Supplemental Materials:

Supplemental Material and Methods

Isolation and culture of KSL cells- Whole bone marrow was isolated from femurs of *Fancc* mutant mice or wild-type controls, and low-density cells obtained by Ficoll gradient separation (Ficoll-Paque PLUS, GE Healthcare Life Sciences, Piscataway, NJ). For Kit+/Sca1+/Lin- (KSL) cells, low-density cells from two mice were pooled and stained with a cocktail of phycoerythrin (PE)-conjugated anti-mouse lineage markers (B220, CD3e, CD4, CD5, CD8a, CD11b, Gr-1, and Ter119), allophycocyanin (APC)-conjugated anti-mouse CD117 (c-Kit), and fluorescein isothiocyanate (FITC)-conjugated Sca-1 in a solution of Hank's buffered salt solution/ 0.5%BSA/10mM HEPES. All antibodies were obtained from BD Biosciences (San Diego, CA). Cells were sorted on a FACSVantage flow cytometer (BD Biosciences) then cultured for 24 hours in RPMI containing 10%FCS, 20 ng/ml IL-6, 10 ng/ml IL-11, 50 ng/ml Flt3L, and 10 ng/ml SCF with or without 10 ng/ml TNF-alpha (all from R&D Systems, Inc. Minneapolis, MN). Experiments were performed in triplicate.

RNA isolation and gene expression profiling- Total RNA was extracted from KSL cells using the RNeasy Micro Kit (QIAGEN, Valencia, CA) and cDNA synthesized and amplified from 5-15 ng RNA using the Ovation V2 kit (NuGEN, San Carlos, CA). Gene expression profiling was performed by the OHSU Affymetrix Microarray Core with GeneChip Mouse Genome 430 2.0 Arrays (39,000 transcripts). Data analysis was performed using GeneSifter (Geospiza, Seattle, WA). CEL files and probe set signals, as well as detailed methods and quality control measures, have been deposited in NCBI's Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo, accession number GSE30318).

Gene expression pattern and ontological analyses- Transcriptomal analysis of KSL cells was conducted on RNA obtained from two genotypes under two conditions; WT cells exposed to growth factors with and without simultaneous exposure to TNF-alpha for 24 hours and *Fancc*-deficient cells exposed to the same two conditions for the same period of culture. There were three biological replicates for each of the four conditions. Seeking genes whose expression was influenced by TNF-alpha in WT but not mutant cells, we used a pattern reading tool (GeneSifter) to apply two filters. The first identified a list of genes induced (>1.2 fold, $p<0.05$, ANOVA) or suppressed (by 0.8 fold) in WT cells by TNF-alpha treatment. The second filter was applied to the first list, seeking only those genes that were not significantly influenced by TNF-alpha in *Fancc*-deficient KSL cells.

In vitro ubiquitination assay- GST tagged fusion proteins were purified as described with minor changes.[1] Ubiquitination assays were performed as described with minor modifications in 20 µl reactions containing 200 nM human recombinant E1 (Boston Biochem, Cambridge, MA), 500 nM E2-conjugating enzyme Ube2t (Boston Biochem), 2 µg of HA-Ub (Boston Biochem), 2 µg purified human GST-beta-catenin, and 2 µg of either wild-type or a ligase-inactive mutant (C307A) purified human GST-FANCL in reaction buffer (50 mM Tris [pH 7.5], 0.5 mM DTT, 25 µM $ZnCl_2$ and 2.5 mM Mg-ATP).[2] Reactions were incubated at 37°C for 2 hours and terminated by adding SDS sample buffer supplemented with 2-mercaptoethanol. Reactions were resolved by SDS-PAGE. The C307A-FANCL mutant was generated by QuickChange II XL (Agilent).

Measurement of protein stability- [^{35}S]-methionine and -cysteine (PerkinElmer, Waltham MA) pulse-chase labeling was performed essentially as described with minor modifications.[3] Cells were pulsed for 30 minutes (methionine- and cysteine-free media supplemented with labeled [^{35}S]-methionine and -cysteine and chased with complete media at the indicated times. Beta-catenin was immunoprecipitated from whole cell extracts and resolved by SDS-PAGE and transferred to PVDF membrane. Signal was detected using xray film and a Typhoon phosphorimager system (GE Healthcare Life Sciences) and quantified by ImageJ.

Chromosome breakage analysis- Cells were treated with 0, 10, or 30 ng/ml of mitomycin C (MMC) for 48 hours. Colcemid (0.05 µg/ml) (Invitrogen) was added for the last 3 hours of MMC incubation. Cells were then removed from flasks, pelleted, treated with 3 ml hypotonic solution (0.075 M KCl, 5% fetal calf serum) for 10 min, and fixed with 3 ml 3:1 methanol:acetic acid. Slides prepared for metaphase spreads were then stained

with 0.2% Wright's stain (Fisher Scientific) for 3.5 min. 50 metaphases for each sample were scored for breaks/cell and radial content on a Nikon Eclipse E800 photoscope. Representative photographs were taken using CytoVision software from Applied Imaging. Sample identification was not revealed to the interpreter until after the data were collected.

List of antibodies for immunoblot analysis and immunoprecipitation studies

Target	Source
Actin	Calbiochem, #CP01, (San Diego, California)
Alexafluor555 goat anti-rabbit	Invitrogen, #A21429, (Carlsbad, CA)
Alpha-tubulin	Sigma-Aldrich, #T6074, (St. Louis, Missouri)
beta-catenin	Santa Cruz Biotechnology, #SC-7199, (Santa Cruz, California)
FANCC	Novus, #NBP-18974, (Littleton, Colorado)
FANCL	Santa Cruz Biotechnology, #SC-137067, (Santa Cruz, California)
FITC conjugated CD34 antibody	Miltenyi, #130-081-001, (Bergisch Gladbach, Germany)
Hemagglutinin	Covance, #MMS101P, (Princeton, NJ)
Protein G plus	Santa Cruz Biotechnology, #SC-2002, (Santa Cruz, California)
Sp1	Millipore, #07-645, (Billerica, Massachusetts)
V5	AbD Serotec, #MCA-1360, (Kidlington, UK)

List of qRT-PCR primers

Target	Forward (5'→3')	Reverse (5'→3')	Reference
AXIN2	GCAAACTTTCGCCAACCGTG	CTCTGGAGCTGTTTCTTACTGCCC	[4]
CTNNB1	TGTTAAATTCTTGGCTATTACGACA	CCACCACTAGCCAGTATGATGA	This study
c-MYC	CTCTCAACGACAGCAGCCCG	CAACATCGATTTCTTCCTCATCTTC	[5]
CCND1	GCGGAGGAGAACAAACAG	TGAGGCGGTAGTAGGACAGG	This study
FANCL	CCATAACATCCCAGAATGCC	CTGTTCCATTTTGTGCCTCC	This study
GUSB	CAGAAGCCCATTATTCAGAGCGAGT	GCACTCTCGTCGGTGACTGT	Stephanie Willis

Supplemental Table S1

Probe Set ID	Gene Name	UG Cluster	Gene ID
1418925_at	Cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, Drosophila)	Mm.22680	*Celsr1*
1439949_at	Glycogen synthase kinase 3 beta (cDNA clone MGC:6814 IMAGE:2648507)	Mm.394930	*Gsk3b*
1447400_at	Receptor-like tyrosine kinase (cDNA clone MGC:40871 IMAGE:5370100)	Mm.335391	*Ryk*
1437393_at	Protein kinase C, alpha	Mm.222178	*Prkca*
1448661_at	Phospholipase C, beta 3 (cDNA clone MGC:18404 IMAGE:4235343)	Mm.273204	*Plcb3*
1423825_at	G protein-coupled receptor 177	Mm.6766	*Gpr177*
1424220_a_at	Porcupine homolog (Drosophila), transcript variant Mporc-d	Mm.443425	*Porcn*
1422486_a_at	MAD homolog 4 (Drosophila)	Mm.100399	*Smad4*
1417301_at	Frizzled homolog 6 (Drosophila) (cDNA clone MGC:14004 IMAGE:3983985)	Mm.4769	*Fzd6*
1430384_at	Groucho-related protein 4	Mm.103638	*Tle4*
1418532_at	Frizzled 2	Mm.36416	*Fzd2*

Table S1. Wnt-pathway related genes differentially expressed in TNF-alpha-exposed KSL cells from wildtype and *Fancc*-deficient mice. Two hundred and nine genes were differentially responsive to TNF-alpha exposure in the pairwise comparison of wild-type (WT) to *Fancc*-deficient KSL cells. The majority (157) were induced by TNF-alpha in WT but not in *Fancc*-deficient KSL cells. In an analysis of the 157 genes using the KEGG pathway analysis tool (GeneSifter, Geospiza, Seattle, WA) the Wnt signaling pathway was the most highly ranked over-represented ontological category (Z = 4.07). Eleven of the 209 genes were related to the Wnt pathway (Supplementary Table 1). Only one of these (GSK3-beta) was induced by TNF-alpha to a greater extent in *Fancc*-deficient KSL cells than in WT cells. The other genes were induced in WT cells but not in *Fancc*-deficient cells, suggesting Wnt pathway dysfunction in *Fancc*-deficient cells.

Supplemental Figure S2

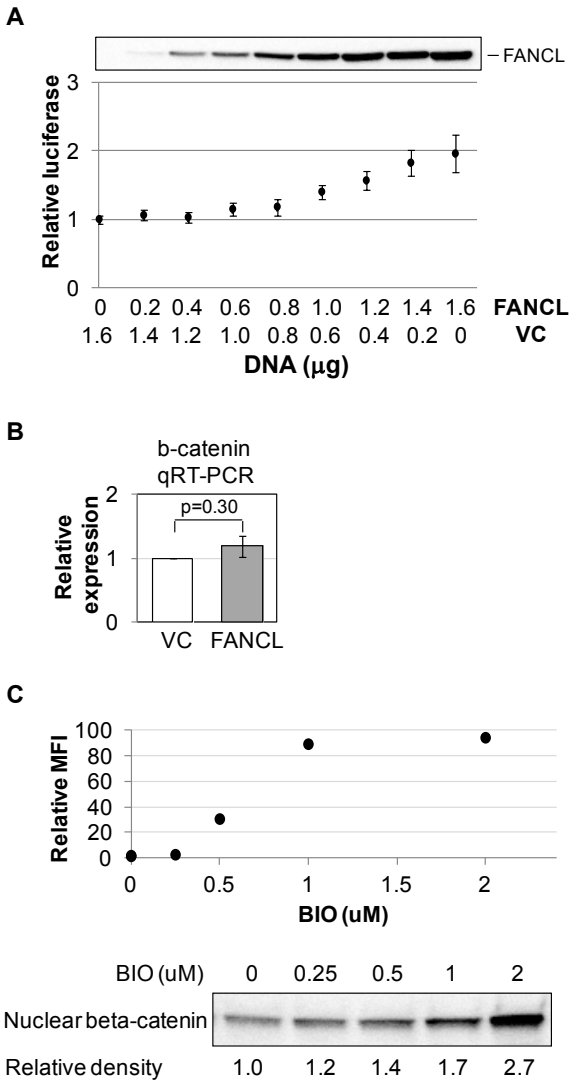

Figure S2. (a) Additional LEF-TCF-luciferase reporter assays were carried out to confirm the specificity that FANCL enhances beta-catenin activity. In these experiments, we increased FANCL expression incrementally using vector-control as the control DNA. Similar results were obtained using *LacZ* as the control DNA (data not shown). Arbitrary luciferase units were normalized to cells transfected with vector-control only. Shown are the average relative luciferase units from four experiments with error bars showing standard deviation. A Western blot is shown to confirm increasing FANCL expression in these assays. (b) No significant difference in beta-catenin mRNA expression was detected by qRT-PCR suggesting that FANCL overexpression regulates beta-catenin at a post-transcriptional level. Error bar represents ±SEM. (c) Changes in nuclear beta-catenin expression were correlated with changes in LEF-TCF-eGFP activity with increasing BIO concentrations.

Supplemental Figure S3

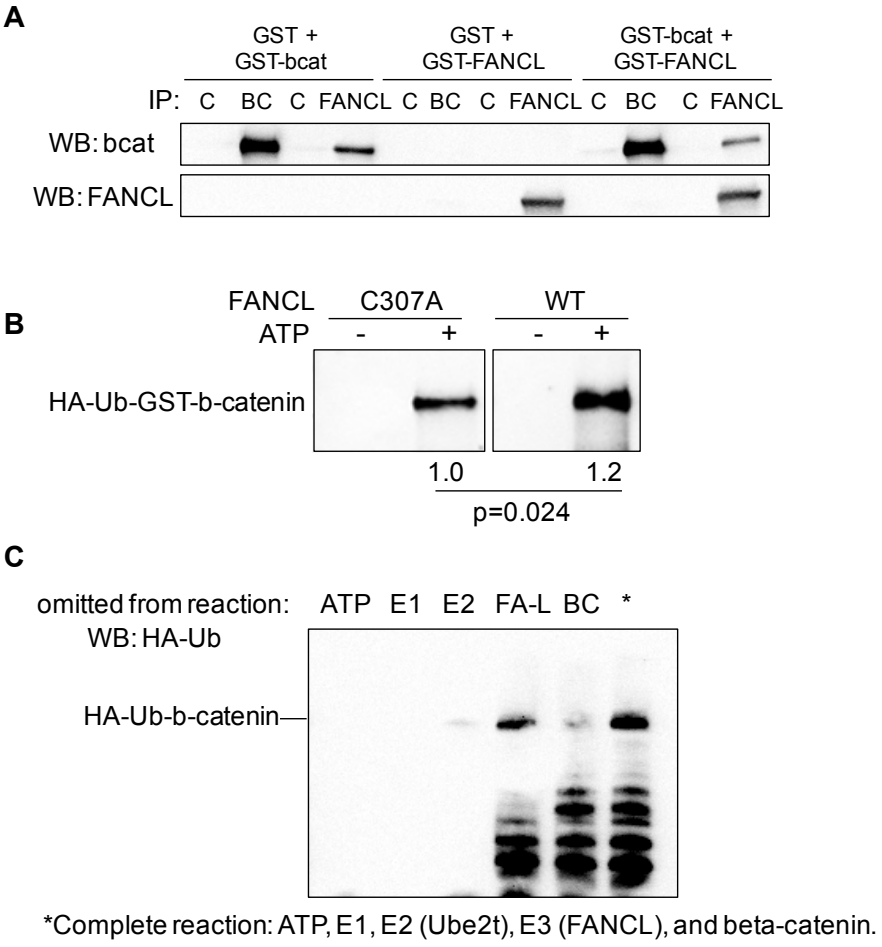

Figure S3. (a) Immunoprecipitation studies using GST fusion proteins. C: isotype control antibody; BC: beta-catenin antibody; FANCL: FANCL antibody. The FANCL antibody pulled down trace amounts of GST-beta-catenin protein non-specifically but there was no difference whether FANCL was included in the reaction or not. No direct interaction between FANCL and beta-catenin was observed. (b) *in vitro* ubiquitination assays were carried out using GST-tagged wild-type (WT) FANCL or a ligase-inactive mutant C307A-FANCL.[6] Shown is a representative experiment with and without ATP in the reaction. In reactions with WT FANCL, we observed increased HA-Ub-beta-catenin above background. Four experiments were performed. (c) There was consistently background level of beta-catenin ubiquitination carried out by the cooperation of E1 and E2 (Ube2t), even in reactions without an E3 ligase. This was determined by carrying out reactions with one component missing, as indicated. The lane labeled with the (*) is a complete reaction containing all components.

Supplemental Figure S4

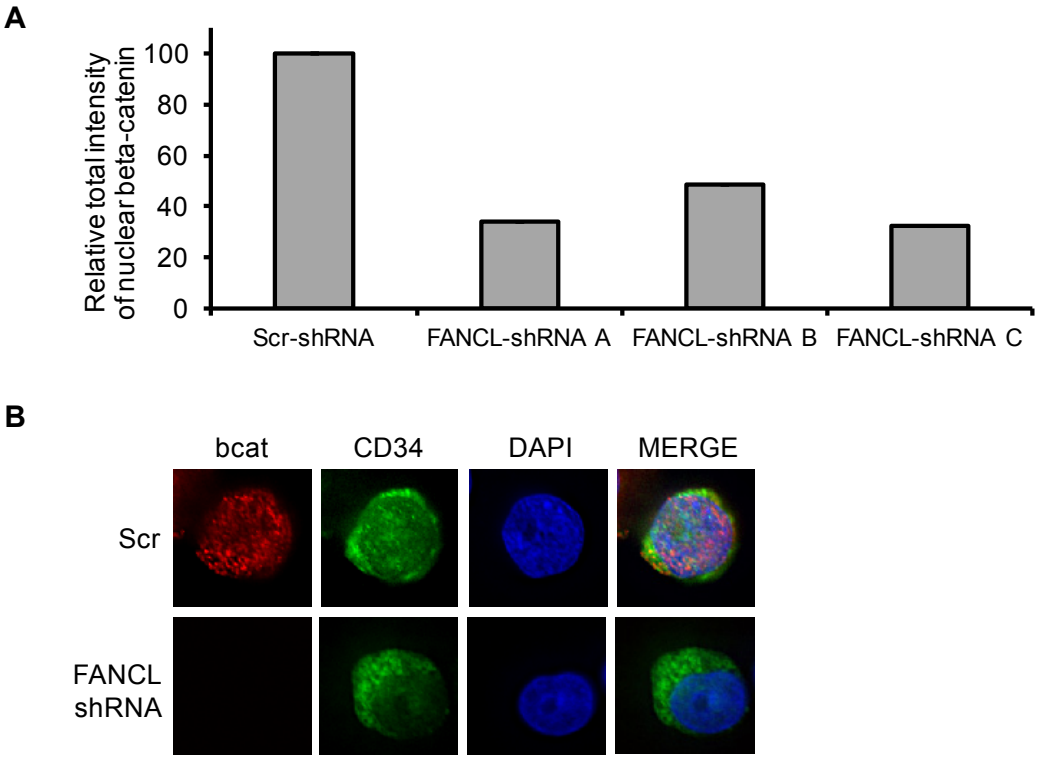

Figure S4. (a) Immunofluorescence staining [beta-catenin (TRITC), CD34 (FITC), and DAPI (blue)] of fixed cells was carried out to quantify beta-catenin expression in CD34+ cord blood stem and progenitor cells transduced with scrambled (Scr) or FANCL shRNA. The data shown are from one experiment quantified by MetaMorph software indicating total nuclear staining of beta-catenin in CD34+ cells. (b) Images showing a CD34+ cell expressing beta-catenin in cells transduced with a scrambled shRNA control (top row) versus a cell not expressing beta-catenin in cells transduced with FANCL shRNA construct C (bottom row). However, there was a wide spectrum of staining intensities.

References for Supplemental Materials

1. Einarson MB, Pugacheva EN, Orlinick JR. Preparation of GST Fusion Proteins. *CSH Protoc.* 2007;2007:pdb prot4738.

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3. Briggs MW, Li Z, Sacks DB. IQGAP1-mediated stimulation of transcriptional co-activation by beta-catenin is modulated by calmodulin. *J Biol Chem.* Mar 1 2002;277(9):7453-7465.

4. Dehner M, Hadjihannas M, Weiske J, Huber O, Behrens J. Wnt signaling inhibits Forkhead box O3a-induced transcription and apoptosis through up-regulation of serum- and glucocorticoid-inducible kinase 1. *J Biol Chem.* Jul 11 2008;283(28):19201-19210.

5. Ou CY, Kim JH, Yang CK, Stallcup MR. Requirement of cell cycle and apoptosis regulator 1 for target gene activation by Wnt and beta-catenin and for anchorage-independent growth of human colon carcinoma cells. *J Biol Chem.* Jul 31 2009;284(31):20629-20637.

6. Machida YJ, Machida Y, Chen Y, et al. UBE2T is the E2 in the Fanconi anemia pathway and undergoes negative autoregulation. *Mol Cell.* Aug 2006;23(4):589-596.