



Intracellular trafficking, localization, and mobilization of platelet-borne thiol isomerases

Article

Accepted Version

Crescente, M., Pluthero, F. G., Li, L., Lo, R. W., Walsh, T. G., Schenk, M. P., Holbrook, L. M., Loureiro, S., Ali, M. S., Vaiyapuri, S., Falet, H., Jones, I. M., Poole, A. W., Kahr, W. H. A. and Gibbins, J. M. (2016) Intracellular trafficking, localization, and mobilization of platelet-borne thiol isomerases. *Arteriosclerosis Thrombosis and Vascular Biology*, 36 (6). pp. 1164-1173. ISSN 1079-5642 doi: <https://doi.org/10.1161/ATVBAHA.116.307461> Available at <http://centaur.reading.ac.uk/64077/>

It is advisable to refer to the publisher's version if you intend to cite from the work.

To link to this article DOI: <http://dx.doi.org/10.1161/ATVBAHA.116.307461>

Publisher: Lippincott, Williams & Wilkins

All outputs in CentAUR are protected by Intellectual Property Rights law, including copyright law. Copyright and IPR is retained by the creators or other copyright holders. Terms and conditions for use of this material are defined in the [End User Agreement](#).

www.reading.ac.uk/centaur

CentAUR

Central Archive at the University of Reading

Reading's research outputs online

Intracellular trafficking, localization and mobilization of platelet-borne thiol isomerases

*Marilena Crescente¹, *Fred G. Pluthero², Ling Li², Richard W. Lo^{2,3}, Tony G. Walsh⁴, Silvia Louriero¹, Marfoua S Ali¹, Sakthivel Vaiyapuri¹, Michael P. Schenk¹, Lisa M Holbrook¹, Hervé Falet⁵, Ian M. Jones¹, Alastair W. Poole⁴, Walter H. A. Kahr^{2,3,#}, and Jonathan M. Gibbins^{1,#}

¹School of Biological Sciences, University of Reading, Reading, United Kingdom; ²Program in Cell Biology, Research Institute, The Hospital for Sick Children, Toronto, ON, Canada; ³Departments of Paediatrics and Biochemistry, University of Toronto, Toronto, ON, Canada; ⁴School of Physiology & Pharmacology, University of Bristol, Bristol, UK; ⁵Division of Hematology, Brigham and Women's Hospital, Department of Medicine, Harvard Medical School, Boston, MA, USA

*M.C. and F.G.P contributed equally to the work

#W.H.A.K and J.M.G. contributed equally to the work and are co-corresponding authors

Running title: Trafficking of platelet thiol isomerases

Corresponding authors:

Jonathan Gibbins. PhD
Professor of Cell Biology

Institute for Cardiovascular and Metabolic Research
School of Biological Sciences
Harborne Building
University of Reading
Whiteknights
Reading
RG6 6AS
UK

Email: j.m.gibbins@reading.ac.uk
Tel. +44 (0)118 3787082 (Direct)

Walter H.A. Kahr, MD, PhD, FRCPC
Associate Professor
Departments of Paediatrics & Biochemistry
University of Toronto

Division of Haematology/Oncology
Senior Scientist, Program in Cell Biology
The Hospital for Sick Children
PGCRL, Rm 19.9714
686 Bay Street
Toronto, ON, M5G 0A4
CANADA

Email: walter.kahr@sickkids.ca
Tel. 416-813-7977
Internal extension: 307977

Keywords: platelets, megakaryocytes, thiol isomerases, trafficking, granules

Subject codes: cell biology, platelets, thrombosis

Word count: 6352

Number of figures: 6

Number of supplementary figures: 3

Number of videos: 7

Number of references: 58

TOC category: basic research

TOC subcategory: thrombosis

Abstract

Objective - Thiol isomerases facilitate protein folding in the endoplasmic reticulum, and several of these enzymes, including PDI and ERp57, are mobilized to the surface of activated platelets, where they influence platelet aggregation, blood coagulation and thrombus formation.

In this study we examined for the first time the synthesis and trafficking of thiol isomerases in megakaryocytes, determined their subcellular localization in platelets and identified the cellular events responsible for their movement to the platelet surface upon activation.

Approach and Results – Immunofluorescence microscopy (IFM) imaging was used to localize PDI and ERp57 in murine and human megakaryocytes at various developmental stages. IFM and subcellular fractionation analysis were used to localize these proteins in platelets to a compartment distinct from known secretory vesicles that overlaps with an inner cell surface membrane region defined by the endoplasmic/sarcoplasmic reticulum proteins calnexin and SERCA3. IFM and flow cytometry were used to monitor thiol isomerase mobilization in activated platelets in the presence and absence of actin polymerization (inhibited by latrunculin), and in the presence or absence of membrane fusion mediated by Munc 13-4 (absent in platelets from *Unc13d*^{Uinx} mice).

Conclusions – Platelet-borne thiol isomerases are trafficked independently of secretory granule contents in megakaryocytes, and become concentrated in a subcellular compartment near the inner surface of the platelet outer membrane corresponding to the sarco/endoplasmic reticulum of these cells. Thiol isomerases are mobilized to the surface of activated platelets via a process that requires actin polymerization but not SNARE/Munc 13-4-dependent vesicular-plasma membrane fusion.

Nonstandard Abbreviations and Acronyms

PDI= protein disulfide isomerase

ER= endoplasmic reticulum

MK= megakaryocyte

DTS= dense tubular system

SNAREs= soluble N-ethylmaleimide-sensitive fusion protein attachment receptors

MVBs= multivesicular bodies

TFR= Transferrin receptor

VWF= Von Willebrand factor

TSP-1= thrombospondin-1

PF4= Platelet Factor 4

RabGDI= Rab GDP Dissociation Inhibitor

SERCA3 = sarco/endoplasmic reticulum calcium ATPase 3.

TLR9= toll-like receptor 9

WT= wild type

Introduction

Thiol isomerases facilitate the reduction, oxidation and rearrangement of disulfide bonds essential for correct folding of secreted proteins in the endoplasmic reticulum (ER).^{1, 2} Some cells also have thiol isomerases in granular structures³ or at the cell surface,^{4, 5} and in platelets protein disulfide isomerase (PDI), ERp57 and other thiol isomerases are mobilized to the cell surface with procoagulant activation. Inhibition of platelet-borne thiol isomerases reduces aggregation, granule secretion, receptor activation and thrombus formation *in vitro* and *in vivo*.^{3, 4, 6-11} As with platelets, release of thiol isomerases by vascular endothelial cells has been linked to de-encryption of tissue factor, fibrin formation^{10, 12, 13} and neutrophil recruitment to the vessel wall during inflammation.^{8, 14}

Elucidating the role of thiol isomerases in platelet function, blood coagulation and thrombus formation requires understanding how they are packaged and mobilized.^{6, 8, 10, 15-19} In this study we tracked PDI and ERp57 through development of precursor megakaryocytes (MK) and platelets, where we observed that these enzymes are concentrated in a subcellular compartment distinct from known secretory vesicles that overlaps with the dense tubular system (DTS) derived from MK smooth ER, and with the inner surface of the platelet outer membrane where the sarco/endoplasmic reticulum calcium ATPase SERCA3 is known to be localized.²⁰ During platelet activation, actin polymerization facilitates the movement of secretory vesicles to the cell plasma membrane surface,²¹ where the interaction of soluble N-ethylmaleimide-sensitive fusion protein attachment receptors (SNAREs) facilitates membrane fusion mediated by Munc 13-4.²²⁻²⁶ We observed that surface mobilization of thiol isomerases within activated platelets also requires actin polymerization, but proceeds in the absence of Munc13-4-mediated membrane fusion.

Material and Methods

Materials and methods are available in the online-only Data Supplement

Results

Expression and distribution of PDI and ERp57 during MK development

Unlike other mammalian cells, platelets are not derived from individual precursors via cell fission. Their development begins in the bone marrow with MK progenitors that undergo endomitosis to produce large cells (>20 μ M diameter) with multi-lobed nuclei. Maturing MKs synthesize large amounts of proteins and develop elaborate intracellular membrane systems. The synthesized secretory cargo (e.g. Von Willebrand Factor, VWF) moves from the Golgi to multivesicular bodies (MVBs), where it is joined by endocytosed proteins (e.g. fibrinogen) prior to packaging in secretory granules. The platelet DTS is derived from MK smooth ER, while cell surface and open canalicular system (OCS) membranes originate in the MK demarcation membrane system (DMS), composed of flattened cisternae and tubules that develop by processes that involve dynamin 2-dependent endocytosis,²⁷ surface membrane invagination and PACSIN2-mediated membrane tubulation.²⁸ MK development culminates in the production of proplatelet extensions that protrude into the vasculature and release hundreds or thousands of platelets from each cell into blood circulation.²⁹

We examined the intracellular distribution of PDI and ERp57 in developing cultured mouse MKs via high resolution immunofluorescence microscopy (IFM; Figure 1). MKs were identified by positive staining for CD41/integrin α IIb³⁰ and staged by size, shape and nuclear morphology. In early (<10 μ M diameter) and mitotic cells (Fig. 1A; Video 1) both thiol isomerases were extensively distributed through the cell, a pattern that persisted in later cells (i.e. synthetic cells; Fig.1B; Video 2), where ERp57 was sometimes concentrated in perinuclear ribbon-like structures (Fig.1B inset detail). Late-stage cultured MKs typically flatten out prior to producing proplatelet extensions, and in these extending cells (Fig.1C; Video 3) both PDI and ERp57 were observed throughout the extranuclear region and in proplatelet extensions (Fig.1D; Video 4) including the terminal buds, where PDI appeared to be concentrated near the outer membrane. While throughout MK development colocalization of PDI and ERp57 was observed (white in merged images), substantial proportions of these proteins were not in the same locations. Similar subcellular distributions of these thiol isomerases were observed in cultured human MKs, where proplatelet development is not as readily observed (data not shown).

MK trafficking of thiol isomerases is distinct from secretory granules and cargo

Platelet secretory granule protein cargo is synthesized in the MK ER and transported via vesicles derived from the trans-Golgi network³¹ to early MVBs, which according to current models also receive cargo in endocytic vesicles as they mature and give rise to platelet lysosomes and α and dense³² granules.^{31, 33, 34} Transferrin receptor (TFR; CD71) undergoes clathrin-mediated endocytosis and is frequently used as marker of the MK recycling endocytic compartment,³⁵ while TGN46 is a marker of the trans-Golgi network. IFM examination of thiol isomerase expression in human MKs in serum-free culture

revealed little overlap between ERp57, TFR and TGN46 (Figure 2 A; Video 5) or between PDI and the α -granule cargo proteins VWF (Figure S1A; Video 6) and thrombospondin-1 (TSP-1; not shown). At no point did we observe thiol isomerases distributed in granular puncta (e.g. VWF in Figure S1A) or structures resembling MVBs, rather their distribution pattern was consistent with association with intracellular membranes. Thus our observations indicate that throughout MK and proplatelet development thiol isomerases are localized in a cellular compartment that is distinct from secretory granules and their cargo, and that these enzymes are likely associated with future platelet cell surface and/or non-granular membranes.

The trafficking of thiol isomerases within MKs should determine their distribution within platelets. IFM observations showed consistent association of the α -granule membrane protein P-selectin with cargo proteins (e.g. VWF, Figure S1B) but not with PDI or ERp57 (Figure 2B), which, as observed in MKs (Figure 1), partially overlap with each other (mean Pearson's correlation coefficient = 0.6; sd = 0.08, n=12 cells). Similar relationships for PDI, ERp57 and P-selectin were observed in platelets from *Nbeal2^{-/-}* mice lacking α -granules (Figure 2C), and thiol isomerase levels were similar in mouse and human platelets in the presence or absence of α -granules^{30,36} (Figure 2D,E), further indicating their production and trafficking is independent of granules and their cargo.

Platelet thiol isomerases are localized to a non-granular compartment near the cell surface membrane defined by overlap with SERCA3

The association of thiol isomerases with platelet subcellular structures was investigated by fractionating cell lysates via sucrose gradient ultracentrifugation, and then probing fractions via immunoblotting for PDI, ERp57 and cell surface and interior membranes (β 3 integrin), proteins associated with cytosol (Rab GDP Dissociation Inhibitor; RabGDI), α – granule cargo (TSP-1) and the DTS (calreticulin) (Figure 3A). As reported by Jonnalagadda et al³⁷ β 3 integrin was present in the lighter fractions (2-3), but also abundant in the more dense fractions (8-10); corresponding to the plasma membrane and to the membrane of granules and endosomal compartments, respectively. The soluble cytosolic marker RabGDI was mainly concentrated at the top of the gradient as were the fractions containing calreticulin, a marker for the DTS previously used to detect PDI in this compartment in platelets.¹⁹ TSP-1 was abundantly expressed in the heaviest fractions where α – granules are fractionated. Consistent with our imaging and previous results,³⁸ PDI and ERp57 were concentrated in the lightest fractions, indicating association with cell-surface and/or near-surface membrane structures such as the DTS. While fractionation might suggest the presence of thiol isomerases in the platelet cytosol, this is less likely because these proteins are expected to be targeted to the secretory system of cells by virtue of their ER retention motifs (PDI: KDEL, ERp57: QEDL).

We examined the potential interaction of ERp57 with the DTS in human MK co-stained for calnexin, which like thiol isomerases promotes protein folding,^{39,40} but observed little apparent association (Figure S2A; Video 7). In platelets we observed that calnexin is associated with well-defined regions distinct from α -granules (Figure S2B) that likely represent the DTS, which showed partial overlap with ERp57 (Figure S2C), with most of the thiol isomerase detected outside the overlapping regions. Similar results were obtained (not shown) in colocalization analyses with other markers of the DTS (e.g. calreticulin), Golgi (e.g. TGN46) and endosomal compartments (e.g. EEA1), all of which were confined to intracellular locations distinct from those of thiol isomerases.

The sarcoplasmic/endoplasmic reticulum calcium ATPase SERCA3 is a well-studied regulator of platelet calcium loading that has been localized to the inner surface of the platelet outer membrane by cell fractionation, immunoprecipitation and IEM.^{20, 41} The main antibody employed in these studies was a mouse monoclonal (PL/IM430) raised against human platelet membranes that detects all 3 SERCA3 isoforms present in human platelets.⁴¹ Using the same antibody we examined the relationship of ERp57 with SERCA3 via IFM in human platelets, and the results (Figure 3B) show extensive overlap (mean Pearson's correlation coefficient = 0.794; sd = 0.067, n=11 cells) in a compartment near the inner surface of the cell outer membrane delineated by the colocalization of these proteins (Figure 3, colocalization channel). Thus platelet thiol isomerase appears to be concentrated in a compartment that may overlap with the DTS and OCS, but is largely located near the inner cell surface.

Platelet-borne PDI has been reported to associate with a T-granule system defined by toll-like receptor 9 (TLR9).¹⁸ We were unable to replicate this observation by IFM with the same anti-TLR9 antibody used in the original description of the T-granules (data not shown). Consistent with proteomics studies reporting low levels of TLR9 in platelets^{42, 43}, we could not detect TLR9 in platelets by immunoblotting, and consequently, we did not further investigate the presence of thiol isomerases in putative T-granules.

Mobilization of thiol isomerases to the platelet surface requires actin polymerization but not Munc 13-4-mediated membrane fusion

The SERCA3 interaction partner STIM1 mobilizes to the surface of activated platelets via a process that is actin-dependent and associated with cytoskeletal and microdomain rearrangements, but is not thought to involve the fusion of vesicular and cell membranes.⁴⁴ Like other platelet-borne thiol isomerases, PDI and ERp57 move to the surface upon activation,^{6, 17} as can be observed by IFM comparing their intracellular distribution with that of P-selectin (Figure S3A). We examined the role of actin polymerization in this process using latrunculin A, which prevents actin polymerization.⁴⁵ We determined that 200 μ M latrunculin A inhibits P-selectin mobilization in activated platelets by >50% (Figure S3B), and compared by IFM the intracellular/extracellular distributions of P-selectin and the α -granule borne protein VWF (Figure 4A) or of P-selectin and thiol isomerases (Figure 4B), in platelets activated in the presence or absence of this concentration of latrunculin A. The results show that, in the absence of actin polymerization, platelets underwent little or no mobilization of PDI and ERp57 or of P-selectin and VWF, and retained a resting morphology indicated by flat shape and intact tubulin cytoskeletal ring. Using flow cytometry, we observed an increased surface expression of thiol isomerases, as reported previously^{10, 17}. Blocking actin polymerization with latrunculin A significantly inhibited the movement to the surface of both PDI and ERp57 (Figure 4C).

When platelets are stimulated by agonists such as PAR4-activating peptide (PAR4-AP), Munc13-4 facilitates the pairing of SNAREs^{25, 26, 46} involved with the fusion of secretory vesicles with the cell surface membrane. ADP has also been observed to trigger Munc13-4-independent granule secretion.⁴⁶ We investigated the role of Munc13-4-dependent membrane fusion in platelet thiol isomerase mobilization using *Unc13d*^{Jinx} mice, which lack Munc13-4 activity but show equivalent expression to wild type (WT) of platelet thiol isomerases (Figure 5A). Flow cytometry assessment of surface expression of P-selectin and thiol isomerases in resting and agonist-stimulated platelets showed

that cells from *Unc13d^{flinx}* mice had significantly less P-selectin surface expression than WT (Figure 5B) when stimulated with PAR4-AP, and there was partial rescue of response with ADP. When stimulated with PAR4-AP, *Unc13d^{flinx}* platelets showed greater mobilization of PDI relative to WT (Figure 5B), and along with WT cells showed increased PDI mobilization in the presence of ADP. These results indicate that Munc13-4-mediated membrane fusion events are not required for the mobilization/release of platelet thiol isomerases during activation, which may involve a mechanism similar to that proposed for STIM1.⁴⁴

Discussion

In this study we began by tracing the synthesis, trafficking and packaging of platelet-borne thiol isomerases in cultured primary human and murine MKs. The use of murine MKs allowed us to follow platelet development to the proplatelet extension stage, where nascent platelets are loaded with secretory granules and other organelles.⁴⁷ This approach has proven effective in elucidating the origin of other platelet proteins such as glycosyltransferases.⁴⁸ While human MKs derived from peripherally-mobilized CD34+ cells cultured under serum-free conditions do not develop proplatelets as readily as mouse cells, they have also proven useful in studies of the biogenesis of platelet granules.⁴⁹ Their use was important to confirm that, as was expected, thiol isomerases destined for platelets are synthesized by MKs, since they are present in cells grown under serum-free conditions and show little evident association with endocytic pathways (Figure 2A).

Our observations indicate that the bulk of MK ERp57 (and most likely PDI) synthesized by MKs is destined to become platelet cargo (Figure S2A), although we did observe an apparent transient strong association of ERp57 with the ER in mouse MKs undergoing peak protein synthesis (Figure 1B). Our observations also consistently indicate that the trafficking of thiol isomerases is distinct from α -granule cargo proteins at all stages of MK development, in terms of cellular localization (Figure 1 A-D, Videos 1-4), distribution pattern (Figure S1A, Video 5) and interaction with the endocytic and trans-Golgi compartments (Figure 2A). This indicates that thiol isomerase destined for platelets is not trafficked through the MVBs that process granule-bound cargo. Rather their intracellular distribution indicates association with another of the many MK membrane systems, as has been proposed for platelet Golgi-derived glycosyltransferases.⁴⁸ The membrane system associated with thiol isomerases appears to elaborate with proplatelet extensions (Figure 1D).

In platelets, we observed that consistent with our MK observations PDI and ERp57 are localized in a subcellular compartment distinct from the α -granule matrix delineated by P-selectin (Figure 2B). This is consistent with the reported lack of overlap between PDI and lysosomes⁵⁰ or hyaluronidase-2 in human platelets, where the latter enzyme has been localized to α -granules via IFM and subcellular fractionation.⁵¹ The thiol isomerase compartment appears to surround the P-selectin matrix (Figure 2B) and to be contained by the outer cell membrane (Figure 2C), a pattern that holds in GPS human and mouse *Nbeal2^{-/-}* platelets where α -granule cargo is absent (Figure 2C). The observation that α -granule cargo-deficient platelets have normal levels of thiol isomerases (Figure 2D,E) further supports the independence of their trafficking from granule cargo.

Our observations are consistent with reports of thiol isomerase located near the platelet surface^{6, 8, 10, 15-17} and DTS.¹⁹ We saw little association of ERp57 with smooth ER defined by calnexin in mature human MKs (Figure S2A), although we did observe localized overlap between this marker of the platelet DTS and ERp57 (Figure S2C). According to the most recent proteomics data ERp57 is approximately twice as abundant as calnexin in platelets (1736 vs. 890 ppm⁴²), consistent with our observation that the thiol isomerase is more widely distributed in these cells (Figure S2C). Subcellular fractionation data indicate that in addition to the DTS, ERp57 and PDI are also likely associated with the cell surface membrane (Figure 3A). This expectation was confirmed by the observation of extensive overlap between ERp57 and SERCA3, long known to be located on the inner surface of the human platelet outer membrane (Figure 3B)²⁰. In addition, ERp57 has been reported to interact with STIM1⁵², a transmembrane ER protein that upon calcium depletion translocates to subplasmalemmal punctae where it interacts with the calcium channel Orai 1 located in the plasma membrane and allows calcium influx into the cell⁵³. Given that as PDI and ERp57, SERCA 3 is an ER-derived protein^{20, 41}, we cannot exclude the possibility that thiol isomerases co-distribute with SERCA 3 in a non-calnexin subpopulation of DTS (or possibly ER remnant) that is localized at the inner surface of the platelet membrane.

The mobilization of thiol isomerases to the surface of activated platelets facilitates their involvement in clot formation,⁸⁻¹⁰ and studies have shown that PDI and ERp5 move to the platelet surface with similar kinetics to α -granule proteins.⁷ We have extended these observations by demonstrating that as with P-selectin and α -granule cargo,²¹ the mobilization of platelet thiol isomerases requires actin polymerization (Figure 4 and Table 1). Secretion by platelet granules also involves interactions between v-SNAREs present on their surface with t-SNAREs on the plasma membrane,^{54, 55} where membrane fusion is primed by Munc13-4, a limiting factor in platelet granule secretion.^{22-24, 26} We observed that compared to WT mice, platelet thiol isomerase mobilization is stronger in *Unc13d*^{Uinx} mice, where platelets lack Munc13-4 activity and have absent or severely impaired P-selectin mobilization (Figure 5B) and secretion from lysosomes and dense and α -granules, although they do show activation-associated changes in morphology.²⁵ We also observed that thiol isomerase mobilization is enhanced in both mutant and wild type mouse platelets by ADP, which has been shown to stimulate Munc13-4-independent secretion of α -granule contents.⁴⁶ Thus our observations indicate that while platelet thiol isomerase mobilization/secretion requires actin polymerization, it does not involve Munc13-4 mediated membrane fusion. It may be that the cellular rearrangements associated with platelet activation are sufficient to move thiol isomerases to the surface and facilitate their release, as has been proposed for STIM1.⁵⁶ Increased surface exposure of PDI in the absence of Munc 13-4 could also indicate that this protein serves as a brake on the mobilization of platelet thiol isomerases, pointing to a novel aspect of regulation of platelet function.

The results presented here can be combined with current knowledge to produce a model of platelet thiol isomerase trafficking, packaging and mobilization in activated cells. In this scheme (Figure 6; for the sake of simplicity only MK-synthesized proteins are considered) protein destined for platelet α -granules (e.g. VWF, TSP-1) moves from the MK ER to the trans-Golgi network for sorting into MVBs that give rise to mature secretory vesicles. Following a different route, thiol isomerases destined for platelets move from the ER to the developing intracellular membranes. When MK extend proplatelets, secretory cargo is packaged into granules while thiol isomerases associate

with membranes that give rise to the platelet surface and possibly the outer DTS and OCS. In platelets that later undergo activation, thiol isomerases are mobilized to the outer cell surface via a mechanism driven by actin polymerization and cytoskeletal/membrane rearrangements.

Elucidation of the expression and function of platelet thiol isomerases is important because these enzymes have been shown to be key modulators of thrombus formation via their activity on the surface of platelets and vascular endothelial cells. The influence of PDI, ERp57 and ERp5 on platelet function has been primarily attributed to their ability to bind the platelet cell surface receptor integrin $\alpha\text{IIb}\beta\text{3}$ and thus favor its activation,⁶⁻¹⁰ and it is also likely that this receptor assumes a high-affinity conformation in activated platelets that facilitates surface binding of mobilized PDI and ERp57.⁵⁷ A search for other substrates of thiol isomerases is ongoing, as are efforts to develop methods for the selective modulation of thiol isomerases as a therapeutic strategy against thrombotic disease.⁵⁸

Authorship

Contribution: M.C. and F.G.P. designed the research, performed the experiments, and wrote the manuscript; S.L., M.S.A., S.V., M.P.S, L.M.H., I.M.J. provided intellectual contribution, antibodies and contributed to writing the manuscript; L.L., R.W.L. and T.G.W designed and performed some experiments; H.F. performed some experiments and contributed to writing the manuscript; A.W.P., W.H.A.K. and J.M.G designed the research and contributed to writing the manuscript.

Acknowledgements

We thank Dr Sidney W. Whiteheart for kindly providing us the cell-disruption apparatus and Mr Khaled Sahli for helping preparing the manuscript.

Sources of Funding

This work was supported by grants from the Medical Research Council (J002666/1) and British Heart Foundation (PG/11/125/29320) to J.M.G. and from the Canadian Institutes of Health Research to W.H.A.K. (MOP-259952).

Disclosures

The authors declare no competing financial interests

References

1. Ferrari DM, Soling HD. The protein disulphide-isomerase family: Unravelling a string of folds. *Biochem J.* 1999;339 (Pt 1):1-10
2. Jordan PA, Gibbins JM. Extracellular disulfide exchange and the regulation of cellular function. *Antioxid Redox Signal.* 2006;8:312-324
3. Jasuja R, Furie B, Furie BC. Endothelium-derived but not platelet-derived protein disulfide isomerase is required for thrombus formation in vivo. *Blood.* 2010;116:4665-4674
4. Akagi S, Yamamoto A, Yoshimori T, Masaki R, Ogawa R, Tashiro Y. Distribution of protein disulfide isomerase in rat hepatocytes. *J Histochem Cytochem.* 1988;36:1533-1542
5. Kroning H, Kahne T, Ittenson A, Franke A, Ansorge S. Thiol-proteindisulfide-oxidoreductase (proteindisulfide isomerase): A new plasma membrane constituent of mature human b lymphocytes. *Scand J Immunol.* 1994;39:346-350
6. Holbrook LM, Sasikumar P, Stanley RG, Simmonds AD, Bicknell AB, Gibbins JM. The platelet-surface thiol isomerase enzyme erp57 modulates platelet function. *J Thromb Haemost.* 2012;10:278-288
7. Jordan PA, Stevens JM, Hubbard GP, Barrett NE, Sage T, Authi KS, Gibbins JM. A role for the thiol isomerase protein erp5 in platelet function. *Blood.* 2005;105:1500-1507
8. Kim K, Hahm E, Li J, Holbrook LM, Sasikumar P, Stanley RG, Ushio-Fukai M, Gibbins JM, Cho J. Platelet protein disulfide isomerase is required for thrombus formation but not for hemostasis in mice. *Blood.* 2013;122:1052-1061
9. Passam FH, Lin L, Gopal S, Stopa J, Bellido-Martin L, Huang M, Furie BC, Furie B. Both platelet- and endothelial cell-derived erp5 supports thrombus formation in a laser-induced mouse model of thrombosis. *Blood.* 2015
10. Wang L, Wu Y, Zhou J, Ahmad SS, Mutus B, Garbi N, Hammerling G, Liu J, Essex DW. Platelet-derived erp57 mediates platelet incorporation into a growing thrombus by regulation of the α IIb β 3 integrin. *Blood.* 2013;122:3642-3650
11. Wu Y, Ahmad SS, Zhou J, Wang L, Cully MP, Essex DW. The disulfide isomerase erp57 mediates platelet aggregation, hemostasis, and thrombosis. *Blood.* 2012;119:1737-1746
12. Cho J, Furie BC, Coughlin SR, Furie B. A critical role for extracellular protein disulfide isomerase during thrombus formation in mice. *J Clin Invest.* 2008;118:1123-1131
13. Reinhardt C, von Bruhl ML, Manukyan D, Grahl L, Lorenz M, Altmann B, Dlugai S, Hess S, Konrad I, Orschiedt L, Mackman N, Ruddock L, Massberg S, Engelmann B. Protein disulfide isomerase acts as an injury response signal that enhances fibrin generation via tissue factor activation. *J Clin Invest.* 2008;118:1110-1122

14. Hahm E, Li J, Kim K, Huh S, Rogelj S, Cho J. Extracellular protein disulfide isomerase regulates ligand-binding activity of alphabeta2 integrin and neutrophil recruitment during vascular inflammation. *Blood*. 2013;121:3789-3800, s3781-3715
15. Essex DW, Chen K, Swiatkowska M. Localization of protein disulfide isomerase to the external surface of the platelet plasma membrane. *Blood*. 1995;86:2168-2173
16. Furie B, Flaumenhaft R. Thiol isomerases in thrombus formation. *Circ Res*. 2014;114:1162-1173
17. Holbrook LM, Watkins NA, Simmonds AD, Jones CI, Ouwehand WH, Gibbins JM. Platelets release novel thiol isomerase enzymes which are recruited to the cell surface following activation. *Br J Haematol*. 2010;148:627-637
18. Thon JN, Peters CG, Machlus KR, Aslam R, Rowley J, Macleod H, Devine MT, Fuchs TA, Weyrich AS, Semple JW, Flaumenhaft R, Italiano JE, Jr. T granules in human platelets function in tlr9 organization and signaling. *J Cell Biol*. 2012;198:561-574
19. van Nispen Tot Pannerden HE, van Dijk SM, Du V, Heijnen HF. Platelet protein disulfide isomerase is localized in the dense tubular system and does not become surface expressed after activation. *Blood*. 2009;114:4738-4740
20. Kovacs T, Berger G, Corvazier E, Paszty K, Brown A, Bobe R, Papp B, Wuytack F, Cramer EM, Enouf J. Immunolocalization of the multi-sarco/endoplasmic reticulum ca²⁺ atpase system in human platelets. *Br J Haematol*. 1997;97:192-203
21. Flaumenhaft R, Dilks JR, Rozenvayn N, Monahan-Earley RA, Feng D, Dvorak AM. The actin cytoskeleton differentially regulates platelet alpha-granule and dense-granule secretion. *Blood*. 2005;105:3879-3887
22. Betz A, Okamoto M, Benseler F, Brose N. Direct interaction of the rat unc-13 homologue munc13-1 with the n terminus of syntaxin. *The Journal of biological chemistry*. 1997;272:2520-2526
23. Feldmann J, Callebaut I, Raposo G, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (fhl3). *Cell*. 2003;115:461-473
24. Madison JM, Nurrish S, Kaplan JM. Unc-13 interaction with syntaxin is required for synaptic transmission. *Curr Biol*. 2005;15:2236-2242
25. Ren Q, Wimmer C, Chicka MC, Ye S, Ren Y, Hughson FM, Whiteheart SW. Munc13-4 is a limiting factor in the pathway required for platelet granule release and hemostasis. *Blood*. 2010;116:869-877
26. Savage JS, Williams CM, Konopatskaya O, Hers I, Harper MT, Poole AW. Munc13-4 is critical for thrombosis through regulating release of adp from platelets. *J Thromb Haemost*. 2013;11:771-775
27. Bender M, Giannini S, Grozovsky R, Jonsson T, Christensen H, Pluthero FG, Ko A, Mullally A, Kahr WH, Hoffmeister KM, Falet H. Dynamin 2-dependent endocytosis is required for normal megakaryocyte development in mice. *Blood*. 2015;125:1014-1024
28. Begonja AJ, Pluthero FG, Suphamungmee W, Giannini S, Christensen H, Leung R, Lo RW, Nakamura F, Lehman W, Plomann M, Hoffmeister KM, Kahr WH,

- Hartwig JH, Falet H. Flna binding to pacsin2 f-bar domain regulates membrane tubulation in megakaryocytes and platelets. *Blood*. 2015;126:80-88
29. Junt T, Schulze H, Chen Z, Massberg S, Goerge T, Krueger A, Wagner DD, Graf T, Italiano JE, Jr., Shivdasani RA, von Andrian UH. Dynamic visualization of thrombopoiesis within bone marrow. *Science*. 2007;317:1767-1770
 30. Kahr WH, Lo RW, Li L, Pluthero FG, Christensen H, Ni R, Vaezzadeh N, Hawkins CE, Weyrich AS, Di Paola J, Landolt-Marticorena C, Gross PL. Abnormal megakaryocyte development and platelet function in nbeal2(-/-) mice. *Blood*. 2013;122:3349-3358
 31. de Larouziere V, Brouland JP, Souni F, Drouet L, Cramer E. Inverse immunostaining pattern for synthesized versus endocytosed alpha-granule proteins in human bone marrow megakaryocytes. *Br J Haematol*. 1998;101:618-625
 32. Ambrosio AL, Boyle JA, Di Pietro SM. Mechanism of platelet dense granule biogenesis: Study of cargo transport and function of rab32 and rab38 in a model system. *Blood*. 2012;120:4072-4081
 33. Blair P, Flaumenhaft R. Platelet alpha-granules: Basic biology and clinical correlates. *Blood Rev*. 2009;23:177-189
 34. Heijnen HF, Debili N, Vainchencker W, Breton-Gorius J, Geuze HJ, Sixma JJ. Multivesicular bodies are an intermediate stage in the formation of platelet alpha-granules. *Blood*. 1998;91:2313-2325
 35. Beardmore J, Howell KE, Miller K, Hopkins CR. Isolation of an endocytic compartment from a431 cells using a density modification procedure employing a receptor-specific monoclonal antibody complexed with colloidal gold. *J Cell Sci*. 1987;87 (Pt 4):495-506
 36. Kahr WH, Hinckley J, Li L, et al. Mutations in nbeal2, encoding a beach protein, cause gray platelet syndrome. *Nat Genet*. 2011;43:738-740
 37. Jonnalagadda D, Sunkara M, Morris AJ, Whiteheart SW. Granule-mediated release of sphingosine-1-phosphate by activated platelets. *Biochim Biophys Acta*. 2014;1841:1581-1589
 38. Niessen J, Jedlitschky G, Grube M, Bien S, Strobel U, Ritter CA, Greinacher A, Kroemer HK. Subfractionation and purification of intracellular granule-structures of human platelets: An improved method based on magnetic sorting. *J Immunol Methods*. 2007;328:89-96
 39. Benham AM. Protein secretion and the endoplasmic reticulum. *Cold Spring Harb Perspect Biol*. 2012;4:a012872
 40. Caramelo JJ, Parodi AJ. Getting in and out from calnexin/calreticulin cycles. *The Journal of biological chemistry*. 2008;283:10221-10225
 41. Kovacs T, Felfoldi F, Papp B, Paszty K, Bredoux R, Enyedi A, Enouf J. All three splice variants of the human sarco/endoplasmic reticulum ca2+-atpase 3 gene are translated to proteins: A study of their co-expression in platelets and lymphoid cells. *The Biochemical journal*. 2001;358:559-568
 42. Kim MS, Pinto SM, Getnet D, et al. A draft map of the human proteome. *Nature*. 2014;509:575-581

43. Martens L, Van Damme P, Van Damme J, Staes A, Timmerman E, Ghesquiere B, Thomas GR, Vandekerckhove J, Gevaert K. The human platelet proteome mapped by peptide-centric proteomics: A functional protein profile. *Proteomics*. 2005;5:3193-3204
44. Lopez JJ, Salido GM, Pariente JA, Rosado JA. Interaction of stim1 with endogenously expressed human canonical trp1 upon depletion of intracellular ca²⁺ stores. *J Biol Chem*. 2006;281:28254-28264
45. Spector I, Braet F, Shochet NR, Bubb MR. New anti-actin drugs in the study of the organization and function of the actin cytoskeleton. *Microsc Res Tech*. 1999;47:18-37
46. Harper MT, van den Bosch MT, Hers I, Poole AW. Platelet dense granule secretion defects may obscure alpha-granule secretion mechanisms: Evidence from munc13-4-deficient platelets. *Blood*. 2015;125:3034-3036
47. Patel SR, Hartwig JH, Italiano JE, Jr. The biogenesis of platelets from megakaryocyte proplatelets. *J Clin Invest*. 2005;115:3348-3354
48. Wandall HH, Rumjantseva V, Sorensen AL, Patel-Hett S, Josefsson EC, Bennett EP, Italiano JE, Jr., Clausen H, Hartwig JH, Hoffmeister KM. The origin and function of platelet glycosyltransferases. *Blood*. 2012;120:626-635
49. Lo B, Li L, Gissen P, Christensen H, McKiernan PJ, Ye C, Abdelhaleem M, Hayes JA, Williams MD, Chitayat D, Kahr WH. Requirement of vps33b, a member of the sec1/munc18 protein family, in megakaryocyte and platelet alpha-granule biogenesis. *Blood*. 2005;106:4159-4166
50. Zbidi H, Jardin I, Woodard GE, Lopez JJ, Berna-Erro A, Salido GM, Rosado JA. Stim1 and stim2 are located in the acidic ca²⁺ stores and associates with orai1 upon depletion of the acidic stores in human platelets. *J Biol Chem*. 2011;286:12257-12270
51. Albeiroti S, Ayasoufi K, Hill DR, Shen B, de la Motte CA. Platelet hyaluronidase-2: An enzyme that translocates to the surface upon activation to function in extracellular matrix degradation. *Blood*. 2015;125:1460-1469
52. Prins D, Groenendyk J, Touret N, Michalak M. Modulation of stim1 and capacitative ca²⁺ entry by the endoplasmic reticulum luminal oxidoreductase erp57. *EMBO reports*. 2011;12:1182-1188
53. Muik M, Frischauf I, Derler I, Fahrner M, Bergsmann J, Eder P, Schindl R, Hesch C, Polzinger B, Fritsch R, Kahr H, Madl J, Gruber H, Groschner K, Romanin C. Dynamic coupling of the putative coiled-coil domain of orai1 with stim1 mediates orai1 channel activation. *The Journal of biological chemistry*. 2008;283:8014-8022
54. Rothman JE, Warren G. Implications of the snare hypothesis for intracellular membrane topology and dynamics. *Curr Biol*. 1994;4:220-233
55. Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE. Snarepins: Minimal machinery for membrane fusion. *Cell*. 1998;92:759-772
56. Lopez JJ, Jardin I, Bobe R, Pariente JA, Enouf J, Salido GM, Rosado JA. Stim1 regulates acidic ca²⁺ store refilling by interaction with serca3 in human platelets. *Biochem Pharmacol*. 2008;75:2157-2164

57. Bennett JS, Zigmond S, Vilaire G, Cunningham ME, Bednar B. The platelet cytoskeleton regulates the affinity of the integrin alpha(iib)beta(3) for fibrinogen. *The Journal of biological chemistry*. 1999;274:25301-25307
58. Flaumenhaft R, Furie B, Zwicker JI. Therapeutic implications of protein disulfide isomerase inhibition in thrombotic disease. *Arteriosclerosis, thrombosis, and vascular biology*. 2015;35:16-23

Highlights

- Platelet-borne thiol isomerases play important roles in hemostasis and in pathological thrombosis.
- We examined for the first time the synthesis of thiol isomerases during megakaryocyte development showing that they are trafficked independently of granule cargo.
- Within platelets thiol isomerases localize near the inner surface of the plasma membrane and partially overlap with the DTS
- Thiol isomerases are released on the surface of activated platelets in an actin-dependent process that does not require Munc13-4 mediated membrane fusion.
- Defining the synthesis, trafficking, location and mechanism of mobilization/secretion of platelet thiol isomerases may aid the development of new antithrombotic strategies.

Figures

Figure 1

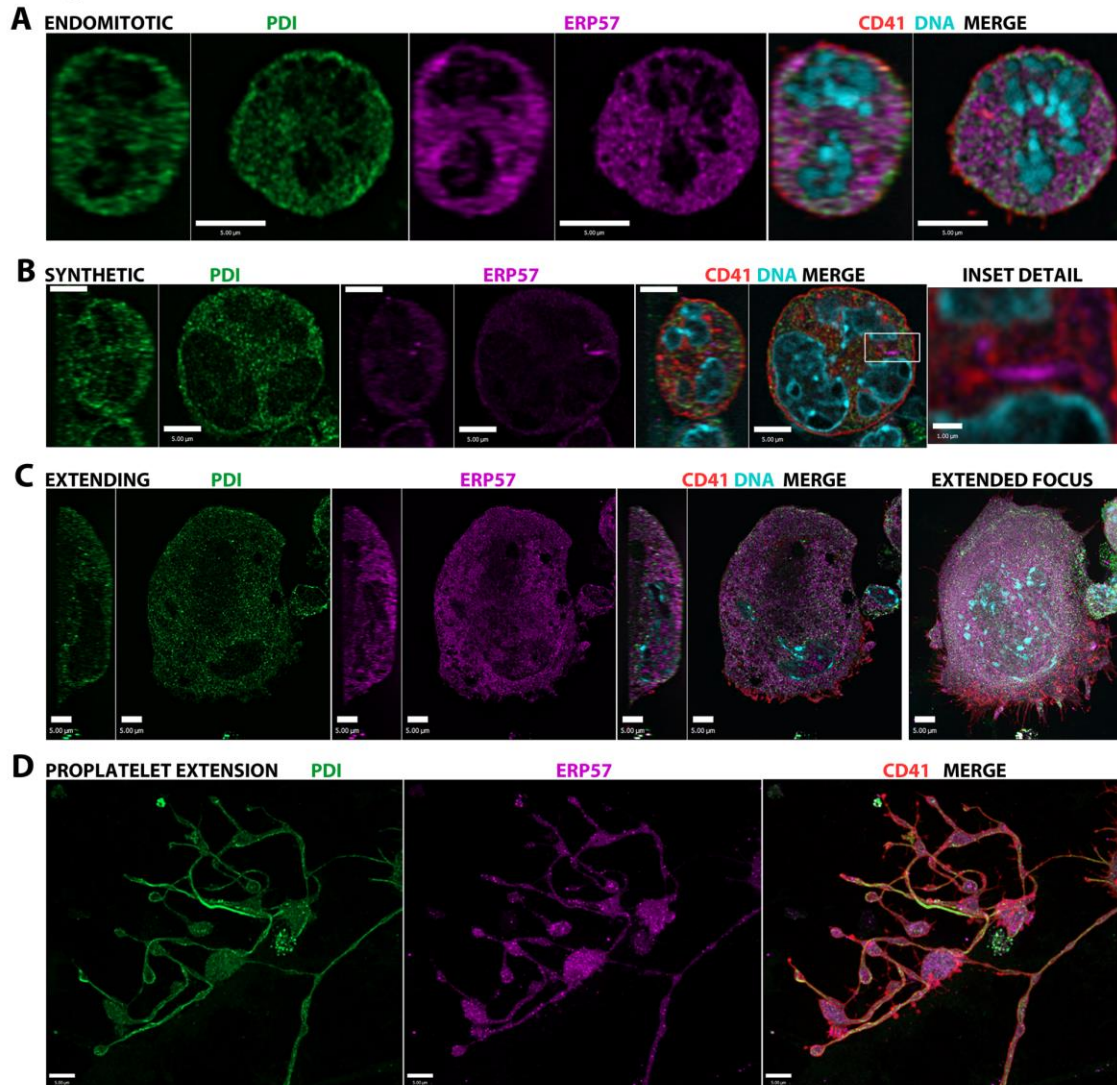


Figure 1. Intracellular distribution of PDI and ERp57 during MK development. IFM images (mid-cell Z-sections with YZ and XY profiles or extended focus renders) are shown for representative cultured mouse MK stained for ERp57 (magenta), PDI (green), CD41 (red) and DNA (blue; bars = 5 μ m except where noted). **A**) In early endomitotic cells ERp57 and PDI show a similar extranuclear distribution (see Video 1). **B**) In larger cells undergoing maximal protein synthesis PDI remains widely distributed, and in some cells ERp57 is highly concentrated in ribbon-like structures that may represent ER (inset detail bar = 1 μ m; see Video 2). **C**) As late-stage MKs flatten out, both thiol isomerases are present throughout the extranuclear region (see Video 3) and **D**) in proplatelet extensions (main cell body at right out of field), where they are present throughout the shafts and in terminal buds (see Video 4).

Figure 2

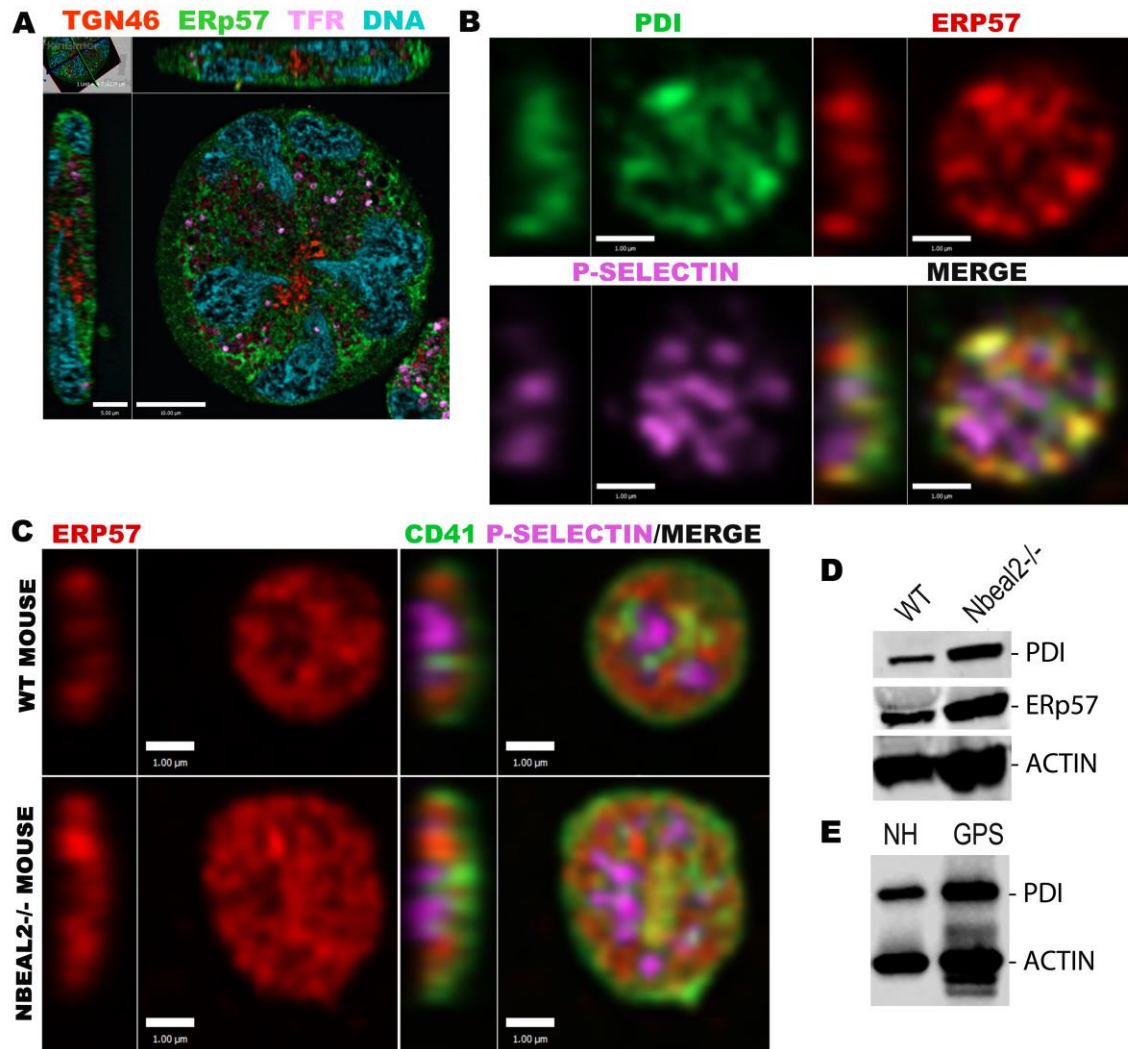


Figure 2. Thiol isomerases are not associated with MK trans-Golgi network or recycling endosomal compartments, or with platelet α -granules. **A**) IFM imaging of a representative mature human MK shows ERp57 (green) distributed throughout the cell having little overlap with trans-Golgi defined by TGN46 (red) or recycling endosomes containing TFR (magenta; mid-cell Z-section, bar = 10 μ m). **B**) In a human platelet, PDI (green) and ERp57 (red) overlap with each other but not with P-selectin (magenta; mid-cell Z-sections, bars = 1 μ m). **C**) Platelets stained for CD41/integrin α IIb (green), ERp57 (red) and P-selectin (magenta) from WT and *Nbeal2*^{-/-} mice (the latter lacking α -granule cargo) show similar intracellular distributions of thiol isomerase (mid-cell Z-sections, bars = 1 μ m). **D**) Immunoblot analysis shows similar levels of PDI and ERp57 in WT and *Nbeal2*^{-/-} mouse platelet lysates, and **E**) similar expression of PDI in lysates from normal human (NH) and Gray platelet syndrome (GPS) patient platelets (actin used as loading control).

Figure 3

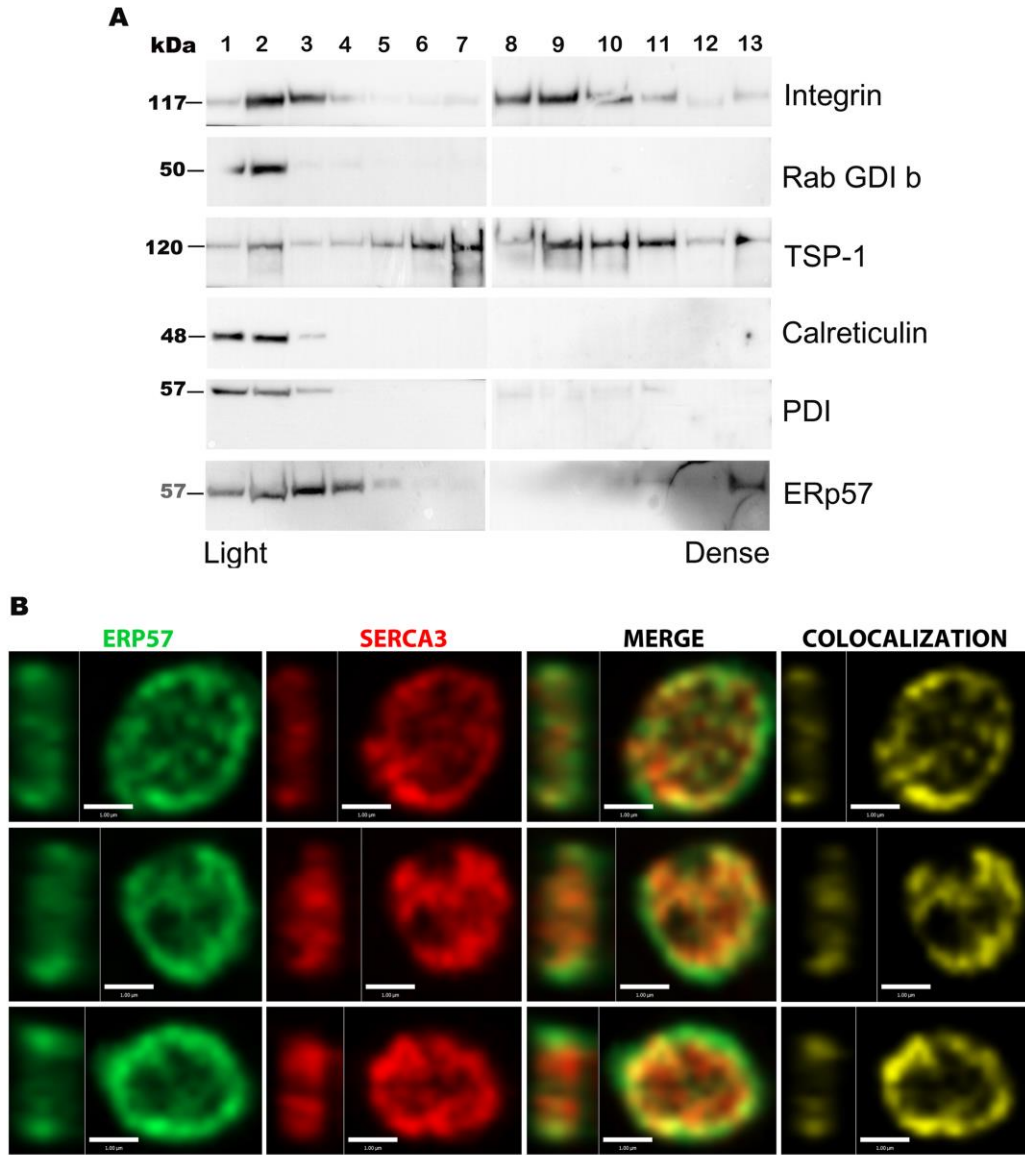


Figure 3. Platelet thiol isomerases associate with cell-surface membrane structures and overlap with SERCA3 on the inner surface of the cell outer membrane. A) Human platelet lysates were fractionated by sucrose gradient and fractions (1-13) analyzed by immunoblotting for PDI, ERp57, β 3 integrin, RabGDI b, TSP-1 and calreticulin (marker sizes in kilodaltons at left). Peak concentrations of PDI and ERp57 overlapped with β 3 integrin corresponding to the cell surface membrane fraction, with cytosolic RabGDI b, and the DTS marker calreticulin and showed little overlap with α -granule borne TSP-1 (Figure representative of 3 experiments). **B)** IFM imaging of fixed resting human platelets stained for ERp57 (green) and SERCA3 (red) shows overlap in a compartment defined by the latter on the inner surface of the cell outer membrane, delineated by the colocalization channel (yellow; mid-cell Z-sections, bars = 1 μ M).

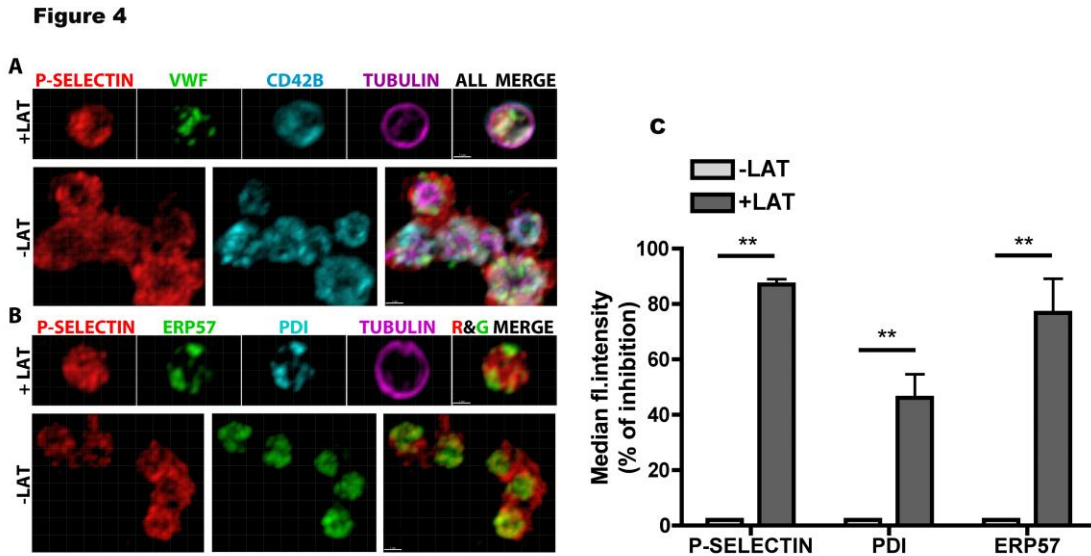


Figure 4. Mobilization of thiol isomerases in activated platelets requires actin polymerization. IFM imaging of human platelets activated with 4 μ M U46619 in the presence (+LAT) or absence (-LAT) of latrunculin A, fixed and stained for tubulin (magenta), P-selectin (red) and either: **A**) CD42B/GP1b (light blue) and VWF (green), or **B**) PDI (light blue) and ERp57 (green). Reorganization of cytoskeletal tubulin and surface mobilization of P-selectin, VWF and ERp57 is evident in platelets not treated with latrunculin, while treated cells show intracellular protein distribution patterns similar to unactivated platelets (Fig.2B). Maximum intensity renders, bars = 1 μ M. **C**) The effect of latrunculin treatment on the surface mobilization of thiol isomerases, as compared to that of P-selectin, was quantified by flow cytometry. Platelets were activated with 1U/ml of thrombin, the binding of the primary antibody for thiol isomerases was detected using a FITC-conjugated species-specific secondary antibody, the PE/Cy5 anti-human CD62P was used to detect P-selectin. The percentage of inhibition of the Median Fluorescence Intensity was calculated (** $P < 0.01$, one-way ANOVA, followed by Dunnett's test, $n = 3$).

Figure 5

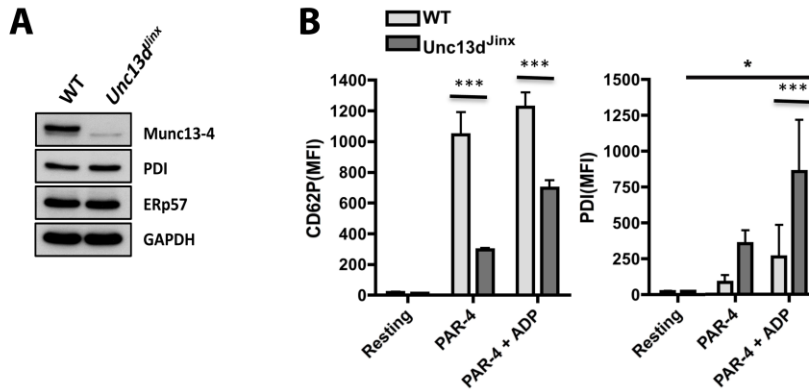


Figure 5. Surface mobilization of platelet thiol isomerase is independent of Munc13-4. **A)** Immunoblot analysis of platelet lysates from wild type (WT) and *Unc13d^{Jinx}* mice shows lack of Munc13-4 in the latter and similar levels of PDI and ERp57 (GAPDH loading control). **B)** Flow cytometry measurement of surface P-selectin (CD62P) and PDI in platelets under resting conditions or after stimulation with PAR4-AP in the presence/absence of ADP shows that P-selectin mobilization is significantly impaired in *Unc13d^{Jinx}* platelets but PDI mobilization is greater than WT. ADP significantly increased PDI mobilization as compared to WT or resting *Unc13d^{Jinx}* platelets (* $P < 0.05$, *** $P < 0.001$, one-way ANOVA, followed by Dunnett's test or two-way ANOVA, followed by Bonferroni's test, $n = 4$).

Figure 6

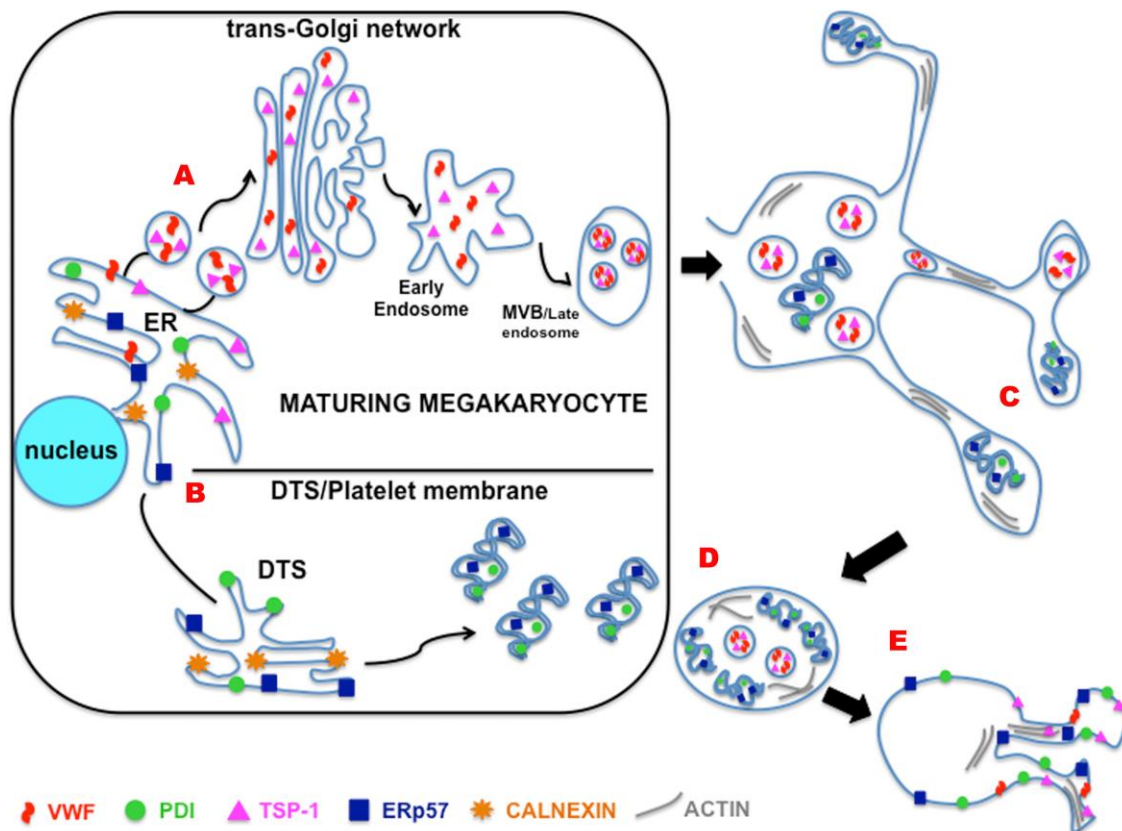


Figure 6. Model comparing synthesis, trafficking, packaging and mobilization of platelet thiol isomerases and α -granule cargo.

A) In the maturing MK, proteins destined for α -granules (e.g. VWF, TSP-1) are trafficked from the ER via a pathway that leads through the trans-Golgi network to multivesicular bodies. **B)** Thiol isomerases associate with membranes destined for the platelet DTS and cell surface. **C)** As proplatelet extensions form secretory granule cargo is packaged, while thiol isomerases move with membranes into forming platelets. **D)** In mature platelets, thiol isomerases are concentrated near the surface membrane and in the near-surface DTS. **E)** In activated platelets actin polymerization and membrane fusion facilitates the surface mobilization and secretion of granule cargo, while thiol isomerases mobilized without the need for membrane fusion.