Fanconi anemia (FA) is an autosomal recessive syndrome characterized by pancytopenia, predisposition to cancer, and a wide range of congenital malformations. Cells from patients with FA exhibit elevated spontaneous chromosome breakage and are hypersensitive to DNA cross-linking agents, including mitomycin C and diepoxybutane. FA is a genetically heterogeneous disorder comprising at least 8 complementation groups (FA-A, -B, -C, -D1, -D2, -E, -F, -G). The genes for 6 of which (FA, FANCC, FANCG, FANCD2, FANCE, FANCF) have been cloned. Most recently biallelic inactivation of BRCA2 was identified in FA cell lines from complementation groups FA-B and FA-D1.

With the exception of FANCD2, which has conserved sequences in Arabidopsis thaliana, Drosophila melanogaster, and Caenorhabditis elegans, FANC protein orthologs have not been identified in nonvertebrates. In addition, despite the similar clinical phenotype generated by their individual absence, the FANC proteins do not bear any sequence similarity to each other. This phenomenon has given rise to the question of how, when, and where these orphan proteins might interact to maintain genomic integrity.

Database searches have revealed few identifiable protein-interaction motifs among the FANC proteins. Neither FANCC nor FANCF contain any such motifs. FANCE has a putative nuclear localization signal (NLS) in the central region of its sequence. FANCA, FANCC, and FANCG proteins immunoprecipitated only with FANCE. Direct interaction between FANCE and FANCF, as well as between monomers of FANCA and FANCF, has been demonstrated in the yeast 2-hybrid system. This interaction involving an amino-terminal region of FANCF may provide a link between the FA protein complex and its downstream targets.

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of the N-terminal region of FANCA with FANCG has been well documented. Interactions of FANCG with FANCC, as well as FANCC with FANCC, have also been demonstrated. The possibility of weak or transient interaction between other FANC protein pairs, including FANCA/FANCA, FANCG/FANCG, FANCA/FANCC, FANCA/FANCF, and FANCG/FANCF has been suggested by some yeast 2-hybrid studies.

To gain a better understanding of the architecture of the FA protein complex, as well as its connection to FANC22,26,30, a GAL4 yeast 2-hybrid system was used to identify direct interactions between full-length FANC proteins and to map the contact points of these interactions. The data suggest the impact of patient-derived missense mutations on protein interactions was also investigated. As FANCG was demonstrated to interact directly with both FANCA and FANCF, the yeast 3-hybrid system was used to investigate the ability of FANCG to mediate interactions between the other FANC proteins, uncovering a possible role for FANCG at multiple stages of complex assembly.

**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 all plasmid constructs. *E. coli* was propagated in Luria-Bertani (LB) media at 30°C for FANC22 and FANCF at 30°C and for other expression constructs. *Saccharomyces cerevisiae* strain PJ69-4A (American Type Culture Collection, Manassas, VA) was used in the yeast 2-hybrid assay. *S. cerevisiae* strains AH109 and Y187 (Clontech, Palo Alto, CA) were used in the 3-hybrid assay. Yeast was grown at 30°C in YPD or synthetic dropout minimal media (SC).

**Vectors and expression constructs**

All constructs were made using vectors supplied in the Matchmaker GAL4 Vectors and expression constructs pairs, including FANCA/FANCA, FANCG/FANCG, FANCA/FANCC, FANCA/FANCF, and FANCG/FANCF, as shown. Cotransformants were assayed for activation of the 2 nutritional markers (Table 1). Interaction was observed between the AD-FANCC and BD-FANCF combination, as well as the reciprocal pairing of AD-FANC and BD-FANCF. AD-FANC also interacted with BD-FANCD2. Interactions were also observed between AD-FANCG and BD-FANCA and BD-FANCF. The BD-FANCG construct autoactivated all 3 reporters strongly. Nonetheless, measurement of β-galactosidase activity of the pairings by liquid ONPG assay revealed that the LacZ reporter activation differed markedly from the basal activity of the BD-FANCG construct when it was paired with AD-FANCA or AD-FANCF (Table 2), consistent with the reciprocal assays. When cotransformed with AD-FANCA, the LacZ activation was considerably above background, in agreement with the interaction seen between the reciprocal pairing of BD-FANCA and AD-FANCG. The basal LacZ autoactivation was actually lowered for the BD-FANCG/AD-FANCF pairing, suggesting that FANCG may interfere with the strong ability of FANCG to autoactivate. The AD-FANCD2 construct also showed mild autoactivation of reporters and was not used in further analysis.

To better understand the nature of these interactions among the FA proteins, deletion constructs were used to map contact sites for the binding of FANCA/FANCG, FANCF/FANCG, FANCC/FANCE, and FANCG/FANCF demonstrated in the yeast 2-hybrid system. The amino-terminal 40 amino acids of FANCA encompassing the NLS motifs were sufficient for interaction with full-length FANCG.

**Results**

**Yeast 2-hybrid system**

In the PJ-694A strain of *S. cerevisiae*, 3 reporters are used to assess interaction in the GAL4-based yeast 2-hybrid system: GAL2p-ADE2, GAL1p-HIS3, and GAL7p-LacZ. Cotransformants expressing full-length FANC proteins fused to the GAL4 activation domain (AD) or DNA-binding domain (BD) were assayed pairwise for activation of the 2 nutritional markers (Table 1). Interaction was observed between the AD-FANCC and BD-FANCF combination, as well as the reciprocal pairing of AD-FANC and BD-FANCC. AD-FANC also interacted with BD-FANCD2. Interactions were also observed between AD-FANCG and BD-FANCA and BD-FANCF. The BD-FANCG construct autoactivated all 3 reporters strongly. Nonetheless, measurement of β-galactosidase activity of the pairings by liquid ONPG assay revealed that the LacZ reporter activation differed markedly from the basal activity of the BD-FANCG construct when it was paired with AD-FANCA or AD-FANCF (Table 2), consistent with the reciprocal assays. When cotransformed with AD-FANCA, the LacZ activation was considerably above background, in agreement with the interaction seen between the reciprocal pairing of BD-FANCA and AD-FANCG. The basal LacZ autoactivation was actually lowered for the BD-FANCG/AD-FANCF pairing, suggesting that FANCG may interfere with the strong ability of FANCG to autoactivate. The AD-FANCD2 construct also showed mild autoactivation of reporters and was not used in further analysis.

**Table 1. Pairwise analysis of interaction between full-length FANC proteins**

<table>
<thead>
<tr>
<th></th>
<th>AD-FANCA</th>
<th>AD-FANCG</th>
<th>AD-FANCF</th>
<th>AD-FANCD2</th>
<th>BD-FANCA</th>
<th>BD-FANCG</th>
<th>BD-FANCF</th>
<th>BD-FANCD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD-FANCA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>AD-FANCC</td>
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<tr>
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<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Growth (+) or – (−) of PJ69-4A cotransformants on -Ade -His media. The AD-FANCD2 and BD-FANCG constructs are not included because they autoactivated the reporters.
FANCG. As a polypeptide lacking only the first 35 amino-terminal amino acids of FANCA could not sustain this interaction, no secondary independent sites for FANCG binding were detected (Figure 1A). Of the truncated AD-FANCG polypeptides tested, only an AD-FANCG lacking the 142 C-terminal amino acids retained any ability to interact with FANCA (Figure 1B). The carboxyl-terminal region of FANCF was sufficient for interaction with FANCG (Figure 1C). None of a series of truncated polypeptides spanning the length of FANCG were able to mediate interaction with FANCF (Figure 1D). The effect of patient-derived Leu71Pro and Gly546Arg missense mutations of FANCG on interaction with both FANCA and FANCF was examined. AD-FANCGLeu71Pro was unable to interact with BD-FANC, and the reporter activation in the AD-FANCGLeu71Pro/BD-FANCA cotransformants was much reduced compared with that observed for wild-type AD-FANCG/BD-FANCA. AD-FANCGGly546Arg retained the ability to interact with both BD-FANCA and BD-FANCF. LacZ reporter activation was lower for AD-FANCG Gly546Arg/BD-FANCA cotransformants than for wild-type AD-FANCG/BD-FANCA.

The central portion of FANCE encompassing amino acids 150 to 371 was sufficient for interaction with FANCC, although the use of constructs encoding additional amino acids on either side activated the reporters more strongly (Figure 2A). None of the amino- or carboxyl-terminal–truncated FANCC polypeptides tested were capable of interaction with FANCE. The AD-FANCCLeu554Pro mutant also failed to interact with BD-FANCE (Figure 2B). This result was confirmed by assaying cotransformants of the BD-FANCCLeu554Pro/AD-FANCE combination (data not shown).

The amino-terminal 291 amino acids of FANCD2 were sufficient for interaction with FANCE, although this region alone activated the reporters less strongly than larger segments of the amino-terminus. Further truncation resulted in loss of activation (Figure 2C). The more carboxyl-terminal regions of FANCD2 were not eliminated as possible secondary sites of interaction.

Yeast 3-hybrid system

As FANCG was identified as able to bind more than one other FA protein in the 2-hybrid system, the yeast 3-hybrid system was used to investigate the ability of FANCG to act as a molecular “bridge” in mediating interaction between the FA proteins. The BrAG construct encodes a constitutively expressed BD-FANCA together with a HA-tagged FANCG under the control of the methionine repressible Met25 promoter. 35,42 BrAG autoactivated all 3 reporters only when HA-FANCG was coexpressed in the absence of methionine. As FANCA and FANCG have been shown to interact, and BD-FANCG to autoactivate, this autoactivation was not surprising as the DNA-binding domain would be brought into contact with FANCG. However, elevated β-galactosidase activity on conditional expression of HA-FANCG in the presence of AD-FANCA revealed the ability of HA-FANCG to mediate an interaction between monomers of BD-FANCA and AD-FANCA (Figure 3). The presence of AD-FANCG reduced the activation of the BrAG construct, just as AD-FANCG reduced the autoactivation of BD-FANCG in the 2-hybrid assay. BrAG paired with an interaction between monomers of BD-FANCA and AD-FANCA.

### Table 2. LacZ reporter activation in cotransformants of BD-FANCG with AD-FANCC constructs

<table>
<thead>
<tr>
<th>Full-length protein fused to GAL4-AD</th>
<th>β-Galactosidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No insert</td>
<td>41.2 ± 1.5</td>
</tr>
<tr>
<td>FANCA</td>
<td>100.1 ± 5.2</td>
</tr>
<tr>
<td>FANCC</td>
<td>42.8 ± 3.3</td>
</tr>
<tr>
<td>FANCD2</td>
<td>41.0 ± 2.6</td>
</tr>
<tr>
<td>FANCE</td>
<td>44.5 ± 6.9</td>
</tr>
<tr>
<td>FANCF</td>
<td>23.2 ± 1.6</td>
</tr>
<tr>
<td>FANCG</td>
<td>42.7 ± 1.4</td>
</tr>
</tbody>
</table>

*Units of β-galactosidase activity in the liquid ONPG assay were calculated as 1000 × optical density at 420 nm (OD420) divided by the product of the volume (milliliter) × time (minutes) × OD600.

![Figure 1](image1.png)

**Figure 1. Mapping binding sites for the interactions of FANCG with FANCA and FANCF.** (A) Mapping the FANCG binding region on FANCA. (B) Mapping the FANCG binding region on FANCF. (C) Mapping the FANCF binding region on FANCG. Yeast strain Pu69-4A was cotransformed with the expression constructs indicated. Constructs activating the reporter genes above background levels are shaded. The dotted lines represent internal deletions. (+), (+), and (–) indicate good, weak, and absent growth, respectively. β-Galactosidase activity units in the liquid ONPG assay were calculated as 1000 × OD600 divided by the product of the volume (milliliter) × time (minutes) × OD600. The values shown indicate the mean and SE for 4 assays, each done in triplicate. Nd indicates that no β-galactosidase assay was performed because of nonactivation of the ADE2 and HIS3 reporters. NLS indicates nuclear localization signal; LZ, leucine zipper.

![Figure 2](image2.png)

**Figure 2. Mapping binding sites for the interactions of FANCE with FANCC and FANCD2.** (A) Mapping the FANCE binding region on FANCC. (B) Mapping the FANCE binding region on FANCD2. Yeast strain Pu69-4A was cotransformed with the expression constructs indicated. Constructs activating the reporter genes above background levels are shaded. (+), (+), and (–) indicate good, weak, and absent growth, respectively. β-Galactosidase activity units were calculated as in Figure 1. The values shown indicate the mean and SE for 4 assays, each done in triplicate. Nd indicates that no β-galactosidase assay was performed because of nonactivation of the ADE2 and HIS3 reporters. NLS indicates nuclear localization signal; LZ, leucine zipper.
The PJ69-4A strain of *S. cerevisiae* was designed with 3 reporter genes under the control of different GAL4-responsive promoters to decrease the incidence of false positives while remaining a highly sensitive method of detecting protein-protein interaction. Pairwise analysis of FANC proteins in this version of the 2-hybrid system revealed 4 interactions (FANCA/FANCG, FANCF/FANCE, FANCC/FANCE, and FANCD2/FANCE). Comparison of reporter activation between these pairings of FANC proteins may not be a reliable indicator of relative interaction strength, as differences in the expression level and toxicity of proteins will affect reporter activation. However, all interactions were adequate to permit the use of deletion studies to map contact points involved in binding.

Identification of the NLS region of the FANCA protein as a binding site for FANCG concurred with reports from site-directed mutagenesis studies that amino acids within the NLS are important for FANCG binding. Failure of the amino-terminal–truncated FANCA lacking only amino acids 1 to 35 also agreed with an earlier study demonstrating that an epitope-tagged FANCA missing only the most amino-terminal NLS region failed to immunoprecipitate with FANCG. The ability of a carboxyl-terminal–truncated FANCG to sustain interaction with FANCA was consistent with the reported immunoprecipitation of FANCG amino acids 1 to 428 with full-length FANCA. Retention of the FANCA/FANCG interaction in FA cell lines in which the larger FA nuclear complex is absent, together with failure of an NLS mutant of FANCA to immunoprecipitate with FANCC, suggests this interaction may be an early requirement for complex formation.

Direct binding of FANCG to the NLS of FANCA may explain why FANCG has been shown to promote the nuclear accumulation of FANCA.

The detrimental effect of the Leu71Pro mutation on FANCG protein interactions is not surprising, given it occurs in a region described as having similarity to a leucine zipper, and substitution of a proline could be expected to disrupt α-helical secondary structure. In addition to the disruption of FANCF binding, reduced activation of all 3 reporters suggested that the FANCG/FANCA interaction could also be partially affected. Reduced activation of the nutritional reporters is particularly telling, as they exert strong selective pressure for interaction. The ability of the Gly546Arg mutant to bind both FANCA and FANCF is interesting, given that, when previously expressed in FA-G cells, this mutant cDNA was unable to correct mitomycin C (MMC) sensitivity. It is difficult to assess the relevance of the 2-fold decrease in LacZ reporter activation for AD-FANCG^G546R/BD-FANCA cotransformants, particularly in the absence of effects on the nutritional reporters, as differences in the expression level or toxicity of mutants could affect reporter activation. One study reported that in vivo, the base change predicted to cause this glycine to arginine substitution actually results in an in-frame deletion, presumably due to altered RNA processing. Nevertheless, the inability of a cDNA-based expression construct to correct MMC sensitivity suggests that FANCG^Gly546Arg is deficient in some aspect of FANCG function required to maintain genomic integrity.
The failure of the FANCC<sup>Leu554Pro</sup> mutant to interact with FANC! is interesting, given that overexpression of this mutant in 293 cells has been shown to induce MMC sensitivity. One possible explanation is that the failure of FANCC<sup>Leu554Pro</sup> to bind FANC! may result in the sequestering of other FANCC-binding proteins away from FANC and possibly the FA protein complex. FANCC<sup>Leu554Pro</sup> fails to localize to the nucleus in HeLa cells, and recently FANC was found to promote the nuclear accumulation of wild-type FANCC but not FANCC<sup>Leu554Pro</sup>. Thus, FANC binding may be required for nuclear import or retention of FANC, consistent with the failure of FANC to form a nuclear complex in FANC cell lines. The smallest region of FANC mapped as sufficient for FANC binding included the putative NLS. However, unlike the interaction of FANC with a minimal NLS region of FANCA, a smaller construct still fully encompassing the putative NLS of FANC was not sufficient to interact with FANC. Thus, either the NLS region does not interact with FANC, or, although this construct was expressed in yeast, it could not assume the correct conformation for FANC binding. The failure of any of a series of truncated FANCC polypeptides spanning the entire FANC protein to interact with FANC suggests that noncontiguous regions of FANCC may be involved in this interaction.

The direct interaction of FANC with FANCD2 provides a possible link between the FA nuclear protein complex and the downstream activity of the FANCD2 protein. The amino-terminal fragments of FANCD2 able to maintain FANC binding are known to contain biologically significant residues. These residues include the site of monoubiquination (Lys561) as well as the site of ionizing radiation-inducible ataxia telangiectasia mutated (ATM)-dependent phosphorylation (Ser222), which is located in the most truncated polypeptide able to sustain interaction. However, these posttranslational protein modifications may not occur in yeast, and the possibility of a role for these residues in the FANCD2/FANC interaction requires further investigation. It also remains to be determined whether FANC is capable of binding both FANCC and FANCD2 simultaneously, or if the interactions occur sequentially as part of a cascade, as FANC is the only FANC protein demonstrated to immunoprecipitate with FANCD2. Elucidation of the FANCD2 binding region of FANC may provide further insight into this question. The recent identifications of BRCA2 mutations in FA-D1 cell lines provides another interesting candidate for FANC protein interaction studies, as FA-D1 cells
have intact FA nuclear complex formation and FANCd2 mono-
ubiquination pathways. 11,23,25

In summary, this study mapped the binding sites involved in 3 protein-protein interactions between members of the FA nuclear protein complex (FANCA/FANCG, FANCF/FANCC, and FANCC/ FANCE). A fourth demonstrated interaction, that of FANCE with the amino-terminal region of FANCd2, provides a possible link between the FANCAC/CE/FG complex and the downstream action of the FANCd2 protein. The ability of FANCd2 to mediate interaction between monomers of FA as well as between FA and FANCf suggests that the FANCg protein may play an important structural role in the architecture of the FA protein complex with involvement at multiple stages of complex assembly.

A model for the architecture of the nuclear FA complex based on these observations is presented in Figure 6. Taken together, these results demonstrate an increased level of complexity in the web of interactions among proteins underlying the molecular pathogenesis of Fanconi anemia.

Acknowledgments

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