Award Number: W81XWH-19-1-0262

TITLE: A Novel FANCD2-Chromatin Bound Complex in Replication Stress Response and HSC Maintenance

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REPORT DATE: NOVEMBER 2022

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED *(From - To)*
NOVEMBER 2022	Final	*15JUL2019 - 14JUL2022*

4. TITLE AND SUBTITLE

A Novel FANCD2-Chromatin Bound Complex in Replication Stress Response and HSC Maintenance

5a. CONTRACT NUMBER
W81XWH-19-1-0262

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT: Due to the ongoing COVID-19 pandemic, we faced repeated disruption of our research activities in the past several months. However, we have mostly finished Aims 1&2 in the second funding year. We have generated the *Fanca-KO;Fancd2-KI* mice, which are deficient for the essential component of the FA core complex (*Fanca*). We have performed preliminary experiments to determine whether expression of the 3XFLAG-*Fancd2 KI* allele in *Fanca-KO* mice would alter the FA hallmark phenotype; DNA damage and homologous recombination (HR) repair. We isolated LSK ($Lin^-Sca1^+c\text{-}kit^+$; enriched for HSCs) from *Fanca-KO;Fancd2-KI* mice and the *WT;Fancd2-KI* control mice, and exposed the cells to MMC (5 ng/ml) to assess MMC-induced γH2AX and RAD51 foci formation. We observed a significant increase in MMC-induced formation of γ-H2AX foci in *Fanca-KO;Fancd2- KI* LSK cells compared to those from the *WT;Fancd2-KI* control. MMC-induced HR repair, as determined by the formation of RAD51 foci, was evidently defective in *Fanca-KO;Fancd2-KI* LSK cells.

Our studies on the functional consequence of the interaction between the FANCD2-RNF40 and FANCD2-EDD has the potential to establish the functional link between the FANCD2/FA pathway and replication stress response in genomic maintenance. We also expect these functional data to uncover whether these interacting pathways may be viable targets for therapy in FA BM failure.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
U	U	U	UU	23	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

The goals of the project are to delineate the function of FANCD2-chromatin-bound complex in the recognition and stabilize stalled DNA replication forks, namely 1) to identify and characterize novel FANCD2 binding partners in response to replication stress and examine the mechanistic insights into regulation of the FANCD2 complex recruitment (or being recruited by the FANCD2 complex) to stalled/collapsed replication forks on newly replicated DNA, and 2) functional evaluation of the link between Fancd2-novel replication stress factors interactions and HSC function.

2. KEYWORDS:

Fanconi Anemia
FANCDI-FANCD2 complex
Acute Myeloid Leukemia
Mitomycin C
Hydroxyurea
Hematopoietic stem cells
Bone marrow failure
Immunoprecipitation
Stalled replication forks

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**
  - Subtask 1

    A) Isolation of Proteins that bind to nascent DNA at stalled/collapsed replication forks- For this purpose, thymidine analogs (CIdU or IdU) will be used to label ongoing DNA synthesis, HU treatment and later immunoprecipitated by it from cross-linked chromatin to analyze proteins present with the nascent DNA at stalled/collapsed replication forks. **B) Isolation of FANCD2 bound/enriched nascent DNA/protein complex under replicative stress.** To further delineate the pivotal role of FANCD2 in resolving stalled/collapsed forks during early replication, next we will isolate a FANCD2 containing nascent DNA/protein complex under replicative stress conditions by combining the nascent DNA pull down by IdU immunoprecipitation followed by RIME using FANCD2 antibody. The mass-spectrometry analysis will be carried out for protein identification.

  - Subtask 2

    Tissue wide Proteomic analysis of FANCD2-associated proteins in the mouse: Perform a tissue-wide RIME analyses of the FANCD2 chromatin-bound complexes from both *Fancd2-KI* and *Fancd2K559R- KI* mice

- Subtask 3
  Delineate the exact mechanisms of their recruitment to nascent DNA at stalled/collapsed forks: between FANCD2+TFII-I, FANCD2+RNF20/40, FANCD2+FEN1 and FANCD2+EDD; binding-deficient mutants will be used in add-back experiments (**CRISPR-Cas9-mediated gene knockout** and complementation/addback analysis using wildtype and various mutants of corresponding proteins) in the same experimental settings. Using cell-based and in vitro systems a component of the complex that physically links with a component complex will be identified. Deletion constructs or point mutants of epitope-tagged FANCD2 will be expressed in FANCD2-deficient cells, and then teste for their ability to 1) bind complex, 2) load the complex to chromatin and 3) promote its foci formation. Analyze the functional consequences of mutations of FANCD2, RNF20/40, FEN1 and EDD in FA and non-FA pathways.

- Subtask 4
  To assess the genetic and functional consequences of the interaction between FANCD2 and the novel replication stress factors
  We will first utilize primary cells from three KO/KI mouse models (*Fancd2-KO, Fanca-/-Fancd2-KI,* and *Fancd2K559R-KI*) and FA patients to determine the structural elements of the biochemical interaction between FANCD2 and two of the novel replication stress-responsive binding partners (RNF40 and EDD) that we recently identified, and the requirement for these novel interactions in the maintenance of DNA replication integrity and genomic stability. We will then employ the *Fancd2-KO* mouse model and FA patient BM CD34+ HSPCs to assess the functional consequence of the FANCD2-RNF40 and FANCD2-EDD interactions in HSC maintenance.

- **What was accomplished under these goals?**

  - Due to the ongoing COVID-19 pandemic, we faced repeated disruption of our research activities in the past several months. However, we have made progress in **Aims 1& 2**.

  - We have found several FANCD2 associated proteins at stalled replication forks and we also determined the ubiquitination status of these proteins in chromatin under replication stress condition. We used a Ubiquitin Remnant Motif (K–∑–GG) Antibody Bead Conjugate (Cell Signaling technology), a proprietary ubiquitin branch ("K–∑–GG") antibody with specificity for a di-glycine tag that is the remnant of ubiquitin left on protein substrates after trypsin digestion, to enrich ubiquitinated peptides from trypsin digested chromatin samples (shNT vs shFANCA) under stalled replication forks condition (by treating with 1mM HU). This enrichment is followed by LC-MS/MS analysis for quantitative profiles of hundreds to over a thousand nonredundant ubiquitinated sequences**.**

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  -

    We Isolated proteins that bind to nascent DNA at stalled/collapsed replication forks. For this purpose, we used thymidine analogs (CIdU or IdU) to label ongoing DNA synthesis, HU treatment, and later immunoprecipitated by it from formaldehyde (1%) cross-linked chromatin to analyze proteins present with the nascent DNA at stalled/collapsed replication forks. Then, we isolated the FANCD2 containing nascent DNA/protein complex under replicative stress conditions by combining the nascent DNA pull-down by IdU immunoprecipitation (IP) followed by IP using FANCD2 antibody (**Figure 1**).

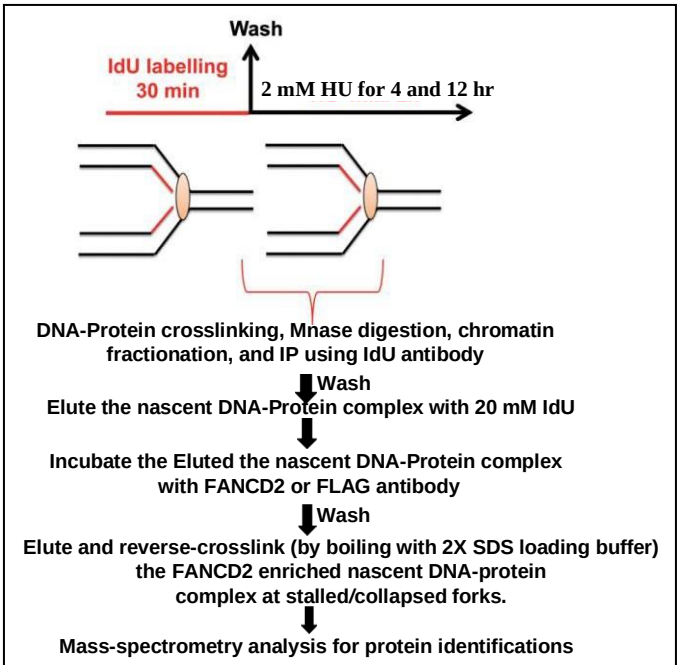

Figure 1. Schematic showing the experimental design for isolation of nascent DNA-FANCD2 protein complex at stalled/collapsed replication forks. HeLa/U2OS cells will be labeled with IdU for 30 min, followed by a chase into 1.5 mM HU-containing medium for the indicated times. Cells will be cross-linked, and the chromatin fraction will be isolated and subjected to IP using anti-IdU antibody (Idu-IP; to pull down nascent DNA), the eluate will be then subjected to FANCD2-IP using FANCD2/FLAG antibody to specifically pull down only FANCD2-nascent DNA complex.

- We have carried out Mass-spectrometry analysis to ID the specific proteins in the resulted complex. In addition to all the FANCD2 interaction proteins such as TFII-1, EDD, RNF40, we found many other proteins involved in DNA-replications and repair. Notably, TOP2A (DNA topoisomerase 2-alpha) was highly enriched in this FANCD2 containing nascent DNA/protein complex (**Table 1**). Interestingly, we also found that TOPO2A is ubiquitinated at multiple lysine residues in response to HU by our Ubiscan analysis (**Table 2**).

	A	B	C	D	E	F
1	Unique Peptides	Total Peptides	reference Protein	Gene Sym	MWT(kDa	AVG
2	55	105	Q9NVI1_FANCI_HUMAN	FANCI	149.23	2.8484
3	51	172	Q9BXW9_FACD2_HUMAN	FANCD2	164.02	2.7577
4	31	37	P11388_TOP2A_HUMAN	TOP2A	174.28	2.8807
5	23	23	O95071_UBR5_HUMAN	EDD	309.16	2.8053
6	22	26	P52701_MSH6_HUMAN	MSH6	152.69	2.9005
7	22	23	P43246_MSH2_HUMAN	MSH2	104.68	2.3266
8	21	12	Q5VTR2_BRE1A_HUMAN	RNF20	113.59	2.7382
9	21	10	Q5UIP0_RIF1_HUMAN	RIF1	274.29	2.7684
10	21	9	O75150_BRE1B_HUMAN	RNF40	113.58	2.9102

Table 1. FANCD2-interacting Proteins Identified by Mass spectrometry analysis.

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Table #1: *Human Nuclei/Chromatin; Trypsin Digest; Ubiquitin K-GG Remnant Motif Antibody #5562*

Samples: *AD = CS 26913, 26914; B7 = CS 26915, 26916; C5 = CS 26917, 26918; DC = CS 26919, 26920*

Legend: ** - ubiquitination, # - oxidized methionine, § - published site,* ***Blue Text*** *- CST antibody available,* ***Bold In***

			Normalized Fold Change						
	Index	Index in Detail	Chromatin shA : shNT	Nuclei shA : shNT	Max Abundance	Max % CV	Gene Name	Protein Name	Site
2818	2807	6154	-1.6	-1.8	76,926,457	23%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	1159;1185;1195;§12
2819	2808	6155	-8.1	-10.6	22,531,341	74%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	1196;1222;1232;127
2820	2809	6156	-3.1	-2.3	**41,991,782**	43%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	1259;1285;§1295;13
2821	2810	6158	-1.6	-1.9	35,401,676	24%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	§397;397;433;478
2822	2811	6159	-2.6	-2.2	25,577,231	3%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	§466;492;502;547
2823	2812	6162	-2.0	-2.0	80,409,564	49%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	§655;681;691;736
2824	2813	6163	-1.4	-2.3	149,366,894	17%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	§723;749;759;804
2825	2814	6165	-1.6	-10.6	**30,988,847**	33%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	1047;1073;1083;112
2826	2815	6167	-1.3	-3.0	12,585,874	19%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	336;336;372;336
2827	2816	6168	1.6	-1.7	381,741,440	22%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	;131;§131;§131;§15
2828	2817	6169	-1.1	-1.4	36,096,540	55%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	9;515;525;570;§510;
2829	2818	6170	-1.1	-2.6	12,614,003	44%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	9;545;555;600;§540;
2830	2819	6172	-1.6	-	**22,557,605**	31%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	5;561;571;616;§556;
2831	2820	6174	-1.6	-2.8	**11,206,658**	29%	TOP2A;TOP2A;TOP2A;	TOP2A;TOP2A iso2;TC	2;538;548;593;533;5

Table 2. The amount of ubiquitinated TOP2A are significantly reduced in Hela cells (nuclei and chromatin) depleted of FANCA.

- By Chip-seq analysis of the isolated complex (we used 1% formaldehyde to cross-link protein-protein and protein-DNA, prior to complex isolation), we found that most of the FANCD2 binding sites in the genome are mapped to the early origin of DNA replication (**Figure 2**).

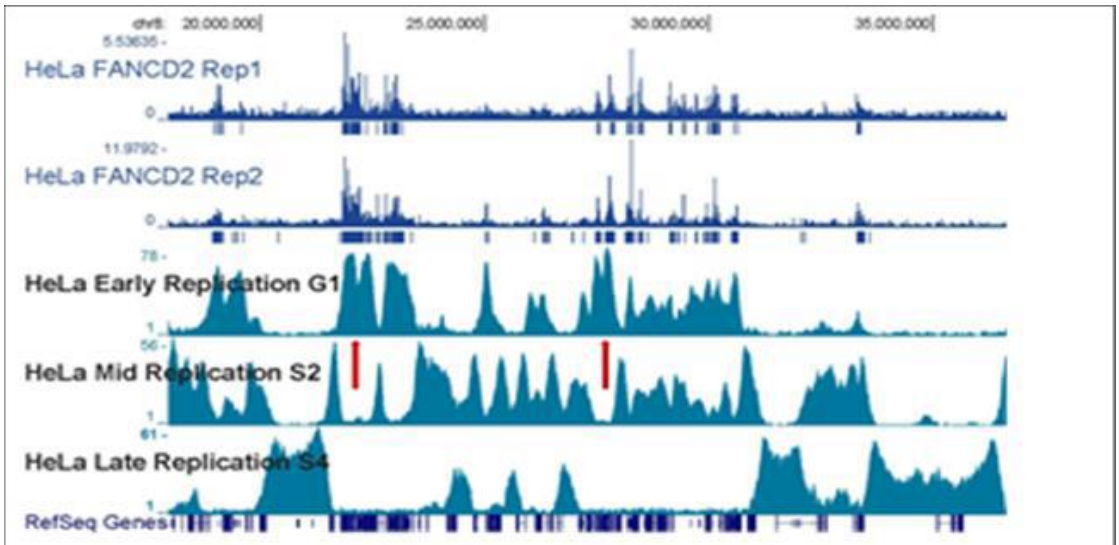

Figure 2. FANCD2 is recruited to the sites of early origin of replication in hydroxyurea (HU) treated HeLa cells. FANCD2 binding sites were identified by ChIP-Seq in HU-treated HeLa cells (biological replicates are shown) and compared to HeLa Repli-Seq data from ENCODE. Peaks of FANCD2 binding (red arrows) correlate with the sites of early replication suggesting that FANCD2 binds to replication origins.

- We also made several truncated mutants of FANCD2 (**Figure 3**) and we will use these mutants to delineate the exact domain required for FEN1, TFII-I and TOPO2A binding. We have generated mutants of FANCD2 that no longer binds to RNF40 and EDD (**Figures 4&5**). We also show that

both RNF40 and EDD binding deficient FANCD2-mutants (FANCD2$^{\Delta 772-820}$ and FANCD2$^{\Delta 672-690}$ mutants respectively) failed to rescue the MMC sensitivity of FAND2 KO cells (**Figures 4B & 8B**).

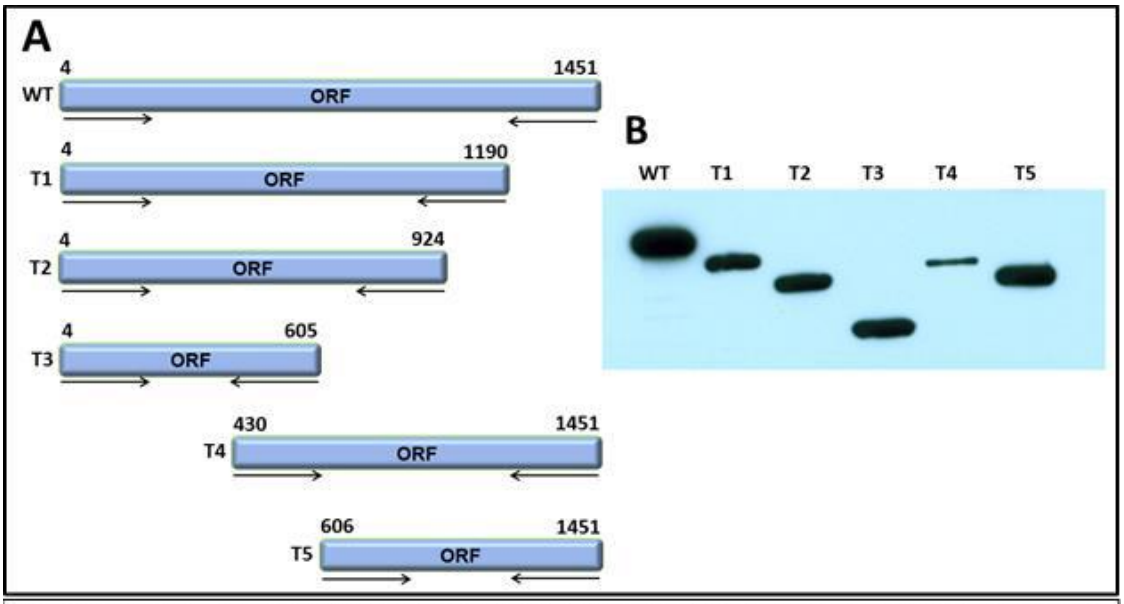

Figure 3. Construction of FANCD2 truncated mutants. A) Schematic showing the ORFs and **B**) Immunoblots showing the expressed truncated FANCD2 mutants. Indicated FLAG-tagged cDNAs were cloned into pMIEG3 retroviral vector and transduced to HEK293 cells. FLAG antibody was used for the immunoblots.

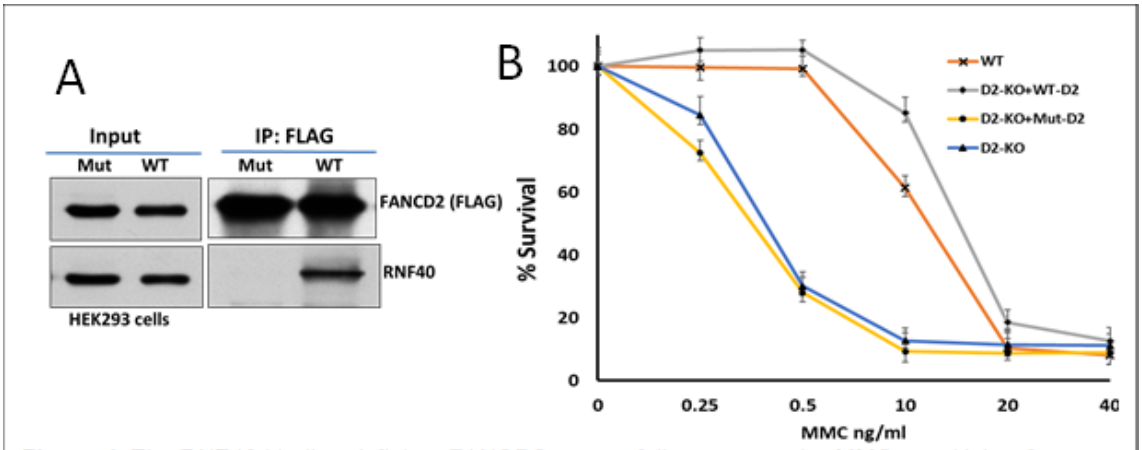

Figure 4. The RNF40 binding deficient FANCD2-mutant fails to rescue the MMC sensitivity of FAND2 KO cells **A)** Immunoblots using the indicated antibodies. 293T cells were transiently transfected with plasmids expressing FLAG-FANCD2$_{WT}$ and FLAG- FANCD2$^{\Box 772-820}$ mutant;). After 48 hours post transfection, cells were treated with 1 mM HU for 16 hours. Cell extracts were immunoprecipitated with M2-agarose beads, and immunoblotted with the indicated antibodies. **B)** MMC kill curves. Wild type (WT) and Mut-FANCD2, (FANCD2$^{\Delta 772-820}$ mutant; cDNAs were cloned into pMIEG3 retroviral vector and transduced to KO FANCD2 HAP1 cells.

- We performed RIME using mouse 3XFLAG-HA-Fancd2-KI testes extracts by two step immuno-affinity purification; FLAG antibody IP, eluted with 3XFLAG peptide followed by HA antibody (instead of FANCD2 antibody, since FANCD2 antibody to human protein does not work well with mouse protein in IPs) (**Figure 6**). The first batch of Mass-spectrometry data is currently awaiting. Next, we will compare the proteomic and DNA binding profiles obtained from 3XFLAG-HA-Fancd2K559R-KI (a non-ubiquitinable mutant) testes extracts in a similar experimental setting.

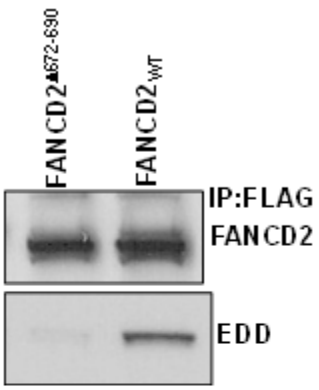

Figure 5. A FANCD2 mutant ($FANCD2^{\Delta 672\text{-}690}$ mutant) **that is deficient in EDD binding.** 293T cells were transiently transfected with plasmids expressing FLAG-$FANCD2_{WT}$ and FLAG-$FANCD2^{\Delta 672\text{-}690}$ mutant). After 48 hours post transfection, cells were treated with 1 mM HU for 16 hours. Cell extracts were immunoprecipitated with M2-agarose beads, and immunoblotted with the indicated antibodies.

- We examined the level of FANCD2 in 293T cells transfected with either wild-type Flag-EDD or ligase defective mutant Flag-C2768A-EDD and found that indeed, E3 ligase deficient mutant (C2768A-EDD) increased the steady state levels of FANCD2 indicating that E3 ligase activity of EDD is required for FANCD2 degradation (**Figure 7**).

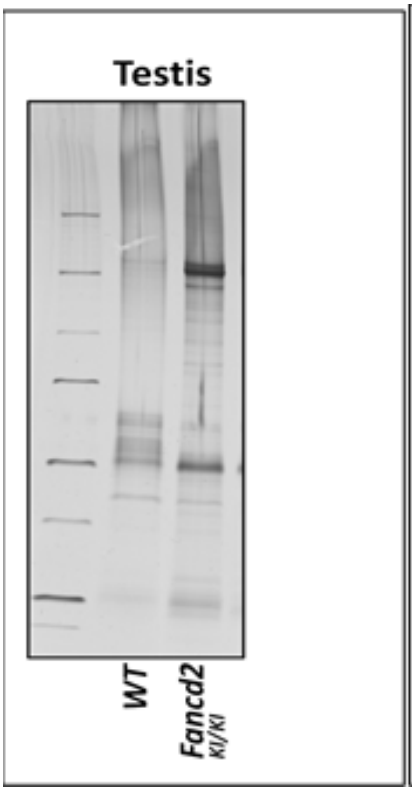

Figure 6. A silver stained gel showing FANCD2 binding proteins in the purified complex after formaldehyde crosslinks. 3XFLAG-HA-*Fancd2-KI* total mouse Testes cells were treated with 1.5 mM HU for then protein/protein/DNA complex was stabilized by crosslinking the cells with 1% formaldehyde for 8mins prior to chromatin extraction, *Micrococcal Nuclease (MNase)* digestion and soluble chromatin preparation. FANCD2 multiprotein/DNA bound complex was purified by a 2-step immunoprecipitation with anti-FLAG and anti-HA antibodies, reversed the crosslinks (by boiling with 2 X SDS sample loading buffer), and polypeptides were resolved onto 8-16% SDS-PAGE. Gel pieces were sliced and subjected to mass-spectrometry for protein identification. WT lane represent mock purification from of wild-type mouse testes cells.

- **Cells:** HAP1, HeLa, U2OS, 293T, PD20 and HSC93. These cell lines were obtained from ATCC and were tested regularly for mycoplasma contamination. 3XFLAG-HA Fancd2 knock in mouse ES cell. CRISPR-Cas9-mediated gene knockout. HAP1 cells knockouts of FANCD2, EDD, TFII-I, and RNF40 were generated in the pSpCas9 (BB)-2A-GFP (PX458) plasmid and created by the Transgenic Core (CCHMC).

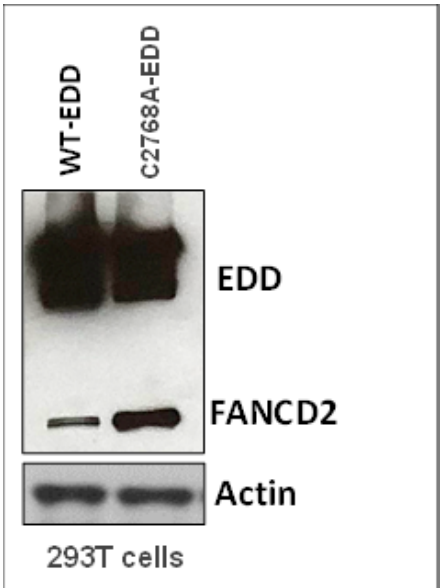

Figure 7. . E3 ligase deficient mutant (C2768A-EDD) of EDD increased the steady state levels of FANCD2. Both Wild type and C2768A-EDD were transiently transfected in 293T cells and the levels of endogenous FANCD2 were assayed by immunoblotting after 48 hr post transfection.

- We have made progress in **Aim 2** in last funding year. First, we completed the studies on the identification of the structural elements in mouse FANCD2 responsible for the interaction with RNF40 and EDD. We have generated mutants of FANCD2 that no longer binds to RNF40 and EDD (**Figures 4&5**). We also show that both RNF40 and EDD binding deficient FANCD2-mutants (FANCD2$^{\Delta 772\text{-}820}$ and FANCD2$^{\Delta 672\text{-}690}$ mutants respectively) failed to rescue the MMC sensitivity of FAND2 KO cells (**Figures 4B & 8B**) We have also successfully constructed mouse FANCD2 mutants lacking the RNF40-interacting domain or the EDD-interacting domain, which will be expressed as FLAG- or MYC-tagged protein pairs and subjected to immunoprecipitation and functional assays. We are now in the process of assessing whether FANCD2-RNF40 or FANCD2-EDD

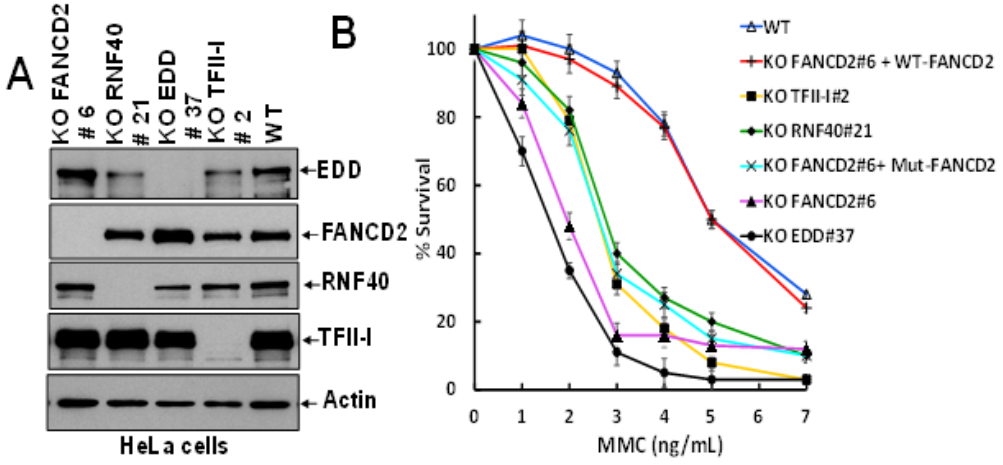

Figure 8. CRISPR-Cas9-mediated gene knockout in HeLa cells. CRISPR-Cas9 knockouts of FANCD2, EDD, TFII-I, and RNF40 were generated using pSpCas9(BB)-2A-GFP (PX458) vector containing gRNAs and the constructs were made by the Transgenic Core (CCHMC). Sequences of gRNAs used were: *FANCD2*: 5'-TTGTGAGCGTCTGCAGGATG-3'; *EDD*: 5'-GAATAACTCGAGTCTGCCGG-3'; *TFII-I*: 5'-GGCCAAGTCCAAAGCCGAAG -3'; *RNF40*: 5'-GCGATTGACGATGAGGAGTG-3'. **A)** Immunoblots using the indicated antibodies, and **B)** MMC kill curves. Wild type (WT) and Mut-FANCD2, (a human FANCD2$^{\Delta 672\text{-}690}$ mutant; corresponding to Δ670-688 mouse) cDNAs were cloned into pMIEG3 retroviral vector and transduced to KO FANCD2 HeLa cells.

interaction requires Fancd2 monoubiquitination and an intact FA core complex using the *Fancd2-KI* and *Fanca* mouse models, as proposed in Aim 2. Second, we have successfully performed CRISPR-Cas9-mediated knockout of *FANCD2, EDD*, and *RNF40*. (**Figure 8**).

- The reagents generated from these efficient knockouts will be critical for CRISPR/Cas9-mediated *FANCD2, RNF40* and *EDD* knockout in BM $CD34^+$ HSPCs from healthy donors, which we proposed in Aim 2 to investigate the functional consequence of the FANCD2-RNF40/EDD interaction in HSC maintenance under replication stress. Third, we proposed in Aim 2 to examine whether FANCD2-RNF40 or FANCD2-EDD interaction is required for the maintenance of DNA replication integrity and genomic stability. In the last funding year, we have been working on the optimal conditions for expression of a WT FANCD2 or mutant FANCD2 lacking the RNF40 -interacting domain or the EDD-interacting domain in *Fancd2-KO* BM $Lin^-Sca1^+c\text{-}kit^+$ (LSK) cells and FA patient BM $CD34^+$ HSPCs, as well as the optimal dose range of the DNA cross-linker MMC to induce DNA replication stress. Our preliminary results show that we obtained high efficiency of transduction with the lentivirus expressing eGFP alone or eGFP/FANCD2 in *Fancd2-KO* BM LSK cells (**Figure 9**). We were able to

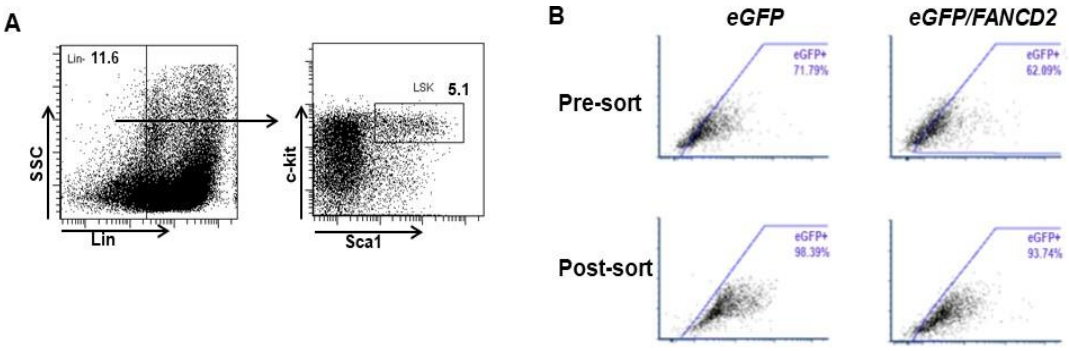

Figure 9. (A) Gating strategy for flow cytometry sorting BM LSK ($Lin^-Sca1^+c\text{-}kit^+$) cells. (B) Transduction efficiency of *Fancd2-KO* BM LSK cells with the lentivirus expressing eGFP alone or eGFP/FANCD2. FACS analyses demonstrate eGFP expression on the infected cells prior (Pre-sort) and after (Post-sort) flow sorting.

demonstrate that *Fancd2-KO* BM LSK cells transduced with the eGFP/FANCD2 lentivirus significantly reduced the well-established DNA damage signatures γH2AX (**Figure 10**) and

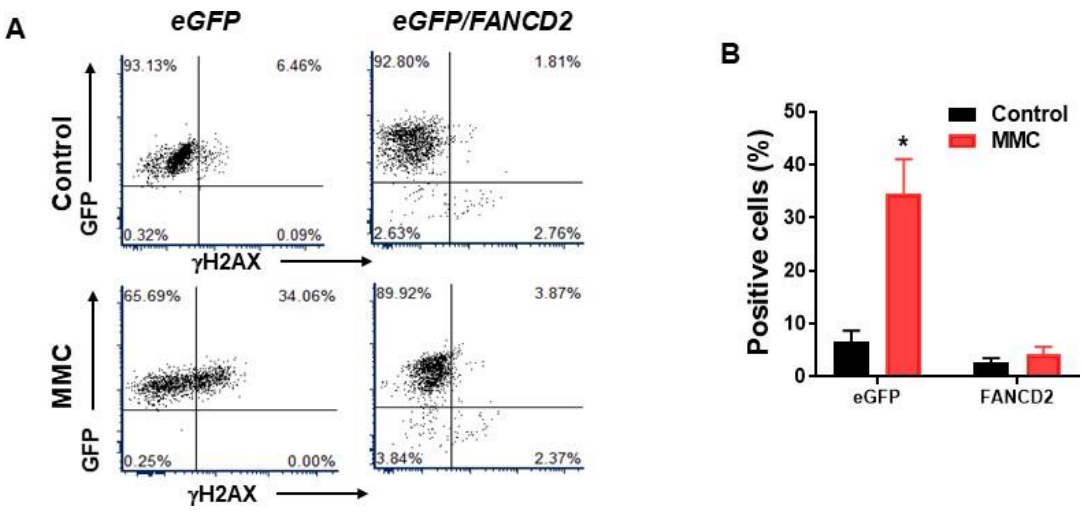

Figure 10. *Fancd2-KO* BM LSK cells transduced with the eGFP/FANCD2 lentivirus reduce DNA damage. *Fancd2-KO* BM LSK cells transduced with eGFP or eGFP/FANCD2 lentivirus were treated with MMC (5 ng/ml) for 8h and analyzed for the DNA damage signatures H2AX. Representative flow plots (A) and quantification (B) are shown. Results are mean ± SD of three independent experiments.

DNA strand breaks induced by the replication-stressing agent MMC (**Figure 11**). Furthermore, *Fancd2-KO* BM LSK cells transduced with the eGFP/FANCD2 lentivirus

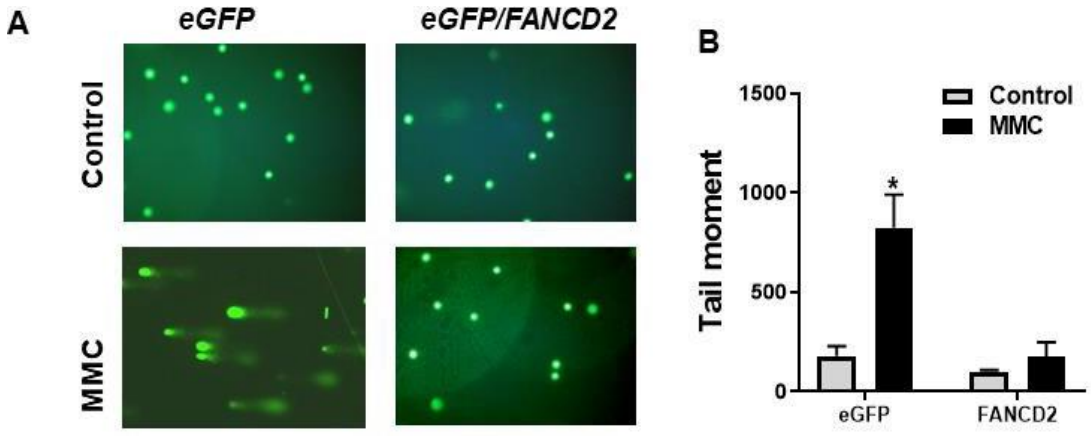

Figure 11. *Fancd2-KO* BM LSK cells transduced with the eGFP/FANCD2 lentivirus reduce DNA strand breaks. *Fancd2-KO* BM LSK cells transduced with eGFP or eGFP/FANCD2 lentivirus were treated with MMC (5 ng/ml) for 8h and analyzed for DNA strand breaks by Comet assay. Representative comet images (A) and quantification (B) are shown. Results are mean ± SD of three independent experiments and 50 cells were evaluated from each experiment. Larger tail moment represents higher levels of DNA strand breaks.

significantly increased repopulation of both MMC-treated and untreated cells in BM transplantation assays (Figure 12). These data suggest that the eGFP/FANCD2 lentivirus

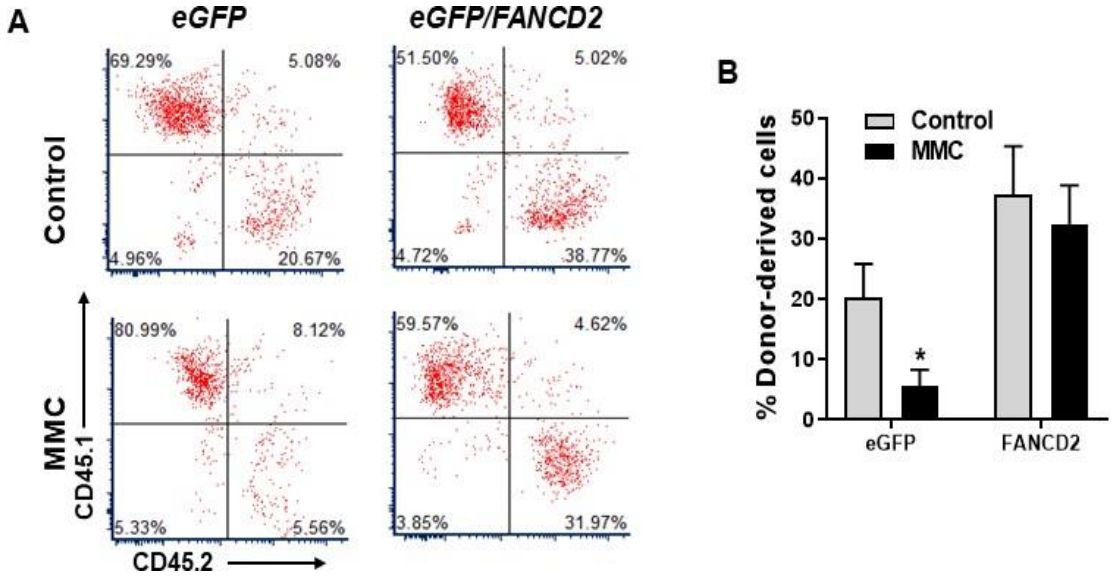

Figure 12. *Fancd2-KO* BM LSK cells transduced with the eGFP/FANCD2 lentivirus increase repopulation of both MMC-treated and untreated cells. *Fancd2-KO* BM LSK cells transduced with eGFP or eGFP/FANCD2 lentivirus were treated with MMC (5 ng/ml) for 2h and transplanted into lethally irradiated BoyJ recipient mice. Donor (CD45.2+) cell engraftment was analyzed by flow cytometry 4 months post-transplant. Representative flow plots (A) and quantification (B) are shown. Results are mean ± SD of three independent experiments (n = 8-10 per group).

constructs are functional both *in vitro* and *in vivo*. We believe that we can utilize these primary cells and improved techniques to achieve three goals; that is, to analyze (1) stalled replication forks using RPA, RAD51, and γH2AX foci formation; (2) the requirement of an intact FA core complex for FANCD2-RNF40 or FANCD2-EDD interaction using the *Fanca-KO;Fancd2-KI* mice; (3) the functional link between the FANCD2-RNF40 and FANCD2-EDD interactions and HSC function under replication stress using progenitor plating and serial BM transplantation assays.

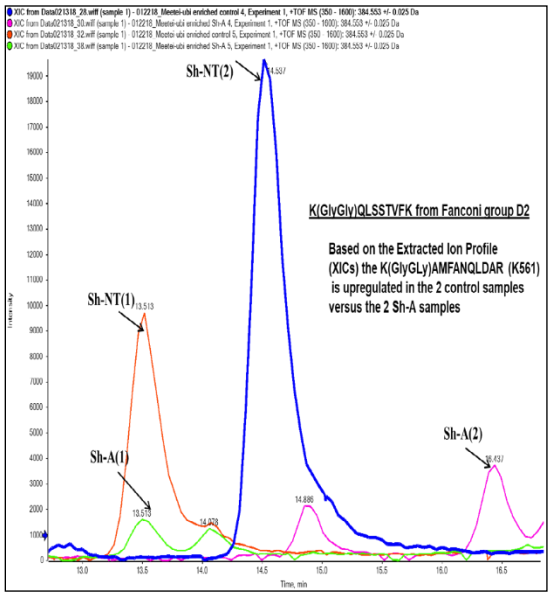

Figure 13. Mass spectrometry intensity plot showing the amount of ubiquitinated FANCD2 peptide is significantly reduced in Hela chromatin depleted of FANCA (sh-NT;shcontrol and sh-A;shFANCA).

Two biological repeats are shown.

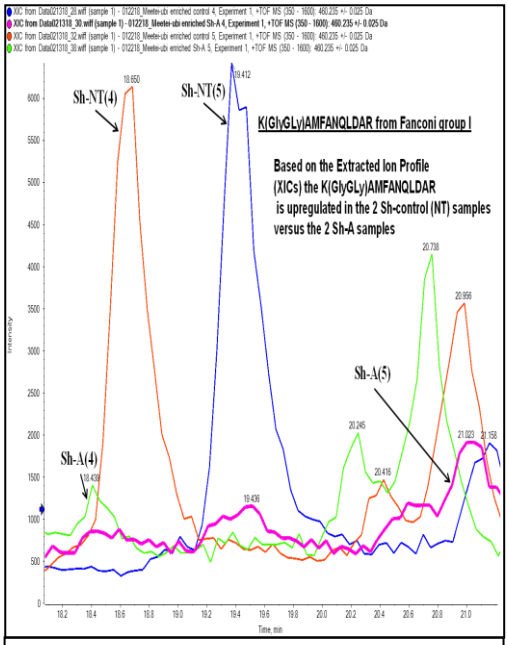

Figure 14. Mass spectrometry intensity plot showing the amount of ubiquitinated FANCI peptide is significantly reduced in Hela chromatindepleted of FANCA (sh-NT;shcontrol and sh-A;shFANCA). Two biological repeats are shown.

As shown in **figures 13,14&15**, we were successful to demonstrate that under steady state condition (without proteasome inhibitor treatment) the ubiquitinated forms of both FANCD2 and FANCI proteins at chromatin(under replication stress condition) are much higher in shcontrol (shNT) hela cells.

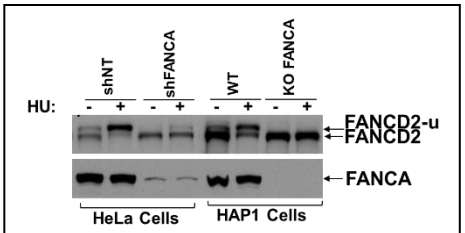

Figure 15. Immunoblots showing the lack of ubiquitinated FANCD2 (FANCD2-ub) in both Hela cells depleted of FANCA (via ShRNA) and KO-FANCA HAP1cells (via CRISPR-Cas9, a kind from Dr. Loizou, **CeMM,** Vienna, Austria) in response to HU (12 hr).

Since there are numerous ubiquitinated proteins found to be dysregulated in our UbiScan analyses, we will use the following criteria to select the target proteins based on; a) **-**fold changes, and b) proteins that are known to participate in the DNA repair signaling pathways. For example, we selected both H1B and its corresponding transcription activator ZBED1 to begin our validation (**table 3**) since the amount both ubiquitinated H1B at K49 and ubiquitinated ZBED1 at K80 were found to be significantly less in FANCA depleted cells (chromatin). We also selected histone H4 since the amount of ubiquitinated H4 at four sites (K,6,9,13,17) simultaneously were found to be significantly less in FANCA depleted cells (chromatin). Whereas H4 ubiquitination at K60, K78 and K92 were found to be unchanged.

- We used multiple shRNAs or CRISPR-Cas9 mediated KO (FANCA and FANCL) cells to detect the endogenous protein ubiquitination, we also Immunoprecipitate the target protein to improve the quality of the data.

Normalized Fold Change						
Chromatin shA : shNT	Nuclei shA : shNT	Max Abundance	Max % CV	Gene Name	Protein Name	Site
-568.6	-270.2	468,626,255	11%	HIST1H1B	H1B	§49
-531.7	-4.7	81,777,917	66%	ZBED1	ZBED1	80

Table 3. The amount of ubiquitinated Histone H1B and its transcription factor ZBED1 are significantly reduced in Hela cells (nuclei and chromatin) depleted of FANCA.

Normalized Fold Change										
Chromatin shA : shNT	Nuclei shA : shNT	Max Abundance	Max % CV	Gene Name	Protein Name	Site	Description	Accession	URL	kD	
-72.4	-2.2	11,131,150	54%	HIST1H4A	H4	6, 9, 13, 17	Histone H4	P62805	http://www.phosphos	11.4	GK*GGK*GLGK*GGAK*R
-1.4	-1.9	189,778,110	16%	HIST1H4A	H4	60	Histone H4	P62805	http://www.phosphos	11.4	GVLK*VFLENVIR
1.5	1.3	466,751,903	10%	HIST1H4A	H4	78	Histone H4	P62805	http://www.phosphos	11.4	DAVTYTEHAK*R
-1.4	1.2	104,925,965	9%	HIST1H4A	H4	92	Histone H4	P62805	http://www.phosphos	11.4	TVTAM#DVVYALK*R

Table 4. The amount of ubiquitinated (quadrupled ubiquitinations at K,6,9,13,17) Histone H4 significantly reduced in Hela cells (chromatin) depleted of FANCA.

- **Transient transfection and Immunoprecipitation:** We transiently transfected HAP1 wild type and FANCA KO cells with HA-tagged ubiquitin plasmids. Then cells were treated with hydroxyurea (HU) and endogenous proteins were immunoprecipitated by specific antibodies. The ubiquitination status of the immunoprecipitated proteins were detected by HA immunoblots. As shown as in **figure 16**, we now successfully demonstrated that mono-ubiquitinated FANCD2 is detected only in wild type cells but not in FANCA KO cells. Interestingly, we also show that ubiquitinated histone H4 is significantly reduced in FANCA KO cells (**figure 17**). These results are consistent with our UbiScan results.

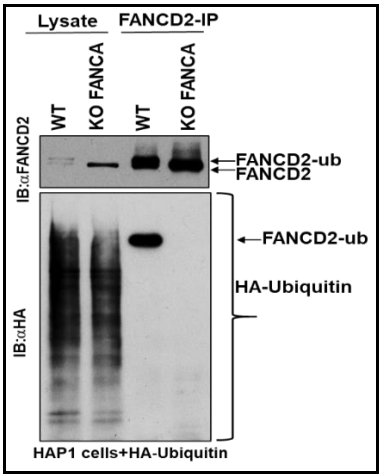

Figure 16: Mono-ubiquitinated FANCD2 is absent in FANCA KO HAP1 cells. Cells were transiently transfected with HA-tagged ubiquitin plasmids, treated with 1mM HU and endogenous FANCD2 was immunoprecipitated by anti FANCD2 antibody and immunoblotted with the indicated antibodies.

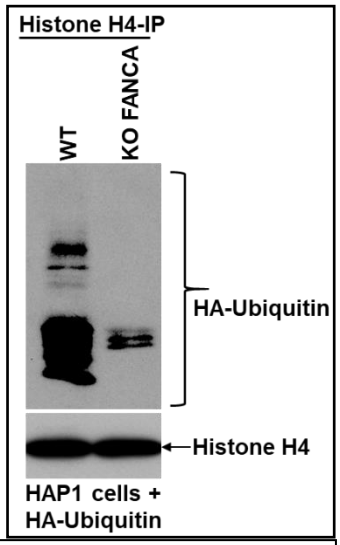

Figure 17: Ubiquitinated H4 is significantly reduced in FANCA KO HAP1 chromatin. Cells were transiently transfected with HA-ubiquitin plasmids, treated with 1mM HU and endogenous H4 was immunoprecipitated from chromatin fraction by CHIP grade anti H4 antibody and immunoblotted with the indicated antibodies.

- **Transient transfection:** For this purpose, we have established HEK293 cell lines stably expressing WT, K63R and K48R mutants of HA-tagged ubiquitin. As shown in **figure 18,** we have found that K49 of Histone H1B is the major site of ubiquitination.

- **To assess FA pathway functions:** We also found that nuclear foci formation of EDD requires the presence of functional FANCD2 protein as shown in **figure 19**.

- **Cells:** HAP1, HeLa, U2OS, 293T, PD20 and HSC93. These cell lines were obtained from ATCC and were tested regularly for mycoplasma contamination. 3XFLAG-HA Fancd2 knock in mouse ES cell. CRISPR-Cas9-mediated gene knockout. HAP1 cells knockouts of FANCD2, EDD, TFII-I, and RNF40 were generated in the pSpCas9 (BB)-2A-GFP (PX458) plasmid and created by the Transgenic Core (CCHMC).

- We have made progress in **Aim 2** in last funding year. First, we completed the studies on the identification of the structural elements in mouse FANCD2 responsible

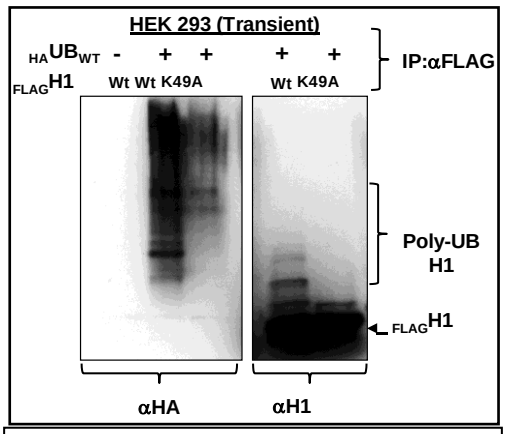

Figure 18. Immunoblots showing the K49 of Histone H1B is the major site of ubiquitination. Flag-tagged Wildtype and K49R mutant of H1were transiently transfected in HEK293 cells stably expressing HA-tagged ubiquitin. Cells were treated with 1mM HU for 8 hr. Flag proteins were immunoprecipitated by Flag antibody and immunoblotted with the indicated antibodies.

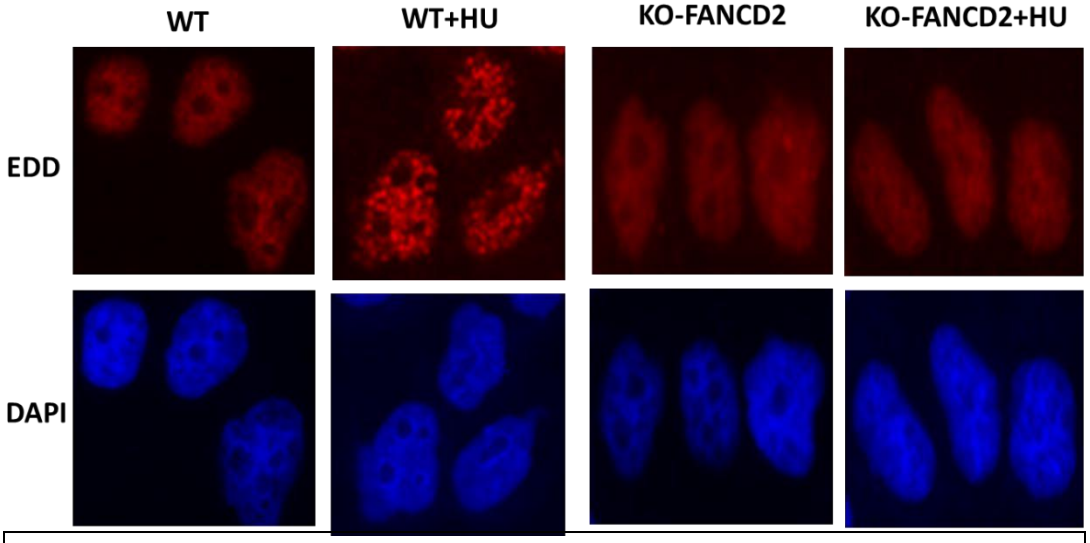

Figure 19. Immunofluorescence analysis showing EDD can form HU induced nuclear Foci only in HAP1 WT cells but not in FANCD2 KO cells. Cells were probed with DAPI (blue) and EDD antibodies for (red).

for the interaction with RNF40 and EDD. We have generated mutants of FANCD2 that no longer binds to RNF40 and EDD. We also show that both RNF40 (Mut1) and EDD (Mut2) binding deficient FANCD2-mutants failed to rescue the MMC sensitivity of FAND2 KO cells (**Figure 20)**.

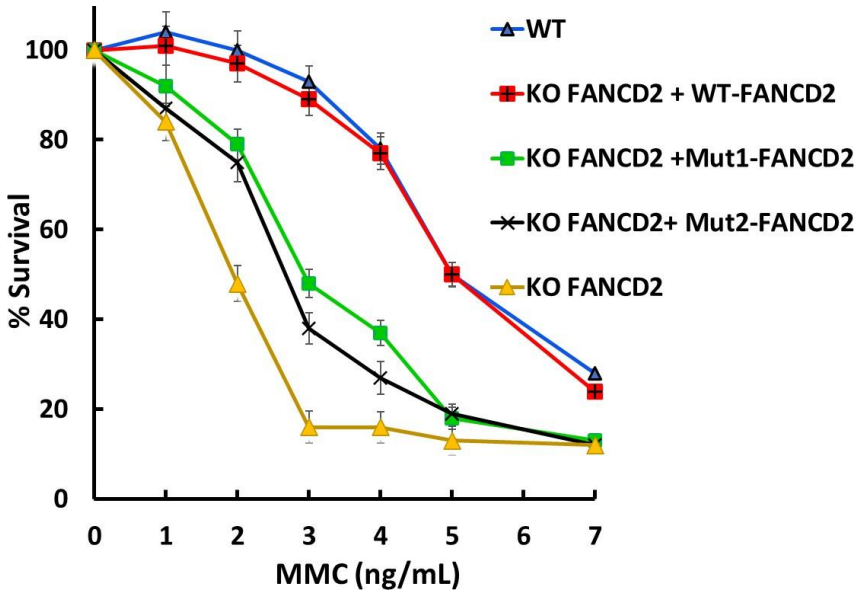

Figure 20: HAP1 KO cells and KO of FANCD2 and add-back experiments as described above.

- We have also proposed to determine whether FANCD2-RNF40 or FANCD2-EDD interaction requires an intact FA core complex. We have generated the *Fanca-KO;Fancd2-KI* mice, which are deficient for the essential component of the FA core complex (*Fanca*). We have performed preliminary experiments to determine whether expression of the 3XFLAG-*Fancd2 KI* allele in *Fanca-KO* mice would alter the FA hallmark phenotype; DNA damage and homologous recombination (HR) repair. We first confirmed the expression of the 3XFLAG-*Fancd2* KI protein in *Fanca-KO* mice (Figure 21**A**). We then isolated LSK ($Lin^-Sca1^+c\text{-}kit^+$; enriched for HSCs) from *Fanca-KO;Fancd2-KI* mice and the *WT;Fancd2-KI* control mice, and exposed the cells to MMC (5 ng/ml) to assess MMC-induced γH2AX and RAD51 foci formation. We observed a significant increase in MMC-induced formation of γ-H2AX foci in *Fanca-KO;Fancd2-KI* LSK cells compared to those from the *WT;Fancd2-KI* control (Figure 21**B**). MMC-induced HR repair, as determined by the formation of RAD51 foci, was evidently defective in *Fanca-KO;Fancd2-KI* LSK cells (Figure 21**C**). These preliminary data indicate that the 3XFLAG-*Fancd2 KI* allele does not alter the FA cellular hallmarks of *Fanca-KO* mice. We will be using these mice to examine the FANCD2-RNF40 or FANCD2-EDD complex formation in the absence of *Fanca*.

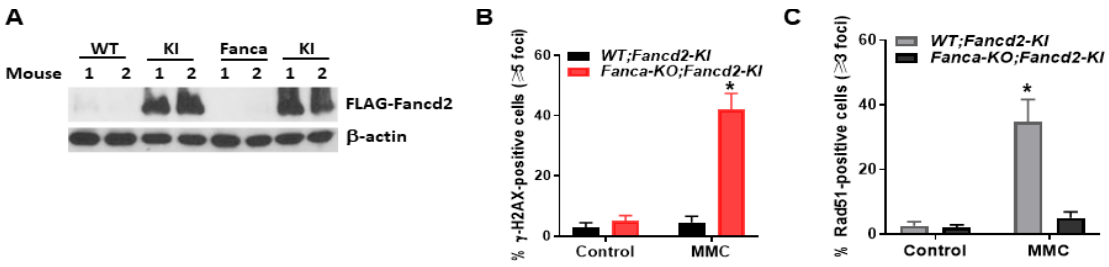

Figure 21. Formation of γ-H2AX and Rad51 foci in HSPCs of *Fancd2-KI* mice. (A) Western blot analysis of the FLAG-tagged Fancd2 protein in BM cells from *Fanca-KO;Fancd2-KI* mice and the *WT;Fancd2-KI* control mice, using anti-FLAG (M2; Sigma) antibodies. (B) Formation of γ-H2AX and Rad51 foci in HSPCs of *Fancd2-KI* mice. BM LSK ($Lin^-Sca1^+c\text{-}kit^+$) cells were isolated from *Fanca-KO;Fancd2-KI* mice and the *WT;Fancd2-KI* control mice and exposed to MMC (5 ng/ml) for 8 hours followed by immunofuorescence staining for γ-H2AX. (C) Formation of Rad51 foci in HSPCs of *Fancd2-KI* mice. BM LSK cells were isolated from *Fanca-KO;Fancd2-KI* mice and the *WT;Fancd2-KI* control mice and exposed to MMC (5 ng/ml) for 8 hours followed by immunofuorescence staining for Rad51.

- **What opportunities for training and professional development has the project provided?**
  - *"Nothing to Report."*
- **How were the results disseminated to communities of interest?**
  - "Nothing to Report."
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Not applicable since funding period was ended.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**
  - Our studies on the functional consequence of interaction between the FANCD2-RNF40 and FANCD2-EDD has the potential to establish the functional link between the FANCD2/FA pathway and replication stress response in genomic maintenance. Indeed, utilizing primary

hematopoietic stem/progenitor cells from Fancd2-KO mice and FA patients expressing the FANCD2 mutants lacking the RNF40 -interacting domain or the EDD-interacting domain, we will provide the first line of valuable information demonstrating that the FANCD2-RNF40 and FANCD2-EDD interactions serve to maintain HSC function defined by supporting HSC proliferation and repopulation. We also expect these functional data to uncover whether these interacting pathways may be viable targets for therapy in FA BM failure.

- **What was the impact on other disciplines?**
  - *"Nothing to Report."*

- **What was the impact on technology transfer?**
  - *"Nothing to Report."*
- **What was the impact on society beyond science and technology?**
  - *"Nothing to Report."*

5. CHANGES/PROBLEMS:

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - Projects have been significantly delayed due COVID-19 related disruption in the research activities. Mouse colonies perished during Covid-19 pandemic making it impossible to re-start it on time.
    I could not perform some of the experiments such as transplantation μsing FA derived Bone marrow primary cells due to Covid Lockdown, no cells were available due to clinic shutdown, mouse colony was totally shut down, and my collaborator Dr. Pang retired before performing the proposed experiments. We could not perform the interaction studies between FEN1-EDD since in vitro expression and purification of the recombinant EDD was unsucessful.

6. PRODUCTS:

- "Nothing to Report."

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**
  -

Name:	*Ruhikanta Meetei*
Project Role:	*PI*

-

Name:	*Qishen Pang*

Project Role:	*Co-PI*

-

Name:	*A*beer Najjar
Project Role:	*Research Assistant III*
Name:	Srinivas Chatla
Project Role:	*Research Associate*

  - **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
    - "No Change."
  - **What other organizations were involved as partners?**
    - "Nothing to Report."

8. SPECIAL REPORTING REQUIREMENTS

- **N/A**.

9. APPENDICES:

- **Transition Plan Questionnaire**

Transition Plan Questionnaire

Directions: Please answer all questions that apply for each product under development. Please fill out one document per product.
This is not an application for funding; however, answers will help us understand the outcomes and products from your award.

1. After the award closes, would you be willing to periodically provide voluntary information (via email) regarding the project status (i.e. where the research is headed)? **Yes** ☐ or **No** ☑
These responses will help CDMRP demonstrate the return on its investments and will help demonstrate that the CDMRP is a responsible and successful steward of federal research funding.

2. What **conclusion(s)** does your final data support?

Our studies on the functional consequence of the interaction between the FANCD2-RNF40 and FANCD2-EDD has the potential to establish the functional link between the FANCD2/FA pathway and replication stress response in genomic maintenance. We also expect these functional data to uncover whether these interacting pathways may be viable targets for therapy in FA BM failure.

3. Will you/have you applied for/obtained follow-on-funding for this project? **If yes**, please list (a) funding organization, (b) total budget requested/obtained, and (c) title of the funded proposal. *This information will be recorded as an outcome to this award*.

NA

4. What will be **the next step(s)** for this project?

Further investigation is needed to uncover the disease mechanisms of this severe bone marrow failure and childhood cancer.

5. How would you classify your **lead candidate product**? Please choose best option
(a) Therapeutic (Small Molecule, Biologic, Cell/Gene Therapy): Please choose, if applicable
(b) Diagnostic
(c) Device
(d) Research Tool to Address a Research Bottleneck
(e) Knowledge Product (Non-material product such as a compound library, database, something that improves clinical practice, education, etc.)
(f) Other - Please Specify: Research tool to address research bottleneck(d)

6. How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

NA

7. Therapy / Product Development, Transition Strategies, and Intellectual Property

Describe the steps and relevant strategies required to move the candidate product (knowledge or tangible) to the next phase of development and/or commercialization. Please address any issues with intellectual property.

PIs are encouraged to explore the technical requirements and the current regulatory strategies involved in product development as well as to work with their organization's Technology Transfer Office (or equivalent regulatory/legal office), federal/international regulatory experts, to develop the transition plan and to explore developing relationships with industry, DoD advanced developers (e.g. USAMMDA), and/or other funding agencies to facilitate moving the product into the next phase.

NA