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# *KMT2A-MAML2* Rearrangement Emerged and Regressed During Neuroblastoma Therapy without Leukemia After 12.8-Year Follow-up

**Carolyn A. Felix**[1,2,9], **Diana J. Slater**[1,3], **James W. Davenport**[1], **Xiang Yu**[4], **Brian D. Gregory**[4], **Marilyn M. Li**[2,5,6,7], **Eric F. Rappaport**[3], **Nai-Kong V. Cheung**[8]

[1]Division of Oncology, The Children's Hospital of Philadelphia, Center for Childhood Cancer Research, Philadelphia, PA 19104

[2]Department of Pediatrics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104

[3]Nucleic Acids and PCR Core Facility, The Children's Hospital of Philadelphia, Philadelphia, PA 19104

[4]Biology Department, University of Pennsylvania, Philadelphia, PA 19104

[5]Department of Pathology and Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, 19104

[6]Division of Genomic Diagnostics, Children's Hospital of Philadelphia, Philadelphia, PA, 19104

[7]Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia, Philadelphia, PA, 19104

[8]Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

## Abstract

[9]Corresponding Author: Carolyn A. Felix, M.D., Professor of Pediatrics, Joshua Kahan Endowed Chair in Pediatric Leukemia Research, Division of Oncology, The Children's Hospital of Philadelphia, Colket Translational Research Building, Room 4006, 3501 Civic Center Blvd., Philadelphia, PA 19104-4318, (215) 590-2831, felix@chop.edu.

**Current Affiliations**: D.J.S., Center for Applied Genomics, The Children's Hospital of Philadelphia; X.Y., School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai, China

ETHICS STATEMENT

Molecular analyses were performed under IRB approved protocols of the Children's Hospital of Philadelphia (Protocol 99-001792) and MSK (Clinicaltrials.gov NCT00588068), and written consent was properly documented.

CONFLICT OF INTEREST STATEMENT

C.A.F. and E.F.R. are named inventors on the following issued patent filed by the Children's Hospital of Philadelphia that has not been licensed: Methods and Kits for Analysis of Chromosomal Rearrangements Associated with Leukemia. United States of America 6,368,791. 2002 April 09. C.A.F. is a named inventor on the following issued patents filed by the Children's Hospital of Philadelphia that have not been licensed: Compositions and Methods for the Detection of DNA Topoisomerase II Complexes with DNA. United States of America 8,642,265 B2. 2014 February 04. CYP3A4 NFSE Variant and Methods of Use Thereof - United States of America 6,174,684. 2001 January 16. N-K.C. is the inventor and owner of issued patents licensed by MSK to Y-mabs Therapeutics, Biotec Pharmacon, and Abpro-labs. Hu3F8 and 8H9 were licensed by MSK to Y-mabs Therapeutics.

N-K.C. reports receiving commercial research grants for work outside of this work from Y-mabs Therapeutics and Abpro-Labs Inc., holding ownership interest/equity in Y-Mabs Therapeutics Inc., holding ownership interest/equity in Abpro-Labs, and owning stock options in Eureka Therapeutics. Both MSK and N-K.C. have financial interest in Y-mabs. N-K.C. is an advisory board member for Abpro-Labs and Eureka Therapeutics.

DATA SHARING

The *KMT2A-MAML2* transcript was deposited in GenBank (Accession no. KY584083).

Twelve patients without therapy-related leukemia were studied after completing TOP2 poison chemotherapy in a high-risk neuroblastoma regimen. One patient harbored an inv(11) that was a *KMT2A* rearrangement. The *KMT2A-MAML2* transcript was expressed at low level. The patient was prospectively followed. The inv(11) was undetectable in ensuing samples. Leukemia never developed after a 12.8-year follow-up period. Enriched etoposide-induced TOP2A cleavage in the relevant *MAML2* genomic region supports a TOP2A DNA damage mechanism. After completing TOP2 poison chemotherapies, covert *KMT2A*-R clones may occur in a small minority of patients; however, not all *KMT2A* rearrangements herald a therapy-related leukemia diagnosis.

**Keywords**

*KMT2A-MAML2*; TOP2 poison chemotherapy; TOP2A cleave-ome; neuroblastoma; therapy-related leukemia

Therapy-related leukemias with balanced translocations attributed to TOP2 poisons, most of which involve *KMT2A*, were recognized when epipodophyllotoxins came into use in the late 1980s[1]. Discontinuing the more leukemogenic epipodophyllotoxin teniposide, and dose and schedule modifications lessened risk, but TOP2 poisons are mainstay chemotherapies and TOP2 poison-related leukemias remain a significant problem[1].

Therapy-related leukemia occurred following dose-intensive multi-modality MSK metastatic neuroblastoma treatments. MSK-N6, MSK-N7 and MSK-N8 all emphasized dose-intensive TOP2 poison- and alkylating agent-containing induction chemotherapy, but the number of cycles was successively reduced, and post-induction treatments changed[2]. On MSK-N6 (1990–1993), which used 7 chemotherapy cycles during induction, leukemia incidence was 7%[3,4]. About 40% of cases exhibited chromosome 5 and/or 7 loss attributed to alkylators; 40% had *KMT2A* translocations[3]. On MSK-N7 (1994–1999), which included 7 but later 5 chemotherapy cycles during induction, leukemia incidence was 5.6% after ≥12 years[5–7]. MSK-N8 (2000–2007) used 5 cycles[8–10]. After changing to 5 cycles, leukemia incidence was 0 after induction[7]. Occasional patients requiring additional chemotherapy for relapse developed leukemia/myelodysplasia, but the incidence is <7%[5,7]. The differences in therapies likely contributed to the decreased incidence of leukemia.

The MSK neuroblastoma experience affords unique opportunities to trace when *KMT2A* translocations originate relative to treatment and leukemia diagnosis because marrow surveillance is required[3,6,7]. MSK-N7 specified multi-site marrow samplings at diagnosis, stem cell harvest or second look surgery, end-induction[11], every 3–6 months thereafter until 2 years from diagnosis, and periodically for a few more years.

There are five therapy-related *KMT2A*-R leukemia cases altogether where tracing the translocation in sequential samples was reported[12–16]. Our studies of two children treated on MSK regimens who developed therapy-related leukemia showed that *KMT2A* translocations can emerge early or late during treatment, and that latency to leukemia can be short or protracted[12,13].

Here we describe a *KMT2A* rearrangement that emerged and regressed during neuroblastoma treatment without ever manifesting as leukemia. The prolonged prospective follow-up is uniquely longer than for any patients described to date. We investigate how often *KMT2A*-R occurs during chemotherapy without leukemia by studying 12 patients with metastatic neuroblastoma at an identical treatment timepoint, and study the *KMT2A* partner gene as a chemotherapy-induced TOP2A cleavage target.

## RESULTS AND DISCUSSION

A 17-year-old European Caucasian female was treated on MSK-N7[6,7] (Fig. 1) for Stage 4N neuroblastoma with disseminated lymph node disease without marrow or extranodal metastases[17]. Induction chemotherapy comprised four cycles of CAV, and three cycles of PVP. At diagnosis, the marrow karyotype was normal, and lymph node tumor showed a hyper-tetraploid karyotype and 4–5 copies of MYCN by FISH (Fig. 1). At surgical resection three months later, 100% of primary tumor cells showed 4 copies of MYCN by FISH, but all cells after culture showed a 46,XX,t(8;15)(p21;q26) karyotype (Fig. 1). The marrow karyotype after all seven chemotherapy cycles (10 months from diagnosis) demonstrated 46,XX,t(8;15)(p21;q26),inv(11)(q21q23)[cp10];46,XX,t(8;15)(p21;q26)[cp10] (Fig. 1). Except for this sample, there was no karyotypic evidence of inv(11) (Fig. 1). A blood sample before myeloablative radiolabeled 3F8 antibodies and autologous PBSC rescue (11 months from diagnosis) showed 46,XX,t(8;15)(p21;q26)[13];46,XX[3] (Fig. 1); however, marrow was not sampled. The t(8;15) was never detected afterwards (Fig. 1). The conditioning did not include chemotherapy and was not leukemia-directed.

These data suggest that the t(8;15) and inv(11) were transient clonal abnormalities, that the clone with t(8;15) and inv(11) was more short-lived than the clone with just t(8;15), and that these abnormalities were acquired during treatment and originated in the marrow. The tumor cells were hyper-tetraploid, but the cells with t(8;15) cultured from the tumor pseudodiploid (Fig. 1), suggesting that the t(8;15) was from contaminating blood cells in the tumor. The two clones with t(8;15) in the marrow 10 months from diagnosis, one of which contained inv(11), suggest that the clone with both abnormalities evolved from the clone with t(8;15) alone. The normal karyotypes of all other marrows, and cells with a normal karyotype besides cells showing t(8;15) in the blood 11 months from diagnosis (Fig. 1), indicate that the t(8;15) was non-constitutional.

During 12.8 years of prospective follow-up from detecting the inv(11) (13.7 years from starting treatment), far beyond the typical latency of therapy-related *KMT2A*-R leukemias of ~1.5 to 3 years from primary cancer diagnosis[18], there were never clinical signs of leukemia, increased blasts or morphologic hematopoietic abnormalities.

Unlike 11 other patients with metastatic neuroblastoma whom we studied after completing all seven chemotherapy cycles who also did not develop leukemia, Southern blot analysis of this patient's marrow showed *KMT2A* gene rearrangement (Fig. 2A). Therefore, the inv(11) disrupted *KMT2A,* and the *KMT2A*-R clone expanded to Southern blot detection level (using a $^{32}$P radiolabeled probe, ~1/100 cells[19]). cDNA panhandle PCR[12] identified the *KMT2A* partner gene at band 11q21 as *MAML2*. Two independent cDNA panhandle

PCRs were performed using first strand cDNA prepared from bone marrow RNA after chemotherapy cycle 7 (Fig. 1). The transcript joining *KMT2A* exon 9 to *MAML2* exon 2 was found in just one of 94 total subclones (Fig. 2B,C), indicating that expression was low-level.

Four cases of therapy-related T-cell ALL[15,20,21], two cases of therapy-related AML[16,22], and one case each of therapy-related MDS[23], therapy-related B-cell ALL[24], *de novo* MDS[16], and T-cell ALL that underwent lineage switch to AML[23] with *KMT2A-MAML2* or inv(11)(q21q23) have been reported. *KMT2A-MAML2* also occurs in aggressive thymoma subtypes[25]. The eight patients with therapy-related *KMT2A-MAML2* leukemia/MDS differ from this patient. The primary cancer was leukemia[15,16,21–23] except in two patients[20,24]. None had neuroblastoma. Latencies were 25–86 months. The latter is longer than usual for therapy-related *KMT2A*-R leukemia, but far shorter than the observation time in this patient. Like other *KMT2A* rearrangements traced in sequential samples manifesting as leukemia[12–14], *KMT2A-MAML2* remained detectable after first appearing until therapy-related leukemia/MDS diagnosis[15,16]. In contrast, in this patient, inv(11) never appeared again after first being detected.

MAML2 is one of three *Mastermind-Like* Notch receptor transcriptional co-activators, each having a conserved amino-terminal basic domain that binds the ankyrin domain within the intracellular domain of Notch receptors, and carboxyl-terminal TAD[26,27]. The in-frame 5'-*KMT2A-MAML2*-3' transcript we identified predicts a fusion protein (Fig. 2D) with potential for Notch signaling perturbation.

We surveyed existing TOP2A cleave-ome datasets for CEM (Yu and Davenport, unpublished) and K562[28] cells to gain insight into the *MAML2* disruption. Although the genomic breakpoint junction of the inv(11) was not cloned, the *MAML2* fusion point in the 5'-*KMT2A-MAML2*-3' transcript at the start of exon 2 (Fig. 2C), indicates a genomic breakpoint upstream of exon 2. Accordingly, both cell lines exhibited enriched TOP2A cleavage compared to vehicle along *MAML2* intron 1 in the presence of etoposide (Fig. 2E), indicating that *MAML2* is a TOP2 poison-induced TOP2A cleavage target. In K562 cells we reported etoposide-enriched TOP2A cleavage along the *KMT2A* bcr[28], further highlighting plausibility of TOP2 poison-induced TOP2A cleavage as the damage mechanism.

Thus, we followed a patient for 12.8 years after *KMT2A-MAML2* emerged and regressed during neuroblastoma treatment without onset of leukemia. The case series of 12 patients at the same MSK-N7 treatment timepoint in which just this patient had *KMT2A*-R, suggests that after receiving TOP2 poisons a small minority of patients harbor clinically silent clonal *KMT2A* rearrangements. The 11 other patients were not an age-matched cohort (Fig. 2A) because the median age at neuroblastoma diagnosis is 22 months and 90% of cases occur before age 5[29]. The literature suggests that clonal hematopoiesis during pediatric anticancer treatment is uncommon. Targeted next generation sequencing did not detect clonal hematopoiesis in peripheral blood DNA of 84 survivors of heterogeneous pediatric cancers (age at diagnosis 0–25.4 years), including 17 survivors of neuroblastoma (age at diagnosis 0.3–15.4 years), after median follow-up of 6 y[30]. In pediatric patients with ALL remission (age range 2–16 years), hematopoiesis proved polyclonal[31]. This is

the second reported covert *KMT2A*-R clone during anticancer treatment without evidence of leukemia. A *KMT2A-ARHGEF17* rearrangement occurred after primary pediatric AML treatment in a 5-year old without therapy-related leukemia developing by 30 months from diagnosis[32]. However, in the patient studied here, the follow-up was much longer. The dynamics of hematopoiesis during chemotherapy in pediatric patients who do and do not develop therapy-related leukemia after TOP2 poisons warrants further study.

Why leukemia did not occur is unknown. The identical transcript occurred in therapy-related leukemias[16,21] and thymomas[25]. Why this transcript was associated with leukemia in other patients, and why expression was low-level are uncertain. Expression is affected by transcriptional and post-transcriptional regulation; decreased production or transcript instability cause low-level expression. *MAML2* translocations not involving *KMT2A* in indolent benign and malignant neoplasms[27,33–42] raise questions about oncogenic potency of *MAML2* disruption. Additional mutational events may have been required. The marrow milieu or target cell may have been unfavorable. The *KMT2A*-R clone may have been obliterated immunologically[43–49] or by ensuing myeloablative treatment; however, therapy-related *KMT2A*-R leukemia has occurred on MSK-N7[5]. The latency may prove even longer. Our finding that *KMT2A-MAML2* did not herald therapy-related leukemia challenges the paradigm that all *KMT2A*-R clones connote leukemia. It has important implications about whether careful follow-up *vs*. aggressive intervention is prudent for clinically silent *KMT2A* rearrangements. This patient suggests that transient *KMT2A*-R during chemotherapy may not have the significance that clonal *KMT2A* rearrangement has historically implied for leukemia prediction.

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## ABBREVIATIONS

| | |
|---|---|
| **3F8*** | Radiolabeled anti-GD2 monoclonal antibody |
| **3F8** | Cold anti-GD2 monoclonal antibody |
| **ACK** | Ammonium-Chloride-Potassium |
| **ALL** | Acute lymphoblastic leukemia |
| **AML** | Acute myelogenous leukemia |
| **bcr** | breakpoint cluster region |
| **BM** | Bone marrow |
| **CAV** | cyclophosphamide 4200 mg/m$^2$, adriamycin 75 mg/m$^2$, vincristine 1.5 mg/m$^2$ |
| **cDNA PH PCR** | cDNA panhandle PCR |

| | |
|---|---|
| **COG** | Children's Oncology Group |
| **[cp]** | Composite karyotype |
| **Dx** | Diagnosis |
| **FISH** | Fluorescence *in situ* hybridization |
| **g/m$^2$** | grams per square meter |
| **GITC/CsCl** | guanidinium isothiocyanate/Cesium Chloride |
| **IGV** | Integrative Genomics Viewer |
| **IRB** | Institutional Review Board |
| ***KMT2A*-R** | *KMT2A*-rearranged |
| **MDS** | myelodysplastic syndrome |
| **mo** | month |
| **mos** | months |
| **MSK** | Memorial Sloan Kettering Cancer Center |
| **NCBI** | National Center for Biotechnology Information |
| **no.** | number |
| **PBSC** | peripheral blood stem cell |
| **PVP** | cisplatin 200 mg/m$^2$, etoposide 600 mg/m$^2$ |
| **TAD** | Transcriptional Activation Domain |
| **TOP2** | Topoisomerase II |
| **VP16** | Etoposide |
| **WBCs** | white blood cells |
| **y** | Year/years |
| **XRT** | local radiation therapy to neck and chest and to abdomen and hip |

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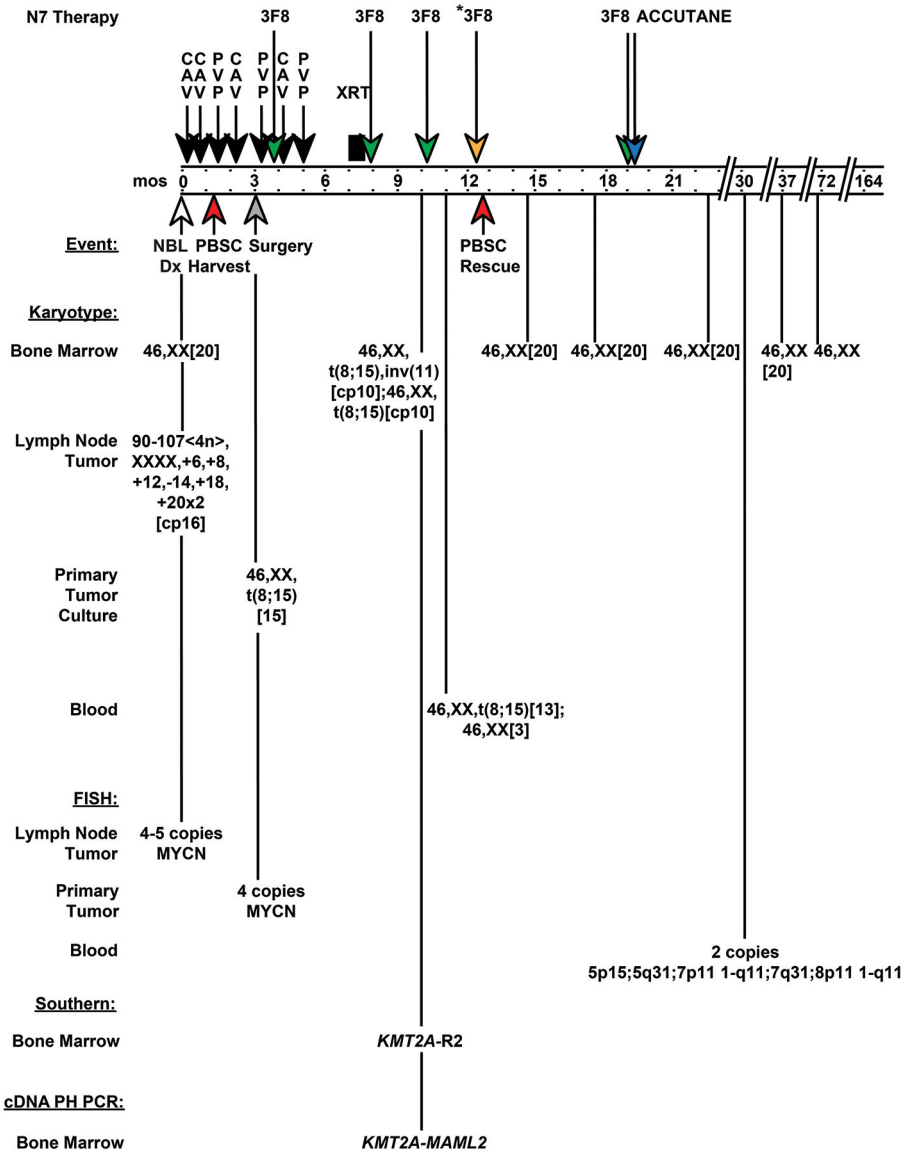


**Fig. 1.**

Treatment, clinical course, and timeline of cytogenetic and molecular findings in patient with Stage 4N neuroblastoma enrolled to MSK-N7 trial. Schematic shows when inv(11)(q21q23) resulting in *KMT2A-MAML2* rearrangement occurred and disappeared relative to treatment. The treatment included 7 dose-intensive cycles of induction chemotherapy [4 cycles of cyclophosphamide, adriamycin, and vincristine (CAV); 3 cycles of cisplatin and etoposide (PVP)], 3 cycles of cold anti-GD2 monoclonal antibodies (3F8) and local radiation before myeloablation with radiolabeled 3F8 monoclonal antibodies (3F8*) and autologous PBSC rescue. Autologous PBSCs were harvested after cycle 2, which was CAV. Following PBSC rescue, a final cycle of adjuvant 3F8 began, but accutane was substituted for 3F8 due to allergic reaction. At neuroblastoma diagnosis (time 0 months), note hyper-tetraploid karyotype of lymph node tumor tissue, and normal marrow karyotype. At surgical resection (time 3 months) the primary tumor cells contained 4 copies of MYCN

by FISH, whereas the karyotype after culture was 46,XX,t(8;15)(p21;q26)[15], suggesting contaminating blood cells in the tumor. After all 7 chemotherapy cycles (time 10 months), all cells in the marrow had a pseudodiploid karyotype with t(8;15)(p21;q26), and half also had inv(11)(q21q23). At time 11 months the peripheral blood karyotype was 46,XX,t(8;15)(p21;q26)[13];46,XX[3]. All subsequent marrow karyotypes were normal. FISH studies and results are shown. Abbreviations: mos, months; CAV, cyclophosphamide 4200 mg/m$^2$, adriamycin 75 mg/m$^2$, vincristine 1.5 mg/m$^2$; PVP, cisplatin 200 mg/m$^2$, etoposide 600 mg/m$^2$; 3F8*, radiolabeled anti-GD2 monoclonal antibody; 3F8, cold anti-GD2 monoclonal antibody; NBL, neuroblastoma; XRT, local radiation therapy to neck and chest and to abdomen and hip; PBSC, peripheral blood stem cell; *KMT2A*-R2, two rearranged *KMT2A* bcr fragments; cDNA PH PCR, cDNA panhandle PCR.

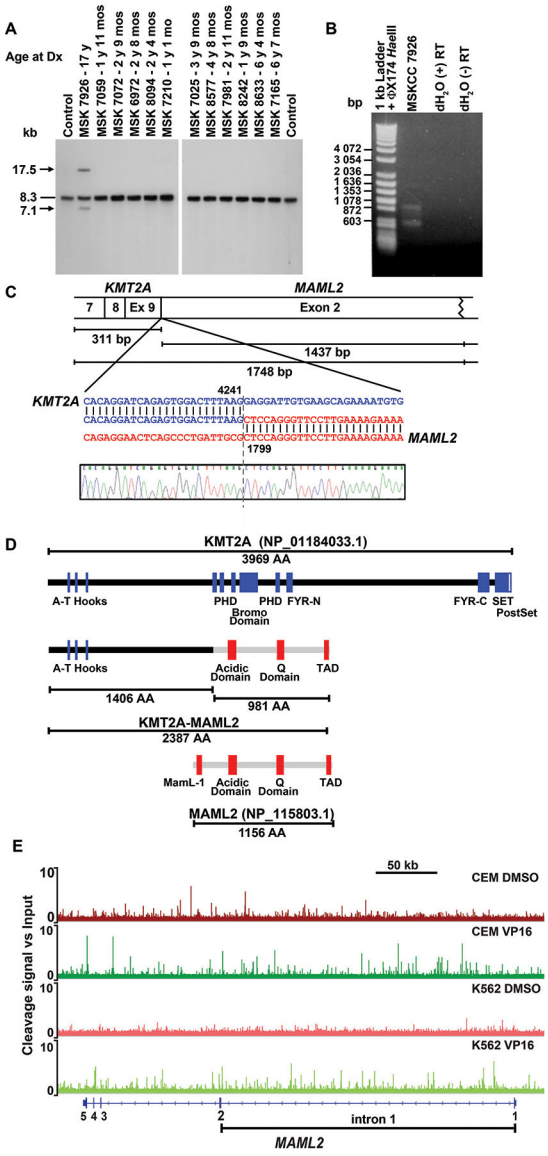


**Fig. 2.**

Molecular analysis of *KMT2A* rearrangement. (A) Southern blot analysis of bone marrow samples of 12 pediatric patients who did not develop leukemia by time of completion of all 7 cycles of MSK-N7 chemotherapy. Arrows show rearrangements; dash, germline bands. Genomic DNA and total RNA were prepared on GITC/CsCl gradients. Southern blot analysis used 5 μg *Bam*HI-digested DNA. B859 fragment of *KMT2A* cDNA was radiolabeled with $^{32}$P by nick translation[12,13]. Normal adult peripheral WBCs isolated by ACK lysis of red blood cells in whole blood were control. (B) cDNA panhandle PCR[12] identification of unknown 5'-*KMT2A-partner*-3' transcript. First strand cDNA prepared from bone marrow RNA after chemotherapy cycle 7 using 5'-*KMT2A*-NNNNNN-3' primers[12], was amplified in two independent cDNA panhandle PCRs. Gel shows products of one reaction, which vary in size as expected[12]. Products of the two cDNA panhandle PCRs were subcloned by recombination PCR[12], 31 and 63 subclones from which, respectively,

were sequenced. (C) Fusion transcript sequence compared to native *KMT2A* (GenBank accession no. NM_001197104.1) and the full-length 5-exon 7115 bp *MAML2* (GenBank accession no. NM_032427.3) mRNA reference sequences with coordinates at point of fusion indicated. One subclone contained transcript sequence joining *KMT2A* exon 9 in-frame to *MAML2* exon 2. Exon numbering is according to NCBI. Ninety-two subclones contained *KMT2A* transcript sequence only, with normally spliced, alternatively spliced and scrambled exons. One subclone was vector only. (D) Schematic of predicted fusion protein (middle) compared to native KMT2A and MAML2 (top and bottom). Amino acids 1 to 1406 from KMT2A are joined to acidic domain, Q domain and carboxyl-terminal TAD of MAML2. The first of three poly-glutamine tracts comprising the Q domain, in which the number of glutamine (Q) repeats is known to vary, contains 30 Q residues in the fusion protein compared to 34 in the reference sequence (GenBank accession no. NM_032427.3). This is accounted for in number of amino acids in the fusion protein. (E) TOP2A cleave-ome data for K562[28] and CEM (Yu and Davenport, unpublished) cell lines illustrating cleavage along Chromosome band 11q21 in *MAML2* gene body. The Y axis shows fold-change in TOP2A cleavage signal relative to input. Note many peaks in intron 1 (bracketed) in presence of etoposide (VP16) not detected in vehicle treated samples, indicating enriched cleavage with etoposide, of interest because 5'-*KMT2A* exon 9-*MAML2* exon 2–3' transcript (C) signifies a *MAML2* genomic breakpoint upstream to start of exon 2. Fold-change in cleavage signal was plotted and tracts along *MAML2* captured using IGV.