Dematin Regulates Thrombosis and Early Akt Activation

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Abstract

The complex intrinsic and extrinsic pathways contributing to platelet activation exert profound impact on hemostasis and thrombosis. Detailed cellular mechanisms that regulate calcium mobilization, Akt activation, and integrin signaling in platelets remain incompletely understood. Dematin is a broadly expressed actin binding and bundling cytoskeletal adaptor protein regulated by phosphorylation via cAMP-dependent protein kinase. Here, we report the development of a conditional mouse model specifically lacking dematin in platelets. Using the new mouse model termed PDKO, we provide direct evidence that the genetic deletion of dematin in platelets inhibits the early phase of Akt activation in response to collagen and thrombin agonists in platelets. The aberrant platelet shape change and reduced *in vivo* thrombosis observed in PDKO mice will enable future characterization of dematin-mediated integrin activation mechanisms in thrombogenic as well as non-vascular pathologies.

Acknowledgment

Thank authors of references for their literature support. Thank my advisor, Dr. Athar Chishti, for his constructive comments and patient guidance and Dr. Daniel Fritz who trained me, provided the data and gave me helpful advice. Thank Dr. Lidija Covic for reading my thesis and giving precious advices. Thank Dr. Toshihiko Hanada for generating the PDKO mice and Dr. Glenn Merrill-Skoloff as well as Dr. Robert Flaumenhaft (Harvard Medical School) for performing and advising the *in vivo* thrombosis experiments. Thank Dr. Edward Plow (Cleveland Clinic) and Dr. Stephen C. Bunnell (Tufts University) for kindly providing anti-Kindlin-3 antibodies. Thank Ansel Link, Minxin Huang and Wanting Huang for supporting me for the genotyping experiments. Thank Donna-Marie Mironchuk for her contributions in the administrative organization of the project, Proofreading and improvements of figures. Finally thank my parents for always supporting and encouraging me. Their willingness to give me their time so generously has been much appreciated.

Table of Contants

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List of Abbreviations

bp= Base Pair
CBC= Complete Blood Count
CRP= Collagen Related Peptide
FLKO= Full Length of Dematin Knockout
HPKO= Headpiece Knockout
KO= Knockout
PDKO= Platelet Dematin Knockout
Pf4= Platelet Factor 4
PRP= Platelet Rich Plasma
RBC= Red Blood Cells
WT= Wild Type

Chapter 1: Introduction

1.1 Background Information

Dematin is an F-actin binding cytoskeletal adaptor protein essential for maintaining erythrocyte (RBC) shape and membrane stability (Khanna et al. 2002; Lu et al. 2016). Its modular structure of ~50 kDa consists of a C-terminal headpiece domain shared with the villin family of cytoskeletal proteins (Rana et al. 1993; Azim et al. 1995; Vardar et al. 2002). The large N-terminal core domain of dematin is somewhat unique, showing sequence similarity to its closest homologue abLIM (Fig. 1A) (Rana et al. 1993; Roof et al. 1997) but not with other members of the villin family. Dematin's broad expression in most tissues along with its regulation by phosphorylation by multiple protein kinases merit its functional evaluation in non-erythroid cells (Husain-Chishti et al. 1988; Husain-Chishti et al. 1989; Vugmeyster and McKnight 2009; Chen L et al. 2013). Furthermore, the location of the dematin gene (*DMTN* or *EBP49*) on human chromosome 8p21.3 (Kim et al. 1998), a region frequently mutated in tumors including B-cell lymphomas (Rubio-Moscardo et al. 2005), is notable and increases the putative translational applications of further investigation of dematin function.

Using immunoelectron microscopy, our previous studies visualized dematin as well as adducin at the spectrin-actin junctions that are critical for the regulation of RBC membrane mechanical properties (Derick LH et al. 1992; Chen H et al. 2007). A similar spectrin-based network attaches and supports the plasma membrane in

anucleate platelets (Patel-Hett et al. 2011). Unlike mature RBCs, platelets serve as a relatively tractable cell system to evaluate cell shape as well as multiple signaling pathways elicited by a variety of agonists (Hsu-Lin et al. 1984; Shattil and Newman 2004; Varga-Szabo et al. 2009; Wieschhaus et al. 2012; Durrant et al. 2017). Since dematin is abundant in both human and mouse platelets (Faquin et al. 1988; Wieschhaus et al. 2012), we have utilized mouse gene deletion approaches to interrogate dematin's function in platelets. Originally, we developed a mouse model with the systemic deletion of the headpiece domain of dematin (Khanna et al. 2002; Wieschhaus et al. 2012). The headpiece knockout (HPKO) mice expressing the core domain of dematin showed a mild phenotype of RBC shape change and hemolysis (Khanna et al. 2002; Wieschhaus et al. 2012). The relatively mild anemia in HPKO mice (Khanna et al. 2002) enabled us to evaluate the functional role of dematin's headpiece domain in platelets. Our previous studies using HPKO mice demonstrated that dematin lacking its headpiece domain is unable to regulate calcium mobilization in platelets (Wieschhaus et al. 2012). While these studies were informative, a valid issue was raised about the functional role of the core domain of dematin that was stably expressed in the HPKO platelets (Wieschhaus et al. 2012). To address this concern, we generated a conditional mouse knockout (KO) of the full length of dematin (FLKO) (Lu et al. 2016). Remarkably, the systemic deletion of full length dematin resulted in nearly complete fragmentation of RBCs, acute hemolysis, and unsustainable anemia in long term (Lu et al. 2016). While these studies demonstrated an essential role of the core domain in maintaining dematin function in RBCs, the

FLKO model was not suitable to study platelet functions due to massive hemolysis *in vivo* (Lu et al. 2016).

Here, we report the generation and characterization of the first tissue-specific deletion of the dematin gene in platelets. Using a multi-stage Cre-LoxP gene deletion strategy, we generated a platelet-specific dematin knockout model termed PDKO by employing a megakaryocyte-specific platelet factor 4 (Pf4) promoter-driven genetic recombination of the *DMTN* gene. This genetic approach enabled us to perform platelet function analysis without the deletion of dematin in other cell types, including RBCs. Our results demonstrate a functional role of dematin in platelet shape change, calcium mobilization, integrin signaling, and *in vivo* thrombosis. Importantly, dematin emerges as a positive regulator of early Akt activation in platelets with implications in signaling and actin cytoskeletal reorganization in other cell types.

1.2 Statement of Contributions

This chapter is revised by Dr. Chishti.

Chapter 2: Materials and Methods

2.1 Materials, antibodies, and reagents

The dematin monoclonal antibody used in this study was originally developed by the Chishti Lab in collaboration with BD Biosciences/Transduction Laboratories (#D77620). Multiple antibodies purchased from Cell Signaling Technologies (Danvers, MA, USA) are as follows: PLCγ2 (#3872); p-PLCγ2 (Y759) (#3874); Akt (#4691); p-Akt (S473) (#4058); p38 MAPK (#8690); p-p38 (T180/Y182) (#4511); p44/42 MAPK (ERK1/2) (#4695); p-p44/42 MAPK (ERK1/2) (T202/Y204) (#4377). Thrombin (#60-519) was purchased from EMD Millipore. U46619 (#16450) was purchased from Cayman Chemical Company. Collagen (#NC9533954) was purchased from Chrono-Log. ADP (#A2754) was purchased from Sigma. Platelet agonist TRAP4 was generously provided by Dr. Lidija Covic (Tufts Medical Center, Boston, MA). Collagen related peptide (CRP) was generously provided by Dr. Peter Newman (Milwaukee, WI). PGE1 (#BML-PG006) was purchased from Enzo Life Sciences.

2.2 Mice and genotyping

PDKO mice were derived from the FLKO model as previously described (Lu et al. 2016). Mice homozygous for the mutated *DMTN* allele were crossed with FRT-expressing lines to generate *DMTN* floxed/floxed (*DMTN* f/f) alleles. PDKO mice were generated by crossing *DMTN* f/f mice with *DMTN* f/f mice heterozygous for Cre driven by the megakaryocyte-specific Pf4 promoter (Pf4-Cre). PDKO (*DMTN* f/f; Cre

+/-) mice were produced from this cross at a Mendelian ratio of 1:1. Mice were genotyped using DNA digested from tail tip tissue and tested with two primer pairs. Cre expression was tested with Pf4-Cre forward primer: *CCA AGT CCT ACT GTT TCT CAC TC* and Pf4-Cre reverse primer: *TGC ACA GTC AGC AGG TT* resulting in a 400-base pair (bp) amplicon. *DMTN* f/f genotyping was performed using *DMTN* f/f forward primer: *GCC GGC TGA CTT AAG TGG GAT CC* an *DMTN* f/f reverse primer: *GTT TTC CAG GGT GAC AGC TGT TCA* resulting in a 351 bp amplicon. *DMTN* f/f; Pf4-Cre +/- mice were designated as "PDKO", while *DMTN* f/f; Pf4-Cre -/- mice were designated as "WT". Primers were designed and generated by Integrated DNA Technologies. Both WT and PDKO mice were apparently healthy upon visual inspection, with no weight or behavior disparities. No pathological mortality was observed. Differential dematin expression was further confirmed by Western blotting of purified washed platelets lysates and red blood cell membrane lysates and compared with a loading control antibody using standard techniques.

2.3 Platelet isolation

Blood was collected through venipuncture of the inferior vena cava using a 22-gauge needle and collected into 150 µL of acid citrate dextrose solution (trisodium citrate 2.5%, glucose 1.5%, citric acid 0.4%) supplemented with PGE1 (0.1 µg/mL). Blood samples were then mixed with an equivalent volume of modified Tyrode's buffer (137 mM NaCl, 10 mM HEPES, 12 mM $NaHCO_3$, 5 mM glucose, 2.5 mM KCl, 1.0 mM $MgCl_2$), incubated at 37°C for 10 minutes, centrifuged at 200g for 15

minutes in a swinging bucket centrifuge with no centrifuge braking to separate the platelet rich plasma (PRP). PRP was harvested and incubated with 1.0 mM EDTA, rested at room temperature for 10 minutes, spun at 600g for 10 minutes to pellet the platelets. The platelet pellet was washed and resuspended with fresh Tyrode's buffer and then rested at 37^{o}C prior to quantification. Platelet samples were quantified based on OD600 measurements using the formula: 6.23/ ((2.016 - OD600)-3.09) *Dilution Factor*108 = platelets/mL. Samples were subsequently diluted and prepared for specific experiments.

2.4 CBC (complete blood count)

500 μL of blood was harvested in EDTA tubes and submitted for comprehensive complete blood count analysis using IDEXX commercial service (product # 61330). Results shown are representative of 3 separate samples submitted for CBC analysis.

2.5 Platelet aggregation

Freshly harvested platelets were diluted to a concentration of 3E8 platelets/mL. Using a Chrono-Log model 700 aggregometer set to 37^{o}C and stirrer spinning at 1,200 rpm, platelet samples were supplemented with calcium (1.0 mM) immediately prior to agonist stimulation. Platelet aggregation was tested using various agonists indicated in the Results section. Platelets were pretreated with indomethacin (10 μM) 3 minutes prior to the agonist stimulation for the designated experiments. Dense granule release was assessed using the Chrono-Lume reagent (Product #395) added

and preincubated for 3 minutes before the agonist addition according to manufacturer's specifications. Results shown are representative of at least 3 separate experiments.

2.6 Platelet protein lysates and Western blotting

Washed platelets were adjusted to 4E8 platelets/mL, and platelet suspension was incubated in a Chrono-Log model 700 aggregometer at 37°C and spinning at 1,200 rpm. Platelets were supplemented with 1.0 mM calcium and stimulated by the indicated agonists or left resting. At corresponding time points, platelet stimulation was halted by cell lysis through the addition of 5X reducing sample buffer (0.5 M sucrose, 15% SDS, 312.5 mM Tris-HCl, pH 6.9, 10 mM EDTA). SDS-PAGE and Western blotting were performed using standard techniques. Anti-Kindlin-3 (D817V) rabbit mAb (Cell Signaling Technology) and Anti-Kindlin-3 (2F3) mouse mAb (Dr. Edward Plow, Cleveland Clinic) were used to detect Kindlin-3 in PDKO platelets. Results shown are representative of at least 3 separate experiments.

2.7 Tail bleeding hemostasis assay

Mouse tail bleeding assays were performed as described before (Novak et al. 1988) by cutting approximately 2 mm of mouse tail tip tissue using surgical scissors. The tail wound was submerged in 37°C saline and bleeding time was recorded. Statistical medians were determined using GraphPad Prism software.

2.8 Induction of *in vivo* Thrombosis

Laser-induced thrombosis in mice was performed as previously described (Falati et al. 2002). Briefly, mouse platelets were labeled using a platelet specific β3 integrin antibody conjugated to DyLight 649 (Emfret Analytics, Eibelstadt Germany). The progression of fibrin formation was detected using a fibrin-specific antibody (clone 59D8, labeled with DyLight 488). Laser-induced injuries triggered platelet accumulation and fibrin deposition at the site of injury, and fluorescent intensities over time were recorded. Three mice were analyzed for each condition with 10-15 observations per mouse.

2.9 Statistics

Statistical significance for all methods with the exception of laser-induced thrombosis, was determined using a Student's unpaired t-Test where $*P < 0.05$ and $**P < 0.01$. Laser-induced thrombosis significance was determined using the Mann-Whitney test on area under the curve median fluorescent intensity values of either platelet accumulation or fibrin deposition over time.

2.10 Statement of Contributions

This chapter is revised by Dr. Chishti.

Chapter 3: Results

3.1 Generation and validation of platelet dematin null mouse model (PDKO)

Previously, our laboratory generated the FLKO mouse model using a targeted gene insert in the *DMTN* (*EBP49*) gene to induce a null allele (Lu et al. 2016). This mouse model contained FRT and LoxP sites for flippase and Cre recombination. Breeding of this mouse line with a flippase-expressing line generated an allele with integrated loxP sites flanking exons 5 and 8 (Fig. 1B, top). The allele was transcriptionally intact thus retaining normal expression of dematin protein. A series of crosses with platelet factor 4-Cre mice (Pf4-Cre) resulted in the generation of mice with the following genotypes: Pf4-Cre +/-; *DMTN* f/f mice and Pf4-Cre -/-; *DMTN* f/f (Fig. 1B). The Cre recombination of exons 5-8 yielded a frame shift and a premature stop codon in exon 10, preventing translation of dematin protein (Fig. 1B, bottom). The use of the Pf4 promoter restricted Cre expression to the megakaryocyte lineage. Mice lacking dematin in platelets were designated as “platelet dematin knockout” (PDKO) and mice not expressing Pf4-Cre were designated “WT ". Mice were genotyped for Pf4-Cre (400 base pair expected size; Fig. 1C, lanes 2 and 3) and *DMTN* f/f alleles (351 base pair expected band size; Fig. 1C, lane 4). PDKO mice genotyped positive for Pf4-Cre (Fig. 1C, lane 2), while WT mice genotyped negative for Pf4-Cre (Fig. 1C, lane 3). Dematin protein expression was detected in WT and PDKO RBCs, however dematin expression was not observed in purified, washed PDKO platelets, validating that dematin expression in platelets was lost (Fig. 1D,

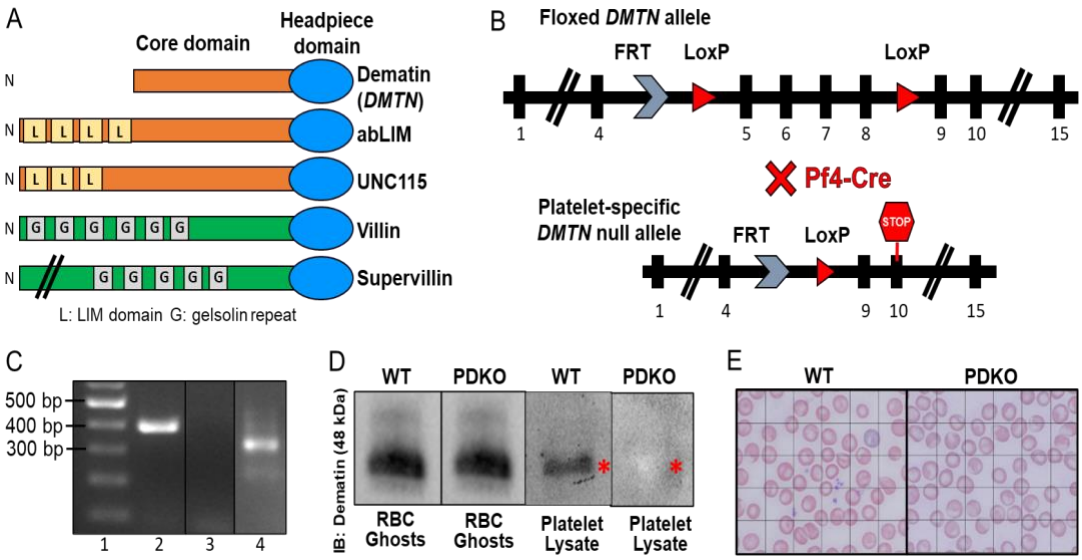

FIG. 3.1. Generation of the PDKO mouse model.

(A) Dematin is homologous to the Villin-family of proteins. Dematin shares its headpiece domain with Villin and Supervillin, but its core domain. Unique gelsolin repeats are labeled as "G" in Villin and Supervillin. Dematin shares its core domain with abLIM and UNC115 but lacks the LIM domains labeled as "L". (B) Mice homozygous for the *DMTN* (dematin) floxed allele containing loxP sites are crossed with Pf4-Cre expressing mice to recombine exons 5 through 8 of the *DMTN* gene. This yields a frame shift creating a premature stop codon in exon 10 generating an unstable dematin transcript that degrades and is not translated. (C) Genotyping strategy for PDKO mice. Pf4-Cre genotyping was performed using the Pf4-Cre forward primer: *CCA AGT CCT ACT GTT TCT CAC TC* and the Pf4-Cre reverse primer *TGC ACA GTC AGC AGG TT* resulting in an expected 400 bp amplicon. *DMTN* floxed/floxed genotyping was tested using *DMTN* floxed/floxed forward primer: *GCC GGC TGA CTT AAG TGG GAT CC* and *DMTN* floxed/floxed reverse primer: *GTT TTC CAG GGT GAC AGC TGT TCA* resulting in an expected 351 bp amplicon. Lane 1: DNA marker, Lane 2: PDKO mouse Pf4-Cre genotyping, Lane 3: WT mouse Pf4-Cre genotyping, Lane 4: *DMTN* floxed/floxed genotyping. (D) Dematin protein expression in WT and PDKO RBCs and platelets. The expected 48 kDa size band is marked by a red asterisk in platelet samples. (E) Wright stain of WT and PDKO blood smears.

asterisk indicates expected protein size of 48 kDa). As expected, RBC morphology was unaltered in the PDKO mice (Fig. 1E). Together, these data provide experimental evidence for the generation of the platelet-specific dematin null mouse model used in this study.

3.2 Hematological analysis of PDKO model

PDKO mice were bred on a C57B/6J genetic background. PDKO mice were born at the expected Mendelian ratio of 1:1, and adult mice appeared healthy with no discernible anomalies. Routine necropsy did not show any discernible changes in the anatomy of major organs of mutant mice (data not shown). Complete blood count (CBC; IDEXX BioAnalytics) of adult PDKO mice under steady state conditions revealed normal blood cell parameters, including the platelet count and volume (Fig. 2A). For subsequent platelet characterization, it was necessary to evaluate the status of RBCs due to the emerging evidence for physiological interactions between platelets and RBCs (Cines et al. 2014). Wright-Giemsa staining and SDS-PAGE analysis showed no distinct changes in the RBC shape and membrane proteins of

A

Hematology	WT	PDKO	Hematology	WT	PDKO
Platelet count (K/µL)	612.5	545	WBC (K/µL)	9.65	8.3
RBC (M/µL)	6.87	6.95	Lymphocytes (%)	85.05	87.15
MCV (fL)	46.5	47.5	Neutrophils (%)	8.9	7.15
HCT (%)	31.9	32.65	Monocytes (%)	3.6	3.85
HGB (g/dL)	10	10.25	Eosinophils (%)	2.45	1.85
MCHC (g/dL)	31.35	31.35	MPV (fL)	6.9	6.7
Absolute reticulocyte (K/µL)	182	225		6.1	6.6
Reticulocyte (%)	2.65	3.2	PDW(fL)	8.1	6.9
MCH (pg)	14.6	14.75		7.4	7.8

B

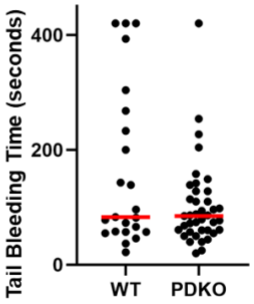

FIG. 3.2. Hematological analysis of PDKO mice.

(A) Complete Blood Count (CBC) of WT and PDKO blood samples. RBC (red blood cells), MCV (mean corpuscular volume), HCT (hematocrit), HGB (hemoglobin), MCHC (mean corpuscular hemoglobin concentration), MCH (mean corpuscular hemoglobin), WBC (white blood cells), MPV (Mean Platelet Volume), PDW (Platelet Distribution). (B) Tail bleeding time assay with statistical median: WT 80 seconds, PDKO 82 seconds. 25-30 mice of each genotype were used for the tail bleeding measurements. WT: 124.50±102.1; PDKO: 93.76±52.66, p-value 0.1346

PDKO mice (data not shown). No nucleated RBCs and Heinz bodies were detected in the peripheral blood of PDKO mice.

Finally, the PDKO mice were examined for any bleeding diathesis. No statistical difference in the tail bleeding time was observed between wild type (WT) and PDKO mice (Fig. 2B). Unlike our previous dematin headpiece and full-length whole-body knockout mouse models (Khanna et al. 2002; Lu et al. 2016), the PDKO mice do not

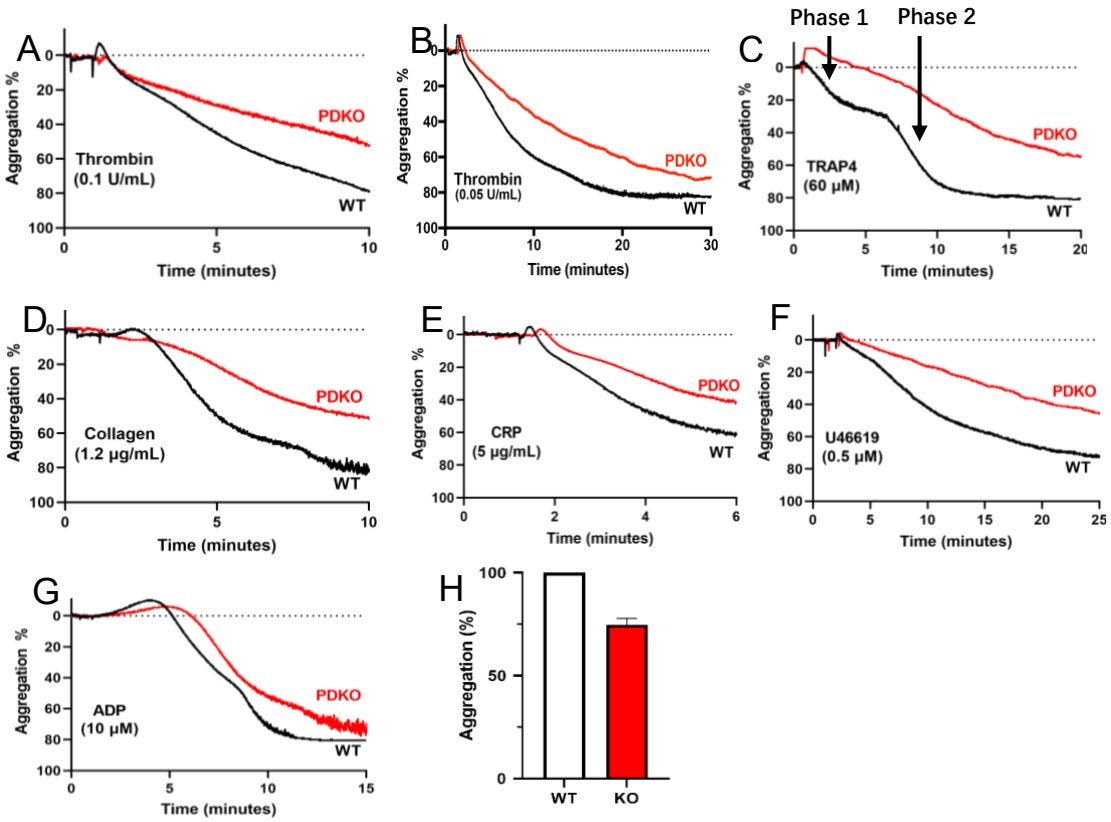

FIG. 3.3. Platelet aggregation responses to agonists in WT and PDKO.

Platelet aggregation response to (A) Thrombin (0.1 U/mL), (B) Thrombin (0.05U/mL), (C) TRAP4 (60 µM), (D) Collagen (1.2 µg/mL), (E) CRP (5 µg/mL), (F) U46619 (0.5 µM), and (G) ADP 10 µM. TRAP4-induced activation of WT platelets shows two distinct aggregation phases marked by arrows 1 and 2. TRAP4 (Thrombin receptor activating peptide 4, a PAR4 specific agonist). CRP (Collagen related peptide). U46619 (a synthetic agonist of thromboxane A2). (H) Quantification of platelet aggregation at two concentration of thrombin (0.1 and 0.05 U/ml) in WT and PDKO mice. For statistical analysis, the extent of WT platelets aggregation at each agonist concentration was taken as 100%. PDKO platelets mean value: 74.77±3.04; p-value 0.0072.

show any hemolysis concerns and therefore serve as a suitable model system for platelet function studies as outlined below.

3.3 PDKO mice exhibit broad platelet aggregation defects

Given that the core domain of dematin is critical for maintaining the cell shape and membrane stability (Mohseni and Chishti 2008; Lu et al. 2016), we hypothesized that deletion of full-length dematin may lead to functional defects in platelets. To investigate this possibility, we measured platelet aggregation response of multiple agonists by transmission light aggregometry (Fig. 3). PDKO platelets showed attenuated aggregation response to Thrombin (Figs. 3A, 3B), TRAP4 (thrombin receptor activating peptide 4, a PAR4 specific agonist (Fig. 3C), Collagen (Fig. 3D), CRP (Collagen Related Peptide) (Fig. 3E), U46619 (a synthetic agonist of thromboxane A_2) (Fig. 3F), and ADP (Fig. 3G). PDKO platelet aggregation tracings by multiple agonists indicated two potential nodes of functional defects. PDKO platelets showed reduced shape change in response to Thrombin (Fig. 3A) and Collagen (Fig. 3D) as indicated by a brief decrease in light transmission immediately after agonist stimulation. This finding is consistent with the essentiality of dematin's core domain in the regulation of cellular shape in RBCs and fibroblasts (Mohseni and Chishti 2008; Lu et al. 2016). Furthermore, the deletion of dematin predominantly impaired the progression of secondary platelet aggregation response by agonists (Fig. 3C; arrow depicts phase 2). These findings suggest that dematin may play a functional role in the regulation of downstream signaling pathways common to multiple platelet

agonist receptors, which may be impacted by the secretion of platelet granules.

3.4 Dematin is a novel mediator of early activation of Akt in platelets

Akt, also known as protein kinase B, is a potent signal transducer of multiple signaling functions in platelets. Upon stimulation by a variety of agonists, Akt activation occurs rapidly and mediates several downstream signaling cascades, including degranulation and PLCγ2 signaling, contributing to platelet activation (Yin et al. 2008; Woulfe 2010; O'Brien et al. 2012). Active Akt promotes outside-in signaling mediated by integrin αIIb/β3 upon post-ligand binding (O'Brien et al. 2012).

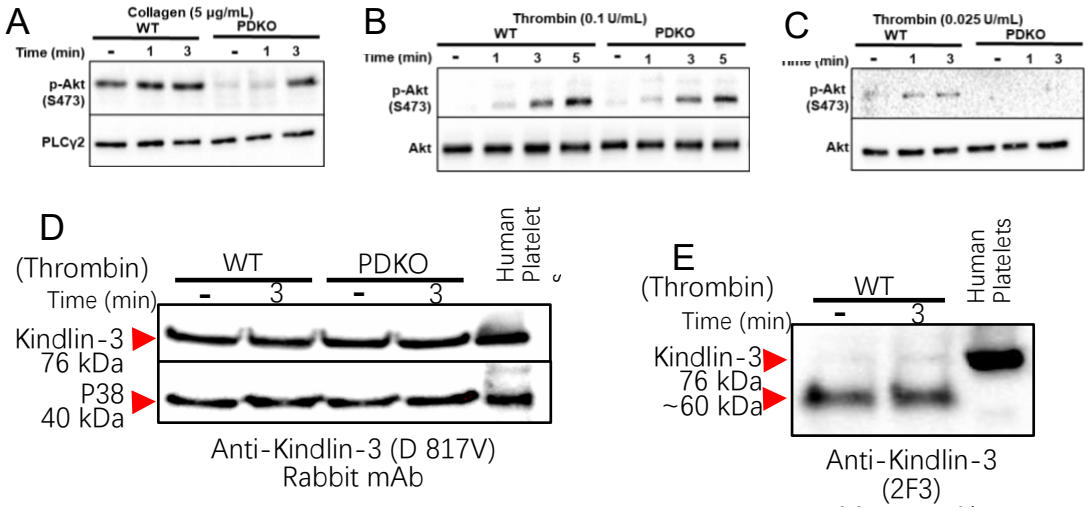

FIG. 3.4. Mechanistic analysis of dematin's regulation of platelet activation pathways.

(A-C) Phosphorylation of WT and PDKO Akt (S473) using collagen (5 µg/mL) (A), and thrombin (0.025 U/mL) (B), and thrombin (0.1 U/mL) (C). Phosphorylation of platelets was measured under resting conditions and upon stimulated at 1-, 3-, and 5-minute timepoints as indicated. (D-E) Kindlin-3 protein expression in WT and PDKO platelets. Platelets under resting and Thrombin (0.1 U/mL) stimulation (3 minutes) conditions were tested by Western blotting. (D) Anti-Kindlin-3 (D817V) rabbit mAb. (E) Anti-Kindlin-3 (2F3) mouse mAb. Note: Rabbit monoclonal anti-Kindlin-3 (D817V) detected both mouse and human kindlin-3 at ~76 kDa. Mouse monoclonal anti-Kindlin-3 (2F3) detected human Kindlin-3 but not mouse platelet Kindlin-3. The ~60 kDa band is non-specific in mouse platelets.

To investigate the status of Akt activation in PDKO platelets, we measured Akt phosphorylation (S473) at the early stage of platelet activation by multiple agonists. In response to collagen (5 μg/mL), PDKO platelets showed a near absence of Akt phosphorylation at basal levels and this inhibition persisted during the early phase of platelet activation (Fig. 4A). This Akt inhibition is in contrast to WT platelets that undergo rapid phosphorylation of Akt after 1 minute of agonist stimulation (Fig. 4A). At 3 minutes of post stimulation of platelets by collagen, the PDKO platelets eventually restored Akt phosphorylation to WT levels (Fig. 4A).

To further confirm impaired Akt activation with other agonists, we measured platelet Akt phosphorylation (p-Akt) in response to thrombin. Upon stimulation by low concentrations of thrombin (0.025 U/mL), p-Akt was inhibited in PDKO platelets both at 1- and 3-minute time points post-agonist stimulation (Fig. 4B). However, the p-Akt phosphorylation defect in PDKO platelets was rescued to WT levels at higher concentration of thrombin (0.1 U/mL) (Fig. 4C). These results indicate that dematin is required for the early activation of Akt in platelets. To our knowledge, this finding is the first demonstration that dematin regulates Akt signaling in platelets with broad implications in the regulation of cell shape change, aggregation, degranulation, and clot retraction pathways in platelets.

3.5 No effect of dematin deletion on Kindlin-3 expression

An unexpected linkage between dematin, Kindlin-3, and integrin has been previously identified vis-à-vis platelet activation and hemolytic anemia (Kruger et al.

2008; Moser et al. 2008). Kindlin-3 is expressed in erythrocytes, and Kindlin-3 mutant mice show a dramatic reduction of dematin in the erythrocyte membranes (Kruger et al. 2008). Since both dematin and Kindlin-3 are abundantly expressed in platelets, we decided to examine the impact of dematin deletion on Kindlin-3 in PDKO mice. Western blot analysis showed no effect of dematin deletion on Kindlin-3 expression in the resting and thrombin-activated platelets (Fig. 4D, E). Given that beta-adducin is also substantially reduced in kindlin-3 mutant erythrocytes (Kruger et al. 2008), there is a possibility that kindlin-3 deficient platelets may also show reduction in the beta-adducin (Hartwig and DeSisto 1991). Therefore, we cannot rule out the possibility that the generation of double knockouts mice lacking both dematin and adducin might produce synergistic effects on platelet integrin activation, aggregation, degranulation, shape change, clot retraction, and spreading as observed in the PDKO mice.

3.6 Dematin in platelets attenuates *in vivo* Thrombosis

To determine the role of platelet dematin in thrombus formation *in vivo*, we employed a well-established laser-induced thrombosis model (Falati et al. 2002; Furie and Furie 2005). The mice lacking platelet dematin generated smaller thrombi as measured by both platelet accumulation (WT: 122 x 10^{15} RFU: KO: 3.72 x 10^{15} RFU; $p < 0.001$) and fibrin deposition (WT: 12.7 x 10^{15}; 1.32 x 10^{15} RFU; $p < 0.001$) than their litter-mate controls (Figure 5). Thrombus formation *in vivo* was analyzed over

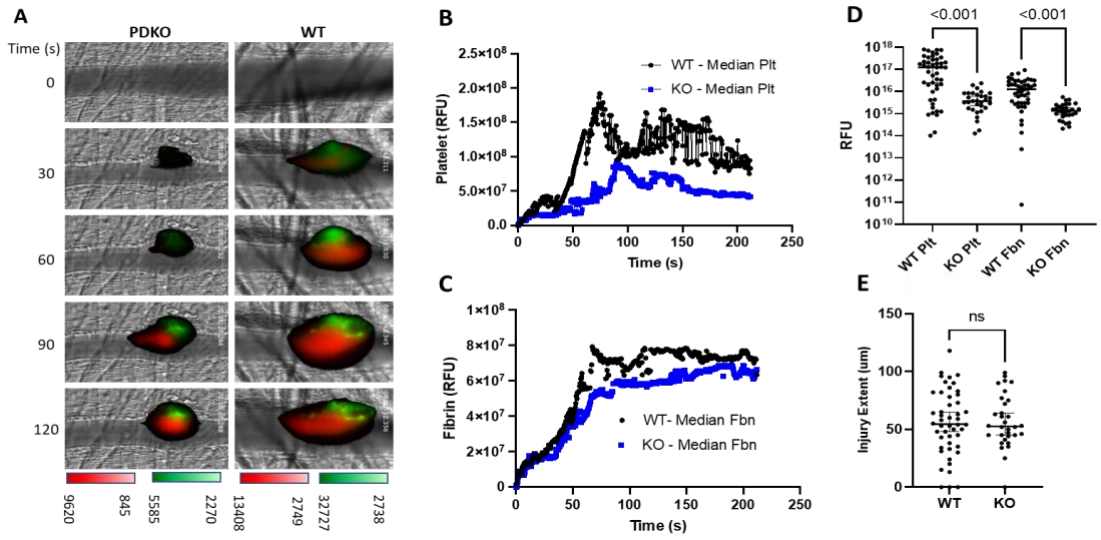

Figure. 3.5 Thrombus formation is decreased in mice with platelets lacking dematin.

Platelet accumulation and fibrin deposition following laser ablation of cremasteric arterioles were monitored in mice with a platelet/megakaryocyte-specific deficiency in dematin (PDKO) and in Cre-negative littermate control mice. (**A**) Representative images of thrombus formation taken at 30 seconds intervals. Platelets are represented in red and fibrin in green. Median curves of (**B**) platelet accumulation and (**C**) fibrin deposition in PDKO (blue squares) and WT controls (black circles) were plotted following laser ablation at time 0. (**D**) Areas under the curve from 0 to 200 seconds were calculated and plotted for all curves (PDKO: n=32 thrombi; WT: n=48 thrombi). (**E**) Injury size following laser ablation was measured to assess whether differences in platelet accumulation and fibrin deposition were due to differences in injury size.

220 seconds, and representative pictures of the first 120 seconds are shown (Figure 5A). In summary, these data suggest that mice lacking dematin in their platelets have a deficiency in clot formation.

3.7 Statement of Contributions

Blood smear and tail bleeding experiments are done by Dr. Fritz. Part of the genotyping, aggregation experiments and western blots are done by Dr. Fritz as well. *In vivo* thrombosis experiments are done by Dr. Merrill-Skoloff and advised by Dr.

Flaumenhaft. The blood count is done by IDEXX. All the other genotyping, Western blotting, aggregation experiments and data analysis are done by Yiwen Ding. This chapter is revised by Dr. Chishti.

Chapter 4: Discussion

4.1 New Mouse Model with Targeted Deletion of Full Length Dematin in Platelets

To withstand extreme shear stress during circulation, anucleate RBCs and platelets rely on their flexible spectrin-actin cytoskeletal network regulated by multiple adaptor proteins. Actin is a major cytoskeletal component constituting ~15-20% of the total protein in platelets (Bearer et al. 2002). Therefore, there is considerable interest in understanding the regulation of actin reorganization in platelets, with functional implications in both shape change and downstream signaling pathways essential for hemostasis and thrombosis. Adducin and dematin are two major actin-binding proteins sharing structural and functional similarities between RBCs and platelets (Barkalow et al. 2003; Lu et al. 2016). Here we report the first characterization of a mouse model with targeted deletion of full length dematin in platelets.

4.2 Early Akt Activation Affected

Akt activation is a crucial early timepoint signaling nexus that mediates agonist-induced signaling cascades in platelets and potentially in other cells. Akt plays an important role in GPIb-IX-mediated early signals that lead to inside-out integrin activation in platelets (Yin et al. 2008; Woulfe 2010). Related on previous dense-granule release experiments, the second phase of ATP secretion is completely ablated in PDKO platelets when activated by the thromboxane analog. The second phase is a

PI3K/Akt-dependent process. Thus, we hypothesize that dematin is able to regulate the activation of Akt. This hypothesis is consistent with the inhibition of agonist-induced early Akt phosphorylation that recovered 3 minutes after stimulation of PDKO platelets (Fig. 4A-C).

4.3 Thrombosis Affected

When dematin is deleted from platelets, both the accumulation of platelets and the deposition of fibrin are attenuated at the site of a laser induced injury in KO mice when compared to their littermate controls, indicating that the deletion of dematin in platelets can attenuate in vivo thrombosis. Both the median accumulation curves (Figure 5 B, C) and areas under all curves (Figure 5D) for both platelet and fibrin support this conclusion (Figure 5 A-D). The size measurements of injuries, as determined by assessing the longitudinal length of vessel wall damage caused by laser ablation, are similar in both the experimental and control conditions (Figure 5 E). These *in vivo* data are consistent with the platelet function studies demonstrating impaired platelet aggregation in platelets lacking dematin.

In summary, the selective and complete deletion of dematin in the PDKO model demonstrates a functional role of dematin in early phosphorylation of Akt post-agonist activation and thrombosis in platelets. The absence of a bleeding phenotype in PDKO mice is relevant for therapeutic targeting of dematin in future pharmacological and translational applications.

4.4 Statement of Contributions

This chapter is revised by Dr. Chishti.

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