Efficient CRISPR-Cas9 mediated gene disruption in primary erythroid progenitor cells

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SUPPLEMENTAL DOCUMENT

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Author Contributions

H.L., J.S., and H.F.L. designed the experiments. H.L., J.S., N.J.H, N.P., A.N., and J.C.E. performed the experiments. H.L., J.S., and H.F.L. wrote the manuscript.

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Supplemental Methods

Plasmids

The SpCas9 cDNA was PCR amplified from plasmid pX330[16] (a kind gift of Dr. Feng Zhang, Broad Institute) using Phusion polymerase (New England Biolabs) and cloned into the pXZ201 MSCV retroviral vector plasmid[17] using EcoRI (New England Biolabs) and XhoI (New England Biolabs) restriction sites. The 2A-GFP cDNA was PCR amplified from pXZ201 using Phusion polymerase and an extended primer encoding the 2A cDNA and cloned in 3' to the SpCas9 cDNA using a SalI (New England Biolabs) restriction site. The self-cleaving 2A peptide sequence was constructed as previously described.[18] The U6 promoter, BbsI restriction site, sgRNA construct was PCR amplified from plasmid pX330 using Phusion polymerase and cloned in reverse orientation 3' to the GFP cDNA using a NotI (New England Biolabs) restriction site. Specific guide RNAs were synthesized as complimentary oligos (Integrated DNA Technologies), phosphorylated with T4 polynucleotide kinase (New England Biolabs), annealed, and cloned into the BbsI (New England Biolabs) site.

Retrovirus production

The 293T cells used for transfection were split and plated at 6 million cells per 10 cm plate on day -1 in antibiotic - free Dulbecco's Modified Eagle Medium (DMEM) with added 15% Fetal Bovine Serum (FBS) and 2 mM L-Glutamine (Invitrogen). On day 0, 10 ug plasmids were transfected into 293T cells together with 5 µg

packaging vector (pCL-Eco, IMGENEX) using Fugene 6 (Promega). Six hours later, the culture medium was replaced with DMEM with added 15% FBS, 2 mM L-Glutamine and 1× Pen Strep (Invitrogen). On day 1, fresh virus - containing supernatant was collected, filtered through 0.45 μm filter (Millipore), and used immediately to infect the murine lineage negative fetal liver cells.

Isolation of erythroid progenitors from murine E14.5 fetal liver cells

Day 14.5 pregnant C57BL/6J mice were anesthetized by carbon dioxide. The embryos were isolated and the entire fetal livers were carefully collected in Phosphate Buffered Saline (PBS) with 2% Fetal Bovine Serum (FBS) and 100 μM EDTA. After suspension by pipette tips and filtration through a 70 μm filter (BD), the fetal liver cells were incubated with Ammonium Chloride Solution (Stemcell) for lysis of red blood cells. Ten minutes later, the remaining fetal liver cells were centrifuged at 300x(g) for 5 minutes, and resuspended in PBS. Following the manufacturer's protocol, lineage negative cells were obtained after depletion of lineage positive cells magnetically using BD Biotin Mouse Lineage Panel (559971) and BD Streptavidin Particles Plus – DM (557812). The detail protocol was described previously.[5] One fetal liver resulted in the isolation of 500,000 lineage-negative progenitor cells.

Viral infection and culture of erythroid progenitors

After isolation, lineage negative fetal liver cells were plated in 24-well plates with 100,000 cells per well, covered by 1 ml virus containing supernatant, and centrifuged at 400x(g) for 90 min at 37°C. After spin-infection, we replaced the virus supernatant with erythroid maintenance medium (StemSpan-SFEM (StemCell Technologies) with added recombinant mouse stem cell factor (100 ng/ml SCF, R&D), recombinant mouse IGF-1 (40 ng/ml, R&D), dexamethasone (100 nM, Sigma) and erythropoietin (2 u/ml, Amgen)). The cells were cultured overnight or for up to three days for recovery and expression of transgenes. Then infected cells were pooled and sorted for green fluorescence by FACS sorting at flow rate 2. The GFP+ cells were cultured in Epo-only erythroid differentiation medium (Iscove modified Dulbecco's medium (IMDM) containing 15% FBS (Stemcell), 1% detoxified bovine serum albumin (BSA) (Stemcell), 500 μg/mL holo-transferrin (Sigma-Aldrich), 0.5 U/mL Epo (Amgen), 10 μg/mL recombinant human insulin (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen) and 1× Pen Strep (Invitrogen)).

Flow cytometry (FACS) sorting and analysis

The cells were pooled, washed once in PBS and resuspended at density of 5-10 million/ml in PBS with 1 μg/ml PI for FACS sorting or analysis. Ter119 staining was performed using APC Rat Anti-Mouse TER-119 (BD Biosciences) and CD71 staining was performed using PE Rat Anti-Mouse CD71 (BD Biosciences) as per manufacturer's instructions. FACS was performed using an ARIA-II sorter

(Becton-Dickinson) and flow cytometry was performed using a Fortessa flow cytometer (Becton-Dickinson).

Protein isolation and Western blotting

One million cells after 48 hours of differentiation were pelleted, resuspended in RIPA lysis buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.25% sodium deoxycholate), and incubated for 30 min on ice. After centrifugation at 14,000 rpm in an Eppendorf table-top centrifuge for 5 min at 4°C to remove debris, the supernatant was transferred to a new tube, mixed with sample loading buffer (Invitrogen), and incubated for 10 min at 90°C. The NuPAGE Bis-Tris gel system (Invitrogen) was used to separate proteins, which subsequently were transferred to a nitrocellulose membrane using NuPAGE transfer buffer (Invitrogen). Membranes were blocked with 3% BSA-PBST for 1 h and probed with primary antibody (anti-BCL11A(CTIP1) from Abcam, anti-FLAG from Sigma, anti-Lamin A/C from cell signaling technology, anti-Lamin B from Santa Cruz, anti-alpha globin from Santa Cruz, and anti-GAPDH from Abcam) at 1:1,000 dilution in 3% BSA-PBST overnight at 4°C. Membranes were washed three times with PBST, incubated with corresponding peroxidase-coupled secondary antibodies at a 1:10000 dilution in 3% BSA-PBST for 1 h at room temperature, washed twice with PBST, and incubated for 1 min with Western Lightning Plus-ECL substrate (Perkin Elmer). Proteins were visualized by exposure to scientific imaging film (Kodak). Densitometry was calculated using the ImageJ (freely available from NIH) program.

RNA isolation and real-time PCR

RNA from 500,000 cells after 48 hours of differentiation was isolated using Trizol and purified using RNeasy columns (Qiagen). Real-time PCR was performed using a 7900HT RT-PCR machine (Applied Biosystems) and SYBR green 2X PCR master mix (Applied Biosciences). Mouse epsilon-gamma transcripts were amplified using primers Exon1 For- TGGCCTGTGGAGTAAGGTCAA, and Exon2 Rev- GAAGCAGAGGACAAGTTCCCA, and normalized using the delta-delta Ct method to GAPDH transcripts amplified using primers For-AGGTTGTCTCCTGCGACTTCA and Rev- CCAGGAAATGAGCTTGACAAA.

Detection of gene disruption frequency

DNA from 1 million cells 72 hours post-transduction was isolated using the MasterPure complete DNA purification kit (Epicentre Biotechnologies). Specific genetic loci were PCR amplified using Accuprime Supermix II (Invitrogen) and the corresponding primers listed in Supplemental Table 1. PCR reactions were then treated with Surveyor nuclease (Integrated DNA technologies) as previously described,[19] resolved on a 2% agarose gel, and visualized with ethidium bromide. Images were captured using a UV detection camera (Canon) and band intensities were quantified using the ImageJ (freely available from NIH) program.

Terminal differentiation analysis
Cell numbers used for cellular proliferation calculations were counted using a standard hemocytometer. Hemoglobin content was measured using Drabkin's reagent and compared to a standard curve.

Gene expression changes in erythroid differentiation by RNA-seq
RNA sequencing expression data for *Lmna* and other structural proteins from experiments by Wong, et al.[2] were accessed through Gene Expression Omnibus database under the accession number GSE27893.

Statistics
Standard deviations were calculated using Microsoft Excel and Graphpad Prism. Statistical significance between comparison groups was calculated using Student's T-Test.

Methods References

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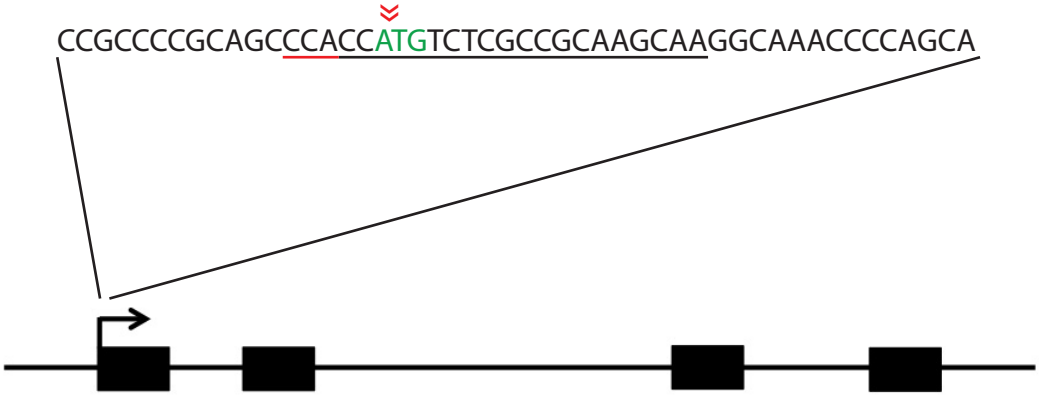
Supplemental Table S1

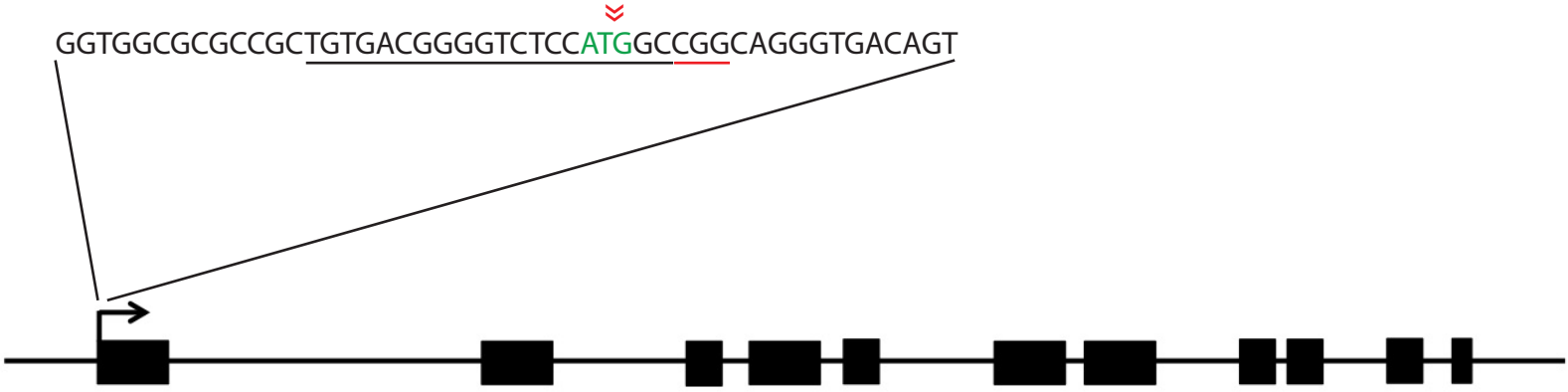
Gene	Guide sequence	Surveyor assay forward primer	Surveyor assay reverse primer
Bcl11a	TTGCTTGCGGCGAGACATGG	CCCTGCGCCATCTTTGTATTATTTCT	CTCAAAAGTGCACACGGTTCATG
Gata3	GAGCACAGCCGAGGACATGG	GTTAAAAAGTACGTCCACCTCTTCCG	GGGATCGCCCTCATTCTTTTCTT
Lcp2	CGGGACATTCTTCAAGGCCA	GCAGAAAAGAGTCCTCTTCAACAAGG	TGTAGCTGAGGGCCCTCC
Lmna	TGTGACGGGGTCTCCATGGC	CGCACGCGATCGATGTACA	CCCTGTAGAGGAGGGCCTATTAG

CRISPR DNA sequences. For all 4 genes targeted, guide sequences and primers used for the Surveyor nuclease assay are listed.

Supplemental Figure S1



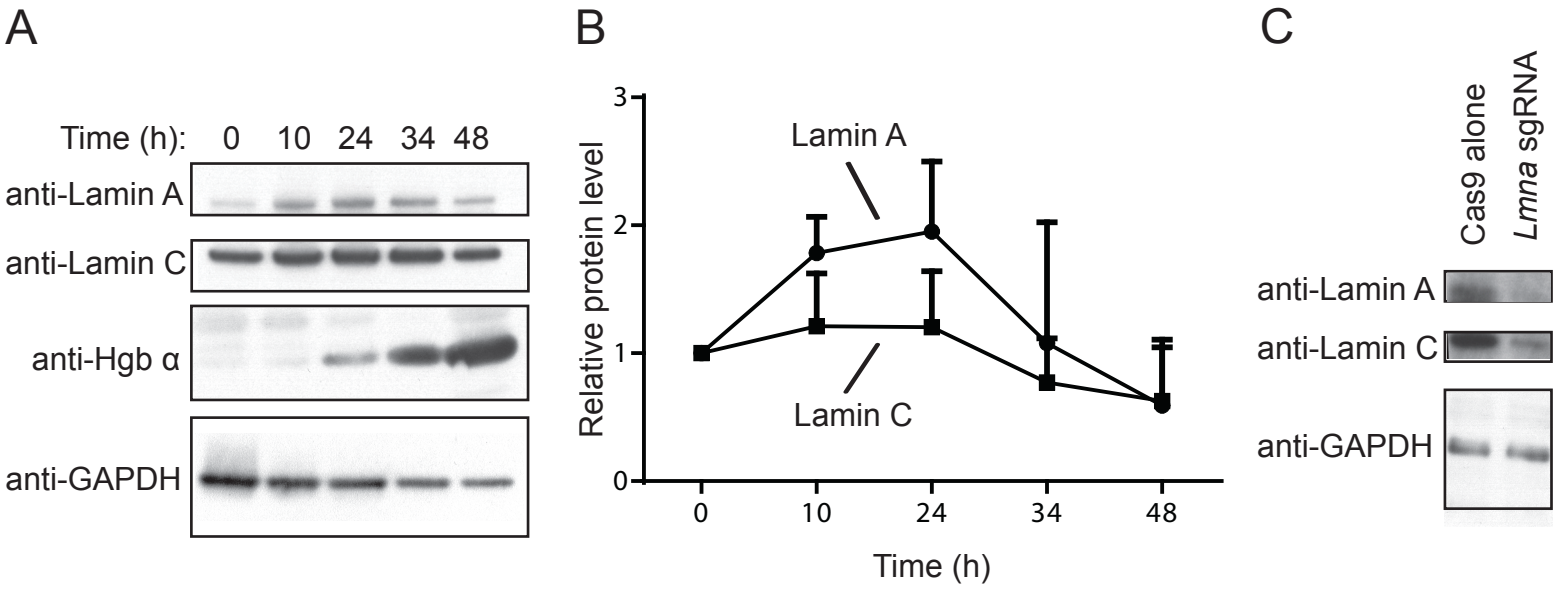
Bcl11a



Lmna

CRISPR-mediated cleavage sites for *Bcl11a* and *Lmna.* For both genes, sgRNA guide sequence is underlined in black. Protospacer adjacent motif (PAM) is underlined in red. Start codon is highlighted in green. Cleavage site denoted with double red arrow.

Supplemental Figure S2



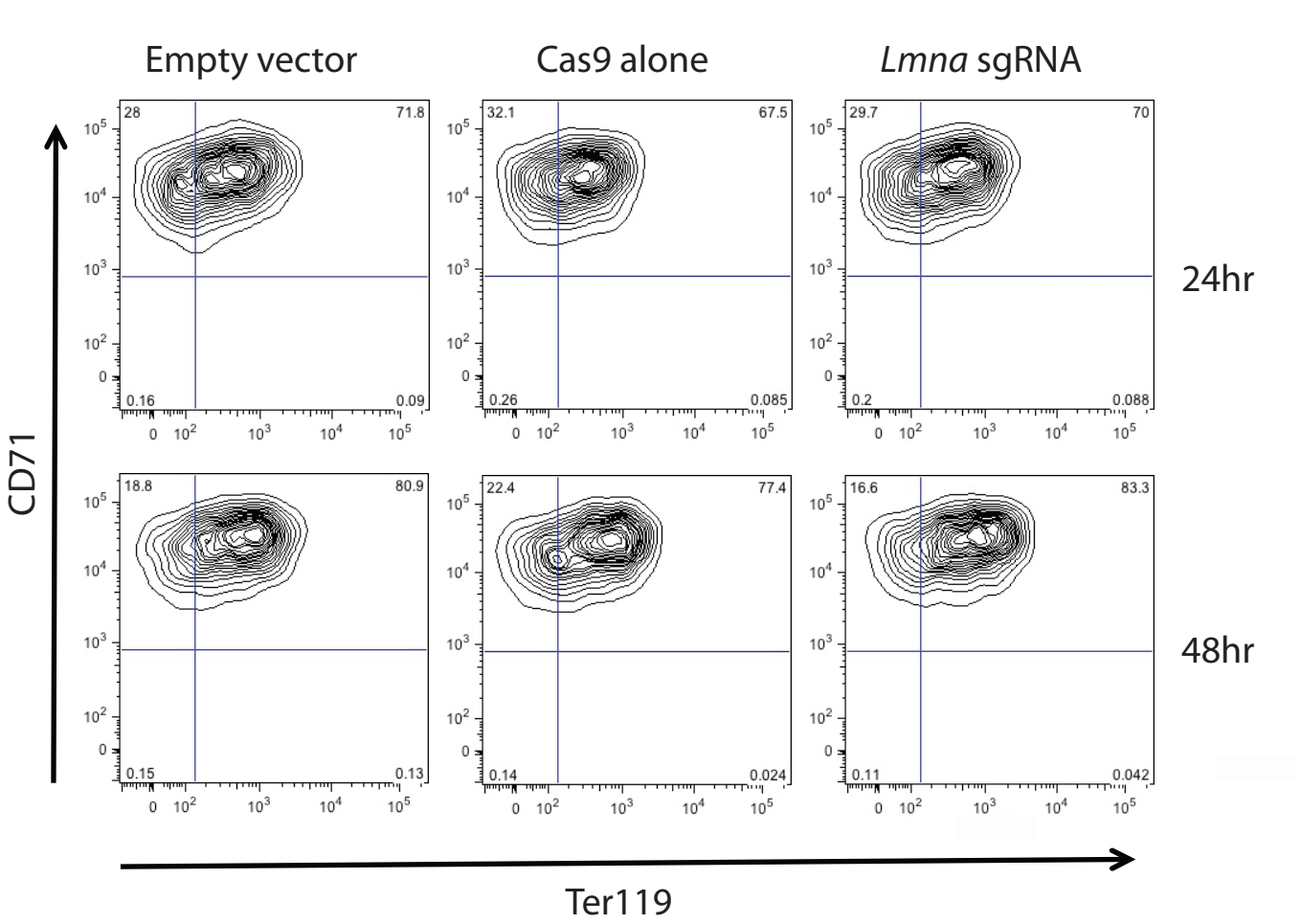
Supplemental Figure S2 – More dynamic regulation of Lamin A than Lamin C during erythroid differentiation and expression loss following CRISPR-Cas9-mediated *Lmna* disruption.

(A) Western blot comparing Lamin A and C protein levels following transition of fetal liver erythroid progenitors to differentiation culture. One million cells were loaded per lane.

(B) Densitometry quantification of relative changes in Lamin A and C protein levels following transition of fetal liver erythroid progenitors to differentiation culture. Each data point represents the mean of 3 biological replicates with error bars denoting standard deviation.

(C) Western blot assessing levels of Lamin A and C protein in fetal liver erythroid progenitor cells following delivery of Cas9 with and without an sgRNA targeting *Lmna*.

Supplemental Figure S3



Supplemental Figure S3 – CD71 and Ter119 expression during erythroid differentiation.
(A) Representative flow cytometry plots of CD71 and Ter119 at various time points during erythroid differentiation culture following 3 days in erythroid maintenance culture.
(B) Percentage of cells expressing both CD71 and Ter119 after 3 days in erythroid maintenance culture and 48 hours in erythroid differentiation culture. Data represent the mean of 3 biological replicates and error bars denote standard deviation. Differences between the 3 conditions did not reach statistical significance when calculated using Student's T-test. Data at 24 hours was similar to 48 hours.