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The *scat* mouse model highlights RASA3, a GTPase activating protein, as a key regulator of vertebrate erythropoiesis and megakaryopoiesis

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Although significant progress has been made in the past decades in our understanding of bone marrow failure syndromes and anemia, many pathological conditions of unknown origin remain. Mouse models have significantly contributed to our understanding of normal erythropoiesis and the pathogenesis of erythroid disorders. Recently, we identified in the *scat* (severe combined anemia and thrombocytopenia) mouse model a missense mutation (G125V) in the *Rasa3* gene, encoding a Ras GTPase activating protein (GAP). RASA3 is lost during reticulocyte maturation through the exosomal pathway and is, therefore, absent in mature erythrocytes. In wild type reticulocytes, RASA3 is bound to the plasma membrane, a prerequisite for its GAP activity, but is mislocalized to the cytosol in *scat*. This mislocalization leads to RASA3 loss of function and higher levels of Ras-GTP, the active form of Ras, are consistently found in *scat* mature red cells. Finally, RASA3 function is conserved among vertebrates, since erythropoiesis and thrombopoiesis are impaired in zebrafish in which *rasa3* is knocked-down by morpholinos, and RASA3 is expressed in human erythroleukemia cells as well as in primary cells. In this commentary, we highlight the critical, conserved and non-redundant function of RASA3 in the context of vertebrate erythropoiesis and megakaryopoiesis. We notably discuss the mechanism of RASA3 downregulation and speculate on the most intriguing part of the phenotype observed in *scat*; the transient remission period.

Keywords: mouse model, RASA3, Ras, erythropoiesis, megakaryopoiesis, development, anemia, thrombocytopenia

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In this era of intricate, precise genetic engineering in the mouse and other model organisms, it is worth remembering the enormous contributions of naturally occurring, spontaneous mutations in biomedical research. Excellent historical reviews of the mouse in biomedical research are available.[1,2] Both targeted, engineered mutations (i.e., the gene-driven approach) and spontaneous, co-isogenic mutations (the phenotype-driven approach) in the laboratory mouse have played key roles in our understanding of the etiology of inherited anemia.[3] However, the knowledge gained from the two approaches, though often complementary, is by no means the same. A prime example is *scat* (severe combined anemia and thrombocytopenia), a spontaneous, autosomal recessive mutation co-isogenic with the BALB/cBy inbred mouse strain.[4] The defective gene in *scat, Rasa3,* is embryonic lethal when null; homozygous knockout out mice die at E12.5–13.5 due to massive hemorrhaging resulting at least in part from under-developed adherens junctions between capillary endothelial cells.[5] *Scat* mice homozygous for a spontaneously occurring *Rasa3* allele, however, survive on average for ~30 d after birth, sometimes much longer, and present a very different, fascinating phenotype.[4,6,7]

Unlike other models of anemia and/or thrombocytopenia, the phenotype is cyclic in *scat*. Indeed, homozygotes exhibit successive episodes of crisis and remission.

During crisis, mice are severely anemic and thrombocytopenic but a significant decrease in total B- and T-cells numbers is also observed, reflecting leukopenia. However, the disease is not lymphocyte mediated; the *scat* phenotype is recapitulated when doubly homozygous with immunodeficient mutations such as *scid* and *Rag1*$^{tm1Mom\ -/-}$.[7] These results suggest that most hematopoietic lineages are affected by the mutation, raising the question of a defect at the Hematopoietic Stem Cell (HSC) level. Studies of HSCs and multipotent progenitors are in progress and will help us to better understand the mechanism of the *scat* disease.

Breeding of heterozygous *scat*/+ mice leads to only 15% *scat/scat* liveborn neonates which, according to Mendel's laws, demonstrates in utero loss and strongly suggests that the first crisis episode starts before birth. This first crisis episode lasts for about 9 d after birth, and is followed by a remission period in which the phenotype reverts to normal. However, this remission is transient, and animals enter a second crisis. Ninety-four percent of the *scat* homozygotes are dead by 30 days of age mainly as result of a catastrophic bone marrow failure. The same cyclic pattern then ensues for the very few animals that survive the second crisis.

Bone marrow failures syndromes are associated with an impairment of erythropoiesis notably in conjunction with thrombocytopenia. We investigated the *scat* anemia phenotype by monitoring erythropoiesis in mice during crisis episodes. Using CD44 and Ter119 and CD44 and FSC as markers of differentiation, we found a significant delay during the late stages of erythropoiesis, at the polychromatophilic and orthochromatophilic stages. However, the differentiation process is not blocked, since mature red cells are still produced in *scat*. As one would expect in thrombocytopenia, megakaryopoiesis is also affected in *scat* and evidence of delayed megakaryocyte differentiation is observed. As a result, very few platelets are found in the peripheral blood of *scat* animals in crisis. The phenotypic evidence supports the likelihood that, in addition to delayed differentiation, survival of the few mature red cells that are produced is adversely affected as well. We are currently investigating this latter possibility notably through the involvement of reactive oxygen species production, which are detrimental to red cell survival.

The gene defect in *scat* is a transversion (G→T) in exon 5 of the *Rasa3* gene.[6] This transversion leads to a missense mutation wherein the glycine at position 125 is replaced by valine. RASA3 is a Ras-GTPase Activating Protein (GAP) belonging to the GAP1 family and therefore negatively regulates the small GTPase Ras.[8] Four members compose the GAP1 family: GAP1m, RASA3, CAPRI, RASAL. Membrane localization is essential for the GAP activity of all members, and they share a common structure organized in five domains.[9,10] Two C2 domains (C2A and C2B) are involved in membrane binding and calcium-dependent activation. However, RASA3 fulfills its function in a calcium independent manner. Catalytic GAP activity is located within the RasGAP domain. Constitutive membrane localization for RASA3 comes from interactions of the Pleckstrin Homology (PH) domain with PIP2 and PIP3.[11-13] Activation of RASA3 is induced by binding of the Bruton's tyrosine kinase (Btk) domain to IP4 upon stimulation.[12]

Ras has been shown to play multiple roles during hematopoiesis, notably in erythropoiesis and megakaryopoiesis. Indeed, Ras is a critical mediator of cytokine-dependent signaling (EPO, erythropoietin; SCF, stem cell factor). Upon receptor tyrosine kinase engagement, Ras is activated by GEFs (guanine exchange factors), which induce dissociation of GDP and allows GTP to bind. However, this activation is transient, and GAPs downregulate Ras activity by accelerating GTP hydrolysis.[14]

We found that in *scat*, expression levels of RASA3 are normal compared with WT. However, the protein is mislocalized and found within the cytosol. The *scat* mutation lies in between the two C2 domains. Although a molecular model for the PH domain has been defined,[15] the crystal structure for RASA3 is not known yet. But one may speculate that the mutation induces a conformational change, preventing its binding to the membrane. Previous studies showed that RASA3 has a dual specificity for Ras and Rap-1.[10] However, knockout studies of Rap-1a and Rap-1b fail to show erythropoietic defects,[16,17] and we could not detect the presence of Rap-1 in the most mature stages.

Very interestingly, we found that, both in WT and in *scat* mice, RASA3 is downregulated at the reticulocyte stage, and is virtually absent from the mature red cell. Reticulocyte maturation is the last step of erythropoiesis. At that stage, the immature erythrocyte has already lost its nucleus, but still contains mitochondria as well as the endosomal compartment. Extensive membrane remodeling is responsible for giving the erythrocyte its characteristic biconcave shape and most of the membrane proteins are downregulated through the exosomal pathway.[18] Exosomes are small membrane vesicles (50–100 nm) secreted by reticulocytes among other cell types.[19] The fusion of a so-called multivesicular endosome with the plasma membrane leads to the release of the small intraluminal vesicles, called at that point exosomes. Historically, the transferrin receptor-1 (CD71) was the first protein shown to be downregulated by the exosomal pathway.[20] We found that RASA3 is actually lost from reticulocytes in association with exosomes. However the sorting signal is still unclear. Some studies have demonstrated mono-ubiquitination as one sorting signal,[21-23] and a recent publication describing the so-called "Ubiquitome" identified RASA3 as substrate.[24] It is, therefore, tempting to speculate that exosomal secretion of RASA3 is triggered by its ubiquitination. Downregulation of RASA3 through the exosomal pathway is harder to envision in *scat*. Indeed, despite the fact that the protein is absent from the mature stage, loss of its membrane attachment suggests another mechanism for its removal during the maturation process. Nevertheless, ubiquitination of RASA3 is still an attracting hypothesis as signal in the process of downregulation, with involvement of the proteasome in that case.[25] The ubiquitin/proteasome pathway is one of the main degradation pathways for cytosolic proteins and is still active at the reticulocyte stage.[26] Indeed, a recent study described the proteasomal degradation of tubulin during reticulocyte maturation.[27] Future studies will examine the ubiquitin/proteasome pathway in *scat*.

As mentioned above, the glycine in position 125 is a highly conserved amino acid in the RASA3 sequence. At the same time, G→V is a conservative amino acid change to say the least, and initially raised the issue of whether *Rasa3* was really the gene underlying the dramatic *scat* phenotype. We wondered if altering *Rasa3* in another animal model would lead to the same consequences. For that, we used morpholinos technologies to knock down *rasa3* in the zebrafish and produced dramatic anemia and thrombocytopenia. Disruption of the *rasa3* mRNA with splice-blocking morpolinos in tandem showed a profound reduction in hemoglobinized cells when stained with o-dianisidine. Semi-quantitative RT-PCR showed efficient disruption of the rasa3 mRNA. Although zebrafish lack megakaryocytes, zebrafish thrombocytes function as hemostatic equivalent of mammalian platelets.[28] Monitoring the expression of the integrin CD41 allows quantitation of thrombocytes. Knock down of *rasa3* in the *Tg(cd41:GFP)* transgenic line confirmed thrombocytopenia by flow cytometry of the GFP-tagged thrombocytes.

Despite our progress in the identification of *Rasa3* as the gene underlying *scat,* and our explanation of one of the mechanisms leading to the dramatic erythroid phenotype, many questions remain. Among others, and probably one of the most puzzling, the mechanism by which animals enter remission has yet to be explained. One may suggest that compensation occurs through upregulation of another GAP. In that view, RASA2 (GAP1m) is also expressed in the spleen, although its expression levels are much lower (BioGPS: http://biogps.org). In mice under stress conditions (e.g., anemia), the spleen becomes erythropoietic, and an increase in cellularity is observed.[29] It is, therefore, tempting to suggest that RASA2 levels are increased, concomitant with the increased spleen cellularity, leading in turn to the remission period. However, when the spleen cellularity returns to normal, levels of RASA2 are decreased, cannot compensate any further and mice finally enter the second crisis. This mechanism remains to be tested directly.

Finally, one may wonder to what extent these studies are relevant to human pathophysiology. To date, no pathology involving *RASA3* have been identified in human hematopoiesis. However, the etiology of bone marrow failure syndromes is only known in about 60–70% of the total cases.[30] Furthermore, recapitulation of the *scat* phenotype in the zebrafish in which *rasa3* has been knocked-down strengthens a conserved function for RASA3 in vertebrate hematopoiesis, and we detected RASA3 both in a human erythroleukemic cell line (HEL cells,[6]) and in primary erythroid progenitors (our unpublished results). It is, therefore, tempting to speculate that some bone marrow failure syndromes for which the etiology has not been uncovered yet may find their origin in a mutation in *RASA3.*

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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