Establishing a hematopoietic genetic network through locus-specific integration of chromatin regulators

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The establishment and maintenance of cell type-specific transcriptional programs require an ensemble of broadly expressed chromatin remodeling and modifying enzymes. Many questions remain unanswered regarding the contributions of these enzymes to specialized genetic networks that control critical processes, such as lineage commitment and cellular differentiation. We have been addressing this problem in the context of erythrocyte development driven by the transcription factor GATA-1 and its coregulator Friend of GATA-1 (FOG-1). As certain GATA-1 target genes have little to no FOG-1 requirement for expression, presumably additional coregulators can mediate GATA-1 function. Using a genetic complementation assay and RNA interference in GATA-1–null cells, we demonstrate a vital link between GATA-1 and the histone H4 lysine 20 methyltransferase PR-Set7/SetD8 (SetD8). GATA-1 selectively induced H4 monomethylated lysine 20 at repressed, but not activated, loci, and endogenous SetD8 mediated GATA-1–dependent repression of a cohort of its target genes. GATA-1 used different combinations of SetD8, FOG-1, and the FOG-1–interacting nucleosome remodeling and deacetylase complex component Mi2β to repress distinct target genes. Implicating SetD8 as a context-dependent GATA-1 corepressor expands the repertoire of coregulators mediating establishment/maintenance of the erythroid cell genetic network, and provides a biological framework for dissecting the cell type-specific functions of this important coregulator. We propose a coregulator matrix model in which distinct combinations of chromatin regulators are required at different GATA-1 target genes, and the unique attributes of the target loci mandate these combinations.

Traffic assignment: GATA: epigenetics, genomics, erythroid

The precise regulation of complex transcriptional networks ensures the fidelity of critical developmental processes. A fundamental component of this regulation involves epigenetic mechanisms that impose stringent constraints to restrict cis-element occupancy by trans-acting factors and postchromatin occupancy mechanisms involving the recruitment of broadly expressed chromatin modifying and remodeling enzymes that chemically alter or reposition nucleosomes. Histone modifications, such as acetylation and methylation, confer transcriptional repression or activation in a context-dependent manner. Although numerous enzymes modify and remodel chromatin, and knowledge on their biochemical mechanisms has advanced tremendously, many questions remain regarding how they establish and maintain genetic networks that control essential processes, including stem cell self-renewal, lineage commitment, and cellular differentiation.

Given the crucial red blood cell functions and common therapeutic scenarios demanding modulation of erythropoiesis (1), it is instructive to consider how epigenetic mechanisms control hematopoietic stem cell (HSC) differentiation into multipotent progenitors, lineage-committed progenitors, and ultimately erythrocytes. The transcription factor GATA-2 (2, 3) is required for the genesis and maintenance of HSCs (4), whereas GATA-1 (5, 6) is crucial for erythrocyte, megakaryocyte, mast cell, and eosinophil development (7, 8). During erythropoiesis, GATA-1 replaces GATA-2 at Gata2 chromatin sites, thus conferring repression (9–11). GATA switches occur at numerous loci and are frequently associated with altered transcriptional output. The GATA–1–interacting coregulator Friend of GATA-1 (FOG-1) mediates GATA-1–dependent activation and repression in a context-dependent manner (12, 13). FOG-1 facilitates GATA-1 chromatin occupancy (14, 15) and interacts with the nucleosome remodeling and deacetylase (NuRD) chromatin remodeling complex (16) containing the ATPase CHD4 (Mi2β), which is required for development of erythroid and other hematopoietic lineages (17, 18). GATA-1 also recruits the chromatin remodeler BRG1 (19, 20), the histone acetyltransferase CBP/p300 (21), and the mediator complex component Med1 (22, 23). BRG1 promotes expression of adult α- and β-like globin genes in erythroid cells (19, 24), CBP/p300 mediates GATA-1 function in at least certain contexts (21), and Med1 amplifies GATA-1 activity at select target genes (22). Given the crucial developmental functions of FOG-1 factors, it is reasonable to assume that the requisite coregulator machinery is complex and involves considerable functional redundancy to ensure developmental fidelity. Because GATA-1 target genes differ in their requirements for FOG-1 (11, 12) and GATA-1 K137 sumoylation, a modification that enhances GATA-1 activity at loci requiring FOG-1 (25), the ensemble of coregulators mediating GATA factor function appears to be locus-specific.

Unraveling mechanisms underlying locus-specific GATA factor actions will provide key insights into how GATA factors function uniquely in distinct cell types and developmental stages. To address this problem, we conducted in silico data mining of the BioGPS database (http://biogps.gnf.org) (26) to identify chromatin

Significance

Broadly expressed enzymes commonly change chromatin structure and function. How ubiquitous chromatin regulators establish specialized patterns of gene activity is not understood. We identified an important link between a histone methyltransferase and a transcription factor (GATA-1) that controls red blood cell development. We found that distinct combinations of this enzyme and additional chromatin regulators are required for GATA-1 to control transcription at different genetic loci. The resulting regulatory “matrix” provides a conceptual framework for understanding how cell-restricted factors use broadly expressed chromatin regulators to confer specialized gene-expression patterns that control important biological processes.
regulators enriched in erythroid cells, which may imply an important erythroid function and identify novel GATA-1 coregulators. This analysis revealed SetD8, the sole methyltransferase that catalyzes H4K20 monomethylation (H4K20me1) (27). Although the SetD8 catalytic mechanism has been elucidated (28), many questions remain regarding its cell type-specific functions. SetD8 and H4K20me1 are dynamically regulated throughout the cell cycle, and SetD8 degradation promotes cell cycle progression (29). Targeted deletion of SetD8 blocks embryogenesis at the four- and eight-cell stages and impairs hematopoietic maturation (30). H4K20me1 has been correlated with transcriptional activation and repression (27). H4K20me1 localizes predominantly to nontranscribed hematopoietic regions on Drosophila polytene chromosomes (31) and to E2F-repressed genes in HeLa cells (32). Functional studies in Drosophila provide evidence for SetD8-dependent repression mechanisms (33). In contrast, analyses in HeLa cells and T lymphocytes revealed that H4K20me1 resides at actively transcribed hematopoietic loci (34, 35). We demonstrate that SetD8 is a context-dependent GATA-1 corepressor and provide evidence for locus-specific mechanisms that integrate ensembles of hematopoietic regulators, which we term a coregulator matrix model of GATA factor function.

Results

SetD8-Dependent Target Gene Ensemble in Erythroid Cells. Given the crucial SetD8 activity for early development, and the common H4K20me1 mark in diverse systems, presumably SetD8 controls a broad spectrum of biological processes. As cell type-specific SetD8 mechanisms and SetD8 function/regulation in the hematopoietic system are largely unexplored, we conducted siRNA-based loss-of-function and genetic complementation analysis to evaluate its function in a physiologically relevant model of erythroid cell maturation, G1E-ER-GATA-1 cells (36) (Fig. 1A). G1E-ER-GATA-1 cells were derived from GATA-1–nullizygous ES cells, resemble normal proerythroblasts, and stably express a conditionally active allele of GATA-1 (ER-GATA-1) (37). Because GATA-1 knockdown altered the capacity of GATA-1 to regulate target genes, we generated a new GATA-1–expressing GATA-1–regulated and SetD8–regulated gene dataset, because prior GATA-1 datasets were obtained with a different microarray platform and different functional studies in nonspecific hematopoietic systems revealed no enrichments at gross chromosomal features (e.g., telomeres or centromeres) (Fig. 2E).

SetD8 as a Context-Dependent GATA-1 Corepressor. Comparison of GATA-1– and SetD8-regulated gene cohorts by real-time RT-PCR revealed two modes by which SetD8 mediates GATA-1 function. First, SetD8 was required for GATA-1 to repress Klf3 expression (Fig. 2C) and knocking-down SetD8 abrogated the repression. Second, SetD8 repressed expression of certain GATA-1–activated genes. GATA-1 activated Scamp5 (Fig. 2C), and knocking-down SetD8 yielded Scamp5 hyperactivation. A distinct gene cohort, exemplified by Ak1, was SetD8–, but not GATA-1–, regulated (Fig. 2C).

To more rigorously establish interrelationships between GATA-1– and SetD8–regulated genes, we generated a new GATA-1–regulated gene dataset, because prior GATA-1 datasets were obtained with a different microarray platform and different cultures of G1E-ER-GATA-1 cells (38, 40). We conducted transcriptional profiling in nonspecific siRNA-transfected cells, uninduced, and β-estradiol-induced G1E-ER-GATA-1 cells to control for potential influences of nucleoefection on ER-GATA-1 activity. Because GATA-1–regulated and SetD8–activated genes did not overlap, we focused only on SetD8–repressed genes. A total of 47 genes were coregulated by SetD8 and GATA-1 (2% of all GATA-1 targets). The SetD8–repressed genes segregated based on ER-GATA-1–responsive genes, GATA-1–activated, and GATA-1–insensitive (Fig. 3A and B). Although the majority (52%) of SetD8-regulated genes were GATA-1–insensitive, 32% were GATA-1–repressed (Fig. 3A and B), and 16% were GATA-1–activated (Fig. 3A and B). GO analysis of the SetD8/GATA-1–corepressed cohort revealed some of these genes function in “B-cell proliferation,” including Cd81 and Tnfrsf13b (Fig. 3A and B). Two SetD8-repressed/GATA-1–activated genes were involved in “protein amino acid ADP-ribosylation (Fig. 3A and B). SetD8–regulated, GATA-1–insensitive genes were linked
to diverse cellular processes, including response to wounding, stress, and apoptosis, (Fig. 3 A and B). Thus, expression profiling demonstrated that SetD8 is a GATA-1 corepressor at a restricted cohort of GATA-1 target genes, and its quantitative contribution to GATA-1 function can be considerable. Because our analysis revealed genes that have not been studied in erythroid cells, we mined expression data obtained from murine primary adult erythroblasts of differing maturation stage (44), focusing on SetD8/GATA-1–corepressed genes that will be the focus of subsequent mechanistic analyses. The expression of Vim, Clec10a, Rgs19, and Limd2 resembled Gata2 and c-Kit, direct GATA-1 target genes whose expression declines upon transition from proerythroblasts to reticulocytes (Fig. 3C). SetD8 was highly expressed at all maturation stages.

Although GATA-1–mediated repression can involve FOG-1 (12), GATA switches (10), and reduced occupancy of the hematopoietic transcription factor Scl/TAL1 (11), many questions remain unanswered regarding the underlying mechanisms. In addition, we are unaware of reports in which SetD8 functions as a corepressor for any cell type-specific activator. To ask whether GATA-1 directly controls the SetD8-regulated gene cohort, we tested whether endogenous GATA-1 occupies the respective loci. Analysis of an endogenous GATA-1 ChIP-seq dataset from mouse erythroleukemia cells generated with our anti-GATA-1 antibody revealed GATA-1 occupancy at, or in the vicinity of, the SetD8/GATA-1–corepressed genes (Fig. 4A). GATA-1 occupied MyoIg and Rgs19 intronic sites, Limd2, Vim, and Kank3 distal sites, and Vim, Kank3, and Clec10a promoter sites (Fig. 4A). Analysis of histone modifications that demarcate enhancers (H3 acetylation at K27 and H3 monomethylation at K4) and promoters (H3 trimethylation at K4) from a mouse erythroleukemia cell ChIP-seq dataset revealed patterns largely predictable from the genomic location of GATA-1 occupancy (Fig. 4A). Of the 31 SetD8/GATA-1–corepressed genes, GATA-1 peaks were detected at or in the vicinity of 24 genes (77%) (Fig. S2). Of these 24 genes, GATA-1 occupied the promoter or gene body of 14 genes (promoter occupancy, 9 genes; gene body, 11 genes).

To further assess whether the SetD8/GATA-1–corepressed genes occupied by endogenous GATA-1 are direct GATA-1 targets, we analyzed the kinetics of GATA-1–mediated repression of these genes. β-Estradiol treatment of G1E-ER-GATA-1 cells rapidly reduced primary transcript levels for the previously established direct GATA-1 targets Gata2 (9) and Ly1 (45) by 70% after 12 h (Fig. 4B). MyoIg, Vim, and Clec10a primary transcript levels were reduced rapidly by 1–2 h, and to a similar extent 12-h postestra diol treatment, consistent with these genes being direct GATA-1 targets (Fig. 4B). Kank3 and Rgs19 repression was slightly slower (Fig. 4B). However, significant repression was apparent at these loci by 1 and 2 h for Kank3 and Rgs19, respectively.

Considering the SetD8 catalytic mechanism, presumably SetD8 mediates repression of the GATA-1 targets by catalyzing H4K20me1. We tested whether ER-GATA-1 induces H4K20me1 in SetD8/GATA-1–corepressed genes. To address this theory, we conducted quantitative ChIP analysis with untreated or β-estradiol–treated G1E-ER-GATA-1 cells using an anti-H4K20me1 antibody. At the SetD8-sensitive, GATA-1–repressed genes Vim, Clec10a, Kank3, and Rgs19, β-estradiol induced promoter-associated H4K20me1 (Fig. 5A). At GATA-1–activated promoters, H4K20me1 levels were reduced or unaffected by ER-GATA-1 activation. ER-GATA-1 significantly reduced H4K20me1 at the Alas2 promoter by 3.4-fold. H4K20me1 declined at the Slc4a1 promoter, but not significantly (P = 0.058). Low-level H4K20me1 resided at the Hbb-b1 and Hba-a1 promoters, which was unaffected by β-estradiol treatment. ER-GATA-1 did not regulate H4K20me1 at the constitutively repressed Necdin promoter, which is insensitive to β-estradiol and SetD8 knockdown.

To assess the mechanism by which ER-GATA-1 activation induces H4K20me1 at SetD8/GATA-1–regulated genes, we determined the relationship between SetD8, GATA-1, and H4K20me1 occupancy. Quantitative ChIP analysis was conducted in β-estradiol–treated and untreated G1E-ER-GATA-1 cells using anti–GATA-1, anti-H4K20me1, and anti-SetD8 antibodies. At the Vim locus, β-estradiol-induced GATA-1 occupancy at sites −0.6 kb and +1.2 kb from the Vim transcription start site (TSS) (Fig. 5B). SetD8 occupancy was also detected at the Vim locus, and was maximal at the +1.2-kb GATA-1 binding site (Fig. 5B). This finding indicates that SetD8 and GATA-1 can be cross-linked to overlapping chromatin regions.
Although β-estradiol treatment did not affect SetD8 occupancy, H4K20me1 levels increased across a ∼7-kb region around the TSS (Fig. 5B). GATA-1 occupied a site −0.4 kb from the Clec10a TSS (Fig. 5C), and similar to Vim, SetD8 and GATA-1 occupancy overlapped (Fig. 5C). Also similar to Vim, β-estradiol treatment yielded a broad zone of H4K20me1 enrichment, whereas SetD8 occupancy was unaffected (Fig. 5C). These data indicate that SetD8 precedes GATA-1 occupancy at these target genes, and are consistent with a model in which GATA-1 occupancy stimulates SetD8 activity to induce H4K20me1 at the respective loci in a manner that is not restricted to the GATA-1 occupancy site.

**Locus-Specific Integration of Chromatin Regulators: Evidence for a Coregulator Matrix Model of GATA Factor Function.** The activation or repression of GATA-1 target genes can be SetD8–sensitive or -insensitive (11, 12). Thus, GATA-1–mediated repression of SetD8 target genes might be SetD8–sensitive, SetD8–insensitive, or both at distinct loci. We knocked-down FOG-1 in G1E-ER-GATA-1 cells to assess SetD8–sensitive of SetD8/GATA-1–corepressed genes (Fig. 6A). Whereas ER-GATA-1–mediated repression of Clec10a, Myo1g, and Ly1l was mildly sensitive to the knockdown, Kank3, Rgs19, Gata2, and c-Kit repression was abrogated (Fig. 6A). As an alternative approach, we evaluated FOG-1 sensitivity by stably expressing ER-GATA-1 or a mutant (ER-GATA-1(V205G)) defective in FOG-1 binding (12) in G1E cells. Four clones of ER-GATA-1(V205G)–expressing cells were compared with an ER-GATA-1–expressing clone. Because clonal lines typically express ER-GATA-1(V205G) at levels lower than our conventional G1E-ER-GATA-1 line (46), we used a clonal line expressing lower levels of ER-GATA-1 to ensure that ER-GATA-1 did not exceed expression levels of the mutants. Semiquantitative Western blotting demonstrated that ER-GATA-1(V205G) protein was expressed at at least as high as ER-GATA-1 (Fig. 6B). Because of the low-level ER-GATA-1 expression, however, the magnitude of ER-GATA-1 responses at certain loci is less than with our typical G1E-ER-GATA-1 line. Whereas ER-GATA-1 repressed Gata2, Kank3, and Rgs19, repression was lower in all of the ER-GATA-1 (V205G) clones (Fig. 6B). In contrast, ER-GATA-1(V205G) repressed Lyl1, Rgs18, Myo1g, Clec10a, and Vim to an equal or greater extent than ER-GATA-1, indicating that disrupting the ER-GATA-1–FOG-1 interaction did not affect repression of these genes (Fig. 6B). In aggregate, these results demonstrate that SetD8/GATA-1–corepressed genes are not dedicated to a single transcriptional mode involving FOG-1, but rather are FOG-1–sensitive or -insensitive.

The NuRD complex associates with FOG-1 (16) and is an important determinant of GATA-1–mediated regulation of transcription and hematopoiesis (17, 18). We predicted that FOG-1–regulated, SetD8/GATA-1–corepressed genes would also require Mi2β, a key ATPase subunit of the NuRD complex (47, 48). siRNA-mediated knockdown of Mi2β mRNA nearly ablated Mi2β protein (Fig. 7A, Left) and abolished GATA-1–mediated repression of the FOG-1–insensitive SetD8/GATA-1–corepressed gene Clec10a (Fig. 7A, Right). Repression of the FOG-1–sensitive genes Gata2, c-Kit, and Rgs19 was significantly, but modestly, reduced (Fig. 7A, Right). The FOG-1–sensitive
Fig. 3. SetD8 as a context-dependent GATA-1 corepressor. The set of genes up-regulated by SetD8 knockdown was subjected to sorting into one of three categories based on their response to GATA-1 activation: GATA-1-activated, GATA-1-repressed, or GATA-1-insensitive. GATA-1-sensitivity values are based on microarray analysis comparing cells receiving control siRNA, and either no treatment or treatment with GATA-1 activator Kank3 or repressor Mi2. Knocking down Mi2 activated 100 genes and down-regulated 768 genes. Seventeen percent of GATA-1-activated genes were also repressed by Mi2, including Hbb-bh1 and Clec10a (Fig. 7B). Only 12 genes were repressed by GATA-1, Mi2β, and SetD8. No SetD8-activated genes were also activated by GATA-1, and only one gene was activated by SetD8 and Mi2β (Fig. 7B). These data suggest that although Mi2β can positively and negatively coregulate genes with GATA-1, SetD8 is exclusively involved in GATA-1-mediated repression. The terminal coregulator requirements for GATA-1-mediated repression constitute a matrix (Fig. 8A), supporting a model in which GATA factor function requires different coregulator combinations at distinct endogenous loci (Fig. 8B).
Discussion

We describe evidence that the H4K20me1 methyltransferase SetD8 is a context-dependent GATA-1 corepressor. A prior analysis had implicated SetD8 as a coactivator for the Wnt pathway factor LEF1/TCF4 (49). SetD8 overexpression and knockdown in 3T3 cells increased and decreased Axin2 expression, respectively (49). SetD8 knockdown in HEK293 cells reduced expression of several Wnt target genes, and SetD8 regulates Wnt target genes in zebrafish (49). However, it was unknown whether SetD8 mediates transcriptional control by a large or highly restricted cohort of trans-acting factors, whether other developmental regulators use SetD8 to instigate cell type-specific transcriptional programs,
Control GATA-1-Activated Hba-a1 Alas2 -estradiol Hbb-b1

that selectively repress the embryonic/fetal up-regulated mouse fetal and embryonic globin genes. Factors intriguingly, GATA-1 target genes differ in their requirements for SetD8 dominantly as a corepressor in G1E-ER-GATA-1 cells. Intriguingly or a corepressor (31, 33).

and whether SetD8 commonly functions as a coactivator (34, 35, 49) or a corepressor (31, 33). and whether SetD8 commonly functions as a coactivator (34, 35, 49) or a corepressor (31, 33).

Our results demonstrate that endogenous SetD8 functions predominantly as a corepressor in G1E-ER-GATA-1 cells. Intriguingly, GATA-1 target genes differ in their requirements for SetD8 and other coregulators. Reducing the level of endogenous SetD8 up-regulated mouse fetal and embryonic globin genes. Factors that selectively repress the embryonic/fetal β-like globin genes are of great interest, as increasing embryonic/fetal β-like globin gene expression in human hemoglobinopathies involving mutated or reduced levels of adult β-globin is efficacious (43); existing clinical strategies are relatively nonspecific. Genome-wide expression analysis revealed that SetD8 repressed a restricted cohort of genes in erythroid cells, some of which are GATA-1-regulated (e.g., murine embryonic/fetal β-like globin genes). SetD8 also repressed genes that were not GATA-1-regulated, including genes implicated in B-cell biology. The majority of SetD8-repressed genes in erythroid cells were nonerythroid genes. GATA-1 occupied SetD8/GATA-1–corepressed genes, and kinetic studies imply direct GATA-1 regulation. GATA-1 used SetD8 at genes that are FOG-1–sensitive or -insensitive and those that are Mi2β-sensitive or -insensitive.

Our loss-of-function studies with SetD8 and other coregulators provide evidence for a coregulator matrix model of GATA factor function. This model assumes that distinct combinations of coregulators confer target-gene regulation in a locus-specific manner. Conceptually, this model differs from the paradigm in which an activator or repressor function via a common mechanism in diverse contexts. It is attractive to propose that the local chromatin environment, higher-order chromatin structure, or subnuclear neighborhood mandate the locus-specific mechanistic requirements for transcriptional control. At a rudimentary level involving a single coregulator, FOG-1, FOG-1–sensitive GATA-1 target genes are expelled from the nuclear periphery upon activation, whereas FOG-1–insensitive GATA-1 target genes constitutively reside at the periphery (25, 50). Our coregulator matrix constitutes a unique foundation that can be extended to yield a genome-wide perspective of the complex relationships between GATA-1 and requisite coregulators at target genes with unique attributes, including local chromatin environment, higher-order chromatin structure, and subnuclear neighborhood. This matrix will be an exceptionally powerful tool to dissect parameters dictating context-dependent GATA factor functions and will permit sophisticated modeling to evaluate how alterations in the regulatory parameters influence GATA factor-dependent genetic networks and downstream physiological and pathophysiological outputs.

Erythroid development requires cell-intrinsic and -extrinsic mechanisms that control commitment of multipotent hematopoietic precursors, massive gene-expression changes, and sequential maturation steps, including gross organelle remodeling, that prepare for enucleation (1, 44, 51). Analogous to GATA-1, FOG-1 is a master regulator of erythropoiesis with broad roles to establish the erythroid cell phenotype. We predict that select GATA-1 coregulators have more specialized functions to confer specific components of the GATA-1–dependent genetic network, such as components dedicated to controlling the induction of autophagy

![Fig. 5.](image)

**A** Quantitative ChIP analysis of promoter-associated H4K20me1 levels at GATA-1 target genes (n = 3, ± SE). *P < 0.05. (B and C) Quan-

titative ChIP analysis of ER-GATA-

1, SetD8, and H4K20me1 as a function of distance from the (B) Vim TSS and (C) Clec10a TSS (n = 4, ± SE) An asterisk indicates a significant difference (P < 0.05) between the untreated and j-estradiol–treated value for each amplified site. The TSS is depicted as a vertical dashed line. Rabbit pre-immune serum (for GATA-1 and H4K20me1) and purified mouse IgG (for SetD8) are graphed as a horizontal dashed line, which represents the average value from all sites at each respective locus. Vim and Clec10a loci are depicted at the top of B and C, respectively, with marks indicating the position of each amplicon.
(52), which is required for organelle remodeling (53, 54), or enucleation (55, 56). The G1E-ER-GATA-1 system recapitulates a normal window of maturation (38), but ER-GATA-1 activation in this system does not drive efficient enucleation. Furthermore, regulatory events underlying the genesis of proerythroblasts cannot be studied in this system. Although SetD8 was not required for Ter119 induction in G1E-ER-GATA-1 cells grown under conventional conditions in a 2-d maturation assay, it repressed embryonic/fetal β-like globin genes and additional genes expected to have important roles in cellular physiology. Our results establish the molecular underpinnings of a pivotal biological mechanism in which Vm (encoding Vimentin) is down-regulated during erythropoiesis as a key step in maturation-associated cytoskeletal remodeling (57). The loss of Vimentin-containing intermediate filaments has been proposed to be a prerequisite for enucleation (58). Extending our studies to interrogate coregulator requirements for specific components of the GATA-1–dependent genetic network in more complex systems will almost certainly reveal additional SetD8/GATA-1–coregulated biological processes, and the work described herein provides foundational insights to guide such studies.

Beyond GATA-1 mechanisms, we expect that the coregulator matrix model can be extrapolated to the actions of trans-acting

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**Fig. 6.** SetD8/GATA-1–corepressed genes differentially require FOG-1. (A) FOG-1 knockdown in G1E-ER-GATA-1 cells. Real-time RT-PCR quantitation of mRNA levels of FOG-1–sensitive and –insensitive genes, as well as SetD8/GATA-1–corepressed genes, was performed by qPCR. (B) Gene expression analysis of GATA-1-target genes in G1E-ER-GATA-1 or G1E-ER-GATA-1(V205G) mutant cells matched for ER-GATA-1 expression. White bars, untreated; black bars, β-estradiol-treated. One ER-GATA-1 and four ER-GATA-1(V205G) clonal lines. Each untreated sample was normalized to a value of 1. For G1E-ER-GATA-1 samples, error bars represent SD from two technical replicates. For G1E-ER-GATA-1(V205G) samples, error bars represent SE from four biological replicates. *P < 0.05.

**Fig. 7.** SetD8/GATA-1–corepressed genes differentially require the NuRD component, Mi2β. (A) Mi2β knockdown in G1E-ER-GATA-1 cells. (Left) Western blot to detect Mi2β protein levels. (Right) Real-time RT-PCR quantitation of mRNA of GATA-1 target genes that are FOG-1–sensitive, FOG-1–insensitive, and SetD8-sensitive (n = 5 ± SE). *P < 0.05. (B, Left) Venn diagram depicting the extent of overlap between GATA-1–repressed, Mi2β–repressed, and SetD8-repressed genes. (Right) Venn diagram depicting overlap of GATA-1–activated, Mi2β–activated, and SetD8-activated genes.
factors functioning at endogenous loci in diverse biological contexts. However, further studies on endogenous coregulator actions at endogenous loci are required. Comparative analyses of factor-specific or tissue-specific matrices will almost certainly uncover broadly important principles.

Materials and Methods

Cell Culture. G1E-ER-GATA-1 and G1E-ER-GATA-1(205G) cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Gibco) containing 15% (vol/vol) FBS (Gibco), 1% penicillin-streptomycin (Gibco), 2 μM erthropoietin, 120 μM monothioglycerol (Sigma), 0.6% conditioned medium from a Kit ligand-producing CHO cell line, and 1 μg/mL puromycin (Gibco). ER-GATA-1 activity was induced by treating cells with 1 μM estradiol (Steraloids). FOG-1-null hematopoietic precursor cells were maintained in IMDM (Gibco) containing 15% FBS (Gibco), 1% antibiotic/antimycotic (Gibco), and 10 ng/mL IL-3 (R&D Systems). Mouse erythroleukemia cells were cultured in DMEM (Gibco) supplemented with 5% (vol/vol) FBS (Gibco).

RNA Interference. Dharmacon siGenome SmartPool siRNAs targeting mouse SetDb, Fog1, and Mi2β were electroporated into 3 × 10^6 G1E-ER-GATA-1 cells using an Amaza Nucleofector (Lonza) coupled with Nucleofection Kit R (Lonza), as described previously (22, 50). Nontargeting siRNA (Dharmacon) served as a control. siRNA transfections were conducted twice (at 0 h and at 24 h). For mRNA and protein analysis, transfectant cells were treated with β-estradiol at 24 h and harvested at 48 h. For flow cytometric analysis, cells were treated with β-estradiol at 24 h and harvested at 72 h.

Real-Time RT-PCR. Total RNA was purified with TRIzol (Invitrogen). To prepare cDNA, 1 μg RNA was annealed with 250 ng of a 5:1 mixture of random hexamer and oligo(dT) primers by heating to 68 °C for 10 min. The annealed RNA/primers were incubated with murine Moloney leukemia virus reverse-transcriptase (Invitrogen), 10 mM DTT (Invitrogen), RNAsin (Promega), and 0.5 mM deoxynucleoside triphosphates (dNTPs) at 42 °C for 1 h in a total reaction volume of 20 μL. This mixture was heat-inactivated at 95 °C for 5 min, and then diluted to a final volume of 100 μL. RT-PCR reactions contained 1.5 μL cDNA, 10 μL Power SYBR Green Master Mix (Applied Biosystems), appropriate primers, and water to a total volume of 20 μL. PCR product accumulation was monitored by SYBR green fluorescence. Relative expression was determined from a standard curve of serial dilutions of cDNA sample. As an internal control, all RNA measurements were normalized to 18S RNA levels.

Transcriptional Profiling. RNA samples from three independent SetDb-knockdown and Mi2β-knockdown experiments in G1E-ER-GATA-1 cells were used for microarray analysis. mRNA was isolated and used to synthesize Amino Allyl RNA (aRNA). aRNA was labeled and hybridized to 8 × 60K Mouse Whole Genome arrays (Agilent), and read using a G-2505C DNA Microarray Scanner with Surescan High Resolution (Agilent). Data were analyzed using EDGE3, a Web-based two-color microarray analysis software, coupled with Microsoft Excel. Heat maps were generated using Java TreeView software.

Quantitative ChIP. ChIP analysis in G1E-ER-GATA-1 cells was conducted as described previously (59). Briefly, samples containing 5 × 10^6 cells were crosslinked in 1% formaldehyde for 10 min. H4K20me1 was immunoprecipitated using rabbit polyclonal anti-H4K20me1 antibody (Millipore). SetDb was immunoprecipitated using a mouse monoclonal antibody (Abcam ab3798) and GATA-1 was immunoprecipitated using a rabbit polyclonal antibody developed by the Bresnick Lab. Rabbit preimmune serum (Covance) was used as a control. Samples were quantitated using RT-PCR (Applied Biosystems Viia 7). Quantity of DNA was determined by SYBR green fluorescence, and the amount of product was determined relative to a standard curve created from serial dilution of input chromatin.

Protein Analysis. Protein samples were isolated by centrifugation of 1 × 10^6 cells from each condition, washing with cold PBS, and lysing in 1× SDS sample buffer (25 mM Tris, pH 6.8, 2% β-mercaptoethanol, 3% SDS, 0.005% bromophenol blue, 5% glycerol). Samples were boiled for 10 min and stored at −80 °C. Samples were resolved by SDS/PAGE, and proteins were detected by semiquantitative Western blotting with ECL Plus (GE Healthcare). Antibodies used were anti-SetDb (Millipore 07–316), anti–GATA-1 (Santa Cruz Biotechnology; sc-265), anti–α-tubulin (Millipore; clone DM1A, 05–829). Secondary antibodies included goat anti-mouse-IgG-HRP, goat anti-rabbit-IgG-HRP, or goat anti-rat-IgG-HRP (Santa Cruz Biotechnology; sc-2005, sc-2030, sc-2032).

Flow Cytometry. For flow cytometry, 1 × 10^6 cells were isolated by centrifugation (6 min, 168 × g), washed with ice-cold PBS, and resuspended in 100 μL Annexin V Binding Buffer (Invitrogen). Cells were incubated with 5 μL Alexa Fluor 350-conjugated Annexin V (Invitrogen A23202) at room temperature for 15 min in the dark. Ice-cold Annexin V binding buffer was added (400 μL), followed by 30 μL of 150 μg/mL propidium iodide solution in PBS. Samples were maintained on ice and were analyzed using a BD LSR II Flow Cytometer. Annexin V Alexa Fluor 350 was detected with the UV laser (detector at 355 nm, filter at 450/50 nm), and propidium iodide was detected with the green laser (detector at 561 nm, filter at 450/50 nm). Data were analyzed using FlowJo 9.5.2 software.

Statistical Analysis. Statistical significance was determined by Paired Student’s t test using web-based GraphPad software (www.graphpad.com). Statistical analysis of genome-wide expression data were conducted using EDGE3 software (60). Statistical significance of GO terms was conducted with the Web-based National Institutes of Health DAVID tool (http://david.abcc. nci.nih.gov).

ChIP-Seq. ChIP-seq profiles for GATA-1, H3K4me1, H3K27ac, and H3K4me1 in mouse erythroleukemia cells were generated using the University of California at Santa Cruz Genome Browser (http://genome.ucsc.edu). Endogenous GATA-1 ChIP-seq data were generated by Sherman Weissman (Yale University, New Haven, CT) with an anti–GATA-1 antibody developed by the Bresnick laboratory (GEO accession GSM912907). Data for H3K4me1 (Abcam ab8895, GEO accession GSM1000073), H3K4me3 (Millipore 07–473 GEO accession GSM1000087), and H3K27ac (Abcam ab4729, GEO accession GSM1000142) were generated by Bing Ren (University of California, San Diego, La Jolla, CA).
ACKNOWLEDGMENTS. We thank members of the E.H.B. group for critical comments. This study was funded in part by National Institutes of Health Grant DK50107, and National Institutes of Health Grant T32 GM081061 (to A.W.D.), and a University of Wisconsin Comprehensive Cancer Center Support Grant P30 CA014520.

Supporting Information

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Fig. S1. Murine erythroleukemia (MEL) cells were transfected with histone lysine methyltransferase (SetD8) siRNA or control siRNA. SetD8 and erythroid transcription factor (GATA-1) mRNA levels were quantified by RT-PCR (n = 3, mean ± SE) *P < 0.0001.

<table>
<thead>
<tr>
<th>GATA-1-Repressed</th>
<th>ChIP-Seq Peaks</th>
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<tr>
<td>Vim</td>
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</table>

Fig. S2. GATA-1 occupancy at GATA-1/SetD8-corepressed genes in MEL cells. –, no GATA-1 peaks; +, GATA-1 occupancy in the gene body, promoter, or the surrounding chromatin.