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**Functional redundancy between RAP1 isoforms in murine platelet production and function**

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**Key points**

- Deletion of both *Rap1a* and *Rap1b* impairs platelet production and abolishes platelet adhesion at sites of mechanical trauma.
- Platelet RAP1 signaling is dispensable for vascular integrity during development and at sites of inflammation.

**Abstract**

RAP GTPases, important regulators of cellular adhesion, are abundant signaling molecules in the platelet/megakaryocytic lineage. However, mice lacking the predominant isoform, RAP1B, display a partial platelet integrin activation defect and have a normal platelet count, suggesting the existence of a RAP1-independent pathway to integrin activation in platelets and a negligible role for RAP GTPases in megakaryocyte biology.

To determine the importance of individual RAP isoforms on platelet production and on platelet activation at sites of mechanical injury or vascular leakage, we conditionally deleted *Rap1a* and/or *Rap1b* in the megakaryocytic lineage (mKO). Interestingly, *Rap1a/b-mKO* mice displayed a marked macrothrombocytopenia due to impaired pro-platelet formation by megakaryocytes. In platelets, RAP isoforms had both redundant and isoform-specific functions. Deletion of RAP1B, but not RAP1A, significantly reduced α-granule secretion and activation of the cytoskeleton regulator RAC1. Both isoforms significantly contributed to thromboxane $A_2$ generation and the inside-out activation of platelet integrins. Combined deficiency of RAP1A and RAP1B markedly impaired platelet aggregation, spreading and clot retraction. Consistently, thrombus formation in physiological flow conditions was abolished in *Rap1a/b-mKO,* but not *Rap1a-mKO* or *Rap1b-mKO* platelets. *Rap1a/b-mKO* mice were strongly protected from experimental thrombosis and exhibited a severe defect in hemostasis after mechanical injury. Surprisingly, *Rap1a/b-mKO* platelets were indistinguishable from controls in their ability to prevent blood-lymphatic mixing during development and hemorrhage at sites of inflammation.

In summary, our studies demonstrate an essential role for RAP1 signaling in platelet integrin activation and a critical role in platelet production. While important for hemostatic/thrombotic plug formation, platelet RAP1 signaling is dispensable for vascular integrity during development and inflammation.

## Introduction

Platelets are specialized cells generated from the cytoplasmic fragmentation of megakaryocytes to ensure the integrity of the vascular system upon mechanical injury or any other vascular breach, occurring for instance at sites of inflammation[1-3]. Platelet stimulation triggers intracellular signaling cascades that promote cytoskeletal remodeling, secretion of granules, release of eicosanoids, and conversion of integrin receptors from a low- to high-affinity state for their ligands (inside-out activation). Once active, integrins mediate platelet adhesion to the exposed extracellular matrix and aggregation to adjacent active platelets. Auto/paracrine agonists released from activated platelets, such as adenosine diphosphate (ADP) and thromboxane (Tx) $A_2$, locally perpetuate platelet activation and promote the formation of a stable shear-resistant hemostatic plug[4,5].

The signaling machinery controlling these responses must be tightly regulated to prevent pathological thrombosis or bleeding. RAP GTPases (Ras-related proteins) are among the most abundant signaling proteins expressed in platelets[6-9]. They are molecular switches that cycle between a GDP-bound OFF state and a GTP-bound ON state, under the tight control of guanine-nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs)[10]. Once GTP-loaded, RAP GTPases undergo conformational changes that enable specific binding to effectors, which in turn control a wide range of biochemical events, most notably cell adhesion[11], cytoskeletal dynamics[12] and mitogen-activated protein kinase (MAPK) cascades[13].

Vertebrates express two RAP1 and three RAP2 isoforms encoded by separate genes. *Rap1a* and *Rap1b* share a 95% sequence identity, suggesting that they may have redundant functions during cellular activation. However, there is also growing evidence that these two closely-related isoforms differentially regulate cellular functions such as NADPH oxidase activity[14,15] and cadherin-mediated adhesion[16]. In platelets, RAP1A and RAP1B are the most highly expressed members of the RAS superfamily[6-9]. Our previous studies demonstrated that both the activity state of RAP1 and platelet adhesiveness are controlled by the antagonistic balance between two upstream RAP regulators[17,18]. In circulating platelets, RAP proteins are maintained inactive by the RAP-GAP RASA3[18]. At sites of injury, when platelets are stimulated, the RAP-GEF CalDAG-GEFI mediates rapid RAP activation triggered by the near-immediate rise of cytosolic calcium concentrations[17,19-21]; however, this response is transient in nature and RAP can quickly reverse back to the OFF state. Thus, signaling from secreted ADP via the P2Y12 receptor is critical to inactivate RASA3 and enable sustained RAP activation[18,22]. Simultaneous loss of both RAP stimulatory pathways leads to marked defects in integrin activation[22], RAC1-mediated cytoskeletal dynamics [20,23], MAPK ERK signaling and $TxA_2$ generation[19].

These studies provide indirect evidence that RAP signaling is critical for overall platelet activation and hemostatic plug formation. The only direct evidence supporting an important role for RAP1 signaling in platelet activation comes from studies in mice with systemic deletion of the predominant RAP1 isoform in platelets, RAP1B. Surprisingly, *Rap1b*$^{-/-}$ platelets displayed a rather mild platelet phenotype, including partially decreased agonist-induced fibrinogen binding and aggregation[24], reduced cytoskeleton-regulated responses such as secretion and spreading, and no defect in MAPK signaling[25]. To date, the role of RAP1A in the platelet hemostatic response has not been resolved, although Chrzanowska-Wodnicka and colleagues reported unpublished observations that platelet aggregation is normal in RAP1A-null mice[24]. Mice lacking RAP1A have impaired immune cell functions, which were not compensated for by RAP1B, suggesting that these isoforms may indeed have distinct roles[26]. Interestingly, the peripheral platelet count and size was normal in *Rap1a*$^{-/-}$ and *Rap1b*$^{-/-}$ mice, suggesting a minimal role for RAP1 signaling in platelet production. This finding is surprising considering that RAP1 GTPases are highly

expressed in platelet progenitor cells[27]. However, very little[28,29] has been done so far to investigate their role in megakaryocyte biology and platelet production.
In the present study, we characterized mice with targeted deletion of *Rap1a* and *Rap1b* selectively in the platelet/megakaryocyte lineage to investigate the specific contribution of the individual RAP isoforms in the regulation of platelet production, platelet activation and hemostatic/thrombotic plug formation. We demonstrate that RAP isoforms have both redundant and isoform-specific functions in platelets and that they are essential for the regulation of hemostasis at sites of mechanical injury, but dispensable at sites of inflammation. Moreover, we identify a previously unrecognized role of RAP1 signaling in platelet production.

## Methods

### Mice

Generation of *Rap1a*$^{fl/fl}$*Rap1b*$^{fl/fl}$ [30] and *Talin1*$^{fl/fl}$[31] *mice* has been described previously. Megakaryocyte-specific *Rap1a* knockout (*Rap1a-mKO*), *Rap1b* knockout (Rap1b-mKO), *Rap1a/b* double knockout mice (*Rap1a/b-mKO*) and their respective control mice were generated by crossing *Rap1a*$^{fl/fl}$*Rap1b*$^{fl/fl}$ mice with *C57BL/6-Tg(Pf4-Cre*$^{+}$*)* mice[32]. Experimental procedures were approved by the Institutional Animal Care and Use Committees.

### Megakaryocyte studies

Proplatelet formation was studied in mouse bone marrow–derived megakaryocytes as described recently[18].

### Platelet studies

Platelet count, size and lifespan, surface receptor expression, integrin αIIbβ3 activation and α-granule secretion were determined as previously described[18]. Integrin β1 activation was determined by measuring the binding of an antibody directed against the active form of murine β1 (clone 9EG7)[33]. Aggregometry and RAP2 pull-down assays were performed as described in [23]. Ex vivo flow studies were performed in a collagen-coated microfluidic chamber as described in [22].

### Thrombosis and hemostasis studies

Ferric chloride($FeCl_3$)-induced thrombosis to the common carotid artery was performed as described previously[34]. Tail bleeding time and blood loss volume were measured as described in[22]. Hemostatic plug formation and bleeding time upon laser-induced injury to the saphenous vein was described in [35].

### Vascular integrity studies

The contribution of platelets to vascular integrity at sites of inflammation was determined by challenging mice with the reverse passive Arthus (rpA) reaction as described previously[36]. Preparation and immunohistochemistry of embryos at ~E16.5 to assess blood-lymphatic mixing (BLM) was performed as described in [18].

### Statistics

Results are reported as mean ± standard error of the mean (SEM) and statistical significance was assessed by ANOVA test, unless otherwise indicated. A P value less than 0.05 was considered significant.

Detailed methods are presented in the online supplement.

## Results

### Combined deficiency of RAP1A and RAP1B in the platelet/megakaryocytic lineage leads to macrothrombocytopenia.

Systemic deletion of both RAP1 isoforms is not viable in mice[37]. To investigate the respective role of RAP1A and RAP1B in platelet biology, we crossed floxed *Rap1a* and *Rap1b* mice[30] with *C57BL/6-Tg(Pf4-Cre)* mice[32], where the *Cre* recombinase expression is driven by the *Pf4* promoter. Megakaryocyte-specific *Rap1a/b* double knockout mice *(Rap1a/b-mKO, Rap1a$^{fl/fl}$Rap1b$^{fl/fl}$Pf4-Cre+)* were viable and fertile and appeared overall healthy, with no spontaneous bleeding seen to date. Mice were routinely genotyped to detect the floxed *Rap1* alleles and the *Cre* recombinase gene (**Supplementary Figure 1A**). Western blot analysis confirmed the complete ablation of both RAP1 isoforms in the *Rap1a/b-mKO* platelets and deletion of RAP1A or RAP1B in *Rap1a-mKO (Rap1a$^{fl/fl}$Rap1b$^{+/+}$Pf4-Cre+)* and *Rap1b-mKO (Rap1a$^{+/+}$Rap1b$^{fl/fl}$Pf4-Cre+)* mice, respectively (**Supplementary Figure 1B**). Both platelet count and platelet size were normal in *Rap1a-mKO* mice (data not shown). *Rap1b-mKO* mice exhibited a normal platelet count and a slight increase in platelet size (**Figure 1A,B**). Concomitant deletion of one allele of *Rap1a* led to a further increase in platelet size (**Figure 1B**). *Rap1a/b-mKO* displayed a ~40% reduction in platelet count and a ~50% increase in platelet size. The marked thrombocytopenia observed in *Rap1a/b-mKO* mice is likely the result of impaired platelet production, as we observed no reduction, but rather a slight increase, in the half-life of *Rap1a/b-mKO* platelets in circulation (**Figure 1C**) and a significant defect in proplatelet formation by megakaryocytes ex vivo (**Figure 1D,E**).

### Signaling by both RAP1A and RAP1B contributes to platelet integrin activation.

Given the documented role of RAP1 signaling in cell adhesion[11], we next investigated the role of both RAP1 isoforms in integrin activation. Deletion of both isoforms did not impair the surface expression of important adhesion receptors, including αIIbβ3 and β1 integrins (**Supplementary Figure 1C**). Agonist-induced activation of platelet integrins was first evaluated by flow cytometry using conformation-sensitive antibodies against activated αIIbβ3 (**Figure 2A**) and β1 (**Figure 2B**). Consistent with studies in conventional *Rap1b* knockout mice[24], integrin activation was partially reduced in *Rap1b-mKO* platelets activated with various agonists. A more modest, but significant defect in integrin activation was observed in *Rap1a-mKO* platelets, revealing a previously unrecognized role for this isoform in platelet inside-out signaling. Importantly, combined deficiency of RAP1A and RAP1B led to an almost complete (80-90%) inhibition of αIIbβ3 and β1 integrin activation, even at high doses of strong agonists.

### Limited integrin activation, mediated by TALIN1, in platelets lacking both RAP1 isoforms is not mediated by RAP2 GTPases.

TALIN binding to the integrin β tail is the final common step that mediates integrin activation. Elegant studies in heterologous cell lines stably expressing αIIbβ3[38-40] demonstrated that RAP1 mediates integrin activation by targeting the cytoskeletal protein TALIN to the plasma membrane. To test whether RAP1 is essential for TALIN-mediated integrin activation, we next compared *Rap1a/b-mKO* and *Talin1-mKO* platelets[31]. Consistent with previous reports[31,41,42], both agonist-induced JON/A-PE binding and aggregation response were virtually abolished in *Talin1-mKO* platelets **(Figure 3A,B)**. When compared to *Talin1-mKO* platelets, *Rap1a/b-mKO* platelets exhibited a small but significant increase in JON/A-PE binding and delayed aggregation in response to high doses of agonists (except ADP). The maximum extent of aggregation was always reduced in *Rap1a/b-mKO* platelets when compared to control platelets and aggregates of *Rap1a/b-mKO* platelets appeared smaller by visual inspection (not shown). Residual aggregation

of *Rap1a/b-mKO* platelets was inhibited by pre-incubation with a function blocking antibody to murine αIIbβ3 **(Figure 3B)**. Thus, while *Talin1-mKO* platelets were intrinsically incapable of activating αIIbβ3 integrin, a limited amount of integrin-mediated aggregation was observed in platelets lacking RAP1A and RAP1B.
Platelets also express all RAP2 isoforms, RAP2A, RAP2B and RAP2C[6-8], which are under the control of the same GEF[23] and are 70% homologous to RAP1[43]. Thus, we investigated whether RAP2 GTPases could be responsible for the RAP1-independent, TALIN1-dependent integrin activation elicited by high doses of agonists in *Rap1a/b-mKO* platelets. We stimulated Rap1a/b-mKO, *Talin1-mKO* and control platelets for 5 minutes with two distinct concentrations of agonist, above or below the aggregating threshold of *Rap1a/b-mKO* platelets, and then measured RAP2-GTP levels in these samples **(Figure 3C)**. We detected comparable levels of active RAP2 in platelets from all genotypes. Moreover, we found no correlation between RAP2 activation and the level of platelet aggregation reached by these platelets, i.e. RAP2 was activated in both aggregating and non-aggregating platelets. Thus, it is unlikely that RAP2 signaling mediates integrin activation in platelets deficient in RAP1.

**Loss of both RAP1A/B isoforms impairs platelet spreading and clot contraction**
Following inside-out activation of αIIbβ3 integrin, ligand binding triggers outside-in signaling which plays an essential role in platelet spreading and clot contraction by linking integrin activation with cytoskeletal mechanical forces[44]. Previous work has reported that fibrinogen binding induces RAP1 GTP-loading[25], and that *Rap1b*$^{-/-}$ platelets exhibit defects in spreading[24,25] and clot contraction[25]. To analyze the ability of our RAP mutant platelets to spread, cells were added to fibrinogen-coated wells, activated with ADP at 37ºC, and both the platelet area and the percentage of fully spread platelets were quantified. Under these conditions, approximately 85-90% of control platelets were fully spread. Both *Rap1a-mKO* and *Rap1b-mKO* platelets spread normally compared to controls, while *Rap1a/b-mKO* platelets had ~50% reduction in platelet area and displayed an abnormal, non-spread morphology **(Figure 3D)**. Clot contraction was also partially impaired in *Rap1a/b-mKO* platelets **(Figure 3E)**. In both the platelet spreading and clot contraction assays, the defect in *Rap1a/b-mKO* platelets was less severe than that of *Talin1-mKO* platelets.

**Specific RAP isoforms differentially regulate the release of second-wave mediators.**
Deficiency in CalDAG-GEFI affects not only platelet integrin activation but also the release of second wave mediators[17,19,20,23], which are crucial for full platelet activation and hemostatic plug formation. Therefore, we next investigated the contribution of RAP1 isoforms to granule secretion and $TxA_2$ generation. As previously described for conventional *Rap1b*-KO mice[25], RAP1B deficiency in platelets impaired α-granule secretion in response to GPVI, but not PAR4 receptor stimulation **(Figure 4A,B)**. In contrast, deletion of Rap1A alone did not affect P-selectin exposure, and *Rap1a/b-mKO* platelets displayed the same secretion phenotype as *Rap1b-mKO* platelets. Our previous studies suggested important cross-talk between RAP1 and RAC1 in the regulation of actin dynamics and granule secretion in platelets activated via GPVI[23]. Consistently, *Rap1b-mKO and Rap1a/b-mKO* platelets, but not *Rap1a-mKO* platelets, failed to activate RAC1 in response to convulxin stimulation at early time points **(Figure 4C,D)**. RAC1-GTP levels were also significantly reduced in *Rap1a/b-mKO* platelets at the later time point after addition of the agonist.
Our previous studies also demonstrated impaired $TxA_2$ generation in platelets lacking CalDAG-GEFI[19]. Consistently we found that both RAP1 isoforms contributed to $TxA_2$ generation, with *Rap1b-mKO* platelets displaying a phenotype almost comparable to that

of the *Rap1a/b-mKO* platelets and RAP1A playing a minor role (**Figure 4E**). However, while simultaneous loss of both RAP stimulatory pathways (*Caldaggef1*$^{-/-}$*P2y12*$^{-/-}$) completely blocked GPVI-mediated $TxA_2$ generation, the defect in this response was partially overcome in *Rap1a/b-mKO* platelets stimulated with a high concentration of agonist (**Figure 4F**).

**RAP1A and RAP1B are critical for platelet adhesion under shear conditions and the formation of both thrombotic and hemostatic plugs.**

To assess the contribution of RAP1A and RAP1B to thrombus formation under physiological shear stress conditions, we next studied platelet adhesion to collagen *ex vivo* in a microfluidics chamber assay (**Figure 5A**). Under these conditions, combined deficiency of RAP1A and RAP1B strongly inhibited platelet adhesion to collagen and completely prevented platelet-platelet cohesion (**Figure 5B,C**). Expression of a single *Rap1a* allele significantly improved platelet adhesion compared to the *Rap1a/b-mKO* platelets, and it allowed for the formation of few very small thrombi. Both platelet adhesion and platelet-platelet cohesion were further improved in blood from mice expressing only a single copy of *Rap1b*, confirming that RAP1B is more effective than RAP1A in supporting integrin-mediated platelet adhesion.

Consistent with the ex vivo studies, we observed severely reduced thrombotic plug formation after ferric chloride injury to the carotid artery in *Rap1a/b-mKO* mice (**Figure 5D**). No defect in arterial thrombosis was observed in *Rap1a-mKO* mice, while a small but significant prolongation in the occlusion time was observed in *Rap1b-mKO* mice. Thus, both RAP1A and RAP1B contribute to platelet adhesion and thrombus formation under flow conditions *ex vivo* and *in vivo*.

To determine the contribution of individual RAP1 isoforms to hemostatic plug formation in vivo, we challenged our Rap1 knockout mice in the standard tail transection model **(Figure 6A,B)** and our recently developed laser injury-induced saphenous vein hemostasis model[35]**(Figure 6C,D)**. While expression of a single allele of *Rap1b* was sufficient to promote hemostasis in both models, a significant prolongation in the bleeding time and an increase in the blood loss volume from the severed tail were observed in mice expressing one allele of *Rap1a* and in *Rap1a/b-mKO* mice **(Figure 6A,B)**. Interestingly, only *Rap1a/b-mKO* showed a significant increase in the bleeding time after laser injury, comparable to that observed in *Talin1-mKO* mice **(Figure 6C).** Compared to the tail transection model, laser–induced injuries to the endothelial lining are small (~50µm in diameter). Platelets expressing one allele of *Rap1b* were able to plug these small lesions, but platelet plugs were significantly smaller than in control mice **(Figure 6D and supplementary videos).** In conclusion, these *ex vivo* and *in vivo* studies demonstrate that signaling by RAP1A can partially compensate for loss of RAP1B during hemostatic and thrombotic plug formation.

**RAP1 GTPases, but not TALIN, play a minor role for embryonic vascular development and vascular integrity at sites of inflammation in the adult.**

In addition to their classical role in the prevention of excessive blood loss at sites of mechanical injury, platelets also safeguard vascular integrity during development and at sites of inflammation[45-47]. To test whether platelet RAP1-TALIN signaling is important during development, we studied embryos from *Rap1a/b-mKO* and *Talin1*-mKO mice for signs of blood-lymphatic mixing (BLM). Compared to controls, ~E16.5 *Rap1a/b-mKO* embryos showed very mild signs of BLM as shown by macroscopic observation of whole embryos and immunostaining of embryo sections (**Figure 7A**). Markedly more severe BLM was observed in some *Talin1*-mKO embryos, while others showed only mild defects. Similar to our observations in the developing embryos, *Talin1*-mKO mice, but not Rap1a/b-mKO mice, exhibited significant hemorrhage in a model of immune complex–mediated

inflammation in the skin (rpA reaction) (**Figure 7B**). Hemorrhage at sites of inflammation in *Talin1*-mKO mice, however, was significantly less than in control mice rendered thrombocytopenic (<2% platelet count).

**Discussion**

Integrin inside-out activation is critical for platelet adhesion and hemostatic plug formation at sites of vascular injury. The dogma in the field is that integrin activation requires the assembly of the integrin activation complex, including RAP1, TALIN1, and KINDLIN3, at the cytoplasmic tail of the integrin β subunit[48]. However, while platelet integrin activation was completely abolished in mice deficient in *Talin1*[31,41] or *Kindlin3*[49], studies in germline knockout mice identified a significant, but not crucial role for RAP1B in this process[24,25]. This led to the belief that there is a RAP1-independent mechanism that facilitates TALIN/KINDLIN3-mediated integrin activation in platelets[50]. Alternative mechanisms for RAP1-independent TALIN recruitment in proximity of the integrin were suggested, including TALIN binding to acidic phospholipids[51]. Recent advances in proteomic profiling, however, demonstrated that murine platelets also express ~20,000 RAP1A molecules[52], a copy number that may be high enough to facilitate integrin activation in the absence of RAP1B. Our studies in mice lacking *Rap1a* and/or *Rap1b* in the megakaryocytic lineage provide the first definitive proof that both RAP1A and RAP1B regulate the activation of αIIbβ3 and β1 integrins in platelets. Consistent with previous reports[24], integrin activation in platelets from *Rap1b-mKO* mice was reduced by >50%. However, we also observed a significant contribution of RAP1A to β1 and β3 integrin activation in stimulated platelets, which accounted for >70% of the integrin activation observed in platelets lacking RAP1B. The residual integrin activation observed in platelets from mice deficient in both RAP1 isoforms allowed for limited platelet aggregation as measured in standard aggregometry. We and others described similar results for mice expressing low levels of CalDAG-GEFI[34], mice expressing a loss-of-function (LOF) mutant of TALIN1[42], or mice expressing a LOF mutant of KINDLIN3[53]. Our studies suggest that the minimal integrin activation observed in *Rap1a/b-mKO* platelets is not mediated by other RAP proteins, as RAP1-independent platelet aggregation did not correlate with RAP2 activation. Future studies will have to determine whether small GTPase-dependent or -independent mechanisms mediate limited integrin activation in the absence of RAP1 proteins. Irrespective of the exact mechanism, however, the patho-physiological significance of this RAP1-independent pathway of integrin activation in arterial thrombosis and classical hemostasis is limited considering that thrombus formation under physiological flow conditions was abolished in *Rap1a/b-mKO* mice. Our data indicates that expression of at least one *Rap1* allele is necessary to allow for the minimal amount of integrin activation that supports the formation of three-dimensional hemostatic plugs capable of ensuring hemostasis. While expression of a single *Rap1b* allele in mice was sufficient to facilitate hemostasis at sites of small (laser injury) and large injuries (tail-clip), expression of a single *Rap1a* allele could only support hemostatic plug formation after small injuries. At the moment, it is not clear how RAP1A and RAP1B mediate platelet integrin activation. It was suggested that the RAP1 effector RIAM was necessary to connect RAP1 and TALIN, thereby enabling the recruitment of TALIN to the plasma membrane where active RAP1 is localized[40]. This hypothesis was disproved, however, as RIAM was found dispensable for β1 and β3 integrin activation, adhesion and aggregation of murine platelets[54,55]. Recent in vitro studies suggest that active RAP1 can directly bind TALIN[56], without an intermediate effector, but whether this is true also in platelets needs to be confirmed.

Our studies also provide indirect evidence for non-overlapping roles of RAP1A, RAP1B and RAP2 in platelet activation (**Supplementary Figure 2**). While both isoforms exhibit

redundant function in integrin activation and $TxA_2$ formation, RAP1B primarily facilitates cross-talk with RAC1 and solely regulates α-granule secretion in platelets stimulated via the collagen receptor, GPVI. Interestingly, $TxA_2$ formation was not completely abolished in *Rap1a/b-mKO* platelets as it was in platelets lacking CalDAG-GEFI and P2Y12, pathways that control the activity state of both RAP1 and RAP2[23]. Consistent with a role for RAP2 in this process, proteomics profiling[6,52] revealed that platelets express several RAP2-specific effectors[57,58], including MINK1. Platelets from mice deficient in MINK1 are defective in ERK and p38 MAPK signaling[59], pathways that contribute to $TxA_2$ generation[60]. Thus, while RAP2 does not seem to be involved in the regulation of integrin activation, it is a likely player in the regulation of MAPK-dependent $TxA_2$ generation. Alternatively, $TxA_2$ production may, in part, depend on a RAP-independent signaling pathway. *Rap1a/b-mKO* platelets, but not *Rap1a-mKO* and *Rap1b-mKO* platelets, were also defective in cellular responses depending on integrin outside-in signaling. Our data on the *Rap1b-mKO* platelets is not consistent with previous work showing defects in outside-in signaling in platelets from *Rap1b*$^{-/-}$ mice[25]. Aside of methodological differences, we currently do not have an explanation for the conflicting phenotypes. It is also important to remember that integrin inside-out activation has to precede outside-in signaling in spreading and clot contraction assays. Both *Rap1a-mKO* and *Rap1b-mKO* platelets, but not *Rap1a/b-mKO* platelets, showed robust inside-out activation of αIIbβ3 integrin. Thus, the defect in spreading and clot contraction observed in *Rap1a/b-mKO* platelets may primarily be a reflection of impaired integrin activation in these cells. Consistent with this conclusion, *Rap1b-mKO* platelets exhibited a defect in RAC1 activation at an early time point after agonist stimulation, while no defect was seen later in the activation process. RAC1 activation was markedly diminished in *Rap1a/b-mKO* platelets throughout the activation process. These findings could be explained by normal outside-in signaling in RAP1 mutant platelets. More in-depth studies will be required to clarify this point.

While well-established in platelets, surprisingly little is known about the contribution of RAP GTPase signaling to megakaryocyte development and platelet production. It is known that ERK is activated in response to megakaryocyte stimulation with thrombopoietin, a key cytokine regulator of megakaryocyte development[61,62], and that RAP and RAS GTPases mediate ERK activation in megakaryocyte cell lines via engagement of their downstream effectors B-RAF and RAF-1[63,64]. While mice deficient in RAF-1 did not show defects in megakaryocyte biology[65], deficiency in B-RAF led to impaired hematopoietic progenitor cell development and altered megakayopoiesis[66]. Furthermore, the cross-talk between RAP1 and RAC1 may also affect platelet production, as RAC1 is an important regulator of proplatelet formation in mature megakaryocytes[67]. Deletion of RAC1 in mice, however, did not affect the peripheral platelet count, unless another Rho GTPase, CDC42, was also deleted. Previous preclinical and clinical data did not suggest a role for RAP in megakaryocyte biology as deficiency in CalDAG-GEFI or RAP1B did not affect the peripheral platelet count in mice, dogs, or humans[17,20,24,68]. Consistent with these findings, a normal platelet count was observed in mice with megakaryocyte-specific deficiency in *Rap1a* or *Rap1b.* However, *Rap1a/b-mKO* mice exhibited a significant macrothrombocytopenia and a marked defect in proplatelet formation *in vitro*. Thus, we here provide the first definitive proof that RAP is a critical player during platelet production. Studies in progress will address if RAP1A and RAP1B have redundant or unique functions during megakaryocyte development and proplatelet formation. Given its crucial role in platelet function, it is surprising that CalDAG-GEFI is dispensable in megakaryocytes. However, while RAP1 activation in platelets depends on a highly sensitive and rapidly-activated GEF like CalDAG-GEFI, megakaryocytes do not depend on the same kinetics of cellular activation. Future studies should investigate the

role of other RAP-GEFs, such as PDZ-GEF and EPAC, as regulators of RAP signaling in megakaryocytes.

In addition to their role in thrombus formation at sites of injury, platelets contribute to hemostasis by ensuring vascular integrity where the endothelial barrier has been breached without mechanical injury, for instance during development or at sites of inflammation in adulthood[69]. In both cases it is currently not clear how platelets affect vascular integrity. However, previous studies identified mechanistic similarities as mice with defects in platelet ITAM signaling show BLM during embryonic development and marked hemorrhage at sites of inflammation[36,47,70-72]. No bleeding was observed in mice with defects in receptors and signaling molecules important for classical hemostatic plug formation, including PAR and P2Y receptors, GPIb-V-IX, αIIbβ3, and CalDAG-GEFI[47]. Interestingly, degranulated platelets failed to prevent tumor-associated inflammatory bleeding[73], suggesting that granule release but not integrin-mediated adhesion is critical for vascular integrity in inflammation and during development, a conclusion that was recently challenged by studies in mice genetically engineered to be defective in granule secretion[74]. The studies reported here shift the focus back on integrins, as we observed significant BLM and inflammatory bleeding in *Talin1-mKO* mice. However, vascular integrity was intact in *Rap1a/b-mKO* mice. Considering this partial TALIN1 dependency and recent studies demonstrating that single platelets, not platelet aggregates, plug holes in the inflamed vascular wall[72], we propose that integrins other than αIIbβ3 or other TALIN1-regulated adhesion receptors contribute to platelet adhesion in this form of hemostasis. The very limited ability of *Rap1a/b-mKO* platelets to activate integrin receptors, however, seems sufficient to prevent hemorrhage during embryonic development and inflammation. Alternatively, it is possible that TALIN1 has RAP1-independent functions that are critical for platelet-mediated vascular integrity.

In humans, loss-of-function mutations in CalDAG-GEFI[20,21,75-77] or P2Y12[78], the main RAP stimulating pathways, lead to a marked defect in hemostasis. Interestingly, no patients with mutations in one of the RAP1 isoforms have been identified to date. Studies in RAP1 mutant mice[24] suggested that loss-of-function in *Rap1b* may cause embryonic lethality in humans, while mutations in *Rap1a* would be without significant impact on platelet function and hemostasis. It is important, however, that RAP1A is much more abundant in human than in mouse platelets, with the expression ratio for RAP1B to RAP1A being 2/1 in humans[6] and 10/1 in mice[52]. Our findings of functional redundancy between the two RAP isoforms in the inside-out activation of platelet integrins provides an alternative, more plausible explanation for the lack of loss-of-function mutations in either *Rap1a* or *Rap1b* in humans. We propose that both isoforms would have to be defective simultaneously for classical hemostasis to be impaired. The same conclusions can be drawn for the role of RAP1 in platelet production and the effect of RAP1 mutations on the peripheral platelet count. Lastly, our findings also have important implications for the development of novel antiplatelet therapies, as targeting of the pathways regulating RAP1 activity or of individual RAP1 isoforms would be expected to provide significant protection from thrombosis without jeopardizing the platelet count or hemostasis.

In conclusion, our studies demonstrate that RAP1 GTPases have both redundant and isoform-specific functions in platelets and MKs. Mice deficient in both *Rap1a* and *Rap1b* exhibit significant macrothrombocytopenia due to impaired proplatelet formation, strongly impaired integrin activation in platelets, and marked defects in hemostasis after mechanical injury. In contrast, platelet RAP1 signaling is dispensable for the maintenance of vascular integrity during development and at sites of inflammation in mice.

**Authorship**
L.S designed research, performed research, analyzed data and wrote manuscript, R.H.L. performed experiments, analyzed data and wrote manuscript, D.S.P. performed experiments, E.C.O. performed experiments and analyzed data, D.G. performed experiments, C.I.J. performed experiments and analyzed data, Y.B. performed experiments, K.O.P. performed experiments and provided technical assistance, R.P. performed experiments, D.O.K. performed experiments, K.M.C. analyzed data, K.M.H contributed vital reagents, J.M.G. contributed vital reagents and analyzed data, W.B. designed research and wrote manuscript.

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**Disclosures**
The authors have declared that no conflict of interest exists.

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## Figures

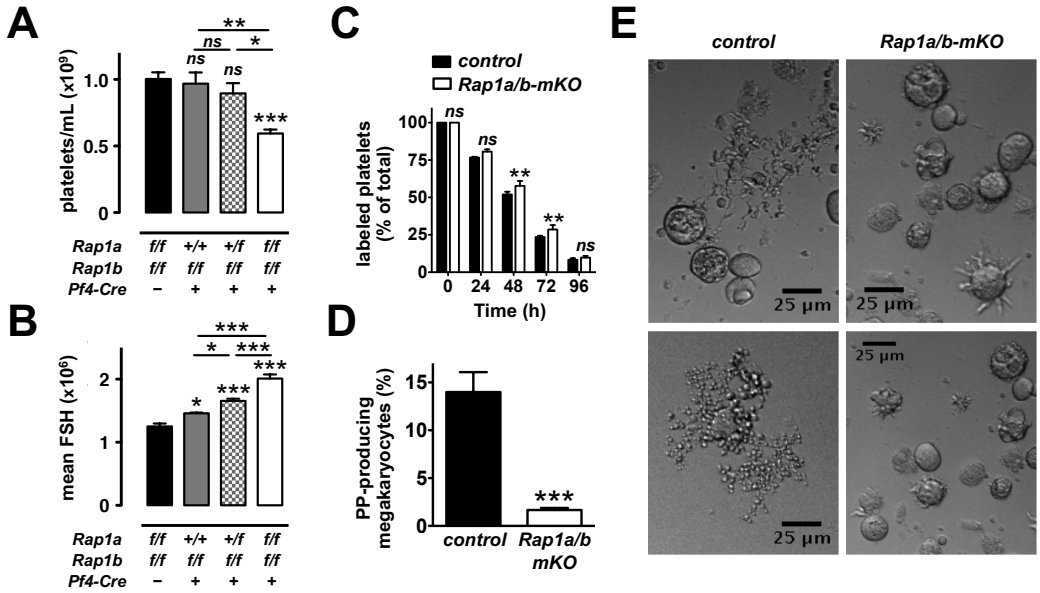


**Figure 1. Megakaryocyte-specific deletion of the *Rap1a* and *Rap1b* genes leads to macrothrombocytopenia.** Flow cytometric analysis of the (**A**) peripheral platelet count and (**B**) platelet size (forward scatter height, FSH) in mice lacking one or both alleles of the *Rap1a* and *Rap1b* genes in the platelet/megakaryocyte lineage as indicated below each column (n=12). (**C**) Platelet lifespan assay. Endogenous platelets were labeled by infusion of Alexa Fluor 488–conjugated antibody to GPIX, and the percentage of GPIX-labeled platelets remaining was determined by flow cytometry every 24 hours. (**D**) *In vitro* pro-platelet (PP) formation in bone marrow–derived megakaryocytes. (**E**) Representative images of megakaryocytes for PP formation assay, acquired on an Olympus IX-81 wide-field microscope with a Photometrics CoolSnap HQ2 camera. *p<0.05, **p<0.01, ***p<0.001.

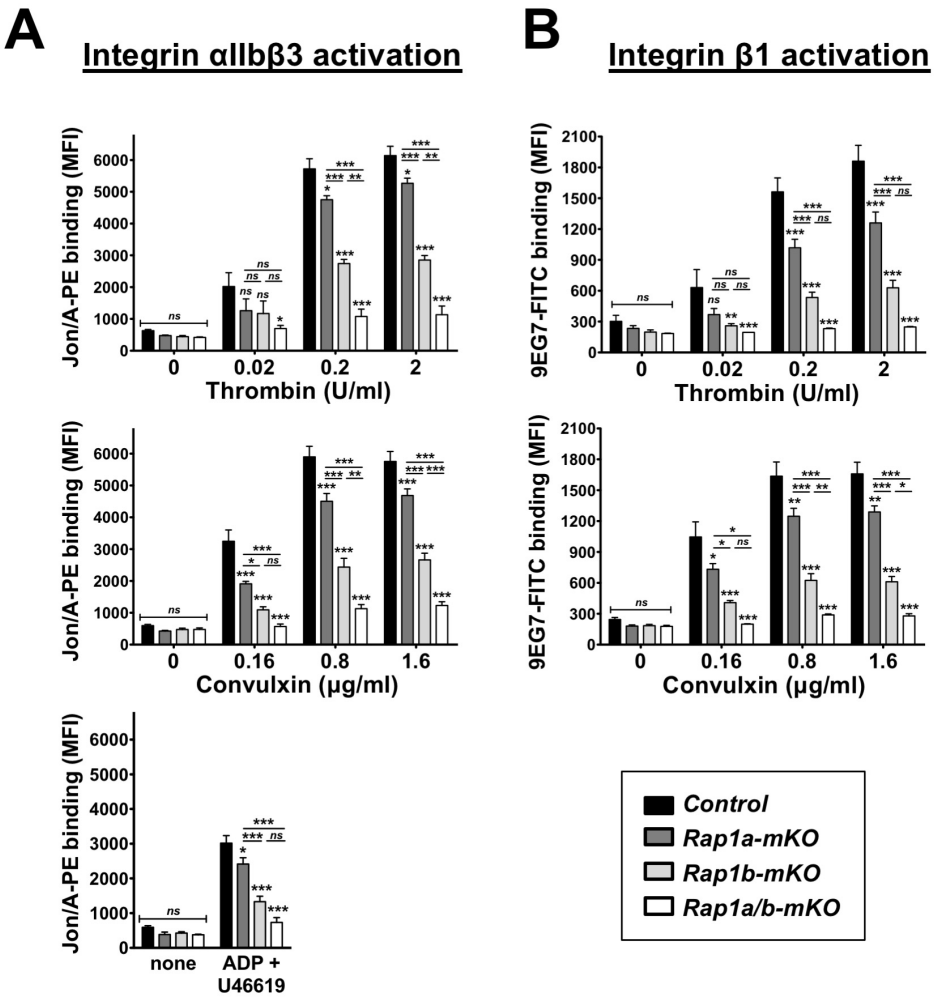


**Figure 2. Both RAP1A and RAP1B contribute to platelet integrin activation.** Flow cytometric analysis of (**A**) integrin αIIbβ3 activation (binding of Jon/A-PE, clone Leo.H4, Emfret Analytics) and (**B**) integrin β1 activation (binding of 9EG7-FITC, BD Biosciences) in response to increasing concentrations of thrombin, the GPVI-agonist convulxin, or the combination of ADP and the thromboxane (Tx)$A_2$ analog U46619. Data shown are mean fluorescence intensities (MFI) ± SEM of control *(Rap1a$^{fl/fl}$Rap1b$^{fl/fl}$Pf4-Cre-), Rap1a-mKO (Rap1a$^{fl/fl}$Rap1b$^{wt/wt}$Pf4-Cre+), Rap1b-mKO (Rap1a$^{wt/wt}$Rap1b$^{fl/fl}$Pf4-Cre+)* and *Rap1a/b-mKO (Rap1a$^{fl/fl}$Rap1b$^{fl/fl}$Pf4-Cre+)* platelets (n = 6); *p<0.05, **p<0.01, ***p<0.001.

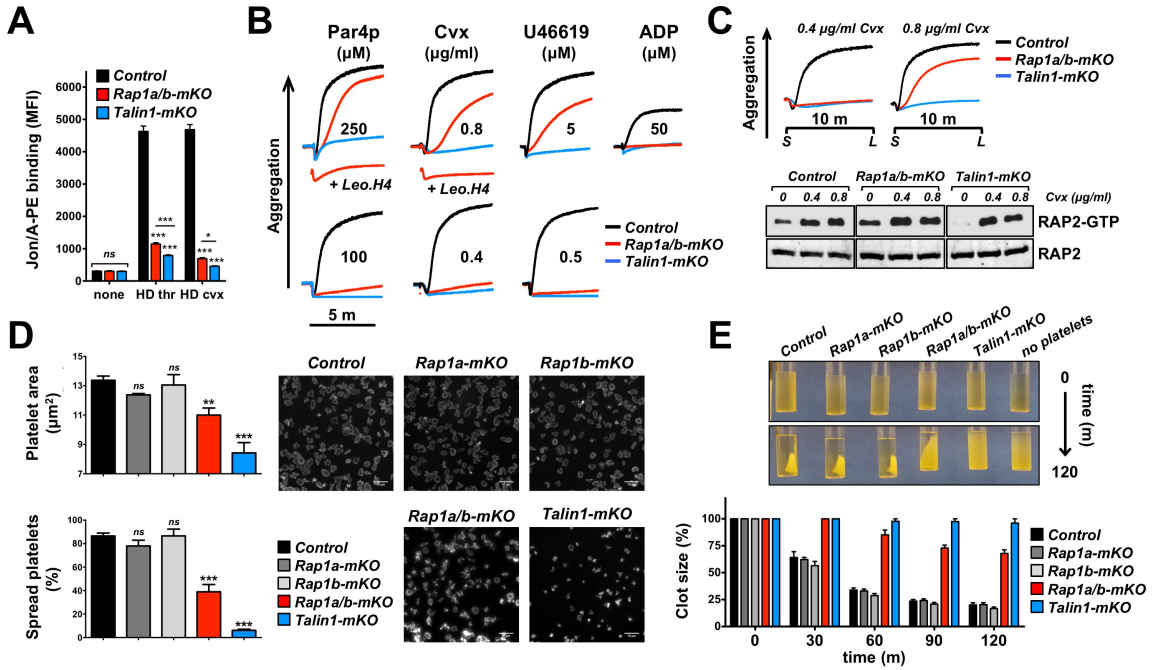


**Figure 3. Limited TALIN1-mediated integrin activation in platelets lacking both RAP1 isoforms is not mediated by RAP2 GTPase.** (**A**) Flow cytometric analysis of αIIbβ3 integrin activation (JON/A-PE antibody binding) in *Rap1a/b-mKO (Rap1a$^{fl/fl}$Rap1b$^{fl/fl}$Pf4-Cre+)* and *Talin1-mKO (Talin1$^{fl/fl}$Pf4-Cre+)*, platelets (n=5); *p<0.05, ***p<0.001. (**B**) Aggregation response of platelets from *Rap1a/b-mKO* and *Talin1-mKO* platelets (representative of 4 independent experiments). For high dose Par4p and Cvx aggregation, the residual aggregation response of *Rap1a/b-mKO* platelets was completely abolished by pre-incubation with 30 μg/ml of the αIIbβ3 blocking antibody Leo.H4. (**C**) Rap2-GTP pulldown assay. Washed platelets stimulated (S) in standard aggregometry with 0, 0.4 μg/mL or 0.8 μg/mL of the GPVI-agonist convulxin (Cvx) were lysed (L) after 10 minutes, to measure the levels of active RAP2 by pull-down assay; n=3. Total RAP2 was determined as loading control. (**D**) Platelet spreading on fibrinogen. Platelets were incubated in fibrinogen-coated wells, activated with ADP (100 μM), and the extent of platelet spreading was determined after 45 minutes. Representative images of phalloidin-stained platelets are shown (n=3-5). (**E**) Clot contraction assay. Washed platelets from indicated knockout mice were added to human platelet-poor plasma (PPP) in ACD containing 5 mM $Ca^{2+}$ and 0.2 U/ml thrombin in siliconized cuvettes (n=3). Representative images of clots at time 0 and 120 mins are shown; "no platelets" sample contained all components except washed platelets. *p<0.05, **p<0.01, ***p<0.001.

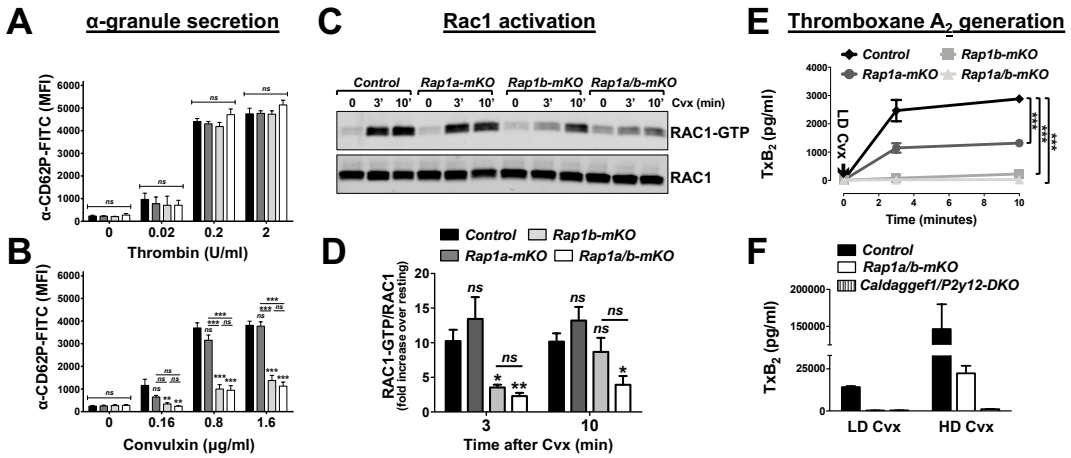


**Figure 4. Specific RAP isoforms regulate granule secretion, RAC1 activation and $TxA_2$ generation.** (**A, B**) Dose response studies of (A) thrombin- or (B) convulxin-induced P-selectin exposure (anti-CD62P binding by flow cytometry, clone RB40.34, BD Biosciences). Data shown are mean fluorescence intensities (MFI) ± SEM of control *(Rap1a$^{fl/fl}$Rap1b$^{fl/fl}$Pf4-Cre-,* black*), Rap1a-mKO (Rap1a$^{fl/fl}$Rap1b$^{wt/wt}$Pf4-Cre+,* dark grey*), Rap1b-mKO (Rap1a$^{wt/wt}$Rap1b$^{fl/fl}$Pf4-Cre+,* light grey*)* and *Rap1a/b-mKO (Rap1a$^{fl/fl}$Rap1b$^{fl/fl}$Pf4-Cre+,* white*)* platelets (n=6). (**C, D**) RAC1-GTP pulldown assay. (C) RAC1 activation was determined by pulldown assay in platelets of the indicated genotype stimulated for 0, 3 or 10 minutes with convulxin (Cvx). Total RAC1 was determined as loading control. Western blot images are representative of 3 independent experiments. (D) The ratio of RAC1-GTP over RAC1 band intensity for the three experiments is shown as fold increase over resting. (**E, F**) Thromboxane (Tx)$B_2$ generation assay. (E) Washed platelets from control *(*black diamond*), Rap1a-mKO (*dark grey circle*), Rap1b-mKO (*light grey square*)* and *Rap1a/b-mKO (*lighter grey triangle*)* mice were stimulated in standard aggregometry (not shown) with low doses of convulxin (LD Cvx; 0.16 μg/ml). After 0, 3 and 10 minutes of stimulation samples were withdrawn to measure the levels of $TxB_2$, the stable product of $TxA_2$. (F) In similar experimental conditions, *Rap1a/b-mKO* and *Caldaggef1$^{-/-}$P2y12$^{-/-}$* platelets were stimulated for 10 minutes with low (LD Cvx; 0.16 μg/ml) or high (HD Cvx; 1.6 μg/ml) doses of convulxin. *p<0.05, **p<0.01, ***p<0.001.

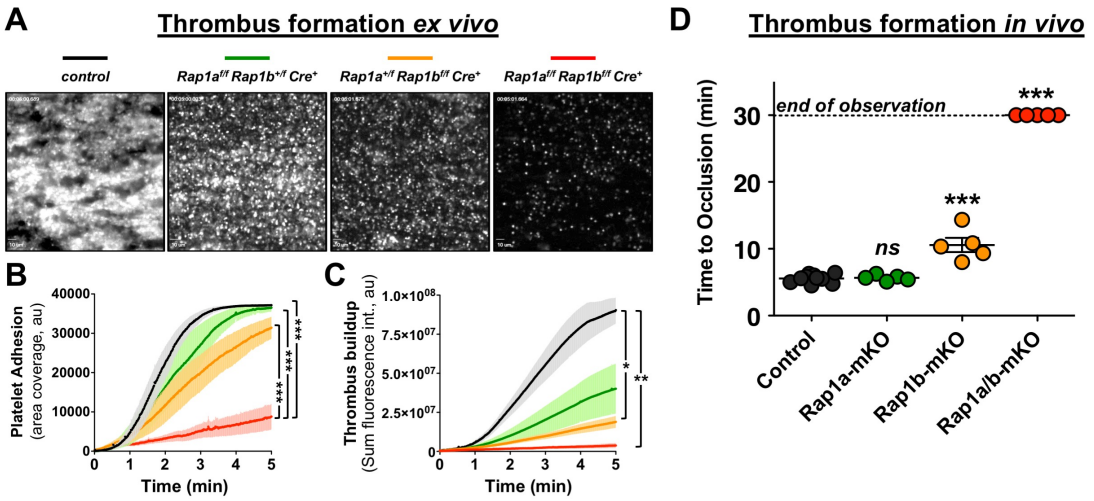


**Figure 5. Both RAP1A and RAP1B are critical for platelet adhesion and thrombus formation under shear conditions *ex vivo* and *in vivo*.** (**A, B, C**) Platelet adhesion to fibrillar collagen ex vivo. Anticoagulated whole blood from control (*Rap1a*$^{fl/fl}$*Rap1b*$^{fl/fl}$*Pf4-Cre-*, black), *Rap1a*$^{fl/fl}$*Rap1b*$^{+/fl}$*Pf4-Cre+* (green), *Rap1a*$^{+/fl}$*Rap1b*$^{fl/fl}$*Pf4-Cre+* (orange) or *Rap1a*$^{fl/fl}$*Rap1b*$^{fl/fl}$*Pf4-Cre+* (red) mice was perfused over fibrillar collagen type I (200 µg/mL) at venous shear rates (400$^{-s}$). Adhesion of platelets was monitored continuously. Shown are (A) representative images after 5 minutes of perfusion of Alexa Fluor 488–α-GPIX–labeled platelets obtained on a Nikon TE300 (Nikon, Tokyo, Japan) microscope equipped with a QImaging Retiga Exi camera (Qimaging, Surrey, BC, Canada), (B) platelet adhesion determined as the area of adhesion coverage ± SEM and (C) thrombus buildup quantified as sum fluorescence intensity ± SEM. Analysis were performed using Slidebook 5.0 Software (Intelligent Imaging Innovations, Denver, CO). (**D**) $FeCl_3$-induced thrombosis in the carotid artery. Data shown is the scatter dot plot (line at median) of the time of occlusion (minutes). Blood flow velocity was monitored for 30 minutes with a 0.5 mm Doppler flow probe connected to a TS420 Transonic flowmeter (Transonic, Ithaca, NY); time to occlusion was recorded when blood velocity reached 25% of baseline velocity. All *Rap1a/b-mKO* vessels never dropped below 25% of baseline flow, thus they were assigned a time of 30 minutes. *p<0.05, **p<0.01, ***p<0.001.

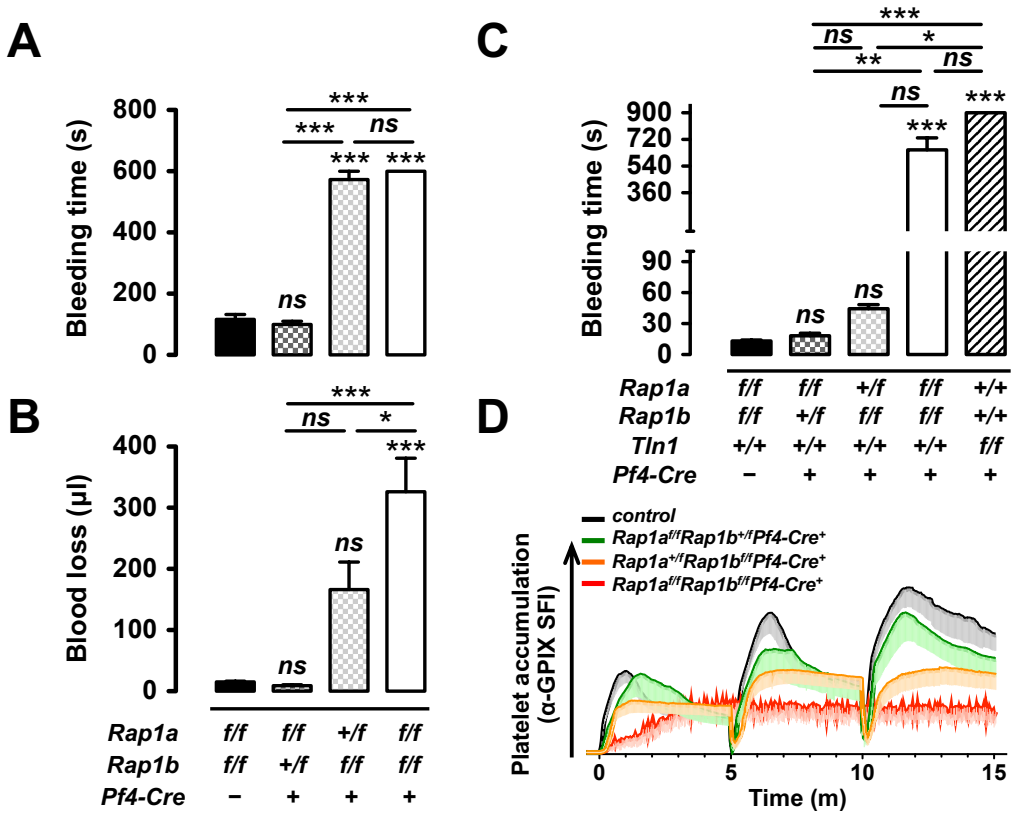


**Figure 6. Platelet RAP1 signaling is critical for hemostasis at sites of injury.** Determination of hemostasis using (**A, B**) tail clip or (**C, D**) saphenous vein laser injury model in control (*Rap1a*$^{fl/fl}$*Rap1b*$^{fl/fl}$*Pf4-Cre-*, black bar, black line), *Rap1a*$^{fl/fl}$*Rap1b*$^{+/fl}$*Pf4-Cre+* (checkered dark grey bars, green line), *Rap1a*$^{+/fl}$*Rap1b*$^{fl/fl}$*Pf4-Cre+* (checkered light grey bars, orange line) and *Rap1a/b-mKO* (*Rap1a*$^{fl/fl}$*Rap1b*$^{fl/fl}$*Pf4-Cre+,* white bars, red line) mice. Tail bleeding time (A) and blood loss volume (B) was determined following tail clipping. (C) Repeated laser injuries were made to the saphenous vein using an Ablate! photoablation system equipped with an attenuable 532nm pulse laser (Intelligent Imaging Innovations). Bleeding time was assessed as time (seconds) to stable hemostatic plug formation (no leakage of blood for more than 60 seconds) within the laser injury-induced vascular lesion. *Talin1*-mKO mice (*Talin1*$^{fl/fl}$*Pf4-Cre+,* striped bar), which bleed for the entire observation period, are shown for comparison. (D) Platelet accumulation was recorded using a Zeiss Axio Examiner Z1 microscope (Intelligent Imaging Innovations, Denver, CO) equipped with a 20x/1 numerical aperture water immersion objective lens and determined as sum fluorescence intensity (SFI) of GPIX-labelled platelets ± SEM at the site of injury over time; data were analyzed using Slidebook 5.0 software (Intelligent Imaging Innovations). *p<0.05, **p<0.01, ***p<0.001.

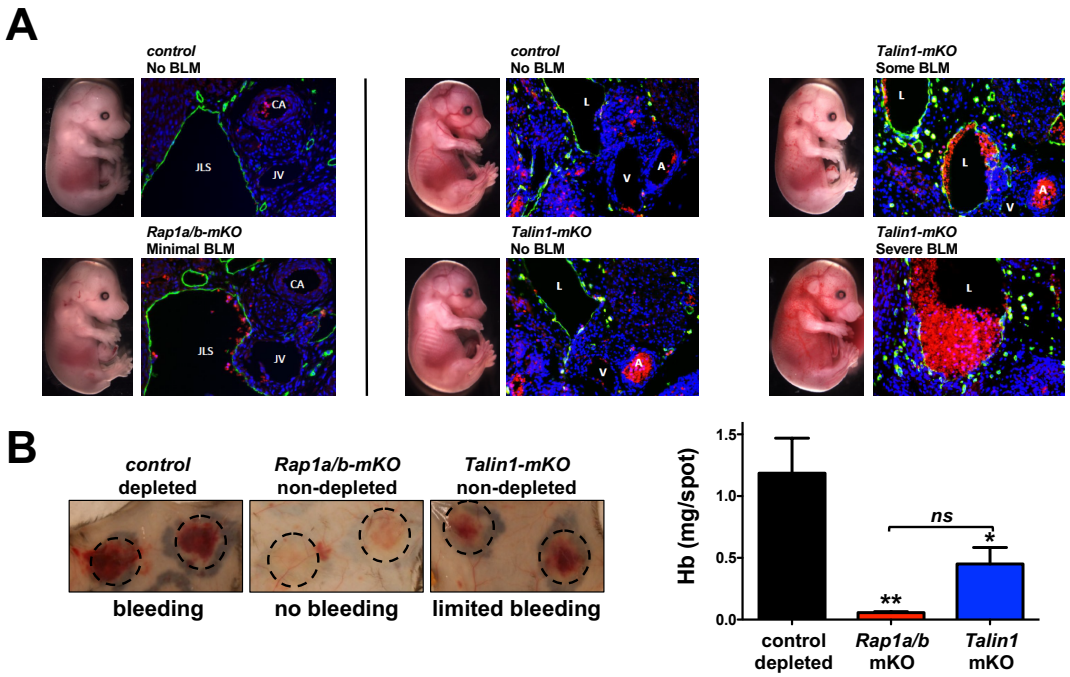


**Figure 7. Platelet RAP1 signaling minimally contributes to the maintenance of vascular integrity during development and inflammation.** (**A**) Contribution to vascular integrity during development was determined by assessing blood/lymphatic mixing (BLM) in embryos at ~E16.5 by macroscopic observation, using a Leica MZ16FA dissecting stereoscope, and immunofluorescence staining of embryos sections. Embryos were sectioned at the jugular lymph sac (JLS) and lymphatic endothelial cells (green) were stained with primary antibody, polyclonal rabbit anti-mouse LYVE-1 overnight, followed by donkey anti-rabbit Alexa Fluor 488 and DAPI. RBCs were visualized by autofluorescence (red). Images were acquired on a Nikon E800 microscope with a Hammamatsu camera with Metamorph software (Molecular Devices Corp.). (**B**) Contribution to vascular integrity at sites of inflammation was determined by reverse passive Arthus (rpA) reaction in the skin of control (*Pf4-Cre-*) mice depleted of all circulating platelets (by intravenous injection of antibodies against GPIbα), compared to non-depleted Rap1a/b-mKO or non-depleted *Talin1*-mKO mice. Representative images of rpA sites are shown (indicated by dashed outlines). Hemorrhage at sites of inflammation was quantified by measuring hemoglobin (Hb) levels in skin lesions 4 hours after rpA challenge (n=5-7). *$p<0.05$, **$p<0.01$.