FANCC, FANCE AND FANCD2 FORM A TERNARY COMPLEX ESSENTIAL TO THE INTEGRITY OF THE FANCONI ANEMIA DNA DAMAGE RESPONSE PATHWAY*  

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Fanconi anemia (FA) is a genetically heterogeneous disorder characterized by bone marrow failure, cancer predisposition, and increased cellular sensitivity to DNA-crosslinking agents. The products of seven of the nine identified FA genes participate in a protein complex required for monoubiquitination of the FANCD2 protein. Direct interaction of the FANCE protein with both fellow FA complex component FANCC and the downstream FANCD2 protein has been observed in the yeast two-hybrid system. Here we demonstrate the ability of FANCE to mediate interaction between FANCC and FANCD2 in the yeast three-hybrid system and confirm the FANCE-mediated association of FANCC with FANCD2 in human cells. A yeast two-hybrid based screen was devised to identify randomly mutagenized FANCE proteins capable of interaction with FANCC but not with FANCD2. Exogenous expression of these mutants in an FA-E cell line and subsequent evaluation of FANCD2 monoubiquitination and DNA-crosslinker sensitivity indicate a critical role for the FANCE/FANCD2 interaction in maintaining FA pathway integrity. Three-hybrid experiments also demonstrated the ability of FANCE to mediate interaction between FA core complex components FANCC and FANCF, indicative of a further role for FANCE in complex assembly. Thus FANCE is shown to be a key mediator of protein interactions, both in the architecture of the FA protein complex and in connection of complex components to the putative downstream targets of complex activity.  

Fanconi anemia (FA) is a rare disorder characterized by bone marrow failure, cancer susceptibility, congenital malformations, chromosomal instability, and cellular hypersensitivity to DNA-crosslinking agents. Genetically heterogeneous, FA is comprised of at least eleven complementation groups (1). Nine of the corresponding genes (FANCA, B, C, D1, D2, E, F, G and L) have been identified, with the encoded protein product sequences bearing no similarity to each other, and in most cases, no obvious clues as to protein function (2-8).  

The FA protein core complex, first suggested to exist by the reciprocal coimmunoprecipitation of several FA proteins (9-12), was subsequently isolated from nuclear extracts of HeLa cells as a subcomponent of a 1.5 to 2MDa BLM-associated multiprotein complex (13). Thus far, seven FA proteins including FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, and FANCL have been identified in this complex (13). Evidence of FA core complex involvement in smaller BLM-independent complexes was also presented (13), and a more recent study identified four forms of the FA protein complex in an immortalized fibroblast cell line, two of which are present in the nucleus, and two in the cytoplasm (14).  

The absence of any individual core complex protein results in loss of full complex formation and lack of monoubiquitination of the FANCD2 protein, which correlates with failure of FANCD2 to form nuclear foci in response to DNA crosslinking agents (15). In chromatin, monoubiquitinated FANCD2 associates with BRCA2, identified as the FANCD1 protein (16). In addition, absence of a single core complex component often results in reduced stability or altered subcellular localization of remaining complex members, particularly direct binding partners (8,11,12,17-19).  

FANCE has been coimmunoprecipitated with other components of the FA protein complex
as well as the downstream FANCD2 and FANCD1/BRCA2 proteins (12,19). The interaction of FANCD2 with FANCE in human cells has been shown to involve either the monoubiquitinated long form (FANCD2-L), or the nonubiquitinated short form (FANCD2-S), depending on the subnuclear fraction analyzed (12,16). Using the yeast two-hybrid (Y2H) system, FANCE interactions with FANCC and FANCD2 have been shown to involve direct binding (20,21). FANCE has thus become a focus of interest as a connection between the core complex and other elements of the FA pathway, although its simultaneous binding to FANCC and FANCD2, providing an actual physical link, has not been demonstrated. In this study, the role of FANCE in mediating interactions between other components of the FA pathway is tested in the yeast three-hybrid (Y3H) system. Both a FANCC/FANCE/FANCF ternary interaction, indicative of a role for FANCE in complex assembly, and a FANCC/FANCE/FANCD2 ternary interaction linking another member of the core complex to the downstream components of the FA pathway, are identified. The FANCE-mediated FANCC/FANCD2 interaction is also observed between endogenous proteins in human cell lines. A Y2H-based random mutagenesis strategy was developed to generate FANCE mutants with selective loss of the FANCD2, but not the FANCC, interaction for further analysis in the mammalian system. Loss of the FANCE/FANCD2 link is associated with the absence of FANCD2 monoubiquitination and the presence of DNA-crosslinker sensitivity, indicative of the importance of this interaction to the function of the FA DNA damage response pathway.

MATERIALS AND METHODS

Bacterial and yeast strains - Escherichia coli strains DH10B (Invitrogen, Carlsbad, CA) or XL1-Blue (Stratagene, La Jolla, CA) were used in the construction and propagation of plasmid constructs unless otherwise indicated. E. coli was propagated in LB media at 30°C for FANCD2 and FANCE constructs, and 37°C for other constructs. Saccharomyces cerevisiae strain PJ69-4A (22) (ATCC, Manassas, VA) was used in the yeast two-and three-hybrid assays. Yeast was grown at 30°C in YPDA or synthetic dropout media.

Yeast vectors and expression constructs - GAL4 binding domain (BD) and activation domain (AD) constructs were generated previously (21) using vectors supplied in the Matchmaker GAL4 two-hybrid systems (Clontech, Palo Alto, CA). Constructs for constitutive expression of influenza A hemagglutinin (HA)-tagged proteins were generated by excision of ORFs together with a 5’ HA-tag sequence from GAL4-AD constructs made in the pGADT7 vector and insertion into the p426ADH (23) vector, which has a URA3 nutritional selection marker. Western blotting to confirm expression of GAL4-fusion or HA-tagged proteins used in analyses (data not shown) was as previously described (21).

Yeast three-hybrid (Y3H) assay - A version of the Y3H system in which a HA-tagged protein is constitutively expressed from a third vector (24), in this case p426ADH, was used in this study. PJ69-4A was transformed first with the p426ADH constructs, followed by simultaneous cotransformation with the GAL4-AD and GAL4-BD constructs, using the lithium acetate method as described in the Matchmaker Yeast Protocols Handbook (Clontech). Cotransformants growing on both –Ade and –His selective media and controls were assayed for β-galactosidase activity by a liquid ONPG (o-nitrophenol-β-D-galactoside) sarkosyl permeabilization method (25).

Generation and screening of a randomly mutagenized AD-FANCE Y2H library - The strategy for generation and use of a library of randomly mutagenized AD-FANCE constructs is outlined in Figure 1. Propagation of the AD-FANCE construct in the E. coli mutator strain XL1-Red (Stratagene) was performed according to the specifications of the manufacturer. Transformation of mutant AD-FANCE library into yeast strain PJ69-4A and subsequent isolation of plasmid DNA from yeast was performed as described in the Yeast Protocols Handbook of the Matchmaker system (Clontech). Western blotting to evaluate expression of AD-FANCE mutants (data not shown) was performed as previously described for the Y2H system (21).

Mammalian expression constructs - The myc-FANCE retroviral constructs were made in the pBabe-puro vector (26). c-Myc-epitope sequence was added to the 5’ end of the ORF by polymerase
chain reaction (PCR) using Pwo polymerase (Roche, Indianapolis, IN). All PCR products and linker regions were verified by DNA sequencing. pBabe-myc-FANCE mutants were generated by replacement of the pBabe-myc-FANCE ORF with ORFs excised from the pGADT7-FANCE mutant yeast constructs. Presence of mutations identified in pGADT7-FANCE was verified in the pBabe-myc-FANCE constructs by DNA sequencing.

**Cell lines and transduction** - The PA317 packaging cell line (27) was maintained in DMEM with 10% FCS. Previously described (1,5) normal (HSC93) and FA (HSC72, FA-A; HSC230, FA-B; HSC536, FA-C; HSC62, FA-D1; EUFA130, FA-E; EUFA409, FA-E; EUFA410, FA-E; EUFA121, FA-F; EUFA143, FA-G) Epstein-Barr virus (EBV)-immortalized human lymphoblastoid cell lines (LCLs) were maintained in RPMI 1640 media with 15% FCS. The EUFA409 and EUFA410 FA-E LCLs used for retroviral transduction are homozygous for the 421 C>T (R141X) mutation (5). Cell lines were grown at 37°C with 5% CO2 unless otherwise indicated. PA317 was stably transfected with pBabe-puro constructs using Lipofectamine with PLUS reagent (Gibco), with selection in 2µg/ml puromycin (Sigma, St. Louis, MO) Following removal of PA317 producer lines from selection, retroviral supernatant was collected from cells grown at 34°C, filtered through a 0.4 µm filter, and used to transduce LCLs in the presence of 8µg/ml polybrene (Sigma). LCLs were exposed to fresh supernatant twice daily for 4 days, followed by selection in 1µg/ml puromycin.

**Generation of FANCE antisera** - The region of the FANCE ORF encoding amino acids 371 to 536 was cloned into the pGEX3X (Pharmacia) vector for generation of GST-FANCE(Cterm) fusion protein. GST fusion protein was produced in the E. coli strain JM109 by induction with isopropyl β-D-thiogalactopyranoside (IPTG). Protein was purified by affinity chromatography on glutathione sepharose 4B (Amersham, Piscataway, NJ) according to the instructions of the manufacturer, and eluted protein was dialyzed against phosphate buffered saline (PBS). New Zealand White rabbits were injected with a 1:1 mixture of GST-FANCE(Cterm) fusion protein and complete Freund adjuvant, followed by monthly boosters of antigen in incomplete Freund adjuvant. The polyclonal antiserum was purified with immobilized Protein A (Pierce, Rockford, IL) according to the directions of the manufacturer, followed by depletion of antibodies directed against GST by chromatography with glutathione sepharose 4B containing immobilized GST. Antiserum specificity was validated by testing for recognition of recombinant protein and endogenous protein (58kDa) in cellular extracts from normal versus FA-E cell lines. The animal experiments were approved by the Animal Care Committee of The Hospital for Sick Children.

**Subcellular fractionation, whole cell extract preparation, immunoprecipitation and immunoblotting** - Subcellular fractionation of LCLs was performed using the NucBuster protein extraction system (Novagen, Madison, WI). For preparation of whole cell extracts (WCEs), LCLs were washed with PBS and resuspended in NETN lysis buffer (20mM Tris-HCL pH8.0, 150mM NaCl, 1mM EDTA, 0.4% NP-40) supplemented with protease inhibitor cocktail (Roche) and 1mM sodium orthovanadate, followed by shearing with a 25 gauge needle and centrifugation at 10000xg at 4°C for 10min. Protein concentration of clarified supernatants was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). For immunoprecipitation, WCEs were diluted to adjust NP-40 concentration to 0.1%, antibodies were added at a concentration of 1µg/mg protein extract (for rabbit polyclonal antibodies or purified preimmune serum) or 0.8 µg/mg protein (for goat antibodies or goat control IgG) and incubated for 1 hour at 4°C. Immobilized Protein A or G (Pierce) was then added and incubation continued overnight 4°C, followed by three washes with NETN containing 0.15% NP-40 and addition of Laemmli sample buffer. Antisera used in immunoprecipitation include the polyclonal anti-FANCE antibody described above, a rabbit polyclonal anti-FANCC antibody generously provided by H. Youssoufian and described previously (28) and goat anti -FANCC (C14), anti-FANCE (L21) and anti-FANCD2 (E19) antibodies (Santa Cruz, Santa Cruz, CA). WCEs, subcellular fractions, or immunoprecipitates were resolved by SDS-PAGE on 10% Tris-glycine, 7% Tris-acetate, or, for resolution of the monoubiquitinated form of FANCD2, 3-8% Tris-acetate gels (Invitrogen), and transferred to Immobilon-P membrane (Millipore, Bedford, MA). Antibodies used in Western analysis include the rabbit antibodies against
FANCC and FANCE described above, and mouse antibodies against FANCD2 (F117), c-myc (9E10) (Santa Cruz), and β-tubulin (Roche) followed by IgG-peroxidase conjugate and chemiluminescent detection (Amersham).

MMC viability assay - LCLs (10^5/ml) were seeded in 24-well plates. After 24 hours of growth, medium supplemented with MMC (Roche) was added at concentrations up to 300nM. After 8 days, cells were counted using a Coulter counter (Beckman Coulter, Fullerton, CA). Cell counts were plotted against MMC concentrations.

RESULTS

Y3H Analysis of FANCE-mediated protein interactions. Use of the Y3H system to examine the role of FANCE in mediating interactions between other FA proteins revealed the participation of FANCE in two ternary complexes (Table 1). FANCE was able to mediate interaction between FANCC and FANCD2, the two proteins with which FANCE interacts directly in the Y2H system. Nutritional and LacZ reporter gene activation was observed for both AD-FANCC/BD-FANCD2 and BD-FANCC/AD-FANCD2 pairings (Table 2, Figure 2A). The Y3H interaction was abrogated by the L554P mutation (29) in the C-terminus of FANCC (Table 2), previously shown to disrupt the interaction and colocalization of FANCC with FANCE (12,21).

The HA-FANCE protein was also able to mediate an interaction between the FANCC and FANCF proteins, again with reporter activation observed for both AD-FANCC/BD-FANCF and BD-FANCC/AD-FANCF pairings (Table 3, Figure 2B). Some reporter activation was observed for the BD-empty vector/HA-FANCE/AD-FANCF cotransformants, but the levels were below those seen for the BD-FANCC/HA-FANCE/AD-FANCF combination (Table 3, Figure 2B). The L554P mutant of FANCC (29) also failed to participate in this ternary interaction.

The FANCC/FANCE/FANCD2 interaction in human cells. Following the demonstration of this ternary complex in the Y3H system, which is designed to bring heterologously expressed proteins together in large quantities, it was important to demonstrate that the interaction reflected occurrence in human cells. Consistent with the Y3H result, novel reciprocal coimmunoprecipitation of endogenous FANCC and FANCD2 proteins from a normal human lymphoblastoid cell line (LCL) was observed, as were the previously described constituent FANCC/FANCE and FANCE/FANCD2 interactions (Figure 3A,B,C). Only antibodies against the C-terminus of FANCD2 coimmunoprecipitated FANCE and FANCC, as shown, whereas antibodies to the N-terminus of FANCD2 failed to immunoprecipitate either protein. Absence of FANCC/FANCD2 interaction in FA-E cells is recovered by exogenous expression of FANCE with an N-terminal c-myc epitope tag (myc-FANCE) demonstrating the FANCE-dependent nature of the interaction (Figure 3D). Interaction was reduced or absent in FA patient-derived LCLs from complementation groups corresponding to core complex members, but maintained in a FA-D1 LCL, which is deficient for a downstream component of the pathway. Loss of the FANCC/FANCD2 interaction accompanied loss or reduction of the FANCE/FANCD2 interaction, also consistent with mediation of the FANCC/FANCD2 interaction by FANCE (Figure 3E). FANCF, which the FANCE/FANCC/FANCF Y3H interaction indicates may be proximal to the FANCE/FANCC pairing in the FA core complex, was not detected in FANCD2 immunoprecipitates from normal LCLs (data not shown).

Generation of FANCE mutants with selective loss of FANCD2 interaction. To further investigate the contribution of FANCE interactions to the cellular phenotype of FA cells, a mutagenesis study of the FANCE protein was undertaken. In the absence of patient-derived missense mutants, recognizable motifs involved in protein interaction, or full sequence information from multiple species for comparison, a Y2H-based method was designed to identify randomly-mutagenized FANCE mutants with selective loss of the FANCD2 interaction, but maintenance of the FANCE interaction. A flow chart outlining this screening strategy is shown in Figure 1. Of five mutants selected for exogenous expression in human LCLs, stable expression of three was observed. A schematic representation of these three mutants used in further analyses is shown in Figure 4A. All were unable to mediate the FANCC/FANCD2 interaction in the Y3H system, not surprising given the disruption of the
FANCE/FANCD2 Y2H interaction. One mutant, FANCE-E263K, did retain the ability to participate in the FANCC/FANCE/FANCF interaction (Figure 4B).

Immunoblotting of subcellular fractions revealed that the two point mutants, FANCE-L348M and FANCE-E263K, were expressed in the nuclear compartment of the FA-E LCL EUFA409, as was wild type myc-FANCE. This is consistent with previous reports that FANCE, which has putative nuclear localization signals (5), localizes primarily to the nucleus (19). The truncation mutant generated by a single base pair insertion resulting in a frameshift (FANCE-312X) was observed only in the cytoplasm (Figure 4C).

The ability of myc-FANCE mutants exogenously expressed in EUFA409 to restore the FANCC/FANCE/FANCD2 interaction and reconstitute elements of the FA pathway, namely monoubiquitination of FANCD2 and correction of MMC sensitivity, was also examined. Wild type myc-FANCE restored the FANCC/FANCE/FANCD2 interaction (Figure 4D), as well as FANCD2 monoubiquitination (Figure 5A) and MMC resistance, which was assayed as the viability of transduced cell lines at increasing concentrations of MMC and shown relative to the HSC93 wild-type LCL. (Figure 5B). The FANCE-L348M mutant, shown to retain some ability to interact with FANCD2 in human LCLs (Figure 4D), and perhaps weakly mediate the FANCC/FANCE/FANCD2 interaction, showed weak FANCD2 monoubiquitination (Figure 5A) and corrected MMC sensitivity, although not as fully as wild-type myc-FANCE (Figure 5B). Failure to detect the FANCE/FANCD2 interaction of the FANCE-L348M mutant in the Y2H screen may have been due to the lower expression level of this mutant in yeast, relative to wild type FANCE or FANCE-E263K (data not shown), and introduces the possibility that failure to detect the FANCC/FANCE/FANCF interaction was also a false-negative result. The FANCE-312X and FANCE-E263K mutants were unable to restore the FANCE/FANCD2 interaction (Figure 4D) or monoubiquitination of FANCD2 (Figure 5A), and had little effect on the poor viability of FA-E cells exposed to MMC (Figure 5B).

**DISCUSSION**

In this study we demonstrate the ability of FANCE to mediate interaction between its binding partners FANCC and FANCD2 both in yeast and human cells, and evaluate the effect of FANCE mutants with selective loss of the FANCD2 interaction on reconstitution of the FA pathway in FA-E cells. The ability of FANCE to mediate interaction between FANCC and FANCF in the Y3H system is also demonstrated.

Detection of the FANCC/FANCE/FANCF ternary interaction is interesting given that FANCF could not be shown to bind directly in either FANCC or FANCE in the Y2H system (20,21). The FANCC/FANCE interaction may alter the conformation of one or both partners, allowing for FANCF binding, or, alternatively, FANCF may bind to both FANCC and FANCE simultaneously, with one-on-one interaction being too unstable for detection in the Y2H system. Another possibility is that FANCE transiently interacts with, and modifies, either FANCC or FANCF in some manner, turn allowing a direct FANCC/FANCF interaction to occur. However, the presence of these three proteins together in the larger FA core complex (13) makes this scenario less likely. Furthermore, a previous study identifying functional domains within the FANCF protein demonstrated a requirement for the FANCF N-terminus in binding of both FANCE and FANCC to the FA core complex, suggestive of ternary complex formation (30), and indicating that FANCC/FANCE/FANCF interaction is an important step in the assembly of the larger FA core complex.

The ability of FANCE to mediate an interaction between FANCC and FANCD2 in the Y3H system is most simply explained by formation of a ternary complex in which FANCE simultaneously forms the direct interactions with FANCC and FANCD2 previously reported in Y2H studies (20,21). The maintenance of this FANCC/FANCE/FANCD2 interaction between endogenous levels of proteins in human cells demonstrates that a physical link between FANCD2 and a second component of the FA core complex can exist at natural protein expression levels and stoichiometry. Mediation of the FANCC/FANCE/FANCD2 interaction by FANCE in yeast is mirrored by the situation in human cells. First,
the FANCC/FANCD2 interaction is absent in LCLs lacking the FANCE/FANCD2 interaction, either in FA-E cells exogenously expressing FANCE mutants, or in cells from other FA complementation groups. Second, exogenous expression of FANCE restored the FANCC/FANCD2 interaction in FA-E cells. Finally, antibodies against the N-terminus of FANCD2 that failed to coimmunoprecipitate FANCE both in this study and in an earlier report (19) failed to immunoprecipitate FANCC in parallel. The interaction of FANCE with FANCD2 maps to the N-terminus (21), thus FANCD2 bound to FANCE may not be accessible to this antibody, whereas an antibody directed against the C-terminal region of FANCD2 coimmunoprecipitated FANCE and FANCC in tandem.

Despite lack of evidence for interaction of FANCD2 with other FA core complex proteins such as FANCF, the FANCC/FANCE/FANCD2 interaction appears to be reduced in cell lines lacking these proteins. Reduced stability of FANCC or FANCE in the absence of core complex formation could be a contributing factor. The levels of both FANCF and FANCC proteins are reduced in FA-E LCLs (12,19), and this is corrected by exogenous expression of FANCE, suggesting that formation of the FANCC/FANCE/FANCF ternary complex may have a stabilizing effect on its components (19). Correction of FA-A lines has also been reported to increase the expression of FANCC (10). However, maintenance of the interaction at reduced levels in at least some core complex complementation groups suggests formation of the entire core complex is not an absolute prerequisite for the FANCC/FANCE/FANCD2 interaction.

Interaction of the FANCE-312X mutant with FANCC in the Y2H system further narrows the C-terminal boundary of the minimal region of FANCE sufficient for FANCE binding, previously defined as amino acids 150 to 371 (21). Also interesting is the cytoplasmic localization of this mutant, despite retaining the putative NLSs. While this may suggest the motifs are non-functional, it is also possible that C-terminal truncation counteracts the activity of functional motifs, as is observed for the FANCA protein (31).

A FANCE mutant (E263K) that retains the observed interactions with core complex proteins in the Y2H and Y3H systems, but not the interaction with FANCD2, did not restore FANCD2 monoubiquitination or correct MMC sensitivity when exogenously expressed in a FA-E cell line. Failure of this mutant with potential for core complex formation to reconstitute the FA pathway suggests the FANCE/FANCD2 interaction is an absolute requirement; ablation of this interaction alone may be capable of generating the FA cellular phenotype, by severing the connection between the intact complex and a downstream element of the FA pathway.

Only the FANCE-L348M mutant that retains some ability to interact with FANCD2 in LCLs was able to weakly reconstitute the FANCD2 monoubiquitination feature of the FA pathway, and partially correct the MMC sensitivity of FA-E cells. This is reminiscent of the findings of a study of patient-derived variants of the FANCA gene in which those that partially reconstituted elements of the FA pathway, including FANCD2 monoubiquitination, were able to confer some MMC resistance (32). Previous studies have also demonstrated the failure of patient-derived missense mutants of FANCC to interact with FANCE (12,21). Taken together, this evidence highlights the importance of formation of the FANCC/FANCE/FANCD2 ternary complex to the function of the FA damage response pathway.

The requirement for FANCE to mediate interaction between FANCC and FANCF in the Y3H system suggests a role for this protein in the assembly of the FA complex, in addition to its interaction with FANCD2 (12,21) and role in the subcellular localization of FANCC (12,19). There is currently no biochemical evidence to show that FANCE mediates FANCC/FANCF and FANCC/FANCD2 interaction simultaneously, which would connect at least one additional core complex component, FANCF, to FANCD2. A yeast four-hybrid system (33) could prove useful in determining whether such a higher order interaction may exist, or whether FANCE and FANCC must exit one ternary complex to form the other. A report of FANCE association with BRCA2/FANCD1 (16) also raises the question of whether FANCD2 mediates this interaction between its direct binding partners, and, if so, whether the presence of FANCC is maintained at this point in the FA pathway. Connection of FANCE to BRCA2, together with identification of
the additional core complex components FANCB and FANCL (3,8), provides additional avenues via which the crucial roles for FANCE in the FA DNA damage response pathway demonstrated in this study could be expanded.

REFERENCES


FOOTNOTES

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1The abbreviations used are: FA, Fanconi anemia; Y2H, yeast two-hybrid; Y3H, yeast three-hybrid; LB, Luria-Bertani; ATCC, American Type Culture Collection; BD, binding domain; AD, activation domain; ORF, open reading frame; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; LCL, lymphoblastoid cell line; PBS, phosphate buffered saline; PCR, polymerase chain reaction; GST, glutathione-S-transferase; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; MMC, mitomycin C; NLS, nuclear localization signal; WCE, whole cell extract; wt, wild type
FIGURE LEGENDS

Figure 1. Y2H-based strategy devised for the generation of FANCE mutants with selective disruption of the FANCE/FANCD2 interaction and maintenance of the FANCE/FANCC interaction. Following the reverse Y2H screen for mutants with loss of FANCE/FANCD2 interaction (step 2), selection of clones positive for interaction with FANCC (step 4) eliminates mutants lacking FANCE expression due to mutations in the promoter, mutants with very premature FANCE protein truncation, unstable mutant proteins, or non-functional GAL4-activation domains. Growth assays in steps 2 through 4 were repeated to verify the results. In addition, plasmids isolated in step 3 maintaining interaction with BD-FANCC in step 4 were retransformed to PJ69-4A containing BD-FANCD2 to reconfirm loss of FANCE/FANCD2 interaction. Five unique mutants from step 5 were selected for use in the final step, with stable expression of three mutants observed in human cell lines.

Figure 2. Mediation of FA protein interactions by HA-FANCE in the Y3H system: LacZ reporter activation. A) FANCC/FANCE/FANCD2 ternary interaction. B) FANCC/FANCE/FANCF ternary interaction. GAL4-AD and -BD fusion proteins were co-expressed in the presence or absence (empty vector) of HA-FANCE. Activation of the LacZ reporter gene was measured by ONPG liquid β-galactosidase assay. Activity units are expressed as 1000 x OD420 divided by the product of the volume (ml) x time (min) x OD600. The results shown indicate the mean and SD for 4 assays, each done in triplicate.

Figure 3. Demonstration of the FANCC/FANCE/FANCD2 interaction in human cells. Endogenous FANCC and FANCD2 communoprecipitate from whole cell extract (WCE) of normal (wt) HSC93 LCLs. A) Immunoprecipitation with anti-FANCE antibodies, blots probed with anti-FANCC or FANCD2 antibodies. B) Immunoprecipitation with anti-FANCC antibodies, blots probed with anti-FANCD2 or FANCE antibodies. C) Immunoprecipitation with anti-FANCD2 antibody, blots probed with anti-FANCC or FANCE antibodies. Immunoprecipitations in panels A to C were performed with goat (anti-FANCE Ab1, anti-FANCC Ab1 and anti-FANCD2) or rabbit (anti-FANCE Ab2, anti-FANCC Ab2) antibodies from 3mg whole cell extract. The electrophoretic conditions used do not resolve the monoubiquitinated from the nonubiquitinated form of FANCD2. D) Exogenous expression of myc-FANCE in the EUFA410 FA-E LCL restores communoprecipitation of FANCD2 with FANCC (left panel) absent in the mock-transduced EUFA410 FA-E LCL (right). Immunoprecipitation with rabbit anti-FANCC antibody was from 3mg whole cell extract, and blots are probed with anti-FANCD2 antibody. E) Status of the FANCE/FANCD2 and FANCC/FANCD2 interactions in FA LCLs representing seven of the eleven known FA complementation groups. Immunoprecipitation with goat anti-FANCE (top) or anti-FANCC (bottom) antibodies was from 5mg whole cell extract, and blots are probed with anti-FANCD2 antibody.

Figure 4. Characteristics of FANCE mutants exogenously expressed in FA-E cells. A) Schematics of FANCE mutants stably expressed in FA-E cells. Mutations identified in the FANCE ORF and their predicted effect on amino acid sequence are indicated, together with the ability of FANCE mutants to mediate known FANCE interactions in the Y2H and Y3H systems. B) Mediation of the FANCC/FANCF interaction by in the Y3H system is at least partially maintained by the FANCE-E263K mutant. LacZ reporter activation is shown relative to that generated by wild type HA-FANCE and the empty vector control. Activation of the LacZ reporter gene was measured by β-galactosidase assay as in Figure 2. The results shown indicate the mean and SD for 4 assays, each done in triplicate. C) Expression and subcellular localization of myc-tagged FANCE mutants exogenously expressed in the FA-E LCL EUFA409. The molecular mass of the FANCE-312X mutant is below the range of the left panel, so a
second panel is shown on the right. Both blots were probed with an anti-c-myc antibody. FANCD2 and β-tubulin are shown as nuclear and cytoplasmic markers, respectively. C=cytoplasmic fraction, N=nuclear fraction.  

D) Ability of mutants to maintain the FANCC/FANCE/FANCD2 interaction. Coimmunoprecipitation of FANCD2 with FANCE (top) and FANCC (bottom) from retrovirally-transduced EUFA409 whole cell extracts. Immunoprecipitation with anti-FANCE or anti-FANCC antibodies was from 3mg (FANCE) or 4mg (FANCC) whole cell extract of EUFA409 LCLs transduced with the myc-FANCE vectors indicated. Both panels are probed with anti-FANCD2 antibody.

Figure 5. Reconstitution of the FA pathway in EUFA409 FA-E cells stably expressing wild type or mutant myc-FANCE.  

A) Monoubiquitination status of FANCD2. Whole cell lysates of retrovirally-transduced EUFA409 or normal control HSC93 were analyzed after 24hr treatment with 25nM of the DNA-crosslinking agent MMC, a known inducer of monoubiquitination, or in the absence of MMC. Electrophoretic conditions allow resolution of the monoubiquitinated long form of FANCD2 (FANCD2-L), which is 7kDa larger, from the nonubiquitinated short form (FANCD2-S).  

B) MMC viability assay. Each dosage response curve represents the average of three experiments. Error bars have been excluded for clarity.
Table 1. Ability of the FANCE protein to mediate interactions between other FA proteins in the Y3H system. HA-FANCE protein was constitutively co-expressed in the yeast strain PJ69-4A with pairs of GAL4 activation domain (AD-) and DNA-binding domain (BD-) FA protein fusions. (+) indicates pairings for which activation of the ADE2 and HIS3 reporter genes, as assessed by growth on selective media, was observed, but seen only in the presence of HA-FANCE, not in the absence of HA-FANCE (i.e. reporter activation can only be attributed to Y3H interaction, not to a direct Y2H interaction). (-) indicates pairings with no growth, or growth in the absence, as well as the presence, of HA-FANCE (i.e. growth explainable by Y2H interaction). *The reciprocal BD-FANCG construct was not included due to strong autoactivation of reporters.

<table>
<thead>
<tr>
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<th>BD-FANCA</th>
<th>BD-FANCC</th>
<th>BD-FANCD2</th>
<th>BD-FANCE</th>
<th>BD-FANCF</th>
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Table 2. **Y3H analysis of the FANCC/FANCE/FANCD2 interaction.** Activation of ADE2 and HIS3 nutritional reporter genes as determined by growth on -Ade -His media; (-) no growth, (+++) strong growth. Strong growth was characterized by the appearance of growth within 5 days of plating, with the consistent formation of robust colonies.

<table>
<thead>
<tr>
<th>Protein Fused to AD</th>
<th>HA-tagged protein</th>
<th>Protein Fused to BD</th>
<th>Growth on -Ade -His</th>
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Table 3. **Y3H analysis of the FANCC/FANCE/FANCF interaction.** Activation of ADE2 and HIS3 nutritional reporter genes as determined by growth on -Ade -His media; (-) no growth, (+) weak growth, (++) strong growth as defined in Table 2.

<table>
<thead>
<tr>
<th>Protein Fused to AD</th>
<th>HA-tagged protein</th>
<th>Protein Fused to BD</th>
<th>Growth on -Ade -His</th>
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Figure 1:

1. Pass AD-FANCE construct through the DNA repair-deficient E. coli strain XL1-Red to create a mutant AD-FANCE library by random mutagenesis.

2. Transform the library to yeast strain PJ69-4A containing BD-FANC2, plate on media non-selective for interaction, then replica plate to selective media.

3. Identify colonies failing to grow on selective media (lacking FANC2 interaction), and isolate mutant AD-FANCE plasmids.

4. Retransform plasmids to PJ69-4A containing BD-FANCC and plate to media selective for interaction to identify mutants capable of maintaining interaction with FANCC.

5. Western blot positives to evaluate expression of mutant AD-FANCE; select clones to identify sequence variation.

6. Clone ORF of unique mutants into retroviral vector for to generate isogenic cell lines.
Figure 2:

A

B

Fusion Protein Combination

Fusion Protein Combination

β-Galactosidase Activity Units

β-Galactosidase Activity Units
Figure 3:
Figure 4:

A

<table>
<thead>
<tr>
<th>Mutant Name</th>
<th>Nucleotide Alteration in ORF</th>
<th>Predicted Amino Acid Alteration</th>
<th>Schematic Diagram N-NLS</th>
<th>Ability to maintain Y2H and Y3H interactions</th>
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B

Fusion Protein Combination

- AD-C
- HA-E
- BD-F

C

D

(my)FANCE (68kDa)

FANCED2

β-TUBULIN

(my)FANCE 312X (34kDa)
Figure 5:

A

B

![Experiment A Diagram]

![Experiment B Graph]

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FANCC, FANCE and FANCD2 form a ternary complex essential to the integrity of the Fanconi anemia DNA damage response pathway
Susan M. Gordon, Noa Alon and Manuel Buchwald

J. Biol. Chem. published online August 26, 2005

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