role of EZH2 and EZH1 in different tumors is undoubtedly multifaceted with aberrancies involving gain-of-function mutations, loss-of-function mutations, and protein overexpression.[8] Remarkably, in B-cell lymphomas, wild-type EZH2 must be present for gain-of-function mutations to exhibit a survival benefit—a unique phenomenon among human cancers.[9] Nevertheless, it was the discovery of gain-of-function mutations in B-cell lymphomas, mainly found in those of germinal center origin such as follicular lymphoma and a majority of diffuse large B-cell lymphomas, which led to the development of several EZH inhibitors.[7,8]

Li et al now describe an additional role of EZH2 in extranodal NKTL. Previous work from the same group described an alternative pathway whereby JAK3-mediated phosphorylation of EZH2 results in decreased methylation of histone H3 on Lys27 (H3K27). In other words, this site-specific phosphorylation transforms EZH2 into a transcriptional activator rather than a transcriptional repressor, the classic downstream function of EZH2 mutations found in other nodal lymphomas (see figure). Consequently, anti-tumor response in vitro with several EZH inhibitors that inhibit methyltransferase activity through S-adenosyl-methionine (SAM) competitive inhibition or inhibit other proteins of the polycomb repressive complex 2 (PRC2) were not effective.[10] The Li et al group now report the discovery of the pathogenic link between EZH2 and MELK. The authors begin with the discovery that MELK and EZH2 are concordantly and significantly overexpressed in extranodal NKTL patient samples when compared with healthy NK cells. Both therapeutic inhibition of MELK and MELK knockdown led to decreased EZH protein levels, but not messenger RNA expression, suggesting that MELK is involved in EZH2 protein stability and/or function. Li et al later identified a unique site-specific MELK-mediated phosphorylation at S220 of EZH2, which required the addition of recombinant MELK to EZH2 for phosphorylation. At the same time, they found K222 on EZH2 to be the exclusive site for ubiquitination. Given the close proximity of K222 to S220, it was speculated that phosphorylation of S220 led to deubiquitination of K222 and thus conservation of the EZH2 protein. USP36 was observed to be the deubiquitinase responsible for maintaining a ubiquitin-free pocket, thereby allowing for ongoing phosphorylation at

S220 driven by MELK. Finally, the authors describe increased sensitivity of extranodal NKTL to the proteasome inhibitor (PI) bortezomib in the absence of functional MELK, but this was not seen with the second-generation PI carfilzomib.

In summary, Li et al provide us with an additional piece to the puzzle of the oncogenesis of extranodal NKTL. Although in vitro studies showed little benefit for methyltransferase-targeted EZH2 inhibitors in extranodal NKTL, this has not been confirmed in clinical trials. However, given the work by Li and colleagues, the addition of MELK or JAK3 inhibitors may prove useful and is worth further study in extranodal NKTL. We are just beginning to unlock the full potential of epigenetic manipulation for the treatment lymphoma. One example would be combining EZH pathway inhibitors with other epigenetic modifiers such as the histone deacetylase inhibitors. This and other combinations warrant further investigation, particularly in EBV-driven lymphomas such as extranodal NKTL.

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REFERENCES

1. Li B, Yan J, Phyu T, et al. MELK mediates the stability of EZH2 through site-specific phosphorylation in extranodal natural killer/T-cell lymphoma. *Blood.* 2019;134(23):2046-2058.

2. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood.* 2016;127(20):2375-2390.

3. Au WY, Weisenburger DD, Intragumtornchai T, et al; International Peripheral T-Cell Lymphoma Project. Clinical differences between nasal and extranasal natural killer/T-cell lymphoma: a study of 136 cases from the International Peripheral T-Cell Lymphoma Project. *Blood.* 2009;113(17):3931-3937.

4. Yamaguchi M, Suzuki R, Oguchi M, et al. Treatments and outcomes of patients with extranodal natural killer/T-cell lymphoma diagnosed between 2000 and 2013: A cooperative study in Japan. *J Clin Oncol.* 2017;35(1):32-39.

5. Yamaguchi M, Kwong YL, Kim WS, et al. Phase II study of SMILE chemotherapy for newly diagnosed stage IV, relapsed, or refractory extranodal natural killer (NK)/T-cell lymphoma, nasal type: the NK-Cell Tumor Study Group study. *J Clin Oncol.* 2011;29(33):4410-4416.

6. Wang JH, Wang H, Wang YJ, et al. Analysis of the efficacy and safety of a combined gemcitabine, oxaliplatin and pegaspargase regimen for NK/T-cell lymphoma. *Oncotarget.* 2016;7(23):35412-35422.

7. McCabe MT, Ott HM, Ganji G, et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. *Nature.* 2012;492(7427):108-112.

8. Lue JK, Amengual JE. Emerging EZH2 inhibitors and their application in lymphoma. *Curr Hematol Malig Rep.* 2018;13(5):369-382.

9. Sneeringer CJ, Scott MP, Kuntz KW, et al. Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. *Proc Natl Acad Sci U S A.* 2010;107(49):20980-20985.

10. Yan J, Li B, Lin B, et al. EZH2 phosphorylation by JAK3 mediates a switch to noncanonical function in natural killer/T-cell lymphoma. *Blood.* 2016;128(7):948-958.

PLATELETS AND THROMBOPOIESIS

*Comment on Lentaigne et al, page 2070*

Pegasus causes inherited thrombocytopenia

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In this issue of *Blood*, Lentaigne et al identified novel pathogenetic missense mutations in the IKAROS family zinc finger 5 (*IKZF5*) gene that were associated with isolated thrombocytopenia in 3 pedigrees, affecting a total of 20 individuals and representing de novo mutations also associated with isolated thrombocytopenia in 3 individuals from 2 pedigrees.[1]

IKZF5 (also known as Pegasus [a winged horse in Greek mythology]) is one of the zinc finger transcription factors encoded by genes belonging to the *IKAROS* family that includes genes named after 4 additional mythological divinities: the founding member *IKZF1* (Ikaros), *IKZF2* (Helios), *IKZF3* (Aiolos), and *IKZF4* (Eos).[2] The best
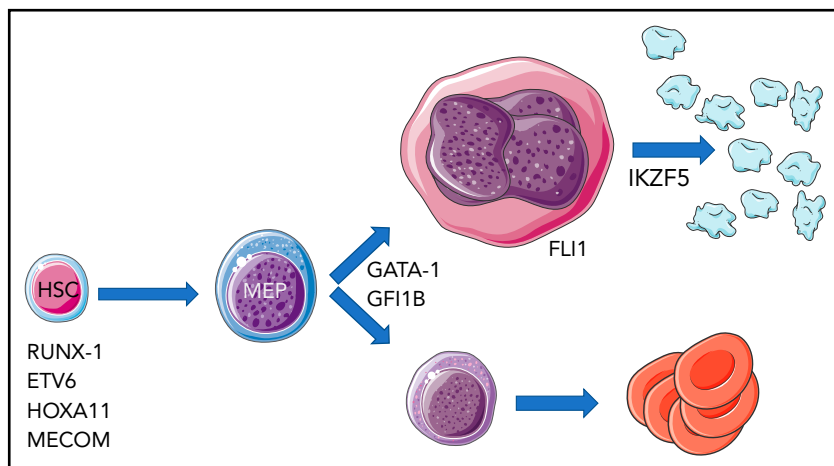
Hereditary thrombocytopenia associated with defects in transcription factor genes. The cellular level at which abnormal activity of mutated transcription factor is exerted is largely inferred by the associated hematologic abnormalities. HSC, hematopoietic stem cell; MEP, megakaryocyte-erythroid progenitor.

known *IKAROS* family members (ie, *IKZF1, -2,* and *-3*) are involved in lymphocyte development, and somatic mutations of *IKZF1* and *IKZF3* are associated with hematologic malignances.[3] Conversely, the functions of *IKZF4* and *IKZF5* remained elusive until the Lentaigne et al study established a role for *IKZF5* in megakaryocytopoiesis.

Hereditary thrombocytopenias (HTs) are rare and presumably underdiagnosed diseases if, according to an Italian study, their prevalence is estimated at almost 3 of every 100 000 individuals.[4] HT should be suspected in the presence of an evocative familial history, concomitant congenital defects, if present, and/or the detection of morphologic and/or volumetric and/or functional platelet abnormalities. Assessment of these platelet abnormalities requires considerable expertise and complex laboratory tests, and these platelet abnormalities are subjected to high phenotypic variability. Major advances in the understanding of HTs came from the application of whole-exome sequencing or whole-genome sequencing (WGS) to phenotypically well-characterized kindreds. This approach has identified an unexpectedly high number of involved genes.[5] This knowledge is promoting a shift in the classification of HTs from schemes based on associated (or not) syndromic features, the size and volume and/or functional defects of platelets, or the main pathophysiologic mechanisms responsible for thrombocytopenia to a genetic defect-based classification. However, it is estimated that only about 50% of HTs

can currently be associated with defined genetic variants. It is noteworthy that a joint effort of the American Society of Hematology and the National Institutes of Health–funded ClinGen resource (http://clinicalgenome.org) for HT variant classification is underway. Accurate diagnosis of HTs, notwithstanding how infrequent they are, is clinically relevant because some patients may be long misdiagnosed as having autoimmune thrombocytopenia, which leads to inappropriate exposure to prolonged cortisone therapy, use of thrombopoietin mimetics, and/or splenectomy.[6] Adopting the phenotypic criteria commonly used in clinical practice, thrombocytopenia associated with *IKZF5* mutations might be classified as an isolated mild thrombocytopenia without additional clinical features, with normally sized platelets and modest, variable platelet functional abnormalities, likely the result of a defect in the very late stages of megakaryocytopoiesis.

There are several lessons that can be learned from the work of Lentaigne et al. The first is that *IKZF5* mutations must be added to the list of autosomal (*HOXA11, RUNX1, FLI1, GFI1B, ETV6, MECOM*) and X-linked (*GATA1*) transcription factor–encoding genes whose germ line variants are associated with HT (see figure). Of importance, *IKZF5*-associated thrombocytopenia seems to be minimally symptomatic, with mild if any bleeding tendency, and to have an overall favorable outcome. This is quite different from other forms of HTs that are the result of transcription factor variants, in particular, the leukemia-predisposing

diseases associated with *RUNX1* and *ETV6*[7] mutations that pose clinical and ethical issues regarding the identification, counseling, and management of affected individuals.

The second is that the newly discovered mutations in *IKZF5* are rare genetic determinants of HT; indeed, only 5 variants were identified among the 233 patients with isolated thrombocytopenia in the WGS data from 13 037 individuals enrolled in the National Institute for Health Research BioResource. Although the study by Lentaigne et al has the value of describing a novel molecularly characterized entity of HT, it leaves largely unchanged the number of patients with HT still in search of a genetic explanation for their platelet disorder.

Third, the functional analysis of mutated IKZF5 performed in their study establishes Pegasus as a novel transcriptional regulator of thrombocytopoiesis and opens avenues for further research. Detailed studies of gene expression profiles by RNA sequencing in platelets of *IKFZ5*-mutated individuals highlighted that almost 10% of the >10 000 expressed RNAs were abnormally regulated compared with just 4 RNAs in purified CD4$^+$ T cells, monocytes, and neutrophils, which makes a strong case for IKZF5 being highly specific for the megakaryocytic lineage, in spite of being widely expressed in hematopoietic cells. Therefore, *IKZF5*-mutated megakaryocytes might represent a powerful model for elucidating mechanisms of platelet granule formation and platelet release via proplatelet formation. On the clinical side, this lineage specificity of action explains isolated thrombocytopenia as the only hematologic abnormality in patients with an *IKZF5* mutation, at odds with patients who have mutations in *GATA1* and *GLI1B*, who also present with anemia of various severities, or those with *HOXA11* and *MECOM* abnormalities, in which thrombocytopenia is part of a more generalized hematopoietic failure. Thrombocytopenia seems to be the only hematologic abnormality that also appears in the Paris-Trousseau syndrome as a result of hemizygous *FLI1* deletion, which is associated with the additional manifestations of the Jacobsen syndrome, or homozygous missense mutation leading to isolated thrombocytopenia (see figure).

Finally, the Lentaigne et al study represents an illuminating example of the incredible

amount of information that population genome sequencing efforts have the potential to generate for the understanding of rare diseases, provided the right questions are asked and the right approach to interpretation of genetic data is implemented.

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REFERENCES

1. Lentaigne C, Greene D, Sivapalaratnam S, et al. Germline mutations in the transcription factor IKZF5 cause thrombocytopenia. *Blood.* 2019; 134(23):2070-2081.

2. Heizmann B, Kastner P, Chan S. The Ikaros family in lymphocyte development. *Curr Opin Immunol.* 2018;51:14-23.

3. Churchman ML, Mullighan CG. Ikaros: exploiting and targeting the hematopoietic stem cell niche in B-progenitor acute lympho-blastic leukemia. *Exp Hematol.* 2017;46:1-8.

4. Noris P, Pecci A. Hereditary thrombocytopenias: a growing list of disorders. *Hematology Am Soc Hematol Educ Program.* 2017;2017: 385-399.

5. Lentaigne C, Freson K, Laffan MA, Turro E, Ouwehand WH; BRIDGE-BPD Consortium and the ThromboGenomics Consortium. Inherited platelet disorders: toward DNA-based diagnosis. *Blood.* 2016;127(23):2814-2823.

6. Rodeghiero F, Pecci A, Balduini CL. Thrombopoietin receptor agonists in hereditary thrombocytopenias. *J Thromb Haemost.* 2018; 16(9):1700-1710.

7. Di Paola J, Porter CC. ETV6-related thrombocytopenia and leukemia predisposition. *Blood.* 2019. doi.org/10.1182/blood.2019852418.

THROMBOSIS AND HEMOSTASIS

The next(gen) step in coagulation testing

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In this issue of *Blood*, Downes et al provide an updated report on the use of a high-throughput screening (HTS) panel developed by the ThromboGenomics group in the United Kingdom for genetic analysis of patients with coagulation, platelet, or thrombotic disorders.[1]

Clinical hematologists across the globe find that unexplained bleeding or clotting disorders make up a substantial part of new patient evaluations. In the 1980s, the genes encoding factor VIII, factor IX, and von Willebrand factor were characterized, which opened the door to genetic analysis at dedicated research centers. As a result, pathogenic gene variants were identified in about 95% of cases of hemophilia A or B and about two-thirds of those with type 1 von Willebrand disease.[2,3] A short time later, various inherited thrombophilias were also described, leading to the demonstration of factor V Leiden, prothrombin gene mutation, antithrombin deficiency, protein C deficiency, or protein S deficiency in about 10% to 20% of patients with venous thromboembolism.[4] In 2004, the World Health Organization created international standards for common genetic tests, beginning with factor V Leiden, reflecting the high demand for clinical thrombophilia testing around the world.

Despite these advances, the vast majority of patients with thrombotic or bleeding disorders, including platelet function defects, do not have one of the above genotypes. Until recently, a genetic diagnosis has largely remained elusive for these patients.

In the last decade, the advent of next-generation sequencing (NGS) led to the discovery of many new genes with potential roles in hemostasis and platelet function. In 2016, a landmark study by the ThromboGenomics group described the use of a 63-gene HTS panel in 296 patients with bleeding, platelet, or thrombotic disorders, with encouraging results, although the study population was weighted toward individuals with platelet function defects.[5,6] Inclusion of genes in the ThromboGenomics panel and interpretation of genetic test results were determined by a multidisciplinary team of laboratory and clinical specialists. The likelihood of establishing a genetic diagnosis was dependent on pretest probability as gauged by clinical laboratory testing. In this setting, other investigative groups, including our own, also developed expanded NGS panels for use in patients with bleeding or thrombotic disorders, all with promising findings.[7-9]

The updated study by Downes et al improves upon their prior work in several key ways. The number of genes in the HTS panel has now been expanded to 96, reflecting the discovery and validation of new genes involved in hemostasis and platelet function over the intervening years. Methodologic changes have vastly improved the detection of copy number and intronic variants. The panel has now been tested in 2390 patients, representing a broad distribution of hemostatic, platelet, and thrombotic disorders. The consensus criteria for establishing a genetic diagnosis as determined by the multidisciplinary team have been refined.

In their new study, Downes et al were able to identify a genetic diagnosis in 37.3% of all patients (see figure). Interestingly, the likelihood of establishing a genetic diagnosis was a function of disease phenotype. The highest diagnostic rate was seen in patients with coagulation disorders (63.6%), followed by thrombotic disorders (48.9%), thrombocytopenia (47.8%), and platelet function defects (26.1%). By contrast, very few patients with unexplained bleeding achieved a genetic diagnosis (3.2%).

What implications do these findings have in the clinic? The high diagnostic yield of the ThromboGenomics panel in patients with coagulation disorders is as interesting as the very poor diagnostic yield in those with unexplained bleeding. Although numerous patients and family members who undergo ThromboGenomics testing will now have the satisfaction of being able to define their diseases genetically, the fact that more than half of patients have a negative ThromboGenomics evaluation suggests either that other genes not included in the HTS panel may be involved or that epigenetic, proteomic, or non-genetic factors may have important roles