among patients with intermediate risk cytogenetics, most patients with high INPP4B expression were FLT3 and nucleophosmin 1(NPM1) wild-type (WT). Importantly, in patients <65 years of age, a higher INPP4B expression predicted a lower complete response rate (39% vs 71% in low expressers). Moreover, high INPP4B remained an independent factor of poor OS in multivariate analysis performed with or without censoring at transplantation.

Rijal et al went on to explain why INPP4B overexpression impacted on prognosis focusing on chemoresistance, since high expressers more often experienced primary induction failure. First, they demonstrated that overexpressed INPP4B was catalytically active in AML cells. However, a striking observation was the absence of correlation between INPP4B protein levels and the phosphorylation of protein kinase B (AKT) on serine 473, a surrogate marker of PI3K activation, suggesting that although INPP4B has been shown to be a negative regulator of the PI3K pathway, it could play a different role in AML. Next, using ectopically INPP4B overexpression in low-expressing or specific INPP4B depletion in high-expressing AML cells, they provided evidence that INPP4B is an important player in the resistance to drugs used in the management of AML (cytarabine, daunorubicin, and etoposide). They judiciously used a catalytically inactive mutant of INPP4B (INPP4B-C842A) and unexpectedly, showed that it also mediated chemoresistance. Thus, these results demonstrated that INPP4B mediates chemoresistance through a novel gain-of-function mechanism independent of its phosphatase activity in AML. Lastly, they validated in vivo the role of both INPP4B and INPP4B-C842A overexpression using xenografts in immunocompromised mice.

The first question we generally ask over such clinical results concerns its reproducibility in validation cohorts. Interestingly, other investigators very recently reported a similar clinical impact of INPP4B overexpression through the analysis of several publicly available AML datasets, strengthening the findings of Rijal et al.[6] Higher induction failure was also observed in this study, which is of particular interest with respect to our limited ability to predict resistance based on routinely available pretreatment covariates.[7] However, further

studies are needed to address several issues raised by this study: (1) What is the expression level of INPP4B in residual cells after chemotherapy or at relapse after clonal evolution (especially in patients with low expression at diagnosis)? (2) Which molecular mechanisms lead to its overexpression in AML? (3) Are there any correlations between INPP4B expression and other molecular markers since a higher expression was found in patients with WT FLT3 and NPM1? (4) What is the role of INPP4B in normal hematopoiesis? It would be interesting to determine if INPP4B also drives resistance in AML patients treated with hypomethylating agents.

How does INPP4B drive chemoresistance in AML? The field is open. Because INPP4B reduces the activity of drugs with various mechanisms of action, it is likely that it could be involved in apoptotic response rather than in drug-specific metabolisms. INPP4B overexpression in leukemic cells did not impact the phosphorylation of AKT or the expression of antiapoptotic members of the Bcl-2 family. Thus, critical downstream targets of INPP4B remain to be determined. The finding that INPP4B could act independently of its phosphatase activity raises several clues to investigate how cells resist to chemotherapy. Phosphoinositide enzymes have both a catalytic and a molecular adapter activity that are crucial to organizing multimolecular complexes. As discussed by the authors, INPP4B contains an N-terminal C2-lipid binding domain, which interacts with membranes. It also

contains a Nervy homology 2 domain known to mediate oligomerization (ie, AML1-ETO oligomerization) or protein-protein interaction.[8] Thus, the findings of Rijal et al pave the way to perform further molecular studies of the INPP4B interactome in order to identify new therapeutic targets aimed at unlocking chemoresistance in AML.

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● ● ● **RED CELLS, IRON, AND ERYTHROPOIESIS**

Toward a BETter grasp of acetyl-lysine readers

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In this issue of *Blood*, Stonestrom et al describe the unanticipated complexity of the distinct yet overlapping activities of acetyl-lysine–binding bromodomain and extraterminal motif (BET) proteins bromodomain-containing 2-4 (BRD2-4) in erythropoiesis, in the context of rising interests in BET pharmacologic inhibitors.[1]

Epigenetic modifications dictate chromatin structure and affect gene expression. It has been long established that aberrant activity

of the enzymes controlling the deposition, recognition, and removal of these chromatin marks often leads to cancer. Indeed, since

the first report of a link between the acetyltransferase CREB-binding protein (CBP), the oncoprotein E1A, and cell proliferation/differentiation,[2] large numbers of translocations and mutations involving epigenetic modifiers have been reported in various cancers.[3]

Lysine acetylation is a major posttranslational histone modification, associated with chromatin opening and positive regulation of gene expression. While attention was initially focused on writers (CBP, p300) and erasers (histone deacetylases) of this modification as potential drug targets, epigenetic readers of acetylation have recently been in the limelight. The BET motif family includes ubiquitously expressed BRD2, BRD3, and BRD4.[4] These proteins possess 2 highly conserved tandem bromodomains mediating binding to acetylated histones and transcriptional regulators; BETs also interact with components of the basal transcription machinery.[5] Among their targets are genes controlling progression of the cell cycle. BETs are thus functionally and mechanistically associated with the control of gene expression and cell growth.

Involvement of BETs in the pathophysiology of various cancers came as no surprise. As chromosomal translocation partners or cofactors of transcriptional complexes nucleated by oncogenic proteins, BRD2/3/4 have appeared as suitable pharmacologic targets in a range of malignancies such as carcinomas, leukemias, and prostate cancer.[6] Although protein/protein interactions are notoriously difficult to disrupt, recent technological advances have led to the development of numerous BET inhibitors, including the acetyl-lysine mimetics JQ1 and I-BET, currently being evaluated in clinical trials.[7] These molecules interact with the highly conserved acetyl-lysine–binding pockets of BETs, leading to efficient disruption of their interaction with acetylated targets.

BET inhibitors have therefore been identified as promising therapeutic molecules. However, it is becoming clear that not all BET targets are sensitive to BET inhibitors and that BETs may operate through distinct mechanisms; yet there has been little study of the functional differences between BETs. What are the active targets of individual BETs? Do BETs have overlapping functions? Why do BET inhibitors exhibit restricted activity

on some gene targets? It is essential to start answering these questions to be able to fully understand the biology of BETs, dissect the mechanisms underlying the activities of current inhibitors, and develop new inhibitors specifically targeting biologically relevant BETs.

To better understand epigenetic and transcriptional regulation, Gerd Blobel's group recently studied the role of BETs in the activity of the key transcription factor, GATA1, in erythropoiesis.[8] They demonstrated that BETs bind acetylated GATA1 and established a functional relationship between BETs and GATA1 association with its chromatin targets.[8] Through genome-wide chromatin immunoprecipitation (ChIP) and transcriptomic analyses combined with pan vs selective inhibition of BETs, Stonestrom et al now further dissect the mechanisms of action of BETs in erythropoiesis.[1]

A well-established model of erythroid differentiation relying on inducible expression of GATA1 (G1ER cells)[9] allowed the authors to test BET activities in absence or presence of GATA1 and to compare the effects of JQ1 inhibition vs depletion of individual BETs. They uncover a complex landscape of BET chromatin occupancy and transcriptional activities supporting the association of GATA1 with chromatin and the regulation of its target genes.

At the heart of this study is the discovery that individual BETs use distinct mechanisms of action in erythropoiesis, suggesting functional interplay. Indeed, BRD3 was present at the greatest number of GATA1-occupied sites and was strongly influenced by the presence of GATA1. In contrast, recruitment of BRD4 and BRD2 correlated less well with GATA1 occupancy, with BRD4 binding strongly linked to histone acetylation levels. Therefore, it was unexpected that CRISPR-Cas9–mediated BRD3 deletion produced no measurable phenotype, suggesting functional compensation by other BETs. Given the high conservation of structural motifs among BETs, it is now important to identify what determines the specific and overlapping activities of BETs and understand the role of their bromodomains in chromatin vs transcription factor binding.

The authors then probed the functional relationships between BETs, GATA1

chromatin occupancy, and transcriptional activation. Pulsed application of JQ1 not only showed destabilization of GATA1 binding to different extents genome-wide, but also highlighted BET transcriptional activities post-GATA1 chromatin occupancy, presumably through interactions with the transcriptional machinery.

To establish reliable relationships between BETs and GATA1 targets, the authors repeated transcriptome analyses in G1ER cells using spike-in controls: remarkably, only 220 genes were activated more than twofold in response to GATA1 induction, whereas 5094 genes were repressed. Comprehensive analyses of gene expression combined with ChIP data led to 4 major conclusions: (1) BET inhibition overwhelmingly reduced transcription activation with little effects on inhibition by GATA1, (2) not all BET-bound genes were JQ1-sensitive, (3) gene activation by GATA1 predicted JQ1 sensitivity much better than BRD4 occupancy (thereby raising the question of how reliable the so-called BRD4-bound superenhancers[10] are in predicting a response to BET inhibition), and (4) BETs and GATA1 chromatin binding as well as sensitivity of GATA1 to JQ1 all seemed to correlate with co-occupancy by GATA1 protein partners. Overall, this study offers a platform to identify distinct classes of GATA1 targets and to dissect the molecular mechanisms underlying JQ1 sensitivity likely to be influenced by the multiprotein complexes that include GATA1.

Finally, despite its high degree of colocalization, depletion of BRD3 had little effect on GATA1 activity unless in the context of BRD2 depletion. Moreover, BRD3 overexpression partially restored the defects caused by BRD2 loss. The ability of BETs to partially compensate for each other needs to be taken into consideration when linking pharmacologic BET inhibition to any given BET protein.

In conclusion, this thorough analysis paves the way for further molecular dissection of the activities of these critical epigenetic readers and should motivate similar studies in other cellular systems. It is essential to fully describe the biology of BETs for an informed optimization of inhibitors targeting specific oncogenic mechanisms.

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● ● ● **THROMBOSIS AND HEMOSTASIS**

Comment on Tanimura et al, page 2835

HLA class II meets β₂-glycoprotein I

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In this issue of *Blood*, Tanimura et al describe an interaction between certain human leukocyte antigen (HLA) class II alleles and misfolded β₂-glycoprotein I (β₂GPI). This complex is expressed on the surface of HLA class II–expressing placental endothelial cells, and it is a target for the autoantibodies against β₂GPI seen in patients with antiphospholipid syndrome (APS), providing a mechanistic basis for pregnancy-related morbidity in these patients.[1]

In antigen-presenting cells, HLA class II molecules present peptide antigens derived from extracellular proteins by the endocytic pathway to the CD4 T cells via the peptide-binding groove.[2] Following their assembly in the endoplasmic reticulum with an invariant chain (Ii), the Ii/HLA class II complexes are transported to a late endosomal compartment called the major histocompatibility complex (MHC) class II compartment. Here, Ii is proteolytically processed and removed, allowing peptide loading to the antigen-binding groove in the HLA class II complex. The HLA class II complex is then transported to the plasma membrane to present the peptide cargo to CD4 T cells.

In previous publications, these authors have shown that the HLA class II molecules can also transport certain intact misfolded proteins such as the immunoglobulin G heavy chain from the endosomal compartment to the cell surface.[3,4] Compared with HLA class I molecules, the peptide-binding groove of HLA class II molecules is open, and it can accommodate longer peptides. By using 293T cells transfected with complementary

DNAs for β₂GPI and HLA class II complex, the authors showed that misfolded β₂GPI was bound to HLA class II molecules inside the cell. This interaction presumably occurs in the endosomal compartment, and the complex is transported to the cell surface. The binding of the β₂GPI/HLA class II complex depends on the HLA-DR alleles. Certain APS-susceptible alleles such HLA-DR7 and HLA-DR4 bound to β₂GPI more effectively than other alleles. Although these studies were performed in transfected 293T cells with forced expression, the authors demonstrated the association of β₂GPI/HLA class II complex on endothelial cells in the placenta of patients with APS but not in the placenta of patients without APS. HLA class II–bound misfolded β₂GPI is not only a target of antibody-induced injury but is also a potent inducer of antigen-specific B cells and may play a role in the persistence of these antibodies in APS patients.

These novel findings raise several interesting questions, which have a direct bearing on the mechanism of the procoagulant state associated with APS. Endothelial cells, the most extensively studied target, express

class II antigens only after stimulation. This raises the question of whether only inflamed endothelial cells are the targets of the antiphospholipid antibodies. However, most patients with APS do not have evidence of vasculitis or other inflammatory conditions. Macrophages, the professional antigen-presenting cells, express HLA class II molecules and internalize β₂GPI/phosphatidylserine–containing vesicles and platelet microparticles,[5] and they can potentially express this epitope on their surface. In monocytes, antiphospholipid antibodies induce tissue factor, the major initiator of the coagulation system.[6] Furthermore, the HLA class II molecules can transmit outside-in signals by triggering multiple pathways,[7] and several signal transduction cascades have been shown in endothelial activation by β₂GPI–dependent antiphospholipid antibodies.[8]

Despite a large number of studies on this subject, the precise mechanism of the procoagulant state in APS is still elusive.[9] The Tanimura et al study provides yet another potential cell surface receptor for β₂GPI that may be involved in the induction of the procoagulant state.

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