Single-nucleotide human disease mutation inactivates a blood-regenerative GATA2 enhancer

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Graphical abstract

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The development and function of stem and progenitor cells that produce blood cells are vital in physiology. GATA-binding protein 2 (GATA2) mutations cause GATA-2 deficiency syndrome involving immunodeficiency, myelodysplastic syndrome, and acute myeloid leukemia. GATA-2 physiological activities necessitate that it be strictly regulated, and cell type–specific enhancers fulfill this role. The +9.5 intronic enhancer harbors multiple conserved cis-elements, and germline mutations of these cis-elements are pathogenic in humans. Since mechanisms underlying how GATA2 enhancer disease mutations impact hematopoiesis and pathology are unclear, we generated mouse models of the enhancer mutations. While a multi-motif mutant was embryonically lethal, a single-nucleotide Ets motif mutant was viable, and steady-state hematopoiesis was normal. However, the Ets motif mutation abrogated stem/progenitor cell regeneration following stress. These results reveal a new mechanism in human genetics, in which a disease predisposition mutation inactivates enhancer regenerative activity, while sparing developmental activity. Mutational sensitization to stress that instigates hematopoietic failure constitutes a paradigm for GATA-2 deficiency syndrome and other contexts of GATA-2–dependent pathogenesis.

Introduction

Development of the hematopoietic system involves massive genome remodeling and the establishment of complex genetic networks. The transcription factor GATA-binding protein 2 (GATA-2) establishes and maintains genetic networks governing hematopoietic stem and progenitor cell (HSPC) genesis and function (1–5) and is a major determinant of all blood cell lineages (6, 7). Human GATA2 deficiency syndrome resulting from germline coding or enhancer (+9.5) mutations includes monocytopenia and mycobacterial infection syndrome (2, 8, 9); DC, monocyte, B, and NK lymphoid deficiency (10); Emberger syndrome (primary lymphedema with myelodysplastic syndromes [MDS]) (8, 10–12); and familial MDS/acute myeloid leukemia (AML) (8, 10–12); and NK lymphoid deficiency (10); Emberger syndrome (primary lymphedema with myelodysplastic syndromes [MDS]) (8, 10–12); and familial MDS/acute myeloid leukemia (AML) (11). These pathologies might extend beyond germline mutations, as a patient and familial MDS/acute myeloid leukemia (AML) (11). These pathologies might extend beyond germline mutations, as a patient

patients often harbor additional mutations, e.g., in ASXL transcriptional regulator 1 (ASXLI) (20), or cytogenetic abnormalities such as monosomy 7 (18).

Since murine Gata2 ablation is lethal at approximately E10.5 (6), conditional (21, 22), heterozygous (23, 24), and enhancer-mutant mice (1, 2, 4, 5, 25) have been used to elucidate GATA-2 regulation and function. Transcriptional control of GATA-2 requires cell type–specific enhancers 9.5 kb downstream and 77 kb upstream of the Gata2 start site (2, 5, 26). Whether these enhancers function and are essential in regenerative contexts is unknown. The +9.5 triggers hematopoietic stem cell (HSC) emergence from hemogenic endothelium and confers vascular integrity (1, 2) (Figure 1). While Gata2-mutant mice have not previously been found to have malignancies, GATA-2 mediates leukemogenesis in a Tet2 ablation and FLT3–internal tandem duplication–overexpression (FLT3-ITD–overexpression) AML mouse model (27). Thus, normal hematopoiesis requires stringent GATA-2 regulation.

The +9.5 enhancer contains multiple transcription factor motifs, including an E-box–spacer–WGATAR and Ets motif (Figure 1). These include canonical binding motifs for the heptad transcription factors and cofactors governing the generation of HSPCs (28), including stem cell leukemia/T cell acute lymphoblastic leukemia (SCL/TALI) (E-box: CANNTG), GATA-2 (WGATAR), and the Ets factors ETS-related gene (ERG) and FlI-1 proto-oncogene (FLI1) (GGAW). The Gata2 +9.5 E-box, GATA, and downstream motifs (including the Ets motif) are essential for +9.5 site enhancer activity in reporter assays (29, 30), and human
patients with heterogeneous disruptions of the +9.5 E-box or Ets motifs exhibit decreased GATA2 expression (2, 9). Deletion of the E-box-spacer-WGATAR (+9.5−) composite element reduced chromatin accessibility and abrogated occupancy of SCL/TAL1 and its cofactor LIM domain–binding 1 (LDB1) in fetal liver cells (31), revealing +9.5 enhancer–dependent chromatin accessibility and regulatory complex assembly at the Gata2 locus.

Patients with GATA-2 deficiency syndrome with enhancer mutations can lack the E-box or harbor single-nucleotide Ets mutations (2, 9, 32) that disrupt the GGAW motif, which is essential for high-affinity DNA binding (33). Both the E-box and Ets motif mutations reduce GATA2 expression (2, 9). The Ets 1017+572C>T transition was detected in at least 6 families and is the most common noncoding GATA2 mutation described to date (9, 34–36). Neither of these mutations is predicted to introduce known transcription factor–binding motifs (37). Since E-box- and Ets motif–mutant patients share phenotypes, +9.5 cis–elements might contribute equivalently to enhancer activity, including transcriptional complex assembly. Alternatively, different +9.5 cis–elements may exert qualitatively or quantitatively distinct functions, with essentially any corruption of +9.5 enhancer activity being pathogenic.

Here, we generated mouse strains to test these models and found differential cis–element contributions to +9.5 enhancer function. Mutation of E-box and Ets motifs, with retention of WGATAR, abrogated HSC emergence and was embryonically lethal, resembling mice lacking the E-box-spacer-WGATAR composite element (2). Though the Ets motif was dispensable for embryogenesis, it enhanced Gata2 upregulation and HSPC regeneration in response to myeloablation. These results revealed the human genetics concept that disease mutation segregates regenerative versus developmental functions of an enhancer. Furthermore, our analyses with the unique in vivo models provided evidence for a paradigm of GATA-2–dependent pathogenesis.

Results

Multiple cis–elements within the Gata2 +9.5 enhancer collectively support HSPC genesis. The +9.5 deletion is embryonically lethal at approximately E14 (1, 2). It was unclear whether individual motifs within the +9.5 are essential or dispensable for enhancer activity. To test models to determine how distinct cis–elements contribute to +9.5 enhancer activity, we generated a mouse strain lacking the E-box and Ets, but retaining WGATAR (+9.5(E-box Ets)−/−) (Figure 2, A–C). We asked whether WGATAR confers any +9.5 activities in diverse cellular and developmental contexts, if it recapitulates the defects of +9.5− embryos, or if unique phenotypes emerge. By E13.5, +9.5(E-box Ets)−/− embryos showed a decrease of approximately 90% in fetal liver cellularity and reduced fetal liver cellularity and size (Figure 2, D and E), resembling +9.5− embryos. We found that homozygous mutants had severe hemorrhages (100% penetrance) and variable edema (Figure 2F) and died by E14.5 (Figure 2F and Table 1). Gata2 mRNA levels in +9.5(E-box Ets)−/− fetal liver from live embryos were 23-fold lower than those in WT littermates (Figure 2G), also resembling +9.5− embryos.
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compensation for the LT-HSC reduction. These results indicate that WGATAR is insufficient to support developmental hematopoiesis without the neighboring E-box and Ets motifs.

The Gata2 +9.5 enhancer Ets motif is dispensable for HSPC genesis. To test models for how the human disease Ets mutation (1017+572C>T) impacts GATA-2 regulation and function, we generated a mouse strain harboring the disease mutation (Figure 4, A and B). Whereas +9.5 –/– and +9.5(E-box Ets) –/– embryos exhibited severe anemia and hemorrhaging at E12.5 and died by E14.5 (Figure 2F and Table 1) (2), +9.5(Ets) –/– mice lacked hemorrhages (Figure 4C) and were born at Mendelian ratios (Table 2). The cellularity of WT and +9.5(Ets)–/– fetal livers was not significantly different (Figure 4D), although Gata2 expression was approximately 50% lower in the mutants (Figure 4E). Importantly, the lack of an Ets requirement for embryogenesis distinguishes the +9.5(Ets) –/– mutant from all other +9.5 mutants described (Figure 2 and Figure 3) (2).

While +9.5+/– embryos are morphologically normal (2), HSC emergence (4), immunophenotypic and long-term–repopulating fetal liver HSCs, and Gata2 expression are approximately 50% lower than in WT mice (1, 2). To determine whether +9.5(Ets) –/– embryos resemble +9.5+/– embryos, we analyzed HSC emergence. CD31+c-Kit+ cell numbers decreased by approximately 50% in +9.5(Ets) –/– embryos (Figure 5, A and B). To assess whether this defect persisted throughout embryogenesis, we quantified immunophenotypic HSCs and MPPs in E15.5 fetal liver (Figure 5, C–E). We found that the percentages of +9.5(Ets) –/– fetal liver HSCs and MPPs were approximately 70% and 50% lower, respectively, than in WT mice. While the percentage of +9.5(Ets) +/– fetal liver HSCs did not differ significantly from that in WT embryos, the percentage of MPPs was approximately 2-fold higher in +9.5(Ets)+/– embryos. Thus, unlike the E-box-spacer-WGATAR HSC genesis in the +9.5–/– aorta-gonad-mesonephros (AGM) is defective, given the lack of HSC-containing clusters and depletion of long-term repopulating HSCs (LT-HSCs) (1). To determine whether the +9.5(E-box Ets)–/– mutation impacts the endothelial-to-hematopoietic transition, we performed 3D confocal analysis of embryos to quantify emerging hematopoietic cells. Endothelial and hematopoietic cells express CD31, and hematopoietic, but not endothelial, cells, express c-Kit (38). While CD31-c-Kit+ clusters were abundant in E10.5 WT AGM, +9.5(E-box Ets)–/– embryos lacked clusters (Figure 3, A and B), indicating an HSC emergence defect.

At E13.5, the +9.5–/– fetal liver is devoid of immunophenotypic and LT-HSCs (Lin CD48 CD41 Scal Kit+ Mac1 CD150+) (2). We found that E13.5 +9.5(E-box Ets)–/– fetal liver was depleted of immunophenotypic HSCs (Figure 3, C and D). The percentage of multipotent progenitors (MPPs) (Lin CD48 CD41 Scal Kit+ Mac1 CD150–) was 5-fold lower than in WT litters (Figure 3E). At this stage, the percentage of LT-HSCs was approximately 40% lower in +9.5(E-box Ets)–/– embryos than in WT embryos. MPP percentages were 2-fold higher in +9.5(E-box Ets)–/– embryos (Figure 3E), which may reflect compensation for the LT-HSC reduction. These results indicate that WGATAR is insufficient to support developmental hematopoiesis without the neighboring E-box and Ets motifs.

The Gata2 +9.5 enhancer Ets motif is dispensable for HSPC genesis. To test models for how the human disease Ets mutation (1017+572C>T) impacts GATA-2 regulation and function, we generated a mouse strain harboring the disease mutation (Figure 4, A and B). Whereas +9.5–/– and +9.5(E-box Ets)–/– embryos exhibited severe anemia and hemorrhaging at E12.5 and died by E14.5 (Figure 2F and Table 1) (2), +9.5(Ets)–/– mice lacked hemorrhages (Figure 4C) and were born at Mendelian ratios (Table 2). The cellularity of WT and +9.5(Ets)–/– fetal livers was not significantly different (Figure 4D), although Gata2 expression was approximately 50% lower in the mutants (Figure 4E). Importantly, the lack of an Ets requirement for embryogenesis distinguishes the +9.5(Ets)–/– mutant from all other +9.5 mutants described (Figure 2 and Figure 3) (2).

Table 1. Genotypes of embryos from +9.5(E-box Ets) heterozygous matings at developmental stages and weaning

<table>
<thead>
<tr>
<th>Genotype</th>
<th>E12.5</th>
<th>E13.5</th>
<th>E14.5</th>
<th>E15.5</th>
<th>Weaning</th>
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<td>12</td>
<td>13 (1)</td>
<td>0 (6)</td>
<td>0 (9)</td>
<td>0</td>
</tr>
</tbody>
</table>

P < 1 × 10–4, by χ² test. Numbers in parentheses indicate the number of dead embryos.
insult might exacerbate such alterations and trigger phenotypic alterations that do not arise with either the predisposition mutation alone or the secondary aberration or insult alone. Alternatively, the predisposition mutation might be insufficient to derail molecular processes in the steady state, and only after a secondary aberration or insult would measurable molecular alterations be manifested.

To distinguish between these mechanisms, we used RNA-Seq to compare transcriptomes of Lin−Sca1+c-Kit+ (LSK) cell populations isolated from WT and +9.5(Ets)−/− mice. After filtering out the outlying genes, the data were processed with DEseq at a FDR of 0.05 and a fold-change minimum of 2. Strikingly, of the 53,342 transcripts quantitated, only 11 were differentially expressed (Figure 6B).

Figure 3. The +9.5 enhancer WGATAR motif is insufficient for HSC genesis and function. (A) Whole-mount immunostaining of E10.5 dorsal aorta (DA). CD31+ cells are shown in magenta and c-Kit+ cells in green. Scale bars: 100 μm. (B) c-Kit+ cell quantification within the dorsal aorta from +9.5(E-box Ets)+/+ (n = 3), +9.5(E-box Ets)+/− (n = 4), and +9.5(E-box Ets)−/− (n = 4) mice. Data are from 2 experiments. (C) Flow cytometric analysis of E13.5 fetal liver HSCs (Lin−CD41−CD48−Mac1−Sca1−Kit+CD150+) and MPPs (Lin−CD41−CD48−Mac1−Sca1−Kit−CD150−). FSC, forward scatter. (D and E) HSC and MPP quantification (percentage of live fetal liver cells from +9.5(E-box Ets)+/+ (n = 12), +9.5(E-box Ets)+/− (n = 19), and +9.5(E-box Ets)−/− (n = 4) mice. Data are from 3 experiments. Quantitative data are represented as box-and-whisker plots, with bounds from the 25th to 75th percentiles, the median line, and whiskers ranging from minimum to maximum values. *P < 0.05, **P < 0.01, and ***P < 0.001, by 2-tailed, unpaired Student’s t test with Benjamini-Hochberg correction.

Human disease GATA2–mutant mouse model: combinatorial impact of a predisposition mutation and myeloablative stress on pathogenesis. Since the Ets motif was dispensable for embryogenesis, we tested whether the Ets motif mutation affected adult hematopoiesis. Peripheral WBC, RBC, and platelet numbers were comparable between +9.5(Ets)−/− and WT mice (Figure 6A).

In the steady state, a predisposition mutation might generate measurable but functionally “silent” molecular alterations. In this scenario, a secondary genetic aberration(s) or environmental insult might exacerbate such alterations and trigger phenotypic alterations that do not arise with either the predisposition mutation alone or the secondary aberration or insult alone. Alternatively, the predisposition mutation might be insufficient to derail molecular processes in the steady state, and only after a secondary aberration or insult would measurable molecular alterations be manifested. To distinguish between these mechanisms, we used RNA-Seq to compare transcriptomes of Lin Sca1−c-Kit+ (LSK) cell populations isolated from WT and +9.5(Ets)−/− mice. After filtering out the outlying genes, the data were processed with DEseq at a FDR of 0.05 and a fold-change minimum of 2. Strikingly, of the 53,342 transcripts quantitated, only 11 were differentially expressed (Figure 6B).
Thus, we determined that the predisposition mutation had little to no impact on the LSK cell transcriptome, consistent with a model in which the predisposition mutation creates a “silent” defect that is manifested only upon a secondary aberration or insult.

The silent nature of the predisposition mutation led us to question what triggers the emergence of pathogenic phenotypes. We reasoned that an increased demand on the hematopoietic system involving the transition of quiescent-to-proliferating HSCs and HSPC regeneration might unveil deleterious phenotypes in mutants harboring the predisposition mutation. To test whether myeloablation-induced stress reveals Ets activity, we used 5-fluorouracil (5-FU) to kill cycling cells and promote HSC proliferation, thereby regenerating the hematopoietic system (39). Two doses of 5-FU (250 mg/kg) were administered with an eleven-day interval for maximal stimulation of HSPC expansion (40). The +9.5(Ets)–/– mice had a reduced median survival of 14 days versus 22 days for WT mice (Figure 6C).

During regeneration, Gata2 transcripts are induced in the BM LSK cell population (40). We performed quantitative reverse transcription PCR (qRT-PCR) of BM and RNA-Seq analysis of LSK cells and found that Gata2 transcript levels did not differ significantly in BM from untreated +9.5(Ets)–/– mice compared with that from WT mice (Figure 6D). In contrast, 9 days after 5-FU treatment, Gata2 expression increased by approximately 15-fold in BM LSK cells, and the remaining 32.8% were unresponsive to 5-FU mutant LSK cells. While 423 of these were also differentially expressed between vehicle-treated WT and 5-FU–treated LSK cells detected 2974 genes (754 upregulated, 2220 downregulated) at a FDR of 0.05 and a minimum fold-change of 2 (Figure 6F). Of these genes, only 423 were differentially expressed between vehicle-treated and 5-FU–treated mutant LSK cells (Figure 6G). Thus, the predisposition mutation altered the responsiveness of 85.8% of the genes that were 5-FU responsive in WT LSK cells. Gene Ontology analysis indicated that genes dysregulated by the Ets motif mutation included those linked to cell-cycle regulation (e.g., G1/M checkpoint) and cellular proliferation (e.g., Myc and E2F targets) (Table 3). The analysis detected 630 differentially expressed genes between vehicle-treated mutant and 5-FU–treated mutant LSK cells. While 423 of these were also differentially expressed between vehicle-treated WT and 5-FU–treated WT LSK cells, the remaining 32.8% were unresponsive to 5-FU treatment in the WT cells. The predisposition mutation corrupted the LSK transcriptome in the context of hematopoietic injury followed by regeneration, but not in the steady state. These results provide strong evidence that the Ets motif mutation generates a disease predisposition.

To establish how defective Gata2 induction impacts BM, we analyzed histological sections 9 and 11 days after 5-FU treatment (Figure 7A). No gross differences between untreated samples were apparent, consistent with the peripheral blood counts. While both WT and +9.5(Ets)–/– mice were hypocellular 9 days after treatment, we detected regeneration 11 days after treatment in WT sections (Figure 7, A and B). In contrast, there was still considerable hypocellularity 11 days after treatment of +9.5(Ets)–/– animals, confirming the delayed regeneration in the mutants.

**Table 2. Genotypes of embryos from +9.5(Ets) heterozygous matings at weaning**

<table>
<thead>
<tr>
<th>Weaning</th>
<th>Number</th>
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</tr>
<tr>
<td>+/−</td>
<td>126</td>
</tr>
<tr>
<td>−/−</td>
<td>55</td>
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</table>

P = 0.23, by χ² test.
To analyze +9.5(Ets)–/– BM cellularity, we quantified immunophenotypic HSPCs in untreated and treated (9 and 11 days after 5-FU) mice. In the untreated mice, we observed that +9.5(Ets)–/– and WT HSCs (Lin–Sca1+ Kit+ CD48– CD150+, common myeloid progenitors (CMPs) (Lin– Sca1– Kit+ FcγR– CD34+), granulocyte macrophage progenitors (GMPs) (Lin– Sca1– Kit+ FcγR+ CD34+), and megakaryocyte-erythrocyte progenitors (MEPs) (Lin– Sca1 Kit+ FcγR– CD34–) were unaffected (Figure 8, A and B). MPP (Lin– Sca1+ Kit+ CD48– CD150–) percentages were 9-fold lower in mutants (Figure 8, A and B). Nine days after 5-FU treatment, HSC percentages were 12-fold higher in WT animals and were maintained at 11 days after treatment (Figure 8, A and B). The disease mutation abrogated the HSC increase. While all immunophenotypic progenitor cell populations were severely depleted by 5-FU after 9 days, 11 days after 5-FU treatment, WT progenitor percentages were 3.6- to 5.9-fold higher than those in untreated animals (Figure 8, A and B). In contrast, mutant progenitor percentages were restored only to the steady-state level. Thus, Ets confers Gata2 expression as a vital step in HSPC regeneration. To assess HSC function, BM from untreated animals was competitively transplanted into lethally irradiated mice. Sixteen weeks after transplantation, we observed that the +9.5(Ets)–/– BM multi-lineage repopulating activity was 3.0-fold lower (P < 0.0001) than that of WT BM (71 ± 4.6% and 23 ± 6.0% for mutant vs. WT BM, respectively) (Figure 9A). Analysis of donor-derived hematopoietic precursors in BM reconstituted with +9.5(Ets)–/– cells revealed significant reductions in LSK, HSC, LS–K, CMP, GMP, and MEP percentages relative to those in WT donor BM (Figure 9B).

Regeneration after hematopoietic injury, such as that caused by 5-FU–induced myeloablation, involves increased expression of the HSC-regulatory genes Scl, Gata2, and Ets variant 2 (Etv2) (40), each of which contains binding motif(s) within the +9.5 enhancer. ETV2 occupies the +9.5 enhancer in mouse embryonic stem cell-derived embryoid bodies (Figure 10A, irreplicable discovery rate [IDR] <0.01), and the +9.5 Ets motif mutant phenocopied the conditional deletion of Etv2 throughout the hematopoietic system (40, 41). While ETV2 deficiency does not affect progenitors or blood cells in the steady state, vascular (42) or hematopoietic (40) injury induces ETV2, which then functions to promote recovery. Given the hundreds of ETV2 chromatin occupancy sites detected by ChIP-Seq, it is striking that the single-nucleotide mutation in the +9.5 enhancer disrupts hematopoietic regeneration in a manner analogous to the conditional deletion of ETV2 throughout the hematopoietic system. To test...
whether there is a hierarchical relationship between ETV2 and Gata2 in the hematopoietic injury response, we quantified Gata2 expression in regenerating HSPCs from WT and Tie2-Cre Etv2 fl/fl conditional–knockout mice after 5-FU injury. We found that Gata2 expression was 1.5-fold lower ($P = 0.001$) in Etv2fl/fl LSK cells than in WT cells after 5-FU treatment (Figure 10B), suggesting that ETV2 functions upstream of GATA-2 in the injury response.

Discussion
Recurring GATA2 mutations in human pathologies (11, 14, 43) highlight vital GATA-2 functions (18, 32, 44), some of which were predictable on the basis of prior studies of Gata2-mutant mouse models. As decreased (2, 6, 9) or elevated GATA-2 (45, 46) levels disrupt hematopoeisis, establishing and maintaining GATA-2 expression within a physiological window is crucial. Though this control is accomplished, in part, via +9.5 and –77 enhancer–dependent transcriptional induction of GATA-2 expression, many questions remain unanswered regarding the underlying mechanisms, including the relative contributions (qualitative and quantitative) of the individual cis-elements constituting the enhancers to enhancer function in diverse developmental and adult contexts, as well as the constitution of regulatory factors conferring or suppressing enhancer activity.

Mechanistic and pathological insights have emerged from analyses of Gata2 mouse models, including Gata2 enhancer
mutants with impaired HSPC levels and functions (1, 2, 4, 5, 21, 23, 24). Here we dissected models governing +9.5 enhancer function and discovered that the WGATAR motif, without E-box and Ets motifs, was insufficient for embryogenesis and developmental hematopoiesis; WGATAR required additional cis-elements to confer the critical +9.5 enhancer activity and the control of developmental hematopoiesis (Figure 11). While the Ets motif was dispensable in embryos and adults in the steady state, the Ets motif disease mutation abrogated myeloablation-dependent GATA-2 induction and hematopoietic regeneration in BM. Myeloablation increased GATA-2 expression and HSC numbers in the BM of WT, but not +9.5(Ets)−/−, mice. Combined with our comparison of WT and +9.5(Ets)−/− LSK cell transcriptomes, these results establish a pathogenesis paradigm, in which the Ets motif disease mutation is, in effect, “silent” and singularly does not corrupt the LSK transcriptome or steady-state hematopoiesis. Because the mutation altered the capacity of 5-FU to induce transcriptomic changes, the mutation predisposed the cells to a secondary insult that instigated hematopoietic failure.

It is instructive to consider what qualitative insults and quantitative changes initiate and/or accelerate pathogenesis. While we used mild or severe myeloablation, does a damage threshold constitute a binary switch to trigger pathogenesis, or does modest stress induce phenotypes that are reversible or compensated

Table 3. Gene Ontology (Database for Annotation, Visualization, and Integrated Discovery [DAVID], NIH) of genes dysregulated by Ets motif mutation

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</tr>
<tr>
<td>E2F targets</td>
<td>0.0053</td>
</tr>
<tr>
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</tr>
<tr>
<td>G0/G1/M checkpoint</td>
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</tr>
<tr>
<td>mTORC1 signaling</td>
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</tr>
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</table>

P values were determined by Fisher’s exact test.
Figure 8. +9.5 enhancer Ets motif–dependent HSPC regeneration. BM was harvested 9 or 11 days after vehicle (PBS) or 5-FU (250 mg/kg) treatment. (A) Flow cytometric analysis of LSK cells (Lin−CD48−Sca1−Kit+), HSCs (Lin−CD48−Sca1−Kit−CD150+), MPPs (Lin−CD48−Sca1−Kit−CD150+), LS–K cells (Lin−Sca1−Kit+), MEPs (Lin−Sca1−Kit−FcγR+CD34+), CMPs (Lin−Sca1−Kit−FcγR+CD34+), and GMPs (Lin−Sca1−Kit−FcγR+CD34+). (B) LSK, HSC, MPP, LS–K, MEP, CMP, and GMP quantification (percentage of live BM cells). n = 8–10 per genotype and treatment; data are from 5 experiments. Quantitative data are represented as box-and-whisker plots, with bounds from the 25th to 75th percentiles, the median line, and whiskers ranging from minimum to maximum values. *P < 0.05, **P < 0.01, and ***P < 0.001, by Tukey’s multiple comparisons test.
physiological and pathological mechanisms. Studies illustrate the power of microdissecting enhancers to unveil regulatory mechanisms, leading to hematopoietic failure, a feature of GATA-2-deficiency syndrome in humans. Furthermore, these cis-elements predispose mutations, which require multiple cis-elements. Dissecting this mechanism in the unique mouse model described here established a paradigm in which the cis-element predisposition mutation. This mechanism in the unique mouse model described here established a paradigm in which the cis-element predisposition mutation.

We provide evidence for a new mechanism in human genetics, in which a disease mutation of a cis-element inactivates the regenerative activity of an enhancer, while sparing its developmental activities, which require multiple cis-elements. Dissecting this mechanism in the unique mouse model described here established a paradigm in which the cis-element predisposition mutation, combined with a secondary insult, corrupts hematopoietic-regulatory mechanisms, leading to hematopoietic failure, a feature of GATA-2-deficiency syndrome in humans. Furthermore, these studies illustrate the power of microdissecting enhancers to unveil physiological and pathological mechanisms.

Methods

Statistics. Results are presented as either the mean ± SEM or as box-and-whisker plots, with bounds from the 25th to the 75th percentiles, the median line, and whiskers ranging from minimum to maximum values. Multiple independent cohorts were used in each experiment. Statistical comparisons were performed using 2-tailed Students t tests (significance cutoff of \( P < 0.05 \)) with correction of statistical overrepresentation of functions calculated using the Benjamini-Hochberg multiple tests correction procedure or Tukey’s multiple comparisons test. A log-rank test was performed on the Kaplan-Meier survival curve. GraphPad Prism (GraphPad Software) was used to perform the statistical analyses.

Generation of mutant mice. Pronuclear injection was conducted on a C57BL/6J background at the 1-cell zygote stage at the UW-Madison Biotechnology Center using recombinant Cas9, guide RNA (gRNA) (5′-TCGTGTATCTGTTCCGAGCG-3′), and repair oligonucleotide (5′-TTTCAAAACAGCCCGAGAAGGCGAGG-3′). We provide evidence for a new mechanism in human genetics, in which a disease mutation of a cis-element inactivates the regenerative activity of an enhancer, while sparing its developmental activities, which require multiple cis-elements. Dissecting this mechanism in the unique mouse model described here established a paradigm in which the cis-element predisposition mutation, combined with a secondary insult, corrupts hematopoietic-regulatory mechanisms, leading to hematopoietic failure, a feature of GATA-2-deficiency syndrome in humans. Furthermore, these studies illustrate the power of microdissecting enhancers to unveil physiological and pathological mechanisms.

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Generation of mutant mice. Pronuclear injection was conducted on a C57BL/6J background at the 1-cell zygote stage at the UW-Madison Biotechnology Center using recombinant Cas9, guide RNA (gRNA) (5′-TCGTGTATCTGTTCCGAGCG-3′), and repair oligonucleotide (5′-TTTCAAAACAGCCCGAGAAGGCGAGG-3′). We provide evidence for a new mechanism in human genetics, in which a disease mutation of a cis-element inactivates the regenerative activity of an enhancer, while sparing its developmental activities, which require multiple cis-elements. Dissecting this mechanism in the unique mouse model described here established a paradigm in which the cis-element predisposition mutation, combined with a secondary insult, corrupts hematopoietic-regulatory mechanisms, leading to hematopoietic failure, a feature of GATA-2-deficiency syndrome in humans. Furthermore, these studies illustrate the power of microdissecting enhancers to unveil physiological and pathological mechanisms.

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Histological analysis. Femurs were fixed with 10% neutral-buffered formalin (Dot Scientific, DSF10800-500) and embedded in paraffin using standard procedures. Sections (10-μm) were stained with H&E. The hematopoietic content of serial images along the entire length of the femurs was quantified.

Flow cytometric analysis. Fetal liver or BM was dissociated and resuspended in IMDM with 2% FBS and passed through 25-μm cell strainers to obtain single-cell suspensions prior to antibody staining. All antibodies were purchased from ebioscience (Thermo Fisher Scientific) unless otherwise stated. Lineage markers for the LSK cell populations were stained with the FITC-conjugated antibodies B220 (11-0452), CD3 (11-0031), CD4 (11-0041), CD5 (11-0051), CD8 (11-0081), CD41 (11-0411) (for fetal liver only), CD48 (11-0481), Gr-1 (11-5931), and TER-119 (11-5921). Other surface proteins were detected with PE-conjugated CD150 (BioLegend, 115904; PE-Cy7–conjugated Mac1, 25-0112 [for fetal liver only]); PerCP-Cy5.5–conjugated Sca1, and allophycocyanin-conjugated (APC-conjugated) c-Kit (2B8, 17-1171) antibodies. Analysis of myeloid progenitors was conducted as described in Johnson et al. (5). Lineage markers were stained with FITC-conjugated B220, CD3, CD4, CD5, CD8, CD19 (11-0193), IgM (11-5890), Il7Ra (11-1271), AA4.1 (11-5892), and TER-119 antibodies. Other surface proteins were detected with PE-conjugated FcγR (12-0161), eFluor 660–conjugated CD34 (50-0341), PerCP-Cy5.5–conjugated Sca1 (45-5981), and PE-Cy7–conjugated c-Kit (BioLegend, 105814). After staining, cells were washed with PBS and resuspended in IMDM, 2% FBS, and DAPI and analyzed on a LSR II Flow Cytometer (BD Biosciences).

RNA-Seq. BM from 5-FU– or PBS-treated animals (10 days after treatment) was dissociated, resuspended in IMDM with 10% FBS and passed through 25-μm cell strainers to obtain single-cell suspensions prior to antibody staining. LSK cells (B220–CD3–CD4–CD5–Sca1+ c-Kit+) were collected on a FACSAria II cell sorter (BD Biosciences). RNA was purified using an RNAeasy Micro Kit (QIAGEN, 74004). RNA libraries were prepared for sequenc-
ing using a standard NuGEN Ovation protocol. Library sequencing was conducted at the UW-Madison Biotechnology Center using an Illumina HiSeq 2500 (1 x 100). Data processing was conducted as described previously (48), and data were deposited in the NCBI’s Gene Expression Omnibus ( GEO) database ( GEO GSE123080).

**BM transplantation.** Adult C57BL/6 recipient mice (CD45.1+, 6 to 8 weeks old; stock no. 002014, The Jackson Laboratory) were lethally irradiated using an XRAD 320 irradiator for a single dose of 8.5 Gy. BM cells were harvested from individual 8-week-old animals (CD45.2+). A total of 10⁶ BM cells were mixed with the same number of CD45.1+ BM cells and injected into individual irradiated CD45.1+ recipients. The transplanted recipient mice were maintained on a Irradiated Uni-

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**Author contributions**

AAS and EHB conceived and designed the research. AAS, CM, JW, MC, and KDJ conducted experiments. YZ and SK analyzed RNA-Seq data. IH analyzed histology. YZ and JZ facilitated the transplants. KC generated ETV2 ChiP-Seq data. PL analyzed the ChiP-Seq data. AAS and EHB wrote the manuscript. AAS, EHB, CM, KDJ, SK, KC, and JZ edited the manuscript.