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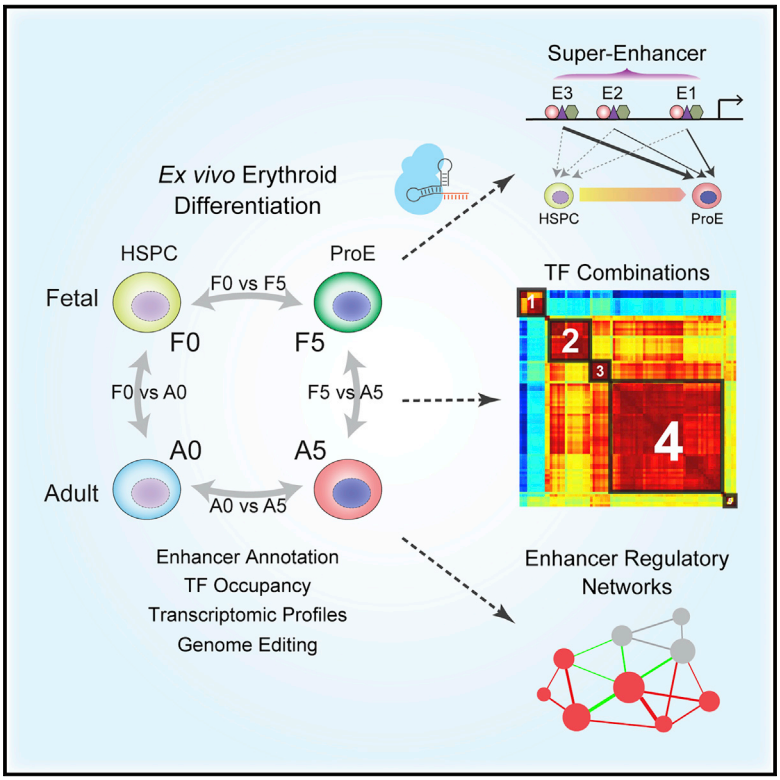
Dynamic Control of Enhancer Repertoires Drives Lineage and Stage-Specific Transcription during Hematopoiesis

Graphical Abstract



Highlights

- Pervasive changes in enhancer landscapes occur during blood stem cell specification
- Analysis of developmentally regulated enhancers uncover driver TFs and combinations
- Genomic editing reveals functional hierarchy of super-enhancer constituents
- Functionally divergent GATA switch enhancers cooperate within enhancer clusters

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In Brief

Enhancers are crucial determinants of cell identity. Huang et al. compare enhancer landscapes in human hematopoietic stem/progenitor cells and erythroid progenitors. They uncover driver transcription factors and their combinatorial patterns in enhancer turnover. Genomic editing of constituent enhancers reveals functional hierarchy and diversity of enhancer clusters during hematopoiesis.

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Dynamic Control of Enhancer Repertoires Drives Lineage and Stage-Specific Transcription during Hematopoiesis

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SUMMARY

Enhancers are the primary determinants of cell identity, but the regulatory components controlling enhancer turnover during lineage commitment remain largely unknown. Here we compare the enhancer landscape, transcriptional factor occupancy, and transcriptomic changes in human fetal and adult hematopoietic stem/progenitor cells and committed erythroid progenitors. We find that enhancers are modulated pervasively and direct lineage- and stage-specific transcription. GATA2-to-GATA1 switch is prevalent at dynamic enhancers and drives erythroid enhancer commissioning. Examination of lineage-specific enhancers identifies transcription factors and their combinatorial patterns in enhancer turnover. Importantly, by CRISPR/Cas9-mediated genomic editing, we uncover functional hierarchy of constituent enhancers within the *SLC25A37* super-enhancer. Despite indistinguishable chromatin features, we reveal through genomic editing the functional diversity of several GATA switch enhancers in which enhancers with opposing functions cooperate to coordinate transcription. Thus, genome-wide enhancer profiling coupled with in situ enhancer editing provide critical insights into the functional complexity of enhancers during development.

INTRODUCTION

Stem cell self-renewal and differentiation require precisely regulated tissue-specific and developmental stage-specific gene expression. Enhancers are *cis*-acting DNA sequences that can increase the transcription of genes through cooperative and synergistic binding of transcription factors (TFs), DNA binding effectors of signaling pathways, and chromatin-modifying complexes (Banerji et al., 1981). Enhancers function from distal regions in an orientation-independent manner, and harbor distinct chromatin features including increased chromatin accessibility, characteristic histone modifications and DNA hypomethylation, and bidirectional transcription (Bulger and Groudine, 2011). Although major progress has been made toward genome-wide annotation of candidate enhancers (Andersson et al., 2014; Heintzman et al., 2007, 2009; Visel et al., 2009a), the molecular processes controlling enhancer activation and deactivation during lineage commitment remain poorly understood.

A defining feature of enhancers is their ability to function as integrated platforms for TF binding, where cell-intrinsic and -extrinsic signaling cues are interpreted in a highly lineage- and context-dependent manner (Buecker and Wysocka, 2012). Despite recent advances in profiling enhancer-associated biochemical features, the biological importance of individual enhancers in lineage differentiation is often limited by lack of insights in enhancer regulation in vivo and in molecular details of enhancer structure-function in situ. The fundamental questions related to enhancer function and mechanisms remain unanswered: how do enhancers regulate precise spatiotemporal gene expression patterns? How are enhancers organized and regulated in a high-dimensional chromatin environment? How do enhancers communicate with their target genes in vivo during development? Furthermore, how do mutations and genetic variations in enhancers influence human disease?

Recently, intensely marked clusters of enhancers or super-enhancers containing an exceptionally high degree of enrichment of master TFs, Mediator, and chromatin marks have been identified in a broad range of mammalian cell types (Hnisz et al., 2013; Parker et al., 2013; Whyte et al., 2013). Super-enhancers

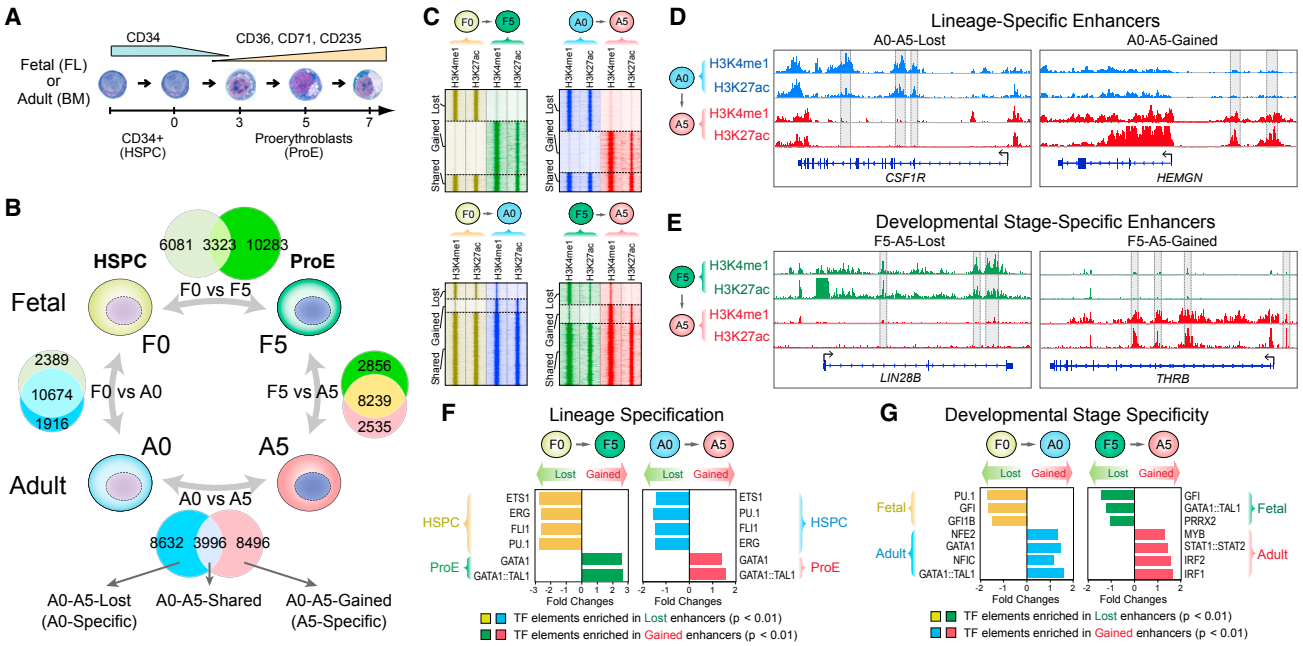


Figure 1. Comparative Analysis of Enhancer Repertoires during Human Erythropoiesis

(A) Ex vivo erythroid differentiation of fetal liver (FL) or adult bone marrow (BM) CD34+ HSPCs. Cells at matched stages of differentiation (HSPC: F0 and A0; ProE: F5 and A5) were collected for transcriptomic profiling and ChIP-seq analyses.

(B) Identification of lineage or developmental stage-specific enhancers. Venn diagram shows the overlap between HSPC and ProE, or fetal and adult enhancers. The numbers of lost, shared, or gained enhancers in each comparison are shown.

(C) ChIP-seq density heatmaps for H3K4me1 and H3K27ac within lost, gained, and shared enhancers in each comparison.

(D) Representative lineage-specific enhancers are shown. The putative active enhancers are depicted by shaded lines.

(E) Representative developmental stage-specific enhancers are shown. The putative active enhancers are depicted by shaded lines.

(F) Enrichment of lineage-defining TFs in lineage-specific enhancers. The top enriched TF motifs in lost or gained enhancers between HSPCs and ProEs at fetal or adult stage are shown. p values were calculated using the hypergeometric test.

(G) Enrichment of distinct coregulators in stage-specific enhancers. The top enriched TF motifs in lost or gained enhancers between fetal and adult HSPCs (F0 versus A0) or ProEs (F5 versus A5) are shown. p values were calculated using the hypergeometric test.

See also Figure S1.

differ from regular enhancers in both the size and intensity of the associated chromatin features, and are frequently found in genomic proximity of cell identity genes and disease-associated variants. Super-enhancers are also found at key oncogenic drivers, thus selective inhibition of oncogenes may be achieved by disruption of super-enhancers (Loven et al., 2013). These studies suggested a model that a relatively small set of lineage-defining super-enhancers might determine cell identity in development and disease. Despite the proposed prominent roles, the regulatory components and functional features associated with super-enhancers are currently unknown. The critical questions are whether the super-enhancer represents a single functional unit in vivo, and how the individual constituent enhancers contribute to maximal enhancer activity through cooperativity or interaction within their native chromatin environment (Pott and Lieb, 2015).

We reasoned that modeling human fetal and adult-stage erythropoiesis combined with epigenomic enhancer annotation and analysis of the underlying DNA sequences can be used as an unbiased approach to identify causative TFs driving enhancer temporal activities in lineage specification. Comparing and contrasting TF binding associated with developmentally regulated enhancers facilitates identification of lineage-regulating factors and their combinatorial rules as putative drivers of enhancer turnover during differentiation. GATA2-to-GATA1 switch is a critical molecular driver of developmentally dynamic enhancers during erythroid specification. Most importantly, through CRISPR/Cas9-mediated loss-of-function analysis, we uncover functional hierarchy and complexity of constituent enhancers within the same enhancer clusters. Thus, genome-wide enhancer profiling coupled with in-depth enhancer editing provides important insights into the regulatory components controlling enhancer functions during erythropoiesis.

RESULTS

Pervasive Changes in Enhancer Landscape during Human Erythropoiesis

Primary fetal- and adult-stage human CD34+ hematopoietic stem/progenitor cells (HSPCs) were expanded and differentiated ex vivo into highly enriched stage-matched populations of erythroid progenitor cells (proerythroblasts or ProEs) (Figure 1A). We selected fetal or adult-stage HSPCs (F0 or A0; Figure 1B), and lineage-committed ProEs (F5 or A5) for genome-wide enhancer annotation and expression profiling. Specifically, we analyzed active enhancer-associated histone modifications H3K4me1

and H3K27ac by chromatin immunoprecipitation sequencing (ChIP-seq) and transcriptomic profiles in four distinct populations of human HSPCs or ProEs at the fetal or adult stage (Figures 1B–1E and S1A).

Comparative analysis of the enhancer landscape illustrates pervasive temporal changes in enhancer usage underlying lineage and developmental stage specificity. For example, 8,632 enhancers are lost (A0-A5-lost) and 8,496 enhancers are acquired (A0-A5-gained) upon differentiation of adult HSPCs (A0) to ProEs (A5), whereas only 3,996 enhancers are preserved (A0-A5-shared, Figures 1B and 1C). The number of developmentally dynamic enhancers is much higher than that of differentially expressed genes (Figure S1B), suggesting that the enhancer landscape undergoes more extensive turnover than transcriptomic changes during lineage specification. Furthermore, we identified 1,916 to 2,856 enhancers uniquely active at fetal or adult stage in HSPCs or ProEs, indicating a substantial change in genome-wide regulatory architecture across developmental stages within the erythroid lineage (Figure 1B). During the transition from HSPCs to ProEs, lost enhancers at both fetal and adult stages are significantly enriched in recognition sites (or motifs) for HSPC-regulating TFs including ETS1, ERG, FLI1, and PU.1 (Orkin and Zon, 2008; Wilson et al., 2010), whereas gained enhancers are enriched in motifs for erythroid master regulators GATA1 and TAL1 (Cantor and Orkin, 2002) (Figure 1F). By contrast, the comparison between fetal and adult stage-specific enhancers reveals enrichment of distinct TF motifs (Figure 1G). Of note, the most enriched motifs in F5-A5-gained enhancers are IRF1, IRF2, and STAT1/2, consistent with recent studies demonstrating a role of inflammatory signaling pathways in establishing HSPC programs (Espin-Palazon et al., 2014; He et al., 2015; Li et al., 2014; Xu et al., 2012). Taken together, these results indicate that the lineage-defining TFs are functionally conserved within enhancers during lineage specification; however, they cooperate with distinct stage-specific cofactors to modulate enhancer landscape for fetal and adult erythropoiesis.

Enhancers Control Lineage and Stage-Specific Transcription

To directly examine the correlation between enhancer activities and lineage or developmental stage-specific gene expression, we mapped enhancers to target genes using the "nearest neighbor gene" approach (Heintzman et al., 2009; Visel et al., 2009a; Xu et al., 2012) and compared these with lineage or stage-specific gene expression (Figure 2A and Table S3). Importantly, the erythroid lineage-specific enhancers (F0-F5-gained and A0-A5-gained) strongly associate with genes induced during erythropoiesis (F0-F5-up and A0-A5-up), whereas the HSPC-associated enhancers (F0-F5-lost and A0-A5-lost) strongly associate with downregulated genes (F0-F5-down and A0-A5-down). Similarly, the presence of stage-specific enhancers highly correlates with gene expression changes in the respective fetal or adult stage (Figure 2A).

Of note, by focusing on representative A0-A5-up genes, we observed that genes with an increasing number of enhancers display faster expression kinetics and higher mRNA levels during differentiation (Figure 2B). We then compared on a global scale the expression kinetics of genes associated with single and multiple lost or gained lineage-specific enhancers (Figure 2C). These analyses demonstrate that an increasing number of enhancers strongly correlate with more rapid kinetics and pronounced transcriptional changes. It has been suggested that clustered enhancers, including super-enhancers, associate with critical developmental or cancer-associated transcription units (Hnisz et al., 2013; Loven et al., 2013; Whyte et al., 2013). Consistent with this notion, we observed that the lineage-specific super-enhancers correlate with more robust transcriptional changes than regular enhancers (Figure 2D).

Enhancers can act from distance to regulate transcription. We then compared the expression of genes targeted by enhancers located at varying distance (Figure 2E). Interestingly, the proximal enhancers correlate with more rapid kinetics and larger quantitative changes in gene expression compared with distal enhancers. Taken together, these analyses illustrate critical roles of enhancers in modulating lineage- and stage-specific gene expression patterns, and suggest that the quantity and physical proximity of enhancers can influence the transcriptional robustness of their gene targets during development.

In Situ Genomic Editing of the *SLC25A37* Super-enhancer

Intensely marked enhancer clusters or super-enhancers, consisting of multiple discrete enhancers spanning larger chromatin domains, have been proposed to control genes essential for cell identity. The enhancer cluster upstream of the *SLC25A37* gene, consisting of three distinct constituent enhancers as measured by H3K4me1 and H3K27ac ChIP-seq, is defined as an erythroid-specific super-enhancer in both human (A5 ProE) and mouse (G1ER) erythroid cells (Figure 3A and Table S4). The orthologous mouse super-enhancer displays high primary sequence homology, syntenic position, and similar chromatin signature and TF occupancy (Figure 3A). The *SLC25A37* gene encodes Mitoferrin 1 that functions as an essential mitochondrial importer for iron metabolism and heme biogenesis. A genetic deficiency of *SLC25A37* results in profound hypochromic anemia in vertebrate species (Amigo et al., 2011; Shaw et al., 2006). The expression of *SLC25A37*, but not the neighboring *ENTPD4* gene, is progressively and significantly induced during human and mouse erythropoiesis (Figures S2A and 3C), suggesting that it is *trans*-activated through the upstream super-enhancer.

To define the regulatory components of the *SLC25A37* super-enhancer, we asked whether the function of each constituent enhancer depends on the activity of neighboring enhancers in situ. We employed site-directed loss-of-function analysis of the *SLC25A37* super-enhancer constituents using CRISPR/Cas9-mediated genomic engineering. We focused on the orthologous mouse super-enhancer in the murine G1E/G1ER erythroid cell model (Welch et al., 2004). Analogous to the ex vivo erythroid maturation of human HSPCs, the *Gata1*-null G1E cells are maintained in an undifferentiated state and express a high level of *Gata2*. Upon activation of the Gata1-ER transgene by β-estradiol treatment in G1ER cells, *Gata1* mRNA was progressively elevated whereas *Gata2* expression was sharply downregulated, resulting in a "GATA switch" (Figures S3A and S3B).

We designed sequence-specific single-guide RNAs (sgRNAs) flanking each constituent enhancer (E1, E2, and E3) or the

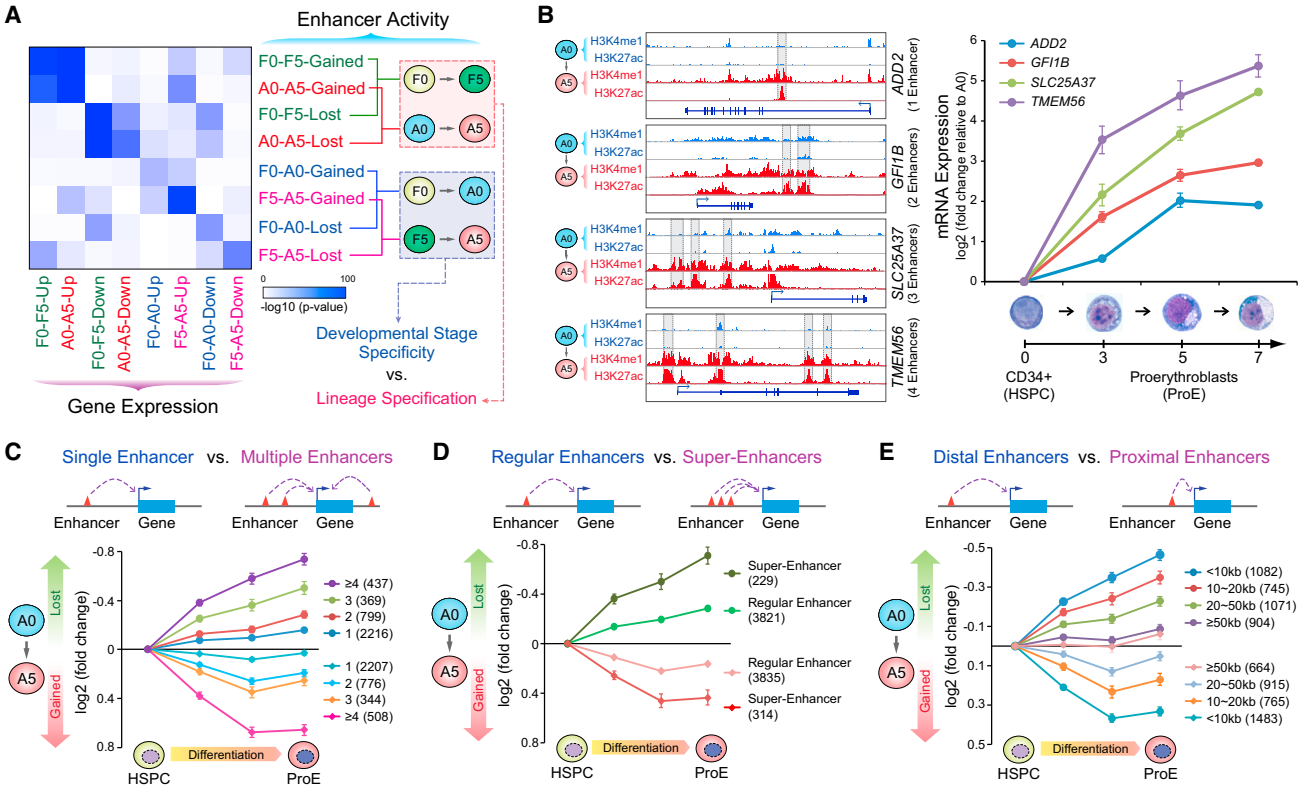


Figure 2. Enhancers Control Lineage- and Stage-Specific Transcription

(A) Enhancers positively associate with lineage or stage-specific gene expression changes. The enrichment significance of differentially expressed genes (columns) harboring different types of enhancers (rows) is calculated using Fisher's exact test.

(B) Representative genes targeted by one, two, three, and four "A0-A5-gained" enhancers are shown, respectively. The putative enhancers are depicted by shaded lines. The mRNA expression of each gene is shown on the right.

(C) mRNA expression of genes harboring single versus multiple enhancers.

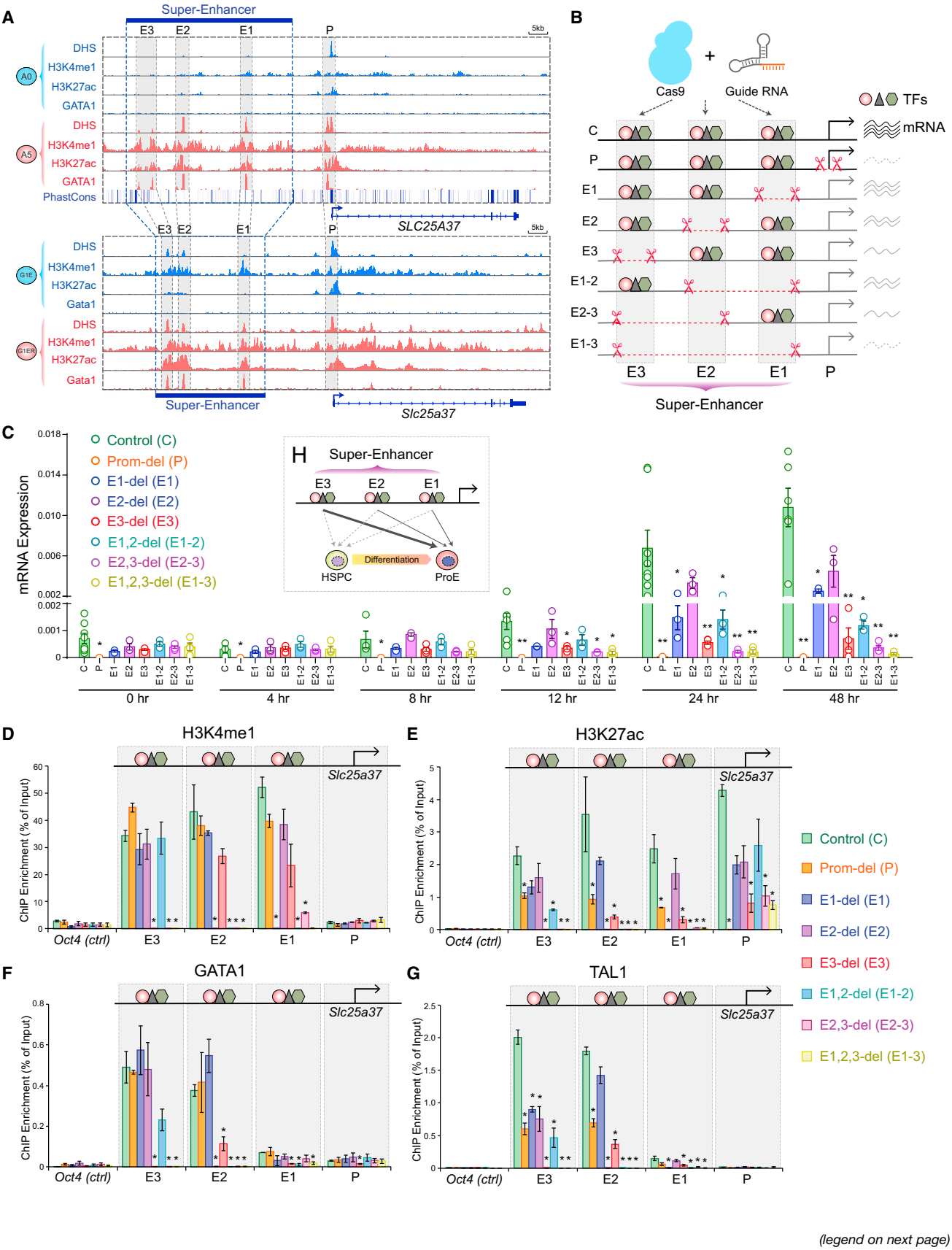
(D) The correlation between mRNA expression and regular enhancers versus super-enhancers.

(E) mRNA expression of genes harboring enhancers with varying distance.

Values are shown as mean ± SEM between replicates.

promoter (P) (Figure 3B). Upon transfection into undifferentiated G1E cells together with an SpCas9-expressing construct, we screened and obtained multiple independent single-cell-derived clones containing biallelic deletion of each enhancer (Figures S2B and S2C). Surprisingly, knockout of individual enhancers confers markedly varying effects on *Slc25a37* expression. Specifically, while deletion of E1 or E2 individually only modestly or slightly impairs *Slc25a37* activation during differentiation, respectively, E3 deletion abolishes its activation, resulting in a 15-fold decrease in expression (48 hr after β-estradiol treatment; Figure 3C). The expression of the neighboring *Entpd4* gene remains low and unchanged in control and enhancer-deletion cells (Figure S2D). ChIP-seq analysis of CTCF reveals two CTCF binding sites between *Slc25a37* and *Entpd4* genes (Figure S2E), suggesting that the *Slc25a37* super-enhancer does not control *Entpd4* transcription in erythroid cells. The differentiation kinetics of G1E cells, as measured by *Gata1* and *Gata2* expression, appear ostensibly normal in the absence of *Slc25a37* enhancers (Figures S3A and S3B).

Of note, by using combinations of sgRNAs, we also obtained G1E cells containing deletion of multiple constituent enhancers (Figures 3B, S3C, and S3D). Consistent with a prominent role of E3 in super-enhancer activation, combined deletion of E2 and E3 (E2-3) or all three enhancers (E1-3) completely abolishes *Slc25a37* expression upon differentiation (Figure 3C). Importantly, deletion of individual or multiple constituent enhancers has only a subtle effect on *Slc25a37* baseline expression in G1E cells (Figures 3C, S3C, and S3D), indicating that enhancer usage is highly context specific. Loss of E3 leads to near absence of H3K27ac, GATA1 and TAL1 occupancy at the neighboring E1 and E2 enhancers, whereas loss of E1 or E2 has minimal impact on H3K27ac or GATA1/TAL1 binding at neighboring enhancers (Figures 3D–3G). These results strongly suggest that, despite the indistinguishable chromatin features and TF occupancy at the *Slc25a37* constituent enhancers, the E3 enhancer is functionally more potent than its neighboring enhancers in directing transcriptional activation. Thus, our in-depth in situ enhancer-deletion analyses demonstrate that the *Slc25a37* super-enhancer is composed of a functional hierarchy containing both critical and dispensable constituent components (Figure 3H).



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Control of Enhancer Activities by Distinct Combinations of Transcription Factors

A common approach to identifying enhancer-associated TFs is to survey for the presence of TF binding consensus sequences (or motifs), without knowing whether the putative motifs are occupied by the cognate TFs in the cell type of interest. We reasoned that uncovering the functionally relevant TFs that associate with developmentally dynamic enhancers should help to infer lineage-specific regulators and their combinations in controlling cellular identity. To this end, we compared the motif enrichment and ChIP-seq occupancy of a panel of lineage-regulating TFs (GATA1, TAL1, FLI1, and PU.1) and the ubiquitously expressed CTCF (Figure 4A). Strikingly, while only 3% (22,570 of 707,718) of the GATA1 motif-matched loci are covered by GATA1 ChIP-seq at a genome scale in ProEs, 34% of identified GATA1 motifs are covered by GATA1 within the enhancer context. Furthermore, 55% of GATA1 motifs at A0-A5-gained enhancers are occupied by GATA1, in contrast to only 9% in A0-A5-lost enhancers, consistent with a prominent role of GATA1 in erythroid specification. A similar pattern is observed for another principal erythroid regulator, TAL1 (Figure 4A). In stark contrast, the opposite patterns are observed for HSPC-regulating FLI1 and PU.1. Of note, comparable frequencies of CTCF motif-matched loci are covered by CTCF ChIP-seq in both cell types. These analyses demonstrate that functionally relevant TF motifs are highly enriched in lineage-selective enhancers of lineage-relevant cell types.

To comprehensively identify TFs involved in developmentally dynamic enhancers, we performed motif enrichment analysis and identified 86 TF motifs that are significantly enriched in at least one enhancer type across 12 types of lineage or stage-specific enhancers (Figures 4B and S4A). Specifically, GATA1 and GATA1:TAL1 motifs are highly enriched in the erythroid-specific F0-F5-gained and A0-A5-gained enhancers. In contrast, PU.1, RUNX1, ETS1, and FLI1 motifs are highly enriched in the HSPC-specific F0-F5-lost and A0-A5-lost enhancers. To validate the motif analysis, we performed 30 additional ChIP-seq analyses of identified TFs in both HSPCs and ProEs, including GATA1, GATA2, TAL1, PU.1, and RUNX1 in HSPCs (F0 and A0) and/or ProEs (F5 and A5) (Figures 4C, 5F, and Table S1). We also re-analyzed 45 previously published ChIP-seq datasets of TFs and chromatin regulators obtained in the same cell populations (Abraham et al., 2013; Beck et al., 2013; Dogan et al., 2015; Su et al., 2013; Xu et al., 2012, 2015) (Table S1). Consistent with the motif analysis, ChIP-seq of a panel of identified TFs clearly demonstrates enrichment of distinct TF clusters within each enhancer type (Figures 4C and S4B).

To explore the TF combinatorial rules, we determined the co-association between TFs within the enhancer repertoires (Figure 4D). By hierarchical clustering analysis, we identified five distinct TF combinatorial modules, including expected associations such as GATA1 and TAL1, and some less expected associations such as NFE2 and MYB. Importantly, we found that the ubiquitous "housekeeping" E2F-SP1 and AP1 modules, and the HSPC-specific RUNX1-FLI1-PU.1-ETS module, are interconnected but dissociable, suggesting that distinct TF combinations cooperate to modulate the spatiotemporal activities of enhancers in a highly context-specific manner. Taken together, these analyses uncover TF combinatorial regulatory patterns with known and unknown roles as putative drivers of enhancer turnover during erythropoiesis.

Enhancer-Centered TF Combinatorial Regulatory Networks

To illustrate the temporal control of enhancer activities, we developed a computational methodology to delineate enhancer-mediated transcriptional networks by connecting motif enrichment, TF occupancy, and enhancer activity (Figure 5A). In brief, to quantify the relative importance of individual TF on enhancer specificity, we calculated the enrichment score as the significance of the enrichment of TF ChIP-seq peaks (or motif-matched loci) within each type of lineage or stage-specific enhancers using the whole genome as background. To quantify the likelihood of TF cooperation, we calculated the combinational score as the frequency of co-occurrence of two TFs at the same enhancer relative to genome background. We then selected TF/motif pairs with significant combinational scores to assemble the networks for each enhancer type in Cytoscape (Shannon et al., 2003).

Notably, the A0-A5-lost network is predominantly modulated by the PU.1-RUNX1-FLI1-ETS-GATA2 combinatorial interactions. In contrast, the A0-A5-gained network is dominated by GATA1-TAL1 together with an IRF2-STAT1-STAT2 interaction module, suggesting that the temporal changes in TF occupancy control differential enhancer activity (Figure 5B). Distinct sets of TF combinatorial modules were found at lineage or stage-specific enhancers (Figure S5 and Table S5). Importantly, the

Figure 3. CRIPSR/Cas9-Mediated Deletion Analysis of the *SLC25A37* Super-enhancer

(A) Chromatin signatures and TF occupancy within the human or mouse *SLC25A37* locus in HSPC (A0) versus ProE (A5) or undifferentiated G1E versus differentiated G1ER cells are shown, respectively. The *SLC25A37* constituent enhancers (E1, E2, and E3) and the proximal promoter (P) are depicted by shaded lines. Super-enhancers were called using Rose (Whyte et al., 2013) base on the H3K27ac ChIP-seq signal. The sequence conservation by PhastCons analysis is shown.

(B) Schematic of CRISPR/Cas9-mediated genomic editing to dissect the *SLC25A37* super-enhancer. The scheme is adapted from Pott and Lieb (2015). The scissors indicate DNA double-strand breaks induced by CRISPR/Cas9. The red dashed lines indicate the deleted genomic DNA.

(C) Expression of *Slc25a37* mRNA in unmodified (control) and enhancer-deletion G1E/G1ER cells at various time points (0–48 hr) after β-estradiol treatment. The mRNA expression levels relative to GAPDH are shown. Each colored circle represents an independent single-cell-derived biallelic enhancer or promoter-deletion clone. Results are means ± SD of multiple independent clones. The p value measures the statistical significance between control (C) and each experimental group. $^{*}p < 0.01$; $^{**}p < 0.001$.

(D–G) ChIP-qPCR analysis of H3K4me1, H3K27ac, GATA1, and TAL1 in control and enhancer-deletion cells. Primers against *Slc25a37* promoter (P) and each constituent enhancer (E1, E2, and E3) are used. *Oct4* promoter is analyzed as a negative control. Results are means ± SD of multiple independent clones. $^{*}p < 0.01$.

(H) Schematic of the hierarchical structure of the *SLC25A37* super-enhancer.

See also Figures S2 and S3.

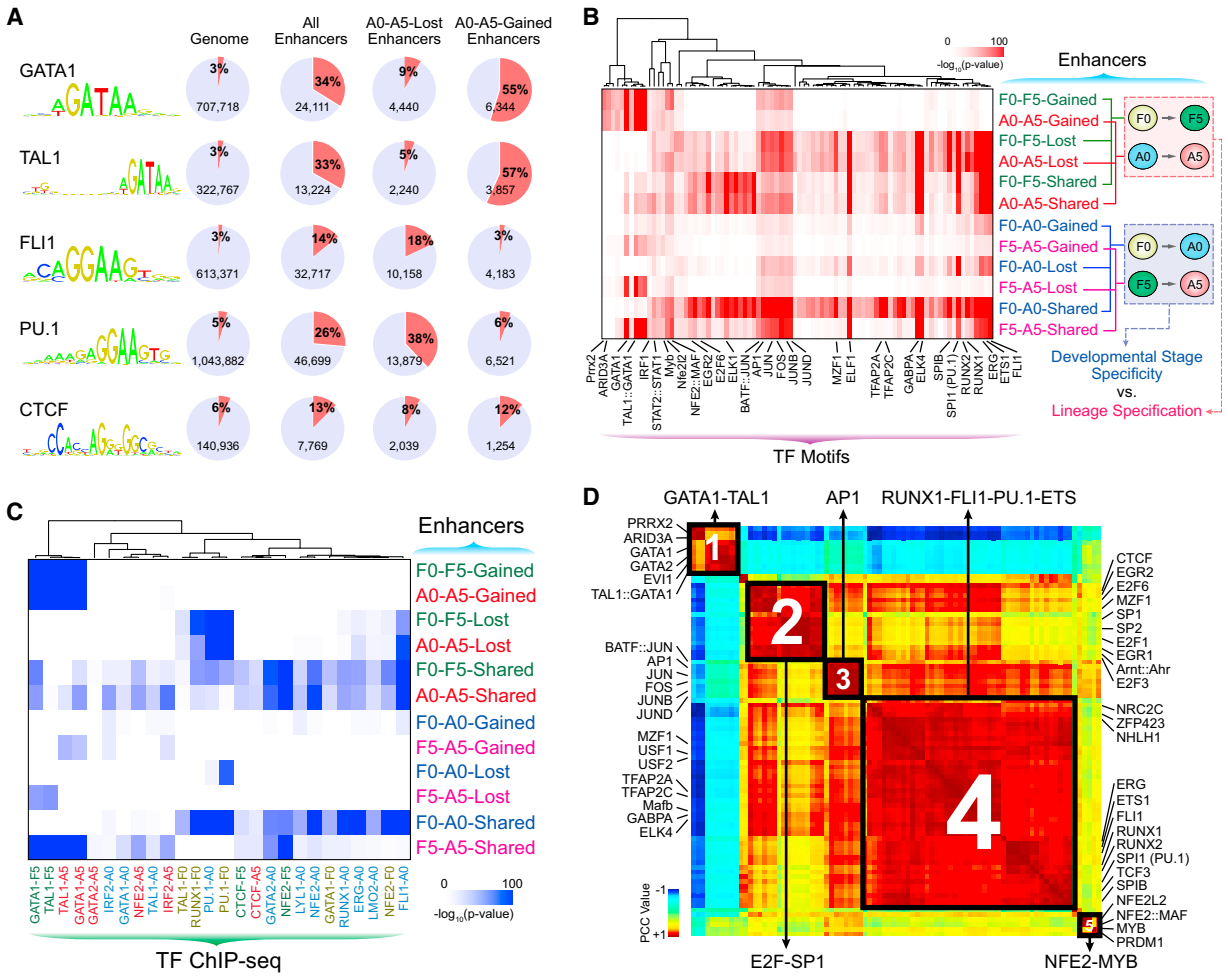


Figure 4. Combinatorial Control of Enhancers by Transcription Factors

(A) Enrichment of functionally relevant TF motifs. The numbers of identified motif-matched loci and the percentage of motifs overlapped with ChIP-seq peaks are shown.

(B) Hierarchical clustering of 86 TF motifs significantly enriched in the lineage- and/or stage-specific enhancers. Heatmap shows the enrichment score of TF motifs.

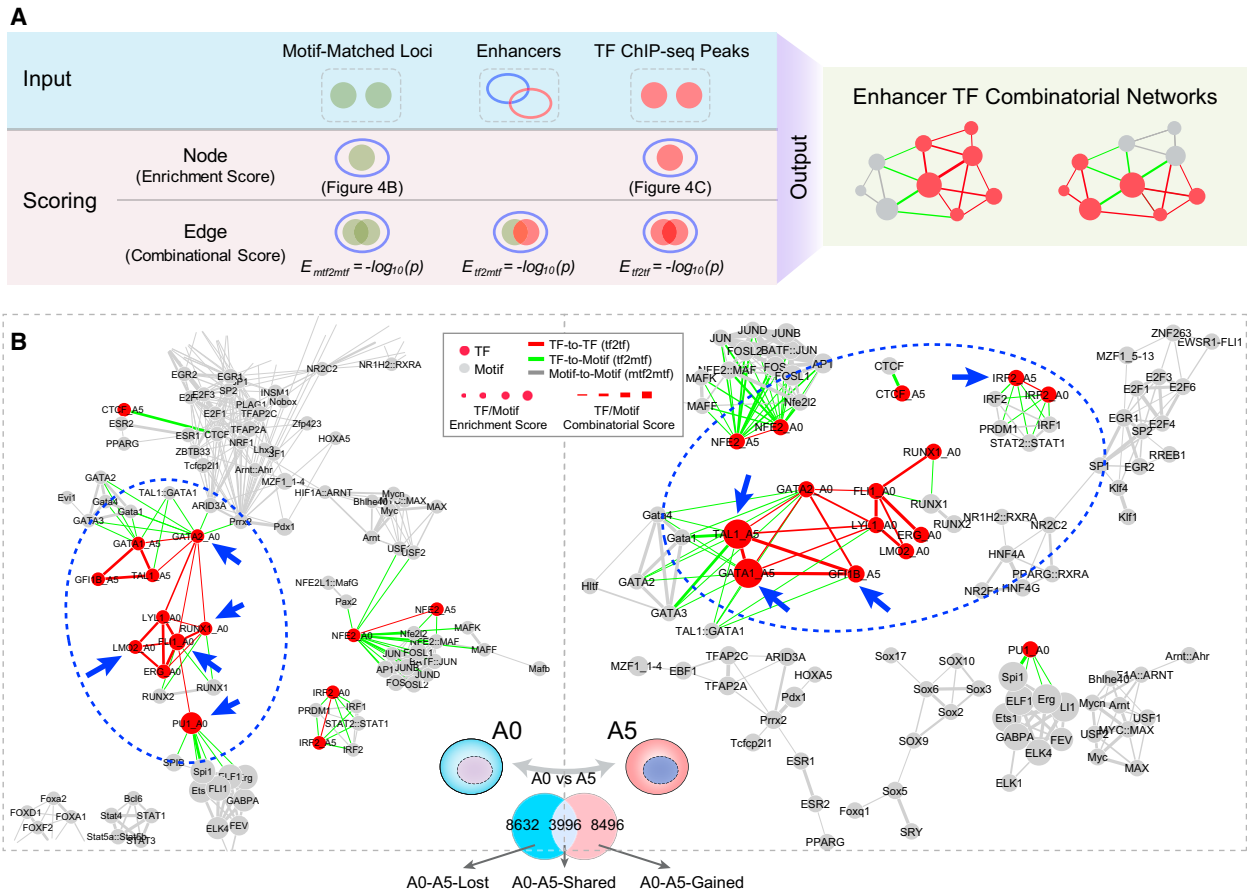
(C) Hierarchical clustering of ChIP-seq occupancy of the indicated TFs within enhancers. Heatmap shows the enrichment score based on the overlap significance calculated by Fisher's exact test.

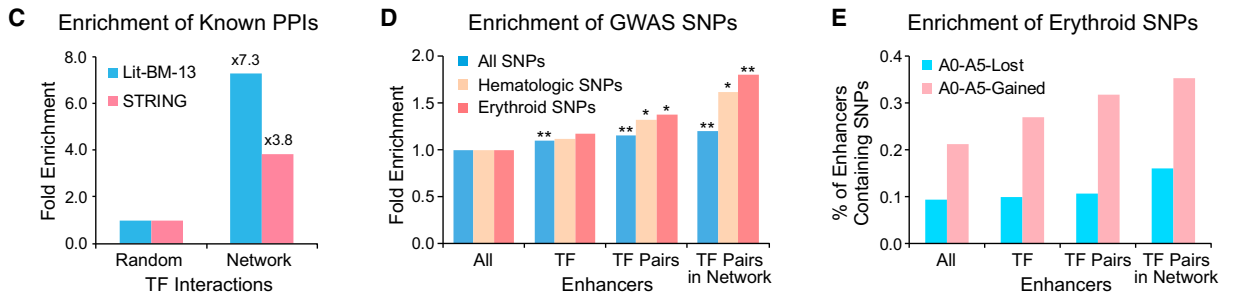
(D) Combinatorial TF modules within enhancers during erythropoiesis. TF modules were defined by hierarchical clustering of enriched TF motifs based on their enrichment score across all 12 enhancer types. Five distinct TF motif modules are shown for (1) GATA1-TAL1, (2) E2F-SP1, (3) AP1, (4) RUNX1-FLI1-PU.1-ETS, and (5) NFE2-MYB. The color code indicates the Pearson correlation coefficient (PCC) of the enrichment scores.

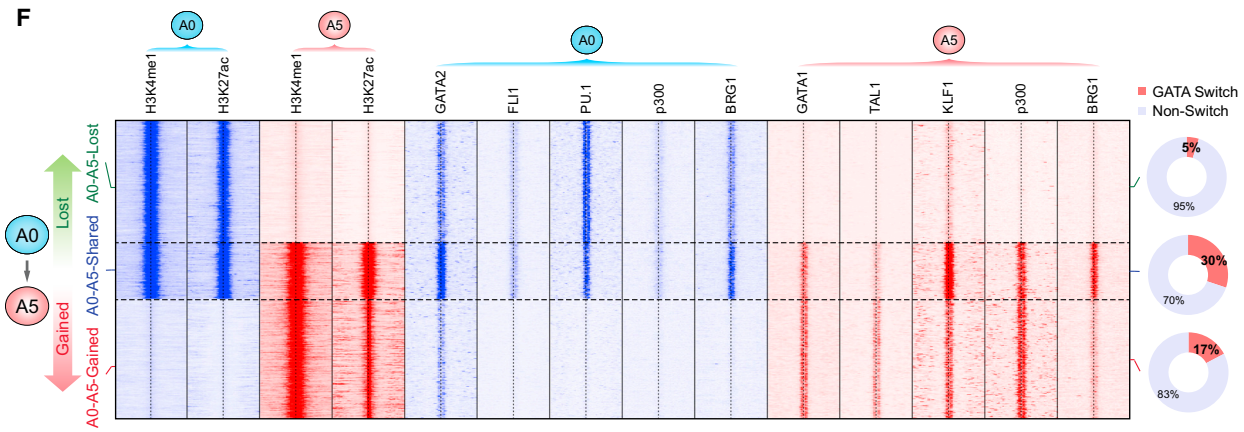
See also Figure S4.

GATA2 and GATA1 combinatorial interactions are present in both A0 and A5 networks, with GATA2 dominating the A0 network while GATA1 dominates the A5 network (Figure 5B). Thus, our data suggest that the combinatorial assembly of lineage-defining TFs and transcriptional coregulators at lineage- and stage-specific enhancers directs temporal regulation of transcriptional networks during erythroid specification.

To validate the network predictions, we first compared the network-identified interactions with known protein-protein interactions (PPIs) from Lit-BM-13 (Rolland et al., 2014) and STRING (Szklarczyk et al., 2015) databases. We observed that the network-predicted TF interactions are 7.3- and 3.8-fold more enriched of known PPIs in Lit-BM-13 and STRING databases, respectively, compared with random interactions (p values 8.0×10^{-33} and 1.9×10^{-66} by Fisher's exact test; Figure 5C). We then investigated the extent to which disease-associated SNPs occur in enhancers containing network-predicted TF pairs (Figure 5D). We observed that enhancers containing network-predicted TF interactions (TF pairs in network; Figure 5D) are highly enriched in GWAS SNPs, particularly the hematologic and erythroid trait-associated SNPs. Furthermore, A0-A5-gained erythroid-specific enhancers are more significantly enriched in erythroid SNPs than A0-A5-lost HSPC-specific enhancers (Figure 5E). These analyses confirm that the enhancer-centered TF combinatorial networks can be used to identify functionally relevant and disease-associated regulatory interactions.







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In addition, we generated ChIP-seq density heatmaps within the lost, shared, and gained enhancers in HSPCs (A0) and ProEs (A5). Strikingly, while the HSPC-regulating TFs (FLI1 and PU.1), p300, and BRG1 exclusively associate with HSPC-specific (A0-A5-lost) and shared enhancers, GATA2 can occupy a subset of erythroid-specific (A0-A5-gained) enhancers prior to their activation (Figure 5F). A significant portion of A0-A5-gained (17%) and -shared (30%) enhancers display GATA2-to-GATA1 switch. These results, together with the network analysis, strongly suggest that GATA switch plays a major role in modulating enhancer turnover during erythroid specification.

GATA2-to-GATA1 Switch Functions as a Molecular Driver of Enhancer Turnover

To further dissect the molecular processes controlling enhancer turnover, we focused on enhancers that are lost or gained between adult HSPCs (A0) and ProE (A5). Specifically, we subdivided A0-A5-lost enhancers into two groups depending on whether they have lost H3K27ac ("active → primed"), or both H3K27ac and H3K4me1 ("active → silent") (Figure S6A). Similarly, we subdivided A0-A5-gained enhancers into two groups depending on the prior chromatin states in HSPCs ("silent → active" and "primed → active"). Notably, the active → silent enhancers are highly enriched for TF motifs required for HSPC identity such as RUNX1, PU.1, FLI1, and ETS1. In contrast, the silent → active enhancers are highly enriched for GATA1 and TAL1 motifs. We next examined the occupancy of various lineage-specific TFs and chromatin regulators at the enhancer groups. Strikingly, GATA2-to-GATA1 switch (or GATA switch) is highly prevalent within transcriptionally primed enhancers, consisting of 30% of active → primed lost enhancers and 41% of the primed → active gained enhancers (Figure S6B). By network (Figure S6C) and motif analysis (Figure S6D), we also identified GATA switch as the major TF combinatorial module in primed enhancers. These data strongly suggest that GATA switch functions as a major molecular driver of enhancer turnover during erythropoiesis.

Genomic Features of GATA Switch Enhancers

To further dissect the role of GATA switch in enhancer turnover, we compared GATA2 and GATA1 occupancy within A0 and A5 enhancers by ChIP-seq. Specifically, we enumerated the distribution of enhancers occupied by GATA2-only, GATA1-only, or GATA2-to-GATA1 switch (GATA switch) (Figure 6A and Table S6). By overlap analysis, we identified 3,101 GATA switch enhancers and the remainder as GATA2-only (2,697) or GATA1-only (4,348) enhancers, respectively. Importantly, the GATA switch enhancers consist of 39% of A0-A5-shared and 47% of A0-A5-gained enhancers, and only 14% of A0-A5-lost enhancers, consistent with the role of GATA switch as a major driver for enhancer activation.

We then examined the differentially enriched TF motifs within GATA switch and GATA1/2-only (or non-switch) enhancers. We found that GATA motifs are highly enriched in GATA switch and GATA1-only enhancers, but not in GATA2-only enhancers. By contrast, the GATA2-only enhancers are highly enriched with TF motifs known to be important for HSPC identity (Figure 6B). These analyses indicate that the recruitment of GATA factors within the GATA switch enhancers are mediated primarily through GATA motifs (motif-driven). However, GATA2 binding to the non-switch GATA2-only enhancers are mediated largely through transcriptional cofactors (cofactor-driven). We then compared the mRNA expression of genes targeted by GATA2-only, GATA switch, or GATA1-only enhancers. The GATA2-only targets are progressively downregulated, whereas the GATA1-only targets are progressively upregulated during erythropoiesis. Interestingly, the expression levels of GATA switch enhancer targets are much higher than that of genomic average, and remain largely unchanged during differentiation (Figure 6C). These results suggest that, on the global scale, GATA switch enhancers function to maintain the high level of gene expression critical for cellular differentiation and housekeeping functions, in contrast to GATA1-only or GATA2-only enhancers (Figure 6D).

In Situ Genomic Editing of GATA Switch Enhancers in Lineage Differentiation

To gain functional insights into the role of GATA switch in regulating enhancer activities, we employed CRISPR/Cas9-mediated genomic editing to remove GATA switch or non-switch enhancers at multiple independent loci in G1E cells (Figures 7 and S7). Strikingly, loss of GATA switch and non-switch enhancers results in pleiotropic effects on target gene expression during erythroid differentiation. At the *Pinx1* gene, biallelic deletion of the GATA2-only enhancer (E1) markedly impairs *Pinx1*

Figure 5. TF Combinatorial Regulatory Networks within Enhancers

(A) Schematic of the construction of enhancer-mediated TF combinational regulatory networks.

(B) Representative TF combinational regulatory networks in adult HSPCs (A0, left) and ProEs (A5, right). In the network, the nodes represent TF with ChIP-seq data (red) or motif information (gray). The size of node represents the enrichment score. The color of edges represents different types of combination (red: TF-to-TF; green: TF-to-Motif; gray: Motif-to-Motif). The width of edges represents the combinatorial score. The blue arrows and the areas outlined with dashed lines indicate the most significantly enriched TF interactions.

(C) Enrichment of known protein-protein interactions (PPIs) in network-predicted TF interactions. "Network" on the x axis represents TF interactions predicted in A0 or A5 network, whereas "Random" represents all possible interactions among TFs shown in the network.

(D) Enrichment of hematologic and erythroid trait-associated SNPs in enhancers containing network-predicted TF interactions. All, all A0-A5-gained and A0-A5-lost enhancers; TF, enhancers occupied by at least one TF based on ChIP-seq analysis; TF Pairs, enhancers occupied by TF pairs; TF Pairs in Network, enhancers occupied by TF pairs identified in the A0 or A5 network. The p value measures statistical significance between all enhancers and each enhancer group. *$p < 0.05$; **$p < 0.001$.

(E) A0-A5-gained enhancers are highly enriched for erythroid SNPs. The y axis shows the percentage of enhancers containing erythroid trait-associated SNPs. The x axis is the same as in (D).

(F) ChIP-seq density heatmaps are shown for H3K4me1, H3K27ac, GATA1, GATA2, FLI1, PU.1, TAL1, KLF1, p300, and BRG1 within the indicated lost or gained enhancers. The percentage of GATA2-to-GATA1 switch enhancers is shown on the right.

See also Figures S5 and S6.

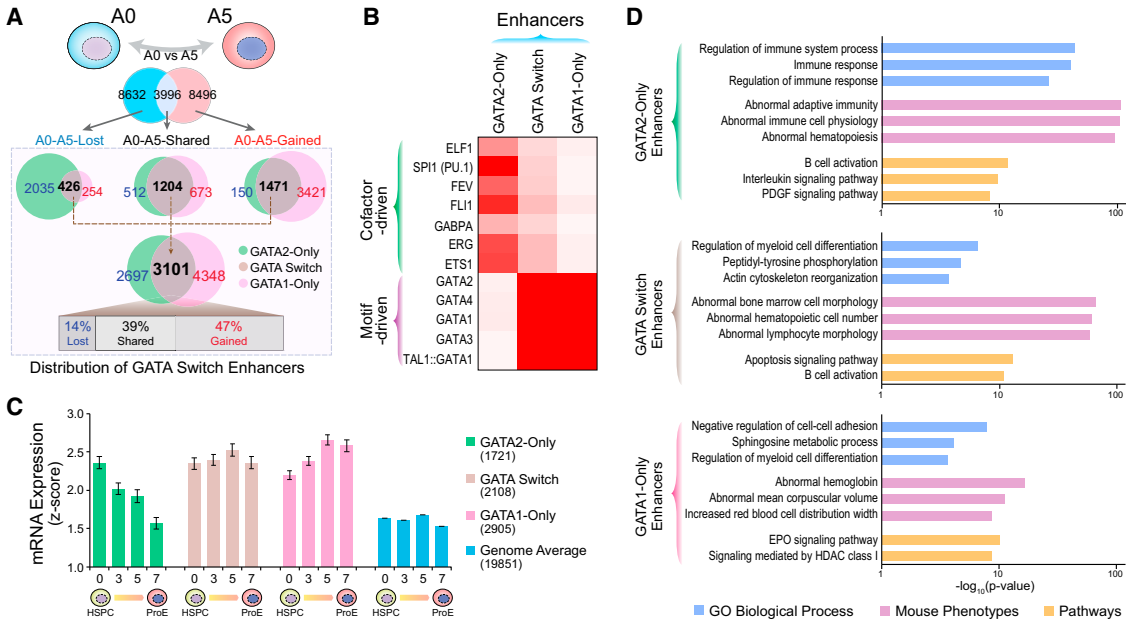

Figure 6. GATA Switch Controls Enhancer Activities during Erythroid Specification

(A) Distribution of GATA switch and non-switch enhancers within the lost, gained, or shared enhancers in adult HSPCs (A0) and ProEs (A5). The numbers of GATA1, GATA2, or both (GATA switch) occupied enhancers are shown.

(B) Differential enriched TF motifs in GATA2-only, GATA switch, or GATA1-only enhancers.

(C) mRNA expression of genes associated with GATA2-only, GATA switch, or GATA1-only enhancers during erythroid differentiation of adult HSPCs (A0) to ProEs (A5). The normalized expression level in particular cell types (*Z* score) were calculated using barcode (McCall et al., 2014).

(D) GREAT analysis (McLean et al., 2010) of GATA2-only, GATA switch, or GATA1-only enhancers. Top enriched gene ontology (GO) biological processes, mouse phenotypes, and pathways are shown, respectively.

See also Figure S6.

expression in G1E cells and during early erythroid differentiation (4 hr and 8 hr after β-estradiol treatment; Figures 7A–7C), whereas *Pinx1* is activated at a comparable level as in the unmodified control cells during late differentiation (24 hr and 48 hr). In contrast, deletion of the GATA switch enhancer (E2) significantly and selectively impairs *Pinx1* expression during late differentiation. The differentiation kinetics of G1E cells remain ostensibly normal in the absence of *Pinx1* enhancers (Figures S7A–S7D). Similarly, biallelic deletion of the *Mrto4* GATA2-only enhancer (E1) impairs baseline *Mrto4* expression, whereas deletion of the GATA switch enhancer (E2) selectively diminishes *Mrto4* expression upon differentiation (Figures 7B, 7D, 7E, and S7E–S7H). The distinct effects upon loss of GATA2-only versus GATA switch enhancers strongly suggest that multiple spatially or temporally distinct enhancers cooperate to control the optimal expression of target genes.

We then employed genomic editing to dissect the requirement of GATA switch enhancers within the paradigmatic *Gata2* locus (Bresnick et al., 2010; Dore et al., 2012; Kaneko et al., 2010). *Gata2* is a potent regulator of hematopoietic stem/progenitor cells and is highly expressed in HSPCs, then rapidly silenced in erythroid cells (Bresnick et al., 2010; Tsai et al., 1994) (Figure 7H). The transcription of *Gata2* gene is controlled by multiple distal regulatory elements including an enhancer cluster immediately upstream of the *Gata2* transcriptional start site (TSS) (E1, E2, and E3; Figures 7F and 7G). The upstream enhancers display indistinguishable chromatin features, such as the enrichment of histone marks H3K4me1 and H3K27ac, and occupancy by GATA2 in HSPCs (or G1E) and GATA1 in ProEs (or G1ER), respectively (Figure 7F). Surprisingly, deletion of E1 has no effect on *Gata2* expression in G1E and during early differentiation, but *Gata2* expression is markedly induced during late differentiation (Figures 7G and 7H). In contrast, deletion of E2 or E3 significantly impairs *Gata2* expression in G1E, with minimal effects on its expression upon differentiation. Of note, these findings are consistent with previous studies using engineered mouse models lacking critical GATA motifs at the *Gata2* upstream enhancers. In mice lacking a single palindromic GATA motif 1.8 kb upstream of the *Gata2* TSS, which overlaps with the E1 enhancer, *Gata2* expression is reactivated in late-stage erythroblasts, resulting in defective erythropoiesis (Snow et al., 2010). By contrast, in mice lacking GATA motifs 2.8 kb upstream of *Gata2* TSS, which overlaps with the E2 enhancer, *Gata2* expression is compromised in HSPCs (Snow et al., 2011).

Together with prior studies, our analyses demonstrate that qualitatively distinct and functionally divergent GATA switch enhancers cooperate within the same enhancer cluster at the *Gata2* locus. While the E2 and E3 enhancers are indispensable for maximal *Gata2* activation in stem/progenitor cells, the E1 enhancer is required to maintain *Gata2* repression in committed erythroid cells. Thus, despite the indistinguishable chromatin features among the GATA switch enhancers at the *Gata2* locus, we reveal through in situ genomic editing the functional diversity of GATA switch enhancers whereby enhancers with opposing

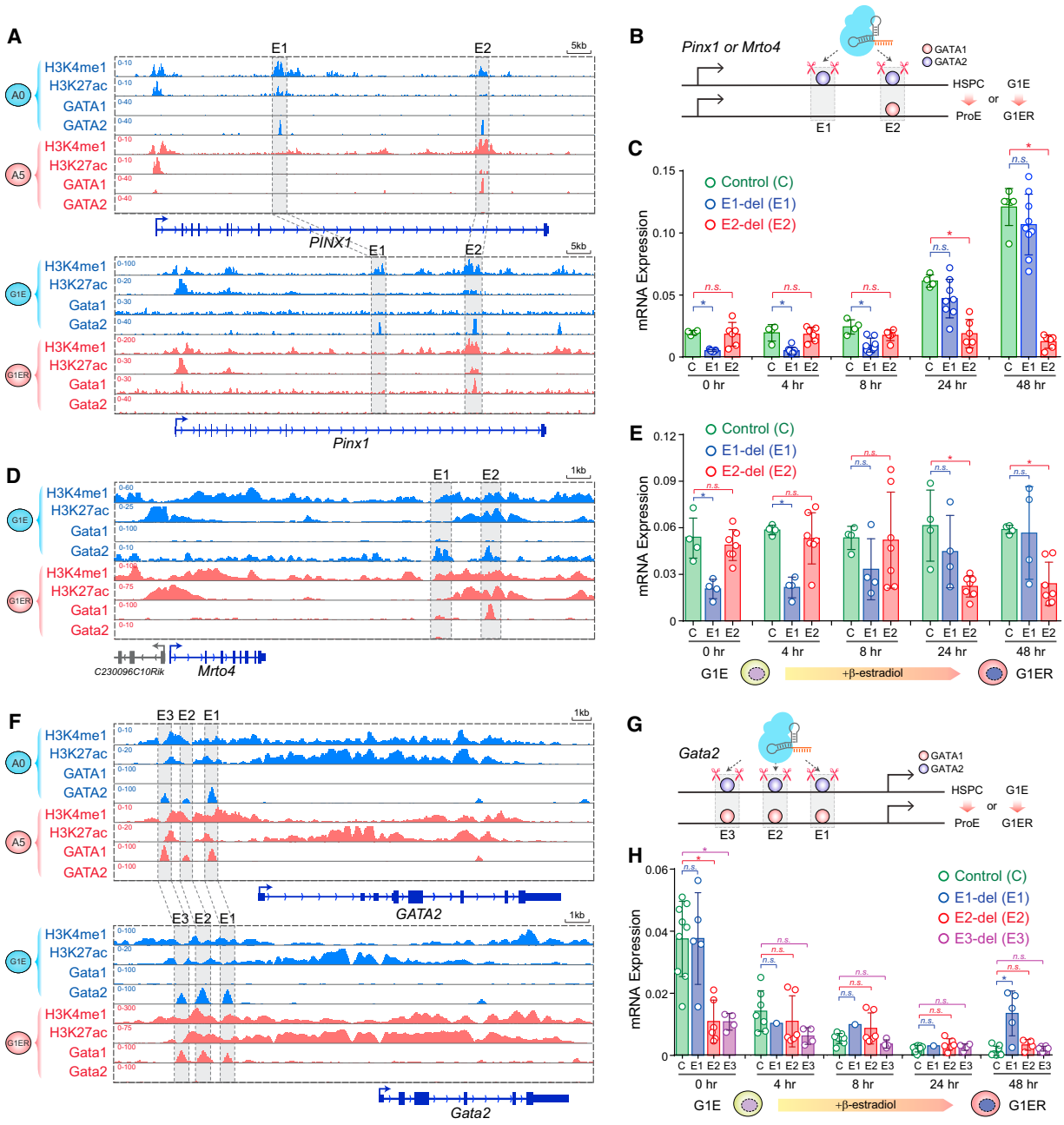

Figure 7. Genome Editing of GATA Switch-Mediated Enhancer Turnover

(A) Chromatin signatures and TF occupancy within the human or mouse *PINX1* locus. The putative GATA2-only (E1) and GATA switch (E2) enhancers are shown. The putative active enhancers are depicted by shaded lines.

(B) Schematic of CRISPR/Cas9-mediated deletion of *Pinx1* or *Mrto4* enhancers. The scissors indicate DNA double-strand breaks induced by CRISPR/Cas9.

(C) Expression of *Pinx1* mRNA in unmodified (control) and enhancer-deletion cells at various time points (0–48 hr) after β-estradiol treatment. Each colored circle represents an independent biallelic enhancer-deletion clone. Results are means ± SD of multiple independent clones. *p < 0.01; n.s., not significant.

(D) Chromatin signatures and TF occupancy within the mouse *Mrto4* locus. The putative GATA2-Only (E1) and GATA switch (E2) enhancers are shown. The putative active enhancers are depicted by shaded lines.

(E) Expression of *Mrto4* mRNA in control and enhancer-deletion cells. Results are means ± SD of multiple independent clones. *p < 0.01; n.s., not significant.

(F) Chromatin signatures and TF occupancy within the human or mouse *GATA2* locus. The putative GATA switch (E1, E2, and E3) enhancers are shown. The putative active enhancers are depicted by shaded lines.

(G) Schematic of CRISPR/Cas9-mediated deletion of *Gata2* enhancers. The scissors indicate DNA double-strand breaks induced by CRISPR/Cas9.

(H) Expression of *Gata2* mRNA in control and enhancer-deletion cells. Results are means ± SD of multiple independent clones. *p < 0.01; n.s., not significant.

See also Figure S7.

functions cooperate to orchestrate gene expression during cellular differentiation.

DISCUSSION

In Situ Perturbation of Enhancers

The term ''transcriptional enhancer'' was first introduced to describe the effects of SV40 DNA on the transcription of the rabbit β-globin gene (Banerji et al., 1981). Since then, enhancers have been routinely identified and characterized in ectopic heterologous reporter assays or by sequence analysis (Bulger and Groudine, 2011; Pennacchio et al., 2013; Visel et al., 2007). With the advent of genome-scale chromatin profiling, many putative enhancers have been identified in the human genome (Heintzman et al., 2007, 2009; Visel et al., 2009a, 2009b). While the biological importance of enhancers could be inferred from these genome-scale studies, it remains challenging to ascertain the precise in vivo function of individual enhancers using conventional loss-of-function methods. Thus, advances in genome engineering technologies hold enormous promise for systematic evaluation of the functional significance of enhancers in physiologically relevant contexts (Bauer et al., 2013; Canver et al., 2015; Groschel et al., 2014; Hnisz et al., 2015; Mansour et al., 2014; Xu et al., 2015).

In this study, we employed the CRISPR/Cas9 system to examine the functional requirements of a panel of discrete enhancers in an experimental model recapitulating normal erythroid development. Our results reveal functional hierarchy of enhancer regulation within their native chromatin. We first demonstrate the distinct roles of GATA switch and GATA2-only (or non-switch) enhancers in the temporal control of gene transcription. Moreover, by systematic dissection of three distinct GATA switch enhancers at the *Gata2* locus, our studies uncover functional divergence of enhancers cooperating within the same cluster. Despite the indistinguishable chromatin features based on ChIP-seq analyses, the neighboring GATA switch enhancers display distinct requirements for the precise control of *Gata2* transcription. Hence, our studies highlight the power and necessity of combining genome-scale enhancer annotation with genomic editing for analyzing enhancer cooperation during lineage differentiation. We speculate that further in-depth studies of enhancer regulation through high-throughput, high-resolution in situ enhancer editing will likely uncover novel regulatory principles underlying the context-specific actions of enhancers in mammalian genomes.

Hierarchical Composition of Erythroid Super-enhancers

Highly marked clusters of enhancers or super-enhancers have been identified in various cell types (Hnisz et al., 2013; Parker et al., 2013; Whyte et al., 2013). Despite the proposed roles of super-enhancers in gene regulation and disease, the functional significance of enhancer clustering within their native chromatin environment remains largely unexplored. More specifically, it is unclear whether super-enhancer represents a simple assembly of regular enhancers or whether it behaves as a single functional unit through cooperative activities of its constituent enhancers (Pott and Lieb, 2015).

By focusing on the *Slc25a37* super-enhancer, our studies demonstrate that the *Slc25a37* clustered enhancers are composed of a functional hierarchy of constituent components, with some significantly stronger than others for target gene expression during cellular differentiation. Strikingly, although all constituent enhancers possess similar levels of enhancer-associated histone marks and TF occupancy, deletion of each enhancer has a markedly distinct effect on *Slc25a37* transcription. Our findings on the distinct requirement of enhancer constituents are consistent with a recent analysis of several super-enhancers in mouse embryonic stem cells (Hnisz et al., 2015). One critical difference, however, is that our studies were performed in cells undergoing lineage differentiation. Hence, we were able to analyze and compare the effects of each enhancer deletion during the spectrum of erythroid differentiation. Importantly, while deletion of individual *Slc25a37* constituent enhancers has minimal effects at the undifferentiated stage, deletion of E3 has a profound impact on *Slc25a37* expression at later differentiation (Figure 3C). The developmental stage-specific requirement of enhancer functions would have been overlooked if one had only analyzed the steady-state undifferentiated cells.

Moreover, our results suggest a cooperative behavior in the recruitment of master TFs at the *Slc25a37* super-enhancer. While deletion of E1 or E2 has a minimal effect on histone marks and TF occupancy at neighboring enhancers, loss of E3 substantially reduces H3K27ac and completely abolishes GATA1/TAL1 binding at the neighboring enhancers. Combined deletion of E3 and neighboring enhancers further diminishes the transcriptional activity, the level of H3K27ac, and TF occupancy at *Slc25a37* enhancer and promoter regions, suggesting that E3 cooperates with other constituent enhancers to achieve maximal activity. Interestingly, despite the profound impact on H3K27ac level, loss of individual enhancers or their combinations does not affect H3K4me1, suggesting that the level of H3K27ac is more sensitive and predictive to transcriptional activities. Taken together, our results indicate that at least a subset of super-enhancers is organized in a hierarchical structure composed of enhancer constituents with non-redundant functions. Some constituent enhancers may possess significantly stronger effects on transcription while others cooperate with the dominant enhancers for maximal activity. These findings also raise the possibility that further in-depth dissection of super-enhancers by high-resolution genomic editing may unravel the regulatory components and distinct vulnerabilities underlying enhancer clustering in gene regulation.

TF Combinatorial Rules Underlying Enhancer Dynamics during Erythropoiesis

Enhancers are known to function as multifactorial platforms for binding of lineage-regulating TFs, chromatin regulators, and signaling effectors (Buecker and Wysocka, 2012; Xu and Smale, 2012). A long-standing question is how enhancers acquire the ability to translate intra- and extracellular signals to cell-type-specific transcriptional responses in development and disease. On the other hand, it is estimated that there are 2,000–3,000 sequence-specific DNA binding TFs encoded by the human genome (Babu et al., 2004), with 200–300 TFs being expressed in each cell type (Vaquerizas et al., 2009). However, the underlying principles by which TFs collaborate to regulate enhancer dynamics are poorly understood.

In this study, we developed a new network approach to connect TF occupancy, motif enrichment, and enhancer activity for illustration of the combinational regulatory mechanisms in complex differentiation processes. The rationale rests on accumulating evidence that enhancers are critical modulators of lineage- and stage-specific gene expression, and enrich for binding sequences (or motifs) for lineage-regulating TFs (Figure 4A) (ENCODE Project Consortium, 2012; Neph et al., 2012; Xu et al., 2012). Using an ex vivo model for human erythropoiesis, we identified distinct sets of TF combinatorial modules as putative drivers of enhancer turnover during erythroid differentiation. Of note, the HSPC-specific enhancer network is predominantly modulated by the PU.1-RUNX1-FLI1-ETS-GATA2 combinatorial interactions. In contrast, the erythroid-specific enhancer network is dominated by GATA1-TAL1 together with IRF2-STAT1-STAT2 interactions (Figures 5, S5, and Table S5). More importantly, we identified previously unrecognized TF interaction modules including NFE2-MYB and PRRX2-ARID3A-GATA2 within the enhancer context, thus providing a platform for substantive future investigations. Finally, in-depth experimental validation by ChIP-seq and CRISPR/Cas9-mediated loss-of-function studies not only validated the overall approach but also provided novel insights into the structure-function relationship of enhancer dynamics during erythroid specification. Taken together, our studies demonstrate that the integrative analysis of genome-wide enhancer annotation coupled with in situ enhancer editing has the potential to identify the underlying regulatory components of enhancer-directed cellular phenotypes in complex developmental processes.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture

Primary human adult and fetal CD34 + HSPCs were isolated as previously described (Van Handel et al., 2010; Xu et al., 2012). Primary fetal or adult committed proerythroblasts (ProEs) were generated ex vivo as previously described (Sankaran et al., 2009; Xu et al., 2012). G1E/G1ER cells were cultured as described by Welch et al. (2004).

ChIP-Seq and Data Analysis

ChIP-seq was performed as described by Xu et al. (2012). Other ChIP-seq datasets were obtained from previous publications (Abraham et al., 2013; Beck et al., 2013; ENCODE Project Consortium, 2012; Dogan et al., 2015; Pinello et al., 2014; Su et al., 2013; Trompouki et al., 2011; Xu et al., 2012, 2015).

Enhancer Annotation

Putative active enhancers were annotated using the peaks of H3K4me1, H3K27ac, and H3K27me3. In brief, H3K27ac peaks were used to define the enhancer boundary, followed by filtering based on the criteria: (1) excluded H3K27ac peaks not overlapped with H3K4me1 peaks; (2) excluded H3K27ac peaks located within ±2-kb region of RefSeq-annotated promoters; (3) excluded H3K27ac peaks overlapped with H3K27me3 peaks.

Identification of Lineage or Developmental Stage-Specific Enhancers

Lineage-specific enhancers were identified by comparing A0 and A5, or F0 and F5 enhancers. Similarly, stage-specific enhancers were identified by comparing F0 and A0, or F5 and A5 enhancers. The overlapped ($\geq$1 bp) enhancers were considered as "shared" enhancers and the remainder as "lost" or "gained" enhancers. To obtain the high-confidence enhancers, we filtered the enhancers using MAnorm (Shao et al., 2012; Xu et al., 2012) for H3K27ac (referred as $M_{H3K27ac}$). For lineage or stage-specific enhancers, we only keep enhancers with $|M_{H3K27ac}| > 1$; for shared enhancers we keep enhancers with $|M_{H3K27ac}| \leq 1$.

Motif Enrichment Analysis

The position weight matrices of 196 core vertebrate motifs were downloaded from the JASPAR database (Mathelier et al., 2014). The motif enrichment score was defined as $-\log_{10}$(p value), where p value is the significance of observed over-representation of each motif in enhancer regions compared with randomly selected control regions. Motif modules were detected based on the hierarchical clustering of motif enrichment scores across lineage or stage-specific enhancers (Table S2).

Construction of Enhancer-Mediated TF Regulatory Networks

The enrichment score was calculated to measure the significance of the enrichment of TF ChIP-seq peaks (or motif-matched loci) within each enhancer type relative to the genome background. The combinational score was calculated to measure the frequency of co-occurrence of two TFs at the same enhancer compared with the genome background. TF or motif pairs with significant combinational scores were selected to assemble the networks in Cytoscape (Shannon et al., 2003).

Enhancer Editing by CRISPR/Cas9

The clustered regularly interspersed palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9 nuclease system was used for enhancer-deletion analyses following published protocols (Cong et al., 2013; Mali et al., 2013).

ACCESSION NUMBERS

All ChIP-seq and microarray datasets have been deposited in GEO under accession numbers NCBI-GEO: GSE70660, GSE36985, GSE52924, and GSE59087.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.12.014.

AUTHOR CONTRIBUTIONS

X.L., D.L., H.C., and J.X. performed experiments and analyzed the data. J.H., X.L., Z.S., Y.Z., G.C.Y., and J.X. performed bioinformatics analyses. E.T., T.V.B., and L.I.Z. contributed the GATA2 ChIP-seq datasets and analyzed the data. J.H., X.L., S.H.O., and J.X. wrote the manuscript. S.H.O. and J.X. supervised the project.

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