Supporting Information

Wang et al. 10.1073/pnas.1002603107

SI Results

Fanconi Anemia Complementation Group Core Activity Is Not Required for C/EBPδ-Mediated Nuclear Import of Fanconi Anemia Complementation Group D2 Protein. In cells that lack Fanconi anemia complementation group A (FANCA), an essential component of the Fanconi anemia (FA) ubiquitin ligase core complex (1–3), mitomycin C (MMC) still promoted nuclear translocation of FANCD2, which was impaired again by silencing of CCAAT/enhancer binding protein δ (C/EBPδ) (Fig. S5). Consistent with the loss of FANC core complex function, only the FANCD2-S isoform was observed, unless the cells were reconstituted with FANCA (Fig. S5*B*). These data show that monoubiquitination of FANCD2 is not required for nuclear translocation, consistent with the notion that this modification occurs within the nucleus (1–3). In FANCA-deficient cells, MMC also induced C/EBPδ expression, which therefore is independent of the FA pathway (Fig. S5*A*).

C/EBPδ Is Not Required for MMC-Induced FANCD2 Foci. Because monoubiquitinated FANCD2 specifically binds to sites of DNA damage (4, 5), we also analyzed the effect of C/EBPδ depletion on the subcellular localization of FANCD2 in FANCD2-null PD20 fibroblasts reconstituted with FANCD2 (PD20-D2) cells (Fig. S6). MMC-induced focal localization of FANCD2 was observed even when C/EBPδ was silenced, although the overall intensity of staining was reduced. These data are consistent with a role for C/EBPδ in nuclear translocation of FANCD2 and suggest that C/EBPδ is not required for the binding of FANCD2-L to sites of DNA damage. This experiment also showed that C/EBPδ does not colocalize with FANCD2 to the sites of DNA-damage (Fig. S6).

SI Materials and Methods

Cells. WT and C/EBPδ-KO mouse embryo fibroblasts (MEFs) were generated as described (6). The cell lines PD20, PD20-D2, PD220, and PD220RV were obtained from the Fanconi Anemia Research Fund. 293-C/EBPδ cells were as described (7). All cell lines were maintained in DMEM (Invitrogen) supplemented with 10% (15% for PD220) FBS (Invitrogen) and were incubated in a 5% CO_2 humidified atmosphere at 37 °C. Cells were transiently transfected with expression constructs using TransIT-LT1 (Bio LLC #2300A; Mirus). Extracts were prepared 24 h later if cells were untreated. Otherwise, cells were treated the following day with MMC at 1 μg/mL for 24 h, unless indicated otherwise. Cells were transfected with empty vector as control.

Plasmids. To generate the amino acids 1–151, amino acids 140–215, and C/EBPδ-ΔDBD constructs, the corresponding regions were PCR amplified and subcloned into shuttle vector 5′Flag-SK+ by Nhe I and Bam HI. The Flag-tagged C/EBPδ cassettes were excised by Sal I and Xba I and subcloned into pMEX mammalian expression vector by blunt-end ligation into Sma I. To generate the C/EBPδ mutant lacking the FANCD2 interaction domain (ΔD2ID), PCR was performed using a 5′ primer containing a NotI restriction enzyme site and DNA sequence coding for amino acids 192–194 and a 3′ primer harboring an EcoRI site downstream of the coding region. The PCR product was then inserted into a NotI- and EcoRI-digested WT expression construct, replacing the WT sequence. All final products were verified by sequencing.

Cell Survival and Colony Formation Assays. Cells were seeded in 96-well plates at 3,000–5,000 cells/well and were treated the next day with MMC and/or tetracycline as indicated. Each experiment was done with triplicate wells. Cells were incubated with 10% Alamar blue (BioSource) for 3 h, and fluorescence was quantified with a NovaStars (BMG) plate reader. Mouse bone marrow cell colony assays were performed using MethoCult (Stem Cell Technologies) according to the manufacturer's protocol, seeding 25,000 cells per 35-mm dish. Colonies were counted after 10–14 d of incubation, blinded to the genotype and treatment.

Flow cytometric Analysis. Cells were trypsinized, fixed in 70% ethanol, stained with propidium iodide, and analyzed by fluorescence-activated cell sorting using FACSCalibur and CellQuest software (Fig. 2*D*) or FACSCanto II and DiVa software (Fig. 2*E*) (Becton Dickinson).

Immunocytochemistry. WT and C/EBPδ KO MEFs were seeded at ≈30% confluence on coverslips. The cells were washed with PBS 16 h later and were fixed with 2% formaldehyde (Electron Microscopy Sciences) for 20 min. Cells were permeabilized with 0.3% Triton X-100 in PBS for 15 min and quenched with 1% glycine in PBS for 5 min. The phosphorylated form of H2AX (γH2AX) was detected with polyclonal anti-γH2AX (1:600; Millipore) and Alexa Fluor 488 goat anti-rabbit IgG (1:1,000; Invitrogen) in 3% BSA. C/EBPδ and FANCD2 were detected with monoclonal anti-C/EBPδ (1:1,000; BD Biosciences) and polyclonal anti-FANCD2 (1:1,000; Novus), Alexa Fluor 594 goat anti-mouse IgG (1:1,000, Invitrogen), and Alexa Fluor 488 goat anti-rabbit IgG (1:1,000, Invitrogen) in 3% BSA. Cell nuclei were detected with 0.1% DAPI (Invitrogen). Cells were visualized with an LSM 510 META confocal fluorescence microscope (Zeiss).

Protein Extracts and Western Analysis. Cells were lysed in lysis buffer (30 mM Tris, pH 8.0, 75 mM NaCl, 10% glycerol, 1% Triton X-100) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/mL phosphatase inhibitor I and phosphatase inhibitor II, 10 μg/mL protease inhibitor mixture) for immunoprecipitation. Whole-cell lysates were prepared with SDS sample buffer. Cytoplasmic and nuclear fractionation was done essentially according to methods described (8). The proteins were resolved by SDS/PAGE and transferred onto PROTRAN nitrocellulose membranes (Schleicher & Schull). Membranes were blocked with 5% skim milk in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5% Tween20 (TBS-T). After incubation with primary antibodies and secondary antibodies in TBS-T, the protein signals were visualized by Lumiglo Reserve Chemiluminescence reagent (KPL Inc.) according to the manufacturer's instructions. Antibodies were from Novus (FANCD2, NB-100-182), Santa Cruz Biotechnology Inc. (FANCD2, H-300; GAPDH, 0–411), BD Biosciences Pharmingen (C/EBPδ, BD69319), Fanconi Anemia Research Fund, Inc. (FANCA, R6512), Proteintech Group (IPO4, 11679–1-AP), Rockland Immunologicals Inc. (tubulin, 600–401-880; C/EBPδ, 600–401-A61), Millipore (Ser139 phosphorylated H2AX, 07–164), Pierce (anti-mouse and anti-rabbit HRP-conjugated IgG, #1858413 and #1858415), Bethyl Laboratories Inc. (A300-083A), and Thermo Scientific (anti-goat HRP-conjugated IgG, #31400). Quantification of FANCD2-L and -S ratios was done using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij/).

Coimmunoprecipitation. Cell lysate (1 mg) was mixed with antibody (5 μg) and rotated overnight at 4 °C. An equivalent aliquot of lysate for specific antibodies as indicated was used for immunoprecipitation with IgG as a negative control. Protein A/G beads were blocked with 1% BSA and 1 mg/mL ssDNA overnight. Preblocked

beads (40 μL) were added to the lysate and antibody mixture for 4 h at 4 °C before centrifugation at 1,000 × *g* rpm (Eppendorf) for 30 s, followed by five washes with lysis buffer. Preboiled sample buffer (40 μL) (Bio-RAD) was added to each immunoprecipitation mixture and denatured in 100 °C for another 5 min. Then 10 μL of each immunoprecipitation product was loaded.

RNAi. On-Target plus SMARTpool siRNA for IPO4 (catalog #L-009516-01) and C/EBPδ (catalog# L-010453-00) were purchased from Dharmacon RNAi Technologies (Thermo Scientific) along with Accell Red nontargeting siRNA (catalog #D-001960-01-05) as a negative control and were applied according to the manufacture's protocol. Briefly, MDA-MB-468 cells were transfected with 100 nM On-Target plus SMARTpool siRNA at 2×10^6/100 μL in Nucleofector solution V (Amaxa Biosystems/Lonza) according to the manufacturer's instructions. For Fig. 3*E*, PD20-D2 cells were transfected with 0.5 μg expression vectors for C/EBPδ shRNA (7, 9) or GFP as control (10) at 1×10^6cells/100 μL Nucleofector solution V according to the manufacturer's instructions. The pMaxGFP expression construct (200 ng, Amaxa Biosystems/Lonza) was included to estimate transfection efficiency.

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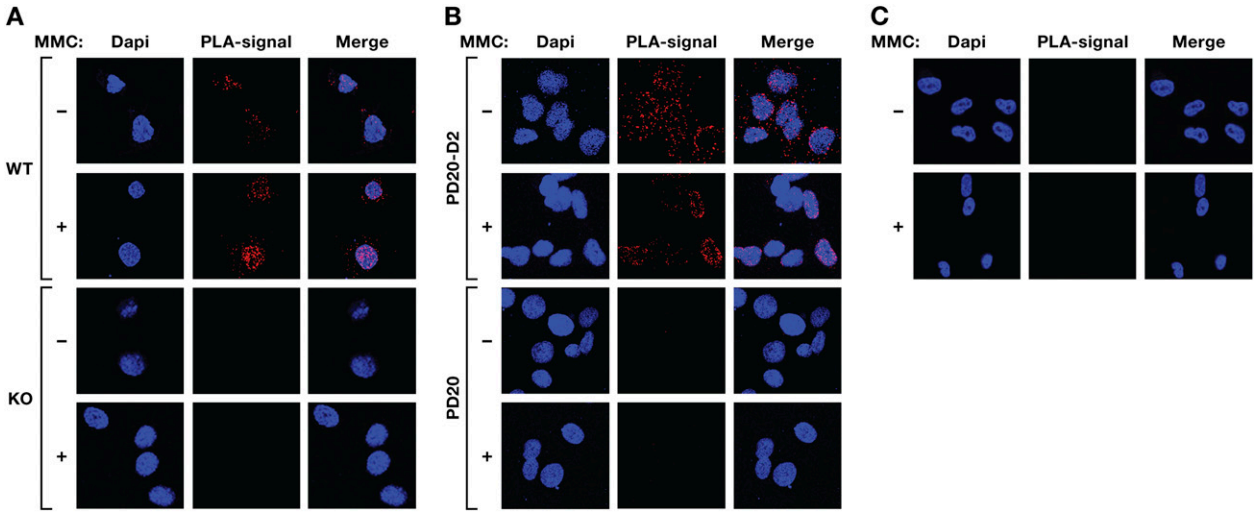

Fig. S1. Interaction of endogenous C/EBPδ and FANCD2. (*A*) WT and KO MEFs were treated for 3 h with 5 μg/mL MMC as indicated. Cells were fixed and incubated with antibodies against C/EBPδ and FANCD2 followed by OLink in situ proximity ligation assay (PLA) and fluorescence microscopy using appropriate filters. Merged images of antibody proximity (red) and nuclei (blue) are shown on the right. (*B*) FANCD2-deficient cells (PD20) and FANCD2-deficient cells reconstituted with WT FANCD2 (PD20-D2) were treated for 20 h with 500 ng/mL MMC before processing as described in *A*. The merged images are the same as in Fig. 1 *D* and *E*. (*C*) PD20-D2 cells were treated as in *B*. The cells then were incubated with antibodies against C/EBPδ and FANCA followed by OLink PLA assay as described in *A*.

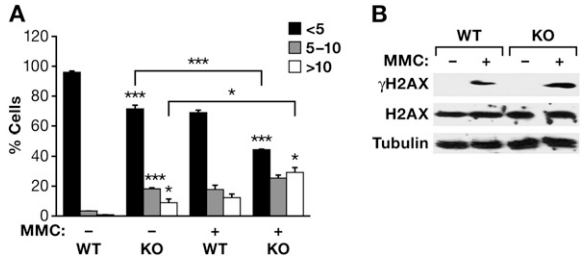

Fig. S2. Analysis of γH2AX in response to DNA damage. (*A*) Quantification of DNA damage-response foci containing γH2AX. WT and C/EBPδ-null (KO) primary MEFs cultured on coverslips were fixed and immunostained for γH2AX after 3 h of MMC (5 μg/mL) treatment. For each sample, at least 100 nuclei were examined by fluorescence microscopy and categorized as having <5, 5–10, or >10 foci. The percentage of cells in each category is shown as mean ± SEM of three independent WT-MEF and KO-MEF preparations, respectively. *P* values refer to comparison of WT and KO samples of the same treatment group unless indicated otherwise; $*P < 0.05$; $***P < 0.001$. (*B*) H2AX phosphorylation in response to MMC. Immortalized WT and KO MEFs were harvested after 24 h of MMC (1 μg/mL) treatment, followed by Western blot analysis of the indicated proteins in whole-cell lysates. Untreated cells were used as controls.

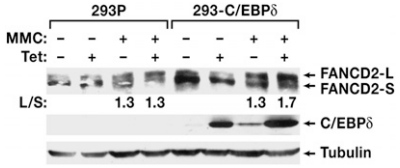

Fig. S3. C/EBPδ augments FANCD2 monoubiquitination in response to MMC. Western blot analysis of FANCD2 and C/EBPδ in whole-cell lysates from HEK293 cells with tetracycline-inducible C/EBPδ expression (293-C/EBPδ) and the parental cell line (293P) treated with tetracycline (Tet; 500 ng/mL) and/or MMC (1 μg/mL) for 24 h. L/S, quantification of the FANCD2-L/S ratio in lysates of MMC-treated cells.

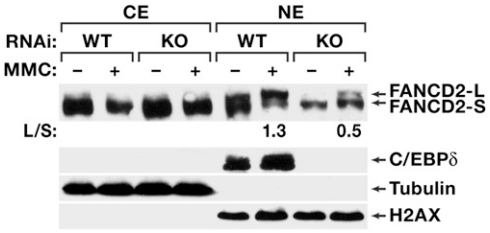

Fig. S4. C/EBPδ augments nuclear localization of FANCD2 in MEFs. WT and C/EBPδ-KO MEFs were treated with 1 μg/mL MMC for 20 h as indicated, followed by Western blot analysis of protein expression in cytoplasmic (CE) and nuclear (NE) extracts. Tubulin and H2AX were used as markers for cytoplasmic and nuclear extracts, respectively. L/S, quantification of the FANCD2-L/S ratio in lysates of MMC-treated cells.

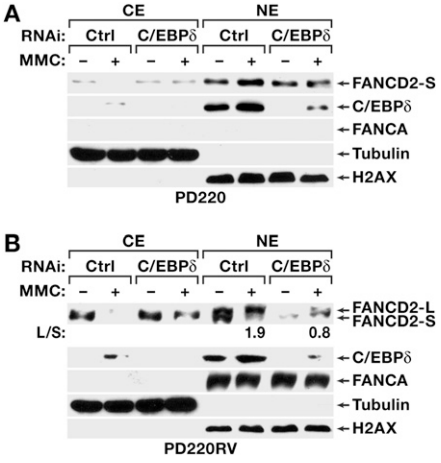

Fig. S5. C/EBPδ augments nuclear import of FANCD2 in FANC core-deficient cells. (*A*) FANCA-deficient cells (PD220) and (*B*) PD220 cells reconstituted with WT FANCA (PD220RV) were transiently transfected with siRNA against C/EBPδ or with scrambled oligos as control. MMC (500 ng/mL) was added 24 h later, and cells were incubated for another 20 h followed by Western blot analysis of protein expression in cytoplasmic (CE) and nuclear (NE) extracts. Tubulin and H2AX were used as markers for cytoplasmic and nuclear extracts, respectively. L/S, quantification of the FANCD2-L/S ratio in lysates of MMC-treated cells.

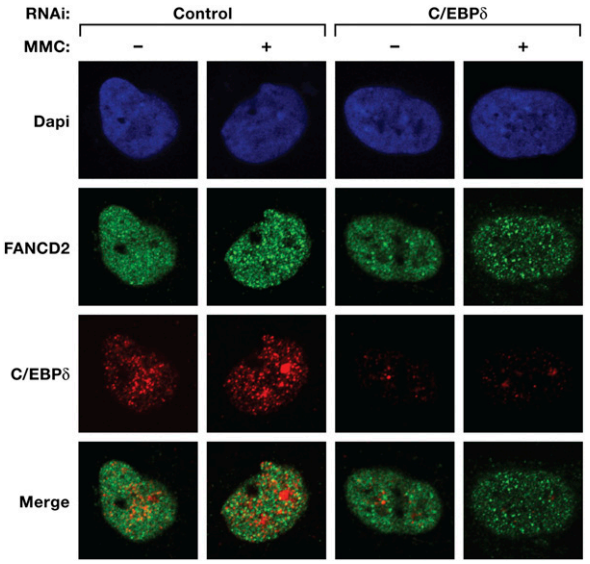

Fig. S6. Subcellular distribution of FANCD2 and C/EBPδ in PD20-D2 cells. PD20-D2 cells were transiently transfected with siRNA against C/EBPδ or with scrambled oligos as control. MMC (500 ng/mL) was added 24 h later, and cells were incubated for another 20 h. The fixed cells were incubated with mouse anti-C/EBPδ and rabbit anti-FANCD2 antibodies, followed by respective fluorescence-labeled secondary antibodies and confocal microscopy using appropriate filters. Merged images of C/EBPδ (red) and FANCD2 (green) are shown in the bottom row. A representative nucleus is shown for each condition. This assay is not sensitive enough to detect the colocalization of C/EBPδ and FANCD2 demonstrated by PLA (Fig. 1 *D* and *E* and Fig. S1 *A* and *B*).

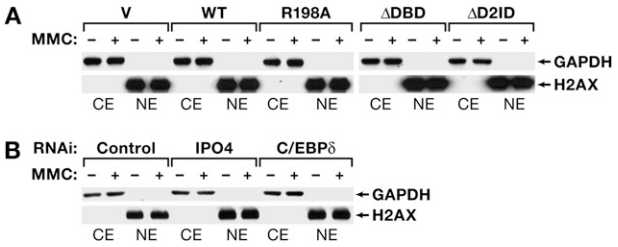

Fig. S7. Quality control of nuclear versus cytoplasmic protein fractions. Western blot analysis of cytoplasmic (CE) and nuclear (NE) protein extracts from (*A*) HEK293 cells transfected with the indicated C/EBPδ constructs as shown in Fig. 4 *B* and *C* and (*B*) MDA-MB-468 cells nucleofected with the indicated siRNA as shown in Fig. 6 *A* and *B*. The same membranes were probed with antibodies against cytoplasmic GAPDH and nuclear H2AX.

Table S1. Proteins interacting with C/EBPδ by mass spectrometry

Gene name	Accession no.	Unique peptides identified using MS[†]	Total peptide count[‡]	Common name(s), aliases	Function
FANCD2	Q9BXW9	K.AIEEIAGVGVPELINSPK.D	13	Fanconi anemia group D2 protein	DNA repair
		K.DASSSTFPTLTR.H	13		
		K.DGGPVTSQESGQK.L	3		
		K.GILDYLDNISPQQIR.K	5		
		K.LLLGIDILQPAIIK.T	5		
		K.LPEYFFENK.N	14		
		K.TGESQNQLAVDQIAFQK.K	10		
IPO4	Q8TEX9	K.AGLLVLAVLSDGAGDHIR.Q	9	Importin-4 (Importin 4b) (Imp4b) Ran-binding protein 4 (RanBP4)	Nuclear import
		K.LLGLLFPLLAR.E	13		
		K.SFAVGTLAETIQGLGAASAQFVSR.L	2		
		K.SLILTALQR.E	12		
		R.EVMPLLLAYLK.S	9		
		R.LLPVLLSTAQEADPEVR.S	27		
		R.NAALFALGQFSENLQPHISSYSR.E	3		
		R.RATEQLQIVLR.A	9		
		R.TLTTMAPYLSTEDVPLAR.M	9		
		R.VVPSYMQAVNR.E	9		
SPAG5	Q96R06	K.ELQEVIQQQNEK.I	6	Sperm-associated antigen 5 (Astrin) Mitotic spindle associated protein p126	Mitotic spindle
		K.ILEQIDK.S	6		
		K.LASTIADNQEQDLEK.T	10		
		K.LGLQEGSNNSSPVDFVNNK.R	3		
		K.SGELISLR.E	6		
		K.STNTSQTGLVGTK.H	7		
		R.ELTLQPGALTNSGK.R	3		
		R.NVMQSWVLISK.E	6		
TRIM26	Q12899	K.AQQPAAELMQDTR.D	4	Tripartite motif protein 26 Zinc finger protein 173 (ZNF173), Acid finger protein (AFP) RING finger protein 95 (RNF95)	Putative ubiquitin E3 ligase
		K.ENIRPVWQLASLVENIER.L	4		
		K.GEADILAALK.K	3		
		K.LEQELTEGR.E	8		
		R.LALVISELEGK.A	7		
		K.AQQPAAELMQDTR.D	4		
		K.GEADILAALK.K	8		
		R.RLVPFLWLK.W	4		
UBR5	O95071	K.IVLLSANSIR.A	4	Ubiquitin protein ligase E3 component n-recognin 5 Ubiquitin–protein ligase EDD1 Hyperplastic discs protein homolog (hHYD)	Ubiquitin E3 ligase
		K.LEHTAQTYSELQGER.I	8		
		K.YNHLVYSQIPAAVK.L	3		
		R.AYPAAITILETAQK.I	4		
		R.LLTATNLVTLPNSR.G	6		
		R.NVAIFTAGQESPIILR.D	10		
XPO1	O14980	K.AIIASNIMYIVGQYPR.F	11	Exportin-1 Chromosome region maintenance 1 protein homolog	Nuclear export
		K.DFEEYPEHR.T	11		
		K.EFAGEDTSDLFLEER.E	11		
		K.LFVTGLFSLNQDIPAFK.E	11		
		K.LISGWVSR.S	11		
		K.LISTLIYK.F	11		
		K.LLSEEVFDFSSGQITQVK.S	11		
		K.LVLDSIIWAFK.H	22		
		K.NVDILKDPETVK.Q	11		
		K.YYGLQILENVIK.T	13		

Cycling 293T cells expressing Pyo-tag'd wild-type C/EBPδ or vector control-transfected cells were lysed in Triton X-100 lysis buffer (20 mM Tris [pH 8.0], 50 mM NaCl, 10% glycerol, 1% Triton X-100, 0.15 U/mL aprotinin, 1 mM PMSF, 20 δM leupeptin, and 5 mM sodium vanadate). Immunoprecipitates with anti-pyo antibodies were resolved by PAGE. Each lane was cut into 20 gel fractions and proteins were isolated and analyzed by mass spectrometry as previously described (1). Only one sample was run per condition. Because of this preliminary nature of the experiment, only those proteins are shown that were represented by more than five independent peptides and more than 10 counts total. Proteins of peptides that were also detected in vector control cells or immunopreicipated with other pyo-tagged proteins (2) were not included in this list. FANCD2 and IPO4 are subject of this publication. Based on these data, XPO1 is the likely mediator of C/EBPδ export form the nucleus. UBR5 and TRIM26 are candidates for targeting C/EBPδ for proteasomal degradation. The interaction with SPAG5 is also intriguing due to the potential role of SPAG5 in the DNA-damage response (3) and will be subject of future studies.

[†]Identity of unique peptides identified for the protein listed.

[‡]Number of times the unique peptide was identified using mass spectrometry.

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