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Global analysis of induced transcription factors and cofactors identifies Tfdp2 as an essential coregulator during terminal erythropoiesis

Cynthia Chen[1] and **Harvey F. Lodish**[1,*]

[1]Whitehead Institute for Biomedical Research, and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142, USA

Abstract

Key transcriptional regulators of terminal erythropoiesis, such as GATA1 and TAL1, have been well characterized, but transcription factors and cofactors and their expression modulations have not yet been explored on a global scale. Here we use global gene expression analysis to identify 28 transcription factors and 19 transcriptional cofactors induced during terminal erythroid differentiation and whose promoters are enriched for binding by GATA1 and TAL1. Utilizing protein-protein interaction databases to identify cofactors for each transcription factor, we pinpoint several co-induced pairs, of which *E2f2* and its cofactor *Tfdp2* were the most highly induced. TFDP2 is a critical cofactor required for proper cell cycle control and gene expression. GATA1 and TAL1 are bound to the regulatory regions of *Tfdp2* and upregulate its expression, and knockdown of *Tfdp2* results in significantly reduced rates of proliferation, as well as reduced upregulation of many erythroid-important genes. Loss of Tfdp2 also globally inhibits the normal downregulation of many E2F2 target genes, including those that regulate the cell cycle, causing cells to accumulate in S phase and resulting in increased erythrocyte size. Our findings highlight the importance of TFDP2 in coupling the erythroid cell cycle with terminal differentiation and validate this study as a resource for future work on elucidating the role of diverse transcription factors and coregulators in erythropoiesis.

Keywords

transcription factors; cofactors; erythropoiesis; cell cycle; E2F

*Corresponding author: Prof. Harvey F. Lodish, Whitehead Institute for Biomedical Research, Suite 601, 9 Cambridge Center, Cambridge, MA 02142, lodish@wi.mit.edu, Tel: 617 258 5216, Fax: 617 258 6768.

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Introduction

The adult human generates roughly 2.4 million red blood cells every second, a process that requires the intricately regulated proliferation and differentiation of hematopoietic stem cells into mature erythrocytes. Much of this regulation occurs during terminal erythroid differentiation, the highly coordinated final step that begins with the committed erythroid progenitor CFU-E, the colony-forming unit erythroid, and is characterized by several cellular phenomena, including heme biosynthesis, 3–5 cell divisions followed by permanent cell cycle exit, chromatin condensation, and enucleation [1–3].

Close regulation at the mRNA level of erythroid genes is essential for proper erythroid maturation, and global studies of the changing transcriptional landscape have yielded insight into gene regulatory networks during terminal erythropoiesis [4, 5]. The role of key transcription factors and coregulators, notably GATA1 and its cofactor FOG1 (*Zfpm1*) [6, 7], in controlling gene expression have also been well-characterized [8, 9], although a comprehensive view of all transcriptional regulators is lacking.

To this end, we analyzed expression levels of 276 transcription factors and 213 transcriptional coregulators during distinct stages of erythroid differentiation [4], and identified 28 induced transcription factors and 19 induced cofactors, the promoters for the vast majority of which are themselves bound by factors GATA1 and TAL1. Protein interaction analysis [10] was then used to identify cofactors for each induced transcription factor. Several interacting partners were found to be jointly upregulated and bound by GATA1 and TAL1 during terminal erythropoiesis, including the known essential erythroid factors Tal1, Lmo2, and Gata1, each with Fog1, validating the use of this global study as a resource for finding potential critical transcriptional regulators.

As further validation of this study, we functionally characterized the role of cofactor Tfdp2, whose interaction with E2f2 constituted the top candidate pair. The E2F family of transcription factors is essential for proper cell cycle control in many cell types and has also been shown to play critical roles in many differentiation pathways [11–13]. Specifically, loss of E2F family member E2F2 results in defective erythroid maturation, anemia, larger red blood cell (RBC) size, arrest in S phase, and other hematopoietic defects [14, 15]. E2Fs are usually bound to DNA in a heterodimer with one of three functional DNA-binding cofactors, TFDP1, 2, or 3 [16–20]; however, the specific role of these coregulators in E2F-mediated cell cycle control during terminal erythropoiesis has not been explored.

Here we use a primary mouse erythroid cell culture system to show that loss of TFDP2 results in ineffective erythropoiesis. GATA1 and TAL1 bind to regulatory regions of *Tfdp2* and *E2f2* genes to upregulate their expression levels. In contrast, expression levels of known E2F2 target genes decrease during terminal erythropoiesis, suggesting that E2F2 acts as a transcriptional repressor in terminally dividing erythroblasts. Consistent with this hypothesis, knockdown of *Tfdp2* results in higher than normal levels of these cell cycle genes, causing cells to stall in S phase and fail to mature. Guided by our bioinformatics study, these findings suggest a novel model by which cells can coordinate their cell cycle

with differentiation and serve as a roadmap for future functional and mechanistic studies of transcriptional regulators in erythropoiesis.

Methods

Bioinformatics analyses

Protein-protein interactions for a list of all expressed transcription factors and cofactors, defined by gene ontology [21] as 'sequence-specific DNA binding transcription factor activity' and 'transcription cofactor activity', were obtained from the STRING database [10] containing known and predicted physical and functional protein associations. Interactions were filtered to remove those between two transcription factors or two cofactors.

Publicly available chromatin-immunoprecipitation sequencing (ChIP-seq) databases for H3K9ac in mouse erythroleukemia cells (GEO accession GSM1000141), H3K27ac in mouse E14.5 fetal liver (GSM1000113), and H3K4me1 (GSM946536), H3K4me3 (GSM946524), GATA1 (GSM923575), and TAL1 (GSM923582) in E14.5 Ter119+ mouse erythroblasts were analyzed on the UCSC Mouse Genome Browser, mm9 assembly using the model-based analysis of ChIP-seq (MACS) [22].

Cells

293T cells were used as the retrovirus-packaging cell line and were maintained in DMEM with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin/streptomycin (P/S). For MCF-7 cells, 293T culture medium was supplemented with 10 μg/mL human insulin (Sigma).

Plasmid constructs

The shRNA sequences targeting mouse *Tfdp2* were obtained from the Broad Institute RNAi consortium shRNA library. shRNA sequences were then cloned into the *BbsI* sites of the MSCV-pkgGFP-U3-U6P vector, which coexpresses GFP from a PGK promoter. The following are the shRNA sequences: shTFDP2a, AaaaCCCTGTTCATTCAACGATGAAgtcgacTTCATCGTTGAATGAACAGGG; shTFDP2b, AaaaCCACAGGACCTTCTTGGTTAAgtcgacTTAACCAAGAAGGTCCTGTGG. An shRNA against the firefly luciferase gene was also cloned into the same vector as a positive control.

For the luciferase reporter assay, putative promoter and enhancer regions of *Tfdp2* (chr9: 96158706-96159362 and chr9:96167467-96168140) were amplified from mouse genomic DNA and cloned into the pGL3-Basic luciferase reporter vector (Promega). The XZ-GATA1-IRES-GFP and XZ-TAL1-IRES-GFP constructs were made by cloning the ORF of *Gata1* or *Tal1* into the XZ vector.

Luciferase reporter assays

24 hours prior to transfection, MCF-7 cells were seeded into 96 well plates at a density of 50,000–100,000 cells per well. For transfection of MCF-7 cells in each well, Lipofectamine

2000 (Invitrogen) was used to co-transfect 10 ng pGL3-Basic luciferase reporter containing either the *Tfdp2* putative promoter or enhancer regions detailed above, or a control empty vector plasmid, together with 90 ng each of XZ-GATA1 and XZ-TAL1 into MCF-7 cells, followed by culture for 48 hours. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

Mouse fetal liver erythroid progenitor isolation, retrovirus infection, and in vitro culture

To obtain erythropoietin-dependent erythroid progenitors of high purity, homogenized mouse E14.5 fetal liver cells were labeled with the mixture of biotin-conjugated antibodies in the Lin-negative Kit (BD 559971) and subsequently incubated with Streptavidin Particles (BD 557812). Magnetic-based negative selection was then used to deplete mature erythrocytes and non-erythroid cells [23].

Standard retroviral infection of purified erythroid progenitors with shRNA constructs was then performed and the cells were cultured in EPO-containing differentiation medium at 37°C until experimental analysis [24].

Flow cytometry analysis and sorting

For analysis of enucleation, standard immunostaining followed by flow cytometry analysis was performed [23]. Enucleated cells were identified as Ter119-positive, Hoechst-negative.

For analysis of apoptosis, standard immunostaining using the apoptosis detection kit (BD Biosciences Pharmingen) was performed according to the manufacturer's protocol [25]. Cells undergoing apoptosis were identified as PE Annexin V-positive and 7-aminoactinomycin D (7-AAD)-negative.

For flow cytometry sorting of the R1 to R5 stages of late erythroid cells, homogenized mouse E14.5 fetal liver cells were stained with 1 μg/mL propidium iodide (PI), 1:100 APC-conjugated Ter119, 1:300 PE-conjugated CD71 and were sorted into 5 fractions (R1-R5) [26]. For flow cytometry sorting of GFP+ cells, *in vitro* cultured erythroid cells were stained with PI (1μg/mL). GFP-positive/PI-negative cells were sorted. For flow cytometry sorting of enucleated cells, *in vitro* cultured erythroid cells were stained with PI (1μg/mL), 1:100 APC-conjugated Ter119, and Hoechst (1μg/mL). GFP-positive/PI-negative/Ter119-positive/Hoechst-negative cells were sorted.

May-Grünwald Giemsa staining

Approximately 200,000 erythroid cells were centrifuged at 300 rpm for 4 min onto polylysine-coated slides in a cytocentrifugation apparatus. Standard May-Grünwald Giemsa staining was then performed [26]. Images were taken under a fluorescence microscope (40X objective; Zeiss AxioPlan2 upright microscope). Quantification of cell areas was performed using Image J software.

Cell-cycle and proliferation analyses

In vitro cultured erythroid cells at indicated time points were pulsed with 10 μM BrdU for 30 min. using a BrdU flow kit (BD Pharmingen, 552598) according to the manufacturer's

protocol. Pulsed cells were fixed and permeabilized, treated with DNase to expose incorporated BrdU epitopes, and stained with APC-conjugated anti-BrdU antibodies and 7-AAD for total DNA content. Cells were analyzed by flow cytometry [24].

Lineage-negative mouse fetal erythroid progenitor cells were infected with Tfdp2 shRNA and Luc control shRNA viruses. Immediately after infection, the number of seeded mouse fetal liver cells was determined using a hematocytometer. After 12 hours of infection, the GFP+ percentages were measured by flow cytometry analysis, and multiplied by the number of cells seeded; this was taken to be the day 0 cell number. The number of cells after 24 and 48 hours of culture were counted using a hematocytometer. GFP+ percentages for those time points were measured by flow cytometry analysis.

RNA isolation, quantitative RT-PCR, and microarray experiments

Erythroid cells were collected at specified time points using QIAzol (Qiagen) and total cellular RNA was isolated using the miRNeasy minikit (Qiagen) according to the manufacturer's protocol. Reverse transcription was carried out using SuperScript II Reverse Transcriptase (Invitrogen). Relative transcript levels of different genes were quantified by real-time PCR, using SYBR Green (Applied Biosystems) and the ABI 7900 Machine Real-Time PCR system. Results were normalized to 18S (criteria for differences in 18S Ct values for control and experimental sample set at <1.5). Primer sequences are found in Supplemental Table 1.

Microarray experiments were performed and processed using the Affymetrix Mouse Genome 430 2.0 array at the Whitehead Institute Genome Core.

Statistical analysis of experimental data

Pairwise comparisons of experimental results were performed. Unpaired one-tailed Student's t-tests for experimental data with corresponding biological replicates were used to determine the statistical significance of data. P values <0.05 were considered significant.

Results

Global gene expression analysis identifies many transcription factors and cofactors induced during terminal erythroid differentiation

Transcription factors and their cofactors play important roles in regulating terminal erythroid differentiation. To obtain a global view of these transcriptional regulators in this process, we analyzed the expression pattern of 276 transcription factors and 213 cofactors defined by gene ontology [21] as 'sequence-specific DNA binding transcription factor activity' and 'transcription cofactor activity' respectively. Based on RNA-sequencing data on R1-R5 populations, which represent consecutive stages of *in vivo* erythropoiesis from CFU-Es to mature reticulocytes, the majority of transcription factors and cofactors are downregulated during terminal differentiation, while only a small number are upregulated (Figure 1A, Supplemental Figure 1). Several upregulated factors, such as Sox6 and Zfpm1, have previously been shown to be indispensable for erythropoiesis [27, 28]; however, the majority of these induced factors remain to be characterized.

Many of these induced factors may also be targeted by erythroid-important transcription factors, specifically GATA1 and TAL1 [29, 30]. By mining existing ChIP-seq data for the promoter, exon, and intron regions of all induced and repressed factors, we found that the regulatory regions of 18 out of 28 (64%) genes encoding induced transcription factors and 12 out of 19 (63%) induced cofactors are co-bound by GATA1 and TAL1 in mouse erythroblasts, compared to only 37 out of 226 (16%) repressed transcription factors and 41 out of 180 (22%) repressed cofactors (Figure 1B, C). Co-occupancy of gene regulatory regions by GATA1 and TAL1 for transcription factors and cofactors is therefore significantly associated with gene induction, consistent with previous studies [31]. These ChIP-seq findings, together with analysis of gene expression patterns, serve as a resource to provide promising candidates for further functional analysis of their roles in erythroid transcriptional regulation.

Since transcription factors and their cofactors normally function together to regulate biological processes, we further probed the expression correlation of paired interacting transcription factors and cofactors using the STRING protein-protein interaction database [10], and pinpointed several pairs as jointly upregulated (Figure 2A). Our analysis yielded Zfpm1 (Fog1) and its previously identified interacting transcription factors Tal1, Lmo2, and Gata1 [1, 32, 33], as co-induced pairs, thus validating our approach. Regulatory regions of genes encoding many of these co-induced factors are also bound by GATA1 and TAL1, and binding peaks are shown for both the known Tal1 and Zfpm1 pair (Figure 2B), and a novel candidate pair, Ddit3 and Trib3 (Figure 2C).

Tfdp2 and E2f2 mRNA are highly induced during terminal erythropoiesis by erythroid-specific transcription factors GATA1 and TAL1

Our global analysis revealed transcription factor E2F2 and cofactor TFDP2 as the most highly induced pair (Figure 2A). Since TFDP2 has never been linked with erythropoiesis, we aimed to functionally characterize the potential role of TFDP2 in regulating erythroid differentiation. Based on RNA-sequencing gene expression data [4], expression of both *Tfdp2* and *E2f2* mRNA were highly upregulated *in vivo* and followed similar patterns, with the greatest induction occurring at the R2 to R3 transition (Figure 3A). The expression patterns for *Tfdp2* and *E2f2* were confirmed by quantitative RT-PCR (Figure 3B). In contrast, expression of *Tfdp1*, the first TFDP family member identified, decreased ~2 fold from R2 to R3 and over 5 fold from R3 to R4 (Figure 3A); there was no detectable expression of *Tfdp3* at any stage. The significant upregulation of *Tfdp2* expression, as opposed to the downregulation of *Tfdp1* expression, suggests that TFDP2 plays an important role in regulating terminal erythropoiesis.

In parallel with our bioinformatics analysis, we utilized publicly available ChIP-seq databases for GATA1 and TAL1 and found two regions of co-occupancy in both *E2f2* and *Tfdp2* genes (Figure 3C–D). We also analyzed the pattern of histone modifications, using histone 3 Lys 9 acetylation (H3K9ac) as a marker of active chromatin, histone 3 Lys 4 monomethylation (H3K4me1) as a marker of enhancer elements, and histone 3 Lys 4 trimethylation (H3K4me3) as a marker of active promoter regions. The two peaks within the *E2f2* gene (1 and 2) are likely in enhancer regions, as they are found in introns and in

H3K4me1-positive regions (Figure 3C; Supplemental Figure 2A). Peak 1 in the first intron of *E2f2* has been shown in previous studies as a region that is occupied by GATA1 and that likely modulates *E2f2* expression [34]. In the region surrounding the *Tfdp2* gene, we discovered a single peak (3) present in a possible promoter region that is 5′ of the transcriptional start site, H3K4me3-positive and H3K4me1-negative. A second peak (4) lies within the first intron of *Tfdp2*, possibly representing an enhancer element (Figure 3D; Supplemental Figure 2B).

To determine whether GATA1 and TAL1 binding is functionally significant for increased expression of *Tfdp2*, we performed luciferase reporter assays on the two peaks in the *Tfdp2* gene. Both the regions in the putative promoter of *Tfdp2*, peak 3, as well as in the first intron, peak 4, were responsive to GATA1 and TAL1, with a ~3 fold increase in relative luciferase activity after addition of GATA1 and TAL1 (Figure 3E).

Tfdp2 is required for induction of multiple erythroid-important genes and eventual enucleation

To characterize the role of *Tfdp2* in terminal erythroid differentiation, we used *in vitro* culture of primary mouse fetal liver (FL) erythroid cells and abrogated *Tfdp2* expression through retroviral-expressed shRNA hairpins. Both shRNAs (shTFDP2a and shTFDP2b) significantly reduced expression of *Tfdp2* mRNA in FL cells after both 1 and 2 days of culture (Figure 4A).

TFDP2 is critical for proper induction of erythroid-important genes, as knockdown reduced the expression of the alpha and beta hemoglobin chains (Hbb-b1 and Hba-a1), GATA1, the erythrocyte membrane protein Epb4.1, enzymes required for heme biosynthesis, and other markers of late-stage differentiation (Figure 4B). Consistent with impaired differentiation, TFDP2 knockdown cells also contained abnormally high mRNA levels of the transcription factor and proto-oncogene c-Myc (Figure 4B), whose normal downregulation is essential for terminal erythropoiesis [35].

Given the reduced expression of key erythroid-important genes we expected that subsequent enucleation would also be inhibited. FL cells were therefore analyzed by flow cytometry after 24, 48, and 72 hours of culture. Both shRNA constructs reduced the percentage of enucleated cells (Ter119-positive, Hoechst-negative), and quantification of enucleation rates in three biological replicates for all time points revealed that both shRNA constructs reduced enucleation to less than 40% of normal levels (Figure 4C–D). The number of enucleated cells in knockdown cultures remained low even after 72 hours of culture, suggesting that loss of *Tfdp2* does not result in delayed erythroid maturation, but rather in a blockage of this process.

To determine whether TFDP2 affects apoptosis, we stained FL cells after 2 days of culture and found only a slight increase in the percentages of apoptotic cells in TFDP2 knockdown samples (Figure 4E; Supplemental Figure 3). Collectively, these results suggest that normal upregulation of *Tfdp2* is specifically required for proper erythroid differentiation and eventual enucleation.

TFDP2 regulates cell cycle progression and cell size during terminal erythroid differentiation

Loss of *Tfdp2* resulted in an over 2-fold decrease in cell proliferation (Figure 5A). May-Grünwald Giemsa staining of the total population of cells after a 2-day culture showed that, after *Tfdp2* knockdown, the cells appeared larger with aberrations in cellular morphology and reduced enucleation (Figure 5B). An increase in overall cell size after *Tfdp2* knockdown was confirmed by quantifying the mean forward scatter area (FSC-A), a metric of erythroblast size and shape [36] (Figure 5C); this increase is also shown using FSC-A histograms and cumulative distribution functions (Figure 5D). Additionally, we examined the sizes of enucleated cells following May-Grünwald Giemsa staining of a sorted pure enucleated cell population. There is not only a subtle increase in cell size in the knockdown samples, but also a wider range of cell sizes, with some comparable to the control, and others markedly larger (Figure 5E). To confirm this finding, we quantified the areas of individual reticulocytes, and found that knockdown with either shRNA resulted in cells with larger areas ($P < 0.001$) and with a wider range of sizes (Figure 5F). This finding is consistent with the increased RBC volume observed in E2F2 knockout mice [14].

To understand the underlying mechanism behind these differentiation, proliferation, and cell size defects, we investigated TFDP2's effect on the cell cycle of terminally differentiating erythroblasts utilizing a bromodeoxyuridine (BrdU) incorporation assay. *Tfdp2* knockdown resulted in accumulation of cells in S phase, with progressively increasing S phase percentages over the control during the 2 day culture (~1.2 fold increase at day 1 and ~1.5 fold increase at day 2) (Figure 5G; Supplemental Figure 4). Overall, for both knockdown and control samples, more cells were in S phase at day 1 of culture compared to day 2, as expected [37]. Also as expected, by the end of the second day the majority of control cells had exited the cell cycle and accumulated in G0/G1. Following *Tfdp2* knockdown however, fewer cells were in the G1 phase and more had accumulated in the S phase of the cell cycle (Figure 5G). In summary, cells lacking *Tfdp2* undergo fewer cell divisions than normal and are defective in exiting the cell cycle, leading to fewer and larger cells.

TFDP2 is essential for normal global downregulation of E2F2 target genes

In many cell types, E2Fs controls the cell cycle by directly regulating the expression levels of many cell cycle important genes [38]. To understand the involvement of E2F-regulated genes in terminal erythroid differentiation, we profiled the expression levels of several genes, known in other cells to be direct E2F targets, in the R1-R5 phases of differentiation. The expression of key genes important for DNA replication (*Dhfr* and *Mcm3*), as well as cyclin A2 and two cyclin-dependent kinases, *Cdc2* and *Cdc6*, are normally downregulated during terminal erythropoiesis (Figure 6A). A more global analysis comparing the expressions of 130 verified E2F2 target genes [38] in CFU-E (Ter119-negative) versus Ter119-positive mature erythrocytes [39] extends the results in Figure 6A, as 112 (86%) of these target genes are more highly expressed in CFU-Es (Figure 6B), demonstrating a significant global repression of E2F2 target genes during terminal differentiation.

As a mechanistic explanation for the effects of *Tfdp2* knockdown on erythroid cell cycle and differentiation, we hypothesize that TFDP2, a known E2F binding partner that associates

with E2F2 in erythroid cells (Supplemental Figures 5–6), likely plays a role in the physiological repression of these E2F2 targets. To probe the transcriptional effects of *Tfdp2* knockdown on a global scale, we used unbiased DNA microarrays to compare the global gene expression profiles of cells infected with luciferase control shRNA with *Tfdp2* knockdown cells. The DAVID Bioinformatics database [40] was used to analyze functional gene ontology (GO) terms assigned to genes whose expressions were significantly increased after *Tfdp2* knockdown, and to calculate enrichment scores for individual functional categories. Expression of the 'cell cycle' gene category was most highly enriched, supporting TFDP2's important role in repressing expression of cell cycle genes (Figure 6C).

To visualize any global shifts in expression of subsets of genes, cumulative distribution function curves, generated by calculating the ratio of log2 expression values in knockdown cells to those in control cells across specified gene sets, were plotted. On average, there is no change in expression of the totality of genes probed by the microarray. In contrast, the average expression level of E2F2 target genes undergoes a statistically significant increase, demonstrated by the rightward shift of the curve compared to the curve representing all genes probed by the microarray (Figure 6D). This microarray data is confirmed by quantitative RT-PCR of five selected E2F2 target genes (Supplemental Figure 7).

The cumulative distribution curves depicted in Figure 6E make a related point and extend the results shown in Figure 4B. The global expression levels of the 664 most highly induced genes during erythroid terminal differentiation ('Induced genes') decreases following *Tfdp2* knockdown, shown by the leftward shift of the curve, whereas the average expression of the 9584 most highly repressed genes during erythroid differentiation from the CFU-E to Ter119-positive stage [39] ("'Repressed genes'") is increased, shown by the rightward shift of the curve.

Altogether, these findings suggest that mechanistically, TFDP2 mediates the normal repression of E2F2 targets during the final stages of erythroid differentiation; knockdown of *Tfdp2* therefore results in insufficient repression of E2F2 target genes, leading to stalled cell cycle progression, which in turn disrupts transcriptional and cellular events that characterize terminal erythropoiesis.

Discussion

Differentiating erythroblasts undergo vast transcriptional changes, closely orchestrated by a network of transcription factors and their cofactors. Our bioinformatics-based study provides a global view of all factors and their expression modulations in erythropoiesis, and utilizes protein interaction databases to pinpoint novel and known pairs of transcription factors and their respective cofactors that are critical for proper erythroid maturation. This work supplements recent advances in our understanding of key erythroid transcriptional regulators, notably GATA1, a master regulator of gene expression in terminally differentiating erythroblasts [1, 6, 29, 32]. GATA1 targets genes involved in heme biosynthesis, erythropoietin signaling, and anti-apoptotic and proliferation pathways, as well as other transcription factors and cofactors, giving rise to the theory of regulatory hierarchies, whereby GATA1 exerts its ultimate effects by modulating expression of

intermediary regulators [6, 41–43]. The upregulation of the alpha and beta globin genes by GATA1 for example, requires a feed-forward loop, in which GATA1 induces FOG1, which then cooperates with GATA1 to induce globin gene expression [6]. Many of the transcription factors and cofactors found to be induced and bound by GATA1 in our study may also represent an essential intermediate layer of transcriptional regulation, with TFDP2 serving as a validated example.

We uncover a novel mechanism involving TFDP2, by which the cell can control cell cycle progression and couple it with terminal differentiation (Figure 7). In brief, joint upregulation of *Tfdp2* and *E2f2* by GATA1 and TAL1 is essential for normal downregulation of many E2F2 target cell cycle genes during differentiation, for normal induction of many erythroid-important genes, and for terminal cell divisions. This discovery helps answer the elusive question of how erythroid cells coordinate their cell cycle with their differentiation program, and sheds light on the specific role of E2F2 in terminal erythropoiesis. Although E2F2 is known to be essential for terminal erythropoiesis [14, 15], its detailed mechanism had not been clearly explicated. Erythroid progenitors from E2F2 knockout mice exhibit defective S phase progression and fail to exit the cell cycle and mature[14], suggesting that E2F2 is required for cell cycle progression, without which terminal erythroid differentiation cannot occur. Previous work has also suggested the formation of a triprotein complex composed of E2F2, GATA1, and the tumor suppressor Rb, that stalls cell proliferation and drives erythroid precursors towards differentiation [44]. Other E2F2-associated proteins, including Rb and the erythroid-specific transcription factor EKLF, are also critical for terminal erythropoiesis [15, 45, 46], further highlighting the central role of E2F2 in regulating the erythroid cell cycle.

Our experiments establish that E2F2 acts as a transcriptional repressor in terminally differentiating erythroid cells to facilitate cell cycle exit. Although E2F2 is traditionally characterized as an activating E2F essential for normal cell proliferation [47], more recent evidence has suggested that E2F2 can switch from an activator in intestinal stem cells to a repressor in terminally differentiating intestinal cells [48], consistent with our working model (Figure 7). We show that TFDP2 is a key link between erythroid differentiation and cell cycle control, since loss of TFDP2 allows continued gene activation by E2F2, causing cells to stall in S phase, reducing the number of erythroid cell divisions, and resulting in ineffective erythropoiesis. TFDP2 may also mediate its effects through interactions with other transcriptional regulators identified in our bioinformatics analyses and known to function in erythropoiesis, including transcription coactivators p300 and CBP [49–51], and histone deacetylase 1 (Hdac1) [52–54].

In conclusion, we utilize predictions from our bioinformatics study on expression changes of all transcription factors and cofactors, and their interactions to uncover the novel gene, TFDP2 as a crucial molecule that allows terminally differentiating erythroid cells to couple cell cycle control and differentiation. Our global analysis and functional study of TFDP2 provide both a validated resource and roadmap for future studies on transcriptional regulators in erythropoiesis, helping to enhance our molecular understanding of red blood cell production in both physiological and pathophysiological states, and to develop more targeted therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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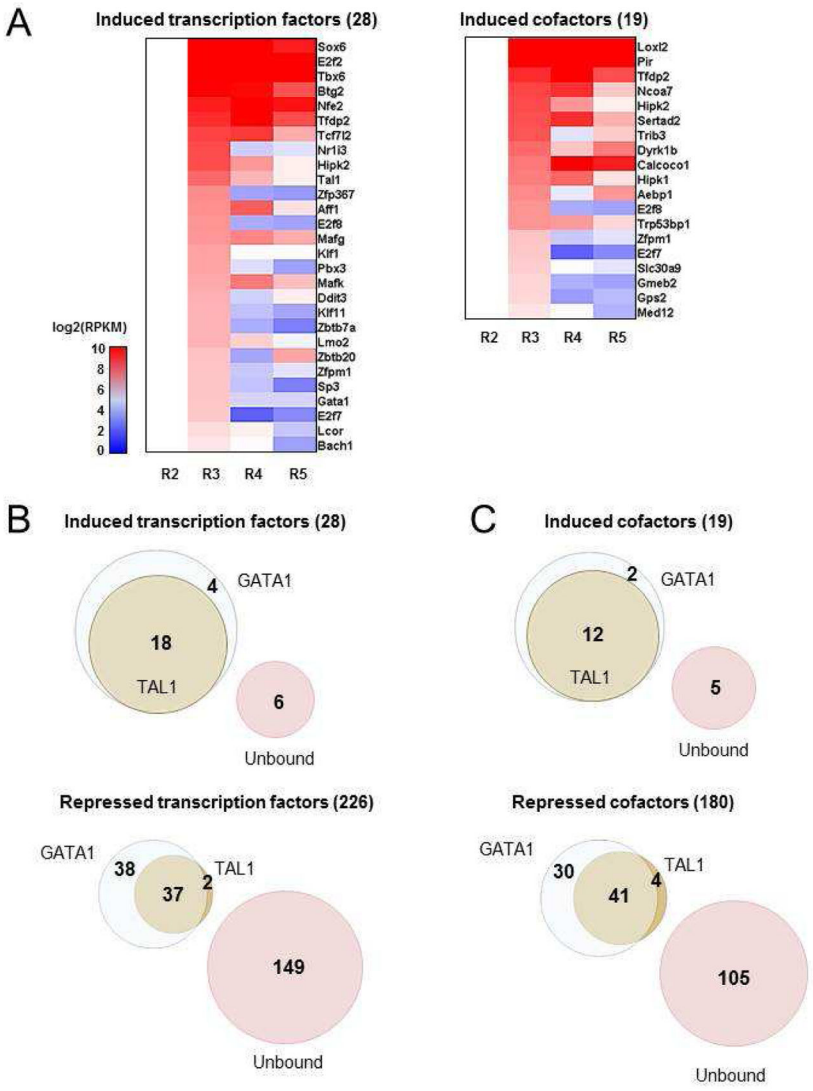

Figure 1. Expression and ChIP-seq analysis of transcription factors and cofactors induced during terminal erythroid differentiation

(A) Freshly isolated E14.5 mouse fetal liver erythroid progenitors were analyzed by FACS using fluorescent antibodies for CD71 and Ter119. R2 through R5 represent consecutive stages in terminal erythroid differentiation, defined by staining patterns of the different stages. mRNA expression profiles of the 28 highest induced transcription factors and 19 highest induced cofactors are shown over the R2 to R5 stages, as determined by RNA-seq deep sequencing of the total mRNA expressed by each population [4]. All expression values are calculated as ratios compared with R2 values. (B–C) Binding of GATA1 and TAL1 within promoter, exon, and intron regions of either repressed or induced transcription factors (B) and cofactors (C) shown as Venn diagrams. ‘Unbound’ represents genes exhibiting no binding by either GATA1 or TAL1.

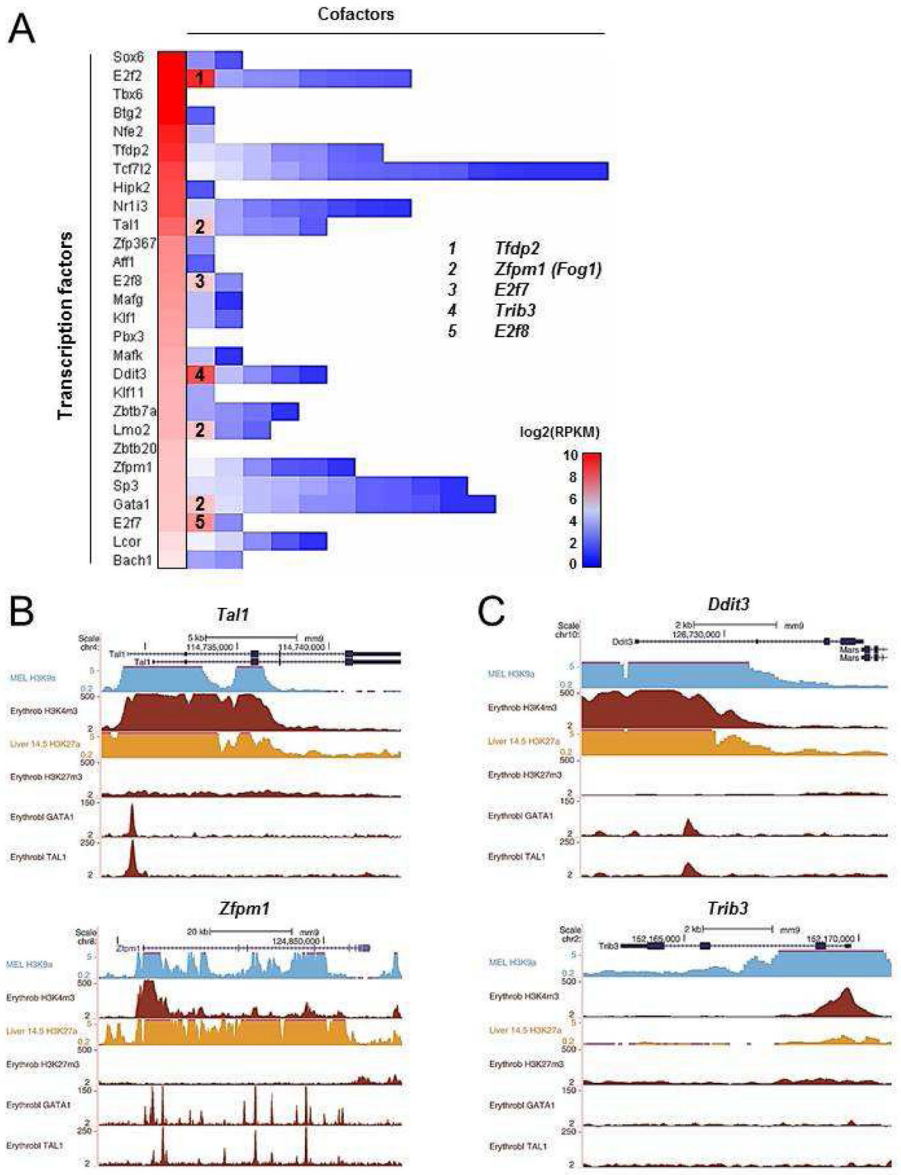

Figure 2. Identification of pairs of transcription factors and cofactors that are co-induced and possess co-occupancy by GATA1 and TAL1

(A) Ratios of R3 over R2 expression values are shown as a heat map for 28 induced transcription factors and their respective cofactors. Induced cofactors (1–5) are numbered and labelled with their gene names. (B–C) ChIP-seq (chromatin IP coupled with deep sequencing) data for H3K9ac, H3K4me3, H3K27ac, H3K27me3, GATA1, and TAL1 are shown as images from the UCSC Genome Browser in the *Tal1* and *Zfpm1* (B), and *Ddit3* and *Trib3* (C) genes. ChIP-seq signals are depicted as the density of processed signal enrichment. H3K9ac marks active chromatin, H3K4me3 marks active promoters, H3K27ac marks active enhancers, and H3K27me3 marks regions of transcriptional repression.

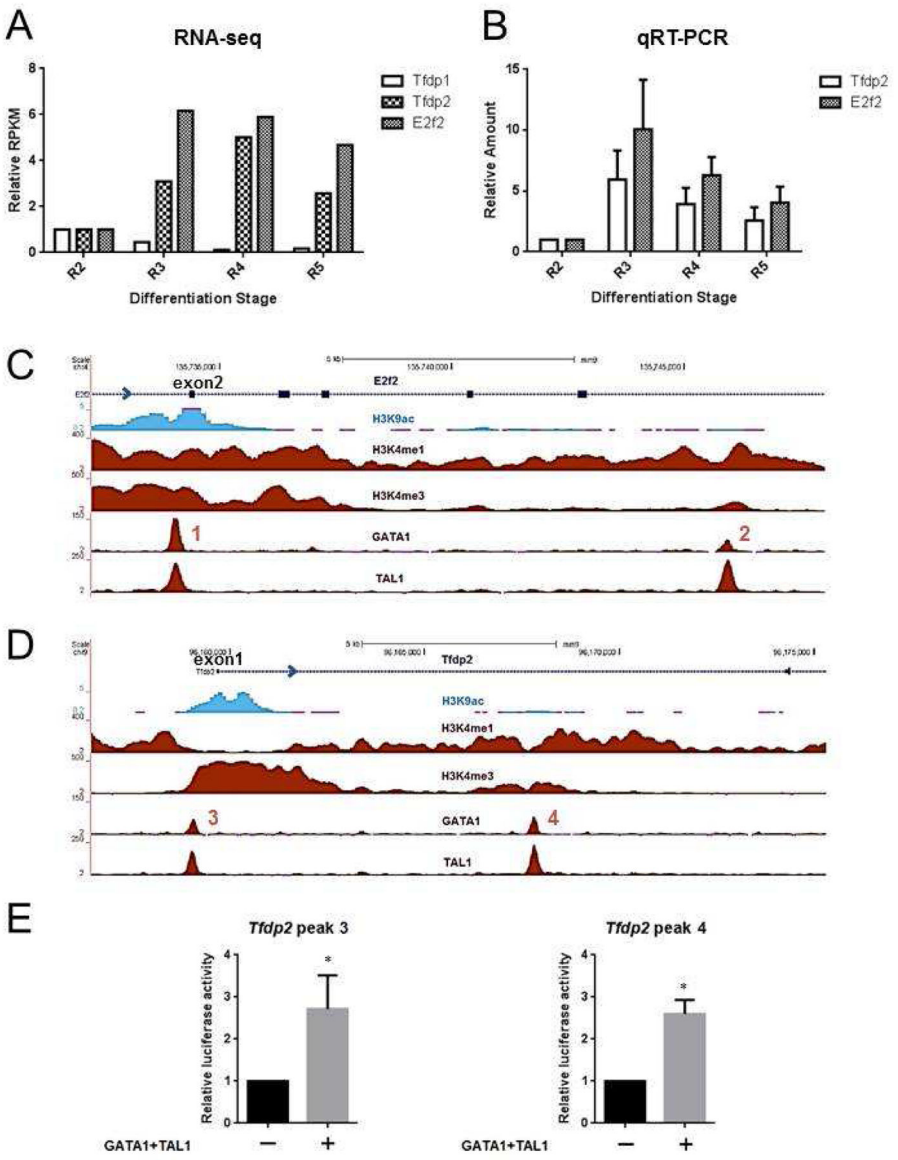

Figure 3. *Tfdp2* and *E2f2* expression are upregulated by GATA1 and TAL1 during *in vivo* terminal erythropoiesis

(A) *Tfdp1, Tfdp2,* and *E2f2* mRNA expression profiles are shown over the R2 to R5 stages. The y-axis represents the amount of expressed mRNA as RPKM (reads per kilobase of exon per million mapped reads) in each differentiation stage (x-axis). (B) *Tfdp2* and *E2f2* mRNA expression profiles are shown over the R2 to R5 stages, as determined by quantitative RT-PCR. Expression levels were normalized to 18S. Error bars represent standard deviation (SD; n=3). (C–D) ChIP-seq data for H3K9ac, H3K4me1, H3K4me3, GATA1, and TAL1 are shown in the region of the *E2f2* and *Tfdp2* genes, respectively, surrounding two GATA1 and TAL1 peaks (labeled 1–4) Exon numbers are labeled, and arrows indicate direction of transcription. (E) The luciferase reporter pGL3-Basic vector cloned with either *Tfdp2* peaks 3 and 4 (see Methods) or an empty vector were co-transfected with 90 ng each of XZ-GATA1 and XZ-TAL1 into MCF-7 cells. Luciferase activities were measured 2 days later

and were normalized to *Renilla* luciferase activity. Error bar is standard deviation (SD; n=3). (*) represents P <0.05 in student T-test.

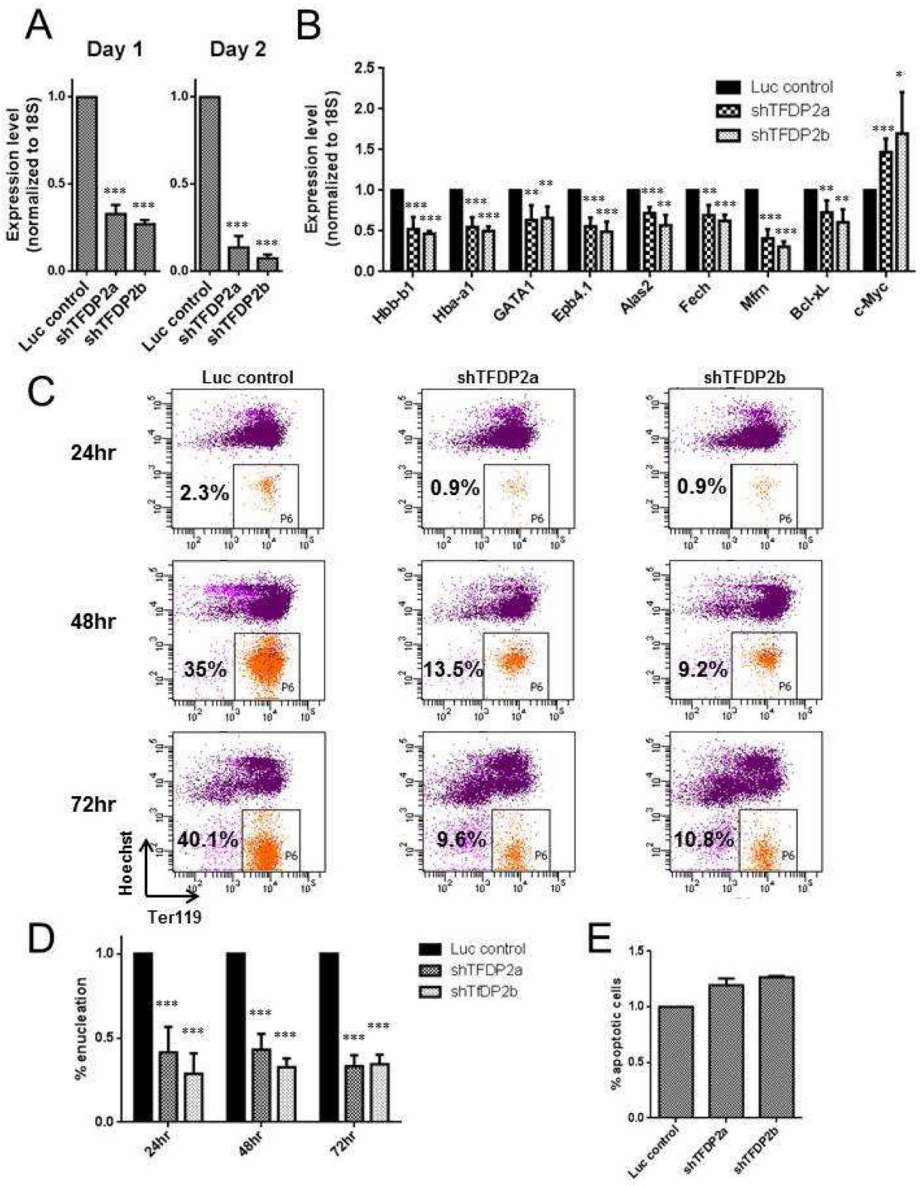

Figure 4. Specific *Tfdp2* knockdown results in differentiation and enucleation defects

shRNA hairpins designed to specifically target *Tfdp2* were cloned into retroviruses and subsequently infected into Ter119-negative fetal liver erythroid progenitors. Cells were then cultured in Epo-containing media for a specified amount of time before analyses. A nonspecific shRNA hairpin against the luciferase gene was used as a control. (A) Quantitative RT-PCR on RNA isolated from cells after 24 (Day 1) and 48 (Day 2) hours of culture was performed using primers against *Tfdp2*. Expression levels were normalized to 18S and compared with control-infected cells. Error bars represent standard deviation (SD; n=3). (***) represents P <0.001 in student T-test. (B) Percentages of enucleated cells after 24, 48, and 72 hours after culture are shown. Results for knockdown cells were normalized to those in control samples. Error bar is SD (n=3). (***) represents P<0.001. (C) *In vitro* cultured fetal liver erythroid progenitors were stained with Ter119-APC and Hoechst at 24,

48, and 72 hours. Representative FACS plots are shown (n=3); percentages represent percentage of enucleated cells. (D) mRNA expression changes of essential erythroid development genes after 48 hours of *Tfdp2* knockdown are shown as measured by quantitative PCR analysis. Error bar is SD (n=3). (*) represents P <0.05, (**) represents P <0.01, (***) represents P <0.001. (D) Percentages of apoptotic cells in day 2 samples as determined by Annexin V-PE and 7AAD staining are shown, normalized to those in control samples. Error bar is SD (n=3).

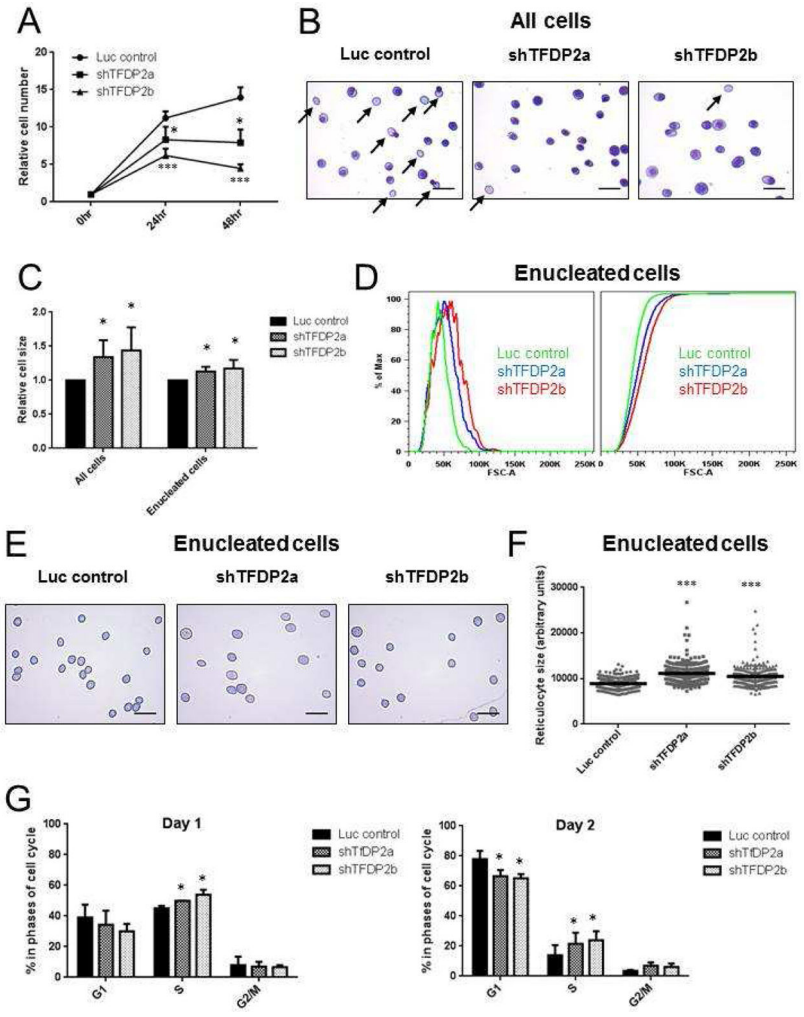

Figure 5. Loss of *Tfdp2* causes an increase in cell size and an accumulation in S phase

(A) Proliferation assays were performed for shRNA-infected cells and control cells. Relative cell numbers at 24 and 48 hours were normalized to day 0 cell numbers. Error bar is SD (n=3). (*) represents P <0.05, (***) represents P <0.001. (B) May-Grunwald Giemsa staining was performed on day 2 *in vitro* cultured progenitors; representative images (n=3) are shown; scale bar represents 25um. Arrows mark enucleated, or enucleating, reticulocytes. (C) Relative cell size after 48 hours of culture, determined by normalizing the mean FSC-A (forward scatter area) of the knockdown samples to the control, is plotted for all infected cells and enucleated cells. Error bar is SD (n=3). (*) represent P <0.05 in student T-test. (D) Representative forward scatter area and plots of the cumulative distribution function are shown for enucleated cells in the control and two shRNA-infected samples. (E) May-Grunwald Giemsa staining was performed on day 2 sorted enucleated cells; representative images (n=3) are shown; scale bar represents 25um. (F) Cell sizes for (E) were quantified by image processing. (***) represent P <0.001. (G) Day 1 and Day 2 cultured erythroid progenitors were pulsed with BrdU and subsequently stained with anti-BrdU-APC and 7AAD. Distribution of cells in different phases of the cell cycle after 24 and 48 hours of culture was quantified. Error bar is SD (n=3). (*) represents P <0.05.

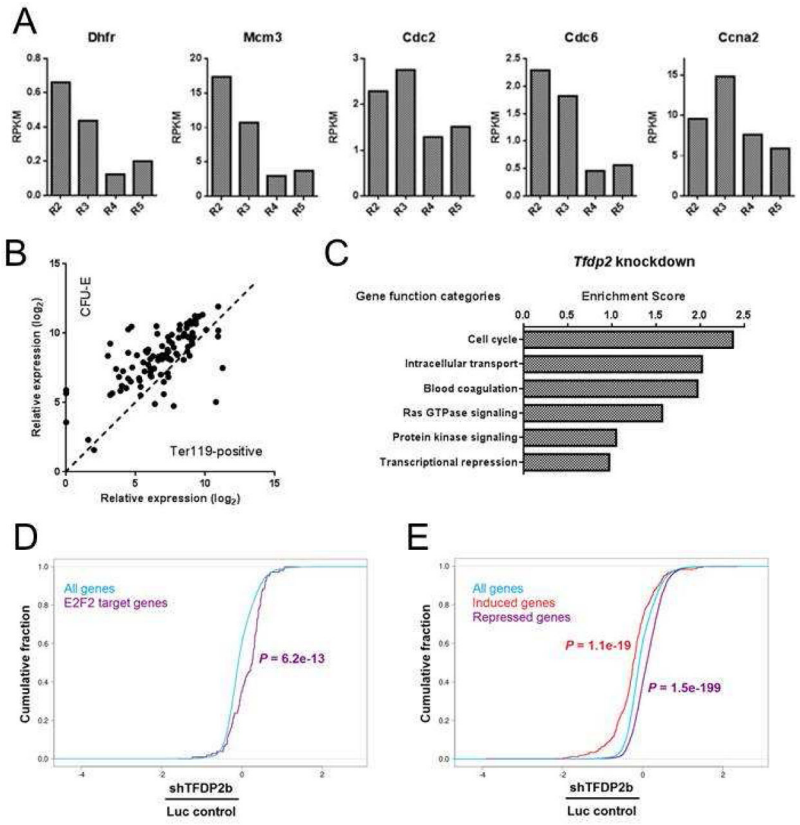

Figure 6. Expression of E2F2 target genes is globally repressed during terminal differentiation and loss of TFDP2 releases that repression

(A) mRNA expression profiles for five E2F2 target genes are shown over the R2 to R5 stages. The y-axis shows the amount of expressed mRNA as RPKM (reads per kilobase of exon per million mapped reads) in each differentiation stage (x-axis). (B) Scatter plot comparing expression of 130 known E2F2 target genes [38], in CFU-Es and Ter119-positive cells are shown [39]. Dashed line represents x=y. (C) Total RNA was isolated from fetal liver erythroid cells cultured in Epo-containing media for 48 hours after infection with either shTFDP2b or the Luc control. Isolated RNA was reverse transcribed, labeled, and hybridized onto Affymetrix mouse genome expression arrays. Samples were isolated and processed in duplicates. Functional ontology classifications for genes whose expressions were significantly increased ($p<0.05$, log2 ratio>0.7) in TFDP2 knockdown cells are shown. (D–E) Plots of the cumulative distribution function are shown as a comparison of gene expression changes in infected cells and control. The x axis is the relative expression of each gene, defined as the ratio of the log2 expression values of knockdown cells to those of control cells. The y axis is the cumulative fraction as a function of the relative expression (x axis). ‘All genes’ represent all the genes probed by the microarray; ‘E2F2 target genes’ represent 130 known targets of E2F2; ‘Induced genes’ and ‘Repressed genes’ represent 664 most highly induced genes and 9584 most highly repressed genes during erythroid differentiation from the CFU-E to Ter119-positive stage. P-values were calculated using the Kolmogorov-Smimov test.

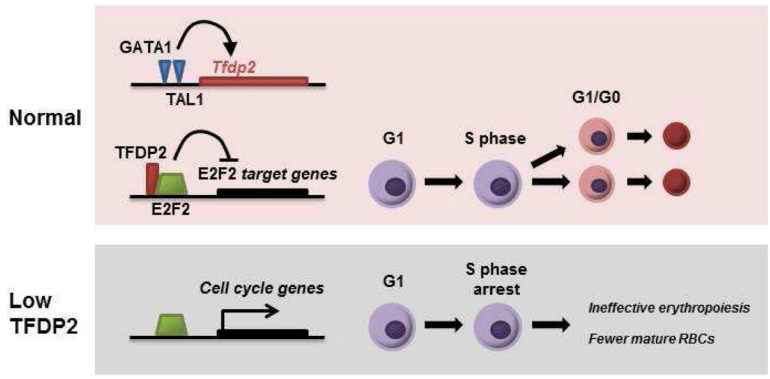

Figure 7. Model of how TFDP2 acts as a key regulator in the coordination of differentiation and cell cycle control in erythropoiesis

During normal terminal erythroid differentiation, erythroid transcription factors GATA1 and TAL1 jointly upregulate expression of *Tfdp2*. TFDP2 and E2F2 then associate to repress expression of E2F2 target genes, many of which are important cell cycle genes. Proper regulation of their expression levels allow for proper S phase progression, subsequent cell cycle exit, and finally maturation into enucleated RBCs. Loss of *Tfdp2* compromises E2F2's ability to act as a repressor, leading to continued expression of normally repressed cell cycle genes. This causes aberrant entry into and arrest in S phase, ultimately resulting in ineffective erythropoiesis and fewer mature RBCs. This model is supported by mouse genetic and cellular studies presented here.