

Supplementary Information:

Figure S1. Characterization of anti-EGFR nanobodies identifies four non-overlapping epitope groups.

A Schematic overview of the generation and screening strategy for EGFR-specific nanobodies following alpaca immunization. **B** Standard ELISA assessing the binding capacity of the indicated nanobodies to the recombinant EGFR extracellular domain (EGFR-ECD). **C** Competitive ELISA mapping the epitopes recognized by the nanobodies. Plates were coated with the recombinant EGFR-ECD. Unconjugated competitor antibodies (20 µg/mL) were applied at a tenfold molar excess over the biotin-conjugated detection antibodies (2 µg/mL). Binding was detected using HRP-conjugated streptavidin. **D** Flow cytometry analysis of nanobody binding to cell surface EGFR in the EGFR-mutant lung cancer cell lines H1975 (EGFR-L858R/T790M) and PC9 (EGFR-ΔE746-A750), as well as MC38 murine cells overexpressing wild-type EGFR.

Figure S2. Functional and biophysical characterization of anti-EGFR nanobodies.

A, B Surface plasmon resonance (SPR) sensorgrams and fitted binding curves for nanobodies binding to the recombinant EGFR-ECD (**A**), and the affinity (KD) values derived from SPR kinetics (**B**). **C** Western blot analysis of total EGFR and phospho-EGFR (Y1068) levels in H1975 cells following 24-hour treatment with 5 µg/mL of the indicated antibodies or 300 nM osimertinib. **D** Western blot analysis of total EGFR and phospho-EGFR (Y1068) levels. The lung carcinoma cell line A549 (wild-type EGFR)

was pre-incubated with 5 µg/mL of antibodies for 20 minutes at 4 °C, stimulated with 100 ng/mL of EGF at 37 °C under 5% CO₂ for 20 minutes, and then harvested for analysis.

Figure S3. Optimized triparatopic hexameric antibodies inhibit EGFR signaling and proliferation.

A Western blot analysis of total EGFR and phospho-EGFR (Y1068) levels in H1975 (up) or PC9 cells (down) following 24-hour treatment with 5 µg/mL of triparatopic antibodies fused to cetuximab, or with 300 nM osimertinib. **B, C** Proliferation of H1975 (**B**) or PC9 (**C**) cells after 5-day treatment with 5 µg/mL of triparatopic antibodies fused to cetuximab, or with 300 nM osimertinib (n = 3 independent biological samples).

Figure S4. DB5A1-induced EGFR degradation is time- and dose-dependent and inversely correlates with baseline EGFR levels.

A Western blot analysis of total EGFR and phospho-EGFR (Y1068) levels in H1975 (left) or PC9 cells (right) following 24-hour treatment with a concentration gradient of DB5A1, or treatment for the indicated durations with 5 µg/mL of DB5A1. **B** Western blot analysis of total EGFR and phospho-EGFR (Y1068) levels in H1975, PC9 or HCC827 cells following 24-hour treatment with 5 µg/mL of DB5A1 or cetuximab, or 300 nM osimertinib.

Figure S5. Binding characterization of cetuximab and multiparatopic antibodies.

A, B Surface plasmon resonance (SPR) sensorgrams and fitted binding curves for antibodies binding to the recombinant EGFR-ECD (**A**), and the affinity (KD) values derived from SPR kinetics (**B**). **C** Flow cytometry analysis of PC9 cells co-stained with 50 µg/mL of nanobodies and 5 µg/mL of AF647-conjugated cetuximab, confirming that the nanobodies bind to epitopes that do not overlap with those of cetuximab.

Figure S6. Validation of the EGFR-C797S knock-in PC9 clone 10B1.

A Western blot analysis of total EGFR and phospho-EGFR (Y1068) levels in EGFR-C797S knock-in PC9 single-cell clone cell lines following 24-hour treatment with or without 300 nM osimertinib. **B** Genomic DNA was extracted from the EGFR-C797S knock-in PC9 single-cell clone cell line 10B1 and subjected to PCR amplification. The resulting products were cloned into a vector, transformed into *E. coli*, and multiple individual colonies were picked for Sanger sequencing.

Figure S7. DB5A1 and osimertinib exhibit an additive effect across computational models.

A, B Combination Index (CI) scores were calculated from gradient concentrations using the Highest Single Agent (HSA), Loewe additivity, Bliss independence, and Zero Interaction Potency (ZIP) models. An additive effect, defined by CI values approximately between -10 and 10, was observed.

Figure S8. Time-dependent internalization of pHrodo-conjugated EGFR-targeting antibodies.

A, B Flow cytometry histograms of H1975 (**A**) and PC9 (**B**) cells treated with 5 $\mu\text{g/mL}$ of pHrodo-conjugated multiparatopic antibodies or cetuximab for 0, 10, 30, or 60 minutes.

Figure S9. Enhanced endocytosis of multiparatopic antibodies compared to cetuximab.

A Confocal imaging of PC9 cells following 10-, 30- or 60- minute treatment with 5 $\mu\text{g/mL}$ of AF647-conjugated multiparatopic antibodies or cetuximab.

Figure S10. Internalization of multiparatopic antibodies is inhibited by endocytic pathway blockers.

A, B Flow cytometry analysis of H1975 (**A**) or PC9 (**B**) cells following 1-hour treatment with 5 $\mu\text{g/mL}$ of pHrodo Deep Red-conjugated Ce-Fc-B6A1 or DB5A1, with or without endocytic pathway inhibitors ($n = 3$ independent biological samples). **C** Representative flow cytometry histograms corresponding to the analyses shown in (**A, B**).

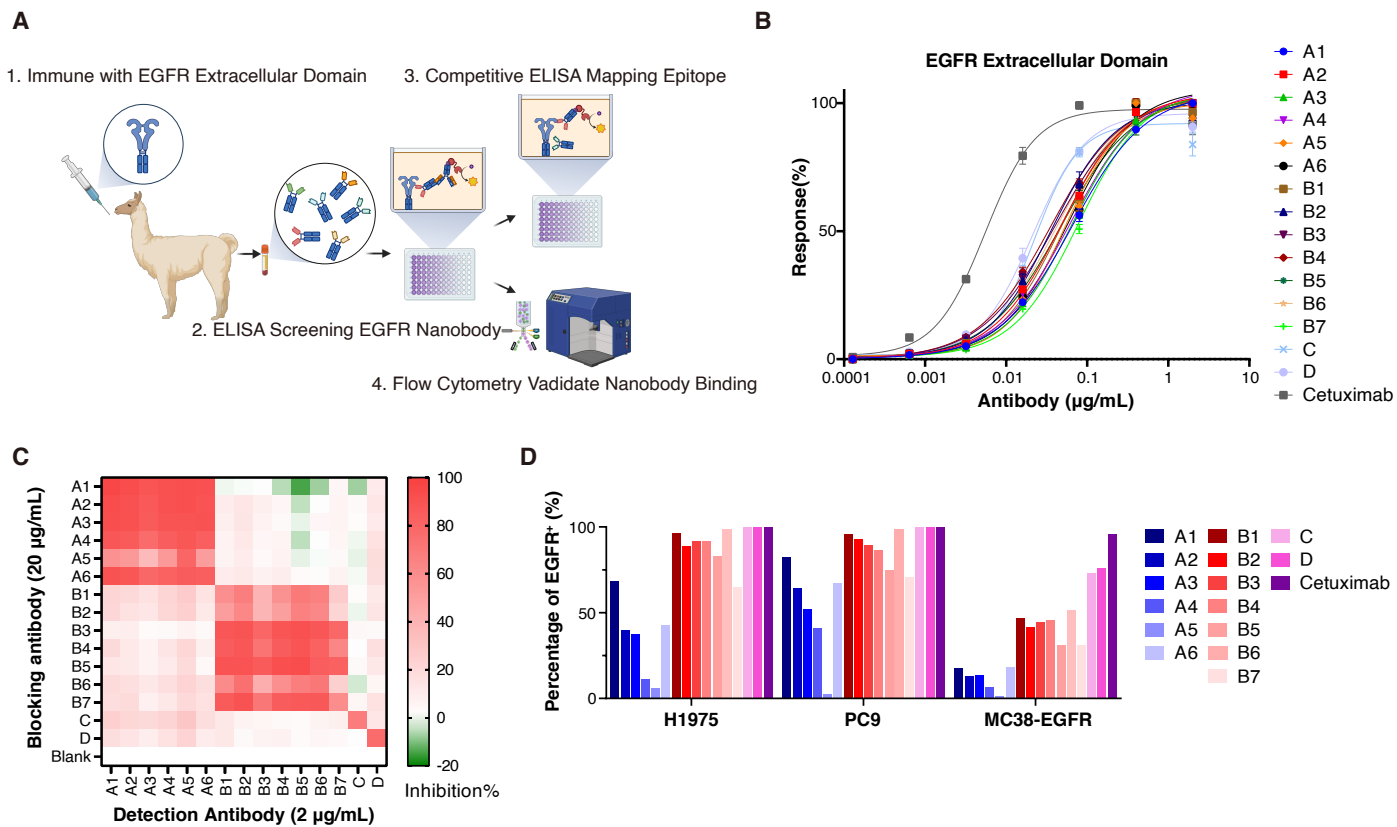
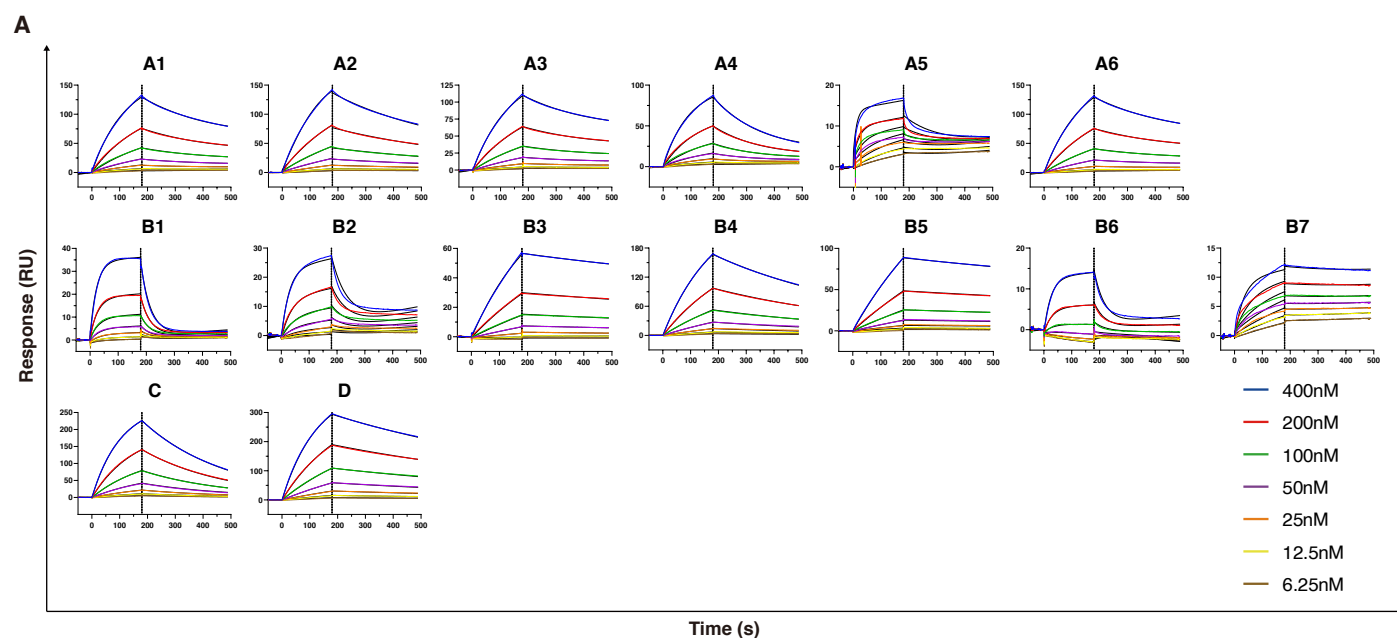


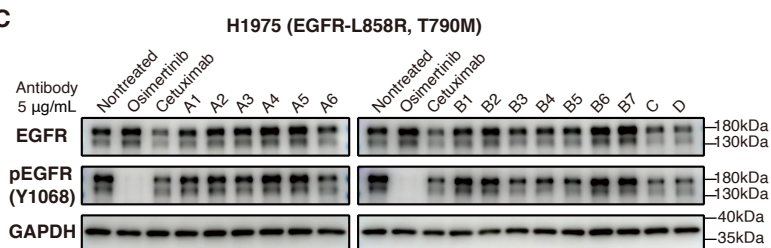
Fig. S1



B

Abs	k_a (1/Ms)	k_d (1/s)	KD (M)
A1	1.61E+04	2.91E-03	1.81E-07
A2	3.93E+04	2.77E-03	7.05E-08
A3	1.41E+04	2.71E-03	1.92E-07
A4	1.64E+04	5.13E-03	3.13E-07
A5	2.01E+05	6.69E-03	3.32E-08
A6	1.41E+04	2.56E-03	1.81E-07
B1	3.66E+04	2.81E-02	7.67E-07
B2	4.73E+04	1.77E-02	3.73E-07
B3	9.33E+03	5.77E-04	6.18E-08
B4	1.28E+04	1.75E-03	1.36E-07
B5	5.47E+03	4.10E-04	7.49E-08
B6	3.48E+04	2.62E-02	7.54E-07
B7	7.74E+04	1.54E-03	1.99E-08
C	1.61E+04	3.34E-03	2.08E-07
D	1.75E+04	1.01E-03	5.76E-08

C



D

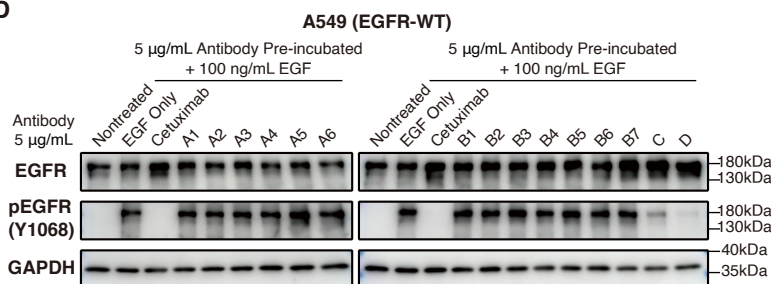


Fig. S2

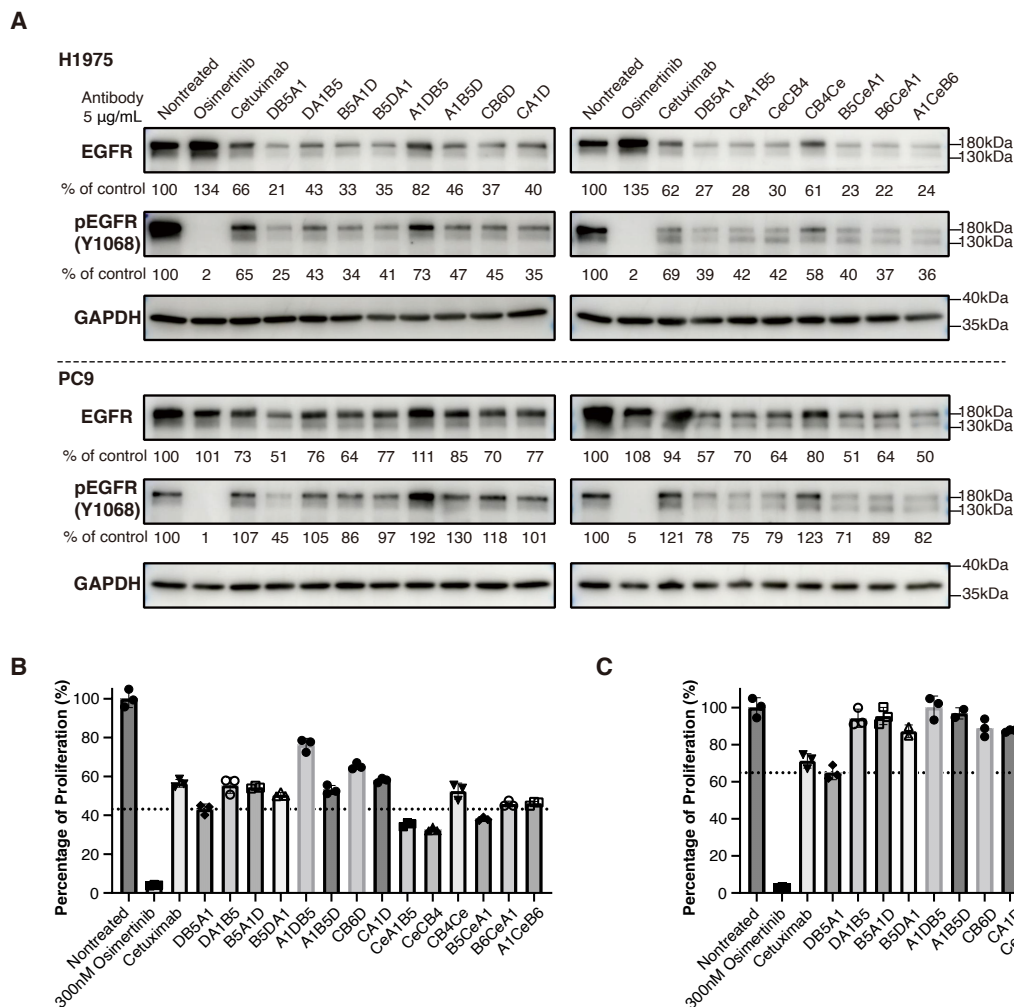


Fig. S3

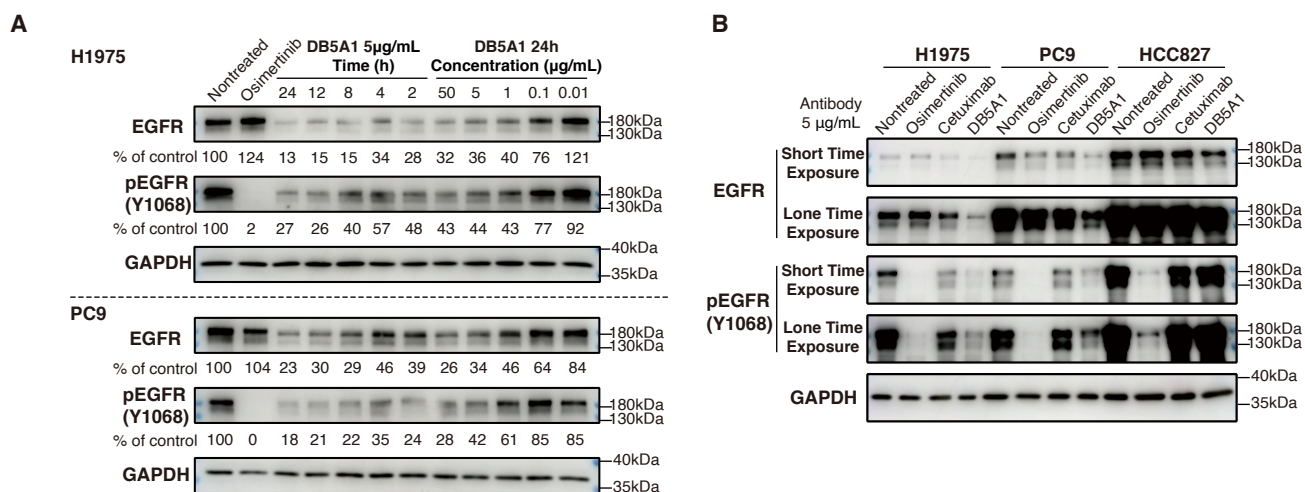


Fig. S4

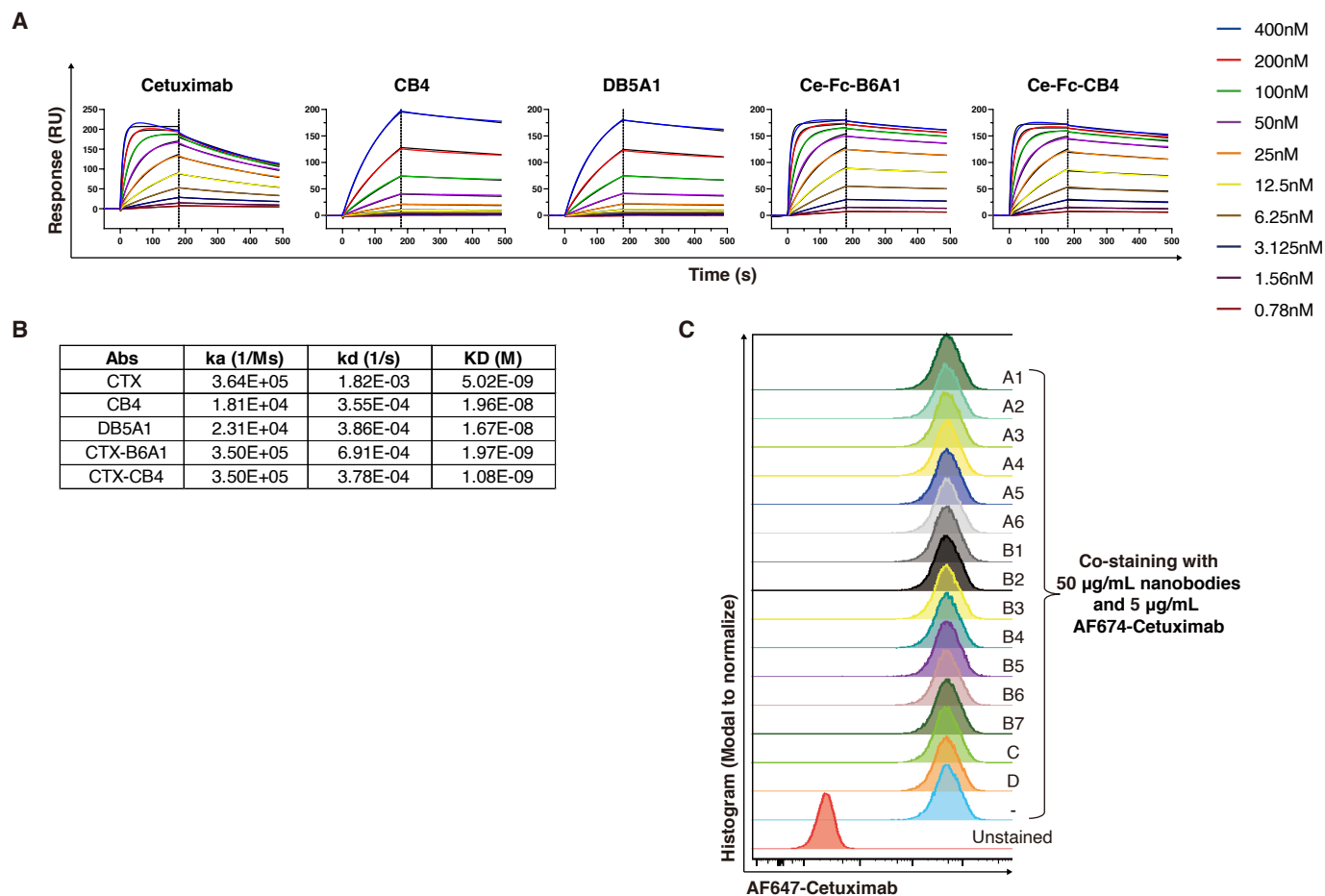


Fig. S5

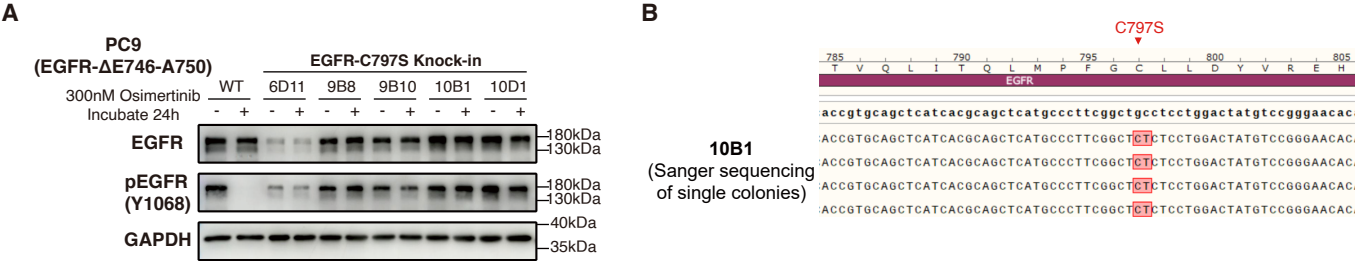


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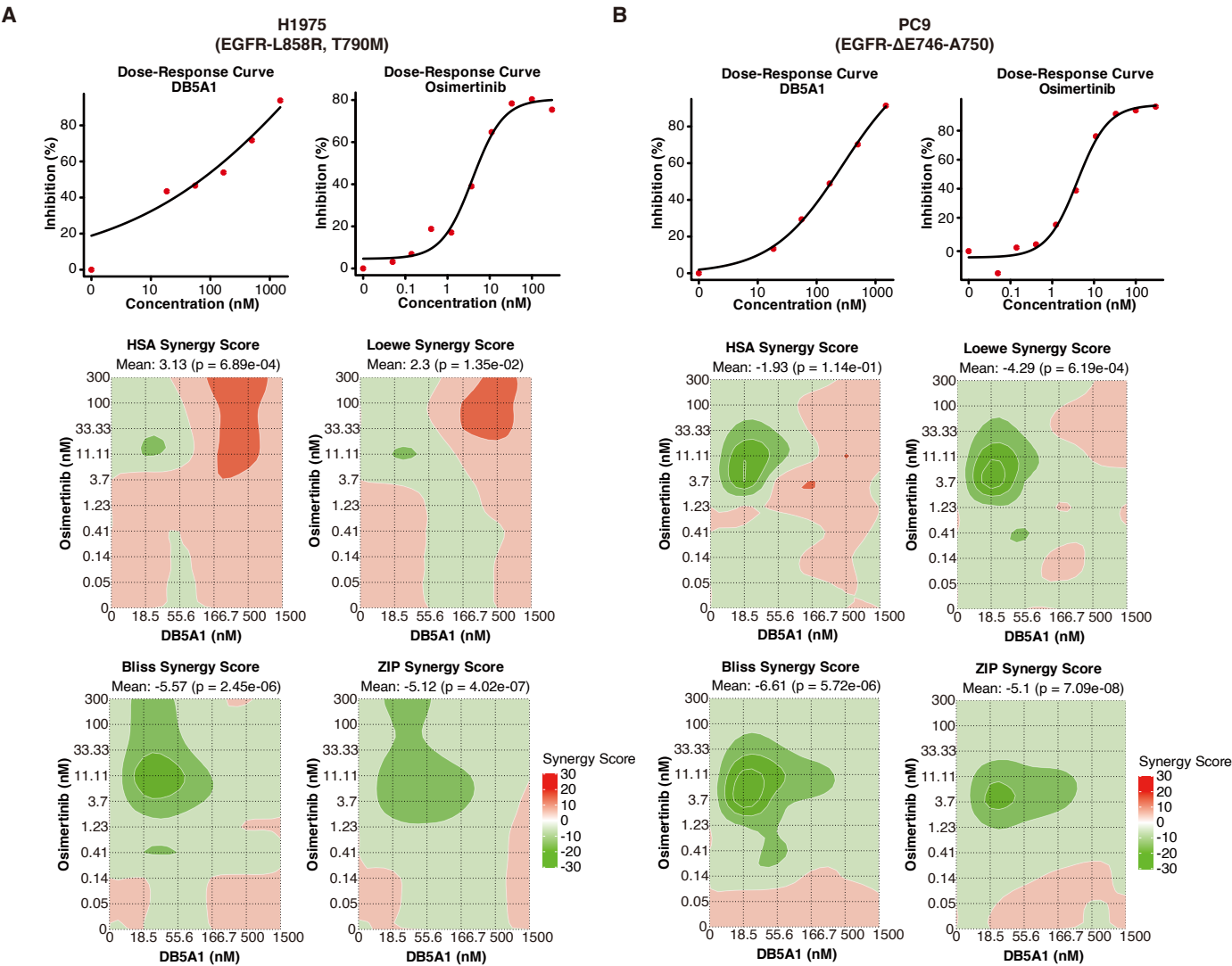
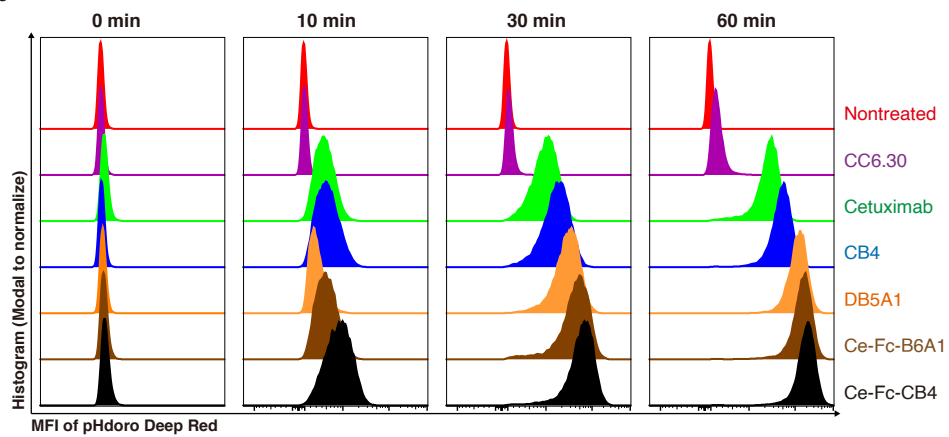


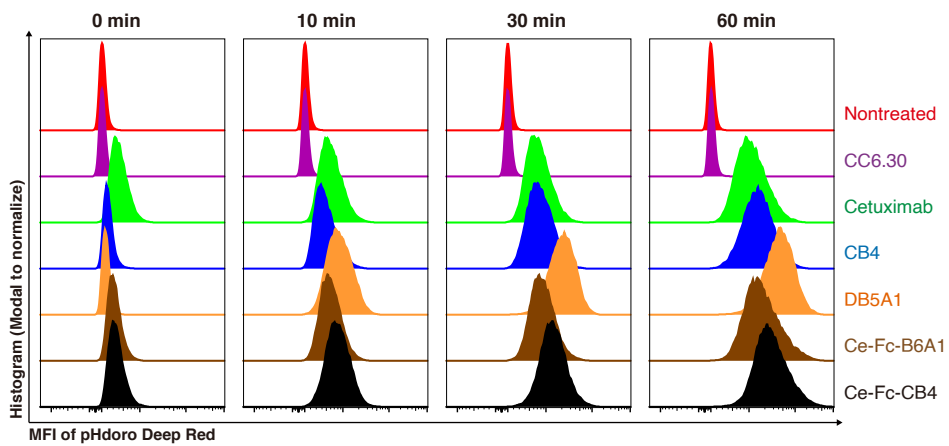
Fig. S7

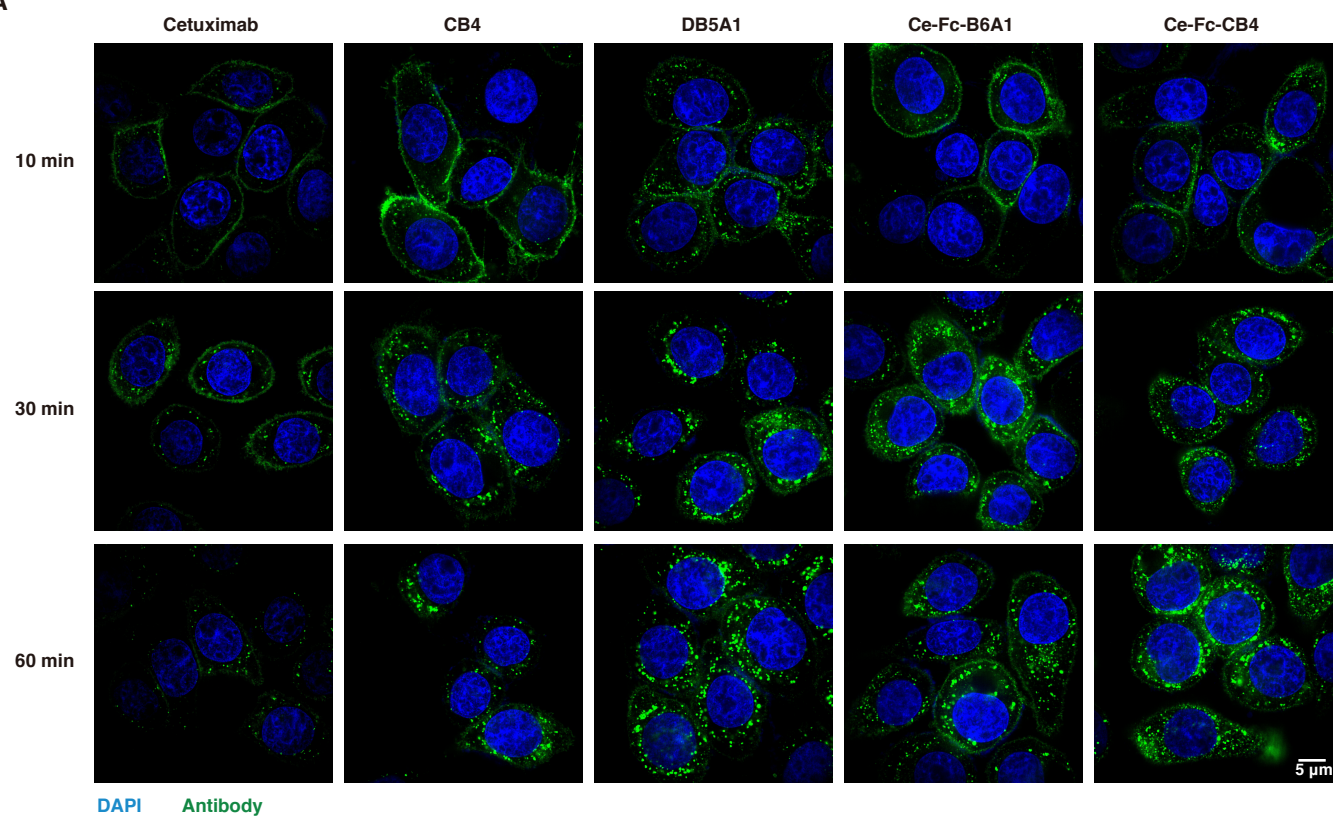
A

H1975

**B**

PC9

**Fig. S8**

A**Fig. S9**

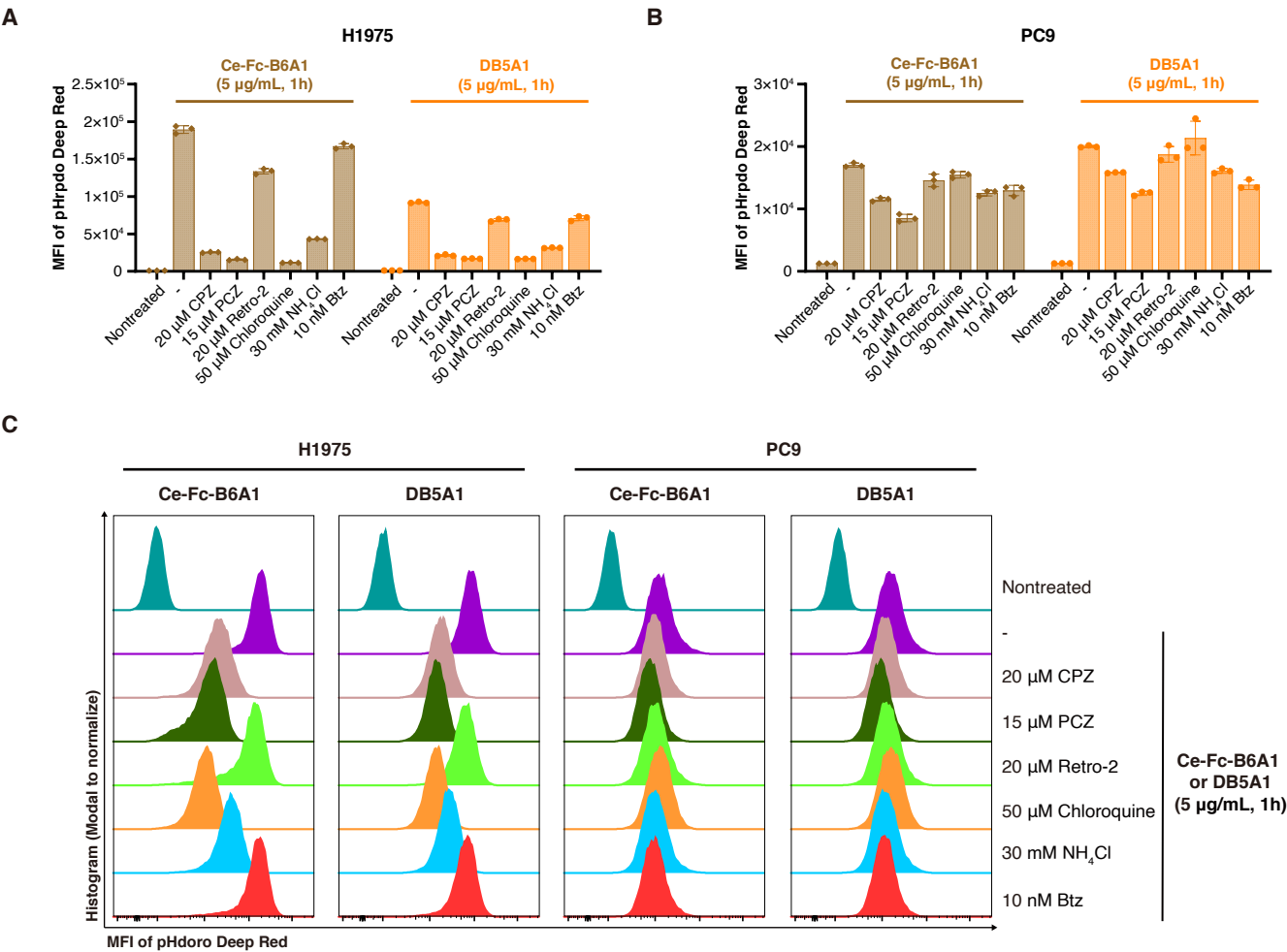


Fig. S10