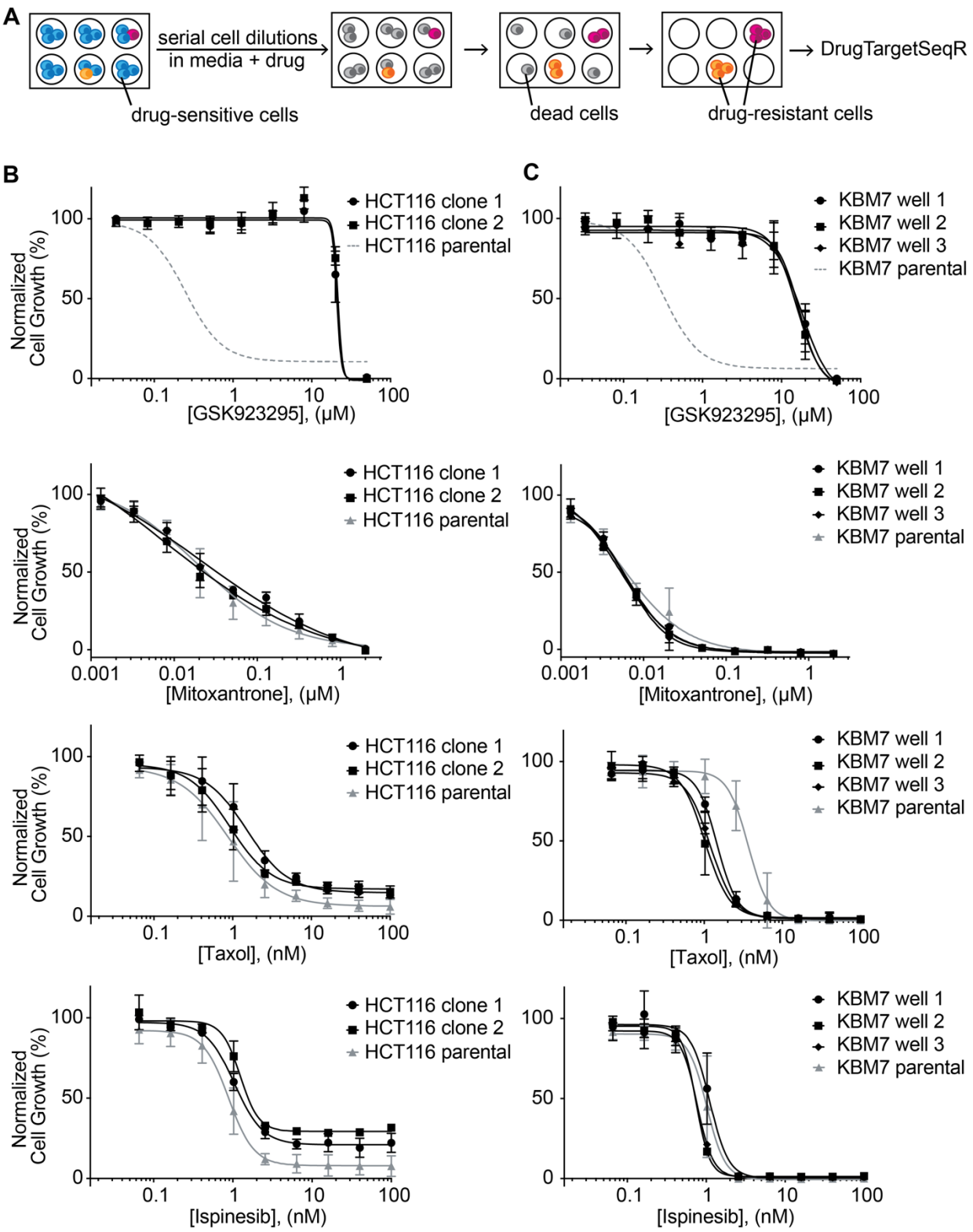

Figure S1. Characterizing GSK923295-resistant clones in HCT116 and KBM7 cells. Related to Figure 1.

(A) Schematic of cell dilution-based protocol for selection of drug-resistant cells growing in suspension (e.g. KBM7).

(B-C) Concentration-dependent inhibition of cell growth of parental and GSK923295-resistant HCT116 and KBM7 cells. Cell proliferation was determined using Alamar Blue assay (72 h, see STAR Methods for details). Data were fit to a sigmoidal dose-response equation and LD_{50} values were determined (LD_{50} values are shown in Table S1). For comparison, data for parental cells treated with GSK923295 are also shown (from Figure 1B, gray dotted lines).

KBM7

	LD_{50}:	parental	well 1	well 2	well 3
GSK923295	average (μM)	0.31	18.93	16.03	16.92
	s.d.	0.02	0.95	3.60	3.92
Ispinesib	average (nM)	0.98	1.10	0.71	0.77
	s.d.	0.26	0.22	0.21	0.06
Taxol	average (nM)	3.78	1.46	1.24	0.78
	s.d.	1.23	0.10	0.08	0.32
Mitoxantrone	average (nM)	5.77	5.99	5.12	6.34
	s.d.	0.55	0.55	1.04	0.30

HCT116

	LD_{50}:	parental	clone 1	clone 2
GSK923295	average (μM)	0.25	20.30	21.50
	s.d.	0.04	1.13	0.70
Ispinesib	average (nM)	0.93	1.11	1.24
	s.d.	0.20	0.05	0.11
Taxol	average (nM)	0.92	1.38	0.86
	s.d.	0.46	0.50	0.40
Mitoxantrone	average (nM)	23.32	21.00	9.80
	s.d.	9.10	14.16	8.79

Table S1. Characterizing GSK923295-resistant clones in KBM7 and HCT116 cells. Related to Figure 1 and Figure S1.

LD_{50} values were determined as described in STAR Methods. Corresponding dose-response curves are shown in Figure 1B, C, D and Figure S1B, C.

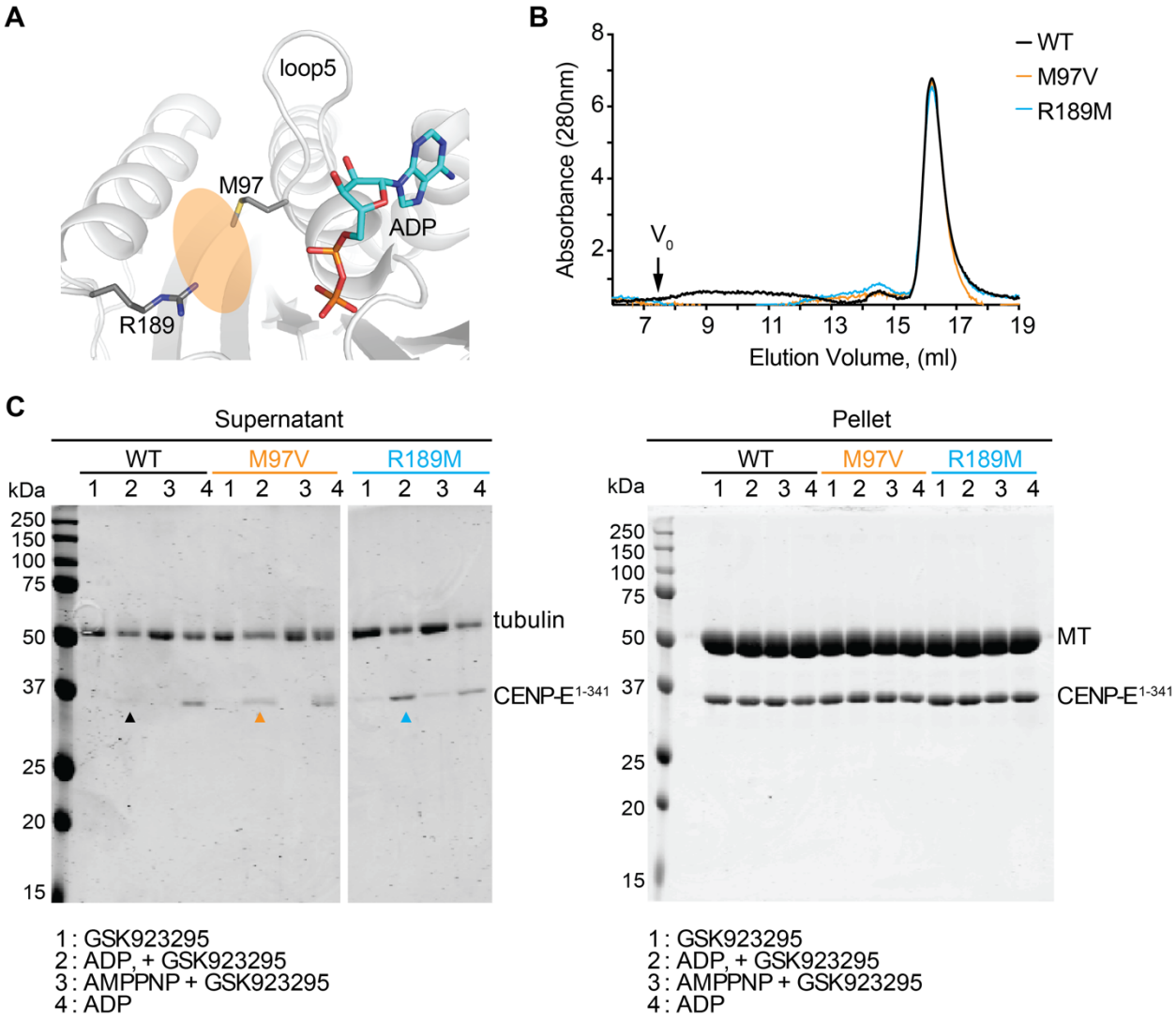

Figure S2. Characterizing mutant alleles of CENP-E motor domain. Related to Figure 2.

(A) Structural model (ribbon diagram) of human CENP-E motor domain (PDB: 1T5C). Adenosine-5'-diphosphate (ADP, stick representation), selected structural motif (loop 5), and side chains of selected residues (M97 and R189) are shown. Proposed binding site of GSK923295 is highlighted (orange oval).

(B) Size-exclusion chromatography traces (Superdex 200 Increase 10/300 GL column, GE Healthcare) of CENP-E motor domain constructs (WT, M97V and R189M; aa 1-341). V_0, void volume.

(C) SDS-PAGE analysis of soluble supernatant and pellet fractions from a cosedimentation assay of CENP-E motor domain ($CENPE^{1-341}$; 3 μM) with microtubules (MT, 6 μM). See STAR Methods

for details. The presence or absence of nucleotide (ADP or AMPPNP, 1 mM) and the presence or absence of the inhibitor (GSK923925, 20 μM) are indicated. Motor domains ($CENPE^{1-341}$) in the presence of GSK923925 and ADP are indicated with colored arrows (WT: black, M97V: orange, R189M: cyan).

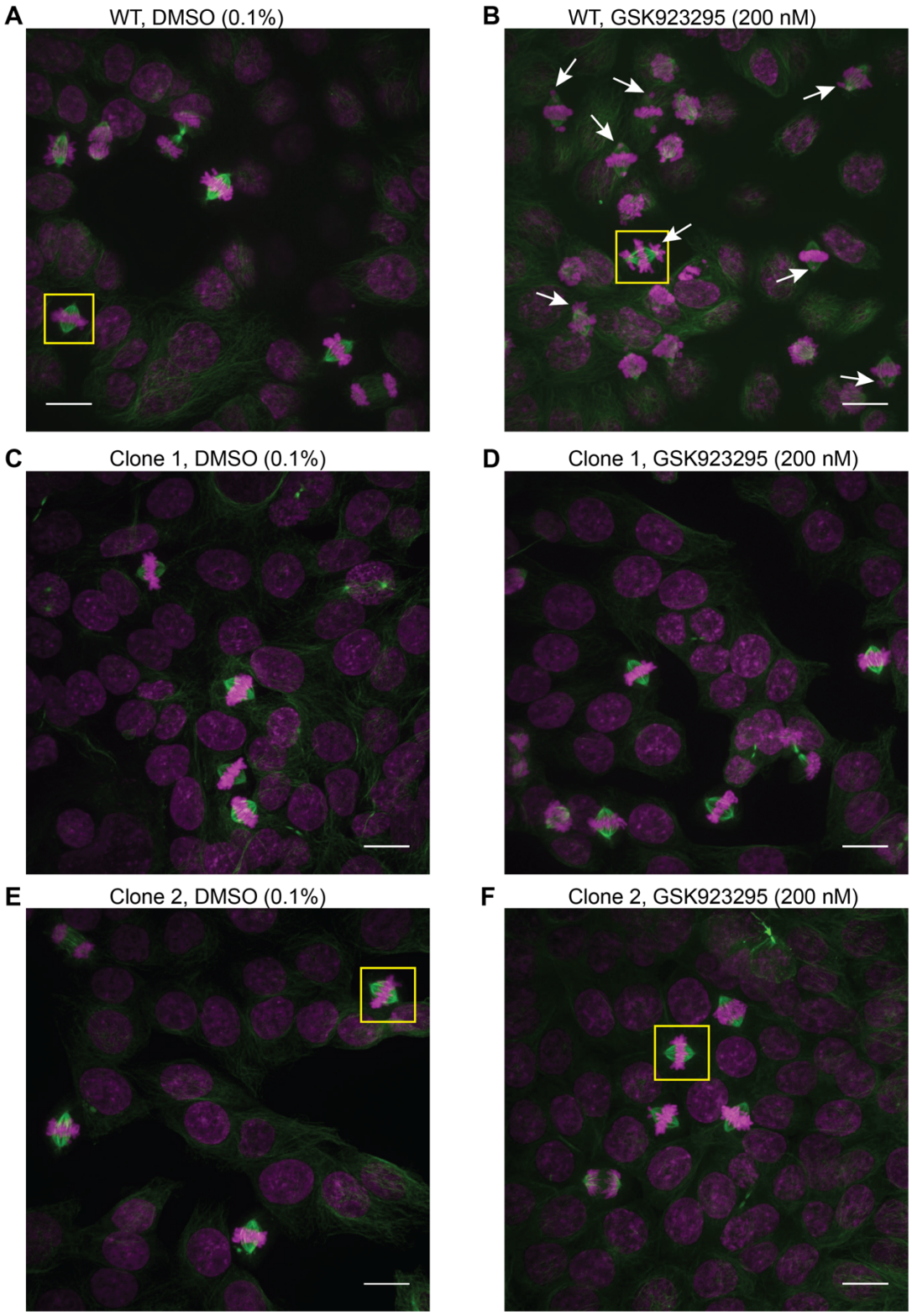

Figure S3. Characterizing parental and GSK923295-resistant HCT116 cells. Related to Figure 3.

(A-F) Representative immunofluorescence images of parental and GSK923295-resistant HCT116 cells. Parental (A-B), GSK923295-resistant clone 1 (C-D) and clone 2 (E-F) cells were treated with DMSO vehicle control (0.1%, A, C, E) or GSK923295 (200 nM, B, D, F). After treatment (4h) the cells were fixed, stained with FITC-conjugated anti-tubulin antibody and DNA-binding dye and imaged using confocal fluorescence microscopy. Maximum intensity confocal projections of DNA (magenta) and tubulin (green) are shown (see STAR Methods for details). Bipolar spindles were counted and manually classified as aligned or containing pole-stuck chromosome(s). DMSO-treated parental cells with aligned chromosomes: 62, pole-stuck chromosomes: 1. GSK923295-treated parental cells with aligned chromosomes: 12, pole-stuck chromosomes: 132. DMSO-treated resistant cells (clones 1 and 2) with aligned chromosomes: 91, pole-stuck chromosomes: 5. GSK923295-treated resistant cells (clones 1 and 2) with aligned chromosomes: 81, pole-stuck chromosomes: 8; data from three experiments. Mitotic spindles shown in Figure 3A-D are highlighted (yellow rectangles). Pole-stuck chromosomes are indicated with white arrows. Scale bars: 15 μm.

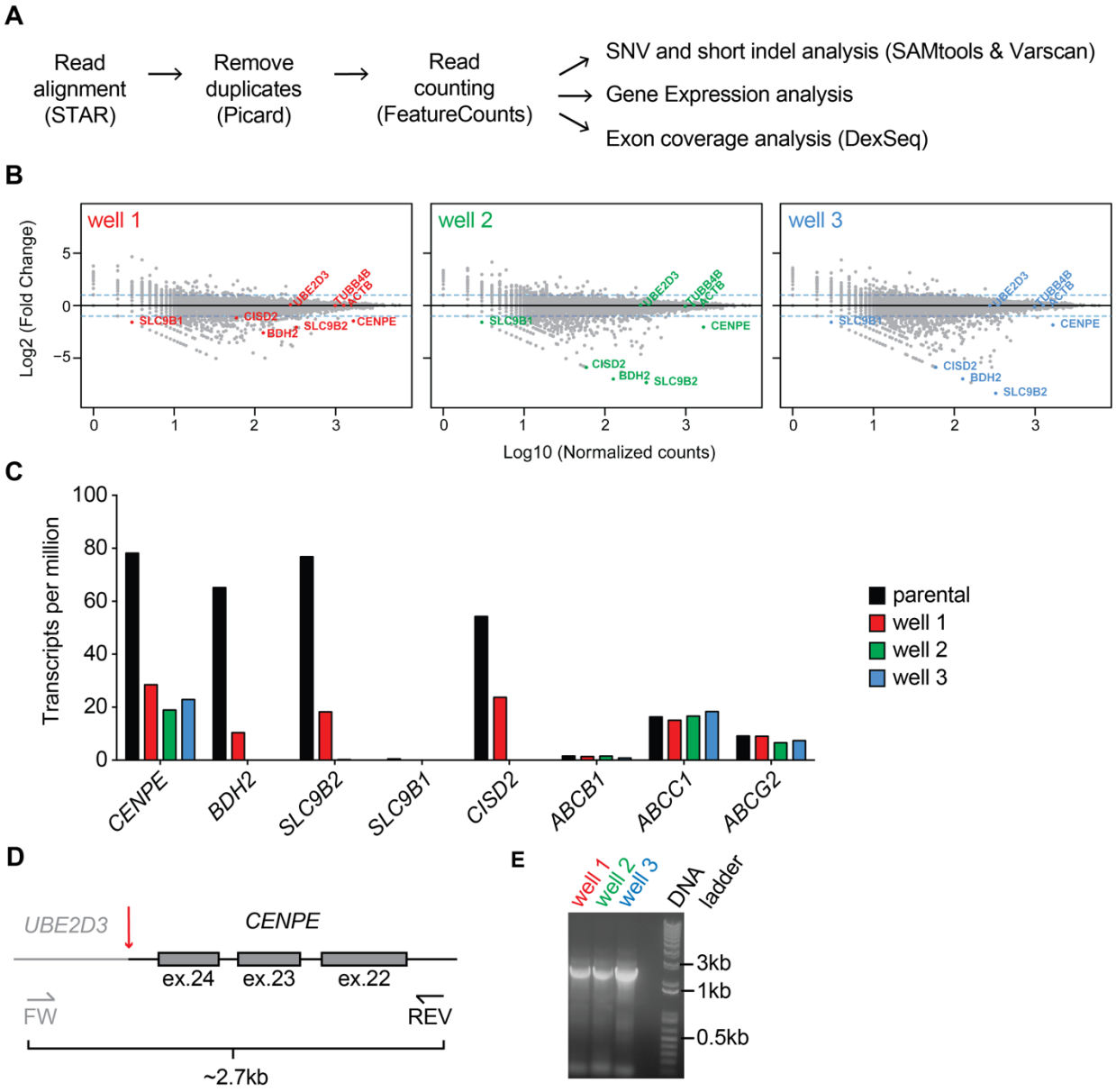

Figure S4. Characterizing GSK923295-resistant KBM7 cells. Related to Figure 4.

(A) Schematic of pipeline used for the analysis of transcriptome sequencing data. Software used in each step is indicated in brackets (STAR aligner (Dobin et al., 2013), Picard (http://broadinstitute.github.io/picard/), featureCounts (Liao et al., 2014), SAMtools (Li et al., 2009), Varscan2 (Koboldt et al., 2013), DexSeq (Anders et al., 2012))

(B) Plots showing normalized read counts versus fold change for genes in GSK923295-resistant KBM7 cells in comparison to parental cells (gray and colored dots). *CENPE* and the neighboring genes (*BDH2, CISD2, SLC9B1, SLC9B2* and *UBE2D3*) are indicated (colored dots and text). Tubulin (*TUBB4B*) and actin (*ACTB*) genes are also shown.

(C) Plot showing transcripts per million (TPM) values corresponding to *CENPE* and the neighboring genes (*BDH2, CISD2, SLC9B1, SLC9B2* and *UBE2D3*) in parental and GSK923295-resistant KBM7 cells (see STAR Methods for details). Multi-drug resistance genes *ABCB1* (also known as P-glycoprotein (P-gp) or multidrug resistance protein 1 - MDR1), *ABCC1* (also named multidrug-associated protein 1 - MRP1) and *ABCG2* (also known as breast cancer resistance protein - BCRP) are also shown.

(D) Schematic of the deletion/fusion locus on chromosome 4 in GSK923295-resistant KBM7 cells (not to scale). *UBE2D3* and *CENPE* genomic regions are indicated (*UBE2D3* - gray, *CENPE* - black, deletion/fusion site - red arrow). Primers used for amplification of the genomic deletion/fusion region are indicated (FW - forward primer, REV - reverse primer).

(E) Agarose gel showing amplified PCR products (~2.7kb) of the deletion/fusion locus from GSK923295-resistant KBM7 cells.