HEMATOPOIESIS AND STEM CELLS

GATA2 haploinsufficiency caused by mutations in a conserved intronic element leads to MonoMAC syndrome

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Key Points

- Mutations in a conserved intronic enhancer element lead to *GATA2* haploinsufficiency.
- Mutations in *GATA2*, regardless of mutation type, lead to decreased *GATA2* transcript levels and a common global transcriptional profile.

Previous reports of *GATA2* mutations have focused on the coding region of the gene or full gene deletions. We recently identified 2 patients with novel insertion/deletion mutations predicted to result in mRNA nonsense-mediated decay, suggesting haploinsufficiency as the mechanism of GATA2 deficient disease. We therefore screened patients without identified exonic lesions for mutations within conserved noncoding and intronic regions. We discovered 1 patient with an intronic deletion mutation, 4 patients with point mutations within a conserved intronic element, and 3 patients with reduced or absent transcription from 1 allele. All mutations affected *GATA2* transcription. Full-length cDNA analysis provided evidence for decreased expression of the mutant alleles. The intronic deletion and point mutations considerably reduced the enhancer activity of the intron 5 enhancer. Analysis of 512 immune system genes revealed similar expression profiles in all clinically affected patients and reduced *GATA2* transcript levels. These mutations strongly support the haploinsufficient nature of GATA2 deficiency and identify transcriptional mechanisms and targets that lead to MonoMAC syndrome. (*Blood*. 2013;121(19):3830-3837)

Introduction

GATA2 deficiency is characterized by monocytopenia; B, natural killer (NK), and dendritic cell lymphopenia; and mycobacterial, fungal, and viral infection.[1-3] It has been called both MonoMAC, for monocytopenia and *Mycobacterium avium* complex, and DCML deficiency, for dendritic cell, monocyte, B and NK lymphoid deficiency. Patients may present with myelodysplastic syndrome (MDS)/acute myelogenous leukemia (AML) or pulmonary alveolar proteinosis. Unlike typical MDS, the marrow in patients with GATA2 deficiency is typically hypocellular and contains atypical and micro-megakaryocytes.[4] Since GATA2 also plays a critical role in the development of the vascular and lymphatic systems,[5] patients may present with lymphedema along with monosomy 7 and MDS, a triad known as Emberger syndrome.[6,7]

The *GATA2* mutations reported previously cluster into 2 main groups. Mutations within the highly conserved C-terminal zinc finger include missense changes and deletions that result in loss of the C-terminus. They are predicted to allow production of a stable mRNA that is translated into an abnormal protein. In contrast, the other group of mutations includes full gene deletions, as well as frame shift or early stop mutations, predicted to cause nonsense-mediated decay (NMD) of the mRNA, as reported in both MonoMAC and Emberger syndrome. However, several patients with clear MonoMAC phenotype lacked mutations within the *GATA2* exonic sequence or large intragenic deletions. Of the 16 families reported by Vinh et al,[1] mutations were only identified in 12.[2] In view of the phenotypic homogeneity of the MonoMAC syndrome between our mutation-positive and mutation-negative families, we investigated whether distinct mechanisms explain GATA2 deficiency in patients lacking mutations.

Methods

Probands with clinical presentations consistent with MonoMAC syndrome and their family members gave informed consent on institutional review board-approved protocols at the National Institutes of Health between 1996 and 2012. Diseased controls were patients enrolled in the same approved protocols with similar infections but without MonoMAC phenotype and having wild-type *GATA2*. This study was conducted in accordance with the Declaration of Helsinki.

DNA and RNA were isolated from whole blood or isolated cells using Puregene DNA isolation kit (Qiagen) or RNeasy (Qiagen). Genomic

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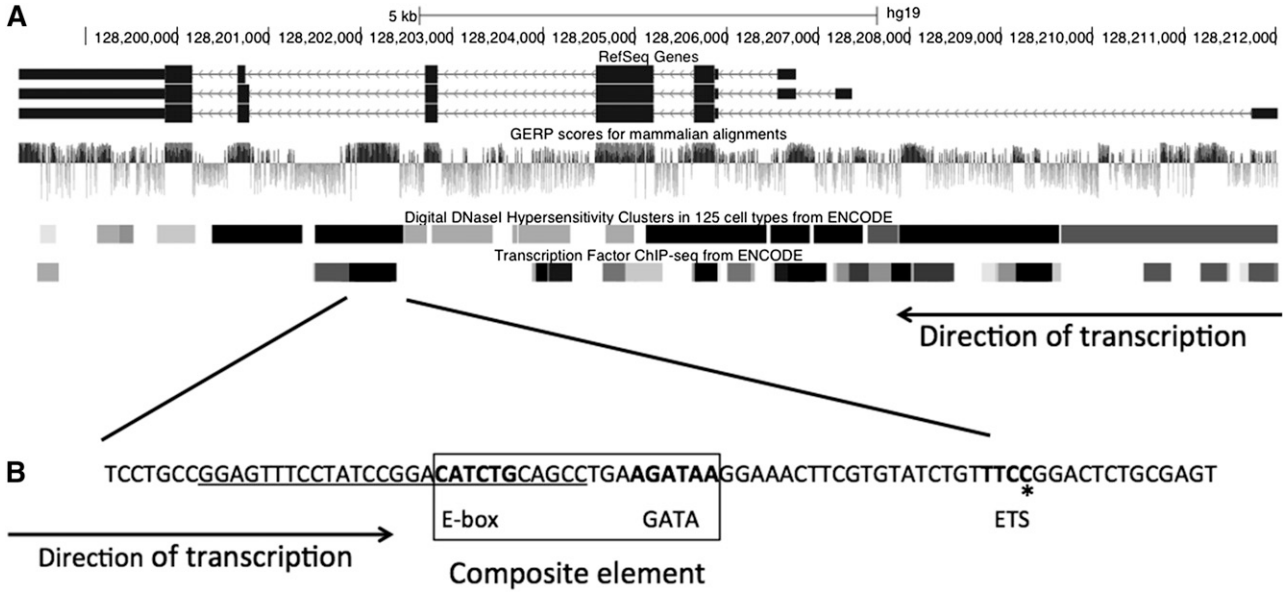


Figure 1. Organization and conservation within the *GATA2* locus. (A) *GATA2* locus. The 3 identified isoforms of human *GATA2* are shown with the associated GERP, DNaseI hypersensitive sites, and reported transcription factor binding sites.[36,37] (B) The conserved region within intron 5 including the composite element encompassing an E-box and GATA motifs and the ETS motif. Bold text denotes motifs for transcription factor binding. Underline denotes deletion in patient 6.II.1; *recurrent ETS motif point mutation, c.1017+572C>T. Figure modified from UCSC browser.

amplification and sequencing were performed as described previously.[2] cDNA amplification of *GATA2* (NM_001145661.1) was performed using Superscript III One-Step RT-PCR with Hi Fidelity Platinum Taq kit (Life Technologies), 5% dimethylsulfoxide, and primers 196F 5′-GCGCCAGGG CGGCCGGAGGATG-3′ and 1963R 5′-GTGTCGGCCTTCGGGAAATGC TGGGCTGCTAAG-3′. Sequencing primers are available upon request.

Transient transfection analysis

The intron 5 enhancer reporter construct was constructed using a polymerase chain reaction (PCR) fragment of the wild type or 28 base deletion intron 5 enhancer cloned upstream of the *GATA2* isoform 2 exon 1 promoter in the pGl3 luciferase reporter plasmid (Promega). The C to T substitution in the E-twenty six (ETS) motif site was introduced by PCR-mediated mutagenesis, and all resulting constructs were sequence verified. Reporter plasmids were purified using the Purelink HQ miniprep kit (Life-Technologies), and 2 independent plasmid preparations were used for each construct. Plasmids were introduced into K562 cells using Lipofectamine reagent (Invitrogen). For each reporter construct, 2×10^5 cells were transfected with 500 ng of reporter plasmid and 50 ng control *Renilla* Luciferase. Forty-eight hours posttransfection, cells were lysed in accordance with the Dual-Luciferase reporter assay kit (Promega), and the relative luciferase values were measured using the 20/20^n luminometer (Turner Biosystems/Promega).

Cell sorting

Ficoll-separated peripheral blood mononuclear cells (PBMCs) were stained with anti-CD3 (Becton Dickenson) and sorted on a FACS Aria (Becton Dickenson), collecting $CD3^+$ and $CD3^-$ fractions. The granulocyte pellet from the Ficoll was treated with ACK lysis buffer (Lonza), washed, and lysed for RNA.

Relative allele expression

Chromatogram peaks from single nucleotide polymorphisms (SNPs) identified by genomic sequence were measured using Pixelstick (PlumAmazing, Princeville, HI). The relative peak percentage was calculated as described[8] using the peak height of 1 allele divided by the sum of the peak heights of both alleles. The relative genomic SNP peak height was compared with the same peak sequenced from full-length cDNA transcripts.

Gene expression panel

For the gene expression panel, 250 ng total RNA isolated from Epstein-Barr virus (EBV) transformed B-cell lines (RNeasy, Qiagen) was hybridized with reporter and capture probes for the nCounter GX Human Immunology kit (Nanostring Technologies) and/or a custom probe set according to manufacturer's instructions, prepared on an nCounter Prep station and analyzed on an nCounter Analysis system. Data were normalized to spiked positive controls and housekeeping genes (nSolver Analysis system). Transcript counts less than the mean of the negative control transcripts plus 2 standard deviations for each sample were considered background on the human immunology panel; mean plus 1 standard deviation was considered background on the custom panel. Differences between sample groups were compared by 2-tailed Student *t* test with Welch approximation using MeV software.[9,10]

Results

There are 3 known isoforms of *GATA2* (Figure 1A) shown with the genomic evolutionary rate profile (GERP) score for each nucleotide, a measure of evolutionary constraint on each base. Regions with high GERP scores suggest putative functional elements.[11,12] The *GATA2* exons are highly conserved across species, as is the intron 5 region. The regions of intron 5 with high GERP scores have high DNaseI hypersensitivity scores as well as multiple occupied *cis*-elements demonstrated by ChIP-Seq (Figure 1B). Specifically, there is a composite *cis*-element, consisting of a Tal1/SCL-binding E-box motif, a spacer, and a GATA motif (WGATAA),[13-16] followed by a conserved ETS motif.

We conducted genomic sequencing of phenotypically identified MonoMAC patients (Table 1) lacking recognized mutations and their at-risk family members. The proband of each family had cytopenias, infections, and MDS. Their presentations were indistinguishable from those of patients with null alleles reported previously.[2] All exons (coding and noncoding) of *GATA2* as well as conserved intronic regions from each proband were sequenced. We identified

Table 1. Clinical features and *GATA2* mutations identified in MonoMAC patients

Pedigree No.	Cytopenia	Age*	Infection	MDS	Mutation	Mutation class	Ref.
Proband							
4.II.1	T/B/NK/mono	26	CMV, MAC, recurrent pneumonias, histoplasmosis	MDS	c.1017+572C>T	i5	1
25.I.1	T/B/NK/mono	13	HPV, *Mycobacterium kansasii*, MAC	MDS	c.1017+572C>T	i5	
11.II.1	B/NK/mono	9	HPV, VZV, treatment-resistant *Candida* thrush and vaginitis	+Abnormal megakaryocytes	c.1017+572C>T	i5	1
28.I.1	B/NK/mono	21	CMV pneumonia	+Abnormal megakaryocytes	c.1017+572C>T	i5	
6.II.1	B/NK/mono	22	HPV, Group C Strep, M. tuberculosis	MDS	c.1017+512del28	i5	1, 17
23.I.1†	T/B/NK	21	MAC	MDS	c.761C>T/unknown†	Unknown	2
7.I.1	T/B/NK/mono	51	HPV, *M. kansasii*	MDS	Unknown	Unknown	1
29.I.1	T/B/NK/mono	45	HPV	RAEB2	Unknown	Unknown	
8.I.1	B/NK/mono	28	HPV, MAC, *Aspergillus*	MDS	c.243_244delAinsGC	Haplo	2
13.II.1	T/B/NK/mono	33	HPV, MAC, histoplasmosis, *Neosartorya udagawae*	MDS	c.1-200_871+527 del 2033bp	Haplo	1, 2, 7
20.I.1	T/B/NK/mono	13	*Molluscum contagiosum*, HPV, MAC	MDS	c.769_778dup	Haplo	2
22.I.1	T/B/NK/mono	25	HPV, *M. kansasii*	MDS	c.941_951dup	Haplo	2
26.I.1	B	15	HSV, CMV	AML	c.302delG	Haplo	
27.I.1	T/B/NK/mono	46	HPV, *M. kansasii*	None	c.586_593dup	Haplo	
41.I.1	B/NK/mono	9	HSV	MDS	c.1009C>T; R337×	Haplo	
2.II.3	T/B/NK/mono	36	NTM, histoplasmosis	MDS	c.1192C>T; R398W	Mis	1
5.II.1	T/B/NK/mono	19	NTM	RAEB2	c.1061C>T; T354M	Mis	1
9.III.1	B/NK/mono	8	Warts	MDS	c.1192C>T; R398W	Mis	1
15.I.1	B/NK/mono	3	NTM, HSV	MDS	c.1186C>T; R396W	Mis	1,2
19.II.1	B/NK/mono	20	NTM, EBV	None	c.1061C>T; T354M	Mis	2
30.II.1	B/NK/mono	21	NTM, HSV	None	c.1163T>C; M388T	Mis	
37.I.1	T/B/NK/mono	32	*M. kansasii*, MAC	MDS	c.1081C>T; R361C	Mis	
Family member							
4.II.5	B/NK	19	HPV	MDS	c.1017+572C>T	i5	1
4.I.1	Monocytosis	78	None	CMML	c.1017+572C>T	i5	1
4.III.2	NK	23	None	None	c.1017+572C>T	i5	
4.III.3	NK	21	None	None	c.1017+572C>T	i5	
6.I.1	ND	13	Lymphedema	ND	c.1017+512del28	i5	
6.III.2	None	1.5	None	ND	c.1017+512del28	i5	
33.II.1	Thrombo	50	None	ND	c.1-276T>G‡	Unknown	
13.I.2	T/B/NK/mono	61	HPV, lymphedema	+Abnormal megakaryocytes	c.1-200_871+527 del 2033bp	Haplo	1, 2, 7
1.II.5	T/B/NK/mono	49	NTM, HPV	CMML	c.1192C>T; R398W	Mis	1
30.I.1	ND	65	None	None	c.1163T>C; p.M388T	Mis	
33.III.3	Mono	9	None	ND	c.1099insG; D367fs c.1-276T>G‡	Mis	
40.I.1	B	54	None	None	c.1187G>A; R396Q	Mis	

Abbreviations: CMML, chronic myelomonocytic leukemia; CMV, cytomegalovirus; Haplo, haploinsufficiency; HPV, human papillomavirus; HSV, herpes simplex virus; i5, intron 5 mutation; Mis, missense mutation.

*Approximate age (y) at presentation or age at diagnosis if initial presentation unknown.

†Previously reported as missense mutation; however, functional studies have demonstrated loss of expression of 1 allele.

‡Change in 5′ UTR of undetermined significance.

small frameshift mutations within exon 4, c.302delG and c.586_593dup, in patients 26.I.1 and 27.I.1, respectively (Table 1). These mutations were predicted to result in loss of expression of the mutant allele through NMD. Within the highly conserved region of intron 5 we identified a single point mutation in probands from 4 unrelated families. The mutation, c.1017+572C>T (i5C>T), is predicted to disrupt an ETS motif within intron 5 following the composite *cis*-element. Additionally, within the same region, 1 patient was identified with a 28 base deletion of intron 5 that eliminated the E-box and 5 bp of the spacer of the E-box/GATA composite element.[17]

At-risk family members were subsequently screened for the presence of the mutations found in the probands. We identified 6 additional individuals with intron 5 mutations. Four individuals from family 4 had i5C>T point mutations: the father (4.I.1), sister (4.II.5), and 2 adult children (4.III.2, 4.III.3). The father (6.I.1) of the proband in family 6 as well as the proband's 18-month-old son (6.III.2) were heterozygous for the 28 base deletion spanning the start of the E-box/GATA composite element. Onset and phenotype varied between mutation-positive family members, ranging from full MonoMAC phenotype (4.II.5), to monocytosis and chronic myelomonocytic leukemia at age 78 years without previous infection history (4.I.1), to isolated reduced NK cell numbers (4.III.2, 4.III.3). Patient 6.III.2 had normal monocyte and lymphocyte counts and percentages but mildly reduced neutrophils. Only 2 relatives in the cohort, 6.I.1 and 13.I.2,

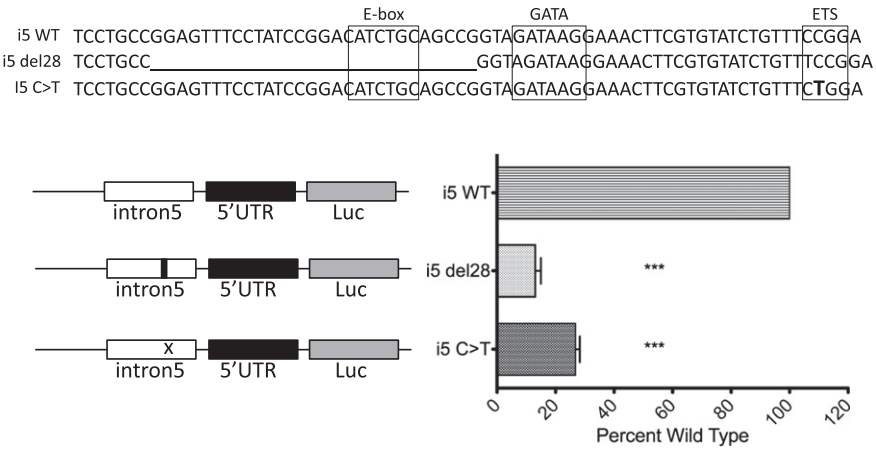
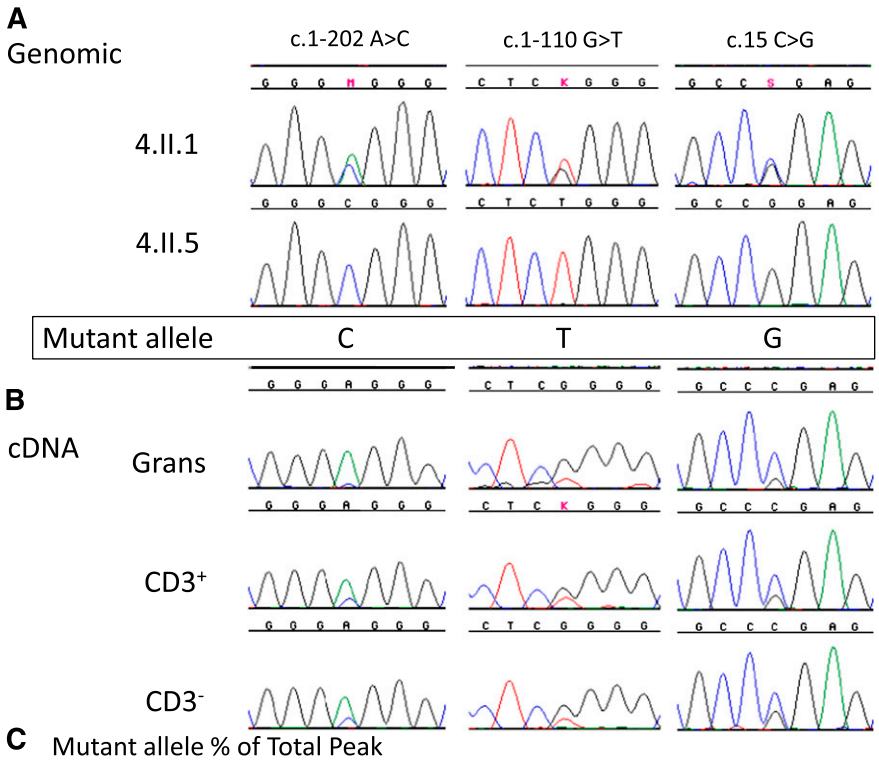


Figure 2. Mutation of conserved ETS motif or deletion of the composite element reduces intron 5 enhancer activity. Luciferase activity from exon 1 preceded by the wild-type intron 5 enhancer set and exon 1 preceded by the intron 5 enhancer containing either the 28 base deletion seen in patient 6.II.1 or the ETS motif C>T point mutation seen in patients 4.II.1, 11.II.1, 25.I.1, and 28.I.1. ***$P < .001$.

displayed lymphedema. The mutation in the 8 patients with i5C>T disrupts an ETS motif (Figure 1, asterisk), while the mutation in the patient with the 28 bp deletion eliminates the E-box and 5 bp of the spacer from the composite element (Figure 1, underlined).

Earlier studies in mice demonstrated that the intronic region spanning the composite element (referred to as the +9.5 enhancer element) is sufficient to drive reporter gene expression in fetal liver and vascular endothelium in transgenic mice.[13] Both the E-box and GATA motif were necessary for the enhancer activity of the +9.5 element,[13,14] whereas the requirement for the ETS motif was not addressed. We tested whether the ETS motif mutation or 28 base deletion influenced the GATA factor-dependent enhancer activity in the human intron 5 enhancer element. Luciferase vector constructs containing the wild-type human intron 5 enhancer, the 28 base deletion, or the C>T substitution in the ETS motif coupled to the untranslated first exon of *GATA2* (NM_032638) were transfected into K562 cells that express endogenous GATA2 (Figure 2). With the wild-type enhancer construct set as 100% luciferase activity, both the i5C>T mutated enhancer and the 28 base deletion had significantly lower activity ($P < .001$). Therefore, the ETS motif site and the E-box-GATA composite element are both required in *cis* to maximize the activity of the intron 5 enhancer.



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Mutant allele % of Total Peak			
Genomic	40	58	45
Grans cDNA	11	27	24
$CD3^+$ cDNA	26	35	29
$CD3^-$ cDNA	26	32	33

Figure 3. Reduced expression of mutant allele in a patient with c.1017+572C>T. (A) Genomic sequences of *GATA2* exons in patient 4.II.1 and her sister 4.II.5 indicate the phase of the SNPs and the mutation. M, K, and S refer to base calls of mixed nucleotide bases at a single site, A/C, G/T, and C/G, respectively. (B) *GATA2* cDNA sequences from isolated granulocytes (Grans), $CD3^+$ T-cells ($CD3^+$), and $CD3^-$ PBMCs ($CD3^-$) demonstrate reduced expression of c.1017+572C>T allele. (C) Quantitation of relative peak heights of patient 4.II.1 mutant allele as a percentage of the combined peak height at that site.

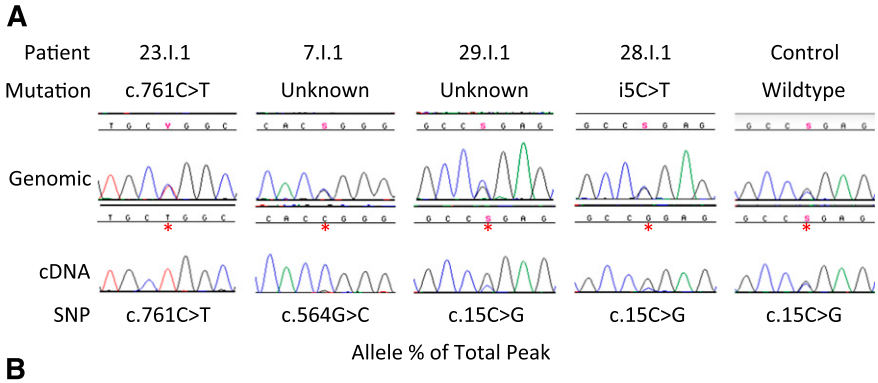

Figure 4. Reduced allelic expression of GATA2 in MonoMAC patients. (A) Genomic vs cDNA *GATA2* sequence for patients 23.I.1, 7.I.1, 29.I.1, 28.I.1, and a healthy control. Shown is a portion of the transcript in which the patient is heterozygous at the genomic level with reduced or absent expression of 1 allele by full-length cDNA sequence. Y, S refer to base calls of mixed nucleotide bases at a single site, C/T and C/G, respectively. (B) Quantitation of relative peak heights of patient's mutant allele as a percentage of the combined peak height at that site.

To test whether the i5C>T mutation influences transcription, we sequenced genomic DNA as well as cDNA from sorted PBMCs in patient 4.II.1. Due to the lack of B, NK, and monocyte cells in peripheral blood, we separated $CD3^+$ cells from $CD3^-$ cells and also analyzed the cells within the granulocyte pellet. Patient 4.II.1 is heterozygous by genomic sequence at several known SNPs within the *GATA2* cDNA, while her sister, 4.II.5, who also carries the i5C>T mutation, is homozygous at the same SNPs (Figure 3A). This homozygosity allowed us to determine the phase of mutation with cDNA SNPs and thereby permitted evaluation of relative allele expression. The mutation in this family resides on the CTG haplotype allele. Sequencing of full length *GATA2* cDNA in 4.II.1 demonstrated reduced levels of the mutation-bearing CTG haplotype allele compared with her wild-type allele (Figure 3B). In the genomic sequence, the heterozygous peak heights were similar, whereas in the cDNA sequence, the CTG allele accounted for roughly one third of the total peak height compared with the wild-type AGC allele seen in both $CD3^+$ and $CD3^-$ cells as well as granulocytes.

Patients with intron 5 mutations and those with known *GATA2* mutations that should cause NMD had reduced *GATA2* allelic expression. Based on these observations, we pursued reduced allelic expression of *GATA2* as the basis for other phenotypic MonoMAC patients. Three patients, 23.I.1 (c.761C>T causing P254L nonsynonymous change, patient 23 from Hsu et al[2]) and 2 without identified mutations in *GATA2* exons or conserved intronic or promoter regions, 7.I.1 and 29.I.1 (patient 7 from Vinh et al[1] and unpublished, respectively), all had significantly reduced expression of 1 allele by cDNA analysis, similar to the allelic expression of patients with the i5C>T mutation (Figure 4). While the P254L change is predicted by PolyPhenII[18] to be deleterious, cDNA sequence shows expression of only the c.761T transcript, suggesting that this patient also carries a mutation on the other allele, leading to haploinsufficiency, and only expresses *GATA2* mRNA from the P254L allele. The function of the P254L protein is unclear; however, expression from a single allele is insufficient for long-term normal hematopoiesis[19,20] and leads to the MonoMAC syndrome in humans[2,6] and abnormal bone marrow repopulation in mouse models.[19]

Using chromatogram peak height measurements, patient 7.I.1 had equal quantities of the G and C alleles at c.564 in the genomic sequence. However, when full-length cDNA was examined, the G allele represented only 12% of the total peak height, indicating loss of expression of that allele. Likewise, patient 29.I.1 exhibited similar allele peak heights by genomic sequence, while 1 allele was present at only 28% of the total peak by cDNA. This is similar to patient 28.I.1 with i5C>T mutation, and only 25% of the total peak is from the mutant allele and in contrast to a healthy normal with even allele ratios in both genomic and cDNA sequence. Thus, in patients with the MonoMAC phenotype lacking an identified mutation, uniallelic cDNA expression provided further evidence for *GATA2* haploinsufficiency.

We screened an additional 15 patients with informative SNPs and available EBV lines. All informative patients and family members with mutations in the intronic enhancer region demonstrated skewed allelic expression, including the previously reported patient with the 28 base deletion of the intron 5 composite element,[17] as did a patient with a premature termination predicted to result in NMD. We screened 6 patients and 2 family members with known missense changes, 6 of whom (5.I.1, 15.I.1, 19.II.1, 30.I.1, 30.II.1, 37.I.1) demonstrated equal representation of both alleles, while 2 (2.II.3 and 40.I.1) had skewed allelic expression at a level similar to that of the intron 5 patients. Three individuals with wild-type *GATA2* had equal representation of both alleles (data not shown).

We tested whether the i5C>T point mutations were transcriptionally equivalent to identified missense *GATA2* mutations. We used the Nanostring Human Immunology Panel to quantitate expression of 512 immune system genes and 15 housekeeping genes. We used EBV-transformed B-cell lines from patients and healthy normals as the source of RNA since they provide a renewable, homogeneous cell population without the inherent differences in lymphocyte and monocyte subsets in patient PBMCs. Additionally, acute events in patients that can drive transcriptional profiles, such as infections and neoplasms, probably do not differentially affect EBV lines. No significant differences in mRNA transcript expression between the missense and i5C>T mutated EBV-B cells were apparent. Principal component analysis of the full dataset separates *GATA2* patients from both healthy normals as well as diseased controls, showing distinct clustering of their transcript profiles (Figure 5A). Samples from healthy normal controls are clearly different from the phenotypic MonoMAC patients, including the 2 MonoMAC patients lacking recognized mutations who had reduced cDNA expression from 1 *GATA2* allele. This clustering is not simply due to the patients having a defect in an immune gene per se, since disease control samples with similar infections—disseminated mycobacteria, fungi, or cryptosporidia—yet wild-type *GATA2* sequence, clustered separately from both *GATA2* patients and normal controls.

Given the clustering among MonoMAC patients regardless of specific genotype, we compared transcript levels in healthy normals and those affected. We found significant ($P < .05$) differences in

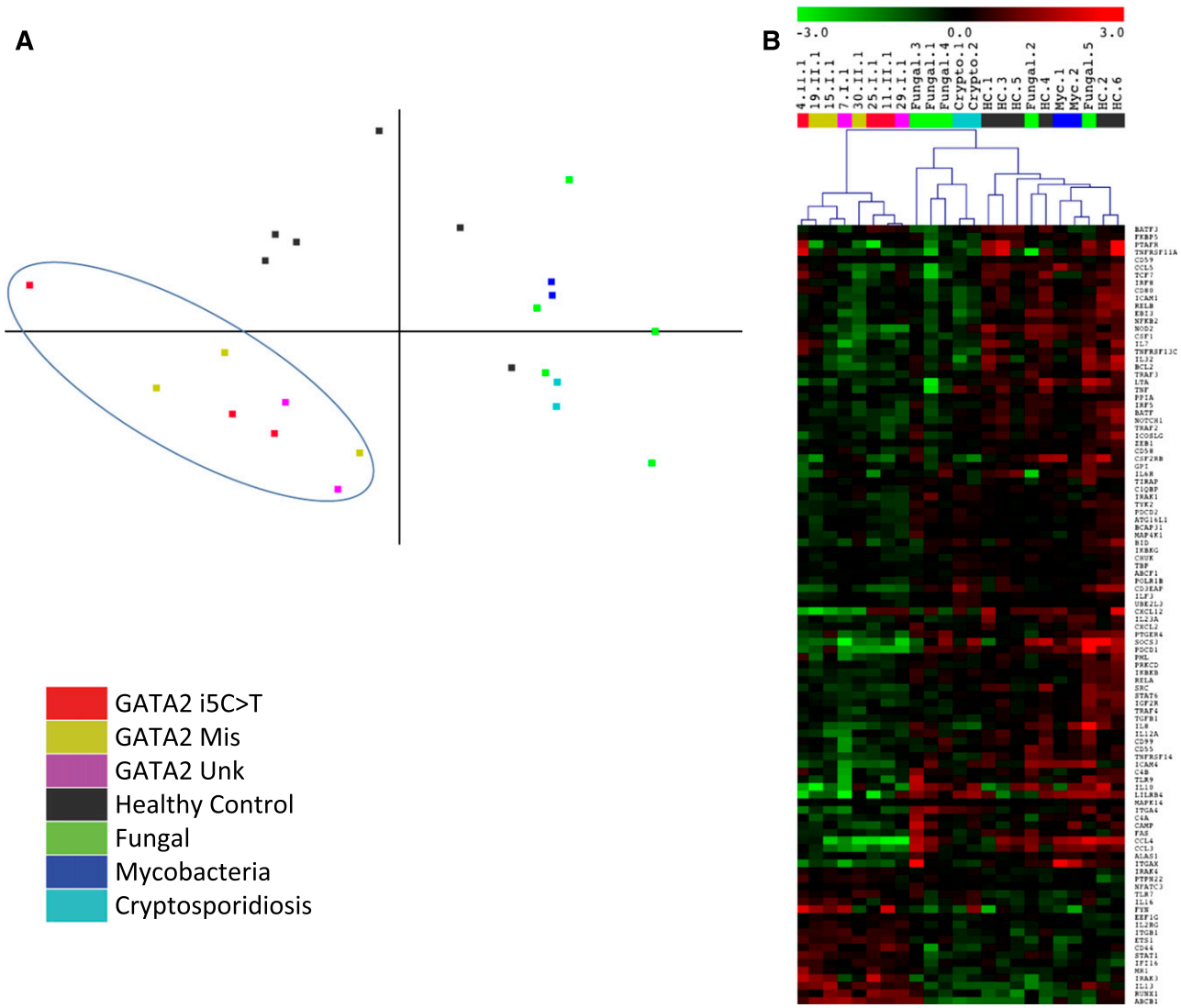

Figure 5. *GATA2* patients have unique transcript profiles compared with healthy normals and diseased controls. Normalized transcript counts from nCounter analysis used for (A) 2-dimensional principal component analysis of patients with GATA2 i5C>T (patients 4.II.1, 11.II.1, 25.I.1), missense mutation (GATA2 Mis; patients 15.I.1, 19.II.1, 30.II.1), and reduced allelic expression without identified GATA2 mutation (GATA2 Unk; patients 7.I.1, 29.I.1) compared with healthy controls and diseased controls with similar infection types but wild-type *GATA2*. Fungal, disseminated coccidioidomycosis or histoplasmosis; Mycobacteria, disseminated mycobacteria; Cryptosporidiosis, disseminated cryptosporidium. (B) Hierarchical clustering of transcripts differentially regulated ($P < .05$) between healthy normals and *GATA2* mutated patients irrespective of genotype. Samples and color labels correspond to mutations as in A.

expression levels of 102 genes (Figure 5B): 18 had increased expression and 84 decreased expression (supplemental Table 1). Genes with altered expression include *FYN, RUNX1*, and *ETS1* transcription factors (increased) and *CXCL12*, *SRC*, and *NOTCH1* (decreased). Using Ingenuity IPA core analysis, the subset of differentially expressed genes was analyzed in terms of systems, diseases, and disorders as well as molecular and cellular processes (supplemental Table 2).

We then designed a custom Nanostring code set to directly query *GATA2* transcript levels. The MonoMAC patients, regardless of mutation type, had decreased *GATA2* transcript levels compared with healthy controls and pulmonary nontuberculous mycobacterial patients with wild-type *GATA2* (68%, $P = .0218$ and 49%, $P = .0177$, respectively). Both the haplo and missense groups were significantly decreased compared with healthy controls ($P = .0220$, 0.0083, respectively), while transcript levels between mutation types were not significant (Figure 6). We examined several genes with significantly altered transcript levels between patients and controls and found conserved GATA2 binding sites with demonstrated GATA2 chromatin occupancy localized near the gene (supplemental Figures 1 and 2). While *GATA2, IKBKG*, and *FERMT3* have decreased transcript levels in GATA2 patients, *RUNX1* transcript counts were elevated in the patients (Figure 6). Reduced levels of *Fermt3* have been found in PECAM1+ embryonic cells in mice with either homozygous or heterozygous deletion of the +9.5 intronic element.[17]

Discussion

One third of previously reported patients with defects in *GATA2* have mutations predicted to cause loss of protein from the mutant allele, either through small insertions/deletions that result in nonsense-mediated decay[2,3,6] or through intragenic[2] or full gene

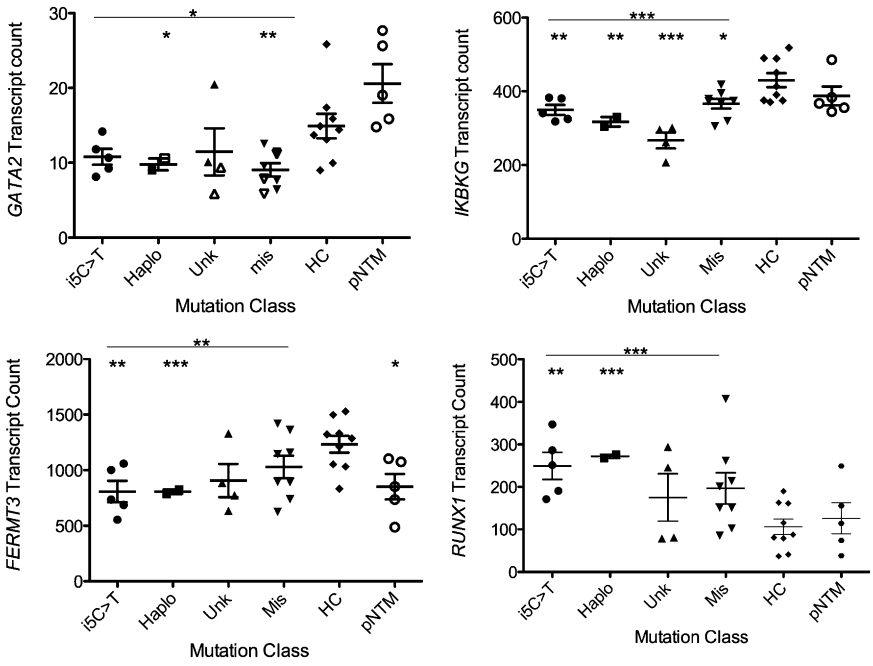

Figure 6. Transcript counts of *GATA2, IKBKG, FERMT3*, and *RUNX1* from patients segregated by mutation class. i5C>T (n = 5, patients 4.II.1, 4.II.5, 6.II.1, 11.I.1, 25.I.1); Haplo, identified mutations resulting in loss of expression of 1 allele (n = 2, patients 13.I.2, 41.I.1); Unk, patients without identified pathogenic mutations (n = 4, patients 7.I.1, 23.I.3, 29.I.1, 33.II.2); Mis, identified *GATA2* mutations predicted to result in a single amino acid change or a late frameshift with demonstrated mRNA stability (n = 8, patients 1.II.5, 5.I.1, 9.II.1, 15.I.1, 30.II.1, 33.III.3, 37.I.1, 40.I.1); compared with healthy controls (HC) (n = 9) or patients with pNTM (n = 5) with wild-type *GATA2*. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$.

deletions.[7] These patients are predicted to have reduced GATA2 levels, which lead to clinical disease and constitute haploinsufficiency. Heterozygous knockout mice with reduced endogenous levels of *Gata2* are born at normal Mendelian ratios but exhibit an approximately 50% reduction in the number of adult bone marrow hematopoietic stem cells (HSCs); those *Gata2*$^{+/-}$ HSCs have reduced repopulating potential.[19,20] We therefore predicted that patients with mutations resulting in significantly reduced expression from 1 allele would mimic previously characterized heterozygous *GATA2* missense mutations.[2,3,6,21]

The intron 5 mutations we identified reside within a region conserved since *Xenopus* (divergence ~350 million years ago[22]), a criterion that can imply functional importance. GATA2 does not act independently, rather there are several cooperating transcription factors that occupy closely linked chromatin locations.[23] In mice, Gata2, stem cell leukemia protein/T-cell acute lymphocytic leukemia protein (Scl/TAL1), and the ETS family member, Fli1, form a core recursive network,[24] and multiple factors, including GATA2, FLI1, and Scl/TAL1, occupy endogenous chromatin sites.[14,25-27] Mutations of the murine +9.5 kb site E-box, spacer, or GATA motif strongly reduce the enhancer activity of the composite element in cultured erythroid precursor cells expressing endogenous Gata2.[13] Furthermore, transgenic analysis provided evidence that the +9.5 kb site enhancer is active in the endothelium and fetal liver of developing mouse embryos.[14,16] Importantly, targeted deletion of the endogenous +9.5 kb site revealed its crucial role in the genesis of fetal liver HSCs and for conferring *Gata2* expression in fetal liver and embryonic PECAM1+ cells.[17] It is interesting to note that +9.5$^{-/-}$ embryos died between E13.5 and E14.5 of development and were characterized by ablation of long-term repopulating HSCs and progenitors in the fetal liver and severe hemorrhaging. The heterozygous mutation of the +9.5 site also reduced HSC numbers, long-term repopulating activity, and *Gata2* expression, but +9.5$^{+/-}$ embryos were born in Mendelian ratios.[17]

The mutations we identified in the intron 5 region occur at a LIM domain binding protein 1 (Ldb1) complex binding site, similar to others present in a high percentage of genes critical for HSC maintenance.[28,29] Examining a compendium of mouse ChIP-Seq data, the intron 5 region is occupied by multiple components of the GATA2-Scl/TAL1-FLI1 complex, including LIM domain only 2 (Lmo2), Gata2, Fli1, and Scl[30] as well as Ldb1.[28] Based on the disruption of the composite element in 1 patient and the ETS motif mutations, which occur at a consensus FLI1 binding site[31] adjacent to the composite element, it is possible that these mutations disrupt the assembly and/or function of the Scl/TAL1-GATA-2, FLI1 multimeric complex, providing a mechanism that underlies these patients' GATA2 haploinsufficiency. Mathematical modeling indicates tightly controlled protein levels[32] supported by <30-minute half-life of GATA2.[33] Given the established concentration-dependent actions of Gata2 in mice,[19,20] relatively modest decreases in the total level of Gata2 are likely to translate into significant molecular and cellular deficits. As well, the heterozygous +9.5 mutation in mice caused defects in Gata2 target gene expression.

We identified several genes with differential transcription patterns in GATA2 mutant cell lines. While several of the genes, including *RUNX1, NOTCH1, ETS1*, and *IKBKG,* have GATA2 binding sites within the gene region, GATA2 is classified as a remote element preferential transcription factor and commonly occupies chromatin in nonpromoter regions that can be intronic or large distances (>20 kb) away.[15,34,35]

Of the original 16 patients with MonoMAC syndrome described by Vinh et al,[1] 12 had identified *GATA2* mutations.[2] We have demonstrated that the 4 probands not previously associated with exonic *GATA2* mutations have *GATA2* haploinsufficiency due either to intron 5 mutations or reduced expression of 1 *GATA2* allele. As would be predicted, analysis of *GATA2* transcript levels has shown that mutations causing loss of expression of 1 allele result in reduced *GATA2* transcript level. Likewise, mutations predicted to result in a nonfunctional protein also result in reduced levels of *GATA2* transcripts. The consistency of the large-scale transcript expression data across mutation types demonstrates that haploinsufficiency at the transcript level results in similar alterations of target genes when compared with patients with missense mutations. Given the reduced *GATA2* transcript levels, similar transcriptional profile of immune-related genes, and comparable clinical presentation of probands, regardless of mutation type, we propose that GATA2 deficiency is

a disease of haploinsufficiency, whether by loss of production of protein at the transcript level or production of a nonfunctional protein that fails to drive transcription of the *GATA2* gene.

We identified 11 patients from 5 unrelated families with mutations disrupting critical functional units in intron 5, as well as 3 patients with significant loss of expression from 1 *GATA2* allele, all yielding similar clinical phenotypes, *GATA2* transcript levels, and global transcriptional profiles. Reduced expression of GATA2 is a common underlying cause of the syndromes variously known as MonoMAC, DCML, and Emberger and is due to various defects in the coding and noncoding regions of the gene.

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Authorship

Contribution: A.P.H. and K.D.J. designed and performed experiments; A.P.H., K.D.J., E.L.F., and J.E.L. analyzed data; E.L.F. and R.S. created the human intron 5 enhancer and murine +9.5 constructs, respectively; L.S. collected patient clinical data; J.C.-R., D.D.H., C.S.Z., and S.M.H. provided clinical care and patient samples; A.P.H, wrote the manuscript; and K.D.J., E.H.B., J.C.-R., and S.M.H revised the manuscript.

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