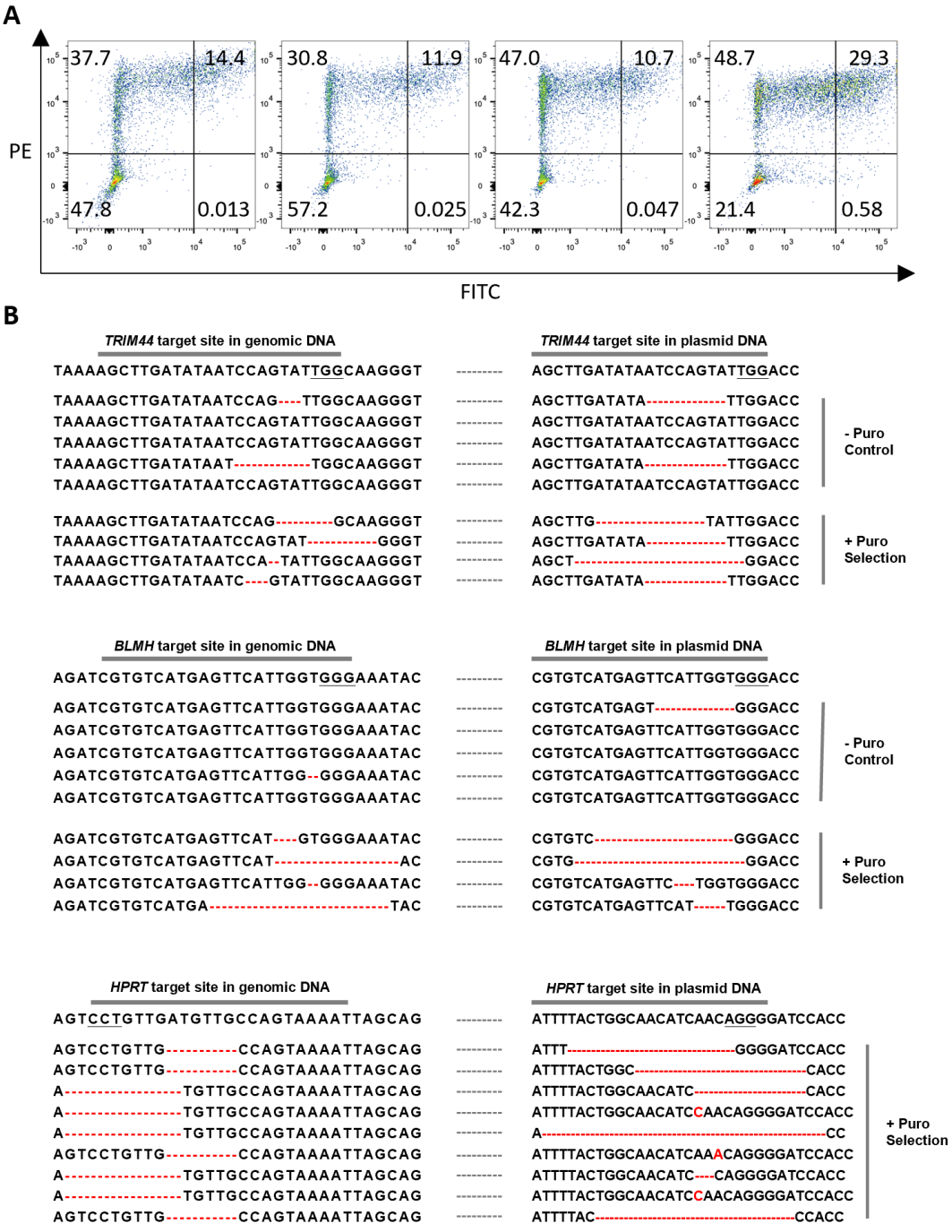


Fig. S1. The surrogate reporter enables selection of genome targeted cells. (A) Flow cytometry analysis of PK15 colonies with *CMAH* edits in genomic DNA (PE positive) and plasmid DNA (FITC positive). (B) DNA sequence analysis of *TRIM44*, *BLMH,* and *HPRT* target sites in both genomic DNA and plasmid DNA from single-cell clones.

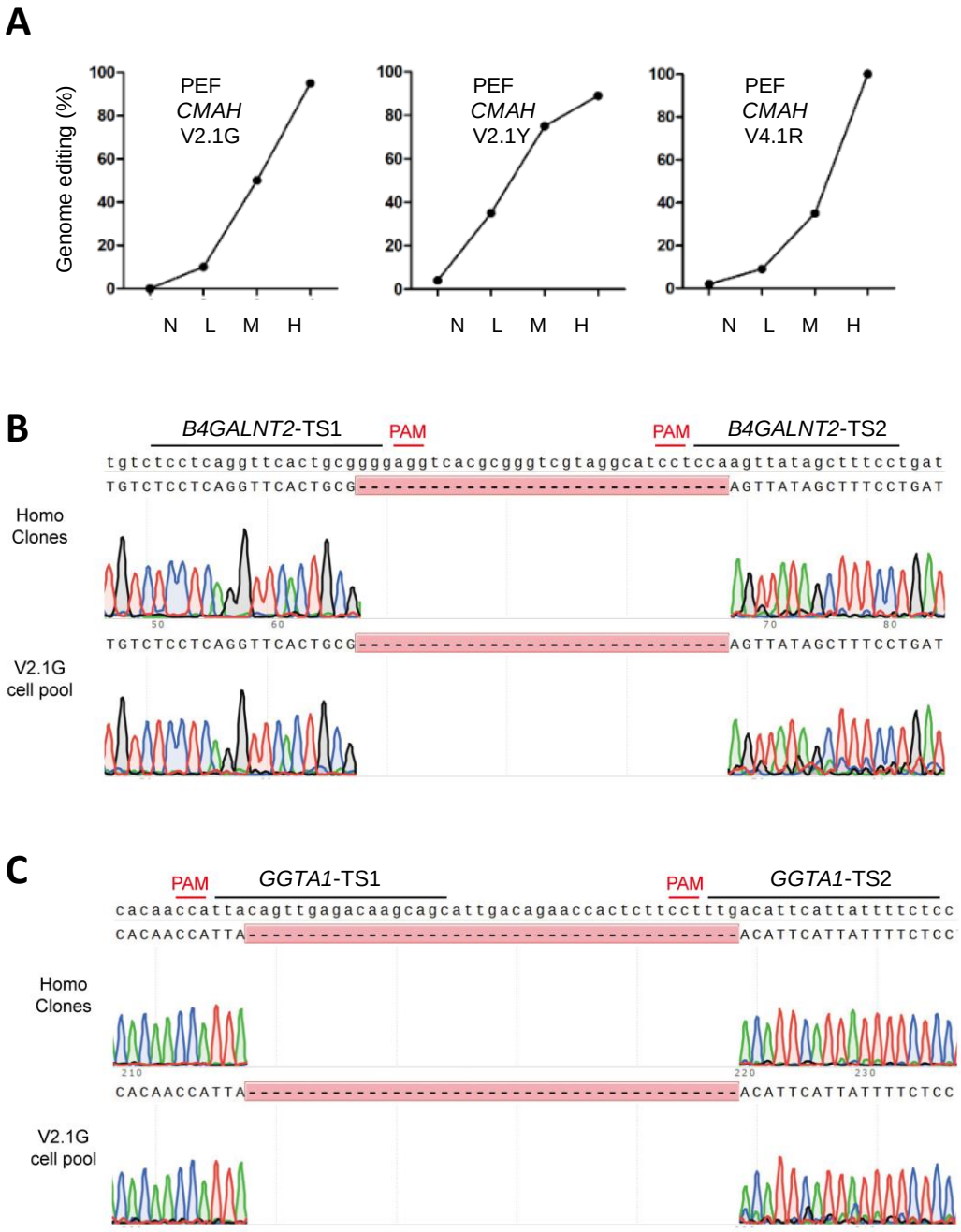


Fig. S2. The targeting efficiency of cell pools selected by surrogate reporter. (A) Targeting efficiency of the H (highest FITC intensity), M (medium FITC intensity), L (lowest FITC intensity), and N (FITC negative) groups for *CMAH* in PEFs using V2.1G, V2.1Y, and V4.1R, with *eGFP*, *eYFP* and *tdTomato* as the reporter genes, respectively. In the V4.1R plasmid, tdTomato is expressed followed by *BFP*, with the target site inserted between them. (B,C) Representative Sanger sequences of *B4GALNT2* and *GGTA1* target sites in homozygous cell clones and cell pools selected by V2.1G.

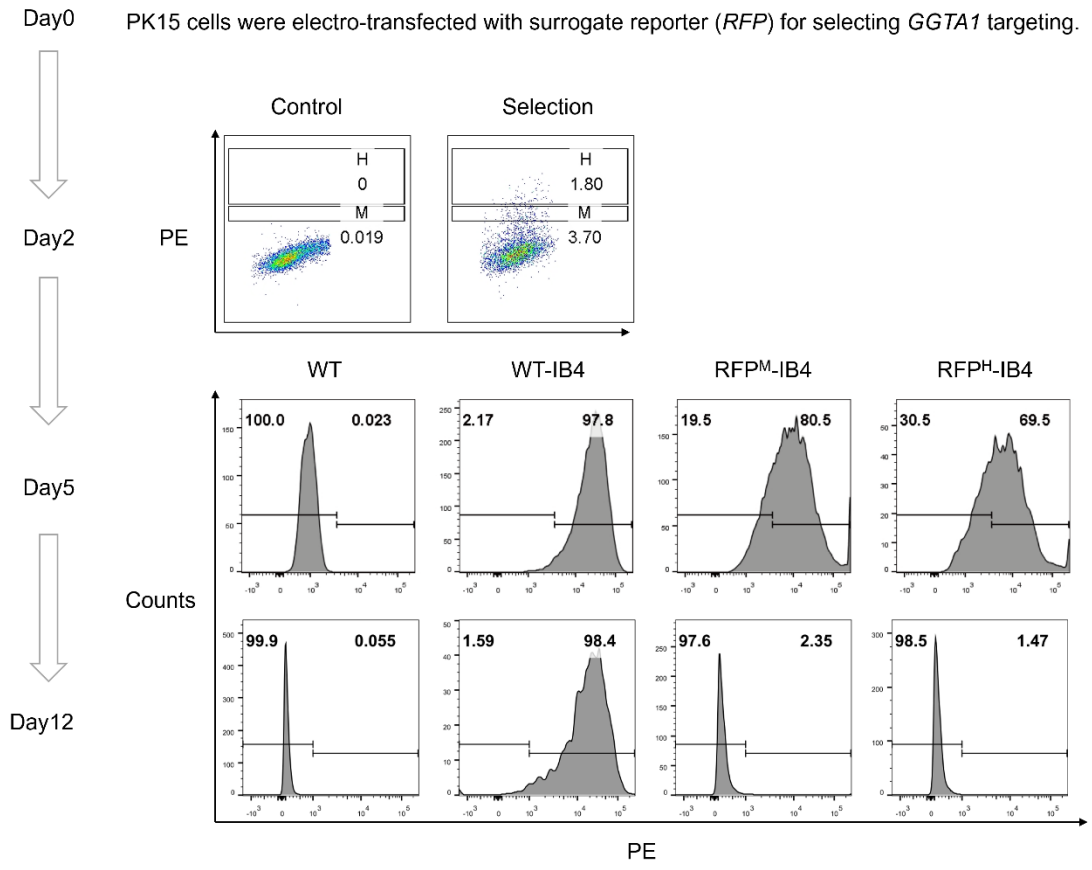


Fig. S3. Timeline for the elimination of α-gal epitopes. A timeline showing the analysis of *GGTA1* knockout. On day 0, PK15 cells were electro-transfected with three plasmids: pM3-Cas9 for expressing Cas9, pCRISPR-sg6-GGTA1 for expressing GGTA1 sgRNA, pV4.1R-GGTA1 for reporting the targeting of *GGTA1*. On day 2, RFP^{H} and RFP^{M} cells were sorted and cultured. On day 5 and day 12, cells were stained with IB4 lectin to detect the α-Gal epitopes encoded by *GGTA1*.

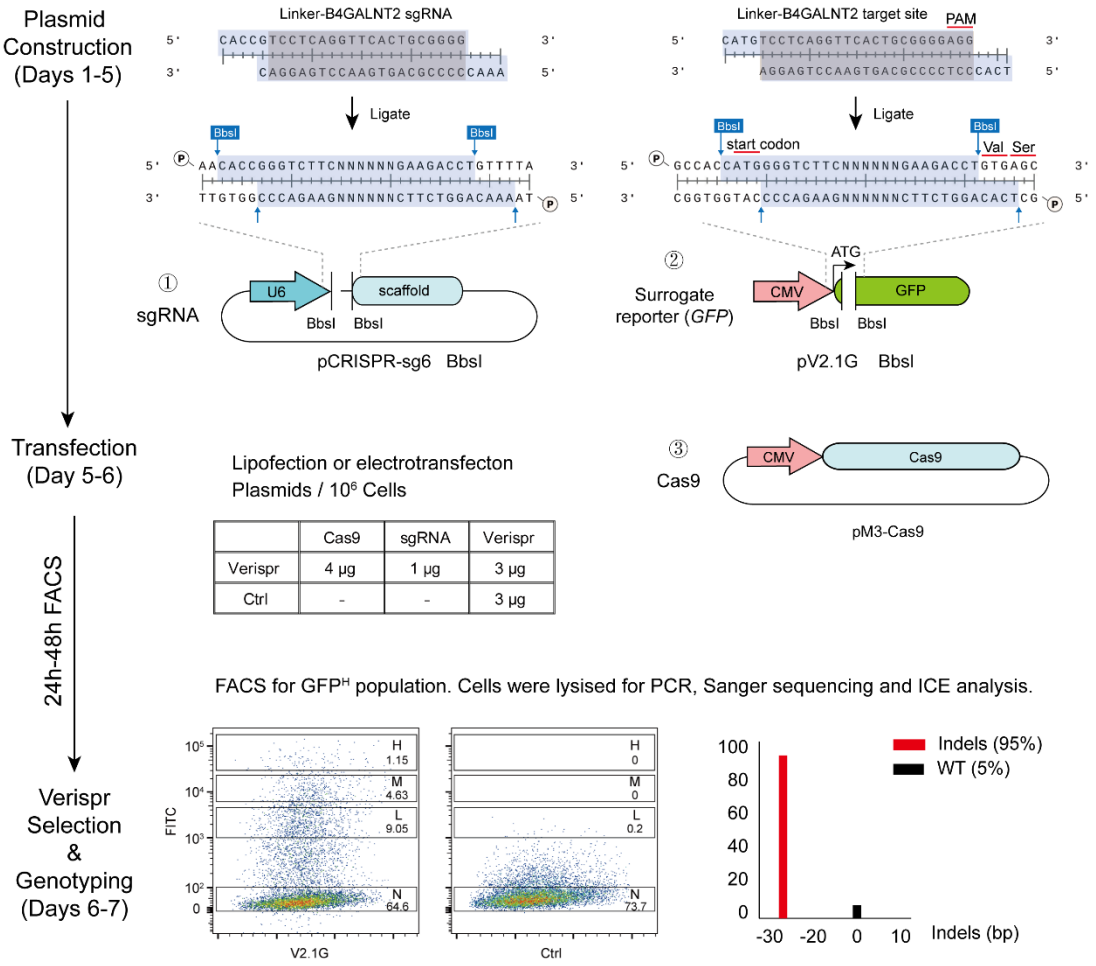


Fig. S4. Workflow of enrichment for knockout of single genes. This experiment involves three types of plasmids: a Cas9 expression plasmid (pM3-Cas9), sgRNA expression plasmid (pCRISPR-sg6-TS), and a surrogate reporter plasmid (pV2.1G-TS). Specific sgRNAs were designed using CHOPCHOP, and synthesized oligos were annealed and ligated into the pCRISPR-sg6 backbone cut by BbsI. To construct the surrogate reporter plasmid, the specific target site sequence, containing the PAM sequence, was synthesized, annealed, and ligated into the pV2.1G backbone cut by BbsI. For example, these plasmids were built for the target gene *B4GALNT2*. For genes with poor targeting efficiency, paired sgRNAs are recommended. The three kinds of plasmids are co-transfected into cells, and GFP-positive (GFP^H) populations were sorted by FACS after 24 - 48 hours. The mixed cell pools can be directly used for the next-step.

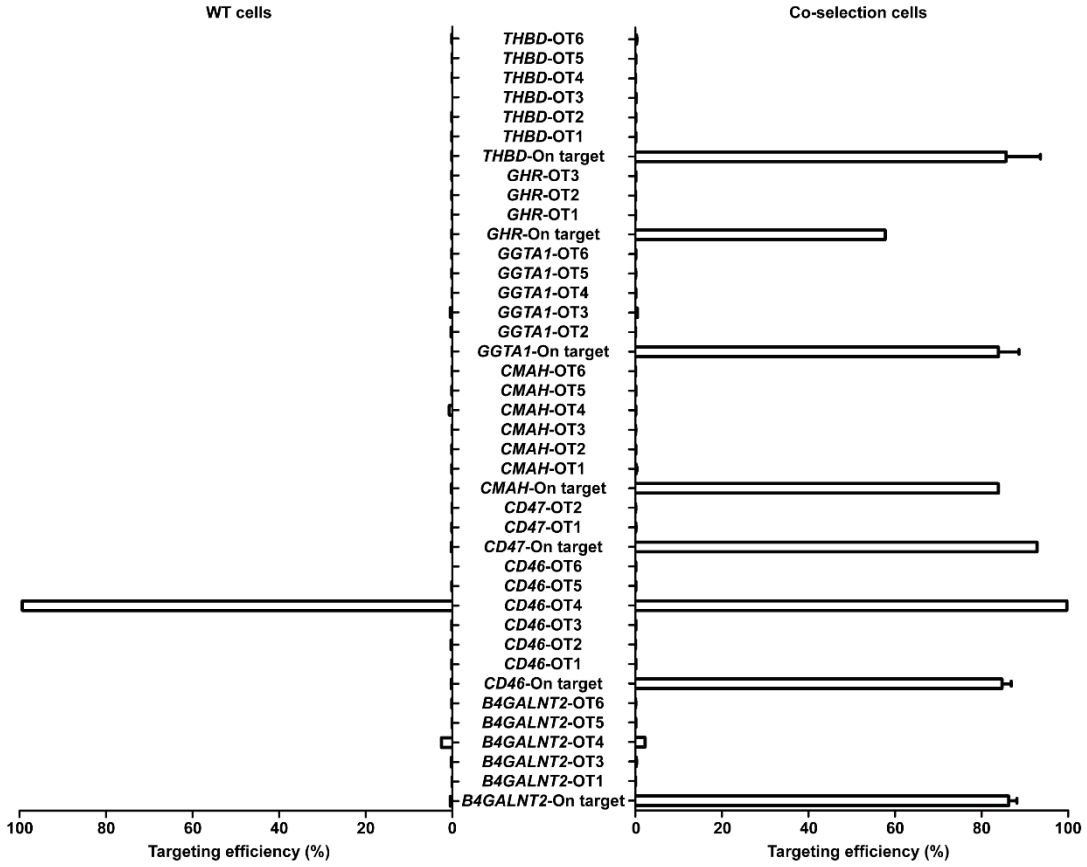
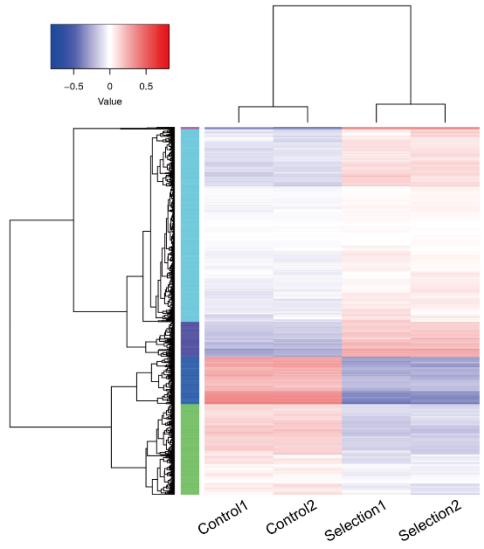
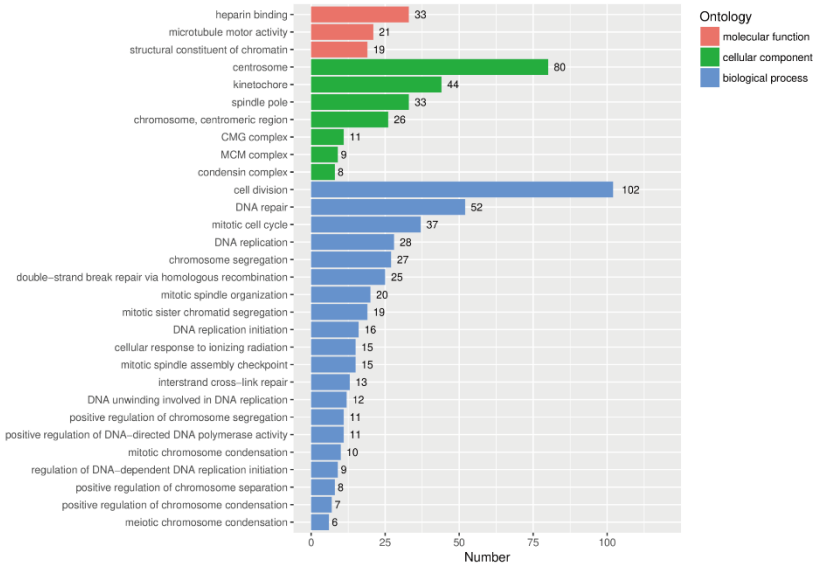


Fig. S5. Off-target analysis in cells with seven genes simultaneously targeted. The co-selection cells represent the highest FITC intensity population of porcine fetal fibroblasts (PFFs) edited for seven genes: *GGTA1*, *CMAH*, *B4GALNT2*, *THBD*, *CD46*, *CD47* and *GHR* (Fig. 4). Potential off-target sites for each gene were predicted using Cas-OFFinder. The analysis was conducted in triplicate (n=3).

A


−0.5
0
0.5
Value
Control1
Control2
Selection1
Selection2

B


heparin binding 33
microtubule motor activity 21
structural constituent of chromatin 19
centrosome 80
kinetochore 44
spindle pole 33
chromosome, centromeric region 26
CMG complex 11
MCM complex 9
condensin complex 8
cell division 102
DNA repair 52
mitotic cell cycle 37
DNA replication 28
chromosome segregation 27
double−strand break repair via homologous recombination 25
mitotic spindle organization 20
mitotic sister chromatid segregation 19
DNA replication initiation 16
cellular response to ionizing radiation 15
mitotic spindle assembly checkpoint 15
interstrand cross−link repair 13
DNA unwinding involved in DNA replication 12
positive regulation of chromosome segregation 11
positive regulation of DNA−directed DNA polymerase activity 11
mitotic chromosome condensation 10
regulation of DNA−dependent DNA replication initiation 9
positive regulation of chromosome separation 8
positive regulation of chromosome condensation 7
meiotic chromosome condensation 6
0
25
50
75
100
Number
Ontology
molecular function
cellular component
biological process

C

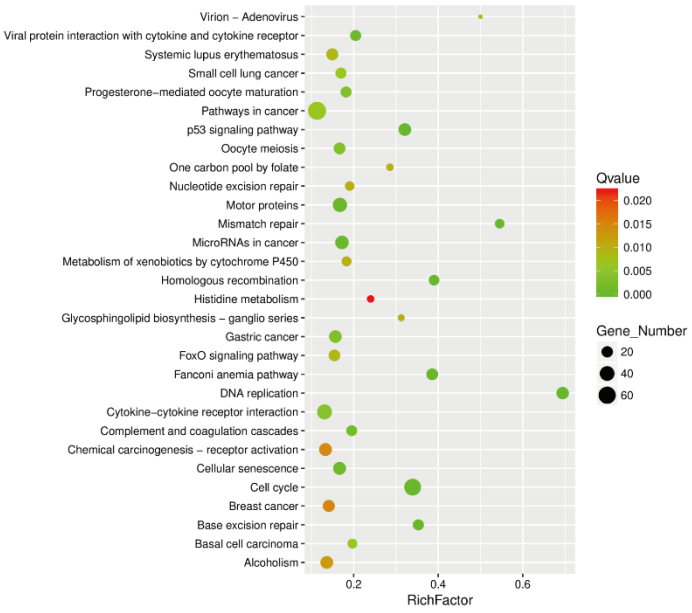
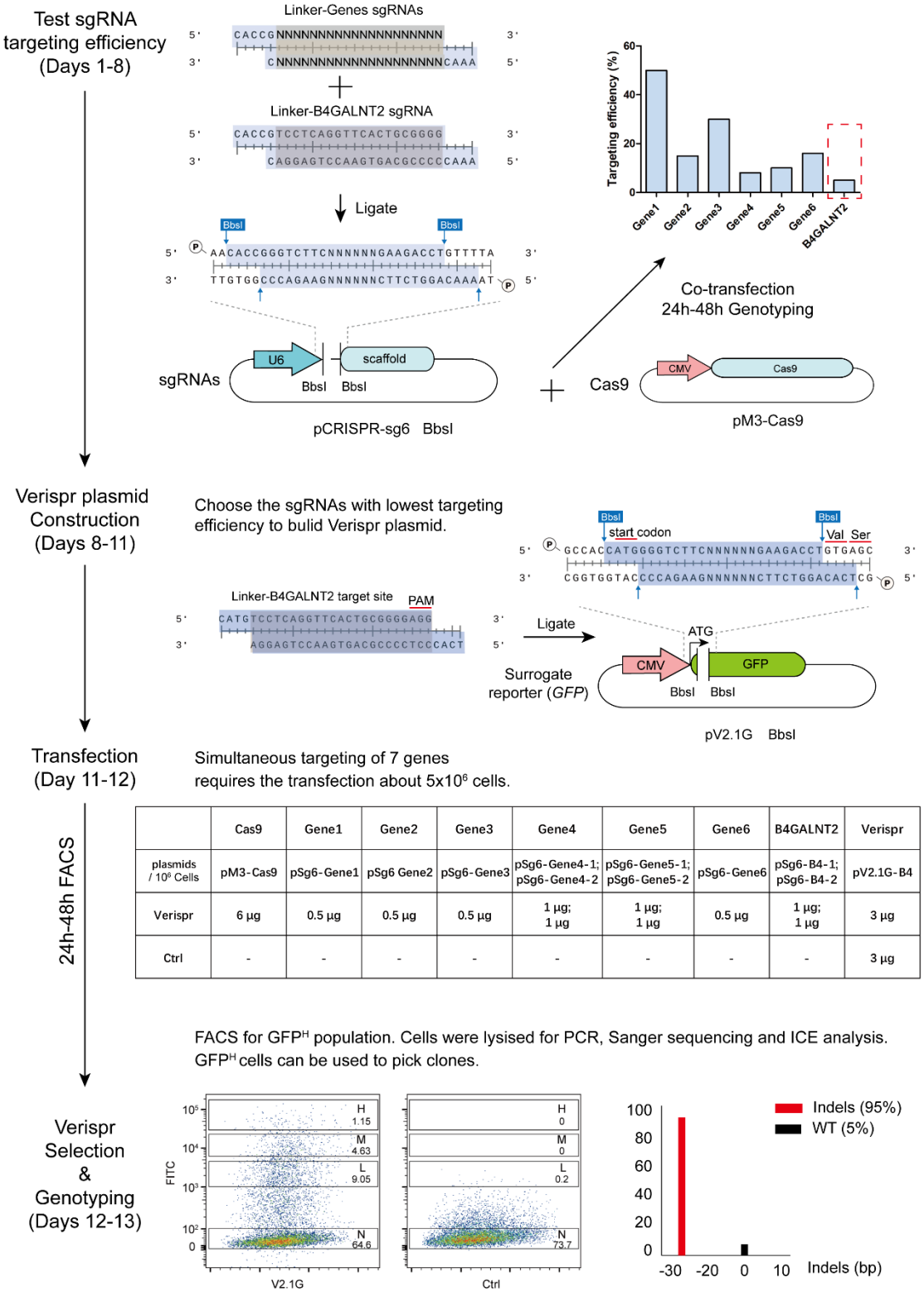

Virion − Adenovirus
Viral protein interaction with cytokine and cytokine receptor
Systemic lupus erythematosus
Small cell lung cancer
Progesterone−mediated oocyte maturation
Pathways in cancer
p53 signaling pathway
Oocyte meiosis
One carbon pool by folate
Nucleotide excision repair
Motor proteins
Mismatch repair
MicroRNAs in cancer
Metabolism of xenobiotics by cytochrome P450
Homologous recombination
Histidine metabolism
Glycosphingolipid biosynthesis − ganglio series
Gastric cancer
FoxO signaling pathway
Fanconi anemia pathway
DNA replication
Cytokine−cytokine receptor interaction
Complement and coagulation cascades
Chemical carcinogenesis − receptor activation
Cellular senescence
Cell cycle
Breast cancer
Base excision repair
Basal cell carcinoma
Alcoholism
0.2
0.4
0.6
RichFactor
Qvalue
0.020
0.015
0.010
0.005
0.000
Gene_Number
20
40
60

Fig. S6. RNA-seq analysis of cells simultaneously targeting seven genes. (A) Clustered heatmap of differential gene expression in control (negative FITC) and selected (highest FITC) PFFs edited for *GGTA1*, *CMAH*, *B4GALNT2*, *THBD*, *CD46*, *CD47* and *GHR*. RNA sequencing was performed on micro-extraction samples (n=2). (B) GO term enrichment analysis for the selection group of cells simultaneously targeting seven genes. (C) KEGG pathway enrichment analysis for the selection group of cells simultaneously targeting seven genes.



	Cas9	Gene1	Gene2	Gene3	Gene4	Gene5	Gene6	B4GALNT2	Verispr
plasmids / 10^6 Cells	pM3-Cas9	pSg6-Gene1	pSg6 Gene2	pSg6-Gene3	pSg6-Gene4-1; pSg6-Gene4-2	pSg6-Gene5-1; pSg6-Gene5-2	pSg6-Gene6	pSg6-B4-1; pSg6-B4-2	pV2.1G-B4
Verispr	6 μg	0.5 μg	0.5 μg	0.5 μg	1 μg; 1 μg	1 μg; 1 μg	0.5 μg	1 μg; 1 μg	3 μg
Ctrl	-	-	-	-	-	-	-	-	3 μg

Fig. S7. Workflow of enrichment for knockout of multiple genes. To identify optimal target sites for surrogate reporter plasmid construction, the targeting efficiency of various sgRNAs was tested and ranked. The target site, including the PAM sequence for the least effective sgRNA, was then integrated into the pV2.1G backbone digested with BbsI. As with the single-gene knockout workflow, three plasmids were used: a

Cas9 expression plasmid (pM3-Cas9), an sgRNA expression plasmid (pCRISPR-sg6-TS), and a Version 2.1-eGFP-target site plasmid (pV2.1G-TS). The plasmids were co-transfected into cells, and the GFP-positive (GFPH) population was sorted by FACS 24 – 48 hours later. The mixed cell pools were used for subsequent analysis.

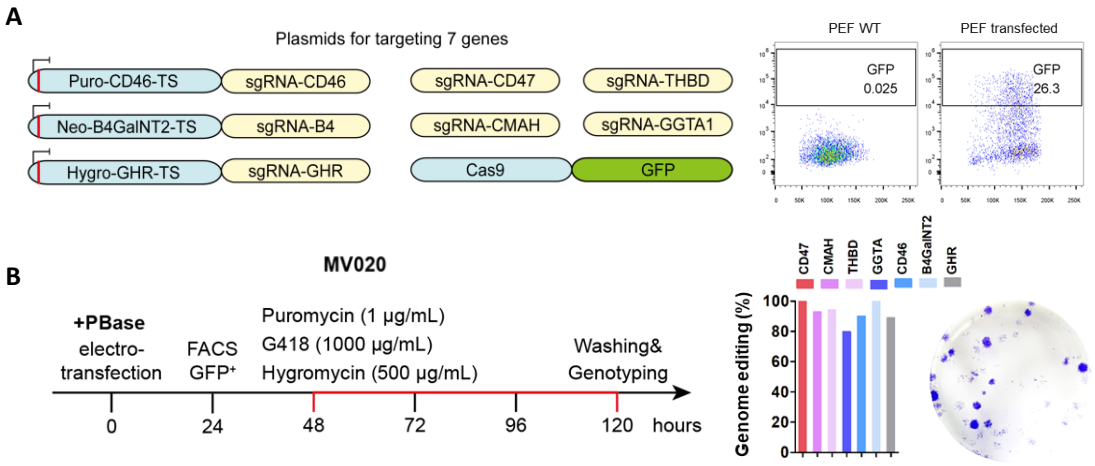


Fig. S8. Simultaneous targeting of seven genes using integrated drug-resistant surrogate plasmids. (A) Schematic representation of plasmids designed to target seven endogenous genes in porcine fetal fibroblasts (PFFs). The *Puro*, *Neo*, and *Hygro* surrogate plasmids are utilized for the selection of *CD46*, *B4GALNT2*, and *GHR* targeted cells, respectively. (B) Co-transfection of these plasmids with PBase into PEFs. GFP-positive populations were sorted by FACS within 24-48 hours post-transfection and subjected to puromycin, G418, and hygromycin selection for 4 days. Surviving cells were analyzed for genome editing efficiency across the seven genes and their ability to form cell colonies, which were then stained with crystal violet.

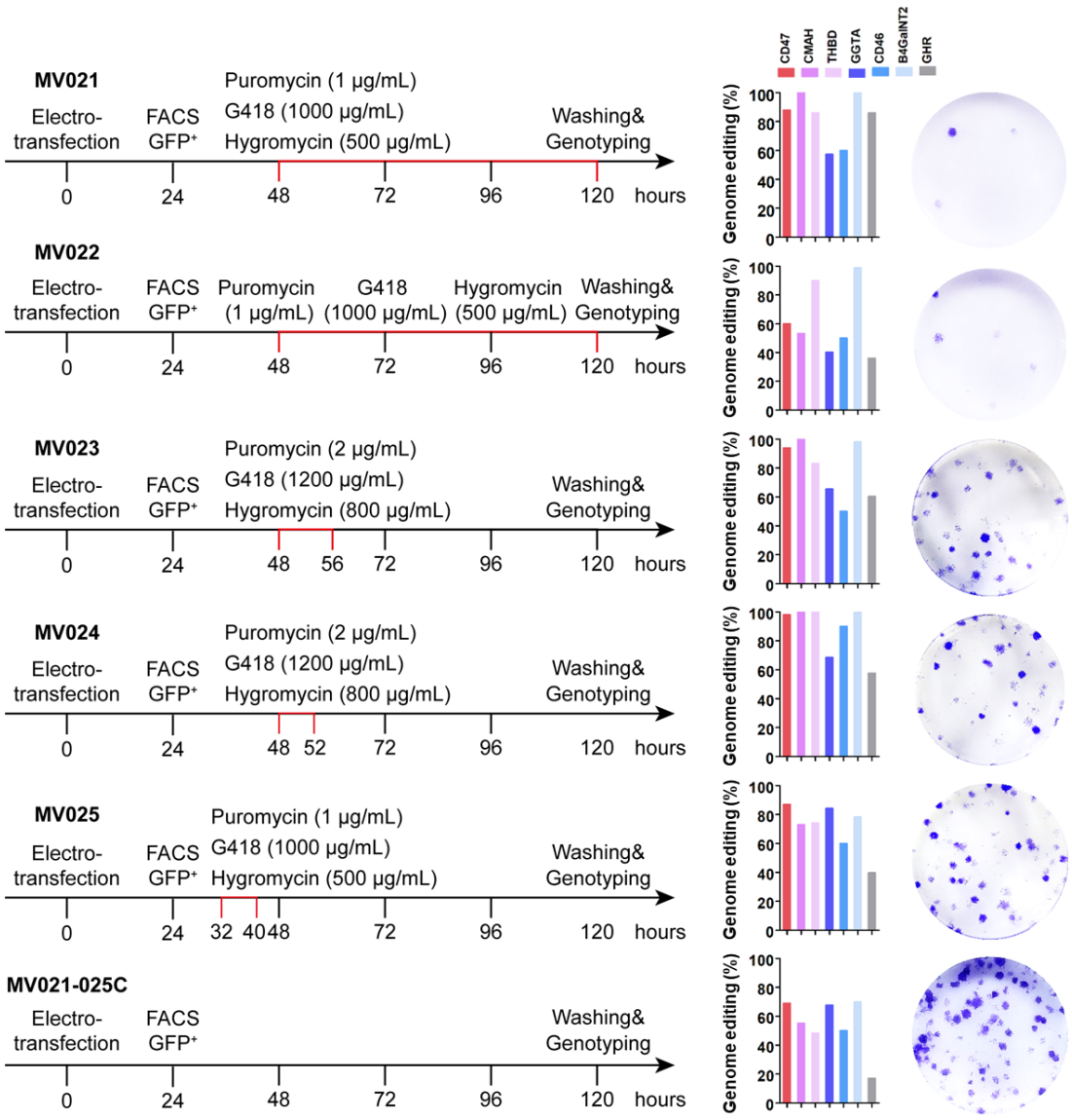


Fig. S9. Simultaneous targeting of seven genes using unintegrated drug-resistant surrogate plasmids under rigorous drug treatments. The plasmids depicted in Supplemental Fig. S7A were electro-transfected into PFFs without the PBase plasmid. GFP-positive cells were subjected to different drug treatments (MV021-MV025) or not (MV021-025C). The red line indicates the treatment duration. The remaining cells were analyzed for genome editing efficiency in seven genes and cell colony formation ability. The right panel shows numbered the cell colonies stained by crystal violet.