Supplemental Methods and Figures

The pAAVS1-Gp1ba construct

The proximal 2.6 kb of the murine *Gp1ba* promoter followed by its 5' UTR/1st intron had been previously spliced to the SV40 splice acceptor/5' UTR followed by a coding region of interest and then the SV40 3' UTR and polyadenylation signal and used in transgenic mice models to express human factor VIII in platelets[1]. This expression construct was now cloned into the Sal1 restriction site of the AAVS1 targeting plasmid (Addgene; *pAAVS1-SA-2A-puro-pA* donor*)* using the iProof High Fidelity DNA Polymerase (BioRad) and In-Fusion Advantage PCR Cloning kit (Clontech) according to the manufacturers instructions. The coding region of the *Gp1ba* promoter construct was replaced with either eGFP (Addgene pFUGW) or αIIb cDNA[2] by restriction digestion and PCR/In-fusion at the labeled Not1 sites (Supplemental Figure 2A). The additional Not1 site in the *pAAVS1-SA-2A-puro-pA* donor backbone was mutated by PCR overlap[3] for facile cloning.

Cell culture

Induced pluripotent stem (iPS) cells were maintained in human ES (hES) media: DMEM/F12 (Gibco) containing 20% knockout serum replacement (Gibco), 2 mM L-glutamine (Cellgro), 1,000 IU/mL penicillin/ 1mg/mL streptomycin (P/S, Cellgro, 100x), 1X non-essential amino acids (Gibco, 100x), 0.5 mM sodium pyruvate (Gibco), 0.12% sodium bicarbonate (Gibco), 0.1 mM β-mercaptoethanol (Gibco), and 10 ng/mL basic fibroblast growth factor (R&D Systems). All iPS cell lines were maintained on 0.1% gelatin (Sigma) coated 6-well culture plates containing irradiated mouse embryonic fibroblasts (iMEFs). Cultures were split every 3-5 days after incubation with TrypLE (Invitrogen) for 3-4 minutes, and then mechanically disaggregated and plated on fresh iMEFs with 10 μM Rock inhibitor (Cayman Chemical). Cultures were maintained at 37°C, 5% CO_2, 5% O_2, and 90% (vol/vol) N_2.

Generation of iPS cells

Peripheral blood from a healthy control (WTPB2-3/Control 1) and two patients with Type I GT (GTPB1-1/GTP1, GTPB2-1/GTP2) was collected, expanded, and reprogrammed as described[4]. CD34(+)-selected bone marrow cells were purchased from the Stem Cell Core at the University of Pennsylvania and used to generate a control iPS cell line (WTBM1-8/Control 2) as described[5]. Briefly, mononuclear cells were infected with lentiviral vectors pHAGE-Tet-hSTEMCCA and pHAGE2-CMV-rTTA encoding the reprogramming genes OCT4/SOX2/KLF4/MYC and reverse tetracycline transactivator protein (rTTA), respectively[6]. The iPS cell lines were generated in the presence of doxycycline 1 μg/mL starting Day 1 of reprogramming and removed when iPS cell clones were selected to silence reprogramming gene expression.

To establish iPS cell lines, doxycycline-resistant clones were handpicked about 2-3 weeks post-infection based on cell morphology and size. Clones were subsequently passaged and expanded in hES medium for a minimum of 20 passages to erase residual epigenetic memory[7]. Characterization of the clones included karyotype, teratoma formation, flow cytometry for pluripotency surface markers (SSEA3/4, TRA-1-81 and TRA-1-60), and qPCR analysis for pluripotency gene expression (Supplemental Figure 1).

RNA isolation and real-time quantitative PCR analysis

The qPCR procedure and primers for iPS cell pluripotency characterization are listed in Mills et al[8]. Additional primer sequences for the housekeeping gene cyclophillin, transgene construct (SV40 5' UTR), and megakaryocytes are as follows:

CYCLOPHILIN:

F' 5'-GAAGAGTGCGATCAAGAACCCATGAC-3'

R' 5'-GTCTCTCCTCCTTCTCCTCCTATCTTTACTT-3'

SV40 5' UTR:

F' 5'-CCGGTGGTGGTGCAAATCAAAGAA-3'

R' 5'-AGCAGAAGTAACACTTCCGTACAGGC-3'

ITGA2B:

F' 5'-GCTTTGGGTTGGAGCTGTTCCATT-3'

R′ 5'-GGGCTCAGTCTCTTTATTAGGCAGCA-3'

ITGB3:

F′ 5'-TCTACCTCTTGGCTGCCTTGTGAA-3'

R′ 5'-ACACGTGCTGATACAACTGACCCA-3'

GP1BA:

F′ 5'-GCACACAATTTCAGTCCCAGCCAA-3'

R′ 5'-TGGGAACCATGCTGTATTCTGCCA-3'

GP1BB:

F′ 5'-TTTCTTCCGTTGTGAATGCCGCGT-3'

R′ 5'-TGCAGCGGCAGATAGCACACTA-3'

GP9:

F′ 5'-TGTATCCCATAGAGTTGCCACCCA-3'

R′ 5'-TGTGAAGCCTTTGGTCAGGTGCT-3'.

Targeting the AAVS1 locus in iPS cells

AAVS1-specific ZFN sequences were cloned into an expression vector under the control of the PGK promoter *(pPGK-ZFN-L and pPGK-ZFN-R)*[9]. Pluripotent stem cells were transiently transfected with both ZFN plasmids and the AAVS1 donor plasmid containing the *Gp1ba* promoter construct driving either eGFP or αIIb cDNA. Approximately 1x10^5 iPS cells/well were plated onto puromycin-resistant DR4 MEFs (GlobalStem) with 10 μM Rock inhibitor in a 1:3 Matrigel (BD Biosciences) coated 6-well culture dish and then re-fed the next morning with hES media with 20 ng/mL of bFGF without P/S. The donor pAAVS1-*Gp1ba* construct (3 μg), 1 μg of each ZFN plasmid, and 4 μL of Plus reagent (Invitrogen) were added to 250 μL of DMEM/F12 and incubated at RT for 5 minutes (use sterile plasmids at ~1 μg/μL in tissue culture grade water). A total of 16 μL of Lipofectamine LTX (Invitrogen) was added to 250 μL of DMEM/F12, which was then combined with the 250 μL DNA/Plus mixture and incubated at RT for 30 minutes. The transfection mixture (500 μL) was then added drop wise per well and incubated 4-6 hours at 37°C and then washed and re-fed with hES media. Puromycin selection (0.5 μg/mL) was added 24-48 hours later for ~3-4 days. After ~1-2 weeks individual puromycin-resistant clones were hand picked and expanded.

Southern blot for AAVS1 targeting

To detect a single, hemizygous insertion at the AAVS1 locus, a 480 bp internal 5' probe aligned with the left homology arm (HA-L, Supplemental Figure 2B) was synthesized by a BamH1 restriction digest of 100 μg of *pAAVS1-SA-2A-puro-pA* donor plasmid. The probe was extracted using the Qiaquick PCR Purification Kit (Qiagen) and ^{32}P labeled using the Prime-It II Random Primer Labeling Kit (Agilent). Southern blotting was preformed on an Sph1 restriction digest (Supplemental Figure 2B) of 10 μg of genomic DNA extracted using the DNeasy Blood and Tissue Kit (Qiagen). A targeted insertion within the AAVS1 locus (Supplemental Figure 2C) was detected by the 5' probe as a 3.8 kb fragment[9]. A non-targeted allele was detected as a 6.5 kb fragment. Hemizygous insertions contained both the 6.5 and 3.8 kb fragments. Homozygous insertions only contained a single 3.8 kb fragment. Fragments of other sizes represent off-target insertions. The *pAAVS1-SA-2A-puro-pA* donor plasmid contains a second Sph1 restriction site outside of the HA-L, which gives a 4.3 kb plasmid-derived fragment due to non-homologous insertion of the plasmid DNA[10].

Hematopoietic differentiation

iPS cells were feeder depleted and differentiated into hematopoietic cells on 1:3 Matrigel coated 6-well plates using a previously described serum free, adherent monolayer protocol[8]. Adult enriched CD34+ bone marrow cells were differentiated into megakaryocytes as previously described[11].

Flow cytometry

Antibodies used included anti-CD41a phycoerythrin (PE), anti-CD41a allophycocyanin (APC), anti-CD42a PE, anti-CD235a APC, anti-CD18 APC, anti-Tra-1-60 PE (BD Biosciences); anti-CD31 PE-Cyanine7, anti-CD45 Pacific blue, anti-SSEA-3 AlexaFluor 488, anti-SSEA-4 AlexaFluor 647, anti-Tra-1-81 AlexaFluor 647 (BioLegend); anti-KDR PE (R&D). Cells were stained as previously described[8] and analyzed on a FacsCanto (BD Biosciences) with FlowJo software (Tree Star).

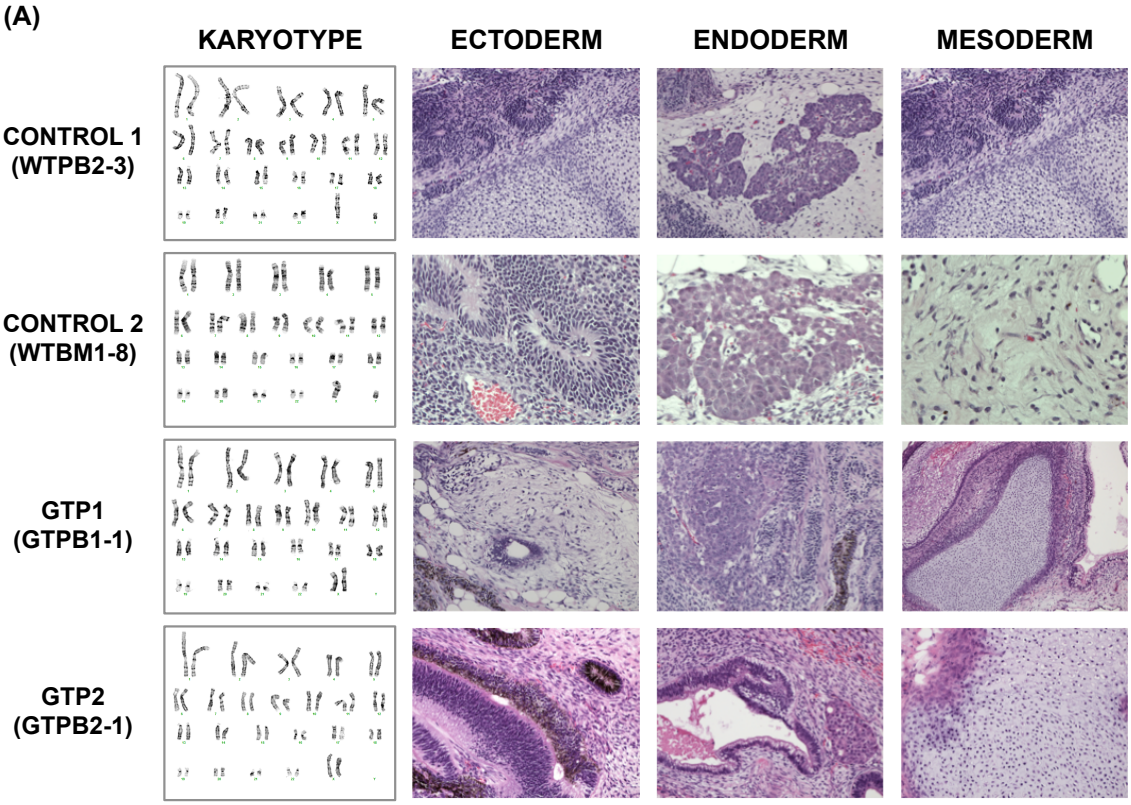
PAC-1 and fibrinogen binding

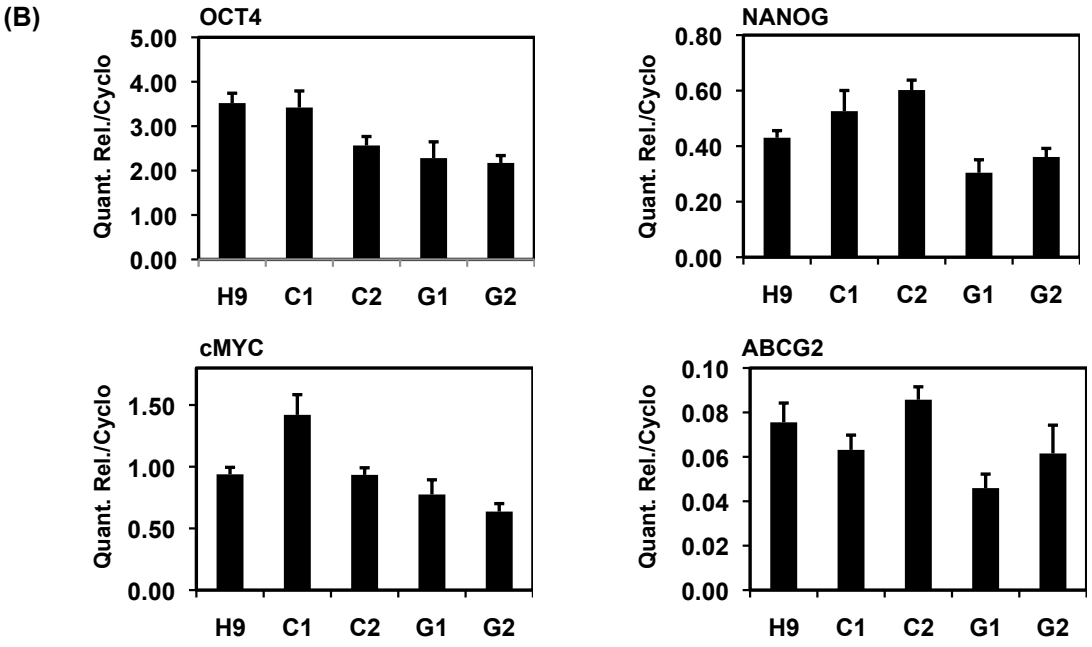
Megakaryocytes spun at 200 RCF for 5 minutes and re-suspended in Tyrode's salt solution (Sigma) with 0.1% bovine serum albumin (BSA) at room temperature (RT) to a final concentration of ~1-4x10^6 cells/mL. The cells were added to 5 mL polystyrene tubes containing 2 μL CD42a PE, ± 10 μM tirofiban, ± 10 μM ethylenediaminetetraacetic acid (Invitrogen), 10 μg/mL of AlexaFluor 647-conjugated fibrinogen (Molecular Probes) or 10 μL PAC-1 Fluorescein isothiocyanate (BD Biosciences), and convulxin (Enzo) 500 ng/mL to give a final reaction volume of 50 μL. The tubes were incubated for 15 minutes at RT, then placed on ice and diluted with 450 μL of ice-cold Tyrode's salt solution with 0.1% BSA. Fibrinogen and PAC-1 binding were analyzed by flow cytometry[12,13].

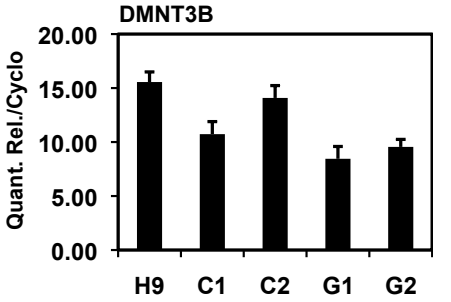
Western blots

Megakaryocytes were lysed with 1% Triton-X100 lysis buffer (150 mM NaCl, 1% TX-100, 0.02% NaN3, 0.01M Tris [tris(hydroxymethyl) aminomethane] buffer pH 8.0). Lysate protein concentrations were determined using the bicinchoninic acid protein assay. Sodium dodecyl sulfate (SDS) loading buffer was mixed with protein samples and loaded into 4-12% precast gels (Invitrogen). Proteins were separated by SDS polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. Membranes were blocked in 5% nonfat dry milk in PBS with Tween 20 (PBST) for 1 hour at RT, followed by primary antibody incubation in 1% nonfat dry milk in PBST overnight at 4°C. Membranes were washed with PBST for 15 minutes 3 times to remove excess antibody. Incubation with the appropriate secondary antibody conjugated to horseradish peroxidase at a 1:10000 dilution was done at RT for 1 hour. After 3 washes with PBST, membranes were developed using the ECL Plus detection system. Antibodies used: anti-rabbit polyclonal SEW-8 anti-αIIb (0.6 μg/mL); mouse monoclonal β3 (1:200, Santa Cruz Biotechnology, sc-365679); anti-mouse monoclonal β-actin (1:100,000, Sigma-Aldrich, A1978); Peroxidase-AffiniPure goat anti-rabbit IgG (1:10,000, Jackson Immunoresearch, 111-035-003); and Peroxidase-AffiniPure goat anti-mouse IgG (1:10,000, Jackson Immunoresearch, 115-035-003).

Sullivan et al. Supplemental Figure 1



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10.00
5.00
0.00
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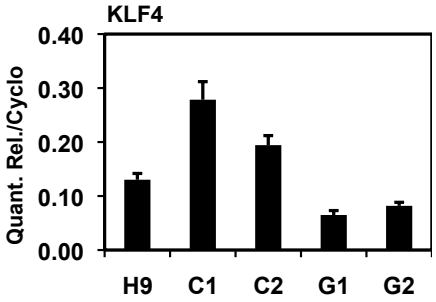
C1
C2
G1
G2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0.00
H9
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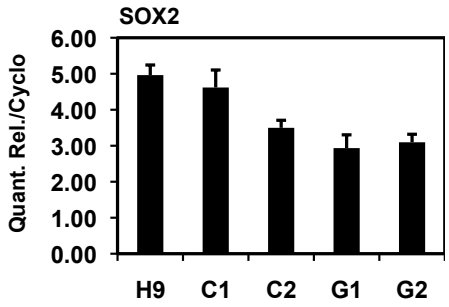
C2
G1
G2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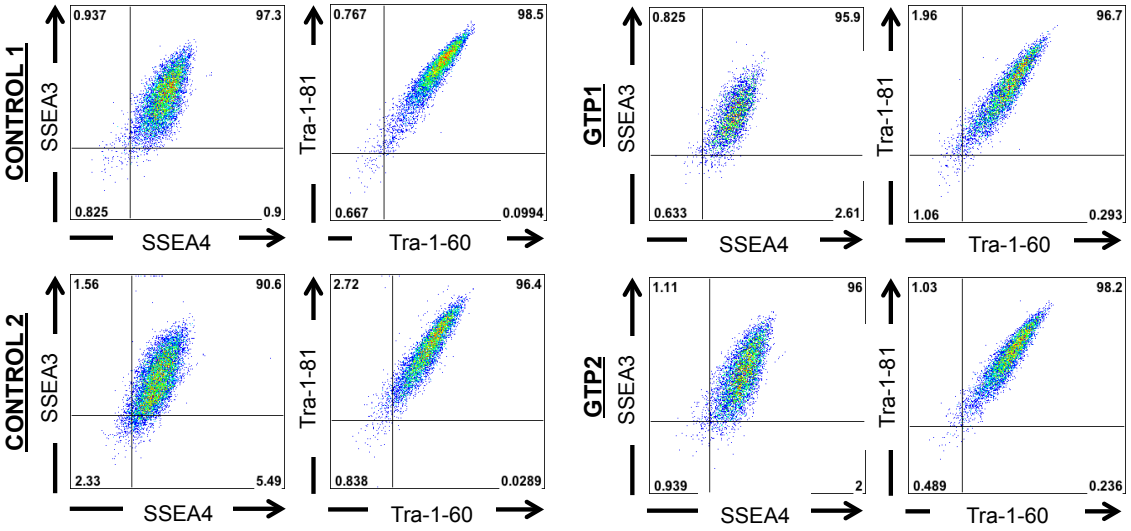
0.00
H9
C1
C2
G1
G2

(C)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SSEA4
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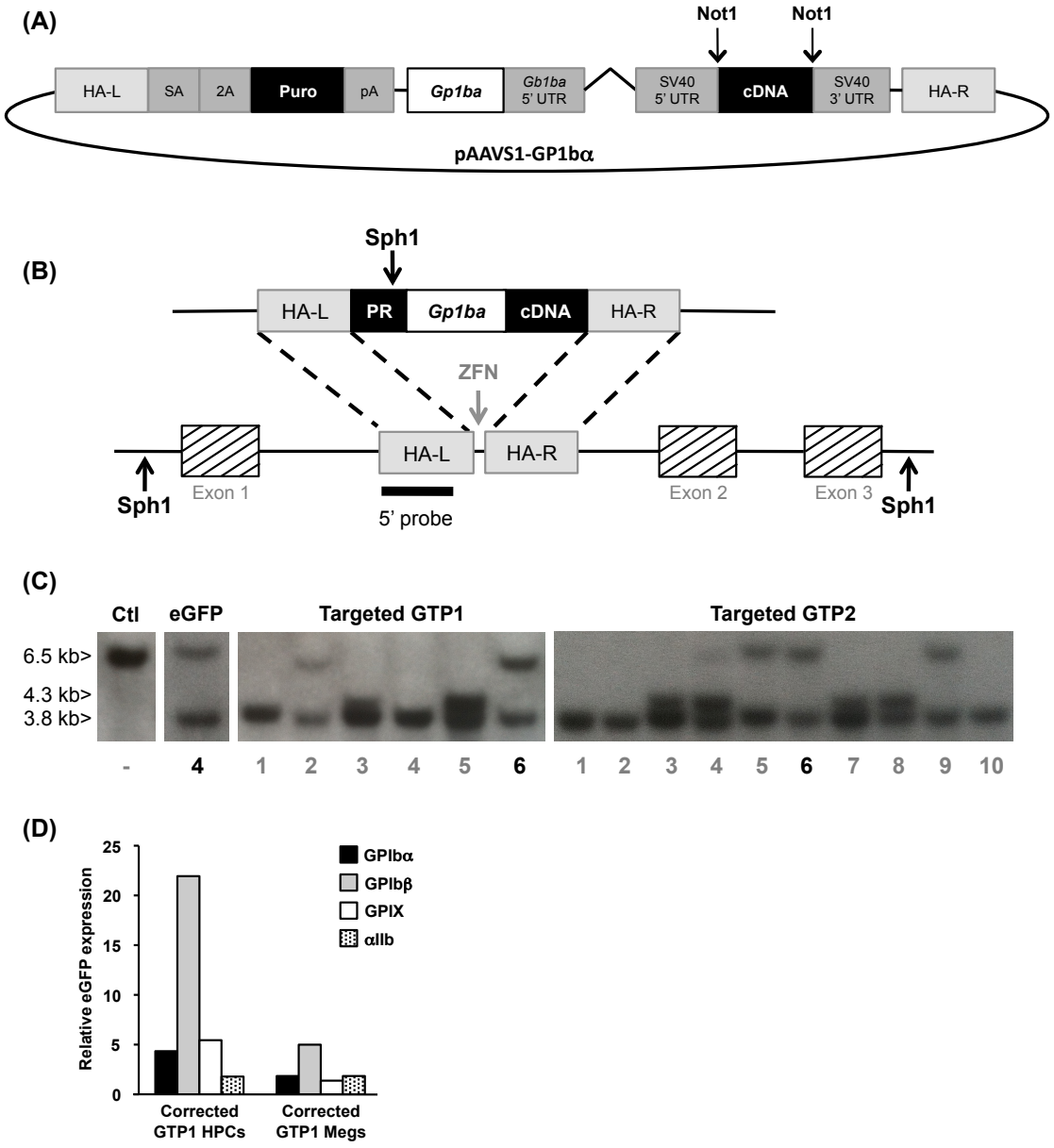
0.825
0.9
Tra-1-81
Tra-1-60
0.767
98.5
0.667
0.0994
GTP1
0.825
95.9
0.633
2.61
1.96
96.7
1.06
0.293
CONTROL 2
1.56
90.6
2.33
5.49
2.72
96.4
0.838
0.0289
GTP2
1.11
96
0.939
2
1.03
98.2
0.489
0.236

Supplemental Figure 1. **Characterization of iPS cell lines**.

(A) Karyotypes and generation of all three germ layers in a teratoma assay: ectoderm, endoderm, and mesoderm (original magnification, X20-40). WTPB2-3, WTBM1-8, GTPB1-1, GTPB2-1 are the names of the iPS cell lines used in this study. (B) Molecular profiles of iPS cell lines (C1 – Control 1, C2 – Control 2, G1 – GTP1, G2 – GTP2). Real-time PCR analysis shows the expression of pluripotency genes: OCT4, NANOG, cMYC, ABCG2, DNMT3B, KLF4, and SOX2 relative to cyclophilin (Cyclo). (C) Pluripotent surface antigen expression: SSEA3+/SSEA4+ and TRA-1-81+/TRA-1-60+.

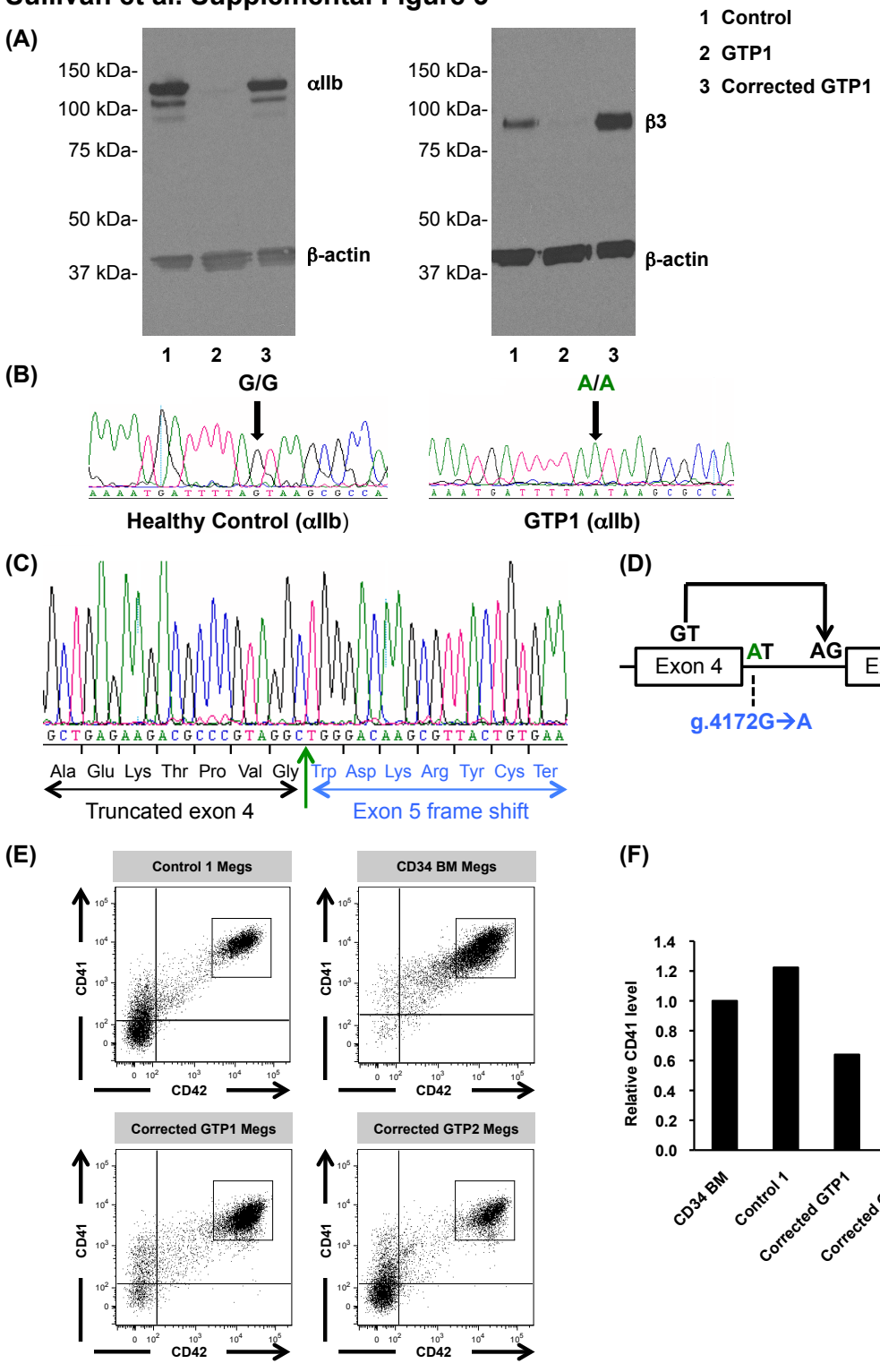
Sullivan et al. Supplemental Figure 2



Supplemental Figure 2. **iPS cell targeting strategy with the *pAAVS1-Gp1ba* vector, Southern blot analysis, and eGFP expression relative to endogenous gene expression**.

(A) AAVS1 targeting plasmid (*pAAVS1-Gp1ba*) containing the puromycin resistance gene (Puro) followed by the murine *Gp1ba* promoter/5' UTR linked to the SV40 SA/5'UTR; followed by the coding region of a cDNA of interest; followed by the SV40 3'UTR/pA. The transgene is flanked by AAVS1 homologous sequences (HA-L: homology arm left, HA-R: homology arm right). (B) Integration site of the transgenic construct within the AAVS1 locus (1st intron of *PPP1R12C)*. The grey arrow points to the double-strand DNA break created by site-specific AAVS1 ZFNs. Sph1 restriction sites (black arrows) and internal 5' probe (black bar) are also shown (PR - puromycin resistance gene). (C) Southern blots of a control iPS cell line (Control 2) targeted with the *Gp1ba* promoter construct driving eGFP and GTP1 and GTP2 iPS cells targeted with the *Gp1ba* promoter construct driving αIIb cDNA. A 6.5 kb fragment is the untargeted AAVS1 allele; a 3.8 kb fragment is the targeted AAVS1 allele[9]; and a 4.3 kb fragment is the non-homologous insertion of plasmid DNA[10]. Hemizygous insertions are represented by both the 6.5 and 3.8 kb fragments (~30% for selected GT clones). Homozygous insertions are represented by a single 3.8 kb fragment (~30% for selected GT clones). ~40% of selected GT clones demonstrated non-homologous insertion of plasmid DNA. Lanes are noted as follows: "–" for an untargeted clone; numbers under blot are for all clones tested; and bold numbers are the eGFP, GTP1, and GTP2 clones studied in this paper. (D) mRNA expression of eGFP relative to endogenous GPIbα, GPIbβ , GPIX, and αIIb in iPS cell-derived HPCs and megakaryocytes (megs). *Gp1ba*-driven eGFP is expressed ~2 to 5-fold higher than endogenous GPIbα, GPIX, and αIIb and ~22-fold higher than GPIbβ in HPCs. In megakaryocytes, *Gp1ba*-driven eGFP is expressed 5-fold higher than GPIbβ.

Sullivan et al. Supplemental Figure 3



Supplemental Figure 3. **Characterization and correction of the thrombasthenic mutation in GTP1**. (A) Western blots of αIIb and β3 in control and GTP1 iPS cell-derived megakaryocytes. (B) Sequencing of GTP1 *ITGA2B* genomic DNA compared to a normal control shows that the patient is homozygous for an IVS4(+1)G>A (g.3956G>A) mutation at the exon 4 invariant splice donor site. (C) Sequence of αIIb cDNA derived from GTP1 platelet RNA shows that the mutation results in utilization of a cryptic splice site within exon 4 continuing out-of-frame in exon 5. (D) Schematic representation of the mutation in blue at the splice donor site of exon 4 of the *ITGA2B* gene with utilization of a cryptic exon 4 splice donor site to the normal splice acceptor site of exon 5. (E) Analysis of CD41 versus CD42 expression in enriched human CD34+ bone marrow stem and progenitor cell-derived megakaryocytes (CD34 BM Megs), control iPS cell-derived megakaryocytes, and corrected GT iPSC cell-derived megakaryocytes by flow cytometry. (F) Expression of CD41 of control and corrected GT iPS cell-derived megakaryocytes relative to CD34+ BM-derived megakaryocytes.

References

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