Kinesin-1, a new actor involved in platelet secretion and thrombus stability by interacting with the granule Slp4/Rab27b effector complex.

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ABSTRACT

Objective: Platelet secretion is crucial for many physiological platelet responses. Even though several regulators of the fusion machinery for secretory granule exocytosis have been identified in platelets, the molecular mechanisms of trafficking on the cytoskeleton have not been fully characterized.

Approach and results: By studying a mouse model (cKO^{Kif5b}) lacking Kif5b (the heavy chain of kinesin-1) in its megakaryocytes and platelets, we evidenced unstable hemostasis characterized by an increase of blood loss associated to a marked tendency to rebleed in a tail clip assay, and thrombus instability in an *in vivo* thrombosis model. This instability was confirmed *in vitro* in a whole-blood perfusion assay under blood flow conditions. Aggregations induced by thrombin and collagen were also impaired in cKO^{Kif5b} platelets. Furthermore, P-selectin exposure and ATP release after thrombin stimulation were impaired in cKO^{Kif5b} platelets, highlighting the role of kinesin-1 in α-granule and dense granule secretion, respectively. Importantly, exogenous ADP rescued normal thrombin induced-aggregation in cKO^{Kif5b} platelets, which indicates that impaired aggregation was due to defective release of ADP and dense granules. Lastly, we demonstrated that independently of platelet activation, kinesin-1 interacts with the molecular machinery comprising the granule-associated Rab27b and the synaptotagmin-like protein 4 (Slp4; SYTL4) adaptor protein.

Conclusion: Our results indicate that a kinesin-1 dependent process plays a role for platelet function by acting into the mechanism underlying α-granule and dense granule secretion.

ABBREVIATIONS

- Kif5b Heavy chain of kinesin-1
- KLC Light chain of kinesin -1
- SLP4 / SYTL4 Synaptotagmin-like protein 4
- MK Megakaryocyte
- GP Glycoprotein

INTRODUCTION

Regulated secretion is an essential part of platelet function in hemostasis and thrombosis processes, as well as, in inflammation, angiogenesis, wound healing, and the response to invading microbes.[1] Classically, platelets contain three types of secretory granule: α-granules, dense granules and lysosomes. The importance of platelet cargo secretion is highlighted by the existence of bleeding pathologies with granule storage pool deficiencies, such as grey platelet syndrome and Hermansky-Pudlak syndrome, characterized respectively by a lack of α-granule or dense granule content.[2,3] Even though several components of the molecular machinery responsible for platelet secretion have been identified, the underlying molecular mechanisms have yet to be fully characterized. Indeed, these secretory processes involve (i) fusion of granules with the plasma membrane, (ii) regulatory mechanisms and (iii) granule transport. The fusion of granule with plasma membrane is controlled by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs); these enable the binding and fusion between secretory vesicles/granules (v-SNAREs) and the plasma/target membrane (t-SNAREs).[4-9] In platelets, the v-SNAREs vesicle-associated membrane proteins 7 and 8 (VAMP-7 and VAMP-8) and the t-SNAREs syntaxin-8, syntaxin-11 and synaptosomal-associated protein 23 (SNAP-23) are predominantly involved in α-granule and dense granule secretion.[10-17] Furthermore, the formation of SNARE complexes requires chaperone proteins, such as Munc 18-2 and Munc 13-4, which are thought to regulate vesicle "tethering" and "priming" prior to secretion.[8, 18-20] Upstream of SNARE complex formation, the small GTPase Rab27b and the effector molecule synaptotagmin-like protein 4 (Slp4; also called SYTL4 or granuphilin) regulate granule-membrane docking events.[6, 21] Lastly, granule transport within the cell constitutes the third important mechanism involved in platelet secretion. In many nucleated cells, three superfamilies of motor proteins control vesicle exocytosis: the myosins interact with the actin cytoskeleton during the terminal step in membrane fusion,[22-24] whereas the dyneins and kinesins interact with microtubules and act as molecular motors by transporting granules along the microtubule network. However, the dyneins' and kinesins' exact roles in platelet granule transport have not been defined.

The present study is the first to have evaluated the role of kinesin-1, the archetypal member of the kinesin family, in platelet function. This heterotetrameric protein is composed of two heavy subunits (Kif5a, Kif5b or Kif5c isoforms) and two light chains (KLC1, KLC2, KLC3 or KLC4 isoforms).[25] Using *Kif5b*$^{fl/-}$*; Vav1-Cre* conditional knockout (cKO^{Kif5b}) mice lacking *Kif5b* in all their hematopoietic lineages, including megakaryocytes (MKs) and platelets, we showed that kinesin-1 represents a new actor involved in the secretion of α-granules and dense granules from platelets, capable of influencing thrombus stability. Moreover, we identified kinesin-1's molecular partners in platelet secretion, and found that kinesin-1 interacts with granule-associated Rab27b and the Slp4 adaptor protein in this context.

MATERIAL AND METHODS

Materials and Methods are available in the online-only Data Supplement.

RESULTS

Kif5b is expressed in megakaryocytes and platelets but is not required for thrombopoiesis

To investigate the molecular machinery involved in platelet secretion, we focused on Kif5b, the major heavy chain isoform of the molecular motor kinesin-1 in MK lineages, as demonstrated by qPCR (Figure 1A). To elucidate, the role of Kif5b in platelet function, we generated a conditional Kif5b-deficient mouse model (Kif5b$^{fl/-}$/Vav1-Cre; cKO^{Kif5b}) by crossing a Kif5b$^{fl/-}$ mouse[26] with a Vav1-Cre mouse. These animals lack Kif5b in all their hematopoietic cell lineages (including MKs and platelets) but do not display any obvious abnormalities in the behavior, development or maturation of their lymphoid and myeloid lineages.[27] Quantitative real-time PCR assays confirmed the absence of Kif5b in cKO^{Kif5b} MKs and also

the absence of compensatory up-regulation of the other isoforms (Figure 1A). The absence of Kif5b protein in cKO^{Kif5b} platelets was also confirmed by Western blotting (Figure 1B). In order to evaluate hemostatic parameters and identify possible abnormalities of hematopoiesis in cKO^{Kif5b} mice, we performed complete blood counts. There were no significant differences in platelet, leucocyte, and red blood cell counts, hematocrit or hemoglobin concentration, relative to wild-type (WT) animals (Table 1). The cKO^{Kif5b} mice's mean platelet volume was also normal. Transmission electron microscopy showed that the cKO^{Kif5b} platelets had a normal discoid shape, a normal size and normal organelle counts (Figures 1C-F). There were no significant differences in the number, size or morphology of α-granules, relative to WT platelets. Similarly, no significant difference in the number of dense granules was noticed by whole mount transmission electron microscopy (Figure 1G). Taken as a whole, these results indicate that kinesin-1 does not have a crucial role in thrombopoiesis and platelet morphology.

Hemostasis is unstable in cKO^{Kif5b} mice

To investigate Kif5b's involvement in hemostasis and thrombosis *in vivo*, we first compared the bleeding times of WT and cKO^{Kif5b} mice in a tail clip assay. Although the bleeding time was similar in the two genotypes (Figure 2A), a significant increase of blood loss ($p<0.05$; Figure 2B) and of the frequency to rebleed (29% of animals *versus* 0% in WT mice; $p<0.05$; Figure 2C) were observed in cKO^{Kif5b} mice. We next assessed the *in vivo* role of Kif5b in a $FeCl_3$-induced thrombosis model, using intravital microscopy of mesenteric vessels. We observed that 37% of the cKO^{Kif5b} mice exhibited an abnormal absence of occlusion in venules *versus* 0% in WT mice ($p<0.05$; Figure 2D). Moreover, a higher frequency of embolization of thrombi were noticed in venules of cKO^{Kif5b} mice (50% of animals *versus* 30% in WT mice) and arterioles (33% of animals *versus* 17% in WT mice), suggesting that the thrombi formed in cKO^{Kif5b} mice could be less stable over time than those in WT mice. In order to confirm the *in vivo* instability of thrombi in these mice, we next assessed adhesion and thrombus formation of cKO^{Kif5b} platelets in a whole-blood perfusion assay over a collagen matrix under blood flow conditions (Figure 2E). Just after whole-blood perfusion, the adhesion of cKO^{Kif5b} platelets was similar to that of WT platelets (9.3 ± 0.8% *versus* 9.1 ± 0.8%, respectively; $p=0.99$). However, the perfusion of Tyrode's buffer over adhered platelets for 5 minutes (the washing step) revealed significantly greater detachment (29.8 ± 1.3% decrease in adhesion; $p<0.05$) for the cKO^{Kif5b} platelets than for WT platelets (9.7 ± 7.3% decrease in adhesion; $p=0.66$); this was suggestive of impaired stability in cKO^{Kif5b} platelets.

Given that thrombus formation and platelet aggregation are closely associated, we next evaluated kinesin-1's involvement in aggregation after the activation of washed platelets by thrombin (Figure 3A) and collagen (Figure 3B). At a low dose of thrombin (50 mU/mL), the aggregation of cKO^{Kif5b} platelets was significantly lower (by 77.5 ± 7.3%; $p<0.001$) than for WT platelets. At an intermediate dose of thrombin (70 mU/mL), the aggregation of cKO^{Kif5b} platelets was still defective (37.7 ± 6.1%; $p<0.001$), relative to WT platelets. Aggregation was normal at a higher dose of agonist (100 mU/mL) only (Figure 3A). Similar results were observed for collagen-induced aggregation. Indeed, the aggregation of cKO^{Kif5b} platelets was impaired by 48.8 ± 6.2% *versus* WT ($p<0.001$; Figure 3B) at a low dose of agonist (0.5 µg/mL) but not at a high dose (1 µg/mL).

Taken as a whole, these *in vivo* and *in vitro* results demonstrate that kinesin-1 has a role in platelet function and thrombus stability.

Kinesin-1 regulates dense granule and α-granule secretion in platelets

The release of dense granules and α-granules from platelets has an essential role in platelet adhesion and aggregation, and thrombus stability. We next investigated the role of Kif5b in dense granule secretion by monitoring the release of ATP from platelets during thrombin-induced aggregation (Figure 4A). At a low agonist concentration (50 mU/mL), cKO^{Kif5b} platelets showed strong impaired dense granule secretion by 75 ± 4% ($p<0.001$). At an intermediate thrombin concentration (70 mU/mL), the impairment was 32 ± 1% ($p<0.001$). At a high concentration (100 mU/mL), the impairment was no longer observed. This defect

could not be attributed to a lack of dense granule cargo, since similar ATP release was observed with a very high thrombin concentration (2 U/mL) for WT and cKO^{Kif5b} platelets (Figure 4A). Furthermore, the total serotonin content of dense granules was similar in cKO^{Kif5b} and WT platelets (2.63 ± 0.15 µg/mL *vs.* 2.59 ± 0.20 µg/mL, respectively; n=7; p=0.91). To confirm kinesin-1's role in dense granule secretion, we used videomicroscopy to analyze in real-time granule release by mepacrine-labeled platelets (Figures 4B-C; Video 1). Whereas the mepacrine labeling disappeared rapidly after WT platelet activation by thrombin (75 mU/mL), secretion was significantly delayed in cKO^{Kif5b} platelets. Taken as a whole, these results confirmed that dense granule release was impaired in cKO^{Kif5b} platelets.

ADP release from dense granules constitutes an autocrine signal that amplifies platelet aggregation, notably at low agonist concentrations. Hence, in order to confirm that defective release of ADP (and thus dense granules) was responsible for the impaired aggregation of cKO^{Kif5b} platelets, we assessed the effect of adding subthreshold concentration of ADP (5 µM) on aggregation induced by a low dose of thrombin (60 mU/mL). Indeed, while ADP alone did not induce platelet aggregation (not shown), the incubation of platelets with ADP reversed the aggregation defect observed in cKO^{Kif5b} platelets by thrombin (Figure 4D). Hence, the impaired aggregation was likely due to defective release of dense granule contents, including ADP.

To determine whether kinesin-1 is also involved in α-granule exocytosis, we used flow cytometry to investigate P-selectin (CD62P) surface exposure on thrombin-stimulated cKO^{Kif5b} platelets (Figure 4E). Relative to WT platelets, cKO^{Kif5b} platelets displayed significant delays of P-selectin exposure at thrombin concentrations up to 200 mU/mL. These defects in P-selectin exposure were overcome by very high dose of thrombin (1 U/mL). Moreover, the quantification by immunoblotting of von Willebrand factor (VWF) and thrombospondin-1 (TSP-1) levels, two proteins stored in α-granules, revealed no significant difference of expression between WT and cKO^{kif5b} platelets (Figure I). Therefore, these data indicate that the defect resulted from impaired exocytosis rather than reduced P-selectin exposure, due to defective platelet activation, or a lack of α-granule protein contents. As α-granule secretion is strongly dependent on dense granule secretion,[28] we next determined whether kinesin-1 directly acts on α-granule exocytosis, or whether the observed defect in CD62P exposure is the consequence of the defect of dense granule secretion in cKO^{Kif5b} platelets. To address this question, we measured P-selectin exposure in the presence of apyrase and indomethacin, in order to prevent amplification of CD62P exposure by released ADP and synthetized thromboxane A_2, respectively (Figure 4F). Significant delays of P-selectin exposure by cKO^{Kif5b} platelets were still observed, proving the direct involvement of kinesin-1 on α-granule exocytosis (Figure 4F).

Taken as a whole, these results demonstrate that kinesin-1 in platelets has a significant role in both the secretion of dense granules and α-granules.

Secretion defects in Kif5b-deficient platelets are not caused by impaired platelet signaling

To further elucidate the role of Kif5b in platelet function in general and platelet secretion in particular, we hypothesized that the motor protein kinesin-1 might act directly on the intracellular granule transport. However, in order to rule out a possible defect in cell signaling, we evaluated receptor expression and signaling events in cKO^{Kif5b} platelets.

Firstly, the cell surface expression levels of GPIbα, GPVI and integrin $\alpha_{IIb}\beta_3$ and α_2 were found to be normal in cKO^{Kif5b} platelets (Figure II-A). We then looked at whether defective calcium signaling might account for impaired function in cKO^{Kif5b} platelets, since calcium mobilization from intracellular stores is essential for platelet activation and secretion.[29] When comparing WT and cKO^{Kif5b} platelets, no significant differences in intracellular calcium mobilization were noted at either low or high doses of thrombin (Figure II-B), indicating a normal calcium signaling in cKO^{Kif5b} platelets. Next, we evaluated protein kinase C (PKC) activity, since PKC family members are significant regulators of platelet function; in particular, the conventional PKC isoform, PKCα, is the main regulator of dense granule secretion.[30] After thrombin activation and the blockade of $\alpha_{IIb}\beta_3$ engagement using the Leo.H4 antibody,

the serine phosphorylation profile of PKC substrates (Figure II-C) did not reveal any differences between WT and cKO^{Kif5b} platelets, indicating that the secretion defects were not due to the impairment of PKC signaling by kinesin-1. Similar results were obtained when tyrosine-phosphorylated proteins were investigated in thrombin-activated WT and cKO^{Kif5b} platelets (Figure II-D).
Lastly, we used flow cytometry and the JON/A monoclonal antibody, which binds specifically to activated mouse integrin $\alpha_{IIb}\beta_3$, to evaluated its activation. Whereas cKO^{Kif5b} platelets displayed impaired $\alpha_{IIb}\beta_3$ activation at low thrombin concentrations (Figure II-E), in line with the aggregation experiments in Figure 3A, there were no differences between WT and cKO^{Kif5b} platelets after stimulation with either low or high thrombin concentrations in the presence of apyrase and indomethacin (used to prevent the amplification of integrin activation by released ADP and synthetized thromboxane A_2, respectively) (Figure II-F). These results indicate that Kif5b deficiency did not act directly on the integrin activation, but Kif5b modulated this activation *via* the secretion.
Taken as a whole, the defective secretion observed in cKO^{Kif5b} platelets result from impaired secretion machinery and not from impaired signaling.

Characterization of the kinesin-1-dependent secretory machinery in platelets
Given that kinesin-1 is known to regulate the transport of Rab27/Slp-associated vesicles along microtubules in nucleated cells,[27,31,32] we next looked at whether this molecular machinery was also involved in kinesin-1-dependent granule secretion in platelets. Firstly, qPCR assays showed that Rab27b was the main Rab27 family member expressed in MKs, whereas Rab27a was expressed at very low levels only (Figure 5A); this is in line with the literature data on defective secretion by platelets in Rab27b KO mice.[21] Secondly, analysis of the expression of the five Slp members revealed that only Slp4 was strongly expressed in MKs (Figure 5B). The presence of Rab27b, Slp4 and the kinesin-1 light chain (KLC1) in platelets was also confirmed by Western blotting (Figure 5C). We therefore investigated Rab27b and Slp4's contributions to the kinesin-1-dependent secretory machinery in platelets. Co-immunoprecipitation of Slp4 and Kif5b in both resting and thrombin-activated platelet lysates indicated that the two proteins interacted constitutively (Figure 5D). Interestingly, Rab27b also co-immunoprecipitated with Kif5b and Slp4 in platelet lysates (Figure 5E).
To further identify interactions between proteins in the complex, we overexpressed various combinations of tagged proteins in HEK 293T cells. A strong interaction between Rab27b and Slp4 was confirmed by co-immunoprecipitation of the two proteins (Figure 5F). Interestingly, we also confirmed the interaction between Slp4 and the light chain (KLC1; Figure 5G) and heavy chain (Kif5b; Figure 5H) of kinesin-1.
Lastly, we investigated the subcellular localization of Kif5b in platelets. To facilitate these observations, we performed immunofluorescence analysis in human platelets instead of murine platelets, as human platelets display a better cellular spreading after activation, facilitating co-localization analysis. In resting platelets, endogenous Kif5b was distributed along the marginal band, a circumferential microtubule bundle located beneath the plasma membrane, and within granular structures (Figure 6A). After activation, Kif5b was associated with centralized granular structures and weakly associated with peripheral tubulin labeling.
To further evaluate Kif5b interactions with platelet granules, we analyzed its distribution by probing ADP-activated human platelets for the α-granule marker CD62P and the dense granule/lysosome marker CD63. Kif5b was strongly co-localized with the markers (Figures 6B-C), confirming its interaction with platelet granules.

DISCUSSION
In addition to its role in maintaining vascular homeostasis, platelet granule secretion is involved in stabilizing platelet adhesion and promoting thrombus growth and stability.[33] Furthermore, platelet granules contain several chemokines, growth factors and cytokines[34,35] with crucial roles in inflammation reactions, wound repair and vascular remodeling.[36] Accordingly, dysfunctions of platelet secretion can lead to bleeding disorders, thrombosis,

and impaired inflammation and immunological responses.[37] Although the mechanisms that control the fusion between platelet granules and the plasma membrane have been well investigated, little is known about the molecular machinery responsible for trafficking granules to the secretion site. The present study provided the first *in vitro* and *in vivo* evidence to show that the Kif5b heavy chain isoform of the kinesin-1 motor protein is a new actor involved in the mechanisms of the secretion of α-granules and dense granules. We further demonstrated that independently of platelet activation, kinesin-1 links microtubules to α-granules and dense granules through the molecular machinery composed of the granule-associated Rab27b and the Slp4 adaptor protein. Moreover, we showed that (i) Kif5b is not required for thrombopoiesis, platelet morphology or platelet signaling, and (ii) the absence of Kif5b does not affect the cargo content.

Several lines of evidence indicate that kinesin-1 is critical for the secretion of dense granules, since a lack of Kif5b (in cKO^{kif5b} mice) dramatically impaired the release of ADP from granules and slowed the time course of dense granule secretion (visualized by videomicroscopy). The defect in dense granule secretion resulted in impaired platelet aggregation. The aggregation response to many agonists requires an amplification cascade, notably through the release of ADP from dense granules.[38] Here, we confirmed that kinesin-1 has a role in dense granule secretion because the addition of exogenous ADP reversed the aggregation defect in cKO^{kif5b} platelets. We also demonstrated that kinesin-1 is directly involved in α-granule exocytosis, as shown by the delays of P-selectin exposure by cKO^{Kif5b} platelets. Moreover, it is also accepted that platelet secretion contributes to thrombus formation and stability.[39] Interestingly, cKO^{kif5b} mice increase blood loss and tended to rebleed in a tail-clip assay. In addition, kinesin-1 deficiency displayed a delay of thrombus occlusion and an increased frequency of embolization in a $FeCl_3$-induced thrombosis model; these observations suggest that kinesin-1 participates in thrombus stability by acting on granule secretion. Although the *in vivo* hemostasis defect could not be restricted to a platelet defect, since our conditional knockout mice (*$Kif5b^{fl/-}$; Vav1-Cre)* was under the control of the Vav1 promoter which is also expressed in endothelial cells,[40] all the *in vitro* experiments (platelet aggregations, platelet adhesion under flow and platelet secretion) confirmed the involvement of platelet kinesin-1 in the secretion of α-granules and dense granules.

It is noteworthy that in the absence of Kif5b, the release of α-granule and dense granule contents was only partially impaired, notably at low concentrations of agonist. Possible mechanisms to explain the absence of strong defect associated to kinesin-1 deficiency in platelet is that kinesin-1 could participate to granule transport only during the early phase of signaling events, before a full platelet activation which is associated to cytoskeletal reorganization and platelet shape change, leading to the centralization of the granules. The relocalization of the granules to a limited central area can facilitate a direct fusion of the granules with channels of the surface-connected open canalicular system,[41] which limits the requirement for kinesin-1-dependent granule secretion. Furthermore, recent research has shown that two types of fusion occur in platelets: single exocytosis of α-granules and dense granules predominates in moderately stimulated platelets, whereas compound exocytosis (during which only α-granules fuse with each other before reaching the plasma membrane) predominates in strongly stimulated platelets.[42] These sequential fusion events may facilitate the release of α-granule contents by limiting vesicle trafficking prior to fusion with the plasma membrane. Furthermore, we cannot exclude that kinesin family members other than kinesin-1 could also regulate granule secretion in platelets and could partially compensate for the loss of kinesin-1. Furthermore, we found that the hemostasis defect in cKO^{Kif5b} mice was less marked *in vivo* than *in vitro*. This difference could be explained by the *in vivo* involvement of high local concentrations of agonists, thus bypassing the secretory defect. Indeed, the *in vitro* defects in platelet secretion and aggregation were reversed by high doses of agonist.

It has been reported that the small GTPase Rab27b regulate the number and secretion of dense granules in platelets.[21] Unlike cKO^{kif5b} mice, Rab27b-deficient mice exhibit a severe bleeding defect, and have half the normal number of dense granules, while α-granule secretion is normal. If Kif5b and Rab27b interact and have a crucial role in platelet secretion, how can this phenotypic difference be explained? In fact, this discrepancy may be related to

(i) Rab27b's broader role in dense granule biogenesis and/or packaging in proplatelets, and (ii) the mice's differing genetic backgrounds. Rab27 proteins are known to regulate the transition from microtubule-mediated to actin-mediated transport or to the vesicle transport along the peripheral actin cytoskeleton in various cell types, including melanocytes, pancreatic beta-cells and endothelial cells;[43-45] this suggests that Rab27b regulates functions in platelets that do not involve kinesin-1 and so might explain the more profound platelet defect in Rab27b deficiency. Unlike Rab27b's relationship with dense granules, its relationship with α-granules is less well defined. Indeed, although some studies have shown that Rab27b is mainly localized in the limiting membranes of α-granules,[46, 47] its involvement in α-granule secretion has not been unambiguously demonstrated.

Of the five different Slp proteins known to interact with the GTP-bound form of Rab27a and Rab27b,[48,49] we found that Slp4 was predominantly expressed in MKs and platelets, without excluding the presence and a role of others isoforms such as Slp1, as previously described.[50] Here, we observed a previously unknown, activation-independent interaction between Slp4 and kinesin-1 in platelets. In contrast to the situation in mast cells, where kinesin-1 is recruited to the Rab27b/Slp3 complex upon activation,[27] it seems that the complex in platelets is already preformed. This may correspond to the requirement for rapid granule release.

Furthermore, a recent study showed that the SNARE VAMP-7 regulates the link between α-granule/dense granule secretion and actin reorganization by interacting with the VPS9-domain ankyrin repeat protein (VARP) and Arp2/3.[14] Interestingly, VARP is also known to be an effector for kinesin-1's Kif5 heavy chains.[51] Future research should seek to determine whether the molecular trafficking complex Rab27b/Slp4/kinesin-1 can interact with VARP, and whether formation of the complex depends on platelet activation. Given that platelet activation dissociates VARP from VAMP-7 and ARP2/3, it would be interesting to study the putative effect of VARP/kinesin-1 complex formation on platelet function.

Studies from the past have shown a controversial role of microtubules in regulating platelet secretion.[52-56] Our recent study has highlighted the role of the microtubule-dependent motor protein kinesin-1 to participate to the secretion of α-granules and dense granules. As it is known that kinesin-1 allows the anterograde movement going from the minus (-) end to the plus (+) end of the microtubule, future study should assess again the requirement of microtubule in the secretion process of platelet.

Lastly, Sadoul's group has shown that reorganization of the cytoskeleton during platelet activation requires precise coordination; a balance between the kinesin and dynein families is required to keep the marginal band in the resting state.[57,58] In the present study, kinesin-1 deficiency was not associated with defects in platelet morphology, suggesting that maintenance of the platelet's discoid shape mainly involve the dynein family and/or kinesins other than kinesin-1.

In conclusion, the present study is the first to demonstrate that kinesin-1 plays a role in platelet function, thrombus stabilization and regulation of α-granule and dense granule secretion by interaction with the Rab27b/Slp4 complex. Overall, our results provide a better understanding of the molecular mechanisms that regulate platelet secretion, a crucial hemostatic function, and suggest the existence of novel pharmacological targets for regulate platelet secretion, in particular in the context of thrombotic risk.

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HIGHLIGHTS

- Kinesin-1 participates in the secretion of dense granules and α-granules in platelets, and consequently the thrombus stability.
- Kinesin-1 acts on platelet secretion only at low doses of agonist, suggesting its likely role in the initiation of platelet activation, by participating in the first steps of platelet secretion.
- The role of kinesin-1 in platelet secretion involved the interaction of the granule-associated Rab27b and the Slp4 adaptor protein.

TABLE

	WT	cKO^{Kif5b}
Platelets (x10^3/µL)	1031 ± 26	970 ± 26
Red blood cells (x10^6/µL)	9.5 ± 0.2	9.4 ± 0.2
Leucocytes (x10^3/µL)	6.7 ± 0.4	6.6 ± 0.3
Hematocrit (%)	44.2 ± 1.0	43.9 ± 0.9
Hemoglobin (g/dL)	13.6 ± 0.3	13.5 ± 0.3
Mean platelet volume (µm^3)	5.3 ± 0.1	5.4 ± 0.1

Table 1: **Hematologic parameters**
Data on hematologic parameters are quoted as the mean ± SEM. None of the differences between WT and cKO^{Kif5b} mice were statistically significant (n=24; unpaired Student's *t* test).

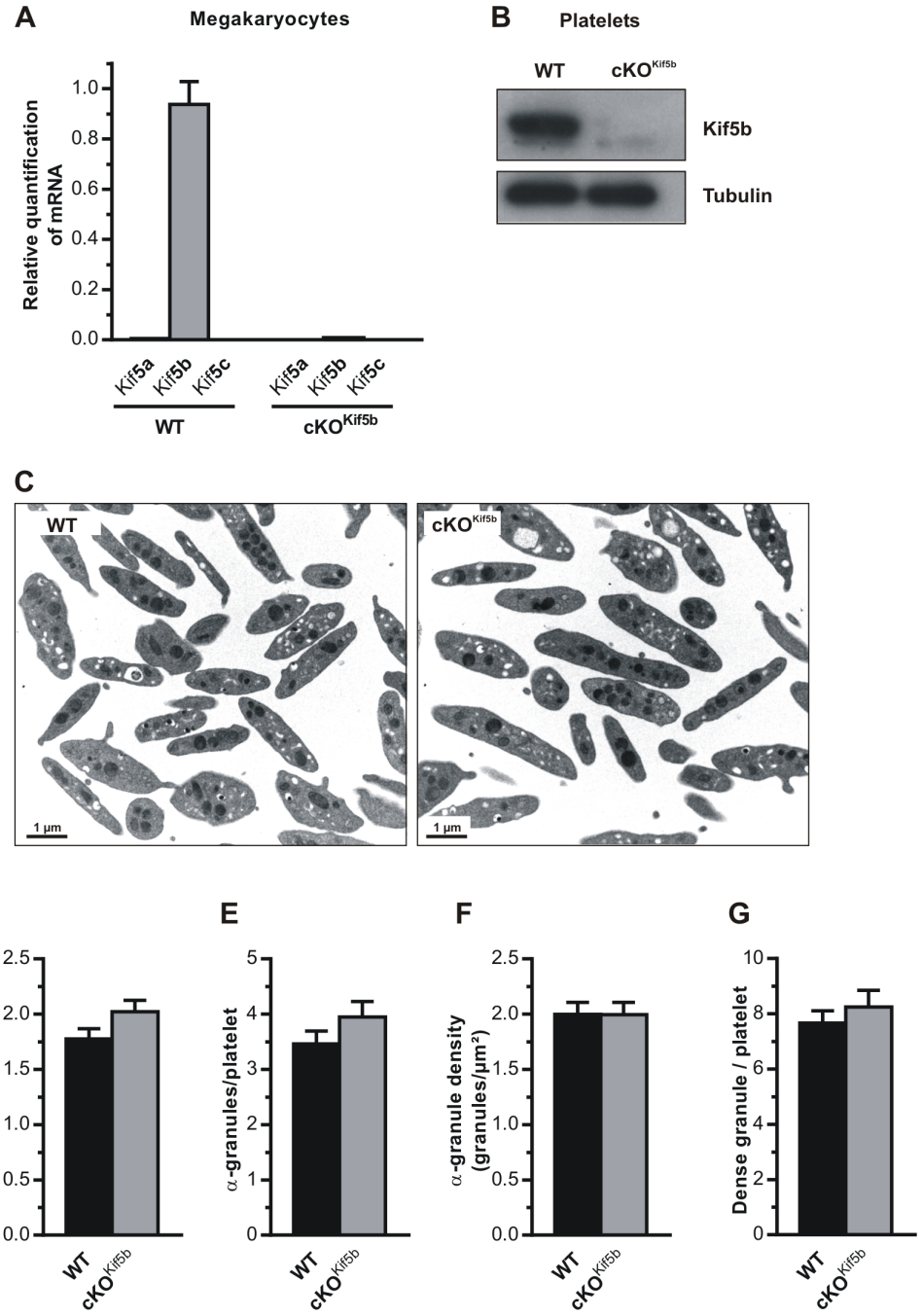

Figure 1. Kif5 expression in WT and cKO^{Kif5b} megakaryocytes and platelets, and platelet morphology in WT and cKO^{Kif5b} mice.

(A) Relative quantification of Kif5a, Kif5b and Kif5c transcripts by real-time PCR in MKs from WT and cKO^{Kif5b} mice. Transcript levels in each sample were expressed as a proportion of the mean value for Kif5b. The data are representative of three independent experiments performed in triplicate. (B) WT and cKO^{Kif5b} platelet lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were immunoblotted with anti-Kif5b and anti-tubulin antibodies. The blots are representative of three independent experiments. (C) Transmission electron microscopy of resting WT and cKO^{Kif5b} platelets. The scale bars correspond to 1 µm. (D-F) Analysis of transmission electron microscopy images for the following parameters: platelet area, number of α-granules per platelet, and α-granule density. (G) Quantification of the number of dense granules in WT and cKO^{Kif5b} platelets by whole mount transmission electron microscopy. Results are expressed as the mean ± SEM (n=100 platelets for D-F; n=140 platelets for G; unpaired Student's *t* test).

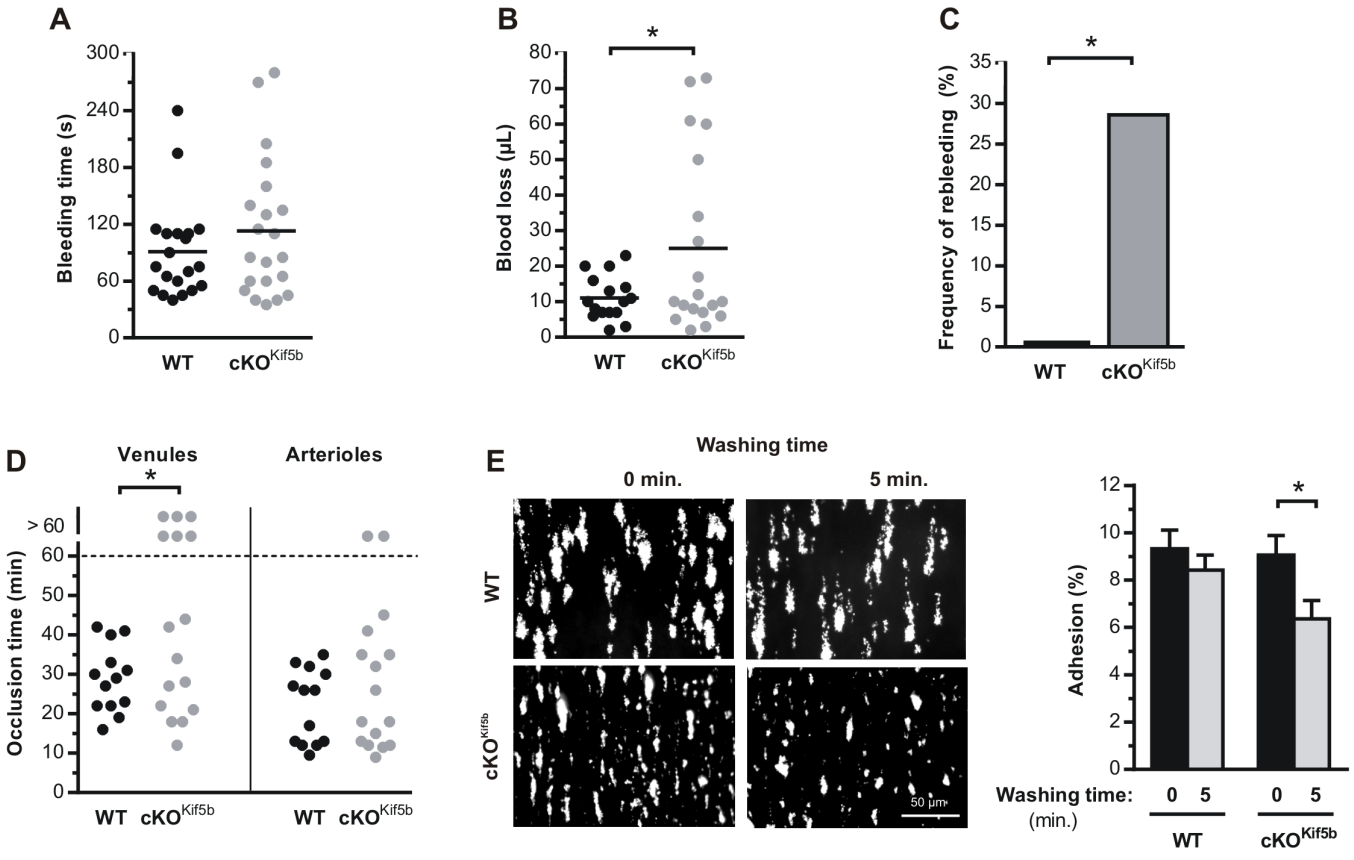

Figure 2. Kinesin-1 is involved in platelet function and thrombus stability.
Tail bleeding time (A) and blood loss (B) in WT and cKO^{Kif5b} mice. Each dot represents one animal. A total of 20 WT and 21 cKO^{Kif5b} mice were used. Statistical significance was determined by unpaired Student's *t* test (* $p<0.05$). (C) Rebleeding was assessed for 1 minute, following the initial bleeding arrest. Comparison of frequencies of rebleeding between WT and cKO^{kif5b} mice were analyzed by Fisher's exact test (* $p<0.05$). (D) *In vivo* thrombosis in the mesenteric vessels (venules and arterioles) of WT and cKO^{Kif5b} mice after $FeCl_3$-induced injury. The adhesion of fluorescently-labeled platelets and subsequent thrombus formation were monitored with intravital microscopy. The graph shows the vessels' occlusion time. Statistical significance was determined by 2-tailed Mann-Whitney test. (E) *In vitro* adhesion of WT and cKO^{Kif5b} platelets was measured in a whole-blood perfusion assay, using a fibrillar collagen matrix, at a shear rate of 1200 seconds^{-1} for 1 minute, followed by 5 minutes of perfusion with Tyrode's buffer (the washing step). Images represent platelet adhesion before and after the washing step. The graph shows the quantification of platelet adhesion. The results are expressed as the mean ± SEM percentage of the total area covered with platelets in five independent experiments. Statistical significance was determined in a one-way ANOVA, followed by Sidak's correction for multiple comparisons (* $p<0.05$). The scale bar corresponds to 50 µm.

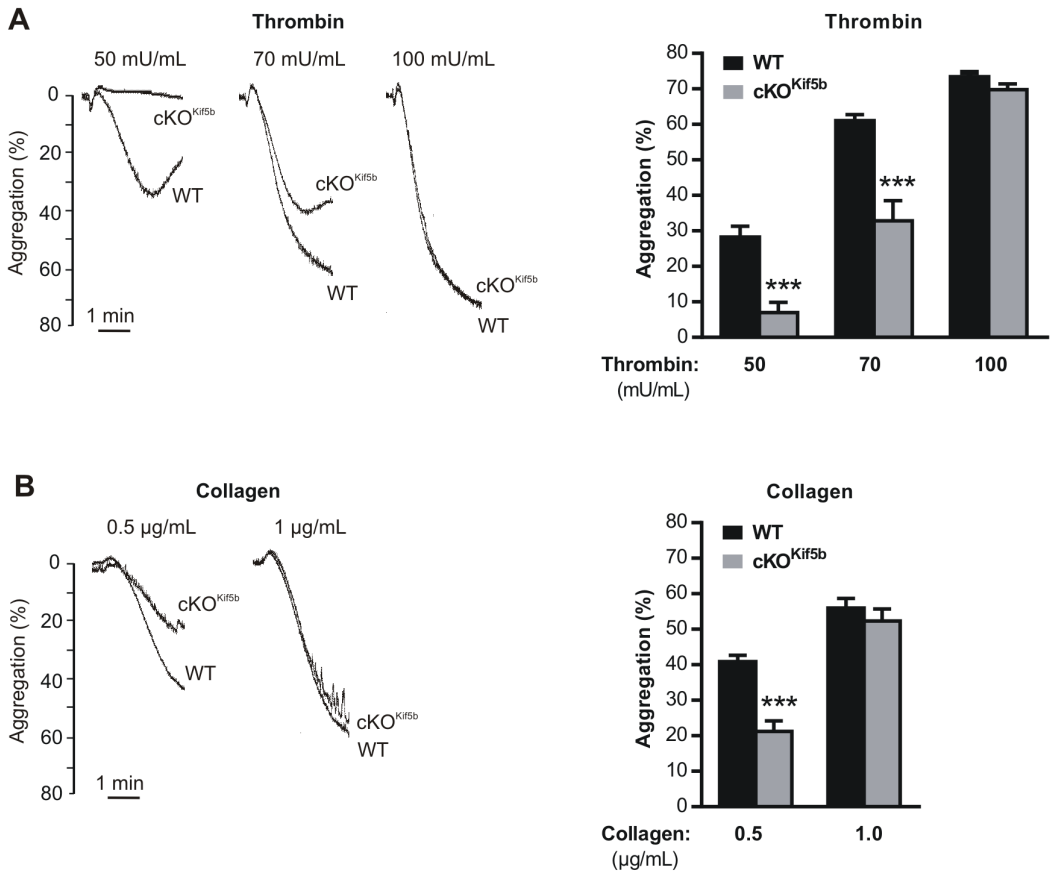

Figure 3. Kinesin-1 plays a role in platelet aggregations.
Aggregation of washed WT and cKO^{Kif5b} platelets induced by thrombin (50 to 100 mU/mL; A) or collagen (0.5 or 1 µg/mL; B). The traces are representative of at least three independent experiments, and the graphs show the mean ± SEM of aggregation (n=9; *** $p<0.001$ in an unpaired Student's *t* test).

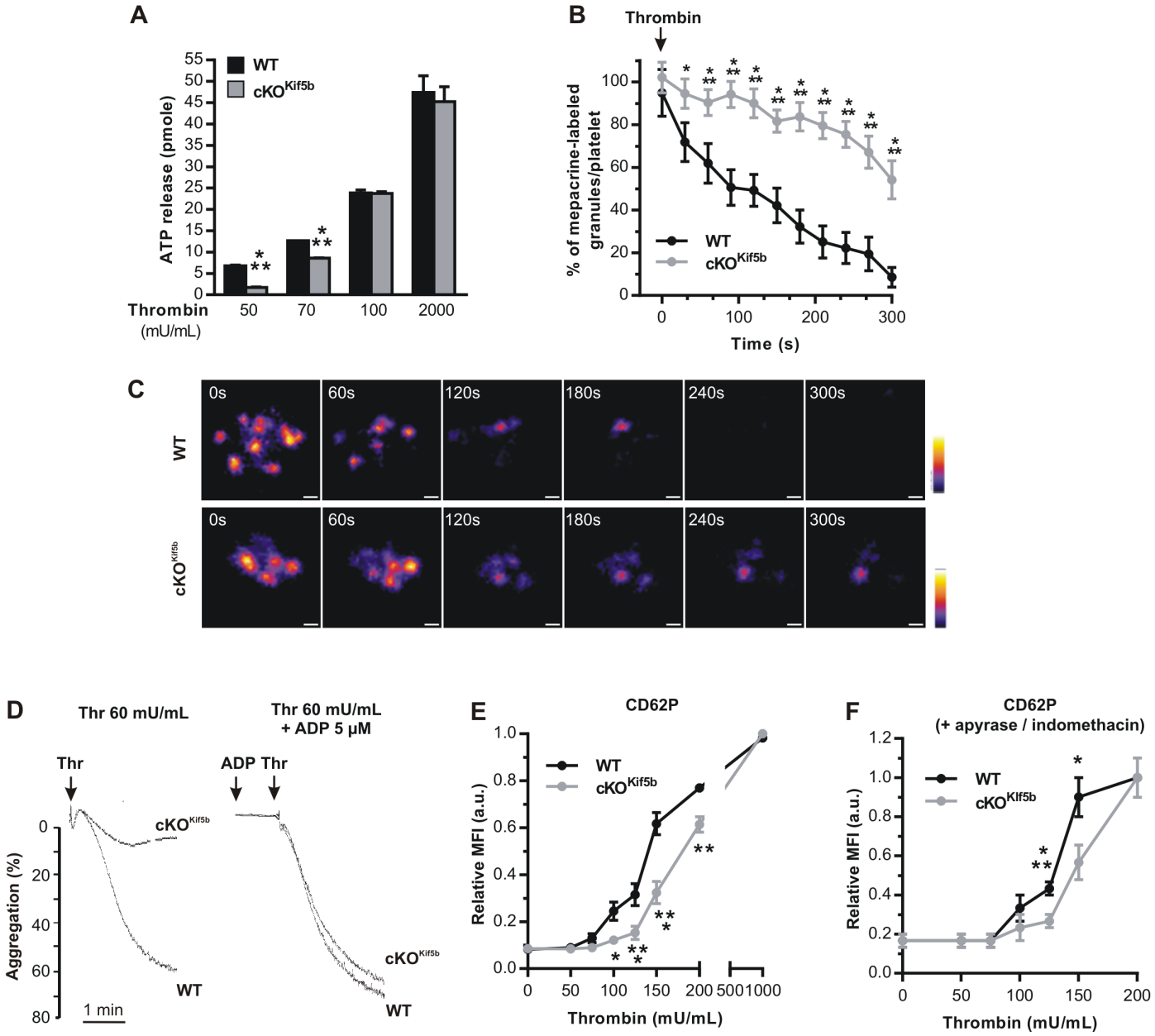

Figure 4. Kinesin-1 regulates the secretion of α-granules and dense granules in platelets.

(A) Dense granule secretion from platelets aggregated after stimulation with thrombin (50 to 2000 mU/mL) was assessed by measuring ATP release from WT platelets (black bars) and cKO^{Kif5b} platelets (grey bars). The results are expressed as the mean ± SEM of ATP released (in picomoles) from 3x10^6 platelets in at least three independent experiments. Statistical significance was determined using an unpaired Student's *t* test (*** p<0.001). (B-C) Dynamic secretion of dense granules upon thrombin activation (75 mU/mL) of mepacrine-labeled WT and cKO^{Kif5b} platelets was observed with real-time videomicroscopy. Representative series of images are shown every minute from t=0 (when thrombin was added) to t=300 seconds. The pseudocolor scale goes from blue (very weak staining) to white (intense staining). A mepacrine-containing granule was defined as a strong-intensity signal (orange to white intensity) with a minimum size of 4 pixels (1 pixel=0.13 µm). The scale bars correspond to 0.5 µm. The graph shows the percentage of mepacrine-labeled granules *per* platelet acquired during 300 seconds of videomicroscopy. Fifteen cells were analyzed for each condition, and an unpaired Student's *t* test was used to determine statistical significance (* p<0.05; *** p<0.001). (D) Exogenous ADP bypasses the defect in thrombin-induced aggregation of cKO^{Kif5b} platelets. WT and cKO^{Kif5b} platelets were incubated with a subthreshold concentration of ADP (5 µM) prior to stimulation with a low thrombin concentration (60 mU/mL). Exposure to ADP reversed the aggregation defect in cKO^{Kif5b} platelets. Platelets exposed to ADP alone failed to aggregate. The traces are representative of three independent experiments. (E-F) Expression of the α-granule membrane marker P-selectin (CD62P) following WT and cKO^{Kif5b} platelet stimulation with several thrombin concentrations was assessed using flow cytométrie in absence (E) or in presence of apyrase (2.5 U/mL) and indomethacin (4.5 µM) (F) in order to prevent the amplification of CD62P exposure by released ADP and synthetized thromboxane A_2, respectively. The data are quoted as the mean ± SEM expression in 8 separate experiments. Statistical significance was determined in a one-way ANOVA, followed by Sidak's correction for multiple comparisons (* p<0.05; ** p<0.01; *** p<0.001).

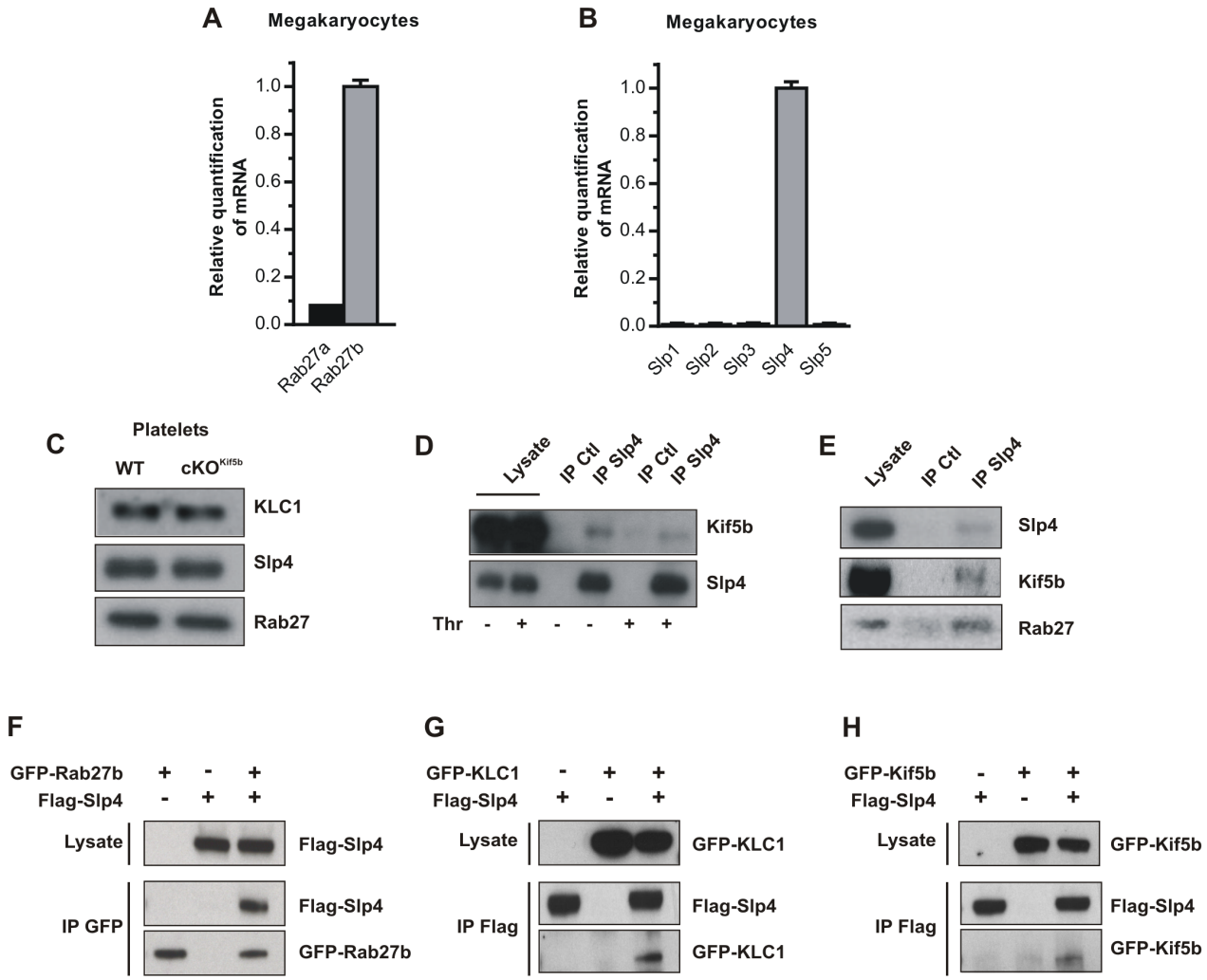

Figure 5. Characterization of the kinesin-1-dependent secretory machinery in platelets.
(A) Real-time PCR quantification of the relative levels of Rab27a and Rab27b transcripts in MKs. Transcript levels in each sample were expressed as a proportion of the mean value for Rab27b. (B) Real-time PCR quantification of the relative levels of Slp1, Slp2, Slp3, Slp4 and Slp5 transcripts in MKs. Transcript levels in each sample were expressed as a proportion of the mean value for Slp4. (C) WT and cKO^{Kif5b} platelet lysates were separated by SDS-PAGE and then immunoblotted with anti-KLC1, anti-Slp4 and anti-Rab27 antibodies. (D) Human platelets ($4x10^9$) were either not stimulated (-) or stimulated (+) with thrombin (Thr; 0.5 U/mL) for 10 minutes without stirring prior to be lysed. Cell lysates were immunoprecipitated with a polyclonal anti-Slp4 antibody (IP Slp4) or with an isotype control antibody (IP Ctl). The immunoblots were analyzed using anti-Kif5b and anti-Slp4 antibodies. The data are representative of three independent experiments. The immunoblots were analyzed using anti-Kif5b and anti-Slp4 antibodies. The data are representative of three independent experiments. (E) WT platelets ($2.5x10^9$) were lysed and immunoprecipitated with a polyclonal anti-Slp4 antibody (IP Slp4) or an isotype control antibody (IP Ctl). The immunoblots were analyzed using anti-Kif5b, anti-Slp4 and anti-Rab27 antibodies. The data are representative of three independent experiments. (F) GFP-Rab27b and Flag-Slp4 were co-expressed in HEK 293T cells. Cell lysates were immunoprecipitated with an anti-GFP antibody and separated using SDS-PAGE. Co-precipitated Rab27b and Slp4 were immunoblotted with anti-Flag and anti-GFP antibodies. The blots are representative of three independent experiments. (G) GFP-KLC1 and Flag-Slp4 were co-expressed in HEK 293T cells. Cell lysates were immunoprecipitated with an anti-Flag antibody (M2 beads) and then separated using SDS-PAGE. Co-precipitated KLC1 and Slp4 were immunoblotted with anti-Flag and anti-GFP antibodies. The blots are representative of three independent experiments. (H) GFP-Kif5b and Flag-Slp4 were co-expressed in HEK 293T cells. Cell lysates were immunoprecipitated with an anti-Flag antibody (M2 beads) and then separated using SDS-PAGE. Co-precipitated Kif5b and Slp4 were immunoblotted with anti-Flag and anti-GFP antibodies. The blots are representative of three independent experiments.

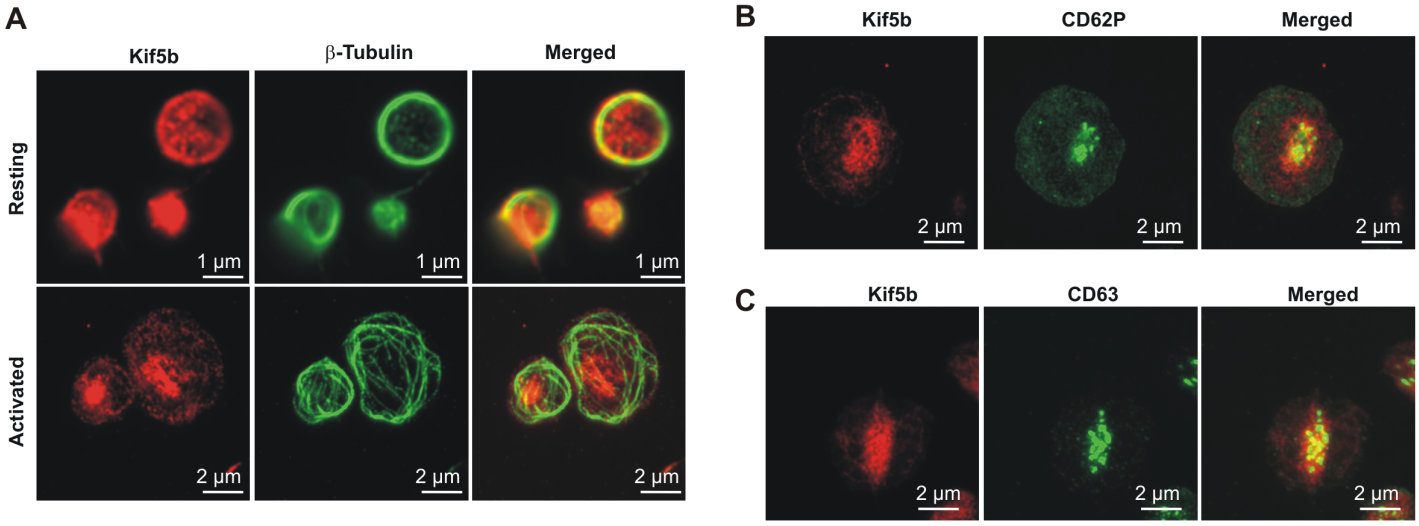

Figure 6. Subcellular localization of kinesin-1 in platelets.
(A) Immunofluorescence analysis of the localization of Kif5b in platelets. Human platelets were adhered to fibrinogen-coated glass coverslips and were either not stimulated (resting) or stimulated with ADP (20 μM) for 30 minutes at 37°C. Platelets were then fixed, permeabilized and stained with antibodies against β-tubulin and Kif5b or β-tubulin. All images are representative of at least three independent experiments. (B, C) Immunofluorescence analysis of the colocalization of Kif5b with CD62P (an α-granule marker) or CD63 (a dense granule/lysosome marker). Human platelets were adhered to fibrinogen-coated glass coverslips and stimulated with ADP (20 μM) for 30 minutes at 37°C. Platelets were then fixed, permeabilized and stained with an anti-CD62P or anti-CD63 antibody and an anti-Kif5b antibody. All images are representative of at least three independent experiments.