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It is entitled:
Uncovering the roles of RNF8 ubiquitin signaling networks and BRCA1 in recruiting Fanconi Anemia proteins to DNA damage

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Uncovering the roles of RNF8 ubiquitin signaling networks and BRCA1 in recruiting Fanconi Anemia proteins to DNA damage

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ABSTRACT

Fanconi Anemia is a multigenic DNA damage repair disorder and cancer predisposition syndrome. The 20 proteins in the FA pathway are all linked by their mutual necessity in responding to DNA interstrand crosslinks, such as those which arise from mitomycin C; however, there are clear distinctions between the proteins as well. For instance, loss of some proteins, such as FANCA or FANCD2 does not dramatically impact the cellular resistance to ionizing radiation, while loss of others such as BRCA1, PALB2 or BRCA2 leaves cells exquisitely sensitive. This distinction is echoed by differences in clinical phenotypes as well as biochemical properties. FANC A, B, C, E, F, G, L, and M, interact in a large complex, called the FA core complex, which monoubiquitinates FANCD2 and FANCI. BRCA1, PALB2, BRCA2, FANCJ, RAD51C, XRCC2, and RAD51 also form complexes which are important for homologous recombination. In this dissertation, we examine how these groups of proteins are recruited to DNA damage through protein-protein interactions and ubiquitin signaling. First, we examine the role of the PALB2 coiled coil domain in recruitment of itself and RAD51 to DNA damage. We show that PALB2 coiled coil domain interacts with BRCA1 and PALB2 coiled coils, it is dispensible for PALB2 mediated DNA repair, when PALB2 is fused to the BRCA1 BRCT-repeat domain. This domain is essential for BRCA1’s recruitment to DNA damage foci through the MDC1, RNF8, RAP80 and ABRAXAS ubiquitin signaling cascade. We show that both endogenous PALB2 and BRCT fusion PALB2 are also dependent on this pathway. This indicates the PALB2 is recruited via a coiled coil mediated interaction with BRCA1, allowing it to recruit other downstream complex proteins. Concomitant work showed that RNF8 plays a role in recruiting the upstream FA protein FANCD2. Our work shows links have been identified which may coordinate the different FA proteins to facilitate the specific steps of DNA repair. As such, we tested whether FANCD2 and PALB2 could be coordinately recruited to crosslinks via shared
RNF8 mediated regulation. We showed that FANCD2 and PALB2 colocalize after crosslink induction and that both also colocalize with RNF8 dependent ubiquitin chains. Interestingly, while we show that RNF8 is indeed important for the regulation of both FANCD2 and PALB2, other proteins such as MDC1, RAP80, and the ubiquitin binding component of the FA-Core complex, FAAP20, differentially regulate PALB2 or FANCD2, respectively. This indicates that although RNF8 is a shared regulator of FANCD2 and PALB2 the mechanism through which it acts is distinct. This is interesting given that FANCD2 and PALB2 are involved in different steps in the repair of DNA crosslinks. More work must be done to understand what these mechanisms are and why they are distinct. Because of the continued use of DNA damaging agents such as crosslinkers and radiotherapy in the treatment of cancer, as well as to better help FA patients themselves, this work sheds light on important and possibly targetable regulatory pathways.
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Chapter 1. INTRODUCTION

1. Discovery, Diagnosis, and Treatment of Fanconi Anemia

In 1927, an Austrian physician named Guido Fanconi reported on several sets of siblings displaying similar congenital anomalies. The symptoms of this syndrome, now called Fanconi Anemia (FA), included short stature, bone morphogenetic defects, most prominently of the thumbs and radii, hyperpigmentation (café-au-lait spots), and pancytopenia (Auerbach 2009). While the prominence in siblings suggested a genetic cause, he noted that within sets of siblings the penetrance and severity of these phenotypes was inconsistent, a property which even today hinders the diagnosis of new patients who may not present with congenital abnormalities (Auerbach 2009). Currently, the presumed incidence of FA is between 1 and 5 per million individuals; however, the carrier frequency is much higher (.3-1%) as FA is polygenic and heterozygous loss of an allele comes with relatively few risks for most of the genes (Joenjje and Patel 2001).

Initially, patients were only identifiable by associating their congenital defects with the defects described by Fanconi, as well as some additional ones described later such as gastrointestinal and central nervous system defects such as hydrocephalus and mental retardation (Auerbach 2009; Smith and Wagner 2012). For patients who present with mild or no congenital malformations, diagnosis may not occur until the patient presents with aplastic anemia, leukemias, or the identification of an effected sibling. Due to inconsistencies in patient presentations, diagnosis of patients relies on cellular phenotypes which are shared regardless of the severity of the clinical presentation (Auerbach 1993). Cells from all FA subtypes are hypersensitive to DNA interstrand cross-linking agents, but less so to other DNA damaging agents like UVC light (Kalb et al. 2004). The cellular phenotypes tested in response to
crosslinking agents include an increased rate of chromosomal abnormalities post-crosslinker treatment and accumulation of cells in a diploid G2/M state. Two radial chromosome formations from DNA crosslinker treated FA patient cells can be seen in Figure 1. A non-FA patient cell should average about 0-1 radial chromosomes per metaphase after treatment with crosslinkers. FA patient cells tend to have around 6-10 (Auerbach et al. 1989). To assay the G2/M arrest, cells are treated with a cross-linker and then cell cycle analysis is performed. Because FA cells fail to repair the DNA damage caused by ICLs there is a characteristic accumulation at the 4N peak suggesting that cells have completed replication despite the presence of DNA damage and are paused prior to entering mitosis (Centurion et al. 2000; Sala-Trepat et al. 2000; Akkari et al. 2001; Phelps et al. 2007). In order to confirm the subtype of FA, patient cells are corrected with the suspected gene and subjected to the above tests as well as survival assays. Correction should rescue sensitivity to MMC, as well as reduce the number of radial chromosomes and the G2/M arrest.

Fig. 1. Examples of radial chromosomes in uncorrected FANC-U patient cells after treatment with Mitomycin C. Image credit Jung-Young Park

Treatments are available to alleviate some of the symptoms of FA, however no cure is available. FA patients are routinely monitored for signs of cytopenias and bone marrow failure, as well as leukemic transformation. If the patient presents with leukemia or bone marrow failure, they will likely require a bone marrow transplant. FA patients are very sensitive to the DNA damaging agents used to myeloablate the general population, but the introduction of the nucleoside analog fludarabine has made transplants safer and more effective for FA patients (Shimamura and Alter 2010). Increases in transplant effectiveness have allowed FA patients to survive longer; however a new trend has emerged in transplant recipients, namely head and neck squamous cell carcinoma (HNSCC). The risk of HNSCC has been estimated at about 4.4 fold higher in FA
patients who received transplant than those who did not (Rosenberg et al. 2008). Only 11% of
the cohort studied for this analysis was treated with the improved fludarabine regimen however,
so this number may prove to be lower as more patients are treated with the less toxic
fludarabine regimens. Unfortunately, there are few ways to prevent the developmental issues
that arise in utero, which can also be devastating. The complexity of this syndrome makes
treatment difficult and requires a deeper understanding of the mechanisms that are failing in FA
patients in order to develop improved therapies.

FA is a multi-genic disorder, associated with biallelic loss of 18 different genes, or in the
cases of FANCB and FANCR sex-linked or dominant negative mutations, respectively (Meetei
et al. 2004; Shamseldin et al. 2012; Schneider et al. 2014; Sawyer et al. 2015; Wang et al.
2015; Park et al. 2016). Although 20 genes have been identified, a large majority of the known
cases are attributable to just three; FANCA (60%), FANCC (16%) and FANCG (10%), with the
remainder of the genes accounting for all but a few percent of the known patients (Auerbach
2009; Shimamura and Alter 2010; Kee and D’Andrea 2012). The remaining patients have still
not been assigned to a subtype, and the mutated genes are unknown. Hopefully, with the
advent of new technologies such as high throughput sequencing, new FA genes and mutations
can be identified.

The known FA genes are distributed widely across the genome, and the proteins are
expressed in every tissue although at very different levels (Uhlén et al. 2015). They are
expressed at the highest levels in the male germ cell development, which correlates well with
the ubiquitous infertility in male patients (Auerbach 2009). While the precise regulation of
expression of the FA genes isn’t well mapped, it is likely that cell cycle status is a primary
regulatory event. One such event has been explored in 293 cells, in which the FANCC protein is
degraded during G1 phase and accumulates through M phase, with no noticeable difference in
mRNA levels (Heinrich et al. 2000).
2. Molecular contributions to FA pathology

2.a. Reactive aldehydes

The physiological reasons for the congenital anomalies in FA patients are poorly understood, and, given that mouse models do not readily phenocopy the patients, studying the developmental issues of FA patients is difficult. It is expected that DNA damage and/or replication stress play a role; however, it is unclear which endogenous or environmental agents are responsible. As the subject of recent work, many endogenous sources of stress have come to the surface, some of those which have come up are reactive aldehydes and reactive oxygen species (ROS), as well as non-DNA damage stressors like Tumor Necrosis Factor α (TNF-α), and Interferon-γ (IFN-γ).

Genetic data from patients with severe phenotypes have found a link to insufficiency of the protein acetaldehyde dehydrogenase 2 (ALDH2), an important enzyme in reducing the levels of acetaldehyde (Hira et al. 2013). Aldehydes are naturally occurring byproducts of metabolism that can cause a large number of lesions to nearly any type of biomolecule if left unchecked (Voulgaridou et al. 2011). Initial studies in FA deficient DT40 chicken cells showed that DT40 cells deficient in Fancl, but not several other DNA repair proteins, such as Ku70, Xpa, Rev1, and Xrcc2, were exquisitely sensitive to formaldehyde and acetaldehyde (Langevin et al. 2011; Rosado et al. 2011). Additional work confirmed that Fancd2 deficient DT40 cells as well as FANCC and FANCG deficient human cells are also hypersenstive to biologically relevant levels of formaldehyde (Ridpath et al. 2007). Interestingly, XRCC2 is now FANCU and REV1 has been shown to be related to the FA pathway (Shamseldin et al. 2012; Kim et al. 2012; Park et al. 2016). The Patel group also showed that loss of Adh5, which is vital for processing formaldehyde, but not Aldh2 is synthetically lethal with Fancd2 loss in DT40 cells, suggesting that the FA pathway is required for responding to formaldehyde (Rosado et al. 2011).
To directly test whether Aldh2 is important in limiting FA phenotypes in vivo, Fancd2 deficient mice were crossed with Aldh2 deficient mice to generate Fancd2-/- Aldh2-/- mice. While Fancd2-/- mice are already born at lower than expected ratios, no double knockouts were born to mothers who were also Aldh2-/-, however, they were born to Aldh2+/- mothers. This suggests that in order for fetal development of Fancd2-/- embryos, either the mother or the embryo must be able to efficiently catabolize acetaldehyde. Double knockout mice obtained from Aldh2 +/- mothers were subjected to ethanol challenges, since ethanol is catabolized into acetaldehyde. Ethanol challenge caused a decrease in blood counts and colony forming units only in the double knock out mice. When allowed to develop normally, mice showed signs of leukemia-like pathology such as splenomegaly and expansion of pre-T-cell clones by 6 months. The mice which did not develop malignancies were later shown to eventually succumb to bone marrow failure with high levels of DNA damage restricted to the HSC compartment as tested by γH2AX fluorescence (Garaycoechea et al. 2012). This work together suggests that acetaldehyde and possibly other types of reactive aldehydes such as formaldehyde may damage the DNA of hematopoietic stem and progenitor cells left vulnerable by loss of the FA pathway.

2.b. Reactive oxygen species

Like aldehydes, reactive oxygen species are formed as a natural byproduct of metabolism, especially mitochondrial metabolism. Complexes I and III of the electron transport chain are among the largest producers of ROS in the cell, by leaking electrons to oxygen molecules (Murphy 2009). Due to their reactive nature ROS can cause damage to nearly all bio-molecules DNA, RNA, proteins, lipids etc. (Klaunig and Kamendulis 2004). The best known effect of ROS on DNA is the generation of 8-hydroxyguanine, which is expected to be repaired by the base excision repair pathway, however there are many additional mechanisms of DNA damage (Cooke et al. 2003). During replication for instance, a single strand break (SSB) caused
by reactive oxygen molecule attacking a phosphate linker on DNA can become a double strand break, which could activate the FA pathway. In addition to causing SSBs, ROS may lead to interstrand cross-links through lipid peroxidation. While this event would likely be rare, its effect on FA deficient cells could be drastic. To start, an oxygen radical reacts with a lipid which generates a product such as malondialdehyde, which can form DNA crosslinks (Niedernhofer et al. 2003). These types of reactive products may also be found in cigarette smoke and automobile exhaust as well as after the metabolism of alcohol (Sapkota and Wyatt 2015). Given the increased incidence and early onset of oropharangeal tumors in FA patients, these environmental agents could be important causes of malignany (Romick-Rosendale et al. 2013).

Several observations have suggested that ROS can contribute to FA pathology. First, several groups have reported that FA patient cells grow better and have fewer spontaneous chromosomal abnormalities in hypoxic than normoxic conditions (Joenje et al. 1981; Schindler and Hoehn 1988). This suggests that the presence of atmospheric oxygen was able to cause damage which would normally be repaired by the FA pathway. Of course, very few cells in the body— including those most affected by FA, the hematapoietic cells, are exposed to such high concentrations of oxygen; however, many studies of FA deficient mice support the cell culture data. Mice deficient in many of the FA proteins largely show no overt FA phenotypes other than infertility (Cheng et al. 2000; Noll et al. 2002); however, when Fancc-/- mice were crossed with Superoxide dismutase (Sod-/-) mice, the resulting adults showed bone marrow hypocellularity which is expected to be a precursor to bone marrow failure (Hadjur et al. 2001). Since Sod is required to limit the levels of ROS, the cells in Sod-/- mice have higher levels of ROS, levels which are even higher in double knockout cells. This increase may be a result of FA proteins acting to increase the levels of other anti-oxidant genes such as glutathione peroxidases and thiodoxin reductases (Zhang et al. 2007). Additional evidence of the interaction of FA and ROS comes from Fancc-/- and Fancd2-/- mice which have been crossed with Foxo3A -/- mice.
Foxo3A is a transcription factor which activates transcription of genes like Sod which decrease levels of ROS in the cell (Storz 2011). These mice were shown to have several hallmark problems associated with FA, not just bone marrow hypocellularity but more prominently, hydrocephalus and neural stem cell death (Li et al. 2014). In addition, many of the phenotypes can be at least partially rescued through treatment with anti-oxidants (Li et al. 2014). In fact, the use of antioxidants such as quercetin in treatment of FA patients is an ongoing interest for clinical FA researchers (FDA and Children’s Hospital Medical Center). Whether these strategies will prove useful for preventing the worsening of FA phenotypes is still unknown.

2.c. Non-DNA damage drivers of FA pathology

While the above work has demonstrated that ROS and aldehydes could be endogenous sources of stress, additional work has uncovered the role of the cytokines TNF-α and Interferon-γ in inducing ROS and exacerbating Fanconi phenotypes (Ventura et al. 2004; Pang and Andreassen 2009). These two cytokines have been implicated in non-FA aplastic anemia (Dubey et al. 2005), and are present at increased levels in FA patients (Dufour et al. 2003). Ex vivo lymphoid cells from Fanconi patients are exquisitely sensitive to the addition of TNF-α, and conversely, culturing FA cells in the presence of a TNF-α inhibitor leads to increased colony formation in bone marrow cells from FA patients but not healthy controls (Dufour et al. 2003). Murine models of FA treated with Tnf-α or Ifn-γ showed reduced peripheral blood cell counts and could receive HSC transplant without additional conditioning (Whitney et al. 1996; Rathbun et al. 1997; Haneline et al. 1998; Li et al. 2004). Impressively, prolonged treatment of ex vivo FA HSCs with Tnf-α was able to drive clonal expansion of Tnf-α resistant cells, suggesting that Tnf-α may also have a role in leukemic transformation in FA patients (Li et al. 2007). Interestingly, this phenomenon may be unrelated to DNA damage. Pang et al. (2001) described a patient mutation in FANCC which generates an N-terminally truncated protein. This protein is unable to correct classical MMC phenotypes; however, it does correct cytokine sensitivity. TNF-α
stimulation works partially through ROS release within cells, suggesting that the pathology driven by ROS in FA patients is more complex than DNA damage induction alone (Zhang et al. 2007). Sumpter et al. (2016) have recently described an additional function of Fanconi proteins in clearing damaged mitochondria by mitophagy. Aged FA mice showed clear persistence of misshapen mitochondria in heart and brain, suggesting this phenotype occurs in vivo. The mechanism by which FA proteins function in mitophagy still requires work, but this seems to agree with a widely observed phenomenon of mitochondrial dysfunction in FA patients (Pagano et al. 2014). While the causal relationship between these DNA damage and non damage factors and FA pathology are unclear, these findings are clarifying important questions for Fanconi researchers.

3. Mechanisms for sensing and repairing DNA damage

As Fanconi Anemia is caused at least partially by failure to accurately or efficiently repair damaged DNA, it is important to understand the cellular responses to DNA damage, collectively termed the DNA damage response (DDR). Any unwanted chemical or physical modification of the DNA activates the DDR. This includes, but is not limited to, single and double strand breaks, base modifications, nucleotide mismatches, crosslinking of the DNA to itself or to other molecules, and even telomere shortening. Depending on the type of damage, the cell activates different responses. Not only is the type of damage important in determining the cellular response, the context of the damage is also important. For instance, a DNA double strand break occurring in heterochromatin is more likely to be repaired by homologous recombination than DSBs in euchromatin (Goodarzi et al. 2010). Also, if the lesion is encountered during transcription or replication the response may be different from lesions encountered outside of these processes. FA proteins are believed to be largely associated with responding to different
types of replication blocking lesions. These lesions include DNA double strand breaks, interstrand crosslinks and stalled replication forks, which will be the focus of our discussion as well as the work in chapters two and three.

3.a. Sensing DNA double strand breaks

DNA double strand breaks, such as those caused by ionizing radiation are highly lethal to cells if left unrepaired. The Mre11-Rad50-NBS1 (MRN) ternary complex is thought be first in sensing the damaged ends (Fig. 2) (Lee and Paull 2004; Lee and Paull 2005; Yuan and Chen 2010). This initial activation of MRN allows for recruitment of many additional proteins including the ATM kinase, the Mediator of the DNA damage Checkpoint 1 (MDC1), and BRCA1/FANCS (Lee and Paull 2004; Lee and Paull 2005; Sawyer et al. 2015). These proteins all work together to activate cell cycle checkpoints and recruit downstream effector proteins. ATM phosphorylates CHK2 kinase as well as H2AX. The CHK2 kinase phosphorylates several checkpoint and DNA repair proteins such as RB, p53 and BRCA1 (Reinhardt and Yaffe 2009). Phosphorylated

![Figure 2. Simplified model of double strand break initiated DNA damage response. Double strand breaks are initially bound by the MRN complex which then recruits the ATM kinase. ATM autophosphorylates and phosphorylates Checkpoint Kinase 2 (CHK2) and the histone variant H2AX. CHK2 and ATM also activate several other proteins through phosphorylation; such as RB, MDC1, p53, and BRCA1. Phospho-H2AX, also called γH2AX recruits MDC1 through MDC1’s BRCT domain. Phospho-MDC1 can then recruit the E3 ligase RNF8 to ubiquitinate histones such as γH2AX. These ubiquitin chains serve to recruit repair proteins. MDC1 can also recruit additional MRN trimers through a separate phospho-site, which likely serves to amplify the ATM-CHK2 signaling cascade.](image-url)
H2AX or γH2AX (the phosphorylation is on the gamma serine in the c-terminus), is bound by MDC1 (Goldberg et al. 2003; Stewart et al. 2003). MDC1 can recruit additional MRN ternary complexes, which has been suggested to be important in tethering large chromatin regions nearby damage sites (Williams et al. 2008; Hari et al. 2010). MDC1 recruits the E3 ubiquitin ligase RNF8 which ubiquitinates histones such as H2A and H2AX (Huen et al. 2007; Mailand et al. 2007; Kolas et al. 2007; Wang and Elledge 2007). This action then feeds back into the pathway by activating additional ATM molecules, which can phosphorylate additional H2AX proteins amplifying the cycle (Wu et al. 2011). In addition to regulating ATM activation, RNF8 also facilitates the recruitment of additional DNA damage proteins such as BRCA1, 53BP1, and the MRN complex (Huen et al. 2007; Mailand et al. 2007; Kolas et al. 2007; Wang and Elledge 2007; Lu et al. 2012).

3.b. Repairing DNA double strand breaks

The repair of double strand breaks follows two primary mechanisms non-homologous end joining (NHEJ) and homologous recombination (Fig. 3) as well as two less understood mechanisms: single strand annealing and microhomology mediated end joining. Non-homologous end joining has been shown to be the most common form of DSB repair (Goodarzi et al. 2011).

Figure 3. Models of DNA repair mechanisms of non-homologous end joining and homologous recombination following a double strand break. In non-homologous end joining (NHEJ), two broken DNA ends are combined to seal the gap. Homologous recombination (HR) requires formation of single stranded DNA through end resection. This single strand DNA is then used by RADS1 (R51) to find a homologous sequence on an undamaged chromosome, shown in blue. When homology is discovered, missing DNA is filled in and the crossed over DNA strands must be cleaved and religated to separate the two undamaged chromosomes. While NHEJ can occur in G1, S and G2, Homologous recombination requires a guide template usually a sister chromatid and occurs mainly in S and G2.
It is mechanistically simple and can respond quickly. The DNA ligase, LigIV, is recruited to the broken DNA ends and simply ligates the ends (Grawunder et al. 1997). LigIV makes no use of sequence information and so it is possible that unrelated sequences can be fused to generate a chromosomal rearrangement. Homologous recombination is more complex and occurs more frequently in heterochromatic regions (Shrivastav et al. 2008; Chapman et al. 2012). Mechanistically, the double stranded DNA must be resected to form single stranded DNA, which serves as a platform for recruiting the single strand binding protein, Replication Protein A (RPA) (Sartori et al. 2007). This step is highly regulated as end resection could cause a loss of sequence information. The RPA filament can activate ATR signaling which may activate the FA pathway (Fig. 4) (Andreassen et al. 2004; Shiotani and Zou 2009). The proteins in the FA-BRCA pathway, such as PALB2 and BRCA1, replace the RPA filament with a Rad51 filament (Godthelp et al. 2006; Rahman et al. 2007; Zhang et al. 2009b; Zhang et al. 2009a; Sy et al. 2009c; Feng and Zhang 2012). Rad51 uses the single strand to search for homologous sequences in nearby undamaged DNA. Once a match is found, new DNA is synthesized to prevent any loss or addition of DNA. This invasion process creates a Holliday Junction (HJ) (Fig. 3 bottom), which must be resolved by nucleolytic incision and unhooking of the two double stranded chromosomes.

### 3.c. Sensing DNA damage through replication stress

The actual mechanism through which the Fanconi Anemia pathway recognizes interstrand crosslinks is unclear, but, as will be discussed later, is likely partially by the sensing of replication stress. Much of the work done to understand the basic replication stress response has focused on two replication inhibiting drugs, hydroxyurea and aphidicolin. Hydroxyurea inhibits nucleotide biosynthesis by inhibiting the ribonucleotide reductase enzyme (Yarbro 1992), while aphidicolin inhibits Pol-α, which generates RNA primers for replication initiation and Okazaki fragments on the lagging strand (Baranovskiy et al. 2014). When the replication
machinery stalls, the helicase which unwinds DNA produces large tracts of single stranded DNA (Fig. 4). This leads to accumulation of RPA, facilitating the stable recruitment of the RPA binding protein ATRIP (ATR interacting protein) (Zou and Elledge 2003). As its name suggests, ATRIP binds and recruits the checkpoint kinase ATR (Ataxia Telangiactasia Mutated and Rad3 related protein) (Zou and Elledge 2003). ATR’s major checkpoint target is the Checkpoint Kinase 1 (CHK1) (Liu et al. 2000; Brown and Baltimore 2003), which phosphorylates proteins such as p53 and Cdc25A that freeze the cell cycle or force cells into senescence or apoptosis (Stracker et al. 2009; Reinhardt and Yaffe 2009). ATR also phosphorylates the translesion synthesis polymerase Polη, which drives the switching from normal polymerases to DNA damage polymerases (Chen et al. 2008b; Göhler et al. 2011). Importantly, ATR also phosphorylates FANCD2 and FANCI, which is essential for the mono-ubiquitination of FANCD2 (Andreassen et al. 2004; Ishiai et al. 2008). This dependency on ATR signaling helps explain how FA proteins are both cell cycle regulated and related to protection of replication forks.
3.d. Repairing DNA interstrand crosslinks

Like double strand breaks, the mechanism for repairing ICLs is context dependent whether within a replication fork or not (Fig. 5). Both mechanisms require cleavage near the ICL to free one side of the DNA. During replication this causes a double strand break which is repaired by homologous recombination. The DSB-DNA must be resected to create a 3’ overhang which can be used for RAD51 mediated strand invasion and homologous recombination. Meanwhile, the ICL, which is now a monoadduct, is bypassed by translesion synthesis machinery and removed by base excision repair (Godthelp et al. 2006; Rahman et al. 2007; Zhang et al. 2009b; Zhang et al. 2009a; Sy et al. 2009c; Feng and Zhang 2012). During non recombination repair of ICLs, such as occurs during G1, cleavage of one side of DNA creates a single strand gap across from a mono-adduct. This can be repaired via a stepwise

Figure 5. Simplified models of interstrand crosslink repair outside and inside of a replication fork. When an interstrand crosslink is encountered outside of replication, such as in G1 it is thought to be repaired through a dual step nucleotide excision repair in which one side of the DNA will be cleaved and translesion synthesis will fill in the gap. Next the mono-adduct left from the first step is removed and the gap is filled by DNA synthesis. When encountered in the context of replication during S-phase, again one strand is cleaved, producing a double strand break like structure. This break is resected and RAD51 is loaded to prepare for homologous recombination. The uncleaved side of the replication fork, which contains the mono-adduct, is filled in by translesion synthesis creating a template for homologous recombination. The mono-adduct is likely removed through a similar method as outside the replication fork.
process which requires translesion synthesis, likely directed by Polymerase-ζ or REV-1 (Sarkar et al. 2006; Hicks et al. 2010), nucleotide excision, and gap synthesis by Polymerase-δ, however the relationship of this mechanism to the FA pathway is still unclear (Sarkar et al. 2006). The complexity of repairing this lesion requires coordination of all of these steps to avoid chromosomal rearrangements or sequence loss. This is the role of the many proteins in the FA pathway.

4. FA proteins and their functions

4.a. The Fanconi core complex and Ube2T

As stated previously, the many FA proteins are all essential in responding to DNA interstrand crosslinks. The role each protein plays in that response, however, is not necessarily shared. The FA-core complex (FA-CC), made up of FANC -A, B, C, E, F, G, L, M, and the FA associated proteins- FAAP20, and FAAP100, is believed to act as a sensor for the interstrand crosslink and activate FANCD2 and FANCI through ligation of a single ubiquitin moiety at lysine 561 and 523 respectively (Garcia-Higuera et al. 2001; Smogorzewska et al. 2007; Sims et al. 2007). This mono-ubiquitination event is catalyzed by FANCL and the E2 ligase UBE2T to monoubiquitinate FANCD2 and FANCI. UBE2T, recently identified as FANCT (Rickman et al. 2015; Virts et al. 2015), is not a member of the core complex and localizes to chromatin constitutively, unlike the FA-CC which is induced to chromatin localization by DNA damage (Fig. 6) (Alpi et al. 2007). It has been suggested therefore that the regulation of FANCT activity occurs primarily at the level of recruitment of the core complex to damage.
The contribution of the individual proteins in the FA core complex is a matter of ongoing work. The core complex has been suggested to be divided into three subcomplexes which can be observed independently through column chromatography (Huang et al. 2014). Although it is difficult to discern whether these subcomplexes are meaningful or artefactual, the evidence purports that FANCL, FANCB and FAAP100 act as the main E3 ligase sub-complex, while FAAP20, FANCA, and FANCG; as well as FANCC, FANCE, FANCF, and FANCM are hypothesized to recruit, stabilize and activate the E3 ligase complex. FA core complex purified from DT40 cells lacking Fanca, Fancc, Fancf or Fancg, possesses *in vitro* ubiquitin ligase activity toward FANCD2. Complexes purified from cells lacking Fancl, Fancb or Faap100; however show no D2 directed ligase activity (Rajendra et al. 2014). Instead, the members of the non-ligase subcomplexes likely serve to recruit and regulate the FANCL subcomplex. FANCL failed to localize to ICLs in FANCG or FANCF deficient HCT116 cells; however FANCL deficiency showed no effect on the crosslink association of FANCG and only a moderate impact on global chromatin association of FANCG (Huang et al. 2014). FANCE has been shown to directly interact with FANCD2, and may serve to bridge FANCD2 and FANCL in the chromatin environment (Pace et al. 2002). The mechanisms which direct recruitment of the core complex to ICLs is a matter of ongoing study, including work discussed in Chapter 3.
FANCM is a prime candidate for an initial sensor of replication stress for the FA core complex. Chromatin loading of the FA core complex is depleted in FANCM deficient cells, however FANCM is observed largely in chromatin fractions (Fig. 6) (Kim et al. 2008). FANCM has been shown to interact with a single strand binding protein FAAP24 (Ciccia et al. 2007), as well as MHF1 and MHF2 which are histone-fold containing proteins that bind to double stranded DNA (Singh et al. 2010; Yan et al. 2010). Experiments with purified FANCM, show that it promotes migration of branched DNA structures such as those that form during replication stalling, as well as Holliday junctions which result from homologous recombination (Gari et al. 2008a; Gari et al. 2008b). FANCM can also promote lesion bypass synthesis at stalled forks and ICLs (Blackford et al. 2012; Huang et al. 2013). This could be essential for generating recombination templates and allowing for replication progression. It is tempting to speculate that the prolonged existence of forked structures during replication of damaged DNA may be essential for activating FANCM at stalled but not progressing replication forks.

Interestingly, FANCM depletion only partially reduces FA pathway activation and chromatin association, indicating the presence of a secondary means of activation. When cells are exposed to psoralen UVA laser stripes, FANCM only localizes to these stripes during S phase, however FANCA, FANCD2, and the Fanconi associated protein FAAP20 all localize to stripes in non-S phase cells (Yan et al. 2010; Yan et al. 2012). While this method is controversial due to the intensity of the laser and the clustered distribution of damage, replication independent and dependent recruitment of Fanconi proteins has also been shown using engineered crosslinks in episomes driven by EBNA (Shen et al. 2009). These experiments showed that both FANCA and FANCD2 can be recruited to crosslinks independently of a replication fork, while BRCA2 was localized only to replication competent episomal crosslinks. Interestingly, recruitment of the FANCM interacting proteins MHF1 and FAAP24 to ICLs are differentially governed by replication as well, with FAAP24 being recruited
to ICLs independent of replication (Yan et al. 2010). The E3 ligase protein RNF8 and its E2 enzyme, UBC13, are essential for the recruitment of FAAP20, FANCD2 and FANCA to laser stripes, however FANCM localized regardless of RNF8 status (Yan et al. 2012). Since RNF8 and UBC13 catalyze K63 linked ubiquitin chain formation (Lok et al. 2011) and FAAP20 is the only known core complex protein which binds K63 chains (Kim et al. 2012; Leung et al. 2012; Ali et al. 2012) it is suspected that RNF8 may recruit the core complex by erecting chains which FAAP20 binds. This suggests that FANCM may sense stalled ICLs forks during replication, while RNF8 may signal the presence of ICLs through FAAP20 recruitment independent of replication.

Recent work has suggested that the FA core complex may have additional roles outside of FANCD2 and FANCI activation in the response to replication stress. For instance, FANCM translocase activity is able to allow stalled replication forks to bypass interstrand crosslinks and allow for replication to continue, independently of FANCF (Blackford et al. 2012; Huang et al. 2013). Further, FAAP20 and FANCA have both been shown to important for recruiting the translesion polymerase REV1 (Mirchandani et al. 2008; Kim et al. 2012). The significance of these mechanisms in protecting cells is unclear, but they suggest that the core complex may function to quickly rescue replication forks and allow for repair without requiring recombination mediated mechanisms. The ability of the core complex to localize to crosslinks outside of replication supports a role in non-recombination mediated repair (Fig. 5). This could be an important mechanism for cells to avoid excessive damage signaling and apoptosis that could result.

4.b. FANCD2 and FANCI

Although it is clear that FANCD2 mono-ubiquitination is a vital step in the response to ICLs, the biochemical function of mono-ubiquitination has remained elusive (Garcia-Higuera et al. 2001). FANCI mono-ubiquitination is less vital for repair of crosslinks, the reason for this
being poorly understood (Smogorzewska et al. 2007). Recent work has shown that non-ubiquitinated FANCI, but not FANCD2, recruits FA-CC to foci, suggesting FANCI acts upstream of core-complex recruitment (Castella et al. 2015). Complementation of FANCD2 or FANCI deficient fibroblasts with WT and non-ubiquinatable mutants suggest that these proteins are dependent upon mono-ubiquitination in order to re-locate to nuclear foci as well as the detergent insoluble chromatin fraction after cell lysis (Garcia-Higuera et al. 2001; Smogorzewska et al. 2007; Sims et al. 2007). Interestingly, FANCD2 and FANCI are important for maintaining one another’s levels in the cells and monoubiquitination status (Smogorzewska et al. 2007; Sims et al. 2007). The mechanism by which FANCD2 and FANCI regulate one another has been informed by both biochemical and structural work. Initially, it was observed that both wild type and ubiquitin mutant, K561R and K523R respectively, FANCD2 and FANCI interact in cells, suggesting that these proteins interact in the non-ubiquitinated states (Smogorzewska et al. 2007; Sims et al. 2007). However, because they were dependent on one another for ubiquitination and they both contain ubiquitin binding domains, it was hypothesized that ubiquitination may strengthen the interaction by allowing the ubiquitin binding domains to interact with the ubiquitin moiety and protect it from de-ubiquitination (Smogorzewska et al. 2007). This later turned out to be untrue, as it was discovered that FANCD2 and FANCI interact exclusively in their unmodified forms (Sareen et al. 2012). This is supported by crystallographic evidence which shows that the ubiquitinated lysines on FANCD2 and FANCI are buried in their interface, suggesting that interaction of FANCD2 and FANCI actually inhibits monoubiquitination (Joo et al. 2011). Instead, FANCI phosphorylation by ATR dissociates it from FANCD2, allowing FANCL to catalyze mono-ubiquitination of the former complex members (Fig. 5) (Meetei et al. 2003; Sareen et al. 2012). Further, FANCI typically shows lower levels of ubiquitination than FANCD2, and this ubiquitination tends to arise later in the damage response; when compared with the resistance of FANCI-ubiquitin mutant cells to MMC, it suggests different functions of ubiquitination of FANCI and FANCD2 (Smogorzewska et al. 2007; Sareen et al. 2012). However
it is likely that tight regulation of D2 mono-ubiquitination is important, given that loss of the D2-deubiquitinating enzyme USP1 leads to extreme MMC sensitivity (Oestergaard et al. 2007).

Interestingly, while this evidence clarifies how FANCD2 and FANCI regulate each other’s activation, it doesn’t answer why FANCD2 and FANCI are required for maintenance of their own mono-ubiquitination (Smogorzewska et al. 2007; Sims et al. 2007).

After initial discovery, the regulation and function of FANCD2 were mysteries given the lack of clear functional and structural domains. FANCD2 has an nuclear localization signal (NLS), a ubiquitin binding CUE domain, a DNA interacting domain called an EDGE domain, and a PIP (PCNA interacting protein) box (Montes de Oca et al. 2005; Howlett et al. 2009; Rego et al. 2012). As would be expected, loss of the NLS prevents FANCD2 from accumulating in the nucleus (Boisvert et al. 2013). Mutation of the ubiquitin binding CUE domain results in deficiency in FANCI binding, FANCD2 protein instability, and MMC resistance; however the CUE domain appears to be dispensible for D2 monoubiquitination (Rego et al. 2012). Mutation of the PIP box, which facilitates FANCD2’s interaction with PCNA, inhibits monoubiquitination, focus formation, and rescue of MMC resistance in FANCD2 deficient cells (Howlett et al. 2009). Additionally, Rad18, the E3 ligase responsible for PCNA ubiquitination during replication stress, acts upstream of FANCD2 ubiquitination (Hoege et al. 2002; Nakajima et al. 2006; Williams et al. 2011; Palle and Vaziri 2011), and FANCD2 colocalizes with PCNA after DNA damage (Hussain et al. 2004; Howlett et al. 2005). Because of these findings, it was hypothesized that PCNA would be one of the primary activators of FANCD2; however, knockdown of PCNA, unlike mutation of the PIP box, does not impact FANCD2 mono-ubiquitination (Williams et al. 2011). Whether mono-ubiquitinated FANCD2 was able to form foci after PCNA knockdown was not tested, so it may still be possible that ub-PCNA may play a role in recruiting FANCD2 to foci.

Together these data suggest that the PIP box mutations inhibited FANCD2 mono-ubiquitination by some non-PCNA mediated function, and the CUE domain is largely important to support
stability and FANCI binding. Again, these results tell us little about FANCD2’s biochemical functions in the maintenance of genome integrity.

In addition to their well known role in protecting the genome from interstrand crosslinks, FANCD2 and FANCI are important in the maintenance of replication, particularly at what are termed common fragile sites (CFSs). Common fragile sites are regions of the genome which are frequent sites of replication errors. They have been shown to be overrepresented as sister chromatid exchange sites, regions lost during cancer progression, sites of viral integration, and regions of chromosomal translocations (Arlt et al. 2006). FANCD2 and FANCI localize to highly expressed CFSs and ultrafine DNA bridges, regions of failed replication that connect two daughter chromosomes during mitosis (Chan et al. 2009; Naim and Rosselli 2009). Importantly, loss of FANCD2 or FANCA exacerbates CFS phenotypes, indicating that the FA pathway is active at CFSs and protects them from aberrations that arise during replication (Howlett et al. 2005). Besides the FA pathway’s role in protecting CFSs during the replication, some evidence suggests that FANCD2 may interact with MCM helicases, which unwind DNA ahead of the replication fork, to assist efficient fork firing (Song et al. 2010; Lossaint et al. 2013; Panneerselvam et al. 2014). The importance of FANCD2 to replication initiation in healthy cells is unclear given that FANCD2 deficient PD20 patient fibroblasts proceed from G1 through S into G2 phase at a near equal rate as corrected cells either untreated or treated with MMC (Taniguchi et al. 2002a). This contradiction, that although D2 is clearly activated in S-phase independent of damage (Taniguchi et al. 2002b), unperturbed cells still replicate DNA in the absence of FANCD2 confounds the work on non-ICL mediated functions of FANCD2.

While FANCD2 and FANCI’s functions in repairing ICLs still need clarification, several putative functions have been found using co-immunoprecipitation studies. These include nuclease recruitment, translesion synthesis, and histone chaperoning. FAN1, (FA Associated Nuclease 1) is a nuclease that has been found to bind to FANCD2 and FANCI – binding which
is enhanced by monoubiquitination of either protein (MacKay et al. 2010; Smogorzewska et al. 2010; Liu et al. 2010). Additional work confirms that monoubiquitination of FANCD2 and FANCI are required for FAN1 localization to DNA damage foci. FAN1 possesses nuclease activity for DNA arms particularly when tested against a 5’ flap, a structure expected to occur at an ICL during replication (Fig 7) (Kratz et al. 2010; MacKay et al. 2010; Shereda et al. 2010; Smogorzewska et al. 2010; Liu et al. 2010). Further, both a nuclease dead mutant and an ubiquitin binding mutant of FAN1 were unable to correct cellular sensitivity to MMC (Liu et al. 2010). FANCD2 has also been implicated in the recruitment of the Fanconi Protein SLX4/FANCP to sites of DNA damage. Initial work showed that an exogenous GFP tagged SLX4 formed foci which colocalized with FANCD2, and cells which had impaired FANCD2 activation (FANCC−/− or FANCD2K561R) showed impaired SLX4 focus formation as well (Yamamoto et al. 2011). This finding, however, has been contradicted by a separate study which shows no dependence on the Fanconi pathway for recruitment of SLX4 to sites of DNA damage (Lachaud et al. 2014). Given SLX4’s role in organizing and coordinating other nuclease such as XPF (FANCQ) and Mus81, clarifying if and how FANCD2 regulates SLX4, and possibly FANCQ and Mus81, will be important information for the field. In addition to these endonucleases, FANCD2 also has been shown to
interact with and regulate the exonuclease CtIP (Yeo et al. 2014) which is responsible for resecting double stranded DNA to generate single stranded DNA overhangs during homologous recombination and replication stress (Sartori et al. 2007; Duquette et al. 2012). Finally, FANCD2 itself has been proposed to contain some nucleolytic activity, however in the 3’-5’ direction (Pace et al. 2010).

Non-nuclease related functions of FANCD2 have also been described. The translesion synthesis polymerase Pol-η has been shown to interact with FANCD2 which may be essential for recruitment of Pol-η to DNA lesions (Fu et al. 2013; Chen et al. 2015). Pol-η may synthesize new DNA past the ICL, which could create a template for homologous recombination (Fig. 8). Direct comparisons of Pol-η and REV1/Pol-ζ have shown that REV1/Pol-ζ, which are recruited by members of the FA core complex, may be more important for TLS of ICLs (Hicks et al. 2010). Pol-η has also been proposed to synthesize DNA during homologous recombination (McIlwraith et al. 2005), suggesting that recruitment of Pol-η may be more important for completing HR and secondarily important for TLS. An additional proposed function of the FANCD2 protein has to do with histone chaperoning. This activity was initially identified during a screen for proteins which interact with H3-H4 complex and confirmed using a superhelical formation assay (Sato et al. 2012). A patient derived mutation R302W was identified which was able to be ubiquitinated and localize to chromatin, however failed to act as a histone chaperone in vitro and rescue cisplatin sensitivity. The function of this histone chaperoning activity, however, remains to be clarified. All together, this suggests that the primary function of FANCD2 may be to regulate nuclease activity and generate chromatin structures including newly synthesized DNA, which can be repaired by homologous recombination. The interplay between all of these functions is unclear, but suggests that FANCD2’s role involves removing damaged nucleotides and preparing DNA for downstream events such as homologous recombination.
4.c. BRCA1

While the core complex and FANCD2 and FANCI are essential for crosslink repair through homologous recombination, they seem to be dispensable for double strand break initiated homologous recombination and resistance to ionizing radiation (Kalb et al. 2004; Nakanishi et al. 2011). This is in stark contrast with the FA-BRCA proteins which are dispensible in FANCD2 monoubiquitination. Five of these proteins, FANCS/BRCA1, FANCN/PALB2, FANCD1/BRCA2, FANCO/RAD51C, and XRCC2/FANCU play integral roles in facilitating RAD51/FANCR filament formation and strand invasion (Godthelp et al. 2002; Vaz et al. 2010; Buisson et al. 2010; Dray et al. 2010; Carreira and Kowalczykowski 2011; Wang et al. 2015). BRCA1 also works in a complex with FANCJ/BACH1/BRIP1, a DNA helicase required for several aspects of chromosome stability, including efficient HR. When most of these proteins are mutated in a heterozygotic manner, they cause familial breast and ovarian cancer, or BRCA, syndrome (Miki et al. 1994; Wooster et al. 1995; Cantor et al. 2001; Rahman et al. 2007; Meindl et al. 2010; Park et al. 2012). Heterozygous loss of core complex members (aside from FANCM (Kiiski et al. 2014)), FANCD2 or FANCI has not yet been associated with any increased cancer susceptibility, suggesting that these proteins may not function in the same breast and ovarian cancer protective pathway as the FA/BRCA proteins. Interestingly, the FA patients deficient in these breast cancer associated (BRCA) genes, have some distinct clinical features. The two patients identified with biallelic BRCA1-loss presented with either breast or ovarian cancer in their twenties (Domchek et al. 2013; Sawyer et al. 2015). FANCD1 and FANCN patients tend to present with solid tumors such as Wilms’s tumor as well as leukemic transformation very early in childhood, usually 1-3 years (Hirsch et al. 2004; Reid et al. 2007; Myers et al. 2012). Patients deficient in core complex members tend to present with leukemia later, usually in the early teens if at all (Alter 2014). The functions of these proteins have been largely worked out in response to DSBs, but most work (including work presented in this dissertation) has suggested that many of these functions apply in the response to ICLs as well.
BRCA1/FANCS is among the best known DNA damage response proteins. It was initially implicated as a DNA damage response protein when shown to colocalize with the Rad51 and PCNA before and after treatment with a variety of DNA damage agents (Scully et al. 1997a; Scully et al. 1997b). BRCA1 mediates many functions of the DNA damage response including checkpoint activation (Zhu and Dutta 2006), loading Rad51 filaments for DNA strand invasion (Greenberg et al. 2006), and recruitment of downstream proteins such as FANCJ (Cantor et al. 2001). Many of these functions are involved in priming DNA for homologous recombination, and as would be expected, loss of BRCA1 reduces the efficiency of homologous recombination (Moynahan et al. 1999). Interestingly, the loss of the pro-NHEJ protein, 53BP1, is able to correct many of the phenotypes of BRCA1 loss in BRCA1Δ11 mice (Cao et al. 2009). These phenotypes include embryonic survival and tumor resistance (Cao et al. 2009), as well as Rad51 foci formation, homologous recombination and resistance to ionizing radiation (Bunting et al. 2010). This finding suggests that BRCA1 has a vital role in making the switch from end-joining pathways to homologous recombination pathways.
Studies of the different domains of BRCA1 have clarified the mechanisms it uses in the DDR (Fig. 9). The N-terminus of BRCA1 contains an E3 ligase RING domain which facilitates formation of the constitutive dimer BARD1 (Wu et al. 1996; Hashizume et al. 2001). The central region of BRCA1 is made of a single huge exon (exon 11) which can be spliced out to create a shortened semi-functional version of the protein, which still contains all of the known motifs aside from the nuclear localization signal (Huber et al. 2001). After exon 11 is a coiled-coil domain that facilitates BRCA1's interaction with the FA-BRCA protein PALB2/FANCN. As well as a string of ATM and ATR phospho-target sites which are partially phosphorylated during S-Phase and then hyper-phosphorylated after induction of DNA damage (Chen et al. 1996; Scully et al. 1997a; Gatei et al. 2001). Mutation of the phospho-sites reduces HR efficiency and sensitizes cells to IR (Cortez et al. 1999; Beckta et al. 2015). Finally, the extreme C-terminus of BRCA1 contains phosphoprotein binding repeat domain now termed BRCA1 c-terminal repeats or (BRCT repeats) (Rodriguez et al. 2003; Yu et al. 2003).

The N-terminal RING domain allows BRCA1 to heterodimerize with BARD1, which is essential for BRCA1 protein stability (Joukov et al. 2001; McCarthy et al. 2003). The function of the ubiquitinating activity of the RING domain remains controversial. Two point mutants inhibiting the catalytic activity have been engineered into mice with different results. The I26A mutant preserves Bard1 binding and shows normal developmental progression (Shakya et al. 2011); however the Brca1 C61A mutation, which does not preserve Bard1 binding (Brzovic et al. 2003), causes embryonic lethality (Drost et al. 2011). Reid and colleagues (2008) showed that mouse ES cells expressing the I26A variant had wild type like resistance to MMC, and Shakya et al. (Shakya et al. 2011) show that Murine embryonic fibroblasts, (MEFs) that express Brca1 I26A have no increase in MMC sensitivity. Tian et al. (Tian et al. 2013); however, show that the I26A mutation leaves MEFs MMC-sensitive to a similar degree as a commonly used exon 11 deletion. In addition to the controversial catalytic function, the RING domain functions as a
protein binding unit, most notably with the MRN complex (Chen et al. 2008a). Interaction with this complex is important orchestrating end resection and the removal of damaged nucleotides (Yu et al. 2006; Chen et al. 2008a). The MRN complex is essential for FANCD2 stability and resistance to interstrand crosslinks (Roques et al. 2009), which is interesting given that BRCA1 has been shown to act upstream of FANCD2 activation (Garcia-Higuera et al. 2001). This leads to the hypothesis that BRCA1 may regulate FANCD2 indirectly through regulating other proteins, including proteins which bind the BRCA1 BRCT repeats.

The BRCA1 BRCT repeats are a phosphoprotein binding motif (Rodriguez et al. 2003; Yu et al. 2003), which has three known interacting partners, CtIP, FANCJ, and Abraxas. All of these proteins are phosphorylated in a DNA damage independent manner allowing them to bind to the repeats (Yu et al. 2003; Kim et al. 2007; Liu et al. 2007; Buis et al. 2012). The BRCA1 C-
terminal Interacting Protein, CtIP, has been shown to stimulate MRE11’s 5’ exonuclease activity in the response to double strand breaks and interstrand crosslinks (Sartori et al. 2007; Duquette et al. 2012). This exonuclease activity seems to be important for creating single stranded DNA for RAD51 (Fig. 7), but is also important for the G2/M checkpoint through the RPA-ATR-CHK1 axis (Greenberg et al. 2006; Yuan and Chen 2010). Knockdown of CtIP in cells treated with psoralen-UVA stripes, which cause interstrand crosslinks, led to decreases in the accumulation of γH2AX, RPA, ATR, and FANCD2, although this was not seen when cells were treated with BrdU+UVA stripes which causes double strand breaks (Duquette et al. 2012). Recruitment of CtIP to ICL stripes, but not DSB stripes was dependent on FANCM, consistent with a model suggesting FANCM is an early sensor of crosslinks (Fig. 6). This finding conflicts with other work showing that IR induced RPA foci do require CtIP (Yuan and Chen 2010), but it could be that UVA stripes produce large amounts of damage in a single line and may be more readily visible than IR foci. Surprisingly, FANCD2 deficient cells also show reduced CtIP accumulation, suggesting that FANCD2 functions both upstream and downstream of CtIP (Yeo et al. 2014).

Interestingly, homozygous loss and expression of dominant negative mutants of CtIP have been observed in patients with Seckel syndrome, which can also be caused by ATR deficiency (O’Driscoll et al. 2003; Qvist et al. 2011). This suggests that a key role of CtIP in vivo may be to activate ATR signaling by generating single stranded DNA.

The function of CtIP’s interaction with BRCA1 appears to be context dependent. It has been reported that BRCA1 ubiquitinates

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Fig. 10. FANCI interaction with BRCA1 and MLH1 drives repair mechanism selection. FANCI recruitment to ICLs or DSBs is differentially regulated by the FA-Core complex or the MRN complex respectively. At ICLs FANCI is required for inhibiting MLH1 directed mismatch repair. This inhibition can drive BRCA1 interaction dependent homologous recombination, or BRCA1 independent Pol-η dependent translesion synthesis.
CtIP, facilitating its chromatin localization after ionizing radiation (Yu et al. 2006). The results of the BRCA1-RING domain mutant studies described above, however, call the importance of this finding into question. Regardless of ubiquitination, expression of Brca1 binding mutant Ctip in DT40 cells showed that disrupting the Ctip-Brca1 interaction yielded intermediate sensitivity to ionizing radiation (Yun and Hiom 2009), as well as in human U2OS cells (Chen et al. 2008a). A similar experiment in Mouse embryonic stem cells expressing a Brca1 interaction deficient Ctip display near wild-type resistance to MMC and camptothecin and barely reduced DSB induced HR (Reczek et al. 2013). Further, rescue of Brca1 mutation by deletion of 53bp1 is dependent upon Ctip mediated end resection, suggesting that Ctip can function independently of Brca1 (Polato et al. 2014). In the DT40 cell model, it has been shown that Ctip interaction with Brca1 has greater importance in facilitating homologous recombination at sites with monoadducts such as those damaged by camptothecin and etoposide (Nakamura et al. 2010). This has also been seen in xenopus extracts (Aparicio et al. 2016). As such, the importance of the BRCA1-CtIP interaction is likely damage specific.

The FANCJ/BACH1/BRIP1 helicase also binds to the BRCT-repeats of BRCA1 and like BRCA1 is a familial breast cancer gene (Cantor et al. 2001). The helicase activity of FANCJ has been shown to be effective against forked DNA and D-loops, like those formed during homologous recombination (Gupta et al. 2005). FANCJ has also been shown to unwind difficult regions such as G-quadruplex DNA (Castillo Bosch et al. 2014). Interestingly, recruitment of FANCJ to double strand breaks, but not ICLs, appears to be dependent on MRE11 mediated end resection. Conversely, FANCJ was dependent on the FA core complex for recruitment to ICLs but not DSBs (Fig. 10) (Suhasini et al. 2013). This could be due to FANCJ needing a single strand overhang in order to function as a helicase and different mechanisms for generating the appropriate overhang based on the type of lesion. FANCJ, along with BRCA1, is required for efficient FANCD2 focus formation in response to ICLs but less so to DSBs.
Mutants of FANCJ that inhibit BRCA1 interaction do not support FANCD2 focus formation, suggesting that BRCA1’s role in recruiting FANCD2 to ICL foci may involve FANCJ as well as possibly CtIP (Zhang et al. 2010; Chen et al. 2014). Surprisingly, expression of BRCA1 binding defective FANCJ in FANCJ null cells fully rescues MMC sensitivity. Instead, FANCJ interaction with the mismatch repair (MMR) protein MLH1 is essential for resistance (Peng et al. 2007). To better understand this phenomenon, Xie et al. analyzed FANCJ mutants and showed that while the BRCA1 binding S990A mutant can complement MMC sensitivity, the repair mechanism of choice seems to be altered from homologous recombination to Polymerase-η dependent translesion synthesis. In accordance with this, the MMC resistance of S990A corrected cells was completely abrogated by knockdown of polymerase-η, while cells corrected with wild-type FANCJ retained their resistance despite Pol-η knockdown (Xie et al. 2010). Together, this suggests that FANCJ has dual roles in promoting MMC resistance, the mechanism of which may be dependent on BRCA1 interaction (Fig. 10). FANCJ upregulates TLS when not bound to BRCA1 and promotes homologous recombination when bound to BRCA1, in addition to its role in unwinding difficult regions during replication.

The final BRCT binding phospho-protein, Abraxas, is a component of a 5 member complex that is recruited by the ubiquitin binding protein RAP80 to ubiquitinated histones (Wang et al. 2007). The other three members of this complex include MERIT40, BRCC36, and BRCC45, and all are required for stability and function of the complex (Dong et al. 2003; Chen et al. 2006; Wang and Elledge 2007; Wang et al. 2009; Shao et al. 2009b). This complex seems to play a balancing role in the DNA damage response. For instance, BRCC36 is a de-ubiquitinase which can degrade the K63 chains created by RNF8 (Shao et al. 2009a; Feng et al. 2010). RAP80 may also be an important signal to prevent excessive end resection (Hu et al. 2011; Coleman and Greenberg 2011). When cells lose RAP80, a concomitant increase in the size of RPA foci can be seen after treatment with ionizing radiation. This suggests that larger
than normal tracts of single stranded DNA are being formed, but the mechanism behind this is still under investigation. In addition to these functions, this complex, through phospho-Abraxas, recruits BRCA1 to DNA damage foci (Wang et al. 2007; Liu et al. 2007; Wang and Elledge 2007). Further, as will be seen in chapters 2, because BRCA1 interacts with the BRCA-Fanconi protein PALB2/FANCN through BRCA1’s coiled-coil domain, Abraxas and RAP80 are also important for recruiting PALB2 to these foci.

4.d. PALB2 and BRCA2

The BRCA1 coiled-coil domain facilitates BRCA1’s interaction with the RAD51 loading proteins BRCA2 and PALB2, which are also essential for homologous recombination (Fig. 9) (Zhang et al. 2009b; Sy et al. 2009a; Zhang et al. 2009a). BRCA2, as its name implies was the second gene found to be a causative agent in familial breast and ovarian cancer syndrome (Wooster et al. 1995). PALB2 (Partner and Localizer of BRCA2) was discovered to be the protein essential for recruiting BRCA2 to DNA damage (Xia et al. 2006) and is also implicated in familial breast and ovarian cancer syndrome (Rahman et al. 2007; Erkko et al. 2007). PALB2 interestingly, may not show loss-of heterozygosity events in tumors (Tischkowitz et al. 2007), and PALB2 heterozygosity has been associated with increases in DNA damage responses (Nikkilä et al. 2013). BRCA1 and BRCA2 cancer and surrounding non-malignant tissue show a high rate of LOH events (Osorio et al. 2002; Cavalli et al. 2004). Unlike BRCA1 to date, PALB2 and BRCA2 have also been noted to play a role in predisposing to pancreatic cancer as well (Roy et al. 2012; Pauty et al. 2014). These three proteins seem to form a recruitment chain, by which BRCA1 recruits PALB2 via interactions in their coiled coil domains (Fig. 9) (Zhang et al. 2009b; Sy et al. 2009a; Zhang et al. 2009a). PALB2 then recruits BRCA2 by interactions in PALB2’s C-terminal WD40 repeats with the N-terminus of BRCA2 (Xia et al. 2006; Xia et al. 2007; Park et al. 2014a). BRCA2 contains several motifs called BRC repeats, (distinct from BRCT repeats) which play a primary role in loading Rad51 onto single stranded DNA and
replacing RPA (Wong et al. 1997; Pellegrini et al. 2002; Carreira and Kowalczykowski 2011). In vitro work has shown that a smaller version of BRCA2 can stimulate this loading of RAD51 as well as strand invasion. Further, this work showed that PALB2, when added to the reaction, could further stimulate loading and strand invasion (Buisson et al. 2010; Dray et al. 2010). Suggesting that not only does PALB2 simply recruit BRCA2, it plays a supportive role in BRCA2’s RAD51 loading function. In support of this, PALB2 also forms complexes with RAD51 paralogs, such as XRCC3 and RAD51C/FANCO (Park et al. 2014a). It is known that the different RAD51 paralogs modulate and enhance RAD51’s functions in homologous recombination and are essential for resistance to DNA damage (Takata et al. 2000; Takata et al. 2001). Given the central role of RAD51 in HR it is perhaps unsurprising that it is regulated by so many support proteins and PALB2 is central in this process.

That PALB2 interacts directly with so many proteins suggests it may play a role in coordinating all of these FA and BRCA proteins in the DNA damage response (Park et al. 2014b). As such, PALB2 is also regulated in several ways, in addition to BRCA1 mediated recruitment. For instance, PALB2 is also regulated by the chromatin remodeling protein MRG15 (Sy et al. 2009b; Hayakawa et al. 2010). MRG15 is a member of the TIP60-histone-acetyltransferase complex, and is required for efficient recruitment of both 53BP1 and PALB2 to IR induced foci (Garcia et al. 2007; Hayakawa et al. 2010). PALB2 interacts with MRG15 through a central region of PALB2, unlike BRCA1 or BRCA2 and RAD51C, which it interacts with in either the extreme N or C terminus, respectively. While it is clear that MRG15 is important for resistance to DNA damage (Hayakawa et al. 2010), the mechanism it plays is unclear. One report suggests that loss of MRG15 reduces homologous recombination as measured by a DR-GFP reporter (Hayakawa et al. 2010). In conflict with this, loss of MRG15 or expression of an MRG15 binding deficient PALB2 strongly increased the number of gene conversions, a separate methodology to assess global homologous recombination (Sy et al. 2009b). PALB2
also contains a centrally located Chromatin Associated Motif or ChAM domain which allows it to interact with nucleosomes but not histones or DNA exclusively (Fig. 9) (Bleuyard et al. 2011). Deletion of this motif reduces chromatin association of PALB2, as well as PALB2 and RAD51 focus formation and MMC resistance (Bleuyard et al. 2011). The single strand binding protein RPA has also been implicated in recruiting PALB2 to damage (Murphy et al. 2014). This could help signal to the recombination machinery that end resection has occurred and ssDNA is available for formation of RPA filaments. PALB2 is also regulated by KEAP1-CULLIN3 binding during the G1 phase. This complex ubiquitinates PALB2 on the coiled-coil, preventing BRCA1 dimerization and recruitment to foci (Orthwein et al. 2015). Since sister chromatids do not exist during G1, cells repress this repair mechanism outside of S and G2 phase. Given the evidence that PALB2 is a central player in recruiting RAD51 and RAD51 loading proteins, these multiple levels of regulation are essential for preventing inappropriate recombination, which could cause oncogenic chromosomal rearrangements.

Additional work suggests that PALB2 plays a role in regulating the transcription of ROS responsive genes. Ma and colleagues identified KEAP1 as an interacting partner of PALB2 (2012). KEAP1 targets NRF2, which positively regulates ROS responsive genes, targeting it for degradation (Pang and Andreassen 2009; Taguchi et al. 2011). The interaction of PALB2 with KEAP1 was shown to compete with NRF2 for KEAP1 interaction (Ma et al. 2012). This competition prevents KEAP1 from ubiquitinating NRF2 allowing it to activate transcription of ROS responsive genes. While this activity did not require PALB2 to interact with BRCA1 or BRCA2, BRCA1 has also been shown to interact with NRF2, protecting it from KEAP1 mediated degradation (Gorrini et al. 2013). The confluence of BRCA1 and PALB2 in NRF2 transcriptional activation may be key to understanding breast and ovarian cancers which arise from these mutants.
5. Rationale and findings in this dissertation

Given PALB2’s central role in promoting RAD51 mediated homologous recombination, understanding its regulation is essential to our knowledge of BRCA mediated recombination repair. However, when the interaction of PALB2 and BRCA1 was demonstrated via their coiled coil domains (Zhang et al. 2009b; Zhang et al. 2009a), PALB2’s coiled-coil was also shown to support homo-dimerization (Sy et al. 2009c). This left uncertainty about the functions and importance of the heterodimer versus the homodimer. What was known is that BRCA1 is required for efficient PALB2 localization to foci, and interruption of the PALB2 coiled-coil inhibited its localization and its ability to rescue DNA damage sensitivity. Since the only known coiled-coil function was localization we sought to test whether rescuing localization capacity in a coiled coil mutant would rescue the function of PALB2 in localizing RAD51 to foci and repairing damaged DNA. To answer this we fused the BRCA1 BRCT repeats to coiled-coil mutant PALB2 and assessed PALB2 function. Our BRCT-PALB2 fusion coiled-coil mutant was indeed able to rescue all tested functions of PALB2 in the response to DNA damage, suggesting that the primary function of the coiled-coil was to localize PALB2 to sites of DNA damage. We then sought to use this fusion to assess the mechanism by which the RAD51 loading PALB2/BRCA2 complex was recruited to nuclear foci. Interestingly, both the fusion protein and endogenous PALB2 foci were shown to be dependent on ABRAXAS, RAP80, RNF8, and MDC1 in response to DSBs. This is in agreement with RAP80’s role in localizing BRCA1. This work was published in 2012 (Zhang et al. 2012) and can be found in chapter 2 entitled “MDC1 and RNF8 function in a pathway that directs BRCA1-dependent recruitment of PALB2 required for homologous recombination”.

After discovering that PALB2 is dependent on the MDC1-RNF8 ubiquitin signaling pathway, we became interested in the possibility of RNF8 signaling being a central player in coordinating the upstream FA pathway and the FA-BRCA pathway. The fact that processing of
ICLs by the FA pathway creates double strand breaks, single stranded DNA overhangs and monoadducts throughout the process suggests that it could be highly deleterious to the cell if all of the steps of repair were not coordinated. RNF8 was a prime candidate to regulate both pathways. We first show that, although FANCD2 and PALB2 colocalize at multiple time points, their recruitment to foci is independent of one another. We also show that RNF8 mediated ubiquitin signaling is important for recruiting both FANCD2 and PALB2 to interstrand crosslinks. Interestingly, MDC1, which recruits RNF8 to double strand breaks, is essential only for PALB2 recruitment to interstrand crosslinks and double strand breaks. Further, we found that the ubiquitin binding proteins FAAP20 and RAP80 served to specifically recruit either FANCD2 or PALB2, respectively. This was the first study to test the regulation of upstream-FA and FA-BRCA protein recruitment side by side and suggests two different ubiquitin signaling pathways which recruit upstream-FA or FA-BRCA proteins. Clarifying the regulation of the FA and BRCA pathways in a side by side manner has created a deeper understanding of how the pathways might function together in order to repair DNA during replication and thereby maintain genomic stability. This work was published in 2016 (Bick et al. 2016) and can be found in Chapter 3 entitled “Coordination of the recruitment of the FANCD2 and PALB2 Fanconi Anemia proteins by an ubiquitin signaling network.” Our work sheds light on the mechanisms which regulate FA protein networks which protect cells from accumulating DNA damage causing cell death or transformation, making this work germane to the understanding of a variety of different health and disease states.
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Chapter 2. MDC1 AND RNF8 FUNCTION IN A PATHWAY THAT DIRECTS BRCA1-DEPENDENT LOCALIZATION OF PALB2 REQUIRED FOR HOMOLOGOUS RECOMBINATION

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Summary

The PALB2 protein is associated with breast cancer susceptibility and Fanconi anemia. Notably, PALB2 is also required for DNA repair by homologous recombination (HR). The mechanisms that regulate PALB2, and the functional significance of its interaction with the BRCA1 breast cancer susceptibility protein, are poorly understood, however. Here, to better understand these processes, we fused PALB2, or the PALB2(L21P) mutant which cannot bind to BRCA1, with the BRCT repeats that are present in, and which localize, BRCA1. Our results yield important insight into the regulation of PALB2 function. Both fusion proteins can bypass BRCA1 to localize to sites of DNA damage. Further, the localized fusion proteins are functional, as determined by their ability to support the assembly of RAD51 foci, even in the absence of the capacity of PALB2 to bind BRCA1. Strikingly, the localized fusion proteins mediate DNA double-strand break (DSB)-initiated HR and resistance to mitomycin C in PALB2-deficient cells. Additionally, we show that the BRCA1-PALB2 heterodimer, rather than the PALB2-PALB2 homodimer, mediates these responses. Importantly, we offer the first insight into how BRCA1-dependent recruitment of PALB2 is integrated with other DNA damage signaling pathways. We find that PALB2 localization depends on the presence of MDC1, RNF8, RAP80, and Abraxas upstream of BRCA1. Thus, PALB2 may link HR to a key ubiquitin-related signaling pathway that responds to DSBs.
1. Introduction

PALB2 is encoded by a breast cancer susceptibility (Erkko et al., 2007; Rahman et al., 2007) and Fanconi anemia (FA) (Reid et al., 2007; Xia et al., 2007) gene. We and others have demonstrated that PALB2 functionally links the products of the two major breast cancer susceptibility genes, BRCA1 and BRCA2, respectively, by direct interactions with its N- and C-termini (Sy et al., 2009a; Zhang et al., 2009a; Zhang et al., 2009b). Further, PALB2 mediates BRCA2-dependent oligomerization of the RAD51 recombinase required for strand invasion during homologous recombination (HR) and also directly interacts with RAD51 (Buisson et al., 2010; Dray et al., 2010).

BRCA1 is a large protein, including a N-terminal RING domain, a coiled-coil domain that interacts with PALB2 (amino acids 1391-1424), and two C-terminal BRCT repeats (amino acids 1646-1863) (Moynahan and Jasin, 2010). The two BRCT repeats are required for the localization of BRCA1 to sites of DNA damage (Scully et al., 1999) and mediate interactions with phosphoproteins, including CtIP, BRIP1/FANCJ, and Abraxas (Wang et al., 2007; Yu et al., 2003; Yu et al., 1998).

While BRCA1 is involved in HR (Moynahan et al., 2001), its exact function in this process and the role of the PALB2-BRCA1 interaction are not well understood. Among the possibilities, it has been suggested that the PALB2-BRCA2-RAD51 complex interacts with BRCA1 after it is independently recruited to sites of DNA damage (Sy et al., 2009a). Alternatively, the interaction with BRCA1 may localize PALB2 (Zhang et al., 2009a; Zhang et al., 2009b). A coiled-coil motif at the N-terminus of PALB2 (amino acids 9-44) mediates both homo-oligomerization of PALB2 and its hetero-oligomerization with BRCA1 (Sy et al., 2009a; Sy et al., 2009b; Zhang et al., 2009a; Zhang et al., 2009b). The specific role of these homo- and hetero-oligomers in mediating the various functions of PALB2 is unclear.
The MDC1 checkpoint protein is localized to sites of DNA damage by binding to γH2AX (Stewart et al., 2003; Stucki et al., 2005). RNF8, in turn, binds to MDC1, and mediates protein ubiquitination in response to DNA damage (Huen et al., 2007; Huen et al., 2010; Kolas et al., 2007; Mailand et al., 2007). RAP80 and are also components of this signaling pathway. RAP80 is an ubiquitin-binding protein that forms a complex with Abraxas and BRCA1 (Kim et al., 2007b; Wang et al., 2007). The role of RAP80 and Abraxas in HR has remained controversial (Coleman and Greenberg, 2011; Hu et al., 2011; Wang et al., 2007), but they have a clear function in regulating the G2 DNA damage checkpoint (Kim et al., 2007a; Sobhian et al., 2007; Wang et al., 2007). As a measure of the complexity of DNA damage responses, other proteins, including CtIP and the MRE11-RAD50-NBS1 complex, are also involved in HR. CtIP and the MRE11-RAD50-NBS1 complex are important for end resection (Huen et al., 2010; Sartori et al., 2007).

Given controversy about the importance of the PALB2-BRCA1 interaction, and to better understand the regulation of PALB2 function, and of DNA repair by HR, we generated a PALB2 fusion protein containing the BRCT repeats of BRCA1. Subsequent mutation of the PALB2 coiled-coil domain yielded an additional fusion protein, BRCT-PALB2(L21P), which is incapable of binding to BRCA1 but which has the potential to bypass BRCA1 and localize to sites of DNA damage via BRCA1-derived BRCT repeats. The results have yielded important insights into the regulation and function of the products of the BRCA1 and PALB2 tumor suppressor genes in cellular responses to DNA damage. We clearly demonstrate that BRCA1 mediates HR, in part, by localizing PALB2. Further, we find that the BRCA1-PALB2 hetero-oligomer, and not the PALB2-PALB2 homo-oligomer, is essential for PALB2-dependent DNA repair. Additionally, our results suggest that BRCA1-dependent localization of PALB2 may link DNA repair by HR into a pathway with the MDC1-RNF8-RAP80-Abraxas signaling cascade. Thus, in this manner, PALB2-dependent HR may be responsive to signals generated at DSBs.
2. Results

2.a. The BRCT-PALB2 and BRCT-PALB2(L21P) fusion proteins support the formation of PALB2 and RAD51 foci

To resolve whether BRCA1 is required to localize PALB2, we fused the BRCT repeats of BRCA1 to PALB2 or the L21P mutant of PALB2 that cannot bind to BRCA1 (Zhang et al., 2009a). The localization of BRCA1 to sites of DNA damage is mediated by this BRCT domain (Scully et al., 1999). The fusion proteins containing PALB2 or PALB2(L21P), and the BRCT repeats of BRCA1, are diagrammed in Fig. 1A. We also generated a control fusion with PALB2 (BRCT-C-PALB2), based upon the truncated and non-functional BRCT domain present in HCC1937 breast cancer cells (Scully et al., 1999).

HCC1937 cells, in which BRCA1 does not localize to foci normally (Scully et al., 1999), were stably transduced with various constructs containing PALB2 or with BRCA1 itself. BRCT-PALB2, BRCT-PALB2(L21P), and BRCT-C-PALB2 were expressed at similar levels (Fig. 1B). The fusion proteins were expressed at higher levels than endogenous PALB2. Transduction with BRCA1 by itself increased the levels of total BRCA1 (Fig. 1B), since the mutant BRCA1 that is endogenous in HCC1937 shows the same mobility as the exogenously expressed form of the protein.

Examples of foci assembled in each case, detected with anti-PALB2 antibodies, following exposure to IR are shown in Fig. 1C. Strikingly, many of the PALB2 foci showed colocalization with γH2AX, a marker for DSBs, in cells reconstituted with either the BRCT-PALB2 or BRCT-PALB2(L21P) fusion proteins or with BRCA1. Foci were assembled by γH2AX in response to IR in cells that contained either of the fusion proteins, BRCA1, the BRCT-C-PALB2 control, or vector alone, regardless of whether PALB2 foci were detected. These results suggest that BRCA1-derived BRCT repeats can correctly address PALB2 to sites of DNA
damage in the absence of intact BRCA1 that is itself localized.

Quantification of the assembly of foci, detected with anti-PALB2 antibodies, in each of the reconstituted HCC1937 cells is shown in untreated populations or following exposure to IR (Fig. 1D). Both BRCT-PALB2 and BRCT-PALB2(L21P), and exogenously expressed BRCA1, supported the assembly of PALB2 foci but the fusion protein with truncated BRCT repeats (BRCTΔC-PALB2) did not.

Since PALB2 is required for the assembly of the RAD51 recombinase into foci (Xia et al., 2007; Zhang et al., 2009a), we also assayed the formation of RAD51 foci as a measure of the function of the various fusion proteins (Fig. 1E). The assembly of RAD51 foci paralleled the results for PALB2 foci, demonstrating that fusion with the BRCT repeats is sufficient for targeting a functional PALB2 to DNA damage foci. This is even true for the BRCT-PALB2(L21P) mutant fusion protein that cannot bind to BRCA1. It should be noted that while there is a minimal level of PALB2 foci in HCC1937 cells prior to transduction with the fusion proteins, RAD51 foci display an intermediate level. This is presumably due to BRCA1-independent mechanisms which upregulate the levels of RAD51 protein and foci, and which may thereby compensate for the deficiency in BRCA1 (Martin et al., 2007).

To further support the conclusion that fusion of PALB2 to the BRCT repeats of BRCA1 can mediate recruitment of functional PALB2 in the absence of BRCA1, we expressed the fusion proteins in U2OS cells depleted of endogenous BRCA1. Unlike HCC1937 cells, U2OS cells express full-length BRCA1. Consistent with results obtained in HCC1937 cells, both the wild-type (WT) and L21P mutant fusion proteins, and siRNA resistant BRCA1, supported the assembly of foci by endogenous PALB2 or RAD51 (supplementary material Fig. S1). PALB2 alone or PALB2 fused to the truncated BRCT domain, however, did not support the assembly of PALB2 or RAD51 foci in U2OS cells depleted of full-length BRCA1. In fact, expression of PALB2 or BRCTΔC-PALB2 in cells depleted of BRCA1 was associated with lower levels of
RAD51 foci than in BRCA1-depleted cells which contained the empty vector alone (supplementary material Fig. S1C). BRCT-C-PALB2 did not similarly affect RAD51 foci in a different BRCA1-deficient background found in HCC1937 cells, however (Fig. 1C).

We next utilized EUFA1341 FA-patient derived fibroblasts that contain a truncated PALB2 (Y551X) (Xia et al., 2007) to address the question of fusion protein localization in cells with an inherited deficiency for PALB2 function. BRCA1 is intact in EUFA1341 cells. The different forms of PALB2 were expressed at similar levels (Fig. 2A). Examples from cells exposed to IR demonstrate that the BRCT-PALB2(L21P) fusion protein assembled into foci that colocalized with γH2AX foci as well or better than normal PALB2 that was exogenously expressed in EUFA1341 cells (Fig. 2B). Quantification demonstrates that the BRCT-PALB2 and BRCT-PALB2(L21P) fusion proteins, along with exogenously expressed PALB2, assembled into...
foci, detected with anti-HA antibodies, in untreated EUFA1341 cells (Fig. 2C). Further, assembly of foci was increased following exposure to IR (Fig. 2C). BRCT-PALB2 displayed increased

Figure S1. The BRCT-PALB2(L21P) fusion protein assembles into foci and supports the assembly of RAD51 foci in cells depleted of endogenous, full-length BRCA1. (A) U2OS cells stably expressing the BRCT-PALB2 fusion protein [Fu], BRCT-PALB2(L21P) [Fu(L21P)], PALB2, BRCA1, or the pMMP vector alone, along with a N-terminal Flag-HA epitope tag, were transfected with a control siRNA (siLacZ) or a siRNA targeting BRCA1. Depletion and reconstitution of BRCA1 is shown by blotting for this protein. Levels of PALB2 or either fusion protein were detected by immunoblotting with anti-HA antibodies. Actin is shown as a loading control. (B,C) Quantification of the assembly of PALB2 (B) and RAD51 (C) foci in U2OS cells depleted of BRCA1 and stably reconstituted with the indicated proteins. Cells were fixed 16 h after exposure to 10 Gy IR. Both the BRCT-PALB2 and BRCT-PALB2(L21P) fusion proteins, but not BRCT-C-PALB2 or PALB2 alone, supported the assembly of PALB2 and RAD51 foci in cells depleted of full-length BRCA1.
assembly into foci, as compared to BRCT-PALB2(L21P) or PALB2, apparently because it can localize by two pathways: 1) via the BRCT repeats or 2) by binding to BRCA1. In contrast PALB2(L21P) did not assemble into foci (Fig. 2C). Similarly, BRCT-PALB2, BRCT-PALB2(L21P) and PALB2, but not PALB2(L21P), supported the assembly of RAD51 foci both in untreated populations and following exposure to IR (Fig. 2D). Together, these results suggest that the mutant fusion protein can bypass BRCA1, and that localization of PALB2 is both necessary and sufficient for the recruitment of RAD51 foci to sites of DNA damage.

To further test the specific requirement for the BRCA1 BRCT repeats in localizing functional PALB2, as a control, we fused PALB2-L21P to the BRCT repeats of the BRCA1 partner, BARD1 (diagrammed in supplementary material Fig. S2). The BRCT repeats of BARD1 are similar to those in BRCA1 but are not exactly conserved. Foci formation by BRCT[BARD1]-PALB2(L21P) was greatly diminished as compared to BRCT[BRCA1]-PALB2(L21P) (Fig. 2E). We refer to BRCT[BRCA1]-PALB2(L21P) as BRCT-PALB2(L21P) elsewhere. While these results suggest that the BRCA1 BRCT repeats have a specific role in recruiting PALB2, there was a small degree of foci assembled by BRCT[BARD1]-PALB2(L21P). This may reflect the fact that BARD1 also forms DNA damage foci (Brodie and Henderson, 2010) and, thus, its BRCT domain may have some affinity for proteins at sites of DNA damage.

2.b. The BRCT-PALB2(L21P) mutant fusion protein supports resistance to MMC and DSB-initiated HR in PALB2-deficient cells

Given that PALB2 is required for resistance to MMC and for DSB-initiated HR (Xia et al., 2006), as a measure of the functional importance of BRCA1-dependent localization of PALB2,
we assayed these processes in PALB2-deficient cells reconstituted with BRCT-PALB2(L21P). Again, PALB2(L21P) does not bind to BRCA1, so any function of the mutant fusion protein can be attributed to the BRCT repeats of BRCA1. BRCT-PALB2(L21P), as well as BRCT-PALB2 or PALB2 alone, restored resistance of EUFA1341 (PALB2-deficient cells) to MMC (Fig. 3A). In contrast, cells reconstituted with PALB2(L21P) displayed the same sensitivity to MMC as cells containing only the empty vector. Thus, localization of PALB2 to sites of DNA damage, in this case by fusion to the BRCT repeats of BRCA1, is linked with the cellular response to DNA interstrand crosslinking agents.

U2OS-DR cells containing the DR-GFP HR reporter, and various forms of exogenously-expressed PALB2, were depleted of endogenous PALB2. Cells reconstituted with PALB2(L21P) or BRCTC-PALB2(L21P) were deficient for DSB-initiated HR, while BRCT-PALB2(L21P) restored recombination to the same levels as PALB2 itself (Fig. 3B). Taken together, results in
Figs. 1-3 suggest that BRCA1-mediated localization of PALB2 is necessary for HR. Perhaps related to its increased assembly into nuclear foci, as compared to PALB2 or BRCT-PALB2(L21P) (Fig. 2C), BRCT-PALB2 mediated higher levels of HR.

Neither BRCT-PALB2(L21P), nor BRCT-PALB2, was able to correct the HR deficiency of U2OS-DR cells depleted of BRCA1, however (Fig. 3C). Thus, while the interaction of PALB2 with BRCA1 is necessary for HR (Sy et al., 2009a; Zhang et al., 2009a; Zhang et al., 2009b), it is not by itself sufficient to mediate DNA repair by HR.

2.c. PALB2 recruitment and function in homologous recombination is dependent only on the PALB2-BRCA1 interaction, and not the PALB2-PALB2 interaction

It was previously demonstrated that PALB2 can form a homo-oligomer. Deletion of the N-terminal 42 amino acids of PALB2, which contains most of its coiled-coil domain, abrogated the PALB2 oligomer and disrupted various PALB2-dependent functions (Sy et al., 2009b). Whether this mutant was also deficient for interactions with BRCA1 was not examined, however. Given that fusion to the BRCT repeats of BRCA1 can restore the function of a mutant of the PALB2 coiled-coil domain [BRCT-PALB2(L21P)], we sought to determine the effect of the L21P mutation on both PALB2-PALB2 and BRCA1-PALB2 interactions. For this purpose, we transiently expressed Flag-tagged WT or PALB2(L21P) in 293T cells along with either WT HA-PALB2 or MYC-tagged BRCA1 (Fig. 4). WT PALB2 interacted with PALB2 and BRCA1, as expected, but PALB2(L21P) was deficient

Fig. 4. The L21P mutant of the PALB2 coiled-coil is deficient for interactions with PALB2 itself or with BRCA1. Wild-type (WT) PALB2 or the L21P mutant were transiently expressed in 293T cells along with an N-terminal Flag-epitope tag. Cells were also co-transfected with N-terminally tagged HA-PALB2-WT or Myc-BRCA1-WT and subjected to immunoprecipitation with M2 anti-Flag antibodies. Inputs and immunoprecipitations (IP) are shown, and were immunoblotted to detect interactions. Thus, BRCT-PALB2(L21P) restored various PALB2 functions in Figs 1–3 without binding to BRCA1 and without forming a PALB2 homodimer.
for interactions with both proteins. Thus, BRCT-PALB2(L21P) restores PALB2 function through fusion to the BRCT repeats of BRCA1, without the capacity for PALB2-PALB2 or BRCA1-PALB2 interactions. Together our results (Figs. 1-4) suggest that the BRCA1-PALB2 interaction alone, and not the PALB2-PALB2 interaction, is sufficient to support the assembly of RAD51 foci, and for DNA repair by HR and for resistance to MMC.

2.d. MDC1, RNF8, RAP80 and Abraxas act upstream of BRCA1 to promote recruitment of PALB2

The BRCT domain of BRCA1 was capable of accurately localizing PALB2(L21P) when fused to it (Figs. 1B and 2B). This suggested a potential role in localizing the mutant fusion protein [BRCT-PALB2(L21P)] for one of the three DNA damage response proteins that are known to bind to the BRCT domain of BRCA1: CtIP, FANCJ, and Abraxas (Wang et al., 2007; Yu et al., 2003; Yu et al., 1998). As a first test of this possibility, we immunoprecipitated epitope-tagged versions of PALB2 or BRCT-PALB2(L21P) stably expressed in U2OS cells (Fig. 5A). Indeed, CtIP, FANCJ, and Abraxas, each co-immunoprecipitated with BRCT-PALB2(L21P). The specificity of these interactions for the BRCT repeats present in the fusion protein is demonstrated by the fact that none of these proteins co-immunoprecipitated with PALB2 alone or the vector control that contained only the HA-Flag epitope tags. In contrast, BRCA2 co-immunoprecipitated both with PALB2 and BRCT-PALB2(L21P), which is consistent with the fact that its N-terminus binds to PALB2 directly (Oliver et al., 2009).
To determine whether the interaction of the BRCT repeats with CtIP, FANCJ, and/or Abraxas is involved in the recruitment of BRCT-PALB2(L21P) to nuclear foci, we depleted each protein from U2OS-DR cells (Fig. 5B). Foci formed by the fusion protein containing PALB2(L21P) were not influenced by depletion of CtIP or FANCJ (Fig. 5C). Depletion of Abraxas, however, significantly decreased assembly of the mutant fusion protein into foci following exposure to IR (Fig. 5C).

Importantly, depletion of Abraxas, but not depletion of CtIP or FANCJ, also significantly decreased the assembly of foci by endogenous PALB2 in U2OS cells that lacked the fusion protein construct (Fig. 5D). Effects of depletion of Abraxas on foci formation by the mutant fusion protein were more dramatic than the effects on endogenous PALB2, presumably because the BRCT repeats make the fusion protein more dependent upon Abraxas.
To further elucidate the upstream pathway that regulates recruitment of PALB2 to sites of DNA damage we depleted NBS1, which is part of the MRE11-RAD50-NBS1 complex required for end resection (Sartori et al., 2007). Alternatively, we depleted several different proteins, MDC1, RNF8, and RAP80, which function in an ubiquitin-dependent signaling cascade that responds to DSBs (Fig. 6).

As shown by representative images, depletion of RAP80, RNF8, and MDC1 decreased the assembly of endogenous PALB2, but not γH2AX, into foci following exposure to IR (Fig. 6A). RAP80 is an ubiquitin-binding protein that forms a complex with Abraxas (Kim et al., 2007b; Wang et al., 2007). Consistent with results obtained for depletion of Abraxas in Fig. 5C,D, quantification demonstrates that depletion of RAP80, but not NBS1, decreased the assembly of
foci by endogenous PALB2 in response to IR (Fig. 6B). As further support for the role of RAP80 in regulating the recruitment of PALB2 following exposure to IR, we expressed a siRNA-resistant form of RAP80 in U2OS cells (Fig. 6C). Assembly of PALB2 foci in cells depleted of endogenous RAP80 and expressing the siRNA-resistant form of the protein was recovered to the same levels as cells transfected with the control siRNA (Fig. 6D).

The conclusion that RAP80 regulates recruitment of PALB2 in response to DSBs is also supported by our observation that depletion of RAP80 decreased the percentage of cells that were positive for PALB2 foci at each time point tested, ranging from 1-16 h after exposure to IR (Fig. 7A). It should also be noted that RAP80 appears to regulate basal levels of PALB2 foci, since PALB2 foci were also decreased in untreated populations of U2OS cells depleted of RAP80 (Fig. 7A). Depletion of RAP80 decreased the percentage of cells with PALB2 (Fig. 7A) or BRCA1 (Fig. 7B) foci to similar degrees, consistent with the possibility that RAP80 regulates BRCA1-dependent recruitment of PALB2 to sites of DNA damage.

Since MDC1 and RNF8 are known to function upstream of RAP80, we also tested their role in the recruitment of PALB2 in response to DNA damage. MDC1 is a checkpoint mediator that binds to γH2AX at DSBs, while RNF8 is an E3 ubiquitin ligase that binds to MDC1 and ubiquitinates histones and other proteins (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). A representative image demonstrates that depletion of RNF8 compromised PALB2 recruitment to DSBs following exposure to IR, as determined by decreased colocalization γH2AX foci (Fig. 6A). Further, quantification demonstrates that depletion of either MDC1 or
RNF8 dramatically decreased the percentage of cells that displayed assembly of endogenous PALB2 into foci after exposure to IR (Fig. 6B). Given the roles of MDC1 and RNF8, and of RAP80, in ubiquitinating histone and binding to it, respectively, these results suggest that histone ubiquitination regulates PALB2 recruitment. In further support of this possibility, we find that PALB2 strongly colocalized with ubiquitin after exposure of cells to IR (Fig. 6E). It has been demonstrated previously that ubiquitin accumulates at DSBs after treatment with IR (Sobhian et al., 2007).

It should be noted that depletion of MDC1, RNF8, or RAP80 also strongly inhibited the assembly of foci by the BRCT-PALB2(L21P) fusion protein (supplementary material Fig. S3). Since deficient foci formation by PALB2(L21P) can be rescued through fusion to the BRCT repeats of BRCA1 (Figs. 1C, 2C, and supplementary material Fig. S1B), this result suggests that MDC1, RNF8, and RAP80 regulate PALB2 through their actions on BRCA1. Because PALB2 recruits BRCA2 and RAD51 to sites of DNA damage, our results suggest that PALB2 may integrate the MDC1-RNF8-RAP80-Abraxas network, which is initiated after detection of DSBs, with HR.
3. Discussion

Previous reports have demonstrated that BRCA1 and PALB2 interact through a coiled-coil domain on each protein and that this interaction is required for DNA repair by HR (Sy et al., 2009a; Zhang et al., 2009a; Zhang et al., 2009b). How this interaction mediates HR and resistance to MMC has remained controversial, however. There have also been discrepant reports as to whether BRCA1 recruits PALB2 to sites of DNA damage (Sy et al., 2009a; Zhang et al., 2009a; Zhang et al., 2009b). To address these issues, we have utilized a fusion protein that contains PALB2(L21P), which is incapable of binding to BRCA1, and the BRCT repeats that localize BRCA1. We find that this fusion protein is recruited to sites of DNA damage, both in BRCA1- and PALB2-deficient cells, and can therefore bypass the normal requirement for BRCA1 and its capacity to bind PALB2. The fusion protein localizes correctly to DSBs following exposure to IR, as determined by partial colocalization with γH2AX foci. Importantly, BRCT-PALB2(L21P) mediates the formation of RAD51 foci, both in BRCA1- and PALB2-deficient cells. As an important control, we find that fusion of PALB2(L21P) to incomplete BRCT repeats which are found in a breast cancer cell line and which are incapable of localizing BRCA1 (Scully et al., 1999), does not support the assembly of DNA damage foci by the fusion protein or by RAD51. On the basis of these results, we conclude that PALB2 is localized by its interaction with BRCA1.

Furthermore, we find that the BRCT-PALB2(L21P) mutant fusion protein supports HR and resistance to MMC in a PALB2-deficient background. Thus, our results suggest that correct, BRCA1-dependent localization of PALB2 and the machinery for HR may be linked to DNA repair by HR. In this context, it is noteworthy that we also find that the L21P mutant of PALB2 is defective for interactions either with other PALB2 molecules or with BRCA1. Together, these observations make the novel point that the BRCA1-PALB2 hetero-dimer, and not the PALB2-PALB2 homo-dimer, is required to mediate PALB2 functions such as assembly of RAD51 foci,
HR, and resistance to MMC.

The BRCT-PALB2 (L21P) mutant fusion protein has also been an important tool for understanding how recruitment of PALB2 is linked to upstream DNA damage signaling processes. We find that both BRCT-PALB2 (L21P) and endogenous PALB2 are recruited by a network, including MDC1, RNF8, RAP80, and Abraxas, that generates and responds to ubiquitin signals at DSBs. This pathway is initiated following the generation and detection of DSBs. Thus, PALB2 is part of a pathway that may link DNA repair by the core machinery for HR to various signals generated at the DSB. The potential importance of this pathway is underscored by the fact that RAP80 (Akbari et al., 2009; Nikkila et al., 2009), in addition to BRCA1, PALB2, and BRCA2, is linked to an inherited susceptibility to breast cancer.

3.a. BRCA1 recruits PALB2 to sites of DNA damage

Together, the results on foci formation by cells reconstituted with BRCT-PALB2(L21P) suggest that BRCA1 has an important role in localizing PALB2, either spontaneously or in response to DNA damage induced by IR. Further, combined with previous results (Xia et al., 2006; Zhang et al., 2009a), it appears that PALB2 recruits BRCA2 and RAD51 after it is localized by BRCA1. We suggest that recruitment of PALB2 and RAD51 requires two domains on BRCA1: 1) the BRCT domain that initially localizes it and 2) the coiled-coil domain that binds to PALB2. It should be noted that breast cancer-associated mutations of BRCA1 are observed in both domains of the protein (Gayther et al., 1996). Further, the fact that BRCA1 mediates PALB2 localization and function in HR may explain the shared association of mutations of BRCA1 or PALB2 with breast cancer.

While BRCA1-dependent localization of PALB2 is required for HR (Fig. 3B), the BRCT-PALB2(L21P) fusion protein does not support DSB-initiated HR in BRCA1-deficient cells (Fig.
3C). We therefore speculate that another domain of BRCA1, in addition to the BRCT repeats and the coiled-coil domain, is also required for HR. One possibility is the N-terminal interaction of BRCA1 with the MRE11-RAD50-NBS1 complex (Chen et al., 2008). E3 ligase activity at the N-terminus of BRCA1 is not required for HR, however (Reid et al., 2008; Shakya et al., 2011).

3.b. PALB2 is recruited by a network involving MDC1, RNF8, RAP80, and Abraxas

We propose that the ubiquitin-related signaling cascade initiated at DSBs by binding of MDC1 to γH2AX (Stewart et al., 2003; Stucki et al., 2005) is linked to HR by mediating BRCA1-dependent recruitment of PALB2. It appears that RNF8 is subsequently recruited by binding to MDC1 and generates ubiquitin-dependent binding sites for RAP80. In fact, RNF8 mediates histone ubiquitination at DSBs (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007) and RAP80 binds to ubiquitinated histone (Mailand et al., 2007; Wu et al., 2009). It should be noted, in this context, that deficiency for MDC1 or RNF8 (Huang et al., 2009; Zhang et al., 2005), like deficiency for PALB2 (Xia et al., 2006), compromises DSB-initiated HR.

Importantly, Abraxas is recruited to DSBs via its interaction with RAP80 and binds to BRCA1 (Kim et al., 2007b; Wang et al., 2007). Thus, RAP80 and Abraxas potentially couple BRCA1-dependent recruitment of PALB2 to the upstream ubiquitin-dependent signaling cascade. Consistent with this possibility, and in agreement with others (Hu et al., 2011; Kim et al., 2007a; Sobhian et al., 2007; Wang et al., 2007), we find that BRCA1 foci were reduced by depletion of RAP80 (Fig. 7). Further, PALB2 foci were reduced to a similar degree as BRCA1 foci (Fig. 7). Also, depletion of Abraxas resulted in decreased assembly of PALB2 foci (Fig. 5D). Additionally, depletion of Abraxas decreases the assembly of BRCA1 foci in response to IR (Kim et al., 2007b). Considering all of this together, we propose that a primary function of RAP80 and Abraxas is to promote the BRCA1-dependent recruitment of PALB2.
Depletion of RAP80 appears to have opposite effects on the recruitment of PALB2 (Fig. 6A) as compared to two proteins that bind to the BRCT repeats of BRCA1: CtIP and FANCJ (Hu et al., 2011). PALB2 foci were not tested in this previous study. We find that depletion of RAP80 in the same cell line and at the same time points they utilized resulted in decreased assembly of PALB2 foci after exposure to IR (Fig. 7).

Abraxas, CtIP and FANCJ bind to the BRCT domain of BRCA1 in a mutually-exclusive manner (Wang et al., 2007). Because PALB2 binds through a different domain of BRCA1, depletion of RAP80 may affect PALB2 differently. In accord with this possibility, Hu et al. (2011) reported that depletion of RAP80 did not decrease levels of RAD51 foci (Hu et al., 2011). Like PALB2, RAD51 does not bind to the BRCT repeats of BRCA1. Interestingly, since BRCA1 is a component of both the CtIP-MRN (Chen et al., 2008) and PALB2-BRCA2-RAD51 (Sy et al., 2009a; Zhang et al., 2009a; Zhang et al., 2009b) complexes, respectively, RAP80-dependent recruitment of BRCA1 could coordinate the end resection and strand invasion activities that are together required for HR.
4. Materials and methods

Cell culture

EUFA1341, HCC1937 and U2OS cell lines were cultured at 37°C in a 5% CO₂ environment and irradiated as described previously (Zhang et al., 2009a).

Cloning

N-terminally tagged (HA-Flag) proteins were generated in pOZ as described previously and subcloned into pMMP, where appropriate (Zhang et al., 2009a). PALB2 was fused to the BRCT domain of BRCA1 [amino acids 1629-1863] (BRCT-PALB2) or a truncated BRCT domain [amino acids 1629-1794] (BRCT-C-PALB2). BRCT-PALB2(L21P) was then generated by subsequent site-directed mutagenesis according to our published procedures (Zhang et al., 2010). As a control (Supplemental Figure S2), we introduced the BRCT domain of BARD1 [amino acids 550-777] in front of PALB2(L21P).

Transduction

Retroviral transduction and selection with interleukin-2 beads (pOZ) or with puromycin (pMMP) were as described previously (Zhang et al., 2009a; Zhang et al., 2010). Unless otherwise noted PALB2 and its fusions, and BRCA1, were transduced using pOZ and pMMP retroviruses, respectively. No differences in foci formation or HR were noted in cells transduced into cells using the empty pOZ or pMMP vectors.
**SiRNAs and antibodies**

Cells were stably transduced with a retrovirus for siRNA-resistant BRCA1 [UCACAGUGUCCUUUAUGUA; (Ganesan et al., 2002)] or siRNA-resistant RAP80 [GUAUUGACUCGGAGACAAA; (Hu et al., 2011)] that were generated by site-directed mutagenesis. Cells were transiently depleted of endogenous PALB2, or BRCA1 or RAP80, using siRNAs against the 3'-UTR or the coding sequence, respectively, as described (Ganesan et al., 2002; Hu et al., 2011; Zhang et al., 2009a). For other depletion experiments, siRNAs (5'-3') directed against FANCJ (GUACAGUACCCACCUUAU) (Zhang et al., 2010), CtIP (GCUAACACGAACGAGAACU) (Yu and Chen, 2004), Abraxas (GAGAAAAGUGUGGAGCAAGA) (Liu et al., 2007), RNF8 (GGACAAUAUGGAGCACCACAA) (Mailand et al., 2007), MDC1 (UCCAGUGAUCUUGAGGU) (Lou et al., 2003), and NBS1 (GGAGGAAGAUGUCAAUGUU) (Yoo et al., 2009) were utilized as previously described. All siRNAs were purchased from Dharmacon.

The following antibodies were utilized: mouse anti-HA (HA.11; Covance), mouse anti-Flag (M2, Sigma), rabbit anti-PALB2 (Zhang et al., 2009a), rabbit anti-BRCA1 (Millipore), goat anti-CtIP (Santa Cruz Biotechnology), rabbit anti-FANCJ (Zhang et al., 2010), rabbit anti-Abraxas (Bethyl Laboratory), rabbit anti-RAP80 (Bethyl Laboratory), mouse anti-γ-H2AX (Millipore), and mouse anti-ubiquitin conjugates [FK2; Millipore; (Huen et al., 2007)].

**Immunofluorescence microscopy**

Cells were prepared and imaged as previously described (Zhang et al., 2009a). For each data point, three independent counts of at least 150 cells each were made. Cells that had three or more nuclear foci were scored as positive.

Labeled cells were observed with a Leica DMI6000 microscope, and images were
collected with a Hamamatsu Camera using Openlab software (Improvision). Images were processed into figures using Photoshop (Adobe).

**Immunoblotting**

Immunoblotting was as previously described (Zhang et al., 2009a).

**Immunoprecipitation to assay oligomerization**

293T cells were transiently transfected with Flag-PALB2 or PALB2(L21P), and HA-PALB2 or MYC-BRCA1, extracts prepared, and immunoprecipitations with anti-Flag antibodies performed as described previously (Zhang et al., 2009a).

**Mitomycin C sensitivity assay**

Survival was calculated in triplicate, relative to the average corrected absorbance for each untreated cell line, as described (Zhang et al., 2009a).

**Assay of DNA DSB-initiated HR**

U2OS-DR cells containing an integrated reporter for HR that stably expressed various constructs described above were depleted of endogenous PALB2 or BRCA1, transfected with pCBASce encoding the I-SCEI endonuclease, and assayed as described previously (Zhang et al., 2009a). The ratio of recombination with and without depletion of PALB2 or BRCA1, as appropriate, was calculated for each cell line and condition.
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References


Chapter 3. COORDINATION OF THE RECRUITMENT OF THE FANCD2 AND PALB2 FANCONI ANEMIA PROTEINS BY A UBIQUITIN SIGNALING NETWORK

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Abstract

Fanconi anemia (FA) is a chromosome instability syndrome and the 19 identified FA proteins are organized into two main arms which are thought to function at distinct steps in the repair of DNA interstrand crosslinks (ICLs). These two arms include the upstream FA pathway, which culminates in the monoubiquitination of FANCD2 and FANCI, and downstream breast cancer (BRCA) associated proteins that interact in protein complexes. How, and whether, these two groups of FA proteins are integrated is unclear. Here, we show that FANCD2 and PALB2, as indicators of the upstream and downstream arms, respectively, colocalize independently of each other in response to DNA damage induced by mitomycin C (MMC). We also show that ubiquitin chains are induced by MMC and colocalize with both FANCD2 and PALB2. Our finding that the RNF8 E3 ligase has a role in recruiting both FANCD2 and PALB2 also provides support for the hypothesis that the two branches of the FA-BRCA pathway are coordinated by ubiquitin signaling. Interestingly, we find that the RNF8 partner MDC1, as well as the ubiquitin binding protein, RAP80, specifically recruit PALB2, while a different ubiquitin binding protein, FAAP20, functions only in the recruitment of FANCD2. Thus, FANCD2 and PALB2 are not recruited in a single linear pathway, rather we define how their localization is coordinated and integrated by a network of ubiquitin-related proteins. We propose that such regulation may enable upstream and downstream FA proteins to act at distinct steps in the repair of ICLs.
1. Introduction

Fanconi anemia (FA) is a pathologically and genetically heterogeneous chromosome instability syndrome associated with a variety of congenital defects that can affect every organ system. FA patients also frequently display bone marrow failure and a predisposition to cancer (Longerich et al. 2014; Walden and Deans 2014). There are 19 identified genes, which, when mutated in a biallelic, sex-linked, or dominant negative manner give rise to FA (Walden and Deans 2014; Ameziane et al. 2015; Hira et al. 2015; Rickman et al. 2015; Sawyer et al. 2015; Virts et al. 2015; Wang et al. 2015). These genes share a common role in the repair of DNA interstrand crosslinks (ICLs), such as those caused by mitomycin C (MMC), and in preventing both spontaneous and ICL-induced chromosome instability (Andreassen and Ren 2009; Longerich et al. 2014). Understanding the repair of ICLs is important for defining the molecular basis of FA and because ICLs can be induced by commonly used chemotherapeutic agents, as well as endogenous and environmental substances (Andreassen and Ren 2009; Clauson et al. 2013).

Eleven of the FA genes encode proteins involved in a biochemical pathway termed the FA pathway, which acts “upstream” in the repair of ICLs. Eight of these proteins, FANC- A, B, C, E, F, G, L and M, are components of the FA nuclear core complex, which, along with the E2 ligase UBE2T/FANCT, is required for the monoubiquitination of FANCD2 and FANCI (Garcia-Higuera et al. 2001; Sims et al. 2007; Smogorzewska et al. 2007). This monoubiquitination event enables the recruitment of the FANCD2 and FANCI proteins to chromatin and nuclear foci at sites of DNA damage (Garcia-Higuera et al. 2001; Wang et al. 2004; Montes de Oca et al. 2005; Sims et al. 2007; Smogorzewska et al. 2007). The central role of FANCD2 monoubiquitination to the collective function of FA pathway proteins is demonstrated by the inability of the non-ubiquitinated K561R mutant of human FANCD2 to form nuclear foci or
correct the sensitivity of FANCD2-deficient cells to MMC (Garcia-Higuera et al. 2001; Montes de Oca et al. 2005).

The other eight FA genes are referred to as “downstream” because they are not required for FANCD2 monoubiquitination (Andreassen and Ren 2009; Walden and Deans 2014; Ameziane et al. 2015; Wang et al. 2015). The products of six of these genes, including BRCA2, PALB2, RAD51, RAD51C, BRCA1 and BRIP1, interact together in protein complexes, which are necessary for their roles in repairing DNA damage (Xia et al. 2006; Kumaraswamy and Shiekhattar 2007; Sy et al. 2009; Zhang et al. 2009a,b; Buisson et al. 2010; Dray et al. 2010; Park et al. 2014a,b). Additionally, heterozygous mutation of any of this subset of FA genes, except RAD51, is also associated with an increased risk for breast cancer in the general population (Miki et al. 1994; Wooster et al. 1995; Seal et al. 2006; Erkko et al. 2007; Rahman et al. 2007; Meindl et al. 2010). In contrast, the upstream FA pathway genes are not generally associated with an increased risk of inherited breast cancer (Berwick et al. 2007), suggesting that the upstream and downstream pathways may have distinct functions in DNA repair. Together, the upstream and downstream FA proteins have been termed the “FA-BRCA” pathway.

One of the “downstream” FA proteins, PALB2, was first identified as a partner and localizer of BRCA2 (Xia et al. 2006). Importantly, PALB2 functionally links BRCA1 and BRCA2 into a network that mediates double strand break (DSB)-initiated homologous recombination (HR) and resistance to MMC (Sy et al. 2009; Zhang et al. 2009a,b). PALB2 also binds the RAD51 recombinase (Buisson et al. 2010; Dray et al. 2010) and the RAD51 paralog, RAD51C (Park et al. 2014a), which along with BRCA2 and BRCA2, are the products of FA genes. PALB2 appears to coordinate these “downstream” FA proteins into a network of breast cancer susceptibility proteins (Park et al. 2014b) which we refer to here as the ‘BRCA pathway of FA proteins’. 
While there are commonalities among each of the FA proteins, such as roles in mediating resistance and chromosome stability in response to MMC (Andreassen and Ren 2009; Walden and Deans 2014), important differences between upstream FA pathway genes/proteins and the downstream BRCA pathway also suggest some distinct functions. Notably, biallelic mutation of BRCA2 or PALB2 results in a dramatically earlier onset of cancer than in other FA complementation groups (Hirsch et al. 2004; Wagner et al. 2004; Reid et al. 2007). Further, DNA damage-induced assembly of RAD51 into nuclear foci, and cellular resistance to ionizing radiation, requires BRCA1, BRCA2, PALB2 and RAD51C but not components of the “upstream” FA pathway (Abbott et al. 1998; Scully et al. 1999; Takata et al. 2001; Godthelp et al. 2006; Xia et al. 2007; Park et al. 2014a). In accord with these findings, it has been suggested that monoubiquitinated FANCD2, and thus the FA pathway, acts at different steps in the repair of ICLs than do BRCA pathway proteins such as PALB2 and BRCA2 (Andreassen and Ren 2009; Longerich et al. 2014). Indeed, the repair of ICLs requires multiple steps, including recognition, incision, translesion synthesis to restore duplex DNA, and homologous recombination. The upstream FA pathway mediates early steps in ICL repair, including incision and translesion synthesis (Knipscheer et al. 2009), while the downstream BRCA pathway reconstructs the replication fork (Long et al. 2011).

It is possible that there is a mechanism which coordinates the recruitment of proteins in the upstream and downstream branches of the FA-BRCA pathway to orchestrate the multi-step repair of ICLs. In support of such coordination, we demonstrate that FANCD2 and PALB2 colocalize in response to treatment with MMC in a manner that is independent of the status of the other. This coordination involves, at least in part, ubiquitin signaling. We show here that MMC induces foci composed of ubiquitin chains, dependent upon the RNF8 E3 ubiquitin ligase, which colocalize with both FANCD2 and PALB2. Importantly, we also demonstrate that a network that generates and ‘reads’ ubiquitin signals acts to coordinate the recruitment of FANCD2 and PALB2. While independent reports implicated RNF8 in having a role in recruiting
FANCD2 or PALB2 in response to DNA damage (Yan et al. 2012; Zhang et al. 2012), by performing a direct comparison in response to a particular type of DNA damaging agent, we establish that RNF8 functions in coordinating the recruitment of an upstream FA protein and a downstream FA protein in response to ICLs. Our finding that MDC1 has a role in recruiting PALB2 but not FANCD2 in response to MMC, or ionizing radiation (IR), demonstrates that there are also distinct aspects to the coordination of the recruitment of proteins in the FA and BRCA branches of the FA-BRCA pathway. This ubiquitin signaling network also involves distinct ubiquitin binding proteins, FAAP20 and RAP80, as adaptors that specifically mediate the recruitment of FANCD2 and PALB2, respectively, in response to ICLs. We propose that this mechanism mediates the recruitment of upstream and downstream FA proteins to the same site of damage, but permits some degree of independence that enables their function at different steps in the repair of ICLs.
2. Results

2.a. FANCD2 and PALB2 colocalize in a manner that is independent of the other

Loss of FA and BRCA pathway proteins results in similar pathological and cellular phenotypes associated with Fanconi anemia (Andreassen and Ren 2009). Thus, we hypothesize that there may be a shared regulatory pathway that regulates these proteins and leads to their recruitment to DNA damage foci. Given that FA cells are hypersensitive to DNA interstrand crosslinking agents (Andreassen and Ren 2009; Longerich et al. 2014), we focused on the recruitment of FA proteins in response to such insults. We utilized FANCD2 and PALB2 foci as representative of the FA and BRCA pathways, respectively. We selected FANCD2 because it functions at the end of the FA pathway (Garcia-Higuera et al. 2001), and because it has been reported to have a role early in the repair of ICLs such as incision and/or translesion synthesis (Knipscheer et al. 2009). For its part, PALB2 has a central role in the BRCA pathway and is required for HR (Xia et al. 2006; Park et al. 2014b), which is a later step in the repair of ICLs. As a potential measure of coordinated recruitment, we first sought to determine whether FANCD2 and PALB2 colocalize. Indeed, FANCD2, detected via an epitope tag, and PALB2 displayed strong colocalization after treatment with MMC (Fig. 1a). To better visualize foci, black-white images are shown for single channels in all but Figure 3, while combined channels/merged images are displayed in color.

As further support for the coordinated recruitment of FANCD2 and PALB2, we find that each progressively assembled into nuclear foci at a similar rate following the addition of MMC to the culture medium (Fig. 1b). Also in agreement with the possibility of coordinated recruitment of FANCD2 and PALB2, quantification demonstrated strong colocalization of these proteins at both an earlier and a later time point of treatment with MMC (Fig. 1c).
Since ICLs are repaired in a step-wise manner (Andreassen and Ren 2009; Longerich et al. 2014), this raised the possibility that the localization of one protein, particularly FANCD2 since it is thought to act earlier in the repair of ICLs, might be required for the localization of the other. To test this possibility, we examined foci formation in uncomplemented and complemented pairs of PD20 and EUFA1341 FA cells that are genetically deficient for FANCD2 and PALB2, respectively. As is evident both from the examples shown (Fig. 2a) and from counts (Fig. 2b), the presence or absence of FANCD2 (Fig. 2c) in PD20 cells did not influence the assembly of PALB2 into foci or the levels of PALB2 present in cells. Conversely, proficiency or deficiency for PALB2 in EUFA1341 cells did not affect the assembly of FANCD2 foci (Figs. 2d-f).
Ubiquitin signaling has a role in coordinating the recruitment of the FANCD2 and PALB2 FA proteins

One possible mechanism that could act upstream of the recruitment of FA-BRCA pathway proteins in response to ICLs is ubiquitin signaling. It was previously reported that K63-linked ubiquitin chains are detected in foci by immunofluorescence microscopy after cells are exposed to ionizing radiation (IR) (Sobhian et al. 2007). Whether MMC induces ubiquitin
signaling, detected as foci, was unknown, however. Thus, we tested this possibility and indeed discovered that MMC induced polyubiquitin foci, detected with the FK2 antibody, as compared to untreated populations of cells (Fig. 3a). This further established ubiquitin signaling as a candidate that can mediate coordination of the recruitment of FANCD2 and PALB2. As further support for this possibility, we then sought to determine whether FANCD2 and PALB2 colocalize with ubiquitin foci detected with the FK2 antibody in cells treated with MMC. In accord with this hypothesis, both FANCD2 (Fig. 3b) and PALB2 (Fig. 3c) displayed clear colocalization with FK2
foci. Additionally, quantification demonstrates that colocalization of FANCD2 and PALB2 with FK2 foci was strongly induced by MMC, as compared to untreated populations (Fig. 3d-e).

As an additional test of the role of ubiquitin signaling in regulating the coordination of the recruitment of FANCD2 and PALB2, in parallel experiments, we tested the role of RNF8. RNF8 is an E3 ubiquitin ligase known to mediate the assembly of ubiquitin foci in response to DNA double-strand breaks (DSBs) (Huen et al. 2007; Mailand et al. 2007). We utilized siRNAs to

![Image of experiment results]

Figure 4. The recruitment of both FANCD2 and PALB2 in response to MMC involves the RNF8 E3 ubiquitin ligase. 

a Represents immunoblot showing knockdown of RNF8 in HeLa cells. b-c Representative black-and-white images of FANCD2 foci induced by MMC in HeLa cells transfected with either siLacZ (control) or RNF8 siRNAs (b) and treated with 0.5 μM MMC for 1.6 hr, and quantification (c) of the percentage of cells with five or more foci. FANCD2 foci are also shown in merged images in b with FANCD2 foci in red and DAPI signal in blue to indicate the position of nuclei. d Representative black-and-white images of PALB2 foci induced by MMC in cells transfected with either siLacZ or RNF8 siRNAs. PALB2 foci are also shown in merged images where PALB2 foci are red and DAPI signal is shown in blue. e Quantification of the percentage of HeLa cells with five or more PALB2 foci following transfection with siRNAs directed against LacZ or RNF8 and treatment with MMC. f Quantification of the percentage of MMC-treated cells which display FK2 foci after transfection with a control siRNA or siRNAs against RNF8. (c, e-f) Each value represents the mean of three independent counts of at least 150 cells each ± standard deviation. * indicates p<0.005.
deplete RNF8 (Fig. 4a) and treated the cells with MMC. In support of a role for RNF8 in coordinating the recruitment of FANCD2 and PALB2, we indeed found that foci assembled by each FA protein was decreased when RNF8 was depleted with either of two distinct siRNAs. A decrease in the percentage of cells with FANCD2 foci, and of their intensity, can be seen in the examples shown in Fig. 4b and by quantification (Fig. 4c). Two siRNAs/shRNAs for each target protein were utilized here, and in other experiments, to minimize the possibility of off-target effects. RNF8 knockdown also led to a strong reduction in PALB2 foci. Representative images are shown in Fig. 4d and quantification is shown in Fig. 4e. As further support for these results, we then tested another human cell line, U2OS, and similarly found that RNF8 is involved in regulating the formation of both FANCD2 and PALB2 foci in response to MMC (Fig. S1).

Together, our data on the colocalization of both FANCD2 and PALB2 with ubiquitin foci induced by MMC (Fig. 3), and our finding that the RNF8 E3 ubiquitin ligase has a role in regulating both the recruitment of FANCD2 and PALB2 (Fig. 4), support a role for ubiquitin signaling in coordinating the recruitment of these two FA proteins. As further support for a role for RNF8 and ubiquitin signaling in coordinating the recruitment of the FA and BRCA branches
of FA proteins, we make the novel observation that RNF8 is required for ubiquitin foci induced by MMC (Fig. 4f).

2.c. MDC1 has a role in the recruitment of PALB2, but not FANCD2, in response to MMC

Given the common role of RNF8 in the recruitment of both FANCD2 and PALB2 in response to the DNA interstrand crosslinking agent MMC (Fig. 4), next we tested whether MDC1 also has a function in this process. MDC1, which forms a complex with, and colocalizes with γH2AX at DSBs induced by IR (Goldberg et al. 2003; Stewart et al. 2003), binds to RNF8 and recruits it to DSBs (Huen et al. 2007; Kolas et al. 2007; Mailand et al. 2007). The role of MDC1 in the response to ICLs has not been determined, however.

To address the role of MDC1 in the recruitment of FANCD2 and PALB2, we transfected HeLa cells with a control siRNA directed against LacZ or with two independent siRNAs directed against MDC1. Depletion of MDC1 is shown by immunoblots in Figure 5a. Surprisingly, depletion of MDC1 with either siRNA did not affect the assembly of FANCD2 foci in cells treated with MMC. This is seen both in the examples given (Fig. 5b) and by counts (Fig. 5c). In contrast, depletion of MDC1 with either siRNA clearly inhibited the assembly of PALB2 foci in response to MMC (Figs. 5d-e). A similar effect of depletion of MDC1 on PALB2 foci but not FANCD2 foci was also observed in another cell type, U2OS (Fig. S1). Taken together, in contrast to the shared role for RNF8, the specific role of MDC1 in recruiting PALB2 but not FANCD2 in response to MMC indicates that there is a network that functions with some independence in the regulation of the recruitment of these distinct FA proteins. Thus, there are both common and distinct layers to the coordination of the recruitment of FANCD2 and PALB2.

MDC1 is recruited to DSBs induced by IR (Goldberg et al. 2003; Stewart et al. 2003) but does not appear to be involved in recruiting FANCD2 in response to MMC (Fig. 5). Therefore, it is interesting that quantification demonstrates that nearly all corrected PD20 cells which contained FANCD2 foci displayed colocalization with γH2AX foci at either 4 or 16 hr of
Figure 5. The recruitment of PALB2, but not FANCD2, to ICLs is dependent on MDC1. a. Representative immunoblot showing knockdown of MDC1. b–c Representative black-white images (b) and quantification (c) of FANCD2 focus formation in HeLa cells transfected with a control siRNA or two distinct siRNAs targeting MDC1 and treated with 0.5 μM MMC for 16 hr. FANCD2 foci are also shown in merged images in b with FANCD2 foci in red and DAPI signal in blue to indicate the position of nuclei. d–e Representative black-white images of MMC-induced PALB2 foci (d) in cells transfected with either siLacZ or MDC1 siRNAs and quantification (e) of the percentage of HeLa cells with five or more PALB2 foci. PALB2 foci are also shown in merged images in d with PALB2 foci in red and DAPI signal in blue. f Immunoblot showing FANCD2 monoubiquitination status in cells treated with a siRNA directed against RNF8 or MDC1. The intensity of the upper monoubiquitinated band divided by the intensity of the lower ubiquitinated band is shown for each lane. g Quantification of the percentage of HeLa cells with five or more ubiquitin foci following transfection with siRNAs and treatment with MMC. Values in c,e,g represent the mean of three independent counts of at least 150 cells each + standard deviation; * indicates p<0.005.
treatment with MMC (Fig. S2a). Further, while levels of γH2AX foci reached greater than 98% of cells at 16 hr of treatment with MMC and FANCD2 foci were only detected in 80% of cells by 32 hr of treatment, γH2AX and FANCD2 foci, as well as PALB2 foci, all accumulated with treatment over time (Fig. S2b). Additionally, cells that were positive for FANCD2 and PALB2 foci displayed strong colocalization with γH2AX foci (Fig. S2c-d). Thus, both FANCD2 and PALB2 foci have some relationship to DSBs in cells treated with MMC.

FANCD2 monoubiquitination is required for its assembly into DNA damage foci (Garcia-Higuera et al. 2001; Montes de Oca et al. 2005). Thus, to better understand the mechanistic

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**Fig. S2 FANCD2 and PALB2 foci display colocalization with γH2AX foci following treatment with MMC.** a Quantification of the percentage of corrected FANCD2-deficient PD20 fibroblasts with FANCD2 nuclear foci which display five or more colocalized γH2AX foci at 4 or 16 hr of treatment with 0.5 μM MMC. b The percentage of FANCD2-deficient cells, PD20 fibroblasts, corrected with HA-FANCD2, that display three or more FANCD2 (HA), PALB2, or γH2AX foci at different time points of treatment with 0.5 μM MMC. Results for γH2AX foci are added here to those already shown in Fig. 1b for FANCD2 and PALB2. Values in a-b represent the mean ± standard deviation of three counts of 150 cells each. c-d Representative images of FANCD2 (c) and PALB2 (d) foci in which colocalization with γH2AX foci is detected. Images of DAPI-stained cells are shown to indicate the position of nuclei. Single channel images are shown in black-white, while merged images with FANCD2 or PALB2 foci in red and γH2AX foci in green are also included to display colocalization. Colocalization is indicated by a yellow signal in the merged images.
basis for the differential role of RNF8 and MDC1 in regulating FANCD2 foci in response to the treatment of cells with MMC, we examined the effects of depletion of RNF8 or MDC1 on FANCD2 activation (monoubiquitination) using immunoblots (Fig. 5f). MMC induced FANCD2 monoubiquitination in cells transfected with a control siRNA (siLacZ), as compared to untreated populations, as expected. Depletion of RNF8 but not MDC1 diminished the levels of monoubiquitinated FANCD2 in cells treated with MMC. This is seen as a decrease in the ratio of the monoubiquitinated and unubiquitinated forms of FANCD2, and supports the conclusion that RNF8, but not MDC1, has a role in controlling FANCD2 foci in response to the DNA interstrand crosslinking agent MMC.

Interestingly, depletion of MDC1 strongly decreased the levels of ubiquitin foci detected with the FK2 antibody in cells treated with MMC (Fig. 5g), similar to the effect of depletion of
RNF8 (Fig. 4f). Since MDC1 has a role in the assembly of PALB2 but not FANCD2 foci, we suggest that MDC1 has a role at later steps in the processing of ICLs. Further, because PALB2 has an important role in the repair of DNA DSBs (Xia et al. 2006), it is possible that ubiquitin foci induced by MMC could be related to the formation of DSBs during the processing of ICLs (Andreassen and Ren 2009).

Because FANCD2 monoubiquitination and focus formation are strongly related to cell cycle status (Taniguchi et al. 2002), it was important to confirm that knockdown of RNF8 or MDC1 does not impact cell cycle progression. To test this, we labeled unperturbed HeLa cells depleted of RNF8 or MDC1 with bromodeoxyuridine (BrdU) and performed a flow cytometric analysis. Dot plots are shown in Fig. S3a and cell cycle distributions are quantified in Fig. S3b. Importantly, neither depletion of RNF8 nor MDC1 led to a significant change in any phase of the cell cycle. Thus, the effects of depletion of RNF8 and MDC1 described above are not an indirect effect of cell cycle alterations.

As discussed above, MDC1 and RNF8 have been implicated in the response to agents such as IR that directly induce DSBs. It is known that DSBs also arise during the processing of ICLs, where nucleases cleave the DNA and produce DSBs that are then repaired by homologous recombination (Raschle et al. 2008). Thus, we wanted to test whether MDC1 and RNF8 have roles in the recruitment of FANCD2 and PALB2 to IR-induced foci similar to our findings on their roles in the response to ICLs. As for treatment with DNA interstrand crosslinking agents (Figs. 4-5), again, RNF8 knockdown, but not MDC1 knockdown, had a profound effect on FANCD2 foci formation (Fig. S4). In contrast, depletion of either RNF8 or MDC1 reduced PALB2 foci following exposure to IR, similar to our findings with DNA interstrand crosslinking agents (Figs. 4-5). These data suggest that the role for RNF8 and MDC1 in the recruitment of PALB2 is not damage specific, but may occur at any lesion that is repaired by homologous recombination. Conversely, the MDC1-independent RNF8 signal, which activates
the FA protein FANCD2, may be involved in the response to any lesion that activates FANCD2 and the FA pathway.

2.d. The ubiquitin binding proteins, FAAP20 and RAP80, independently recruit FANCD2 and PALB2, respectively, to MMC-induced foci

Next, the question arose as to the role of ubiquitin-binding proteins, such as FAAP20 and RAP80, in the coordination of the recruitment of FANCD2 and PALB2. Thus, we treated HeLa cells with siRNAs and/or shRNAs directed against these two “ubiquitin readers” (Sobhian et al. 2007; Ali et al. 2012; Yan et al. 2012). Depletion of FAAP20 and RAP80 with a siRNA directed against either target is shown in Fig. 6a. Consistent with a previous report (Ali et al. 2012), depletion of FAAP20 resulted in a marked reduction in the percentage of cells with FANCD2 foci following treatment with MMC. Cells transfected with siRAP80, however, showed no significant change in the levels of FANCD2 focus formation (Fig. 6b). In striking contrast, the opposite result was seen for PALB2 foci. Depletion of RAP80 reduced the percentage of cells...
that were positive for PALB2 foci; however, depletion of FAAP20 did not significantly affect PALB2 foci in MMC-treated cells (Fig. 6c). These results have been confirmed using RNAi-mediated depletion of either FAAP20 or RAP80 based upon a second target sequence for each. Using a shRNA that targets a different sequence resulted in a reduction in FAAP20 levels and a significant decrease in FANCD2 foci, but not PALB2 foci, in cells treated with MMC (Fig. 6d-e). In contrast, an alternative siRNA directed against RAP80 led to a significant decrease in PALB2 foci (Fig. 6f), but not FANCD2 foci (Fig. 6g). Thus, FAAP20 and RAP80 appear to function in an
ubiquitin signaling network as specific readers of ubiquitin chains that are individually dedicated to the recruitment of distinct FA proteins, FANCD2 and PALB2, respectively.
3. Discussion

When the data presented here are considered together, they point toward a mechanism for the coordinated recruitment of two FA proteins: FANCD2, which functions in the upstream FA pathway, and PALB2, which is in the downstream BRCA pathway. In particular, we focus here on the response to MMC, which induces DNA interstrand crosslinks. In this context, it should be noted that cells deficient for any FA protein, whether it is in the FA pathway or is a ‘downstream’ protein, are characteristically hypersensitive to ICLs and display ICL-induced chromosome instability (Andreassen and Ren 2009; Longerich et al. 2014). Thus, FA proteins have a common function in the repair of ICLs, a highly deleterious lesion that impedes DNA replication and transcription.

Importantly, the repair of ICLs is thought to involve multiple steps, including recognition of the lesion, incision of the lesion, translesion synthesis to restore duplex DNA, and homologous recombination to restart the stalled replication fork (Andreassen and Ren 2009; Longerich et al. 2014). In fact, FA pathway proteins such as FANCD2 are believed to function in earlier steps of ICL repair, including incision and translesion synthesis. Downstream HR proteins such as PALB2 instead have a role in reconstructing the replication fork and in repairing DSBs that result from previous steps in ICL processing (Andreassen and Ren 2009; Long et al. 2011; Longerich et al. 2014). Thus, a coordinated mechanism for recruitment of these FA proteins may be integral to orchestrating ICL repair.

Further, it must be recognized that a mechanism for coordination may be necessary given that the various FA proteins form distinct protein complexes, including the FA core complex, the FANCD2-FANCI complex, and a BRCA1-PALB2-BRCA2-RAD51-RAD51C complex (Park et al. 2014b; Walden and Deans 2014). More pertinent to our current work, PALB2 was not identified as a component of FANCD2 complexes in a previous study which utilized mass spectrometry (Lossaint et al. 2013).
Our results demonstrate that the recruitment of FANCD2 and PALB2 occurs in a similar time frame (Fig. 1b), presumably as cells enter S phase and arrest prior to entry into mitosis (Taniguchi et al. 2002). Further, FANCD2 and PALB2 foci strongly colocalize, when either is present in cells, both at an earlier and a later time point of treatment with MMC (Fig. 1c). This contrasts with a report which demonstrates that foci of the XPF FA protein assemble later than FANCD2 foci but also disappear more rapidly (Zhang et al. 2016). Thus our findings suggest a more intimate relationship of FANCD2 and PALB2 foci, as compared to XPF foci. In particular, the observed assembly of FANCD2 and PALB2 into foci at similar rates, and their colocalization, could represent recruitment at distinct, but proximal, steps in the processing and repair of ICLs. For FANCD2 this may be just before the generation of DSBs and for PALB2 perhaps just after.

Indeed, we find that both FANCD2 and PALB2 foci appear to be related to DSBs (Fig. S2). For this purpose, we utilized γH2AX as a marker for DSBs. Still, although FANCD2 displays a high degree of colocalization with γH2AX foci in corrected PD20 cells, FANCD2 is not necessarily recruited directly to the DSB itself but could be recruited to some other DNA structure. In contrast, because of the involvement of MDC1, which binds to γH2AX (Stewart et al. 2003), PALB2 may be recruited to DSBs at processed ICLs. In any case, FANCD2 and PALB2 may be recruited to different DNA structures that occur during the processing of ICLs.

Some possible mechanisms of coordination of FANCD2 and PALB2 are diagrammed in Figure 7. We demonstrate that FANCD2 and PALB2, as representative of the FA pathway and downstream HR proteins, respectively, do strongly colocalize in response to treatment with MMC (Figs. 1a,c). This rules out the possibility, diagrammed in Figure 7a, that FANCD2 and PALB2 are not recruited to similar sites of DNA damage. Instead, the colocalization we observe raises the possibility of coordinated recruitment of FANCD2 and PALB2. By examining the localization of FANCD2 and PALB2 in PALB2-deficient and FANCD2-deficient cells, respectively, we also exclude the possibility that one acts first as a pre-requisite for the recruitment of the other to DNA damage foci (Fig. 7b). Such a situation might have been
imagined if processing of the lesion by one of the proteins, for example, FANCD2, generated a new structure required for the recruitment of the other.

Our finding that RNF8 has a role in recruiting both FANCD2 and PALB2 supports there being a mechanism of coordination, rather than the proteins being regulated by unrelated mechanisms despite their colocalization (Fig. 7c). The involvement of MDC1 in recruiting PALB2 but not FANCD2, and a role for distinct ubiquitin-binding proteins in recruiting FANCD2 and PALB2, argues against there being a single regulatory pathway that recruits both proteins (Fig. 7d). Instead, MDC1, RNF8, FAAP20 and RAP80 act as a network of ubiquitin-related proteins, external to the FA-BRCA pathway, which coordinates the recruitment of FANCD2 and PALB2 (Fig. 7e). This network has both common elements, such as RNF8, and distinct elements, such as MDC1, and FAAP20 and RAP80, that cooperate to mediate the recruitment of FANCD2 and PALB2. Such a mechanism may serve to recruit these, and perhaps other, FA proteins to the DNA lesion to act at different steps in repair, but may also permit some degree of independence that enables each protein to carry out different roles in that repair.

RNF8 is known as an E3 ubiquitin ligase and it has been implicated previously in recruiting FA proteins (Yan et al. 2012; Zhang et al. 2012), but this is the first side-by-side comparison of its role in recruiting two distinct FA proteins in response to the same type of DNA damage. As a result of this comparison, we came to the novel conclusion that RNF8, by having a role in recruiting both FANCD2 and PALB2 in response to ICLs, may serve to coordinate the recruitment of these FA proteins.

Importantly, by examining FANCD2 foci in response to either DNA interstrand crosslinking agents or IR, we find that RNF8 has a MDC1-independent role in cellular responses to DNA damage. This was unexpected because of the canonical role of MDC1 in recruiting RNF8 to mediate the localization of DNA repair proteins, such as BRCA1 and 53BP1, in response to DNA double-strand breaks induced by IR (Huen et al. 2007; Mailand et al. 2007). Confidence in our finding of an MDC1-independent function of RNF8 in the recruitment of
FANCD2 is increased by the fact that we show that both MDC1 and RNF8 are required for the recruitment of another FA protein, PALB2, in parallel experiments. Although a previous study suggested a link between RNF8 and the recruitment of FANCD2, whether MDC1 also has a role was not considered (Yan et al. 2012). Thus, the observation that RNF8 has a MDC1-independent function in recruiting FANCD2 is novel. While it is currently unknown whether RNF8 has MDC1-independent roles that extend beyond FANCD2 and the FA pathway, nonetheless, this finding enhances understanding of cellular responses to DNA damage.

Future work will be required to understand how RNF8 and MDC1 are dissociated in the recruitment of FANCD2 while appearing to cooperate in the recruitment of PALB2. One possibility is that RNF8 is recruited when the replication fork encounters an ICL, but that MDC1 is recruited only after a DSB is formed by incision of the lesion. This might then lead to subsequent DSB-dependent signaling by RNF8 and the recruitment of BRCA pathway proteins. At present, it is unknown whether RNF8 assembles into DNA damage foci in response to ICLs, but this is not necessarily required for a role in mediating FANCD2 recruitment. We propose that binding to a different chromatin-associated protein may be involved in MDC1-independent
regulation of FANCD2 by RNF8, but the identification of that protein(s) is beyond the scope of the current work.

Finally, our findings suggest that two different ubiquitin-binding proteins, FAAP20 and RAP80, act distally in a ubiquitin signaling network as ubiquitin “readers” that independently and specifically recruit FANCD2 and PALB2, respectively. Thus, we propose that FAAP20 and RAP80 cooperate with RNF8 in coordinating the recruitment of the FA pathway and the BRCA pathway of FA proteins.

While FAAP20 has been reported to have a role in the recruitment of FANCD2 in response to DNA damage (Ali et al. 2012; Leung et al. 2012; Yan et al. 2012), it was previously unknown whether it might also recruit downstream FA proteins such as PALB2. Instead, we show here that as a ubiquitin ‘reader’, FAAP20 has a specific role in recruiting FANCD2. In particular, by recruiting FANCA, and along with it other FA nuclear core complex proteins, to chromatin, FAAP20 may lead to FANCD2 monoubiquitination and assembly into foci (Ali et al. 2012; Leung et al. 2012). In contrast, RAP80 has a specific function in recruiting PALB2 but not FANCD2. The RAP80 complex, which also includes Abraxas, may recruit PALB2 through its interacting partner BRCA1 (Zhang et al. 2012).

In summary, a ubiquitin signaling network that includes the RNF8 E3 ligase and distinct ubiquitin-binding proteins coordinates the recruitment of an upstream FA protein, FANCD2, and a downstream FA protein, PALB2. Such regulation may permit FANCD2 and PALB2, and related proteins, to have related, but distinct, DNA repair activities.
4. Materials and methods

Cell culture and treatments
HeLa, U2OS, and PD20 cells and derivatives, were grown in Dulbecco’s Modified Eagle Medium (GE Healthcare) supplemented with 10% calf serum and were kept in an humid incubator with 5% CO\textsubscript{2}. EUFA1341 cells from a FA patient with restoration of PALB2, or which contained the empty pOZ vector (Zhang et al. 2009a), were grown in a 1:1 mixture of F10 and DMEM medium containing 10% calf serum. MMC treatment was performed at 0.5 μM. Exposure of cells to 10 Gy IR was as previously described (Zhang et al. 2009a).

RNA interference
SiRNAs targeted the following sequences for transient knockdowns: RNF8 #1 – GGACAAUUAUGGACACAACAA (Mailand et al. 2007), RNF8 #2 – CAGAGAAGCUUACAGAUGU (Huen et al. 2007), MDC1 #1 – UCCAGUGAAUCCUUGAGGU (Lou et al. 2003), MDC1 #2 – AATCTGAGACCTCCTAAGGT (Goldberg et al. 2003), RAP80 #1 – GUUUGACUCGGAGACAAA (Hu et al. 2011), RAP80#2 GCACAAAGACUUCAGAUGCA Hu et al. 2011), and FAAP20 siRNA targeting the 3’ UTR (Yan et al. 2012) (Dharmacon, D-018651-18-0002). All siRNAs were purchased from Dharmacon. Transfection of the siRNAs was done using Lipofectamine 2000 according to the manufacturer’s online protocols (Life Technologies -11668). Short hairpin RNAs were expressed from the PLKO.1 vector. The FAAP20 sequence cloned into the vector was described in (Ali et al. 2012) as shFAAP20 sequence #2.

Antibodies
Primary antibodies utilized for immunofluorescence microscopy and immunoblotting were as follows: FK2 (EMD Millipore, 04-263), FANCD2 (E35) (Garcia-Higuera et al. 2001), PALB2
(Zhang et al. 2009a), γH2AX (EMDMillipore, JBW301), RNF8 (Santa Cruz, sc271462), MDC1 (Novus Biologicals, NB100-395), FAAP20 (Ali et al. 2012), RAP80 (Bethyl Laboratory, A300-763A), and anti-HA (Covance, 16B12). Secondary antibodies included: FITC-conjugated donkey anti-Mouse IgG (Jackson Immunoresearch, 715-095-150) and Rhodamine-conjugated Donkey anti-Rabbit IgG (Jackson Immunoresearch, 711-296-152) for immunofluorescence microscopy. HRP-conjugated sheep anti-mouse IgG (GE Healthcare, NA931) and HRP-conjugated donkey anti-Rabbit IgG (GE Healthcare, NA934) secondary antibodies were utilized for immunoblotting. Antibodies for cell cycle analysis were as follows: anti-BrdU antibody (GE Healthcare, RPN202) and Alexa-488 conjugated anti-mouse antibody (Jackson Immunoresearch, 715-545-150) as primary and secondary antibodies, respectively.

**Immunofluorescence Microscopy**

Immunofluorescence microscopy was as described previously (Zhang et al. 2009a). Briefly, cells were grown on poly-lysine coated coverslips. Cells were fixed in 2% paraformaldehyde for 20 minutes and then permeabilized with 0.2% Triton X-100 in PBS. In certain experiments, to more readily visualize PALB2 foci, cells were fixed for 30 min in PBS containing 4% paraformaldehyde and 0.5% Triton X-100 (Castella et al. 2015). Antibodies were diluted in PBS containing 3% bovine serum albumin, 0.2% Tween-20, and 0.05% Sodium Azide. FANCD2 antibody was diluted 1:200, PALB2 antibody was diluted 1:100, HA antibody was diluted 1:250, and FK2 antibody was diluted 1:1000. Fluorescent secondary antibodies were diluted 1:200. Coverslips were mounted in Vectashield mounting medium containing DAPI (Vector Laboratories) and imaged using a Leica DMI6000 microscope fitted with a Hamatsu Camera. Images were collected in Openlab (Improvision) and figures were prepared using Adobe Photoshop. Quantification was performed by counting cells which had five or more foci as positive. Three counts of 150 cells each were made for each cell type and condition.
**Immunoblotting**

Immunoblotting was performed using poly-acrylamide gel electrophoresis. Membranes were blocked in 5% milk in PBS + 0.2% Tween-20 for 1 hr. Primary antibodies were incubated overnight at 4°C and were diluted as follows: Actin (1:5000), RNF8 (1:400), MDC1 (1:1000), FANCD2 (1:2000), FAAP20 (1:2000), and RAP80 (1:4000). Following washes, membranes were incubated for 1 hr at RT with secondary antibodies diluted 1:5000. Proteins were then detected with ECL detection reagent (GE Healthcare RPN2232). Band intensity was determined using ImageJ.

**Cell Cycle Analysis**

Thirty minutes prior to collection, subconfluent cells were treated with 30 μM bromodeoxyuridine (BrdU) for incorporation into DNA. Cells were harvested and fixed in methanol at -20°C for 30 min. Cells were then treated with 2 M HCl in PBS containing 0.5% Triton-100 for 30 min at RT. Subsequently, cells were collected by centrifugation and were neutralized with 0.1 M sodium tetraborate at pH 8.5 in PBS. Cells were then resuspended in 300 μL of antibody buffer (PBS + 0.05% Tween-20 and 3% BSA) containing 10 μL anti-BrdU antibody for 1 hr at 37°C. Cells were washed once with PBS and resuspended in antibody buffer containing secondary antibody (1:500) for 30 min at 37°C. Following this, cells were collected by centrifugation and resuspended in PBS containing 50 μg/mL propidium iodide (PI) and 30 units/mL RNase A for 10 min at 37°C. Cells were then analyzed using a FACSCalibur II instrument. Cells which stained positive for BrdU were counted as S phase. Cells which stained negative for BrdU were classified as either G1 or G2 based on PI signal intensity.

**Statistical Analysis**

The significance of counts of foci was determined using Chi-squared tests. For each experiment, three independent counts were used to determine the average and standard
deviation. Of these three counts, the counts closest to the average were used in the chi-square test. Each experiment was repeated at least twice.
5. Acknowledgements

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

The authors declare that they have no competing interests.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.
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Chapter 4. OVERALL DISCUSSION

Given the importance of understanding the cellular response to DNA damage, we sought to better clarify the regulatory pathways which recruit and activate Fanconi Anemia proteins. Our findings suggest that cells respond to double strand breaks and interstrand crosslinks by activating ubiquitin signaling through RNF8, allowing the recruitment of different protein networks which carry out distinct functions to protect the genome. We show that two networks of Fanconi Anemia proteins, one in which MDC1, RNF8, RAP80, and BRCA1 recruit PALB2, and the other in which RNF8 and the FA core complex, but not MDC1, recruit and activate FANCD2. Clarification of these pathways will lead to better chemo-therapies for patients with cancers, as well as improve predictive and therapeutic possibilities for FA patients.

When PALB2 was found to recruit BRCA2 and RAD51 to sites of DNA damage, it was unclear what mechanisms regulated its recruitment (Xia et al. 2006). Later, it was discovered that BRCA1 is vital for the recruitment of PALB2 to DNA damage, however BRCA1 itself has several different regulators including Abraxas and the MRN complex (Wang et al. 2007; Liu et al. 2007; Wang and Elledge 2007; Yuan and Chen 2010). Further, the coiled coil domain of PALB2 was shown to mediate both BRCA1 binding and homo-dimerization which obscured its role. We began with the hypothesis that the primary function of the PALB2 coiled-coil was to interact with BRCA1 allowing for BRCA1 dependent localization (Chapter 2). By fusing the BRCT repeats of BRCA1 to coiled-coil mutant PALB2, we showed that this fusion was able to localize itself and RAD51 to DNA damage foci and fully correct the DNA damage sensitivity of PALB2 mutant cells, indicating that localization was indeed the primary, if not the only, role of the coiled coil in the DNA damage response. It further suggested that binding to BRCA1 may be sufficient to allow for appropriate localization (Fig. 1A).
We then determined that the BRCA1 regulatory protein Abraxas, also regulates PALB2. We showed that Abraxas and RAP80 were important for both endogenous PALB2 and BRCT-PALB2 mutant focus formation. Further, we showed that the other BRCA1-BRCT binding proteins, FANCJ and CtIP were dispensable for focus formation. Because RAP80 is an ubiquitin binding protein, we also tested and showed that the E3 ligase RNF8 and MDC1 are essential for recruitment of PALB2 and the BRCT-PALB2 fusion protein to DNA damage foci. This work suggests that γH2AX sets up a pathway whereby MDC1 is recruited to γH2AX where it brings RNF8 to erect K63 linked chains, likely on histones like H2A and H2AX. These ubiquitin chains then recruit the RAP80-Abraxas complex which brings in the BRCA1-PALB2-BRCA2 RAD51 loading complex (Fig. 1A). The BRCT-PALB2 fusion protein however was able to bypass BRCA1 interaction by directly interacting with phosphorylated Abraxas (Fig. 1B).
the coiled-coil is predominantly involved in localization, it is possible that BRCA1 may only play a partial role and that PALB2, once localized through a BRCA1 interaction may be able to localize more PALB2 without the help of BRCA1. Since, PALB2 can be recruited to DNA by MRG15 (Hayakawa et al. 2010), and PALB2 directly interacts with DNA via the ChAM domain (Bleuyard et al. 2011) or direct interaction with DNA outside of the ChAM domain (Buisson et al. 2010; Dray et al. 2010; Bleuyard et al. 2011), one of these other binding activities may take over, after BRCA1 initially localizes PALB2 via the coiled-coil domain.

Interestingly, a mouse model has been generated which expresses a coiled-coil mutant of Palb2 (Simhadri et al. 2014). Oddly, although the protein seems to have lost nearly all HR activity in cell systems, the mouse develops relatively normally, with the exception of male (but not female) infertility. This is in stark contrast to Palb2 knockout mice which are embryonically lethal, even when crossed to a p53 mutant background (Rantakari et al. 2010; Bouwman et al. 2011). Two different interpretations exist, either the mutant form of Palb2 used in this study retains sufficient coiled-coil function to support unchallenged mouse development, or the coiled-coil is dispensable for Palb2’s function in supporting embryonic viability and healthy lifespan but not for supporting male fertility. At this point, this question remains unanswered.

While our work described the E3 ligase RNF8 as an important factor for recruiting PALB2 to DNA damage, it has also been implicated in recruiting FANCD2 to DNA damage through the ubiquitin binding protein FAAP20. Side by side, the work described in Chapter 2 and in Yan et al (2012), suggested that RNF8 may serve to link the two best described arms of the FA pathway – the upstream arm which consists of the FA core-complex, FANCD2 and FANCI; and the BRCA arm which consists of BRCA1, PALB2, and the other HR proteins. Our second paper started with the hypothesis that RNF8 and MDC1 serve to co-recruit both arms of this pathway to crosslinks, which may better coordinate the multi-step repair of this complex lesion.
We hypothesized that MDC1 and RNF8 work to recruit FANCD2 and PALB2 to crosslinks in a manner similar to that described for PALB2 at double-strand-breaks in our earlier work (Zhang et al 2012) (Fig. 2A). In support of this hypothesis, FANCD2 and PALB2 colocalize independently of one another in an RNF8 dependent manner. However, we showed that while RNF8 recruits both FANCD2 and PALB2 to crosslinks – MDC1 appears to only function in the recruitment of PALB2 (Fig. 2B). We also showed this to be the case for a second type of crosslink, psoralen+UVA light (Accessory Figs. 1 and 2). Additionally, we have shown that MMC induced BRCA1 foci also dependend on both RNF8 and MDC1, like PALB2 (Accessory Fig. 3). To our knowledge, this is the first work directly implicating an MDC1-independent role for RNF8 in the DNA damage response. We further showed that even in the case of double strand breaks, MDC1 but not RNF8 is dispansable for FANCD2 focus formation. We also showed that FAAP20 and RAP80 function in the recruitment of either FANCD2 or PALB2 to foci, but not vice versa. We conclude that although RNF8 is involved in recruiting both FANCD2 and PALB2, the pathways in which it acts appear to be distinct.

Our work indicates that RNF8’s role in FA activation functions in an unclear pathway. We see several hypotheses which could explain RNF8’s distinct role from MDC1 in the recruitment of FANCD2. First, RNF8 could be recruited to interstrand crosslinks by a non-MDC1 interacting partner (Fig. 2C). Second, RNF8 could be acting at a distance (Fig. 2D), such as through transcriptional regulation of FANCD2 regulating proteins and therefore would not need to be recruited to DNA damage sites to recruit FANCD2, but may need to be recruited to facilitate PALB2 recruitment. A third possibility, is that MDC1 but not RNF8 plays an inhibitory role in FANCD2 recruitment, but a positive role through recruiting RNF8 (Fig. 2E). This hypothesis would suggest that depletion of MDC1 would remove both a positive recruitment signal, recruitment through RNF8, and an unknown signal which normally works against recruitment. This would also mean that FANCD2 can be recruited in the absence of the MDC1-RNF8 signal,
which has some support in the fact that our experiments suggested only a partial dependence of FANCD2 on RNF8. While we have not directly tested the mechanism by which RNF8 knockdown reduces FAAP20 mediated FANCD2 activation, experiments testing the role of other RNF8 binding partners and ubiquitinating targets will be essential to unraveling this mystery. Chromatin immunoprecipitation of DNA damage proteins at sites of crosslinks has yielded intriguing results about the DNA replication independent accumulation of FA proteins (Shen et al. 2009). While proteins in both the FA and BRCA pathways were present in replication

Fig. 2. Models of RNF8 coordination of two groups of Fanconi Anemia proteins. (A) Incorrect model of MDC1 and RNF8 cooperatively recruiting FANCD2 and PALB2 through FAAP20 or RAP80 ubiquitin binding complexes. (B) Established model showing MDC1 and RNF8 work together to recruit PALB2 through RAP80. (C) Hypothetical model in which a non-MDC1 protein recruits RNF8 to interstrand crosslinks to activate the FAAP20-FANCD2 pathway. (D) Hypothetical model in which RNF8 acts at a distance from the crosslink in order to activate the FAAP20-FANCD2 pathway. (E) Hypothetical model where MDC1 functions to both positively and negatively regulate the FAAP20-FANCD2 pathway, possibly by recruiting an inhibitory factor as well as the pro-FANCD2 protein RNF8.
competent plasmids, the non-BRCA FA proteins were also observed at crosslinks in non-replicating plasmids. This suggests that replication forks may be a distinct signal which differentiates between upstream FA proteins and FA-BRCA proteins, similar to MDC1. If RNF8 but not MDC1 is recruited to ICLs outside of replication forks, this could be an important new area of study of the FA pathway.

While FA patients are difficult to treat because many of the phenotypes develop in utero, these results also provide us with a new window into cancer. Although many steps have been made to produce drugs which inhibit specific mitogenic signals, rather than widely toxic DNA damaging agents, many tumors are still treated with DNA damaging agents such as ionizing radiation or interstrand crosslinking agents such as mitomycin C or cisplatin. The work described in the previous pages sheds further light on how cells respond to such agents, and further suggests new connections between these proteins and pathways. For instance, that MDC1 and RNF8 play different roles in recruiting FA pathway members to DNA damage, or that RAP80 recruits PALB2 to DNA damage through recruitment of its interacting partner, BRCA1. Although ubiquitin ligases have been largely refractory to small molecule inhibition, recent findings have shown that the E2 ubiquitin ligase dimer UBC13/UEV1A can be inhibited (Pulvino et al. 2012; Ushiyama et al. 2012). This heterodimer functions as the E2 for TRAF6, which functions in the Toll-Like Receptor signaling cascade. Interestingly, the E2 ligase for RNF8 is an UBC13 and MMS2 dimer (Lok et al. 2011). Whether the compounds which inhibit UBC13/UEV1A inhibit the UBC13/MMS2 interaction and RNF8 activity is unknown but is an attractive investment opportunity, with the potential to sensitize cancer cells to several damaging agents. This could be especially helpful when dealing with targeted agents such as ionizing radiation. We expect that RNF8 loss would likely be more problematic in repairing double strand breaks in the context of replication given its relationship to the FA-BRCA proteins.
as well as the upstream proteins. This could widen the window of efficacy for these toxic treatments.

With cancer and FA patients awaiting novel therapies, time is well spent improving our understanding of the pathways which respond to DNA damage. These pathways act as guardians of the genome which can protect our cells, but can also protect cancerous cells from chemotherapies. Clear understanding of these pathways is essential to protect patients both from failures of these pathways and their co-opting by cancers. Our results will inform future work aimed at curing or controlling these challenging diseases.
References


**Accessory Data**

**Accessory Fig. 1.** RNF8 and MDC1 knockdown differentially impact FANCD2 recruitment to psoralen + UVA foci. (A-B) HeLa were cells treated with control siRNA or siRNAs directed against RNF8 (A) or MDC1 (B) described in chapter 3. Cells were then treated with 5 μM angelicin or trioxsalen in PBS for 10 minutes. Cells were then subjected to 60 kJ/M² of UVA light twice with two PBS washes between the UVA treatments. This is intended to increase the proportion of crosslinks to monoadducts. Cells were returned to growth media, and 16 hours later, cells were fixed and stained as described in Chapter 3. Angelicin is not capable of forming crosslinks and serves as a control for the monoadducts which form in response to treatment with either angelicin or trioxsalen. Treatment with trioxsalen+UVA shows a much stronger induction of FANCD2 foci than Angelicin + UVA, suggesting that treatment does indeed induce ICLs. Knockdown of RNF8 (A) but not MDC1 (B) shows a significant reduction in FANCD2 foci, as was seen with MMC and IR in Chapter 3.
Accessory Fig. 2. RNF8 and MDC1 knockdown reduce PALB2 recruitment to psoralen + UVA foci. (A-B) HeLa were cells treated with control siRNA or siRNAs directed against RNF8 (A) or MDC1 (B) described in chapter 3. Cells were then treated with 5 μM angelicin or trioxsalen in PBS for 10 minutes. Cells were then subjected to 60 kJ/M² of UVA light twice with two PBS washes between the UVA treatments. This is intended to increase the proportion of crosslinks to monoadducts. Cells were returned to growth media, and 16 hours later, cells were fixed and stained as described in Chapter 3. Treatment with trioxsalen+UVA shows a much stronger induction of PALB2 foci than Angelicin + UVA, suggesting that treatment does indeed induce ICLs. Knockdown of both RNF8 (A) and MDC1 (B) show a significant reduction in PALB2 foci, as was seen with MMC and IR in Chapter 3.
Accessory Fig. 3. RNF8 and MDC1 knockdown reduce MMC induced BRCA1 foci. HeLa cells were treated with siRNAs targeting LacZ as a control, RNF8, or MDC1 as in chapter 3. Cells were exposed to 0.5 μM MMC for 16 hours and were fixed and stained as in chapter 3. (A) Representative images of MMC induced BRCA1 foci. (B) Quantification of cells from (A) with 5 or more BRCA1 foci. Mouse anti-BRCA1 antibody (clone D-9, sc-6954), was purchased from Santa Cruz and used at a dilution of 1:200.