Supplementary Figure Legends

Figure S1. (A-F) Optimization of the CD3-bispecific antibody CRISPR screens. *CD123* was knocked out in MOLM13 cells ($CD123^{KO}$) to mimic antigen loss induced resistance. RTCC assay (n=3) of Scramble and $CD123^{KO}$ MOLM13 cells in response to CD123-DART (left panel) and Isotype CD3-bispecific antibody (right panel) was conducted (A). A companion qPCR assay was developed to quantify sgRNA transduced cells with high specificity. This assay was used to quantify Scramble and CD123 targeting guides in the genomic DNA using guide specific PrimeTime qPCR probe sets (n=3) (B). In a pilot test, $CD123^{KO}$ cells were mixed 1:9 with wild-type Scramble cells followed by RTCC assay using the CD123-DART antibody. The relative ratio between $CD123^{KO}$ and Scramble cells was quantified used the qPCR assay, and the best differential was achieved at 1pM of CD123-DART treatment and a T-cell to target cell ratio (E:T) of 1:1 (n=4) (C). Actual CRISPR screen with CD123-DART treatment at 1pM and 10pM. Note that the10pM group performed significantly worse than1pM group (D), which echoed the results in the qPCR-based optimization (C). The same qPCR optimization steps were performed for SEM (n=5) and HCC827 cells (n=4) using the CD123-DART and PCAD-DART antibodies (E and F). (G) Gene ontology analysis of the resistance hits and sensitizing hits from the CRISPR screens in all three cell lines. (H) Pathway map of the highest enriched resistance hit pathway "Inflammation Interferon Signaling".

Figure S2. (A) Western blot validation of *JAK1* and *STAT1* knockout in MOLM13, SEM, and HCC827 cells. The JAK1 blot in MOLM13 cells was omitted due to no detectable signal. (B) Sanger sequencing validation of *JAK1* knockout in MOLM13 cells. The validation was performed by mapping the sequencing data with the sgRNA targeting reference sequence using the TIDE tool (2). (C) Western blot validation of *PTPN2* knockout in MOLM13 cells. (D) Quantification of IFNγ in the supernatant of the RTCC assay in MOLM13, SEM and HCC827 cells (n=5). (E) RTCC assay (n=4) of B2M knockout MOLM13, SEM and HCC827 cells. (F) Volcano plot of the gene expression from the cells following IFNγ treatment (n=3). The CRISPR screen overlapping hits were highlighted in boxes. (G) FACS analysis of the ICAM1 expression in Scramble and $JAK1^{KO}$ MOLM13 cells treated by titrated doses of IFNγ. (H) RTCC assay (n=4) of ICAM1 overexpressed Scramble or $JAK1^{KO}$ MOLM13 cells at 1pM of CD123-DART antibody. (I) Killing of SEM cells (n=3) with combination treatment of IFNg (10ng/ml) and FasL (6ng/ml).

Figure S3. (A) LCA staining of the MOLM13 and SEM cells 4 days post indicated doses of L-fucose treatment. (B) RTCC assay of MOLM13 and SEM cells post L-fucose treatment (n=4). (C) FUT8 overexpression in MOLM13 and SEM cells (upper panel) and LCA staining (low panel). (D) RTCC assay of FUT8 overexpressed MOLM13 and SEM cells compared to the parental groups (n=3). (E) Western blot validation of FUT8 knockout by two guides (left panel) and silver staining of LCA pull down products from the 3 groups (right panel). (F) RTCC assay of $FUT8^{KO}$ MM1S, HCC827 and HCC1954 cells using the BCMAxCD3 and PCAD-DART CD3-bispecific antibodies (n=4).

Figure S4. (A) Quantification of the afucosylation status of CD123His-Scramble and CD123His-$FUT8^{KO}$ protein samples. The proteins were treated with PNGase and the released glycan species were analyzed by capillary electrophoresis. Peaks indicate different glycan structures according to selected glycan standards. Two sipping were performed for each sample. (B) SPR analysis of CD123-Scramble and CD123-$FUT8^{KO}$ binding to 6H6 and CD123-$DART_{IgG}$, and original CD123-DART. The proteins were captured on the chips and the antibodies were subsequently flowed through. (C) Schematics of CD123 protein, highlighting the NTD, D2, D3 domains in the ECD, as well as 3 putative N-glycosylation sites (left panel). Antibody staining of human-mouse CD123 domain chimeras as indicated by their names (right panel). (D) Binding competition assay of 6H6 and CD123-$DART_{IgG}$ antibodies. MOLM13 and SEM cells were stained with AF647 labeled 6H6, CD123-$DART_{IgG}$ or isotype antibodies in the presence of unlabeled forms. Inhibition% of binding was calculated by normalizing to the Isotype group, and presented in the heatmap. (E) Pair distance distribution functions calculated using GNOM for CD123His-Scramble (black) and CD123His-$FUT8^{KO}$ (red). (F) Guinier approximations for CD123His-Scramble (black) and CD123His-$FUT8^{KO}$ (red).

Figure S5. (A) SPR analysis of human IL-3 binding to the CD123-Scramble and CD123-$FUT8^{KO}$ proteins. (B) FACS validation of FUT8 and CD123 knockout in TF-1 cells. (C) Growth of $FUT8^{KO}$ and $CD123^{KO}$ TF-1 cells with titrated doses of GM-CSF treatment (n=4). (D) Western blot of phospho-STAT5 and total STAT5 in $FUT8^{KO}$ and $CD123^{KO}$ TF-1 cells treated with 10ng/ml IL-3. (E) Western blot and FACS validation of FUT8 knockout in CD34+

stem cells by two guides. (F) Growth of Scramble and IL3RA-g3 CD34+ stem cells in different cytokine cocktails at day 5 (n=3). *P<0.05, **P<0.01.

Figure S6. (A) Illustration of the mixed killing RTCC assay setup. Cells of different genotypes were labeled with different fluorescent proteins and spiked in wild-type (Scramble) cells. Each cell population can be tracked by FACS. The right panel shows the analysis of MOLM13 Scramble-BFP/FUT8^{KO}-RFP/JAK1^{KO}-GFP cells at Day 0 of the assay. (B) Tumor volumes of each group at Day 14 and Day 16 from the *in vivo* study described in Figure 6E (n=5). *P<0.05, **P<0.01, n.s. not significant.

1. Paulo JA, O'Connell JD, Gaun A, and Gygi SP. Proteome-wide quantitative multiplexed profiling of protein expression: carbon-source dependency in Saccharomyces cerevisiae. *Mol Biol Cell.* 2015;26(22):4063-74.
2. Brinkman EK, Chen T, Amendola M, and van Steensel B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res.* 2014;42(22):e168.

Figure S1

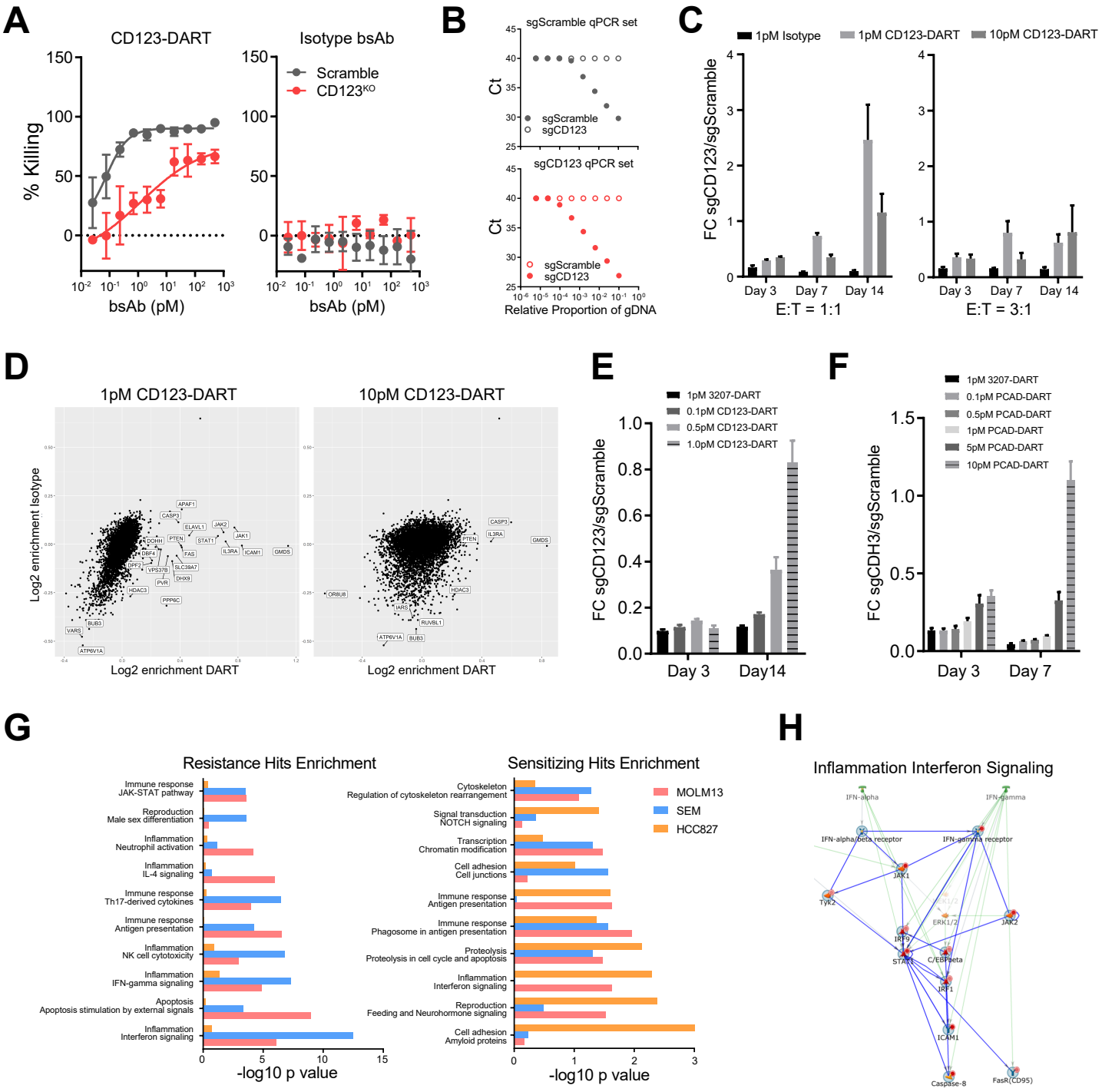

A
CD123-DART
Isotype bsAb
Scramble
CD123KO
% Killing
bsAb (pM)
B
sgScramble qPCR set
sgCD123 qPCR set
sgScramble
sgCD123
Ct
Relative Proportion of gDNA
C
1pM Isotype
1pM CD123-DART
10pM CD123-DART
FC sgCD123/sgScramble
Day 3
Day 7
Day 14
E:T = 1:1
E:T = 3:1
D
1pM CD123-DART
10pM CD123-DART
Log2 enrichment Isotype
Log2 enrichment DART
E
1pM 3207-DART
0.1pM CD123-DART
0.5pM CD123-DART
1.0pM CD123-DART
FC sgCD123/sgScramble
Day 3
Day14
F
1pM 3207-DART
0.1pM PCAD-DART
0.5pM PCAD-DART
1pM PCAD-DART
5pM PCAD-DART
10pM PCAD-DART
FC sgCDH3/sgScramble
Day 3
Day 7
G
Resistance Hits Enrichment
Sensitizing Hits Enrichment
MOLM13
SEM
HCC827
-log10 p value
H
Inflammation Interferon Signaling

Figure S2

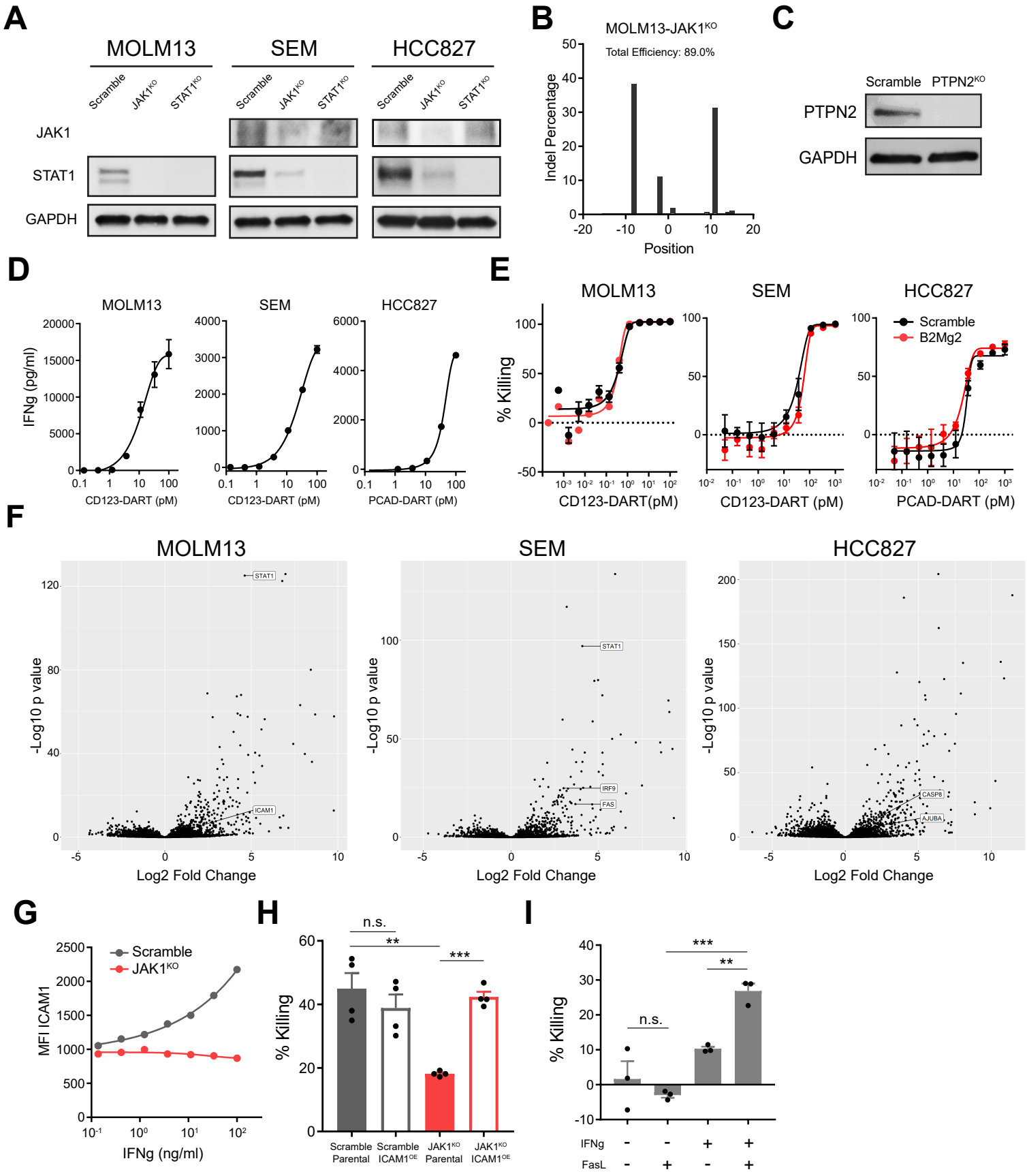

A
MOLM13
SEM
HCC827
Scramble
JAK1KO
STAT1KO
JAK1
STAT1
GAPDH
B
MOLM13-JAK1KO
Total Efficiency: 89.0%
Indel Percentage
Position
C
Scramble
PTPN2KO
PTPN2
GAPDH
D
MOLM13
SEM
HCC827
IFNg (pg/ml)
CD123-DART (pM)
CD123-DART (pM)
PCAD-DART (pM)
E
MOLM13
SEM
HCC827
% Killing
Scramble
B2Mg2
CD123-DART(pM)
CD123-DART (pM)
PCAD-DART (pM)
F
MOLM13
SEM
HCC827
-Log10 p value
Log2 Fold Change
STAT1
ICAM1
IRF9
FAS
CASP8
AJUBA
G
Scramble
JAK1KO
MFI ICAM1
IFNg (ng/ml)
H
n.s.
**
***
% Killing
Scramble Parental
Scramble ICAM1OE
JAK1KO Parental
JAK1KO ICAM1OE
I
***
**
n.s.
% Killing
IFNg
FasL

Figure S3

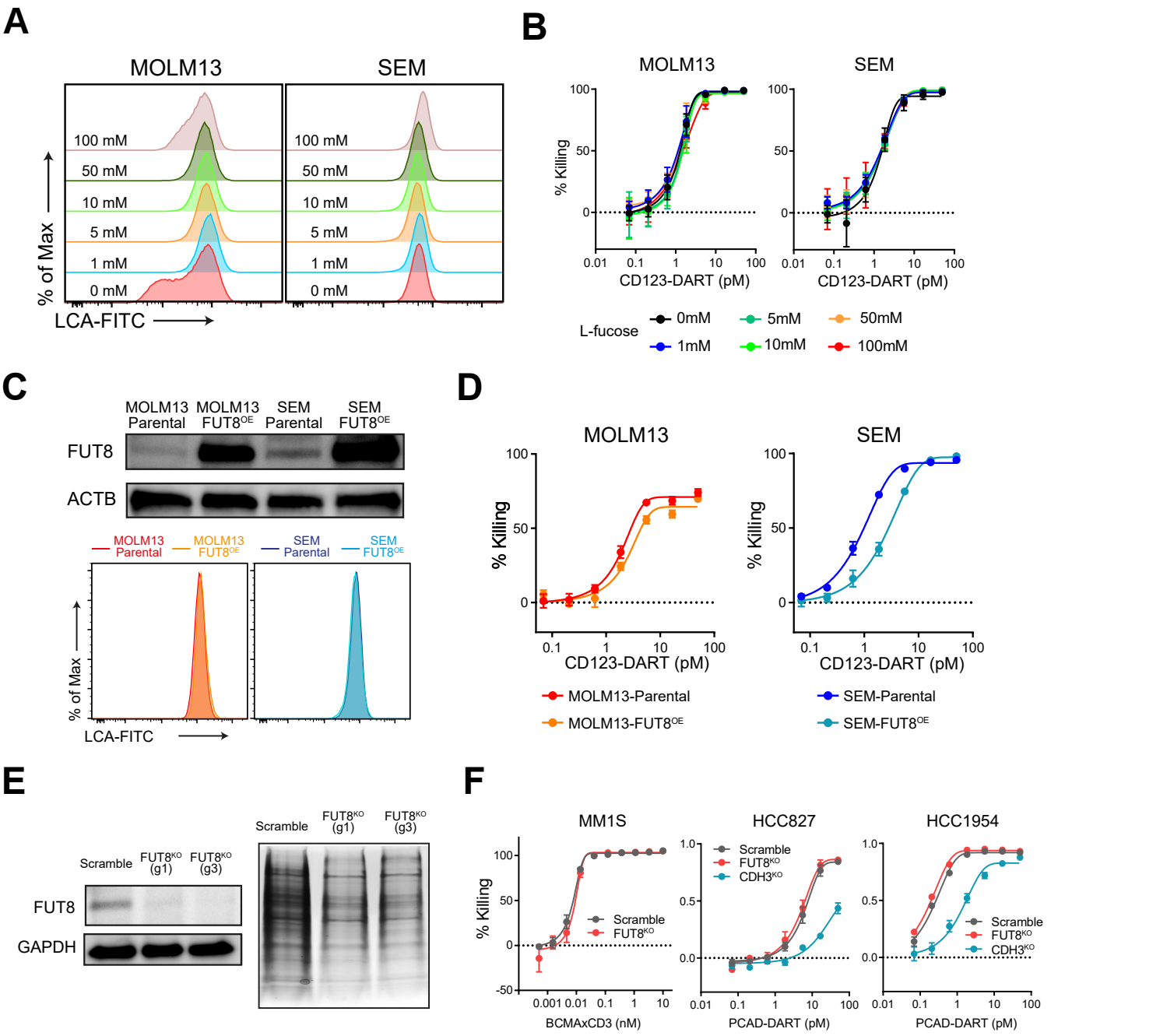

A
MOLM13
SEM
100 mM
50 mM
10 mM
5 mM
1 mM
0 mM
% of Max
LCA-FITC
B
MOLM13
SEM
% Killing
CD123-DART (pM)
L-fucose
0mM
1mM
5mM
10mM
50mM
100mM
C
MOLM13 Parental
MOLM13 FUT8OE
SEM Parental
SEM FUT8OE
FUT8
ACTB
% of Max
LCA-FITC
D
MOLM13
SEM
% Killing
CD123-DART (pM)
MOLM13-Parental
MOLM13-FUT8OE
SEM-Parental
SEM-FUT8OE
E
Scramble
FUT8KO (g1)
FUT8KO (g3)
FUT8
GAPDH
F
MM1S
HCC827
HCC1954
% Killing
Scramble
FUT8KO
CDH3KO
BCMAxCD3 (nM)
PCAD-DART (pM)

Figure S4

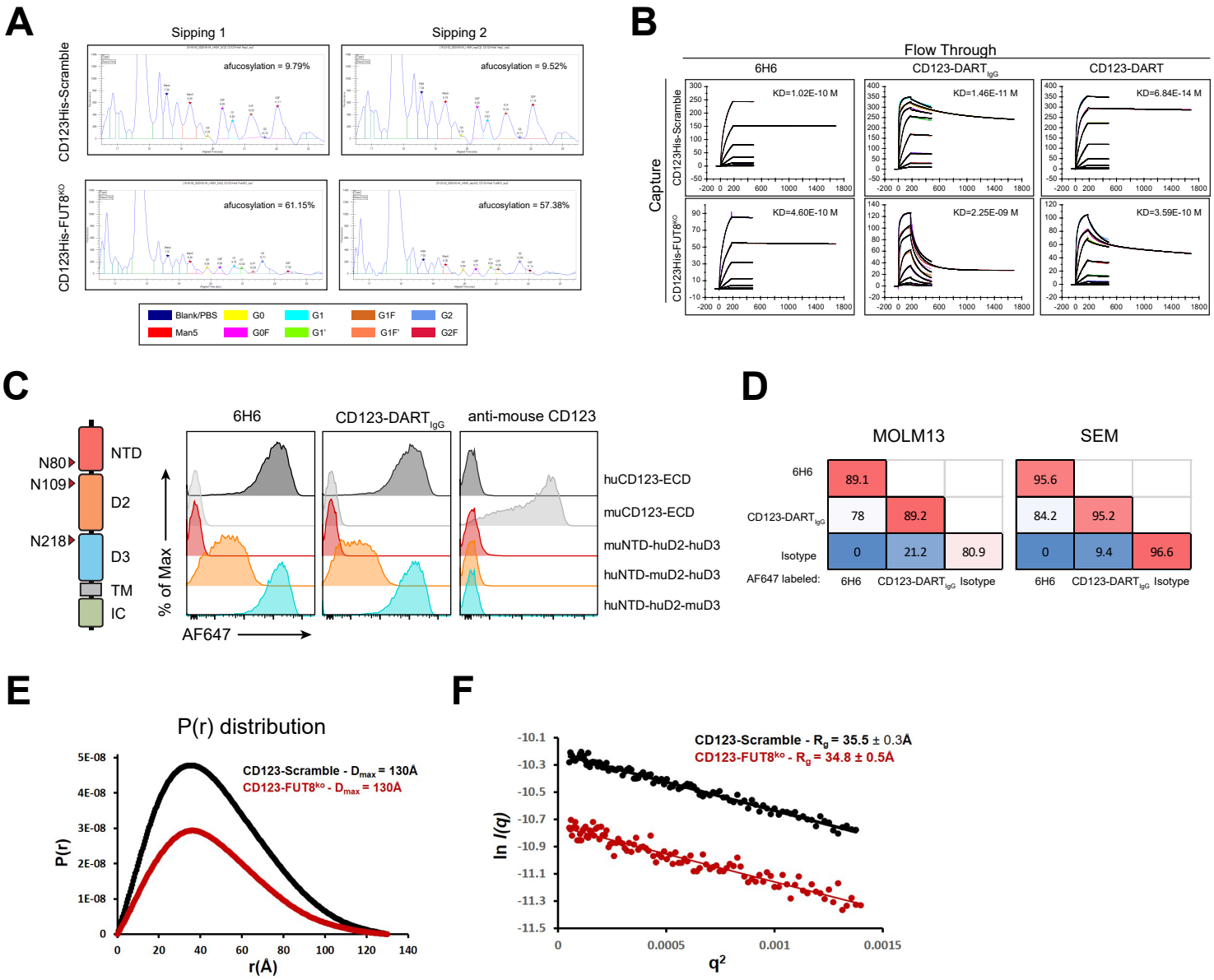

A
Sipping 1
Sipping 2
CD123His-Scramble
CD123His-FUT8KO
afucosylation = 9.79%
afucosylation = 9.52%
afucosylation = 61.15%
afucosylation = 57.38%
Blank/PBS
G0
G1
G1F
G2
Man5
G0F
G1'
G1F'
G2F
B
Flow Through
6H6
CD123-DARTIgG
CD123-DART
Capture
KD=1.02E-10 M
KD=1.46E-11 M
KD=6.84E-14 M
KD=4.60E-10 M
KD=2.25E-09 M
KD=3.59E-10 M
C
6H6
CD123-DARTIgG
anti-mouse CD123
N80
N109
N218
NTD
D2
D3
TM
IC
% of Max
AF647
huCD123-ECD
muCD123-ECD
muNTD-huD2-huD3
huNTD-muD2-huD3
huNTD-huD2-muD3
D
MOLM13
SEM
6H6
CD123-DARTIgG
Isotype
AF647 labeled:
89.1
78
89.2
0
21.2
80.9
95.6
84.2
95.2
0
9.4
96.6
E
P(r) distribution
CD123-Scramble - Dmax = 130Å
CD123-FUT8ko - Dmax = 130Å
P(r)
r(Å)
F
CD123-Scramble - Rg = 35.5 ± 0.3Å
CD123-FUT8ko - Rg = 34.8 ± 0.5Å
ln I(q)
q2

Figure S5

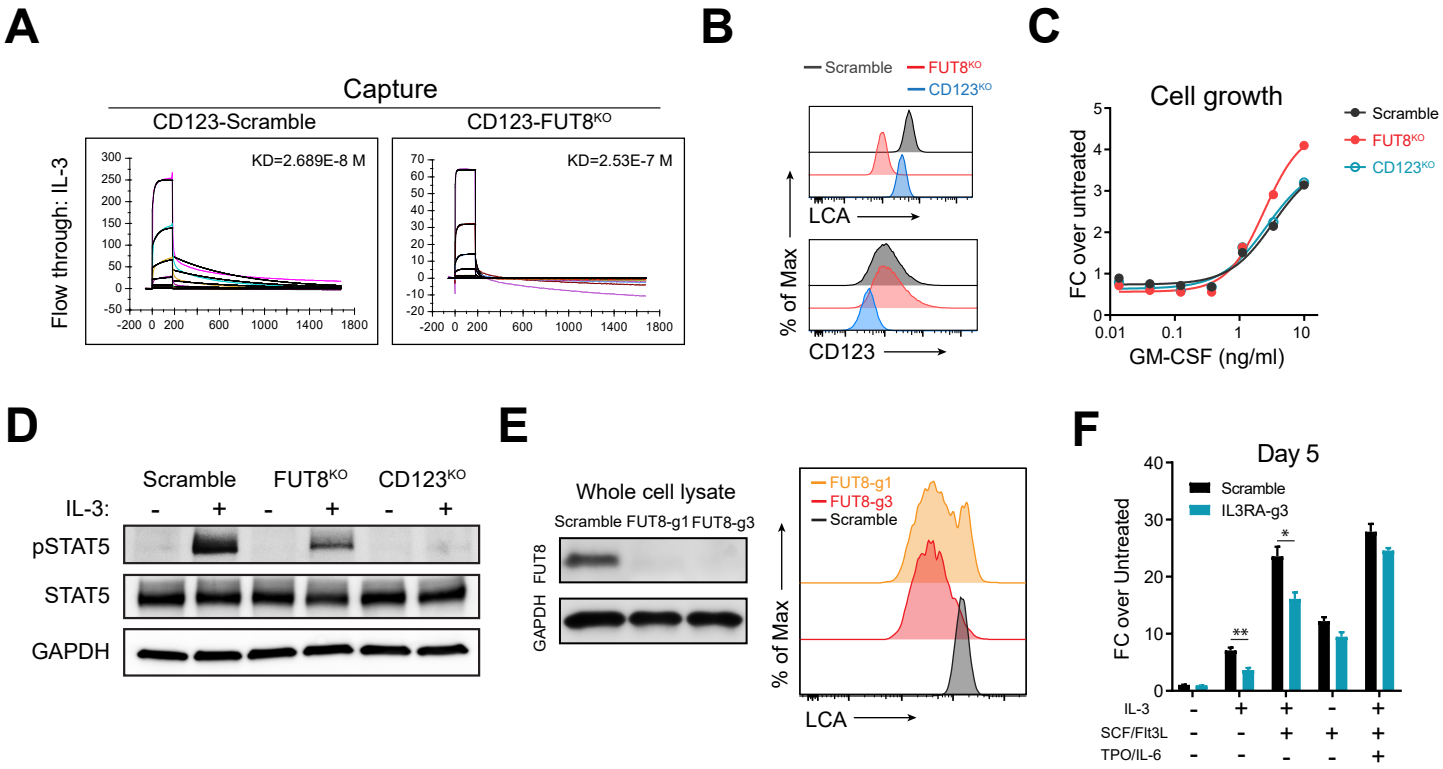
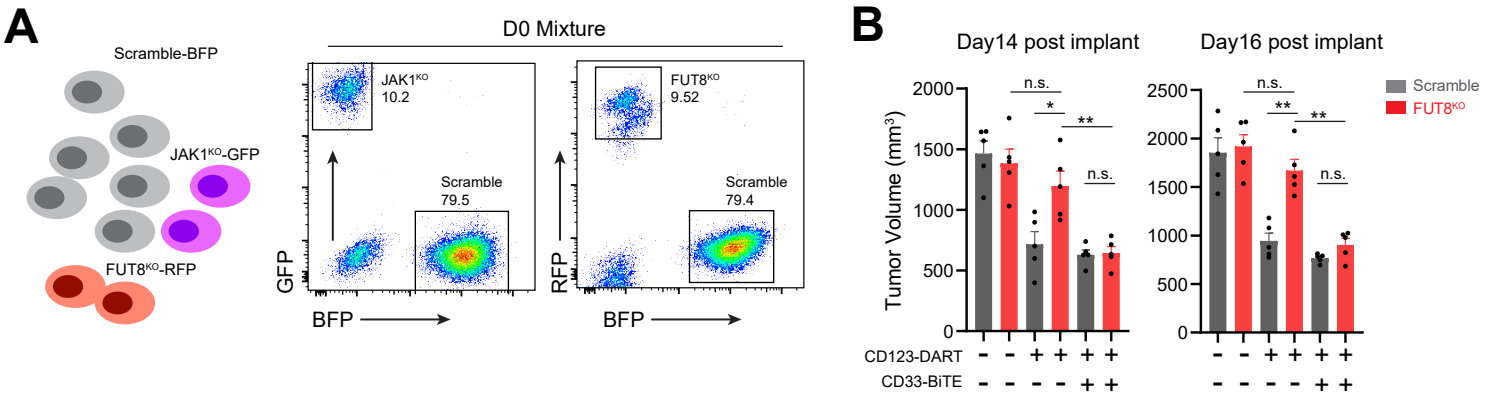

A
Capture
CD123-Scramble
CD123-FUT8KO
Flow through: IL-3
KD=2.689E-8 M
KD=2.53E-7 M
B
Scramble
FUT8KO
CD123KO
LCA
% of Max
CD123
C
Cell growth
Scramble
FUT8KO
CD123KO
FC over untreated
GM-CSF (ng/ml)
D
Scramble
FUT8KO
CD123KO
IL-3:
pSTAT5
STAT5
GAPDH
E
Whole cell lysate
Scramble FUT8-g1 FUT8-g3
FUT8
GAPDH
FUT8-g1
FUT8-g3
Scramble
% of Max
LCA
F
Day 5
Scramble
IL3RA-g3
FC over Untreated
IL-3
SCF/Flt3L
TPO/IL-6

Figure S6


A
Scramble-BFP
JAK1KO-GFP
FUT8KO-RFP
D0 Mixture
JAK1KO
10.2
Scramble
79.5
GFP
BFP
FUT8KO
9.52
Scramble
79.4
RFP
BFP
B
Day14 post implant
Day16 post implant
Tumor Volume (mm3)
2000
1500
1000
500
0
2500
n.s.
*
**
n.s.
CD123-DART
CD33-BiTE
Scramble
FUT8KO