Fanconi anemia signaling network regulates spindle assembly checkpoint

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*- equal contribution

Supplemental Information

Supplemental Table. Mutations found in the FA patient-derived primary fibroblasts used in this work.

FA gene	Mutation 1	Mutation 2	Reference
FANCA	c.856C>T (p.Q286X)	c.3976C>T (p.Q1326X)	Gross et al 2002(1)
FANCA	c.3163C>T (p.1055W)	c.4124-4125 delCA (p.T1375fsX1423)	This study (Bai)
FANCB	Dup chrX: c.14788000-14797000 (ex2+3)		Chandrasekharappa et al (2)
FANCC	c.377_378delGA, p.R126fsX127	c.377_378delGA, p.R126fsX127	This study (Bai)
FANCE	c.1111 C>T (p.R371W)	c.1111 C>T (p.R371W)	Neveling 2007(3)
FANCF	c.349-395del47	c.16C>T (p.Q6X)	De Winter et al 2000(4)
FANCG	c.313 G>T (p.E105X)	IVS 9+1 G>A	Demuth et al 2000(5)
FANCL	c.920G>A (p.C307Y)	c.217-20T>G	This study (Schindler)
FANCD2	c.1948-16TrG (ex22 skipping)	c. 2775_2776CC>TT (p.R926X)	Kalb et al 2007(6)
FANCI	c.3853C>G (p.R1285G)	c.3853C>G (p.R1285G)	Scheckenbach et al (7)
FANCD1/BRCA2	c.706-15 del10	c.706-15 del10	Rischewski et al 2002(8)
FANCJ/BRIP1	c.2533C>T (p.R798X)	80037A>T	Levran et al 2005(9)
FANCN/PALB2	c.2393_2394insCT, p.T799fs	c.3350+4A>G, r.3350insGCAG/p.F1118fs	Reid et al 2007(10)

Supplemental References

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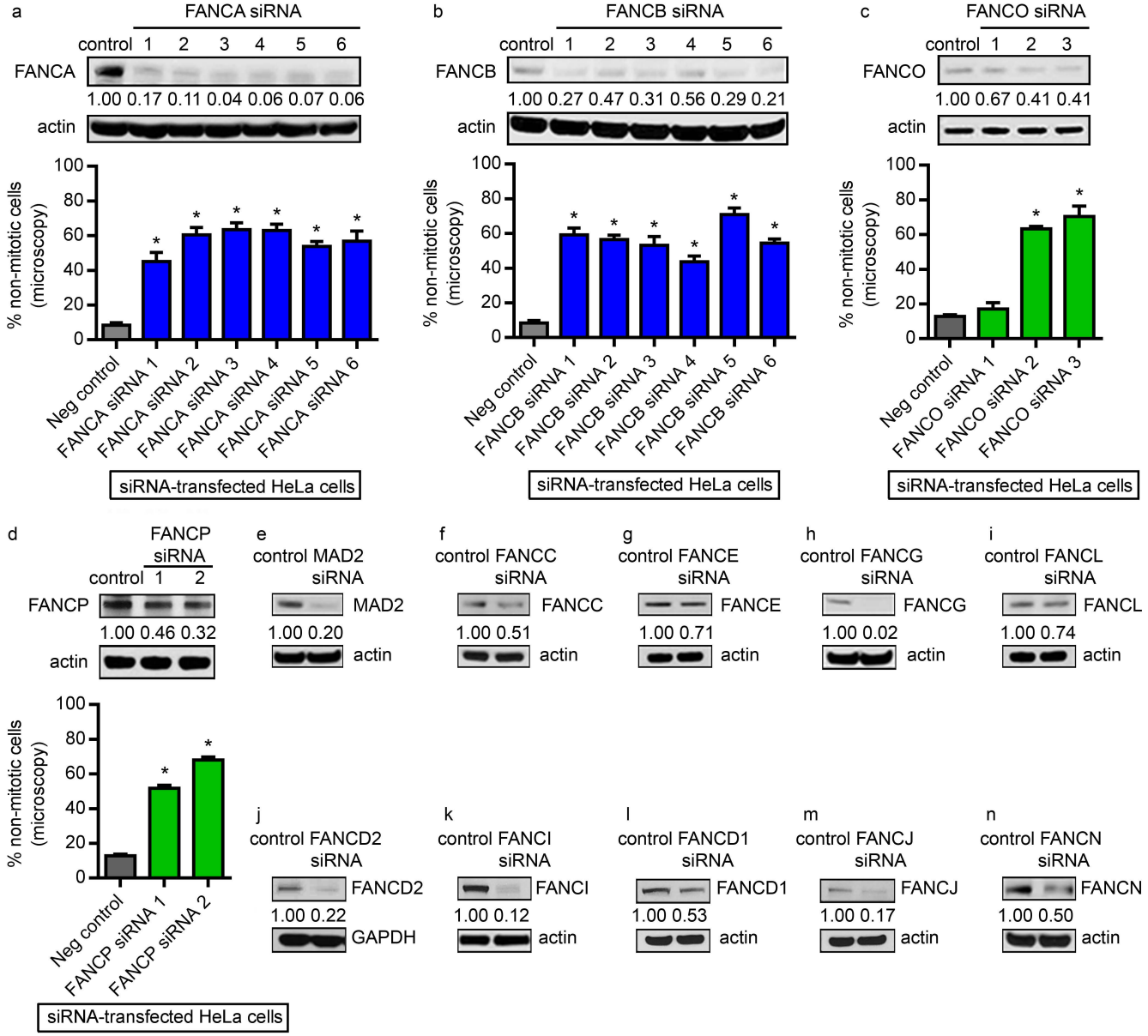
Nalepa, Enzor et al Supplemental Figure 1

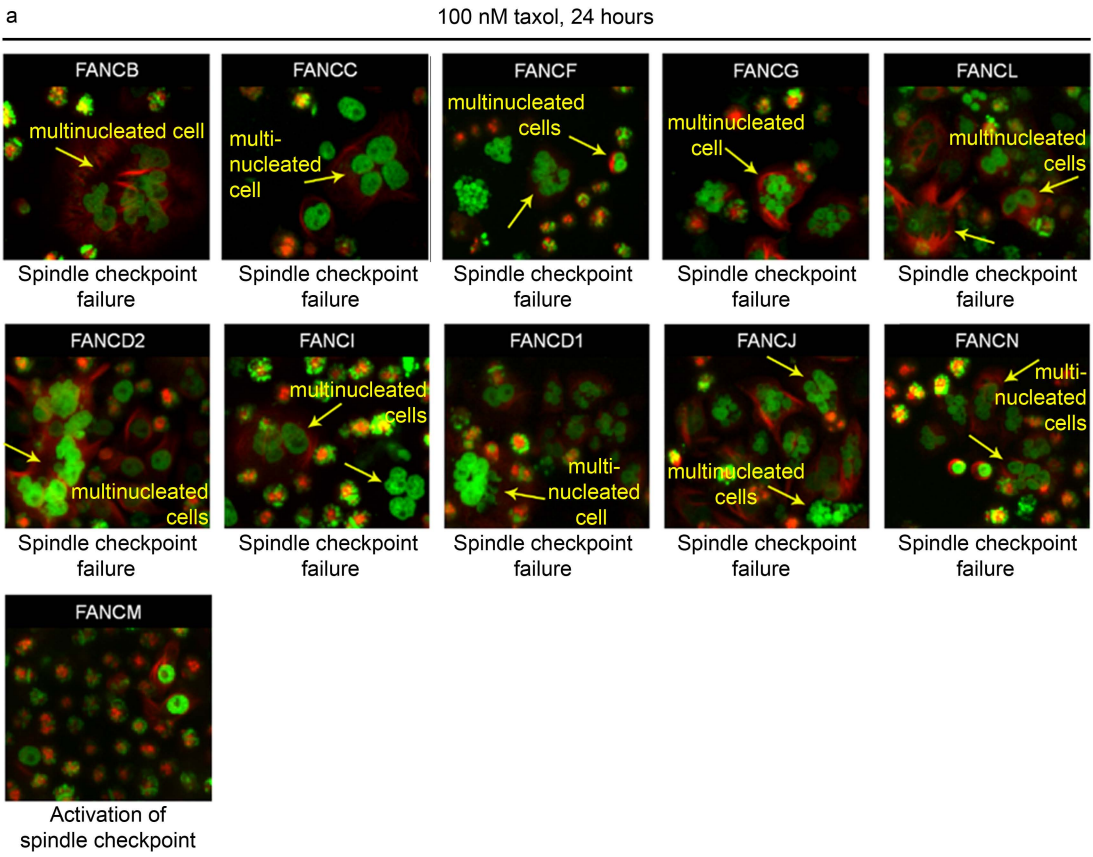


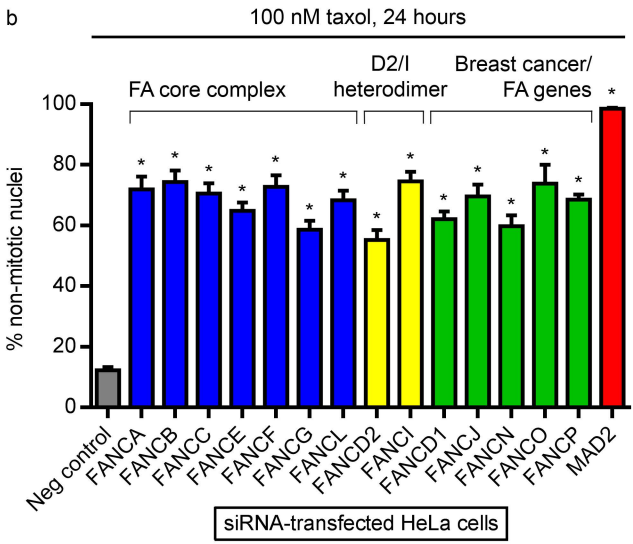
Verification of target protein knockdown by siRNAs used in the FA RNAi spindle checkpoint screen.

(a-n) Western blotting verification of target protein knockdown by indicated siRNAs. Transfection with 6/6 FANCA siRNAs **(a)**, 6/6 FANCB siRNAs **(b)**, 2/3 FANCO siRNAs **(c)**, and 2/3 FANCP siRNAs **(d)** led to target FA protein knockdown that correlated with the spindle checkpoint failure induced by respective siRNAs (shown by bar graphs underneath each Western blot image). In **a-d**, the SAC phenotype was quantified by nuclear counts as described in Fig. 1; at least three independent counts were performed for each siRNA shown. Asterisks indicate $p<0.0001$ (one-way ANOVA with Bonferroni's post-hoc multiple comparison test), and error bars show SEM.

MAD2 protein knockdown by positive control siRNA is shown in **(e)**. Numbers on Western blots indicate band intensities normalized to target band intensity in cells transfected with negative control siRNA. Actin or GAPDH was used as a loading control.

Nalepa, Enzor et al. Supplemental Figure 2



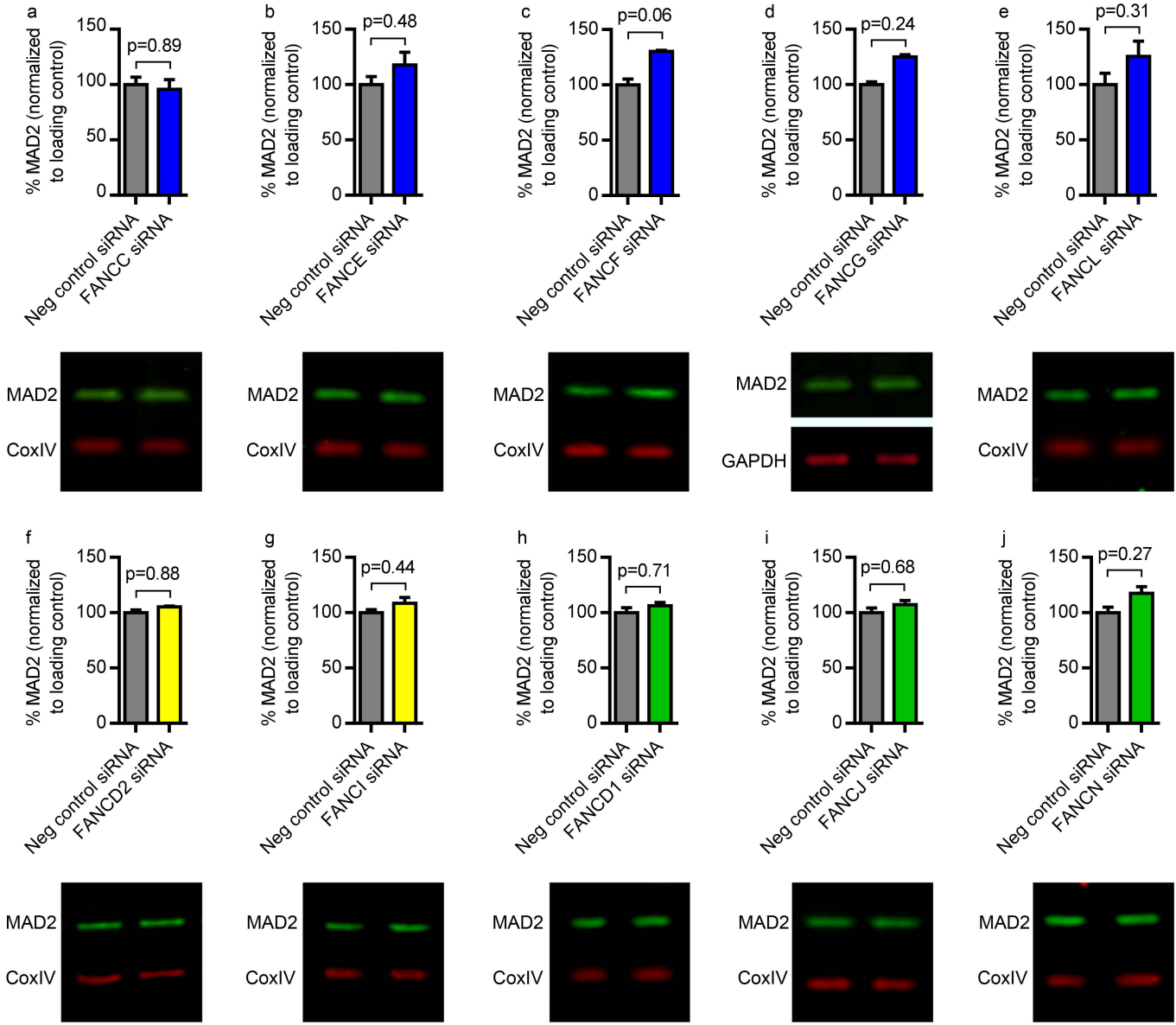


RNAi-mediated knockdown of FA pathway genes disrupts the spindle assembly checkpoint.

(a) Additional microscopy images showing taxol-induced multinucleation in cells transfected with indicated siRNAs against the FA network genes. Note that all FA siRNAs except for FANCM siRNA disrupt the SAC, as shown by generation of multinucleated cells in the presence of taxol. Magnification 200x (BD Pathway 855).

(b) Quantification of non-mitotic nuclei in cells transfected with indicated siRNAs and treated with taxol 48 hours post-transfection as indicated. Asterisks denote $p<0.0001$ (one-way ANOVA with post-hoc Bonferroni's multiple comparison test). n=9 counts per siRNA. All bars represent mean values +/− SEM.

Nalepa, Enzor et al. Supplemental Figure 3

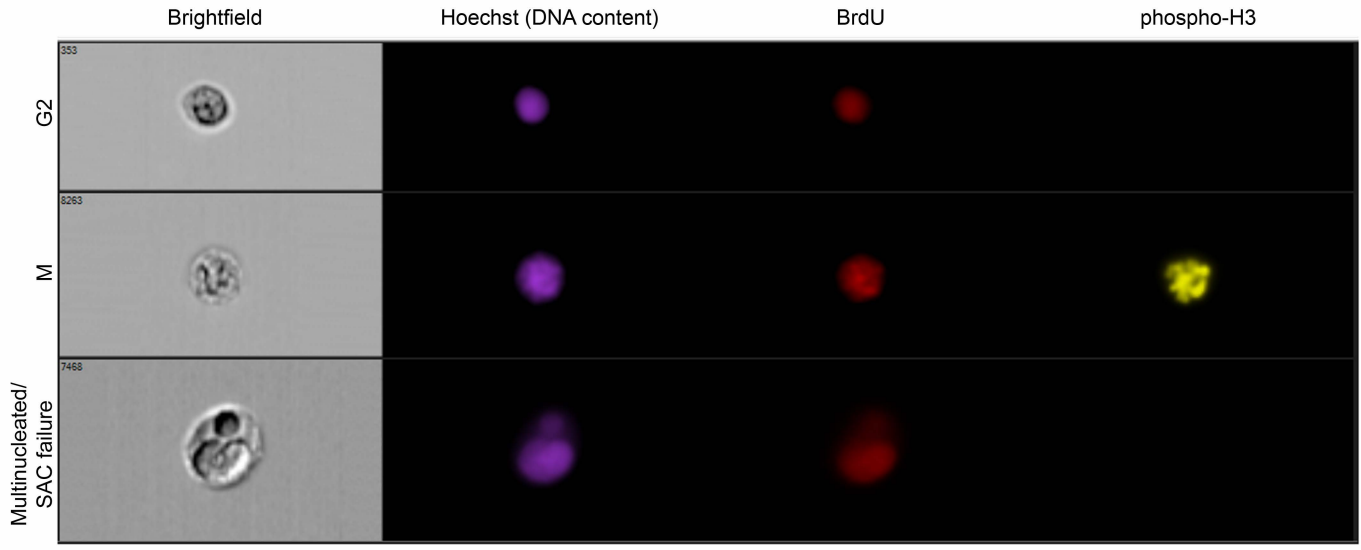


Verification that MAD2 is not non-specifically knocked down by siRNAs used in the FA RNAi spindle checkpoint screen.

(a-j) Western blotting verification that MAD2 is not non-specifically knocked down by the indicated FA siRNAs. In **a-j**, the level of MAD2 after transfection with the respective FA siRNA is not significantly different from the level of MAD2 after transfection with negative control siRNA ($p>0.05$ by two-tailed t-test). Three independent transfections and immunoblots were performed for each siRNA, and error bars show SEM.

Quantitative Western blots were performed using a LI-COR Odyssey CLx imager. MAD2 target band intensities were normalized to loading control and scaled so that the MAD2 intensity for negative control siRNA-transfected cells was 100%. CoxIV or GAPDH was used as a loading control.

Nalepa, Enzor et al. Supplemental Figure 4

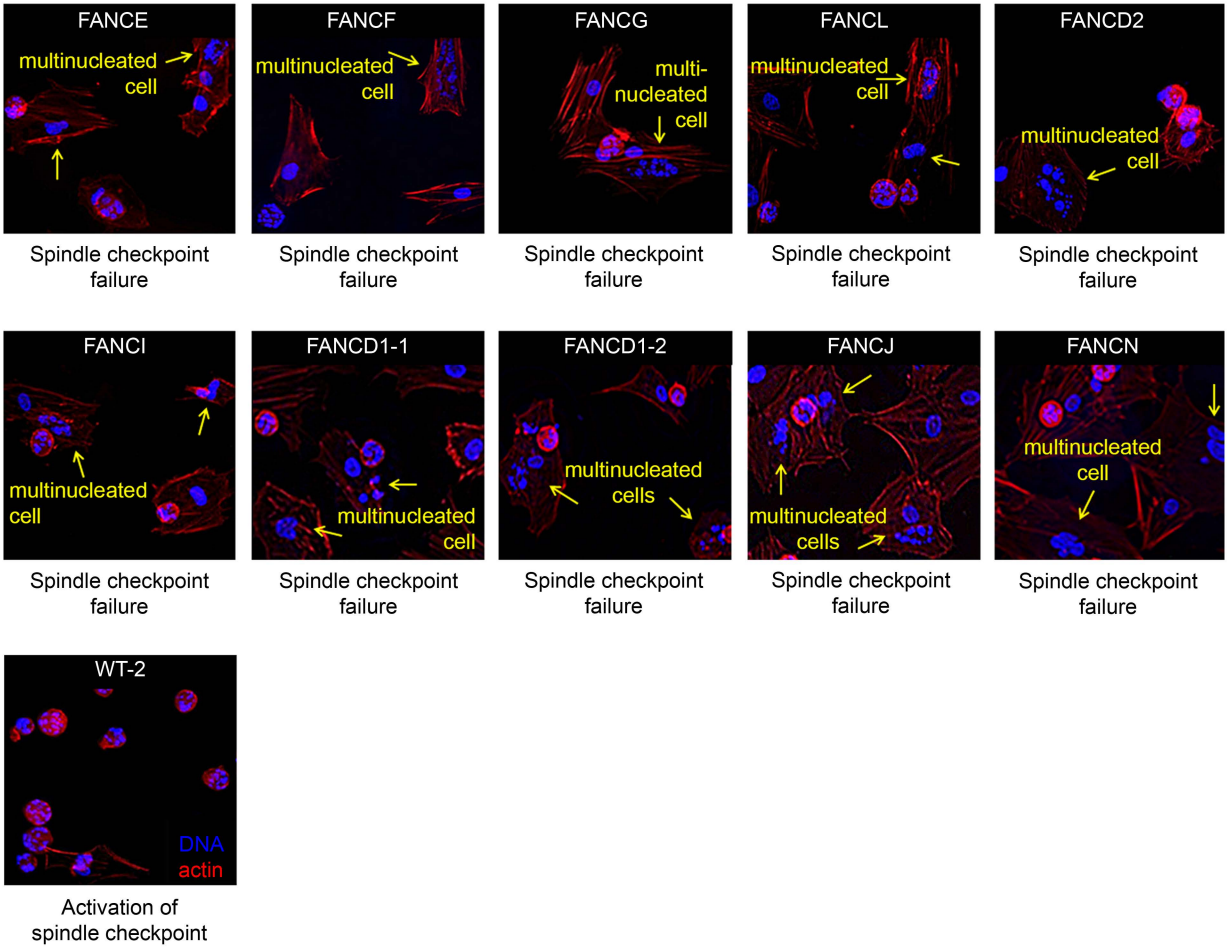


Phenotype examples for CD34+ cells that are BrdU+ and have 4N DNA content.

CD34+ cells transduced with either scrambled shRNA or FANCA shRNA were labeled with BrdU for 4 hours and then treated with taxol for 24 hours to activate the SAC. Images of single cells were collected using an ImageStreamX Mark II imaging flow cytometer. When BrdU+ G2/M cells were gated, the phenotypes observed included interphase cells (G2), prometaphase cells (SAC arrest), and multinucleated cells (SAC failure). Magnification 400x (ImageStreamX Mark II).

Nalepa, Enzor et al Supplemental Figure 5

200 nM taxol, 24 hours

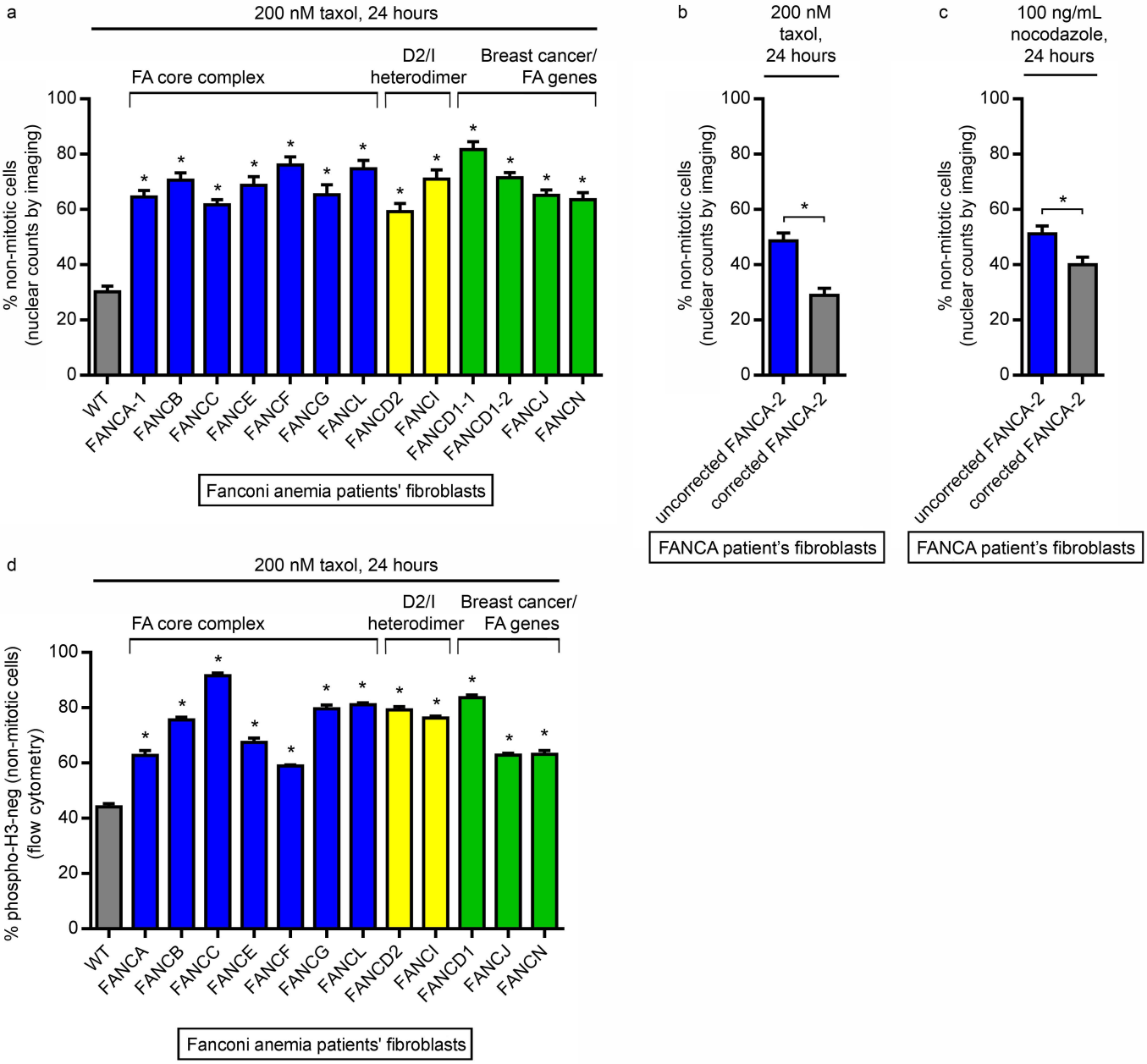


Spindle assembly checkpoint malfunction in Fanconi anemia patients' primary fibroblasts.

Gallery of microscopy images showing disrupted spindle assembly checkpoint (as evidenced by generation of multinucleated cells in the presence of taxol) in cells obtained from FA patients of indicated complementation groups. Mitotic arrest in wild-type (WT) taxol-treated human fibroblasts is shown for comparison. Magnification 200x (Applied Precision personalDX).

The two FANCD1 cell lines shown (FANCD1-1 and FANCD1-2) were isolated from siblings who carry the same FANCD1 truncating mutation (exon 6 c.706-15 del10). See Supplemental Table for full list of patients' mutations.

Nalepa, Enzor et al Supplemental Figure 6

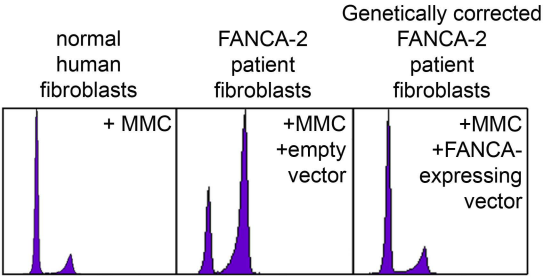


Spindle checkpoint failure in primary human FA cells.

(a-c) Quantification of FA patients' cells imaged by deconvolution microscopy. Weakened SAC in FA fibroblasts results in a higher proportion of non-mitotic cells in response to taxol or nocodazole. In **a**, asterisks denote p<0.0001(one-way ANOVA with post-hoc Bonferroni's correction); n=10-15 counts per genotype. In **b**, ectopic expression of FANCA rescues the SAC defect in FA-A fibroblasts. P<0.0001 (two-tailed t-test); n=15 counts. In **c**, FANCA patient fibroblasts fail to arrest at the SAC in response to nocodazole.P=0.0096 (two-tailed t-test); n=15 counts. All bars represent mean values +/− SEM.

(d) Flow cytometry confirms weakened SAC in FA fibroblasts. Phospho-H3-negative fraction represents non-mitotic cells. Asterisks denote p<0.001 (one-way ANOVA with Bonferroni's correction), error bars show SEM, and n=3 flow assays per FA genotype.

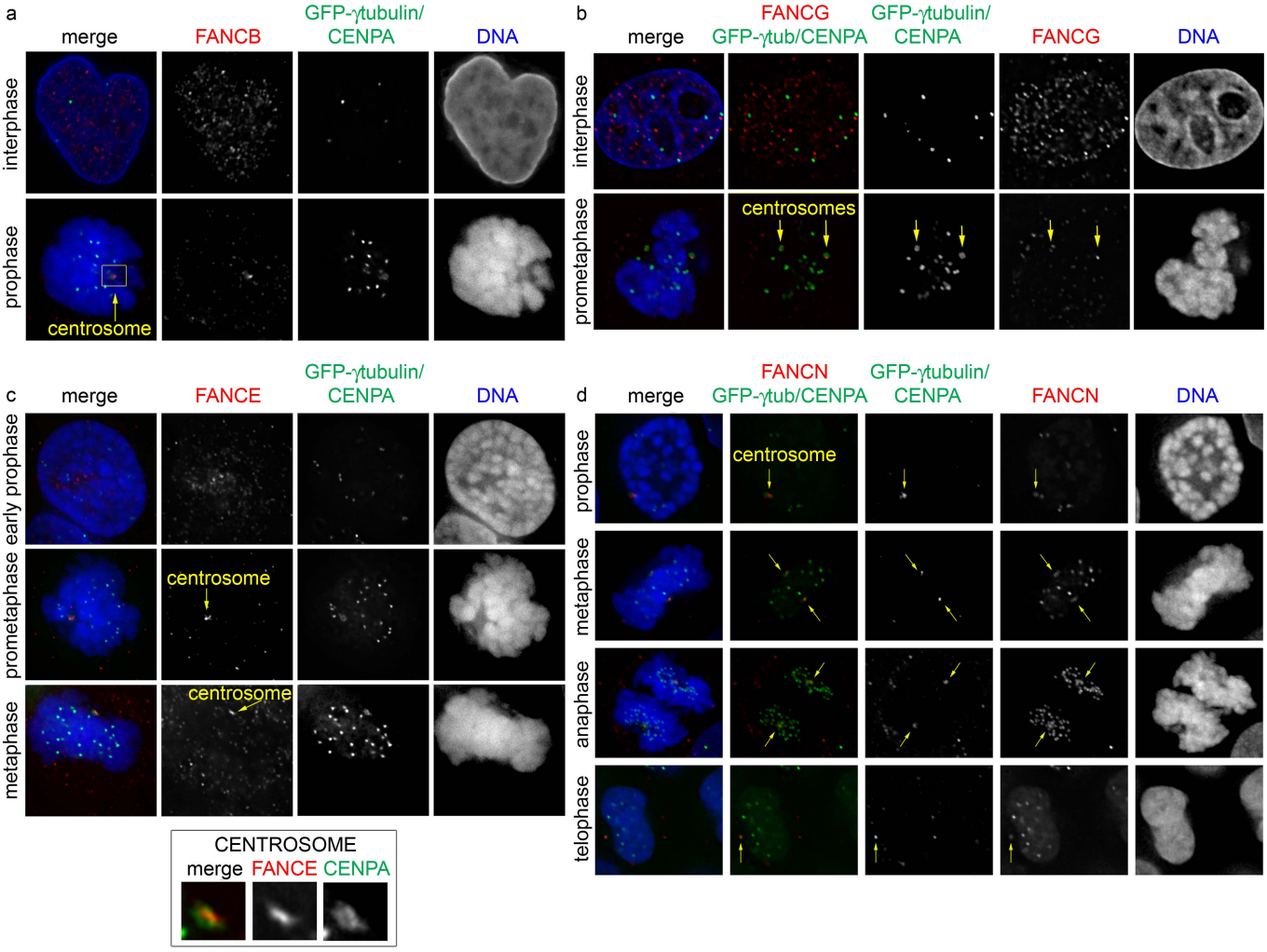
Nalepa, Enzor et al. Supplemental Figure 7



Functional correction of FANCA patient fibroblasts via stable ectopic expression of wild-type FANCA.

Cells were treated with 33 nM mitomycin C (MMC) for 24 hours. Cell cycle profiles were obtained through flow cytometry. Note MMC-induced G2/M arrest in FANCA patient fibroblasts, but not in normal fibroblasts or FANCA patient fibroblasts stably transduced with vector expressing wild-type FANCA.

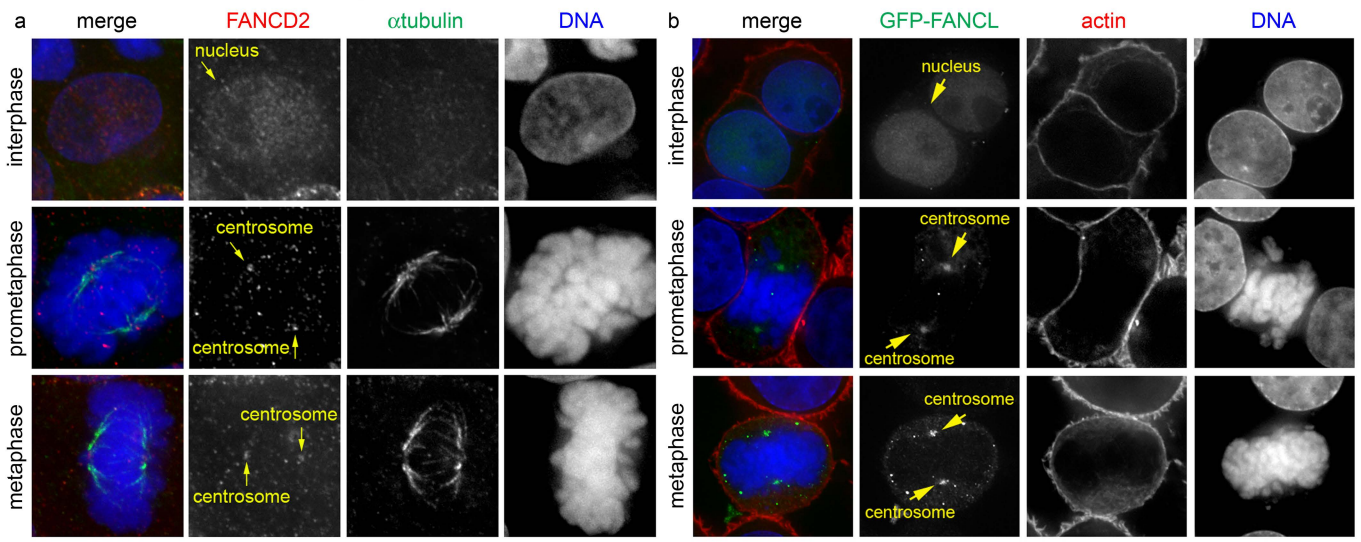
Nalepa, Enzor et al Supplemental Figure 8



Endogenous FANCB, FANCG, FANCE, and FANCN proteins localize to centrosomes during mitosis.

Note co-localization of (a) FANCB, (b), FANCG, (c), FANCE and (d) FANCN with the centrosome marker GFP-γtubulin/CENPA during cell division. The FA proteins are labeled red, while GFP-γtubulin/CENPA is shown in green. Representative microscopy images are shown. Magnification 1000x (Applied Precision personalDX).

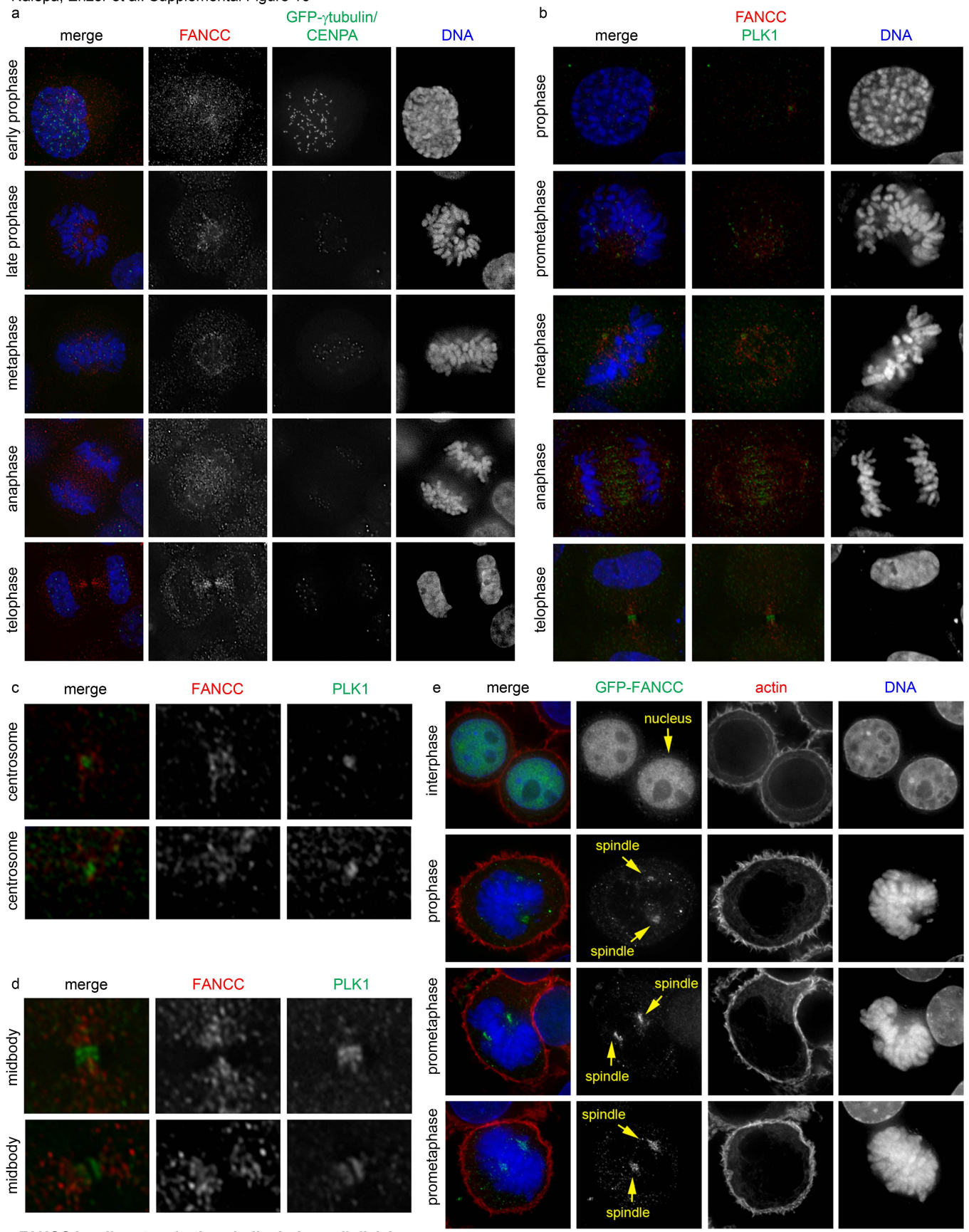
Nalepa, Enzor et al Supplemental Figure 9



FANCD2 and FANCL proteins localize to centrosomes during mitosis.

(a) Endogenous FANCD2 and **(b)** GFP-FANCL associate with centrosomes during cell division. Endogenous αtubulin was stained green in **(a)** to visualize the mitotic spindle; FANCD2 is labeled red. Actin was stained with Alexa594-phalloidin (red) to outline cell borders in **(b)**; GFP-FANCL is green. Representative images are shown. Magnification 1000x (Applied Precision personalDX).

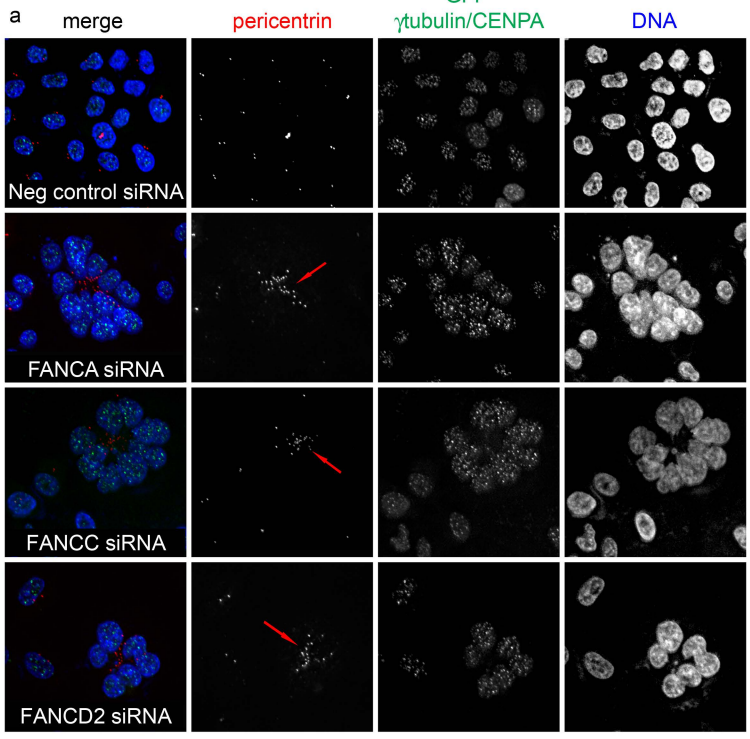
Nalepa, Enzor et al. Supplemental Figure 10

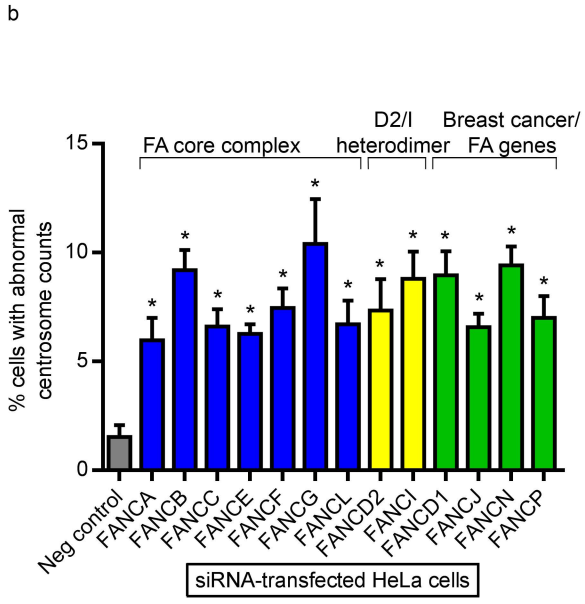


FANCC localizes to mitotic spindle during cell division.

(a) HeLa cells stably expressing GFP-γtub/CENPA were immunostained with antibody against endogenous FANCC. Note FANCC association with the mitotic spindle. **(b)** HeLa cells were co-immunostained with antibodies recognizing endogenous FANCC (red) and PLK1 (green). PLK1 and FANCC localize in close proximity around centrosomes in early mitosis **(c)** and midbody at mitotic exit **(d)**. **(e)** Overexpressed GFP-FANCC localizes to the nucleus in interphase and to the mitotic spindle during cell division. Representative images are shown. Magnification 1000x (Applied Precision personalDX).

Nalepa, Enzor et al Supplemental Figure 11





RNAi knockdown of FA pathway genes leads to accumulation of supernumerary centrosomes in HeLa cells.

(a) Representative examples of cells that spontaneously accumulate extra centrosomes and undergo multinucleation (red arrows) upon transfection with validated siRNAs against FA pathway genes. Note normal number of centrosomes and nuclear structure in cells transfected with negative control siRNA. HeLa cells stably expressing GFP-γtubulin/CENPA were transfected with indicated siRNAs, grown in the absence of spindle poisons for 72 hours, stained and imaged as indicated. Magnification 200x (Applied Precision personalDX). (b) Increased fraction of cells with abnormal centrosome counts resulting from RNAi knockdown of FA pathway genes.

Asterisks indicate $p<0.05$ compared with WT (one-way ANOVA with post-hoc Bonferroni's multiple comparison test).