A functional screening platform for engineering chimeric antigen receptors with reduced on-target, off-tumour activation

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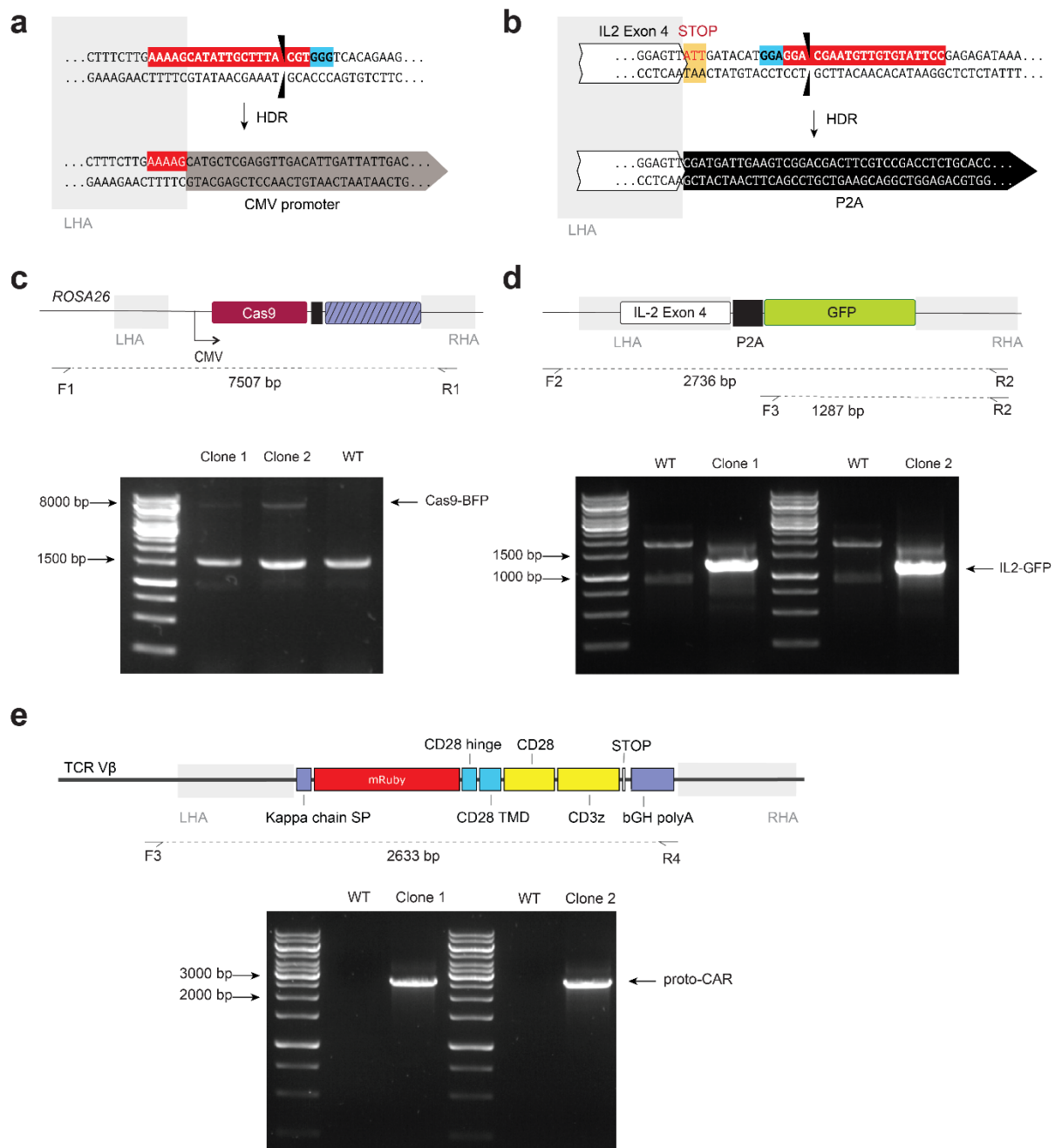
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SUPPLEMENTARY INFORMATION

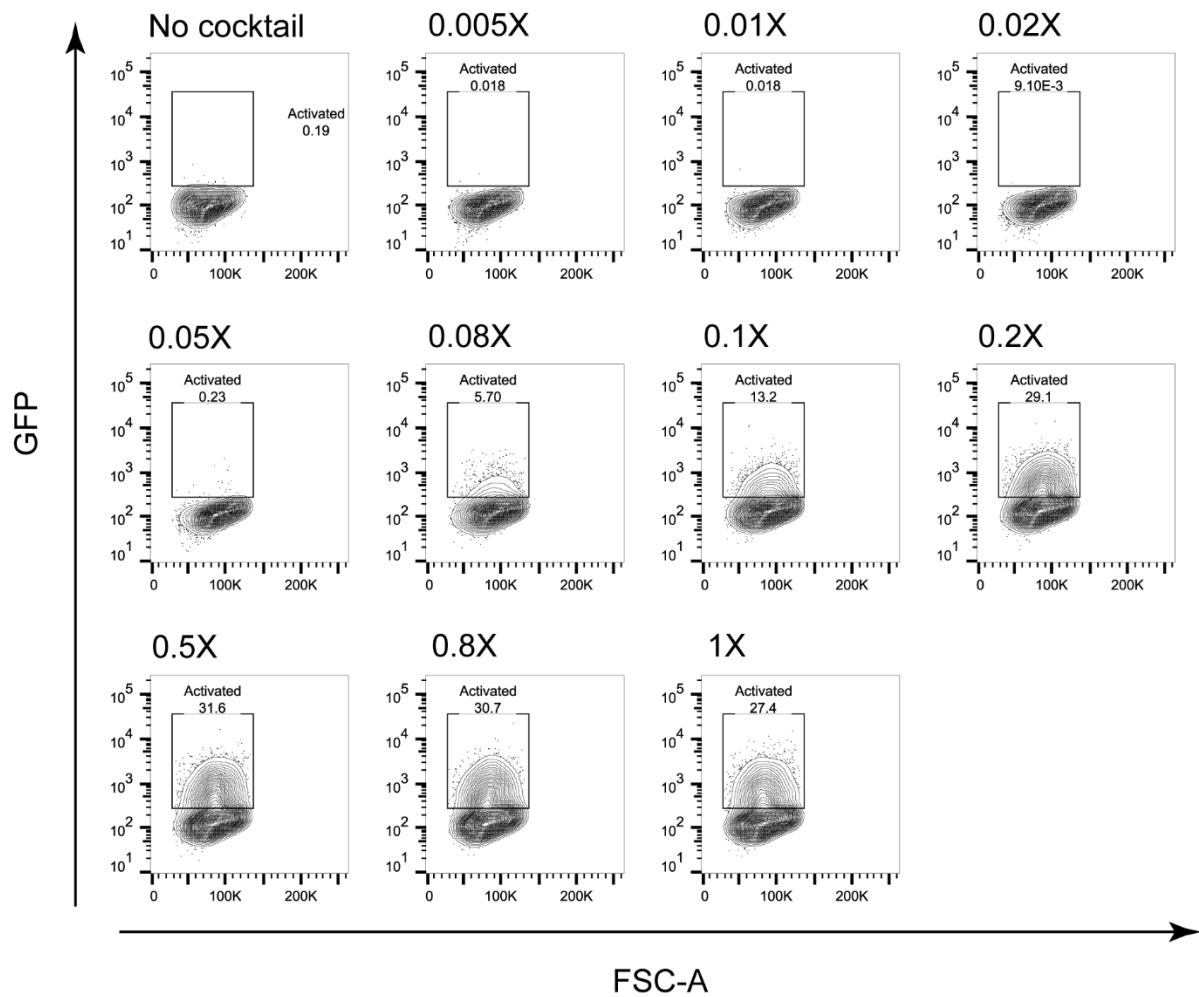SUPPLEMENTARY FIGURES 1 – 6

SUPPLEMENTARY TABLES 1 and 2

3   **Supplementary Figure 1: Genomic integration strategy and PCR confirmations for engineering the CAR T cell**

4   **platform. a** CRISPR-Cas9 cut site in the *ROSA26* locus and post-HDR sequence of the integrated Cas9-BFP

5   expression cassette. **b** CRISPR-Cas9 cut site in the IL-2 locus and post-HDR sequence of the integrated GFP ORF. **c-**

6   **e** PCR amplification strategy to confirm the genomic integration of the Cas9-BFP cassette, GFP ORF or proto-CAR

7   expression cassette. DNA gels show the sequenced amplimers.

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10 **Supplementary Figure 2: Representative flow cytometry plots of GFP expression in engineered T cells**

11 **following PMA/ionomycin stimulation.** Following overnight incubation with varying concentrations of the cell

12 stimulation cocktail PMA/ionomycin (1X: 2 µL/mL), cells were collected and assayed. The fraction of GFP-positive

13 cells was used to plot the dose-response curve of Fig. 1**d**.

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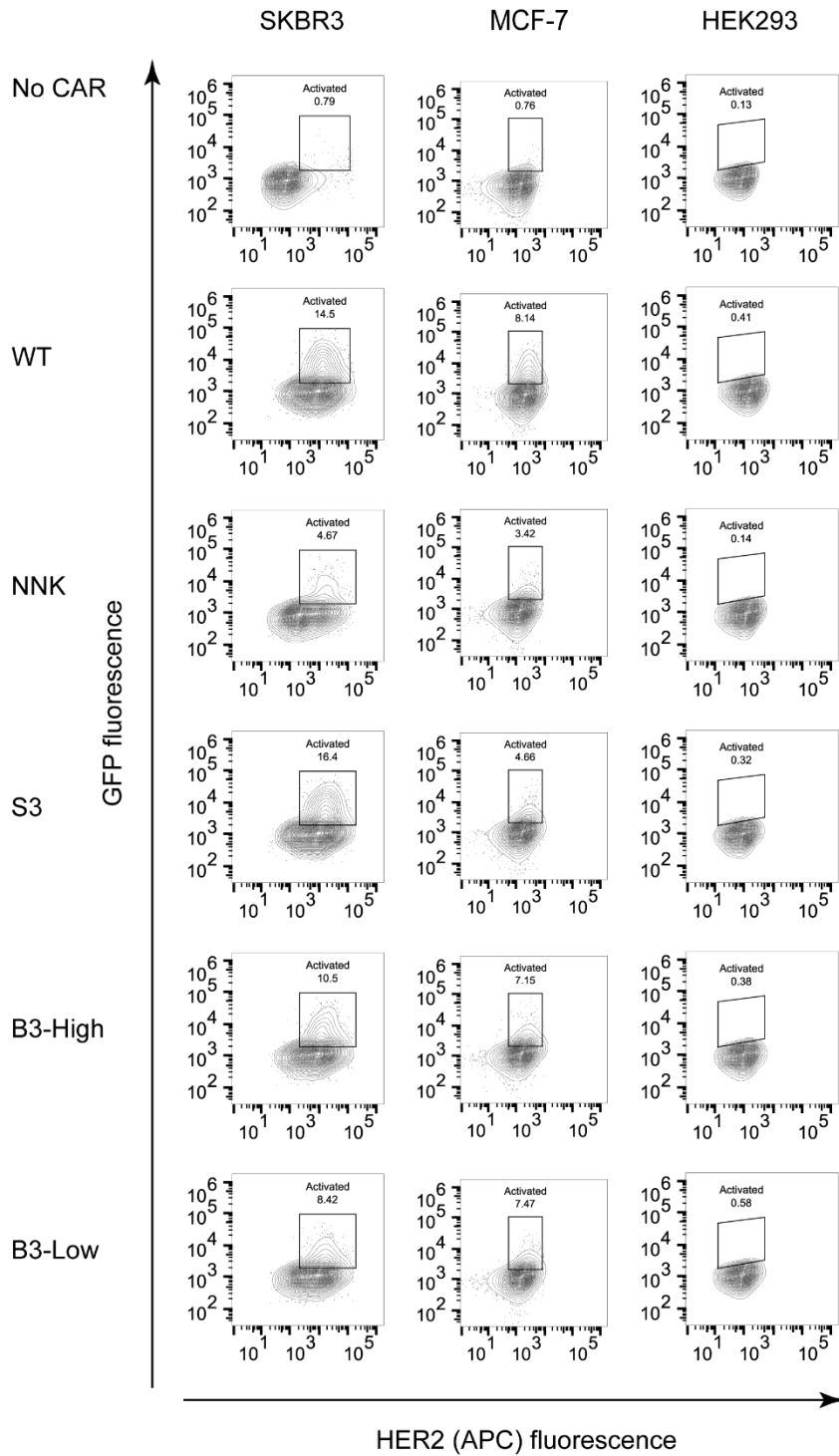
Supplementary Figure 3: Representative flow cytometry plots of GFP expression in post-selection CAR T cell libraries following co-culture with HER2-expressing cell lines. CAR T cell libraries were incubated overnight in co-culture with SKBR3, MCF-7 or HEK293. Cells were collected and labeled with monoclonal anti-HER2 antibody (APC-conjugated) to distinguish CAR T cells from target cells on the basis of HER2 cell surface expression. The fraction of GFP-positive cells was used to plot the histogram of Fig. 4**a**.

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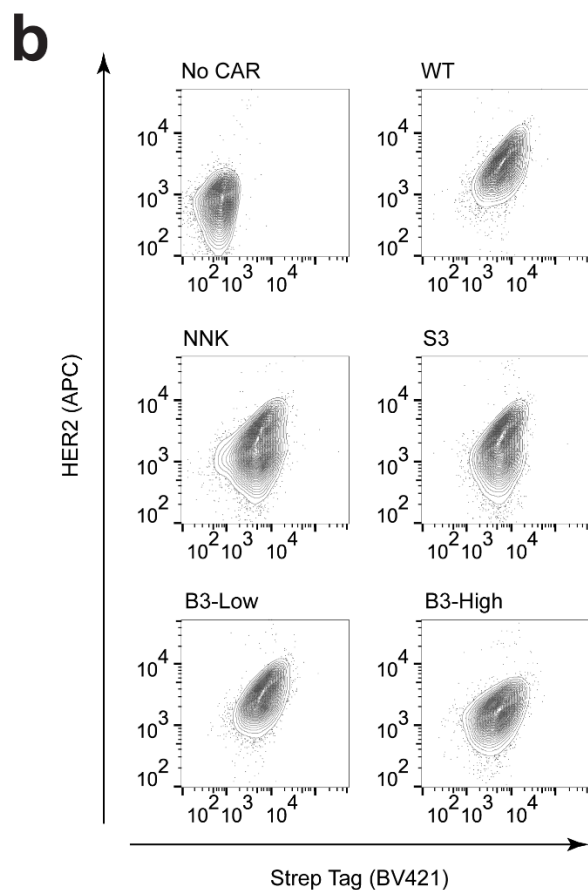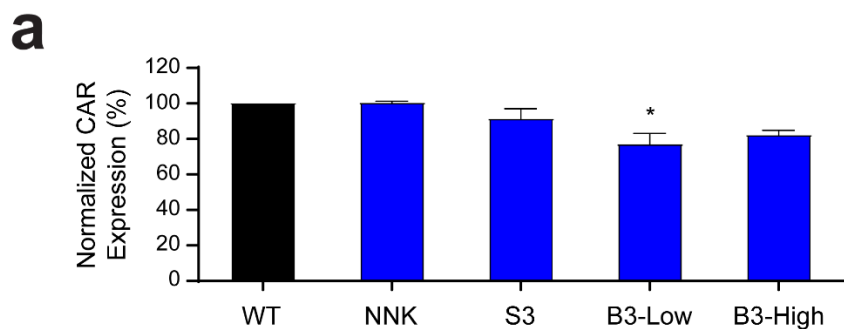
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24 **Supplementary Figure 4: CAR cell surface expression shows little difference across variant libraries. a** Bar

25 plot of CAR surface levels from CAR T cell platform libraries following selection. Mean fluorescence for each was

26 obtained from two independent experiments normalized to the WT of that experiment after subtracting background

27 fluorescence. For assessing significance, libraries were compared to the WT and Dunnett's multiple comparisons test

28 was used with the following indicators: * P < 0001, ** P < 0.001, *** P < 0.0001. **b** Representative flow cytometry

29 plots of CAR T cells used to generate the bar plot in **a**. Cells were labeled with a monoclonal anti-Strep tag (BV421-

30 conjugated) and a combination of soluble HER2 antigen and monoclonal anti-HER2 antibody (APC-conjugated).
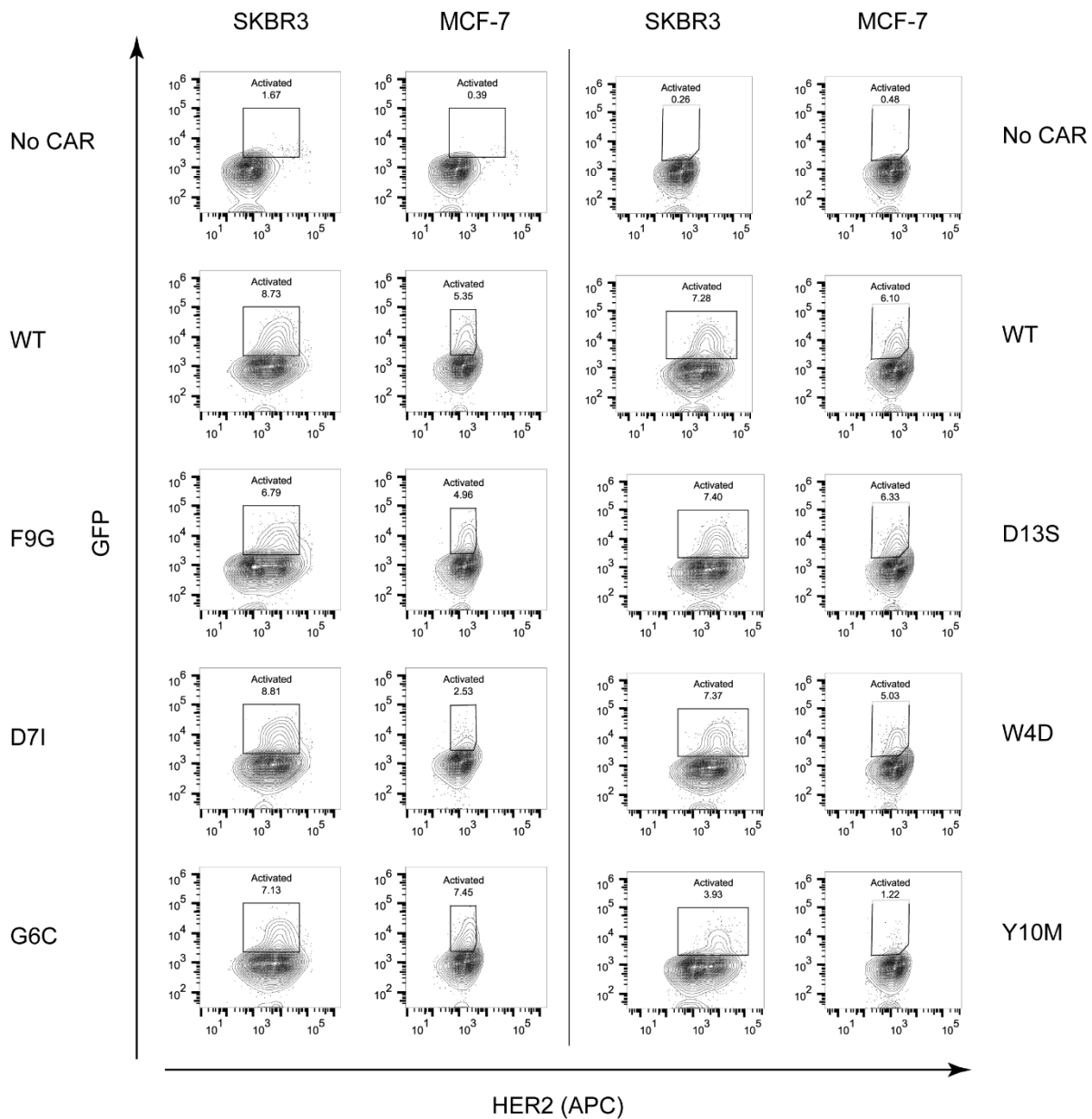
**Supplementary Figure 5: Representative flow cytometry plots of GFP expression in CAR T cell variants following co-culture with HER2-expressing cell lines.** Selected CAR T cell variants were incubated overnight in co-culture with SKBR3 or MCF-7 cells. Cells were collected and labeled with monoclonal anti-HER2 antibody (APC-conjugated) to distinguish CAR T cells from target cells on the basis of HER2 surface expression. The fraction of GFP-positive cells was used to plot the bar plot of Fig. 5**c** and **d**.
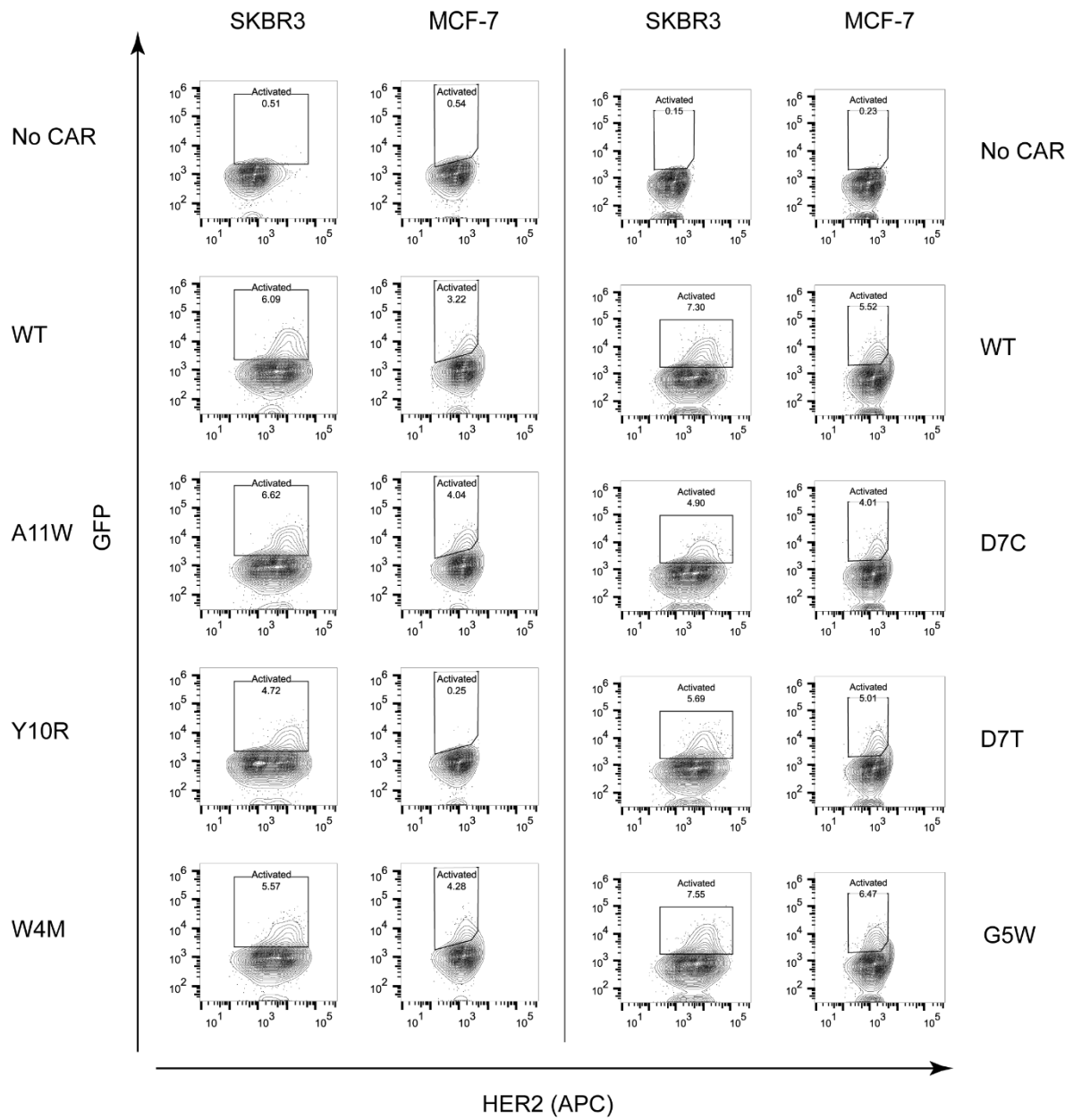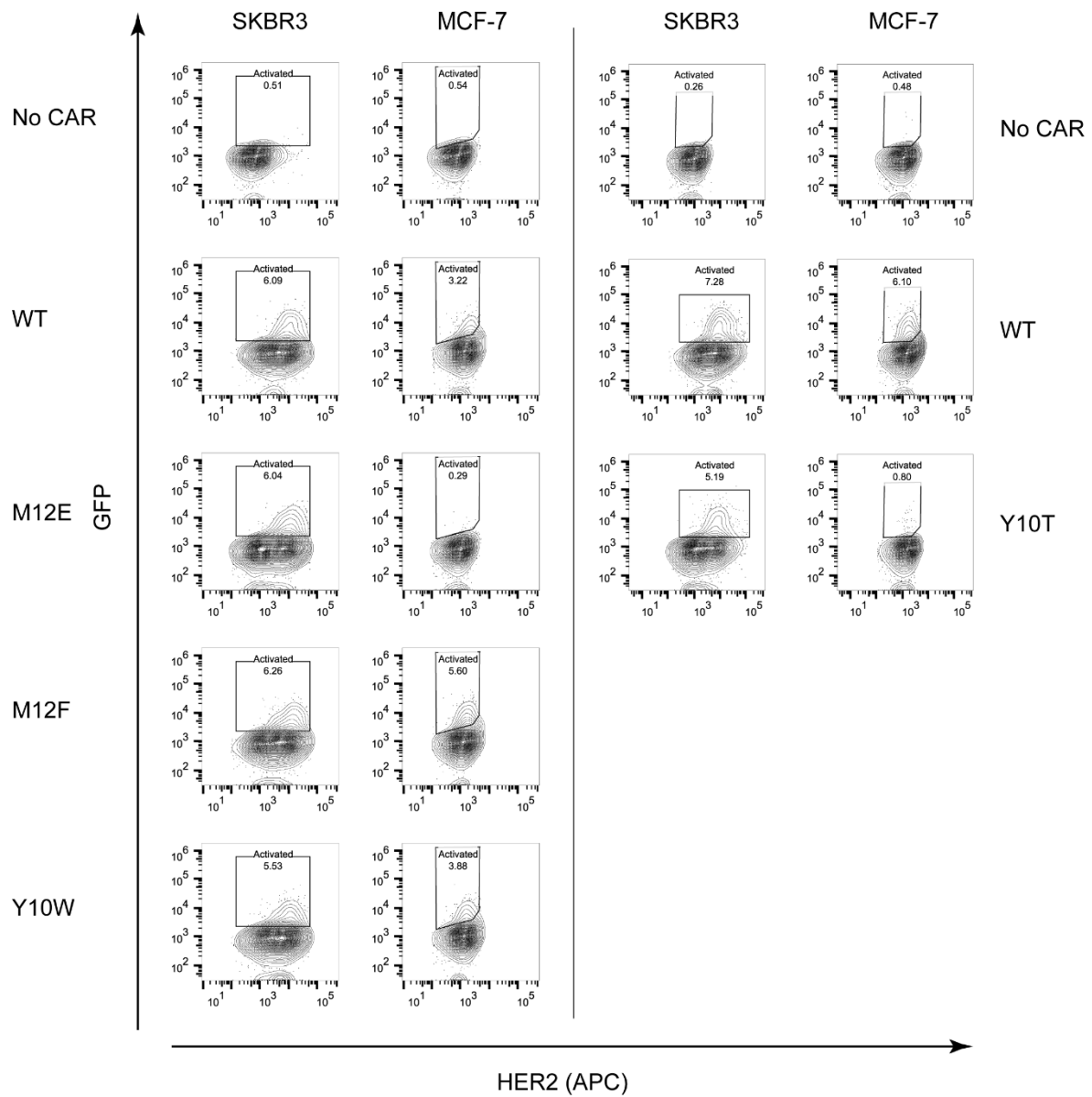
Supplementary Figure 5 (continued)
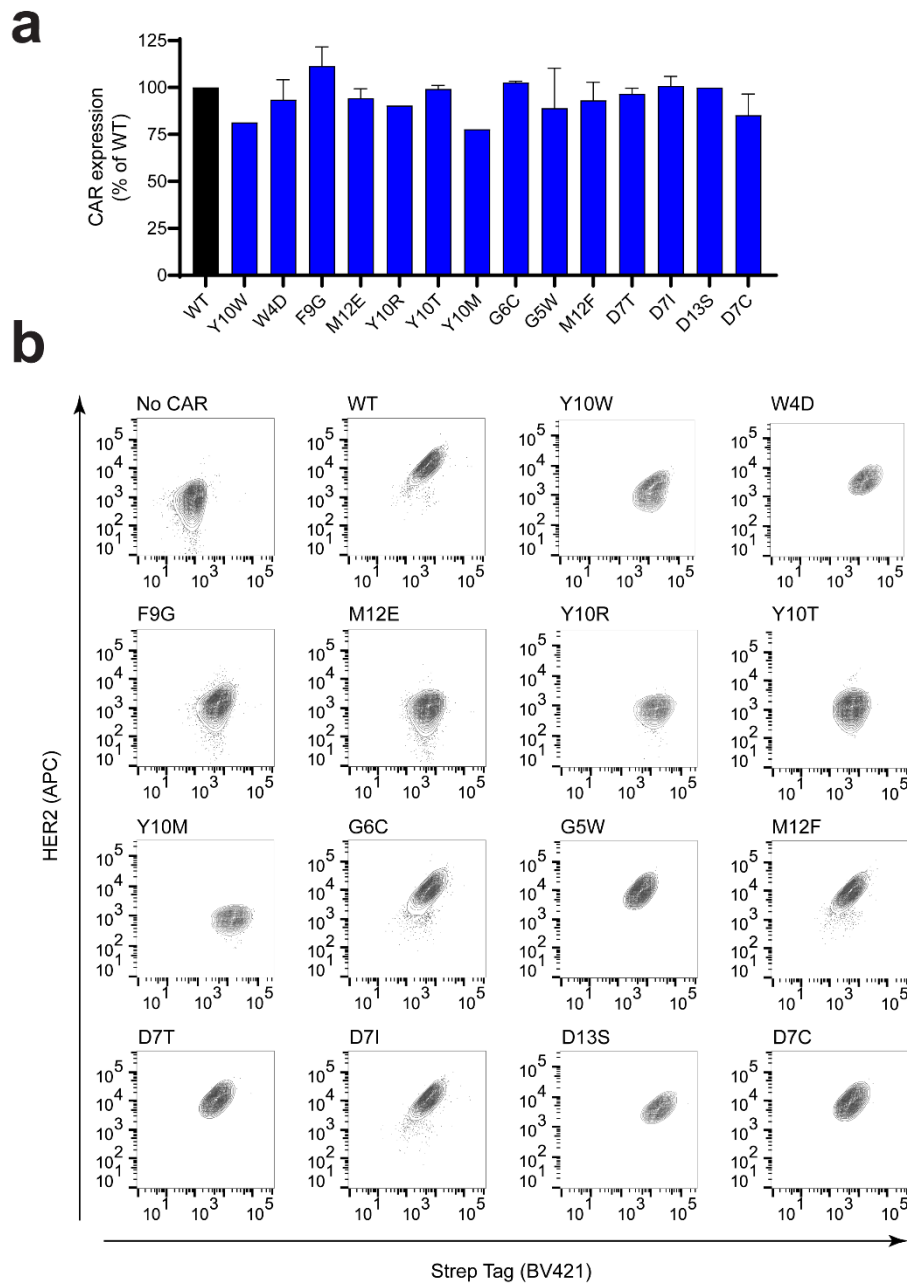
Supplementary Figure 5 (continued)

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Supplementary Figure 6: CAR surface expression shows little difference across variants. a Bar plot of CAR surface expression from selected CAR T cell variants. Mean fluorescence for each was obtained from two independent experiments normalized to the WT of that experiment after subtracting background fluorescence. For assessing significance, variants were compared to the WT and Dunnett's multiple comparisons test was used with the following indicators: * P < 0001, ** P < 0.001, *** P < 0.0001. **b** Representative flow cytometry plots of CAR T cells used to generate the bar plot in **a**. Cells were labeled with a monoclonal anti-Strep tag (BV421-conjugated) and a combination of soluble HER2 antigen and monoclonal anti-HER2 antibody (APC-conjugated).

53 SUPPLEMENTARY TABLES

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55 Supplementary Table 1: Oligonucleotides used in this study

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Name	Sequence (5' to 3')	Purpose
F1	CTGTATTAGTTGAAGGAAAGTGT	To amplify Cas9-BFP in the *ROSA26* locus for sequencing
R1	TTTACTGAACAAATGAACTACTTTTT	
F2	TTGCCTTTGCCAAAATAAGATTTTA	To amplify IL2-GFP in the IL-2 locus for sequencing
R2	AAGAGGGTGTGGGCTAGTCCTTTCC	
F3	ATGGTGAGCAAGGGCGAGG	
F4	GAGCCCCTTTTTTTTTTGGTCTG	To amplify the CAR in the TCR Vβ locus
R3	CTTACCCATGCCTGCTATTCTCTTCCCAATC	
F5	GGTCAGACAAGCTCCCGGAAAAGGA	To amplify the CDRH3 of the anti-HER2 CAR for sequencing
R4	TTCCATTGCTCCTCTCGTTGTCTAGG	

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59 Supplementary Table 2: crRNA sequences used in this study

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Genomic target	crRNA sequence (5' to 3')
ROSA26	AAAAGCAUAUUGCUUUACGU
BFP	CACUUCAAGUGCACAUCCGA
IL-2	GGAAUACACAACAUUCGUCC
mRuby2	GUUUACCACGUCCAAGUCAG
4D5 CDRH3	GGGAGAUGGGUUUUACGCUA
4D5 CDRH3 (with deletion)	GUUUUACUAUGGACGUCUGG

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