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Direct interactions of the five known Fanconi anaemia proteins suggest a common functional pathway

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Fanconi anaemia (FA) is an autosomal recessive inherited disorder associated with a progressive aplastic anaemia, diverse congenital abnormalities and cancer. The condition is genetically heterogeneous, with at least seven complementation groups (A–G) described. Cells from individuals who are homozygous for mutations in FA genes are characterized by chromosomal instability and hypersensitivity to DNA interstrand crosslinking agents. These features suggest a possible role for the encoded proteins in the recognition or repair of these lesions, but neither their function nor whether they operate in a concerted or discrete functional pathways is known. The recent cloning of the *FANCF* and *FANCE* genes has allowed us to investigate the interaction of the proteins encoded by five of the seven complementation groups of FA. We used the yeast two-hybrid system and co-immunoprecipitation analysis to test the 10 possible pairs of proteins for direct interaction. In addition to the previously described binding of FANCA to FANCG, we now demonstrate direct interaction of FANCF with FANCG, of FANCC with FANCE and a weaker interaction of FANCE with both FANCA and FANCG. These findings show that the newly identified FANCE protein is an integral part of the FA pathway, and support the concept of a functional link between all known proteins encoded by the genes that are mutated in this disorder. These proteins may act either as a multimeric complex or by sequential recruitment of subsets of the proteins in a common pathway that protects the genomic integrity of mammalian cells.

INTRODUCTION

Fanconi anaemia (FA) is a rare autosomal recessive disorder with a diverse clinical phenotype, including congenital malformations, progressive bone marrow failure and increased incidence of acute myeloid leukaemia and solid tumours. Cells from FA patients display chromosomal instability and increased sensitivity to DNA cross-linking agents such as mitomycin C and diepoxybutane, which is used to confirm the clinical diagnosis of FA (1). Complementation studies using somatic cell fusion techniques have identified seven different complementation groups, FA-A through FA-G (2–4). The earlier cloning of the *FANCC*, *FANCA* and *FANCG* genes (5–8) has now been followed by the cloning of *FANCF* and *FANCE* (9,10), and *FANCD* has been mapped to a small region of chromosome 3p (11,12). The cloned genes display no sequence similarity to each other, and no significant homologies to any known protein or nucleotide sequences other than their direct mammalian orthologues that have been reported. Since the clinical features of patients with mutations in different complementation groups overlap, it is thought that the FA proteins may function in a common pathway. A variety of functional roles have been proposed for the FA genes to explain the underlying defect in FA, including involvement in cell cycle regulation, apoptosis, DNA repair or in the metabolism of genotoxic agents such as oxygen radicals.

In order to elucidate the function of the FA proteins, studies have focused on their expression, sub-cellular localization and potential interactions. The mouse orthologues of FANCA and FANCC are ubiquitously expressed at low levels in adult tissue. During mouse embryogenesis, Fancc is expressed in whisker follicles, brain, kidney, lung, gut and stomach, in addition to osteogenic and haematopoietic lineages (13). The expression pattern of Fanca was similar to Fancc, except for the lung and gut where no expression was detected, and it was also highly expressed in lymphoid tissue, testis and ovary (14,15). These findings are consistent with the wide pattern of phenotypic defects observed in FA patients. Initial studies of the cellular localization of FANCC by cell fractionation and immunofluorescence indicated a predominantly cytoplasmic location (16,17). However, more recent work suggested partial localization to the nucleus (18), and placed FANCC in a nuclear complex with FANCA (19). Similarly, FANCA was initially thought to be cytoplasmic (20), but has now been identified in both the nuclear and cytoplasmic fractions (19–21). FANCF and FANCG have been reported to be predominantly nuclear (22,23), and FANCE contains two putative nuclear localization signals (10). Initial studies to determine interactions between known FA proteins were conflicting. Interaction of FANCA

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with FANCC was reported using co-immunoprecipitation (19,21,22), but this was not replicated by Kruyt and Youssoufian (24). Further evidence for their interaction has since been reported, but it may be weak or indirect (25–29). The interaction of FANCA with FANCG is undisputed, and has been shown by both co-immunoprecipitation and yeast two-hybrid analysis (23,25,28–30).

The recent cloning of two novel FA genes, *FANCE* and *FANCF* (9,10), has allowed us to carry out a comprehensive survey of interactions among the five known FA proteins, FANCA, FANCC, FANCE, FANCF and FANCG. The yeast two-hybrid system (31) detects direct interactions between pairs of proteins expressed in yeast, and has the advantage that reporter genes may be activated by weak or transient interactions that might not be detectable by other physical methods such as co-immunoprecipitation. However, interactions of proteins that are dependent on post-translational modifications that occur only in mammalian cells would not be detected. We have used this system to test for direct interaction between all 10 possible pairs of the five FA proteins, and also tested the interactions by *in vitro* co-immunoprecipitation of pairs of fusion proteins with epitope tags. We have observed four novel interactions, revealing that every known FA protein binds to at least one other FA protein. Our findings support the hypothesis that these proteins act in a common pathway to maintain genomic stability in mammalian cells.

RESULTS

FANCF binds FANCG in yeast two-hybrid analysis

We have used the yeast two-hybrid system to test for interactions between FANCF and all other known FA proteins. Full-length *FANCF* was subcloned into both the activation domain (AD) and the binding domain (BD) vectors, and tested against full-length FANCA, FANCC, FANCE and FANCG in both the BD and AD vectors. We also tested for non-specific interaction of FANCF with a series of control plasmids. No activation of the reporter genes was observed when FANCF was tested with human lamin C, murine p53, SV40 large T antigen or plasmid vectors containing only GAL4 domains. We observed no interaction of FANCF with FANCA, FANCC or FANCE, or when tested against itself. However, co-transformation of FANCF with FANCG activated the reporter genes, indicating direct interaction of FANCF with FANCG (Fig. 1). This interaction was found with FANCF in the GAL4–AD vector and FANCG in the GAL4–BD vector, and was confirmed when the proteins were tested with exchanged GAL4 domains (Fig. 2, Table 1). In addition, reporter gene activation was found when mating singly transformed yeast strains to co-express bait and prey proteins. The relative strength of this interaction was assessed using a semi-quantitative analysis, which incorporates data on the number of colonies, and the time and intensity of colour development (see Materials and Methods). This analysis showed that reporter gene activation for the FANCF/FANCG pair was approximately half that previously observed for the FANCA/FANCG pair (28), and that interaction strength was similar when the GAL4 domains were exchanged (Fig. 2). The site of interaction in FANCG was examined by testing three previously constructed *FANCG* fragments (28) containing the N-terminal half (amino acid residues 1–313), the C-terminal half (305–622) and a fragment spanning the central region (195–510), against FANCF. No activation of the reporter genes was observed with any of these fragments of FANCG. We have previously observed that N- and C-terminal FANCG fragments interact with FANCA in the yeast two-hybrid assay (28).

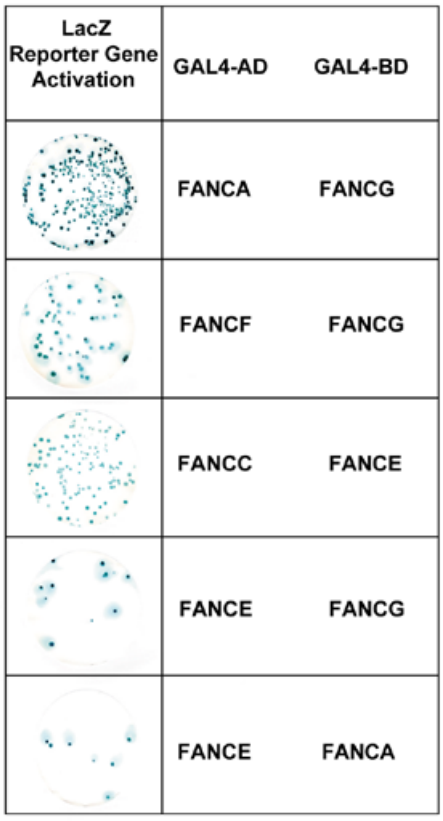

Figure 1. Yeast two-hybrid reporter gene activation by FA protein pairs. Typical results observed with a β-galactosidase filter lift assay, visualizing activation of the *LacZ* reporter gene.

FANCF co-immunoprecipitates with FANCG *in vitro*

In order to confirm the interaction by an alternative method, the *FANCF* and *FANCG* genes were transcribed and translated *in vitro* to express the fusion proteins HA–FANCF and c-*myc*–FANCG. Mixing approximately equal amounts of the expressed proteins followed by addition of anti-c-*myc* antibody resulted in co-immunoprecipitation of FANCF with FANCG (Fig. 3, lane 2), thus confirming the interaction of this protein pair. Although the amount of co-precipitated FANCF protein was low, this result was reproduced in three experiments. Confirmation of reciprocal co-immunoprecipitation was hindered by the small amount of HA–FANCF routinely precipitated from the mixture by anti-HA (Fig. 3, lane 3). Since this antibody was used successfully in the immunoprecipitation of HA–FANCA and HA–FANCE (Fig. 3, lanes 6 and 7), it is possible that the HA tag on FANCF may be partially shielded by the molecular conformation of the fusion protein.

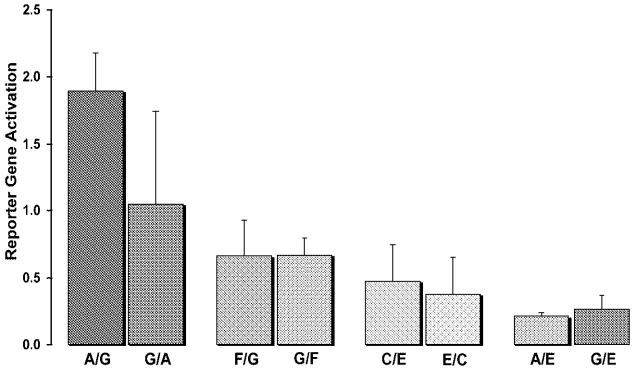

Figure 2. Relative strengths of reporter gene activation. The equation given in the Materials and Methods section allows a direct comparison of the relative strength of reporter gene activation of the FA protein pairs. Results are from an average of five (2–9) independent experiments. Error bars show the standard deviations from the mean. The labels display the respective fusion proteins used in each experiment, where the first named corresponds to the BD and the latter to the AD, e.g. A/G = BD–FANCA/AD–FANCG. Values for reporter gene activation for protein pairs FANCA–BD/FANCE–AD and FANCG–BD/FANCE–AD have been determined from only those experiments that showed positive results.

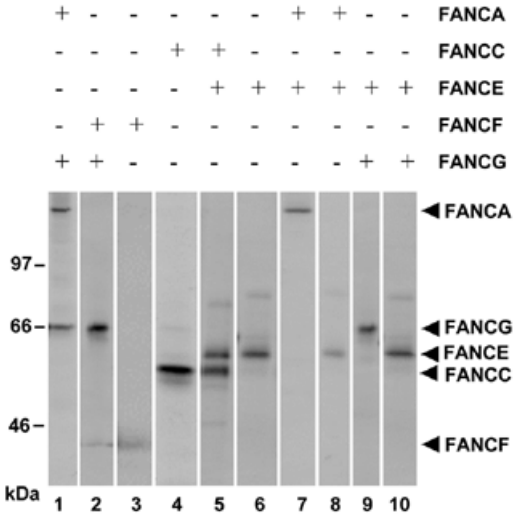

Figure 3. Co-immunoprecipitation of FA protein pairs. Radiolabelled protein pairs were mixed and allowed to form complexes. Subsequently an antibody against one or other of the protein construct tags was added and binding was analysed by SDS–PAGE. Lane 1, HA–FANCA + c-*myc*–FANCG immunoprecipitated by anti-HA antibody; lane 2, HA–FANCF + c-*myc*–FANCG immunoprecipitated by anti-c-*myc* antibody; lane 3, HA–FANCF immunoprecipitated by anti-HA antibody; lane 4, c-*myc*–FANCC immunoprecipitated by anti-c-*myc* antibody; lane 5, HA–FANCE + c-*myc*–FANCC, immunoprecipitated by anti-HA antibody; lane 6, HA–FANCE immunoprecipitated by anti-HA antibody; lane 7, HA–FANCE + c-*myc*–FANCA, immunoprecipitated by anti c-*myc* antibody; lane 8, HA–FANCE + c-*myc*–FANCA, immunoprecipitated by anti-HA antibody; lane 9, HA–FANCE + c-*myc*–FANCG, immunoprecipitated by anti-c-*myc* antibody; lane 10, HA–FANCE + c-*myc*–FANCG, immunoprecipitated by anti-HA antibody. The upper panel indicates which translated proteins were (+) or were not (–) added prior to immunoprecipitation.

Table 1. Yeast two-hybrid analysis of FA protein interactions

	BD–FANCA	BD–FANCC	BD–FANCE	BD–FANCF	BD–FANCG
AD–FANCA	+	–	–	–	+++
AD–FANCC	–	–	++	–	–
AD–FANCE	+	++	–	–	+
AD–FANCF	–	–	–	–	++
AD–FANCG	+++	–	–	++	+

–, no activation of reporter; +, weak activation; ++, stronger activation; +++, strongest activation.

Interactions of FANCE in the yeast two-hybrid system

The yeast two-hybrid system was then used to test FANCE for interaction with FANCA, FANCC, FANCF and FANCG. As with FANCF, the full-length cDNA was subcloned into both the BD and AD vectors. These constructs were tested for non-specific activation of the reporter genes prior to testing against the other FA proteins as described above, and none was found. We observed no indication of dimerization when testing FANCE with itself, and no interaction between FANCE and FANCF. However, co-transformation of FANCE with FANCC resulted in activation of the reporter genes. This interaction was observed with FANCE in the GAL4–BD vector and FANCC in the GAL4–AD vector (Fig. 1). Activation was also observed on exchanging the vectors into which the genes were cloned (Fig. 2), thus confirming complex formation of FANCE with FANCC (Table 1). FANCE was also tested for interaction against the other known FA proteins in a yeast-mating assay. This confirmed the interaction of FANCE with FANCC, and also detected two new interactions that had not been observed using the co-transformation technique. These were interactions of FANCE in the GAL4–AD vector with both FANCG and FANCA in the GAL4–BD vector (Fig. 1). These were considered to be weak but reproducible, since they were observed in two out of six and four out of six independent mating experiments respectively. Controls including human lamin C, murine p53, SV40 large T antigen or plasmid vectors only containing GAL4 domains were carried out in all experiments (six out of six) and found to be negative. The relative strength of reporter gene activation for the interaction of FANCE with FANCC was slightly less than that for FANCF and FANCG (Fig. 2). Reporter gene activation for both FANCE/FANCA and FANCE/FANCG was much lower than in the other interactions, and reciprocal GAL4 domain fusions for the two protein pairs did not show reporter gene activation. A summary of all of the yeast two-hybrid interaction data is shown in Table 1.

FANCE co-immunoprecipitates with FANCC *in vitro*

The FANCE interactions that we observed in the yeast two-hybrid system were tested by *in vitro* transcription and translation of FA constructs, followed by co-immunoprecipitation of the fusion proteins using antibodies against epitope tags, as for FANCF above. Anti-HA antibody resulted in precipitation of c-*myc*–FANCC with HA–FANCE when these two proteins were mixed (Fig. 3, lane 5), and this result was reproduced in five experiments. The electrophoretic mobilities of FANCE

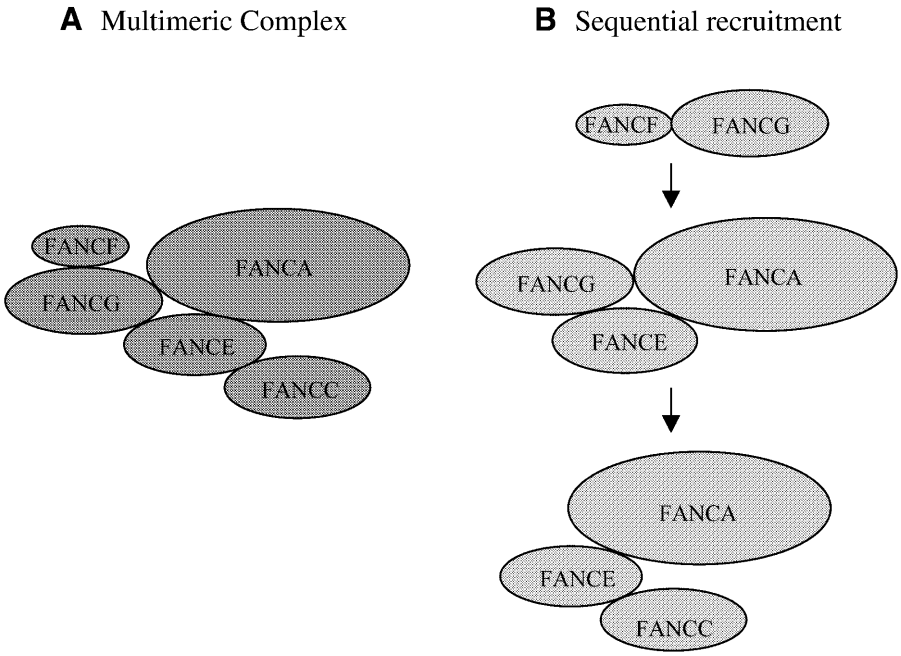

Figure 4. Interaction models of FA proteins. All FA proteins bind at least one other of the FA proteins. This is compatible with a model in which all FA proteins act within a multimeric complex (**A**), or where they are involved in sequential recruitment of subsets of these proteins (**B**) for specific functions. The direct interactions depicted were all detected by yeast two-hybrid analysis, and some were confirmed by co-immunoprecipitation of the relevant protein pairs (this study).

and FANCC were inverted relative to their predicted molecular weights of 60 and 63 kDa respectively. This is evident in Figure 3 (lanes 4 and 6), which shows the migration of c-*myc*–FANCC and HA–FANCE when precipitated with antibodies to their respective epitope tags. Immunoprecipitation of this pair with anti-c-*myc* pulled down FANCC but did not precipitate detectable amounts of HA–FANCE (not shown). The interaction of FANCE with FANCA was tested by mixing HA–FANCE with c-*myc*–FANCA, followed by precipitation with either anti-c-*myc* or anti-HA antibodies. Co-immunoprecipitation was not observed (Fig. 3, lanes 7 and 8). Similarly, we did not observe co-immunoprecipitation of HA–FANCE with c-*myc*–FANCG (Fig. 3, lanes 9 and 10). Also, co-immunoprecipitation did not detect interaction between pairs of proteins that were negative in the yeast two-hybrid assay (not shown).

DISCUSSION

The rapid progress in the genetics of FA, which has seen the cloning of the genes for five of the seven known complementation groups, has not led to immediate insight into the functional mechanisms that are disrupted in this condition. All five encoded proteins are 'orphans', with no significant similarity to other known proteins or to each other. The nuclear localization signal observed in FANCA does appear to contribute to the nuclear location of this protein (25,32), but functional evidence for other putative motifs is lacking. The lack of similarity between the five known FA proteins raises the question of whether they function in discrete molecular pathways to protect chromosomal integrity, or whether some or all are part of a common pathway. In the latter case, it would be expected that at least some of them would interact with each other. Some evidence for this hypothesis has been provided by reports of weak or indirect binding of FANCA to FANCC, and direct interaction between FANCA and FANCG (19,21,23–30). In a previous study we used the yeast two-hybrid system to study the interaction of FANCA with FANCC and FANCG. This provides a sensitive test for the interaction of pairs of proteins that may not be detected by other methods (33). We observed no direct interaction of FANCA with FANCC, but we did detect strong interaction between FANCA and FANCG (28). In this study we have used the opportunity provided by the recent cloning of the *FANCE* and *FANCF* genes to carry out a detailed analysis of all possible interactions between the five known FA proteins using the yeast two-hybrid system.

We first detected a novel direct interaction between FANCF and FANCG, which was confirmed by co-immunoprecipitation analysis. We sought to delineate the domain of FANCG that was involved in this interaction by testing three large FANCG fragments, spanning amino acid residues 1–313, 195–510 and 305–622, against FANCF. No interaction was detected between FANCF and any of these fragments, in contrast to the weak interaction of the N- and C-terminal FANCG fragments with FANCA reported previously (28). This suggests either that these fragments disrupt the binding region, or that two or more distinct regions may be required for binding. This situation is similar to what we observed for the interaction of FANCA with FANCG, where reporter gene activation was greatly reduced in interactions between FANCA and the N- or C-terminal halves of FANCG (28). A second novel interaction was identified between FANCC and FANCE, and this was confirmed by co-precipitation of c-*myc*–FANCC with immunoprecipitated HA–FANCE. We failed to detect co-precipitated HA–FANCE when c-*myc*–FANCC was immunoprecipitated.

This may reflect shielding of the c-*myc* tag in the FANCC/FANCE complex, thus allowing only for precipitation of unbound c-*myc*–FANCC. The FANCF/FANCG and FANCC/FANCE interactions were highly reproducible in both co-transformation and mating yeast two-hybrid assays. Semi-quantitative analysis of the degree of reporter gene activation for these two interaction pairs indicated that these interactions were not as strong as that between FANCA and FANCG. However, reporter gene activation in this system is affected by multiple factors, and an accurate assessment of binding strength by biophysical studies is required. We also detected evidence for weak interaction of FANCE with FANCA, and FANCE with FANCG. Although these latter interactions were reproduced several times in the yeast two-hybrid mating assay, some mating experiments were negative. These positives were never observed in the co-transformation assay, and were not confirmed by co-immunoprecipitation. It might be argued that they resulted from 'leaky' transcription of the reporter genes, but we carried out controls in each of these experiments and they were clearly negative. We thus conclude that these interactions are weak and/or transient in nature and may be detectable only when a threshold of protein expression within the yeast cells is achieved. This is consistent with the negative co-immunoprecipitation result, since this method cannot always detect weak or transient binding (34). In this context, even much stronger yeast two-hybrid interactions such as FANCF with FANCG (Figs 1 and 2) may result in low amounts of immunoprecipitated protein (Fig. 3, lane 2). We also investigated both the FANCE and FANCF proteins for potential dimerization, by testing for reporter gene activation when each of these molecules was cloned into both the BD and AD vectors. No activation was detected, indicating that neither protein forms dimers (Table 1). In our previous study we observed that both FANCA and FANCG bound themselves, indicating potential dimer formation of these molecules (28).

This study is the first demonstration that all of the five known FA proteins interact with at least one other FA protein (Table 1). The interaction that we detected between FANCF and FANCG is consistent with the known nuclear location of FANCG (23,25) and the very recent report of a nuclear location for FANCF and its co-immunoprecipitation from cell extracts with FANCG (22). It also demonstrates that this pair of proteins bind directly to each other. The clear and direct interaction that we observed between FANCE and FANCC is of particular interest in view of the suggestion that FANCC might function in a different cellular compartment to other FA proteins (24), and the fact that the majority of FANCC is localized in the cytoplasm (18). While our data do not rule out multiple functions for FANCC, they do provide novel and strong evidence that at least part of its function is related to the mainstream FA pathway. The cellular location of FANCE remains to be determined, but the presence of two putative nuclear localization signals in its sequence (10) is consistent with at least a partial nuclear location. The weaker or transient interactions of FANCE with both FANCA and FANCG could provide a temporary link between FANCC and the main FA protein complex. Again, these novel interactions suggest that the protein encoded by the newly described *FANCE* gene is intimately connected to the main FA pathway. FANCG may have a central role in this complex, since it binds strongly to both FANCA and FANCF, and weakly to FANCE. In this context, a recent genotype–phenotype study of clinical outcome in patients from different complementation groups found a more severe haematological progression, with a higher occurrence of leukaemia, in patients from complementation group G (35). In addition to the positive interactions detected, our data also indicate a lack of direct interaction of FANCA with FANCC or FANCF, of FANCC with FANCF or FANCG and of FANCE with FANCF (Table 1). We cannot exclude such interactions because they might be dependent on post-translational modifications that do not occur in yeast cells. However, it is also possible that they do not interact, or that the interaction is indirect via other proteins.

This study supports the concept of a functional link between the products of all of the FA genes described to date. Our findings are compatible with models that involve all five FA proteins binding in a multimeric complex at a site of functional action (Fig. 4A), or with sequential recruitment of subsets of these proteins along a functional pathway (Fig. 4B). In the multimeric model one of the FA proteins, such as FANCG, could bind to the site of action of the complex and then recruit FANCA and FANCF, which we have shown to interact directly with FANCG. The weak or transient interactions that we detected between FANCE and FANCA and between FANCE and FANCG may combine to stabilize the recruitment of FANCE to the complex, which in turn recruits FANCC by direct interaction. In the sequential model, one of the proteins (shown here as FANCF) could bind to the site of action (such as an interstrand DNA crosslink), recruit FANCG and then dissociate. FANCG would then recruit additional members of the complex to carry out specific functions, in a series of steps involving binding and dissociation of single proteins. The sequential model appears to be favoured in, for example, the nucleotide excision repair pathway, in order to explain the diverse actions of the multiple proteins involved (36). Distinguishing between these possibilities in FA is likely to be challenging, but powerful experimental tools such as mass spectrometry are now being used to dissect such complexes (37). Finally, although the severity of the clinical phenotype of FA may differ between patients and families, and between different complementation groups and mutation types, the defects are very similar both at the cellular and clinical level. This is consistent with the evidence that we have presented for the existence of a common functional pathway in FA.

MATERIALS AND METHODS

Bacterial and yeast strains

The bacterial strain *Escherichia coli* DH5α was used for propagation of plasmid constructs. The yeast strains *Saccharomyces cerevisiae* AH109 and Y187 (Clontech) were used as hosts in the two-hybrid assay. AH109 contains two nutritional reporter genes for adenine and histidine. Both AH109 and Y187 contain the *LacZ* reporter gene.

Plasmids

Cloning vectors used in the two-hybrid assay were purchased from Clontech. pGBKT7 [*GAL4*(1–147) DNA–BD, *TRP1*, kan^r, c-*myc* epitope tag] or pAS2.1 [*GAL4* (1–147) DNA–BD, *TRP1*, amp^r, CYH^{s}2] were used for creating bait constructs,

and pGADT7 [*GAL4* (768–881) DNA–AD, *LEU2*, amp^r, HA epitope tag] or pACT2 [*GAL4* (768–881) DNA–AD, *LEU2*, amp^r, HA epitope tag] were used for creating prey constructs. Two-hybrid constructs containing full-length *FANCA*, *FANCC*, *FANCG* and the *FANCG* fragments were created as described previously (28). Full-length *FANCF* and *FANCE* cDNAs were amplified using the Expand High Fidelity PCR System (Boehringer Mannheim) from a pREP4 construct as template. The following primer sequences were used for the introduction of restriction sites and amplification of *FANCF*: FANCF 5′, 5′-GCGGCCGC**catatg**GAATCCCTCTGCAGC-3′, and FANCF 3′, 5′-CTATATATTCT**ggatcc**AAGTAATAAC-3′. The product was digested and ligated using the *Nde*I and *Bam*HI sites of the two-hybrid vectors. Similarly, the following primers were used for the amplification of and restriction site creation in *FANCE*: FANCE 5′, 5′-CGGCGCGG**catatg**TGCCCCGGC-3′, and FANCE 3′, 5′-GCCCTTCAG**gaattc**GGTGATGGAGCACC-3′. The product was ligated into *Nde*I and *Eco*RI sites of the two-hybrid vectors. Fluorescent sequencing on an ABI PRISM 377 DNA Sequencer confirmed the *FANCF* and *FANCE* sequences and linker regions. Control plasmids to test for non-specific interaction were also purchased from Clontech, pGBKT7-LAM which encodes human lamin C fused to the GAL4–BD, pGBKT7-p53 which encodes the murine p53 fused to the GAL4–BD and pGADT7 which encodes SV40 large T antigen fused to the GAL4–AD. As template for *in vitro* transcription and translation of FANCC, a construct coding for the full-length sequence was produced by PCR amplification. This was followed by digestion and ligation into the *Eco*RI and *Xho*I sites of pcDNA3.1/Myc His (Invitrogen). The sequence was verified by sequencing as described above.

Yeast two-hybrid assay

The MATCHMAKER Two-Hybrid System 3 (Clontech) was used for yeast two-hybrid analysis according to the manufacturer's instructions. In brief, bait and prey constructs were sequentially transformed into AH109 by a modified lithium acetate procedure (Clontech). Cells were allowed to recover in YPD for 2 h prior to plating onto synthetic dropout (SD) medium lacking tryptophan, histidine, leucine and adenine, to select for interacting proteins. Yeast mating was used as an alternative method to test interactions. The bait construct was transformed into the yeast strain Y187, and the prey construct into strain AH109. Mating cultures were plated onto SD (–trp –leu –his –ade), and diploid colonies allowed to grow. Colonies were transferred onto filters, and X-gal was added as substrate to test for expression of β-galactosidase. Previous experience using a semi-quantitative liquid assay was unsatisfactory for analysis of the weak interactions since they yielded similar values to the background levels obtained with negative controls (28). Therefore in order to perform a semi-quantitative assessment of our yeast two-hybrid experiments we used the following equation, the basis of which has been described in detail previously (28): reporter gene activation = $n \times C/t \times \ln T$ where n refers to the number of blue colonies (n = 1 for <5 colonies, n = 2 for 6–50 colonies, n = 3 for 51–500 colonies, n = 4 for >500 colonies), C is the intensity of the blue colour (dark = 2; light = 1), t is the time of colony growth prior to testing for *LacZ* expression (1 week = 1; 2 weeks = 2) and T is the time in minutes taken for colour development.

In vitro co-immunoprecipitation of protein pairs

In vitro translation of the FA proteins was performed using the T_NT-coupled rabbit reticulocyte lysate system (Promega), according to manufacturer's instructions. FA constructs (as described above) were used as templates to create fusion proteins with N-terminal c-*myc* and HA epitope tags. Constructs also contained a T7 promoter upstream of the tags, to allow proteins to be transcribed and translated *in vitro*. Approximately equal quantities of translated proteins were mixed and incubated at 30°C for 60 min. Buffer A (20 mM Tris–HCl pH 7.8, 50 mM NaCl, 3.5 mM EDTA and 0.1% IPEGAL), protease inhibitors, and either anti-HA or anti-c-*myc* antibodies (both rabbit polyclonals from Santa Cruz, supplied by Autogen Bioclear) were subsequently added to the protein mix. In addition an approximately equal quantity of each translated protein was incubated with the reciprocal antibody, buffer A and protease inhibitors in order to control for non-specific binding to the opposing anti-tag antibody. After an overnight incubation at 4°C on a rotating wheel, Dynabeads Protein A (Dynal) were added, followed by an additional incubation for 2 h at 4°C. Dynabeads and attached proteins were extracted from the solution by a magnet and washed three times with Buffer A. Proteins were mixed in SDS sample buffer, boiled and separated on a SDS 10% polyacrylamide gel. Incorporated ^{35}S-methionine during transcription and translation allowed detection of the proteins by autoradiography.

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