Dematin Regulates Calcium Mobilization and Signaling in Platelets

BY
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THESIS
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This thesis is dedicated to my friends and family, without whom it would never have been accomplished.
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<tr>
<td>ACD</td>
<td>Acid citrate dextrose</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<td>DKO</td>
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<td>Dense tubular system</td>
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<td>IP_{4}</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PDI</td>
<td>Phosphate disulfide isomerase</td>
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<td>PGE_{1}</td>
<td>Prostaglandin E_{1}</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PRP</td>
<td>Platelet rich plasma</td>
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<td>SOCE</td>
<td>Store-operated calcium entry</td>
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<td>STIM_{1}</td>
<td>Stromal interaction molecule-1</td>
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<td>TRAP_{4}</td>
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<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiocyanate</td>
</tr>
<tr>
<td>TxA₂</td>
<td>Thromboxane A₂</td>
</tr>
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<td>vWF</td>
<td>Von Willebrand factor</td>
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<td>WT</td>
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1. LITERATURE REVIEW

1.1 DEMATIN

1.1.1 An introduction to dematin

Dematin, also known as protein 4.9, is a peripheral membrane protein that links the spectrin-actin junctions to the plasma membrane in erythrocytes (Khan et al., 2008, Khanna et al., 2002). Dematin is also a substrate of multiple protein kinases, and phosphorylation by cAMP-dependent protein kinase (PKA) reversibly abolishes its actin bundling function in vitro (Husain-Chishti et al., 1988, Husain-Chishti et al., 1989).

1.1.2 The primary structure of dematin

Biophysical measurements including nuclear magnetic resonance studies of the primary structure of dematin have shown a fully folded headpiece domain linked to a primarily unfolded core domain (Chen et al., 2009, Frank et al., 2004). The sequence of the carboxyl-terminal headpiece domain is highly homologous to the headpiece domain found in the villin-type family of cytoskeletal proteins (Khanna et al., 2002). Additionally, the amino-terminal core domain of dematin shows sequence similarity to the LIM domain containing proteins termed ABLIMs (Roof et al., 1997). Native dematin purified from human erythrocyte membranes forms a trimer in solution composed of two polypeptides of 48 kDa and one polypeptide of 52 kDa (Husain-Chishti et al., 1989, Siegel et al., 1985). These two isoforms of dematin are generated by alternative RNA splicing of a single gene located on human chromosome 8p21 (Azim et al., 1995). The 48 kDa isoform of dematin, composed of 383 amino
acids, predicts a protein with an isoelectric point of 9.54, whereas the 52 kDa isoform consisting of 405 amino acids is generated by a 22-amino acid insertion within the headpiece domain (Khanna et al., 2002). It is to be noted that although the 383 amino acids of dematin predict a protein of 43 kDa, the two isoforms of dematin isolated from erythrocytes migrate as 48 kDa and 52 kDa bands on SDS-polyacrylamide gels (Husain-Chishti et al., 1988). This anomalous electrophoretic migration is likely due to the presence of a cluster of negatively charged residues within the core domain shared between the two isoforms of dematin (Khanna et al., 2002). While the headpiece domain contains the major actin-binding site, the core domain contains a second minor actin-binding site as well as mediates dematin's interactions with the plasma membrane (Azim et al., 1995, Chen et al., 2007, Khan et al., 2008).
FIGURE 1: Structural representation of the 52 kDa isoform of dematin. In the N-terminal core domain there exists a PEST motif (containing multiple proline residues) that is targeted in proteolysis, a cysteine residue involved in dematin complex formation (C194), and a PolyAcidic motif (containing multiple negatively charged residues) believed to be important for subcellular targeting. In the C-terminal headpiece domain there exists an actin binding site, a 22 amino acid variable insert (that differentiates 48 and 52 kDa isoforms) containing an additional cysteine residue (C336), and a PKA phosphorylation site (S403).

---

PEST Sequence
STSPPSPPEVWE
PolyAcidic Motif
GAEEEEEEEDDDSGEEM
Actin Binding Motif
SRLQSTEFSPSGSET
PKA Phosphorylation Site
C194
C336
S403
N
0
C
405
Core Domain
Headpiece Domain
Insert Region
1.1.3 The expression and function of dematin

Dematin is located at the spectrin-actin junction in the erythrocyte membrane (Derick et al., 1992). The spectrin-actin junction, also called the junctional complex, is critical for maintaining the mechanical properties of the erythrocyte membrane (Bennett et al., 2001). A multiprotein complex consisting of spectrin, actin, dematin, adducin, protein 4.1R, tropomyosin, tropomodulin, calmodulin, and p55/MPP1 assembles at each junction. In erythrocytes, this multiprotein complex is linked to the plasma membrane by several transmembrane proteins including glycophorin C, band 3, and glucose transporter-1 (GLUT1) in a species-specific manner (Bruce et al., 2003, Khan et al., 2008).

To determine the physiological function of dematin in vivo, we generated a dematin headpiece knockout (HPKO) mouse model (Khanna et al., 2002). The HPKO mice are characterized as having defects in erythrocyte shape, membrane stability, and membrane-cytoskeletal interactions (Khanna et al., 2002). Later, we generated a double knockout mouse model lacking both the headpiece domain of dematin and β-adducin (DKO) (Chen et al., 2007). The DKO mice exhibit severe hemolytic anemia with further increased abnormalities in erythrocyte shape and membrane stability compared to HPKO mice (Chen et al., 2007). These findings demonstrated that dematin and adducin perform a critical overlapping function at the spectrin-actin junctions regulating erythrocyte shape and membrane stability in vivo (Chen et al., 2007).

Despite our improved understanding of dematin’s function in mature erythrocytes, its function in non-erythroid cells has just begun to be appreciated. Dematin is a widely expressed protein with immunoreactive polypeptides present in heart, brain, skeletal muscle, lung, and
kidney (Faquin et al., 1988, Khanna et al., 2002). Using the HPKO mouse model, we recently demonstrated that dematin headpiece domain deletion results in impaired wound healing in vivo, delayed cell migration, and enhanced fibroblast adhesion (Mohseni et al., 2008, Mohseni et al., 2009). Moreover, dematin was identified as a suppressor of RhoA activation in mouse fibroblasts (Mohseni et al., 2008, Mohseni et al., 2009). This finding is consistent with the role of ABLIM family of proteins in the regulation of Rho family of GTPases and the actin cytoskeleton (Barrientos et al., 2007). Interestingly, both adducin and RhoA have been functionally implicated in the regulation of the actin cytoskeleton and signaling pathways in platelets (Barkalow et al., 2003, Leng et al., 1998).
1.2 THE PLATELET

1.2.1 Biogenesis and anatomy

One of the cell types that has shown a high expression of the dematin protein is the platelet (Faquin et al., 1988, Zahedi et al., 2008). Platelets are anuclear hematopoietic cells produced by megakaryopoiesis (Breton-Gorius et al., 1986, Junt et al., 2007). In megakaryopoiesis, large polyploid cells in the bone marrow called megakaryocytes rearrange their entire cytoplasm to produce pseudopod protrusions known as proplatelets (Italiano et al., 1999).

At rest, platelets exhibit a discoid shape with a diameter of approximately 3 μm and a thickness of 0.5 μm. Once produced, these cells circulate at levels of approximately 150-400 x 10⁹/L in the blood (Cardigan et al., 2005). Under normal physiological conditions, a platelet can circulate within the body for up to 10 days. At that point, the cell will be cleared from the peripheral circulation and destroyed by splenic macrophages.

Notable features of a platelet include various receptors, a clear cytoplasm, a cytoskeletal network, glycogen, organelles, and a complex membrane system. In order to respond to a diverse assortment of stimuli, platelets have been endowed with a host of receptors. These assorted glycoproteins are found on the plasma membrane and classified into different types including tyrosine kinase receptors, integrin family receptors, and G-protein-coupled receptors (GPCRs) (Rivera et al., 2009).

GPCRs are the largest and most diverse collection of membrane receptors found in platelets (Vassilatis et al., 2003). Due to their diversity, they are responsible for inducing an
array of signaling pathways depending on the stimulus. An extracellular N-terminus, seven transmembrane α-helices, and an intracellular C-terminus characterize this family of integral proteins. Upon binding to an external signaling molecule, the GPCR undergoes a conformational change, which triggers a reaction between the receptor and a nearby G protein. A G protein is a heterotrimeric guanine nucleotide binding protein located at the plasma membrane, composed of α, β, and γ subunits. Upon activation of the GPCR and subsequent G protein engagement, G\textsubscript{α} exchanges GDP for GTP and the G protein trimers (α and β-γ) dissociate. Following trimer separation, the two subunits interact with other membrane proteins to propagate the signal transduction cascade. Together, GPCR-mediated signaling events are responsible for platelet shape change, secretion, and aggregation.

The platelet cytoskeleton is built from long actin filaments cross-linked by accessory proteins (Hartwig, 2006). Although this network is found throughout the platelet, there is an increased concentration at the cell periphery (Boyles et al., 1985). Additionally, a long, coiled microtubule lies beneath the plasma membrane and is responsible for maintaining the platelet’s discoid shape. Finally, a spectrin-based membrane skeleton is located beneath the lipid bilayer (Fox et al., 1988, Hartwig et al., 1999). In a resting platelet, the complete skeletal network protects the cell against the oppressive shear force experienced during circulation. In activated and aggregating platelets, the cytoskeleton interacts with the internal signaling molecules and receptors to induce rapid cytoskeletal reorganizations required for platelet signaling (Fox, 1985, Fox et al., 1996).

Platelets contain a large amount of glycogen, which can be metabolized as an energy source for the cell. The cytoplasm, in particular, is rich in glycogen. Here, glycogen particles are
randomly distributed both individually and en masse (Murata et al., 1977). In addition to glycogen, various organelles are dispersed randomly within the cytoplasm. These include a limited number of mitochondria (4-7), which consist of a simple, tubular structure. These platelet mitochondria power oxidative phosphorylation, energy metabolism, and the regulation of other cellular functions (Fukami et al., 1973). Although there are alternative sources of energy, the mitochondria are sufficient to support any energy requirements of the platelet on their own.

Also within the platelet are three types of granules: alpha granules, dense granules, and lysosomes. The alpha granules (α-granules) represent the most abundant of the platelet organelles. These secretory granules contain heterogeneous cargo due to their unique compartmentalization feature. Their cargo includes both membrane-bound (integrins, receptors, and P-selectin) and soluble proteins (fibrinogen, vWF, β-thromboglobulin, platelet factor 4, and albumin).

Dense granules (δ-granules) are another secretory organelle found in platelets. These granules are relatively smaller than alpha granules and possess electron dense cores. Similar to the mitochondria, dense granules are found in a limited number ranging from 4-8 within the platelets. Dense granules contain small non-protein molecules such as the adenines (ADP and ATP) and ionized calcium. Additionally, dense granules contain basic compounds such as serotonin and epinephrine. Upon platelet activation, these granules release their effector molecules to stimulate paracrine activation of nearby platelets through positive feedback. Interestingly, dense granules require relatively higher agonist concentrations to stimulate release than alpha granules (Kaplan et al., 1979).
The lysosome (λ-granules) is also a platelet secretory organelle. Whereas alpha granules and dense granules are only found in megakaryocytes and platelets, lysosomes are present in all cell types. These granules contain acid hydrolases, which can degrade proteins, carbohydrates, lipids, and nucleic acids. Upon secretion, lysosomes release more slowly and incompletely than alpha or dense granules (Holmsen et al., 1982).

Two distinct membranous channel systems exist in platelets, the open canalicular system and the dense tubular system. The open canalicular system (OCS) is an elaborate system of tunneling invaginations that is contiguous with the platelet plasma membrane (White, 1972). Upon platelet activation, the OCS acts as a conduit for granule secretion by enabling granule fusion with its membrane and cargo extrusion. Concomitantly, the OCS rapidly enlarges resulting in a loss of its tubular shape, and increases the overall platelet surface area, an important feature for thrombosis (Klinger, 1996).

The second membrane system, the dense tubular system (DTS), is derived from the smooth endoplasmic reticulum of megakaryocytes or nucleated cells (White, 1972). The DTS is the primary site for sequestration, storage, and regulation of calcium (Menashi et al., 1984, Robblee et al., 1973). Additionally, prostaglandin endoperoxides (Prostaglandin G2, Prostaglandin H2) and TxA2 are synthesized within the DTS (Gerrard et al., 1976, Hamberg et al., 1973, Needleman et al., 1976).
FIGURE 2: Platelet Anatomy. Components of the platelet include the plasma membrane (PM) and underlying filaments of the membrane skeleton. A perimeter is constructed beneath the plasma membrane by a banded microtubule (MT) coil. Indentations in the plasma membrane form the channels of the open canalicular system (OCS). Glycogen is randomly dispersed in the clear cytoplasm as well as organelles including mitochondria, alpha granules (α), dense granules (δ), and lysosomes (λ). The dense tubular system (DTS) is a site of calcium storage. Multiple platelet receptors function in adhesion (GPIb-IX-V, GPVI, α2β1), activation (PAR, TPR, P2YR), and aggregation (αIIbβ3).
1.2.2 Hemostasis

The primary function of a platelet is to act as a surveillance cell that monitors the integrity of blood vessels. Under physiological conditions, platelets circulate within the blood as flat, oval discs in a quiescent state. Following an arterial blood-vessel injury, matrix proteins of the underlying subendothelium are uncovered. One of these proteins, collagen, immobilizes and unfolds circulating plasma von Willebrand factor (vWF), a multimeric glycoprotein. This vWF unfolding exposes the macromolecule’s A1 domain (Barg et al., 2007, Siedlecki et al., 1996). The unfolded A1 domain can then tether subendothelial collagen to the platelet glycoprotein Ibα (GPIα) receptor on circulating platelets (Andrews et al., 1999, Ruggeri, 1999). However, this complex dissociates rapidly preventing stable adhesion and causing platelet rolling (Savage et al., 1998). Therefore, both GPVI and integrin α2β1 receptors serve as additional contacts on the platelet to bind collagen and secure the platelet to the site of injury (Moroi et al., 1996, Nieswandt et al., 2003, Santoro, 1986).

Platelet adhesion receptors do more than stably anchor platelets to the site of vascular injury; they also perform an important role in transducing signals necessary for efficient platelet activation. Many of these platelet responses occur simultaneously in a mechanism known as inside-out signaling (Shattil et al., 2004). Morphological changes, including shape change and spreading, are primarily driven by internal actin transformations (Leistikow, 1996). Shape change is considered to be the first measurable physiological response produced by platelets, following exposure to an agonist. During shape change, the activated platelet expands from a biconcave discoid to a compact spheroid. This rounding effect is primarily due to depolymerization of the microtubule coil by gelsolin, a calcium-activated filament-severing
protein. This transition is accompanied by an increase in F-actin content by severing actin polymers originally concentrated at the cell center. Gelsolin is then removed, and the Arp 2/3 complex assembles the F-actin into long, thin actin bundles called filopodia. These filopodia radiate outward from the cell periphery and serve to detect other platelets and strands of fibrin (Hartwig, 2006).

To adhere closer to the vessel wall, platelets undergo additional cytoskeletal reorganizations and flatten out. This flattening forces the platelet granules and organelles into the center of the cell. The closely adherent platelet extends many flat, cytoplasmic lamellipodia between the two banks of the wounded vessel. The multiple lamellipodia coalesce to form a monolayer over the wound and seal the gap. Although the cellular volume remains constant, there is an increase in the surface area of up to 400% after platelet spreading (Raucher et al., 2000).

The mechanism of exocytosis in platelets is similar to other cells such as neurons. The cargo of granules includes soluble effector molecules such as ADP and precursors of Thromboxane A2 (TxA2). In the case of dense granules, secretion is initiated by the rapid generation of TxA2 (T_{1/2} of TxA2 = 30 seconds) by either of two methods. Primarily diglyceride lipase hydrolyzes DAG, resulting in the liberation of arachidonic acid (AA), which is a substrate for cyclooxygenase-1 (COX-1) (Bell et al., 1979). The product of COX-1, prostaglandin H$_2$, is then metabolized to TxA2 by thromboxane synthase. An alternative method is by the activation of cytosolic Phospholipase A$_{2\alpha}$, which also liberates AA and produces TxA2 (McKean et al., 1981). Once TxA2 is generated, platelet secretion consists of the movement of granules in proximity to the plasma membrane, the fusion of membranes, and the release of contents.
Additionally, other granule contents including adhesive receptors and clotting factors are translocated to the platelet surface. Here, factor assembly and activation take place leading to a localized coagulation cascade and the generation of the terminal protease, thrombin (Tocantins, 1957). Together, these steps function to enhance platelet activation and thrombus formation through autocrine or paracrine recruitment.

Inside-out signaling events ultimately result in conformational changes in $\alpha_{\text{IIb}}\beta_3$, the predominant integrin in platelets. These changes lead to an up-regulation in the adhesive function of the $\alpha_{\text{IIb}}\beta_3$ receptor, which can then bind vWF, fibrinogen, fibrin, and fibronectin (Bennett et al., 1979, Hantgan, 1988, Lopez et al., 2005, Ni et al., 2000, Ruggeri et al., 1982). In particular, the binding of bivalent soluble fibrinogen by activated $\alpha_{\text{IIb}}\beta_3$ generates a cross linking of platelets and forms stable aggregates (Jackson, 2007).

Ligand binding of fibrinogen to $\alpha_{\text{IIb}}\beta_3$ receptors generates receptor-mediated signaling cascades in what is known as outside-in signaling (Shattil, 1999). Briefly, activated integrin $\alpha_{\text{IIb}}\beta_3$ tethers actin filaments and filopodia. With the aid of contractile proteins, stress fibers pull and retract the strings of fibrin between platelets. This contraction reduces the clot volume by up to 90% and functions to prevent premature platelet disaggregation by stabilizing the thrombus. The successful culmination of these events results in the successful formation of a thrombus, a vital event in limiting bleeding at the site of injury (Li et al., 2010).
FIGURE 3: Platelet responses. (A) Inactive circulating platelets are minimally adherent to the blood vessel wall. (B) However, upon injury the wall is denuded and a platelet can contact the exposed adhesive matrix proteins. (C) The adherent platelet undergoes cytoskeletal rearrangements and projects pseudopods, (D) which help to form a complete monolayer over the injury. (E) Platelet secretion releases effector molecules to attract additional cells and increases membrane receptors to strengthen the adhesive abilities of the platelet. (F) The binding of fibrinogen amongst activated platelets generates stable aggregates. (G) Finally, stress fibers pull inwardly to retract the strings of fibrin between platelets, and (H) form a stable clot.
1.2.3 Thrombosis

Platelets play an important role in both health and disease, and although platelet responses to vascular injury are vital they must also be tightly controlled. In diseased blood vessels, the rupture of atherosclerotic plaques denudes the endothelium and results in unwanted platelet adhesion and activation (Stoll et al., 2008). Additionally, too much or unnecessary platelet activation can lead to the unregulated propagation of an intravascular thrombus. A complete occlusion of the vessel will block blood flow and lead to hypoxia and infarction of distal tissues (Varga-Szabo et al., 2008). The thrombotic occlusion of a coronary artery results in acute myocardial infarction, whereas the thrombotic occlusion of a cerebral artery causes an acute ischemic stroke. Importantly, these two pathologies are among the leading causes of mortality in industrialized countries (Packham, 1994, Ruggeri, 2002).
**FIGURE 4:** The development of thrombosis. Adhesion of platelet to the vascular endothelium causes platelet activation. Consequently, activated platelets secrete their chemokines and express adhesion molecules. Circulating cells are recruited and adhere to the platelet surface. Too much platelet activation can lead to the unregulated propagation of an intravascular thrombus. An acute occlusion of the vessel will block blood flow and lead to hypoxia and infarction of distal tissues.
1.3 CALCIUM

1.3.1 Calcium regulation in platelets

Cells contain a multitude of diverse signaling pathways that govern their activities and functions. The calcium (Ca\(^{2+}\)) ion is a ubiquitous second messenger common to several of these cellular processes. In platelets, Ca\(^{2+}\) regulates many essential responses including granule secretion, integrin activation, and phosphatidylserine (PS) exposure (Harper et al., 2010). Of the total platelet Ca\(^{2+}\), only a small fraction is freely available in the ionized form. This low cytosolic concentration of Ca\(^{2+}\) (50-100 nM) is used to keep platelets in their resting state or to aid in restoring the resting state after activation. To maintain intracellular levels of Ca\(^{2+}\), the platelet uses many pumps and channels including Ca\(^{2+}\)/ATPases (SERCAs and PMCAs) and Na\(^{+}/Ca^{2+}\) exchangers (NCXs) (Dean et al., 1994).

The largest fraction of platelet Ca\(^{2+}\) is sequestered within the dense tubular system (DTS), an internal membranous structure. However, lower levels of Ca\(^{2+}\) has been detected in organelles or bound to membranes and cytoplasmic proteins (Lopez et al., 2005). During platelet signaling, the DTS-compartmentalized calcium is released into the cytosol to propagate multiple signal transduction cascades. Afterwards, an additional supply of calcium is influxed from the extracellular medium to consolidate and supplement further platelet signaling (Varga-Szabo et al., 2011). Furthermore, calcium is released from the dense granules during secretion as a positive feedback mechanism to facilitate aggregation.
**1.3.2 Platelet signaling leads to increases in cytosolic calcium**

To initiate intracellular signaling cascades, an agonist binds to its corresponding receptor located on the surface of the platelet. This interaction causes an isoform of the PLC enzyme to be activated. In the case of GPCRs (G-protein coupled receptors), the PLC-β isoform is activated through the Gq protein. Alternatively, integrins and receptors coupled to an immunoreceptor tyrosine-based activation motif (ITAM) activate the PLC-γ isoform through Syk (Daniel et al., 1994, Offermanns, 2000, Watson et al., 2001). These newly activated PLC isoforms catalyze hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to produce the second messengers, inositol 1,4,5-trisphosphate (IP$_3$), and 1,2-diacylglycerol (DAG). While IP$_3$ is involved in the release of Ca$^{2+}$ from the intracellular stores, DAG induces the influx of Ca$^{2+}$ from the extracellular medium and initiates dense granule secretion (Bell et al., 1979, Berridge, 1993, Bird et al., 2004).

Soluble IP$_3$ freely diffuses through the cytosol and directly docks to the IP$_3$ receptor (IP$_3$-R). The IP$_3$-R is a membrane glycoprotein complex that channels Ca$^{2+}$ ions and is divided into 3 classes. Type 1 and 2 IP$_3$-Rs are the most predominant members in platelets and are found on the intracellular membranes. The lesser-expressed type 3 receptors are located at the plasma membrane and function to regulate cation levels (El-Daher et al., 2000).

Although the IP$_3$-R pathway is well characterized, the physiological inhibition of this pathway is less well understood. One mode of pathway regulation is proposed to occur through inhibitory phosphorylation of the IP$_3$-R (Ferris et al., 1991, Komalavilas et al., 1994, Supattapone et al., 1988). A second and more attractive method of pathway regulation is by preventing IP$_3$ from reaching its target receptor. It is well established that inositol
trisphosphate 3-kinase (IP$_3$K) metabolizes IP$_3$ to its lesser active form, inositol 1,3,4,5-tetakisphosphate (IP$_4$) in an ATP-dependent manner (Berridge et al., 1989). Out of the three isoforms of IP$_3$Ks, inositol trisphosphate 3-kinase B (IP$_3$KB) is the only isoform that is almost ubiquitously expressed in mammalian cells. More specifically, IP$_3$KB is localized exclusively at the membranes of Ca$^{2+}$ storage sites (Schell et al., 2006, Soriano et al., 1997). Furthermore, IP$_3$KB regulates cytoplasmic Ca$^{2+}$ signals in multiple cells (Miller et al., 2007, Nalaskowski et al., 2011, Stokes et al., 2006). Currently, the anthracyclin drug doxorubicin is successfully used as an IP$_3$K inhibitor (da Silva et al., 1994). Inhibition of IP$_3$K results in the increase in intracellular Ca$^{2+}$ (Chang et al., 2002). Unfortunately, very little is known regarding the mechanism of IP$_3$KB phosphorylation and its effects on Ca$^{2+}$ mobilization in platelets at this time.

### 1.3.3 The extracellular medium is a source for additional cytosolic calcium

The initial increase of cytosolic Ca$^{2+}$ from the finite intracellular stores is supplemented by Ca$^{2+}$ entry from the external medium (Sage, 1997). This secondary influx of calcium from the extracellular medium is achieved by store-operated Ca$^{2+}$ channels (SOCC) in a mechanism termed “store operated calcium entry (SOCE)” (Varga-Szabo et al., 2011). There are two key components of SOCE. Calcium-release activated calcium modulator 1 (Orai1) is a plasma membrane protein that mediates Ca$^{2+}$ influx across the plasma membrane by forming porous ion channels (Bergmeier et al., 2009). The second key player, stromal interaction molecule 1 (STIM1) is a transmembrane protein expressed on the DTS. STIM1 serves as a Ca$^{2+}$ sensor to regulate Orai1 (Varga-Szabo et al., 2008). When the intraluminal Ca$^{2+}$ concentration is reduced in the DTS, STIM1 relocates and directly interacts with Orai1, triggering SOCE. The relative
contributions of these different Ca\textsuperscript{2+} entry pathways to agonist-induced Ca\textsuperscript{2+} signaling and Ca\textsuperscript{2+}-dependent platelet responses are not well understood. Moreover, how Ca\textsuperscript{2+} signaling is regulated during platelet activation to provide sufficient signal is not known.

1.3.4 Intracellular calcium propagates platelet responses

During platelet activation, cytosolic Ca\textsuperscript{2+} can reach 1-2 μM depending on the agonist (Tsien et al., 1982). This heightened calcium level is responsible for a variety of allosteric enzyme activations within the platelet. For example, calcium can liberate arachidonic acid from membrane phospholipids (Gilliam et al., 1991). Additionally, Ca\textsuperscript{2+}-dependent myosin light-chain kinase phosphorylates myosin light chain protein allowing for cytoskeletal rearrangements essential in platelet shape change (Paul et al., 1999). Furthermore, the calcium-dependent enzyme, calpain, proteolyzes many cytoskeletal and membrane substrates including integrin α\textsubscript{IIb}β\textsubscript{3} (Schoenwaelder et al., 2000). This proteolysis of integrin α\textsubscript{IIb}β\textsubscript{3} is necessary for the transition from its resting to active state, the hallmark of platelet activation (Shattil et al., 2004).
**FIGURE 5: Calcium signaling in platelets.** Receptor activation by various agonists activates isoforms of PLC. PLC hydrolyzes PIP$_2$ into IP$_3$ and DAG. IP$_3$ activates the IP$_3$R and causes Ca$^{2+}$ release from the DTS. Once internal stores are depleted, STIM1 opens Orai1 channels allowing for extracellular Ca$^{2+}$ influx. Ca$^{2+}$ flux is also regulated by plasma membrane Ca$^{2+}$ ATPases (PMCA), DTS Ca$^{2+}$ ATPase pumps (SERCA), Na$^+$/Ca$^{2+}$ exchangers (NCX), and ATP gated channels (P2X$_3$s).
2. PURPOSE OF STUDY

Although dematin is a widely expressed protein, its function in non-erythroid cells has just begun to be appreciated. Prior work from our lab indicates that dematin plays important roles in a wide array of cellular functions including cytoskeletal reorganization, membrane stability, wound healing, migration, adhesion, and RhoA inhibition (Mohseni et al., 2009).

One of the cell types showing a relatively high expression of the dematin protein is the blood platelet; however its role in these cells remains to be determined. Interestingly, proteins similar to dematin, such as adducin, have been functionally implicated in the regulation of the actin cytoskeleton and signaling pathways in platelets (Schmidt et al., 1998). Therefore, the purpose of this study is to provide the first comprehensive characterization of dematin's function in platelets. In this dissertation, I used a dematin headpiece null mouse as a model system to explore the functional consequences of dematin deficiency in platelets. Our results demonstrate a novel function of dematin in the regulation of calcium flux in platelets upon stimulation by a variety of physiological agonists. These findings place dematin as a key modulator of calcium homeostasis and thus platelet activation. We believe these findings can improve our understanding of platelet signaling pathways and provide new insights into the regulation of platelet functions.
3. MATERIALS AND METHODS

3.1 Antibodies and reagents. Anti-Rho antibody (ARH01) was purchased from Cytoskeleton Inc., anti-Rac1 antibody was purchased from EMD Inc., and HRP-conjugated secondary antibodies were purchased from Upstate Biotechnology and Santa Cruz Biotechnology. Bovine Thrombin, Apyrase, Prostaglandin E\(_1\), Leupeptin, Aprotinin, and the anti β-actin antibody were purchased from Sigma. Antifade and Phalloidin-Alexa-594 were obtained from Molecular Probes.

3.2 Isolation of platelets. Blood was collected from healthy human donors and adult mice. Generally, 5-6 wild type (WT) and headpiece knockout (HPKO) mice were anesthetized by inhalation of 3% isoflurane and blood was collected from the inferior vena cava. Blood was anticoagulated with \(1/7\)th volume (\(-15\%\)) of ACD (85 mM Trisodium citrate, 83 mM Dextrose, and 21 mM Citric acid), and platelet-rich plasma (PRP) was harvested by centrifugation of the blood at 180 x g for 15 minutes at room temperature. The PRP was centrifuged for 10 minutes at 600 x g in the presence of 0.1 \(\mu\)g/ml PGE\(_1\), and 5 mM EDTA. Washed platelets were resuspended in modified Tyrode’s buffer (10 mM HEPES, 12 mM NaHCO\(_3\), 137 mM NaCl, 2.5 mM KCl, 5.0 mM glucose, 1.0 mM MgCl\(_2\), and 0.35% BSA). Platelet count was adjusted to 0.2-20 \(\times\) \(10^8\)/ml. Platelets were allowed to rest for 60 minutes, and 1.0 mM CaCl\(_2\) was added prior to the start of each experiment.
3.3 Platelet spreading assay. Glass coverslips were coated with 100 µg/ml human fibrinogen in 0.1 M NaHCO₃ at 4°C overnight, and then blocked with 5% BSA for 90 minutes. Washed WT and HPKO mouse platelets (5 x 10⁷ platelets/ml) were pre-incubated with DMSO or 10 µM indomethacin and 2.0 U/ml apyrase for 30 minutes. Platelets were added to each well and allowed to incubate for up to 90 minutes at 37°C with 5% CO₂. The wells were washed and fixed with 4% paraformaldehyde in PBS. Platelets were treated with permeabilization buffer (100 mM Tris-HCl, pH 7.4, 10 mM EGTA, 154 mM NaCl, 5.0 mM MgCl₂, 0.5 mM Leupeptin, 1.0 mM PMSF, and 0.1% Triton X-100) and blocked with 5% BSA. Phalloidin-Alexa 594 at 2.0 µg/ml was added to visualize F-actin. The coverslips were mounted to glass slides and immunofluorescence images were recorded using a 100X objective on a Nikon TE-2000E microscope. Platelet surface area was calculated using MetaMorph® software. For quantification of platelet surface area, 10 random fields of acquired images were analyzed and statistical analysis was performed using the Student’s t-test.

3.4 Immunofluorescence analysis. Glass coverslips were coated with 100 µg/ml human fibrinogen in 0.1 M NaHCO₃ (pH 8.3) at 4°C overnight, and wells were blocked with 5% BSA. Washed human platelets (5 x 10⁷ platelets/ml) were added to each well and incubated for 60 minutes at 37°C. After washing, platelets were fixed with 4% paraformaldehyde in PBS, treated with the permeabilization buffer (100 mM Tris-HCl, pH 7.4, 10 mM EGTA, 154 mM NaCl, 5 mM MgCl₂, 0.5 mM Leupeptin, 1.0 mM PMSF, and 0.1% Triton X-100), and blocked with 5% BSA. Dematin monoclonal, PDI monoclonal, and dematin polyclonal antibodies were used with the appropriate secondary antibodies. Finally, the coverslips were mounted to slides
and immunofluorescence images were recorded using a 100X objective on a Nikon TE-2000E inverted microscope.

**3.5 Western blotting.** Western blotting of dematin in human (\(3 \times 10^8\) platelets/ml) and mouse (\(2 \times 10^9\)/ml) platelets was performed using multiple antibodies directed against the core domain of dematin (Husain-Chishti et al., 1988). The dematin antibodies used in this study recognized both human and mouse dematin in a comparable fashion, as indicated in our previous studies (Chen et al., 2007, Khan et al., 2008). Immunoblotting of mouse dematin was performed according to the protocol previously developed in our laboratory for mouse fibroblasts (Mohseni et al., 2008). Briefly, washed WT and HPKO platelets were treated with the proteasome inhibitors, Lactacystin (10 \(\mu\)M) and MG-132 (5 \(\mu\)M), for 30 minutes at 4°C. Platelet fractions were then isolated by sonication and centrifugation. The membrane fraction was probed with a dematin polyclonal antibody and a polyclonal \(\beta\)-tubulin antibody from Sigma. Human platelet fractions were isolated using a previously published protocol (Kovacs et al., 1997). Platelet fractions were probed with a dematin monoclonal antibody, a PDI monoclonal antibody to detect the dense tubular system (DTS), and a monoclonal antibody against GPIb\(\alpha\) to detect the plasma membrane fraction. A monoclonal antibody (sc-100385) against human IP\(_3\)KB was purchased from Santa Cruz Biotechnology, and a monoclonal antibody against RhoA was used as a loading control.

**3.6 Measurement of cAMP in platelets.** cAMP measurements were performed using a protocol as previously described (Gilman, 1970, Srinivasan et al., 2009). Briefly,
phosphodiesterase inhibitor Ro 20-1724 (100 μM) was added to WT and HPKO platelets. The platelets were centrifuged and the pellet fraction was snap frozen in liquid nitrogen. The platelet pellet was resuspended in 50 mM sodium acetate buffer, sonicated, boiled for 4 minutes, and then snap frozen to precipitate proteins. Upon thawing, the samples were centrifuged and the supernatant was transferred to a new tube to quantify cAMP. A standard curve of cAMP was generated to quantify cAMP levels in the samples. Samples were incubated with \(^3\)H-cAMP, a PKA inhibitor, and PKA. Potassium phosphate (20 mM) was then added, and samples were filtered to collect the cAMP-PKA complex. Filters were then dissolved with Cellosolve and radioactivity was measured using a Beckman LS 6500 liquid scintillation counter.

3.7 Dense granule secretion measurements. Platelet secretion was measured using the Luciferin/Luciferase reagent (Chrono-lume) to detect ATP release from platelet dense granules. WT and HPKO platelets (2 x 10^8 platelets/ml) were incubated for 3 minutes at 37°C prior to the addition of Chrono-Lume reagent. After 2 minutes of incubation, the platelets were stimulated with specific agonists (0.6-1 μg/ml collagen, 0.25-0.5 μM U46619, and 60-80 μM TRAP4), and platelet secretion response was recorded. All experiments were recorded in real time in a model 700 Chrono-log lumiaggregometer at 37°C with stirring (1,000 rpm).

3.8 Aggregation measurements. Platelet aggregation was measured by detecting changes in light transmission. Washed mouse platelets (2 x 10^8 platelets/ml) were treated with a specific agonist at a range of concentrations to establish a dose response curve. The following agonists
were used: 0.6-2.0 μg/ml Collagen, 0.25-1.0 μM U46619, 5-10 μM ADP, and 40-100 μM TRAP4. All experiments were recorded in real time in a model 700 Chrono-log Lumiaggregometer at 37°C with stirring (1,000 rpm). Statistical significance was determined using a Student’s t-test.

3.9 RhoA Activation Assay. RhoA activity was assessed using a G-LISA according to the manufacturer’s protocols (Cytoskeleton, Inc.). Briefly, WT and HPKO washed platelets (2x10⁸/ml) were stimulated with thrombin (1 U/ml), lysed, and centrifuged. Binding buffer was added to each tube, and the sample was transferred to a RhoA assay plate. Anti-RhoA 1° antibody (1:250), and Anti-mouse 2° antibody (1:5000) were added to the wells and the sample was analyzed using a microplate reader. Total RhoA was determined by Western blotting.

3.10 Platelet fractionation. Platelet fractions were isolated similar to a previously established procedure (Kovacs et al., 1997). Briefly, washed human and mouse platelets (2 x 10⁹ platelets/ml) were incubated in lysis buffer (1% NP-40, 50 mM Tris-HCl, 154 mM NaCl, 1 mM Na₃VO₄, 1 mM EGTA, 1 mM NaF, with protease inhibitors), and centrifuged at 19,000 x g for 25 minutes to remove intact platelets, granules, and mitochondria. The supernatant was centrifuged at 100,000 x g for 60 minutes. The resulting pellet was resuspended and represents the mixed membrane fraction, and the high speed supernatant represents the cytosolic fraction. The two fractions were adjusted to equivalent protein concentrations for Western blotting using a BCA protein assay (Pierce). For additional studies, the fraction containing mixed membranes was layered over a 40% sucrose gradient and centrifuged at 100,000 x g for 120 minutes. The resulting higher density band represents the dense tubular system (DTS),
and the band at the sucrose interface represents the plasma membrane fraction. Material in each fraction was washed and re-centrifuged before the Western blotting analysis.

3.11 Flow cytometry. Washed WT and HPKO platelets (1 x 10⁸ platelets/ml) were stimulated with an agonist and incubated for 5 minutes at 37°C without stirring. Platelets were incubated with reagents conjugated to a fluorophore (P-selectin or JON/A) for 15 minutes at room temperature under cover. Finally, PBS was added to each sample to terminate the reaction, and the samples were immediately analyzed with a FACSCalibur flow cytometer (BD Biosciences) to measure FSC (platelet size), P-selectin expression (α-granule secretion), and JON/A expression (integrin α₁β₃ activation). Statistical significance was determined using a Student’s t-test.

3.12 Clot retraction. Blood from 6 WT and 6 HPKO mice was anticoagulated with 1/10th volume of 3.8% sodium citrate. PRP was isolated by centrifugation of the whole blood at 100 x g for 15 minutes. The platelet count in PRP was adjusted with platelet-poor plasma (PPP), and packed erythrocytes were added to enhance the contrast of the clot. After resting for 60 minutes, 2.0 mM CaCl₂ was added and the PRP was stimulated with thrombin (0.4 U/ml) and gently mixed. Platelets were allowed to incubate at 37°C for up to 12 hours. Two-dimensional size of the retracted clots was quantified using the NIH Image J software, and clot retraction was expressed as the percent retraction [1-(final clot size/ initial clot size)]. Statistical significance was determined using a Student’s t-test.
3.13 Tail bleeding assay. Bleeding time was measured by cutting off the tip (5.0 mm) of the tail from each WT and HPKO adult (8-12 weeks) mouse. The tail was immediately immersed in PBS at 37°C. The time until the stable cessation of bleeding was recorded. Bleeding experiments were terminated after 420 seconds if the tail bleeding did not stop. Statistical significance was determined using a Student’s t-test.

3.14 Measurement of cytoplasmic Ca\(^{2+}\) concentration. Washed platelets (7.5 x 10\(^8\) platelets/ml) from WT and HPKO mice were resuspended in modified Tyrode’s buffer. 15 µM FURA-PE3/AM conjugate (Calbiochem) was added to each sample, and they were allowed to incubate for 30 minutes at RT in the dark. The samples were centrifuged and the platelets were resuspended in modified Tyrode’s buffer (containing 0.1% BSA) and diluted to 1.0 x 10\(^8\) platelets/ml. At this point, 1.0 mM CaCl\(_2\), 200 µM EGTA, or 10 µM Doxorubicin were added depending on the experiment. The platelets were loaded onto a 96-well assay plate and incubated for one hour. The agonists (Collagen, U46619, TRAP\(_4\), A23187, and Thapsigargin) were added robotically and the samples were analyzed using a FlexStation II (Molecular Devices). Platelet samples were excited at 340/380 nm and calcium fluorescence was measured at the emission wavelength of 510 nm. The relative Ca\(^{2+}\) fluorescence (FL) value was computed by dividing the fluorescence at 340 nm by the fluorescence at 380 nm for each time point. Statistical significance was determined using a Student’s t-test.

3.15 Proteomics and immunoprecipitation. Washed platelets were resuspended in lysis buffer and the lysate was incubated on ice for 30 minutes to promote complete lysis. The cell
lysate was centrifuged for 25 minutes at 15,000 x g to remove any intact and aggregated platelets. Immunoprecipitation was then performed on the lysates using a Crosslink-IP kit from Pierce. Briefly, the lysate supernatant was pre-cleared with control beads for one hour to remove any non-specific interactions. The pre-cleared lysate was incubated with Protein A/G beads cross-linked to dematin monoclonal antibody for 2 hours at room temperature. A monoclonal antibody against GAKIN was used as a negative control under the same conditions. The beads were washed, and bound proteins were eluted and analyzed by SDS-PAGE. The gel was stained with colloidal Coomassie Blue, and prominent protein bands were excised and analyzed by mass spectrometry as described before (Khan et al., 2008). The presence of dematin in the immunoprecipitate was confirmed by Western blotting using a dematin monoclonal antibody and TrueBlot secondary antibody from eBioscience. To detect the dematin-IP₃KB complex, platelet lysate was immunoprecipitated with a monoclonal antibody against IP₃KB, and dematin in the immunoprecipitate was detected using a monoclonal antibody against dematin followed by the TrueBlot secondary antibody (as described above). This biochemical association was confirmed with a reverse co-IP experiment in which dematin-immunoprecipitated from human platelet lysates was probed with an IP₃KB monoclonal antibody and TrueBlot secondary anti-mouse antibody.
4. RESULTS

4.1 Dematin is abundantly expressed in platelets

Previous immunoblotting and proteomics studies have indicated the presence of dematin in platelets (Faquin et al., 1988, Zahedi et al., 2008), but its function in platelets has not been investigated. Using a panel of monoclonal and polyclonal antibodies against human and mouse dematin, which have been previously characterized in our laboratory (Chen et al., 2007, Mohseni et al., 2008), we examined the expression of dematin in platelets by immunoblotting and immunofluorescence assays. Human platelets were solubilized with SDS-sample buffer, and total protein fraction was analyzed by Western blotting using a monoclonal antibody directed against the core domain of dematin. As compared to human erythrocytes where the 48 kDa isoform of dematin is dominant (Fig. 6A, left lane), human platelets predominantly express the 52 kDa isoform of dematin (Fig. 6A, right lane). A similar expression profile of dematin was observed in both human and mouse platelets (Fig. 6B). Quantification of Western blots indicated the relative expression of dematin in mouse platelets is 40.1% as compared to human platelets. At a higher exposure of the Western blots, a second, faint band of 48 kDa is also detectable in both human and mouse platelets (Fig. 6A and B). Using a dematin monoclonal antibody, immunofluorescence analysis confirmed abundant expression of dematin in human platelets (Fig. 6C). The subcellular punctate distribution of dematin appears to be concentrated at the cell periphery as well as at the center of the platelet. In addition, biochemical fractionation of human platelets indicated that dematin is associated with both membrane and cytosolic compartments (Fig. 6D). Densitometry-based
quantification of Western blots indicated that 68.0% of dematin is associated with the membrane fraction, whereas 32.0% is recovered in the cytosolic fraction of human platelets under resting conditions.
FIGURE 6: Dematin expression in human and mouse platelets. (A) Human RBC membrane lysate and human platelet (3 x 10⁶/ml) lysate were analyzed by Western blotting using a dematin-specific monoclonal antibody directed to the core domain. The same material was blotted using an antibody against RhoA. (B) The larger 52 kDa isoform of dematin was detected in both the human platelet lysate and mouse platelet membrane. The same material was blotted using an antibody against RhoA. (C) Immunofluorescence analysis of dematin in human platelets. An abundant punctate staining pattern of dematin is detected at the membrane as well as in the center of the cell. Scale bar = 5 μm. (D) Lane 1, human platelet lysate; lane 2, membrane; lane 3, cytosol fractions were normalized for protein and analyzed for relative dematin and RhoA expression by Western blotting.
4.2 Status of dematin in HPKO platelets

The abundant expression of dematin implied a functional role in platelet physiology. To investigate dematin’s function in mature platelets, we utilized our HPKO mouse model system (Khanna et al., 2002). These mice have a genetically deleted headpiece domain of dematin (Fig. 7A). The membrane fraction from WT and HPKO platelets was subjected to Western blotting using an affinity-purified polyclonal antibody against the core domain of mouse dematin. As expected, the mutant dematin protein was truncated in the HPKO platelets as compared to the WT platelets (Fig. 7B). Densitometry analysis indicated ~65% reduction in the expression of truncated dematin in the HPKO platelets, as compared to WT platelets (Fig. 7C). These observations suggest that the truncated core domain of dematin is either unstable or the expression of truncated dematin is reduced in the HPKO mice, and therefore the amount of truncated dematin is significantly decreased in the HPKO platelets. These findings are consistent with our previous observations showing only partial retention of the truncated dematin in the HPKO erythrocyte membranes (Chen et al., 2007, Khanna et al., 2002). Further hematological and biochemical analysis of resting platelets indicated no measurable differences in the platelet number, volume, basal cAMP level, and integrin αIIbβ3 expression in the HPKO and WT platelets (Table 1).
FIGURE 7: Dematin expression in HPKO platelets. (A) Presence of truncated dematin in HPKO platelets. (B) Platelet lysates from WT and HPKO mice were subjected to Western blotting using a polyclonal antibody directed against the core domain of dematin. β-tubulin was used as a loading control. (C) Quantification of dematin core domain expression in the WT and HPKO platelets using densitometry (Bio-Rad Quantity One®). Data represent the mean of two independent experiments.
<table>
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<td>1394 ± 44</td>
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<td>Mean Platelet Volume, fL</td>
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<td>Basal cAMP, pmoles/10^8 platelets</td>
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<td>Surface expression of integrin α_{IIb}β_{3/} MFI</td>
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<td>29.8 ± 3.4</td>
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**TABLE 1: Platelet analysis of dematin HPKO mice.** Data are mean plus or minus SEM (n=5). MFI = mean fluorescence intensity.
4.3 HPKO platelet spreading on fibrinogen matrix

Dematin has been implicated as a modulator of actin dynamics in erythrocytes and fibroblasts (Azim et al., 1995, Mohseni et al., 2008). This view is consistent with the observation that the headpiece domain of dematin shows sequence similarity to the villin headpiece domain, a known regulator of actin reorganization in response to various stimuli (Ferrary et al., 1999). Because of functional similarities in the components of the membrane-skeleton between erythrocytes and platelets (Barkalow et al., 2003, Patel-Hett et al., 2011), we investigated the effects of headpiece domain deletion on platelet spreading and actin reorganization. First, platelets from WT and HPKO mice were spread on a fibrinogen-coated surface for up to 90 minutes, and platelet spreading was monitored by staining with TRITC-Phalloidin to detect F-actin. A measurable defect in platelet adhesion and spreading was observed in HPKO mice, mainly at the earliest time point (Fig. 8). In contrast, when a similar spreading assay was performed using platelets that were treated with the secretory inhibitors apyrase and indomethacin, the HPKO platelets did not show any measurable difference in their ability to spread or adhere on the fibrinogen-coated surface as compared to WT controls (Fig. 9).
FIGURE 8: HPKO platelets display abnormal platelet spreading in the absence of secretory inhibitors. WT and HPKO mouse platelets (5 x 10^7 platelets/ml) were spread up to 90 minutes on fibrinogen-coated coverslips. Immunofluorescence analysis was performed using Phalloidin-Alexa 594 to detect F-actin. Scale bar represents 10 μm. Images are representative of three separate experiments. (B) Adhesion was determined by random counts of platelet number per field of view and graphed (n=10). (C) Cell surface area (mean ± SEM) of WT and HPKO platelets using the Metamorph® software was determined and graphed. Error bars represent the SEM from three separate experiments.
FIGURE 9: HPKO platelets display normal platelet spreading in the presence of secretory inhibitors. WT and HPKO mouse platelets (5 x 10^7 platelets/ml) were incubated with 2.0 U/ml apyrase and 10 μM indomethacin, and spread up to 90 minutes on fibrinogen-coated coverslips. (A) Immunofluorescence analysis was performed using Phalloidin-Alexa 594 to detect F-actin. Scale bar represents 10 μm. (B) Graph representing adhesion, as determined by random counts of platelet number per field of view (n=10). (C) Cell surface area (mean + SEM) of WT and HPKO platelets determined using the Metamorph® software. Error bars represent the SEM from three separate experiments.
4.4 HPKO platelets exhibit defective granule secretion

A platelet spreading phenotype that is dependent upon the release of internal agonists would imply a defect in platelet dense granule secretion (Naik et al., 2003). Dense granules contain many secretory components such as ATP, ADP, serotonin, epinephrine, and calcium. We measured ATP release to determine the functional effect of dematin headpiece domain deletion on dense granule secretion. WT and HPKO platelets were stimulated with multiple agonists (Collagen, U46619, and TRAP4), and ATP release was quantified using a luciferase assay. Our results indicated that dense granule secretion was significantly impaired in the HPKO platelets as compared to WT platelets (Fig. 10). The impaired platelet secretory response was observed at 0.6 µg/ml Collagen (Fig. 10A), 0.5 µM U46619 (Fig. 10B), and 60 µM TRAP4 (Fig. 10C). Interestingly, the HPKO platelet secretion defect was partially restored at high concentrations of agonists (Fig. 10D). It is to be noted that except for TRAP4, the effect remained significant for all agonists.

Alpha granules represent the second secretory organelle responsible for maintaining hemostasis in platelets. These granules are storage sites for P-selectin, von Willebrand factor, and various clotting proteins (Chen et al., 2005). We measured the surface expression of P-selectin to determine the effect of dematin headpiece deletion on platelet alpha granule secretion. WT and HPKO platelets were stimulated with multiple agonists (Collagen, U46619, and TRAP4), incubated with a PE-conjugated monoclonal antibody against P-selectin, and flow cytometry was used to quantify P-selectin expression. Our results indicated that alpha granule secretion was also defective in the HPKO platelets. Specifically, the expression of P-selectin was impaired at 1.0 µg/ml collagen, 0.25 µM U46619, and 60 µM TRAP4 (Fig. 10E).
Again, increasing the concentration of agonists partially restored the granule secretion defect in HPKO platelets. These findings suggest that dematin is important for the normal secretion of both alpha and dense granules in mouse platelets.
FIGURE 10: Abnormal platelet secretion in HPKO platelets. Dense granule secretion was evaluated by measuring the ATP release from WT (blue) and HPKO (red) platelets induced by (A) 0.6 μg/ml collagen (38.9 ± 3.9 in WT vs. 12.1 ± 1.2 in HPKO, P < 0.05); (B) 0.5 μM U46619 (51.3 ± 5.4 in WT vs. 29.8 ± 3.3 in HPKO, P < 0.05); and (C) 60 μM TRAP4 (31.2 ± 2.9 in WT vs. 17.5 ± 1.8 in HPKO, P < 0.05). (D) The maximum ATP release by three agonists is shown in a histogram. (E) Alpha granule secretion was determined by flow cytometry of P-selectin exposure on WT or HPKO platelets. Platelets were activated with collagen (0.6 and 1.0 μg/mL) (for 1.0 μg/mL, the values were 50.0 ± 5.4 in WT vs. 23.4 ± 3.7 in HPKO, P < 0.05), U46619 (0.25 and 0.5 μM) (for 0.25 μM U46619, the values were 30.7 ± 1.8 in WT vs. 11.9 ± 1.1 in HPKO, P < 0.05), or TRAP4 (60 and 80 μM) (for 60 μM TRAP4, the values were 38.1 ± 2.9 vs. 30.5 ± 1.8 in HPKO, P < 0.05), and labeled with FITC-labeled anti-P-selectin mAb. The level of P-selectin exposed on the surface of platelets is expressed as mean fluorescence intensity (MFI). Error bars represent the SEM from three separate experiments. * represents a p-value of less than 0.05.
4.5 Platelet aggregation is reduced in HPKO mice

Granule secretion is necessary for amplifying the platelet aggregation responses (Remijn et al., 2002). As a consequence, defective granule secretion in the HPKO platelets would suggest a functional abnormality in the platelet aggregation reaction. To test this prediction, WT and HPKO platelets were stimulated with multiple agonists (Collagen, U46619, ADP, and TRAP4), and the platelet aggregation responses were measured by monitoring a change in light transmission upon agonist stimulation. Our results indicated that platelet aggregation was inhibited in the HPKO platelets activated by multiple agonists (Fig. 11).

Reduced platelet aggregation was observed at 0.6 µg/ml Collagen (Fig. 11A), 0.5 µM U46619 (Fig. 11B), 10 µM ADP (Fig. 11C), and 60 µM TRAP4 (Fig. 11D). Similar to the secretion response, inhibition of platelet aggregation in the HPKO mice was more prominent at low concentrations of Collagen, U46619, and TRAP4 (Fig. 11E).
FIGURE 11: Aggregation response in HPKO platelets. WT (blue) and HPKO (red) platelets were analyzed for their aggregation response to (A) 0.6 µg/ml collagen (56.2% ± 6.2 in WT vs. 15.5% ± 4.1 in HPKO, P < 0.05), (B) 0.5 µM U46619 (79.8% ± 10.0 in WT vs. 41.2% ± 5.3 in HPKO, P < 0.05), (C) 10 µM ADP (75.3% ± 4.7 in WT vs. 60.8% ± 4.5 in HPKO, P < 0.05), and (D) 60 µM TRAP4 (69.8% ± 4.6 in WT vs. 43.3% ± 3.8 in HPKO, P < 0.05). (E) Data for maximum aggregation are shown in the histogram. Error bars represent the SEM from three separate experiments. *represents a p-value of less than 0.05.
4.6 RhoA and Integrin α\textsubscript{IIb}β\textsubscript{3} activation are defective in HPKO platelets

Impaired platelet secretion and aggregation responses in HPKO mice suggested the existence of additional signaling defects in the mutant platelets. Besides secretion, platelet inside-out signaling exerts a concomitant effect on RhoA activation and integrin α\textsubscript{IIb}β\textsubscript{3} activation (Shattil et al., 2004). The small GTPase RhoA, a critical regulator of the cytoskeleton, is believed to aid in platelet aggregate formation (Morii et al., 1992). To test the effect of dematin deficiency on RhoA, we examined the status of platelet RhoA activation. WT and HPKO platelets were activated by thrombin, and a G-LISA assay was used to measure RhoA activation. Consistently, we observed a decrease in the activation of RhoA (Fig. 12A).

Additionally, we measured integrin α\textsubscript{IIb}β\textsubscript{3} activation in HPKO mice. WT and HPKO platelets were activated by multiple agonists (Collagen, U46619, ADP, and TRAP4), and incubated with the PE fluorophore conjugated to JON/A, an antibody that specifically recognizes the activated form of integrin α\textsubscript{IIb}β\textsubscript{3}. Integrin activation was measured by flow cytometry as represented by the mean fluorescence intensity (MFI). The results indicated that the activation of integrin α\textsubscript{IIb}β\textsubscript{3} is significantly reduced in HPKO platelets in response to multiple agonists (Fig. 12B). Together, these observations suggest that dematin plays an important role in platelet inside-out signaling as confirmed by defects in cell secretion, aggregation, RhoA, and integrin α\textsubscript{IIb}β\textsubscript{3} activation.
**FIGURE 12: RhoA and integrin activation in HPKO platelets.** To further assess the status of inside-out signaling, WT (blue) and HPKO (red) platelets were analyzed for RhoA activation in response to 1 U/ml thrombin (0.98 ± 0.23 in WT vs. 0.55 ± 0.06 in HPKO, P < 0.05). (B) Integrin α_{IIb}β_{3} activation was assessed by flow cytometry of WT and HPKO platelets. Platelets were activated by collagen (0.6, 1.0 μg/ml) (0.6 μg/ml collagen: 51.2 ± 12.8 in WT vs. 31.1 ± 8.5 in HPKO, P < 0.05), U46619 (0.25, 0.5 μM) (0.25 μM U46619: 98.4 ± 17.7 in WT vs. 38.8 ± 20.9 in HPKO, P < 0.05), ADP (5, 10 μM), and TRAP4 (60, 80 μM) (60 μM TRAP4: 125.3 ± 13.6 in WT vs. 79.1 ± 10.3 in HPKO, P < 0.05). PE-labeled anti-integrin α_{IIb}β_{3} mAb (JON/A) was used to specifically detect the activated conformation of mouse integrin α_{IIb}β_{3}. Error bars represent the SEM from three separate experiments. *represents a p-value of less than 0.05.
4.7 HPKO mice show defects in the maintenance of hemostasis

Integrin $\alpha_{IIb}\beta_3$ activation regulates the initiation of outside-in signaling in platelets (Chen et al., 1994, Flevaris et al., 2007, Law et al., 1996, Shattil et al., 1998). The outside-in signaling pathway in turn regulates clot retraction and tail bleeding phenotypes. Therefore, we examined the status of the outside-in signaling pathway in HPKO mice using a clot retraction assay. For this assay, platelet-rich plasma from the WT and HPKO mice was stimulated with thrombin (0.4 U/ml), and allowed to incubate at 37°C for up to 12 hours (Fig. 13). The clot volume was measured at each time point, and percent retraction was quantified. The results indicated that clot retraction was markedly inhibited in the HPKO platelet-rich plasma (Fig. 13). Statistical analysis showed that the clot volume difference was significant at every time point (Fig. 13G).

It is well-known that addition of MnCl$_2$ can directly activate integrin $\alpha_{IIb}\beta_3$, and thus bypass integrin activation via the inside-out signaling pathway (Haling et al., 2010). Consistent with this mechanism, the addition of MnCl$_2$ rescued the clot retraction defect in HPKO mice (Fig. 14). These findings suggest that the clot retraction defect observed in HPKO mice originates from integrin $\alpha_{IIb}\beta_3$ inactivation, and is unlikely to be a consequence of defective outside-in signaling in mutant platelets.

Improper platelet-mediated plug formation in the vessel wall leads to impaired hemostasis in mice. Generally, the tail bleeding assay can provide one measure of the in vivo hemostatic function. Therefore, a bleeding assay was performed by cutting the tip of the tail from WT (n=40) and HPKO (n=38) mice, and immersing the tip in saline at 37°C. The time until the stable cessation of bleeding occurred was recorded to determine the mean bleeding time.
The HPKO mice displayed slightly longer bleeding times as compared to WT mice (65.2 seconds ± 8.2 in WT vs. 73.5 seconds ± 7.6 in HPKO) (Fig. 14C).
FIGURE 13: HPKO mice display features of unstable thrombosis. (A) Clot retraction phenotype of WT and HPKO mice. Platelet-rich plasma was incubated at 37°C with thrombin (0.4 U/ml) for (A) 0, (B) 30 minutes, (C) 45 minutes, (D) 60 minutes, (E) 120 minutes, and (F) 720 minutes. (G) The clot volume of each time point is plotted in a histogram. Error bars represent the SEM from three separate experiments. *represents a p-value of less than 0.05
FIGURE 14: Outside-in signaling is normal in HPKO platelets (A) Clot retraction phenotype of WT and HPKO mice. Platelet-rich plasma of WT and HPKO mice was incubated with 0.5 mM MnCl₂ to activate the integrin αIIbβ₃ receptor. Thrombin (0.4 U/ml) was added, the platelets were incubated for up to 720 minutes, and clot retraction was reassessed. (B) The clot volume with MnCl₂ of each time point is plotted in a histogram. Error bars represent the SEM from three separate experiments. * represents a p-value of less than 0.05 (C) The role of dematin in thrombosis was investigated using a tail-bleeding assay. Bleeding times for WT (n=40) and HPKO mice (n=38) are shown. Horizontal lines indicate the mean bleeding time for each population.
4.8 HPKO platelets exhibit defective intraplatelet calcium flux

Previous studies have shown that defects in the pathway of integrin $\alpha_{\text{IIb}}\beta_3$ activation do not necessarily correspond to defects in the platelet secretion pathways (Zhang et al., 2011). This indicates that there are multiple subpathways involved in inside-out signaling. Therefore, it is a likely possibility that dematin may regulate a shared step in inside-out signaling that is upstream of these divergent signaling outcomes. Calcium mobilization is one such signaling event that occurs prior to both platelet secretion and integrin $\alpha_{\text{IIb}}\beta_3$ activation in platelets (Jantzen et al., 2001). In fact, platelet activation by any stimulatory agonist results in the elevation of cytosolic calcium (El-Daher et al., 2000). As a result, calcium release in platelets is vital for the transduction of converging pathways into subsequent inside-out signaling events. To investigate this possibility, we examined the status of calcium signaling response in HPKO platelets. Washed platelets in the presence of 1.0 mM CaCl$_2$ were labeled with FURA-PE3/AM, a cytosolic calcium-sensitive dye, and platelets were activated by various agonists (Collagen, U46619, TRAP$_4$, ADP, and A23187). Calcium fluorescence measurements indicated that there was a significant defect in calcium mobilization upon agonist stimulation in HPKO platelets (Fig. 15). Furthermore, the addition of calcium ionophore, A23187, induced a complete response in both WT and HPKO platelets (Fig. 15E). Interestingly, the HPKO platelets were hyperreactive to this agonist. This finding indicates that HPKO platelets compensate for deficiencies in internal calcium flux by increasing their ability to transport calcium across the plasma membrane. The calcium mobilization defect in HPKO platelets was somewhat tempered as the concentration of agonists was raised (Fig. 15F).
FIGURE 15: HPKO platelets show a significant defect in total calcium flux. WT (blue) and HPKO (red) washed platelets in the presence of 1.0 mM CaCl\(_2\) were analyzed for calcium mobilization in response to: (A) 1.0 µg/ml collagen (2.31 ± 0.76 in WT vs. 1.52 ± 0.72 in HPKO, P < 0.05), (B) 0.5 µM U46619 (2.13 ± 0.22 in WT vs. 1.61 ± 0.21 in HPKO, P < 0.05), (C) 60 µM TRAP4 (2.42 ± 0.13 in WT vs. 1.61 ± 0.22 in HPKO, P < 0.05), and (D) 5 µM ADP (1.51 ± 0.12 in WT vs. 1.13 ± 0.21 in HPKO, P < 0.05). (E) As an internal positive control, addition of 10 µM calcium ionophore A23187 induced a complete response in both WT and HPKO platelets. The values reflect relative calcium fluorescence (FL). (F) Peak relative calcium fluorescence is plotted displaying a range of agonist concentrations. Error bars represent the SEM from three separate experiments.
4.9 HPKO platelets show impaired intraplatelet calcium release

An imbalance in the total calcium flux can originate either from the impaired influx of extracellular calcium through the platelet plasma membrane or by the inhibition of calcium release from the internal platelet stores. Therefore, by removing the extracellular calcium from the medium, the only detectable cellular calcium will be released from the intra-platelet pool. Therefore, a calcium mobilization assay was performed in WT and HPKO platelets by omitting the calcium in the Tyrode’s buffer. For this assay, TRAP4 and U46619 were used to exert the agonist-induced calcium release in platelets. The HPKO platelets exhibited a significant defect in the calcium mobilization upon stimulation by agonists (Fig. 16). The calcium mobilization defect was observed by stimulation with 0.5 µM U46619 (Fig. 16A) and 60 µM TRAP4 (Fig. 16B).

Thapsigargin is often used to induce the release of calcium from internal platelet pools (Alfonso et al., 2005). Specifically, thapsigargin inhibits SERCAs, the ATPases that sequester cytosolic Ca$^{2+}$ into the DTS. By inhibiting these ATPases, DTS stores of Ca$^{2+}$ are released into the cytosol. Using 200 nM Thapsigargin, we found the targeted release of calcium from the DTS was defective in HPKO platelets (Fig. 16C). The defects in the release of intracellular calcium were observed at multiple doses of each agonist in HPKO platelets (Fig. 16D). These results indicate that dematin headpiece domain regulates the flux of calcium across the dense tubular system in mouse platelets.
FIGURE 16: HPKO platelets show a significant defect in internal calcium release. The effect of the removal of extracellular calcium was investigated using (A) 0.5 μM U46619 (1.38 ± 0.19 in WT vs. 1.10 ± 0.13 in HPKO), (B) 60 μM TRAP4 (1.99 ± 0.32 in WT vs. 1.21 ± 0.09 in HPKO), and (C) 200 nM Thapsigargin (1.52 ± 0.71 in WT vs. 1.28 ± 0.18 in HPKO; P < 0.05). (D) Peak relative calcium fluorescence is plotted for various agonists. Error bars represent the SEM from three separate experiments.
4.10 Dematin is located at the DTS in platelets

As indicated before, immunofluorescence analysis identified a fraction of dematin concentrated at an unidentified central region in platelets (Fig. 6C). Since calcium release from the DTS appeared to be defective in the HPKO platelets (Fig. 16), the localization of dematin was evaluated relative to the DTS in platelets. Human platelets were examined with a monoclonal antibody to detect the endogenous dematin and a polyclonal antibody to detect protein disulfide isomerase (PDI). PDI has been shown to concentrate predominantly in the platelet DTS (van Nispen Tot Pannerden et al., 2009). Although a substantial portion of total dematin is detected at other sites in platelets, most notably the plasma membrane, the PDI co-localization data suggests that a significant fraction of dematin is located at the DTS in human platelets (Fig. 17A). To further confirm this observation, human platelets were biochemically fractionated into plasma membrane and intracellular membrane fractions. The intracellular membrane fraction is devoid of granule and mitochondria membranes, and therefore is composed primarily of the DTS. Western blot analysis indicated that a significant amount of dematin is recovered in the fraction containing intracellular membranes (DTS), thus supporting the co-localization of dematin with PDI in platelets (Fig. 17B). It is of note that the detection of a higher molecular weight band of dematin in the plasma and intracellular membranes is likely due to the differential propensity of disulfide bond formation of dematin polypeptides, thus resulting in dimeric or higher oligomeric species, as is the case with erythrocyte dematin (Khanna et al., 2002).
FIGURE 17: Dematin is located at the plasma membrane and dense tubular system (DTS) in platelets. (A) Co-localization analysis was performed using dematin (green) and PDI (red), a marker of the DTS in platelets. Scale bar = 5 µm. (B) Human platelets were washed and fractionated. Lane 1, total platelet membranes; lane 2, plasma membrane; lane 3, intracellular membranes. Each fraction (20 µg protein) was probed with antibodies against dematin, PDI (to detect DTS), and GPIbα (to detect plasma membrane). A significant expression of dematin was detected in the intracellular membranes. A slight mobility shift of dematin may indicate a post-translational modification.
4.11 Dematin-associated proteins at the DTS in platelets

To further investigate the mechanisms that give rise to reduced calcium mobilization in dematin HPKO platelets, we performed immunoprecipitation (IP) of dematin from platelets and identified dematin-associated proteins by mass spectrometry. It is to be noted that an IP of dematin in platelets poses unique technical challenges because of the tight association of dematin with the membrane fraction. The disruption of the dematin biochemical complex under harsh conditions and its re-association under mild detergent conditions may prevent identification of some physiologically relevant interactions by this approach. This is one reason why the dematin-binding proteins in erythrocytes were not identified by conventional immunoprecipitation and proteomics approaches. Nonetheless, suitable biochemical conditions were optimized to immunoprecipitate dematin-associated proteins in platelets. Proteomics analysis of the dematin immunoprecipitated proteins revealed several calcium-sensitive proteins that could be potentially associated with the DTS in platelets and contribute to the release of calcium. These proteins include protein disulfide-isomerase A5, inositol 1,4,5-trisphosphate 3-kinase isoform B (IP$_3$KB), caldesmon, and calmodulin-like protein 5. Of these candidate proteins, only IP$_3$KB has been shown to directly regulate calcium release in cells (Soriano et al., 1997, Yu et al., 2005). IP$_3$KB functions to phosphorylate IP$_3$ into IP$_4$, thereby eliminating the ability of IP$_3$ to activate its IP$_3$ receptor and liberate calcium from intracellular pools (Xia et al., 2005). To validate our proteomics findings, we performed immunoprecipitation analysis using antibodies against IP$_3$KB and dematin in platelets. The results demonstrate that both dematin and IP$_3$KB exist in the same biochemical complex in human platelets (Fig. 18A, B).
FIGURE 18: Dematin is associated with IP$_3$KB in human platelets (A) Dematin Western blotting of endogenous co-IP of dematin and IP$_3$KB in human platelets. Lane 1, co-IP with an unrelated monoclonal antibody, GAKIN; lane 2, co-IP with IP$_3$KB monoclonal antibody; lane 3, pre-cleared lysate. The same material was detected by Coomassie-blue after SDS-PAGE. (B) IP$_3$KB Western blotting of endogenous co-IP of dematin and IP$_3$KB in human platelets. Lane 1, co-IP with an unrelated monoclonal antibody, GAKIN; lane 2, co-IP with dematin monoclonal antibody. The same material was detected by Coomassie-blue after SDS-PAGE.
4.12 Dematin sequesters IP3KB in mouse platelets

Loss of the dematin headpiece in mouse platelets results in a significant decrease of IP3KB detected in the platelet membrane fraction (Fig. 19A). The reduced level of IP3KB at the membrane translates to an increase in liberated IP3KB detected within the cytosol in the HPKO platelets (Fig. 19A). Furthermore, administration of doxorubicin (DOX), a known inhibitor of IP3KB (da Silva et al., 1994, Zhu et al., 2000), produced a significant rescue in HPKO intraplatelet calcium release (Fig. 19B). This indicates that aberrant IP3KB activity was responsible for the inhibition of DTS-originated calcium signaling in HPKO platelets. With the biochemical and mechanistic validation of the dematin-IP3KB complex in platelets, we propose that this novel interaction may offer a mechanism for the modulation of intraplatelet calcium by dematin, with broad implications in cells where both dematin and IP3KB are abundantly expressed.
FIGURE 19: Dematin sequesters IP₃KB in mouse platelets (A) IP₃KB expression in WT and HPKO platelet fractions. *Lane 1, total WT platelet lysate; lane 2, total HPKO platelet lysate; lane 3, WT membrane fraction; lane 4, HPKO membrane fraction; lane 5, WT cytosolic fraction; lane 6, HPKO cytosolic fraction. Each fraction (6 μg protein) was probed with an antibody against IP₃KB and total protein levels were normalized using a β-actin control. (B) The effect of IP₃KB inhibition by Doxorubicin (DOX) on internal calcium release was investigated in WT and HPKO platelets. Peak relative calcium fluorescence is plotted for various agonists with or without 10 μM DOX.
5. DISCUSSION

The spectrin-based membrane skeleton in erythrocytes has served as a paradigm for cytoskeletal organization in many non-erythroid cells including platelets (Barkalow et al., 2003, Bennett et al., 1993, Fox et al., 1988). For example adducin, a component of the erythrocyte spectrin-actin junctions where dematin is also located, plays an important role in the reorganization of the membrane-cytoskeleton in platelets (Barkalow et al., 2003, Gilligan et al., 2002). Recently, our studies have shown a compensatory role of adducin and dematin in the regulation of erythrocyte shape and membrane properties (Chen et al., 2007). Therefore, we initiated the present study to investigate the role of dematin in blood platelets. In this work, we provide the first comprehensive characterization of dematin function in platelets. Our results demonstrate a novel function of dematin in the regulation of calcium flux in platelets upon stimulation by a variety of physiological agonists. These findings place dematin as a key modulator of platelet secretion and activation pathways.

Initially, Western blotting indicated that dematin is abundantly expressed in both human and mouse platelets, a finding in agreement with other studied cells. This abundant expression of dematin suggested that it may play a role in key platelet functions. Another similarity was the discovery that dematin migrates predominantly as a 52 kDa polypeptide on SDS-gel electrophoresis (Fig. 6). Immunofluorescence analysis revealed a distinct punctate pattern of dematin in platelets, which suggested a coordinated localization of the protein. Biochemical fractionation assays detected dematin distributed within both plasma membrane and cytosol, which suggested the possibility of protein re-localization as a means to regulate protein function (Fig. 6).
The generation of dematin HPKO mice in our laboratory (Khanna et al., 2002) provided an experimental tool to investigate the function of dematin in platelets. Consistent with our previous findings on HPKO erythrocytes, the HPKO platelets also express a truncated form of dematin encoded by its core domain (Fig. 7). A marked reduction of truncated dematin in the HPKO platelets indicates that deletion of the headpiece domain causes instability of the protein, and therefore results in further degradation of the remaining core domain. An alternative explanation is that the genetic mutation of dematin causes reduced synthesis and membrane association of truncated dematin during platelet development.

In the HPKO mice, the resting platelet morphology, number, and other basal platelet parameters are not affected (Table 1). This finding suggests that dematin does not affect platelet biogenesis or other quiescent properties. Functionally, the HPKO platelets exhibited a minor defect in the early stages of platelet adhesion on immobilized fibrinogen, an integrin $\alpha_{IIb}\beta_3$ dependent process (Fig. 8). Consistently, there was a slight reduction in platelet spreading at this time point. However, the HPKO platelets did not display any measurable spreading or adhesion defects in the presence of known secretion inhibitors, at any time point (Fig. 9). Taken together, these findings suggest a functional role of dematin in the regulation of platelet secretion rather than integrin $\alpha_{IIb}\beta_3$-mediated outside-in signaling pathways.

Platelet secretion is a critical step in hemostasis that provides key effector molecules and proteins to amplify and stabilize the platelet thrombi at the site of vascular injury (Reed et al., 2000). Conversely, secretion is also essential for the pathological conditions of thrombosis (Graham et al., 2009). In platelets, the secretory components are contained within two principal organelles: the dense and alpha granules. Consistent with our spreading results, both
alpha and dense granule secretion pathways are attenuated in HPKO platelets upon stimulation by multiple agonists (Fig. 10). This finding reinforces our hypothesis positioning dematin as a key regulator of the secretory process in platelets.

Since secretion amplifies thrombus propagation, it stands to reason that a defect in this release reaction would affect overall thrombus formation. Assays of aggregation provide a tool to measure this platelet response in suspended platelets. Our findings detect defects in aggregation with low doses of multiple agonists in the HPKO platelets (Fig. 11). Under these low agonist conditions, granule release is necessary to further the activation of the platelet population. This defect is eliminated at higher doses of agonist, which indicates that the involvement of dematin in the platelet aggregation pathways is primarily driven by its functional role in the dense granule secretion. Ostensibly, an inhibition of dense granule secretion indicates a likely reduction in TxA2 production in the HPKO platelets.

A similar deficiency may exist in platelet RhoA activation and integrin activation in HPKO platelets. This prediction is based on the commonality that the aforementioned events all occur during platelet inside-out signaling. Consistent with this prediction, RhoA activation was decreased in the dematin HPKO platelets (Fig. 12A). Furthermore, although the basal expression of integrin αIIbβ3 is unaltered in HPKO platelets (Table 1), integrin αIIbβ3 activation is attenuated in the HPKO platelets simulated with multiple agonists (Fig. 12B). These findings predicted that attenuated integrin αIIbβ3 activation influences outside-in signaling processes. This hypothesis is confirmed by the delayed clot retraction phenotype observed in the HPKO mice (Fig. 13). To validate that the defects we observed in outside-in signaling are exclusively from inside-out signaling (and therefore integrin inactivation), we directly activated integrin
α_{IIb}β_3 with MnCl\textsubscript{2} in our clot retraction studies. As a result, there was nearly complete retraction in the HPKO platelets (Fig. 14). In addition, the tail bleeding times of HPKO mice were slightly increased (Fig. 14). These findings are consistent with the platelet adhesion and spreading studies, and confirm the outside-in signaling pathway is not defective in dematin HPKO platelets.

The presence of multiple deficiencies in the secretion, aggregation, and integrin α_{IIb}β_3 activation pathways places dematin at an unknown central intersection of the inside-out signaling cascade in platelets. One well-established fundamental event in inside-out signaling is calcium mobilization (Varga-Szabo et al., 2009). Calcium is a key secondary messenger that acts on a broad range of signaling enzymes and proteins in multiple platelet pathways (Bergmeier et al., 2009). In resting platelets, the amount of cytosolic calcium is limited to prevent premature activation. However, after ligand-receptor binding this pool of calcium is rapidly increased to aid in platelet responses (Varga-Szabo et al., 2009). Therefore, a defect in the regulation of calcium mobilization could explain the pleiotropic effects caused by dematin deficiency in platelets.

Interestingly, we found a marked reduction in the mobilization of intracellular calcium of HPKO platelets stimulated by multiple agonists (Fig. 15). This calcium mobilization assay detects both the efflux of calcium from the dense tubular system (DTS) to the cytosol, and the following influx of extracellular calcium through the platelet plasma membrane (Cutler et al., 1978, Dean, 1984, Enouf et al., 1989, Kaser-Glanzmann et al., 1978, Menashi et al., 1982, Rybicki et al., 1983). Interestingly, our results show the calcium mobilization defect is independent of the level of calcium in the extracellular medium (Fig. 16). Therefore, these
findings suggest that the calcium mobilization defect in HPKO platelets originates from the release of calcium from the membrane-bound pools in platelets.

There are multiple sources of calcium within the platelet, which include acidic organelles and the dense tubular system (DTS). However, the co-localization of dematin with a DTS marker (PDI) provides evidence that a significant fraction of dematin is located at the DTS in particular (Fig. 17). To better understand the relationship between dematin and the DTS, we utilized proteomics and immunoprecipitation techniques. Here, we identified and confirmed a biochemical association between dematin and inositol 1,4,5-trisphosphate 3-kinase isoform B (IP$_3$KB) in platelets (Fig. 18). Like dematin, IP$_3$KB is ubiquitously expressed and membrane associated. Furthermore, IP$_3$KB has been described as a major regulator of calcium homeostasis, and is localized at the cytoplasmic face of the ER membrane - a counterpart of the DTS in platelets (Xia et al., 2005).

Interestingly, our fractionation results indicate a deficiency in platelet dematin alters the localization of IP$_3$KB from the DTS to the cytosol (Fig. 19A). One possible mechanism to explain this observation is a model whereby dematin tethers IP$_3$KB to the DTS membrane, either directly or indirectly, to regulate the inhibitory activity of IP$_3$KB on Ca$^{2+}$ release. This hypothesis is in agreement with previously published work, which suggested that modulating the localization of IP$_3$KB offers a means to regulate IP$_3$ levels in the cell (Lloyd-Burton et al., 2007, Yu et al., 2005). We validated this proposed mechanism by utilizing DOX, an inhibitor of IP$_3$KB (da Silva et al., 1994, Zhu et al., 2000) and calcium release (Kim et al., 2011). Our results demonstrate that this inhibition ameliorated the calcium mobilization defect that originated from a dematin deficiency in platelets (Fig. 19B).
To integrate our findings, we propose a model illustrating a critical role of dematin in the mobilization of calcium at the DTS of platelets (Fig. 20). Upon agonist stimulation, receptor activation results in PLC activation. This leads to the cleavage of membrane-bound PIP$_2$ into soluble IP$_3$ and DAG in platelets. The newly generated IP$_3$ then activates its IP$_3$ receptor located at the DTS, and induces the release of calcium into the cytosol. The liberated calcium then activates many important downstream signaling events such as integrin $\alpha_{IIb}\beta_3$ activation, granule secretion, and platelet aggregation. Our model predicts dematin tightly regulates the localization of IP$_3$KB, a terminator of calcium signaling via the phosphorylation of IP$_3$ into IP$_4$. The cytoskeletal tethering of IP$_3$KB to the DTS prevents unwanted and premature phosphorylation of IP$_3$, thus maintaining calcium mobilization in platelets. Alternate mechanisms may entail the regulation of dematin-IP$_3$KB interactions by phosphorylation and calpain-mediated proteolysis in activated platelets.
FIGURE 20: Role of dematin in the calcium mobilization in platelets. Proposed model for the function of dematin in the platelet signaling cascade. The model predicts that dematin regulates the release of internal calcium at the DTS through a biochemical interaction with IP$_3$KB. Regulation of intracellular calcium release would impact multiple downstream signaling events including granule secretion and integrin α$_{\text{IIb}}$β$_3$ activation.
Recent studies have shown an enrichment of IP₃KB within the cortical actin filaments (Nalaskowski et al., 2011). However, the precise mechanism of IP₃KB localization at the membrane sites via cortical actin structures is not known (Nalaskowski et al., 2011). Our findings offer one potential mechanism for the cytoskeletal anchor of IP₃KB not only in platelets but also in many other cell types. Interestingly, the internal calcium release is not completely ablated in the HPKO platelets (Fig. 16). Therefore, either the residual core domain of dematin provides a low level of calcium release activity or there is a compensatory mechanism for calcium release in the absence of dematin headpiece domain in mouse platelets. These questions will be addressable in future using platelets completely deficient of full-length dematin.

In erythrocytes, dematin is an excellent substrate of calpain-1, and its phosphorylation by cAMP-dependent protein kinase regulates its association with actin filaments (Rana et al., 1993). Whether these post-translational modifications modulate the function of dematin in platelets remains to be determined. Additionally, in erythrocytes dematin and adducin form a complex at the membrane skeleton to link the plasma membrane receptors GLUT1 and Band 3 to the underlying actin cytoskeleton (Khan et al., 2008). Our immunofluorescence staining of dematin in platelets revealed a significant fraction of dematin is located at the plasma membrane (Fig. 6), and therefore may perform a secondary role in platelets to link the membrane skeleton to a plasma membrane protein. However, neither the transmembrane protein GLUT1 nor Band 3 is detected in platelets. Therefore, dematin and adducin may complex with an unknown transmembrane protein and provide a new mechanism for the linkage of the underlying cytoskeleton with the platelet plasma membrane. A more detailed
investigation of this association could introduce new insights into platelet biology and therefore provide a new avenue for the design of platelet therapeutics.

Since dematin is expressed in many non-erythroid tissues, including heart, brain, kidney, and skeletal muscle, it would be important to determine whether dematin performs a similar function regulating calcium homeostasis outside of platelets. In this study, we have provided the first evidence that dematin functions as a novel regulator of internal calcium release in platelets, and plays an important role in the regulation of multiple platelet functions. Our findings are likely to provide new insights into the modulation of calcium mobilization and regulation of platelet functions in vivo.

The positive effect of dematin on intracellular calcium release has larger implications on promoting vascular thrombosis. Intracellular calcium has been shown to have a key role in regulating the recruitment of translocating cells to the initial adherent platelets at the site of injury (Nesbitt et al., 2003). Initial platelet signaling upon contact with exposed vWF in the subendothelium triggers the activation of calcium release from the DTS. Our studies have shown that dematin is a global regulator of this calcium mobilization. The released calcium activates the integrin receptor and allows vWF (in alpha granules) to fuse to the plasma membrane. The newly activated integrin receptor strongly tethers the platelet to the vessel wall and outside-in signaling causes further release of intracellular calcium and full platelet activation. Platelets in the circulation now adhere to the surface-expressed vWF on the adherent platelet. Consequently, intracellular calcium is released within the recently tethered platelet and the platelet-platelet contacts are strengthened. The further amplification of these signaling cascades in newly recruited platelets results in platelet aggregation and eventually
thrombus formation. Importantly, pathological conditions of platelet dysfunction and thrombus dysregulation contribute to the progression of cardiovascular disease, heart attacks, and stroke (Gregg et al., 2003). Specifically, aberrant calcium signaling in platelets has been reported in diabetic and hypertensive individuals, and may be a factor in their disease progression (El Haouari et al., 2008, El Haouari et al., 2009). Although intracellular Ca$^{2+}$ is possibly the most widely used means of controlling cellular functions (Petersen et al., 2008), dematin represents a promising candidate and a more specific target in platelets. Future research will elucidate the precise details delineating the molecular mechanisms of how dematin positively regulates IP$_3$KB and therefore calcium release from the DTS in platelets.

In conclusion, further characterization of platelet signaling pathways would aid not only in the diagnosis of patients but also in a more targeted drug design. The involvement of platelets in the pathology and progression of cardiovascular disease, a major cause of death in industrialized countries, makes these cells highly relevant for fundamental research. Many drugs that target platelet signaling pathways have been proven to be beneficial in the treatment of cardiovascular disease as anti-thrombotics. Therefore, creating cell-permeable peptides and small molecules to target the newly characterized proteins such as dematin represents a novel therapeutic option with the potential of increased efficacy and clinical benefit.
**FIGURE 21:** The release of intracellular calcium drives platelet thrombosis. Initial platelet signaling upon contact with exposed vWF in the subendothelium triggers the activation of calcium release from the DTS. Cytosolic calcium activates the integrin receptor and initiates granule exocytosis. Circulating platelets adhere to the surface-expressed vWF on the adherent platelet. Consequently, intracellular calcium is released within the newly tethered platelet and the platelet-platelet contacts are strengthened. The amplification of these signaling cascades in newly recruited platelets results in platelet aggregation and thrombus formation (modified from Nesbitt et al., 2003).
6. CITED LITERATURE


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