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Further Insights Into the Regulation of the Fanconi Anemia FANCD2 Protein

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FURTHER INSIGHTS INTO THE REGULATION OF THE FANCONI ANEMIA FANCD2 PROTEIN

BY

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ABSTRACT

Fanconi anemia (FA) is a rare autosomal and X-linked recessive disorder, characterized by congenital abnormalities, pediatric bone marrow failure and cancer susceptibility. FA is caused by biallelic mutations in any one of 16 genes. The FA proteins function cooperatively in the FA-BRCA pathway to repair DNA interstrand crosslinks (ICLs). The monoubiquitination of FANCD2 and FANCI is a central step in the activation of the FA-BRCA pathway and is required for targeting these proteins to chromatin. Despite their critical role in ICL repair, very little is known about the structure, function, and regulation of the FANCD2 and FANCI proteins, or how they are targeted to the nucleus and chromatin. The goal of this dissertation is to study the mechanisms and regulation of FANCD2. Through this research, we have uncovered a nuclear localization signal (NLS) in FANCD2. Mutation of this NLS region impairs FANCD2 and FANCI monoubiquitination and inhibits the recruitment of FANCD2 and FANCI to chromatin. In addition, we have identified a putative CDK phosphorylation site cluster in FANCD2. Taken together, we believe these findings have enhanced our understanding of this important DNA repair protein and will allow for further investigation of this rare cancer susceptibility syndrome.

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PREFACE

The following dissertation has been prepared in manuscript format. Chapter 1, "The Fanconi Anemia ID2 Complex: Dueling Saxes at the Crossroads", was published in *Cell Cycle* and is a review encompassing our current understanding of the regulation and function of the FANCD2 and FANCI proteins. Chapter 2, "Coordinate Nuclear Targeting of the FANCD2 and FANCI Proteins *via* a FANCD2 Nuclear Localization Signal" was published in *PLoS One*. Chapter 3, "Characterization of a Putative CDK Phosphorylation Site Cluster in the FANCD2 Protein" was prepared for submission to *PLoS Genetics*.

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The Fanconi Anemia ID2 Complex: Dueling Saxes at the Crossroads

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The Fanconi Anemia ID2 Complex: Dueling Saxes at the Crossroads

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Keywords

Fanconi anemia, DNA repair, ubiquitin, FANCD2, FANCI, DNA interstrand crosslink repair

***F*anconi *a*nemia (FA) is a rare recessive genetic disease characterized by congenital abnormalities, bone marrow failure and heightened cancer susceptibility in early adulthood. FA is caused by biallelic germ-line mutation of any one of 16 genes. While several functions for the FA proteins have been ascribed, the prevailing hypothesis is that the FA proteins function cooperatively in the FA-BRCA pathway to repair damaged DNA. A pivotal step in the activation of the FA-BRCA pathway is the monoubiquitination of the FANCD2 and FANCI proteins. Despite their importance for DNA repair, the domain structure, regulation, and function of FANCD2 and FANCI remain poorly understood. In this review, we provide an overview of our current understanding of FANCD2 and FANCI, with an emphasis on their posttranslational modification and common and unique functions.**

The Discovery of FANCD2 and FANCI

FANCD2

The *FANCD2* gene was identified in 2001 by the Grompe, D'Andrea, and Moses Laboratories using a positional gene cloning approach, following many years of collaborative efforts.[1-4] The PD20 FA patient line was originally assigned to the FA-D complementation group because of its inability to complement the *m*ito*m*ycin *C* (MMC) hypersensitivity of the FA-D reference line HSC62.[4-6] Using a microcell-mediated chromosome transfer approach, the *FANCD* gene was initially mapped to 3p22-26.[4] Hejna and colleagues subsequently narrowed the critical region to ~200 kb, a region which harbored three candidate genes: *TIGR-A004X28*, *SGC34603*, and

AA609512.[2] Timmers and colleagues then used *r*apid *a*mplification of *c*DNA *e*nds (RACE) to obtain the respective full-length cDNAs and sequenced these three genes in the PD20 cells.[1] No sequence changes were uncovered in *A004X28* and *AA609512*. However, five sequence changes were found in *SGC34603*; three of which represented common polymorphisms and two represented *bona fide* mutations. One sequence alteration was a maternally inherited A to G transition at nucleotide 376, which resulted in a S to G missense change. This transition was also associated with missplicing and insertion of 13 nt from intron 5 into the mRNA, causing a frameshift and production of a severely truncated protein. A second paternally inherited sequence change in *SGC34603* resulted in an R to H missense and hypomorphic change at position 1236 (R1236H),[1] strongly suggesting that *SGC34603* was indeed the *FANCD* gene. Confirming this, transduction of PD20 cells with full-length wild-type *FANCD* rescued the MMC hypersensitivity of these cells.[1] No sequence changes in *SGC34603* however were uncovered in HSC62. These findings led to the delineation of two FA-D complementation groups, FA-D1 and FA-D2, with biallelic mutations in *SGC34603/FANCD2* causative for the FA-D2 complementation group.[1] Early biochemical studies of the FANCD2 protein uncovered two isoforms: an unmodified form and a higher molecular mass isoform posttranslationally modified through the covalent attachment of a single ubiquitin polypeptide, referred to as monoubiquitination.[3]

FANCI

The existence of the FA-I complementation group was first established by Levitus and

colleagues, on the basis of somatic cell hybrid complementation analyses,[7] and the *FANCI* gene was subsequently identified by three groups in 2007.[8-10] Using a two-step genome-wide linkage approach with four genetically informative families, Dorsman and colleagues identified four candidate regions encompassing 39.4 Mb and comprising 351 genes. The sequencing of several candidate DNA repair genes failed to reveal sequence alterations. However, two strong alternative candidate genes - *KIAA1794/NP_060663* and *C15orf42/NP_689472* - were interrogated due to their resemblance to known FA genes in their extent of evolutionary conservation and the presence of nuclear localization signals, as many FA proteins, including FANCD2, were known to be nuclear.[3, 11-14] Sequencing of the *KIAA1794* gene in all eight FA-I patients uncovered pathogenic mutations.[8]

Employing a phosphoproteomics screen to identify substrates of the DNA damage response kinases ATM (*a*taxia-*t*elangiectasia *m*utated) and ATR (ATM and *R*ad3-related), the Elledge group identified KIAA1794 as an ionizing radiation-inducible phosphoprotein.[15] An amino acid sequence alignment of KIAA1794/FANCI and FANCD2 uncovered a modest overall 13% identity and 20% primary sequence similarity. However, a higher degree of sequence conservation was revealed in the region encompassing KIAA1794/FANCI K523 and that flanking FANCD2 K561, the site of FANCD2 monoubiquitination.[3, 10] Subsequent experiments established that, like FANCD2, FANCI is monoubiquitinated following treatment of cells with DNA damaging agents and during S phase of the cell cycle.[10] While full-length KIAA1794/FANCI protein was detected by immunoblotting in BD0952, an Epstein-Barr virus-transformed lymphocyte line from an FA patient of unassigned

complementation group, monoubiquitinated FANCI was not detected in these cells. DNA sequencing revealed homozygosity for two base substitutions in *KIAA1794/FANCI* in BD0952 cells. These included a C to T transition leading to a P55L amino acid change, and a G to A transversion leading to a R1285Q change, the latter representing the pathogenic mutation.[10]

In an alternative strategy, Sims and colleagues performed a BLAST search using the conserved amino acid sequence flanking the site of FANCD2 monoubiquitination K561, LVIRK, to identify proteins with similar monoubiquitination 'consensus' sequences.[9] A highly similar sequence - LVLRK - was uncovered in the uncharacterized protein KIAA1794, which, upon further examination, displayed approximately 40% sequence similarity within the regions surrounding KIAA1794 K523 and FANCD2 K561.[9] KIAA1794/FANCI was shown to undergo DNA damage-inducible monoubiquitination, and, importantly, this monoubiquitination was dependent on both the presence and monoubiquitination of FANCD2.[9] Sims and colleagues subsequently sequenced all of the exons and intron-exon boundaries of the *FANCI* gene for several FA patients of unassigned complementation group with defective FANCD2 monoubiquitination. Biallelic mutations in *FANCI* were uncovered in four patients. Patient EUFA592 expressed an amino-terminal truncated FANCI as a result of a homozygous T to A transition in exon 2 leading to the creation of a cryptic start site at M94.[9] Patient F010191 was compound heterozygous for *FANCI* mutations, with one allele encoding a R614X variant and the other encoding FANCI R1299X. Only the latter variant was detectable by immunoblotting. F010191 cells were defective for both FANCI and FANCD2

monoubiquitination indicating an important role for the extreme carboxy-terminus of FANCI in the regulation of this posttranslational modification. Importantly, all FA-I patients examined expressed some FANCI protein suggesting that *FANCI* null mutations may lead to embryonic lethality.[9]

FA-D2 and FA-I Clinical Aspects

FA is a clinically heterogeneous disease characterized by a wide spectrum of congenital abnormalities, a predisposition to develop early-onset progressive *b*one *m*arrow *f*ailure (BMF), and an increased risk of developing solid tumors in early adulthood. The carrier frequency of FA was previously estimated at 1:300 with an expected incidence rate of approximately 1:360,000.[16] However, a more recent report cites a higher carrier frequency of 1:156 - 1:209, leading to an expected incidence rate of 1:130,000.[17] A much higher carrier frequency of approximately 1:100 is found in certain populations, including Spanish Romani, Ashkenazi Jews, and Afrikaaners; this increase being attributed to the founder effect.[17]

It had previously been estimated that the FA-D2 complementation group accounts for ~1-3% of all FA patients.[18, 19] However, more recently it has been reported that this complementation group accounts for approximately 6% of all FA patients.[20] Patients belonging to the FA-D2 complementation group display the common FA congenital malformations including skin hypo- or hyper-pigmentation, short stature, and microcephaly (**Table 1**).[20-22] Importantly, these physical abnormalities, along with skeletal irregularities such as radial ray deformities, often allow for early clinical diagnosis. One notable difference between the FA-D2

Table 1. Summary of clinical data available for FA-D2 patients													
Patient	Micro-cephaly	Growth retardation	Skin pigmentation	Radial-ray defects	Micro-phthalmia	Renal anomalies	Ear Malformations	Brain anomalies	Genital anomalies	Hip dysplasia	Heart anomalies	Gastrointestinal malformations	BMF onset (yr)
EGF096 **	NI	NI	+	-	NI	-	-	NI	-	NI	-	-	NI
1	+	+	+	+	-	+	+	+	-	+	+	-	2.3
2	+	+	+	+	+	+	-	-	-	+	-	-	7.6
3	+	+	+	+	-	-	-	-	+	-	-	-	11.0
4	+	+	+	+	+	-	-	+	-	-	-	-	2.5
5	+	+	-	-	+	-	-	-	-	-	-	-	3.25
6	+	+	-	+	+	+	+	+	+	+	-	-	2.75
7	+	+	+	+	+	-	+	-	-	-	-	-	4.5
8	+	-	+	+	-	-	+	-	-	-	-	-	4.0
9	+	+	-	+	-	+	-	+	+	-	+	+	2.8
10	+	+	+	+	+	+	+	+	-	+	-	-	2.0
11	+	+	+	+	-	+	-	-	-	-	-	-	10.8
12	+	+	-	+	+	-	+	+	+	-	-	+	0.04
13	+	+	+	-	+	-	-	-	-	-	-	-	1.4
14	+	+	-	+	-	-	-	-	-	-	-	-	-
15	+	+	-	+	-	-	-	-	-	+	-	-	-
16	-	-	+	+	-	-	-	-	+	-	+	-	7.0
17	-	-	+	+	+	-	-	-	+	-	-	-	-
18	+	+	+	-	+	-	-	-	-	-	-	+	5.25
19 *	NA	+	NA	+	NA	NA	NA	+	NA	NA	NA	NA	NA
20	+	+	+	-	+	-	-	-	-	-	-	-	2.0
21	+	+	+	+	+	+	+	-	+	-	-	+	-
22	-	-	+	+	-	-	+	-	-	-	+	-	1.3
23	+	+	-	+	+	+	-	-	-	-	-	-	-
24	+	+	+	-	+	-	-	-	-	-	-	-	4.5
25	+	+	+	-	+	+	-	-	-	-	-	-	4.0
26	+	+	+	+	+	+	+	+	-	+	-	-	5.0
27	+	+	+	+	-	-	-	+	-	-	-	-	2.1
28	+	+	+	-	-	-	-	-	-	-	-	-	8.4
29	+	+	+	-	+	-	-	-	-	-	-	-	8.7

* – Terminated with diagnosis
+ – Exhibits congenital abnormalitiy
- – Does not display phenotype
NI – No information
NA – Not applicable
All patient data is from Kalb *et al*, 2007 unless otherwise noted.[xv]

complementation group and others is the penetrance of congenital abnormalities: while it is estimated that ~30% of individuals with FA do not display any congenital abnormality, every FA-D2 patient in one study exhibited a congenital defect.[20] Distinct phenotypes have also been observed in *Fancd2* knockout mice that are not observed in *Fanca, Fancc, or Fancg* mutant mice. [23] Houghtaling and colleagues discovered that *Fancd2*-/- mice have a high prevalence of micropthalmia, an increased incidence of epithelial tumors, severe hypogonadism, as well as perinatal lethality.[23] In addition to the congenital abnormalities, an earlier mean age of onset of BMF is observed for FA-D2 patients, compared to patients from all other FA complementation groups - 4.5 y compared to 8.0 y.[20, 24] Similar to that mentioned above for FA-I patients, residual FANCD2 protein expression is detected in the vast majority of FA-D2 patient cells, suggesting that only hypomorphic *FANCD2* mutations are compatible with viability. The majority of *FANCD2* mutations detected among FA-D2 patients are homozygous missense or compound heterozygous for a missense mutation and nucleotide change affecting splicing, often leading to monoallelic expression of low levels of both non- and monoubiquitinated FANCD2.[1, 20]

To date, only 12 patients from the FA-I complementation group have been described, compared to 30 FA-D2 patients. Much less is known about FA-I patients' clinical manifestations, and this complementation group only accounts for an estimated less than 1% of all FA cases.[18] Of the few FA-I patients with available clinical data, all have growth retardation (**Table 2**).[8, 9] In addition, approximately 50% of FA-I patients exhibit radial ray defects as well as both kidney and heart anomalies. Furthermore, the age of onset of hematological manifestations among FA-I patients

Table 2. Summary of clinical data available for FA-I patients

Patient	Micro-cephaly	Growth retardation	Skin pigmentation	Radial-ray defects	Micro-phthalmia	Renal anomalies	Ear Malformations	Brain anomalies	Genital anomalies	Hip dysplasia	Heart anomalies	Gastrointestinal malformations	BMF onset (yr)
F010191[9]	NI	+	NI	+	+	NI	+	NI	NI	NI	NI	NI	NI
F010095[9]	NI	+	NI	+	+	+	+	NI	NI	NI	NI	NI	6.0
IFAR663[9]	NI	+	NI	+	+	+	+	+	NI	NI	+	NI	NI
EUFA592[7,8,9]	NI	+	NI	+	NI	+	NI	NI	NI	NI	+	NI	2.5
BD952[7,8,10]	NI	+	NI	-	NI	-	NI	NI	NI	NI	-	NI	7.3
1428[8]	NI	+	NI	-	NI	-	NI	NI	NI	NI	-	NI	7.3
EUFA816[7,8]	NI	+	NI	-	NI	-	NI	NI	NI	NI	-	NI	6.0
EUFA480[8]	NI	+	NI	+	NI	-	NI	NI	NI	NI	-	NI	4.8
EUFA961[7,8]	NI	+	NI	+	NI	+	NI	NI	NI	NI	+	NI	8.0
EUFA1399[8]	NI	+	NI	-	NI	+	NI	NI	NI	NI	+	NI	1.0
Patient 6[20]	-	-	-	-	-	-	-	-	-	-	-	-	-
EUFA695[8]	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI

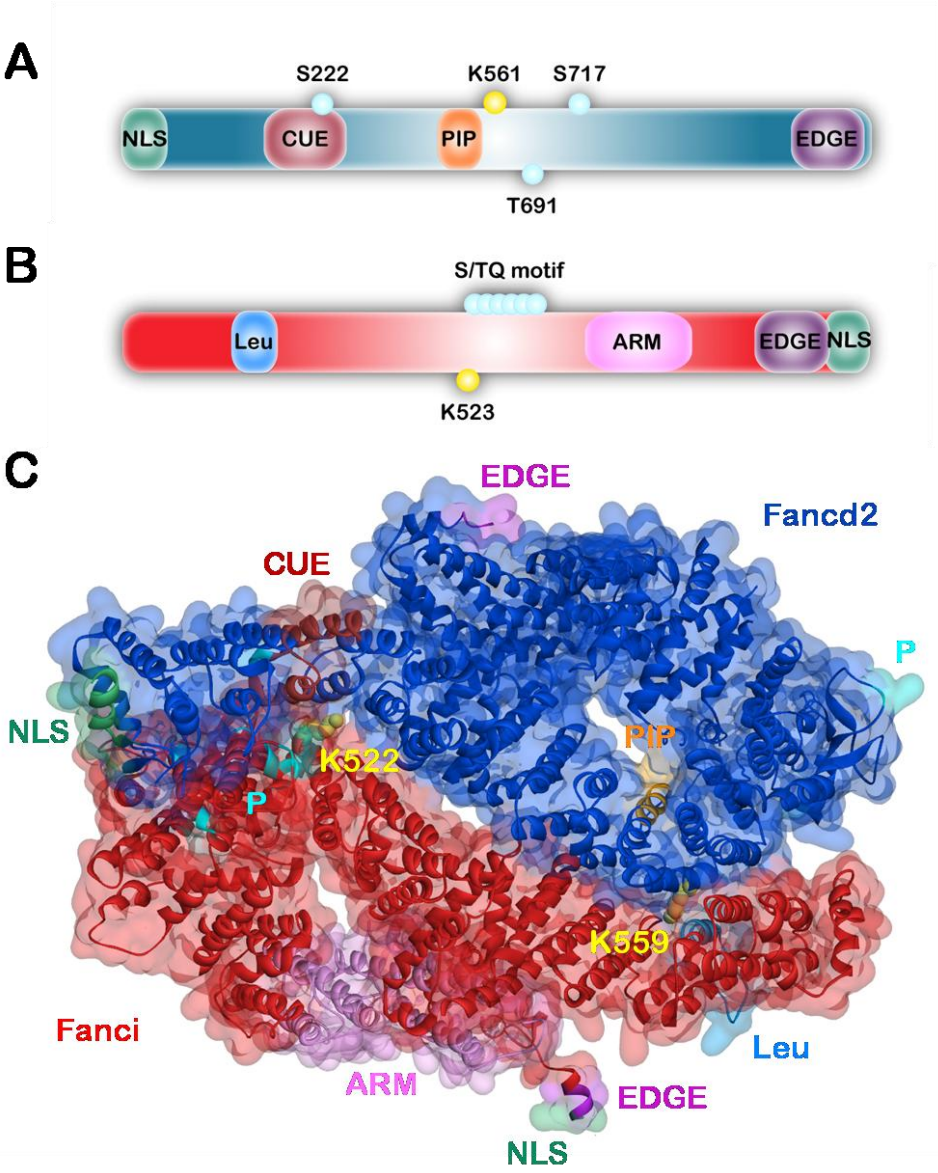
+ – Exhibits congenital abnormalitiy
- – Does not display phenotype
NI – No information

varies widely, ranging from 1 - 8 y, and the average age of onset of BMF for the small number of FA-I patients documented is 5.4 y.[8] Of the limited FA-I population size examined to date, to our knowledge no unique clinical manifestations have been observed.

Domain Architecture and Structure of FANCD2 and FANCI

FANCD2 is a 1,451 amino acid protein with a molecular mass of 164 kDa. When originally identified, FANCD2 was described as an orphan protein, as no recognizable domains or enzymatic motifs were evident.[1] To date, only four FANCD2 domains/motifs have been experimentally characterized (**Figure 1A)**. The carboxy-terminal EDGE motif, named for the single letter amino acid code, is not required for FANCD2 monoubiquitination or nuclear foci formation. However, this motif is necessary for complementation of the ICL-sensitivity of FA-D2 patient cells.[25] An interaction between FANCD2 and the major DNA processivity factor PCNA has also been described.[26] FANCD2 contains a highly conserved PIP-motif (*P*CNA-*i*nteracting *p*rotein motif) which is necessary for the association of FANCD2 with PCNA (*p*roliferating *c*ell *n*uclear *a*ntigen), and for efficient FANCD2 monoubiquitination, nuclear foci formation and ICL repair, suggesting that PCNA may function as a molecular scaffold to facilitate FANCD2 function.[26] FANCD2 also harbors an amino-terminal CUE (*c*oupling of *u*biquitin conjugation to *e*ndoplasmic reticulum degradation) *u*biquitin-*b*inding *d*omain (UBD).[27] The CUE domain adopts a triple α-helical configuration and mediates noncovalent ubiquitin binding. The CUE domain is important for interaction with FANCI, for the retention of monoubiquitinated

Figure 1. Domain architecture and structure of FANCD2 and FANCI. (**A**) Schematic of the FANCD2 protein indicating the amino-terminal NLS (*n*uclear *l*ocalization *s*ignal) domain (green), CUE (*c*oupling of *u*biquitin conjugation to *e*ndoplasmic reticulum degradation) domain (maroon), PIP-box (*P*CNA-*i*nteracting *p*rotein motif) (orange), and the carboxy-terminal EDGE motif (purple). Functionally-characterized phosphorylation sites (teal) and K561 monoubiquitination site (yellow) are indicated by small circles. (**B**) Schematic of the FANCI protein indicating the Leu (*leu*cine zipper) domain (light blue), ARM (*arm*adillo repeat) domain (pink), and carboxy-terminal EDGE motif (purple) and NLS domain (green). The S/TQ motif (teal) and K523 site of monoubiquitination (yellow) are indicated by small circles. (**C**) Mouse Fanci-Fancd2 heterodimer crystal structure represented as both surface and ribbons with domains indicated. This structure was solved by the Pavletich group in 2011(PDB ID: 3S4W).[35]


A
S222
K561
S717
NLS
CUE
PIP
EDGE
T691
B
S/TQ motif
Leu
ARM
EDGE
NLS
K523
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EDGE
Fancd2
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NLS
K522
PIP
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K559
Fanci
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FANCD2 and FANCI in chromatin, and for efficient ICL repair.[27] In addition, the amino terminal 58 amino acids of FANCD2 harbors a *n*uclear *l*ocalization *s*ignal (NLS).[28] This sequence is required for the efficient nuclear localization of both FANCD2 and FANCI, and for their monoubiquitination and function in ICL repair.[28]

FANCI is a 1,328 amino acid protein with a molecular mass of 149 kDa (**Figure 1B**). FANCI has an *arm*adillo (ARM) repeat domain, which form superhelical folds involved in mediating protein-protein interactions.[10, 29] FANCI also possesses an amino-terminal leucine zipper domain, a domain capable of mediating protein-protein or DNA-protein interactions.[30, 31] The FANCI leucine zipper domain does not bind directly to DNA, but does contribute to FANCI protein stability.[31] The conserved carboxy-terminus of FANCI includes a putative EDGE motif and NLS, which have been shown to enhance DNA binding and to promote the nuclear localization of FANCI, respectively.[31, 32] FANCI also possesses numerous S/TQ motifs, known ATM/ATR phosphorylation consensus sites, and mass spectrometry revealed that at least five of these sites are phosphorylated in human and mouse FANCI. [15, 33, 34] The alignment of human and chicken FANCI sequences revealed that eight S/TQ motifs are highly conserved, with six of these motifs clustering proximal to K523, the site of FANCI monoubiquitination.[9, 10, 33]

Importantly, the 3.4Å crystal structure of the murine Fancd2-Fanci heterodimer (ID2) was recently solved by the Pavletich laboratory. While this structure continues to be a major resource for FA researchers, it is important to recognize that structural information remains lacking for several peptide stretches of both proteins.[35] Fancd2 and Fanci are structurally highly similar and consist largely of antiparallel α-helices

that form αα-superhelical structures referred to as solenoids. Fancd2 and Fanci fold into back-to-back, inverted saxophone-like structures with trough-like partially open ends (**Figure 1C**). Multiple positively charged grooves within these troughs are capable of accommodating single and double-stranded DNA. The sites of monoubiquitination are located in the ID2 interface within solvent accessible tunnels. However, these tunnels are predicted to be too small to accommodate the ubiquitin ligase machinery, raising challenging questions about the temporal, kinetic, and spatial aspects of FANCD2 and FANCI monoubiquitination.[35]

FANCD2 and FANCI Monoubiquitination

Protein ubiquitination is a multi-step post-translational modification that results in the covalent attachment of a single ubiquitin molecule (*monoubiquitination*) or chain of linked ubiquitin molecules (*polyubiquitination*) to a lysine residue on a substrate protein. Mono- and poly-ubiquitination signal a wide range of cellular instructions, the former being most typically associated with the targeting of proteins to specific sub-cellular localizations, while the canonical role of the latter is to target proteins for degradation by the proteasome.[36, 37] In the general process of ubiquitination, an E1 ubiquitin-activating enzyme activates ubiquitin by forming a thioester bond between a catalytic C of E1 and the carboxy-terminal G76 of ubiquitin. Ubiquitin is then transferred from the E1 to an active site C of an E2 ubiquitin-conjugating enzyme. Ubiquitin can be further transferred to the catalytic site of a HECT (*h*omologous to *E*6-AP *c*arboxy *t*erminus) domain-containing E3 ubiquitin ligase, and then subsequently transferred to a target K on the substrate. Alternatively, through the

action of a RING (*really interesting new gene*) domain-containing E3 ubiquitin ligase, ubiquitin is transferred directly from the E2 to the substrate. The human genome encodes two E1 ubiquitin-activating enzymes, ~40 E2 ubiquitin-conjugating enzymes, and >600 E3 ubiquitin ligases.[38]

As mentioned above, when the FANCD2 protein was first characterized by Garcia-Higuera and colleagues in 2001, two isoforms of FANCD2 were detected by immunoblotting; a short form approximately 164 kDa and a longer form approximately 173 kDa.[3] The latter isoform was determined to represent FANCD2 covalently linked to a single ubiquitin polypeptide on K561.[3] Similar to FANCD2, a slower migrating isoform of FANCI was also detected by immunoblotting, following treatment of cells with DNA damaging agents.[9] This slower migrating FANCI isoform was subsequently determined to represent FANCI monoubiquitinated on K523.[9, 10] The monoubiquitination of FANCD2 and FANCI is required for their assembly into discrete nuclear foci, where they co-localize with several proteins including the protein product of one of the major breast and ovarian cancer susceptibility genes *BRCA1*.[3, 9, 10] This seminal finding provided an important clue as to a potential function for FANCD2, FANCI, and the FA-BRCA pathway, as BRCA1 had previously been determined to play a key role in DNA repair by *homologous recombination* (HR).[39-41] HR is an error-free and conservative DNA repair pathway that utilizes an intact homologous template, such as a sister chromatid or homologous chromosome, to repair DNA damage.[42] The ability of FANCD2 and FANCI to promote error-free HR repair was assessed using the DR-GFP HR reporter assay system developed by the Jasin laboratory.[43, 44] This reporter assay comprises two

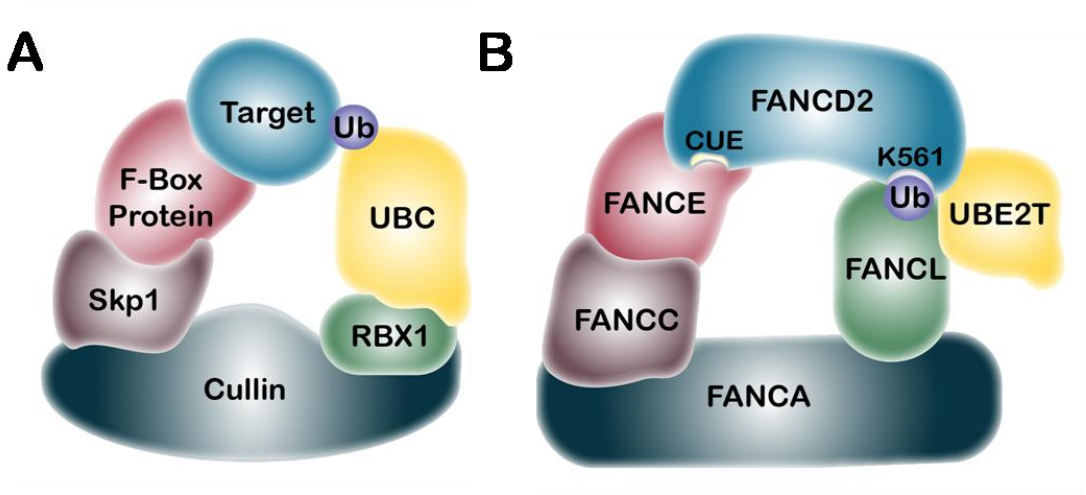
differentially mutated nonfunctional *GFP* genes oriented in tandem, and separated by a drug selection marker. One *GFP* gene is interrupted with the rare 18-bp I-*Sce* I restriction endonuclease recognition sequence, while the other is an 812-bp internal fragment. Following induction of a DSB with the I-*Sce* I endonuclease, a gene conversion HR event results in the expression of GFP, which can be assayed by flow cytometry or fluorescence microscopy.[43] Knockdown of either FANCD2 or FANCI, as well as mutation of FANCD2 K561 or FANCI K523, which precludes the covalent attachment of ubiquitin, leads to decreased HR efficiency.[9, 10, 44] Taken together, this data strongly suggests that monoubiquitinated FANCI and FANCD2 function cooperatively to promote error-free HR repair. Importantly, FANCD2 and FANCI monoubiquitination and nuclear foci formation are interdependent.[9, 10] For example, siRNA-mediated depletion of FANCI results in decreased FANCD2 monoubiquitination and nuclear foci formation, as well as a reduction in steady state levels of FANCD2.[9, 10] These findings were suggestive of a physical interaction between FANCD2 and FANCI, and indeed this was verified by co-immunoprecipitation studies as well as by co-crystallization of the murine ID2 heterodimer.[10, 35] Mutation of FANCD2 K561 renders this protein incompetent for nuclear foci formation, and abrogates its ability to rescue the ICL hypersensitivity of FA-D2 patient cells. Mutation of FANCI K523 also precludes its assembly into nuclear foci.[9, 10] However, somewhat surprisingly, in contrast to FANCD2 K561, mutation of FANCI K523 does not overtly affect its function in ICL repair.[3, 9, 10, 33] *In vitro* studies have also revealed that mutation of FANCD2 K561 leads to impaired

FANCI monoubiquitination, whereas mutation of FANCI K523 has little effect on the monoubiquitination of FANCD2.[45]

The E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase for FANCD2 and FANCI monoubiquitination are UBE2T and the RING domain-containing FANCL, respectively.[46, 47] Biallelic mutations in the *FANCL* gene underlie the FA-L complementation group, however, FA patient-derived mutations in *UBE2T* have yet to be uncovered.[46, 47] In addition to UBE2T and FANCL, the monoubiquitination of FANCD2 and FANCI requires the assembly of the FA core complex, comprising FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, and FANCM, as well as numerous accessory proteins including FAAP20, FAAP24, FAAP100, MHF1, and MHF2.[48-53] The precise roles of these ancillary proteins in FANCD2/FANCI monoubiquitination remain to be determined. Indeed, *in vitro* FANCD2/FANCI monoubiquitination reactions have been reconstituted with FANCL in the absence of these FA core complex components.[54-56] Recent studies have identified three functional modules within the FA core complex: the FANCB-FANCL-FAAP100 module, which provides the critical catalytic activity, and the FANCA-FANCG-FAAP20 and FANCC-FANCE-FANCF modules, which independently promote the recruitment of the core complex to chromatin.[57, 58] Accordingly, using isogenic single and double mutants of the HCT116 colorectal carcinoma cell line, Huang *et al.* established that FANCD2/I monoubiquitination is completely abrogated in the absence of FANCB, FANCL, or FAAP100, and only partially abrogated in the absence of FANCA, FANCG, FANCE, or FAAP20.[57] Furthermore, in contrast to FANCD2, the purified FA core complex fails to robustly promote the monoubiquitination of FANCI

in the absence or presence of DNA.[58] To gain further insight into the relationship between the FA core complex members and FANCD2/I monoubiquitination, it is useful to draw comparisons with other large multi-subunit ubiquitin ligase protein complexes, such as the *S*kp1/*C*ullin/*F*-box protein complex (SCF) (**Figure 2**).[59] SCF, along with the *a*naphase-*p*romoting *c*omplex or *c*yclosome (APC/C), plays an intricate and critical role in cell-cycle regulation.[60] The F-box protein of the SCF complex functions in substrate recognition and recruitment.[59] A good candidate for an FA-pathway protein analogous to the SCF F-box protein is FANCE, a FA core complex member that has been demonstrated to interact with FANCC, FANCF, and FANCD2, thereby bridging the core ubiquitin ligase machinery and the substrate.[61] The FANCE-FAND2 interaction, *via* FANCE F522, is critical for efficient FANCD2 monoubiquitination and ICL repair.[62] The Cullin protein functions as a molecular scaffold for the SCF complex, bridging the catalytic RING finger subunit RBX1 to the adaptor and F-box proteins. The FANCA protein, with a molecular mass of 163 kDa, may play a similar structural role in the FA-BRCA pathway. FANCA interacts with FANCC, FANCG, and FAAP20.[12, 57, 58, 63, 64] The Skp1 protein of the SCF complex functions as an adaptor between the Cullin protein and the F-box protein.[65] FANCC interacts with FANCA, FANCE, and FANCF and thus represents a strong candidate Skp1 analog.[57, 58, 61] FANCM is present in very low abundance in the native FA core complex, consistent with its role in replication fork remodeling and chromatin loading of the core complex.[58, 66-68] The core complex members FANCM, FANCG, and FANCF do not appear to have analogous SCF components. FANCL is analogous to the SCF E3 ubiquitin ligase RBX1. As mentioned above, FANCL, FANCB, and

Figure 2. Comparison of the SCF multi-subunit ubiquitin ligase protein complex and the FA core complex. (**A**) The Skp1/Cullin/F-box protein (SCF) complex includes the Cullin protein, which acts as a scaffold to bridge the catalytic E3 ubiquitin ligase RBX1 to the adaptor protein Skp1, and the F-box protein.[59] The F-box protein recognizes and recruits the target protein for ubiquitination by the E2 ubiquitin-conjugating enzyme, UBC. (**B**) We propose that the FANCA protein is structurally analogous to Cullin, and may link the E3 ubiquitin ligase FANCL with the putative adaptor protein FANCC. FANCC has been shown to interact with both FANCA and FANCE, indicating that it may function analogously to Skp1.[61] FANCE may be analogous to the F-box protein. FANCE is known to interact directly with FANCD2 and may facilitate its monoubiquitination of FANCL and UBE2T.[62]


A
Target
Ub
F-Box
Protein
UBC
Skp1
RBX1
Cullin
B
FANCD2
CUE
K561
Ub
UBE2T
FANCE
FANCL
FANCC
FANCA

FAAP100 comprise the E3 monoubiquitin ligase catalytic module.[57, 58] FANCL and RBX1 are both RING E3 ubiquitin ligases, with the RING domain facilitating recruitment of the E2 ubiquitin-conjugating enzyme, UBE2T in the case of the FA-BRCA pathway and UBC3, UBC4, or UBC5 for SCF.[46, 65, 69] FANCL binds to FANCD2 *via* its DRWD domain, and to UBE2T *via* its RING domain, to facilitate monoubiquitination.[69, 70]

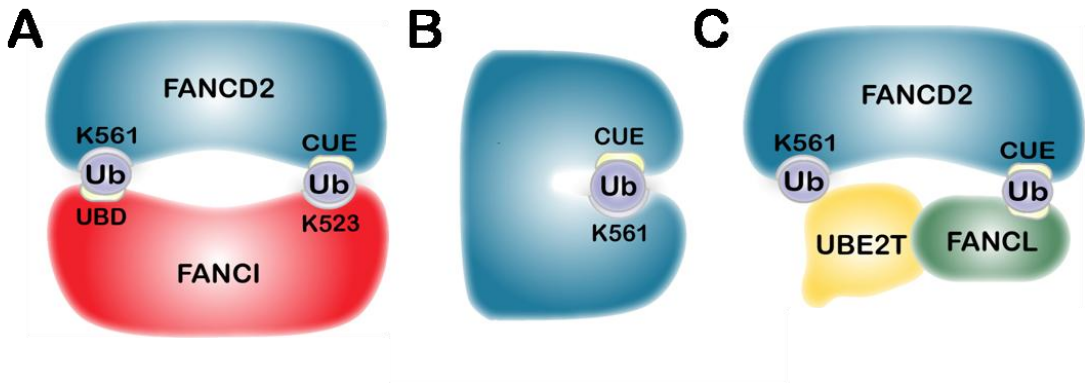
Ubiquitination: Mono- versus Poly-

To date, only the monoubiquitination of FANCD2 has been observed, begging the question; how is monoubiquitinated FANCD2 restricted from being polyubiquitinated? Insights into this question can be gained from extensive studies of regulators of endocytosis. Many endocytic proteins are monoubiquitinated in a process referred to as coupled monoubiquitination, whereby substrate recognition and ubiquitination are regulated, at least in part, through noncovalent interactions between ubiquitin and substrate *u*biquitin-*b*inding *d*omains (UBDs). One such example is EPS15 (*e*pidermal *g*rowth factor receptor pathway *s*ubstrate *15*), an important regulator of the endocytosis of ligand-inducible receptors of the *r*eceptor *t*yrosine *k*inase (RTK) family, in particular EGFR.[71] EPS15 is monoubiquitinated by the NEDD4 HECT E3 ubiquitin ligase and by the Parkin RING E3 ubiquitin ligase. EPS15 harbors two *u*biquitin-*i*nteracting *m*otifs (UIMs) in its carboxy-terminus that are critical for its regulation. One proposed model for why EPS15 is solely monoubiquitinated is that once ubiquitinated, EPS15 undergoes an intramolecular rearrangement enabling covalently attached ubiquitin to engage in a noncovalent

interaction with one of its two carboxy-terminal UIMs, generating a closed conformation refractory to further ubiquitination.[72, 73] The crystal structure of the murine ID2 heterodimer has revealed that the mouse Fancd2 K559 and Fanci K522 sites of monoubiquitination are buried within the ID2 interface. A solvent accessible tunnel adjacent to these sites is predicted to be too small to accommodate UBE2T, suggesting that monoubiquitination either precedes heterodimerization or occurs subsequent to ID2 dissociation. Similar to the EPS15 UIM, one possibility is that ID2 heterodimerization occurs following monoubiquitination, and that the ID2 heterodimer is stabilized through a noncovalent interaction between monoubiquitin covalently linked to FANCI K523 and the FANCD2 CUE domain, and quite possibly a reciprocal interaction between monoubiquitinated FANCD2 K561 and an UBD in the carboxy-terminus of FANCI (**Figure 3A**).[27] Thus, FANCD2 and FANCI might be shielded from further ubiquitination *via* intermolecular association. It is also possible that monoubiquitination promotes an intramolecular association between ubiquitin covalently attached to K561 and the amino-terminal CUE domain,[27] potentially blocking the ubiquitin on K561 from further ubiquitination, similar to that for EPS15 (**Figure 3B**). The latter model would more closely align with recent findings from the Sobeck laboratory indicating that activation of the FA-BRCA pathway coincides with dissociation of FANCD2 and FANCI.[74] ID2 dissociation is triggered by ATM/ATR-mediated phosphorylation of a cluster of at least six FANCI SQ/TQ motifs, and is followed by the monoubiquitination of FANCD2, *see below.*[33, 74]

The NEDD4 E3 ubiquitin ligase can mono- and polyubiquitinate substrates, and the balance between these two posttranslational modifications is determined, at

Figure 3. Models for FANCD2 and FANCI monoubiquitination. The schematics depict several potential outcomes upon monoubiquitination of FANCD2 and FANCI, which would preclude further ubiquitination. (**A**) The ID2 heterodimer inactivation model. Following monoubiquitination, ID2 heterodimerization occurs and is stabilized through a noncovalent interaction between monoubiquitin covalently linked to FANCI K523 and the FANCD2 CUE domain. There is also possibly a reciprocal interaction between monoubiquitinated FANCD2 K561 and an UBD in the carboxy-terminus of FANCI, shielding FANCD2 from further ubiquitination. (**B**) The FANCD2 self-inactivation model. Monoubiquitination could promote an intramolecular association between ubiquitin covalently attached to K561 and the amino-terminal CUE domain, resulting in a closed conformation. (**C**) The E3 ubiquitin ligase dissociation model. Once FANCL is autoubiquitinated, the ubiquitin moiety may interact noncovalently with the CUE domain on FANCD2 enabling monoubiquitination of FANCD2 on K561. This interaction is predicted to be weak and short-lived, leading to rapid dissociation of FANCL and FANCD2, precluding further ubiquitination.


A
FANCD2
K561
CUE
Ub
Ub
UBD
K523
FANCI
B
CUE
Ub
K561
C
FANCD2
K561
CUE
Ub
Ub
UBE2T
FANCL

least in part, by the nature of the enzyme-substrate interaction. The tryptophan-tryptophan (WW) motif of NEDD4 can interact with relatively high affinity to a substrate PPxY (*where x is any amino acid*) motif, leading to substrate polyubiquitination.[75, 76] EPS15 lacks a PPxY motif and instead interacts with NEDD4 *via* a weak noncovalent interaction between ubiquitin covalently conjugated to NEDD4 and one of its carboxy-terminal UIMs.[77, 78] Similarly, EPS15 interacts with the Parkin RING E3 ubiquitin ligase *via* a weak interaction between an *u*biquitin-*l*ike *d*omain (UbL) in Parkin and one of its UIMs.[79] Previous studies have indicated that the FANCL E3 ubiquitin ligase, as well as the UBE2T E2 ubiquitin-conjugating enzyme, undergoes autoubiquitination.[54, 80] Therefore, the FANCD2 CUE domain may interact with ubiquitin conjugated to FANCL (*or UBE2T*) and, because of the weak nature of this interaction, FANCD2 and FANCL may rapidly dissociate following ubiquitination, precluding further ubiquitination (**Figure 3C**).

Another major factor influencing mono- versus poly-ubiquitination is the nature of the E2 ubiquitin-conjugating enzyme. There are ~40 E2 ubiquitin-conjugating enzymes encoded by the human genome, 28 of which are classified as monoubiquitinating E2s. The FANCD2/I E2 ubiquitin-conjugating enzyme UBE2T is classified as a monoubiquitinating E2.[46, 54, 56, 80] Several other E2s function as polyubiquitinating E2s and display ubiquitin linkage-specificity. For example, UBE2G1, UBE2G2, UBE2K, UBE2R1, and UBE2R2 promote the assembly of K48-linked polyubiquitin chains, while UBE2S and UBE2N promote the assembly of K11- and K63-linked polyubiquitin chains, respectively.[81] In the case of UBE2N, K63-linked polyubiquitin chain assembly is facilitated by the cofactor proteins UBE2V1

and UBE2V2, which structurally align K63 of the acceptor ubiquitin toward the active site C of UBE2N.[82, 83] Thus, the monoubiquitination fate of FANCD2 and FANCI may simply be determined by the mechanistic nature of UBE2T. Nevertheless, much remains to be learned about the mechanism, structure, and regulation of UBE2T.

Lastly, the concentration of an E3 ubiquitin ligase relative to the concentration of its substrate has also been shown to play an important role in determining if a protein is monoubiquitinated or polyubiquitinated. One example of this mechanism of regulation applies to the ubiquitination of the p53 tumor suppressor protein by its E3 ubiquitin ligase MDM2: When levels of MDM2 are low, p53 is largely monoubiquitinated. Conversely, when levels of MDM2 are high, p53 is predominantly polyubiquitinated.[84] Likewise, it is plausible that the cellular concentration of FANCL is sufficient for FANCD2/I monoubiquitination only, precluding further ubiquitination.

FANCD2 and FANCI Deubiquitination

The site-specific monoubiquitination of FANCD2 and FANCI is a reversible process. The deubiquitination of monoubiquitinated FANCD2 and FANCI is mediated by *u*biquitin-*s*pecific *p*rotease *1* (USP1). This important function for USP1 was uncovered in a DUB (*d*e*ub*iquitinating enzyme) gene family RNA interference library screen, whereby knockdown of USP1 resulted in a significant increase in monoubiquitinated FANCD2.[85] Deletion of murine Usp1 results in increased perinatal lethality, male infertility, and an FA phenotype.[86] Usp1-/- *m*ouse *e*mbryonic *f*ibroblasts (MEFs) exhibit elevated levels of Fancd2 monoubiquitination and impaired Fancd2

nuclear foci formation and are defective in HR repair.[86] USP1 is activated upon formation of a complex with the UAF1 (*USP1 associated factor-1*) protein.[87] The amino-terminus of UAF1 has a WD40 domain, which binds and activates USP1, while the carboxy-terminus contains two *SUMO-like domains* (SLD1 and SLD2) that mediate substrate binding.[87, 88] FANCI harbors a highly conserved *SUMO-like domain-interacting motif* (SLIM) that has been shown to interact with SLD2 of UAF1, targeting USP1/UAF1 to the ID2 complex.[88] USP1/UAF1 activity is tightly regulated: upon exposure to UV irradiation, USP1 undergoes inhibitory autocleavage.[89] The carboxy-terminal fragment of autocleaved USP1 is subsequently degraded by the R/N-end rule pathway, leading to the effective elimination of deubiquitinating activity.[90] The *USP1* gene is also transcriptionally repressed upon cellular exposure to DNA damaging agents, a process that requires the function of the p21 cyclin-dependent kinase inhibitor.[87, 91] Both negative regulatory mechanisms facilitate the timely accumulation of monoubiquitinated FANCD2 and FANCI following the induction of DNA damage.[89]

FANCD2 Phosphorylation

The FANCD2 protein is also subject to posttranslational modification by phosphorylation. The ATM kinase is a member of the *phosphatidylinositol-3-OH-kinase-like family of protein kinases* (PIKK). Bilallelic mutations in the *ATM* gene cause the autosomal recessive disorder *Ataxia- telangiectasia* (AT).[92] AT is clinically characterized by progressive cerebellar ataxia, telangiectasias, immune defects, and increased susceptibility to hematologic cancers of B and T cell origin, as well as

central nervous system tumors.[93, 94] The ATM kinase plays a major coordinating role in the cellular response to DNA DSBs and phosphorylates multiple substrates to halt cell cycle progression and initiate DNA repair. For example, the ATM kinase phosphorylates T68 of the CHK2 kinase following exposure to *i*onizing *r*adiation (IR) to initiate cell cycle arrest at the G1-S boundary.[95] In 2002, Taniguchi and colleagues discovered that ATM phosphorylates FANCD2 on S222, S1401, S1404, and S1418 in response to IR exposure.[96] Phosphorylation of FANCD2 S222 is required for the establishment of the IR-inducible S phase checkpoint, an ability to pause DNA synthesis in the presence of chromosomal DSBs.[97] However, ATM-mediated FANCD2 S222 phosphorylation is dispensable for FANCD2 monoubiquitination, nuclear foci formation, and ICL resistance.[96]

An important role for the ATR kinase in the regulation of FANCD2 has also been established.[98, 99] While ATM primarily responds to DNA DSBs, ATR is a major regulator of the DNA replication checkpoint, primarily responding to agents that disrupt DNA replication, e.g. hydroxyurea, an inhibitor of deoxyribonucleotide reductase, *aph*idicolin (APH), a processive DNA polymerase inhibitor, and UV irradiation, which promotes the formation of cyclobutane pyrimidine dimers.[100, 101] Mutations in *ATR* underlie *S*e*ck*e*l* syndrome (SCKL1) a rare autosomal recessive disorder characterized by severe growth retardation, short stature, microcephaly, mental retardation, and increased risk for *a*cute *m*yeloid *l*eukemia (AML), *m*elo*d*y*s*plasia (MDS), and aplastic anemia, similar to FA.[102-104] Following exposure to DNA crosslinking agents, ATR and FANCD2 co-localize in nuclear foci.[98, 105] ATR also promotes efficient DNA damage-inducible FANCD2 monoubiquitination and

nuclear foci formation.[98] Accordingly, similar to FA patient cells, ATR-deficient SCKL1 patient cells exhibit increased chromosome aberrations, including radial formations, following exposure to MMC.[98, 104] The D'Andrea laboratory established that ATR phosphorylates FANCD2 on T691 and S717 *in vitro* and *in vivo*.[98, 99] A FANCD2-T691A/S717A double mutant fails to undergo efficient DNA damage-inducible monoubiquitination and fails to correct the MMC hypersensitivity of FA-D2 patient cells.[99] How phosphorylation of these two sites promotes FANCD2 monoubiquitination remains unclear. Phosphorylated T691 and S717 may stimulate interaction with the core complex or with FANCL or UBE2T, promoting monoubiquitination. Alternatively, phosphorylation may promote dissociation from FANCI, allowing access to K561, similar to that described for FANCI, *see below*. Phosphorylation could also promote the interaction of FANCD2 with DNA in chromatin, which has been shown to lead to increased efficiency of monoubiquitination.[56, 106]

FANCI Phosphorylation

FANCI was originally identified as an ATM/ATR substrate in a large-scale SILAC (*s*table *i*sotope *l*abeling with *a*mino acids in *c*ell culture) proteomics screen.[15] As described above, six S/TQ motifs are positioned proximal to K523, the sites of monoubiquitination. These S/TQ sites become phosphorylated in the absence of prior monoubiquitination, indicating that this posttranslational modification acts upstream of monoubiquitination.[33] Importantly, mutation of these S/TQ sites results in abrogation of FANCI and FANCD2 monoubiquitination and nuclear foci formation,[33]

indicating that phosphorylation of the FANCI S/TQ cluster functions as a molecular switch to activate the FA-BRCA pathway. However, the underlying molecular mechanism remains unclear. Recently, it has been suggested that activation of the pathway coincides with disassociation of the ID2 heterodimer, and that ID2 disassociation is triggered by ATM/ATR-mediated phosphorylation of the S/TQ cluster.[106] Using cell-free *Xenopus laevis* egg extracts, Sareen and colleagues demonstrated that a FANCI S/TQ cluster phosphorylation-dead mutant failed to dissociate from FANCD2 and failed to undergo monoubiquitination. Conversely, a phosphorylation-mimetic mutant failed to associate with FANCD2 and exhibited increased monoubiquitination.[106] Taken together, these findings indicate that phosphorylation of FANCI at the conserved S/TQ cluster is a key mechanism in the activation of the FA-BRCA pathway. The introduction of a cluster of negatively charged phosphate groups may result in a localized charge repulsion that promotes ID2 disassociation, enabling access of the previously occluded ubiquitin ligase machinery.

FANCD2 and FANCI Function

Replisome Surveillance

Early studies indicated that, in addition to being monoubiquitinated following exposure to DNA damaging agents, FANCD2 and FANCI monoubiquitination also occurs during unperturbed cell cycle progression. In addition, monoubiquitinated FANCD2 co-localizes with BRCA1 and RAD51 in nuclear foci during S phase.[97] These findings strongly suggested that monoubiquitinated FANCD2 might play an

important role in maintaining genome stability during the process of DNA replication.[10, 97] Accordingly, the FANCD2 and FANCA proteins are required for the maintenance of common *c*hromosomal *f*ragile *s*ite (CFS) stability.[107] CFS are chromosomal loci that are prone to breakage when cells are cultured under conditions of DNA replication stress, for example following treatment with low concentrations of the DNA polymerase inhibitor APH.[108] CFSs are hot spots for *s*ister *c*hromatid *e*xchanges (SCE), chromosomal translocations, and viral integration, and are frequently rearranged or deleted in cancer.[109] The physical localization of FANCD2 and FANCI to FRA3B and FRA16D, the most frequently expressed CFSs, has also been demonstrated.[110] Combined immunofluorescence microscopy and FISH analysis of metaphase chromosomes revealed the presence of paired FANCD2/I-stained CFSs on sister chromatids linked by BLM helicase-associated *u*ltra-*f*ine DNA *b*ridges (UFBs).[110, 111] These paired FANCD2/I sister foci form during S phase and persist into mitosis, and their presence strongly suggest that FANCD2 and FANCI participate in the resolution of stalled or collapsed DNA replication forks that recurrently arise at particularly unstable genomic loci.[107, 110, 111] In further support of an important role for FANCD2 in the resolution of stalled or collapsed replication forks, *Fancd2-/-* MEFs are ICL hypersensitive yet do not display increased sensitivity to IR.[23] Consistent with a role for the FA-BRCA pathway in the DNA replication stress response, FANCD2 has been shown to interact with several components of the DNA replisome. For example, as mentioned earlier, FANCD2 interacts with the DNA polymerase processivity factor PCNA, *via* a conserved PIP-box, and this interaction is necessary for efficient FANCD2 monoubiquitination and ICL repair.[26] In addition, using a

method called iPOND (*i*solation of *p*roteins *o*n *n*ascent *D*NA), the Cortez group recently discovered that FANCD2 and FANCI, in addition to ATR, MRE11 and other proteins, are highly enriched at stalled and collapsed replication forks following depletion of deoxyribonucleotide pools.[112] Interestingly, FANCI, and not FANCD2, was also shown to accumulate at active replication forks prior to fork staling, suggesting common and independent functions for these proteins. [112] In addition, the Vaziri group has recently shown that FANCD2 is necessary for efficient initiation of DNA replication in primary human fibroblasts.[113] An unbiased proteomics screen of chromatin-bound FANCD2 immune complexes revealed that FANCD2 physically associates with the *m*ini*c*hromosome *m*aintenance 2-7 (MCM2-MCM7) replicative helicases.[114, 115] FANCD2 interacts with MCM2-MCM7 in response to ATR-mediated DNA replication stress signaling independently of the core FA complex and monoubiquitination.[115] In the absence of FANCD2, replication forks stall stochastically and primary cells exhibit increased senescence, indicating that FANCD2 is required for replication fork progression.[115] Schlacher and colleagues also showed that FANCD2 is necessary to protect stalled replication forks from MRE11-mediated degradation, and that fork protection in the absence of FANCD2 can be rescued by RAD51.[116] Moreover, it was recently discovered that FANCD2, in cooperation with BRCA1 and MRE11, recruits and interacts with CtIP (*Ct*BP-*i*nteracting *p*rotein) at stalled replication forks to promote replication restart.[117, 118] CtIP was originally identified on the basis of its ability to bind to CtBP (*C*-*t*erminal *b*inding *p*rotein), a transcriptional co-repressor.[118] However, several recent studies have established that CtIP interacts with the MRN (*M*RE11/*R*AD50/*N*BS1) complex and BRCA1 and

facilitates the DNA strand resection step of HR repair.[118-121] This evidence supports a key role for FANCD2 and FANCI in coordinating the recruitment of HR proteins to sites of stalled replication forks, enabling timely replication fork resolution and progression.

In addition to being directly regulated by the FANCL-UBE2T ubiquitin ligase machinery, several studies have established that FANCD2/I monoubiquitination is indirectly regulated by the RAD18-RAD6 E3/E2-ubiquitin ligase complex.[122-126] RAD18-RAD6 catalyzes the monoubiquitination of PCNA on K164, a molecular event necessary for DNA polymerase switching during *t*rans*l*esion DNA *s*ynthesis (TLS).[127, 128] TLS polymerases possess larger active sites and exhibit reduced fidelity, and have the ability to catalyze DNA polymerization opposite DNA templates harboring aberrantly modified nucleobases.[129] RAD18 and FANCD2, as well as FANCL, function epistatically in the cellular ICL response.[122, 126] Genetic disruption of RAD18 or RAD18 depletion *via* siRNA leads to decreased ICL-induced FANCD2/I monoubiquitination and chromatin binding.[122-126] Furthermore, RAD18-RAD6 mediated monoubiquitination of PCNA on K164 is necessary for efficient FANCD2 monoubiquitination.[122] FANCL was also shown to bind to PCNA directly and PCNA K164 monoubiquitination was shown to be required for FANCL chromatin loading.[122] While the molecular mechanism underlying this regulation remains unclear, these findings suggest that the FANCL-UBE2T and RAD18-RAD6 ubiquitin ligases cooperatively regulate FANCD2/I and PCNA monoubiquitination, respectively, to facilitate the coordinated recruitment of HR and TLS factors to stalled or collapsed replication forks.

Independent Functions of FANCD2 and FANCI

BLM Complex Chromatin Assembly

Since the discovery of FANCI, it has generally been accepted that FANCD2 and FANCI function cooperatively in the resolution of ICLs. However, several recent studies have suggested independent functions for these proteins. For example, it has recently been determined that FANCD2, and not FANCI, is an important regulator of the recruitment of the BLM helicase to chromatin.[130] Biallelic mutations in the *BLM* gene underlie *B*loom *s*yndrome (BS), a rare recessive disorder characterized by growth retardation, sunlight sensitivity, telangiectasia, hypo- or hyper-pigmentation of the skin, and increased susceptibility to hematologic cancers.[131] The BLM helicase is a member of the RecQ family of helicases, which also includes the WRN and RECQ4 proteins, and possesses ATP-dependent 3'-5' DNA helicase activity.[132] Similar to FA patient cells, BS patient cells are hypersensitive to the cytotoxic effects of DNA crosslinking agents as well as agents that inhibit DNA replication.[133, 134] In addition, FANCD2 and BLM co-localize in nuclear foci following exposure to DNA damaging agents.[134] Pichierri and colleagues established that the DNA damage-inducible phosphorylation of BLM and its ability to assemble into nuclear foci are compromised in FA-C and FA-G patient cells.[134] In contrast, FANCD2 monoubiquitination and nuclear foci formation, as well as the DNA damage-inducible chromatin localization of FANCA and FANCC, are intact in BS patient cells.[134] Similarly, using *Xenopus* egg extracts, Chaudhury *et al* recently showed that immunodepletion of both FANCD2 and FANCI leads to a failure of the BLM helicase, and its binding partners TOP3a, RMI1, RM2, and RPA1-3, to associate with chromatin. Notably, FANCD2 alone is

sufficient to rescue this defect.[130] Furthermore, neither FANCI nor FANCD2 monoubiquitination are required to promote BLM complex chromatin assembly, indicating that this function is monoubiquitination and FANCI-independent.[130]

Nucleosome Assembly Activity

FANCD2 was recently identified in a proteomics screen of histone H3/H4-interacting proteins.[135] Recombinant FANCD2 was subsequently confirmed to bind to H3/H4 *in vitro*.[135] These findings suggested that FANCD2 might play an active role in chromatin dynamics and/or reorganization. Indeed, using a topological assay and a nucleosome assembly assay, FANCD2 was shown to promote nucleosome assembly to an extent comparable to that of the known nucleosome assembly protein Nap1. Similar to the promotion of BLM complex chromatin localization, FANCD2 histone binding and nucleosome assembly activity appear to be FANCI and monoubiquitination-independent.[135] However, while FANCI fails to promote nucleosome assembly by itself, it can stimulate FANCD2-mediated nucleosome assembly at low FANCD2 concentrations. These results suggest that the ID2 complex may promote chromatin reorganization during ICL repair. Consistent with this hypothesis, FANCD2 was previously discovered to interact with the *60* kDa *T*at-*i*nteractive *p*rotein (TIP60) chromatin remodeling acetyltransferase and menin, a chromatin remodeling tumor suppressor.[136, 137] Depletion of TIP60 using siRNA sensitizes cells to ICL cytotoxicity.[137] TIP60 is a subunit of the NuA4 chromatin remodeling complex, which comprises multiple enzymatic subunits including TIP60, the p400 motor ATPase, and the RUVBL1 and RUVBL2 helicase-like proteins.[138]

Upon DSB generation, TIP60 is rapidly recruited to sites of damage where it acetylates multiple substrates including H2A, H4, p53, and ATM.[139-142] Menin has also been shown to function in chromatin remodeling, and DNA repair.[136, 143, 144] Cells lacking menin are more sensitive to DNA damage and the interaction between FANCD2 and menin is increased following exposure to IR.[136] Collectively, these findings suggest that FANCD2 may function in a chromatin remodeling capacity during the early stages of ICL repair.

FANCD2: Recruitment of Nucleases

Several recent studies have provided important mechanistic insight into the function of monoubiquitinated FANCD2 in ICL repair. The previously uncharacterized KIAA1018 protein was simultaneously identified by several groups as a FANCD2-interacting partner and an important cellular determinant of ICL repair efficiency.[145-147] The Jiricny group identified KIAA1018 as a component of the mismatch repair MLH1 and PMS2 interactomes. This protein was predicted to harbor an amino-terminal UBZ (*u*biquitin-*b*inding *z*inc finger) UBD and a carboxy-terminal PD-D/E(X)K-type nuclease domain.[146] Similarly, the Huang and Rouse groups identified KIAA1018 on the basis of its UBZ domain and a VRR_nuc (*v*irus-type *r*eplication-*r*epair *nuc*lease) domain, which contains a motif frequently found in bacterial and bacteriophage restriction nucleases.[145, 148] Importantly, UBZ domains have been uncovered in several DNA damage response proteins, including RAD18 and DNA *pol*ymerase *κ* (POL κ).[149, 150] In another approach, Smogorzewska and colleagues discovered KIAA1018 in an unbiased genome-wide shRNA screen of proteins

necessary for efficient ICL repair.[147] KIAA1018 was found to co-localize with FANCD2 in nuclear foci following ICL exposure and to interact with FANCD2 *via* co-immunoprecipitation analysis, and was hence renamed FAN1 (*F*ANCD2-*a*ssociated *n*uclease *1*).[145-148] FAN1 was shown to display 5'-3' exonuclease activity and structure-specific endonuclease activity, preferentially toward 5' flap structures.[145-148] Furthermore, the FAN1 UBZ domain was shown to interact noncovalently with monoubiquitin conjugated to FANCD2 K561. The FAN1 UBZ domain and the monoubiquitination of FANCD2 were shown to be required for the recruitment of FAN1 to sites of DNA damage, and for efficient ICL repair.[145] While *FAN1* mutations have yet to be uncovered in FA patients, mutations in *FAN1* have been identified as a cause of karyomegalic interstitial nephritis, a chronic kidney disease.[151]

Mutations in the *SLX4* gene were recently uncovered in FA complementation group P.[152-154] FANCP/SLX4 is a ~200 kDa multidomain protein that, similar to FAN1, harbors an amino-terminal UBZ domain.[155] FANCP/SLX4 functions as a molecular platform to coordinate the activities of the structure-specific endonucleases XPF-ERCC1, MUS81-EME1, and SLX1, with XPF-ERCC1 playing a major role in ICL repair, and MUS81-EME1 and SLX1 playing less critical roles.[156-160] Similar to FAN1 and FANCD2, FANCP/SLX4 and FANCD2 co-localize in nuclear foci and co-immunoprecipitate.[154] While Yamamoto and colleagues established that the FANCP/SLX4 UBZ domain and the monoubiquitination of FANCD2 are required for FANCP/SLX4 targeting to nuclear foci, a recent report has contradicted these findings and shown that SLX4 recruitment occurs independent of FANCD2

monoubiquitination.[154, 161] Taken together, these findings suggest that one major function for chromatin-bound monoubiquitinated FANCD2 may be the recruitment of structure-specific endonucleases that mediate key incision steps during the process of ICL repair.[162]

FANCD2 and FANCI: DNA Binding

Several studies have established that FANCD2 and FANCI physically bind to DNA. Using FANCD2 purified from baculovirus-infected SF9 insect cells, the Parvin group originally determined that full-length FANCD2 binds to dsDNA and *H*olliday *j*unctions (HJs), quadruplex heteroduplex DNA intermediates that arise during HR.[163] Similarly, the Zhang group purified full-length FANCI and demonstrated that it can bind to multiple DNA substrates, including HJs, dsDNA, ssDNA, and flap structures, without any apparent structural preference.[31] Longerich *et al* also demonstrated that purified FANCI is capable of binding to dsDNA, ssDNA, and HJs.[80] Importantly, however, co-purified ID2 displayed a much lower affinity for dsDNA and ssDNA, yet maintained a robust binding activity towards HJs and various branched DNA structures.[31] Consistent with these findings, the Pavletich group described the structure of co-crystals of FANCI bound to splayed Y DNA at a resolution of 7.8 Å.[35] Based on this structure and the structural homology between FANCD2 and FANCI, the authors proposed that the ID2 heterodimer could have two sets of pseudo-symmetrical dsDNA/ssDNA binding sites, structures that could arise upon stalling of one or two replication forks at an ICL.[35] Notably, the Patel group discovered that, in addition to binding to dsDNA and ssDNA, FANCD2 exhibits 3' to 5' exonucleolytic activity

towards ssDNA in the presence of Mg^{2+} or Mn^{2+}.[164] However, the functional significance for this activity remains to be established.

In addition, recent studies have established that FANCD2 monoubiquitination is strongly stimulated through DNA binding. For example, the Kurumizaka and Sung laboratories demonstrated that several forms of DNA, including dsDNA, ssDNA, and Holliday junctions, could robustly stimulate the *in vitro* monoubiquitination of FANCD2.[45, 56] Of note, this DNA-dependent stimulation of FANCD2 monoubiquitination required both the presence of FANCI and its ability to bind to DNA.[56] Using *Xenopus* egg extracts, Sobeck and colleagues also demonstrated that the monoubiquitination of FANCD2 could be stimulated with linear and branched dsDNA structures but not with ssDNA or Y-shaped DNA.[165] Expanding on these studies, the Sobeck group recently demonstrated that while FANCD2 monoubiquitination is strongly stimulated by circular and linear dsDNA, the monoubiquitination of FANCI is strongly stimulated by ssDNA and only weakly induced by circular and linear dsDNA, indicating a dichotomy in substrate specificity for both proteins.[74] In addition, through a time course study, the same group determined that the monoubiquitination of FANCD2 in the presence of circular dsDNA and ssDNA occurs significantly earlier than the monoubiquitination of FANCI.[74] FANCD2 was also found to localize to chromatin earlier than, and independent of, FANCI. In contrast, the localization of FANCI to chromatin is FANCD2-dependent.[74]

Finally, recent studies from the D'Andrea laboratory have established that FANCD2 promotes *RAS* oncogene-induced senescence in part by promoting the transcriptional activation of the *TAp63* tumor suppressor gene.[166] Monoubiquitinated

FANCD2 and FANCP/SLX4 bind to the *TAp63* promoter and cooperate in the transcriptional activation of TAp63.[166] Genome-wide *ChIP seq*uencing (ChIP-seq) analysis revealed that following exposure to DNA damaging agents, FANCD2-Ub binds to *cis*-regulatory sequences of numerous genes essential for epithelial development and morphogenesis.[166] This newly discovered function for FANCD2 in transcriptional regulation is supported by other studies describing the binding of FANCD2 to the *TNF*α gene as well as the antioxidant genes *GPX1* and *TXNRD1*, and constitutes a novel dimension to the portfolio of FANCD2 functions.[167, 168]

Conclusions

The FANCD2 and FANCI proteins function at a pivotal point in the FA-BRCA pathway and play an essential role in maintaining chromosome stability. Both proteins are critical for hematopoietic stem cell homeostasis and the prevention of cellular transformation. These key functions are achieved through what could be considered disparate means: the promotion of error-free HR repair through the recruitment of structure-specific endonucleases, replisome surveillance and resolution, nucleosome remodeling, as well as transcriptional regulation. However, these functions are not necessarily mutually exclusive and it is conceivable that FANCD2 and FANCI facilitate these processes through the recruitment of multiple binding partners and/or *via* a fundamental chromatin remodeling capacity. Many outstanding questions remain to be addressed, including 1) the identification and characterization of structural and functional domains of FANCD2 and FANCI, 2) cataloging the full spectrum and functional significance of additional FANCD2 and FANCI posttranslational

modifications, 3) identification of the FANCI catalytic ubiquitin ligase machinery, 4) determination of the function of monoubiquitinated FANCI (*and FANCD2*), and 5) elucidation of the roles of FANCB and FAAP100 in the regulation of FANCL activity. Much work remains to be done to clearly elucidate the structural and regulatory mechanisms of these functions. The achievement of these goals will no doubt greatly inform our understanding of the molecular etiology of FA and offer much needed direction in the urgent quest for potential therapeutics.

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Manuscript – II

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Coordinate Nuclear Targeting of the FANCD2 and FANCI Proteins *via* a FANCD2 Nuclear Localization Signal

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Abstract

Fanconi anemia (FA) is a rare recessive disease, characterized by congenital defects, bone marrow failure, and increased cancer susceptibility. FA is caused by biallelic mutation of any one of sixteen genes. The protein products of these genes function cooperatively in the FA-BRCA pathway to repair DNA interstrand crosslinks (ICLs). A central step in the activation of this pathway is the monoubiquitination of the FANCD2 and FANCI proteins. Monoubiquitinated FANCD2 and FANCI localize to discrete chromatin regions where they function in ICL repair. Despite their critical role in ICL repair, very little is known about the structure, function, and regulation of the FANCD2 and FANCI proteins, or how they are targeted to the nucleus and chromatin. In this study, we describe the functional characterization of an amino-terminal FANCD2 nuclear localization signal (NLS). We demonstrate that the amino terminal 58 amino acids of FANCD2 can promote the nuclear expression of GFP and is necessary for the nuclear localization of FANCD2. Importantly, mutation of this FANCD2 NLS reveals that intact FANCD2 is required for the nuclear localization of a subset of FANCI. In addition, the NLS is necessary for the efficient monoubiquitination of FANCD2 and FANCI and, consequently, for their localization to chromatin. As a result, FANCD2 NLS mutants fail to rescue the ICL sensitivity of FA-D2 patient cells. Our studies yield important insight into the domain structure of the poorly characterized FANCD2 protein, and reveal a previously unknown mechanism for the coordinate nuclear import of a subset of FANCD2 and FANCI, a key early step in the cellular ICL response.

Introduction

Fanconi anemia (FA) is a rare autosomal and X-linked recessive disease, characterized by congenital abnormalities, pediatric bone marrow failure, and heightened cancer susceptibility [1]. FA is caused by biallelic mutations in any one of 16 genes (*FANCA, -B, -C, -D1/BRCA2, -D2, -E, -F, -G, -I, -J/BRIP1, -L, -M, -N/PALB2, -P/SLX4, O/RAD51C, Q/ERCC4*) [2-4]. The protein products of these genes function cooperatively in the FA-BRCA pathway to repair DNA interstrand crosslinks (ICLs) and to maintain chromosome stability. A central step in the activation of the FA-BRCA pathway is the monoubiquitination of the FANCD2 and FANCI proteins. Monoubiquitination of FANCD2 and FANCI is catalyzed by the FA core complex, which is comprised of FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM, in addition to the E2 ubiquitin conjugating enzyme UBE2T, as well as FAAP100, FAAP24, and FAAP20 [4,5]. Following monoubiquitination, FANCD2 and FANCI co-localize in discrete nuclear foci with several well characterized DNA repair proteins including BRCA1 and NBS1 [6-9]. However, importantly, the domain structure, regulation, and function of both FANCD2 and FANCI are poorly described. Furthermore, the mechanism(s) by which FANCD2 and FANCI are localized to the nucleus remains unknown.

Several studies have established that the FANCD2 and FANCI proteins function proximal to or within chromatin [6,8-10]. As FANCD2 and FANCI are relatively large proteins, with molecular weights of 164 and 149 kDa, respectively, the nuclear import of these proteins necessitates an energy-dependent active transport mechanism. The most common nuclear protein transport mechanism involves the

recognition of nuclear localization signals (NLSs) by members of the importin (Imp) superfamily of nuclear transporters, followed by translocation through the nuclear pore complexes [11]. NLSs generally comprise short stretches of basic amino acids either alone (monopartite) or separated by one (bipartite) or two (tripartite) mutation-tolerant linker regions of 10-12 amino acids (bipartite). NLSs in the cargo protein are recognized by the Imp α subunit of the Imp α/β heterodimer or by Imp β alone, and nuclear transport is coupled to GTP hydrolysis [11].

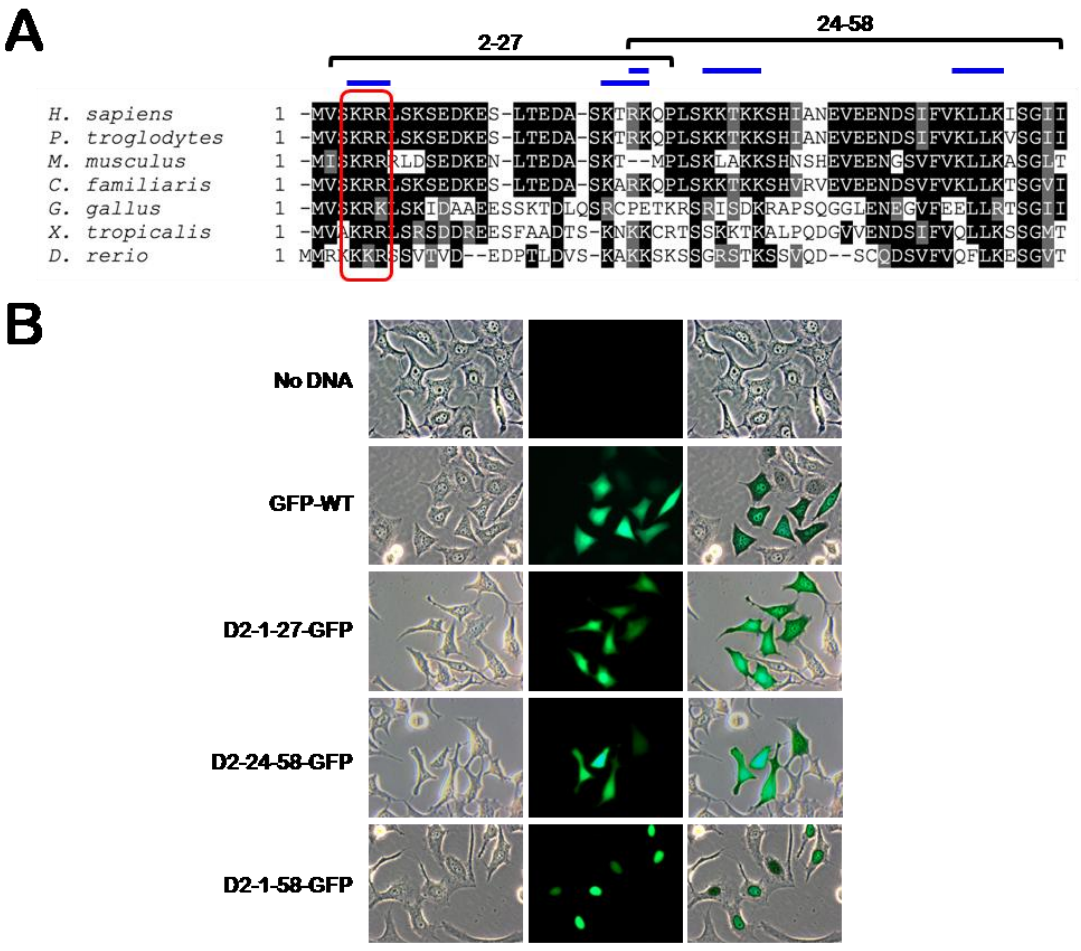
In this study, we describe the identification and functional characterization of a NLS in the amino terminus of FANCD2. We demonstrate that the amino terminal 58 amino acids of FANCD2 can promote the nuclear expression of green fluorescent protein (GFP). In addition, using deletion and site-directed mutagenesis strategies we establish that the amino terminal 58 amino acids of FANCD2 are necessary for the nuclear and chromatin localization of FANCD2 in FA-D2 patient-derived cells. Importantly, we also demonstrate that the nuclear and chromatin localization of a subset of the cellular pool of FANCI is dependent on the nuclear import of FANCD2. Moreover, the FANCD2 NLS is required for the efficient DNA damage-inducible monoubiquitination of FANCD2 and FANCI. Consequently, the FANCD2 NLS mutants fail to rescue the ICL hypersensitivity of FA-D2 patient-derived cells. Our results suggest that a subset of FANCI is translocated to the nucleus in a piggyback mechanism with FANCD2, dependent on its amino-terminal NLS, and suggest that FANCD2 and FANCI are targeted to the nucleus as a heterodimer. These findings lend important insight into the structure and regulation of two poorly characterized tumor suppressor proteins with key early roles in the cellular ICL response.

Results

FANCD2 contains a highly conserved amino-terminal nuclear localization signal, which facilitates nuclear expression of GFP

In silico analysis using cNLS mapper uncovered several high-scoring Imp α/β-dependent bipartite NLSs within the amino-terminal 58 amino acids of FANCD2 (**Figs. S1A and B**) [12]. In contrast, cNLS mapper did not predict any high scoring NLSs in FANCI. A sequence alignment of FANCD2 from multiple species illustrates strong evolutionary conservation in general (**Fig. S1C**). However, in contrast to the sequence divergent carboxy-terminus, the alignment illustrates strong conservation of several blocks of basic amino acids within the amino terminal 58 amino acids (**Figs. 1A and S1C**). To determine if this entire region or amino acids 1-27 or 24-58 - harboring the two highest scoring predicted NLSs - were sufficient for nuclear localization, we fused amino acids 1-58 (D2-1-58-GFP), 1-27 (D2-1-27-GFP), or 24-58 (D2-24-58-GFP) of FANCD2 to the amino terminus of GFP (**Fig. 1B**). HeLa cells were transiently transfected with wild type GFP (GFP-WT) and the FANCD2-GFP fusion constructs and cells were analyzed by inverted fluorescence microscopy. HeLa cells transiently expressing GFP-WT, D2-1-27-GFP, or D2-24-58-GFP all exhibited uniform cytoplasmic and nuclear fluorescence (**Fig. 1B**). Conversely, cells transiently expressing D2-1-58-GFP primarily exhibited nuclear fluorescence (**Fig. 1B**). Similar findings were observed with IMR90 cells (**Fig. S1D**). These results demonstrate that the amino terminal 58 amino acids of FANCD2 are necessary to promote exclusive nuclear GFP localization. In support of an importin α/β-dependent mechanism of

Figure 1. The amino terminal 58 amino acids of FANCD2 contain a highly conserved nuclear localization signal, which facilitates nuclear expression of GFP. (**A**) A Clustal Omega multiple sequence alignment of the amino terminus of FANCD2 demonstrates strong evolutionary conservation of several blocks of basic amino acids of this region, indicated by blue bars. The red box indicates the highly conserved K4, R5, and R6 residues selected for mutagenesis. (**B**) The amino terminal 58 amino acids of FANCD2 are required to promote exclusive nuclear expression of GFP. HeLa cells were transiently transfected with wild type GFP (GFP-WT), amino acids 1-27 (D2-1-27-GFP), 24-58 (D2-24-58-GFP), or 1-58 (D2-1-58-GFP) of FANCD2 fused to GFP followed by analysis by inverted fluorescence microscopy.

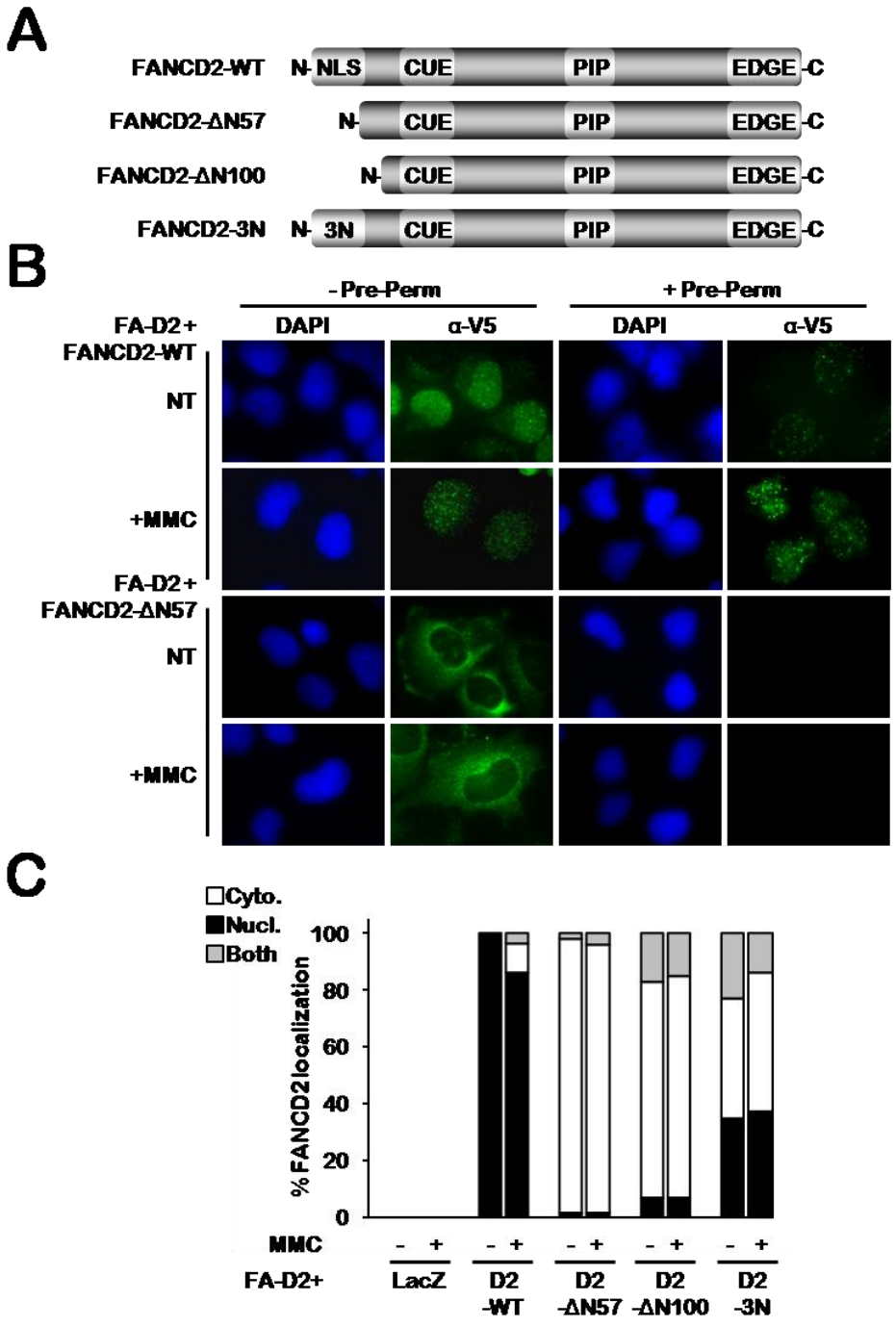

A
2-27
24-58
H. sapiens 1 -MVSKRRLSKSEDKES-LTEDA-SKTRKQPLSKKTKKSHIANEVEENDSIFVKLLKISGII
P. troglodytes 1 -MVSKRRLSKSEDKES-LTEDA-SKTRKQPLSKKTKKSHIANEVEENDSIFVKLLKVSGII
M. musculus 1 -MISKRRRLDSEDKEN-LTEDA-SKT--MPLSKLAKKSHNSHEVEENGSVFVKLLKASGLT
C. familiaris 1 -MVSKRRLSKSEDKES-LTEDA-SKARKQPLSKKTKKSHVRVEVEENDSVFVKLLKTSGVI
G. gallus 1 -MVSKRKLSKIDAAEESSKTDLQSRCPETKRSRISDKRAPSQGGLENEGVFEELLRTSGII
X. tropicalis 1 -MVAKRRLSRSDDREESFAADTS-KNKKCRTSSKKTKALPQDGVVENDSIFVQLLKSSGMT
D. rerio 1 MMRKKKRSSVTVD--EDPTLDVS-KAKKSKSSGRSTKSSVQD--SCQDSVFVQFLKESGVT
B
No DNA
GFP-WT
D2-1-27-GFP
D2-24-58-GFP
D2-1-58-GFP

nuclear import, treatment with ivermectin, a broad-spectrum inhibitor of importin α/β-dependent nuclear import [13], inhibited the exclusive nuclear localization of D2-1-58-GFP, herein referred to as D2-NLS-GFP (**Figs. S1E and F**). In addition, mass spectrometry analysis of FANCD2 immune complexes revealed the presence of importin β1, as well as the nuclear pore complex proteins NUP160 and NUP155 (**Supplemental Table 1**). Using a chromatin fractionation approach we also observed that the majority of GFP-WT resided in a soluble cytoplasmic and nuclear fraction (S) (**Fig. S1G, lane 5**). While a large proportion of D2-NLS-GFP also resided in a soluble cytoplasmic and nuclear fraction, a higher relative proportion of D2-NLS-GFP was detected in a chromatin-associated nuclear fraction (C) (**Figs. S1G, lane 9 and H**). Taken together these results demonstrate that the amino-terminal 58 amino acids of FANCD2 harbors a *bona fide* NLS that can promote exclusive nuclear GFP localization.

The FANCD2 NLS is required for the nuclear localization of FANCD2

To determine the functional significance of the FANCD2 NLS, we next generated deletion and missense mutations of this amino acid sequence. Two amino-terminal deletion mutations, FANCD2-ΔN57, lacking amino acids 2-58, and FANCD2-ΔN100, lacking amino acids 2-101, were generated (**Fig. 2A**). In addition, using a site-directed mutagenesis approach, amino acids K4, R5, and R6, the most highly conserved basic amino acids within this region (**Fig. 1A**), were mutated to N4, N5, and N6, herein referred to as FANCD2-3N (**Fig. 2A**). These *FANCD2* cDNAs were cloned into the pLenti6.2 lentiviral vector, which contains a carboxy-terminal V5 tag, and lentivirus

Figure 2. The FANCD2 NLS is required for the nuclear localization of FANCD2. (**A**) Schematic diagram of the FANCD2 constructs generated in this study. In addition to the NLS, FANCD2 harbors a CUE (for coupling of ubiquitin conjugation to endoplasmic reticulum degradation) ubiquitin-binding domain [47], a PCNA-interaction motif or PIP-box [19], and a carboxy-terminus EDGE motif [10]. (**B**) FA-D2 cells stably expressing FANCD2-WT and FANCD2-ΔN57 were incubated in the absence (NT) or presence of 40 nM MMC for 24 h. Cells were then incubated in the absence (-Pre-Perm) or presence (+Pre-Perm) of pre-permeablization buffer, fixed, stained with mouse monoclonal anti-V5 antibody (green) and counterstained with DAPI (blue). (**C**) FA-D2 cells stably expressing LacZ, FANCD2-WT, FANCD2-ΔN57, FANCD2-ΔN100, and FANCD2-3N were incubated in the absence (NT) or presence of MMC for 24 h, fixed, and stained with rabbit polyclonal anti-FANCD2 antibody, and counterstained with phalloidin and DAPI. At least 300 cells were scored for cytoplasmic (Cyto.), nuclear (Nucl.), and both cytoplasmic and nuclear (Both) localization of FANCD2.

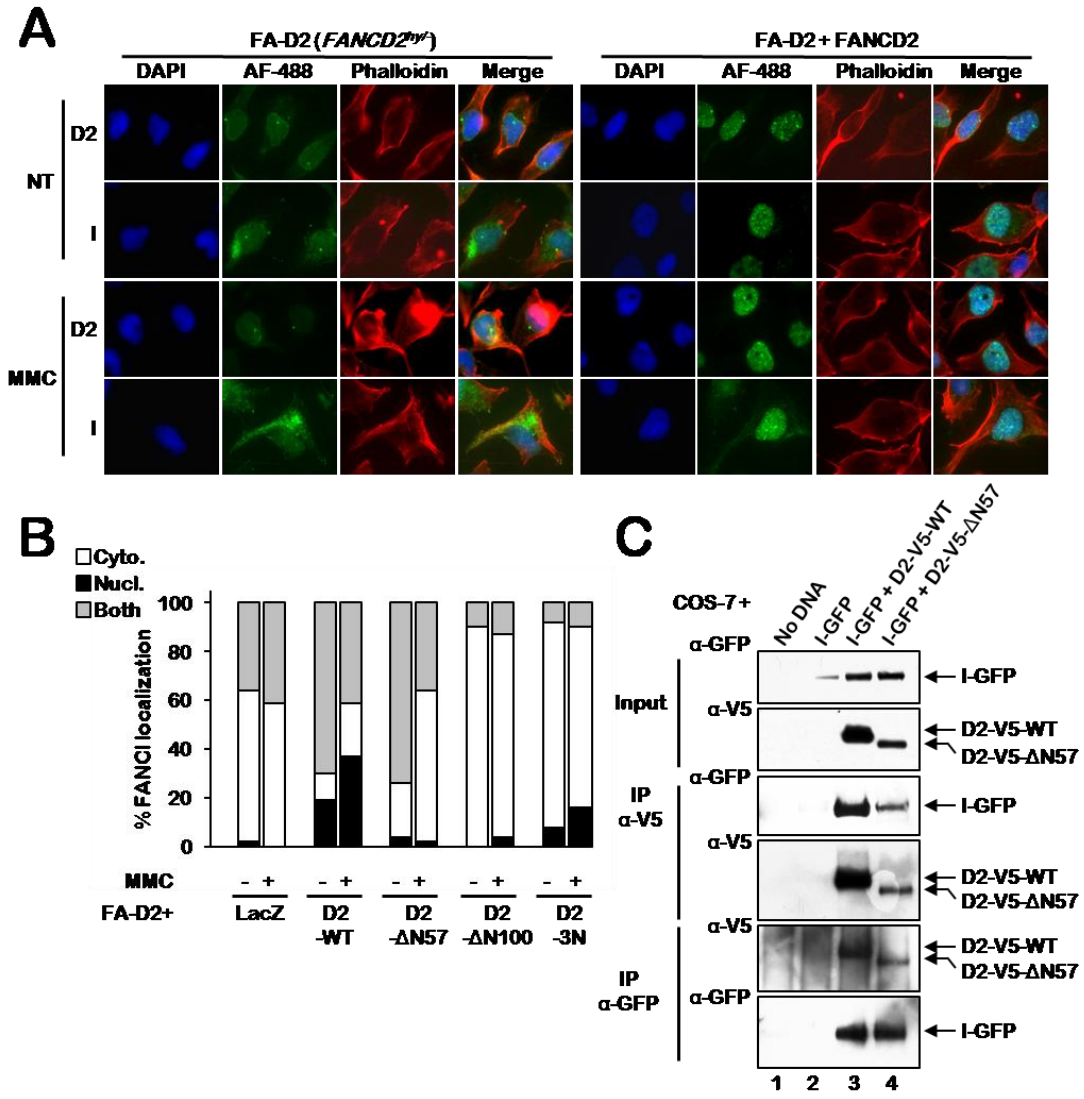

A
FANCD2-WT
N-
NLS
CUE
PIP
EDGE
-C
FANCD2-ΔN57
FANCD2-ΔN100
FANCD2-3N
3N
B
- Pre-Perm
+ Pre-Perm
FA-D2+
DAPI
α-V5
FANCD2-WT
NT
+MMC
FANCD2-ΔN57
C
Cyto.
Nucl.
Both
% FANCD2localization
100
80
60
40
20
0
MMC
FA-D2+
LacZ
D2 -WT
D2 -ΔN57
D2 -ΔN100
D2 -3N

was used to generate a series of PD20 FA-D2 patient-derived cells stably expressing wild type or mutant FANCD2. These FA-D2 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 mutation leading to a R1236H change [14]. Immunofluorescence microscopy (IF) revealed that deletion of the FANCD2 NLS resulted in exclusive cytoplasmic localization of FANCD2, in contrast to wild type FANCD2, which exhibited both diffuse and focal nuclear localization (**Figs. 2B and C**). Furthermore, permeabilization of FA-D2 cells expressing the FANCD2-ΔN57 mutant with non-ionic detergent resulted in complete loss of fluorescent signal indicating the high solubility of cytoplasmic FANCD2-ΔN57 (**Fig. 2B**). In contrast, nuclear and focal localization of wild type FANCD2 was largely resistant to permeabilization (**Fig. 2B**). Similar findings were obtained with FA-D2 cells expressing FANCD2-ΔN100 (**Fig. 2C**). In addition, a partial yet significant defect in nuclear localization was observed for the FANCD2-3N mutant, compared to wild type FANCD2 (**Fig. 2C**).

The FANCD2 NLS is required for the nuclear localization of a subset of FANCI

Next, we examined the sub-cellular localization of FANCI in our FA-D2 cell series. It is important to note that the FA-D2 cells used in this study, like all known FA-D2 patient-derived lines, harbor hypomorphic *FANCD2* mutations and express residual FANCD2 protein [15]. IF analysis revealed that FANCI was uniformly present in both the cytoplasm and nucleus of FA-D2 cells in the absence or presence of MMC (**Figs. 3A and B**). Importantly, complementation of FA-D2 cells with wild type FANCD2

Figure 3. The FANCD2 NLS is required for the nuclear localization of a subset of FANCI. (**A**) FA-D2 patient cells or FA-D2 cells stably expressing FANCD2-WT were incubated in the absence (NT) or presence of MMC for 24 h, fixed, stained with rabbit polyclonal anti-FANCD2 or anti-FANCI antibody and counterstained with phalloidin and DAPI. AF-488, Alexa Fluor 488. (**B**) FA-D2 cells stably expressing LacZ, FANCD2-WT, FANCD2-ΔN57, FANCD2-ΔN100, and FANCD2-3N were incubated in the absence (NT) or presence of MMC for 24 h, fixed, and stained with rabbit polyclonal anti-FANCI antibody, and counterstained with phalloidin and DAPI. At least 300 cells were scored for cytoplasmic (Cyto.), nuclear (Nucl.), and both cytoplasmic and nuclear (Both) localization of FANCI. (**C**) COS-7 cells were transiently transfected with no DNA, FANCI-GFP, FANCI-GFP plus FANCD2-V5-WT, or FANCI-GFP plus FANCD2-V5-ΔN57. Whole-cell lysates were immunoprecipitated with anti-V5 or anti-GFP antibodies and immune complexes immunoblotted with anti-GFP and anti-V5 antibodies.

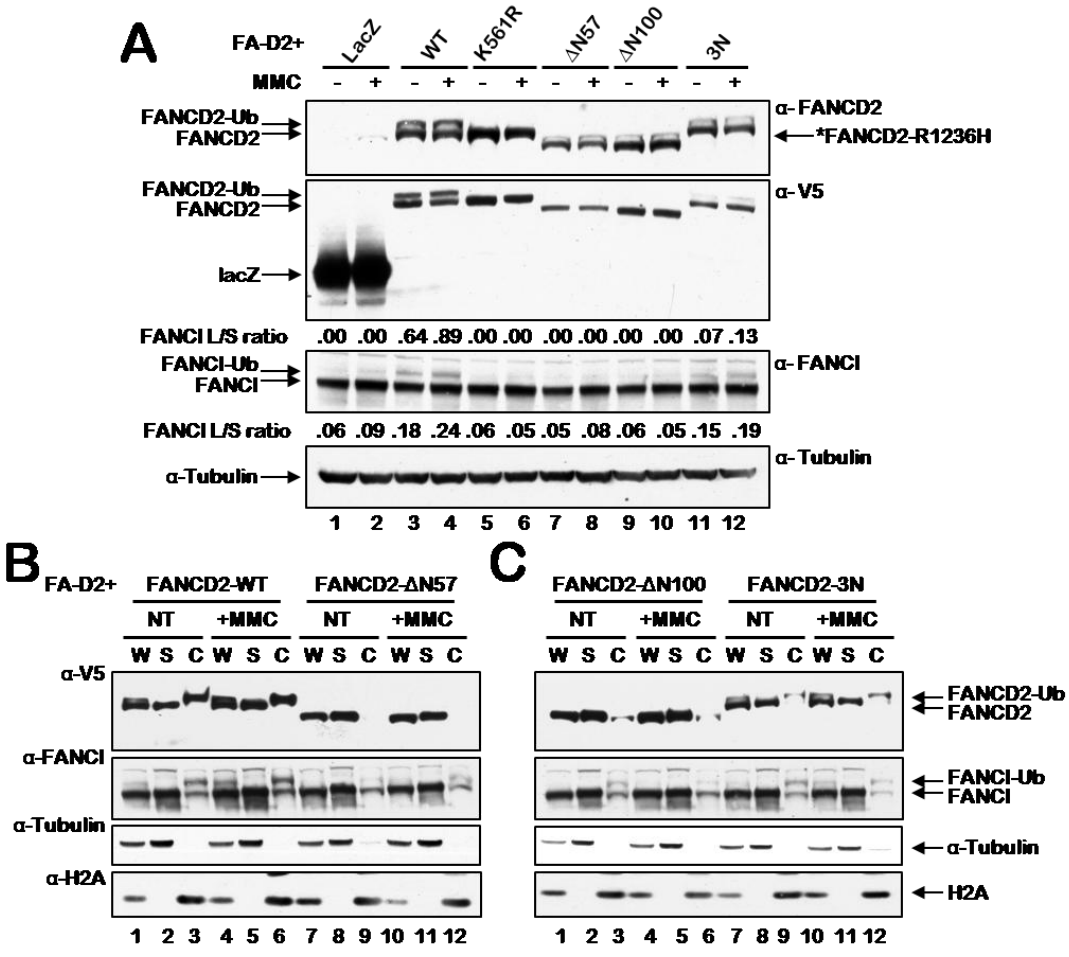

A
FA-D2 (FANCD2hyf/-)
FA-D2 + FANCD2
DAPI
AF-488
Phalloidin
Merge
NT
MMC
D2
I
B
Cyto.
Nucl.
Both
% FANCI localization
100
80
60
40
20
0
MMC
- + - + - + - + - +
FA-D2+
LacZ
D2 -WT
D2 -ΔN57
D2 -ΔN100
D2 -3N
C
COS-7+
No DNA
I-GFP
I-GFP + D2-V5-WT
I-GFP + D2-V5-ΔN57
α-GFP
α-V5
Input
IP α-V5
IP α-GFP
I-GFP
D2-V5-WT
D2-V5-ΔN57
1 2 3 4

(FANCD2-WT) led to a large increase in exclusive nuclear localization of FANCI (**Figs. 3A and B**). Furthermore, MMC-inducible FANCI nuclear foci formation was restored in these cells, consistent with previous studies [8,9] (**Fig. 3A**). Similar results were obtained with hTERT-immortalized mutant and FANCD2-complemented KEAE FA-D2 patient-derived cells [15] (**Fig. S2A**). In contrast to wild type FANCD2, both the FANCD2-ΔN57 and -ΔN100 NLS mutants failed to promote the exclusive nuclear localization of FANCI (**Figs. 3B and S2B**). Furthermore, a partial defect in FANCI nuclear localization was observed for FA-D2 cells expressing FANCD2-3N (**Fig. 3B**). To rule out the possibility that deletion of the amino-terminus of FANCD2 prevented its heterodimerization with FANCI, we tested the ability of the FANCD2-ΔN57 mutant to physically interact with FANCI by transiently transfecting COS-7 cells with GFP-tagged FANCI and V5-tagged FANCD2-WT or FANCD2-ΔN57 and examining their ability to interact by co-immunoprecipitation. Co-expression of both FANCD2-WT and FANCD2-ΔN57 with FANCI led to its stabilization (**Fig. 3C, upper panel, lanes 3 and 4**). Furthermore, the FANCD2-ΔN57 mutant was capable of interacting with FANCI and, when corrected for levels of protein input, no appreciable difference in the level of interaction between FANCI and FANCD2-ΔN57 and FANCI and FANCD2-WT was observed (**Fig. 3C, lower panels, lanes 3 and 4**). Together, these results establish that the nuclear localization of a subset of FANCI is dependent in part on FANCD2, and suggest a piggyback mechanism of nuclear FANCI targeting that is dependent on the FANCD2 amino-terminal NLS.

The FANCD2 NLS is required for efficient FANCD2 and FANCI monoubiquitination and chromatin association

Next, we examined the consequences of disruption of the FANCD2 NLS on the monoubiquitination and chromatin localization of FANCD2 and FANCI. Similar to the FANCD2-K561R mutant that cannot be monoubiquitinated [6], mutation of the FANCD2 NLS had a significant impact on its monoubiquitination: the ΔN57 and ΔN100 NLS deletion mutants failed to undergo spontaneous or MMC-inducible FANCD2 monoubiquitination (**Fig. 4A, lanes 7-10**). A faint band of similar molecular weight to wild type FANCD2 can be seen in the α-FANCD2 panel for FA-D2 cells expressing FANCD2-ΔN57 and -ΔN100 (**Fig. 4A, lanes 7-10**). This is most likely the hypomorphic FANCD2-R1236H missense mutant (*see Materials and Methods*) [14], as this band is also present in FA-D2 cells expressing LacZ (lanes 1 and 2) and is not recognized by the anti-V5 antibody (**Fig. 4, lanes 7-10**). The FANCD2-3N mutant also exhibited considerably reduced spontaneous and MMC-inducible monoubiquitination (**Fig. 4A, lanes 11 and 12**). Previous studies have demonstrated that FANCD2 and FANCI monoubiquitination are interdependent [8,9]. Accordingly, FANCI monoubiquitination was largely abrogated in FA-D2 cells stably expressing the ΔN57 and ΔN100 NLS deletion mutants, similar to that observed in cells expressing FANCD2-K561R (**Fig. 4A, lanes 5-10**). A very modest statistically insignificant decrease in FANCI monoubiquitination was also observed for FA-D2 cells expressing the FANCD2-3N mutant (**Fig. 4A, lanes 11 and 12**). In addition, a chromatin fractionation approach revealed that the FANCD2-ΔN57, -ΔN100, and -3N mutants were largely defective in chromatin localization compared to FANCD2-WT

Figure 4. The FANCD2 NLS is required for efficient FANCD2 and FANCI monoubiquitination and chromatin association. (**A**) FA-D2 cells stably expressing LacZ, FANCD2-WT, FANCD2-K561R, FANCD2-ΔN57, FANCD2-ΔN100 and FANCD2-3N were incubated in the absence and presence of 250 nM MMC for 18 h, and whole-cell lysates were immunoblotted with antibodies to FANCD2, V5, FANCI and α-tubulin. The FANCD2 and FANCI L/S ratios are the ratios of monoubiquitinated to nonubiquitinated protein, and were calculated by measuring protein band intensities using ImageJ image processing and analysis software (http://rsb.info.nih.gov/ij/). (**B and C**) FA-D2 cells stably expressing FANCD2-WT, FANCD2-ΔN57, FANCD2-ΔN100 and FANCD2-3N were treated as above and cell pellets were fractionated into soluble (S) and chromatin-associated (C) fractions. Fractions were immunoblotted with antibodies against V5, FANCI, α-tubulin and H2A. W, unfractionated whole cell extract.

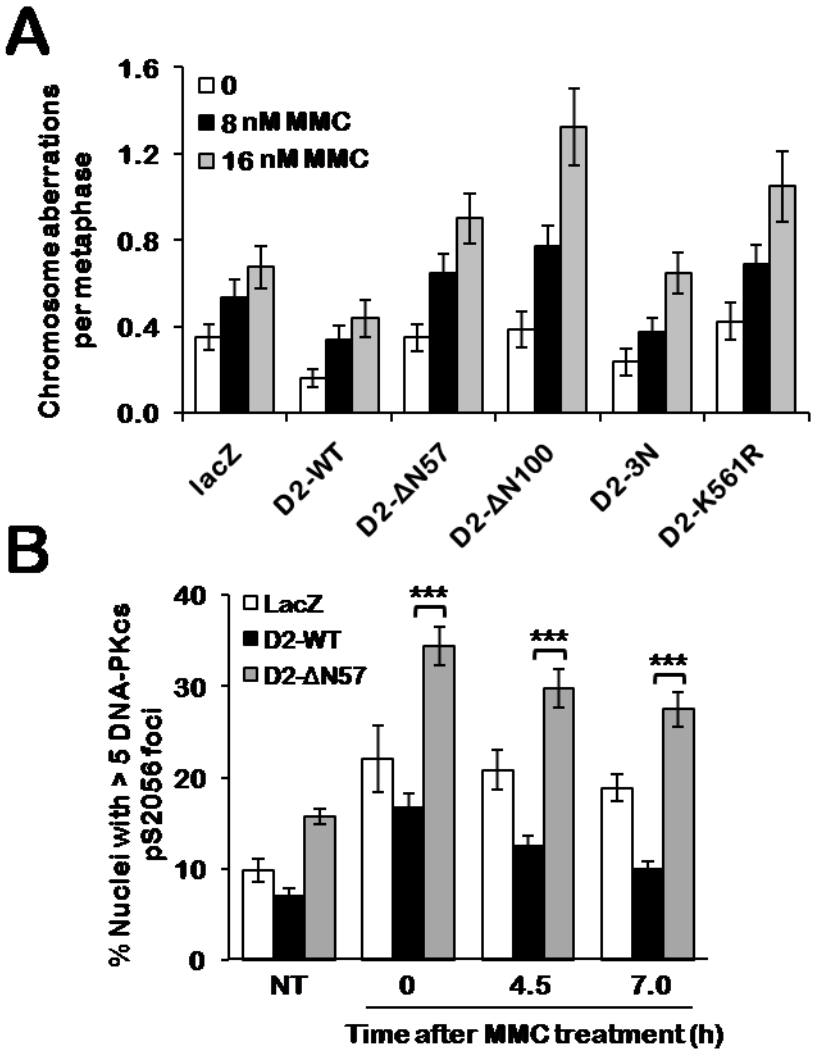

A
FA-D2+
LacZ
WT
K561R
ΔN57
ΔN100
3N
MMC
- + - + - + - + - + - +
FANCD2-Ub
FANCD2
α-FANCD2
*FANCD2-R1236H
FANCD2-Ub
FANCD2
α-V5
lacZ
FANCI L/S ratio .00 .00 .64 .89 .00 .00 .00 .00 .00 .00 .07 .13
FANCI-Ub
FANCI
α-FANCI
FANCI L/S ratio .06 .09 .18 .24 .06 .05 .05 .08 .06 .05 .15 .19
α-Tubulin
α-Tubulin
1 2 3 4 5 6 7 8 9 10 11 12
B
FA-D2+
FANCD2-WT
FANCD2-ΔN57
NT
+MMC
NT
+MMC
W S C W S C W S C W S C
α-V5
α-FANCI
α-Tubulin
α-H2A
1 2 3 4 5 6 7 8 9 10 11 12
C
FANCD2-ΔN100
FANCD2-3N
NT
+MMC
NT
+MMC
W S C W S C W S C W S C
FANCD2-Ub
FANCD2
FANCI-Ub
FANCI
α-Tubulin
H2A
1 2 3 4 5 6 7 8 9 10 11 12

(**Figs. 4B and C**). Furthermore, while robust FANCI chromatin localization was observed for cells expressing FANCD2-WT, in comparison, FANCI chromatin localization was considerably diminished in cells expressing the FANCD2-ΔN57, -ΔN100, and -3N mutants (**Figs. 4B and C and S3**). It is important to note that this particular fractionation protocol does not distinguish between true chromatin and the nuclear matrix.

The FANCD2 NLS mutants fail to correct the ICL sensitivity of FA-D2 patient cells

FA patient-derived cells are hypersensitive to ICL-induced cytotoxicity and clastogenicity [16]. Therefore, we next assessed the ability of the FANCD2 NLS mutants to rescue the MMC sensitivity of FA-D2 cells. In a MMC clastogenicity assay, FA-D2 cells expressing the ΔN57 and ΔN100 NLS mutants exhibited markedly elevated levels of chromosome aberrations, including gaps and breaks, dicentrics, and radial formations, compared with FA-D2 cells complemented with wild type FANCD2 (p = 0.001 and 1.1 x 10^{-5}, respectively for 16 nM MMC) (**Fig. 5A**). In addition, in a MMC cytotoxicity assay, similar to FA-D2 cells expressing FANCD2-K561R [6], cells expressing the FANCD2-ΔN57 and -ΔN100 NLS mutants displayed increased MMC sensitivity compared with cells expressing wild type FANCD2 ($p < 0.05$ at 50 nM for FANCD2-ΔN57, -ΔN100, and -K561R compared with wild type FANCD2) (**Fig. S4**). Recent studies have demonstrated increased error-prone nonhomologous DNA end joining (NHEJ) in FA patient cells [17]. Therefore, we also examined the recruitment of DNA-PK_{CS} to nuclear foci in FA-D2 cells expressing LacZ, wild type

Figure 5. The FANCD2 NLS mutants fail to correct the ICL sensitivity of FA-D2 patient cells. (A) FA-D2 cells stably expressing LacZ, FANCD2-WT, FANCD2-ΔN57, FANCD2-ΔN100, FANCD2-3N, or FANCD2-K561R were incubated in the absence or presence of 8 or 16 nM MMC for 24 h and the numbers of chromosome aberrations including gaps and breaks, dicentrics, and complex chromosome aberrations, including radial formations, were scored. Metaphase spreads were analyzed using a Zeiss AxioImager.A1 upright epifluorescent microscope with AxioVision LE 4.6 image acquisition software. At least 80 metaphases were scored per treatment. Error bars represent the standard error of the means. (B) FA-D2 cells stably expressing LacZ, FANCD2-WT and FANCD2-ΔN57 were incubated in the absence (NT) or presence of 40 nM MMC for 18 h, and allowed to recover for 0, 4.5 or 7 h. Cells were then fixed, stained with rabbit polyclonal anti-DNA-PK_{CS} pS2056, and counterstained with DAPI. At least 300 cells were scored for nuclei with > 5 DNA-PK_{CS} foci. Error bars represent the standard error of the means from two independent experiments. ***, $p < 0.001$.


A
Chromosome aberrations per metaphase
1.6
1.2
0.8
0.4
0.0
0
8 nM MMC
16 nM MMC
lacZ
D2-WT
D2-ΔN57
D2-ΔN100
D2-3N
D2-K561R
B
% Nuclei with > 5 DNA-PKcs pS2056 foci
40
30
20
10
0
LacZ
D2-WT
D2-ΔN57
***
***
***
NT
0
4.5
7.0
Time after MMC treatment (h)

FANCD2 or the FANCD2 ΔN57 mutant, using an antibody raised against DNA-PK_{CS} phosphorylated on S2056, a marker of NHEJ [17,18]. Persistent increased DNA-PK_{CS} pS2056 nuclear foci formation was observed in FA-D2 cells expressing LacZ following treatment with MMC, and this phenotype was rescued by wild type FANCD2 (**Fig. 5B**). In contrast, we observed markedly increased MMC-inducible DNA-PK_{CS} pS2056 nuclear foci formation in FA-D2 cells expressing FANCD2 ΔN57, compared to cells expressing LacZ or wild type FANCD2 (**Fig. 5B**). Taken together, these results demonstrate that the FANCD2 NLS is essential for the correct function of the FA-BRCA pathway in the cellular ICL response.

Discussion

Despite their critical role in the cellular ICL response and their tumor suppressor function, very little is known about the structure, function, and regulation of the FANCD2 and FANCI proteins. For FANCD2, a large 1451 amino acid protein, only two functional motifs, a PCNA-interaction motif, or PIP box, and a carboxy-terminus EDGE motif, have been described to date [10,19]. Our laboratory has also recently identified and characterized a CUE ubiquitin-binding domain in the amino-terminus of FANCD2, which mediates noncovalent binding to ubiquitin, and is essential for efficient cellular ICL repair [20]. In this study we describe the functional characterization of an amino terminal FANCD2 NLS. While a previous study reported the existence of a FANCD2 NLS, this study failed to examine the functional consequences of its disruption in a FA-D2 patient-derived cell system [21]. Here, we demonstrate that fusion of amino acids 1-58 of FANCD2 to the amino terminus of

GFP drives its exclusive nuclear localization. cNLS mapper identified several high scoring putative bipartite NLSs within this region, in particular, amino acids 2-27 and 24-55. However, in contrast to amino acids 1-58, fusion of either sequence to GFP failed to drive exclusive nuclear GFP expression. Furthermore, mutation of K4, R5, and R6, the most highly conserved block of basic amino acids within this region, reduced, but did not abrogate, nuclear FANCD2 localization in FA-D2 cells. These results establish that the functional NLS elements are harbored within the amino terminal 58 amino acids. While the classical bipartite NLS comprises two clusters of basic amino acids separated by a 10-12 amino acid linker region, exemplified by the NLS of nucleoplasmin [22,23], unconventional bipartite NLSs with extended linker lengths have also been described [24-26]. However, cNLS mapper searches for both conventional and unconventional bipartite NLSs and only detected the former [12]. In addition to monopartite and bipartite NLSs, at least two other classes of NLS have been described: tripartite containing three clusters of basic amino acids similar to those found in L-periaxin and the epidermal growth factor receptor (EGFR) family [27,28], as well as NLSs containing dispersed basic residues within a random coil structure such as that found for 5-lipoxygenase [29]. These NLSs are poorly characterized in comparison with their mono- and bi-partite counterparts and are not predicted by cNLS mapper or PSORT II amino acid prediction algorithms. While the crystal structure of the murine Fanci-Fancd2 heterodimer (ID2) has been solved, the majority of the NLS described in this study was not crystallized precluding speculation about the structure of this region [30]. It is also important to note that FANCD2 harbors several putative phosphorylation sites within the amino terminal 58

amino acids (PhosphoSitePlus), which may also contribute to the regulation of its nuclear localization [31].

Our studies suggest that FANCD2 is imported to the nucleus *via* an importin α/β-dependent mechanism as treatment with ivermectin, a broad-spectrum inhibitor of importin α/β-dependent nuclear import [13], results in markedly decreased exclusive nuclear localization of D2-NLS-GFP. Furthermore, using mass spectrometry we have recently detected importin β1, as well as the nuclear pore complex proteins NUP160 and NUP155, in FANCD2 immune complexes (**Supplemental Table 1**). In summary, our functional analyses have revealed the following important points: 1) the NLS is necessary for the nuclear localization of FANCD2, 2) the FANCD2 NLS is required for the nuclear localization of a subset of FANCI, 3) the NLS is necessary for the efficient monoubiquitination of both FANCD2 and FANCI, and 4) the NLS is required for the localization of both FANCD2 and FANCI in chromatin. Consequently, FA-D2 cells expressing FANCD2 NLS deletion mutants are defective in the repair of ICLs. Our studies provide additional important insight into the domain structure of FANCD2, and suggest a novel FANCD2-dependent piggyback mechanism of FANCI nuclear import. Furthermore, our results suggest that FANCD2 and FANCI are targeted to the nucleus as a heterodimer. These findings lend important insight into the structure and regulation of two poorly characterized tumor suppressor proteins with key early roles in the cellular ICL response.

Here we have established that FANCI is, at least partially, dependent on FANCD2 for both its nuclear localization and chromatin association: In FA-D2 patient cells, as well as FA-D2 cells expressing the FANCD2 NLS mutants, FANCI localized

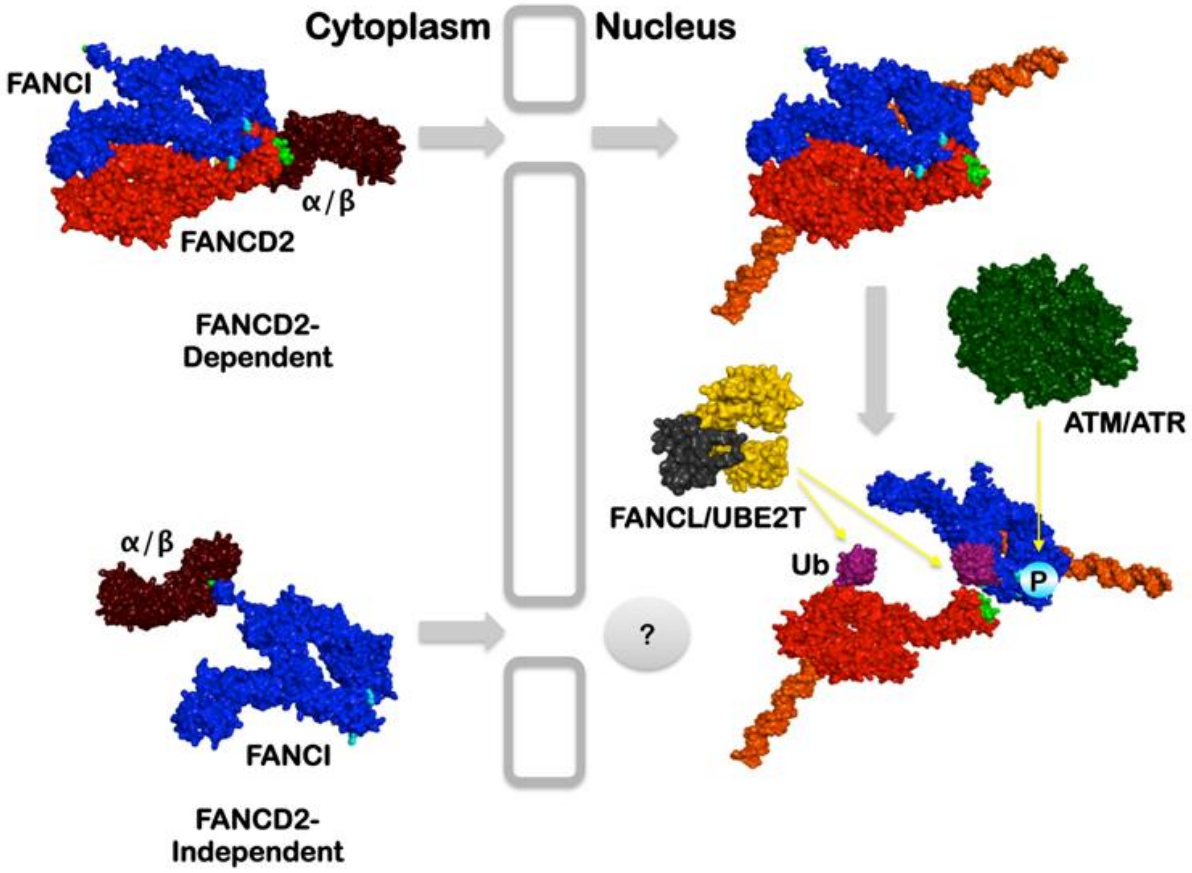
diffusely to the cytoplasm and nucleus. The introduction of wild type FANCD2 into these cells resulted in a large increase in exclusively nuclear FANCI as well as its chromatin localization, particularly following exposure to MMC. In contrast, we, and others, have observed robust nuclear localization of FANCD2 in FA-I cells, indicating that FANCD2 is not dependent on FANCI for its nuclear localization [32]. A previous study of the patient-derived FANCI R1299X nonsense mutant, which lacks its carboxy-terminal 30 amino acids, demonstrated that FANCI harbors a monopartite NLS in this region [32]. While loss of this NLS reduced FANCI nuclear accumulation, this NLS was not completely necessary for FANCI or FANCD2 nuclear accumulation, strongly suggesting the existence of alternative nuclear import mechanisms for both proteins, consistent with our data [32]. The elucidation of the crystal structure of the ID2 heterodimer indicates that the FANCD2 and FANCI NLSs are spatially separated within this structure [30], arguing against the simultaneous contribution of both NLSs to nuclear import of the ID2 complex. Taken together, these results suggest that FANCI localizes to the nucleus *via* FANCD2-independent and -dependent mechanisms (**Fig. 6**). These findings are also consistent with the observation that only a minor fraction of the cellular pools of FANCD2 and FANCI physically interact [8,9], reinforcing the concept of ID2 complex-independent functions for both proteins, such as that recently described by Chaudhury and colleagues [33]. A recent study has also established that a fraction of FANCD2 is transported to the nucleus following MMC exposure *via* an indirect interaction with importin 4 (IPO4), which is mediated by the C/EBPδ transcription factor [34]. While clearly important for ICL repair, this mechanism in unlikely to be the major mechanism of FANCD2 nuclear import as

robust levels of nuclear FANCD2 were observed in C/EBPδ-null mouse embryonic fibroblasts as well as cells depleted of IPO4 and C/EBPδ [34]. Nevertheless, this C/EBPδ/IPO4-dependent FANCD2 nuclear import mechanism could account for the low levels of nuclear FANCD2-ΔN57 and FANCD2-ΔN57 observed in our studies.

Interestingly, we observed markedly increased MMC-inducible chromosome aberrations and DNA-PK_{CS} pS2056 nuclear foci formation in FA-D2 cells expressing FANCD2-ΔN57, compared to FA-D2 cells expressing LacZ. These results suggest that the FANCD2-ΔN57 mutant may act in a dominant-negative manner. The FA-D2 patient-derived cells used in this study are compound heterozygous for *FANCD2* mutations (*see Materials and Methods*). This variant is detectable by immunoblotting (*see* **Fig. 4A, top panel**) and is predicted to retain residual or partial function. Indeed, the vast majority of FA-D2 patient-derived cells retain residual FANCD2 function with complete loss of FANCD2 predicted to result in embryonic lethality [15]. Our results suggest that the FANCD2-ΔN57 mutant interferes with residual FANCD2 R1236H function, perhaps competing with FANCD2 R1236H for heterodimerization with FANCI, or in a manner analogous to missense *p53* mutations, by assembling into nonfunctional homo-oligomers, the formation of which has been suggested by previous studies [30,35].

Based on our findings, and those of several other groups, we propose the following model for early steps in the FA-BRCA pathway of ICL repair (**Fig. 6**). A subset of the total cellular pools of FANCD2 and FANCI associate in the cytoplasm to assemble into the ID2 heterodimer. The ID2 heterodimer is transported to the nucleus most likely *via* an importin α/β-mediated transport process, using the amino terminal

Figure 6. FANCD2-dependent and -independent mechanisms of FANCI nuclear localization. We propose that a subset of FANCI (blue) associates with FANCD2 (red) in the cytoplasm, and that the ID2 heterodimer is transported to the nucleus *via* an importin α/β (brown)-mediated transport mechanism, using the amino terminal FANCD2 NLS (light green). Nuclear ID2 binds to DNA (orange) and is also phosphorylated by the ATM/ATR kinases (dark green). One or both of these events may trigger ID2 complex restructuring, facilitating FANCD2 and FANCI monoubiquitination by FANCL (black), UBE2T (yellow) and the FA core complex (not shown).


Cytoplasm
Nucleus
FANCI
α/β
FANCD2
FANCD2-
Dependent
ATM/ATR
FANCL/UBE2T
Ub
P
α/β
?
FANCI
FANCD2-
Independent

NLS of FANCD2. Once inside the nucleus the ID2 heterodimer is targeted to sites of ICL damage possibly *via* the association of FANCD2 with PCNA and the replication fork machinery [19]. Recent *in vitro* studies have demonstrated that FANCI binding to DNA is necessary for robust stimulation of the monoubiquitination of FANCD2 [36]. However, analysis of the ID2 crystal structure indicates that the FANCD2 K561 side chain, the site of monoubiquitination, is embedded within the ID2 interface [30]. Furthermore, a solvent accessible tunnel adjacent to FANCD2 K561 is predicted to be too small to accommodate the active site of the UBE2T ubiquitin-conjugating enzyme [30,37]. Therefore, either 1) monoubiquitination occurs on FANCD2 and FANCI monomers prior to ID2 heterodimerization or 2) binding of the ID2 complex to DNA leads to a conformational change in the ID2 structure leading to the exposure of K561R and FANCI K523, and their subsequent monoubiquitination, as has been proposed [36]. A recent study by Sareen and colleagues suggests that activation of the FA-BRCA pathway coincides with dissociation of FANCD2 and FANCI [38]. ID2 dissociation is triggered by ATR/ATM-mediated phosphorylation of a cluster of at least six FANCI SQ motifs, and is followed by the monoubiquitination of FANCD2 [38,39]. Once monoubiquitinated, FANCD2 can then facilitate the recruitment of several structure specific nucleases, including FAN1 and FANCP/SLX4, initiating the process of ICL removal [40-46].

Materials and Methods

Cell culture

COS-7, HeLa, and IMR90 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 12% v/v FBS, L-glutamine and penicillin/streptomycin. 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*$^{hy/-}$) 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 [14]. To generate stable lines expressing wild type or mutant FANCD2, FA-D2 cells were infected with pLenti6.2-FANCD2 (Invitrogen) lentivirus, followed by selection in DMEM supplemented with 12% v/v FBS, L-glutamine, penicillin/streptomycin and 2 μg/ml blasticidin. KEAE FA-D2 cells and KEAE FA-D2 + FANCD2 cells were a kind gift from Detlev Schindler of the University of Würzburg [15]. These cells were telomerase immortalized using pBABE-hTERT and grown in DMEM supplemented with 12% v/v FBS, L-glutamine, penicillin/streptomycin and 0.75 μg/ml puromycin.

Antibodies and immunoblotting

For immunoblotting analysis, cell pellets were washed in PBS and lysed in 2% w/v SDS, 50 mM Tris-HCl, 10 mM EDTA. 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 (Dr. Patrick Sung, Yale University), H2A (07-146; Millipore), and mouse monoclonal sera against α-tubulin (MS-581-PO; Neomarkers), GFP (sc-9996; Santa Cruz), and V5 (R96025; Invitrogen).

Immunofluorescence microscopy

For immunofluorescence microscopy (IF) analysis, cells were seeded in 4-well tissue culture slides (BD Falcon) and treated with mitomycin C (MMC) for 18 h. Soluble cellular proteins were pre-permeabilized with 0.3% v/v Triton X-100 and cells were fixed in 4% w/v paraformaldehyde and 2% w/v sucrose at 4°C followed by permeabilization in 0.3% v/v Triton X-100 in PBS. Fixed cells were blocked for 30 minutes in antibody dilution buffer (5% v/v goat serum, 0.1% v/v NP-40, in PBS) and incubated with primary antibody for 1 h. Cells were washed three times in PBS, as well as permeabilization buffer, and incubated for 30 min at room temperature with an Alexa Fluor 488-conjugated secondary antibody combined with Texas Red labeled phalloidin. The slides were counterstained and mounted in vectashield plus 4'6-diamidine-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories). Nuclear foci were analyzed using a Zeiss AxioImager.A1 upright epifluorescent microscope with AxioVision LE 4.6 image acquisition software. Primary antibodies used for IF were anti-FANCD2 (NB100-182; Novus Biologicals), anti-FANCI (Dr. Patrick Sung, Yale University), anti-DNA-PK_{CS} pS2056 (ab18192; Abcam), and anti-V5 (R960-25; Invitrogen).

Plasmids, site-directed mutagenesis, and transient transfections

The full length, ΔN57, and ΔN100 *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. The *FANCD2-KRR4NNN* (*FANCD2-3N*) cDNA was generated by site-directed mutagenesis of the wild type *FANCD2* cDNA using the Quikchange Site-directed Mutagenesis Kit (Stratagene). The forward and reverse oligonucleotide sequences used are as follows: FP, 5'-TTCACCATGGTTTCCAACAACAACCTGTCAAAATCTGAGG-3'; RP, 5'-CCTCAGATTTTGACAGGTTGTTGTTGGAAACCATGGTGAA -3'. The FANCD2 GFP fusion vectors D2-1-27-GFP, D2-24-55-GFP, and D2-1-58-GFP were generated by PCR amplifying the coding sequences of amino acids 1-27, 24-55, or 1-58 of FANCD2 and directionally cloning these fragments into the multiple cloning site of pEGFP-N1 (Clontech). The FANCI-GFP construct was a gift from Tony Huang in the Department of Biochemistry at New York University School of Medicine. COS-7, HeLa, and IMR90 cells were transiently transfected with plasmid DNA using Fugene 6 (Roche) at a 1:3 ratio (μg DNA:μL Fugene 6) in Opti-MEM. After incubating for 24 h, GFP fluorescence was monitored using a Zeiss AxioImager X-Cite series 120Q inverted fluorescence microscope with AxioVision LE 4.8 image acquisition software. Ivermectin (Sigma) was added to a final concentration of 25 μM 4 h following transfection.

Cellular fractionation

Soluble proteins were removed by extraction in cytoskeletal buffer (CSK) (10 mM PIPES pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% v/v Triton-X-100) for 10 minutes at 4^{o}C. Pellets were washed once with CSK buffer, lysed in SDS sample buffer (2% w/v SDS, 50 mM Tris-HCl pH 7.4, 10 mM EDTA), boiled for 15 min, followed by sonication for 10 s at 10% amplitude using a Fisher Scientific Model 500 Ultrasonic Dismembrator.

Chromosome breakage analysis

Cells were incubated in the absence or presence of MMC for 18 h. Prior to harvesting, cells were treated with 0.1 ug/ml Colcemid (Gibco/Invitrogen) for 2 h; pellets were then incubated in 0.075 M KCl at 37°C for 18 min, followed by fixation in Carnoy's fixative (3:1 methanol:glacial acetic acid). Cells were dropped onto chilled slides, air-dried, and then stained with 2.5% w/v Giemsa solution (Sigma). Metaphases were analyzed using a Zeiss AxioImager.A1 upright epifluorescent microscope with Axio Vision LE 4.6 image acquisition software.

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Author Contributions

RAB, MAR, and NGH conceived and designed the experiments. RAB, MAR, PAA, and MM performed the experiments and analyzed the data. RAB and NGH wrote the manuscript.

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Manuscript – III

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Characterization of a Putative CDK Phosphorylation Site Cluster in the FANCD2 Protein

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Characterization of a Putative Cyclin Dependent Kinase Phosphorylation Site Cluster in FANCD2

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Running title: FANCD2 Phosphorylation by Cyclin Dependent Kinase

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Abstract

Fanconi anemia (FA) is a rare genetic disease characterized by bone marrow failure, congenital defects, and increased cancer susceptibility. FA is caused by mutation in any of 19 known genes. The protein products of these genes function collectively in the FA-BRCA pathway to repair DNA interstrand-crosslinks (ICLs). Upon exposure to ICL-inducing agents, the FA core complex catalyzes the monoubiquitination of the FANCD2 and FANCI proteins, activating these proteins for ICL repair. FANCD2 is also posttranslationally modified by phosphorylation following exposure to DNA damaging agents and upon disruption of DNA replication. The DNA damage-inducible posttranslational regulation of the FA pathway has been extensively studied, however, few studies have explored the regulation of the FANCD2 and FANCI proteins independent of DNA damage. In this study, we have determined that FANCD2 is phosphorylated in a DNA damage-independent manner, which appears to be mediated by CDK and is maximal during S phase, coinciding with FANCD2 monoubiquitination. We have also identified a putative CDK phosphorylation site cluster proximal to K561. Phosphorylation at this cluster has a negative regulation of FANCD2 monoubiquitination. We hypothesize that CDK-mediated FANCD2 phosphorylation is critical for the efficient regulation of FANCD2 function during S phase.

Introduction

Fanconi anemia (FA) is a rare recessive disease, characterized by congenital abnormalities, progressive pediatric bone marrow failure, and heightened cancer

susceptibility [1]. FA is caused by mutation in any one of 19 genes (*FANCA, -B, -C, -D1/BRCA2, -D2, -E, -F, -G, -I, -J/BRIP1, -L, -M, -N/PALB2, -P/SLX4, O/RAD51C, Q/ERCC4, R/RAD51, S/BRCA1, T/UBE2T*) [2-4]. The FA proteins function cooperatively in the FA-BRCA pathway to repair DNA interstrand crosslinks (ICLs) and to prevent chromosome instability. The core complex, comprised of FANCA/B/C/E/F/G/L, along with the E2 conjugating enzyme FANCT, FANCM, FAAP100/24/20, and MHF1/2 catalyze the monoubiquitination of the FANCD2 and FANCI proteins [4,5]. This central activation step promotes FANCD2 and FANCI co-localization to discrete nuclear foci with several downstream DNA repair proteins, including FANCS/BRCA1 and NBS1 [6-9]. Monoubiquitinated FANCD2 has been shown to recruit the structure specific endonucleases FAN1 and FANCP/SLX4, which suggests that this modified form of FANCD2 functions to promote one or more incision steps during the ICL repair process [10,11]. FANCD2 and FANCI are also posttranslationally modified by phosphorylation in response to DNA damage by ATM (*A*taxia-*t*elangiectasia *m*utated) and ATR (*AT*M and *R*ad3-related). ATM phosphorylates FANCD2 in response to IR exposure independently of monoubiquitination [12]. Phosphorylation of FANCD2 by ATR promotes FANCD2 monoubiquitination in response to exposure to ICL-inducing agents and is important in its function in ICL repair [13]. FANCI phosphorylation by ATM/ATR on six SQ/TQ residues has been linked to activation of the FA-BRCA pathway, potentially by promoting the dissociation of the FANCD2-FANCI heterodimer [14,15].

Although the main focus concerning FANCD2 has been based on DNA damage activation, FANCD2 has also been shown to be monoubiquitinated during

unperturbed cell cycle progression in S phase, where it co-localizes with BRCA1 and RAD51 [16]. With the addition of hydroxyurea (HU), which arrests DNA replication, FANCD2 has been shown to protect stalled replication forks from degradation by MRE11, and to promote replication restart at stalled replication forks by recruiting and interacting with CtIP (CtBP-interacting protein) [17,18]. These studies suggest that FANCD2 has many roles outside of the more recognized DNA damage inducible function, specifically during the cell cycle.

In this study, we have determined that FANCD2 is phosphorylated in a DNA damage-independent manner, which we believe is essential for the regulation of FANCD2 function during unperturbed S phase. Many DNA repair proteins are regulated by cyclin-dependent kinase (CDK) mediated phosphorylation, such as BRCA1 and NBS [19,20]. Here we show that FANCD2 is a substrate of CDK through immunoprecipitation studies as well as CDK inhibitor experiments. Importantly, we have discovered and are beginning to characterize a putative CDK site cluster in FANCD2 that is positioned proximal to FANCD2s site of monoubiquitination. Missense mutations of these sites yields unexpected results, a phospho-mimetic mutant fails to induce a response to DNA replication or damaging agents and a phospho-dead mutant is constitutively activated. The findings from this study establish novel phosphorylation of FANCD2 in unperturbed S phase and reveal a CDK phosphorylation site cluster that is posttranslationally modified to negatively regulate FANCD2 monoubiquitination.

Results

FANCD2 is phosphorylated in the absence of DNA damage

Initial findings from a previous study using FANCD2 patient cells expressing FANCD2-WT, FANCD2-K561R, and truncation mutants, suggested that FANCD2 may be phosphorylated in the absence of DNA damage (data not shown). In order to analyze the phosphorylation of FANCD2 under these conditions, a lambda phosphatase assay was performed. Lambda phosphatase cleaves phosphorylated serine, threonine, and tyrosine residues [21]. HeLa, U2OS, COS7, and BJ-TERT cells were treated with and without MMC and lysates were incubated in the presence and absence of lambda phosphatase (**Fig. 1A**). When comparing incubation with and without lambda phosphatase, the electrophoretic mobility of FANCD2 increases in both the presence and absence of MMC, indicating that FANCD2 is phosphorylated independent of DNA damage. It is interesting to note that this is not seen with FANCI.

To ensure this finding is due to phosphorylation, a lambda phosphatase assay was done using HeLa, FA-D2 patient-derived cells stably express the empty vector, FANCD2-WT, and FANCD2-K561R, which were treated for the indicated times with lambda phosphatase (**Fig. 1B**). Reaction products were then run on a Phos-tag gel; Phos-tag is a novel phosphate-binding tag that binds phosphorylated proteins [22]. Treatment with lambda phosphatase causes the stabilization of non-phosphorylated FANCD2 and limits the isoforms present when compared to lack of treatment. To ensure the molecular weight difference in FANCD2 was due to the cleavage of phosphate groups and not an unforeseen interaction with the lambda phosphatase enzyme, protein phosphatase 1 (PP1) enzyme was used (**Fig. 1C**). This enzyme

Figure 1. FANCD2 is phosphorylated in the absence of DNA damage. (**A**) HeLa, U2OS, COS-7, and BJ-TERT cells were incubated in the absence (-) and presence (+) of 250 nM MMC for 18 h and whole-cell lysates were subsequently treated with (+) and without (-) lambda phosphatase for 2 h 30°C and immunoblotted with antibodies against FANCD2, FANCI, and α-tubulin. (**B**) HeLa, FA-D2 cells with empty plasmid or stably expressing FANCD2-WT, and FANCD2-K561R were incubated with lambda phosphatase for 2, 4, 16 h at 30°C and run on a SuperSep Phos-tag gel. They were immunoblotted against FANCD2 and V5. (**C**) HeLa and U2OS cells were incubated with lambda phosphatase or PP1 for 2 h at 30°C and immunoblotted against FANCD2. Δ↓, molecular weight shift difference between nonphosphorylated and phosphorylated FANCD2.

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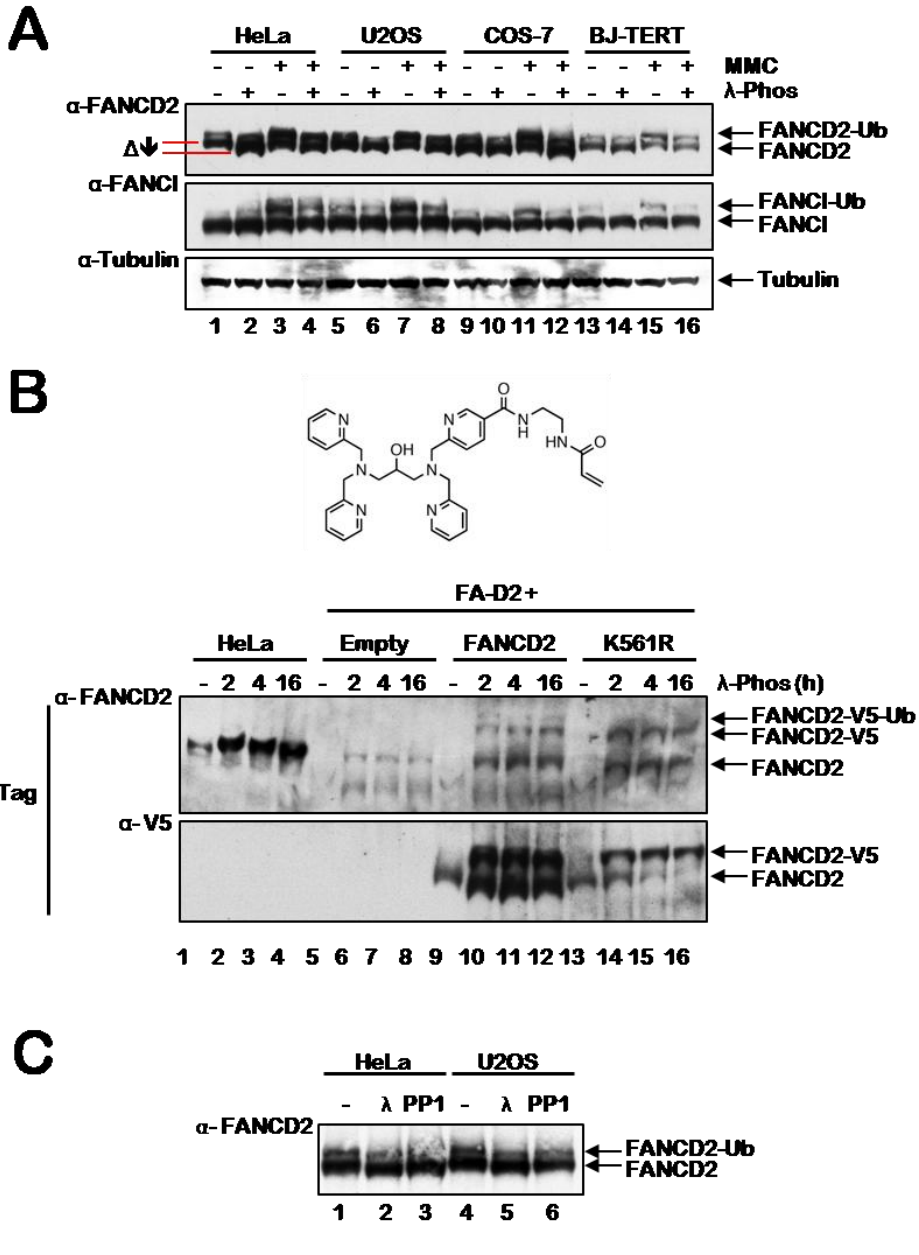
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B
FA-D2+
HeLa
Empty
FANCD2
K561R
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α-FANCD2
Phos-Tag
α-V5
FANCD2-V5-Ub
FANCD2-V5
FANCD2
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U2OS
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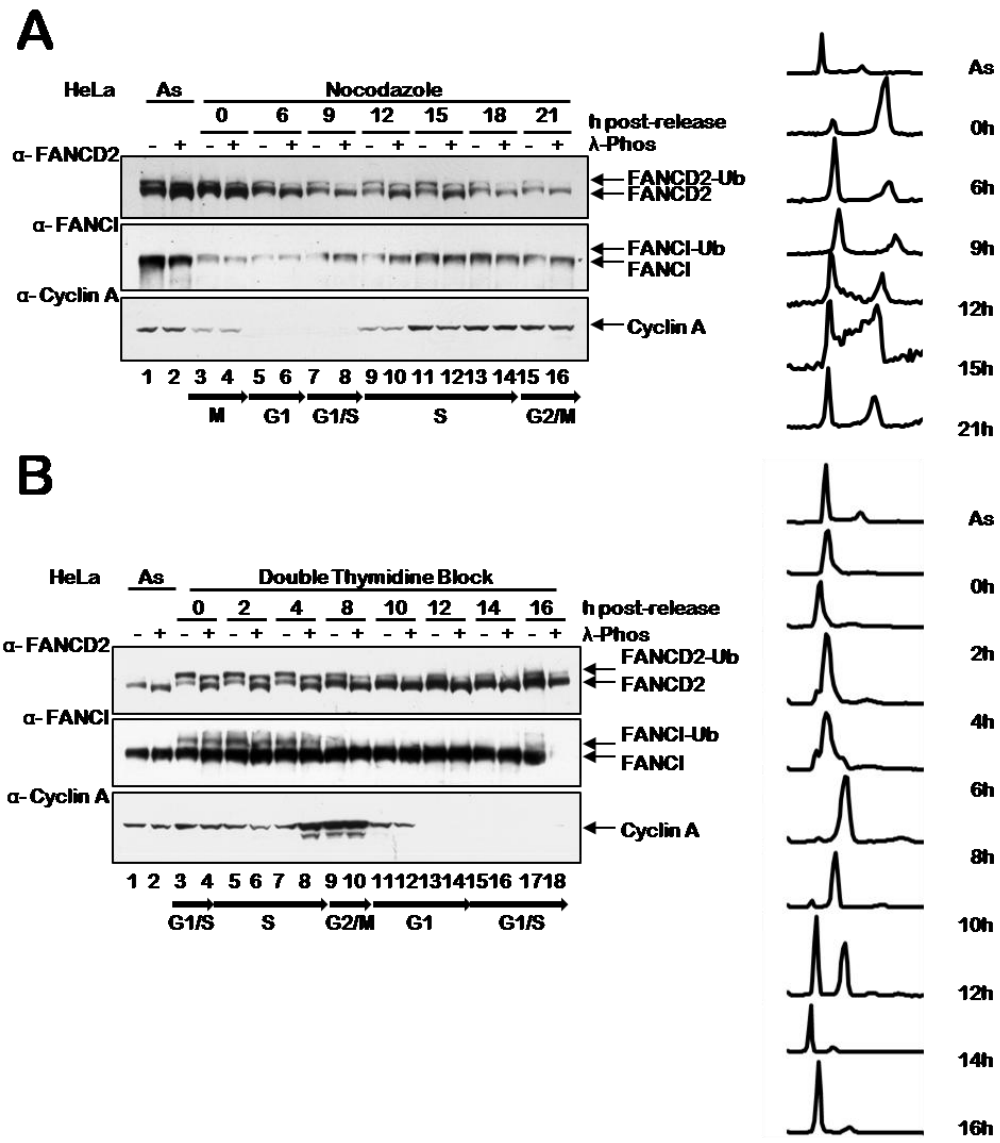
cleaves phosphates from serine and threonine residues. Much like lambda phosphatase, PP1 also caused FANCD2 to migrate faster in the gel in both HeLa and U2OS cells, indicating a true phosphorylation pattern. Taken together, this data indicates that FANCD2 is phosphorylated on several residues, in a DNA damage-independent manner.

FANCD2 phosphorylation is maximal in S phase

It is known that FANCD2 is activated through monoubiquitination during S phase of the cell cycle; however, the regulation behind this cycle dependent posttranslational modification is currently unknown [16]. To determine if FANCD2 is differentially phosphorylated throughout the cell cycle, several cell synchronizations were performed. Using HeLa cells, a nocodazole block was performed, blocking the cells in early M phase (**Fig. 2A**). Cells were then released for the indicated times and cell pellets were separated into three, treating with and without lambda phosphatase for 2 h, and the third was fixed and stained with propidium iodide for FACS analysis. Although there is a molecular weight shift difference when looking at FANCD2 *via* western blot, this shift in migration is most prominent at 12 and 15 h post-release, correlating with S phase of the cell cycle. Again, when probing for FANCI, no molecular weight shift was observed. Cyclin A was used as a cell cycle marker, as it is active and upregulated in S and G2 phases of the cell cycle [23]. To explore FANCD2 phosphorylation during the cell cycle, a second synchronization method was performed. Using both HeLa (**Fig. 2B**) and U2OS cells (**Fig. S1**), a double thymidine block arrest was used to synchronize in G1/S phase of the cell cycle. A lambda

Figure 2. FANCD2 phosphorylation is maximal in S phase. (**A**) HeLa cells were synchronized by nocodazole block or double thymidine block (**B**). The cell cycle phase was determined by flow cytometric analysis of DNA content and whole-cell lysates were incubated in the absence (-) and presence (+) of lambda phosphatase for 2 h at 30°C and immunoblotted with antibodies against FANCD2, FANCI, and Cyclin A. AS, asynchronous whole-cell lysate.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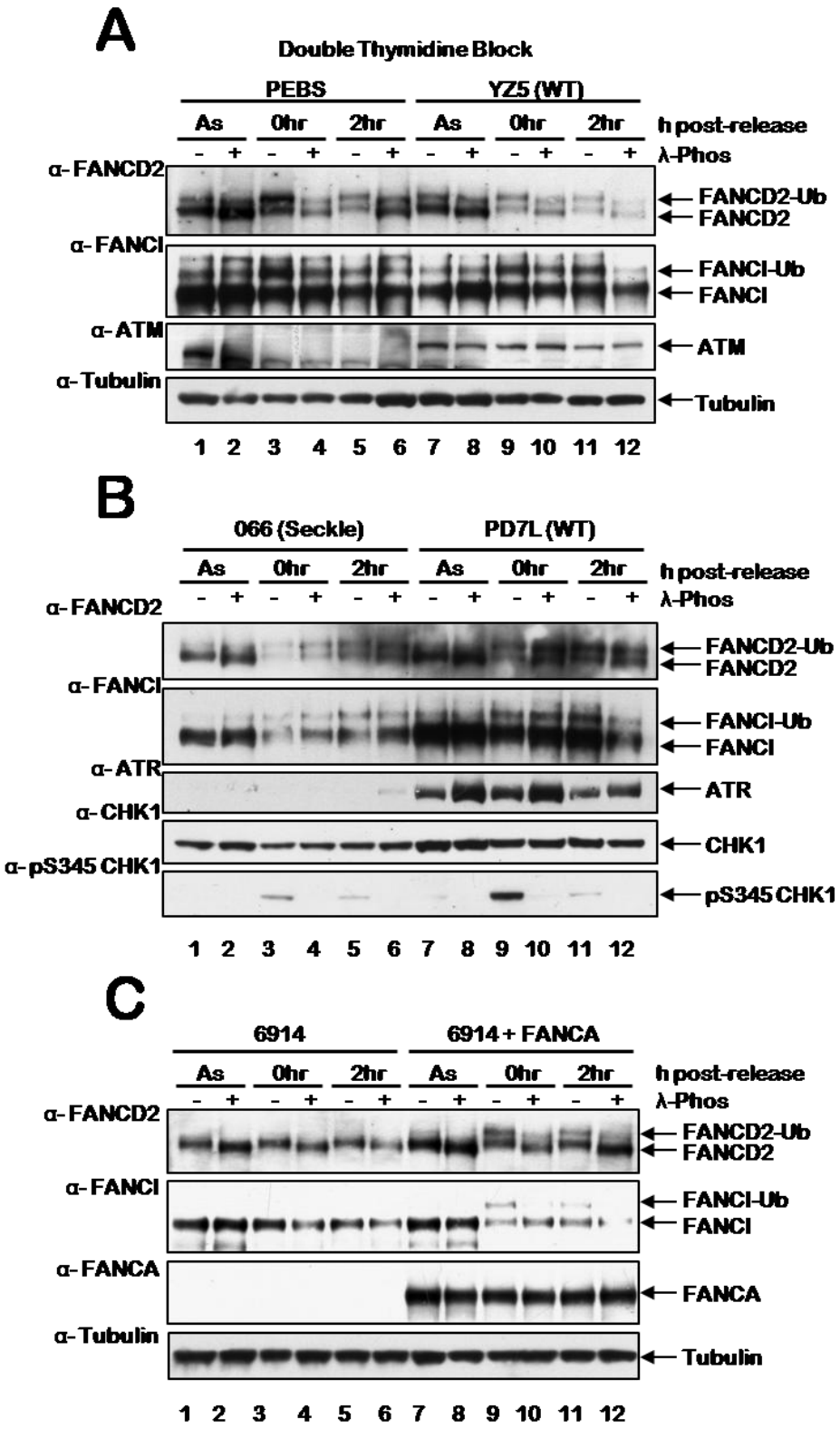
14h
16h

phosphatase assay and FACs analysis was then performed, and in a similar manner as the nocodazole block, a maximal molecular weight shift difference is seen 0-4 h post-release. This is indicative of S phase of the cell cycle, suggesting there is a DNA damage independent phosphorylation of FANCD2 that correlates with its monoubiquitination.

Non-DNA damage inducible phosphorylation of FANCD2 is independent of ATM, ATR, and the core complex member FANCA

It is known that FANCD2 and FANCI are phosphorylated by the DNA damage response kinases ATM and ATR [12-14]. To test if ATM was the kinase responsible for the phosphorylation of FANCD2 in S phase seen *via* the lambda phosphatase assay, PEBS (ATM-/-) and YZ5 (ATM+/+) fibroblasts were used for a double thymidine block and subsequent lambda phosphatase assay, as described previously (**Fig. 3A**). If ATM is essential for this posttranslational modification, the molecular weight difference in FANCD2 treated with and without lambda phosphatase would be absent or reduced. In comparing PEBS and YZ5 cells, there is no difference in FANCD2s electrophoretic mobility with the addition of the lambda phosphatase enzyme, indicating this phosphorylation is largely independent of ATM. To determine ATRs importance in FANCD2s phosphorylation in the absence of DNA damage and in S phase of the cell cycle, 066 Seckel patient lymphoblasts (ATR-/-) and wild-type lymphoblasts were then used (**Fig. 3B**). Similar to the results seen in ATM deficient cells, ATR does not appear to be crucial for this mobility in FANCD2. The FA core complex is responsible for orchestrating FANCD2s monoubiquitination;

Figure 3. Non-DNA damage inducible phosphorylation of FANCD2 is independent of ATM, ATR, and the core complex member FANCA. (**A**) PEBS (ATM-/-) and YZ5 (ATM+/+) fibroblasts were synchronized by double thymidine and released for 0 and 2 h followed by treatment with lambda phosphatase for 2 h at 30°C and immunoblotted with antibodies to FANCD2, FANCI, ATM, and α-tubulin. (**B**) 066 (ATR-/-) and PD7L (WT) lymphoblasts were synchronized by double thymidine block and released for 0 and 2 h followed by treatment with lambda phosphatase for 2 h at 30°C and immunoblotted with antibodies against FANCD2, FANCI, ATR, CHK1, and pS345 CHK1. (**C**) 6914 (FA-A) and 6914 + FANCA were synchronized by double thymidine block and released for 0 and 2 h followed by treatment with lambda phosphatase for 2 h at 30°C and immunoblotted with antibodies against FANCD2, FANCI, FANCA, and α-tubulin.


A
Double Thymidine Block
PEBS
YZ5 (WT)
As
0hr
2hr
As
0hr
2hr
h post-release
- + - + - + - + - + - +
λ-Phos
α-FANCD2
FANCD2-Ub
FANCD2
α-FANCI
FANCI-Ub
FANCI
α-ATM
ATM
α-Tubulin
Tubulin
1 2 3 4 5 6 7 8 9 10 11 12
B
066 (Seckle)
PD7L (WT)
As
0hr
2hr
As
0hr
2hr
h post-release
- + - + - + - + - + - +
λ-Phos
α-FANCD2
FANCD2-Ub
FANCD2
α-FANCI
FANCI-Ub
FANCI
α-ATR
ATR
α-CHK1
CHK1
α-pS345 CHK1
pS345 CHK1
1 2 3 4 5 6 7 8 9 10 11 12
C
6914
6914 + FANCA
As
0hr
2hr
As
0hr
2hr
h post-release
- + - + - + - + - + - +
λ-Phos
α-FANCD2
FANCD2-Ub
FANCD2
α-FANCI
FANCI-Ub
FANCI
α-FANCA
FANCA
α-Tubulin
Tubulin
1 2 3 4 5 6 7 8 9 10 11 12

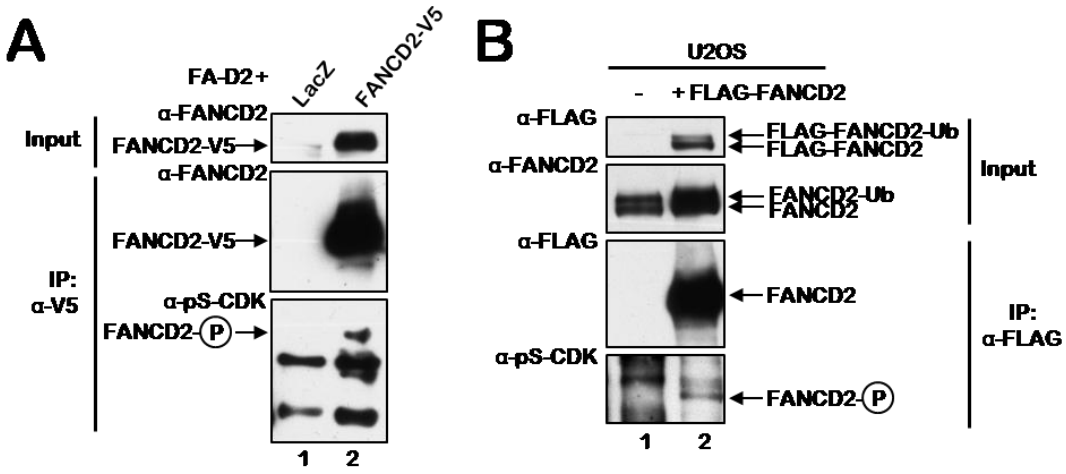
therefore, we next assessed the ability of FANCD2 to become phosphorylated in the absence of FANCA. FA-A (6914) and FA-A corrected cells (6914 + FANCA) were arrested in early S phase by double thymidine treatment, followed by incubation in the presence and absence of lambda phosphatase (**Fig. 3C**). As seen in the ATR and ATM deficient cells, in the absence of FANCA, there is still a phosphorylation shift visualized. These results combined suggest that the DNA damage response kinases are not responsible for FANCD2s phosphorylation in the cell cycle and this phosphorylation event occurs either upstream or independently of FANCD2s monoubiquitination. To corroborate FANCD2 phosphorylation acting upstream or independently of FANCD2s monoubiquitination, FA-D2 + empty, FANCD2, and K561R cell lines were used incubated with lambda phosphatase for 2, 4, and 16 h (**Fig. S2**). FANCD2 K561R is unable to become monoubiquitinated; however, a molecular weight shift difference is still seen. This indicates that monoubiquitination is not essential for FANCD2s phosphorylation.

FANCD2 is a potential substrate of CDK phosphorylation

Cell cycle progression is regulated through phosphorylation by CDKs [24]. We next assessed whether FANCD2 was a substrate of CDK phosphorylation by using

FA-D2 patient cells stably expressing LacZ-V5 or FANCD2-V5 and performing an immunoprecipitation (IP) (**Fig. 4A**). V5 agarose was used to pull down FANCD2-V5. A pan phospho serine antibody was then used, which recognizes phosphorylated substrates of CDK, and FANCD2 is seen in the FA-D2 patient cell line expressing FANCD2. A second IP was performed using a different cell system, U2OS cells stably

Figure 4. FANCD2 is a potential substrate of CDK phosphorylation. (**A**) FA-D2 cells stably expressing LacZ-V5 and FANCD2-V5 were immunoprecipitated with anti-V5 agarose and immune complexes were immunoblotted with anti-FANCD2 and anti-pSCDK. (**B**) U2OS and U2OS cells stably expressing 3X FLAG FANCD2-WT were immunoprecipitated with anti-FLAG agarose and immune complexes were immunoblotted with anti-FANCD2, anti-FLAG, and anti-pSCDK. (**C**) U2OS 3X FLAG FANCD2 immunoprecipiated as shown in (**B**), was used for phospho enrichment and further mass spectrometry analysis revealed several phosphorylated FANCD2 isoforms.



C

Protein	Phosphosite(s)	Peptide Length
FANCD2	S8	17
FANCD2	S126	15
FANCD2	S592	11
FANCD2	S1404	23
FANCD2	S1404 S1412	23
FANCD2	S1407 S1412	23
FANCD2	S1412	23
FANCD2	S1435	16

expressing 3XFLAG tagged FANCD2 (**Fig. 4B**). Here we used FLAG agarose and performed an IP with U2OS and U2OS 3X FLAG-FANCD2 fibroblasts. Similar results were obtained as with FA-D2 FANCD2-V5; FANCD2 is a substrate of CDK as seen through the pan phospho serine antibody. The U2OS 3XFLAG FANCD2 IP was then run on a commassie gel in triplicate and the FANCD2 bands were combined and a tryptic digestion was performed in preparation for mass spectrometry (**Fig. S3**). Using TiO_2 phosphopeptide enrichment, these samples were analyzed for phospho peptides (**Fig. 4C**). Several known ATM/ATR sites were found in this sample, as well as one serine protein site, S692, which resides in the phosphoCDK cluster proximal to K561. The CDK consensus sequence is [S/T*]PX[K/R], however, known DNA repair proteins, such as BRCA1, have been shown to be phosphorylated by CDK through only containing S/P of the consensus sequence.

Treatment with CDK inhibitor purvalanol A alters FANCD2s phosphorylation shift

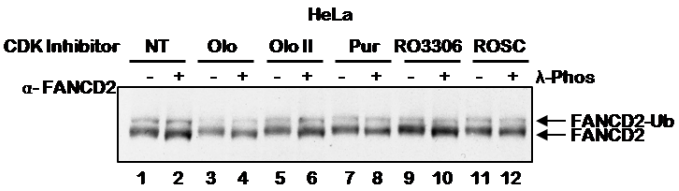
As it was determined that FANCD2 is a potential substrate of CDK, we wanted to establish which CDK was responsible. If maximal FANCD2 phosphorylation occurs during S phase of the cell cycle, CDK2 could be the potential kinase responsible. In order to determine a specific CDK was responsible for FANCD2s non-DNA damaging phosphorylation, various CDK inhibitors with differing specificities were utilized (**Fig. 5A**). HeLa cells were incubated with each indicated inhibitor for 24 h and a lambda phosphatase assay was performed to determine if a specific CDK is required for FANCD2s phosphorylation (**Fig. 5B**). Two chemicals that comprise CDK2

Figure 5. Treatment with CDK inhibitor purvalanol A alters FANCD2s phosphorylation shift. (**A**) Table depicting CDK inhibitors and their specific targets. (**B**) HeLa cells were treated for 24 h with 10 μM Olomoucine, 10 μM Olomoucine II, 10 μM Purvalanol A, 10 μM RO-3306, and 20 μM Roscovatine and whole-cell lysates were treated in the absence (-) and prescence (+) of lambda phosphatase for 2 h at 30°C and immunoblotted with antibodies against FANCD2. (**B**) HeLa cells were treated with 10 uM Purvalanol A and 10 uM RO-3306 for 2, 4, 8, 24 h and incubated with (+) and without (-) lambda phosphatase. Immunoblotting was performed and antibodies against FANCD2 and α-tubulin were used.

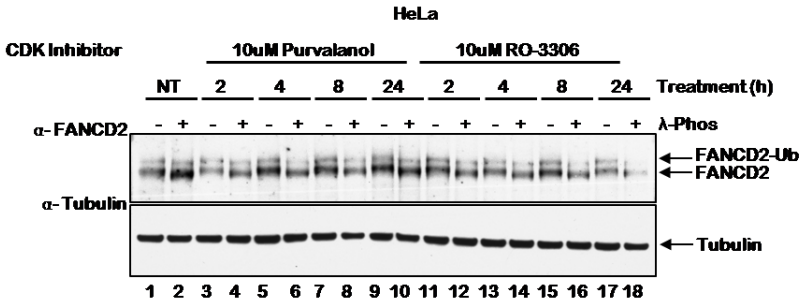
A

Inhibitor/CDK	CDK1	CDK2	CDK3	CDK4	CDK5	CDK6	CDK7	CDK8	CDK9
Olomoucine		X	X	X	X	X	X	X	X
Olomoucine II	X	X							
Purvalanol A	X	X							
RO3306	X								
Roscovotine	X	X			X		X		X
SNS032		X					X		X

B



C

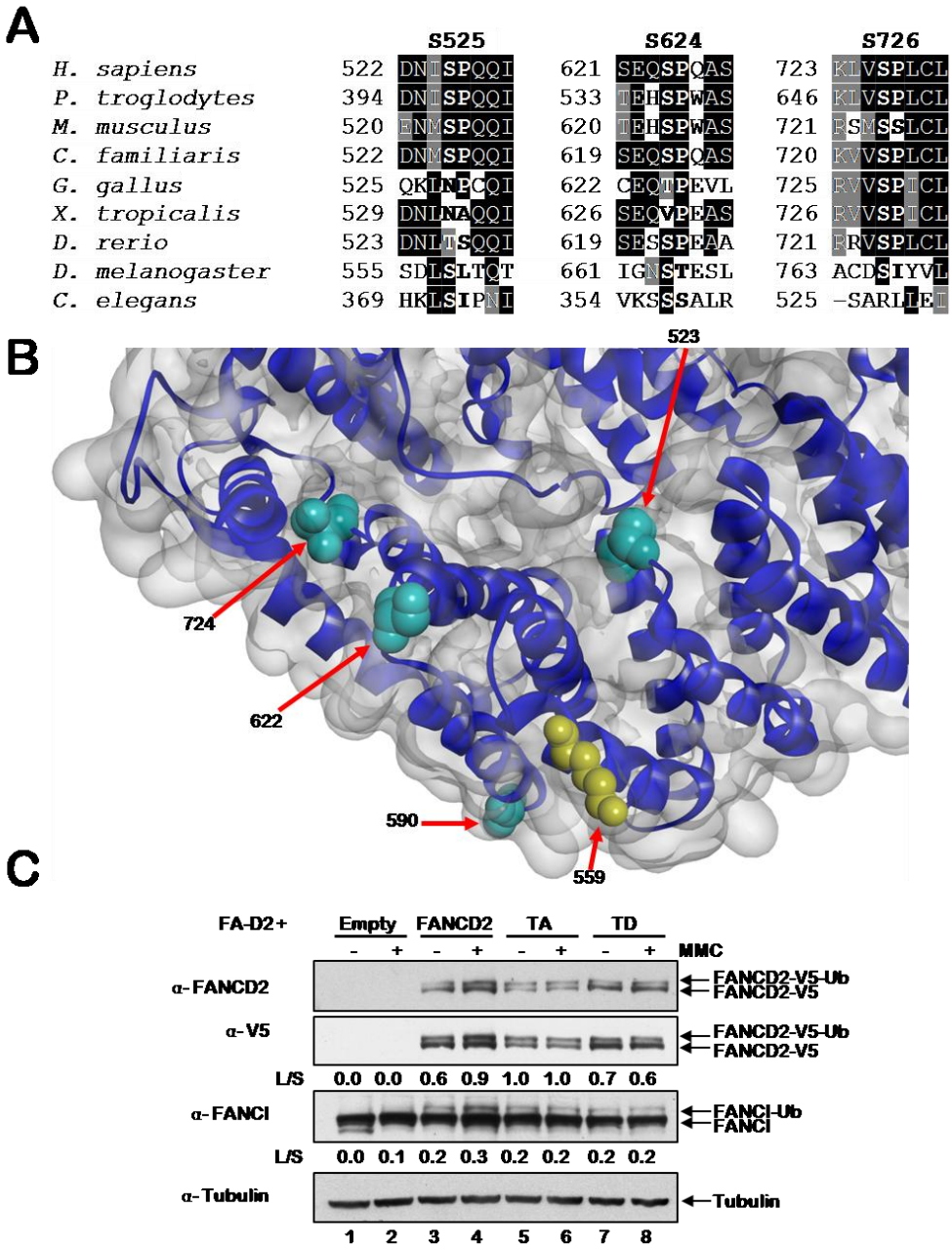


inhibition, Purvalanol A and Roscovitine, were shown to reduce FANCD2s mobility with the addition of the lambda phosphatase enzyme, indicating CDK2 could be needed. To further examine treatment with CDK inhibitors, HeLa cells were incubated for 2, 4, 8, or 24 h with either the CDK2 inhibitor Purvalanol A or the CDK1 specific inhibitor RO-3306 (**Fig. 5C**). A lambda phosphatase assay was then performed. As expected from the previous result, RO-3306 does not have an effect on FANCD2s phosphorylation. However, treatment with Purvalanol A, specifically for 4 and 8 h, depletes the mobility shift of FANCD2 with the addition of the enzyme. These results indicate CDK2 could be the potential kinase resulting in FANCD2s phosphorylation during S phase of the cell cycle.

FANCD2 contains a conserved putative CDK consensus site cluster

As the previous results suggest that FANCD2 is a substrate of CDK, the FANCD2 sequence was analyzed for potential CDK phosphorylation sites. A multiple sequence alignment of FANCD2 found strong evolutionary conservation in a cluster of three S/P sites: S525, S624, S726 (**Fig. 6A**). Interestingly, when looking at the murine crystallized structure of FANCD2, this putative CDK cluster is situated in close proximity to FANCD2s monoubiquitination site (**Fig. 6B**). As previous results have established a connection between maximal FANCD2 phosphorylation occurring during S phase, which is when FANCD2 is monoubiquitinated during the cell cycle, the position could shed light on FANCD2s activation. It is important to note that the S/P site found through mass spec analysis, S692, is also situated within this cluster, albeit on a short uncrystallized loop. To determine the functional significance of the

Figure 6. FANCD2 contains a conserved putative CDK consensus site cluster. (**A**). A Clustal Omega multiple sequence alignment of three putative CDK phosphorylation sites, S525, S624, S726, in FANCD2 demonstrates strong evolutionary conservation. (**B**) Region of the mouse Fancd2 crystal structure represented as both surface and ribbons with S/P CDK consensus (teal) and the monoubiquitination site (yellow) indicated, using the program Discovery Studio. (**C**) FA-D2 cells stably expressing empty, FANCD2-WT, FANCD2-TA, and FANCD2-TD were incubated in the absence (-) and presence (+) of 250 nM MMC for 18 h, and whole-cell lysates were immunoblotted with antibodies to FANCD2, V5, FANCI and α-tubulin. The FANCD2-V5 and FANCI L/S ratios are the ratios of monoubiquitinated to nonubiquitinated protein, and were calculated by measuring protein band intensities using ImageJ image processing and analysis software (http://rsb.info.nih.gov/ij/).


A
S525
S624
S726
H. sapiens 522 DNISPQQI 621 SEQSPQAS 723 KLVSPLCL
P. troglodytes 394 DNISPQQI 533 TEHSPWAS 646 KLVSPLCL
M. musculus 520 ENMSPQQI 620 TEHSPWAS 721 RSMSSLCL
C. familiaris 522 DNMSPQQI 619 SEQSPQAS 720 KVVSPLCL
G. gallus 525 QKLNPCQI 622 CEQTPEVL 725 RVVSPICL
X. tropicalis 529 DNLNAQQI 626 SEQVPEAS 726 RVVSPICL
D. rerio 523 DNLTSQQI 619 SESSPEAA 721 PRVSPLCL
D. melanogaster 555 SDLSLTQT 661 IGNSTESL 763 ACDSIYVL
C. elegans 369 HKLSIPNI 354 VKSSSALR 525 -SARILEI
B
523
724
622
590
559
C
FA-D2+
Empty
FANCD2
TA
TD
- + - + - + - +
MMC
α-FANCD2
FANCD2-V5-Ub
FANCD2-V5
α-V5
FANCD2-V5-Ub
FANCD2-V5
L/S 0.0 0.0 0.6 0.9 1.0 1.0 0.7 0.6
α-FANCI
FANCI-Ub
FANCI
L/S 0.0 0.1 0.2 0.3 0.2 0.2 0.2 0.2
α-Tubulin
Tubulin
1 2 3 4 5 6 7 8

putative CDK cluster, we generated two missense mutants in S525, S624, S726. These sites were mutated into either phospho-dead triple alanine (TA) or phospho-mimetic triple aspartic acid (TD) mutants. The *FANCD2* cDNAs were cloned into the pLenti6.2 lentiviral vector that contains a carboxy-terminal V5 tag. FA-D2 patient-derived cells were used to stably express the empty vector, wild type FANCD2 and the missense mutants. To determine the consequences of mutating the putative CDK site cluster, the FA-D2 cell lines were then treated with and without MMC to determine their monoubiquitination efficiency (**Fig. 6C**). Although these mutations did not abrogate FANCD2 monoubiquitination, the TA mutant is constitutively activated and the TD mutant is unable to elicit a monoubiquitination response with the addition of damage. FANCI monoubiquitination is unaffected by the mutation of the FANCD2 phosphorylation sites. To further study the functional effect of mutating these conserved phosphorylation residues, FA-D2 + empty, FANCD2-WT, TA, and TD cells were incubated with and without either HU or aphidicolin (APH) (**Fig. S5**). As we believe these residues are important in non-DNA damage inducible activation of FANCD2, it is important to explore DNA replication inhibitors, as FANCD2 is active during S phase of the cell cycle. HU transiently stalls replication by causing an imbalance in the deoxyribonucleoside triphosphate pool and APH inhibits DNA polymerase α [25,26]. Similar to the results obtained when treating with MMC, the TA mutant appears to be constitutively monoubiquitinated, while the TD mutant fails to become monoubiquitinated following exposure to the replicative inhibitors. Interestingly, when looking at CHK1 pS345 and RPA pS3/4, both mutants have an increase without the addition of treatment. These are markers for blocked DNA

replication, suggesting that mutating these phosphorylation sites causes inefficient DNA replication. Taken together, these results demonstrate the importance of these sites in FANCD2 function and further analysis must be performed to determine their mechanism of action and specific purpose in DNA replication.

Discussion

Although FANCD2 has been shown to function in unperturbed S phase of the cell cycle, how this is regulated has not been studied [16]. In this study, with the use of lambda phosphatase assays, we have revealed novel DNA damage-independent phosphorylation of FANCD2. Several cell lines show FANCD2 having an increase in electrophoretic mobility through an SDS Page gel with the addition of lambda phosphatase, indicating the cleavage of phosphate groups from the protein during incubation. This variance is not seen in FANCI, even after the addition of MMC. Although we know FANCI is phosphorylated by ATM/ATR with the addition of damage, this inability to visualize a molecular weight shift difference, due to phosphate cleavage, could be explained by how extensive the phosphorylation is [15]. Currently, six S/TQ sites have been described to be phosphorylated by ATM/ATR [14]. The limited number of residues phosphorylated could be the reason for no visible shift difference seen in this assay. FANCD2 and FANCI are very large proteins, 1451 and 1328 amino acids, respectively [27]. In order to see this slight shift in protein molecular weight, due to their size, the immunoblot must be extensively run and the ability of FANCD2 phosphorylation to be visualized could mean it is extensively phosphorylated. It is important to note that the shift is also seen with incubation with

the second phosphatase, PP1. Interestingly, we have found that maximal FANCD2 phosphorylation, as seen through the lambda phosphatase assay, occurs at S phase. This has been visualized by double thymidine block, as well as a nocodazole block. This suggests that FANCD2s function in the cell cycle could be regulated through phosphorylation.

DNA damage inducible FANCD2 phosphorylation has been widely studied, so it is necessary to determine if the key regulatory proteins involved in this well-known posttranslational modification are essential for the non-DNA damaging regulation of FANCD2 [12,16,28]. With the use of patient fibroblast and lymphoblasts, we found that the extensive phosphorylation of FANCD2, in S phase, is unaffected by the absence of ATM, ATR, and FANCA. Due to FANCD2s monoubiquitination in S phase and, now seen, phosphorylation, we next wanted to determine if these posttranslational modifications were reliant on one another [16]. In using FA-D2 patient cells expressing FANCD2-K561R, the monoubiquitination deficient mutant, this shift in FANCD2 in the presence of lambda phosphatase is still observed. This would indicate that this phosphorylation event occurs upstream of monoubiquitination or is monoubiquitination-independent. Importantly, with the addition of the phosphatase throughout this study, monoubiquitinated FANCD2 is still present. This suggests that this phosphorylation does not affect the stabilization of monoubiquitinated FANCD2 and monoubiquitination is not essential for phosphorylation.

If FANCD2 is indeed phosphorylated during the cell cycle, the potential kinase responsible is a CDK. As mentioned, several DNA repair proteins are phosphorylated

by CDKs, so we decided to determine if FANCD2 was a substrate of CDK phosphorylation [19,20]. In using two different cell systems and an antibody, that specifically recognizes substrates of CDKs through its known consensus sequence; we found that FANCD2 is potentially a substrate of CDK. To try to determine which residues are phosphorylated and narrow down our sites of interest, we used the U2OS 3XFLAG FANCD2 immunoprecipitation to enrich for phospho peptides and analyze by mass spectrometry. Several ATM/ATR sites were enriched, however, a putative CDK consensus site that has not been studied due to its lack of sequence conservation was also found, S592. We then optimized several CDK inhibitors to try to focus on a specific CDK responsible. Correlating with our finding that FANCD2s phosphorylation is maximal during S phase, the inhibitor Purvalanol A seemed to limit FANCD2s molecular weight shift with the addition of lambda phosphatase. This inhibitor is specific towards CDK2, with some inhibition of CDK1 [29]. We further compared the CDK1 specific inhibitor, RO-3306, and Purvalanol A and found that incubation with the CDK2 inhibitor for 4 and 8 h limited FANCD2s phosphorylation. This suggests that FANCD2s phosphorylation during S phase is regulated by CDK2.

Through initial sequence and structural analysis, several important putative CDK phosphorylation sites have been discovered. The CDK S/P residues S525, S624, and S726 were highly conserved amongst various species and are situated proximal to FANCD2s monoubiquitination site and form a cluster with the mass spectrometry phospho peptide found, S592. This site, however, is located on a short uncrystallized loop in FANCD2. Through site-directed mutagenesis, S525, S624, S726 were mutated to alanines (TA), phospho-dead, or aspartic acid (TD), phospho-mimetic, residues.

Originally, due to FANCD2s extensive phosphorylation occurring when it is monoubiquitinated in S phase, we hypothesized that these sites may activate FANCD2. Surprisingly, when FA-D2 patient cells expressing the phosphorylation mutants were treated with MMC, the phospho-dead mutant is constitutively monoubiquitinated and the phospho-mimetic mutant is unable to induce monoubiquitination after damage. This would indicate, not only the importance of these residues in FANCD2s function, but also that phosphorylation at these sites may inhibit FANCD2 monoubiquitination. Although maximal FANCD2 phosphorylation is seen during S phase, these sites may not play a role in that function, but instead have a novel regulatory effect on FANCD2 for its inactivation. As we are interested in DNA damage-independent regulation of FANCD2 and we know it functions at stalled replication forks in unperturbed S phase, the FA-D2 cells were treated with two DNA replication inhibitors. With the addition of APH or HU, FANCD2-TA is constitutively monoubiquitinated and FANCD2-TD is unable to show a response. Both mutations, however, have an increase in CHK1 pS345 and RPA pS4/8, indicating replication is blocked in both cases. Based on these findings, we predict that although these sites may not be linked to FANCD2s cell cycle phosphorylation, they have an important effect on FANCD2 function in response to DNA damaging and replicative stress inducing agents. Further analysis on these mutants must be done in order to (A) determine if CDK2 is the kinase responsible for this phosphorylation, (B) establish the function of this posttranslational modification on the FA-BRCA pathway, and (C) extensively characterize how mutation of these residues affect FANCD2 function.

Materials and Methods

Cell culture

COS-7, HeLa, U2OS, U2OS 3X FLAG FANCD2, BJTERT, PEBS, YZ5, 6914, and 6914 + FANCA cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 12% v/v FBS, L-glutamine and penicillin/streptomycin. 066 and PD7L cells were grown in Roswell Park Memorial Institute medium (RPMI) supplemented with 18% v/v FBS, L-glutamine and penicillin/streptomycin. 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*$^{hy/-}$) 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 [30]. To generate stable lines expressing wild type or mutant FANCD2, FA-D2 cells were infected with pLenti6.2-FANCD2 (Invitrogen) lentivirus, followed by selection in DMEM supplemented with 15% v/v FBS, L-glutamine, penicillin/streptomycin and 2 μg/ml blasticidin. Cells were treated with the CDK inhibitors Olomoucine (Acros), Olomoucine II (Santa Cruz), Purvalanol A (Santa Cruz), RO-3306 (Santa Cruz), and Roscovitine (Acros) as indicated.

Antibodies and 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 using a Fisher Scientific Model 500 Ultrasonic Dismembrator. 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), ATR (ab10312, Abcam), ATM (C2C1, Genetech), CHK1 (2345, Cell Signaling), FANCA (ABP-6201, Cascade), and pS CDK (9477, Cell Signaling), RPA (NA18, Calbiochem), FLAG (F7425, Sigma),and mouse monoclonal sera against α-tubulin (MS-581-PO, Neomarkers), cyclin A (SC751, Santa Cruz), CHK1 pS345 (2348, Cell Signaling), RPA pS4/8 (A300-245A, Bethyl), and V5 (13202, Cell Signaling).

Immunoprecipitation

FA-D2 + LacZ, FA-D2 + FANCD2-V5, U2OS, and U2OS 3XFLAG FANCD2 cells were lysed in Triton-X lysis buffer (50 mM Tris.HCl pH 7.5, 1% v/v Triton X-100, 200 mM NaCl, 5mM EDTA, 2mM Na_3O_4V, Protease inhibitors (Roche), and 0.153g B-glycerophosphate) on ice for 15 min followed by sonication for 10 s at 10% amplitude using a Fisher Scientific Model 500 Ultrasonic Dismembrator. V5 or FLAG-agarose were washed and blocked with NETN100 (20 mM Tris-HCl pH 7.5, 0.1% NP-40, 100 mM NaCl, 1 mM EDTA, 1 mM Na_3O_4V, 1 mM NaF, protease inhibitor (Roche)) + 1% BSA and the final wash and resuspension was done in Triton-X lysis buffer. Lysate was then incubated with appropriate agarose at 4°C for 2 h, nutating. Beads were then washed in Triton-X 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.

Plasmids, site-directed mutagenesis, and transient transfections

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. The *TA* and *TD* cDNA were generated by site-directed mutagenesis of the wild type *FANCD2* cDNA using the Quikchange Site-directed Mutagenesis Kit (Stratagene). The forward and reverse oligonucleotide sequences used are as follows: TA: S726A-FP 5'- AGGCCAAAAATTGGTGGCTCCGCTGTGCCTGGC-3', S624A-FP 5'- TCCTGCAGTGAGCAGGCTCCTCAGGCCTCTGCAC-3', S525A-FP 5'-CTGGATAACATAGCCCCTCAGCAAATACGAAAACTCTTCTATGTTCTCAGCAC-3'; S726A-RP 5'- GCCAGGCACAGCGGAGCCACCAATTTTTGGCCT-3', S624A-RP 5'- GTGCAGAGGCCTGAGGAGCCTGCTCACTGCAGGA-3', S525A-RP 5'-GTGCTGAGAACATAGAAGAGTTTTCGTATTTGCTGAGGGGCTATGTTATCCAG -3'. TD: S726D-FP 5'-AGGCCAAAAATTGGTGGATCCGCTGTGCCTGGC-3', S624D-FP 5'- TCCTGCAGTGAGCAGGATCCTCAGGCCTCTGCAC-3', S525D-FP 5'-CTGGATAACATAGACCCTCAGCAAATACGAAAACTCTTCTATGTTCTCAGCAC-3'; S726D-RP 5'-GCCAGGCACAGCGGATCCACCAATTTTTGGCCT-3',

S624D-RP 5’- GTGCAGAGGCCTGAGGATCCTGCTCACTGCAGGA-3’, S525D-RP 5’-GTGCTGAGAACATAGAAGAGTTTTCGTATTTGCTGAGGGTCTATGTTATCCAG-3’.

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_3VO_4 and 5 mM NaF for 15 min at 4^oC 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 pr 10 U of PP1for 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), or 6% SuperSep Phos-tag (Wako Pure Chemical Industries) and transferred to polyvinylidene difluoride (PVDF) membranes. Larger proteins were resolved for 5 h on ice.

Cell-cycle synchronization

HeLa cells or the indicated fibroblast or lymphoblast lines were synchronized by double thymidine block method. Cells were treated with 2.5 mM thymidine for 18 hours, thymidine-free media for 10 hours, and 2.5 mM thymidine for 18 hours to arrest the cell cycle at G_1/S. Cells were washed twice with phosphate-buffered saline (PBS)

and released in DMEM + 12% FBS, L-glutamine, and penicillin/streptomycin and analyzed at various time intervals by lambda phosphatase assay and immunoblotting, or flow cytometry. For synchronization in M phase, a nocodazole block was used. Cells were treated with 0.1 μg/mL nocodazole (Sigma) for 15 hours, and the nonadherent cells were collected and washed with PBS, followed by resuspension in DMEM without nocodazole and replated in DMEM 12% FBS, L-glutamine, and penicillin/streptomycin.

Cell-cycle analysis

Cells collected from synchronization were resuspended in 0.1 mL PBS and fixed by adding 1 mL ice-cold ethanol and stored at -20^{o}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^{o}C, then stored on ice. 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.

Acknowledgements

We thank members of the Howlett laboratory for critically reading this manuscript and for helpful discussions. This work was supported by National Institutes of Health/National Heart, Lung and Blood Institute grant R01HL101977 (NGH); Department of Defense Bone Marrow Failure Research Program New Investigator Award W81XWH-11-1-0318 (NGH); Rhode Island IDeA Network of Biomedical Research Excellence (RI-INBRE) grant P20RR016457-09 from the National Center

for Research Resources (Zahir A. Shaikh, PI/NGH, faculty development); and National Science Foundation EPSCoR grant #1004057.

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APPENDIX I

Conclusion

Although FANCD2 is essential for proper function of the FA-BRCA pathway and subsequent preservation of chromosome stability, there are many facets concerning its regulation, function, and domain architecture that remain elusive to date. Thus far, key FANCD2 functions include recruitment of the structure-specific endonucleases, SLX4 and FAN1, nucleosome remodeling and assembly, transcriptional activation of the tumor suppressor gene *TAp63*, replisome surveillance of chromosomal fragile sites, as well as, resolution of stalled replication forks. FANCD2 is an orphan protein, meaning there are no recognizable domains found in the protein sequence. Several groups, our lab included, have focused on analyzing the domain structure of FANCD2 and have discovered important domains in FANCD2. An EDGE motif was found to be essential for ICL-sensitivity [1]. A PIP-motif was discovered to be necessary for the interaction between FANCD2 and PCNA and is required for efficient FANCD2 monoubiquitination and repair [2]. A CUE ubiquitin binding domain in FANCD2 was needed for interaction with FANCI, as well as, efficient ICL repair [3]. It was known that FANCD2 functions in the nucleus; however, no experiments prior to my graduate research had examined how FANCD2 is transported for ICL repair.

One project that was comprehensively studied during my graduate research, focused on the identification and characterization of a putative NLS in the amino terminus of FANCD2 [4]. In this research, through initially fusing a predicted NLS region to GFP, we discovered that the first 58 amino acids of FANCD2 are required to

promote GFP nuclear localization. This region contained two bipartite NLSs, however, both were found to be essential for localization. To determine if FANCD2 is transported in an importin-α/β manner, treatment with a known inhibitor of this pathway, ivermectin, caused GFP localization to be abolished even with the NLS fused. In creating FANCD2 truncation mutants, localization and FANCD2 function was abrogated without the FANCD2 NLS. FANCD2 was unable to form nuclear foci, become monoubiquitinated, or bind to chromatin. Deletion of the NLS also had an effect on FANCIs chromatin localization and monoubiquitination, albeit it was not abolished, indicating that a subset of FANCI was transported to the nucleus *via* FANCD2. Mutating three highly conserved, positively charged residues, in FANCD2 caused an intermediate phenotype, suggesting these sites are important for FANCD2s localization. Together, these findings suggest that FANCD2 and a subset of FANCI are transported to the nucleus through FANCD2s amino terminus NLS and its subsequent interaction with importin-α/β. Once in the nucleus, FANCD2 and FANCI bind to DNA and are phosphorylated by ATM/ATR, triggering the monoubiquitination of FANCD2 and FANCI by the core complex.

As mentioned, FANCD2 and FANCI are phosphorylated by ATM/ATR in response to DNA damaging agents in order to facilitate the repair of DSBs [5-7]. However, it has been demonstrated that FANCD2 is monoubiquitinated during unperturbed S phase of the cell cycle and functions to protect stalled replication forks from MRE11 degradation [8,9]. FANCD2s DNA damage-independent regulation has not been described. Through the utilization of lambda phosphatase assays in my most recent project, I found that FANCD2 is extensively phosphorylated, specifically in S

phase of the cell cycle. This led us to hypothesize that this is how FANCD2 is being regulated in the absence of damage. In synchronizing various cell lines, we found that this phosphorylation pattern is independent of the major DNA damage kinases, ATM and ATR, as well as the core complex member FANCA. Interestingly, we also showed that the ability of FANCD2 to become monoubiquitinated did not alter FANCD2s phosphorylation and the monoubiquitinated form of FANCD2 persists even with the addition of the phosphatase enzyme. This demonstrates that the DNA damage independent phosphorylation of FANCD2 is also independent of its monoubiquitination. As this phosphorylation is maximal at S phase, we found that FANCD2 is indeed a substrate of CDK phosphorylation. To narrow down which residues in FANCD2 could be phosphorylated by CDK, we found several conserved SP sites that were surprisingly clustered around FANCD2s site of monoubiquitination. These sites were then mutated to phospho-dead alanine (TA) residues or phospho-mimetic asparagine (TD) residues. Currently, we have found that these sites do alter FANCD2s ability to become monoubiquitinated with the addition of MMC or DNA replication inhibitors. The TA mutant is constitutively monoubiquitinated and the TD mutant does not elicit a response. These preliminary findings suggest that these sites are phosphorylated in order to inhibit FANCD2s monoubiquitination. These FANCD2 mutants need to be further studied to determine the function of FANCD2 monoubiquitination inhibition. Ultimately, due to the importance of FANCD2 in aspects of DNA repair and DNA replication, this protein should be further characterized to potentially discover significant therapeutic implications for FA that allow for tailored prophylactic and chemotherapeutic measures to be adopted.

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APPENDIX II

Supplemental information for manuscript II: "Coordinate Nuclear Targeting of the FANCD2 and FANCI Proteins via a FANCD2 Nuclear Localization Signal"

Supplemental materials and methods

Generation of FANCD2-GFP fusion vectors

To fuse amino acids 1-27 of FANCD2 to the amino terminus of GFP to generate D2-1-27-GFP, we PCR amplified the coding sequence of amino acids 1-27 using the following forward and reverse primers: FP, 5'-AAAGAGCTCCACCATGGTTTCC-3'; RP, 5'-TATCTGCAGTGGTTGCTTCCTGGT-3'. Similarly, D2-24-58-GFP was generated by PCR amplifying amino acids 24-55 of FANCD2 using the following primers: FP, 5'-ATAGAGCTCCACCATGAGGAAGCAACCACT-3'; RP, 5'-GGCGCTGCAGAATAATTCCTGATATCTTAAGA-3'. D2-1-58-GFP was generated by PCR amplifying amino acids 1-58 of FANCD2 using the following primers: FP, 5'-AAAGAGCTCCACCATGGTTTCC-3'; RP 5'-CCCCTGCAGAATAATTCCTGATATC-3'. All amplified fragments were directionally cloned into the *SacI-PstI* site of pGFP-N1 (Clontech). DNA fragments were amplified using an Eppendorf Mastercycler ep thermal cycler using the following conditions: Thirty cycles of denaturation at 95°C for 30 sec, annealing at 59-65°C for 1 min, and extension at 68°C for 1 min, followed by cooling to 4°C.

Supplemental Figures

Figure S1. FANCD2 contains a highly conserved amino-terminal nuclear localization signal, which facilitates nuclear expression of GFP. (**A**) cNLS mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) was used to analyze the FANCD2 amino acid sequence for importin α/β-dependent nuclear localization signals (NLSs), and identified amino acids 2-58 as harboring several putative high scoring bipartite NLSs (**B**). (**C**) D2-1-58-GFP localizes primarily to the nucleus. IMR90 cells were transiently transfected with the indicated GFP constructs and analyzed by inverted fluorescence microscopy. (**D**) HeLa cells were transiently transfected with wild type GFP (GFP-WT) or GFP fused to amino acids 1-58 of FANCD2 (D2-NLS-GFP), incubated in the absence or presence of 25 μM ivermectin for 20 h, followed by analysis by inverted fluorescence microscopy. (**E**) The % of cells exhibiting both cytoplasmic and nuclear (Cyto. + Nucl.) and exclusive nuclear (Nuclear) staining were scored and plotted. Error bars represent the standard errors of the means from two independent experiments. ***, $p < 0.001$. (**F**) HeLa cells were transiently transfected with the indicated GFP constructs and 24 h later cell pellets were fractionated into soluble (S) and chromatin (C) fractions. Fractions were resolved by SDS-PAGE and immunoblotted with antibodies to GFP, α-tubulin, and H2A. W, unfractionated whole-cell extract. (**G**) The integrated densities of the protein bands from **Fig. S1F** were quantified using ImageJ image processing and analysis software, and plotted. While the integrated band densities for a single experiment are shown, these experiments were repeated several times with very similar findings. WCE, whole-cell extract.

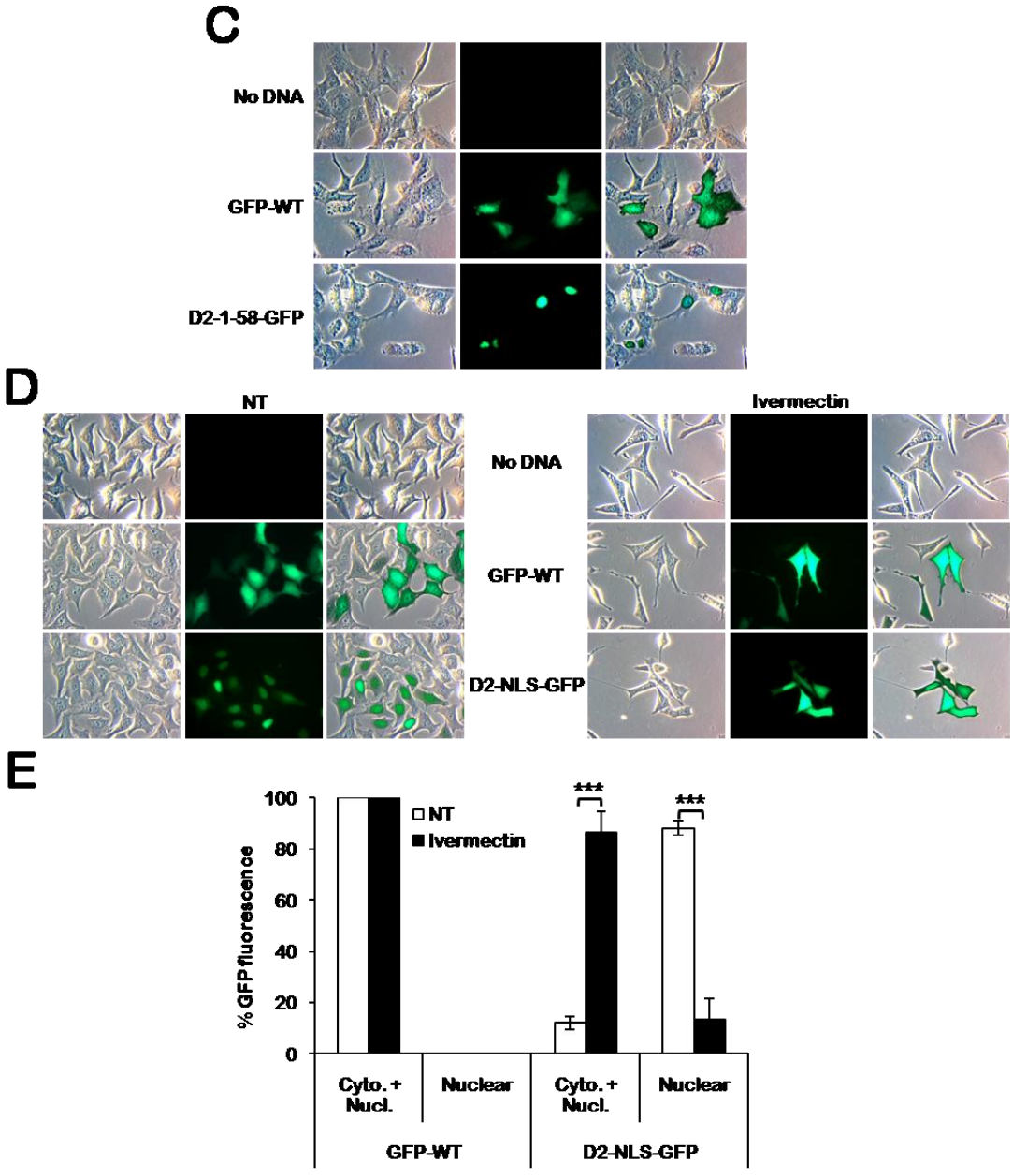
A

Predicted NLSs in query sequence

```
MVSKRRLSKSEDKESLTEDASKTRKQPLSKKTKKSHIANEVEENDSIFVK 50
LLKISGIILKTGESQNQLAVDQIAFQKKLFQTLRRHPSYPKIIEEFVSGL 100
ESYIEDEDSFRNCLLSCERLQDEEASMGASYSKSLIKLLLGIDILQPAII 150
KTLFEKLPEYFFENKNSDEINIPRLIVSQLKWLDRVVDGKDLTTKIMQLI 200
SIAPENLQHDIITSLPEILGDSQHADVGKELSDLLIENTSLTVPILDVLS 250
SLRLDPNFLLKVRQLVMDKLSSIRLEDLPVIIKFILHSVTAMDTLEVISE 300
LREKLDLQHCVLPSRLQASQVKLKSKGRASSSGNQESSGQSCIILLFDVI 350
KSAIRYEKTISEAWIKAIENTASVSEHKVFDLVMLFIIYSTNTQTKKYID 400
RVLRNKIRSGCIQEQLLQSTFSVHYLVLKDMCSSILSLAQSLLHSLDQSI 450
ISFGSLLYKYAFKFFDTYCQQEVVGALVTHICSGNEAEVDTALDVLLELV 500
VLNPSAMMMNAVFVKGILDYLDNISPQQIRKLFYVLSTLAFSKQNEASSH 550
IQDDMHLVIRKQLSSTVFKYKLIGIIGAVTMAGIMAADRSESPSLTQERA 600
NLSDEQCTQVTSLLQLVHSCSEQSPQASALYYDEFANLIQHEKLDPKALE 650
WVGHTICNDFQDAFVVDSCVVPEGDFPFPVKALYGLEEYDTQDGIAINLL 700
PLLFSQDFAKDGGPVTSQESGQKLVSPLCLAPYFRLLRLCVERQHNGNLE 750
EIDGLLDCPIFLTDLEPGEKLESMSAKERSFMCSLIFLTLNWFREIVNAF 800
CQETSPEMKGKVLTRLKHIVELQIILEKYLAVTPDYVPPLGNFDVETLDI 850
TPHTVTAISAKIRKKGKIERKQKTDGSKTSSSDTLSEEKNSECDPTPSHR 900
GQLNKEFTGKEEKTSLLLHNSHAFFRELDIEVFSILHCGLVTKFILDTEM 950
HTEATEVVQLGPPELLFLLEDLSQKLESMLTPPIARRVPFLKNKGSRNIG 1000
FSHLQQRSAQEIVHCVFQLLTPMCNHLENIHNYFQCLAAENHGVVDGPGV 1050
KVQEYHIMSSCYQRLLQIFHGLFAWSGFSQPENQNLLYSALHVLSSRLKQ 1100
GEHSQPLEELLSQSVHYLQNFHQSIPSFQCALYLIRLLMVILEKSTASAQ 1150
NKEKIASLARQFLCRVWPSGDKEKSNISNDQLHALLCIYLEHTESILKAI 1200
EEIAGVGVPELINSPKDASSSTFPTLTRHTFVVFFRVMMAELEKTVKKIE 1250
PGTAADSQQIHEEKLLYWNMAVRDFSILINLIKVFDSHPVLHVCLKYGRL 1300
FVEAFLKQCMPLLDFSFRKHREDVLSLLETFQLDTRLLHHLCGHSKIHQD 1350
TRLTQHVPLLKKTLELLVCRVKAMLTLNNCREAFWLGNLKNRDLQGEEIK 1400
SQNSQESTADESEDDMSSQASKSKATEDGEEDEVSAGEKEQDSDESYDDS 1450
D                                                    1451
```

B

Position	Sequence	Score
2-27	VSKRRLSKSEDKESLTEDASKTRKQP	10.1
24-55	RKQPLSKKTKKSHIANEVEENDSIFVKLLKIS	7.2
2-30	VSKRRLSKSEDKESLTEDASKTRKQPLSK	6.8
24-58	RKQPLSKKTKKSHIANEVEENDSIFVKLLKISGII	5.7
30-55	KKTKKSHIANEVEENDSIFVKLLKIS	5.6


C
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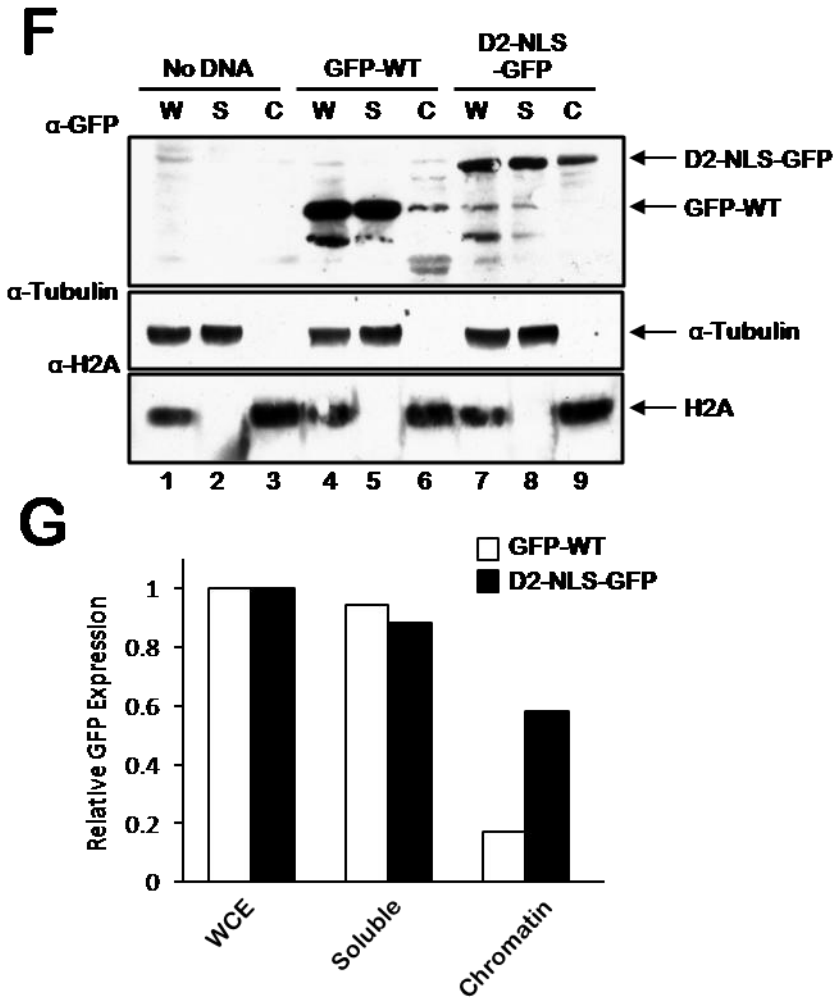
GFP-WT
D2-1-58-GFP
D
NT
Ivermectin
No DNA
GFP-WT
D2-NLS-GFP
E
% GFP fluorescence
100
80
60
40
20
0
NT
Ivermectin
***
***
Cyto. + Nucl.
Nuclear
Cyto. + Nucl.
Nuclear
GFP-WT
D2-NLS-GFP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GFP-WT
D2-NLS
-GFP
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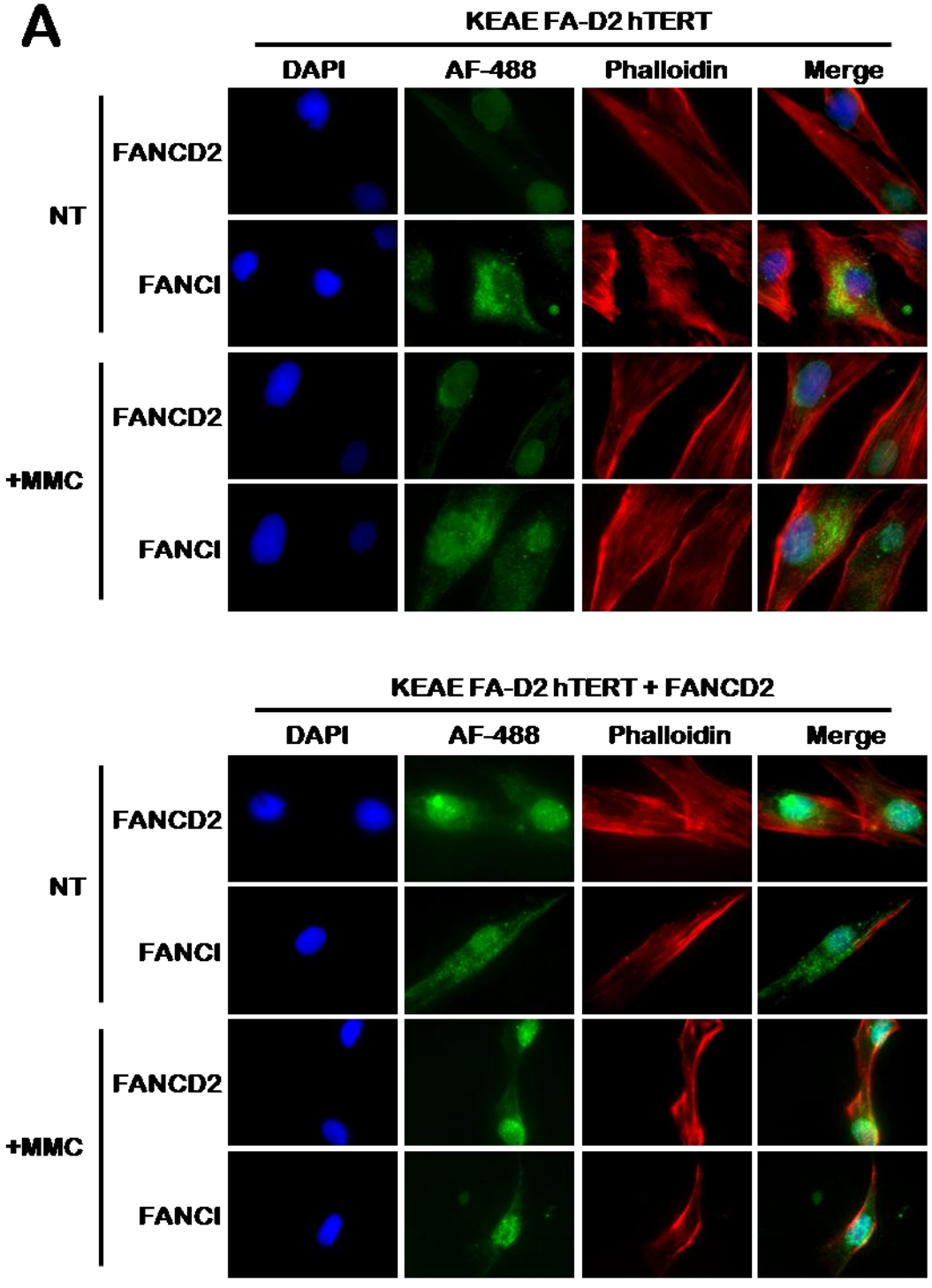
α-GFP
D2-NLS-GFP
GFP-WT
α-Tubulin
α-Tubulin
α-H2A
H2A
1 2 3 4 5 6 7 8 9
G
GFP-WT
D2-NLS-GFP
Relative GFP Expression
1
0.8
0.6
0.4
0.2
0
WCE
Soluble
Chromatin

Figure S2. The FANCD2 NLS is required for the nuclear localization of a subset of FANCI. (**A**) KEAE FA-D2 hTERT cells or KEAE FA-D2 hTERT cells stably expressing FANCD2-WT were incubated in the absence (NT) or presence of MMC for 24 h, fixed, stained with rabbit polyclonal anti-FANCD2 or anti-FANCI antibody and counterstained with phalloidin and DAPI. AF-488, Alexa Fluor 488. (**B**) FA-D2 cells stably expressing FANCD2-WT, FANCD2-ΔN57, or FANCD2-ΔN100 were incubated in the absence (NT) or presence of MMC for 24 h, fixed, stained with mouse monoclonal anti-V5 (red) to detect V5-tagged FANCD2 and rabbit polyclonal anti-FANCI (green) and counterstained with DAPI (blue). IF microscopy was performed with (+ Pre-Perm) and without (No Pre-Perm) a pre-permeabilization step (*see Materials and Methods*). The pre-permeabilization step leads to complete loss of fluorescent signal for FANCD2-ΔN57 and FANCD2-ΔN100 because of the high solubility of these proteins (*see* **Fig. 2B**), while this step is necessary for the resolution of FANCI fluorescence signal.


A
KEAE FA-D2 hTERT
DAPI
AF-488
Phalloidin
Merge
NT
FANCD2
FANCI
+MMC
FANCD2
FANCI
KEAE FA-D2 hTERT + FANCD2
DAPI
AF-488
Phalloidin
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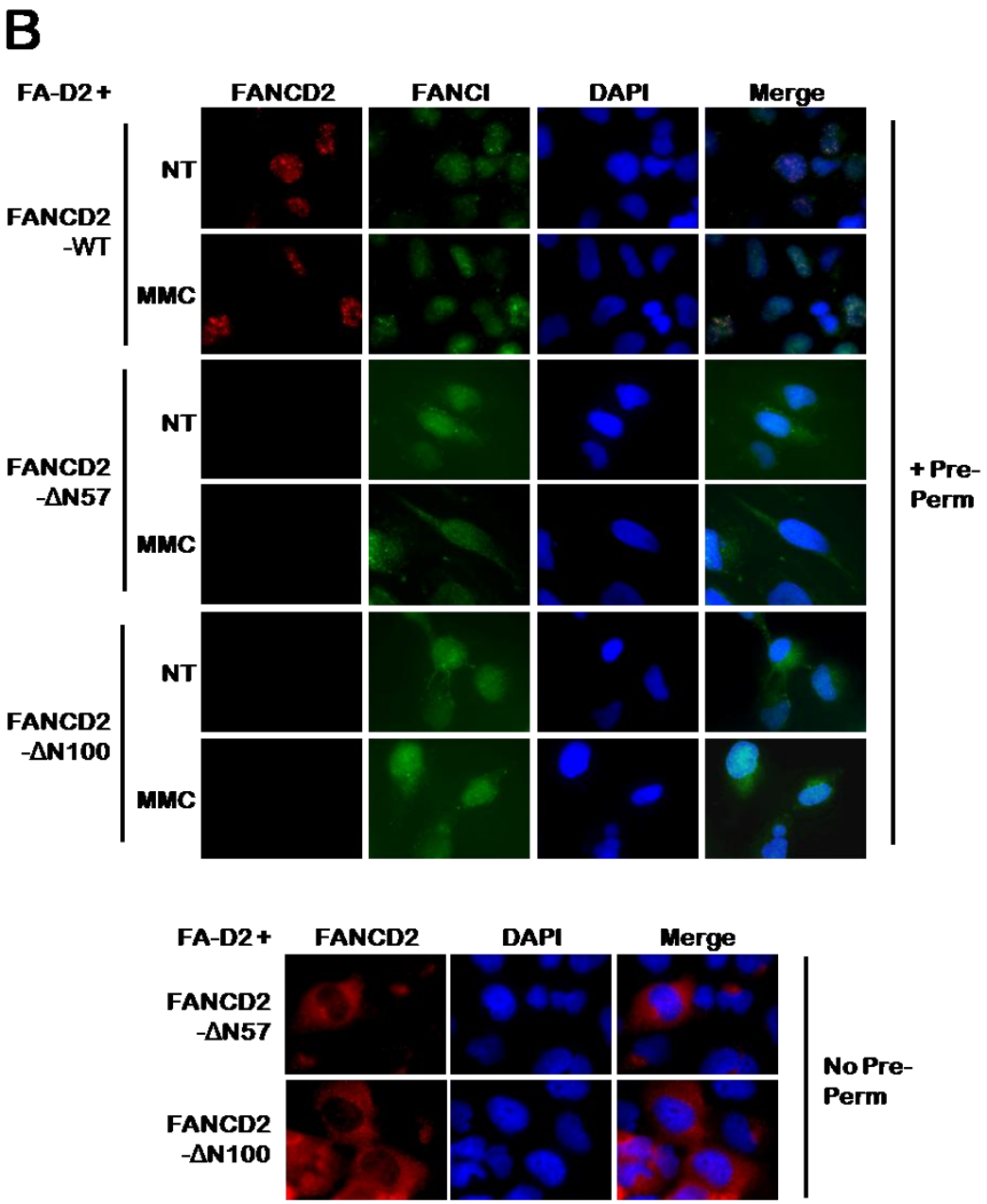
FANCI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FANCI
DAPI
Merge
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NT
MMC
FANCD2 -ΔN57
NT
MMC
FANCD2 -ΔN100
NT
MMC
+ Pre-Perm
FA-D2+
FANCD2
DAPI
Merge
FANCD2 -ΔN57
FANCD2 -ΔN100
No Pre-Perm

Figure S3. The FANCD2 NLS is required for efficient FANCI chromatin association. FA-D2 cells stably expressing FANCD2-WT (WT), FANCD2-ΔN57 (ΔN57), FANCD2-ΔN100 (ΔN100) or FANCD2-3N (3N) were incubated in the absence or presence of MMC for 18 h and cell pellets were fractionated into soluble and chromatin-associated fractions (*see* **Figs. 4B and C**). The total integrated densities of the chromatin-associated (C) nonubiquitinated and monoubiquitinated FANCI protein bands were quantified using ImageJ image processing and analysis software, and plotted. While the integrated band densities for a single experiment are shown, these experiments were repeated several times with similar findings. NT, not treated; MMC, MMC-treated.

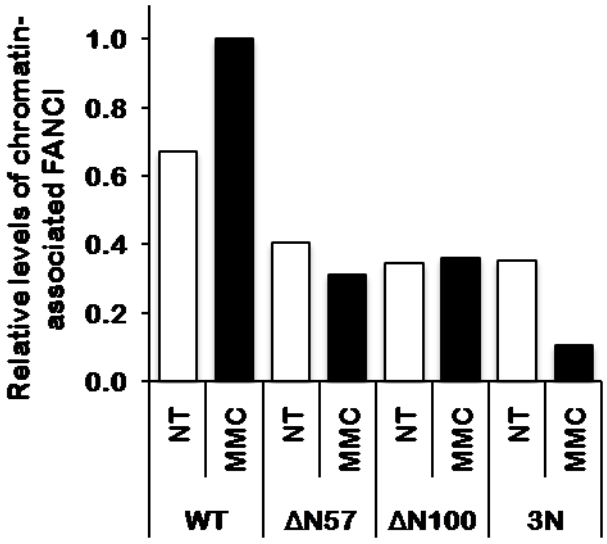
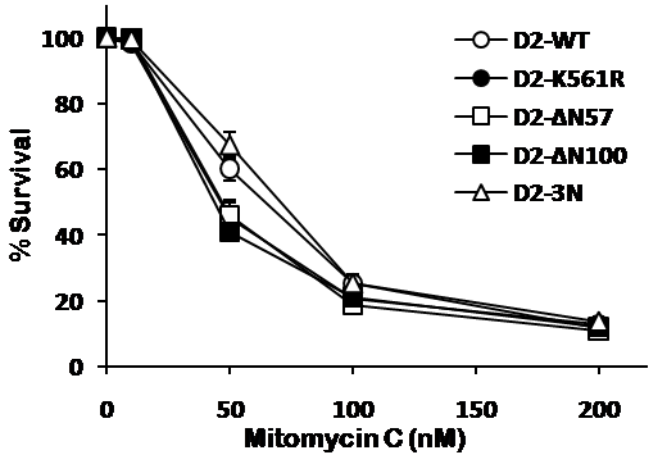

Relative levels of chromatin-associated FANCI
1.0
0.8
0.6
0.4
0.2
0.0
NT
MMC
NT
MMC
NT
MMC
NT
MMC
WT
ΔN57
ΔN100
3N

Figure S4. The FANCD2 NLS deletion mutants fail to rescue the MMC sensitivity of FA-D2 cells. FA-D2 cells stably expressing FANCD2-WT, FANCD2-K561R, FANCD2-ΔN57, FANCD2-ΔN100, or FANCD2-3N were treated with the indicated concentrations of MMC for 7-10 days. Cells were fixed and stained with crystal violet and percent survival calculated and plotted. Each measurement was performed in triplicate and experiments were performed multiple times with similar results. The 20% trimmed means ($\overline{X}_{20\%}$) for all recorded measurements were calculated and plotted. Error bars represent the standard errors of the means.


100
80
60
40
20
0
% Survival
0
50
100
150
200
Mitomycin C (nM)
D2-WT
D2-K561R
D2-ΔN57
D2-ΔN100
D2-3N

Supplemental Table 1. Detection of importin subunit β1, NUP160 and NUP155 in FANCD2 immune complexes. FANCD2 immune complexes were analyzed using a LTQ Orbitrap Velos hybrid mass spectrometer.

Protein Name	# of Unique Peptides	Peptide Sequences
Importin subunit beta-1	2	R.VLANPGNSQVAR.V, K.TTLVIMER.L
Nuclear pore complex protein NUP160	2	R.SEDGEIVSTPR.L, R.SFVELSGAER.E
Nuclear pore complex protein NUP155	4	R.AILSAK.S, K.AAPQSPSVPK.K, K.ANELLQR.S, R.YGGEAQMR.F

APPENDIX III

Supplemental information for manuscript III: “Characterization of a Putative CDK Phosphorylation Site Cluster in the FANCD2 Protein”

Supplemental Figures

Figure S1. FANCD2 phosphorylation is maximal in S phase. U2OS cells were synchronized double thymidine block and the cell cycle phase was determined by flow cytometric analysis of DNA content. Whole-cell lysates were incubated in the absence (-) and presence (+) of lambda phosphatase for 2 h at 30°C and immunoblotted with antibodies against FANCD2, FANCI, and Cyclin A. AS, asynchronous whole-cell lysate.

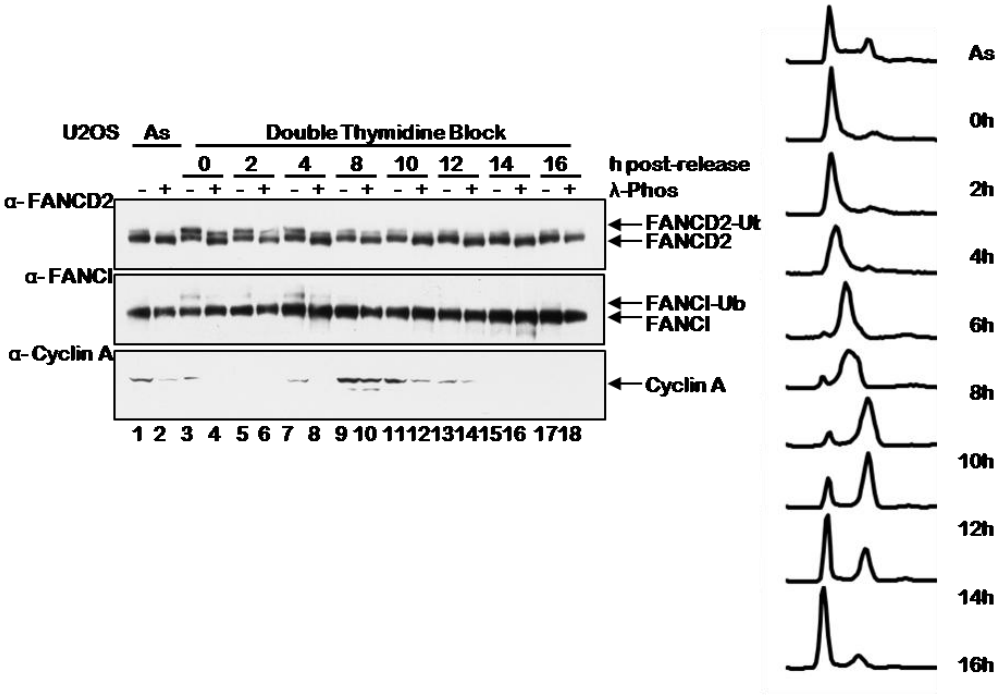

U2OS
As
Double Thymidine Block
0
2
4
8
10
12
14
16
h post-release
λ-Phos
α-FANCD2
FANCD2-Ub
FANCD2
α-FANCI
FANCI-Ub
FANCI
α-Cyclin A
Cyclin A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
As
0h
2h
4h
6h
8h
10h
12h
14h
16h

Figure S2. Non-DNA damage inducible phosphorylation of FANCD2 is independent of FANCD2 monoubiquitination. HeLa, FA-D2 cells with empty plasmid or stably expressing FANCD2-WT, and FANCD2-K561R were incubated with lambda phosphatase for 2, 4, 16 h at 30°C and immunoblotted against FANCD2.

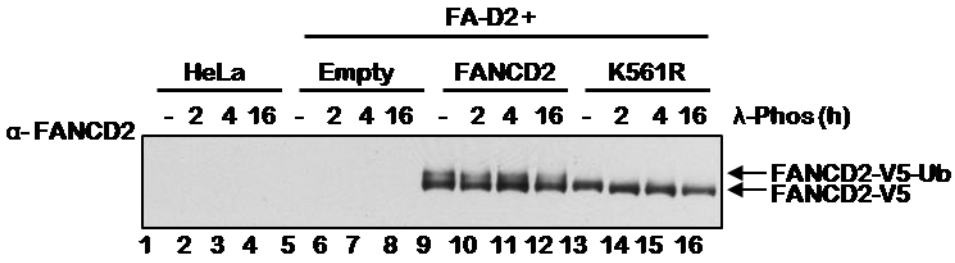

FA-D2+
HeLa
Empty
FANCD2
K561R
- 2 4 16 - 2 4 16 - 2 4 16 - 2 4 16
λ-Phos (h)
α-FANCD2
FANCD2-V5-Ub
FANCD2-V5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure S3. FANCD2 is a potential substrate of CDK phosphorylation. U2OS and U2OS cells stably expressing 3X FLAG FANCD2-WT were immunoprecipitated with anti-FLAG agarose and immune complexes were run on a coomassie gel. 3X FLAG-FANCD2 bands were then cut out and sent for phospho enrichment and mass spectrometry analysis.

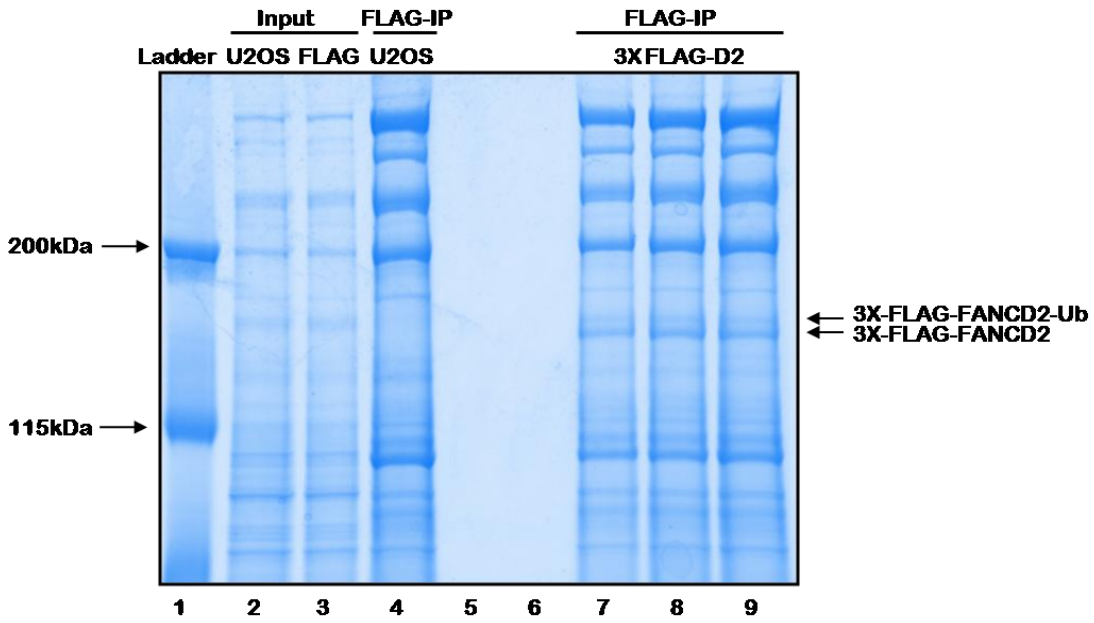
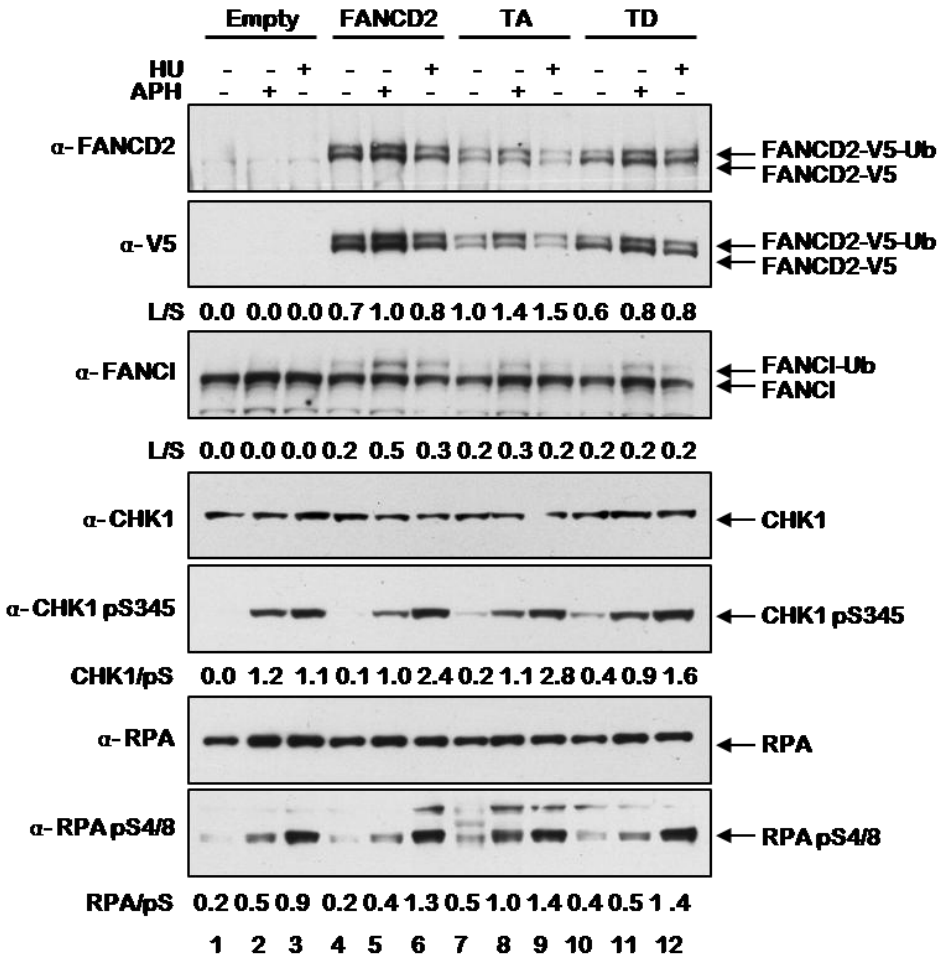

Input
FLAG-IP
FLAG-IP
Ladder
U2OS
FLAG
U2OS
3XFLAG-D2
200kDa
115kDa
3X-FLAG-FANCD2-Ub
3X-FLAG-FANCD2
1
2
3
4
5
6
7
8
9

Figure S4. FANCD2 contains a conserved putative CDK consensus site cluster. FA-D2 cells stably expressing empty, FANCD2-WT, FANCD2-TA, and FANCD2-TD were incubated in the absence (-) and presence (+) of 1 μM APH and 1 mM HU for 24 h, and whole-cell lysates were immunoblotted with antibodies to FANCD2, V5, FANCI, CHK1, CHK1 pS345, RPA, and RPA pS4/8. FANCD2-V5 and FANCI L/S ratios were then determined as described.


Empty
FANCD2
TA
TD
HU
APH
α-FANCD2
FANCD2-V5-Ub
FANCD2-V5
α-V5
FANCD2-V5-Ub
FANCD2-V5
L/S 0.0 0.0 0.0 0.7 1.0 0.8 1.0 1.4 1.5 0.6 0.8 0.8
α-FANCI
FANCI-Ub
FANCI
L/S 0.0 0.0 0.0 0.2 0.5 0.3 0.2 0.3 0.2 0.2 0.2 0.2
α-CHK1
CHK1
α-CHK1 pS345
CHK1 pS345
CHK1/pS 0.0 1.2 1.1 0.1 1.0 2.4 0.2 1.1 2.8 0.4 0.9 1.6
α-RPA
RPA
α-RPA pS4/8
RPA pS4/8
RPA/pS 0.2 0.5 0.9 0.2 0.4 1.3 0.5 1.0 1.4 0.4 0.5 1 .4
1 2 3 4 5 6 7 8 9 10 11 12