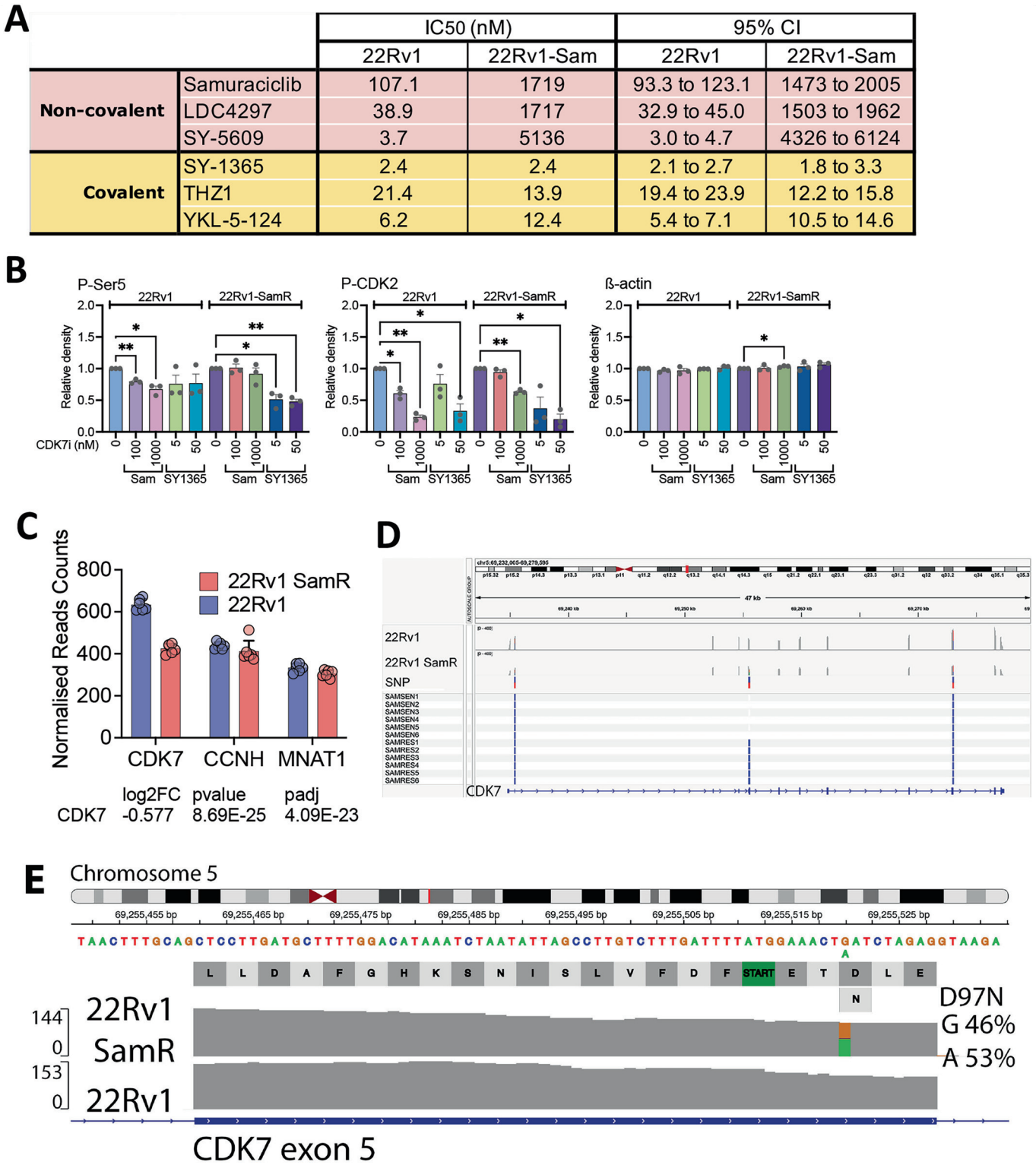
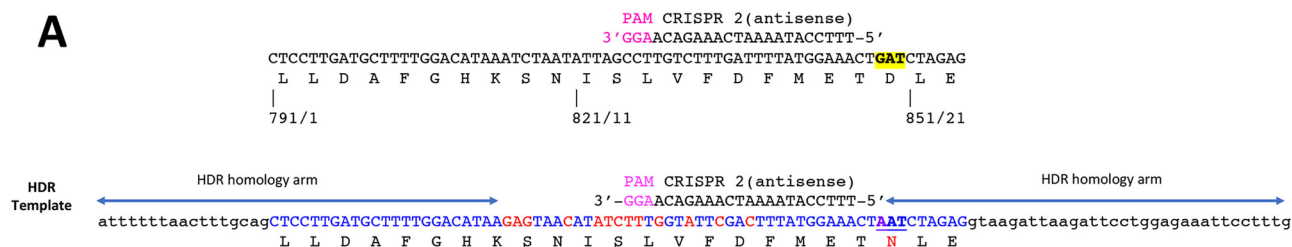
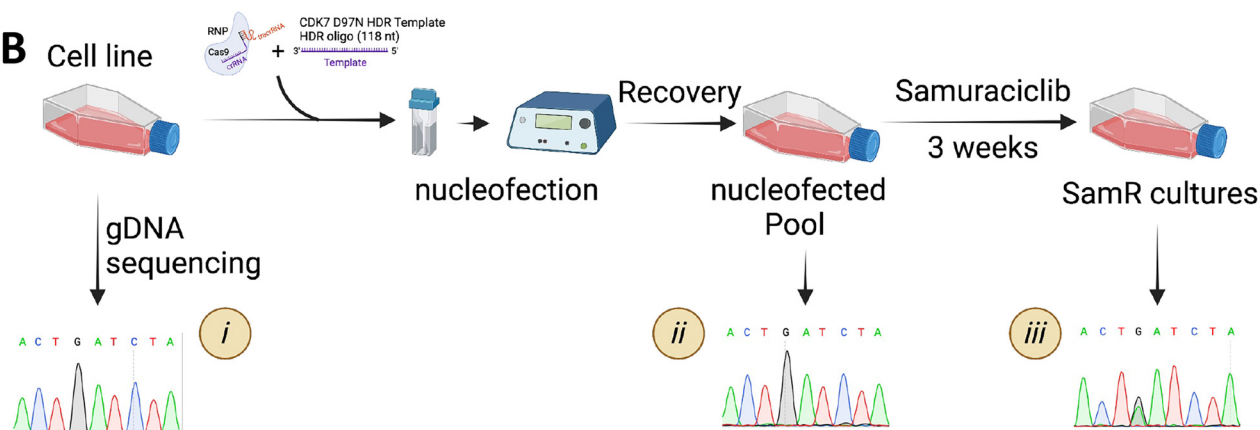
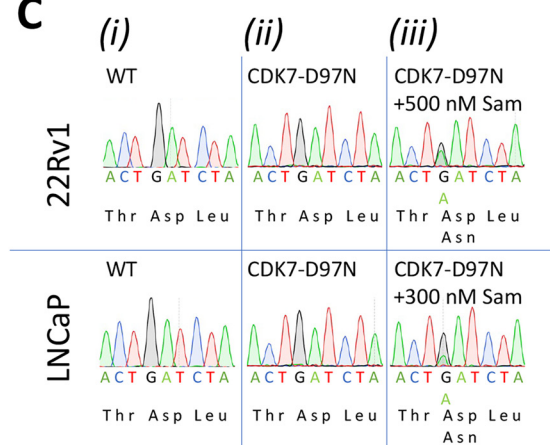
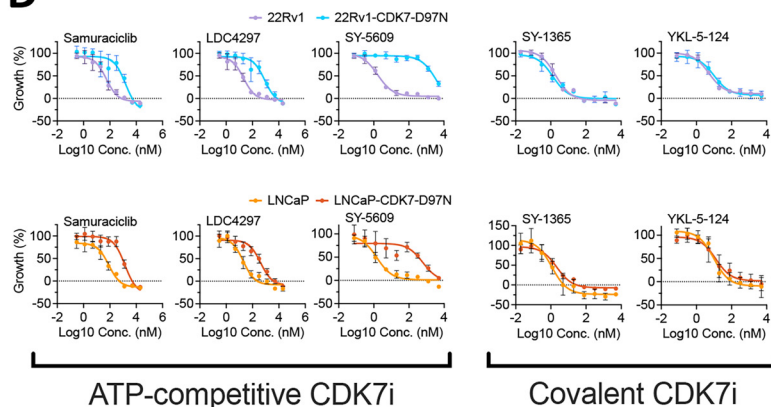
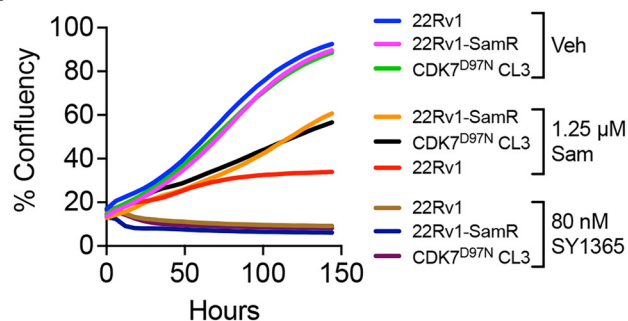
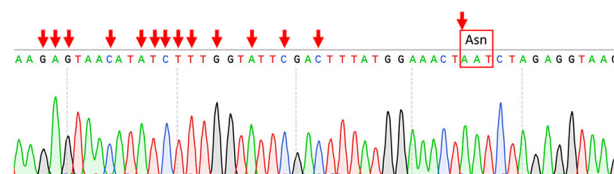


Expanded View Figures

Figure EV1. Samuraciclib-resistant 22Rv1 cells are cross-resistant to other ATP-competitive CDK7 inhibitors.

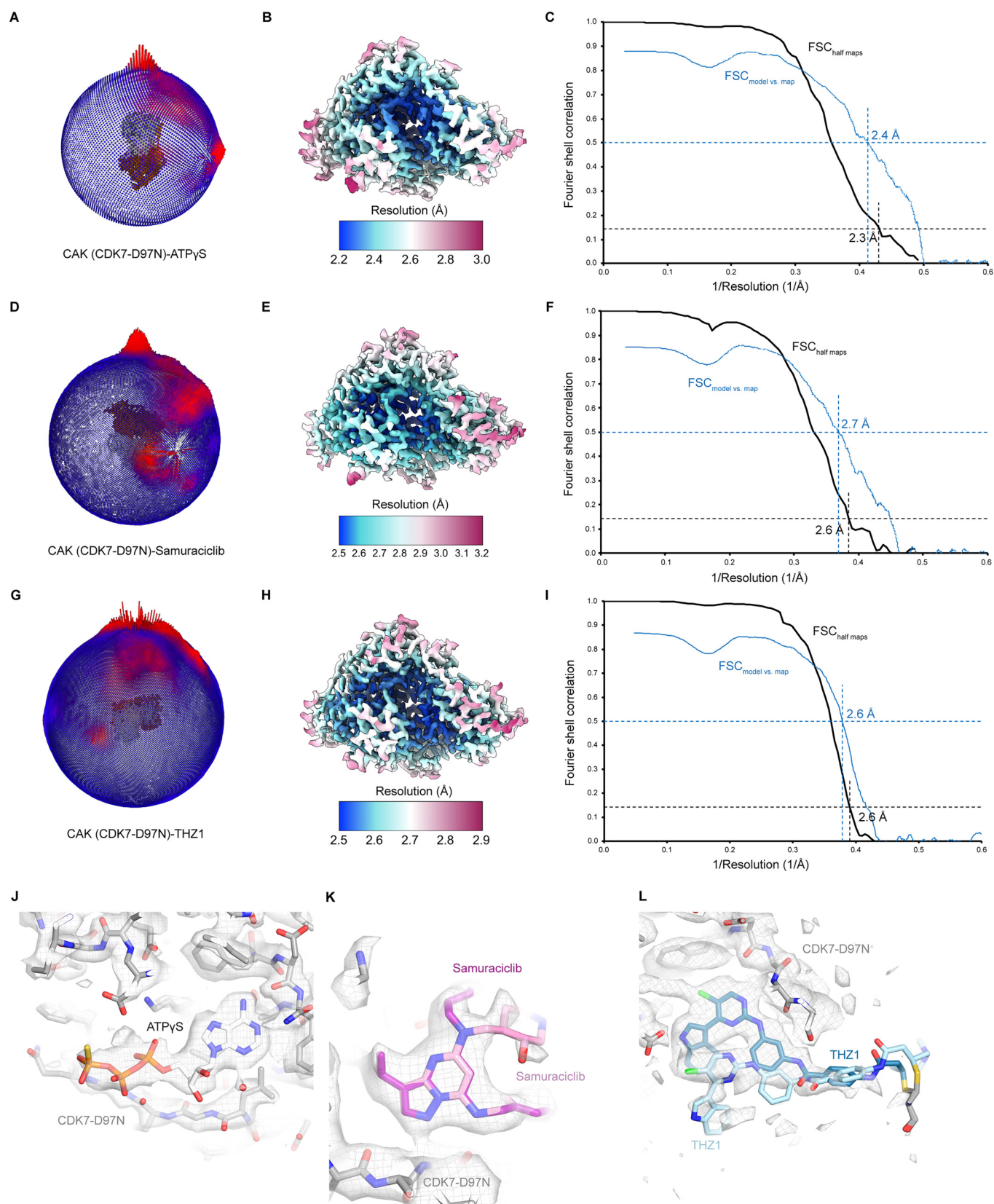
(A) Mean IC_{50} values from $n = 3$ independent experiments for 22Rv1 and 22Rv1-SamR cells. These results are summarised in Fig. 1. Error bars = SEM. (B) Densitometric quantification of immunoblots from $n = 3$ independent experiments in which 22Rv1 and 22Rv1-SamR cells were treated with the indicated concentrations of Samuraciclib or SY1365. Signal intensities are shown relative to the appropriate vehicle (0 nM) controls. One of the immunoblots used for the quantification is shown in Fig. 1D. Error bars show SEM. Pairwise comparisons between treated groups and vehicle were performed using repeated measures of one-way ANOVA followed by Fisher's LSD post hoc test (uncorrected). Asterisks indicate significance (* $P < 0.05$, ** $P < 0.01$). P value for P-Ser5, 22Rv1 cells: Veh vs Sam (100 nM) $P = 0.0093$; Veh vs Sam (1000 nM) $P = 0.0232$. 22Rv1-SamR cells: Veh vs SY1365 (5 nM) $P = 0.0227$; Veh vs SY1365 (50 nM) $P = 0.0052$. For P-CDK2, 22Rv1 cells: Veh vs Sam (100 nM) $P = 0.0148$; Veh vs Sam (1000 nM) $P = 0.0017$; Veh vs SY1365 (50 nM) $P = 0.0268$. 22Rv1-SamR cells: Veh vs Sam (1000 nM) $P = 0.0027$; Veh vs SY1365 (50 nM) $P = 0.0102$. For β -actin, 22Rv1-SamR cells: Veh vs sam (1000 nM) $P = 0.0441$. No asterisk indicates a non-significant difference ($P > 0.05$). (C) RNA-seq was performed using six RNA samples prepared from 22Rv1 and 22Rv1-SamR cells. Shown are normalised read counts for CDK7 and its interacting partners cyclin H (CCNH) and MAT1 (MNAT1). Circles represent the normalised read counts for each of the six biological replicate RNA samples. (D, E) Genome browser snapshots (hg38) of RNA-seq data for 22Rv1 (SAMSEN) and 22Rv1-SamR (SAMRES) cells are shown. The vertical bars show positions of two known CDK7 SNPs (rs2972388 (exon 2) and rs34584424 (exon 10)), which are present in the Sam-sensitive and resistant cells at a ratio of 1:1, suggestive of the presence of 2 CDK7 alleles in 22Rv1 cells. Also evident is a single nucleotide difference (exon 5; c.289G>A) in the codon encoding aspartate 97 (p.Asp97Asn), which is seen only in 22Rv1-SamR cells. Source data are available online for this figure.



A**B** Cell line**C****D****E****F**

◀ **Figure EV2. Development of CDK7-D97N mutation in cancer cell lines using CRISPR/Cas9 mutagenesis.**

(A, B) Shown are the CRISPR sequences and the sequence of the homology-directed recombination (HDR) template for introduction of the CDK7-D97N mutation. The sequences in blue show the CDK7 exon 5 sequences, and the sequences in red represent silent changes introduced in the template for facilitating screening for the mutation. Underlined is the codon encoding asparagine at position 97 to create the p.Asp97Asn (D97N) mutation in the CDK7 gene. (B) The strategy for enrichment of cells encoding CDK7-D97N. (C) Sequencing chromatograms for the region around Asp97 in unmutated cells (i) in gDNA prepared from 22Rv1 and LNCaP cells. Sequencing chromatograms for cells following nucleofection and culturing in the absence (ii) or the presence (iii) of Sam. (D) Growth inhibition for increasing concentrations of the indicated drugs was carried out using Sam-selected D97N knock-in cells (22Rv1-CDK7-D97N, LNCaP-CDK7-D97N (iii)) alongside parental Sam-sensitive cells; means and SEM from $n = 2$ independent experiments are shown. (E) The cell lines were cultured in the presence of the indicated drugs, with confluency being measured every 6 h using an Incucyte Zoom. Non-linear regression analysis was used for curve fitting. The results of $n = 3$ independent experiments are summarised, with error bars omitted for ease of viewing. Calculated doubling times with 95% confidence intervals (h) for vehicle-treated cells: 22Rv1 (49.4; 45.3–54.1), 22Rv1-SamR (45.4; 42.2–49.0), 22Rv1 homozygous CDK7-D97N CL3 (48.8; 45.1–53.0). (F) Sanger sequencing chromatogram for gDNA prepared from the 22Rv1 CDK7-D97N CL3. Source data are available online for this figure.



◀ Figure EV3. Validation measures for the cryo-EM reconstructions and additional ligand views.

(A) Plot of the orientation distribution of the particle images entering the CAK D97N-ATPyS cryo-EM reconstruction. (B) Local resolution estimation for the CAK D97N-ATPyS cryo-EM reconstruction. (C) FSC plots to assess the overall resolution of the CAK D97N-ATPyS cryo-EM reconstruction (black line) and the model-to-map fit of the refined coordinate model (blue line). FSC thresholds of $FSC = 0.143$ and $FSC = 0.5$ were used for half-map and model-to-map comparisons, respectively (Rosenthal and Henderson, 2003). (D-F) As (A-C), but for the CAK D97N-Samuraciclib cryo-EM reconstruction. (G-I) As (A-C), but for the CAK D97N-THZ1 cryo-EM reconstruction. (J-L) Additional views of the ligands in the cryo-EM density. Source data are available online for this figure.

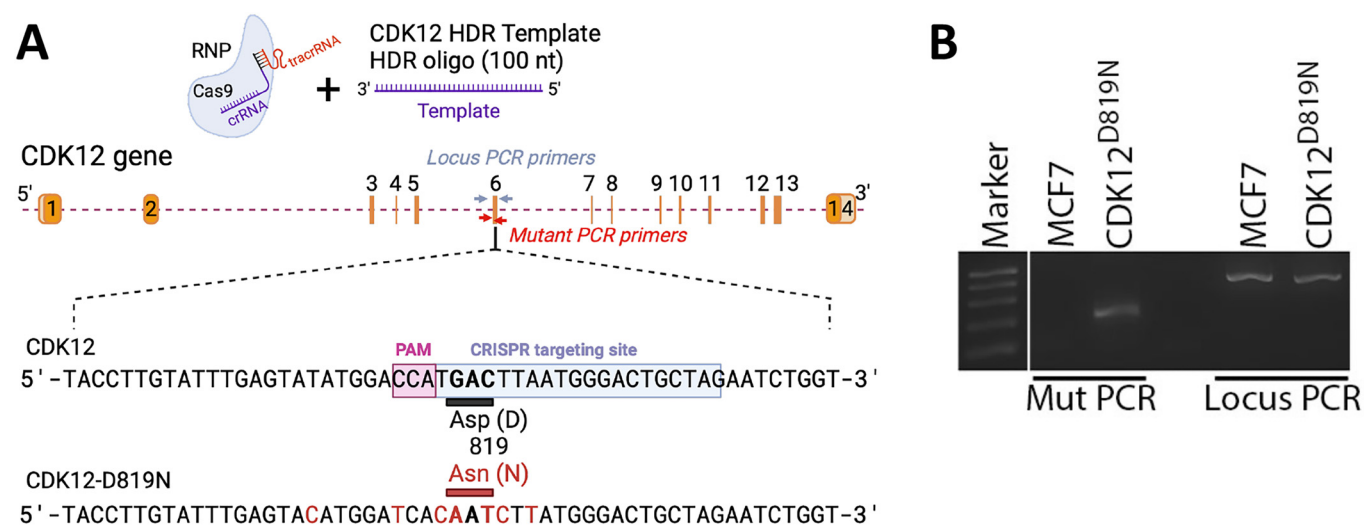


Figure EV4. CDK12-D819N CRISPR-Cas9 knock-in in MCF7 cells.

(A) Strategy for generating the D819N mutation in the CDK12 gene. The full sequence of the donor template is: 5'-TTCGTCTTTATGTAGGTGCCTTTTACCTTG-TATTTGAGTACATGGATCACAATCTTATGGGACTGCTAGAATCTGGTTTGGTGCACTTTTCTGAGGACCA-3'. (B) PCR was carried out using gDNA and primers amplifying the region around exon 6 ("locus PCR"; 5'-CGCCCAGCCACAGAAGATTA-3' and 5'-GAGGAGAAGAGGAAAGTGCTTAA-3', product size: 458 bp). PCR using primers, one of which is located within the region containing base changes incorporated in the donor template ("mutant PCR"; 5'-GGACTTGAGGCATTGTTATTT-3' and 5'-CCATAAGATTGTGATCCATG-3', product size: 116 bp), was carried out with gDNA prepared from cells following RNP CRISPR knock-in but prior to selection with (R)-CR8.

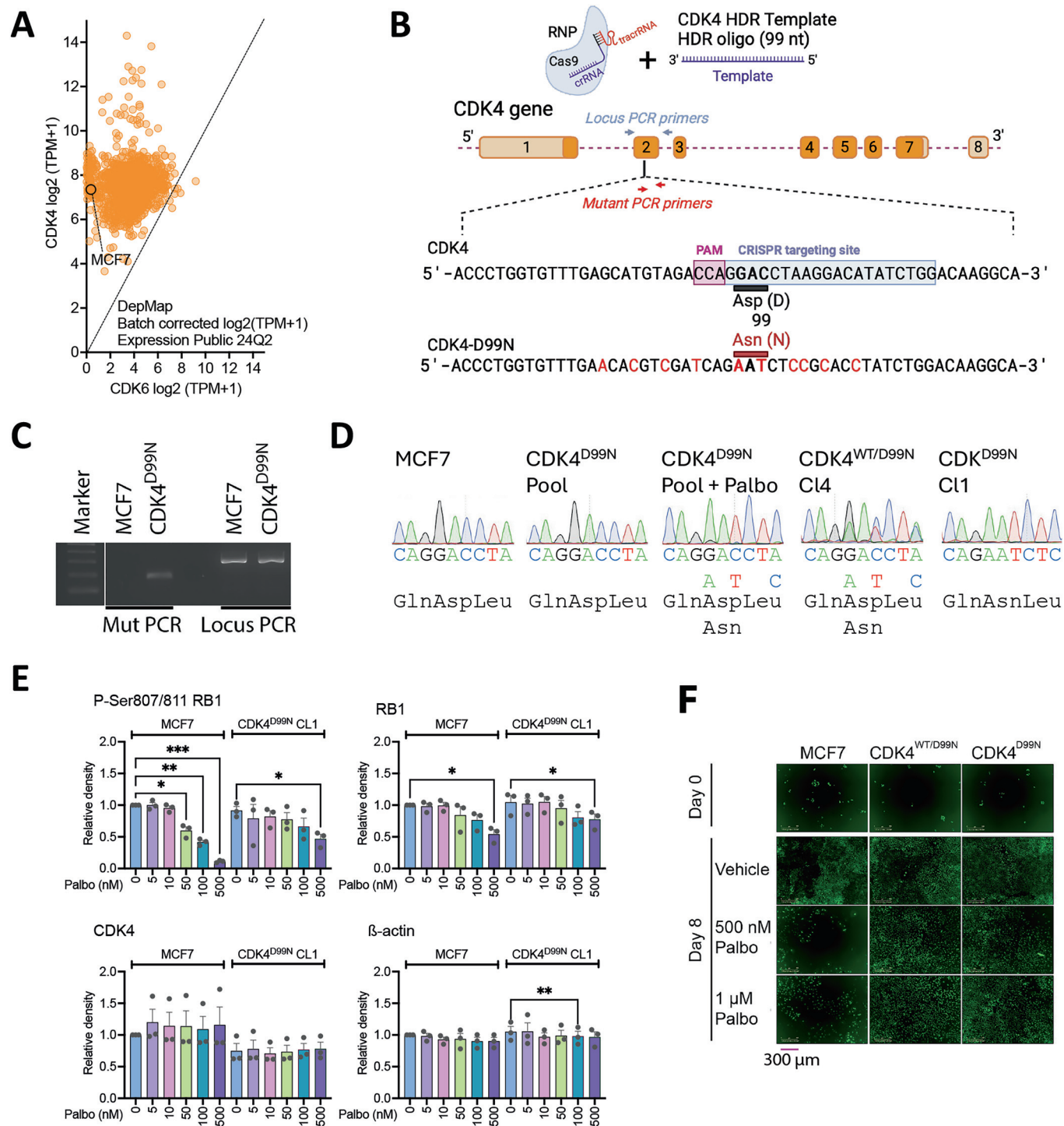



Figure EV5. CDK4-D99N CRISPR-Cas9 knock-in in MCF7 cells.

(A) Batch corrected expression public 24Q2 data was acquired for CDK4 and CDK6 from the DepMap portal. (B) Strategy for generating the D99N mutation in the CDK4 gene. The full sequence of the donor template is: 5'-CCCGAACTGACCGGGAGATCAAGGTAACCTGGTGTGTTGAACACGTCGATCAGAATCTCCGCACCTATCTGGA-CAAGGCACCCCCACCAGGCTTGCCAGCCGAAA-3'. (C) Knock-ins were confirmed by PCR of gDNA using mutant-specific primers having the sequences, 5'-ACACGTCGATCAGAATCTCC-3' and 5'-ATCCACCTCTCAATGCCTAC-3'. Locus-specific primers used for gDNA PCR had the sequences 5'-AGGTGGGGTGTGAT-GATCTG-3', 5'-AAGGGGAGGTACAGATGCAC-3'. (D) Sanger sequencing of DNA generated using locus PCR primers. Sequencing was performed using gDNA prepared from MCF7 cells after nucleofection (CDK4-D99N Pool), gDNA prepared from the CDK4-D99N Pool after culturing in the presence of 1 μ M Palbociclib for 9 passages over a period of 3 months. Individual clones were isolated from the Palbociclib-selected pools; sequences of heterozygous and homozygous mutant clones are shown. (E) ImageJ was used to quantify the immunoblotting results for $n = 3$ independent experiments in which MCF7 cells and MCF7-CDK4-D99N CL1 cells were treated with Palbociclib for 48 h. Error bars = SEM. Pairwise comparisons between treated groups and vehicle were performed using repeated measures of one-way ANOVA followed by Fisher's LSD post hoc test (uncorrected). For P-Ser807/811 RB1 MCF7 cells: Veh vs Palbo (50 nM) $P = 0.0213$; Veh vs Palbo (100 nM) $P = 0.0026$; Veh vs Palbo (500 nM) $P = 0.0001$. CDK4^{D99N} CL1 cells: Veh vs Palbo (500 nM) $P = 0.0302$. For RB1, MCF7 cells: Veh vs Palbo (500 nM) $P = 0.0236$. Veh vs Palbo (500 nM) $P = 0.0143$. For β -actin, CDK4^{D99N} CL1 cells: Veh vs Palbo (100 nM) $P = 0.0063$. No asterisk indicates a non-significant difference ($P > 0.05$). (F) Representative images of cultures 24 h after seeding, when Palbociclib was added (day 0) and 8 days following the start of treatment. Source data are available online for this figure.