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Two effects of *GATA2* enhancer repositioning by 3q chromosomal rearrangements

Running title: **Leukemogenesis by *GATA2* enhancer repositioning**

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Abstract

Chromosomal inversion and translocation between 3q21 and 3q26 [inv(3)(q21.3q26.2) and t(3;3)(q21.3;q26.2), respectively] give rise to acute myeloid leukemia (AML) and myelodysplastic disorder (MDS) with poor prognosis. The chromosomal rearrangements reposition a *GATA2* distal hematopoietic enhancer from the original 3q21 locus to the *EVI1* (also known as *MECOM*) locus on 3q26. Therefore, the *GATA2* enhancer from one of two *GATA2* alleles drives *EVI1* gene expression in hematopoietic stem and progenitor cells, which promotes the accumulation of abnormal progenitors and induces leukemogenesis. On the other hand, one allele of the *GATA2* gene loses its enhancer, which results in reduced *GATA2* expression. The *GATA2* gene encodes a transcription factor critical for the generation and maintenance of hematopoietic stem and progenitor cells. *GATA2* haploinsufficiency has been known to cause immunodeficiency and myeloid leukemia. Notably, reduced *GATA2* expression suppresses the differentiation but promotes the proliferation of *EVI1*-expressing leukemic cells, which accelerates *EVI1*-driven leukemogenesis. A series of studies have shown that the *GATA2* enhancer repositioning caused by the chromosomal rearrangements between 3q21 and 3q26 provokes misexpression of both *EVI1* and *GATA2* genes and that these two effects coordinately elicit high-risk leukemia.

Introduction

Chromosomal translocation and inversion are collectively referred to as chromosomal rearrangements. These chromosomal rearrangements often alter gene conformation around the breakpoints. If two genes are combined within gene bodies as a result of the rearrangement, an abnormal fusion protein such as BCR-ABL (1) is formed. In contrast, if rearrangement occurs in a gene regulatory region, it does not produce abnormal fusion proteins but affects gene expression regulation. Chromosomal rearrangement that combines one gene (proto-oncogene) with an enhancer of another gene results in ectopic expression and/or overexpression of the proto-oncogene. We refer to this phenomenon as enhancer repositioning, and another group has referred to this phenomenon as enhancer hijacking (2). Chromosomal translocation or inversion between 3q21 and 3q26 is one of the prototypical examples of enhancer repositioning.

In 1992, a chromosomal rearrangement was reported to be associated with overexpression of the *EVI1* (also known as *MECOM*) proto-oncogene on 3q26 (3). However, the enhancer on 3q21 that is responsible for *EVI1* overexpression has been unknown for more than 20 years. In 2014, the *GATA2* gene enhancer on 3q21 was identified as an enhancer that induces *EVI1* overexpression (4,5). Discovery of the *GATA2* enhancer-mediated induction of *EVI1* overexpression revealed two effects of the chromosomal rearrangements between 3q21 and 3q26 provoke: *EVI1* overexpression and *GATA2* haploinsufficiency. Both these phenomena are caused by repositioning of the *GATA2* enhancer. As these observations open new avenues for the study of enhancer rearrangement, in this review, we introduce details regarding these two effects.

Acute myeloid leukemia with chromosomal rearrangements between 3q21 and 3q26

Chromosomal translocation or inversion between 3q21 and 3q26 are observed in 1-2% of all AML cases (6,7). The 3q rearrangements are also observed in myelodysplastic syndrome (MDS) cases. AML and MDS cases with chromosomal rearrangements between 3q21 and 3q26 show poor prognosis (8,9). While the blasts in the bone marrow (BM) of AML patients with the rearrangement are morphologically variable, 7-22% of the AML patients with the rearrangement presented marked thrombocythemia (10,11).

Golomb *et al.* reported in 1976-1978 that AML patients with the 3q abnormality showed high platelet numbers; therefore, a gene associated with megakaryocyte maturation or platelet production might be located in the 3q region (12,13). In 1985, Bitter *et al.* reported that AML patients with chromosomal rearrangements involving 3q21 and 3q26 showed high platelet counts (14). Notably, in 1992, Morishita *et al.* reported that the *EVI1* (*MECOM*) gene on 3q26 is overexpressed in AML with 3q26 rearrangements (3). After the report, *EVI1* was focused on as an oncogene of AML with chromosomal rearrangements between 3q21 and 3q26, and a number of reports have been published (see below). However, the precise functional contribution of 3q21 remained unclear for more than 20 years.

In 2014, back-to-back studies by Gröschel *et al.* and Yamazaki *et al.* shed light on this enigma, via a human leukemia study and a mouse model study, respectively. These two studies have clarified that a *GATA2* distal hematopoietic enhancer (G2DHE) on 3q21 activates *EVI1* gene expression (4,5). Notably, Gröschel *et al.* also found that *GATA2* gene expression is decreased in AML patients with 3q rearrangements (5), suggesting that the *GATA2* haploinsufficiency may contribute to the leukemogenesis of patients. Consistent with this notion, Katayama *et al.* successively clarified in 2017 by using mouse models that the *GATA2* haploinsufficiency in fact accelerates the leukemogenesis associated with 3q rearrangements (15).

Based on these studies, our current understanding of the abnormality in gene expression caused by chromosomal rearrangements between 3q21 and 3q26 has been improved and advanced. In the 3q-rearranged allele, G2DHE on 3q21 is brought into proximity to the *EVI1* gene on 3q26. Because of this repositioning of G2DHE, *EVI1* gene expression is induced in hematopoietic stem cells (HSCs) and progenitor cells via the influence of G2DHE, while *GATA2* gene expression is reduced due to loss of G2DHE (Figure 1A). We will introduce the expression profiles and functions of two misexpressed genes, namely, *EVI1* (*MECOM*) and *GATA2,* in leukemic cells with 3q rearrangements.

MECOM (MDS1 and EVI1 complex locus)

The *MECOM* (MDS1 and EVI1 complex locus) gene is located on 3q26. Historically, it was recognized that two independent genes, namely, *MDS1* and *EVI1,* were localized in the *MECOM* locus. However, recent research has revealed that *MDS1* and *EVI1* are isoforms of one gene, *MECOM*. *EVI1* was identified as a gene upregulated by retrovirus integration (*EVI1* is an abbreviation for the ecotropic virus integration 1 site) (16). The *EVI1* gene encodes the DNA-binding protein EVI1.

As shown in Figure 1B, the EVI1 protein has two sets of DNA-binding zinc finger domains, which are referred to as the N-terminal and C-terminal zinc finger domains and contain 7 and 3 zinc fingers, respectively (17,18). The N-terminal and C-terminal zinc finger domains bind to GATA-binding site-like and ETS-binding site-like motifs, respectively (19,20). The MDS1-EVI1 isoform has a PR (PRDI-BF1 and RIZ) domain at the N-terminus of EVI1 (21). The PR domain is a subtype of the SET [Su(var)3-9, enhancer-of-zeste and trithorax] domain associated with histone methyltransferase activity. The MDS1-EVI1 isoform is known to serve as a methyltransferase for H3K9me1 (monomethylated histone H3 lysine 9) and convert H3K9me1 to H3K9me3 (trimethylated H3K9), which contributes to heterochromatin formation (22). While both EVI1 and MDS1-EVI1 isoforms are detected in the cells of AML patients, EVI1 expression is associated with a poor prognosis (23). Therefore, we will focus on the EVI1 isoform hereafter.

In hematopoietic lineage cells, the endogenous *EVI1* gene is highly expressed in long-term HSCs (LT-HSCs), and the expression of this gene is sharply decreased during differentiation (24). Mouse model studies of an *EVI1* loss-of-function mutant revealed that the numbers of HSCs and progenitors decreased in *EVI1*-deficient mice, indicating that EVI1 is critical for HSC formation (25). In contrast, once formed, *EVI1*-deficient progenitors can differentiate into erythrocytes, granulocytes and B-lymphocytes. However, platelet numbers are decreased in *Evi1*-deficient mice, indicating that *EVI1* plays a role in platelet formation. Based on these studies, EVI1 is thought to play critical roles in HSCs and progenitors as well as platelets (25).

Notably, abnormalities in HSCs and platelets are also observed in EVI1 heterozygous knockout mice (25). Highly consistent with mouse studies, heterozygous loss-of-function mutations or deletions of the *MECOM* gene have been identified in radioulnar synostosis with amegakaryocytic thrombocytopenia patients (26-28). The patients suffer from amegakaryocytic thrombocytopenia followed by BM failure, as well as other symptoms, including radioulnar synostosis, clinodactyly, cardiac and renal malformations, B cell deficiency and presenile hearing loss.

MECOM is an oncogene

In several cancers and leukemia, elevation of *MECOM* gene expression has been reported. In hematological neoplasms, approximately 10% of AML cases show high expression of the *EVI1* gene (29), which is associated with poor prognosis (30,31). Chromosomal

rearrangements between 3q21 and 3q26 are observed in approximately 10-20% of the AML cases with high EVI1 expression (*i.e.,* 1-2% of total AML cases). In addition, the MLL (mixed-lineage leukemia) fusion protein has been reported to contribute to high-level expression of EVI1 (32). In most other cases, however, the causes of high-level EVI1 expression remain unknown.

Because *EVI1* overexpression has been known to be a main cause of AML with chromosomal rearrangements between 3q21 and 3q26, several mouse models recapitulating *EVI1* overexpression have been established. In 2000, Louz *et al.* generated a transgenic mouse model expressing *EVI1* in HSCs and progenitor cells under the control of the Sca1 promoter (33). This transgenic line of mice showed a few salient phenotypes, including expansion of progenitors, defects in erythropoiesis, and lineage skewing from lymphoid to myeloid lineages. While this line of transgenic mice did not develop leukemia, mutagenesis by viral infection induced the development of leukemia in mice (33). In 2004, Buonamici *et al.* established another mouse model by means of transplantation of BM cells transduced with *EVI1*-expressing retroviruses into lethally irradiated wild-type mice (34). While recipient mice showed pancytopenia in the peripheral blood, the recipient mice concomitantly showed BM hypercellularity and erythroid differentiation defects. These mice died 10-12 months after transplantation because of the pancytopenia but did not suffer from leukemia.

In contrast to the two preceding model mouse lines, *EVI1*-expressing mouse models established by Yoshimi *et al.* (in 2011), Yamazaki *et al.* (in 2014) and Ayoub *et al.* (in 2018) all developed myeloid leukemia, in which $CD11b^+$ or $Gr1^+$ myeloid leukemia cells are expanded (4,35,36). These mouse models were established by BM transplantation or the transgenic mouse approach (see below in detail). Nevertheless, it takes a long time (approximately 3-12 months) for these *EVI1*-overexpressing mouse models to develop leukemia, implying that another hit (DNA mutation) is required for leukemogenesis of the *EVI1*-expressing cells.

Based on a series of studies on *EVI1*-overexpressing mice, *EVI1* overexpression in HSCs and progenitor cells forms a basis for myeloid leukemogenesis through expansion of progenitors as well as defects of erythroid and lymphoid lineage differentiations. In addition, it is known that EVI1 upregulates the expression of *PBX1* and *PML* and downregulates *PTEN* expression, which promotes leukemogenesis by suppressing apoptosis and inducing proliferation, respectively (35,37,38). EVI1 also upregulates *GATA2* gene expression, as mentioned in a later section (39).

In addition to leukemia, amplification of the *MECOM* gene has been observed in solid tumors such as ovarian cancer, lung cancer and oral squamous cell carcinoma, suggesting that high-level expression of the *MECOM* gene promotes tumorigenesis in several solid tumors (40-43).

Structure, function and genetic mutations of GATA2

Intriguingly, the gene encoding *GATA2* is localized on 3q21. GATA2 is a member of the GATA family of transcription factors (44). Similar to the other vertebrate GATA family members, GATA2 has two zinc fingers (N-terminal finger and C-terminal finger) (Figure 1C). The functions of these zinc fingers have been revealed by analyses of another GATA family transcription factor, GATA1. The C-terminal finger is critical for binding to DNA through the GATA motifs (45-48), while the N-terminal finger mediates interactions with cofactors (*e.g.,* FOG1) and stabilizes DNA binding (49-51).

In the hematopoietic lineages, *GATA2* is highly expressed in HSCs and progenitor cells

and some other cell lineages (52,53). Studies utilizing various genetically engineered mice, including *Gata2*-null, conditional knockout, heterozygous knockout, knockdown and enhancer-deficient mouse models, unequivocally demonstrate that GATA2 plays critical roles in generating and maintaining HSCs and progenitor cells (54-62). Notably, heterozygous GATA2 germline mutations have been identified in four related syndromes in humans: MonoMAC (monocytopenia/ mycobacterium avium complex) (59,63,64), DCML deficiency (dendritic cell, monocyte, B and natural killer lymphoid deficiency) (65), Emberger's syndrome (66) and a familial MDS/AML (67). Patients with these diseases have similar symptoms, such as immunodeficiency, myelodysplasia, myeloid leukemia and lymphatic vascular dysfunction, suggesting the presence of a similar molecular basis for these syndromes. Mutations in coding regions were identified in both C-terminal and N-terminal finger domains. It has been reported that mutations in the C-terminal finger impair DNA binding directly, while mutations in the N-terminal finger decrease GATA2 chromatin occupancy (68). In addition to the mutations in coding regions, deletion of a *GATA2* gene enhancer has also been identified as a cause of MonoMAC, resulting in reduced *GATA2* expression (59). Based on this broad range of observations, loss of *GATA2* function has been recognized as a main cause of these diseases. Meanwhile, several GATA2 mutants promote myeloid differentiation and proliferation more powerfully than wild-type GATA2, suggesting that gain of GATA2 function may also contribute to the pathogenesis of these diseases (68).

GATA2 gene regulation and the distal enhancer G2DHE

In hematopoietic lineages, *Gata2* gene expression is regulated by GATA2 itself and by GATA1 in feedforward and feedback manners, respectively (69). GATA2 binds to GATA-binding sequences in the *Gata2* gene when *Gata2* is expressed in progenitors, implying that GATA2 positively regulates the *Gata2* gene. Meanwhile, *Gata2* gene expression is repressed by GATA1 when GATA1 expression starts increasing in the erythroid lineage. Therefore, during the differentiation of hematopoietic progenitors to the erythroid lineage, transition of GATA factors from GATA2 to GATA1 occurs, which is referred to as GATA factor switching (69,70). During GATA factor switching, GATA2 and GATA1 share their binding sites (GATA-switching sites) (71,72). The *Gata2* locus has multiple GATA-switching sites at –77, –3.9, –2.8, –1.8 and +9.5 kb from the transcription start site (TSS) (73). These sites have specific and redundant functions for *Gata2* gene expression (55,59,73-76).

The GATA-switching site at –77 kb from the TSS is located between the *RPN1* gene and breakpoints of 3q chromosomal rearrangements. Homozygous mice with the –77 enhancer deficiency show defects in definitive hematopoiesis and embryonic lethality at the late embryonic stage (61). While *Gata2* expression levels are maintained in lineage$^-$Sca1$^+$c-Kit$^+$ (LSK) fractions containing HSCs and multipotent progenitors (MPPs) of the enhancer-deficient mice compared with wild-type mice, *Gata2* expression in common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs) is decreased, indicating that the –77 enhancer is required for *Gata2* expression in CMPs, GMPs and MEPs.

On the other hand, we constructed a line of bacterial artificial chromosome (BAC) reporter mice that contained the 180-kb upstream region of the *Gata2* gene but did not retain the +9.5 enhancer. Deletion of the –77 enhancer results in reduced reporter expression in the LSK fraction (4). Based on these observations, the –77 enhancer seems to contribute to *Gata2* gene expression in both LSK and myeloid progenitors, while the +9.5 downstream enhancer is able to compensate for endogenous *Gata2* expression in LSK cells when the –77 enhancer is not present. We refer to the –77 enhancer as G2DHE.

Two consequences of the repositioning of G2DHE

We identified two consequences of the repositioning of G2DHE in AML with chromosomal rearrangements between 3q21 and 3q26. One consequence is the overexpression of EVI1 in the hematopoietic cell lineages, and the other is the haploinsufficiency of GATA2 (4,5). Recent analyses described below revealed that both consequences are important and contribute to leukemogenesis (15).

In 2014, two research groups independently and concomitantly discovered evidence that supports the contention that G2DHE contributes to EVI1-related leukemogenesis. These two groups analyzed the contribution of G2DHE to *EVI1* expression by means of transgenic mouse model analyses (4) and human patient cell analyses (5). Gröschel *et al.* identified G2DHE as an interacting region with the *EVI1* gene promoter in human patient-derived leukemic cells with the 3q-rearranged allele (5). Deletion of G2DHE suppresses *EVI1* expression and promotes differentiation of leukemic cells with the 3q-rearranged allele.

On the other hand, as shown in Figure 2, we have established a mouse model (3q21q26 mouse) harboring a transgene recapitulating the 196-kb human 3q chromosomal inverted allele that contains G2DHE and the *EVI1* gene (4) using a BAC linking technique (77). To generate the 3q21q26 model mouse line, two BAC clones containing the 64-kb 3q21 and 132-kb 3q26 regions were combined by simultaneous Cre-mediated recombination between the *loxP514* sites and between the *loxP511* sites selectively. While every type of mouse body cell shares the transgene in 3q21q26 mice, we found that the *EVI1* gene is specifically expressed in hematopoietic lineage cells and found that G2DHE, a hematopoietic stem-progenitor cell-specific enhancer, drives *EVI1* gene expression. Notably, 3q21q26 mice develop leukemia after 6 months of age. Targeted deletion of G2DHE from the BAC construct represses *EVI1* expression and leukemogenesis in 3q21q26 mice, demonstrating that the *GATA2* enhancer G2DHE is critical for *EVI1* gene expression in the 3q-rearranged allele.

It is important to note that several *EVI1*-overexpressing mouse models recapitulate only this *EVI1* overexpression consequence, as described in the previous section. However, loss of G2DHE results in one additional consequence, that is, haploinsufficiency of GATA2 in 3q21q26 cells. Indeed, it was found that *GATA2* gene expression is reduced in leukemic cells with 3q chromosomal rearrangements, concomitant with the promotion of EVI1 overexpression (5).

To establish this situation in model mice, as shown in Figure 3A, the 3q21q26 mice were crossed with the *Gata2* heterozygous knockout mice to obtain 3q21q26::*Gata2*+/– compound mutant mice that recapitulated both *EVI1* overexpression and *GATA2* haploinsufficiency (15). While *Gata2* heterozygous knockout mice did not develop leukemia, 3q21q26::*Gata2*+/– mice developed leukemia significantly earlier than 3q21q26 mice (Figure 3B). Detailed analyses showed that while 3q21q26 mice developed leukemia with myeloid maturation, 3q21q26::*Gata2*+/– mice frequently developed leukemia without maturation. Based on these studies, the *Gata2* heterozygous deletion accelerates *EVI1*-driven leukemogenesis.

Our initial hypothesis is that the two consequences of G2DHE repositioning, *i.e., EVI1* expression driven by the *GATA2* enhancer and reduced *GATA2* expression due to loss of the enhancer, both contribute to malignant leukemia. These analyses utilizing the 3q21q26::*Gata2*+/– mutant mouse model strongly support this hypothesis.

Leukemic cell phenotypes in the 3q21q26 mouse models

Through analyses of two mouse models of AML with 3q rearrangements, it has been found

that leukemic cell populations containing leukemia-initiating cells express the surface marker B220 (15,35). While B220 is commonly used as a B cell lineage marker in mouse models, the $B220^+$ cells in leukemic 3q21q26 mouse BM are negative for another B-lineage marker, CD19, and do not express the transcription factors EBF1 and PAX5 that are critical for B-lineage differentiation (15). In addition, the $B220^+$ cells in leukemic 3q21q26 mice are positive for the immature progenitor marker c-Kit and myeloid markers CD11b and FcγR II/III, which is a similar marker profile to that of GMP. Indeed, the $B220^+$ leukemia cells have the potential to differentiate into $Gr1^+$ myeloid leukemia cells, indicating that the $B220^+$ leukemia cells harbor myeloid progenitor-like potential (Figure 4A, upper panel).

Notably, heterozygous deletion of *GATA2* alters the characteristics of $B220^+$ leukemic cells in 3q21q26 mice. The $B220^+$ leukemic cells in 3q21q26::*Gata2*+/– mice are positive for c-Kit, IL7Ra and Flt3 but negative for myeloid markers CD11b and FcγR II/III. This profile shows similarity to that of lymphoid-primed multipotent progenitors (LMPP)-like cells (15) (Figure 4A, lower panel). Notably, $B220^+$ leukemic cells in 3q21q26::*Gata2*+/– mice fail to differentiate into myeloid lineages and proliferate strongly in the BM (15), indicating that heterozygous deletion of *GATA2* suppresses myeloid differentiation of *EVI1*-expressing leukemia cells and promotes proliferation of leukemic blasts, which results in acceleration of leukemogenesis. An intriguing observation related to this leukemia-initiating cell is that, in addition to the 3q21q26 mouse model, mouse models of CALM/AF10-positive myeloid leukemia and FLT3/ITD and NUP98-HOXD13 double-positive myeloid leukemia retain $B220^+$ leukemia-initiating cells similar to those of 3q21q26 mice (78,79).

An anti-B220 antibody (clone RA3-6B2) recognizes an isoform of CD45 (CD45R). CD45 is a protein tyrosine phosphatase that is associated with the immunoregulatory process (80). The N-terminal region of CD45 contains domains dependent on alternative splicing of exons 4 (also known as exon A), 5 (exon B), and 6 (exon C) (81). While the epitope of the RA3-6B2 antibody was not precisely identified, we confirmed that an isoform containing exon A was transcribed in $B220^+$ leukemic cells of both 3q21q26 and 3q21q26::*Gata2*+/–mice (15) (Figure 4B). In humans, B220 does not serve as a pan-B cell marker (81). An anti-CD45RA antibody that detects the CD45 isoform containing exon A is used for cell-type profiling of T cells, B cells and monocytes. Notably, an anti-CD45RA antibody has been identified for one of the markers of leukemic stem cells (LSCs) in human AML. In fact, Goardon *et al.* identified two distinct $CD34^+$ LSC populations in AML patients by utilizing the anti-CD45RA antibody: LMPP-like LSC ($lineage^-CD34^+CD38^-CD90^-CD45RA^+$) and GMP-like LSC ($lineage^-CD34^+CD38^+CD123^+CD45RA^+$) (82). Thus, $B220^+$ leukemic cells in 3q21q26 mice appear to be phenocopies of human AML leukemic stem cells.

Interaction between EVI1 and GATA2

As mentioned in the previous sections, both the *EVI1* and *GATA2* genes encode zinc finger transcription factors. Although the interaction between EVI1 and GATA2 has not been reported, EVI1 is known to suppress erythroid differentiation by inhibiting GATA1 function via two mechanisms. One mechanism is competition between GATA1 and EVI1 for DNA binding, while the other is direct protein-protein interaction between EVI1 and GATA1.

With regard to the former mechanism, EVI1 has been reported to compete with GATA1 for DNA binding (83), since EVI1 binds to GATA and ETS motifs on DNA. Because GATA1 and GATA2 share similar binding sites to each other (71,72), EVI1 may compete with GATA2 for DNA binding, as is the case for GATA1; however, this point remains to be verified. For the latter mechanism, an important prior observation is that EVI1 binds to the C-terminal finger domain of GATA1 (84). The C-terminal finger domain of GATA1 is highly homologous to

that of GATA2, as well as to those of the other GATA family factors. Therefore, EVI1 may interact with GATA2.

Based on these observations, we surmise that EVI1 and GATA2 may physically interact with each other and control leukemogenesis by mutual modification of activity. An additional mechanism for the EVI1-mediated interference of GATA2 activity is that EVI1 may directly upregulate *GATA2* gene expression (39), which results in an imbalance of the EVI1 and GATA2 protein ratio in leukemic cells. To clarify the mechanisms underlying EVI1-GATA2-related leukemogenesis, not only the individual functions of EVI1 and GATA2 but also the interaction between EVI1 and GATA2 should be taken into consideration.

Conclusion

Notably, reciprocal translocation or inversion gives rise to abnormalities at "two" genetic loci. One site produces an abnormal fusion protein or induces misexpression of a proto-oncogene, and this site usually receives more attention, while the other site tends to be overlooked. Recent identification of the G2DHE revealed the importance of the second site; *GATA2* haploinsufficiency accelerates leukemogenesis in AML with chromosomal rearrangements between 3q21 and 3q26. This advancement in the identification of molecular mechanisms will promote the development of therapies for patients with AML with 3q rearrangements, who show an extremely poor prognosis. Concomitant with the progress in the analyses of 3q rearrangement and leukemia, alternative sites of reciprocal chromosomal rearrangement, rather than those with widely recognized effects, have emerged as important contributors to leukemogenesis. Advances in this research will lead to the elucidation of the detailed pathogenesis of translocation- or inversion-associated diseases. Consideration of the other site of reciprocal rearrangement will provide an improved understanding of these leukemias.

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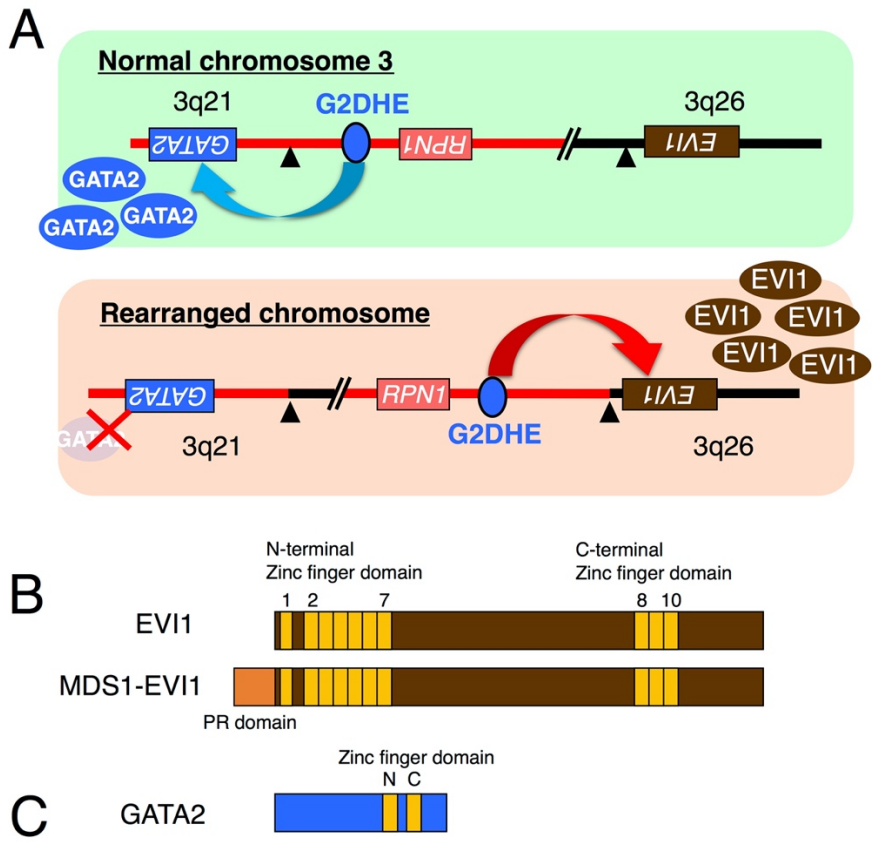

Figure 1. Misexpression of *EVI1* and *GATA2* by 3q chromosomal rearrangement.
(A) *EVI1* and *GATA2* gene regulation in normal chromosome 3 and rearranged chromosome 3. *GATA2* distal hematopoietic enhancer (G2DHE) activates expression of the *GATA2* gene on 3q21 in normal chromosome 3 (upper panel). In the rearranged (translocated or inverted) chromosome 3 (lower panel), G2DHE shifts from the *GATA2* gene on 3q21 to the *EVI1* gene on 3q26, which results in reduced *GATA2* expression and increased *EVI1* expression. (B, C) Molecular structures of the EVI1 (B) and GATA2 (C) proteins. The *MECOM* gene has two isoforms, *i.e.,* EVI1 and MDS1-EVI1. The EVI1 and MDS1-EVI1 proteins have two sets of DNA-binding zinc finger domains containing 7 (1-7th) and 3 (8-10th) zinc fingers, while the GATA2 protein has two zinc fingers (N-terminal (N) and C-terminal (C) fingers). The zinc finger domains and PR domain are depicted as yellow boxes and orange boxes, respectively.

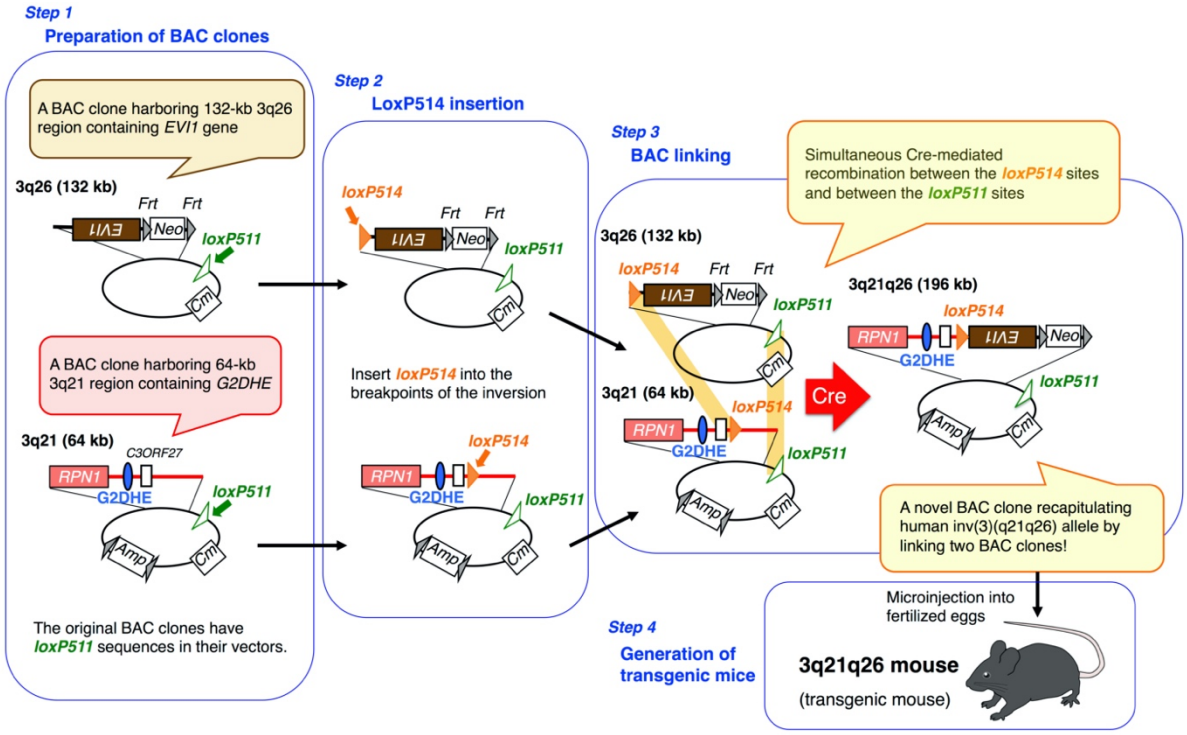

Figure 2. Generation of the 3q21q26 transgenic mouse model by linking two BAC clones. Precise construction strategies for BAC clones are depicted. Two BAC clones of a human genomic library, containing the 132-kb 3q26 region (*EVI1* side) and 64-kb 3q21 region (G2DHE side), have *loxP511* sites (green) in their vectors (step 1). *LoxP514* sites (orange) were inserted into the breakpoints of the inversion (step 2). Two BAC clones were linked by simultaneous Cre-mediated recombination (step 3). By microinjection of the linked BAC clone into fertilized eggs, 3q21q26 transgenic mice were generated (step 4). *Amp* (ampicillin resistance gene), *Cm* (chloramphenicol resistance gene) and *Neo* (neomycin/kanamycin resistance gene) were used for selection of recombinant clones. This protocol is an expansion of the original detailed protocol described in Yamazaki *et al.* (4).

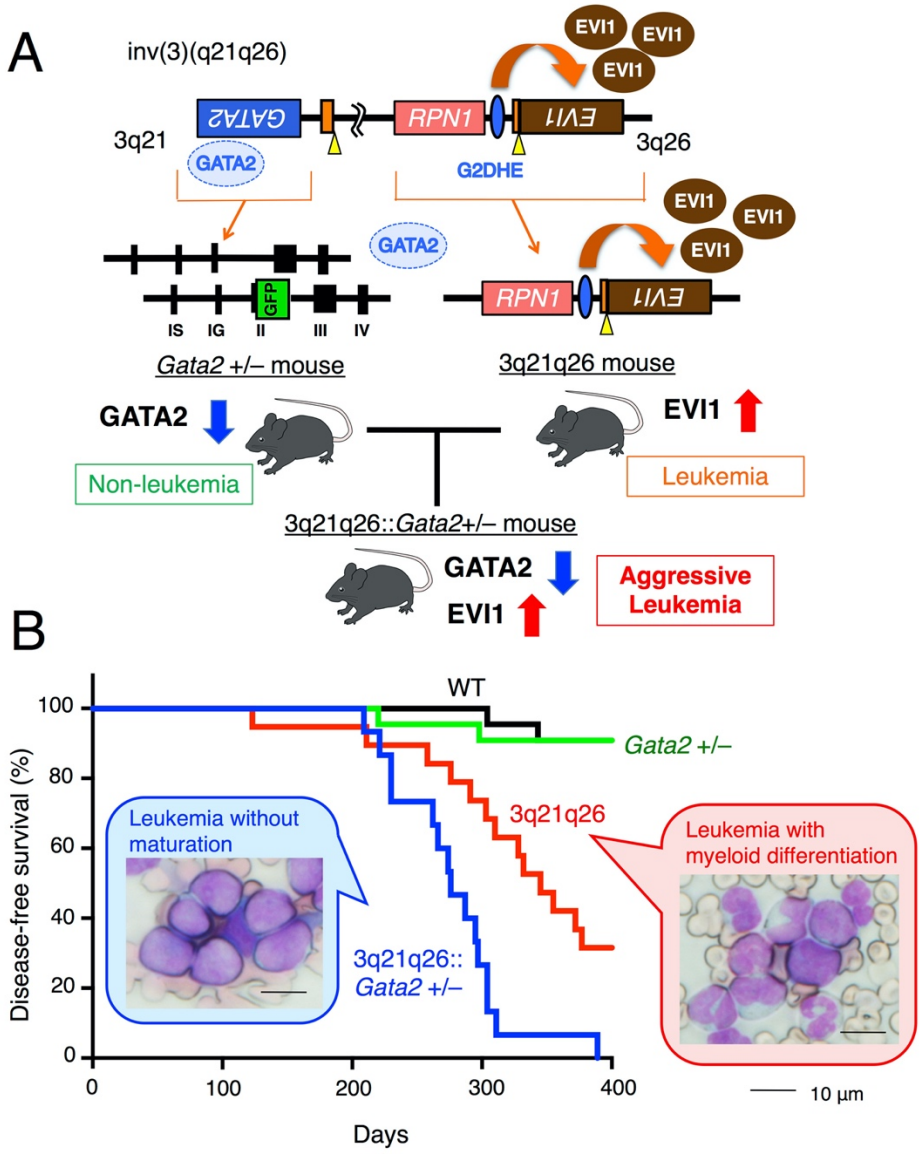

Figure 3. Mouse model recapitulating the two effects of the repositioning of the *GATA2* enhancer.

(A) Mouse model recapitulating both *EVI1* overexpression and reduced *GATA2* expression by crossing *Gata2* heterozygous knockout (*Gata2*+/–) mice and 3q21q26 mice. *Gata2*+/– mice did not develop leukemia. 3q21q26 mice developed leukemia containing myeloid leukemia cells. Notably, the compound mutant (3q21q26::*Gata2*+/–) mice developed much more aggressive leukemia than the 3q21q26 mice, in which blast cells were expanded. (B) Survival curves of WT, *Gata2*+/–, 3q21q26 and 3q21q26::*Gata2*+/– mice modified from Katayama *et al.* (15). The 3q21q26 and 3q21q26::*Gata2*+/– mice developed leukemia with myeloid differentiation and leukemia without maturation, respectively.

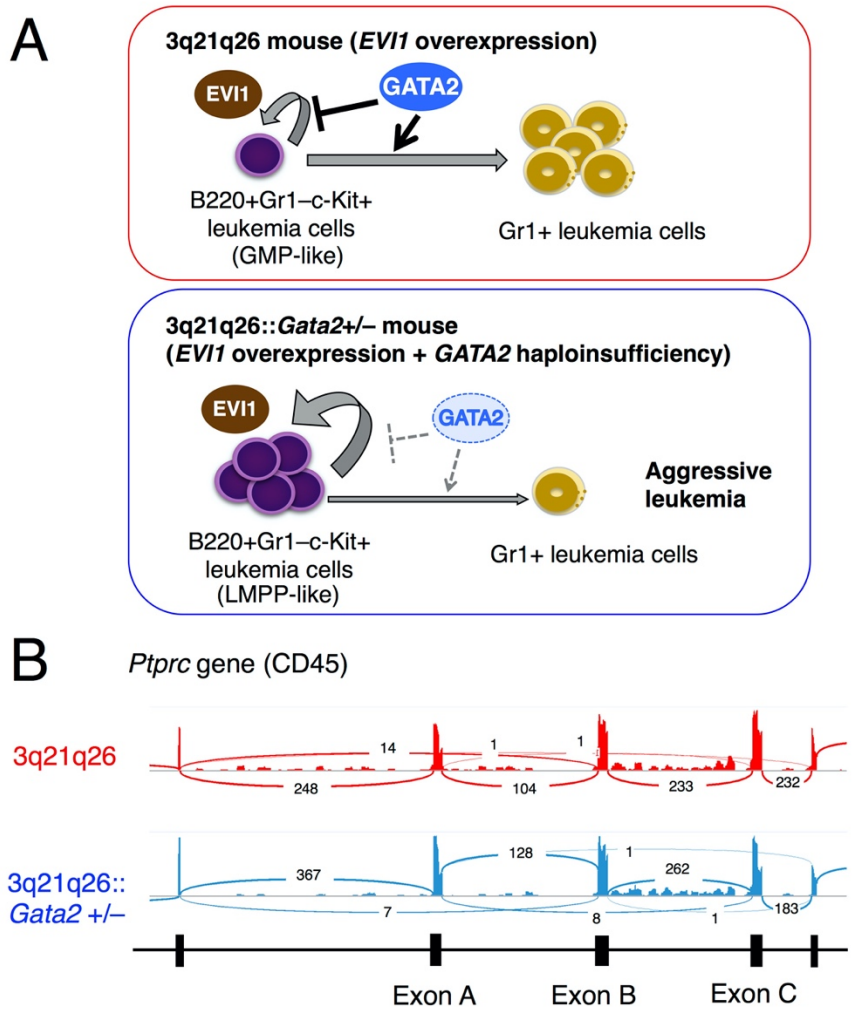

Figure 4. Contribution of *EVI1* overexpression and *GATA2* haploinsufficiency to leukemic phenotypes.
(A) The B220^{+}Gr1^{-}c-Kit^{+} population contains leukemia-initiating cells in both leukemic 3q21q26 mice (recapitulating only *EVI1* overexpression) and 3q21q26::*Gata2*+/– mice (recapitulating both *EVI1* overexpression and *GATA2* haploinsufficiency). While B220^{+}Gr1^{-} c-Kit^{+} cells in 3q21q26 mice differentiate into Gr1^{+} myeloid leukemia cells, those in 3q21q26::*Gata2*+/– mice fail to differentiate, but their proliferation is enhanced, which results in aggressive leukemia. (B) Sashimi plots of the RNA sequencing data for the *Ptprc* gene, encoding CD45, obtained using B220^{+} leukemic cells from 3q21q26 and 3q21q26::*Gata2*+/– mice. The RNA sequencing data are described in Katayama *et al.* (15). Numbers in the sashimi plots indicate numbers of junction reads. Isoforms containing exon A were transcribed in B220^{+} leukemic cells of both 3q21q26 and 3q21q26::*Gata2*+/– mice.