

12-21-2017

CK2b regulates thrombopoiesis and Ca²⁺-Triggered platelet activation in arterial thrombosis

Patrick Münzer
Universität Tübingen

Britta Walker-Allgaier
Universität Tübingen

Sascha Geue
Universität Tübingen

Friederike Langhauser
Julius-Maximilians-Universität Würzburg

Eva Geuss
Julius-Maximilians-Universität Würzburg

See next page for additional authors

Follow this and additional works at: <https://ir.lib.uwo.ca/biochempub>

Citation of this paper:

Münzer, Patrick; Walker-Allgaier, Britta; Geue, Sascha; Langhauser, Friederike; Geuss, Eva; Stegner, David; Aurbach, Katja; Semeniak, Daniela; Chatterjee, Madhumita; Menendez, Irene Gonzalez; Märklin, Melanie; Quintanilla-Martinez, Leticia; Salih, Helmut R.; Litchfield, David W.; Buchou, Thierry; Kleinschnitz, Christoph; Lang, Florian; Nieswandt, Bernhard; Pleines, Irina; Schulze, Harald; Gawaz, Meinrad; and Borst, Oliver, "CK2b regulates thrombopoiesis and Ca²⁺-Triggered platelet activation in arterial thrombosis" (2017). *Biochemistry Publications*. 256.
<https://ir.lib.uwo.ca/biochempub/256>

Authors

Patrick Münzer, Britta Walker-Allgaier, Sascha Geue, Friederike Langhauser, Eva Geuss, David Stegner, Katja Aurbach, Daniela Semeniak, Madhumita Chatterjee, Irene Gonzalez Menendez, Melanie Märklin, Leticia Quintanilla-Martinez, Helmut R. Salih, David W. Litchfield, Thierry Buchou, Christoph Kleinschnitz, Florian Lang, Bernhard Nieswandt, Irina Pleines, Harald Schulze, Meinrad Gawaz, and Oliver Borst

PLATELETS AND THROMBOPOIESIS

CK2 β regulates thrombopoiesis and Ca²⁺-triggered platelet activation in arterial thrombosis

Patrick Münzer,^{1,*} Britta Walker-Allgaier,^{1,*} Sascha Geue,¹ Friederike Langhauser,² Eva Geuss,² David Stegner,³ Katja Aurbach,^{3,4} Daniela Semeniak,^{3,4} Madhumita Chatterjee,¹ Irene Gonzalez Menendez,⁵ Melanie Märklin,⁶ Leticia Quintanilla-Martinez,⁵ Helmut R. Salih,^{6,7} David W. Litchfield,⁸ Thierry Buchou,⁹ Christoph Kleinschnitz,^{2,10} Florian Lang,^{1,11} Bernhard Nieswandt,³ Irina Pleines,^{3,4} Harald Schulze,^{3,4} Meinrad Gawaz,¹ and Oliver Borst¹

¹Department of Cardiology and Cardiovascular Medicine, University of Tübingen, Tübingen, Germany; ²Department of Neurology, University of Würzburg, Würzburg, Germany; ³Rudolf Virchow Center, Research Center for Experimental Biomedicine, Würzburg, Germany; ⁴Institute for Experimental Biomedicine, University of Würzburg, Würzburg, Germany; ⁵Department of Pathology, and ⁶Department of Hematology and Oncology, University of Tübingen, Tübingen, Germany; ⁷Clinical Cooperation Unit Translational Immunology, German Cancer Consortium, German Cancer Research Center Partner Site Tübingen, Tübingen, Germany; ⁸Department of Biochemistry, University of Western Ontario, London, ON, Canada; ⁹INSERM, Institute for Advanced Biosciences, University Joseph Fourier Grenoble, La Tronche, France; ¹⁰Department of Neurology, University of Essen, Essen, Germany; and ¹¹Department of Physiology, University of Tübingen, Tübingen, Germany

Key Points

- CK2 β is critically required for thrombopoiesis by regulating tubulin polymerization, MK fragmentation, and proplatelet formation.
- CK2 β facilitates inositol triphosphate-mediated increase of cytosolic Ca²⁺ and is essential for platelet activation in arterial thrombosis in vivo.

Platelets, anucleated megakaryocyte (MK)-derived cells, play a major role in hemostasis and arterial thrombosis. Although protein kinase casein kinase 2 (CK2) is readily detected in MKs and platelets, the impact of CK2-dependent signaling on MK/platelet (patho-) physiology has remained elusive. The present study explored the impact of the CK2 regulatory β -subunit on platelet biogenesis and activation. MK/platelet-specific genetic deletion of CK2 β (*ck2 β ^{-/-}*) in mice resulted in a significant macrothrombocytopenia and an increased extramedullary megakaryopoiesis with an enhanced proportion of premature platelets. Although platelet life span was only mildly affected, *ck2 β ^{-/-}* MK displayed an abnormal microtubule structure with a drastically increased fragmentation within bone marrow and a significantly reduced proplatelet formation in vivo. In *ck2 β ^{-/-}* platelets, tubulin polymerization was disrupted, resulting in an impaired thrombopoiesis and an abrogated inositol 1,4,5-triphosphate receptor-dependent intracellular calcium (Ca²⁺) release. Presumably due to a blunted increase in the concentration of cytosolic Ca²⁺, activation-dependent increases of α and dense-granule secretion and integrin $\alpha_{IIb}\beta_3$ activation, and aggregation were abrogated in *ck2 β ^{-/-}* platelets. Accordingly, thrombus

formation and stabilization under high arterial shear rates were significantly diminished, and thrombotic vascular occlusion in vivo was significantly blunted in *ck2 β ^{-/-}* mice, accompanied by a slight prolongation of bleeding time. Following transient middle cerebral artery occlusion, *ck2 β ^{-/-}* mice displayed significantly reduced cerebral infarct volumes, developed significantly less neurological deficits, and showed significantly better outcomes after ischemic stroke than *ck2 β ^{+/+}* mice. The present observations reveal CK2 β as a novel powerful regulator of thrombopoiesis, Ca²⁺-dependent platelet activation, and arterial thrombosis in vivo. (*Blood*. 2017; 130(25):2774-2785)

Introduction

Platelets are small, anucleated cells derived from proplatelets, which are generated by polyploid megakaryocytes (MKs) within sinusoids of the bone marrow (BM).¹ Microtubules, polarized tubular filaments of α - and β -tubulin heterodimers, are the major structural components involved in proplatelet formation.^{2,3} Polymerization of microtubules is necessary to support the enlarging proplatelet mass.^{4,5} β 1-Tubulin expression is restricted in the MK/platelet lineage and is essential for late MK differentiation and platelet biogenesis.⁶ Genetic loss of β 1-tubulin, the major β -tubulin isoform in MKs, results in macrothrombocytopenia due to pathological MK fragmentation, impaired

proplatelet formation, and release of large, immature platelets with an attenuated response to activation.^{6,7}

The microtubule plus end-binding protein 3 (EB3) transiently binds to growing polymerized microtubules by recognizing the GTP-bound state of β -tubulin.^{3,8,9} EB3 provides an essential hub for assembly of plus-end-tracking proteins that facilitate interactions of microtubules with the endoplasmic reticulum (ER).^{8,10} The ER is the major intracellular calcium (Ca²⁺) store in platelets and is continuously remodeled through its interactions with microtubules.¹¹ Inositol 1,4,5-triphosphate receptors (IP₃Rs) within ER membranes facilitate

Submitted 12 May 2017; accepted 12 September 2017. Prepublished online as *Blood* First Edition paper, 19 September 2017; DOI 10.1182/blood-2017-05-784413.

*P.M. and B.W.-A. contributed equally and are joint first authors.

The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2017 by The American Society of Hematology

rapid Ca²⁺ release from internal stores on binding of Inositol 1,4,5-triphosphate (IP₃) generated in activated platelets.^{12,13} Clustering of IP₃Rs in the ER membranes is essential for IP₃R activity and intracellular Ca²⁺ release.^{14,15} Microtubule cytoskeleton dynamics are crucial to IP₃R clustering and IP₃R-driven release of Ca²⁺ from internal stores.^{8,16,17} Microtubule-associated EB3 binds to IP₃Rs, thus promoting IP₃R clustering and Ca²⁺ release.⁸

Platelet adhesion, activation, and aggregation are essential for primary hemostasis, but are also critically involved in acute arterial thrombotic occlusion, leading to myocardial infarction or ischemic stroke.¹⁸ Moreover, platelets participate in the orchestration of vascular inflammation and atherogenesis.^{19,20} Subendothelial collagen, collagen-related peptide (CRP), and thrombin are the main triggers of platelet activation, characterized by granule release, integrin $\alpha_{IIb}\beta_3$ activation, aggregation, and consecutive thrombus formation.^{12,21} All those platelet responses depend on a rapid increase in the concentration of cytosolic Ca²⁺ ([Ca²⁺]_i), which is accomplished by IP₃-mediated Ca²⁺ release from intracellular stores triggering subsequent extracellular Ca²⁺ influx via the stimulation of Orai1-mediated store-operated Ca²⁺ entry.²²

Casein kinase 2 (CK2) is a constitutively active serine-threonine protein kinase with a tetrameric structure, in which 2 regulatory β -subunits dimerize to link the 2 catalytic subunits α or α' .²³ By bridging the 2 catalytic subunits, CK2 β is required for the assembly of tetrameric CK2 complexes, thus enhancing CK2 holoenzyme stability to modulate catalytic activity and substrate specificity.²⁴⁻²⁶ The regulatory CK2 β -subunit plays an essential role in development because ubiquitous *Ck2 β* -deficiency in mice causes lethality before embryonic day 7.5.²⁷ CK2 controls various cellular processes, such as cell cycle progression, proliferation, apoptosis, and signal transduction, involving phosphatidylinositol 3-kinase (PI3K)/Akt and JAK/STAT pathways in a number of human cancers, including hematological malignancies.²⁸ Recently, cell-specific deletion of CK2 β in regulatory T cells identified CK2 as an important modulator of immune response.²⁹ Growing evidence points to an important role of CK2 in the maintenance of cell morphology as well as in the regulation of tubulin cytoskeleton and microtubule dynamics.^{23,25,30} Although CK2 is strongly expressed in platelets,³¹ its role in thrombopoiesis as well as platelet activation and platelet-dependent arterial thrombosis still remains poorly defined.

The present study explored the role of CK2 β in MK and platelet biology as well as its impact on platelet biogenesis and function. To circumvent prenatal death and to investigate the MK/platelet-specific effects of CK2 β on mechanisms regulating thrombopoiesis, hemostasis, and arterial thrombosis in vivo, mice expressing *ck2 β* alleles flanked with the *loxP*-Cre excision sequence (*cskn2b^{fl/fl}*) were used to achieve MK/platelet-specific deletion of *ck2 β* (*ck2 β ^{-/-}*).

Material and methods

Mice

CK2 β -floxed mice (*cskn2b^{fl/fl}*) were generated by Thierry Buchou (INSERM, Grenoble, France). Origin, breeding, and genotyping of *cskn2b^{fl/fl}* mice were described previously.²⁷ For platelet-specific deletion of CK2 β , *cskn2b^{fl/fl}* mice were crossed with PF4-Cre mice (The Jackson Laboratory) to obtain *cskn2b^{fl/fl} pf4-Cre⁺* (*ck2 β ^{-/-}*) and *cskn2b^{fl/fl} pf4-Cre⁻* (*ck2 β ^{fl/fl}*) littermates. Mice were studied at the age of 6 to 12 weeks. All animal experiments were performed according to the German animal protection law and were in accordance with the recently published Animal Research: Reporting In Vivo Experiments guidelines (National Centre for the Replacement, Refinement and Reduction of Animals in Research) and approved by the local authorities. Animal handling and care complied with published regulations by the German law for the protection of

animals, and experiments were approved by the local authorities (Regierungspräsidium Tübingen).

Methods

Platelet preparation, in vitro differentiation, and cultivation of fetal liver- and BM-derived MKs, platelet and MK immunoblot analysis, determination of reticulated platelets, platelet life span assay, determination of thrombopoietin (TPO) levels and analysis of thrombopoiesis after TPO treatment, immunohistochemistry of murine BM and spleens, immunofluorescence staining on femora cryosections and cultured MKs, ultrastructural transmission electron microscopy (TEM) analysis of MKs, determination of MK ploidy, multiphoton intravital microscopy analysis of in vivo proplatelet formation, microtubule sedimentation, real-time polymerase chain reaction, spectrofluorometry for Ca²⁺ measurements, flow cytometry, platelet aggregometry and adenosine triphosphate (ATP) release, in vitro thrombus formation and stabilization, tail bleeding time, in vivo thrombus formation in mesenteric arterioles and in carotid arteries, ischemic stroke in mice (transient middle cerebral artery occlusion [tMCAO]), and the statistical analysis are described in detail in supplemental Material and methods, available on the *Blood* Web site.

Results

Genetic deletion of CK2 β results in embryonic lethality in mice. Therefore, to specifically delete *ck2 β* in the MK lineage, *cskn2b^{fl/fl}* mice were crossed with transgenic mice expressing Cre-recombinase under the control of the MK- and platelet-specific platelet factor 4 (PF4-Cre) to obtain *cskn2b^{fl/fl} pf4-Cre⁺* (*ck2 β ^{-/-}*) and *cskn2b^{fl/fl} pf4-Cre⁻* (*ck2 β ^{fl/fl}*) littermates. Real-time polymerase chain reaction analysis revealed that CK2 β was totally ablated in *ck2 β ^{-/-}* platelets on the transcript level, whereas CK2 β expression in other tissues was unaffected (Figure 1A; supplemental Figure 1). Immunoblotting confirmed that platelets and MKs derived from *ck2 β ^{-/-}* mice completely lacked CK2 β protein abundance (Figure 1B). *Ck2 β ^{-/-}* mice were viable and fertile, but, notably, mice with the *ck2 β* -deficient MK lineage developed distinct macrothrombocytopenia without obvious signs of spontaneous hemorrhage. Macrothrombocytopenia in *ck2 β ^{-/-}* mice was reflected by a reduction of platelet counts of 55% and a significant increase in platelet volume (Figure 1C). To examine whether the macrothrombocytopenia in *ck2 β ^{-/-}* mice resulted from increased platelet turnover, platelet life span was determined in *ck2 β ^{-/-}* mice. Clearance of *ck2 β* -null platelets was only moderately accelerated, which may have contributed to, but does not fully explain, the severely reduced platelet counts of *ck2 β ^{-/-}* mice (Figure 1D). Further, according to the determination of major surface glycoproteins by flow cytometric analysis (Table 1), CK2 β deficiency resulted in a mildly but significantly reduced expression of von Willebrand factor (vWF) receptor complex subunits, whereas the expression of other surface receptors was comparable in *ck2 β ^{-/-}* and *ck2 β ^{fl/fl}* platelets.

The significant thrombocytopenia that was observed despite the only moderately reduced platelet life span prompted us to assess platelet production. To this end, we labeled blood samples ex vivo with thiazole orange. As revealed by flow cytometry, we found a significantly higher amount of larger young reticulated platelets in *ck2 β ^{-/-}* mice (Figure 1E). Because TPO is the main trigger of (compensatory) megakaryopoiesis and its plasma concentrations are reciprocally regulated by total platelet mass, we measured plasma TPO levels in thrombocytopenic *ck2 β ^{-/-}* mice. TPO concentrations were slightly but significantly increased in *ck2 β ^{-/-}* mice (Figure 1F). TPO injection was followed by significant elevations in the platelet counts of *ck2 β ^{fl/fl}* mice after 96 hours, whereas exogenous TPO did not sufficiently elevate the platelet counts of *ck2 β ^{-/-}* mice and failed to prevent

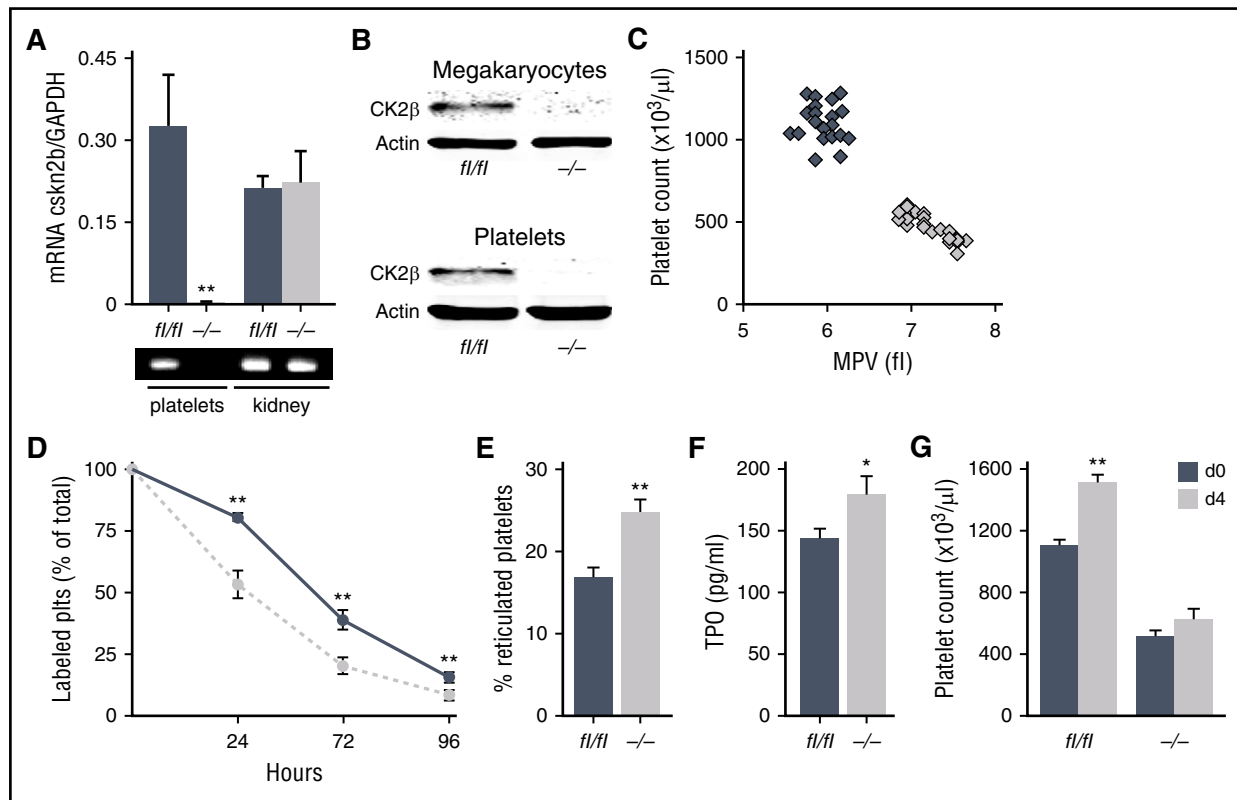


Figure 1. CK2 β is strongly expressed in MKs and platelets, and CK2 β deficiency results in macrothrombocytopenia, reduced platelet life span, and an increased amount of immature reticulated platelets. (A) Representative image (bottom) and arithmetic means \pm standard errors of the means ($n = 6$; top) of CK2 β transcript levels in platelets and kidneys from $ck2\beta^{fl/fl}$ and $ck2\beta^{-/-}$ mice indicating a cell-specific knockout of CK2 β in the MK/platelet lineage. (B) Representative immunoblots of CK2 β protein levels of MKs (top) and platelets (bottom) from $ck2\beta^{fl/fl}$ and $ck2\beta^{-/-}$ mice ($n = 4$) confirming a complete knockout of CK2 β in the MK/platelet lineage. (C) Count and mean platelet volume (MPV) of $ck2\beta^{fl/fl}$ (blue diamonds) and $ck2\beta^{-/-}$ (gray diamonds) platelets ($n = 24$). Each diamond represents 1 individual mouse. (D) Endogenous survival of $ck2\beta^{fl/fl}$ (blue trace) and $ck2\beta^{-/-}$ (gray trace) platelets measured by the determination of the percentage of fluorescently labeled platelets in vivo at indicated time points after injection of DyLight 488 α -GPIX ($n = 6$). (E) Flow cytometric quantification of new, reticulated platelets as a percentage of total platelets in $ck2\beta^{fl/fl}$ and $ck2\beta^{-/-}$ mice. Arithmetic means \pm standard errors of the means ($n = 6$) are shown. (F) TPO levels of $ck2\beta^{fl/fl}$ (blue bar) and $ck2\beta^{-/-}$ (gray bar) mice. Arithmetic means \pm standard errors of the means ($n = 15$) are shown. (G) Platelet counts of $ck2\beta^{fl/fl}$ and $ck2\beta^{-/-}$ mice before and 4 days after treatment with TPO (2 μ g/animal per day). Arithmetic means \pm standard errors of the means ($n = 8$) are shown. Unpaired Student t test in panels A and D-G. * $P < .05$; ** $P < .01$.

thrombocytopenia in these mice (Figure 1G). Of note, protein expression levels and surface coverage of the TPO receptor myeloproliferative leukemia virus oncogene were similar in platelets and MKs (supplemental Figure 2). Platelet sialylation and hepatic TPO transcript levels were comparable in $ck2\beta^{-/-}$ and $ck2\beta^{fl/fl}$ mice as well (supplemental Figure 3), indicating that Ashwell-Morell receptor-dependent platelet clearance is not affected by CK2 β .

As revealed by immunohistological analysis, the BM of $ck2\beta^{-/-}$ mice showed remarkable MK hyperplasia, reflected by a significantly increased number of MKs within the BM (Figure 2A). Moreover, a significant number of $ck2\beta^{-/-}$ BMMKs displayed signs of fragmentation

with abrogated cellular demarcation. Further examination of MKs flushed out of BM revealed a significantly reduced ploidy in $ck2\beta$ -null MKs with a significant reduction of 16N- and 32N-containing MKs (Figure 2D), indicating that CK2 β -deficiency results in the accumulation of immature MKs. Although only a mild splenomegaly was observed in $ck2\beta^{-/-}$ mice (Figure 2C), after spleen immunostaining, we found drastically increased extramedullary thrombopoiesis in $ck2\beta^{-/-}$ mice, with distortion of splenic architecture strongly enriched with GPIb-positive MKs (Figure 2B). Nevertheless, $ck2\beta^{-/-}$ mice displayed neither a modification of the hematopoietic stem and progenitor cell compartment in the BM and spleen nor an excess of hematopoietic progenitors in the peripheral blood (supplemental Figure 4). Hematoxylin and eosin (H&E) staining of formalin-fixed, paraffin-embedded spleens confirmed a markedly increased number of MKs in the spleens from $ck2\beta^{-/-}$ mice (Figure 2B), whereas BM and spleen fibrosis was not accelerated in CK2 β -deficient mice (supplemental Figure 5). GPIb and H&E staining of the lungs ruled out any difference in the presence of MKs and platelets in the lung circulation or parenchyma in $ck2\beta^{-/-}$ mice as compared with wild-type mice (supplemental Figure 6). Because $ck2\beta^{-/-}$ MKs were hardly identifiable due to their altered morphology and demarcation from surrounding cells, we analyzed the ultrastructure of BM MKs by TEM, revealing impaired DMS formation and prominent granule-free zones in $ck2\beta^{-/-}$ MKs (Figure 3A). To evaluate the functional consequence

Table 1. Glycoprotein surface expression of $ck2\beta^{fl/fl}$ and $ck2\beta^{-/-}$ platelets

Platelet GP	$ck2\beta^{fl/fl}$	$ck2\beta^{-/-}$	P
GPIb α	343 \pm 10	311 \pm 4	<.01
GPV	145 \pm 3	121 \pm 2	<.001
GPVI	66 \pm 3	62 \pm 2	.08
GPIX	175 \pm 5	145 \pm 1	<.001
Integrin β_1	147 \pm 5	141 \pm 7	.50
Integrin β_3	184 \pm 4	182 \pm 3	.83

Results are expressed as mean fluorescence intensity \pm standard error of the mean for 10 mice per group.

GPIb α , glycoprotein Ib α .

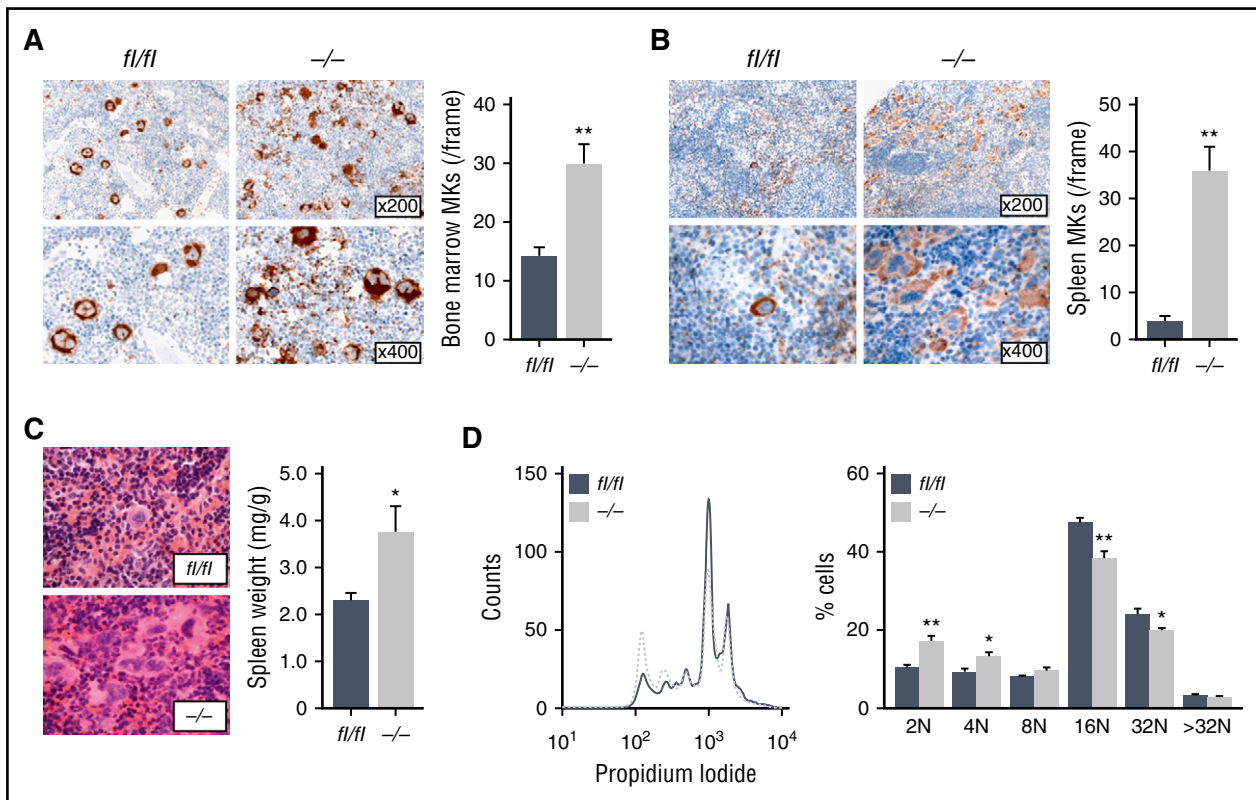


Figure 2. *Ck2 β ^{-/-}* mice display BM MK hyperplasia and an upregulation of extramedullary thrombopoiesis. (A) Representative images of GPIb immunostaining of BM sections from 2-month-old *ck2 β ^{fl/fl}* and *ck2 β ^{-/-}* mice (left). Quantification of *ck2 β ^{fl/fl}* and *ck2 β ^{-/-}* BM MKs per visual field. Arithmetic means \pm standard errors of the means (n = 6; right) are shown. (B) Representative images of GPIb immunostaining of spleen sections from 6-month-old *ck2 β ^{fl/fl}* and *ck2 β ^{-/-}* mice (left). Quantification of *ck2 β ^{fl/fl}* and *ck2 β ^{-/-}* spleen MKs per visual field. Arithmetic means \pm standard errors of the means (n = 6; right) are shown. (C) Representative H&E staining of spleen sections from 6-month-old *ck2 β ^{fl/fl}* and *ck2 β ^{-/-}* mice (left). Arithmetic means \pm standard errors of the means (n = 8; right) of relative spleen weight in *ck2 β ^{fl/fl}* and *ck2 β ^{-/-}* mice. (D) Ploidy histogram of *ck2 β ^{fl/fl}* and *ck2 β ^{-/-}* MKs (left) and arithmetic means \pm standard errors of the means (n = 6; right) are shown. Unpaired Student t test. * P < .05; ** P < .01.

of these findings, we next visualized MKs in BM cryosections of intact femora. Immunostaining clearly indicated that *ck2 β* -null MKs had less contact with BM sinusoids and displayed markedly increased fragmentation (Figure 3B). To further study the role of CK2 β in MK-dependent platelet biogenesis, we performed in vitro proplatelet formation assays. Accordingly, significantly fewer numbers of *ck2 β ^{-/-}* MKs formed proplatelets (Figure 3C). For examination of these findings in vivo, we analyzed proplatelet formation under steady-state conditions, visualizing the BM in the mouse skull by using 2-photon intravital microscopy (2P-IVM). 2P-IVM confirmed that *ck2 β ^{-/-}* MKs undergo premature ectopic fragmentation and are unable to efficiently release proplatelets into the vascular sinusoids in vivo because $40.6\% \pm 7.6\%$ of the *ck2 β* -deficient MKs had an altered morphology, appearing fragmented as if the MKs were somewhat unstable, whereas only $11.5\% \pm 2.6\%$ of the *ck2 β ^{fl/fl}* MKs were fragmented (Figure 3D; supplemental Videos 1 and 2). As further revealed by 2P-IVM, premature platelet release by *ck2 β ^{-/-}* MKs is probably caused by defective in vivo proplatelet formation. Only $3.2\% \pm 2.9\%$ of *ck2 β* -null MKs produce proplatelets in vivo compared with $8.5\% \pm 2.7\%$ of *ck2 β ^{fl/fl}* MKs, indicating that CK2 β is required for proplatelet formation and is a critical regulator of thrombopoiesis (Figure 3D). To eliminate MK fragmentation of *ck2 β ^{-/-}* MKs as consequence of increased apoptosis in these cells, we determined caspase 3/7 activity. As illustrated in supplemental Figure 7, caspase 3/7 activity in *ck2 β ^{-/-}* MKs was similar to that in *ck2 β ^{fl/fl}* MKs.

Microtubules and tubulin polymerization are essential for platelet structure and function. Because CK2 β has been described as a critical regulator of tubulin and microtubule architecture, we next examined tubulin structure and polymerization. Of note, α -tubulin filaments

appeared fragmented in many cultured *ck2 β ^{-/-}* MKs, indicating that filament stability or polymerization were affected in the absence of CK2 β (Figure 4A). Along those lines, treatment with the microtubule-stabilizing toxin, paclitaxel, resulted in an increase of polymerized β -tubulin in *ck2 β ^{fl/fl}*, whereas *ck2 β ^{-/-}* platelets failed to stabilize polymerized tubulin (Figure 4B), pointing to a critical role of CK2 β in platelet microtubule stability. Presumably as a result of impaired tubulin polymerization, *ck2 β ^{-/-}* platelets also showed significantly less EB3 binding (Figure 4C).

Because we know from other cell types that EB3 may act as an important linker between polymerized microtubules and the regulation of internal Ca^{2+} stores,⁸ we next aimed to elucidate the impact of CK2 β -driven tubulin polymerization on intracellular Ca^{2+} release and subsequent Ca^{2+} -dependent platelet activation. To discriminate between intracellular Ca^{2+} store release and Ca^{2+} influx from extracellular space, we used spectrofluorometry to determine the activation-dependent changes of $[\text{Ca}^{2+}]_i$ in the absence or presence of extracellular Ca^{2+} (Figure 5A-B). Before stimulation, the cytosolic Ca^{2+} concentration was similar. By comparison, in the presence and in the absence of extracellular Ca^{2+} , *ck2 β ^{-/-}* platelets displayed a significantly blunted $[\text{Ca}^{2+}]_i$ increase in response to stimulation with CRP or thrombin, pointing to an impaired intracellular regulation of cytosolic Ca^{2+} release in *ck2 β* -null platelets. IP_3 production occurs due to the activation of phospholipase C (PLC) induced by thrombin downstream of G protein-coupled signaling or after stimulation of the tyrosine kinase-linked collagen receptor glycoprotein VI (GPVI) by collagen or CRP.³² To test if the decreased intracellular Ca^{2+} release in *ck2 β ^{-/-}* platelets could have been due to defective PLC-triggered IP_3

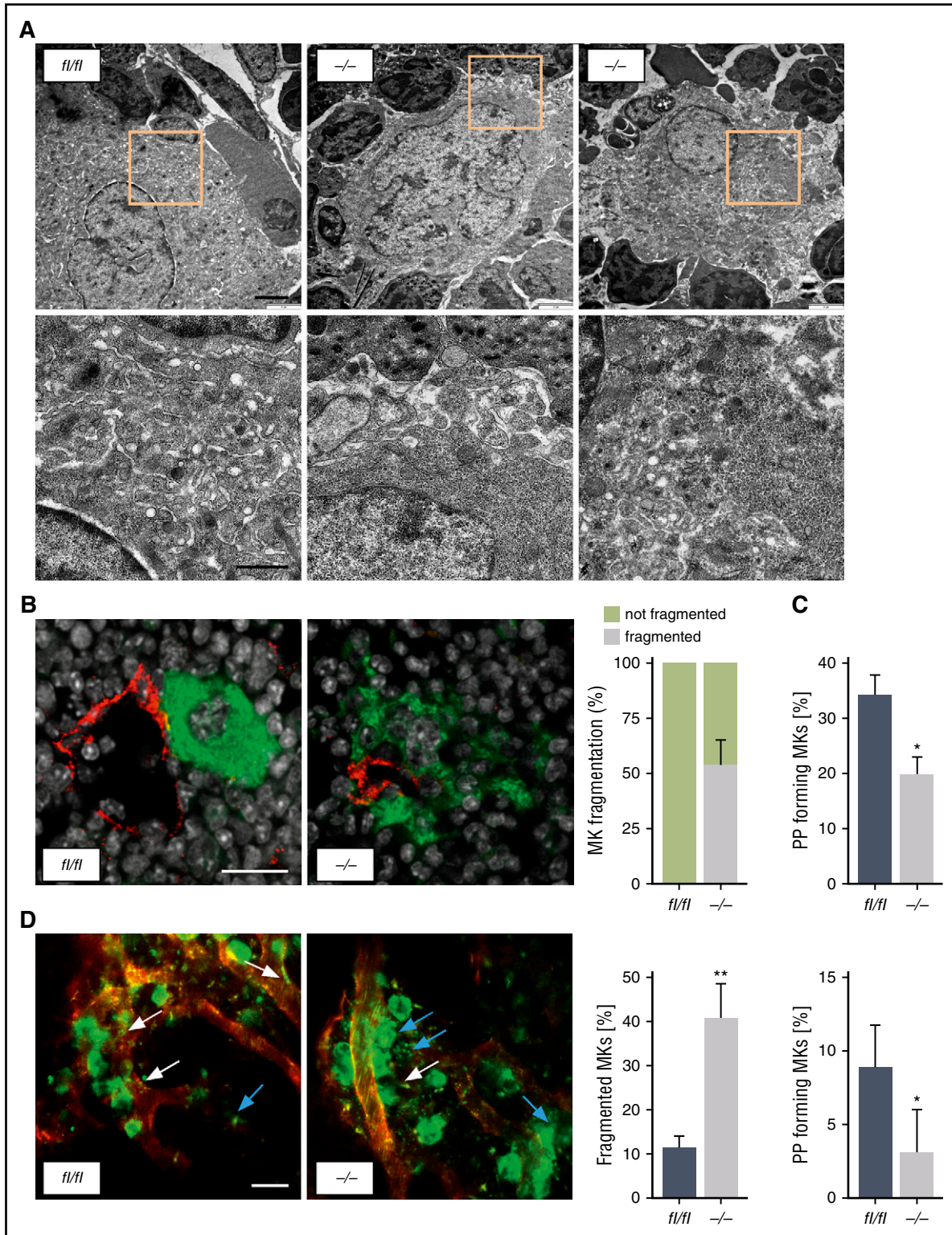
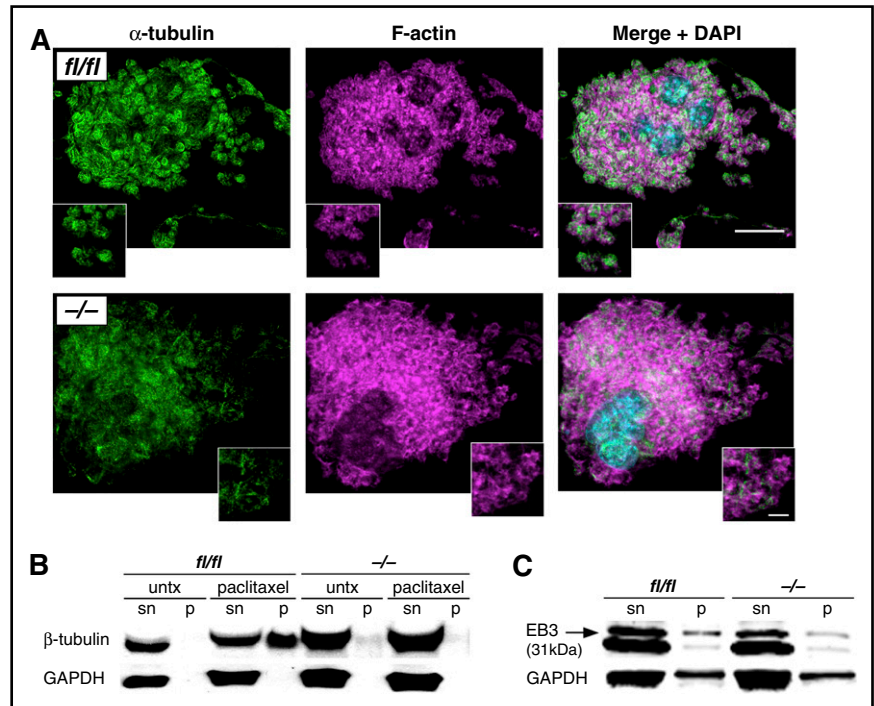


Figure 3. *Ck2b*^{-/-} MKs exhibit abnormal ultrastructure, resulting in premature MK fragmentation in the BM and impaired proplatelet formation. (A) Representative TEM images of *ck2b*^{fl/fl} (left) and *ck2b*^{-/-} (middle and right) MKs in the BM showing increased fragmentation, decreased presence of demarcation membranes, and large granule-free zones on *ck2b* deficiency. Upper, overview (bar represents 3 μ m). Lower, detail (bar represents 1 μ m). (B) Representative confocal microscopy images of immunostained BM sections (left) and arithmetic means \pm standard errors of the means (n = 6; right) of MK fragmentation in the BM of *ck2b*^{fl/fl} and *ck2b*^{-/-} mice (n = 6). Green, MKs (GPIb); red, sinusoids (CD105); gray, nuclei (4',6-diamidino-2-phenylindole). Bar represents 20 μ m. (C) Quantification of the percentage of proplatelet-forming fetal liver cell-derived MKs from *ck2b*^{fl/fl} and *ck2b*^{-/-} mice in vitro on day 4 of culture. *Ck2b*^{fl/fl} and *ck2b*^{-/-} MKs extending proplatelets were counted 18 hours after bovine serum albumin gradient and expressed as percentage of total MKs. Arithmetic means \pm standard errors of the means (n = 10) are shown. (D) 2P-IVM revealing MK instability and reduced proplatelet formation in *ck2b*^{-/-} MKs in vivo. Platelets and MKs were stained with anti-GPIX antibodies (green), and the vessel lumen was labeled using fluorescein isothiocyanate-bovine serum albumin and anti-CD105 antibodies (red). Proplatelet-forming MKs (white arrows indicated proplatelets) were counted, and the ratio per mouse was assessed (n = 5). MK morphology was categorized by a blinded experimenter in normal and fragmented (blue arrows). Representative images from the BM of *ck2b*^{fl/fl} and *ck2b*^{-/-} MKs (left) and arithmetic means \pm standard errors of the means (n = 5; right) of proplatelet-forming MKs and of MKs with altered morphology are shown. Bar represents 50 μ m. Unpaired Student *t* test in panels C and D. **P* < .05; ***P* < .01.

Figure 4. CK2 β is essential for microtubule architecture and tubulin polymerization as well as EB3 binding to polymerized tubulin. (A) Representative immunofluorescence confocal microscopy images of cultured fetal liver–derived *ck2b^{fl/fl}* and *ck2b^{-/-}* MKs. α -Tubulin staining appears fragmented in *Ck2b^{-/-}* MKs undergoing proplatelet formation. Bar represents 20 μ m (overview) and 5 μ m (detail). (B) Representative immunoblots of 3 independent experiments of free and polymerized tubulin in *ck2b^{fl/fl}* and *ck2b^{-/-}* platelets ($n = 3$) in the absence and presence of paclitaxel (10 μ M) in supernatant (sn) and pellet (p). (C) Representative immunoblots of EB3 bound to polymerized tubulin in *ck2b^{fl/fl}* and *ck2b^{-/-}* platelets ($n = 3$) after treatment with paclitaxel (10 μ M) in supernatant (sn) and pellet (p).



production, we investigated whether IP₃ production or activation of PLC is regulated by CK2 β . Neither activation-dependent myo-inositol 1 phosphate production, reflecting the levels of the unstable IP₃, nor PLC phosphorylation were significantly affected in *ck2b^{-/-}* platelets (supplemental Figure 8). However, as revealed by light pulse-triggered activation of caged IP₃, the IP₃-dependent release of Ca²⁺ from intracellular stores was significantly blunted in *ck2b*-deficient platelets (Figure 5C), pointing to CK2 β -sensitive regulation of IP₃R activation on platelet stimulation. The total expression of platelet IP₃R and phosphorylation of IP₃R1, mainly located in the ER of platelets, were comparable (supplemental Figure 9). However, the abundance of IP₃R1 bound to the fraction of polymerized tubulin was significantly diminished in *ck2b^{-/-}* platelets (supplemental Figure 9).

To assess the functional significance of impaired IP₃R activation and the corresponding defective [Ca²⁺]_i increase in *ck2b^{-/-}* platelets, we determined platelet degranulation, integrin $\alpha_{IIb}\beta_3$ activation, and aggregation after platelet stimulation via ITAM-coupled (CRP) or G-protein-coupled (thrombin) receptors. As quantified by flow cytometry and luminescence analysis, the activation-dependent secretion of platelet α and dense-granules after stimulation with low and intermediate concentrations of CRP or thrombin were significantly decreased in *ck2b^{-/-}* platelets (Figure 5D-E). Moreover, integrin $\alpha_{IIb}\beta_3$ activation, which is required for fibrinogen binding, was significantly diminished in *ck2b^{-/-}* platelets after stimulation with CRP or thrombin (Figure 5F). Light transmission aggregometry was used to explore whether impaired degranulation and integrin $\alpha_{IIb}\beta_3$ activation would translate into reduced activation-dependent platelet aggregation. After stimulation with increasing concentrations of CRP or thrombin, aggregation of *ck2b*-null platelets was significantly blunted (Figure 5G). Defective degranulation, integrin $\alpha_{IIb}\beta_3$ activation, and aggregation in *ck2b^{-/-}* platelets were overcome by high agonist concentrations. Interestingly, as illustrated in supplemental Figures 10-14, comparable with our findings in murine *ck2b^{-/-}* platelets, the treatment of mature murine and human platelets with the CK2 inhibitors (E)-3-(2,3,4,5-tetrabromophenyl)acrylic acid (TBCA)

and CX-4945 significantly affected platelet Ca²⁺ influx, secretion, and aggregation in response to CRP.

A further series of experiments elucidated the consequences of impaired activation of *ck2b*-deficient platelets for platelet adhesion to collagen-coated surfaces under flow at high arterial shear rates (1700 s⁻¹). Although *ck2b^{fl/fl}* platelets rapidly adhered to collagen and formed stable thrombi, thrombus formation by *ck2b^{-/-}* platelets was significantly decreased because the thrombus surface coverage was reduced by ~71% (Figure 6A). Because Ca²⁺-dependent platelet activation may contribute to pathological thrombus formation in vivo, we next assessed the time to occlusion of mesenteric arterioles and carotid arteries due to thrombus formation after ferric chloride (FeCl₃)-induced injury in mice with a platelet-specific deletion of CK2 β . Arterial thrombotic occlusion was significantly delayed and decreased in *ck2b^{-/-}* mice after FeCl₃-triggered vascular injury of mesenteric arterioles (Figure 6B). Seventy-five percent of the *ck2b^{-/-}* mice failed to form an occlusive thrombus within 25 minutes, whereas in *ck2b^{fl/fl}* mice, the mean time to thrombotic vascular occlusion was ~14 minutes. *Ck2b^{-/-}* mice were similarly protected from acute arterial thrombosis after FeCl₃-induced injury of carotid arteries. Whereas all *ck2b^{fl/fl}* mice displayed a rapid and complete vascular occlusion, the time to occlusion was significantly prolonged in *ck2b^{-/-}* mice. No discontinuation of blood flow was found in 42% of the *ck2b^{-/-}* mice due to the absence of thrombotic occlusion at the end of the 20-minute observation period (Figure 6C). Real-time imaging of thrombus formation under flow that was achieved by capturing images of fluorescently labeled platelets indicated a consistent and gradual increase in fluorescence intensity from the deposited *ck2b^{fl/fl}* platelets (Figure 6D). With platelets from the *ck2b^{-/-}* mice, the slope of fluorescence increase was diminished, and the time traces pointed to a slow and insufficient thrombus buildup. These findings from both in vitro and in vivo models of arterial thrombosis indicate that CK2 β -mediated activation of IP₃R is required for efficient platelet activation underlying thrombotic vascular occlusion. To test whether the defect in *ck2b^{-/-}* platelets was associated with impaired hemostasis,

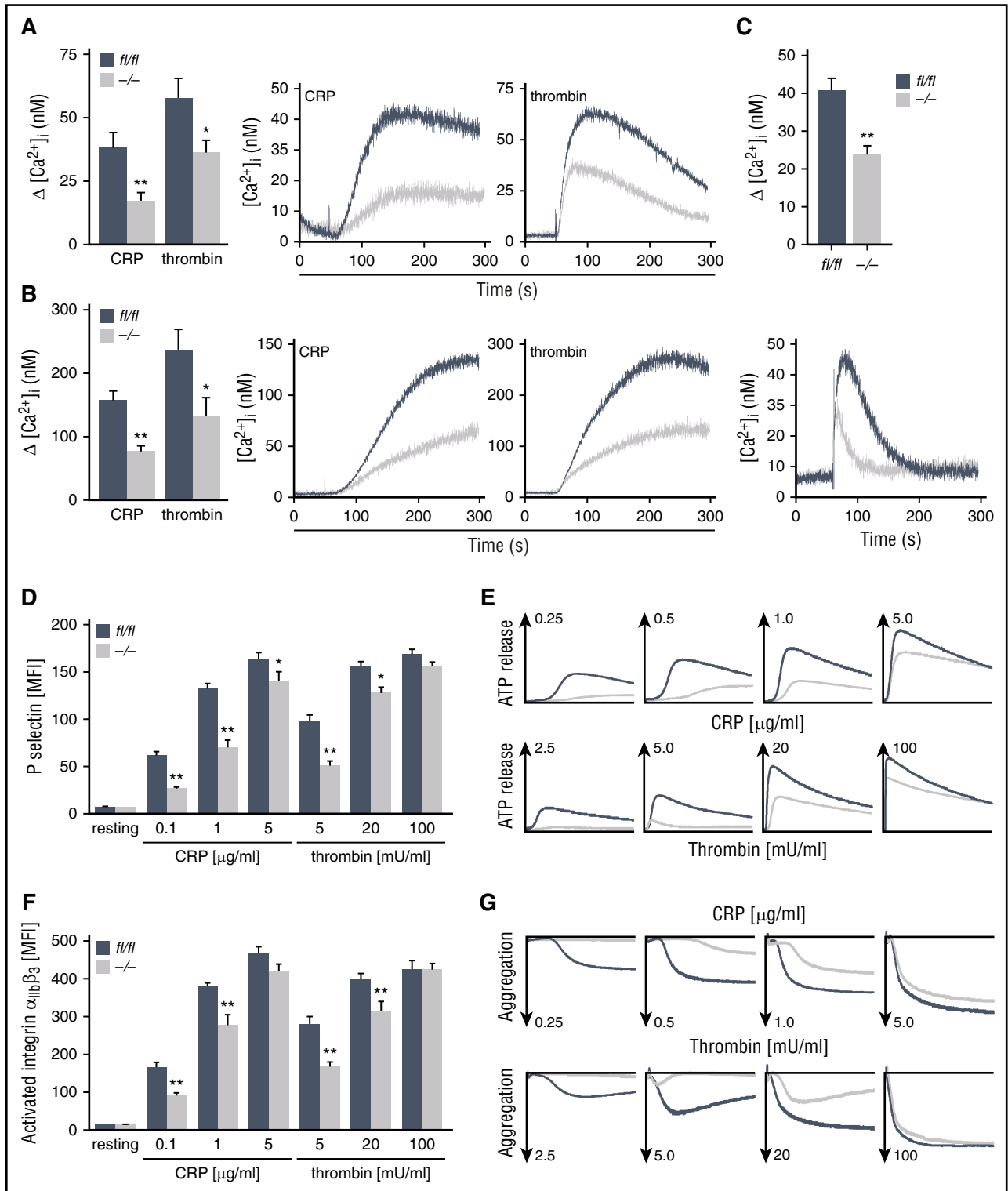


Figure 5. CK2 β is a critical regulator of platelet IP₃-triggered Ca²⁺ release from internal stores with subsequent extracellular Ca²⁺ influx, resulting in impaired granule secretion, integrin $\alpha_{IIb}\beta_3$ activation, and aggregation in *ck2 β -null* platelets. (A) Representative tracings of Fura-2-fluorescence reflecting the cytosolic Ca²⁺ concentration [Ca²⁺]_i (right) and arithmetic means of maximal $\Delta[Ca^{2+}]_i$ \pm standard deviations (n = 6; left) of *ck2 β ^{fl/fl}* (blue line) and *ck2 β ^{-/-}* (gray line) platelets before and after stimulation with CRP (5 μ g/ml) and thrombin (20 mU/ml) in the absence (0.5 mM EGTA) of extracellular Ca²⁺. (B) Representative tracings of Fura-2-fluorescence reflecting the cytosolic Ca²⁺ concentration [Ca²⁺]_i (right) and arithmetic means of maximal $\Delta[Ca^{2+}]_i$ \pm standard deviations (n = 6; left) of *ck2 β ^{fl/fl}* (blue line) and *ck2 β ^{-/-}* (gray line) platelets before and after stimulation with CRP (5 μ g/ml) and thrombin (20 mU/ml) in the presence (1 mM Ca²⁺) of extracellular Ca²⁺. (C) Representative tracings of Fura-2-fluorescence reflecting cytosolic Ca²⁺ concentration [Ca²⁺]_i (lower) and arithmetic means of maximal $\Delta[Ca^{2+}]_i$ \pm standard deviations (n = 6; upper) of *ck2 β ^{fl/fl}* (blue line) and *ck2 β ^{-/-}* (gray line) platelets before and after stimulation with IP₃ (2.5 μ M). The cytosolic Ca²⁺ concentration before and after flash photolysis (365 nm) in the absence of IP₃ was measured as a control (tracing not shown). (D) Flow cytometric analysis of P-selectin exposure reflecting α -granule release in *ck2 β ^{fl/fl}* (blue bars) and *ck2 β ^{-/-}* (gray bars) platelets in response to increasing concentrations of CRP (in micrograms per milliliter) or thrombin (in microunits per milliliter). Arithmetic means \pm standard errors of the means (n = 6) are shown. (E) Luminescence analysis of ATP release reflecting the secretion of dense granules in response to increasing concentrations of CRP

we measured tail bleeding time. As illustrated in Figure 6E, *ck2 β ^{-/-}* mice showed a minor bleeding tendency without a significantly prolonged bleeding time as compared with *ck2 β ^{fl/fl}* mice (Figure 6E).

As a logical extension of our findings that CK2 β can modulate arterial thrombosis, we investigated the impact of platelet CK2 β on responses and outcome after ischemic stroke. Mice with a platelet-specific knockout of *ck2 β* were studied in the tMCAO model. As determined by 2,3,5-triphenyltetrazolium chloride staining (Figure 7A), brain infarct volumes were significantly reduced in *ck2 β ^{-/-}* mice 24 hours after tMCAO. Moreover, the platelet-specific CK2 β deficiency resulted in a significantly decreased Bederson score reflecting the loss of global neurological function and a significantly improved grip test, assessing motor function and coordination. Collectively, these indicators demonstrate that the marked reduction of infarct volumes in *ck2 β ^{-/-}* mice was functionally relevant (Figure 7B). Histological analysis of brain sections revealed significantly less thrombotic-occluded vessels in *ck2 β ^{-/-}* mice (Figure 7D). To confirm the protective effect of a genetic CK2 β knockout on ischemic brain infarct development, magnetic resonance imaging was performed on living mice. As illustrated by serial T2-weighted magnetic resonance imaging, hyperintense ischemic brain infarcts were markedly reduced in *ck2 β ^{-/-}* mice after tMCAO, a protective effect that was sustained over 7 days (Figure 7C-E). *ck2 β ^{-/-}* mice further displayed significantly improved outcomes because CK2 β deficiency resulted in increased survival 7 days after tMCAO. As illustrated by the Kaplan-Maier curve, 53.8% of *ck2 β ^{-/-}* mice survived the 7-day observation period after tMCAO, whereas 82.5% of the *ck2 β ^{fl/fl}* mice died within 7 days after ischemic stroke. Notably, we found no intracranial hemorrhages in *ck2 β ^{-/-}* mice after tMCAO (Figure 7F).

Discussion

The results of the present study disclose a key role for the protein kinase CK2 in the tightly regulated signaling processes in MKs and platelets. CK2 β is required for thrombopoiesis and Ca²⁺-triggered platelet activation and, by the same token, contributes to acute arterial thrombotic occlusion and consecutive ischemic diseases, such as acute myocardial infarction and ischemic stroke, the leading causes of death and permanent disability in industrialized countries.¹⁸

ck2 β ^{-/-} mice suffer from severe macrothrombocytopenia resulting from defective thrombopoiesis due to premature MK fragmentation with prematurely released, enlarged platelets and crippled proplatelet formation, potentially driven by significantly altered tubulin polymerization and impaired microtubule assembly. Similar macrothrombocytopenia was observed in mice lacking other regulators of platelet tubulin organization and microtubule dynamics, such as Filamin A (FlnA)³³ or the GTPases, Rac1/Cdc42.³⁴ Similar to *Rac1/Cdc42*-null platelets or platelets with a genetic ablation of FlnA, *ck2 β ^{-/-}* platelets harbor less vWF receptor complex abundance than *ck2 β ^{fl/fl}* platelets (Table 1). FlnA cross-links actin filaments, connecting vWF receptor GPIb-IX-V to the platelet cytoskeleton, and interacts with Syk, regulating platelet ITAM-mediated receptor signaling and function.^{33,35} Similar to *ck2 β ^{-/-}* platelets, *FlnA*-null platelets are relatively insensitive to activation.³⁵ However, neither FlnA expression (supplemental

Figure 15) nor Syk phosphorylation (supplemental Figure 8C) were significantly affected in CK2 β -deficient platelets. Double knockout of 2 additional major regulators of tubulin organization, Rac1 and Cdc42, resulted in altered MK morphology and uncontrolled MK fragmentation,³⁴ similar to *ck2 β ^{-/-}*. Nevertheless, the activity of Rac1 and Cdc42 was unaffected in *ck2 β ^{-/-}* platelets (supplemental Figure 16), excluding Rac1/Cdc42 as potential downstream targets of CK2 in MKs or platelets.

In vivo visualization of thrombopoiesis in MK/platelet-specific knockout of CK2 β revealed significant alterations in the MK cytoskeleton, resulting in premature ectopic M-fragmentation and severely affected proplatelet formation. During MK maturation, polymerization of microtubules assembled from α - and β -tubulin heterodimers is essential for maintaining reorganization of the MK cytoskeleton underlying proplatelet formation and subsequent platelet production.^{4,5} Recently, tubulin has been reported as a direct target of CK2.^{25,36} Accordingly, CK2 directly binds tubulin heterodimers, thus strongly stabilizing microtubule polymerization.²³ Even the microtubule-stabilizing agent paclitaxel failed to stabilize polymerized tubulin and to prevent depolymerization of β 1-tubulin in *ck2 β ^{-/-}* MKs. β 1-tubulin, restrictively expressed in the MK lineage,³⁷ is the major tubulin isotype in platelets and is indispensable for microtubule assembly and thus platelet production in MKs.⁶ Loss of β 1-tubulin in mice and loss-of-function β 1-tubulin polymorphisms in humans both result in severely impaired proplatelet formation with significant macrothrombocytopenia due to deformed microtubules, premature MK fragmentation, and release of large platelets,^{6,7,37,38} a phenotype virtually identical to the deletion of CK2 β in the MK/platelet lineage reported in this article. Due to the abrogated tubulin polymerization, binding of EB3 to polymerizing microtubules was impaired in *ck2 β ^{-/-}* platelets and MKs. Accordingly, proplatelet maturation and platelet release in MKs may be affected by CK2 β deficiency. However, despite the analogy between loss of β 1-tubulin and CK2 β in the MK/platelet lineage for platelet biogenesis, the phenotype regarding platelet activation is different. As reported recently,³⁷ even if aggregation of β 1-tubulin^{-/-} platelets was blunted after stimulation with a low dose of thrombin, thrombus formation in mice lacking platelet β 1-tubulin was not as strongly impaired as in *ck2 β ^{-/-}* mice, suggesting that CK2 deficiency affects additional mechanisms in platelets that are essential for activation and thrombus formation beyond modulating platelet β 1-tubulin.

ck2 β ^{-/-} mice compensate for defective proplatelet formation and insufficient thrombopoiesis by increasing BM megakaryopoiesis and extramedullary thrombopoiesis. Similar to what was observed in other mice with impaired platelet production as a result of abolished microtubule assembly,³⁹ splenomegaly was only mild in *ck2 β ^{-/-}* mice despite the profound spleen MK hyperplasia. The reticuloendothelial system in the liver and spleen clears platelets from the circulation due to altered cellular morphology³⁴ or platelet apoptosis.^{40,41} In theory, CK2 could suppress apoptosis by the stimulation of PI3K/Akt signaling,⁴² direct interaction with the Bcl-2-associated death promoter (BAD),⁴³ or decreased susceptibility of caspase substrates, such as the BH3 interacting-domain death agonist (BID), to caspase cleavage.^{36,44} However, phosphorylation of Akt and its downstream target, BAD, both critical elements in prosurvival signaling of platelets,⁴⁵ were not significantly affected in *ck2 β ^{-/-}* platelets (supplemental Figure 17).

Figure 5 (continued) (in micrograms per milliliter) or thrombin (in microunits per milliliter). Representative ATP release tracings of *ck2 β ^{fl/fl}* (blue lines) and *ck2 β ^{-/-}* (gray lines) mice are shown (n = 6). (F) Flow cytometric analysis of α _{IIb} β ₃ integrin activation in *ck2 β ^{fl/fl}* (blue bars) and *ck2 β ^{-/-}* (gray bars) platelets in response to increasing concentrations of CRP (in micrograms per milliliter) or thrombin (in microunits per milliliter). Arithmetic means \pm standard errors of the means (n = 6) are shown. (G) Light transmission aggregometry after stimulation with increasing concentrations of CRP (in micrograms per milliliter) or thrombin (in microunits per milliliter). Representative aggregation tracings of *ck2 β ^{fl/fl}* (blue lines) and *ck2 β ^{-/-}* (gray lines) platelets are shown (n = 4). Unpaired Student *t* test in panels A-D. **P* < .05; ***P* < .01.

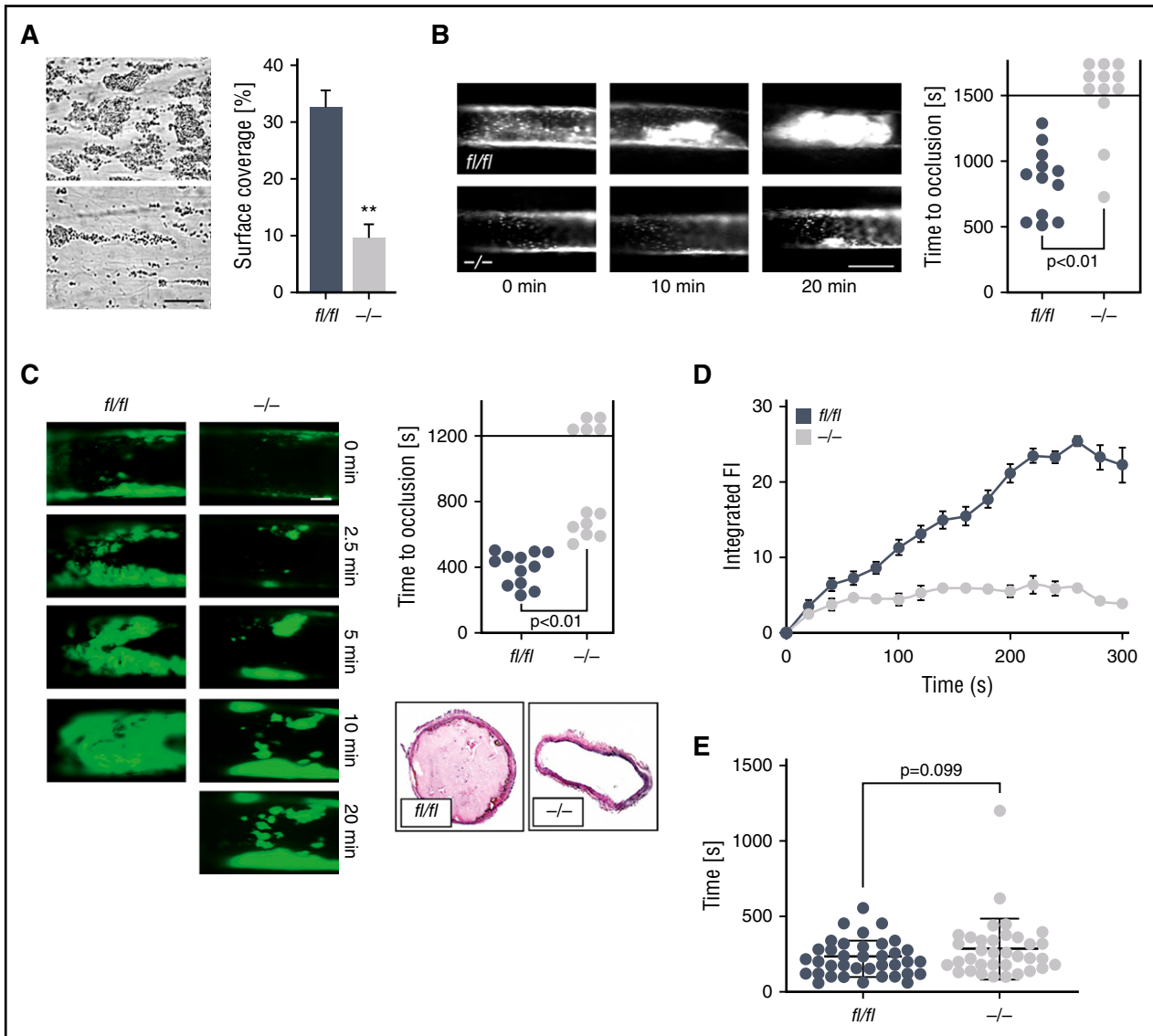


Figure 6. *Ck2β* deficiency results in significantly reduced platelet adhesion and thrombus stability under high arterial shear rates as well as abrogated thrombotic vascular occlusion *in vivo*, whereas primary hemostasis is not significantly affected. (A) Flow chamber analysis of platelet adhesion to collagen and thrombus formation *in vitro* under high arterial shear rates. Whole blood from *ck2β^{fl/fl}* and *ck2β^{-/-}* mice was perfused over a collagen-coated surface for 5 minutes at a shear rate of 1700 s^{-1} . Arithmetic means \pm standard errors of the means ($n = 6$; right) and representative phase contrast images (left) of surface coverage are shown. Bar represents $50 \mu\text{m}$. (B) Time to arterial occlusion after FeCl_3 -induced injury of mesenteric arterioles (right, $n = 12$) and representative images of occlusive *in vivo* thrombus formation after 0, 10, and 20 minutes (left) in *ck2β^{fl/fl}* and *ck2β^{-/-}* mice. Each dot represents 1 individual mouse. Bar represents $50 \mu\text{m}$. (C) Representative images of *in vivo* thrombus formation after 0, 2.5, 5, 10, and 20 minutes (left) and time to arterial occlusion after FeCl_3 -induced injury of carotid arteries (upper right, $n = 12$) and representative immunohistological H&E staining (lower right) of occlusive *in vivo* thrombus formation after 7 minutes in *ck2β^{fl/fl}* and *ck2β^{-/-}* mice. Each dot represents 1 individual mouse. Bar represents $100 \mu\text{m}$. (D) Arithmetic means \pm standard errors of the means ($n = 6$) of integrated fluorescence intensity of adherent platelets after perfusion of whole blood from *ck2β^{fl/fl}* (blue dots) and *ck2β^{-/-}* (gray dots) mice over a collagen-coated surface representing thrombus growth *in vitro* over 5 minutes. (E) Tail bleeding time measured after amputating the tail tip of *ck2β^{fl/fl}* and *ck2β^{-/-}* mice. Each dot represents 1 individual mouse ($n = 36$). Unpaired Student *t* test in panels A-E. ***P* < .01.

Besides the critical role of CK2 in thrombopoiesis, the present study revealed that CK2 is essential for the activation-dependent $[\text{Ca}^{2+}]_i$ increase via the regulation of IP_3 -dependent Ca^{2+} release from internal stores. IP_3 is generated by several PLC isoforms and mediates intracellular Ca^{2+} release by activating IP_3Rs located on the ER.^{13,46} Although platelet activation by collagen and CRP culminates in $\text{PLC}\gamma 2$ -activation downstream of GPVI , stimulation with thrombin activates $\text{PLC}\beta$ via G-protein-coupled signaling.⁴⁷ IP_3R function is presumably regulated in part by the modulation of IP_3R expression, clustering, and phosphorylation.^{8,13} Cyclic nucleotide cyclic adenosine monophosphate (cAMP) mediates its inhibitory effects on platelet activation potentially by phosphorylating IP_3Rs with subsequent

inhibition of intracellular Ca^{2+} release.^{13,46} However, even though CK2 has recently been identified as a negative regulator of cAMP generation,⁴⁸ neither cAMP levels (data not shown) nor phosphorylation of $\text{IP}_3\text{R1}$, mainly expressed in the ER of platelets, were significantly affected in *ck2β^{-/-}* platelets (supplemental Figure 9). Total IP_3R protein abundance was similar in *ck2β^{-/-}* platelets and *ck2β^{fl/fl}* platelets (supplemental Figure 9). The major regulators of IP_3 -induced Ca^{2+} -signaling also includes microtubule-associated EB3, which directly interacts with the S/TxIP motif within IP_3Rs , thus facilitating IP_3R clustering and activation.⁸ Because *ck2β^{-/-}* platelets display blunted tubulin polymerization as well as abrogated binding of EB3 and $\text{IP}_3\text{R1}$ to the fraction of polymerized tubulin, a

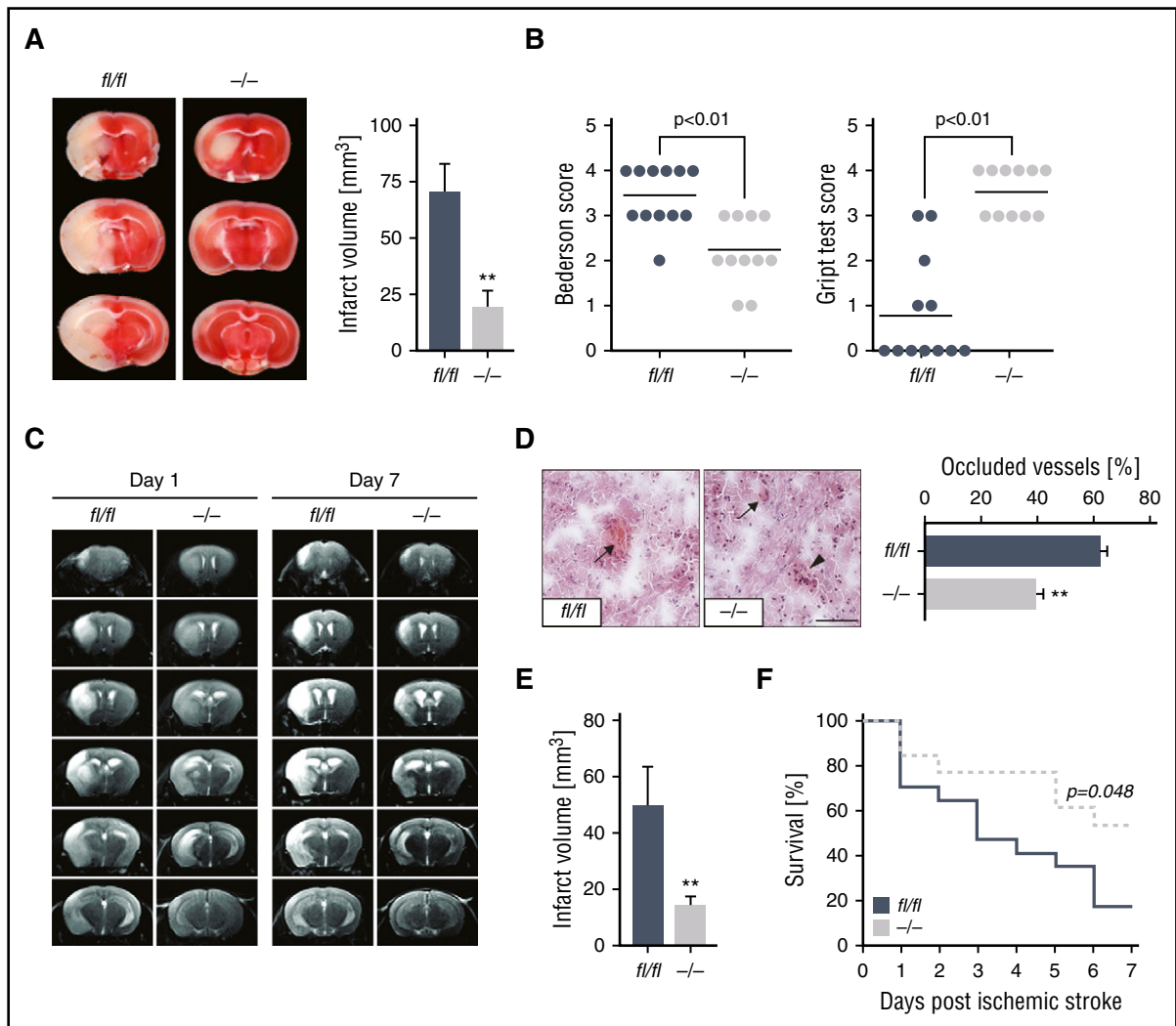


Figure 7. *Ck2 β ^{-/-}* mice are protected from ischemic stroke in vivo and display less thrombotic cerebral vascular occlusions with a significantly better neurological outcome and survival after stroke. (A) Representative images of 3 corresponding coronal sections of 2,3,5-triphenyltetrazolium chloride–stained brains from *ck2 β ^{fl/fl}* and *ck2 β ^{-/-}* mice 24 hours after tMCAO (left). Arithmetic means \pm standard errors of the mean (n = 11–12) of brain infarct volumes in *ck2 β ^{fl/fl}* (blue bar) and *ck2 β ^{-/-}* mice (gray bar) 24 hours after tMCAO (right). (B) Bederson score reflecting global neurological deficits (0 indicates best, 5 indicates worst; left) and grip test indicating motor functional and coordination deficits (0 indicates worst, 5 indicates best; right) assessed 24 hours after tMCAO. Each dot represents 1 individual mouse. (C) Representative coronal T2-weighted magnetic resonance imaging images of hyperintense ischemic brain infarct lesions of *ck2 β ^{fl/fl}* and *ck2 β ^{-/-}* mice at day 1 and day 7 (infarct maturation) after tMCAO (n = 3–7/group). Hypointense areas (reflecting intracerebral hemorrhage) were not observed. (D) Determination of thrombosis index by quantification of thrombotic occluded vs nonoccluded vessels within the ischemic hemisphere 24 hours after tMCAO. Arithmetic means \pm standard errors of the means (n = 5, right) and representative images (left) of H&E-stained cryosections are shown. Bar represents 100 μ m. (E) Arithmetic means \pm standard errors of the means (n = 6) of infarct volume in *ck2 β ^{fl/fl}* and *ck2 β ^{-/-}* mice 7 days after tMCAO (infarct maturation). (F) Analysis of survival of *ck2 β ^{fl/fl}* (blue line) and *ck2 β ^{-/-}* (gray line) mice 7 days after ischemic stroke (n = 13–17). Student *t* test in panels A–D and log-rank (Mantel-Cox) analysis in panel F. ***P* < .01.

disordered IP₃R clustering may contribute to impaired Ca²⁺ release from intracellular stores in *ck2 β ^{-/-}* platelets. These findings are in line with previous studies reporting that the disruption of the cortical microtubule system results in abrogated intracellular Ca²⁺ release.⁴⁹

In platelets, IP₃-mediated Ca²⁺ release from the ER triggers Ca²⁺ entry from the extracellular compartment, potentiating the [Ca²⁺]_i increase and thus facilitating subsequent platelet activation and aggregation with consecutive thrombus formation. Intracellular Ca²⁺ store depletion is sensed by STIM1, which activates store-operated Ca²⁺ entry via the pore-forming Ca²⁺ release-activated channel Orai1.⁴⁷ Similar to *ck2 β ^{-/-}* mice, animals expressing mutant Stim1 displayed macrothrombocytopenia, MK hyperplasia, and decreased platelet life span, pointing to an important role of intracellular Ca²⁺ regulation in MKs/platelets for thrombopoiesis.³² In STIM1-deficient

platelets, the Ca²⁺ response on platelet activation with CRP and thrombin is blunted,⁴⁷ mimicking the phenotype of *ck2 β ^{-/-}* platelets. However, STIM1 expression was similar in *ck2 β -null* platelets and wild-type platelets (supplemental Figure 18).

Although CK2 is readily detected in platelets, its role in platelet function remained ill defined. CK2 is constitutively active in platelets, suggesting that its effects are tightly modulated by phosphatases.³¹ According to pharmacological studies using CK2 inhibitors, CK2 may play a role in platelet activation.^{50–52} However, the precise role of CK2 in platelet function and arterial thrombosis has remained elusive due to the lack of selectivity of the reagents available to target CK2 and its subunits. Pharmacological approaches suggested that CK2 modulates PI3K-Akt-dependent signaling in platelets.^{51,52} In our genetic knockout approach, we did not find evidence for CK2-dependent activation of the PI3K downstream effectors Akt and GSK3 β in platelets

(supplemental Figure 17). We do not have a precise explanation for these apparent discrepancies other than the limited selectivity of the pharmacological CK2 inhibitors used.⁵³⁻⁵⁵ For example the CK inhibitor 5,6-dichloro-1-(β -D-ribo-furanosyl) benzimidazole, used in some of previous studies on platelets, inhibits both CK2 and CK1 isoforms as well as other cellular targets.⁵⁰ In the present study, we were able to define CK2 β as a critical regulator of Ca²⁺-triggered platelet activation using MK/platelet-specific deletion of the CK2 regulatory β -subunit. Similar to our observations in *ck2 β -null* platelets, treatment of murine and human platelets with TBCA and CX-4945 significantly affected platelet Ca²⁺ influx, secretion, and aggregation in response to CRP (supplemental Figure 10-14) without affecting Akt phosphorylation (supplemental Figure 19), indicating that CK2 is crucial for GPVI-dependent signaling without involving Akt-dependent intracellular pathways. Because TBCA and CX-4945 sufficiently blocked platelet Akt phosphorylation after activation with the Par-4 agonist AYPGKF or 2-methylthioadenosine diphosphate (2-MeSADP), we speculate that CK2 may play a role in modifying Akt signaling downstream of PAR-4 or the purinergic receptor P2Y₁₂. However, further studies will be necessary to clarify the role of CK2 in signaling downstream of purinergic receptors, in particular P2Y₁₂. Inhibition of CK2 in thrombin-driven platelet activation did not affect Akt phosphorylation (supplemental Figure 19), pointing to Akt-independent CK2 signaling in response to platelet stimulation with thrombin. Nevertheless, consistent with the findings by Ampof et al,⁵⁶ using the CK2 inhibitor CX-4945, we show for the first time that CK2 β is an essential player in thrombopoiesis and arterial thrombosis in vivo.

According to our observations, activation-dependent Ca²⁺ release is mainly under the control of the constitutively active CK2. As a consequence of the severely impaired Ca²⁺ response, platelet adhesion, secretion, and integrin $\alpha_{IIb}\beta_3$ activation are significantly abolished in *ck2 β ^{-/-}* platelets. The marked knockdown of integrin $\alpha_{IIb}\beta_3$ activation in response to thrombin or CRP in platelets lacking CK2 β paralleled a deficit in the ability of these platelets to undergo aggregation at submaximal agonist concentrations. Increasing the agonist concentrations dissipated the differences between *ck2 β ^{-/-}* and *ck2 β ^{fl/fl}* platelets, indicating that other signaling processes are able to bypass intracellular platelet pathways that are controlled by CK2. The defective activation of *ck2 β ^{-/-}* platelets in vitro translated into a significantly blunted arterial occlusive thrombus formation in vivo, revealing CK2 β to be a potential drug target for antithrombotic therapy, because tail bleeding time, partially reflecting primary hemostasis, was not significantly affected. Platelet-specific deletion of CK2 β in mice resulted in a significant reduction of intravascular thrombosis and a profound protection against ischemic neuronal damage, with significantly improved neurological outcomes and survival 7 days after ischemic stroke. However, it is important to note that the data obtained in the murine tMCAO model cannot be extrapolated to humans without reservations due to possible differences in the underlying pathomechanisms.⁴⁷ Of note, not only does CK2 regulate platelet-triggered thrombus formation, but also a considerable number of proteins involved in the coagulation cascade have been identified as substrates of CK2.⁵⁷ CK2 phosphorylates several coagulation factors, tissue factor

pathway inhibitor, and fibrinogen, suggesting that CK2 may also directly interfere with plasmatic coagulation.^{57,58}

In conclusion, using MK/platelet-specific deletion of the CK2 regulatory β -subunit, we provide strong evidence that CK2 is essential for platelet biogenesis and activation. CK2 is critically required for the regulation of MK maturation and fragmentation, both underlying sufficient proplatelet formation, due to the fact that tubulin polymerization is controlled by CK2. Further, CK2 was identified as a major player in the activation-dependent [Ca²⁺]_i increase by facilitating the release from stores as well as Ca²⁺-dependent platelet activation and arterial thrombus formation in vivo. These findings may have important implications for the development of novel antithrombotic therapies because the absence of CK2 β provides profound protection from occlusive arterial thrombosis and ischemic stroke in vivo with substantially improved outcomes. Further, this study may deepen the understanding of the (patho-)physiological mechanisms underlying proplatelet formation, thrombopoiesis, and the development of macrothrombocytopenia.

Acknowledgments

The authors thank the microscopy platform of the Bioimaging Centre (Rudolf Virchow Centre) for providing technical infrastructure and support. The authors also thank Daniela Eißler for excellent technical assistance.

This work was supported by Deutsche Forschungsgemeinschaft grants BO 3786/1-1 (O.B.), KFO274 (M.G., O.B., and F.L.), SFB688 (D. Stegner and B.N.), the Fortune Research Programme (grants 2133-0 and 1895-0), the German Cardiac Foundation (grant F16/15), and the Dr Karl Kuhn Foundation.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authorship

Contribution: P.M., B.W.-A., S.G., F.L., M.G., and O.B. designed the research; P.M., B.W.-A., S.G., F.L., E.G., D. Stegner, K.A., D. Semeniak, M.C., I.G.M., M.M., and L.Q.-M. conducted the experiments; P.M., B.W.-A., S.G., F.L., E.G., D. Stegner, D. Semeniak, M.C., L.Q.-M., H.R.S., C.K., I.P., H.S., and O.B. analyzed the data; D.W.L., T.B., F.L., B.N., and H.S. provided reagents; and P.M., B.W.-A., D.W.L., F.L., B.N., I.P., H.S., M.G., and O.B. wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Oliver Borst, Department of Cardiology and Cardiovascular Medicine, University of Tübingen, Otfried Müller-Str10, 72076 Tübingen, Germany; e-mail: oliver.borst@med.uni-tuebingen.de.

References

- Patel SR, Hartwig JH, Italiano JE Jr. The biogenesis of platelets from megakaryocyte proplatelets. *J Clin Invest*. 2005;115(12):3348-3354.
- Italiano JE Jr, Lecine P, Shivdasani RA, Hartwig JH. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol*. 1999;147(6):1299-1312.
- Bender M, Thon JN, Ehrlicher AJ, et al. Microtubule sliding drives proplatelet elongation and is dependent on cytoplasmic dynein. *Blood*. 2015;125(5):860-868.
- Thon JN, Montalvo A, Patel-Hett S, et al. Cytoskeletal mechanics of proplatelet maturation and platelet release. *J Cell Biol*. 2010;191(4):861-874.
- Patel SR, Richardson JL, Schulze H, et al. Differential roles of microtubule assembly and sliding in proplatelet formation by

- megakaryocytes. *Blood*. 2005;106(13):4076-4085.
6. Schwer HD, Lecine P, Tiwari S, Italiano JE Jr, Hartwig JH, Shivdasani RA. A lineage-restricted and divergent beta-tubulin isoform is essential for the biogenesis, structure and function of blood platelets. *Curr Biol*. 2001;11(8):579-586.
 7. Kunishima S, Kobayashi R, Itoh TJ, Hamaguchi M, Saito H. Mutation of the beta1-tubulin gene associated with congenital macrothrombocytopenia affecting microtubule assembly. *Blood*. 2009;113(2):458-461.
 8. Geyer M, Huang F, Sun Y, et al. Microtubule-associated protein EB3 regulates IP3 receptor clustering and Ca(2+) signaling in endothelial cells. *Cell Reports*. 2015;12(1):79-89.
 9. Maurer SP, Fourniol FJ, Bohner G, Moores CA, Surrey T. EBs recognize a nucleotide-dependent structural cap at growing microtubule ends. *Cell*. 2012;149(2):371-382.
 10. Akhmanova A, Steinmetz MO. Microtubule +TIPs at a glance. *J Cell Sci*. 2010;123(Pt 20):3415-3419.
 11. Pendin D, McNew JA, Daga A. Balancing ER dynamics: shaping, bending, severing, and mending membranes. *Curr Opin Cell Biol*. 2011;23(4):435-442.
 12. Münzer P, Walker-Allgaier B, Geue S, et al. PDK1 determines collagen-dependent platelet Ca²⁺ signaling and is critical to development of ischemic stroke in vivo. *Arterioscler Thromb Vasc Biol*. 2016;36(8):1507-1516.
 13. El-Daher SS, Patel Y, Siddiqua A, et al. Distinct localization and function of (1,4,5)IP(3) receptor subtypes and the (1,3,4,5)IP(4) receptor GAP1 (IP4BP) in highly purified human platelet membranes. *Blood*. 2000;95(11):3412-3422.
 14. Shuai JW, Jung P. Optimal ion channel clustering for intracellular calcium signaling. *Proc Natl Acad Sci USA*. 2003;100(2):506-510.
 15. Pantazaka E, Taylor CW. Differential distribution, clustering, and lateral diffusion of subtypes of the inositol 1,4,5-trisphosphate receptor. *J Biol Chem*. 2011;286(26):23378-23387.
 16. Bélièveau E, Guillemette G. Microfilament and microtubule assembly is required for the propagation of inositol trisphosphate receptor-induced Ca²⁺ waves in bovine aortic endothelial cells. *J Cell Biochem*. 2009;106(2):344-352.
 17. Taufiq-Ur-Rahman, Skupin A, Falcke M, Taylor CW. Clustering of InsP3 receptors by InsP3 retunes their regulation by InsP3 and Ca²⁺. *Nature*. 2009;458(7238):655-659.
 18. Jackson SP. Arterial thrombosis—insidious, unpredictable and deadly. *Nat Med*. 2011;17(11):1423-1436.
 19. Gawaz M, Langer H, May AE. Platelets in inflammation and atherogenesis. *J Clin Invest*. 2005;115(12):3378-3384.
 20. Borst O, Münzer P, Gatidis S, et al. The inflammatory chemokine CXC motif ligand 16 triggers platelet activation and adhesion via CXC motif receptor 6-dependent phosphatidylinositolide 3-kinase/Akt signaling. *Circ Res*. 2012;111(10):1297-1307.
 21. Borst O, Schmidt EM, Münzer P, et al. The serum- and glucocorticoid-inducible kinase 1 (SGK1) influences platelet calcium signaling and function by regulation of Orai1 expression in megakaryocytes. *Blood*. 2012;119(1):251-261.
 22. Bergmeier W, Oh-Hora M, McCarl CA, Roden RC, Bray PF, Feske S. R93W mutation in Orai1 causes impaired calcium influx in platelets. *Blood*. 2009;113(3):675-678.
 23. Lim AC, Tiu SY, Li Q, Qi RZ. Direct regulation of microtubule dynamics by protein kinase CK2. *J Biol Chem*. 2004;279(6):4433-4439.
 24. Zhang C, Vilk G, Canton DA, Litchfield DW. Phosphorylation regulates the stability of the regulatory CK2beta subunit. *Oncogene*. 2002;21(23):3754-3764.
 25. Canton DA, Litchfield DW. The shape of things to come: an emerging role for protein kinase CK2 in the regulation of cell morphology and the cytoskeleton. *Cell Signal*. 2006;18(3):267-275.
 26. Huillard E, Ziercher L, Blond O, et al. Disruption of CK2beta in embryonic neural stem cells compromises proliferation and oligodendrogenesis in the mouse telencephalon. *Mol Cell Biol*. 2010;30(11):2737-2749.
 27. Buchou T, Vernet M, Blond O, et al. Disruption of the regulatory beta subunit of protein kinase CK2 in mice leads to a cell-autonomous defect and early embryonic lethality. *Mol Cell Biol*. 2003;23(3):908-915.
 28. Piazza F, Manni S, Ruzzene M, Pinna LA, Gurrieri C, Semenzato G. Protein kinase CK2 in hematologic malignancies: reliance on a pivotal cell survival regulator by oncogenic signaling pathways. *Leukemia*. 2012;26(6):1174-1179.
 29. Ulges A, Klein M, Reuter S, et al. Protein kinase CK2 enables regulatory T cells to suppress excessive TH2 responses in vivo. *Nat Immunol*. 2015;16(3):267-275.
 30. Faust M, Schuster N, Montenarh M. Specific binding of protein kinase CK2 catalytic subunits to tubulin. *FEBS Lett*. 1999;462(1-2):51-56.
 31. Hoyt CH, Oh CJ, Beekman JB, Litchfield DW, Lerea KM. Identifying and characterizing casein kinase II in human platelets. *Blood*. 1994;83(12):3517-3523.
 32. Grosse J, Braun A, Varga-Szabo D, et al. An EF hand mutation in Stim1 causes premature platelet activation and bleeding in mice. *J Clin Invest*. 2007;117(11):3540-3550.
 33. Jurak Begonja A, Hoffmeister KM, Hartwig JH, Falet H. FlnA-null megakaryocytes prematurely release large and fragile platelets that circulate poorly. *Blood*. 2011;118(8):2285-2295.
 34. Pleines I, Dütting S, Cherpokova D, et al. Defective tubulin organization and proplatelet formation in murine megakaryocytes lacking Rac1 and Cdc42. *Blood*. 2013;122(18):3178-3187.
 35. Falet H, Pollitt AY, Begonja AJ, et al. A novel interaction between FlnA and Syk regulates platelet ITAM-mediated receptor signaling and function. *J Exp Med*. 2010;207(9):1967-1979.
 36. Olsen BB, Guerra B. Ability of CK2beta to selectively regulate cellular protein kinases. *Mol Cell Biochem*. 2008;316(1-2):115-126.
 37. Italiano JE Jr, Bergmeier W, Tiwari S, et al. Mechanisms and implications of platelet discoid shape. *Blood*. 2003;101(12):4789-4796.
 38. Basciano PA, Matakas J, Pecci A, et al. β -1 tubulin R307H SNP alters microtubule dynamics and affects severity of a hereditary thrombocytopenia. *J Thromb Haemost*. 2015;13(4):651-659.
 39. Kosoff RE, Aslan JE, Kostyak JC, et al. Pak2 restrains endomitosis during megakaryopoiesis and alters cytoskeleton organization. *Blood*. 2015;125(19):2995-3005.
 40. Kile BT. The role of apoptosis in megakaryocytes and platelets. *Br J Haematol*. 2014;165(2):217-226.
 41. Josefsson EC, James C, Henley KJ, et al. Megakaryocytes possess a functional intrinsic apoptosis pathway that must be restrained to survive and produce platelets. *J Exp Med*. 2011;208(10):2017-2031.
 42. Shehata M, Schnabl S, Demirtas D, et al. Reconstitution of PTEN activity by CK2 inhibitors and interference with the PI3-K/Akt cascade counteract the antiapoptotic effect of human stromal cells in chronic lymphocytic leukemia. *Blood*. 2010;116(14):2513-2521.
 43. Klumpp S, Mäurer A, Zhu Y, Aichele D, Pinna LA, Kriegstein J. Protein kinase CK2 phosphorylates BAD at threonine-117. *Neurochem Int*. 2004;45(5):747-752.
 44. Duncan JS, Turowec JP, Duncan KE, et al. A peptide-based target screen implicates the protein kinase CK2 in the global regulation of caspase signaling. *Sci Signal*. 2011;4(172):ra30.
 45. Chatterjee M, Borst O, Walker B, et al. Macrophage migration inhibitory factor limits activation-induced apoptosis of platelets via CXCR7-dependent Akt signaling. *Circ Res*. 2014;115(11):939-949.
 46. Varga-Szabo D, Braun A, Nieswandt B. Calcium signaling in platelets. *J Thromb Haemost*. 2009;7(7):1057-1066.
 47. Varga-Szabo D, Braun A, Kleinschnitz C, et al. The calcium sensor STIM1 is an essential mediator of arterial thrombosis and ischemic brain infarction. *J Exp Med*. 2008;205(7):1583-1591.
 48. Rebholz H, Nishi A, Liebscher S, Nairn AC, Flajolet M, Greengard P. CK2 negatively regulates Galphas signaling. *Proc Natl Acad Sci USA*. 2009;106(33):14096-14101.
 49. Walford T, Musa FI, Harper AG. Nicergoline inhibits human platelet Ca(2+) signalling through triggering a microtubule-dependent reorganization of the platelet ultrastructure. *Br J Pharmacol*. 2016;173(1):234-247.
 50. Nakanishi K, Komada Y, Hayashi T, Suzuki K, Ido M. Protease activated receptor 1 activation of platelet is associated with an increase in protein kinase CK2 activity. *J Thromb Haemost*. 2008;6(6):1046-1048.
 51. Nakanishi K, Toyoda H, Tanaka S, et al. Phosphoinositide 3-kinase induced activation and cytoskeletal translocation of protein kinase CK2 in protease activated receptor 1-stimulated platelets. *Thromb Res*. 2010;126(6):511-516.
 52. Ryu SY, Kim S. Evaluation of CK2 inhibitor (E)-3-(2,3,4,5-tetrabromophenyl)acrylic acid (TBCA) in regulation of platelet function. *Eur J Pharmacol*. 2013;720(1-3):391-400.
 53. Duncan JS, Gyenis L, Lenehan J, et al. An unbiased evaluation of CK2 inhibitors by chemoproteomics: characterization of inhibitor effects on CK2 and identification of novel inhibitor targets. *Mol Cell Proteomics*. 2008;7(6):1077-1088.
 54. Gyenis L, Kuš A, Bretner M, Litchfield DW. Functional proteomics strategy for validation of protein kinase inhibitors reveals new targets for a TBB-derived inhibitor of protein kinase CK2. *J Proteomics*. 2013;81:70-79.
 55. Gyenis L, Turowec JP, Bretner M, Litchfield DW. Chemical proteomics and functional proteomics strategies for protein kinase inhibitor validation and protein kinase substrate identification: applications to protein kinase CK2. *Biochim Biophys Acta*. 2013;1834(7):1352-1358.
 56. Ampofo E, Müller I, Dahmke IN, et al. Role of protein kinase CK2 in the dynamic interaction of platelets, leukocytes and endothelial cells during thrombus formation. *Thromb Res*. 2015;136(5):996-1006.
 57. Singh LS, Kalafatis M. Sequencing of full-length cDNA encoding the alpha and beta subunits of human casein kinase II from human platelets and megakaryocytic cells. Expression of the casein kinase IIalpha intronless gene in a megakaryocytic cell line. *Biochemistry*. 2002;41(28):8935-8940.
 58. Kalafatis M. Identification and partial characterization of factor Va heavy chain kinase from human platelets. *J Biol Chem*. 1998;273(14):8459-8466.