Integrated protein quality control pathways regulate free α globin in β-thalassemia

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SUPPLEMENTAL METHODS

Insoluble globin analysis

10 μL of washed red blood cells were lysed and pellets washed extensively in ice-cold 0.05X PBS. Membrane lipids were extracted with 56 mM sodium borate, pH 8.0 at 4°C. Precipitated globins were dissolved in 8M urea, 10% acetic acid, 10% β-mercaptoethanol, and 0.04% pyronin, fractionated by Triton-acetic acid-urea (TAU) gel electrophoresis and stained with Coomassie brilliant blue. Soluble hemoglobin fractions were analyzed as loading controls. Gel electrophoresis was performed using a Mini-PROTEAN Tetra Cell apparatus (Biorad).

Reticulocyte pulse-chase analysis

Freshly collected circulating mouse erythrocytes and reticulocytes were incubated in methionine/cysteine-free DMEM (Invitrogen), 2 mM L-glutamine, 10% dialyzed FBS, and 0.1 mM non-essential amino acids for 37°C for 1 hour. 50 μCi of ^{35}S-labeled methionine and cysteine (PerkinElmer) were added and the cells labeled for 20 minutes at 37°C. The cells were washed with PBS, and resuspended in chase medium supplemented with 2mM unlabeled methionine and cysteine. When indicated, chase medium contained 100 μM chloroquine (Sigma), 0.5 μM epoxomicin, 10 μM MG132 (Enzo Life Sciences) or 0.1% DMSO as control. For ATP depletion, chase was done in glucose-free DMEM containing 20 mM 2-deoxyglocose and 0.2 mM 2,4-dinitrophenol (Sigma). Soluble and insoluble fractions were analyzed as above. Gels

were dried, and images were acquired using a phosphor screen and Storm 865 scanner (GE Healthcare). Bands were quantified using National Institutes of Health ImageJ software.

Denaturing immunoprecipitation

Erythrocytes were resuspended in 50 mM Tris pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 1mM EDTA, supplemented with 1 mM DTT, 0.1 mM PMSF, 2 mM N-ethylmaleimide, 10 μM MG132, and protease inhibitor cocktail. After addition of SDS to 1%, samples were boiled for 5 minutes, cooled, and diluted to 0.1% SDS and 1% Triton X-100. Samples were pre-cleared with protein G beads (Invitrogen). For immunoprecipitations, equal amounts of protein were incubated overnight with pre-immune serum, anti-α globin serum, anti-poly ubiquitin FK2 antibody (MBL),[1] or mouse IgG. Protein G beads were added and incubated for 1 hour. Beads were then washed with PBS containing 0.2% NP-40 and 0.5M LiCl, and boiled in 2X Laemmli buffer. For competitive IPs, 5 μg of purified human α or β hemoglobin were denatured as above and added to the IP mixture.

Western blotting

Protein concentrations were determined using Pierce BCA protein assay kit (Thermo Scientific), and 20 μg of protein was resolved on a SDS-PAGE gel. For insoluble fractions, loading was normalized based on concentration of soluble fraction. After transfer to 0.2 μm PVDF membranes (Whatman), proteins were detected with specific primary antibodies (see table) and HRP-conjugated secondary antibodies and SuperSignal West ECL substrate (Thermo). For polyubiquitin western blots, membranes were first denatured in 6M guanidine hydrochloride

in 50 mM Tris pH 7.4 for 30 minutes. For LC3b western blots, proteins were resolved on NuPage 12% bis-tris gels using MES running buffer (Invitrogen).

Primary Antibodies for western blots

Protein	Antibody	Source	Dilution
α globin	Rabbit polyclonal	Covance	1:1000
β-actin	HRP conjugated, mouse monoclonal	Sigma	1:50,000
p62	Mouse monoclonal	Abnova	1:2500
LC3	Rabbit polyclonal	Novus Biologicals	1:500
LC3	Mouse monoclonal 4E12	MBL	1:250
Ubiquitin	FK2, mouse monoclonal	MBL	1:1000
Ubiquitin	Mouse monoclonal	Covance	1:1000

Flow cytometry

For fetal liver, bone marrow, and spleen analysis, $5x10^5$ cells were stained with 5 μM Hoescht33342 (Sigma) for 1 hour in fetal liver differentiation medium. After washing, cells were stained with Live/Dead near-IR fixable dead cell stain (Invitrogen) for 30 minutes in PBS, followed by antibody staining for 30 minutes in PBS with 2% FCS. Antibodies used were CD71-PE, Ter119-PerCPCy5.5, and CD44-APC (Biolegend). For proteasome activity quantification, cells were first stained with 1 μM MV151 (Chemical Proteomics Reagents, Leiden Institute for Chemistry, Leiden, Netherlands) for 4 hours in fetal liver differentiation medium.[2] Fluorescence minus one controls included all antibody and viability stains, but no MV151. Human patient erythrocytes were similarly treated, but were stained with Hoescht33342 and thiazole orange (ReticCOUNT, BD) instead of specific antibodies. Cells were analyzed on

an LSRII or an LSRFortessa instrument (BD Biosciences) maintained by the Flow Cytometry Core Laboratory of The Children's Hospital of Philadelphia Research Institute.

Microarray analysis.

Samples were processed for microarray analysis using the Mouse Gene 1.0ST Array (Affymetrix) by the microarray core facility at the University of Pennsylvania (http://www.med.upenn.edu/microarr/Data%20Analysis/Affymetrix/methods.htm). The original probes of Affymetrix 430 2.0 platform were mapped to the current version of mouse coding sequences through BRAINARRAY (http://brainarray.mbni.med.umich.edu/brainarray/default.asp). RefSeq genes were further mapped to 24,418 unique Entrez genes. The raw array data in .CEL files was processed by the R/Bioconductor implementation of Robust Multi-chip Average (RMA) method.[3] Differences between sample groups are expressed as $\log_2$-ratio. Data processing steps and statistical analyses were performed within R/Bioconductor statistical environment (http://www.bioconductor.org). A 3-group ANOVA analysis was performed to identify genes differentially expressed between any of the 3 groups. 1,480 genes have p-values less than 0.01. Heatmap of those genes shows the difference mostly exists between homozygous mutations and the other 2 groups. The comparison between any 2 groups was performed by SAM method, a variation of Student's t test. The test reports p value and FDR of differential expression. Top 250 most differentially expression genes with p less than 0.05 on both sides were identified and analyzed with DAVID. Mouse genes were mapped to human gene symbols through Homologene database for GSEA analysis using the default gene set collections.[4]

Real-time RT-PCR

RNA was extracted using TRIzol reagent, DNAse treated, and used for random hexamer-primed cDNA synthesis from 1 μg of RNA with the Superscript First Strand kit (Invitrogen) or with the iScript cDNA kit (Biorad). Quantitative real-time PCR was done using the standard curve method using SYBR green dye on an ABI 7900 real-time machine (PE Applied Biosystems). Target gene expression was normalized to the average of Actb and Hprt values. Primer sequences are described in supplemental methods.

Real-time PCR primers

Gene	Forward Primer	Reverse Primer	Source
ACTB	CCT TCC TTC TTG GGT ATG GAA TC	AGC ACT GTG TTG GCA TAG AGG T	
HMOX1	AAGCCGAGAATGCTGAGTTCA	GCCGTGTAGATATGGTACAAGGA	PrimerBank 6754212a1
HPRT	TCA GTC AAC GGG GGA CAT AAA	GGG GCT GTA CTG CTT AAC CAG	
Hsp105	GGT CCC AAT GAA AAA TGG TG	TCA GCA GCA TGG CTG TTA TC	
Hsp90aa1	TCC AAA GTC CCG AGA ACA AC	CAG AAT GTG ATT GGG CAC TG	
Hspa1a	TGC ATG GTG GTT GCA CTG TAG G	ACT GAA CAC ATG CTG GTG CTG TC	
Hspa1b	TCG AGG AGG TGG ATT AGA GG	GCA GCT ATC AAG TGC AAA GAG	
NFE2L1	CTGAGTCTGATTGGGGTTCGG	GAACTGGGTCTGGGTATAGGC	PrimerBank 31982173a1
NFE2L2	GCCCACATTCCCAAACAAGAT	CCAGAGAGCTATTGAGGGACTG	PrimerBank 6754832a2
NQO1	GCAGTTTCTAAGAGCAGAACG	GTAGATTAGTCCTCACTCAGCCG	
PsmA4	AGGTCGCTTATACCAAGTGGA	ACGTTAGCATCAGATGTTATGCC	PrimerBank 6755196a1
PsmA5	GAGTACGACAGGGGTGTGAAT	GCTCCATTAGTGGGGAGGTAA	PrimerBank 7106387a1
PsmA6	AACGGAAAGCATTGGCTGTGT	TGCACAGCATGTCCACAGGAA	
PsmA7	CGGCCCTAATTGTGGGTTTTG	GTTTTTGCCACCTGACTGGAC	
PsmB3	CAGCGTCTCAAGTTCCGACTG	CGCTTCTCATACAGGAGGTTG	PrimerBank 6755202a1
PsmB6	GCAGGCTGGACCCTCAAGAA	CAAGCGGATCACCCCTCCACT	
PsmC1	CCCAACTAGAGTGGGGAAAAAG	AAGGTTCCTACAGACATGGGG	PrimerBank 6679501a1
PsmC2	GATGTTGGTGGCTGTAAGGAA	CCCAATAACTCGAATGAAGCA	
PsmC3	CCACTCACGGAAGATGAATGT	TCAACTTTAGCCCTCAGACCA	
PsmD11	AGGCAGACAGAAGCATTGAAA	GGTCCAAAATCCCATGAAACT	
PsmD12	GTGGCCGAACCTGGGGTACAG	TCTTGAAGCCGCCCTTCCTTG	
PsmD3	CGGCTCTGTGCCTGGTCTCTG	TCCCAGCTGCTGCCTCCTCTT	

When indicated, primer sequences were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/). Otherwise, proteasomal subunit primer sequences

were from Lee et al.[5] Heat shock protein sequences were designed using Primer Express, based on data from Trinklein et al[6] and Hee-Jung et al.[7]

shRNA sequences:

Clones were purchased from OpenBiosystems as pGipZ vectors and cloned into the MSCV-PIG retroviral vector.[8] The sense strand is indicated in red, antisense in blue, loop in green, and the common miR-30 context in black.

shLuciferase (RHS1705)
TGCTGTTGACAGTGAGCGCCCGCCTGAAGTCTCTGATTAATAGTGAAGCCACAGATG
TATTAATCAGAGACTTCAGGCGGTTGCCTACTGCCTCGGA

mNrf1-sh1 (V2LMM_28202)
TGCTGTTGACAGTGAGCGAGGCCACAATCATACATACAATTAGTGAAGCCACAGAT
GTAATTGTATGTATGATTGTGGCCCTGCCTACTGCCTCGGA

mNrf1-sh1 (V3LMM_501203)
TGCTGTTGACAGTGAGCGACAAGTTGGACACCATCCTAAATAGTGAAGCCACAGAT
GTATTTAGGATGGTGTCCAACTTGCTGCCTACTGCCTCGGA

mNrf2-sh1 (V3LMM_515621)
TGCTGTTGACAGTGAGCGCTAGCAATAATATGAAACTTTATAGTGAAGCCACAGATG
TATAAAGTTTCATATTATTGCTATTGCCTACTGCCTCGGA

mNrf2-sh2 (V2LMM_1622)
TGCTGTTGACAGTGAGCGACCGAATTACAGTGTCTTAATATAGTGAAGCCACAGATG
TATATTAAGACACTGTAATTCGGGTGCCTACTGCCTCGGA

For cloning shRNAs into the MSCV-PIG vector BglII and XhoI sites, the following primers were used:

BglII-miR30sh-F AGATCTAGATCTTGCTGTTGACAGTGAGCG
XhoI-miR30sh-R CTCGAGCTCGAGTCCGAGGCAGTAGGC

Proteasome activity assays

Circulating RBCs were lysed in 50 mM HEPES (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 2 mM ATP. 100 μg of protein were used for each assay. Proteasome activity was determined by release of AMC fluorescence from Suc-LLVY-AMC substrate, and quantified against a standard curve, and background fluorescence from parallel reactions containing proteasome inhibitor lactacysteine was subtracted. All reagents were from the 20S proteasome activity assay kit (Millipore).

Supplemental References

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3. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res*. 2003;31(4):e15.
4. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A*. 2005;102(43):15545-15550.
5. Lee CS, Lee C, Hu T, et al. Loss of nuclear factor E2-related factor 1 in the brain leads to dysregulation of proteasome gene expression and neurodegeneration. *Proc Natl Acad Sci USA*. 2011;108(20):8408-8413.
6. Trinklein ND, Chen WC, Kingston RE, Myers RM. Transcriptional regulation and binding of heat shock factor 1 and heat shock factor 2 to 32 human heat shock genes during thermal stress and differentiation. *Cell Stress Chaperones*. 2004;9(1):21-28.
7. Kim HJ, Joo HJ, Kim YH, et al. Systemic analysis of heat shock response induced by heat shock and a proteasome inhibitor MG132. *PLoS ONE*. 2011;6(6):e20252.
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Supplemental Table 1: Low dose bortezomib treatment does not alter erythropoiesis.

Complete blood counts for wild type and Th3/+ mice prior to treatment (A), treated with 0.25 mg/kg bortezomib (B), and 0.5 mg/kg bortezomib (C). $p < 0.001$ by ANOVA for +/+ Ctrl vs Th3/+ Ctrl for RBC, Hb, MCV, MCH, RDW, and retics. *: $p < 0.05$ vs +/+ control; **: $p < 0.01$ vs +/+ control; ***: $p < 0.001$ vs +/+ control; #: $p < 0.05$ vs Th3/+ control; ##: $p < 0.01$ vs Th3/+ control; ###: $p < 0.001$ vs Th3/+ control

(A) Pre-treatment

β globin (*Hbb*) genotype	+/+		Th3/+	
Treatment	Control	Bortezomib	Control	Bortezomib
Number of mice	12	10	11	10
RBC (10^6/uL)	9.82 ± 0.92	9.82 ± 0.42	8.72 ± 0.72	8.88 ± 0.56
Hemoglobin (g/dL)	12.76 ± 1.07	12.88 ± 0.75	8.53 ± 0.67	9.10 ± 0.49
MCV (fL)	49.99 ± 1.56	49.95 ± 1.00	37.95 ± 2.04	38.95 ± 1.29
MCH (pg)	13.01 ± 0.44	13.14 ± 0.62	9.82 ± 0.71	10.26 ± 0.52
MCHC (g/dL)	26.03 ± 0.76	26.29 ± 0.99	25.80 ± 0.83	26.35 ± 0.69
RDW (%)	17.38 ± 0.55	17.18 ± 0.53	28.51 ± 3.28	30.78 ± 1.92
Reticulocytes (%)	2.43 ± 0.37	2.54 ± 0.39	14.41 ± 2.53	14.91 ± 1.70

(B) 2 weeks at 0.25 mg/kg

β globin (*Hbb*) genotype	+/+		Th3/+	
Treatment	Control	Bortezomib	Control	Bortezomib
Number of mice	12	10	11	10
RBC (10^6/uL)	8.994 ± 0.89	9.67 ± 0.55	8.775 ± 0.82	8.18 ± 0.81
Hemoglobin (g/dL)	12.66 ± 1.11	13.50 ± 0.39	9.08 ± 0.75	9.31 ± 0.56
MCV (fL)	50.51 ± 1.97	49.84 ± 2.22	39.07 ± 2.38	40.00 ± 1.43
MCH (pg)	15.73 ± 3.30	14.00 ± 0.60	11.40 ± 2.91	11.45 ± 0.92
MCHC (g/dL)	31.30 ± 7.40	28.15 ± 2.32	29.17 ± 7.51	28.65 ± 2.45
RDW (%)	16.67 ± 0.64	17.77 ± 0.64	28.38 ± 3.38	31.84 ± 1.93 ###
Reticulocytes (%)	2.61 ± 0.42	1.97 ± 0.46	13.72 ± 4.09	11.53 ± 1.54

(C) 2 weeks at 0.50 mg/kg

β globin (*Hbb*) genotype	+/+		Th3/+	
Treatment	Control	Bortezomib	Control	Bortezomib
Number of mice	12	10	11	10
RBC (10^6/uL)	9.87 ± 0.63	9.65 ± 0.45	9.11 ± 0.61	8.60 ± 0.25
Hemoglobin (g/dL)	12.40 ± 0.73	12.04 ± 0.68	8.60 ± 0.64	8.87 ± 0.50
MCV (fL)	45.60 ± 1.05	44.97 ± 0.96	36.41 ± 1.68	39.30 ± 0.98 ###
MCH (pg)	12.58 ± 0.36	12.48 ± 0.53	9.46 ± 0.55	10.33 ± 0.60 ##
MCHC (g/dL)	27.58 ± 0.56	27.74 ± 0.98	25.97 ± 0.93	26.25 ± 1.26
RDW (%)	17.90 ± 0.75	19.98 ± 0.83 *	27.51 ± 2.79	31.54 ± 1.02 ###
Reticulocytes (%)	2.98 ± 0.57	3.68 ± 1.45	13.93 ± 2.90	13.26 ± 2.37

Supplemental Table 2: *In vivo* proteasome inhibition non-specifically impairs erythropoiesis.

Complete blood counts for wild type and Th3/+ mice treated with 1 mg/kg bortezomib at 2 weeks of treatment (A) and 5 weeks (B). $p < 0.001$ by ANOVA for +/+ Ctrl vs. Th3/+ Ctrl for RBC, Hb, MCV, MCH, RDW, and retics. *: $p < 0.05$ vs +/+ control; **: $p < 0.01$ vs +/+ control; ***: $p < 0.001$ vs +/+ control; #: $p < 0.05$ vs Th3/+ control; ##: $p < 0.01$ vs Th3/+ control; ###: $p < 0.001$ vs Th3/+ control

(A) 2 weeks at 1.0 mg/kg

β globin (*Hbb*) genotype	+/+		Th3/+	
Treatment	Control	Bortezomib	Control	Bortezomib
Number of mice	12	10	11	10
RBC (10^6/uL)	9.81 ± 0.59	9.06 ± 0.55 *	8.91 ± 0.68	8.03 ± 0.63 #
Hemoglobin (g/dL)	12.03 ± 0.48	11.46 ± 0.85	8.41 ± 0.37	8.52 ± 0.78
MCV (fL)	45.27 ± 1.13	45.27 ± 0.63	36.05 ± 1.79	39.90 ± 1.70 ###
MCH (pg)	12.28 ± 0.39	12.66 ± 0.62	9.47 ± 0.59	10.62 ± 0.54 ###
MCHC (g/dL)	27.13 ± 0.54	27.97 ± 1.45	26.26 ± 0.64	26.60 ± 1.173
RDW (%)	17.99 ± 0.66	21.95 ± 0.62 ***	27.20 ± 2.91	32.21 ± 0.92 ###
Reticulocytes (%)	3.32 ± 0.52	4.70 ± 0.70	14.44 ± 4.40	18.85 ± 3.20 ##

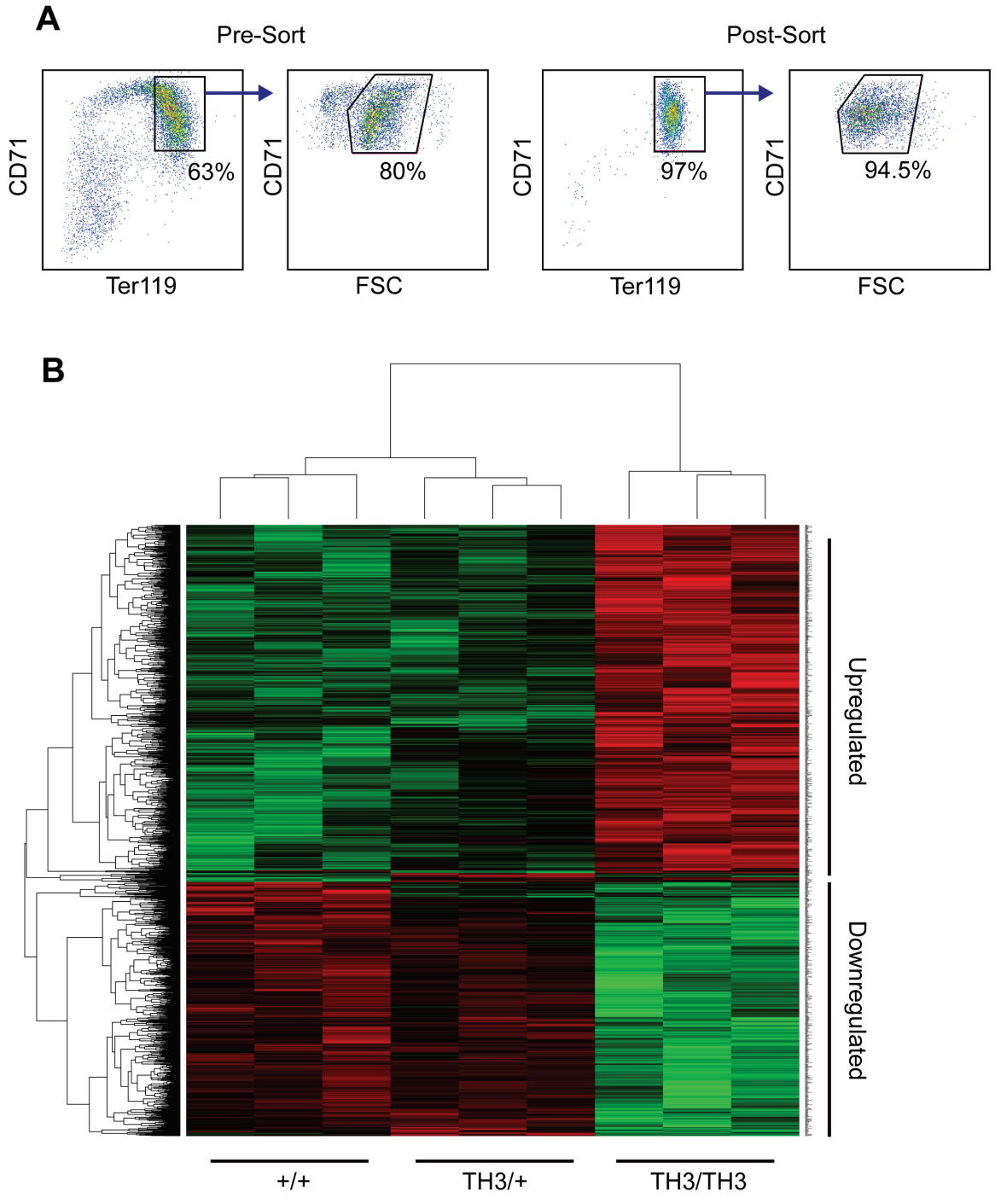
(B) 5 weeks at 1.0 mg/kg

β globin (*Hbb*) genotype	+/+		Th3/+	
Treatment	Control	Bortezomib	Control	Bortezomib
Number of mice	6	6	7	6
RBC (10^6/uL)	9.51 ± 0.55	8.37 ± 0.48 *	8.27 ± 0.60	7.10 ± 0.73 ##
Hemoglobin (g/dL)	12.48 ± 0.51	11.37 ± 0.64 **	8.27 ± 0.33	7.82 ± 0.56
MCV (fL)	47.73 ± 1.11	50.02 ± 0.77 **	38.07 ± 1.27	40.95 ± 1.13 ###
MCH (pg)	13.15 ± 0.55	13.58 ± 0.39	10.04 ± 0.59	11.07 ± 0.60 #
(g/dL)	27.55 ± 0.79	27.15 ± 0.77	26.34 ± 0.83	26.98 ± 0.92
RDW (%)	18.47 ± 0.49	22.42 ± 1.17 **	29.33 ± 2.24	30.27 ± 1.67
Reticulocytes (%)	3.23 ± 0.51	2.50 ± 0.72	14.99 ± 2.77	11.78 ± 2.52 #

Supplemental Figure 1: Gene expression profiling of β-thalassemic erythroblasts.

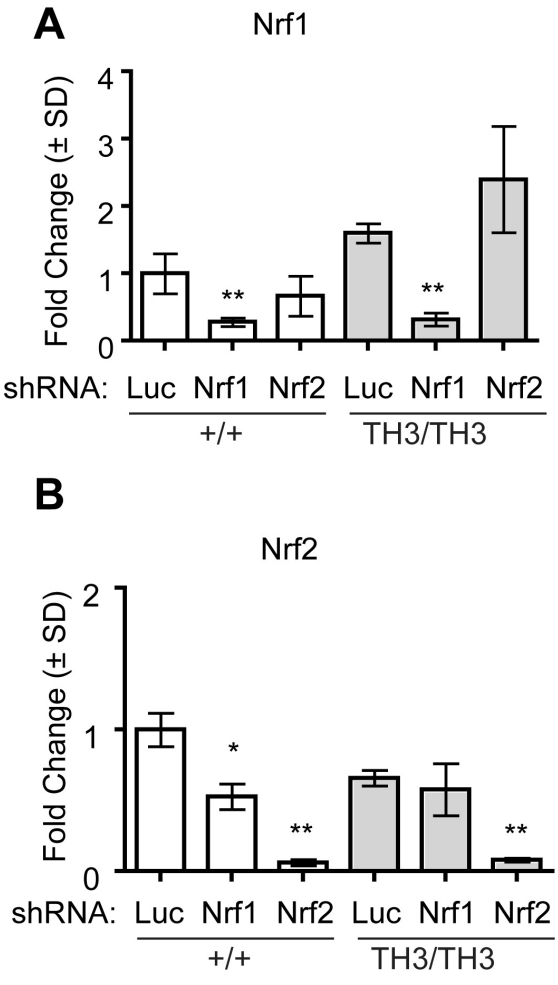
(A) Strategy for flow cytometry purification of $Ter119^{+}CD71^{+}FSC^{high}$ fetal liver erythroblasts from E14.5 embryos of Th3/+ x Th3/+ intercrosses. Sample pre-sort and post-sort flow plots are shown.

(B) Hierarchical clustering of differentially expressed genes ($p < 0.01$ by ANOVA) in erythroblasts of indicated genotypes.



Supplemental Figure 2: Efficacy of Nrf1 and Nrf2 shRNAs

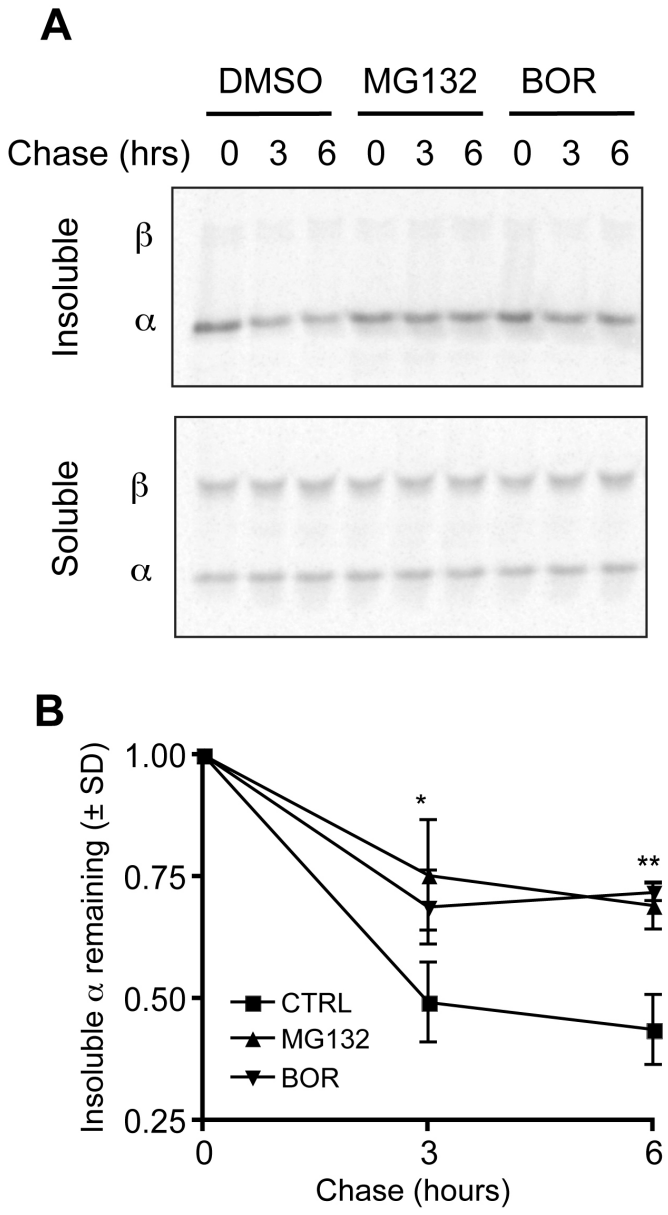
Extent of *Nrf1* **(A)** and *Nrf2* **(B)** knockdown shown for experiments in Figure 4H of main text. mRNAs were quantified by real time RT-PCR and normalized to *β-actin* and *Hprt*. Expression in different experimental samples is compared with luc shRNA from wild type (+/+) mice assigned an arbitrary value of 1. N = 3 embryos per group. * $p < 0.05$ vs Luc; ** $p < 0.01$ vs Luc; *** $p < 0.001$ vs Luc.



Supplemental Figure 3: Bortezomib impairs α globin turnover *ex vivo.*

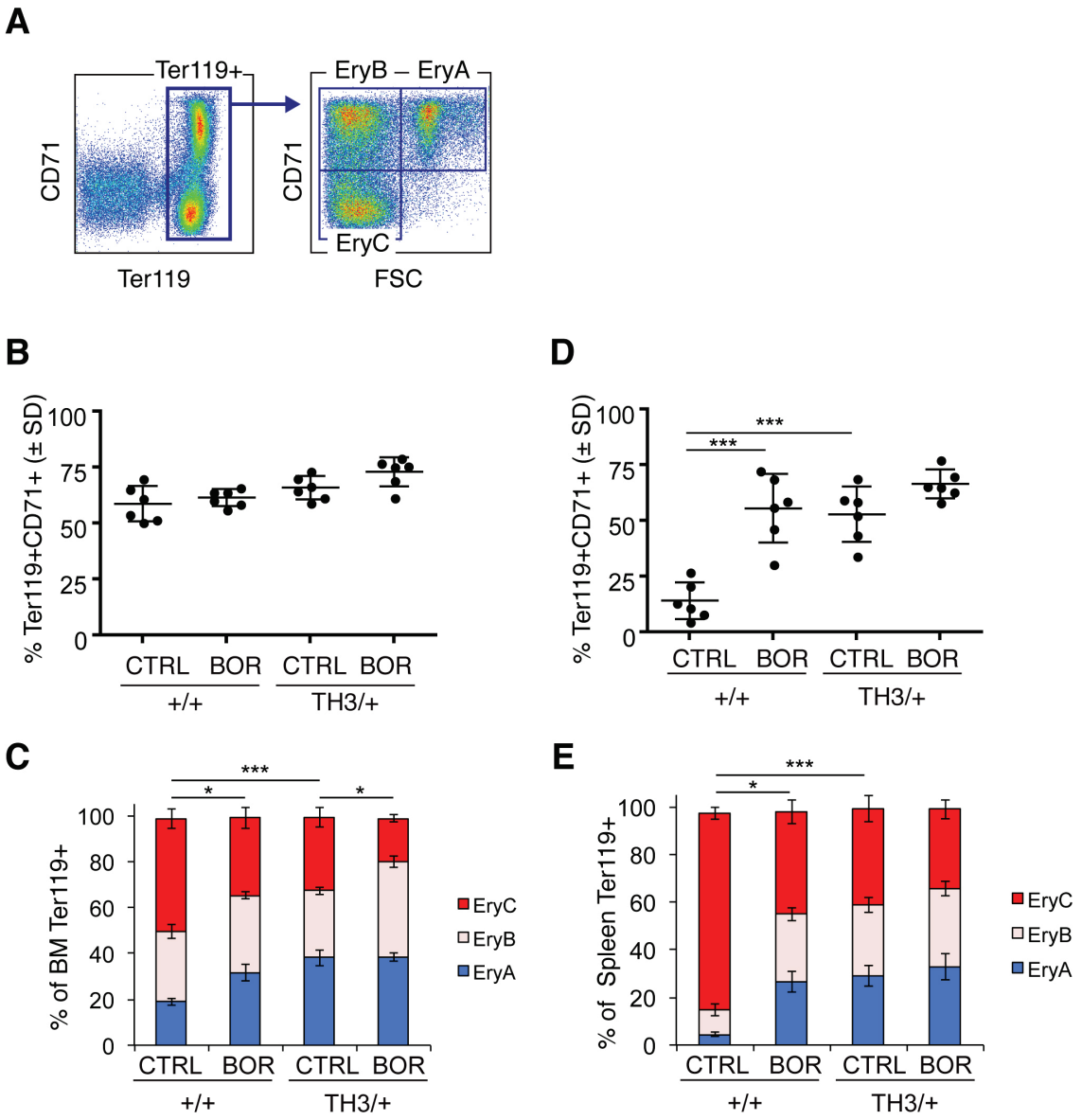
(A) Th3/+ reticulocytes were pulse labeled with ^{35}S-methionine and ^{35}S-cysteine and chased with unlabeled amino acids for the indicated periods of time in the presence or absence of proteasome inhibitors MG132 (10 μM) or bortezomib (100 nM). Soluble and insoluble fractions were obtained by differential centrifugation and analyzed by triton acetic acid urea gel electrophoresis followed by autoradiography.

(B) Quantification of autoradiographs from (A). n = 3 mice, *: $p < 0.05$ vs control (MG132 and bortezomib); ** $p < 0.01$ vs control (MG132 and bortezomib), ANOVA.



Supplemental Figure 4: Bortezomib treatment non-specifically impairs bone marrow and spleen erythropoiesis *in vivo*.

(A) Flow cytometry scheme for analyzing bone marrow and splenic erythroblasts. **(B)** Bone marrow $CD71^{+}Ter119^{+}$ erythroblast populations in bortezomib-treated (BOR) and control (CTRL) mice. **(C)** Bone marrow Ery A, B and C erythroblast populations in bortezomib-treated and control mice. n = 6 mice/group; *: $p < 0.05$; *** $p < 0.001$. **(D)** Spleen $CD71^{+}Ter119^{+}$ erythroblast populations in bortezomib-treated and control mice. n = 6 mice/group; *** $p < 0.001$. **(E)** Splenic Ery A, B and C erythroblast populations in bortezomib-treated and control mice. n = 6 mice/group; *: $p < 0.05$; *** $p < 0.001$.



Supplemental Figure 5: ATP depletion in reticulocytes increases insoluble globin accumulation.

(A) Th3/+ reticulocytes were pulse labeled with ^{35}S-methionine and ^{35}S-cysteine and chased with unlabeled amino acids for the indicated periods of time in the presence or absence of proteasome inhibitor (MG132) or ATP depletion. Soluble and insoluble fractions were obtained by differential centrifugation and analyzed by triton acetic acid urea gel electrophoresis followed by autoradiography.

(B) Quantification of autoradiographs from (A). n = 3 mice, *: $p < 0.05$ vs control; ** $p < 0.01$ vs control or MG132, ANOVA.