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# NRAS palmitoylation and oncogenic fitness

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**In this issue of *Blood*, Zambetti et al validate that palmitoylation of mutant NRAS is essential for its oncogenic function by using a genetic mouse model. Their study highlights that the palmitoylation/depalmitoylation cycle is an attractive candidate for therapeutic intervention in hematologic malignancies with NRAS mutations.[1]**

A refined understanding of the mechanistic underpinnings driving neuroblastoma-RAS (*NRAS*) oncogenic function is critical for the development of effective cancer therapies. NRAS is frequently mutated in hematologic malignancies, primarily in multiple myeloma, myelodysplastic syndrome/myeloproliferative neoplasia (MDS/MPN) overlap syndromes, and acute myeloid leukemia.[2] These mutations lock the enzyme in an active conformation and drive ligand-independent anti-apoptotic signals.[3]

Like other mutant RAS proteins, oncogenic NRAS has been considered undruggable because it lacks deep pockets suitable for stable binding of small molecule inhibitors. Alternatively, the disruption of posttranslational modifications that activate NRAS function may define molecular targets of clinical value. Efforts to inhibit farnesylation, a lipid modification at cysteine-186 (C186) that is necessary for its biologic and oncogenic function, have been largely unsuccessful because *RAS*-mutant cancer cells become resistant by using geranyl-geranylation. This alternative modification, which is not normally used at this site in hematopoietic progenitors, reactivates oncogenic NRAS (and KRAS) function in the neoplastic cells.[4]

Farnesylated NRAS is palmitoylated at C181 in the Golgi apparatus and is then transported to the plasma membrane by the VPS35 chaperone protein. In contrast to farnesylation, palmitoylation is reversible, and NRAS undergoes rapid palmitoylation and depalmitoylation that shuttles the protein between an active and palmitoylated form in the plasma membrane and an inactive and depalmitoylated form in the Golgi apparatus.[5]

Despite the well-documented implication of palmitoyl modifications in NRAS function, previous studies have not been able to establish the essentiality of palmoitylation in hematologic malignancies. Studies by Cuiffo and Ren[6] and Xu et al[7] used a retroviral transduction approach with overexpressed palmitoylation-defective oncogenic Nras ($Nras^{G12D,C181S}$) protein to demonstrate that C181 is important for its oncogenic activity and for its proper subcellular localization.

Notably, $Nras^{G12D,C181S}$ expression had a strong negative impact on signaling activation in hematopoietic cells. However, data showing that expression levels of Ras oncoprotein affect specificity and function have shed doubts on studies in which nonphysiologic levels of Nras expression were used. Indeed, inducible and physiologic expression levels of the oncogenic $Nras^{G12D}$ from its endogenous locus ($Nras^{LSL\text{-}G12D}$) resulted in the development of a variety of hematologic malignancies in mice in a dose-dependent manner.[8,9]

To clearly define the relevance of palmitoylation in hematopoietic transformation by $Nras^{G12D}$, Zambetti et al developed an $Nras^{LSL\text{-}G12D,C181S}$-inducible double knockin mouse strain, in which physiologic levels of the palmitoylation-defective oncogenic Nras protein ($Nras^{G12D,C181S}$) are expressed from the *Nras* locus. The authors' compelling results demonstrate that the suppression of C181 palmitoyl chains is essential for $Nras^{G12D}$ oncogenic activity.

An important point that emerges from the study is that genetic suppression of palmitoylation completely abrogates $Nras^{G12D}$-driven neoplasia. Because *NRAS* mutations are heterozygous in practically all cases of human hematologic malignancies, these results suggest that efficient inhibition of palmitoylation could hinder oncogenic fitness and reverse neoplastic expansion. Notably, this suppression was effective when either 1 or both alleles express $Nras^{G12D,C181S}$ for lymphoid and myeloid neoplasms, suggesting that palmitoylation may be an attractive target for different hematologic malignancies.

Previous studies have reported that increased gene dosage drives $Nras^{G12D}$-dependent aggressive MPNs in vivo.[7,9] Zambetti et al evaluated the impact of reduced palmitoylation on the latency of *Nras*-driven MPNs by using mice with either 2 copies of G12D or 1 copy of G12D and 1 copy of G12D,C181S mutations at the *Nras* locus. They determined that the C181S mutation rendered the encoded protein non-oncogenic, because mice with C181S showed an intermediate disease latency. Further analysis unexpectedly revealed a clonal outgrowth of cells that had reverted the C181S mutation in G12D,G12D background in mice that developed aggressive MPN and other hematologic diseases. These data indicate that suppression of palmitoylation may result in a fitness disadvantage.

Hence, the essentiality of the palmitoylation/depalmitoylation modifications in hematopoietic neoplasias with *NRAS* driver mutations provides proof-of-principle evidence for therapeutic intervention. However, further mechanistic and functional studies are necessary before embarking on clinical studies. For example, there is a limited understanding of which (and how many) enzymes are involved in the palmitoylation/depalmitoylation cycle, and whether there is a redundancy in different hematopoietic compartments. Targeting the ABHD17 family of serine hydrolases, which depalmitoylate NRAS,[10] is an alternative potential therapeutic strategy. Finally, the identification of revertant aggressive clones with growth advantage may indicate that C181 is under high selective pressure, and that pharmacologic interference of palmitoylation may promote the emergence of resistant clones that escape this block and reactivate neoplastic outgrowth. This could be envisioned by a mutation in the targeted palmitoyl-transferase that excludes the inhibitor and reestablishes

palmitoylation, a mutation in an alternative pathway that reactivates MAPK signaling, or by the use of alternative enzymes and/or lipid moieties, as was found when using farnesylation inhibitors.

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**RED CELLS, IRON, AND ERYTHROPOIESIS**

*Comment on Sparkenbaugh et al, page 1783*

# Sickle cell vaso-occlusion: the clot thickens

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**In this issue of *Blood*, Sparkenbaugh et al reported that inhibition of tissue factor, the initiator of the extrinsic coagulation pathway, or of protease-activated receptor-1 (PAR-1), the endothelial receptor for thrombin, attenuates microvascular occlusion in murine sickle cell disease, suggesting anticoagulation should be explored for the prevention of vaso-occlusive events.[1]**

Painful vaso-occlusive events that frequently require emergency department visits and hospitalization are the most pressing concern for patients with sickle cell disease. The exact cause of these events and how to prevent them has been the subject of intensive research for decades. Progress is being made, but our knowledge is incomplete and there is much room for new discoveries.

Deformed erythrocytes that result from polymerization of deoxygenated hemoglobin S initiate the obstruction of microvessels of the bone marrow and other organs.[2] Adherence to the microvascular endothelium by these misshaped erythrocytes as well as by neutrophils and platelets appears to be fundamental to these painful events.[3] The pharmacologic agents that have been approved by the US Food and Drug Administration to prevent vaso-occlusive events in sickle cell disease provide potential insights into the pathogenesis of these events.

Hydroxyurea prevents hemoglobin S polymerization by raising the concentration of hemoglobin F within erythrocytes; it also reduces inflammation and promotes the production of nitric oxide.[4] Endari is a pharmacologic grade of L-glutamine, an amino acid important for the production of the antioxidant, glutathione.[5] Crizanlizumab inhibits endothelial expression of the adhesion molecule, P-selectin.[6]

Thus, sickling-induced changes in the erythrocyte membrane, inflammation, deficient nitric oxide signaling, oxidative stress, and abnormal expression of cellular adhesion molecules seem to be part of a complex process that leads to painful vaso-occlusive events. None of these approved pharmacologic agents provide full protection from painful events, and there must be other processes involved in the pathogenesis of vaso-occlusion.

The findings by Sparkenbaugh et al provide a potential path forward. Activation of coagulation is an important feature of sickle cell disease.[7] The Sparkenbaugh et al paper builds on a series of publications over the past 7 years that explored the role of the coagulation system in vaso-occlusive complications of sickle cell disease, mostly in murine models. The present paper shows that inhibition of tissue factor, at the initiation of the extrinsic pathway of coagulation, and inhibition of nonhematopoietic PAR-1, which mediates some of the responses of endothelial cells to thrombin, has effectiveness in preventing microvascular vaso-occlusion in murine models of sickle cell disease.

Specifically, the investigators explored the contributions of thrombin-dependent endothelial PAR-1 activation on microvascular stasis in murine models of sickle cell disease. Using intravital microscopy of dorsal skinfold chambers and real-time quantitative fluorescence intravital lung microscopy, they visualized the amount of microvascular stasis induced by stroma-free hemoglobin when mice were pretreated with various interventions. These modalities included anti-tissue factor antibody, anticoagulants targeting factor Xa (rivaroxaban) and thrombin (dabigatran), and an inhibitor of PAR-1 (vorapaxar). The investigators observed a marked attenuation of microvascular stasis with each of the interventions tested. PAR-1 null sickle mice were also resistant to microvascular stasis induced by stroma-free hemoglobin. In PAR-1 null sickle cell mice, the investigators noted decreased von Willebrand factor and P-selectin expression in lung tissue, in addition to decreased arteriolar neutrophil-platelet microemboli.[1]

The finding that antithrombotic agents currently used in the clinic induced a marked reduction in microvascular stasis