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ORIGINAL ARTICLE

C-terminal *BRE* overexpression in 11q23-rearranged and t(8;16) acute myeloid leukemia is caused by intragenic transcription initiation

AE Marneth[1,9], KHM Prange[2,9], ASA Al Hinai[3], SM Bergevoet[1], N Tesi[2], EM Janssen-Megens[2], B Kim[2], N Sharifi[2], ML Yaspo[4], J Kuster[2], MA Sanders[3], ECG Stoetman[3], J Knijnenburg[5], TCJM Arentsen-Peters[6], CM Zwaan[6], HG Stunnenberg[2], MM van den Heuvel-Eibrink[6,7], T Haferlach[8], M Fornerod[6], JH Jansen[1], PJM Valk[3], BA van der Reijden[1,9] and JHA Martens[2,9]

Overexpression of the *BRE* (*brain and reproductive organ-expressed*) gene defines a distinct pediatric and adult acute myeloid leukemia (AML) subgroup. Here we identify a promoter enriched for active chromatin marks in *BRE* intron 4 causing strong biallelic expression of a previously unknown C-terminal BRE transcript. This transcript starts with *BRE* intron 4 sequences spliced to exon 5 and downstream sequences, and if translated might code for an N terminally truncated BRE protein. Remarkably, the new BRE transcript was highly expressed in over 50% of 11q23/*KMT2A* (lysine methyl transferase 2A)-rearranged and t(8;16)/*KAT6A-CREBBP* cases, while it was virtually absent from other AML subsets and normal tissues. In gene reporter assays, the leukemia-specific fusion protein KMT2A-MLLT3 transactivated the intragenic *BRE* promoter. Further epigenome analyses revealed 97 additional intragenic promoter marks frequently bound by KMT2A in AML with C-terminal *BRE* expression. The corresponding genes may be part of a context-dependent KMT2A-MLLT3-driven oncogenic program, because they were higher expressed in this AML subtype compared with other groups. C-terminal BRE might be an important contributor to this program because in a case with relapsed AML, we observed an ins(11;2) fusing *CHORDC1* to *BRE* at the region where intragenic transcription starts in *KMT2A-rearranged* and *KAT6A-CREBBP* AML.

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INTRODUCTION

Oncogene activation contributes to cancer onset and can be associated with treatment response. For instance, in acute myeloid leukemia (AML), high *MECOM* (*MDS1-EVI1 complex*) expression predicts poor outcome.[1] Chromosome 3 rearrangements juxtaposing a *GATA2* super-enhancer to the *MECOM* locus cause *MECOM* overexpression,[2,3] but *MECOM* overexpression also occurs in samples without chromosome 3 rearrangements. *MECOM* activation is caused by independent and recurrent chromosomal abnormalities, strongly suggesting its contribution to disease pathogenesis. Indeed, inhibition of *MECOM* expression resulted in cellular differentiation and growth inhibition in AML models.[2,4–6] *MECOM* overexpression lacking chromosome 3 abnormalities is recurrently found in AML with *KMT2A* (lysine methyl transferase 2A, *MLL*) translocations. *MLLT3* (*mixed-lineage leukemia translocated to chromosome 3*, *AF9*) is the most frequent fusion partner of *KMT2A*[7,8] and KMT2A-MLLT3 may contribute to *MECOM* overexpression.[9] Since the majority of *KMT2A-MLLT3* AML samples lack *MECOM* overexpression,[10,11] *MECOM* activation by KMT2A-MLLT3 may be context dependent. Earlier we reported that *KMT2A-MLLT3* AML is characterized by mutually exclusive *MECOM* and *BRE* (*brain and reproductive organ-expressed*) overexpression.[10–12] These findings are relevant, because both pediatric and adult *KMT2A-MLLT3* AML with *BRE* overexpression are associated with a favorable outcome.[10–12] *BRE* is relatively highly expressed in blood cells in general, suggesting an important function in hematopoiesis. BRE functions in BRCA-1 (breast cancer 1)-mediated DNA damage repair, BRISC (BRCC3 isopeptidase complex)-mediated deubiquitination and in protection from apoptosis and senescence.[13–20] Its exact function in hematopoiesis remains to be determined. Importantly, it is currently unknown how *BRE* overexpression is caused in *KMT2A-MLLT3* AML. Here, we generated RNA-sequencing (RNA-seq) and chromatin immunoprecipitation-sequencing (ChIP-seq) profiles from *KMT2A-MLLT3* samples to obtain more insight into the mechanisms causing *BRE* overexpression.

MATERIALS AND METHODS

Human samples

Bone marrow and peripheral blood samples were collected at diagnosis and relapse. The study was performed in accordance with the Code of

[1]Department of Laboratory Medicine, Laboratory of Hematology, Radboud Institute for Molecular Life Sciences (RIMLS), Radboud University Medical Center, Nijmegen, The Netherlands; [2]Department of Molecular Biology, Faculty of Science, RIMLS, Radboud University, Nijmegen, The Netherlands; [3]Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands; [4]Max Planck Institute for Molecular Genetics, Berlin, Germany; [5]Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands; [6]Pediatric Oncology/Hematology, Erasmus University Medical Center-Sophia Children's Hospital, Rotterdam, The Netherlands; [7]Princess Maxima Center for Pediatric Oncology, Utrecht, The Netherlands and [8]MLL Munich Leukemia Laboratory, Munich, Germany. Correspondence: Dr BA van der Reijden, Department of Laboratory Medicine, Laboratory of Hematology, Radboud Institute for Molecular Life Sciences (RIMLS), Radboud University Medical Center, Geert Grooteplein zuid 8, Nijmegen 6525GA, The Netherlands or Dr JHA Martens, Department of Molecular Biology, Faculty of Science, Radboud Institute for Molecular Life Sciences, Radboud University, Geert Grooteplein zuid 8, 6525 GA Nijmegen, The Netherlands.
E-mail: Bert.vanderReijden@radboudumc.nl or J.Martens@ncmls.ru.nl
[9]These authors contributed equally to this work.
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Conduct for Responsible use of Human Tissue in Medical Research and institutional guidelines and regulations (institutions and ethical approval numbers, Radboudumc: CMO 2013/064 and 2010/348, Erasmus University Medical Center: MEC2015-155, Munich Leukemia Laboratory: Bavarian Medical Association 05117 and Stichting Kinderoncologie Nederland (SKION)). For many samples used in this study, sample collection has been described before.[11,21–23] Table 1 summarizes characteristics of samples used for ChIP-seq and RNA-seq studies.

Cell culture, RNA isolation, cDNA synthesis, RT-(q)PCR, 5′-RACE and luciferase reporter assays

Extensive methods for cell culture, RNA isolation, cDNA synthesis, real-time quantitative reverse transcription PCR (RT-(q)PCRs), 5′-Rapid Amplification of cDNA Ends (RACE) and luciferase reporter assays are described in the Supplementary Information.

ChIP, ChIP-seq and Illumina high-throughput sequencing

Methods for H3K4me1, H3K4me3, H3K9me3, H3K27ac, H3K27me3, MECOM and KMT2A ChIP-seq/qPCR, RNA-seq and bioinformatic analyses are described in the Supplementary Information.

RESULTS

The epigenome of human *KMT2A-MLLT3* AML

To gain more insight into the epigenetic regulation of the *MECOM* and *BRE* loci in *KMT2A-MLLT3* AML, we generated H3K4me1, H3K4me3 and H3K27ac ChIP-seq profiles from two primary *KMT2A-MLLT3* AML samples (Figure 1a, one *MECOM* and one *BRE* overexpression (patients P1a and P2a; Table 1)). This revealed ~30 000 and ~26 000 active promoters (H3K27ac/H3K4me3) as well as ~40 500 and ~46 000 active enhancers (H2K27ac/H3K4me1) for P1a and P2a, respectively. The relative abundance and genomic distribution of active promoters and active enhancers was similar between P1a and P2a (Figure 1b and Supplementary Figure 1a). The common promoter and enhancer regions (>65% of active promoters/enhancers) showed comparable average ChIP-seq signal intensities (Figure 1b). In contrast, patient-specific active promoters and enhancers showed markedly higher average signals (Supplementary Figures 1B and C). Remarkably, for H3K4me3/H3K27ac peaks, only 50% overlapped with annotated (RefSeq hg19) promoters, suggesting that *KMT2A-MLLT3* samples might harbor many alternate transcription start sites. Thus, the epigenomes of *MECOM*- and *BRE*-overexpressing *KMT2A-MLLT3* AML samples are comparable, and also contain unique features.

Deposition of active histone marks at annotated promoters *in MECOM*-overexpressing *KMT2A-MLLT3* AML

The sample with *MECOM* overexpression exhibited higher H3K4me3 signals than the sample with *BRE* overexpression at the two annotated *MECOM* promoter regions (Supplementary Figure 1D). Additionally, H3K27ac was only detected at the H3K4me3 peaks in P1a, while no signal was found in P2a, which is in line with the low *MECOM* expression in P2a. These observations were subsequently confirmed in additional primary *KMT2A-MLLT3* AML samples: by H3K4me3 ChIP-seq ($n=2$ *MECOM*, $n=5$ *BRE* overexpression), RNA-seq ($n=2$ *MECOM*, $n=2$ *BRE* overexpression) and a H3K27ac ChIP-seq profile (*BRE* overexpression, P4; Supplementary Figure 1E). Taken together, these results show that *MECOM* overexpression is associated with increased deposition of active promoter marks at its two annotated promoters.

Intragenic promoter marks in *BRE* in *KMT2A-MLLT3* AML

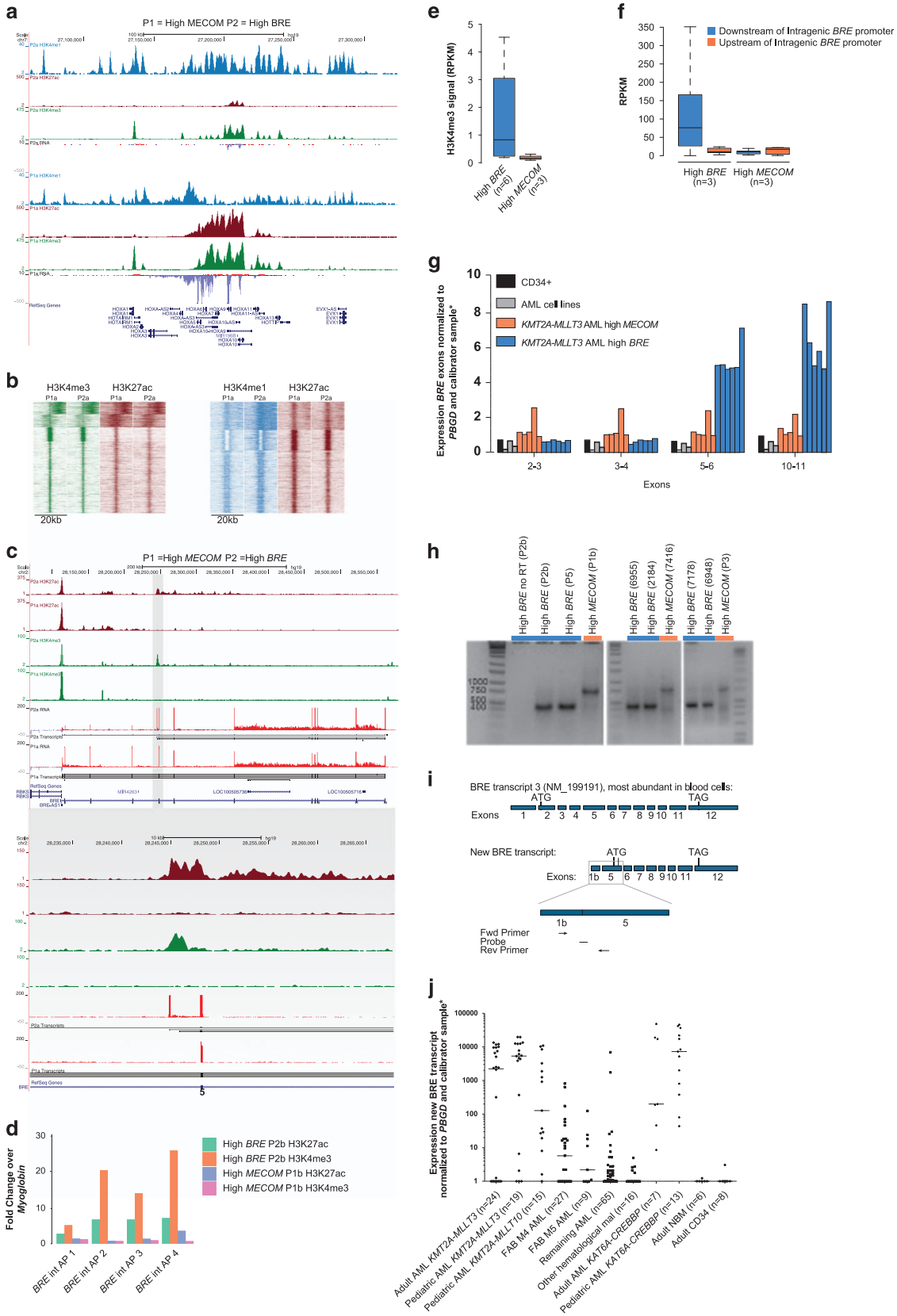
Subsequently, the *BRE* locus was examined. No differences were detected in active enhancers around the *BRE* locus between the two initial *KMT2A-MLLT3* AML samples (not shown). Interestingly, apart from enrichment on the annotated promoter, the sample

Table 1. Characteristics of *KMT2A-MLLT3* samples used for ChIP-seq and RNA-seq experiments

Sample number	*Age (years)*	*Sex*	*Tissue*	*Blast percentage*	*High* BRE/MECOM	*ChIP-seq/RNA-seq*	*Study number*
P1a/b[a]	25	Male	BM	96	High *MECOM*	RNA-seq,[25] H3K4me1 (only P1a), H3K4me3, H4K9me3 (only P1a), H3K27ac (only P1a), H3K27me3 (only P1a) ChIP-seq	AML_MLLAF9_1[25]
P2a/b[a]	27	Female	BM	62	High *BRE*	RNA-seq,[25] H3K4me1 (only P2a), H3K4me3, H3K9me3 (only P2a), H3K27ac (only P2a), H3K27me3 (only P2a) ChIP-seq	AML_MLLAF9_4[25]
P3	45	Female	BM	45	High *MECOM*	RNA-seq,[25] H3K4me3 ChIP-seq	AML_MLLAF9_3[25]
P4	40	Female	BM	95	High *BRE*	KMT2A,[25] H3K4me3, H3K27ac ChIP-seq	5358[21]
P5	1	Male	BM	45	High *BRE*	RNA-seq,[25] H3K4me3 ChIP-seq	AML_MLLAF9_2[25]
P6	69	Female	BM	89	High *BRE*	H3K4me3 ChIP-seq	11-030076
P7	77	Male	BM	76	High *BRE*	H3K4me3 ChIP-seq	12-040773

Abbreviations: BM, bone marrow; *BRE, brain and reproductive organ-expressed*; ChIP-seq, chromatin immunoprecipitation-sequencing; *KMT2A-MLLT3, lysine methyl transferase 2A-mixed-lineage leukemia translocated to chromosome 3*; *MECOM, MDS1-EVI1 complex*; RNA-seq, RNA-sequencing. [a]Samples a and b are technical replicates.

with *BRE* overexpression contained intragenic active promoter marks (H3K4me3/H3K27ac) in *BRE* intron 4 that were absent in the sample with normal *BRE*/high *MECOM* expression (Figure 1c). These data were confirmed using ChIP-qPCRs on two *KMT2A-MLLT3* cell lines and primary samples (Figure 1d and Supplementary Figure 2A). Analysis of all H3K4me3 ChIP-seq



profiles revealed that 5/6 samples with *BRE* overexpression harbored an intragenic H3K4me3 peak in *BRE* (the peak in P6 was not called, likely due to the higher background levels), whereas none of the three samples with *MECOM* overexpression did (Figure 1e and Supplementary Figures 2B and C). A second H3K27ac profile generated from a sample with *BRE* overexpression (P4) also showed a clear peak in the same region (not shown). These results show that the *BRE* intragenic active promoter marks were specific to *KMT2A-MLLT3* samples with *BRE* overexpression.

Intragenic transcription activation causes high downstream *BRE* expression in *KMT2A-MLLT3* AML

Next, we investigated whether the intragenic *BRE* promoter was accompanied by the expression of a specific transcript. Indeed, RNA-seq from sample P2a (*BRE* overexpression) showed expression of intronic sequences adjacent to the active promoter marks, ~3 kb upstream of exon 5 (Figure 1c). Downstream exons were expressed at a higher level compared with upstream exons. RNA-seq and RT-qPCR in a larger cohort showed that exons upstream of the intragenic promoter were equally expressed in all KMT2A-MLLT3 samples (Figures 1f and g). In contrast, downstream exons were five- to ninefold higher expressed in samples with *BRE* versus *MECOM* overexpression (Figure 1g). The start of the new BRE transcript and fusion of intragenic reads to exon 5 as determined by RNA-seq (Figure 1c) was confirmed by 5′-RACE (Figure 1h) followed by Sanger sequencing. The new transcript contained a ~100 bp region within intron 4 (exon 1b) fused to exon 5. As several samples harbored a heterozygous single-nucleotide polymorphism within exon 1b, which was expressed, we could determine that alternate BRE expression was biallelic (not shown). Besides exons 1b and 5, the novel BRE transcript contained exons 6–12 explaining high expression of these downstream exons (Figures 1c and i and not shown). Importantly, the new transcript was not detected by 5′-RACE in three KMT2A-MLLT3 samples with *MECOM* overexpression. Instead, the expected transcript containing BRE exons 1–7 was amplified (~870 bp; Figure 1h). Exon 5 codes for two methionines that potentially serve as translation start sites (Figure 1i), which, if used, might result in the formation of a 138 or 154 amino-acid N terminally truncated BRE protein. In conclusion, these results show that *BRE* overexpression in KMT2A-MLLT3 AML is caused by biallelic intragenic transcription activation from an alternate promoter in intron 4.

To study whether MECOM directly repressed the intragenic *BRE* promoter, we performed MECOM ChIP-qPCRs in a KMT2A-MLLT3 sample with high *MECOM* expression. The *SPI1* enhancer region showed a clear enrichment for MECOM compared with control regions (Supplementary Figure 3A). MECOM binding was also found on the normal *BRE* promoter, yet no enrichment was detected at the intragenic *BRE* promoter (Supplementary Figure 3A). This suggests that MECOM does not directly repress the intragenic *BRE* promoter. Additionally, there is no indication for heterochromatin formation at the intragenic *BRE* promoter in samples with high *MECOM* expression, as the repressive promoter marks H3K27me3 and H3K9me3 were not detected (Supplementary Figure 3A).

Novel BRE transcript in *KMT2A*-rearranged and *KAT6A-CREBBP* AML

Our next goal was to study whether *BRE* intragenic transcription activation is unique to *KMT2A-MLLT3* AML. We previously described samples with *BRE* overexpression without detectable rearrangements involving *KMT2A*.[12] To determine whether *BRE* overexpression in these samples is caused by intragenic transcription activation, we performed 5′-RACE on two such samples (patients 2184 and 6948 from Valk *et al*.[21]). Identical fragments were identified in these samples as in *KMT2A-MLLT3* AML with *BRE* overexpression (Figure 1h), suggesting that expression of the new BRE transcript may occur independently of *KMT2A* rearrangements. We developed an RT-qPCR for detection of the new BRE transcript (Figure 1i) to determine its prevalence in AML in general (Figure 1j). The new BRE transcript was highly expressed in approximately half of the adult *KMT2A-MLLT3* (13/24) as well as ~70% and ~50% of pediatric *KMT2A-MLLT3* (13/19) and *KMT2A-MLLT10* AMLs (7/15), respectively. As most *KMT2A*-rearranged AML samples have French–American–British M4 or M5 morphology, additional samples were screened for expression of the novel BRE transcript. Two of the 36 M4/M5 samples exhibited high expression of the new BRE transcript. Thus, morphological classification *per se* does not correlate with high expression of the new BRE transcript. The novel BRE transcript was only sporadically highly expressed in remaining AML samples (2/65) and was not found in other hematological malignancies ($n = 16$; Figure 1j).

As *BRE* overexpression was previously detected in one *KAT6A-CREBBP* AML sample,[11] additional samples were screened for the new BRE transcript. Indeed, 3/7 adult and 10/13 pediatric *KAT6A-CREBBP* samples exhibited high new BRE transcript levels (Figure 1j). The new BRE transcript was hardly detected in normal primary hematopoietic cells (Figure 1j and 20 different human tissues, not shown). Alternate BRE expression or the intragenic *BRE* H3K4me3/H3K27ac peak that we identified were not found in publicly available databases (not shown). We conclude that high expression of the new BRE transcript is largely confined to *KMT2A*-rearranged and *KAT6A-CREBBP* AML and is not present in healthy hematopoietic or non-hematopoietic cells.

Figure 1. Intragenic transcription activation in *BRE* in primary *KMT2A-MLLT3* samples with *BRE* overexpression. (**a**) Representation of the *HOXA* locus, a well-known KMT2A-MLLT3 target. H3K4me1, H3K27ac and H3K4me3 ChIP-seq profiles of two primary *KMT2A-MLLT3* AML samples: P1a (*MECOM* overexpression) and P2a (*BRE* overexpression). (**b**) ChIP-seq signal intensity at active promoter loci (H3K4me3/H3K27ac, left) and active enhancer loci (H3K4me1/H3K27ac, right) for samples P1a and P2a. (**c**) ChIP-seq and RNA-seq results at the *BRE* locus showing an intragenic active promoter (H3K27ac/H3K4me3, gray box) and enhanced expression of adjacent and downstream sequences in P2a but not P1a. (**d**) H3K4me3/H3K27ac ChIP-qPCRs at the *BRE* intragenic active promoter (int AP) over background (*Myoglobin*) in P2b and P1b. Four different qPCRs were used. (**e**) Normalized H3K4me3 signal at the *BRE* intragenic active promoter in *KMT2A-MLLT3* profiles ($n = 6$ *BRE* overexpression, $n = 3$ *MECOM* overexpression). (**f**) Expression of *BRE* downstream and upstream exons relative to the intragenic active promoter based on RNA-seq data from three samples with *BRE* overexpression (P2a, P2b and P5) and three samples with *MECOM* overexpression (P1a, P1b and P3). (**g**) Expression of *BRE* exons 2–3, 3–4, 5–6 and 10–11 (RT-qPCR) normalized to *PBGD* expression and a *KMT2A-MLLT3* AML sample with *MECOM* overexpression (calibrator sample, P3). AML cell lines: NB4, OCI-AML3 and THP-1. (**h**) 5′-RACE using *BRE* exon 8 reverse primer for cDNA synthesis and *BRE* exon 7 reverse primer for PCR. Samples 6955, 2184, 7416, 7178 and 6948 are from Valk *et al*.[21] (**i**) Schematic overview of the most abundant BRE transcript in blood cells (NM_199191) and the new BRE transcript. The new BRE transcript contains two potential translation start sites in exon 5 that are in-frame with the protein encoded by transcript NM_199191. RT-qPCR primers and probe used in (**j**) are indicated. (**j**) Expression of the new BRE transcript (RT-qPCR) in indicated samples normalized to *PBGD* expression and a calibrator sample (P3). The novel BRE transcript was higher expressed in 'Adult *KMT2A-MLLT3*', 'Pediatric *KMT2A-MLLT3*', 'Pediatric *KMT2A-MLLT10*', 'FAB M4 AML', 'Adult *KAT6A-CREBBP*' and 'Pediatric *KAT6A-CREBBP*' samples compared with 'remaining AML' samples ($P < 0.004$). Mal, malignancies.

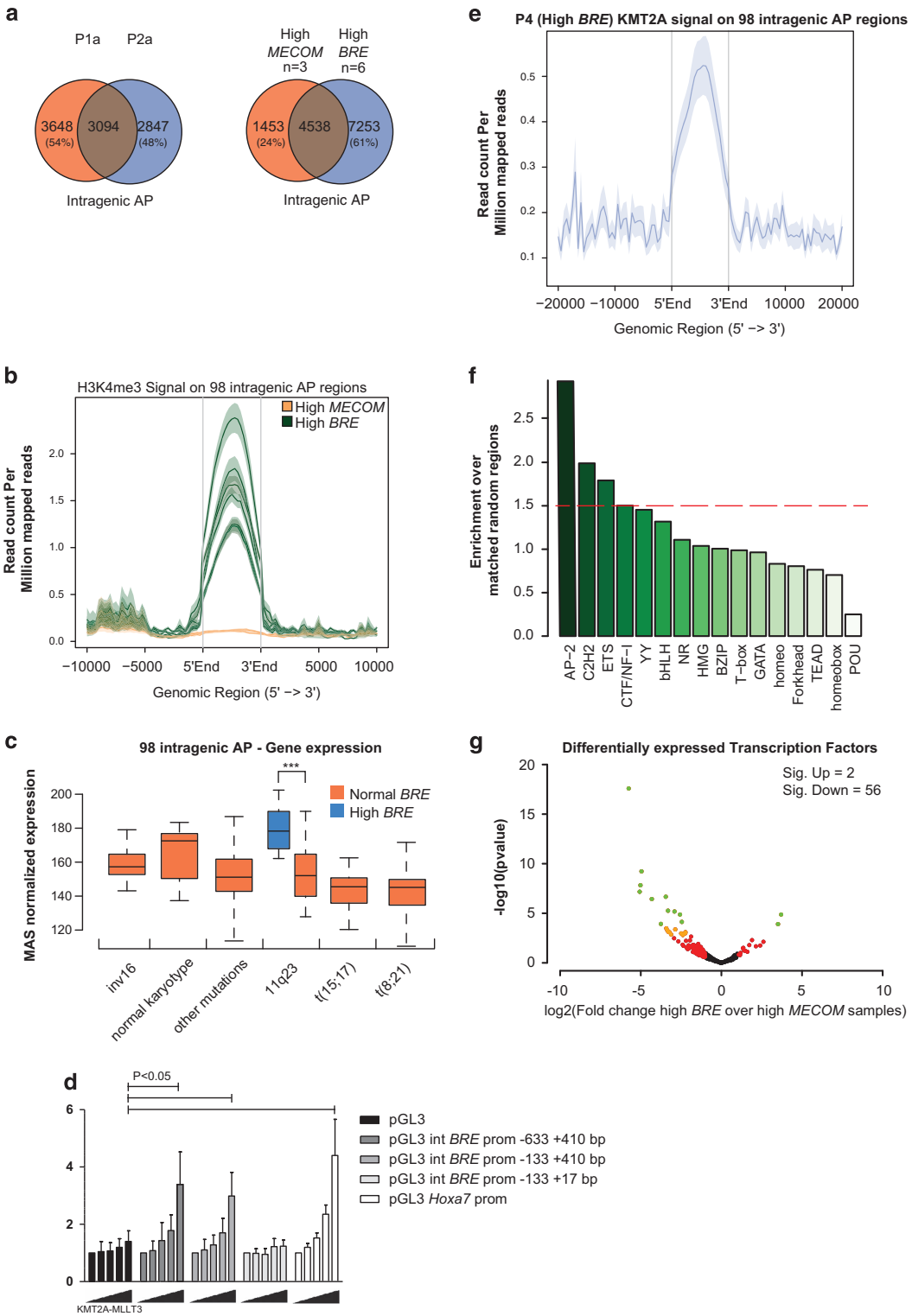


Figure 2. Genome-wide intragenic active promoter marks in *BRE*-overexpressing primary *KMT2A-MLLT3* samples. (**a**) Overlap of intragenic active promoter peaks in *KMT2A-MLLT3* samples with *MECOM* overexpression (P1a) and *BRE* overexpression (P2a, left). Overlap of intragenic H3K4me3 peaks including an additional five samples with high *BRE* expression and two samples with high *MECOM* expression (right). (**b**) Average H3K4me3 signal at 98 intragenic active promoter sites for nine *KMT2A-MLLT3* AML samples (*BRE* overexpression $n = 6$, *MECOM* overexpression $n = 3$). (**c**) Expression of 90 genes containing 98 intragenic active promoter marks, in various subtypes of AML. (**d**) Reporter assays showing Renilla normalized Firefly luciferase signal from a control vector (pGL3 basic), from the *Hoxa7* promoter and from three fragments of the new *BRE* promoter upon increasing concentrations of KMT2A-MLLT3. Mean ± s.d. (**e**) Average KMT2A signal at 98 intragenic promoter mark regions (**b** and Supplementary Figure S3B) in a *KMT2A-MLLT3* sample with *BRE* overexpression (P4). (**f**) TF family motifs enriched under 98 intragenic active promoter marks. (**g**) Differential expression of TFs belonging to enriched motif families (AP-2, C2H2 and ETS, panel f) in *BRE*-overexpressing versus *MECOM*-overexpressing samples. Red dots: ($n = 168$) fold change > 2; orange dots: ($n = 23$) fold change > 2, Bonferonni-adjusted *P*-value < 0.05; green dots: ($n = 35$) fold change > 4, Bonferonni-adjusted *P*-value < 0.01. AP, active promoter; int *BRE* prom, intragenic *BRE* promoter; Sig., significant; TF, transcription factor.

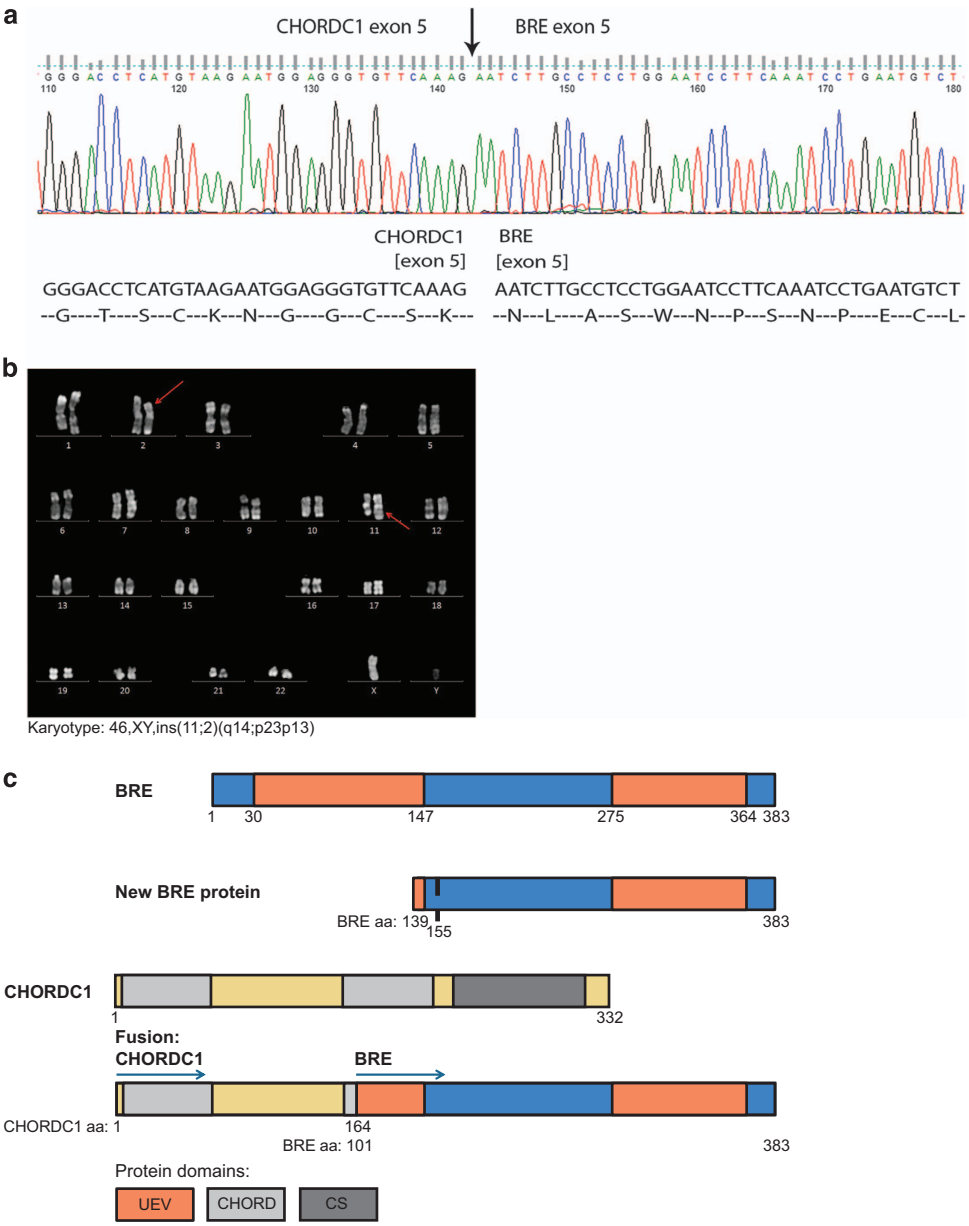


Figure 3. A novel t(11;2) translocation fuses *CHORDC1* exon 5 to *BRE* exon 5. (**a**) Sanger sequencing of the CHORDC1-BRE fusion transcript. (**b**) Karyotype of AML at the time of relapse: 46,XY,ins(11;2)(q14;p13p23)[10]. (**c**) Schematic representation of the full-length and predicted new BRE proteins, CHORDC1 and the CHORDC1-BRE fusion protein (UEV: ubiquitin E2 variant, CHORD: cysteine- and histidine-rich, CS: interaction module named after CHORD-containing proteins and SGT1). Amino-acid numbers refer to proteins encoded by BRE transcript 3 (NM_199191) and CHORDC1 transcript 1 (NM_012124).

Genome-wide intragenic promoter marks in *BRE*-overexpressing *KMT2A-MLLT3* AML

To examine whether intragenic transcription activation is unique for *BRE*, we performed genome-wide analyses revealing ~7000 H3K4me3/H3K27ac intragenic peaks that were specific for cases with *BRE* overexpression (Figure 2a). To avoid false positives, we narrowed down the group of specific intragenic active promoters among others by including only those with a marked difference in the average H3K4me3 signal (Figure 2b and Supplementary Figures 3B and C). This analysis yielded intragenic active promoter marks that were unique to samples with *BRE* overexpression in 98 regions, residing in 90 genes (Figure 2b, Supplementary Figure 3C and Supplementary Table 1). For *MECOM*-overexpressing samples, similar filtering revealed eight (Supplementary Figure 3B and Supplementary Table 1) specific intragenic active promoter regions. In conclusion, *BRE*-overexpressing *KMT2A-MLLT3* AML is characterized by specific genome-wide intragenic active promoters.

Genes with intragenic active promoter marks are activated in *BRE*-overexpressing *KMT2A-MLLT3* AML

We analyzed the expression levels of the 90 genes with intragenic promoters in a large adult AML cohort.[24] This showed significantly higher expression of these genes in *BRE*-overexpressing samples as opposed to 11q23/*KMT2A*-rearranged *MECOM*-overexpressing samples ($P = 0.015$) and a trend towards higher expression compared with other genetically defined patient subsets (Figure 2c). Remarkably, RNA-seq profiles described above indicated that only five (*BRE*, *PAN3*, *SMYD3*, *MS4A7* and *TGM5*) of the 90 genes with intragenic promoters had $\geqslant$2-fold increased

expression of downstream versus upstream exons relative to their intragenic active promoter mark (not shown). These data suggest that intragenic active promoter marks are found in actively transcribed genes, but do not necessarily cause transcription initiation at that position. Alternatively, intragenic transcription initiation might occur, but the expression or stability of downstream sequences may be so low that it does not result in a significant increase in read count.

The high expression of downstream *SMYD3*, *PAN3* and *TGM5* sequences in *KMT2A-MLLT3* samples with versus without *BRE* overexpression was confirmed by RT-qPCR, but this was not found for *KAT6A-CREBBP* AML samples (Supplementary Figure 3D). These results could imply that *KAT6A-CREBBP* and *KMT2A-MLLT3* AML do not harbor a similar genome-wide intragenic active promoter signature.

KMT2A-MLLT3 transactivates the intragenic *BRE* promoter

To determine whether KMT2A-MLLT3 contributes to intragenic *BRE* transcription, gene reporter assays were performed. Both the *Hoxa7* promoter (positive control) and *BRE* fragments containing sequences from +17 to +410 bp relative to the novel transcription start site were transactivated in a dose-dependent manner by KMT2A-MLLT3, whereas a fragment containing the region −133 to +17 bp was not transactivated (Figure 2d). To determine whether KMT2A or KMT2A-fusion proteins regulate the 98 intragenic active promoter marks, we analyzed three KMT2A ChIP-seq profiles,[25] one from a primary *KMT2A-MLLT3* sample with *BRE* overexpression and two from the *KMT2A*-rearranged cell lines THP-1 and MV4-11 (Supplementary Figure 4A). KMT2A signals were enriched at the 98 intragenic active promoter marks in all three samples (Figure 2e and Supplementary Figure 4B). The level of KMT2A enrichment at the 98 intragenic promoter marks was similar to KMT2A enrichment in previously defined[25] KMT2A-fusion binding sites in THP-1 and MV4-11 cells (Supplementary Figures 4C and D). These data suggest that KMT2A/KMT2A-fusion proteins bind to the intragenic active promoter marks to contribute to high expression of the respective genes.

AP-2, C2H2-Znf and ETS TF motif enrichment at intragenic promoter marks

As only part of the *KMT2A-MLLT3* AML samples have *BRE* overexpression, other factors besides KMT2A-MLLT3 might be involved in expression of the new BRE transcript. To identify candidates that may bind intragenic active promoter marks, we performed transcription factor (TF) motif analysis. This showed enrichment of motifs for AP-2, C2H2-Znf and ETS TF families (Figure 2f). RNA-seq data from two *BRE*- and two *MECOM*-overexpressing samples revealed significant differential expression of 58 TFs belonging to these families (Figure 2g). Only two TFs were higher expressed in *BRE* compared with *MECOM*-overexpressing samples (PLAG1 and ZNF595). Possibly, PLAG1 and ZNF595 are involved in enhancing transcription of the 90 genes with intragenic promoters.

A novel ins(11;2) fuses *CHORDC1* to *BRE* exon 5

As described above, the *BRE* gene is actively transcribed from an intragenic promoter preceding exon 5. To find other changes in *BRE* in AML, we screened an RNA-seq library (32 paired diagnosis-relapse samples) for alterations in the *BRE* gene. Interestingly, an in-frame fusion transcript between *CHORDC1* exon 5 (chromosome 11) and *BRE* exon 5 (chromosome 2) was found in a relapsed AML case. This fusion was confirmed by RT-PCR followed by Sanger sequencing, and an insertion of chromosome 2 into 11 was confirmed by karyotyping (Figures 3a and b). Heterozygous Chordc1 (Morgana) inactivation in mice results in spontaneous atypical chronic myeloid leukemia.[26] The CHORDC1 fusion to BRE exon 5 is remarkable because the alternate BRE transcript starts with exon 1b also fused to exon 5. This means that if the CHORDC1-BRE and alternate BRE transcripts would be translated, they might contain a similar C-terminal part of the BRE protein (Figure 3c). *BRE* and *CHORDC1* were equally expressed in the diagnosis and relapse samples (stranded RNA-seq data, not shown). The CHORDC1-BRE fusion was not detected at diagnosis nor in 200 randomly selected *de novo* AML cases (not shown), indicating that this fusion is rare in *de novo* AML and may be associated with relapsed AML. Thus, leukemia-specific expression of the C terminus of *BRE* may be caused by intragenic transcription activation or chromosomal insertions.

DISCUSSION

Here, we report abnormal expression of C-terminal *BRE* sequences specific for AML, caused by distinct, non-random events, by a chromosomal insertion and by an intragenic transcription initiation in 11q23/*KMT2A*-rearranged and t(8;16)/*KAT6A-CREBBP* AML. The intragenic transcriptional activation cannot be explained by recurrent chromosomal abnormalities affecting the *BRE* locus itself, because the alternate transcript was expressed from both alleles. Instead, the oncofusion protein KMT2A-MLLT3 could be involved in intragenic *BRE* activation. In *KMT2A-MLLT3* AML harboring the intragenic *BRE* promoter, we observed active intragenic promoter marks in 89 additional genes. KMT2A/KMT2A-fusion protein enrichment was observed at these promoter marks and the corresponding genes were overexpressed in *KMT2A*-rearranged AML with high *BRE* expression. Moreover, KMT2A-MLLT3 activated the intragenic *BRE* promoter in reporter assays. These findings suggest that KMT2A-MLLT3 contributes to alternate transcription initiation in *BRE*. It should, however, be noted that especially in adult AML a significant number of samples with these two fusion oncoproteins lack high expression of the new BRE transcript. Thus, intragenic *BRE* activation may be dependent on additional factors. Motif analyses of *KMT2A-MLLT3* AML showed an overrepresentation of AP-2, C2H2-Znf, and ETS TF binding sites at intragenic promoter marks with C2H2-Znf TFs PLAG1 and ZNF595 being highly expressed in *BRE* overexpression cases. It would be interesting to test the contribution of especially PLAG1 to alternate *BRE* expression, as PLAG1 has been implicated in AML development.[27,28]

Earlier we reported that patients with *BRE* overexpression have a superior outcome compared with *MECOM*-overexpressing patients. The unique 5′ end of the novel BRE transcript allows for reliable detection by RT-qPCR enabling an optimal recognition of both subtypes. Importantly, the association of alternate *BRE* expression with good clinical outcome appears to be restricted to *KMT2A*-rearranged AML, as patients with t(8;16)/*KAT6A-CREBBP* have a very dismal outcome in general.

KMT2A-MLLT3 and KAT6A-CREBBP may contribute to AML pathogenesis by altering gene expression.[29–34] We observed that the genes with active intragenic promoter marks were bound by KMT2A/KMT2A-fusion proteins, and, indeed, the respective genes showed highest expression compared with other AML subtypes. These genes might represent a newly identified oncogenic program. It is tempting to speculate that alternate BRE contributes to this oncogenic program for various reasons. Abnormal *BRE* expression is exclusively observed in AML and caused by distinct mechanisms. The novel BRE transcript contains two putative translation start sites in exon 5, which if used might possibly lead to N terminally truncated BRE isoforms. The in-frame CHORDC1-BRE fusion includes a similar C-terminal part of BRE and Chordc1 functions as a tumor suppressor in mice.[26] BRE is required for proper function of the BRCA-1-A and BRISC complexes, which are involved in DNA repair and cell cycle regulation.[14,17–20,35,36] Another important constituent of these complexes is BRCC3. Mutations in *BRCC3* and other components of these two

complexes have recently been observed in myeloid malignancies, including AML.[37] *BRE* overexpression is also found in other malignancies and correlates with higher grade tumors.[38,39] In addition to AML, BRE has prognostic impact in breast cancer and a translocation including downstream *BRE* sequences has been observed in adrenocortical carcinoma.[40,41] We conclude that it will be interesting to determine whether leukemia-specific alternate BRE transcripts are translated into protein and contribute to disease pathogenesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

AEM, KHMP, BAR and JHAM coordinated research and wrote the manuscript. AEM, KHMP and SMB conducted and analyzed most experiments. KHMP, EMJM, BK, NS, MLY, JK, HGS and JHAM contributed to ChIP-seq and RNA-sequencing of *KMT2A-MLLT3* samples (BLUEPRINT consortium). KHMP, NT and MAS did bioinformatic analyses. ASAAH, ECGS, JK, MAS and PJMV detected CHORDC1-BRE fusion. TCJMA-P, CMZ, HGS, MMH-E, TH, MF, JHJ, PJMV, BAR and JHM provided patient samples and funding. All authors critically revised the paper and approved the final version.

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