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Thymosin β4 is essential for thrombus formation by controlling the G-actin/F-actin equilibrium in platelets

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DISCLOSURES

The other authors declare no competing financial interests.

KEY POINTS

- Deficiency of thymosin β4 causes macrothrombocytopenia, defective proplatelet formation and impaired platelet activation downstream of GPVI
- Thymosin β4 controls actin dynamics in platelets

ABSTRACT

Coordinated rearrangements of the actin cytoskeleton are pivotal for platelet biogenesis from megakaryocytes (MKs) but also orchestrate key functions of peripheral platelets in hemostasis and thrombosis, such as granule release, the formation of filopodia and lamellipodia, or clot retraction. Along with profilin (Pfn) 1, thymosin β4 (encoded by *Tmsb4x*) is one of the two main G-actin sequestering proteins within cells of higher eukaryotes, and its intracellular concentration is particularly high in cells that rapidly respond to external signals by increased motility, such as platelets. Here, we analyzed constitutive *Tmsb4x* knockout (KO) mice to investigate the functional role of the protein in platelet production and function. Thymosin β4 deficiency resulted in a macrothrombocytopenia with only mildly increased platelet volume and an unaltered platelet life span. MK numbers in the bone marrow (BM) and spleen were unaltered, however, *Tmsb4x* KO MKs showed defective proplatelet formation in vitro and in vivo. Thymosin β4 deficient platelets displayed markedly decreased G-actin levels and concomitantly increased F-actin levels resulting in accelerated spreading on fibrinogen and clot retraction. Moreover, *Tmsb4x* KO platelets showed activation defects and an impaired immunoreceptor tyrosine-based activation motif (ITAM) signaling downstream of the activating collagen receptor glycoprotein (GP) VI. These defects translated into impaired aggregate formation under flow, protection from occlusive arterial thrombus formation in vivo and increased tail bleeding times. In summary, these findings point to a critical role of thymosin β4 for actin dynamics during platelet biogenesis, platelet activation downstream of GPVI and thrombus stability.
INTRODUCTION
Platelets are small anucleate cells circulating in the blood stream that are essential for hemostasis and maintenance of vascular integrity but are also critically involved in thrombosis under pathological conditions. They are derived from giant precursor cells, the megakaryocytes (MKs), residing in the bone marrow (BM). Mature polyploid MKs extend long protrusions, so-called proplatelets, into the sinusoidal vessel lumen, which are shed off by shear forces and further fragment into platelets in the blood stream (1), a process that requires extensive microtubule and actin rearrangements (2, 3). Whereas the actin cytoskeleton is thought to regulate proplatelet branching, microtubule sliding ensures proplatelet elongation (4). In circulating platelets, the actin cytoskeleton is essential to maintain cell morphology and to exert key functions upon activation, such as granule release, as well as the formation of filopodia and lamellipodia (5). The critical role of the actin cytoskeleton for platelet production and function is demonstrated by platelet disorders in humans and mice resulting from defects in proteins regulating actin dynamics (6-8). However, the complex protein network orchestrating actin dynamics in MKs and platelets remains incompletely understood.

β-thymosins are a family of proteins with a molecular weight of approximately 5 kDa that are widely expressed. Of the 15 existing, highly homologous β-thymosins, thymosin β4 is the most abundant isoform (9). Thymosin β4 was first isolated from calf thymus and therefore thought to exert hormone-like activities (10, 11). Indeed, various paracrine effects of the protein have been reported, including cardiac protection, angiogenesis, wound healing and immunomodulatory effects (9, 12-14) although the exact underlying mechanisms have not been fully elucidated.

The intracellular concentration of thymosin β4 is particularly high in cells that rapidly respond to external signals by increased motility such as neutrophils and macrophages or by profound shape changes like platelets (~140,000 copies per platelet in mice and 320,000 copies per platelet in humans) (15-17). For the dynamic spatiotemporal regulation of globular (G)-actin polymerization into filamentous (F)-actin networks, cells rely on a large reservoir of actin monomers sequestered by the actin monomer-binding proteins thymosin β4 and profilin (Pfn) 1 (18). The main intracellular function of thymosin β4 is to bind G-actin, thereby inhibiting actin polymerization (19). Thymosin β4 exclusively binds to actin monomers and not to the filament ends or alongside the filament, and its affinity is 50-fold higher for ATP-than ADP-bound actin monomers maintaining a polymerization-ready pool in reserve (20, 21). In resting cells, thymosin β4 complexes about half of the total G-actin (22). Whereas thymosin β4 prevents actin polymerization, Pfn1 promotes filament assembly (19). Both proteins bind actin monomers transiently with a stoichiometry of 1:1 (23). While Pfn1
catalytically accelerates nucleotide exchange, thymosin β4 strongly inhibits the exchange of
the nucleotide bound to actin monomers by blocking its dissociation (23).

Conditional MK- and platelet-specific Pfn1 KO mice reproduced key features of Wiskott–
Aldrich syndrome (WAS) patients including microthrombocytopenia, due to impaired
proplatelet formation (PPF), cytoskeletal alterations and accelerated platelet clearance (24). Pfn1 KO MKs produced less and smaller-sized platelets into the circulation, which had a
thicker marginal band and a partially disrupted actin cytoskeleton leading to accelerated
integrin inactivation and hence impaired platelet function in vitro and in vivo (25).

As dynamic actin reorganization is crucial for both platelet biogenesis and function (24-26),
we investigated the role of thymosin β4 in these processes by studying Tmsb4x KO mice. Deficiency of thymosin β4 resulted in macrothrombocytopenia and defective proplatelet
formation due to a dysregulated G-to F-actin ratio. Moreover, Tmsb4x KO platelets displayed
impaired ITAM signaling downstream of GPVI, leading to defective aggregate formation
under flow, protection from in vivo thrombus formation, and increased tail bleeding times.
METHODS

Animals
All animal studies were approved by the district government of Lower Franconia (Bezirksregierung Unterfranken). Tmcb4x<sup>-/-</sup> mice were created by MRC mouse network by injection of embryonic stem cell clone Tmcb4x<sup>tm2a(EUCOMM)/Wtsi</sup> into C57Bl/6/129/SvJ blastocysts and afterwards transferred to our animal facility where the KO mice were kept on the mixed background. 8 to 16 weeks-old KO mice and matching WT were used for experiments if not stated otherwise.

Detailed protocols for platelet preparation, determination of platelet lifespan, count and size, aggregometry, flow adhesion assays, actin polymerization, tail bleeding time, IVM models, 2-photon microscopy, spreading assays and clot retraction, as well as MK differentiation and culture, histology, staining procedures and immunoblotting can be found in the Supplemental Materials and Methods.

Data analysis
The presented results are mean ± standard deviation (SD) from at least three independent experiments per group, if not stated otherwise. Data distribution was analyzed using the Shapiro-Wilk-test and differences between control and knockout mice were statistically analyzed using unpaired, two-tailed Student’s t-test, one-way ANOVA or Fisher’s exact test as indicated in the legends. P-values < 0.05 were considered as statistically significant * P<0.05; ** P<0.01; *** P<0.001. Results with a P-value > 0.05 were considered as not significant (ns).
RESULTS

Impaired proplatelet formation and macrothrombocytopenia in thymosin β4 deficient mice

Constitutive Tmsb4x KO mice were born in the expected Mendelian ratios, viable and fertile. The complete absence of thymosin β4 in platelets was confirmed by an automated quantitative capillary-based immunoassay platform, Jess (Figure 1A). Tmsb4x KO mice showed no change in basal blood parameters except for platelet count and size (Supplemental Table 1). Platelet counts were significantly reduced in Tmsb4x KO mice compared to WT control (Figure 1B). Mutant platelets exhibited a small, but significant increase in mean platelet volume (Figure 1C). Transmission electron microscopy (TEM) images revealed that platelet size was quite variable in mutant mice, with platelets comparable to WT size as well as big roundish platelets and platelets with normal shape but increased size (Figure 1D). α- and dense granule numbers were comparable between WT and Tmsb4x KO platelets (Supplemental Figure 1A, B). Notably, platelet lifespan in Tmsb4x KO mice was unaltered as compared to the WT control (Supplemental Figure 2A). Moreover, analysis of hematoxylin and eosin-stained (H&E) femora and spleen sections (Figure 1E, F and Supplemental Figure 2B) as well as cryosections of femora (Supplemental Figure 2C, D) revealed comparable MK numbers in WT and Tmsb4x KO mice. MK ploidy levels in mutant mice were not significantly altered compared to WT animals as assessed by flow cytometric analysis of freshly isolated BM MKs (Supplemental Figure 2E). TEM of BM MKs revealed a slightly altered demarcation membrane system (DMS) morphology with more and smaller invaginations (Supplemental Figure 3).

To elucidate whether the macrothrombocytopenia in Tmsb4x KO mice was caused by impaired platelet biogenesis, we analyzed the ability of BM-derived MKs (27) to form proplatelets in vitro. We found that proplatelet formation (PPF) was significantly reduced in the absence of thymosin β4 (*P<0.05, Figure 1G, H). Microscopic analysis of proplatelet-forming MKs revealed the presence of thickened and shortened proplatelet shafts and tips, suggesting that defective cytoskeletal organization may account for the reduced PPF (Figure 1I). To visualize PPF in vivo, we imaged MKs in the BM of WT and Tmsb4x KO mice by two-photon intravital microscopy of the skull. As shown in Supplemental Video 1, proplatelet-forming MKs in WT mice formed protrusions reaching into the vessel sinusoids that were rapidly shed off by the blood flow (Supplemental Video 1). In marked contrast, large, abnormally thick protrusions were observed in Tmsb4x KO mice (Supplemental Video 2). Interestingly, these aberrant proplatelets dissociated less frequently from the MK and appeared to be more firmly attached to its mother cell (Supplemental Video 2). Notably, we did not observe ectopic release of proplatelet-like particles into the BM, as previously
reported for other proteins involved in actin dynamics such as Pfn1 (24), ADAP (28) or WASP (29) KO mice. This suggests that thymosin β4-deficiency induces pronounced cytoskeletal defects resulting in impaired platelet biogenesis in vivo but not ectopic platelet release into the BM compartment. Actomyosin contractility plays a crucial role for the fragmentation of protrusions from MKs into the blood stream (30). Thus, we assessed myosin levels in MKs and platelets. Although non-muscle myosin IIA (NMIIa) levels were comparable in MKs (Supplemental Figure 4A), Tmsb4x KO platelets displayed significantly decreased NMIIa (Supplemental Figure 4B, C) and myosin light chain 2 (MLC2; Supplemental Figure 4D, E) levels compared to WT. These findings indicate that in the absence of thymosin β4, myosin enrichment during the late stages of MK/proplatelet maturation is affected. This, together with the disturbed actin dynamics, which play a crucial role for proplatelet branching (1), might explain the existence of abnormal, elongated proplatelets without swellings in Tmsb4x KO mice.

**Defective actin dynamics in thymosin β4 deficient platelets**

As thymosin β4 is one of the major G-actin sequestering proteins, we analyzed F-actin content and found that resting Tmsb4x KO platelets exhibited a significant increase in F-actin levels, as assessed by flow cytometry using fluorescently labeled phalloidin (*P<0.05, Figure 2A). Strikingly, Tmsb4x KO platelets were not able to efficiently assemble further F-actin upon activation with different agonists (Figure 2B). Furthermore, by separating the actin cytoskeleton into monomeric and polymeric fractions using ultracentrifugation (24), we observed reduced actin protein levels in the supernatant fraction of Tmsb4x KO platelets indicating lower G-actin levels compared to WT platelets (**P<0.01, Figure 2C, D). In line with these observations, G-actin content was reduced by 50% in Tmsb4x KO platelets spread on fibrinogen as determined by staining with DNase I to label G-actin (31) (**P<0.01, Figure 2E, F).

As Pfn1 might functionally compensate for the loss of thymosin β4, we determined Pfn1 protein levels by Western blotting, and found them unaltered compared to the WT. Moreover, assessment of the activation-dependent phosphorylation of Pfn1 on tyrosine 129, which is known to increase the affinity of Pfn1 towards actin monomers and its actin polymerization activity, revealed an unaltered activity of Pfn1 (Supplemental Figure 5).

**Accelerated spreading and clot retraction of thymosin β4 deficient platelets**

Platelet spreading on fibrinogen is highly dependent on functional cytoskeletal dynamics. Therefore, we next assessed the ability of Tmsb4x KO platelets to form filo- and lamellipodia on a fibrinogen matrix. As shown in Figure 3A-C, we found overall accelerated spreading of
KO platelets, which was most evident at the 15 min time point. We speculate that the formation of protrusions in the KO platelets is facilitated due to the increased F-actin content. We further analyzed the actin filaments of spread platelets on fibrinogen using phalloidin-647 and an anti-tubulin-488 antibody staining (Supplemental Figure 6) or platinum replica electron microscopy (PREM) (Figure 3C, D). Of note, we compared earlier time points of spread Tmsb4x KO with later time points of WT platelets to compensate for the faster spreading.

When comparing WT platelets spread for 15 min with Tmsb4x KO platelet spread for 5 min in the PREM assay (or 30 min WT to 15 min Tmsb4x KO for the staining), we found unaltered F-actin structures. Strikingly, clot retraction of Tmsb4x KO platelets was also enhanced (Figure 4A, B), indicating faster contraction of the actin cytoskeleton, generating forces transmitted to the external fibrin clot, resulting in retraction and fibrin clot shrinkage. To further analyze the dynamics of clot retraction, we have performed immunofluorescence staining of the fibrin meshwork of the clots (32). Clots were analyzed by confocal microscopy and strikingly, as shown in Figure 4C, Tmsb4x KO platelets displayed less and shorter fibrin fibers and they also look less branched. These findings indicate that Tmsb4x KO platelets are able to bend and shorten fibrin fibers faster than WT platelets, which might explain, or at least contribute to, the observed faster clot retraction.

**Defective activation and aggregation of thymosin β4 deficient platelets**

Next, we sought to investigate the effect of thymosin β4 deficiency on platelet activation. Washed platelets were stimulated with various agonists and the activation of αIIbβ3 integrin (JON/A-PE) as well as degranulation-dependent surface exposure of P-selectin was determined. Although expression levels of prominent surface glycoproteins were comparable between mutant and WT platelets (Supplemental Table 2), slightly reduced αIIbβ3 activation was consistently detected in Tmsb4x KO platelets in response to stimulation with ADP, thrombin and the snake venom toxin rhodocytin which activates the hemITAM coupled C-lectin like receptor 2 (CLEC-2), whereas the response to the GPVI-specific agonist, collagen-related peptide (CRP), was strongly reduced (Figure 5A). To exclude pre-activation of Tmsb4x KO platelets, we performed FACS analysis of JON/A-PE binding upon stimulation with epinephrine (Supplemental Figure 7) showing no pre-activation.

P-selectin expression was also reduced in response to CRP, but not in response to other agonists, in Tmsb4x KO platelets, even at high agonist concentrations (Figure 5B). This selective secretion defect was also confirmed when ATP release was determined by luminoaggregometry. In response to thrombin and U46619 (a stable thromboxane analogue), ATP release was slightly increased in Tmsb4x KO platelets compared to WT. In contrast,
ATP release in response to CRP was significantly reduced in the mutant platelets (e.g. 62±2.5 μM in WT vs. 25±4 μM in Tmsb4x KO for 0.1 μg mL−1 CRP, ***P<0.001; Figure 5C).

Tmsb4x KO platelets also exhibited a marked aggregation defect upon thrombin and CRP as well as convulxin (CVX) stimulation, whereas aggregation in response to ADP and U46619 stimulation was unaltered (Figure 5D). Of note, aggregation responses of Tmsb4x KO platelets following stimulation of CLEC-2 were comparable to WT platelets (Supplemental Figure 8). Analysis of GPVI signaling revealed a reduced phosphorylation of the FcRγ chain in the mutant platelets (Figure 5E). Moreover, Jess analysis showed decreased phosphorylation of the tyrosine kinase Syk in mutant platelets, indicating a very early signaling defect downstream of GPVI that explains the in vitro platelet defects (Figure 5F, G). Of note, we observed an increase in total Syk protein levels in Tmsb4x KO platelets using the Jess assay where platelet lysates are centrifuged before immunoblotting, however, when analyzing whole platelet lysates, we could not detect differences in total Syk levels (data not shown).

**Thymosin β4 is required for thrombus formation under flow conditions**

To test the functional consequences of thymosin β4-deficiency under more physiological conditions, we analyzed platelet adhesion and thrombus formation on a collagen-coated surface under flow in a whole-blood perfusion system. Under high (1700 s−1), intermediate (1000 s−1), and low (150 s−1) shear conditions, WT platelets rapidly adhered to the collagen surface and recruited additional platelets from the blood stream resulting in the formation of stable three-dimensional aggregates (Figure 6A, upper panel). In sharp contrast, aggregate formation of Tmsb4x KO platelets was significantly decreased at all tested shear rates (150 s−1, *P<0.05; 1000 s−1, ***P<0.001; 1700 s−1, *P<0.05; Figure 6A-C). To exclude that the decreased aggregate formation of Tmsb4x KO platelets was a result of the lower platelet count in these mice, we adjusted platelet counts to WT level, and, strikingly, thrombus volume and platelet surface coverage were still significantly reduced (1700 s−1, *P<0.05; Figure 6A-C; plt. count adj.). Platelet adhesion and aggregate formation under medium and high shear rates are dependent on the interaction between the mechanoreceptor GPIb and immobilized vWF (33). To investigate a possible involvement of thymosin β4 in GPIb-mediated tethering/ adhesion, we perfused blood from WT and Tmsb4x KO animals over a vWF-coated surface at high shear (1700 s−1). However, the number of adherent Tmsb4x KO platelets on immobilized vWF was comparable to the number of WT platelets (Supplemental Figure 9), suggesting unaltered GPIb-vWF interaction in mutant platelets.
These results demonstrated that thymosin β4 is essential for the formation of stable three-dimensional platelet aggregates on collagen under flow conditions, which appears to be mainly based on a GPVI signaling defect.

**Impaired thrombosis and hemostasis in thymosin β4 deficient mice**

To test whether the observed thrombus formation defect translates into an in vivo phenotype, mice were first subjected to a model of occlusive arterial thrombus formation in the mechanically injured aorta, which has been shown to be partly collagen-driven (34). After a transient increase directly after injury, blood flow progressively decreased for several minutes in all animals. In all WT mice, this decrease resulted in complete and irreversible occlusion of the artery within 7 min after injury (Figure 6D, E). Out of the 14 tested Tmsb4x KO mice, 7 displayed a transient decrease in blood flow, which increased again to normal and led to essentially normal flow rates in the injured vessel at the end of the observation period (30 min; occlusion times: **P<0.01). The other group of KO mice showed a progressive decrease in blood flow, which resulted in full occlusion of the vessel within 8 minutes after injury, almost comparable to WT mice. The protection in the subgroup of the mutant mice was most likely due to embolization, a phenomenon that was also observed in a second thrombosis model. Upon FeCl₃-induced injury of the mesenteric arterioles, Tmsb4x KO mice displayed variable time to occlusion that was overall prolonged and displayed a higher rate of embolization (WT embolization: 3/17 arterioles, KO embolization: 12/19 arterioles analyzed; Supplemental Figure 10, Supplemental Videos 3-4). These results demonstrate that thymosin β4 is essential for stable occlusive arterial thrombus formation in vivo.

To assess the hemostatic function of Tmsb4x KO platelets, we performed a tail bleeding assay. Notably, tail bleeding times were overall significantly increased in KO mice demonstrating that thymosin β4 is also required for normal hemostasis (11.6±6.1 min in KO mice versus 5.4±2.7 min in WT; **P<0.01, Figure 6F).
DISCUSSION

It is already known from studies by others and us that actin-binding proteins have crucial functions during platelet biogenesis and activation (25, 26, 35). The involvement of thymosin β4 in MK/platelet cytoskeletal dynamics, however, has not been assessed to date. Our results reveal that thymosin β4 is required for actin remodeling during PPF by MKs, and in platelets in response to agonist-induced activation. In line with previous studies on cytoskeletal regulators, the deletion of thymosin β4 resulted in a thrombocytopenia with a modest but significant increase in platelet volume, which could be attributed to a defect in proplatelet-formation. However, in clear contrast to Pfn1 deficiency (24), we did not observe premature ectopic release of platelet-like particles in the BM of Tmsb4x KO mice and the lifespan of peripheral platelets was unaltered in these animals. Instead, analysis of Tmsb4x KO MKs revealed abnormal proplatelet shafts and tips, suggesting that defective actin dynamics caused the reduced PPF and thus the observed macrothrombocytopenia. Moreover, we found reduced NMIIa and MLC2 levels in Tmsb4x KO platelets, indicating that during PPF NMIIa recruitment/enrichment is impaired in the absence of thymosin β4. This might contribute to the abnormal appearance of proplatelets as actomyosin contractility plays a crucial role for the process of fragmentation of membrane extensions from MK into the blood flow (30).

Agonist-induced F-actin assembly was significantly reduced in Tmsb4x KO platelets, which was also reported in Pfn1 deficient platelets, although to a lesser extent. This finding was particularly striking as total F-actin levels were increased in resting Tmsb4x KO platelets. Sedimentation of the actin cytoskeleton of Tmsb4x KO platelets revealed a marked reduction in the content of G-actin, which is in line with studies suggesting that thymosin β4 complexes about half of the monomeric actin in resting cells (36). Thus, the robust reduction of the G-actin pool likely accounts for the reduced agonist-induced F-actin assembly in Tmsb4x KO platelets.

Tmsb4x KO platelets also showed accelerated platelet spreading on fibrinogen, which may in part be explained by increased F-actin levels under resting conditions as this process is highly dependent on actin dynamics (37) and the overload of existing actin filaments might facilitate the formation of filo- and lamellipodia. Moreover, clot retraction was significantly enhanced in Tmsb4x KO mice. During blood clot development, platelets interact with fibrin polymers, with contractile force generated internally within the platelet transmitted to the external fibrin clot, resulting in retraction and fibrin clot shrinkage (38). Platelet-mediated contractile forces and hence the level of platelet-mediated clot shrinkage are opposed by the rigidity of the 3D fibrin network (38). In line with this, Tmsb4x KO platelets showed fewer, less branched and shorter fibrin fibers, indicating that
these platelets are able to bend and shorten fibrin fibers faster than WT platelets, which might explain the observed faster clot retraction.

In line with other studies on actin-binding proteins (25, 35), Tmsb4x KO platelets showed significantly impaired \( \alpha IIb\beta3 \) integrin activation and degranulation. We speculate that the increased F-actin content in Tmsb4x KO platelets facilitates shape change but disturbs actin dynamics necessary for integrin inside-out signaling during platelet activation and for clot stability. Moreover, these findings point to a specific role of the G-actin/F-actin ratio and the related actin dynamics in \( \alpha IIb\beta3 \) activation, granule secretion and aggregation. We assume that the increased ATP release results from an enhanced granule mobilization, which might be attributed to the higher F-actin content of Tmsb4x KO platelets. In case of GPVI agonists the defective GPVI signaling ‘overrules’ the accelerated degranulation observed with other agonists. This selective defect in ATP release is responsible for the reduction of integrin activation downstream of GPVI, which may be a consequence of reduced Syk phosphorylation. In line with this, Tmsb4x KO platelets showed significantly reduced FcRγ chain phosphorylation. Therefore, an explanation for the reduced GPVI signaling in Tmsb4x KO platelets might be that lack of thymosin \( \beta 4 \) prevents GPVI receptor clustering (39), as this presents a mechanism for sustained GPVI signaling essential for prolonged platelet activation trough inhibition of GPVI shedding (40-42).

Under flow, defective GPVI signaling leads to impaired aggregate formation on collagen and protection from arterial thrombus formation (43, 44), which was also evident in Tmsb4x KO mice. Grb2-deficient platelets show a GPVI-signaling defect that is slightly more severe than that of Tmsb4x KO platelets and consequently form even smaller platelet aggregates under flow conditions. Interestingly, however, arterial thrombus formation following mechanical injury of the abdominal aorta was only mildly affected by the lack of Grb2 (45). Consequently, the defective GPVI-signaling clearly contributes to the reduced thrombus formation of Tmsb4x KO mice, but other factors are most likely involved. Of note, both FlnA-deficient mouse (46) as well as human (47) platelets show comparable defects in integrin signaling, thrombus formation under flow and GPVI signaling. Here, an interaction between Syk and FlnA was shown to regulate ITAM receptor signaling and platelet function, which could explain the observed defects in FlnA deficient platelets. However, upon thymosin \( \beta 4 \) deficiency, the defects seem more complex, as the reduction in integrin activation is not limited to GPVI signaling and Tmsb4x KO platelets show enhanced integrin outside-in signaling. GPVI signaling is critical for the procoagulant activity of platelets (48) and procoagulant platelets are predominantly localized at the thrombus surface, as a result of their contraction-driven extrusion from the inner core of the thrombus and that such distribution results in surface-enhanced fibrin generation (49). Thus, the reduced platelet
activation in response to GPVI stimulation in Tmsb4x KO platelets, might also affect the fibrin meshwork further destabilizing formed thrombi and thereby enhancing embolization. In addition, to the defective GPVI signaling and enhanced embolization, the accelerated clot retraction is another factor that contribute to the reduced rate of occlusive arterial thrombi observed in Tmsb4x KO mice as compared to WT mice.

Increased tail bleeding times have also been observed in other KO models of actin binding proteins such as Pfn1 (25) and Cot1 (50) KO or Twf1/Cof1 (26) DKO mice. It seems that thrombus stability is reduced in these mouse lines, potentially due to the disturbed actin dynamics. Therefore, we think that the combined defect in the actin regulating function of thymosin β4, the defective GPVI signaling and the reduced thrombus stability led to the increased tail bleeding times.

In summary, we show that thymosin β4 controls the polymerization-ready G-actin pool in the megakaryocytic lineage, which inevitably impacts on MK and platelet function. MKs displayed abnormal proplatelet shafts and tips, and reduced PPF whereas platelets exhibited accelerated spreading and clot retraction, but reduced GPVI-mediated platelet activation. Our findings highlight that the regulation of the G-actin/F-actin ratio by thymosin β4 is not only relevant during platelet biogenesis and activation but is also necessary for the formation of stable thrombi (Supplemental Figure 11). Our findings may thus contribute to a better understanding of the molecular pathways orchestrating actin dynamics in cells.
REFERENCES


FIGURE LEGENDS

Figure 1. Thrombocytopenia and impaired proplatelet formation in thymosin β4 KO mice. (A) Protein levels of thymosin β4 and integrin β1 in WT and Tmsb4x KO platelets were analyzed by an automated quantitative capillary-based immunoassay platform; Jess (ProteinSimple). Platelet count (B) and volume (C) were determined using an automated blood cell analyzer (SciVet). Mean ± SD (n = 4, 3 independent experiments). Unpaired, two-tailed Student’s t-test. ***P<0.001. (D) Representative transmission electron microscopic images of one WT mouse and 3 Tmsb4x KO mice: (2) platelets comparable to WT size, (3) big roundish platelets, (4) platelets with increased size (4) Scale bars: 2 µm. (E, F) Hematoxylin-Eosin stainings of femur paraffin sections of WT and Tmsb4x KO mice (E) and quantification of MK numbers (F). Arrow heads indicate the MKs. Scale bars: 100 µm. Values are mean ± SD (n = 3). (G, H) Proplatelet formation of BM MKs after lineage depletion and culturing in rHirudin- and TPO-conditioned medium. On day 4, proplatelet-forming MKs were counted. Average of 5 analyzed visual fields per MK culture of 3 animals/genotype is shown. Values are mean ± SD. Unpaired, two-tailed Student’s t-test. *P<0.05. (I) Proplatelets were visualized using an α-tubulin antibody and phalloidin and analyzed by confocal microscopy (40x objective, Leica TCS SP8) using a 40x objective. Scale bar 20 µm.

Figure 2. Impaired actin equilibrium and assembly in thymosin β4 KO platelets. (A, B) Relative F-actin content of resting and activated platelets was determined by flow cytometry. Values are mean ± SD of 4 mice per group. The values are displayed as the ratio of MFI from activated and resting platelets. Unpaired Student’s two-tailed Student’s t-test. *P<0.05, **P<0.005, ***P<0.001. (C, D) The actin cytoskeleton was isolated by ultracentrifugation, immunoblotted with an anti-β-actin antibody and analyzed for the content of monomeric vs. filamentous actin using densitometry. GAPDH served as loading control. Values are mean ± SD (n = 3). P: pellet, S: supernatant, T: total protein. Unpaired Student’s two-tailed Student’s t-test. **P<0.005. (E, F) Visualization of the cytoskeleton of spread platelets (15 minutes) on fibrinogen, which were stained with DNase I-AlexaF488 (green) to label G-actin and Phalloidin-atto647N (red) for visualization of F-actin and analyzed by confocal microscopy. Bar, 2 µm. Values are mean ± SD (n = 3). Unpaired, two-tailed Student’s t-test. **P< 0.005.

Figure 3. Accelerated spreading of thymosin β4 KO platelets. (A, B) Washed platelets were stimulated with 0.01 U mL⁻¹ thrombin and allowed to spread (5, 15, 30 min) on fibrinogen (100 µg mL⁻¹). DIC pictures were taken (A) and phase abundance was determined (B). Images are representatives of at least 6 animals per group. Scale bar: 3 µm. (C, D)
Representative images of the platelet cytoskeleton ultrastructure of WT and Tmsb4x KO mice on fibrinogen after 5 min (C) and 15 min (D). Scale bar: 500 nm.

**Figure 4. Accelerated clot retraction of thymosin β4 KO platelets.** (A) Clot retraction of WT and Tmsb4x KO PRP was determined in response to 4 U mL⁻¹ thrombin and monitored over time. (B) Residual volume at the end of the experiment. Values are mean ± SD (n = 6 per group). Unpaired, two-tailed Student’s t-test. *P<0.05. (C) Analysis of the fibrin meshwork of WT and Tmsb4x KO clots. Washed platelets were labelled with an anti-GPIX Alexa 647 derivative and added to a mix of unlabeled fibrinogen (2 mg ml⁻¹) and Alexa Fluor A488-labelled fibrinogen (50 µg ml⁻¹ f.c.). Platelets were stimulated with 0.1 U ml⁻¹ thrombin and clotting was initiated by addition of 5 mM CaCl₂. The mixture was immediately transferred to an uncoated 8 Well Chamber Slide (Ibidi), and allowed to clot. Images were obtained using a Leica SP8 inverted microscope with a 63x oil immersion lens. Optical z-stacks were deconvolved and are shown as maximum projection. Images are representatives of at least 2 z-stacks per mouse and 4 animals per group.

**Figure 5. Altered αIIbβ3 integrin activation, degranulation and aggregation of thymosin β4 KO platelets.** (A, B) Activation of platelet αIIbβ3 integrin (JON/A-PE) (A) and degranulation (α-P-selectin-FITC) (B) in WT and Tmsb4x KO platelets upon stimulation with the indicated agonists was determined by flow cytometry (n = 12). U46, U46619; CRP, collagen-related peptide; Rhd, rhodocytin. Unpaired, two-tailed Student’s t-test. *P<0.05, **P<0.005. (C) Dense granule secretion was assessed by luminometric measurement of released ATP of activated WT and Tmsb4x KO platelets. Results are given as mean ATP concentration [µM] ± SD (n = 12 per group). Unpaired, two-tailed Student’s t-test. *P<0.05, **P<0.005, ***P< 0.001. (D) Aggregation responses of washed platelets or platelet-rich plasma (PRP) in turbidometric aggregometry (n = 6). (E) Western Blot analysis of phosphotyrosine levels in resting and CVX-stimulated WT and Tmsb4x KO platelets using the 4G10 antibody. GAPDH served as loading control. CVX, convulxin; K: Tb4-/-; W: WT. (F, G) Phosphorylation and total protein levels of Syk in resting and CVX-stimulated WT and Tmsb4x KO platelets were analyzed (E) and quantified (F) by an automated quantitative capillary-based immunoassay platform. Values are mean ± SD (n = 3). Unpaired, two-tailed Student’s t-test. *P<0.05, **P<0.005, ***P< 0.001.

**Figure 6. Thymosin β4 is required for thrombus formation and stability in vitro and in vivo.** (A-C) Assessment of platelet adhesion (A, B) and aggregate formation (A, C) on Horm collagen (200 µg mL⁻¹) under flow (150, 1000 and 1700 s⁻¹) in heparinized whole blood or platelet-count adjusted blood of WT and Tmsb4x KO mice. Values are mean ± SD (n = 12).
Scale bar, 50 μm. (D) Representative graph of blood flow of one WT and two Tmsb4x KO mice after mechanical injury of the abdominal aorta. (E) Occlusion times after mechanical injury of the abdominal aorta. Data are mean ± SD of at least 8 mice per group. Fisher’s exact test. **P< 0.005. (F) Tail bleeding times in WT and Tmsb4x KO mice (filter paper method). Each symbol represents one individual. Unpaired, two-tailed Student’s t-test. **P< 0.005.
**Figure A:** Phalloidin-FITC analysis showing the mean fluorescence intensity (MFI) of WT and Tb4/− cells in resting state. 

**Figure B:** Bar graphs representing the effect of ADP, Thrombin, CRP, and RhD on Phalloidin-FITC (active/resting) in WT and Tb4/− cells. The results are presented in MFI units. 

**Figure C:** Western blot analysis of actin and GAPDH in WT and Tb4/− cells. The molecular weight (kDa) is indicated. 

**Figure D:** Bar graph showing the actin content in WT and Tb4/− cells. 

**Figure E:** Fluorescence micrographs showing G-actin and F-actin in WT and Tb4/− cells. 

**Figure F:** Bar graph displaying the DNase I-488 per plt [% of WT] in WT and Tb4/− cells.
Thymosin β4 is essential for thrombus formation by controlling the G-actin/F-actin equilibrium in platelets

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**Running head: Thymosin β4 in platelet biogenesis and function**

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SUPPLEMENTAL METHODS

**Blood parameters**
For assessment of platelet count, size and basic blood parameters, mice were bled into EDTA-coated tubes and undiluted blood was immediately measured at an automated cell counter (ScilVet, scil animal care company GmbH, Germany) (1).

**Platelet preparation**
Mice were bled into heparin (20 U mL\(^{-1}\), Ratiopharm) under isoflurane anesthesia and blood was washed twice using Tyrode-HEPES buffer as described previously (1). Platelet-rich plasma (PRP) was supplemented with 2 μL mL\(^{-1}\) apyrase (0.02 U mL\(^{-1}\); A6410, Sigma-Aldrich) and 5 μL mL\(^{-1}\) PGI\(_2\) (0.1 μg mL\(^{-1}\); P6188, Sigma-Aldrich) and platelets were pelleted by centrifugation for 5 min at 2800 g, washed twice with Tyrode-HEPES buffer (134 mM NaCl, 0.34 mM Na\(_2\)HPO\(_4\), 2.9 mM KCl, 12 mM NaHCO\(_3\), 5 mM HEPES, 5 mM glucose, 0.35% BSA, pH 7.4) containing 2 μL mL\(^{-1}\) apyrase and allowed to rest for 30 min prior to experiments.

**Flow cytometry**
For assessment of platelet activation, 50 μL blood were withdrawn under isoflurane anesthesia, washed twice with Tyrode-HEPES buffer and finally diluted (1:20) in Tyrode-HEPES buffer containing 2 mM Ca\(^{2+}\). Samples were activated with the indicated platelet agonists and concentrations. Activation of αIIbβ3 integrin (JON/A-PE, Emfret, Germany) (2) and P-selectin exposure (WUG 1.9-FITC) were determined using fluorophore-conjugated antibodies (15 min at 37°C). α-granule release and integrin activation were measured after 15 min. Analyses were performed at a FACSCalibur (BD Biosciences) (3).

**Platelet lifespan**
Platelet lifespan was assessed by i.v. injection of a non-cytotoxic Dylight-488-labeled antibody derivative directed against GPIX and measurement of labeled platelets over 5 consecutive days using a FACSCalibur (BD Biosciences).

**Aggregometry**
Washed platelets or PRP (5 x 10\(^5\) platelets per μL) were either supplemented with 100 μg mL\(^{-1}\) fibrinogen (Sigma-Aldrich) or left untreated and light transmission upon activation with the indicated agonists was monitored over time using a four-channel aggregometer (APACT, Laborgeräte und Analysensysteme, Hamburg).
Determination of ATP release

Washed platelets were resuspended to a concentration of $5 \times 10^5$ per µL in Tyrode-HEPES without Ca$^{2+}$. 80 µL of this platelet suspension were diluted into 160 µL Tyrode’s buffer with 2 mM Ca$^{2+}$. After addition of 25 µL Chrono-lume luciferase reagent, agonists were added to the continuously stirred (1,000 rpm) platelet suspension. Light transmission and luminescence were recorded on a 700 Whole Blood/Optical Lumi-Aggregometer (Chrono-log) over 10 min. Results were shown in arbitrary units with buffer representing 100% transmission and washed platelet suspension 0% transmission. ATP release was calculated using an ATP standard and the AggroLink 8 software.

Platelet clot retraction

Mice were bled up to 700 µL in 70 µL sodium citrate (0.129 mM) and PRP was isolated by centrifugation at 1,800 rpm for 5 min. Plasma was collected and platelets were resuspended in 1 mL Ca$^{2+}$-free Tyrode-HEPES supplemented with 2 µL of apyrase (0.02 U mL$^{-1}$) and 5 µL PGI$_2$ (0.1 µg mL$^{-1}$). Platelet count was determined and 7.5 x $10^7$ platelets were resuspended in 250 µL plasma. PRP (3 x $10^5$ platelets per µL) was recalcified by adding 20 mM CaCl$_2$ and supplemented with 1 µL of red blood cells to visualize the clot. Clot formation was initiated by the addition of 4 U mL$^{-1}$ human thrombin (Sigma). Clot formation and retraction was recorded up to 4 h and the residual volume was determined.

Transmission electron microscopy of platelets

Mice were bled into 300 µl heparin (20 U mL$^{-1}$ in TBS). Platelets were washed and fixed using 2.5% glutaraldehyde (16210; Electron Microscopy Sciences) in 50 mM cacodylate buffer (pH 7.2; 1220 AppliChem) containing 2.5 mM MgCl$_2$ and 50 mM KCl. Samples were embedded in Epon 812 (14900, Electron Microscopy Sciences), ultrathin sections were generated and stained with 2% uranyl acetate (22400, Electron Microscopy Sciences) and lead citrate (17800, Electron Microscopy Sciences). Images were acquired on a Zeiss EM900 electron microscope.

Platinum replica electron microscopy.

Washed platelets were allowed to spread on 100 µg/mL fibrinogen-coated coverslips, permeabilized and fixed at the respective timepoint and finally sequentially incubated with 1% glutaraldehyde, 0.1% tannic acid and 0.2% uranyl acetate. Dehydration was performed by transferring samples through graded acetone. Critical point drying was done in a Leica EM CPD300. Samples were finally coated with 1.2 nm of platinum with rotation at 45°C and 3 nm of carbon at 90°C without rotation under high vacuum in a Leica EM ACE600. Replicas were floated, picked up on formvar-carbon-coated grids and examined on a JEOL JEM-2100.
F-Actin assembly
Assessment of F-actin polymerization was performed as described previously (4). Washed platelets were incubated with a DyLight-649-labeled anti-GPIX antibody derivative (20 μg mL⁻¹). Subsequently, platelets either remained resting or were stimulated with the indicated agonists for 2 min. Platelets were fixed with 0.55 volume of 10% paraformaldehyde in PHEM buffer and permeabilized with 0.1 volume 1% Triton™ X100. Subsequently, platelets were stained with 10 μM phalloidin-FITC (P5282, Sigma-Aldrich) for 30 min and immediately analyzed on a FACSCalibur.

F-actin sedimentation
Washed platelets (3 x 10⁵ per μL) in Tyrode-HEPES without Ca²⁺ were lysed by addition of 20 μL 10x PHEM buffer containing 1% Triton X-100, 60 μM taxol, 20 μM phalloidin and protease inhibitors. One set (105 μL) served as whole cell lysate. The other set (105 μL) was separated into a polymerized and a soluble fraction by centrifugation at 56,000 rpm (microtubules) or 75,000 rpm (actin) for 30 min at 37°C in a TLA-100 rotor (Beckman Coulter) (5). Total platelet lysates (T), soluble supernatant (S) and insoluble pellets (P) were supplemented with 2x Loading Dye and subsequently subjected to SDS-PAGE and immunoblotting.

Platelet spreading on fibrinogen
Rectangular coverslips (24 x 50/60 mm) were coated with 100 μg mL⁻¹ human fibrinogen o/N at 4°C in a humid chamber. Slides were blocked with 1% BSA for 1 h at RT. 30-50 μL washed platelets (3 x 10⁵ per μL) were mixed with 50-70 μL Tyrode-HEPES with Ca²⁺, activated with 0.01 U mL⁻¹ thrombin (10602400001, Roche) and immediately allowed to spread on the fibrinogen-coated coverslips. After different time points, the adherent platelets were fixed with 300 μL 4% PFA in PBS for 5 min and differential interference contrast (DIC) microscopy pictures were taken using an inverted microscope Zeiss HBO 100 (Axiovert 200M, Zeiss). For analysis, the phase abundance of the different spreading stages (1, resting; 2, formation of filopodia; 3, formation of filopodia and lamellipodia; 4, fully spread) was determined.

Immunostaining of spread platelets
Coverslips were either coated with Poly-L-Lysine (P8920, Sigma-Aldrich) or fibrinogen (100 μg mL⁻¹; F4883, Sigma-Aldrich) overnight at 4°C. Resting platelets were seeded onto poly-L-lysine-coated slides while the remaining platelets were allowed to spread on fibrinogen in the presence of 0.01 U mL⁻¹ thrombin (10602400001, Roche). At the indicated time points, platelets were fixed and permeabilized in PHEM buffer (60 mM piperazine-N,N-bis-2-ethanesulfonic acid (PIVES), 25 mM N-2-hydroxyethyl-piperazine-N'2-ethanesulfonic acid (HEPES), 10 mM ethylene glycol tetraacetic acid (EGTA), 2 mM MgCl₂, pH 6.9) supplemented
with 4% para-formaldehyde (PFA) and 1% IGEPAL® CA-630 and either analyzed by DIC microscopy at a Zeiss Axiovert 200 inverted microscope or further stained with phallloidin-Atto647N (170 nM, 65906, Fluka) and anti-α-tubulin Alexa F488 (3.33 μg mL⁻¹, 322588 (B-5-1-2), Invitrogen) or DNaseI-488 (0.3 μM, D12371, Thermo Fisher) (6) Samples were mounted with Fluoroshield (F6182, Sigma-Aldrich) and images acquired using a Leica TCS SP5 confocal microscope (Leica Microsystems).

Fibrin formation
Washed platelets (156.000/μl f.c.) were labeled with an anti-GPIX Alexa 647 derivative and added to a mixture of unlabeled fibrinogen (2 mg/ml) and Alexa Fluor A488-labeled fibrinogen (50 μg/ml f.c.). Platelets were stimulated with 0.1 U/ml thrombin and clotting was initiated by addition of 5 mM CaCl₂. The mixture was immediately transferred to an uncoated 8 Well Chamber Slide (Ibidi GmbH, Gräfelfing, Germany), and placed in a dark humidity chamber for 2 h at room temperature to allow clots to form. Images were obtained using a Leica SP8 inverted microscope with a 63x oil immersion lens. Optical z-stacks (15 μm, 0.1 step size, Nyquist conform) were deconvolved (Huygens Essential Software) and are shown as maximum projection (Image J software). Adapted from Campbell et al., Blood 2009 (7).

Immunoblotting
For analysis of platelet signaling, washed platelets (1 x 10⁶ mL⁻¹) were immediately lysed after centrifugation in the respective volume of IP buffer and lysed for 30 min on ice at the indicated time-points. Samples were centrifuged and the supernatant was kept at -80°C until analysis.

Denatured proteins were separated by SDS-PAGE and blotted onto PVDF membranes. Membranes were probed for α-tubulin (1 μg mL⁻¹, T6074, Sigma-Aldrich), P-Pfn1 (1 μg mL⁻¹, PK6930, ECM Biosciences), Pfn1 (1 μg mL⁻¹, PK6930, ECM Biosciences), NMIIa (1 μg mL⁻¹, #3403, Cell Signaling Technology (CST)), phosphotyrosine (clone 4G10, 1 μg mL⁻¹, 05-321, Merck Millipore), GAPDH (1 μg mL⁻¹, G5262, Sigma-Aldrich) and β-actin (1 μg mL⁻¹, #4970, CST) and bound antibodies were detected using horseradish-peroxidase-conjugated secondary antibodies (0.33 μg mL⁻¹) and enhanced chemiluminescence solution (JM-K820-500, MoBiTec). Images were acquired with an Amersham Image 680 (GE Healthcare).

Protein levels of thymosin β4, myosin light chain 2 (MLC2), integrin β1, Syk and phosphorylation state of Syk were assessed with an automated capillary-based immunoassay platform (Jess, ProteinSimple) according to the manufacturers’ recommendations. Separation matrix loading time was set to 200 s stacking matrix loading time to 15 s, sample loading time to 9 s, separation time to 30 min, separation voltage to 375 V, antibody diluent time to 5 min, primary antibody incubation time to 90 min and secondary antibody incubation time to 30 min. For recording of the chemiluminescent signal a High Dynamic Range (HDR) profile was used.
The optimized antibody dilutions were as follows: anti-thymosin β4 (19850-1-AP Proteintech) 1:20, anti-integrin β1 (#34971, CST) 1:10, Syk (#12358, CST) 1:40, Syk p-Tyr525/6 (#2711 CST) 1:10, and MLC2 (#3672, CST) 1:10.

Platelet adhesion on collagen under flow
Mice were bled into heparin and platelets and red blood cells (RBCs) were washed using Tyrode-HEPES or RBC wash buffer (140 mM NaCl, 10 mM HEPES, glucose), respectively. Washed platelets and RBCs were mixed and diluted in Tyrode-HEPES containing 2 mM Ca^{2+} and 250 µg mL^{-1} fibrinogen and platelets were fluorescently labeled for 5 min at 37 °C using a Dylight488-labeled anti-GPIX antibody derivative (0.1 µg mL^{-1}). Reconstituted blood was perfused over collagen I-coated coverslips at a shear rate of 1000 s^{-1} and aggregate formation was monitored using a Leica DMI600 (63x objective). Analysis was done using ImageJ software (National Institute of Health, USA).

Histology
3 µm sections of paraformaldehyde-fixed and paraffin-embedded femora or spleens were stained with hematoxylin (MHS32, Sigma-Aldrich) and eosin (318906, Sigma-Aldrich). MK numbers were counted at a Leica DMI 4000 B microscope.

Immunofluorescence staining of whole femora cryosections
Femora and spleen of mice were isolated, fixed with 4% PFA (A3813, AppliChem) and 5 mM sucrose (S0389, Sigma-Aldrich), transferred into 10% sucrose in PBS and dehydrated using a graded sucrose series. Subsequently, the samples were embedded in Cryo-Gel (39475237, Leica Biosystems) and shock frozen in liquid nitrogen. Frozen samples were stored at -80°C. Cryosections were prepared using the CryoJane tape transfer system (Leica Biosystems) and probed with Alexa488-conjugated anti-GPIb antibodies (13G12, 7A9, 1.33 mg mL^{-1}), to specifically label platelets and MKs, and Alexa647-conjugated anti-CD105 antibodies (3.33 mg mL^{-1}, 120402 (MJ7/18), Biolegend) to stain the endothelium. Nuclei were stained using DAPI (40,6-diamidino-2-phenylindole; 1 mg mL^{-1}, D1306, Invitrogen). Femora were visualized using a Leica TCS SP8 confocal microscope (Leica Microsystems).
Megakaryocyte differentiation for spreading assays and staining

Bone marrow (BM) from femora was flushed (8), passed through a cell strainer and cultured for 2 days in StemPro Medium (Gibco) containing 50 ng mL\(^{-1}\) stem cell factor (SCF) (R&D Systems). On day 2, medium was changed to StemPro containing 50 ng mL\(^{-1}\) SCF and 50 ng mL\(^{-1}\) thrombopoietin (TPO). On day 4, medium was again changed to StemPro containing only TPO. On day 5, the megakaryocytic fraction was isolated by a BSA density gradient. Cells were cultured for another day in StemPro containing 50 ng mL\(^{-1}\) TPO.

For adhesion experiments, coverslips were coated with fibrinogen (100 µg mL\(^{-1}\), F4883, Sigma-Aldrich) for 3 h at 37°C. After 30 min of incubation at 37°C, cells were centrifuged and resuspended in StemPro medium containing 50 ng mL\(^{-1}\) TPO. MKs were then seeded onto coated coverslips and adhesion was allowed for 3 h. Cells were washed with PBS, fixed and permeabilized for 30 min in 4% PFA in PBS containing 1% NP-40. MKs were stained overnight using anti-α-tubulin Alexa F488 (3.33 mg mL\(^{-1}\), 322588 (B-5-1-2), Invitrogen) and phalloidin-Atto647N (170 nM, 65906, Fluka) antibodies. MKs were washed with PBS the following day and slides were mounted with DAPI-containing Fluoroshield (1 μg mL\(^{-1}\), D1306, Invitrogen). Samples were observed at a Leica TCS SP8.

PPF of BM MKs

Male and female mice were anaesthetized and a 1 cm incision was made along the midline to expose the frontoparietal skull, while carefully avoiding damage to the bone tissue. The mouse was placed on a customized metal stage equipped with a stereotactic holder to immobilize its head (9). BM vasculature was visualized by injection of BSA-Alexa Fluor 546 and anti-CD105-Alexa Fluor 546 (6 µg and 0.6 µg per gram bodyweight, respectively). Platelets and MKs were antibody stained (0.6 µg per gram body weight anti-GPIX-Alexa Fluor 488). Images were acquired with a fluorescence microscope equipped with a 20x water objective with a numerical aperture of 0.95 and a TriM Scope II multiphoton system (LaVision BioTec), controlled by ImSpector Pro-V380 software (LaVision BioTec). Emission was detected with HQ535/50-nm and ET605/70-nm filters. A tunable broad-band Ti:Sa laser (Chameleon, Coherent) was used at 760 nm to capture Alexa Fluor 488 and Alexa Fluor 546 fluorescence. ImageJ software (NIH) was used to generate movies.

Transmission electron microscopy of BM MKs

Bones were isolated, cut into 3 mm long pieces and fixed in Karnovsky fixative (2% PFA, 2.5% glutaraldehyde in 0.1 M cacodylate buffer) overnight at 4°C. Subsequently, bones were decalcified using 10% EDTA/ PBS over 5 consecutive days. Afterwards, fatty components of the samples were fixed with 2% osmium tetroxide in 50 mM sodium cacodylate (pH 7.2), stained with 0.5% aqueous uranyl acetate, dehydrated with a graded ethanol series and
embedded in Epon 812. Ultra-thin sections were stained with 2% uranyl acetate (in 100% ethanol) followed by lead citrate. Images were acquired on a Zeiss EM900 TEM.

**Determination of MK ploidy**

To determine BM MK ploidy, both femora were isolated, and the BM was flushed and homogenized. Unspecific binding sites were blocked by incubation of the cell suspension with 0.02 mg mL$^{-1}$ anti-FcgR antibody (553,142 (2.4G2), BD Pharmingen). Afterwards, MKs were stained using a fluorescein isothiocyanate-conjugated anti-GPIIb antibody (10 mg mL$^{-1}$, clone MWReg30). Finally, the cells were fixed, permeabilized and the DNA was stained using 50 mg mL$^{-1}$ propidium iodide (P3561, Invitrogen) staining solution with 100 mg mL$^{-1}$ RNaseA (EN0202, Fermentas) in PBS. Analysis was performed by flow cytometry and FlowJo software (Tree Star Inc., Ashland, USA).

**Two-photon intravital microscopy of the BM**

Male and female mice were anaesthetized and a 1 cm incision was made along the midline to expose the frontoparietal skull, while carefully avoiding damage to the bone tissue. The mouse was placed on a customized metal stage equipped with a stereotactic holder to immobilize its head (9). BM vasculature was visualized by injection of BSA-FITC and anti-CD105-Alexa Fluor 488 (100 µg and 20 µg, respectively). Platelets and MKs were antibody stained (0.6 mg per gram body weight anti-GPIX-Alexa Fluor 546). Images were acquired with a fluorescence microscope equipped with a 20x water objective with a numerical aperture of 0.95 and a TriM Scope II multiphoton system (LaVision BioTec), controlled by ImSpector Pro-V380 software (LaVision BioTec). Emission was detected with HQ535/50-nm and ET605/70-nm filters. A tunable broad-band Ti:Sa laser (Chameleon, Coherent) was used at 760 nm to capture FITC/Alexa Fluor 488 and Alexa Fluor 546 fluorescence. ImageJ software (NIH) was used to generate movies.

**Tail bleeding time**

Mice were anaesthetized and a 2 mm segment of the tail tip was removed using a scalpel. Tail bleeding was monitored by gently absorbing blood on a filter paper at 20 s intervals without touching the wound site. Bleeding was determined to have ceased when no blood was observed on the paper. Experiments were stopped after 20 min by cauterization. Differences between the mean bleeding times were statistically assessed using unpaired Student’s t-test and differences between occluded and non-occluded vessels were determined by Fisher’s exact test.
Aorta injury model
The abdominal cavity of anaesthetized mice was opened to expose the abdominal aorta. An ultrasonic flow probe (0.5PSB699; Transonic Systems, USA) was placed around the abdominal aorta, and thrombus formation was induced by a single firm compression with a forceps upstream of the flow probe. Blood flow was monitored for 30 minutes or until vessel occlusion occurred (blood flow stopped for > 5 minutes). Differences between occluded and non-occluded vessels were statistically assessed using the Fisher’s t-test.

FeCl₃-induced injury of mesenteric arterioles
The mesentery of 3- to 4-week old anaesthetized mice was exteriorized by a midline abdominal incision and endothelial damage in mesenteric arterioles was induced by topical application of a filter paper soaked with 20% FeCl₃. Arterioles were visualized using a Zeiss Axiovert 200 inverted microscope equipped with a 100-W HBO fluorescent lamp source and a CoolSNAP-EZ camera (Visitron). Digital images were recorded and analyzed using the Metavue software. Adhesion and aggregation of fluorescently labeled platelets (Dylight488-conjugated anti-GPIX derivative) was monitored until complete occlusion occurred (blood flow stopped for > 1 min).
SUPPLEMENTAL REFERENCES

**SUPPLEMENTAL TABLE**

**Supplemental Table 1. Basic blood parameters of WT and Tmsb4x KO platelets.** For assessment of platelet count, size and basic blood parameters, mice were bled into EDTA-coated tubes and undiluted blood was immediately measured at an automated cell counter. Results are given as mean ± SD of at least 4 mice per group (3 independent experiments).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>Tmsb4x KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets [nl-1]</td>
<td>920±125</td>
<td>595±44 ***</td>
</tr>
<tr>
<td>Mean platelet volume [fl]</td>
<td>5.3±0.1</td>
<td>5.8±0.2 *</td>
</tr>
<tr>
<td>Red blood cells [pl-1]</td>
<td>9±0</td>
<td>9±1      ns</td>
</tr>
<tr>
<td>Hemoglobin [g dl-1]</td>
<td>15±1</td>
<td>15±1      ns</td>
</tr>
<tr>
<td>Hematocrit [%]</td>
<td>44±3</td>
<td>43±4      ns</td>
</tr>
<tr>
<td>Mean red blood cell volume [fl]</td>
<td>50±2</td>
<td>50±1      ns</td>
</tr>
<tr>
<td>WBC</td>
<td>5±3</td>
<td>3±1      ns</td>
</tr>
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</table>
**Supplemental Table 2. Platelet surface glycoprotein expression in WT and Tmsb4x KO platelets.** For assessment of platelet glycoprotein expression, diluted whole blood was stained with fluorophore-labeled antibodies and analyzed on a FACSCalibur (Becton Dickinson, Heidelberg). Platelets were gated by FSC/SSC characteristics. Results are given as MFI ± SD of at least 4 mice per group (3 independent experiments).

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>WT</th>
<th>Tb4&lt;sup&gt;+&lt;/sup&gt;</th>
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</tr>
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<tr>
<td>GPIb</td>
<td>276±11</td>
<td>260±21</td>
<td>ns</td>
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<tr>
<td>GPV</td>
<td>155±11</td>
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<td>GPIIX</td>
<td>263±29</td>
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<td>544±56</td>
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<td>GPVI</td>
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<tr>
<td>αIIbβ3</td>
<td>270±23</td>
<td>283±6</td>
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Supplemental Figure 1. Unaltered granule content in Tmsb4x KO mice. Analysis of α-granule (A) and dense granule (B) numbers in WT and Tmsb4x KO platelets from transmission electron microscopic images. Values are mean ± SD of at least 80 platelets per genotype.
Supplemental Figure 2. Unaltered platelet lifespan, MK number and ploidy in Tmsb4x KO mice. (A) Platelet lifespan was assessed by flow cytometric measurement of the fluorescence-positive platelet population at the indicated time points after injection of a fluorophore-conjugated anti-GPIX antibody derivative (n = 6). (B) Hematoxylin-Eosin stainings of spleen paraffin sections of WT and Tmsb4x KO mice and (C) quantification of MK numbers. Arrow heads indicate the MKs. Values are mean ± SD (n = 3). (D) Confocal fluorescence microscopic images of femora cryo-sections of WT and Tmsb4x KO mice (Leica TCS SP5). Scale bars: 50 µm. MKs, proplatelets and platelets are shown by GPIb staining in green. CD105 staining (red) labels vessels. Nuclei were counterstained using DAPI (blue). (E) Flushed BM MKs were stained with a megakaryocyte-specific antibody (anti-αIIbβ3 integrin) and DNA was labeled using propidium iodide. DNA distribution was determined by flow cytometric analysis at a FACSCalibur (BD Biosciences) (n = 6). Values are mean ± SD.
Supplemental Figure 3. Altered MK morphology in Tmsb4x KO mice. BM MKs from WT and Tmsb4x KO mice were analyzed by transmission electron microscopy. Scale bars: 1 µm. Representative images of at least 4 animals per group.
Supplemental Figure 4. Reduced non-muscle myosin IIA (NMIIa) and MLC2 levels in thymosin β4-deficient platelets. MKs (A) and platelets (B) were left untreated, lysed, and processed for immunoblotting. Total NMIIa was probed with the respective antibody and analyzed by densitometry (C). GAPDH served as loading control. Values are mean ± SD (n = 3) and given as relative expression compared to WT levels. Unpaired, two-tailed Student’s t-test. **P< 0.005. (D, E) Protein levels of MLC2 in WT and Tmsb4x KO platelets were analyzed (D) and quantified (E) by an automated quantitative capillary-based immunoassay platform. Integrin β1 served as loading control. Values are mean ± SD (n = 3). Unpaired, two-tailed Student’s t-test. ***P< 0.001.
Supplemental Figure 5. Loss of thymosin β4 does not affect the expression levels of Profilin 1. Platelets were left untreated, lysed, and processed for immunoblotting. Phosphorylated Pfn1 and total Pfn1 were probed with the respective antibodies. GAPDH served as loading control.
Supplemental Figure 6. No major changes in the F-actin structure in Tmsb4x KO platelets. Confocal fluorescence microscopy images of resting (poly-L-lysine) and spread platelets (15 and 30 min) on fibrinogen (100 μg mL⁻¹) immunostained for F-actin (red) and α-tubulin (green). Scale bar: 3 μm. Images were acquired with a TCS SP5 confocal microscope (100x/1.4 oil STED WHITE objective, Leica Microsystems) and are representative of at least 3 mice per group.
Supplemental Figure 7. Tmsb4x KO platelets are not pre-activated. Activation of platelet αIIbβ3 integrin (JON/A-PE) in WT and Tmsb4x KO platelets upon stimulation with the indicated agonists was determined by flow cytometry (n = 4). U46, U46619; Epi, epinephrine. Unpaired, two-tailed Student's t-test. *P<0.05, **P<0.005.
Supplemental Figure 8. Unaltered aggregation of thymosin β4 KO platelets in response to CLEC-2 stimulation. Aggregation responses of washed platelets in turbidometric aggregometry in response to the anti-CLEC-2 antibody INU1 (10), or Rhd (rhodocytin). Displayed are representative traces of 6 mice per group.
Supplemental Figure 9. Tmsb4x KO platelets adhere to vWF under flow. (A, B) Heparinized whole blood of WT and Tmsb4x KO mice was perfused over a vWF-coated cover slip for 4 min at a shear rate of 1700 s\(^{-1}\). (A) Representative phase contrast images taken at the end of the perfusion time and (B) analysis of the number of adherent platelets per mm\(^2\) ± SD was performed. Images were acquired with a Zeiss Axiovert 200 inverted microscope (40x/0.6 oil objective). Images are representative of at least 12 animals per group. Scale bar: 50 μm. Unpaired, two-tailed Student’s t-test.
Supplemental Figure 10. FeCl₃-induced arterial thrombosis. (A, B) Adhesion of platelets (A) and occlusion of mesenteric arterioles (B) in WT and Tmsb4x KO mice upon FeCl₃-induced injury of the endothelial barrier. Each symbol represents 1 mesenteric arteriole (n = 9 mice per genotype).
Supplemental Figure 11. Thymosin β4 is essential for platelet formation and function. Loss of thymosin β4 results in defective proplatelet formation in vitro and in vivo leading to a macrothrombocytopenia with only mildly increased platelet volume and an unaltered platelet life span. Thymosin β4 deficient platelets display markedly decreased G-actin levels and concomitantly increased F-actin levels resulting in accelerated spreading on fibrinogen. Moreover, Thymosin β4 deficient platelets show enhanced clot retraction, activation defects and an impaired immunoreceptor tyrosine-based activation motif (ITAM) signaling downstream of the activating collagen receptor glycoprotein (GP) VI. Together, these defects translate into impaired aggregate formation under flow, protection from occlusive arterial thrombus formation in vivo and increased tail bleeding times.
Supplemental Video 1. Rapid proplatelet formation in WT mice. Intravital two-photon microscopy of BM MKs in the skull of a control animal. MKs were labeled using an anti-GPIX antibody derivative coupled to Alexa Fluor 488. Vessels were visualized using BSA-Alexa Fluor 546 as well as an anti-CD105 antibody coupled to Alexa Fluor 546. (20x objective; Frame: 5450.52 ms; 1017x1017 pixel). Scale bar: 25 µm.

Supplemental Video 2. Thickened proplatelet protrusions in Tmsb4x KO mice. Altered proplatelet morphology of BM MKs in the skull of Tmsb4x KO mice as visualized by two-photon microscopy. MKs and vessels were stained as described above. (20x objective; Frame: 5568.42 ms; 1039x1039 pixel). Scale bar: 25 µm.

Supplemental Video 3. FeCl₃-induced arterial thrombus formation in WT mice. Intravital microscopy of arterial occlusive thrombus formation after FeCl₃-induced injury of a mesenteric arteriole. Platelets were labeled using an anti-GPIX antibody derivative coupled to Dylight488. (10x objective; Zeiss Axiovert 200 inverted microscope equipped with a 100-W HBO fluorescent lamp source and a CoolSNAP-EZ camera). Images were acquired every second.

Supplemental Video 4. FeCl₃-induced arterial thrombus formation in Tmsb4x KO mice. Intravital microscopy of arterial thrombus formation and embolization after FeCl₃-induced injury of a mesenteric arteriole. Platelets were labeled using an anti-GPIX antibody derivative coupled to Dylight488. (10x objective; Zeiss Axiovert 200 inverted microscope equipped with a 100-W HBO fluorescent lamp source and a CoolSNAP-EZ camera). Images were acquired every second.