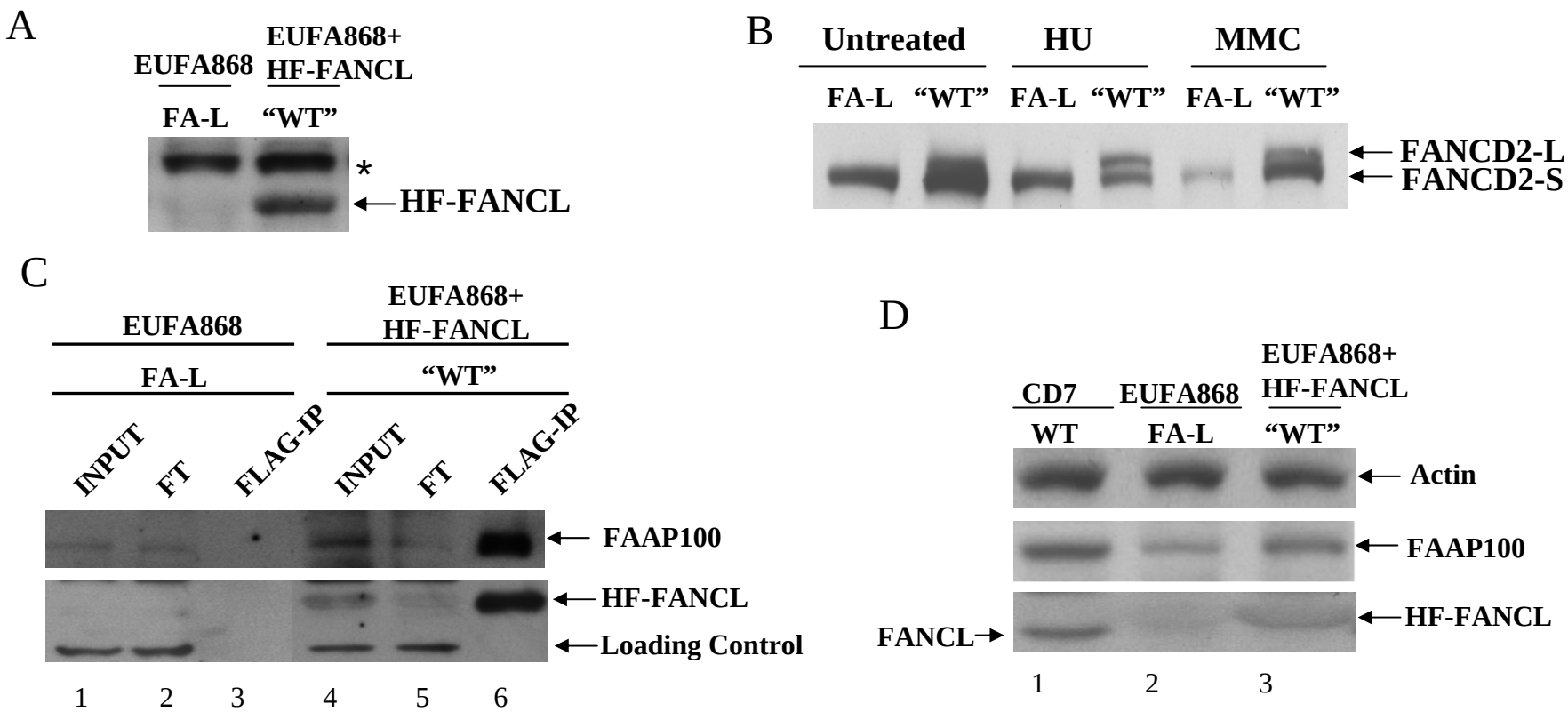
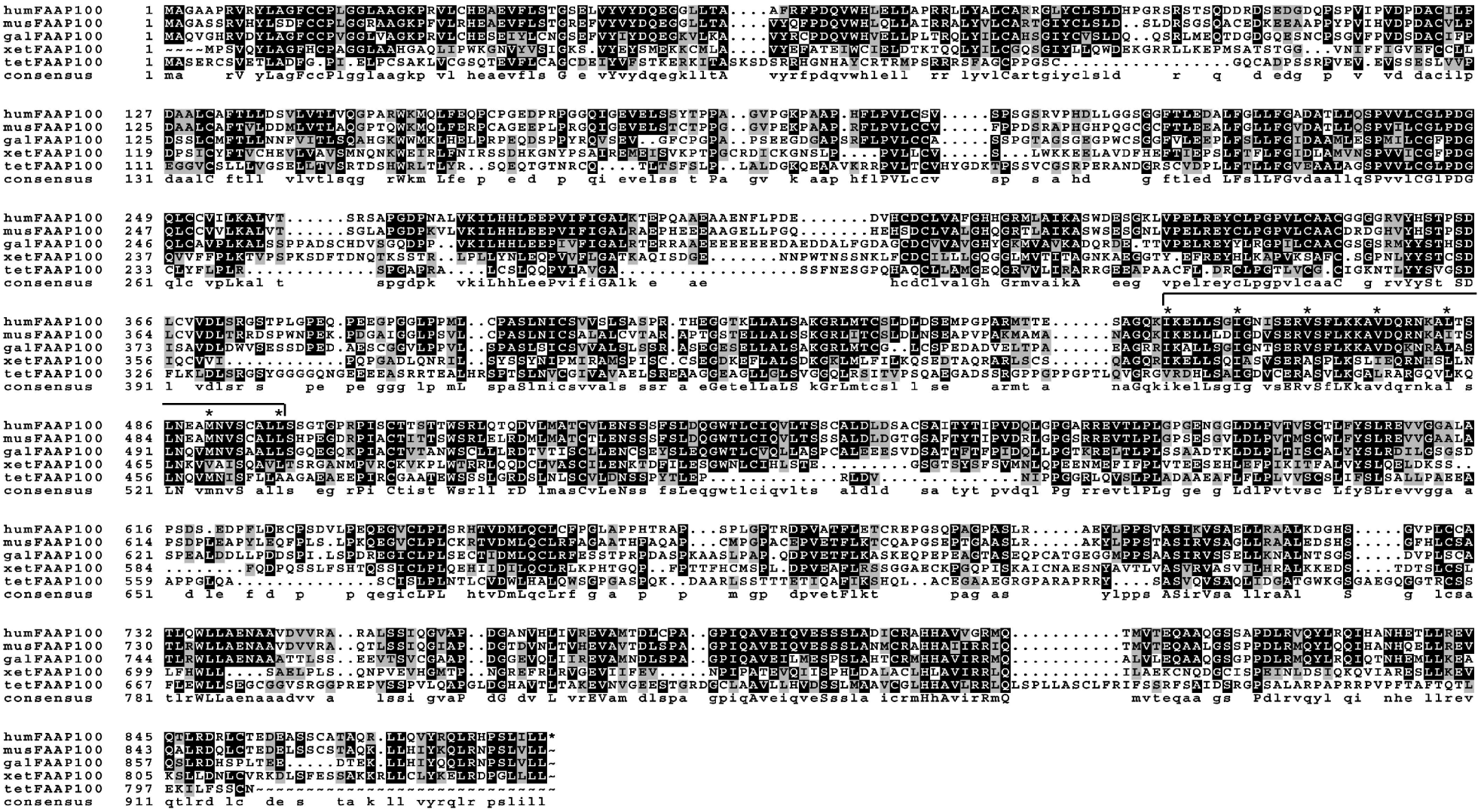
# HF-FANCL complemented FA-L patient cells and co-immunoprecipitates with FAAP100; FAAP100 protein level is reduced in FANCL-deficient cells



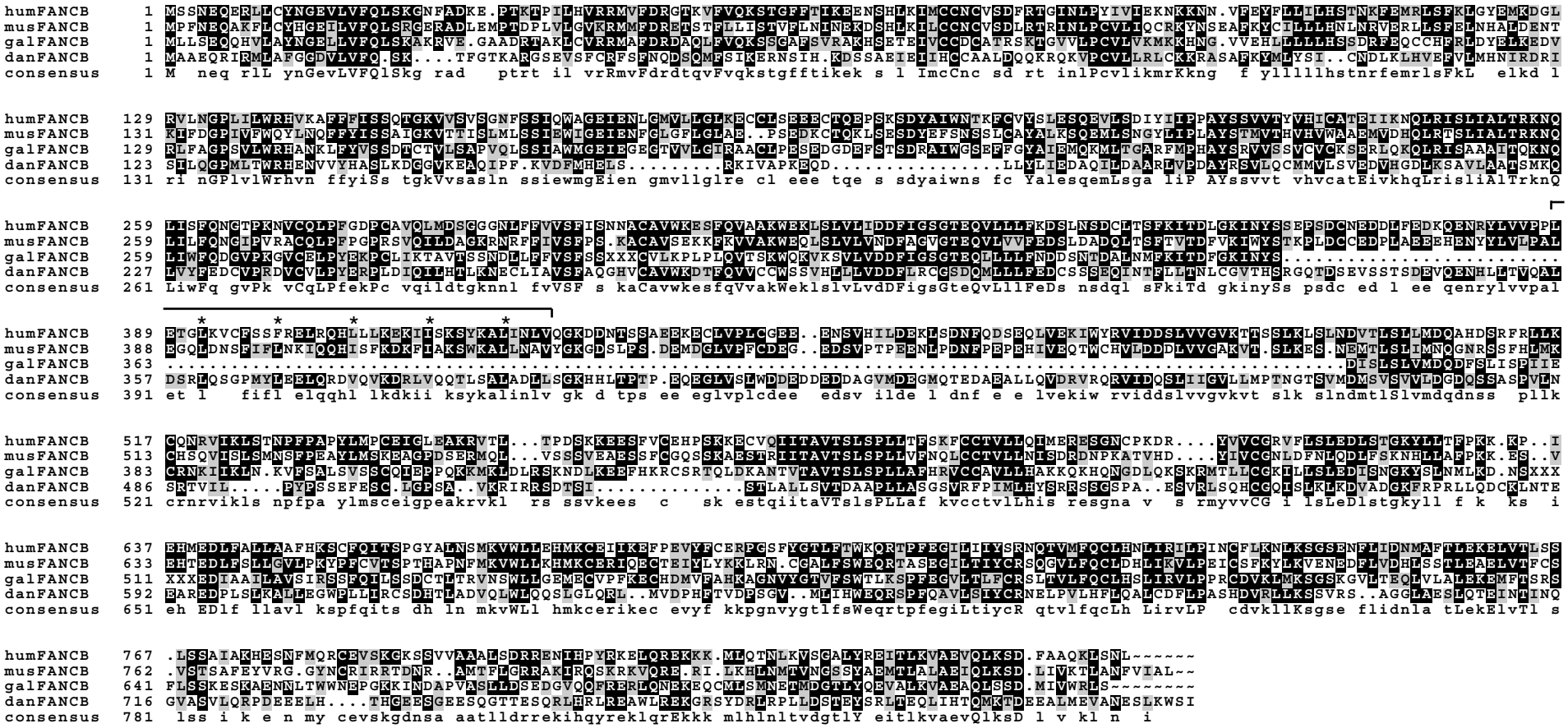

**Legend.** **(**A**)** Immunoblotting showing expression of FANCL tagged with 6xhistidine and a FLAG epitope (HF-FANCL) in FA-L cells after retroviral transduction. A crossreactive band (*) can be used as a loading control. (B) Immunoblotting shows that FANCD2 monoubiquitination (FANCD2-L) was corrected after FA-L cells were transduced by HF-FANCL. Cells were treated with hydroxyurea (HU), mitomycin C (MMC), or left untreated. (C) Immunoblotting shows that majority of FAAP100 co-immunoprecipitated with HF-FANCL, as evidenced by strong reduction of HF-FANCL in the flowthrough (FT) after immunoprecipitation (IP). (D) FAAP100 protein level is reduced in total lysate from the FA-L cells. After the same cell line was corrected by expression of HF-FANCL, the level of FAAP100 is increased.

# FAAP100 contains a potential coiled-coil domain and is highly conserved in Vertebrates



Legend: The alignment of FAAP100 sequences from multiple vertebrate species include: human (hum; DQ989324), mouse (mus; NP_082256), chicken (gal; DDBJ accession number AB270761), Xenopus tropicalis (xet; JGI scaffold_560), and Tetraodon nigroviridis (tet; CAG04431). The bracket indicates the coiled-coil predicted by the STABLECOIL program. The positions of the hydrophobic residues in the heptad repeats are indicated with asterisks.

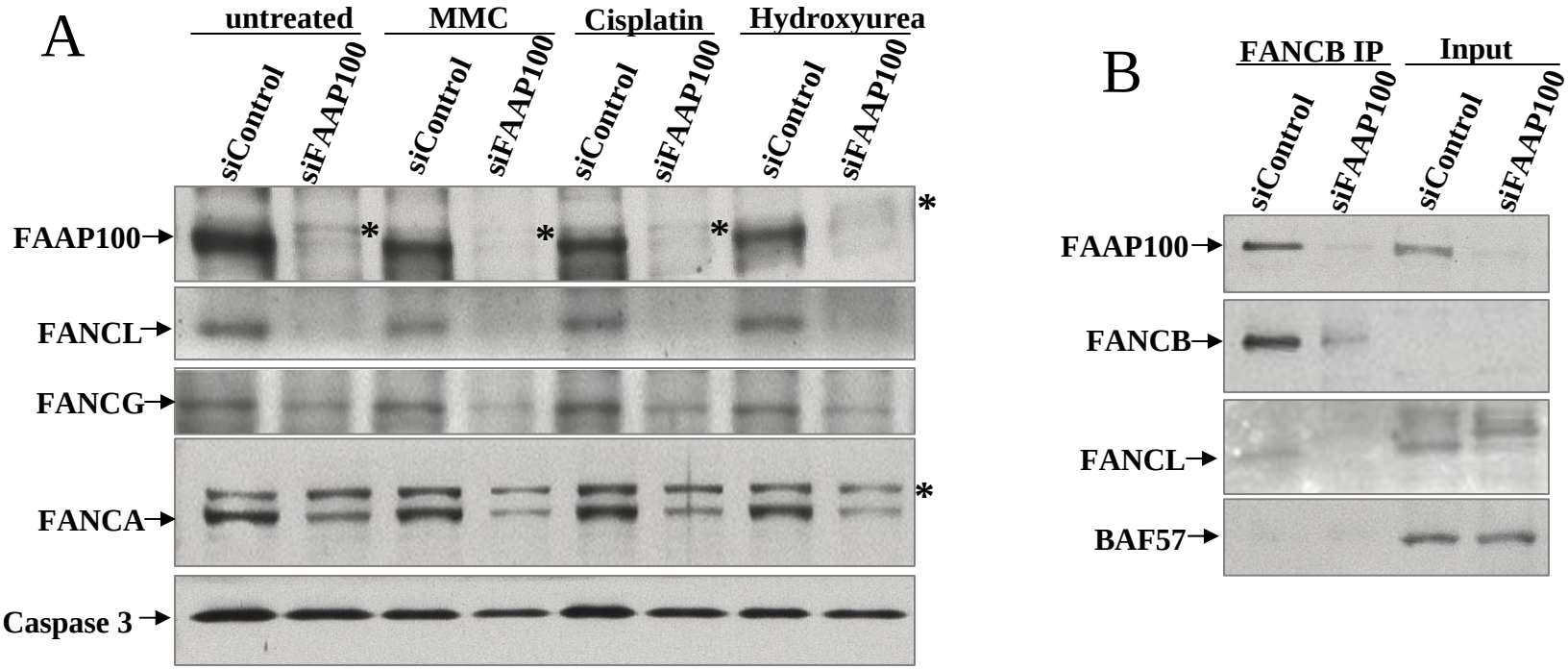
# FANCB contains a putative coiled-coil domain



Legend: The alignment of FANCB sequences from multiple vertebrate species include: human (hum), mouse (mus), chicken (gal), and zebra fish (dan) The bracket indicates the coiled-coil predicted by the STABLECOIL program. The positions of the hydrophobic residues in the heptadrepeats are indicated with asterisks. The chicken sequence is likely missing some exon sequences.

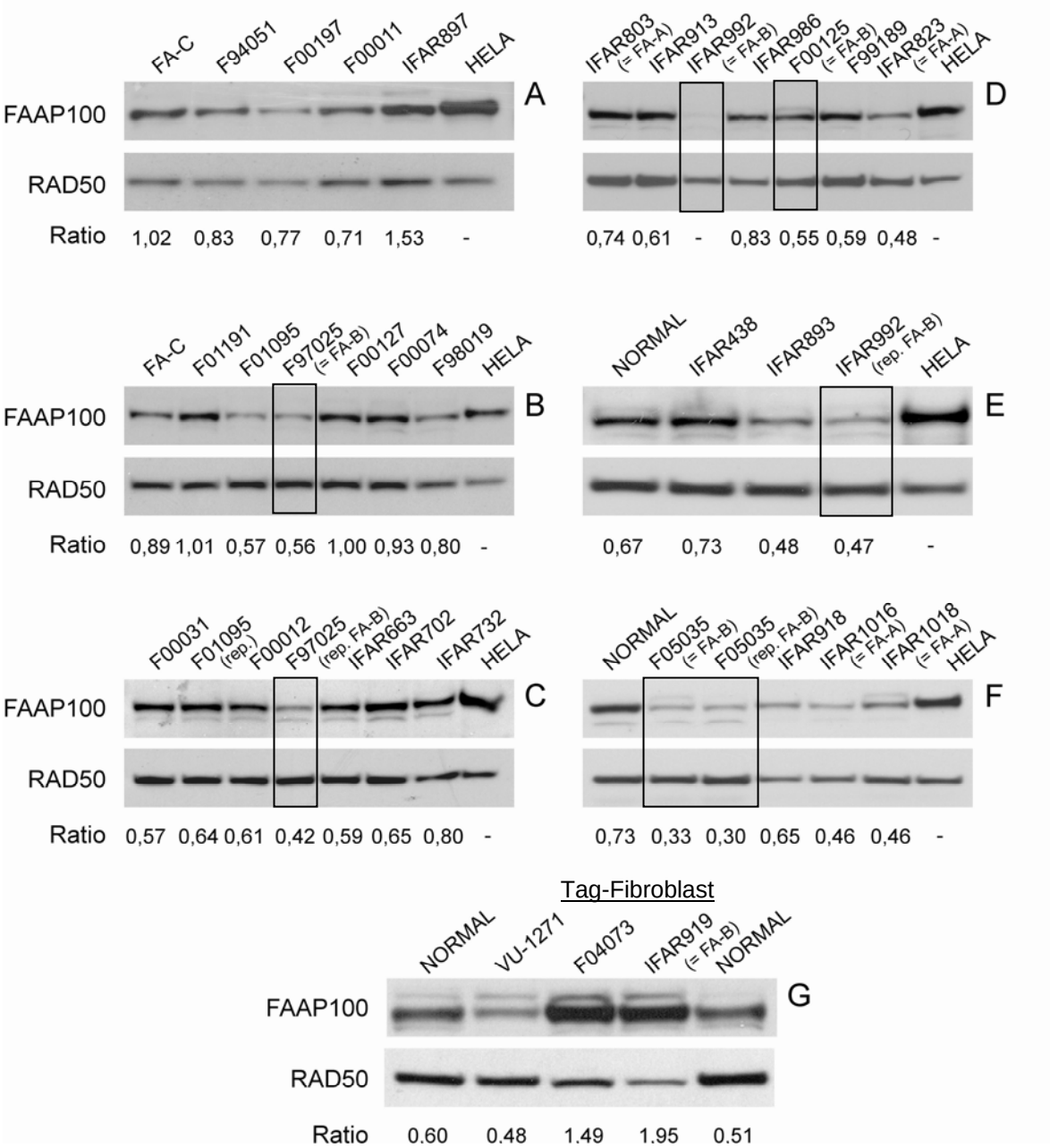
Supplementary Figure 4

# Depletion of FAAP100 by On-Targetplus SMARTpool siRNAs decreases the stability of the Fanconi anemia Core complex



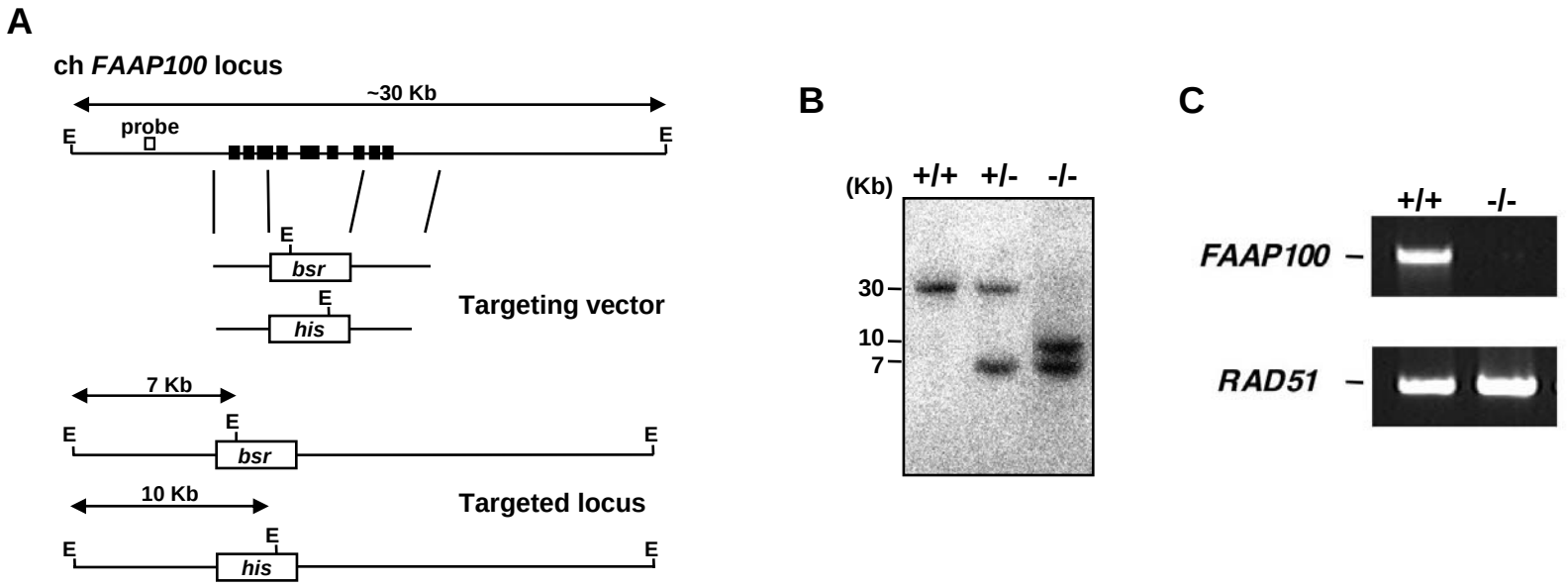

**Legend.** (A) Immunoblotting shows that HeLa cells depleted of FAAP100 by On-Targetplus SMARTpool siRNAs (siFAAP100) decreased stability of several components of the FA core complex. The siRNA and the control (siControl) were from Dharmacon, which are designed by bioinformatics tools and chemically modified to significantly reduce the off-target effects. Note that the FAAP100 level in depleted cells is similar to that of the nonspecific polypeptide (marked by an asterisk), indicating that its level is near the detection limit of the antibody. The image of FAAP100 depletion (top) and the loading control (caspase 3) were reproduced from Figure 3D for comparison purpose. (B) Immunoprecipitation-coupled immunoblotting shows co-immunoprecipitation of FANCB and FAAP100 from lysate of HeLa cells depleted of FAAP100, but their levels are reduced compared to cells treated with control siRNAs. The level of FANCL that co-immunoprecipitated with FANCB was similarly reduced and beyond the detection limit of our antibody. The level of FANCB in this lysate (input) was very low, so that it cannot be detected by direct immunoblotting analysis (Meetei et al. 2004). BAF57 is included as a loading control.

# Screening of FA patients failed to identify individuals with FAAP100 mutation; and FAAP100 protein level is generally lower in FA-B patient-derived lymphoblastoid cell lines




Legend: immunoblots from 31 initially unassigned Fanconi anemia lymphoblast and fibroblast cell lines. Designations are: NORMAL, normal control; FA-C, Fanconi anemia group C control; HELA, HeLa cell nuclear extract; rep., repeated; F, University of Wurzburg; IFAR, International Fanconi Anemia Registry, VU Free University of Amsterdam. Lines with F, IFAR or VU numbers only have remained unassigned; those with numbers and FA designations in parentheses were assigned later in the course of the study. RAD50 was used as the loading control. The densitometrical ratios FAAP100/RAD50 are comparable only within one and the same blot and are not valid for HELA. Samples from FA-B patient-derived lymphoblastoid lines are indicated with boxes. Two SV40 T-antigen (Tag) transformed fibroblast lines are marked. We found that transformation of primary fibroblasts strongly induce FAAP100 protein expression (data not shown).

# Targeted disruption of *FAAP100* Gene in chicken DT40 cells




**Legend.** (A) Schematic representation of *chFAAP100* locus, the gene targeting constructs, and the configuration of targeted alleles. The black box indicates the positions of exons. E, EcoRI site. (B) Southern blot analysis of EcoRI-digested genomic DNA from cells with indicated genotypes by using flanking probes as shown in panel A. (C) RT-PCR analysis of mRNA expression of FAAP100 or control (RAD51) in wild-type and *faap100* cells.

**Supplementary Note**

**Screening of Fanconi anemia patients failed to identify individuals with FAAP100 mutations.**

**Methods**

*Cell lines.* Peripheral blood lymphocytes or cultured fibroblasts from FA patients were immortalized using EBV or large T-antigen, respectively, to establish the lines used for FA complementation screens. Lines deficient in FANCD2 monoubiquitination (Shimamura et al., 2002) were assayed for complementation with cDNAs corresponding to the proteins of the FA nuclear core complex using retroviral gene transfer (Hanenberg et al., 2002). Lines not primarily assigned to any of the reported FA groups upstream of FA-D2 were included into FAAP100 immunodetection studies.

*FAAP100 immunoblotting and detection.* Cultured lymphoblasts or fibroblasts were harvested and washed in PBS at 4 °C. Whole cell protein extracts were obtained from cell pellets taken up in 50 to 100 µL of lysis buffer (50 mM TRIS, pH 7.4; 150 mM NaCl; 2mM EGTA; 2 mM EDTA; 40 mM NaF; 25 mM glycerol 2-phosphate disodium salt pentahydrate; 0.1 mM trisodium o-vanadate; 0.2% Triton X100; 0.3% NP-40; protease inhibitor cocktail, complete, 1:25, Roche, Mannheim, Germany) for 45 min on ice. Samples containing 50 µg total protein each were prepared with LDS Sample Buffer and Sample Reducing Agent (both Invitrogen, Karlsruhe, Germany), denatured for 2 min at 94 °C, loaded onto 3-8% NuPage Tris-Acetate polyacrylamide gels (Invitrogen) and electrophoresed at 120 V for 4 h at 4 °C in an XCell SureLock Mini-Cell chamber (Invitrogen). Blotting was in an XCell II Blot Module (Invitrogen) at 20 V o/n at 4 °C onto Hybond-P PVDF membranes (Amersham Biosciences, Freiburg, Germany). The latter were blocked with 5% (w/v) skim milk in PBS-T for 1 h at rt and washed with PBS-T three times for 5 min. The membranes were probed with the primary rabbit polyclonal anti-FAAP100 antibody at a concentration of 1:2000 or the mouse monoclonal anti-Rad50 antibody (13B3, GeneTex, San Antonio, CA) at 1:5000 in skim milk/PBS-T for 1 h at rt, again washed three times, exposed to the secondary ECL donkey anti-rabbit IgG horseradish peroxidase-linked whole antibody (GE Healthcare, Little Chaffort, UK) at 1:5000 or for RAD50 to the ECL

sheep anti-mouse IgG horseradish peroxidase-linked $F(ab')_2$ (GE Healthcare) at a concentration of 1:3000 for 1 h at rt and finally washed three times. Detection was by the chemiluminescence technique using the ECL system (Amersham, San Francisco,CA, USA). Intensities of the FAAP100 and RAD50 bands were scanned from X-ray films using a densitometer (Type 2222-020 UltroScan XL Laser Densitometer, LKB, Bromma, Sweden).

*Mutation screen.* Genetic screen for mutations was performed by exon-scanning sequencing of FAAP100 including the nine exons and adjacent intron regions of FAAP100, exons 3 and 5 in two overlapping fragments. The primers and PCR conditions were from Johan de Winter. The reactions were performed with Pfx (exons 1 and 2) and Taq DNA polymerase (Invitrogen). The PCR products were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham, Freiburg, Germany). Automated direct DNA sequencing of the PCR products was by the ABI-PRISM big-dye terminator chemistry (BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1) on an ABI PRISM 310 Genetic Analyzer instrument (Applied Biosystems, Darmstadt, Germany).

**Results**

A total of 31 FA cell lines that were initially unassigned to a FA complementation group and deficient of FANCD2 monoubiquitination were screened for the presence of FAAP100 protein. In the course of the studies, 22 of them remained unassigned, 9 were secondarily assigned including 4 previously unrecognized FA-A lines and 5 lines newly assigned to FA-B after the FA-B gene had been reported. After the first blots, the FAAP100 gene of 2 lines was sequenced, that from IFAR992 because of immunologically markedly reduced FAAP100 and that of F000125 because of a faint extra band running slightly higher than FAAP100. No mutations in the FAAP100 exons or adjacent intron region were detected. However, six polymorphisms were identified. IFAR 992 revealed c.-26T>C (rs 4076968), c.2301(IVS6)+214A>G, c.2419(IVS7)-85A>C (rs 2228698), c.2419(IVS7)-63T>C and c.2505(IVS8)+72C>T, all homo- or hemizygous, and F000125 was heterozygous for the silent base substitution c.402C>T in exon 3 (T134T). Faint extra bands running slightly higher than FAAP100 have irregularly been observed also in a few

other unassigned lines and normal controls and thus were considered unrelated to the FAAP100 status. Faint bands running slightly lower than FAAP100 were thought to be due to partial protein degradation due to freeze-thawing of extracts. Immunoblots of 4 of the unassigned lines with FAAP100 bands that had low ratios to the loading control RAD50 were repeated. In unassigned lines that had previously been conspicuous by poor growth, band strength and ratio recovered to normal while their growth was restored (e.g., F01095). Increasing band strength with better cell growth also held true in lines that turned out to be FA-B in retrospect, but 4 of these 5 FA-B lines with frameshift mutations in *FANCB* retained low ratios in repeated studies of LCLs (F97025, F00125, IFAR992 and F05035). Such did not hold true for the FA-B transformed fibroblast line IFAR919, which is not to be compared to the LCLs. Thus, two conditions were associated with reduced FAAP100 protein levels, poor cell growth and FA-B deficient status. However, none of the unassigned lines showed a total and permanent deficiency of FAAP100.

## Discussion

During the course of this study, about one thousand FA patient derived cell lines have been established in the European Fanconi Anemia Research Consortium cell repository, the International Fanconi Anemia Registry, and other repositories. Two unrelated patients were assigned to FA-L, and two siblings from a single family were assigned to FA-M. Thus, if FAAP100 is a FA gene with similar very low prevalence as FANCL and FANCM, their respective patients may not have been found solely for statistical reasons.

## References

Hanenberg H, Batish SD, Pollok KE, Vieten L, Verlander PC, Leurs C, Cooper RJ, Göttsche K, Haneline L, Clapp DW, Lobitz S, Williams DA, Auerbach AD. Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. Exp Hematol 2002, 30: 410-420.

Shimamura A, de Oca RM, Svenson JL, Haining N, Moreau LA, Nathan DG, D'Andrea AD. A novel diagnostic screen for defects in the Fanconi anemia pathway. Blood 2002; 100: 4649-4654.