Expanded View Figures

Figure EV1. Synthesis and characterization of CCB02.

A Chemical synthesis of CCB02.

B CCB02 the derivative of HTS1 exhibits a dose-dependent reduction of CPAP-tubulin AlphaScreen signal. Error bars indicate data from a triplicate determination.

C CPAP-GST pull-down assay to support the findings of AlphaScreen assay. This CPAP-GST pull-down assay uses purified components of CPAP PN2-3 and tubulin. Semi-quantitative Western blot shows that CCB02 perturbs CPAP PN2-3–tubulin interaction in a dose-dependent manner with an approximately estimated IC_{50} value of 0.441 μM. The bait protein GST-tagged PN2-3 domain of CPAP is shown in Coomassie (bottom panel). The results derived from at least three independent experiments for intensity calculations (i). CPAP PN2-3-GST but not GST alone interacts with tubulin. CCB02 (1 μM) perturbs CPAP PN2-3–tubulin interaction. Purified GST-tagged PN2-3 domains of CPAP and GST are shown in Coomassie (bottom panel) (ii). Similar CPAP-GST pull-down assay using cell extracts to show CCB02 prevents cellular tubulin binding to GST-tagged PN2-3 domain of CPAP. CCB02 at 2 and 5 μM (top panel Western blot) prevents binding of cellular tubulin to PN2-3 (iii). We used CPAPΔT, a variant that will not interact with tubulin as a negative control. Addition of DMSO instead of CCB02 is a positive control.

D To evaluate the specificity, CCB02 was profiled against a broad panel of human protein kinases, including cancer-relevant mutant kinases. Of the 469 kinases tested, CCB02 showed no significant inhibitory activity against family of centrosomal and other cell cycle-related kinases even at higher concentration of CCB02 (5 μM). Note that our cell-based experiments used concentration ranging from 1 to 2 μM. Experimental values are average of three independent experiments. (N) = 3. The kinases that represent < 10% of inhibitory activity compared to control are considered significant. Based on this, CCB02 does not seem to affect the activity of centrosomal and cell cycle-related kinases. The complete result of *in vitro* kinases profiling is given in Table EV2 in the article. Western blot at right panel: Cell extracts treated with 2 μM of CCB02 were analyzed for phosphorylated substrates such as p-PCNT, p-CPAP, p-P53, and p-EGFR that are phosphorylated by PLK1, Aurora A, CDK2 (other like, CHK1 or CHK2 or ATM or ATR) and EGFR, respectively. Treatment with CCB02 does not alter the phosphorylation status of these proteins, indicating that the mechanism of CCB02 is not through inhibiting any of these cell cycle- or centrosome-related kinase activities.

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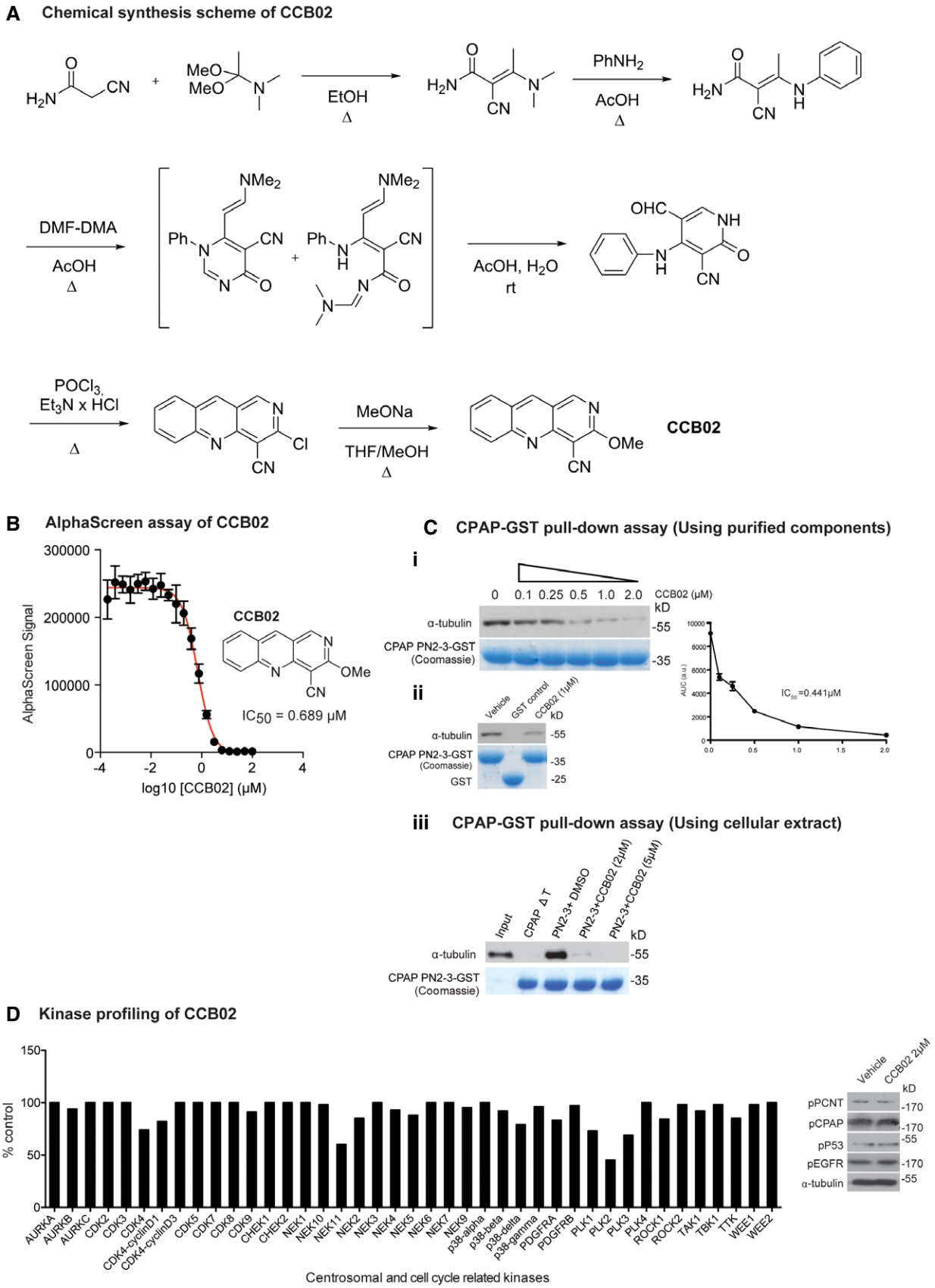

Figure EV1.

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Figure EV2. Related to Fig 3: CCB02 impairs proliferation of extra centrosomes-containing cells.

A Dose–response curves of control (fibroblast and RPE) and cancer cell lines (separated as part 1 and 2) in response to CCB02 treatment. IC_{50} values were calculated after 72 h of treatment. MTS assay was used to calculate IC_{50} values (detailed description is given in the Materials and Methods section). Each data point is representative of three independent experiments. (N) = 3. Error bars, mean ± SEM.

B Correlation of IC_{50} values to the percentage of cells containing extra centrosomes. An enhanced sensitivity of CCB02 is observed with cells harboring extra centrosomes. While cells with extra centrosomes segregate to the right side of the graph exhibiting lower IC_{50} values, two centrosome-containing control cells segregate to the upper left side of the graph exhibiting higher IC_{50} values. Data represent mean ± SEM. (N) = 3. Pearson correlation coefficient was calculated for XY pairs ($r = -0.86$). ***$P < 0.0001$. Also refer Appendix Fig S1Aii for percentages of cells exhibiting extra centrosomes.

C–G CCB02 treatment prevents extra centrosomes of cancer cells from clustering by activating them to nucleate an enhanced level of microtubules prior to mitosis (Arrows). Activated centrosomes fail to cluster, instead of causing multipolar mitosis. $H1975^{T790M}$, POP10, PC9, MDA-MB-231, and BT549 cancer cells exhibiting extra centrosomes were used. For cell-based assays, CCB02 was used in the range of 1–2 μM concentrations depending on the estimated IC_{50} values for respective cell lines. All these cells were stained with Cep152 (green), CPAP (magenta), microtubules (α-tubulin, red), and DNA (DAPI blue). Scale bar, 2 μm.

H Bars show percentages of multipolar interphase (MI) and multipolar mitotic cells (MM) after 48 h of CCB02 treatment. Compared to vehicle treatment, CCB02 treatment causes multipolar spindles in both interphase and mitosis. (N) = 3 with total number of cells > 300 in each cell line. Error bars, mean ± SEM. Unpaired t-test. *$P < 0.01$, **$P < 0.001$. Note that CCB02 does not affect two centrosome-containing MCF10A wild-type cells.

I Bar graphs show MT intensities of interphase cells from the figure panels (C–G). Error bars, mean ± SEM. Two-way ANOVA test. ***$P < 0.0001$.

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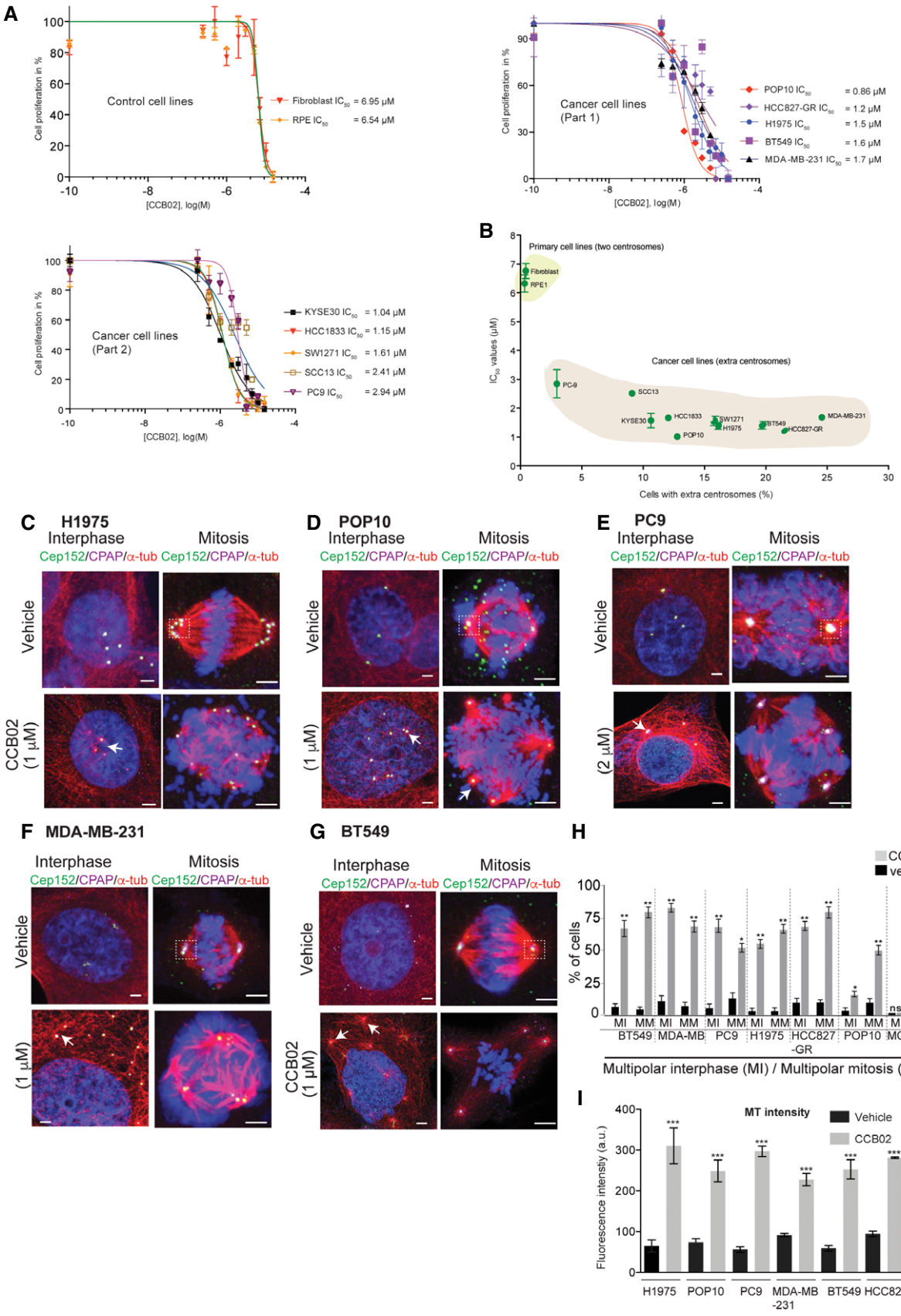

Figure EV2.

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Figure EV3. Related to Fig 3: Inhibiting CPAP–tubulin interaction activates spindle assembly checkpoint in extra centrosomes-containing cells.

A Vehicle treatment. SAC components Bub1 (i, green) and Mad1 (ii, red) mostly accumulate in prophase but not in bipolar mitotic (two centrosomes-containing) or pseudobipolar mitotic (extra centrosome containing) cells. MCF10A cells containing two centrosomes (−Dox, two centrosomes) and extra centrosome (+Dox, extra centrosomes) were used. Cells were stained for Bub1 (green) and Mad1 (red), γ-tubulin (magenta), microtubules (α-tubulin, red) and DNA (DAPI, blue). Scale bar, 2 μm. Bar diagram at right quantifies SAC activation only in metaphase cells (both bipolar and pseudobipolar). Data represent mean ± SEM of three independent experiments, (N) = 3 (n = 220 cells per condition). P-values were obtained using one-way ANOVA. $^{**}P < 0.001$.

B CCB02 treatment or CPAPΔT overexpression. In contrast to bipolar metaphase cells (as observed above in panel A), Bub1 (i) and Mad1 (ii) proteins accumulated in multipolar metaphase cells where CPAP-tubulin interaction is perturbed either by CCB02 treatment or CPAPΔT overexpression. MCF10A (−Dox, two centrosomes) and MCF10A (+Dox, extra centrosomes) cells were used. MCF10A cells (+Dox, extra centrosomes) expressing CPAPΔT were used as control where CPAP-tubulin interaction is genetically perturbed. Bar diagram at right quantifies SAC activation only in metaphase cells (both bipolar and multipolar). Data represent mean ± SEM of three independent experiments (N) = 3 (n = 250 cell per condition). P-values were obtained using one-way ANOVA. $^{**}P < 0.001$.

C SAC activation assay in MDA-MB-231 cells. Bar diagram at right quantifies SAC activation only in metaphase cells (both bipolar and multipolar). Data represent mean ± SEM of three independent experiments. (N) = 3 (n = 240 cells per condition). P-values were obtained using one-way ANOVA. $^{**}P < 0.001$.

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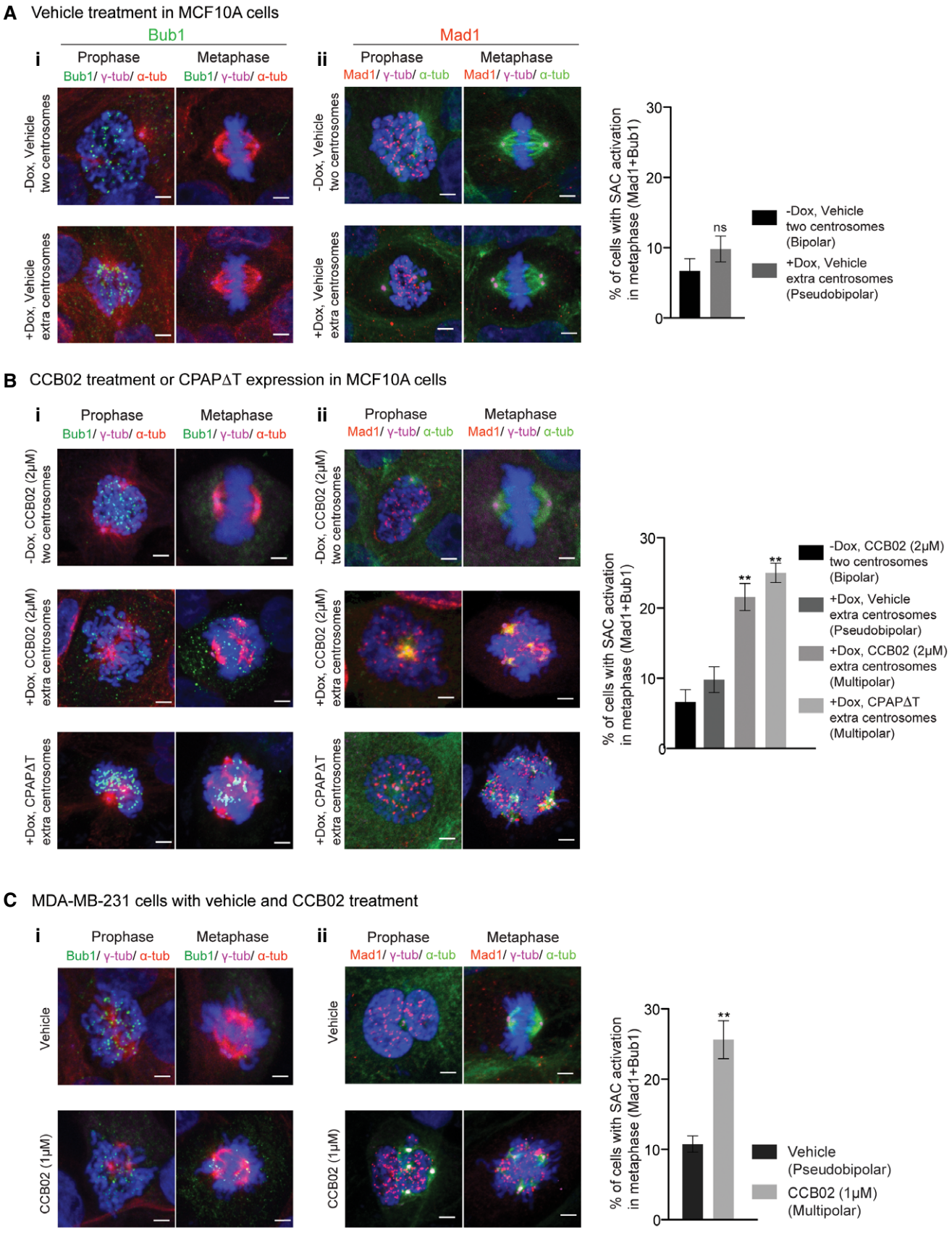

Figure EV3.

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Figure EV4. Related to Fig 5: CCB02 does not induce centrosome fragmentation.

A, B Fragmented centrosomes in extra centrosomes-containing MDA-MB231 and MCF10A cells (+Dox, extra centrosomes) before and after CCB02 treatment. Fragmented centrosomes in these cells are determined by PCNT-negative centrin dots (centrin-3). No increase in centrosome fragmentation is observed after CCB02 treatment.

C Similar experiment was performed in two centrosome-containing MCF10A cells. This was because, in centrosome-amplified cells, extra centrosomes might mislead us in distinguishing them from fragmented centrosomes. No centrosome fragmentation is identified before and after CCB02 treatment. All these cells were stained for centrin-3 (green) and PCNT (red), and DNA (DAPI, blue). Scale bar, 2 μm and insets, 0.5 μm. Bar diagrams at right quantify the findings. (N) = 3. At least 200 cells were used for quantifications from each cell line. Error bars, mean ± SEM. Unpaired t-test.

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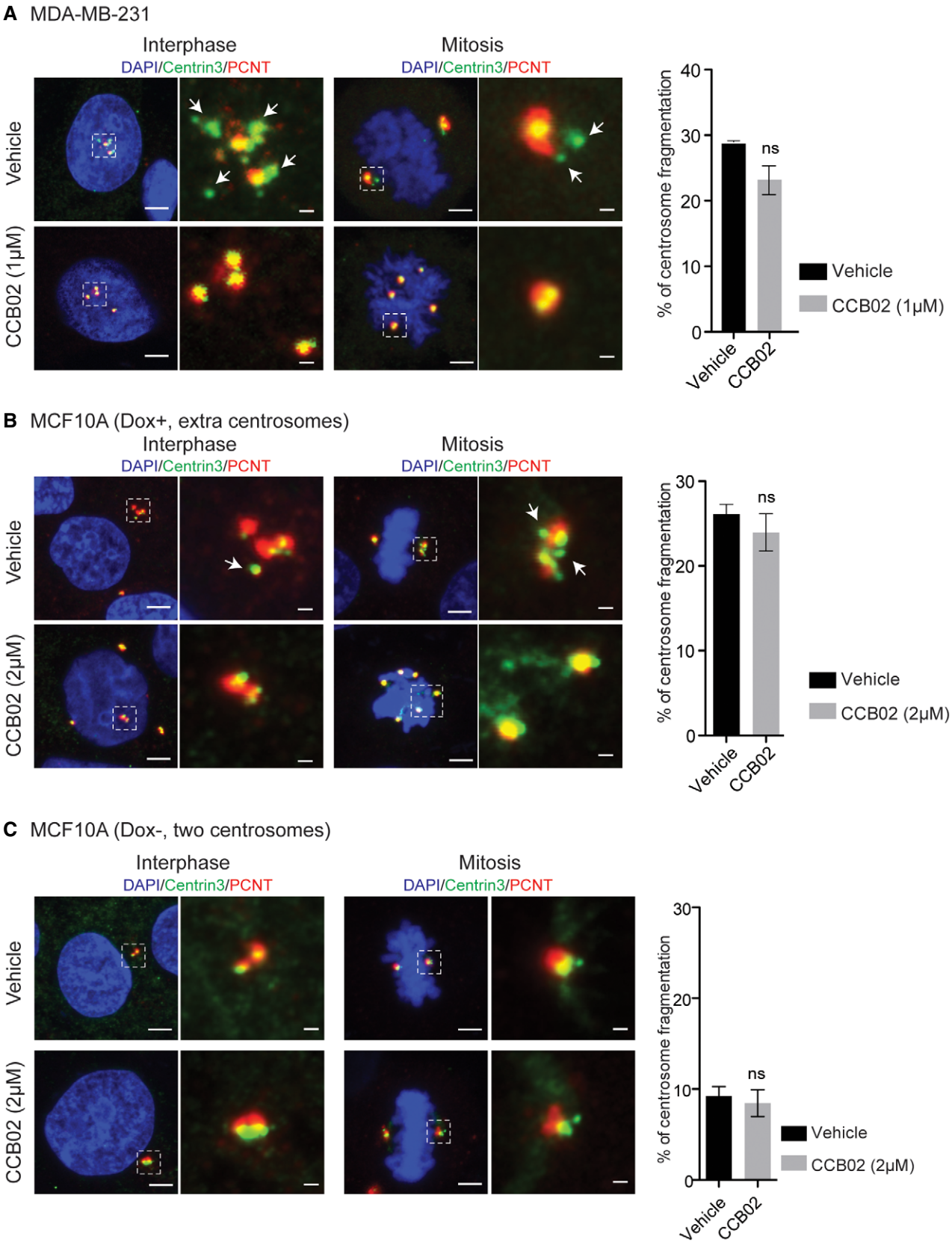

Figure EV4.

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Figure EV5. Related to Fig 8: CCB02 prevents MDA-MB-231 cell migration and induces multipolar mitosis in mouse xenografts.

A Experimental scheme of wound-healing assay using MDA-MB-231 cells.

B Snapshot of live cell images shows wound closure at various time points (0, 10, 24, and 48 h). Relative to vehicle treatment, CCB02 delays wound closure. Dashed lines mark cell-free empty space. Scale bar, 100 μm. Bar diagrams at right quantify the percentage of relative wound closure. (N) = 3. Error bars, mean ± SEM. P-values were obtained using two-way ANOVA. ***$P < 0.0001$.

C Image shows H&E immunohistochemistry staining of representative xenograft tumor samples. In contrast to vehicle control xenograft tumors that show bipolar spindles (red arrows, top panel), CCB02 xenograft-treated tumors show increased frequencies of multipolar mitotic cells, indicating mitotic catastrophe (red arrows, bottom panel). Scale bar, 10 μm. Table shows the percentage of multipolar mitotic cells. Data are represented as mean ± SEM. At least 350 mitotic cells were scored for vehicle and CCB02 treatment from independent xenograft tumors.

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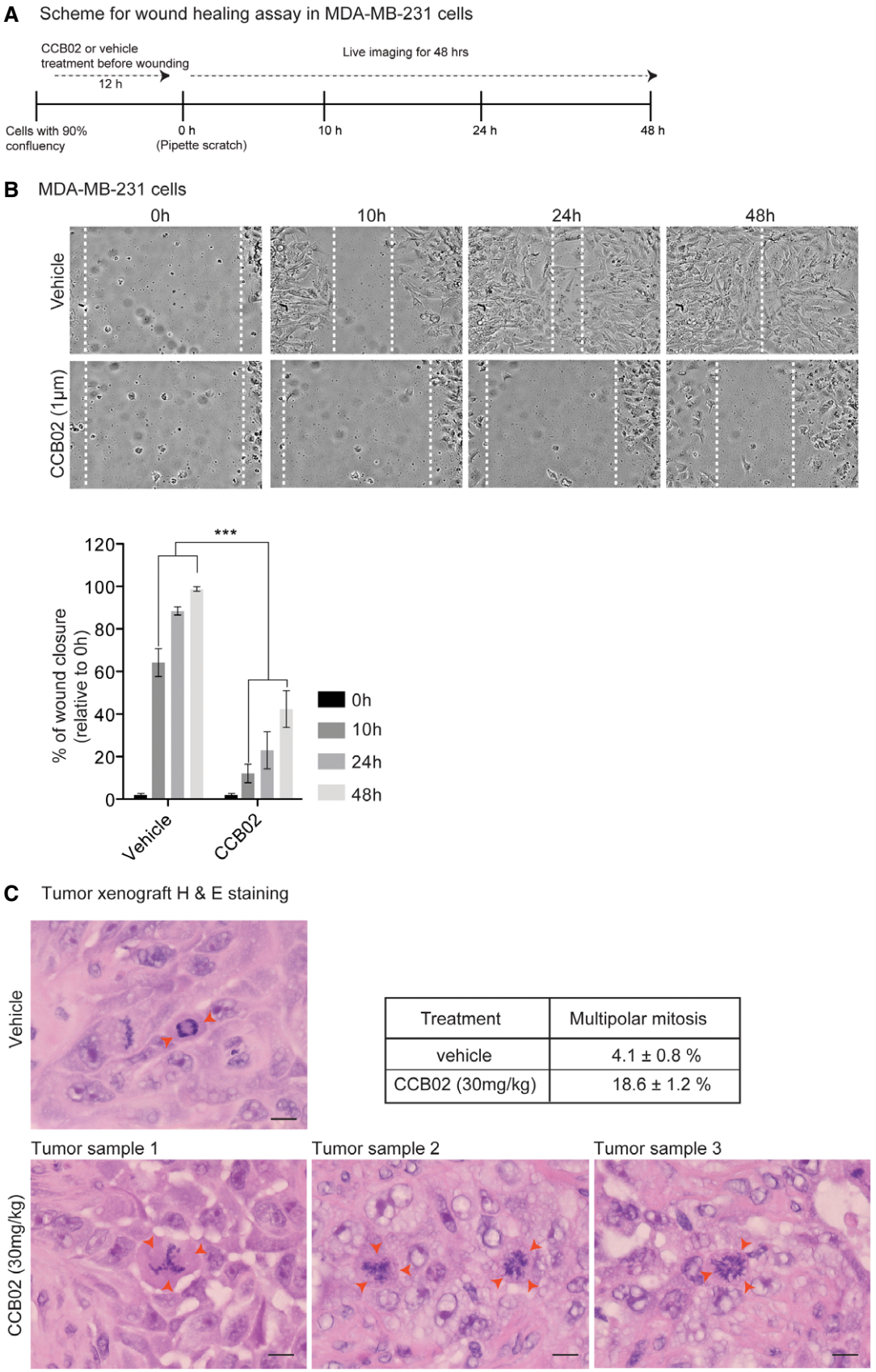

Figure EV5.

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