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Peer Review File

Human Disabled-2 regulates thromboxane A_2 signaling for efficient hemostasis in thrombocytopenia

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors analyzed the role of the adaptor protein DAB2 in platelet function by generating a mouse with conditional overexpression of the human DAB2 isoform in platelets. Hemostasis, thrombus formation, and platelet function were comparable in DAB2 Knock-in mice with respect to controls. Enhanced thromboxane A2 signaling and ADP release were revealed in Dab2 KI platelets together with a reduced bleeding time of DAB2 KI during experimental thrombocytopenia. The conclusion drawn is that Dab2 might play a role in regulating bleeding severity associated with thrombocytopenia. Mechanistically, the authors showed that Dab2 sustains the formation of a complex with phospholipids and AKT leading to an increase in AKT phosphorylation in U46619-stimulated platelets and ADP release.
Comments:
-The bleeding phenotype in ITP is heterogeneous and only partly dependent on the severity of thrombocytopenia. However, before claiming a potential role for DAB2 in regulating bleeding phenotype in thrombocytopenic patients, this should be experimentally evaluated.
-Most of these data are obtained in engineered mice that overexpress a Dab2 isoform only found in human platelets: how are these data relevant from a biological and clinical point of view? Evidence of Dab2 involvement in TxA2 signaling in human platelets should be provided. This would strengthen the story.
-Dab2 was previously reported to bind sulfatides. This is not the case in experiments performed in Fig. 6A. How do the authors explain this? Moreover, binding experiments with Dab2 should also be evaluated by surface plasmon resonance.
-It appears that AKT is not activated in the control cells, as indicated by the absence of Akt pSer phosphorylation in U46619-stimulated control platelets, as shown in Figure 5D. Can we confirm this observation? Additionally, it is noteworthy that basal Dab2KI platelets exhibit lower levels of total AKT.
-It is not clear at which time point of CD41-antibody-dependent thrombocytopenia experiments were performed. After 24 hours? It would be interesting to correlate Dab2-dependent platelet function with platelet counts in this model. Maybe including the recovery phase.
-Dab2 KI increases platelet aggregation upon U46619-stimulation at lower platelet concentrations. Any explanations?
-Fig. 4A: is ADP able to increase the aggregation of U46619-stimulated platelets? This should be evaluated.
-The statistics in Fig 7A are not clear.

Reviewer #2 (Remarks to the Author):

In this manuscript, Tsai and colleagues studied how the human adaptor protein Disabled-2 (hDab2) affects the tendency to bleed in individuals with thrombocytopenia. They generated megakaryocyte lineage-restricted hDab2 knock-in (hDab2-KI) mice by first establishing a mouse strain (ROSA26fl/fl mice) with site-specific insertion of conditional

hDab2 expression cassette into the ROSA26 locus followed by cross-breeding the mice with the PF4-Cre mice. The researchers investigated how platelet hDab2 affects mice's hemostasis, thrombus formation, and platelet function. They found that mice with hDab2-KI had reduced bleeding under thrombocytopenic conditions, which could be reversed using the ADP receptor P2Y12 antagonist clopidogrel. Further studies indicated that hDab2 did not affect protease-activated receptor 4 peptide- and collagen-stimulated platelet activation but did enhance thromboxane A2 (TXA2) mimetic U46619-induced platelet aggregation, integrin activation, CD62P expression, and δ-granule secretion at a low platelet number. They found that hDab2 is crucial to hemostasis in thrombocytopenia by functional interplay with ADP/ATP release underlying TxA2 signaling.

The topic of this study is clinically relevant; however, it contains several limitations that the authors should carefully address.

Major points

The authors found that Dab2 p82 was expressed in hDab2-KI platelets but not in ROSA26fl/fl platelets, indicating the success of their approach. However, they could not detect the expression of p59-Dab2 in platelets from both hDab2-KI and ROSA26fl/fl mice. This contradicts previous research that suggests p59-Dab2 is present in mouse platelets. The authors should provide more clarity on this discrepancy.

Dab2 p82 was comparable in the lung, spleen, and intestine in ROSA26fl/fl and hDab2-KI mice. It is important to know the role of p82-Dab2 in the cardiovascular system and whether hDab2-KI mice showed altered vascular responses to platelet activation.

It would be helpful to characterize the systemic biosynthesis of prostanoids by assessing the urinary levels of 2,3-dinor-TXB2 and 2,3-dinor-6-keto-PGF1a. They are the major metabolites of TXA2 and PGI2 and represent an index of the generation of the two prostanoids in vivo, mainly from platelets and the vascular cells, respectively, in ROSA26fl/fl and hDab2-KI before and after peritoneal injection of anti-CD41 antibody to cause immune thrombocytopenia (ITP).

It would be important to verify the impact of low-dose aspirin administration in ROSA26fl/fl and hDab2-KI before and after peritoneal injection of anti-CD41 antibody on bleeding time.

They suggest that platelets from hDab2-KI have an enhanced U46619-stimulated platelet integrin activation, α-granule secretion, and δ-granule secretion compared to the ROSA26fl/fl platelets. It is important to note that the concentrations of U46619 used in the study may not reflect the actual rate of TXA2 synthesis in the body. In humans, TXA2 generation typically amounts to around 0.75 microM in 24 hours.

Although TxB2 generation in the resting stage and in response to PAR4 peptide or collagen were similar between ROSA26fl/fl and hDab2-KI platelets, this finding does not necessarily apply to in vivo situations. In fact, the biosynthesis in platelets in vitro after stimulation is an index of the maximal capacity of platelet to generate the prostanoid. Thus, this model is inappropriate for detecting biosynthesis differences between ROSA26fl/fl and hDab2-KI. As I

reported above, they should assess the systemic biosynthesis in vivo of TXA2.

They should verify their finding in patients with thrombocytopenia. In particular, the hDab2 expression/function in patients with thrombocytopenia should be studied.

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors analyzed the role of the adaptor protein DAB2 in platelet function by generating a mouse with conditional overexpression of the human DAB2 isoform in platelets. Hemostasis, thrombus formation, and platelet function were comparable in DAB2 Knock-in mice with respect to controls. Enhanced thromboxane A2 signaling and ADP release were revealed in Dab2 KI platelets together with a reduced bleeding time of DAB2 KI during experimental thrombocytopenia. The conclusion drawn is that Dab2 might play a role in regulating bleeding severity associated with thrombocytopenia. Mechanistically, the authors showed that Dab2 sustains the formation of a complex with phospholipids and AKT leading to an increase in AKT phosphorylation in U46619-stimulated platelets and ADP release.

Comments:

The bleeding phenotype in ITP is heterogeneous and only partly dependent on the severity of thrombocytopenia. However, before claiming a potential role for DAB2 in regulating bleeding phenotype in thrombocytopenic patients, this should be experimentally evaluated.

We agree with the Reviewer's point of view. However, we are not able to provide a comprehensive answer to this comment in this manuscript. With the incidence rate of immune thrombocytopenic (ITP) in Taiwan being about 2-5.4 per 100,000 individuals[1], we need to collaborate with the doctors in multiple medical centers to recruit enough patients with ITP for correlation of Dab2 expression and bleeding phenotype. It is unlikely for us to obtain data in a short period of time for inclusion in this manuscript. We hope the Reviewer can understand our difficulty in providing such information herein. Hence, we discuss/suggest but not claim a potential role for Dab2 in regulating the bleeding phenotype in thrombocytopenic patients in this manuscript. We'll continue to evaluate the potential role of Dab2 in regulating the bleeding phenotype in thrombocytopenic patients.

Although we are not able to provide the study outcome with ITP patients at this stage, several experiments using human platelets were performed and include in the revised manuscript. We showed that Dab2 expression was variable by more than 4.3-fold among different individuals (Supplementary Fig. 5). U46619-induced ATP/ADP release is also highly variable when compared to thrombin- and collagen-induced ATP/ADP release (Supplementary Table 2). The lower the platelet concentrations, the higher the variability of U46619-stimulated ATP/ADP release among different individuals. For example, at the platelet concentration of $0.1x10^8$/ml, the ratio of maximum and minimum ATP/ADP release and the coefficient of variation among platelets from different individuals was 12.3 and 88.8%, respectively. Proteomic analysis of human platelets further revealed U46619 treatment altered the profiles of hDab2-interacting proteins in human platelets, implying that hDab2 is downstream of TxA_2 signaling in human platelets (Supplementary Fig. 6, Supplementary Table 3, and Supplementary methods). This information sets a stage for further clinical study and is included in the Discussion of the revised manuscript (page 15, lines 367-383). We hope the Reviewer can accept our explanation and agree with our point of view. Please let us know if more information is required by the Reviewer.

Most of these data are obtained in engineered mice that overexpress a Dab2 isoform only found in human platelets: how are these data relevant from a biological and clinical point of view? Evidence of Dab2 involvement in TxA2 signaling in human platelets should be provided. This would strengthen the story.

We appreciate the Reviewer's comments and suggestions. We have added a paragraph in the Discussion section to discuss the data from a biological point of view (page 12, lines 287-307). It has been demonstrated that the effects of collagen and thrombin are platelet density-dependent and are decreased in thrombocytopenia[2,3]. Based on the findings of this study, TxA_2 signaling is postulated as a key pathway contributing to platelet activation and hemostasis in

stopping bleeding under thrombocytopenic condition. Facilitating TxA_2 signaling and the subsequent ADP/ATP release by hDab2 thereby reduces the bleeding of mice with thrombocytopenia with no effect on normal hemostasis. This not only implies the importance of TxA_2 signaling in hemostasis under thrombocytopenia condition, but also indicates the levels of Dab2 expression in platelets is among the determining factors of bleeding risk for mice, and likely for humans too, with thrombocytopenia. A new insight into the biological function of TxA_2 signaling and Dab2 function in the regulation of hemostasis under thrombocytopenic condition was revealed. Because hDab2 expression in platelets has no effect on TxA_2 generation and TP expression and does not cause thrombocytopenia or alter the normal vascular response to platelet activation, enhancing TxA_2 signaling and the subsequent ADP/ATP release by expression of Dab2 in platelets or by other mechanisms may provide a means to control bleeding associated with thrombocytopenia.

As indicated in the reply to the first comment by the Reviewer, no clinical data are reported in this study. The difficulties for providing clinical data have been explained. Nevertheless, several experiments using human platelets were performed and reported in the revised manuscript. These include the experiments to address whether hDab2 expression and U46619-induced ATP release are variable among different individuals. A paragraph was added in the Discussion section of the revised manuscript to discuss the findings in human platelets (page 15, lines 367-383).

To provide evidence of Dab2 involvement in TxA_2 signaling in human platelets, proteomic analysis of Dab2-interacting proteins in resting and U46619-stimulated human platelets was performed. The profiles of hDab2-interacting proteins were altered by treatment with U46619, implying hDab2 underlies TxA_2 signaling in human platelets. KEGG pathway analysis further showed that hDab2-interacting proteins were mainly involved in the regulation of the actin cytoskeleton and platelet activation (Supplementary Fig. 6, Supplementary Table 3, and Supplementary methods). These findings were described in the Discussion section of

the revised manuscript (page 15, lines 367-383).

Together, we have discussed the biological and clinical relevance of our findings in the revised manuscript. We also provide a new set of data to provide evidence of Dab2 involvement in TxA_2 signaling in human platelets. We believe this has strengthened the story and we would appreciate that the Reviewer agrees with our point of view.

Dab2 was previously reported to bind sulfatides. This is not the case in experiments performed in Fig. 6A. How do the authors explain this? Moreover, binding experiments with Dab2 should also be evaluated by surface plasmon resonance.

In the revised manuscript, we followed the Reviewer's suggestion and performed kinetic analysis of Dab2-PA interaction by the surface plasmon resonance assay. The equilibrium binding constant K_D between PA and GST-Dab2N (residues 1-234) was 0.67 ± 0.24 nM (Fig. 7c, page 10, lines 253-255). With the estimated K_D of 0.67 nM for hDab2-PA binding and 0.6 μM for hDab2-sulfatide binding[4], hDab2 has a higher binding affinity with PA than with the sulfatide. This may explain why hDab2-PA but not the hDab2-sulfatide interaction was observed in our protein lipid overlay assay.

It appears that AKT is not activated in the control cells, as indicated by the absence of Akt pSer phosphorylation in U46619-stimulated control platelets, as shown in Figure 5D. Can we confirm this observation? Additionally, it is noteworthy that basal Dab2KI platelets exhibit lower levels of total AKT.

Thanks for the Reviewer's comment. In Fig. 5d, a slight increase in AKT-Ser473 phosphorylation for the U46619-stimulated control ($ROSA26^{fl/fl}$) platelets was noted when compared to the platelets at the resting stage. To confirm this observation, the levels of AKT-Ser473 phosphorylation from four independent experiments were quantified followed by statistical analysis. There was a 2.6-fold increase in AKT-Ser473 phosphorylation for the

U46619-stimulated control (ROSA26$^{fl/fl}$) platelets when compared to the platelets at the resting stage **(Figure R1 and R2, $p < 0.05$)**.

On the other hand, the total AKT for basal hDab2-KI platelets appeared lower when compared to the other three treatment groups as indicated by the Reviewer. To address this issue, the protein lysates from the ROSA26$^{fl/fl}$ and hDab2-KI platelets were re-quantified followed by SDS-PAGE analysis. The levels of total AKT for basal hDab2-KI platelets and the other three treatment groups were comparable, as confirmed by quantitative analysis of total AKT in four independent experiments **(Figure R1 and R3)**. We conclude that the levels of total AKT were comparable for the platelets from basal/control hDab2-KI and other treatment groups.

To avoid any misunderstanding by the readers, the original total AKT and AKT-Ser473 phosphorylation Western blot image was replaced with the new Western blot results in the revised manuscript (Fig. 6d) and stated clearly in the figure legends that the representative Western blot images may be derived from different experiments.

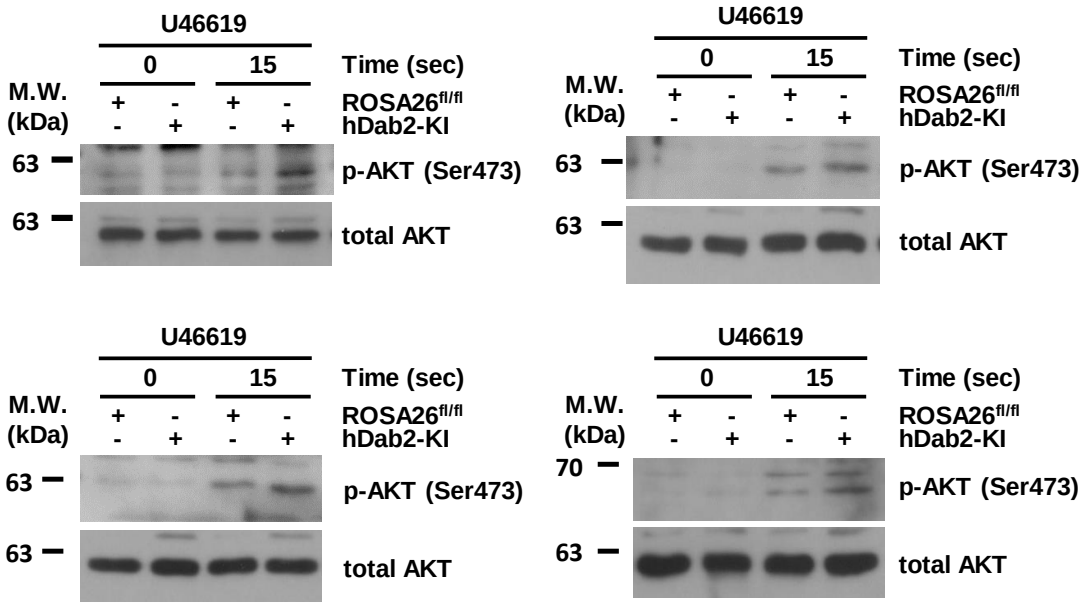

Figure R1. Total AKT expression and AKT-Ser473 phosphorylation in ROSA26$^{fl/fl}$ and hDab2-KI platelets. Washed platelets were stimulated by 0.25 μM U46619 for 15 sec. Platelet lysates were analyzed by Western blot using the indicated antibodies. Four independent experiments are shown.

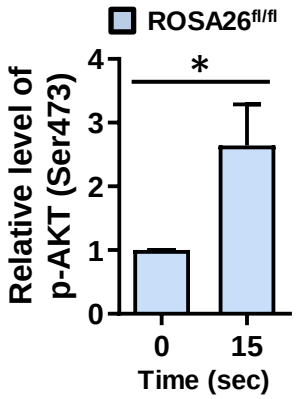

Figure R2. AKT is activated in U46619-stimulated ROSA26$^{fl/fl}$ platelets. The relative level of AKT-Ser473 phosphorylation in resting and U46619-stimulated ROSA26$^{fl/fl}$ platelets was quantified by ImageJ software. Data represent the mean ± SEM of 4 independent experiments. *, $p < 0.05$.

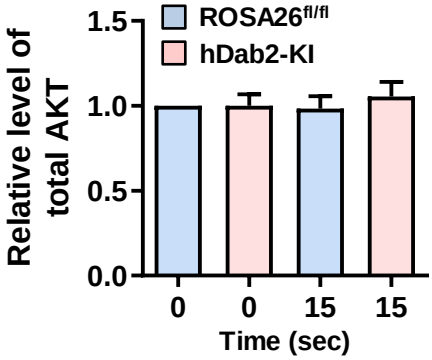

Figure R3. The relative level of total AKT in resting and U46619-stimulated ROSA26$^{fl/fl}$ or hDAb2-KI platelets. The relative level of total AKT in resting and U46619-stimulated ROSA26$^{fl/fl}$ or hDAb2-KI platelets was quantified by ImageJ software. Data represent the mean ± SEM of 4 independent experiments.

It is not clear at which time point of CD41-antibody-dependent thrombocytopenia experiments were performed. After 24 hours? It would be interesting to correlate Dab2-dependent platelet function with platelet counts in this model. Maybe including the recovery phase.

Thanks to the Reviewer for pointing out this issue. We rewrote the section of the anti-CD41 antibody-dependent thrombocytopenia animal model in the revised manuscript (page 18, line 442-453). Briefly, mice received daily intraperitoneal injection of anti-CD41 antibody[5] (MWReg30, 81.6 μg/kg/day for female mice and 122.4 μg/kg/day for male mice in 100 μl 1X PBS, pH 7.2) for 2 days to induce ITP. Experimentation was performed at 24 h after the final

injection of anti-CD41 antibody when the platelet count was below $5x10^8$/ml. Alternatively, experimentation at the recovery phase (RP) was performed at 72 h after the final injection of anti-CD41 antibody. The platelet count at this phase was usually in the range of $5.2x10^8$/ml to $13x10^8$/ml. We hope the new description of the animal model for thrombocytopenia is easier to understand.

We followed the Reviewer's suggestion to analyze the bleeding time when mice were at the recovery phase in the revised manuscript (page 6, lines 142-148). Briefly, at RP, 35% and 7.7% of ROSA26$^{fl/fl}$ and hDab2-KI mice continued to bleed for more than 10 min. The bleeding tendency was slightly but not significantly different between the ROSA26$^{fl/fl}$ and hDab2-KI mice ($p = 0.0981$). These data indicate that the effect of platelet hDab2 expression on reducing mice bleeding is related to the status of thrombocytopenia and the platelet count. We would appreciate that the Reviewer agrees with our point of view.

Dab2 KI increases platelet aggregation upon U46619-stimulation at lower platelet concentrations. Any explanations?

Fig. 4A: is ADP able to increase the aggregation of U46619-stimulated platelets? This should be evaluated.

As supported by the data presented in the manuscript, enhanced U46619-stimulated ADP release by hDab2 caused platelet aggregation at a lower platelet concentration. To strengthen this notion, we followed the Reviewer's suggestion and added a new data set in the revised manuscript showing that ADP further increases the aggregation of the U46619-stimulated hDab2-KI platelets (Fig. 5, page 8, lines 198-201). In addition, the amount of collagen-induced ADP release is highly related to platelet count[6]. The effect of platelet adhesion to collagen and thrombin generation are platelet density-dependent and are decreased in thrombocytopenia[7,8]. Therefore, TxA_2 signaling and the subsequent ADP/ATP release may contribute mainly to the pool of ADP release during platelet activation. This may explain why hDab2 expression in

platelets increases platelet aggregation upon U46619 stimulation at lower platelet concentration but not at normal physiological platelet concentration. We would appreciate that the Reviewer agrees with our point of view.

The statistics in Fig 7A are not clear.

Thanks for pointing out the error. Some information was missing when converting into the PDF file for submission. The error is corrected and the statistics are shown in Fig. 8b of the revised manuscript.

Reviewer #2 (Remarks to the Author):

In this manuscript, Tsai and colleagues studied how the human adaptor protein Disabled-2(hDab2) affects the tendency to bleed in individuals with thrombocytopenia. They generated megakaryocyte lineage-restricted hDab2 knock-in (hDab2-KI) mice by first establishing a mouse strain (ROSA26$^{fl/fl}$ mice) with site-specific insertion of conditional hDab2 expression cassette into the ROSA26 locus followed by cross-breeding the mice with the PF4-Cre mice. The researchers investigated how platelet hDab2 affects mice's hemostasis, thrombus formation, and platelet function. They found that mice with hDab2-KI had reduced bleeding under thrombocytopenic conditions, which could be reversed using the ADP receptor $P2Y_{12}$ antagonist clopidogrel. Further studies indicated that hDab2 did not affect protease-activated receptor 4 peptide- and collagen-stimulated platelet activation but did enhance thromboxane A2 (TxA_2) mimetic U46619-induced platelet aggregation, integrin activation, CD62P expression, and δ-granule secretion at a low platelet number. They found that hDab2 is crucial to hemostasis in thrombocytopenia by functional interplay with ADP/ATP release underlying TxA_2 signaling.

The topic of this study is clinically relevant; however, it contains several limitations that the

authors should carefully address.

Major points

The authors found that Dab2 p82 was expressed in hDab2-KI platelets but not in ROSA26$^{fl/fl}$ platelets, indicating the success of their approach. However, they could not detect the expression of p59-Dab2 in platelets from both hDab2-KI and ROSA26fl/fl mice. This contradicts previous research that suggests p59-Dab2 is present in mouse platelets. The authors should provide more clarity on this discrepancy.

In the original manuscript, the faint p59-Dab2 band may cause the Reviewer misunderstanding that p59-Dab2 is not expressed in ROSA26$^{fl/fl}$ and hDab2-KI platelets. We have re-probed the PVDF membrane from Fig. 1e with the anti-Dab2 antibody followed by a longer exposure time to the X-ray film to enhance Dab2 signal. As shown in Fig. 1e of the revised manuscript, p59-Dab2 was detectable in both ROSA26$^{fl/fl}$ and hDab2-KI platelets (page 5, line 122-123). The data indicate that p59-Dab2 is expressed in platelets from both ROSA26$^{fl/fl}$ and hDab2-KI mice, consistent with our previous research. We hope this can ease the Reviewer's concern.

Dab2 p82 was comparable in the lung, spleen, and intestine in ROSA26$^{fl/fl}$ and hDab2-KI mice. It is important to know the role of p82-Dab2 in the cardiovascular system and whether hDab2-KI mice showed altered vascular responses to platelet activation. It would be helpful to characterize the systemic biosynthesis of prostanoids by assessing the urinary levels of 2,3-dinor-TxB$_2$ and 2,3-dinor-6-keto-PGF1α. They are the major metabolites of TxA$_2$ and PGI$_2$ and represent an index of the generation of the two prostanoids in vivo, mainly from platelets and the vascular cells, respectively, in ROSA26$^{fl/fl}$ and hDab2-KI before and after peritoneal injection of anti-CD41 antibody to cause immune thrombocytopenia (ITP).

Thanks for the Reviewer's comments. We followed the Reviewer's suggestion to assess the urinary level of 2,3-dinor-6-keto-$PGF_{1\alpha}$, an indicator of systemic biosynthesis of prostanoids *in vivo* in vascular cells, in $ROSA26^{fl/fl}$ and hDab2-KI mice (Fig. 4d). The data indicate that the vascular response to platelet activation is not altered in hDab2-KI mice. We further followed the Reviewer's suggestion and assessed the urinary levels of 2,3-dinor-TxB_2 and 2,3-dinor-6-keto-$PGF_{1\alpha}$ in $ROSA26^{fl/fl}$ and hDab2-KI mice before and after peritoneal injection of anti-CD41 antibody. Our data indicate that the urinary levels of 2,3-dinor-TxB_2 and 2,3-dinor-6-keto-$PGF_{1\alpha}$ of hDab2-KI mice were comparable to the $ROSA26^{fl/fl}$ mice (Fig. 4d, page 7, lines 179 to page 8, lines 191). It indicates that *in vivo* TxA_2 generation in platelets and the PGI2 biosynthesis in vascular cells are not affected by hDab2 expression in platelets. Moreover, injection of anti-CD41 caused a slight but not significant increase in urinary 2,3-dinor-TxB_2 for both $ROSA26^{fl/fl}$ and hDab2-KI mice. We would appreciate that the Reviewer agrees with our point of view.

It would be important to verify the impact of low-dose aspirin administration in $ROSA26^{fl/fl}$ and hDab2-KI before and after peritoneal injection of anti-CD41 antibody on bleeding time.

Thanks for the Reviewer's comments. We followed the Reviewer's suggestion and verified the impact of low-dose aspirin on bleeding time before and after peritoneal injection of anti-CD41 antibody (Fig. 4 in the revised manuscript). Low-dose aspirin inhibited TxA_2 generation as revealed by the reduced serum concentration of TxB_2 (a stable metabolite of TxA_2) in mice (Fig. 4a). The bleeding time for $ROSA26^{fl/fl}$ and hDab2-KI mice without injection of the anti-CD41 antibody was comparable regardless whether or not the mice were administered aspirin (Fig. 4b). In the absence of aspirin, the hDab2-KI mice injected with the anti-CD41 antibody displayed thrombocytopenia with a reduced bleeding tendency when compared to the $ROSA26^{fl/fl}$ mice as reported in Fig. 2. Aspirin administration prolonged the

bleeding of hDab2-KI mice with thrombocytopenia (Fig. 4b, c, p = 0.012) (page 7, lines 170-178). These data indicate that TxA_2 is crucial for the reduced bleeding of the hDab2-KI mice with thrombocytopenia. hDab2 regulates intracellular signaling downstream of TxA_2 receptor but not *in vivo* TxA_2 generation.

They suggest that platelets from hDab2-KI have an enhanced U46619-stimulated platelet integrin activation, α-granule secretion, and δ-granule secretion compared to the ROSA26fl/fl platelets. It is important to note that the concentrations of U46619 used in the study may not reflect the actual rate of TxA_2 synthesis in the body. In humans, TxA_2 generation typically amounts to around 0.75 microM in 24 hours.

Although TxB_2 generation in the resting stage and in response to PAR4 peptide or collagen were similar between ROSA26fl/fl and hDab2-KI platelets, this finding does not necessarily apply to *in vivo* situations. In fact, the biosynthesis in platelets *in vitro* after stimulation is an index of the maximal capacity of platelet to generate the prostanoid. Thus, this model is inappropriate for detecting biosynthesis differences between $ROSA26^{fl/fl}$ and hDab2-KI. As I reported above, they should assess the systemic biosynthesis in vivo of TxA_2.

Thanks for the Reviewer's comments. The concentration of U46619 used in the study was mainly 0.25 μM. It may not reflect the actual rate of TxA_2 synthesis in the body, but is generally used to perform *in vitro* platelet functional studies such as integrin activation, α-granule secretion, and δ-granule secretion. In the revised manuscript, we followed the Reviewer's suggestion and assessed the systemic biosynthesis of TxA_2 *in vivo* by measuring urinary 2,3-dinor-TxB_2 of $ROSA26^{fl/fl}$ and hDab2-KI mice by LC-MS/MRM technology. $ROSA26^{fl/fl}$ and hDab2-KI mice had comparable urinary levels of 2,3-dinor-TxB_2 (Fig. 4d, page 7, lines 179 to page 8, lines 191). This indicates that *in vivo* TxA_2 generation is not affected by hDab2 expression in platelets. We would appreciate that the Reviewer agrees with our point of view.

They should verify their finding in patients with thrombocytopenia. In particular, the hDab2expression/function in patients with thrombocytopenia should be studied.

We agree with the Reviewer's point of view. However, we are not able to provide a comprehensive answer to this comment in this manuscript. With the incidence rate of immune thrombocytopenic (ITP) in Taiwan of about 2-5.4 per 100,000 individuals[1], we need to collaborate with the doctors in multiple medical centers to recruit enough patients with ITP for correlation of Dab2 expression and bleeding phenotype. It is unlikely to obtain data in a short period of time for inclusion in this manuscript. We hope the Reviewer can understand our difficulty in providing such information herein. Hence, we discuss/suggest but not claim a potential role for Dab2 in regulating the bleeding phenotype in thrombocytopenic patients in this manuscript. We will continue to evaluate the potential role of Dab2 in regulating bleeding phenotype in thrombocytopenic patients.

Although we are not able to provide the study outcome with ITP patients at this stage, several experiments using human platelets were performed and reported in the revised manuscript. We showed that Dab2 expression was variable by more than 4.3-fold among different individuals (Supplementary Fig. 5). U46619-induced ATP/ADP release is also highly variable when compared to thrombin- and collagen-induced ATP/ADP release **(**Supplementary Table 2**)**. The lower the platelet concentration, the higher the variability of U46619-stimulated ATP/ADP release among different individuals. For example, at the platelet concentration of $0.1x10^8$/ml, the ratio of maximum and minimum ATP/ADP release and the coefficient of variation among platelets from different individuals was 12.3 and 88.8%, respectively. Proteomic analysis of human platelets further revealed that U46619 treatment altered the profiles of hDab2-interacting proteins in human platelets, implying hDab2 is downstream of TxA_2 signaling in human platelets (Supplementary Fig. 6, Supplementary Table 3, and Supplementary methods). This information sets a stage for further clinical study and is included in the Discussion of the revised manuscript (page 15, lines 367-383). We hope the Reviewer

can accept our explanation and agree with our point of view. Please let us know if more information is required by the Reviewer.

References

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The revised manuscript addresses the concerns that this reviewer raised for the first submission and is significantly improved. However, the authors did not perform experiments to prove the clinical relevance of their findings. The authors should determine the role of DAB2 in thrombocytopenic patients. I think that these experiments are feasible.

Reviewer #2 (Remarks to the Author):

The manuscript by Tsai showed the role of platelet hDab2 in reducing the bleeding time of mice with thrombocytopenia. Disabled-2 knock-in (hDab2-KI) mice were generated. hDab2-KI mice display normal appearance, platelet biogenesis, hemostasis, and thrombus formation but a reduced bleeding time in mice with thrombocytopenia. Decreased bleeding time was reversed by the ADP receptor antagonist clopidogrel and low-dose aspirin treatment. Thromboxane A2 (TxA2) generation and the vascular response to platelet activation in vivo were normal. However, hDab2 augmented TxA2 mimetic U46619- but not other agonist-stimulated granule secretion, integrin activation, and aggregation at a lower platelet concentration in vitro. They propose that hDab2 acts as an adaptor protein to facilitate the formation of the PA-hDab2-AKT complex, leading to an increase in U46619-stimulated AKT-Ser473 2phosphorylation and the subsequent first-wave of ADP/ATP release, which is crucial for the reduced bleeding of hDab2-KI mice with thrombocytopenia.

Major points
-Rousson et al. evidenced the existence of an enhanced PGI2 and TxA2 urinary metabolites excretion in the group of idiopathic thrombocytopenic purpura (ITP) patients. Moreover, the production of serum TxB2 per platelet was decreased in the ITP group. Here, the authors assessed serum TXB2. It would be interesting to know if the levels of serum TXB2 were reduced in their mice models in association with a reduced number of platelets. In fact, serum TXB2 levels are dependent on the number of platelets in whole blood.

-The revised version of the study is an improvement. However, there is still an important limitation regarding the relevance of their findings in vivo for patients with immune thrombocytopenia. It is crucial to verify the expression of hDab2 in platelets and the TP signaling. This verification should be carried out in a limited number of individuals. The authors could collaborate with other international centers or seek collaborations within their own country for this purpose.

- I wonder if the mouse tail bleeding time assessment can be used to mimic the bleeding complications seen in human immune thrombocytopenia, ranging from skin bruises to life-threatening intracranial hemorrhage.

Reviewer #1 (Remarks to the Author):

The revised manuscript addresses the concerns that this reviewer raised for the first submission and is significantly improved. However, the authors did not perform experiments to prove the clinical relevance of their findings. The authors should determine the role of DAB2 in thrombocytopenic patients. I think that these experiments are feasible.

Thanks for the Reviewer's comments. In the revised manuscript, we have recruited thrombocytopenic patients and performed experiments to investigate whether there is a correlation between hDab2 expression in platelets and the intensity of U46619-stimulated ATP release. Our data indicate that hDab2 expression in platelets displayed a strong positive correlation with the intensity of U46619-indcued ATP release which in turn elicited a strong negative correlation with the bleeding score of ITP patients. Furthermore, the expression of Dab2 had a moderate inverse correlation with the bleeding score of ITP patients. Consistent with our findings in the animal work, these data indicate that higher hDab2 expression links to higher U46619-stimulated ATP release which is associated with a lower bleeding score in ITP patients. These findings were included in the Results and Discussion section of the revised manuscript (page 7, line 228 to page 8, line 237, page 11, lines 334-338 and page 13, lines 420-426). The new set of data provide evidence of hDab2 involvement in TxA_2 signaling in thrombocytopenic patients. We believe this has strengthened the story and we would appreciate that the Reviewer agrees with our point of view.

Reviewer #2 (Remarks to the Author):

The manuscript by Tsai showed the role of platelet hDab2 in reducing the bleeding time of mice with thrombocytopenia. Disabled-2 knock-in (hDab2-KI) mice were generated. hDab2-KI mice display normal appearance, platelet biogenesis, hemostasis, and thrombus formation but a reduced bleeding time in mice with thrombocytopenia. Decreased bleeding time was reversed by the ADP receptor antagonist clopidogrel and low-dose aspirin treatment. Thromboxane A_2 (TxA_2) generation and the vascular response to platelet activation in vivo were normal. However, hDab2 augmented TxA_2 mimetic U46619- but not other agonist-stimulated granule secretion, integrin activation, and aggregation at a lower platelet concentration in vitro. They propose that hDab2 acts as an adaptor protein to facilitate the formation of the PA-hDab2-AKT complex, leading to an increase in U46619-stimulated AKT-Ser473 2phosphorylation and the subsequent first-wave of ADP/ATP release, which is crucial for the reduced bleeding of hDab2-KI

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Major points

-Rousson et al. evidenced the existence of an enhanced PGI2 and TxA_2 urinary metabolites excretion in the group of idiopathic thrombocytopenic purpura (ITP) patients. Moreover, the production of serum TxB_2 per platelet was decreased in the ITP group. Here, the authors assessed serum TxB_2. It would be interesting to know if the levels of serum TxB_2 were reduced in their mice models in association with a reduced number of platelets. In fact, serum TxB_2 levels are dependent on the number of platelets in whole blood.

In the revised manuscript, we followed the Reviewer's suggestion and determined the levels of serum TxB_2 in our mice models. Consistent with the findings of ITP patients by Rousson et al., both $ROSA26^{fl/fl}$ and hDab2-KI mice injected with anti-CD41 antibody had a lower serum TxB_2 when compared to the mice injected with the vehicle control. As indicated by the Reviewer, serum TxB_2 levels are indeed dependent on the number of platelets in whole blood. The data are reported in the Figure 4d of the revised manuscript.

-The revised version of the study is an improvement. However, there is still an important limitation regarding the relevance of their findings in vivo for patients with immune thrombocytopenia. It is crucial to verify the expression of hDab2 in platelets and the TP signaling. This verification should be carried out in a limited number of individuals. The authors could collaborate with other international centers or seek collaborations within their own country for this purpose.

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signaling in thrombocytopenic patients. We believe this has strengthened the story and we would appreciate that the Reviewer agrees with our point of view.

- I wonder if the mouse tail bleeding time assessment can be used to mimic the bleeding complications seen in human immune thrombocytopenia, ranging from skin bruises to life-threatening intracranial hemorrhage.

We agree with the Reviewer's point of view. Bleeding time assessment certainly cannot mimic the bleeding complications in human immune thrombocytopenia. However, the mouse tail bleeding time assessment and the bleeding manifestations in patients with ITP may share some common underlying mechanisms of bleeding. That is part of the reason to use the mouse tail bleeding time for eliciting the function of platelet hDab2 in thrombocytopenia. Through the study, we unveiled the potential role of hDab2 in TxA_2 signaling in mice. In addition, we showed that hDab2 expression in platelets was correlated with the intensity of TxA_2-stimulated ATP release which in turn inversely correlated with the bleeding tendency (bleeding score) of ITP patients. These findings imply that there is a common involvement of hDab2 and TxA_2 signaling in the bleeding of mice and patients with ITP. Hence, we believe it is still worthy to perform a study using the thrombocytopenic mouse model. We hope the Reviewer can accept our explanation and agree with our point of view.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed the reviewers' concerns. However, the clinical significance of this paper remains unclear.

Reviewer #2 (Remarks to the Author):

The revised manuscript by Tsai et al. has been improved. The authors performed an additional study, suggested by the reviewers, on patients with immune thrombocytopenic purpura. The results show that hDab2 expression in platelets from patients with immune thrombocytopenic purpura was positively correlated with U46619-stimulated ATP release, which in turn is inversely correlated with their bleeding tendency. These data support the results in the hDab2-KI mice that hDab2 appears crucial in regulating bleeding severity associated with thrombocytopenia by a functional interplay with ADP/ATP release underlying TXA2 signaling.

There are minor changes

- On page 6, line 198, and page 7, lines 199-201, please change the sentence with this: “Serum TxB2 is an ex vivo index of the maximal capacity of platelets to generate TXA2 in response to thrombin (generated endogenously) strictly dependent on the number of platelets (Alessandrini et al. Thromb Res . 1985 Jan 1;37(1):1-8). We found a decrease of serum TXB2 generation in ROSA26fl/fl and hDab2-KI mice injected with anti-CD41 antibody compared to mice injected with the vehicle control ($p < 0.001$) (Fig. 4d). These results reflect the decreased number of platelets in the mice injected with anti-CD41 antibody”.

-On page 10, lines 327-331:
Despite the observations that the generation of TxB2 ex vivo (serum) is relative to the platelet number in mice and humans 40,41 (add ref # Alessandrini et al. Thromb Res . 1985 Jan 1;37(1):1-8), in vivo TxA2 generation was not altered and even slightly increased in the mice with thrombocytopenia. This is consistent with the finding that platelet cyclooxygenase activity in vivo is enhanced in chronic ITP 42.

Reviewer #1 (Remarks to the Author):

The authors have addressed the reviewers' concerns. However, the clinical significance of this paper remains unclear.

Thanks for the Reviewer's comments. The clinical significance of this paper is supported by the data of ITP patients presented in Figure 7 and has been discussed in the Discussion section of the manuscript. We agree that it warrants to further increase the number of ITP patients for investigating to confirm the clinical significance of hDab2 expression in the bleeding severity of ITP patients. We hope the Reviewer accepts our explanation and agrees with our point of view.

Reviewer #2 (Remarks to the Author):

The revised manuscript by Tsai et al. has been improved. The authors performed an additional study, suggested by the reviewers, on patients with immune thrombocytopenic purpura. The results show that hDab2 expression in platelets from patients with immune thrombocytopenic purpura was positively correlated with U46619-stimulated ATP release, which in turn is inversely correlated with their bleeding tendency. These data support the results in the hDab2-KI mice that hDab2 appears crucial in regulating bleeding severity associated with thrombocytopenia by a functional interplay with ADP/ATP release underlying TXA2 signaling. There are minor changes

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Thanks for the Reviewer's comments. In the revised manuscript, we followed the Reviewer's suggestion to change sentence and add reference in the section of serum TxB_2 generation (page

6, line 204 to page 7, line 209 and page 10, lines 334-338).