A Single Oncogenic Enhancer Rearrangement Causes Concomitant EVI1 and GATA2 Deregulation in Leukemia

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SUMMARY

Chromosomal rearrangements without gene fusions have been implicated in leukemogenesis by causing deregulation of proto-oncogenes via relocation of cryptic regulatory DNA elements. AML with inv(3)/t(3;3) is associated with aberrant expression of the stem-cell regulator EVI1. Applying functional genomics and genome-engineering, we demonstrate that both 3q rearrangements reposition a distal GATA2 enhancer to ectopically activate EVI1 and simultaneously confer GATA2 functional haploinsufficiency, previously identified as the cause of sporadic familial AML/MDS and MonoMac/Emberger syndromes. Genomic excision of the ectopic enhancer restored EVI1 silencing and led to growth inhibition and differentiation of AML cells, which could be replicated by pharmacologic BET inhibition. Our data show that structural rearrangements involving the chromosomal repositioning of a single enhancer can cause deregulation of two unrelated distal genes, with cancer as the outcome.

INTRODUCTION

Chromosomal inversions and translocations play a central role in the pathogenesis of almost all types of cancers, frequently by formation of oncogenic genes via rearrangement of coding sequences of the involved partner genes (Fröhling and Döhner, 2008; Mitelman et al., 2004, 2013). Mechanisms of transformation remain largely unknown in malignancies arising from chromosomal inversions/translocations that do not cause fusion products, although it is thought that destabilization of cryptic regulatory elements affects genes in the vicinity of the structural rearrangement, as has been shown in Burkitt’s (Polack et al., 1993) or follicular lymphoma (Bakhshi et al., 1985; Tsujimoto et al., 1985).

In the World Health Organization (WHO) category of myeloid malignancies with inv(3)(q21q26.2) or t(3;3)(q21;q26.2) deregulation of the proto-oncogene EVI1 (also termed MECOM or PRDM3) at 3q26.2 is speculated to occur via juxtaposition of a cryptic enhancer of the housekeeping gene RPN1 from 3q21 (Suzukawa et al., 1994). However, this hypothesis has not been experimentally validated and the molecular basis of this prognostically unfavorable subtype of malignancies remains obscure. EVI1 expression and function is indispensable for proper regulation of the hematopoietic stem cell compartment and genomic integrity (Goyama et al., 2008; Kataoka et al., 2011; Pinheiro et al., 2012). The gene was originally described as a hotspot for proviral integration in retrovirally induced murine myeloid leukemias (Morishita et al., 1988) and also represents an important insertional mutagenesis site in humans following gene therapy for X-linked granulomatous disease (Stein et al., 2010).

We tested the hypothesis that rearrangements causing the transcriptional activation of EVI1 involve the reallocation of an enhancer element to the ectopic 3q26.2/EVI1 target site, which may possibly coincide with a loss of enhancer activity at its endogenous location. We applied an integrated functional genomics and genome-engineering approach to identify a distal enhancer of the GATA2 gene that, upon chromosomal 3q rearrangements, ectopically activates EVI1 expression. Simultaneously, the removal of this enhancer from its natural genomic context causes functional GATA2 haploinsufficiency, i.e., reduced GATA2 expression only from the remaining normal allele.
RESULTS

An 18 kb Noncoding Region near RPN1 Commonly Translocates to EVI1 in inv(3)/t(3;3) Disease

We performed targeted next generation sequencing (NGS) of the long arm of chromosome 3 (3q-seq) using genomic DNA isolated from 41 samples with confirmed EVI1 overexpression (EVI1+ and harboring an inv(3)(q21;q26.2) or a t(3;3)(q21;q26.2) (inv(3)/t(3;3)). The samples included 38 primary bone marrow samples from patients, i.e., AML (n = 33), CML-BC (n = 2), and MDS cases (n = 3), as well as three cell lines (MUTZ-3, MOLM-1, and UCSD-AML1) (Table S1 available online). Chromosomal breakpoint positions and novel junction sequences of each case were determined by a breakpoint detection algorithm in conjunction with a de novo assembly tool and validated by Sanger sequencing. Characteristic breakpoint patterns emerged at both 3q21 and 3q26.2 breakpoint cluster regions (Figure 1A). At the 3q26.2 site, samples harboring an inv(3) exclusively exhibited breakpoints in the last intron or downstream of EVI1. Breakpoints in t(3;3) cases distinctively mapped upstream of EVI1, i.e., within the gene locus of the longer splice variant that includes MDS1-EVI1 (Figure 1A). At the 3q21 site, breakpoints occurred in a 130 kb region between GATA2 (centromeric) and RPN1 (telomeric). A minimal 18 kb noncoding region 3’ of RPN1 demarcated by chromosomal breakpoints was identified as a commonly translocated segment (hereafter referred to as CTS) (Figure 1B), which in all cases underwent transpositioning to the vicinity of EVI1 due to the inv(3)/t(3;3) rearrangement. This converging tell-tale pattern of 3q21 breakpoints toward an unaffected 18 kb genomic segment led us to predict the presence of potent regulatory elements within the CTS, essential for aberrant activation of EVI1 upon rearrangement.

The EVI1 Promoter and the 18 kb CTS Physically Interact

A hallmark of distal enhancer elements is their engagement in chromatin loops physically contacting with promoters to induce transcription factor assembly and polymerase II recruitment (Deng et al., 2012; Sanyal et al., 2012; Thurman et al., 2012; Tolhuis et al., 2002). To test whether the CTS harbored elements physically interacting with the EVI1 promoter, we performed high-resolution chromosome conformation capture sequencing (4C-seq) experiments (van de Werken et al., 2012). Using viewpoint-placed on the EVI1 promoter in viable inv(3)/t(3;3) AML samples and cells, we identified a genomic segment of approximately 9 kb size within the 18 kb CTS contacting with the EVI1 promoter (Figures 1C and 1D). Other contact regions located centromeric of this 9 kb contact hotspot in closer distance to the EVI1 promoter after the rearrangement, as observed in individual samples with different breakpoint positions, were considered less likely enhancer candidates. These regions were nonoverlapping across different samples and thus represented less specific contacts, which became more evident after integrative analysis of all 3q-rearranged AML samples (Figure 1D). Reciprocal 4C-seq experiments with the putative 9kb region as viewpoint showed that the interaction with the EVI1 promoter area was also evident (Figure S1). As expected, no substantial chromatin interactions with the distant EVI1 promoter could be detected in 4C-seq experiments with non-3q-rearranged control (U937), suggesting an inv(3)/t(3;3) disease-specific feature (Figures 1C and S1).

A p300-Bound Genomic Element in the 18kb CTS Is Essential for EVI1 Activation

In order to identify a more defined, targetable key enhancer element within the 9 kb EVI1-promoter-contact part of the 18 kb CTS, we integrated data from 4C-seq with ChIP-seq data obtained from inv(3) cell lines MOLM-1 and MUTZ-3 (Figures 2A and S2). Prominent deposition of H3K27ac, H3K4me3, and H3K4me1 was observed within the 18 kb CTS, as well as strong binding of p300 to two regions of approximately 1 kb size in MOLM-1 (Figure 2A). In MUTZ-3 cells only one p300-interacting region was identified (Figure S2), which is the p300 peak located in the 9 kb EVI1-promoter-contact part of the CTS as determined by 4C-seq. Comparison with ENCODE ChIP-seq data of various nonmyeloid cell lines and transcription factor motif analysis pointed to a 1 kb myeloid hematopoiesis-specific enhancer (Figures 2A and S2). This p300-binding site was chosen as the most likely candidate enhancer element responsible for ectopic EVI1 activation after the rearrangement event. We placed the two candidate enhancers (first and second p300 site) into an EVI1-promoter luciferase reporter construct to study their potential enhancer activity (Figure 2B). A strong induction of reporter gene activity could be observed specifically in inv(3) myeloid cell lines MUTZ-3 and MOLM-1 using the first candidate enhancer element, whereas the second candidate enhancer element (second p300 site) located within the 18 kb CTS, but outside of the 9 kb EVI1-promoter-contact region, showed no enhancer activity. No activity was found for two distinct CTCF-interacting CpG islands colocalizing to the CTS. Moreover, EVI1-promoter reporter assays demonstrated no substantial enhancing effect of the candidate 1 kb enhancer in nonmyeloid HEK293T or Jurkat cells, pointing to a myeloid-specific transcription factor repertoire required for successful enhancer-EVI1-promoter engagement.

Genome Editing of the Translocated p300-Interaction Domain Leads to EVI1 Silencing and Growth Inhibition of inv(3) AML Cells

We next undertook a TALE nuclease genome-editing approach to target the ectopic EVI1 enhancer locus in the MUTZ-3 cell line and to examine whether EVI1 transcriptional activity in inv(3) AML cells is dependent on the presence of the rearranged candidate enhancer (first p300 peak). TALE nucleases were assembled as previously published (Sanjana et al., 2012), and targeting of the minimal ectopic enhancer site was performed in a 2 x 2 design (details in Experimental Procedures), directing TALEN heterodimers to enhancer-flanking recognition sequences to induce a segmental deletion by double-strand breaks (DSB) (Figure 3A). Mutation-specific primers allowed for allelic detection of the deletion event (Figure 3B) and for screening of clones using an informative SNV in the candidate enhancer locus of MUTZ-3 (Figures 3B, S3A, and S3B). Overall targeting efficiency was 1% with 4/384 single-cell derived clones harboring a monoallelic enhancer deletion on the inv(3) allele.
Enhancer-targeting effectively attenuated EVI1 mRNA expression in deletion clones as compared with nontargeted MUTZ-3 control clones taken along in the same targeting process (Figure 3C). RPN1 and GATA2 mRNA expression remained unchanged in inv(3)-targeted clones. Notably, all four TALEN-modified MUTZ-3 clones showed severely impaired colony-forming and replating capacity compared to nontargeted clones (Figure 3D).

See also Figure S1 and Table S1.
Deletion of the Ectopic EVI1 Enhancer Releases the Maturation Block of inv(3) AML Cells

Genome editing in MUTZ-3 AML cells using TALENs did not allow for high yields of viable cells lacking the enhancer because of the low targeting efficiency of this genetic tool in the inv(3) cell line model followed by a long and indirect selection process of growth-impaired deletion clones. To better characterize the cellular phenotype and fate after enhancer deletion, we designed an alternative targeting approach using the CRISPR/Cas9 genome-editing system with a site-specific homology-directed repair (HDR) donor for direct labeling and tracking of successfully targeted cells (Figures 4A and 4B). In brief, enhancer deletions were induced by two short guide-RNAs (gRNA) directing hSpCas9 to two enhancer-flanking recognition sequences for DSB formation and HDR of the induced segmental deletion by means of a cotransfected HDR donor construct containing a conditional (loxP-) GFP selection cassette directed against the enhancer (details in Experimental Procedures). The genomic CMV-GFP insertion was subsequently removed by using exogenous cell-permeant Cre recombinase (TAT-Cre). This approach enabled us to isolate sufficient cell numbers for phenotypic characterization. Deletion events and transcriptional changes were confirmed by Sanger sequencing and qPCR (Figures 4C and S3D). Compared to untargeted control MUTZ-3 cells, targeted cells exhibited a markedly reduced proliferative rate as assessed by viable cell count using trypan blue staining (Figure 4D). Cell cycle analysis showed depletion of S phase and G2/M phase combined with higher rates of cell death.
(sub-G0/G1 peak) and a stationary G0/G1 cycle arrest (Figure 4E). Remarkably, flow cytometric immunophenotyping of engineered cells using a panel of informative myeloid differentiation markers (see Extended Experimental Procedures for detailed list) according to published guidelines (van Dongen et al., 2012) revealed a substantial skew toward a more mature, myelomonocytic stage as per cMPO and CD14 expression levels 3 weeks after genome editing (Figure 4F). Cytologic evaluation of enhancer-targeted MUTZ-3 cells in week 3 after genomic modification confirmed morphologic changes from the predominantly immature, myelomonocytic appearance of untargeted cells toward a more differentiated, monocyte/macrophage-like shape (Figure 4G). This also translated into a higher apoptotic rate of CRISPR-targeted MUTZ-3 cells 3 weeks after enhancer deletion (Figure 4H).

Off-target mutagenesis at alternative in silico predicted sites was excluded by Sanger sequencing (Figure S3E). The phenotype observed upon enhancer deletion by genome-editing tools was highly comparable to what was found with small hairpin RNA (shRNA)-mediated EVI1 knockdown in the MUTZ-3 cell line (Figures S4A–S4H), emphasizing that MUTZ-3 cells are addicted to EVI1 and blocked in their differentiation. We did not observe outgrowth of biallelic enhancer deletion or monoallelic mutants of the nonrearranged chromosome 3 allele, hinting toward negative selection of these mutants upon disruption of the enhancer at its natural genomic location.

The Candidate Enhancer Translocated to EVI1 Is an Original Upstream Enhancer of GATA2

The most likely candidate for off-target mutagenesis at the original enhancer-associated domain on the normal chromosome 3 allele appeared to be RPN1 due to its immediate proximity to the candidate enhancer. Concordantly, RPN1 has therefore generally been the assumed origin of ectopic EVI1 regulatory elements, since it is located in the proximity of the chromosome 3 breakpoint cluster (Suzukawa et al., 1994; Wieser et al., 2003). Thus, disturbance of the housekeeping function of RPN1 on the remaining normal allele would most likely be deleterious. However, analysis of our 4C-seq profiling data instead revealed substantial interactions between the candidate enhancer and the promoter of GATA2 rather than with the promoter of RPN1 (Figures 5A, 5B, and S5A). GATA2 is a crucial hematopoietic stem cell regulator, located on the contralateral side of the 3q21 breakpoint cluster. This suggests that the candidate enhancer is an original upstream regulatory element for GATA2, rather than RPN1. Hi-C data confirm that the candidate enhancer is together with the GATA2 locus in a topological domain, physically segregated from the more proximal RPN1 promoter (Dixon et al., 2012) (Figure S5B).

Consequently, we first aimed to examine the effect of loss of the candidate enhancer in a human cell line without 3q rearrangements and the functional impact on either RPN1 or GATA2 expression. We generated custom CRISPR/Cas9 nucleases against the candidate enhancer locus in the GATA2-expressing erythroleukemia cell line K562 (Figure 6A). We effectively deleted the candidate enhancer in K562 cells and observed profoundly reduced levels (10.8-fold) of GATA2 mRNA in targeted K562 pools (Figure 6B), whereas RPN1 expression levels remained unchanged. Luciferase GATA2-promoter reporter studies confirmed strong GATA2-specific enhancer activity of the candidate locus in a myeloid context (Figure 6C). Thus, the candidate ectopic enhancer, which upon translocation is repositioned to the EVI1 locus, is a strong enhancer of GATA2 in its original chromosomal context.

Rearrangement of GATA2 Enhancer to EVI1 Causes Functional Haploinsufficiency of GATA2

To study the effects of the enhancer translocation on GATA2 expression in inv(3)/t(3;3) AML samples, we analyzed allele frequencies of informative SNPs in the GATA2 locus by combining 3q-seq and RNA-seq data. This integrative analysis revealed a monoallelic expression pattern of GATA2 in all 36 inv(3)/t(3;3) cases studied (Figures 6D and S6). Non-3q-rearranged AML patient samples and cell lines, as well as variant 3q-rearranged AML cases (e.g., inv(3)(q21q25); t(3;7)(q26;p15)) displayed a normal, biallelic GATA2 expression pattern (data not shown). To ascertain monoallelic GATA2 expression originating from the normal chromosome 3 allele, we performed an allele-specific chromosome conformation capture sequencing approach (see Experimental Procedures for details), in which captured informative SNPs of the GATA2 locus can only be amplified by allele-specific primers on the nonrearranged, linear chromosome 3 template. Results were validated by long-range, breakpoint-spanning PCR and Sanger sequencing. By integration of results from these NGS platforms (3q-seq, RNA-seq, and allele-specific 4C; Figure 6D), we found monoallelic GATA2 expression as a consequence of GATA2 inactivation on the rearranged allele in cases harboring inv(3) or t(3;3). Notably, GATA2 expression levels in primary inv(3)/t(3;3) AML cases and cell lines (n = 78) were found to be significantly reduced as compared to controls (213 AML patients) (Figure 6E). Thus, our data indicate that the inversion/translocation event in inv(3)/t(3;3) malignancies reorganizes an originally upstream regulatory element of the GATA2 domain, causing reduced and monoallelic expression of GATA2.

The 18 kb CTS and p300-Interaction Domain Are Part of a Translocation-Derived Supereenhancer

We have shown that targeting of the candidate enhancer site in inv(3) AML cells by genome-editing techniques is feasible, based on the premises that the enhancer element interacts with the EVI1 promoter, binds the transcriptional coactivator p300, and is embedded in a region of open, potentially regulatory chromatin, and thereby also accessible for endonucleases. However, ChIP-seq data obtained from the inv(3) cell line MOLM-1 manifested a large segment of H3K27ac deposition that extends beyond the entire 18 kb CTS and p300-interaction domain, covering a region of approximately 40 kb (Figures 2A and 7A). These exceptionally large enhancer domains with high levels of H3K27ac and the chromatin regulator BRD4 have recently been characterized as superenhancers (Lovén et al., 2013; Whyte et al., 2013). Using the bioinformatic analysis tool ROSE (Lovén et al., 2013), the 40 kb H3K27ac-deposition region was identified as a superenhancer, ranking second among 291 superenhancers in the MOLM-1 genome (Figures 7A and 7B). RNA-seq analysis revealed the presence of intense read-through
A

![Diagram of TALEN targeting sites and RNA interference]

- CMV
- NLS
- TALEN
- target repeats
- Fok1
- SV40 pA

Human 3q21 locus

p300

1 kb

TAL EN binding sites

EVI1-promoter-interacting region

RPN1

B

![Image of gel electrophoresis]

- TALENs
- left
- right
- both

1559 bp

308 bp

Deletion frequency

2.4%

C

![Bar graphs of normalized mRNA levels]

- EVI1
- GATA2
- RPN1

D

![Images of colony numbers]

- Deletion targeted MUTZ-3 clones
- Control MUTZ-3 clones

- Clone 1
- Clone 2
- Clone 3
- Clone 4
- Clone A
- Clone B
- Clone C
- Clone D

(legend on next page)
enhancer RNAs (eRNAs) spanning the entire superenhancer area including the 18 kb CTS in MOLM-1 (Figure 7A). Of note, read-through transcription commenced precisely at the breakpoint positions, representing the fusion point of 3q21 with 3q26/EVI1 segments. RNA-seq carried out in all available inv(3)/t(3;3) leukemia samples disclosed identical patterns of long read-through areas of eRNAs (Figure 7A). Consistently, BRD4-occupancy was found at the superenhancer site in 3q-rearranged samples, particularly in the p300-interaction domain (Figure S7A). Non-3q-rearranged samples entirely lacked traces of transcriptional read-through (Figure 7A) and exhibited no H3K27ac deposition, or, if any at all, only in a confined 3–4 kb region immediately downstream of the RPN1 gene, as shown by comparison with ENCODE ChIP-seq data of various non-3q-rearranged cell lines (Figures 2A and S2). Furthermore, combining 3q-capture DNA-seq with ChIP-seq data of MOLM-1 showed the presence of informative heterozygous SNPs in the putative 3q21 superenhancer locus on genomic DNA level, whereas the chromatin after H3K27ac pull-down revealed a skew in the allelic ratio of these SNPs in the same locus (Figure 7C). These observations suggest the presence of an active, rearranged superenhancer in inv(3)/t(3;3) leukemia samples, as was previously observed for MYC rearrangements in multiple myeloma (Lovén et al., 2013).

**BET Inhibition Leads to EVI1 Silencing and Growth Arrest of inv(3)/t(3;3) AML Cells**

Our genome-editing results underline that EVI1 is the key oncogenic driver in inv(3)/t(3;3) AML and vulnerable to interference with its ectopic enhancer. As reported previously, BET-bromodomain inhibition of superenhancers represents a novel therapeutic avenue to target genes particularly regulated by superenhancers (Lovén et al., 2013; Whyte et al., 2013). The observation that the p300-binding ectopic EVI1 enhancer is embedded in a large 3q21 superenhancer complex (Figures 7A and 7B) prompted us to investigate whether EVI1 transcription in inv(3)/t(3;3) AMLs is sensitive to enhancer interference by treatment with a BET-bromodomain inhibitor (JQ1). Exposure of MUTZ-3 and MOLM-1 cells, as well as primary inv(3)/t(3;3) AML samples to JQ1 profoundly inhibited proliferation with concentrations >50 nM (Figures 7D and S7C). EVI1-expressing K562 cells (no 3q rearrangement), however, were not responsive to BET-bromodomain inhibition of superenhancer site on genomic DNA level, whereas the chromatin after JQ1 treatment, allowing for transient EVI1 expression, partly rescued MUTZ-3 from JQ1 cytotoxicity, arguing for relative selectivity of JQ1 for the EVI1 superenhancer as opposed to globally inhibiting other putative oncogenic drivers (Figure 7H).

**DISCUSSION**

In summary, inv(3)/t(3;3) chromosomal rearrangements cause dysregulation of two specific AML predisposition genes by aberrant activity of a single enhancer element in its ectopic chromatin environment: (1) Overexpression of EVI1 is caused by inappropriate transcriptional control of the ectopic GATA2 regulatory element, while (2) GATA2 transcriptional impairment results from the removal of that same enhancer from its genomic origin. These dual events mediated by a single enhancer rearrangement, without formation of an oncogenic fusion product, highlight the vulnerability of genome organization into long-range regulatory interaction domains in case of a chromosomal break. The enhancer we identified appears to originally control transcription of the 110 kb distant GATA2 gene at 3q21, and not the nearby gene RPN1. Our finding is in accordance with reports demonstrating a highly homologous—77 kb enhancer element to constitute a component of the murine Gata2 master regulatory complex (Grass et al., 2006) and that this element is indeed leukemogenic via EVI1 activation in transgenic mice harboring the human 3q21q26-rearranged allele (Yamazaki et al., 2014). In case of an inv(3)/t(3;3), the rearranged enhancer engaged in chromatin loops with the EVI1 promoter, in certain samples over a distance of more than 200 kb. Our data emphasize that the function of an enhancer is not only determined by its location, but in particular by its ability to physically bind to an appropriate promoter.
Figure 4. Genomic Enhancer Excision Induces Proliferative and Differentiation Changes in inv(3) AML Cells

(A) Schematic representation of the CRISPR/Cas9 licensing gRNAs with protospacer-adjacent motifs (PAM) highlighted in blue, the target locus, and the donor construct for site-directed homology repair using a conditional, floxed pCMV-GFP selection cassette.

(B) Timeline of genomic targeting of MUTZ-3 AML cells.

(C) Detection of deletion events by genomic PCR of sequential cell fractions. Representative Sanger sequencing tracks of purified PCR amplicons of the GFP-insertion band (2.3 kb) and a remaining lower-running, normal allele band of 1.5 kb size are shown (from GFP+ fraction of day 14), revealing a monoallelic deletion indicated by a loss of heterozygosity of the SNV present in the targeted enhancer locus (red asterisk).

(D) Proliferation of untargeted control and targeted cells was measured by counting of viable cells using trypan blue.

(E) Cell-cycle analysis of control and genome-edited MUTZ-3 cells harvested after 3 weeks of selection.
which can even occur in a different chromosome topology. Our findings show that not RPN1, as reported in the nomenclature of the WHO2008 classification (inv(3)/t(3;3)/RPN1-EVI1), but rather the GATA2 locus is the source of the ectopic enhancer activating EVI1 in this type of leukemia. Besides aberrant EVI1 activation, rewiring of parts of the GATA2 and EVI1 domains led to a reduction of GATA2 expression levels. EVI1 activation in this subtype of AML argues for a primitive HSC defect (de Pater et al., 2013; Goyama et al., 2008; Kataoka et al., 2011; Ling et al., 2004; Orlic et al., 1995; Spinner et al., 2013; Tsai and Orkin, 1997). Since GATA2 is a critical hematopoietic stemness factor, primitive hematopoietic precursors will be particularly susceptible to disturbances of GATA2 homeostasis. Thus, GATA2 deficiency may provide the right spatiotemporal context for EVI1 oncogene activation, i.e., in the right cell at the right stage of differentiation for subsequent malignant transformation. Functional haploinsufficiency arising from inactivating mutations in GATA2 DNA-binding domains or in GATA2 regulatory sequences represents a well-established underlying cause of MDS/AML and Emberger/MonoMAC syndromes (Hahn et al., 2005), of which the latter are characterized by monocytopenia, immune deficiency, and predisposition to myeloid leukemia with frequent monosomy 7. AML with inv(3)/t(3;3) most commonly associates with monosomy 7 and trilineage dysplasia, and, as demonstrated here, it is accompanied by impaired GATA2 expression as well. It will be of particular interest to investigate whether in Emberger and MonoMAC patients 3q26 defects and consequently aberrant EVI1 expression are also drivers of disease progression toward AML/MDS. Of note, the enhancer-containing 3q21 locus is rarely, but consistently involved in other chromosomal rearrangements with PRDM homologs of the EVI1 gene (e.g., BLIMP1/PRDM1 or MEL1/PRDM16) and their aberrant activation (Lugthart et al., 2010). Both disease categories (inv(3)/t(3;3) and other t(3q21) AMLs) resemble each other by their high white blood cell and exceptionally high platelet counts at diagnosis. Further studies using in vivo models are warranted to investigate how the combined effects of GATA2 haploinsufficiency and overexpression of EVI1 or its homologs cooperate in malignant transformation of primitive hematopoietic progenitors.

The ectopic EVI1 enhancer was embedded in a genomic region exhibiting large deposition of active chromatin marks

(F) Immunophenotyping of control and enhancer-targeted MUTZ-3 cells. The left panel includes two dot plots per sample (CD34/CD14 and CD34/cMPO) that show the myelomonocytic maturation. The right panel shows the distribution of the various maturation stages, simplified in three stages: immature (CD34+/CD14-) = blast cell; intermediate (CD34+/CD14+) = promonocyte; mature (CD34-/CD14+) = monocyte.

(G) Representative images of May-Grünwald-Giemsa staining of control and enhancer-targeted MUTZ-3 cells (100× magnification).

(H) Assessment of apoptosis in control (top) and enhancer-targeted MUTZ-3 cells (bottom). Representative flow cytometry plots for Annexin V and 7-AAD staining with percentages for each gate are shown.

See also Figures S3 and S4.

Figure 5. The Nonrearranged Candidate Enhancer Is Part of the GATA2 Enhancer Complex

(A) Representative 4C data (n = 7 biological replicates) showing the local contact profile using a window of 21 with the first p300 peak site as viewpoint (red dashed line).

(B) Integrative 4C analysis using a viewpoint from the GATA2 promoter region (n = 7 biological replicates). In the top panel (main trend), the contact intensity (black line) is calculated by using a running median analysis of normalized read counts with a 5 kb sliding window. The 20th and 80th percentile are visualized as a gray trend graph. In the bottom panel, contact intensities are computed using linearly increasing sliding windows (scaled 2–50 kb) and displayed as a color-coded heatmap of positive 4C signal (maximum of interaction set to 1). Local color changes are log-scaled to indicate changes of statistical enrichment of captured sequences, corresponding to the enhancer-promoter interaction (red dashed lines).

See also Figure S5.
The presence of so-called superenhancers (Whyte et al., 2013), which represent large open chromatin regions of >10 kb in size with key regulatory function for cellular identity and oncogene regulation in cancer. The observation that the GATA2 enhancer region upon translocation had acquired characteristics of a superenhancer, dominantly ranking in the MOLM-1 genome, provided the rationale for treatment with bromodomain/BET inhibitors (Love´ n et al., 2013). The presence of a 3q21 superenhancer might also explain why JQ1 is effective in inv(3)/t(3;3) cell lines as opposed to various non 3q-rearranged AML cell lines with EVI1 overexpression (Zuber et al., 2011). The effects seen after JQ1 treatment recapitulated the observations obtained by genome-editing experiments involving the translocated p300-interaction domain. Remodeling of the cancer genome by and presence of read-through transcripts. This class of DNA elements has recently been recognized as called superenhancers (Whyte et al., 2013), which represent large open chromatin regions of >10 kb in size with key regulatory function for cellular identity and oncogene regulation in cancer.

Figure 6. Loss of the GATA2 Candidate Enhancer Leads to Functional Haploinsufficiency of the Affected GATA2 Allele

(A) Schematic of the CRISPR nuclease design for candidate enhancer targeting. Arrows indicate primer locations for PCR analysis. For each construct, the protospacer sequence and the Cas9-specific proximal-adjacent motif (PAM; magenta highlight) are indicated.

(B) Upon transfection of the candidate enhancer-flanking CRISPR constructs, K562 cells were analyzed by deletion-specific PCR (gel image). Unsorted cells represented pools of CRISPR-targeted and nontargeted cells. GFP-sorted and isolated deletion clones harbored predominantly biallelic deletion mutants. GATA2 and RPN1 mRNA expression was analyzed by qPCR (right). Error bars denote SD.

(C) The p300-binding core enhancer region and an adjacent control region (second p300 peak region) were cloned into a GATA2-promoter luciferase reporter construct, and luciferase activity was measured 48 hr after transfection of indicated cell lines. GATA2+ MUTZ-3 cells, as well as GATA2- nonmyeloid HEK293T and Jurkat cells were assayed. Relative luciferase induction is plotted as fold change compared to enhancer-empty control vector (mean ± SEM).

(D) Integrated analysis of 3q-DNA-seq, RNA-seq, and allele-specific 4C-seq data of a representative inv(3) AML case reveals monoallelic expression of GATA2 mRNA from the intact chromosome 3q21 allele.

(E) GATA2 expression level analysis by qPCR in inv(3)/t(3;3) AML (n = 78) and unselected, non-3q-rearranged AMLs (n = 213; Mann-Whitney-U test, p = 0.002). See also Figure S6.
using in vivo nuclease as applied in this study helped to experimentally validate EVI1 as an oncogenic driver lesion and warranted further pharmacologic experiments interfering with enhancer activity. These experiments emphasized that targeting EVI1 transcriptional regulation using drugs directed against enhancer complexes could have therapeutic potential for this
highly refractory subgroup of AML and diseases driven by similar mechanisms.

**EXPERIMENTAL PROCEDURES**

**Subjects**

Patient recruitment and sample processing were performed according to protocols from the German-Austrian Acute Myeloid Leukemia Study Group (AMLSG trials 06-04, 07-04, HD93A, HD98A/B) and the Dutch-Belgian Hematology/Oncology Cooperative Group (HOVON trials 04/A, 29, 42, 43, 81, 92). All studies were approved through institutional human ethics review board, and all patients provided written informed consent in accordance with the Declaration of Helsinki.

**Generation of TALEN Constructs**

Construction of TALE DNA-binding domains directed to selected genomic loci was performed as described previously (Sanjana et al., 2012). Genomic target coordinates were selected and filtered for off-target sites using the TAL Effector Nucleotide Targeter 2.0 tool (https://tale-tc.taccornell.edu/node/add/talen). Spacer length was defined within a range of 16–20 bp, and repeat array length was set to 20 bp. The NN repeat variable domain targeting base G was chosen in the assembly. In brief, hexamer modules were assembled from a PCR-amplified monomer library using a hierarchical digestion-ligation reaction and subsequently cloned into a full-length TALEN construct. Plasmids were verified by Sanger sequencing and tested for functionality upon transfection in HEK293T cells. To induce a genomic deletion, two TALEN pairs were transfected owing to dimerization requirement of the FokI nuclease for double-strand break formation. Repair of chromat in cleavage at the left/upstream and right/downstream boundaries of the target locus relies on nonhomologous end joining (NHEJ) in the absence of a repair donor and results in the deletion of a TALEN-targeted DNA segment.

**Generation of CRISPR Constructs**

In this study, the RNA-guided endonuclease genome-editing system was employed in experiments involving the cell lines MUTZ-3 and K562 owing to its cell-line-specific superior targeting efficiency compared with TALENs genome-editing approaches (Mali et al., 2013). Publicly available plasmids expressing the CRISPR (clustered regularly interspaced short palindrome repeats)/Cas9 system were used for cloning of targeting constructs following recently published protocols (Cong et al., 2013; Mali et al., 2013). In brief, custom target-specific oligonucleotides were cloned into a chimeric guide RNA array of an hSpCas9-expressing targeting vector. Oligonucleotides for site-specific chromat in cleavage of genomic target regions were designed following described cloning procedures and selected for uniqueness using a bioinformatic filtering tool (http://www.genome-engineering.org/crispr/). To induce segmental deletions of candidate regulatory DNA regions, two CRISPR plasmids were transfected into cells. Each construct was directed to flanking target site positions of the intervening DNA segment for induction of NHEJ-mediated repair upon DSB formation. Cells were screened for deletion events 48 or 72 hr later by mutation-specific PCR analogous to TALEN experiments.

**Clone Screening and Sequencing**

Upon expansion of TALEN- or CRISPR-targeted clones, genomic DNA was isolated with the QuickExtract DNA Extraction Solution (Epicenter) and screened for deletion events by mutation-specific PCR using primers spanning the breakpoint junction. A shift in amplicon size visualized by appearance of a lower running band on gel electrophoresis indicated successful targeting, and candidate clones were subsequently checked for mono-clonality. The native ampiclon and novel fusion fragment of candidate clones were separately purified, and sequences of informative, heterozygous SNVs in the target region were determined by Sanger sequencing. Monoallelic targeting was confirmed by loss of heterozygosity at the SNV-specific nucleotide site. Monoallelic bi-allelic deletion mutants were detected by loss of the native ampiclon and presence of a single, novel fusion fragment represented by the lower running band.

**ACCESSION NUMBERS**

ChiP-seq and RNA-seq data derived from cell lines are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession numbers E-MTAB-2224 and E-MTAB-2225.

4C-seq data and 3q-seq data derived from cell lines have been deposited at the European Nucleotide Archive (ENA, http://www.ebi.ac.uk/ena), which is hosted by the EBI, under accession numbers PRJEB5236 and PRJEB5233. 3q-seq data and RNA-seq data derived from patient specimens have been deposited at the European Genome-phenome Archive (EGA, https://www.ebi.ac.uk/ega/), which is hosted by the EBI, under accession number EGAS00000100669.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, seven figures, two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.02.019.

**AUTHOR CONTRIBUTIONS**


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