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Supplementary information

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STRAIGHT-IN Dual: a platform for dual single-copy integrations of DNA payloads and gene circuits into human induced pluripotent stem cells

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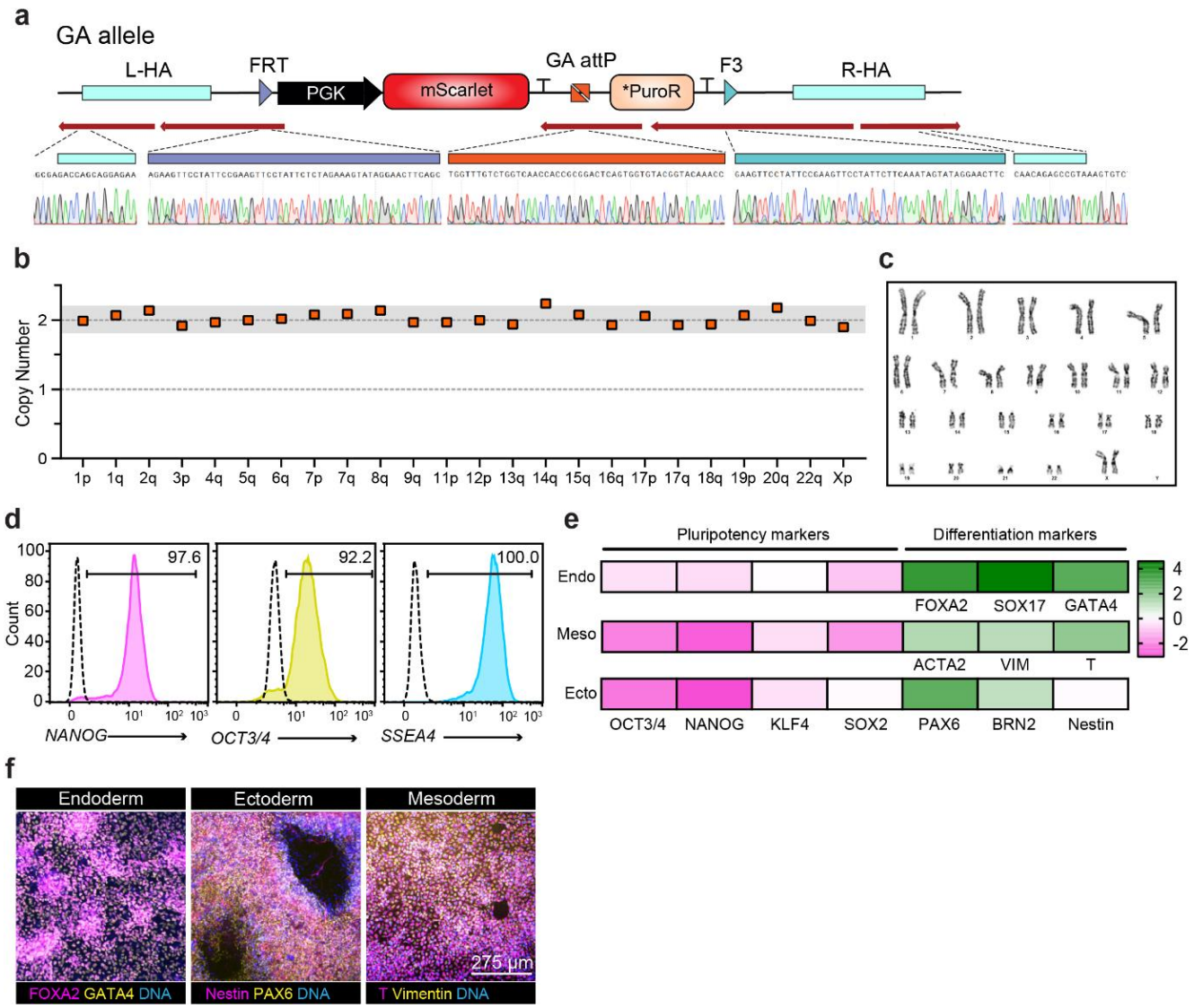


Supplementary Figure 1. Characterization of the STRAIGHT-IN Dual hiPSC line

(a) Sanger sequencing confirming correct targeting of the GA allele at the *CLYBL* locus, including sequences of the *FRT*, *F3* and GA *attP* sites. Red arrows indicate alignment of the sequencing chromatograms with the reference sequence. L-HA, left homology arm; R-HA, right homology arm.

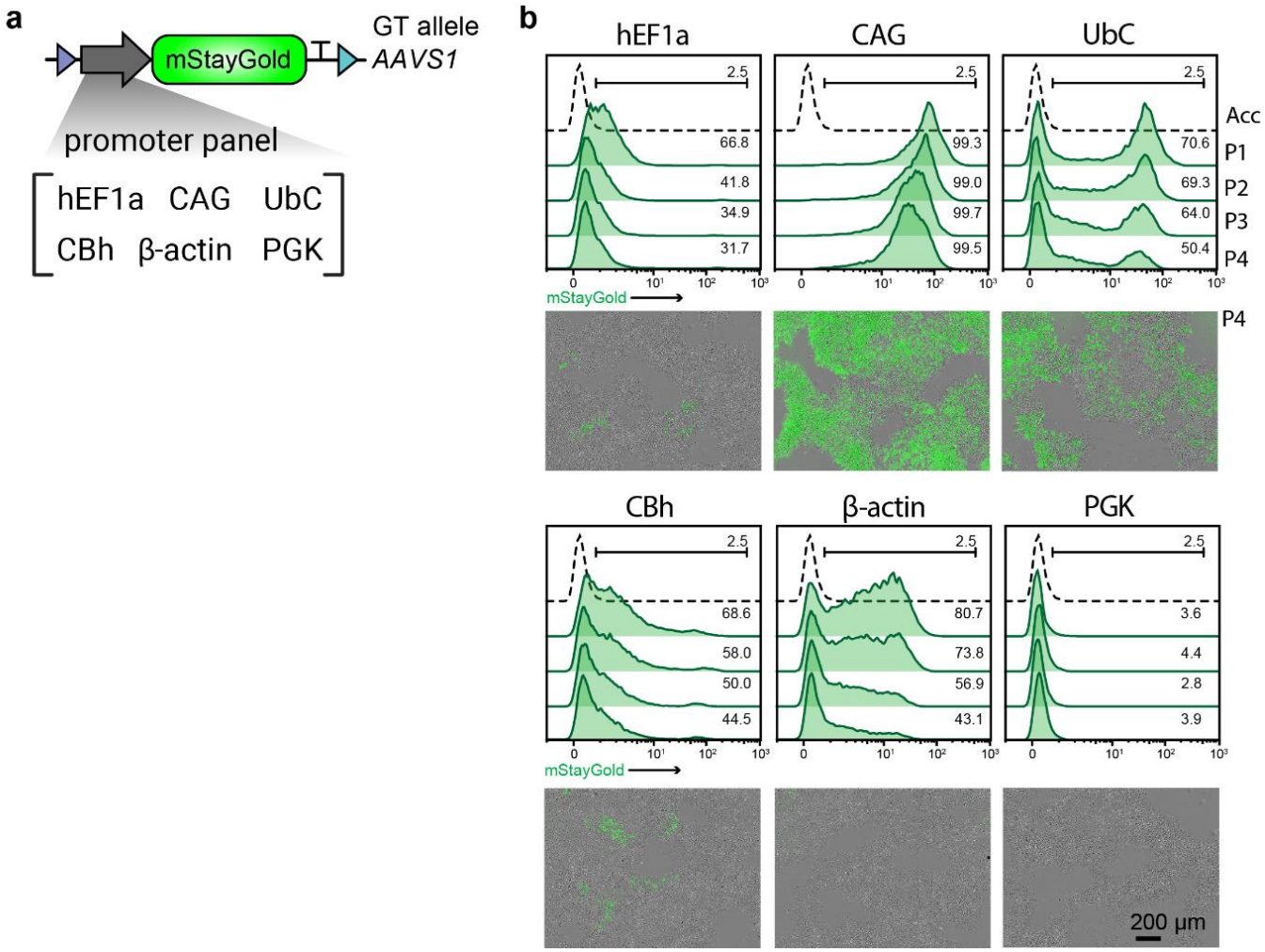
(b) ddPCR analysis of 24 genomic loci covering >90% of the most common genetic abnormalities in hiPSCs. The STRAIGHT-IN Dual line showed a normal copy number (2) at all loci screened.

(c) G-banding karyogram confirming the STRAIGHT-IN Dual hiPSC line has a normal karyotype.

(d) Flow cytometry analysis of pluripotency-associated markers NANOG, OCT3/4 and SSEA-4 in the STRAIGHT-IN Dual hiPSC line. Dashed lines indicate unstained hiPSCs.

(e) Gene expression analysis of pluripotency-associated and lineage-specific markers following differentiation into the three germ layers. Values are normalized to *RPL37A* and presented relative to undifferentiated hiPSCs (log10-transformed). N=3 independent differentiation.

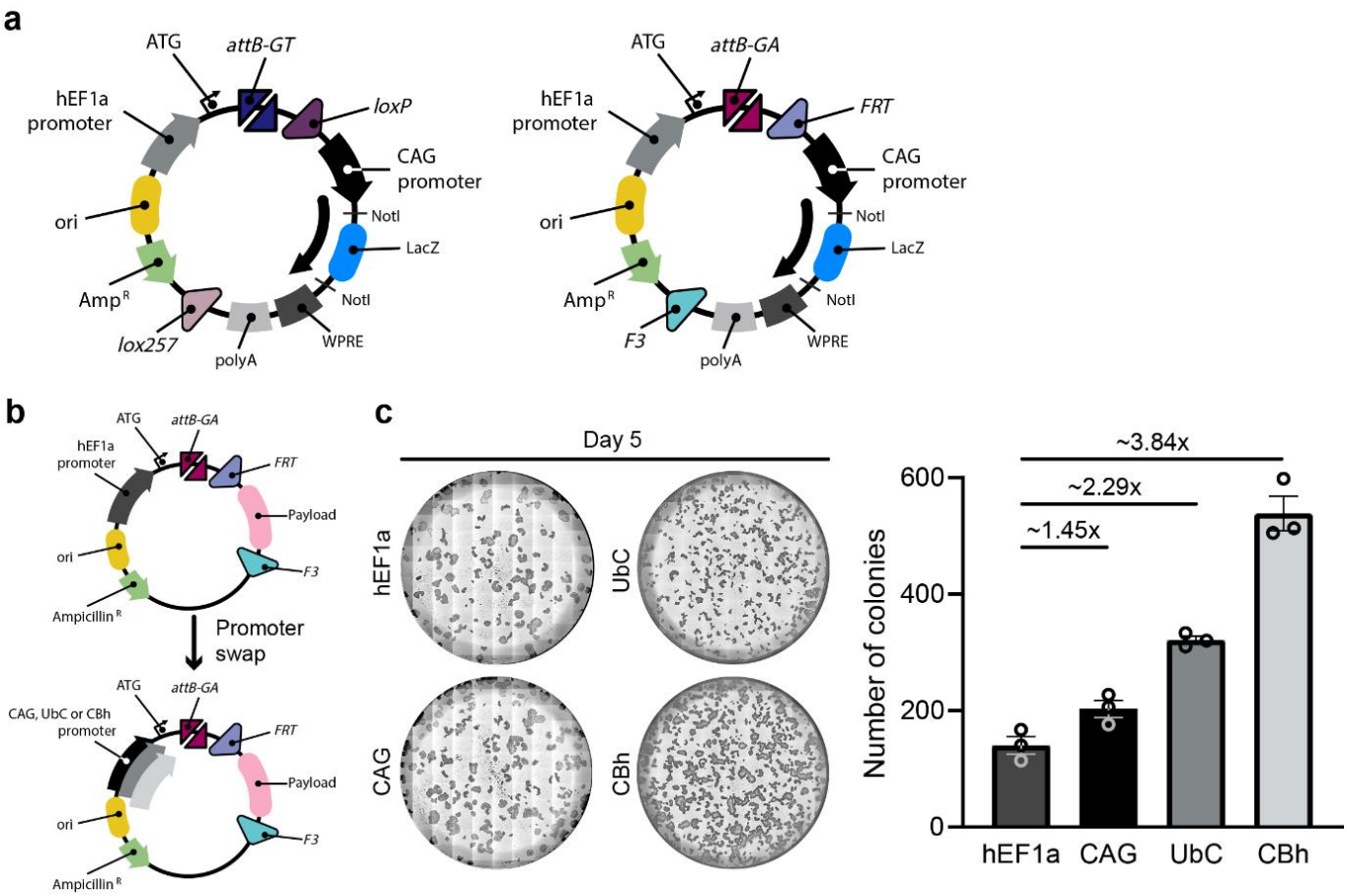
(f) Representative immunofluorescence images showing expression of germ layer markers: FOXA2 and GATA4 (endoderm), Nestin and PAX6 (ectoderm), and T and Vimentin (mesoderm) in differentiated STRAIGHT-IN Dual hiPSCs.



Supplementary Figure 2. Characterization of promoter activity at the *AAVS1* locus

(a) Schematic of the promoter panel, each driving expression of a *mStayGold* reporter and integrated at the *AAVS1* locus in STRAIGHT-IN *AAVS1* v2 acceptor hiPSCs.

(b) Flow cytometry analysis over 4 passages (*top*), and representative fluorescence/phase-contrast images at passage 4 (*bottom*), of hiPSCs expressing mStayGold from the different promoters indicated and integrated at the *AAVS1* locus, prior to excision of the auxiliary sequences. Dashed lines represent untransfected hiPSCs.



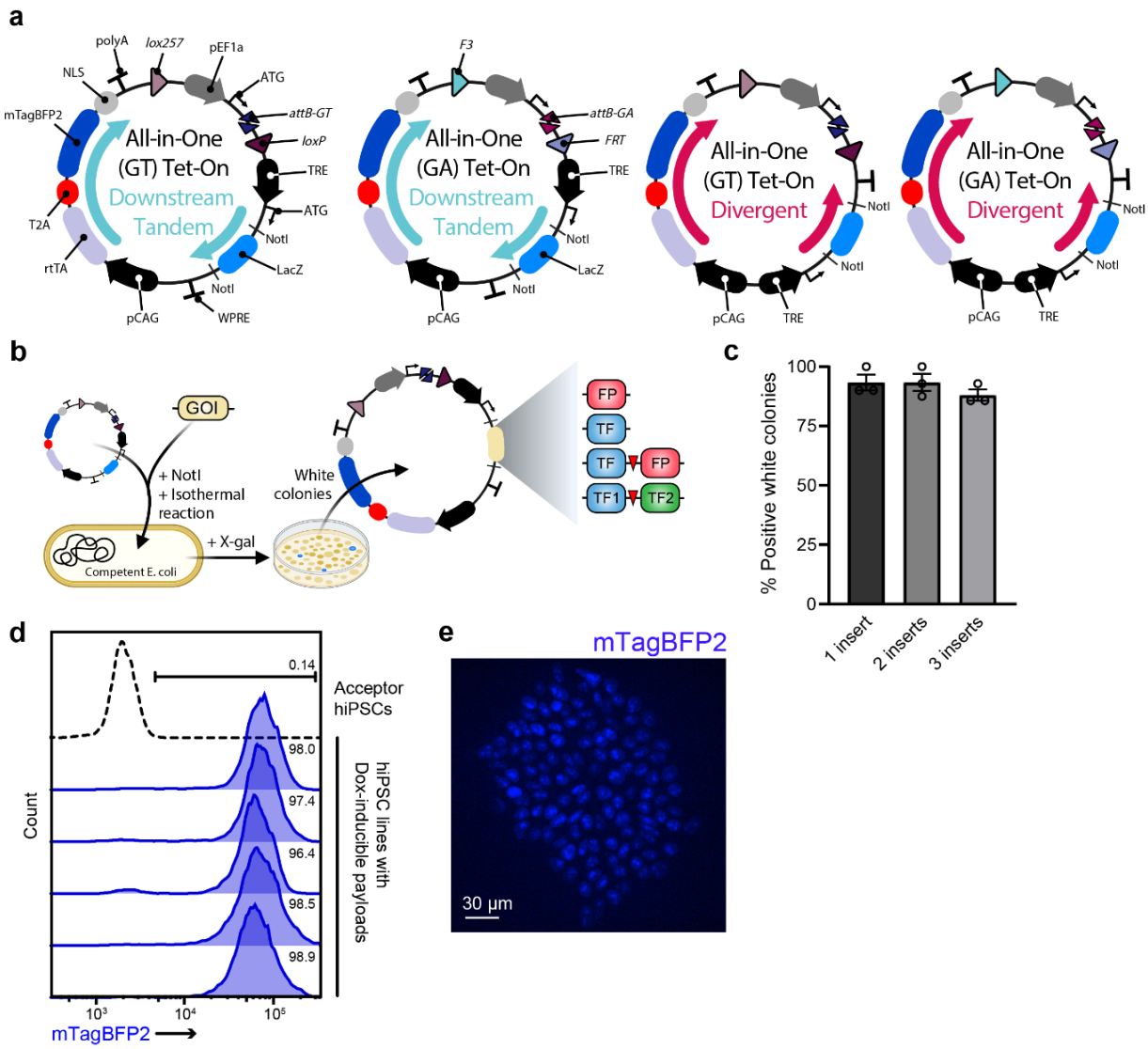
Supplementary Figure 3. Optimization of STRAIGHT-IN donor plasmid backbone for improved transgene expression and integration efficiency

(a) Schematic of the GT and GA donor plasmids containing a CAG promoter, WPRE and bGH polyadenylation signal for improved constitutive gene of interest (GOI) expression. The *LacZ* cassette is replaced with the GOI via NotI digestion followed by isothermal assembly or T4 ligation. White colonies on IPTG/X-gal agar plates identify clones containing the GOI.

(b) Schematic of GA donor plasmids in which the hEF1a promoter was substituted for CAG, UbC or CBh promoter sequences to assess the impact on integration efficiency.

(c) Representative phase contrast images (*left*) showing puromycin-resistant hiPSC colonies following donor plasmid integration, and quantification of the mean colony number per well (*right*). N=3 independent transfections; error bars, ±SEM.

Parts of the figure were created in BioRender. Blanch Asensio, A. (2026)



Supplementary Figure 4. Modular cloning platform for assembling downstream tandem and divergent Tet-ON 3G donor plasmids

(a) Schematic of GT and GA donor plasmids encoding all-in-one Tet-On 3G expression systems in either downstream tandem or divergent orientations.

(b) Overview of the cloning workflow, in which the *LacZ* cassette is replaced with one or more genes of interest (GOIs) via NotI digestion and isothermal assembly. IPTG/X-gal blue-white screening enables identification of clones containing the GOIs (white colonies).

(c) Mean percentages of white bacterial colonies that correctly assembled 1, 2, or 3 inserts into the downstream tandem donor plasmid, as confirmed by colony PCR. N=3 replicates; error bars, ±SEM.

(d) Flow cytometry analysis of mTagBFP2 expression in hiPSC lines with integrated downstream tandem Tet-On 3G GT donor plasmids containing various GOIs. Dashed line represents untransfected hiPSCs and values indicate the percentage of mTagBFP2^{+} cells.

(e) Immunofluorescence image showing nuclear-localized mTagBFP2 expression in hiPSCs containing an integrated downstream tandem Tet-On 3G GT donor plasmid.

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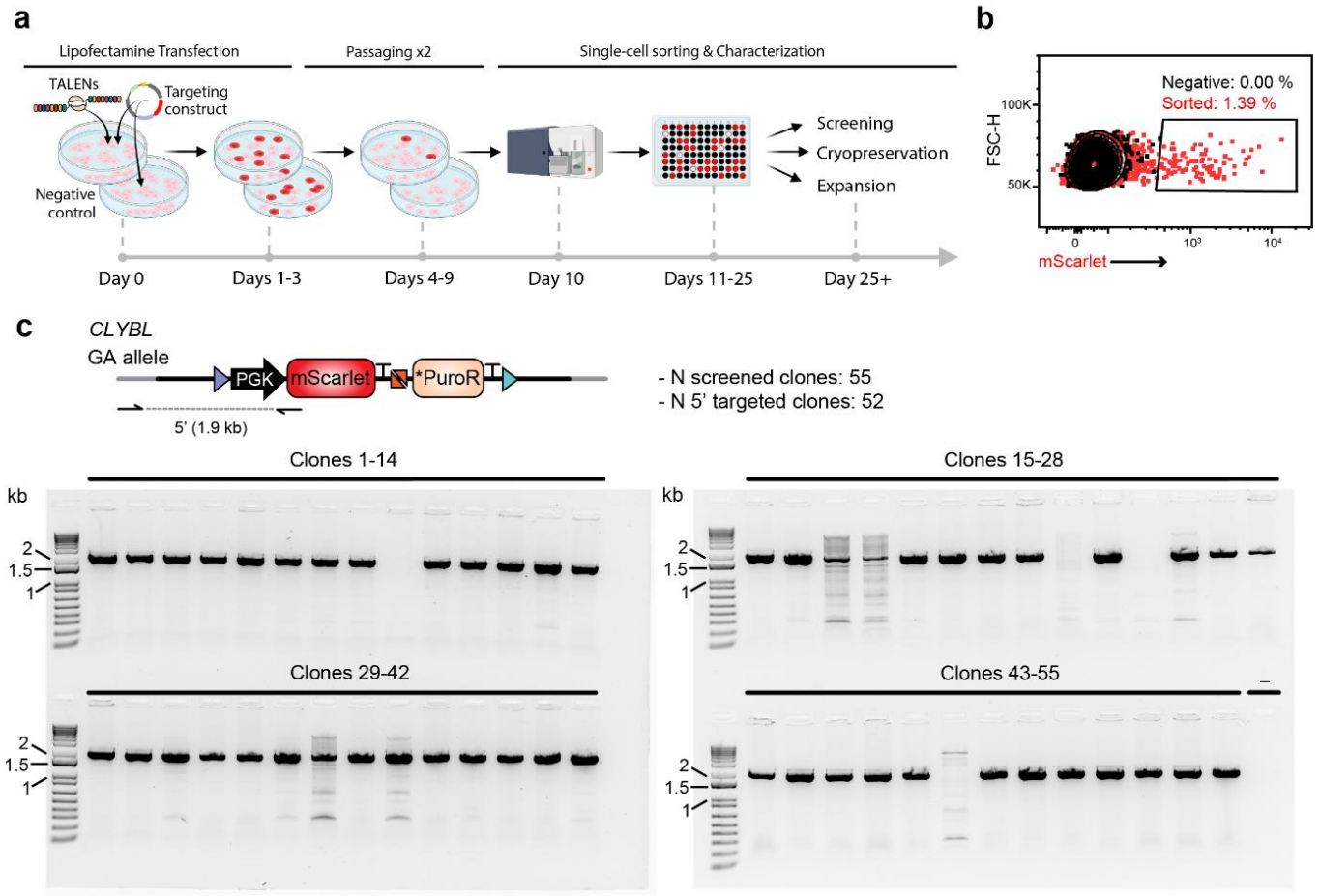

Supplementary Figure 5. Targeting of bxb1-GA *attP* LP into the *CLYBL* locus

(a) Schematic of gene targeting workflow, lipofectamine-mediated co-transfection of the *CLYBL*-targeting construct and TALEN expression plasmids, followed by single-cell sorting and downstream clonal characterization.

(b) Flow cytometry analysis of hiPSCs expressing the mScarlet reporter from the GA allele.

(c) PCR screening results from 55 hiPSC clones using the primer pair indicated in the schematic to detect correct 5' junction integration.

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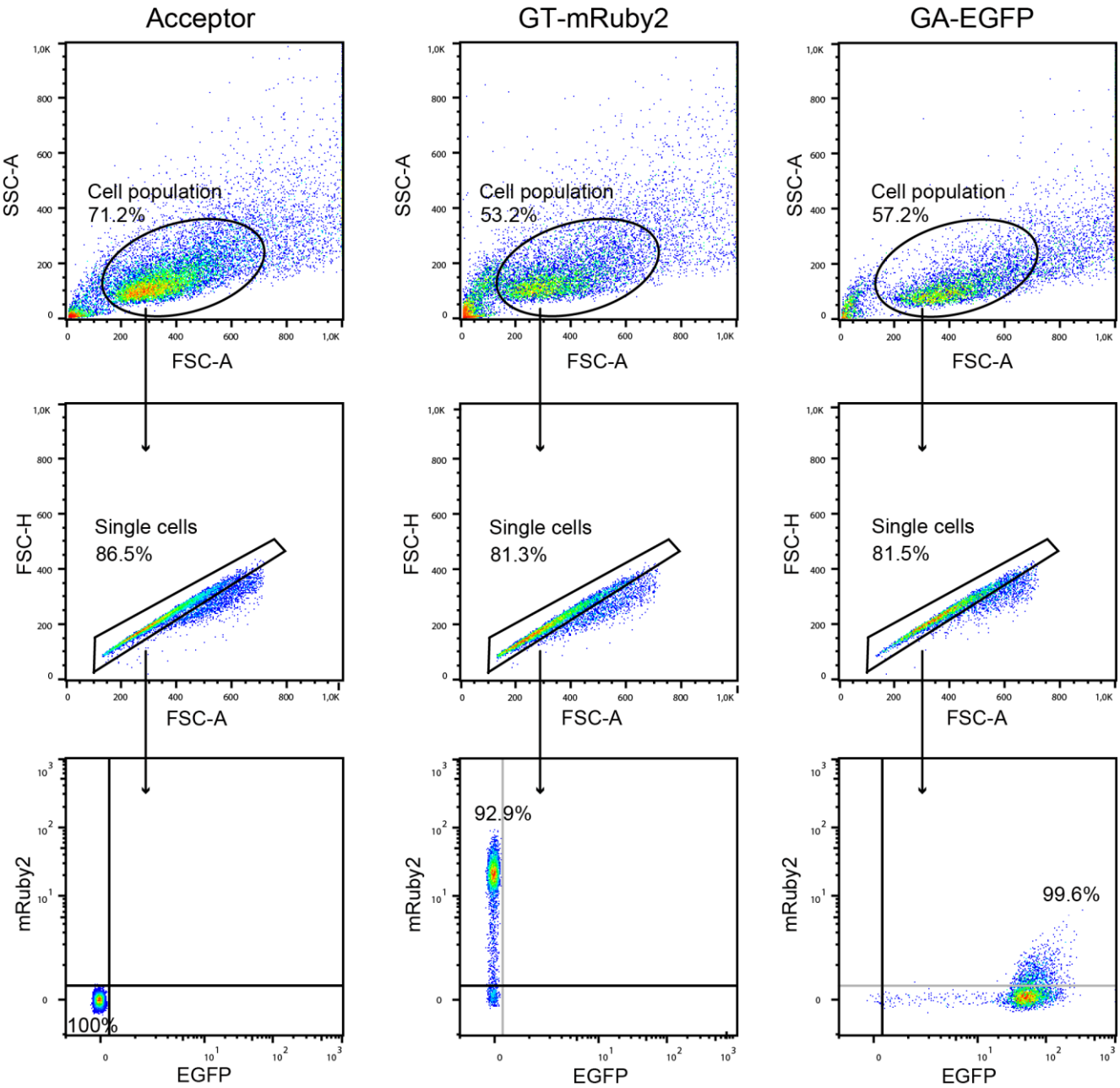

Supplementary Figure 6. Examples of gating strategy used for flow cytometry analysis of hiPSCs

Dot plots showing the gating workflow. Forward and side scatter was used to identify the cell population, with forward scatter area versus height used to distinguish single cells. Side scatter versus the appropriate fluorescence channel was used to gate mRuby2^{+} or EGFP^{+} hiPSCs.

Supplementary Table 1. Genotyping PCR Oligonucleotides

Purpose	Sequence Forward Primer (5' - 3')	Sequence Reverse Primer (5' - 3')
GT allele – 5' junction	AGATCATCCAGCCCTAGTCAAG	CGGTGGTGCAGATGAACTTC
GT allele – 3' junction	AGCAAAGACCCCAACGAGAA	TGGAGCAGTGGATGACAACTT
GA allele – 5' junction	AGATCATCCAGCCCTAGTCAAG	CCGTCCTCGAAGTTCATCAC
GA allele – 3' junction	GACATCACCTCCCACAACGA	TGGAGCAGTGGATGACAACTT

Supplementary Table 2. ddPCR Primer-Probe Sets

Target Gene	Assay type	Primer/Probe	Sequence (5'- 3')	Fluorophore-Quencher	Source
RPP30	Copy number	Forward Primer Reverse Primer Probe	GATTTGGACCTGCGAGCG GCGGCTGTCTCCACAAGT CTGACCTGAAGGCTCT	 HEX-ZEN-IBFQ	38
EBFP2	Copy number	Forward Primer Reverse Primer Probe	GCCGACAAGCAGAAGAACG GGGTGTTCTGCTGGTAGTGG AGATCCGCCACAACATCGAGG	 FAM-ZEN-IBFQ	38
mScarlet	Copy number	Forward Primer Reverse Primer Probe	TGGCAACCTGACTTGTATCG GTCCATCACTGTCCTTCACTATC CGACAGGTGCTTCTCGATCTGCAT	 FAM-ZEN-IBFQ	This study
BleoR	Copy number	Forward Primer Reverse Primer Probe	AGTTGACCAGTGCCGTTCC CGAAGTCGTCCTCCACGAAG AGCCGGTCGGTCCAGAAC	 FAM-ZEN-IBFQ	38
PuroR	Copy number	Forward Primer Reverse Primer Probe	CGCCTTCCTGGAGACCTC TTGCGGGTCATGCACCAG CTCGGCTTCACCGTCACCG	 FAM-ZEN-IBFQ	This study
TurboGFP	Copy number	Forward Primer Reverse Primer Probe	TGATGGGCTACGGCTTCTAC CACCCGC+AT+CGAGAAGTACG ATCACCTTGAAGTCGCCGATC	 FAM-ZEN-IBFQ	This study
AmpR	Copy number	Forward Primer Reverse Primer Probe	TTTCCGTGTCGCCCTTATTCC ATGTAACCCACTCGTGCACCC TGCCTTCCTGTTTTTGCTCACCCA	 FAM-ZEN-IBFQ	38
pUC Ori	Copy number	Forward Primer Reverse Primer Probe	CGATAAGTCGTGTCTTACCG GCTTTCTCATAGCTCACGC TGCACACAGCCCAGCTTG	 FAM-ZEN-IBFQ	38
mTagBFP2	Copy number	Forward Primer Reverse Primer Probe	GGAGAACATGCACATGAAGCTGT CCTGGGTAGCGGTCAGCA AGGGCACCGTGGACAACC	 FAM-ZEN-IBFQ	This study
attP/attR GT allele	Integration	Forward Primer Reverse Primer Reverse Primer Probe Probe	GCATTCTAGTTGTGGTTTGTCC GCACTGGTCAACTTGGCATAT CCTGCTTGCCGAATATCATG CGTGGTTTGTCTGGTCAACCA TCTCCGTCGTCAGGATCATCC	 HEX-ZEN-IBFQ HEX-ZEN-IBFQ	23
attP/attR GA allele	Integration	Forward Primer Reverse Primer Reverse Primer Probe Probe	CGTGGTTTGTCTGGTCAACCA CGTGGGCTTGTACTCGGTAA CTTAATTAACTAGTCGATGCCTGC CGTGGTTTGTCTGGTCAACCA ACTCCGTCGTCAGGATCATCC	 HEX-ZEN-IBFQ HEX-ZEN-IBFQ	This study

+ symbol indicates the following nucleotide is a locked nucleic acid

Supplementary Table 3. Oligonucleotides used for NGS library

Gene	Sequence (5' - 3')	Annealing temperature
hEF1a_Fwd	GTTCAGAGTTCTACAGTCCGACGATCNNNNNNAGGAAAAGGGCCTTTCCGTCC	67.2°C
βactin_Fwd	GTTCAGAGTTCTACAGTCCGACGATCNNNNNNCCTCCGACCAGTGTTTGCCT	71.1°C
CpGfree_Fwd	GTTCAGAGTTCTACAGTCCGACGATCNNNNNNAGTACTCCCTCTCAAAAGCTGGC	71.1°C
PGK_Fwd	GTTCAGAGTTCTACAGTCCGACGATCNNNNNNCTCCGCCCCTAAGTCGGGAA	67.2°C
RSV_Fwd	GTTCAGAGTTCTACAGTCCGACGATCNNNNNNGGTAACGATGAGTTAGCAACATGCC	67.2°C
UbC_Fwd	GTTCAGAGTTCTACAGTCCGACGATCNNNNNNGTGAGGCGTCAGTTTCTTTGGTCG	67.2°C
SV40_Fwd	GTTCAGAGTTCTACAGTCCGACGATCNNNNNNGTTAATTAAGTACTTACTGCAGGCAGAA	67.2°C
CMV_Fwd	GTTCAGAGTTCTACAGTCCGACGATCNNNNNNGCACCAAAATCAACGGGACTTTCC	67.2°C
CAG_Fwd	GTTCAGAGTTCTACAGTCCGACGATCNNNNNNGTTCGGCTTCTGGCGTGTGA	67.2°C
CBh_Fwd	GTTCAGAGTTCTACAGTCCGACGATCNNNNNNAAGAGGTAAGGGTTTAAGGGATGGT	67.2°C
Generic_Rev	TTCCTTGGCACCCGAGAATTCCACGTGGAACCAGTTCTTCAGGC	67.2 or 71.1°C

Supplementary Table 4. qPCR Oligonucleotide Pairs

Gene	Sequence Forward Primer (5' - 3')	Sequence Reverse Primer (5' - 3')
RPL37A	GTGGTTCCTGCATGAAGACAGTG	TTCTGATGGCGGACTTTACCG
OCT4	GTGGAGGAAGCTGACAACAA	ATTCTCCAGGTTGCCTCTCA
NANOG	AGCAGATGCAAGAACTCTCCAA	TGAGGCCTTCTGCGTCACAC
KLF4	ATAGCCTAAATGATGGTGCTTGG	AACTTTGGCTTCCTTGTTTGG
SOX2	GCTACAGCATGATGCAGGACCA	TCTGCGAGCTGGTCATGGAGTT
TUBB3	CAACCAGATCGGGGCCAAGTT	CCGAGTCGCCCACGTAGTT
MAP2	AGACTGCAGCTCTGCCTTTAG	AGGCTGTAAGTAAATCTTCCTCC
SYN1	CCCTGGGTGTTTGCCCAGAT	ACCACGGGGTACGTTGTACT
BRN2	ACCCGCTTTATCGAAGGCAA	CCTCCATAACCTCCCCCAGA
GRIA4	GGCCAGGGAATTGACATGGA	AACCAACCTTTCTAGGTCCTGTG
VGLUT2	GTAGACTGGCAACCACCTCC	CCATTCCAAAGCTTCCGTAGAC
SYP	ACCTCGGGACTCAACACCTCGG	GAACCACAGGTTGCCGACCCAG
Ngn2	TATGCACCTCACCTCCCCATAG	GAAGGGAGGAGGGCTCGACT
ISL1	AAGGTGGAGCTGCATTGGTTTG	TAAACCAGCTACAGGACAGGCC
ChAT	CTCAGCTACAAGGCCCTGCT	ACCAGCGTGTCCTGGGTATG
FOXA2	GGAGCGGTGAAGATGGAA	TACGTGTTCATGCCGTTCAT
PDX1	GATGAAGTCTACCAAAGCTCACG	GTTCAACATGACAGCCAGCTC
SOX17	CGCACGGAATTTGAACAGTA	GGATCAGGGACCTGTCACAC
ACTA2	GTGATCACCATCGGAAATGAA	TCATGATGCTGTTGTAGGTGGT
VIM	AGTCCACTGAGTACCGGAGAC	CATTTCACGCATCTGGCGTTC
T	TGTTTATCCATGCTGCAATCC	CCGTTGCTCACAGACCACAG
PAX6	CGAGATTTCAGAGCCCCATA	AAGACACCACCGAGCTGATT
LHX3	ACAGACACTGGCACAGCAAG	AGCAGTGCAGATGGTACACG
HB9	GCCTAAGATGCCCGACTTCAAC	CGCGACAGGTACTTGTTGAGCT
VAChT	GCTGTTTGCTTCCAAGGCTATCC	GAAGGCGAACAGGACTGTAGAG
LHX4	TGGCGGACAGGTGCTTCTCCA	AGGTGGTAGACAAAGTCCTGGG
CDH5	CCTACCAGCCCAAAGTGTGT	TGTCCTTGTCTATTGCGGAGA
CD31	ATGCCGTGGAAAGCAGATAC	CTGTTCTTCTCGGAACATGGA
TIE2	ACGACCATGACGGCGAATG	CGGCAGCCTGATATGCCTG
FLK1	CGGACAGTGGTATGGTTCTTG	GCCACAGACTCCCTGCTTT
TAL1	CCAACAATCGAGTGAAGAGGA	CCGGCTGTTGGTGAAGATAC