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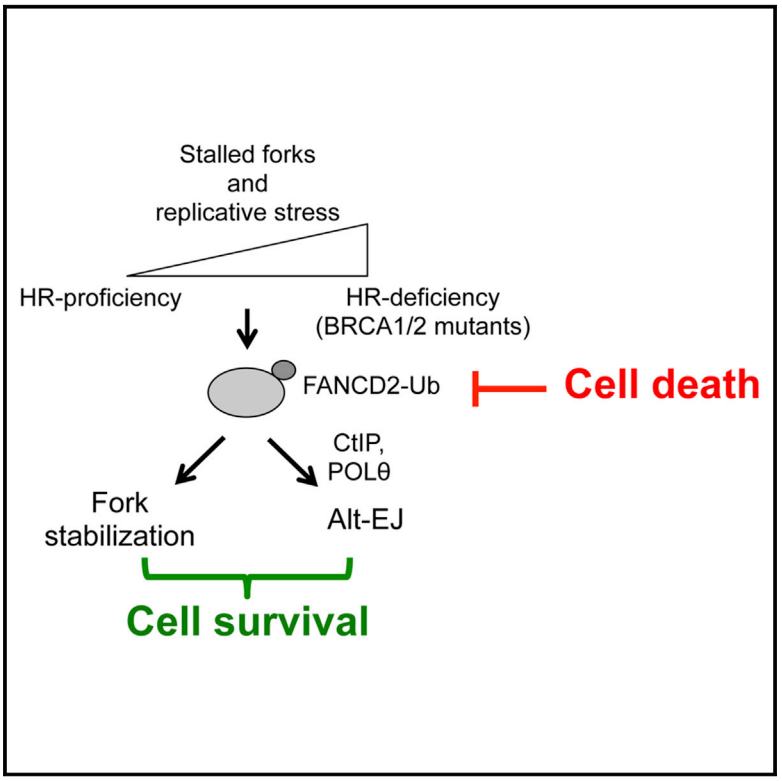
FANCD2 Maintains Fork Stability in BRCA1/2-Deficient Tumors and Promotes Alternative End-Joining DNA Repair

Graphical Abstract



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In Brief

Kais et al. show that BRCA1/2-deficient tumors have a compensatory increase in FANCD2 activity. FANCD2 stabilizes stalled replication forks and promotes alternative end joining (alt-EJ) in BRCA1/2-deficient tumors. Loss of FANCD2 in these tumors results in severe DNA repair defects and enhanced cell death.

Highlights

- Loss of FANCD2 and BRCA1/2 is synthetic lethal
- FANCD2 maintains fork stability in BRCA1/2-deficient cells
- FANCD2 promotes alternative end joining (alt-EJ)
- FANCD2 overexpression confers resistance to PARP inhibitors

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FANCD2 Maintains Fork Stability in BRCA1/2-Deficient Tumors and Promotes Alternative End-Joining DNA Repair

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SUMMARY

BRCA1/2 proteins function in homologous recombination (HR)-mediated DNA repair and cooperate with Fanconi anemia (FA) proteins to maintain genomic integrity through replication fork stabilization. Loss of BRCA1/2 proteins results in DNA repair deficiency and replicative stress, leading to genomic instability and enhanced sensitivity to DNA-damaging agents. Recent studies have shown that BRCA1/2-deficient tumors upregulate Polθ-mediated alternative end-joining (alt-EJ) repair as a survival mechanism. Whether other mechanisms maintain genomic integrity upon loss of BRCA1/2 proteins is currently unknown. Here we show that BRCA1/2-deficient tumors also upregulate FANCD2 activity. FANCD2 is required for fork protection and fork restart in BRCA1/2-deficient tumors. Moreover, FANCD2 promotes Polθ recruitment at sites of damage and alt-EJ repair. Finally, loss of FANCD2 in BRCA1/2-deficient tumors enhances cell death. These results reveal a synthetic lethal relationship between FANCD2 and BRCA1/2, and they identify FANCD2 as a central player orchestrating DNA repair pathway choice at the replication fork.

INTRODUCTION

Multiple mechanisms cooperate in cells to ensure the fidelity of DNA replication and to maintain genome integrity. Exogenous DNA damage and/or endogenous replication stress cause stalling of replication forks, leading to the recruitment of multiple proteins that stabilize stalled forks, repair DNA lesions, and restart replication (Branzei and Foiani, 2007, 2010; Michel et al., 2004). Failure to arrest replication forks at damaged sites or to restart replication once the repair is completed affects both genomic stability and cell survival (Cox et al., 2000). Indeed, damaged DNA, such as double-strand breaks (DSBs) and interstrand crosslinks (ICLs), and replication fork collapse are the main forces that drive genome instability (Aparicio et al., 2014; Deans and West, 2011).

BRCA1 and BRCA2 (BRCA1/2) proteins have a dual role in protecting genomic integrity. On the one hand, BRCA1/2 proteins promote homologous recombination (HR)-mediated DNA repair (Moynahan et al., 1999, 2001). On the other hand, these proteins also limit replication stress by controlling the stability of stalled replication forks (Lomonosov et al., 2003; Pathania et al., 2014; Schlacher et al., 2011; Willis et al., 2014). Another DNA repair pathway carrying repair-independent functions during replication is the Fanconi anemia (FA) pathway (Gari et al., 2008; Kim and D'Andrea, 2012). Indeed, BRCA1/2 and some FA proteins, such as FANCD2, localize to stalled replication forks, protect nascent strands from excessive nucleolytic degradation (Lossaint et al., 2013; Schlacher et al., 2011, 2012), and facilitate replication restart once DNA repair is complete (Lossaint et al., 2013; Schwab et al., 2015). For these reasons, the FA and BRCA1/2 proteins play a central role in limiting replication stress (Chan et al., 2009; Howlett et al., 2005; Naim and Rosselli, 2009). According to a conventional model, FANCD2 and BRCA1/2 proteins cooperate in an epistatic pathway, namely the FA/BRCA pathway, to both repair DNA lesions and stabilize replication forks (Kim and D'Andrea, 2012).

In accordance with the DNA repair and fork stabilization functions of BRCA1/2 proteins, BRCA1/2-deficient tumor cells exhibit both increased genomic instability and replicative stress (Cancer Genome Atlas Research Network, 2011; Schlacher et al., 2011; Zeman and Cimprich, 2014). As a result, BRCA1/2-deficient cells are hypersensitive to chemotherapeutic agents, such as PARP inhibitors (PARPis) (Bryant et al., 2005; Farmer et al., 2005; Konstantinopoulos et al., 2015), and to replication stress-inducing poisons (Howlett et al., 2005).

In BRCA1/2-deficient cells, unstable replication forks lead to chromosomal translocation and copy number variation (Hastings et al., 2009). Although genomic instability is critical to tumor progression, its excess can limit cell survival (Bartkova et al., 2005; Negrini et al., 2010). As a consequence, BRCA1/2-deficient cells have evolved mechanisms to tolerate replication stress and genomic instability, with the ultimate goal of ensuring DNA replication and cell survival (Ceccaldi et al., 2016). As an

example, BRCA1/2-deficient cells upregulate the error-prone Polθ/PARP1-mediated alternative end-joining (alt-EJ) DNA repair pathway, thereby compensating for defective HR (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015). Polθ is a translesion synthesis polymerase (Yousefzadeh and Wood, 2013) that prevents RAD51 assembly on single-stranded DNA (Ceccaldi et al., 2015; Newman et al., 2015), and it concurrently mediates PARP1-dependent alt-EJ to resume DNA replication (Kent et al., 2015). As a consequence, BRCA1/2-deficient cells are dependent on alt-EJ for survival. Inhibition of proteins functioning in alt-EJ, such as PARP1 or Polθ, is synthetically lethal in tumors with inactivated BRCA1/2 (Bryant et al., 2005; Ceccaldi et al., 2015; Farmer et al., 2005; Mateos-Gomez et al., 2015). Besides intrinsically promoting tumor cell survival, the hyperactivation of mechanisms counteracting the onset of genomic instability also can lead to drug resistance (Bouwman and Jonkers, 2014; Lord and Ashworth, 2013). For example, secondary intragenic BRCA1/2 mutations can restore enzyme functionality and rescue HR, thus representing the most common acquired mechanism of resistance to cisplatin or PARPi (Edwards et al., 2008; Sakai et al., 2008).

Despite extensive research on mechanisms conferring resistance to PARPi (Bouwman and Jonkers, 2014; Lord and Ashworth, 2013), how BRCA1/2-deficient tumors limit their replication stress remains unknown. Many DNA repair proteins are indeed recruited to stalled forks upon replication stress (Alabert et al., 2014; Sirbu et al., 2013). For instance PARP1, whose function is required for BRCA1/2-deficient cell survival, is present at the stalled replication fork and contributes to fork stabilization and recovery (Bryant et al., 2009; Ying et al., 2012, 2016). Whether other factors contribute to fork stabilization and survival of BRCA1/2-deficient cells is currently unknown.

Here we demonstrate that BRCA1/2-deficient tumors exhibit a compensatory increase in FANCD2 expression and activity. Upon loss of BRCA1/2 proteins, FANCD2 localizes to stalled replication forks where it promotes replication fork protection and restart. FANCD2 recruits Polθ and CtIP to stalled forks and is required for alt-EJ. As a consequence, FANCD2 overexpression in BRCA1/2 mutant cells confers resistance to PARPi through replication fork stabilization. Finally, double-knockdown experiments of FANCD2 and BRCA1/2 lead to tumor cell death in vitro and in vivo, revealing a synthetic lethal relationship between these proteins. Our findings identify FANCD2 as a critical factor required for the viability of BRCA1/2-deficient tumors and for orchestrating DNA repair pathway choice at replication forks.

RESULTS

FANCD2 Is Upregulated in BRCA1/2-Deficient Cells

To identify gene candidates essential for the survival of BRCA1/2-deficient tumors, we examined gene expression profiles in cancers frequently associated with defects in HR (i.e., mutations in *BRCA1/2* genes) (Cancer Genome Atlas Network, 2012; Cancer Genome Atlas Research Network, 2011). Publicly available microarray databases revealed specific overexpression of *FANCD2* in subgroups of ovarian, breast, and uterine cancers associated with HR deficiency and high genomic instability (Figure 1A). *FANCD2* expression was upregulated in a grade-dependent manner, correlated with KI67 expression, and increased in tumors harboring alterations (mutations or epigenetic silencing) in *BRCA1* or *BRCA2* (Figures S1A–S1D). Since FANCD2 and BRCA1/2 proteins are known to function epistatically in DNA repair and fork protection (Kim and D'Andrea, 2012; Lossaint et al., 2013; Schlacher et al., 2011, 2012), we analyzed the relationship between FANCD2 activation and *BRCA1/2* alterations. Knockdown of BRCA1 or BRCA2 (BRCA1/2) with small interfering RNA (siRNA) resulted in increased FANCD2 monoubiquitination (FANCD2-Ub), similar to the level observed by depletion of the deubiquitinase enzyme USP1 (Nijman et al., 2005) (Figures S1E and S1F). Subcellular fractionation of HeLa cells revealed that BRCA2 depletion increased FANCD2-Ub enrichment on the chromatin (Figure 1B). Knockdown of BRCA1/2 proteins increased basal and damage-inducible FANCD2 expression and foci formation (Figures 1C and S1G–S1I). Taken together, these data suggest that FANCD2 expression and activation are increased in the setting of BRCA1/2 deficiency.

FANCD2 Stabilizes Replication Forks in BRCA1/2-Deficient Cells

Loss of BRCA1/2 hinders fork stability and increases replication stress (Schlacher et al., 2011). Given that FANCD2 has emerged recently as a key factor in stabilizing replication forks (Lossaint et al., 2013; Schlacher et al., 2012) and since we observed a compensatory increase in FANCD2 activity in cells lacking BRCA1/2, we tested whether FANCD2 could have a primary role in stabilizing replication forks in this genetic setting. Two known replication stress proteins, TopBP1 and PCNA, form nuclear foci at sites of halted DNA replication (Howlett et al., 2009; Yan and Michael, 2009). We found that, in response to hydroxyurea (HU)-induced replicative stress, FANCD2 foci colocalize with TopBP1, similar to what is observed with PCNA foci (Howlett et al., 2009). Interestingly, BRCA2 knockdown increased FANCD2 colocalization with TopBP1 and PCNA (Figure 1D). Moreover, siRNA-mediated BRCA1 and BRCA2 depletion in FANCD2-deficient cells (PD20) caused a significant increase in fork degradation and fork collapse after fork stalling by HU (Figures 1E, 1F, S1J, and S1K). To assess the importance of FANCD2 monoubiquitination in the stabilization of the replication fork in BRCA1/2-deficient cells, we evaluated the ability of wild-type (WT) or the ubiquitination mutant of FANCD2 (K561R-FANCD2) to complement the siBRCA1/2-mediated fork instability. Expression of WT FANCD2 in FANCD2-deficient (PD20) cells rescued fork degradation and fork restart, while K561R-FANCD2 did not (Figures 1E, 1F, S1J, and S1K). Collectively, these data indicate that loss of BRCA1/2 triggers localization of FANCD2-Ub to stalled replicative sites, where it maintains fork stability and promotes replication fork restart.

Synthetic Lethal Interaction between BRCA1/2 and FANCD2

Given that the stability of replication forks is essential for genomic integrity (Zeman and Cimprich, 2014), we hypothesized that FANCD2 is required for maintaining genomic stability in BRCA1/2-deficient cells. HR-deficient ovarian tumor cell lines, A2780-shBRCA1 and A2780-shBRCA2 cells, were generated and subjected to siRNA-mediated FANCD2 depletion, and

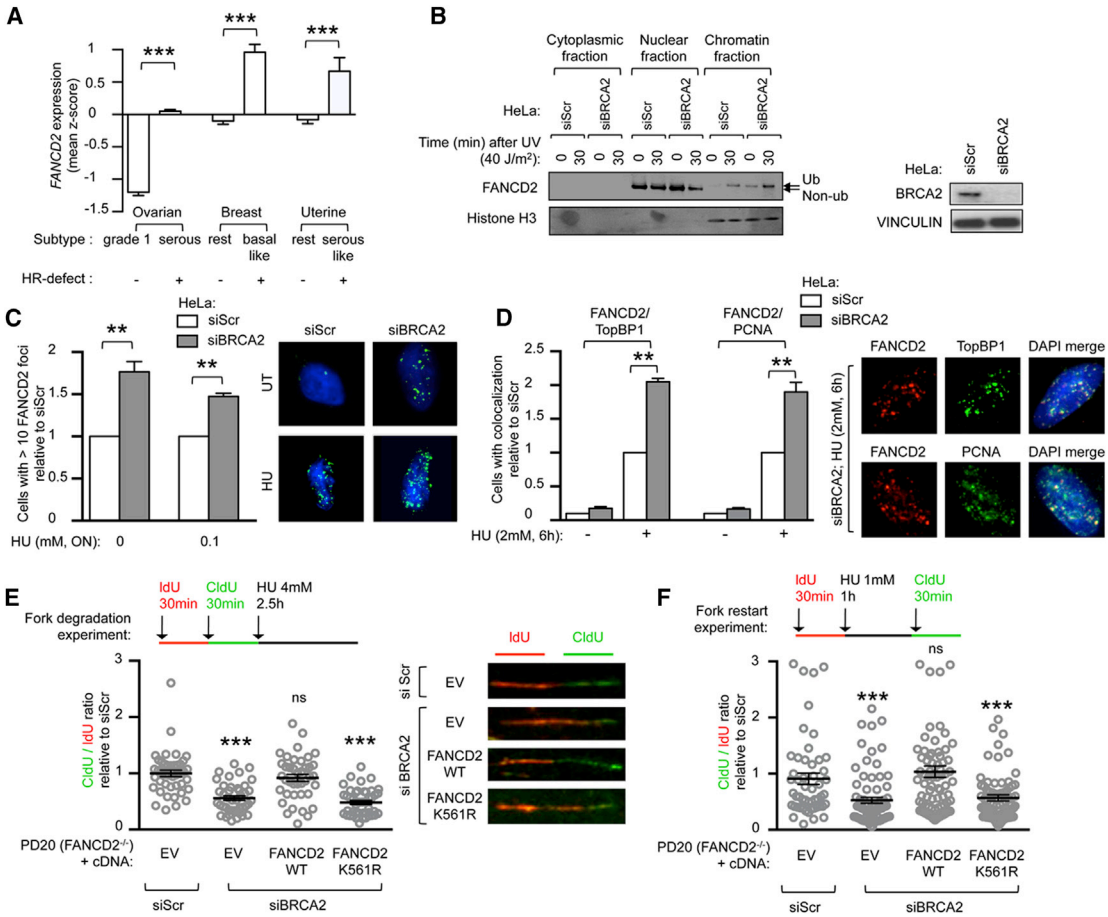

Figure 1. Upregulated FANCD2 Protects Replication Forks in BRCA2-Deficient Tumor Cells

(A) *FANCD2* gene expression in subtypes of ovarian, breast, and uterine cancers. For each tumor group, expression values are represented as the mean of *Z* scores.

(B) FANCD2 immunoblot in HeLa cells after siRNA depletion of BRCA2, at indicated time points after UV treatment and cellular fractionation. Immunoblot shows BRCA2 depletion efficiency.

(C) Quantification of baseline and damage (HU)-induced FANCD2 foci in HeLa cells after siRNA depletion of BRCA2. Representative images are shown. Statistics were performed on n ≥ 150 cells per condition and expressed as relative to siScr that was conventionally set to 1.

(D) Baseline and damage (HU)-induced FANCD2 TopBP1 and FANCD2 PCNA immunofluorescence in HeLa cells after siRNA depletion of BRCA2. Representative images are shown. Statistics were performed on n ≥ 150 cells per condition and expressed as relative to siScr that was conventionally set to 1.

(E and F) Schematic for the labeling of FANCD2-deficient (PD20) cells with IdU and CldU for fork degradation (E) and for fork restart experiments (F). Scatter dot plots for CldU to IdU ratio of cells expressing indicated cDNA and transfected with indicated siRNA are shown. Statistics were performed on n ≥ 100 fibers per condition and expressed as relative to EV that was conventionally set to 1. Representative images are shown.

Data in (C)–(F) represent mean ± SEM over three independent experiments. Data in (A) and (C)–(F) were analyzed using Student's t test. ON, overnight; ns, non significant; EV, empty vector; WT, wild-type.

clonogenic survival was measured (Figure S2A). FANCD2 depletion reduced the survival of BRCA1/2-deficient cells but had no effect on the isogenic HR-proficient cells (Figure 2A). Moreover, tumor cells expressing small hairpin RNAs (shRNAs) against both BRCA2 and FANCD2 exhibited increased chromosomal aberrations and reduced survival after Mitomycin C (MMC) treatment as compared to shScr control cells (Figures 2B, 2C, and S2B). Consistent with these findings, a BRCA2-depleted (VU423) cell line showed increased basal and MMC-induced chromosomal aberrations together with reduced clonogenic survival after siRNA-mediated FANCD2 depletion (Figures 2D and S2C). Since FANCD2 forms a heterodimer with FANCI (Smogorzewska et al., 2007), we evaluated the potential synthetic lethality between FANCI and BRCA1/2 proteins. Similar to FANCD2, FANCI depletion specifically reduced BRCA1/2-deficient cell survival without affecting the survival of BRCA1/2-proficient cells (Figures S2D). Collectively, our data show that FANCD2 limits the extent of genomic instability in BRCA1/2-deficient cells and is required for their survival.

FANCD2 Depletion Reduces Survival of BRCA1/2-Deficient Tumors In Vitro and In Vivo

To further analyze the synthetic lethal relationship between FANCD2 and BRCA1/2, we measured the clonogenic survival of

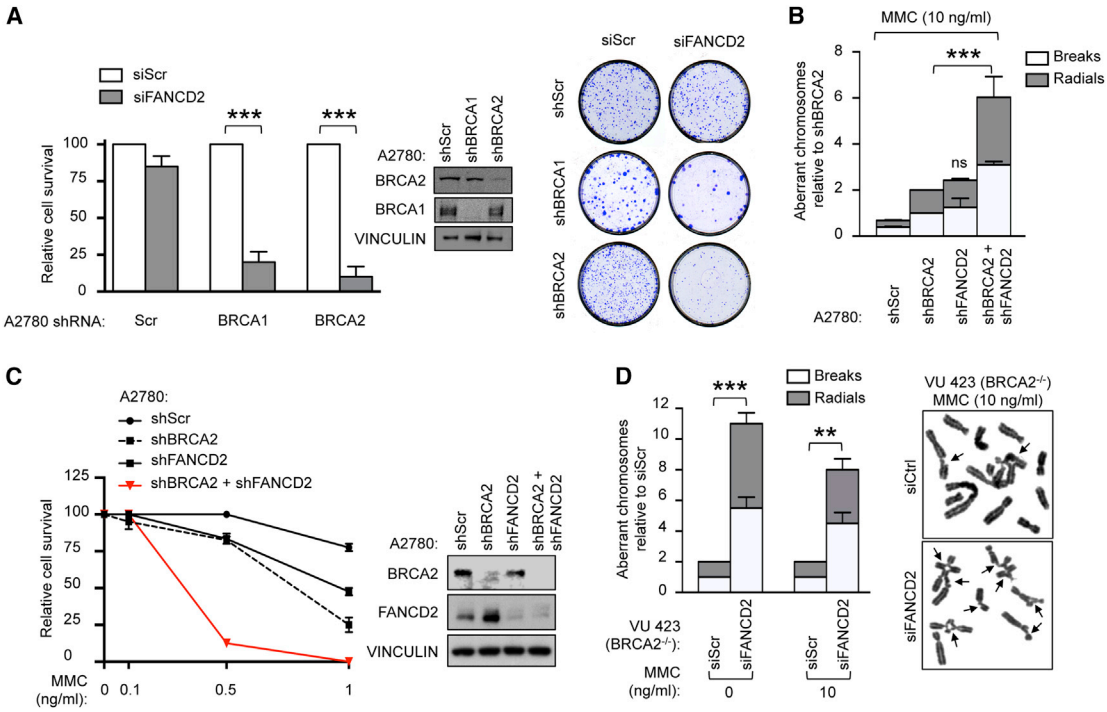

Figure 2. FANCD2 Maintains Genomic Stability in BRCA1- and BRCA2-Deficient Tumors

(A) Clonogenic formation of A2780 cells expressing the indicated shRNA together with the indicated siRNA. Immunoblot showing knockdown efficiencies of siRNAs and representative images for clonogenic formation are shown.

(B) Chromosome breakage analysis of A2780 cells expressing the indicated shRNA and treated with MMC is shown.

(C) Clonogenic formation of A2780 cells expressing the indicated shRNA under increasing concentrations of MMC. Survival is shown as relative to the untreated sample (MMC 0 ng/ml). Immunoblot shows silencing efficiency of shRNAs.

(D) Chromosome breakage analysis of BRCA2-deficient (VU 423) cells transfected with the indicated siRNA. Representative images are shown. Arrows indicate chromosomal aberrations.

Data in (A)–(D) represent mean ±SEM over three independent experiments and were analyzed by using the chi-square test for trend in proportions (A) or Student's t test (B and D). Data in (A) and (D) are displayed as relative to siScr samples, while in (B) they are displayed as relative to shBRCA2 sample.

a panel of 18 breast and 36 ovarian cell lines (Table S1) subjected to FANCD2 depletion. Depletion of FANCD2 primarily affected survival of cell lines with *BRCA1/2* alterations (Figures 3A and S3A). FANCD2 depletion also affected BRCA1-deficient tumor growth in vivo. The BRCA1-mutated (MDA-MB-436) breast cell line, expressing a doxycycline-inducible FANCD2 shRNA, was xenotransplanted into athymic nude mice. Once tumors reached palpable sizes (100–200 mm^3), mice were treated with doxycycline and tumor volumes were measured for 6 weeks. FANCD2 depletion significantly impaired tumor growth in vivo (Figures 3B and 3C). Taken together, these data confirm that BRCA1/2-deficient tumors are hypersensitive to the loss of FANCD2.

To determine whether FANCD2 monoubiquitination is required for the survival of BRCA1/2-depleted cells, we performed complementation studies in BRCA1/2-deficient cells. Expression of WT, but not K561R-FANCD2, in FANCD2-deficient (PD20) cells in which BRCA2 had been depleted rescued clonogenic survival and MMC toxicity (Figures 3D and 3E). Similarly, in BRCA1-mutated (MDA-MB-436) cells in which the endogenous FANCD2 had been depleted, expression of WT, but not K561R-FANCD2, rescued clonogenic survival (Figure S3B). Taken together, our findings indicate that FANCD2 monoubiquitination is required to maintain genomic stability and cell survival in BRCA1/2-deficient tumors.

FANCD2 Is Required for Polθ and CtIP Foci Assembly and Promotes Alt-EJ in BRCA1/2-Deficient Tumors

FANCD2 expression highly correlates with *Polθ* expression (Figure S4A), an alt-EJ factor strongly upregulated in BRCA1/2-deficient cells (Ceccaldi et al., 2015). Moreover, tumors with high *FANCD2* expression exhibit an increase in point mutation frequency and copy-number alteration, suggesting an increase in error-prone alt-EJ repair (Figures S4B and S4C). Knockdown of BRCA2 increased DNA damage-inducible Polθ foci formation (Figure 4A), confirming that alt-EJ activity is upregulated in BRCA1/2-deficient tumors (Ceccaldi et al., 2015). Recent evidence has suggested a role of FA proteins in alt-EJ (Howard et al., 2015). Moreover, the FA protein FANCA is required for class switch recombination (Nguyen et al., 2014), a process that relies, at least in part, on alt-EJ for rejoining DSBs (Deriano and Roth, 2013). Nonetheless, the mechanism by which the FA pathway interacts with alt-EJ remains unknown. In accordance with previous findings, in a cell-based assay that measures the efficiency of recombination of two GFP alleles (alt-EJ assay)

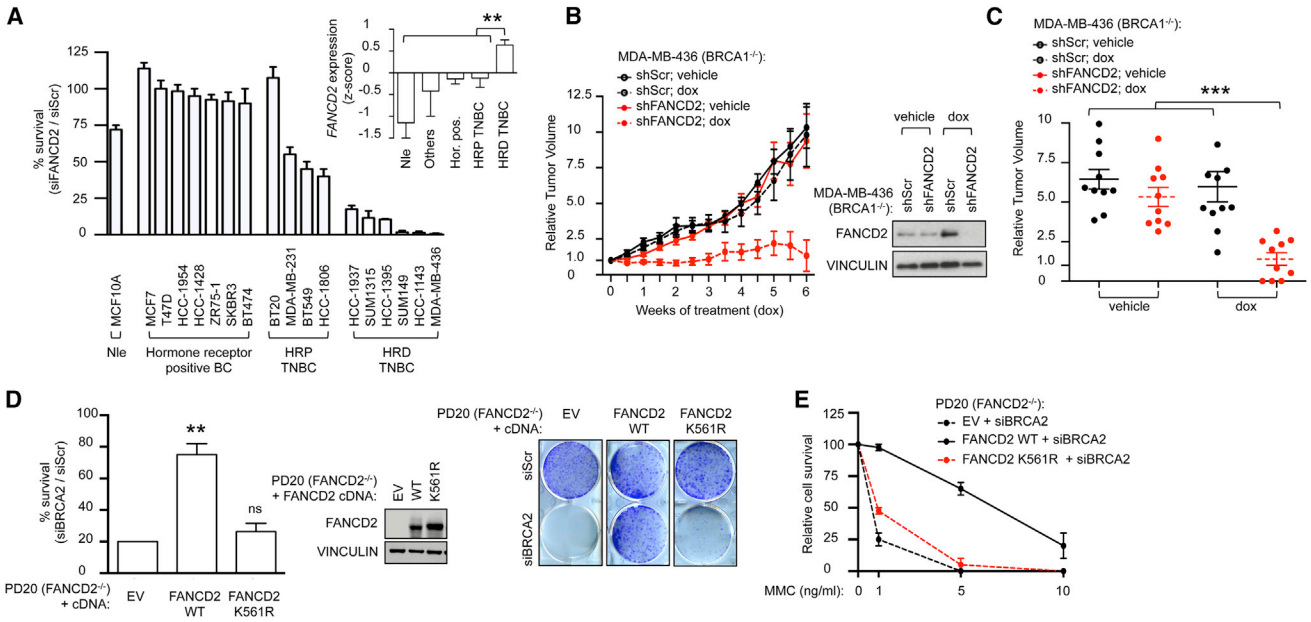

Figure 3. BRCA1- and BRCA2-Deficient Tumor Cells Are Hyperdependent on Monoubiquitinated FANCD2 for Survival

(A) Clonogenic formation of a panel of 18 breast cell lines transfected with Scr or FANCD2 siRNA. Percentage survival of cells transfected with siFANCD2 versus siScr is plotted. The genetic status of the cell line analyzed is indicated (Nle, normal, non-transformed mammary epithelial cell line; TNBC, triple negative breast cancer; HRP, HR-proficient; and HRD, HR-deficient). *FANCD2* gene expression in the 58 breast cell lines from the CCLE collection includes 16 of the 18 breast cell lines tested. Cell lines are grouped based on their genetic status. For each group, expression values are represented as the mean of *Z* scores. (Hor. pos., hormone positive breast cancer). A non-parametric test (Mann-Whitney-Wilcoxon) was used to compare the value of the HRD TNBC group with the rest of the groups.

(B) Growth of MDA-MB-436 cells expressing doxycycline (dox)-inducible FANCD2 or scrambled (Scr) shRNA in athymic nude mice. Immunoblot shows silencing efficiency.

(C) Relative tumor volumes (RTVs) for individual mice treated in (B) after 5 weeks of dox treatment. Each group represents n $\geq$ 10 tumors from n $\geq$ 6 mice while each dot represents data from one tumor. Data are shown as mean ±SEM. Student's t test was used to compare each sample to shFANCD2;dox.

(D) Clonogenic formation of FANCD2-deficient (PD20) cells expressing EV or FANCD2 cDNA constructs and transfected with BRCA2 siRNA. Percentage survival of cells transfected with BRCA2 versus Scr siRNA is plotted. An immunoblot shows expression of FANCD2 cDNA constructs. Representative images are shown.

(E) Clonogenic formation of FANCD2-deficient (PD20) cells expressing EV or FANCD2 cDNA constructs and transfected with BRCA2 siRNA in increasing concentrations of MMC. Percentage survival relative to non-treated cells (MMC 0 ng/ml) is plotted.

Data in (A), (D), and (E) represent mean ±SEM over three independent experiments. Data in (D) were analyzed using the chi-square test for trend in proportions.

(Bennardo et al., 2008) (Howard et al., 2015), FANCD2 depletion reduced alt-EJ efficiency similar to the reduction observed following PARP1 or Polθ depletion (Figure 4B). Conversely, FANCD2 depletion had no effect on single-strand annealing (SSA), another DSB repair mechanism, thus arguing for a specific role of FANCD2 in alt-EJ (Figure S4D).

Since FANCD2 and the Polθ-mediated alt-EJ pathway are upregulated and essential for BRCA1/2-deficient cell survival (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015), we next evaluated whether FANCD2 could cooperate with Polθ in promoting alt-EJ. Interestingly, FANCD2 and Polθ colocalized at damage foci and at laser-induced γH2AX-positive DNA breaks (Figures 4C and 4D). The endonuclease CtIP cooperates with BRCA1 to promote DNA end resection and HR repair at DSBs (Escribano-Díaz et al., 2013; Sartori et al., 2007), but it also is required for alt-EJ (Badie et al., 2015; Zhang and Jasin, 2011). In addition, CtIP and FANCD2 proteins physically interact (Murina et al., 2014; Unno et al., 2014; Yeo et al., 2014). Based on these findings, we determined whether CtIP might cooperate with FANCD2 and Polθ in alt-EJ repair. CtIP, like FANCD2, colocalized with Polθ at sites of DNA breaks (Figure 4E). We next aimed at better elucidating the functional hierarchy among these alt-EJ factors. Of note, FANCD2 inhibition reduced Polθ accumulation at laser-induced DNA breaks and Polθ foci (Figures 4F, 4G, and S4E), similar to the reduction observed upon depletion of the alt-EJ factor PARP1 (Audebert et al., 2004; Ceccaldi et al., 2015; Mateos-Gomez et al., 2015). Analogous to MMC treatment (Murina et al., 2014), knockdown of FANCD2 impaired UV light- and HU-induced CtIP foci formation, together with its localization to laser-induced DNA breaks (Figures S4F and S4G). Knockdown of Polθ did not affect FANCD2 foci formation (Figure S4H), and depletion of CtIP by siRNA neither affected FANCD2 and Polθ foci formation nor their localization to DNA breaks (Figures S4I and S4J). Taken together, these data establish a functional hierarchy among alt-EJ factors, and they suggest that FANCD2 functions upstream of Polθ and CtIP in the alt-EJ pathway by orchestrating their recruitment to sites of DSB repair.

To further dissect how FANCD2 mediates alt-EJ, we asked whether its monoubiquitination is necessary. To this goal, we first assessed the ability of WT or K561R-FANCD2 to complement the

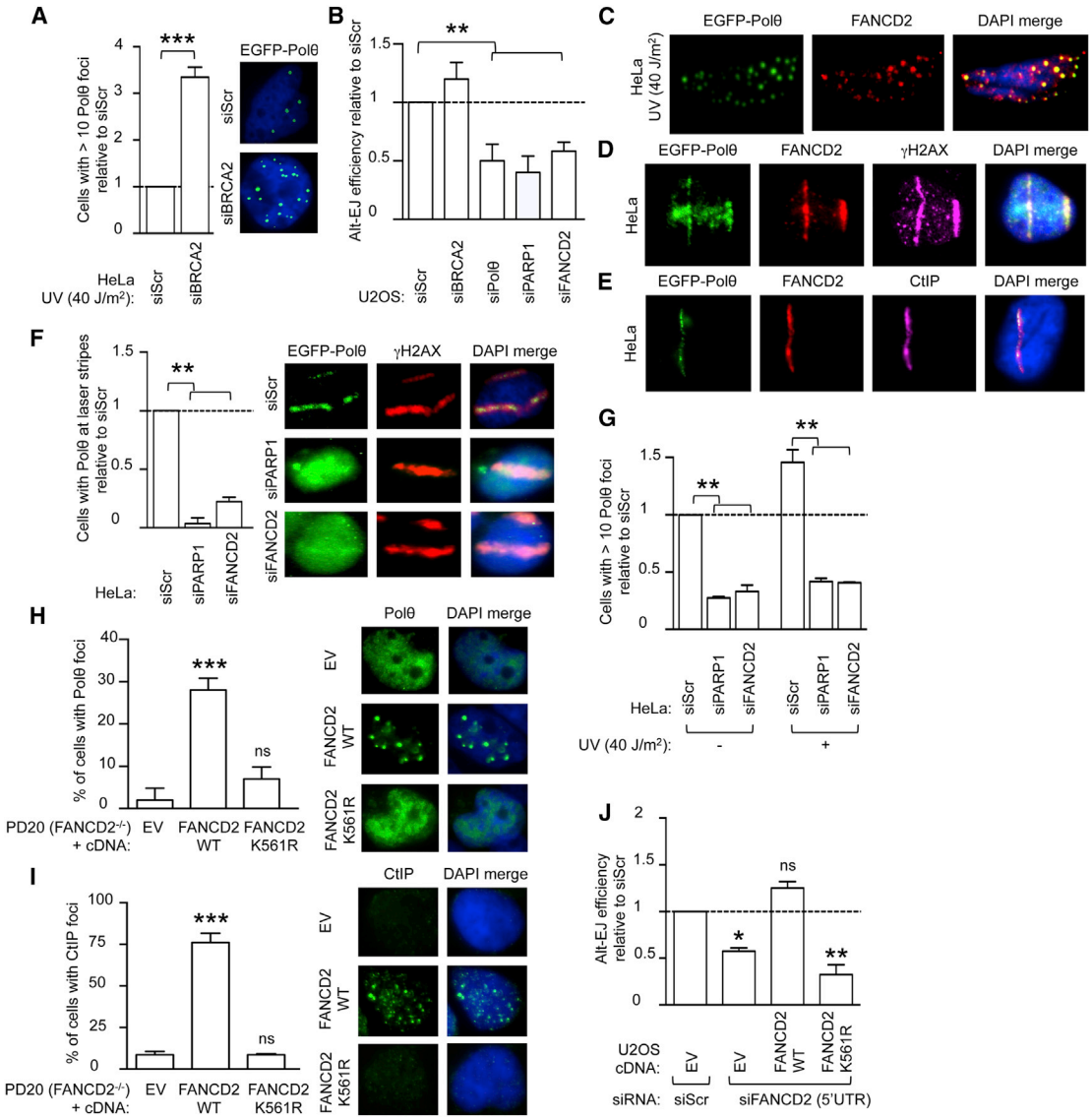

Figure 4. Monoubiquitinated FANCD2 Is Required for Polθ and CtIP Foci Formation and for Alt-EJ

(A) Quantification of damage (UV)-induced Polθ foci in HeLa cells after siRNA depletion of BRCA2 is shown.

(B) EJ reporter assay in EJ2-U2OS cells transfected with the indicated siRNA is shown.

(C) FANCD2 immunofluorescence of HeLa cells transfected with GFP-tagged full-length Polθ and subjected to UV damage. Representative images are shown.

(D) FANCD2 and γH2AX immunofluorescence in HeLa cells transfected with GFP-tagged full-length Polθ after laser micro-irradiation. Representative images are shown.

(E) FANCD2 and CtIP immunofluorescence in HeLa cells transfected with GFP-tagged full-length Polθ after laser micro-irradiation. Representative images are shown.

(F) Quantification of Polθ localization at laser micro-irradiation sites in HeLa cells transfected with GFP-tagged full-length Polθ and with indicated siRNA. γH2AX immunofluorescence serves as a marker of laser-induced DNA breaks. Representative images are shown.

(G) Quantification of baseline and damage (UV)-induced Polθ foci in HeLa cells transfected with indicated siRNA is shown.

(H and I) Quantification of Polθ foci (H) and CtIP foci (I) formation in FANCD2-deficient (PD20) cells expressing EV, WT, or K561R FANCD2 cDNA constructs. Representative images are shown.

(J) EJ reporter assay in EJ2-U2OS cells expressing indicated cDNAs and transfected with Scr or 5′ UTR-FANCD2 siRNA.

Data in (A), (B), and (F)–(J) represent mean ±SEM over three independent experiments. Data in (A), (B), (F), (G), and (J) are displayed as relative to siScr-transfected sample, whose value is set to 1, and were analyzed using Student's t test. Data in (H) and (I) were analyzed using the chi-square test for trend in proportions.

siFANCD2-mediated decrease in Polθ and CtIP foci and their localization to laser-induced DNA stripes. WT FANCD2, but not K561R-FANCD2, complemented the cells (Figures 4H, 4I, and S4K). In parallel, to evaluate whether FANCD2 monoubiquitination is necessary for alt-EJ activity, we assessed the ability of WT or K561R-FANCD2 to complement the siFANCD2-mediated

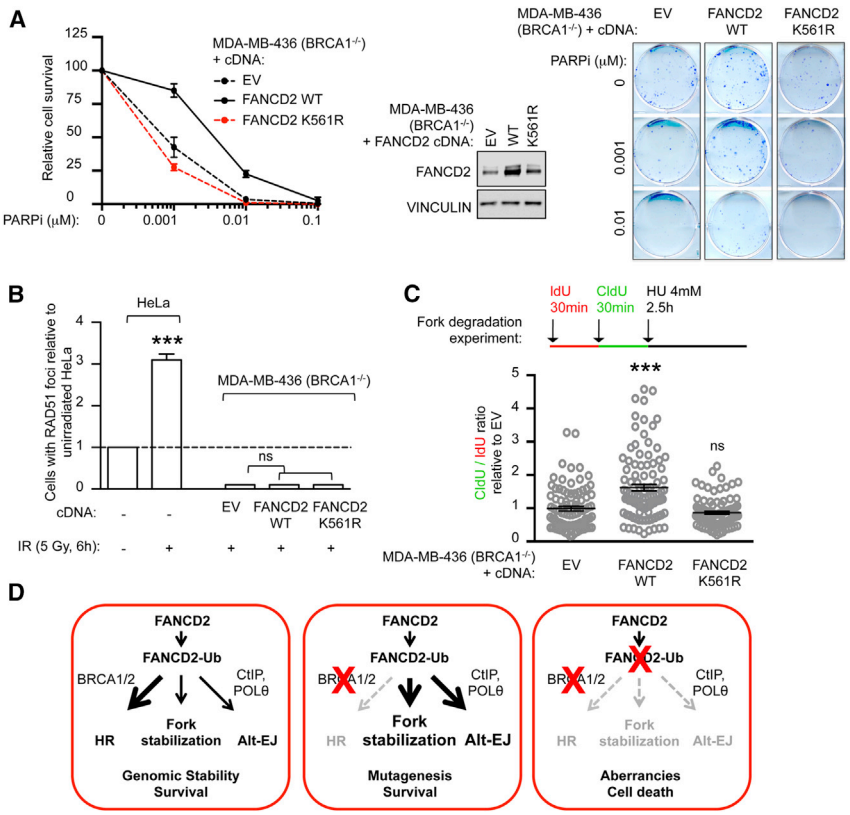

Figure 5. FANCD2 Overexpression in BRCA1-Mutated Breast Cell Line Confers Resistance to PARPi

(A) Clonogenic formation assay of BRCA1-mutated (MDA-MB-436) breast cell line expressing EV or FANCD2 cDNA constructs treated with increasing concentrations of PARPi. FANCD2 immunoblot shows cDNA construct expression. Representative images for clonogenic formation assays are shown. Survival of each sample is expressed as percentage relative to non-treated (PARPi, 0 μM).

(B) Quantification of baseline and damage (IR)-induced RAD51 foci in HeLa and BRCA1-mutated (MDA-MB436) cells expressing indicated cDNA. Data are displayed as relative to unirradiated HeLa cells.

(C) Schematic for the labeling of MDA-MB-436 cells with IdU and CldU for fork degradation experiments. Scatter dot plot for CldU to IdU ratio upon HU treatment for BRCA1-mutated (MDA-MB-436) breast cell line expressing indicated cDNA is shown. Statistics were performed on n ≥ 100 fibers per condition and expressed as relative to EV that was conventionally set to 1.

(D) Model for FANCD2 functions in BRCA1- and BRCA2-deficient tumors. FANCD2 functions in HR and alt-EJ repair pathways and also participates in genomic maintenance by protecting fork stability. Although all pathways function in cycling cells, HR is the predominant pathway under normal conditions and the alt-EJ pathway is thought to play only a minor role (left panel). Inactivation of BRCA1 or BRCA2 has no consequence on cell survival, but it induces both the localization of FANCD2 to stalled forks and the hyperactivation of the alt-EJ pathway (middle panel). As a consequence, loss of both BRCA1/2 and FANCD2 pathways induces cell death, defining a synthetic lethal interaction between the BRCA and the FA pathways (right panel).

Data in (A)–(C) represent mean ± SEM over three independent experiments and were analyzed using Student's t test.

decrease in alt-EJ activity. WT FANCD2, but not K561R-FANCD2, rescued the alt-EJ activity, suggesting that FANCD2-Ub is required for alt-EJ (Figure 4J). Collectively, these data show that FANCD2 monoubiquitination promotes alt-EJ at damaged sites in a mechanism dependent on Polθ and CtIP recruitment.

Since a synthetic lethal relationship exists between BRCA1/2 and FANCD2 (Figure 2A) as well as between BRCA1/2 and Polθ (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015), we evaluated whether also CtIP knockdown affected BRCA1/2-deficient cells' survival. Unlike FANCD2 depletion, CtIP depletion did not reduce BRCA2-deficient (VU 423) cell survival (Figure S5A). These data were recapitulated in isogenic pairs of BRCA1/2-proficient and deficient HeLa cells that were subjected to siRNA-mediated FANCD2 or CtIP depletion and assessed for clonogenic survival (Figure S5B). All together, our data support a role for FANCD2 as an upstream alt-EJ regulatory factor required for both CtIP and Polθ recruitment to damage sites and for BRCA1/2-deficient cell survival.

FANCD2 Overexpression Confers Resistance to PARPis through Replication Fork Stabilization

In BRCA1/2-deficient tumors, reduction of genomic instability results in resistance to PARPis (Bouwman and Jonkers, 2014; Lord and Ashworth, 2013). Since FANCD2 enables BRCA1/2-deficient cell survival, we determined whether FANCD2 overexpression could confer resistance to PARPis. We assessed the clonogenic survival of BRCA1- and BRCA2-mutated cells (MDA-MB-436 and VU 423, respectively) overexpressing WT or K561R-FANCD2 cDNAs and grown in the presence of PARPi. Overexpression of WT, but not K561R-FANCD2, increased the PARPi resistance of BRCA1/2-mutated cells (Figures 5A and S5C). Restoration of HR functionality represents the most common acquired mechanism of PARPi resistance in BRCA1/2-deficient tumors (Edwards et al., 2008; Sakai et al., 2008). We thus assessed whether FANCD2 overexpression restored HR in BRCA1-mutated (MDA-MB-436) cells. Neither WT nor K561R-FANCD2 improved HR function as measured by ionizing radiation (IR)-induced RAD51 foci formation (Figure 5B), suggesting that FANCD2-mediated PARPi resistance occurs independently of HR restoration.

Given that FANCD2-Ub stabilizes replication forks in BRCA1/2-deficient cells (Figure 1E), we tested whether the FANCD2-mediated PARPi resistance correlated with increased fork protection. Of note, WT, but not K561R-FANCD2, improved fork stabilization in BRCA1-mutated (MDA-MB-436) cells, as measured by fork degradation after HU exposure (Figure 5C). Accordingly, in BRCA1/2-deficient cells, increased FANCD2-Ub represents a mechanism of PARPi resistance mediated by replication fork protection.

DISCUSSION

The ability to stabilize and restart stalled replication forks is essential to maintaining genomic stability and cell survival. The importance of this survival mechanism is even more pronounced in cancer cells, characterized by increased replication stress levels (Bartkova et al., 2005; Gorgoulis et al., 2005). BRCA1/2 and FA proteins are known to cooperate in the repair of DNA lesions, causing replication stalling such as ICLs, by promoting end resection and HR (Garcia-Higuera et al., 2001; Wang et al., 2004). In the context of replication stress, BRCA1/2 and FA proteins also cooperate in the protection of stalled replication forks by limiting the endonuclease-dependent degradation of nascent DNA strands (Schlacher et al., 2011, 2012). Besides cooperating with BRCA1/2, FANCD2 also cooperates with BLM and FAN1 to promote fork recovery (Chaudhury et al., 2013, 2014; Lachaud et al., 2016).

BRCA1/2 mutations, and other HR gene mutations, are found in a large proportion of ovarian and breast cancers (Cancer Genome Atlas Network, 2012; Cancer Genome Atlas Research Network, 2011). Importantly, these tumors are characterized by high levels of replication stress (Cancer Genome Atlas Research Network, 2011; Pathania et al., 2014; Schlacher et al., 2011; Zeman and Cimprich, 2014). Elevated replication stress renders these tumors hyperdependent on additional protective mechanisms that might reduce the stress, thus promoting tumor cell survival.

To identify new survival mechanisms of BRCA1/2-deficient cancers, we analyzed gene expression profiles in subsets of tumors frequently associated with defects in HR. FANCD2 mRNA and protein were specifically overexpressed in BRCA1- and BRCA2-mutated ovarian and breast tumors, compared to HR-proficient cancers. While FANCD2 normally functions epistatically with BRCA1/2 to promote repair of ICL lesions (Figure 5D, left panel), these results reveal a nonepistatic function of FANCD2 and BRCA1/2 proteins in fork stabilization. Indeed, in the case of tumors defective in HR (BRCA1/2 mutated), FANCD2 localizes at sites of stalled replication and is monoubiquitinated (FANCD2-Ub) to a higher extent. Moreover, the upregulation of FANCD2-Ub at stalled forks prevents fork degradation and facilitates fork restart (Figure 5D, middle panel). These results are consistent with previous studies that show that FANCD2 has a role in fork stabilization (Lossaint et al., 2013; Schlacher et al., 2012) and fork restart by cooperating with the BLM helicase (Chaudhury et al., 2013; Lossaint et al., 2013). Concomitant with replication fork stabilization, FANCD2 mediates the survival of BRCA1/2-mutated tumors by counteracting the onset of genome instability events. These events arise from the collapse or the instability of stalled forks. Indeed, loss of FANCD2 in BRCA1/2-deficient cells hinders the stability of stalled replication forks and causes severe and disabling damage that compromises genomic integrity and cell survival. These findings reveal a mechanism of synthetic lethality between BRCA1/2 and FANCD2 (Figure 5D, right panel). Of note, the survival dependency of BRCA1/2-mutated cancers on FANCD2 is displayed in a large panel of breast and ovarian cancer cell lines and in in vivo models of breast tumor xenografts.

While our results reveal a requirement for FANCD2-mediated stabilization and restart of replication forks in BRCA1/2-deficient cells, the mechanism by which these cells repair stalled forks also was examined. FANCD2 was required for Polθ and CtIP recruitment to damaged sites in order to promote alt-EJ (Figure 5D, middle panel). Alt-EJ is a highly error-prone DNA repair pathway, requiring CtIP-mediated end resection and PARP1/Polθ-mediated microhomology-based repair (Kent et al., 2015; Lee-Theilen et al., 2011; Simsek and Jasin, 2010; Wang et al., 2006; Zhang and Jasin, 2011). While alt-EJ is a backup pathway for the repair of DSBs in cells deficient for classical non-homologous EJ (C-NHEJ), it has emerged recently as an integral DSB repair pathway contributing to the survival of HR-deficient cancer cells (Aparicio et al., 2014; Bunting and Nussenzweig, 2013; Ceccaldi et al., 2015; Deriano and Roth, 2013; Mateos-Gomez et al., 2015).

Usually relying on HR for error-free repair (Couch et al., 2013), stalled forks also are repaired through mutagenic pathways, such as C-NHEJ or alt-EJ itself in the absence of HR (Ceccaldi et al., 2016). This hypothesis is supported by evidence for a direct role of PARP1 in fork stabilization and recovery after replicative stress (Bryant et al., 2009; Ying et al., 2012, 2016). In this study, we show that FANCD2 also acts as an alt-EJ factor. Indeed, FANCD2 colocalizes with CtIP and Polθ at bona fide DNA damage sites and promotes microhomology-mediated DNA repair in a GFP-based cell reporter system for alt-EJ, similar to PARP1 and Polθ. We next investigated the role of FANCD2 in alt-EJ by establishing a functional hierarchy among alt-EJ proteins. FANCD2 precedes and mediates the binding of CtIP and Polθ to DNA damages sites. Interestingly, siRNA-mediated CtIP depletion does not limit the number of FANCD2 and Polθ foci after damage, nor does it cause synthetic lethality in BRCA1/2-deficient cells. These data are in accordance with previous studies on the mutual contributions of CtIP and FANCD2 in the control of fork recovery (Yeo et al., 2014). They also support the hypothesis of a specific role for FANCD2 in fork protection and ultimately the survival of HR-deficient cells.

While BRCA1 and BRCA2 proteins harbor distinct functions in HR-mediated repair (Prakash et al., 2015), their deficiency similarly leads to fork instability and high replicative stress (Pathania et al., 2014; Schlacher et al., 2011). Moreover, FANCD2 is analogously upregulated in BRCA1- and BRCA2-deficient tumors. For these reasons, we examined the role of FANCD2 upon loss of BRCA1 or BRCA2 as a whole. Previous data showed that BRCA1 was required for FANCD2 activation. For instance, loss of BRCA1 leads to a decrease in IR-dependent induction of FANCD2 foci (Bunting et al., 2012; Duquette et al., 2012; Garcia-Higuera et al., 2001), and, in *Xenopus* egg extracts, BRCA1 is required for FANCD2 recruitment to a site-specific ICL (Long et al., 2014). Nevertheless, BRCA1-deficient cells retain the ability to form distinct FANCD2 foci (Garcia-Higuera et al., 2001), and the loss of BRCA1 increases the level of FANCD2-Ub. Importantly, these data suggest that, even in the absence of functional BRCA1, FANCD2-Ub is still loaded onto chromatin. While FANCD2 is required for the survival of BRCA1-deficient cells independently of the established BRCA1 role in activating FANCD2, further studies should address whether BRCA1 is upstream or downstream of FANCD2. A possibility could be that FANCD2 and BRCA1 present different functional interdependencies based on the genome stability pathway taken into

consideration. For instance, while BRCA1 seems to function upstream of FANCD2 in ICL repair (Long et al., 2014), our data suggest that, in fork protection, FANCD2 functions upstream of BRCA1.

Overall, our study demonstrates a dependency of HR-deficient tumors on FANCD2 for cellular survival. In accordance with previous studies (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015), survival of HR-deficient tumors is promoted by mutagenic alt-EJ DNA repair pathways, of which FANCD2 is identified as an upstream component. Moreover, we propose that the dependency on FANCD2 of these tumors is mediated by its fork stability and recovery functions. In contrast with the common view where BRCA1/2 and FA proteins cooperate in the repair of DNA lesions by HR, we here present evidence for a compensatory fork stabilization activity of FANCD2 in the absence of BRCA1/2 proteins. Aware of the limitations of the genetic, repair-deficient context that we have investigated, we believe that characterizing the role of FANCD2 and its partners in fork protection might shed light on the unexplored mechanisms of fork stabilization as a widespread mechanism of tumor cell survival. More broadly, it will be of interest to investigate whether FANCD2 represents a fork stabilization and/or a survival factor only in BRCA1/2-mutated cancers or also in other cancers that are characterized by high replication stress levels, for example, as a consequence of oncogene activation.

Other questions remain to be answered, such as whether an inverse compensatory mechanism exists between the BRCA1/2 and FA proteins where BRCA1/2 proteins could compensate for FA pathway deficiency, as in the case of FA patients. Further studies also should aim to understand in greater depth what aspect of FANCD2 (fork stabilization or alt-EJ repair) is responsible for the survival of BRCA1/2-deficient tumors. In this regard, our data indicate that FANCI depletion induces synthetic lethality in BRCA1/2-deficient cells, similar to FANCD2 depletion. Since FANCI recently was shown to promote fork restart upon replication stress (Chen et al., 2015), our results enforce the hypothesis that the fork protection function of FA pathway members is a major regulator of BRCA1/2-deficient cells survival. In addition, while previous studies on Polθ suggest that the alt-EJ pathway is required for BRCA1/2 survival (Ceccaldi et al., 2015; Mateos-Gomez et al., 2015), this is not the case for other alt-EJ factors such as CtIP, whose depletion, while decreasing alt-EJ efficiency, does not confer synthetic lethality with BRCA1/2 deficiency. Clinical applications relying on synthetic lethality relationships are just beginning to emerge, and understanding these relationships can shed light on unexpected connections between biological pathways. Therefore, it is of critical importance to dissect their molecular basis from both basic and translational perspectives.

EXPERIMENTAL PROCEDURES

Bioinformatic Analysis

The Cancer Genome Atlas (TCGA) datasets were accessed through the public TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). For mutation count, we accessed data from tumors included in the TCGA datasets for which gene expression and whole-exome DNA sequencing were available. Data were accessed through the public TCGA data portal and the cBioPortal for Cancer Genomics (http://www.cbioportal.org). For each TCGA dataset, non-synonymous mutation count was assessed in tumors with high *FANCD2* expression (top 50%) and compared to tumors with low *FANCD2* expression (the remaining 50%). In the uterine TCGA, we curated all tumors except the ultra and hyper-mutated group (that is, POLE and MSI tumors). In the ovarian and breast TCGA datasets, all tumors were analyzed. *FANCD2* expression analysis shown in Figure S1C was extracted from the ovarian serous carcinoma datasets (GEO: GSE14001, GSE14007, GSE18520, GSE16708, and GSE10971) and compared to the ovarian clear cell carcinoma dataset (GEO: GSE29450).

Immunoblot Analysis, Cellular Fractionation, and Immunofluorescence

Cells were lysed with 1% NP40 lysis buffer (1% NP40, 300 mM NaCl, 0.1 mM EDTA, and 50 mM Tris [pH 7.5]) supplemented with protease inhibitor cocktail (Roche), resolved by NuPAGE (Invitrogen) gels and transferred onto nitrocellulose membrane, followed by detection using the LAS-4000 Imaging system (GE Healthcare Life Sciences). For cellular fractionation, cells were incubated with low-salt permeabilization buffer (10 mM Tris [pH 7.3], 10 mM KCl, and 1.5 mM MgCl2) with protease inhibitor on ice for 20 min. Following centrifugation, nuclei were resuspended in 0.2 M HCl and the soluble fraction was neutralized with 1 M Tris-HCl (pH 8.0). Nuclei were lysed in 150 mM NaCl, and, following centrifugation, the chromatin pellet was digested by micrococcal nuclease (Roche) for 5 min at room temperature. See the Supplemental Experimental Procedures for details on immunofluorescence procedures.

Antibodies, Chemicals, Plasmids, and Transfection

Antibodies used in this study included the following: anti-FANCD2 (FI-17, for immunoblotting), anti-histone H3 (FL-136), anti-PCNA (PC-10), anti-TopBP1 (B-7), anti-vinculin (H-10), and anti-RAD51 (H-92) (all from Santa Cruz Biotechnology); anti-BRCA1 and anti-BRCA2 (both Ab-1, Calbiochem); anti-FANCD2 (NB100-182, for immunofluorescence, Novus Biologicals); anti-phospho-Histone-H2AX (p-Ser139) (05-636, EMD Millipore and 2572, Cell Signaling Technology); and anti-CtIP (14-1, Active Motif). MMC and HU were purchased from Sigma. See the Supplemental Experimental Procedures for details on siRNAs, shRNAs, and plasmids.

Cell Culture

HeLa, U2OS, VU 423 (BRCA2$^{-/-}$), PD20 (FANCD2$^{-/-}$), and PD20-corrected cells were maintained in DMEM plus 10% fetal calf serum (FCS), L-glutamine (2 mM), and penicillin-streptomycin (1%). MDA-MB-436 (BRCA1-mutated) cells were maintained in RPMI medium plus 10% FCS, L-glutamine (2 mM), and penicillin-streptomycin (1%). Breast and ovarian cell lines used in the Figures 3A and S3A were cultured according to recommendations present in the literature (Table S1). HR, alt-EJ, and SSA efficiencies were measured using U2OS cell line expressing the DR-GFP (HR efficiency), EJ2-GFP (alt-EJ efficiency), and SA-GFP (SSA efficiency) reporter assay and performed as previously described (Bennardo et al., 2008; Nakanishi et al., 2005).

Cell Survival Assays

To assess clonogenic survival, cells were transfected twice with the indicated siRNAs for 48 hr, then harvested and seeded at low density into six-well plates in triplicates. For clonogenic survival under MMC or PARPi (rucaparib), cells were treated continuously with the indicated drug concentrations. Survival at each drug concentration was plotted as a percentage relative to the survival in drug-free media. Colony formation was scored 14 days after drug treatment or siRNA transfection using 0.5% (w/v) crystal violet in methanol. Survival curves were expressed as a percentage ±SEM over three independent experiments and relative to control cells (siScr). MMC was purchased from Sigma while the PARPi rucaparib (AG-014699) was purchased from Selleckchem.

Chromosomal Breakage Analysis

A2780 expressing the indicated shRNAs and VU 423 cells twice transfected with the indicated siRNAs were incubated for 48 hr with or without the indicated concentrations of MMC. Cells were exposed for 2 hr to 100 ng/ml colcemid, treated with a hypotonic solution (0.075 M KCl) for 20 min, and fixed with 3:1 methanol/acetic acid. Slides were stained with Wright's stain and 50 metaphase spreads were scored for aberrations. The relative number of chromosomal breaks and radials was calculated relative to control cells (siScr) or shBRCA2, as indicated in the figure legends.

FANCD2 Gene Expression

RNA samples extracted using the TRIzol Reagent (Invitrogen) were reverse transcribed using the Transcriptor Reverse Transcriptaze kit (Roche) and oligo dT primers. The resulting cDNA was used to analyze FANCD2 expression by qRT-PCR using the QuantiTect SYBRGreen kit (QIAGEN) in an iCycler machine (Bio-Rad). *FANCD2* gene expression values were normalized to expression of the housekeeping gene *GAPDH*, using the ΔCt method, and are shown on a log2 scale. See the Supplemental Experimental Procedures for details on primers and the *FANCD2* expression data shown in Figure 3A.

DNA Fiber Analysis

For the fork degradation analysis, PD20 cells expressing empty vector (EV), WT FANCD2, or K561R-FANCD2 were incubated with 250 μM iododeoxyuridine (IdU) (Sigma, I7125) for 30 min, followed by 25 μM chlorodeoxyuridine (CldU) (Sigma, C6891) for 30 min. Cells were then incubated with 4 mM HU for 2.5 hr. For the fork restart analysis, cells were incubated with 250 μM IdU for 30 min. Cells were then treated with 1 mM HU for 1 hr and incubated in 25 μM CldU for 30 min after washout of the drug. Spreading of DNA fibers on glass slides was done as previously reported (Jackson and Pombo, 1998). Glass slides were then washed in distilled water and in 2.5 M HCl for 120 min followed by three washes in PBS. The slides were incubated for 1 hr in blocking buffer (PBS with 1% BSA) and then for 1 hr at 37°C in rat anti-BrdU antibody (Abcam, ab6326) and mouse anti-BrdU antibody (BD Biosciences, 347580) diluted in blocking buffer. After washes in PBS, the slides were incubated for 1 hr at 37°C in goat anti-rat Alexa 488 antibody (Life Technologies, A-11006) and chicken anti-mouse Alexa 594 (Life Technologies, A-21201) diluted in blocking buffer. The slides were then washed with PBS and mounted with DAPI. At least 100 fibers were counted per condition. Pictures were taken with an Olympus confocal microscope and fiber length was measured by ImageJ software.

Studies of Xenograft-Bearing CrTac:NCr-Foxn1nu Mice

In vivo studies were performed as previously described (Ceccaldi et al., 2015), under approval of the Animal Resource Facility at the Dana-Farber Cancer Institute. See the Supplemental Experimental Procedures for more details.

Statistical Analysis

Data are represented as mean ±SEM over three independent experiments. Unless otherwise stated, significance was calculated using the unpaired Student's t test or the chi-square test for trend in proportions. Analyses were performed by the use of GraphPad Prism 6 software. A p value less than 0.05 was considered statistically significant (ns, not significant; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). All the in vivo experiments were run with at least ten tumors from six mice for each condition.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.031.

AUTHOR CONTRIBUTIONS

Z.K., B.R., A.D.D., and R.C. designed the study, performed experiments, and wrote the manuscript. A.H. performed chromosomal breakage analysis. Z.K., C.O., and D.K. performed mice work. A.D.D. and R.C. supervised the study. All authors approved the final version of the manuscript.

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