Molecular Analysis of the Replication Stress Response Caused by DDX11 Dysfunction and Overexpression

Thesis

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Molecular analysis of the replication stress response caused by DDX11 dysfunction and overexpression

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Doctor of Philosophy

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IFOM Fondazione-The FIRC Institute of Molecular Oncology
Milan, Italy
ABSTRACT

DDX11 is a conserved iron-sulfur cluster DNA helicase, dysregulated in various cancers and important for normal development. While DDX11 plays roles in DNA repair and mitotic chromosome structure, the molecular mechanism by which DDX11 affects DNA damage response and tumorigenesis is still elusive. We report that loss/depletion of DDX11 in various cancer cell lines, but not in transformed normal cells, confers sensitivity to chemotherapeutic drugs, including Olaparib and Cisplatin. We established DDX11 knockout (KO) and performed synthetic lethality drug screens, uncovering that DDX11 loss sensitizes cells to ATR inhibitors, TOPII poisons and p53 stabilizers. DDX11 averts accumulation of double strand breaks in unperturbed and chemotherapeutic drug treated conditions and plays a crucial role in protecting against MRE11-mediated nascent strand degradation upon replication stress and fork stalling conditions.

Focusing on the mechanism by which DDX11 facilitates cellular survival upon DNA damage, we find that DDX11 promotes homologous recombination (HR)-mediated double strand break (DSB) repair by facilitating single stranded DNA (ssDNA) formation and subsequently RPA and RAD51 focus formation. By quantitively measuring ssDNA upon induction of DSBs at specific loci, we find that loss of DDX11 significantly impairs DNA end resection. DDX11 inactivation is synergistic to chemotherapeutic drugs in BRCA1/2-deficient cells by aggravating the DNA damage accumulation. Notably, loss of 53BP1 in DDX11 KO cells partially rescues the HR-defects and their sensitivity to Olaparib and Cisplatin. Mechanistically, DDX11 works downstream of the NHEJ factor 53BP1 to facilitate DNA end resection, RAD51 focus formation and to mediate HR-mediated DSB repair non-redundantly with the HR mediators BRCA1 and BRCA2. Importantly, downregulation of DDX11 re-sensitized BRCA1/2-mutated drug-resistant cancer cells that regained HR proficiency and drug resistance to PARP inhibitors and platinum drugs.
Altogether, our results indicate DDX11 as a potential target for both BRCA1/2-deficient as well as HR proficient and drug resistant cancer cells.
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Thank you!!!
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>DDX11</td>
<td>DEAD/H-Box helicase 11</td>
</tr>
<tr>
<td>ESCO2</td>
<td>Establishment of Sister Chromatid Cohesion N-Acetyltransferase 2</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer type1 susceptibility protein</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer type2 susceptibility protein</td>
</tr>
<tr>
<td>BARD1</td>
<td>BRCA1-associated RING domain protein 1</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly [ADP-ribose] polymerase</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase catalytic subunit</td>
</tr>
<tr>
<td>CHK1</td>
<td>Checkpoint kinase 1</td>
</tr>
<tr>
<td>CHK2</td>
<td>Checkpoint kinase 2</td>
</tr>
<tr>
<td>RAD51</td>
<td>RAD51 recombinase</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia-telangiectasia and Rad3-related</td>
</tr>
<tr>
<td>FANCIJ</td>
<td>Fanconi Anemia group J protein</td>
</tr>
<tr>
<td>FANCC</td>
<td>Fanconi Anemia group C protein</td>
</tr>
<tr>
<td>FAM35A</td>
<td>Shieldin complex subunit 2</td>
</tr>
<tr>
<td>C20orf196</td>
<td>Shieldin complex subunit 1</td>
</tr>
<tr>
<td>Rev7</td>
<td>DNA polymerase zeta</td>
</tr>
<tr>
<td>T53BP1</td>
<td>Tumor protein P53 binding protein 1</td>
</tr>
<tr>
<td>RIF1</td>
<td>Replication timing regulatory factor 1</td>
</tr>
<tr>
<td>CST</td>
<td>Cdc13, Stn1 and Ten1</td>
</tr>
<tr>
<td>RNF168</td>
<td>Ring finger protein 168</td>
</tr>
<tr>
<td>PALB2</td>
<td>Partner and localizer of BRCA2</td>
</tr>
<tr>
<td>EXO1</td>
<td>Exonuclease1</td>
</tr>
<tr>
<td>DNA2</td>
<td>DNA replication helicase/nuclease 2</td>
</tr>
<tr>
<td>MRE11</td>
<td>Meiotic recombination 11 homolog</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PRIMPOL</td>
<td>Primase and DNA directed polymerase</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>Gamma H2AX</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Alpha tubulin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>AKT</td>
<td>AKT serine/threonine kinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic target of rapamycin</td>
</tr>
<tr>
<td>TOPII</td>
<td>Topoisomerase-II</td>
</tr>
<tr>
<td>AID</td>
<td>Auxin inducible degron</td>
</tr>
<tr>
<td>FA</td>
<td>Fanconi Anemia</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscopy</td>
</tr>
<tr>
<td>WABS</td>
<td>Warsaw Breakage Syndrome</td>
</tr>
<tr>
<td>DR-GFP</td>
<td>Direct repeat green fluorescent protein</td>
</tr>
<tr>
<td>GCRs</td>
<td>Gross chromosomal rearrangements</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded DNA</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>DSB</td>
<td>Double stranded break</td>
</tr>
<tr>
<td>SSB</td>
<td>Single strand break</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous Recombination</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-Homologous End Joining</td>
</tr>
<tr>
<td>Alt-EJ</td>
<td>Alternative non-homologous end joining</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication Protein A</td>
</tr>
<tr>
<td>TLS</td>
<td>Trans-lesion synthesis</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>APC/C</td>
<td>Anaphase Promoting Complex</td>
</tr>
<tr>
<td>SDSA</td>
<td>Synthesis-dependent strand annealing</td>
</tr>
<tr>
<td>RITA</td>
<td>Reactivation of p53 and induction of tumor cell apoptosis</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2’- deoxyuridine</td>
</tr>
<tr>
<td>CldU</td>
<td>5-Chloro-2’deoxyuridine</td>
</tr>
<tr>
<td>IdU</td>
<td>5-Iodo-2’deoxyuridine</td>
</tr>
<tr>
<td>MMS</td>
<td>Methyl methanesulfonate</td>
</tr>
<tr>
<td>CDDP</td>
<td>Cisplatin</td>
</tr>
<tr>
<td>HU</td>
<td>Hydroxyurea</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxy nucleoside triphosphate</td>
</tr>
<tr>
<td>rNTP</td>
<td>ribo nucleoside triphosphate</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast Peptone Dextrose</td>
</tr>
<tr>
<td>Ctrl</td>
<td>Control</td>
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1. INTRODUCTION

Faithful DNA replication and proper DNA repair processes ensure genome stability and cell survival. Genomic DNA is constantly threatened by various environmental sources that can be physical or chemical such as irradiation (IR) and ultraviolet light (UV) from sunlight. On the other hand, cytotoxic chemicals such as Platinum drugs, Mitomycin-C and alkylating agents affect genome integrity. Moreover, spontaneous DNA damage generated during DNA metabolism such as generation of reactive oxygen species (ROS) that results in DNA breaks, misincorporation of dNTPs and rNTPs during DNA replication, DNA depurination and alkylation (1-3) pose a major potential threat to the maintenance of genome integrity. If not properly repaired, these lesions can have deleterious consequences causing genetic mutations, chromosomal instability, dysregulation of DNA replication and cellular homeostasis. It has been estimated that every cell encounters up to $\sim 10^5$ spontaneous DNA lesions on a daily basis (4-6). These lesions should be precisely dealt by various enzymatic DNA repair factors to ensure the proper maintenance of genome integrity; however, their regulatory functions are strictly controlled in spatiotemporal manner to prevent deleterious alterations to genomic DNA. To repair such lesions, cells evolved with various DNA repair pathway choices used in accordance with the type of lesion and cell cycle stage (7, 8) (Fig 1.1).
Figure 1.1 DNA DSB repair pathway choice (7). A-D, DNA end resection is primarily acting as critical step for the engagement of DSB repair pathways. C-NHEJ pathway takes place when end resection is blocked. Other three pathways, namely HR, SSA and Alt-EJ mediate DSB repair once the end resection occurs. These pathways have different outcome in the context of genetic outcome, such as insertions or deletions. The genetic outcome of each pathway is shown in the figure.

From the genetic point of view, activation of tumor oncogenes and/or inactivation of tumor suppressor genes associates with increased risk for cancer (9-11). Moreover, cancer cells can exhibit increased activity of DNA damage repair pathways to cope up replication stress and to mediate chemotherapeutic drug resistance. Moreover, inactivation or loss of function mutations in DNA repair factors leads to increased mutation rates and chromosomal rearrangements. Inherited or acquired mutations in factors involved in DNA replication and repair are highly correlated with tumor predisposition and linked with various genetic disorders (12). In recent years, these mutations are exploited to selectively target cancer cells using small molecule inhibitors and also m used as genetic biomarkers in clinical applications to assess the risk for developing cancers (13-15). Moreover, new genetic biomarkers are identified by genomic analysis of tumor cells and are exploited for the development of synthetic lethal drugs to selectively target cancer cells and improve therapy. In particular, it has been clinically well documented that mutation in homologous recombination mediators such as BRCA1 and BRCA2 confers sensitivity to platinum drugs and PARP inhibitors (16-19) (Fig 1.2, 1.10 and 1.11).
Inhibition or trapping of PARP leads to conversion of SSBs to DSBs. Accumulation or error prone-mediated repair of DSBs results in cell death in HR-deficient cells including BRCA1/2 mutated cells.

To date genes that are involved in DNA damage repair are clinically represented as cancer-susceptibility genes and documented as one of the main risk factors for cancer initiation. Among them, helicases are one such crucial DNA repair factor becoming dysregulated and mutated in various cancers and indicating that helicases can be potential druggable target and/or inhibited to enhance the chemotherapeutic sensitivity of cancer cells (20). So far, it is well known that mutations in clinically relevant tumor suppressor FANCJ helicase are associated with the high risk of developing various cancers including ovarian and breast cancer (21, 22). On the other hand, various studies have shown that the RecQ DNA helicase WRN is genetically linked with the Werner Syndrome and frequently mutated in various cancers. Interestingly, recent studies have demonstrated that inactivation of WRN helicase is synthetically lethal with microsatellite unstable cell lines (23, 24). Altogether,
helicases can be promising potential drug targets and can be used for synthetic lethal strategies to selectively target cancer cells in cancer therapy.

**DNA topological structures**

Accurate DNA replication and repair ensures faithful duplication of DNA and genome stability. However, ongoing DNA replication fork encounters unusual DNA topological structures and genotoxic agents that affect fork integrity and may cause chromosome fragility (Fig 1.3). DNA replication fork stalling or collapse leads to formation of SSBs and/or DSBs and results in activation of S phase checkpoint to resolve the replication stress and to restart replication fork (25, 26). Moreover, genomic DNA is highly complex in nature and contains repetitive elements such as microsatellites, minisatellites and also transposable elements. Approximately 50% of the human genome comprises repetitive sequences that tend to form various secondary structures including hairpin, slipped strand DNA, triplex DNA and G4-structures (27) (Fig 1.3). If the cells fail to properly address the challenge this can lead to tumorigenesis and/or cell death. Moreover, the DNA replicative helicase and polymerases get stalled when DNA replication encounters replication blocks or bulky lesions. However, DNA lesions are bypassed by error free/prone HR and DNA damage tolerance pathways such as translesion synthesis (TLS), template switch and salvage pathways to restart replication fork to ensure the successful DNA replication fork progression (28, 29).
Figure 1.3 Replication stress and fork stalling caused by DNA topological structures (27). Schematic representation of a wide variety of secondary structures that threaten ongoing DNA replication and can cause aberrant mutations, deletions and genomic rearrangements.

DNA secondary structures are resolved by DNA repair networks activated upon different types of insult. Studies revealed that the human genome can form approximately 10,000 to 700,000 G4-structures across the genome as estimated using in vitro polymerase stop assay (30). Dysregulation formation of G4-structures is highly correlated with genomic instability and cancer. G4-stabilizing ligands induce DNA damage response pathways by inducing R-loops and DNA breaks (31, 32) (Fig 1.3). Moreover, various studies have shown that G4-structures pose a threat to the ongoing DNA replication fork and cause stalling of the replication machinery (Fig 1.3 and 1.4). Different regulatory pathways resolve/bypass G4-structures to prevent fork collapse including DNA helicases-mediated G4 resolving, homologous recombination (HR), PrimPol-mediated repriming downstream of the G4-structure and PolQ-mediated Alt-EJ recovery pathway (28, 29, 33) (Fig 1.4 and 1.9C). BRCA1/2 and RAD51C-deficient cells result in telomere fragility and show sensitivity to various G4 stabilizing ligands. Various specialized DNA repair factors including Fe-S cluster family of DNA helicases such as FANCJ, BLM, DDX11 and RTEL1 are critical players in unwinding G4 structures to promote genome stability (Fig 1.4 and 1.17). Moreover, various biochemical studies demonstrated that these helicases unwind G4-structures in vitro. Inactivation of the above-mentioned helicases results in loss/deletion of G4 structures in the genome and also results in fragility at telomeres (34-38). Furthermore, human telomeric regions are highly enriched with G bases (TTAGGG) and these repetitive guanines-rich sequence tends to form non-canonical helical shape G4-structures (39). Altogether, cells that acquired mutations in G4 helicases and genes involved in HR often show increased sensitivity to G4- stabilizing ligands such as Pyridostatin and Telomestatin.
Figure 1.4 DNA repair pathways that metabolize G4-structures to prevent replication collapse (33). (A) Helicases and nucleases resolve/unwind G4-structures at the replication fork (B) Loss of FANCJ results in deletion of G4-structures causing gaps that are filled in via the PolQ-mediated Alt-EJ pathway. (C) Absence of HR factors BRCA1 and BRCA2 results in defective in HR-mediated fork restart.

DNA replication fork stability

Replication fork reversal, also known as chicken foot, has complex roles in replication fork integrity in higher eukaryotes. When DNA replication fork encounters replication blocks including genotoxins and/or structured DNA, remodeling of replication fork from three-way into four-way junction can initially stabilize the replication fork (25, 40) (Fig 1.5A and B). Genotoxins that cause replication stress/damage results in replication fork stalling or slowing that leads to increased fork reversal, a process mediated by the family
of SWI/SNF2 DNA translocases such as SMARCAL1, HLTF and ZRANB3 (41, 42). These helicase-like chromatin remodeling factors are recruited in an ATP-dependent manner at stalled forks by interacting with the ssDNA binding protein RPA and/or via the checkpoint clamp PCNA (Fig 1.5A and B). Notably, DNA translocases should be tightly regulated, as excessive fork reversal can be coupled with nascent strand degradation and genome instability. For example, recruitment of SMARCAL1 on chromatin is regulated by phosphorylation at S652 mediated by the checkpoint kinase ATR to limit fork regression and avert excessive fork processing (43). On the other hand, loss of the chromatin remodeling factors ZRANB3, SMARCAL1 and HLTF in BRCA1-deficient cells restores the fork protection function caused by BRCA1 deficiency and causes drug resistance (42, 44, 45). Apart from chromatin remodelers, other factors including Fanconi Anemia factors FANCM and FANCJ promote fork reversal upon DNA damaging conditions (46, 47) (Fig 1.5A and B). Moreover, other factors have also been identified that promote fork reversal but it is not clear whether they work together in the same or different pathways in remodeling the fork (Fig 1.5A, B and 1.6). As mentioned above, reversed forks should be tightly controlled as they are prone to degradation by a wide variety of nucleases. Notably, cells are endowed with various fork protection functions (Fig. 1.5B).
25

Figure 1.5 Replication fork reversal and fork protection function (41). (A) Upon replication fork stalling/stress conditions ssDNA is coated with RPA and along with PCNA it recruits chromatin remodelers such as SMARCAL1, HLTF and ZRANB3 that mediate replication fork reversal. Stable RAD51 nucleofilament protects reversed fork from nuclease degradation. Accumulation of ssDNA at the replication fork results in activation of ATR which limits SMARCAL1 remodeling activity via phosphorylation events. (B) Reversed forks are highly vulnerable to MRE11, DNA2, EXO1 and MUS81-mediated nascent strand degradation. In general, reversed forks are protected by stable RAD51 nucleofilaments facilitated by Fanconi Anemia factors and homologous recombination. Moreover, nascent strand degradation is protected by other factors including CtIP, WRNIP1, RIF1, DONSON and ABRO1. Nuclease mediated nascent-strand degradation and parental strand reannealing results in stalled fork backtracking.

Recent studies support the notion that fork reversal is a physiological mechanism induced by stalling. Reversed forks are susceptible to degradation by nucleases including DNA2, MRE11 and MUS81 (Fig 1.5B) (41, 48). In contrast to that, cells have evolved with different fork protection mechanisms to protect the nascent strand degradation from the nuclease to prevent genomic instability. Previous landmark papers reported that loss of BRCA2 results in MRE11-mediated fork degradation upon HU induced fork stalling conditions. Moreover, BRCA2 mutated/null cells are associated with extensive fork degradation upon HU conditions and increased chromosome abnormalities (Fig 1.5B and
Apart from the crucial functions of BRCA1/2 in HR they have other important roles in protecting nascent strand from nuclease-mediated degradation. This role also involves their ability to stabilize RAD51 nucleofilaments at stalled forks (Fig 1.5B). Notably, mutational analysis of BRCA2 uncovered its C-terminus is important for the fork protection function by stabilizing the RAD51 nucleofilament on reversed forks even though is dispensable for HR-mediated DSB-repair (52, 53). On the other hand, loading of RAD51 on the reversed fork is regulated by antagonist RADX to prevent excessive fork remodeling. Moreover, loss of RADX increases the loading of RAD51 in BRCA2 deficient cells thus preventing fork degradation and conferring drug resistance to PARP inhibitor in BRCA2 deficient cells (53-55). RAD51 binds on the reversed fork to protect nascent strand degradation from nuclease such as MRE11 and MUS81 (53, 56-58). In addition, EM analysis unveiled RAD51 prevents MRE11-mediated degradation of newly synthesized DNA and also prevents post replicative ssDNA accumulation (58). Moreover, RAD51-T131P mutation has been identified in FA-patient cells and confers hypersensitivity to DNA crosslinking agents (59-61). Notably, RAD51-T131P shows structural changes in the RAD51 nucleofilament formation and fails to protect nascent strands from MRE11 nuclease upon replication fork stalling (59). *in vitro* and electron microscopic analysis revealed that RAD51-T131P mutant shown destabilization in nucleofilament formation and aberrant binding of ATP (59). Altogether, these findings indicate that RAD51 nucleofilament formation at reversed forks plays critical roles in fork protection and resistance to chemotherapeutic drugs.

HR and fork protection functions of BRCA2 are functionally separated by the BRCA2-S3219A mutation that renders cells proficient in HR but defective in fork protection (62). Moreover, this mutant failed to facilitate RAD51 nucleofilament formation at the stalled fork resulting in defective fork protection from MRE11-mediated nascent strand degradation. Similar to BRCA2, BRCA1 has also implicated in fork protection upon HU-
induced fork stalling (Fig 1.6) (50, 62, 63) and later upon cisplatin treatment (64). Inhibition of MRE11 by Mirin rescued fork degradation in the above-mentioned conditions. Mechanistically, it has been shown that PIN1 regulates the isomerization of the BRCA1-BARD1 heterodimer, which enhances the direct interaction with RAD51 and facilitates loading of RAD51 at the nascent strand. Notably, mutations in BARD1-BRCT domain affecting the interaction with BRCA1 result in failure of BRCA1-BARD1 complex recruitment at the nascent strand upon replication stress. In the above-mentioned study, it was shown that mutation in BRCA1 at phosphorylation site S114A affects cell viability and correlates with increased chromosomal breaks upon exposure to chemotherapeutic drugs. BRCA1-S114A is proficient in HR-mediated DSB repair, but fails to protect the nascent strands against degradation upon HU-induced prolonged fork stalling conditions. On the other hand, BRCA1-S114D phospho-mimicking mutant restored the fork protection function upon replication fork stalling. Moreover, patient-derived cancer cells bearing clinically relevant mutations in phosphorylation/isomerization sites in BRCA1 and RAD51 interaction domain of BARD1 confer sensitivity to HU and show defects in fork protection (65-67). Altogether, this study unveiled that posttranslational modification of BRCA1 plays critical role in separating its HR and fork protection functions. Furthermore, growing evidence has shown that other DNA repair factors also play crucial roles in fork protection including Fanconi Anemia factors and helicases such as WRN (Fig 1.5B) (68).
Figure 1.6 BRCA1/2 are crucial players in replication fork protection upon fork stalling conditions. Genotoxic-mediated prolonged replication fork stalling results in fork stalling. In the absence of HR mediators BRCA1/2, excessive nascent strand degradation mediated by MRE11 causes genome instability.

Homologous recombination

Mammalian cells are equipped with various DNA repair mechanisms to deal with a wide variety of DNA lesions generated by endogenous and exogenous events. One of the main cytotoxic lesions is DNA double strand breaks (DSBs) that threatens genome stability (69). In particular these lesions are caused by cytotoxic chemicals and irradiation; however, it can also occur upon DNA replication fork stall or fork collapse. In the clinical scenario, approximately 50% of cancer patients undergo radiation therapy. Radiotherapy confers successful outcome in curing almost 40% of cancers (70, 71). Ionizing radiation (IR) generates a variety of DNA lesions including SSBs, DSBs and oxidative damage. Failure to repair DSBs leads to accumulation of DNA damage or if the breaks are repaired by error-prone pathways, cells accumulate genetic mutations, chromosomal rearrangements and fusions that result in cell death (72, 73).

Homologous recombination (HR) is an error-free DNA repair pathway that predominantly occurs at mid-S and mid-G2 phases of the cell cycle when the DNA replication rate is very high and the sister template is available as substrate for DNA repair (74) (Fig 1.7). Upon DSB formation, the MRN complex, bearing both exonuclease and endonuclease activities, initiates the resection and acts as scaffold protein for ATM. MRE11 nicks the free 5’ strand away from the break site by its endonuclease activity and it extends the nick by its 3’-5’ exonuclease activity till the end of the break (75). MRE11 interacts with CtIP and together they mediate short range resection (76) (Fig 1.7). Moreover, Ku70/80 at the DSB sites are removed by the MRN exonuclease activity. Removal of the NHEJ factors
KU70/80, makes an entry point for EXO1, DNA2 and BLM to mediate long range resection and the formation of a 3’ long tail (77, 78). The resected ssDNA is rapidly coated with the RPA complex containing RPA1, 2 and 3. RPA limits excessive resection and unwinds the secondary structure formation and also acts as barrier for RAD51 nucleofilament formation (7, 79, 80) (Fig 1.7).

In later steps, BRCA2 binds ssDNA by competing with RPA, resulting in disassembly of RPA. RPA displacement occurs by BRCA2 interaction with ssDNA and the recombinase RAD51 monomer. This is coupled with BRCA1-BARD1 complex that together with PALB2 cause the formation of a dynamic RAD51 nucleofilament (Fig 1.7) (81). The RAD51 coated ssDNA invades dsDNA for homology search and strand invasion. This RAD51-mediated strand invasion results in the formation of three stranded helix known as the synaptic complex. Once the synapse gets stabilized by base pairing, it matures into a D-loop in which RAD51 is disassembled from the ssDNA by RAD51-mediated by ATP hydrolysis. In the next steps, DNA polymerase is recruited at the 3’ of the invading strand for successful synthesis by copying from the donor template, whereas the DSB on the other end will be processed to finish the repair by synthesis-dependent strand annealing (SDSA) and double strand break repair (DSBR) mechanisms with formation of double Holliday Junctions (82) (Fig 1.7).
Figure 1.7 Homologous recombination DSB repair pathway (83). KU70/80 engagement with the DNA break site is dissociated by DNA end resection which acts as critical step for HR. The MRN complex facilitates resection by its endonuclease activity and KU70/80 is dissociated from the DNA by MRE11 3’-5’ exonuclease activity. On the other hand, the 3’ ssDNA tail is the result of 5’-3’ exonuclease activity of EXO1 together with the DNA2-BLM heterodimer. Later, the resulting ssDNA is coated with RPA, followed by RAD51 recombinase displacement. In eukaryotes, BRCA2 is the critical mediator of HR, where it...
works jointly with PALB2 and BRCA1-BARD1. RAD51 nucleofilament coated ssDNA invades the sister strand for homology search and base pairs with complementary sequences resulting in synapse. This synapsis influences the two sub pathways for the HR, the SDSA and the double Holliday junction branch resulting in non-crossovers and crossover outcomes (82, 83).

**Molecular functions of PARP1 in DNA repair and its pharmacological inhibition in cancer therapy**

**Molecular mechanisms of PARP1 in DNA damage repair**

Cells are exposed to various endogenous and exogenous lesions that threaten genome integrity (Fig 1.3). DNA lesions are precisely and accurately dealt with by different DNA repair pathways to prevent genome instability. To repair DNA lesions, a variety of DNA repair factors are recruited to break sites. One of the earliest response is associated with recruitment of PARP1 (Fig 1.8) (84). PARP1 is an evolutionary conserved protein and is also known as molecular sensor for SSBs and DSBs. Among 17 members in the PARP family, PARP1 is a well-studied factor in the context of DNA repair where it PARylates itself and other target proteins upon DNA damage (Fig 1.8) (84). The posttranslational modification PARylation (Poly (ADP-ribosylation) action) is a reversible process and is catabolized by macrodomain protein called PARG glycosylase. PARP1 is predominantly enriched in the nucleus where it directly binds to DNA breaks to promote efficient DNA repair by synthesizing PAR chains (85, 86). Previous reports showed that loss of either PARP1 or PARP2 in mice is compatible with life, whereas double knockout of PARP1 and PARP2 causes embryonic lethality, indicating redundant functions essential for embryonic development (87-89). In contrast to that, double knockout of PARP1 and PARP2 in mammalian cells allows proliferation in both non-cancerous and cancerous cell line models. Moreover, PARP1 and PARP2 knockout cell lines are sensitive to a wide variety of DNA
damaging drugs, indicating the crucial roles of PARP1/2 in DNA damage repair (90, 91). SSBs, DSBs, and stalled forks activate and stimulate PARP1 and PARP2 (5). These genomic insults result in PARP-mediated catalyzation of Poly (ADP-ribose) chains for the recruitment of other DNA repair factors at the break sites to promote DNA damage repair by HR or by NHEJ (Fig 1.8 and Fig 1.9).

**Figure 1.8 Action of PARP1 in DNA damage repair (85).** Upon induction of SSB/DSBs, PARP1 is activated and results in PARylation of itself and target proteins.

SSBs occur spontaneously and also as intermediates of other DNA repair and metabolic processes, such as oxidative DNA damage, formation of abasic sites and base modifications. SSBs are sensed and recognized by PARP1 that undergoes autoPARylation and mediates recruitment of XRCC1, a scaffold protein involved in single strand break repair. XRCC1 interacts with PARylated PARP1 and PARP2 via its BRCT domain and results in recruitment of XRCC1 and other repair factors at the break sites (Fig 1.9). Next, downstream factor Pol-β is recruited by XRCC1 via its N-terminal domain to replace base at the nick site and final ligation is mediated by DNA ligase 3, LIG3 (84). Exposure to γ-irradiation and other DNA damaging agents cause DSB lesions, with these breaks being repaired by HR and NHEJ pathways. Upon DNA damage, PARP1 promotes recruitment of the MRN complex to the DSB sites facilitating along CtIP, short range DNA end resection, followed by long range resection (92). Following this, RAD51 loading results in channeling of DSBs into HR-mediated DSB repair (Fig 1.9). PARP1 also recruits BRCA1 at break sites
to facilitate RAD51 loading and promote HR-mediated DSB break repair. However, when sister strand is not available for HR, PARP1 regulates NHEJ by directly interacting with DNA-PKcs to stimulate its kinase activity via its PARylation activity. In downstream steps, PARP1 recruits CHD2, a chromatin remodeling factor that facilitates chromatin reorganization for the LIG4 mediated cNHEJ (Fig 1.9) (84, 93).

On the other hand, alternative NHEJ (aNHEJ), also known as microhomology mediated end joining (MMEJ) is a mutagenic DNA repair pathway where two microhomology arms ligate together after DSBs are partly processed by the MRN complex. In general, PARP1 and KU complex compete each other for the bind at DSB break sites. Limited resection of DSB ends leads to PolQ-mediated aNHEJ, with breaks are ligated by LIG3 (Fig 1.9) (84). Moreover, HR-deficient cancers often have overexpression of POLQ with high PolQ-mediated aNHEJ resulting in deletions, genomic rearrangements, telomeric fusions and drug resistance (94, 95). Notably, genome wide CRISPR/Cas9 screening revealed that BRCA-deficient cells are synthetically lethal with POLQ loss (16, 94, 96). Interestingly, recent study has found a previously established antibiotic called Novobiocin to inhibit POLQ activity and to selectively kill HR-deficient cells (97). Furthermore, small molecule inhibitor ART558 has been developed recently and shown to inhibit POLQ polymerase and MMEJ, eliciting DNA damage response and causing synthetic lethality in HR-deficient cells (98).
Figure 1.9 Molecular functions of PARP1 in detection and repair of DSB (84). (A) Upon DSBs, PARP1 is recruited at the DNA break site and mediates DNA damage response by directly interacting with MRE11 and ATM. (B) PARP1 plays important role in HR by recruitment of MRE11 at the break sites to promote DNA end resection and the resected ssDNA is coated with RPA. Moreover, it recruits BRCA1, which along BARD1, stimulates HR. PARP1-mediated HR is suppressed by limiting the DNA end resection, potentially via PARylated BRCA1 and association with RAP80 that limits strand invasion. (C) PARP1 also plays vital roles in promoting cNHEJ pathway. PARP1 stimulates catalytic activity of DNA-PKcs by direct interaction. PARP1 recruits chromodomain helicase CHD2 facilitates chromatin remodeling within the cNHEJ pathway, necessary for the recruitment of other
factors, such as XRCC4 and LIG4 for the ligation of DNA ends. (D) In the absence/inactivation of cNHEJ, PARP1 regulates aNHEJ. PARP1 recruits MRE11 for the resection resulting in joining of microhomology arms and filling of gaps via POLQ, followed by LIG3-mediated ligation. However, the recruitment of POLQ by PARP1 is not well studied yet.

**Clinical candidate PARP inhibitor in cancer treatment**

Targeting DNA repair machinery by small molecule inhibitors and pharmacologically exploitable synthetic lethal strategies opened a new arena for dissecting the DNA repair networks and come up with novel chemotherapeutic drug targets for cancer treatment. An interesting DNA repair factor is PARP1, coupling repair of DNA breaks with intracellular signaling mechanism (Fig 1.9)(5). Unrepaired SSBs are converted into DSBs, majorly repaired by error free HR that supports cellular fitness and survival (Fig 1.10). In contrast to that, accumulation of DSBs and/or channeling the breaks to error prone NHEJ-mediated repair results in cell death or cancer initiation (84). It emerged that inhibition of PARP1 with small molecule inhibitors leads to cell death in HR-deficient cells, such as those lacking the HR mediators BRCA1 and BRCA2 (19). PARP inhibitors show promising outcome in selectively killing HR-deficient cells and other DNA repair factors (Fig 1.2 and 1.10). Previous evidence has shown that loss of DNA repair-related factors including cohesin subunits STAG1/2, DDX11, SMC5/6 complex and XRCC3 show greater sensitivity to PARP inhibitors (37, 99-102).

Recent publication from our lab has shown that loss of either BRCA1 and BRCA2 in DDX11 KO cells is additive to various chemotherapeutic drugs including Olaparib. In addition to that loss of DDX11 in BRCA1/2 drug resistant cancer cells are re-sensitized by PARP inhibitor (Fig 3.9). Notably, recent genome wide CRISPR/Cas9 synthetic lethality screens with PARP inhibitor revealed that loss of ALC1 and LIG3 conferred enhanced sensitivity to PARP inhibitor in BRCA mutated cells by increased PARP1 and PARP2
trapping on chromatin and elevated replication gap formation (103-105). Previous landmark studies have shown that PARP inhibitor accelerates DNA replication fork speed which correlates with an increased accumulation of post replicative gaps identified by DNA fiber and EM analysis (106, 107). Moreover, replication gaps arise when cells experience oncogene induced replication stress and upon various genotoxic drug treatment. Error prone TLS polymerases play crucial roles in bypassing the post replicative gaps induced by cancer chemotherapies and in contrast to that, inhibition of scaffold protein REV1 by small molecules resulted in synergism with gap-inducing chemotherapeutic drugs (108, 109). Recent evidences strongly suggest that replication gaps are one of the key determinants and a hallmark to predict the response to chemotherapeutic drug including PARP inhibitors (107, 110). Thus, PARP inhibitors exhibit great response alone and/or in combination with other drug targets to selectively target cancer cells that are deficient in HR or carry mutations in other DNA repair-related genes (Fig 1.2 and 1.10) (111-113).

**Figure 1.10 Roles of PARP1 in DNA repair and its inhibition is synthetic lethal with HR-deficient cells.** PARP1 senses DNA breaks including SSBs/DSBs and inhibition/trapping of PARP1 by small molecule inhibitors leads to cell death in HR-deficient cells. However, cells that successfully repair DNA lesions are proficient in survival.
In the clinical scenario, PARP inhibitors emerged as a promising candidate in cancer treatment and considered as the most outstanding synthetic lethal drug with HR-deficient cancers (Fig 1.2 and 1.10). Second generation of PARP inhibitors that trap PARP1 at the site of DNA lesion resulted in highly toxic DNA-protein crosslinks that affect DNA replication and prevent recruitment of downstream DNA repair factors to the break site. Moreover, PARP inhibitors such as Olaparib, Talazoparib, Niraparib and Rucaparib cause DNA-protein crosslinks but each PARP inhibitor has different PARP trapping activity (Fig 1.11) (114-118). Notably, Olaparib mainly traps PARP1 and also PARP2 on chromatin, as identified using human and chicken DT-40 cell line models. In line with the above notion, loss of PARP1 rescued cell lethality to Olaparib more than the PARP2 loss, indicating that trapping of PARP1 to chromatin underlies cellular cytotoxicity (118, 119). Olaparib was the first clinically approved synthetic lethal chemotherapeutic drug targeting HR-deficient breast and ovarian cancers (120-122). Recently it also showed promising outcome in metastatic prostate cancer patients (114). To date, a variety of PARP inhibitors are in different stages of clinical trials and approved for the treatment of BRCA-mutated breast and ovarian cancers (Fig 1.11). Clinically relevant PARP inhibitor Olaparib was approved by EMA and FDA for the treatment of advanced ovarian cancer patients bearing germline mutation in *BRCA1/2* and FDA approved Olaparib as maintenance therapy for BRCA-mutated ovarian cancer patients (Fig 1.11) (123-125). A previous clinical study revealed that Rucaparib causes longer median survival in BRCA1/2 mutated patients with high grade recurrent ovarian cancer than placebo, which resulted in FDA approving of Rucaparib for the BRCA-mutated advanced ovarian cancers in 2016 (Fig 1.11) (126). Another PARP inhibitor called Niraparib increased the progression free survival of patients with platinum recurrent ovarian cancer and also showed greater potential in newly diagnosed ovarian cancers irrespective of its BRCA status (127, 128).
Table depicts the application of different PARP inhibitors in clinics for cancer treatment (123).

**PARP inhibitor drug resistance molecular mechanisms**

Somatic or Germline mutation in tumor suppressor genes BRCA1 and BRCA2 are frequently observed in various cancers including breast, ovarian, pancreatic and other cancers. Among gynecological cancers ovarian cancer is considered as highly lethal and overall, 50% of high grade serous ovarian cancers (HGSOCs) are deficient in HR (129, 130). Although PARP inhibitors showed great potential as maintenance therapy for various cancers including ovarian and breast cancer, they have limitations for the treatment of cancers. Indeed, it is important to take in consideration that not all cancer patients respond to PARP inhibitor or platinum-based chemotherapies and there is a high chance to get chemotherapeutic drug resistance that results in tumor relapse (Fig 1.12) (131). So far, several studies have shown underlying molecular mechanisms/factors that promote drug resistance and suggested strategies to overcome it. To date, various mechanisms govern drug resistance. They include: (i) elevated drug efflux, (ii) decreased/defective PARP trapping, (iii) HR restoration, (iv) Replication fork stabilization (Fig 1.12, 1.13, 1.14, and 1.15) (132).
**Figure 1.12 Modes of PARP inhibitor resistance mechanism (132).** Tabular column indicates the mode and cause of PARP inhibitor resistance mechanisms. On the right, clinical evidence of PARP inhibitor resistance indicated in preclinical or PDX models is summarized.

BRCA1/2 deficient cells render chemotherapeutic drug sensitivity to wide variety of DNA damaging drugs such as DNA crosslinkers (Cisplatin Carboplatin and Oxaliplatin) and PARP inhibitors (Fig 1.10). Ovarian cancer patients bearing mutation in *BRCA1/2* showed better prognosis to platinum-based chemotherapy and PARP inhibitor than placebo, however ultimately BRCA1/2 mutated tumors eventually develop drug resistance (Fig 1.12) (131, 132). Previous studies have uncovered several mechanisms that conferred drug resistance upon Cisplatin/PARP inhibitor treatment in BRCA2-mutated cell lines. Pancreatic cancer Capan-1 and ovarian cancer PEO1 harbor mutation in BRCA2 alleles with no wild type allele, rendering these cell lines deficient in HR and sensitive to chemotherapeutic drugs including Cisplatin and PARP inhibitor (133-136). In previous studies, culturing Capan-1 and PEO1 cell lines under the presence of either Cisplatin or Olaparib chemotherapeutic
drugs led to isolation of drug resistant cell lines (Fig 1.13). By sequence analysis of the
BRCA2 genomic locus, it was uncovered that most of the drug resistant clones had
secondary/reversion mutation in BRCA2, causing restoration of a functional BRCA2 protein
(Fig 1.13). Moreover, acquired drug resistant clones are proficient in HR as judged from
RAD51 focus formation upon ionization radiation and restored homology directed repair of
DSB analyzed by I-Scel-dependent DR-GFP reporter assay (134).

On the other hand, case reports have shown restoration of BRCA1 by acquired
secondary mutations in BRCA1-mutated ovarian cancer patients (137, 138). Loss of function
CRISPR/Cas9 genetic screens in mammalian cells revealed that suppression/knockout of
PARP leads to PARP inhibitor drug resistance in BRCA2 deficient cells (Fig 1.13). This
occurrence of drug resistance in BRCA2- deficient cells are defined by restoration of partial
PARP1 downstream signaling mechanisms (Fig 1.13) (139). Unbiased CRISPR/Cas9 based
genetic screen unveiled that loss of ubiquitin ligase HUWE1 restored RAD51 focus
formation in BRCA2 knockout cancer cells, however the underlying molecular mechanism
is not well known. In addition to that, loss of acetyltransferase KAT5 regulates the binding
of 53BP1 to DSBs and restores the fork protection function upon HU-mediated fork stalling
conditions in BRCA2 KO cells (102).
Figure 1.13 Models for acquired drug resistance mechanism by restoration of BRCA1/2 and loss of PARG in BRCA2 (139, 140). (A) Acquired Cisplatin/PARP inhibitor drug resistance mediated by restoration of BRCA1/2 by acquiring secondary mutation in ovarian and/or pancreatic cancers. (B) Knockout/depletion of PARG glycosylase renders PARP inhibitor drug resistance by restoration of PARP1 downstream signaling mechanism in BRCA2 deficient/mutated cells.

Acquired drug resistance is one of the major challenges in cancer treatment. Previous landmark papers demonstrated that loss/knockout of 53BP1 in BRCA1-mutated/deficient cancer cells leads to drug resistance by restoration of DNA end resection and improved HR repair (141, 142). Apart from roles in regulating resection and promoting NHEJ, 53BP1 also plays crucial role in protecting the nascent strand degradation at stalled replication forks (143, 144). Similarly, loss/reduced expression of chromatin remodelers and DNA repair factors such as PTIP, CHD4 and EZH2 affects the recruitment of nucleases including MRE11, MUS81 and EXO1 at the replication fork (57, 145-148). This failure in recruitment of nucleases results in the protection of nascent strand degradation upon replication stress and fork stalling conditions (Fig 1.5). Indeed, defective fork protection is a hallmark of BRCA1-deficient cells (Fig 1.6) and is restored by loss of 53BP1. On the other hand, 53BP1 knockout restored lethality in BRCA1-mutated cells as well as in mouse embryonic stem
cells. Remarkably, various genetic interaction studies highlight that BRCA1 and 53BP1 antagonize each other at DSB break sites (149, 150). Moreover, concomitant loss of regulatory factors including 53BP1 and CST/Pol-α complex in BRCA1-deficient/mutated cells, but not in BRCA2-deficient cells, results in restoration of HR, leading to PARP inhibitor drug resistance (Fig 1.14) (142, 151). It has been shown that loss of 53BP1 downstream factors RIF1 and REV7 leads to PARP inhibitor resistance in BRCA1-deficient tumors and in preclinical models (Fig 1.12 and 1.14). However, the exact underlying molecular mechanisms of PARP inhibitor drug resistance is not well understood except for the connection with DNA end resection (Fig 1.14) (152-155).

![Figure 1.14](image_url)

**Figure 1.14 Mechanism of shieldin complex prevents DNA end resection in BRCA1-deficient cells** (132). Upon DSBs, 53BP1-dependent recruitment of RIF1 and Shieldin complex inhibit DNA end resection. Shieldin has different roles in inhibiting the end resection process (1) Shieldin recruits the CST/Polo complex at the break sites to fill the overhanging ssDNA. (2) Shieldin complex binds at the overhanging ssDNA via SHLD2 OB fold domain by which it prevents the access of EXO1 and DNA2-BLM for long-range resection of DNA breaks.
To understand and identify the underlying molecular mechanisms behind PARP inhibitor resistance, various groups performed proteomic studies and genome scale CRISPR/Cas9-based functional screens in BRCA1 knockout non-cancerous and cancerous cell lines. Genetic screens uncovered the previously uncharacterized Shieldin complex that emerged as major inhibitor for 5'-3' DNA end resection in 53BP1-dependent manner. Shieldin complex consists of SHLD1 (C20orf196), SHLD2 (FAM35A), SHLD3 (CTC-534A2.2) and TLS polymerase REV7, proposed to be the critical regulators of HR and NHEJ (Fig 1.14) (152). Shieldin components play critical roles in promoting DNA damage repair upon IR, bleomycin and etoposide treatment. Moreover, loss of REV7 confers sensitivity to DNA crosslinking agents. REV7 bridges Shieldin components by interacting with SHLD3 and SHLD2. 53BP1 and RIF1 recruit Shieldin and SHLD2 binds at the ssDNA to prevent DNA end resection to channel the breaks to NHEJ-mediated DSB repair (Fig 1.14). Loss of 53BP1 and its downstream Shieldin components results in restoration of RAD51 foci accrual upon ionization radiation and restoration of homology-directed repair of DSBs in BRCA1-mutated cells. Moreover, reduced SHLD1, SHLD2, 53BP1 and/or PARP1 mRNA expression in BRCA1-mutated breast cancer and in-patient derived xenografts correlate with drug resistance to PARP inhibitor. In addition to that, BRCA1 KO mouse embryonic cells are not viable, however loss of SHLD1 and SHLD2 in BRCA1-deficient p53 KO mouse embryonic cells allows viability. Notably, loss of 53BP1 in BRCA1-deficient/mutated cells results in restoration of HR and it occurs in a BRCA1-independent manner (155-158).

Mechanistically, BRCA1-independent HR is mediated by the ubiquitin ligase RNF168 that promotes RAD51 loading on resected ssDNA by assisting BRCA2 and PALB2 (150, 159). CRISPR/Cas9 genetic screen identified that loss of previously uncharacterized factor DYNLL1 conferred drug resistance in BRCA1-mutated/knockout cells by restoration of DNA end resection to allow HR. Moreover, previously it has been identified that DYNLL1
interacts with 53BP1, however it does not regulate 53BP1 focus formation in unperturbed and DNA damaging conditions (Fig 1.15). Mechanistically, DYNLL1 limits the end resection by directly interacting with MRE11, but how it impairs the recruitment and nuclease activity is not well understood (Fig 1.15) (158, 160, 161).

![Figure 1.15 Mechanism of DYNLL1 prevents DNA end resection in BRCA1-deficient cells](image)

**Figure 1.15 Mechanism of DYNLL1 prevents DNA end resection in BRCA1-deficient cells** (132). DYNLL1 has dual roles in inhibiting the DNA end resection to prevent HR. (1) DYNLL1 directly binds and prevents end resection machineries EXO1, DNA2/BLM and MRE11. (2) DYNLL1 inhibits end resection by promoting 53BP1 stabilization and dimerization at the DNA break sites.

**Introduction to Helicases**

Helicases are a renowned class of genome caretakers defined as molecular motors with crucial roles in unwinding various DNA structures by translocating on the DNA in 5’-3’ or 3’-5’ directions (162). Helicases collaborate with various DNA repair machineries to cope up with endogenous and exogenous stress that threatens genome integrity (92, 163, 164). Our human genome encodes approximately 95 different helicases including DNA and RNA helicases. They are broadly classified into two different superfamilies SF1, SF2 and 4
different smaller superfamilies. In this family, Fe-S cluster and RecQ DNA helicases play important roles to avert chromosomal instability by contributing to DNA repair and telomeric DNA maintenance via their catalytic functions and by interaction with other proteins. Notably, dysregulation and mutation in Fe-S helicases has been implicated in various developmental disorders and in predisposition to cancer (165-169). Various studies reported that helicases are overexpressed in a wide variety of cancers, correlating with cancer cell proliferation and chemotherapeutic drug resistance. Mainly, helicases are important for cell viability upon chemotherapeutic drug treatment, and loss of those helicases compromise cell viability to DNA damaging chemotherapeutic drugs (20, 37, 123). So far, considerable amount of effort has been made to understand the molecular functions of helicases using molecular, cellular and biochemical studies. However, the roles of helicases in DNA damage response and replication stress still need to be understood.

**DDX11 in development**

DDX11 (DEAD/DEAH box helicase 11) is an ATP dependent 5’-3’ DNA helicase which slides on ssDNA, resolving various substrates including DNA replication forks and secondary structures (170, 171). It belongs to the conserved iron-sulphur [Fe-S] cluster family of helicases, along three other helicases, namely FANCJ, XPD and RTEL1 (Fig 1.4) (172). Mutation in this family of helicases is linked with various genetic disorders. Mutation in DDX11 causes a genetic disorder called Warsaw breakage syndrome (WABS). This genetic disorder is also classified as cohesinopathy, characterized by defective sister chromatid cohesion. Moreover, WABS patient cells resemble in several aspects the Fanconi Anemia disorder for the cellular sensitivity phenotype. WABS patients’ clinical features include microcephaly, growth retardation, hearing loss, bone marrow failure, defective skin pigmentation (173-178). Overall, 23 DDX11 WABS patients have been reported so far and the majority of the pathogenic missense mutations are at the conserved helicase motifs and in the Fe-S domain of DDX11 (Fig 1.16) (178, 179). Apart from the roles of DDX11 in
genome stability maintenance and sister chromatid cohesion, it also plays important role in the proper functioning of mitochondrial metabolism. By closely examining WABS patient-derived primary cells, there is a severe defect in mitochondrial metabolism which is similar to that found in Fanconi Anemia patient cells (180).

Very recently, seven new WABS patients were clinically reported and the underlying mutations were studied using various cellular and biochemical studies. In these WABS patients’ new point mutations in DDX11 has been reported. Broadly, this case study reported hypomorphic mutants where the DDX11 proteins is unstable (R140Q) and mutations that affect the nuclear localization of DDX11 (G57R, S857R and C705Y) in primary cells (Fig 1.16) (179). In previous studies, it was shown that DDX11 localizes and is highly enriched in nucleolus where it positively regulates rRNA transcription (181). At the cellular level, patient-derived and cancer cell lines conferred sensitivity to various DNA damaging drugs such as G4-stabilizing drugs, ATR inhibitors, inter/intra strand crosslinking agents (ICLs), topoisomerase-I and PARP inhibitors (Fig 3.4 and 3.9). Cellular sensitivity to DNA damaging drugs also correlates with the increased DNA damage markers and aberrant chromosomal breaks in patient cells and in DDX11 KO cell line models. To understand the developmental defect, various laboratories attempted to establish the DDX11 mouse models, however DDX11 is indispensable for mouse embryonic development (37, 99, 179, 182-184). Generation of hypomorphic mutation in mouse models resulted in embryonic lethality, which indicates that the G57R mutation in DDX11 impairs helicase activity and causes loss of function (179). Furthermore, morpholino-mediated loss of DDX11 in zebrafish resulted in similar phenotypes that have been previously observed in WABS patients (185). These studies indicate that loss or mutation in DDX11 leads to developmental abnormalities and overlaps with DNA damage related syndromes.
Figure 1.16 Mutations in DDX11 helicase identified in Warsaw breakage syndrome patients.

**DDX11 in cancer**

Genomic instability is observed in most cancer malignancies and is considered as one of the important hallmarks of cancer. Factors involved in DNA damage repair act as tumor suppressors in particular cancer types by preventing genomic instability (186, 187). On the other hand, mutation/loss of DNA repair factors correlates with increased mutation rates, copy number variations and aberrations at chromosomal level. So far mutation or inactivation of various DNA repair factors have been clinically well documented as this event is considered as one of the major risk factors for tumor initiation (188). Mutations in DDX11 associate with the WABS developmental disorder and resemble FA in regard to chromosome anomalies and sensitivity to DNA damaging drugs. Moreover, recent studies have shown oncogenic functions of DDX11 in cancers and revealed strong ties between DDX11 high expression and tumor progression and cancer metastasis. DDX11 is overexpressed and hyper-amplified in various cancers and its dysregulation is highly correlated with cancer metastasis and poor prognosis in various cancers that include melanoma, lung adenocarcinoma, multiple myeloma, cell-renal and hepatocellular carcinoma. Moreover, DDX11 helicase is overexpressed and hyper-amplified in ovarian serous cystadenocarcinoma and its overexpression is highly correlated with the decreased overall survival (Fig 3.1) (189-192). DDX11 is overexpressed in various cancers in
comparison with the normal cells but the pathophysiological functions of *DDX11* overexpression associated with tumorigenesis is not well understood.

*DDX11* expression is positively correlated with genes involved in cell cycle and DNA replication. Previous studies have shown that *DDX11* is transcriptionally suppressed by JunB, resulting in premature sister chromatid separation in non-Hodgkin lymphoma. Moreover, JunB is stabilized by the inactivation of GSK3B, which is a critical mediator for *DDX11* mRNA expression, causing dysregulation of cell cycle and defects in chromosomal segregation (193). *DDX11* overexpression is highly correlated with an increase in tumor burden and metastasis in hepatocellular carcinoma. In general, PI3K/AKT/mTOR pathways are highly dysregulated in various cancers to promote tumorigenesis, cellular proliferation and invasion. Previous studies in hepatocellular carcinoma (HCC) have shown that *DDX11* is overexpressed and is positively correlated with increased HCC tumorigenicity *in vivo*. Moreover, *DDX11* overexpression is significantly correlated with increased cellular proliferation, tumor multiplicity and poor prognosis, however loss of *DDX11* impairs cancer cell proliferation (194). Altogether, these studies indicate that DDX11 may act as a novel biomarker and therapeutic target in a wide variety of cancers.

**In vitro biochemical and molecular studies of DDX11**

Various *in vitro* biochemical studies were carried out to uncover the potential activities of DDX11 in DNA replication associated events. Initial report has shown that DDX11 helicase translocates on to the DNA and slides in 5’-3’ direction in an ATP-dependent manner. It resolves DNA substrates that include duplex forks, secondary structures such as RNA-DNA hybrids, D-loops and G-quadruplex structures (Fig 1.17) (36, 175). Mutation in helicase motif (K50R) and Fe-S domain affects DDX11 helicase and DNA
binding activity, respectively (Fig 1.18A). Mutations in these domain/motifs conferred sensitivity to DNA damaging agents such as Olaparib and Mitomycin-C (Fig 3.5.2) (36, 37, 195). Moreover, a WABS-related hypomorphic mutation at DDX11-G57R located near the Walker A motif failed to resolve duplex DNA substrate in vitro. However, DDX11-R140Q mutation identified in WABS showed helicase activity and sister chromatid cohesion at similar level to the wildtype (175). Furthermore, loss of DDX11 affects the telomere length and heterochromatin organization, suggesting critical roles of DDX11 in the regulation of G-rich repetitive sequences that tend to form G-quadruplex secondary structures (Fig 1.17) (196, 197). Moreover, it was shown that DDX11 is also important for the maintenance of satellite regions containing highly repetitive sequences.

![Figure 1.17 Roles of DDX11 in resolving DNA topological structures.](image)

DDX11 helicase shown specificity and activity towards resolving replication fork, RNA-DNA hybrid, D-loop, G4-Structures.

Previous studies have demonstrated that DDX11 helicase and Fe-S domain are important for the DDX11 functions in the context of DNA replication and repair (Fig 3.5.2) (37). However, it is not yet clear whether DDX11 promotes smooth DNA replication and protects from replication stress. Recent studies have shown that DDX11 associates with the replisome component via its interaction with replication fork stabilizer Timeless/Tipin, and
AND1/Ctf4 (195, 198, 199). Evolutionarily conserved AND1/Ctf4 acts as central factor that bridges various replisome components such as the CMG helicase and DNA polymerase alpha. Moreover, it promotes HR-mediated DSB repair and cell cycle progression mainly in S-phase. Loss of AND1 in chicken DT-40 cells affects replication fork speed and causes accumulation of ssDNA at replication fork junctions (200). However, the biological significance of the interaction between DDX11 and AND-1 is not well understood. On the other hand, DDX11 helicase interacts and associates with the replisome component Timeless, promoting replication fork stability and establishment of sister chromatid cohesion. DDX11 interaction motif with Timeless was identified by performing peptide microarray. Mutating amino acid residues EYE to KAE/KAK results in loss of interaction between DDX11 and Timeless, as confirmed by immunoprecipitation assays. In addition, Timeless enhances and stimulates DDX11 activity to resolve secondary structures and fork duplex in vitro, however at the cellular level, DDX11 – KAE timeless interaction mutant rescued cell viability and DNA damage focus formation upon chemotherapeutic drug treatment (Fig 1.18A and 3.5.2) (37, 201). Altogether, DDX11 helicase activity averts chemotherapeutic drug sensitivity and accumulation of DNA damage largely independently of its binding to the replisome complex (Fig 1.18B).
**Figure 1.18 Mutations in DDX11 affects its activity.** (A) DDX11-K50R mutation in walker-A motif affects its helicase activity. DDX11-R263Q mutation in Fe-S domain affects the DNA binding activity. DDX11-KAE mutation affects interaction with replication protection factor Timeless. (B) Schematic representation depicts DDX11 associating with the replisome by interacting with AND-1 and Timeless.

**DDX11 in DNA replication and repair**

Various groups reported important roles of DDX11 in resolving topological structures via interactions with replisome components (183, 195). However, the underlying molecular mechanisms of DDX11 in DNA replication and repair is still elusive. Previous publication from our laboratory has shown that chicken DT40 cells lacking *DDX11* are hypersensitive to various DNA damaging drugs and defective in immunoglobulin gene diversification (183). Moreover, we have also shown functions of DDX11 helicase activity in facilitating sister chromatid exchange and HR repair, in part by facilitating RAD51 focus.
formation (Fig 3.6). Loss of Fanconi Anemia factors such as FANCJ and FANCC in DDX11 KO cells are additive and failed to recover from the DNA damage. The results indicated that DDX11 acts as backup for Fanconi Anemia pathway and also jointly works together with the 9-1-1 complex to repair bulky lesions and facilitate replication through abasic sites (Fig 1.19A) (183, 202). Furthermore, DDX11 has been found to interact with Fen1 endonuclease, stimulating its activity to resolve DNA flaps in vitro. Consequently, Fen1 and DDX11 were proposed to process Okazaki fragments and displace forming DNA flaps to repair damage (203).

My PhD work focused on mechanistically uncovering roles of DDX11 in DNA damage repair and probing its potential as therapeutic target in cancer treatment. As it will be described in the Results section of this study, we demonstrated that loss of DDX11 sensitizes ovarian and other cancer cell lines to Cisplatin and Olaparib but not transformed normal cell lines. Moreover, by performing synthetic lethality drug screens with a panel of 64 FDA- approved drugs, we uncovered that DDX11 KO cells are sensitive to various DNA damaging drugs, most of which require processing via checkpoint cascades and HR machineries for genome integrity. For instance, we found ATR inhibitors and TOPII poisons as top hits and validated the clinical candidate drug ATR inhibitor (VE-821 and AZD6738) to effectively kill DDX11 KO cells at very lower concentrations. In addition, we have found that U2OS DDX11 KO cells are sensitive to small molecules RITA ( Reactivation of p53 and Induction of apoptosis) and Nutlin-3A from the drug screens (204-207). Both these inhibitors prevent the interaction between MDM2 and p53 results in restoration and stabilization of p53. Previously, it has been known that RITA induces p53 target gene expression and induce apoptosis in p53 wild type cells (208, 209).

In line with previous studies, DDX11 KO in HeLa and U2OS cells showed sensitivity to DNA damaging drugs such as ICLs and PARP inhibitors. By closely examining the activities of DDX11 involved in these processes, we found that DDX11 helicase activity and
Fe-S domain are critical for cell viability and avert DNA damage focus formation upon DNA damaging conditions. Moreover, we have shown that DDX11 promotes HR by facilitating RAD51 accrual formation and homology-directed repair of DSBs. In addition, we have also shown that loss of DDX11 significantly affected RPA focus formation in both unperturbed and DNA damaging conditions. Moreover, by quantitatively measuring ssDNA using U2OS DiVA cells, we found that loss of DDX11 significantly affects ssDNA formation upon AsiSI-induced DSBs in two different genomic loci. We proposed that DDX11 promotes ssDNA formation during DNA repair by resolving various secondary structures such as G4 and hairpin structures to facilitate RAD51-mediated HR repair of DSBs (Fig 1.19) (37, 210).

![Figure 1.19](image.png)

**Figure 1.19** DDX11 facilitates HR-mediated DSB repair and repair of bulky lesion (37, 183). (A) In the model, DDX11 and 9-1-1 complex work jointly to repair bulky lesions and abasic sites. Mechanistically, DDX11 unwind the stalled 3’ end and the 9-1-1 complex facilitates RAD51 focus formation at post replicative gaps. Loss of 9-1-1 strongly impair HR and upregulates TLS-mediated gap filling. (B) DDX11 facilitates ssDNA formation by promoting end resection and later results in RAD51 nucleofilament formation by replacing
RPA. DDX11 may promote ssDNA formation by unwinding secondary structures such as G4 and hairpin structures to facilitate HR- repair.

In a different work, we showed that SMC5/6 complex acts jointly with the FA pathway to avert genome instability by promoting DNA repair and by allowing cellular fitness upon DNA damage drug treatment (211). By using chicken DT40 and mammalian cell line models, we have shown that loss of FA components FANCJ, FANCD2, FANCM and FANCC act together with the SMC5/6 complex to prevent DNA damage accumulation and cell death. Moreover, at cellular level, DDX11 KO cells resemble FA phenotypes. Genetic interaction studies revealed epistatic relationship between DDX11 and SMC5/6 complex in DNA damage repair (211). Moreover, DDX11 KO and FANCJ KO cells are sensitive to G4 stabilizing drugs and double knockout cells are additive towards G4 stabilizing drugs and DNA crosslinking agents (179, 183).

*In vitro* biochemical studies have shown that DDX11 helicase can bypass and unwind damaged DNA and secondary structures. Moreover, DDX11 deficient cells are sensitive to small molecule inhibitors that stabilize G4 structures and triplex structures (178, 212, 213). These results indicate possible roles of DDX11 in DNA replication fork progression by bypassing or unwinding obstacles encountered during replication (212, 214). Previous studies have shown that siRNA or CRISPR/Cas9-mediated loss of DDX11 in mammalian cells affects DNA replication fork speed in unperturbed conditions. Notably, DNA fiber analysis in WABS patients carrying mutation in DDX11 revealed slower replication fork speed, with the defect rescued by complementing with DDX11 WT (174, 215). Recent studies highlighted DDX11 associated with the replisome by interacting with replisome components Timeless and AND1 (198, 216, 217). Furthermore, DDX11 helicase activity was greatly increased *in vitro* to resolve/unwind wide range of DNA substrates by reconstitution with Timeless (198, 212).
Loss of both the DDX11 or Timeless affects DNA replication and restart upon HU-induced replication stress condition. However, loss of both the DDX11 and Timeless resulted in defective fork speed and restart, similar to single mutants (212). It indicates that DDX11 and Timeless possibly work together in a similar pathway to mitigate replication stress and DNA damage repair (212). Moreover, conversion of conserved amino acids EYE to KAE on DDX11 abolished the interaction with Timeless confirmed by pull down and \textit{in vitro} reconstitution studies (198, 217). Recent study has shown that DDX11 works together with a component of fork protection complex Timeless in the maintenance of epigenetic stability at G4 structures where the promoters and enhancers contain G-rich sequences (36, 198). DDX11 helicase-dead and Timeless interaction defective mutant showed an increased loss of G4 structure, indicating that DDX11 helicase and Timeless interaction domains are essential for G4 structure maintenance. In line with previous studies, loss of both DDX11 and FANCJ showed additivity in the context of G4 stability indicating that DDX11 works non-redundantly with FANCJ in G4 stability maintenance (36, 179, 212). Altogether, DDX11 acts in a wide variety of biological process associated with DNA replication and repair. However, its molecular mechanisms in promoting genome stability and in developmental processes are still elusive.
2. MATERIALS AND METHODS

2.1 Cell culturing conditions and genome editing in cancer cell lines

2.1.1 Cell culture conditions and media used to culture cell lines

U2OS (ATCC® HTB-96™), HeLa (ATCC® CCL-2™), PEO1, PEO1-R (C4-2), Capan-1 and Capan-1 R C2-6 (cell lines from Taniguchi lab) were cultured in DMEM high glucose L-glutamine, antibiotics, supplemented with 10% FBS. Ovarian cancer cell lines UWB1.289+BRCA1 (ATCC® CRL-2945) cell line were grown in complete 50% RPMI-1640 and 50% MEGM medium. Stable cell line UWB1.289+BRCA1 was cultured with G418 (400 µg/ml). All mammalian cell lines were maintained with 5% CO₂ at 37°C. All cell lines were checked for mycoplasma contamination frequently.

2.1.2 Establishment of stable cell lines

For the establishment of DDX11 KO cell lines stably expressing different variants, pCDNA3.1 (+) empty vector (EV), DDX11 -WT, DDX11-K50R, DDX11-R263Q and DDX11-KAE variants were transfected in U2OS DDX11 KO cells lines using Lipofectamine 2000 (Thermofisher Scientific). After 48-72 hours post transfection, cells were strongly selected with G418 (1000 µg/ml). After selection, cells were cultured with lower concentration of G418 (500 µg/ml). Western blot was performed to check the expression level of DDX11 variants in the corresponding cell lines.

2.1.3 Engineering DDX11 KO cell line models using CRISPR/Cas9

To establish U2OS and HeLa DDX11 KO cell lines, SpCas9 expressing construct PX459 Addgene (#62988) was transfected in cells using Lipofectamine 2000. After 48 hours post transfection Alt-R synthetic paired guide RNAs (IDT™) that target DDX11 exon 7 (GAGGTGAAGAAGAGCCCCTT) and exon 9 (GGGCTGCAGGGATGGCAAGG) were transfected using RNAiMaX and the cells were expanded for clonal populations in 96 well
plates 48-72 hours of post transfection. The established clonal populations were subjected for genotyping. Western Blot and Sangers sequencing were performed to confirm the knockout cells. The paired guide RNAs were designed by using online tool web tools CRISPRscan (https://www.crisprscan.org/) and CRISPOR (http://crispor.tefor.net/).

2.2 siRNA-mediated knockdown of target genes

For all the experiments cells were transfected with 20-30 nM of siRNAs (listed below in table) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol.

2.2.1 List of siRNAs used in this study

<table>
<thead>
<tr>
<th>Sl. no</th>
<th>siRNAs</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>ON-TARGETplus siRNA siDDX11</td>
<td>L-011843-00-0020</td>
</tr>
<tr>
<td>2</td>
<td>ON-TARGETplus Non-targeting Pool</td>
<td>D-001810-10-05</td>
</tr>
<tr>
<td>3</td>
<td>ON-TARGETplus siRNA siBRCA1</td>
<td>L-003461-00-0005</td>
</tr>
<tr>
<td>4</td>
<td>ON-TARGETplus siRNA siBRCA2</td>
<td>L-003462-00-0005</td>
</tr>
<tr>
<td>5</td>
<td>si53BP1</td>
<td>SASI_Hs_00024578</td>
</tr>
<tr>
<td>6</td>
<td>siREV7</td>
<td>SASI_Hs02_00329127</td>
</tr>
<tr>
<td>7</td>
<td>siC20orf196</td>
<td>SASI_Hs01_00102807</td>
</tr>
<tr>
<td>8</td>
<td>siFAM35A</td>
<td>SASI_Hs02_00352632</td>
</tr>
</tbody>
</table>

2.2.2 siRNA transfection protocol to silence target genes

1. Cells were seeded at a concentration of 1-1.5x10^5 cells/well in 6-well plate and allowed to adhere to the plate.

2. siRNAs at a concentration of 20-30 nM were added to 250μl of serum free media and mixed gently, followed by incubation for 20 min at room temperature.
3. To another 1.5ml eppendorf tube, 4-5µl of lipofectamine-RNAiMAX was added to the 250µl of serum free media and mixed gently, followed by incubation for 20 min at room temperature

3. After the incubation, the RNAi duplex-Lipofectamine RNAiMAX were mixed gently to form complex. Then the complex was added to the plate containing 2 ml of complete media without antibiotics to a final volume of 2.5 ml

4. Then the plate was mixed by rocking back and forth and subsequently incubated at 24-72 hours at 37°C in CO₂ before proceeding with specific assay.

2.3 Cell viability assay upon chemotherapeutic drug treatment

2.3.1 List of chemicals used in this study

<table>
<thead>
<tr>
<th>Sl no.</th>
<th>Drugs/Reagents</th>
<th>Catalogue No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Olaparib</td>
<td>Selleckchem #S1060</td>
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<tr>
<td>2</td>
<td>Cisplatin</td>
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<td>3</td>
<td>Telomestatin</td>
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<td>Pyridostatin</td>
<td>Merck #SMLO678</td>
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<td>5</td>
<td>AZD6738</td>
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<td>7</td>
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</tr>
<tr>
<td>8</td>
<td>IdU</td>
<td>Sigma #I7125</td>
</tr>
</tbody>
</table>

2.3.2 Colony formation assay

For colony formation assays, cells were treated with indicated Cisplatin drug concentrations for 1 h at 37°C. The cells were washed with 1XPBS thrice to wash off the drug and then cells were detached with trypsin. For plating, approximately 400-500 cells were seeded in 10 cm dishes with technical replicates and then cells were allowed to grow for 10-15 days. For PARP inhibitor mediated sensitivity assays, approximately 100 cells were seeded in 6 cm
dishes with technical replicates and the plated cells were incubated overnight in incubator. Next day, cells were treated with indicated Olaparib drug concentrations and allowed to grow for 10-15 days to form individual colonies. Then cells were stained with crystal violet and the fixed colonies were counted manually. The plating efficiency and surviving fraction was identified by normalizing with the colonies from untreated cells.

2.3.3 Cell sensitivity assay

For cell sensitivity assays, approximately 1500-2000 cells/well were seeded in 96 well plates and allowed to adhere. This was followed by acute/chronic drug treatment with indicated concentrations of Mitomycin-C, Telomestatin, Pyridostatin, and Olaparib and 3-5 days of incubation. After the incubation, cells were stained with crystal violet to derive cell viability.

For siRNA-mediated cell viability assays, briefly cells were transfected with indicated siRNAs and the cells were plated in 96 well plates for the assays after 24-48 hours of post transfection. The cells were incubated in the presence/absence of the indicated drug concentration and the viability was calculated after 3-5 days of incubation. Western blot for indicated proteins were performed 48 hours after siRNA transfection.

2.4 Western blot and immunofluorescence analysis of selected factors

2.4.1 List of antibodies used for Western blot and immunofluorescence analysis

anti-DDX11 (Santa Cruz #sc-271711) (1:1000), anti-BRCA2 (ab12349) (1: 1000), anti-pChk2 (pT68) (CST #2661) (1:1000), DNA PKcs (pS2056) (Epitomics #3892-1) (1:1000), anti-DNA PKcs (Epitomics #1579-1) (1:1000), anti-CHK2 (Santa Cruz #sc-17747) (1:1000), anti-BRCA1 (Santa Cruz #sc-6954 )(1:1000), anti-γ-H2AX (Millipore #05-636) (1:500), anti-53BP1 (Novus Biologicals #NB100-304) (1:1000), anti-MAD2B (REV7) (BD Biosciences 612266) (1:1000), α-tubulin (Santa Cruz #8035) (1: 5000), anti-RAD51 (Santa Cruz #sc-17747) (1:50), anti-rabbit HRP-linked (1:5000 CST), anti-mouse HRP-
linked (1:5000 CST) and Alexa Fluor 488 anti-mouse (IF 1:400) Invitrogen, Alexa Fluor Cy3-conjugated anti-rabbit (IF 1:400) Invitrogen were used.

2.4.2 Lysate preparation for Western blot analysis
1. Whole cell lysate extract was prepared using RIPA lysis buffer (50 mM Tris HCl, 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 1 mM EDTA, 0.1% SDS, 0.01% sodium azide at pH 7.4) or AZ lysis buffer (50 MM Tris pH 8, 250 mM NaCl, 1% NP-40, 0.15 SDS, 5 mM EDTA, 10 mM Na4P2O7, 10 mM NaF) supplemented with protease inhibitor cocktail (Roche) and PhosSTOP
2. Lysate was centrifuged at 15000 RPM for 15 to 20 minutes at 4°C and the supernatant was quantified using BIO-RAD reagent
3. Quantified cell extract was mixed with the Laemmlli buffer with β-Mercaptoethanol and heated at 95°C for 15 minutes (~30-40 µg of total protein extract was used for Western blot analysis)
4. After the incubation, samples were loaded on to the 4-20% Bio-Rad polyacrylamide gels and allowed to resolve at 100-140 V for 1 to 1.30 hours
5. Proteins were transferred to the nitrocellulose membrane in 1X Transfer buffer (1X glycine, 0.02M Tris base, 20% Methanol) at 30 V overnight
6. After the transfer was complete the membrane was stained with ponceau
7. Ponceau staining was removed by washing with 1XPBS-T (1M Tris HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) and the membrane was blocked with 5% skim milk for 30 minutes to 1 hour
8. Membrane was incubated with the primary antibody for 2 hours and then the membrane was washed with 1XPBS-T thrice (For probing BRCA1 and BRCA2 the blot was incubated with respected antibody overnight at 4°C)
9. After that, membrane was incubated with secondary antibody for 1 hour and washed thrice with 1XPBS-T
10. Blots were developed in chemidoc using West Femto Maximum Sensitivity substrate (Thermo Scientific)

2.4.3 Immunofluorescence analysis

For immunofluorescence analysis of 53BP1 and γ–H2AX, U2OS cells were grown in sterile coverslips and Cisplatin (2.5 µM) drug treatment was given for 1 hour, then the cells were washed with 1XPBS thrice to wash off the drug. The cells were fixed for 15 to 20 min with 4% formaldehyde containing 1XPBS and the samples were fixed at the indicated time points. After that, the coverslips were briefly washed thrice with 1XPBS and the cells were permeabilized with 0.3% Triton X-100 in 1XPBS for 5 min at RT and the coverslips were washed thrice with 1XPBS, followed by blocking with 10% horse serum for 1 hour. The immunofluorescence of RAD51 was performed as previously described in (218). Briefly, coverslips were incubated with indicated primary antibodies (53BP1 (1:1000), RAD51 (1:50) and γ–H2AX (1:500)) for 2 hours at room temperature with moisture and then washed thrice with 1XPBS. After washing, the coverslips were incubated with secondary antibodies (Alexa Fluor 488 and Cy3 (1:400)) for 1 hour. Coverslips were washed with 1XPBS thrice after the incubation of secondary antibodies and the cells were counterstained with DAPI for 20 min at RT. Immunofluorescence images were acquired using the confocal microscope Leica TCS SP2 AOBS inverted. Cell profiler software was used to analyze the 53BP1, RAD51 and γ–H2AX foci. For foci analysis, at least 100 nuclei were analyzed.

For micronuclei/mitotic catastrophe analysis, cells were grown on coverslips. The cells were treated with Cisplatin (2.5 µM) for 1 hour and briefly washed thrice with 1XPBS to wash off the drug. For PARPi, cells were grown in the presence of Olaparib (1 µM), followed by fixation of cells using 4% paraformaldehyde for 20 min at RT at the indicated time points. After fixation, cells were washed with 1XPBS thrice to remove all residual PFA. Cells were
counterstained with DAPI and then subjected for imaging to score the indicated phenotypes. For micronuclei/mitotic catastrophe, at least 50-100 nuclei were counted.

2.5 Single molecule DNA fiber analysis

1. DNA fiber analysis was performed as described previously with slight modifications. Cells were seeded at a density 1x10⁵ cells/well in 6 well plates.

2. CldU 1μL/mL (25 mM stock, final 25 μM) was added to the cells and incubated for the indicated time.

3. Briefly cells were washed twice with ice cold 1XPBS.

4. IdU 1μL/mL (250 mM stock, final 250 μM) was added to the cells and incubated for the indicated time.

5. Briefly cells were washed twice with 1xPBS and detached with trypsin.

6. Cells were collected with ice cold DMEM media and centrifuged at 4000 RPM for 1 min (4°C).

7. Cells were resuspended with ice cold PBS and spotted on to the glass slide.

8. Cells were lysed with cell lysis buffer and glass slides were tilted for spreading.

9. Cells were fixed with Carmoy solution for 3 min.

   *Prepare Carmoy solution (MeOH: acetic acid = 3: 1)*

10. The slides were incubated with 70% ethanol and then washed twice with 1XPBS.

11. The slides were incubated with fresh 2.5M HCl (25 mL of 12M HCL + 95 mL MilliQ) for 30 minutes at RT to denature DNA.

12. Then slides were treated with Neutralization buffer of 0.1M NaBorate for 3 min.

13. Slides were washed twice with 0.05% Tween containing PBS.

   Prepared primary antibody (2 μL of mouse BrdU antibody & 0.67 μL of rat CldU antibody in 100 μL of 1% BSA PBS) and cut parafilm, Tray with wet paper.

14. Slides were incubated with primary antibody for 1 to 2 hours at RT.

15. Slides were washed twice with 1XPBST for 1 min each and a final wash was given for
Prepared secondary antibody (1 μL of anti-mouse 488, 0.25 μL of anti-rat Cy3 in 100 μL of 1% BSA PBS)

16. Slides were incubated with secondary antibody for 1 hour and washed with 1XPBST
17. Slides were mounted with Permount Mounting medium
18. Images were taken in wide-field microscope and acquired images were analysed using Fiji software.

**DNA fiber lysis buffer**

<table>
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<tr>
<th>Components</th>
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<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% SDS</td>
<td>20%</td>
<td>1.25ml</td>
</tr>
<tr>
<td>200mMTris HCl (pH7.4)</td>
<td>1M</td>
<td>10ml</td>
</tr>
<tr>
<td>50mM EDTA</td>
<td>0.5M</td>
<td>5ml</td>
</tr>
<tr>
<td>MilliQ</td>
<td></td>
<td>up to 50ml</td>
</tr>
</tbody>
</table>

**Neutralization buffer**

0.1M Na Borate (Sodium tetraborate)

| Na$_2$B$_4$O$_7$    | 10.061g         |
| MilliQ              | up to 500ml     |
| Autoclave           |                 |
2.6 DR-GFP assay to assess HR-mediated repair of DSBs

1. U2OS TRI DR-GFP cells (220) were seeded at a density of 1x10^5 cells/well in 6-well plate and cells were allowed to adhere to the plate overnight.

2. Cells were transfected with the indicated siRNAs (20-30 nM) using Lipofectamine RNAiMAX (Invitrogen).

3. I-SceI endonuclease was induced using doxycycline (5 µg/ml) addition 48 hours of post siRNA transfection.

4. Cells were fixed with paraformaldehyde solution 72 hours after induction.

5. The fixed cells were analysed using Attune Nxt Flow cytometer and FACS analysis was performed using the FlowJo software.
2.7 Resection assay to quantitatively measure ssDNA tracts

1. U2OS DivA (AID-AsiSI-ER-U2OS) cells were plated at a concentration of 1x10^5 cells/well in 6-well plates and cells were allowed to adhere to the plate overnight.

2. Cells were transfected with the indicated siRNAs (20-30 nM) using Lipofectamine RNAiMAX™.

3. After 48 hours of siRNAs transfection, tamoxifen (300 nM) was added to the media for 4 hours to induce AsiSI-dependent DSBs.

4. Cells were collected and lysed after the induction of AsiSI.

5. DNA was extracted using DNAeasy kit (Qiagen) as per the manufacturers’ protocol.

6. ~500-1000 ng of isolated genomic DNA was digested using Ban1 restriction enzyme (NEB) at 37°C overnight.

7. The enzyme cuts ~200 bp from the DSB-KDEL3 and ~740bp for DSB-ASXL at the genomic DNA. Digested and undigested samples were treated with RnaseH and Ban1 was heat inactivated at 65°C for 20 min.

8. DNA samples were analyzed by real time PCR using the following primers:

   DSB-KDEL3_200 FW: ACCATGAACGTGTCCGAAT;

   DSB-KDEL3_200_REV: GAGCTCCGCAAAGTTTCAAG;

   DSB-ASXL1_740 FW: GTCCCCTCCCCACTATTT;

   DSB-ASXL1_740_REV: ACGCACCTGGTTTAGATTGG;

   DSB-KDEL3_20kb_FW: CACTCATCCTGATACATCAG;

   DSB-KDEL3_20kb_REV: TACAGTACTAATTGGGAGGC

ssDNA% was calculated as described in (221, 222). DNA quantity was normalized for each sample using a control region at 20 kb away from AsiSI cut site at the KDEL3 locus.
2.8 Synthetic lethality drug screening

For high content U.S. FDA approved drug screens, drug plates were prepared with various concentrations in 384 well plates using a robot. After the generation of drug plates, HeLa Ctrl and DDX11 KO cells were seeded on 384 well plates using Multidrop 384 dispenser Titertek (Thermo LabSystems Inc.) at a concentration of 500 cells/well. The cells were incubated for 72 hours with gradient concentrations of drugs. Cell viability was analysed by using CellTiter-Glo® and readings were taken in 384-well StorPlate-384V (#6008598, Perkin Elmer Inc.).

2.9 Statistical methods

Statistical analysis was performed using GraphPad prism software. Statistical differences for immunofluorescence experiments were identified by unpaired t-test.
3. RESULTS

Part-A DDX11 is dysregulated in cancers

3.1 DDX11 overexpression correlates with poor prognosis of patients with ovarian and lung cancers

DDX11 was found to be dysregulated in cancers and to promote tumorigenesis (37, 196, 223). Previous studies highlighted that DDX11 is overexpressed in various cancers in comparison with normal tissue and its overexpression is highly correlated with poor survival of patients with lung, melanoma, liver, cell renal and hepatocellular carcinoma (194, 196, 223-225). However, DDX11 overexpression in ovarian cancer and whether it negatively correlates with patient survival is not well known yet. Using the cBioportal online web tool, we found that DDX11 is overexpressed and hyper-amplified in 21% of ovarian cystadenocarcinoma (TCGA Pan-Cancer Atlas) and 13% of lung adenocarcinoma (TCGA Pan-Cancer Atlas (Fig. 3.1A and B). Moreover, we used the Kaplan-Meier survival plotter web tool to analyze whether DDX11 overexpression is associated with poor survival outcome of patients with ovarian and lung cancer. We split ovarian and lung cancer patients into two different groups based on DDX11 median expression to generate Kaplan-Meier overall survival plot. Interestingly, we found that DDX11 overexpression significantly correlates with poor prognosis in patients with ovarian and lung cancer at various clinical stages (Fig. 3.1A and B). Altogether, these in silico bioinformatic analyses indicate overexpression of DDX11 as a bad prognostic marker in patients with ovarian and lung cancer.
Figure 3.1 DDX11 overexpression correlates with poor prognosis of patients with ovarian and lung cancer. A and B, Oncoprint (cBioportal) and Kaplan–Meier overall survival plot of patients with ovarian and lung cancer, stratified by DDX11 median expression level. Patient sample numbers are indicated below the Kaplan-Meier plot.
3.2 Loss of DDX11 affects cell viability in a panel of Ovarian cancer cells

DDX11 overexpression has strong ties with tumorigenesis and has poor prognosis for patient survival in ovarian cancer (Fig. 3.1A). Almost 50% of epithelial ovarian cancers are defective in HR. The HR defect is often caused by mutations in tumor suppressor BRCA1 and BRCA2 genes, which often confer therapeutic vulnerability to PARP inhibitors. However, because of emerging chemoresistance, there is an important need to identify novel therapeutic drug targets that sensitize both HR proficient and deficient cancer cells. To probe whether DDX11 represents such a target, we selected a panel of ovarian cancer cell lines to examine whether loss of DDX11 sensitizes the cells to baseline chemotherapeutic drugs such as Cisplatin and Olaparib. By performing cell viability assays, we found that siRNA mediated knockdown of DDX11 broadly sensitized a panel of ovarian cancer cell lines that includes UWB1.289+BRCA1, OVCAR8, IGROV1 and COV362 to Olaparib and Cisplatin (Fig. 3.2A-C). From a therapeutic point of view, we further examined whether loss of DDX11 sensitizes transformed normal cell lines to chemotherapeutic drugs. We thus carried out cell viability assays in transformed non-cancerous cell lines from different tissue types, namely BJ, RPE-1 and MCF10A to Olaparib drug treatment. By performing cell viability assay, we observed mild or no sensitivity to Olaparib in BJ and RPE-1, however MCF10A cell line showed mild sensitivity to Olaparib at higher concentrations upon siDDX11 (Fig. 3.2.1.A-C). Thus, our results suggest that DDX11 loss selectively kills tested ovarian cancer cell lines to DNA damaging drugs.
Figure 3.2. Loss of DDX11 affects cell viability in a panel of Ovarian cancer cells. A, Cell sensitivity assay of ovarian cancer cell line UWB1.289 + BRCA1 transfected with indicated siRNAs. Briefly cells were treated with DNA damaging drugs Olaparib and Cisplatin with for 5-6 days (n=3). Western blot image is shown on the right. Error bars show average ± SEM. B, Cell viability assay of a panel of ovarian cancer cell lines transfected with indicated siRNAs. Briefly cells were treated with Olaparib with the indicated drug.
concentrations (n=3). Cell viability was obtained using crystal violet staining after 5 days incubation of cells with drug. Error bars show average ± SEM.

Figure 3.2.1 Loss of DDX11 does not affect cell viability on normal cell lines. A-C, Cell sensitivity assay of a panel of normal cell lines hTERT RPE-1, BJ and MCF-10A cells transfected with indicated siRNAs. Briefly cells were treated with indicated concentrations of Olaparib (n=3). Cell viability was obtained using crystal violet staining after 5 days incubation of cells with drug. Error bars show average ± SEM.
3.3 Establishment and confirmation of DDX11 knockout in HeLa and U2OS cell lines using CRISPR/Cas9

Next, we engineered DDX11 KO in cancer cell lines to investigate the molecular mechanism of DDX11 in the maintenance of genome stability and tumorigenesis. To establish DDX11 KO cells we designed high scored CRISPR paired guide RNAs using CRISPRscan and CRISPOR online web tools targeting DDX11 genomic loci at exon 7 and 9 (Fig. 3.3A). Briefly, we transfected CRISPR/Cas9 expressing plasmid construct (PX459) followed by transfection of synthetic paired guide RNAs after 48 hours of plasmid transfection to establish DDX11 KO in HeLa and U2OS cancer cell lines. After 72 hours of transfection of paired guide RNAs, we established clonal populations for the generation and validation of DDX11 KO clones. Moreover, established clonal populations were subjected to analysis and validation of DDX11 KOs using Western blot analysis and a monoclonal antibody that recognizes the C-terminus of DDX11 (Fig. 3.3B). We further validated DDX11 KO cells using immunofluorescence analysis and Sangers sequencing of DDX11 genomic loci to identify the nature of insertion or deletion in DNA caused by the Cas9 complex (Fig. 3.3C and D). Next, we performed sensitivity assay to validate the DDX11 KO cells by challenging cells to various DNA damaging drugs. In line with previous studies from our group and others (183, 211, 226), we found that HeLa and U2OS DDX11 KO cells are highly sensitive to DNA damaging drugs such as Cisplatin, Olaparib and Mitomycin-C chemotherapeutic drugs (Fig.3E-F and 3.5.2B). Thus, we successfully established DDX11 KO in HeLa and U2OS cell lines.
Figure 3.3 Establishment and validation of DDX11 knockout in HeLa and U2OS cell lines using CRISPR/Cas9. A, Diagrammatic representation CRISPR paired guide RNAs targeting DDX11 genomic locus at exons 7 and 9 to generate DDX11 knockout (KO) in U2OS and Hela cell lines. B, DDX11 Western blot analysis in U2OS and HeLa cell lines. C, Sequencing of DDX11 genomic loci in both U2OS and HeLa Ctrl and DDX11 KO cells respectively. D, Immunofluorescence analysis of DDX11 in indicated cell lines. E and F,
Cell sensitivity assay of HeLa Ctrl and DDX11 KO cells treated with the indicated concentrations of Cisplatin and Olaparib (n=3). Cell viability was obtained using crystal violet staining after 5 days incubation of cells with drug. Error bars show average ± SEM.

3.4 Synthetic lethality drug screens and validation of top hits

Cells lacking DDX11 are highly sensitive to baseline chemotherapeutic drugs such as Cisplatin and Olaparib. Cisplatin is a clinically approved standard first-line chemotherapeutic drug and also used as maintenance therapy in patients. On the other hand, Olaparib is a potent PARP inhibitor with promising chemotherapeutic response in BRCA1/2 mutated cancers (115, 116, 120, 227). Even though DDX11 KO cells are sensitive to the above-mentioned drugs, we were interested to identify other drugs that are synthetic lethal with DDX11 loss. To explore synthetic lethal drug targets, we performed synthetic lethal drug screens using a series of 64 U.S. FDA approved drugs with various concentrations in cancer cell lines lacking DDX11 (Fig. 3.4A). After 72 hours of chronic treatment with the indicated drug concentrations, we analyzed the cell viability using Cell Titer-Glo. Notably, in our screen we have used a panel of chemical compounds/inhibitors that affect genome integrity and target different pathways (Table 6). Moreover, to validate our drug screen, Olaparib and Cisplatin were used as positive controls and indeed both these drugs appeared as hits. Interestingly, we found various clinically approved drugs that sensitized HeLa DDX11 KO cells, including ATRi, PARPi, platinum drugs, bleomycin, and topoisomerase II poisons (Supplementary Tables 1 and 2). Among top hits, we validated ATR inhibitors (AZD6738 and VE-821), Olaparib and Cisplatin using colony formation assays (Fig. 3.4B-E). Moreover, we found that HeLa DDX11 KO cells are broadly sensitized by chemotherapeutic drugs that cause DNA crosslinks, topoisomerase trappers and agents that cause DSB formation.
Figure 3.4. Synthetic lethality drug screens and validation of top hits. A, Diagrammatic representation of chemical screens with FDA approved drugs in HeLa Ctrl and DDX11 KO.
cells. Cell viability was determined using CellTiter-Glo after 72 hours of drug treatment with gradient concentrations (n=2). B-E, Colony formation assay of HeLa Ctrl and DDX11 KO cells treated with indicated concentrations of Olaparib (n=2), Cisplatin (n=3) and ATR inhibitors (AZD6738 and VE-821) (n=3). Briefly, crystal violet staining was performed to visualize colonies after 10 to 15 days of incubation. For Cisplatin drug treatment, cells were grown in normal conditioned media after 1hour drug treatment. Error bars show average ± SEM.

3.4.1 p53+ DDX11 KO cells are sensitized by p53 stabilizers by inducing DNA damage

We also performed synthetic lethal drug screens using a series of 64 U.S. FDA approved drugs with various concentrations in p53 positive U2OS Ctrl and DDX11 KO cell line (Fig 3.4.1A). From the drug screen, we found various small molecule inhibitors that sensitized U2OS DDX11 KO cells. p53 stabilizers including RITA and Nutlin-3A appeared as top hits in our screen. By performing cell viability assays, we validated that RITA sensitized U2OS DDX11 KO at a very lower concentration (Fig 3.4.1B). Moreover, other p53 stabilizer, Nutlin-3A, significantly sensitized U2OS DDX11 KO cells (Fig 3.4.1C). Next, we examined whether the sensitivity of DDX11 KO cells to RITA drug treatment is associated with elevated DNA damage response. To address this, we performed immunofluorescence analysis for γ-H2AX and 53BP1 at the indicated time points upon chronic RITA drug treatment. Interestingly, we observed significant increase in γ-H2AX and 53BP1 focus formation in both the unperturbed and RITA drug treated conditions (Fig 3.4.1D). Altogether, our results suggest that p53 positive DDX11 KO cells are sensitized by p53 stabilizers.
Figure 3.4.1. p53+ DDX11 KO cells are sensitized by p53 stabilizers by inducing DNA damage. A, Diagrammatic representation of synthetic lethal chemical screen with FDA approved drugs in U2OS Ctrl and DDX11 KO cells. Briefly, cell viability was examined using CellTiter-Glo after 72 hours of drug treatment (n=2). B-C, Cell sensitivity assay of
U2OS Ctrl and DDX11 KO cells treated with RITA and Nutlin-3A. Cell viability was measured using crystal violet after 4-5 days of incubation in the presence of the drug at the indicated concentrations (n=3). Error bar shows average ± SEM. D, Representative images of 53BP1 and γ-H2AX focus formation in U2OS Ctrl and DDX11 KO cells upon RITA (10 nM) drug treatment with the indicated time points (scale bar, 10 μM). Foci quantification is shown. Statistical analysis was performed using Students t-test. Error bar shows average ± SEM.
Part-B Molecular mechanism and activities of DDX11 in DNA damage repair and fork protection upon chemotherapeutic drug treatment

3.5 DDX11 promotes DNA repair and averts genomic instability upon chemotherapeutic drug treatment

Next, we investigated whether the sensitivity of DDX11 KO cells to DNA damaging drugs correlates with accumulation of DNA damage and increase in chromosomal instability. To address this, we treated HeLa Ctrl and DDX11 KO cells with Cisplatin for 1 hour with the indicated drug concentration to induce DNA damage and then cells were allowed to recover in normal media. By performing Western blot analysis at the indicated time points, we found an increase in DNA damage checkpoint markers CHK2-P and γ-H2AX phosphorylation, which indicates an increased accumulation of DSBs (Fig. 3.5A). Moreover, we observed an increased activation of the NHEJ factor DNA-PKcs as detected by autophosphorylation at pS2056 (Fig. 3.5A). Furthermore, we counterstained nuclei using DAPI to quantify the micronuclei, which are a result of chromatin bridges and lagging chromosomes during mitosis. Interestingly, we observed an increase in genomic instability markers such as micronuclei and mitotic catastrophes in U2OS DDX11 KO cells upon acute Cisplatin drug treatment at the indicated timepoints (Fig. 3.5B). Next, we asked if Olaparib induced DNA damage and cellular sensitivity in DDX11 KO cells associates with genome instability markers. We treated HeLa Ctrl and DDX11 KO cells with the indicated Olaparib drug concentration and scored for genomic instability markers such as anaphase bridges, lagging chromosomes, micronuclei formation, and mitotic catastrophes at the indicated time points (Fig. 3.5C and D). In line with the sensitivity assay, we observed an increase in chromosomal instability in both untreated and drug treated conditions in DDX11 KO cells. Thus, our results suggest that DDX11 prevents DSB formation and chromosomal instability.
Figure 3.5 DDX11 promotes DNA repair and averts genomic instability upon chemotherapeutic drug treatment.  

A, HeLa Ctrl and DDX11 KO cells were treated for 1 hour with the indicated Cisplatin drug concentration and cells were allowed to recover for 72 hours. Western blot analysis of indicated DNA damage markers were examined with the indicated time points (n=2).  

B, Micronuclei and mitotic catastrophes quantification in U2OS Ctrl and DDX11 KO cells in both untreated and Cisplatin drug treated conditions with the indicated concentration (scale bar, 10 µm). Error bars show average ± SEM.  

C and D, Lagging chromosomes and mitotic catastrophes quantification in HeLa Ctrl and DDX11 KO
cells in both untreated and Olaparib (1 µM) treated conditions with the indicated time points. Error bar shows average ± SEM.

3.5.1 DDX11 averts persistent DNA damage accumulation upon chemotherapeutic drug treatment

We found that loss of DDX11 affects genomic stability in both unperturbed and drug treated conditions. Notably, we observed an increase in DSBs and genome instability markers such as micronuclei upon chemotherapeutic drug treatment in DDX11 KO cells (Fig. 3.5A and B). Next, we examined whether this genome instability correlates with an increase in DNA damage markers such as 53BP1 and γ-H2AX foci in DDX11 KO cells. To address this, we treated U2OS Ctrl and DDX11 KO cells with Cisplatin drug for 1 hour and allowed the cells to recover in normal media. Interestingly, by performing immunofluorescence analysis at the indicated time points, we observed a significant increase in DSB makers such as 53BP1 and γ-H2AX focus formation in both unperturbed and Cisplatin drug treated conditions in U2OS DDX11 KO cells (Fig. 3.5.1A). Moreover, we gave chronic Olaparib drug treatment to U2OS Ctrl and DDX11 KO cells with the indicated drug concentration and the immunofluorescence was performed after 24 hours of drug treatment. In line with the sensitivity of DDX11 KO cells to Olaparib, we observed significant increase in 53BP1 and γ-H2AX focus formation (Fig. 3.5.1B). Together, the results indicate that loss of DDX11 results in increased DSB formation in both unperturbed and upon chemotherapeutic drug treatment.
Figure 3.5.1 DDX11 averts persistent DNA damage accumulation upon chemotherapeutic drug treatment. A, Representative images and quantification of DNA damage markers 53BP1 and γ-H2AX focus in both U2OS Ctrl and DDX11 KO cells after Cisplatin drug treatment with with the indicated time timepoint (scale bar, 10 µm), n=2. Statistical analysis of foci was performed using Students t-test. Error bars show average ± SEM. B, Representative images and quantification of DNA damage markers γ–H2AX and 53BP1 focus formation in both U2OS Ctrl and DDX11 KO cells after 24 hours of indicated
Olaparib drug concentration (scale bar, 10 µM) (n=2). Statistical analysis was performed using Students t-test. Error bar shows average ± SEM.

3.5.2 DDX11 helicase activity and iron-Sulphur (Fe-S) motif are important for cell viability and avert DNA damage accumulation upon chemotherapeutic drug treatment

Previously, various in vitro biochemical studies showed that both the DDX11-K50R and DDX11-R263Q mutations affecting the helicase activity and DNA binding activity in vitro failed to resolve the DNA replication fork. However, at higher protein concentration, DDX11-R263Q mutant showed partial activity in vitro. Interaction between DDX11 and Timeless is important for normal DNA replication fork progression via resolving DNA secondary structures such as G-Quadruplex and facilitating sister chromatid cohesion (36, 198, 199). Moreover, DDX11-KAE mutation affects the interaction between DDX11 and the Timeless–Tipin replisome complex. However, molecular mechanisms of how these mutations affect DDX11 functions in vivo are still elusive. To address this, we introduced these previously reported point mutants by site directed mutagenesis in a plasmid construct that contains DDX11-WT protein encoding gene. Briefly, U2OS DDX11 KO cells were complemented with DDX11-WT and the point mutants that affect helicase activity (DDX11-K50R), Fe-S domain (DDX11-R263Q), and Timeless interaction (DDX11-KAE) (Fig. 3.5.2A). By performing cell viability assay, we found that both helicase activity and Fe-S domain are important for the cell viability upon both Olaparib and Mitomycin-C drug treated conditions. Moreover, DDX11–WT and DDX11-KAE complemented U2OS DDX11 KO cells showed similar sensitivity level to U2OS Ctrl cell line harboring DDX11-WT (Fig. 3.5.2A and B). Next, we inquired whether the sensitivity of the point mutants is associated with defective DNA damage repair upon chemotherapeutic drug treatment. In line with the sensitivity assay, we observed an increase in 53BP1 and γ-H2AX focus formation in U2OS DDX11 KO cells complemented with EV, helicase dead (DDX11-K50R) and Fe-S domain mutant (DDX11-R263Q), but not Timeless binding mutant (DDX11-KAE) upon acute
Cisplatin drug treated condition (Fig. 3.5.2C). Altogether, our results indicate that both DDX11 helicase and Fe-S domain are important to prevent DNA damage accumulation and cell death upon chemotherapeutic drug treatment.

Figure 3.5.2 DDX11 helicase and iron-Sulphur (Fe-S) motif is important for cell viability and averts DNA damage accumulation upon chemotherapeutic drug
treatment. A, Diagrammatic representation of point mutants (right) and immunoblot analysis of U2OS Ctrl and DDX11 KO cells complemented with the indicated plasmid constructs. B, Cell sensitivity assay of indicated complemented U2OS Ctrl and DDX11 KO cells upon Olaparib and Mitomycin-C drug treated conditions. Cell viability was measured after 5 days of incubation using crystal violet (n=3). Error bar shows average ± SEM. C, Representative and quantification DNA damage markers 53BP1 and γ-H2AX focus in DDX11 variants complemented U2OS Ctrl and DDX11 KO cells. Immunofluorescence analysis was performed after 24 hours with the indicated timepoints of Cisplatin (2 µM) for 1 hour (scale bar, 10 µm), n=2. Statistical analysis was performed using Students t-test. Error bar shows average ± SD.

3.6 DDX11 promotes homologous recombination by facilitating RAD51 focus accrual

Cells that are defective in HR often show sensitivity to chemotherapeutic drugs such as Cisplatin and Olaparib. Previous studies from our lab showed that chicken DT40 cells lacking DDX11 are defective in sister chromatid exchange and in RAD51 focus formation upon Mitomycin-C induced DNA damaging condition (183). We next addressed whether the hypersensitivity of DDX11 KO cells to chemotherapeutic drugs is associated with defects in HR-mediated DSB repair. By performing immunofluorescence, we found that U2OS DDX11 KO cells are significantly defective in RAD51 focus formation in both unperturbed and upon recovery from Cisplatin treatment (Fig. 3.6A). Moreover, we asked whether the reduced efficiency in RAD51 focus formation in U2OS DDX11 KO cells is associated with reduction in the efficiency of homology-directed repair upon DSB induction. To address this, we used a DR-GFP based reporter assay to quantitatively measure homology-directed repair using the engineered cell line called U2OS DR-GFP (220). Briefly, the I-SceI nuclease was induced in cells by addition of doxycycline 48 hours after transfection with the indicated siRNAs, followed by FACS analysis after 72 hours of induction. Interestingly, FACS
analysis revealed a significant decrease in homology-directed repair of GFP reporter in siDDX11 cells in comparison with siCtrl cells. However, the observed HR defect in siDDX11 cells is not as severe as in siBRCA1 cells (Fig. 3.6B).

Previous studies highlighted that loss of 53BP1 in BRCA1 mutated cells confers drug resistance to chemotherapeutic drugs by restoration of HR. Since cells that lack DDX11 show a BRCAness state regarding sensitivity to chemotherapeutic drugs and defects in homology-directed repair of DSBs, we asked whether loss of 53BP1 restores homology-directed repair of DSBs and correlates with drug resistance to chemotherapeutic drugs such as Olaparib and Cisplatin. By performing DR-GFP based reporter assay we found that loss of 53BP1 significantly restored homology-directed repair of DSBs in siDDX11 cells in comparison with single siDDX11 cells (Fig. 3.6B). Moreover, we found that loss of 53BP1 in DDX11 depleted cells showed resistance to DNA damaging drugs such as Cisplatin and Olaparib (Fig. 3.10A and B). Altogether, these results indicate that DDX11 promotes homology-directed repair of DSBs by facilitating RAD51 focus formation.
Figure 3.6 DDX11 promotes homologous recombination by facilitating RAD51 focus accrual. A, Representative micrographs and quantification of RAD51 focus formation in U2OS Ctrl and DDX11 KO cells treated with the indicated Cisplatin (2.5 µM) after 1-hour acute drug treatment (scale bar, 10 µM), n=2. Statistical analysis was performed using
Students \( t \)-test. Error bar shows average ± SD. B, Briefly, U2OS DR-GFP cells were transfected with indicated siRNAs and doxycycline was added after 48 hours of siRNA transfection to induce Sce-I. After 72 hours of doxycycline addition FACS analysis was performed to assess HDR of DSBs (n=3). Statistical analysis was performed using Students \( t \)-test. Error bar shows average ± SEM.

### 3.7 DDX11 facilitates RPA focus formation by promoting resection upon DNA damaging conditions

We next investigated the molecular mechanism by which DDX11 facilitates RAD51 focus formation to promote HR-mediated DSB repair. Previous studies highlighted that DDX11 helicase translocates on ssDNA with 5’-3’ direction in an ATP-dependent manner and resolves various substrates including DNA replication forks, DNA secondary structures such as D-loops and G-Quadruplex structures (171, 175, 228, 229). We next investigated whether defects in RAD51 focus formation in \( DDX11 \) KO cells (Fig. 3.6A) upon DNA damage are due to failure in efficient ssDNA formation. Notably, generation of ssDNA is important for the loading of RPA, which later is replaced by RAD51 nucleofilaments. Thus, we examined RPA focus formation in both unperturbed and chemotherapeutic drug treated conditions. Interestingly, by performing immunofluorescence, we found significant reduction in RPA focus formation in both unperturbed and upon Cisplatin-induced DNA damaging conditions in U2OS \( DDX11 \) KO cells (Fig. 3.7A). Moreover, we quantitatively measured the ssDNA formation at site specific DSBs using U2OS DIvA cells. Specifically, we analyzed two different DSB sites (KDELR3 ~200 bp and ASXL1 ~74 0bp) downstream of AsiSI induced break sites (Fig. 3.7B). Briefly, U2OS DIvA cells were transfected with siCtrl and si\( DDX11 \) using lipofectamine followed by induction of AsiSI endonuclease by using 4-OHT at a concentration of 300 nM for 4 hours after 48 hours of siRNAs transfection. We ascertained efficient DSB induction by performing immunofluorescence for 53BP1 and \( \gamma \)-H2AX focus formation (Fig. 3.7B). We further performed quantitative RT-PCR for the
above mentioned two different break sites and found significant reduction in ssDNA formation in DDX11 knockdown cells compared to control cells (Fig. 3.7B). Altogether, our results suggest that DDX11 helicase facilitates RAD51 focus formation by promoting ssDNA formation.

Figure 3.7 DDX11 facilitates RPA focus formation by promoting resection upon DNA damaging conditions. A, Representative micrographs and quantification of RPA focus
formation in U2OS Ctrl and DDX11 KO cells after 1 hour Cisplatin (2.5 µM for 1 hour) drug
treatment with the indicated time point (scale bar, 10 µm), n=2. Statistical analysis of foci
was performed using Students \( t \)-test. Error bars show average ± SD. B, U2OS DlvA cells
were transfected with the indicated siRNAs. After 48 hours post transfection, 4-OHT (300
nM) was added to induce DSBs mediated bt AsiSI. Two different independent regions
(ASXL1 and KDEL R3) were analyzed for the resection efficiency using RT-PCT after 4
hours of 4-OHT addition. All the obtained values were normalized with the amount of
ssDNA detected in control cells prior to 4-OHT treatment, n≥3. Its corresponding
immunofluorescence analysis (scale bar, 10 µm) and Western blot for AsiSI induced DSBs
is shown in right. Statistical analysis was performed using Students \( t \)-test. Error bars show
average ± SEM.

3.8 DDX11 protects replication forks from MRE11-mediated nascent strand
degradation

Extensive nascent strand degradation by nucleases results in increased genome
instability and often confers sensitivity to chemotherapeutic drugs. Tumor suppressor
proteins BRCA1 and BRCA2 are critical factors for fork protection from nuclease
degradation upon replication stress and fork stalling conditions. Another HR factor called
RAD51 is recruited to reversed forks to protect nascent strands from nuclease degradation,
including MRE11-mediated fork degradation. Moreover, DDX11 KO cells are characterized
by a BRCAness state regarding chemotherapeutic drug response and are defective RAD51
focus accrual (Fig. 3.6A). We asked whether defective RAD51 focus formation in DDX11
KO cells correlates with defective fork protection upon HU and Cisplatin induced replication
stress. To address this, we performed single molecule DNA fiber analysis upon replication
stress and fork stalling conditions. Briefly, we pulse labelled ongoing replication fork with
CldU and IdU labels for 30 min each, followed by addition of HU at higher concentration (4
mM) to induce replication fork stalling. Interestingly, we observed significant decrease in IdU/CldU ratio (<1) indicating that there is an increased nascent strand degradation upon HU-induced fork stalling in DDX11 KO cells than control cells (Fig. 3.8A). Moreover, we found that inhibition of the MRE11 nuclease by Mirin rescued nascent strand degradation upon HU-induced fork stalling (Fig. 3.8A).

BRCA1 deficient cells are sensitive to Cisplatin and are defective in fork protection upon Cisplatin conditions. Moreover, cells lacking DDX11 are sensitive to Cisplatin at very low concentrations and show persistence of DNA damage. We examined the fork protection function of DDX11 upon Cisplatin (150 μM) induced DNA damaging conditions. We pulse labeled the nascent strand DNA with CldU for 30 min, followed by a second label with IdU in the presence of Cisplatin together with addition or absence of Mirin for 1 hour. In this assay, we measured the length of CldU tracts, as readout for nascent strand degradation upon Cisplatin treated conditions. Interestingly, we found a decrease in CldU length upon Cisplatin drug treated conditions, which was rescued by inhibition of MRE11 with Mirin in DDX11 KO cells (Fig. 3.8B). Thus, our results uncovered roles of DDX11 in protecting replication forks from MRE11-mediated fork degradation upon replication stress and fork stalling conditions.
Figure 3.8 DDX11 protects replication forks from MRE11-mediated nascent strand degradation. A, Schematic representation for single molecule DNA fibers in HeLa Ctrl and DDX11 KO cells, IdU/CldU tract ratio upon HU (4 mM) treatment for 5 hours ± Mirin (50 µM), n=3. Dot plot represents IdU/CldU ratio. B, HeLa Ctrl and DDX11 KO cells were pulse labelled with CldU analogue for 30 min, followed by IdU for 1 hour in the presence or absence of Cisplatin (150 µM) ± Mirin (50 µM). Dot plot represents IdU tract length. Statistical analysis was performed using Students t-test. Error bars show average ± SD.
Part-C DDX11 acts complementary with BRCA1/2 in DNA damage repair and loss of DDX11 re-sensitizes BRCA1/2 drug resistant cancer cells to chemotherapeutic drugs

3.9 DDX11 acts complementarily with BRCA1 and BRCA2 in DNA damage repair

DDX11 is involved in HR-mediated DSB repair by facilitating RAD51 focus formation and is involved in fork protection against MRE11-mediated nascent strand degradation. This made us interested in addressing the functional relationship between DDX11 and BRCA1/2 in DNA damage repair upon chemotherapeutic drug treatment. To address the functional interaction, we performed sensitivity assay by depleting BRCA1 and BRCA2 with siRNAs in HeLa Ctrl and DDX11 KO cells. In line with previous studies, loss of BRCA1 and BRCA2 rendered cells sensitive to various chemotherapeutic drugs, including Olaparib, Cisplatin and G-Quadruplex stabilizing drugs such as Pyridostatin and Telomestatin. Moreover, we found that loss of either BRCA1 and BRCA2 in HeLa DDX11 KO cells increase sensitivity in comparison with single knockdown cells to the above-mentioned DNA damaging drugs at much lower drug concentrations (Fig. 3.9A-B). Altogether, these results suggest that DDX11 works in parallel with BRCA1 and BRCA2 to repair DNA damage upon drug treated conditions.
Figure 3.9 DDX11 acts complementary with BRCA1 and BRCA2 in DNA damage repair. A and B, Cell sensitivity assay of HeLa Ctrl and DDX11 KO cells, transfected with the indicated siRNAs. Briefly, cells were treated with the indicated concentration of Olaparib, Cisplatin, Pyridostatin and Telomesatin with for 72 hours. Cell viability was determined using crystal violet staining (n=3). Its corresponding Western blot shown below. Statistical analysis was performed using Students t-test. Error bars show average ± SEM.
3.9.1 DDX11 participates in homologous recombination and in cohesion associated pathways

Cells lacking DDX11 are synergistic to chemotherapeutic drugs upon loss of BRCA1 and BRCA2, which indicates that DDX11 facilitates survival in non-redundant ways. To get more insights on the molecular mechanism of DDX11 in HR and in other pathways, we performed genetic studies using siRNA in HeLa Ctrl and DDX11 KO cells. To address this, we performed and analyzed cell viability of double mutants (DDX11 KO siBRCA1 and DDX11 KO siBRCA2) versus triple mutants (DDX11 KO siBRCA1 siBRCA2) to various DNA damaging drugs such as Olaparib, Cisplatin and G-quadruplex stabilizing drug Telomestatin (Fig. 3.9.1A and B). Unexpectedly, this genetic study revealed non-additive nature between double and triple mutants. Previous publications from our laboratory showed that DDX11 helicase is important for sister chromatid cohesion and for proliferation and centromere integrity in cells defective in ESCO2, a factor involved in the establishment of sister chromatid cohesion (215, 230). Using siRNA for ESCO2, we reproduced synthetic lethality with DDX11 KO, accompanied by increased DNA damage and DSB formation (Fig 3.9.2A-C). Recently, similar results were also published by a different group (215, 231). However, the genetic relationship between DDX11 and cohesion associated pathways is not fully understood.

We were also interested to understand the genetic relationship between DDX11 and a cohesin subunit-STAG2 which is frequently mutated in cancers cells, among which bladder cancer and Ewing sarcoma. Previous studies that performed high throughput siRNA screens found that inactivation of DDX11 did not show synthetic sickness in STAG2 mutated cancer cell lines. To understand the genetic interaction between DDX11 and STAG2, we depleted STAG2 by using siRNA in HeLa Ctrl and DDX11 KO cells and cell viability assay was performed upon chemotherapeutic drug treatment. Interestingly, we found siSTAG2 in HeLa DDX11 KO cells caused much more sensitivity than single mutants
to chemotherapeutic drugs (Fig. 3.9.1C). Double mutants between \textit{DDX11} KO with \textit{siBRCA1}, \textit{siBRCA2} and \textit{siSTAG2} in HeLa cells showed similar sensitivity levels to PARP inhibitor Olaparib (Fig. 3.9.1C). Altogether, our results indicate that DDX11 participates in HR-mediated DSB repair and cohesin related pathways. Moreover, these results indicate that DDX11 can be a novel therapeutic target for STAG2 mutated cancers in the presence of chemotherapeutic drugs that cause DNA damage.
Figure 3.9.1 DDX11 participates in homologous recombination and in cohesion associated pathways. A-C, Cell sensitivity assay of HeLa Ctrl and DDX11 KO cells, transfected with indicated siRNAs. Briefly, cells were treated with the indicated drug concentrations of Olaparib, Cisplatin and Telomestatin for 72 hours, n=3. Cell viability was measured using crystal violet staining. Error bars show average ± SEM.

![Figure 3.9.1](image)

Figure 3.9.2 Loss of ESCO2 is synthetic lethal with DDX11. A, siRNA mediated knockdown of ESCO2 in HeLa Ctrl and DDX11 KO cells. The representative phase contrast images have shown in the above figure. B, Cell proliferation assay of HeLa Ctrl and DDX11 KO cells transfected with the siCtrl and siESCO2 with the indicated time points. C, Western blot analysis of indicated markers after the 72 hours of siRNA transfection in HeLa Ctrl and DDX11 KO cells.
3.10 Acquired drug resistance in HeLa DDX11 KO cells upon loss of 53BP1

Previous studies highlighted that loss of 53BP1 in BRCA1 mutated cells confers drug resistance to various DNA damaging agents including Olaparib by restoration of HR and/or fork protection. Since DDX11 is involved in HR-mediated DSB repair and loss of 53BP1 in DDX11 depleted cells increased the efficiency of HR-mediated DSB repair compared to DDX11 single knockdown cells, we next examined whether loss of 53BP1 in DDX11 KO cells confers drug resistance to chemotherapeutic drugs such as Olaparib and Cisplatin. In line with the DR-GFP based reporter assay, by performing both colony formation and cell viability assays, we found that loss of 53BP1 in DDX11 KO cells caused drug resistance to Olaparib and Cisplatin (Fig. 3.10A and B). However, the improvement in cell viability upon loss of 53BP1 in HeLa DDX11 KO cells is not up to the level of HeLa Ctrl siBRCA1 si53BP1 cells. Thus, our results indicate that DDX11 participates in HR-mediated DSB repair and loss of 53BP1 confers drug resistance in DDX11 KO cells by partial restoration of HR.
Figure 3.10 Acquired drug resistance in HeLa DDX11 KO cells upon loss of 53BP1.

A, Colony formation assay of HeLa Ctrl and DDX11 KO cells, transfected with the indicated siRNAs. Cell were treated with the indicated concentration of Cisplatin (n=3) and Olaparib.
(n=2) for 10 to 15 days. Crystal violet staining was performed to visualize colonies. Error bar shows average ± SEM. B, HeLa Ctrl and DDX11 KO cells, transfected with the indicated siRNAs and cells were incubated with the indicated concentrations of Olaparib and Cisplatin for 5-6 days. Cell viability was determined using crystal violet staining n=3. Error bars show average ± SEM.

Figure 3.11 Loss of DDX11 re-sensitizes BRCA1-deficient/mutated cells that acquired by loss of 53BP1. A and B, Cell sensitivity assay of HeLa Ctrl and DDX11 KO cells, transfected with the indicated siRNAs. Briefly, cells were treated with indicated drug concentrations of Olaparib and Cisplatin for 5-6 days (n=3). Cell viability was measured by
using crystal violet staining. Its corresponding plates and Western blot shown below. Error bars show average ± SEM.

3.11 Loss of DDX11 re-sensitizes BRCA1-deficient/mutated cells that acquired resistance by loss of 53BP1

Previous studies highlighted that loss of 53BP1 and downstream Shieldin components REV7, SHLD1 (C20orf196), SHLD2 (FAM35A), and SHLD3 (CTC.534A2.2) in BRCA1 mutated cancers causes drug resistance to Olaparib and Cisplatin. Acquiring drug resistance to baseline chemotherapeutic drugs is a major challenge in clinic and there is an important need to identify novel drug targets that selectively kill cancer cells including those that acquired resistance. To this end, we asked whether loss of DDX11 can re-sensitize BRCA1 mutated drug resistant cancer cells to chemotherapeutic drugs. In line with previous studies, cell viability assays revealed that loss of 53BP1 in BRCA1 mutated cells caused drug resistance to Olaparib and Cisplatin. However, loss of DDX11 compromised cell viability in BRCA1 mutated drug resistant cancer cells via loss of 53BP1 to both the chemotherapeutic drugs Olaparib and Cisplatin (Fig. 3.11A). Altogether, our studies highlight that acquired drug resistance in BRCA1 mutated cells via loss of 53BP1 are largely dependent on DDX11. Notably, DDX11 can be a novel target for cancer treatment to overcome chemotherapeutic drug resistance (Fig. 3.12D).

3.12 Loss of DDX11 re-sensitizes BRCA1-deficient/mutated cells that acquired resistance by loss of REV7 and Shieldin components FAM35A and C20orf196

The Shieldin complex consists of C20orf196, FAM35A, CTC-534A2. 2, and REV7 and acts downstream of 53BP1 in DSB repair. Loss of Shieldin components in BRCA1 mutated cancers leads to drug resistance via restoration of HR. In this study, we found that loss of DDX11 re-sensitizes siBRCA1 si53BP1 drug resistant cancer cells (Fig. 3.11A). We further asked whether loss of DDX11 can re-sensitize BRCA1 cancer cells rendered resistant
via loss of Shieldin components. In line with previous studies, loss of Shieldin components such as *FAM35A, REV7* and *C20orf196* in si*BRCA1* cells caused drug resistance to Olaparib. Moreover, cell viability assay revealed that loss of *DDX11* re-sensitized these cells to Olaparib and Cisplatin (Fig. 3.12A-C). Interestingly, we also found that loss of Shieldin components (*FAM35A,* and *C20orf196*) and *REV7* showed different levels of additivity towards Olaparib and Cisplatin drug treated conditions in *DDX11* KO cells, differently from their effect in *BRCA1* mutant cells. Thus, our study revealed that DDX11 is important for the cell viability of BRCA1 mutated cells that acquired drug resistance via loss of Shieldin components to chemotherapeutic drugs.
Figure 3.12 Loss of DDX11 re-sensitizes BRCA1-deficient/mutated cells that acquired by loss of REV7 and shieldin components FAM35A and C20orf196. A-C, Cell sensitivity assay of HeLa Ctrl and DDX11 KO cells, transfected with the indicated siRNAs. Briefly, cells were treated with indicated drug concentrations of Olaparib and Cisplatin for 5-6 days (n=3). Cell viability was measured by using crystal violet staining. Its corresponding plates and Western blot shown below. Error bars show average ± SEM. D, Diagrammatic representation indicates that loss of DDX11 confers cell sensitivity in BRCA1 drug resistant and BRCA2 restored cancer cells.

3.13 Accumulation of DNA damage upon BRCA2 loss in DDX11 KO cells

DDX11 and BRCA2 are additive to various DNA damaging drugs. Moreover, BRCA2 acts as a critical mediator for DNA damage repair in BRCA1 mutated drug resistant cancer cells. Next, we investigated the DNA damage accumulation upon loss of siBRCA2 in DDX11 KO cells in unperturbed conditions. Interestingly, by performing
immunofluorescence we found an increase in 53BP1 and γ-H2AX focus formation upon BRCA2 depletion in U2OS DDX11 KO cells (Fig. 3.13A). Moreover, we observed significant increase in genome instability marker such as micronuclei in U2OS DDX11 KO cells upon loss of BRCA2 in unperturbed condition (Fig. 3.13B). Thus, loss of BRCA2 in DDX11 KO cells aggravates genomic instability.

Figure 3.13. Accumulation of DNA damage upon BRCA2 loss in DDX11 KO cells.
A, Micrographs and quantification of DNA damage markers 53BP1 and γ-H2AX focus in U2OS Ctrl and DDX11 KO cells after 72 hours of post transfection with the indicated
siRNAs (scale bar, 10 µm), n=2. Statistical analysis was performed using Students \( t \)-test. Error bars show average ± SD. B, Micrographs and quantification of micronuclei in U2OS Ctrl and \( DDX11 \) KO cells after 72 hours of post transfection with the indicated siRNAs, n=2. Statistical analysis was performed using Students \( t \)-test. Error bars show average ± SEM.

3.14 DDX11 loss re-sensitizes BRCA2 reverted ovarian and pancreatic tumor cell lines

BRCA2 mutated cancers are hypersensitive to platinum and PARP inhibitors including Olaparib. However, BRCA2 mutated cancers acquire drug resistance by restoration of BRCA2 via secondary mutation that restore HR proficiency. Ovarian cancer cell line PEO1 and pancreatic cancer cell line Capan-1 are hypersensitive to chemotherapeutic drugs such as Cisplatin and Olaparib owing to mutation in BRCA2 tumor suppressor gene. In previous studies \( BRCA2 \) mutated cancer cells were cultured in media under selection pressure containing either Cisplatin or Olaparib chemotherapeutic drugs to establish drug resistant cancer cells. In the majority of isolated drug resistant clones, BRCA2 truncation was reverted by secondary mutations that resulted in restoration of the BRCA2 protein. We were interested to examine whether loss of DDX11 can sensitize BRCA2 mutated cancer cells and if drug resistant BRCA2 cancer cells can be re-sensitized to Olaparib by DDX11 loss. In line with this other results in this study, we found that loss of \( DDX11 \) sensitized BRCA2 mutated ovarian (PEO1-S) and pancreatic cancer (Capan-1 S) cells to Olaparib at a much lower concentration than drug resistant cancer cells (Fig. 3.14A and B). Moreover, loss of \( DDX11 \) affected the cell viability of BRCA2 drug resistant cancer cell lines (PEO1-R and capan-1 R) to Olaparib (Fig. 3.14A and B). Thus, our results confirm that DDX11 can be a potential target for BRCA2 mutated and drug resistant cancer cells.
Figure 3.14 DDX11 loss re-sensitizes BRCA2 reverted ovarian and pancreatic tumor cell lines. A, Cell sensitivity assay of PEO1 (S) and C4-2 (R) cells transfected with the indicated siRNAs and cells were treated with indicated Olaparib drug concentrations for 5-6 days (n=3). Cell viability was determined using crystal violet staining. Diagrammatic representations of mutations in BRCA2. Its corresponding Western blot of DDX11 and BRCA2 variants shown (right). Error bars show average ± SEM. B, Cell viability assay of Capan-1 (S) and Capan-1 (R) cells transfected with the indicated siRNAs and cells were treated with indicated Olaparib drug concentrations for 5-6 days (n=3). Cell viability was determined using crystal violet staining. Diagrammatic representations of mutations in BRCA2. Its corresponding Western blot of DDX11 and BRCA2 variants shown (right). Error bars show average ± SEM.
4. DISCUSSION

The genome is constantly threatened by a plethora of endogenous and exogenous sources. Cancer cells are characterized by an intrinsically highly unstable genome, with this genome instability being considered a hallmark of cancer (186, 187). Cells bearing mutation in genes that are involved in DNA replication and repair often confer sensitivity to DNA damaging agents. Because these genes are also often mutated in cancers, chemotherapy often relies on DNA damaging drugs to selectively kill cancer cells. Acquired drug resistance to the baseline chemotherapy highlights that novel drug targets are urgently needed in the clinical scenario. Drug resistance is one of the well-known and considerable limiting factors in cancer therapy that can be mediated by dysregulation in DNA repair mechanisms. Successful chemotherapies exploit cancer vulnerabilities to selectively target cancer cells. Thus, understanding replication stress and DDR mechanisms opens a new arena to exploit cancer specific vulnerabilities. Notably, it is dysregulation of cancer signaling pathways and DNA repair pathways that make possible targeted therapies in synthetic lethal approaches. In my PhD work, I focused on DDX11 helicase as a potential target in chemotherapy.

4.1 Roles of DDX11 in DNA damage repair and fork protection

So far various in vitro biochemical and molecular studies have shown crucial roles of DDX11 in promoting genome/chromosomal stability by unwinding various DNA topological structures and in the establishment of proper sister chromatid cohesion (171, 179, 229, 232). However, the exact molecular mechanism of DDX11 in DNA damage response and in replication is not well understood. Here, we report that DDX11 is upregulated in ovarian cancers and targeting/loss of DDX11 causes replication stress and genome instability in a wide variety of cancer cell lines (37). Moreover, loss of DDX11 in a panel of ovarian cancer cell lines affects cell viability to various DNA damaging drugs irrespective of the genetic background. In addition, we have shown that DDX11 helicase
activity and Fe-S domain are indispensable for DNA damage repair, but its interaction with
the Timeless component of the replisome is not. How these different DDX11 activities affect
its ability to mediate normal fork speed and fork protection, is a question for future studies
that will reveal also if the DNA repair and fork maintenance functions of DDX11 are coupled
or not.

Notably, our results highlight that DDX11 averts accumulation of DSBs and
subsequent mitotic instability. We provide evidence that DDX11 facilitates RAD51 focus
formation and promotes homology direct repair of DSBs. Mechanistically, we have
uncovered that upon DSB induction, DDX11 promotes ssDNA formation, the substrate for
RPA loading and then RAD51 nucleation (37, 183). Currently, how DDX11 facilitates DNA
end resection of DSBs but prevents MRE11-mediated resection of stalled replication forks
still needs to be understood, and it will be a question for the future.

DDX11 averts accumulation of DSBs in unperturbed and DNA damaging conditions.
One possible mechanism behind this action of DDX11 is its ability to mediate HR-mediated
repair of DSBs (37) and possibly HR-mediated replication fork restart that leads to sister
chromatid exchanges (183). However, the primary source of DNA lesions on which DDX11
acts upon, their relationship to fork stalling, and their genomic location, remains to be
investigated. It is of interest to inquire if in the absence of DDX11, DSBs would accumulate
in regions prone to forming specific types of secondary structures andr stalling of the
replication fork, as it was for example proposed for ATR mutant cells (233). This latter
question is relevant also in the context of understanding the sensitivity of DDX11 KO cells
to ATR inhibitors to understand how their actions synergize to promote genome
maintenance.

Cells that are defective in formation of RAD51 nucleofilament are highly associated
with the failure of fork protection function (52). Here, we have uncovered a previously
unknown function of DDX11 in replication fork protection function from MRE11-mediated
nascent strand degradation upon prolonged fork stalling/replication stress conditions (Fig
This observed fork protection function of DDX11 can be most simply explained by its ability to promote or stabilize ssDNA on the reversed arm of the fork, for RPA and RAD51 loading, resulting in the protection of the nascent strands from MRE11-mediated degradation (Fig 4.1). However, other possibilities, invoking differential DDX11-mediated regulation of nuclease activities at DSBs versus stalled forks are possible.

Figure 4.1 DDX11 is crucial for fork protection function upon replication stress and fork stalling conditions.

4.2 Replication gaps can be a determinant of drug response and TLS acts as backup pathway in DDX11 KO cells

It is well known that cells harboring mutation in tumor suppressor BRCA1, BRCA2 and other HR-related genes confer sensitive to various DNA damaging drugs including PARP inhibitors and ICL agents (18). Both BRCA1 and BRCA2 promote HR-mediated DSB repair and protect stalled replication fork from nuclease degradation. Apart from this, recent studies highlighted that replication gaps can be considered as the hallmark of chemotherapeutic response in BRCA1 and BRCA2 mutated cancers (110, 234, 235). Previous landmark studies uncovered that PARP inhibitor accelerates fork speed, resulting in replication stress and DDR (106, 234). Moreover, studies uncovered post-replicative gaps
as determinant of PARP inhibitor response in cells exhibiting a BRCAness status. On the other hand, G4 structures are considered as blockers of DNA replication fork progression and cells that are deficient in BRCA1/2 are sensitive to G4 ligands. Moreover, replication encountered obstacles are bypassed by PRIMPOL-mediated repriming downstream to G4 structures as well as DNA damage sites, resulting in post-replicative gaps. When replicative polymerases are blocked or not functional, translesion synthesis TLS polymerases are recruited at the damage sites to bypass the lesions (28, 108, 109, 236, 237). Notably, post replicative gaps should timely be filled in by DDT mechanisms. One major DDT pathway is mediated by TLS polymerases and is often activated in cancer cells to suppress the replication gap formation and to promote chemoresistance. When instead these gaps are not filled in, they can result in DSBs and cell death.

Interestingly, *DDX11* KO cells are sensitive to various gap-inducing chemotherapeutic drugs such as Cisplatin, ATR and PARP inhibitors. Observed sensitivity to chemotherapeutic drug is possibly mediated by the persistence of post-replicative gaps in *DDX11* KO cells, as proposed by our group from studies in DT40 cells (183), however further studies are needed to uncover this hypothesis (Fig 4.2). Here, by performing genetic interaction studies, we found that loss of TLS polymerase REV7 in *DDX11* KO cells causes increased sensitivity to Cisplatin, but only mild additivity to Olaparib. This indicates that TLS can act as backup pathway in *DDX11* KO cells upon chemotherapeutic drug treated conditions (37). Possibly this additivity to DNA damaging drugs upon REV7 loss in *DDX11* KO can be explained by increased gap formation, which correlates with the observed response to chemotherapeutic drugs. Moreover, it has been demonstrated that inhibition of TLS polymerase REV1 synergizes with the gap inducing drugs such as ATR inhibitor. Inhibition of ATR by small molecule inhibitor results in replication stress by affecting fork reversal and inducing replication gaps (109). Interestingly, synthetic lethal drug screens performed in this study revealed that *DDX11* KO cells are sensitized by ATR inhibitors. This
sensitivity can be mediated by increased formation of post replicative gaps (Fig 4.2), but other explanations are possible as also discussed above.

From our chemical screen of drugs that sensitize DDX11 KO cells, we identified as top hits DNA crosslinkers, including PARP trapper (Olaparib) and platinum drugs, and TOPII poisons or trappers, including Mitoxanthrone, Etoposide and Doxorubicin. (Tables 6.1 and 6.2). Recent genetic screen from yeast studies identified that CHL1 is synthetic lethal with nuclease defective hFEN1 by causing cytotoxic accumulation of DNA-protein crosslinks (238, 239). Altogether, our current work and previous studies indicate that DDX11 is involved in the repair of DNA-DNA and DNA-protein crosslinks, which are toxic to the cells and can result in the formation of DSBs. Further studies are need to uncover the exact roles of DDX11 in preventing DSB accumulation and facilitating DNA repair.
Figure 4.2 Replication gaps can be a determinant of chemotherapeutic response in DDX11 deficient cells.

4.3 DDX11 as therapeutic target in STAG2-mutated Ewing sarcoma

Previous studies highlighted that STAG2 is mutated in 17% of Ewing sarcoma and is associated with poor prognosis (240). Loss of STAG2 is not synthetic lethal with DDX11 KO, which however shows synthetic lethality with ESCO2 inactivating mutations (215, 231). Our study uncovered that loss of STAG2 in DDX11 KO cells confers additivity to DNA damaging drugs Cisplatin and Olaparib. Loss of BRCA1/2 or STAG2 in DDX11 KO cells caused additivity and similar degree of sensitivity to DNA damaging drugs. Thus, DDX11 roles in HR and cohesin-related pathways could be targeted in increasing chemotherapeutic sensitivity of cohesin defective cancers (i.e. STAG2 mutated) or HR defective ones (i.e.
BRCA1/2 mutated). Altogether, our study highlights that DDX11 can be an effective therapeutic target for the combinatorial treatment of Ewing sarcoma and other cancers bearing STAG2 mutation.

4.4 DDX11 unwinds G4 structures and is involved in telomere maintenance

DNA has the ability to fold and form alternative secondary structures such as G-quadruplex, triplex, hairpin and cruciforms across the human genome. So far various studies have highlighted that helicases such as FANCJ, DDX11, RTEL1 and WRN can resolve G4-structures to prevent genome instability (241). Cells lacking these helicase activities show increased cellular sensitivity to G4-stabilizing ligands, which also induce replication stress and DDR by affecting replication and the binding of the Shelterin complex to telomeres, the genomic region most prone to G4 structure formation. Interestingly, Fe-S cluster family of helicases comprising DDX11, RTEL1, FANCJ and XPD can all resolve G4 structures to mitigate replication stress and genome instability (34, 36, 242, 243) and RTEL1 has well known functions in various aspects of telomere maintenance (244). Failure to resolve G4-structures at telomeric regions result in fragility, fusion and shortening. Previous studies highlighted that DDX11 plays important roles in heterochromatin organization at telomeric region and its loss resulted in reduction of telomere length (196, 197). However, the exact molecular mechanism of DDX11 in telomere maintenance is not clearly understood. A simple explanation is that DDX11 promotes telomere stability by resolving G4-structures for smooth telomeric replication (Fig 4.3).

The roles of DDX11 in the replication and unwinding of G4 structures is interesting to pursue scientifically, as this could provide new options of therapy in a subset of situations. It was shown that DDX11 and Timeless work together to unwind G4 structures and maintain epigenetic stability of loci prone to G4 formation (198). Double knockout of DDX11 and FANCJ confers additivity to G4 stabilizing ligands and exhibits increased loss of G4 regions compared to single mutants (179, 245). Thus, DDX11 may unwind G4 secondary structures,
in parallel to FANCJ, to promote genome stability. For instance, many cancers carry mutations in FANCJ that do not associate with clear sensitivity to cisplatin. It is possible that combination of these FANCJ mutations with DDX11 loss, and/or with deficient recruitment of DDX11 to the replisome via defective interaction with Timeless, will result in marked sensitivity to cisplatin and/or G4 stabilizing agents. A secondary application relates to our finding that cells deficient in DDX11 are sensitive to G4 stabilizing ligands, with this sensitivity being further aggravated by loss of BRCA1/2. Thus, inhibition or loss of DDX11 can be coupled with G4 stabilization chemotherapy in BRCA1/2 defective cancers. Whether DDX11 inactivation would be effective also upon restoration of HR in BRCA1/2 cancers by 53BP1 and Shieldin mutations is an interesting and straightforward question to address.

Mechanistically, how sensitivity to G4 ligands ensues in DDX11 defective cells is interesting to understand. DNA fiber analysis upon G4-ligand treatment in DDX11 KO cells revealed no defect in the replication fork speed, but an increase in asymmetric fibers was observed (179). G4-structures can pause ongoing DNA replication forks, but they can be overcome, with formation of post-replicative gaps, by repriming (28). It will be interesting to examine whether the asymmetry in replication fibers and the observed sensitivity of DDX11 KO cells to G4 ligands is due to PRIMPOL- mediated repriming downstream to G4 structures that result in replication gap formation.
Figure 4.3 Potential roles of DDX11 in telomere maintenance based on its reported roles in G4 replication.

4.5 Roles of DDX11 in homologous recombination DSB repair

HR and NHEJ are two main pathways of DSB repair, the choice of which is directed by DNA end resection. This latter event is influenced by MRN and 53BP1 binding to the break site. Once resection takes place, the pathway will be directed to HR-mediated DSB repair, or in case resection is limited, to alternative NHEJ or MMEJ. 53BP1 binding at the DSB site prevents the loading of MRN complex and results in NHEJ-mediated DSB repair (7). In our study, DR-GFP cell-based assay revealed that loss of DDX11 associates with significant reduction in HR repair of DSBs. Loss of 53BP1 significantly restored this defect, highlighting an early action of DDX11 downstream of 53BP1, at the same time with MRN and CtIP-mediated short-range resection. Most often restoration of HR in BRCA1-deficient cells by loss of 53BP1 confers drug resistance (158, 218). Interestingly, we found that restoration of HR in DDX11 KO cells by loss of 53BP1 improves, albeit just partly,
Chemotherapeutic drug resistance to Olaparib and Cisplatin. Possibly drug resistance in DDX11 KO cells upon loss of 53BP1 may be due to partial restoration of HR by RAD51 nucleofilament formation mediated by BRCA2-PALB2-RNF168 complex. The dependency on MMEJ factors, such as DNA polymerase theta, for which recent inhibitors have been published (97, 98), is also worth testing. Further studies are required to uncover the drug resistance mechanism in DDX11 KO upon 53BP1 loss by performing genetic interaction studies with RNF168 and PALB2. However, drug resistance can also be mediated by the restoration of fork protection function, possibilities that need experimental testing.

4.6 DDX11 loss sensitizes BRCA1 and BRCA2 drug resistant cancer cells

Chemotherapeutic drug resistance is one of the biggest limitations in clinics. While BRCA1 mutations associate with high sensitivity to PARP inhibitors and Platinum drugs, loss of 53BP1 and its downstream Shieldin complex results in drug resistance (158, 218). On the other hand, restoration of full length BRCA2 protein by acquired secondary mutation result in drug resistance in BRCA2 defective cancers (133, 134). Therefore, there is a high need to uncover novel drug targets that can re-sensitize cancer cells with acquired drug resistance. Here, we uncovered that loss of DDX11 greatly increases the sensitivity of BRCA1 and BRCA2-mutated cancer cell lines to various chemotherapeutic drugs, even if they carry additional mutations that render them resistant (Fig 4.4).

Interestingly, triple mutant - loss of BRCA1 and BRCA2 in DDX11 KO cells showed similar degree of sensitivity with double mutants of DDX11 KO with either BRCA1 or BRCA2. This genetic interaction study indicates that the observed sensitivity upon loss of BRCA1/2 in DDX11 KO is relative to defective HR, because triple mutant was non-additive in comparison with double mutant. Altogether our results are indicating that DDX11 can be a novel and effective target for therapeutic applications in clinics (Fig 4.4) and future studies will elucidate its mode of action.
Figure. 4.4 Loss of DDX11 re-sensitizes drug resistant BRCA1/2 mutated cells
5. APPENDIX-1

I published part of my PhD work in the following journal

Journal : PNAS Proceedings of the National Academy of Sciences of the United States of America

Date : 20\textsuperscript{th} April 2021

DOI : 10.1073/pnas.2024258118/-/DC Supplemental

Manuscript is attached with the thesis

Title:

DDX11 loss causes replication stress and pharmacologically exploitable DNA repair defects

ABSTRACT

DDX11 encodes an iron–sulfur cluster DNA helicase required for development, mutated, and overexpressed in cancers. Here, we show that loss of DDX11 causes replication stress and sensitizes cancer cells to DNA damaging agents, including poly ADP ribose polymerase (PARP) inhibitors and platinum drugs. We find that DDX11 helicase activity prevents chemotherapy drug hypersensitivity and accumulation of DNA damage. Mechanistically, DDX11 acts downstream of 53BP1 to mediate homology-directed repair and RAD51 focus formation in manners nonredundant with BRCA1 and BRCA2. As a result, DDX11 down-regulation aggravates the chemotherapeutic sensitivity of BRCA1/2-mutated cancers and resensitizes chemotherapy drug–resistant BRCA1/2-mutated cancer cells that regained homologous recombination proficiency. The results further indicate that DDX11 facilitates recombination repair by assisting double strand break resection and the loading of both RPA and RAD51 on single-stranded DNA
substrates. We propose DDX11 as a potential target in cancers by creating pharmacologically exploitable DNA repair vulnerabilities.
6. APPENDIX -2

During my PhD studies, I have contributed to previously published article from our lab by collaborating together with my lab colleagues. The work is published in the following journal

Journal : EMBO reports
Date : 21st November 2019
DOI : 10.15252/embr.201948222

Manuscript is attached with the thesis

Title:
SMC5/6 acts jointly with Fanconi Anemia factors to support DNA repair and genome stability

ABSTRACT

SMC5/6 function in genome integrity remains elusive. Here, we show that SMC5 dysfunction in avian DT40 B cells causes mitotic delay and hypersensitivity toward DNA intra- and inter-strand crosslinkers (ICLs), with smc5 mutants being epistatic to FANCC and FANCM mutations affecting the Fanconi anemia (FA) pathway. Mutations in the checkpoint clamp loader RAD17 and the DNA heli- case DDX11, acting in an FA-like pathway, do not aggravate the damage sensitivity caused by SMC5 dysfunction in DT40 cells. SMC5/6 knockdown in HeLa cells causes MMC sensitivity, increases nuclear bridges, micronuclei, and mitotic catastrophes in a manner similar and non-additive to FANCD2 knockdown. In both DT40 and HeLa systems, SMC5/6 deficiency does not affect FANCD2 ubiquity- lation and, unlike FANCD2 depletion, RAD51 focus formation. SMC5/6 components further physically interact with FANCD2-I in human
cells. Altogether, our data suggest that SMC5/6 functions jointly with the FA pathway to support genome integrity and DNA repair and may be implicated in FA or FA-related human disorders.
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Table 6.2 Synthetic lethal drug screening result

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**Abbreviation:**

Sensitization (S), Resistance (R), Moderate (M)
REFERENCES


140.


DDX11 loss causes replication stress and pharmacologically exploitable DNA repair defects

Nanda Kumar Jegadesan* and Dana Branzei†,‡

*The FIRC Institute of Molecular Oncology, Italian Foundation for Cancer Research, 20139, Milan, Italy; and †Istituto di Genetica Molecolare, Consiglio Nazionale delle Ricerche, 27100, Pavia, Italy

Edited by Richard D. Kolodner, Ludwig Institute for Cancer Research, La Jolla, CA, and approved March 24, 2021 (received for review November 23, 2020)

DDX11 encodes an iron–sulfur cluster DNA helicase required for development, mutated, and overexpressed in cancers. Here, we show that loss of DDX11 causes replication stress and sensitizes cancer cells to DNA damaging agents, including poly ADP ribose polymerase (PARP) inhibitors and platinum drugs. We find that DDX11 helicase activity prevents chemotherapy drug hypersensitivity and accumulation of DNA damage. Mechanistically, DDX11 acts downstream of S3BP1 to mediate homology-directed repair and RAD51 focus formation in manners nonredundant with BRCA1 and BRCA2. As a result, DDX11 down-regulation aggravates the chemotherapy sensitivity of BRCA1/2-mutated cancers and resensitizes chemotherapy drug-resistant BRCA1/2-mutated cancer cells that regained homologous recombination proficiency. The results further indicate that DDX11 facilitates recombination repair by assisting double strand break resection and the loading of both RPA and RAD51 on single-stranded DNA substrates. We propose DDX11 as a potential target in cancers by creating pharmacologically exploitable DNA repair vulnerabilities.

Significance

Replication stress can affect development and is a hallmark of cancers. Warsaw breakage syndrome is a developmental disorder caused by mutations in the conserved DDX11 DNA helicase. Here, using human cellular models of DDX11 deficiency, we report that DDX11 helicase prevents replication stress and mediates homology-directed repair via homologous recombination. Mechanistically, DDX11 promotes resection, enabling RPA and RAD51 focus formation, and acts nonredundantly with the RAD51 mediators BRCA1 and BRCA2. As a result, targeting DDX11 confers improved chemotherapy responsiveness in both chemotherapy-sensitive and drug-resistant BRCA1/2-mutated cancers that regained homologous recombination proficiency by suppressor mutation or somatic reversion. The results pinpoint DDX11 as a critical replication stress mitigating factor whose targeting can improve chemotherapeutic response in a range of cancers.

Author contributions: N.K.J. and D.B. designed research; N.K.J. performed research; N.K.J. and D.B. analyzed data; and N.K.J. and D.B. wrote the paper.

The authors declare no competing interest.

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https://doi.org/10.1073/pnas.2024258118 1 of 12
DDX11 Loss Causes Persistent DNA Damage Accumulation. We next examined whether the sensitivity of HeLa DDX11 KO cells to chemotherapeutic drugs associates with the accumulation of DNA damage. The recovery from acute cisplatin treatment in HeLa DDX11 KO cells resulted in increased γ–H2AX and phosphorylated CHK2 (CHK2-P), indicative of persistent DSBs (Fig. 2A). Moreover, DDX11 KO cells showed increased activation of DNA-dependent protein kinase, catalytic subunit (PKcs) involved in nonhomologous end joining (NHEJ) (24) and BRCA1 helicase activity along its Fe–S domain (Fig. 2B). We found that the helicase activity and Fe–S domain are critical for cellular viability upon olaparib and mitomycin C drug treatment, as these mutants showed similar sensitivity levels to U2OS DDX11 KO cells carrying EV (empty vector) (Fig. 3B). Of interest, the Timeless interaction was not essential in this process (Fig. 3B). In the same line with the cellular viability results, we found that K50R and R263Q DDX11 variants are severely defective in preventing γ–H2AX and 53BP1 DNA damage foci accumulation upon recovery from cisplatin treatment, whereas the KAE mutant had only minor effects in this regard (Fig. 3C). Thus, DDX11 helicase is critical to prevent DNA damage accumulation and chemotherapy sensitivity.

DDX11 Promotes Homology-Directed Repair of DSBs and RAD51 Foci Formation Nonredundantly with BRCA1 and BRCA2. Cells defective in HR often show sensitivity to olaparib and cisplatin drugs. Studies in chicken DT40 cells proposed a role for DDX11 in HR-mediated alignment and RAD51 focus formation upon replication damage (17). However, no defect in RAD51 focus accrual was observed in DDX11-deficient lymphoblasts derived from a WBS patient (18). To address whether the accumulation of DNA damage and hypersensitivity in DDX11 KO cells may stem from a defect in HR, we analyzed RAD51 foci in U2OS cells in both experimental conditions (Fig. 4A). To address whether the observed decrease in RAD51 foci in DDX11 KO cells exposed to DNA damage relates to a role for DDX11 in DSB repair by homology-directed repair mechanisms in cancer cells, we performed synthetic lethality drug screens with ovarian and lung cancers (17, 18, 23). Silencing of DDX11 loss causes replication stress and pharmacologically exploitable DNA repair defects.
Fig. 1. Establishment of DDX11 KO in cancer cell lines and identification of synthetic lethal drugs. (A) Cell viability assay of ovarian cancer cell line UWB1.289 + BRCA1 transfected with siCtrl and siDDX11. Cells were treated with olaparib and cisplatin with the indicated drug concentrations (n = 3). (Right) Corresponding Western blot. Error bars show average ± SEM. (B) Schematic representation of DDX11 genomic locus, targeted by the CRISPR-paired guide RNAs at exons 7 and 9 to establish KO in HeLa and U2OS cell lines. (C) Western blot analysis of DDX11 in HeLa and U2OS cell lines to assess DDX11 targeting with CRISPR-paired guide RNAs. (D) Schematic representation of synthetic lethal drug screen (Food and Drug Administration [FDA]-approved drugs) in HeLa Ctrl and DDX11 KO cells in which cell viability was determined 72 h after drug treatment with 64 FDA-approved drugs at different concentrations using CellTiter-Glo (n = 2). (E) Colony formation assay of HeLa Ctrl and DDX11 KO cells exposed to olaparib (n = 2), cisplatin (n = 3), and ATRi AZD6738 (n = 3) with the indicated drug concentrations. Colonies were stained with crystal violet after 10 to 15 d of incubation. For cisplatin, after 1 h of acute treatment, cells were allowed to grow in normal media. Error bars show average ± SEM.
Fig. 2. DDX11 loss associates with persistent DNA damage accumulation and micronucleation. (A) HeLa Ctrl and DDX11 KO cells were treated with cisplatin (1 μM) for 1 h and allowed to recover for 72 h, during which DNA damage markers were analyzed by Western blotting at the indicated time points (n = 2). (B) Quantification of micronuclei and mitotic catastrophes in U2OS Ctrl and DDX11 KO cells in untreated conditions and upon recovery from an acute cisplatin treatment (2.5 μM for 1 h). Error bars show average ± SEM. (C) Quantification and representative micrographs of γ-H2AX and 53BP1 foci in U2OS Ctrl and DDX11 KO cells recovering from an acute treatment with cisplatin (2.5 μM for 1 h). (Scale bar, 10 μm.) n = 2. Statistical analysis of foci was performed using Student’s t test. Error bars show average ± SD.
Fig. 3. DDX11 helicase activity averts DNA damage accumulation and damage sensitivity. (A) Western blot analysis of U2OS Ctrl and DDX11 KO cells complemented with EV, WT DDX11, DDX11 helicase dead (K50R), mutation in iron–sulfur cluster domain (R263Q), and DDX11-Timeless interaction defective motif (KAE). (Right) Schematic representation of DDX11 and its associated mutations. (B) Cell viability of U2OS Ctrl and DDX11 KO cells complemented with EV and DDX11 variants upon mitomycin C and olaparib drug treatment. Cell viability was measured using crystal violet after 5 d of incubation in the presence of the drugs at the indicated concentrations (n = 3). Error bar shows average ± SEM. (C) Quantification and representative micrographs of 53BP1 and γ-H2AX foci in U2OS Ctrl and DDX11 KO cells complemented with different DDX11 variants after 24 h of recovering from 1 h treatment with cisplatin (2 μM). (Scale bar, 10 μm.) n = 2. Statistical analysis was performed using Student’s t test. Error bar shows average ± SD.
repair, we investigated the efficiency of the latter using a direct repeats GFP (DR-GFP) assay (27). We found that siDDX11 cells had significantly lower homology-directed repair efficiency compared with control cells but were not as drastically defective as siBRCA1 cells (Fig. 4B and SI Appendix, Fig. S5A). The loss of 53BP1 can enhance HR repair by allowing resection (28, 29). Notably, we found that si53BP1 rescued the homology-directed repair defect of siDDX11 cells in the DR-GFP assay (Fig. 4B and SI Appendix, Fig. S5A). Colony formation and cell viability assays further revealed that the loss of 53BP1 in DDII1 KO cells partly rescued their cisplatin and olaparib sensitivity (SI Appendix, Fig. S5B and see below). Because BRCA1 and BRCA2 are critical RAD51 mediators, we analyzed the functional interaction with DDX11. We found that double mutants between DDII1 KO and either siBRCA1 or siBRCA2 are more sensitive than single mutants toward olaparib, cisplatin, and G4 stabilizing drugs, such as Pyridostatin and Telomestatin, shown previously to sensitize DDX11 and BRCA1/2 mutant cells (8, 30) (Fig. 4C and SI Appendix, Fig. S6). Thus, DDX11 participates in the homology-directed repair of DSBs and RAD51 focus formation but acts nonredundantly with BRCA1 and BRCA2 in DNA repair.

DDX11 Loss Resensitizes BRCA1-Deficient Cells with Acquired Drug Resistance. Previous studies highlighted that the loss of 53BP1 in BRCA1 mutated/null tumors leads to drug resistance (31). Because si53BP1 caused higher improvement in the viability of siBRCA1 cells compared with DDII1 KO treated with DNA damage (SI Appendix, Fig. S7), we asked whether DDX11 is required for the HR pathway activated in siBRCA1 si53BP1 cells. Strikingly, the loss of DDX11 sensitized si53BP1 siBRCA1 cells toward both olaparib and cisplatin (Fig. 5A). Moreover, we assessed whether DDX11 is required for the viability of BRCA1-depleted cells rendered resistant to chemotherapeutic drugs via removal of other shieldin components (reviewed in ref. 21). Depletion of REV7 and shieldin components FAM35A and C20orf196 rendered BRCA1-depleted cells resistant to olaparib and cisplatin but did not rescue the sensitivity of DDII1 KO cells (Fig. 5B and SI Appendix, Fig. S8). Importantly, the viability of BRCA1-depleted cells rendered resistant by mutations in REV7 and shieldin largely depended on DDX11 (Fig. 5B and SI Appendix, Fig. S8). Thus, DDX11 is required for viability in response to chemotherapy in BRCA1-depleted cells that acquire resistance via inactivation of 53BP1, REV7, and shieldin components.

DDX11 Is Complementary with BRCA2 in Facilitating DNA Repair and Genome Stability. We further analyzed the functional interaction between DDXII1 and BRCA2, as BRCA2 is critical for DNA repair in siBRCA1 si53BP1 cells (28, 32), whose viability also depends on DDX11 (Fig. 5A). We found that DDII1 KO increases spontaneous DNA damage in BRCA2-depleted U2OS cells as observed by increased 53BP1 and γ-H2AX DNA damage foci (Fig. 6A) and higher levels of micronucleation (Fig. 6B) in double mutants compared with the single inactivation of DDII1 and BRCA2. To analyze the functional interaction between DDII1 and BRCA2 in tumor cells upon chemotherapy drug treatment, we examined the effect of siDDX11 in ovarian and pancreatic tumors that carry BRCA2 mutations (PEO1 and Capan-1) and are sensitive to chemotherapy as well as the corresponding tumor cell lines that acquired PARP resistance by secondary mutations, resulting in restoration of BRCA2 (PEO1 C4-2 and Capan-1 C2-6) (33, 34). Notably, knockdown of DDII1 in PEO1 and Capan-1 sensitive (S) as well as resistant (R) clones rendered the BRCA2-mutated tumor cells sensitive to olaparib (Fig. 6C and D). Thus, DDX11 loss sensitizes both HR-deficient and HR-proficient cancer cells to agents that necessitate repair via HR.

DDX11 Facilitates DSB Resection and RPA Loading. DDII1 KO cells show a BRCA1/2 deficient state in terms of chemotherapeutic sensitivity and RAD51 focus formation upon DNA damage. To understand whether DDX11 affects a step related to RAD51 loading or filament extension/stability or may influence steps upstream of RAD51 nucleation, we examined RPA32 focus formation. We found a significant decrease in RPA32 foci in U2OS DDII1 KO cells in both unperturbed conditions and upon recovery from cisplatin drug treatment (Fig. 7A). To inquire if DDII1 may facilitate the levels of single-stranded DNA (ssDNA) substrates to which RPA is loaded, we used the DSB inducible via the AsiSI (DIVA) U2OS cell line, which allows to induce clean DSBs throughout the genome (35). In this cell line, 4-hydroxytamoxifen (4OHT) treatment induces the relocalization of a stably expressed restriction enzyme (AsiSI) that triggers the production of multiple DSBs at annotated positions across the genome and increased 53BP1 and γ-H2AX foci (Fig. 7B). We quantitatively measured the formation of ssDNA upon DSB induction by AsiSI using quantitative PCR at two different DSB break sites, KDELRS (~200 base pairs [bp]) and ASXLI (~740 bp), downstream of the AsiSI-induced break site (35), observing a significant decrease in ssDNA formation in sDDII1 U2OS DIVA cells at both loci (Fig. 7B). Altogether, these results reveal that DDII1 promotes the formation of a ssDNA substrate suitable for RPA loading and subsequently for RAD51 nuclease filamentation and HR-mediated repair (Fig. 7C).

Discussion
The genome of cancer cells is highly unstable and acquires resistance to the backbone therapies, which suggests that alternative approaches are needed. Moreover, development of new biomarker assays beyond BRCA4 mutations of chemotherapeutic responsiveness would facilitate efforts to optimize current therapies (36). The present work meets both needs. We uncover DDII1 as a potential target in tumors, including BRCA1/2-mutated cancers that acquired chemotherapeutic resistance, and pinpoint DDII1 mutations/loss as a useful biomarker of responsiveness to platinum drugs, PARP inhibitors, and ATRI.

Successful chemotherapies exploit fundamental vulnerabilities of cancer cells to increase replication stress, causing specific cell death. Here, we find that targeting DDII1, a gene whose up-regulation in several cancers correlates with poor patient prognosis (9–11), sensitizes ovarian, uterine, and other cancers to PARP inhibitors and platinum chemotherapies by causing an accumulation of DNA damage and mitotic instability. These phenotypes could result from DDII1 roles in repairing replication-associated lesions and/or preventing their formation. Here, we provide evidence that DDII1 facilitates homology-directed DNA repair of DSBs and potentially of other replication-associated lesions by promoting RPA loading on ssDNA substrates and subsequently RAD51 focus formation. This role of DDII1 in mitigating replication stress relies on its helicase activity and is especially relevant in replication stress conditions induced by DNA damaging drugs, correlating with the ability of cancer cells to tolerate the chemotherapeutic-induced DNA damage.

Once DSBs are formed, their repair choice is being directed by 53BP1 binding to the break ends, which favors NHEJ-mediated repair while preventing resection (22, 28, 32). End resection, favored by BRCA1 engagement of the DSBs, shuts the break into an HR-mediated repair pathway while preventing 53BP1 binding. We find that the depletion of DDII1 impairs homology-directed repair of DSBs, and this effect is rescued by concomitant 53BP1 silencing, suggesting a role for DDII1 in coupling resection of DSBs with effective HR repair. Two main postresection pathways of RAD51 focus formation have been described, one mediated by BRCA1-PALB2-BRCA2 and the other one by the ASXL1 (35). In this cell line, 4-hydroxytamoxifen (4OHT) treatment promotes the formation of ssDNA substrate suitable for RPA loading and subsequently for RAD51 nuclease filamentation and HR-mediated repair (Fig. 7C).
Fig. 4. DDX11 promotes homology-directed repair of DSBs and RAD51 foci formation nonredundantly with BRCA1 and BRCA2. (A) Quantification and representative micrographs of RAD51 focus formation in U2OS Ctrl and DDX11 KO recovering from 1 h treatment with cisplatin (2.5 μM). (Scale bar, 10 μM). n = 2. Statistical analysis was performed using Student’s t test. Error bar shows average ± SD. (B) U2OS TRI DR-GFP cells were transfected with indicated siRNAs, and Sce-I was induced by adding doxycycline after 48 h of siRNA transfection. Fluorescence-activated cell sorting analysis was performed after 72 h of doxycycline induction (n = 3). Schematic representation of the assay is shown above. Error bars show average ± SEM. (C) Cell viability assay of HeLa Ctrl and DDX11 KO cells transfected with siCtrl, siBRCA1, and siBRCA2. Cells were treated with olaparib and Pyridostatin with the indicated drug concentrations for 72 h, and cell viability was measured using crystal violet staining (n = 3). (Bottom Left) Corresponding Western blot is shown. Statistical analysis was performed using Student’s t test. Error bars show average ± SEM.
Fig. 5. DDX11 loss resensitizes siBRCA1 cells that acquired drug resistance via 53BP1 or shieldin loss of function. (A) Cell viability assay of HeLa Ctrl and DDX11 KO cells transfected with indicated siRNAs. Cells were treated with olaparib and cisplatin with the indicated drug concentrations for 5 to 6 d (n = 3). Cell viability was determined by using crystal violet staining. (Right) Corresponding plates are shown. Its corresponding Western blot is shown in SI Appendix, Fig. S7. Error bars show average ± SEM. (B) Cell viability assay of HeLa Ctrl and DDX11 KO cells transfected with indicated siRNAs. Cells were treated with olaparib and cisplatin with the indicated drug concentrations for 5 to 6 d (n = 3). Cell viability was determined by using crystal violet staining. (Top Left) Corresponding Western blot is shown. Error bars show average ± SEM. (Top Right) Model for DDX11 loss in BRCA1 mutant cancer cells are synergistic, and resensitization of BRCA1 drug-resistant cancer cells to chemotherapeutic drugs.
DDX11 is complementary with BRCA2 in facilitating DNA repair and genome stability (A) Quantification and representative micrographs of γ-H2AX and 53BP1 foci in U2OS Ctrl and DDX11 KO cells after 72 h of siCtrl and siBRCA2 transfection. (Scale bar, 10 μm.) n = 2. Statistical analysis was performed using Student’s t test. Error bars show average ± SD. (B) Quantification and representative micrographs of micronuclei in U2OS Ctrl and DDX11 KO cells after 72 h of indicated siRNAs transfection, n = 2. Statistical analysis was performed using Student’s t test. Error bars show average ± SEM. (C) Cell viability assay of PEO1 (S) and C4-2 (R) cells transfected with siCtrl and siDDX11. Cells were treated with olaparib with the indicated drug concentrations for 5 to 6 d (n = 3). Cell viability was determined using crystal violet staining. (Top Right) Schematic representations of BRCA2 mutations and (Bottom Right) Western blot of DDX11 and BRCA2 variants. Error bars show average ± SEM. (D) Cell viability assay of Capan-1 (S) and Capan-1 (R) cells transfected with indicated siRNAs. Cells were treated with olaparib with the indicated drug concentrations for 5 to 6 d (n = 3). Cell viability was determined using crystal violet staining. (Top Right) Schematic representations of BRCA2 mutations and (Bottom Right) Western blot of DDX11 and BRCA2 variants. Error bars show average ± SEM.
Fig. 7. DDX11 facilitates resection and RPA loading to ssDNA substrates. (A) Quantification and representative micrographs of RPA32 foci in U2OS Ctrl and DDX11 KO cells recovering from an acute treatment with cisplatin (2.5 μM for 1 h) after 24 h. (Scale bar, 10 μm.) n = 2. Statistical analysis of foci was performed using Student’s t test. Error bars show average ± SD. (B) U2OS DivA cells were transfected with siCtrl and siDDX11 followed by the addition of 4OHT (300 nM) to induce AsiSI-mediated DSBs after 48 h of post-transfection. Resection assay at the two different regions (KDELR3 and ASXL1) were analyzed after 4 h of 4OHT by real-time PCR. Values were normalized against the amount of ssDNA detected in control cells prior to 4OHT treatment, n ≥ 3. Corresponding Western blot and immunofluorescence for AsiSI-induced DSBs is shown. (Scale bar, 10 μm.) Statistical analysis was performed using Student’s t test. Error bars show average ± SEM. (C) Model for the DDX11 proposed role in resolving secondary structures upon DSB end resection to facilitate RPA loading and subsequently RAD51 nucleofilament formation required for homologous recombination-mediated DSB repair (see text for details).
Mechanistically, we show that DDX11 facilitates ressection and formation of a ssDNA substrate suitable for RPA binding, later replaced by RAD51 (Fig. 7C), therefore explaining its nonoverlapping functions with BRCA1 and BRCA2. We propose that the DDX11 role in facilitating robust RPA and RAD51 focus formation is manifested by its ability to unwind certain DNA substrates, such as those containing G4 secondary structures on which DDX11 was shown to act (8, 16) and hairpins (Fig. 7C). Altogether, the results indicate that DDX11 targeting is useful in several cancers by increasing their sensitization to replication stress-induced chemotherapy. DDX11 loss greatly sensitizes both BRCA1- and BRCA2-deficient cancer cells to chemotherapeutic drugs, generating more DNA damage and genomic instability. Moreover, both BRCA1- and BRCA2-mutated tumors that acquired resistance via inactivation of 53BP1 and shieldin components or BRCA2 functional restoration are sensitized by DDX11 loss. We propose that DDX11 provides a mechanism of replication stress tolerance, which sustains survival of cancers, including BRCA1- and BRCA2-deficient cells, and can be exploited therapeutically through the development of specific inhibitors of DDX11 helicase activity.

Materials and Methods

Cell Lines and Establishment of Stable Cell Lines. HeLa (ATCCCL-2), U2OS (ATCC-HTB-96), PE01, PE01-C1-2, C-1, and C-1-2 C6 (gifts from the Taniguchi laboratory (33, 34)) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS), L-glutamine, and penicillin and streptomycin. The U2OS D5A (AID-Aldi_US-ER-U2OS) cell line from the Legube laboratory (35) was cultured in DMEM high glucose containing G418 (800 μg/mL). The UW121829 + BRCA1 (ATCCCL-2945) cell line was grown in complete 50% Roswell Park Memorial Institute 1640, and 50% mammary epithelial cell growth medium containing G418 (400 μg/mL) was used for the maintenance. All cell lines were tested for mycoplasma contamination and maintained at 37 °C with 5% CO2.

For the generation of stable DXX11 KO cell lines expressing different variants, U2OS DXX11 KO cells were transfected with plasmids as follows: pcDNA3.1(+), EV, DXX11 WT, DXX11 K50R, DXX11 R263Q, and DXX11 KAE variants. The transfections were performed using Lipofectamine 2000 (Thermo Fisher Scientific). The transfected cells were selected with G418 (1 mg/mL) and cultured in the presence of lower concentration of G418 (500 μg/mL). The expression level of DXX11 variants were analyzed by Western blot.

siRNA Transfections. For siRNA transfections, the cells were transfected with 20 to 30 nM of siDDX11 of Dharmacon ON-TARGETplus siRNA (L-011843-00-0002), ON-TARGETplus Non-Targeting Pool (D-001810-10-05), siBRCA1 (SASI_Hs_H02_00231217), siIC200f108 (SASI_Hs_00102807), siBRCA2A (SASI_Hs_00335253), siBRCA1A (L-003461-00-0005), and siBRCA2 (L-003462-00-0005) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol. The depletion was analyzed by immunoblot 48 h post-transfection.

Generation of DXX11 KO Cell Lines by CRISPR/Cas9. To generate HeLa and U2OS DXX11 KO cell lines, cells were transfected with SpCas9 expressing construct PX459 Addgene (#29288) using Lipofectamine 2000 followed by transfection of Alt-R control guide RNA and Alt-R synthetic paired guide RNAs (Integrated DNA Technologies) that target DXX11 exon 7 (GAGGTTG AAGAAAGAGCCCCCT) and exon 9 (GGGCGTCCAGGGTGGCAAGG) and then expanded for clonal populations in 96-well plate. The clonal populations were screened by genotyping and the KO cells were confirmed by Western blot and Sanger sequencing. For U2OS DXX11 KO cell lines, the DXX11 genomic loci were PCR amplified by high fidelity Q5 DNA polymerase (New England Biolabs), and the amplified PCR product was cloned in a Zero Blunt TOPO PCR Cloning Kit (Invitrogen). At least 10 to 15 colonies were sequenced to identify frameshifts and deletions. The guide RNAs were designed by using CRISPRscran (https://www.crisprscan.org) and the CRISPOR online tool (crispor.tefor.net).

Colony Formation and Cell Viability Assays. For colony formation assays, the cells were treated with cisplatin at the indicated concentrations for 1 h and washed thrice with 1× phosphate-buffered saline (PBS) before detachment of the cells with trypsin. Around 400 to 500 cells were seeded in 10 cm dishes and allowed to grow for 10 to 15 d. For PARPi and ATRi sensitivity, ~100 cells were seeded in 10 cm dishes and incubated overnight to allow adherence to the plates. The following day, the cells were treated with olaparib at indicated concentrations and grown for 10 to 15 d to form individual colonies. The cells were stained with 0.5% crystal violet containing 20% methanol for 30 min at room temperature, and plates were washed with deionized water, and colonies were counted manually. The plating efficiency and surviving fraction was determined by normalizing with untreated cells.

For cell viability assays, 500 to 1,500 cells/well were seeded in 96-well plates and then allowed to adhere to the plates. The drug treatments were chronically given with indicated concentrations of Pyridostatin, Telomestatin, mitomycin C and olaparib for 3 to 5 d. Cell viability was determined using the 0.5% crystal violet staining containing 20% methanol and normalization with the untreated cells. For siRNA-mediated cell viability assays, cells were incubated in the presence of drugs, and the viability was calculated after 3 to 5 d of incubation. The corresponding Western blots were performed after 48 h of siRNA transfection.

Western Blot Analysis. Cell extracts were prepared using radioimmunoprecipitation assay or lysis buffer (50 mM Tris, 250 mM NaCl, 1% Igepal, 0.5 mMEDTA, 10 mM Na2PO4, 10 mM NaF) supplemented with Protease Inhibitor Cocktail (Roche) and PhosSTOP, resolved by BioRAD sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and transferred to a nitrocellulose membrane followed by incubation with indicated primary and secondary antibodies. All Western blots were performed using at least two independent biological replicates.

Antibodies for Western Blots and Immunofluorescence. As primary antibodies, anti-DDX11 (Santa Cruz sc-217711) (1:1,000), anti-BRCA1 (Santa Cruz sc-6954) (1:1,000), anti-BRCA2 (ab123491) (1:100), anti-CH2 (Santa Cruz sc-17747) (1:100), anti-RAD51 (Abcam ab2056) (1:1,000), and DNA PKcs (Epitomics #15719–1) (1:1,000), anti-DNA PKcs (p20506) (Epitomics #3892–1) (1:1,000), anti-rH2AX (Millipore #05–636) (1:500), anti-SB3 (Novus Biologicals #NB100–304) (1:1,000), anti-MAD2B (R&D Biosciences 612266) (1:1,000), anti-rRAD51 (Santa Cruz sc-17747) (1:50), anti-rRPA (RPA32) (Thermo Fisher #MA1–26418), and anti–tubulin (Santa Cruz #8035) (1:5,000) were used. For secondary antibodies, anti-mouse-anti-HRP-linked (1:5,000 cell signaling technology), anti-rabbit-anti-HRP-linked (1:5,000 cell signaling technology), and Alexa Fluor 488 anti-mouse (immunofluorescence 1:400) Invitrogen, Alexa Fluor Cy3-conjugated anti-rabbit (immunofluorescence 1:400) Invitrogen were used.

Chemicals. The following chemicals were used: olaparib (Selleckchem #S1906), cisplatin (Sigma-Aldrich #479306), telomestatin (Chemexpress 265114–54–3), pyrrolidin (Merck #5ML0767), AZD7367 (Selleckchem #57683), VE-821 (Selleckchem #S8007), and tamoxifen (4OH)-tamoxifen (MedChemExpress #HY-13575A–1g).

Immunofluorescence. For 53BP1 and γ-H2AX foci, U2OS cells were grown in coverslips and treated with cisplatin (2.5 μM) for 1 h and then washed thrice with 1× PBS followed by fixation with 4% formaldehyde in 1× PBS for 15 to 20 min with samples taken at the indicated time points. The coverslips were washed thrice with 1× PBS followed by permeabilization with 0.3% Triton X-100 in 1× PBS for 5 min at room temperature and washed thrice with 1× PBS and then blocked with 10% horse serum after the washes. RAD51 and RPA32 immunofluorescence was performed as described in ref. 22. Coverslips were incubated with indicated primary antibodies (SB31 [1:1,000], RAD51 [1:30], and γ-H2AX [1:500]) for 2 h in room temperature and then washed thrice with 1× PBS before incubation with secondary antibodies (Alexa Fluor 488 and Cy3 [1:400]) for 1 h. After the incubation with secondary antibodies, the coverslips were washed with 1× PBS thrice and stained with DAPI for 20 min, and images were taken with a confocal microscope (Leica TCS SP2 AOBS inverted). The SB31, RAD51, and γ-H2AX foci were analyzed by Fiji and cell profiler software. For foci analysis, at least 100 nuclei were analyzed for each time point except for Fig. 7A in which at least 75 nuclei were analyzed.

For analysis of micronuclei/mitotic catastrophe, the cells were grown on coverslips. The cells were treated with cisplatin (2.5 μM) for 1 h and washed thrice with 1× PBS before incubation with secondary antibodies (Alexa Fluor 488 and Cy3 [1:400]) for 1 h. After the incubation with secondary antibodies, the coverslips were washed with 1× PBS thrice and stained with DAPI for 20 min, and images were taken with a confocal microscope (Leica TCS SP2 AOBS inverted). The SB31, RAD51, and γ-H2AX foci were analyzed by Fiji and cell profiler software. For foci analysis, at least 100 nuclei were analyzed for each time point except for Fig. 7A in which at least 75 nuclei were analyzed.
post siRNA transfection. The cells were fixed with a formaldehyde solution after 72 h of doxycycline addition. All samples were processed as described in ref. 37 and analyzed by fluorescence-activated cell sorting.

Resection Assay. U2OS DIVa (AID-AsiSI-ER-U2OS) cells were transfected with the indicated siRNA using Lipofectamine RNAiMAX. After 48 h of post-transfection, cells were treated with 4OHT (300 nM) for 4 h to induce AsiSI-dependent Resection Assay. The collected cells were lysed, and DNA was extracted using a DNAeasy kit (Qiagen). Briefly, in total, 500 to 1,000 ng of isolated genomic DNA was digested using a BanI restriction enzyme (New England Biolabs) at 37 °C overnight. The restriction enzyme cuts genomic DNA ∼200 bp from the DSB-KDELR3 and ∼740 bp for DSB-ASXL1. RNase treatment was given to digested and undigested genomic DNA samples and incubated at 65 °C for 20 min for the heat inactivation of enzymes. Genomic DNA samples were analyzed by real-time PCR using the following primers:

DSB-KDELR3, 200 FW: ACCATGAACTGTTCCGAAT,
DSB-KDELR3, 200 REV: GAGTCTCGAGAAGTCTTCC,
DSB-ASXL1, 740 FW: GTCCCTCTCCACCATTT,
DSB-ASXL1, 740 REV: AGCCACCTGTTAGAGTG,
DSB-KDELR3, 20kb FW: CACCTCCTGATACACATCG,
DSB-KDELR3, 20kb REV: TGTAATCAACTGTTGGAGGC.

siDNA percentage was calculated as described in ref. 38. The DNA amount was normalized for each sample using a control region at 20 kb away from the Asil3 cut at the KDELR3 locus.

Synthetic Lethality Chemical Drug Screens. For high content drug screen, the drug plates were prepared with various concentrations in 384-well plates. HeLa Ctrl and DDX17 KO cells were seeded on a 384-well plate using a Multidrop 384 dispenser. Titertek (Thermo LabSystems, Inc.) and incubated for 72 h in the presence of drugs. Cell viability was analyzed by using CellTiter-Glo, and readings were taken in 384-well StopPlate-384V (#6008598, PerkinElmer, Inc.). For Fig. 1D, the image was prepared using the BioRender online software.

Statistical Methods. Statistical analysis was performed using GraphPad prism software. Statistical differences for experiments are mentioned in figure legends. Prism software versions 8 and 9 were used to prepare graphs and analyze statistical significance.

Kaplan-Meyer Curves. We used the Kaplan–Meier Plotter web tool (https://kmplot.com/analysis/) to compare the overall survival of patients divided in two groups by DDX17 median expression in ovarian and lung cancer data.

Data Availability. Source data have been deposited in Mendeley (DOI: 10.17632/tz4zy2ybrz.1). All other study data are included in the article and/or supporting information.

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SMC5/6 acts jointly with Fanconi anemia factors to support DNA repair and genome stability

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Abstract

SMC5/6 function in genome integrity remains elusive. Here, we show that SMC5 dysfunction in avian DT40 B cells causes mitotic delay and hypersensitivity toward DNA intra- and inter-strand crosslinkers (ICLs), with smc5 mutants being epistatic to FANCC and FANCM mutations affecting the Fanconi anemia (FA) pathway. Mutations in the checkpoint clamp loader RAD17 and the DNA helicase DDX11, acting in an FA-like pathway, do not aggravate the damage sensitivity caused by SMC5 dysfunction in DT40 cells. SMC5/6 knockdown in HeLa cells causes MMC sensitivity, increases nuclear bridges, micronuclei, and mitotic catastrophes in a manner similar and non-additive to FANCD2 knockdown. In both DT40 and HeLa systems, SMC5/6 deficiency does not affect FANCD2 ubiquitylation and, unlike FANCD2 depletion, RAD51 focus formation. SMC5/6 components further physically interact with FANCD2-I in human cells. Altogether, our data suggest that SMC5/6 functions jointly with the FA pathway to support genome integrity and DNA repair and may be implicated in FA or FA-related human disorders.

Keywords DNA repair, Fanconi anemia, intra- and inter-strand crosslinks; mitotic instability; SMC5/6

Subject Categories Cell Cycle; DNA Replication, Recombination & Repair; Molecular Biology of Disease

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Introduction

Genomic integrity is safeguarded by multiple genome caretakers that are often implicated in chromosome metabolism reactions induced by various types of replication stress. The structural maintenance of chromosomes (SMC) complexes, including cohesin (SMC1/3), condensin (SMC2/4), and SMC5/6, are critical for chromosome transactions that ensure normal proliferation and genome integrity. Structurally, SMC complexes form molecular rings that can entrap genomic DNA [1]. The SMC5/6 complex ring is composed of two coiled-coil SMC heterodimers SMC5 and SMC6, which associate with a kleisin component, NSMCE4 [2]. In addition, SMC5/6 contains several peripheral subunits. The NSMCE1–NSMCE3 heterodimer has ubiquitin ligase activity and interacts with NSMCE4 and SMC6, while NSMCE2 has SUMO ligase activity and interacts with SMC5 [3]. The NSMCE1–NSMCE3–NSMCE4 sub-complex presents double-stranded (ds) DNA-binding activity, without preference for structured DNA [4]. SMC5/6 also interacts with SLF1 and SLF2, which are functional orthologs of budding and fission yeast Nse5 and Nse6, respectively [5]. The budding yeast Nse5/6 heterodimer is a component of the SMC5/6 complex (essential for proliferation in budding yeast, but not in fission yeast) and has single-stranded (ss) DNA-binding activity [6]. In human cells, SLF1 and SLF2 physically link RAD18 to the SMC5/6 complex, defining a pathway for SMC5/6 recruitment to sites of DNA damage [5].

In terms of molecular functions, SMC5/6 contributes to DNA repair [7], facilitates DNA topological transitions [8], and promotes timely resolution of DNA cruciform structures arising during homologous recombination repair [9,10]. In unperturbed conditions, budding yeast SMC5/6 is required for the organization and segregation of repeat elements [1,11,12] and promotes replication through difficult to replicate regions known as natural pausing sites [13]. Understanding the functions of SMC5/6 in genome integrity and DNA repair is important, because mutations in SMC5/6 have been linked to different human disease conditions that may derive from impaired DNA metabolism reactions. Specifically, mutations in SMC5/6 components increase overall breast cancer risk [14], promote brain metastasis development [15], and result in debilitating diseases associated with severe developmental defects. To date, two genetic disorders caused by mutations in SMC5/6 have been reported: the NSMCE2-associated syndrome featuring primordial dwarfism and deregulation of glucose metabolism [16], and the NSMCE3-associated disorder also known as LICS, characterized by...
increased chromosome breakage and defective T- and B-cell function [17]. At the cellular level, the two syndromes are characterized by increased replication stress, micronuclei formation, and defects in homologous recombination (HR). While the involvement of SMC5/6 in preventing replication-stress accumulation is consistent with the results obtained in other model systems and likely linked to its function in DNA repair, the molecular roles of SMC5/6 in DNA repair are incompletely understood.

Here, we set out to investigate the roles of SMC5/6 that assist genome integrity in vertebrate and mammalian cells, by studying the consequences of SMC5/6 depletion in avian DT40 cells and HeLa cells. Both knockout and conditional depletion of SMC5 in DT40 cells allowed cellular proliferation, as previously reported [18]. smc5 mutant cells showed sensitivity toward DNA intra- and inter-strand crosslinkers (ICLs). Notably, the repair defect of smc5 DT40 cells toward cisplatin was genetically epistatic with mutations in the Fanconi anemia (FA) components, FANCC and FANCM, and with mutations in the FA-like pathway defined by the DNA damage checkpoint clamp loader, RAD17, and the DDX11 helicase [19,20]. Moreover, smc5 mutants were additive to mutations in KU70, mediating non-homologous end-joining (NHEJ) repair of double-strand breaks (DSBs). SMC5/6 knockdown in human cells also caused ICL sensitivity, micronuclei, and aberrant mitoses, in a manner similar and non-additive with FANCD2 knockdown [21]. However, SMC5/6 dysfunction did not interfere with FANCD2 ubiquitylation, and unlike FANCD2 depletion, it did not affect RAD51 focus formation in unperturbed or replication-stress conditions. Combining co-immunoprecipitation and mass-spectrometry approaches, we detected physical interaction between several SMC5/6 components and FANCD2-I in human cells, both when SMC5/6 was overexpressed and with endogenous proteins. The results support the notion that vertebrate SMC5/6 functions jointly with the FA pathway in the repair of DNA lesions to prevent genome instability. We propose that mutations in SMC5/6 in humans may be implicated in FA or FA-like disorders.

Results

SMC5/6 function is required for normal proliferation in DT40 cells

Here, we applied the auxin-inducible degron (AID) system, which enables rapid degradation of target proteins by the proteasome [22,23] to establish conditional depletion of SMC5. We used the DT40 avian B-cell line that stably expresses TIR1, an essential component in the auxin degron system [24] in which we C-terminally tagged the endogenous SMC5 gene with the 3AID-6FLAG tag, using the Flip-In system for insertion of epitope tags [25] (Fig EV1A). As SMC5 is present on chromosome Z, with only one replacement we generated SMC5-3AID-6FLAG cells expressing TIR1 (hereafter referred to as smc5-aid) cells. The functionality of the AID tag inserted to the carboxyl-terminus of SMC5 was confirmed by Western blotting (Fig 1A). After auxin addition, the SMC5-3AID-6FLAG protein was strongly reduced within 24 h (Fig 1A). In regard to proliferation, smc5-aid cells behaved similarly with the wild-type (WT) control in the absence of auxin, but addition of auxin caused slower proliferation (Fig 1B).

To rule out that remaining low levels of SMC5-AID are sufficient for proliferation, albeit with lower speed, we established constitutive knockout of SMC5, employing a construct that causes complete disruption of the open-reading frame (Fig EV1B). SMC5 knockout cells (hereafter referred to as smc5) proliferated slower than WT cells and had a longer doubling time (Fig 1C), showing results similar to the ones observed upon SMC5-AID depletion.

We further investigated whether the observed slower proliferation phenotype of smc5 cells is complemented by expressing WT copies of chicken SMC5 (cSMC5) tagged with HA. To estimate the level of introduced cSMC5-HA vs. endogenous SMC5 present in WT, we also established an smc5-HA cell line using the Flip-In system and used both Western blotting against HA and quantification of mRNA levels (Fig EV1C). The results revealed that cSMC5-HA introduced for complementation was expressed 3–4 times higher than endogenous SMC5 and fully complemented the growth defect of smc5 cells (Fig EV1C). Next, we assessed the cell cycle distribution profile, and found a statistically significant increase in the G2/M population in smc5 mutants (about 25% in smc5 compared with 18% in WT), which was associated with a concomitant decrease in the S-phase population (Fig 1D). We considered the scenario in which smc5 mutants endure replication stress and as a result accumulate lesions that cause G2/M arrest and/or problems in completing mitosis. Supportive of this notion, addition of caffeine, which inhibits damage checkpoint kinases, reduced the population of smc5 cells arrested in G2/M (Fig EV1D). Thus, SMC5 is dispensable for viability in avian DT40 cells [18], but is required for physiological levels of fast proliferation.

**Figure 1.** SMC5 dysfunction causes slower proliferation.

A WB analysis of smc5-aid clones in the absence or presence of auxin using α-FLAG. Tubulin is used as loading control and WT cells are used as a negative control for the FLAG tag.

B Proliferation curve of WT and smc5-aid cells in the presence or absence of auxin. The data represent means ± SD of three independent experiments.

C Proliferation curves of WT and two independent smc5 clones grown at 39.5°C, with the estimated doubling time on the right. Data represent means ± SD of four experiments. Asterisks indicate P value ≤ 0.05, as derived from unpaired t-test type 3, two-sample unequal variance, carried out to check statistical significance between smc5 clones and WT. smc5 Clones 1 vs. WT, P = 0.016, smc5 Clones 2 vs. WT, P = 0.04

D Bidimensional FACS analysis shows significant accumulation in G2/M and decrease in the S phase in smc5 mutants. The data represent means ± SD of three independent experiments for smc5 clones 2 and 3, and 4 independent experiments for WT and smc5 clones 1. Asterisks indicate P value ≤ 0.05, detailed below, using paired t-test. S phase (smc5 clones 1, 2, 3 vs. WT: P = 0.0031, P = 0.0002, P = 0.004, respectively). G2/M (smc5 clones 1, 2, 3 vs. WT: P = 0.029, P = 0.0014, P = 0.02, respectively).

Source data are available online for this figure.
Figure 1.
Figure 2.
SMC5 acts jointly with the FA protein FANCC in the repair of ICLs

SMC5 function has been linked to HR repair in several model systems, including budding and fission yeast, proliferating germ cells of Caenorhabditis elegans and avian DT40 cells [9,10,18–29]. In chicken DT40 cells, smc5 mutants are sensitive to IR (ionizing radiation) and MMS (methyl methanesulfonate) [18]. In regard to IR sensitivity, smc5 cells are epistatic to rad54, defective in HR, but additive to ku70, defective in the non-homologous end-joining (NHEJ) repair of double-strand breaks (DSBs) [18]. However, more information on the roles of SMC5/6 in DNA repair in vertebrate cells is currently missing, especially in regard to lesions other than DSBs.

We investigated the sensitivity of DT40 smc5 mutants to several DNA damaging agents that cause replication-associated lesions, using Celltiter-Glo assay that measures the amount of ATP levels in DNA damaging agents that cause replication-associated lesions, using Celltiter-Glo assay that measures the amount of ATP levels in

...
Figure 3.
Figure 4.
analyzed whether these signaling pathways were functional in smc5 mutants. We found that both CHK1 phosphorylation, used as read-out for ATR activity, and FANCD2 ubiquitylation, used as readout of FA pathway functionality, proceeded unaffected in smc5 mutants (Fig 2E and F). Thus, SMC5 functions downstream of FANCD2 ubiquitylation in a manner non-redundant with FANCN, potentially to support the DNA repair function of the FA pathway.

**SMC5 acts jointly with FANCN and the FA-like pathway involving the RAD17 checkpoint clamp loader and the DDX11 DNA helicase**

FANCN is a component of the FA core presenting DNA translocase and branch-migrating activities and a degenerate C-terminal ERCC4 nuclease domain that binds to DNA structures in vitro [46]. FANCN has functions within the FA pathway related to the recruitment of the FA core complex to chromatin and in triggering checkpoint activation, as well as functions outside the FA pathway related to lesion bypass potential by fork reversal (reviewed in Ref. [47]).

We established double mutants between fancm and smc5 knockouts and found these cells to be proliferating similarly with the smc5 single mutants (Fig 3A). In regard to cisplatin sensitivity measured again by the CellTiter-Glo assay, smc5 mutants are more sensitive than fancm, while smc5 fancm mutants showed reduced cisplatin sensitivity compared with smc5 (Fig 3A), a situation recapitulating the phenotype observed with fanc (Fig 2D), but in a situation in which smc5 and fancm mutants proliferated equally well (although worse than WT). Moreover, long-term clonogenic assays revealed a similar epistatic relationship between fancm and smc5 mutations in regard to cisplatin sensitivity (Fig 3B). A backup pathway for the FA pathway involves the conserved DDX11 helicase and the checkpoint clamp loader RAD17, which act jointly to facilitate recombination-mediated repair of bulky lesions in vertebrate cells [19,20]. In human cells, RAD17 also affects FANC2 ubiquitylation [48]. To study the relationship between this FA-related pathway and SMC5 function, we established double mutants between smc5 (and smc5-aud) and rad17, ddx11 mutations. The double mutants proliferated similarly with smc5 single mutants, and both rad17 and ddx11 showed epistasis to smc5 mutants toward cisplatin (Fig 3C and D). ddx11 showed similar non-additive relationship with depletion of SMC5-AID in regard to proliferation and repair (Fig EV3A and B). Thus, SMC5 functions jointly with FANCN and FA-related pathway involving DDX11 and RAD17 in DNA repair.

**SMC5/6 acts jointly with FANC2 to mediate DNA repair and prevent genomic instability in human cells**

To validate and extend our previous observations made in the chicken DT40 cell system, we analyzed the consequence of SMC5/6 depletion in HeLa cells. siSMC5 depletion and siSMC6 depletion were very efficient by 48 h post-transfection and validated by using different siRNA sequences to avoid off-target consequences of the siRNA approach. SMC5 or SMC6 depletion strongly affected the stability of the other partner (Fig 4A). Notably, their depletion was associated with an increased level of FANC2, and conversely, FANC2 depletion was associated with increased SMC5/SMC6 levels (Fig 4A).

Cell survival in response to mitomycin C (MMC) exposure, measured using crystal violet staining, revealed that SMC6-depleted HeLa cells are slightly more sensitive than control cells but significantly less sensitive than FANC2- or FANCJ-depleted cells. The simultaneous depletion of SMC6 and FANCJ had minimal effect on the MMC sensitivity of FANCJ-depleted cells, whereas the downregulation of SMC6 in FANCJ-depleted cells increased their resistance to MMC (Fig 4B). These results were obtained using smart siRNA pools, but qualitatively similar results were obtained using individual different siRNAs for SMC6 and a single siRNA for FANC2 and FANCJ (Fig EV4). Thus, also in human cells, SMC6 acts in a DNA repair pathway non-redundant with the one mediated by the FA proteins FANCJ and FANCN.
Figure 5.

A

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<th>siFANCD2</th>
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<td>HU</td>
<td>MMC</td>
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</tbody>
</table>

**SMC6**

**FANCD2-Ub**

**FANCD2**

**Vinculin**

**kDa**

| 130   |

B

**sLacZ**

**siSMC6**

**siFANCD2**

**DNA (DAPI)**

**RAD51**

**NT**

**HU**

**MMC**

**Percentage of RAD51 foci positive cells**

- P < 0.01
- P < 0.05
Figure 6.
SMC6 depletion using different siRNAs did not affect FANCD2 monoubiquitylation in either untreated or replication-stress conditions involving hydroxyurea (HU), MMC, or aphidicolin (APH) treatment (Figs 4A and 5A, and EV5) nor FANCD2 foci assembling in S/G2 phases of the cell cycle (Fig EV5), supporting the notion that SMC5/6 acts independently or downstream of FANCD2 activation.

The FANCD2 monoubiquitylation is important for optimal RAD51 focus formation [49–51], and DDX11 deletion in DT40 that does not affect FANCD2 monoubiquitylation also reduces RAD51 focus formation [19]. We assessed the consequences of SMC6 depletion on the formation of RAD51 foci in MMC- or HU-treated HeLa cells. Differently from FANCD2 depletion, siSMC6 did not alter RAD51 foci per se and did not modify the frequency of RAD51 foci-positive cells observed in the absence of FANCD2 (Fig 5B). Thus, our data suggest that SMC5/6 acts independently or downstream of RAD51 focus assembly, and downstream of FANCD2 and DDX11 that facilitate efficient RAD51 focus assembly.

It was proposed that FANCD2 foci assembled during S phase at stalled/delayed replication forks persist into G2/M and up to the end of mitosis to rescue under-replicated or untangled replicated regions, with this action serving to avoid anaphase bridges and genomic instability in daughter cells [21]. Indeed, FANCD2 loss of function is associated with an increased frequency of mitotic catastrophes and nuclear bridges at anaphase-telephase as well as with increased frequency of post-mitotic cells presenting micronuclei [21]. Notably, SMC6-depleted cells revealed a similar level of mitotic abnormalities with FANCD2-depleted cells, and the frequency of these abnormalities remains unchanged in SMC6 and FANCD2 double-depleted cells (Fig 6A and B). The results thus suggest joint action between SMC6 and FANCD2 in preventing genomic instability in mitotic and post-mitotic cells.

### Discussion

Studies of the Smc5/6 complex in budding and fission yeast are consistent with the involvement of this complex in dynamic DNA processes, such as stabilization of stalled replication forks and regulation of recombination intermediate resolution [9–11,13]. The essential functions of the Smc5/6 complex, at least in budding yeast, primarily manifest after the bulk of replication is complete, and involve processes mediating replication termination and DNA synthesis through difficult to replicate regions [11,13], such as ribosomal DNA, rDNA [12,53]. The postreplicative or late replication nature of the essential processes requiring Smc5/6 suggests that this complex may be especially important during late replication, in a subset of DNA metabolism reactions with repercussions on the mitotic chromosome structure and its compaction [54]. In budding yeast, those processes may be particularly relevant to DNA condensation and segregation [12], whereas in vertebrates they may encompass several classes of fragile sites or structures that remain among endogenous proteins. As we experienced low IP efficiency with commercial SMC5 antibodies, we tagged endogenous SMC5 alleles C-terminally with the mAID-EGFP tag in human TK6 cells using CRISPR-Cas9, using the strategy reported in Ref. [52]. Using SMC5-mAID-EGFP TK6 cells, we found that SMC5-EGFP pull-down using CRISPR-Cas9, using the strategy reported in Ref. [52]. Using SMC5-mAID-EGFP TK6 cells, we found that SMC5-EGFP pull-down led to co-immunoprecipitation of FANCD2, and vice versa, independently of induced DNA damage (Fig 7B and C). These results suggest that SMC5/6 physically interacts with FANCD2-I in vivo.

### SMCS/6 physically interacts with FANCD2-I in human cells

To examine potentially informative physical interactions, we transiently expressed FLAG-tagged SMC5, SMC6, and NSMCE2 in HEK293 cells and immunoprecipitated the complexes with an anti-FLAG antibody. Mass spectrometry identified the other SMCS/6 components in the immunoprecipitate of SMC5, along with a few other proteins of interest, among which FANC1 (Table 1).

**Table 1.** FLAG-SMCS IP-MS.

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*The numbers of peptides are averaged over two technical replicates.

**Figure 6.** SMC6 acts jointly with FANCD2 to prevent micronuclei and mitotic abnormalities.

A Left panel: representative images of anaphase HeLa cells with micronuclei (white arrows). Forty-eight hours after transfection with indicated siRNA, cells were incubated 18 h with 2 μg/ml cytochalasin B to block cytokinesis. The scale bar represents 20 μm. Right panel: histograms presenting the percentage of binucleate cells with micronuclei. Data represent the mean ± SEM of three independent experiments. t-test, unpaired. siSMC6 vs. siLacZ, P = 0.0085; siFANCD2 vs. siLacZ, P = 0.0057; and siSMC6 + siFANCD2 vs. siLacZ, P = 0.0019.

B Top: examples of HeLa mitotic catastrophes and anaphase bridges as observed 48 h after siRNA transfection. The scale bar represents 20 μm. Bottom: histograms presenting the percentage of cells with mitotic abnormalities. Data represent the mean ± SEM of three independent experiments. t-test, unpaired. siSMC6 vs. siLacZ: aberrant mitosis P = 0.0025, nuclear bridges P = 0.0098, mitotic catastrophes P = 0.0145; siFANCD2 vs. siLacZ: aberrant mitosis P = 0.0005; nuclear bridges P = 0.0002, mitotic catastrophes P = 0.0011; and siSMC6 + siFANCD2 vs. siLacZ: aberrant mitosis P = 0.0012, nuclear bridges P = 0.0116, mitotic catastrophes P = 0.0033.

Source data are available online for this figure.
Figure 7.
to be identified. Nevertheless, the partners of SMCS/6 in these repair processes remain poorly understood, especially in vertebrate cells.

In this study, we undertook a dual approach to better understand the functions of vertebrate/mammalian SMCS/6 in DNA repair and genome stability. One approach aimed at identifying new interacting partners of SMCS/6 in mammalian cells, while the other addressed the molecular functions of SMCS/6 in DNA repair and genome integrity by examining several mutant combinations in DT40 and upon knockdown in HeLa cells. Remarkably, both our approaches intersected on the FA pathway and particularly FANCD2-I, already reported to play roles in facilitating replication through common fragile sites (CFSS) [21,55–57] and for preventing fragile site expression in the presence or absence of replication stress [45,58–60]. Here, we find that SMCS/6 components interact physically with FANCD2-I and function jointly with FA proteins (FANCC, FANCN, FANCJ, FANCD2) and FA-related pathways (RAD17, DDX11) in the repair of ICLs created by cisplatin and mitomycin C. Considering that SMCS/6 depletion does not impair FANCD2 ubiquitylation and either FANCD2 or RAD51 focus formation, these results indicate roles of SMCS/6 in the late steps of HR, perhaps by resolving emerging recombination intermediates (Fig 7D). This could explain the effects of SMCS/6 depletion on anaphase bridge and micronuclei formation, which resemble in extent and are non-additive with the ones of FANCD2 depletion. FANCD2 counteracts NHEJ, and inhibition of NHEJ in FA rescues survival and genetic instability in FA [33,34]. Our new results suggest that this rescue happens because cells become able to funnel lesions in the HR pathway dependent on DDX11 and SMCS/6 (Fig 7D). Because ICL repair resembles replication termination [61] and termination regions are predisposed to fragility [62,63] and protected by budding yeast SMC5/6 [13], we propose joint roles of SMCS/6 and FANCD2-I at these regions and possibly at other subsets of CFSs.

Our findings can be accommodated by a model envisaging two functional interaction points between the FA pathway and SMCS/6: one in which SMCS/6 function/recruitment to the DNA lesion or terminal sites of replication is facilitated by FA components such as FANCN, and a subsequent one in which SMCS/6 facilitates the repair function of the FA pathway (Fig 7D). The role of SMCS/6 in facilitating DNA repair may be manifested via recruitment and/or Sumoylation of BLM, already reported to act jointly with FANCD2 and SMCS/6/NSMCE2 in certain conditions of replication stress [21,64]. Alternatively, SMCS/6 may downregulate the fork transversal function of FANCN and/or activate the degradation/mobilization of other replication, repair, or structural factors to facilitate lesion bypass and subsequently replication completion. This latter envisaged function may involve the ubiquitin and SUMO ligase activity of the SMCS/6 complex and potentially facilitate replication disassembly during replication termination [65] or cohesin removal to facilitate repair completion and normal chromosome structure in prophase [66]. We thus envisage that there remain undiscovered genetic disorders caused by mutations in genes of the SMCS/6 complex that lead to FA or FA-like disorders and in which the physiological role SMCS/6 in mediating DNA repair and replication termination becomes more apparent.

Materials and Methods

Cell lines and genotoxic treatments

All DT40 cell lines used in the study were derived from the DT40 WT clone 18 [67] and reported in Table 2.

HeLa cells were purchased from ATCC and routinely maintained in 75-cm² flasks in DMEM (Gibco®) + 13% fetal calf serum (Gibco®) + 1% penicillin-streptomycin (Gibco®) with reseeding every 4 days after trypsinization.

TK6 cells expressing TIR1 are a gift from Shunichi Takeda at Kyoto University. TK6 cells were cultured in RPMI 1640 (Cat. No. BE12-167F, Lonza), horse serum 5% (Cat. No. X0550, Microtech or Euroclone, Cat. No. LOBE17605F), sodium pyruvate 1.8 mM (Cat. No. M0503, Sigma-Aldrich), cisplatin (CDDP, Cat. No. P4394, Sigma-Aldrich), hydroxyurea (HU, Cat. No. A0781, Sigma-Aldrich), aphidicolin (APH, Cat. No. A0781, Sigma-Aldrich), or formaldehyde (Cat. No. F8775, Sigma-Aldrich) at the indicated concentrations and time periods for chronic and acute treatments.

DNA restriction enzymes and DNA ligase

All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Frankfurt, Germany) and used according to the manufacturer’s specification. GeneArt® Seamless Cloning and Assembly enzyme mix (Cat. No. A14606, Thermo Fisher Scientific) and GeneArt® Seamless Plus Cloning and Assembly enzyme mix (Cat. No. A14603, Thermo Fisher Scientific) were used to clone the homologous recombination arms for SMCS-C-terminus tagging in DT40 cells.

Plasmids

The vectors used for DT40 cloning were previously reported [68,69]. Vectors used for SMCS tagging with HA and AID were
SMC5-3xMID-6xFLAG Flip-In was generated from genomic PCR products combined with pXmxMID-6xFLAG containing histidinol D selection marker cassette. Genomic DNA sequences were amplified using primers 5'-ATAAAAGTCCAGGAGAGCTTAAATTTGTTGGA C-3' (containing Sall restriction enzyme site) and 5'-ACATTGGTACA GCTTGTCACTTCCATTTCC-3' (containing NotI restriction enzyme site). Amplified PCR product was purified by gel extraction and digested with Sall high-fidelity and NotI high-fidelity restriction enzymes, then purified again, and ligated in pXmxMyc vector [72]. Then, they were cloned with the same strategy in 3xmMID-6xFLAG vector [24]. The Flip-In vector was then linearized at one restriction enzyme site in the middle of the homology region and transfected to DT40 cells as previously described [25].

SMC5-HA Flip-In was generated from genomic PCR products combined with pXmxMID-6xFLAG containing Ectop selector marker cassette. Genomic DNA sequences were amplified using primers 5'-ATAAAAGTCCAGGAGAGCTTAAATTTGTTGGA C-3' (containing Sall restriction enzyme site) and 5'-AGGCGGGCTCAAGCTTAACT GGAGTCTATGGATGCTTCCACG-3' (containing sequence coding HA tag and AscI restriction enzyme site). Amplified PCR product was digested with Sall and AscI restriction enzymes and purified by gel extraction, and then ligated into pXmxMID-6xFLAG vector. The Flip-In vector was then linearized by SnaBl restriction enzyme before transfection.

SMC5 knockout construct was generated by modifying the SMC5 KO-Neo vector published in Ref. [18], which was digested with BamHI in order to cut exon 1 from the 5' arm, purified by gel extraction, and ligated with loxP (histidinol D) selection marker cassette. Then, the vector was digested with PvuII and BamHI restriction enzymes, purified by gel extraction, and ligated with loxP (puromycin) selection marker cassette. SMC5-KO-Puro vector was then linearized with NotI before being transfected to DT40 cells. rad17 and ddx11 mutations were established as previously reported [19].

To construct the targeting vectors for introducing mEID-EF Tag to the C-terminus of the SMC5 gene in TK6 cells, the 5' arm was amplified by using primers 5'-AGGCGGGCTCAAGCTTAACT GGAGTCTATGGATGCTTCCACG-3' and 5'-TGGCGGCCGCTCAC CGAGTCCAAGGTTGATGCTTAACTTCC-3' (3' arm was amplified by using primers 5'-CGAAATTATAGCTCCCTCGTGC AACTATAACGCTTCCTCC-3' and 5'-CGAAACAAAAAGCTCGG GAACCTCTTCGTTCAAGACATGCTTGG-3'. (Primers contain homology sequence to the backbone vectors.)

The 5' and 3' arms were assembled with pBS-mAID-GFP-loxP- Neo (neomycin-resistant cassette) or Hyg (bupyrromycin-resistant cassette) [52] digested with EcoRV and Smal using GeneArt ™ Seamless PLUS Cloning and Assembly Kit (Cat# 14609, Thermo Fisher Scientific) according to the manufacturer’s protocol. To construct the CRISPR/Cas9 vector for targeting SMC5, annealed primers 5' CA CCGTATGCTCATGCTTCAATAAAT-3' and 5'-AATCTTTTATCAGT TGAGCCCATC-3' were inserted into the BbsI site of pX5458 vector (Cat# 48138, Addgene). The two resulting targeting vectors containing Neo and Hyg antibiotic markers and the pX5458-pRNA vector were transfected into wild-type expressing the TIR1 gene.

TK6 cells expressing TIR1 were electroporated using Neon ™ Transfection System MRP5000 (voltage/width/number of pulses: 1,350 V/10 ms/3), with medium containing no PBS. Cells were selected 24 h after electroporation, using the drugs corresponding to

Table 2. DT40 cell lines used in the study.

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</tbody>
</table>

Modified in the laboratory starting from a backbone received from the Hirota Lab [25]. The px458 CRISPR/Cas9 vector reported in Ref. [70] was used in this study to establish fancm and fancm knockouts in WT and smc5 backgrounds. Guide DNA targeting for fancm and fancm was purchased from Sigma-Aldrich and cloned in the px458 vector (https://www.addgene.org/48138/), generating vectors FR18# (for fancm) and FR20# (for fancm), respectively. FR18# was transiently co-transfected with vector #122 [71] in smc5 and WT, respectively, in order to induce double-strand breaks to enhance HR and the insertion of the resistance marker contained in the vector in order to KO fancm. FR20# was transiently co-transfected with vectors #12-31 and #12-29 in smc5 or WT in order to induce double-strand breaks to enhance HR and the insertion of the resistance marker contained in vector in order to KO fancm.
the resistance marker(s) used, and then, the cells were plated in 96 wells to get single-cell clonal population. Cells were grown in medium until a single colony/well was visible, then picked up with a filter tip, and moved to a 24-well dish. Then, genomic DNA was extracted, the genotype was established by PCR, and ultimately confirmed by WB.

Antibodies

As primary antibodies for DT40 cell extracts, we used the following: anti-α-tubulin, mouse monoclonal antibody, clone B-5-1-2 (Cat. No. T5168, Sigma-Aldrich); goat anti-Chk1 (G-4), mouse monoclonal antibody (Cat. No. sc-8408, Santa Cruz Biotechnology); 1:1,000 for WB; anti-CHK1-P S345, rabbit polyclonal antibody (Cat. No. 13333S, #2341, Cell Signaling); 1:1,000 for WB; anti-FANC2D, rabbit polyclonal antibody; the Takata Laboratory, Kyoto medical University [20], 1:4,000 in BSA 5% TBS-T 0.1% for WB; anti-Flag M2, mouse monoclonal Ab (Cat. No. F1365 Sigma-Aldrich); 1:3,000 for WB; anti-Histone H2B, rabbit polyclonal (Cat. No. ab1790, Abcam); 1:3,000 for WB; anti-HA, rat monoclonal (Cat. No. 3F10, Sigma-Aldrich), 1:5,000 for WB; and donkey anti-mouse FITC (Cat. No. 11-095-150, Jackson ImmunoResearch), 1:50 for FACS. For experiments shown in Fig EV4, siSMC-b and siSMC-a with sequences indicated above were tested together with siRNA for FANC2D and FANCJ (indicated above) in sensitivity assays shown in Fig EV4.

Growth curve

For proliferation curves, cells were plated with a starting concentration of 10^5 cells/ml in triplicate. They were counted at the indicated time points with the Burker chamber. Before counting, they were stained with erythrosine (1 volume of medium containing cells and 1 volume of erythrosine) to mark viable cells. Raw data were plotted in Excel to derive growth curves and to calculate the doubling time of individual cell lines.

Auxin treatment

When indicated, auxin was added to the medium to a final concentration of 500 µM. Cells were treated with auxin for the indicated time, dependent on the assay. In order to check the depletion or the recovery of the expression of the target protein, 1 × 10^6 cells were pelleted, then washed 1 × with PBS 1 ×, and lysed with sample buffer 1 ×. Since auxin is reported to be degraded in the media, during proliferation experiments, auxin concentration was maintained by diluting the culture with fresh medium containing auxin. In Cell-titer-Glo ATP sensitivity assays, cells were supplemented with auxin after 24 h of treatment.
Quantitative PCR for mRNA

Total RNA was isolated from DT40 cells by using TRizol RNA Isolation Reagents (Invitrogen) according to the manufacturer’s protocol. cDNA library was prepared from total RNA by using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol. Quantitative PCR was performed using GoTaq qPCR Master Mix (Promega) and LightCycler® 96 Instrument (Roche) according to the manufacturer’s protocols. ACTB was used as house-keeping gene. Used primers are described below.

ACTB fw: 5’-CGTGCCTGTTCACCTATCGTG-3’

ACTB rv: 5’-TACCTCTTTTGCTCTGGGCTTCATC-3’

SMC5 fw: 5’-CCAGGGCTGTTGAAAGGCTACTG-3’

SMC5 rv: 5’-CGTCTCCGTGATTGATGCCCAGTGAC-3’

Drug sensitivity assays (Celltiter-Glo, colony survival, and crystal violet assays)

Sensitivity assays in DT40 were carried out treating 2 × 10⁶ cells/ml with the indicated drug concentration for 48 h. To assess cellular sensitivity, we measured the amount of ATP levels using Celltiter-Glo® assay (Cat. No. G7571, Promega). Luminescence levels were detected using Victor3 TM (PerkinElmer). Control cells were treated with the vehicle diluted at the same concentration of the drug used in the assay. We have carried out each biological experiment in technical triplicates, and in certain occasions, eliminated outliers. We defined as outlier the measurement having a ratio bigger than 25% of each of the other two measurements and with their averages, dividing the smaller data with the bigger data. The other two data should not have a ratio bigger than 25% among themselves. Each experiment was conducted in at least three independent biological experiments.

To determine cisplatin sensitivity in DT40 by colony survival assay, an appropriate number of DT40 cells were inoculated in a medium supplemented with 1.5% (w/v) methylcellulose, 15% fetal bovine serum, and 1.5% chicken serum. Indicated concentration of cisplatin was added and mixed for 6 h before inoculating cells. Colonies were counted after 14 days, and the percent survival was determined relative to the number of colonies of untreated cells. Two independent biological experiments and two independent clones for smc5 fancm were used in the analysis shown in Fig 3B.

For cell viability assays in HeLa (Figs 4B and EV4), 2–3 × 10⁴ cells/well were seeded in 48-well plates and allowed to adhere for 8–10 h. Chronic drug treatment was given to cells with indicated concentrations for 5 days. The cell viability was measured by staining the cells using 0.5% crystal violet containing 20% methanol, and the graph was plotted by normalizing with untreated cells. Three independent biological experiments were performed.

SDS–PAGE and Western blot

DT40 cells were harvested by trypsin treatment and lysed or fractionated according to the experimental aim. Protein quantification was obtained using the Bradford reagent assay (Cat N., Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer’s instructions. Samples were boiled in sample buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 1.6% SDS, 0.1 M DTT, 0.1% bromophenol blue). Whole-cell lysates (WCLs) were separated on 4–12% Blot™ Bis-Tris Plus (NW04125BOX, Thermo Fisher Scientific) or Criterion TGX Stain-Free (#5678094, Bio-Rad). Resolved samples were transferred to Hybond-ECL nitrocellulose membrane (Amer sham Bioscience, Glattbrugg, Switzerland), 1 h at 100 V or overnight at 30 V (4°C). Then, the membrane was saturated with 5% non-fat milk Tris-buffered saline (TBS 10 mM Tris-HCl PH 7.4, 100 mM NaCl) supplemented with 0.1% Tween-20 (TBS-T). The membranes were incubated with primary antibodies for 1 h at room temperature or overnight at 4°C. Then, the membranes were washed as mentioned above and incubated with the horseradish peroxidase (HRP)- conjugated secondary antibody for 1 h at room temperature. The luminescent signal was detected by PICO or FEMTO reagent (Cat No. #308080, #34095, Thermo Fisher Scientific) on Amersham Hyperfilm™ ECL films (Cat No. 28906836 GE Healthcare UK, Little Chalfont, UK) or Chemidoc XR5+ System (Cat No. 1708265; Bio-Rad Laboratories, Hercules, CA, USA). Western blot bands were analyzed by ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2009) for film acquisitions.

FACS analysis

Bidimensional FACS analysis was carried out pulsing the cells with BrdU, final concentration 20 μM for 15 min at 37°C in 10 μM stock, Cat. No. B9285, Sigma-Aldrich. After pelleting 1 × 10⁶ cells, cells were resuspended in 50 μl ice-cold PBS, and then 1 ml of 70% ethanol (ice cold) was added. Cells were washed once in 1 ml PBS 1% BSA (4°C in all the steps if not differently indicated), then resuspended in 1 ml denaturing solution (2N HCl), and incubated at room temperature for 25 min.

Cells were washed twice with 1 ml PBS 1% BSA. Then, cells were stained with primary mouse anti-BrdU antibody (B44, Cat. No. 5295722, BD), diluted 1:5 in PBS 1% BSA, and incubated 1 h at room temperature, light-protected. They were washed once in 1 ml PBS 1% BSA, then the pellet was resuspended in 100 μl anti-mouse secondary goat anti-FITC antibody diluted 1:50 in PBS 1% BSA, and incubated for 1 h at room temperature, light-protected (Cat. No. F0257, Sigma-Aldrich). Cells were then washed once in 1 ml PBS 1% BSA, then resuspended in 1 ml propidium iodine F.C. F. 2.5 g/ml (50 μg/ml, Cat. No. P4170, Sigma-Aldrich) containing RNase I F.C. 250 μg/ml, and incubated overnight at 4°C or 30 min at 37°C. Data were acquired using FACSCalibur (BD, Biosciences). Data analysis was carried out using Cell Quest.

Note: If not differently indicated, all the solutions were ice-cold. Cells were spun down at 300 × g at 4°C, for 5 min.

Immunofluorescence

Cells were seeded at 2 × 10⁵ cells/well in a 6-well plate on glass slides. After treatment, if any, cells were washed with PBS and then incubated in pre-extraction buffer CSKU100 (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 0.2% Triton X-100, PIPES pH 6.8 10 mM, anti-protease) for 5 min at room temperature and then fixed for 10 min with PBS + 4% formaldehyde, and finally permeabilized with PBS + 0.1% Triton X-100 for 10 min at RT. The cells were incubated 1 h with PBS + 5% BSA and then 1 h at 37°C with the primary antibodies, washed in PBS, and incubated again for 1 h at 37°C in the dark with the secondary
antibodies coupled to Alexa 488 fluorophore (green) at 1/1,000. After a final wash with PBS, the slides were mounted with the DAPI-DAKO solution. The labeling was revealed using an epifluorescence microscope (Zeiss Axio Observer Z1). The following antibodies and conditions were used: rabbit anti-FANCD2 (ab180928 Abcam, 1/1,000); rabbit anti-RAD51 (PC.130 Calbiochem, 1/500); and mouse anti-α-tubulin (TS168 Sigma 1/2,000). A minimum of 200 cells for each condition was calculated to percent the age of RAD51-positive cells.

Detection and scoring of micronuclei were performed using an adapted cytokinesis-block assay. Cells were incubated with cytochalasin-B (3 μg/ml; Sigma) for 18 h before fixation. Micronuclei were scored in 40–80 bi-nucleated cells for each condition.

Mitotic catastrophes, anaphase bridges, and post-mitotic cells with nucleocytoplasmic bridges were scored on cells prepared for immunofluorescence analysis looking at nuclei stained with DAPI. A minimum of 10 fields (100–300 cells) were recorded for each condition to score the percentage of abnormal mitotic figures in relation to the totality of the mitoses in the microscopic fields.

Mass spectrometry and co-immunoprecipitation

For immunoprecipitation, HEK293 suspension cells were transiently transfected with expression plasmids of FLAG-tagged SMCS, SMCG, and NSMC2 using polyethyleneimine. Specifically, SMCS, SMCG, and NSMC2 genes from hORFmone (V8.1) were transferred to mammalian expression destination plasmid pDEST26-FLAG with LR reaction (Invitrogen). For transfection, HEK293 suspension cells were cultured in SMM 293-TI medium (Sino Biological Inc.) supplemented with 1% Gibco FBS and 1% P/S in an 37°C incubator with shaking at 140 r.p.m.

Cells were directly lysed with NTEN buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 0.5% NP40, 10 mM NaF, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin] 2 days after transfection. The lysates were ultra-centrifuged at 440,000 × g for 15 min, and then, the supernatant was incubated with anti-Flag M2-conjugated beads (Sigma-Aldrich) for 3–4 h at 4°C. The beads were washed twice with NTEN buffer (20 mM Tris–HCl pH 7.5), 150 mM NaCl, MgCl2, 5 mM, 10% glycerol, 0.1% NP40, 1 mM DTG, and 1 mM PMSF and then incubated with IP buffer containing 400 μg/ml 3XFlag peptide for 1–2 h. Subsequently, the eluted complexes were analyzed by SDS–PAGE and mass spectrometry.

For immunoprecipitating SMCS-mAID-EGFP, 5 × 10⁶ of TK6 cells were harvested and washed by cold PBS twice. Cells were then lysed with 5 ml of CSK buffer [0.3% Triton X-100, 100 mM NaCl, 3 mM MgCl2, 300 mM sucrose, 1 mM EDTA, 10 mM PIPES, 1 mM PMSF, 1 x complete] for 10 min on ice. Lysates were subsequently sonicated by Bandelin SONOPULS (Sigma) [20%, 10 s, five cycles]. After sonication, lysates were centrifuged at 20,000 × g for 5 min at 4°C. Supernatants were then incubated with 3 μg of magnetic beads protein A (Invitrogen, 10001D) which were pre-incubated with 5 μg of anti-GFP OriGene, TP401) antibody. After 2 h of incubation with rotation at 4°C, beads were washed by CSK buffer once and incubated with 1 μl of CSK buffer containing benzonase (Novagen, 70746, final: 50 U/ml) for 20 min at 37°C. Beads were then washed by CSK buffer twice and boiled with sample buffer (10% glycerol, 60 mM Tris–HCl pH 6.8, 2% SDS, 0.025% bromphenol blue) for 10 min to elute proteins.

To immunoprecipitate FANCID2, 1 × 10⁶ TK6 cells expressing SMCS-mAID-EGFP were lysed with 1 ml of CSK buffer. After centrifugation at 20,000 × g for 5 min at 4°C, supernatants were incubated with 3 μg of magnetic beads anti-mouse IgG (Invitrogen, 11301) which were pre-incubated with either 5 μg of anti-FANCID2 antibody (sc20022) or mouse IgG.

Data are available upon request or uploaded as Source Data.

Expanded View for this article is available online.

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Author contributions

FiR executed experiments shown in Figs 1–3 (except for Fig 3B) and EV1–EV3 and established cell lines shown in Table 2 with contributions from RK and TA. AH-L, together with PD and FiR, designed and executed experiments shown in Figs 4A, 5, 6 and EVS. RK conducted experiments shown in Figs 3B, EVLC and 7C, NK) the experiments shown in Figs 4B and EV4. XX and DX provided the results shown in Table 1 and Fig 7A. TA and MT provided useful reagents and advice. B. Szakal (acknowledged), RK, FiR, and DB made the figures starting from data provided by co-authors. DB designed the study, supervised the work, and wrote the paper. All authors commented and provided feedback on figures and article content.

Conflict of interest

The authors declare that they have no conflict of interest.

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