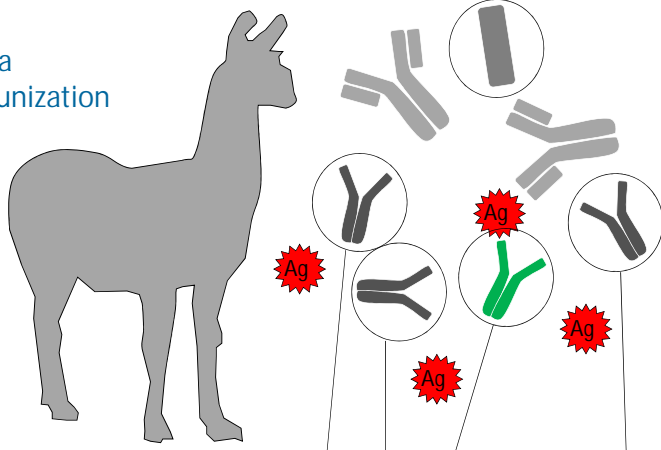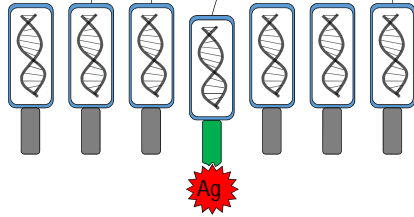**Llama Immunization**

VHH Cloning & Phage Library Construction
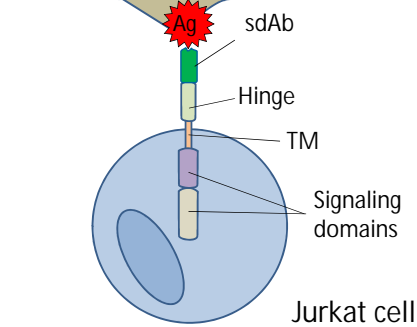
Target Panning

High Throughput Cell Binding Screen

Target

CAR-T Cloning

sdAb

Hinge

TM
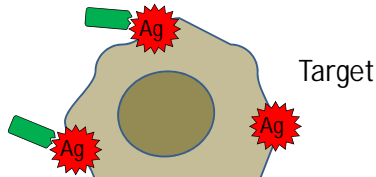
**High Throughput CAR-J Screening (+ and – targets)**

Signaling domains

Jurkat cell

Fine tune Signaling Through Hinge Truncation

Target cell

Target Antigen
Membrane-proximal target epitope
sdAb
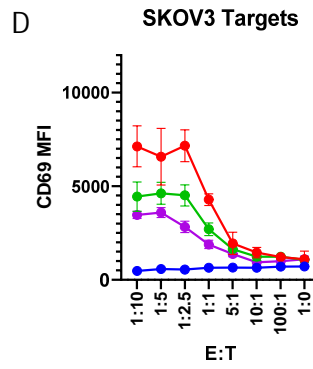Hinge
Transmembrane
Co-stimulation
CD3zeta
T cell

CAR Signaling
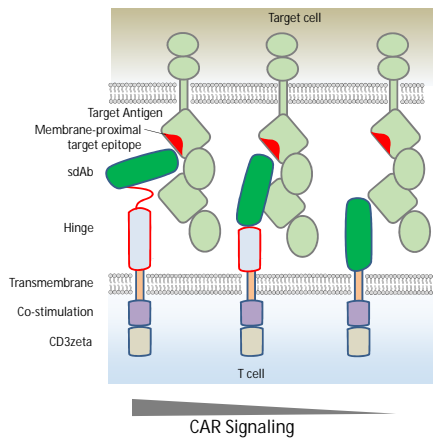
In vitro and *in vivo* confirmatory testing using primary human CAR-T cells

Supplementary figure 1: Workflow diagram for discovery and optimization for novel tumour selective CAR constructs
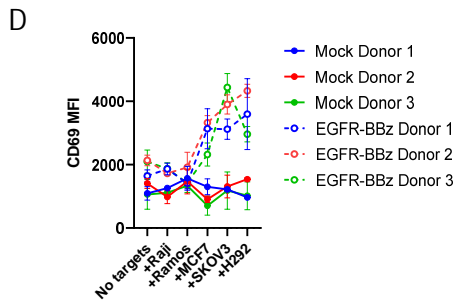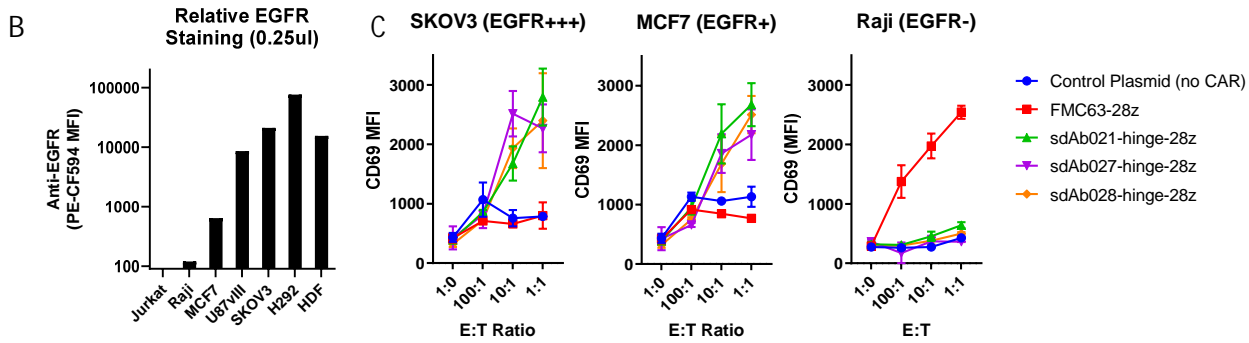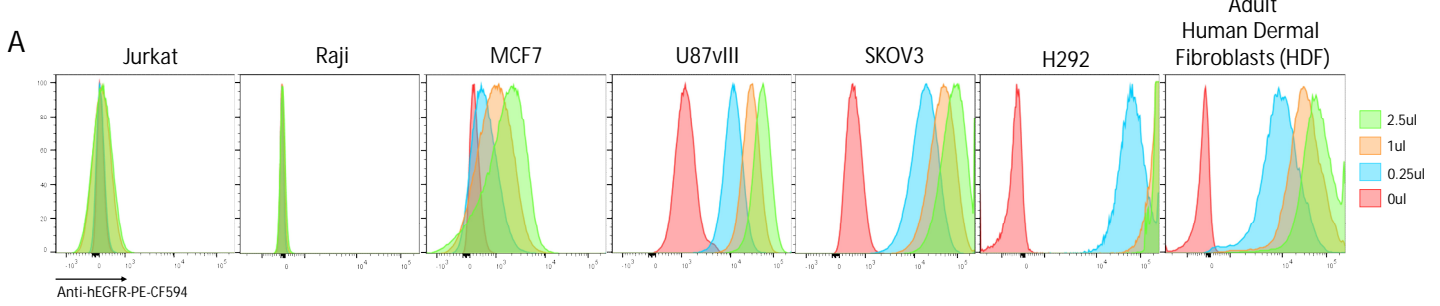
Supplemental Figure 2: EGFR-CAR-Jurkat and primary CAR-T cells can respond to EGFR-positive cells with a wide range of antigen expression
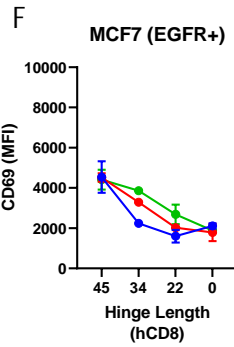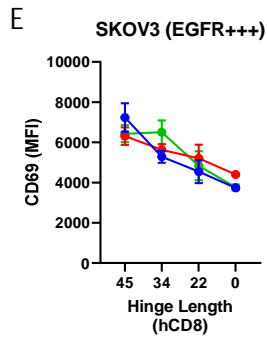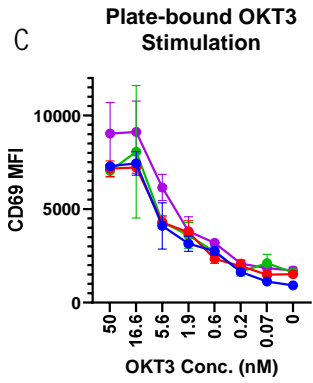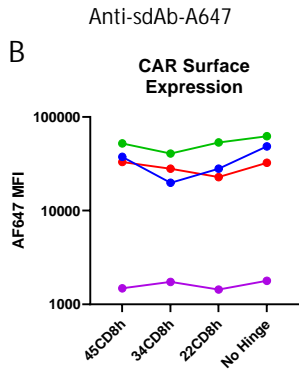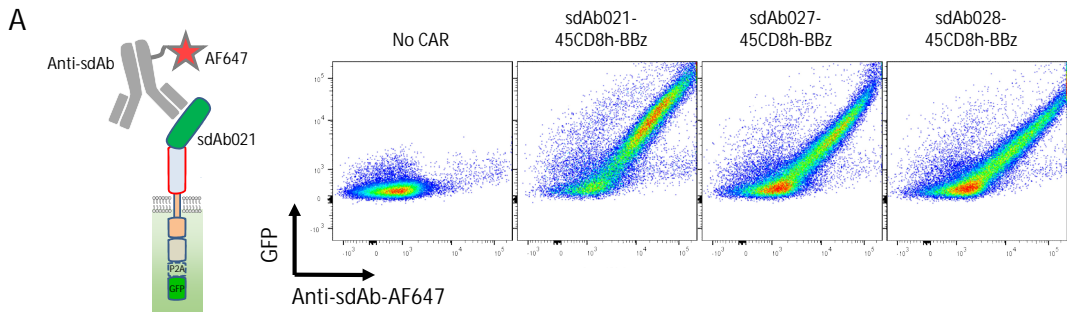
Supplemental Figure 3: CAR Surface expression is not altered with hinge domain modification
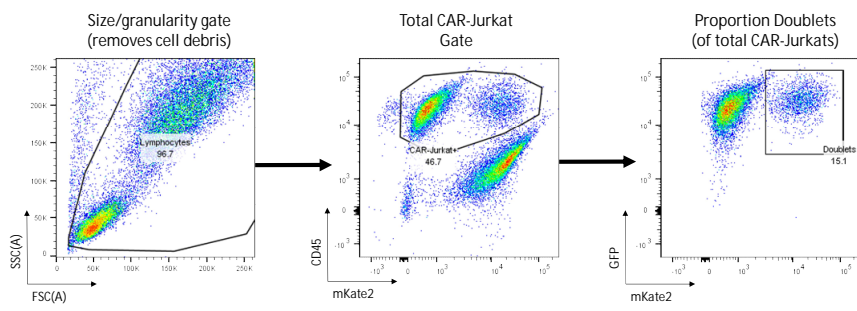
Supplemental Figure 4: Gating scheme for CAR-Jurkat target cell doublet formation assay
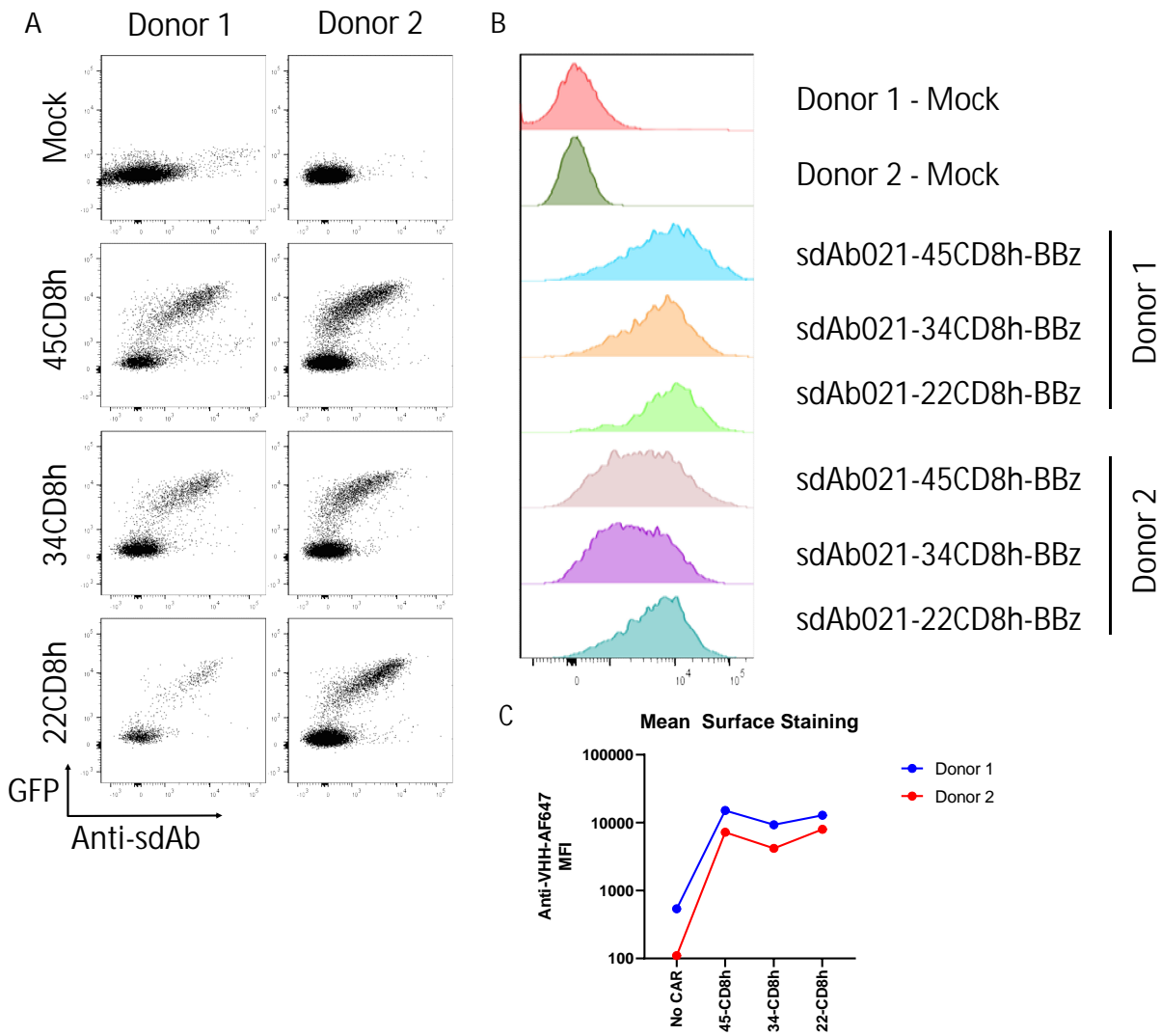
Supplemental Figure 5: CAR Surface expression in primary CAR-T cells does not correlate with hinge length
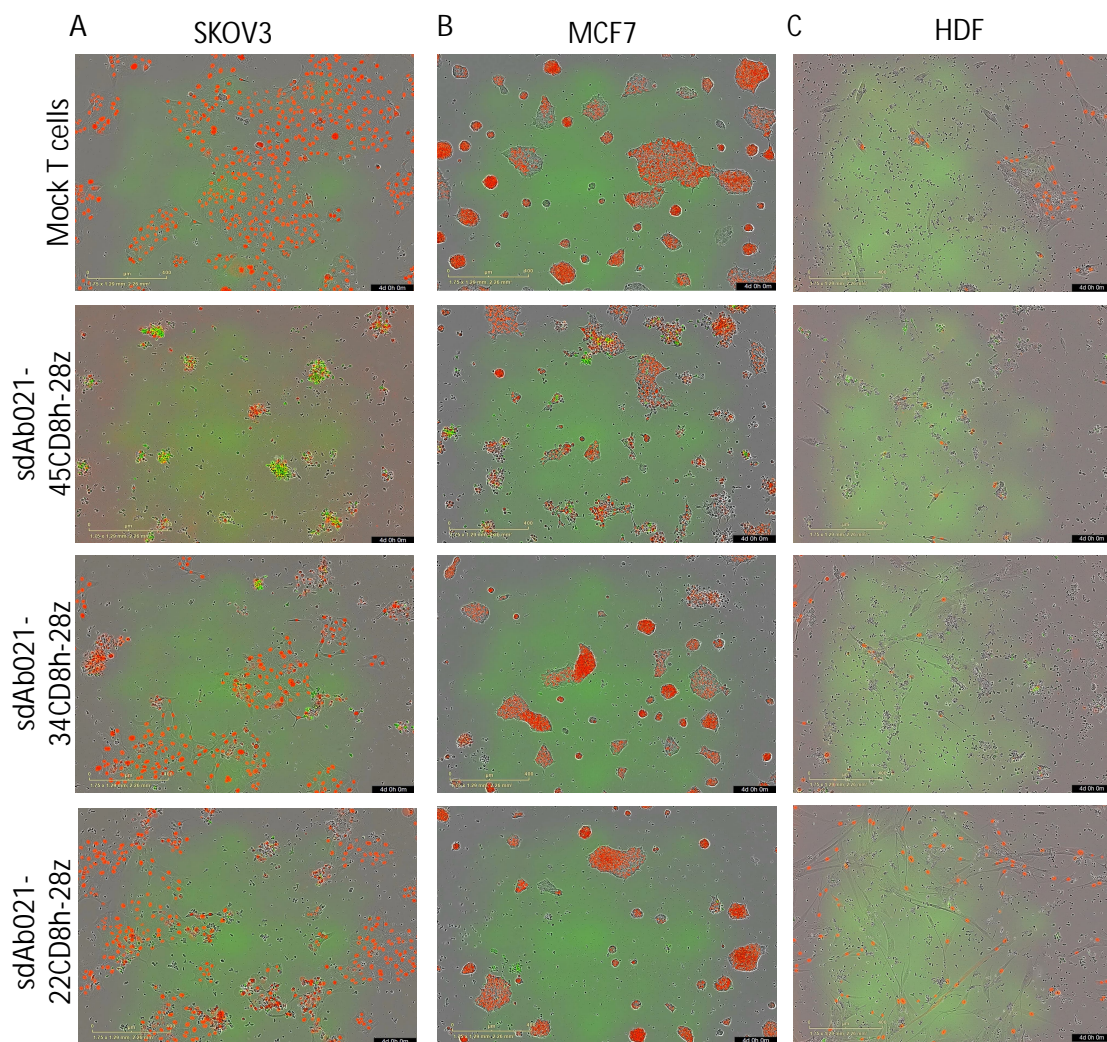
Supplemental Figure 6: Hinge truncation results in progressively reduced EGFR-sdCAR antigen responses and enhanced selectivity for EGFR-high targets

SUPPLEMENTAL Figure 7: Hinge truncated CAR-T cells maintain selectivity for target overexpressing cells following re-challenge

A No T cells

SKOV3 (red) and HDF (unmarked) in co-culture
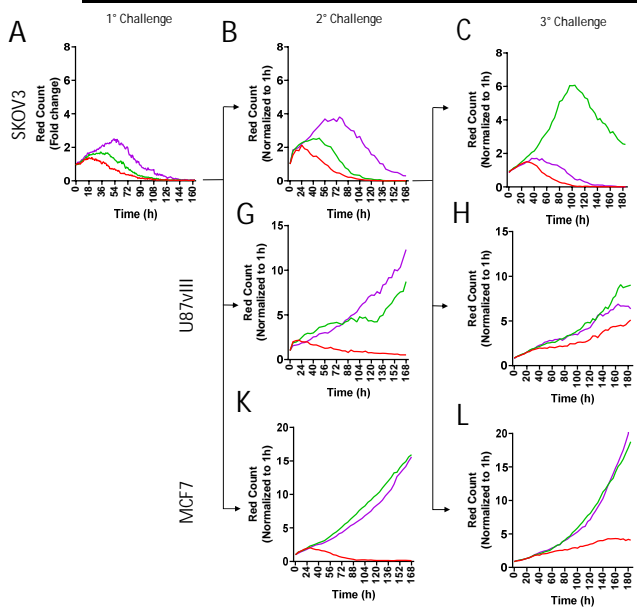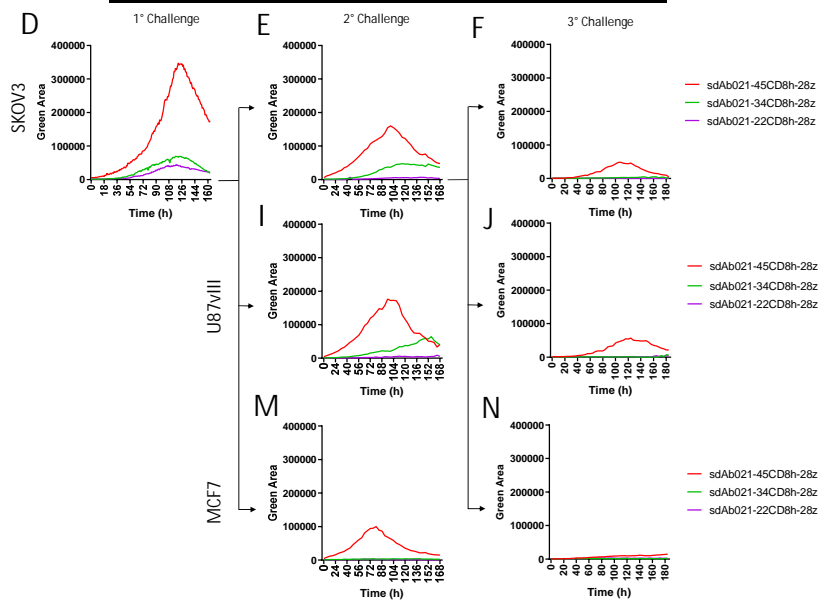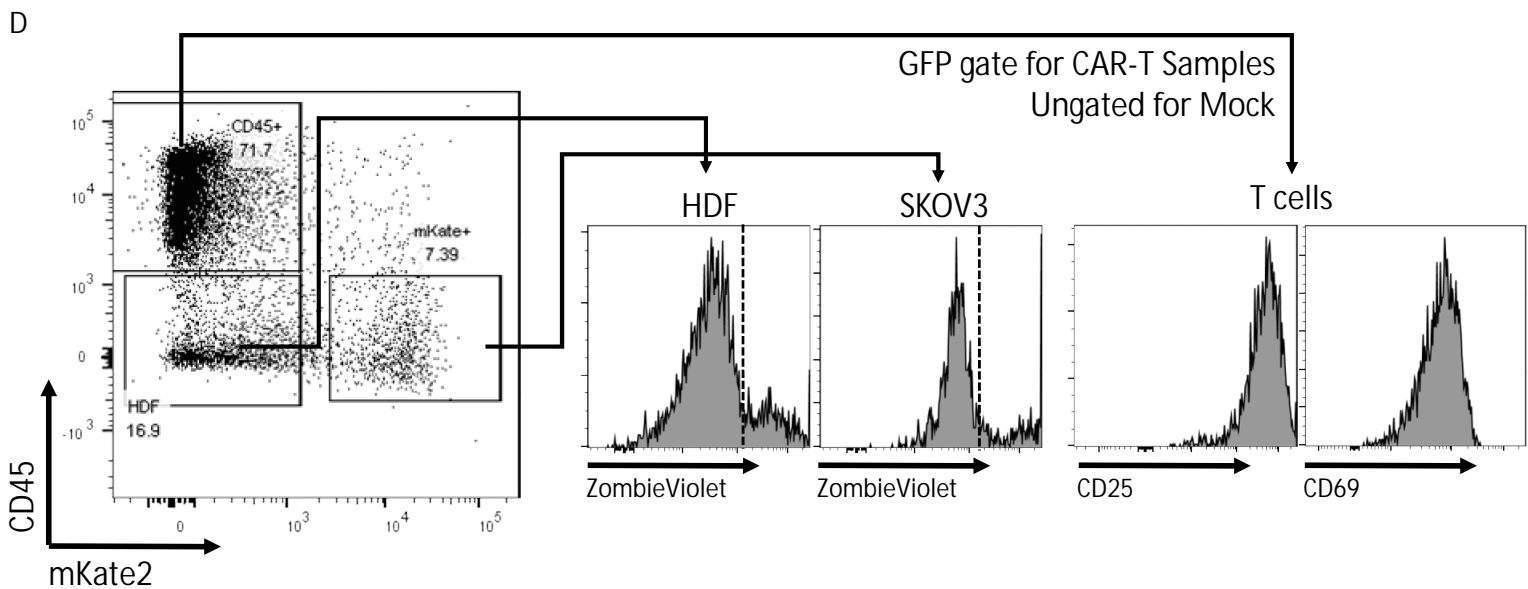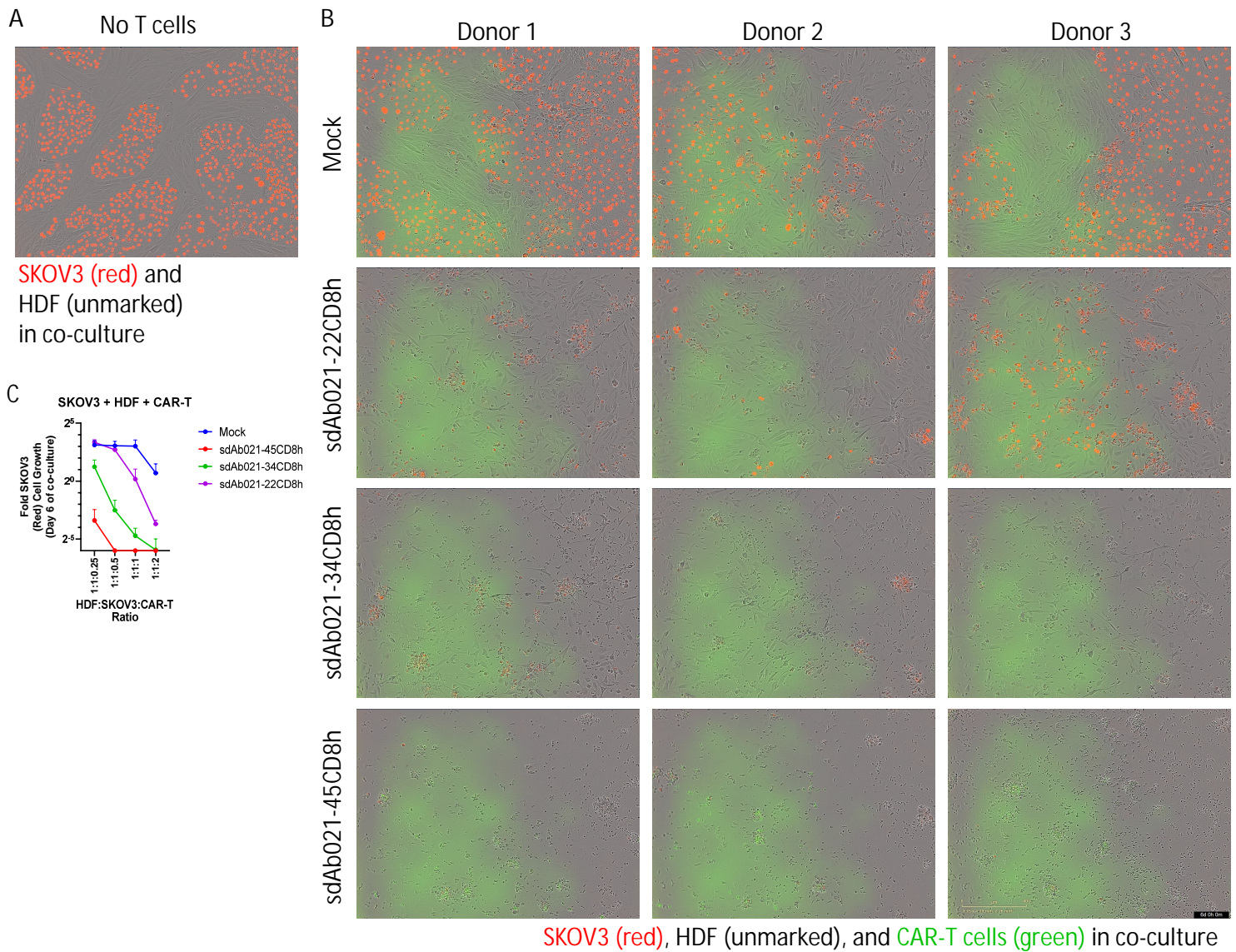
B Donor 1   Donor 2   Donor 3

Mock

sdAb021-22CD8h

sdAb021-34CD8h

sdAb021-45CD8h

SKOV3 (red), HDF (unmarked), and CAR-T cells (green) in co-culture

C

SKOV3 + HDF + CAR-T

Fold SKOV3 (Red) Cell Growth (Day 6 of co-culture)

- Mock
- sdAb021-45CD8h
- sdAb021-34CD8h
- sdAb021-22CD8h

HDF:SKOV3:CAR-T Ratio: 1:1:0.25 1:1:0.5 1:1:1 1:1:2

D

CD45

mKate2

CD45+ 71.7

mKate+ 7.39

HDF 16.9

GFP gate for CAR-T Samples
Ungated for Mock

HDF — ZombieViolet

SKOV3 — ZombieViolet

T cells — CD25

CD69

Supplemental Figure 8: Hinge truncated CAR-T cells maintain enhanced tumour selectivity in triple co-cultures with both healthy donor cells