Supplemental Information for "An IGF2BP3-Cdk9 Pathway Governs the Human Fetal-Adult Megakaryocyte Transition" by Elagib et al.

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Table S1 *Elagib et al.,*

Table S1. Mkl1 expression in megakaryocytes in human marrow samples.

Case #	Patient Age	Transplant (Y/N), Source*	Patient History**	Mkl1 expression***
1	69 years	YES, umbilical cord blood	MDS/AML	Negative
2	27 years	Yes, umbilical cord blood	NHL	Negative
3	**34 years**	**Yes, umbilical cord blood**	**AML**	**1+**
4	**39 years**	**Yes, adult HSPC**	**AML**	**2-3+**
5	**63 years**	**Yes, adult HSPC**	**MDS**	**2-3+**
6	**9 months**	**No**	**Neuroblastoma**	**1+**
7	**6 months**	**No**	**Storage disorder**	**1+**
8	9 weeks	No	Adrenal mass	Negative
9	4 weeks	No	Neuroblastoma	Negative
10	2 weeks	No	Neuroblastoma	Negative

*HSPC: hematopoietic stem and progenitor cells
**MDS: myelodysplastic syndrome; AML: acute myeloid leukemia; NHL: non-Hodgkin lymphoma
***Immunohistochemical scoring system: 1+ denotes weak signal in 10-50% megakaryocytes; 2+ denotes weak signal in >50% megakaryocytes or strong signal in 10-50% megakaryocytes; 3+ denotes strong signal in >50% megakaryocytes.

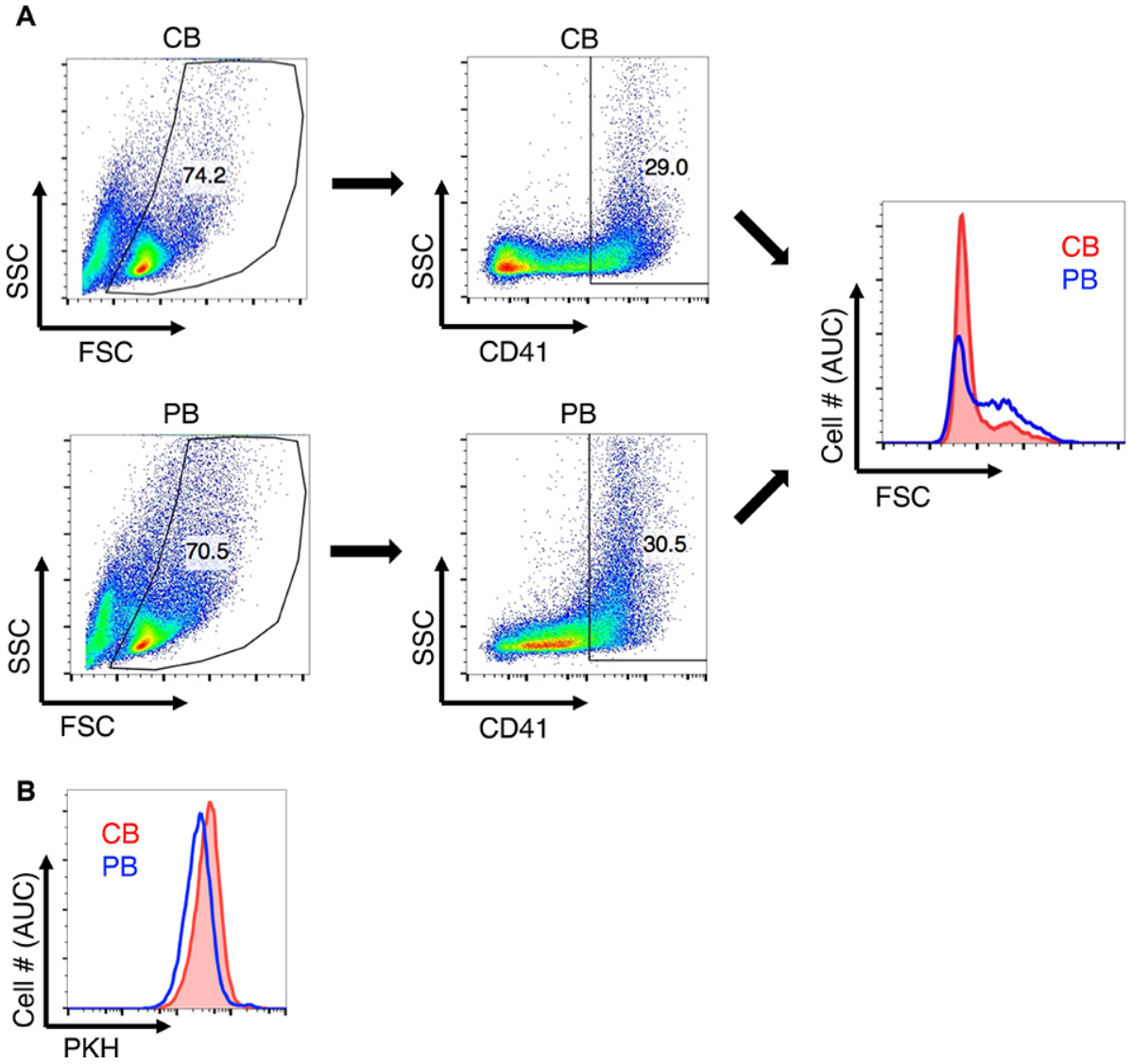

Figure S1. Flow cytometry gating strategy and comparison of initial PKH loading.
(A) Illustration of flow cytometry gating strategy. Neonatal (CB) and adult (PB) progenitors were cultured for six days in megakaryocytic medium as in Figure 1A. Populations were gated first on viable fraction based on FSC/SSC characteristics, followed by gating on CD41+ cells to be used in histogram overlay. Ploidy analysis used a similar strategy but also included a doublet discrimination step after the CD41+ gating to eliminate cell aggregates. (B) Comparison of initial PKH loading efficiency. Samples from the neonatal (CB) and adult (PB) progenitor cultures depicted in Figure 1C underwent flow cytometry four hours post PKH staining, with gating on total viable population.

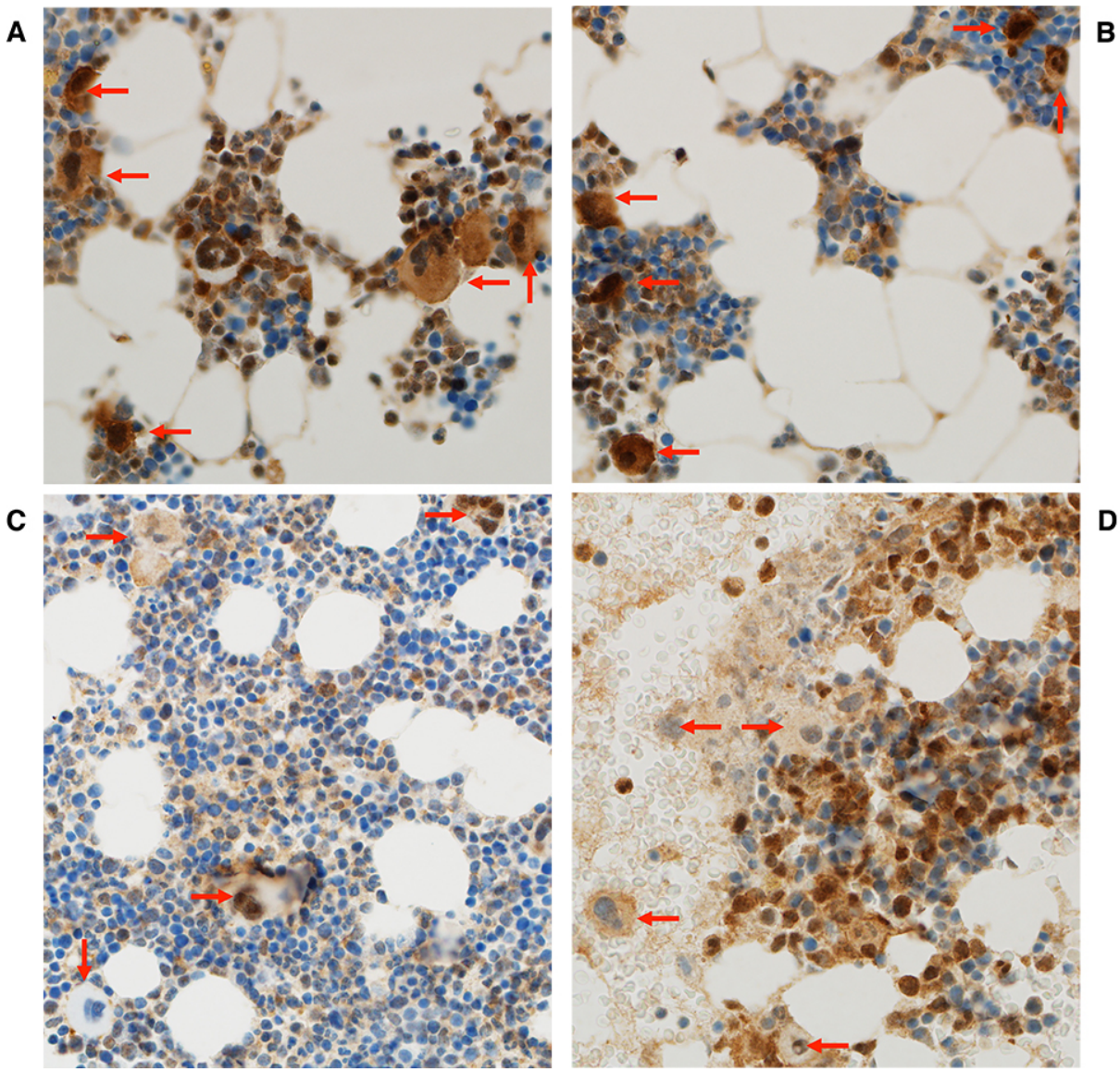

Figure S2: Expression of Mkl1 in megakaryocytes from human transplant recipients of adult and neonatal HSPC.
Immunohistochemical staining for Mkl1 on bone marrow biopsies obtained from adult transplant recipients after receiving either adult HSPC (A-B) or umbilical cord blood HSPC (C-D). Arrows denote megakaryocytes (original magnification 400X).

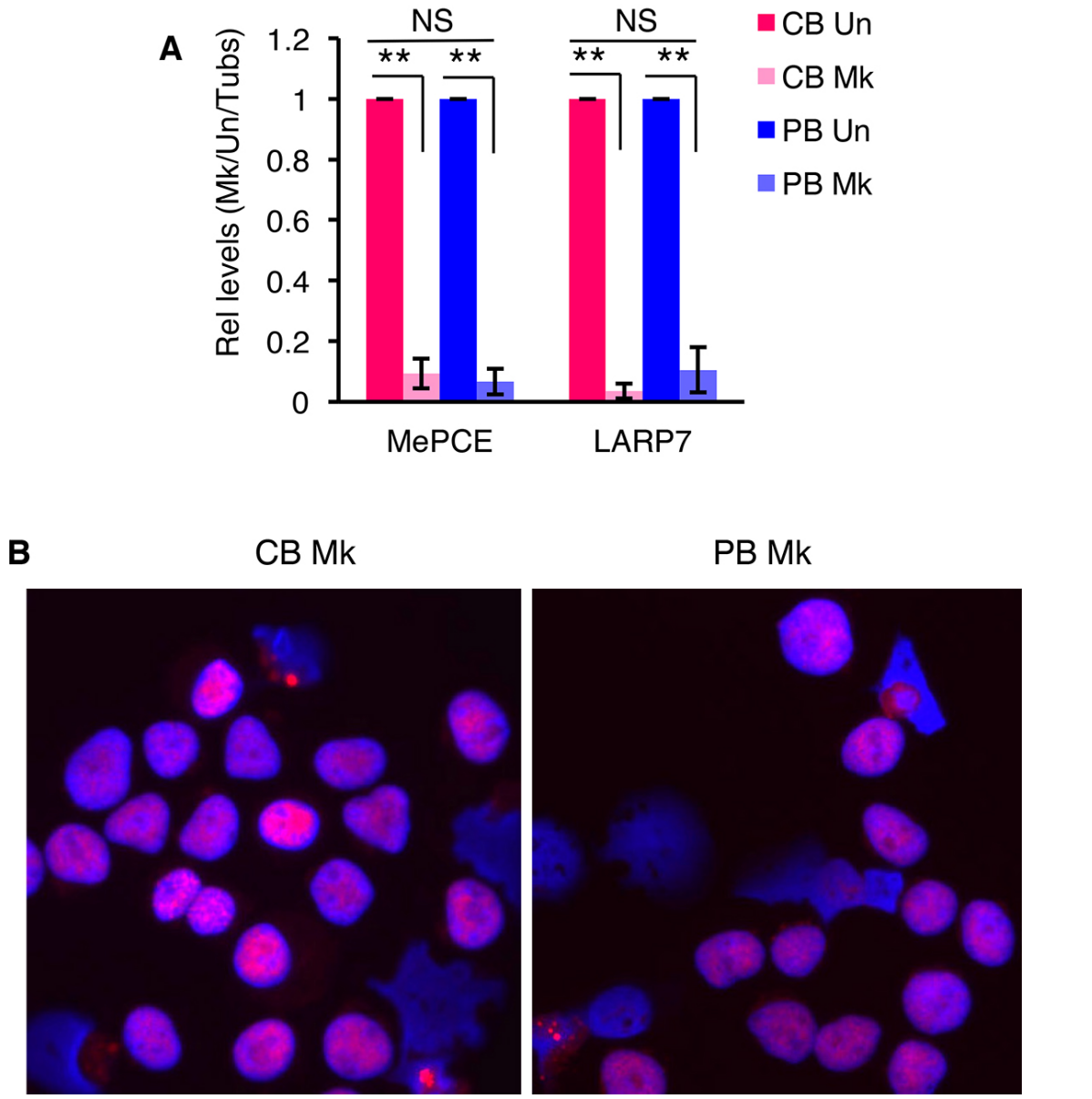

Figure S3: Neonatal megakaryocytes (CB Mk) downregulate 7SK stabilizing factors but retain high levels of 7SK.

(A) Primary human neonatal and adult progenitors, either undifferentiated or subjected to 6 days of Mk culture, underwent immunoblot of whole cell lysates. The graph represents scanning densitometry results comparing undifferentiated and Mk expression of MePCE and LARP7 in adult and neonatal progenitors. Results are from three independent experiments, of which Figure 2E in main text is an example, and represent mean $\pm$ SEM for signals normalized to tubulin, with undifferentiated levels set at 1 (two-way ANOVA; $^{**}P < 0.01$; NS: not significant). (B) In situ hybridization on neonatal and adult day 6 Mk, probing for 7SK snRNA using fluorochrome labelled oligonucleotides. Images were captured with Olympus BX40F fluorescence microscope (original magnification 400X).

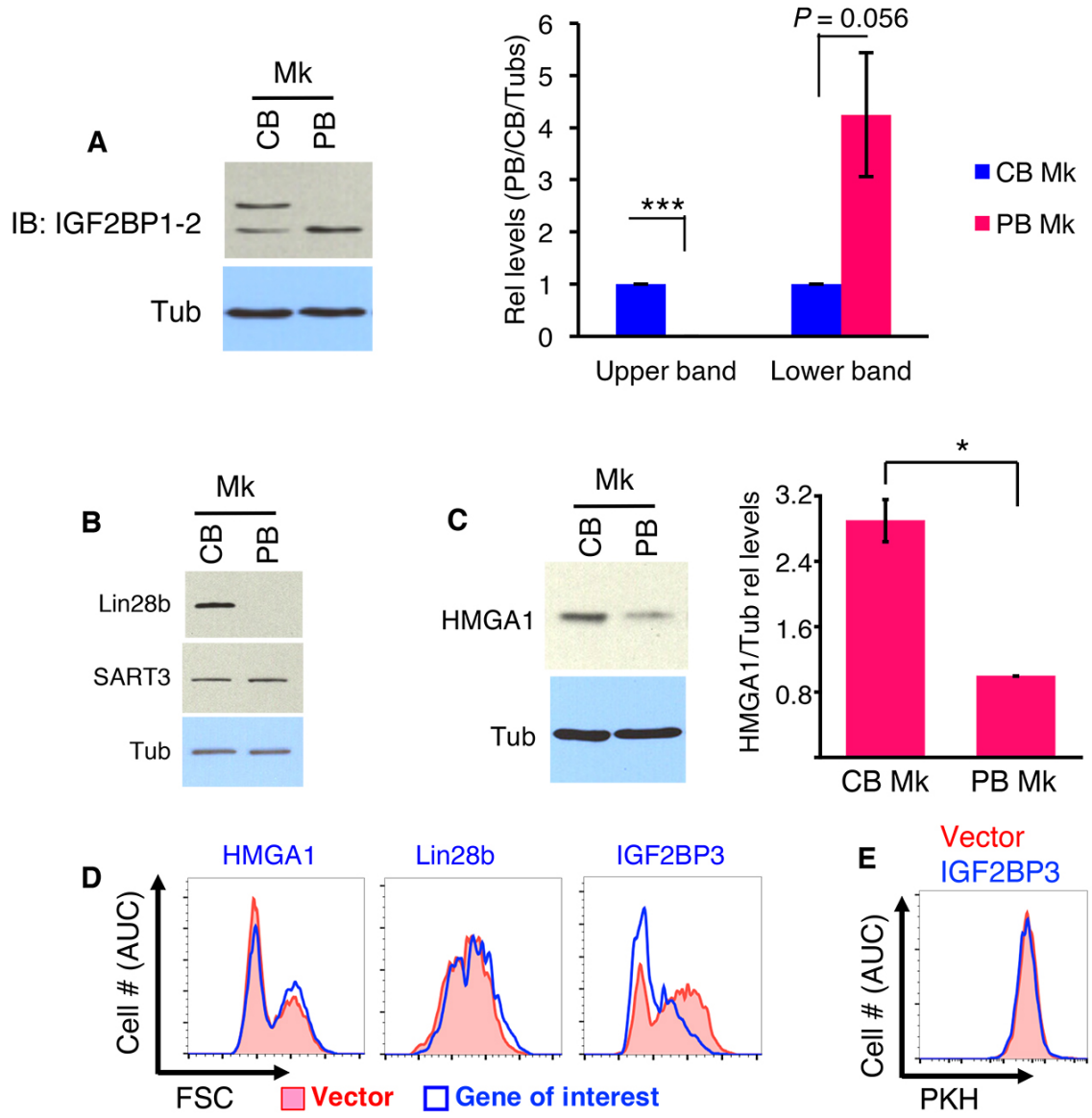

Figure S4: Expression and functional studies of candidate ontogenic regulatory factors.
(A-C) Expression of candidate factors in adult versus neonatal megakaryocytes. Primary adult and neonatal progenitors cultured for 6 days in megakaryocytic (Mk) medium underwent immunoblot for the indicated factors. Graphs show means ± SEM for scanning densitometry values derived from three independent experiments. *** $P < 0.005$. Note that the antibody used in (A) detects both IGF2BP1 and IGF2BP2. (D) Block in adult Mk enlargement by enforced expression of IGF2BP3 but not of HMGA1 or Lin28b. Adult progenitors transduced with retrovirus expressing candidate factors and GFP underwent Mk culture 6 days followed by flow cytometry with gating on viable CD41+ GFP+ cells. Results are representative of 2 experiments for HMGA1 and Lin28b and 3 for IGF2BP3. (E) Comparison of initial PKH loading efficiency. Samples from cultures of transduced adult progenitors in Figure 3C, expressing IGF2BP3 or empty vector, underwent flow cytometry four hours post PKH staining, with gating on viable GFP+ cells. Please note that panel **A** derives from the same immunoblot membrane as **Figure 2A**, **Figure 2E**, and **Figure 3A** and therefore shares the same tubulin control. Please note that panel **B** derives from the same immunoblot membrane as **Figure 2D** and shares the same tubulin control.

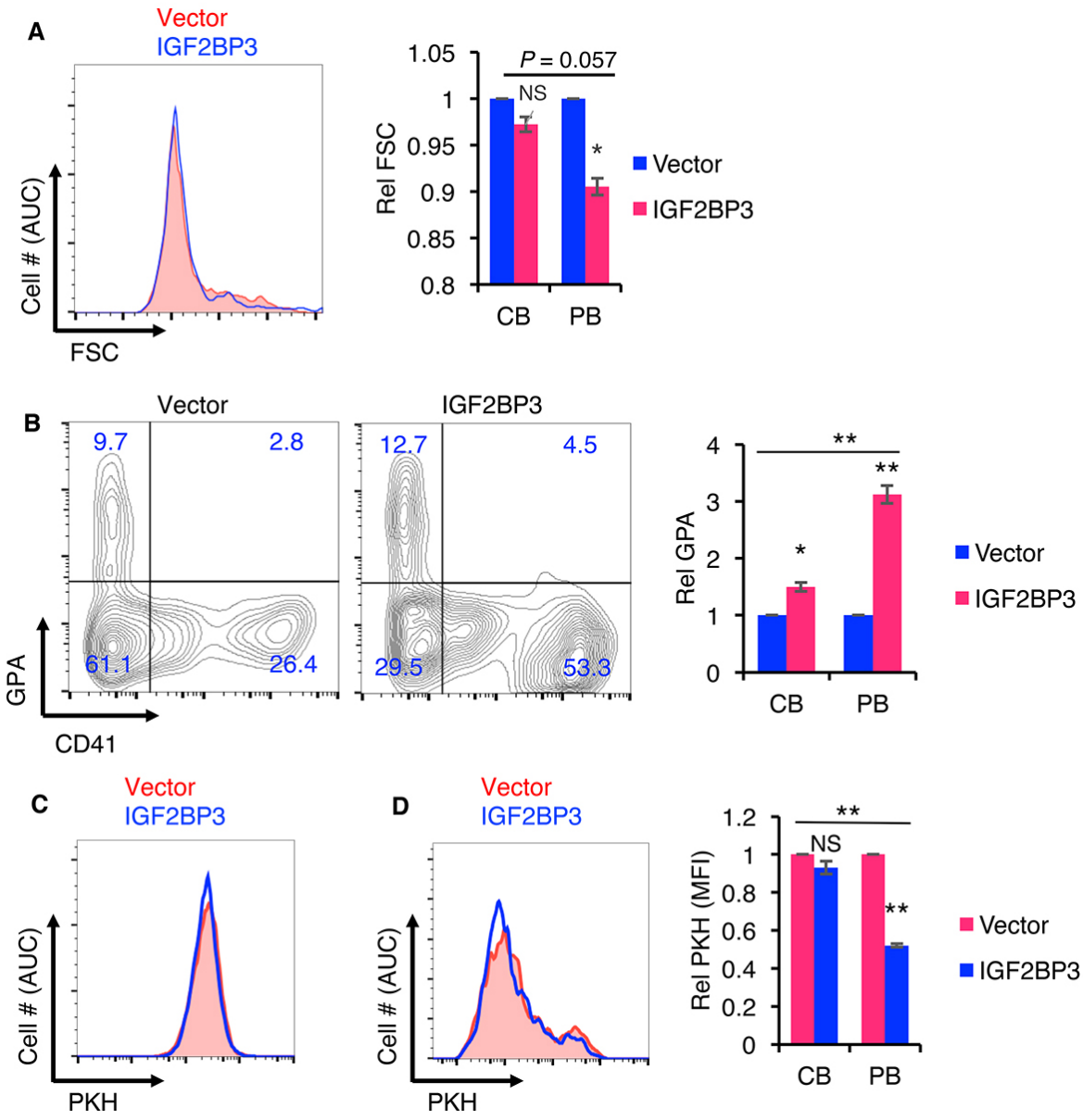

Figure S5. Minimal effect of enforced IGF2BP3 expression in neonatal (CB) as opposed to adult (PB) megakaryocytes.

Cord blood progenitors transduced with retrovirus expressing GFP -/+ IGF2BP3 underwent Mk culture for 6 days followed by flow cytometry with gating on viable CD41+ GFP+ cells as in Figure 3. (A) No significant change in neonatal Mk size as depicted by forward scatter (FSC); (B) relatively minor enhancement of GPA expression. (C-D) No significant effect on proliferation as indicated by PKH dye dilution. (C) Initial PKH loading 4 hours post staining on day 0. Graphs represent mean ± SEM for three independent experiments, comparison between the two groups employed two-way ANOVA, asterisks above the long line indicate the row factor x column factor interaction (*$P < 0.05$; **$P < 0.01$; NS: not significant).

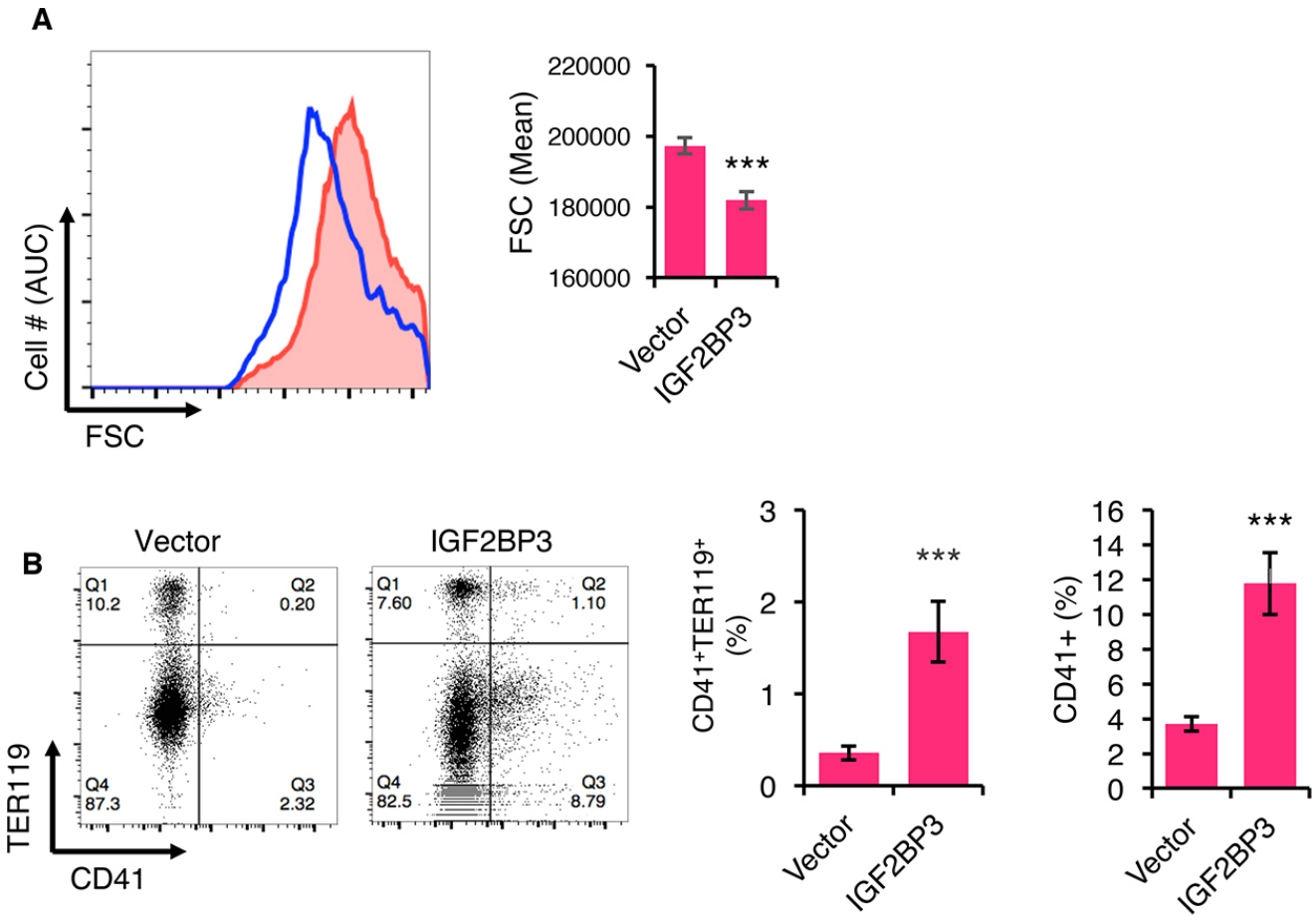

Figure S6. Enforced IGF2BP3 expression in vivo perturbs adult murine megakaryopoiesis. Adult murine marrow transduced with retrovirus expressing GFP -/+ IGF2BP3 was transplanted into lethally irradiated mice. At six weeks post transplant, recipient marrows were analyzed by flow cytometry. (A) Analysis of megakaryocyte size (FSC), with gating on viable GFP+ CD41+ cells. Graph depicts mean FSC ± SEM (n = 6, 5 for Vector and IGF2BP3 groups respectively; t test; ***$P < 0.005$). (B) Marrow expression of CD41 and TER119 with gating on viable GFP+ cells. Graphs depict means ± SEM for the percentages of CD41+ TER119+ double positive cells and of all CD41+ cells (n = 6, 5 for Vector and IGF2BP3 groups respectively; t test; ***$P <$ 0.005).

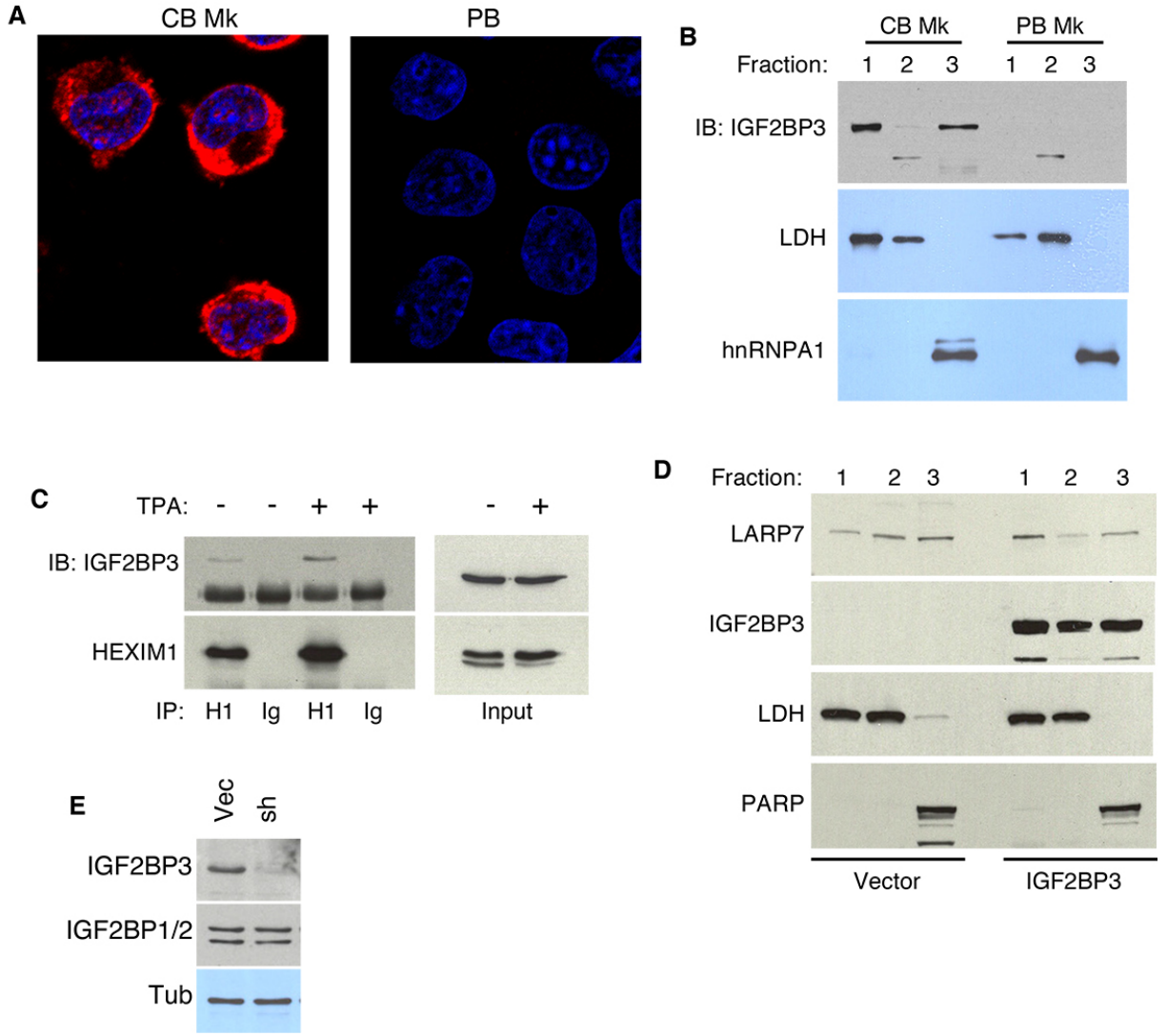

Figure S7: IGF2BP3 subcellular localization, interaction with HEXIM1, influence on LARP7 distribution, and isoform-specific shRNA knockdown.

(A-B) Nuclear and cytoplasmic localization of IGF2BP3 in neonatal megakaryocytes. Adult and neonatal progenitors cultured for 6 days in Mk medium underwent immunofluorescent staining for IGF2BP3 (red) and DAPI staining (blue) (A), or immunoblot of subcellular fractions (B). Cells were visualized by confocal microscopy at a mid-nuclear plane (630x). For immunoblot, fractions 1-2 are cytoplasmic (-/+ 0.5% NP40), and fraction 3 is nuclear. (C) Interaction of IGF2BP3 with HEXIM1 in a cell line model of Mk differentiation. K562 cells were induced with TPA (25 nM 48 hours) as indicated. Cell extracts underwent immunoprecipitation with control Ig or anti-HEXIM1 antibodies, followed by immunoblot. (D) Overexpression of IGF2BP3 redistributes LARP7. 293 cells transfected with control or IGF2BP3 expression vector underwent fractionation by sequential extraction, followed by immunoblot. (E) Isoform-specific knock-down of IGF2BP3 in neonatal megakaryocytes. Neonatal progenitors transduced with lentiviral shRNA vectors underwent puromycin selection and 6 days Mk culture followed by immunoblot.

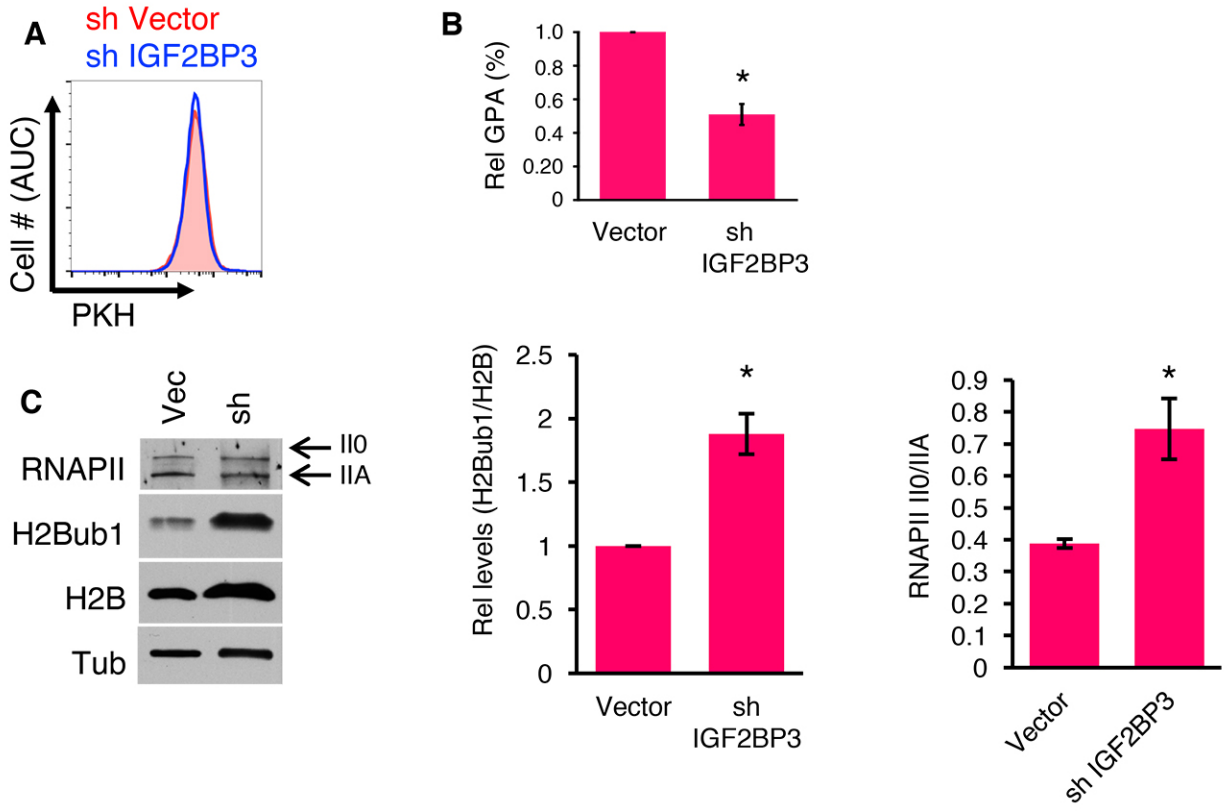

Figure S8: IGF2BP3 knockdown elicits adult phenotypic and molecular features in neonatal megakaryocytes.
(A) Comparison of initial PKH loading efficiency. Samples from cultures of transduced neonatal progenitors in Figure 6B underwent flow cytometry four hours post PKH staining, with gating on viable cells. (B) Decrease in erythroid antigen expression in neonatal progenitors subjected to knockdown of IGF2BP3. Neonatal progenitors transduced with lentiviral shRNA vectors underwent 6 days Mk culture followed by flow cytometry. Graph shows mean fold change $\pm$ SEM for three independent experiments. * $P < 0.05$. (C) Evidence of increased P-TEFb signaling associated with IGF2BP3 knockdown in neonatal megakaryocytes. Cells transduced and cultured as in (A) underwent immunoblot. Graphs show means $\pm$ SEM for scanning densitometry values derived from three independent experiments, with signal normalization as in Figure 6D-E. * $P <$ 0.05.

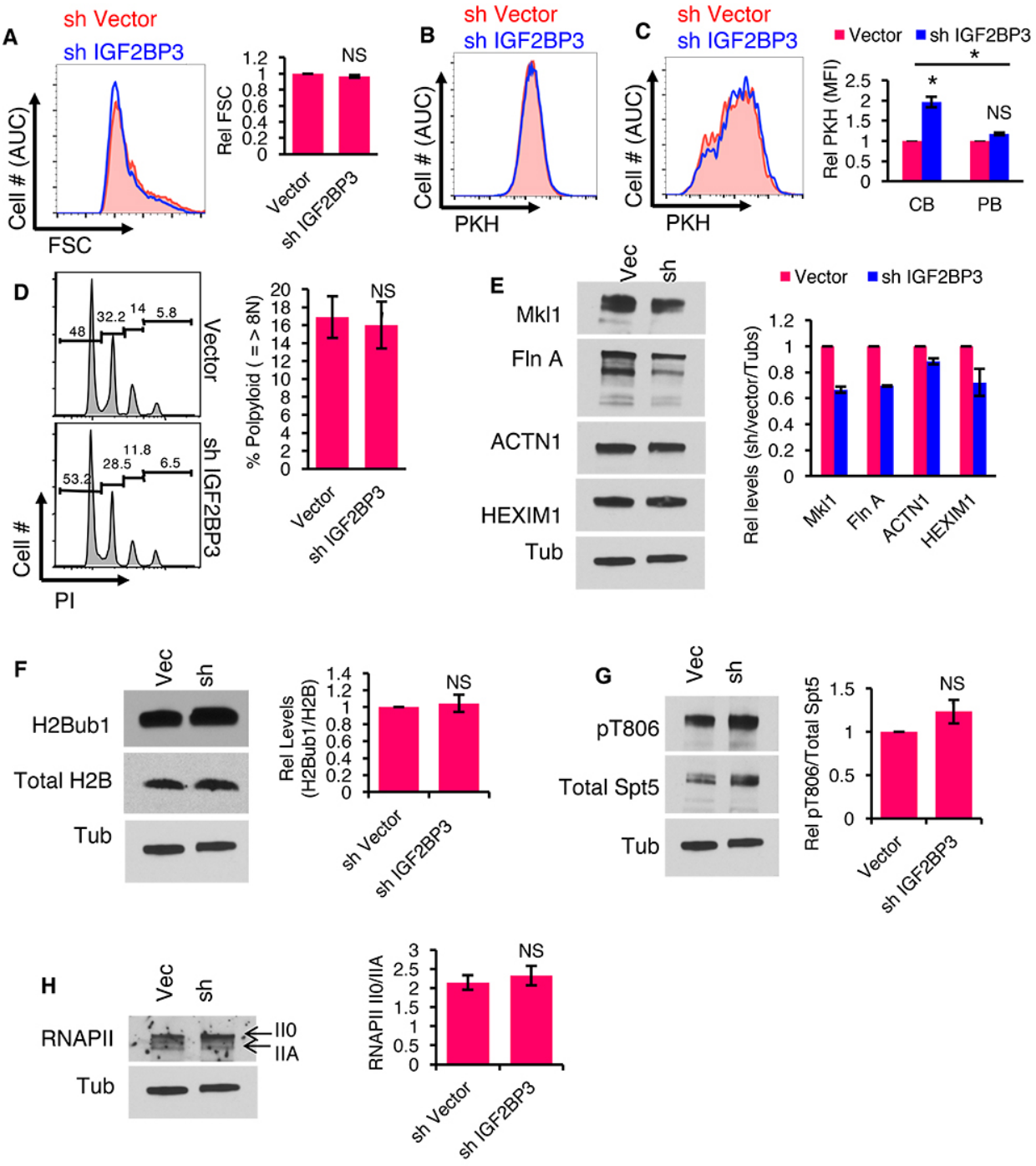

Figure S9. No significant phenotypic or molecular effects of IGF2BP3-targeting shRNA in adult Mk.

(A-D) Adult progenitors transduced with the same shRNA lentiviral vectors as in Figure 6 were puromycin-selected, cultured for 6 days in Mk medium and analyzed by flow cytometry for forward scatter (FSC) (A), PKH dye dilution (B and C), and propidium iodide staining (PI) (D),

with gating on viable CD41+ cells. (B) Comparison of initial PKH loading efficiency. Samples from the cultures of transduced adult progenitors underwent flow cytometry four hours post PKH staining, with gating on viable cells. Graphs represent mean ± SEM for three independent experiments. (*t* test was applied in A and D. Analysis of the groups in C employed two-way ANOVA, asterisk above the long line indicates Row Factor x Column Factor interaction; $*P < 0.05$, NS = not significant). (E-H) Whole cell lysates of cells from (A-D) were immunoblotted for P-TEFb targets (E), H2Bub1 and total H2B (F), pT806 and total Spt5 (G), and total RNAPII (H) as in Figures 6 and S8. Graphs depict means ± SEM for scanning densitometry values derived from three independent experiments, with signal normalization as in Figure 6. (*t* test; NS = not significant). Please note that panels **E**, **F**, **G**, and **H** all derive from the same immunoblot membrane and therefore share the same tubulin control.

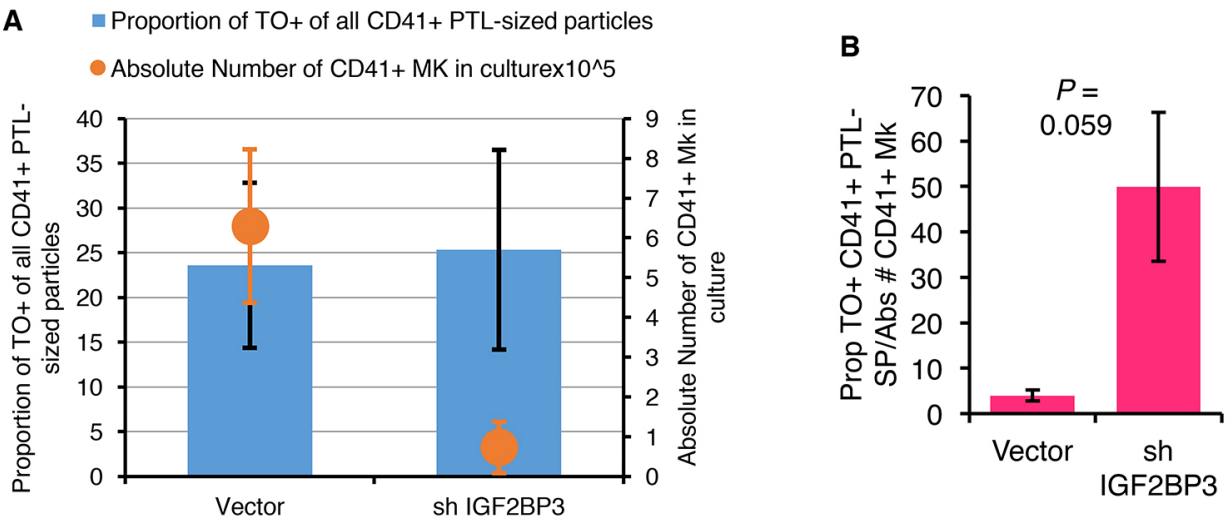

Figure S10: Effect of IGF2BP3 knockdown in neonatal megakaryocytes on efficiency of platelet release.

(A and B) Analysis of platelet release after knockdown of IGF2BP3 in neonatal progenitors. Lentiviral transduction and puromycin selection were conducted as in Figure 6. The cells subsequently underwent megakaryocytic culture for 11 days. Culture-derived platelets in the media supernatants were detected by flow cytometry by staining with anti-CD41 and thiazole orange (TO). An analytical gate for FSC/SSC and CD41/TO was established using fresh normal donor human platelets. (A) Graphs showing means $\pm$ SD from three independent experiments for the proportion of TO positivity within CD41+ platelet (PTL) sized particles (blue) and the absolute number of CD41+ Mk in the culture (brown). (B) Graph representing the proportion (Prop) of TO+ CD41+ platelet sized particles (PTL-SP) normalized to the absolute number (Abs #) of CD41+ Mk in the culture. Shown are mean $\pm$ SEM for three independent experiments. (*t* test).

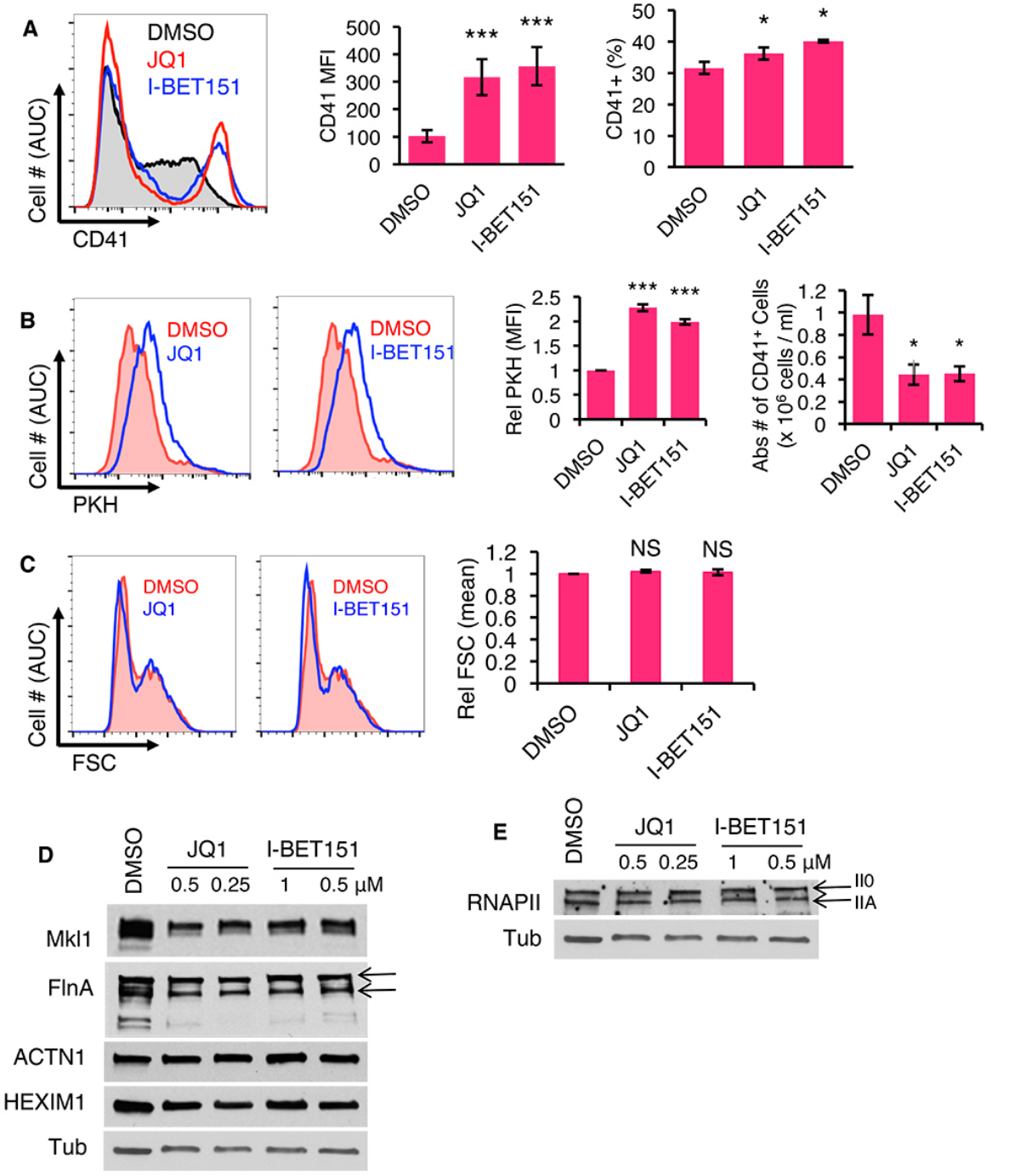

Figure S11: Enhancement of CD41 expression and growth arrest in neonatal megakaryocytes treated with BET inhibitors; BET inhibitors fail to augment morphogenesis program in adult megakaryocytes.

(A) Enhancement of CD41 expression in neonatal megakaryocytes treated with BET inhibitors.

Neonatal progenitors were cultured and analyzed as in Figure 7A. Graphs depict CD41 mean fluorescence intensity (MFI, middle) and percentage (right) as means ± SEM for three independent experiments. (*t* test; **P* < 0.05; *** *P* < 0.005). (B) BET inhibitors enhance growth arrest in neonatal megakaryocytes. Neonatal progenitors cultured as in Figure 7A underwent dye dilution assays, cell counting and flow cytometry for CD41. Graphs depict PKH MFI (middle) and absolute number (Abs #) of CD41+ cells (right) as means ± SEM for three independent experiments. (*t* test; **P* < 0.05; *** *P* < 0.005). (C) BET inhibitors have no effect on adult megakaryocyte size. Adult progenitors grown in megakaryocyte medium 6 days -/+ BET inhibitors as in Figure 7A underwent flow cytometry for FSC with gating on viable, CD41+ population. Graphs represent mean FSC relative to solvent control ± SEM for three independent experiments. (*t* test; NS = not significant). (D-E) BET inhibitors do not upregulate P-TEFb targets or RNAPII hyperphophorylation in adult megakaryocytes. Cells cultured as in (C) underwent lysis and immunoblot analysis as in Figure 8. Arrows in (D): intact filamin A and the ~190 kD cleavage fragment. Arrows in (E): RNAP II0 and IIA isoforms. Representative results from two independent experiments are shown. Please note that panels **D** and **E** derive from the same immunoblot membrane and therefore share the same tubulin control.

Supplemental Methods

Immunohistochemistry, *In Situ* Hybridzation, and Immunofluorescence

Immunohistochemical staining of formalin fixed, paraffin embedded marrow samples used specimens identified by Dr. Alejandro Gru as representing cord blood and adult HSPC recipients at the University of Virginia with adequate marrow cellularity and megakaryocyte content for analysis. Non-transplanted pediatric marrow specimens from University of Arizona were previously described in reference 3 of main text (Fuchs DA, et al. Am J Clin Pathol. 2012;138:140-5). Staining was performed using a DAKO Autostainer (Agilent/DAKO, Santa Clara, CA). Antigens were retrieved at 97° C for 20 minutes in high pH TR Flex-DAKO buffer. Slides were blocked for 10 minutes in DAKO dual endogenous enzyme block buffer. Rabbit anti-Mkl1 (catalog #HPA030782, Sigma) was diluted 1:100 in DAKO antibody diluent and incubated with the slides for one hour. Antigen-antibody complexes were detected by DAKO Envision, anti-rabbit polymer and then developed with DAKO DAB+ substrate. Counterstain employed hematoxylin.

For *in situ* hybridization, CB and PB day 5 megakaryocytes were cytospun and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. The cells were washed with PBS and permeabilized with 2% acetone in PBS for 5 minutes at room temperature, followed by prehybridization for 10 minutes at room temperature in 30% formamide in 2X SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0). The cells underwent hybridization overnight at 42°C with two labelled oligonucleotide probes against 7SK snRNA (5'-/5Cy3/TTGGATGTGTCTGGAGTCTTGGAAGCTTGACTAC/3Cy3Sp/-3' and 5'-/5Cy3/CAGATCAGCCGAATCAACCCTGGC/3Cy3Sp/-3') at 0.1 ng/µl each in RNA FISH hybridization buffer (40% formamide, 2X SSC, 0.2% bovine serum albumin, 10% dextran sulfate, 2 mM vanadyl complex, 1 mg/ml tRNA, and 1 mg/ml salmon sperm DNA). The cells were then washed 3 times in 30% formamide/2X SSC at 42°C followed by DAPI counterstaining in PBS for 5 minutes at room temperature. Images were captured with an Olympus BX40F fluorescence microscope.

For immunofluorescence, similar cytospins preparations were permeabilized with PBS with 0.1% Triton X-100 then fixed 10 minutes in 4% paraformaldehyde/PBS at room temperature. Cells underwent formaldehyde quenching with 100 mM Tris pH 7.5 for 5 minutes, were washed with PBS, blocked 1 hour in PBS with 2% FBS, 2% BSA, and 0.1% Triton X-100, and incubated 1 hour at room temperature with primary antibody at 1:400 in the blocking buffer. After 3 washes with PBS plus Tween-20 (PBST), cells were incubated with Cy5-conjugated secondary antibody in blocking buffer. Single plane mid-nucleus images were captured with Zeiss LSM 700 confocal microscope.

Bone marrow transplantation and analysis

Mice were housed in AALAC-approved, pathogen-free facilities. For marrow donors, six female 7 week old C57BL/6J mice (CD45.2, Jackson Laboratories, Bar Harbor, Maine) received a single IP injection of 5-fluorouracil (Teva Parenteral Medicine, CA) at a dose of 150 mg/kg. One week later bone marrows were harvested, and red blood cells were lysed with Gibco™ ACK lysis buffer (Thermo Fisher Scientific, Waltham, MA). Marrow cells then underwent prestimulation culture in RPMI-1640 with 10% FBS, 50 ng/ml murine SCF, 50 ng/ml murine IL-

6, 20 ng/ml murine IL-3 (all from Peprotech, Rocky Hill, NJ), 50 μM β mercaptoethanol, 100 U/ml penicillin, and 100 U/ml streptomycin. After 24 hours, the cells were transduced by spinoculation with retroviral supernatants for 90 minutes for two consecutive days. The retroviral supernatants contained 50 ng/ml murine SCF, 50 ng/ml murine IL-6, 20 ng/ml murine IL-3 and 4 μg/ml Polybrene (Sigma).

24 recipient mice, consisting of 8 week old female C57BL/6J of CD45.1+ background (B6.SJL-*Ptprc*^{a}*Pepc*^{b}*IBoyJ*, Jackson Labs) were irradiated with two doses of 6 Gy separated by 3 hours using a Mark I Model 68A rodent cesium (137Cs) irradiator. Two hours later each animal received 2 million cells from the spinoculation by tail vein injection (12 animals for MIG and 12 for MIG-IGF2BP3). At day 25 post transplant, we confirmed engraftment in 8/8 animals studied by flow cytometry on peripheral blood, co-staining white cells with APC conjugated anti-Ly6G (granulocyte marker) and Brilliant Violet 421™ conjugated anti-CD45.2 (donor marker) (both from BioLegend, San Diego, CA). Animals were maintained on SulfaTrim water from 3 days prior to transplant until euthanasia. At six weeks post transplant, half of the animals (6/group) were directly euthanized for marrow analysis by flow cytometry. The other half of the animals were treated with murine thrombopioetin (TPO, Peprotech), receiving 2 mg/day by IP injection for 3 consecutive days. Four days after the last injection, the animals were euthanized for marrow and spleen analysis by flow cytometry.

Differential salt extraction

Extraction of protein complexes from cells using different salt concentrations integrated the approaches of Andrews and Faller (1) and of Bartholomeeusen et al. (2), with some modifications. Briefly, progenitors cultured in megakaryocytic medium or HEK293T transfectants were washed in PBS and resuspended in ice cold Buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, protease inhibitor cocktail, 20 μM calpain inhibitor III, 40 U/ml RNase inhibitor), incubated for 10 minutes, and then vortexed for 10 seconds. The suspensions were microfuged for 10 seconds, and supernatants were discarded. Pellets were then resuspended in low salt buffer (10 mM HEPES-KOH pH 7.5, 10 mM KCl, 10 mM MgCl2, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail, 20 μM calpain inhibitor III, 40 U/ml RNase inhibitor) and incubated on ice for 10 minutes. Suspensions were centrifuged at 5000 RPM for 5 minutes, and supernatants consisting of "Fraction 1" were collected. The remaining pellets were washed with low salt buffer, resuspended in low salt buffer with 0.5% NP-40, and incubated on ice for 10 minutes. After centrifugation at 5000 RPM for 5 minutes, supernatants designated "Fraction 2" were collected. Resulting pellets were washed with low salt buffer, resuspended in ice cold high salt buffer (20 mM HEPES-KOH pH 7.5, 450 mM NaCl, 1.5 mM MgCl2, 0.5 mM EDTA, 1 mM DTT, 0.5% NP-40, protease inhibitor cocktail, 20 μM calpain inhibitor III, 40 U/ml RNase inhibitor), incubated on ice for 10 minutes, and centrifuged at 10,000 RPM for 5 minutes. The resulting supernatants consisted of "Fraction 3".

Analysis of culture-derived platelets

Culture-derived platelets were harvested from the supernatants of cell culture suspensions subjected to centrifugation at 140 g for 10 minutes. These supernatants then underwent centrifugation at 2800 g for 10 minutes. The pellet containing culture-derived platelets was re-suspended in phosphate buffered saline (PBS) with 0.1% fetal bovine serum (FBS) and labeled with CD41-APC antibodies for 30 minutes at room temperature. Newly formed reticulated

platelets were identified following incubation with 2 μg/ml thiaozole orange (TO) (Sigma, St Louis, MO) (stock solution of 1 mg/ml in methanol) for 15 minutes at room temperature in the dark. The cells were then washed, re-suspended in PBS with 0.1% FBS, and analyzed by flow cytometry. Fresh peripheral blood (PB)-derived human platelets were immunolabeled in the same manner and used as the control for CD41/TO staining. An analytical gate based on PB-platelet forward and side scatter light properties was initially set and used for the analysis of culture-derived platelets.

Antibodies used

- Rabbit anti-Filamin A, rabbit anti-Mkl1, rabbit anti-HEXIM1, rabbit anti-MePCE, and rabbit anti-LARP7 (Bethyl laboratories), rabbit anti-Mkl1 (HPA030782 - Sigma)
- Rabbit anti-Cdk9, rabbit anti-α-actinin-1, rabbit anti-Lin28B, rabbit anti-SART3, rabbit anti-H2Bub1 and mouse anti-H2B (Cell Signaling Technology)
- Rabbit anti-Cyclin T1, rabbit anti-RNA Polymerase II (H224), Rabbit anti-Spt5 (H300), rabbit anti-LDH, rabbit anti-PARP and mouse anti-hnRNPA1 (Santa Cruz Biotehnology).
- Rabbit anti-IGF2BP3, and rabbit anti-IGF2BP1/2 (EMD Millipore)
- Rabbit anti-pT806 Spt5 (Described in Sanso et al., Genes Dev 30, 117-131, 2016)
- Mouse anti-Hic 5 (BD Transduction Laboratories)
- Mouse anti-tubulin (Sigma-Aldrich)
- Rabbit anti-HMGA1 (Epitomics)

SUPPLEMENTAL REFERENCES

1. Andrews NC, and Faller DC. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucl Acids Res. 1991;19(9):2499.
2. Bartholomeeusen K, Xiang Y, Fujinaga K, and Peterlin BM. BET bromodomain inhibition activates transcription via a transient release of P-TEFb from 7SK snRNP. J Biol Chem. 2012;287(43):36609-16.