SUPPLEMENTARY APPENDIX

Molecular mechanisms of bleeding disorder-associated GFI1B^{Q287*} mutation and its affected pathways in megakaryocytes and platelets

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Supplementary Methods

Lentiviral transduction of MEG-01 cells with GFI1B-GFP and GFI1B^{Q287*}-GFP

GFI1B variant-GFP fusions (wild type GFI1B, Q287*, P2A, and K8A) were cloned in the FUW lentiviral vector. Lentivirus was produced using 293FT cells. After seeding 293FT cells onto 9 cm dishes, cells were transfected at 60% confluency with calcium phosphate. Transfection was performed using 3.68 µg pLP1, 1.84 µg pLP2, and 2.30 µg pLP/VSVG packaging vectors and 14.4 µg FUW construct. After 16-18 hours, the medium was replaced with 5 ml fresh medium. Viral supernatant was harvested ~48 hours after transfection. MEG-01 cells were transduced with FUW, FUW-GFI1B-GFP or FUW-GFI1B^{Q287*} viral supernatant by spinning down the virus on a retronectin coated 6-wells plate. Subsequently, the MEG-01 cells were added and incubated with virus for 24 hours.

Nuclear extracts MEG-01 and GFP-pull down GFI1B interacting proteins

Nuclear extracts from 100 million lentivirally transduced MEG-01 cells were generated according to Dignam *et al*.[1] Cells were harvested and washed twice with PBS. Cells were incubated for 10 minutes at 4°C in 5 volumes of Buffer A (10 mM Hepes KOH pH 7.9, 1.5 mM $MgCl_2$, and 10 mM KCl), and then pelleted at 400*g* for 5 minutes at 4°C. Swollen cells were resuspended in 2 volumes of buffer A supplemented with complete protease inhibitors (Roche) and 0.15% NP40 (Sigma), followed by homogenization on ice using 30 strokes with a type B (tight) pestle. The nuclei were pelleted and washed two times with PBS at 3200*g*, 4°C for 15 minutes. This was followed by incubation in 2 volumes of Buffer C (420 mM NaCl, 20 mM Hepes KOH pH 7.9, 20% v/v glycerol, 2 mM $MgCl_2$, 0.1% NP40, complete protease inhibitors, and 0.5 mM DTT) for 1 hour at 4°C to extract nuclear proteins. The nuclear extract (supernatant) was obtained by centrifugation at 21.000*g* for 30 minutes at 4°C.

Label-free GFP-pulldowns were performed in triplicate as described in Smits *et al*.[2] For GFP-pulldowns, 0.6 mg of nuclear extract was incubated with 7.5 µl GFP-Trap beads (ChromoTek) in incubation buffer (300 mM NaCl, 20 mM Hepes KOH pH 7.9, 20% v/v glycerol, 2 mM $MgCl_2$, 0.2 mM EDTA, 0.25% NP40, complete protease inhibitors, and 0.5 mM DTT) in the presence of 50 µg/ml ethidium bromide (Sigma) to prevent indirect DNA mediated interactions. Beads were washed twice with incubation buffer (0.5% NP40), twice with PBS (0.5% NP40) and finally twice with PBS. Subsequently, the proteins were subjected to on-bead trypsin digestion and peptides were acidified and desalted using C18-Stagetips.

Mass spectrometry and data analysis GFI1B interacting proteins

Just before mass spectrometry analysis, peptides were eluted from the C18 Stagetips and recorded with LC-MS/MS LTXQ-Orbitrap Fusion Tribrid mass spectrometer (ThermoFisher Scientific) at top

speed mode of 3 seconds cycle. Raw data were analyzed by MaxQuant (1.5.7.0) using default settings and blasted against the Human Uniprot database downloaded on 17-02-2017. Statistical outliers for the GFP-pulldown of the empty vector compared to wild type GFI1B or GFI1B^{Q287*} were determined using a two-tailed *t*-test. Multiple testing correction was applied by using a permutation-based false discovery rate (FDR) method in Perseus (from MaxQuant package). Statistical cut-offs (FDR<0.01 and fold change >9.2) were chosen such that no proteins were present as outliers on the empty vector control side of the volcano plot. To determine the stoichiometry of the identified complexes, we compared the relative abundance of interactors by calculating iBAQ intensities (sum of all identified peptide intensities for a certain protein, divided by the number of theoretically observed peptides). Next, the iBAQ intensities were compensated for the iBAQ intensity in the empty vector samples representing the background binding level. Finally, these relative abundance values were normalized by setting the abundance of GFI1B;GFI1 to 1.

Co-immunoprecipitation and western blotting

MEG-01 cells were lentivirally transduced with FUW-GFI1B-(WT, Q287*, P2A, or K8A)-GFP, and when indicated treated with 4µM GSK-LSD1 (Sigma) for 48 hours. Nuclear extracts were made using the NE-PER Nuclear and Cytoplasmic Extraction Kit (ThermoFisher). Co-immunoprecipitation was performed with GFP-Trap beads. For normal western blot, whole cell extracts were prepared in SDS gel-loading buffer (2% SDS, 0.1% bromphenol blue, 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 10% glycerol, water). PVDF membranes were stained with rabbit anti-LSD1 (Abcam), mouse anti-GFI1B (Santa Cruz Biotechnology), or rabbit anti-Actin (Sigma). Luminescence signal was visualized using a ChemiDox XRSþ (Bio-Rad).

Proliferation of GFI1B and GFI1B mutant transduced MEG-01 cells

Flag-tagged GFI1B constructs were cloned into the retroviral vector pMIGR1-IRES-GFP to generate pMIGR1-GFI1B-flag-IRES-GFP. Retrovirus was produced using PhoenixA cells, which were cultured in DMEM supplemented with 10% heat inactivated FCS at 37˚C supplied with 5% CO_2. After seeding onto 9cm dishes, cells were transfected at 60% confluency with calcium phosphate. Transfection was performed using 3.15 µg pCL-Ampho retroviral packaging vector and 18.4 µg pMIGR1 construct. After 16-18 hours, the medium was replaced with 5ml fresh medium. Viral supernatant was harvested 30 hours after transfection. MEG-01 cells were maintained between $2x10^5$ and $8x10^5$ cells/ml in RPMI 1640 (GIBCO) supplemented with 10% heat inactivated FCS, at 37˚C and 5% CO_2. MEG-01 cells were transduced with 2.5 ml pMIGR1 virus. Briefly, 1.25 ml viral supernatant plus $2x10^5$ MEG-01 cells (in 1ml) were added to retronectin (Takara Bio Inc.)-coated non-tissue culture treated 33 mm dishes. After 24 hours, 1.25 ml medium was removed from the plates and 1.25 ml fresh viral supernatant

was added. After an additional 24 hours MEG-01 cells were harvested from retronectin plates, spun down and resuspended in 1 ml fresh culture medium. The GFP% was followed using flow cytometry for 26 days with 2-3 day intervals. GFP percentages were normalized to the start point of the culture (day 5 after transduction) using the following formula: (GFP% day X/(100 - GFP% day X))/(GFP% day 5/(100 - GFP% day 5)) to correct for differences in start GFP%. On day 23, $2x10^5$ GFP^+ cells were FACS-sorted to determine GFI1B expression using quantitative RT-PCR.

Quantitative RT-qPCR

RNA was isolated using the Quick-RNA™ kit (Zymo Research). RNA was treated with DNAse (Zymo Research) and reverse transcribed using M-MLV reverse transcriptase (Invitrogen). For quantitative Real Time Polymerase Chain Reaction (RT-qPCR), cDNA was amplified using Taqman 2x Universal PCR Master Mix (Applied biosystems) as recommended by the manufacturer. GFI1B expression was determined using the following primers and probe: forward primer 5'-CCCGTGTGCAGGAAGATGA, reverse primer 5'-CAGGCACTGGTTTGGGAATAGA, probe 5'-FAM-TTACCCCGGTGCCCAGA-MGB. Endogenous GFI1B expression (5'UTR) was determined using TaqMan Gene Expression Assay Hs01062474_m1 (Applied Biosystems). GAPDH expression was determined using Human GAPDH mix (FAM™ Dye/MGB Probe) (Applied biosystems) to correct for differences in input. RT-qPCR was performed on the 7500 Real Time PCR System (Applied Biosystems). Data were analyzed using 7500 Fast System Software (version 1.3.1).

BEL-5-Cl1/2 iPSC line generation

To generate blood-derived induced pluripotent stem cells (iPSC), patient blood (5-10ml) was collected after written informed consent. Peripheral blood mononuclear cells were expanded and differentiated to erythroblasts within 8 days as described in Heideveld *et al*.[3] The polycystronic Oct4/KLF4/SOX2/c-Myc lentiviral plasmid[4,5] was used to transduce erythroblasts for reprogramming as described in Hansen *et al*.[6] After transduction, cells were cultured in IMDM (Biochrome, Merk) supplemented with 1U/ml erythropoietin, 100ng/ml SCF, 1uM Dexamethason, 10µg/ml polybrene (Sigma) and 2mmol/ml valproic acid (VPA, Sigma) for 1 day. Cells were subsequently plated on 0.1% gelatine (Sigma) coated plates containing a confluent layer of irradiated mouse embryonic fibroblasts (iMEF, GlobalStem). Cells were incubated with VPA for an additional 7 days. The medium was changed to E8 (ThermoFisher Scientific) on day 10 and iPS colonies were harvested between days 14-20. iPSCs were tested for pluripotency as we previously described in Hansen *et al*.[6]

iPSC megakaryocytic differentiation

iPSC lines MML-6838-Cl2[6] and BEL-5-Cl1/2 were maintained on Matrigel coated (BD Biosciences) plates in E8 medium (ThermoFisher Scientific) following manufacturer's instructions. For differentiation, iPS colonies were made single cell with TrypleSelect (ThermoFisher Scientific), and 120-250 cells were seeded in E8 with RevitaCell (ThermoFisher Scientific) on 6cm matrigel coated dishes. Colonies were grown until a size of 400-600µm. Differentiation was initiated with StemLine II (Sigma) supplemented with 50ng/ml bFGF, 40ng/ml VEGF, 20ng/ml BMP4 and insulin transferrin selenium (1:100, ITS) (ThermoFisher Scientific). At day 6 of differentiation, medium was changed to IMDM (Biochrom, Merck) with 10ng/ml VEGF, 20ng/ml BMP4, 10ng/ml IL-1β, 1ng/ml IL-3, 10ng/ml IL-6, 50ng/ml TPO, 50ng/ml SCF and 1:100 ITS. All growth factors were purchased from PeproTech. Cells were harvested from the supernatant between days 14-18, for flow cytometry and on day 18 for sorting $CD41a^+$ cells for mass spectrometry.

Electron Microscopy (EM) iPSC-derived megakaryocytic cells

For EM, tissue culture samples were fixed in Karnovsky's fixative. Postfixation was done with 1% Osmiumtetroxide in 0,1 M cacodylatebuffer, after washing tissues were stained en bloc with Ultrastain 1 (Leica, Vienna, Austria), followed by ethanol dehydration series. Finally the samples were embedded in a mixture of DDSA/NMA/Embed-812 (EMS, Hatfield, U.S.A), sectioned and stained with Ultrastain 2 (Leica, Vienna, Austria) and analyzed with a CM10 electron microscope (Thermo Fisher, Eindhoven, the Netherlands).

Primary megakaryocyte culture

Mobilized peripheral blood was provided by the Sanquin Laboratory for Cell Therapy and obtained from leukopheresis material of healthy donors treated with G-CSF (2x5 µg/kg/day subcutaneously, Filgastrim, Amgen) as described.[7] Informed consent was given in accordance with the Declaration of Helsinki and the Dutch national and Sanquin internal ethical review boards. $CD34^+$ hematopoietic stem and progenitor cells were isolated from mobilized peripheral blood using CD34 Microbeads (Miltenyi Biotec) and magnetic-activated cell sorting according to the manufacturer's instructions. $CD34^+$ cells were differentiated to megakaryocytes in modified IMDM (HEMAdef[8]) supplemented with 50 ng/ml stem cell factor (SCF), 50 ng/ml TPO, 1 ng/ml IL-3 and 20 ng/ml IL-6 for 4 days, followed by culturing in 50 ng/ml TPO and 10 ng/ml IL-1β for 7 more days.[3] All growth factors were from Peprotech. To study the effect of LSD1 inhibition on megakaryocyte differentiation, 4 µM GSK-LSD1 (Sigma) was added to the IL-1β and TPO culture at day 7, and cells were followed for 2-6 days. CD34/CD41a/CD42b expression and expansion was measured after 2 days of GSK-LSD1 treatment. In the experiment with 6 days exposure to GSK-LSD1, CD42b expression and proplatelet formation was

analyzed. The number of proplatelet forming megakaryocytes was based on 6 photos from each culture (n=3) and quantified by ImageJ counting.

Flow cytometry

The Coulter FC500 flow cytometer (Beckman Coulter) was used to determine the percentage of GFP positive MEG-01 cells, and the BD FACSAria (BD Bioscience) to sort these GFP positive cells. Expression of surface molecules on cultured megakaryocytes either originating from mobilized blood $CD34^+$ cells or iPSCs were analyzed by staining with the following fluorochrome-conjugated antibodies: CD34-APC (Biolegend), ITGA2B/CD41a-PE-Cy7 (Biolegend), and GP1BA/CD42b-FITC (Sanquin). After staining, cells were analyzed using the LSR-II (BD Bioscience). FACS beads (BD Bioscience) were taken along to determine the expansion of the $CD34^+/CD41a^+$ megakaryoblast population. Geometric mean fluorescent intensity (MFI) was normalized to isotype control geometric MFI, generating nMFI for normalized MFI.

Platelet isolation

The study was approved by the Medical Ethical Committee of the Radboudumc Nijmegen (2013/064) and conducted in accordance with the Declaration of Helsinki. Venous blood was collected from healthy volunteers and from $GFI1B^{Q287*}$ patients after obtaining written informed consent. The clinical parameters of the $GFI1B^{Q287*}$ patients have been described previously.[9] Platelet-rich plasma was obtained via centrifugation of whole blood for 20 min at 120*g*. Platelets were spun down by centrifugation for 10 minutes at 2000*g*, washed and resuspended in a buffer comprising 36 mM Citric acid, 103 mM NaCl, 5 mM KCl, 5 mM EDTA, 5.6 mM D-glucose, 1 nM Prostaglandin E1 (PGE1) and 10% (v/v) ACD-A (BD-Plymouth) at pH 6.5. Platelets were again washed and resuspended in the same buffer but with 0.1 nM PGE1. Finally, platelets were collected by centrifugation for 10 minutes at 2000*g* and processed for MS analysis.

Sample preparation for proteomics mass spectrometry.

All fine chemicals were from Thermo Scientific unless indicated otherwise. 100 x 10^6 washed platelets per donor or 1 x 10^6 cultured megakaryocytic cells were lysed in 100 µl 8 M Urea, 100 mM Tris-HCl (Life Technologies) at pH 8. For iPSC MK, sorted $CD41a^+$ cells were used. Samples were sonicated for 10 minutes and centrifuged at 12000*g* to remove insoluble material. The protein concentration of the lysates was measured using a Bradford assay. For label-free quantification, three replicates of 5 µg protein from platelets or from cultured megakaryocytic cells were taken. The same amount of protein was used for each iPSC sample. Samples were diluted at least 7-fold (v/v) in 50 mM ammonium bicarbonate (Fluka). Proteins were reduced for 60 minutes at 25°C in 10 mM

dithiothreithol (DTT) and alkylated using 55 mM iodoacetamide (IAM) for 45 minutes at room temperature in the dark. Finally, proteins were proteolysed by trypsin (Promega) overnight at 25°C using a protein:trypsin ratio of 1:20 (mg/mg). Protein samples were acidified with 5 µl 99% formic acid, and prepared for MS analysis using Empore-C18 StageTips.[10]

Proteomics mass spectrometry data acquisition.

Tryptic peptides were separated by nanoscale C18 reverse phase chromatography coupled on-line to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) via a nano-electrospray ion source (Nanospray Flex Ion Source, Thermo Scientific). Peptides were loaded on a 20 cm 75–360 µm inner-outer diameter fused silica emitter (New Objective) packed in-house with ReproSil-Pur C18-AQ, 1.9 µm resin (Dr Maisch GmbH). The column was installed on a Dionex Ultimate3000 RSLC nanoSystem (Thermo Scientific) using a MicroTee union formatted for 360 µm outer diameter columns (IDEX) and a liquid junction. The gradient that was employed to separate the peptides as well as the top-speed method to acquire MS/MS spectra on the Fusion are described previously.[11] All MS data were acquired with Xcalibur software (Thermo Scientific).

Proteomics mass spectrometry data analysis

The RAW mass spectrometry files were processed with the MaxQuant computational platform, 1.5.2.8. Proteins and peptides were identified using the Andromeda search engine by querying the human Uniprot database (89796 entries; downloaded February 2015). Standard settings with the additional options 'match between runs', 'Label Free Quantification (LFQ)', and 'unique peptides for quantification' were selected. The RAW files, MaxQuant search results, and details about the settings are available in the PRIDE repository database[12] with the dataset identifier PXD009020. The generated 'proteingroups.txt' table was imported into Perseus 1.5.1.6 and filtered for 'only identified by site' and reverse hits. The LFQ values were transformed in log2 scale and potential contaminant proteins were removed, except for ALB and THBS1. For primary cultured megakaryocyte and platelet samples, the three technical replicates of samples were grouped, and the proteins were filtered for three valid values in at least one of these groups. The three replicates of the control and GFI1B^{Q287*} platelet samples were then averaged. For the analysis of differentially expressed proteins between GFI1B^{Q287*} and control platelets, only proteins that were detected in four patient and/or four control platelet samples were included. For iPSC-derived samples, proteins detected in at least five control and/or five GFI1B^{Q287*} samples were included. Missing values were imputed by normal distribution (width = 0.3, shift = 1.8), assuming these proteins were close to the detection limit.

Gene ontology term enrichment analysis.

Enrichment of gene ontology (GO) terms based on biological processes, molecular functions and cellular components was performed and visualized with the R package gogadget[13], by comparing up- and down-regulated proteins against the background of total detected proteins. For comparison of significantly enriched proteins to all identified proteins, a p-value cut-off of 0.05 was used. Heatmaps were generated based on overlapping proteins between GO terms, and k-means was determined separately for each clustering. One or two overlapping terms were assigned for each cluster of GO terms. The full lists of GO terms, including cluster numbers, is provided in Supplementary Table 2.

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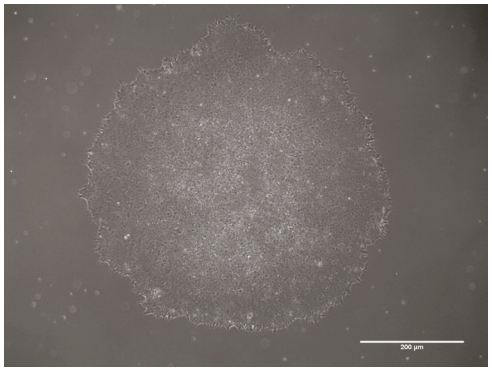

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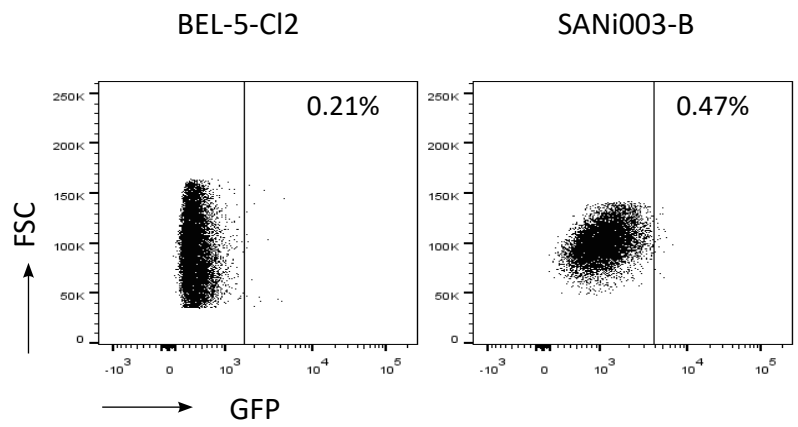

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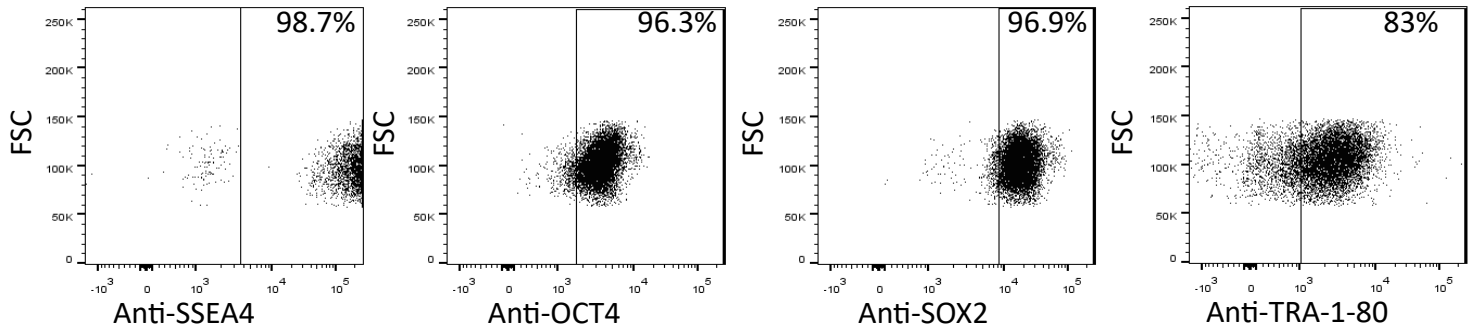

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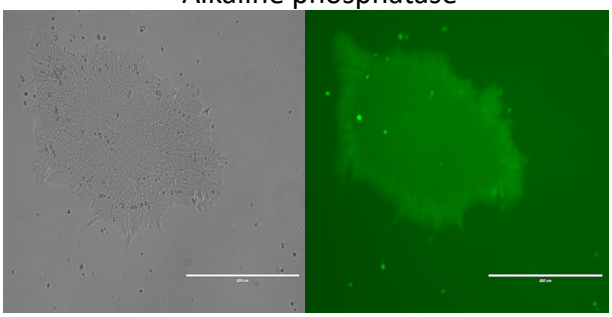

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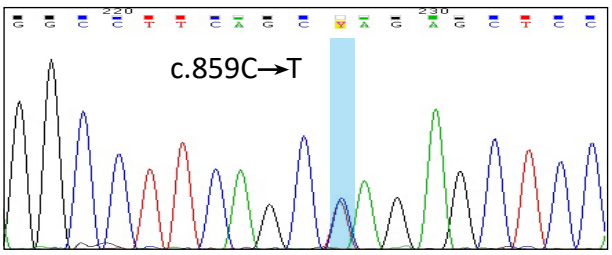

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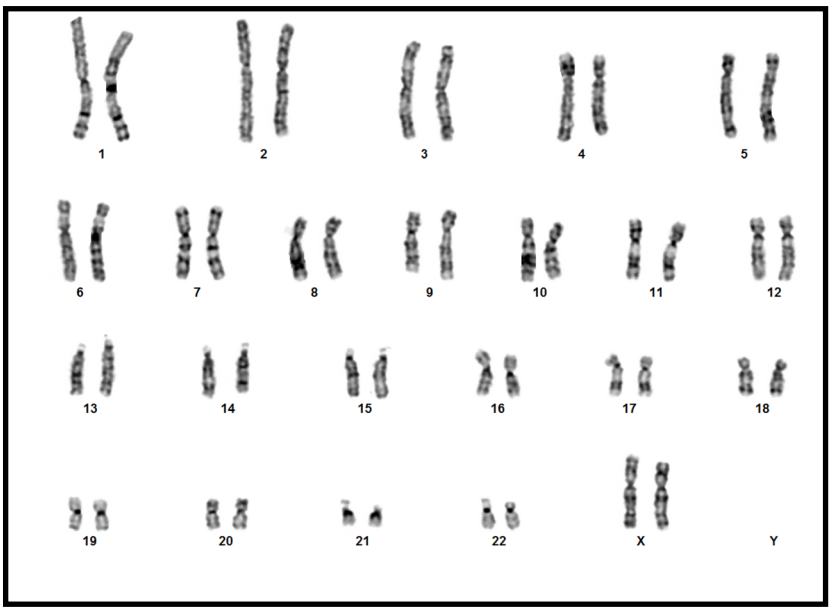

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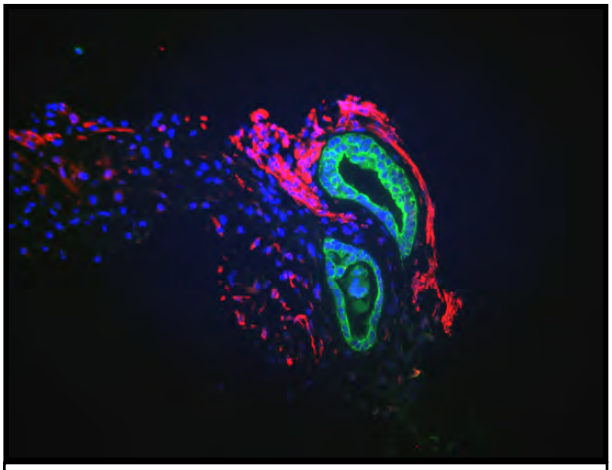

Anti-Alpha- SMA and Anti-Cytokeratin

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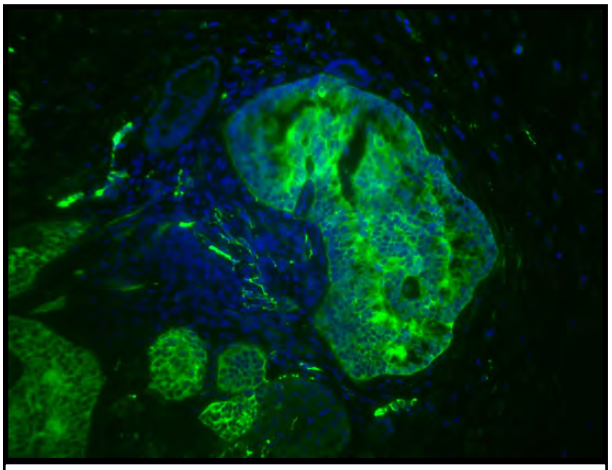
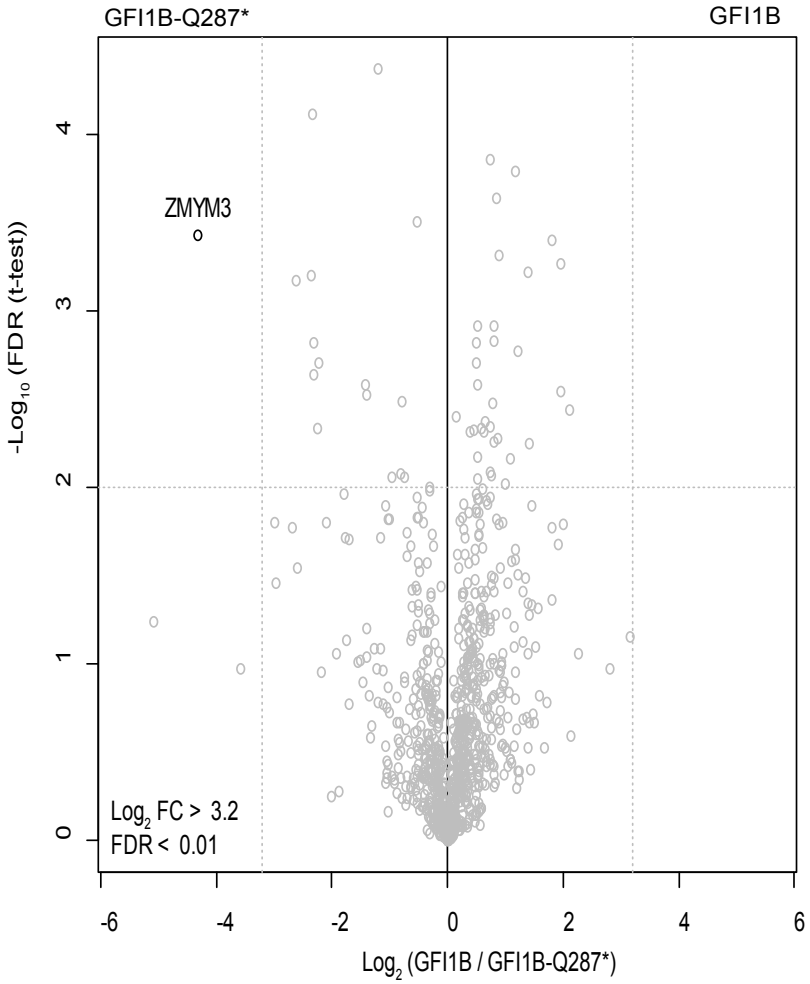
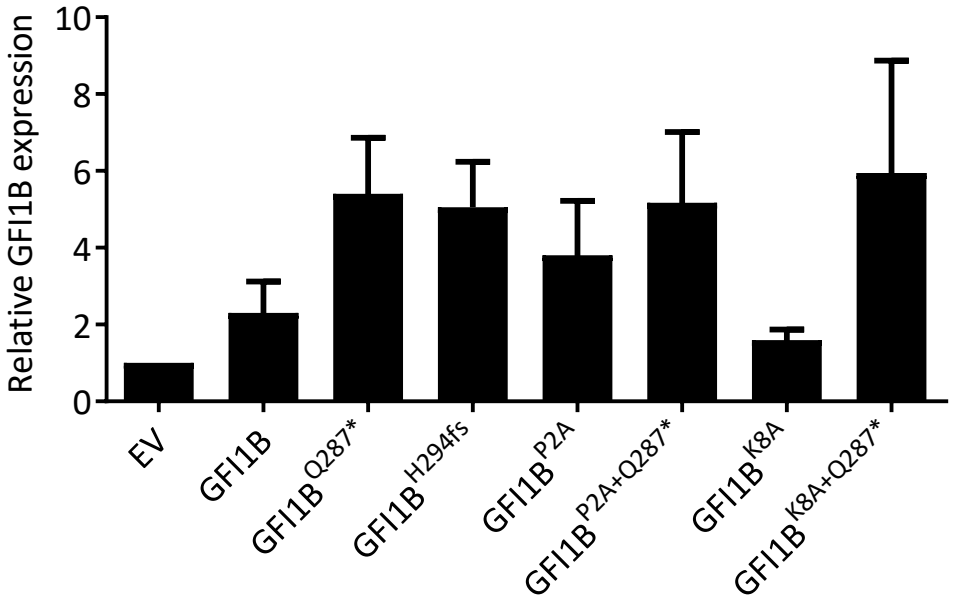


Anti-Beta Tubulin Class III

Supplementary Figure S1. Generation of the GFI1B^{Q287*} iPSC BEL-5-Cl.2 (A) Morphology of BEL-5-Cl2, 4x magnification, EVOS XL (Thermo Fisher Scientific). (B) Silencing of the reprogramming cassette measured by flow cytometry as the absence of green fluorescent protein (GFP; left); an episomal reprogramed iPSC SANi003-B serves as negative control (right). (C) Expression of pluripotency associated markers SSEA4, OCT4, SOX2 and TRA1-81 by flow cytometry. Gating was set based on isotype control. (D) Alkaline phosphatase staining showing bright field (left) and green fluorescence channel (right). (E) Sanger sequencing showing the heterozygous GFI1B^{Q287*} mutation in BEL5 Cl.2 (F) Representative G-banded karyogram. (G) Teratomas stained for mesoderm (Alpha-SMA, red), endoderm (cytokeratin, green), and (H) ectoderm (Beta Tubulin Class III, green) and Dapi (blue).



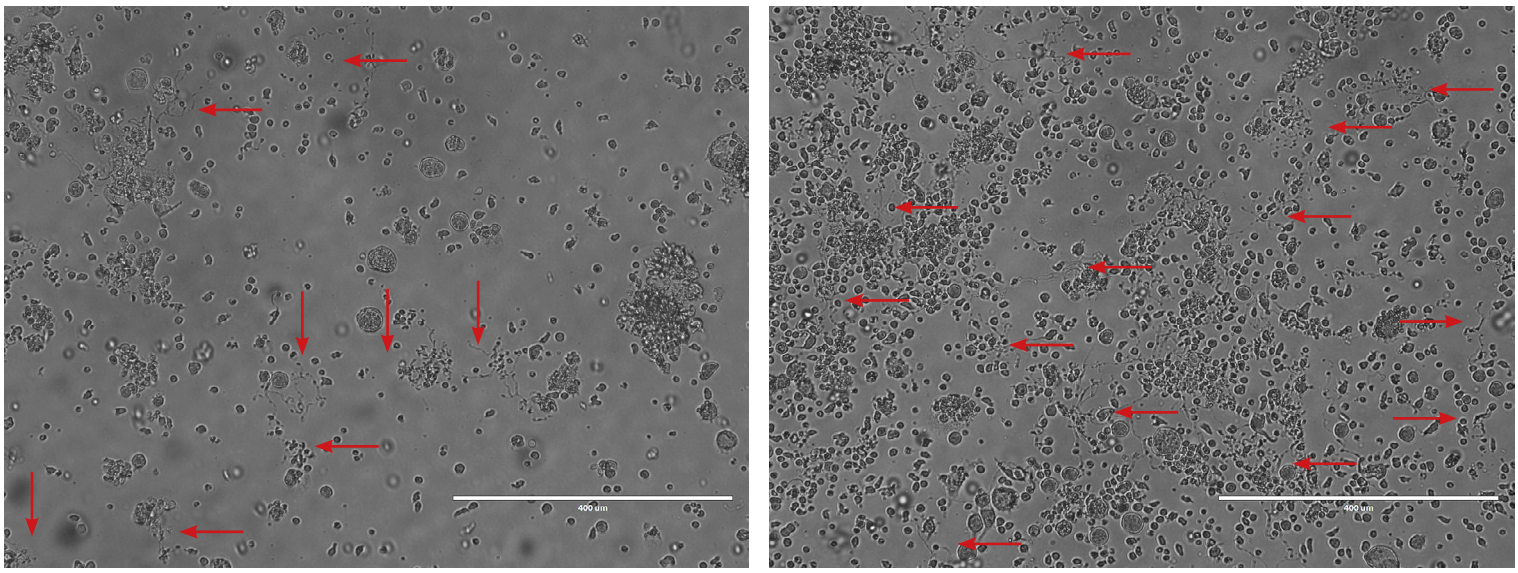
Supplementary Figure S2. ZMYM3 binds significantly different to GFI1B^{Q287*} compared to GFI1B. Nuclear extracts of MEG-01 cells transduced with GFI1B-GFP, or GFI1B^{Q287*}-GFP were analyzed by mass spectrometry. Statistically enriched proteins are identified by a permutation-based false discovery rate (FDR)-corrected *t*-test (FDR<0.01). The volcano plot shows the difference between label free quantification (LFQ) intensities of GFI1B-GFP and GFI1B^{Q287*}-GFP pulldown (log_2-transformed fold change (FC)) on the x-axis, and the -log_{10}-transformed *p*-value on the y-axis. Dotted grey lines represent the statistical cut-offs, which were kept the same as in the other volcano plots in Figure 1.



Supplementary Figure S3. Overexpression of GFI1B in MEG-01 competition cultures. *GFI1B* RNA expression in sorted GFP positive MEG-01 cells transduced with empty vector (EV), GFI1B-flag, GFI1B^{Q287*}-flag, GFI1B^{H294fs}-IRES-GFP, GFI1B^{P2A}-flag, GFI1B$^{P2A+Q287*}$-flag, GFI1B^{K8A}-flag, or GFI1B$^{K8A+Q287*}$-flag at day 23 after transduction. *GFI1B* expression is normalized to *GAPDH* and endogenous *GFI1B* expression in empty vector (EV). Error bars represent mean ± standard deviation (n=3-11).

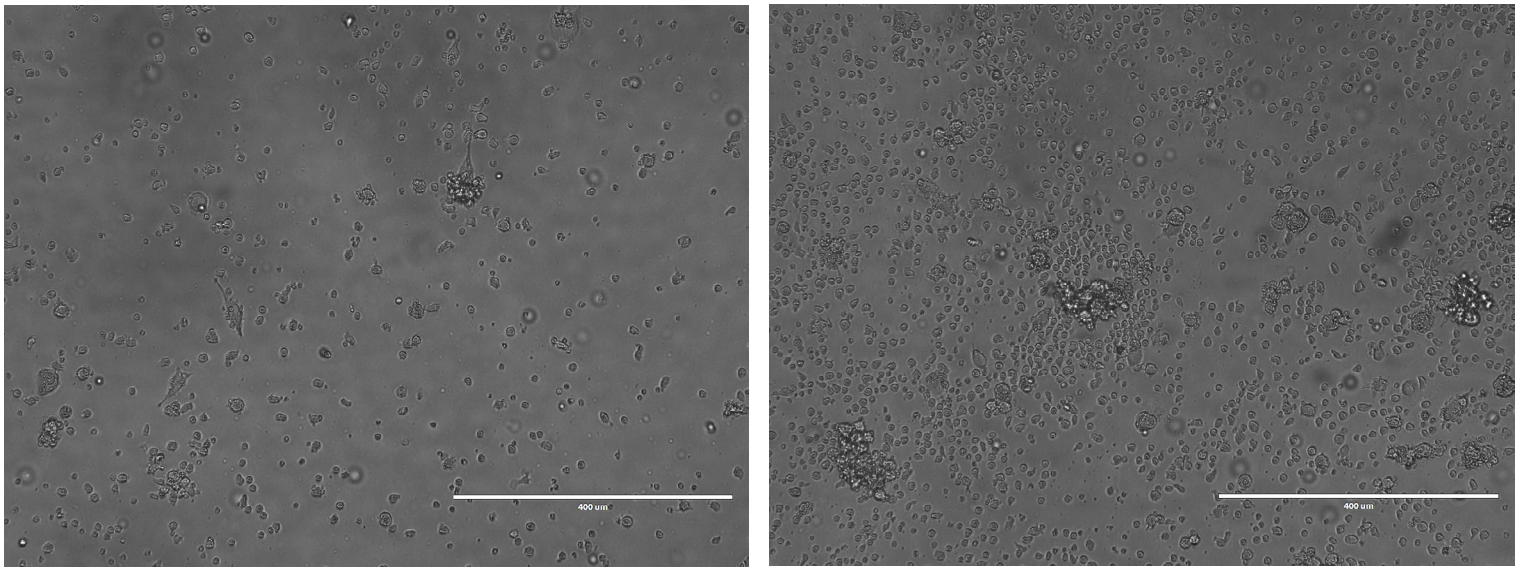
A

Control

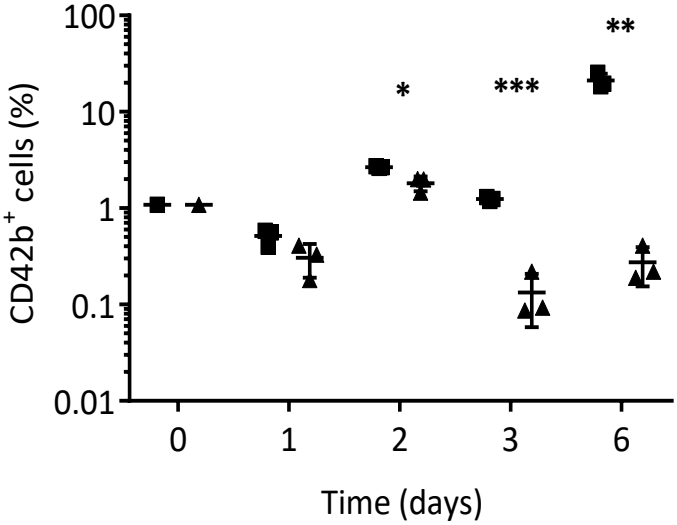


B

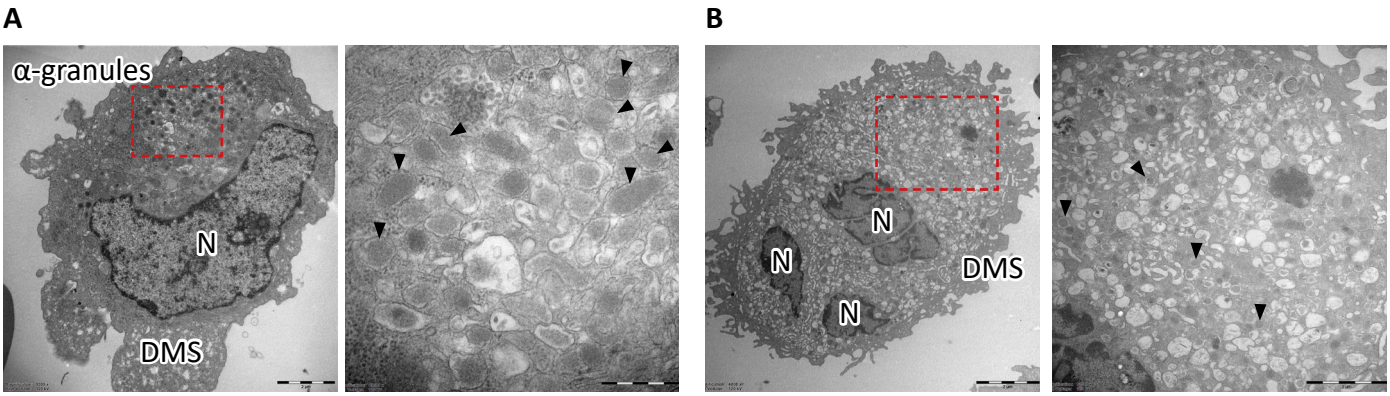
GSK-LSD1



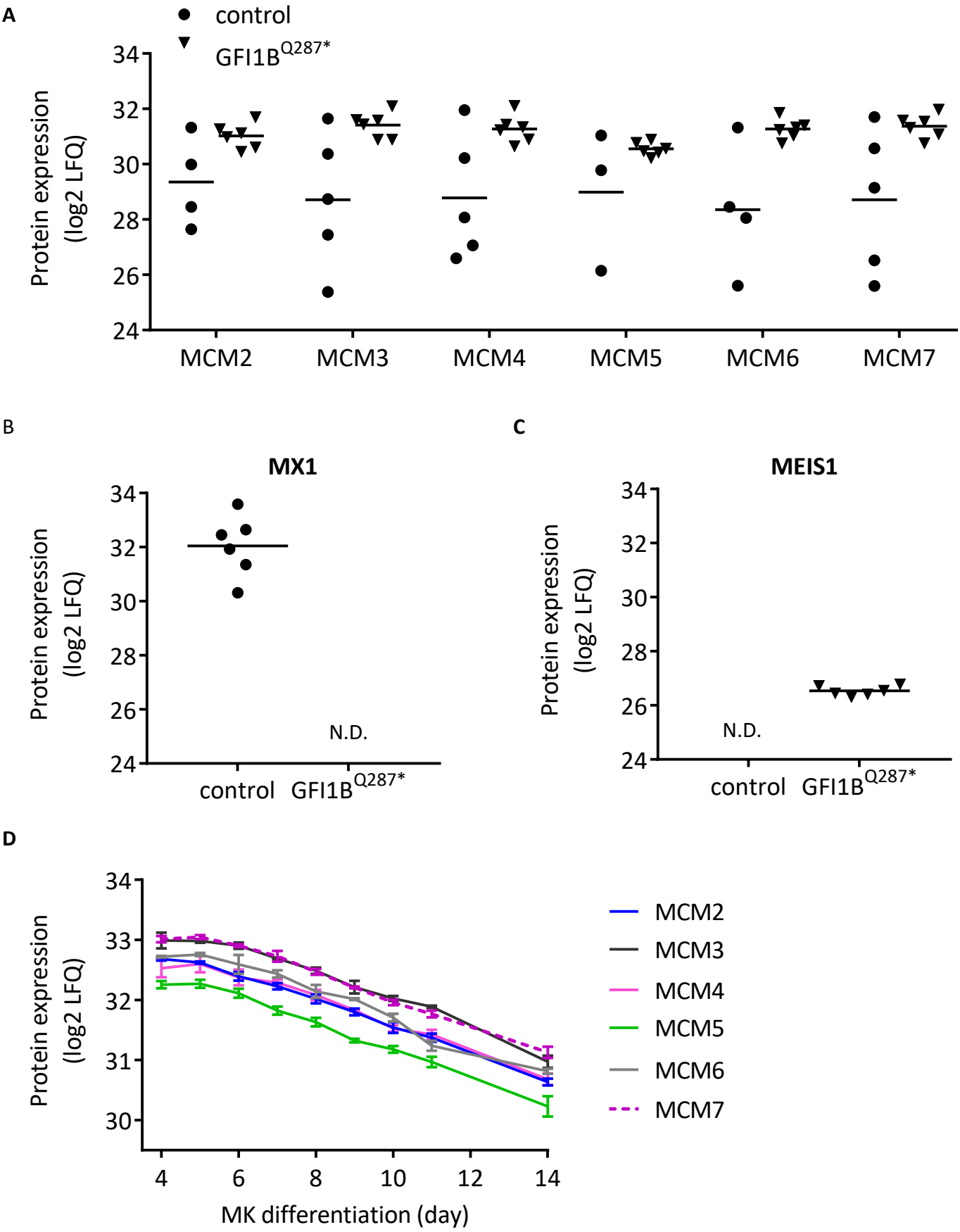
C



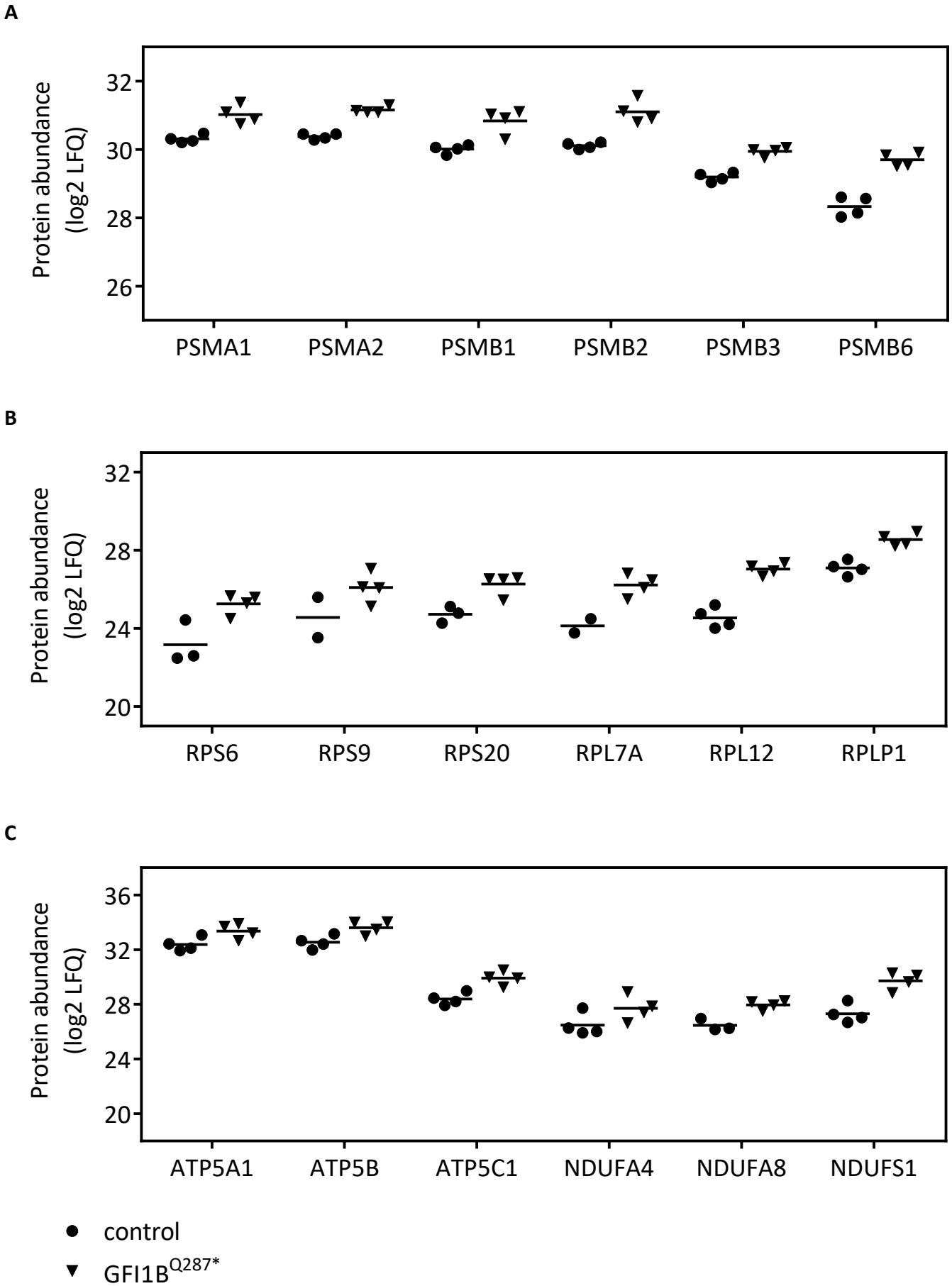
Supplementary Figure S4. LSD1 is essential for proplatelet formation. CD34$^+$ cells were differentiated towards megakaryocytes and treated with GSK-LSD1 for 6 days. On day 6 after addition of GSK-LSD1 photos were taken of (A) DMSO and (B) GSK-LSD1 megakaryocyte cultures. Some of the proplatet forming megakaryocytes are indicated with red arrows. The scale bars represent 400 µm. (C) During GSK-LSD1 treatment the percentage of CD42b cell surface expression was monitored by flow cytometry (n=3, control ■, GSK-LSD1 ▲).



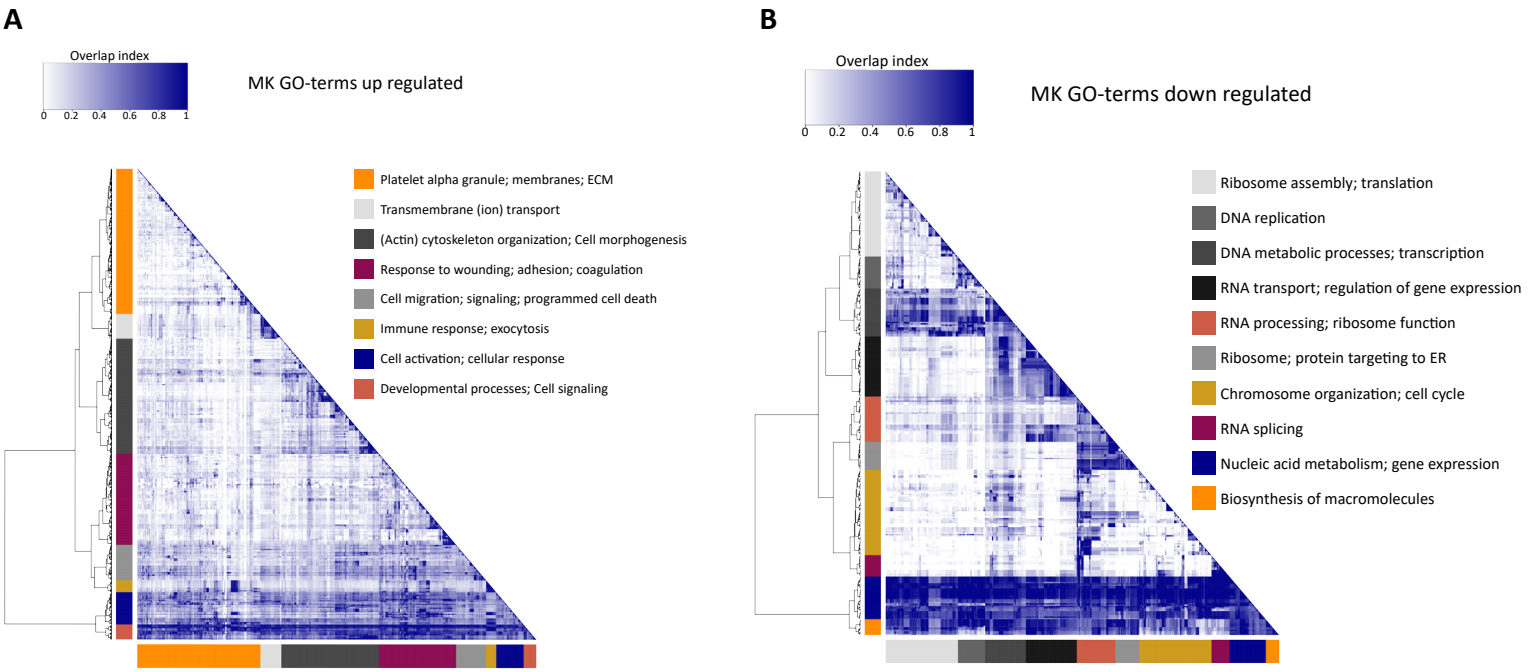
Supplementary Figure S5. Granule formation in iPSC derived megakaryocytic cells. (A) Control iPSC derived megakaryocytic cells have characteristics of a mature megakaryocyte such as the demarcation membrane system (DMS) on the outside of the cell and an intermediary zone close to the nucleus rich of organelles including α-granules . (B) In the GFI1B^{Q287*} iPSC derived megakaryocytic cells the DMS is more pronounced and there are also some α-granules present. Some of the α-granules are indicated with black arrows. The scale bars represent 2 µm except for the zoomed in picture of (A), here it represents 500 nm.



Supplementary Figure S6. MCM complex proteins are elevated in GFI1B^{Q287*} iPSC derived megakaryocytic cells. Relative protein levels (log_2 LFQ values) of (A) the minichromosome maintenance (MCM) complex subunits 2-7, (B) MX1, and (C) MEIS1 in control and GFI1B^{Q287*} iPSC-derived megakaryocytic cells. In case the protein was not detected in one or more of the samples, fewer than six points are shown. N.D. indicates the protein was not detected at all in a sample group. (D) Expression patterns of the MCM proteins during the differentiation of cultured primary megakaryocytes.



Supplementary Figure S7. GFI1B^{Q287*} platelets show elevated levels of proteasomal, ribosomal, and mitochondrial proteins. Relative protein levels (log_2 LFQ values) are shown for the indicated proteins of (A) proteasomal, (B) ribosomal, and (C) mitochondrial complexes in the platelets of control individuals and GFI1B^{Q287*} patients.



Supplementary Figure S8. Full GO term enrichment analysis of the significantly up-regulated and down-regulated proteins during megakaryocyte differentiation. GO terms associated with the up-regulated proteins (A) and down-regulated proteins (B) during megakaryocyte differentiation are presented. For full list of GO terms and clustering see Supplementary Table 2.

Supplementary Tables, see excel files

Supplementary table 1: List of proteins detected in iPSC megakaryocytic cells

Supplementary table 2: GO-terms for significant proteins in iPSC megakaryocytic cells, platelets, and megakaryocyte differentiation

Supplementary table 3: List of proteins detected in platelets

Supplementary table 4: List of proteins detected in megakaryocyte differentiation