Zfp36l2 is required for self-renewal of early erythroid BFU-E progenitors

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Zfp36l2 is required for self-renewal of early erythroid BFU-E progenitors

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Abstract

Stem cells and progenitors in many lineages undergo self- renewing divisions, but the extracellular and intracellular proteins that regulate this process are largely unknown. Glucocorticoids stimulate red cell formation by promoting self-renewal of early erythroid burst forming unit-erythrocyte (BFU-E) progenitors[1-4]. Here we show that the RNA binding protein Zfp36l2 is a transcriptional target of the glucocorticoid receptor (GR) in BFU-Es and is required for BFU-E self-renewal. Zfp36l2 is normally downregulated during erythroid differentiation from the BFU-E stage but its expression is maintained by all tested GR agonists that stimulate BFU-E self-renewal, and the GR binds to several potential enhancer regions of Zfp36l2. Knockdown of Zfp36l2 in cultured BFU-E cells did not affect the rate of cell division but disrupted glucocorticoid-induced BFU-E self-renewal, and knockdown of Zfp36l2 in transplanted erythroid progenitors prevented expansion of erythroid lineage progenitors normally seen following induction of anemia by phenylhydrazine treatment. Zfp36l2 preferentially binds to mRNAs that are induced or maintained at high expression levels during terminal erythroid differentiation and negatively regulates their expression levels. Thus Zfp36l2 functions as part of molecular switch promoting BFU-E self-renewal and thus a subsequent increase in the total numbers of CFU-E progenitors and erythroid cells that are generated.

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Author Contributions

L. Z conceived the project, designed and performed the experiments and bioinformatics analysis, analyzed the data, and wrote the paper. L. P assisted with luciferase reporter assays and BFU-E isolation. V. R. E performed the GR Chip-seq experiment. P. T provided training in bioinformatics analyses. J. F performed the initial experiments with the GR partial agonists. B. L supervised part of the research. H. F. L supervised the research and edited the paper.

Competing financial interests

The authors declare no competing financial interests.

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Humans generate 10^{11} erythrocytes every day, a process regulated by multiple hormones affecting several types of progenitors. Apoptosis, proliferation, and terminal differentiation of CFU-E erythroid progenitors are mainly controlled by erythropoietin (EPO) [5,6]. In contrast, many hormones including EPO, stem cell factor (SCF), interleukin-3 (IL-3), and interleukin-6 (IL-6) regulate the earlier BFU-E progenitors, but we do not know how they interact to control BFU-E quiescence, self- renewal divisions, or cell divisions yielding the later CFU-E progenitors. Under stress conditions such as acute blood loss or chronic anemia, glucocorticoids trigger self-renewal of BFU-E progenitors in the spleen, leading to increased numbers of self- renewal divisions. This results in increased BFU-E numbers and, over time, formation of increased numbers of CFU-E progenitors and subsequently of mature erythrocytes[1-4,7,8].

To identify GR activated genes essential for BFU-E self-renewal, BFU-Es were purified[1] and cultured in a medium (self-renewal medium) containing SCF, EPO, insulin-like growth factor 1 (IGF-1), and several full or dissociated GR agonists. All agonists, except for one dissociated agonist, stimulate BFU-E self-renewal (Supplementary Figure 1). Since the genes upregulated by all functional agonists represent candidates indispensable for BFU-E self-renewal, we performed deep sequencing on mRNAs from BFU-Es cultured with GR agonists for 4 hours, and identified a group of genes upregulated by all functional agonists but not by nonfunctional agonists (Supplementary Table 1). We focused on three genes normally downregulated during erythroid differentiation, *Zfp36l2, Hopx*, and *Nlrp6* (Supplementary Figure 2, b, c). As detailed later, knockdown of *Hopx* and *Nlrp6* resulted in a defect in BFU-E proliferation. In contrast, knockdown of *Zfp36l2*, the most abundant transcript upregulated by GR agonists, did not affect the initial rate of BFU-E division.

During erythroid differentiation *in vivo*, Zfp36l2 is downregulated from the BFU-E stage (Figure 1, a, b). BFU-Es cultured *in vitro* for 4 hours with all functional GR agonists exhibited a ~2.5 fold upregulation of Zfp36l2 that was maintained throughout the culture (Figure 1, c, d, e). Given that Zfp36l2 is upregulated after only 4 hours, Zfp36l2 is likely a direct transcriptional target of the GR. Thus we performed GR chromatin immunoprecipitation sequencing (ChIP-seq) on freshly isolated BFU-Es after 1 hour stimulation with dexamethasone (DEX) and identified five GR binding sites near the *Zfp36l2* transcription start site (TSS), potential enhancers of *Zfp36l2* (Supplementary Figure 3). Four of these sites responded to DEX, indicating that they are functional enhancers regulated by glucocorticoids (Figure 1g). Together, these results suggest that Zfp36l2 is a direct transcriptional target of the GR in BFU-Es.

Zfp36l2 belongs to an RNA binding protein family[9]. Based on RNA-seq gene expression data from purified BFU-E, CFU-E, and Ter119-positive (Ter119+) erythroblasts[1], Zfp36l2 is ~20 times more abundant in erythroid progenitors than its other two family members, Zfp36 and Zfp36l1, and is the only member upregulated by DEX (Figure 1f and Supplementary Figure 4), suggesting that Zfp36l2 is the major family member involved in regulation of BFU-E self-renewal. Furthermore, Zfp36l2 is gradually downregulated from the hematopoietic stem cell (HSC) to the early and late erythroid progenitor stages (Supplementary Figure 5). In summary, glucocorticoid treatment of BFU-Es reverses normal downregulation of Zfp36l2, correlating with glucocorticoid-induced BFU-E self-renewal.

To test whether upregulation of Zfp36l2 is required for glucocorticoid-induced BFU-E self-renewal, we used two shRNAs to knock down expression of Zfp36l2 in BFU-Es (Figure 2, a, b). BFU-Es cultured without DEX stop proliferating at 4 days; as shown previously[1], in the absence of DEX, each BFU-E generates several CFU-Es, each of which generates 10-30 erythroid cells over a 5 to 6 day period. In contrast, BFU-Es cultured in the presence of DEX continue to proliferate and generate over 10 times more mature erythroid cells at 9 days of

culture. As shown previously[1], in the presence of DEX, each BFU-E generates multiple daughter BFU-Es during the first days of culture; over time these BFU-Es generate increased numbers of daughter CFU-Es, each of which generates 10-30 erythroid cells. Importantly, BFU-Es expressing either Zfp36l2 shRNA stop proliferating at day 4, whether or not DEX is included; the proliferation kinetics are similar to those of BFU-Es cultured without DEX (Figure 2c). This suggests that knockdown of Zfp36l2 disrupts DEX-induced self-renewal of BFU-Es.

As one control, knockdown of c-Kit, a cell surface receptor required for the survival of hematopoietic stem and progenitor cells including BFU-Es, resulted in a blockage of BFU-E proliferation after only 1 day of culture (Supplementary Figure 2a). Hopx and Nlrp6 share similar expression patterns as Zfp36l2, are downregulated during erythroid differentiation from the BFU-E stage and upregulated by Dex, and possess promoter regions that, based on our GR ChIP-Seq data, are occupied by GRs. Knockdown of these also resulted in a blockage of BFU-E proliferation after only 1 day of culture. (Supplementary Figure 2 b, c)

To establish that Zfp36l2 is specifically required for BFU-E self-renewal, 3 day BFU-E cultures were tested for their number of daughter BFU-Es by colony assays. Knockdown of Zfp36l2 significantly decreased the number of BFU-Es formed in the presence of DEX (Figure 2d). Knockdown of Zfp36l2 had no influence on apoptosis of BFU-Es (Figure 2e) and, as expected based on its low level of expression in CFU-Es, knockdown of Zfp36l2 had no effect on erythroid differentiation beyond the CFU-E stage (Supplementary Figure 6). These data suggest that Zfp36l2 is specifically required for glucocorticoid-induced BFU-E self-renewal.

Glucocorticoids and GR are required for erythroid lineage cell expansion in the spleen during stress erythropoiesis[2,4]. The data in Figure 3, using a phenylhydrazine (PHZ) induced hemolytic anemia mouse model, shows that Zfp36l2 is required for stress-induced erythroid lineage expansion *in vivo*. Lineage-negative (Lin-) cells were isolated and infected with viruses encoding GFP and either a control shRNA or Zfp36l2 shRNAs, and then transplanted into lethally irradiated recipient mice. 6-8 weeks after transplantation, PHZ or control phosphate buffered saline (PBS) was intraperitoneally injected into transplanted mice at days 0 and 1 to induce hemolytic anemia and erythroid lineage expansion. On day 4, spleens were dissected for flow cytometry analysis for multiple hematopoietic cells, detected by lineage specific markers (Figure 3a). Zfp36l2 shRNAs effectively knocked down the expression of Zfp36l2 in the Lin-cells (Supplementary Figure 7a), and as expected injection of PHZ resulted in a ~10 fold increase in systemic glucocorticoid levels (Figure 3b).

In control mice transplanted with Lin- cells infected with control shRNA, the majority of control transplanted GFP+ splenic cells were Ter119-negative (Ter119-) non-erythroid lineage cells as expected (Supplementary Figures 8 and 9a). PHZ-mediated hemolysis induced a ~20 fold expansion of number of erythroid lineage cells (Figures 3c) and resulted in ~50% of GFP+ cells in the spleen becoming Ter119+ mature erythroid cells (Supplementary Figure 9a).

Importantly, knockdown of Zfp36l2 significantly impaired this erythroid lineage expansion (Figures 3c). In addition, the effects of Zfp36l2 in mediating hemolysis-induced cell expansion is specific to the erythroid lineage, as no other hematopoietic lineages exhibited a difference in the number of donor-derived GFP+ cells between PBS and PHZ injection groups, with or without Zfp36l2 knockdown (Figure 3d, Supplementary Figure 10, a, b, and Supplementary Figure 11, d, e, f). Consistent with the increase in the percentage of Ter119+ erythroid lineage cells of control mice upon PHZ injection, the percentages of other hematopoietic lineages were decreased upon PHZ injection, and these decreases were

eliminated in the absence of Zfp36l2 (Supplementary Figure 10, a, b, c, d, and Supplementary Figure 11, a, b, c).

Zfp36l2 homozygous knockout mice die from HSC failure within two weeks after birth[10], and thus this protein is likely required in early hematopoietic stem and progenitor cells. Consistent with this notion, before PHZ challenge, the percentage of GFP+ splenic cells is lower in mice transplanted with Lin- cells infected with viruses encoding either Zfp36l2 shRNA than with the control shRNA; this difference is not caused by significant differences in infection efficiency of Lin- cells before transplantation (Supplementary Figure 7, b, c).

Importantly, in mice transplanted with Lin- cells expressing the control shRNA the percentage of GFP+ cells in the spleen does not significantly change after PHZ treatment, while in mice transplanted with Lin- cells infected with viruses encoding Zfp36l2 shRNAs, the percentage markedly drops following PHZ treatment (Supplementary Figure 7d), consistent with the loss of erythroid cell expansion (Figure 3c). Together, these data suggest that Zfp36l2 is specifically required for erythroid expansion during stress erythropoiesis *in vivo* and is consistent with the notion that it is essential for glucocorticoid-induced BFU-E self-renewal.

The data in Figure 4 shows that Zfp36l2 contributes to BFU-E self-renewal by repressing expression of genes important for terminal erythroid differentiation. BFU-Es were cultured in self-renewal medium with or without DEX. At day 4, approximately 12% of the cells generated from BFU-Es cultured with DEX differentiated into Ter119+ cells, whereas ~35% of the progeny of BFU-Es cultured without DEX became Ter119+, consistent with a DEX-triggered delay in terminal erythroid differentiation. This DEX-induced differentiation delay was eliminated by Zfp36l2 knockdown; loss of Zfp36l2 results in formation of ~30% Ter119+ cells, similar to the percentage in cultures without DEX (Figures 4a) suggesting that Zfp36l2 is essential for the glucocorticoid-induced delay of erythroid differentiation from the BFU-E stage. Consistent with a differentiation delay evidenced by the Ter119 marker, DEX treatment globally repressed the expression of a group of genes most highly induced during erythroid differentiation, and knockdown of Zfp36l2 eliminated this repression (Figure 4b).

This conclusion is further strengthened by experiments showing that overexpression of Zfp36l2 in BFU-Es significantly reduces the rate of cell proliferation (Supplementary Figure 12, a, b) but also delays erythroid differentiation. When cultured 4 days in the absence of DEX, the fraction of differentiated Ter119+ cells was significantly lower in the overexpression cells than in the controls, and was similar to that from control BFU-Es cultured with DEX (Supplementary Figure 12c). Although overexpression of Zfp36l2 led to a delay in erythroid differentiation, overexpression of Zfp36l2 in BFU-Es cultured without DEX was not able to rescue the self-renewal divisions, suggesting that Zfp36l2 contributes to BFU-E self-renewal by delaying erythroid differentiation (Supplementary Figure 12b).

To identity mRNAs in BFU-Es that directly bind to Zfp36l2, we performed an RNA-binding protein immunoprecipitation, using a verified Zfp36l2-specific antibody (Supplementary Figure 13, a, b), coupled to a microarray (RIP-chip) assay and identified many genes specifically immunoprecipitated by the Zfp36l2 antibody (Supplementary Table 2). Using this unbiased genomic approach, we showed that mRNAs containing AU-rich elements in their 3'UTRs are preferentially incorporated into the antiZfp36l2 immunoprecipitate (Supplementary Figure 14); ~72% of these mRNAs indeed contain the core Zfp36l2 recognition motif ATTTA element in their 3'UTRs. This is consistent with previous reports concerning the binding specificity of Zfp36l2[11,12], but we cannot eliminate the possibility that some mRNAs are binding to other unknown proteins in complexes with Zfp36l2.

Strikingly, we found that in BFU-Es, Zfp36l2 preferentially binds to mRNAs that tend to be induced or maintained at higher than average expression levels during subsequent erythroid differentiation to the CFU-E stage (Figure 4d). In addition, we found that the number of AU-rich elements of mRNAs bound by Zfp36l2 positively correlates with their extent of induction during erythroid differentiation from the BFU-E to CFU-E stage (Supplementary Figure 15, a, b). Thus, the expression pattern of Zfp36l2 negatively correlates with the expression pattern of erythroid differentiation induced genes.

To globally identify functional targets whose expression levels are regulated by Zfp36l2, we analyzed by microarrays the gene expression profile of day 3 *in vitro* cultured BFU-Es, allowing us to identify several genes with lower expression levels in BFU-Es cultured with DEX, compared with their counterparts in BFU-Es cultured without DEX (Figure 4c). Repression of several of these DEX repressed genes is dependent on and presumably mediated by Zfp36l2, since knockdown of Zfp36l2 eliminated this repression (Figure 4c). A group of potential Zfp36l2 functional target genes was then identified by intersecting the set of these repressed genes with the group of genes identified by RIP-chip assay (Supplementary Figure 16 and Supplementary Table 3). As shown in Supplementary Table 4, this group of potential functional targets contains several genes previously known to be important for or related to terminal erythropoiesis, including *Aff1*[13], *Mafk*[14], *Nfe2l1*[15], *Sap30l*[16], *Epb4.1*[17], *Adar*[18], *Mthfd2*[19], and *Mfhas1*[20]. We performed luciferase reporter assays on selected candidates, and found that the 3'UTRs of many of these genes are Zfp36l2 responsive (Supplementary Table 4). In addition, we mutated AU-rich elements in the 3'UTR of *Aff1* and observed a statistically significant 21% increase (p=0.015) of luciferase activity, indicating that AU-rich elements are required for this regulation.

These data illustrate that Zfp36l2 globally negatively regulates the expression of several erythroid differentiation-induced genes, among which some are known to be required for erythroid differentiation and others are unknown but induced. Regulation by Zfp36l2 appears similar to microRNA-mediated posttranscriptional regulation. An individual microRNA binds to multiple target mRNAs and downregulates their expression levels often by only 20- 50%; collectively however, these modulations can have important biological effects. Zfp36l2 is normally downregulated during erythroid differentiation from the BFU-E stage, thus stabilizing many mRNAs required for terminal differentiation. Zfp36l2 transcription is enhanced by glucocorticoids under stress conditions that signal erythroid lineage cell expansion. Upregulation of Zfp36l2 in turn negatively regulates multiple differentiation-induced genes, causing a delay in erythroid differentiation and ultimately contributing to BFU-E self-renewal (Supplementary Figure 17). Altogether, our experiments uncover a novel mechanism that facilitates progenitor self-renewal - delaying differentiation by posttranscriptional downregulation of expression of mRNAs critical for progression to the next differentiation stage.

Methods

Primary BFU-Es and CFU-Es purification, retrovirus infection and *in vitro* culture

Primary BFU-Es and CFU-Es were purified from mouse E14.5 fetal liver[1]. BFU-Es were then placed in virus solution in a 6 well plate, followed by 37°C overnight incubation. After incubation, virus solution was substituted by a medium (self-renewal medium) containing SCF (100ng/ml), EPO (2U/ml), and IGF-1 (40ng/ml) with or without full and partial GR agonists. The cells were then *in vitro* cultured at 37°C for 9 days.

For CFU-E differentiation assay, CFU-Es were placed in virus solution, followed by 37°C spin infection. After infection, virus solution was substituted by an EPO containing differentiation medium, and the cells were then in vitro cultured for two days[21].

Cell number counting assay and BFU-E colony formation assay

In the BFU-E culture system, after infection with viruses encoding GFP and either control shRNA or Zfp36l2 shRNAs, the absolute numbers of GFP+ cells were counted daily by flow cytometry, where the counting beads was used as a internal counting standard. For BFU-E colony formation assay, after 3 days culture, GFP+ cells were flow cytometry sorted and cultured in methylcellulose medium (MethoCult SF M3436 from StemCell Technologies), and the number of BFU-E colonies containing a cluster of more than 20 CFU-E colonies were counted 9 days after culture.

In vivo PHZ induced hemolytic anemia and bone marrow transplantation mouse model

Lin- cells were isolated from mouse E14.5 fetal liver and infected with viruses encoding GFP and either control shRNA or Zfp36l2 shRNAs and transplanted into lethally irradiated recipient mice. 6 to 8 weeks after transplantation, the recipient mice were intraperitoneally injected with 60 mg/kg PHZ or control PBS on day 0 and day 1. On day 4, spleens were dissected and measured for number and percentage of GFP+ cells and of each type of GFP+ hematopoietic lineage cell.

Measurement of corticosterone level

Mice were injected with PHZ or PBS, and plasma were prepared I hour after injection. Corticosterone levels were measured by using an ELISA kit according to instruction by the manufacturer Immunodiagnostic Systems Inc.

RIP-chip and data analysis

RIP-chip was carried out according to the instruction of EZ-Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). BFU-Es were lysed and incubated with Zfp36l2 antibody (Abcam ab70775; 5ug) or control IgG (5ug) conjugated with magnetic beads (50ul) for 4 hours. The beads, protein, and mRNAs complexes were immunoprecipitated and magnetic separated. The mRNAs were purified and analyzed by microarray (Affymetrix mouse gene 1.0 ST array). Ratios of each microarray probe between its intensity in Zfp36l2 antibody immunoprecipitated sample and its intensity in IgG immunoprecipitated sample were calculated.

For the AU-rich elements enrichment analysis, the 2000 microarray probes corresponding to the mRNAs that most preferentially bind by Zfp36l2 antibody are listed as ‘Targets’. The 2000 microarray probes corresponding to the mRNAs that most preferentially bind by control IgG are listed as ‘Non-targets’. The percentages of targets and non-targets containing indicated AU-rich element in their 3’UTR were calculated.

For the cumulative distribution plot, ‘Targets’ represents the 2000 microarray probes corresponding to the mRNAs that most preferentially bind by Zfp36l2 antibody in RIP-chip experiment. ‘Non-targets’ represents all of the rest genes. The relative expressions of each gene calculated as a $\log_2$ ratio between its intensity in CFU-E and its intensity in BFU-E[1] were calculated (x axis). The cumulative fraction is plotted as a function of the relative expression (y axis).

For the correlation analysis, the 2000 transcripts that are most preferentially found in the Zfp36l2 immunoprecipitate in the RIP-chip experiments were ranked based on their relative expression levels calculated as a ratio of their expression levels in CFU-Es relative to BFU-Es, and were classified into 8 groups each with 250 transcripts based on their relative expression level ranking. One-way ANOVA analysis was performed to test the statistical significance of the difference among the average numbers of ATTTA elements in the 3’UTR of each gene of these 8 groups by using GraphPad software. Test for linear trend after

ANOVA was performed to test the statistical significance of the linear trend, the systematical increase of the average number of ATTTA elements in the 3'UTR of each gene of these 8 groups as the rank of average relative expression levels of these 8 group increases, using GraphPad software. The average number of ATTTA elements in the 3'UTR of each gene was plotted together with the average relative expression levels of each group for these 8 groups. The percentage of genes with ATTTA elements in their 3'UTRs was plotted together with the average relative expression levels of each group for these 8 groups. The linear trendlines were drawn and the p values and R^2 values of the Pearson correlation test were calculated for these two plots.

GR Chip-seq

A total of ~7×10^7 primary BFU-E cells were purified from mouse E14.5 fetal liver[1]. Cells were incubated at 37°C for 4 hours in SFEM (Stem Span) medium containing SCF (100ng/ml), EPO (2U/ml), and IGF-1 (40ng/ml); followed by 1 hour stimulation with 100nM dexamethasone. Cells were then chemically cross-linked with 1% formaldehyde solution for 15 minutes at room temperature, lysed and sonicated to solubilize and shear cross-linked DNA in sonication buffer (50 mM HEPES pH 7.5, 40 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% Na-deoxycholate and 0.1% SDS). The glucocorticoid receptor (GR) was immunoprecipitated overnight at 4°C with 10μg of a combination of two antibodies bound to magnetic beads (Dynalbeads, Invitrogen). The antibodies used were mouse monoclonal FiGR (sc-12763) and MAI-510 (BuGR clone, Thermo scientific). The beads containing the GR bound to DNA were washed once with low salt buffer (20mM TRIS pH8, 2mM EDTA, 0.1% SDS, 1% Triton X100, 150mM NaCl), once with high salt buffer (20mM TRIS pH8, 2mM EDTA, 0.1% SDS, 1% Triton X100, 500mM NaCl), and once with LiCl buffer (10mM TRIS pH8, 1mM EDTA, 1% NaDOC, 1% NP40, 150mM LiCl). Bound complexes were eluted from the beads by heating at 65°C overnight in elution buffer (50mM HEPES pH8, 10 mM EDTA, 200mM EDTA and 1% SDS). Whole cell extract DNA was also treated for cross-link reversal and was used as background control. Immunoprecipitated DNA and whole cell extract DNA were then purified by treatment with RNAse A, proteinase K and a phenol:chloroform:isoamyl alcohol extraction.

Purified DNA was prepared for sequencing according to a modified version of the Solexa Genomic DNA protocol. Fragmented DNA was end repaired and adapters were ligated. An additional gel extraction step was added to the Illumina protocol at this step, allowing us to collect the material between 100 and 300 bp. The purified DNA was subjected to 18 cycles of linker-mediated PCR as per the Illumina protocol. Amplified fragments between 200 and 300 bp were isolated by agarose gel electrophoresis and purified. High-quality samples were confirmed by the appearance of a smooth smear of fragments from 100 to 1000 bp, with a peak distribution between 150 and 300 bp.

Sequence reads were aligned to the mouse genome (NCBI Build 37, version mm9) using the model-based analysis of ChIP-Seq (MACS)[22]. Sequences uniquely mapping to the genome with zero or one mismatch were used in further analysis. Genomic bins with a normalized ChIP-Seq density greater than a defined threshold were considered enriched or "bound," based on a p-value of less than 10^{-08}.

Luciferase reporter assay

293T cells were seeded into 96 well plates 24 hours before transfection. For Zfp36l2 enhancer experiment, 10ng luciferase reporter plasmid or control empty vector plasmid were co-transfected with 5ng GR into 293T cells by using Lipofectamine 2000 transfection reagent (Invitrogen). Cells were cultured in a medium containing 1uM DEX and lysed 48 hours after transfection. For 3'UTR experiment, 10ng luciferase reporter plasmid or control

vector plasmid were co-transfected with 150ng of XZ-Zfp36l2 into 293T cells by using Lipofectamine 2000 transfection reagent (Invitrogen) followed with 48 hours culture. Luciferase activities were detected by using a dual luciferase kit (Promega).

RNA-seq, microarray, and qRT-PCR

For RNA-seq, samples were prepared by using the RNA Sample Prep Kit (Illumina) and sequenced by using Illumina genome analyzer at Whitehead Institute.

For microarray experiments, RNA was extracted by using a miRNeasy Mini kit (Qiagen), and microarrays were performed by using the Mouse GE 4×44k microarray (Agilent) at Whitehead Institute.

For RT-PCR, RNA was extracted by using a miRNeasy Mini kit (Qiagen). Reverse transcription was carried out using SuperScript™ II Reverse Transcriptase (Invitrogen). Real-time PCR was performed by using SYBR® Green PCR Master Mix (Applied Biosystems) and 7500 Real-Time PCR System (Applied Biosystems). The following primer sequences were used for real-time PCR: Zfp36l2, forward, GGCCGCACAAGCACAAC, reverse, GAGACTCGAACCAAGATGAATAACG; Aff1, forward, GCCTAACACTTCCTCCTGACACA, reverse, CTGCCTACAGCCCAAAGTCAA; Mafk, forward, GCGGCGCACACTCAAGA, reverse, TTTCTGTGTCACACGCTTGATG; Nef2l1, forward, CCCCAGAAGGCCTTTGTAACT, reverse, TCCAAGAGCATCTTCCCTTCA.

Western blot

Protein was extracted in lysis buffer (150 mM sodium chloride; 1.0% NP-40 or Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 50 mM Tris, pH 8). SDS-PAGE was performed using the NuPAGE® Novex® Bis-Tris Gel Systems (Invitrogen). After electrophoretic transfer, the PVDF membranes with protein were incubated with the first antibody for Zfp36l2 (Abcam # ab70775; at dilution of 1:1000) or with the first antibody for GFP (Abcam # ab290; at dilution of 1:1000) at 4°C overnight. After washing and incubating with HRP conjugated secondary antibody at room temperature for 1 hour, membranes were developed.

Immunoprecipitation experiment

The XZ-Zfp36l2-GFP construct encoding the GFP- Zfp36l2 fusion protein was transfected into 293T cells. Two days after transfection, cells were lysed and immunoprecipitations were performed using either control IgG or Zfp36l2 antibody (Abcam ab70775) with the same amount of input cell lysate according to the instructions for the EZ-Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Briefly, 293T cells were lysed and incubated with control IgG (5ug) or Zfp36l2 antibody (Abcam ab70775; 5ug) conjugated with magnetic beads (50ul) for 4 hours. The beads and proteins complexes were immunoprecipitated and magnetic separated. The proteins were purified and analyzed by Western blot using the Zfp36l2 antibody (Abcam ab70775).

Plasmids

The shRNAs sequences targeting mouse Zfp36l2, c-Kit, Hopx, and Nlrp6 from Broad Institute RNAi consortium shRNA library were cloned into the MSCV-GFP vector. The shRNAs sequences are: Zfp36l2, shRNA1, aaaaCCAAACACTTAGGTCTCAGATgtcgacATCTGAGACCTAAGTGTTTGG; shRNA2, aaaaGCACCACAACTCAATATGAAAgtcgacTTTCATATTGAGTTGTGGTGC; c-Kit, shRNA1, aaaaCGGCTAACAAAGGGAAGGATTgtcgacAATCCTTCCCTTTGTTAGCCG, shRNA2, aaaaCGGATCACAAAGATTTGCGATgtcgacATCGCAAATCTTTGTGATCCG;

Hopx, shRNA1, aaaaGCAGACGCAGAAATGGTTTAAgtcgacTTAAACCATTTCTGCGTCTGC, shRNA2, aaaaAGTACAACTTCAACAAGGTCAgtcgacTGACCTTGTTGAAGTTGTACT, shRNA3, aaaaCCTTCGGAATGCAGATCTGTTgtcgacAACAGATCTGCATTCCGAAGG; Nlrp6, shRNA1, aaaaGACCTCCAAGAGGTGATCAATgtcgacATTGATCACCTCTTGGAGGTC, shRNA2, aaaaCTGGATCATCATAAAGCACAAgtcgacTTGTGCTTTATGATGATCCAG. Sequences from mouse Aff1 3'UTR (5318bp to 6758bp) that are 1441bp in length containing 2 'ATTTA' motifs were PCR amplified from mouse genomic DNA and cloned into the luciferase reporter vector psiCHECK2. Following are the primers used for PCR amplification: forward, GGGCTCGAGTTCTTGGTACCTTGGTTAAATC, reverse, GGGGCGGCCGCCCCAACTCATCTCGAATTTCAC. For mutagenesis experiment, the two 'ATTTA' motifs of Aff1 3'UTR were mutated into 'TGGGC'. Sequences from mouse Mafk 3'UTR (1730bp to 2825bp) that are 1096bp in length containing 5 'ATTTA' motifs were PCR amplified from mouse genomic DNA and cloned into psiCHECK2. Following are the primers for PCR amplification: forward, GGGGTTTAAACGAGCTCTGGGGCCACTGGAC, reverse, GGGGCGGCCGCCATCCCAAACAGGAAATTC. Sequences from mouse Nfe2l1 3'UTR (3736bp to 4614bp) that are 879bp in length containing 1 'ATTTA' motif were PCR amplified from mouse genomic DNA and cloned into psiCHECK2. Following are the primers for PCR amplification: forward, GGGGTTTAAACGCTTCCTCTGCAGGGTCTAAAC, reverse, GGGGCGGCCGCGTCATGTGCTCACAGCATTTC. Zfp36l2 overexpression construct was made by inserting ORF of Zfp36l2 into MICD4 vector. Zfp36l2 enhancer regions (chr17:84500031-84500271; chr17:84515492-84516478; chr17:84518538-84519282; chr17:84585041-84585584; and chr17:84595103-84595494) were each PCR amplified from mouse genomic DNA and cloned into the luciferase reporter vector pGL3-Basic. XZ-Zfp36l2-GFP construct was made by inserting ORF of Zfp36l2 without stop codon followed in frame with ORF of GFP into BglII and NcoI sites of XZ vector. XZ-Zfp36l2-IRES-GFP construct was made by inserting ORF of Zfp36l2 into BglII and EcoRI sites of XZ vector.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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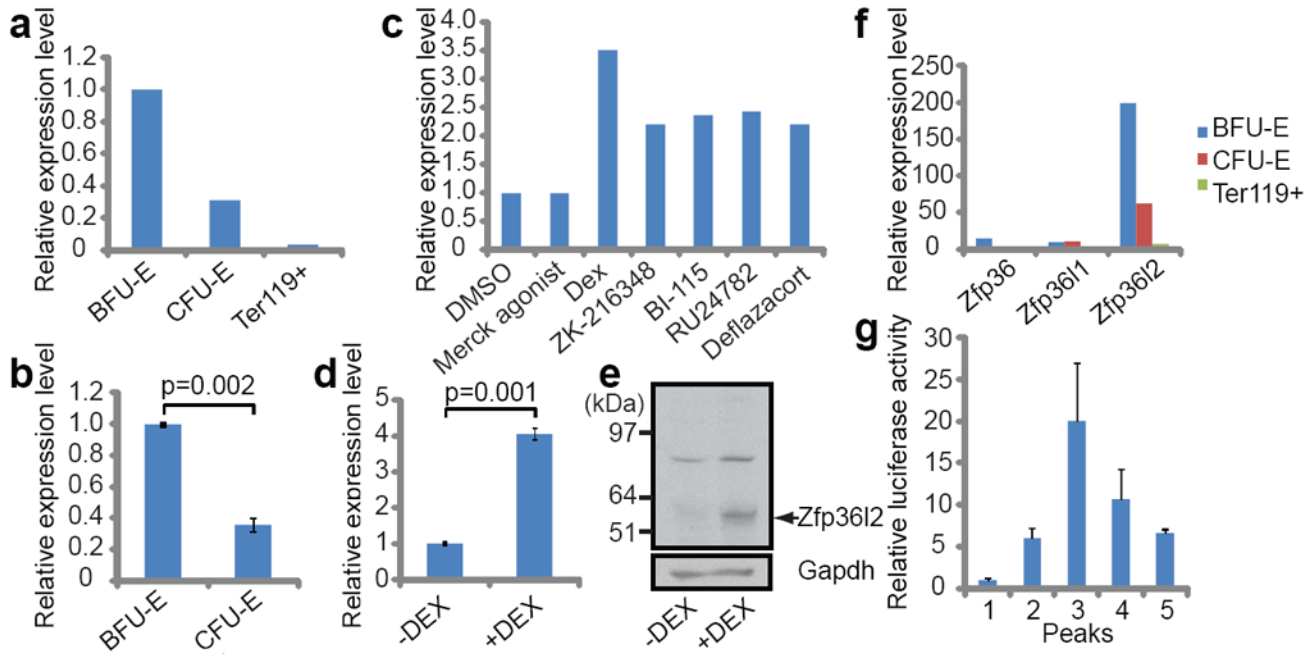

Figure 1. The normal downregulation of Zfp36l2 during erythroid differentiation from the BFU-E stage is reversed by functional GR agonists

a, The expression levels of Zfp36l2 in BFU-Es, CFU-Es, and Ter119+ erythroblasts were measured by RNA-seq.

b, The expression levels of Zfp36l2 in BFU-Es and CFU-Es were measured by RT-PCR normalized to 18 S rRNA. Error bar represents standard deviation (SD) of 3 biological repeats. P-value was calculated using the two-tailed t-test.

c, The expression levels of Zfp36l2 in BFU-Es after 4 hours culture in self-renewal medium with indicated GR agonists were measured by RNA-seq.

d, The expression levels of Zfp36l2 in BFU-Es after 3 days culture in self-renewal medium with or without DEX were measured by RT-PCR normalized to 18 S rRNA. Error bar represents SD of 3 biological repeats. P-value was calculated using the two-tailed t-test.

e, The expression level of Zfp36l2 in BFU-Es after 3 days culture in self-renewal medium with or without DEX was measured by Western blot. Representative data of 3 biological repeats is shown.

f, The expression levels of Zfp36, Zfp36l1, and Zfp36l2 in BFU-Es, CFU-Es, and Ter119+ erythroblasts were measured by RNA-seq[1]; data shown are relative RPKM values normalized to the expression level of Zfp36 in Ter119+ erythroblasts.

g, Luciferase reporter vector cloned with each GR binding site or empty vector were co-transfected with XZ-GR into 293T cells and cultured in a medium containing 1uM DEX, and luciferase activities were measured 2 days later. Error bar represents standard deviation (SD) of 3 biological repeats.

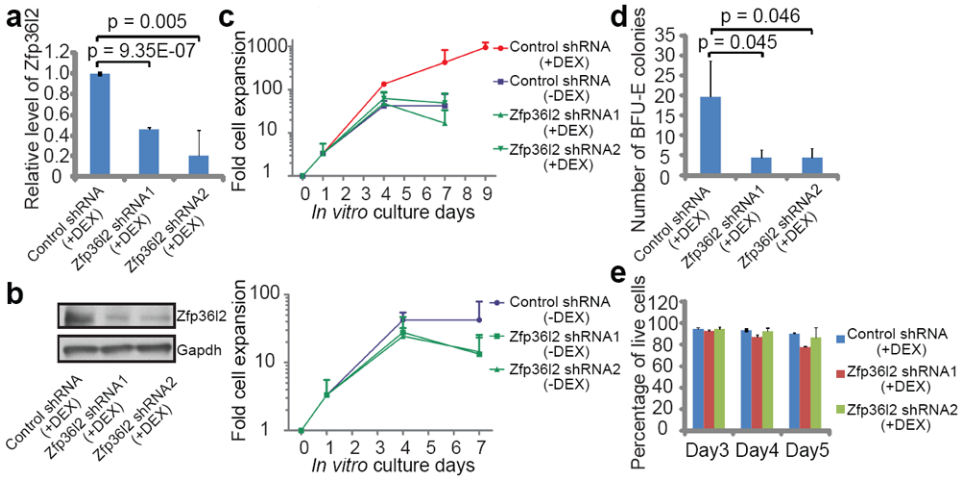

Figure 2. Zfp36l2 is specifically required for BFU-E self-renewal

a, The expression levels of Zfp36l2 in BFU-Es infected with viruses encoding indicated shRNAs followed with 1 day culture in self-renewal medium with DEX were measured by RT-PCR normalized to 18 S rRNA. Error bar represents SD of 3 biological repeats. P-values were calculated using the two-tailed t-test.

b, The expression levels of Zfp36l2 in BFU-Es infected with viruses encoding indicated shRNAs followed by 3 days culture in self-renewal medium with DEX were measured by Western blot. Representative data of 3 biological repeats is shown.

c, BFU-Es were infected with viruses encoding indicated shRNAs and cultured in self-renewal medium with or without DEX. The relative cell numbers throughout the culture are shown. Error bar represents SD of 3 biological repeats.

d, Day 3 cells from this *in vitro* BFU-E culture system were plated in methylcellulose medium; BFU-E colonies were counted 9 days later. Error bar represents SD of 3 biological repeats. P-values were calculated using the two-tailed t-test.

e, Day 3, 4, and 5 cells from this *in vitro* BFU-E culture system were stained with Annexin V and 7-AAD and the percentages of double negative live cells are shown. Error bar represents SD of 3 biological repeats.

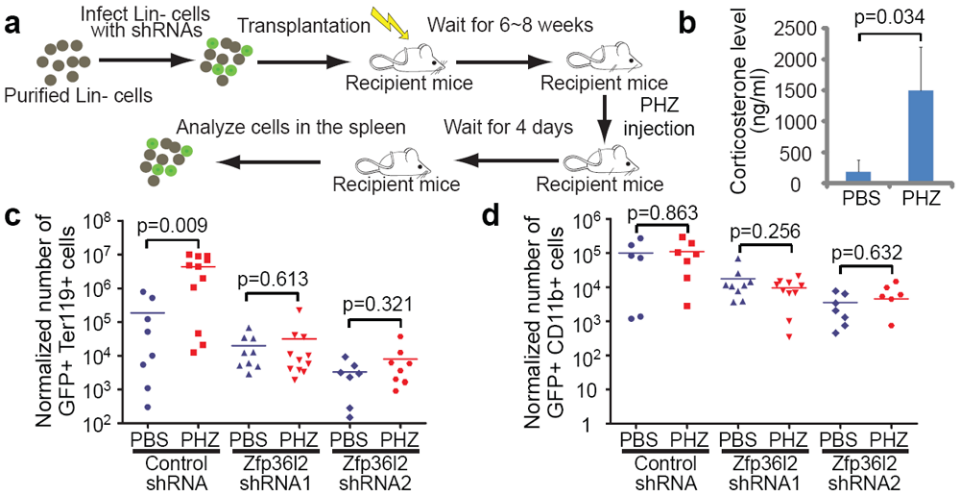

Figure 3. Zfp36l2 is required for erythroid lineage expansion during stress erythropoiesis *in vivo*

a, Schematic diagram shows the *in vivo* bone marrow transplantation and PHZ induced hemolytic anemia mouse model. **b**, Corticosterone levels were measured in mouse plasma 1 hour after PHZ injection. Error bar represents SD of 3 biological repeats. P-value was calculated using the two-tailed t-test.

c, The normalized numbers of each type of GFP+ hematopoietic lineage cells were calculated as a ratio of the number of each type of GFP+ hematopoietic lineage cells in the spleen relative to the percentages of GFP+ cells in the Lin-population before transplantation. In all cases P-values were calculated using the two-tailed t-test. The normalized numbers of GFP+ Ter119+ cells are shown.

d, The normalized numbers of GFP+ CD11b+ cells are shown.

e, The normalized numbers of GFP+ Gr-1+ cells are shown.

f, The normalized numbers of GFP+ B220+ cells are shown.

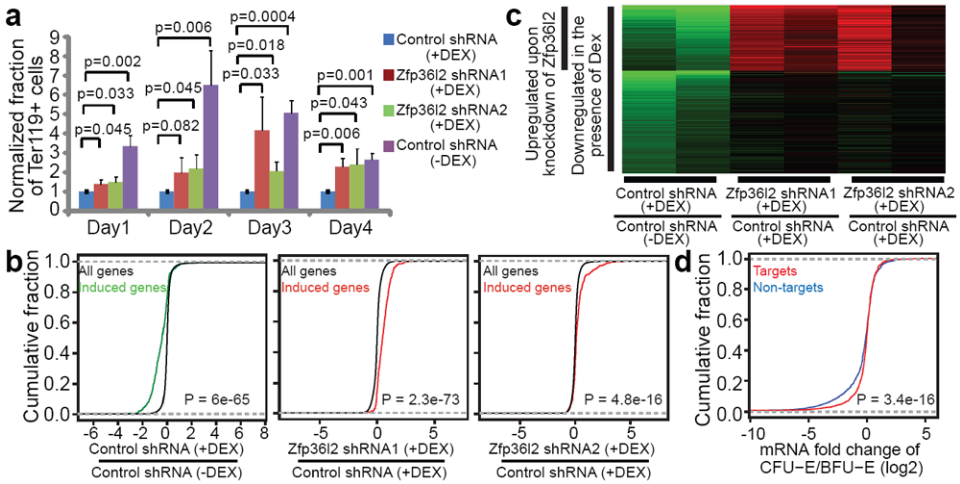

Figure 4. Zfp36l2 delays erythroid differentiation and preferentially binds to several mRNAs that are induced or maintained at higher expression levels during terminal erythroid differentiation

a, BFU-Es were infected with viruses encoding the indicated shRNAs and cultured for 4 days in self-renewal medium with or without DEX. The fraction of Ter119+ cells was measured throughout all of the cultures. The normalized fraction of Ter119+ cells was calculated as a ratio of the fraction of Ter119+ cells in cultures of BFU-Es infected with viruses encoding the indicated shRNAs and cultured under the indicated culture conditions relative to the fraction of Ter119+ cells in cultures of BFU-Es infected with control virus and cultured with DEX. Error bar represents SD of 3 biological repeats. P-values were calculated using the two-tailed t-test.

b, BFU-Es were infected with viruses encoding indicated the shRNAs and cultured for 3 days in self-renewal medium with or without DEX. Microarrays were carried out to profile genomic level expression patterns of these cells. On the x axis is the relative expression of each gene calculated as a $\log_2$ ratio of its expression in the indicated samples. The cumulative fraction (y axis) is plotted as a function of the relative expression (x axis). 'All genes' includes all of the genes in the microarray; 'Induced genes' represent a group of 340 genes most highly induced during erythroid differentiation from the CFU-E stage to the Ter119+ erythroblast stage. P-values were calculated using the Kolmogorov-Smirnov test.

c, BFU-Es were infected with viruses encoding indicated shRNAs and cultured for 3 days in self-renewal medium with or without DEX. Microarrays were carried out to profile genomic level expression patterns of these cells. Genes that are downregulated by at least 20% in the presence of DEX and the subset of these genes that are upregulated by at least 20% upon knockdown of Zfp36l2 are shown.

d, On the x axis is the relative expression of each gene calculated as a $\log_2$ ratio of its expression in CFU-Es relative to BFU-Es. The cumulative fraction (y axis) is plotted as a function of the relative expression (x axis). 'Targets' are the 2000 microarray probes corresponding to mRNAs that most preferentially are found in the Zfp36l2 immunoprecipitate. 'Non-targets' represent all of the remaining genes. P-value was calculated using the Kolmogorov-Smirnov test.