

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

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The study of isolated primary progenitor cells offers great insight into developmental biology and human disease. In particular, *ex vivo* culture of isolated primary erythroid progenitor cells replicates the differentiation events that occur during *in vivo* erythropoiesis. Here we report a high-efficiency method for CRISPR-Cas9 mediated gene disruption in isolated primary erythroid progenitor cells. We use this method to generate the novel result that *Lmna* is required in terminal erythroid differentiation.

Terminal erythroid differentiation is initiated by binding of Erythropoietin (Epo) to Epo receptors on the surface of CFU-E erythroid progenitors, leading to 3 to 6 terminal divisions, induction of ~600 genes, and repression of thousands of others in a defined temporal order. Concomitantly there are major changes in cytoskeletal and nuclear architecture, including condensation of the nucleus to about one-tenth its original volume and, only in mammals, enucleation.^{1,2,3} Numerous human diseases cause disordered erythropoiesis and result in significant morbidity and mortality. In many cases the underlying mechanisms of disease are incompletely understood, and more importantly, identification of disease modifier genes and their function in terminal erythropoiesis is incomplete.⁴

Ex vivo culture and differentiation of isolated primary mouse erythroid progenitor cells faithfully mimics the cellular events that occur during *in vivo* erythropoiesis, including proliferation, upregulation of erythroid-specific genes and cell surface markers, accumulation of hemoglobin, and enucleation.⁵ RNA interference has been used for the study of gene function in erythropoiesis, but the use of small hairpin RNAs is complicated

by difficulty in predicting efficiency of gene knockdown and non-specific effects.⁶ Several genetic determinants of erythropoiesis have been assessed using erythroid progenitors isolated from gene knock-out mice,^{7,8} but this is a laborious process involving *de novo* generation of individual mouse lines that is not amenable to high-throughput assessment of candidate genes.

CRISPR-Cas9 technology has allowed rapid genetic loss of function in many organisms via nuclease-mediated gene disruption.^{9,10} Intracellular delivery of the prokaryotic Cas9 nuclease complexed with a single-guide RNA (sgRNA) molecule results in sgRNA-directed cleavage of a specific genomic site by Cas9. The DNA double strand break induced by Cas9 cleavage is then frequently repaired by the endogenous non-homologous end joining (NHEJ) DNA repair pathway, resulting in insertion and deletion (indel) mutations that disrupt gene expression.^{9,10}

However, successful use of CRISPR-Cas9 in isolated primary erythroid cells has not yet been described. Here we report a highly efficient method for delivering the *Streptococcus pyogenes* Cas9 protein and an sgRNA to isolated primary erythroid progenitor cells. Cas9 and sgRNA delivery with this method results in robust genetic loss of function through gene disruption, and we use this method to identify a novel gene required in terminal erythropoiesis.

To deliver Cas9 and an sgRNA to primary erythroid progenitor cells, we constructed a murine stem cell virus (MSCV)-based retroviral vector co-expressing Cas9 and green

fluorescent protein (GFP) linked by a self-cleaving 2A peptide to mediate equimolar expression of Cas9 and GFP from a single Pol II promoter cassette. This vector also expresses an sgRNA from a Pol III promoter cassette (Figure 1A). Using this vector we transduced primary lineage negative erythroid progenitor cells purified from E14.5 mouse fetal livers, and cultured them in an erythroid progenitor expansion media containing cytokines that promote proliferation but not differentiation. GFP positive cells were isolated by fluorescence activated cell sorting (FACS) between 24 to 72 hours post-transduction depending on the desired degree of cell expansion (Figure 1B). Importantly, our retroviral vector delivering Cas9 and sgRNA was capable of high efficiency transduction such that the majority of the progenitor cell population was successfully transduced (Figure 1C). GFP positive cells were then placed in erythroid differentiation medium for 48 hours (Figure 1B).

To assay gene disruption efficiency we co-delivered Cas9 with three different sgRNAs (Supplemental Table S1), one targeting the start codon of the *Gata3* gene, which encodes a T-lymphocyte specific transcription factor, one targeting the 5' end of the *Lcp2* gene, which encodes a signal-transduction adaptor protein, and one targeting the start codon of the *Bcl11a* gene, which encodes a multi-lineage transcription factor that is expressed in erythroid precursor cells. For all 3 vectors, transduction efficiency was greater than 50% when performing FACS for GFP positive cells (data not shown). Using the Surveyor nuclease assay on GFP positive cells, we detected robust generation of indels in each of these genes (Figures 1D-F).

As the Surveyor nuclease assay suggested the presence of a mixed population of bi-allelically disrupted, haploinsufficient, and wild-type cells, we sought to assess if the observed gene disruption rates resulted in loss of protein expression and an observable phenotype. To determine if gene disruption through indel generation resulted in loss of protein expression, we assessed levels of the protein encoded by the *Bcl11a* gene and found nearly 80% decreased expression in cells transduced with vector expressing Cas9 and an sgRNA targeting *Bcl11a* (Figure 1G), indicating the percent indels reflected by the Surveyor assay only provided a minimum estimate of gene inactivation. As *Bcl11a* controls the developmental switch to adult hemoglobin in mammalian erythropoiesis and mouse models genetically deficient in *Bcl11a* have elevations in $\epsilon\gamma$ globin gene expression,⁷ we determined whether primary erythroid progenitors had elevations in $\epsilon\gamma$ globin gene expression following disruption of the *Bcl11a* gene. In reticulocytes differentiated from progenitor cells transduced with vector expressing Cas9 and an sgRNA targeting *Bcl11a*, there was a greater than fifty-fold increase in $\epsilon\gamma$ globin mRNA, replicating the phenotype observed in mice genetically deficient in *Bcl11a* (Figure 1H).

We next used this system to identify a novel regulator of terminal erythroid differentiation. As prior studies of erythropoiesis had not placed significant emphasis on structural proteins, we analyzed expression data for all genes encoding such types of proteins. *Lmna*, the gene encoding the alternatively spliced nuclear envelope proteins Lamins A and C, exhibited the greatest increase in expression during terminal erythroid differentiation, with a 4 to 6-fold increase in relative expression quantified by RNA-seq methods.² This was in contrast to Lamin B, a protein that complexes with Lamins A and

C to support nuclear membrane architecture and control transcriptional regulation, but is downregulated during terminal erythroid differentiation.^{3,11,12} Interestingly, Lamin A protein exhibited a marked initial increase during terminal differentiation followed by a marked decrease (Figures 2A, 2B), whereas Lamin C protein levels were not as dynamic (Supplemental Figure S2).

We designed an sgRNA targeting the start codon of *Lmna* (Supplemental Figure S1). Retroviral vector transduction resulted in successful delivery of Cas9 and the *Lmna* sgRNA to greater than 50% of isolated primary erythroid progenitors, as assessed by frequency of GFP positive cells when performing FACS (data not shown). Delivery of Cas9 and the sgRNA targeting *Lmna* to primary erythroid progenitors resulted in efficient introduction of indels in the *Lmna* gene (Figure 2C), and in reduction of Lamins A (Figure 2D) and C (Supplemental Figure S2) protein levels. Progenitors with a disrupted *Lmna* gene showed impaired proliferation (Figure 2E) as well as a decreased ability to accumulate hemoglobin (Figure 2F). Many cells underwent apoptosis (data not shown). There was no difference in upregulation of cell surface markers CD71 and Ter119 (Supplemental Figure S3), which are relatively earlier events during terminal erythroid differentiation.¹ These results are the first demonstration that expression of *Lmna* is dynamically regulated during erythropoiesis and that it plays a significant role in terminal red cell differentiation. Although an anemia phenotype has not been reported for *Lmna* -/- mice, they do have decreased growth by 3 weeks and die by 8 weeks of life;¹³ it will be interesting to determine whether young *Lmna* -/- mice do exhibit an anemia phenotype under both normal and stress erythropoiesis conditions.¹⁴ Further biochemical analyses

will be instrumental in determining the underlying mechanism by which *Lmna* regulates terminal erythroid differentiation.

The method we developed for using CRISPR-Cas9 to study genetic loss of function in isolated primary erythroid progenitor cells provides a broadly generalizable, rapid, and facile assessment of gene function in red blood cell development, with a total duration of 3 to 5 days from progenitor cell isolation to phenotypic assessment. Although off-target effects of the CRISPR-Cas9 system are of concern, high-throughput sequencing or array CGH methods can be used to identify off-target cleavage events, and rescue experiments using cDNAs of targeted genes where codons have been substituted to ablate sgRNA binding without changing the amino acid sequence can confirm whether the observed phenotype is due to on-target or off-target gene disruption. Additionally, the use of novel Cas9 variants with high specificity will mitigate many of these concerns.¹⁵ Our system has significant strengths in the high transduction rate of our viral vector co-expressing Cas9 and an sgRNA, and the high efficiency with which gene disruption can be achieved, both of which are key considerations when studying rare and difficult-to-isolate cell populations such as hematopoietic progenitors. Indeed, the ability to achieve high-efficiency gene disruption using this system allows the possibility of robust large-scale genetic loss-of-function screening in erythropoiesis.

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Figure Legends

Figure 1 – CRISPR-Cas9 expression in primary fetal liver erythroid progenitors mediates disruption of multiple genes and loss of *Bcl11a* gene function.

(A). Diagram of MSCV-based retroviral vector co-delivering Cas9-GFP and sgRNA.

(B) Schematic of experimental procedure including cytokines used in different culture phases.

(C) Flow cytometry assessment of efficiency of Cas9 and sgRNA delivery to fetal liver erythroid progenitor cells as quantified by GFP expression.

(D-F) Surveyor nuclease assay of gene disruption efficiency in fetal liver erythroid progenitor cells in the *Gata3*, *Bcl11a*, and *Lcp2* genes following delivery of Cas9 with indicated sgRNAs or of Cas9 alone. Arrows denote cleavage products and lanes without indel quantification indicate less than 1% disruption efficiency.

(G) Western blot assessing level of BCL11A protein and FLAG-tagged Cas9 in fetal liver erythroid progenitor cells following delivery of Cas9 with and without sgRNA targeting *Bcl11a*. Relative expression differences of BCL11A protein quantified by densitometry.

(H) RT-PCR quantification of relative $\epsilon\gamma$ globin mRNA levels in reticulocytes following delivery of Cas9 with *Bcl11a* sgRNA or Cas9 alone. Data are represented as mean of 3 biological replicates and error bars denote standard deviation. P-value calculated using Student's T-test.

Figure 2 – Dynamic regulation of Lamin A during erythroid differentiation and impaired terminal erythroid differentiation following CRISPR-Cas9-mediated *Lmna* disruption.

(A) Western blot of Lamin A 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 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) Surveyor nuclease assay of disruption efficiency of the *Lmna* gene in fetal liver erythroid progenitor cells following delivery of Cas9 with indicated sgRNAs or Cas9 alone. Arrows denote cleavage products and lanes without indel quantification indicate less than 1% disruption efficiency.

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

(E) Proliferation of fetal liver erythroid progenitors in differentiation medium following delivery of empty vector, or Cas9 with and without *Lmna* sgRNA. Data points represent mean of 3 biological replicates and error bars denote standard deviation. P-values comparing Cas9 with *Lmna* sgRNA to other conditions calculated using Student's T-test.

(F) Hemoglobin content per reticulocyte generated from fetal liver erythroid progenitors following delivery of empty vector, or Cas9 with and without sgRNA targeting *Lmna*.

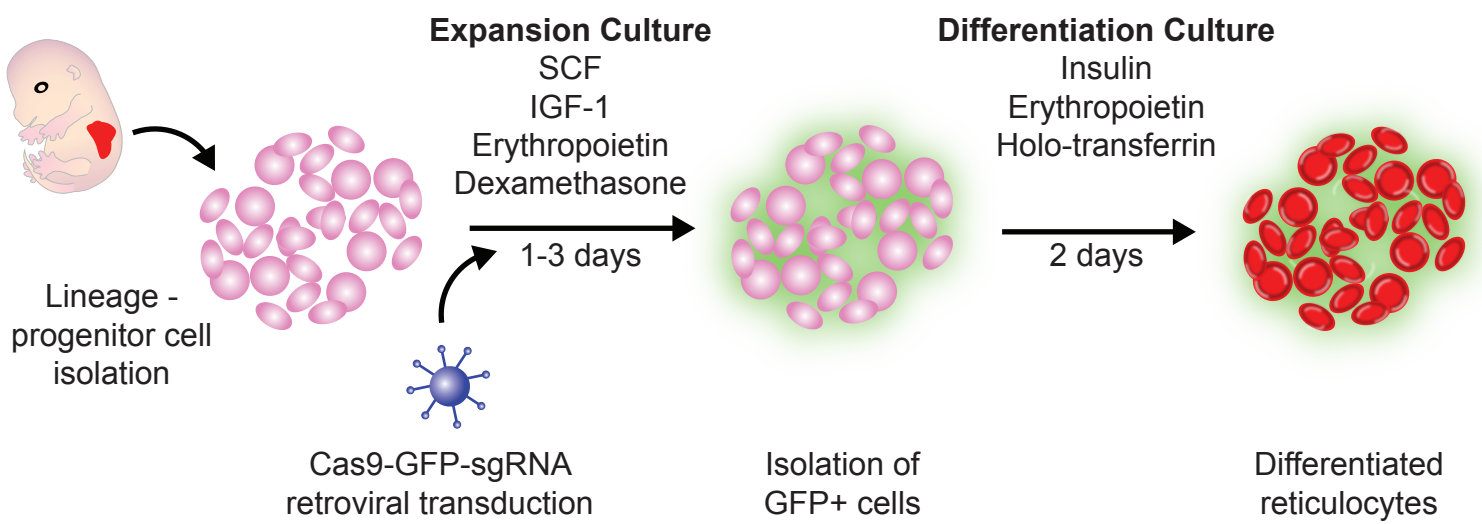
Data represent the mean of 3 biological replicates and error bars denote standard deviation. P-value calculated using Student's T-test.

Figure 1

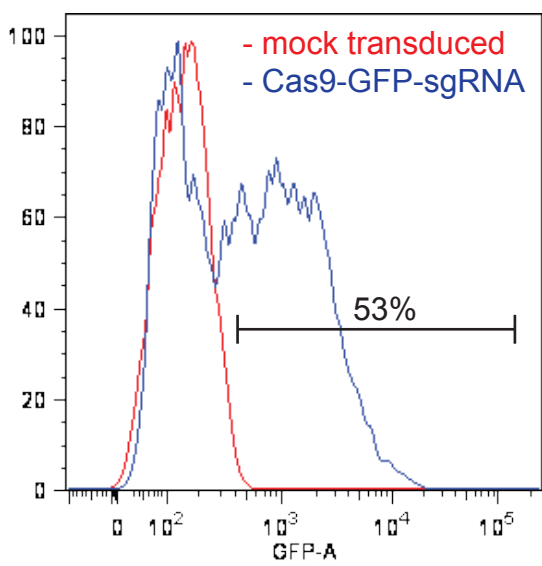
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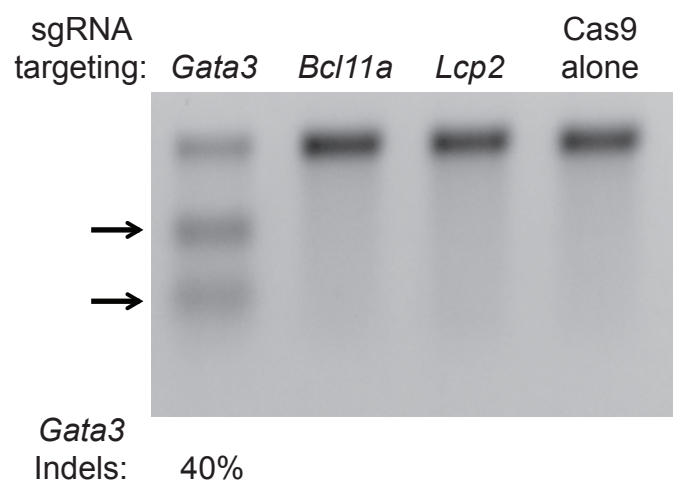
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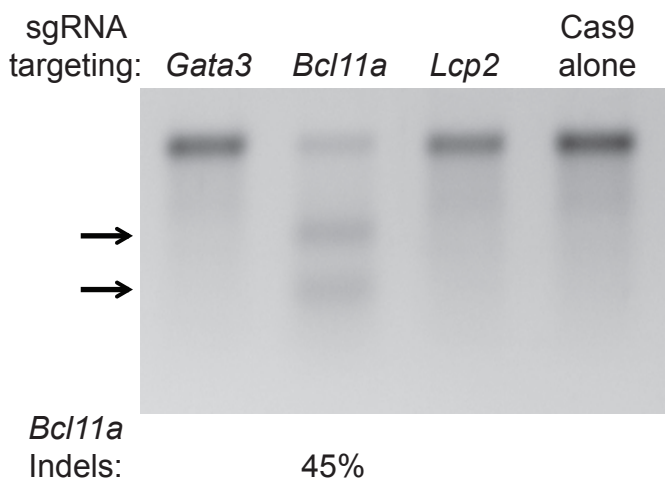
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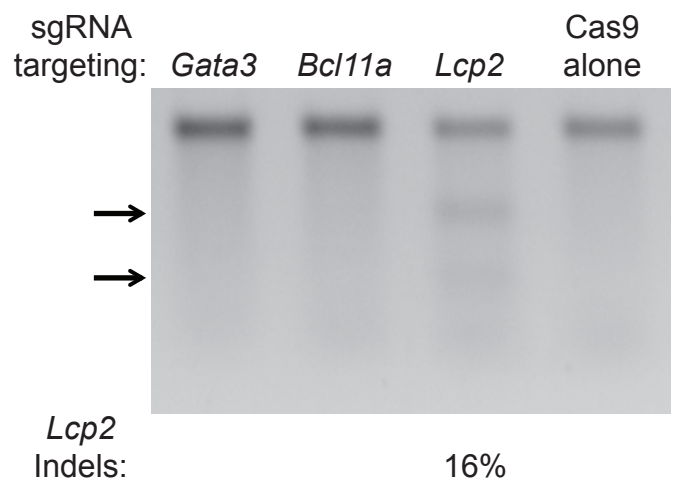
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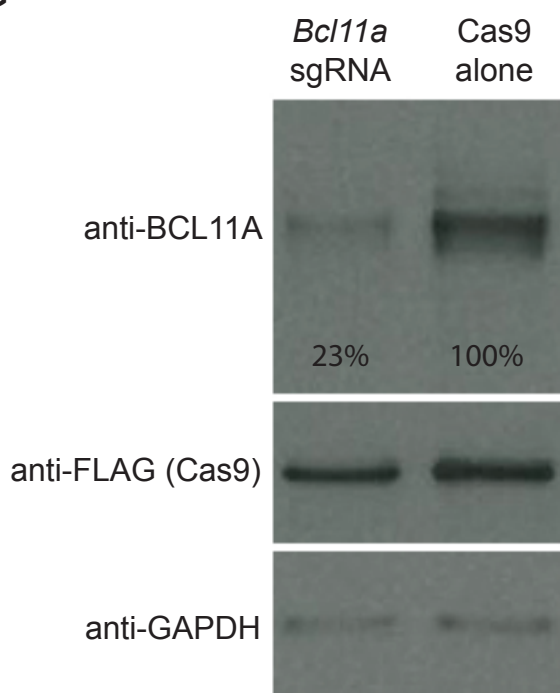
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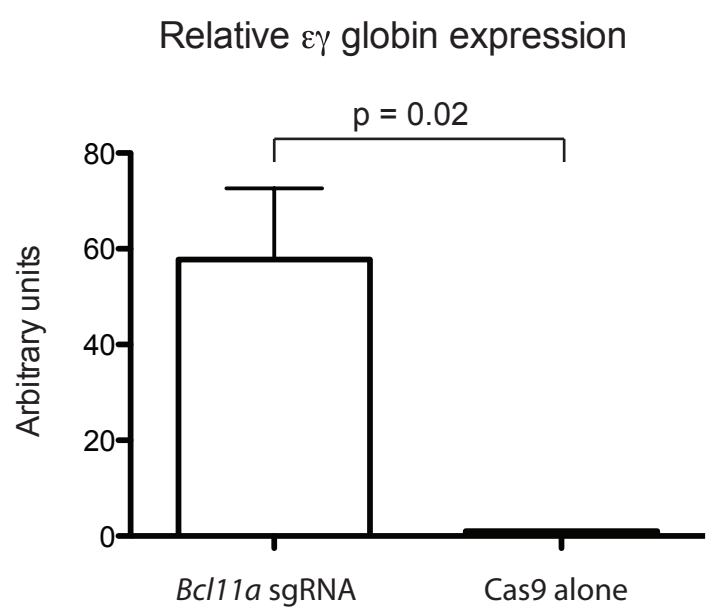
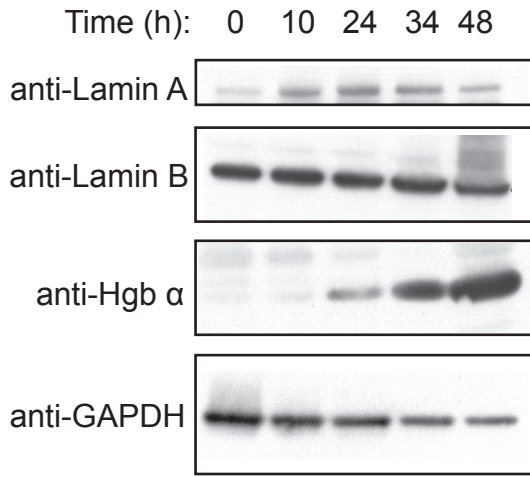
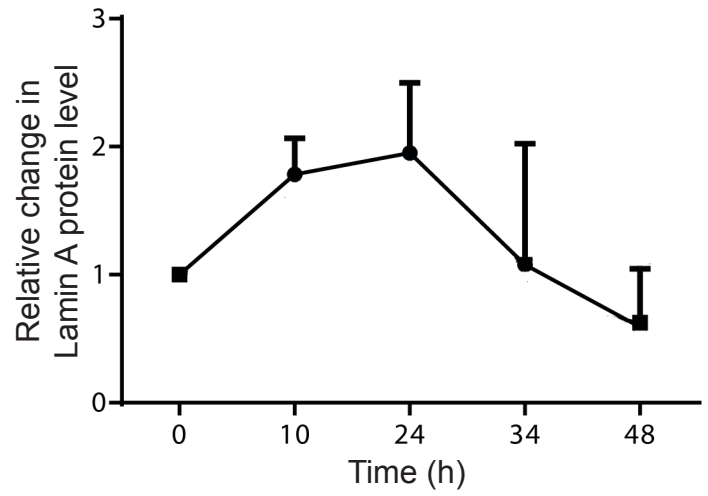


Figure 2

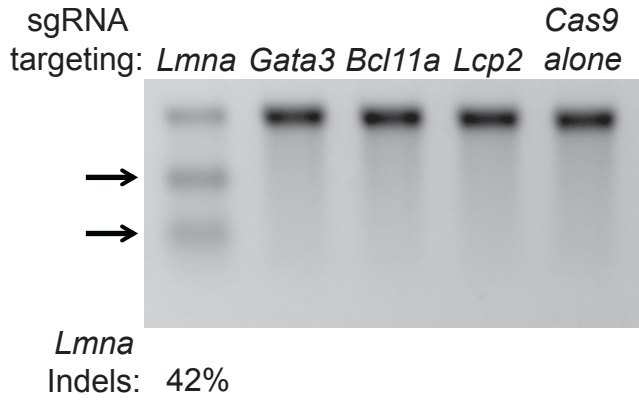
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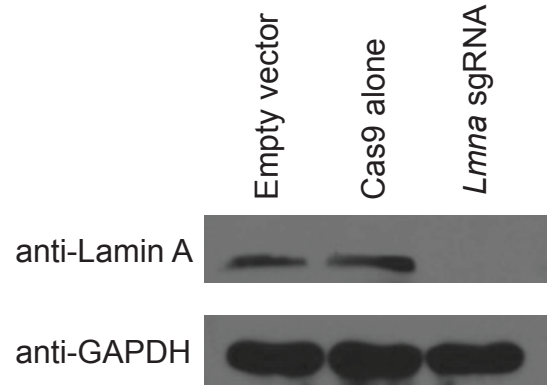
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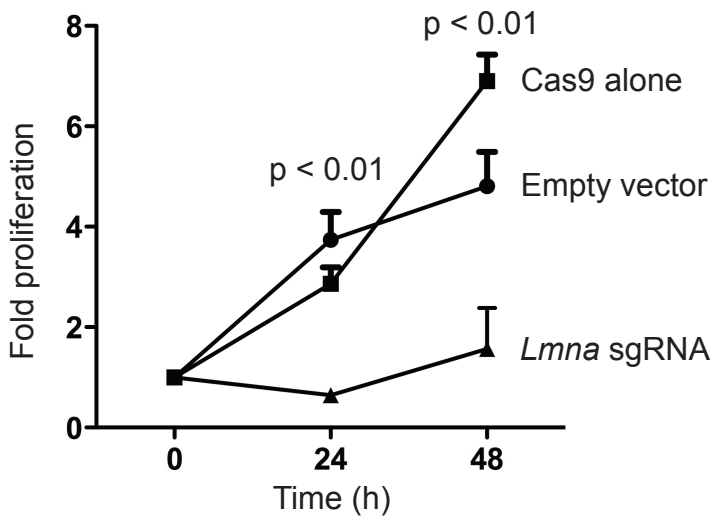
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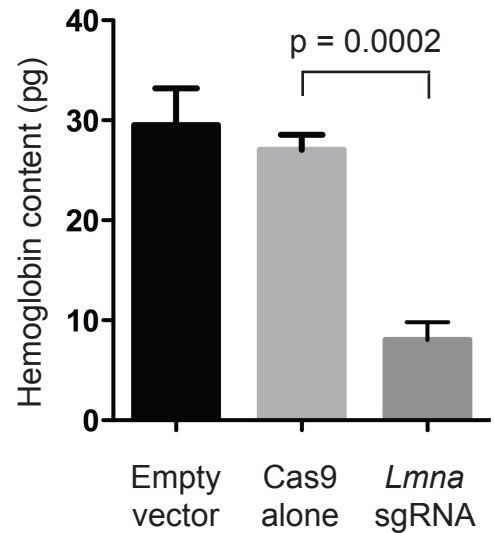
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SUPPLEMENTAL DOCUMENT

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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 pX330¹⁶ (a kind gift of Dr. Feng Zhang, Broad Institute) using Phusion polymerase (New England Biolabs) and cloned into the pXZ201 MSCV retroviral vector plasmid¹⁷ 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 Sall (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

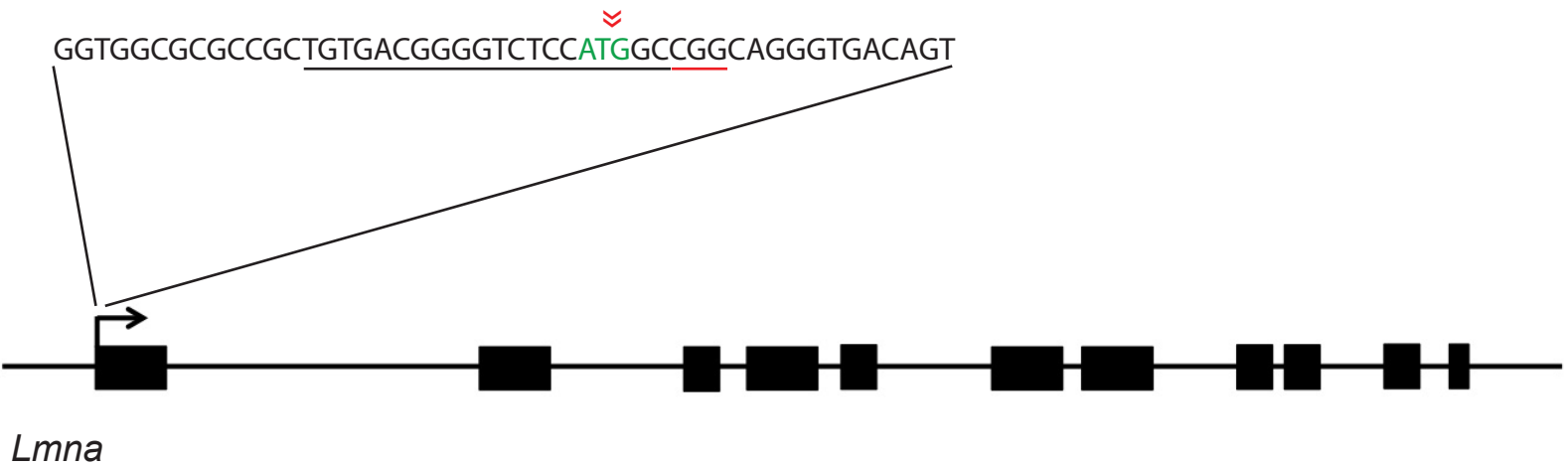
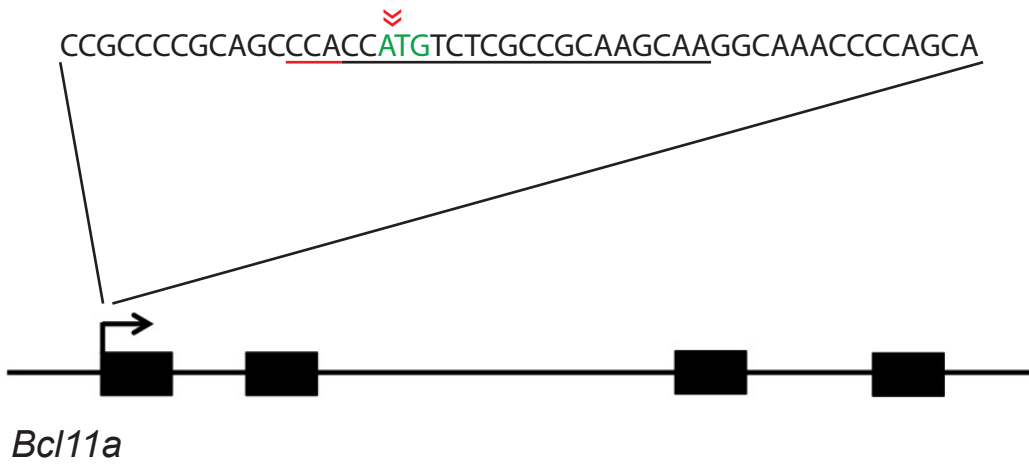
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Supplemental Table S1

Gene	Guide sequence	Surveyor assay forward primer	Surveyor assay reverse primer
<i>Bcl11a</i>	TTGCTTGC GGCGAGACATGG	CCCTGCGCCATCTTTGTATTATTCT	CTCAAAAGTGCACACGGTTCATG
<i>Gata3</i>	GAGCACAGCCGAGGACATGG	GTAAAAAGTACGTCCACCTCTTCCG	GGGATCGCCCTCATTCTTTTCTT
<i>Lcp2</i>	CGGGACATTCTTCAAGGCCA	GCAGAAAAGAGTCCTCTTCAACAAGG	TGTAGCTGAGGGCCCTCC
<i>Lmna</i>	TGTGACGGGGTCTCCATGGC	CGCACGCGATCGATGTACA	CCCTGTAGAGGAGGGCCTATTAG

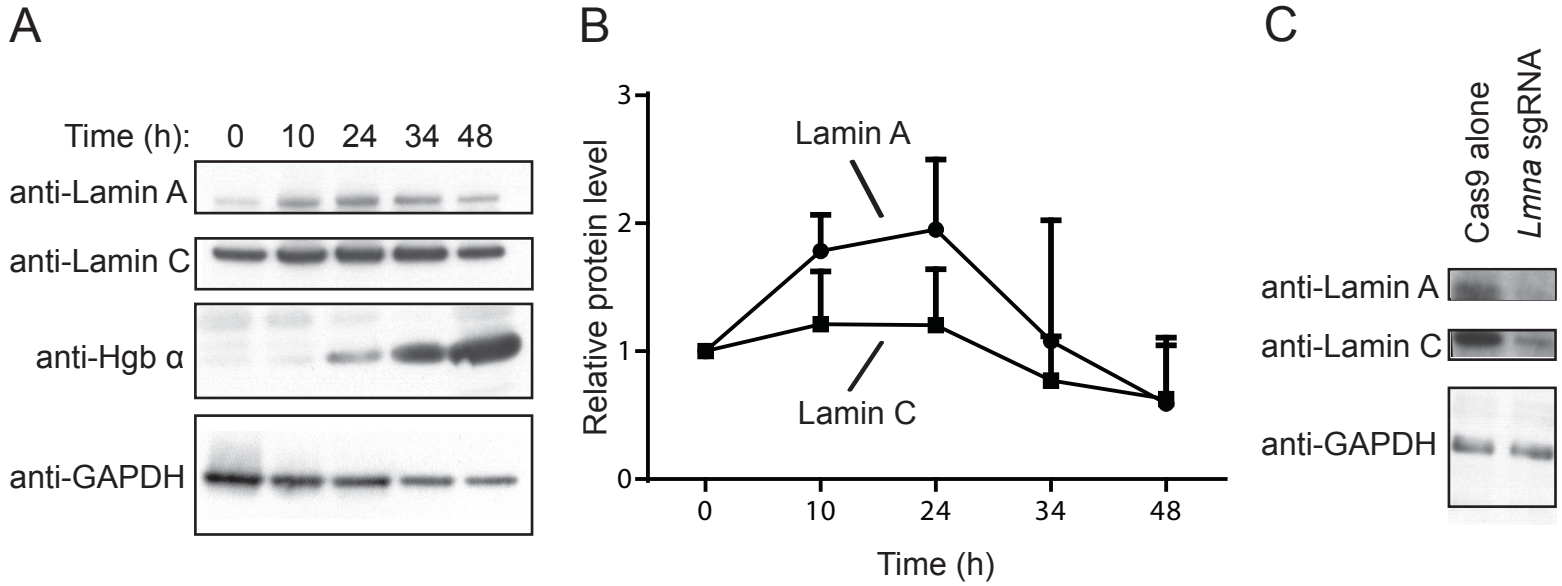
CRISPR DNA sequences. For all 4 genes targeted, guide sequences and primers used for the Surveyor nuclease assay are listed.

Supplemental Figure S1



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



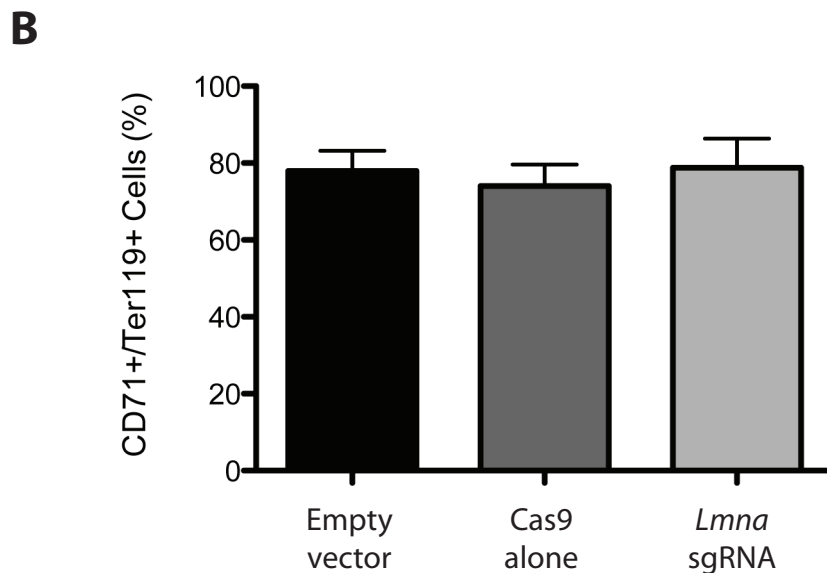
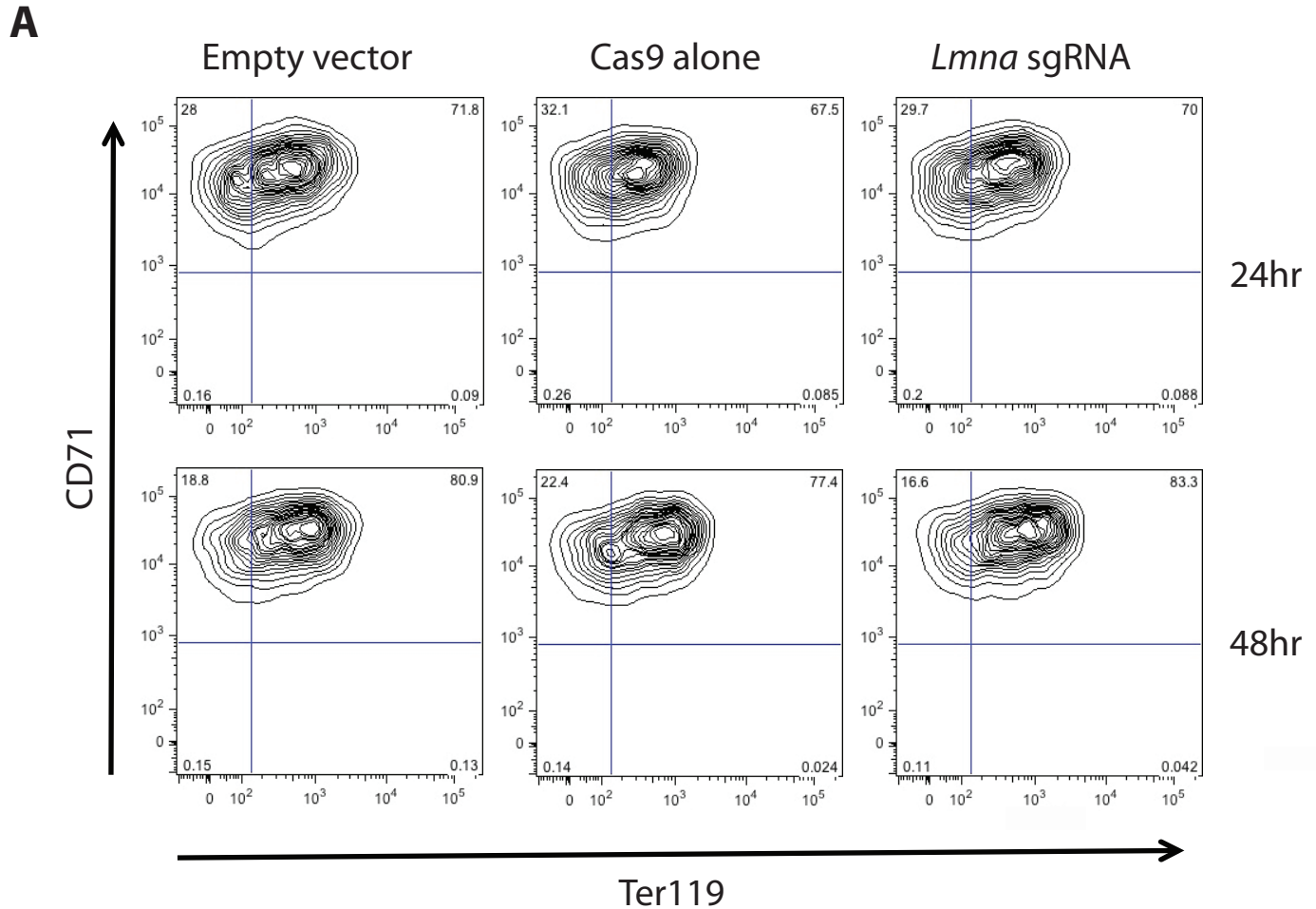
Supplemental Figure S2 – More dynamic regulation of Lamin A than Lamin C during erythroid differentiation and expression loss following CRISPR-Cas9-mediated *Lmna* disruption.

(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.