Constitutive role of the Fanconi anemia D2 gene in the replication-stress response

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In response to DNA cross-linking damage, the Fanconi anemia (FA) core complex activates the FA pathway by monoubiquitinating Fanconi anemia complementation group D2 (FANCD2) for the initiation of the nucleolytic processing of the DNA cross-links and stabilization of stalled replication forks. Given that all the classic FA proteins coordinately monoubiquitinate FANCD2, it is unclear why losses of individual classic FA genes yield varying cellular sensitivities to cross-linking damage. To address this question, we generated cellular knock-out models of FA core complex components and FANCD2 and found that FANCD2-null mutants display higher levels of spontaneous chromosomal damage and hypersensitivity to replication-blocking lesions than Fanconi anemia complementation group L (FANCL)-null mutants, suggesting that FANCD2 provides a basal level of DNA protection countering endogenous lesions in the absence of monoubiquitination. FANCD2’s ubiquitination-independent function is likely involved in optimized recruitment of nucleolytic activities for the processing and protection of stressed replication forks. Our results reveal that FANCD2 has a ubiquitination-independent role in countering endogenous levels of replication stress, a function that is critical for the maintenance of genomic stability.

Fanconi anemia (FA) is an autosomal or X-linked disorder with a spectrum of clinical manifestations, including pancytopenia, cognitive impairment, developmental anomalies, and high susceptibility to a range of cancers (1–3). To date, biallelic mutations in 22 distinct genes have been identified as causative of FA. Cells derived from FA patients are hypersensitive to replication-blocking DNA-damaging agents such as DNA interstrand cross-linkers and exhibit much greater numbers of chromosomal aberrations than normal (4, 5), suggesting that the FA genes constitute a DNA damage response pathway that protects genome integrity from damage-induced DNA replication stress.

Functions of the 22 FA proteins fall into four categories that assemble into the FA pathway. A heterodimeric complex containing FANCD2 and FANCI is monoubiquitinated in response to DNA damage, which marks the activation of the canonical FA pathway. The E3 ligase activity of the monoubiquitination reaction comes from the RING domain protein FANCL (6–8), which is part of the multisubunit FA core complex containing FANCA–C, -E–G, -L, -M, FAPO20, and FAAP100 (9–14). Monoubiquitinated FANCD2 and FANCI (15, 16) have the presumed function of recruiting and orchestrating the nucleolytic processing of DNA interstrand cross-links (ICLs). A third group of FA gene products, including FANCP/XPF and FANCQ/SLX4, are structure-specific endonucleases or nuclease scaffolds that provide DNA cleavage activities to process the lesions (17–23). The resulting DNA double-strand breaks require the homologous recombination group of FA proteins, including FANC1/BRC1, FANCO/RAD51C, FANCJ/BARD1, and FANC/RAD51 (24–31).

In addition to the repair function of DNA cross-linking lesions, FA pathway components are implicated in stabilizing stalled replication forks. FANCD2 (32, 33) and FA-associated nuclease 1 (FAN1) have been shown to prevent stalled replication fork collapse (34, 35). The recombination FA proteins such as Rad51C, BRCA1, and BRCA2 are also important for replication fork protection, suggesting that the FA mechanism has functions both in maintaining the integrity of stalled forks and in processing ICLs. The current models suggest that fork protection is likely rendered by preventing erroneous or excessive processing by nucleases acting upon replication fork stress (32, 33, 36).

FANCD2 monoubiquitination plays a crucial role not only in cellular resistance to cross-linking damage (37) but also in replication fork protection (32, 35, 38) when cells encounter exogenous damages. However, recent evidence has hinted to FANC2 functions that are independent of monoubiquitination (39–42). Whether monoubiquitination is essential for FANCD2 function...
and the mechanistic nature of such functions remain unclear because of the absence of defined genetic model systems.

In this report, we investigated functional distinctions between targeted deletions of FANCD2 and components of the FA core complex and tested whether FANCD2 provides important protection against cross-linking damage independent of FA core complex and monoubiquitination. To this end, we created FA isogenic knock-out and knock-in mutants and found that FANCD2 has a constitutive and indispensable role in providing basal protection against endogenous fork stress. Complete loss of FANCD2 yielded greater chromosomal aberrations and reduced viability during unperturbed cell proliferation compared with loss of other FA genes, including FANCL. Ubiquitination-deficient FANCD2 was able to rescue the phenotypes of FANCD2-null cells. Epistatic analysis showed that FANCD2 is likely the exclusive target for the FA core complex E3 ligase activity in the DNA damage response. These results suggest a bihapic mode of FANCD2 function in which its unmodified form responds to low levels of endogenous replication stress and its monoubiquitinated form responds to elevated levels of exogenous damage.

**Results**

**Deletion of FANCD2 results in more severe DNA damage sensitivity than deletion of FA core complex genes**

To determine the functional contributions of key FA proteins involved directly in the DNA damage–induced FANCD2 monoubiquitination, we generated isogenic null mutants of FANCA, FANCL, and FANCD2 in HeLa cells (supplemental Fig. S1, A–C). FANCA is an integral subunit of the FA core complex. Loss of FANCA severely decreases, but does not eliminate, the E3 ligase function, whereas FANCL loss completely abolishes FA core complex E3 activity (7). We compared the DNA damage sensitivity of FANCA−/−, FANCL−/−, and FANCD2−/− mutants against MMC and observed differential sensitivities among the three in clonogenic survival assays. The FANCD2 deletion mutant displayed the most severe, but rescurable, phenotype (Fig. 1, A and B, and supplemental Fig. S2A). Consistently, the FANCD2−/− mutant also exhibited the greatest sensitivity to psoralen and other types of cross-linking damage (supplemental Fig. S2, B–D).

Of the three mutants, loss of FANCA generated the lowest sensitivity to cross-linking damage, which can be explained by the residual FANCD2 monoubiquitination by the FA core complex as shown previously by us and others (43, 44). The more severe phenotype of the FANCD2−/− mutant, compared with the isogenic FANCL−/− mutant, suggests that FANCD2 plays a role in countering DNA cross-linking damage in the absence of the E3 ligase activity (Fig. 1C). Given that FANCD2 protein is present at normal levels in the FANCL−/− mutant and was completely ablated in the FANCD2−/− mutant, phenotypical distinctions between these two isogenic mutants likely reflect the functional importance of FANCD2 in the absence of FANCL-mediated monoubiquitination.

To establish whether FANCD2 is the main target of the E3 ligase activity of FANCL (45), we generated two FANCL−/−/ FANCD2−/− double knock-out mutants. As shown, hypersensitivity of these two independently derived FANCL−/− FANCD2−/− mutants to MMC did not exceed that of the FANCD2−/− mutant (Fig. 1D), indicating that FANCD2 is the prevalent downstream target of the FA core complex E3 ligase in response to cross-linking DNA damage.

**Complete loss of FANCD2 increases spontaneous endogenous damage and chromosomal aberrations**

FA factors respond coordinately to DNA cross-linking damage and replication stress. Given the epistatic relationship between FANCL and FANCD2, it is unclear why loss of the latter yielded a more severe phenotype than loss of FANCL upon DNA damage. To further assess the impact of loss of different FA factors, we performed immunostaining of γH2AX (Fig. 2, A and C) in FANCA−/−, FANCL−/−, and FANCD2−/− mutant cells exposed to a low/nonlethal dose (100 ng/ml) of MMC to mimic heightened endogenous levels of replication stress. We found that loss of FANCD2 exhibited the highest level of γH2AX foci. Similarly, the number of 53BP1 foci in FANCD2−/− cells was significantly higher than in the FANCL−/− mutant (Fig. 2, B and C), indicating a substantially weakened damage response from loss of FANCD2. Consistently, deletion of FANCD2 yielded higher amplitudes of chromosomal aberrations than deletion of FANCL. (Fig. 2, D and E).

When FANCA−/−, FANCL−/−, and FANCD2−/− cells were exposed to MMC or mock treatment and allowed to recover (Fig. 3, A and B), we found that mock-treated normal proliferating FANCD2−/− cells exhibited a constantly higher basal level of γH2AX than the FANCA−/− and FANCL−/− mutants, further suggesting that cells with complete loss of FANCD2 had elevated endogenous DNA damage during unperturbed cell proliferation. When cells are subjected to low-dose MMC (50 ng/ml) and allowed to recover, γH2AX levels in the FANCD2−/− mutant remain markedly higher than in the FANCL−/− mutant at both 24 and 48 h after exposure. In contrast, the near wild-type basal level of γH2AX in the FANCL−/− mutant indicates that the presence of unubiquitinated FANCD2 is sufficient to suppress such endogenous damage. These observations suggest a FANCD2 function independent of FANCL-mediated monoubiquitination in response to both endogenous and exogenous damages. It is also noticeable that, at the 2-h time point, γH2AX signal is stronger in wild-type cells than in FANCA−/− or FANCL−/− cells. Deficits in the FA pathway are expected to reduce nucleolytic processing of the ICLs. The resulting lack of strand breaks and/or single-stranded DNA necessary for checkpoint activation likely leads to a latent or weakened onset of γH2AX signal.

The higher levels of DNA damage surrogate markers and chromosomal aberrations in the FANCD2−/− mutant suggest that cells completely devoid of FANCD2 are coping with a more frequent occurrence of intrinsic damages. However, FANCD2−/− cells seem to proliferate at a similar rate compared with parental HeLa cells and exhibit a cell cycle profile identical to that of the FANCL−/− mutant (supplemental Fig. S1, G and H). We postulated that FANCD2 deletion in the HeLa cell background is tolerated through compromised apoptosis due to the presence of the human papillomavirus E6 protein, which leads to p53 repression (46). To test this premise, we performed siRNA-mediated knockdown of E6 in HeLa WT,
FANCD2 possesses ubiquitination-independent function

The more severe phenotypes observed in the FANCD2−/− mutant suggest that FANCD2 exhibits monoubiquitination-independent function in countering endogenous DNA damages. To further test this premise, we treated FANCL−/− and FANCD2−/− mutant cells with hydroxyurea to arrest replication forks and determined their clonogenic survival. As shown (Fig. 4A), survival of the FANCD2−/− mutant was markedly lower than that of the FANCL−/− mutant and was accompanied by a much higher occurrence of chromosomal aberrations (Fig. 4B). Importantly, expression of a FANCD2 K561R mutant, which abolishes the monoubiquitination of FANCD2, restored clonogenic survival to a level close to that of the FANCL−/− mutants. This result provides direct evidence that FANCD2 possesses a monoubiquitination-independent function in response to replication stress.

To explore the underlying cause of heightened replication stress sensitivity from FANCD2 loss, we performed replication fork stability analysis by DNA spreading of IdU-labeled replication tracks in cells exposed to hydroxyurea (Fig. 4C). Compared with wild-type HeLa cells (9.45 μM), average replication track lengths were significantly shortened in both FANCL−/− and FANCD2−/− cells (6.96 and 5.15 μM, respectively), indicating reductions of fork stability (Fig. 4D). However, that the greatest reduction in replication...
Figure 2. Complete loss of FANCD2<sup>−/−</sup> leads to increased spontaneous and endogenous DNA damage. A, γH2AX immunostaining in HeLa WT, FANCA<sup>−/−</sup>, FANCL<sup>−/−</sup>, and FANCD2<sup>−/−</sup> cells after 2 h of MMC (100 ng/ml) exposure. B, 53BP1 immunostaining in HeLa WT, FANCA<sup>−/−</sup>, FANCL<sup>−/−</sup>, and FANCD2<sup>−/−</sup> cells after 2 h of MMC exposure (100 ng/ml). Scale bar, 5 μm. C, quantification of γH2AX and 53BP1 foci in A and B, respectively. Error bars represent S.D. D, chromosomal aberrations in HeLa WT, FANCA<sup>−/−</sup>, FANCL<sup>−/−</sup>, and FANCD2<sup>−/−</sup> cells treated with 50 ng/ml MMC for 24 h. E, quantification of chromosomal aberrations in HeLa WT, FANCA<sup>−/−</sup>, FANCL<sup>−/−</sup>, and FANCD2<sup>−/−</sup> cells as shown in D. Red lines mark the mean value of each mutant. p values were generated by Student’s t test.
track length occurred in the FANCD2−/− mutant suggests that FANCD2 can act in a ubiquitination-independent manner to maintain fork stability.

To further validate the monoubiquitination-independent FANCD2 function, we constructed FANCD2−/− and FANCL−/− mutants in HEK293A cells as well as isogenic knock-in mutants harboring homozygous FANCD2 K561R (FANCD2-KR) alleles (Fig. 5A and supplemental Fig. S1, E and F). Examining γH2AX and 53BP1 levels in unperturbed mutant cells showed that the FANCD2−/− mutant exhibits the most severe endogenous damage compared with the FANCL−/− and FANCD2-KR mutants (Fig. 5B and supplemental Fig. S3, A and B). Consistent with this result, upon exposure to MMC, the FANCD2−/− mutant displayed significantly larger numbers of γH2AX foci and chromosomal aberrations than both the FANCL−/− and FANCD2-KR mutants (Fig. 5, C, D, and E). These more severe phenotypes observed in the FANCD2−/− mutant are also reflected by additional defects in replication fork erosion (supplemental Fig. S3, C and D). Collectively, these results indicate a mode of FANCD2 function that can be delivered in the absence of monoubiquitination, most likely in the protection against endogenous genotoxic stress.
FANCD2 possesses ubiquitination-independent function

The exacerbated defects in the damage response and particularly in replication fork protection of FANCD2-null cells suggest that, in the absence of FANCD2, stalled replication forks may be subject to excessive or dysregulated processing by nuclease activities. To verify this premise, we performed siRNA-mediated depletion of nucleases EXO1, DNA2, and...

**Figure 4. FANCD2 deletion leads to increased defects in replication fork protection.**

A, clonogenic survival of HeLa WT and knock-out mutants with the indicated genotypes exposed to hydroxyurea. FANCL+/-, FancL, wild-type cDNA-complemented FANCL+/-, cells; FANCD2+ KR, FANCD2+/- cells complemented with FANCD2 K561R mutant cDNA. Error bars represent S.D. B, chromosomal aberrations in HeLa WT, FANCA+/-, FANCL+/-, and FANCD2+/- cells treated with 1 mM hydroxyurea for 8 h and harvested 18 h after. Red lines mark the mean value for each mutant. p values were generated by Student’s t test. C, preformed IdU track lengths measuring replication fork stability by DNA spreading in HeLa WT, FANCL+/-, FANCL+/-, and FANCD2+/- cells with and without exposure to hydroxyurea (HU). D, median IdU tract lengths derived from C are shown for FANCL+/- and FANCD2+/- mutant cells with and without hydroxyurea treatment. No fewer than 150 replication forks were analyzed for each sample.
Figure 5. FANCD2 possesses replication fork protection function in the absence of monoubiquitination.

A, immunoblotting of FANCD2 in HEK293A cell background with the indicated genotypes. FANCD2 K11002+/+ and FANCD2 K11002−/−, two independent FANCD2 knock-out clones; FANCD2 L, monoubiquitinated form of FANCD2; FANCD2 S, unmodified FANCD2. Cells were treated with 200 ng/ml MMC for 12 h before harvesting for extract preparation.

B, immunoblotting of γH2AX in cells with the indicated genotypes. Protein extracts were prepared from unperturbed proliferating cells.

C, γH2AX immunostaining in 293A WT, FANCL−/−, FANCD2−/−, and FANCD2-KR cells after MMC exposure (100 ng/ml; 2 h). KR1 and KR2 are two independent homozygous FANCD2 K561R knock-in clones. Scale bar, 5 μm.

D, quantification of γH2AX foci in A. Error bars represent S.D. NS, not significant.

E, chromosomal aberrations in 293A WT, FANCL−/−, FANCD2−/−, and FANCD2-KR cells treated with 50 ng/ml MMC for 24 h. Red lines mark the mean value of each mutant. p values were derived by Student’s t test.
MRE11 (supplemental Fig. S4A). The knockdown cells were then exposed to a low concentration of MMC (20 ng/ml) to induce ICL-mediated replication blockage. γH2AX foci were measured after a 24-h recovery period. We found that depletion of DNA2 effectively suppressed the γH2AX levels in the FANCD2−/− mutant and to a much lesser extent in the FANCL−/− mutants. In contrast, knockdown of EXO1 and MRE11 did not produce comparable effects (Fig. 6B). These results suggest that DNA2 most likely carries out excessive or erroneous resection of stalled replication forks in the absence of FA pathway function.

To functionally validate the impact of DNA2 depletion in mitigating the replication stress in FA mutants, FANCD2−/− and FANCL−/− cells with DNA2 knockdown were analyzed for their survival to MMC exposure. As shown (Fig. 6B), depletion of DNA2 alleviated the hypersensitivity of the FANCD2−/− and FANCL−/− mutants, suggesting that a critical function of the FA pathway is to prevent the improper resection activity during replication fork stalling.

**FANCD2 loss compromises stressed cellular survival and leads to increased R-loop formation**

Clonogenicity depends on the initial single-cell survival, a stress condition that may require intensified and programmed transcription. Thus, an increase in stalled and/or damaged replication forks due to the lack of FANCD2 is expected to aggravate hazardous encounters between transcription and stalled replication, resulting in hampered clonogenic growth. Indeed, we found that deletion of FANCD2 caused a drastic reduction of plating efficiency in the 293A cell background (Fig. 7A), whereas cells expressing the FANCD2-KR alleles exhibited substantially improved clonogenic growth, reflecting a significant role of ubiquitination-independent FANCD2 function.

To verify whether loss of FANCD2 indeed leads to disruption of transcription, we performed R-loop staining in unperturbed FANCD2−/− and FANCL−/− cells in both HeLa and 293A backgrounds (Fig. 7B–E). We observed that, although ablation of either gene gave rise to increased R-loop formation, the FANCD2−/− mutants produced significantly higher levels of R-loops than the FANCL−/− mutant. These results validate an important role of the FA pathway in minimizing R-loop formation. More importantly, the diminished plating efficiency and the more severe R-loop phenotype associated with FANCD2 deletion demonstrate an indispensable role of FANCD2 in minimizing endogenous DNA damage dependent of its ubiquitination.

**Discussion**

Monoubiquitination of FANCD2 is the signifying event of FA pathway activation in response to replication stress. This is reflected by the phenotypic severity of FA gene mutations that disrupt the E3 ligase activity of the FA core complex. In this study, however, we discovered through direct comparison of isogenic mutants that deletion of FANCD2 yields stronger defects in the DNA damage response than elimination of the FA E3 ligase activity, strongly suggesting that FANCD2 has a constitutive role in mitigating endogenous damage independent of its monoubiquitination. Further supporting this conclusion are our findings that the phenotypes of the FANCD2-KR mutant closely resemble those of the FANCL-null mutant.

FANCD2−/− cells exhibit much exacerbated replication fork instability than the FANCL−/− mutants (Fig. 4C and supplemental Fig. S3C), indicating a constitutive fork-protecting
function of FANCD2 in the absence of ubiquitination. The fact that DNA2 depletion effectively alleviates FANCD2−/− phenotypes (Fig. 6) suggests that preventing excessive/erroneous fork processing is critical in response to both endogenous and exogenous replication stresses. Accordingly, our results support a biphasic mode of FANCD2 function shown in Fig. 7F. In this model, FANCD2, in its unmodified form, provides basal-level protection against endogenous/low levels of replication fork stress by recruiting the proper nuclease activities such as CTIP and FAN1 to process stalled replication forks, leading to fork stress relief. When levels of exogenous damage escalate, the FA E3 activity is activated, and the monoubiquitinated FANCD2-

Figure 7. Ablation of FANCD2 diminishes clonogenicity and causes R-loop accumulation. A, plating efficiencies of 293A WT and FANCD2−/− and FANCD2-KR mutants as measured by the number of colonies obtained normalized to the number of seeded single cells. Error bars represent S.D. B, immunostaining of R-loop with S9.6 antibody (red) and DAPI counterstain (blue) in unperturbed HeLa cells with the indicated genotypes. C, immunostaining of R-loop with S9.6 antibody (red) and DAPI counterstain (blue) in unperturbed 293A cells with the indicated genotypes. Merges of the two channels are shown for each cell. Scale bars represent 5 μm. D, quantification of S9.6 foci per nucleus in HeLa cells for the experiment described in B. Error bars represent S.D. E, quantification of S9.6 foci per nucleus in 297 cells for the experiment described in C. Error bars represent S.D. F, proposed model for the ubiquitination-independent function of FANCD2 (D2) in replication fork stress.
FANCD2 possesses ubiquitination-independent function

FANCI complex provides increased efficiency of nuclease recruitment and/or turnover to counter larger numbers of stressed replication forks. When FANCD2 is absent, the presence of FANCI alone is insufficient to recruit proper nuclease activities, and the stalled forks are subjected to promiscuous resection by unoptimized nucleases such as DNA2 and MRN, which are capable of long-range resection and are unlikely to be desirable for reversing stalled replication forks (36, 47–49). Excessive processing by long-range or high-processivity nucleases may lead to much higher levels of fork erosion and fork degradation as we observed in FANCD2+/− cells (Fig. 4C and supplemental Fig. S3C). Supporting this model, FANCD2 function in the replication stress response has been shown in recruiting CTIP (41, 42) as well as binding to the MCM2–7 replicative helicase independently of monoubiquitination (51). Although direct FANCD2 recruitment of nucleases is a potential mechanism of preventing fork damage, it is plausible that the RAD51-dependent and homologous recombination-independent mechanism also contributes to fork stability (29). Additionally, the FANCD2–FANCI complex has been found to be enriched at DNA ICL lesions before ubiquitination (39), suggesting a basal level of function in the absence of FANCD2 monoubiquitination. In additional to its fork protection function, FANCD2 was also implicated in replication fork restart in a ubiquitination-independent fashion (42, 52). Thus, the phenotypes of complete FANCD2 knock-out likely reflect the contributions from both.

The constitutive protection from FANCD2 against endogenous stress predicts that cells and organisms with complete loss of FANCD2 would be severely disadvantaged in survival and proliferation (Figs. 3 and 7A) because of severely compromised genomic stability. In fact, of the 34 FANCD2 patients analyzed to date, none presented biallelic null mutations. Instead, each patient carried at least one hypomorphic allele coding full-length mutant proteins that likely confer partial FANCD2 function (53, 54).

From an evolutionary perspective (55, 56), FANCD2 does not seem to co-evolve with the E3 ligase FANCL in an obligatory manner. In Caenorhabditis elegans, for example, no FANCL ortholog has been identified. The Lys-561 residue in human FANCD2 is an unconserved glutamic acid in the C. elegans counterpart. However, C. elegans FANCD2 mutations render significant sensitivity to replication damage (54), suggesting that FANCD2 can function in the absence of monoubiquitination.

Although FANCD2 provides constitutive function in resolving replication fork stress, its role in suppressing R-loop formation may also contribute to genomic instability in unperturbed conditions as reflected by our results (Fig. 7, B–E) and others (40). The accumulation of R-loops may be of particular relevance to the diminished clonogenicity of FANCD2−/− cells and the complete absence of biallelic null mutations in FANCD2 patients. A possible explanation is that stress associated with single-cell growth places a stronger demand on transcription, which results in the aggravation of R-loop formation from encountering more frequently stressed replication forks in the complete absence of FANCD2. This result highlights the physiological significance of FANCD2 function during unperturbed cell proliferation.

Collectively, our studies reveal a mechanism of biphasic FANCD2 function. A constitutive protection against endogenous DNA damage by FANCD2 is essential for maintenance of genome stability and is rendered independently of monoubiquitination mediated by the FA core complex. This mode is perhaps more resource-efficient for cells with a basal level of damage without having to activate the elaborate E3 ligase complex and the reliance on upstream damage-sensing components. When the level of stress surpasses the capacity of constitutive FANCD2 activity, the FA core complex is activated to augment the capacity of the FA pathway. This novel concept establishes a ground-state performance for the FA mechanism and may help guide exploration of potential therapeutic manipulation of the FA pathway.

Experimental procedures

Cell culture and antibodies

HeLa and HEK293A cells were obtained from ATCC. Parental HeLa cells, HEK293A cells, and their knock-out derivatives were maintained in DMEM plus 10% FBS. Antibody against 53BP1 was generated by the Junjie Chen laboratory. Commercial antibodies used in this study were purchased from the indicated sources: anti-human FANCD2 (Santa Cruz Biotechnol- ogy, sc-28394), anti-human FANCA (Bethyl Laboratories, A301-980A), anti-human FANCL (Santa Cruz Biotechnology, H-197), anti-yH2AX (Upstate, 07-164), and anti-human DNA2 (Abcam ab96488). Cells used in this study were negative for Mycoplasma according to biweekly tests by the Tissue Culture core facilities at M. D. Anderson Cancer Center.

Generation of knock-out mutant cell lines

Construction of the knock-out mutants followed the principal procedure described by Church and co-workers (50). Ten to 15 independent PCR product clones were sequenced to genotype each knock-out mutant candidate and subjected to antibody-based screening.

All knock-out mutants in this study were authenticated by the Characterized Cell Line core facility at M. D. Anderson Cancer Center using no less than 13 short tandem repeat markers to confirm isogenicity with the parental cell line. Each variant was complemented by clonogenic survival assay to exclude potential off-target effects.

Clonogenic survival assay

Cells were seeded in triplicates in 6-well plates 24 h prior to exposure to MMC or cisplatin for 1 h, formaldehyde for 2 h, or hydroxyurea for 24 h. After 10–14 days in regular medium, colonies were fixed with 6% (v/v) glutaraldehyde and stained with 0.5% (w/v) crystal violet for visualization.

Mitotic spreading

Cells were seeded in 10-cm plates 24 h prior to treatment and treated with MMC for 24 h. Mitotic spreading was performed according to the standard Colcemid/hypertonic protocol. Slides were stained with 4% Giemsa, and chromosomal abnormalities were quantified by scoring 50–100 metaphase spreads.
**FANCD2 possesses ubiquitination-independent function**

**Immunofluorescence staining**

Cells growing in coverslips were fixed after the specified recovery duration, incubated sequentially with anti-γH2AX or anti-53BP1 antibody and secondary antibodies for 1 h each, and counterstained with DAPI for 2 min. Nuclear foci were counted in at least 100 nuclei in three independent views for each sample.

**DNA fiber assay**

Cells were seeded in 12-well plates. Replication tracts were labeled with 50 μM IdU followed by exposure to hydroxyurea (4 mM) for 5 h. Cells were prepared as described previously (33) to obtain a single DNA molecule. Fibers were imaged using a Nikon Eclipse 90i, measured using ImageJ software, and analyzed by Prism (Version 6).

**R-loop staining**

Cells were fixed by methanol for 5 min and then incubated with the S9.6 antibody against DNA-RNA duplexes at 4 °C overnight. Cells were incubated in a secondary antibody for 1 h and stained with DAPI for 2 min.

**Statistical analysis**

Student’s t test was used for comparisons of two samples. p values ≤ 0.05 are considered significant. Error bars indicate S.D.

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