNA damages, it is followed by the phosphorylation of the histone H2AX. It is phosphorylated by different protein kinases depending on the repair pathway, and phosphorylated H2AX (γ-H2AX) is the first step of recruiting and localizing DNA repair proteins. Unrepaired DSBs result in cells undergoing apoptosis or senescence, failure to repair DSBs properly induces genomic instability and carcinogenesis. There are two major pathways in eukaryotic cells to repair DSBs, and depending on cell cycle stage, cell chooses the most appropriated repair pathway to secure genome stability and integrity. (A) In G1 and early S phase, cells use NHEJ to repair DSBs. Ku70/80 heterodimers recognize and bind to damage site, which helps to bridge the ends close together and maintain the stability of the damage strand. Ku70/80 also serves as a scaffold to recruit the other NHEJ factors, such as DNA-PKcs, Xray cross complementing protein 4 (XRCC4), DNA Ligase IV, and XRCC4-like factor (XLF). Depending on the damage sites, if required, different DNA end processing enzymes would be used to resect DNA ends (Eg. Artemis), fill in gaps, and remove blocking end groups. After end processing, the two ends are ready to be reconnected by DNA Ligase IV to finish the repair. (B) Once cells pass early S phase, the major repair pathway to repair DSBs is HR, it is more accurate compared to NHEJ and at this stage, the homology template is available. HR begins by MRE11-RAD50-NBS1 (MRN) complexes localise to the damage sites to recognize double strand breaks and then activates ATM. After the initiation of the HR pathway, CtIP-mediated nuclease activity is required for end resection, which produce 3'-single strand DNA overhangs. The exposed ssDNA is coated by RPA first then RAD51 nucleoprotein filament is assembled and loaded onto the ssDNA by the guidance of BRCA1, BRCA2 and PALB2 complex. RAD51 then guides invasion of the homology template. DNA synthesis then takes place and Holliday junctions (HJs) are formed, which will be processed by resolvases and processed into either non-crossover or crossover DNA strands. It can also be dissolved by a mechanism involving BLM and TOPOIIIa, in this case, only non-crossover DNA strands are produced.

required for cellular processes. Eukaryotic cells express three different RNA polymerases that each transcribe specific classes of RNA molecules. Independently

of the RNA polymerase involved, the transcription process is subdivided into multiple stages; initiation, elongation, and termination [17, 18]. During each stage, sequential recruitment of transcription-associated factors is required.

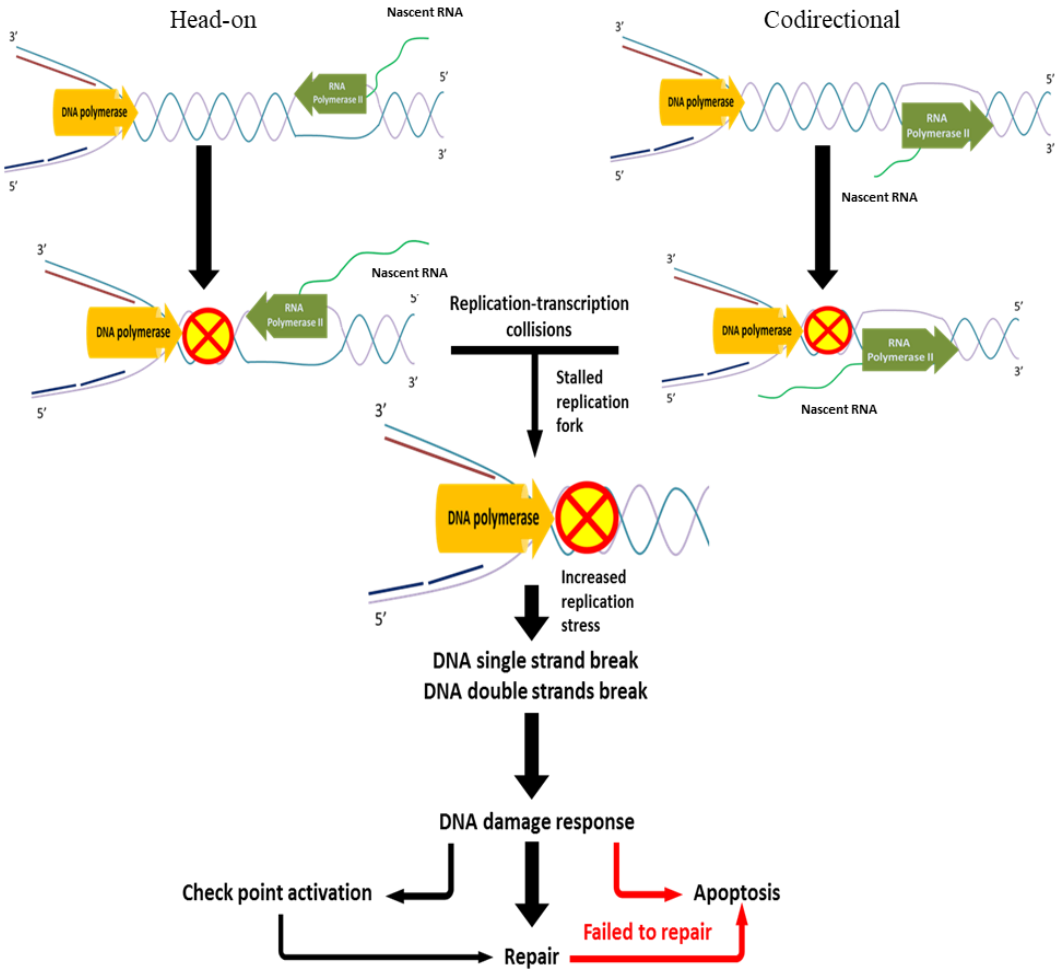


Figure.4 RTC can lead to replication stalling and possible DNA damage. In eukaryotic cells, transcription and replication machineries can occupy the same template at the same time, therefore, collisions may occur under specific circumstances. During replication, depending on the orientation of replicated genes, the replication fork may encounter RNA Polymerase II in either a head-on (left panel) or a codirectional (right panel) way. Both codirectional and head-on collisions can result in replication fork stalling, and possible DNA damage. Subsequently, the DDR is activated, and cell cycle progression stops to fix the problem. If this damage is left unrepaired or not repaired properly, chromosomal recombination events, mutagenesis, and cell death may occur.

In human cells, genetic information encoding genes (i.e. protein-coding genes) are transcribed by RNA polymerase II (RNA Pol II) [17]. The initiation of each individual gene transcription is highly modulated, as such specific regulation is important for maintaining the homeostasis within cells, and the programmed cellular development in different organisms [17, 19]. Transcription initiation begins with activator mediated recruitment of TATA-binding protein (TBP)-containing transcription factor IID complex (TFIID) at the promoter region [20, 21]. The pre-initiation complex (PIC) guides the loading of RNA Pol II onto the transcription start site (TSS) [18, 19]. Following initiation, RNA Pol II in many cases does not go directly into the productive elongation stage of transcription but moves down the DNA template around 20 to 60 base pair (bp) after the TSS, this process is called promoter proximal pausing (PPP) [17, 22, 23, 24]. This pausing process is mediated by the actions of two complexes, which are 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) sensitivity inducing factor (DSIF) and negative elongation factor (NELF), as well as Pol II-associated factor 1 (PAF1) [21-26]. DSIF is comprised by Suppressor of Ty5 (SPT5, SUPT5H in human) and Suppressor of Ty4 (SPT4, SUPT4H1 in human) [27-30], the NELF complex is formed by 4 subunits, NELF-A, NELF-B, NELF-C or D, and NELF-E [30, 31]. To start productive elongation, positive transcription elongation factor b (P-TEFb) phosphorylates the DSIF and NELF complexes as well as the C-terminal domain (CTD) of RNA Pol II [32, 33, 34]. Upon phosphorylation, NELF dissociates from the transcription machinery, while DSIF becomes a positive elongation factor, then RNA Pol II is released from PPP and moves down to the gene body (Fig.5A) [35]. During the elongation stage, the RNA Pol II makes an RNA copy of the template DNA. This nascent RNA will be processed by other factors (e.g. splicing and poly-

adenylation) in order to prepare the mature messenger RNA (mRNA) that will be used as template for the peptide synthesis when target protein synthesis is required. There are many factors that modulate transcription elongation rates, such as topology of DNA template, histone marks and number of exons of the transcribed gene [36]. The variation of transcription elongation rates between and within genes seem to play a role in mRNA splicing and termination, also to a certain extent, it could have a role in maintaining genome stability [36, 37].

Once RNA Pol II approaches the end of the gene, it slows down elongation rate over the termination region. Most eukaryotic mRNA precursors are cleaved in a site-specific manner in the 3'-untranslated region (UTR) followed by polyadenylation of the upstream cleaved product [38]. The cleavage and polyadenylation specificity factor (CPSF) and the cleavage stimulation factor (CSTF) are used to mediate the process of RNA Pol II removal and recycling from the chromatin [39, 40]. Currently, there are two proposed models for the removal and recycling of the RNA Pol II complex from the DNA: the allosteric model and the torpedo model [40, 41]. In the first model, the 3' processing factors causes a conformational change of RNA Pol II elongation complex [40, 41]. This change leads to dissociation of anti-terminator factors and/or binding of termination factors [40, 41]. In the torpedo model however, the 3' pre-mRNA product generated by the cleavage and polyadenylation apparatus is degraded by 5'-3' exoribonuclease, and removal of the polymerase from the template occurs at the same time [40, 41]. However, unlike transcription initiation and elongation, termination is less characterized, and part of the process remains unclear.

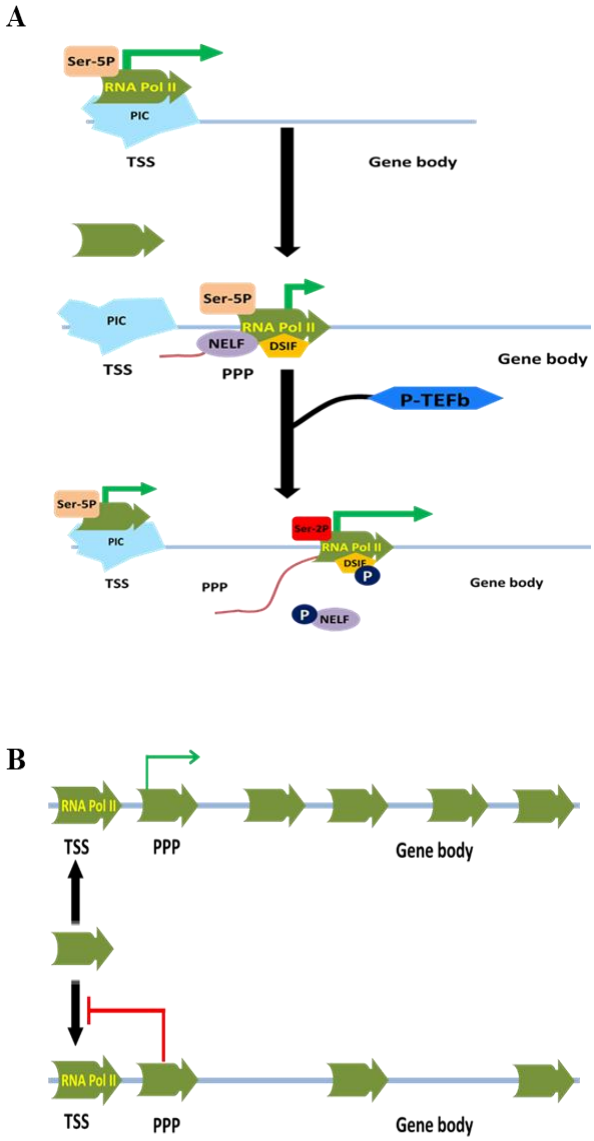


Figure.5 Regulation of RNA Pol II PPP can affect RNA Pol II transcription in general. (A) Transcription begins with loading RNA Pol II onto PIC at the TSS, then RNA Pol II moves 20 to 60 nts downstream from the TSS, where it is then held at pausing position by NELF and DSIF. At this stage, RNA Pol II is highly phosphorylated at Ser-5. P-TEFb is a kinase that removes the inhibition of RNA Pol II elongation by phosphorylating NELF, DSIF and RNA Pol II. Once phosphorylated, NELF dissociates from transcription machinery and DSIF turns into a positive elongation complex that promotes RNA Pol II enter transcription elongation phase and Ser-2 becomes phosphorylated. (B) Regulation of transcription cycle is through loading RNA Pol II, recruitment at TSS and releasing from PPP. Without PPP, RNA Pol II moves into the gene body without delay, which leads to an increase of RNA Pol II within the gene body. When RNA Pol II is stably paused at the PPP region during bursts of transcription, it inhibits the new initiation of transcription by blocking the loading of RNA Pol II to PIC at TSS.

At different stages of transcription, RNA Pol II is phosphorylated at distinctive residues in the CTD repeat of Rbp1, which is the large subunit of RNA Pol II [25, 35, 42-44]. Both biochemical and genomic analyses have shown that transition between transcription initiation and elongation is a multi-step process, and the phosphorylation pattern of RNA Pol II CTD changes during the process. Modification in gene expression in eukaryotes can be regulated at any point between recruitment of RNA Pol II at the TSS with other associated factors, and

RNA Pol II entering productive elongation [18,19,25]. Phosphorylations of specific residues on the extended C-terminal domain (CTD) of RNA Pol II are required for such transition [25]. The CTD is composed of 52 repeats of the heptapeptide sequence YSPTSPS, which is modified through phosphorylation at tyrosine (Tyr), threonine (Thr), serine (Ser) or isomerization at proline residues [25, 42-44]. Among all the modifications of the CTD, Ser-5 and Ser-2 phosphorylation (Ser-5P and Ser-2P) are the best studied, as such modification of the CTD are the most conserved general marks of transcription, and it has a direct link with different steps during transcription [24, 25, 42-45]. In both yeast and mammalian cells, RNA Pol II is recruited to a gene with a hypophosphorylated CTD [46], then Ser-5 and Ser-7 of the CTD are phosphorylated during initiation, and phosphorylation of Ser-2 happens during productive elongation [45, 47]. RNA Pol II is usually phosphorylated at Ser-5 on the CTD at promoter proximal pausing sites [47]. The transition into productive elongation requires the phosphorylation at Ser-2 on the CTD, which is catalysed by cyclin-dependent kinase 9 (CDK9), in human cells, which is part of the P-TEFb complex [45, 47-49].

DSIF and NELF play key roles in RNA Pol II PPP and release

As mentioned above, following initiation, RNA Pol II travels down the template from the TSS for around 20-60 bp and enters the PPP region [15, 22, 23, 24]. Pausing occurs in most of RNA Pol II transcribed genes, and it is proposed to be an extra step for regulating gene expression in metazoans [50, 51]. The concept of PPP was first identified in heat shock protein (*hsp*) genes in Drosophila by Lis and colleagues [51, 52]. They provided the evidence that RNA Pol II was pre-loaded on Drosophila *hsp* genes and it transcribed 20-60 nucleotides before pausing without

heat shock stimulation [50-52]. Once the stimulation occurred, RNA Pol II release was triggered by P-TEFb recruited by heat shock factor (HSF) to promote productive elongation [17, 24, 53]. Studies in mammalian cells have identified paused RNA Pol II on many immediate early genes (IEGs), such as c-myc, c-fos, c-jun and junB under uninduced states [54-57]. Following environmental stimuli, these IEGs can produce a burst of transcripts, and the paused RNA Pol II has been proposed to help these IEGs and environmental responsive genes in general, to produce a quick response when external stress occurs [57]. There is also evidence showing genes with paused RNA Pol II produce more synchronized expression than those without RNA pol II pausing [57], since this type of pausing occurs across different cell types and is shared by different functional genes, potentially representing a common feature for regulating gene expression in metazoans [51, 54, 58].

In metazoans, many rounds of transcription are required to maintain gene expressions levels in response to internal and external stimulation signals, such as growth and environmental signals [18, 19, 59-61]. In this case, RNA Pol II needs to be released from pausing state and travels down the gene body, so that a new RNA Pol II can bind to TSS and initiate the next round of transcription (Fig.5B) [19]. On the other hand, the accumulation of unreleased RNA Pol II at PPP site inhibits the binding and initiation of a new RNA Pol II at the promoter region, which blocks the binding of new RNA Pol II to TSS (Fig.5B) [19]. In human cells, the P-TEFb complex contains CDK9 [18, 21-24, 43, 59, 62], and phosphorylates not only RNA Pol II CTD at Ser-2, but also the SUPT5H subunit of DSIF to convert the complex to a positive elongation factor [21, 25, 28, 35, 43, 62]. It has been

suggested that SUPT5H may enhance the binding between RNA Pol II and the DNA template [32]. The phosphorylation of NELF subsequently causes the dissociation of NELF complex from the transcription and removes its inhibition on DSIF [22, 28, 35, 62]. Subsequently, RNA Pol II escapes from PPP and enters gene body to start productive transcription elongation. PPP is thought to be one of the key steps in transcription regulation as it not only directly regulates the transcription process but is also involved in maintaining chromatin architecture [63, 64]. Especially for highly regulated genes, loss of PPP promotes nucleosome occupancy at promoter regions and leads to repression of the transcription of RNA Pol II [63, 64].

SUPT5H is a highly conserved, ubiquitously expressed RNA Pol II associated factor, which forms the DSIF complex with SUPT4H1 to regulate the pausing and release of RNA Pol II at the PPP region [28, 30, 32, 33]. It has a highly acidic N-terminal, a repetitive C-terminal region which is like the RNA Pol II CTD, and it also contains several Kyprides, Ouzounis, Woese (KOW) motifs to interact with RNA Pol II [29, 30] (Fig.6). On the other hand, SUPT5H also serves as a platform which recruits different co-transcriptional factors to the RNA Pol II complex [32]. Depletion of SPT5 (homolog to human SUPT5H) in yeast causes genome-wide defects of transcription elongation at early stage [39], while in mouse embryonic fibroblasts, losing SUPT5H leads to a reduction in engagement of RNA Pol II complexes during transcription cycle [39]. Therefore, the role of SUPT5H during human cell transcription may contribute to the assembly and maintenance of RNA Pol II complexes at pausing-releasing stage and through the transcription elongation step.

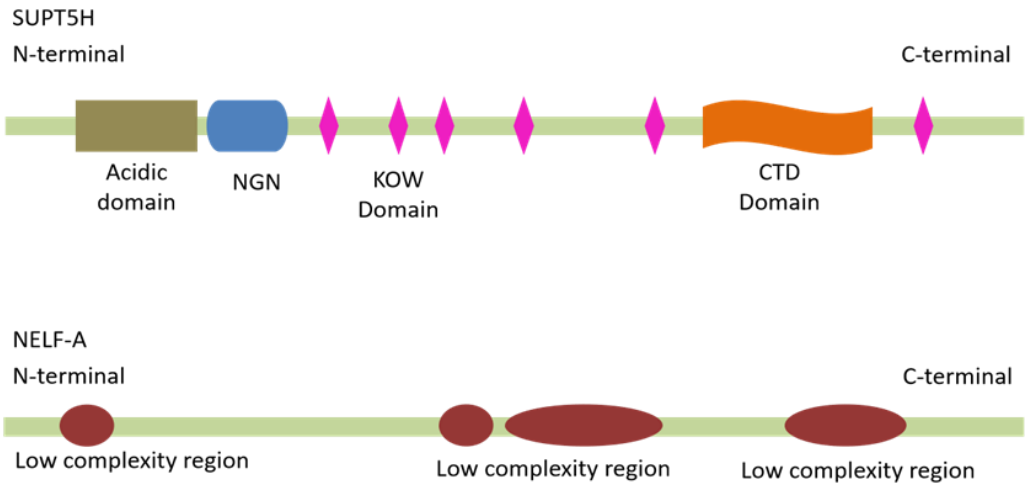


Figure.6 SUPT5H and NELF-A protein structures. SUPT5H is the human homolog of the yeast transcription factors Spt5, encoded by SUPT5H gene located on chromosome 19. It has a highly conserved core in Archea and bacteria (NusG) as well as in eukaryotes. SUPT5H has one NGN domain like NusG, but several KOW domains, one CTD domain, and an acidic N-terminal domain. NGN and KOW regions of SPT5H form the binding site for the interaction with SUPT4H1. (Top). NELF-A belongs to the NELF complex, it is encoded by Wolf-Hirschhorn Syndrome Candidate 2 (WHSC2) gene in the short arm of chromosome 4. The NELF-A subunit has been shown to interact with RNA Pol II and alter the active site of the RNA Pol II to inhibit elongation.

NELF is a complex formed by 4 subunits, which binds specifically to the complex of DSIF and RNA Pol II [30, 34, 65], and such association may be the cause of RNA Pol II pausing shortly after leaving TSS [30, 65]. NELF-A can directly bind to RNA Pol II, and it is encoded by the WHSC2 gene in human (Fig.6) [30]. It was previously shown that NELF-A has an association with a contiguous gene deletion disorder, Wolf-Hirschhorn syndrome [30]. In Wolf-Hirschhorn syndrome patients, it has been proved that insufficiency of NELF-A caused delayed progression from S-phase into M-phase, reduced DNA replication and disruption of chromatin assembly [66]. NELF-B is also known as COBRA1, the cofactor of breast cancer susceptibility gene type 1 (BRCA1) [30, 31, 67]. NELF-B can directly bind to estrogen receptor α (ERα) and reduce the ERα-mediated gene transcription [31, 67]. In breast cancer cells, reduction of endogenous NELF-B leads to an increase in

ERα-mediated gene transcription and cell proliferation [38]. Previous studies have characterised NELF-E structure, finding that it has an N-terminal leucine zipper motif, Arg-Asp dipeptide repeats (RD motif) are located at the centre, and its C-terminal has an RNA recognition motif (RRM) [30]. The interaction between NELF-B and NELF-E is probably through leucine zipper motif, and RRM is critical for NELF complex activity through its binding to a set of RNA sequences [69, 70]. There is also evidence to indicate that knockdown of one subunit of NELF is enough to abolish its structure and function [30, 71] and knockdown of several NELF subunits can cause significant decrease of overall transcription [30, 71, 72].

BRCA1 and BRCA2 function in cellular processes and related cancer risk

It is well known that endogenous and exogenous sources caused DNA damages can be a trigger for carcinogenesis [73]. Especially, mutations in genes involved in HR were found to associate with cancer development, in particular, people with mutations in BRCA1 and BRCA2, are predisposed to breast and/or ovarian cancer, and also to develop prostate, pancreas and other cancers but at a lower frequency [74].

BRCA1 is a large protein encoded by the BRCA1 gene, which is located at 17q21.31, it is comprised of 1863 amino acids and composed of several functional domains that allow interaction with a wide range of proteins during different cellular processes (Fig.7) [75-77]. There are shorter, alternatively spliced isoforms of BRCA1 existing in several different types of tissues [78]. BRCA1 is critical for maintaining genome stability and integrity, as it is involved in DDR, mRNA

transcription, cell cycle regulation, chromatin remodelling, and centrosome duplication [79-87]. During HR, BRCA1 is involved in both early and late stages of repair process: after DSB formation, BRCA1 counteracts NHEJ factor 53BP1, facilitates end resection, which promotes HR in S/G2 phase when sister chromatids are available [74, 82]. To do so, in S/G2 phase, BRCA1 promotes dephosphorylation of 53BP1 by PP4C, which subsequently disrupts the interaction of 53BP1 and RIF1 [74], and leads cell to choose HR to repair the damage as 53BP1 is excluded from core of damage site [74]. BRCA1 also induces the localization of BRCA2 to the repair site through the interaction of PALB2, which facilitates formation of RAD51 filament [74]. The broad involvement of BRCA1 in FA pathway, HR, and NHEJ makes it an important biomarker for cancer development and in BRCA1 deficient cells, factors involved in DDR can be used as therapeutic targets.

Intriguingly, ChIP-Seq data from Gardini and colleagues showed that BRCA1 mirrors the RNA Pol II pattern along genes from TSS to termination site [68], which indicates that BRCA1 not only has an important role in DDR, but also participates in transcription in general. Moreover, BRCA1 interacts with SETX after its recruitment to the R-loop-associated termination regions of RNA Pol II transcribed genes [80]. R-loop is defined as a triple helix structure formed by an RNA: DNA hybrid and the displaced DNA strand. R-loops occur inherently during transcription, but if not processed properly can also threat cellular activities such as transcription and replication inducing replication stress [80]. Moreover, it may damage genome stability and integrity also directly as unsolved R-loop structures make the displaced ssDNA prone to nicking and/or other sources of potential damages [80]. The role

of BRCA1 at transcription termination is known to prevent the possible damage which can arise from the R-loop-associated termination, but the functional role of BRCA1 at the TSS and all along the gene is less clear. However, as mentioned above, there is a known interaction between BRCA1 and NELF-B (COBRA1) [67], and NELF complex regulates RNA Pol II PPP. Knockdown of BRCA1 in mouse embryonic stem cells leads to early embryonic lethality and neuroepithelial abnormalities [87, 88], which indicates that functional BRCA1 is required for cell development and differentiation.

The link between BRCA1 mutations and breast cancer was known from early 1990s [75, 82, 87], ever since then, deeper understanding of both hereditary and non-hereditary breast cancers has been achieved. People carrying BRCA1 germline mutations have increased risk of both breast and/or ovarian cancer development [89]. Though there are breast cancer patients exhibiting non-inherited sporadic types of cancer, many of them show BRCA1 abnormalities [83]. Among these non-inherited breast cancer patients, around 40% of them have a deficiency in BRCA1 expression [83]. Based on the analysis of observational studies worldwide between 1937 and 2011, in 19581 carriers of BRCA1 mutations, 46% of the cohort developed breast cancer, 12% were diagnosed with ovarian cancer and 5% were diagnosed with both breast and ovarian cancer [90]. The mortality of female breast cancer patients shows that breast cancer is the most common cause of death in women aged 20 to 59 and the second most common cause of death in women worldwide [83].

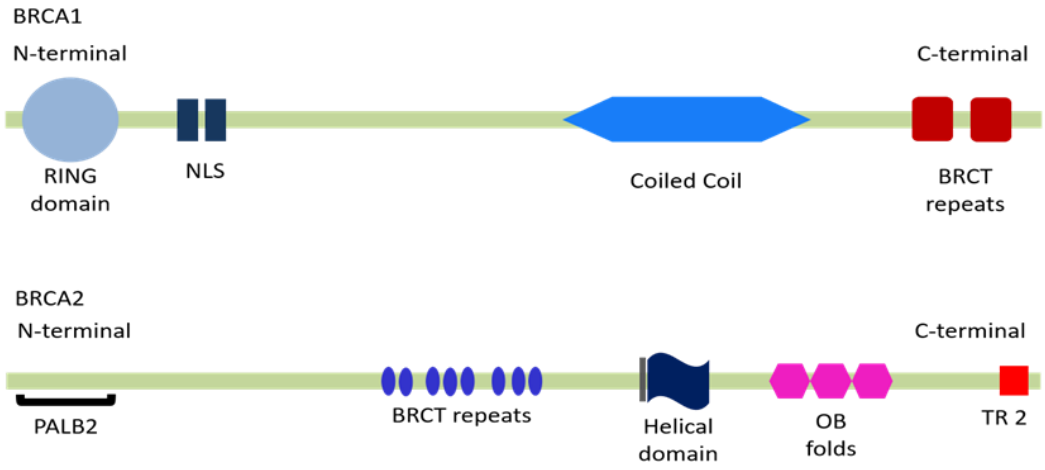


Figure.7 BRCA1 and BRCA2 protein structures. (Top) BRCA1 is a large protein encoded by the BRCA1 gene located on chromosome 17. BRCA1 contains a RING domain which interacts with BRCA1-associated RING domain protein 1 (BARD1), and this RING domain is also responsible for the E3 ubiquitin ligase activity of BRCA1. It also has a nuclear localization sequence (NLS), and two BRCT domains at the C-terminus, which mediate phosphoprotein interactions between BRCA1, and proteins phosphorylated by ATM and ATR. (Bottom) BRCA2 is encoded by the BRCA2 gene located on chromosome 13. The N-terminus of BRCA2 binds PALB2, which helps to localize BRCA2 to the damage site during HR. BRCA2 contains eight BRC repeats, which interact with Rad51. The helical domain of BRCA2 functions as a DNA-binding domain, three oligonucleotide binding (OB) folds may contribute to the binding of both ssDNA and dsDNA. The C-terminus of BRCA2 contains an NLS and a TR2 site, which can also be used for Rad51 binding.

Carriers of germ line BRCA2 mutations instead show around 50% chance of developing breast cancer and a 10-15% chance of developing ovarian cancer [91-95]. Unlike carriers of BRCA1 germ line mutations, which mostly result in female breast and ovarian cancer, male BRCA2 mutation carriers show an increased risk of developing breast, pancreas, and prostate cancers [91, 96]. BRCA2 is an even larger protein than BRCA1, formed by 3418 amino acids encoded by the BRCA2 gene located on chromosome 13q (Fig.7) [97]. BRCA2 contains 8 highly conserved BRC domains which interact with the Rad51 recombinase [91, 93, 98-100], 3 oligonucleotide/oligosaccharide binding (OB) folds which bind to DNA strand during damage repair [91, 93]. The N-terminal region of BRCA2 is known to interact with PALB2 [91], while the C-terminal has a TR2 domain which can also

interact with Rad51 [98, 100]. BRCA2 seems to play a different role in HR of that of BRCA1. The main role of BRCA2 in HR is its ability to interact and localize Rad51 onto single strand DNA and mediate strand invasion [95, 101]. During this process, BRCA2 requires the interaction with PALB2 and BRCA1 for localization to the damage site [91, 95, 97]. After loading Rad51, BRCA2 is thought to stabilize the nucleoprotein filament through its TR2 domain [91]. BRCA2 may also have a role in meiotic recombination, as BRCA2 deficient mice show persistence of Spo11-induced DSBs in gonads at the early meiotic recombination [102]. Moreover, BRCA2 can also interact with DMC1, which is essential for meiotic homologous recombination [101]. BRCA2 is also required for ICL repair, and its function in FA repair pathway is thought to be the same as in HR [103, 104].

Like BRCA1, BRCA2 is therefore also a tumour suppressor gene involved in maintaining genome stability and integrity via DNA damage repair, cell cycle control, but its role in transcription regulation is less clear [91, 98, 99]. Current findings show that product of BRCA2 exon3 can activate transcription, and mutation of Tyr42Cys of BRCA2 can reduce the transactivation potential [100]. Moreover, BRCA2 overexpression is correlated with down regulation of p53 basal transcription [100]. However, BRCA2 may also activate transcription by modulating histone acetylation, and it may recruit histone modifiers and induce transcription [100]. BRCA2 also involved in transcription elongation by prevent R-loop formation, it binds to RNA Pol II via PAF1 and regulate PPP [105].

Synthetic lethality and PARP inhibitors

The concept of synthetic lethality was first introduced in 1922 by Bridges [106] and it can be summarized as a combination of two 'non-lethal' genomic events to induce lethal activity in the cell. For example, in HR-deficient cells, inhibition of key factors in NHEJ would force cells to choose other error-prone repair pathway, and eventually cause cell death due to accumulation of intolerable genome instability [106]. Inhibition of key factors involved in DDR has been successfully exploited in cancer patient treatment and researchers are aiming to identify the best approaches to induce synthetic lethality in cancer cells without causing collateral damages to healthy cells [106]. Recent years of clinical trials have shown that BRCA mutant cells have defects in HR, which promotes the cancer development, but such defects can also be used to develop new cancer treatments. These BRCA mutant cancer cells have defects in DNA damage repair, which makes them more sensitive to DNA damaging agents [89, 90], such as platinum based therapy (e.g. Carboplatin and Cisplatin) [90], and PARP inhibitors (e.g. Olaparib and Talazoparib) [90, 107].

PARP inhibitors (PARPi) are small molecule inhibitors, which are designed to targeting poly (ADP-ribose) (PAR) Polymerase 1 and 2 (PARP1 and PARP2) enzymes [82, 91, 108]. Both PARP1 and PARP2 have multiple functions in cellular activities, such as transcription, apoptosis, and DDR [82, 91-94]. PARP1 is an important sensor and signal transducer in DDR, it binds to the damaged DNA strand, then synthesis branched negatively charged PAR chains (PARylation) and recruits other DDR factors [91-93]. PARP1 can also remodel the chromatin structure around damaged sites to help DNA repair [92, 95]. After repairing DNA damage, PARP1 synthesizes branched PAR chains on itself, which leads to its dissociation from the

DNA strand [91]. Originally, PARPi was used in combination with chemotherapy or radiotherapy, by inhibiting PARP-mediated DNA repair pathway [91]. Such combination of treatment maximizes cell death by increasing unrepaired DNA lesion caused by chemotherapy or radiotherapy. Alternatively, accumulated damages then activate the apoptosis pathway.

The rationale of using PARPi alone to treat BRCA mutant cancer patients is using PARPi to inhibit PARP1 enzymatic activity, and/or depending on the specific PARPi in some cases trap PARP1 on DNA at the break site [82]. Both of PARPi mechanisms can lead to accumulate unrepaired SSBs in cells. Subsequently, if unrepaired SSBs occur in G1 phase and are still present when cells go in S phase, they can lead to replication fork collapse and potentially develop into DSB. DSBs generated from the process can subsequently become cytotoxic lesion causing death. Therefore, HR is essential for repairing of these collapsed replication forks, protect genome stability and maintain cell survival [107]. BRCA mutant cells exhibit defects in HR and NHEJ, hence, when using PARPi as single agent for treatment, a greater cytotoxicity is observed in BRCA mutant cancer cells when compared with cancer cells with wild type BRCA1 and/or BRCA2 (Fig.8) [82, 90, 107]. Over the past decades, as more breakthroughs have been made in the genetic epidemiology of breast cancers, clinical performance of BRCA mutant cancer patients, the prevention and therapeutic treatment for breast cancer have improved. Even though new therapies are developed, drug resistance becomes an obvious challenge when treating certain patients with BRCA mutations [109-113]. Therefore, identification of the mechanism behind PARPi resistance is required to

improve the current treatment strategy. Since the primary tumour samples from BRCA mutant cancer patients remain limited, most of the studies have been done

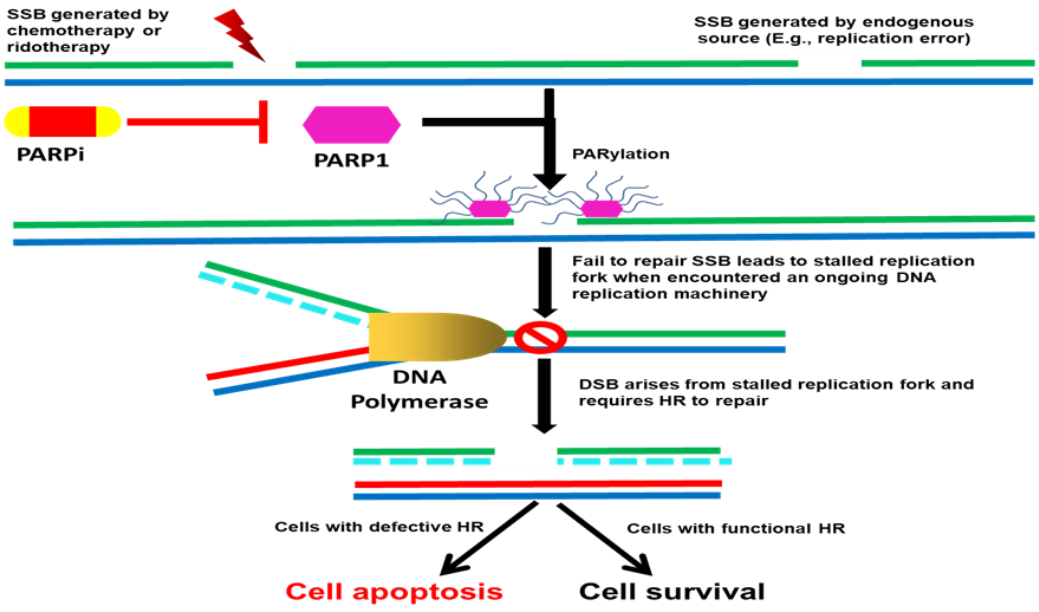


Figure.8 How PARPi can induce synthetic lethality. The original rationale of using PARPi to generate synthetic lethality is by inhibiting PARP1 mediated repair of DNA lesions created by chemotherapy or radiotherapy. SSBs generated by either chemotherapy or radiotherapy require PARP1 enzymatic activity to initiate the repair. When cells are treated with PARPi, SSBs will accumulate and develop into DSBs when they encounter ongoing replication forks in S phase. Hence, in cells with functional HR, DSBs will be repaired and genome stability and integrity will be restored. As there are endogenous sources that can generate SSBs, therefore, PARPi can work alone to treat BRCA mutant cancers. In BRCA1 and BRCA2 mutant cells with defective HR, DSBs are repaired by error prone NHEJ, which may lead to DNA aberrations such as deletion of critical genomic information, which leads to accumulation of intolerable mutations, chromatin remodelling and cell death.

in genetic engineered mouse or cell lines, and during the past years, researchers have revealed several mechanisms that lead to PARPi resistance in BRCA mutant patients. Researchers have found that increased expression of Mdr1 gene leads to the upregulation of drug efflux transporter P-glycoportein (PgP), and subsequently leads to reduce Olaparib response in mice [114, 115]. However, clinically, the connection between induced PgP upregulation and PARPi resistance remains

inconclusive, as PgP expression between human and mice may vary [114]. Secondary mutations in BRCA1/2 gene are also identified to trigger PARPi resistance in BRCA mutant tumours [116, 117]. Jaspers et al. have shown that somatic loss of 53BP1 can partially rescue HR, which generates PARPi resistance in their BRCA1-deficient mammary tumours [114, 116, 118, 119]. Moreover, loss of REV7 function either by deletion or reduced expression of RIF1, also shows increased PARPi resistance [116, 118]. Gupta R et al. have identified that the Shieldin complex acts downstream of 53BP1 pathway in regulating NHEJ, and depletion of Shieldin components also increases PARPi resistance in BRCA1-deficeicent cells [120]. Despite there are many ongoing studies in mechanisms behind PARPi resistance, the complexity merged from different aspects (e.g., genetic, pharmacological, etc.) remain elusive and requires further clinical analysis and study.

Mitotic DNA synthesis and common fragile sites

Common fragile sites (CFSs) in the human genome are regions that are difficult to replicate, they often manifest as gaps or breaks, which makes them also prone to generate copy number variations and chromosomal rearrangements in cancer cells [3, 121]. Due to the nature of these regions, they are normally finished to be replicated in late S phase, or even later when a cell is facing replication stress [3, 121]. Hence, when Minocherhomji et al. treated U2OS cells with aphidicolin (APH) to increase replication stress, they observed that even in M phase, there is 5-ethynyl-2′-deoxyuridine (EdU) labelled DNA synthesis in the nucleus, and incorporated EdU dots predominantly present at CFSs [121]. A similar observation was made in G2/M phase cells when PARPi was used to treat these cells [7]. PARPi has been

shown to increase replication stress in cells by accumulating unrepaired damage [112] and increasing fork progression [7], hence, when cells are treated with PARPi, mitotic DNA synthesis (MiDAS) is used to fix the unrepaired DNA damage and prevent possible cell death [7, 121]. Therefore, the concept of MiDAS was introduced as a repair synthesis pathway: cells unable to complete replication of the whole genome in S phase can use this pathway to complete the duplication of the whole genome before cell division [121]. In this way, cells can preserve genome stability and avoid mutagenesis.

Removal of the transcription machinery in mitosis and G2/M DNA synthesis

To analyse replication and transcription progressions throughout S-phase, we used BrdU-seq to monitor the replication through the incorporation of the nucleotide analogue bromo-deoxyuridine (BrdU), and Chr-RNA-seq to map chromatin bound nascent RNAs produced during transcription. When we combine the BrdU-seq data and RNA-seq data throughout S phase, we can analyse how both processes affect each other [unpublished data, Fig.9A]. Intriguingly, we observed that a replication gap is present and persists at TSS of transcribed genes during S-phase (Fig.9B). This gap indicates that this region is under replicated compared to other regions of the gene. The BrdU gap persisted throughout the S phase and was associated with gene length, as long genes (>100kb) have deeper and wider gaps compared to shorter genes (Fig.9C). Moreover, transcription level shows no significant affect to the gap size or presence, except for the lowest transcribed genes (Fig.9D). Analysing Chr-RNA-Seq data we found an accumulation of RNA Pol II near the TSS site during DNA replication, indicating that the persistence of the RNA Pol II at the TSS might be responsible for the BrdU-Seq gap at the TSS (Fig.9C).

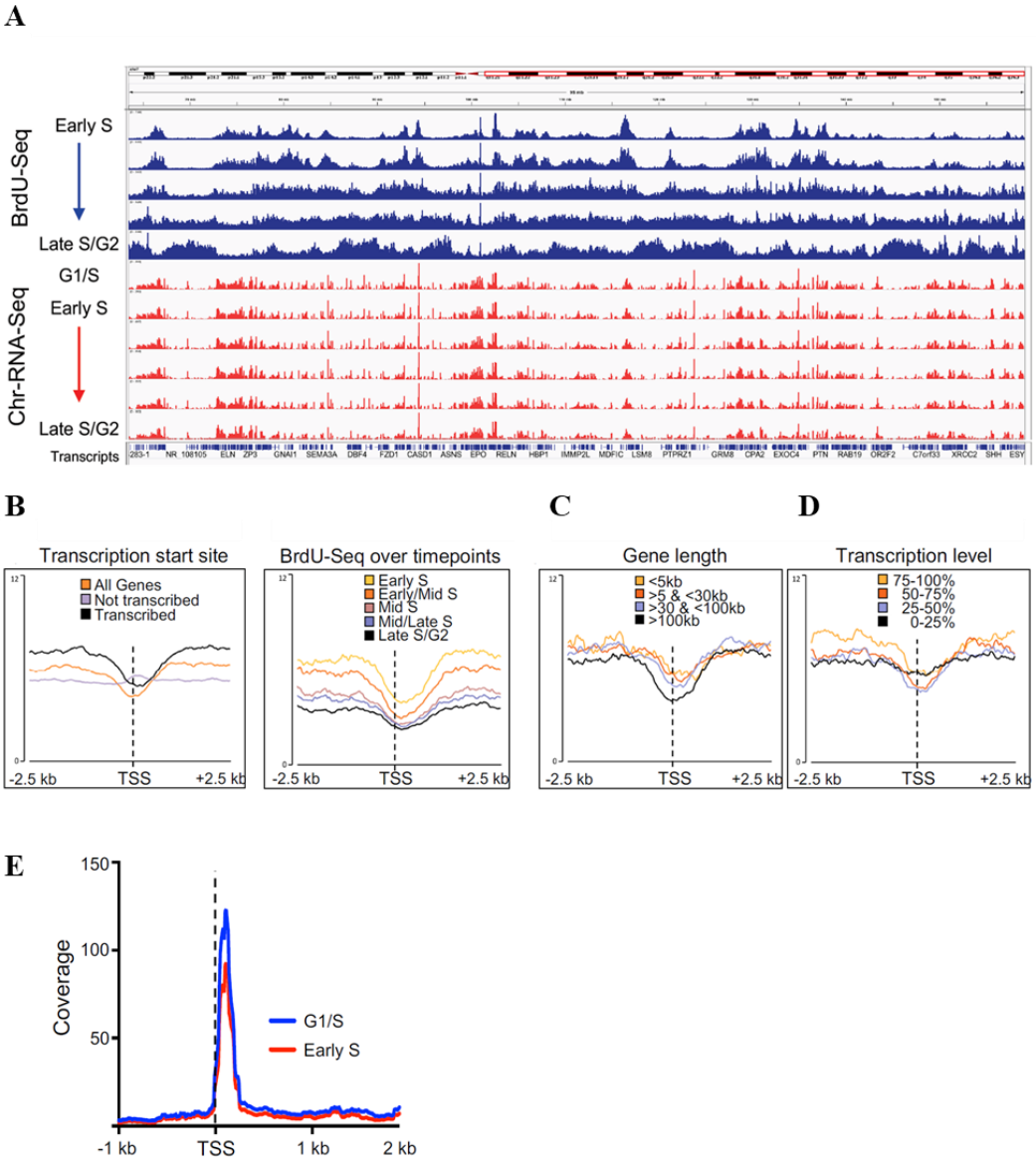


Figure.9 Coordination between transcription and replication shows TSS of transcribed genes are under-replicated during S phase. (A) Snapshot of the long arm of chromosome 7, genomic view of DNA replication analyzed by BrdU-Seq (in blue), and sites of chromatin RNA-Seq (in red), we can observe the progression of transcription and replication from beginning to the end of S phase, replication pattern changes throughout S phase, but there is no significant variation in transcription. (B-D) Comparing average metagene profile of BrdU-Seq level between all genes, transcribed genes ad not transcribed genes, the result shows in early S phase, regions around TSS (+/- 2.5kb) for transcribed genes are under-replicated. For genes replicated in early S phase, the gap persists and is associated with downstream gene length, as longer transcripts presenting larger and deeper gaps than shorter genes. However, transcription level has no effect on the gap excepts for lowest transcribed genes (0-25%). For genes over 100kb replicated in early S phase, we observed the gap persist throughout S phase which indicates that this region around TSS remains under-replicated in S phase. (E) Chr-RNA-Seq result shows that there is accumulation of RNA Pol II near TSS, possibly at PPP site before and during replication in early S phase.

Taken together, these results link TSS gaps during S phase with accumulation of RNA Pol II at TSS. Liang et al. showed the removal of actively engaged RNA Pol II at pausing state in early M phase [122]. However, as mentioned above, DNA synthesis can still occur up until mitosis, especially following replication stress [7].

Hence, we hypothesized that due to the presence of RNA Pol II complex occupying DNA template, TSS region is under replicated during S phase and therefore this region might be 'replicated' in G2/M phase after the clearance of RNA Pol II. This has been tested previously in the lab using synchronized human immortalized fibroblasts (BJ-hTERT) cells. Cells were firstly synchronized at G0/G1 phase using serum starvation, then released into S phase, and synchronized again at G2 phase using CDK1 inhibitor Ro3306. After release from G2 block, EdU was added in the culture medium to label newly synthesised DNA in G2/M phase monitored through Click-iT reaction. In parallel, we reduced RNA Pol II levels at TSS by manipulating the DSIF and NELF complexes. IF images were analysed quantifying G2/M phase cells as labelled by Histone 3 Ser-10 phosphorylation (p-H3), which is a marker used to identify G2/M cells [7], performing DNA synthesis as EdU positive. We found so that knockdown of SUPT5H and NELF-A reduces the number of cells require G2/M DNA synthesis (G-MiDS, Fig.10). Importantly, while MiDAS is a DNA damage repair-associated synthesis, G-MiDS is genetically and functionally separated from MiDAS as it is not dependent on RAD52 like MiDAS [data not shown].

As mentioned above, MiDAS occurs in cells treated with PARPi due to increased replication stress [7, 121], and in parallel PARPi can also lead to an accumulation of RNA Pol II at TSS/PPP region [108]. PARP1 inhibits NELF activity through NELF-E by PARylation, which promotes RNA Pol II entry into productive elongation without pausing [108]. Hence when cells are treated with PARPi, the RNA Pol II is hold at PPP site, and as we have shown that PPP regulates G-MiDS levels, we hypothesise that PARPi treatment would also increase G-MiDS levels.

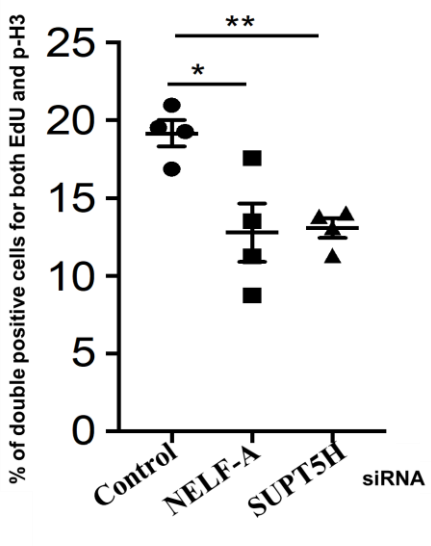


Figure.10 Quantification of G2/M phase cells undergoing G-MiDS. In synchronized BJ-hTERT cells, we analyzed the proportion of G2/M cells that still underwent DNA synthesis. In control group, approximately 20% of cells in G2/M (p-H3 positive) phase were also positive for EdU. However, in cells depleted NELF-A and SUPT5H, only 12.8% and 13.1% cells were undergoing G-MiDS respectively.

One possibility is that PARPi increases replication stress both by creating more unrepaired DNA damages and by increasing the BrdU gaps around TSS due to the connection between PARPi and PPP regulation. Therefore, as reducing by RNA Pol II levels at PPP following the KD of SUPT5H and NELFA, cells are less dependent on G-MiDS to complete the duplication of their genome, perhaps PPP could also be important to regulate how much DNA synthesis cells would be doing in G2/M following PARPi treatment. As such, we could hypothesise that PPP factors could

also be important in regulating cells' sensitivity to PARPi treatment. If that was the case, PPP factors could ultimately have a potential impact also on the hypersensitivity of BRCA1/2 knockdown cells. In this research project I have therefore aimed to validate these hypotheses.

Specific aims of the project:

- Testing whether PPP affects PARPi induced G-MiDS level.
- Testing whether depletion of PPP factors affects PARPi sensitivity in cells.
- Testing whether depletion of PPP factors can potentially rescue also BRCA mutant PARPi hypersensitivity.

Methods and Materials

Cell culture

Human immortalized fibroblasts (BJ-hTERT, BJ cells in short afterward) were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 1% L-glutamine (200 mM 100X stock, Gibco) and 1% penicillin (stock concentration: 5000 units/ml)/streptomycin (stock concentration: 5000 µg/ml) (Gibco) in 5% CO2 at 37°C. Human Bone Osteosarcoma Epithelial Cells (U2OS cells) were cultured in McCoy (Gibco) supplemented with 10% FBS (Gibco), 1% penicillin/streptomycin (Gibco) in 5% CO2 at 37°C.

Cell Synchronization and EdU labelling

BJ cells were seeded at 20% or 30% confluence depending on the experiment and incubated in 5% CO2 at 37°C for 18 hours. To achieve cell synchronization, cells were cultured in serum starvation medium (DMEM supplemented with 0.2 % FBS, 1% L-glutamine and 1% penicillin/streptomycin) for 26 hours. Then cells were released into S-phase with complete DMEM (supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin), 16 hours after release, 1 µM PARPi (Olaprib, AdooqBioscience) was added. 8 hours after adding PARPi, 1 µM RO3306 (Adooq Bioscience) was added to synchronize cells at G2 phase for another 8 hours. Cells were released from the G2 arrest by washing with 1xPBS (Gibco) for 3 times, cultured in complete DMEM supplemented with 10 µM EdU (Sigma-Aldrich) and incubated for 30 minutes.

siRNA transfection

siRNA transfection for BJ and U2OS cells was performed using INTERFERin siRNA Transfection Reagent (Polyplus) following manufacturer's protocol. 37.5 nM of NELFA (SMARTPool, Dharmacon), SUPT5H (SMARTPool, Dharmacon), and Control (against Luciferase, Dharmacon, CTR hereafter) siRNA were used in combination of INTERFERin siRNA Transfection Reagent and Opti-MEM (Sigma-Aldrich) to knockdown target gene expression, while 50 nM of BRCA1 siRNA (SMARTPool, Dharmacon) and 50 nM of BRCA2 siRNA (SMARTPool, Dharmacon) were used to knockdown (KD) BRCA1 and BRCA2 gene expression, respectively. Double knockdown of BRCA1 with SUPT5H and NELFA expressions are were carried out as above. The efficiency of knockdown was verified by RT-PCR and Western blot.

Immunofluorescence (IF) microscope

For IF microscope, BJ cells were seeded at 20% confluence in 6-well plate with poly-lysine (Sigma) treated microslides. Cell transfection was carried out at the same time when cultured in starvation medium. Following the last step of cell synchronization and EdU (Sigma-Aldrich) labelling for 30 minutes, cells were fixed using PTEMF buffer (20 mM PIPES pH 6.8, 10 mM EGTA, 0.2% Triton X-100, 1 mM MgCl2, 4% formaldehyde) at room temperature. Fixed samples were washed 3 times in PBS and stored at 4°C until use. The Click-chemistry reaction was performed using Click-iT RNA Alexa Fluor 594 Imaging Kit (Thermo Fisher Scientific) following manufacturer's instructions. Cells were quickly washed with 1 ml rinse buffer and blocked for 1 hour at room temperature using 10% FBS (Sigma Aldrich, prepared using 1xPBS). Cells were washed 3 times in 1xPBS and

incubated with 1:2000 diluted rabbit monoclonal anti-Phospho-Histone H3 (Ser10) (Cell Signaling Technology) in 1% FBS for 1 hour at room temperature. Cells were then washed 3 times in 1xPBS and incubated with 1:1000 diluted Alexa Fluor488-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific), in 1% FBS for 1 hour at room temperature. Finally, cells were washed 3 times in PBS and mounted with mounting medium with DAPI (GeneTex). Images were taken by Nikon Eclipse E600 Fluorescence Microscope using Nikon Plan Apo x60 (1.3 numerical aperture) oil lens, a Hamamatsu digital camera (C4742-95) and the Volocity acquisition software (Perkin Elmer). Total number of DAPI positive cells was counted excluding from the count cells clearly in any mitotic stage (from prometaphase on); the number of EdU positive and EdU/Ser10-H3 double positive cells was then calculated. At least 150 cells were counted in each sample, and 3 independent repeats were performed.

Fluorescence-activated cell sorting (FACS) and analysis

For FACS, BJ cells were seeded in 100mm petri dish at 30% confluence. Cells were treated as above and following the EdU labelling for 30 minutes, were collected after 2 minutes incubation in 0.25% Trypsin-EDTA 1x (Gibco), followed by 1500 rpm centrifugation. After one wash 1xPBS, cells are transferred into 1.5 ml Eppendorf tubes and fixed using modified FACS specific PTEMF buffer (20 mM PIPES at pH 6.8, 10 mM EGTA, 0.01% Triton X-100, 3 mM MgCl2, 4% formaldehyde) at room temperature. Fixed samples were washed 3 times in PBS and stored at 4°C until use. The Click-chemistry reaction was performed using Click-iT RNA Alexa Fluor 594 Imaging Kit following manufacturer's instructions (Thermo Fisher Scientific) as described above. After the final wash, each sample is

Antibodies	Company	Catalog No.	Dilution
Rabbit monoclonal anti-Phospho-Histone H3 (Ser10)	Cell Signaling Technology	3377S	1:200
Mouse monoclonal anti-Tubulin	The Francis Crick Institute Core Facility	Tat-1	1:5000
Mouse monoclonal anti-NELF-A	Santa Cruz Biotechnology	sc-365004	1:1000
Mouse monoclonal anti-SPT5	Santa Cruz Biotechnology	sc-133217	1:1000
Mouse Monoclonal anti-BACH1	Santa Cruz Biotechnology	sc-271211	1:1000
Mouse Monoclonal ant-ATM	Santa Cruz Biotechnology	sc-135663	1:1000
Mouse Monoclonal anti-BRCA1	Millipore	3017628	1:1000
Rabbit monoclonal anti-PARP	Cell Signaling Technology	9532S	1:2000
Mouse monoclonal ant-BRCA2	Invitrogen	MA5-32896	1:1000
Anti-Rabbit IgG HRP-Linked	Cell Signaling Technology	7074S	1:2000
Anti-mouse IgG HRP-Linked	Cell Signaling Technology	7076S	1:2000
Alexa FlourTM 488 F (ab')2 fragment of goat anti-rabbit IgG (H+L)	Invitrogen	1907301	1:1000

Table.1 Antibodies used in this project.

resuspended in 500µl of 1xPBS with RNase A (Sigma) and Vybrant DyeCycle Violet Stain (Thermo Fisher Scientific). Samples were acquired through CytoFLEX flow cytometer (Beckman Coulter Life Sciences) and analysis was

carried out using both CytoFLEX (Beckman Coulter Life Sciences) and FlowJo (10th version) software.

Western Blot

BJ and U2OS cells were seeded into 60mm petri dish at 20% confluence; cells were incubated in 5% CO2 at 37°C for 24 hours. siRNA transfection was carried out as described above. 24 hours, 48 hours and 72 hours after transfection, cells were collected after incubated in 0.25% Trypsin-EDTA 1x for 2 minutes. Complete medium was added after incubation in Trypsin-EDTA for cell collection. Cells were then washed with 1xPBS for 3 times, followed by adding 2x SDS lysis buffer to cell pellet after final wash. Cells were sonicated using Bioruptor Sonication Device (Diagenode). Cell lysates were boiled at 97°C for 5 minutes. Samples were loaded into 6% stacking gel with 10% separating gel or 4% stacking gel with 5% separating gel depending on the target protein molecular weight. Electrophoresis was set at constant 80V for samples to run through stacking gel and 150V when samples entered separating gel. Gels were transferred onto 0.45 μm nitrocellulose membranes (Merck Millipore) at constant 150V for 3 hours. After transferring, membrane was first incubated in Ponceau (Sigma) to check if the protein transfer was successful. Ponceau was washed away by dH2O and 1xTBST, then the membrane was blocked in 5% skimmed milk (MAVEL) dissolved in 1xTBST buffer for 1 hour. Membranes were incubated with primary antibodies (listed in Table.1) overnight at 4°C followed by 3 washes in 1xTBST buffer for 5 minutes at room temperature. Membranes were incubated with appropriate HRP-linked secondary antibodies (Table.1) at room temperature for 1 hour and washed three times prior to signal detection. Membranes were developed by chemiluminescence

using ECL reagent (Promega) and HRP reagent (Millipore). Buffer used in western blot is listed in Table.2.

2X SDS lysis buffer	
Reagent	Final concentration
sodium dodecyl sulphate	4%
Bromophenol blue	0.20%
Glycerol	20%
β-mercaptoethanol	200 mM
100X Phenylmethylsulfonyl	1 mM
100X Protein inhibitor （PI）	1 mM
1X Tris-Buffered Saline, 0.1% Tween 20 Detergent	
10X TBS	10%
Tween 20	0.10%
10X Tris-Buffered Saline (TBS) buffer	
Tris base	46.2 mM
NaCl	1500 mM
Tris HCl	152.2 mM
Adjust pH to 7.6 with 12 N HCl	

Table.2 Buffer used in western blot.

Colony assay

U2OS cells were seeded into 60 mm petri dish at 20% confluence and incubated in 5% CO2 at 37°C for 24 hours. siRNA transfection was carried out as described

above, 72 hours after transfection, cells were collected into 15 ml centrifuge tube after incubating in trypsin for 2 minutes in 5% CO2 at 37°C, then complete medium was added to collect cells and transfer to 15ml tube for cell counting. Cells were resuspended in the tube and 10 µl of cells was added to Fast-Read 102 plastic counting chamber (Kisker) for cell count. After cell counting, total cells in the 15ml tube was calculated and 300 U2OS cells were seeded into 60mm petri dish, 3 dishes for each drug concentration. 24 hours after cell seeding, 0 µM, 0.5 µM, 1 µM and 3 µM PARPi were added into each petri dish, then cells were incubated in 5% CO_2 at 37°C. After 10 days of incubation, cells were washed with 1xPBS twice, and then 10% crystal violet (Sigma) was added to each dish for colony staining. Crystal violet was removed after an hour incubation in room temperature; excessive crystal violet was washed away by water. Colonies were counted and the average colony numbers were calculated from the triplicate dishes.

RNA extraction

BJ and U2OS cells were seeded to 60 mm petri dish at 20% confluence and incubated in 5% CO2 at 37°C for 24 hours. siRNA transfection was carried out as described above. 24 hours, 48 hours and 72 hours after transfection, cells were collected after trypsinization using Trypsin-EDTA 0.25% and then lysed in RLT buffer (QIAGEN) supplemented with 0.1% β-Mercaptoethanol (VWR). RNA was extracted with RNeasy MiniKit (QIAGEN) according to the manufacturer's instructions. After extraction, RNA concentration and purity were measured using Nanodrop spectrophotometer (Thermo Scientific) and 1 µg of RNA was reverse transcribed with RandomPrimers (Promega) using the SuperScript III Reverse Transcriptase kit (Invitrogen).

Primers	Sequence (5'-3')	Company
BRCA1 Forward	AAG GTC CAA AGC GAG CAA GA	Integrated DNA technologies
BRCA1 Reverse	TTC CAG TTG ATC TGT GGG CA	
RPLPO Forward	AGC CCA GAA CAC TGG TCT C	
RPLPO Reverse	ACT CAG TTC AAT GGT GCC	
SPTAN1 0Kb Forward	GCT TAT AAA AGA GGC GGG GC	
SPTAN1 0Kb Reverse	CCT CTC ACC GGC TCT CGG	
SPTAN1 1Kb Forward	GTG CTT GCA TGG TAT GTG GC	
SPTAN1 1Kb Reverse	CGA CTC GAA AGA CCC AGT CC	
SPTAN1 15Kb Forward	CCA CCG CTT CAA GGA ACT CT	
SPTAN1 15Kb Reverse	TTT GGC AGG AAT CCC AGG AG	
SPTAN1 40Kb Forward	TGT GCA GGA CAC GCA TAA CT	
SPTAN1 40Kb Reverse	GAC CTC TTC CCT TGT GAG CC	
SPTAN1 60Kb Forward	CCT GCT GGC ATC CGA AGA TT	
SPTAN1 60Kb Reverse	CCT GAG GCC ACA ACC TTT GA	
SPTAN1 80Kb Forward	TAT GGT GGA AGA GTC GGG GA	
SPTAN1 80Kb Reverse	GGC CAA GGG AAA AGG GTA CA	

Table.3 Primers used in this project.

Real time PCR (RT-QPCR)

RT-QPCR was carried out using SensiFast SYBR Lo-ROX kit (Bioline) and CFX96 Real-Time System (BIO-RAD) and QuantStudio5 (Thermo Fisher). Primers are listed in Table.3.

cBioPortal analysis

BRCA1 and BRCA2 mutant cancer patient data were obtained from cBioPortal. For Breast cancer study, BRCA1 or BRCA2 mutant patients were selected from Breast Invasive Carcinoma (TCGA, PanCancer Atlas), which contains 1084 patients in total. There were 61 patients with BRCA1 mutation, deletion, or downregulation, within these patients, mRNA expression data of our interested factors involved in transcriptional regulation was extracted. These patients were selected for further analysis. Same selection procedure was applied for BRCA2 mutant breast cancer patients, which provide us 75 patients in total. Ovarian Serous Cystadenocarcinoma (TCGA, PanCancer Atlas) study was used for selecting BRCA1 and BRCA2 mutant carriers, same selection process was applied as in breast cancer study, which provide us 42 and 22 patients with mutations, deletion, or downregulation in BRCA1 or BRCA2 respectively. After removing patients without mRNA expression data, 25 BRCA1 mutant ovarian cancer patients and 22 BRCA2 mutant ovarian cancer patients were left for further analysis. Survival curve and analysis were generated using Prism 8 Graphpad.

Results

Knockdown PPP factors can reduce G-MiDS

As mentioned before, previous research has proven that RNA Pol II is largely removed from the TSS region in M Phase [109]. In parallel, it also confirmed that DNA synthesis can still occur during mitosis, exacerbated following DNA replication stress [7, 108]. Hence, we hypothesize that G-MiDS could be used to complete duplication in G2/M phase across the TSS regions after remove RNA Pol II. Previously in the laboratory, this hypothesis was tested using BJ-hTERT. Cells were firstly synchronized in G0/G1 phase by serum starvation for 26 hours. This approach allowed cells to re-enter cell cycle once released in complete medium. Using this method, it was previously confirmed by fluorescence-activated cell sorting (FACS) and by the increased incorporation of BrdU [unpublished data, Fig.11] that cells started to enter S phase 14 hours after release in complete medium. In parallel with the start of cell serum starvation, siRNA transfection was carried out to deplete SUPT5H or NELF-A, which belong to DSIF and NELF complexes respectively, and both are crucial for regulation of PPP [21-31]. By doing so, we disrupt RNA Pol II PPP, and as a result, there should be less accumulation of RNA Pol II near TSS region as the RNA Pol II is moving more towards the gene body. Cells were released into complete medium to promote cells to enter S phase, then when halfway through S phase synchronized in G2 using CDK1 inhibitor Ro3306 for 16 hours. Following the arrest in G2, cells are released in complete medium with the nucleotide analogue 5-ethynyl-2'-deoxyuridine (EdU) for 30 minutes to label specifically DNA synthesis events after the G2 arrest. Immunofluorescence (IF) images were analysed as the proportion of G2/M cells defined by positivity for H3 Ser-10 phosphorylation (p-H3) that were newly synthesizing DNA in G2/M phase,

as monitored by EdU incorporation by Click-iT reaction (Fig.12A). To confirm data previously generated in the lab [unpublished data], IF experiment was repeated again and the results were consistent to what we observed before: there are approximately 20% of cells doing G-MiDS in CTR siRNA cells, and the percentage of cells requiring G-MiDS decreased when knockdown SUPT5H or NELF-A because of a reduced need of G-MiDS to complete the duplication of TSS regions.

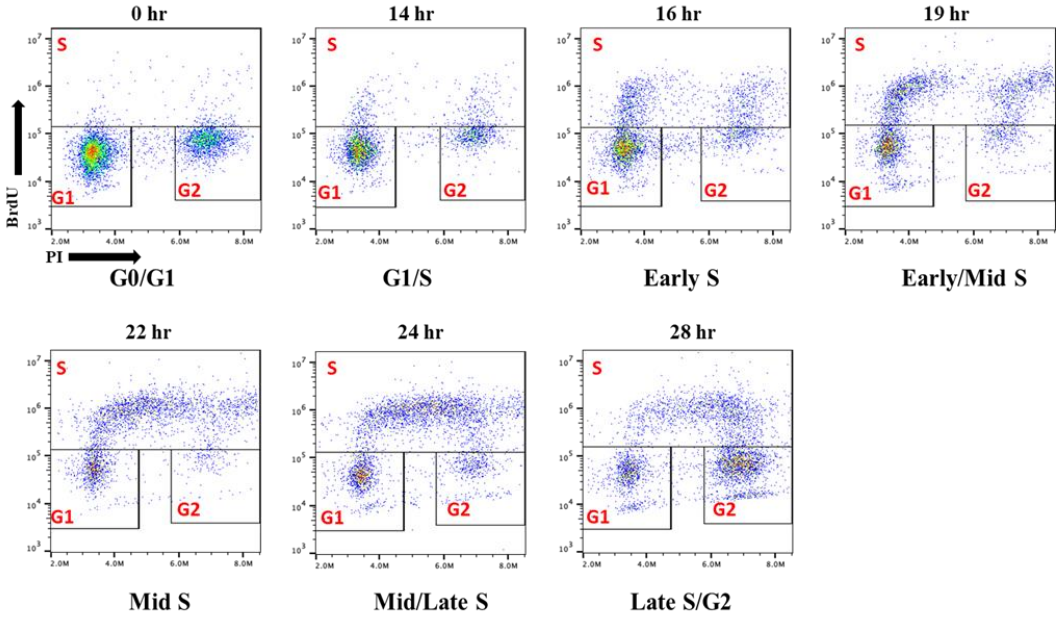


Figure.11 FACS analysis of propidium iodide (PI) and BrdU incorporation was used to monitor S-phase progression. BJ cells were synchronized by serum starvation for 26 hours in G0/G1, once released in complete medium, cells re-enter cell cycle. Cells started to enter S-phase 14 hours after releasing into complete medium, as evidenced by the increased incorporation of BrdU. propidium iodide (PI) was used to label DNA content and used to mark cells in G1 or G2 phase based on the DNA content. Percentage of cells in S phase increased gradually from 3.23 to 67.5 from G0/G1 to mid-S phase and dropped to 35.2 at late S/G2 phase. Cells in G1 phase was 68.4% at 0 hour, and as cell cycle progression, percentage of cells stay in G1 phase gradually decreased to 20.5% in late S/G2 phase.

However, due to the technical issues, the quantification of IF data became difficult later on because of batch to batch variations with the p-H3 antibody, making it difficult to specifically identify G2/M phase cells from p-H3 staining in IF images.

Therefore, we developed an alternative method to reduce the bias of IF quantification and obtain a more reliable data to analysis of cells undergoing G-MiDS. After carrying out standard cell synchronization and cell staining, instead of using IF, FACS was used to sort out the cells based on their DNA content, EdU and p-H3 intensity, with double positive cells for EdU and p-H3 selected based on a consistent gating strategy (Fig.12B). Another advantage of using FACS instead of IF is that we can acquire more than ten thousand cells for analysis within a short period of time. Therefore, FACS analysis not only provides a more robust result, but also saves a great amount of time in acquiring and counting images that contain only hundreds of cells.

FACS analysis was consistent with what we observed previously using IF images, and it confirmed that PPP is directly linked with G-MiDS levels as depletion of PPP factors results in reduction on G-MiDS. In CTR siRNA treated cells 20.75% of p-H3 positive were also EdU positive (Fig 12C). However, the percentage of cells undergoing G-MiDS in SUPT5H and NELF-A knockdown cells was only 6.24% and 13% (Fig.12C). Therefore, KD of SUPT5H and NELF-A both significantly reduce the requirement of G-MiDS. Previous work had shown that following PARPi treatment, more cells were using G2/M to complete DNA synthesis across PARPi-induced sites of DNA damage [7, 117]. However, in parallel, PARPi has been shown to regulate PPP through NELF complex by Gibson et al, as inhibition of PARP1 activity leads to accumulation of RNA Pol II pausing at TSS region [105]. Therefore, when cells are treated with PARPi, we hypothesised that there would be an increase of cells requiring G-MiDS to duplicate TSS region as there would be

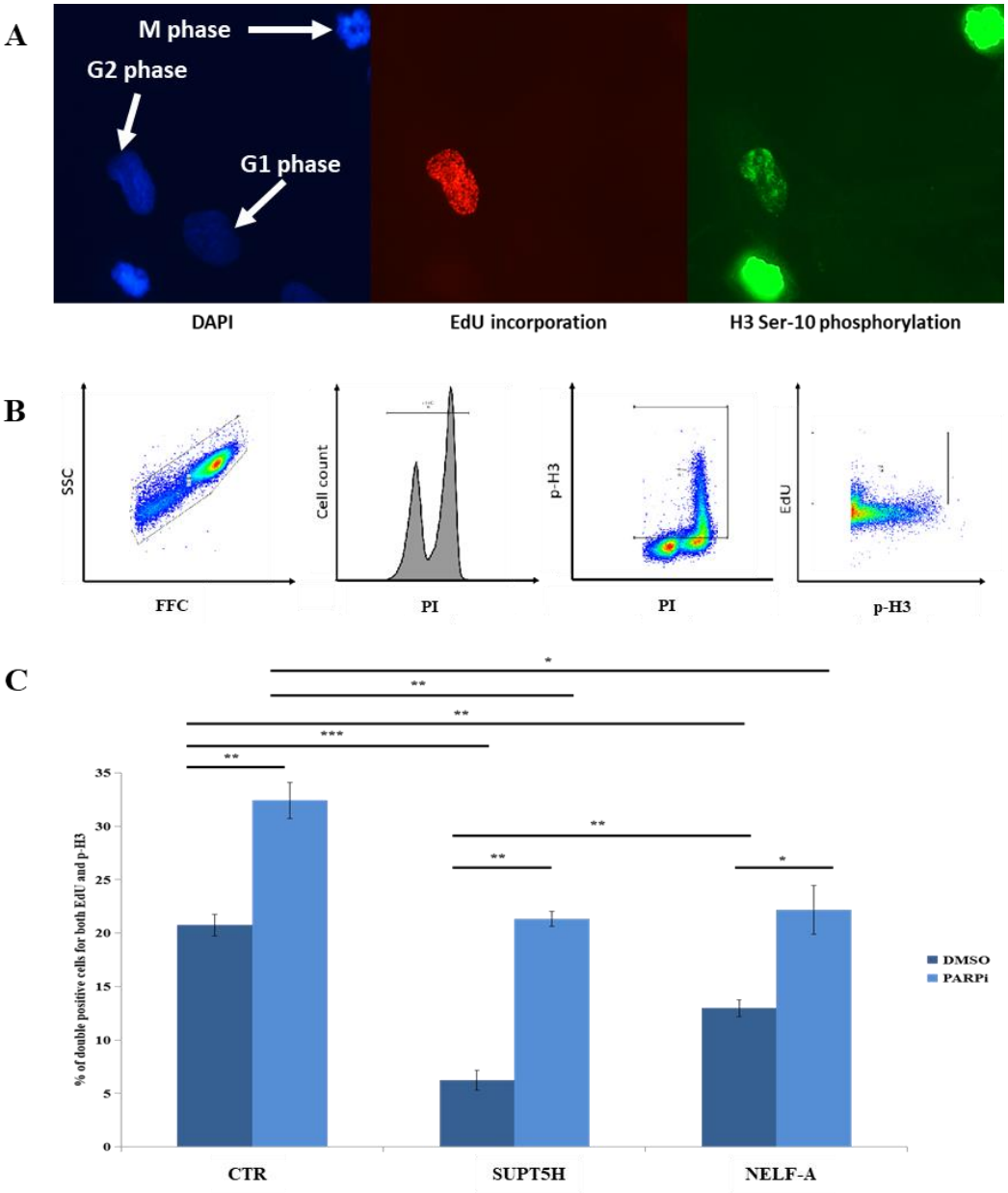
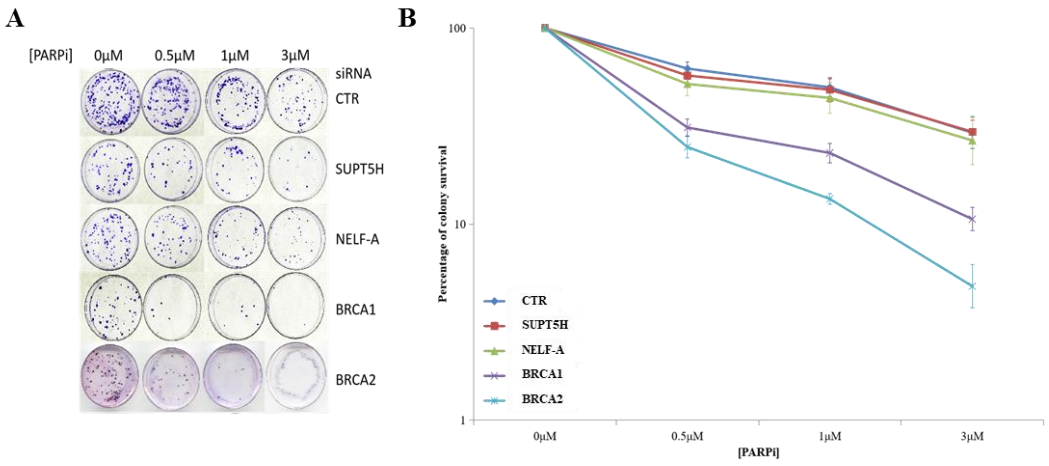


Figure.12 Knockdown of PPP factors can reduce G-MiDS and PARPi induces G-MiDS in cells. (A) In IF images, DAPI (blue) is used to label the nucleus, EdU labelling (red) indicates the newly synthesised DNA after releasing from the G2/M block, H3 Ser10-phosphorylation (green) indicates that cells have entered G2/M phase (M phase cells show a particularly robust fluorescence). (B) To select the double positive cells from FACS, cells are firstly selected according to their size, then sorted by the DNA content to remove all the cell debris and cell doublets. Then cells high in H3 Ser10-phosphorylation (p-H3) are selected also for positivity to EdU labelling; we use the cells that are double positive for both p-H3 and EdU divided by the total cell number from second gate, to calculate the percentage of cells that are doing G-MiDS. (C) FACS data quantification is shown in the bar chart, it shows that when treating cells with PARPi, more cells are doing G-MiDS, but following knockdown of PPP factors, there is a dramatic decrease of G-MiDS levels, this reduction also occurs in cells treated with PARPi. Two-way student T test is used for statistical analysis, experiment was repeated 3 times, and average was shown in the graph, SEM was indicated using error bar. (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$)

more paused RNA Pol II during S phase at TSS, and replication stress increases during S phase because of it. We confirmed that when cells were treated with PARPi, there was an increase of cells that performed G-MiDS, which was consistent with previous finding [7]. We observe 32.24% of CTR siRNA cells being p-H3/EdU double positive after PARPi treatment, in comparison with CTR siRNA cells without PARPi treatment (Fig. 12C). On the other hand, comparing to CTR siRNA cells with PARPi treatment, we observed less G-MiDS positive cells in SUPT5H and NELF-A KD after treatment with PARPi as there were only 21.33% SUPT5H KD cells and 22.17% of NELF-A KD cells doing G-MiDS following PARPi (Fig.12C). Taken together, we have shown that regulation of PPP is directly associated with G-MiDS levels, and we think that PARPi induced G-MiDS is not only through its role in creating replication stress because of DNA damages, but also its function in modulation PPP in transcription and its impact on the formation of under-replicated gaps at TSS.

Knockdown of PPP factors in U2OS cell does not affect sensitivity to PARPi

Given our previous finding that the regulation of PPP affects G-MiDS also following PARPi treatment, we then tested whether knockdown of PPP factors may affect PARPi sensitivity in cells. In particular, in light of the reduced levels of G-MiDS induced by PARPi following the KD of SUPT5H and NELF-A, we wanted to assess whether this was eventually enough to rescue also the hypersensitivity to PARPi of BRCA1 KD cells. Colony assays were used to test this hypothesis, 300 cells were seeded after siRNA transfection, and 24 hours later increasing concentrations of PARPi were added to cells. Cells that are sensitive to PARPi would be affected in their grow, while those that survived PARPi treatment would



	p-value		
	0.5μM	1μM	3μM
CTR vs SUPT5H	ns	ns	ns
CTR vs NELF-A	ns	ns	ns
CTR vs BRCA1	***	**	*
CTR vs BRCA2	**	**	*
SUPT5H vs BRCA1	**	**	***
SUPT5H vs BRCA2	**	**	**
NELF-A vs BRCA1	*	*	*
NELF-A vs BRCA2	*	*	ns
BRCA1 vs BRCA2	ns	*	*

Figure.13 Colony assay results indicate that knockdown of PPP factors does not affect cells' sensitivity to PARPi. (A) Colony assay is used to test PARPi sensitivity in all KD conditions, and each condition is done in triplicates. (B) Quantification of colony assay results from single KD of CTR, SUPT5H, NELF-A, BRCA1 and BRCA2 with increasing concentrations of PARPi. Two-way student T test is used for statistical analysis, 8 repeats were used for CTR siRNA cells and BRCA1 siRNA cells, 4 repeats were used for BRCA2, SUPT5H and NELF-A knockdown cells, average percentage of colony formation ± SEM is shown on the plot. * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$.

grow into visible colonies after 10 days, and a difference in final colony numbers can be used to indicate drug sensitivity or resistance. At first, we used our BJ-hTERT cells, however, due to the nature of fibroblasts, they did not form

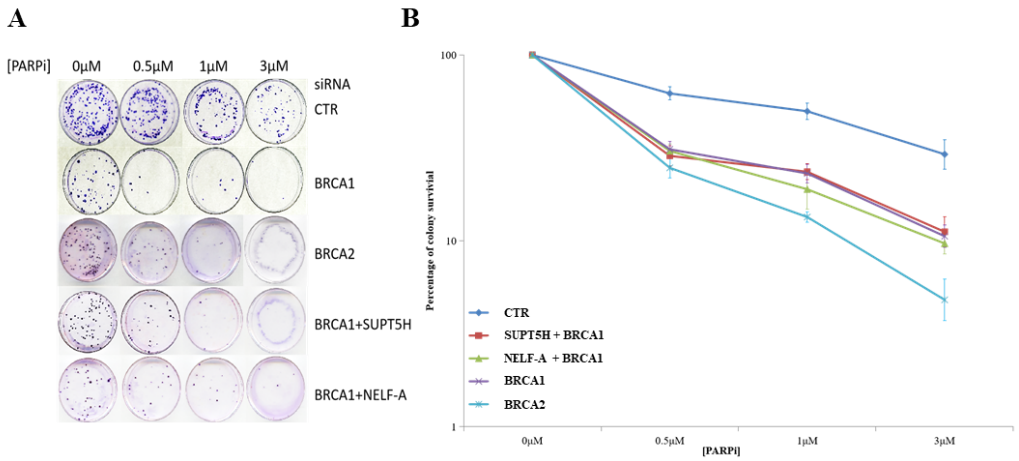
distinguished colonies (data not shown). Then we tried to generate growth curves using BJ-hTERT, but the results were not ideal as the growth time allowed before adding PARPi was too short and cells were stressed (data not shown). Therefore, we used U2OS cells instead, as it is a commonly used cell line for colony assay [119, 120]. After 10 days incubation with the presence of PARPi, we quantify the final colonies that meet restricted measures as only round colonies of more than 50 cells were selected [120]. We did colony survival after PARPi treatment also following the KD of BRCA1 and BRCA2, as both positive controls for conditions hypersensitive to PARPi treatment.

Indeed, in comparison with CTR siRNA cells, BRCA1 and BRCA2 depleted cell showed significantly reduced cell survival rate compared to the CTR siRNA cells, with BRCA2 knockdown cells even more sensitive than the BRCA1 cells to PARPi (Fig.13). However, there was no significant changes in colony survival rate between CTR siRNA cells and SUPT5H or NELF-A depleted cells (Fig.13B). Taken together, colony assay results confirmed that while cells depleted BRCA1 and BRCA2 show hypersensitivity to PARPi treatment, but that upon knockdown of PPP factors cells do not exhibit altered sensitivity to PARPi in comparison with CTR siRNA cells.

PPP knockdown cells show no rescue of BRCA1 sensitivity to PARPi

Previously, we have shown that PPP factors can modulate G-MiDS levels also following PARPi treatment. In parallel, cell knockdown of PPP factors shows same

sensitivity to PARPi in comparison with CTR siRNA cells. Therefore, we want to investigate if depletion of PPP factors can rescue PARPi hypersensitivity in BRCA1



	p-value		
	0.5μM	1μM	3μM
CTR vs BRCA1	***	**	*
CTR vs BRCA2	**	**	*
CTR vs BRCA1+SUPT5H	**	*	ns
CTR vs BRCA1+NELF-A	**	**	*
BRCA1 vs BRCA1+SUPT5H	ns	ns	ns
BRCA1 vs BRCA1+NELF-A	ns	ns	ns
BRCA2 vs BRCA1+SUPT5H	ns	*	Ns
BRCA2 vs BRCA1+NELF-A	ns	ns	*

Figure.14 Colony assay results indicate PPP knockdown does not rescue BRCA1 knockdown PARPi sensitivity. (A) Colony assay is used to test PARPi sensitivity in all KD conditions, and each condition is done in triplicates. (B) Colony survival rate of PPP factors and BRCA1 double KD cells show no significant differences from BRCA1 single knockdown cells when increasing the PARPi dosages. Two-way student T test is used for statistical analysis, 8 repeats were used for CTR and BRCA1 siRNA cells, 4 repeats were used for BRCA2 siRNA cells and double knockdown cells, average percentage of colony formation ± SEM is shown on the plot. * = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$.

deficient cells, as reduced requirement of G-MiDS following PARPi treatment may affect also PARPi sensitivity in BRCA1 knockdown cells. To do so, we repeated colony assay using U2OS cells, and depleted BRCA1 and SUPT5H or NELF-A at the same time (Fig.14), knockdown efficiency was verified using western blot (see next paragraph). For BRCA1 and SUPT5H double knockdown cells, colony

survival rates were 28.67%, 23.54% and 11.19%; BRCA1 and NELF-A double knockdown cells, colony survival rates were 30.46%, 18.96% and 9.69% when 0.5µM, 1µM and 3µM of PARPi was used, very similar to the survival rates of BRCA1 knockdown alone (Fig.14B). This indicated that even though PPP reduced the levels of G-MiDS following PARPi compared to CTR cells, there was no rescue of PARPi sensitivity in BRCA1 deficient cells when PPP factors were also depleted.

SUPT5H knockdown leads to reduction of BRCA1 and BRCA2 protein level

As mentioned above, western blot was used to confirm knockdown efficiency of SUPT5H, NELF-A, BRCA1 and BRCA2. In both BJ (Fig.15A) and U2OS cells (Fig.15C), the amount of siRNA used to deplete SUPT5H, NELF-A, BRCA1 and BRCA2 was sufficient as we observed a clear reduction of target proteins in comparison with CTR siRNA cells. Surprisingly, in SUPT5H depleted U2OS cells, BRCA1 and BRCA2 protein level were also reduced, with BRCA1 levels almost comparable to the BRCA1 knockdown (Fig.15C). However, such a dramatic change in BRCA1 protein level was less pronounced in BJ cells when depleting SUPT5H (Fig.15A). In parallel, western blot for U2OS cells also confirmed that in all knockdown conditions, there was no significant changes in other DDR factors, such as ATM, BACH1 and PARP1 compared to CTR siRNA cells (Fig.15C-D). These findings indicated that the reduction of BRCA1 levels following SUPT5H KD was specific only for BRCA1, and not associated to a more general reduction in the expression of DDR factors. To determine whether the reduced protein expression of BRCA1 was due to reduced gene expression of BRCA1, QPCR was used to measure the mRNA level of BRCA1 in all knockdown conditions. After normalization to a housekeeping gene (RPLP0) and to BRCA1 expression level in

CTR siRNA cells, we confirmed from 24 hours to 72 hours after BRCA1 siRNA transfection, there was a significant reduction of BRCA1 mRNA level. However, it

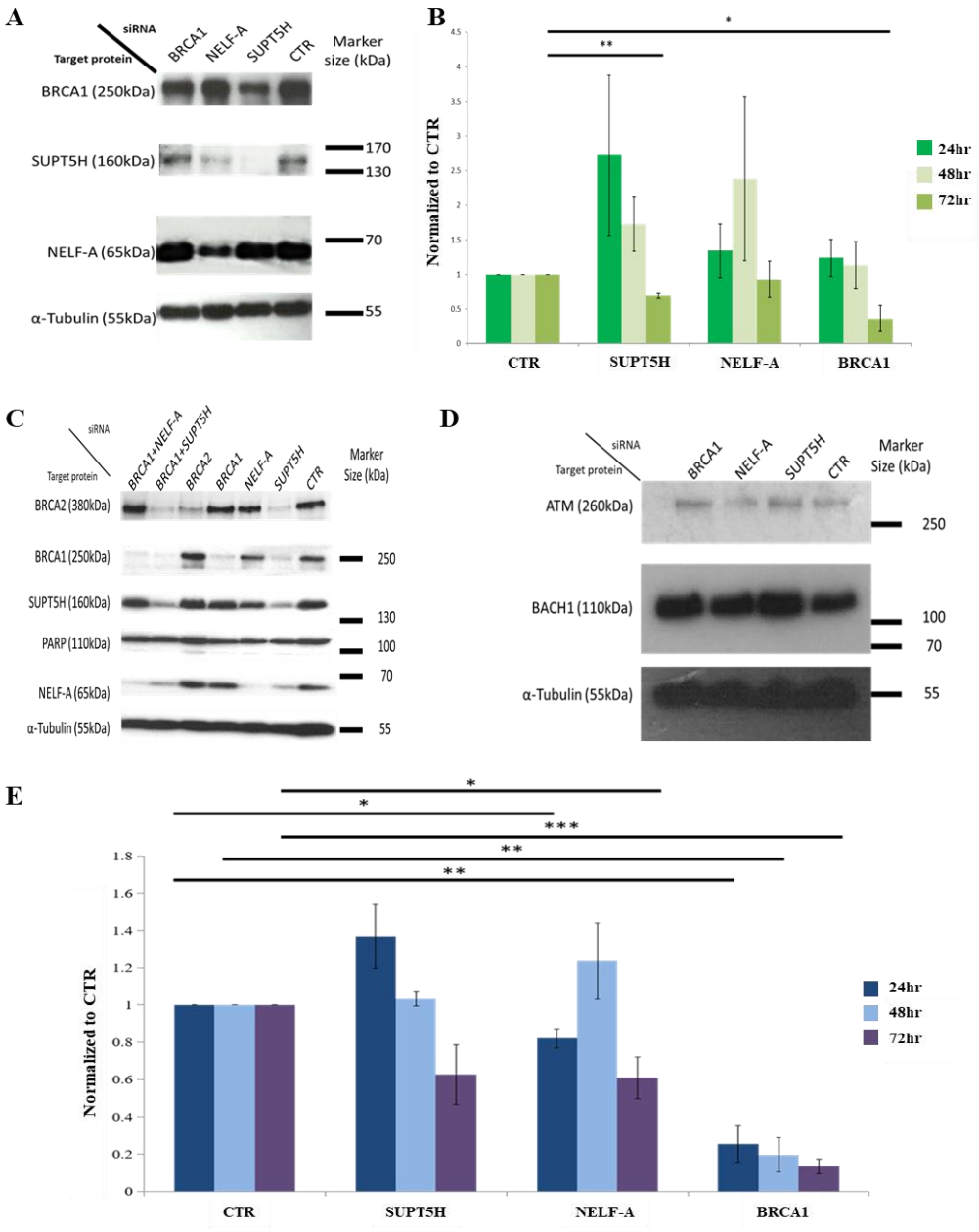


Figure.15 Western blot and QPCR is used to validate the siRNA KD efficiency. (A) Western blot confirmed the siRNA knockdown efficiency in BJ cells. (B) QPCR data after normalization show that in both SUPT5H and BRCA1 knockdown cells, 72 hours post siRNA transfection, the BRCA1 mRNA level is significantly reduced. n= 3, SEM is shown by error bar. (C-D) In U2OS cells, 72 hours after siRNA transfection, western blot for the indicated proteins. (E) QPCR results in U2OS cells show 72 hours post siRNA transfection, there is reduction of BRCA1 mRNA level in SUPT5H, NELF-A and BRCA1 knockdown cells. n = 3, SEM is shown by error bar. * = $p<0.05$, ** = $p<0.005$, *** = $p< 0.0005$.

was intriguing that in BJ cells, BRCA1 mRNA levels also significantly reduced in SUPT5H knockdown cells 72 hours after siRNA transfection (Fig.15B). As well as in U2OS cells, SUPT5H and NELF-A knockdown cells also show slightly reduction of BRCA1 mRNA level comparing to CTR siRNA cells at 72 hours, although not of the same extent as the KD for BRCA1 (Fig.15E). Therefore, KD of SUPT5H and NELFA appear affecting the protein level specifically of BRCA1, both at the transcript and the protein level. The reduction of BRCA1 mRNA level shown in U2OS cells when knockdown SUPT5H may contribute the reduction of overall protein level we observed through western blot. The differences in the impact of the reduction of the protein level between the two cell lines may be explained by differences in the turnover rate of BRCA1, that could be different in BJ cells compared to U2OS cells.

From western blot we confirmed that depletion of SUPT5H caused a reduction of BRCA1 protein level, even though but at the same time, these cells do not show sensitivity to PARPi treatment. Therefore, we believe that BRCA1 mRNA detected by QPCR in SUPT5H depleted U2OS cells was sufficient for protein production and its activity in DDR.

Depletion of PPP factors and BRCA1 is epistatic

Even though PPP factors affected G-MiDS levels following PARPi treatment and that the KD of PPP factors affected also BRCA1 protein levels, KD of PPP did not alter PARPi sensitivity compared to CTR cells nor to BRCA1 KD ones. Therefore, we expanded our analysis on the possible interplay between PPP and BRCA1.

Beyond its role in the DDR, it is well established the pervasive and crucial role of BRCA1 in multiple cellular activities, and indeed knockout BRCA1 leads to embryonic lethality in mice [87, 118].

Importantly, among the many roles of BRCA1 there is also an involvement in RNA Pol II transcription, and BRCA1 can interact with COBRA1 (also known as NELF-B), which belongs to NELF complex [67]. Not to mention that transcription is a key step for cell survival, and we have demonstrated that disruption of PPP is directly linked to G-MiDS, which is essential to maintain genome stability.

Therefore, we investigated whether there was any genetic interaction between BRCA1 and PPP factors. To do this, we assessed whether cell survival rate is affected when knockdown PPP factors is combined with BRCA1 knockdown. We reanalysed colony assay result, that other than showing the PARPi sensitivity in each knockdown condition, also provide a view on overall survival rates. Instead of quantifying the survival rate of cells treated with PARPi, we focused on the colony survival rate in each knockdown condition without PARPi treatment. Considering that we seeded 300 cells per dish, we calculated that the survival rate for untreated CTR siRNA cells was 52.58%, but for SUPT5H and NELF-A, the survival rate was reduced to 20.37% and 39.97%, respectively (Fig.16). The survival rate for untreated BRCA1 depleted cells was 13.34%, similar to the one of BRCA2 knockdown cells that had 17.62%. Double knockdown of BRCA1 and SUPT5H cells had a survival rate of 16.87% but survival rate for BRCA1 and NELF-A cells was only 7.87%, which was the lowest among all conditions. The differences in

survival rate indicated that knockdown of PPP factors or BRCA1 would reduce colony survival as expected, and co-depletion of NELF-A and BRCA1 would induce 'synthetic lethality', indicating that the two factors may have separated functions. However, since there was no significant difference between BRCA1 and SUPT5H double knockdown cells and BRCA1 knockdown alone, this suggests that BRCA1 and SUPT5H may work in the same pathway to maintain cell survival.

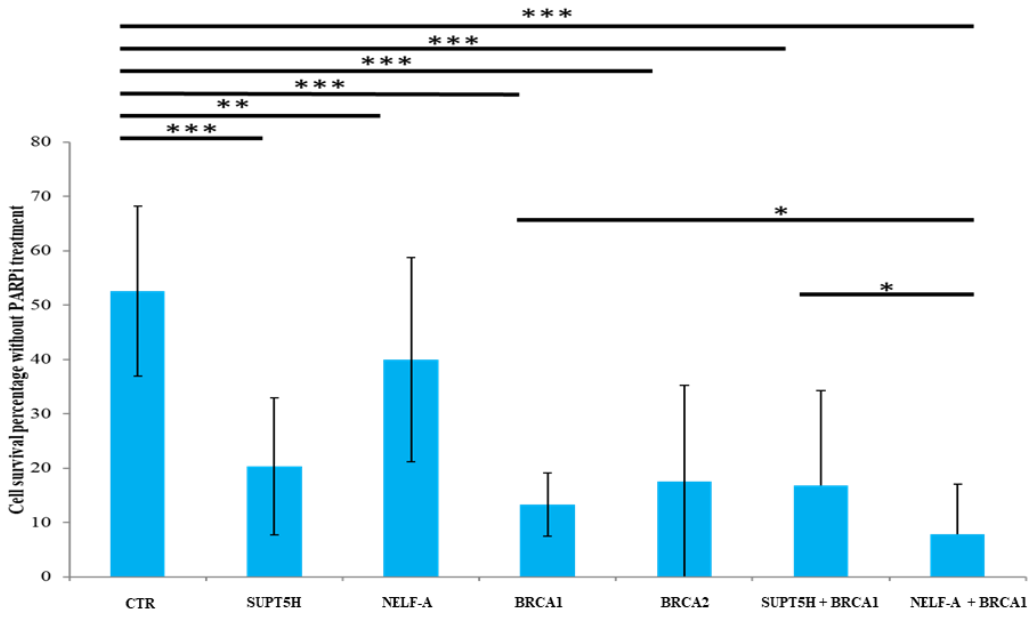


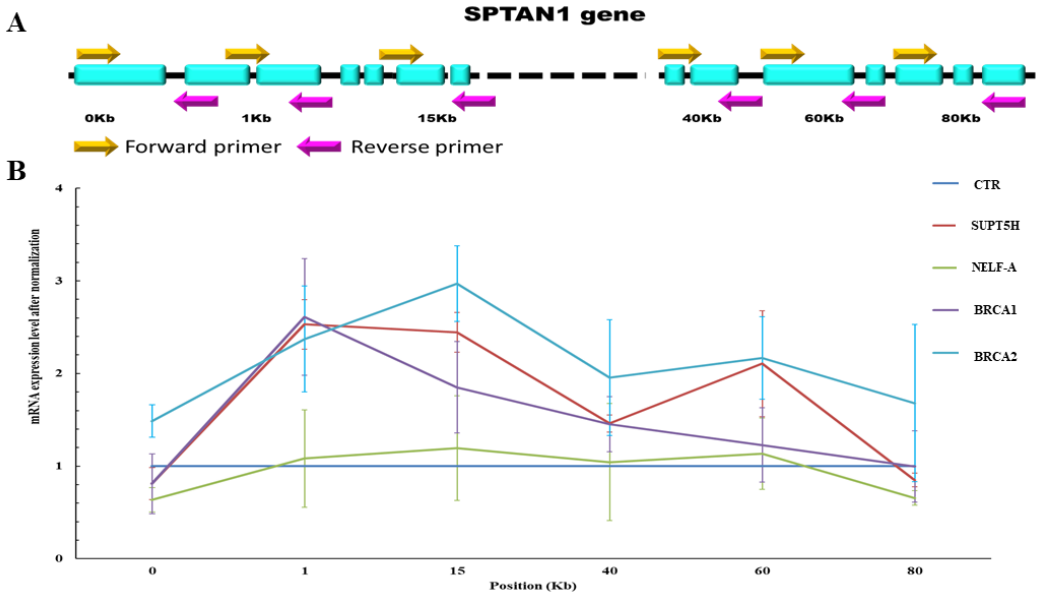
Figure.16 Knockdown of SUPT5H, NELF-A and BRCA1 together can induce synthetic lethality. Colony assay data shows that cell survival rate after depletion of either SUPT5H, NELF-A or BRCA1 alone, or the combination of SUPT5H or NELF-A with BRCA1, can induce synthetic lethality in cells. Two-way student T test is used for statistical analysis, 8 repeats were used for CTR and BRCA1 siRNA cells, 4 repeats were used for cell with other knockdown conditions, SEM is indicated using error bar. * = $p<0.05$, ** = $p<0.005$, *** = $p< 0.0005$.

SUPT5H, BRCA1 and BRCA2 knockdown cells have general transcriptional defects

As mentioned before, Gardini and colleagues showed that the ChIP-Seq distribution of BRCA1 mirrors that of the RNA Pol II from TSS to termination site [66]. On the other hand, our previous data have suggested that BRCA1 and SUPT5H may work together to support cell survival. Therefore, we went on testing what role BRCA1 had during transcription progression, especially compared with cells knockdown of SUPT5H and NELF-A. To do so, we used nascent mRNA production as an indication of transcription progression [126] within the long gene SPTAN1. Primers were designed from the beginning of the gene all along the gene body to monitor transcription progression at different positions (Fig.17A). After normalization and comparison with CTR siRNA cells, accumulations of pre-mRNA occurring at specific positions, could indicate that at that position, there is a defect in transcription progression, very likely due to a slowdown of RNA Pol II transcription (Fig.17B) [126].

In parallel we also assessed transcription progression after the KD of BRCA2, as previously Shivji et al. showed that BRCA2 can prevent unscheduled R-loop accumulation at PPP site [100], hence, knockdown BRCA2 should also exhibit a defect in transcription progression. Unsurprisingly, SUPT5H knockdown cells presented transcriptional defects of transcription in comparison with CTR siRNA cells more pronounced at the early stage (p-value for CTR vs SUPT5H is 0.00007 and 0.000006 at 1Kb and 15 Kb respectively) than later on in the gene body, as SUPT5H is also a more general transcription elongation factor together with its role in regulating PPP (Fig.17B). Indeed, SUPT5H KD showed a 2.5-fold increase in

nascent mRNA levels at positions 1kb and 15kb, potentially indicating that transcription is 2.5 times slower over these positions in SUPT5H KD compared to CTR cells. However, the NELF complex dissociates from transcription machinery after phosphorylation by P-TEFb unlike SUPT5H, which is a positive transcription



	CTR VS SUPT5H	CTR VS NELF-A	CTR VS BRCA1	CTR VS BRCA2	SUPT5H VS NELF-A
0 Kb	ns	ns	ns	**	ns
1 Kb	***	ns	*	*	*
15 Kb	***	ns	*	*	ns
40 Kb	ns	ns	ns	*	ns
60 Kb	ns	ns	ns	*	ns
80 Kb	ns	ns	ns	ns	ns

Figure.17 Cells show transcriptional defects when depleted of PPP factors, but also of BRCA1 and BRCA2. (A) Schematic shows the position of the primers spread along the gene body and designed across exon-intron junctions. (B) ΔΔCt was used to normalize the pre-mRNA expression levels in different knockdown conditions, and then normalized to the control cells levels for comparison. Two-way student T test is used for statistical analysis. n = 3, SEM is indicated by error bar. * = p<0.05, ** = p<0.005, *** = p< 0.0005

elongation factor, and moves along with RNA Pol II, [35]. Consequently, following NELF-A knockdown transcription progression had no significant difference to CTR siRNA cells (Fig.17B). As expected, BRCA2 knockdown cells presented also a severe transcriptional defect from TSS down to 60Kb position, similar to that observed when SUPT5H is KD (Fig.17B). Intriguingly, cells depleted of BRCA1 exhibited a transcription progression pattern similar to that of SUPT5H knockdown cells (Fig.17B, p-value for CTR vs BRCA1 is 0.008 and 0.011 at 1kB and 15 kB respectively), which indicated that BRCA1 may have a similar functional role as SUPT5H during transcription elongation in agreement with the not additive impact on colony survival in untreated conditions. Intriguingly, these data suggest that BRCA1 and BRCA2 are important transcription elongation factors, and in their absence elongation rates could be reduced of up to 2.5-3-fold compared to CTR cells.

Correlation between PPP factors expression and outcome of BRCA1 and BRCA2 mutant breast and ovarian cancer patients

Following on the results above that showed genetic and functional overlap between BRCA1 and BRCA2 and general transcription factors, we wanted to analyse whether PPP factors could have also a direct impact in BRCA1 and BRCA2 mutated cancer patients' outcome. To do this BRCA1 and BRCA2 mutant breast or ovarian cancer patients' data from The Cancer Genome Atlas (TCGA) studies was obtained from cBioPortal.

For each cancer type, patients with BRCA1 or BRCA2 mutations, deletions, and downregulations were selected specifically from the TCGA PanCancer Atlas study. This first filter provided us 42 ovarian and 61 breast BRCA1 mutant cancer patients for further analysis. SUPT5H and NELF-A expression level were also obtained from the same TCGA PanCancer Atlas study, then patients are sorted based on the expression level and split into two group. The two groups of patients were cross checked with the patient lists to filter out the BRCA1 mutant cancer patients without mRNA information for the PPP factors. After cross checking with the previous sub-lists of patients, 25 ovarian and 60 breast BRCA1 mutant cancer patients were used for further analysis. BRCA2 mutant breast or ovarian cancer patient data was obtained and filtered following the same procedure, which left 22 ovarian and 75 breast cancer patients for further analysis.

The low number of patients reduced the ability to perform a significant analysis on the impact of the expression levels of SUPT5H and NELF-A in the survival of BRCA mutated patients. However, even in these instances, it appears that SUPT5H and NELF-A high/low expression levels may have an impact more in breast cancer patients than in ovarian cancer ones (Fig.18). Both in BRCA1 and BRCA2 mutated breast cancer patients SUPT5H high expression correlates with better disease-free survival compared to SUPT5H low expression. In BRCA1 mutated patients the disease-free survival rate for patients that express high levels of SUPT5H is around 91% after 10 years, and for patients with low expression of SUPT5H is around 68% at the same time (p value is 0.56 Log-rank (Mantel-Cox) test). High expression levels of NELF-A correlate with a better overall survival rate and disease-free survival rate in BRCA1 mutant breast cancer patients (Fig.18A). Patients with high

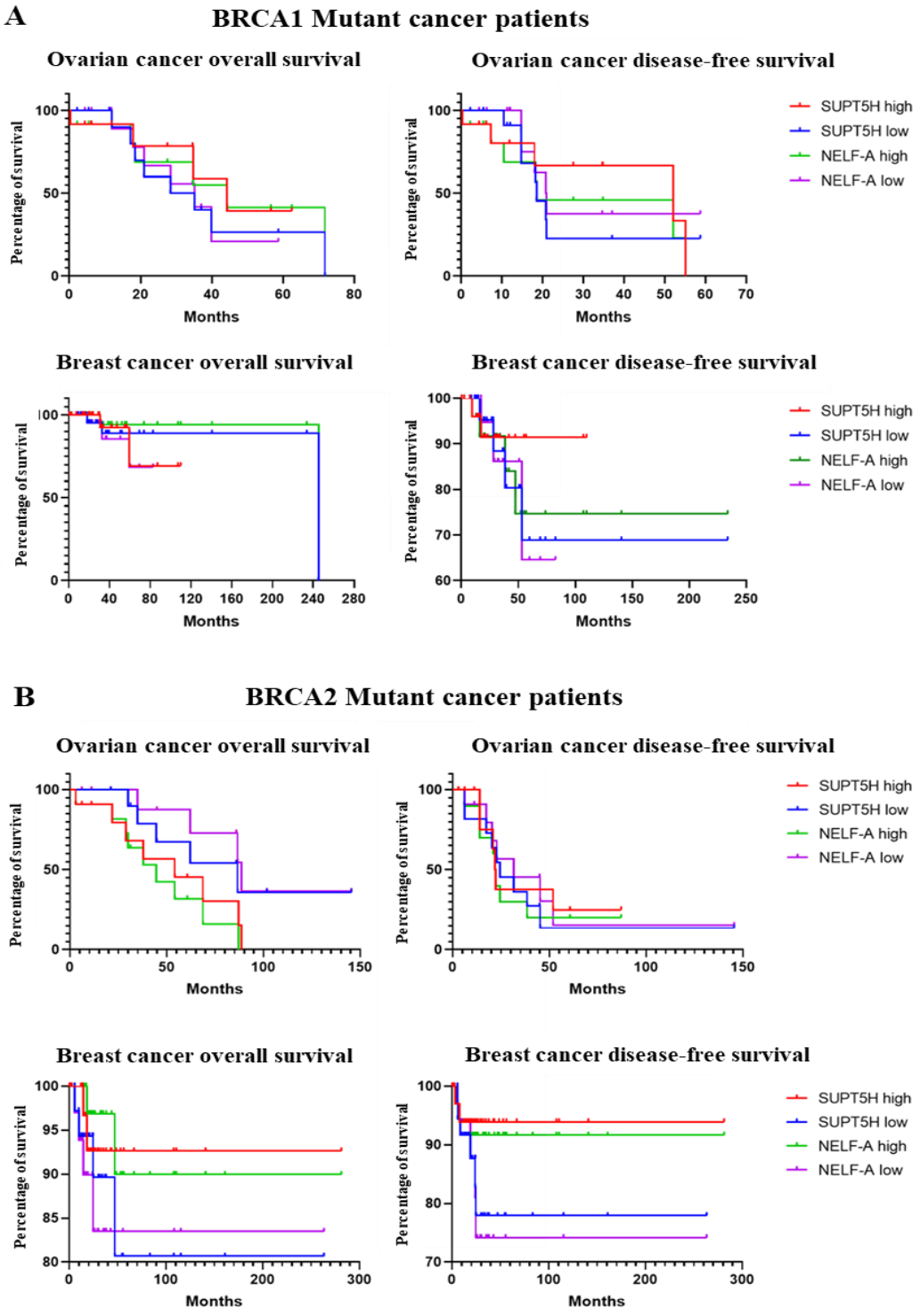


Figure.18 cBioPortal analysis for BRCA1 and BRCA2 mutant ovarian and breast cancer patients. (A) All BRCA1 mutant breast and ovarian cancer patients are separated in two groups indicated as high or low for the expression of SUPT5H and NELF-A and their survival and disease-free survival is analysed. (B) All BRCA2 mutant breast and ovarian cancer patients are separated in two groups indicated as high or low for the expression of SUPT5H and NELF-A and their survival and disease-free survival is analysed.

level of NELF-A expression show approximately 95% overall survival rate and about 75% disease-free survival rate, against 68% and 64% in overall survival (p value is 0.166 Log-rank (Mantel-Cox) test) and disease-free survival rate (p value is 0.932 Log-rank (Mantel-Cox) test) of those with low expression levels of NELF-A.

In BRCA2 mutant ovarian cancer patients, low expression of SUPT5H and NELF-A may associate with a better overall survival rate (p value is 0.172 for SUPT5H, and p value is 0.01 for NELF-A, Log-rank (Mantel-Cox) test, Fig.18B). In BRCA2 mutant breast cancer patients, high expression of SUPT5H and NELF-A correlates with a better overall survival rate and disease-free survival rate (Fig.18B). The overall survival rate for patients with high expression of SUPT5H is around 92.5% after 20 years, while for patients with low expression of SUPT5H it is around 80% (p value is 0.473 Log-rank (Mantel-Cox) test). Meanwhile, patients with high expression of NELF-A show around 90% overall survival rate, and low expression of NELF-A shows around 83% in overall survival rate (p value is 0.147 Log-rank (Mantel-Cox) test). Similarly, the disease-free survival for patients with high expression of SUPT5H and NELF-A is approximately 94% and 91.5% after 20 years, against 78% and 74% in patients with low expression of SUPT5H (p value is 0.182 Log-rank (Mantel-Cox) test) and NELF-A (p value is 0.263 Log-rank (Mantel-Cox) test, Fig.18B) respectively.

Given that SUPT5H and NELF-A belong to the DSIF and NELF complexes respectively, we then conducted the same analysis with the other subunits of these

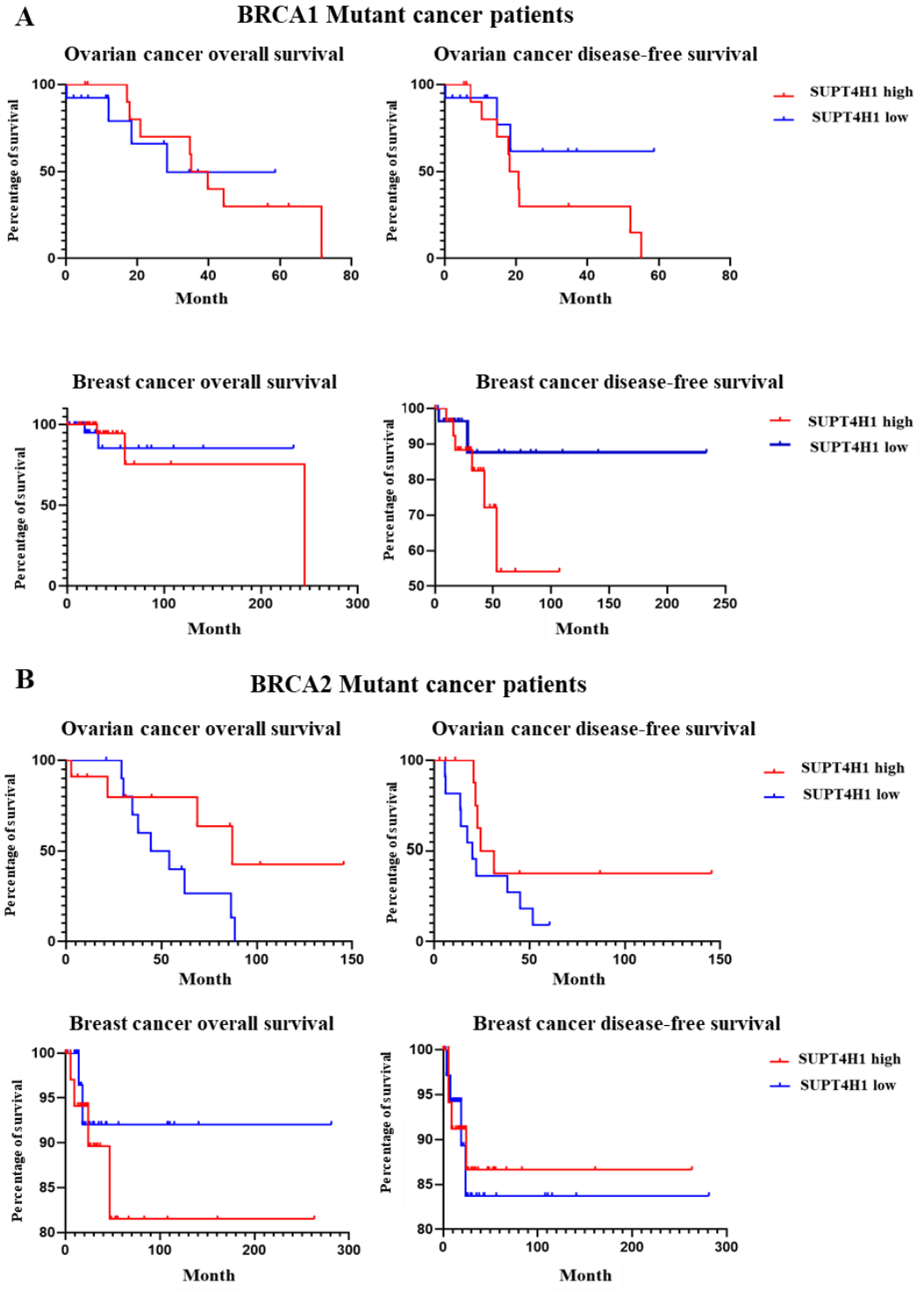


Figure.19 cBioPortal analysis for BRCA1 and BRCA2 mutant ovarian and breast cancer patients. (A) All BRCA1 mutant breast and ovarian cancer patients are separated in two groups indicated as high or low for the expression of SUPT4H1, their survival and disease-free survival is analysed. (B) All BRCA2 mutant breast and ovarian cancer patients are separated in two groups indicated as high or low for the expression of SUPT4H1, and their survival and disease-free survival is analysed.

complexes with the TCGA PanCancer Atlas studies. Also, in these cases, the small patient cohort makes getting statistically significant differences very difficult. In BRCA1 mutant ovarian and breast cancer patients, low expression of SUPT4H1 show no correlation in terms of overall survival (p value for ovarian cancer is 0.586, for breast cancer is 0.814, Log-rank (Mantel-Cox) test) but may associate with better disease-free survival rate (p value for ovarian cancer is 0.141, and 0.1954 for breast cancer, Log-rank (Mantel-Cox) test (Fig.19A).

In BRCA2 mutant ovarian cancer patients, high expression of SUPT4H1 is associated with better overall survival (p value is 0.083 Log-rank (Mantel-Cox) test) and disease-free survival (p value is 0.148 Log-rank (Mantel-Cox) test, Fig.19B), but no effect in BRCA2 mutant breast cancer patients (Fig.19B).

When analysing whether high or low expression levels of NEFL-B and NELF-E correlate with BRCA1/2 mutant patients' outcome, we find actually some statistically significant cases. Patients with high expression levels of NELF-E in BRCA2 mutant ovarian cancer show a better overall survival ($p<0.005$, Log-rank (Mantel-Cox) test) and disease-free survival rate ($p<0.05$, Log-rank (Mantel-Cox) test) than those with low expression of NELF-E (Fig.20A). In BRCA1 mutant breast cancer patients, we did not find clear differences in overall survival rate when patients express high or low level of NELF-B and NELF-E (Fig.20B). However,

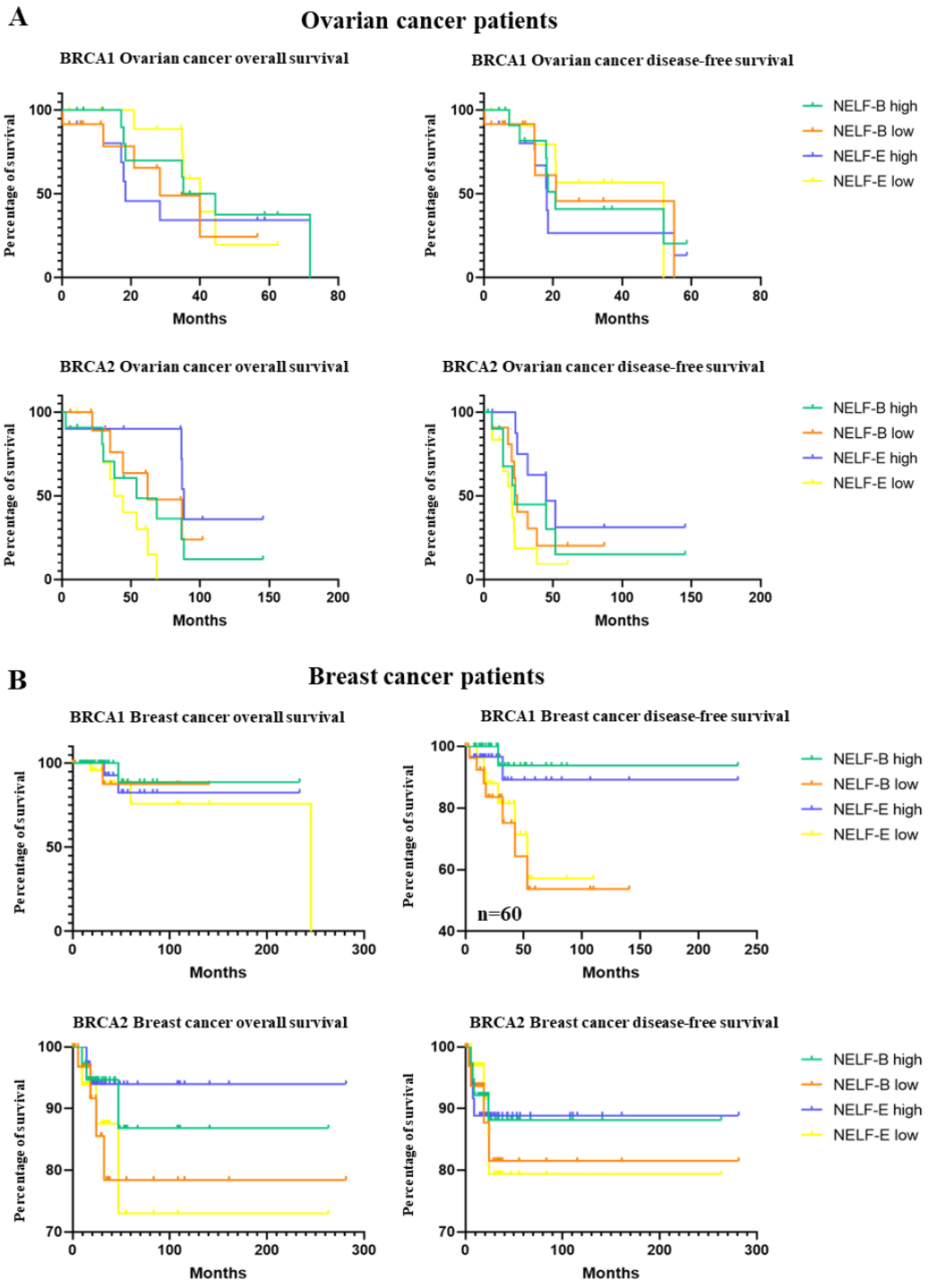


Figure.20 cBioPortal analysis for BRCA1 and BRCA2 mutant ovarian and breast cancer patients. (A) All BRCA1 and BRCA2 mutant ovarian cancer patients are separated in two groups indicated as high or low for the expression of NELF-B and NELF-E, their survival and disease-free survival is analysed. (B) All BRCA1 and BRCA2 mutant breast cancer patients are separated in two groups indicated as high or low for the expression of NELF-B and NELF-E, their survival and disease-free survival is analysed.

the disease-free survival rate of these patients is better when they express high level of NELF-B ($p<0.05$, Log-rank (Mantel-Cox) test, Fig.20B). Similarly, when these patients express high level of NELF-E, they tend to have a higher disease-free survival rate but due to the small patient cohort, there is no statistical significance (Fig.20B).

Altogether, even within this small cohort of BRCA1 and BRCA2 mutant ovarian and breast cancer patients, we have been able to identify particular cases where high or low expression levels of DSIF and NELF complex subunits may associate with an impact on the clinical outcomes. However, there is no clear indication that specifically high or specifically low expression levels for these factors consistently correlate with better or worse clinical outcomes.

Discussion

PARPi and PPP regulation is associated with G-MiDS

MiDAS has been described as the process responsible for the completion of unfinished DNA replication when cells enter M phase, which is exacerbated by replication stress and commonly occurs at CFS and telomeres [7, 121, 127]. Strikingly, we have identified an unusual new DNA synthesis process that occurs in G2/M phase. Previously we discovered that throughout S phase, due to the persistence of RNA Pol II at PPP sites, the TSS region of a gene becomes inaccessible for replication machinery. Therefore, this region remains under-replicated till RNA Pol II is removed from template DNA. Liang et al. have shown that paused RNA Pol II is released from PPP site via transcriptional activation by P-TEFb in G2/M phase [122]. Based on this evidence and the fact that DNA synthesis could occur till late stages in the cell cycle, we hypothesised that the TSS region occupied by RNA Pol II is finished to be duplicated in G2/M phase following the RNA Pol II release from PPP site (Fig.21) [121, 122]. Consequently, we investigated whether the modulation of PPP through the knockdowns of SUPT5H and NELF-A, could reduce the accumulation of RNA Pol II at PPP sites in S phase and subsequently reduce the requirement for G-MiDS. Consistently, as shown previously (Fig. 10, 11), based on the results generated using IF and FACS, we observed a significant reduction of cells undergoing G-MiDS upon knockdown of PPP factors in respect to the control.

At the same time, because the NELF complex is directly affected by PARPi via its component NELF-E [106], we thought that PARPi could also affect G-MiDS levels through its regulation of PPP. Therefore, when cells were treated with PARPi,

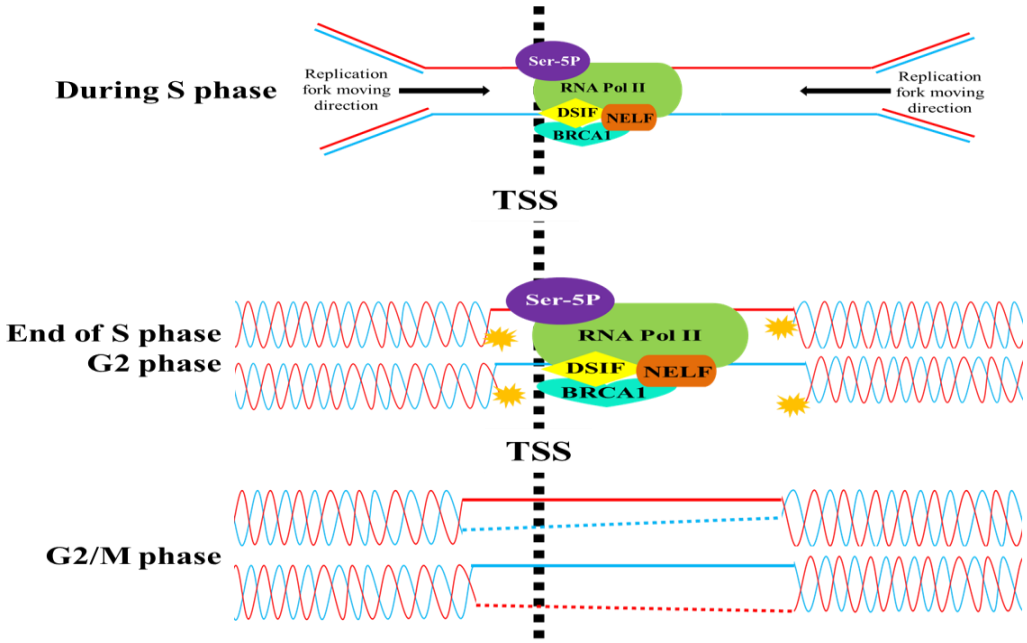


Figure.21 Possible model for G-MiDS. During S phase, due to the occupancy of DNA template by RNA Pol II around TSS region, replication of this region is postponed to G2/M phase. Once RNA Pol II is cleared out from TSS region, cells undergoing G-MiDS to complete duplication of this region.

we saw an increase of the percentage of cells undergoing G-MiDS, which is consistent with the conclusion drawn by Maya-Mendoza, A. et al [7]. Based on FACS experiments, in SUPT5H and NELF-A knockdown cells, we also observed an increase of cells undergoing G-MiDS following PARPi treatment when compared to cells without PARPi. However, in both SUPT5H and NELF-A knockdown cells following PARPi treatment, G-MiDS level is significantly lower than CTR siRNA cells treated with PARPi (Fig.12C). Hence, we confirmed that PPP factors can affect G-MiDS levels, as we showed that this is associated with the replicative gap around TSS region during S phase [data not shown]. Moreover, our data seem to suggest that PARPi can increase G-MiDS level in cells because of increased replication stress, which could depend not solely on the role of PARP in the DDR, but also on the role of PARP in the regulation of transcription via PPP.

Depletion of PPP factors show no rescue of PARPi sensitivity in BRCA1 knockdown cells

Once we confirmed that PARPi can increase G-MiDS levels in cells through regulation of PPP, we speculated that when we modulated G-MiDS level through depletion of PPP factors, cells face less replicative stress in S phase, and it may as well reduce the sensitivity to PARPi. Therefore, we then tested if knockdown of PPP factors can affect cells' sensitivity to PARPi treatment using colony assay. In U2OS cells, BRCA1 and BRCA2 depleted cells presented hypersensitivity to PARPi treatment as expected (Fig.13B, 14B) [82, 90, 107]. In parallel, we found that when we depleted SUPT5H and NELF-A in U2OS cells, there was no significant difference in terms of PARPi sensitivity compared to CTR siRNA cells. Intriguingly from western blot analysis we noticed that in SUPT5H knockdown cells there was an obvious reduction of BRCA1 and BRCA2 protein levels. When combined with the QPCR results in SUPT5H, NELF-A and BRCA1 single knockdown cells, we found that in SUPT5H knockdown cells even if there was a clear reduction in BRCA1 protein level, the mRNA level had not been affected dramatically. This would suggest that the reduction in the protein level of BRCA1 is not only due to defective transcription occurring in SUPT5H knockdown cells, but perhaps due to an increased turnover of BRCA1 following SUPT5H knockdown. Therefore, it was surprising to find that these cells did not show hypersensitivity to PARPi as BRCA1 and BRCA2 depleted cells. We firstly hypothesised that cells showed less sensitivity to PARPi because of the decreased accumulation of RNA Pol II at PPP sites after the knockdown of either SUPT5H and NELF-A, and the consequently reduced requirement of G-MiDS. Therefore, we were keen to know if reduced G-MiDS level could rescue PARPi hypersensitivity in BRCA1 mutant

cells. However, in BRCA1 and SUPT5H or BRCA1 and NELF-A co-depleted cells, when compared with CTR siRNA, the percentage of colony formation was significant decreased. Actually, BRCA1 and PPP factors co-depleted cells showed the same sensitivity to PARPi of BRCA1 single knockdown cells, suggesting that depletion of PPP factors cannot rescue PARPi sensitivity in BRCA1 knockdown cells. Therefore, we are unable to confirm the correlation between G-MiDS and change in PARPi sensitivity because co-depletion of BRCA1 and SUPT5H or NELF-A in cells also exhibit hypersensitivity to PARPi treatment. Therefore, we then hypothesised that in SUPT5H single knockdown cells the levels of BRCA1 mRNA and protein left should be enough to support BRCA1's role in HR. This would explain why even though SUPT5H depleted cells show a great reduction in BRCA1 protein level, they are not sensitive to PARPi.

Depletion of SUPT5H, BRCA1 and BRCA2 causes general transcriptional defects

As we observed an effect of SUPT5H and NELFA on the protein levels of BRCA1 and BRCA2 greater than only due to the defective transcription of SUPT5H and NELFA KD cells, we decided to investigate further the relationship between BRCA1 and BRCA2 with RNA Pol II transcription. Indeed, previous data have established interactions between NELF-B (COBRA1), RNA Pol II and BRCA1, as well as showing that BRCA1 has a role in R-loop-associated transcription termination [80, 87, 88]; moreover also BRCA2 can regulate PPP by preventing R-loop formation [105]. Interestingly, based on the research data from Gardini et al., we know that BRCA1 ChIP-Seq profile mirrors the movement pattern of RNA Pol II throughout transcription [68]. Hence, we wanted to understand how would BRCA1 and BRCA2 participate in transcription initiation and PPP regulation, and

the interplay between BRCA1, BRCA2, DSIF and NELF complexes. To investigate this question, we used an approach similar to Saponaro et al, using nascent mRNA production to monitor transcription progression [126]. Accumulation of nascent mRNA at specific positions along a gene indicates that at that position there is a transcription defect leading to an accumulation of RNA Pol II. It is likely that the accumulation is also associated with changes in transcription elongation rates. In cells depleted of NELF-A, the transcription process along the gene has no dramatic difference comparing to CTR siRNA group. This is because NELF complex is dissociated from transcription machinery after P-TEFb phosphorylation [22, 28, 35, 62], hence, it shows less influence in transcription elongation as its role is specific for PPP. However, SUPT5H is part of the DSIF complex which moves along with RNA Pol II during transcription elongation [28, 33]. Therefore, when we depleted SUPT5H, it affected the function of DSIF as a positive elongation factor and causing higher levels of nascent transcription more pronounced towards the beginning of the gene, indicative of defective transcription. Surprisingly, cells depleted of BRCA1 also show similar transcription defects to the SUPT5H knockdown cells, suggesting that BRCA1 may not only progress with the RNA Pol II as indicated by the Gardini et al., but also might be required to support transcription elongation. Intriguingly, when we knocked down BRCA2, we also observed a pattern similar to SUPT5H and BRCA1 knockdown cells. Previously it was shown that BRCA2 had a role in regulating RNA Pol II levels at the PPP site [105]. However, based on our results we think the similar pattern of transcription defects we observed in SUPT5H, BRCA1 and BRCA2 knockdown cells could indicate that these three factors work in a similar way, or perhaps together, to promote positively transcription elongation, moving all along with RNA Pol II

throughout transcription. In parallel, our result could suggest that BRCA1 could use COBRA1 (part of the NELF complex) only as an initial platform to be loaded onto transcription complex. When NELF complex dissociates from transcription machinery, BRCA1 could remain however attached to the transcription machinery and interacting directly or indirectly with RNA Pol II and/or other transcription factors. However, it is unclear at this stage how much of the accumulation of RNA Pol II in the gene body is caused by the disruption of the PPP and/or whether there is an effect also on RNA Pol II transcription elongation rates, slowing it down. Further experiments will be required in future to distinguish between the two possibilities.

Genetic interaction between SUPT5H and BRCA1

Once we found that the same transcription defects not only occur in SUPT5H knockdown cells but also in BRCA1 and BRCA2 knockdown cells, we were keen to explore if this kind of transcriptional defects could affect cell survival. We reanalysed the colony survival data of the untreated samples. NELF-A knockdown cells did not affect dramatically cell survival rate in respect to the control, which may be related with less transcriptional defects found in those cells as indicated by our data. In SUPT5H, BRCA1 and BRCA2 knockdown cells, the initial survival rate was significantly decreased in comparison with control group. We hypothesised such survival rate differences are related to the impact in the transcription defects occurring in different samples, where less severe transcription defects resulted in increased cell survival. Notably, depletion of BRCA1 with NELF-A induced synthetic lethality in comparison with the control knockdown and single knockdowns of either BRCA1 or NELF-A alone. Indeed, initial survival rate

was better when BRCA1 and SUPT5H were co-depleted than the co-depletion of BRCA1 and NELF-A. This indicates that NELF-A and SUPT5H work on different pathways to maintain cell survival, supported by data in the literature and our data (Fig.17), while SUPT5H and BRCA1 may work synergistically on the same pathway to support cell survival. This is in agreement with the levels of transcription defects we have found in NELF-A, SUPT5H and BRCA1 knockdown cells.

Expression level of PPP factors show some correlation with BRCA1 or BRCA2 mutant cancer patients' survival and relapse rate

Followingly, we then checked if similar survival patterns could be obtained from BRCA1 and BRCA2 mutant cancer patients. We extracted patients' clinical data from cBioPortal and analysed the survival and disease-free survival rate for breast and ovarian cancer patients carrying either BRCA1 or BRCA2 mutations, deletions, and downregulations. Within the patient cohort, we compared the survival and relapse rate when patients express either high or low expression levels of factors belonging to the DSIF and NELF complexes (SUPT4H1, SUPT5H, NELF-A, NELF-B and NELF-E).

Unfortunately, at this point, the clinical data does not show a robust correlation between disruption of DSIF function and survival or relapse rate, and this is at least in part due to the small cohort of samples available for some of the analyses. In BRCA1 and BRCA2 mutant ovarian and breast cancer patients, we observed a slight association between the expression level of SUPT5H and the clinical outcome,

whereas no association was evident between SUPT4H1 expression level and clinical outcome. This could be due to differences between SUPT5H and SUPT4H1 in their transcription elongation roles or it could be speculated that these two factors may have also other roles in additional cellular processes that remain undiscovered.

Due to limited information on mRNA expression level for all subunits within NELF complex, we only obtained the mRNA expression level for NELF-A, NELF-B and NELF-E. We did not observe clear differences in terms of patients' overall survival and relapse rate according to differential mRNA level of NELF complex subunits in general. However, also here there are cases that clearly exhibit better clinical outcomes when the expression level of one of the NELF subunit increases or decreases. Even considering some specific correlations between PPP factors expression levels and BRCA1/2 mutant patients' outcomes, as well as the small patient cohort, the correlation between PPP factor expression level and clinical outcomes in BRCA1 and BRCA2 mutant cancer patients is not consistent and requires further studies. Moreover, the interplay between BRCA1, BRCA2, DSIF and NELF complexes commands more research to decipher the relations between these factors and how they cooperate to regulate transcription at the PPP level and more generally during transcription elongation. All this ultimately is important to inform us whether this interaction can indeed affect BRCA1 and BRCA2 patients' clinical outcomes.

How does under-replicated TSS region avoid activating cell cycle checkpoint?

It is well established that cells employ very restricted checkpoints throughout the cell cycle to protect genome stability and integrity, controlling the progression into different phases of the cell cycle. In mammalian cells, cell cycle progression is governed by specific cyclins and CDK activities, and variation of expression of genes involved in regulation of cell cycle progression subsequently define the phase of cell cycle stage [128]. Once quiescent cells enter cell cycle, checkpoints become essential for detecting and activating appropriate response to genotoxic stress to maintain genome stability and integrity [129]. Especially during DNA replication, cells are vulnerable to genomic instability as replication progress may encounter unrepaired SSBs and blocks, leading to potential fork stalling and collapse [128]. Subsequently, such events would trigger mutagenesis or cell death. Therefore, intra-S checkpoint is highly sensitive to blocks and impediments to replication forks progression [130]. If cell cycle checkpoints fail, increased genetic abnormalities may occur, which is often associated with carcinogenesis [129]. Moreover, it is generally assumed that progression through cell cycle would not be possible without completion of DNA replication nor repairing DNA lesions because of the restriction of cell cycle checkpoint [129]. Nevertheless, the existence of processes like G-MiDS and MiDAS indicates that cells can continue progression into mitosis also without a fully duplicated genome. Therefore, if our working model is correct (Fig.21), when DNA replication machinery approaches the TSS region, replication fork progression would face a block which cannot bypass, and the unresolved replication stress should be able to trigger activation of S phase checkpoint and delay cell cycle progression. At the same time, if the replication machinery is removed from DNA template, that may leave ssDNA and SSBs around TSS region.

In normal circumstances, ssDNA would be covered by replication protein A (RPA) and this together with the SSBs around the TSS region should also activate the ATR mediated intra-S checkpoint and stop cell cycle progression [130]. Therefore, it is surprising that cell cycle progression continues, and a replicative gap is left under-replicated till G2/M phase.

Hence, we tried to understand whether DNA damages were present around TSS regions. To address whether there is indeed increased DNA damage around TSS regions, initially in the lab we performed a ChIP-Seq of γH2AX, a histone modification associated with sites of DNA damage. γH2AX levels were quantified specifically around the TSS (±1Kb) of all the transcribed genes, and of the 449 G-MiDS hotspots we had identified. We found however no indication of increased DNA damage levels specifically at the G-MiDS hotspot TSS (Fig.22). This, together with other data generated in the lab confirmed that G-MiDS is not associated with late cell cycle repair of DNA damage sites, like proposed for MiDAS [unpublished data]. In parallel, inhibition of the DDR factors RAD51, RAD52, ATM and ATR show no impact of G-MiDS levels [data not shown], further supporting that G-MiDS is not associated with canonical DNA damage repair pathways. Altogehter, G-MiDS could therefore be more likely a gap filling process which completes the under-replicated TSS regions in G2/M phase once the RNA Pol II is removed from TSS.

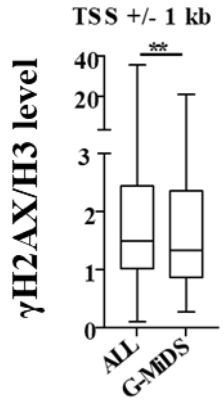


Figure.22 Quantification of γH2AX ChIP-Seq. γH2AX signal was used as an indication of DNA damage, ChIP-Seq for γH2AX was performed. Total H3 level was used to normalize the γH2AX level in cells. Within the range of ±1Kb around TSS, comparing to all genes, G-MiDS hotspots show reduced DNA damage signal.

Based on the current evidence, it is possible that under-replicated TSS regions are not activating the cell cycle checkpoint as there is no ssDNA structure exposed at TSS region, and no DNA damage signal is detected at that region. However, it remains unclear if G-MiDS is essential for cell survival as cells could still MiDAS as last resource to fix unrepaired DNA damages and preserve genome stability and integrity in M phase. Therefore, it is possible that if G-MiDS fail to duplicate the TSS region once RNA Pol II is removed from the template, cells can still complete the duplication of TSS region using MiDAS before going into mitotic division. Indeed, evidence from the lab shows that if RNA Pol II is maintained at the TSS also in G2/M and consequently blocking G-MiDS, this leads to an increase of MiDAS levels (data not shown).

Conclusion

Taken together, we have confirmed that G-MiDS is directly associated with RNA Pol II PPP, as depletion of SUPT5H and NELF-A results in reduction of G-MiDS level in cells. Additionally, PARPi-induced increase in G-MiDS level may be also because PARPi could regulate PPP through the NELF complex affecting the gap formation at TSS; however, this hypothesis requires further research to be fully validated.

Surprisingly, in SUPT5H knockdown U2OS cells, we found there is a clear reduction of BRCA1 and BRCA2 protein levels in comparison with CTR siRNA cells, although BRCA1 mRNA level in SUPT5H knockdown cells shows not such a dramatic reduction. All this suggests that perhaps in the absence of SUPT5H, BRCA1 and BRCA2 protein turnover is increased, perhaps because engaged with supporting RNA Pol II transcription elongation as SUPT5H does, or in place of SUPT5H. Nevertheless, we found that SUPT5H and NELF-A knockdown cells exhibit the same level of PARPi sensitivity as CTR siRNA cells, which is clearly separated from BRCA1 and BRCA2 knockdown cells. At the same time knockdown of SUPT5H or NELF-A and BRCA1 together shows no rescue of BRCA1 depleted cells hypersensitivity to PARPi. In parallel, we found that co-depletion of BRCA1 and SUPT5H or NELF-A can induce synthetic lethality and intriguingly, SUPT5H, BRCA1, and BRCA2 knockdown cells exhibit a similar pattern of transcription defects. Altogether, our data let us hypothesise that BRCA1 and SUPT5H may work synergistically to support cell survival and regulate transcription, but BRCA1 and NELF-A may work at different stages during transcription. By analysing clinical data obtained from cBioPortal, we found in

BRCA1/BRCA2 mutant ovarian and breast cancer patients' instances where there may be a correlation between expression level of PPP factors and patients' overall survival and relapse rate. However, due to the limited information within a small patient cohort, the correlation we identified is not robust and requires further analysis and experimental evidence to confirm.

To conclude, one important future experiment will be to explore how the knockdown of BRCA1 and BRCA2 affect G-MiDS levels, in light in particular or how both factors appear affecting RNA Pol II transcription similarly to SUPT5H knockdown. This would provide a more direct link between these two factors and RNA Pol II PPP regulation. In parallel, it would be interesting to test whether using different types of PARPi may alter G-MiDS levels, as only PARPi that are not trapping PARP1 on the DNA are able to regulate PPP activity. As such, this would confirm how much PARPi-induced G-MiDS are actually dependent on the PPP, but also to determine whether NELFA and SUPT5H may play additional roles in the cellular response to PARPi beyond their role in PPP. As such, it would be important to test this also in BRCA1 and BRCA2 mutant cancer cells.

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