CELL CYCLE-REGULATION OF THE FANCD2 PROTEIN VIA CDK-MEDIATED S592 PHOSPHORYLATION

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CELL CYCLE-REGULATION OF THE FANCD2 PROTEIN

VIA CDK-MEDIATED S592 PHOSPHORYLATION

BY

JUAN A. CANTRES VELEZ

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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ABSTRACT

Fanconi anemia (FA) is a rare genetic disease characterized by increased risk for bone marrow failure and cancer. The FA proteins function together to repair damaged DNA. A central step in the activation of the FA pathway is the monoubiquitination of the FANCD2 and FANCI proteins under conditions of cellular stress and during S-phase of the cell cycle. The regulatory mechanisms governing S-phase monoubiquitination, in particular, are poorly understood. In this study, we have identified a CDK regulatory phospho-site (S592) proximal to the site of FANCD2 monoubiquitination. FANCD2 S592 phosphorylation during S-phase was detected by LC-MS/MS and by immunoblotting with a S592 phospho-specific antibody. Mutation of S592 disrupts S-phase and DNA damage-inducible monoubiquitination. In addition, FA-D2 (FANCD2<sup>−/−</sup>) patient cells expressing S592 mutants display reduced proliferation under conditions of replication stress and increased mitotic aberrations, including nucleoplasmic bridges and multinucleated cells. Our findings describe a novel cell cycle-specific regulatory mechanism for the FANCD2 protein.
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always believed in me and continues to be one of my biggest inspirations no matter how much time passes.
PREFACE

This thesis is submitted in manuscript format. This chapter is in preparation for submission to Molecular and Cellular Biology, a peer-reviewed journal. All references are formatted according to the reference guidelines of the proposed journal.
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CHAPTER 1

CELL CYCLE-REGULATION OF THE FANCD2 PROTEIN VIA CDK-MEDIATED S592 PHOSPHORYLATION

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INTRODUCTION

Protection of the integrity of our genome depends on the concerted activities of several DNA repair pathways that ensure the timely repair of DNA damage. These DNA repair pathways need to be tightly coordinated and regulated upon cellular exposure to exogenous DNA damaging agents, as well as during the cell cycle. Somatic disruption of the DNA damage response leads to mutation, genome instability and cancer. In addition, germline mutations in DNA repair genes are associated with hereditary diseases characterized by increased cancer risk and other clinical manifestations.

Fanconi anemia (FA) is a rare genetic disease characterized by congenital anomalies, increased risk for bone marrow failure and cancer, and accelerated aging (Alter 2014; Ceccaldi, Sarangi, and D’Andrea 2016; Rosenberg, Greene, and Alter 2003). FA is caused by germline mutations in any one of 23 genes. The FA proteins function together in a pathway to repair damaged DNA and to maintain genome stability. A major role for the FA pathway in the repair of DNA interstrand crosslinks (ICLs) has been established (I Garcia-Higuera et al. 2001; Kim and D’Andrea 2012).

The main activating step of the FA pathway is thought to be the monoubiquitination of the FANCD2-FANCI heterodimer (ID2), which is catalyzed by the FA-core complex, comprising FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, UBE2T/FANCT, and the FA associated proteins. Monoubiquitination occurs following exposure to DNA damaging agents and during S-phase of the cell cycle (I Garcia-Higuera et al. 2001; Taniguchi et al. 2002). Recently, it was discovered that the monoubiquitination of FANCD2 and FANCI leads to the formation of a closed ID2 clamp that encircles DNA (Alcón et al. 2020; R.
Wang et al. 2019). Moreover, ubiquitinated ID2 (ID2-Ub) assembles into nucleoprotein filament arrays on double-stranded DNA (Tan et al. 2020). The monoubiquitination of FANCD2 is necessary for efficient interstrand crosslink repair, the maintenance of common fragile site stability and faithful chromosome segregation, events that are all crucial for genomic stability (Meetei et al. 2003; Nakanishi et al. 2005; Vinciguerra et al. 2010).

A DNA damage-independent role for the FA pathway during the cell cycle has been implicated in several studies. For example, FANCD2 promotes replication fork protection during S-phase and ensures the timely and faithful replication of common chromosome fragile sites (Howlett et al. 2005; Madireddy et al. 2016). FANCD2 has also been shown to be involved in mitotic DNA synthesis (MiDAS) during prophase and is present on the terminals of anaphase ultrafine bridges (Chan et al. 2009; Garribba et al. 2018; Vinciguerra et al. 2010). During the cell cycle FANCD2 monoubiquitination is maximal during S-phase and minimal during M-phase (Taniguchi et al. 2002). FANCD2 and FANCI are phosphorylated by the ATR and ATM kinases following exposure to DNA damaging agents, promoting their monoubiquitination (Chen et al. 2015; G. P. H. Ho et al. 2006). However, in general, the function and regulation of FANCD2 and FANCI during the cell cycle remains poorly understood.

Cyclin-dependent kinases (CDKs) play a major role in regulating cell cycle progression, with CDK-mediated hyperphosphorylation of the retinoblastoma protein (pRb) being the primary mechanism of cell cycle regulation (Adams et al. 1999; Ezhevsky et al. 2001). CDKs are also known to regulate DNA repair during the cell
cycle. Several protein components of the homologous recombination repair (HR), translesion DNA synthesis (TLS), non-homologous end joining (NHEJ), and telomere maintenance pathways are CDK substrates (Wohlbold and Fisher 2009). For example, the BRCA2 protein is phosphorylated by CDK1 as cell progress towards mitosis. CDK-mediated phosphorylation of BRCA2 blocks interaction with RAD51 and thereby restricts homologous recombination DNA repair during M-phase (Esashi et al. 2005; Yata et al. 2014). Conversely, CDK1/2 phosphorylate the EXO1 nuclease to promote DNA strand resection and homologous recombination repair during S-phase (Tomimatsu et al. 2014).

In this study we have examined the regulation of the FANCD2 protein by phosphorylation, specifically during the cell cycle. We show that FANCD2 is phosphorylated on S592, a putative CDK site, during S-phase but not during M-phase and is phosphorylated by CDK2-Cyclin A in vitro. Mutation of S592 as well as CDK inhibition disrupts S-phase FANCD2 monoubiquitination. Furthermore, we demonstrate that FA-D2 patient cells stably expressing FANCD2 mutated at S592 display reduced growth in the presence of DNA damaging agents, an altered cell cycle profile, and increased genomic instability manifested as increased micronuclei, mitotic bridges, and aneuploidy. Our results provide further mechanistic insight into the regulation of a key DNA repair/genome maintenance pathway under unperturbed conditions.
RESULTS

FANCD2 is phosphorylated under unperturbed conditions and during S-phase of the cell cycle

To study the phosphorylation of the FANCD2 protein in the absence and presence of DNA damaging agents, we performed a lambda-phosphatase assay with several cell lines following incubation in the absence or presence of the DNA crosslinking agent mitomycin C (MMC). Notably, we observed a large increase in FANCD2 mobility following incubation of lysates with lambda-phosphatase even in the absence of MMC in all cell lines examined (Fig. 1A). These results suggest that FANCD2 is subject to extensive phosphorylation even in the absence of an exogenous DNA damaging agent. A similar change in protein mobility was not observed for FANCI (Fig. 1A). To determine if FANCD2 is subject to phosphorylation during the cell cycle, we performed a double thymidine block experiment causing an early S-phase arrest and analyzed phosphorylation at regular time points following release using the lambda-phosphatase assay. We observed maximal phosphorylation of FANCD2 during S-phase of the cell cycle, with much less phosphorylation observed as cells progressed through G2/M- and G1-phases of the cell cycle (Fig. 1B). Again, we observed no appreciable change in FANCI mobility upon lambda-phosphatase treatment, suggesting that FANCI is not subject to the same level of phosphorylation as FANCD2 during the cell cycle (Fig. 1B). Similar findings were observed with U2OS cells (Fig. S1A and B). We also performed a M-phase arrest using nocodazole and again observed maximal levels of FANCD2 phosphorylation during S-phase (~15 h after release) of the cell cycle (Fig. S1C and D). The FANCA protein is a central
component of the FA core complex, a multi-subunit ubiquitin ligase that catalyzes the monoubiquitination of FANCD2 (I Garcia-Higuera et al. 2001; Irene Garcia-Higuera et al. 2000). FANCD2 and FANCI monoubiquitination are defective in the absence of FANCA (I Garcia-Higuera et al. 2001). To determine if FANCD2 phosphorylation was dependent on the presence of FANCA, we performed a lambda-phosphatase assay with asynchronous and early-S-phase synchronized FA-A (*FANCA-/-*) and FANCA-complemented FA-A cells. S-phase FANCD2 phosphorylation was observed in the absence of FANCA, albeit to a slightly lesser extent than in cells FANCA-complemented FA-A cells, suggesting that phosphorylation of FANCD2 is not strictly dependent on its ubiquitination (Fig. 1C).

**FANCD2 is a CDK substrate**

Phosphorylation of FANCD2 during the cell cycle suggested a role for cyclin-dependent kinases (CDKs) in the regulation of FANCD2. CDKs phosphorylate serine or threonine residues in the consensus sequence [S/T*]PX[K/R], and several DNA repair proteins are known CDK substrates, including BRCA2, FANCJ, and CtIP (Esashi et al. 2005; Huertas and Jackason 2009; Nath and Nagaraju 2020). Both FANCD2 and FANCI have several putative CDK phosphorylation sites with varying degrees of conservation (Fig. S2A and B). To begin to assess the role of CDKs in the regulation of FANCD2, we performed a double-thymidine block in the absence and presence of the CDK inhibitors purvalanol A and SNS-032. At the concentrations tested in this experiment, treatment with both inhibitors resulted in a significant reduction in the levels of FANCD2 and FANCI monoubiquitination observed after
double-thymidine arrest and a modest reduction in FANCD2 phosphorylation (Fig. 2A). Reduced FANCD2 phosphorylation was also observed upon incubation with other CDK inhibitors and upon short-term exposure to purvalanol A (Fig. S2C and D).

To determine if FANCD2 is a CDK substrate, we immunoprecipitated FANCD2 from FA-D2 (FANCD2⁺/-) patient cells stably expressing V5-tagged LacZ or FANCD2 and probed immune complexes with a pan anti-pS/T-CDK antibody. An immune reactive band was detected in immune complexes from cells expressing FANCD2 and not LacZ (Fig. 2B), suggesting that FANCD2 is a CDK substrate. We also performed an in vitro CDK kinase assay with CDK2-Cyclin A and full length FANCD2 purified from High Five insect cells (van Twest et al. 2017). Low levels of CDK-mediated phosphorylation were observed even in the absence of CDK2-Cyclin A and phosphorylation increased markedly upon incubation with CDK2-Cyclin A for 30 min (Fig. 2C). These results indicate that FANCD2 is a CDK substrate and suggest that FANCD2 monoubiquitination may be coupled to CDK phosphorylation.

**FANCD2 is phosphorylated on S592 during S-phase of the cell cycle**

To map the in vivo sites of FANCD2 phosphorylation, we immunoprecipitated FANCD2 from asynchronous U2OS cells stably expressing 3xFLAG-FANCD2 under stringent conditions. Immunoprecipitated FANCD2 bands were combined and subjected to phosphoproteomic analysis using LC-MS/MS (Fig. 3A and B). Under these non-DNA damaging conditions, we observed the phosphorylation of multiple sites including the previously detected ATM/ATR phosphorylation sites S1401 and S1404 (Table 1) (Taniguchi et al. 2002). We also detected phosphorylation of the
putative CDK site S592 (Table 1). To analyze the phosphorylation of FANCD2 during the cell cycle, we synchronized HeLa cells in M-phase using nocodazole, immunoprecipitated FANCD2 immediately upon release and at 15 h post-release and again performed phosphoproteomic analysis using LC-MS/MS (Fig. 3C-F). Under these conditions, we again detected phosphorylation of FANCD2 on S592 in S-phase of the cell cycle and not during M-phase synchronized cells suggesting that the phosphorylation of FANCD2 S592 might have an important regulatory function during S-phase (Fig. 3F). To study FANCD2 S592 phosphorylation more closely, we generated a S592 phospho-specific antibody. We arrested cells in M-phase using nocodazole and analyzed FANCD2 S592 phosphorylation upon release from nocodazole block. A FANCD2 pS592 immunoreactive band was observed at 12 h (S-phase) following release (Fig. 3G), consistent with the results of our LC-MS/MS phosphoproteomic analysis (Fig. 3F, Table 1).

**Mutation of S592 disrupts FANCD2 monoubiquitination during S-phase and upon exposure to DNA damaging agents**

FANCD2 S592 localizes to a flexible loop proximal to K561, the site of monoubiquitination, suggest a potential ubiquitination regulatory function for S592 phosphorylation (Fig. 4A). To begin to characterize the role of S592 phosphorylation, we generated phospho-dead (S592A) and phospho-mimetic (S592D) variants and stably expressed these in FA-D2 (FANCD2$^{-/-}$) patient-derived cells. Mutation of S592 did not overtly impact the stability or overall structure of FANCD2 as evidenced by equal protein expression levels and equal basal levels of FANCD2 monoubiquitination
To analyze the effects of mutation of S592 on DNA damage-inducible FANCD2 monoubiquitination, we incubated cells in the absence and presence of mitomycin C (MMC) and analyzed FANCD2 monoubiquitination by immunoblotting. The FANCD2-S592A mutant showed similar levels of basal and MMC-inducible monoubiquitination compared to FANCD2-WT (Fig. 4B). However, basal levels of FANCD2-S592D monoubiquitination were elevated, and MMC-inducible FANCD2-S592D monoubiquitination was reduced, compared to FANCD2-WT and FANCD2-S592A (Fig. 4B). Under the same experimental conditions, we also examined levels of CDC2 pY15, which is an inhibitory mark of mitotic progression at the G2/M checkpoint of the cell cycle and is expected to increase upon DNA damage conditions. In cells expressing FANCD2-WT, we observed a robust increase in CDC2 pY15 levels following MMC exposure. However, this increase was attenuated in the absence of FANCD2 and in cells expressing the FANCD2 S592 variants (Fig. 4B), suggestive of a defect in the mitotic checkpoint or mitotic progression.

To analyze the effects of S592 mutation on S-phase FANCD2 monoubiquitination (Taniguchi et al. 2002), cells were subject to a double thymidine block, and FANCD2 monoubiquitination was analyzed upon release. Compared to FANCD2-WT, S-phase monoubiquitination was markedly attenuated for both S592 variants (Fig. 4C). We also analyzed levels of the mitotic marker H3 pS10 in these cells. Compared to cells expressing FANCD2-WT, we observed persistent levels of H3 pS10 in FA-D2 cells expressing empty vector and the FANCD2-S592A mutant (Fig. 4C). We observed a similar phenotype of elevated H3 pS10 in HeLa FANCD2+/− cells generated by CRISPR-Cas9 gene editing (Liang et al. 2016) (Fig. S3A-C). In
contrast to cells expressing empty vector and the FANCD2-S592A mutant, we observed a more rapid disappearance of H3 pS10 in cells expressing FANCD2-S592D (Fig. 4C). FACS analysis of FA-D2 patient cells expressing empty vector and the S592 variants upon release from double thymidine block indicated a higher percentage of cells in G2/M at earlier time points, compared to cells expressing FANCD2-WT (Fig. S3D). Taken together, these results demonstrate that mutation of FANCD2 S592 disrupts S-phase monoubiquitination and leads to altered G2-M cell cycle progression.

**Mutation of FANCD2 S592 leads to decreased proliferative capacity and increased mitotic defects**

To assess the functional impacts of mutation of FANCD2 S592, we monitored the proliferation of FA-D2 patient cells stably expressing FANCD2-WT and the S592 variants in the presence of low concentrations of DNA damaging agents for prolonged periods using the xCELLigence real time cell analysis system. The xCELLigence system enables the analysis of cellular phenotypic changes by continuously monitoring electrical impedance. Impedance measurements are displayed as the cell index, which provides quantitative information about the biological status of the cells, including cell number, cell viability, and cell morphology (Hamidi, Lilja, and Ivaska 2017; Kho et al. 2015). Compared to cells expressing FANCD2-WT, cells expressing empty vector and the S592 variants exhibited reduced viability when cultured in the presence of the DNA polymerase inhibitor aphidicolin (APH) (Fig. 5A and S4A). In contrast, mutation of FANCD2 S592 had no impact on growth in the presence of low concentrations of mitomycin C (MMC) (Fig. 5A and S4A). We also analyzed growth
in the presence of the CDK1 inhibitor RO3306. CDK1 inhibition resulted in reduced cell proliferation in cells expressing FANCD2-WT and the S592A variant compared to cells expressing empty vector or the S592D variant (Fig. S4B and C).

To test the effects of S592 mutation on cell division we looked at different types of cytogenetic markers under non-stressed conditions. Micronuclei arise from chromosome fragments that fail to incorporate in daughter nuclei during mitosis and they are a well characterized marker of genomic instability. We counted at least 400 cells and the presence of micronuclei was significantly higher in cells expressing mutation in S592 compared with cells expressing FANCD2-WT suggesting increasing genomic challenges after mutation in S592 (Fig 5B, S6C). We also looked at nuclear buds which are biomarker of elimination of repair complexes and amplified DNA (Fenech 2002; Fenech and Crott 2002). We found a statistically significant higher percent of nuclear buds in S592 mutant cell lines compared to cells expressing FANCD2-WT (Fig. 5B, S6B). A high number of binucleated cells in asynchronous populations suggest difficulties in cell division, and is consistent with that previously observed by Vinciguerra et al., 2010 (Vinciguerra et al. 2010). In addition multinucleated cells (>2 nucleus) are caused by multiplication of centrioles and can promote genomic instability (Duensing et al. 2009; Korzeniewski, Treat, and Duensing 2011). We detected a significant higher percent of binucleated and multinucleated cells expressing the FANCD2-S592 variants compared to cells expressing FANCD2-WT (Fig. 5B, S6A-D). Together these increases in mitotic aberrations suggest that mutation in S592 is promoting mitotic defects and genomic instability under non-stressed conditions.
To test cell division under DNA damage conditions we performed a micronucleus assay. We treated FA-D2 cells expressing WT and S592 mutant versions of FANCD2 with APH and MMC for 24 h followed by a cytochalasin B treatment to block cytokinesis. We counted at least 300 binucleated cells and we did not detect statistically significant differences in percentage of micronuclei in cells expressing FANCD2-S592 mutant versions and FANCD2-WT (Fig. S5 A, C). Next, we counted at least 400 cells and scored nuclear buds and multinucleated cells. We found a statistically significant increase in nuclear buds after treating with MMC in cells expressing FANCD2-S592D compared to WT (Fig. S5 A, D). There were no significant differences in number of multinucleated in cells expressing FANCD2-S592 mutants and FANCD2-WT (Fig. S5 A, E). In addition, we looked at nucleoplasmic bridges (NPBs) which are caused by telomere end-fusion or defective DNA repair (Fenech 2002; Podrimaj-Bytyqi et al. 2018). We score at least 400 cells and detected a significant increase in NPBs after cytokinesis block under non-stressed conditions in cells expressing mutant FANCD2-S592 compared to cells expressing FANCD2-WT (Fig. S5 A-B). These results suggest that the effect of mutation of FANCD2 in S592 is stronger under non-stressed conditions than when adding DNA damage.

**DISCUSSION**

In this study, we have investigated the posttranslational regulation of the central FA pathway protein FANCD2 under unperturbed conditions. The majority of studies to date have focused on the posttranslational regulation of FANCD2 and FANCI following exposure to DNA damaging agents (Cheung et al. 2017; Irene Garcia-
Higuera et al. 2001; Ishiai et al. 2008). We have observed that FANCD2 undergoes extensive phosphorylation during the cell cycle and, in particular, during S-phase of the cell cycle. Notably, in contrast, FANCI, a FANCD2 paralog, does not appear to be subject to the same degree of phosphorylation during the cell cycle. We have also determined that FANCD2 is phosphorylated by CDK2 on S592 both in vivo and in vitro. Phosphoproteomic analysis of human and Xenopus FANCD2 expressed in insect cells, as well as FANCD2 immune complexes from S-phase, but not M-phase, enriched cell populations uncovered phosphorylation of S592. Previous studies have shown that FANCD2 is monoubiquitinated as cells traverse S-phase, and this has been shown to contribute to the protection of stalled replication forks from degradation (Schlacher, Wu, and Jasin 2012; Taniguchi et al. 2002). Here we show that mutation of S592, or CDK inhibition, markedly abrogates S-phase monoubiquitination, strongly suggesting that S592 phosphorylation primes FANCD2 for ubiquitination during S-phase. Recent studies have established that the FANCL RING E3 ubiquitin ligase allosterically alters the active site of the UBE2T E2 ubiquitin-conjugating enzyme to promote site-specific monoubiquitination of FANCD2 (Chaugule et al. 2020). Specifically, FANCL binding to UBE2T exposes a basic triad of the UBE2T active site, promoting favorable interactions with a conserved acidic patch proximal to K561, the site of ubiquitination (Chaugule et al. 2020). We speculate that S592 phosphorylation may also augment interaction with the basic active site of UBE2T. Alternatively, S592 phosphorylation may inhibit FANCD2 de-ubiquitination by USP1, in a manner similar to that previously reported for the FANCI S/TQ cluster (Cheung et al. 2017).
Our studies further emphasize the critical nature of coordinated posttranslational modification of FANCD2. Several studies have previously established intricate dependent and independent relationships between FANCD2 monoubiquitination and phosphorylation. For example, the ATM kinase phosphorylates FANCD2 on several S/TQ motifs following exposure to ionizing radiation, e.g. S222, S1401, S1404, and S1418 - phosphorylation of S1401 and S1404 were also detected under unperturbed conditions in this study. While phosphorylation of S222 promotes the establishment of the IR-inducible S-phase checkpoint, S222 phosphorylation and K561 monoubiquitination appear to function as independent events (Taniguchi et al. 2002). In contrast, phosphorylation of FANCD2 on T691 and S717 by the ATR kinase has been shown to be required for efficient FANCD2 monoubiquitination (Andreassen, D’Andrea, and Taniguchi 2004; Gary P. H. Ho et al. 2006). Phosphorylation of FANCD2 by CK2 in residues 882-889 decreases monoubiquitination of FANCD2 in the absence of DNA damaging agents (Lopez-Martinez et al. 2019).

Multiple studies have showed the coordination of the DNA damage response and the cell cycle. For example BRCA2 is phosphorylated by CDK2 on the carboxy-terminus precluding RAD51 binding, this inactivates homologous recombination prior to mitosis (Esashi et al. 2005). In contrast, CDK1/2 phosphorylation of EXO1 endonuclease positively regulates DNA strand resection and homologous recombination repair during S-phase (Tomimatsu et al. 2014). Another example of positive regulation occurs by CDK phosphorylation of CtIP during S-phase triggering conformational changes and making it a better substrate to ATM phosphorylation at
the C-terminus (H. Wang et al. 2013). This phosphorylation event describes a coordination between the DNA damage response and cell cycle.

A role of FANCD2 during S-phase of the cell cycle is to assist replication through common fragile sites (CFS) (Howlett et al. 2005; Madireddy et al. 2016). The presence of FANCD2 in CFS persist through mitosis where is involved in mitotic DNA synthesis (MiDAS) in prophase and in anaphase ultrafine bridges (Chan et al. 2009; Özer et al. 2018; Vinciguerra et al. 2010). Functional FANCD2 promotes faithful chromosomal segregation. Previous studies showed that cells lacking FANCD2 have increased cytokinesis failure and apoptosis in unperturbed conditions. In this study, mutation in S592 caused elevated levels of binucleated cells multinucleated cells, nuclear buds and micronuclei in the absence of DNA damaging agents. This increase in mitotic defects suggest that mutation in S592 alters normal FANCD2 function during the cell cycle. We propose that CDK-mediated phosphorylation of FANCD2 promotes monoubiquitination during S-phase. This allows FANCD2 to assist in the replication of common fragile sites. We proposed that S592 phosphorylation during S-phase needs to occur to facilitate FANCD2 function in replication and further roles in CFS stability during mitosis. Mutation of this phosphosite disrupts FANCD2 role during CFS replication thus increasing genomic instability. This is translated to disturbances during cell division, causing non-disjunction and chromosome breakages. We propose that S592 phosphorylation of FANCD2 needs to be shut down at the onset of M-phase to promote FANCD2 de-ubiquitination during mitosis. This study increases the understanding of the regulation of FANCD2 during non-stressed conditions and brings new perspectives to the pathogenesis of this
disease.

MATERIALS AND METHODS

Cell culture and Generation of Mutant Cell Lines

HeLa cervical carcinoma and U2OS osteosarcoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v FBS, L-glutamine (2 mM) and penicillin/streptomycin. HeLa D2\(^{-/-}\) generated by CRISPR-Cas9 were provided by Martin Cohn at the University of Oxford. 293FT viral producer cells (Invitrogen) were cultured in DMEM containing 12% v/v FBS, 0.1 mM non-essential amino acids (NEAA), 1% v/v L-glutamine, 1 mM sodium pyruvate and 1% v/v penicillin/streptomycin. PD20 FA-D2 (FANCD2\(^{hy/-}\)) patient cells were purchased from Coriell Cell Repositories (Catalog ID GM16633). These cells harbor a maternally inherited A-G change at nucleotide 376 that leads to the production of a severely truncated protein, and a paternally inherited missense hypomorphic (\(^{hy}\)) mutation leading to a R1236H change (Timmers et al. 2001). PD20 FA-D2 cells were stably infected with pLenti6.2/V5-DEST (Invitrogen) harboring wild-type or mutant FANCD2 cDNAs. Stably infected cells were grown in DMEM complete medium supplemented with 2 \(\mu\)g/ml blasticidin.

Immunoprecipitation

FA-D2 cells stably expressing LacZ or V5-tagged FANCD2, U2OS, and U2OS 3xFLAG FANCD2 cells were lysed in Triton-X100 lysis buffer (50 mM Tris.HCl pH 7.5, 1% v/v Triton X-100, 200 mM NaCl, 5 mM EDTA, 2 mM Na\(_3\)VO\(_4\), Protease...
inhibitors (Roche), and 40 mM β-glycerophosphate) on ice for 15 min followed by sonication for 10 s at 10% amplitude using a Fisher Scientific Model 500 Ultrasonic Dismembrator. Anti-V5 or anti-FLAG-agarose were washed and blocked with NETN100 buffer (20 mM Tris-HCl pH 7.5, 0.1% NP-40, 100 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, protease inhibitors (Roche)) plus 1% BSA and the final wash and resuspension was done in Triton-X100 lysis buffer. Lysates were incubated with agarose beads at 4°C for 2 h with nutating. Agarose beads were then washed in Triton-X100 lysis buffer and boiled in 1× NuPAGE buffer (Invitrogen) and analyzed for the presence of proteins by SDS-PAGE and immunoblotting or stained using Colloidal Blue Staining Kit (Invitrogen) for mass spectrometry.

**Cell Cycle FANCD2 Immunoprecipitation**

S-phase and M-phase synchronized populations of HeLa cells were lysed in lysis buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 400 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.1 mM DTT, 0.1% NP-40, 1 mM PMSF and complete protease inhibitors). 100 units of benzonase was added per 100 µL of lysis buffer. Protein G magnetic beads (Dynabeads, Novex) were crosslinked with anti-FANCD2 (NB100-182, Novus Biologicals) antibody. An equal volume of no salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.2 mM KCl, 0.2 mM EDTA, 0.1 mM DTT, 0.1% NP-40, plus complete protease inhibitors) was added to 2 mg of whole cell lysate. Samples were resuspended in anti-FANCD2-bound beads rotating at 4°C for 4 h. Beads were washed in wash buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.2 mM KCl, 300 mM KCl, 10% glycerol, 0.2 mM EDTA, 0.1 mM DTT, 0.1% NP-40, plus complete protease
inhibitor). The samples were eluted in urea elution buffer (8 M urea, 1 mM Na$_3$VO$_4$, 2.5 mM Na$_2$H$_2$P$_2$O, 1 mM β-glycerophosphate and 25 mM HEPES, pH 8.0). Silver staining was performed to visualize the IP and FANCD2 pulldown was confirmed by western blot. Samples were sent for mass spectrometry at the COBRE Center for Cancer Research Development Proteomics Core at Rhode Island Hospital.

**Lambda Phosphatase Assay**

Cells were harvested and pellets were split into two, lysed in either lambda phosphatase lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 mM EGTA, 1 mM dithiothreitol, 2 mM MnCl$_2$, 0.01% Brij35, 0.5% NP-40, and protease inhibitor) or lambda phosphatase buffer with the addition of phosphatase inhibitors, 2 mM Na$_3$VO$_4$ and 5 mM NaF for 15 min at 4°C followed by sonication for 10 s at 10% amplitude using a Fisher Scientific Model 500 Ultrasonic Dismembrator. Whole-cell lysates were incubated with or without 30 U of lambda phosphatase per 10 U of PP1 for 2 h at 30°C, or for the times indicated. Proteins were resolved on NuPage 3-8% w/v Tris-Acetate, 4-12% w/v Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes. Larger proteins were resolved for 5 h on ice.

**Immunoblotting**

For immunoblotting analysis, cell pellets were washed in PBS and lysed in 2% w/v SDS, 50 mM Tris-HCl, 10 mM EDTA followed by sonication for 10 s at 10% amplitude. Proteins were resolved on NuPage 3-8% w/v Tris-Acetate or 4-12% w/v
Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes. The following antibodies were used: rabbit polyclonal antisera against FANCD2 (NB100-182; Novus Biologicals), FANCI (A301-254A, Bethyl Laboratories), CHK1 pS345 (2345, Cell Signaling), anti-pSP-CDK (9477, Cell Signaling), FLAG (F7425, Sigma), cyclin A (SC751, Santa Cruz), Cdc2 pY15 (4539, Cell Signaling), H3 pS10 (9701, Cell Signaling), FANCD2 pS592 (Pacific Bio), V5 (13202, Cell Signaling) and mouse monoclonal, α-tubulin (MS-581-PO, Neomarkers).

Plasmids

The S592A and S592D cDNA were generated by site-directed mutagenesis of the wild type FANCD2 cDNA using the Quickchange Site-directed Mutagenesis Kit (Stratagene). The forward (FP) and reverse (RP) oligonucleotide sequences used are as follows: S592A FP 5′-CGGCAGACAGAAGTGAAGCACCTAGTTTGACCCAAG-3′; S592A RP 5′-CTTGGGTCAAACTAGGTGCTTCACTTCTGTCTGCCG-3′; S592D FP 5′-GGCGGCAGACAGAAGTGAAGATCCTAGTTTGACCCAAGAG-3′; and S592D RP 5′-CTCTTGGGTCAAACTAGGTGCTTCACTTCTGTCTGCCGCC-3′. The full length FANCD2 cDNA sequences were TOPO cloned into the pENTR/D-TOPO (Invitrogen) entry vector, and subsequently recombined into the pLenti6.2/V5-DEST (Invitrogen) destination vector and used to generate lentivirus for the generation of stable cell lines.
**Immunofluorescence Microscopy**

Hela WT or Hela D2\(^{-/}\) generated by CRISPR-Cas9 were seeded in at a density of 2 x 10\(^{4}\) in chamber slides (Millicell EZ Slide, Millipore) overnight. Cells were treated with 100 nM MMC for 24 h and fixed in fixing buffer (4% w/v paraformaldehyde, 2% w/v sucrose in PBS, pH 7.4) at 4°C for 10 min. Cells were permeabilized using 0.3% v/v Triton-X in PBS for 10 min at room temperature. Cells were blocked in antibody dilution buffer (ADB, 5% v/v goat serum, 0.1% v/v NP-40 in PBS, pH 7.4) and then incubated with mouse-anti H3 pS10 (Cell si1:250) and rabbit-anti-FANCD2 (1:250). Cells were washed with PBS and incubated in secondary goat-anti-mouse Alexa Fluor 594 (1:500, Thermo Fisher) and donkey-anti-rabbit Alexa Fluor 488 (1:500, Thermo Fisher) in ABD for 1 h. Cells were washed in PBS and stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Vector Laboratories).

**Generation of Lentiviral Particles**

5 x 10\(^{6}\) 293FT cells were seeded in 10 cm\(^{2}\) dishes in DMEM + 12% v/v FBS + 1% v/v L-glutamine + 1% v/v sodium pyruvate +2% v/v non-essential amino acids without antibiotics. 5mL of a mix of Opti MEM and Fugene 6 was added dropwise to the cells and then cells were incubated. A mixture of 9 µg of Virapower packaging mix and 3 µg pLenti-6.2-vector in OptiMEM was added to the cells and incubated for 6-8 h. The supernatant was collected and filtered through a 45 µm filter.

**Cell-Cycle Synchronization**
For early S-phase arrest, cells were synchronized by the double thymidine block method (Ma and Poon 2016). Cells (5.0 x 10^5) were seeded in 10 cm^2 dishes and treated with 2 mM thymidine (226740050, Acros Organics) for 18 h. Cells were washed with PBS and released in thymidine-free media for 10 h following by a second incubation in 2 mM thymidine for 18 h. Cells were washed twice with phosphate-buffered saline (PBS) and released into DMEM + 15% FBS, L-glutamine, and penicillin/streptomycin. For M-phase arrest, cells were synchronized using the mitotic shake-off method. HeLa (7.5 x 10^5) cells were seeded in 10 cm^2 dishes. Cells were treated with 100 ng/mL of nocodazole (SML1665, Sigma) at 80-90% confluency for 15 h. Mitotic cells were collected by the shake off method and/or replated in nocodazole-free media for cell cycle progression analysis. For late G2-phase arrest, cells were synchronized by reversible inhibition of CDK1 (Vassilev 2006). Cells (7.5 x 10^5) were seeded in 10 cm^2 dishes and treated with 6 µM R03306 (15149, Cayman Chemicals) for 16 h. Cells were washed with PBS and released in fresh media without CDK inhibitors.

**Cell Cycle Analysis by FACS**

Cells were resuspended in 0.1 mL PBS and fixed by adding 1 mL ice-cold ethanol and stored at -20°C until analysis. Cells were then washed in PBS and incubated in 0.3-0.5 mL RNase solution (1x RNase) for 10 min at 37°C. Cells were then incubated on ice for 5-10 min in 50 µg/mL propidium iodide (Sigma). Cells were analyzed using a BD FACSVerse flow cytometer system. Cell cycle distributions were determined using the FlowJo software (FlowJo, LCC).
**In-vitro CDK Phosphorylation Assay**

Purified FANCD2, pRb and CDK2/Cyclin A proteins were a generous gift from Andrew Deans at the University of Melbourne. In order to remove any previous phosphorylation 2 µg of protein were incubated with 100 U of lambda phosphatase, 10 mM protein metallophosphatases (PMP) and 10 mM MnCl₂ at 30°C for 30 min. Lambda phosphatase was then removed from the reaction using 30K columns. Protein was then resuspended in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, in preparation for the kinase reaction. For the phosphorylation reaction, each reaction tube was composed of the following reagents in this specific order: 10x CDK kinase buffer (250 mM Tris pH 7.5, 250 mM glycerophosphate, 50 mM EGTA, 100 mM MgCl₂), 2 µg of protein, 15, 30, 60, 100nM CDK2:Cyclin A, and 20 µM ATP. Samples were incubated at 30°C for 30 min and the reaction was stopped by adding 10 µL of 10% β-mercaptoethanol in 4x LDS buffer.

**Micronucleus Assay**

For micronuclei analysis, cells were seeded at a density of 2 x 10⁴ in chamber slides (Millicell EZ Slide, Millipore) overnight. On the following day, cells were treated with 0.4 µM aphidicolin (APH) or 40 nM mitomycin C (MMC) for 24 h, then washed with PBS and treated with 4.5 µg/mL cytochalasin B (Sigma) for 24 h to inhibit cytokinesis. Cells were fixed in fixing buffer (4% w/v paraformaldehyde, 2% w/v sucrose in PBS, pH 7.4) at 4°C for 10 min. Permeabilization was performed using 0.3% v/v Triton-X in PBS for 10 min at room temperature. Cells were blocked in
antibody dilution buffer (ADB, 5% v/v goat serum, 0.1% v/v NP-40 in PBS, pH 7.4) and then incubated with mouse-anti-tubulin (1:1,000, NeoMarkers) for 1 h in ADB, washed with PBS and incubated in secondary goat-anti-mouse Alexa Fluor 594 (1:500, Thermo Fisher) in ABD for 1 h. Cells were washed in PBS and stained with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI, Vector Laboratories). At least 300 binucleated cells were scored per group. We tested whether frequency of micronuclei differed across groups by using the base package R-function \textit{t.test} to perform Student’s t-test. All statistical analyses were performed in R version 3.6.1 (R Core team 2015).

\textbf{Cell Proliferation Assay}

For cell proliferation assays we used electrical impedance with the xCELLigence RTCA DP system from Acea Biosciences. Cells were seeded at a density of 5.0 x 10\(^3\) in polyethylene terephthalate (PET) E-plates (300600890, Acea Biosciences). Cells were treated with 20 nM MMC or 0.4 µM APH. Electric impedance measurements were taken every 15 min for 120 h. We tested whether electric impedance measurements differed across timepoints by using the base package R-function \textit{t.test} to perform Student’s t-test. All statistical analyses were performed in R version 3.6.1 (R Core team 2015).

\textbf{G2/M Accumulation Assay}

Cells were seeded at a density of 4.0 x 10\(^5\) in 6 cm\(^2\) dishes and treated with 20 nM and 40 nM MMC for 24 h and 48 h. Cells were harvested, washed with PBS and fixed in
ice cold methanol. Cells were then washed in PBS and incubated in 0.3-0.5 mL RNase solution (1x RNase) for 10 min at 37°C. Cells were then incubated on ice for 5-10 min in 50 μg/mL propidium iodide (Sigma). DNA content was measured by BD FACSVerse flow cytometer system.
Table 1. Immuno-precipitation mass spectrometry analysis of FANCD2.

| Protein   | Peptide Sequence                        | P-site(s) | Putative Kinase(s)* | Previously Detected |
|-----------|-----------------------------------------|-----------|--------------------|---------------------|
| FANCD2    | R.LS*KSEDKESLTDASK.T                   | S8        | PKACa              | Yes\(^1\)           |
| FANCD2    | R.LQDEEAS*M#GASYSK.S                   | S126      | RSK2               | No                  |
| FANCD2    | R.SES*PSLTQER.A                        | S592      | P38/MAPK, CDK1, CDK2 | Yes\(^2\)           |
| FANCD2    | R.DLQGEEIKS*QNSQESTADES*EDDM#SSQASK.S | S1401, S1412 | ATR, DNA-PK, BARK1 | Yes\(^3, 4\)        |
| FANCD2    | K.SQNS*QESTADESEDDM#SSQASK.S           | S1404     | ATR, DNA-PK        | Yes\(^3\)           |
| FANCD2    | K.SQNS*QESTADES*EDDM#SSQASK.S         | S1404, S1412 | ATR, DNA-PK, BARK1 | Yes\(^3, 4\)        |
| FANCD2    | K.SQNSQES*TADES*EDDM#SSQASK.S         | S1407, S1412 | CKIa1, BARK1       | Yes\(^4\)           |
| FANCD2    | K.SKATEDGEEDEV*SAGEK.E                 | S1435     | CKII, CKI          | Yes\(^5\)           |
| FANCD2    | K.ATEDGEEDEV*SAGEK.E                   | S1435     | CKII, CKI          | Yes\(^5\)           |
| FANCD2    | R.SES*PSLTQER.A                        | S592      | P38/MAPK, CDK1, CDK2 | Yes\(^2\)           |
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5 Sacco F, et al. (2016) Glucose-regulated and drug-perturbed phosphoproteome reveals molecular mechanisms controlling insulin secretion. Nat Commun 7, 13250
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Figure 1. FANCD2 is highly phosphorylated under non-stressed conditions and during S-phase of the cell cycle

(A) HeLa, U2OS and COS-7 cells were incubated with or without 200 nM MMC for 24 h. Cells were harvested and lysed in lambda phosphatase lysis buffer, incubated in the absence or presence of lambda phosphatase, and the indicated proteins analyzed by immunoblotting. (B) HeLa cells were synchronized at the G1/S boundary by a double-thymidine block and then released into thymidine-free media. Cells were lysed in lambda phosphatase buffer, incubated in the presence or absence of lambda phosphatase, and lysates analyzed by immunoblotting. For cell cycle stage analysis, cells were fixed, stained with propidium iodide and analyzed by flow cytometry. Cell cycle analysis was performed using FlowJo V10.2 software. (C) FA-A (FANCA-/) and FANCA-complemented FA-A cells were synchronized at the G1/S boundary by a double-thymidine block and released into thymidine-free media. Cells were harvested, lysed in lambda phosphatase buffer, incubated in the presence or absence of lambda phosphatase, and analyzed by immunoblotting.
A

2x Thymidine Block (dT) Release

18 h

Wash

Wash

Wash

18 h

HeLa

| h post-release | AS | 0 | 2 | 0 | 2 | 0 | 2 |
|----------------|----|---|---|---|---|---|---|
| λ-Phos         | -  | + | - | - | + | - | + |
| FANCD2-Ub      |    |   |   |   |   |   |   |
| FANCD2         |    |   |   |   |   |   |   |
| FANCI-Ub       |    |   |   |   |   |   |   |
| FANCI          |    |   |   |   |   |   |   |

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Cell number

Propidium iodide

B

FANCD2-V5

FANCD2-V5

FANCD2-\(\alpha-V5\)

Input

IP:

FA-D2

(\textit{FANCD2}\textsuperscript{\textmd{+}})

\textmd{La52}

\textit{FANCD2-V5}

\textit{α-V5}

C

\textit{α-pS-CDK}

\textit{pRb FANCD2}

\textit{CDK2/Cyclin A}

\textit{FANCD2}

\textit{pRb}

\textit{FANCD2}

\textit{pRb}
Figure 2. FANCD2 is a CDK substrate

(A) HeLa cells were synchronized at the G1/S boundary by a double-thymidine block performed in the absence or presence of the CDK inhibitors Purvalanol A or SNS-032. Cells were lysed in lambda phosphatase buffer, incubated in the absence or presence of lambda phosphatase, and analyzed by immunoblotting. Cells were also fixed, stained with propidium iodide and analyzed by flow cytometry to determine cell cycle stage. (B) FA-D2 (FANCD2−/−) cells stably expressing LacZ-V5 or FANCD2-V5 were immunoprecipitated with anti-V5 agarose and immune complexes were immunoblotted with anti-FANCD2 and anti-pS-CDK antibodies. (C) Purified FANCD2 and pRb were incubated in the absence or presence of CDK2/Cyclin A plus ATP at 30°C for 30 min. Samples were immunoblotted with a pan anti-pS-CDK antibody or stained with SimplyBlue SafeStain.
**A**

- **U2OS**
  - **-**
  - **+**
- **3x FLAG**

*Input*:
- **α-FLAG**
- **α-FANCD2**

*IP: α-FLAG*:
- **α-FLAG**
- **α-FANCD2**

**B**

- **Input**
  - **IP: α-FLAG**
- **Mw**
  - **-**
  - **+**
  - **B**
  - **+**
  - **+**
  - **3x FLAG-FANCD2**

**200 kDa**
**115 kDa**

- **1**
- **2**
- **3**
- **4**
- **5**
- **6**
- **7**
- **8**
- **9**

**C**

- **Shake off**
- **Release**

- **NOCO**
  - **15 h**
  - **12 h**

**2N 4N**

**D**

- **α-FANCD2**
- **Rabbit IgG**

**Mw**
**1**
**2**
**3**
**4**
**5**
**6**
**7**
**8**
**9**
**10**

**E**

- **Input**
  - **α-FLAG**
  - **α-FANCD2**
  - **Rabbit IgG**

**FANCD2**

**1**
**2**
**3**
**4**
**5**
**6**
**7**

**F**

- **α-FANCD2**
- **Rabbit IgG**

**S8**
**S126**
**S592**
**S1401**
**S1404**
**S1407**
**S1412**
**S1435**
**S14XX**

**G**

- **α-FANCD2**
- **α-FANCD2 pS592**
- **α-H3 pS10**
- **α-Cdc2 pY15**
- **α-Tubulin**

**-/-**
**0h**
**12h**
**15h**
**18h**

**FANCD2-Ub**
**FANCD2**
**FANCD2 pS592**
**H3 pS10**
**Cdc2 pY15**
**Tubulin**
Figure 3. FANCD2 is phosphorylated on S592 during S-phase

(A-B) FANCD2 was immunoprecipitated from U2OS cells stably expressing 3xFLAG-FANCD2 under stringent conditions using anti-FLAG agarose. Immune complexes were analyzed by immunoblotting using anti-FLAG and anti-FANCD2 antibodies (A), and by staining with SimplyBlue SafeStain (B). Immunoprecipitated FANCD2 bands were combined and subjected to phosphoproteomic analysis using LC-MS/MS. (C) HeLa cells were synchronized in M-phase by nocodazole block. Mitotic cells were harvested or released into nocodazole-free medium for 12 h (S-phase) prior to harvesting. (D) FANCD2 was immunoprecipitated from M-phase and S-phase synchronized cells using an anti-FANCD2 antibody and visualized using silver staining (D) or by immunoblotting (E). (F) A schematic of the FANCD2 protein indicating phosphorylation sites identified by LC-MS/MS. (G) HeLa cells were synchronized in M-phase by a nocodazole block. HeLa FANC D2−/− cells generated by CRISPR-Cas9 gene editing (Liang et al. 2016) were used as negative control for FANCD2. Immunoblotting was performed with an anti-FANCD2-pS592 antibody, anti-H3 pS10, anti-Cdc2 pY15 and anti-tubulin.
Figure 4. Mutation of S592 disrupts FANCD2 monoubiquitination during S-phase and following exposure to DNA damaging agents

(A) Shown is a partial model of the FANCD2 protein illustrating the site of monoubiquitination K561 in red and S592 in blue. (B) FA-D2 (FANCD2<sup>−/−</sup>) patient cells stably expressing empty vector or V5-tagged FANCD2-WT, FANCD2-S592A, and FANCD2-S592D were incubated in the absence or presence of 200 nM MMC for 24 h, and whole-cell lysates were analyzed by immunoblotting. (C) FA-D2 cells stably expressing empty vector or V5-tagged FANCD2-WT, FANCD2-S592A, or FANCD2-S592D were synchronized at the G1/S boundary by double-thymidine block. Cells were released into thymidine-free medium and whole cell lysates were analyzed by immunoblotting at the indicated times post-release.
A

Cell Index

Time (h)

FA-D2 (FANCD2*)

Empty

FANCD2-WT

FANCD2-S592A

FANCD2-S592D

NT + APH + MMC

B

Buds (%)

Multinucleated (%)

Binucleated (%)

Micronuclei (%)

Empty WT K561R S592A S592D

Empty WT K561R S592A S592D

Empty WT K561R S592A S592D

Empty WT K561R S592A S592D

35
Figure 5. Mutation of FANCD2 S592 leads to decreased proliferation under conditions of replicative stress and increased mitotic defects

(A) FA-D2 cells stably expressing empty vector or V5-tagged FANCD2-WT, FANCD2-S592A, or FANCD2-S592D were incubated in the absence (NT) or presence of 0.4 μM aphidicolin (+APH) or 20 nM mitomycin C (+MMC). Cellular proliferation was monitored by measuring electrical impedance every 15 minutes over a 120 h period using the xCELLigence real time cell analysis system. (B) FA-D2 cells were incubated in the presence or absence of 0.4 APH and 40 nM MMC. Then cells were treated with Cytochalasin-B for cytokinesis block and stained with DAPI. Nucleoplasmic bridges, micronuclei, nuclear buds and multinucleated cells were scored. We tested whether frequency of mitotic defects differed across groups by using the base package R-function `t.test` to perform Student’s t-test. All statistical analyses were performed in R version 3.6.1 (R Core team 2015).

(A-D).
SUPPLEMENTAL MATERIALS

A

U2OS

\[ \begin{array}{c|cccccccccc}
\text{h post-release} & \text{AS} & 0 & 2 & 4 & 8 & 10 & 12 & 14 & 16 \\
\lambda\text{-Phos} & - & + & - & + & - & + & - & + & - \\
\hline
\text{FANCD2-Ub} & \text{G1/S} & & & & & & & & \\
\text{FANCD2} & \text{S} & & & & & & & & \\
\text{FANCI-Ub} & \text{G2/M} & & & & & & & & \\
\text{FANCI} & \text{G1} & & & & & & & & \\
\text{Cyclin A} & \text{G1/S} & & & & & & & & \\
\end{array} \]

B

U2OS

\[ \begin{array}{c|c}
\text{Cell number} & \text{Propidium iodide} \\
\text{AS} & \text{0 h} \\
\text{0 h} & \text{2 h} \\
\text{2 h} & \text{4 h} \\
\text{4 h} & \text{8 h} \\
\text{8 h} & \text{10 h} \\
\text{10 h} & \text{12 h} \\
\text{12 h} & \text{14 h} \\
\text{14 h} & \text{16 h} \\
\text{16 h} & \text{18 h} \\
\end{array} \]
C

HeLa

| h post-release | AS | 0 | 6 | 9 | 12 | 15 | 18 | 21 |
|----------------|----|---|---|---|----|----|----|----|
| λ-Phos         | -  | + | - | + | -  | +  | -  | +  |
| FANCD2-Ub      |    |   |   |   |    |    |    |    |
| FANCD2         |    |   |   |   |    |    |    |    |

Nocodazole arrest

| 0 h post-release | M | G1 | G1/S | S | G2/M |
|------------------|---|----|------|---|------|
| FANCI-Ub         |   |    |      |   |      |
| FANCI            |   |    |      |   |      |
| Cyclin A         |   |    |      |   |      |
| Tubulin          |   |    |      |   |      |

D

A8

Propidium iodide

Cell number

0 h

6 h

9 h

12 h

15 h

21 h
Figure S1. Phosphorylation of FANCD2 during S-phase of the cell cycle

(A) U2OS cells were synchronized at the G1/S boundary by a double-thymidine block and released into thymidine-free media. Cells were harvested, lysed in lambda phosphatase buffer and incubated in the presence or absence of lambda phosphatase. Samples were analyzed by immunoblotting. (B) Cells were fixed, stained with propidium iodide and analyzed by flow cytometry to determine cell cycle stage. (C) HeLa cells were synchronized in M-phase with a nocodazole block. Mitotic cells were physically detached by the shake-off method and released into nocodazole-free media. Cells were harvested, lysed in lambda phosphatase buffer and incubated in the presence or absence of lambda phosphatase. Samples were analyzed by immunoblotting. (D) Cells were fixed, stained with propidium iodide and analyzed by flow cytometry to determine cell cycle stage.
**Figure S2. FANCD2 is a CDK substrate**

(A-B) Shown is an alignment of mouse, human, and chicken FANCD2 (A) and FANCI (B) amino acid sequences using the T-Coffee server, with the secondary structure of mouse FANCD2 or FANCI illustrated. Putative CDK phosphorylation sites are shaded in yellow. (C) HeLa cells were incubated in the absence or presence of 10 μM olumucine, 10 μM olomucine II, 10 μM purvalanol A, 10 μM RO3306 and 20 μM roscovitine for 24 h. Whole cell lysates were incubated in the absence or presence of lambda phosphatase and analyzed by immunoblotting. (D) HeLa cells were treated with 10 μM purvalanol A for the indicated times, lysates were incubated in the absence or presence of lambda phosphatase, and analyzed by immunoblotting.
**A**

HeLa

|       | NT | WT | + MMC | NT | FANCD2<sup>−</sup>/− | + MMC |
|-------|----|----|-------|----|----------------------|-------|
|      DAPI |    | FANCD2 | H3 pS10 | Merge |          |         |

**B**

| HeLa | WT | FANCD2<sup>−</sup>/− |
|------|----|----------------------|
|      | -  | -                    |
|      | +  | +                    |
|      | -  | -                    |
|      | +  | +                    |

α-FANCD2

α-FANCI

α-H3 pS10

α-CHK1 pS345

α-Tubulin

1 2 3 4 5 6

**C**

| HeLa | WT | FANCD2<sup>−</sup>/− |
|------|----|----------------------|
|      | NT |                       |

%G1 63.6 46.9
%G2/M 11.6 26.0

%G1 24.7 3.97
%S 50.8 35.7
%G2/M 24.0 60.1

%G1 27.5 10.1
%S 59.7 59.3
%G2/M 11.2 28.0
### FA-D2

| Time after 2x dT release (h) | 0h | 3h | 6h | 9h | 12h | 15h | 18h | 21h |
|------------------------------|----|----|----|----|-----|-----|-----|-----|
| **(FANCD2) + Empty**        |    |    |    |    |     |     |     |     |
| %G1                          | 37.7 | 32.2 | 6.6 | 8.1 | 32.2 | 54.6 | 54.4 | 47.0 | 32.6 |
| %S                           | 35.5 | 56.8 | 81.9 | 27.7 | 10.9 | 15.2 | 19.3 | 33.6 | 46.7 |
| %G2/M                        | 20.2 | 7.6 | 9.1 | 63.4 | 56.5 | 27.5 | 20.1 | 14.4 | 14.8 |

### FA-D2 + FANCD2-WT

| Time after 2x dT release (h) | 0h | 3h | 6h | 9h | 12h | 15h | 18h | 21h |
|------------------------------|----|----|----|----|-----|-----|-----|-----|
| %G1                          | 37.8 | 23.0 | 12.2 | 9.3 | 39.2 | 52.9 | 49.2 | 39.1 | 30.9 |
| %S                           | 33.2 | 68.3 | 74.0 | 23.2 | 14.7 | 16.4 | 26.8 | 40.7 | 42.2 |
| %G2/M                        | 28.0 | 5.8 | 11.6 | 65.8 | 44.8 | 24.6 | 20.2 | 15.3 | 24.3 |

### FA-D2 + FANCD2-S592A

| Time after 2x dT release (h) | 0h | 3h | 6h | 9h | 12h | 15h | 18h | 21h |
|------------------------------|----|----|----|----|-----|-----|-----|-----|
| %G1                          | 42.6 | 43.6 | 15.2 | 7.2 | 31.9 | 60.5 | 52.8 | 42.9 | 30.2 |
| %S                           | 33.1 | 52.8 | 63.0 | 24.6 | 11.4 | 9.9 | 24.8 | 36.6 | 49.9 |
| %G2/M                        | 20.1 | 1.3 | 21.4 | 66.8 | 54.2 | 27.1 | 20.1 | 15.4 | 16.1 |

### FA-D2 + FANCD2-S592D

| Time after 2x dT release (h) | 0h | 3h | 6h | 9h | 12h | 15h | 18h | 21h |
|------------------------------|----|----|----|----|-----|-----|-----|-----|
| %G1                          | 34.1 | 23.4 | 2.4 | 7.2 | 32.5 | 58.2 | 56.5 | 44.0 | 36.9 |
| %S                           | 35.4 | 72.5 | 64.3 | 20.9 | 10.1 | 12.8 | 16.8 | 33.9 | 42.8 |
| %G2/M                        | 23.2 | 0.7 | 34.2 | 69.4 | 53.9 | 25.5 | 21.9 | 14.7 | 18.0 |
|          | Empty | FANCD2-WT | FANCD2-K561R | FANCD2-S592A | FANCD2-S592D |
|----------|-------|-----------|--------------|--------------|--------------|
| NT       |       |           |              |              |              |
| %G1      | 43.2  | 42.2      | 46.3         | 46.3         | 41.7         |
| %S       | 33.4  | 31.1      | 33.4         | 32.6         | 33.2         |
| %G2/M    | 18.5  | 23.7      | 18.1         | 20.0         | 22.8         |
| 20 nM MMC 24 h |       |           |              |              |              |
| %G1      | 22.3  | 27.7      | 23.4         | 26.7         | 27.6         |
| %S       | 35.8  | 35.9      | 31.9         | 35.5         | 35.2         |
| %G2/M    | 40.3  | 35.5      | 43.6         | 35.6         | 36.2         |
| 20 nM MMC 48 h |       |           |              |              |              |
| %G1      | 24.8  | 30.9      | 21.3         | 27.5         | 31.3         |
| %S       | 26.3  | 29.0      | 32.8         | 30.8         | 30.1         |
| %G2/M    | 47.0  | 38.1      | 44.1         | 38.7         | 36.7         |
| 40 nM MMC 24 h |       |           |              |              |              |
| %G1      | 19.1  | 22.6      | 18.6         | 24.4         | 22.2         |
| %S       | 34.8  | 39.9      | 33.5         | 33.4         | 35.5         |
| %G2/M    | 45.4  | 34.5      | 46.8         | 40.7         | 39.8         |
| 40 nM MMC 48 h |       |           |              |              |              |
| %G1      | 18.0  | 22.3      | 16.9         | 20.3         | 21.5         |
| %S       | 26.8  | 29.6      | 25.4         | 28.6         | 28.3         |
| %G2/M    | 50.7  | 45.5      | 55.3         | 46.7         | 47.6         |
Figure S3. Mutation on S592 affects cell cycle progression / Cells lacking FANCD2 exhibit increased mitotic arrest in unstressed conditions

(A) Wild-type HeLa and HeLa FANCD2−/− cells generated by CRISPR-Cas9 gene editing were incubated in the absence or presence of 100 nM MMC for 24 h. Cells were fixed and co-immunofluorescence microscopy was performed for FANCD2 (green) and H3 pS10 (red). (B) The same cells were treated with 100 nM MMC or 1 mM acetaldehyde for 24 h and whole-cell lysates analyzed by immunoblotting. (C) Cells were incubated in the absence or presence of 100 nM MMC or 0.2 μM APH for 24 h. Cells were fixed, stained with propidium iodide and analyzed by flow cytometry. Cell cycle stage analysis was performed using the FlowJo V10.2 software. (D) FA-D2 cells stably expressing empty vector or V5-tagged FANCD2-WT, FANCD2-S592A, or FANCD2-S592D were synchronized at the G1/S boundary by double-thymidine block, and then released into thymidine-free medium. At the indicated time points, cells were fixed, stained with propidium iodide and analyzed by flow cytometry. Cell cycle stage analysis was performed using the FlowJo V10.2 software. (E) FA-D2 cells stably expressing empty vector or V5-tagged FANCD2-WT, FANCD2-S592A, or FANCD2-S592D were treated with 20 nM and 40 nM MMC for 24 h and 48 h. Cells were fixed with methanol, stained with propidium iodide and analyzed by flow cytometry to determine cell cycle stage.
### Table A

|          | 30 h | 60 h | 90 h | 120 h |
|----------|------|------|------|-------|
| NT       |      |      |      |       |
| E-WT     | 0.038| 0.084| 0.066| 0.131 |
| E-SA     | 0.014| 0.015| 0.011| 0.015 |
| E-SD     | 0.913| 0.292| 0.061| 0.053 |
| WT-SA    | 0.448| 0.022| 0.002| 0.005 |
| WT-SD    | 0.064| 0.363| 0.811| 0.296 |
| SA-SD    | 0.249| 0.035| 0.070| 0.228 |

### Table B

|          | 30 h | 60 h | 90 h | 120 h |
|----------|------|------|------|-------|
| 0.4 µM APH |      |      |      |       |
| E-WT     | 0.359| 0.377| 0.088| 0.015 |
| E-SA     | 0.524| 0.969| 0.632| 0.273 |
| E-SD     | 0.976| 0.786| 0.490| 0.465 |
| WT-SA    | 0.116| 0.176| 0.029| 0.025 |
| WT-SD    | 0.045| 0.053| 0.034| 0.019 |
| SA-SD    | 0.389| 0.625| 0.724| 0.635 |

### Table C

|          | 30 h | 60 h | 90 h | 118 h |
|----------|------|------|------|-------|
| 20 nM MMC |      |      |      |       |
| E-WT     | 0.034| 0.072| 0.010| 0.0004|
| E-SA     | 0.021| 0.021| 0.001| 0.002 |
| E-SD     | 0.009| 0.173| 0.003| 0.006 |
| WT-SA    | 0.923| 0.935| 0.214| 0.096 |
| WT-SD    | 0.085| 0.177| 0.280| 0.052 |
| SA-SD    | 0.052| 0.096| 0.743| 0.463 |
B

C

|          | NT   | 30 h | 60 h | 90 h | 120 h | 140 h |
|----------|------|------|------|------|-------|-------|
| E-WT     | 0.645| 0.492| 0.852| 0.938| 0.505 |
| E-SA     | 0.265| 0.059| 0.027| 0.135| 0.152 |
| E-SD     | 0.859| 0.979| 0.871| 0.838| 0.907 |
| WT-SA    | 0.286| 0.111| 0.059| 0.114| 0.077 |
| WT-SD    | 0.884| 0.616| 0.768| 0.752| 0.360 |
| SA-SD    | 0.265| 0.048| 0.062| 0.076| 0.150 |

|          | 2 µM RO3306 | 30 h | 60 h | 90 h | 120 h | 140 h |
|----------|--------------|------|------|------|-------|-------|
| E-WT     | 0.340        | 0.132| 0.125| 0.088| 0.033 |
| E-SA     | 0.006        | 0.026| 0.016| 0.020| 0.007 |
| E-SD     | 0.889        | 0.604| 0.381| 0.890| 0.542 |
| WT-SA    | 0.419        | 0.431| 0.256| 0.316| 0.297 |
| WT-SD    | 0.402        | 0.092| 0.075| 0.107| 0.091 |
| SA-SD    | 0.068        | 0.042| 0.044| 0.048| 0.031 |
Figure S4. Mutation of FANCD2 S592 leads to decreased proliferation under conditions of replicative stress

(A) FA-D2 cells stably expressing empty vector or V5-tagged FANCD2-WT, FANCD2-S592A, or FANCD2-S592D were incubated in the absence (NT) or presence of 0.4 μM aphidicolin (+APH) or 20 nM mitomycin C (+MMC). Cellular proliferation was monitored by measuring electrical impedance every 15 minutes over a 120 h period using the xCELLigence real time cell analysis system. Student’s t-test was used to compare the means of electrical impedance measurements between populations at 30 h, 60 h, 90 h and 120 h. (B) The same cells were incubated in the absence or presence of 2 μM RO3306 and cellular proliferation was monitored by measuring electrical impedance every 15 minutes over a 140 h period using the xCELLigence real time cell analysis system. (C) Student’s t-test was used to compare electrical impedance measurements between populations at 30 h, 60 h, 90 h, 120 h, and 140 h.
| T-test | NT + CytB | MMC + CytB | APH + CytB |
|--------|-----------|------------|------------|
| Empty-WT | 0.067     | 0.182      | 0.008      |
| Empty-K561R | 0.933     | 0.0003     | 0.001      |
| Empty-S592A | 0.126     | 0.041      | 0.008      |
| Empty-S592D | 0.413     | 0.148      | 0.053      |
| WT-K561R | 0.090     | 0.035      | 0.589      |
| WT-S592A | 0.001     | 0.517      | 0.942      |
| WT-S592D | 0.009     | 0.989      | 0.449      |
| K561R-S592A | 0.115     | 0.140      | 0.510      |
| K561R-S592D | 0.377     | 0.016      | 0.182      |
| S592A-S592D | 0.456     | 0.466      | 0.473      |
### Table C

| T-test          | NT + CytB | MMC + CytB | APH + CytB |
|-----------------|-----------|------------|------------|
| Empty-WT        | 0.526     | 0.412      | 0.060      |
| Empty-K561R     | 0.244     | 0.633      | 0.370      |
| Empty-S592A     | 0.305     | 0.003      | 0.004      |
| Empty-S592D     | 0.047     | 0.088      | 0.091      |
| WT-K561R        | 0.543     | 0.246      | 0.301      |
| WT-S592A        | 0.632     | 0.051      | 0.187      |
| WT-S592D        | 0.140     | 0.465      | 0.890      |
| K561R-S592A     | 0.920     | 0.003      | 0.030      |
| K561R-S592D     | 0.439     | 0.056      | 0.393      |
| S592A-S592D     | 0.396     | 0.157      | 0.171      |

| T-test          | NT-MMC    | NT-APH     |
|-----------------|-----------|------------|
| Empty           | 0.237     | 0.021      |
| WT              | 0.364     | 0.231      |
| K561R           | 0.014     | 0.011      |
| S592A           | 0.516     | 0.883      |
| S592D           | 0.096     | 0.013      |
| T-test  | NT + CytB | MMC + CytB | APH + CytB |
|---------|-----------|------------|------------|
| Empty-WT| 0.011     | 0.040      | 0.945      |
| Empty-K561R| 0.333   | 0.040      | 0.627      |
| Empty-S592A| 0.456   | 0.899      | 0.200      |
| Empty-S592D| 0.164   | 0.219      | 0.119      |
| WT-K561R| 0.099     | 0.929      | 0.638      |
| WT-S592A| 0.163     | 0.058      | 0.304      |
| WT-S592D| 0.244     | 0.002      | 0.213      |
| K561R-S592A| 0.960   | 0.579      | 0.089      |
| K561R-S592D| 0.644   | 0.002      | 0.046      |
| S592A-S592D| 0.669   | 0.183      | 0.846      |
| T-test                  | NT + CytB | MMC + CytB | APH + CytB |
|------------------------|-----------|------------|------------|
| Empty-WT               | 0.00107   | 0.0755     | 0.2683     |
| Empty-K561R            | 0.2759    | 0.642      | 0.5814     |
| Empty-S592A            | 0.09614   | 0.8163     | 0.2553     |
| Empty-S592D            | 0.07672   | 0.675      | 0.2796     |
| WT-K561R               | 0.01768   | 0.02605    | 0.4518     |
| WT-S592A               | 0.08317   | 0.06528    | 0.9826     |
| WT-S592D               | 0.202     | 0.2437     | 0.9641     |
| K561R-S592A            | 0.5319    | 0.8483     | 0.4354     |
| K561R-S592D            | 0.4116    | 0.4066     | 0.473      |
| S592A-S592D            | 0.7997    | 0.5498     | 0.9799     |
**Figure S5. Mutation in S592 causes increased mitotic aberrations**

(A) FA-D2 cells were incubated in the presence or absence of 0.4 aphidicolin (APH) and 40 nM mitomycin C (MMC). Then cells were treated with cytochalasin-B for cytokinesis block and stained with DAPI (B). Nucleoplasmic bridges were scored in 300 binucleated cells. (C) Micronuclei were scored in at least 300 binucleated cells. (D) Nuclear buds were scored in at least 400 cells. (E) Cells with more than 2 nuclei were scored. We tested whether frequency of mitotic defects differed across groups by using the base package R-function `t.test` to perform Student’s t-test. All statistical analyses were performed in R version 3.6.1 (R Core team 2015).
### A

| T-test     | NT  |
|------------|-----|
| Empty-WT   | <0.001 |
| Empty-K561R | 0.002 |
| Empty-S592A | 0.045 |
| Empty-S592D | 0.019 |
| WT-K561R   | <0.001 |
| WT-S592A   | <0.001 |
| WT-S592D   | <0.001 |
| K561R-S592A | 0.172 |
| K561R-S592D | 0.314 |
| S592A-S592D | 0.700 |

### B

| T-test     | NT  |
|------------|-----|
| Empty-WT   | 0.016 |
| Empty-K561R | 0.665 |
| Empty-S592A | 0.413 |
| Empty-S592D | 0.488 |
| WT-K561R   | 0.007 |
| WT-S592A   | 0.030 |
| WT-S592D   | <0.001 |
| K561R-S592A | 0.005 |
| K561R-S592D | 0.119 |
| S592A-S592D | 0.039 |

### C

| T-test     | NT  |
|------------|-----|
| Empty-WT   | <0.001 |
| Empty-K561R | 0.034 |
| Empty-S592A | 0.001 |
| Empty-S592D | 0.033 |
| WT-K561R   | <0.001 |
| WT-S592A   | 0.005 |
| WT-S592D   | <0.001 |
| K561R-S592A | 0.057 |
| K561R-S592D | 0.971 |
| S592A-S592D | 0.066 |

### D

| T-test     | NT  |
|------------|-----|
| Empty-WT   | 0.053 |
| Empty-K561R | 0.100 |
| Empty-S592A | 0.841 |
| Empty-S592D | 0.631 |
| WT-K561R   | <0.001 |
| WT-S592A   | 0.005 |
| WT-S592D   | 0.001 |
| K561R-S592A | 0.080 |
| K561R-S592D | 0.140 |
| S592A-S592D | 0.727 |
Figure S6. Mutation in S592 causes increased mitotic aberrations during non-stressed conditions.

FA-D2 cells were incubated without the presence of DNA damaging agents. Then cells were fixed in ice cold methanol and stained with DAPI. Binucleated, micronuclei, nuclear buds and multinucleated cells were scored. We tested whether frequency of mitotic defects (A-Binucleated, B- Nuclear Buds, C- Micronuclei, D- Multinucleated) differed across groups by using the base package R-function *t.test* to perform Student’s t-test. All statistical analyses were performed in R version 3.6.1 (R Core team 2015).
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