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Marginal band microtubules are acetylated by α TAT1

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Keywords

Tubulin, microtubules, acetylation, post-translational modification, acetyltransferase

Abstract

The discoid shape of resting platelets is maintained by a peripheral, circular bundle of microtubules called marginal band. Marginal band microtubules are acetylated on lysine 40 of the α -tubulin subunits. We have previously shown that the deacetylase HDAC6 is responsible for tubulin deacetylation in platelets and that the hyperacetylated state of the microtubules in *HDAC6KO* platelets correlates with faster activation/spreading kinetics, pointing to a regulatory role of this modification. So far, the question about the reverse enzyme, responsible for tubulin acetylation in platelets, has remained unanswered. Several enzymes have been described as having tubulin acetylation activity. Here we identify α TAT1 as the enzyme responsible for the acetylation of marginal band microtubules. We show that α TAT1 deficiency has only minor consequences for platelet production and function. A residual tubulin acetylation level in α TAT1 deficient platelet lysates suggests the presence of an additional tubulin-acetylation enzyme that is unable to acetylate marginal band microtubules.

Introduction

Platelets are discoid cell fragments patrolling in the circulation to check vessel integrity. In case of vessel injury they undergo activation, change their shape from a disc to a sphere and extend filopodia before spreading on the injured vessel surface. Other incoming platelets get recruited and aggregate to form a hemostatic plug, which they then retract to re-establish unhindered blood flow in the damaged vessel. All these events are highly dependent on the reorganization of the cytoskeleton, especially of actin fibers and microtubules. These reorganizations have to be coordinated in a precise and efficient way to avoid bleeding complications and therefore depend on diverse regulatory mechanisms including reversible post-translational modifications. One of these modifications is the acetylation of marginal band microtubules at the residue lysine 40 of the α -tubulin subunit [1]. We have shown previously that microtubule motor protein actions are implicated in the reorganization of marginal band microtubules during the activation process [2] and it is known that microtubule acetylation can influence the kinetics of motor actions [3, 4]. We have also demonstrated that deacetylation rapidly follows platelet activation and is mediated by the deacetylase HDAC6. Platelets of mice deficient in HDAC6 have hyper-acetylated marginal bands and tubulin acetylation is maintained throughout all steps after platelet activation [5]. Furthermore, there is a positive correlation between the kinetics of platelet activation/spreading and the higher acetylation state. We therefore decided to search for the acetyltransferase able to acetylate marginal band microtubules and identified the major tubulin acetyltransferase α TAT1 [6, 7] as the responsible enzyme. We, thus, characterized platelets and megakaryocytes of mice deficient in α TAT1 and examined the functional consequences of α TAT1 deficiency for platelet production and physiology.

Methods

Reagents and antibodies

The following antibodies and reagents were used: mouse monoclonal anti-acetylated tubulin (clone 6-11B-1, Sigma, T6793); mouse monoclonal anti- α -tubulin (clone B-5-1-2, Sigma, T5168); rabbit monoclonal anti- α -tubulin (clone EP1332Y, Millipore, 04-1117);

mouse monoclonal anti-actin (MP Biomedicals, 691001); rat monoclonal anti-integrin $\beta 3$ (LucA5, Emfret, M030-0); Alexa Fluor 546 goat anti-rat IgG (Invitrogen, A11081); Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, A11029); Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen, A11035); HRP goat anti-mouse IgG (Cell Signaling, 5127); phalloidin-rhodamine (Sigma, P1951); poly-D-lysine (Sigma, P7280); thrombin (Sigma, T4648); pluronic F-127 (Sigma, P2443), Mowiol 4-88 (Calbiochem, 475904)

Mouse breeding

Mice were bred in the High Technology Animal Facility (PHTA) of the Grenoble Alpes University. Mouse experiments were performed in accordance with the institutional guidelines of the European Community and approved by an *Ad Hoc* committee under the registration number 315_UHTA-U1209-KS-01. All investigators directly involved in the project have an official animal-handling authorization obtained after 2 weeks of intensive training and a final formal evaluation. *HDAC6KO* and *α TAT1KO* mice (for KO strategy see [8] and [7], respectively) were backcrossed regularly to C57BL/6 mice and used for experiments between 7 and 10 months of age.

Preparation of mouse platelet rich plasma (PRP)

Blood was collected into 100 μ L 3.2% Na-citrate from anesthetized mice by cardiac puncture. Na-citrate volumes were adjusted according to recovered blood volumes. Blood with identical genotypes was pooled, centrifuged for 15 min, 200 g, no break, at RT to obtain the PRP. Platelets were counted and PRP was adjusted with the corresponding plasma to obtain an equal platelet concentration for all genotypes.

Resting and spread platelets

PRPs were diluted in PBS/1mM CaCl_2 to a concentration of 2.5×10^6 platelets/mL. For the platelet spreading assay, 400 μ L of these suspensions were added into wells of a 24-well plate containing coverslips and centrifuged for 3 min, 600g, at RT to allow simultaneous contact of all platelets with the glass surface for synchronised spreading. The plate was then placed into a cell culture incubator for 15 min. Platelets were then fixed in 4% isotonic formalin. For fixation of resting platelets, 400 μ L of the suspension was added into wells of a 24-well plate containing coverslips (coated with 20 μ g/mL poly-D-lysine) and 800 μ L isotonic formalin. After 10 min of fixation, plates were centrifuged for 5 min, 600g, at RT.

Clot retraction assay

Platelet concentration was adjusted to 2.5×10^8 platelets/mL in 50% plasma/50% PBS and a small amount of erythrocytes was added for color contrast. Clots of 200 μ L were allowed to form by addition of thrombin (final concentration 0.5U/mL) in glass tubes coated with 2% pluronic and containing a plastic loop. After 15-20 min at 37°C clots were removed and transferred into new tubes containing 200 μ L of PBS. The extruded serum volume was quantified and expressed as % of the initial volume. Progression of clot retraction was followed by taking photos at different time points.

Platelet counts

Platelet counts and mean platelet volumes were determined in a Scil Vet ABC Counter (Scil Animal care company, Altorf, France).

MK culture

Bone marrow cells were flushed from femurs and tibias and passed successively through 21-, 23- and 25-gauge needles. Cells were then pelleted at 1200 rpm for 7 min and resuspended at 1×10^8 cells/mL in PBS supplemented with 5% (v/v) rat serum and 2 mM EDTA to perform a negative lineage antigen selection (Lin selection, Stem Cell Technologies). The Lin negative population was adjusted to 5×10^6 cells/mL in DMEM containing 2 mM L-glutamine, penicillin/streptomycin, 10% FBS and 50 ng/mL thrombopoietin with addition of 50 U/mL hirudin. Cultures were usually performed in 12-well tissue culture plates with 700 μ L of cell suspension per well and incubated at 37°C under a 5% CO₂ atmosphere for up to 5 days.

Immunofluorescence

Fixed platelets on coverslips were permeabilized with PBS/0.2% Triton-X-100 for 15 min at RT and then incubated with blocking buffer (3% BSA, 10% goat serum in PBS) for 1 h at RT. Coverslips were then incubated for 2 h with primary antibodies diluted in blocking buffer, then washed 2x PBS and 1x PBS/0.2% Triton-X-100 and incubated for 2 h at RT with secondary antibodies diluted in blocking buffer. After 3 washing steps coverslips were mounted on glass slides using Mowiol. Fluorescent images were acquired using an upright microscope (Olympus BX41) equipped with a color camera DP70 using a 100x oil immersion objective.

Western blot

To prepare platelet lysates, PRP was diluted in PBS and centrifuged for 2 min at 9800g, the platelet pellet was lysed in 2x Laemmli sample buffer and heated at 95°C for 5 min. Proteins of platelet lysates were separated by 10% SDS-PAGE (2×10^6 platelets/10 μ L Laemmli sample buffer/lane) and transferred onto PVDF membranes for chemiluminescence detection of specific proteins using the indicated antibodies. Signals were quantified using the Bio-Rad ChemiDoc imaging system and the Image Lab analysis software.

Statistical analysis

P-values for differences in the clot retraction assay have been calculated using the Wilcoxon matched pairs test and p-values for platelet counts and volumes were determined using an unpaired, two-tailed t-test.

Results

Although several enzymes have been described as having tubulin acetylation activity, α TAT1 appears to be the major tubulin acetyltransferase [9]. We therefore hypothesized that α TAT1 could also be the major tubulin acetyltransferase in platelets. To test this hypothesis, we took advantage of the availability of a mouse line deficient in α TAT1 [7]. In order to compare the tubulin acetylation level in resting platelets from *wild type* (*WT*), *HDAC6KO* and *α TAT1KO* mice, platelet rich plasma (PRP) was prepared from mice of each genotype. Platelet lysates were then analyzed by Western blot for the presence of α -tubulin acetylated on lysine 40. In accordance with our previous publication, we observed tubulin acetylation in *WT* platelets and a much higher acetylation level in HDAC6 deficient platelets, indicating that the enzyme responsible of tubulin acetylation is active in resting platelets [5]. We expected complete absence of tubulin acetylation in *α TAT1^{-/-}* platelets. However, quite a strong residual signal for acetylated tubulin was detected in the *α TAT1* deficient platelets. We performed four independent experiments for the quantification of tubulin acetylation levels and obtained the following ratios: *T^{-/-}/WT*=0.64±0.1, *H^{-/-}/WT*=9.5±1.9, mean±sem (Figure 1A). In order to see whether the signal of acetylated tubulin is enhanced in *WT* and *α TAT1^{-/-}* platelets after deacetylase inhibition, PRP was incubated with TSA (trichostatin A; inhibitor of type I and II deacetylases including HDAC6) and nicotinamide (inhibitor of type III deacetylases). As shown in supplementary figure 1A, an enhanced signal for acetylated tubulin is observed only in *WT* platelets. The fact that the signal is not enhanced in *α TAT1^{-/-}* platelets suggests that the enzymes could be regulated themselves by their acetylation level as it has been shown for certain acetyltransferases and deacetylases [9]. We then decided to use immunofluorescence to evaluate tubulin acetylation levels of the marginal band in resting platelets. While HDAC6 deficient platelets have hyperacetylated marginal bands compared to *WT* platelets, *α TAT1KO* platelets show a complete absence of marginal band acetylation. However, a faint, diffuse signal is visible in the center of resting platelets (Figure 1B). In spread platelets the hyperacetylated state of microtubules persists in *HDAC6^{-/-}* platelets, as shown previously [5], and a faint signal is also visible in the spread *α TAT1^{-/-}* platelets (Figure 1B).

We have previously reported that the higher acetylation level of *HDAC6^{-/-}* platelets correlates with faster activation/spreading kinetics. Accordingly, we wondered whether *α TAT1^{-/-}* platelets would show a retardation of their spreading capacity. We decided to use again a spreading assay to compare the speed of activation and spreading between the different genotypes. HDAC6 deficient platelets spread faster than *WT* platelets confirming our previously published observations, while *α TAT1* deficient platelets behave similar to *WT* platelets under these conditions (Figure 1C). We also used a clot retraction assay to compare the functional efficiency of the three different platelet populations. Clot retraction reflects the capacity of platelets to activate and to perform a "three-dimensional spreading" within the fibrin network as well as their ability to subsequently reorganize their cytoskeleton to contract the fibrin network. While HDAC6 deficient platelets behave similar to *WT* platelets, we observe a slight retardation of clot retraction by *α TAT1KO* platelets (Figure 1D, see also supplementary figure 1B for a typical example of clot retraction kinetics).

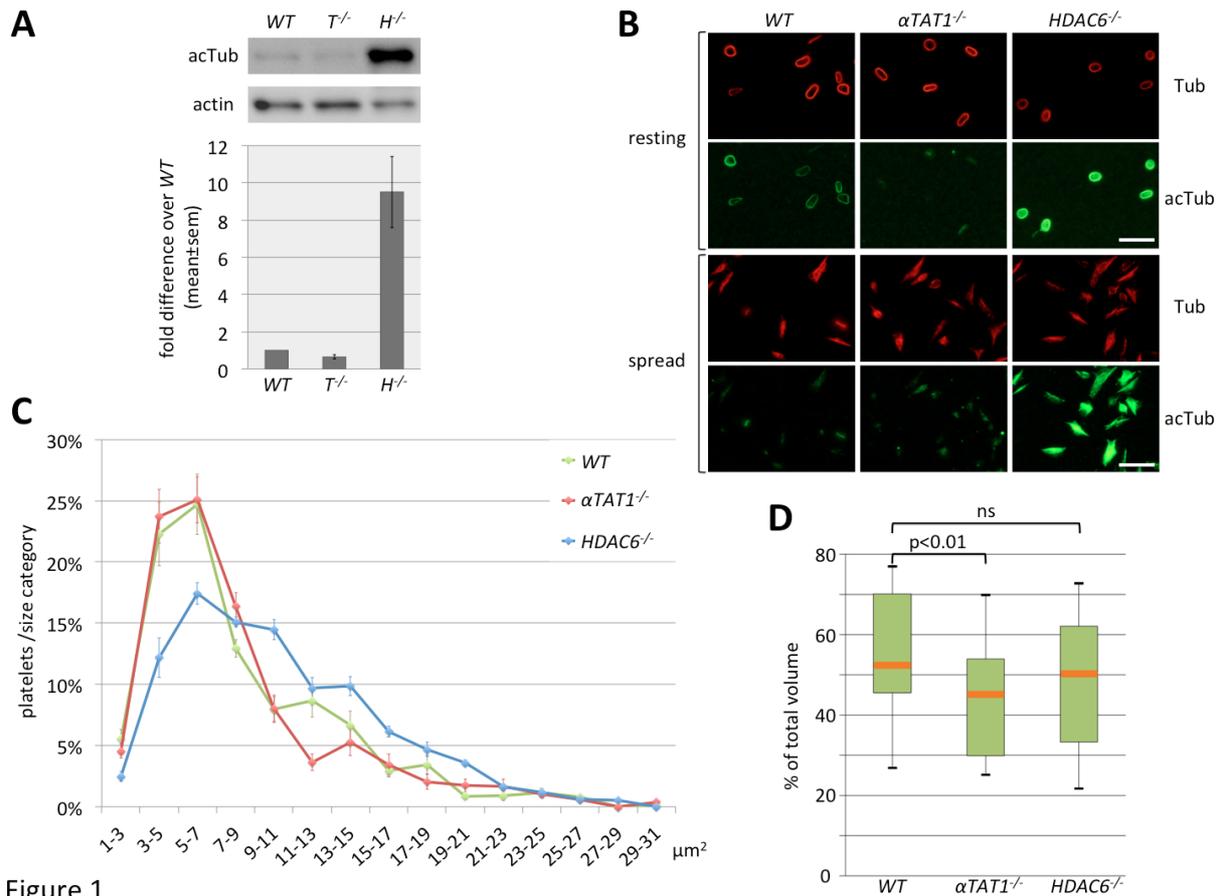


Figure 1

Figure 1: Comparison of platelets from WT, α TAT1 and HDAC6 deficient mice

A) Western blot of platelet lysates from WT, α TAT1^{-/-} (T^{-/-}) and HDAC6^{-/-} (H^{-/-}) mice probed for acetylated α -tubulin and actin as a loading control (2×10^6 platelets/lane). For quantification acetylated tubulin signals were adjusted according to actin loading controls and expressed as fold difference over WT platelets. Ratio of T^{-/-}/WT=0.64±0.1 and H^{-/-}/WT=9.5±1.9; mean±sem of four independent experiments.

B) Resting platelets and platelets spread for 1h from WT, α TAT1^{-/-} and HDAC6^{-/-} mice were fixed and stained for acetylated α -tubulin and total α -tubulin (scale bar 10 μ m).

C) Platelets were allowed to spread on glass coverslips for 15 min, before fixation and tubulin/ β 3-integrin staining. After determination of the surface area occupied by each platelet, the percentage of platelets in different size categories was plotted (values are mean±sem of 6 independent experiments).

D) Clot formation is induced by addition of thrombin to PRP from WT, α TAT1 and HDAC6 deficient mice and the extruded serum volume after 20 min of clot retraction is quantified and expressed as % of the initial volume. Each experiment is performed in triplicates and boxplots (min, max, median, Q1, Q3) are calculated from 12 independent experiments using 8 different PRPs. Values were tested for significance using the Wilcoxon matched pairs test.

It has recently been shown that Chinese hamster ovary cells expressing a mutant, partially active integrin receptor ($\alpha_{IIB}\beta_3D723H$) recapitulate certain steps of megakaryocyte differentiation. They form proplatelet-like extensions when cultured on fibrinogen and tubulin acetylation appears to be essential for the formation of these protrusions [10]. This suggests that platelet production might be influenced by tubulin acetylation levels. Our previously unpublished observations comparing platelet counts of *WT* and *HDAC6KO* mice did not show a difference between the two genotypes ($1240\pm64 \times 10^3/\mu\text{L}$ and $1246\pm79 \times 10^3/\mu\text{L}$, mean \pm sem; 9 and 8 animals, respectively), indicating that tubulin hyper-acetylation has no detectable consequence on platelet production. To test whether the absence of microtubule acetylation might influence platelet production in αTAT1KO mice, we investigated primary megakaryocytes, which form proplatelet extensions after a 4-day differentiation period *in vitro*. The percentage of differentiating megakaryocytes with proplatelet-like extensions within the total population is similar for the two genotypes (Figure 2B). However, maturing *WT* megakaryocytes display a different microtubule network compared to αTAT1KO megakaryocytes. *WT* cells have a higher number of circular elements, while *KO* cells have more loosely arranged proplatelet-like extensions (Figure 2A). Yet, these *in vitro* observations did not translate into major defects in platelet biogenesis since platelet counts and mean platelet volumes were not or only slightly different between *WT* and *KO* mice (Figure 2C,D).

Since there is a dynamic crosstalk between the actin and the microtubule part of the cytoskeleton, we investigated whether the microtubule acetylation level might have an impact on the actin cytoskeleton. However, as shown in the supplementary figure 2, no major differences can be visualized between the different genotypes.

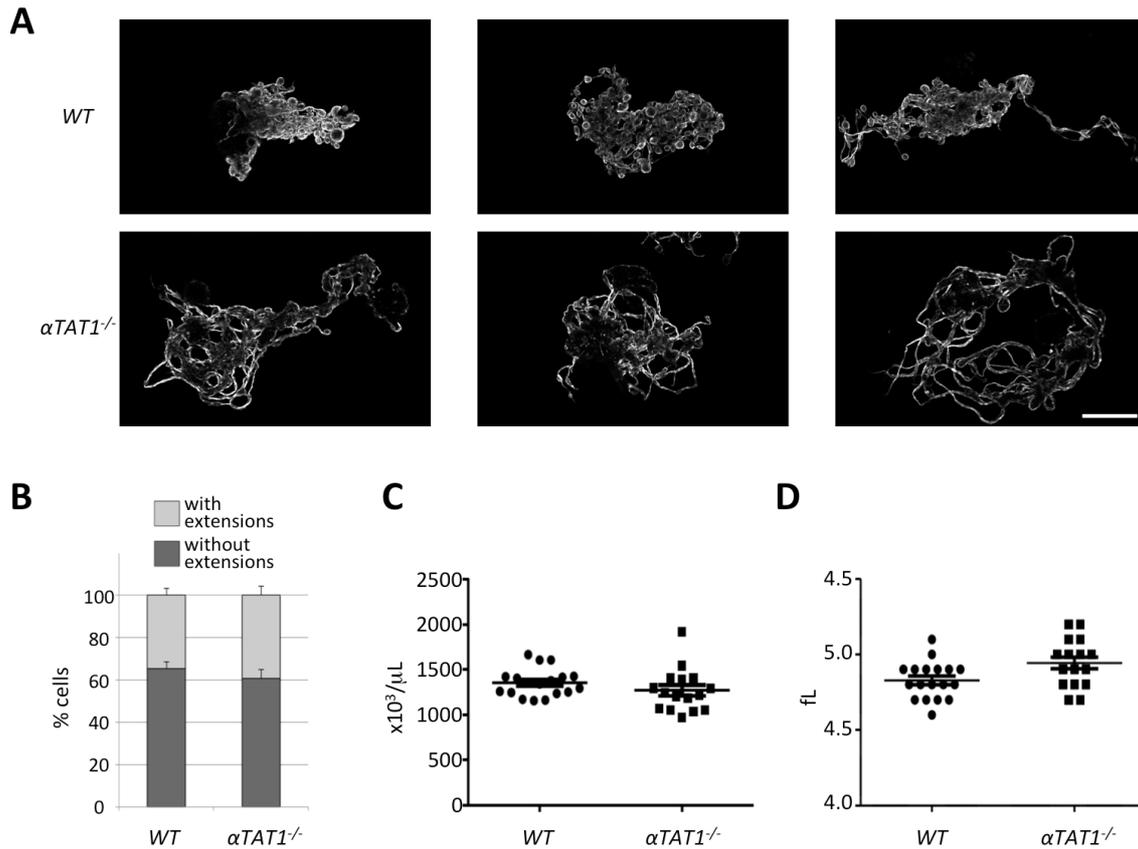


Figure 2

Figure 2: Platelet production in WT and $\alpha TAT1$ KO mice

A) Megakaryocytes from WT and $\alpha TAT1^{-/-}$ mice differentiated for 4d in culture have been stained for α -tubulin, three representative examples are shown for each genotype (scale bar, 25 μm).

B) Percentage of megakaryocytes with proplatelet-like extensions in the total population of Lin negative selected bone marrow cultures from WT and $\alpha TAT1^{-/-}$ mice after 4d of differentiation.

C) Platelet counts in blood from WT and $\alpha TAT1^{-/-}$ mice (WT: $1354 \pm 36 \times 10^3/\mu L$, N=18 $\alpha TAT1^{-/-}$: $1267 \pm 60 \times 10^3/\mu L$; N=16; mean \pm sem; $p=0.276$ Unpaired t-test, Two-tailed).

D) Determination of platelet volumes from WT and $\alpha TAT1^{-/-}$ mice (WT: 4.83 ± 0.03 fL, N=18; $\alpha TAT1^{-/-}$: 4.94 ± 0.04 fL; N=16; mean \pm sem; $p=0.0226$ Unpaired t-test, Two-tailed).

Discussion

Our results indicate that α TAT1 is the acetyltransferase responsible for the acetylation of marginal band microtubules in platelets. However, a residual acetylation level of lysine 40 of the α -tubulin subunit is detected in α TAT1 deficient platelets. This is in agreement with an earlier study characterizing α TAT1KO mice, which show residual tubulin acetylation in different tissues [11]. It has been reported that under certain conditions the mouse monoclonal anti acetylated tubulin antibody, clone 6-11B-1, recognizes deacetylated tubulin by immunofluorescence but not by Western blot [12]. Thus, the possibility that this residual signal detected by Western blot is due to an unspecific recognition of unacetylated tubulin is very unlikely. An additional tubulin acetyltransferase must therefore be expressed in platelets, as well as other tissues. The relatively faint and diffuse acetylated tubulin staining present in the center of α TAT1KO platelets may be due to α -tubulin subunits acetylated by this other acetyltransferase, but clearly this other enzyme is unable to acetylate marginal band microtubules. Further studies will be necessary to identify the enzyme(s) involved. Potential candidates might be GCN5, NAT10 or the ARD1-NAT1 complex [9]. The acetyltransferase p300, which has been shown to play an important role in platelets [13], could also directly or indirectly influence tubulin acetylation levels.

The functional characterization of α TAT1 deficient platelets revealed only minor consequences of deficient microtubule acetylation. The fact that no significant difference in the spreading capacity was detected between platelets from *WT* and α TAT1KO mice but a delay in clot retraction may suggest that the absence of tubulin acetylation is less important for early activation events than for subsequent clot retraction. Several publications have shown a role of microtubules in clot retraction [14-16]. Microtubules could be important for the repeated, successive extensions and retractions of filopodia, which bind to fibrin strands to pull them together, thereby retracting the clot [17]. Extension of filopodia might depend on motor driven sliding of microtubules, as it is the case for the extension of proplatelets by megakaryocytes [18]. This, in turn, may be less efficient in α TAT1KO platelets, since microtubule motor actions are less productive on non-acetylated microtubules [3, 4]. Alternatively, the effect may be due to deficient acetylation of an additional substrate of α TAT1, such as cortactin [19]. An altered inside-out or outside-in signaling can also not be ruled out.

Our results show that the tubulin acetylation level has no impact on platelet production *in vivo*, which is surprising in view of our observation that α TAT1 deficient megakaryocytes differentiated *in vitro* have an altered microtubule network. This may suggest that compensation mechanisms have been established in the mutant mice. The absence of a strong functional phenotype of both, *HDAC6KO* and α TAT1KO platelets, may point to a role of acetylation in the fine-tuning of microtubule reorganizations during platelet activation, spreading and retraction. Alternatively, tubulin acetylation might become important under shear stress conditions, when forces are exerted on microtubules. Indeed, it has been shown that resistance of microtubules to mechanical breakage is conferred by tubulin acetylation [20].

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Disclosure statement

The authors have no declaration of interest to disclose.

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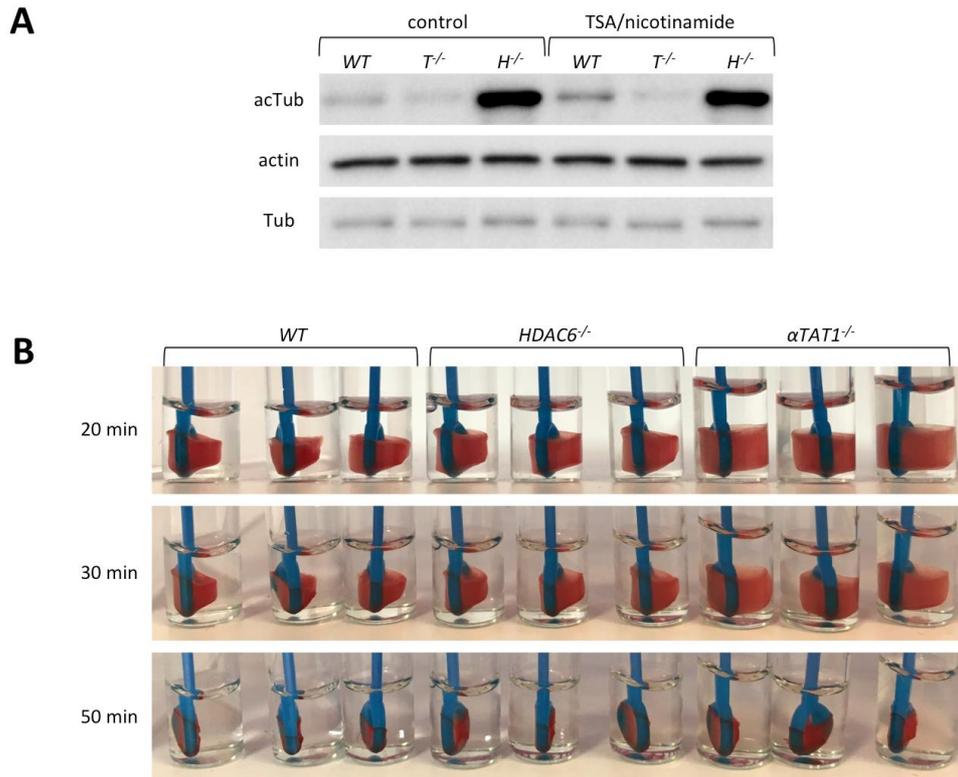
Author contributions

A.-S.R., M.B., C.R., T.B., S.M., K.S. performed experiments. F.L. and K.S. designed experiments and wrote the paper. S.K. and L.L. financially contributed to the study. All authors interpreted results and reviewed the manuscript.

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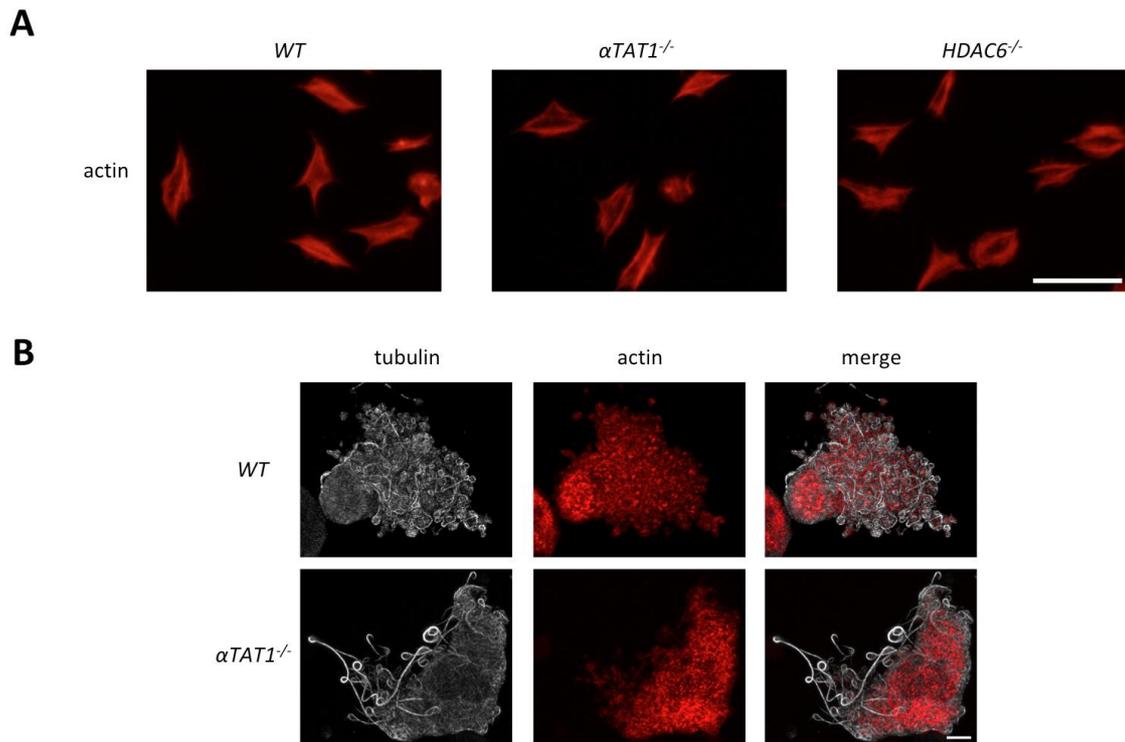


supplementary Figure 1

supplementary Figure 1:

A) Platelets of WT, αTAT1^{-/-} (T^{-/-}) and HDAC6^{-/-} (H^{-/-}) mice were treated or not with TSA (0.1 μg/mL) and nicotinamide (5 mM) for 20 min. Tubulin acetylation levels in platelet lysates were determined by Western blot using an antibody against acetylated α-tubulin and antibodies against tubulin as well as actin as loading controls (2x10⁶ platelets/lane).

B) Thrombin (final concentration 0.5 U/mL) was added to a glass tube containing 200 μL PRP to induce clot formation. After 20 min clots are transferred into new glass tubes containing 200 μL PBS and contraction was allowed to continue for 50 min. The extruded serum volume after 20 min of contraction was measured to quantify clot retraction (see figure 1D).



supplementary Figure 2

supplementary Figure 2:

A) The actin cytoskeleton in platelets of WT, $\alpha TAT1^{-/-}$ and HDAC6 $^{-/-}$ mice after 1h of spreading stained with phalloidin-rhodamine (scale bar, 10 μ m).

B) The actin and tubulin cytoskeleton of megakaryocytes from WT and $\alpha TAT1^{-/-}$ mice differentiated for 4d in culture (scale bar, 10 μ m).