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## Targeting the FANCD1–BRCA1 interaction promotes a switch from recombination to pol $\eta$ -dependent bypass

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### Abstract

BRCA1 and the DNA helicase FANCD1 (also known as BACH1 or BRIP1) have common functions in breast cancer suppression and DNA repair. However, the functional significance of the direct interaction between BRCA1 and FANCD1 remains unclear. Here, we have discovered that BRCA1 binding to FANCD1 regulates DNA damage repair choice. Thus, when FANCD1 binding to BRCA1 is ablated, the molecular mechanism chosen for the repair of damaged DNA is dramatically altered. Specifically, a FANCD1 protein that cannot be phosphorylated at serine 990 or bind BRCA1 inhibits DNA repair via homologous recombination and promotes pol $\eta$ -dependent bypass. Furthermore, the pol $\eta$ -dependent bypass promoted by FANCD1 requires the direct binding to the mismatch repair (MMR) protein, MLH1. Together, our findings implicate that in human cells BRCA1 binding to FANCD1 is critical to regulate DNA repair choice and promote genomic stability. Moreover, unregulated FANCD1 function could be associated with cancer and/or chemoresistance.

### Keywords

BRCA1; FANCD1; DNA repair

### Introduction

BRCA1 function and tumor suppression depend on the BRCA1 C-terminal (BRCT) region, which contains two discrete domains called BRCT repeats. Mutations in the BRCT repeats result in defective DNA damage repair, such as failure to induce cell-cycle checkpoints and sensitivity to DNA double-stranded breaks (DSBs) and interstrand cross-links (ICLs) (Kim and Chen, 2008). These BRCTs also mediate the direct binding of BRCA1 to the FANCD1 DNA helicase (also known as FANCD1/BRIP1) (Cantor *et al.*, 2001). FANCD1 phosphorylation at serine 990 is required to mediate the BRCT-FANCD1 interaction such that a serine to alanine mutation (S990A) ablates this interaction (Yu *et al.*, 2003). Similar to BRCA1, mutations in FANCD1 have been associated with hereditary breast cancer (Cantor *et al.*, 2001; Seal *et al.*, 2006). In addition, FANCD1 is mutated in the rare childhood disease,

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### Conflict of interest

The authors declare no conflict of interest.

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Fanconi anemia (FA), within the FANCF (FA-J) patient complementation group (Levitus *et al.*, 2005; Levran *et al.*, 2005; Litman *et al.*, 2005).

It has been proposed that the FA pathway maintains genomic integrity by coordinating at least two DNA repair mechanisms. One of these mechanisms is homologous recombination (HR), a typically error-free DNA-repair mechanism that uses the homologous sequence in a sister chromatid for repair. HR is critical to repair DSBs, which can form when ICLs fail to be processed and replication forks collapse. Defects in HR characterize BRCA1-, FANCF-, and BRCA2-deficient cells (Moynahan *et al.*, 1999, 2001; Litman *et al.*, 2005). Evidence also suggests that the FA pathway promotes ICL resolution by a mechanism engaging the error-prone translesion synthesis (TLS) polymerases. Supporting this possibility, fewer TLS-like point mutations are present in the genome of FA cells (Patel and Joenje, 2007). Moreover, the TLS polymerases REV1 and REV3 function with the FA protein FANCF to promote ICL resistance (Niedziedz *et al.*, 2004). Consequently, the FA pathway has been proposed to coordinate both TLS and HR to resolve DNA ICLs as well as other DNA lesions thereby limiting the severity of mutagenesis (Niedernhofer *et al.*, 2005; Hinz *et al.*, 2006; Patel and Joenje, 2007).

Current models predict that TLS can function independent of HR. TLS is not expected to repair lesions, but facilitate lesion tolerance or bypass (Dronkert and Kanaar, 2001). In particular, pol $\eta$  has been implicated in recombination-independent repair of ICLs generated by mitomycin C (MMC) (Zheng *et al.*, 2003) as well as in the cellular tolerance to cisplatin (Albertella *et al.*, 2005). Because of the unique structure of its active site, pol $\eta$  replicates through cross-linked DNA (Alt *et al.*, 2007). Depending on the lesion bypassed, TLS can be mutagenic or error-free. For example, pol $\eta$  bypasses ultraviolet (UV) light-induced thymidine–thymidine dimers in an error-free manner and bypasses intrastrand cross-links and ICLs, once unhooked, in an error-prone manner (Zheng *et al.*, 2003; Prakash *et al.*, 2005). Depending on the type and severity of DNA damage, TLS is activated by a Rad6–Rad18-dependent PCNA monoubiquitination (Kannouche and Lehmann, 2004) that loads different TLS polymerases in a lesion-specific manner (Barbour and Xiao, 2003; Papouli *et al.*, 2005).

Given that both FANCF and BRCA1 are critical for HR, genomic stability, breast cancer suppression, and ICL resistance (Cantor and Andreassen, 2006), we hypothesized that these two proteins likely function together in ICL repair. However, recent studies support independent functions. In particular, the ICL sensitivity of FANCF-null chicken and patient cells was rescued with mutant versions of FANCF that cannot interact with BRCA1 (Bridge *et al.*, 2005; Peng *et al.*, 2007). This finding leads one to wonder what is the functional role for the BRCA1–FANCF interaction.

Here, we identify that when uncoupled from BRCA1, FANCF functions to inhibit HR and promote pol $\eta$ -dependent bypass. As such, FA-J patient cells expressing the BRCA1-interaction defective mutant, FANCF<sup>S990A</sup> resist DNA damage in a pol $\eta$ -dependent manner. Furthermore, FANCF<sup>S990A</sup> requires the MLH1 interaction to promote resistance to agents that induce ICLs. Together, our data implicate that in human cells, BRCA1 binding to FANCF is critical to regulate DNA repair and bypass mechanisms to promote genomic stability.

## Results

### The DNA damage response is altered when FANCF is uncoupled from BRCA1

In response to ICLs, FA cells undergo a prolonged G2/M accumulation that correlates with ICL sensitivity (Wang, 2007). Restoration of the missing FA gene, such as FANCF in FA-J

patient cells, restores ICL resistance and reduces the G2/M accumulation, most likely because cells process ICLs and re-enter the cell cycle (Litman *et al.*, 2005; Peng *et al.*, 2007). The finding that correction is also achieved on introduction of the BRCA1-interaction defective mutant FANCI<sup>S990A</sup> (Peng *et al.*, 2007) could suggest that BRCA1 binding is dispensable for FANCI to process ICLs. Alternatively, FANCI<sup>S990A</sup> could promote ICL resistance by a distinct mechanism from FANCI<sup>WT</sup>. For example, the restored ICL resistance and reduced G2/M accumulation in FA-J cells expressing FANCI<sup>S990A</sup> could have resulted from a disruption of the ICL-induced checkpoint. To test this idea, we examined the timing of entry and exit from the ICL-induced G2/M accumulation in FA-J cells complemented with vector, FANCI<sup>WT</sup>, or FANCI<sup>S990A</sup>. Expression was confirmed by western blot and as before both FANCI<sup>WT</sup> and FANCI<sup>S990A</sup> restored ICL resistance (Peng *et al.*, 2007) (Figures 1a and c; Supplementary Figure 1A). Interestingly, the maximum G2/M accumulation in the FANCI<sup>S990A</sup> complemented FA-J cells was ~25%, as compared with FANCI<sup>WT</sup> at ~40% (Figure 1b). Fewer FA-J cells accumulated at G2/M at all times when they were complemented with FANCI<sup>S990A</sup> than when complemented with FANCI<sup>WT</sup> or vector (Figure 1b). However, the growth of untreated cells was not measurably different (Supplementary Figure 1b). Thus, FANCI<sup>S990A</sup> reduced, but did not eliminate, the melphalan-induced G2/M accumulation. More importantly, this finding implicated that FA-J cells complemented with FANCI<sup>S990A</sup> or FANCI<sup>WT</sup> were distinct in the melphalan-induced response.

To further assess whether FANCI<sup>S990A</sup>, as compared with FANCI<sup>WT</sup>, promoted a distinct DNA damage repair response, the complemented FA-J cell lines were treated with different forms of DNA damage. Interestingly, FANCI<sup>S990A</sup>-, as compared with FANCI<sup>WT</sup>-, complemented FA-J cells were slightly more resistant to cisplatin, but dramatically more resistant to UV and 6-thioguanine (6-TG). In contrast, FANCI<sup>S990A</sup>-, as compared with FANCI<sup>WT</sup>-, complemented FA-J cells were more sensitive to zeocin or methyl methanesulfonate (MMS) (Figure 1c). Together with the G2/M accumulation data, it appears that the DNA damage response is distinct in FA-J cells complemented with FANCI<sup>S990A</sup> as compared to FANCI<sup>WT</sup>.

### FANCI<sup>S990A</sup> reduces RAD51 foci and HR

Zeocin induces DSBs and sensitizes HR-defective cells (Delacote *et al.*, 2007). Thus, we reasoned the zeocin sensitivity of FA-J cells complemented with FANCI<sup>S990A</sup> could result from defects in double-strand break repair and/or HR. Formation of DSBs corresponds with nuclear  $\gamma$ -H2AX foci formation. Treatment with zeocin induced the formation of  $\gamma$ -H2AX foci to the same extent in FANCI<sup>WT</sup>- and FANCI<sup>S990A</sup>- complemented FA-J cells as detected by immunofluorescence (Figures 2a and b). In contrast, RAD51 foci were not similarly detected (Figures 2a and c; Supplementary Figure 2). As compared with untreated cells, at 12 h post-zeocin treatment RAD51 foci were induced ~5-fold in FANCI<sup>WT</sup>-, as compared with ~2-fold in FANCI<sup>S990A</sup> or ~3.5-fold in vector complemented FA-J cells (Figure 2c). To rule out the possibility that zeocin differentially affected the FA-J cell lines and the number of cells in S-phase, in which RAD51 foci are most prominent, we measured cell-cycle distributions and found no significant differences (Supplementary Figure 3A).

Next, we examined the consequence of expressing FANCI mutants on DSB-induced HR. DSBs were induced by transient expression of I-Sce1 in an established U2OS cell line containing an integrated copy of the pDR-GP reporter. With this reporter, if HR occurs, GFP is expressed, which is quantifiable by flow cytometric analysis (Pierce *et al.*, 1999). Consistent with the possibility that HR was reduced as a result of more unbound FANCI, expression of FANCI<sup>S990A</sup> reduced the number of cells with restored GFP. Compared with that in U2OS cells with FANCI<sup>WT</sup> or vector, U2OS cells with FANCI<sup>S990A</sup> and FANCI<sup>K52R</sup> reduced HR, 4.5- and 3-fold, respectively (Figure 2d). FANCI species were

expressed similarly (Figure 2d). FANCI<sup>WT</sup> precipitated similar ratio of BRCA1 as endogenous FANCI, and as expected, the FANCI<sup>S990A</sup> did not precipitate BRCA1 (Supplementary Figure 4A). Despite reduced HR due to expression of FANCI<sup>S990A</sup> or FANCI<sup>K52R</sup>, only U2OS cells expressing FANCI<sup>K52R</sup> were sensitive to MMC (Supplementary Figure 4B). Together, these data suggested that expression of FANCI<sup>S990A</sup> in U2OS or FA-J cells reduced RAD51-based HR, but not crosslink resistance.

### MMC-induced pol $\eta$ foci are enhanced in U2OS cells expressing FANCI<sup>S990A</sup>

Given these findings, we hypothesized that resistance to agents that induce ICLs was mediated by a recombination-independent mechanism such as TLS. In particular, the TLS polymerase pol $\eta$  can facilitate bypass of lesions that escape excision repair such as unhooked ICLs and UV lesions (Kannouche and Lehmann, 2004; Nojima *et al.*, 2005). Thus, we sought to examine whether the reduced HR in U2OS cells expressing FANCI<sup>S990A</sup> corresponded with elevated eGFP-pol $\eta$  foci that have been shown to form in response to UV (Kannouche *et al.*, 2001). First, we sought to confirm this UV-dependent eGFP-pol $\eta$  foci formation and its dependence on Rad18 (Watanabe *et al.*, 2004). U2OS cells were cotransfected with eGFP-pol $\eta$  and -siRNA targeting luciferase (*luc*) or Rad18; UV irradiated and cells positive for eGFP-pol $\eta$  foci were counted. In response to UV, pol $\eta$  foci clearly formed in B35% of the cells (Figure 3a). Moreover, the number of cells with eGFP-pol $\eta$  foci was reduced ~4-fold when Rad18 was depleted. Next, the U2OS cells stably expressing vector, FANCI<sup>WT</sup>, or FANCI<sup>S990A</sup> (Figure 3b) were transfected with the eGFP-pol $\eta$  fusion protein, UV irradiated, and analyzed for eGFP-pol $\eta$  foci. Expression of FANCI<sup>S990A</sup> did not affect the number of pol $\eta$  foci in untreated cells, but in UV-irradiated cells expressing FANCI<sup>S990A</sup> or FANCI<sup>WT</sup>, ~30% more cells with eGFP-pol $\eta$  foci were detected as compared with the vector controls (Figure 3b). In contrast to UV, MMC-treated cells showed few cells with pol $\eta$  foci, suggesting that repair of ICLs does not readily activate pol $\eta$ . However, when cells expressed FANCI<sup>S990A</sup>, pol $\eta$  foci were readily observed (Figure 3b) consistent with the finding that FANCI<sup>S990A</sup> enhances TLS in response to MMC. To rule out the possibility that DNA damage differentially affects the number of cells in S-phase, in which pol $\eta$  foci are most prominent (Kannouche *et al.*, 2001), we measured cell-cycle distributions before and after UV or MMC in control or FANCI over-expressed cells and found no significant changes (Supplementary Figure 3b).

### Cells expressing FANCI<sup>S990A</sup> rely on pol $\eta$ for MMC resistance

Given that pol $\eta$  readily bypasses UV-induced thymidine–thymidine dimers as well as MMC-induced ICLs (Zheng *et al.*, 2003; Prakash *et al.*, 2005), we considered that the MMC and hyper UV resistance of FA-J cells expressing FANCI<sup>S990A</sup> (Figure 1c) could rely on pol $\eta$ . To address this possibility, the FA-J cell lines were transfected with siRNA targeting pol $\eta$  or *luc* control. Consistent with FANCI<sup>S990A</sup> promoting DNA cross-link resistance in a manner that requires pol $\eta$ , pol $\eta$ -depletion uniquely reversed the MMC and UV resistance in FANCI<sup>S990A</sup>-, as compared with FANCI<sup>WT</sup>-, or vector complemented FA-J cells (Figures 4a and b). Consistent with this effect being due to pol $\eta$ -depletion and not off-target effects, depletion of pol $\eta$  with either of two distinct siRNAs was substantial B90% (Figure 4b) and also uniquely reversed the MMC sensitivity of FA-J cells expressing FANCI<sup>S990A</sup> (Figure 4c). As Rad18 is required for pol $\eta$  foci formation (Figure 3a) (Watanabe *et al.*, 2004), we tested how Rad18 depletion affected the MMC resistance of the FA-J cell lines. Similar to pol $\eta$ -depletion, Rad18-depletion, uniquely sensitized FA-J cells complemented with FANCI<sup>S990A</sup>, as compared with FANCI<sup>WT</sup> or vector (Figure 4d). Likewise, depletion of Rad18 with either of two distinct siRNAs reversed the ICL resistance of FA-J cells expressing FANCI<sup>S990A</sup> (Supplementary Figure 5A). Furthermore, the effect did not appear restricted to FA-J cells as transient over-expression of FANCI<sup>S990A</sup>, as compared with FANCI<sup>WT</sup>, in U2OS cells also promoted UV and MMC resistance in a Rad18 and pol $\eta$ -

dependent manner (Supplementary Figure 5B and Figure 6). In contrast, Rad54- or Rev1-siRNAs did not affect the MMC resistance of the FA-J cell lines (Figure 4d). Introduction of siRNAs into the FA-J cell lines had minimal affect on cell viability (Supplementary Figure 7). Immunoblot analysis revealed that Rad18, Rad54, and Rev1 were depleted; however, Rad54 and Rev1 expression was lower in vector FA-J cells and only partially reduced by siRNAs (Figure 4e). Thus, the data most clearly show that FA-J cells expressing FANCS<sup>990A</sup>, in contrast to FANCS<sup>WT</sup>, rely on pol $\eta$ -dependent bypass for ICL resistance.

To confirm the pol $\eta$  dependence of our findings, we next tested whether over-expression of FANCS<sup>990A</sup> differentially affected the MMC or UV resistance of pol $\eta$ -null or pol $\eta$ -complemented XPV patient cells (Kannouche *et al.*, 2001). As expected, the pol $\eta$ -complemented XPV cells expressed pol $\eta$  and were more resistant to UV than the non-complemented XPV cells (Kannouche *et al.*, 2001) (Supplementary Figures 8A and B). Next, vector and pol $\eta$ -complemented lines were transfected with vector, FANCS<sup>WT</sup>, or FANCS<sup>990A</sup>, expression was analyzed, and viability in response to MMC or UV was tested in survival assays (Supplementary Figure 8B). Transfection of FANCS<sup>990A</sup>, as compared with FANCS<sup>WT</sup> or vector, enhanced UV and MMC resistance in the pol $\eta$ -complemented XPV cells, but not in the vector-complemented XPV cells (Supplementary Figure 8B). Altogether, the data strongly support that the BRCA1-interaction defective mutant, FANCS<sup>990A</sup> promotes resistance to MMC and UV in a pol $\eta$ -dependent manner.

### FANCS<sup>990A</sup> MLH1 requires binding to promote ICL resistance

Previously, we identified that FANCS<sup>WT</sup> binds directly to MLH1, and this interaction is required for MMC resistance (Peng *et al.*, 2007). MLH1 binds directly to the FANCS helicase domain through lysines 141 and 142. To address whether the MLH1 interaction with FANCS<sup>990A</sup> was also required for MMC resistance, we replaced lysines 141 and 142 of FANCS with alanines (A), which ablates MLH1 binding, but does not alter FANCS helicase function (Peng *et al.*, 2007). FA-J cells were complemented with this triple mutant FANCS<sup>990AK141/142A</sup>, FANCS<sup>990A</sup>, FANCS<sup>K141/142A</sup>, vector, or FANCS<sup>WT</sup>. Expression and ablation of MLH1 and/or BRCA1 binding was confirmed by western blot (Figures 5a and b). In contrast to FANCS<sup>990A</sup>, FANCS<sup>990AK141/142A</sup> complemented FA-J cells were sensitive to MMC (Figure 5c). Furthermore, FA-J cells expressing FANCS<sup>990AK141/142A</sup> underwent a prolonged G2/M accumulation in contrast to FA-J cells expressing FANCS<sup>WT</sup> or FANCS<sup>990A</sup> (Figure 5d), suggesting that FANCS<sup>990A</sup> promotes pol $\eta$ -dependent TLS in a MLH1-dependent manner (Figure 5e).

## Discussion

In this study, we explore the possibility that FANCS binding to BRCA1 is important for ICL repair in mammalian cells. This possibility was proposed based on their direct binding and common functions in breast cancer suppression, HR, and ICL repair. We provide data that support this hypothesis by demonstrating that uncoupling FANCS from BRCA1 alters the DNA damage response. Specifically, (1) cells expressing unbound FANCS (FANCS<sup>990A</sup>), unlike FANCS that can bind BRCA1, are sensitive to DSBs, (2) have reduced RAD51-based HR, (3) survive cross-link DNA damage with a reduced G2/M accumulation, and (4) dependence on the TLS polymerase pol $\eta$ . Notably, depletion of pol $\eta$  sensitized FA-J cells complemented with FANCS<sup>990A</sup>, but not FANCS<sup>WT</sup>, to MMC and reverted the hyper-UV resistance of FA-J or U2OS cells expressing FANCS<sup>990A</sup>. Moreover, pol $\eta$ -null XPV cells transfected with FANCS<sup>990A</sup> were not hyper-UV or MMC resistant unless pol $\eta$  was re-introduced. Together, these data suggest that FANCS has anti-recombination and TLS functions that are normally regulated by BRCA1 binding. Moreover, we find that the BRCA1-bound or -unbound FANCS requires its MLH1 interaction to promote ICL resistance.

FANCI likely has a complex role in HR: contributes to HR when bound to BRCA1 and inhibits HR when unbound to BRCA1. If FANCI functioned only as an anti-recombinase depletion of FANCI would be expected to enhance HR. Instead, FANCI depletion reduces HR, similar to BRCA1 depletion (Litman *et al.*, 2005). Further indicating a positive role for FANCI in HR, in response to DSBs induced by zeocin complementation of FANCI<sup>WT</sup> in FA-J cells enhanced the appearance of DNA damage induced RAD51 foci (Figures 2a and c). Whether the role of FANCI in HR is direct is not clear. Conceivably, FANCI could have an indirect role in HR as a 'place-holder' to prevent other proteins from disrupting HR, such as the anti-recombination helicases BLM or RTEL (Bugreev *et al.*, 2007; Barber *et al.*, 2008). Also consistent with a positive role in HR following ICLs, FANCI deficiency enhanced recombination-independent repair (Shen *et al.*, 2009). Intriguingly, pol $\eta$  also functions in HR to extend D-loop recombination intermediates (Kawamoto *et al.*, 2005; McIlwraith *et al.*, 2005). These dual functions in HR and TLS are likely regulated by the DNA damage response. Similar to pol $\eta$ , UV damage could link FANCI to TLS. Here, the anti-recombination activity of FANCI could be unleashed through loss of BRCA1 binding and gained helicase activity. This unleashed FANCI activity could explain why BRCA1-deficient cells are defective in RAD51 foci formation and HR (Moynahan *et al.*, 1999; Bhattacharyya *et al.*, 2000).

Conceivably, the anti-recombination activity of FANCI<sup>S990A</sup> could indirectly enhance TLS. For example, the yeast helicase Srs2 promotes TLS by binding PCNA and antagonizing HR and recombination bypass pathways. The ability of Srs2 to disrupt recombination and displace Rad51 requires its enzyme activity (Barbour and Xiao, 2003; Papouli *et al.*, 2005). Similar to Srs2, FANCI colocalizes at sites of replication arrest with PCNA and has been shown to translocate DNA, unwind D-loops and displace RAD51 (Gupta *et al.*, 2005; Dupaigne *et al.*, 2008; Sommers *et al.*, 2009). Moreover, UvrD, which is structurally and functionally related to Srs2, binds the MLH1 homologue, MutL (Mechanic *et al.*, 2000). MutL helps to load and activate the UvrD helicase. Perhaps, MLH1 helps to load or activate the FANCI helicase to promote TLS or HR.

FANCI could enhance TLS not only by limiting recombination, but also by potentiating TLS. For example, FANCI could limit negative regulators of TLS, such as mismatch repair (MMR), which detect mismatches generated by mutagenic TLS processing. Mismatch-induced MMR checkpoint signaling could generate the MMC sensitivity in FA-J cells lacking the FANCI/MLH1 interaction. Alternatively, FANCI<sup>S990A</sup> could directly enhance TLS by altering the structure of the stalled DNA replication fork. Failure to separate DNA strands or create a DNA loop at the ICL is thought to block NER-dependent incisions required for recombination-independent repair (Zheng *et al.*, 2003). The unleashed FANCI<sup>S990A</sup> could enhance DNA strand separation in a manner distinct from the BRCA1-bound FANCI to facilitate NER-dependent processing events and recombination-independent repair. Either scenario could explain how FANCI<sup>S990A</sup> promotes TLS without directly binding pol $\eta$  (data not shown).

Together, these findings imply that reduction or loss of BRCA1 binding to FANCI could enable cells to survive toxic chemotherapies and provide a possible route to chemoresistance. If true, targeting FANCI or pol $\eta$  bypass could reverse resistance to agents that induce ICLs in such cancers. Likewise, a possible route to cancer in BRCA1-mutation carriers could result from excess unbound FANCI and mutagenic bypass. Perhaps, this is why FANCI amplification is also linked to malignancy (Sinclair *et al.*, 2003; Eelen *et al.*, 2008). In fact, loss of BRCA1 binding to FANCI could evolve from mutations in either gene, or from loss of DNA-damage signaling components that regulate the association of these two proteins. Future studies are needed to clarify the signaling pathways that participate in regulating the switch between BRCA1-bound and-unbound FANCI.

## Materials and methods

### Cell culture

MCF7, U2OS, HeLa, XP30RO (XP-V)-PCDNA vector, and PCNDA-pol $\eta$  complemented (generous gift of Alan Lehmann) (Kannouche *et al.*, 2001) cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. Stable cells were selected with 20 mg/ml G418. FA-J (EUFA30-F) fibroblasts were cultured in DMEM supplemented with 15% fetal bovine serum and antibiotics. FA-J cells were infected with pOZ vectors and selected as before (Peng *et al.*, 2007).

### Cell growth and G2/M accumulation assays

FA-J cells infected with pOZ (Peng *et al.*, 2007) vectors and U2OS cells were transfected with siRNA using Fugene or Lipofectamine. siRNA reagents for pol $\eta$  (siRNA Pol $\eta$  pool, antisense sequence of #1, AACCCUCAAUGUAA GUGCUU, or antisense sequence of #2, UAGUCCUGGG CUAUUGCUU), Rev1 (siRNA Rev1 pool), Rad18 (siRNA Rad18 pool, antisense sequence of #1, UACCAGUUCAUC UAAUAUGUU, antisense sequence of #2, AAUUAUCC AUUAACCUGCUU, antisense sequence of #3, UUACUG AGGUCAUUAUUCUU, or antisense sequence of #4, UGACUCUAAAGCAAACUGCUU), Rad54 (siRNA Rad54 pool), and luciferase (Luc) were obtained from Dharmacon (Lafayette, CO, USA). The FANCI siRNA reagent was described earlier (Litman *et al.*, 2005). U2OS cells were infected with shRNA against pGIPZ non-silencing control, Rad18 #1 (mature antisense sequence, AAATA TATCCATGTGAGCT), or Rad18 #2 (mature antisense sequence, TTGGTCTTTGCAGCAGGGC). shRNAs were obtained from the UMMS shRNA core facility. Infected cells or XPV complemented with vector or pol $\eta$  were subsequently transfected with V, FANCI<sup>WT</sup>, or FANCI<sup>S990A</sup>. After transfection (24–48 h), 1500–3000 U2OS cells/well or 12 500 complemented FA-J cells/well were seeded into six-well plates, respectively. Complementated FA-J cells were seeded into six-well plates at 8000 cells/well. Seeded cells were incubated overnight and left untreated or treated with MMC (Sigma, St Louis, MO, USA) for 1 h, UV (Stratalinker, Spectronics Corporation, Westbury, NY, USA), cisplatin (Sigma) for 4 h, zeocin (Invitrogen, Carlsbad, CA, USA) for 1 h, 6-TG (Sigma) for 24 h, or MMS (Sigma) for 1 h. Cells were counted after 5–8 days using a hemocytometer. Percent growth was calculated as (treated cells/untreated cells)  $\times$  100. G2/M accumulation was assayed as described (Litman *et al.*, 2005), but at 0.25  $\mu$ g/ml melphalan.

### Immunoprecipitation, western blot and antibodies

Cells were harvested and prepared for immunoprecipitation and western blot as described (Litman *et al.*, 2005). Immunoprecipitation Abs included FANCI (E67) or Myc (9e10). Antibodies for western blot analysis included BRCA1 (ms110) and FANCI monoclonal (2G7 and 2C10) (Cantor *et al.*, 2001) or polyclonal E67 (Cantor *et al.*, 2004). In addition,  $\beta$ -actin (Sigma), Rad18 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Rev1 (H-300, Santa Cruz), pol $\eta$  (Abcam, Cambridge, MA, USA), and Rad54 (Abcam) Abs were used.

### DNA constructs

The FANCI<sup>WT</sup>, FANCI<sup>K52R</sup>, FANCI<sup>K141/142A</sup>, and FANCI<sup>S990A</sup> pCDNA-3myc-6xhis and pOZ vectors have been described earlier (Cantor *et al.*, 2001; Peng *et al.*, 2007). The eGFP-pol $\eta$  construct was described earlier (Kannouche *et al.*, 2001). The FANCI<sup>K141/142AS990A</sup> pOZ vector was generated with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using our published primers (Cantor *et al.*, 2001; Peng *et al.*, 2007).

## Immunofluorescence

FA-J cells were either left untreated or treated with 12.5 µg/ml zeocin and incubated 12 h and processed for immunofluorescence as described (Cantor *et al.*, 2001). Antibodies included RAD51 (Santa Cruz 1:200) and γ-H2AX (Upstate 1:500, Upstate, Temecula, CA, USA). Visualization of eGFP-polη foci was as described (Kannouche *et al.*, 2001). In brief, U2OS cells were transfected with eGFP-polη, incubated for overnight, seeded on cover slips, incubated overnight, and examined 4 h post-UV or for 24 and 48 h post-MMC. Foci counting experiments were conducted blind to the counter and in triplicate as the number of cells with 10 or more foci.

## Homologous recombination

U2OS pDR-GFP cells were obtained from Maria Jasin (Pierce *et al.*, 1999) and  $1.8 \times 10^5$  cells were seeded per well in six-well plates and incubated overnight. The cells were transfected with 0.5 µg of pCDNA3, FANCI<sup>WT</sup>, FANCI<sup>S990A</sup>, or FANCI<sup>K52R</sup> and 2.0 µg of pBABE I-SceI using Fugene. Transfected cells were incubated for 72 h, collected by trypsinization, and analyzed by FACS. The percentage of green positive cells was calculated using Flow Jo software.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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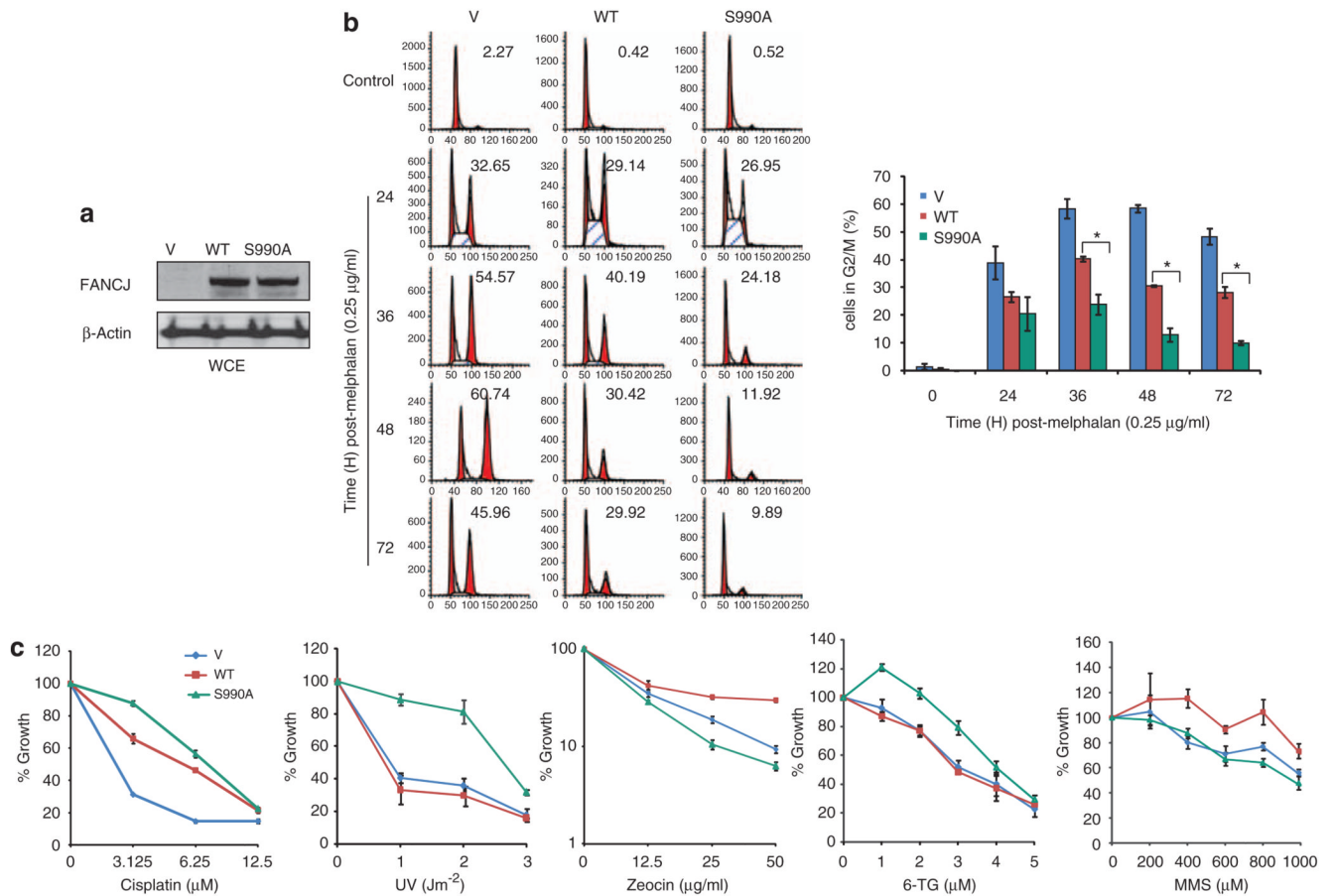
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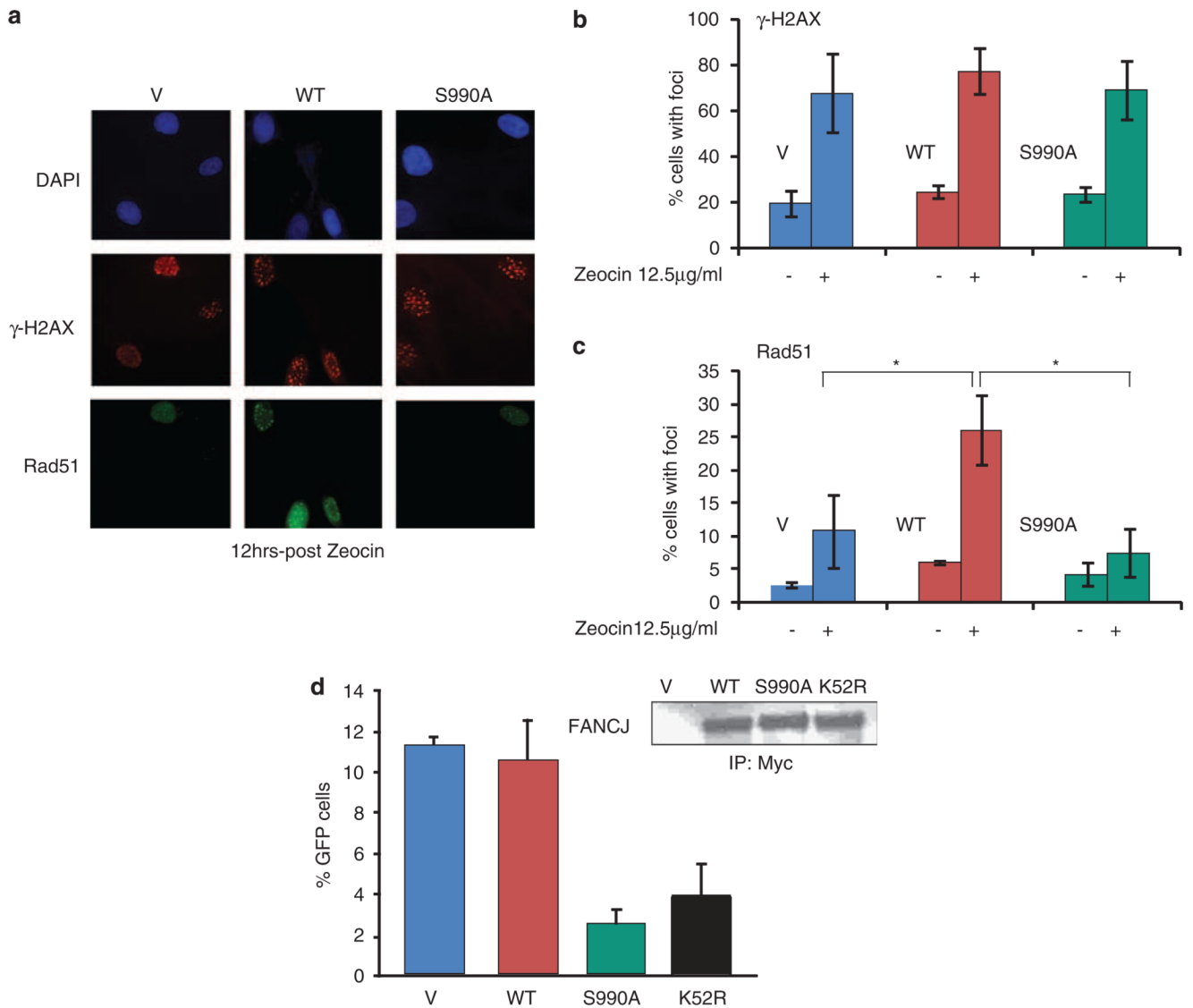


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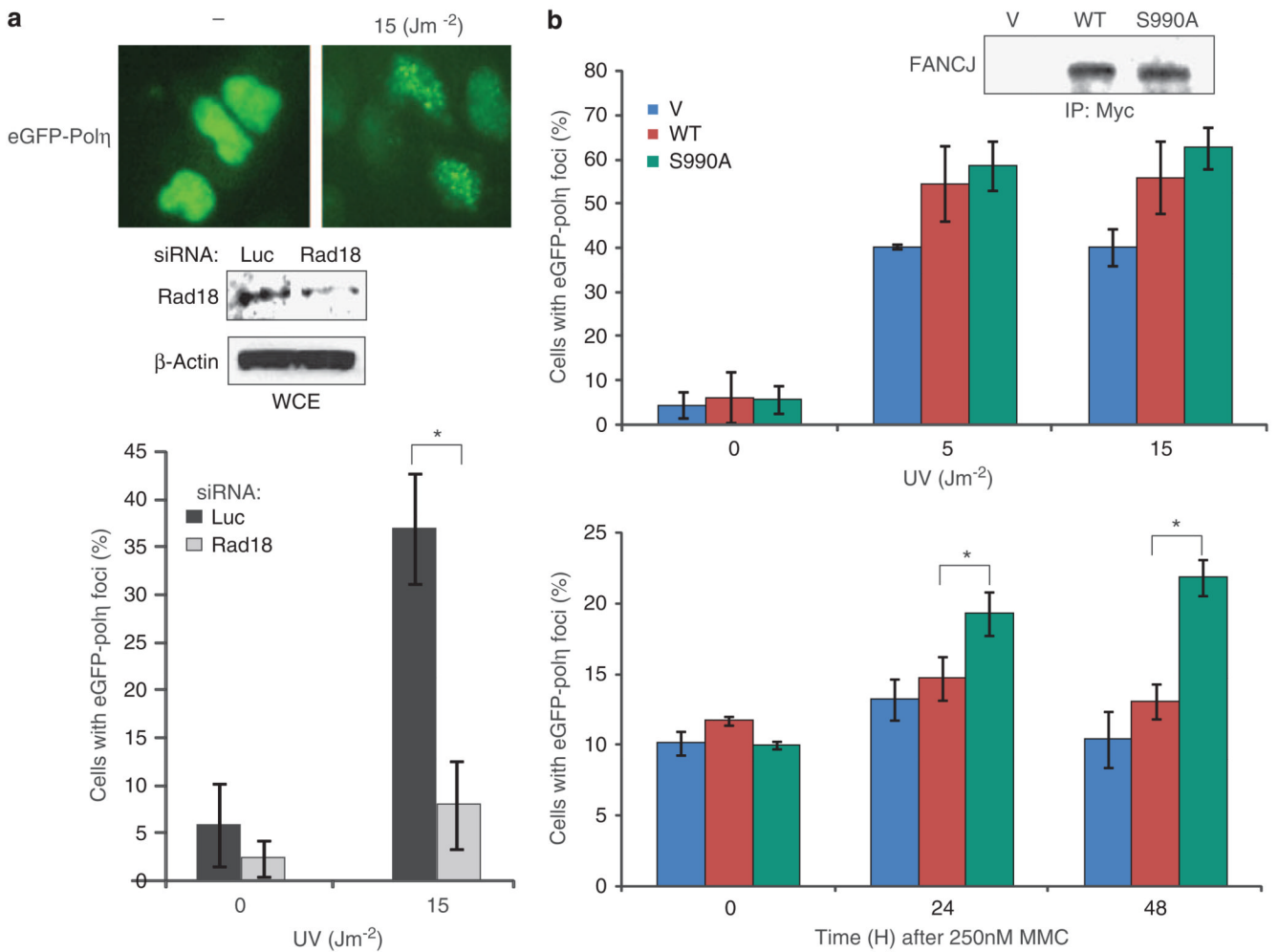
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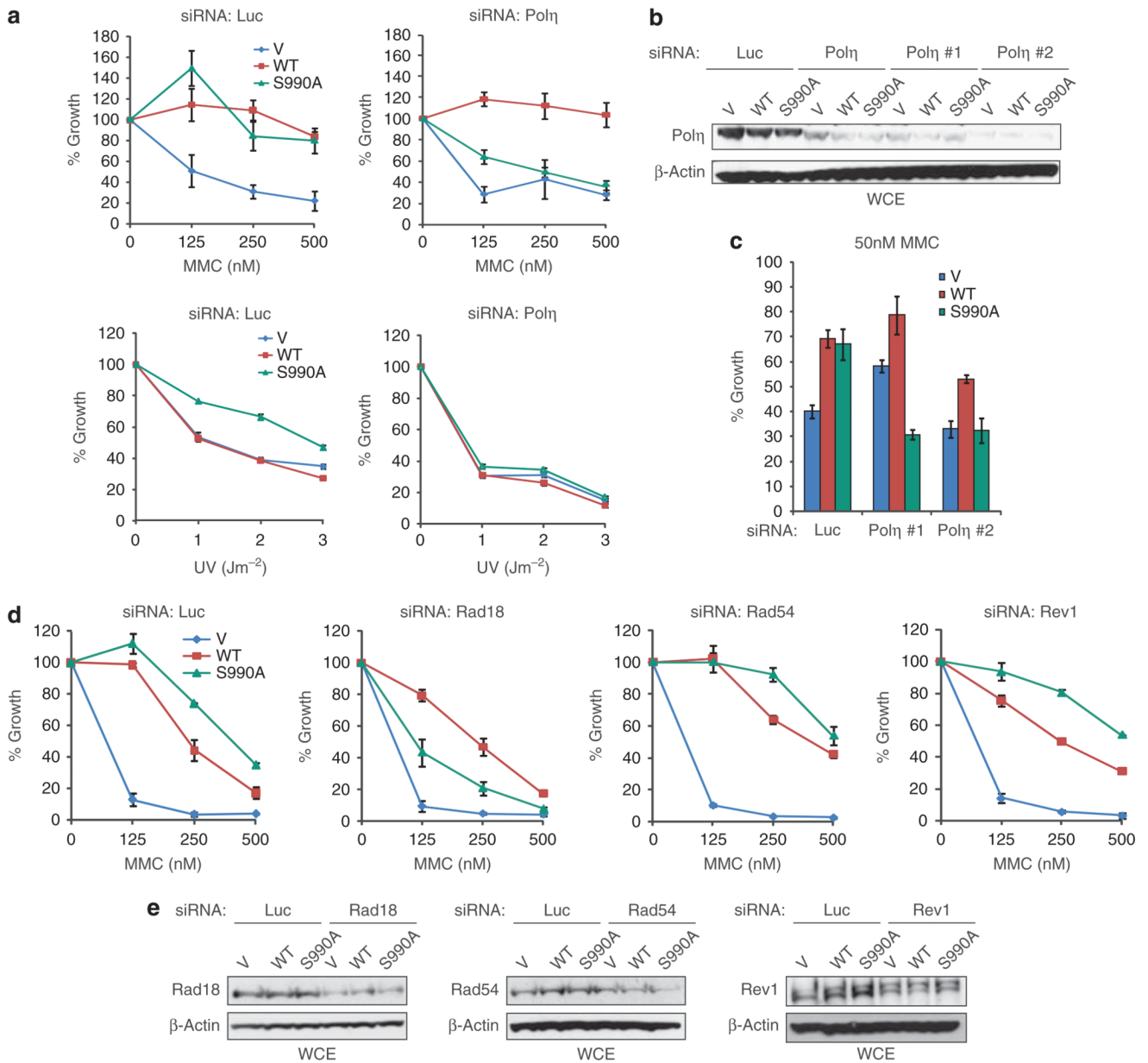


**Figure 1.** FANCD1<sup>S990A</sup>, as compared with FANCD1<sup>WT</sup>, promotes a distinct DNA damage response in FA-J cells. **(a)** FANCD1-null FA-J cells were complemented with vector, FANCD1<sup>WT</sup>, or FANCD1<sup>S990A</sup> and lysates were analyzed by immunoblot with the indicated antibodies (Abs). **(b)** The FA-J cell lines were treated constitutively with 0.25 µg/ml melphalan, collected at the indicated times, and analyzed by FACS to determine the percentage of cells in G2/M. A representative experiment is shown. The bar graph represents mean ± s.d. from three independent experiments. The asterisk indicates a significant difference ( $P < 0.05$ , unpaired  $t$ -test). **(c)** The FA-J cells expressing vector, FANCD1<sup>WT</sup>, or FANCD1<sup>S990A</sup> were plated at low density, treated with the indicated doses of cisplatin, UV, zeocin, 6-thioguanine, or methyl methanesulfonate and allowed to grow for 5–8 days. The cells were then collected and counted to analyze percent growth. Data represent mean percent ± s.d. of growth from three independent experiments.

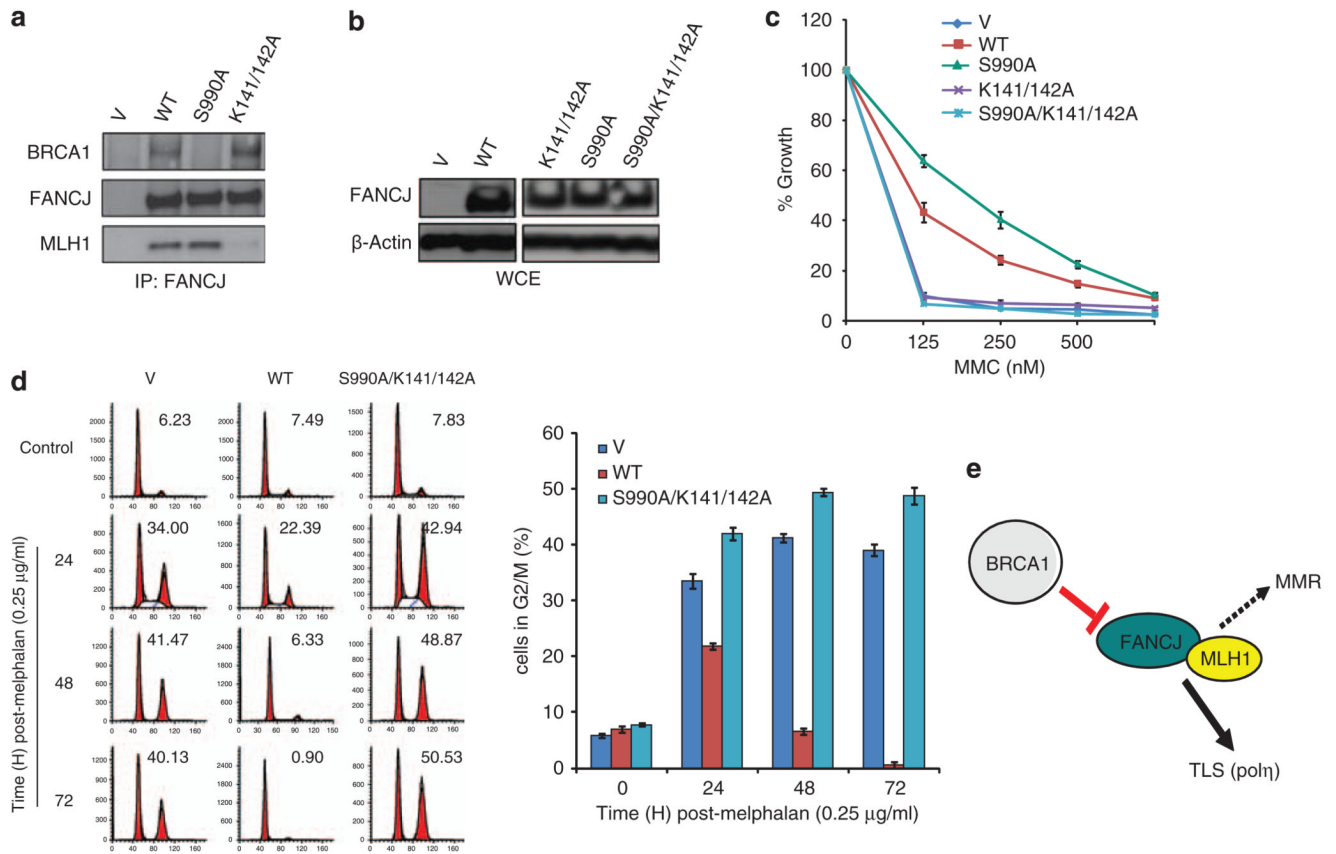


**Figure 2.** FANCD1<sup>S990A</sup> reduces DSB-induced RAD51 foci and HR. (a) The FA-J cell lines were treated with 12.5  $\mu$ g/ml of zeocin and immunofluorescence was performed with  $\gamma$ -H2AX and RAD51 Abs. A representative image is shown to depict how staining was observed. (b) The  $\gamma$ -H2AX and (c) RAD51 foci were quantitated based on a cell being positive (> 10) foci per 300 DAPI positive cells from three independent experiments. Asterisk indicates significant difference ( $P < 0.05$ , unpaired  $t$ -test). (d) DR-U2OS cells were cotransfected with the I-Sce-1 endonuclease and vector, FANCD1<sup>WT</sup>, FANCD1<sup>S990A</sup>, or FANCD1<sup>K52R</sup>, collected and either lysed and immunoprecipitated followed by immunoblot with the indicated Abs or analyzed by FACS. The bar graph shows the percentage of GFP-positive cells.





**Figure 4.** FANCS990A promotes ICL and UV resistance in a polη-dependent manner. **(a)** FA-J cells stably expressing vector, FANCS990A, or FANCS990A (Figure 1a) were transfected with siRNA to Luc or polη, incubated for 48 h, treated with MMC or UV, and percent growth was assessed as in Figure 1. **(b)** FA-J cell lines transfected with siRNA to luc, polη, polη #1, or polη #2 were collected for immunoblot with the indicated Abs. **(c)** The FA-J cell lines with indicated siRNAs were incubated for 48 h, treated with MMC at the IC50 dose, and percent growth was assessed as in Figure 1. Graph shows the percent growth mean±s.d. **(d)** FA-J cells stably expressing vector, FANCS990A, or FANCS990A were transfected with siRNA to luc, Rad18, Rad54, or Rev1 and incubated for 48 h, treated with MMC, and percent growth was assessed as in Figure 1. **(e)** Cells used in **(d)** were collected and lysed for immunoblot with the indicated Abs.



**Figure 5.** FANCS<sup>S990A</sup> requires MLH1 binding to promote pol $\eta$ -dependent bypass. **(a)** FA-J cells stably expressing vector, FANCS<sup>WT</sup>, FANCS<sup>S990A</sup>, or FANCS<sup>K141/142A</sup> were collected and immunoprecipitated with anti-FANCS Abs and blotted with the indicated Abs. **(b)** FA-J cells stably expressing vector, FANCS<sup>WT</sup>, FANCS<sup>S990A</sup>, FANCS<sup>K141/142A</sup>, or FANCS<sup>K141/142A/S990A</sup> were either collected for immunoblot with the indicated Abs or **(c)** treated with the indicated doses of MMC and allowed to grow for 5–8 days. The cells were then collected and counted to analyze percent growth. Data represent mean percent  $\pm$  s.d. of growth from three independent experiments. **(d)** The FA-J cell lines were treated constitutively with 0.25  $\mu$ g/ml melphalan, collected at the indicated times, and analyzed by FACS to determine the percentage of cells in G2/M. A representative experiment is shown. The bar graph represents mean  $\pm$  s.d. from three independent experiments. **(e)** Model summarizes observations of this study. FANCS when uncoupled from BRCA1 promotes pol $\eta$ -dependent TLS in a manner that requires MLH1 binding. Dotted line to MMR is added as a discussion point. To promote TLS, FANCS could limit negative regulators of TLS, such as MMR.