Arteriosclerosis, Thrombosis, and Vascular Biology

BRIEF REVIEW

Orchestration of Primary Hemostasis by Platelet and Endothelial Lysosome-Related Organelles

Ellie Karampini, Ruben Bierings, Jan Voorberg

ABSTRACT: Megakaryocyte-derived platelets and endothelial cells store their hemostatic cargo in α- and δ-granules and Weibel-Palade bodies, respectively. These storage granules belong to the lysosome-related organelles (LROs), a heterogeneous group of organelles that are rapidly released following agonist-induced triggering of intracellular signaling pathways. Following vascular injury, endothelial Weibel-Palade bodies release their content into the vascular lumen and promote the formation of long VWF (von Willebrand factor) strings that form an adhesive platform for platelets. Binding to VWF strings as well as exposed subendothelial collagen activates platelets resulting in the release of α- and δ-granules, which are crucial events in formation of a primary hemostatic plug. Biogenesis and secretion of these LROs are pivotal for the maintenance of proper hemostasis. Several bleeding disorders have been linked to abnormal generation of LROs in megakaryocytes and endothelial cells. Recent reviews have emphasized common pathways in the biogenesis and biological properties of LROs, focusing mainly on melanosomes. Despite many similarities, LROs in platelet and endothelial cells clearly possess distinct properties that allow them to provide a highly coordinated and synergistic contribution to primary hemostasis by sequentially releasing hemostatic cargo. In this brief review, we discuss in depth the known regulators of α- and δ-granules in megakaryocytes/platelets and Weibel-Palade bodies in endothelial cells, starting from transcription factors that have been associated with granule formation to protein complexes that promote granule maturation. In addition, we provide a detailed view on the interplay between platelet and endothelial LROs in controlling hemostasis as well as their dysfunction in LRO related bleeding disorders.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: endothelial cells ■ hemostasis ■ lysosome-related organelles ■ megakaryocytes ■ platelets ■ von Willebrand factor

Lysosome-related organelles (LROs) are specialized storage compartments that include melanosomes in skin and eye melanocytes, lytic granules of cytotoxic T cells and natural killer cells, alpha (α) and dense (δ) granules in platelets, as well as Weibel-Palade bodies (WPB) in endothelial cells.[1] Originally evolving from lysosomes, they have critically adapted to a highly specialized physiological role in the storage, clustering and regulated on demand release of bioactive components.[1] In line with their evolutionary origin LROs, or as recently proposed endo-lysome related organelles,[1] share common features with endosomes.[2] In this review, we focus on the biogenesis, morphological features, and physiological role of LROs present in platelets and endothelial cells which are released in a coordinated fashion during primary hemostasis. Megakaryocytes and platelets contain 2 types of LROs, the α-, and δ-granules, whereas endothelial cells contain only one, the WPBs. Each type of LRO can be considered as the adaptation of the cell's endosomes into functional secretory organelles that have evolved to specific physiological needs related to on demand cargo delivery.[1] Although LROs are similar enough to be placed in the same category, they differ in their stored cargoes, which is highly cell specific. In megakaryocytes, α-granules contain hemostatic proteins like VWF (von Willebrand factor) and Fbg (fibrinogen), whereas δ-granules store small molecules like ATP, Ca^{2+} and serotonin as well as the tetraspanin CD63.[3] Similar to α-granules, WPBs primarily store VWF, along with a diverse cocktail of proinflammatory and angiogenic proteins, but also CD63.[4] Because of their absence or dysfunction in specific bleeding disorders, both platelet

Correspondence to: Prof Jan Voorberg, PhD, Department of Molecular and Cellular Hemostasis, Sanquin Research and Landsteiner Laboratory, Amsterdam, the Netherlands. Email j.voorberg@sanquin.nl

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Nonstandard Abbreviations and Acronyms

AMPK	5′ AMP-activated kinase
AP	adaptor protein
ARM	Armadillo
BEACH	beige and Chediak-Higashi
CK	cysteine knot
Dlg1	discs large 1
ER	endoplasmic reticulum
Fbg	fibrinogen
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
HPS	Hermansky-Pudlak syndrome
KO	knock out
LRO	lysosome-related organelle
MVB	multivesicular body
PF4	platelet factor 4
PI4K	phosphatidylinositol 4 kinase
TGN	trans-Golgi network
VWF	von Willebrand factor
WPB	Weibel-Palade body

granules and WPBs have been positioned as master orchestrators of primary hemostasis.

At the site of vascular damage, endothelial cell activation drives the bulk expulsion of highly condensed VWF from WPBs and their conversion into adhesive strings, that serve as a landing platform for platelets.[5] Platelets are arrested from the circulation by GPIb-V-IX/VWF interaction, which is the predominant receptor-ligand interaction supporting platelet recruitment (Figure 1A).[6,7] Subsequently, adhered platelets interact with exposed subendothelial collagen which initiates an intracellular cascade leading to platelet activation (Figure 1B), which, in turn, results in the α- and δ-granules release and their respective cargoes (Figure 1C).[9] In more detail, ADP released from δ-granules binds to the P2Y12 receptor which strongly potentiates collagen and thromboxane A2–mediated platelet activation, shape changes and aggregation.[10,11] In addition, polyphosphate, whose trafficking mechanisms to δ-granules are still unclear, is released and may promote secondary hemostasis by accelerating the generation of FXIa by thrombin.[12] Release of stored Fbg and VWF from α-granules during platelet activation is likely to contribute to cross-linking of the activation-dependent open conformation of integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa), thereby promoting formation of a stable platelet plug.[13] Apart from Fbg and VWF, multiple growth factors like PDGFB (platelet-derived growth factor subunit B) and VEGF (vascular endothelial growth factor) as well as chemokines like PF4 (platelet factor 4) and RANTES are present in α-granules, suggesting that α-granule release also contributes to restoration of vascular integrity following injury.[14,15] Besides active α- and δ-granules exocytosis, platelets also release their lysosomal content[16]; however, this is beyond the scope of this review.

The exocytotic mechanisms of endothelial and platelet LROs involve a complex interplay of Rab GTPases and proteins of the SNARE complex, with substantial overlap between the endothelial and platelet exocytotic machinery. However, several detailed reviews on this topic are already available,[4,17,18] and this will, therefore, not be part of this review. The importance of WPB and platelet granule release for controlling primary hemostasis is further highlighted by a group of heterogeneous bleeding disorders due to defective biogenesis and maturation of these LROs.

In this review, we summarize new advances in LRO formation, highlighting the interplay and differential regulation of megakaryocyte and platelet localized α- and δ-granules and endothelial cell WPBs. We discuss in depth the pathways involved in the biogenesis and maturation of the aforementioned LROs, as well as disorders that are linked to defects in these pathways.

Highlights

- Primary hemostasis.
- Biogenesis α and δ granules in megakaryocytes.
- Biogenesis of Weibel-Palade bodies in endothelial cells.

BIOGENESIS AND MATURATION OF α- AND δ-GRANULES IN MEGAKARYOCYTES AND PLATELETS

Transcription Factors in α- and δ-Granule Biogenesis

Several transcription factors, including RUNX1, NF-E2, GFI1B, and FLI1, have a crucial role in megakaryopoiesis and platelet formation.[19,20] Their importance is illustrated by defects in platelet function in patients with genetic defects in these transcription factors.[21] *RUNX1* haplodeficiency has been linked to thrombocytopenia and both α- and δ-granule deficiencies, while *RUNX1* knock out (KO) mice lack primary hematopoiesis.[21] Two new studies have demonstrated that *RUNX1* haplodeficiency decreases *RAB1B* and *PLDN* expression levels which sheds light on the mechanisms for α- and δ-granule biogenesis, respectively.[22,23] Rab1b belongs to the small GTPase family and facilitates vesicular endoplasmic reticulum (ER)-to-Golgi transport, and, in the case of megakaryocytes, promotes VWF intracellular trafficking.[22,24] Reduced RUNX1 and Rab1b levels were proposed to lead to defective VWF maturation and decreased targeting to α-granules.[22] RUNX1 also controls the expression of *PLDN* which encodes for pallidin

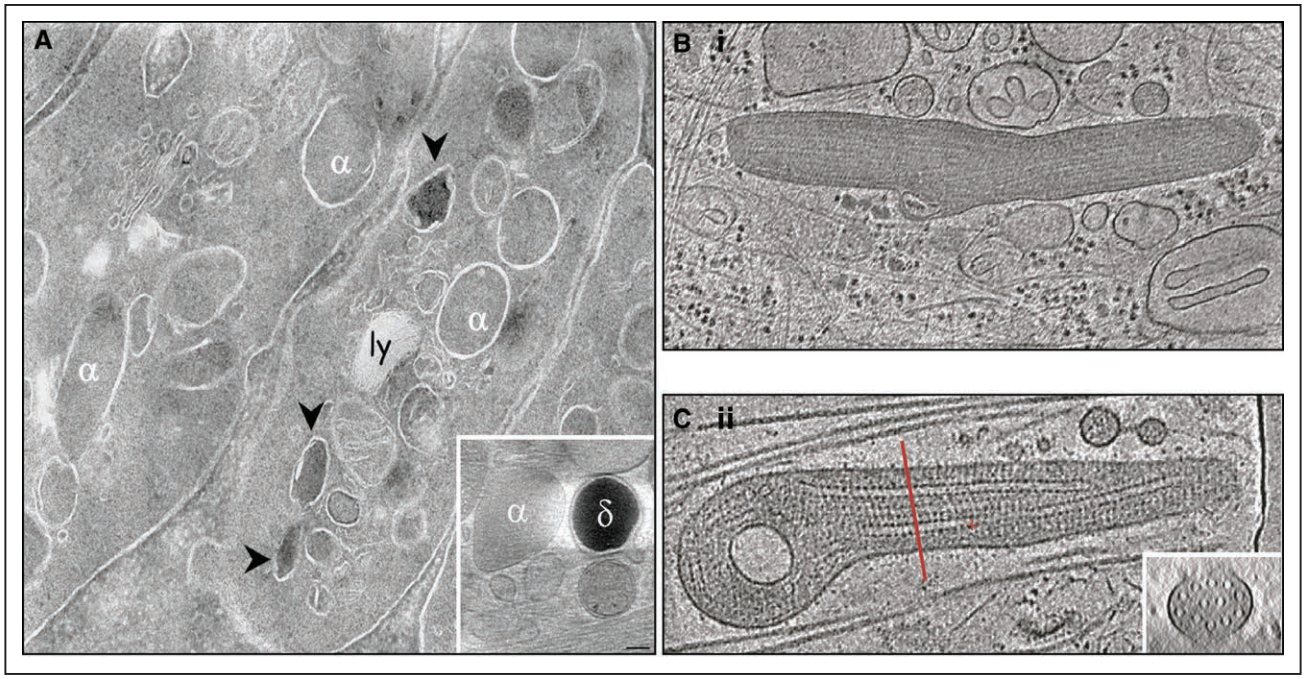

Figure 1. Platelet and endothelial lysosome-related organelles.
A, Transmission electron micrographs of α- and δ-granules in platelets. α-granules are annotated with the respective letter and δ-granules with black arrowheads. Reprinted from Heijnen and van der Sluijs[17] with permission. Copyright ©2015, International Society on Thrombosis and Haemostasis. The inset shows a detailed cryo electron micrograph of an α- and δ-granule (bar 50 nmol/L); this image was kindly provided by Dr H.F.G. Heijnen. **B**, Section of an electron cryotomogram of frozen-hydrated endothelial cells displaying Weibel-Palade bodies (WPBs), both containing intraluminal vesicles. **B**, I, WPB within a dense mesh of cytoskeletal filaments and surrounded by several cytoplasmic vesicles. **B**, ii, Section of an electron cryotomogram of a WPB with typical striations running parallel within the organelle. A cross-section of the WPB at the site of the red line is shown in the **bottom right** inset, which shows a highly organized stack of tubules that consist of condensed VWF (von Willebrand factor) multimers. Reprinted from Streetley et al[8] with permission. Copyright ©2019, American Society of Hematology. ly indicates lysosomes.

or BLOC1S6, a member of the BLOC-1 complex that promotes δ-granule formation.[23,25] Downregulation of pallidin in RUNX1 haplodeficiency was suggested to underlie disrupted CD63 trafficking to δ-granules and impaired δ-granule biogenesis.[23]

NF-E2 has been described to regulate the expression of Rab27b, a small GTPase family that is strongly expressed in the megakaryocyte/ platelet lineage and colocalizes with both α- and δ-granules.[26] *NF-E2* KO megakaryocytes lack Rab27b expression, which has been shown to regulate the number of δ-granules per platelet.[27] The closely related small GTPase Rab27a deficient in ashen (*ash)* mice, a model for Griscelli Syndrome has been implicated in dense granule release.[26–28] Screening of a panel of patients with unexplained thrombocytopenia revealed defects in the gene encoding transcription factor *FLI1*.[29] *FLI1* is localized in the 11q24 arm on chromosome 11q. This region is deleted in patients with Jacobsen and Paris-Trousseau syndrome (collectively called 11q deletion syndromes), whose clinical symptoms include a.o. thrombocytopenia.[30] In accordance with its proposed role in thrombopoiesis, *Fli1*$^{-/-}$ mice are embryonic lethal at day 11.5 due to thrombocytopenia and abnormal vasculature development.[29,30] Absence of *FLI1* has previously been shown to induce macrothrombocytopenia, in addition to abnormal granule biogenesis that results in giant α-granules and complete absence of δ-granules.[31] Based on these findings, it was suggested that FLI1 may play a role in protein packaging in α-granules, whereas FLI1 interactions with Hermansky-Pudlak syndrome (*HPS*) *4* and *RAB27B* may regulate δ-granule biogenesis.[19,31] Dominant-negative mutations in the transcription factor GFI1B have been identified in patients with Gray platelet syndrome.[32,33] Detailed morphological studies of patients with dominant-negative GFI1B mutation revealed lack of δ-granules as well as abnormalities in α-granule contents giving rise to a gray appearance in blood smears in a subset of platelets.[34,35] The precise mechanisms underlying the observed changes in platelet LROs in patients with dominant-negative GFI1B mutations remain to be identified but are most likely caused by dysregulation of developmental programs involved in thrombopoiesis and markedly reduced expression of proteins that reside in α-granules.[24] In summary, several transcription factors have been identified that affect the biogenesis of platelet LROs. Further elucidation of pathways controlling biogenesis of content delivery to platelet LROs is likely to shed light on general mechanisms that drive the formation of LRO compartments in other cell types, including melanocytes and endothelial cells (Figure 2).

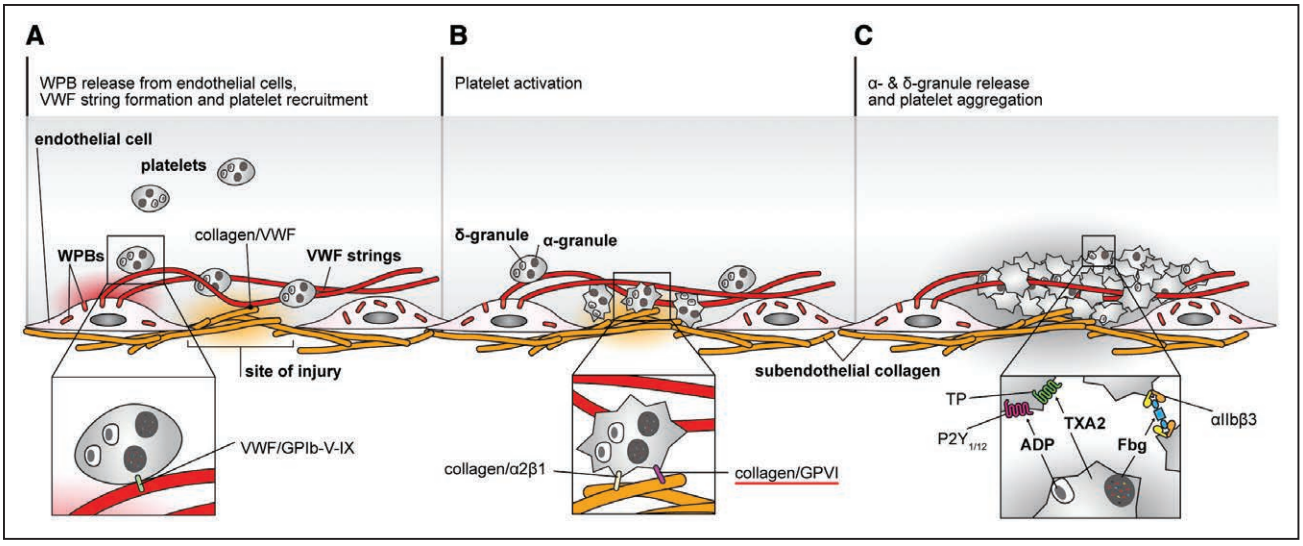

Figure 2. Model for the initiation of the platelet plug during primary hemostasis.
A, Upon vascular damage, endothelial cells secrete their VWF (von Willebrand factor) content from their storage organelles the Weibel-Palade bodies (WPBs), which unfolds in long VWF strings. VWF strings form an adhesive platform for platelets to be arrested from circulation through the receptor/ ligand interaction of platelet GP1b-V-IX and VWF. **B**, Adhered platelets interact with exposed subendothelial collagen primarily through GPVI, which initiates an intracellular cascade leading to platelet activation. **C**, Activated platelets change their share and release their α- and δ-granules. ADP released from δ-granules binds to the P2Y12 receptor, which strongly potentiates collagen and thromboxane A2 (TXA2)–mediated platelet activation. Additionally, release of Fbg (fibrinogen) from α-granules contributes to platelet cross-linking through activation-dependent open conformation of integrin $\alpha_{IIb}\beta_3$ and platelet aggregation.

α-Granule Maturation: the Role of VPS33B/16B Complex and NBEAL2 in Cargo Trafficking and Retention

VPS33B is the first protein to be described as essential for α-granule maturation.[36,37] VPS33B belongs to the family of S/M (Sec1/Munc18-like) proteins and has been proposed to promote assembly of newly forming SNARE complexes by aligning SNARE helices from target and vesicle membranes.[38] Mutations in *VPS33B* cause arthrogryposis-renal dysfunction-cholestasis syndrome, a multisystemic autosomal disorder that includes platelet deficiency.[39] VPS33B deficiency results in the absence of α-granules and reduced levels of α-granule proteins like soluble cargo (Fbg and VWF) and membrane proteins (CD62P) that are presumably degraded in the lysosomes.[36,37] Colocalization studies have shown that VPS33B is not associated with the Golgi, but it is mainly found on the recycling Rab11a positive endosomes, suggesting that the VPS33B is part of the protein sorting machinery.[36,37] Additionally, depletion of VPS33B in megakaryocytes not only results in α-granule deficiency but also multivesicular bodies (MVB) maturation.[40] MVB has 2 distinct stages during maturation: type I and II.[41] The former is characterized by abundant intraluminal vesicles, whereas the latter is identified by an electron-dense core and peripheral intraluminal vesicles.[40,41] During megakaryocyte development, MVB type I mature into type II, which are the progenitors of α-granules.[41] Downregulation of VPS33B results in near absence of MVB type II along with α-granules, suggesting that VSP33B is primarily involved in MVB maturation and subsequent α-granule formation.[40] VPS33B is part of the heterohexameric tethering complexes CORVET (class C core vacuole/endosome tethering) and HOPS (homotypic fusion and vacuole protein sorting) that operate in endosome and lysosome fusion.[42] In addition, together with VPS16B, VPS33B forms the CHEVI (class C homologs in endosome-vesicle interaction) complex which is recruited in a Rab11 dependent manner to recycling endosomes.[43] Similar to *VPS33B,* genetic defects in the VPS16B encoding *VIPAS39* gene are found in patients with the arthrogryposis-renal dysfunction-cholestasis syndrome.[44] Loss of VPS16B results in reduction of VPS33B and α-granule proteins expression as well as defective α-granule formation similar to *VPS33B* KO.[45] In a recent study, VPS33B and VSP16B are found to form a heterodimer smaller than CORVET and HOPS in megakaryocytes, which possibly does not include any other protein.[36] The VPS33B/16B complex localizes on endosomes, which are closely related with the trans-Golgi network (TGN) and promotes protein trafficking towards MVB before finally reaching α-granules.[36]

Another key component controlling α-granule formation is *NBEAL2* (neurobeachin-like protein 2), a gene that encodes Neurobeachin-like 2 and is mutated in the storage pool disorder gray platelet syndrome.[46] NBEAL2 has been described to be involved in protein sorting in megakaryocytes.[47] *NBEAL2* KO megakaryocytes and platelets show greatly reduced intracellular levels of α-granule components, such as Fbg and VWF, that are either sequestered by endocytosis or arrive in the α-granule through biosynthetic pathways.[47,48] Interestingly,

when compared with VPS33B/16B mutations that result in platelets that lack both soluble cargo and membrane proteins like CD62P, *NBEAL2*$^{-/-}$ platelets, despite missing their soluble cargo, still contain a pool of releasable CD62P.[37,45,48] Moreover, VPS33B and VPS16B expression is normal in *NBEAL2* KO platelets, suggesting that NBEAL2 is involved at a later stage in α-granule maturation, rather than promoting the formation of α-granule progenitors (MVB type II).[48] Recently, Lo et al[49] suggested that NBEAL2 is required for the retention of synthesized and endocytosed cargo through interaction with CD62P, potentially as a part of a multimolecular complex, to allow for packaging of cargo into maturing α-granules. In the absence of NBEAL2, α-granule cargoes, such as PF4, VWF, and Fbg, are not sufficiently retained, allowing for their transport to Rab11 positive recycling endosomes which are subsequently exocytosed.[49] Further insights on the NBEAL2-dependent mechanisms can be provided by a recent proteomic study, which identified binding partners of NBEAL2 (Dock7, Sec16a, and Vac14) that may synergistically regulate α-granule formation.[50]

Therefore, the current view on α-granule maturation includes the heterodimeric CHEVI complex, composed of VPS33B and VSP16B, that promotes conversion of endosomes into α-granule progenitors, the MVB type II, and α-granules. After the α-granule cargo has arrived at its destination, NBEAL2 retains it intraluminally, thereby ensuring packaging of cargo into nascent and maturing α-granules (Figure 2).

δ-Granule Maturation: Proteins and Complexes Involved in a "Kiss-and-Run" Maturation

δ-granules, similarly to α-granules, go through a complex maturation process that involves the delivery of specialized cargo catalyzed by several proteins and protein complexes. VPS33A has been identified as a crucial component of multisubunit tethering complexes that control δ-granule formation.[51] *Vps33a*$^{-/-}$ mice display a prolong bleeding time, which is attributed to decreased δ-granule ATP and serotonin secretion, due to their reduced number.[51] Although this suggests that the VPS33 isoforms A and B may differentially traffic cargo towards δ- and α-granules respectively, mutations in *VPS33A* have not yet been reported.[40,51]

Intracellular vesicular trafficking is governed by Rab GTPases, which control specificity and directionality of the vesicles ensuring correct protein delivery to the final destination.[52] In the case of δ-granule maturation, Rab32 and Rab38 play an important role by defining the delivery pathway from the endosomal compartment to the maturing δ-granule.[53] Both Rabs localize on Rab7a-positive MVBs, closely related to immature δ-granule, and are required for tethering and fusion of cargo-containing vesicles with the maturing δ-granule.[53] Double KO of *Rab32* and *Rab38*, but not the single KO, results in morphological defects and decreased presence of δ-granules in mouse platelets, proving that they are both required for the biogenesis of δ-granules from the MVB compartment.[54] Rab GTPases are considered to act as molecular switches, as they are found in an on GTP-bound and off GDP-bound state, each controlled by GEFs (guanine nucleotide exchange factors) and GAPs (GTPase-activating proteins), respectively.[52] Rab32 and Rab38 are activated by BLOC-3, a multisubunit protein complex that promotes the correct localization of Rab32/38 on its target membranes, which is an essential step in the formation and maturation of melanosomes.[55] Mutations in BLOC-3 subunits also lead to subtypes of the δ-storage pool disorder HPS (HPS1 and HPS4)[53,56,57]; however, to date, very little is known on how the BLOC-3 complex regulates formation and maturation of δ-granules.

Similar to BLOC-3, AP (adaptor protein)-3 is another heteromeric protein trafficking complex, which promotes δ-granule formation by delivering proteins, like LAMP2 (lysosome-associated membrane glycoprotein 2), the serotonin transporter VMAT2 (synaptic vesicular amine transporter), and most recently SLC53D3 (solute carrier family 35 member D3), to the forming granule.[53,58] Mutations in AP-3 subunits are associated with subtype HPS2 (defect in *AP3B1*) and HPS10 (defect in *AP3D1*) δ-storage pool disorders, which lead to complete absence or significantly lower numbers of δ-granules along with mislocalization or decreased expression of δ-granule cargo.[58]

Contrary to α-granule formation, δ-granules may follow a kiss-and-run maturation step, where luminal content in addition to membrane-bound proteins are exchanged between two maturing granules.[59] TPC2 (two pore calcium channel protein 2) is a calcium release channel, which operates as a dual sensor for luminal pH and calcium.[60] In megakaryocytes, TPC2 is part of the δ-granule limiting membrane and regulates the granule pH and calcium release, while it orchestrates the kiss-and-run events, through which δ-granules can achieve homogenization and adjustment of their volume.[59] Thus, TPC2 is a key regulator of a novel layer in δ-granule maturation.

Taken together, recent studies have shown that δ-granule maturation is strongly tied to cargo delivery, regulated by Rab32 and Rab38 GTPases. BLOC-3 is a known GEF for both Rabs, and mutations in BLOC-3 complex members result in HPS, therefore, by extension, it may regulate δ-granule formation. Similarly, VPS33A (vacuolar protein sorting-associated protein 33A) is shown to regulate δ-granule formation in mice; however, no current studies have been performed in human megakaryocytes, nor mutations have been described to have pathophysiological significance yet. Finally, δ-granules have an additional layer of a kiss-and-run mechanism

that normalizes their content and promotes their maturation (Figure 2).

BIOGENESIS AND MATURATION OF WPB IN ENDOTHELIAL CELLS

VWF Maturation and ER-to-Golgi Protein Trafficking in the Biogenesis of WPBs

VWF, the main cargo of WPBs, undergoes an extensive and complex maturation process before finally being stored. VWF enters the early secretory pathway, as a single prepro-polypeptide chain, and after the removal of the signal peptide, it is dimerized in the ER by the formation of disulfide bonds between the C-terminal CK (cysteine knot) domains of 2 pro-VWF monomers.[61,62] At this stage, the ER acts as the quality control check point that allows only VWF dimers to proceed to exit and subsequent transport to the Golgi apparatus.[63,64] At the Golgi, VWF dimers are further processed: the propeptide is proteolytically cleaved, and VWF dimers are organized into head-to-head multimers, before condensing into tubules and being packaged in TGN-associated WPBs.[5,61] Blockage of any step during VWF maturation may lead to abnormal formation of WPBs, like in the case of von Willebrand disease (VWD) where mutations in the *VWF* gene affect the WPB formation.[65] Interestingly, mutations have been reported in the D3, and C2 domains of VWF (p.Cys1130Phe and p.Cys2671Tyr, respectively) that affect ER exit of VWF dimers and lead to VWF retention in the ER as well as the rarer occurrence of WPBs.[64] Similar results with ER retention of VWF and formation of abnormal WPBs have been recently shown in Sec22b knockdown endothelial cells.[66] Sec22b is a SNARE protein that regulates the fusion of COPII (coat protein complex II)-coated ER-exiting vesicles.[67] Downregulation of Sec22b affects the rate of ER/Golgi transport of VWF and other proteins, which consequently affects the Golgi morphology and results in the formation of short and stubby WPBs, probably due to Golgi fragmentation.[66,68] Additionally, we and others have shown that Sec22b is paired with STX5 (syntaxin-5) as part of the ER-Golgi SNARE machinery, and depletion of STX5 in endothelial cells strongly affects WPB formation.[69,70] Depletion of STX5 in endothelial cells leads to decreased intracellular VWF storage levels and extensive Golgi dispersal, which results in the formation of shorter and rounder WPBs.[69] It has previously been shown that AP1/clathrin coats control WPB biogenesis at the level of the TGN.[71] The small GTPase Arf1 (ADP-ribosylation factor 1) is crucial for targeting the AP-1 complex to the TGN, and recently, GBF1 (Golgi-specific brefeldinA-resistance guanine nucleotide exchnage factor 1) a guanine exchange factor for members of the Arf family has been implicated in WPB biogenesis.[72] Intriguingly, depletion of GBF1 resulted in accumulation of unprocessed VWF in the ER, whereas very long, often bent WPBs were observed in close proximity to the TGN. The metabolic regulator 5′ AMPK (AMP-activated kinase) has been shown to modulate the activity of GBF1 suggesting that environmental cues can regulate number and WPB size through their effect on ER-to-Golgi.[72] Taken together, these exciting findings indicate that modulation of ER-to-Golgi trafficking of VWF can significantly alter the WPB formation by retaining VWF in the ER and thus decrease the amount of mature VWF that can be stored in WPBs while governing morphology of WPB emerging from the TGN (Figure 3).

TGN Emerging WPBs: Monitoring the Morphology of Immature WPBs

WPBs biogenesis is taking place at the TGN, where VWF units, known as VWF quanta, are tightly co-packaged in the form of tubular segments in newly synthesized WPB.[68] The first factor the regulates WPBs at the TGN site is the actual morphology of the Golgi. Dispersed Golgi and TGN morphology can negatively affect the ability of endothelial cells to incorporate sufficient amount of VWF quanta in the newly synthesized WPBs, limiting their length and affecting their string formation on the endothelial apical side.[66,68,69,73] The second factor that promotes WPB biogenesis is the VWF tubulation state. Endothelial cells that lack HPS6, a subunit of BLOC-2 complex associated with HPS type 6, have abnormal VWF tubulation and misshaped WPBs, potentially due to failed recruitment of proteins involved in acidification of WPB milieu.[74] Although the Golgi/TGN morphology sets the bar for WPB size and VWF tubulation affects the formation itself, more proteins at the site regulate WPB biogenesis like the AP-1 complex.[71] AP-1/clathrin is speculated to act as a corset, stabilizing the forming WPB during VWF-tubule assembly and preventing premature budding.[71] In parallel, Dlg1 (discs large 1) protein, associates with both mature VWF and AP-1 complex on the TGN, suggesting that Dlg1 may link VWF with a yet-unidentified transmembrane partner to the AP-1 complex.[75] Both AP-1 and Dlg1 knockdowns result in ablation of elongated WPBs and restrictive localization of VWF to perinuclear puncta.[71,75] AP-1 complex targeting and docking on the TGN is dependent on phosphatidylinositol 4 phosphate–rich domains, whose levels are determined by Golgi resident PI4K (phosphatidylinositol 4 kinases).[76] In endothelial cells, PI4KII-depletion disturbs the WPB intraorganellar environment due to abnormal VWF folding.[77] The resulting WPBs are shorter, suggesting a critical role for PI4KIIs in WPB biogenesis, as it could recruit AP-1 at the TGN.[77] At early stages of WPB biogenesis, WPBs do not leave the Golgi apparatus fast, but rather maintain multiple connections with it, for cargo delivery.[78] More nontubulated VWF is added to the newly formed WPB together with other proteins until the formation is completed and the clathrin coat is removed.[78] In

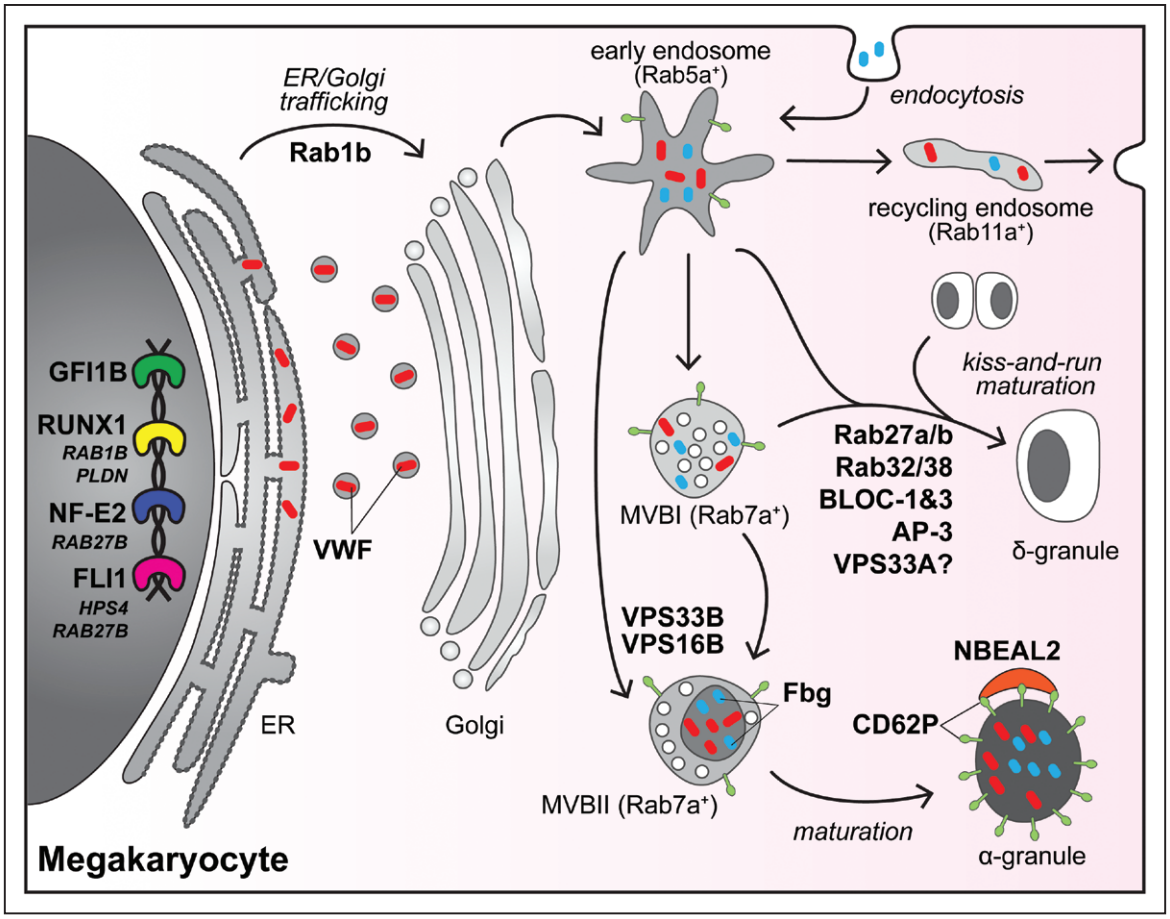

Figure 3. Model for lysosome-related organelles (LRO) formation in megakaryocytes: α- and δ-granule.
In megakaryocyte (MKs), transcription factors which regulate the expression of several genes that encode proteins, which are involved in the biogenesis of LROs. In the case of α-granules, the first identified step of their biogenesis is the Rab1b-dependent endoplasmic reticulum (ER)-to-Golgi protein trafficking. After newly synthesized proteins have been fully processing in the Golgi, they are sorted in Rab5a recycling endosomes, which serve as distribution center for new and recycled proteins. Endocytosed cargo, such as fibrinogen, is also targeted to early endosomes. The VPS33B/16B complex facilitates the transport of proteins to Rab7a positive multivesicular body (MVB) II, the progenitors of α-granules. Cargo retention in the maturing α-granule and prevention of premature release of content is achieved by NBEAL2 (neurobeachin-like protein 2) most likely by virtue of its binding to the cytoplasmic tail of CD62P, thereby providing a content-stabilizing coat for α granules. In the absence of NBEAL2 α-granular content is released through Rab11a positive recycling endosomes. On the other hand, δ-granule progenitors are Rab7a MVBI, that supply the maturing granule with appropriate cargo. Several proteins and protein complexes have been suggested to promote δ-granule maturation, including the well-characterized AP (adaptor protein)-3 complex, BLOC-1, and BLOC-3 and the small GTPases Rab27a/b and Rab32/38, while VPS33A has been implicated in δ granule biogenesis in mice. Additionally, δ-granules have an extra step during maturation, known as kiss-and-run, though which they can exchange content and achieve homogenization while adjusting their volume. Fbg indicates fibrinogen; HPS, Hermansky-Pudlak syndrome; and VWF, von Willebrand factor.

summary, WPB is formed at the TGN, which regulates its size. Their biogenesis is dependent on the recruitment of AP-1 complex at the PI4P-riched TGN domains, where, in conjunction with Dlg1 and clathrin, they create a girdle around the forming WPB. The WPB remains attached to the Golgi apparatus for the further delivery of protein cargo (Figure 3).

WPB Maturation: Acquisition of More Protein Cargo

Similar to α- and δ-granules, WPBs mature through the receipt of transmembrane cargo. WPBs, apart from soluble cargo like VWF, also contain proteins like CD62P and CD63, that follow different pathways arriving to the mature WPB. It has been shown that CD62P is accumulated on the budding WPB at the TGN through its cytoplasmic tail motif, probably while the forming WPB maintains multiple connections with the TGN as described above.[78,79] In contrast, CD63 is trafficked to the WPB at a later stage during maturation, following and AP-3 dependent route.[79] Mutations that affect the AP-3 complex formation lead to abnormal WPB maturation, resulting in aberrant trafficking of CD63 as well as SNARE proteins like VAMP8 (vesicle-associated membrane protein 8) to other cellular compartments.[79,80] Improper WPB formation results in reduced hormone-evoked VWF secretion, indicating

that WPB maturation is required for production of organelles with proper exocytotic behavior.[80] Similar to platelet LROs, WPBs acquire Rab proteins during the maturation process, like Rab3B and 3-dimensional and Rab27a that have been shown to regulate their exocytotic behavior.[81] It has been suggested that Rab27a is maturation marker that arrives at the WPB at a later stage, separating the WPBs into 2 populations: the immature Rab27a negative and the mature Rab27a positive[82] (Figure 3). The recruitment of Rab27a on the WPB membrane is potentially controlled by changes on the lipid/protein local environment driven by VWF.[82] Interestingly, although a new study sheds light on the interacting partners of mature WPB Rabs (Rab3b, Rab3d, and Rab27a), it also identifies proteins that are involved in WPB biogenesis and VWF ER-to-Golgi trafficking, like the aforementioned Sec22b, STX5, and GBF1, suggesting that our knowledge on WPB formation is still incomplete.[83]

DISORDERS ASSOCIATED WITH ABNORMAL α-, δ-GRANULE, AND WPB FORMATION

Several disorders have been described to negatively affect the biogenesis of megakaryocyte/ platelet granules and endothelial cell WPBs (Table). Gray platelet syndrome is a disease that affects the α-granule formation, caused by mutations in the *NBEAL2* gene, and clinically is characterized by macrothrombocytopenia and decreased platelet activity.[84] Similar to Gray platelet syndrome, Quebec platelet syndrome is an α-storage pool disorder that is caused by mutation in *PLAU* (urokinase-type plasminogen activator), resulting in proteolysis of α-granule proteins, reducing their expression.[86] Another disorder that is presented with bleeding tendency, in addition to liver, skin, and central nervous system complications, is arthrogryposis, renal dysfunction, and cholestasis syndrome. Arthrogryposis-renal

Table. Table of Genetic Disorders Affecting Hemostatic LRO Formation

LRO	Disorder	Abbreviation	Subtype	Mutated Gene	Bleeding Phenotype	Reference
α-granule	Gray platelet syndrome	GPS	…	*NBEAL2*	Moderate-severe	Gunay-Aygun et al,[46] Albers et al,[84] Kahr et al[85]
	Quebec platelet syndrome	QPS	…	*PLAU*	Moderate-severe	Hayward et al[86]
	Arthrogryposis, renal dysfunction, and cholestasis syndrome	ARCS	ARCS1	*VPS33B*	Severe	Cullinane et al,[44] Zhou and Zhang[87]
			ARCS2	*VPS16B (VIPAS39)*		
δ-granule	Hermansky-Pudlak syndrome	HPS	HPS1	*HPS1*	Mild-severe	Wei et al[88]
			HPS2	*AP3B1*		Karampini et al,[80] Wei et al,[88] Meng et al[89]
			HPS3	*HPS3*		Wei et al[88]
			HPS4	*HPS4*		Wei et al,[88] Meng et al[89]
			HPS5	*HPS5*		Wei et al[88]
			HPS6	*HPS6*		Ma et al,[74] Wei et al,[88] Sharda et al[90]
			HPS7	*DTNBP1*		Wei et al[88]
			HPS8	*BLOC1S3*		Wei et al[88]
			HPS9	*PLDN*		Wei et al,[88] Meng et al[89]
			HPS10	*AP3D1*		Ammann et al[91]
	Chediak-Higashi syndrome	CHS	…	*LYST*	Mild-moderate	Sharma et al[92]
	Griscelli syndrome	GS	GS1	*MYO5A*	Not reported	Van Gele et al[93]
			GS2	*RAB27A*		Van Gele et al[93]
			GS3	*MLPH*		Van Gele et al[93]
Weibel-Palade body	Von Willebrand disease	VWD	VWD1	*VWF*	Varies according to VWF levels	Leebeek et al[94]
				other genes		
			VWD2	*VWF*	Mild-moderate	Leebeek et al,[94] Nurden et al,[95] Nurden et al[96]
			VWD3	*VWF*	Severe	Leebeek et al,[94] Nurden et al[97]

Genetic disorders and their respective mutated genes that lead to a bleeding phenotype. Most of the disorders are categorized as rare, with the exception of VWD, the most common bleeding disorder, with prevalence of up to 1% of the general population. ARCS, arthrogryposis-renal dysfunction-cholestasis; CHS, Chediak-Higashi syndrome; GPS, gray platelet syndrome; GS, Griscelli syndrome; HPS, Hermansky-Pudlak syndrome; LRO, lysosome-related organelles; LYST, lysosomal-trafficking regulator; NBEAL2, neurobeachin-like protein 2; PLAU, urokinase-type plasminogen activator; QPS, Quebec platelet syndrome; and VWD, von Willebrand disease.

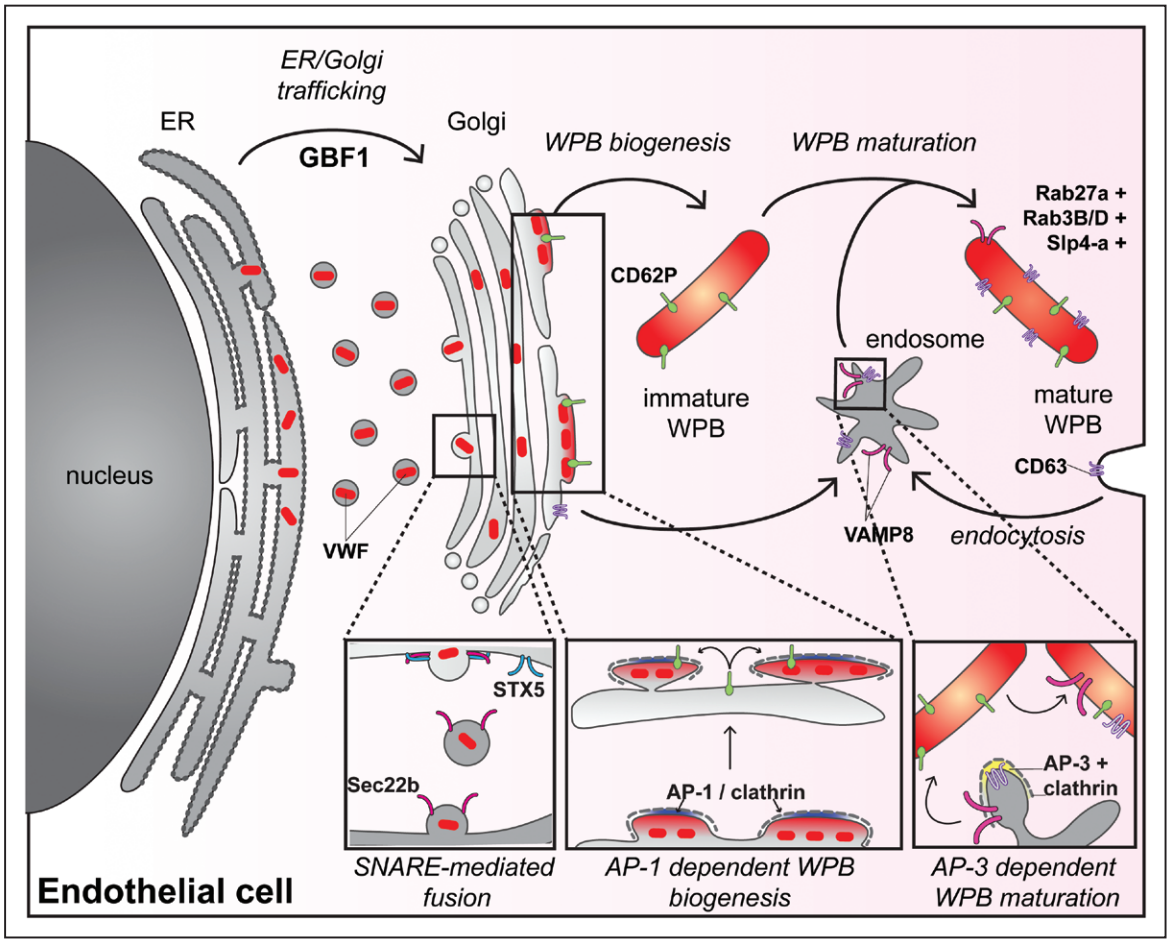

Figure 4. Model for Weibel-Palade bodies (WPB) formation in endothelial cells.
Several transcription factors have been found to positively regulate VWF (von Willebrand factor) expression, the driving force of WPB biogenesis. VWF exits endoplasmic reticulum (ER) to arrive at the Golgi in a GBF1-dependent manner. The fusion of ER-exiting vesicles is dependent on the interaction of Sec22b with the cognate SNARE on the Golgi, STX5. After VWF is fully processed and multimerized, it condenses in tubules and packages in VWF quanta in the emerging granule. At this stage, AP (adaptor protein)-1 localizes the trans-Golgi network (TGN), by means of PI4P-riched rich domains, and acts like a corset for forming WPBs, preventing it from premature budding off. The newly synthesized WPB maintains several connections to the TGN, for the further transfer of proteins potentially like CD62P. The immature WPB undergoes a maturation process, where is acquires more proteins from the endosomal compartment in an AP-3 dependent manner. Mature WPBs are positive for the small GTPases, RAB27A, 3B, and 3D as well as Slp4-a and migrate towards the periphery of the cell, ready to be secreted upon demand.

dysfunction-cholestasis syndrome consists of 2 types that influence the expression of VPS33B and VPS16B, the subunits of CHEVI complex, which is responsible for α-granule maturation.[87] Likewise, HPS is a multisystemic disorder with 10 identified genetic types that affect skin pigmentation, hemostasis, and lung tissue consistency, along with reported immunodeficiency in some types.[88,91] The mutated genes that cause HPS encode proteins that primarily facilitate δ-granule biogenesis and maturation; thus, in most cases, δ-granules are absent from the circulating platelets. Diseases that are characterized by δ-storage pool deficiency, along with hypopigmentation, are Chediak-Higashi and Griscelli syndrome (type 2). Chediak-Higashi is caused by mutations in the *LYST* (lysosomal-trafficking regulator) gene, which results in decreased δ-granule components and dysfunctional platelets, whereas Griscelli syndrome 2 is caused by mutations in *RAB27A*, which supports the retention of δ-granule cargo, but the effects are dependent on the genetic background.[28,92,93] Interestingly, although LYST exhibits great similarity to NBEAL2 in terms of protein structure—as they both contain BEACH (beige and Chediak-Higashi), ARM (Armadillo) and WD40 domains—they participate in different steps during granule formation: the former affecting δ- and the latter affecting α-granule maturation.[85,98] This indicates the great complexity of the pathways involved in the biogenesis of LROs. Finally, WPB biogenesis and maturation is affected in the most common bleeding disorder, known as VWD. VWD is characterized by quantitative or qualitative defects in VWF, the main cargo of WPBs. VWD is subcategorized into 3 types, depending on the mutations and how they alter VWF synthesis and

maturation, which, in turn, can influence the biogenesis and the morphology of WPBs.[65,94]

Either derived from MVBs or directly formed at the TGN, LRO biogenesis and maturation are governed by universal pathways.[99] Hence, there are multisystemic disorders that can alter the biogenesis of >1 LRO. Such a case is that HPS, apart from defective δ-granule formation, has been shown to alter α-granule protein release (HPS2, HPS4, and HPS9), whereas, it has also been described to influence WPB morphology and maturation (HPS2 and HPS6).[74,80,89,90] Additionally, VWD, especially type 2 and 3, has been associated with impaired megakaryopoiesis and platelet dysfunction, implicating VWF/GPIb-V-IX interactions in a novel role during platelet production.[95–97]

Taken together, LRO disorders comprise a heterogeneous group that has a multisystemic phenotype. In most cases, the bleeding tendency caused by these disorders has been attributed to a particular LRO; however, as we understand that cellular mechanisms are shared between cell types, it becomes clearer that >1 LRO can be affected.

PERSPECTIVE

Although we have a better grasp on some aspects, we still need to cover a lot of ground to fathom out the tightly regulated cellular mechanisms involved in the hemostatic LRO formation. What is seemingly the most important factor in LRO biogenesis and maturation is the correct delivery of specialized cargo. It has recently been appreciated that ER-to-Golgi protein influx is the first regulatory step in LRO formation, through which the cell can control the protein supply to progenitor compartments in response to environmental cues like glucose levels. However, the exact molecular mechanisms and protein complexes are poorly defined. Additionally, it has been described that the same type of granules can contain different packaged proteins, giving rise to subpopulations that that may undergo different modes of exocytosis.[100] How is cargo distributed differently? Are the same proteins monitoring different components' trafficking, and if so, what are the molecular cues for differential composition? These are questions that we cannot yet answer, which points out the need for further examination. As far the LRO cargo is concerned, recently, it has been shown that LROs contain also CD63 positive intraluminal vesicles that contribute to intercellular communication and modulate the targeted cells through acute secretion upon stimulation.[8,101] However, whether the process of intraluminal vesicle accumulation in LROs is intentional or unintentional and how the cell may control this acquisition are still unknown.

It is now understood that, despite the different characteristics of LROs types, several pathways can have a universal impact in the formation of >1 granule type. Therefore, disease models that are traditionally connected with one particular LRO can be used to study the formation of another LRO in a different cell type. In such cases, the recent developments in induced pluripotent stem cells can be utilized in LRO biology. Starting from fibroblasts, CD34-positive progenitor cells and blood outgrowth endothelial cells, cells from various disease models can easily be reprogrammed in a pluripotent state and differentiate into different cell types, containing different LROs. In this way, the parallel monitoring of the mechanisms involved in granule formation can highlight the similarities or point out the differences.

Megakaryocyte/platelet and endothelial LROs are the major players in primary hemostasis, regulating cell-cell communication and secreting bulk amounts of hemostatic proteins. Mutation in genes that govern their formation results in abnormal morphology, secretion, and finally, in bleeding disorders. Therefore, improved comprehension of their biogenesis and cargo retention can lead to better and more personalized treatments for patients with bleeding disorders as well as patients with thrombo-inflammatory and other vascular disorders.

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Affiliations

From the Department of Molecular and Cellular Hemostasis, Sanquin Research and Landsteiner Laboratory (E.K., R.B., J.V.) and Experimental Vascular Medicine (J.V.), Amsterdam University Medical Center, University of Amsterdam, the Netherlands; and Hematology, Erasmus University Medical Center, Rotterdam, the Netherlands (R.B.).

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