Crosstalk between Translesion Synthesis, Fanconi Anemia Network, and Homologous Recombination Repair Pathways in Interstrand DNA Crosslink Repair and Development of Chemoresistance

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Abstract

Bifunctional alkylating and platinum based drugs are chemotherapeutic agents used to treat cancer. These agents induce DNA adducts via formation of intrastrand or interstrand (ICL) DNA crosslinks, and DNA lesions of the ICL type are particularly toxic as they block DNA replication and/or DNA transcription. However, the therapeutic efficacies of these drugs are frequently limited due to the cancer cell’s enhanced ability to repair and tolerate these toxic DNA lesions. This ability to tolerate and survive the DNA damage is accomplished by a set of specialized low fidelity DNA polymerases called translesion synthesis (TLS) polymerases since high fidelity DNA polymerases are unable to replicate the damaged DNA template. TLS is a crucial initial step in ICL repair as it synthesizes DNA across the lesion thus preparing the damaged DNA template for repair by the homologous recombination (HR) pathway and Fanconi anemia (FA) network, processes critical for ICL repair. Here we review the molecular features and functional roles of TLS polymerases, discuss the collaborative interactions and cross-regulation of the TLS DNA damage tolerance pathway, the FA network and the BRCA-dependent HRR pathway, and the impact of TLS hyperactivation on development of chemoresistance. Finally, since TLS hyperactivation results from overexpression of Rad6/Rad18 ubiquitinating enzymes (fundamental components of the TLS pathway), increased PCNA ubiquitination, and/or increased recruitment of TLS polymerases, the potential benefits of selectively targeting critical components of the TLS pathway...
pathway for enhancing anti-cancer therapeutic efficacy and curtailing chemotherapy-induced mutagenesis are also discussed.

**Keywords**
Interstrand DNA crosslinks; ubiquitination; Rad6; PCNA

**1. Introduction**

DNA repair mechanisms play a critical role in maintenance of genomic integrity as damage caused by spontaneous mutations, radiation or chemotherapeutic drugs if not accurately repaired can lead to genomic instability. Depending upon the type and location of the lesion, processes handling DNA damage can be classified into four broad classes: nucleotide excision repair (NER), homologous recombination repair (HRR), nonhomologous end joining (NHEJ), and translesion synthesis (TLS) or postreplication repair (PRR). Whereas the former three pathways represent true repair mechanisms, the TLS or PRR pathway enables DNA repair by allowing lesions or structural aberrations blocking replicative DNA polymerases to be tolerated. The repair pathways are highly conserved and are recruited to repair modified nucleotides, DNA strand breaks, or both. The specificity and fidelity of these processes vary but may be mutually compensatory in certain contexts [1].

DNA damaging agents used in cancer therapy induce a variety of toxic DNA lesions. Among these, agents such as bifunctional alkylating drugs, platinum compounds, and psoralen introduce both intrastrand crosslinks (the crosslinking of two bases on the same DNA strand) and interstrand crosslinks or ICLs (the crosslinking of two bases on opposite strands of DNA). Mitomycin C mainly induces ICLs [2], whereas psoralen induces up to 40% ICLs [3]. In contrast, 90% of the crosslinks induced by cisplatin are intrastrand crosslinks and 5-8% are ICLs [3-6]. Doxorubicin, another commonly used chemotherapeutic drug, is a DNA intercalator which prevents topoisomerase from binding DNA and blocks DNA religation at low concentration [7]. In addition, doxorubicin forms covalent adducts that exhibit characteristics of ICLs [8]. Most ICLs produce major distortions to DNA structure, which prevent DNA strand separation. Thus ICLs are particularly deleterious as they block DNA replication and/or DNA transcription, and if unrepaired they can lead to single strand breaks (SSBs), double strand breaks (DSBs), and chromosomal rearrangements [9]. Therefore processes that allow cancer cells to survive in the face of these damaging lesions, such as upregulation of DNA damage response (DDR) and DNA damage tolerance (DDT) pathways are advantageous to cancer cells [10]. It is no surprise then that many cancer cells exhibiting chemoresistance demonstrate upregulated DDR and DDT pathways. TLS, a component of the DDT pathway, constitutes a critical initial step in ICL repair as it prepares the leading template strand for repair by HR pathway. The HRR pathway is essential for stabilization and restart of stalled DNA replication forks. Stalled replication forks activate the Fanconi anemia (FA) pathway which cooperates in a common biochemical FA/BRCA HRR pathway to detect and repair stalled replication forks [11,12]. In this review, we will discuss the TLS pathway in relation to the FA network and HRR pathway,
the contribution of their crosstalk in ICL repair and acquisition of chemoresistance, and the potential value of targeting the TLS pathway to restore chemosensitivity.

2. Translesion synthesis pathway

Most DNA damage is successfully removed or repaired by error-free DNA repair pathways. However, if the DNA lesions are not repaired before replication because of inefficient repair mechanisms or checkpoint controls, the damaged DNA cannot be utilized as a template for replication by high fidelity DNA polymerases as it results in replication fork stalling and replication gaps. To enable completion of DNA replication and cell survival, cells utilize error-free or error-prone lesion bypass mechanisms to synthesize DNA across the lesion. Error-free lesion bypass involves template switch from damaged to undamaged DNA strand for synthesis past the lesion, whereas error-prone lesion bypass utilizes specialized DNA polymerases that tolerate DNA distortions to synthesize DNA across the lesion in a damage tolerance process known as translesion synthesis that occurs at the cost of replication fidelity [13,14].

2.1. The RAD6 gene

The process of DNA damage tolerance has been studied extensively in yeast. RAD6 is a fundamental component of the TLS/PRR pathway, and its ubiquitin conjugating activity is critical for its function in TLS/PRR. Loss of RAD6 catalytic activity or mutations in RAD6 pathway have been linked to hypersensitivity to ionizing and UV radiation, as well as alkylating and DNA crosslinking agents, supporting its important role in DNA damage tolerance [15]. RAD6 participates in both error-prone TLS and error-free damage avoidance repair, and plays an important role in balancing both processes [15,16]. There are two human homologs of yeast RAD6, HHR6A and HHR6B. These proteins share 95% amino acid identity and have ~70% sequence homology to the yeast RAD6 protein. HHR6A and HHR6B are localized on chromosomes Xq24–q25 and 5q23–q31, respectively [17]. Mice defective in both HHR6A and HHR6B are nonviable, indicating that at least one functional RAD6 allele is required for cell survival [18]. Additionally, cysteine 88 in the ubiquitin conjugating catalytic site is essential for RAD6 function as replacement of cysteine 88 with valine, alanine or serine results in loss of RAD6 activity and hypersensitivity to DNA damaging drugs [19].

2.2. The RAD6 Translesion Synthesis Pathway

The RAD6 TLS pathway is initiated in response to DNA damage when RAD6 complexes with RAD18, a RING domain E3 ubiquitin ligase. A critical step in lesion bypass mechanism is RAD6-RAD18 induced posttranslational ubiquitination of proliferating cell nuclear antigen (PCNA) at Lysine 164, which facilitates recruitment of specialized TLS DNA polymerases. Unlike the high fidelity replicative DNA polymerases, low fidelity TLS polymerases are nonprocessive, lack intrinsic exonuclease activity for proofreading capability [20-22], and contain larger active sites that can accommodate distorted DNA bases and base pair mismatches [21]. Thus depending upon the fidelity of the incorporated nucleotides, the outcome can be error-free or mutagenic.
RAD6/RAD18 mediated monoubiquitination of PCNA is the major modification of PCNA in mammals. PCNA polyubiquitination has also been reported in response to UV radiation and other DNA damaging agents, however, it occurs at a rate that is ~20-fold lower than PCNA monoubiquitination [23]. PCNA polyubiquitination is promoted by MMS2-UBC13, a stable complex of an ubiquitin E2 variant (UEV) and an E2, and RAD5 in yeast [24], or by RAD5 human homologs HTLF and SHPRH which serve as E3 ligases [25-28]. MMS2-UBC13 complexes mediate the formation of ubiquitin chains with Lysine-63 linkages, which unlike Lysine-48 linked ubiquitins that tag proteins for 26S proteasomal degradation, promote protein-protein interactions and DNA repair [24]. A recent study showed that PCNA polyubiquitination predominantly occurs via en bloc transfer of preformed ubiquitin chains rather than by extension of the ubiquitin chain on monoubiquitinated PCNA [29]. This study showed that HTLF initially forms a thiol-linked ubiquitin chain on UBC13, which is then transferred to RAD6-ubiquitin to form RAD6-ubiquitin_{n+1}. The resultant chain is subsequently transferred en bloc to PCNA by RAD18 [29]. This model not only connects RAD6 in PCNA polyubiquitination, but highlights the central role of RAD6 in coordinating with RAD18 and HTLF for promoting PCNA mono and polyubiquitinations. Polyubiquitinated PCNA is implicated in error-free damage avoidance pathway likely through a template switch mechanism, although the details of the mechanism remains to be established [23]. It is, however, believed that it involves release of the stalled primer end from the damaged template and annealing with the newly synthesized daughter strand of the sister chromosome [30]. This use of sister duplex information ensures an error-free process by preventing accumulation of mutations in the replicated strand [23]. PCNA from the budding yeast S.cerevisiae is modified by SUMO (small ubiquitin-like modifier protein) at Lysine 164 (the same site as ubiquitination) and Lysine 127 (to a lesser extent) during S phase even in the absence of DNA damage. SUMOylation is mediated by the SUMO-specific E2 conjugating enzyme Ubc9 and the SUMO E3 ligase Siz1. SUMOylated PCNA interacts with Srs2 helicase, and thus prevents Rad51 nucleofilament formation and homologous recombination [24]. Although SUMOylated PCNA has been observed in Xenopus egg extracts [31] and chicken DT40 cells [32], SUMOylated PCNA has not been detected in human cells, raising the question about its presence in human cells or the technical difficulty of detecting SUMOylated PCNA.

2.3. Translesion Synthesis Polymerases

Replicative DNA polymerases are high fidelity enzymes that become stalled at sites of DNA damage. Synthesis of DNA past DNA damage sites requires replacement of the replicative polymerases by low fidelity and low speed TLS polymerases [33]. Unlike the high fidelity DNA polymerases such as Pol α, Pol δ and Pol ε that belong to the classical B-family of DNA polymerases, most of the TLS polymerases including Pol ι, Pol η, Pol κ and Rev1 belong to the Y-family. Pol ζ, another error-prone polymerase, belongs to the B-family of polymerases and is composed of a Rev3/Rev7 heterodimer [23]. Monoubiquitination of PCNA increases its affinity for these low fidelity polymerases at the damage site allowing for a polymerase switch [15]. This was first demonstrated for TLS Pol η, but later shown to apply to other TLS polymerases (Pol ι and Pol κ) that are not present in yeast [34,35]. This enhanced affinity of TLS polymerases for PCNA is due to the presence of ubiquitin-binding domains in these polymerases which interact with the monoubiquitin on PCNA.

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The TLS process requires the exchange of a replicative DNA polymerase stalled at the DNA damage site with a TLS polymerase, and is thought to occur in a multi-step process [36,37]. In the initial step, one of the four Y-family polymerases (Pol κ, Pol η, Pol τ, or Rev1) is recruited to the stalled replication fork or the damage site for replication over the DNA lesion. The recruitment of insertion TLS polymerases is facilitated by DNA damage-induced PCNA monoubiquitination [38-41]. Following incorporation of nucleotide opposite the damage site, the insertion TLS polymerase is replaced by extension polymerase which extends the TLS patch by ~18 nucleotides. This extension step allows the lesion to escape detection by the 3′-5′ exonuclease proofreading activity of a replicative DNA polymerase. The TLS extension is predominantly executed by the heterodimeric Pol ζ complex.

Following extension past the DNA lesion, Pol ζ is switched back to the high fidelity DNA polymerase for resuming processive DNA replication. However, recent studies have shown that the transition from insertion to extension TLS polymerase can be accomplished in one step by the formation of a quaternary TLS polymerase complex that contains both initiation and extension polymerases bridged by Rev1 [42]. Formation of such a megatranslesion polymerase complex as noted in this study would facilitate efficient TLS by circumventing the need for an additional switching mechanism [42]. Recent studies have also shown that Pol31 and Pol32 subunits of Pol β interact with Pol ζ to form a four subunit Pol ζ complex (Rev3-Rev7-Pol31-Pol32, referred to as Pol ζ4), and that complexing with Pol31 and Pol32 is essential for Pol ζ function in PCNA-dependent TLS. The functional requirement of this complex for TLS was supported by evidence that mutations compromising binding of Rev3 to Pol31 abolishes Pol ζ function in TLS [43]. These data not only implicate a role for Pol β in Pol ζ-dependent mutagenesis but also reveal the resemblance between Pol ζ and Pol β in structural complexity and subunit composition [43,44]. Comparison of the catalytic activities and processivities of Pol ζ2 (Rev3-Rev7) vs. Pol ζ4 (Rev-Rev7-Pol31-Pol32) to bypass a 1,2-intrastrand (GG)-cisplatin crosslink showed Pol ζ4 to be more efficient and processive at lesion bypass compared to Pol ζ2 [45]. The authors of this paper also showed that compared to Pol ζ4, Pol η accurately and efficiently inserted a C opposite the 5′G of cisplatin-GG, but unlike Pol ζ, Pol η was less efficient in subsequent primer extension. However, when Pol η and Pol ζ4 were combined, cisplatin-GG bypass occurred more efficiently than with either polymerase alone. These data not only reveal synergistic interactions between the low fidelity polymerases but also emphasize the need for stringent control and engagement of these polymerases only when necessary for lesion bypass and cell survival [45].

3. The Fanconi anemia network

Fanconi Anemia (FA) is a rare autosomal recessive genetic disease caused by mutations in the Fanconi anemia protein cluster. 14 FANC genes have been identified including FANCA, FANCB, FANC, FANCD1 (BRCA2), FANCD2, FANCE, FANCF, FANCN, FANCI, FANCJ (BACH1; BRIP1), FANCL, FANCM, FANCN (PALB2) and FANCP (SLX4). Mutations in RAD51C, a RAD51 paralog, have also been identified in FA patients. Fanconi anemia is characterized by hypersensitivity to DNA ICL agents, chromosomal instability, and predisposition to various cancers including leukemia, breast cancer and head & neck
3. Fanconi Anemia and ICL Repair

The FA network, which functions mostly during S phase, plays an important role in ICL repair. The FA pathway is characterized by assembly of a core complex at the site of ICL, phosphorylation of FANCI followed by monoubiquitination of FANCD2 and FANCI, and subsequent formation of FANCI/FANCD2 complex at the site of damage [47]. These two modified proteins then bind to the ICL and recruit endonucleases for the removal of the ICL through NER. It has been proposed that low fidelity TLS polymerases, initiated by RAD6/RAD18-induced PCNA monoubiquitination, are then recruited to fill in the gap. The exact sequence of events concerning the NER and TLS are still under investigation. It is possible that the FANCI/FANCD2 complex promotes incision at the site of the ICL and TLS subsequently fills in the gap at the lesion. Alternatively, the TLS activity may precede the incision, and the FANCI/FANCD2 complex promotes TLS activity near the ICL [48].

Monoubiquitination of FANCD2 is essential for this repair pathway and is considered a surrogate marker of FA network activation. FANCL, functioning as an E3 ubiquitin ligase, in association with the E2 enzymes, UBE2T and UBE2W, promotes FANCD2 monoubiquitination at Lysine 561 [49,50]. UBE2T association with FANCL also induces ubiquitination of FANCI at Lysine 521. Another important modification of FANCD2 protein in response to ionizing radiation is phosphorylation by ATM and the checkpoint kinase ATR [51]. Although phosphorylation of FANCD2 is not required for FA pathway activation, it helps stabilize the replication forks [52].

4. Homologous recombination repair

In response to double strand breaks (DSBs), cells utilize the HRR pathway which relies on the undamaged sister chromatid as a template for repair. Due to this reliance on the sister chromatid, HRR is active during S and G2 phases of the cell cycle. Throughout the remainder of the cell cycle NHEJ is utilized, which will not be discussed here. Repair by HR requires three major steps: end resection, strand invasion, and resolution. End resection involves the activities of MRE11-RAD50-NBS1 (MRN) complex and exonuclease 1 (Exo1) singly or in combination with the Bloom’s syndrome RecQ helicase like protein (BLM) and DNA replication helicase 2 (DNA2) to resect nucleotides and extend 3’ ssDNA overhangs [53]. The 3’ overhangs are then stabilized with replication protein A (RPA) [53]. Proteins such as BRCA2 in conjunction with its binding partner and localizer PALB2, referred to as recombination mediators [54], facilitate the removal of RPA. These proteins function to load RAD51 on the ssDNA, increase intrinsic stability of the RAD51 presynaptic filament, and protect RAD51 from removal. Once the presynaptic filament is assembled, it interacts with the sister chromatid, samples it for homology and allows for the homologous DNA molecules to align and form the synaptic complex. Upon invasion, the complementary strand of the sister chromatid is displaced leading to the formation of a transient structure known as the D-loop structure [53]. The 3’ overhang of the invading strand is then freed from RAD51 and used as a primer for elongation. At this time it is unclear which replication machinery is used for this elongation though it has been shown that TLS polymerases, Pol η...
in particular, demonstrate affinity for D-loop elongation (discussed below). After elongation, two pathways can be utilized to resolve the D-loop. In the first pathway, referred to as the synthesis-dependent strand-annealing (SDSA) pathway, D-loop extension continues for a short distance and is disassembled by regulator of telomere elongation helicase 1 (RTEL1) and reannealed with the ssDNA associated with the other DSB end [53]. DSB repair is then completed by gap filling and ligation. The second pathway involves BLM helicase, an ATP-dependent 3’-5’ DNA helicase that is able to unwind D-loops [55].

5. PRR pathway, FA network and HRR pathway crosstalk

The PRR pathway can initiate error-prone or error-free repair depending upon the modification made to PCNA, i.e., monoubiquitination or polyubiquitination respectively. Evidence shows that initiation of error-prone and error-free repair by the PRR pathway contributes to increased activation of the FA network and the HRR pathway, which is discussed below.

5.1. Crosstalk Between PRR pathway and FA network

As described above, error-prone repair initiated by the PRR pathway occurs when RAD6 complexes with RAD18 E3 RING ubiquitin ligase to monoubiquitinate PCNA. The RAD6/RAD18 complex has also been demonstrated to induce FANCD2 ubiquitination, as silencing of RAD18 leads to a decrease in FANCD2 ubiquitination and increased sensitivity to ICL inducing agents [56,57]. Furthermore, silencing of RAD6 results in decreased levels of mitomycin C-induced FANCD2 monoubiquitination, whereas overexpression of RAD6 leads to an increase in FANCD2 ubiquitination [58]. These studies suggest that the RAD6/RAD18 complex can directly impact FA network activation via FANCD2 ubiquitination. In addition, studies show that PCNA coimmunoprecipitates with FANCL, the E3 ubiquitin ligase responsible for FANCD2 ubiquitination [56,58] and that monoubiquitinated PCNA is also able to recruit FANCL to the chromatin [56]. Lastly, evidence suggests that PCNA and FANCD2 interact via the FANCD2-PCNA interacting peptide (PIP)-box motif and that a mutation in this motif abrogates FANCD2 monoubiquitination [59]. These studies are suggestive of a role for PCNA as a scaffold protein for recruiting both FANCL and FANCD2 to the chromatin and promoting FANCD2 ubiquitination.

Further evidence for crosstalk between error-prone repair and FA network activation is revealed by the observation that abasic bypass is dramatically decreased in nuclear extracts without FANCD2 compared to those with FANCD2 [57]. Moreover, it has been shown that K561R FANCD2, a mutant FANCD2 unable to be ubiquitinated, is able to reverse colony formation in cells overexpressing RAD6 thus nullifying the oncogenic potential of RAD6 [58 _ENREF_34]. This is in part due to the ability of FANCD2 to recruit Pol η to the sites of ICL. Since FANCD2 interacts with PCNA and undergoes subsequent FANCD2 ubiquitination and interaction with Pol η, we conjecture that FANCD2 monoubiquitination and subsequently FA network activation plays a role in PRR-induced chemoresistance by recruiting Pol η to the sites of damage and potentially facilitating TLS polymerase switching. Deubiquitination of ubiquitinated PCNA is accomplished by ubiquitin specific protease (USP1). Cellular stress induced by UV radiation or DNA damaging agents causes autocleavage of USP1, thus allowing accumulation of monoubiquitinated PCNA and
initiation of TLS at the site of damage [60]. USP1 also negatively regulates FA pathway by deubiquitination of FANCD2 [61]. Since ubiquitinated PCNA and FANCD2 colocalize at stalled DNA replication forks, it underscores the importance of downregulating USP1 under conditions of cellular stress to facilitate TLS. An additional link between FA and TLS was demonstrated by Kim et al [62]. FAAP20, a FA-associated protein and an integral member of the FA core complex, binds to monoubiquitinated-Rev1 via its ubiquitin-binding zinc finger 4 domain, and promotes interaction of the FA core with PCNA-Rev1 TLS bypass complex [62].

5.2. Crosstalk between PRR and HRR

During PRR, PCNA and FANCD2 recruit and interact with Pol η to allow for TLS. In addition to its role in TLS, Pol η has also been implicated in homologous recombination. Cells lacking Pol η showed a 10-fold decrease in HRR while overexpression of Pol η resulted in a 6-fold increase in HR frequency [63,64]. Evidence also indicates that Pol η can extend D-loop structures formed during HRR strand invasion [65]. Furthermore, preincubation of D-loop structures with RAD51 enhances Pol η binding [65]. These studies support a link between PRR and HRR via Pol η, which plays a role in TLS as the polymerase capable of bypassing the ICL and in HRR as the polymerase capable of extending D-loop structures.

Besides Pol η, UBC13 also provides a link between PRR and HRR. In the error-free mode of PRR, monoubiquitinated PCNA is extended by Lysine 63-linked multiubiquitin chain by UBC13/MMS2 and RAD5. UBC13 also plays a role in HRR initiation. Cells deficient in UBC13 show reduced RAD51, BRCA1 and RPA foci formation in response to damage [66]. UBC13 deficient cells also display decreases in BRCA1 interaction with MRE11 and UBCH5c at the sites of DNA damage [66]. These studies indicate that UBC13 functions in both the error-free mode of PRR and in HRR to ensure efficient DSB recognition and end resection.

5.3. Crosstalk between FA and HRR

Crosstalk between the FA network and HRR pathway is evident by the fact that many FA proteins are also involved in HRR. The observation that BRCA2, the protein required for RAD51 loading onto ssDNA, is FANCD1, provides direct evidence for a link between the FA network and HRR. FANCM, a member of the FA core complex, has also been shown to play a role in HRR independent of the FA network. Depletion of FANCM reduced RPA foci formation at ICL, while depletion of the FA core excluding FANCM did not produce a similar response [67]. Moreover, FANCM is needed for proper recruitment of C-terminal binding protein interacting protein (CtIP) and MRN end resection at the site of ICL for HRR [68]. In addition to BRCA2/FANCD1 and FANCM, PALB2 (FANCN) and BRIP1 (FANCJ/BACH1) also participate in HRR by working as a binding partner and regulator of BRCA2 and BRCA1 function, respectively. While the crossover of these proteins between the FA network and the HRR pathway provides evidence for FA and HRR crosstalk, it is important to note that BRCA2, PALB2, and BACH1 are the only FA proteins that are not required for monoubiquitination of FANCD2. This would suggest that their role in the FA network may be a secondary function. However, BRCA2 has been shown to directly interact with
FANCG, a member of the FA core protein complex, and this interaction potentially allows FANCG to colocalize with RAD51 after DNA damage [69]. It has also been demonstrated that inactivation of FANCG decreases HRR [70]. These studies indicate that BRCA2, though not essential to FANCD2 monoubiquitination and thus FA pathway activation, is required to provide crosstalk between the FA network and the HRR pathway. This also gives insight into the possible dual role of PALB2 (FANCN) and BACH1 (FANCJ).

As discussed above, FANCD2 participates in recruiting Pol η, the TLS polymerase capable of extending D-loop structures formed during HRR. In conjunction with this role, FANCD2 expression increases HRR by 2-fold and the monoubiquitination site of FANCD2 is required for this action [71]. This indicates that the interaction between FANCD2 and Pol η plays a functional role in initiating HRR. A recent study by Sharma et al. [72] demonstrated that TLS polymerases Rev1 and Pol ζ (Rev 3 and Rev7) also interact in vivo to promote HR.

Evidence for the HRR pathway linking the FA network is supported by the observation that loss of BRCA1 leads to a decrease in FANCD2 foci formation within the nucleus, but has no effect on FANCD2 ubiquitination [73]. Furthermore, loss of CtIP leads to a decrease in FANCD2 accumulation at the ICLs [68]. This suggests that BRCA1 and CtIP play a role downstream of the FA core complex allowing for FANCD2 foci formation at the site of damage. A role for the MRN complex in FANCD2 stability has also been reported as silencing of MRE11, RAD50 or NBS1 leads to decrease in FANCD2 protein life [74]. Together these data suggest that integral members of HRR are needed for proper FANCD2 function within the FA network.

6. FA/BRCA/HRR/TLS crosstalk and chemoresistance

Among the various chemotherapeutic drugs used for cancer treatment, ICL-inducing agents are most widely used, particularly in treatment of solid tumors. ICL-inducing agents include nitrogen mustards, mitomycin C, platinum and psoralens. Cyclophosphamide, a nitrogen mustard alkylating agent, with trade names Endoxan, Cytoxan, Revimmune, Procyscoy and Neosar are routinely administered as first line treatment for leukemia, lymphoma and metastatic breast cancer [75,76]. Cisplatin is used for treatment of lung cancer, ovarian cancer, lymphoma, and recently for triple negative breast cancer. Carboplatin, a second generation platinum drug is increasingly used for breast cancer treatment. Other platinum based drugs include oxaliplatin, triplatin, satraplatin, mitomycin C and psoralens.

Mitomycin C is used for treatment of esophageal and bladder cancers, and oxaliplatin is administered as a first line treatment for colon cancer. Cancer patients are usually treated with multiple cycles of chemotherapy, however, the durability of treatment response is frequently limited by development of therapy resistance and disease relapse. While several mechanisms such as decreased drug uptake, increased drug efflux or increased drug inactivation can contribute to the emergence of chemoresistant phenotype, elevation of DNA damage tolerance and DNA repair play a major role in development of chemoresistance and disease recurrences.

The TLS, FA network and HRR pathways have all been implicated in cancer development and chemoresistance. Cancer cells deficient in BRCA1 display sensitivity to irofulven, an alkylating anticancer agent that induces DSBs in DNA. On the other hand, expression of
wild type BRCA1 confers resistance to irofulven by controlling S and G2/M checkpoints that is critical for repairing DNA DSBs through RAD51-dependent HR [77]. Secondary mutations that restore the reading frame of BRCA1 or BRCA2 lead to resistance to platinum based drugs in ovarian cancer [78,79], further supporting the contribution of the BRCA/HRR pathway in chemoresistance. RAD51 and RAD51 paralogs, are involved in HRR via their interactions with BRCA1 and BRCA2. Among the RAD51 paralogs, RAD51C has been associated with both Fanconi anemia and familial breast and ovarian cancers [80,81], and RAD51D and RAD51L1 have been associated with breast and/or ovarian cancer risk [82,83]; however, the clinical significance of these findings remain to be validated. Consistent with the role of RAD51 in promoting DNA strand exchange, upregulation of RAD51 results in an increased number of recombinational events and chromosomal rearrangements in mouse embryonic stem cells [84]. Whereas constitutive overexpression of RAD51 increased spontaneous recombination frequency [85,86] and resistance to chemotherapeutic drugs in tumor cells [87,88], treatment with gemcitabine inhibited RAD51 foci formation after irradiation and induced radiosensitivity [89]. Hypoxia associated downregulation of HR has been linked with hypoxia-induced decreases in RAD51 levels. Since solid tumors are characterized by hypoxic environment, aberrant regulation of RAD51 could create heterogeneity in DNA damage response among cells in the tumor and potentially impact response to cancer therapy [90].

Several studies have reported elevated levels of TLS components in different cancer types and contributing to resistance against multiple DNA damaging agents. Expression of Rev3L, the catalytic subunit of the human TLS polymerase ζ, was found to be elevated in clinical gliomas compared to the normal brain tissue, and silencing Rev3L increased cisplatin sensitivity of glioma cells [91]. Suppression of Rev3 in a preclinical model of lung adenocarcinoma induced pronounced sensitivity to cisplatin and increase in the overall survival of treated mice [92]. Furthermore, Rev3 deficient cells exhibited reduced cisplatin induced mutations, implicating the therapeutic potential of TLS inhibition for treatment of chemoresistant malignancies. Similarly, suppression of Rev7 slowed tumor growth and increased sensitivity to DNA damaging drugs in ovarian cancer [93]. In a study using a mouse model of B-cell lymphoma, inhibition of Rev1, another component of TLS, decreased cisplatin and cyclophosphamide induced mutagenesis [94]. Using a nanoparticle-based platform engineered to codeliver cisplatin and Rev1/Rev3-specific siRNAs, Xu et al. demonstrated synergistic inhibition of tumor growth in a human prostate cancer xenograft model [95]. Pol η expression was found to be elevated in 67% of head and neck cancers compared to normal tissue [96]. Alterations in Pol η expression have been correlated with response to platinum based chemotherapy. Low levels of Polη have been associated with better prognosis, and its expression status considered as a potential marker for platinum therapy effectiveness [96]. The involvement of TLS polymerases in chemoresistance offers further proof that error-prone polymerases are crucial for filling gaps caused by chemotherapeutic drugs, thus providing a novel framework for development of therapeutics with chemosensitization potential.

The RAD6 group plays a critical role in damage bypass, and the RAD6 ubiquitin conjugating activity is essential for this process. Analysis of RAD6 protein expression in normal and cancerous human breast cell lines and tissues show low levels in normal breast
cell lines and tissues, and overexpression in invasive and metastatic breast cancers [97]. Consistent with these data, ectopic overexpression of RAD6 in nontransformed breast epithelial cells induced anchorage independent growth, aneuploidy, and formation of hyperplastic lesions in vivo [97,98]. Whereas RAD6 overexpression in normal breast cells confers tolerance to doxorubicin and cisplatin, RAD6 silencing induces hypersensitivity to these chemotherapeutic drugs. Furthermore, this ability to tolerate the DNA damaging drugs is directly associated with PRR activity as RAD6 overexpressing cells are PRR-competent as opposed to RAD6 silenced cells that are PRR-deficient, thus providing support for a direct role for RAD6 in repair of ICLs and acquisition of chemoresistance [99]. Among the protein markers p53, Ki67, Mdr P-glycoprotein and RAD6 analyzed in postneoadjuvant chemotherapy breast tissue samples, only RAD6 expression and distribution was associated with clinical outcome. Strong nuclear RAD6 staining was associated with complete pathologic and clinical response, whereas cytoplasmic RAD6 overexpression and lacking nuclear RAD6 was associated with pathologic and clinical nonresponse [100]. Sequence and ubiquitin conjugation activity analyses of RAD6 in breast cancer cell lines and carcinomas confirmed that RAD6 is normal and functional, linking the oncogenic activity of RAD6 to its ubiquitinating function.

6.1. Exploiting TLS/FA/HRR Pathway Crosstalk as a Cancer Therapy Strategy

The studies reviewed here show the interactive relationships between HRR, FA network, and PRR/TLS pathways, and their active roles in development of chemoresistance. Not only are these pathways or components of these pathways upregulated in cancer cells, but the anticancer agents themselves enhance expression and recruitment of DNA repair proteins to the sites of damage to enable repair of the damaged DNA. Thus the DNA damage response induced in drug treated cells become counterintuitive as it actively participates in development of drug resistance. The RAD6 epistasis pathway accounts for the majority of drug resistance and damage-induced mutagenesis in the yeast [16], thus targeting key genes of this pathway could provide a novel therapeutic approach for overcoming resistance to a broad spectrum of drugs. We propose that RAD6, RAD18, PCNA and FANCD2 play key roles in activating TLS/HRR crosstalk that culminate in polymerase switch and recruitment of TLS polymerases such as Pol η for TLS and subsequent initiation of HRR via Pol η/RAD51 interaction and extension of D loop structures (Fig. 1). Based on this model, we posit that inhibition of RAD6/RAD18, blocking interactions between RAD6 and RAD18, or blocking ubiquitination of PCNA or FANCD2 could lead to downregulation of the crosstalk and restoration of chemosensitivity. Recently, a small molecule inhibitor T2 amino alcohol (T2AA) that blocks PCNA/PIP-box interactions was reported to suppress TLS, inhibit ICL repair and sensitize U2Os cells to cisplatin [101]. T2AA decreased PCNA/Pol η and PCNA/Rev1 chromatin colocalization but had no effect on PCNA monoubiquitination, suggesting that the T2AA induced effects resulted from inhibition of interactions between monoubiquitinated PCNA and Pol η or Rev 1 [101]. Disruption of RAD6 was shown to significantly block ionizing radiation-induced mutagenesis whereas deletion of the entire ORF of RAD18 had no effect [102]. Furthermore, since RAD18 activity is dependent upon the catalytic activity of RAD6, RAD6 may provide a better druggable target for blocking TLS and potentially attenuating HRR activity. Using a structure guided approach, we have recently developed a RAD6 selective small molecule inhibitor that inhibits the ubiquitin
conjugating activity of RAD6 [103]. The availability of such RAD6-selective or TLS polymerase-targeting inhibitors could potentially provide a novel therapeutic strategy for cancer treatment as they can sensitize resistant tumors to lower doses of chemotherapy by attenuating error-free PRR/HRR and blocking damage-induced mutagenesis associated with error-prone TLS.

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Figure 1.

A model for the integral role of the Rad6 DNA damage tolerance pathway in coordinating the activities of Fanconi anemia (FA) network and homologous recombination repair (HRR) pathway in interstrand DNA crosslink (ICL) repair. In conjunction with Rad18 E3 ligase, Rad6 activates the error-prone translesion synthesis (TLS) component of DNA damage tolerance by inducing PCNA monoubiquitination and recruitment of TLS polymerases. Evidence suggests that Rad6/Rad18 can also mediate FANCD2 monoubiquitination and activation of FA network [33, 34]. Besides its role in BRCA-HRR, monoubiquitinated FANCD2 also partakes in TLS by recruiting TLS polymerase Pol η. In addition to their involvement in TLS, PCNA and Pol η enable HRR by regulating extension of D-loop structures. The error-free arm of DNA damage tolerance or template switching is accomplished by PCNA polyubiquitination. Here the K63-linked polyubiquitin chains assembled by Rad6 are transferred en bloc to PCNA via HLTF E3 ligase.