Supplemental Material

Xenopus FA (xFA) homologs contain domains conserved in vertebrates

We searched for *Xenopus laevis* DNA sequences homologous to human FA genes. Partial gene sequences of *xFANCA*, and *xFANCD2* were identified in sequence databases for *Xenopus laevis* and *Xenopus tropicalis*. Full-length sequences for *xFANCA*, and *xFANCD2* were obtained by RT- and RACE-PCR. An image clone containing *xFANCL* was found at NCBI Genbank (BJ095024). The sequence for *xFANCB, xFANCC*, *xFANCE*, *xFANCG,* and *xBRCA2/FANCD1* are also present in *Xenopus laevis* and will be described elsewhere. Comparison of the amino acid sequences of *x*FANCA, xFANCD2 and xFANCL with their human counterparts revealed regions of high homology (Fig 1). xFANCA has an overall homology of 53% compared to human FANCA (Fig 1). A highly homologous region (amino acids 321 – 616), contains the consensus sequence for four SRC-homology-module binding motifs (SH2 and SH3), proposed to be protein-protein interaction domains that could be important for the phosphorylation and subcellular localization of FANCA (Pawson, 1995; van de Vrugt et al., 2000; Yamashita et al., 1998). In addition, the reported phosphorylation target site in the human sequence at Ser 1149 is conserved in xFANCA (Otsuki et al., 2002).
xFANCL is very highly conserved (79%) and contains the characteristic WD40 domains as well as the PHD finger domain, which is thought to be the catalytic site of the E3 ubiquitin ligase for the FA core complex (Meetei et al., 2003; Meetei et al., 2004).
The xFANCF sequence has been reported previously (Leveille 2004) (Fig 1).

Fig 1

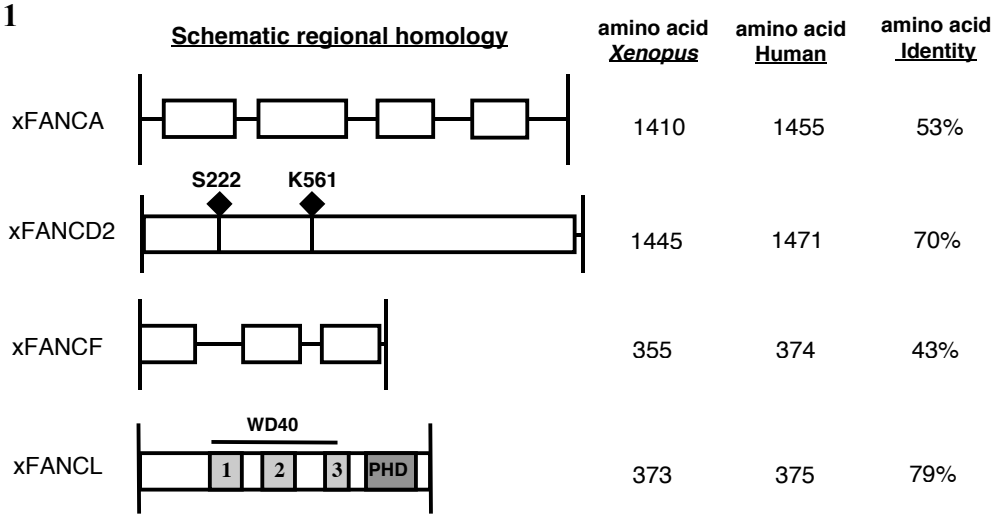

Fig. 1 Regional homologies between *Xenopus* and human FA proteins. (A) Blocks represent regions of the xFA protein with more than 50% sequence identity to the human protein sequence. Predicted sizes for xFA and hFA homologs are listed to the right of the schematic along with the overall amino acid identity.

Characterization of antibodies specific for xFA proteins for subsequent use in functional assays with *Xenopus* egg extracts.

We generated antibodies against xFA proteins for use in western blot, immunoprecipitation, and immunodepletion. Rabbit polyclonal antibodies were generated against: (1) xFANCF using a GST-xFANCF (aa 1-340) fusion protein, xFANCA using a GST-xFANCA (aa #1205-1383) fusion protein. The resulting polyclonal anti-xFANCF, and -xFANCA antibodies specifically recognize the corresponding Flag-tagged antigens in western blotting (Fig 2 A). As shown in Fig 2 B, these xFA antibodies also immunoprecipitate their corresponding antigens.

An antibody generated against the N-terminus of human FANCD2 (Holzel et al., 2003) detected two isoforms of xFANCD2 in lysates from a *Xenopus* tadpole cell line, XTC-2 (Pudney et al., 1973). The isoforms co-migrate with the human FANCD2 short form (hFANCD2-S) and long form (hFANCD2-L), indicated as xFANCD2-S and xFANCD2-L, respectively (Fig 2 C).

Fig 2

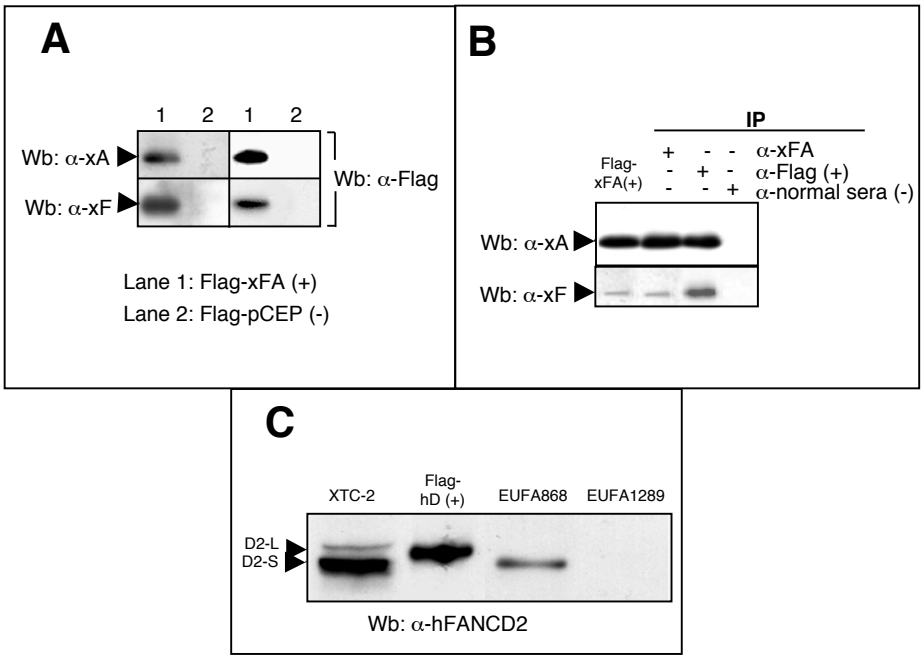

Fig 2. Characterization of antibodies that recognize *Xenopus laevis* FA proteins. **(A)** Anti-xFANCA, and -xFANCF antibodies recognize the corresponding Flag-tagged xFA protein by immunoblot when expressed in transfected 293 EBNA cells (Flag-xFA) but not in mock-transfected Flag-pCEP (-).The same blots were stripped and reprobed with anti-Flag (right panel). **(B)** Immunoprecipitates (IPs) were analyzed by SDS-PAGE and immunoblot as indicated for each Flag-xFANCA, xFANCF, and xFANCL lysates with the corresponding anti-xFANC antibody, anti-Flag as a positive control, and normal rabbit sera as a negative control. A Flag-xFANC lysate is a positive control for each. **(C)** An antibody against hFANCD2 recognizes xFANCD2 on an immunoblot of XTC-2 cell lysate. FA patient fibroblast cells that expresses only short form (EUFA868, FANCL patient cell line) or lack both forms (EUFA 1289, FANCD2 patient cell line) of hFANCD2 were used as controls.

Conservation of the FA pathway in *Xenopus*

DNA damage-induced monoubiquitination of hFANCD2 at residue K561 is a key step in the FA pathway and depends on a functional FA core complex (Garcia-Higuera et al., 2001). We tested if DNA damage-dependent modification of FANCD2 also occurs in *Xenopus*. As shown in Fig 3 A, SDS-PAGE and immunoblot analysis of lysates from XTC-2 cells detected proteins co-migrating with both isoforms of human FANCD2, the shorter non-ubiquitinated form (FANCD2-S) and the larger monoubiquitinated form

(FANCD2-L).

xFANCD2-L was induced in XTC-2 cells treated with DNA damaging agents MMC or hydroxyurea (HU), following a time course comparable to that observed for FANCD2 in human cells (Fig 3 A). In addition, we found that XTC-2 cells (like human cells) show increased formation of chromatid interchanges following MMC-treatment (Table I. and Fig 3 B). Co-immunoprecipitation using specific xFA antibodies revealed that interactions among the CCPs are conserved in egg extracts synchronized in S-phase (Fig 3 C and data not shown), while xFANCD2, like its human counterpart, does not associate with the FA core complex (data not shown). We infer from these data that interactions among the CCPs and the response of FANCD2 to DNA damage, are conserved in *Xenopus*.

Fig 3

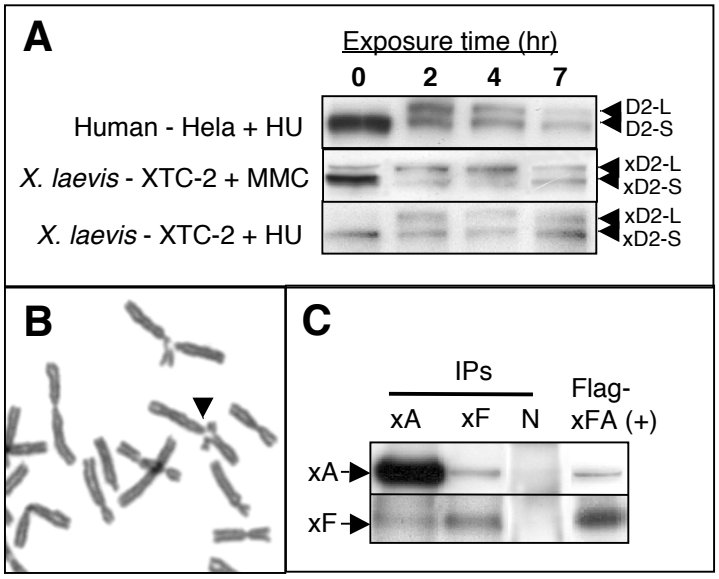

Fig. 3 The central steps of the FA pathway are conserved in *Xenopus*. **(A)** Activation of the long form of xFANCD2 (xFANCD2-L) following exogenous DNA damage. Human HeLa cells and *Xenopus* XTC-2 cells exposed to mitomycin C (MMC, 150 nM) or hydroxyurea (HU; 2mM) from 0 to 72 hours show a comparable time course for induction of FANCD2-S to FANCD2-L. **(B)** Chromatid interchange formation (quadriradial indicated by arrow) in metaphase chromosomes from *Xenopus* XTC-2 cells treated with a DNA crosslinking agent mitomycin C (MMC; 150 nM) and caffeine for 48 hours. **(C)** Interactions between CCPs xFANCA and xFANCF, in *Xenopus* egg extracts. Specific antibodies for xFANCA and xFANCF proteins (as indicated) or normal rabbit anti-sera (indicated as N) were added to equal amounts (2 mg/ml) of egg extract. Resulting immunoprecipitates were analyzed by SDS-PAGE followed by immunoblot as indicated (left). Positive controls: Flag-xFANCA (top), Flag-xFANCF (bottom).

Table I. Cytogenetic analysis of a *Xenopus laevis* tadpole derived cell line, XTC-2

Carcinogen (48 hour exposure)	Carcinogen Concentration	Total Chromatid interchanges	% Chromatid interchanges	Cells with 1 or more breaks	% Breaks	Total Mitotic cells counted	Cells with no breaks/inter-changes
---	0	0	0	2	4	50	48
Caffeine	4 mM	1	3	2	7	29	26
Caffeine, MMC	4 mM, 150 nM	17	34	18	36	50	15
Caffeine, HU	4 mM, 2 mM	1	2	14	28	50	35

Table I. Cytogenetic analysis of *Xenopus* metaphase chromosomes. XTC-2 cells were untreated, treated with caffeine, mitomycin C (MMC) + caffeine or hydroxyurea (HU) + caffeine and metaphase chromosomes were analyzed for chromosomal aberrations.

Materials and Methods

Isolation of *Xenopus laevis* FA gene homologs

Tblastn-based searches were performed at NCBI/JGI as previously described (Blom et al., 2002). Several short homologous protein fragments were identified using a tblastn search with full-length human FANCD2 in a *Xenopus tropicalis* database (JGI). Primers designed from these sequences were used in an RT-PCR to amplify a central region of FANCD2 from a *Xenopus laevis* testis mRNA. The amplified fragment was sequenced and primers were designed for 3' and 5' RACE PCRs. Full-length *Xenopus laevis* FANCD2 (xFANCD2) was amplified using primers; 5'-ATGGTGGCCAAAAGAAAGCTG and 5'-TTAATCTGAATCTTCATCCTCATT.
To isolate *Xenopus laevis FANCA*, a tblastn search was performed with full-length human *FANCA* in the *Xenopus laevis* EST database (NCBI). Four image clones were found and specific primers were designed to PCR-amplify a partial 3' sequence of *FANCA* from *Xenopus laevis* testis tissue. An additional tblastn search of the Sanger *Xenopus tropicalis* database identifed a *Xenopus tropicalis* sequence homologous to the 3' end of human *FANCA*. Primers were designed from these sequences and a PCR was performed to obtain the 5' region of *Xenopus laevis FANCA (xFANCA*). Subsequently,

full-length *xFANCA* was amplified by RT-PCR using primers; 5'-ATGTCCGCTGTGTCAGGGT and 5'-TTAAAAGACAGGCTCAAAGAAC.
A *Xenopus laevis* image clone containing a highly similar sequence to full length human *FANCL* (including extra 5' and 3' flanking sequences) was found at NCBI Genbank (BJ095024). The clone was obtained and full length *xFANCL* was sequenced and submitted to NCBI with accession number AY633666. The *xFANCF* image clone has been previously reported (Leveille et al., 2004)
The full length nucleotide sequence for *xFANCA*, and *xFANCD2*, were submitted to the NCBI Genbank with accession numbers; AY633664, and AY633665, respectively.

Cloning and sequencing of *Xenopus* cDNAs

For cloning of *xFA* cDNAs, a Flag-tagged destination vector (pDEST-Flag/pCEP) was created using the conversion kit for Gateway (Invitrogen) according to the manufacturer's instructions. PCRs were performed with the primers for full-length *xFANCA*, *xFANCD2*, *xFANCF*, and *xFANCL* using "Elongase" DNA polymerase (Invitrogen). PCR products were ligated (via BP reaction) into the pDONR201 vector of the Gateway Cloning System (Invitrogen) according to the manufacturer's instructions. Subsequently, an LR reaction was performed to create three destination vectors; GST (pDEST 15), HIS (pDEST 17), and Flag (pDEST-Flag/pCEP) each for *xFANCA, xFANCD2, xFANCF*, and *xFANCL*.

Cell Culture

The Xenopus tadpole cell line XTC-2 (Pudney et al., 1973) was cultured at room temperature without CO^2 in Leibovitz's L-15 media containing 10% fetal bovine serum. The human 293 EBNA cell line was cultured in Dulbecco's modified eagle medium with 10% fetal bovine serum and transfected with lipofectamine (Invitrogen) according to manufacturer's specifications.

Immunoprecipitation

To immunoprecipitate FA proteins, 2 mg of egg extract was added to 1 ml of lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1% NP40, 0.5% Deoxycholate, 1mM EDTA, 0.5

mg/ml Pefabloc, 1mM DTT). 10 µl of rabbit polyclonal antibody against xFANCA, or xFANCF, were added and samples were mixed by rotating overnight at 4ºC. Then 100 µl of pre-swelled and washed (50% slurry in PBS) sepharose 4B beads (Amersham) were added and rotated for 30 minutes at 4 °C. The beads were pelleted from solution by centrifugation at 2500 rpm for 10 minutes at 4ºC and washed three times with lysis buffer. Beads were mixed with 50 µl 4X SDS-page sample buffer and boiled. 10 µl of each sample was analyzed by SDS-PAGE and immunoblot.

Cytogenetic analysis

Xenopus XTC-2 cells were cultured to approx. 70 % confluence and then incubated for 24 hours in untreated media or media containing either 4mM caffeine (to override MMC-induced S-phase arrest), 4 mM caffeine + 150 nM Mitomycin C (MMC), or 4 mM caffeine + 2 mM hydroxyurea (HU). Next, the cells were exposed to colcemid (10 µg/ml) for 2 hours, collected by trypsinization and exposed to 75 mM KCl for 20 minutes. Cells were washed 3 times and finally fixed onto glass slides in methanol: acetic acid (3:1). Slides were stained with Wright's stain and up to 50 metaphases were evaluated for chromosomal aberrations as previously described (Joenje et al., 1981).

Literature

Blom, E., H.J. van de Vrugt, J.P. de Winter, F. Arwert, and H. Joenje. 2002. Evolutionary clues to the molecular function of fanconi anemia genes. *Acta Haematol*. 108:231-6.

Garcia-Higuera, I., T. Taniguchi, S. Ganesan, M.S. Meyn, C. Timmers, J. Hejna, M. Grompe, and A.D. D'Andrea. 2001. Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol Cell*. 7:249-62.

Joenje, H., F. Arwert, A.W. Eriksson, H. de Koning, and A.B. Oostra. 1981. Oxygen-dependence of chromosomal aberrations in Fanconi's anaemia. *Nature*. 290:142-3.

Leveille, F., E. Blom, A.L. Medhurst, P. Bier, H. Laghmani el, M. Johnson, M.A. Rooimans, A. Sobeck, Q. Waisfisz, F. Arwert, K.J. Patel, M.E. Hoatlin, H. Joenje, and J.P. de Winter. 2004. The Fanconi anemia gene product FANCF is a flexible adaptor protein. *J Biol Chem*. 279:39421-30.

Meetei, A.R., J.P. De Winter, A.L. Medhurst, M. Wallisch, Q. Waisfisz, H.J. Van De Vrugt, A.B. Oostra, Z. Yan, C. Ling, C.E. Bishop, M.E. Hoatlin, H. Joenje, and W. Wang. 2003. A novel ubiquitin ligase is deficient in Fanconi anemia. *Nat Genet*. 35:165-170.

Meetei, A.R., Z. Yan, and W. Wang. 2004. FANCL Replaces BRCA1 as the Likely Ubiquitin Ligase Responsible for FANCD2 Monoubiquitination. *Cell Cycle*. 3:179-81.

Otsuki, T., T. Nagashima, N. Komatsu, K. Kirito, Y. Furukawa, S. Kobayashi Si, J.M. Liu, and K. Ozawa. 2002. Phosphorylation of Fanconi anemia protein, FANCA, is regulated by Akt kinase. *Biochem Biophys Res Commun*. 291:628-34.

Pawson, T. 1995. Protein modules and signalling networks. *Nature*. 373:573-80.

Pudney, M., M.G. Varma, and C.J. Leake. 1973. Establishment of a cell line (XTC-2) from the South African clawed toad, Xenopus laevis. *Experientia*. 29:466-7.

van de Vrugt, H.J., N.C. Cheng, Y. de Vries, M.A. Rooimans, J. de Groot, R.J. Scheper, Y. Zhi, M.E. Hoatlin, H. Joenje, and F. Arwert. 2000. Cloning and characterization of murine fanconi anemia group A gene: Fanca protein is expressed in lymphoid tissues, testis, and ovary. *Mamm Genome*. 11:326-31.

Yamashita, T., G.M. Kupfer, D. Naf, A. Suliman, H. Joenje, S. Asano, and A.D. D'Andrea. 1998. The fanconi anemia pathway requires FAA phosphorylation and FAA/FAC nuclear accumulation. *Proc Natl Acad Sci U S A*. 95:13085-90.