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# Dynamin 2 is required for GPVI signaling and platelet hemostatic function in mice

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## ABSTRACT

Receptor-mediated endocytosis, which contributes to a wide range of cellular functions, including receptor signaling, cell adhesion, and migration, requires endocytic vesicle release by the large GTPase dynamin 2. Here, the role of dynamin 2 was investigated in platelet hemostatic function using both pharmacological and genetic approaches. *Dnm2*$^{fl/fl}$ Pf4-Cre (*Dnm2*$^{Plt-/-}$) mice specifically lacking dynamin 2 within the platelet lineage developed severe thrombocytopenia and bleeding diathesis and *Dnm2*$^{Plt-/-}$ platelets adhered poorly to collagen under arterial shear rates. Signaling *via* the collagen receptor GPVI was impaired in platelets treated with the dynamin GTPase inhibitor dynasore, as evidenced by poor protein tyrosine phosphorylation, including that of the proximal tyrosine kinase Lyn on its activating tyrosine 396 residue. Platelet stimulation *via* GPVI resulted in a slight decrease in GPVI, which was maintained by dynasore treatment. Dynasore-treated platelets had attenuated function when stimulated *via* GPVI, as evidenced by reduced GPIbα downregulation, α-granule release, integrin αIIbβ3 activation, and spreading onto immobilized fibrinogen. By contrast, responses to the G-protein coupled receptor agonist thrombin were minimally affected by dynasore treatment. GPVI expression was severely reduced in *Dnm2*$^{Plt-/-}$ platelets, which were dysfunctional in response to stimulation *via* GPVI, and to a lesser extent to thrombin. *Dnm2*$^{Plt-/-}$ platelets lacked fibrinogen in their α-granules, but retained von Willebrand factor. Taken together, the data show that dynamin 2 plays a proximal role in signaling *via* the collagen receptor GPVI and is required for fibrinogen uptake and normal platelet hemostatic function.

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## Introduction

Receptor-mediated endocytosis (RME), the process by which cells internalize and sort specific extracellular material, plasma membrane proteins, and lipids, contributes to a wide range of cellular functions, including receptor signaling, cell adhesion, and migration.[1] RME requires membrane fission by the large and ubiquitous GTPase dynamin 2 (DNM2), which polymerizes at the neck of budding endocytic vesicles to mediate the GTP-dependent membrane fission required for their release into the cytosol prior to their incorporation into the endosomal compartment.[2] Consistent with its indispensable role in cellular homeostasis, *DNM2* mutations have been associated with Charcot-Marie-Tooth disease, centronuclear myopathy, and early T-cell precursor acute lymphoblastic leukemia (ETP-ALL),[3-5] and *Dnm2* deletion results in early embryonic lethality in mice.[6] While other classical dynamins (DNM1 and DNM3) are critical for activity-dependent vesicle recycling in presynaptic neurons,[7,8] their functions in other cells remain less clear.

The most well-characterized physiological roles of RME are to regulate uptake of nutrients such as cholesterol and iron and to down-modulate cytokine receptor signaling.[1] Lack of DNM2-dependent RME enhances responses to thrombopoietin in platelets and megakaryocytes (MK),[9] and to epidermal growth factor and inter-

leukins 5 and 7 in other cells,[10-12] the proposed mechanism associating *DNM2* loss-of-function mutations and ETP-ALL development.[5] Further, previous studies using pharmacological approaches have suggested that dynamin GTPase activity contributes to receptor desensitization in human platelets, as in the case of the purinergic receptors P2Y1 and P2Y12.[13]

Human platelets express all three classical dynamins,[14,15] including an inactive DNM3 spliced variant, for which a single nucleotide polymorphism has been associated with platelet size.[16] In comparison, mouse platelets express predominantly the ubiquitous DNM2,[14,17] thus providing a valuable model to study DNM2-dependent RME in platelet and MK biology, independent of neuronal DNM1 and DNM3. We have previously shown that $Dnm2^{fl/fl}$ Pf4-Cre ($Dnm2^{Plt-/-}$) mice specifically lacking DNM2 in the platelet lineage develop severe macrothrombocytopenia due to membrane fission arrest and accumulation of clathrin-coated vesicles obstructing the MK demarcation membrane system, the highly organized membrane reservoir for future platelets.[9] Here we investigated the role of DNM2 in platelet hemostatic function using both pharmacological and genetic approaches. Our data show that DNM2 regulates proximal signaling *via* the platelet collagen receptor GPVI and that DNM2-dependent RME is required for the accumulation of plasma fibrinogen into α-granules to facilitate normal platelet hemostatic function.

**Table 1.** Primary antibodies used.

| Target | Host | Type | Company |
|---|---|---|---|
| pTyr (4G10 Platinum) | Mouse | Monoclonal | EMD Millipore |
| pLyn (Y396) | Rabbit | Monoclonal | Boster Biological |
| Lyn | Mouse | Monoclonal | Santa Cruz |
| DNM2 | Mouse | Monoclonal | Santa Cruz |
| β-actin | Rabbit | Polyclonal | Abcam |
| β-tubulin | Mouse | Monoclonal | Sigma-Aldrich |
| GPVI (JAQ1) | Rat | Monoclonal | Emfret Analytics |
| GPIbα (CD42b) | Rat | Monoclonal | Emfret Analytics |
| P-selectin (CD62P) | Rat | Monoclonal | BD Biosciences |
| αIIb (CD41) | Rat | Monoclonal | R&D Systems |
| Fibrinogen | Rabbit | Polyclonal | DAKO |
| vWF | Rabbit | Polyclonal | DAKO |
| Clathrin Heavy Chain | Mouse | Monoclonal | EMD Millipore |
| Cavin 2 (SDPR) | Rabbit | Polyclonal | Proteintech |
| Caveolin 1 | Rabbit | Monoclonal | Cell Signaling |
| Rab5 | Rabbit | Monoclonal | Cell Signaling |
| Rab7 | Rabbit | Monoclonal | Cell Signaling |
| Rab11 | Rabbit | Monoclonal | Cell Signaling |
| Flotillin 1 | Mouse | Monoclonal | BD Biosciences |

pTyr: phosphotyrosine; pLyn: phosphorylated Lyn; vWF: von Willebrand factor.

## Methods

### Mice

$Dnm2^{Plt-/-}$ mice were described previously.[9] Mice were treated according to the National Institutes of Health and Medical College of Wisconsin Institutional Animal Care and Use Committee guidelines.

### Platelet count

Platelet count was measured on a Sysmex XT-2000i automatic hematology analyzer using blood collected by mouse retro-orbital plexus bleeding and immediately diluted in Cellpack (Sysmex) supplemented with EDTA and PGE1.[18]

### Tail bleeding time

Bleeding time was determined by snipping 2 mm of distal mouse tail and immediately immersing the tail in 37°C isotonic saline.[19] A complete cessation of bleeding was defined as the bleeding time.

### *Ex vivo* perfusion assay

Platelet interaction with immobilized type I collagen was performed using the VenaFlux Platform and Vena8Fluor+ biochips (Cellix).[20] Additional information can be found in the *Online Supplementary Methods*.

### Platelet preparation and flow cytometry

Blood was collected by mouse retro-orbital plexus bleeding and was anticoagulated in acid-citrate-dextrose.[19] Platelets were isolated by sequential centrifugation, resuspended at $5x10^8$ platelets/mL, and incubated for 30 minutes (min) at 37°C with 100 μM of the non-competitive inhibitor of dynamin GTPase activity, dynasore (EMD Millipore),[13,21-23] or vehicle (0.1% DMSO).

Platelets were activated or not with collagen-related peptide (CRP; Protein Chemistry Core Laboratory, Blood Research Institute, Versiti, USA) or human thrombin (Roche) for 2-3 min at 37°C and stained with FITC-labeled rat anti-mouse GPIbα or FITC-labeled rat anti-mouse P-selectin antibodies (Table 1) or Oregon Green 488-labeled fibrinogen (Thermo Fisher Scientific).[19] Fluorescence was quantified using an Accuri C6 flow cytometer (BD Biosciences) and FlowJo software. A total of 10,000 events were analyzed for each sample.

### Immunoblot analysis

Platelets were lysed as described.[19] Platelet proteins were separated by SDS-PAGE, transferred onto an Immobilon-P membrane (EMD Millipore), and probed with antibodies directed against proteins of interest (Table 1). Platelet fibrinogen content was quantitated using purified mouse fibrinogen (Enzyme Research) as standard.

Lipid rafts were isolated from human platelets as described.[24] Blood was collected from volunteers by venipuncture and was anticoagulated in acid-citrate-dextrose. Approval was obtained from the Western Institutional Review Board and informed consent was approved according to the Declaration of Helsinki.

### Immunofluorescence microscopy

Samples were imaged on Nikon Structured Illumination Microscopy (N-SIM, NIS-Elements AR v4.40.00 software) and Olympus Confocal FV1000-MPE (FluoView software) platforms under 100x oil objectives.[9,25] Additional information can be found in the *Online Supplementary Methods.*

### Statistical analysis

All experiments were performed at least in triplicate. Results were compared with the unpaired Student *t*-test (simple), two-way ANOVA followed by Bonferroni correction (multiple), or the Kaplan-Meier analysis (time-to-event) using Prism software (GraphPad). $P<0.05$ was considered significant.

## Results

### Hemostatic defects in *Dnm2*$^{Plt-/-}$ mice

*Dnm2*$^{Plt-/-}$ mice developed severe thrombocytopenia, with 152±15x10$^3$ platelets/μL [mean±standard error of mean SEM); n=15], compared to 1,299±54x10$^3$ platelets/μL in control *Dnm2*$^{fl/fl}$ mice (n=18) ($P$<0.0001), an 88% reduction (Figure 1A), as described previously.[9] The role of DNM2 in platelet hemostatic function was evaluated using the tail bleeding time assay (Figure 1B). Control mice had a median tail bleeding time of 1.16 min. By contrast, *Dnm2*$^{Plt-/-}$ mice had a profound bleeding diathesis with all mice studied bleeding for 10 min, our experimental end-point measurement (n=12 in each group) (Log-rank $P$<0.0001).

Following blood vessel injury and disruption of the vascular endothelium, platelets are exposed to basement membrane proteins and soluble agonists, which initiate platelet adhesion and activation, leading to thrombus formation and preventing excessive bleeding. At arterial shear rates, initial platelet adhesion is mediated by collagen-bound von Willebrand factor (vWF) binding to the GPIb-IX complex, followed by platelet activation *via* the collagen receptor GPVI.[26] The functionality of *Dnm2*$^{Plt-/-}$ platelets in whole blood was tested in flow chamber experiments using the VenaFlux platform,[20] where binding to a collagen-coated surface was measured under arterial shear rate (1500 s$^{-1}$) to mediate the interaction of plasma vWF with surface-bound collagen (Figure 1C-F). After 4 min, control platelets covered 18.0±5.1% (mean±SEM; n=7) of the collagen-coated surface (Figure 1E). Adhesion was markedly decreased in *Dnm2*$^{Plt-/-}$ platelets, with only 0.6±0.1% (n=4) ($P$=0.0333) of surface coverage, a 97% reduction.

The dwell time of individual control and *Dnm2*$^{Plt-/-}$ platelets was analyzed under the same experimental conditions (Figure 1F). After initial tethering, control platelets dwelled for a median time of 61 seconds (s) (n=60). *Dnm2*$^{Plt-/-}$ platelets dwelled for a significantly lower median time of 33 s (n=61) (Log-rank $P$=0.0331), indicating that a decreased stability of the GPIbα-vWF interaction contributes to the poor adhesion of *Dnm2*$^{Plt-/-}$ platelets to collagen under arterial shear rates and the profound bleeding diathesis of *Dnm2*$^{Plt-/-}$ mice.

### Impaired GPVI signaling in dynasore-treated and *Dnm2*$^{Plt-/-}$ platelets

Collagen binding to its platelet receptor GPVI initiates a signaling pathway that sequentially involves activation of the Src family tyrosine kinases Fyn and Lyn, phosphorylation of the GPVI-associated FcR γ-chain, and recruitment, tyrosine phosphorylation, and activation of the tyrosine kinase Syk, leading to activation of phospholipase C-γ2 (PLC-γ2).[27] The ability of DNM2 to regulate GPVI signaling was investigated (Figure 2). Control platelets, platelets treated with 100 μM dynasore to inhibit DNM2 GTPase activity pharmacologically, and *Dnm2*$^{Plt-/-}$ platelets were activated with the GPVI agonist CRP. In control platelets,

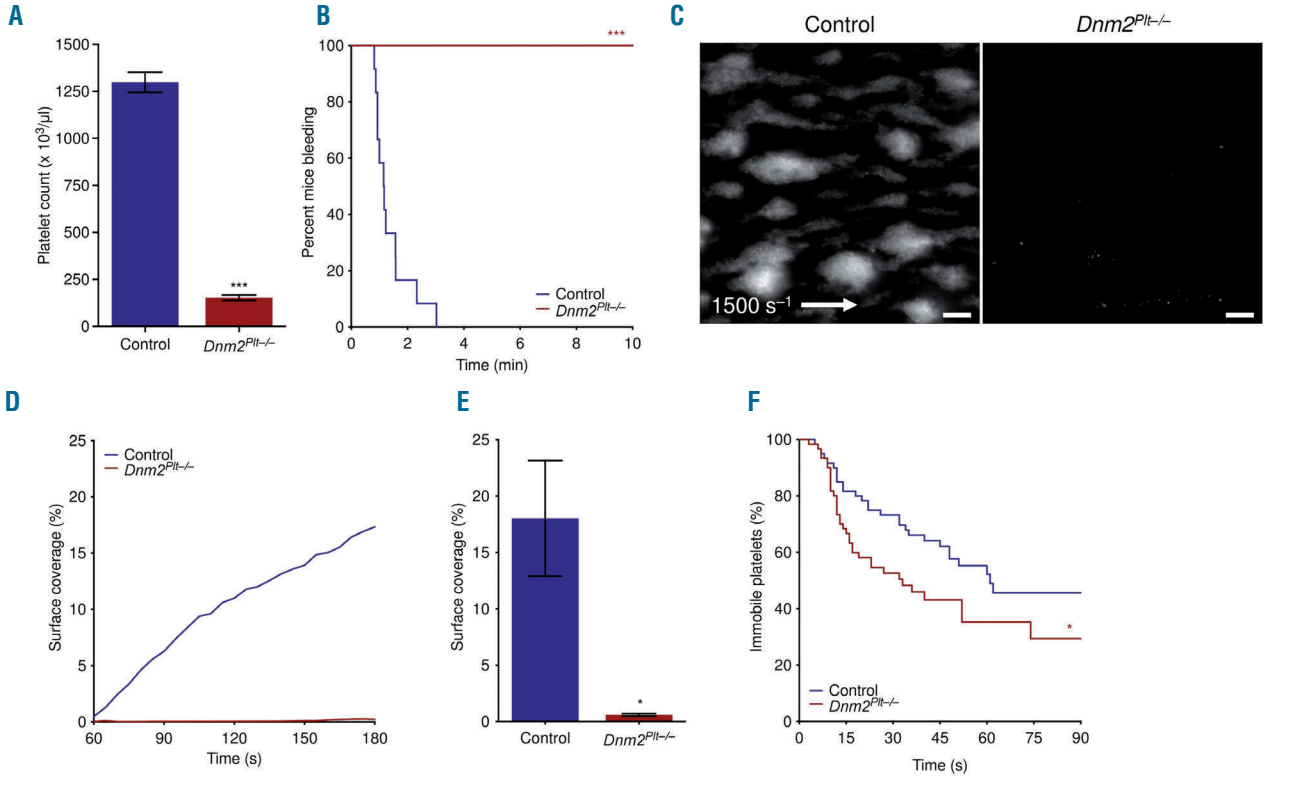


**Figure 1. Hemostatic defects in *Dnm2*$^{Plt-/-}$ mice.** (A) Blood platelet count of control *Dnm2*$^{fl/fl}$ (n = 18) and *Dnm2*$^{Plt-/-}$ mice (n=15; ***$P$<0.0001). (B) Tail bleeding time of control *Dnm2*$^{fl/fl}$ and *Dnm2*$^{Plt-/-}$ mice (n=12 in each group; Log-rank ***$P$<0.0001). (C-F) PPACK-anticoagulated whole blood from control and *Dnm2*$^{fl/fl}$ mice was labeled and perfused on type I collagen-immobilized surface at an arterial shear rate of 1500 s$^{-1}$. (C) Representative still image at 4 min. Scale bars, 100 μm. (D) Representative time-course surface coverage, as labeled platelets accumulate in the field of view. (E) Surface coverage at 4 min (mean ± SEM; 7 control and 4 *Dnm2*$^{Plt-/-}$; *$P$=0.0333). (F) Dwell time of individual control and *Dnm2*$^{Plt-/-}$ platelets (n=60 in each group; Log-rank *$P$=0.0331).

CRP stimulation induced tyrosine phosphorylation of several proteins, including proteins at 125, 72, 68, 56, 52, and 38 kDa (Figure 2A). Dynasore-treated platelets had a moderate reduction of tyrosine phosphorylation of these proteins in response to CRP stimulation, and *Dnm2*$^{Plt-/-}$ platelets failed to increase protein tyrosine phosphorylation, even at high doses of CRP.

Because of its proximal role in the GPVI signaling pathway,[28] Lyn activation was probed using an antibody specifically directed against its phosphorylated activating tyrosine 396 (Tyr396) residue (Figure 2B). Stimulation of control platelets with CRP induced a 62±19% increase in Lyn Tyr396 phosphorylation that peaked at 2.5 μg/mL CRP (mean±SEM; n=3 in each group) ($P$=0.0408). Lyn Tyr396 phosphorylation was attenuated in dynasore-treated platelets and markedly reduced in *Dnm2*$^{Plt-/-}$ platelets.

GPVI expression in control, dynasore-treated, and *Dnm2*$^{Plt-/-}$ platelets was further evaluated by immunoblot analysis using the monoclonal antibody JAQ1 (Figure 2C). Following stimulation of control platelets with 25 μg/mL CRP, JAQ1 signal decreased by 15%, compared to resting levels. Dynasore treatment resulted in a 10% increase in JAQ1 signal, which was maintained following CRP stimulation. Taken together, the data show that DNM2 contributes to GPVI homeostasis at rest and GPVI downregulation and Lyn activation following GPVI ligation. As reported previously, GPVI expression was markedly decreased in *Dnm2*$^{Plt-/-}$ platelets,[9] and was unaffected by CRP.

## Impaired GPIbα downregulation in CRP-stimulated dynasore-treated and *Dnm2*$^{Plt-/-}$ platelets

GPIbα is internalized during platelet activation,[29] a phenomenon that is expected to negatively affect initial platelet adhesion to collagen-bound vWF and for which a role of dynamin has been reported, based on pharmacological inhibition.[22] The role of DNM2 in this process was investigated in response to the GPVI agonist CRP or the soluble G-protein-coupled receptor agonist thrombin (Figure 3). Expression of surface GPIbα decreased to about 30% of resting levels following stimulation of control platelets with CRP (Figure 3A) or thrombin (Figure 3B).

In response to stimulation with CRP, expression of surface GPIbα decreased to 60% of resting levels in dynasore-treated platelets, a 50% reduction compared to controls, and *Dnm2*$^{Plt-/-}$ platelets failed to down-regulate GPIbα (Figure 3A). By contrast, dynasore treatment or *Dnm2* deletion did not affect GPIbα downregulation when platelets were stimulated with thrombin (Figure 3B).

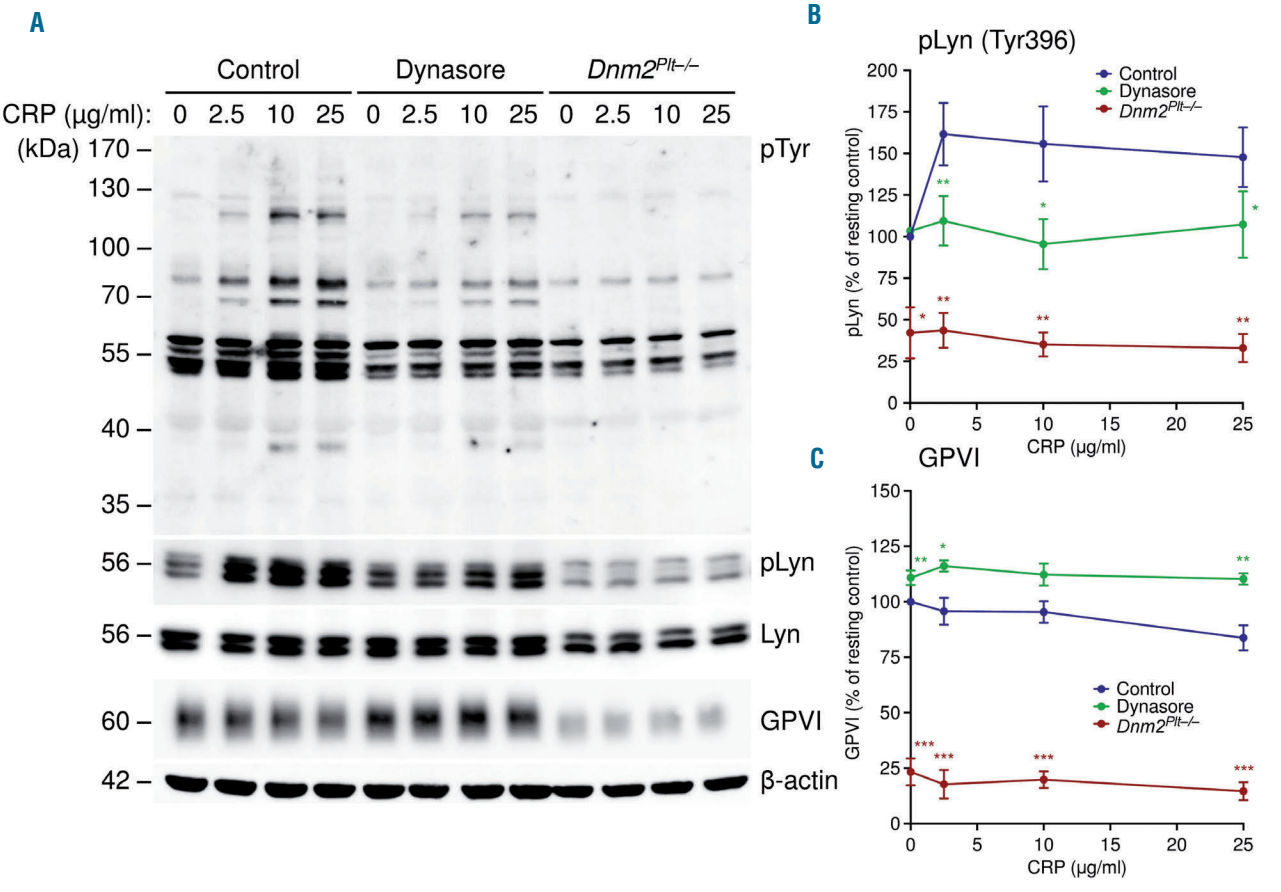


**Figure 2. GPVI signaling defects in dynasore-treated and *Dnm2*$^{Plt-/-}$ platelets.** Control platelets, platelets treated with 100 μM dynasore, and *Dnm2*$^{Plt-/-}$ platelets were activated or not with CRP for 2 minutes at 37 °C as indicated. (A) Platelet lysates corresponding to 2 μg of protein were subjected to SDS-PAGE and probed for phosphotyrosine (pTyr), phosphorylated Lyn Tyr396 (pLyn), Lyn, GPVI, and β-actin as a loading control. Results are representative of five independent experiments. Assessment of Lyn Tyr396 phosphorylation (B) and GPVI expression (C) in CRP-stimulated control, dynasore-treated, and *Dnm2*$^{Plt-/-}$ platelets. Results represent mean±standard error of mean (SEM) of 3-4 independent experiments, and are compared statistically to control (*$P$<0.05; **$P$<0.01; ***$P$<0.001).

The decreased GPIbα surface expression was due to internalization, and not to shedding, as total GPIbα expression was maintained during the course of the experiment, as shown by immunoblot analysis (Figure 3C). Taken together, the data show that DNM2 specifically regulates GPVI signaling, rather than GPIbα downregulation.

### Impaired α-granule secretion and integrin αIIbβ3 activation in CRP-stimulated dynasore-treated and *Dnm2*$^{Plt-/-}$ platelets

Following activation by collagen or soluble agonists, platelets secrete their granule contents and activate their surface integrin αIIbβ3 in order to recruit circulating platelets and mediate platelet aggregation, respectively. The significance of DNM2 in these platelet hemostatic processes was assessed by flow cytometry (Figure 4). Control platelets, dynasore-treated platelets, and *Dnm2*$^{Plt-/-}$ platelets were activated with CRP (Figure 4A and C) or thrombin (Figure 4B and D), and analyzed for P-selectin (CD62P) expression, a marker for α-granule secretion (Figure 4A and B), and binding of fluorescently-labeled fibrinogen, a marker for integrin αIIbβ3 activation (Figure 4C and D).[19] Both CRP and thrombin induced a concentration-dependent increase of α-granule secretion and integrin αIIbβ3 activation in control platelets, reaching about 80% of platelets expressing CD62P or binding fibrinogen with 25 μg/mL CRP or 0.25 U/mL thrombin, respectively. Dynasore treatment resulted in a significant decrease in platelet responses to CRP, as only 30-40% platelets expressed CD62P and bound fibrinogen with 50 μg/mL CRP. By contrast, platelet responses to thrombin were not significantly affected by dynasore treatment. CRP-dependent CD62P expression and fibrinogen binding were completely abolished in *Dnm2*$^{Plt-/-}$ platelets and only about 20% expressed CD62P and bound fibrinogen in response to 0.5 U/mL thrombin.

### Altered spreading of dynasore-treated and *Dnm2*$^{Plt-/-}$ platelets

Following activation, platelets rapidly change shape from resting disc-like entities to morphologically distinct forms, first by rounding, then by extending finger-like filopodia and spreading thin sheet-like lamellipodia.[30] The significance of DNM2 in platelet spreading was examined (Figure 5). Control platelets, dynasore-treated, and *Dnm2*$^{Plt-/-}$ platelets were activated with either 1 μg/mL CRP or 0.01 U/mL thrombin and allowed to adhere onto immobilized fibrinogen. In control platelets, stimulation with CRP or thrombin resulted in filopodia extension and lamellipodia spreading, as evidenced by phalloidin staining, a marker for polymerized actin, with the greatest difference being more angular or rounded appearance, respectively (Figure 5A). Treatment with dynasore mitigated lamellipodia formation in CRP-stimulated platelets (Figure 5B), and to a lesser, non-statistically significant degree in thrombin-stimulated platelets (Figure 5C), although it did not prevent filopodia growth.

*Dnm2*$^{Plt-/-}$ platelets displayed great heterogeneity in shape change with either agonist (Figure 5A), wherein spread platelet surface area varied between below control levels or up to a 5-fold increase in size following stimulation, reflecting the increased size of these platelets.[9] *Dnm2*$^{Plt-/-}$ platelets revealed extreme irregularity in their cytoskeletal and overall morphological arrangement, consistent with altered spreading capacity.

### Absence of fibrinogen in *Dnm2*$^{Plt-/-}$ platelets

The fibrinogen content of platelet α-granules derives from the integrin αIIbβ3-dependent uptake of plasma-

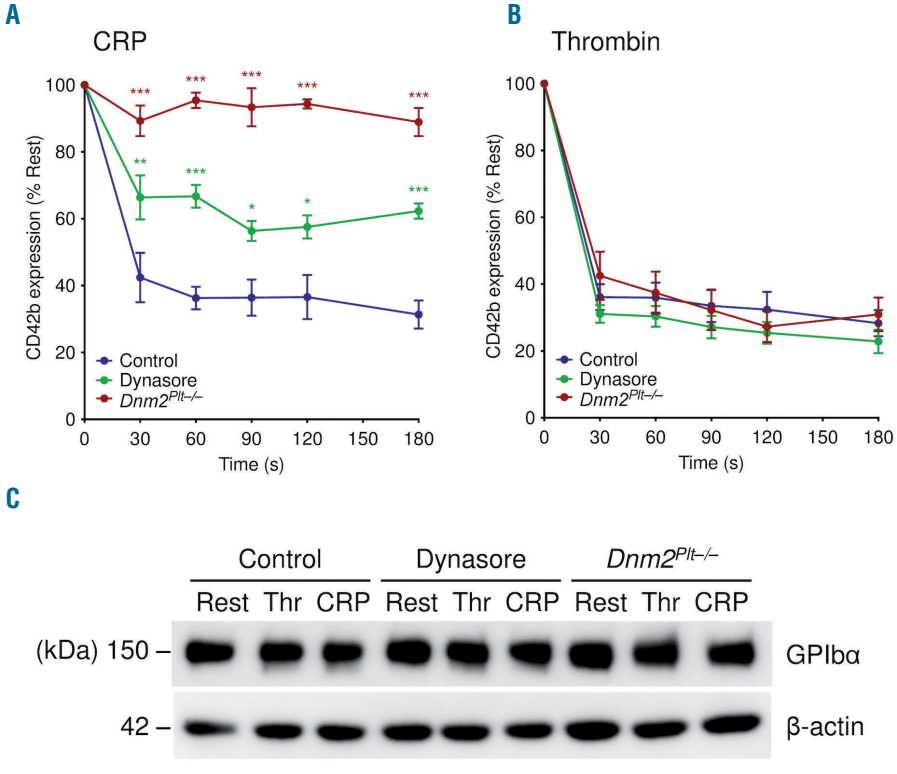


**Figure 3. Impaired GPIbα downregulation in CRP-stimulated dynasore-treated and *Dnm2*$^{Plt-/-}$ platelets.** Control platelets, platelets treated with 100 μM dynasore, and *Dnm2*$^{Plt-/-}$ platelets were activated for 3 minutes (min) at 37°C with 25 μg/mL CRP (A) or 0.1 U/ml thrombin (B), incubated with FITC-labeled anti-mouse CD42b antibody, and analyzed by flow cytometry. Results are expressed as percentage of CD42b expression at rest, represent mean±standard error of mean (SEM) of three independent experiments, and are compared statistically to control (*$P$<0.05; **$P$<0.01; ***$P$<0.001). (C) Control, dynasore-treated, and *Dnm2*$^{Plt-/-}$ platelets were activated or not with 0.1 U/mL thrombin or 25 μg/mL CRP for 3 min at 37°C as indicated. Platelet lysates were subjected to SDS-PAGE and probed for GPIbα and β-actin as loading control, as indicated. Results are representative of three independent experiments.

derived fibrinogen.[31,32] To evaluate the contribution of DNM2-dependent RME in the process, the fibrinogen content of *Dnm2*$^{Plt-/-}$ platelets was compared to that of vWF, which is synthesized by MK and is also stored in platelet α-granules (Figure 6A).[33] Immunoblot analysis showed severe fibrinogen reduction in *Dnm2*$^{Plt-/-}$ platelet lysates, but normal expression of vWF. Quantification of the immunoblots using purified mouse fibrinogen as standard revealed that $10^6$ *Dnm2*$^{Plt-/-}$ platelets contained 71±6 ng fibrinogen, compared to 333±27 ng in $10^6$ control platelets (n=3 in each group) ($P$=0.0007), a 79% decrease (Figure 6B).

The fibrinogen content of control and *Dnm2*$^{Plt-/-}$platelets was further evaluated by structured illumination microscopy and compared to that of the αIIb subunit (CD41) of its receptor, the integrin αIIbβ3 (Figure 6C, top panels). In control platelets fibrinogen was observed in large puncta, consistent with its presence in α-granules.[33] By contrast, *Dnm2*$^{Plt-/-}$ platelets had severely reduced fibrinogen content, with about 90% of *Dnm2*$^{Plt-/-}$ platelets presenting barely detectable fibrinogen positive α-granules. CD41 resided on the platelet surface and in small vesicles or granules within platelets, independent of DNM2 expression, consistent with the association of the integrin αIIbβ3 with multiple intracellular platelet compartments that include α-granules and the open canalicular system.[34] By contrast, vWF was normally packaged in *Dnm2*$^{Plt-/-}$ platelets (Figure 6C, bottom panels). Taken together, the data show that integrin αIIbβ3-dependent uptake of plasma-derived fibrinogen requires DNM2-dependent RME.

## Platelet endocytic and endosomal components

Thin-section electron microscopy studies have subdivided RME into clathrin- and caveolae-mediated endocytosis (CME and CavME, respectively).[1] CME is found in virtually all cells and requires cargo receptor binding to clathrin-associated adaptor protein 2 complexes to form clathrin-coated vesicles.[35] Caveolae are invaginated lipid rafts rich in cholesterol, sphingolipids, and scaffolding proteins called caveolins and cavins that are found in many mammalian cell types.[36] Platelet endocytic and endosomal components were investigated in the presence or absence of DNM2 (Figure 6A). Platelets contained clathrin heavy chain required for CME and its expression was increased in *Dnm2*$^{Plt-/-}$ platelets. Cavin 2 (also known as SDPR, PS-p68) was detected in platelets, but not caveolin 1, which is required for CavME. Ruling out poor antibody reactivity, a strong caveolin 1 signal was observed at the expected molecular weight of 21 kDa with mouse lung tissue lysates (*data not shown*).[37]

As an additional control and because cavin 2 associates

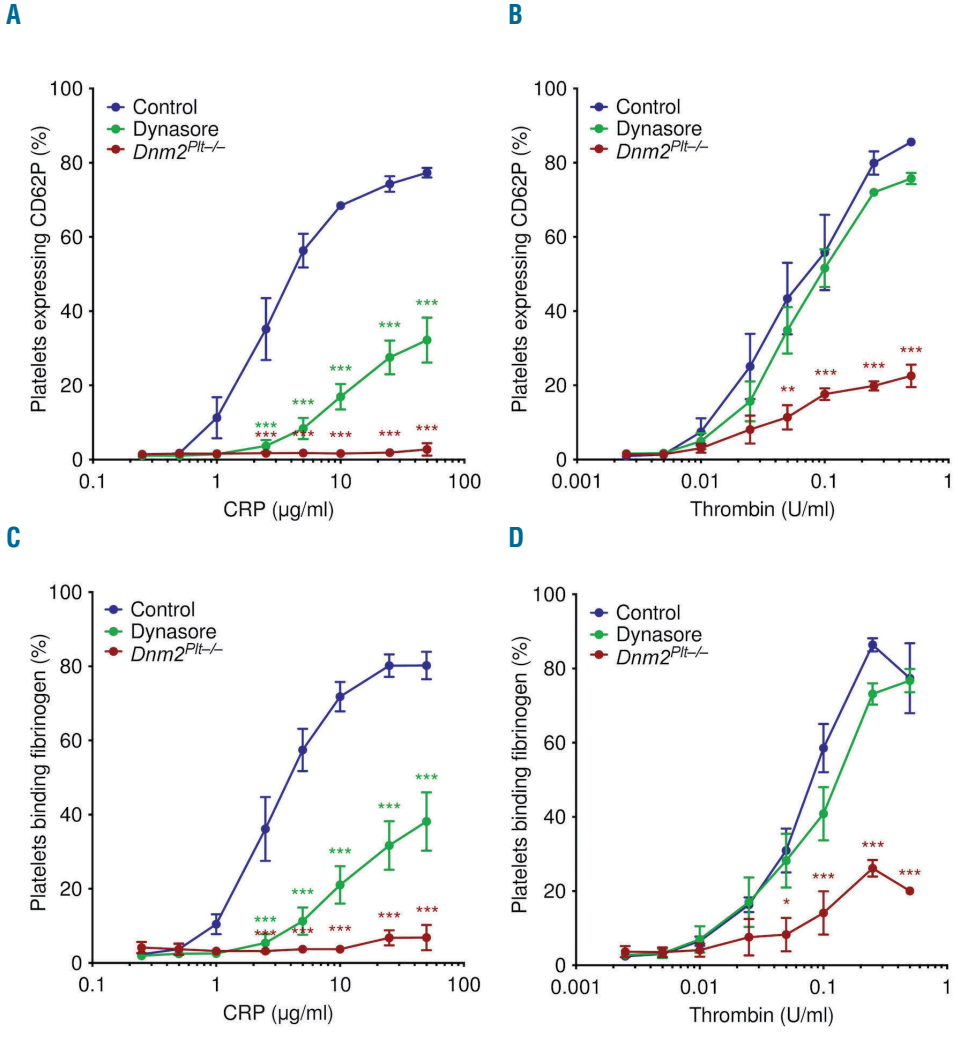


**Figure 4. Hemostatic defects of CRP-stimulated dynasore-treated and *Dnm2*$^{Plt-/-}$ platelets.** Control platelets, platelets treated with 100 μM dynasore, and *Dnm2*$^{Plt-/-}$ platelets were activated for 2 min at 37°C with CRP (A and C) or thrombin (B and D) as indicated. Platelets were then incubated with FITC-labeled anti-mouse CD62P antibody (A and B) or Oregon green 488-labeled fibrinogen (C and D) and analyzed by flow cytometry. Results are expressed as percentage of positive platelets, represent mean±standard error of mean (SEM) of 3-6 independent experiments, and are compared statistically to control (*$P$<0.05; **$P$<0.01; ***$P$<0.001).

with insoluble lipid rafts in cells expressing caveolin 1,[38] the association of cavin 2 with detergent-resistant platelet lipid rafts was investigated using a sucrose gradient in human platelet lysates (Figure 6D), which also lack caveolin 1 (*data now shown*). Cavin 2 did not associate with the insoluble sucrose gradient fractions rich in GM1 ganglioside and flotillin 1. Taken together, the data show that platelets contained the endocytic machinery required for CME, but not for CavME.

Early, late, and recycling endosomal compartments are distinguished by their association with specific members of the Rab family of small GTPases. Platelets contained Rab5 (early), Rab7 (late), and Rab11 (recycling), as described previously,[33,39] and their expression levels were not affected by the lack of DNM2 (Figure 6A).

## Discussion

The cellular mechanisms and proteins regulating platelet and MK RME are poorly understood.[39] Here, using both pharmacological and genetic approaches, we described the role of the endocytic GTPase DNM2 in intracellular signaling *via* the collagen receptor GPVI and platelet hemostatic function.

In control platelets, ligation of the collagen receptor GPVI by its soluble agonist CRP induced an increase in protein tyrosine phosphorylation, including that of the proximal protein tyrosine kinase Lyn on its activating residue Tyr396, and a decrease in GPVI expression. Following dynasore treatment, phosphorylation of Lyn Tyr396 was attenuated and GPVI expression was maintained. Recent studies have shown that common dynamin inhibitors, such as dynasore and Dyngo-4a, not only inhibit dynamin GTPase activity, but also disrupt the organization of cholesterol-rich membrane rafts in a dynamin-independent manner.[40,41] Dynasore-treated platelets elicited defects in GPIbα downregulation, α-granule secretion, integrin αIIbβ3 activation, and spreading onto fibrinogen when stimulated *via* GPVI, but not by thrombin. By contrast, the cholesterol-lowering reagent, methyl-β-cyclodextrin, inhibits GPVI signaling, as well as platelet responses to the G-protein-coupled receptor agonists, thrombin and ADP.[42,43] Hence, the data argue

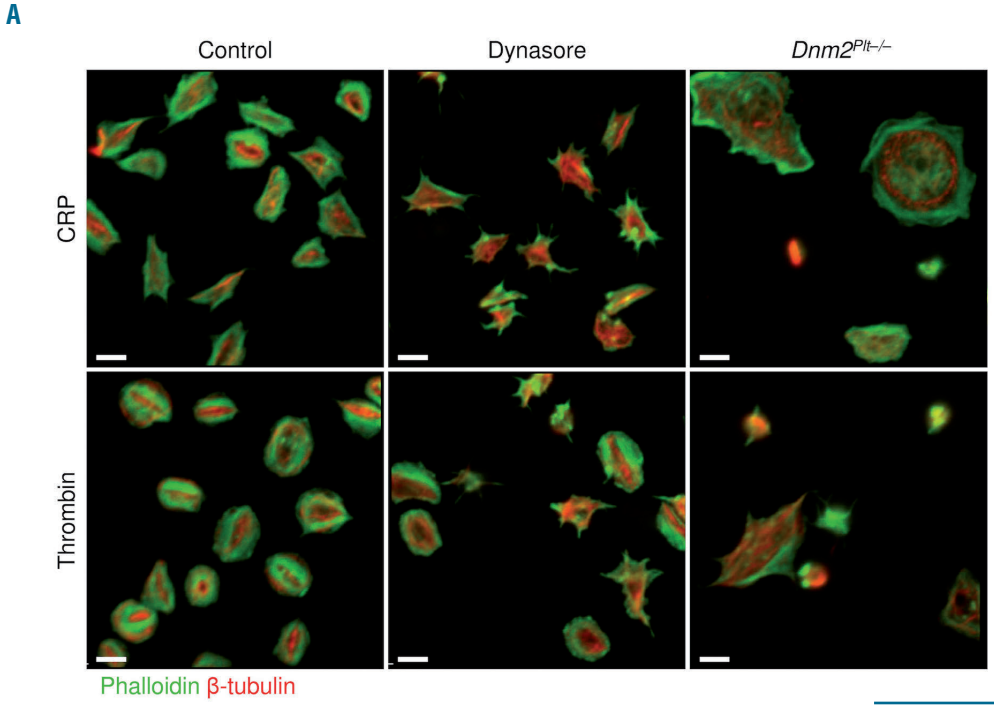


**Figure 5. Altered spreading of CRP-stimulated dynasore-treated and *Dnm2*$^{Plt-/-}$ platelets.** (A) Control platelets, platelets treated with 100 μM dynasore, and *Dnm2*$^{Plt-/-}$ platelets were activated with 1 μg/mL CRP or 0.01 U/mL thrombin and spread onto fibrinogen-coated coverslips for 30 minutes (min) as indicated. Fixed platelets were stained for polymerized actin (phalloidin; green) and β-tubulin (red) and analyzed by confocal microscopy. Images are representative of at least three independent experiments. Scale bars, 3 μm. (B) Assessment of platelet spreading in response to 1 μg/mL CRP. Total platelets scored were 374 control, 275 dynasore-treated, and 187 *Dnm2*$^{Plt-/-}$. (C) Assessment of platelet spreading in response to 0.01 U/mL thrombin. Total platelets scored were 298 control, 226 dynasore-treated, and 192 *Dnm2*$^{Plt-/-}$ (*$P$<0.05; **$P$<0.01; ***$P$<0.001).

against membrane raft disruption and indicate that DNM2 plays a proximal role in GPVI signaling.

The positive role of DNM2 in GPVI signaling contrasts with its commonly reported function in attenuating receptor signaling. Lack of DNM2-dependent RME enhances responses to thrombopoietin in platelets and MK and to epidermal growth factor and interleukins 5 and 7 in other cells.[9-12] Dynasore treatment also inhibits the desensitization of the purinergic receptors P2Y1 and P2Y12 in human platelets.[13] Thus, DNM2 differentially regulates signaling depending on the receptor it is linked to. The GPVI-associated FcR γ-chain contains two putative endocytic YxxL motifs that are present within its immunoreceptor tyrosine-based activation motif. Whether these motifs recruit the endocytic machinery necessary to down-regulate GPVI is unclear, as their mutation in mouse platelets abol-

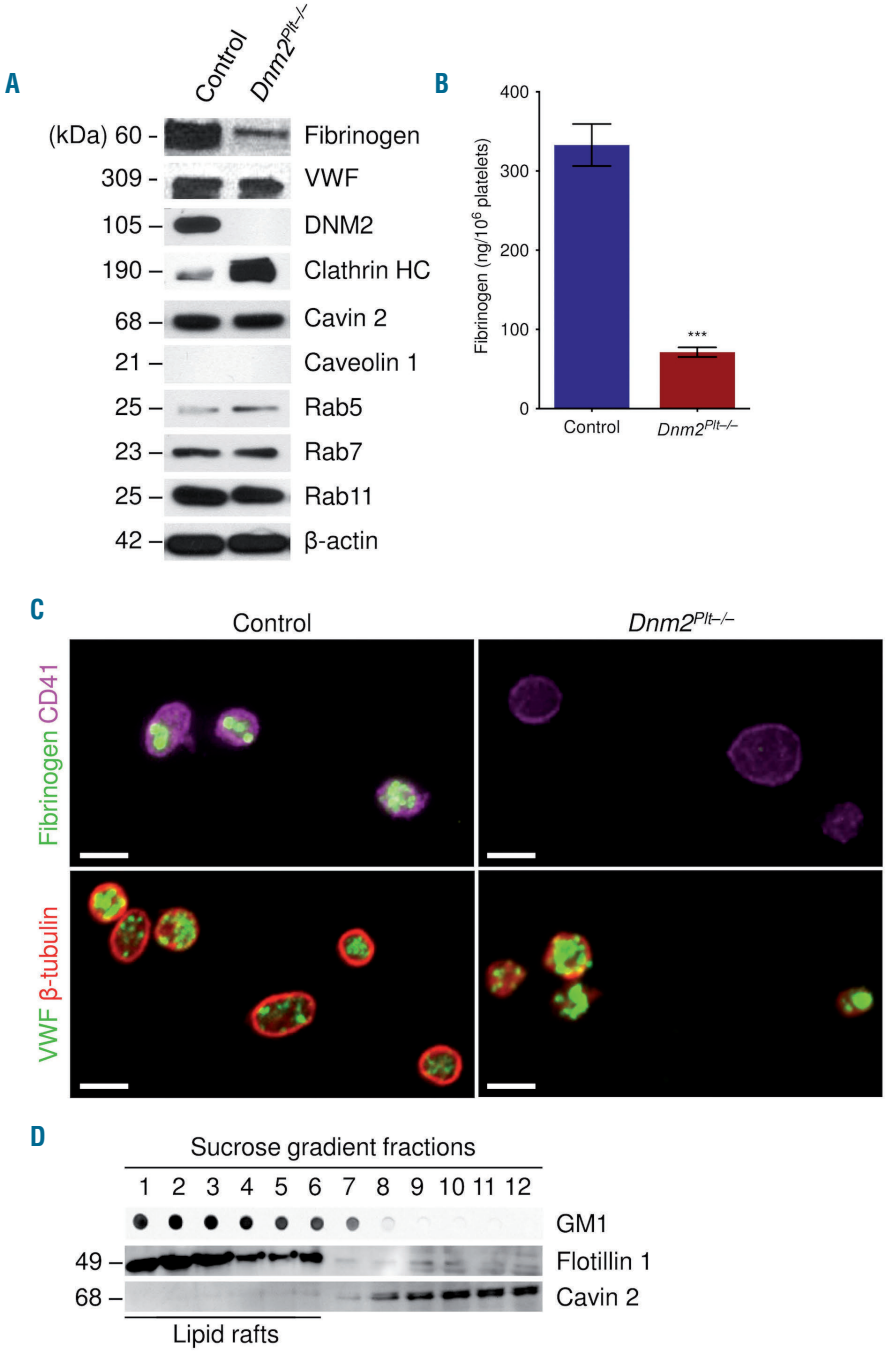


**Figure 6. Cargo, endocytic, and endosomal proteins in *Dnm2*$^{Plt-/-}$ platelets.** (A) Control and *Dnm2*$^{Plt-/-}$ platelet lysates corresponding to 2 μg of protein were subjected to SDS-PAGE and probed for cargo, endocytic, and endosomal proteins, and β-actin as loading control, as indicated. Results are representative of three independent experiments. HC: heavy chain. (B) The fibrinogen content of control and *Dnm2*$^{Plt-/-}$ platelets was evaluated by immunoblot analysis using purified mouse fibrinogen as standard. Results are expressed as ng/$10^6$ platelets and represent mean±standard error of mean (SEM) of three independent experiments (***$P$=0.0007). (C) Structured illumination microscopy analysis of fibrinogen (green) and CD41 (magenta; top panels) and confocal microscopy analysis of vWF (green) and β-tubulin (red; bottom panels) in control and *Dnm2*$^{Plt-/-}$ platelets. Scale bars, 3 μm. (D) Sucrose density fractions of human platelet lysates were dot-blotted and probed with HRP-conjugated cholera toxin B subunit to detect GM1 ganglioside or immunoblotted for flotillin 1 and cavin 2.

ishes both GPVI signaling and internalization.[44] Besides its role in membrane fission during RME, DNM2 can also serve as a scaffolding protein for signaling intermediates.[2] In T cells, DNM2 directly interacts with the guanine nucleotide exchange factor Vav1 to regulate activation of PLC-γ1 and the accumulation of cortical actin at sites of T-cell receptor activation, thereby regulating T-cell receptor signaling.[45] Vav1 and Vav3 play critical but redundant roles in the activation of PLC-γ2 downstream of GPVI,[46] and clustering of GPVI dimers, the mechanism of which depends on a dynamic actin cytoskeleton, contributes to GPVI signaling.[47] It is, therefore, tempting to speculate that platelet DNM2 serves as a scaffolding protein for signaling intermediates and/or actin-regulatory proteins downstream of GPVI.

*Dnm2*$^{Plt-/-}$ mice had a severe bleeding diathesis, which was intrinsic to platelets as *Dnm2* deletion under control of the *Pf4* promoter is specific to the platelet lineage.[9] Consistently, *Dnm2*$^{Plt-/-}$ platelets adhered poorly to collagen under arterial shear rates and were depleted of fibrinogen. Further, α-granule secretion, integrin αIIbβ3 activation, and spreading onto fibrinogen were markedly reduced in *Dnm2*$^{Plt-/-}$ platelets stimulated through GPVI or with thrombin. The lack of GPVI signaling is likely explained by the profound deficit in GPVI expression.[9] Whether this deficit is due to decreased GPVI synthesis in *Dnm2*$^{Plt-/-}$ MK, increased internalization and degradation, or ADAM10-mediated extracellular domain shedding is unclear. The differences between the pharmacological and genetic approaches indicate that either dynasore treatment does not completely inhibit platelet DNM2 GTPase activity or *Dnm2*$^{Plt-/-}$ platelets acquire defects during production affecting their functional responses. Because RME is a critical component of cellular cholesterol homeostasis,[1] it is possible that *Dnm2* genetic deletion impacts cell membrane composition and lipid raft organization, thereby affecting signaling in cholesterol-rich membrane domains. While the tail bleeding time in mice is largely unaffected by severe reduction of platelet count or lack of GPVI,[48,49] it cannot be excluded that the macrothrombocytopenia of *Dnm2*$^{Plt-/-}$ mice combined with the profound deficit in GPVI expression contributes to the bleeding diathesis.

The severe reduction in fibrinogen content of *Dnm2*$^{Plt-/-}$ platelets is consistent with impaired RME and a role for DNM2 downstream of integrin αIIbβ3 function as plasma fibrinogen is taken up by platelets and MK in an integrin αIIbβ3-mediated manner.[31,32] Beside fibrinogen, platelets and MK take up and store in their α-granules a long list of plasma proteins such as coagulation factor V and regulators of angiogenesis.[50,51] While the hypothesis was not tested here, endocytosed proteins are expected to be absent in *Dnm2*$^{Plt-/-}$ platelets due to defective RME, affecting other biological processes aside from hemostasis.

Platelets contained major endocytic and endosomal proteins such as clathrin and Rab GTPases.[33,39] The increased expression of clathrin heavy chain in *Dnm2*$^{Plt-/-}$ platelets likely reflects the accumulation of clathrin-coated vesicles observed in the demarcation membrane system of *Dnm2*$^{Plt-/-}$ MK.[9] Caveolin 1 was not detected in platelets, consistent with mRNA and protein profiling,[14,15,17] and cavin 2, a detergent-insoluble caveolae marker in cells expressing caveolin 1,[38] remained soluble following sucrose gradient of human platelet lysates. While cavin 2 is abundantly expressed in platelets,[14,15,17] where it was originally characterized,[52] its role in the absence of caveolin 1 is unclear. Taken together, the observations show that CME is the primary mechanism for RME in platelets. Consistently, fibrinogen uptake involves the recruitment of clathrin-associated adaptor protein 2 complexes to the β3 subunit of the integrin αIIbβ3 *via* clathrin adaptor proteins such as Dab2 and Numb.[53,54] After its uptake by CME and transport *via* the early (Rab5) and late (Rab7) endosome, fibrinogen is retained within α-granules by mechanisms dependent on NBEAL2, mutated in gray platelet syndrome.[33] The remaining fibrinogen content of *Dnm2*$^{Plt-/-}$ platelets is likely associated with the plasma membrane or trapped in the open canalicular system.

In conclusion, our work provides pharmacological and genetic evidence for a role of DNM2 in GPVI signaling and platelet hemostatic function. DNM2 regulates signaling events downstream of the collagen receptor GPVI, including the activation of the proximal tyrosine kinase Lyn, and is required for the integrin αIIbβ3-mediated accumulation of plasma fibrinogen into α-granules. Our data also show that DNM2 does not contribute to GPIbα downregulation, as dynasore-treated platelets and *Dnm2*$^{Plt-/-}$ platelets down-regulated GPIbα normally in response to thrombin. Whether DNM2 is a reasonable target for antithrombotic therapies remains to be determined.

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