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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.

Keywords

Fanconi anemia; DNA interstrand crosslinks; Chromosome instability; FANCD2; PALB2

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COMPLIANCE WITH ETHICAL STANDARDS

Ethical Approval

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

Conflict of interest

The authors declare that they have no competing interests.

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, FANCA, FANCB, FANCC, FANCE, FANCG, FANCL and FANCM, are components of the FA nuclear core complex, which, along with the E2 ligase UBE2T/FANCT1, 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 Shiekhhattar 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; Erkkö 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 BRCA1 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.

Results

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 Figures 3–5, 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 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).

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 γ H2AX 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 γ H2AX foci at either 4 or 16 hr of treatment with MMC (Fig. S2a). Further, while levels of γ H2AX 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, γ H2AX 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 γ H2AX 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 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.

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.

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 γ H2AX as a marker for DSBs. Still, although FANCD2 displays a high degree of colocalization with γ H2AX 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 γ H2AX

(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.

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% CO₂. 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 μ M. 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 – GGACAAUUAUGGACAACAA (Maidland et al. 2007), RNF8 #2 – CAGAGAAGCUUACAGAUGU (Huen et al. 2007), MDC1 #1 – UCCAGUGAAUCCUUGAGGU (Lou et al. 2003), MDC1 #2 – AATCCTGAGACCTCCTAAGGT (Goldberg et al. 2003), RAP80 #1 – GUAUUGACUCGGAGACAAA (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), γ H2AX (EMD Millipore, 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 μ M 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 μ L of antibody buffer (PBS + 0.05% Tween-20 and 3% BSA) containing 10 μ L 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 μ g/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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. FANCD2 and PALB2 foci accumulate at similar rates and colocalize following treatment of cells with MMC

a FANCD2 (detected with anti-HA antibodies) and PALB2 foci displayed in individual black-white images colocalize following treatment of PD20 fibroblasts that express HA-FANCD2 with 0.5 μ M MMC for 16 hr. FANCD2 and PALB2 are shown in green and red, respectively, in the merged image and colocalization is demonstrated by a yellow signal. DAPI is shown to demonstrate the position of nuclei. **b** The percentage of cells with three or more FANCD2 foci (detected with anti-HA antibodies) and PALB2 foci is quantified at multiple time points following the addition of 0.5 μ M MMC to corrected PD20 fibroblasts. **c** Quantification of cells with five or more colocalized FANCD2 (HA) and PALB2 foci at 4 or 16 hr after beginning treatment of corrected PD20 cells with 0.5 μ M MMC. Values in **b–c** represent the mean of three independent counts of 150 cells each $\pm$ standard deviation.

Figure 2. FANCD2 and PALB2 foci assemble independently of each other

a Representative black-white images of PALB2 foci, detected with anti-PALB2 antibodies, in PD20 fibroblasts complemented with FANCD2 or which contained the empty vector following treatment with 0.5 μ M MMC for 16 hr. PALB2 foci are also shown in merged images, where PALB2 is red and DAPI signal in blue indicates the position of nuclei. **b** Counts of PD20 cells, with or without complementation of FANCD2, that have five or more PALB2 foci following treatment with MMC. **c** Immunoblots demonstrating complementation of PD20 cells with FANCD2 and that this does not influence PALB2 protein levels. **d–e** Similarly, representative black-white images (**d**) and counts (**e**) of FANCD2 foci are shown for EUFA1341 cells complemented with PALB2 or which contain the empty pOZ vector. FANCD2 foci are also shown in merged images, with FANCD2 foci in red and the DAPI signal in blue (**d**). Values in **b,e** represent the mean of three independent counts of at least 150 cells each $\pm$ standard deviation. **f** Immunoblots demonstrating complementation of EUFA1341 cell with PALB2 and that this does not influence the levels of FANCD2 in the cells.

Figure 3. MMC induces ubiquitin foci that colocalize with FANCD2 and PALB2

a Quantification of the percentage of HeLa cells which show five or more FK2 foci before and after treatment with 0.5 μ M MMC for 16 hr. **b–c** Representative color images of FANCD2 foci (**b**) and PALB2 foci (**c**) displaying strong colocalization with FK2 foci in HeLa cells treated with MMC. The positions of nuclei are indicated by DAPI signal in blue. **(d–e)** Quantification of the percentage of cells which show five or more FANCD2 (**d**) or PALB2 (**e**) foci colocalized with FK2 foci, either with or without treatment with 0.5 μ M MMC for 16 hr. Values in **d–e** represent the mean of three independent counts of at least 150 cells each $\pm$ standard deviation; * indicates $p < 0.005$.

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

a Representative immunoblot showing knockdown of RNF8 in HeLa cells. **b–c** Representative images of FANCD2 foci induced by MMC in HeLa cells transfected with either siLacZ (control) or RNF8 siRNAs (**b**) and treated with 0.5 μ M MMC for 16 hr, and quantification (**c**) of the percentage of cells with five or more foci. FANCD2 foci (red) are also shown in merged images in **b** with DAPI signal in blue to indicate the position of nuclei. **d** Representative images of PALB2 foci (red) 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 $\pm$ standard deviation. * indicates $p < 0.005$.

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 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 μ M MMC for 16 hr. FANCD2 foci (red) are also shown in merged images in **b** with DAPI signal in blue to indicate the position of nuclei. **d–e** Representative 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 (red) are also shown in

merged images in **d** with 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 unubiquitinated 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 $\pm$ standard deviation; * indicates $p < 0.005$.

Figure 6. The FAAP20 and RAP80 ubiquitin binding proteins specifically recruit FANCD2 and PALB2, respectively

a Immunoblot demonstrating siRNA mediated knockdown of FAAP20 and RAP80. Actin is shown as a loading control. **b** Quantification of MMC-induced FANCD2 foci in cells transfected with a control siRNA, or with siRNAs directed against FAAP20 or RAP80, 16 hr after exposure to 0.5 μ M MMC. **c** Quantification of MMC-induced PALB2 foci in cells transfected with siRNAs directed against FAAP20 or RAP80. **d–e** Immunoblot (**d**) displaying knockdown of FAAP20 mediated by a shRNA that targets a second sequence and quantification (**e**) of FANCD2 and PALB2 foci in cells expressing this shRNA or a control shRNA. **f–g** Immunoblot (**f**) showing knockdown of RAP80 with a second siRNA and quantification (**g**) of FANCD2 and PALB2 foci. Values in each bar graph represent the mean of three independent counts of at least 150 cells each $\pm$ standard deviation. * indicates $p < .005$, ** indicates $p < 0.05$.

Figure 7. A comparison of different mechanisms that potentially regulate the recruitment of FANCD2 and PALB2 in response to DNA damage

a One possibility is that FANCD2 and PALB2 do not colocalize and are therefore unrelated. FANCD2 is shown diagrammatically in green, while PALB2 is indicated by red foci throughout this figure. **b** Another possibility is that FANCD2 or PALB2 is required for the recruitment of the other and this could result in colocalization. **c** An alternative is that FANCD2 and PALB2 colocalize but are regulated by unrelated mechanisms and are therefore not coordinated. **d** It is also possible that FANCD2 and PALB2 colocalize as a result of regulation by the same pathway. **e** Instead of each of these possible relationships and regulatory possibilities, we find that FANCD2 and PALB2 are colocalized by a branched regulatory network that has both a common element (RNF8) and distinct elements that coordinates the recruitment of these FA proteins.