Supplemental Figures

Figure S1 ***Elagib et al.***

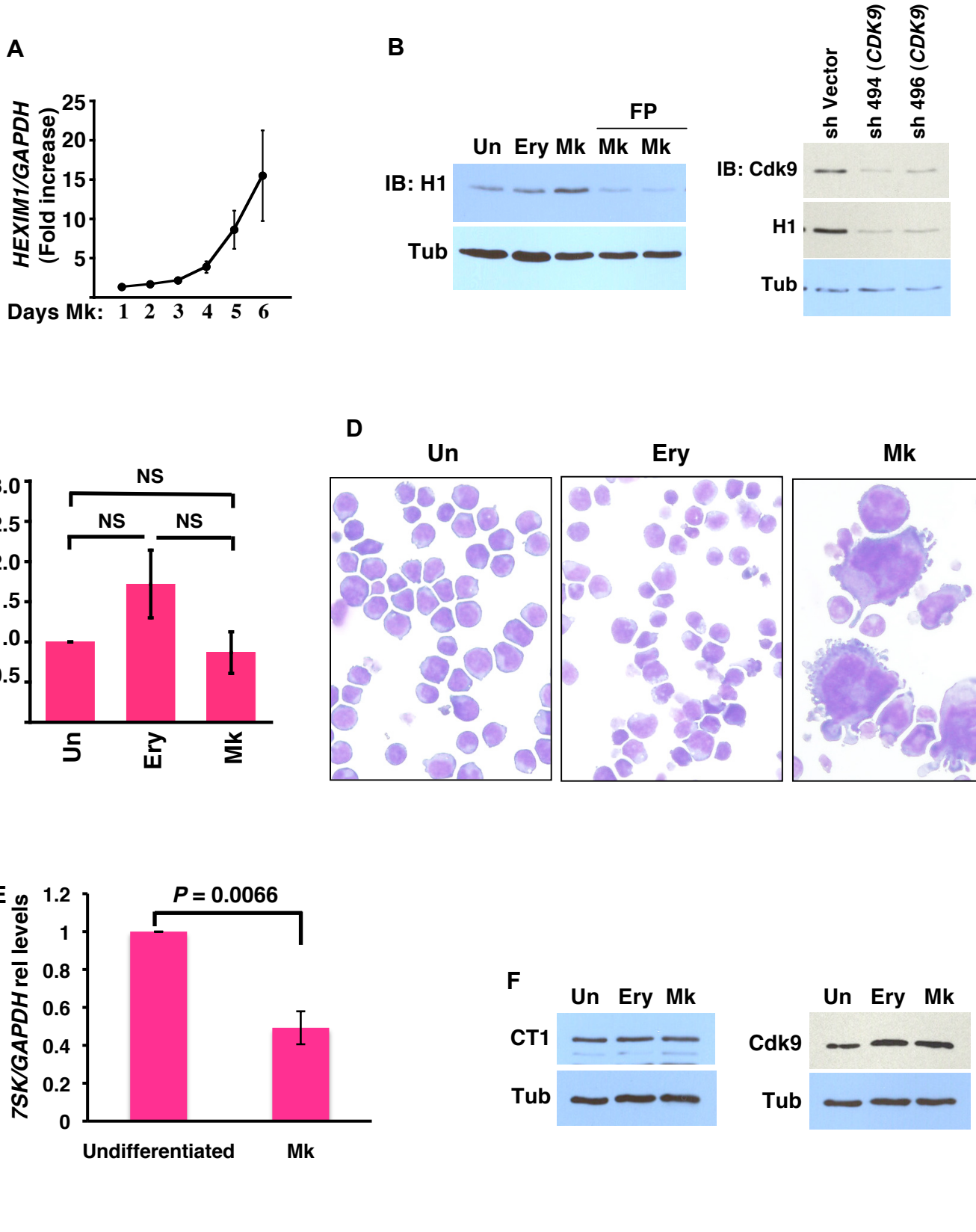

Figure S1, related to Figure 1: Megakaryocytic upregulation of HEXIM1; comparison of MEPCE transcript levels; cytokine responsive differentiation of HPC7 murine cell line; comparison of 7SK transcript levels.

(A) Primary human progenitors in megakaryocytic culture for 1-6 days underwent quantitative RT-PCR for *HEXIM1* levels, normalized to *GAPDH* expression. Results represent mean ± SEM for three independent experiments.

(B) Contribution of P-TEFb to megakaryocytic HEXIM1 (H1) upregulation. (Left panel) Immunoblot analysis of human progenitors cultured as in Figure 1A. Where indicated, cells were treated with 50-100 nM flavopiridol (FP). (Right panel) progenitors transduced with the indicated shRNA constructs underwent megakaryocytic culture followed by immunoblot.

(C) *MEPCE* transcript levels during megakaryocyte differentiation. Cells cultured as in Figure 1A were analyzed by qRT-PCR. Graphs represent mean ± SEM of *MEPCE* normalized to *GAPDH* in four independent experiments. NS, not significant.

(D) Cytokine-responsive erythroid and megakaryocytic differentiation of the non-transformed murine cell line HPC7 undergoes . Wrights-stained cytospins of HPC7 cells cultured as in Figure 1C, with images captured by light microscopy (200X).

(E) Undifferentiated HPC7 cells or grown in megakaryocytic culture were analyzed by quantitative RT-PCR for *7SK* levels normalized to *GAPDH*. Graphs represent mean ± SEM for four independent experiments.

(F) Expression of cyclin T1 and Cdk9 in HPC7. HPC7 cells cultured under the indicated conditions underwent immunoblot analysis as in Figure 1D.

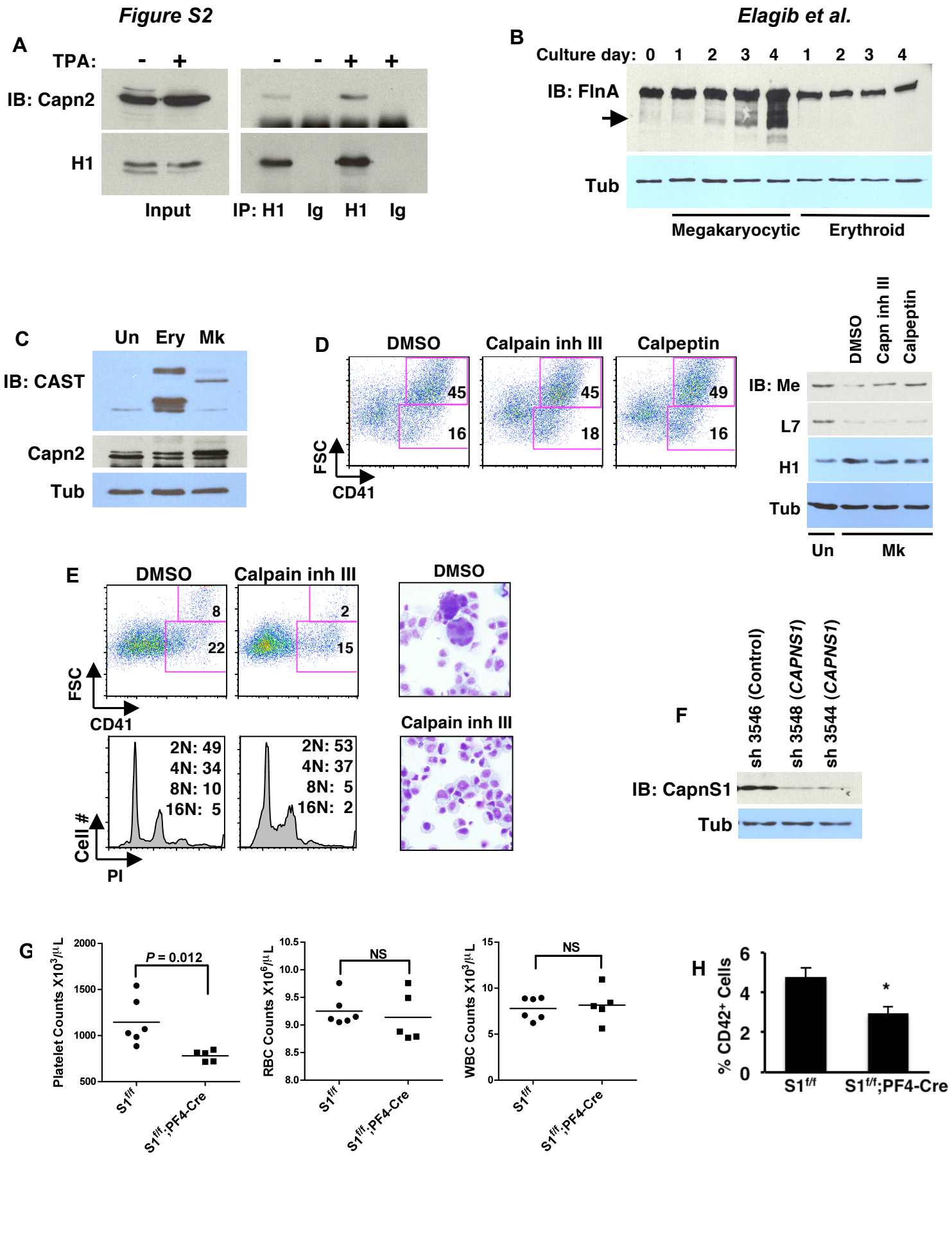
Figure S2
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A
TPA: - + - - + +
IB: Capn2
H1
Input
IP: H1 Ig H1 Ig
B
Culture day: 0 1 2 3 4 1 2 3 4
IB: FlnA
Tub
Megakaryocytic
Erythroid
C
Un Ery Mk
IB: CAST
Capn2
Tub
D
DMSO
Calpain inh III
Calpeptin
45
16
45
18
49
16
FSC
CD41
DMSO
Capn inh III
Calpeptin
IB: Me
L7
H1
Tub
Un
Mk
E
DMSO
Calpain inh III
8
22
2
15
FSC
CD41
2N: 49
4N: 34
8N: 10
16N: 5
2N: 53
4N: 37
8N: 5
16N: 2
Cell #
PI
DMSO
Calpain inh III
F
sh 3546 (Control)
sh 3548 (CAPNS1)
sh 3544 (CAPNS1)
IB: CapnS1
Tub
G
Platelet Counts X10³/μL
P = 0.012
RBC Counts X10⁶/μL
NS
WBC Counts X10³/μL
NS
S1f/f
S1f/f;PF4-Cre
H
% CD42+ Cells
*
S1f/f
S1f/f;PF4-Cre

Figure S2, related to Figure 2: Calpain participation in megakaryocytic differentiation; consequences of megakaryocytic deletion of CAPNS1 in mice.

(A) Analysis of calpain 2 interaction with HEXIM1. K562 cells underwent induction with TPA (25nM 48 hours) where indicated. Cellular extracts underwent immunoprecipitation with control Ig or anti-HEXIM1 antibodies, followed by immunoblot analysis.

(B) Analysis of endogenous calpain activation, reflected by the appearance of a 190 kd filamin A cleavage fragment (arrow) during megakaryocytic but not erythroid differentiation. Primary human progenitors cultured for the indicated number of days underwent immunoblotting of whole cell lysates.

(C) Calpastatin and calpain 2 expression in primary human progenitors. Human $CD34^+$ progenitors cultured 3 days in expansion medium (Un) or 6 days in erythroid or Mk medium underwent immunoblotting of whole cell lysates for calpastatin (CAST), calpain 2 and tubulin. For calpastatin, the larger species are "tissue type" isoforms (100-120 kd) and the smaller ones "erythrocyte type" isoforms (~70 kd) (Takano et al. 1993). For calpain 2, the 2 species represent the isoform 2 (622 aa) and isoform 1 (700 aa).

(D) Effect of delayed calpain inhibition during megakaryopoiesis on MePCE protein levels. Human progenitors underwent megakaryocytic culture for 5 days followed by treatment with solvent (DMSO) or calpain inhibitors (25 μM calpain inhibitor III or 40 μM calpeptin) for 18 hours of culture. Cells were then analyzed by flow cytometry for CD41 expression (left panel) or immunoblot as in Figure 1A (right panel).

(E) Effect of calpain inhibition on megakaryocytic differentiation. Human progenitors were cultured and analyzed as in Figure 2D, but with treatments consisting of DMSO or 25 μM calpain inhibitor III.

(F) Immunoblot assessment of shRNA knockdown of calpain S1 (*CAPNS1*) in human progenitors cultured in megakaryocytic medium as in Figure 2E.

(G-H) Consequences of megakaryocytic deletion of *CAPNS1* in mice. The *CAPNS1*$^{f/f}$ strain was backcrossed 9 generations onto C57BL/6 and crossed with the *PF4Cre* deleter strain, also on a C57BL/6 background. (G) Reduced platelet counts associated with *CAPNS1* deletion. CBC values in adult *CAPNS1*$^{f/f}$ (S1$^{f/f}$) (n = 6) and *CAPNS1*$^{f/f}$;PF4-Cre (S1$^{f/f}$;PF4-Cre) (n = 5) strains. (H) Defects in ex vivo development of megakaryocytes lacking calpain S1. Marrows from *CAPNS1*$^{f/f}$ (n = 4) and *CAPNS1*$^{f/f}$;PF4-Cre (n = 3) mice recovering from 5-FU treatment were cultured in megakaryocytic medium for 72 hours followed by flow cytometry for CD42. Graph represent mean $\pm$ SEM. * $P < 0.05$.

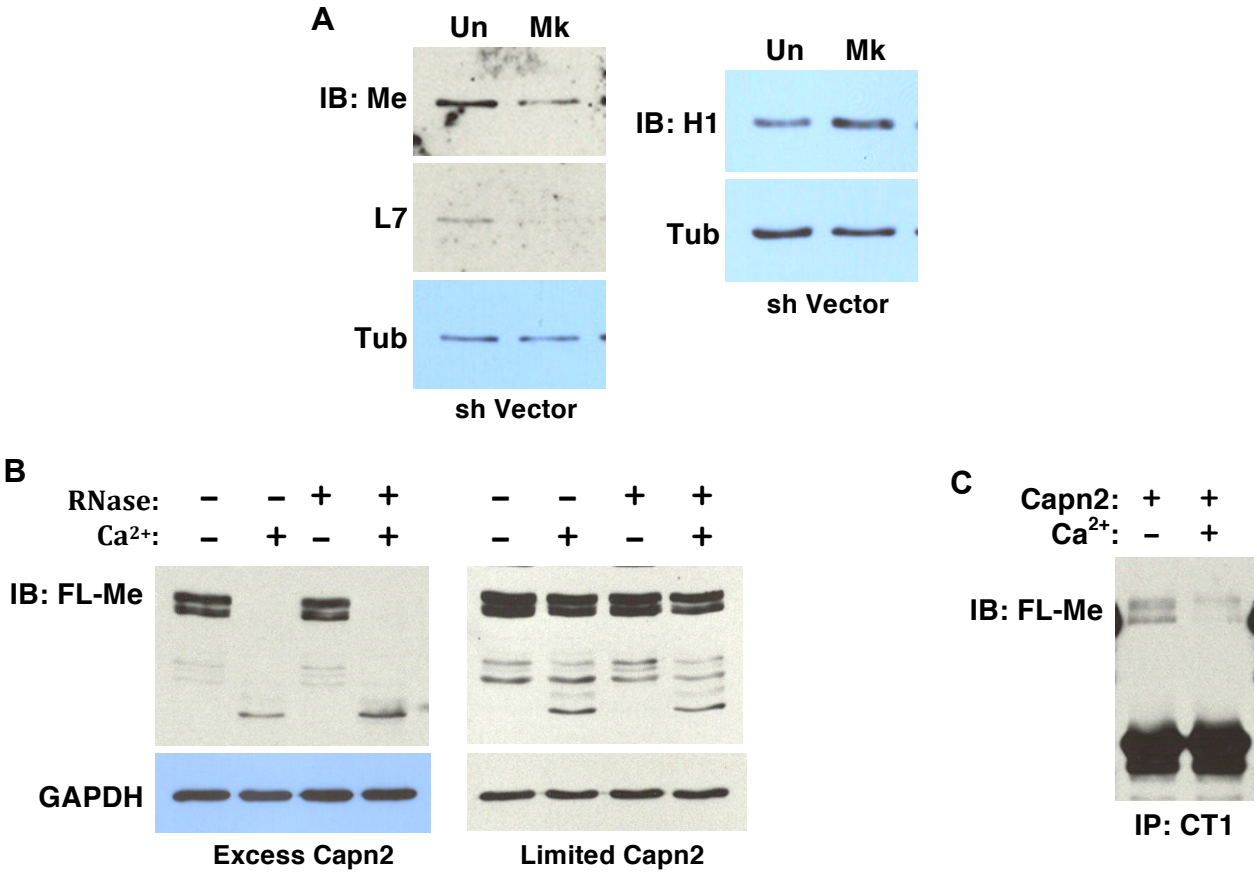

Figure S3, related to Figure 4: Calpain 2 regulation of MePCE.

(A) MePCE expression in human progenitors transduced with control shRNA vector. Human $CD34^+$ cells were analyzed as in Figure 4A. Representative results are shown corresponding to graph in 4A.

(B-C) Assays for calpain 2 cleavage of free MePCE and MePCE in complexes. (B) 293T cells expressing FLAG-MePCE underwent digests of extracts with recombinant calpain 2/S1 $\pm$ Ca^{2+} and $\pm$ RNase A. (C) Extracts as in B underwent immunoprecipitation of cyclin T1, followed by incubation of beads with recombinant calpain 2/S1 $\pm$ Ca^{2+} and then immunoblotting for FLAG-MePCE. Lower bands are IgH.

Figure S4

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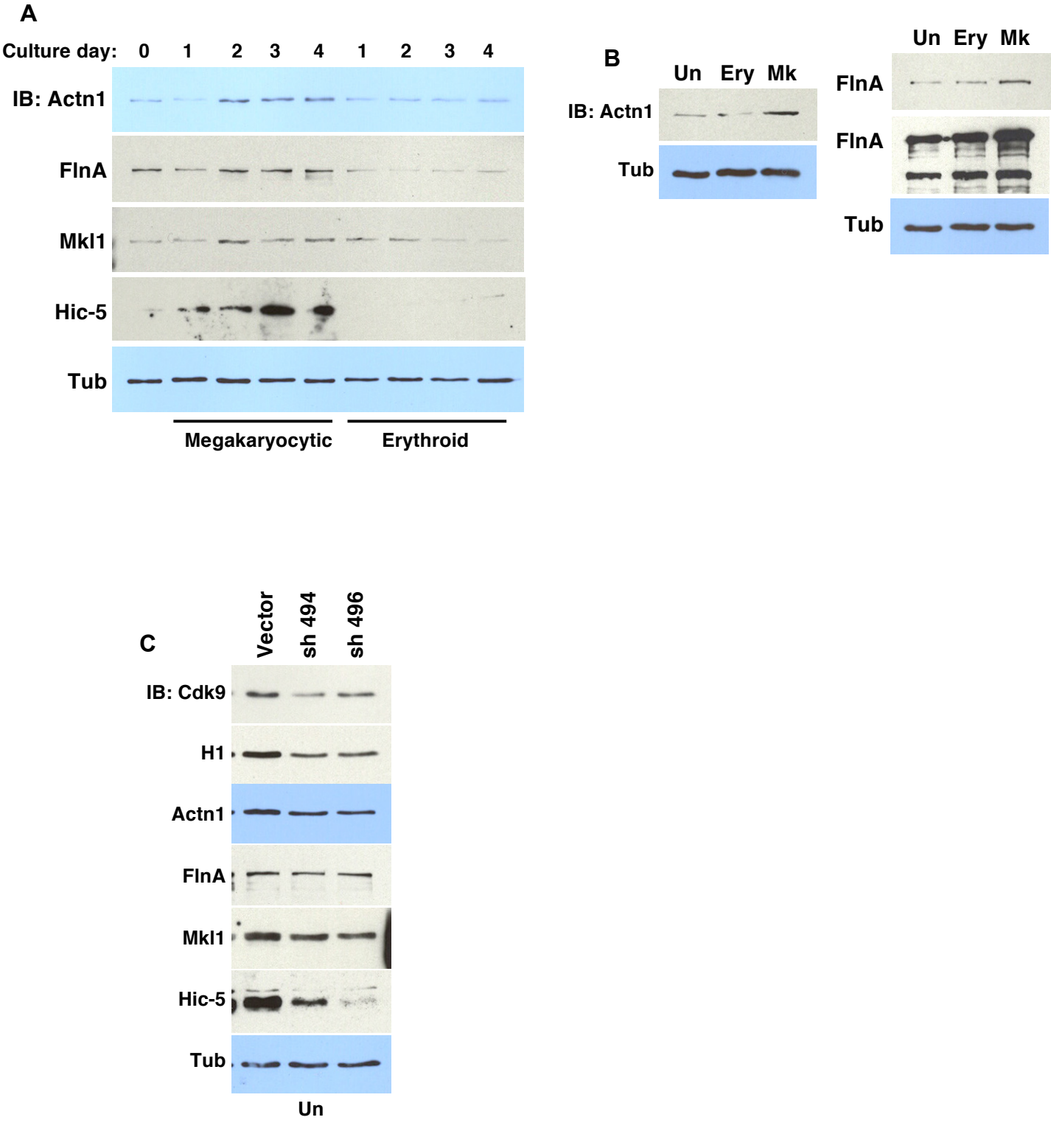

Figure S4, related to Figure 5: Megakaryocyte-specific upregulation of a cohort of cytoskeletal remodeling factors.

(A) Kinetic profiles of cytoskeletal remodeling factor levels during megakaryocytic and erythroid differentiation. Primary human progenitors cultured in megakaryocytic or erythroid medium for the indicated days underwent immunoblotting of whole cell lysates as in Figure 5B.

(B) Expression of α-actinin 1 and filamin A in the murine HPC7 cell line undergoing megakaryocytic or erythroid differentiation. Cells underwent culture followed by immunoblot as in Figure 1D.

(C) Impact of Cdk9 knockdown on expression of cytoskeletal remodeling factors in undifferentiated progenitors. Human $CD34^+$ progenitors transduced with lentiviral shRNA constructs as in Figure 5B were cultured 72 hours in prestimulation medium followed by immunoblotting.

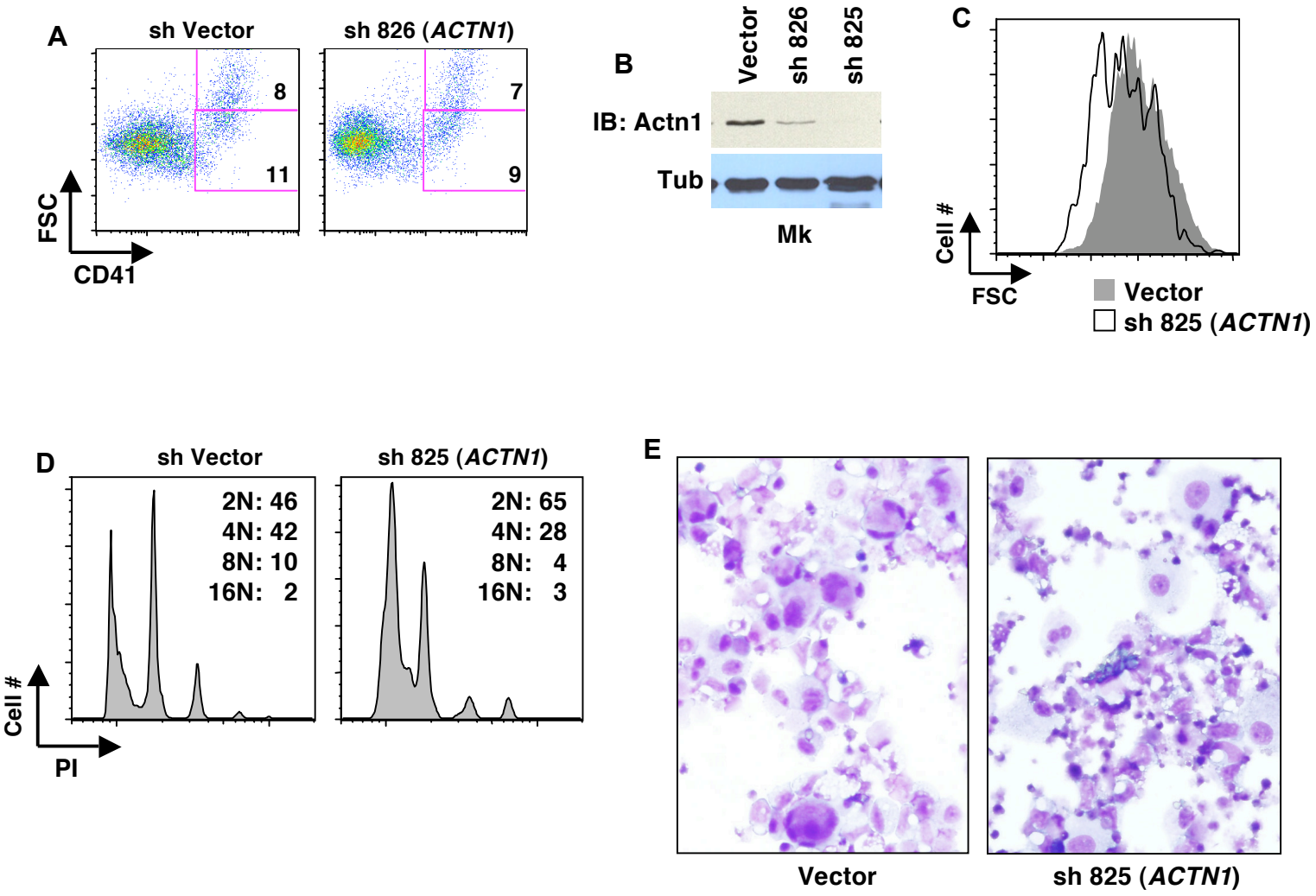

Figure S5, related to Figure 6: α-actinin-1 knockdown inhibits megakaryocytic enlargement and polyploidization.

Human CD34$^+$ cells were subjected to shRNA mediated knockdown of α-actinin-1 followed by megakaryocytic culture as in Figure 6.

(A) Flow cytometric ananlysis of CD41 expression associated with α-actinin-1 knockdown.

(B) Immunoblot comparing extent of α-actinin-1 knockdown by two different shRNAs.

(C) Effect of knockdown of α-actinin-1 using the sh 825 construct on enlargement of $CD41^+$ cells; findings with the sh 826 construct are shown in Figure 6C.

(D) Effect of knockdown of α-actinin-1 using the sh 825 construct on polyploidization of CD41+ cells; findings with the sh 826 construct are shown in Figure 6B.

(E) Effect of knockdown of α-actinin-1 using the sh 825 construct on megakaryocyte morphogenesis; findings with the sh 826 construct are shown in Figure 6D.

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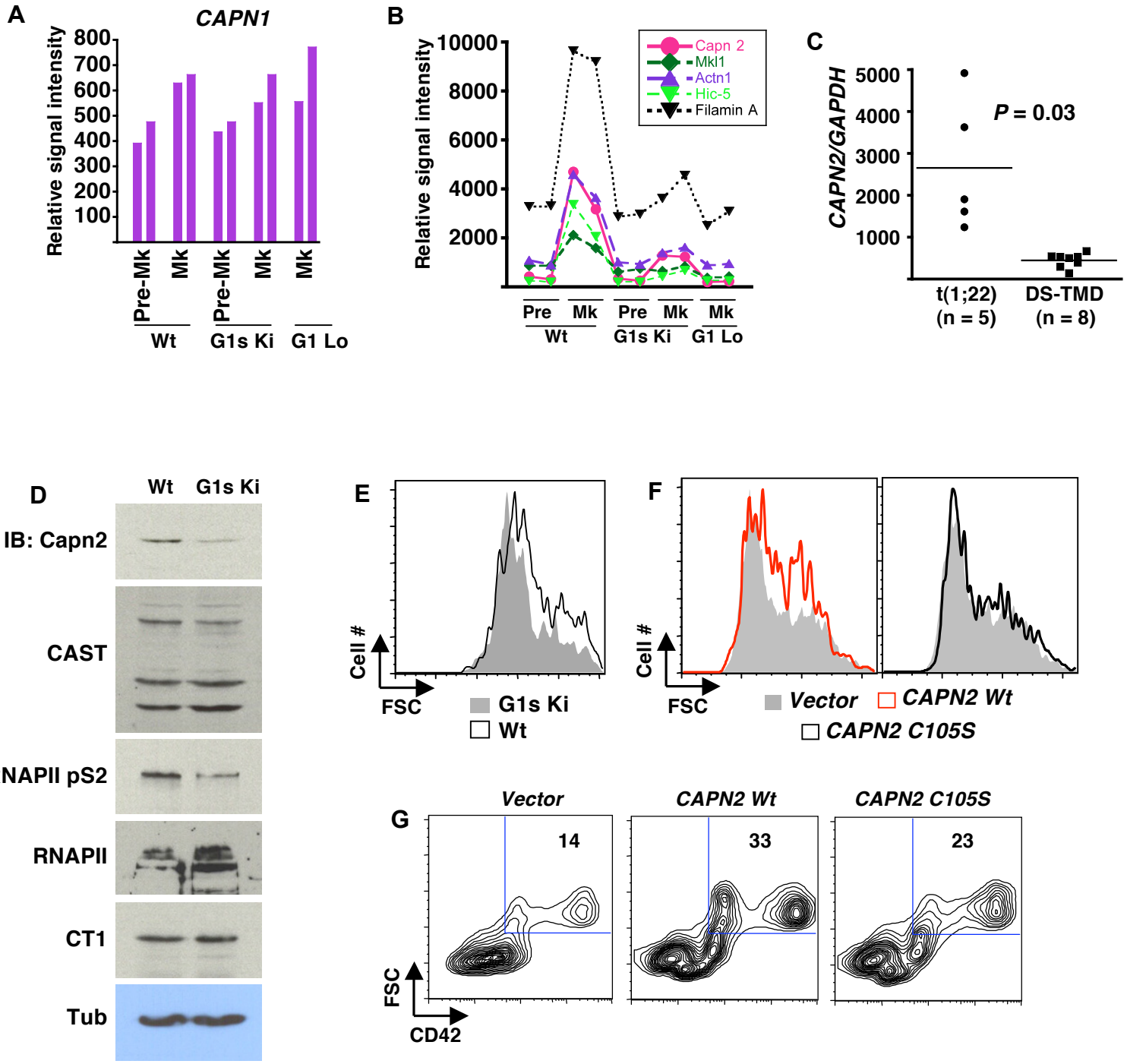

Figure S6, related to Figure 7: Megakaryocytic abnormalities associated with the GATA1s mutation; effects of enforced CAPN2 expression and of MePCE knockdown in fetal liver progenitors with GATA1s mutation.

(A) *CAPN1* expression in fetal liver megakaryocytes with GATA1s mutation (G1s Ki) or in megakaryocytes deficient for GATA1 (G1 Lo). Plotted are normalized signals obtained from GEO DataSet Record GDS1316 comparing relative mRNA levels from murine fetal liver pre-megakaryocytes (Pre-Mk) and megakaryocytes (Mk) of the indicated strains. Paired bars represent two independent experiments.

(B) Expression of cytoskeletal remodeling factors and *CAPN2* in fetal liver megakaryocytes with GATA1s mutation (G1s Ki) or GATA1 deficiency (G1 Lo). Analysis was carried out as in (A).

(C) *CAPN2* expression in Down syndrome-associated transient myeloproliferative disorder (DS-TMD). Comparison of *CAPN2* transcript levels, conducted as in Figure 7B (GEO DataSet Record GSE4119), applied to cases of infantile acute megakaryoblastic leukemia with t(1;22) versus Down syndrome-associated transient myeloproliferative disorder (DS-TMD).

(D) Impact of GATA1s mutation on calpain 2 protein expression and phosphorylation of RNA polymerase II on serine 2 of the carboxy terminal domain. Embryonic day 13.5 fetal liver cells underwent megakaryocytic culture as in Figures 7C-D, followed by immunoblotting of whole cell lysates. Note the multiplicity of calpastatin (CAST) isoforms in these samples, as in human samples (see Figure S2C).

(E) Impact of GATA1s mutation on cellular enlargement in fetal liver megakaryocytes. Day 13.5 fetal liver progenitors from wild type (Wt) and G1s Ki mice underwent megakaryocytic culture followed by flow cytometry as in Figures 7C-D.

(F-G) Role of protease activity in rescue of megakaryocyte differentiation by enforced calpain 2 in fetal liver progenitors with the GATA1s mutation. G1s Ki fetal liver progenitors underwent transduction with lentiviral constructs expressing either wild type (Wt) or protease-dead (C105S) calpain 2 or with control lentiviral vector, followed by megakaryocytic culture and flow cytometry as in Figure 7C-D.

Supplemental Experimental Procedures

Repeats

For all graphs, the number of experimental repeats is provided in figure legends. For other data, representative results are shown from ≥ 2 repeats.

Antibodies used

- Rabbit anti-Filamin A, rabbit anti-Mkl1, and rabbit anti-HEXIM1 (Bethyl laboratories)
- Rabbit anti-Calpain 1, rabbit anti-Calpain 2, rabbit anti-Cdk9 and rabbit anti-a-actinin-1 (Cell Signaling Technology)
- Mouse anti-Calpain S1, rat anti-GATA-1 (N1), rabbit anti-Cyclin T1, rabbit anti-Cdk9, rabbit anti-Calpastatin and rabbit anti-RNA Polymerase II (N20) (Santa Cruz Biotehnology)
- Rabbit anti-MePCE (Proteintech group, Inc)
- Rabbit anti-LARP7 (LifeSpan BioSciences)
- Rabbit anti-HEXIM1 (ABCAM)
- Mouse anti-Hic 5 (BD Transduction Laboratories, San Diego, CA, USA)
- Mouse anti-tubulin (Sigma-Aldrich)
- Rat anti-Phospho-Serine-2 of CTD of RNA Polymerase II (3E10, Millipore)
- Mouse anti-RNA Polymerase II (8WG16, Covance)

PCR for genotyping

For genotyping and verification of deletion of *CAPNS1* floxed alleles genomic DNA extracted using standard buffer containing (100 mM Tris HCL pH 8.5, 0.5 mM EDTA, 200 mM NaCl, 0.2% SDS and 0.1 mg/ml proteinase K), followed by isopropanol/ethanol precipitation and PCR using HotStar Taq Master mix (QIAGEN Inc) and primers described below.

Constructs

The protease dead calpain 2 mutant (C105S), as described by Elce et al. (Elce et al. 1995) was subcloned, packaged, and transduced as described for wild type calpain 2 in Experimental Procedures. Lentiviral shRNA against mouse MePCE was purchased from Open Biosystems (Huntsville, AL).

Primers and cycling parameters for genotyping

Exon 11 3' primer common for all *CAPNS1* alleles: 5' GGGACATGGAAGGAGGAGGAGT 3'

Exon 10 5' primer amplify *CAPNS1* wild type allele 5' GCTTTCAAATCT CTTGACAAAAATGG 3'

Neo 5' primer amplify *CAPNS1* targeted allele 5' TCT CGTCGTGACCCATGGGGAT 3'

Intron 8 5' primer amplify *CAPNS1* deleted allele 5' CACCGTCACTGTACTGGAGATTG 3'

Cycling parameters: Initial denaturation of 94°C for 3 minutes followed by 35 cycles of: 94°C for 30", 55°C for 1 minute, 72°C for 1 minute and a final cycle of 72°C for 2 minutes

Primers and cycling parameters for quantitative RT-PCR

7SK Forward: 5' CTTCGGTCAAGGGTATACGAGT 3'

7SK Reverse: 5' ATGCAGCGCCTCATTTGGATGT 3'

MePCE Forward : 5' ACCTCTAAAGGCTTCCAGCG 3'

MePCE Reverse : 5' GTCCTTATGAGCAGCGAGGG 3'

HEXIM1 Forward: 5' CGATGACGACTTCATGGAAGAAGG 3'

HEXIM1 Reverse: 5' ACTCCTTGATGAGCTCCTGCTTGCTC 3'

GAPDH Forward: 5' AGCCACATCGCTCAGACA 3'

GAPDH Reverse: 5' GCCCAATACGACCAAATCC 3'

7SK and GAPDH cycling parameters: Initial denaturation of 95°C for 2:30 followed by 40 cycles of: 95°C for 15", 55°C for 30", and 72°C for 30".

HEXIM1 and MePCE cycling parameters: Initial denaturation of 95°C for 2:30 followed by 40 cycles of: 95°C for 15", 60°C for 30", and 72°C for 30".

In silico approach for identification of coexpressed genes within gene expression databases

Candidate genes following a specified pattern of expression changes across multiple samples within a given GEO dataset (e.g. GSE2433) were identified applying the Jonckheere-Terpstra test, a rank-based, non-parametric comparison method and using R-

package SAGx software. This group of candidate genes was subsequently refined using the time-series biclustering algorithm of Zhang et al. (Zhang et al. 2005).

Supplemental References

Takano, E., Nosaka, T., Lee, W. J., Nakamura, K., Takahashi, T., Funaki, M., Okada, H., Hatanaka, M., and Maki, M. 1993. Molecular diversity of calpastatin in human erythroid cells. Arch. Biochem. Biophys. 303, 349-354.

Elce, J. S., Hegadorn, C., Gautheir, S., Vince, J. W., and Davies, P. L. 1995. Recombinant calpain II: improved expression systems and production of a C105A active-site mutant for crystallography. Protein Eng. 8, 843-848.

Zhang, Y., Zha, H., and Chu, C-H. 2005. A time-series biclustering alorithm for revealing co-regulated genes. Proeceedings of the International Conference on Information Technology: Coding and Computing (ITCC '05), 0-7695-2315-3/05, pages 1-6.