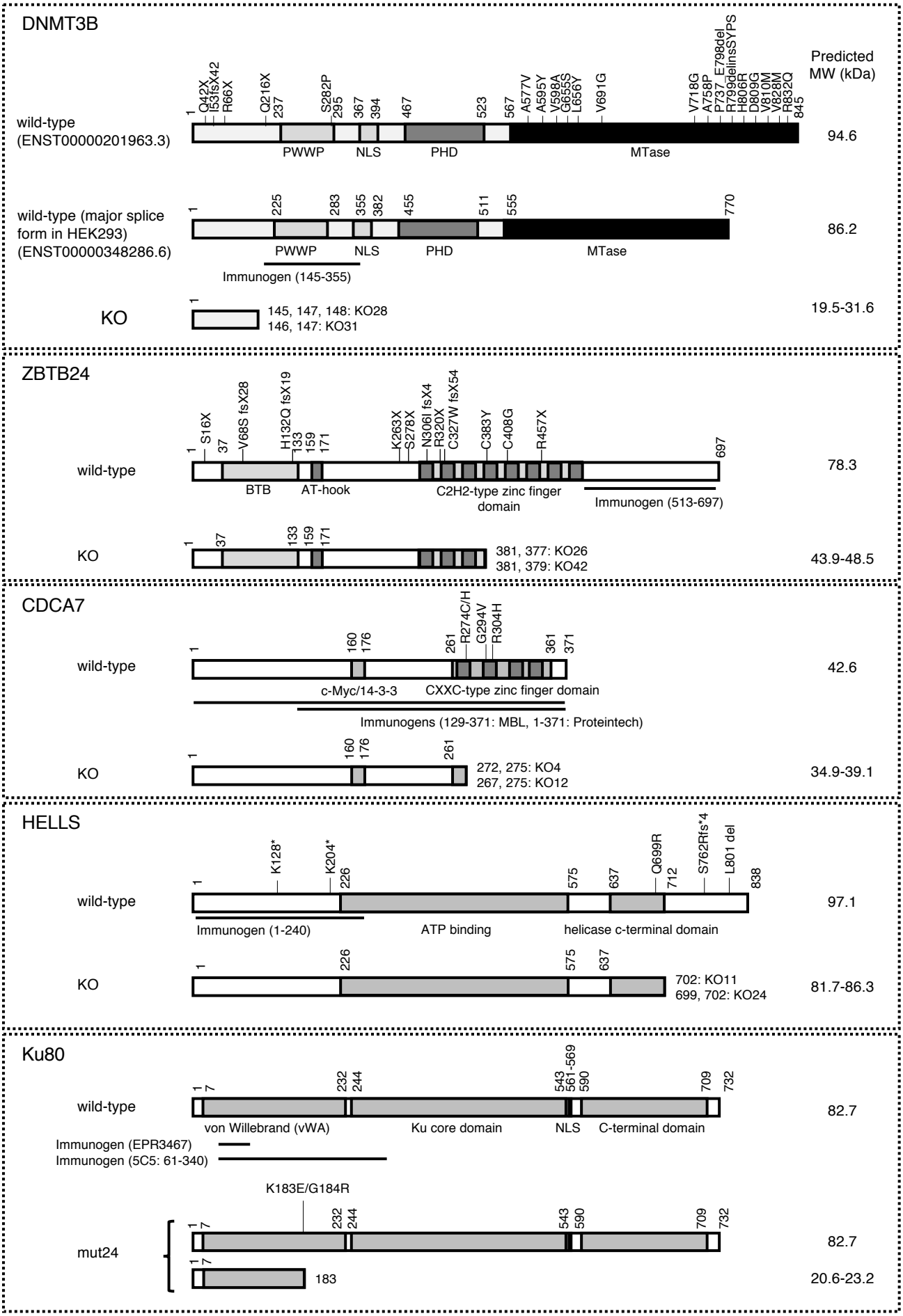
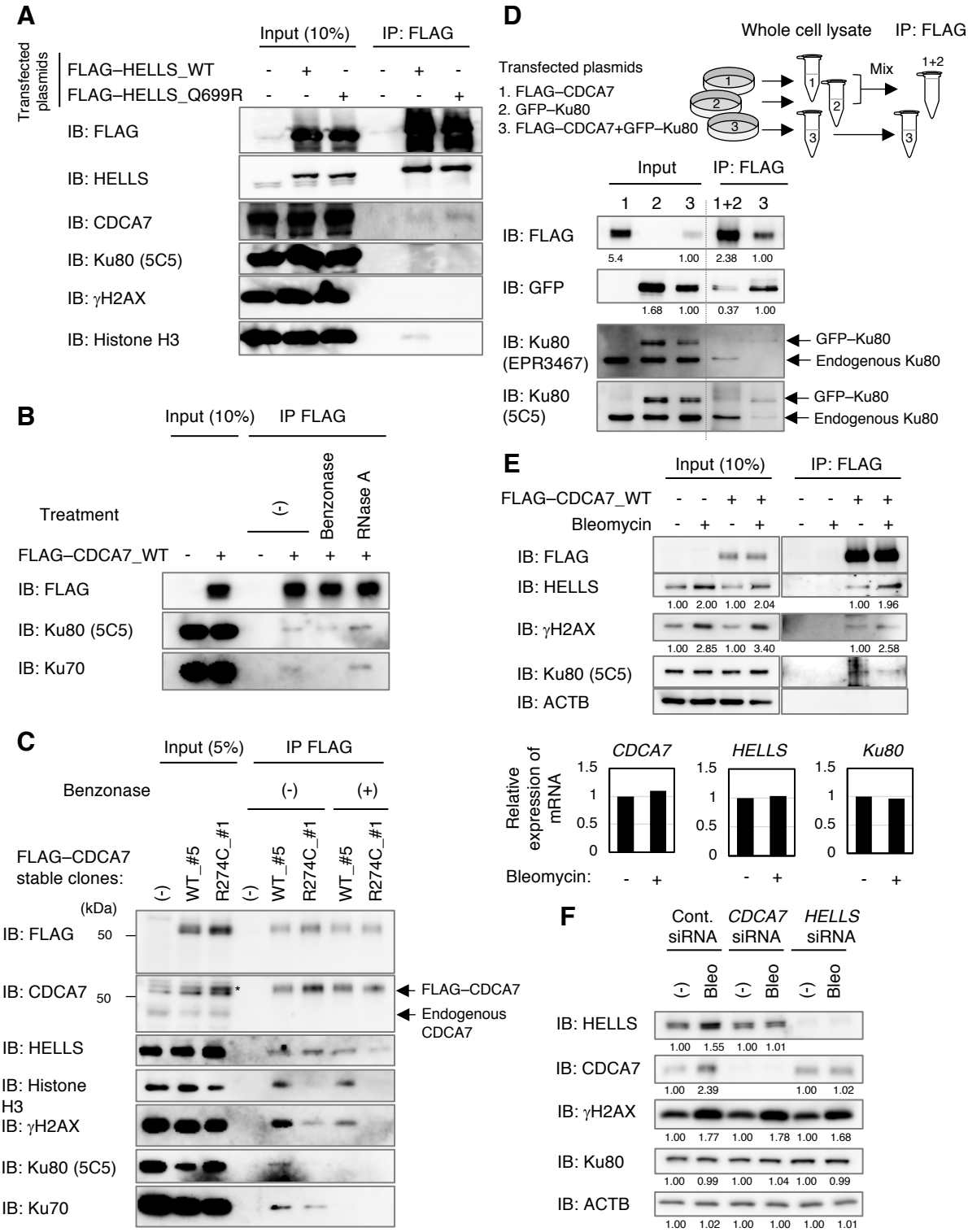
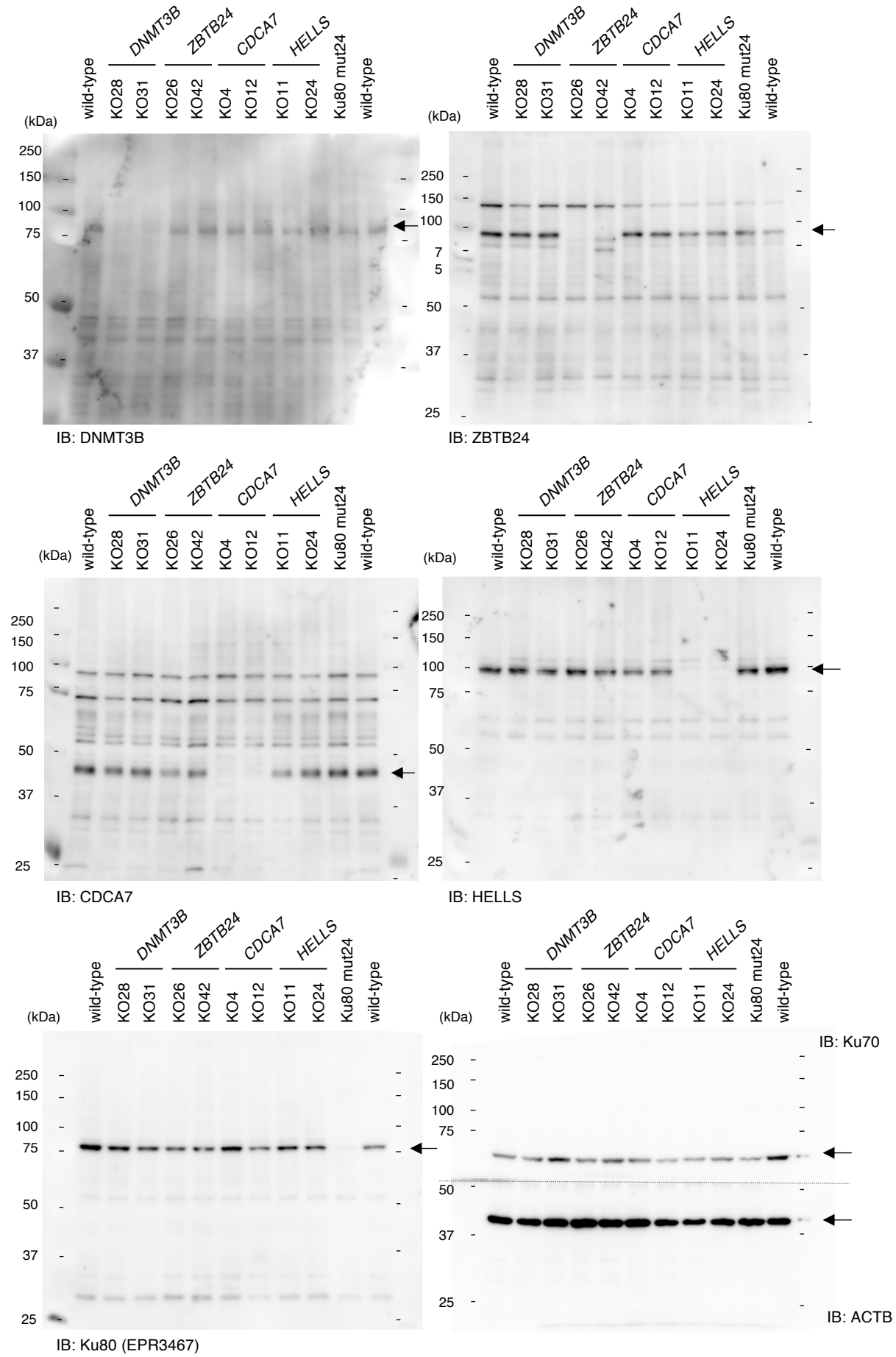
Supplemental data



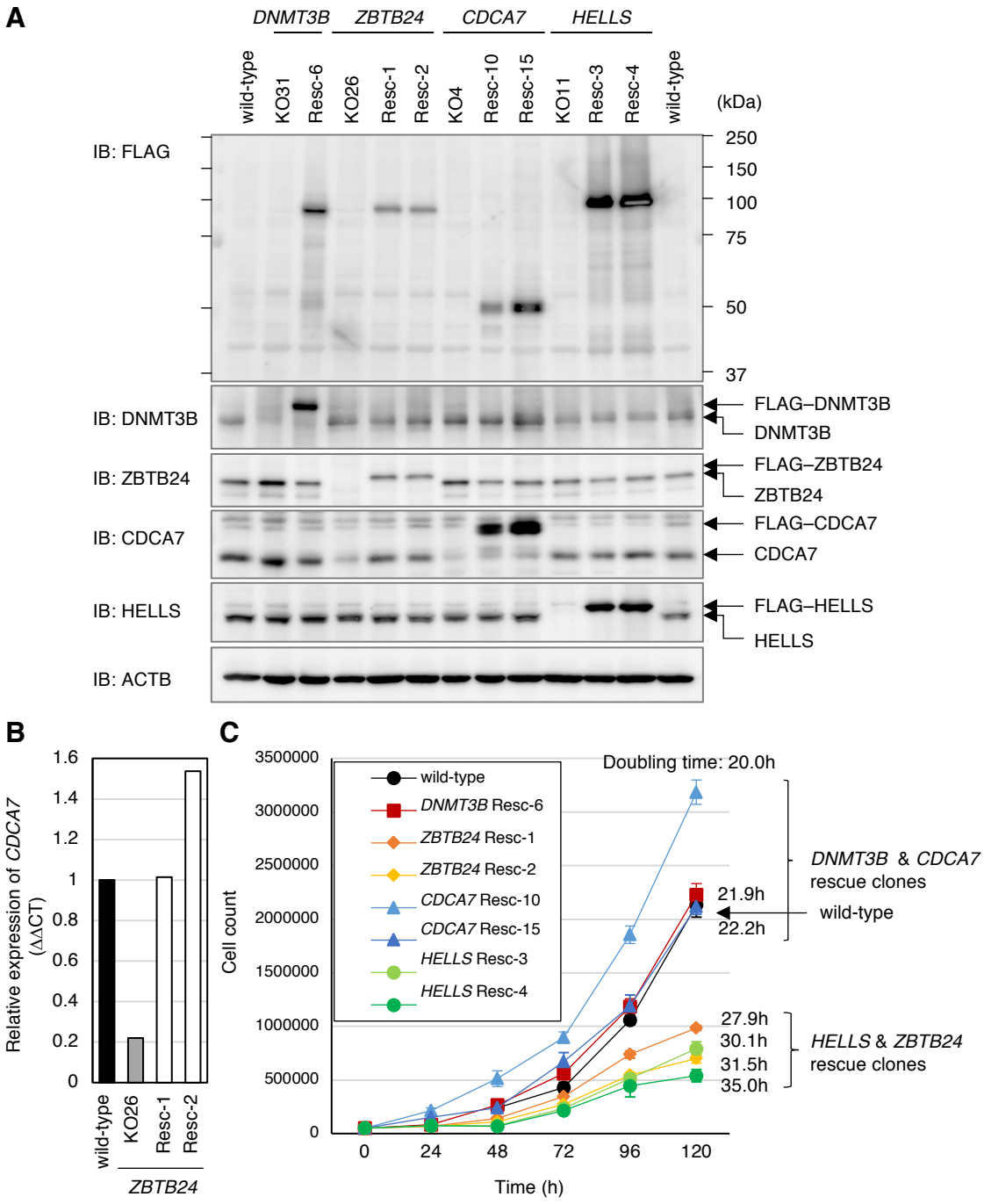
Supplemental Figure 1. Structure of wild-type ICF proteins and Ku80, mutations identified in ICF patients, and predicted truncated proteins in mutant HEK293 cells. Predicted molecular weights (MW) and immunogens recognized by the antibodies used for Western blotting are indicated. See also Supplemental Table 5 for the truncated proteins in mutant cells.



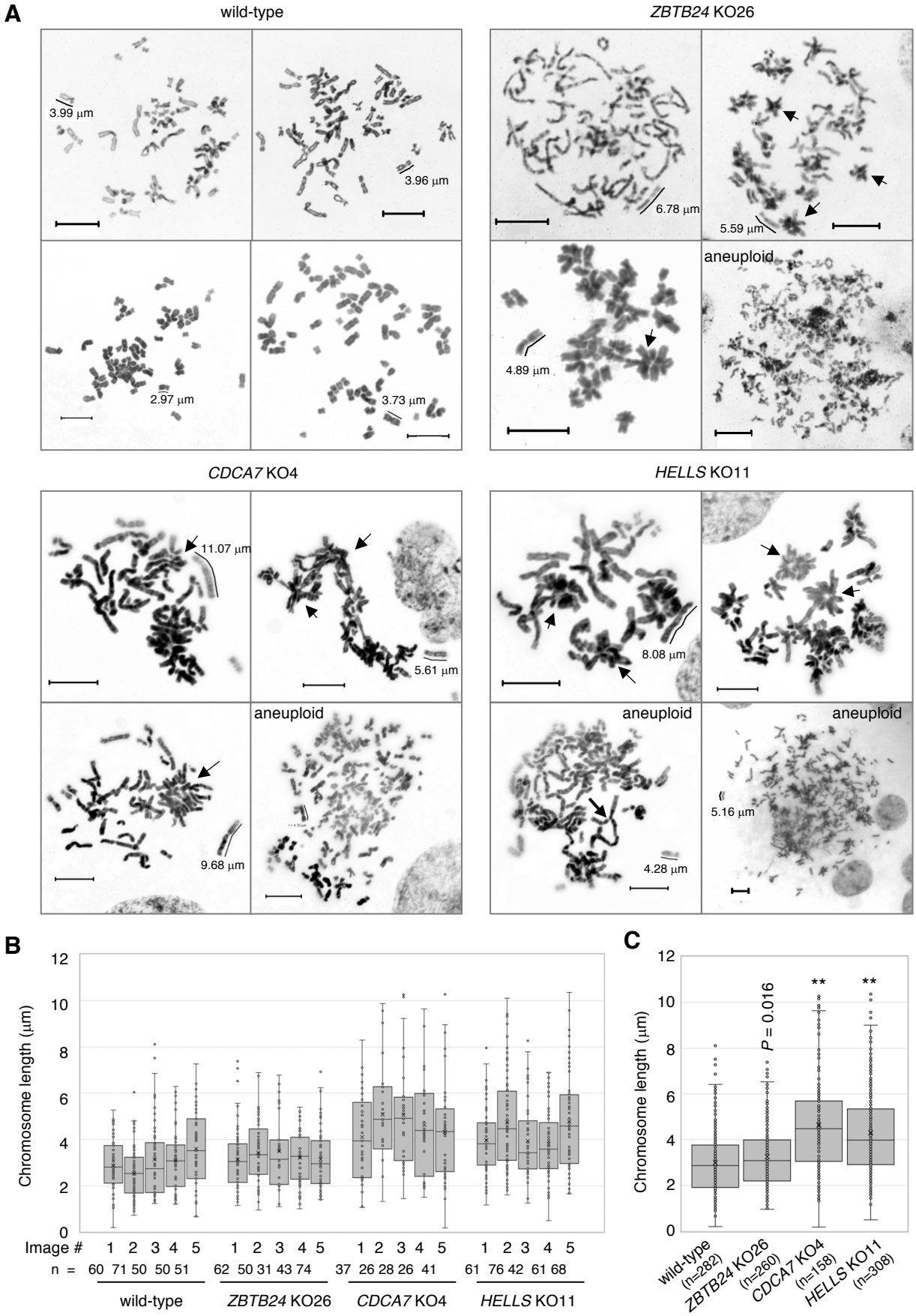
Supplemental Figure 2. Evaluation of coimmunoprecipitation of HELLS, Ku80, and γH2AX with CDCA7 under various conditions. (**A**) Coimmunoprecipitation of indicated proteins with FLAG–HELLS_WT or _Q699R assessed by Western blotting. (**B**) The Ku80/Ku70 heterodimer interacts with CDCA7 via DNA, not RNA. (**C**) Coimmunoprecipitation of indicated proteins detected by Western blotting in HEK293 clones stably expressing FLAG–CDCA7_WT (clone #5) or _R274C (clone #1) at a near endogenous CDCA7 level. An asterisk indicates a non-specific band overlapping with FLAG–CDCA7_WT and _R274C. (**D**) Exclusion of artificial interaction. HEK293T cells were spread into three culture dishes, and transfected with #1 FLAG–CDCA7, #2 GFP–Ku80, or #3 both FLAG–CDCA7 and GFP–Ku80. After 48 h, whole cell lysates were prepared, and those from dish #1 and #2 were mixed. FLAG–CDCA7 was immunoprecipitated with associated proteins to evaluate the possibility of post-preparation interaction between CDCA7 and Ku80. Whole cell lysate from dish #3 was used as a positive control. The signal intensity described below each image was obtained by Image Quant TL software. (**E**) Effect of bleomycin treatment on expression and coimmunoprecipitation of indicated proteins. HEK293T cells were treated with (+) or without (-) bleomycin (5 μM) for 30 min. Coimmunoprecipitation of indicated proteins with FLAG–CDCA7 was assessed by Western blotting (top). Expression of *CDCA7, HELLS,* and *Ku80* mRNAs was detected by qRT-PCR (bottom). *ACTB* was a control. (**F**) Effect of bleomycin treatment on the stability of CDCA7 upon HELLS depletion and vice versa. HEK293T cells were treated with siRNAs against *CDCA7* or *HELLS* 48 h prior to bleomycin treatment (25 μM, 50 min), and subjected to Western blotting. The signal intensity described below each image was obtained by Image Quant TL software.



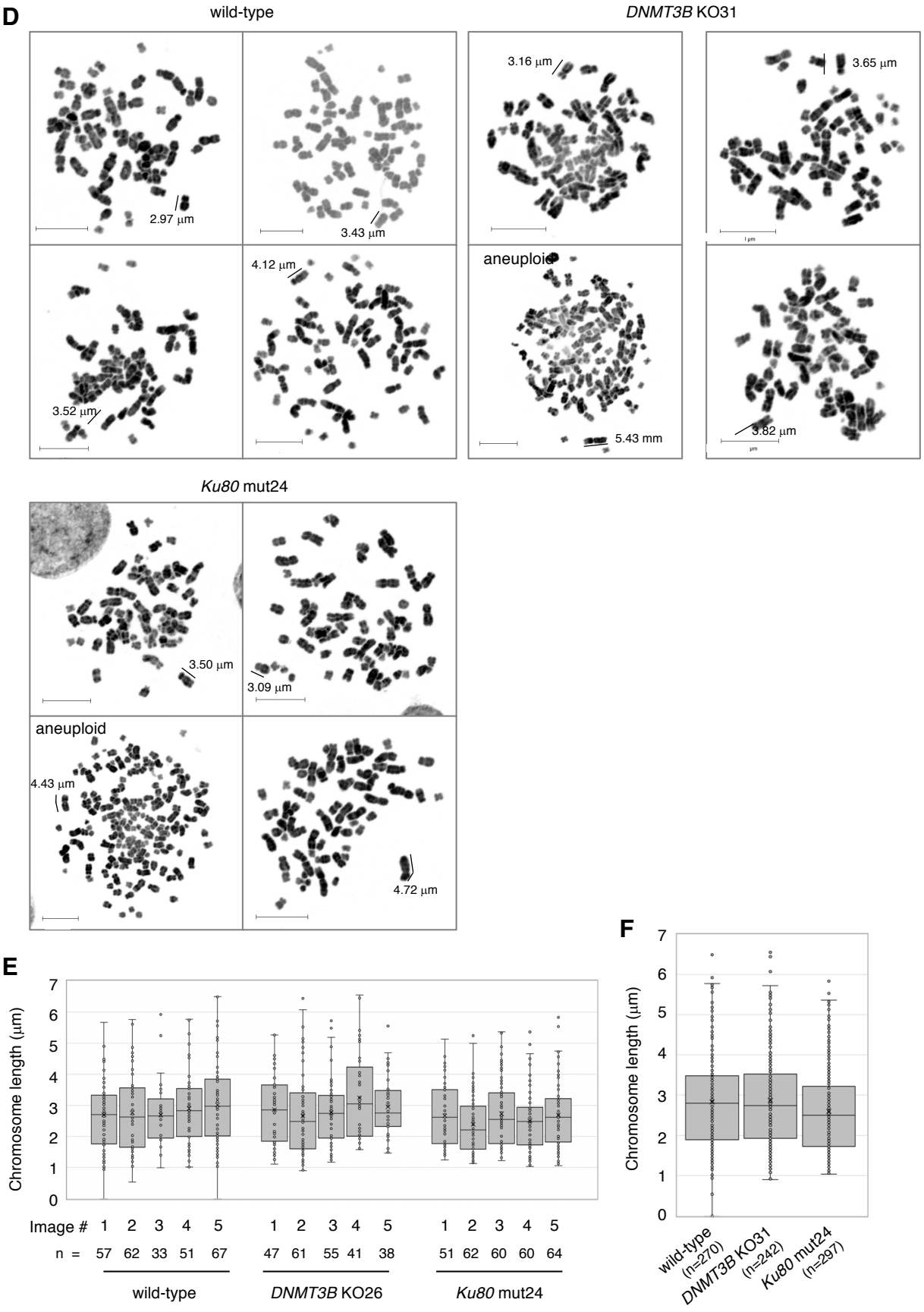
Supplemental Figure 3. Expression of DNMT3B, ZBTB24, CDCA7, HELLS, Ku80, and Ku70 proteins in mutant cells. Western blotting was performed using indicated antibodies. Arrows indicate the band representing the wild-type protein. ACTB was a control. The figure is reshown from Figure 2A .



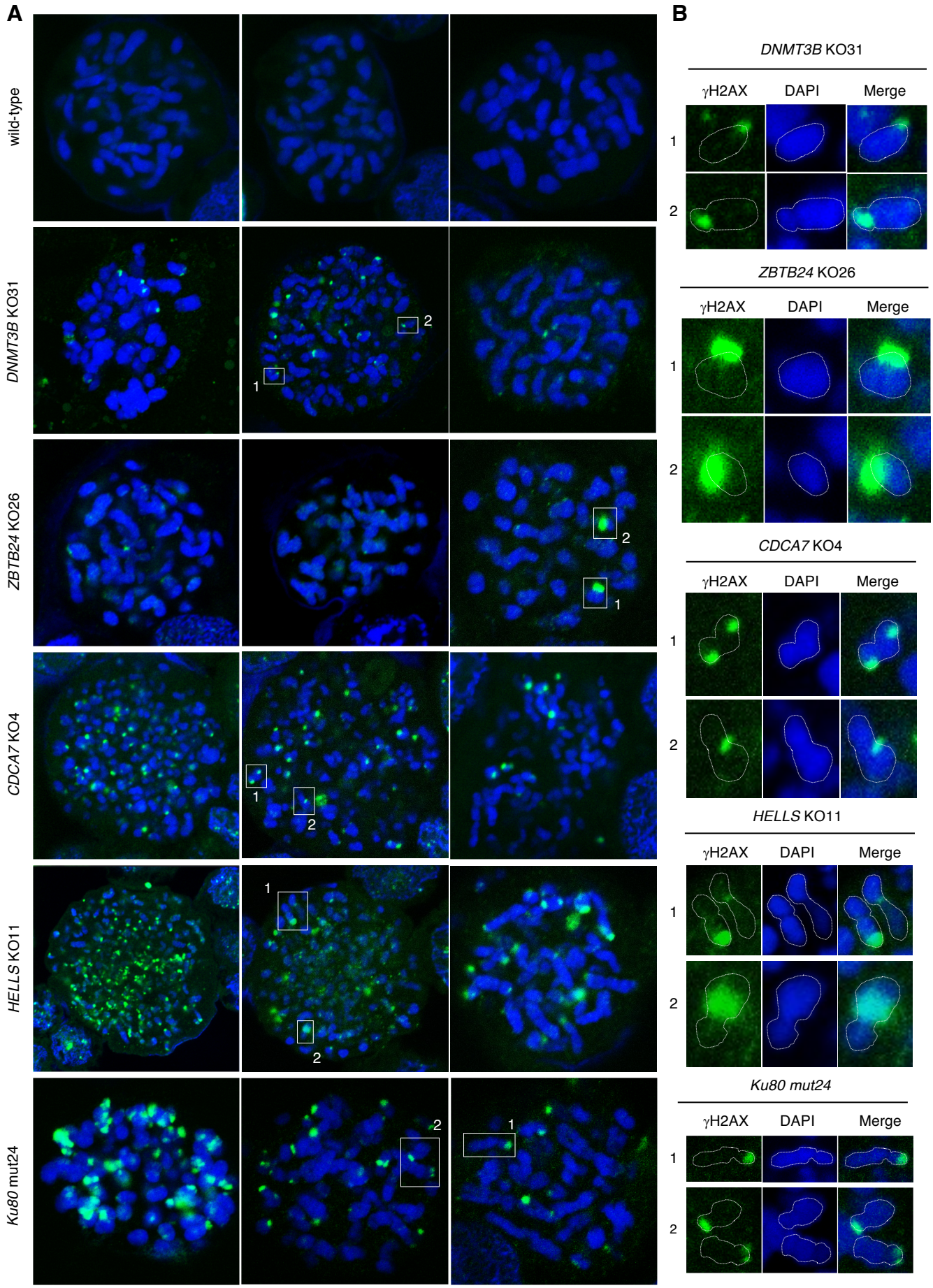
Supplemental Figure 4. Effects of restoration of wild-type proteins in ICF mutant cells. (**A**) Restoration of wild-type proteins confirmed by Western blotting. Two clones expressing FLAG tagged wild-type proteins were established for each mutant clone except for *DNMT3B,* for which only one clone was available. The restored mutant clones were designated "rescue" (Resc) (plus clone number). Western blotting was performed using indicated antibodies. (**B**) Expression of *CDCA7* mRNA in wild-type, *ZBTB24* mutant (KO26), and two rescue clones (Resc-1 and Z Resc-2) was detected by qRT-PCR. *ACTB* was a control. The expression level of *CDCA7* in wild-type cells was set as 1.0. (**C**) Proliferation curves of wild-type and rescue clones. Fifty thousand cells were seeded, cultured, and counted after indicated hours (mean ± SE, n = 4 for each clone). The average doubling time is shown on the right.



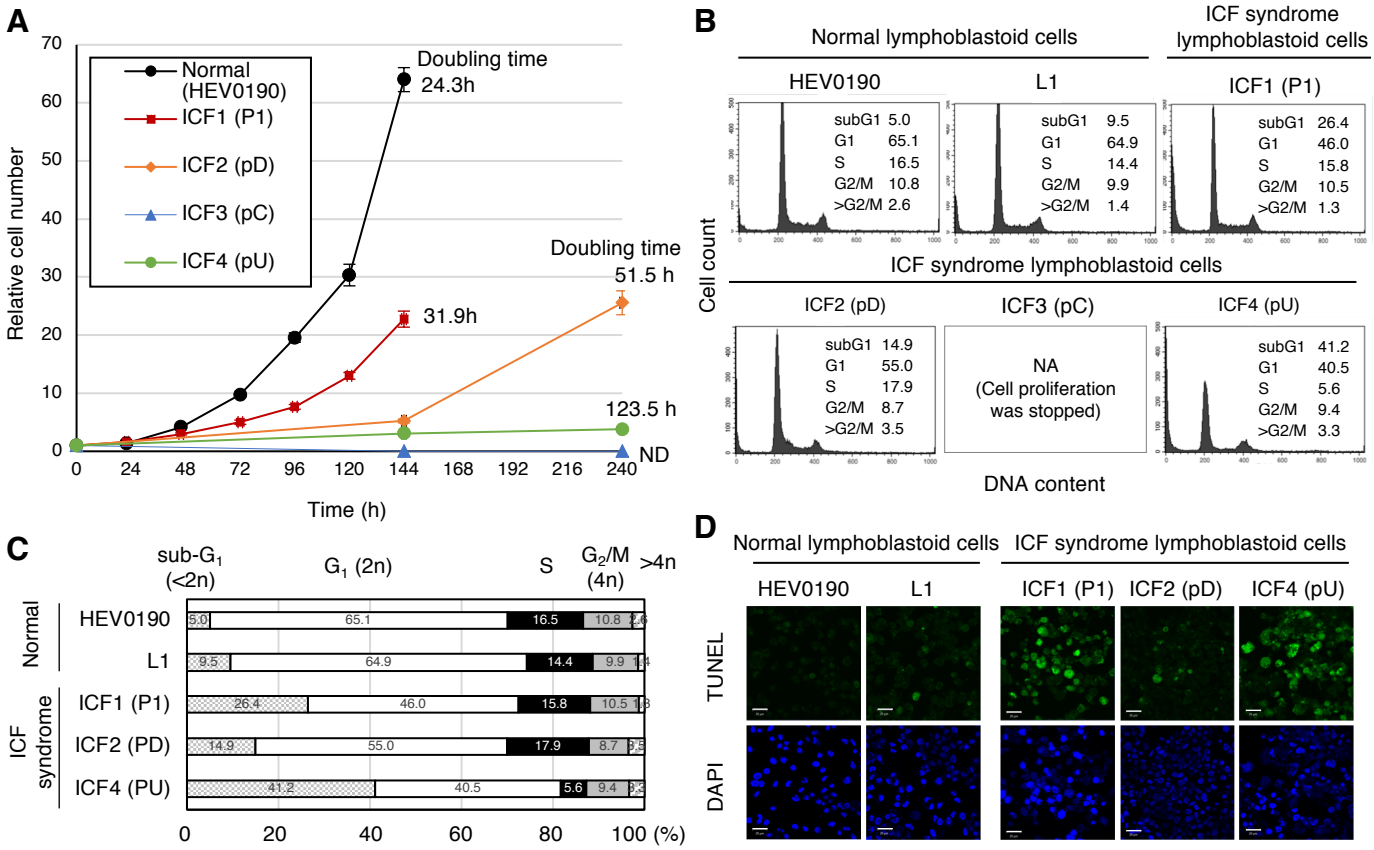
Supplemental Figure 5. Detection of aneuploidy and chromosomal abnormalities in ICF mutant cells. (**A**) Representative images of metaphase spreads (from more than 30 spreads) of wild-type, *ZBTB24* mutant, *CDCA7* mutant, and *HELLS* mutant cells stained by Giemsa or DAPI. Significant increase in aneuploidy was detected in the mutant cells. Many chromosomes were stretched and fragile. Arrows indicate possible chromosomal fusions via centromeric/pericentromeric regions. Scale bars represent 10 μm. (**B**) Average length of chromosomes in the mutant cells. All untangled chromosomes available for measurement (n is described under image#) in five independent spreads were examined per mutant clone. Each box indicates 25–75 percentile and a bar in the box indicates the median. X indicates the mean. (**C**) Combined data presented in (**B**) and statistical significance. $^{**}P < 0.0017$ (Mann-Whitney *U* test) was considered statistically significant at the 1% levels after Bonferroni correction. The exact *P*-value, which is significant ($P < 0.05$) before the correction, is shown for reference.



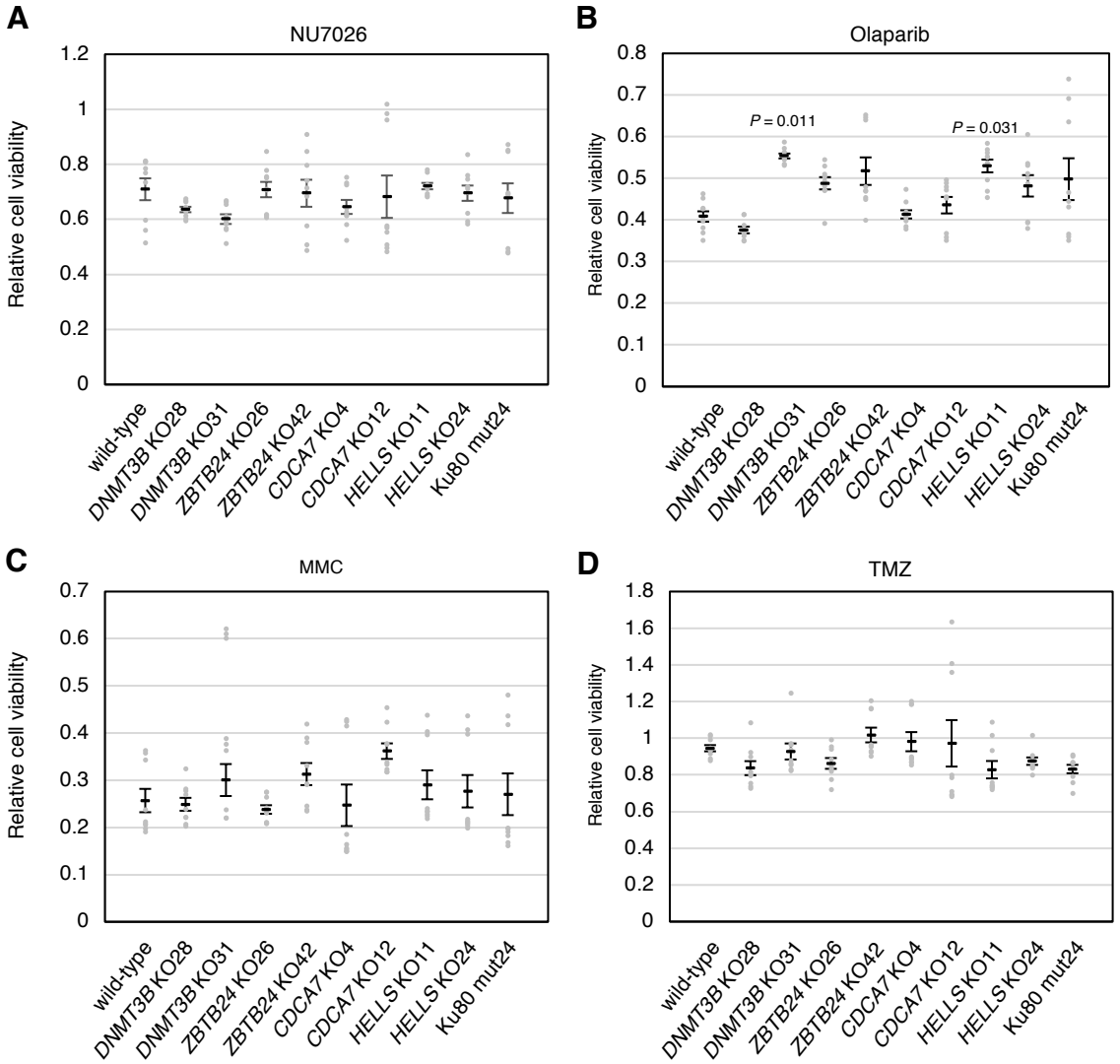
Supplemental Figure 5 (continued). (**D**) Representative images of metaphase chromosome spreads (from 16 spreads) of wild-type, *DNMT3B* mutant, and *Ku80* mutant cells stained by DAPI. Significant increase in aneuploidy was detected, but chromosomes were not stretched or fragile. Scale bars represent 10 μm. (**E**) Average length of chromosomes in mutant cells. Five independent spreads were examined per mutant clone. Each box indicates 25–75 percentile and a bar in the box indicates the median. X indicates the mean. (**F**) Combined data presented in (**E**). There is no statistical significance (Mann–Whitney *U* Test) even before Bonferroni correction.



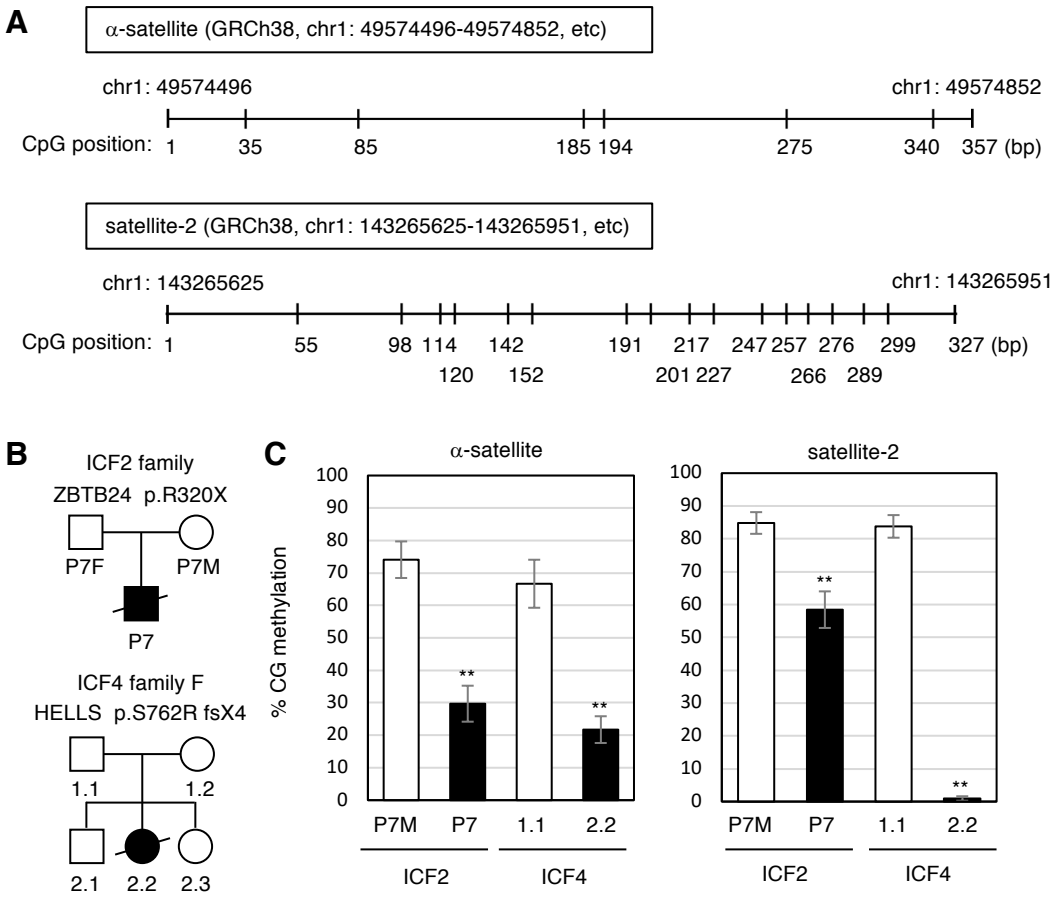
Supplemental Figure 6. Spontaneous and predominant γH2AX staining in centromeric, pericentromeric, and telomeric regions. (**A**) Three representative metaphase chromosome spreads (from more than ten spreads) of wild-type and mutant cells stained with a γH2AX antibody (green) and DAPI. (**B**) Enlarged images of the regions indicated by the boxes numbered 1 and 2.



Supplemental Figure 7. Lymphoblastoid cells from ICF patients display proliferation defects and apoptosis. (**A**) Proliferation curves of lymphoblastoid cells derived from a healthy volunteer and ICF patients. Relative cell numbers (the cell number at 0 h is set as 1.0) and average doubling times are shown (mean ± SE, n = 8 per each clone). Lymphoblastoid cells from an ICF3 patient (pC) stopped proliferation. ND, not determined. (**B** and **C**) Cell cycle analysis of lymphoblastoid cells from healthy volunteers and ICF patients based on DNA content measured by flow cytometry (n = 2). NA, not available. (**D**) Representative images showing apoptotic cells in lymphoblastoid cells from healthy volunteers and ICF patients. For each cell-line, five images were taken and examined. DNA fragmentation was detected by TUNEL assay (green), and nuclei were counterstained with DAPI. Scale bars represent 20 μm.



Supplemental Figure 8. Evaluation of various repair pathways in ICF mutant cells. (A) Relative viability of the mutant cells treated with 10 μM DNA-PK inhibitor, NU7026, for 48 h. The viability was measured by a tetrazolium-based assay and the value for untreated cells was set as 1.0. Welch's *t*-test was done compared against *Ku80* mutant cells. There is no cells showing statistically significant difference even before Bonferroni correction. (**B-D**) Relative viability of the mutant cells treated with 160 μM PARP1/2 inhibitor, olaparib (**B**), 10 μg/ml MMC (**C**) or 100 μM TMZ (**D**) for 48 h. Welch's *t*-test was done compared against wild-type cells. There is no cells showing statistically significant difference after Bonferroni correction (**P* < 0.0011 was considered statistically significant at the 5% level). The exact P-values, which were significant (*P* < 0.05) before the correction, are shown for reference. (**A-D**) All assays were performed in biological triplicate and technical triplicate (n = 9). Data are mean ± S.E.M. MMC; mitomycin C, TMZ; temozolomide.



Supplemental Figure 9. CG methylation of centromeric α-satellite and pericentromeric satellite-2 repeats in ICF patients and their parents. (**A**) Regions of α-satellite and satellite-2 repeats selected for bisulfite sequencing. Positions of CpGs are indicated. There are six and sixteen CpGs in α-satellite and satellite-2 regions, respectively. (**B**) Family trees of ICF2 and ICF4 patients and their parents (14, 15). (**C**) CG methylation (%) of each satellite repeat in an ICF2 patient (P7), his mother (P7M), an ICF4 patient (2.2), and her farther (1.1) determined by bisulfite sequencing. PCR products were cloned into TA-vector and at least 8 clones were sequenced for each sample. Data are mean ± S.E.M. $^{**}P < 0.01$ (Mann–Whitney *U* test).

Supplemental Table 1. Proteins coimmunoprecipitate with FLAG–CDCA7 (mutation-insensitive) (peptide ≥ 2, R274C/WT ≥ 0.6)

Proteins	MW	CDCA7_WT	CDCA7_R274C	R274C/WT
CDCA7	43,458	155	137	0.88
Histone H1.4	21,852	30	21	0.70
Histone H1.3	22,336	26	29	1.12
HUWE1	485,523	26	18	0.69
DDX21	87,804	16	14	0.88
HELLS	97,639	14	11	0.79
HNRH2	49,517	13	14	1.08
1433B	28,179	13	12	0.92
1433Z	27,899	13	10	0.77
1433F	28,372	12	11	0.92
1433G	28,456	10	12	1.20
TOP1	91,125	8	8	1.00
AP2A1	108,561	8	6	0.75
CSNK2A2	41,358	7	6	0.86
NOP2	89,589	6	10	1.67
P53	44,196	6	6	1.00
AP2M1	49,965	6	4	0.67
ZFR	118,079	5	4	0.80
Histone H2B1B	13,942	4	11	2.75
AP2B1	105,398	4	6	1.50
H2A1A	14,225	4	5	1.25
NOM1	96,768	4	5	1.25
2AAA	66,065	4	3	0.75
TCPH	59,842	3	4	1.33
BYST	49,798	3	3	1.00
MOV10	114,512	3	3	1.00
UBP7	129,304	3	3	1.00
LORI	26,828	3	2	0.67
MAP7	84,116	3	2	0.67
NOP56	66,408	3	2	0.67
STAU1	63,428	2	5	2.50
BAG2	23,928	2	2	1.00
DDX18	75,702	2	2	1.00
DDX54	98,819	2	2	1.00
DHX57	157,103	2	2	1.00
MA7D1	93,106	2	2	1.00
PP1G	37,701	2	2	1.00

Supplemental Table 2. Proteins coimmunoprecipitate with FLAG–CDCA7 (mutation-sensitive) (peptide ≥ 2, R274C/WT <0.6)

Proteins	MW	CDCA7_WT	CDCA7_R274C	R274C/WT
Histone H2B1C	13,898	28	7	0.25
Histone H3.1	15,613	16	8	0.50
Histone H4	11,360	16	7	0.44
Ku80	83,222	10	1	0.10
SMARCA5	122,513	9	5	0.56
TCPD	58,401	9	5	0.56
SUPT16H	120,409	7	2	0.29
FBRL	33,877	6	2	0.33
EIF3J	29,159	6	1	0.17
PRKDC	473,749	5	1	0.20
RRBP1	152,780	5	1	0.20
CSNK2A1	45,229	4	2	0.50
RFC2	39,588	4	2	0.50
LA	46,979	4	1	0.25
SYEP	172,080	4	1	0.25
RBBP7	48,132	4	0	ND
RCL1	41,273	4	0	ND
NKRF	78,308	3	1	0.33
LAMC3	176,933	3	0	ND
MYH10	229,827	3	0	ND
NEUA	49,033	3	0	ND
POTEE	122,882	3	0	ND
RBM4	40,688	3	0	ND
RFA1	68,723	3	0	ND
WDR5	37,136	3	0	ND
BMS1	146,571	2	1	0.50
GRSF1	53,606	2	1	0.50
IF4A1	46,353	2	1	0.50
NOG1	74,317	2	1	0.50
PSME3	29,602	2	1	0.50
RALY	32,501	2	1	0.50
REN3B	57,841	2	1	0.50
RFC4	40,170	2	1	0.50
SSRP1	81,367	2	1	0.50
THOC4	26,872	2	1	0.50
UBE2O	142,631	2	1	0.50
ARID1A	242,805	2	0	ND
BUB3	37,587	2	0	ND
DNJA3	53,083	2	0	ND
ECHB	51,547	2	0	ND
EXOC4	111,170	2	0	ND
H1FNT	28,213	2	0	ND
Histone H1X	22,474	2	0	ND
Histone H2AX	15,135	2	0	ND
KDM8	47,867	2	0	ND
LYAR	44,044	2	0	ND
M3K7	67,895	2	0	ND
MAGB1	39,184	2	0	ND
TFAM	29,306	2	0	ND
WDR20	63,709	2	0	ND

Supplemental Table 3. Proteins coimmunoprecipitate with FLAG–HELLS (mutation-insensitive) (peptide ≥ 2, R274C/WT ≥ 0.6)

Proteins	MW	HELLS_WT	HELLS_Q699R	Q699R/WT
HELLS	97,639	283	273	1.0
IMA1	58,168	4	3	0.8
Histone H1.3	22,336	3	4	1.3
LA	46,979	3	3	1.0
GBB1	38,151	3	2	0.7
Histone H3.1	15,613	3	2	0.7
M4A10	30,070	2	5	2.5
MYH10	229,827	2	3	1.5
1433Z	27,899	2	2	1.0
HAT1	49,880	2	2	1.0
POTEE	122,882	2	2	1.0

Supplemental Table 4. Proteins coimmunoprecipitate with FLAG–HELLS (mutation-sensitive) (peptide ≥ 2, R274C/WT <0.6)

Proteins	MW	HELLS_WT	HELLS_Q699R	Q699R/WT
Histone H2A1A	14,225	6	0	ND
MAGB1	39,184	4	1	0.3
DIDO1	245,434	4	0	ND
TBB6	50,281	4	0	ND
GRDN	216,593	3	1	0.3
1433G	28,456	3	0	ND
GBB2	38,048	3	0	ND
KDIS	198,016	3	0	ND
Histone H2B1M	13,981	3	0	ND
Histone H2B1B	13,942	2	1	0.5
Histone H2B1H	13,884	2	1	0.5
ATPA	59,828	2	1	0.5
APC	313,622	2	0	ND
RBBP7	48,132	2	0	ND
RN152	23,141	2	0	ND
SPR2D	8,584	2	0	ND
ZN717	108,091	2	0	ND

Supplemental Table 5. Summary of mutations introduced into HEK293 cells by genome editing (See also Supplemental Figure 1)

Gene	Disease	Chr.	Ensembl Transcript ID	Clone #	Mutations generated by CRISPR/Cas9 system[A]	Targeted exon	Premature stop codon	Total exon numbers
DNMT3B	ICF1	20q11.21	ENST00000348286.6	KO28	c.440_441insA (p.Arg148fsX140), c.439_442delAGAC (p.Arg147fsX43), c.433_442del TCCCTGAGAC (p.Ser145fsX43)	exon 6	exon 8, exon 6, exon 6	23
DNMT3B	ICF1	20q11.21	ENST00000348286.6	KO31	c.438_442delGAGAC (p.Arg147fsX139), c.436_455delCTGAGACGGCGGGCAACAGC (p.Lys146fsX135), c.437_470delTGAGACGGCGGGCAACAGCATCGGCAGGAACGCC (p.Lys146fsX34)	exon 6	exon 8, exon 8, exon 6	23
ZBTB24	ICF2	6p21	ENST00000230122.3	KO26	c.1141_1142insA (p.Asp381fsX50), c.1129_1157delTCTTTTACCTGTGATCAATGCGGAAAATA (p.Ser377fsX44)	exon 4	exon 6, exon 6	7
ZBTB24	ICF2	6p21	ENST00000230122.3	KO42	c.1141_1142insA (p.Asp381fsX50), c.1136_1142delCCTGTGA (p.379ThrfsX12), c.1142_1152delATCAATGCGGA (p.Asp381fsx46)	exon 4	exon 6, exon 4, exon 6	7
CDCA7	ICF3	2q31.1	ENST00000347703.7	KO4	c.824delA (p.Gln275fsX72), c.815_830delAATGCCGTCAGAAGAC (p.Gln272fsX70)	exon 7	exon 8, exon 8	9
CDCA7	ICF3	2q31.1	ENST00000347703.7	KO12	c.824_834delAGAAGACTATT_835G>A (p.Gln275fsX27), c.799_835delGGCTCTACTTGTCATCAATGCCGTCAGAAGACTATTG (p.Gly267fsX68)	exon 7	exon 9, exon 8	9
HELLS	ICF4	10q23.33	ENST00000348459.9	KO11	c.2103_2104delTC (p.Lys702fsX4)	exon 19	exon 19, exon 19	22
HELLS	ICF4	10q23.33	ENST00000348459.9	KO24	c.2095_2104delCAGTCGGATC (p.Gln699fsX43), c.2105_2111delTTCAGGC (p.Lys702fsX41)	exon 19	exon 19, exon 19	22
Ku80	Cancer, SCID	2q35	ENST00000392132.6	mut24	c.252_253insT (p.Lys183fsX21), c.253_254insAATCCTGCGGCCCAACTGGCGATTATCAGCCAGATGAGCAGGCATG_254T>A(p.Lys183X), c.253_259TTAGGT>GAAAGG(p.183_184LysGly>GluArg)	exon 6	exon 6, exon 6, N/A	21

[A]HEK293 cells are hypotriploid.

Supplemental Table 6. Primer sequences used in this study

Gene	Primer name	Sequence	Experiment
Cas9	Forward-1	5´-*TGGACTATAAGGACCACGAC*-3´	Genomic PCR
Cas9	Forward-2	5´-*AAGAGAATGCTGGCCTCTGC*-3´	Genomic PCR
Cas9	Reverse-1	5´-*TCGATCCGTGTCTCGTACAG*-3´	Genomic PCR
DNMT3B	Forward	5´-*CTGAAGCCCATGTTGGAGTG*-3´	qRT-PCR
DNMT3B	Reverse	5´-*ATTTGTCTTGAGGCGCTTGG*-3´	qRT-PCR
ZBTB24	Forward	5´-*AGGCCCGCTGTAAAGACTGT*-3´	qRT-PCR
ZBTB24	Reverse	5´-*GGACCTGTAGCGAGTGCTTC*-3´	qRT-PCR
CDCA7	Forward	5´-*TGGAAGAAATTACAGAGGAGG*-3´	qRT-PCR
CDCA7	Reverse	5´-*ATGACAAGTAGAGCCCAGTG*-3´	qRT-PCR
HELLS	Forward	5´-*ACTGATCAGTCAAATACAGCC*-3´	qRT-PCR
HELLS	Reverse	5´-*CTGCAGCTTCAGATTAACTTC*-3´	qRT-PCR
Ku80	Forward	5´-*GGAGGGGAAGTGCTTCTCTG*-3´	qRT-PCR
Ku80	Reverse	5'-*CACGCCGACTTGAGGATTAG*-3´	qRT-PCR
ACTB	Forward	5´-*CCAACCGCGAGAAGATGA*-3´	qRT-PCR
ACTB	Reverse	5´-*CCAGAGGCGTACAGGGATAG*-3´	qRT-PCR
Uncut and joined DNA	BP-F1	5´-*GTACGGTGGGAGGTCTATATAAG*-3´	Genomic PCR
Uncut and joined DNA	300-DR	5´- *GCCGTCCTCGTACTTCTCGAT*-3´	Genomic PCR
Internal control	2500-DF	5'-*CCCTGAACCTGAAACATAAAATGA*-3´	Genomic PCR
Internal control	2500-DR	5'-*TGTGAAATTTGTGATGCTATTGCTT*-3'	Genomic PCR
satellite-2	Forward	5'-*AATGAAAGGAGTTATTATTTAATGG*-3´	Bisulfite PCR
satellite-2	Reverse	5´-*CATTCCATTCCATTAAATAATTCC*-3´	Bisulfite PCR
α–satellite	Forward	5´-*GTTTTGAGGTTAAAGGTAGAAAAG*-3´	Bisulfite PCR
α-satellite	Reverse	5´-*TTCCTTTTCTACCATTAACCTC*-3'	Bisulfite PCR

Supplemental Table 7. Antibodies used in this study

Name	Company	Catalog#	Clone #	Dilution
anti-FLAG mouse monoclonal antibody	MBL	M185-3L	FLA-1	1:1000
anti-DNMT3B sheep polyclonal antibody	R&D systems	AF7646-SP	-	1:1000
anti-ZBTB24 guinea pig polyclonal antibody	MBL	PM085	-	1:1000
anti-CDCA7 rabbit polyclonal antibody	MBL	MB0148	-	1:1000
anti-HELLS rabbit polyclonal antibody	Santa Cruz Biotechnology	sc-28202	-	1:1000
anti-Ku80 rabbit monoclonal antibody	Abcam	Ab79391	EPR3467	1:1000
anti-Ku80 mouse monoclonal antibody	Abcam	ab119935	5C5	1:1000
anti-Ku70 rabbit monoclonal antibody	Abcam	ab92450	EPR4027	1:1000
anti-p53 rabbit monoclonal antibody	Cell Signaling Technology	2527	7F5	1:1000
anti-H2AX rabbit monoclonal antibody	Cell Signaling Technology	7631	D17A3	1:1000
anti-γH2AX mouse monoclonal antibody	Millipore	05-636	JBW301	1:1000
anti-SMARCA5 rabbit polyclonal antibody	Abcam	Ab3749	-	1:1000
anti-SUPT16H rabbit monoclonal antibody	Abcam	Ab108960	EPR3685	1:1000
anti-Histone H3 rabbit polyclonal antibody	Abcam	Ab1791	-	1:1000
anti-UHRF1 mouse monoclonal antibody	BD Biosciences	612264	28/ICBP90	1:1000
anti-PCNA mouse monoclonal antibody	BioLegend	307902	PC10	1:1000
anti-GFP rabbit polyclonal antibody	MBL	598	-	1:1000
anti-Pericentrin mouse monoclonal antibody	Abcam	ab28144	mAbcam 28144	1:1000
anti-ACTB mouse monoclonal antibody	Santa Cruz Biotechnology	sc-69879	AC-15	1:1000
anti-DDX21 rabbit polyclonal antibody	Proteintech	10528-1-AP	-	1:1000