Sorting nexin 24 is required for α-granule biogenesis and cargo delivery in megakaryocytes

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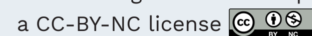
Haematologica material is published under a CC-BY-NC license 

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Supplementary Methods

Preparation of platelet RNA

Platelet RNA was prepared as described previously.[1] Briefly, peripheral blood samples were centrifuged (200g, 20 min) before carefully removing the platelet rich plasma (PRP). Red blood cells and leukocytes were removed by an additional centrifugation (200g, 10 min) step. The supernatant was collected and platelets harvested by centrifugation (800g, 10 min). Platelet pellets were dissolved in 1mL TRIzol (Thermo Scientific) per 100mg of platelets. Platelet RNA was isolated using TRIzol and then further purified using an RNeasy MinElute Cleanup Kit (Qiagen).

Human pluripotent stem cell culture

The Gibco episomal hiPSC line was purchased from Thermo Scientific and cultivated feeder-free in Geltrex coated flasks. Briefly, Geltrex was diluted 1:100 in DMEM F12 and incubated at 37°C for 1h prior to plating cells. hiPSCs were maintained in StemFlex medium (Thermo Scientific). Routine clump passaging was performed every 4-5 days using 0.5mM EDTA following manufacturer's instructions (Sigma). TrypLE was used for single cell seeding during transfection or single cell cloning following manufacturer's instructions (Thermo Scientific).

iPSC differentiation to megakaryocytes

Mature megakaryocytes and proplatelet forming megakaryocytes were derived from hiPSCs.[2,3] Briefly, iPSCs were clump passaged onto Collagen Type IV (Advanced Biomatrix) coated dishes (5µg/cm^2) in Stemflex medium containing RevitaCell (Life Technologies). For phase one of the differentiation (Day 1-6), APELII medium (Stem Cell Technologies) was supplemented with 50ng/mL BMP-4 (ThermoScientific), 50ng/mL FGF-β (Stem Cell Technologies) and 50ng/mL VEGF (Stem Cell Technologies). For phase two of the differentiation (Day 6-12), cells were incubated in APELII containing 25ng/mL TPO (Stem Cell Technologies), 25ng/mL SCF (Stem Cell Technologies), 25ng/mL Flt-3 (Stem Cell Technologies), 10ng/mL Interleukin-3 (Stem Cell Technologies), 10 ng/mL Interleukin-6 (Stem Cell Technologies) and 5U/mL Heparin (Stem Cell Technologies). During Day 6-12, cells were collected by centrifugation at 400 g for 5 min and frozen in 10% FBS/DMSO. For terminal differentiation, all frozen cells were thawed and plated onto low attachment dishes (Corning) containing StemSpanII (Stem Cell Technologies) supplemented with Megakaryocyte Expansion supplement (Stem Cell Technologies) and 5U/mL Heparin.

CRISPR-Cas9 mediated generation of SNX24 KO cell line

The SNX24 KO cell line was generated using the Alt-R RNP system (IDT). SNX24 crRNA (2nmol) was resuspended in 1x TE pH7.4 at a concentration of 100μM. Atto-555 labelled tracrRNAs (5nmol) were resuspended in 1x TE pH7.4 at a concentration of 100μM. 4.4μL of crRNA and 4.4μL of tracrRNA were added to 1.2 μL of Nuclease Free Duplex Buffer. The mixture was incubated at 95°C for 5 min before cooling at -1°C/sec to 25°C. 1μL of crRNA/tracrRNA mix was then added to 0.6 μL of HiFi Cas9 V3 (IDT; Cat # 1081058) at a ratio of 42pmol:37pmol. The samples were incubated for 20 min at room temperature to form stable RNP complexes. Lipofectamine Stem (Life Technologies) was used to transfect RNP complexes into iPSCs, following the manufacturer's instructions for Stemflex conditions.

Single cell cloning

For single cell cloning, Stemflex medium was supplemented with CloneR (Stemcell Technologies) and the manufacturer's instructions were followed. Briefly, 96-well plates were coated with geltrex and the single cell suspension harvested using TrypLE (Thermo Scientific). Cells were sorted on a BD FACSMelody Cell Sorter directly into 96-well plates. Immediately after sorting, the plates were centrifuged at 1000 rpm for 1 min to aid attachment of the cells. Colonies were allowed to develop for 10-14 days before passaging those wells containing iPSC colonies.

Analysis of genome editing in CRISPR generated clones

Genomic DNA was isolated after single cell cloning using QuickExtract DNA Extraction Solution (Lucigen). Oligonucleotide primers were designed to amplify the CRISPR targeted region of *SNX24* and incorporated EcoRI and SalI sites to facilitate cloning. The Alt-R Genome Editing Detection kit (IDT) was used according to the manufacturer's instructions to screen all single cell clones for *SNX24* editing. PCR products with mismatches indicating editing were cloned between the EcoRI and SalI sites of YCplac33 and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen). Plasmid DNA was sequenced to obtain sequences for both alleles (Source Bioscience).

Qualitative Real Time PCR (qPCR)

RNA was extracted using the RNA Purification Plus kit (Geneflow) and reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). PCRs were set up in triplicate, with each 10μl sample containing 1X TaqMan Fast Universal Master Mix (ThermoFisher), 100nM of the forward and reverse primers (Supplementary

Table 1), 100nM of the appropriate probe from the Universal Probe Library (Roche) and 10ng of cDNA (Applied Biosystems). Each UPL probe is indicated in the supplementary primer table. Using an ABI 7900 HT analyser (Applied Biosystems) samples were heated to 50°C for 2 min and denatured at 95°C for 10 min, before being subjected to 40 cycles of 95°C for 15s and 60°C for 1 min. The Ct values obtained were analysed using the delta-delta Ct method and normalised using either *GAPDH* or *ACTB* as housekeeping genes.

Immunofluorescence

Cells were fixed in a solution of 3.7% paraformaldehyde for 15 min at room temperature, and then permeabilised with 0.2% Triton-X in PBS for 15 min at room temperature. Non-specific binding was blocked by incubation for 1 hour in 1% bovine serum albumin (BSA). Cells were then incubated with primary antibodies at a dilution of 1:200, in PBS supplemented with 1% BSA and 0.1% Tween20, either for 1h at room temperature or overnight at 4°C. The following primary antibodies were used in this study: CD62P (Fisher Scientific), CD42/GPIb (Universal Biologicals), CD41 (Thermo Fisher), VWF (Thermo Fisher), Rab5a (CellSignalling), EEA1 (CellSignalling), Rab7a (CellSignalling), SNX24 (Thermo Fisher). Following three washes with PBS, cells were incubated with fluorescent-dye conjugated secondary antibodies diluted 1:200 in PBS supplemented with 1% BSA and 0.1% Tween20. The following secondary antibodies were used in this study: anti-Mouse Alexa fluor 488, anti-Mouse Alexa fluor 568, anti-Rabbit Alexa fluor 488, anti-Rabbit Alexa fluor 594 (Invitrogen). Nuclei were stained by incubating cells with 10µg/ml Hoechst 33342 for 1 hour at room temperature before washing three times with PBS and imaging. Cells that were prepared for confocal imaging were grown on glass coverslips and mounted onto slides with one drop of Vectashield Mounting Medium (Vector Laboratories).

Fluorescence Microscopy

Images were acquired using a Zeiss A1 confocal microscope with a 63x oil immersion lens, and a Hammamatsu camera. LED power and exposure time were the same within each experiment across different samples. Using NIS-Elements software, five images were taken per sample as representative Z-stacks, and presented as a maximum intensity projection. ND2 files containing z-stacks were processed using FIJI ImageJ software. Equal numbers of 0.15µm slices were used to compare wild-type and SNX24 KO cells.

Electron microscopy of platelets

Platelets were fixed and embedded into TAAB EMIX medium grade epoxy resin and 85nm sections stained in saturated uranyl acetate in 99% ethanol, and Reynold's lead citrate. Sections were viewed on a Philips 400 transmission electron microscope.

Electron microscopy of megakaryocytes

Megakaryocytes were fixed in 2.5% Glutaraldehyde/0.1M Sodium Cacodylate buffer, postfixed in 2% aqueous Osmium Tetroxide, dehydrated through a graded series of ethanol, cleared in epoxypropane (EPP) and then infiltrated in a 50:50 mixture of Araldite resin and EPP overnight with rotation. This mixture was replaced twice over 8 hours with fresh Araldite resin mixture before being embedded and cured in a 60°C oven for 48-72 hours. Ultrathin sections, approximately 85nm thick, were cut using a Leica UC6 ultramicrotome onto 200 mesh copper grids, stained with saturated aqueous Uranyl Acetate followed by Reynold's Lead Citrate and examined using an FEI Tecnai Transmission Electron Microscope at an accelerating voltage of 80Kv. Electron micrographs were recorded using a Gatan Orius 1000B digital camera and Gatan Digital Micrograph software.

Karyotyping

Karyotyping by G-banding was performed by the Sheffield Diagnostic Genetics Service (https://www.sheffieldchildrens.nhs.uk/sdgs/). Cells were treated with 0.1µg/ml KaryoMAX Colcemid Solution in PBS (Life Technologies) for 2 - 4h to arrest cells in metaphase. Cells were harvested and resuspended in 0.0375M KCl. Cells were incubated for 10 min in KCl, collected by centrifugation and fixed with methanol:acetic acid. Metaphase cells were prepared on glass microscope slides and stained with 4:1 Gurr's/Leishmann's stain (Sigma-Aldrich). Banded metaphases were imaged on the Leica Biosystems Cytovision Image Analysis system. At least 20 metaphases were analysed.

References

1. Amisten S. A rapid and efficient platelet purification protocol for platelet gene expression studies. Methods Mol Biol. 2012;788:155-72.
2. Feng Q, Shabrani N, Thon JN, et al. Scalable generation of universal platelets from human induced pluripotent stem cells. Stem Cell Reports. 2014;3(5):817-31.
3. Khan AO, Slater A, Maclachlan A, et al. Post-translational polymodification of β1-tubulin regulates motor protein localisation in platelet production and function. Haematologica. 2020;Online ahead of print. doi: 10.3324/haematol.2020.270793. PMID: 33327716.

Supplementary Tables.

Table S1: Primer and probe sequences.

Name	UPL probe number	Sequence
SNX24 crRNA		tctcttcatagcgaaaggac
SNX24 F cloning		atatatgaattcagcccgcagacctgagtc
SNX24 R cloning		atatatgtcgacctgaactaattgtacagaacttg
SNX24 F	1	cgtcctttcgctatgaagaga
SNX24 R	1	ttcttccattcattagcacttctatc
OCT4 F	78	gtggagagcaactccgatg
OCT4 R	78	tgcagagctttgatgtcctg
CD34 F	78	gtgaaattgactcagggcatc
CD34 R	78	cccctgtccttcttaaactcc
CD41a F	60	gagacacccatgtgcagga
CD41a R	60	agctggggcacacatacg
CD42b F	68	ccttcggaggtctttctgc
CD42b R	68	atggggtgggggtgtaag
SELP F	78	acctgccatttctcttgtgac
SELP R	78	cccctggagtaggaagtgatg
GAPDH F	60	agccacatcgctcagacac
GAPDH R	60	gcccaatacgaccaaatcc
CD61 F	19	ccatcatgcaggctacagtc
CD61 R	19	aaacaccagcaagtgggatg
VWF F	68	ttccaggactgcaacaagc
VWF R	68	agcaatggtgtcgcagaag
ACTB F	38	tcgtgcgtgacattaaggag
ACTB R	38	caggcagctcgtagctcttc

EEA1 F	2	gctgaagaatcagtcagaaagtca
EEA1 R	2	ccttctgctcttgtacctggtc
NBEAL2 F	1	tgctaccactggatgagctg
NBEAL2 R	1	aggttcctgcagagaatgatg
Rab5a F	38	ccaaccaggaatcagtgttgt
Rab5a R	38	actgggctggttaaatgctc
Rab7a F	18	gacaggctagtcacaatgcaga
Rab7a R	18	gtagaaggccacaccgagag
FLI1 F	1	agatccagctgtggcaattc
FLI1 R	1	gtgatacagctggcgttgg

Tables S2 to S9: Transcriptome data and annotation cluster analysis

The transcriptome data and results of annotation cluster analysis are provided as a series of tables in a separate excel file. The first sheet in the excel file provides a key to the eight tables, which correspond to sheets 2 to 9 of the file as shown below.

Table S2: Expression of transcripts showing significant upregulation in FLI1-deficient platelets.

Table S3: Expression of transcripts showing significant downregulation in FLI1-deficient platelets.

Table S4: Expression of coding transcripts showing significant downregulation in FLI1-deficient platelets.

Table S5: Expression of coding transcripts showing significant upregulation in FLI1-deficient platelets.

Table S6: Differentially expressed coding transcripts identified by Ensembl name.

Table S7: Results of DAVID analysis showing the four annotation clusters having the highest enrichment scores.

Table S8: Genes associated with GO terms in the four annotation clusters having the highest enrichment scores.

Table S9: Names, descriptions and expression levels of genes associated with GO terms in the four annotation clusters having the highest enrichment scores.

Supplementary Figures

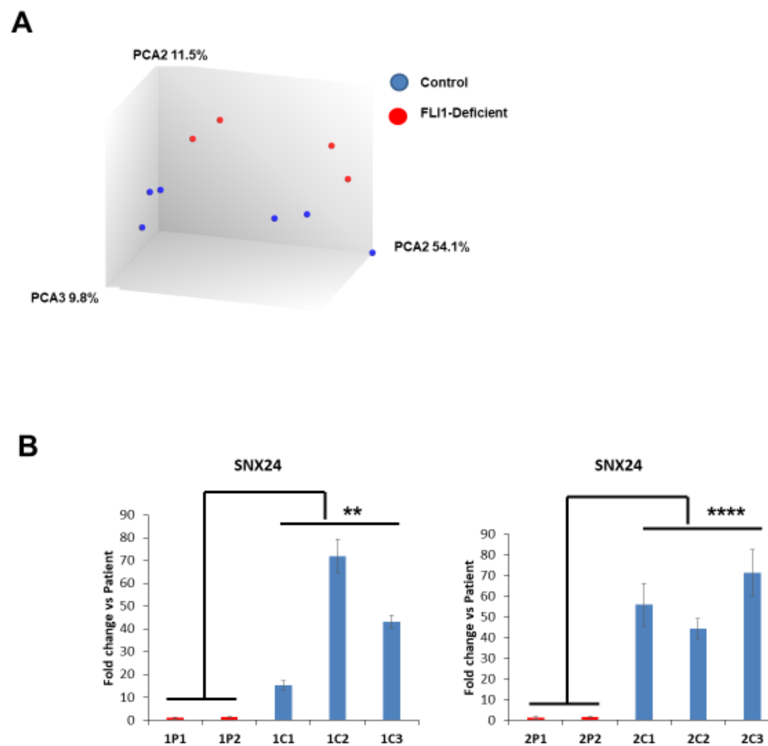

Figure S1: Transcriptome analysis of FLI1-deficient platelets

A. Principal component analysis of FLI1-deficient (red) and control (blue) samples used for microarray analysis.

B. RT-qPCR analysis of *SNX24* expression using independent primers in control and FLI1-deficient platelets. n=3 experiments. Samples were analysed in parallel on two occasions. P<0.0001 = **** and p<0.01 = **, Student's t-test.

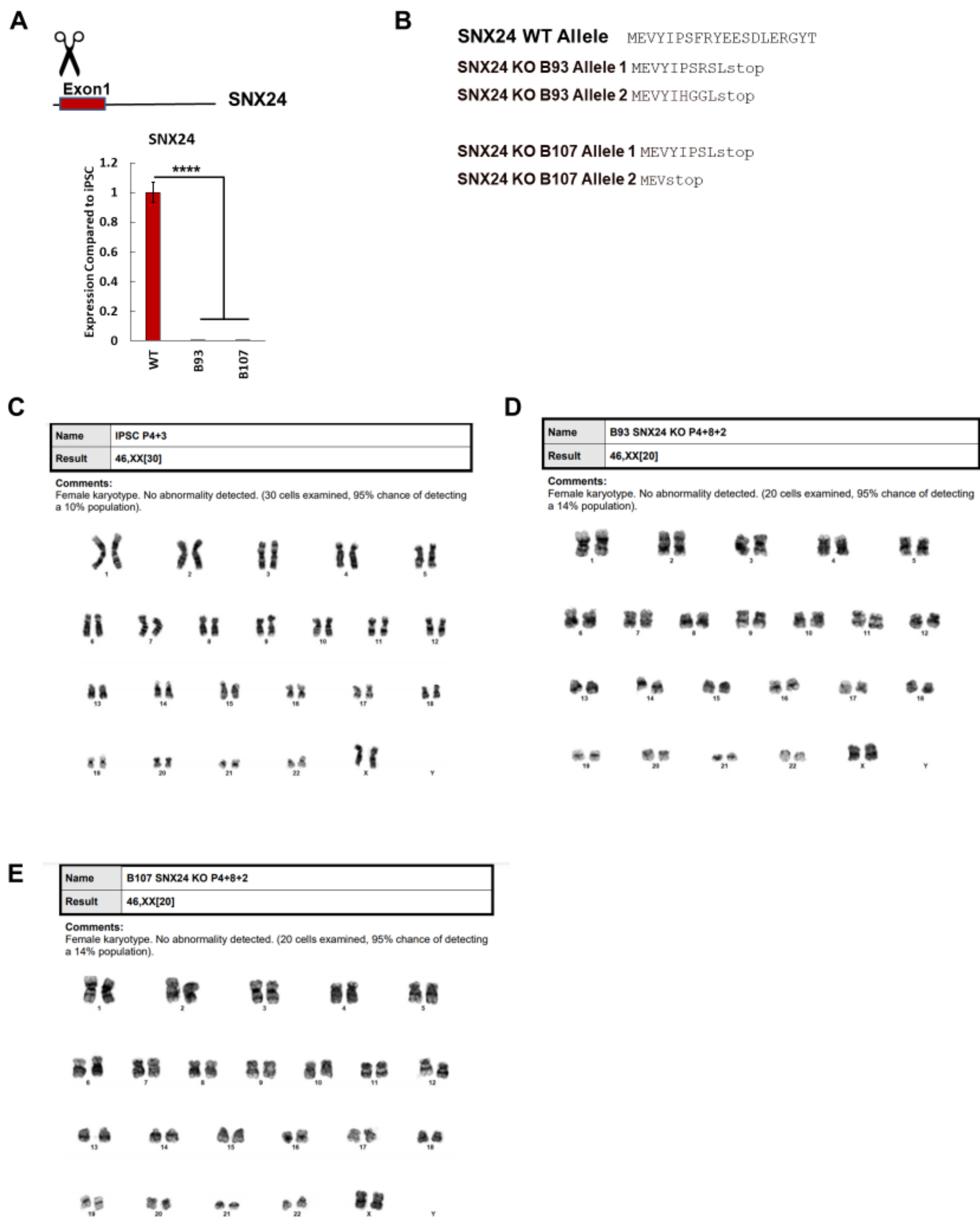

Figure S2: Generation of SNX24 KO cell line

A. CRISPR gene editing of *SNX24* in iPSCs and qPCR analysis of *SXN24* expression in wild-type (WT) and SNX24 KO clones. n=3 experiments. P<0.0001 = ****, Student's t-test
B. Multiple sequence alignment of SNX24 KO clones showing amino acid sequence predicted by both alleles.
C. Karyotype report for wild-type iPSC cells showing a normal karyotype.
D. Karyotype report for SNX24 KO clone B93 showing a normal karyotype.
E. Karyotype report for SNX24 KO clone B107 showing a normal karyotype.

Supplementary Figure S3

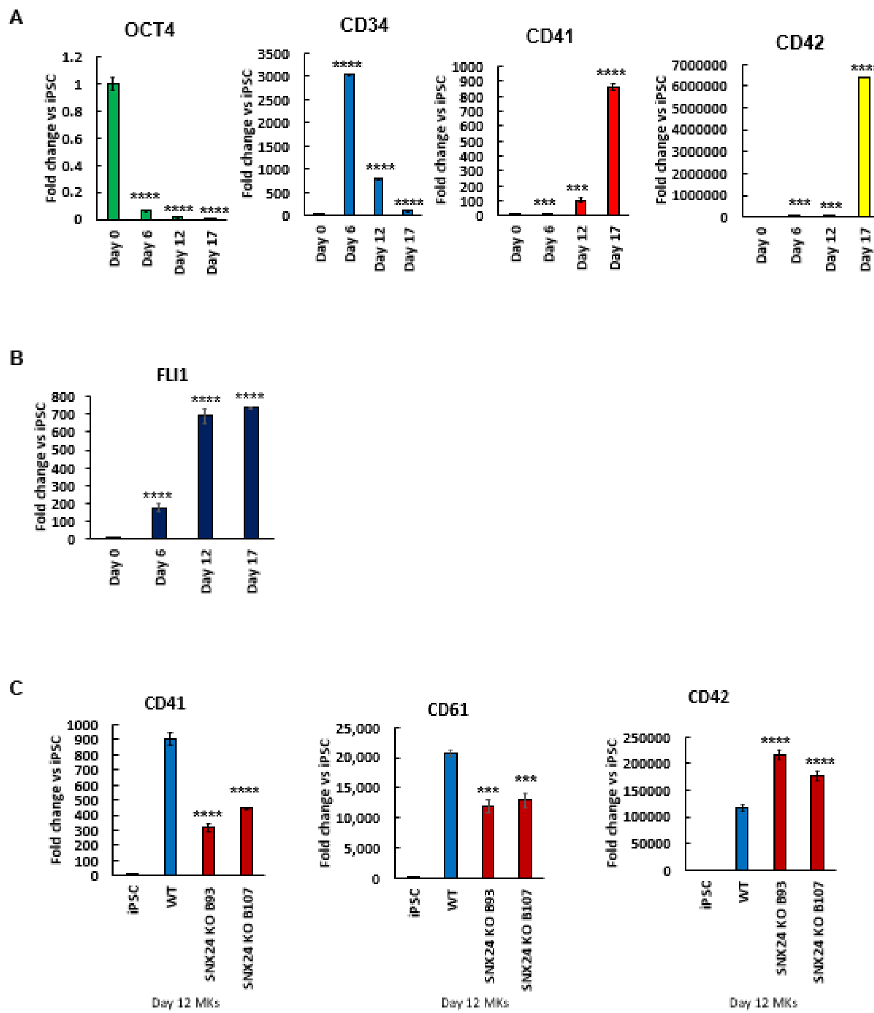

Figure S3: Characterisation of SNX24 KO Megakaryocytes

A. qPCR gene expression analysis of OCT4, CD34, CD41 and CD42 from Day0-Day17 of iPSC differentiation. $P<0.0001$ = **** and $P<0.001$ = ***, Student's t test, n=3.
B. qPCR analysis of *FLI1* expression during iPSC differentiation to megakaryocytes. $P<0.0001$ = ****, Student's t test, n=2.
C. qPCR gene expression analysis of CD41, CD61 and CD42 in WT and SNX24 KO clones (B93 and B107) on Day12 of differentiation $P<0.0001$ **** and $P<0.001$ = ***, Student's t test, n=2.

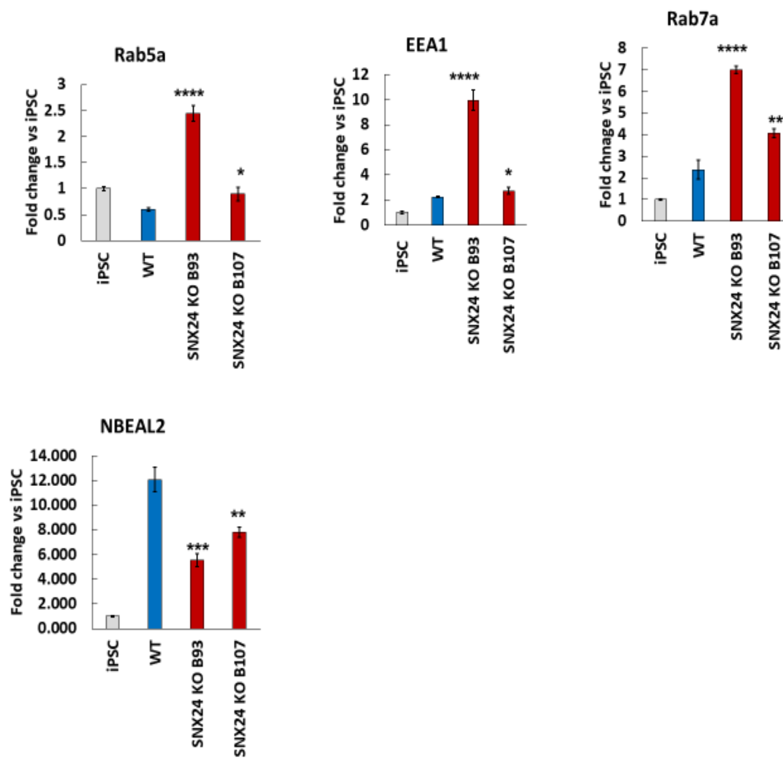

Figure S4: Disruption to endosomal intermediates in SNX24 KO cells

qPCR analysis of EEA1, Rab5a, Rab7a and NBEAL2 expression in Day12 wild-type (WT) and SNX24 KO megakaryocytes. $p<0.0001$ = ****, $p<0.001$ = ***, $p<0.01$ = **, $p<0.05$ = *, Student's t test, n=2