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Single-Nucleotide Human Disease Mutation Inactivates a Blood-Regenerative GATA2 Enhancer

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Abstract

The development and function of stem and progenitor cells that produce blood cells are vital in physiology. GATA2 mutations cause GATA-2-deficiency syndrome involving immunodeficiency, myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). 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 embryonic 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 establishment of complex genetic networks. The transcription factor GATA-2 establishes and maintains genetic networks governing hematopoietic stem cell (HSC) 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 include monocytopenia and mycobacterial infection syndrome (2, 8, 9), dendritic cell, monocyte, B, and natural killer 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 with a somatic GATA2 mutation had similar phenotypes (13). Though mechanisms underlying GATA-2 pathologies and their unpredictable onset are unresolved, a GATA-2 growth-promoting circuit in AML (14) with clinical relevance (15) has been described. The variable onset and multicomponent phenotypes characterizing patients with GATA-2 deficiency syndrome (16-19) suggest that additional genetic and/or environment factors contribute to disease progression. Consistent with this inference, symptomatic patients often harbor additional mutations, e.g. in ASXL1 (20), or cytogenetic abnormalities, such as monosomy 7 (18).

Since murine Gata2 ablation is lethal at ~E10.5 (6), conditional (21, 22), heterozygous (23, 24), and enhancer-mutant mice (2, 4, 25-27) 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, 28). Whether these enhancers function and are essential in regenerative contexts is unknown. The +9.5 triggers HSC emergence from hemogenic endothelium and confers
vascular integrity (1, 2) (Figure 1). While Gata2 mutant mice have not previously revealed malignancies, GATA-2 mediates leukemogenesis in a Tet2 ablation and Flt3(ITD)-overexpression AML mouse model (29). 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 of transcription factors/cofactors governing the generation of HSPCs (30), including SCL/TAL1 (E-box: CANNTG), GATA-2 (WGATAR), and the Ets factors ERG and 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 (31, 32), and human patients with heterozygous disruptions of the +9.5 E-box or Ets motifs exhibit decreased GATA2 expression (2, 33). Deletion of the E-box-spacer-WGATAR (+9.5-/-) composite element reduced chromatin accessibility and abrogated occupancy of SCL/TAL1 and its cofactor LDB1 in fetal liver cells (34), revealing a +9.5 enhancer-dependent chromatin accessibility and regulatory complex assembly at the Gata2 locus.

GATA-2-deficiency syndrome patients with enhancer mutations can lack the E-box or harbor single-nucleotide Ets mutations (2, 33, 35) that disrupt the GGAW motif, which is essential for high-affinity DNA binding (36). Both the E-box and Ets motif mutations reduce GATA2 expression (2, 33). The Ets 1017+572C>T transition was detected in at least 6 families and is the most common noncoding GATA2 mutation described (33, 37-39). Neither of these mutations are predicted to introduce known transcription factor binding motifs (40). 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 derived mouse strains to test these models and demonstrated 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 embryonic 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 a new concept in human genetics - a 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 embryonic lethal at ~E14 (1, 2). It was unclear whether individual motifs within the +9.5 are essential or dispensable for enhancer activity. To test models for 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 2A-C]. We asked whether WGATAR confers any +9.5 activities in diverse cellular and development contexts, if it recapitulates defects of +9.5-/- embryos, or if unique phenotypes emerge. By E13.5, +9.5(E-box;Ets)-/- embryos exhibited a ~90% decrease in fetal liver cellularity and reduced fetal liver cellularity and size (Fig D, E), resembling +9.5-/- embryos. Homozygous mutants had severe hemorrhages (100% penetrance), 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 was 23-fold lower than wild type littermates (Figure 2G), also resembling +9.5-/- embryos.

HSC genesis in the +9.5-/- aorta-gonad-mesonephros (AGM) is defective, based on lack of HSC-containing clusters and depletion of long-term repopulating HSCs (25). To determine if the +9.5(E-box;Ets)-/- mutation impacts the endothelial to hematopoietic transition, 3D confocal analysis of embryos was conducted to quantify emerging hematopoietic cells. Endothelial and hematopoietic cells express CD31, and hematopoietic, but not endothelial, cells, express c-Kit (41). While CD31+c-Kit+ clusters were abundant in E10.5 wild type AGM, +9.5(E-box;Ets)-/- embryos lacked clusters (Figure 3A, B), indicating an HSC emergence defect.
At E13.5, the +9.5+/− fetal liver is devoid of immunophenotypic and long-term repopulating HSCs (Lin−CD48−CD41−Sca1+Kit+Mac1+CD150+) (2). E13.5 +9.5(E-box;Ets)−/− fetal liver was depleted of immunophenotypic HSCs (Figure 3C, D). Multipotent progenitors (MPP; Lin−CD48−CD41−Sca1+Kit+Mac1+CD150−) were 5-fold lower than wild type littermates (Figure 2E). At this stage, LT-HSC levels are ~40% lower in +9.5(E-box;Ets)+/− embryos in comparison with wild type embryos. MPPs are 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.

*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 4A, B). Whereas +9.5−/− and +9.5(E-box;Ets)−/− embryos exhibited severe anemia, 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 wild type and +9.5(Ets)−/− fetal livers was not significantly different (Figure 3D), although *Gata2* expression was ~50% lower in 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.

While +9.5+/− embryos are morphologically normal (2), HSC emergence (4), immunophenotypic and long-term repopulating fetal liver HSCs and *Gata2* expression are ~50% lower than wild type (2, 25). To determine if +9.5(Ets)−/− embryos resemble +9.5+/− embryos, HSC emergence was analyzed. CD31+c-Kit+ cells decreased ~50% in
+9.5(Ets)$^{-/-}$ embryos (Figure 5A, B). To assess whether this defect persisted throughout embryogenesis, we quantified immunophenotypic HSCs and MPPs in E15.5 fetal liver (Figure 5C-E). +9.5(Ets)$^{-/-}$ fetal liver HSCs and MPPs were ~70 and 50% less, respectively, vs. wild type. While +9.5(Ets)$^{+/}$ fetal liver HSC levels did not differ significantly from wild type embryos, MPPs were ~2 fold higher in +9.5(Ets)$^{+/}$ embryos. Thus, unlike the E-box-spacer-WGATAR composite element, which is was essential for developmental hematopoiesis and embryogenesis, the Ets motif was dispensable, yet it contributed to select +9.5 functions.

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 impacted adult hematopoiesis. Peripheral white blood cells, red blood cells, and platelets were comparable between +9.5(Ets)$^{-/-}$ and wild type 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/insult alone. Alternatively, the predisposition mutation might be insufficient to derail molecular processes in the steady-state, and only after a secondary aberration/insult would measurable molecular alterations be manifested. To distinguish between these mechanisms, we used RNA-seq to compare transcriptomes of LSK (Lin$^{-}$,Sca1$^+$,c-Kit$^+$) population cells isolated from wild type and +9.5(Ets)$^{-/-}$ mice. After filtering out outlying genes, the data was processed with DEseq at a false discovery rate 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, 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/insult.

The silent nature of the predisposition mutation raised the question as to 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 if myeloablation-induced stress reveals Ets activity, 5-FU was used to kill cycling cells and promote HSC proliferation, thus regenerating the hematopoietic system (42). Two 5-FU doses (250 mg/kg) were administered with an eleven-day interval to maximally stimulate HSPC expansion (43). +9.5(Ets)−/− mice had a reduced median survival of 14 vs. 22 days for wild type mice (Figure 6C).

During regeneration, Gata2 transcripts are induced in the bone marrow LSK cell population (43). Gata2 transcript levels did not differ significantly between bone marrow from untreated +9.5(Ets)−/− and wild type mice (Figure 3D: qRT-PCR with bone marrow and RNA-seq analysis with LSK cells). In contrast, nine days post 5-FU treatment, Gata2 expression increased ~15 fold in bone marrow of wild type, but not +9.5(Ets)−/−, mice (Figure 6D). The Ets motif was therefore essential for myeloablation-induced Gata2 expression and function. In principle, the Ets motif mutant phenotypes might reflect hypersensitivity to 5-FU-mediated cell death and/or defective regeneration. Using a sublethal 5-FU dose (150 mg/kg), regeneration was delayed in +9.5(Ets)−/− mice (Figure 6E).
Since the predisposition mutation had little to no impact on the LSK transcriptome in the steady-state, we used RNA-seq to globally analyze whether the mutation impacts 5-FU-induced gene expression changes. Differential expression analysis between vehicle-treated wild type and 5-FU-treated LSK cells detected 2974 genes (754 upregulated; 2220 downregulated) at an 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 wild type LSK cells. Gene Ontology analysis indicated that genes dysregulated by the Ets motif mutation included those linked to cell cycle regulation (e.g., G2M 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 wild type and 5-FU-treated wild type LSK cells, the remaining 32.8% were unresponsive to 5-FU treatment in the wild type 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 bone marrow, we analyzed histological sections 9- and 11-days post 5-FU treatment (Figure 7A). No gross differences between untreated samples were apparent, consistent with peripheral blood counts. While both wild type and +9.5(Ets)−/− were hypocellular 9 days post-treatment, regeneration was detected 11 days post-treatment in wild type sections (Figure 7A, B).
In contrast, there was still considerable hypocellularity 11 days post-treatment in +9.5(Ets)^/- animals, confirming the delayed regeneration in the mutants.

To analyze +9.5(Ets)^/- bone marrow cellularity, we quantified immunophenotypic HSPCs in untreated and treated (9 and 11 days post-5-FU) mice. In untreated mice, +9.5(Ets)^/- and wild type HSC (Lin^-Sca1^+Kit^+CD48^-CD150^+, Common Myeloid Progenitors (CMP) (Lin^-Sca1^+Kit^+Fc_R^+CD34^+), Granulocyte Macrophage Progenitors (GMP) (Lin^-Sca1^+Kit^+Fc_R^-CD34^+), and Megakaryocyte-Erythrocyte Progenitors (MEP) (Lin^-Sca1^+Kit^+Fc_R^-CD34^-) were unaffected (Figure 8A, B). MPPs (Lin^-Sca1^+Kit^+CD48^-CD150^-) were 9-fold lower in mutants (Figs. 8A, B). Nine days post-5-FU, HSCs were 12-fold higher in wild type animals, which was maintained at 11 days post-treatment (Figure 8A, B). The disease mutation abrogated the HSC increase. While all immunophenotypic progenitor populations were severely depleted by 5-FU after 9 days, 11 days post-5-FU treatment wild type progenitor levels were 3.6- to 5.9-fold higher than in untreated animals (Figure 8A, B). In contrast, mutant progenitor levels were restored only to the steady-state level. Thus, Ets confers Gata2 expression as a vital step in HSPC regeneration. To assess HSC function, bone marrow from untreated animals was competitively transplanted into lethally-irradiated mice. Sixteen weeks post-transplant, +9.5(Ets)^/- bone marrow multi-lineage repopulating activity was 3.0-fold lower (P < 0.0001) than wild type bone marrow (71 +/- 4.6 and 23 +/- 6.0 for mutant vs. wild type, respectively) (Figure 9A). Analysis of donor-derived hematopoietic precursors in bone marrow reconstituted with +9.5(Ets)^/- cells revealed significant reductions in LSK, HSC, LS-K, CMP, GMP and MEP levels relative to wild type donors (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 Etv2 (43), each of which contains binding motif(s) within the +9.5 enhancer. ETV2 occupies the +9.5 enhancer in mouse ES cell-derived embryoid bodies (Figure 10A, IDR < 0.01), and the +9.5 Ets motif mutant phenocopied the conditional deletion of Etv2 encoding a hematopoietic/vascular-regulatory ETS factor (43, 44). While ETV2 deficiency does not affect progenitors or blood cells in the steady-state, vascular (45) or hematopoietic (43) 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 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, Gata2 expression was quantified in regenerating HSPCs from wild type and Tie2-Cre;Etv2fl/fl conditional knock out mice post-5-FU injury. Gata2 expression was 1.5-fold lower ($P = 0.001$) in Etv2fl/fl LSK cells in comparison with wild type cells post-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, 46) highlight vital GATA-2 functions (18, 35, 47), some of which were predictable based on prior studies of Gata2 mutant mouse models. As decreased (2, 6, 33) or elevated GATA-2 (48, 49) levels disrupt hematopoiesis, establishing and maintaining GATA-2 expression within 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 (2, 4, 21, 23-26). 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 myeloblation-dependent GATA-2 induction and hematopoietic regeneration in bone marrow. Myeloablation increased GATA-2 expression and HSCs in bone marrow of wild type, but not +9.5(Ets)−/− mice. Combined with our comparison of wild type 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 and steady-state hematopoiesis. As the mutation altered the capacity of 5-FU to induce transcriptomic changes, the mutation predisposes to a secondary insult that instigates hematopoietic failure.

It is instructive to consider what qualitative insults and quantitative changes initiate and/or accelerate pathogenesis. While we utilized 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 for? Our model will enable this problem to be rigorously dissected – in the contexts of environmental insults and genetic aberrations. As mimicking viral infection with the type I interferon inducer polyinosinic:polycytidylic acid (pI:pC) in Fanca<sup>−/−</sup> mice resulted in HSC functional defects (50), high levels of DNA damage and bone marrow failure, there may be mechanistic parallels between this system and the defective hematopoietic regeneration caused by the Gata2 Ets motif 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 herein 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 micro-dissecting enhancers to unveil physiological and pathological mechanisms.

**Methods**
**Statistical analysis**

Results are displayed as either mean +/- SEM or as box-and-whisker plots, with bounds from 25th to 75th percentile, median line, and whiskers ranging from minimum to maximum values. Multiple independent cohorts were used in each experiment. Statistical comparisons were performed using two-tailed Students t-tests (significance cutoff of p < 0.05) (GraphPad Prism) with correction of statistical over-representation of functions calculated using Benjamini-Hochberg multiple-test correction procedure or using Tukey’s multiple comparisons test (GraphPad Prism). A log-rank test was performed on the Kaplan-Meier survival curve (GraphPad Prism).

**Mutant mice generation**

Pronuclear injection was conducted in the C57BL/6J background at the zygote (1-cell) stage at the UW-Madison Biotechnology Center using recombinant Cas9, gRNA (5’-TCGTGTATCTGTTCGGACCGG), and repair oligo (5’-TTTCAACAGCCAGGACAGGCAAGGCAGGACTGAGTGGCTGCTGAAAACTTTG CCGGTCCCAGAACAGTACACAGCAGGCTTCTTTCTATTCTACCGGTCTGGTCGTGATCGAGATGTCCGG ATAGGAAACTCCGGC). The C>T transition, without additional mutations, was detected in 4 of 25 live pups by DNA sequencing at and surrounding the mutation site. One additional founder contained the Ets point mutation and a 28 bp deletion corrupting the E-box motif. Further genotyping was performed using a TaqMan Assay (ABI, 6253771_1), or primers *Gata2* -78F CACACAGCGGCACCAAA and *Gata2* +120R AAAGCGGGTGAAACGATTTAAAAC.

**Mouse embryo isolation**
Embryos were obtained from timed matings between heterozygous males and females, with the day of vaginal plug detection considered as day 0.5. Pregnant females were euthanized with CO₂, and fresh embryos were transferred into ice-cold PBS for dissection.

**Quantitative real-time RT-PCR**

Total RNA was purified with TRIzol (Invitrogen). DNase (Invitrogen) treatment was performed on 0.1-1 μg RNA at 25°C for 15 min, followed by addition of 2.5 mM EDTA at 65°C for 10 min. cDNA was prepared by annealing with 250 ng of a 1:5 mixture of random hexamer and oligo (dT) primers incubated with m-MLV Reverse Transcriptase (Invitrogen) with 10 mM DTT, RNasin (Promega), and 0.5 mM dNTPs at 42°C for 1 h, and heat inactivated at 95°C for 5 min. cDNA was analyzed in reactions (20 μl) containing 2 μl of cDNA, primers, and 10 μl of Power SYBR green (Applied Biosystems) by real-time RT-PCR with a Viia7 real-time RT-PCR cycler (Applied Biosystems). Standard curves of serial 1:5 dilutions of cDNAs were prepared from control cDNA with the highest predicted gene expression. Values were normalized to the standard curve and 18S control.

**Whole-embryo confocal microscopy**

E10.5 embryos were fixed, stained and analyzed as described (25, 41). Embryos were stained with Biotinylated anti-PECAM-1 (BD Biosciences, 553371) and anti-c-Kit (BD Biosciences, 553352) antibodies. Samples were mounted in a 1:2 mix of benzyl alcohol (Sigma-Aldrich, 402834) and benzyl benzoate (Acros Organics, 105862500) (BABB) to increase tissue transparency and visualized with a Nikon A1RS Confocal
Microscope. Three-dimensional reconstructions were generated from Z-stacks (80–200 optical sections) using Fiji software.

5-fluorouracil (5-FU) myeloablation model

Myeloablation was induced by 5-FU (Sigma, F6627) administered intraperitoneally with a single dose (150 mg/kg) or two doses (250 mg/kg) at 0 and 11 days. Blood samples were collected by retro-orbital bleeding (~10 μl per collection), and hematologic parameters quantified on a HemaVet complete blood count (CBC) instrument.

Bone marrow isolation

Bone marrow (BM) from femurs and tibias of 8-14 week-old mice was collected by removing epiphyses and flushing the BM with a 25-gauge needle and syringe containing IMDM + 2% FBS.

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 hematoxylin-eosin. The hematopoietic content of serial images along the entire length of femurs were quantified.

Flow analysis

Fetal liver or bone marrow 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/ThermoFisher unless stated.
Lineage markers for the LSK populations were stained with 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 (45-5981); and allophycocyanin conjugated (APC-conjugated) c-Kit (2B8, 17-1171) antibodies. Analysis of myeloid progenitors was conducted as described in Johnson et al., 2015 (26). 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 + DAPI and analyzed on a LSR II flow cytometer (BD Biosciences).

**RNA-sequencing**

Bone marrow from 5-FU- or PBS-treated animals (10 days post-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−,CD8−,Sca1+,c-Kit+; 3,000-10,000 per replicate) were collected on a FACSaria II cell sorter (BD Bioscience). RNA was purified using RNAeasy Micro Kit (Qiagen, Cat. 74004). RNA libraries were prepared for sequencing using a standard NuGEN Ovation protocol. Library sequencing was conducted by the UW-Madison Biotechnology Center using an Illumina Hiseq2500 (1 x 100). Data processing was
conducted as described (51), and has been deposited in the Gene Expression Omnibus database (GSE123080).

**Bone marrow transplantation**

Adult C57BL/6 recipient mice (CD45.1+, 6 to 8 weeks old; Stock # 002014, Jackson Labs) were lethally irradiated using an XRAD 320 irradiator for a single dose of 8.5 Gy. Bone marrow cells were harvested from individual 8-week old animals (CD45.2+). A total of $10^6$ bone marrow cells were mixed with the same number of CD45.1+ bone marrow cells and injected into individual irradiated CD45.1+ recipients. The transplanted recipient mice were maintained on Irradiated Uniprim Diet (Envigo; Cat# TD.06596) for two weeks. Blood obtained from the retro-orbital venous sinus or bone marrow was isolated after transplantation and analyzed using flow cytometry for donor-derived hematopoiesis.

**Study approval**

All animal protocols were approved by the UW-Madison IACUC in accord with AAALAC International (Association for Assessment and Accreditation of Laboratory Animal Care) regulations.
Author Contributions

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References


Figure 1

A. Mouse

+9.5

+9.5 (E-box; Ets)

+9.5 (Ets)

B. Human

c.1017+512del28

c.1017+572C>T

c.1017+512del28 T

c.1017+572C>T

Mouse

Human

GGATCTCCTGCCGGAGTTTCCTATCCGGACATCTGCAGCCGGTAGATAAGGAAACTTCGTGTATCTGTTTCCGGA

GGATCTCCTGCCGGAGTTTCCTATCCGGACATCTGCAGCCGGTAGATAAGGAAACTTCGTGTATCTGTTTCTGGA

+9.5 (E-box; Ets)-/-

+9.5 (Ets)-/-

+9.5 (Ets)+/

+9.5 +/-

+9.5 +/-

+9.5 (+)

+9.5 (Ets)+/

+9.5 (+)
Figure 1. +9.5 enhancer WGATAR motif is insufficient for HSC genesis and function. (A) Mouse +9.5 mutations. Deletion of the E-box spacer GATA composite elements abrogates +9.5 function (2). The ? denotes uncertainty regarding whether the mutation impacts protein occupancy at adjacent sites. (B) Human +9.5 mutations found in GATA-2 deficiency syndrome patients (2, 52).
**Figure 2**

A. +9.5 GATAAGAAGGAACCTCAGTTATTTATCTGTTTCTCCTGGCA
+9.5(E-box;Ets)^+GG- --------------------------------- ATCTGACGCCTAGATAAAGGAACCTCAGTTATTTATCTGTTTCTCCTGGCA

B. GGATCTGCAGGCCGGTAGATAAGGAAACTTCGTGTATCTGTTTCTGGA

E-box

GATA

Ets

C. +/+ +/− −/−

Cells/Liver (x10^7)

D. +/+ +/− −/−

Total Fetal Liver

Gata2 mRNA levels (Relative Units)

E. +/+ +/− −/−

F. +/+ +/− −/−
Figure 2. +9.5 enhancer WGATAR motif is insufficient for embryogenesis. (A) Sequence of wild type (+9.5+/-+) and E-box;Ets mutant [+9.5(E-box;Ets)-/-] mice. (B) Sequence of mutant mice. (C) PCR genotyping. (D) Quantification of E13.5 liver cellularity [+9.5(E-box;Ets+/-+) (n = 11), +9.5(E-box;Ets)+/- (n = 30), and +9.5(E-box;Ets)-/- (n = 6) from 5 experiments]. (E) E13.5 fetal livers from littermates. (F) E13.5 littermates illustrating anemia, hemorrhage, and edema. (G) Gata2 mRNA quantification from fetal liver [+9.5(E-box;Ets)+/- (n = 5), +9.5(E-box;Ets)+/- (n = 9), and +9.5(E-box;Ets)-/- (n = 4) from 3 experiments]. Quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, median line, and whiskers ranging from minimum to maximum values; *P < 0.05 (two-tailed unpaired Student’s t test with Benjamini-Hochberg correction).
Figure 3

A

+9.5

+9.5(E-box;Ets)+/

+9.5(E-box;Ets)-/

B

C

D

E

LT-HSC

MPP

Percent Live Cells

Percent Live Cells

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

A. E-box and GATA motifs

+9.5 \( \text{GGATCTCCTGCCGGAGTTTCTATCCGGACATCTGCAGCCGGTAGATAAGGAAACTTCGTGTATCTGTTTCCGGA} \)

+9.5(Ets) \( \text{GGATCTCCTGCCGGAGTTTCTATCCGGACATCTGCAGCCGGTAGATAAGGAAACTTCGTGTATCTGTTTCTGGA} \)

B. Electropherograms

+9.5 \( \text{TGTTTCCGGACCG} \)

+9.5(Ets) \( \text{TGTTTCTGGACCG} \)

C. Images of fetuses

D. Cells/Liver (x10^7)

E. Gata2 mRNA levels (Relative Units)

**Total Fetal Liver**

+9.5

+9.5(Ets)

+/-

-/-
Figure 4. +9.5 enhancer Ets motif is dispensable for HSC emergence. (A) Sequence of wild type [+9.5(Ets)+/+] and Ets motif-mutant [+9.5(Ets)-/-] mice. Asterisk, C>T transition. (B) Sequence of +9.5+/+ and +9.5(Ets)-/- animals. (C) E15.5 litters. (D) Quantification of E15.5 liver cellularity [+9.5+/+ (n = 18), +9.5(Ets)+/- (n = 20), and +9.5(Ets)-/- (n = 10) from 4 experiments]. (E) Gata2 mRNA quantification [+9.5(Ets)+/+ (n = 4), +9.5(Ets)+/- (n = 7), and +9.5(Ets)-/- (n = 5) from 2 experiments]. Quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, median line, and whiskers ranging from minimum to maximum values; *P < 0.05, **P < 0.01 (two-tailed unpaired Student’s t test with Benjamini-Hochberg correction).
A: +9.5  
+9.5(Ets)-  
+9.5(Ets)+  

B: c-Kit Cells/DA  

C: Lin- Mac1+  
CD41  
CD48  

D: HSC  
MPP  

E: Percent Live Cells  

c-Kit+ Cells/DA  

Figure 5
Figure 5. +9.5 enhancer Ets motif is dispensable for HSC emergence. (A) Whole-mount immunostaining of E10.5 dorsal aorta (DA). CD31+ cells, magenta; c-Kit+ cells, green. Scale bars, 100 μm. (B) c-Kit+ cell quantification within the DA [+9.5+/+(n = 6), +9.5(Ets)+/-(n = 8), and +9.5(Ets)-/-(n = 6) from 4 experiments]. (C) Flow cytometric analysis of E15.5 fetal liver for HSC (Lin−CD41−CD48−Mac1+Sca1+Kit+CD150+) and MPP (Lin−CD41−CD48−Mac1+Sca1+Kit+CD150−). (D, E) HSC and MPP quantification (percentage of live fetal liver cells). [+9.5(Ets)+/+(n = 9), +9.5(Ets)+/-(n = 11), and +9.5(Ets)-/-(n = 8) from 2 experiments]. Quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, median line, and whiskers ranging from minimum to maximum values; *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed unpaired Student's t test with Benjamini-Hochberg correction).
Figure 6

A. White Blood Cells, Red Blood Cells, Platelets

B. Log2 Fold Change (Mut/WT) vs. -Log2(Adjusted P-value)

C. Percent Survival

D. Gata2 mRNA Level (Relative Units)

E. WBCs, RBCs, PLTs over time for different treatments

F. Log2 Fold Change (5-FU/Untreated) vs. -Log2(Adjusted P-value)

G. Gene Counts for different conditions and treatments
Figure 6. +9.5 enhancer Ets motif confers hematopoietic regeneration and survival following stress. (A) Steady-state peripheral blood parameters (n = 18 per genotype). (B) MA plot summarizing RNA-seq analysis of transcripts from bone marrow-derived wild type and +9.5(Ets)^/- LSK cells in the steady-state. (C) Kaplan-Meier survival curve of mice following two 5-FU doses (250 mg/kg, day 0 and 11) [+9.5^+/+ (n = 17), +9.5(Ets)^+/+ (n = 23), and +9.5(Ets)^/- (n = 10) from 2 experiments]. Significance was determined using the Log-rank test. (D) qRT-PCR quantitation of mRNA from bone marrow 9 days post-vehicle (PBS) or 5-FU (250 mg/kg) treatment [+9.5^+/+ (n = 8) and +9.5(Ets)^/- (n = 8) from 4 experiments]. Quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, median line, and whiskers ranging from minimum to maximum values; **P < 0.01 (Tukey multiple comparison test). (E) Hematologic parameters following 5-FU treatment (150 mg/kg; n = 9 per genotype; 3 experiments). (F) MA plot summarizing RNA-seq analysis of transcripts from bone marrow-derived wild type and +9.5(Ets)^/- LSK cells at day 10 post-5-FU treatment. (G) Relationships derived from the RNA-seq data of panel F. Venn diagram comparing 5-FU-regulated (total upregulated and downregulated) transcripts in bone marrow-derived wild type and +9.5(Ets)^/- LSK cells at day 10 post-5-FU treatment. These relationships were further deconvoluted by separating 5-FU-upregulated transcripts from the downregulated transcripts.
Figure 7. +9.5 enhancer Ets motif promotes hematopoietic regeneration after stress. (A) H&E staining of bone marrow after vehicle (PBS), 9 days, or 11 days post 5-FU (250 mg/kg) treatment. Scale bars = 50 μm. (B) Quantification of marrow cellularity as shown in (A) (n = 4 per genotype and condition; 4 experiments). Quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, median line, and whiskers ranging from minimum to maximum values; *P < 0.05 (two-tailed unpaired Student’s t test with Benjamini-Hochberg correction).
Figure 8

A

B

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Graph

- LSK
- LT-HSC
- MPP
- LS-K
- CMP
- GMP
- MEP

Legend

- Bars represent percent live cells
- Comparisons are made between different treatments and time points
- Statistical significance indicated with asterisks (**, ***)

Analysis

- The effects of 5-FU and 5-FUPBS treatments on hematopoietic cell populations are shown
- Changes in cell surface marker profiles are observed over time
- Live cell percentages are quantified for various cell types across different conditions

Conclusion

- Treatment with 5-FU and 5-FUPBS has a significant impact on hematopoietic cell populations
- Further studies are needed to understand the underlying mechanisms
Figure 8. +9.5 enhancer Ets motif-dependent HSPC regeneration. Bone marrow was harvested 9 or 11 days after vehicle (PBS) or 5-FU (250 mg/kg) treatment. (A) Flow cytometry for LSK (Lin⁻CD48⁻Sca1⁺Kit⁺), HSC (Lin⁻CD48⁻Sca1⁺Kit⁺CD150⁺), MPP (Lin⁻CD48⁻Sca1⁺Kit⁺CD150⁻), LS⁻K (Lin⁻Sca1⁻Kit⁺), MEP (Lin⁻Sca1⁻Kit⁺FcγR⁻CD34⁻), CMP (Lin⁻Sca1⁻Kit⁺FcγR⁻CD34⁻) and GMP (Lin⁻Sca1⁻Kit⁺FcγR⁺CD34⁻). (B) LSK, HSC, MPP, LS⁻K, MEP, CMP, and GMP quantification (percentage of live bone marrow cells) (n = 8-10 per genotype and treatment; 5 experiments). Quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, median line, and whiskers ranging from minimum to maximum values; *P < 0.05, **P < 0.01, ***P < 0.001 (Tukey multiple comparison test).
Figure 9

**A**

CD45.2

Monocytes

Granulocytes

B cells

T cells

Percent of CD45.2

Percent of Total

Weeks 0 4 8 12 16

Wild type  Ets⁻/⁻

**B**

LSK

HSC

MPP

LSK

CMP

GMP

MEP

% Donor Derived

% Donor Derived

% Donor Derived

% Donor Derived

P = 0.0149

P = 0.0014

P = 0.2014

P = 0.0005

P = 0.0013

P = 0.0006

P = 0.0002

P = 0.0013

P = 0.0000

P = 0.0000

P = 0.0002

P = 0.0000
Figure 9. +9.5 enhancer Ets motif-dependent regeneration of long-term repopulating HSCs. (A) Multi-lineage repopulating activity 16 weeks post-competitive transplant (n = 10 per genotype) (two-tailed unpaired Student’s t test). (B) Analysis of donor-derived LSK, HSC, MPP, LS*K, CMP, GMP and MEP within BM 16 weeks post-competitive transplant (n = 10 per genotype) (two-tailed unpaired Student’s t test).
A

**Gata2**

Exon 4  +9.5  Exon 5

Rep 1

ETV2 Rep 2

Rep 3

IgG

ACATCTGCAGCCGGTAGATAAGGAAACTTCGTGTATCTGTTTCCG

B

Gata2 mRNA Level (Relative Units)

WT  Tie2:Cre; Etv2^m^
Figure 10. Evidence for an ETV2-GATA-2 regenerative axis. (A) ETV2 ChIP-seq. ETV2 occupancy was detected at the +9.5 enhancer in mouse embryonic stem cell-derived embryoid bodies (GEO GSE59402) (44). (B) Sorted LSK cells from WT (n = 6) or Tie2-Cre;Etv2 conditional knockout mice (n = 6) 10 days post-5-FU injection (250 mg/kg). Gata2 mRNA levels were normalized to β-actin. **P < 0.01 (two-tailed unpaired Student’s t test).
Figure 11
Figure 11. Human disease predisposition mutation segregates developmental versus regenerative enhancer functions. The model depicts the +9.5(E-box;Ets)^/- HSC emergence and fetal liver hematopoiesis defects, which phenocopy the previously described +9.5^-/- phenotype. Both mutations are embryonic lethal. In contrast, the human disease +9.5 Ets motif mutant is not an embryonic lethal mutation. In adult mice, the mutation impedes HSPC regeneration. We propose that the human mutation renders the hematopoietic system vulnerable to subsequent insults that demand HSPC regeneration to re-establish the steady-state. The intimate connection between the predisposition mutation and mechanisms elicited by secondary insults may underlie the variable penetrance of disease onset in GATA-2-deficiency syndrome.

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*P < 1x10^-8*

**Table 1.** Genotypes of embryos from +9.5(E-box;Ets) heterozygous matings at developmental stages and weaning. Parentheses, dead embryos.
Table 2. Genotypes of embryos from +9.5(Ets) heterozygous matings at weaning.

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**Table 3.** Gene Ontology (NIH David) of genes dysregulated by Ets motif mutation.