

Cloning and characterization of a novel druggable fusion kinase in acute myeloid leukemia

Rearrangements of the genes for the platelet-derived growth factor receptor alpha (*PDGFR α*) or the platelet-derived growth factor receptor beta (*PDGFR β*) have been identified in myeloid/lymphoid neoplasms with eosinophilia.¹ Fusions of *PDGFR β* , which encodes a type III receptor tyrosine kinase, result in constitutive kinase activation and promotion of survival, proliferation and cell migration.² Since the description of *ETV6-PDGFR β* in 1994,³ more than 30 *PDGFR β* fusion partners have been described, most of which have been identified in myeloproliferative neoplasms or in a myelodysplastic syndrome/myeloproliferative neoplasm overlap, but only rarely in acute myeloid leukemia (AML).^{1,4,5}

PDGFR β fusions are commonly in-frame mutations that contain an N-terminal fusion partner with dimerization/oligomerization motifs, enabling *PDGFR*-ligand-independent receptor tyrosine kinase activation.^{1,6} *PDGFR β* -rearranged chronic myeloproliferative neoplasms show a favorable response to imatinib with a 10-

year overall survival rate of 90%.⁷ The zinc finger protein “Zinc finger and BTB (broad-complex, tramtrack, bric-à-brac) domain containing 11” (*ZBTB11*) is a transcriptional repressor, which was originally identified as a regulator of metallothionein 2A and recently described as a master regulator of neutrophil development.⁸

Here we report on the cloning, biological and biochemical characterization of a novel *ZBTB11-PDGFR β* fusion gene, which occurred in a therapy-resistant AML patient who relapsed only shortly after allogeneic stem cell transplantation (SCT).

A 53-year old female presented in October 2015 with the primary diagnosis of an AML: nucleophosmin (*NPM1*)-mutated, *FMS-like tyrosine kinase 3 (FLT3)*-wild-type, *DNA (cytosine-5)-methyltransferase 3A (DNMT3A)*-wildtype, normal karyotype, European Leukemia Net (ELN) favorable risk. The patient received induction chemotherapy and one cycle of consolidation chemotherapy. Due to persistence of *NPM1*-positive minimal residual disease (MRD), the patient underwent SCT in March 2016. Three months later, the patient relapsed.

At the time of relapse, bone marrow cytology showed

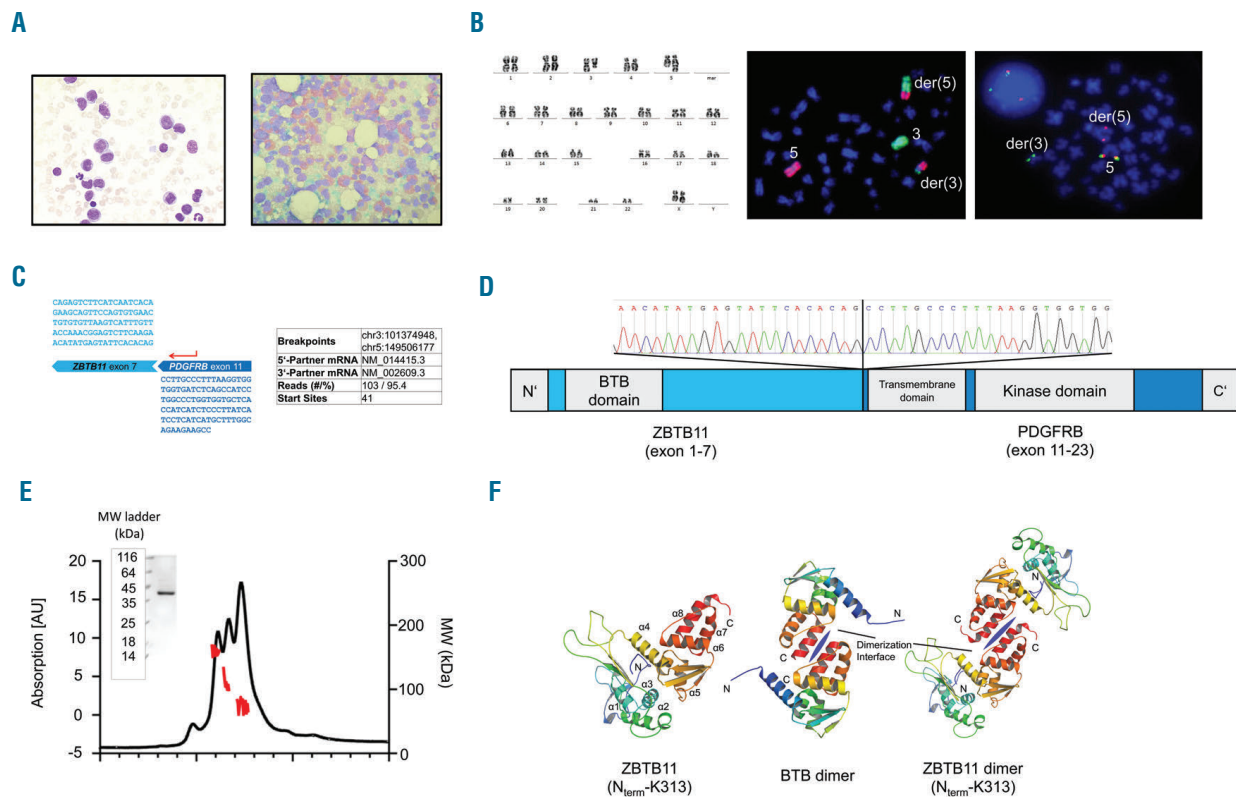


Figure 1. Characterization of the ZBTB11-PDGFR beta (β) fusion gene and protein. (A) Bone marrow Papanheim smears showing immature leukemic blasts at primary diagnosis (left) of acute myeloid leukemia (AML) and at first relapse with atypical eosinophil precursors (right). (B) (Left) G-banded karyogram: 46,XX,t(3;5)(q12;q32). (Middle) Whole chromosome painting with probes for chromosomes 3 (red) and 5 (green). (Right) Interphase nuclei and metaphase hybridized with a dual color break apart probe (MetaSystems, 3'*PDGFR β* labeled in red, 5'*PDGFR β* labeled in green) indicating a *PDGFR β* rearrangement. (C) Identification of the *ZBTB11-PDGFR β* fusion via next generation sequencing. (Left) Graphical representation (not to scale) of the *ZBTB11-PDGFR β* fusion with the approximate location of the gene specific primer that enabled detection of the chimeric reads and the mRNA sequence in the fusion region. (Right) Genomic co-ordinates of the fusion breakpoints and supporting evidence from bioinformatic analysis. (D) Sanger sequencing reveals the formation of a novel *ZBTB11-PDGFR β* fusion gene, which translates into a putative ZBTB11-PDGFR β fusion protein. Functional domains, the N- and C-terminus of this novel fusion kinase are marked in gray. The black line indicates the breakpoint of the fusion gene. (E) Chromatogram of the SEC-MALS-RI experiment. The black graph shows the absorption at a wavelength of 280 nm and refers to the left y-scale. The red line indicates the absolute molecular weight (MW) as determined by MALS-RI and refers to the right y-scale. The Coomassie stained SDS gel of the sample shows a band at approximately 36 kDa corresponding to the denatured monomer. (F) (Left) Phyre2 model of ZBTB11- N_{term} -K313. (Middle) Crystal structure of the BTB homodimer. Each monomer shares high structural homology to the C-terminal domain of ZBTB11- N_{term} -K313 (red). (Right) Structural model of the ZBTB11- N_{term} -K313 homodimer based on the BTB homodimer (compare to the middle panel). The blue line indicates the homodimer interface.

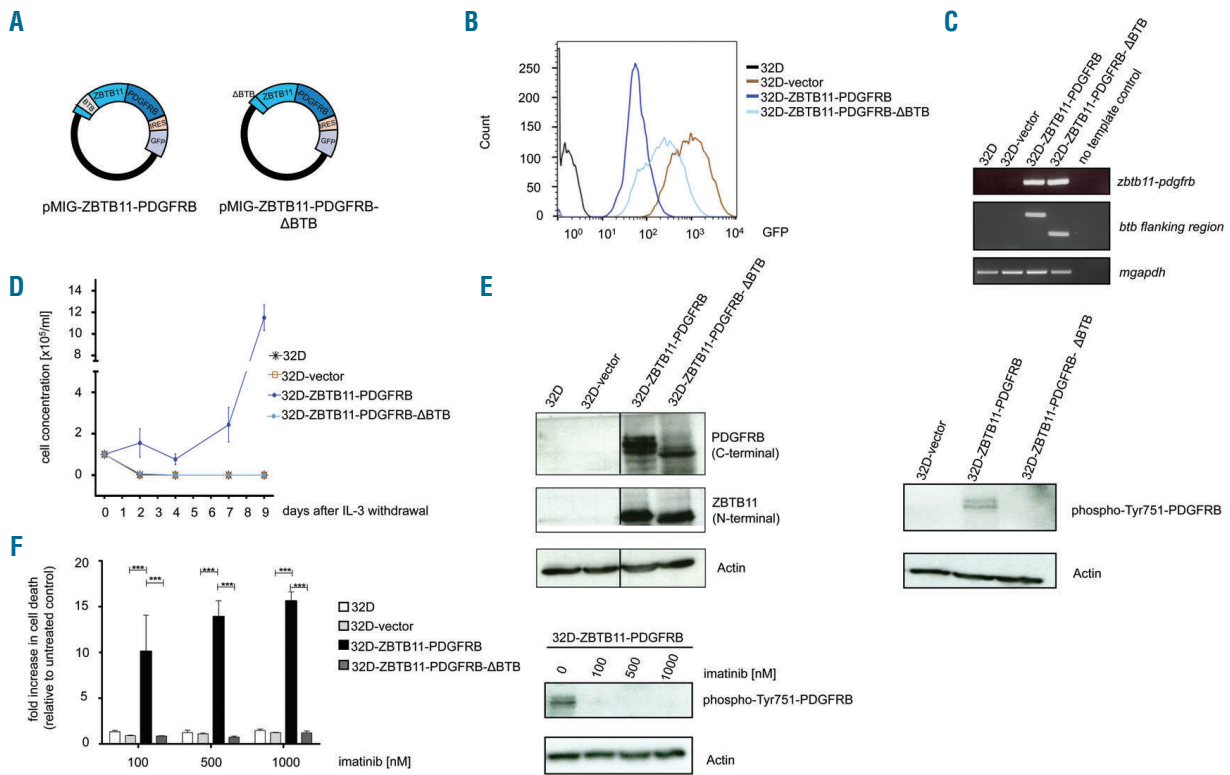


Figure 2. Cloning and expression of ZBTB11-PDGFR β in 32D cells. (A) Retroviral expression vectors pMIG-ZBTB11-PDGFR β and pMIG-ZBTB11-PDGFR β - Δ BTB. IRES: internal ribosomal entry site; GFP: green fluorescent protein. (B) Flow cytometric quantification of the GFP staining intensity (x-axis) as a direct measure of ZBTB11-PDGFR β protein levels or mutant, BTB domain-deleted ZBTB11-PDGFR β (ABTB) protein expression in 32D cells. GFP staining intensities of untransduced 32D cells and 32D-vector cells expressing only GFP are shown as controls. The y-axis displays cellular events. (C) Real-time quantitative polymerase chain reaction with primers specific for the *ZBTB11-PDGFR β* fusion and the BTB domain flanking region in 32D cells transduced with ZBTB11-PDGFR β or ZBTB11-PDGFR β - Δ BTB; murine GAPDH was used as a housekeeping gene. (D) Proliferation curves as assessed by trypan blue exclusion of the indicated cell lines after IL-3 withdrawal. (E, left) Western blot analysis of ZBTB11-PDGFR β expression in the indicated cell lines using antibodies against the C-terminus of PDGFR β and the N-terminus of ZBTB11. (Right) Western blot analysis of phospho-PDGFR β in the indicated cell lines after culture in IL-3-free medium for 8 hours. (F) (Left) Flow cytometric analysis of dead cells using DAPI stain after treatment with imatinib for 48 hours (h) *** $P \leq 0.001$. (Right) Western blot analysis of phospho-PDGFR β in 32D-ZBTB11-PDGFR β cells after imatinib treatment (8 h) with the indicated concentrations. Imatinib treatment leads to a reduction in PDGFR β -phosphorylation.

an increased content of blasts and the appearance of atypical eosinophil precursors (Figure 1A) which were not present at diagnosis. Cytogenetic analysis revealed a translocation t(3;5)(q12;q32). Fluorescence *in situ* hybridization (FISH) suggested that one of the fusion partners of this translocation was *PDGFR β* (Figure 1B). Targeted resequencing identified *ZBTB11* as the 5' fusion partner of *PDGFR β* (Figure 1C). Sanger sequencing confirmed the *ZBTB11-PDGFR β* fusion gene (Figure 1D). Breakpoint regions were downstream of exon7 of *ZBTB11* and upstream of exon 11 of *PDGFR β* . Thus, the fusion transcript retained the coding sequences for the transmembrane and kinase domains of *PDGFR β* , suggesting that these two important functional domains of *PDGFR β* were intact in the putative ZBTB11-PDGFR β fusion protein (Figure 1D).

As PDGFR β fusion proteins can be constitutively activated and cause transformation by oligomerization mediated through the 5' fusion partner,⁹ we hypothesized that the N-terminal part of ZBTB11 with the BTB domain and a dimerization motif¹⁰⁻¹² causes PDGFR β -autophosphorylation, -oligomerization, and hence cellular transformation. Indeed, the N-terminal part of ZBTB11 (N_{term}-K313) forms tetramers (142 kDa), trimers (108 kDa) and dimers (72 kDa) in solution, while no monomers (MW=36 kDa)

were detected (Figure 1E). Data suggest that the most apparent oligomeric state of the protein is the homodimeric state (Figure 1E). The protein showed high structure and sequence similarity with BTB-domains, which have previously been extensively characterized [e.g. Protein Data Bank (PDB)-ID: 2IF5, 3GA1, 4U2N, 3M52] (Figure 1F, left). BTB domains are known to form homodimers of which structural information is available (Figure 1F, middle panel). Correlating this to the findings of the dimerization of a dephospho-CoA kinase (dpCk) from *Aquifex aeolicus* described by Schubot *et al.*,¹³ when superimposing this structure onto our model and generating the symmetry related molecules, a clear dimerization interface could be observed between the α -helix 8 from our model and the dpCk α -helix 6, as well as between the loop regions between α -helices 6 and 7 (Figure 1F).

To address whether the putative gene product of the t(3;5)(q12;q32) translocation causes malignant transformation, full length *ZBTB11-PDGFR β* and a *ZBTB11-PDGFR β* mutant lacking the BTB domain (*ZBTB11-PDGFR β - Δ BTB*) were cloned into retroviral vectors that also encoded a green fluorescent protein (GFP) and were expressed in the myeloid progenitor cell line 32D (Figure 2A-C). 32D cells are immortalized hematopoietic precursors

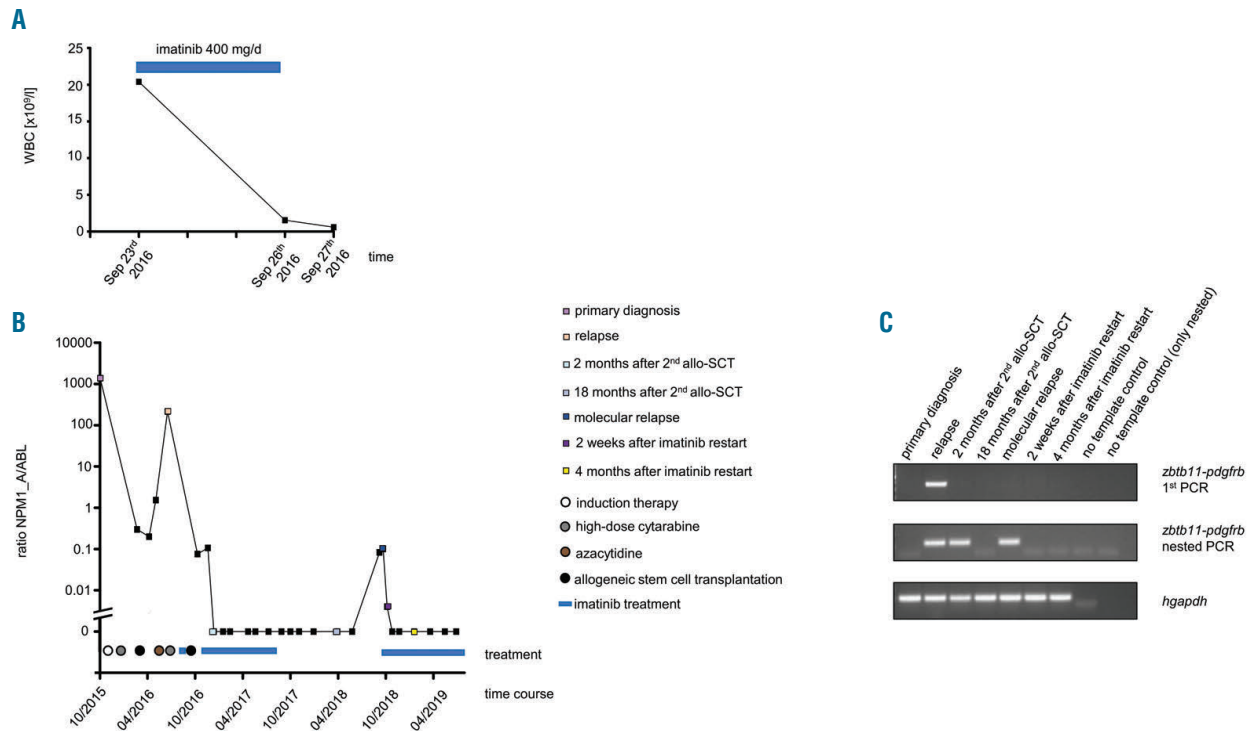


Figure 3. Treatment response to imatinib. (A) Decrease of leukemic blasts in the peripheral blood (PB) after three days of imatinib treatment (400 mg/d) prior to the conditioning therapy before second stem cell transplantation (SCT). (B) Course of *NPM1_A* mutational burden measured from the PB during the treatment course. The patient received one short imatinib treatment with 400 mg/day for three days prior to the second SCT followed by a 10-month imatinib treatment period with 100 mg/day three times a week. After a molecular relapse in September 2018 imatinib was restarted at 100 mg/day. (C) Nested polymerase chain reaction from the PB with primers specific for *ZBTB11-PDGFR β* (human *GAPDH* was used as a housekeeping gene). Of note, the fusion was not detectable at primary diagnosis.

sor cells, which critically require IL-3 to sustain growth and survival. While the ZBTB11-PDGFR β fusion protein was constitutively phosphorylated and caused IL-3-independence as a readout for oncogenic transformation (Figure 2D), the ZBTB11-PDGFR β - Δ BTB kinase was unphosphorylated and failed to induce factor independence in 32D cells (Figure 2E, right panel). Moreover, while inhibition of the PDGFR β kinase using imatinib caused a dose-dependent increase in cell death in 32D-ZBTB11-PDGFR β cells, this was not observed with IL-3-dependent 32D-ZBTB11-PDGFR β - Δ BTB cells (Figure 2E, left panel), suggesting that ZBTB11-PDGFR β transformed 32D cells critically depend on PDGFR β kinase activation to sustain survival. (See the *Online Supplementary Appendix* for full details of the materials and methods used.)

After failing to respond to intensive re-induction chemotherapy in relapse after SCT, our patient was given imatinib 400 mg/d to inhibit PDGFR(β) (Figure 3A). This led to a very fast hematologic response and the patient underwent a second SCT. After this SCT, imatinib was given as maintenance therapy at 100 mg/day three times a week for ten months. In *NPM1*-negative molecular remission, imatinib was suspended and the patient developed a molecular relapse in September 2018 (Figure 3B and C). Imatinib was restarted, and again induced an *NPM1*- and *ZBTB11-PDGFR β* -negative molecular remission. Of note, a nested-polymerase chain reaction suggested that the chemo- and immune-therapy refractory AML clone harboring the *ZBTB11-PDGFR β* fusion gene had been undetectable at the initial AML diagnosis (Figure 3C).

Here we describe the first AML patient with a t(3;5) chromosomal translocation leading to the expression of a chimeric fusion gene, consisting of the *PDGFR β* receptor tyrosine kinase gene and *ZBTB11*. *ZBTB11* encodes a BTB domain containing protein.¹⁴ We show that the BTB domain is essential for the transforming property of ZBTB11-PDGFR β by mediating ZBTB11-PDGFR β -oligomerization and autophosphorylation. On the other hand, myeloid precursor cells that had been transformed by the ZBTB11-PDGFR β oncoprotein become strongly dependent on PDGFR β -signaling for survival *in vitro*, providing a strong rationale for targeting ZBTB11-PDGFR β therapeutically using the PDGFR β tyrosine kinase inhibitor (TKI) imatinib.

Patients with chemotherapy-refractory AML relapse early after SCT have a very unfavorable outcome, with an estimated 3-year overall survival <5%.¹⁵ As the *ZBTB11-PDGFR β* translocation was undetectable at initial diagnosis and clonally expanded or occurred only after failing chemotherapy and allo-immunotherapy, it is tempting to speculate that ZBTB11-PDGFR β plays a pivotal role in the emergence of therapy-resistance in AML. Remarkably, treatment resistance could be effectively and durably overcome by imatinib. This observation is worthy of note, because, in contrast to myeloproliferative disorders harboring PDGFR β translocations,^{1,7} achievement of sustained molecular remissions with TKI monotherapy targeting a single oncogenic kinase in refractory AML is almost unprecedented. Even a second molecular relapse after temporary imatinib cessation remained imatinib-sensitive. We propose, therefore, that

allo-graft-mediated immune effects restrict the occurrence of kinase domain mutations as a simple escape mechanism under imatinib selection pressure. The life-saving response to imatinib in our patient underscores the importance of an in-depth morphological and genetic characterization of AML not only at initial diagnosis, but also at relapse.

Christian Michel,¹ Elisabeth K.M. Mack,¹ Christopher-Nils Mais,² Lea V. Fritz,¹ Ying Wang,¹ Lutz B. Jehn,¹ Sonja K. Hühn,¹ Clara Simon,¹ Sabrina Inselmann,¹ André Marquardt,¹ Jennifer Kremer,¹ Ellen Wollmer,¹ Kristina Sohlbach,¹ Andreas Neubauer,¹ Cornelia A. Brendel,¹ Claudia Haferlach,³ Gert Bange² and Andreas Burchert¹

¹Universitätsklinikum Gießen und Marburg, Campus Marburg, Klinik für Hämatologie, Onkologie und Immunologie, Philipps Universität Marburg, Marburg; ²SYNMIKRO Research Center and Department of Chemistry, Philipps-University Marburg, Marburg and ³MLL Munich Leukemia Laboratory, Munich, Germany

Correspondence: ANDREAS BURCHERT
burchert@staff.uni-marburg.de

doi:10.3324/haematol.2019.237818

Acknowledgments: we thank Gavin Giel, Lisa-Marie Koch and Petra Ross for their excellent technical assistance.

Funding: this work was supported by the foundation P.E. Kempkes, Marburg, Germany. GB thanks the LOEWE excellence initiative of the state of Hesse for support.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

- Reiter A, Gotlib J. Myeloid neoplasms with eosinophilia. *Blood*. 2017;129(6):704-714.
- Cross NC, Reiter A. Tyrosine kinase fusion genes in chronic myeloproliferative diseases. *Leukemia*. 2002;16(7):1207-1212.
- Golub TR, Barker GF, Lovett M, Gilliland DG. Fusion of PDGF receptor beta to a novel ets-like gene, tel, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation. *Cell*. 1994;77(2):307-316.
- Stengel A, Nadarajah N, Haferlach T, et al. Detection of recurrent and of novel fusion transcripts in myeloid malignancies by targeted RNA sequencing. *Leukemia*. 2018;32(5):1229-1238.
- Abe A, Emi N, Tanimoto M, Terasaki H, Marunouchi T, Saito H. Fusion of the platelet-derived growth factor receptor beta to a novel gene CEV14 in acute myelogenous leukemia after clonal evolution. *Blood*. 1997;90(11):4271-4277.
- Steer EJ, Cross NC. Myeloproliferative disorders with translocations of chromosome 5q31-35: role of the platelet-derived growth factor receptor Beta. *Acta Haematol*. 2002;107(2):113-122.
- Cheah CY, Burbury K, Apperley JF, et al. Patients with myeloid malignancies bearing PDGFRB fusion genes achieve durable long-term remissions with imatinib. *Blood*. 2014;123(23):3574-3577.
- Keightley MC, Carradice DP, Layton JE, et al. The Pu.1 target gene Zbtb11 regulates neutrophil development through its integrase-like HHCC zinc finger. *Nat Commun*. 2017;8:14911.
- Medves S, Demoulin JB. Tyrosine kinase gene fusions in cancer: translating mechanisms into targeted therapies. *J Cell Mol Med*. 2012;16(2):237-248.
- Ahmad KF, Engel CK, Prive GG. Crystal structure of the BTB domain from PLZF. *Proc Natl Acad Sci U S A*. 1998;95(21):12123-12128.
- Ahmad KF, Melnick A, Lax S, et al. Mechanism of SMRT corepressor recruitment by the BCL6 BTB domain. *Mol Cell*. 2003;12(6):1551-1564.
- Li X, Peng H, Schultz DC, Lopez-Guisa JM, Rauscher FJ 3rd, Marmorstein R. Structure-function studies of the BTB/POZ transcriptional repression domain from the promyelocytic leukemia zinc finger oncoprotein. *Cancer Res*. 1999;59(20):5275-5282.
- Schubot FD, Tropea JE, Waugh DS. Structure of the POZ domain of human LRF, a master regulator of oncogenesis. *Biochem Biophys Res Commun*. 2006;351(1):1-6.
- Siggs OM, Beutler B. The BTB-ZF transcription factors. *Cell Cycle*. 2012;11(18):3358-3369.
- Bejanyan N, Weisdorf DJ, Logan BR, et al. Survival of patients with acute myeloid leukemia relapsing after allogeneic hematopoietic cell transplantation: a center for international blood and marrow transplant research study. *Biol Blood Marrow Transplant*. 2015;21(3):454-459.