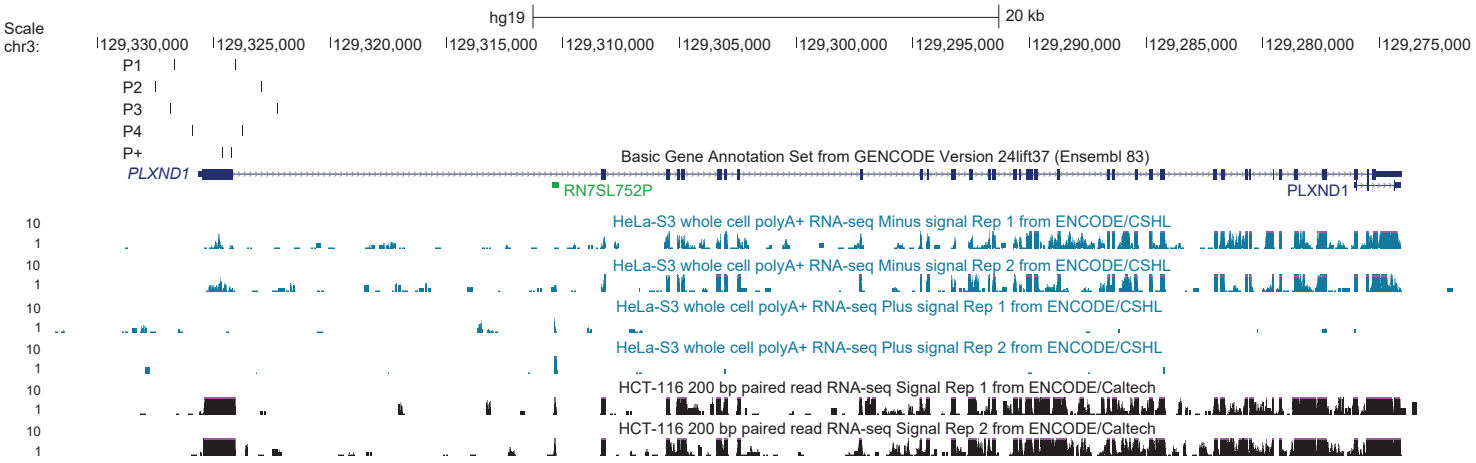
a

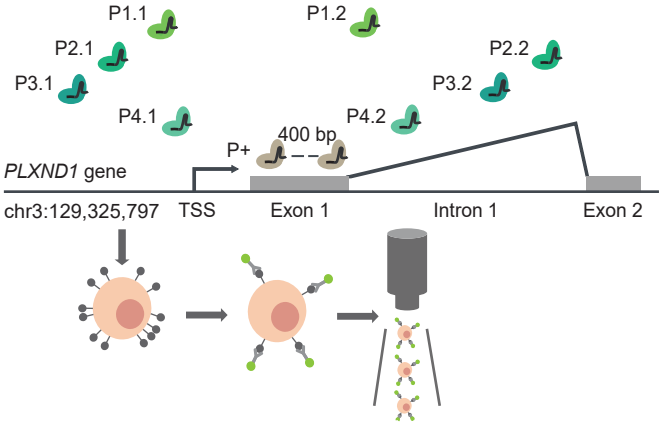


Supplemental Fig S1 | *PLXND1* genomic locus. a, Image from the UCSC Genome Browser (GRCh37/hg19). *PLXND1* gene is shown in dark blue (exons are represented as thick boxes and introns as lines). sgRNA pairs are depicted (*P1*, *P2*, *P3*, *P4*, *P+*) around exon 1. Whole-cell RNA expression in HeLa (ENCODE/Cold Spring Harbor Lab; plus and minus strand) and HCT116 (ENCODE/Caltech).

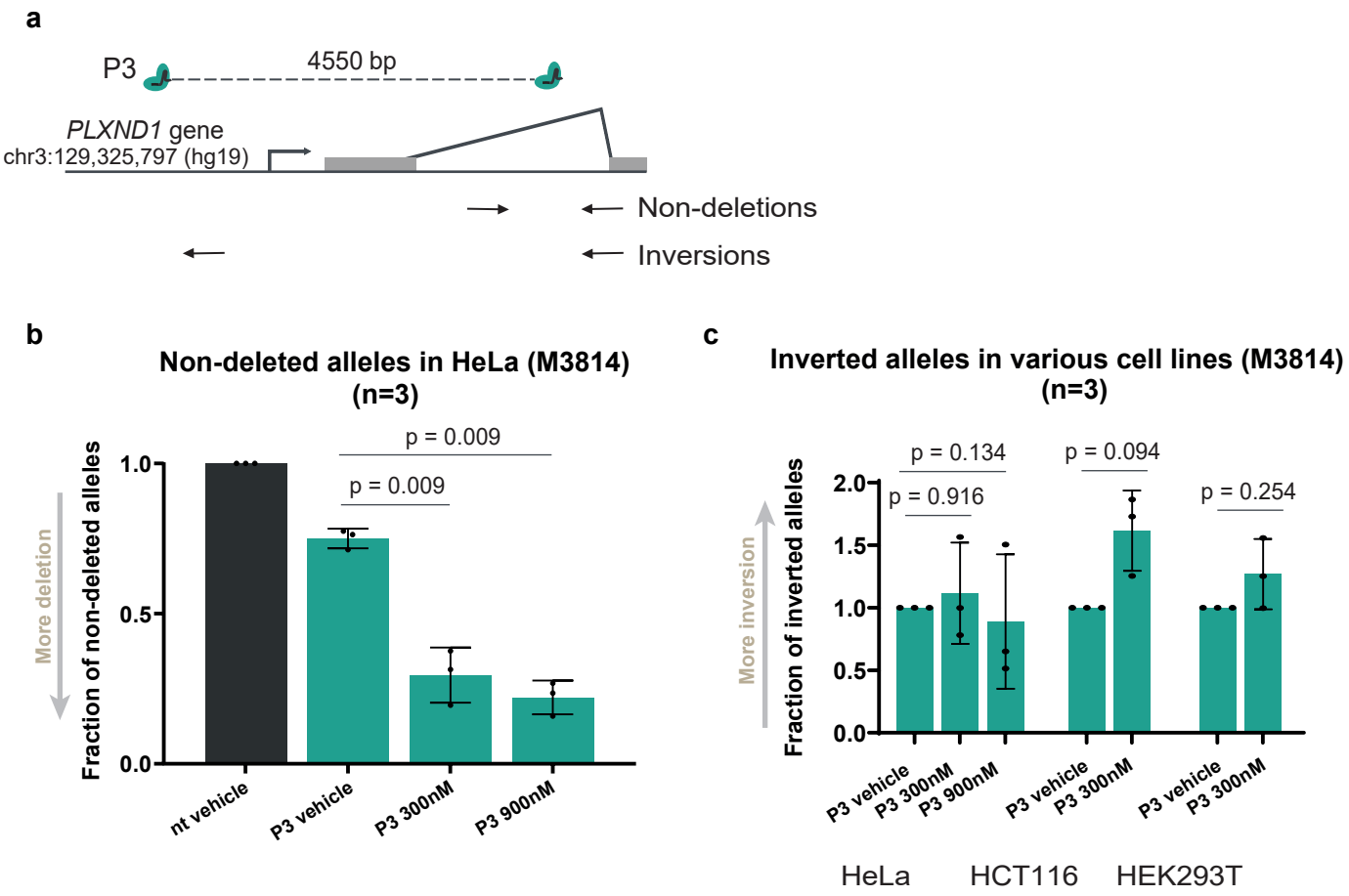
HeLa (M3814)
(n=3)

Fraction of plexin D1 negative cells

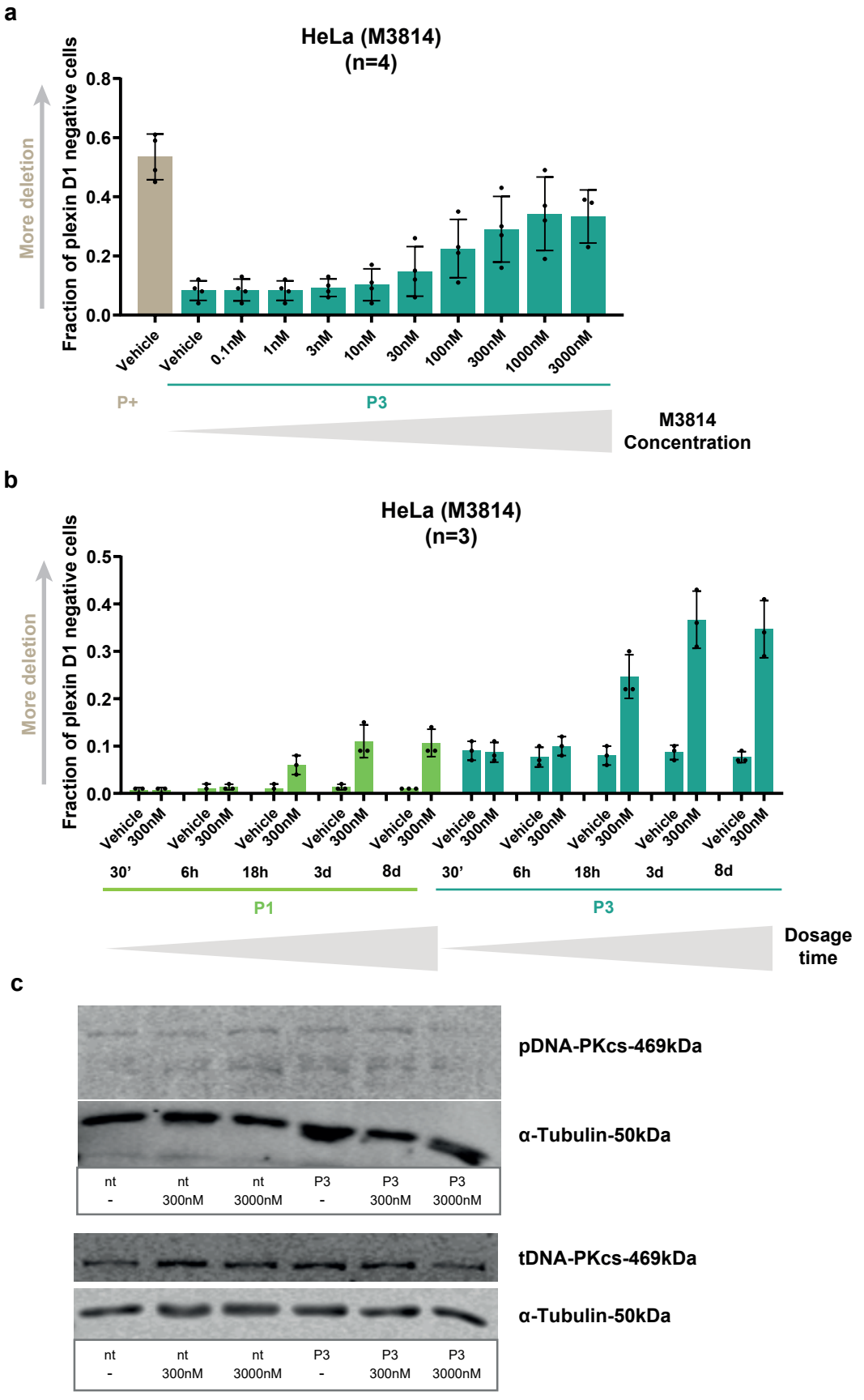
		Average	Standard deviation
P+	Vehicle	0.909	0.013
	300nM	0.896	0.017
P1.1	Vehicle	0.013	0.015
	300nM	0.000	0.008
P1.2	Vehicle	0.009	0.005
	300nM	0.004	0.007
P2.1	Vehicle	0.004	0.011
	300nM	0.002	0.009
P2.2	Vehicle	0.006	0.009
	300nM	0.003	0.009
P3.1	Vehicle	0.006	0.012
	300nM	0.002	0.007
P3.2	Vehicle	0.001	0.009
	300nM	0.000	0.008
P4.1	Vehicle	0.016	0.015
	300nM	0.012	0.012
P4.2	Vehicle	0.016	0.014
	300nM	0.010	0.008



Supplemental Fig S2| Effect of single sgRNAs around *PLXND1* first exon. Left: CRISPR-del efficiency of each single sgRNA as measured by CiDER in HeLa with and without DNA-PKcs inhibition (mean, standard deviation). Right: Scheme of CiDER single sgRNAs.

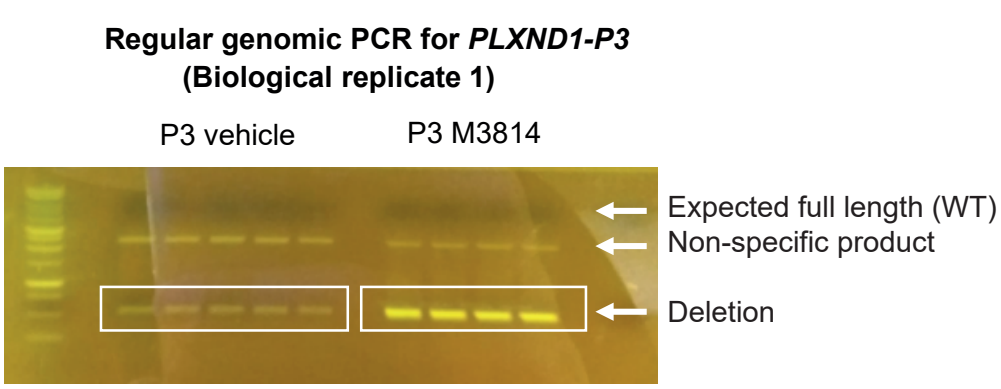


Supplemental Fig S3| . Effect of DNA-PKcs inhibition in the *PLXND1* locus. a, Scheme of the locus showing the sgRNA pair used (P3) and QC-PCR primers used to detect non-deleted and inverted alleles. **b,** CRISPR-del efficiency in HeLa cells. The bar plots show the fraction of non-deleted alleles in the pooled population quantified by qPCR (mean, standard deviation, 1-tailed paired *t*-test on the 2^dCt values previous to normalization to the vehicle samples). The black bar corresponds to a nontargeting control used for normalization in the ratio calculation. Blue bars correspond to non-treated and M3814-treated samples targeting *PLXND1* (P3). **c,** Proportion of inverted alleles in the pooled population quantified by qPCR and normalized to the P3 non-treated (vehicle) samples (mean, standard deviation, 2-tailed paired *t*-test on the 2^dCt values previous to normalization to the vehicle samples).

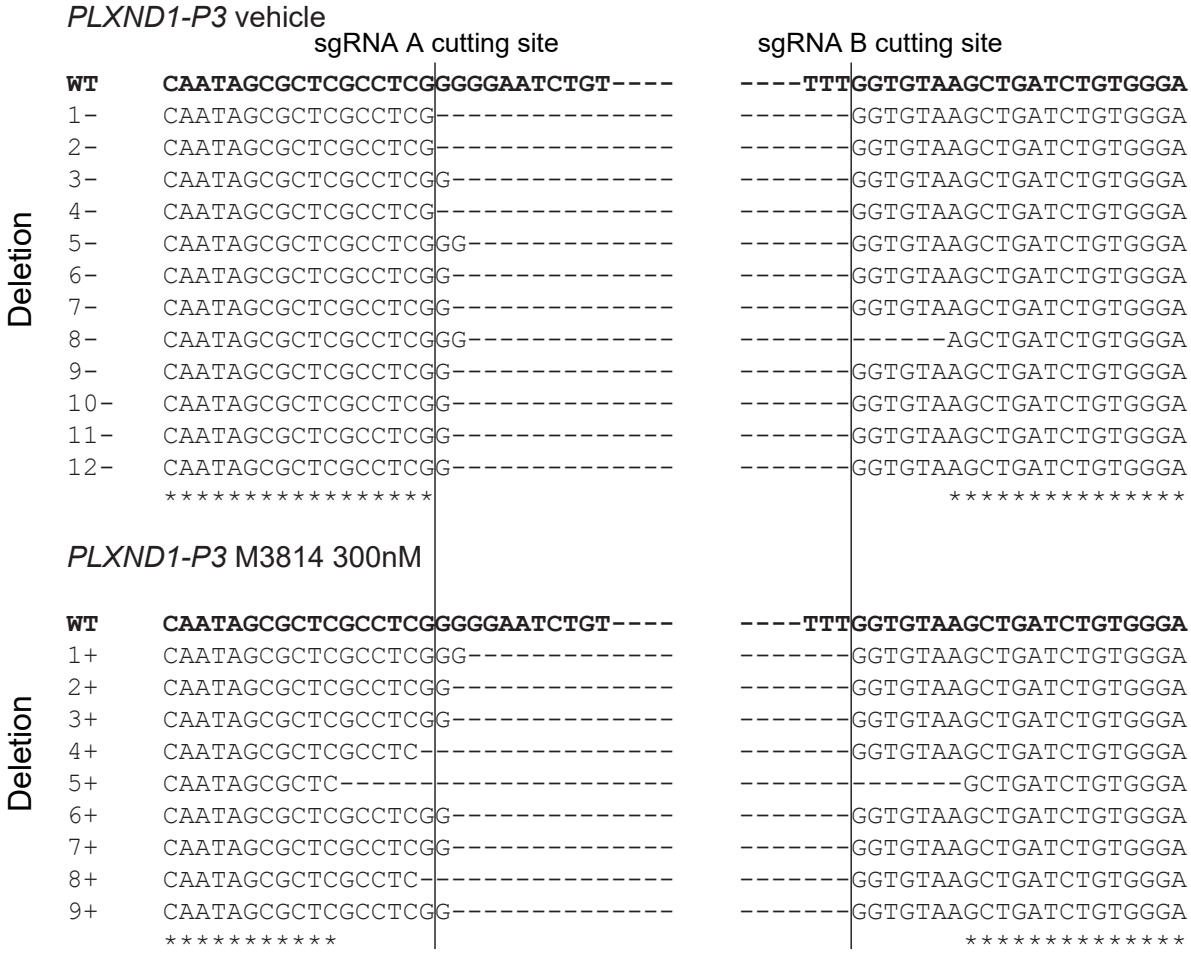


Supplemental Fig S4| Effect of different DNA-PKcs inhibition conditions. a, Dose-response curve performed with by CiDER with pgRNA P3 (green). The positive *PLXND1* ORF-targeting control (P+) is shown in tan. **b,** Effect of different DNA-PKcs inhibition duration on CRISPR-del efficiency (30 minutes, 6 hours, 18 hours, 3 days and uninterrupted inhibition for 8 days). CiDER pgRNAs P1 and P3 were used. The graph displays the fraction of plexin D1 negative cells detected by flow cytometry (mean, standard deviation). **c,** Detection of active phosphorylated DNA-PKcs (pDNA-PKcs) in untreated (-) and M3814-treated (300nM and 3000nM) samples with pgRNAs targeting *PLXND1* (P3) and a nontargeting control (nt). Upper panel: western blot detecting pDNA-PK. α-Tubulin is used as loading control. Bottom panel: total levels of DNA-PKcs (tDNA-PKcs).

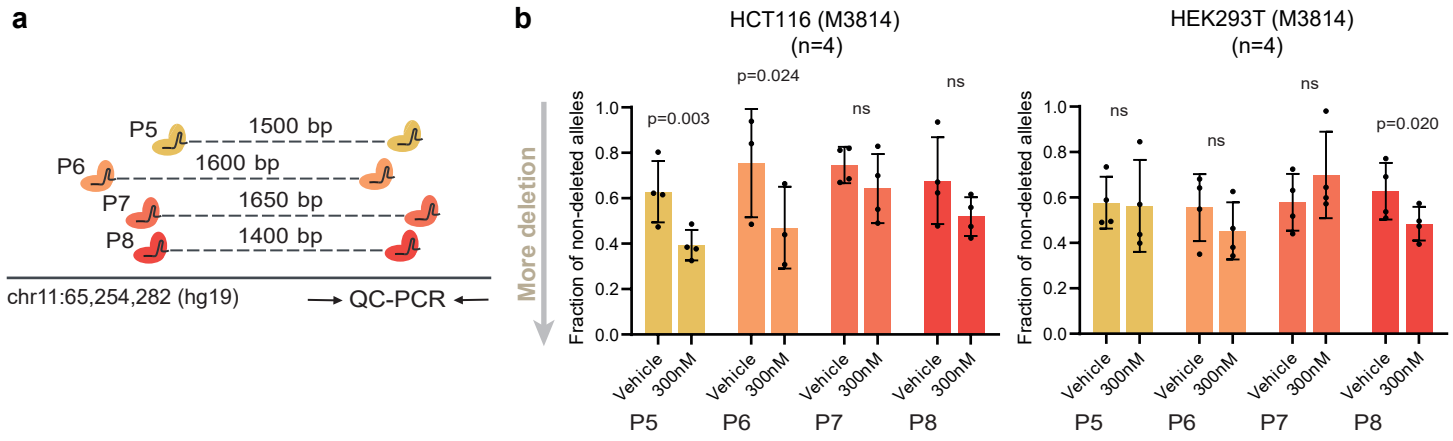
a



b



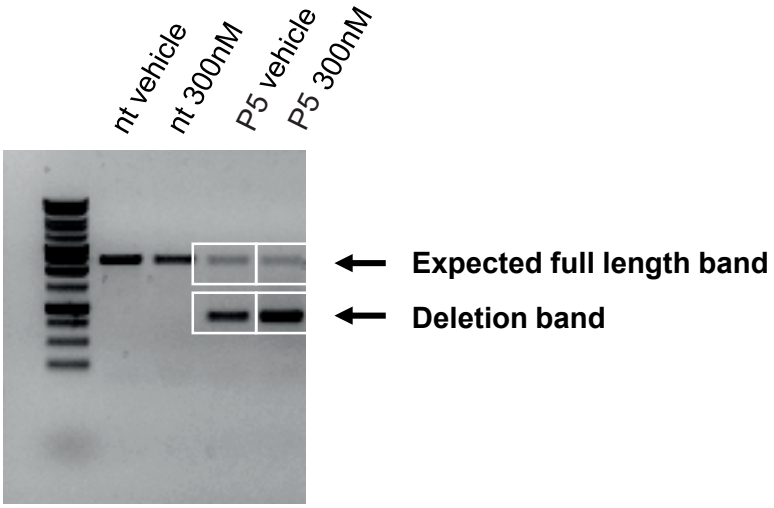
Supplemental Fig S5| CiDER deletion outcomes in DNA-PKcs inhibition untreated and treated cells. a, Genomic PCR amplification of the *PLXND1* gene from untreated and treated CiDER samples using the pgRNA-P3. Deletion bands are boxed. **b,** Sanger sequencing alignment results of TOPO cloned alleles of purified PCR deletion bands from (a). Expected sgRNAs cut sites are indicated by vertical lines.



Supplemental Fig S6| Effect of DNA-PKcs inhibition on deletion of the *MALAT1* enhancer locus. a, Scheme of the sgRNA pairs and QC-PCR primers. **b,** CRISPR-del efficiency in HCT116 and HEK293T upon DNA-PK inhibition. The bar plots show the fraction of non-deleted alleles in the pooled population quantified by qPCR (mean, standard deviation, 1-tailed paired *t*-test).

a

Genomic PCR for *MALAT1*-enhancer-*P5*
(Biological replicate 1)



b

MALAT1-enhancer-*P5* vehicle

```
                                  sgRNA A cutting site      sgRNA B cutting site
WT      TATAGTTCTGCCTCAGCTCAGGACG---- ----GTCTTTACTTGACCAACACTGA
1-      TATAGTTCTGCCTCAGCTCA--------- ----GTCTTTACTTGACCAACACTGA
2-      TATAGTTCTGCCTCAGCTCA--------- ----GTCTTTACTTGACCAACACTGA
3-      TATAGTTCTGCCTCAGCTC---------- ----GTCTTTACTTGACCAACACTGA
4-      TATAGTTCTGCCTCAGCTCA--------- ----GTCTTTACTTGACCAACACTGA
5-      TATAGTTCTGCCTCAGCTCA--------- ----GTCTTTACTTGACCAACACTGA
6-      TATAGTTCTGCCTCAGCTC---------- ----GTCTTTACTTGACCAACACTGA
7-      TATAGTTCTGCCTCAGCTC---------- ----GTCTTTACTTGACCAACACTGA
8-      TATAGTTCTGCCTCAGCTC---------- ----GTCTTTACTTGACCAACACTGA
9-      TATAGTTCTGCCTCAGCTCA--------- ----GTCTTTACTTGACCAACACTGA
10-     TATAGTTCTGCCTCAGCTCA--------- ----GTCTTTACTTGACCAACACTGA
11-     TATAGTTCTGCCTCAG------------- ----------CTTGACCAACACTGA
        ****************                      ***************
```

Deletion

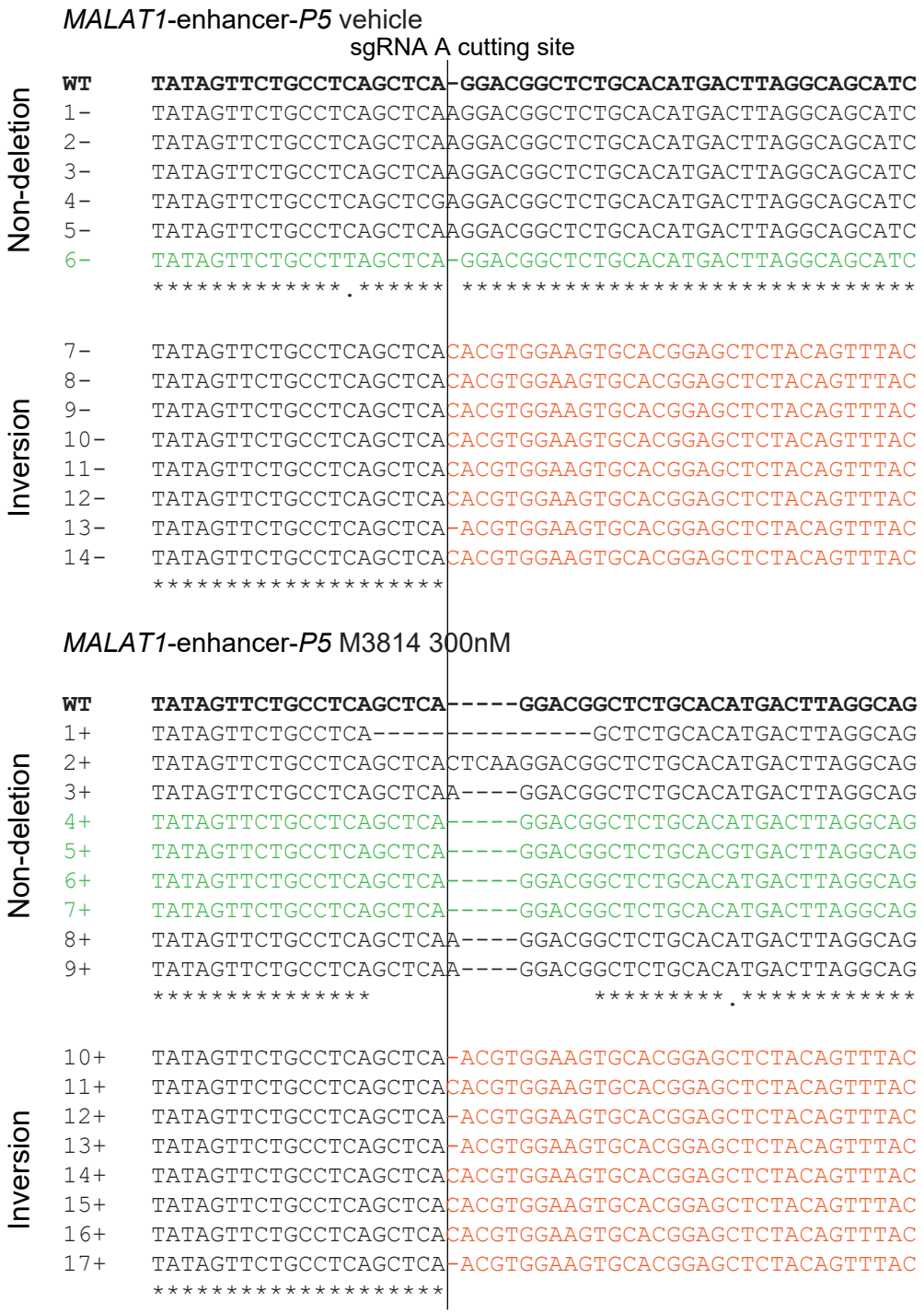
MALAT1-enhancer-*P5* M3814 300nM

```
WT      TATAGTTCTGCCTCAGCTCAGGACG---- ----GTCTTTACTTGACCAACACTGA
1+      TATAGT---------------------- -----TCTTTACTTGACCAACACTGA
2+      TATAGTTCTGCCTCAGCTCA--------- ----GTCTTTACTTGACCAACACTGA
3+      TATAGTTCTGCCTCAGC------------ -----TCTTTACTTGACCAACACTGA
4+      TATAGTTCTGCCTCAGCTC---------- ----GTCTTTACTTGACCAACACTGA
5+      TATAGTTCTGCCTCAGCTCA--------- ----GTCTTTACTTGACCAACACTGA
6+      TATAGTTCTGCCTCAGCTCA--------- ----GTCTTTACTTGACCAACACTGA
7+      TATAGTTCTGCCTCAGCTCA--------- -----TCTTTACTTGACCAACACTGA
8+      TATAGTTCTGCCTCAGCTCA--------- ----GTCTTTACTTGACCAACACTGA
9+      TATAGTTCTGCCTCAGCTCA--------- ----GTCTTTACTTGACCAACACTGA
10+     TATAGTTCTGCCTCAGCT---------- ----------CAACACTGA
11+     TATAGTTCTGCCTCAGCTCA--------- ----GTCTTTACTTGACCAACACTGA
        ******                               *********
```

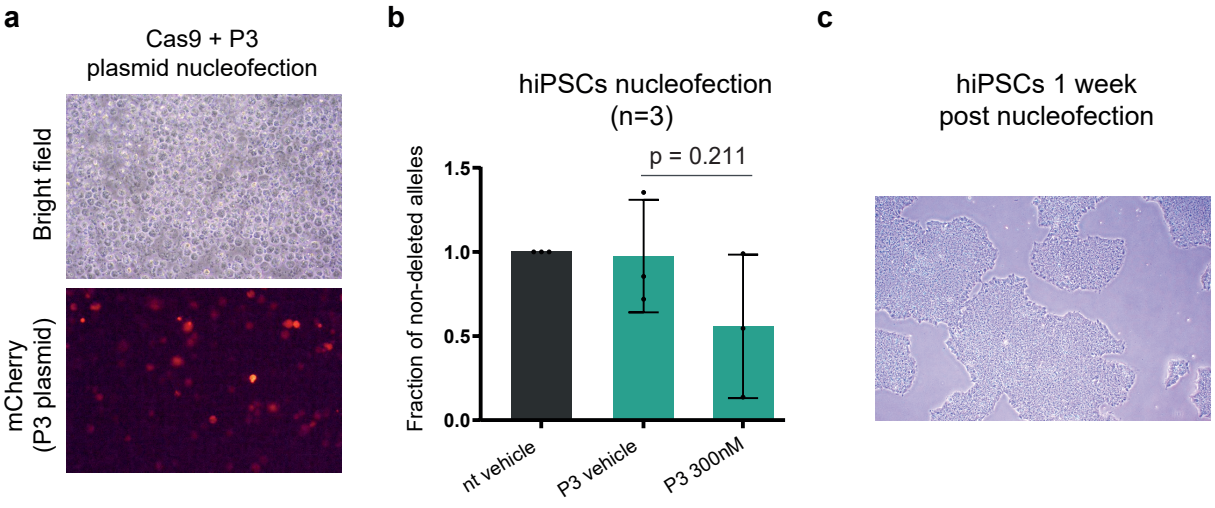
Deletion

***Figure legend in the next page.**

c

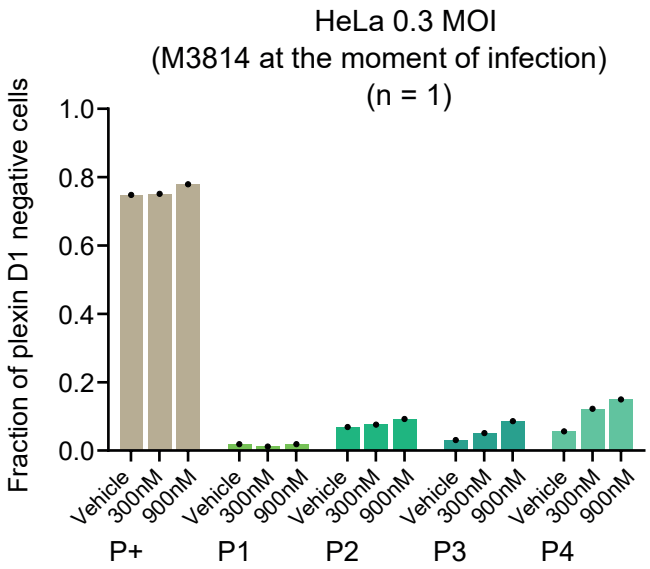


Supplemental Fig S7| Editing outcomes in DNA-PKcs inhibition untreated and treated cells at the *MALAT1* enhancer site. a, Genomic PCR amplification of the *MALAT1* enhancer region from pooled non-treated and treated samples using the pgRNA-P5. Both full-length (including non-deleted and inverted alleles) and deletion bands are boxed. **b,** Sanger sequencing results of TOPO cloning of deletion bands from (a). **c,** Sanger sequencing results of TOPO cloning of full-length bands from (a). WT (unmutated) alleles are shown in green. Inversions are shown in orange. Expected sgRNA cut sites are indicated with vertical line.



Supplemental Fig S8| Effect of DNA-PKcs inhibition in the *PLXND1* locus in hiPSCs. a, Microscope images showing successfull delivery of the pgRNA plasmid (mCherry expression) 24h after nucleofection. **b,** CRISPR-del efficiency in hiPSCs. The bar plots show the fraction of non-deleted alleles in the pooled population quantified by qPCR (mean, standard deviation, 1-tailed paired *t*-test on the 2^dCt values previous to normalization to the vehicle samples). The black bar corresponds to a nontargeting control used for normalization in the ratio calculation. Blue bars correspond to non-treated and M3814-treated samples targeting *PLXND1* (P3). **c,** hiPSCs growth 1 week after nucleofection.

a



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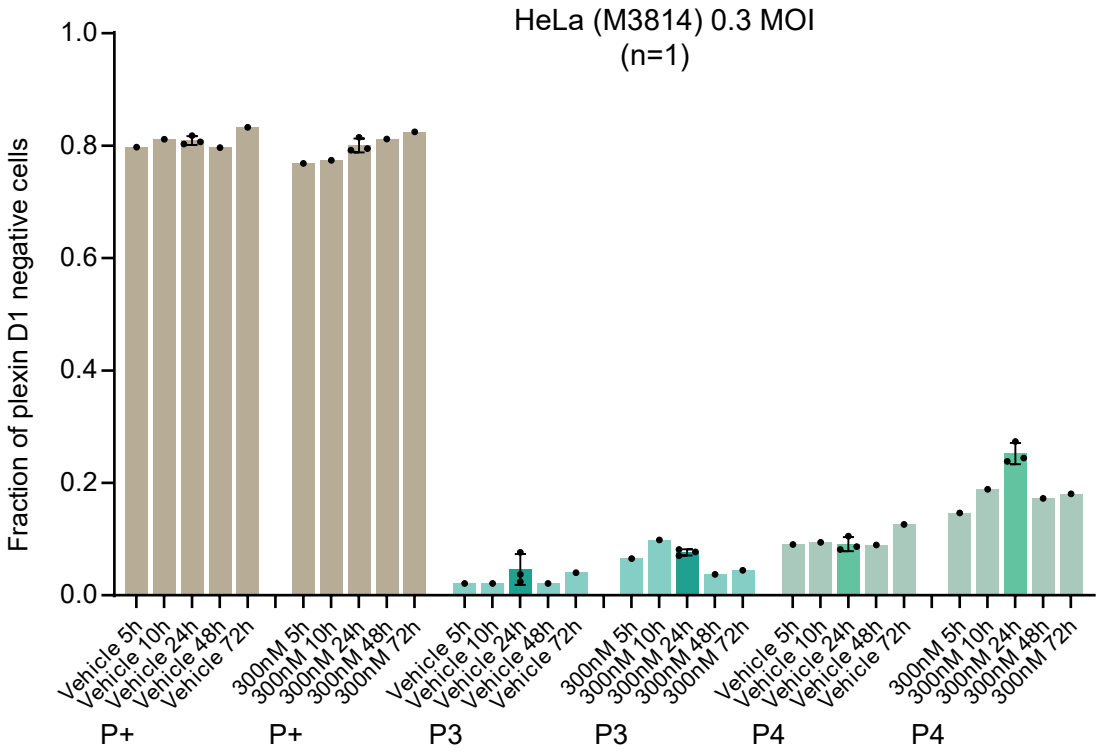
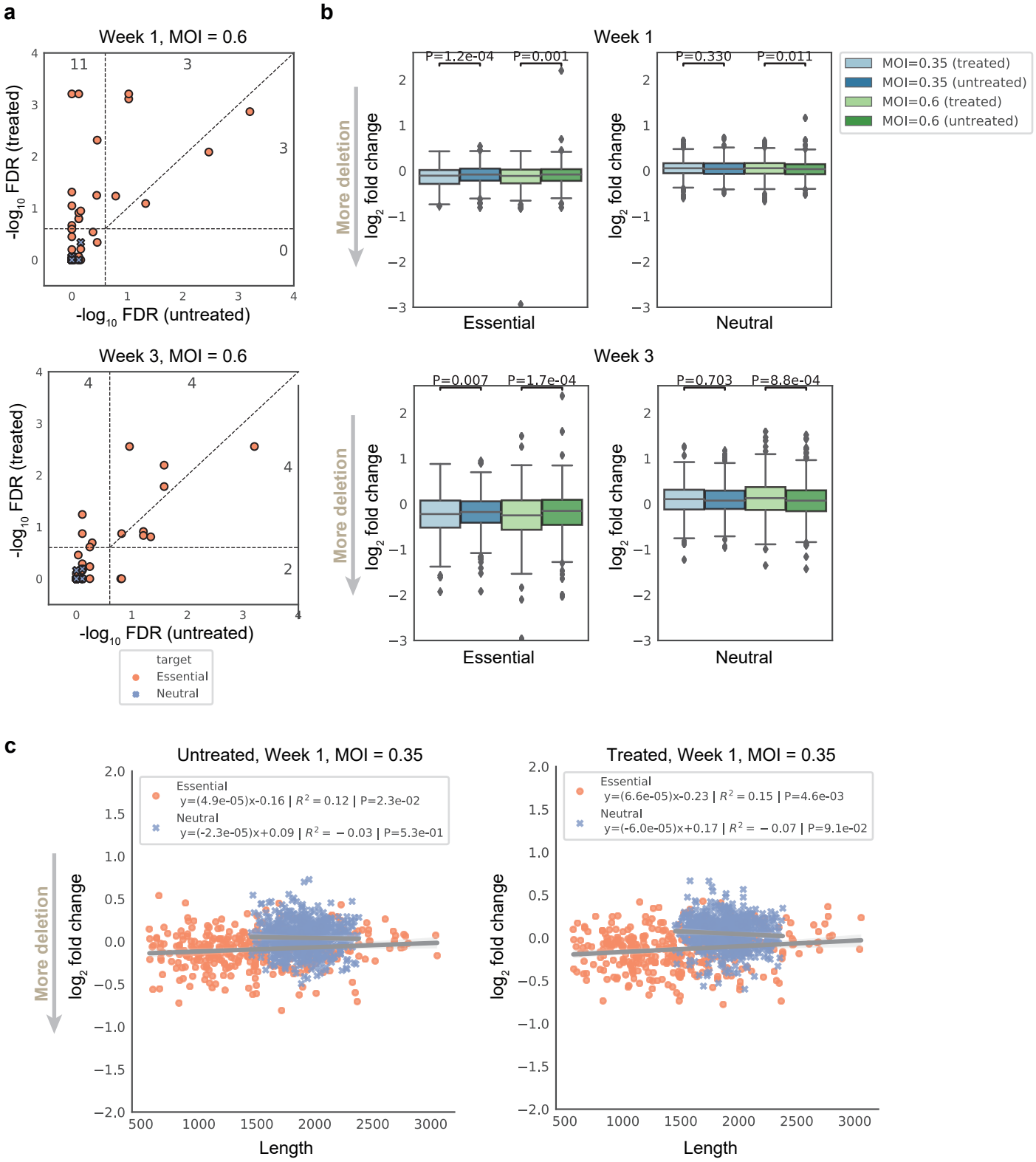


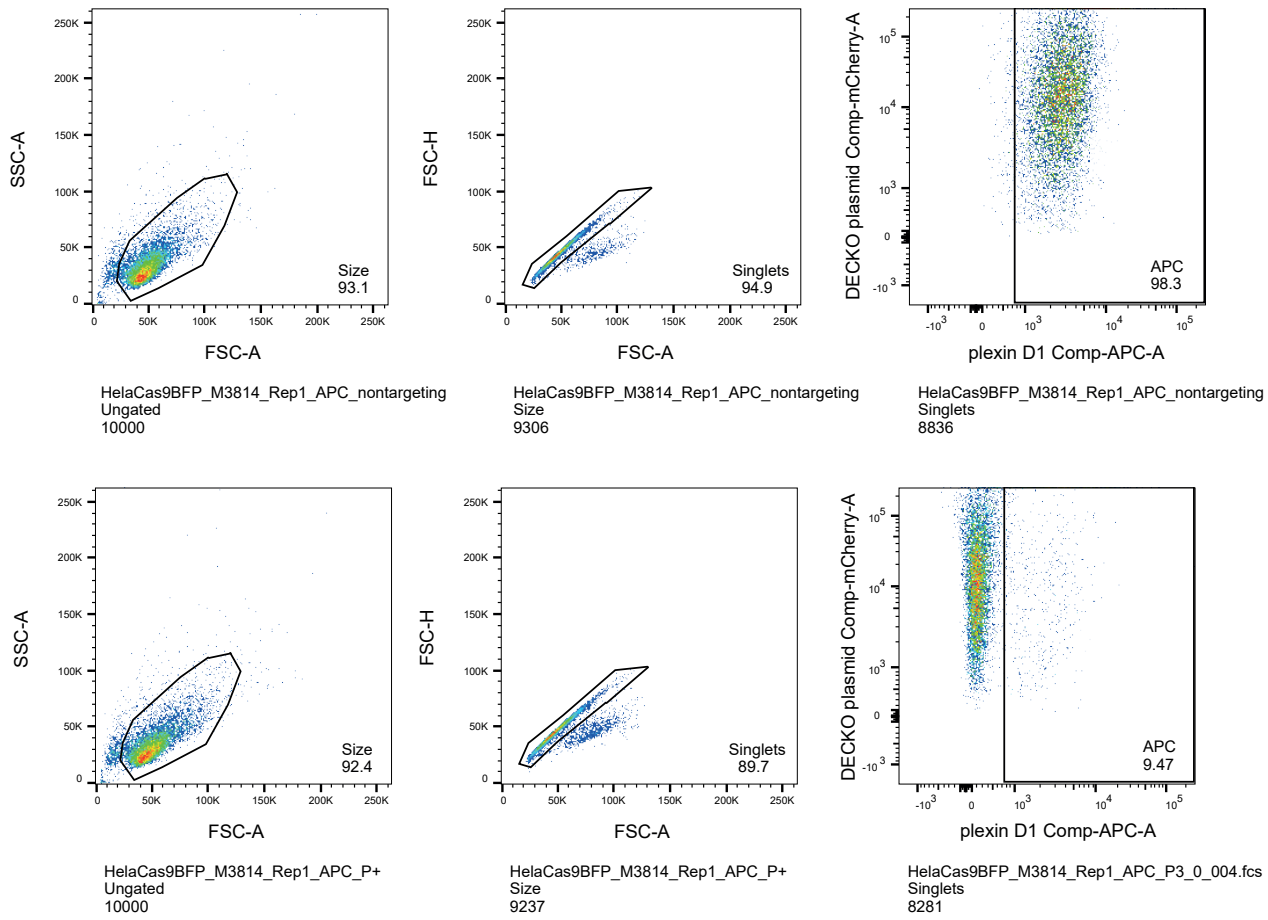
Supplemental Fig S9| Optimising timing of DNA-PKcs inhibition to promote CRISPR-deletion by lentiviral delivery. CiDER measurement of CRISPR-del efficiency in HeLa. **a,** DNA-PKcs inhibition started at the same time of lentiviral infection (MOI=0.3). **b,** DNA-PKcs inhibition was commenced at different time points after lentiviral infection (MOI=0.3).



Supplemental Fig S10| DNA-PKcs inhibition to promote pooled screen performance. a, Hits reported by MAGeCK at two timepoints (Week 1, Week 3) under MOI=0.6. *y*- and *x*- axes record the negative $\log_{10}$ false discovery rate ($-\log_{10}$FDR) for M3814-treated and -untreated samples, respectively. Each point represents a target. A hit is called a true positive (TP) if it is targeting an essential gene and has an FDR below 0.25. Points above the diagonal indicate hits with a lower FDR (higher $-\log_{10}$FDR) in the treated sample. Numbers in plot reflect TPs in each combination of treated/untreated cells. **b,** Log_2 fold change in abundance of indicated pgRNAs compared to Day 0. Significance calculated by 2-tailed *t*-test. **c,** Relation between deletion size and pgRNA $\log_2$ fold change in (treated/untreated) sample. Each point represents a pgRNA. *y*-axis: $\log_2$ fold change in abundance; *x*-axis is the genomic distance between the two sgRNAs in the pair.

		chromosome	start	end	sgRNA_1	chromosome	start	end	sgRNA_2	distance
PLXND1	P+	chr3	129324592	129324615	GCGTAGGACTGTGCACCCGG	chr3	129324215	129324238	GGTGGCGGTGCTCGACAGCG	400
	P1	chr3	129324014	129324037	GTATTCTCGCGTGCACACCT	chr3	129326648	129326671	TGATCTCAAAAGCAGCGTTA	2611
	P2	chr3	129327478	129327501	ATAGGAACAGAGATGGGTGG	chr3	129322928	129322951	CATGTGTGTTGACATGACAA	4527
	P3	chr3	129326800	129326823	TTTCCTCACAGATTCCCCCG	chr3	129322248	129322271	ACAGATCAGCTTACACCAAA	4529
	P4	chr3	129323746	129323769	TCAGAGTTACCATTGCACGT	chr3	129325881	129325904	CGCGAGCGAGGCAGGCCAAG	2112
MALAT1 enh.	P5	chr11	65254685	65254708	GTTGGTCAAGTAAAGACACG	chr11	65256189	65256212	AGTTCTGCCTCAGCTCAGGA	1481
	P6	chr11	65254522	65254545	GCAGGTCACGCAGGCCCCCA	chr11	65256189	65256212	AGTTCTGCCTCAGCTCAGGA	1644
	P7	chr11	65254522	65254545	GCAGGTCACGCAGGCCCCCA	chr11	65256126	65256149	GCACCAGCCCAAGGCTGCAT	1581
	P8	chr11	65254685	65254708	GTTGGTCAAGTAAAGACACG	chr11	65256126	65256149	GCACCAGCCCAAGGCTGCAT	1418
RPS5	P+	chr19	58904535	58904558	GACCTGCTCACAGGCGAGGTA	chr19	58904833	58904856	ACCTGGTTCACACGGCGCAG	321
	P9	chr19	58897261	58897284	GCAGAACTGGGACTTTCCAG	chr19	58900417	58900440	GTACGGAGTAGGAACAAAGT	3133
	P10	chr19	58897115	58897138	CCTCCAACTCGGGATCCGAA	chr19	58899083	58899106	TTATATGACATCAAGGACCA	1945
	P11	chr19	58897115	58897138	CCTCCAACTCGGGATCCGAA	chr19	58900434	58900457	AGGGTATGTGTAAAACCGTA	3296
nontargeting		-	-	-	GAGCTGGACGGCGACGTAAA	-	-	-	CAGAACACCCCCATCGGCGA	-
AAVS1.1		chr19	55628049	55628069	GAGTGCCCTTGCTGTGCCGC	chr19	55626228	55626248	CCTCTGGGGGATGCAGGGGA	1801
AAVS1.2		chr19	55626493	55626513	GACTCTTTAAGGAAAGAAGGA	chr19	55626287	55626307	TCAGTCTGAAGAGCAGAGCC	126

Supplemental Fig S11| Details of all the sgRNA pairs. The genomic location and sequence of each sgRNA pair. Distance between each sgRNA within a pair is also shown. Coordinates from human genome assembly GRCh37 / hg19.



Supplemental Fig S12| Representative example of the CiDER flow cytometry gating strategy. First column: gating for initial cell population; second column: gating for single cells; third column: gating for plexin D1 positive cells. Numbers represent the percentage of cells with respect to the parental population. First row: HeLa with nontargeting pgRNA. Second row: HeLa with P+ pgRNA.

Supplemental Methods

hiPSCs culture. iPSC DF6-9-9T.B (purchased from WiCell) were cultured on mTeSR Plus Medium (StemCell Technologies, 100-0276) supplemented with Antibiotic-Antimycotic (Gibco, Thermo Fisher Scientific, 15240062). Cells were grown at 37°C and 5% O2 in plates coated with Matrigel hESC-Qualified Matrix (Corning, 354277). Cells were passaged approximately every week at 1:8 dilution using ReLeSR (StemCell Technologies, 05872).

hiPSCs nucleofection and DNA-PKcs inhibition. Optimal nucleofection conditions were determined using Amaxa Human Stem Cell Nucleofector Starter Kit (Lonza, VPH-5002) and the Nucleofector 2b Device (Lonza, AAB-1001). Solution 1 and program B16 were chosen as the best conditions. Cells were nucleofected with 2500ng of Cas9 plasmid and 2500ng of pgRNA plasmid following provider's guidelines. Cells were placed in media containing RevitaCell Supplement (100X) (Gibco, Thermo Fisher Scientific, A26445-01) and M3814 300nM or DMSO during 18h. The treatment was finished by replacing the nucleofection media with fresh complete media (RevitaCell Supplement was maintained for 1 more day). At that time, pgRNA plasmid expression was confirmed observing mCherry fluorescence. When confluent, cells were expanded to 6-well plates and (approximately after 1 week, when confluent) collected for gDNA extraction.