How Fanconi Anemia Proteins Promote the Four Rs: Replication, Recombination, Repair, and Recovery

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The genetically complex disease Fanconi anemia (FA) comprises cancer predisposition, developmental defects, and bone marrow failure due to elevated apoptosis. The FA cellular phenotype includes universal sensitivity to DNA crosslinking damage, symptoms of oxidative stress, and reduced mutability at the X-linked HPRT gene. In this review article, we present a new heuristic molecular model that accommodates these varied features of FA cells. In our view, the FANCA, -C, and -G proteins, which are both cytoplasmic and nuclear, have an integrated dual role in which they sense and convey information about cytoplasmic oxidative stress to the nucleus, where they participate in the further assembly and functionality of the nuclear core complex (NCCFA = FANCA/B/C/E/F/G/L). In turn, NCCFA facilitates DNA replication at sites of base damage and strand breaks by performing the critical monoubiquitination of FANCD2, an event that somehow helps stabilize blocked and broken replication forks. This stabilization facilitates two kinds of processes: translesion synthesis at sites of blocking lesions (e.g., oxidative base damage), which produces point mutations by error-prone polymerases, and homologous recombination-mediated restart of broken forks, which arise spontaneously and when crosslinks are unhooked by the ERCC1-XPF endonuclease. In the absence of the critical FANCD2 monoubiquitination step, broken replication forks further lose chromatid continuity by collapsing into a configuration that is more difficult to restart through recombination and prone to aberrant repair through nonhomologous end joining. Thus, the FA regulatory pathway promotes chromosome integrity by monitoring oxidative stress and coping efficiently with the accompanying oxidative DNA damage during DNA replication. Environ. Mol. Mutagen. 45:128–142, 2005.

Key words: DNA replication fork; chromosomal breakage; homologous recombination; translesion synthesis; DNA crosslinks; oxidative damage

BRINGING FANCONI ANEMIA PROTEINS INTO FOCUS

Fanconi anemia (FA) is a rare autosomal chromosomal instability disorder that combines developmental defects, pancytopenia, and cancer susceptibility. Progressive bone marrow failure and a predisposition for acute myelogenous leukemia and solid tumors are hallmarks of FA [Tischkowitz and Hodgson, 2003]. The median patient survival age is ~12 years [Alter, 2003]. The severity of the clinical phenotype can depend on genetic background and environmental factors in addition to the specific mutation [Gillio et al., 1997; Futaki et al., 2000].

Besides increased chromosomal breakage/exchange, the phenotype of FA cells includes elevated apoptosis in hematopoietic cells, increased reactive oxygen species (ROS), and prolongation of the G2 phase [Joenje and Patel, 2001; Bogliolo et al., 2002; Bagby, 2003; D’Andrea and Grompe, 2003; Rosselli et al., 2003]. The chromosome instability includes more rapid telomere shortening [Ball et al., 1998; Adelfalk et al., 2001; Hanson et al., 2001; Callen et al., 2002; Li et al., 2003]. Many studies have suggested that FA cells have defects in oxygen metabolism [Pagano and Youssoufian, 2003], and at least the FANCC protein appears to have a role in redox metabolism [Kruyt et al., 1998; Cumming et al., 2001]. One model for the primary
FA defect has been that elevated ROS leads to genotoxic stress in FA cells. However, the preponderance of evidence indicates that the nuclear functions of FA proteins predominantly determine the FA cellular phenotype. Historically, FA has often been viewed as a DNA repair deficiency disorder, largely because FA cells are consistently hypersensitive to DNA crosslinking agents and have increased chromosome fragility [Sasaki and Tonomura, 1973; Auerbach and Wolman, 1976]. The induction of chromosomal damage by mitomycin C (MMC) or diepoxybutane, typically chromatid-type aberrations including breaks and quadriradials, is used as a diagnostic test for FA [Auerbach, 1993]. However, the evidence for defects in DNA crosslink repair has been inconsistent and contradictory [Fujiwara et al., 1977; Poll et al., 1984; Buchwald and Moustacchi, 1998]. The lack of homology between FA proteins and DNA repair proteins in yeast argues that the FA proteins are not involved in the enzymology of DNA repair.

FA is genetically heterogeneous, consisting of at least 11 complementation groups for which nine genes have been cloned and identified: FANCA, -B, -C, -D1, -D2, -E, -F, -G, and FANCL/PHF9 [D’Andrea and Grompe, 2003; Meetei et al., 2003a, 2004]. The role of the FA protein pathway in the recovery of cells from DNA-damaging agents is under intense investigation. FA proteins interact to form a nuclear complex [Garcia-Higuera et al., 1999; Waisfisz et al., 1999; de Winter et al., 2000; Siddique et al., 2001; Pace et al., 2002; Meetei et al., 2003a, 2003b, 2004]. We refer to the nuclear core complex as NCCFA. Genomic stability requires the full integrity of NCCFA, which promotes monoubiquitination of FANCD2 at K561 following treatment with MMC, ionizing radiation (IR), or hydroxyurea. This monoubiquitination event appears to be essential for normal cellular recovery from exposure to DNA crosslinking chemicals and other agents [Garcia-Higuera et al., 2001; Taniguchi et al., 2002a].

Interestingly, FANCD1, D2, and L are the only FA proteins that are evolutionarily conserved in nonvertebrates [Timmers et al., 2001; Lo et al., 2003; Meetei et al., 2003a]. Recently, the FANCD1 gene was found to be identical to BRCA2 [Howlett et al., 2002], a participant in the formation of RAD51 nucleoprotein filaments during homologous recombination (HR) [Davies et al., 2001; Pellegrini et al., 2002; Yang et al., 2002]. FA-D1 mutant cells are able to monoubiquitinate FANCD2 [Garcia-Higuera et al., 2001], whereas cells from all other complementation groups except J cannot [Levitus et al., 2004]. The amount of FANCD2 protein increases during S-phase (Taniguchi et al., 2002a) and data not shown), and FANCD2 colocalizes with BRCA1 and RAD51 in nuclear foci [Taniguchi et al., 2002a]. Monoubiquitination is required for normal cell cycle progression following cellular exposure to MMC [Taniguchi et al., 2002a]. The intriguing finding that the master-regulator ATM kinase phosphorylates FANCD2 on Ser222 after IR may link the FA pathway with global signaling events that typically occur in response to direct DNA double-strand breaks (DSBs) [Taniguchi et al., 2002b]. Phosphorylation of FANCD2 by ATM in response to IR damage requires the MRE11/RAD50/NBS1 (MRN) complex [Nakanishi et al., 2002] and may contribute to the intra-S-phase checkpoint [Taniguchi et al., 2002b], although cells from the Fancd2 knockout mouse did not show this checkpoint defect [Houghtaling et al., 2003]. Phosphorylation likely contributes to the execution of FANCD2’s functions [Taniguchi et al., 2002b; Pichierri and Rosselli, 2004], an issue that merits attention.

The many complementation groups of FA [Levitus et al., 2004] contrast starkly with the single-gene chromosomal instability disorders involving the genes ATM (ataxia telangiectasia), BLM (Bloom syndrome), WRN (Werner syndrome), NBS1 (Nijmegen breakage syndrome), and MRE11 (AT-like disorder) [Thompson and Schild, 2002; Thompson and Limoli, 2003]. We suggest that the many FA proteins reflect a finely tuned regulatory system that enables DNA replication to cope with a wide variety of polymerase-blocking lesions (i.e., oxidized bases, breaks, and cross-links). This regulation may include the transfer of information on redox status from the cytoplasm to the nucleus to facilitate replication of DNA containing oxidative damage. Since chemical inhibition of DNA replication also activates FANCD2 [Taniguchi et al., 2002a], the complexity of the NCCFA may reflect the versatility needed to cope with many different modes of replication arrest/blockage. The FA proteins may also help coordinate replication fork stabilization and recovery with replication checkpoint functions.

Moreover, several FA proteins interact with other chromosome stability proteins, including BRCA1, ATM, NBS1, and BLM [Nakanishi et al., 2002; Taniguchi et al., 2002b; Meetei et al., 2003b]. Thus, the emerging picture indicates that FA proteins operate in damage-response processes during DNA replication that are centrally important in chromosome stability and cancer biology. We believe that these processes help ensure the physical continuity of the very large vertebrate chromosomes during replication by preventing breakage and rearrangement. Recent evidence suggests that the FA proteins also have an important role in carcinogenesis separate from the cancer-proneness of the FA clinical disorder [Taniguchi et al., 2003; Narayan et al., 2004; Rogers et al., 2004].

INCREASINGLY COMPLEX FA PROTEIN COMPLEXES

As indicated above, the NCCFA consists of FANCA/B/C/E/F/G/L (and likely FANCI), and mutations in several of these proteins cause major disruption and loss of the multimeric interactions. In cells of the FA-D1 and FA-D2 complementation groups, as well as the uncloned FA-J group, the NCCFA appears to remain intact [Levitus et al.,
FANCA, FANCG, and FANCC proteins are required for the interaction of FANCA and FANCC, and mutual dependence for in vivo stability is seen with [Garcia-Higuera et al., 2000; Park et al., 2004]. A similar role in assembling and stabilizing the NCCFA [Pace et al., 2002] is required for binding the FANCC-FANCE subcomplex [Leveille et al., 2004]. FANC potentials, has been characterized in some detail. These proteins interact directly by yeast two-hybrid analysis [Medhurst et al., 2001; Gordon and Buchwald, 2003]. The interaction between FANCA and FANCG results in longer half-lives in vivo for both proteins, and one function of FANCA-FANCG is to promote the nuclear accumulation of NCCFA [Garcia-Higuera et al., 2000; Park et al., 2004]. A similar mutual dependence for in vivo stability is seen with FANC-FANCC [Garcia-Higuera et al., 1999]. FANC facilitates the interaction of FA proteins with FANCE, and FANCA, FANCG, and FANC are required for stabilization of NCCFA [Garcia-Higuera et al., 1999]. The cellular levels of FANCA, -G, and -C all depend on the presence of FANC [Siddique et al., 2001], but the level of FANC expression is similar and unchanged in all non-FA complementation groups when compared with gene-complemented derivatives. Similarly, immunoblot analysis confirmed that FANCE is present in cells with defects in FANCA, -C, -D2, -F, and -G but absent from FA-E cells [Pace et al., 2002]; four YFP-tagged fluorescent FA proteins (A, E, F, and G) localized to the nucleus independently, whereas FANCC nuclear accumulation required coexpression of FANCE. Disease-associated FANCC mutations prevented FANCE nuclear accumulation and binding in the NCCFA [Pace et al., 2002].

FA proteins in HeLa cells reside in large multimeric complexes that include non-FA proteins [Meetei et al., 2003b; Thomashevski et al., 2004]. The composition of these complexes depends on their subcellular location. In the cytoplasm of asynchronous cells, FANCA, -C, -F, and -G exist in a 600 kDa complex, and in mitotic cells in a ∼750 kDa complex. Only phosphorylated forms of FANCG are found in the mitotic complex. The same four FA proteins, together with FANCE, are also found in nuclear and chromatin-bound fractions of 1 and 2 MDa size, respectively [Thomashevski et al., 2004].

There is limited information about the subcellular distribution of FA proteins during the cell cycle. In thymidine-synchronized HeLa cells moving through S-phase, chromatin-bound FANCA and FANCG were elevated compared with the initial G1-S population, and this association was not noticeably enhanced by MMC exposure [Mi and Kupfer, 2004]. In whole cell extracts of thymidine-synchronized cells, the level of FANCC increased as cells progressed into S-phase [Kupfer et al., 1997], but FANCF appeared constant [Siddique et al., 2001]. However, in elutriation-synchronized cells, chromatin-bound whole-cell FANCG did not appear to change significantly through the cycle [Lamerdin et al., 2004]. It is noteworthy that FANCD2 is monoubiquitinated in response to inhibition of DNA replication by thymidine [Taniyaguchi et al., 2002a]. This observation raises concerns about using thymidine synchronization to evaluate FA protein dynamics. Precise information on the association of FA proteins with chromatin during the unperturbed cell cycle is likely to enhance our understanding of the function of these proteins.

In the yeast two-hybrid system, FANCA, -C, and -G were used as bait to identify 69 interacting proteins that were not previously linked to the FA pathway [Reuter et al., 2003]. The significance of these numerous interactions remains unclear. This large screen identified several interactions that were previously known, including the Zn-finger FAZF transcriptional repressor [Hoatlin et al., 1999]. Additional FANC-interacting proteins not identified in this study were reported in other two-hybrid screening studies. FANCA associates with BRG1, a subunit of the SWI/SNF chromatin-remodeling complex, as confirmed by coimmunoprecipitation and colocalization [Otsuki et al., 2001]. (Note that both FANCA [Folias et al., 2002] and BRG1 [Bochar et al., 2000] interact directly with BRCA1, which does not interact with the other FA proteins, namely, FANCC, -D2, -E, -F, and -G [Folias et al., 2002].) Curiously, a molecular chaperone protein, GRP94, identified by mass spectrometry to associate with immunoprecipitated NCCFA [Thomasheskvi et al., 2004], was previously found among BRG1-associated factors in FA-A cells [Otsuki et al., 2001]. These results suggest that GRP94 may have a functional role with respect to FA proteins.

**FANCD2: A SERGEANT WHO GIVES ORDERS TO THE MAJOR, FANCD1/BRCA2**

FA cell lines originally assigned to the D-complementation group were found to subdivide into groups D1 and D2. FANCD2 was cloned by a positional strategy and found to encode a protein of 1451 amino acids [Timmers et al., 2001]. FANCD2 is highly conserved in nonvertebrates, including A. thaliana, C. elegans, and Drosophila.
sugesting that FANCD2 plays a more central role than most other FA proteins. Also, FA-D2 mutant cells seem to be more sensitive to killing by IR than the other non-BRCA2 FA mutants [Taniguchi et al., 2002b; Yamamoto et al., 2004], suggesting that in the nonubiquitinated state FANCD2 may have a residual function that is independent of the other FA proteins. In support of this idea, the Fancd2 null mouse has a more severe phenotype [Houghtaling et al., 2003] than knockouts of Fanca [Cheng et al., 2000; Noll et al., 2002; Wong et al., 2003], Fance [Chen et al., 1996; Whitney et al., 1996], and Fancg [Yang et al., 2001; Koomen et al., 2002].

In dividing asynchronous populations of undamaged cells, a major portion of FANC2 exists in the faster-migrating nonubiquitinated short form (D2-S) [Garcia-Higuera et al., 2001]. In S-phase synchronized cultures, much of FANC2 is in the monoubiquitinated long (D2-L) form (Taniguchi et al., 2002a) and during S-phase in synchronized cells, a major portion of FANCD2 exists in the faster-migrating nonubiquitinated short form (D2-S) [Garcia-Higuera et al., 2001]. This monoubiquitination at K561 is absent in FA cell lines of groups A, B, C, E, F, G, I, and L, indicating that the intact NCCFA is necessary for this modification [Houghtaling et al., 2003] than knockouts of Fanca [Cheng et al., 2000; Noll et al., 2002; Wong et al., 2003], Fance [Chen et al., 1996; Whitney et al., 1996], and Fancg [Yang et al., 2001; Koomen et al., 2002].

FANCD2 is expressed in diverse human tissues, and its expression level is higher in proliferating cells [Holzel et al., 2003].

As mentioned, ATM phosphorylates FANCD2 after 10 Gy of IR but the significance of this event, which occurs independently of ubiquitination [Taniguchi et al., 2002a], is not known. Taniguchi et al. [2002a] state that FANCD2 monoubiquitination and focus formation occur in G1 phase after IR damage, but another group showed data directly contradicting this statement by showing an absence of these events during G1 [Rothfuss and Grompe, 2004]. In our model below, FANCD2 monoubiquitination only occurs in S-phase.

The finding that FANC1 = BRCA2 prompts the question of whether other FA proteins participate directly in HR. This issue is explored below in the context of our model. We suggest that the FA disease may involve two different kinds of defective processes, as occurs in xeroderma pigmentosum (XP). The XP variant complementation group is defective in translesion synthesis and clinically indistinguishable from the other XP groups, which are defective in nucleotide excision repair. In our view, the primary function of the NCCFA and FANCD2 proteins lies upstream of HR-mediated strand exchange, but these FA proteins may have additional ancillary functions during HR. The finding that FANCG and FANCD2 interact directly with BRCA2 [Hussain et al., 2003, 2004], as shown by the yeast two-hybrid system (and coimmunoprecipitation for FANC2), suggests that the NCCFA and FANC2 proteins may help facilitate HR, for example by recruiting BRCA2 to damaged forks so that it can mediate the loading of RAD51 onto single-stranded DNA. Notably, FANC2 foci colocalized ~90% with both RAD51 and BRCA2 in response to MMC treatment in HeLa cells after transient transfection and visualization of a GFP-FANC2 fusion construct, analyzed 3 hr after a 1-hr MMC treatment [Hussain et al., 2003]. BRCA2 and FANCD2 coimmunoprecipitate (co-IP) in both untreated and MMC-treated cells [Hussain et al., 2004]. After IR and MMC damage, an increase occurs in coimmunoprecipitation and colocalization of BRCA2 with FANCD2 in foci, and this is accompanied by increased chromatin-bound BRCA2 and FANCD2-L [Wang et al., 2004]. Wang et al. [2004] argue that the connection between FA proteins and HR is supported by defective RAD51 focus formation in FA-D2 mutant cells, but other laboratories find no such defect [Godthelp et al., 2002; Yamamoto et al., 2004]. (One study reported reduced IR focus formation in all FA groups tested [Digweed et al., 2002].)

In vivo, FANCG appears to be necessary for the interaction of FANCD2 and BRCA2, and this interaction is independent of NCCFA formation and expression of other FA proteins including FANCA and FANCC (data not shown).

**FA PROTEINS ARE WELL CONNECTED**

We have described stable complexes containing FA proteins and mentioned several proteins that interact with
them. Here we examine other FA protein interactions and associations in both the cytoplasm and nucleus, which are particularly relevant to the discussion of our model.

In the Cytoplasm

Only the FANCA, -C, -F, and -G proteins appear to be present in the cytoplasm [Thomashevski et al., 2004]. Cytoplasmic interactions and functions of FANCC and FANCG are documented. FANCC binds to NADPH cytochrome-P450 reductase, a microsomal membrane protein involved in electron transfer, and is proposed to have an important role in attenuating the reductase activity, thereby regulating a major detoxification pathway [Kruyt et al., 1998]. FANCC also interacts with glutathione S-transferase P1-1 (GSTP1) [Cumming and Buchwald, 2001; Cumming et al., 2001], an enzyme that catalyzes the detoxification of xenobiotics and by-products of oxidative stress. This interaction increases GSTP1 activity by preventing the formation of inactivating intermolecular disulfide bonds in GSTP1 during apoptosis in a myeloid progenitor cell line. FANCC also interacts with HSP70 to prevent apoptosis in hematopoietic cells exposed to IFN-γ and TNF-α [Pang et al., 2001a]. FANCC is required for optimal activation of STAT1 during signaling through the JAK/STAT pathway in response to cytokine and growth factors and for suppressing cytokine-induced oxidative DNA damage. However, no increase in ROS was detected in fancg knockout CHO cells [Tebbs et al., 2004] (note that mutant genes are written in lower case). These results suggest that the interaction of FANCG with CYP2E1 could alter redox metabolism and account for the abnormally high levels of 8-oxoguanine in the DNA of FA-G cells (compared with gene-complemented cells) after treatment with H2O2 or MMC [Futaki et al., 2002]. Thus, Futaki et al. [2002] proposed a cytoplasmic role for FANCG in protecting against ROS formation and oxidative DNA damage. However, no increase in ROS was detected in fancg knockout CHO cells [Tebbs et al., 2004] (note that mutant genes are written in lower case).

FANCG interacts directly with CYP2E1, which is associated with the production of ROS and the bioactivation of carcinogens [Futaki et al., 2002]. An abnormally high level of CYP2E1 was present in FA-G lymphoblasts compared with FANCG-complemented FA-G cells. These results suggest that the interaction of FANCG with CYP2E1 could alter redox metabolism and account for the abnormally high levels of 8-oxoguanine in the DNA of FA-G cells (compared with gene-complemented cells) after treatment with H2O2 or MMC [Futaki et al., 2002]. Thus, Futaki et al. [2002] proposed a cytoplasmic role for FANCG in protecting against ROS formation and oxidative DNA damage. However, no increase in ROS was detected in fancg knockout CHO cells [Tebbs et al., 2004] (note that mutant genes are written in lower case). Treatment of transformed human embryonic kidney (293) cells with TNF-α-induced expression of both FANCG and FANCA proteins, and the FANCA-FANCG-containing complex in the nucleus, was increased following TNF-α treatment [Futaki et al., 2001].

In the Nucleus

Linking NCCFA to FANCD2

As a component of NCCFA, FANCE is required for the monoubiquitination of FANCD2 and the downstream recovery events that require this modification [Pace et al., 2002; Taniguchi and D’Andrea, 2002]. Several studies show a direct interaction between FANCE and FANCD2, providing the critical link between the NCCFA and the pivotal FANCD2 protein [Pace et al., 2002; Gordon and Buchwald, 2003]. This interaction involves the N-terminus of FANCD2, whose residues 1–291 (of 1,451 amino acids) interact with FANCE at ~50% maximal efficiency in yeast two-hybrid analysis [Gordon and Buchwald, 2003]. The precise signals causing, and mechanistic basis of, FANCD2 monoubiquitination are currently unknown [Garcia-Higuera et al., 2001].

Role of ATR in activating FANCD2

The phosphorylation of FANCD2 in response to cross-links is mediated by ATR, and the two proteins colococalize in foci in response to psoralen crosslinking damage [Pichierrri and Rosselli, 2004]. Monoubiquitination of FANCD2 also requires ATR as well as RPA (single-strand binding protein) [Andreassen et al., 2004].

Linking NCCFA to MRN (MRE11/RAD50/NBS1) complex

Several studies point to a functional connection between FA proteins and the MRN complex. In wild-type cells, MMC treatment results in colocalization of FANCD2 and NBS1 proteins in subnuclear foci [Nakanishi et al., 2002]. FA-C mutant cells appear to be defective in the formation of MRN nuclear foci in response to MMC or photoactivated 8-methoxypsoralen [Pichierrri et al., 2002], although MRE11 focus formation was normal after IR in cells from eight FA complementation groups [Digweed et al., 2002]. Interestingly, phosphorylation of NBS1 requires the NCCFA, whereas phosphorylation of FANCD2 requires phosphorylated NBS1 in response to both crosslinks [Pichierrri a-and Rosselli, 2004] (summarized in Fig. 1) and IR [Taniguchi et al., 2002b]. These results indicate a functional interdependence of FA proteins and NBS1 analogous to the relationship between ATM and the MRN complex, which are also interdependent for their phosphorylations). Moreover, MRE11 (AT-like disorder) mutant cells showed increased sensitivity to MMC exposure [Nakanishi et al., 2002].

Linking NCCFA to BLM helicase

In addition to NCCFA proteins being present in a BLM-containing complex [Meetei et al., 2003b], a functional association exists between ubiquitinated FANCD2 and BLM, which colocalize and co-IP after treatment with either DNA crosslinking or replication-arresting
agents [Pichierri et al., 2004b]. NCCFA is necessary for BLM phosphorylation and assembly in nuclear foci in response to DNA crosslinking [Pichierri et al., 2004b]. This requirement could be indirect because NCCFA is apparently required for NBS1 phosphorylation [Pichierri et al., 2002, 2004a; Pichierri and Rosselli, 2004], an event that might be needed for phosphorylation of BLM. However, based on siRNA inhibition of MRE11, Pichierri et al. [2004b] concluded that BLM and MRE11 contribute through two separate pathways to ensure cell viability and chromosome integrity after crosslinking treatment.

Linking NCCFA to ERCC1/XPF endonuclease

FANCA colocalizes in nuclear foci with the XPF endonuclease in response to psoralen crosslinking, and FA-A cells show diminished XPF focus formation [Sridharan et al., 2003]. These findings suggest that NCCFA might help recruit XPF to sites of blocked replication forks where ERCC1/XPF performs crosslink unhooking through dual incision in one parental strand [Niedernhofer et al., 2004]. Nonerythroid α-spectrin (αSpΠΣ*) was shown to colocalize in foci and co-IP with both FANCA and XPF. Moreover, αSpΠΣ* focus formation was defective in FA-A cells. A role for αSpΠΣ* as a structural component that promotes XPF relocalization and incision was suggested [McMahon et al., 2001; Sridharan et al., 2003].

Linking NCCFA to DNA replication checkpoint

Studies measuring the rate of DNA synthesis or rate of accumulation of cells in G2 in response to crosslinking treatment (psoralen + UV-A) have provided evidence for a replication checkpoint defect in response to such treatment in four complementation groups of FA (FA-A, FA-B, FA-C, and FA-D1) [Centurion et al., 2000; Salat-Campmany et al., 2000]. This defect was not seen in cells treated with methyl methanesulfonate [Centurion et al., 2000]. FANCD2 is also implicated in the replication checkpoint associated with crosslink damage (Fig. 1) [Pichierri and Rosselli, 2004]. Full implementation of this checkpoint appears to require separate subpathways involving CHK1 activity and normal MRN-FA protein interactions as discussed above. FANCD2 and BRCA2 are also implicated in the intra-S-phase checkpoint that operates in response to IR [Taniguchi et al., 2002b; Wang and D’Andrea, 2004]. Moreover, FANCD2 was found to colocalize tightly with PCNA (proliferating cell nuclear antigen) following treatment of HeLa cells with hydroxyurea [Hussain et al., 2004]. PCNA is suggested to provide communication between replication checkpoint control and DNA replication and repair [Dahm et al., 2004].
Hubscher, 2002]. The G2 checkpoint in response to MMC exposure appeared normal in FA-C cells [Heinrich et al., 1998].

**Regulation of NCC<sub>FA</sub> by cytoplasmic redox status**

FA cells are known to be abnormally sensitive to ambient oxygen and show reversal of their chromosomal instability or apoptosis phenotype in the presence of antioxidants [Joenje et al., 1981; Joenje and Oostra, 1983; Schindler and Hoehn, 1988; Ruppitsch et al., 1997; Saadatzadeh et al., 2004]. Oxidative DNA damage appears to be intimately involved in the overall FA phenotype [Kelley et al., 2001; Zunino et al., 2001], and thioredoxin overexpression in the nucleus is reported to reverse the chromosomal instability and MMC sensitivity of FA cells [Kontou et al., 2002].

The cytoplasmic roles and interactions discussed above for FANCC and FANCG suggest that these proteins might help determine the cellular level of ROS, although this idea has been controversial [Cumming and Buchwald, 2001; D’Andrea, 2001]. In turn, ROS levels are expected to determine the level of oxidative damage in nuclear DNA. We propose that this damage, which is likely responsible for the spontaneous chromosomal breakage and rearrangement in FA cells, requires the appropriate amounts of functional NCC<sub>FA</sub> to cope with such oxidative DNA damage during replication. An important recent study suggests that the rate of formation of NCC<sub>FA</sub> is determined by the ROS status of the cytoplasm [Park et al., 2004]. Specifically, the FANCA and FANCG proteins were shown to be redox-sensitive and to form multimers via intermolecular disulfide linkage in response to oxidative stress (hydrogen peroxide). Oxidative conditions also produced disulfide binding of FANCA with FANCG [Park et al., 2004]. This covalent binding may serve to nuclease the assembly of a cytoplasmic complex composed of FANCA, -C, and -G that undergoes further assembly into NCC<sub>FA</sub>. Thus, a global model in which cytoplasmic, and possibly nuclear, FA proteins act as sensors of redox status to convey information to the nucleus could account for the regulation of NCC<sub>FA</sub> formation in response to cellular oxidative stress. It will be of much interest to determine whether cytoplasmic ROS levels correlate positively with the level of NCC<sub>FA</sub> as ambient oxygen or other ROS-determining parameters are varied.

**Hypomutability of HPRT Gene in FA**

The first indication of an altered rate of mutation in FA cells was seen using Herpes simplex virus DNA in human fibroblasts by measuring spontaneous iododeoxycytidine resistance, which results from inactivation of HSV thymidine kinase [Coppey et al., 1989]. Soon after, Papadopoulo et al. [1990b] showed a decrease in the frequency of psoralen-induced mutation in the Na<sup>+</sup>/K<sup>+</sup>-ATPase and X-linked HPRT gene in FA-A lymphoblasts. In a subsequent study, they found that this hypomutability, also seen in FA-B lymphoblasts, was associated with fewer point mutations relative to deletions when compared to normal cells [Papadopoulo et al., 1990a]. Whereas 77% of the spontaneous HPRT mutants arising from normal cells were point mutations, only 33% of the FA-derived mutants were of this class. After exposure to monofunctional or bifunctional psoralens, HPRT mutations in normal cells were 10% and 0% deletions, respectively; in FA cells, however, deletions accounted for 62% and 39% of the mutations induced by monofunctional or bifunctional psoralens. A higher percentage of HPRT deletions was also seen in circulating T-lymphocytes in FA patients compared with age-matched controls [Laquerbe et al., 1999].

Recently, we made a fancg knockout mutant in CHO cells [Tebbs et al., 2004], which also shows reduced Hprt mutation frequencies. Both the spontaneous and induced (IR, ethynitrosourea) Hprt mutation frequencies are lower in fancg cells than in wild-type or Fangc-complemented fancg cells (data not shown). HPRT hypomutability, a key feature in our model, is interpreted below.

**Dissimilar phenotypes of FANCG and RAD51 paralog mutants**

Because of the high sensitivity of FA cells to DNA crosslinking, it has frequently been suggested that the main function of the FA proteins lies in HR, which is required to repair interstrand crosslinks. Crosslinks are thought to be processed into broken replication forks as they are unhooked by the ERCC1/XPF endonuclease [Dronkert and Kanaar, 2001; McHugh et al., 2001; Niedermhofer et al., 2004]. The discovery that FANCID1 was identical to BRCA2 [Howlett et al., 2002] reinforced the notion that FA proteins may participate in HR. However, our recent characterization of a fancg knockout
mutant of CHO cells provides compelling evidence against the direct involvement of FANCG in HR [Tebbs et al., 2004]. These fancg cells show no detectable defects for endpoints that generally reflect HR proficiency: spontaneous sister-chromatid exchange, spontaneous and IR-induced Rad51 focus formation, and spontaneous chromosomal aberrations [Thompson and Schild, 2002; Tebbs et al., 2004]. Very importantly, the CHO fancg KO40 cells are only 3-fold more sensitive to MMC, whereas the isogenic rad51d knockout is 75-fold more sensitive ([Tebbs et al. 2004] and data not shown). We also conclude that the common feature of cytotoxic lesions in fancg knockout CHO cells is not interstrand crosslinks because these cells are sensitive to agents that produce a variety of lesions [Tebbs et al., 2004], as are the earlier nonisogenic fancg hamster mutants [Wilson et al., 2001]. We conclude that single-strand nicks and gaps arising as repair intermediates (e.g., from methylating agents and 6-thioguanine) may also be a critical class of lesions for the FA pathway.

**Elevated γH2AX Foci in fancg Mutant Cells**

Using elutriation-synchronized cells treated in early S-phase with a subtoxic dose of MMC, we have observed higher levels of γH2AX foci in fancg CHO cells than in parental AA8 cells (data not shown). These results suggest that fancg cells experience more frequent blockage or breakage of DNA replication forks. In the same experiments, we have not seen any consistent difference in Rad51 foci in fancg KO40 cells. This finding is in contrast to RAD51-paralog mutants, which exhibit reduced or no RAD51 focus formation after IR or MMC treatment [Bishop et al., 1998; O’Regan et al., 2001; French et al., 2002].

**FA Proteins Promote Translesion Synthesis**

We propose a new mechanistic model for the role of the FA proteins (Fig. 2). Figure 2A and B depict events occurring in normal cells, whereas Figure 2C and D show events predicted to occur in FA mutant cells. In this model, the primary function of FA proteins is to help stabilize blocked or broken replication forks so that translesion synthesis or HR, respectively, can manage and restart them. Because monoubiquitination of FANCD2 correlates with integrity of the FA pathway, we propose that fork stabilization requires the pivotal FANCD2 monoubiquitination step and operates in two ways. Most important, fork stabilization facilitates the recruitment of mutagenic translesion polymerases, such as REV1, REV3 (POLζ subunit) [Diaz et al., 2003], or POLκ [Gibbs et al., 2000]. These polymerases can produce base substitution mutations at sites of monoadducts, oxidized bases, and base loss. We predict that interactions and associations, such as colocalization, between FANCDD and translesion polymerases will be identified. One example of colocalization of FANCDD and REV1 nuclear foci in response to inhibition of DNA replication by excess thymidine (but not IR or hydroxyurea) was reported [Niedzwiedz et al., 2004].

**Do FA Proteins Promote HR During Normal Replication?**

In untreated normal cells, if translesion synthesis past blocking oxidative lesions does not occur, an alternative pathway may act to bypass the lesion without a mutation occurring. Stabilization of the blocked fork by the FA proteins might be required for an HR-dependent template switching mechanism such as the "chickenfoot" intermediate that arises from fork reversal/regression (Fig. 2A, step c) [Michel et al., 2001; Postow et al., 2001]. The concept of fork reversal was originally proposed by Strauss [Higgins et al., 1976]. Such intermediates are seen in rad53 checkpoint-defective yeast cells [Sogo et al., 2002]. The phenotype of our rad51d knockout CHO cells includes a 20-fold increased spontaneous Hprt mutation rate, which suggests that fork regression could be a major pathway for avoiding point mutations (data not shown). This hypermutability, in contrast with the hypomutability in fancg CHO cells, seems much at odds with a major role for FA proteins in promoting error-free HR to deal with blocked replication forks. In DT40 cells, fancc and fancg mutants show reduced HR as measured by gene targeting and recombination between direct repeat sequences [Yamamoto et al., 2003; Niedzwiedz et al., 2004]. The relevance of these nonphysiological HR substrates to events occurring at replication forks is unclear. It is noteworthy that the DT40 fancg and fancce cells show two- to fourfold increased spontaneous sister-chromatid exchange [Niedzwiedz et al., 2004], which is not a feature of human FA cells [Latt et al., 1975; Sperling et al., 1975] or fancg CHO cells [Wilson et al., 2001; Tebbs et al., 2004].

However, interactions between certain FA proteins and BRCA2 suggest that these FA proteins may enhance the efficiency of some HR events. FANCDD interacts directly with BRCA2, but with none of nine other HR proteins [Hussain et al., 2004]. FANCG is reported to interact directly with BRCA2 and to co-IP with BLM [Hussain et al., 2003; Meetei et al., 2003b]. A BLM-containing complex isolated from HeLa cells contains six proteins of the FA core complex (A, C, E, F, G, and L) [Meetei et al., 2003a, 2003b]. This complex also contains topoisomerase IIIβ and RPA, which interact with BLM and could facilitate DNA unwinding [Meetei et al., 2003b]. Models for BLM helicase invoke its protecting against fork breakdown or restoring productive synthesis of blocked forks, as by resolving chickenfoot structures [Bachrati and Hickson, 2003].
Figure 2.
Subpathways of HR for Restarting Broken Replication Forks

Current models invoke formation of a (one-sided) DSB when the replication fork encounters a crosslink that gets unhooked by the ERCC1/XPF endonuclease [Niedernhofer et al., 2004]. Restarting the resulting broken fork requires HR. We propose that, through monoubiquitination of FANCD2, the FA proteins serve to protect broken forks from collapsing into a configuration that involves spatial dislocation of the free DNA end. Thus, fully collapsed forks compared to newly broken forks will be more difficult to restart through RAD51-mediated recombination (Fig. 2D, step o). After fork collapse, as chromatid replication continues and free ends become amenable to rejoining by non-homologous end joining (NHEJ), misjoining will also occur, resulting in chromosomal translocations (Fig. 2D, steps j and m). The restarting of broken and collapsed forks by HR is presumably associated with RAD51 focus formation, but RAD51 foci after MMC treatment are much less distinct and quantifiable than after IR treatment (data not shown).

Overview of Model

Our model is supported by the three major observations with *fancg* cells discussed above: sensitivity to agents besides crosslinkers that produce polymerase-blocking lesions or broken forks; the highly divergent phenotypes of isogenic *fancg* and *rad51d* mutants; and a specific reduction in spontaneous and chemically induced HPRT point mutations, which we attribute to decreased capacity for translesion synthesis. With defective translesion synthesis in FA mutant cells, blocked forks will undergo more breakage, resulting in either unrepaired DSBs (causing a reduced frequency of recoverable HPRT mutants for this X-chromosome gene) or more NHEJ-mediated repair (causing a higher proportion of both large lethal deletions and viable deletion events among detectable HPRT mutants).

A study of spontaneous mutations in red blood cells in the glycoporphin A (*GPA*) gene, which detects allele loss variant phenotypes arising from mutations in erythroid progenitor cells of *GPA* heterozygous MN individuals, showed hypermutability in FA patients [Sala-Trepat et al., 1993]. The frequency of *GPA* variant cells was elevated 30-fold for hemizygous NO variants and 8-fold for homzygous NN variants in FA patients compared with normal controls. These data are also consistent with our model, which predicts more chromosome breakage and deletion in FA cells. Thus, because the nature of the mutation assay, in terms of locus and cell type, governs the detection of mutations, single-gene mutation rates in FA cells can be both decreased and increased. In either case, deleted chromosomal regions are the underlying cause of the change in mutation frequency or rate.

Our model provides a defined conceptual framework in which to conduct future mechanistic studies. Recently, a study using γH2AX foci as a marker of fork-associated DSBs concluded that FA proteins act after DSBs are formed during replication of psoralen-crosslinked DNA by promoting the recovery of broken forks [Rothfuss and Grömppe, 2004]. Although it has not been established that fork breakage is necessary for ATR-mediated γH2AX foci to form [Ward and Chen, 2001], this conclusion for crosslink damage is consistent with our model.

A recent study of knockout mutants in DT40 cells provides support for our model [Niedzwiedz et al., 2004]. The high cisplatin sensitivity (to killing and chromosomal aberrations) of the *rev1* and *rev3* translesion-synthesis mutants is the same as the sensitivity of the *rev1* *fancg* and *rev3 fancg* double mutants; the *rev1/3* mutants are much more sensitive to cisplatin than the *fancg* mutant. These results
show that FancC operates in the same pathway as Rev1 and Rev3 in processing crosslink damage, in the sense that much of Rev1/3 function depends on Fancc. Similarly, the cisplatin sensitivity of the fanc mutant is the same as the sensitivity of the fanc xrc2 double mutant. In this case, the fanc mutant is more sensitive than the xrc2 mutant. These results are consistent with our model in which NCE\textsuperscript{FA} (including FancC) is required for efficient HR-mediated fork restart after crosslink unhooking. We note that DT40 cells differ from CHO cells in that the RAD51 paralog mutants of CHO are much more sensitive to crosslinking (e.g., MMC) than the fancg mutants [Wilson et al., 2001; Tebbs et al., 2004].

**FA Cell Hypersensitivity to DNA Interstrand Crosslinking**

An intriguing aspect of FA is the universal cellular sensitivity to DNA crosslinking agents (MMC, cisplatin, diepoxybutane, bifunctional psoralens + UV-A). This cardinal feature can be better understood with our model, in which the stabilization of a crosslink-blocked replication fork is needed not only for HR-mediated restart of the broken fork but also for translesion synthesis past the unhooked crosslink. This dual requirement is unique to crosslinking DNA lesions.

**Inhibitor Arrest of DNA Replication**

The potential role of the FA pathway in the cellular survival response to replication stress associated with chemical arrest of DNA replication (e.g., hydroxyurea, aphidicolin, thymidine) is another area of interest. A recent study on BRCA2 mutant cells (not derived from an FA patient) provided direct evidence that BRCA2 is needed for the stabilization of stalled replication forks under conditions of hydroxyurea inhibition of DNA replication [Lomonosov et al., 2003]. BRCA2 mutant cells displayed excessive DSBs arising from fork breakage. Since hydroxyurea treatment causes FANCD2 monoubiquitination, it will be of interest to know whether FA proteins besides BRCA2 are important in the cellular response to arrest of replication under these conditions. In this regard, the CHO fancg knockout mutant shows only slight sensitivity to hydroxyurea and no sensitivity to aphidicolin or excess thymidine [Tebbs et al., 2004].

**CONCLUSION**

The intensive research on FA and the processing of damaged DNA during replication can be expected to evaluate the validity of our model. Many questions arise. How valid are CHO cells as a model for studying the FA pathway? The Tp53 defect in CHO cells [Lee et al., 1997; Hu et al., 1999; Tzang et al., 1999] may influence the way in which HR deals with DNA lesions during replication. Why do FA cells not show high sensitivity to replication-arresting chemical agents, such as hydroxyurea, that cause FANCD2 monoubiquitination? What is the chemical signal that produces activation of FANCD2, and how does this modification of FANCD2 improve replication fork stability? Do chickenfoot structures arise in mammalian cells? Many challenging experiments lie ahead.

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