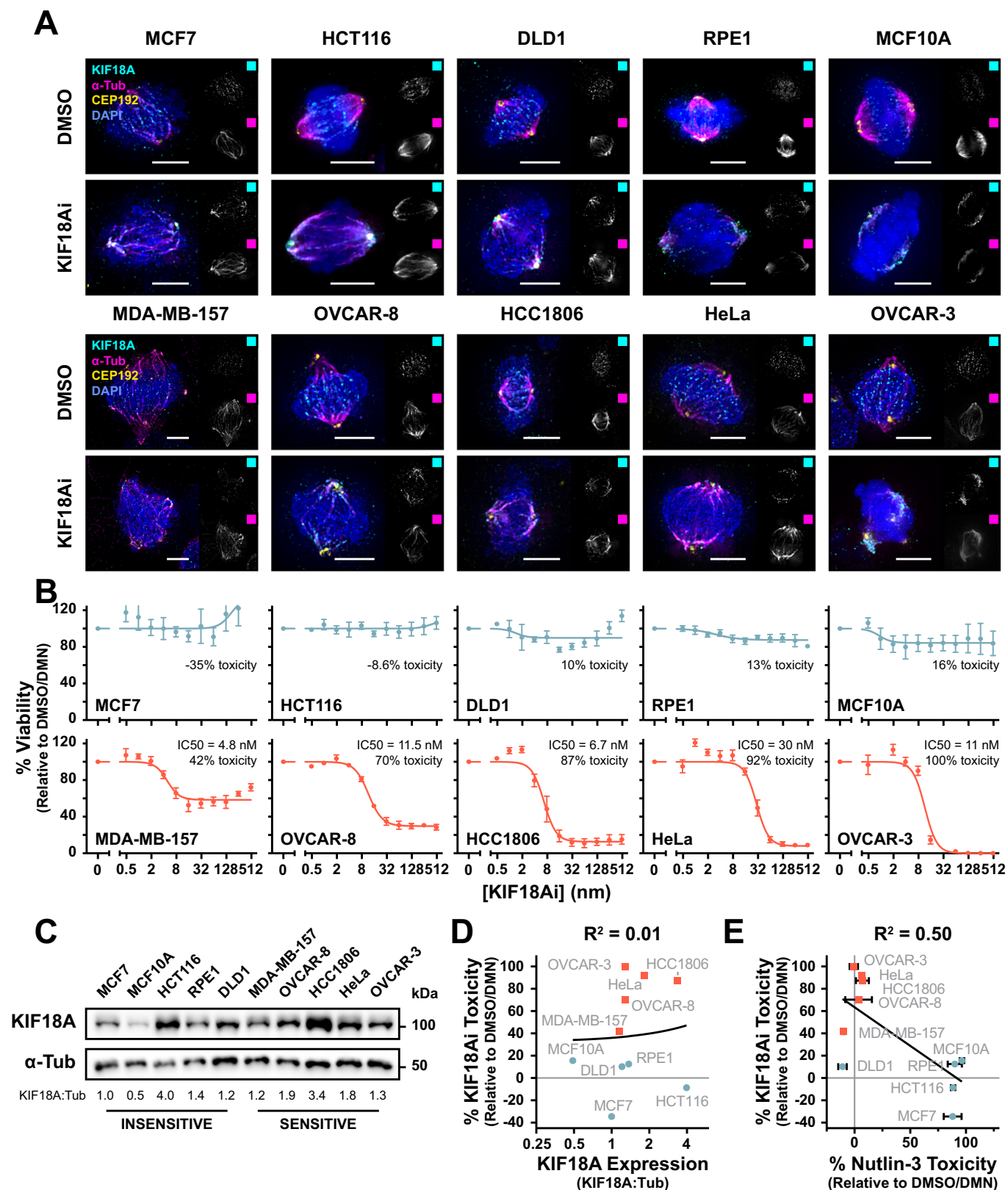


Expanded View Figures

Figure EV1. Extended analysis of cellular response to KIF18Ai.

(A) Wide-field immunofluorescence of KIF18A localization relative to spindle poles (CEP192, yellow) and the mitotic spindle (α -Tubulin, magenta) in response to KIF18Ai treatment across full cell line panel. Scale bar = 5 μ m. (B) Titration of KIF18Ai in a 5-day MTT endpoint viability assay for the panel of sensitive and insensitive cell lines. $N \geq 3$ independent experiments, $n \geq 2$ technical replicates per experiment. Data are represented as mean $\pm$ SEM. (C) Western blot of KIF18A expression levels across the full panel of sensitive and insensitive cell lines. (D) Linear correlation between 5-day KIF18Ai toxicity and normalized KIF18A expression (KIF18A/ α -Tubulin) from (C). Expression is plotted on a $\log_2$ axis. (E) Linear correlation between 5-day KIF18Ai toxicity and Nutlin-3 toxicity from a 5-day MTT endpoint viability assay for the panel of sensitive and insensitive cell lines. $N = 3$ independent experiments, $n = 3$ technical replicates per experiment. Data are represented as mean $\pm$ SD.



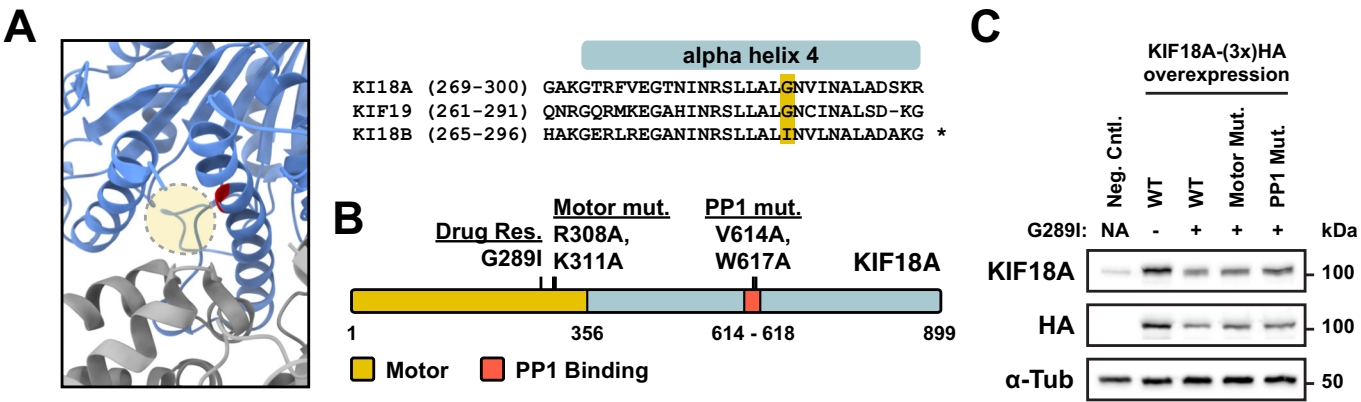
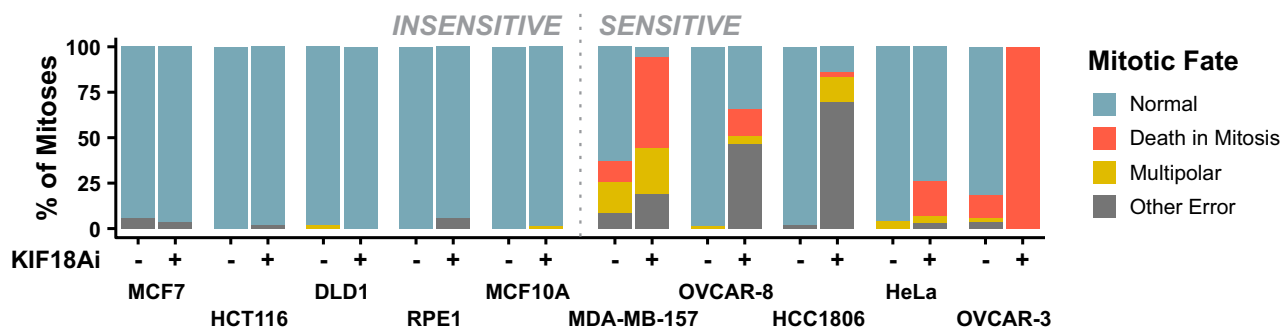
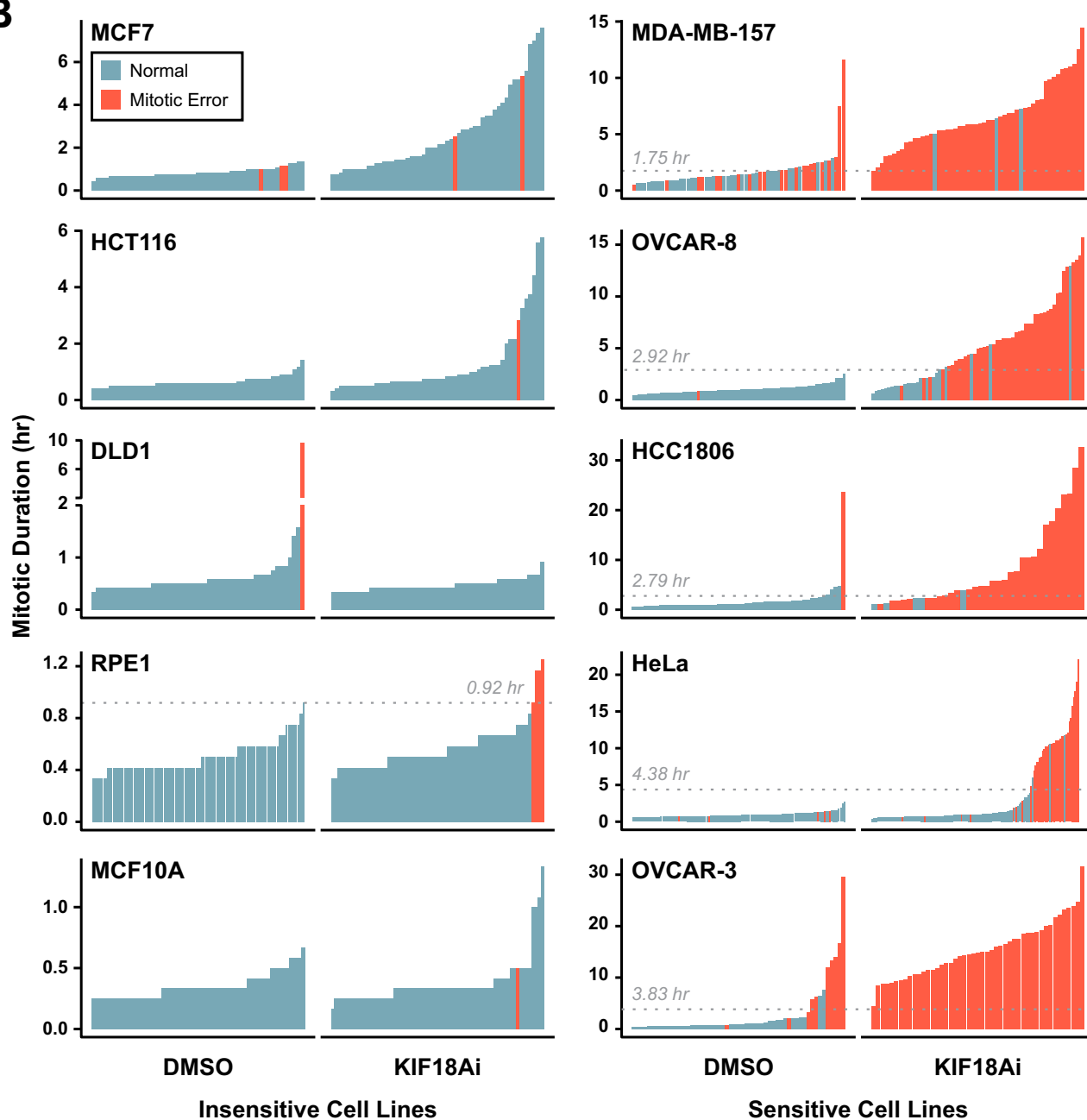


Figure EV2. Validation of drug-resistant KIF18A transgenic HCC1806 cells.

(A) Schematic of KIF18Ai drug binding site from EM structure (PDB: 5OAM). Blue: KIF18Ai motor domain with G289 highlighted in red. Gray: α-tubulin/β-tubulin. Highlighted circle represents drug binding pocket. Protein sequence alignment of drug binding pocket between KIF18A, KIF19, and KIF18B. (B) Schematic of KIF18A protein domains and mutations. (C) Western blot validation of HCC1806 cells constitutively expressing a WT, drug-resistant (G289I), drug-resistant and motor dead (G289I, R308A, K311A), or drug-resistant and PP1-binding deficient (G289I, V614A, W617A) KIF18A-(3x)HA transgene.

A**B**

**Figure EV3. Live-cell analysis of mitotic outcomes from KIF18Ai Treatment.**

(A) Proportion of mitotic fates across the panel of H2B/ α -Tubulin fluorescently tagged cell lines in response to DMSO or KIF18Ai treatment from live-cell wide-field time-lapse microscopy in Fig. 1E. (B) Representation of mitotic error threshold across a panel of H2B/ α -Tubulin fluorescently tagged cell lines in response to DMSO or KIF18Ai treatment from live-cell wide-field time-lapse microscopy in Fig. 1E. Bars represent individual mitotic events. Dotted line represents error threshold.

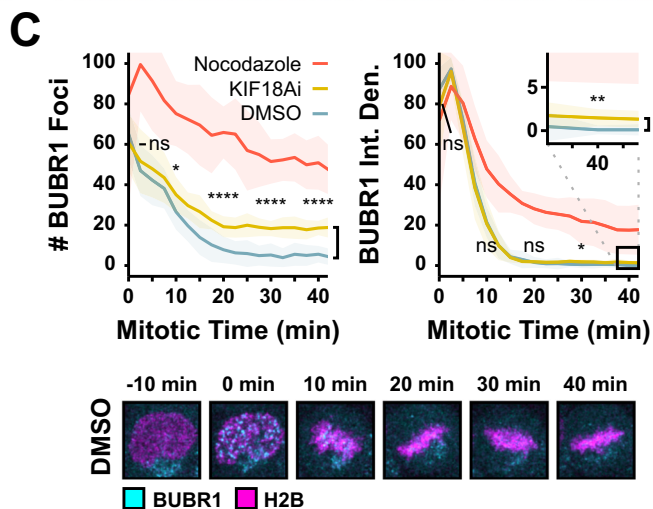
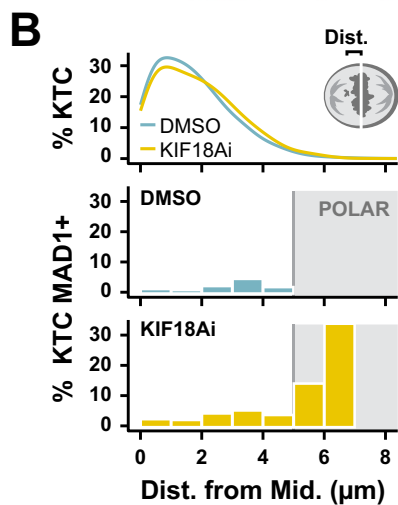
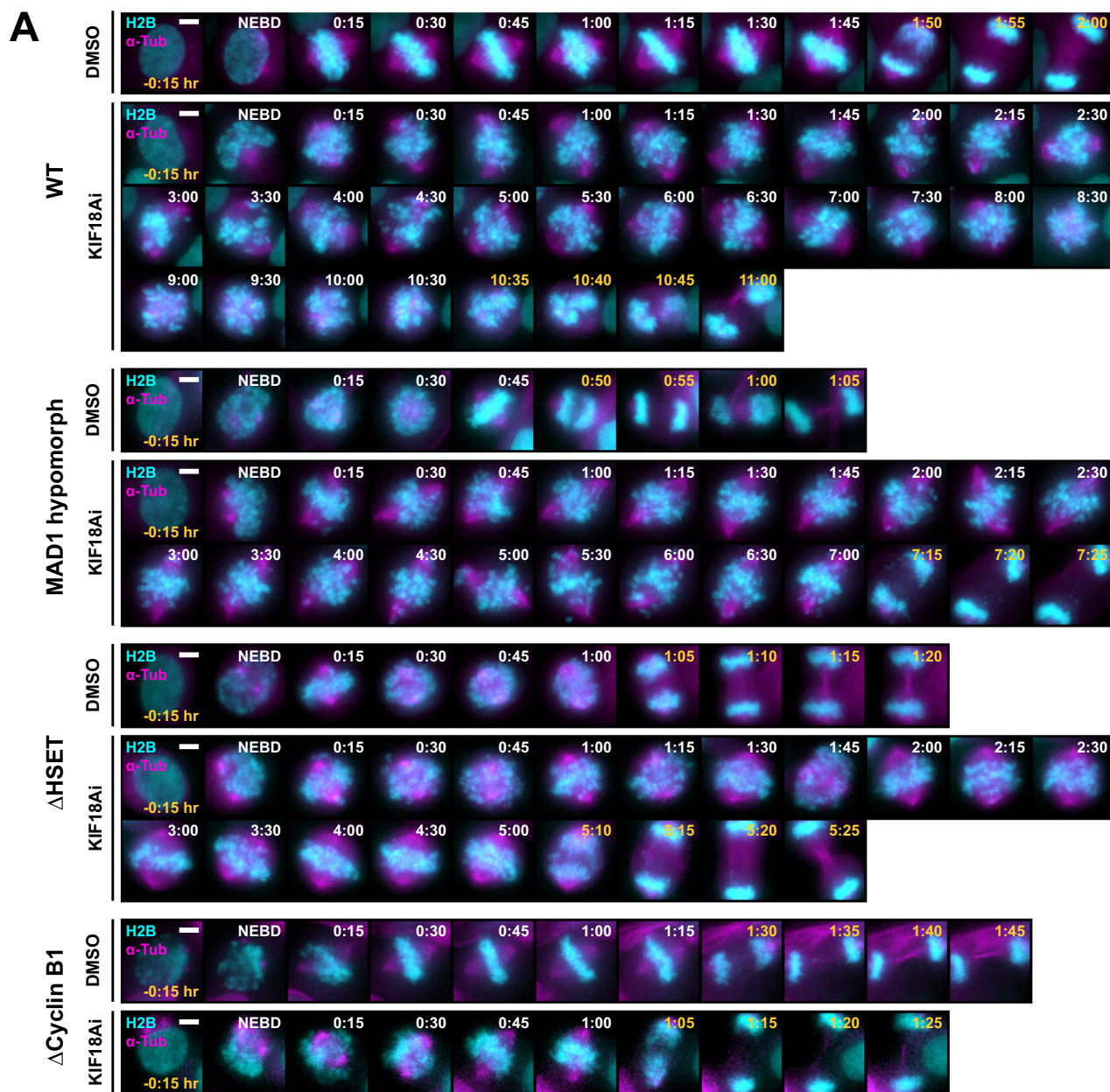


Figure EV4. Extended chromosome alignment and SAC activation data in KIF18Ai.

(A) Representative live-cell time-lapse microscopy stills from HCC1806 cells used to evaluate mitotic chromosome congression. Scale bar = 5 μ m. (B) Top: Kinetochores distribution probability along the spindle axis from Fig. 3C. Bottom: Probability of a kinetochore being MAD1+ relative to its position on the spindle axis in DMSO or KIF18Ai conditions. Polar kinetochores are defined as >5 μ m from the spindle midline. (C) Top: Quantification of BUBR1 foci from live-cell confocal time-lapse microscopy of HeLa EGFP-BUBR1 BAC H2B-iRFP cell lines in DMSO, KIF18Ai, and Nocodazole conditions. Error bars represent mean $\pm$ SD. Statistical significance was determined using an unpaired two-tailed Student's *t* test between DMSO and KIF18Ai conditions at 0, 10, 20, 30, and 40 min. Sample size and full statistical results are listed in Dataset EV2. Bottom: Representative still images from mitotic movies. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

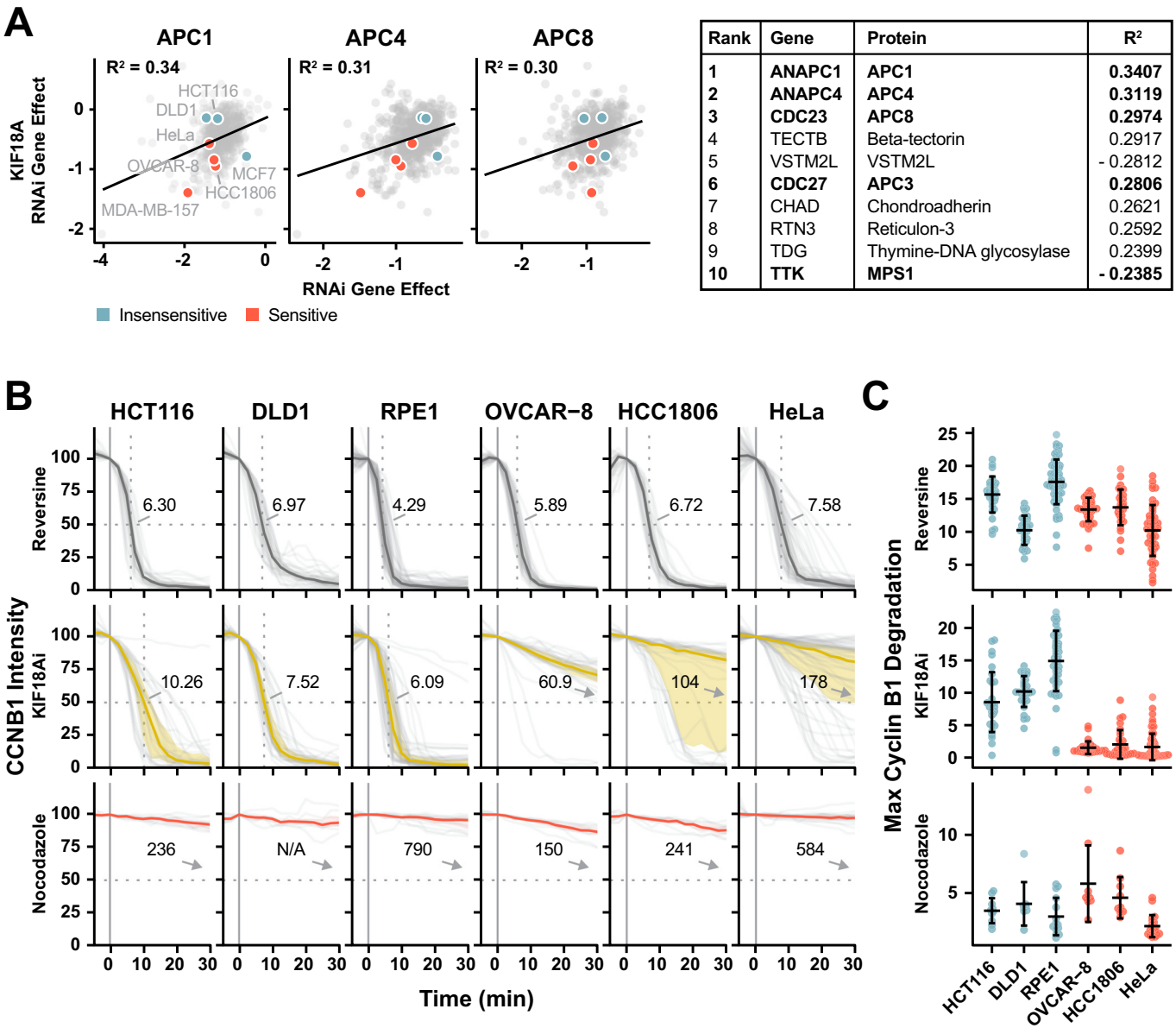


Figure EV5. Extended analysis of APC/C activity relative to KIF18A dependency.

(A) Top 10 RNAi co-dependency relationships from DepMap dataset for KIF18A. Red and blue points represent sensitive and insensitive cell lines respectively from the panel in Fig. 1C. Bolded table entries are APC/C or SAC genes. (B) Quantification of Cyclin B1 degradation rates for endogenously tagged fluorescent cells treated with Reversine, KIF18Ai, or Nocodazole. The median (colored line) of individual traces (gray lines) is plotted, with the shaded region encompassing the first and third quartile of each population. Cyclin B1 signal is normalized to the metaphase inflection point and median $t_{1/2}$ values are listed. Full sample size information is listed in Dataset EV3. (C) Maximum slope of Cyclin B1 degradation at metaphase for endogenously tagged fluorescent cells treated with Reversine, KIF18Ai, or Nocodazole in (B). Rates are calculated relative to the total Cyclin B1 signal at mitotic entry. Data are represented as mean $\pm$ SD. $N \geq 20$ cells per condition (KIF18Ai, Reversine), $N \geq 5$ cells per condition (nocodazole).