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A new look at an old body: molecular determinants of Weibel-Palade body composition and VWF exocytosis

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Running head: Weibel-Palade body content and release

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Abstract

Endothelial cells, forming a monolayer along blood vessels, intricately regulate vascular hemostasis, inflammatory responses, and angiogenesis. A key determinant of these functions is the controlled secretion of Weibel-Palade bodies (WPBs) which are specialized endothelial storage organelles housing a pre-synthesized pool of the hemostatic protein von Willebrand factor (VWF) and various other hemostatic, inflammatory, angiogenic, and vasoactive mediators. This review delves into recent mechanistic insights into WPB biology, including the biogenesis that results in their unique morphology, the acquisition of intraluminal vesicles and other cargo, and the contribution of proton pumps to organelle acidification. Additionally, in light of a number of proteomic approaches to unravel the regulatory networks that control WPB formation and secretion we provide a comprehensive overview of the WPB exocytotic machinery, including their molecular and cellular mechanisms.

Keywords: Weibel-Palade Bodies, von Willebrand Factor, exocytosis, endothelial cells, organelle biogenesis

Introduction

Weibel-Palade bodies (WPBs) are rod-shaped storage organelles found only in the cytoplasm of vascular endothelial cells [1]. The principle soluble component of WPB is the mosaic glycoprotein von Willebrand factor (VWF) [2]. VWF is a secreted protein that functions physiologically in primary and secondary hemostasis. VWF first appeared in chordates [3] at around the same time that important evolutionary steps were occurring in the early blood coagulation system in response to selective pressures posed by more complex, high pressure circulatory systems [4,5]. WPBs are classified as Lysosome Related Organelles (LROs) [6], a class of subcellular organelles that arose in metazoans as cell-specific functional adaptations of the endolysosomal pathway. The adaptable size and unique shape of WPBs facilitates the optimal release of VWF. As secretory organelles, WPBs are also involved in the controlled release of additional cargo, thereby contributing to hemostasis, angiogenesis and inflammation (Figure 1A). Ongoing research aims to unravel the complexities of their cargo composition, including the contribution of organelle acidification to organelle rigidity, exploring how they respond to various signals and investigating the potential existence of subclasses tailored for specific functions. Recent advancements, including insights from techniques like proteomic interactome screening, have significantly expanded our understanding since the last review from our lab [7]. In this updated overview we delve into these recent discoveries, providing novel molecular and mechanistic insights into WPB biology.

Novel mechanistic insights into WPB biogenesis: dependence on Golgi structure and the role of SNAREs and ARFs in ER-Golgi transport

VWF was the first protein identified as cargo molecule within WPB, confirming their function in hemostasis [2]. The formation of the organelles is entirely driven by the expression of VWF [8,9], therefore genetic defects in *VWF*, such as in Von Willebrand

Disease (VWD), can impair WPB biogenesis and morphology [10,11]. The biosynthesis of VWF is extensively reviewed and will not be covered here [12,13]. A number of studies have established that there is a correlation between the length of the WPB and the hemostatic potential of its main cargo VWF, which includes the length of VWF strings formed upon release, the number of platelets that can adhere to these strings and the self-association with VWF from plasma [14,15]. How the size of WPBs, which can range from 0.5 μm up to 5 μm in length, is regulated is now beginning to be understood. Large scale morphometric analysis revealed that WPB lengths seemed to occur at a rhythm of approximately 0.5 μm intervals [16], which is best explained by a scenario that involves an amalgamation of equally-sized building blocks. In the model that was put forward, VWF arrives at the trans-Golgi network (TGN) in discrete "quanta" of protein cargo that are condensed within TGN cisterna [16]. Simultaneous arrival of such quanta in adjacent regions within the TGN would lead to their joint incorporation in a newly budding WPB, its length being determined by the number of quanta that are organized alongside each other. Interestingly, the dimensions of these VWF quanta correspond to those of so-called Golgi mini-stacks, the minimal units from which Golgi cisterna self-organize in endothelial cells [17]. This is supported by the observation that unlinking the Golgi into smaller sized units is accompanied by a similar step-wise reduction in WPB size. In 2D and 3D reconstructions of WPBs imaged by CryoEM densely packed and parallel-arranged VWF tubules can be found that extend from one end of the WPB to the other [18]. VWF tubules are essentially helically condensed ultra-long VWF multimers and to reconcile the existence of such long tubules with the joining of quanta, multimerization needs to take place after their arrival in the TGN with sufficient room and time for rearrangements into the VWF paracrystal. From this model it also follows that Golgi integrity, i.e. an extended Golgi ribbon, and a continuous flux of VWF through the early secretory pathway are essential for the formation of elongated WPBs (Figure 2A). Recently, two protein families

crucial for VWF trafficking from the endoplasmic reticulum (ER) to the TGN have been identified: soluble NSF attachment receptors (SNAREs) and ADP-ribosylation factor (Arf) proteins. Anterograde transport of VWF from the ER requires the longin-SNARE SEC22B and its cognate SNARE Syntaxin-5 (STX5), located on the cis-Golgi membrane (Figure 2B). Depletion of either SNARE results in impaired ER-Golgi trafficking as well as Golgi fragmentation, and leads to loss of elongated WPBs [19,20]. In SEC22B depleted cells dilations of the ER cisterna containing aggregates of VWF were observed by electron microscopy (EM), indicative of defective progression of VWF to the TGN [19]. Arf proteins and the guanine exchange factors (GEFs) that regulate them, are now known to be crucial in VWF trafficking (Figure 2B). The Golgi-specific GEF, GBF1, plays a key role in VWF trafficking and links WPB biogenesis to cellular metabolism. When glucose levels drop, AMP-activated protein kinase (AMPK) activates GBF1, that in turn regulates Arf1 and Arf4 to control anterograde VWF trafficking, leading to smaller WPBs and reduced VWF secretion [21]. The Arf GTPase activating protein (GAP) SMAP1 is also reported to regulate WPB size [22], although the mechanism appears not to involve Arf6, the Arf GTPase associated with SMAP1 [21,22]. The mechanism by which SMAP1 regulates WPB size remains to be established but could be through clathrin recruitment during organelle formation. Importantly, loss of function of SNAREs or Arf proteins associated with WPB formation can affect their morphology in ways that are similar to what is observed in endothelial cells from patients with VWD causing mutations in VWF, emphasizing the relevance of the trafficking machinery in WPB biology and VWF function.

New insights into the composition of WPBs

Although VWF is the primary cargo within WPBs, it has been known for many years that the luminal composition of these storage organelles can be highly complex and highly plastic, changing in response to fluid shear, pro- or anti-inflammatory signals [2,23–36]

(Figure 1A). The diversity of cargo found within WPBs suggests that these organelles can take part in various physiological process beyond hemostasis, notably leukocyte recruitment and angiogenesis [9,37,38]. How all these different soluble cargo molecules enter into and are subsequently retained within WPBs remains of considerable interest (Figure 1B). In several cases the low pH and high calcium environment of the TGN appears to be important. Using *in vitro* binding assays with purified proteins two studies have recently shown that the Tie-2 ligand angiopoetin-2 (Ang2) binds with high affinity to VWF under conditions of low pH- and high Ca^{2+} similar to that of the TGN [39,40], indicating a mechanism similar to that of another soluble WPB cargo, osteoprotegerin (OPG) [25,41]. However, while both studies confirm the binding of Ang2 to VWF, they diverge in their conclusions regarding the specific domains involved. Mobayen et al [39] show binding to the A1 domain, whereas Texier et al [40] suggest a broader interaction encompassing multiple domains in VWF. To what extent such *in vitro* protein-protein interactions can be extrapolated to what happens in the complex mix of proteins within the confines of the Golgi is uncertain. pH-dependent binding of chemokines to VWF may also mediate inclusion in WPBs [42,43], but mobility analysis of EGFP-tagged chemokines inside mature WPBs show that such interactions are at most weak, enabling selectively rapid chemokine secretion during WPB exocytosis [44,45]. A route into the forming granule that is independent of interaction with VWF is also possible, since a variant of FVIII that lacks the high affinity binding site for VWF and even EGFP attached to a signal peptide are able to enter the WPB when ectopically expressed in endothelial cells [26,46]. A key new discovery has been the identification of intraluminal vesicles (ILVs) inside WPBs (Figure 3A-D) and the demonstration of their release as exosomes during regulated exocytosis [47]. The discovery of a hitherto unrecognized pathway for exosome-mediated signaling by the endothelium raises many new questions including when and how WPBs acquire ILVs, what they contain, and what they do once secreted. WPB-ILVs can be

identified in individual organelles as discreet regions of enrichment of the endosomal/exosomal protein CD63. The absence of CD63 in newly forming WPBs connected to the TGN [48], coupled with the absence of ILVs detect in this compartment by ultrastructural studies [49] suggest that CD63-positive ILVs are acquired at a later step in the life cycle of the organelle. Mature WPBs studied by cryo-electron tomography of vitrified cells show a very smooth limiting membrane shrink-wrapping a tightly packed para-crystalline assembly of tubules [18,47]. The absence of membrane ruffles suggests little or no excess membrane for ILV formation and the rigid para-crystalline matrix a physical obstacle to inward budding, which makes it unlikely that ILVs can still be incorporated into WPBs at this stage. In 2D and 3D cryo-EM images ILVs were often seen to be highly distorted/squeezed between the smooth tight wrapped membrane and the para-crystalline tubule matrix (Figure 3D) suggesting compression during organelle dehydration. VWF tubules are inherently flexible but pack to a ridge paracrystal in the mature organelle [18]. Tubule disruption around ILVs indicate they were most likely present before dehydration and tubule packing. Thus, it seems likely that ILVs appear during the post-Golgi maturation phase when WPBs are actively acquiring endosomal components, and sorting away unwanted materials [48–52] prior to final dehydration. The presence of cytoplasmic components inside WPB-ILVs (Figure 3B-C) [47] indicates they form by a process of inward membrane budding, but in which membrane compartment does this process occur? The WPB limiting membrane acquires CD63, along with other integral membrane proteins (e.g. VAMP8) from a late endosomal compartment as part of the post-Golgi maturation process [48,50,51,53]. CD63 delivery to post-Golgi-WPBs involves the adapter complex AP-3 [48,50], Annexin-8 (CD63) [54] and potentially BLOC-2 [52]. While direct fusion and content transfer between endosomes and WPB have yet to be directly visualized, the nature of the proteins trafficked (integral membrane components) along with the intimate associations seen between WPBs and MVBs [18] or

small carrier vesicles make it the likely scenario. Tracking the fate of cell surface labelled CD63 in live cells showed that CD63 in WPB-ILVs (like that in the WPB limiting membrane) is also derived from endosomal trafficking [47]. This key observation gives rise to two possible scenarios for how WPBs acquire ILVs; first, preformed CD63-positive ILVs are transferred from late endosomes/MVBs to WPBs during fusion as part of the organelle's maturation process (Figure 3E model 1). Second, inward budding of the WPB membrane itself captures endosomal derived CD63 after its delivery to the WPB limiting membrane (Figure 3E model 2). Future ultrastructural examination of WPBs in endothelial cells either depleted of CD63 or where endosomal-WPB trafficking is blocked (e.g. in Hermansky-Pudlak syndrome type 2 or 6 [50,52]) will help clarify a role for endosomal derived factors in WPB-ILV formation. Identification of a pore connecting an ILV to the WPB membrane would provide strong evidence for ILV biogenesis within the WPB.

The composition of WPB-ILVs is likely to be complex. Each ILV contains a small biopsy of the cell's cytoplasm with components such as ribosomes and glycogen granules clearly visible [47]. Proteomic and RNA sequence (RNAseq) analysis of EC-derived exosomes has shown a rich diversity of proteins and RNA types, and functional studies implicate EC exosomes in regulating a wide range of processes including cellular protection [55], atheroprotection [56], immune tolerance [57], antitumor response [58] and angiogenesis [59,60]. Interestingly, the angiogenic function of EC exosomes overlaps with those of other soluble WPB cargo proteins such as Ang-2, VWF and IGFBP7 [24,27,37,61] and it is possible that exosome release via the WPB-ILV pathway complements or augments the function of soluble angiogenic mediators in WPBs. Whether the exact composition of WPB ILVs differs from other EC-derived exosomes and if the quantities of cargo released via this novel pathway are of sufficient magnitude to drive biological processes, requires further studies. Establishing how WPB ILVs are formed will be an important step towards elucidating their physiological role. Such insights may also provide new approaches to

selectively promote or interfere with the formation of WPB ILVs, allowing for systematic investigations of their composition using multi-omic profiling and functional studies of (purified) WPB-ILVs.

Molecular basis for WPB acidification

Recent studies have shed new light on the molecular basis for the regulation of intra-WPB pH during organelle formation and maturation [62–64]. During WPB formation, the acidic pH within the TGN (pH 6.5-6.2) facilitates the non-covalent association of VWF dimers through the N-terminal D1-D3 domains leading to formation of tubule like structures [65,66], that position the D3 domains in such a way that allows intermolecular disulfide bond formation and VWF multimerization [67]. As VWF tubules grow they drive formation of the WPB and the order packing of these long tubule structures is thought to be important for the way in which VWF multimers unfurl to form extracellular strings following secretion [68–70]. Since the acidification of TGN is maintained by the activity of vacuolar-type H+ ATPases (v-ATPase) [71], it seemed logical that such a pump operates within the WPB membrane since following scission from the TGN the intra-WPB pH continues to decrease rapidly during maturation to close to the isoelectric point for VWF [69]. v-ATPases consist of two distinct domains that operate in tandem. The V1 domain, composed of 8 subunits, hydrolyses ATP, while the V0 domain, comprising 6 subunits, translocate the resulting H^+ ions through the host membrane [71] (Figure 4A). Recent proteomic analysis of WPB content identified components of this pump [27,72,73]. Blocking the v-ATPase complex has no effect on exocytosis but does reduce VWF unfurling and therefore platelet recruitment, producing results similar to those observed with overall forced neutralization of the WPB interior through the use of ionophores or weak bases [63,70]. So far, the subunits V1G1, V1A, V0c, V0d1, V0a1 and V0a2 have been shown by immunofluorescence or proteomic analysis to be associated with WPBs [27,62,63].

Intriguingly, V0a1 and V0a2, two isoforms of the V0a subunit, appear to localize to WPBs at different maturation stages. V0a1 colocalizes with the maturation marker Rab27a on mature WPBs, while V0a2 is predominantly found in nascent WPBs in the perinuclear region [62] (Figure 4B). The presence of these isoforms, and thus two (or more) v-ATPase complexes with different functions is confirmed by the distinct effects of depleting either of them. V0a1, primarily found on mature WPBs, appears to be crucial for separating WPB buds from the TGN. Whether this is a direct function of V0a1 or mediated by the recruitment of other proteins is still unclear. On the contrary, depletion of the V0a2 subunit results WPB that are able to separate from the TGN but appear to have an altered tubule organization [62]. Their presence at different stages could indicate a regulatory mechanism where the transition from nascent to mature WPBs involves a switch in v-ATPase isoform composition, possibly influenced by signals from the cellular environment or specific cues within the TGN. Overall, it seems like V0a2 is a resident TGN subunit and then V0a1 is recruited for membrane fission. Importantly, the depletion of these subunits not only affects WPBs but also leads to the neutralization of the luminal pH in the Golgi. This perturbation in Golgi pH balance could potentially have additional downstream effects, such as aberrant glycosylation of secretory proteins. Interestingly, in some patients with V0a2 mutations bleeding diathesis and wound healing defects have been reported, as well as a low VWF activity to antigen ratio [74,75].

How might v-ATPase subunits be trafficked to mature WPBs? Rab27a depletion does not disrupt WPB acidification, suggesting that v-HSP6, a component of the BLOC-2 complex responsible for cargo trafficking from endosomes to LROs may be involved in V0d1 delivery to WPBs influencing their acidification and size [63,64]. More work is needed to establish the specific routes by which different v-ATPase subunits are delivered to WPBs. Many questions remain to addressed: Do v-ATPases play a role in initiating acidification, or is their sole purpose to maintain the acidic luminal environment within mature WPBs?

What is the mechanism and timing of complex assembly? Are there distinct subsets of organelles with varying v-ATPase compositions, and if so, how does this diversity affect WPB integrity and exocytosis?

Proteomic insights into WPB maturation and secretion

Newly formed WPBs are secretion-incompetent and acquire their secretion competence as they mature by the recruitment of soluble factors to their membrane [76]. Among these factors are Rab27a and Rab3d, two members of the Ras superfamily of small G proteins [77,78]. Recent research has revealed that the guanine nucleotide exchange factor (GEF) MADD activates both GTPases, resulting in their localization to WPB and the subsequent assembly of effector complexes on the WPB membrane [79–81]. Given its role in WPB maturation, Rab27a has been employed as bait in numerous proteomic studies to identify novel effector proteins that localize to WPB (Figure 5A). We anticipate that these proteomic approaches will continue to unveil both known and previously unexplored proteins relevant to WPB biology. Four studies on the WPB interactome have so far been published that use two different approaches. The first approach involved “classical” pulldown experiments with the Rab27a effector Slp4-a or the SNARE proteins syntaxin-3 (STX3) and syntaxin binding protein (STXBP) 5 (STXBP5, formerly known as tomosyn) [72,82]. The second approach, applied by two different groups, is the use of Rab27a-APEX2 proximity biotinylation [73,83] (Figure 5A). The strength of proteomic studies lies in the combination of results from multiple research teams, thereby enriching the pool of biologically significant discoveries. To pinpoint frequently detected protein interactors, we have combined the results of the four published studies with WPB effector proteins in Figure 5B. While there is not a single common hit across all studies, there are 85 proteins found in multiple of them. 12 out of these 85 proteins have been published in the context of WPB biogenesis (**bold** in Figure 5B [21,63,64,72,76,84–92]). Other hits consist of

proteins involved in various cellular processes, including vesicle transport (such as SNARE, COPII, NRZ), Golgi proteins (some of them previously associated with Golgi fragmentation upon knockdown), Rab and Rho GTPases along with their GEFs and GAPs and the actin cytoskeleton. These findings provide further support for the involvement of these protein groups in the regulation, formation, and function of WPB, highlighting the complexity of WPB trafficking and fusion mechanisms. Notably, biallelic mutations in some the identified proteins, such as ARFGEF1 (BIG1), ATL3, BET1, and WDR11, have been associated with rare and severe diseases with predominantly neurological phenotypes [93–96]. The rarity of these diseases shows the critical importance of intracellular trafficking. However, it has been shown that knocking down ARFGEF1 has no apparent impact on WPB formation [21]. This underscores the need for validation of the specific contribution of these individual proteins to WPB biology and VWF secretion and that such a role cannot be assumed.

Calcium-sensitive proteins and regulators in WPB exocytosis

In endothelial cells, WPB exocytosis is primarily driven by sustained high levels of intracellular calcium ions (Ca^{2+}), which occur during stimulation by physiological agonists or cellular injury. However, our understanding of how these Ca^{2+} increases are sensed and translated into WPB exocytosis remains incomplete, prompting research to focus on this aspect of the process. Prior to stimulated exocytosis WPBs located in the cell periphery are anchored to the actin cytoskeleton via the Rab27a effectors MyRIP and Myosin Va [89,97,98]. This serves to strategically position the organelles close to the plasma membrane whilst preventing premature exocytosis. As calcium levels increase within the cell in response to hormones or injury, a series of calcium-sensitive proteins come into play, contributing to plasma membrane docking and fusion of WPBs. WPBs bind to specific plasma membrane microdomains enriched for phosphatidylinositol 4,5-

bisphosphate (PIP_2) [99]. The formation of these microdomains is orchestrated by calcium-dependent regulation of RalA, which, in turn, promotes Arf6-dependent phospholipase D1 (PLD1) activity [84]. Two Ca^{2+} binding effector proteins of Rab27a, synaptotagmin-like protein 4a (Slp4-a) and Munc13-4 guide WPB localization to the plasma membrane [76,81,100]. Munc13-4 associates with S100A10 that operates in conjunction with Annexin A2 (AnxA2), which is recruited in a Ca^{2+} dependent fashion to newly formed PIP_2 microdomains. Together these proteins form the adapter complex that links WPB to the plasma membrane [81]. Additionally, WPBs dock to the SNARE machinery on the plasma membrane via a Rab27a/Slp4-a/STXBP1 or STXBP3 complex [72,76]. The resulting SNARE complexes that are required for WPB-PM fusion consist of the R-SNAREs VAMP3/VAMP8 (on the organelle) [50,87] and the Q-SNAREs STX3/STX4/SNAP23 (on the plasma membrane) [85,86,101]. While STX2 is frequently identified in genetic association studies with levels of WPB proteins [102,103], its role in this fusion process remains unconfirmed by experimental evidence. A more recent discovery introduces synaptotagmin-like protein 2a (Slp2-a) as a calcium dependent effector of Rab27a. This protein mediates tip-end fusion of the organelle, a noteworthy revelation given their unconventional shape [80]. This mediation is achieved through the Ca^{2+} dependent condensation of Slp2-a, which can bind to PIP_2, at the WPB tip. Although this is beneficial for the release of highly multimeric VWF, it does not significantly impact WPB-PM fusion. This explains the relatively mild inhibitory effect of Slp2a knockdown on total VWF secretion [80].

When WPBs have reached the plasma membrane, the stage is set for fusion to occur, a process intricately linked to the regulation of calcium levels in conjunction with the SNARE fusion machinery. Notably, SNAREs themselves lack inherent sensitivity for Ca^{2+}. Instead, the calcium dependence of exocytosis is widely attributed to synaptotagmins, a family of Ca^{2+}-sensing proteins renowned for their interaction with SNAREs. Among these

synaptotagmin proteins, Synaptotagmin 5 (SYT5) emerged as a key player in the orchestration of WPB exocytosis, functioning as a vesicle-associated calcium sensor [104]. SYT5 exhibits a lower affinity for calcium compared to other synaptotagmins, a trait that seemingly serves as a regulatory mechanism. It is believed that SYT5 predominantly recruits effector molecules when intracellular Ca^{2+} levels increase to higher thresholds, a feature that functions as a safeguard [104]. This selective recruitment strategy effectively reduces the risk of untimely release of high molecular mass forms of VWF and inflammatory mediators when their presence is unwarranted. The link of SYT5 to the SNARE complexes involved in WPB exocytosis has not been found, and it is unknown whether other synaptotagmins play a role in this process. At the final stages of WPB exocytosis, Rab46, a calcium-sensing Rab GTPase, might play a role by redirecting a subpopulation of WPBs away from the plasma membrane to the microtubule organizing center in response to histamine [105].

In summary, the stimulated release of WPBs is a highly orchestrated process involving preparation of the PM by altering the lipid composition, moving the organelle to the PM, granule polarization, and SNARE formation that finally results in fusion and release (Figure 6). Novel key participants have been identified, but the underlying molecular mechanisms governing their functions require further investigation.

Diverse mechanisms of VWF secretion and actomyosin dynamics in WPB exocytosis

There are three ways VWF can be secreted. Constitutive secretion consists of constant, unimpeded release of newly synthesized VWF that is directly released from the TGN via constitutive vesicles without the need for cellular stimulation. Cargo from this route is characterized by short, low molecular weight forms of VWF that are primarily released at the endothelial cell's basolateral side into the subendothelial matrix [106]. This pool of

VWF can bind platelets [107], but consists of short multimers and doesn't form long strings [14]. Basal release, which involves non-stimulated release of regulated cargo, consists of continuous low-rate secretion of higher molecular weight VWF multimers that originate from WPBs and are preferentially released at the apical face of the endothelium [106,108]. This route has been postulated to account for the vast majority of circulating VWF. The regulated secretion pathway, triggered by agonists, releases a rapid bolus of high molecular weight (HMW) VWF in the vessel lumen by simultaneous exocytosis of a large number of WPBs. Ca^{2+} or cAMP serve as second messengers in response to these signals. Regulated secretion of WPB involves two distinct mechanisms. In the majority of WPBs VWF is rapidly expelled from the organelle upon fusion with the plasma membrane. The explosive release of tubularly packed VWF is driven by the entry of water molecules, and increase in WPB luminal pH that occurs immediately upon fusion [69,109]. In a smaller fraction of WPBs, approximately 20-30%, expulsion of VWF is not immediate, but occurs several seconds after fusion and is associated with the formation of an actomyosin ring around the post fusion organelle [80,83,110,111] (Figure 6). Two functions for actomyosin structures are proposed: firstly, to stabilize the fusion pore and contributing to sustained exocytosis; secondly, to facilitate the expulsion of HMW VWF through a mechanical "squeezing" process. The actomyosin ring refers to a complex structure formed by the coordinated interaction between actin filaments and myosin motor proteins. It is not a single protein or entity but rather a composite structure made up of multiple proteins working together. It typically consists of actin filaments that provide the structural framework, myosin motor proteins that generate contractile forces and other regulatory proteins that modulate its structure and activity. It's worth noting that the depletion of Slp2-a , also disrupts actin ring assembly [80]. This suggests that a specific organelle orientation might be a requirement for actin ring formation. Furthermore, ongoing investigations continue to explore the composition and functional nuances of actin rings in the context of

WPB exocytosis, revealing variability based on the specific stimulus employed [69,109–113]. During cAMP-mediated stimulation, the focal adhesion protein zyxin mediates the formation of actin frameworks around the WPB in a myosin II-a dependent manner [112,113]. The knockdown of zyxin does not affect Ca^{2+} mediated VWF secretion, suggesting that different mechanisms of actin nucleation are employed after different stimuli [113]. Other studies revealed that histamine stimulation or wounding, both Ca^{2+} mediated, can induce actin ring formation in a subset of post-fusion WPBs, again confirming that this phenomenon occurs at a later stage than the initial release [110,114]. This process is dependent on RhoA, which potentially activates Arp2/3 or Spire1, effectors of the Rho GTPase that facilitate actin nucleation at WPB fusion sites [114,115]. Additionally, Spire1 interacts with MyoVc and can bind to Rab27a or Rab3 isoforms, thereby facilitating its recruitment to WPB-PM fusion sites. Of note, even with a mutated Rab3-binding site in MyoVc, its recruitment to WPBs remains observable, hinting at additional recruitment modes, potentially involving interactions with Spire1. Depletion of Spire1 or MyoVc results in diminished surface VWF and reduced actin ring formation following histamine stimulation [114]. This underscores their roles in initiating or stabilizing these actin structures. The mechanisms by which Spire1 and MyoVc are activated remain unknown. Septin rings are recruited to fused WPBs before actin rings form. Their primary role is likely to assist in the coordination of myosin proteins (e.g., NMII or Myo1C) or their activators to facilitate actomyosin ring function. The recruitment of septin hetero-oligodimers to WPBs is initiated by p21 activated kinase 2 (PAK2). In the absence of septins, the actomyosin ring exhibits slower contraction kinetics and eventually disassembles [83].

The complexity of actomyosin ring formation is exemplified by the diverse array of processes described, including signaling pathways (involving PAK2 and rho GTPases), actin nucleation (influenced by Spire1 and Arp2/3, as well as zyxin for reorganization),

linking, and actomyosin contraction (involving septins and myosins). However, several fundamental questions remain unanswered: Firstly, the mechanism by which actomyosin attaches to the organelle membrane remains a puzzle. Secondly, the molecular physical route that drives the 'squeezing' process itself remains unknown. Lastly, there is the question surrounding the biological significance of these actomyosin rings, given that the majority of VWF is secreted rapidly in a subsecond expulsion manner that is independent of their function.

Conclusions and future directions

In conclusion, WPBs intricately regulate the storage and release of VWF and an array of additional cargo molecules, responding to environmental cues. From the formation and maturation of WPBs to the precise cargo release mechanisms, there remains much to explore and comprehend. By deciphering the complex pathways that result in WPB formation and expulsion we can potentially intervene in pathological conditions, such as vascular complications seen in COVID-19, and advance treatments for disorders related to aberrant WPB function. Moreover, selective tuning of the endothelial response, including aspects like WPB size selection, cargo inclusion, and the strength of expulsion, remain interesting questions in WPB biology that will be explored further in the next years. The journey through WPB complexity offers great potential for both scientific discovery and clinical application, promising innovative strategies to manipulate these cellular structures for therapeutic benefit.

Contribution

S.H. created the figures, all authors contributed to the writing of the paper.

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Disclosure of Conflicts of Interest

The authors state that they have no conflict of interest.

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Figure Legends

Figure 1: Contents of a Weibel-Palade body (WPB). A; Cargo sorted by function. VWF, Von Willebrand Factor; OPG, osteoprotegerin; tPA, tissue-type plasminogen activator; Eo-3, eotaxin-3; IL-6, interleukin-6; IL-8, interleukin-8; Groα, growth related oncogene α; MCP1, monocyte chemoattractant protein-1; Ang-2, angiopoietin-2; IGFBP7, insulin-like growth factor-binding protein 7; ET-1, endothelin 1; CGRP, calcitonin gene-related peptide. **B**; Mechanisms of cargo inclusion in WPB. The model depicts molecules binding to VWF, independent cargo entry, and the endosome recycling pathway.

Figure 2: WPB size is a regulated process. A; Illustration depicting the regulated biogenesis of WPBs where VWF quanta arrive at the TGN and are co-packaged into newly forming WPBs. B; Close-up view of the selected area highlighting the regulatory role of SNARE proteins SEC22B and STX5, along with Arf proteins, in the regulation of ER-Golgi trafficking.

Figure 3: How may WPBs acquire intraluminal vesicles? A-D; examples of WPBs containing ILVs. B-C; ILVs often contain cytoplasmic components. D; ILVs can be seen compressed between the WPB limiting membrane and the VWF para-crystal. Scale bars 200 nm. E; two possible models by which WPBs may acquire ILVs. Panels A-D reproduced with permission of the rights holder, Elsevier, license numbers 5471370060842 (A-C), 5481321142311 (D).

Figure 4: v-ATPase pump is present in the WPB membrane. A**;** The v-ATPase pump comprises two essential subunits: the soluble V1 domain, responsible for ATP hydrolysis, and the membrane-spanning V0 domain, which transfers resulting H+ ions into the

organelle. The pump functions effectively only when these subunits are together. B; Distinct v-ATPase complexes, characterized by different isoforms of the V0a domain, are localized to different stages of WPB. V0a2 localizes to nascent WPB at the TGN, while V0a1 is only found on mature WPB. The complete pump is shown for reference, with only the subunits identified through immunofluorescence or proteomic studies rendered opaque. Other subunits are depicted transparent.

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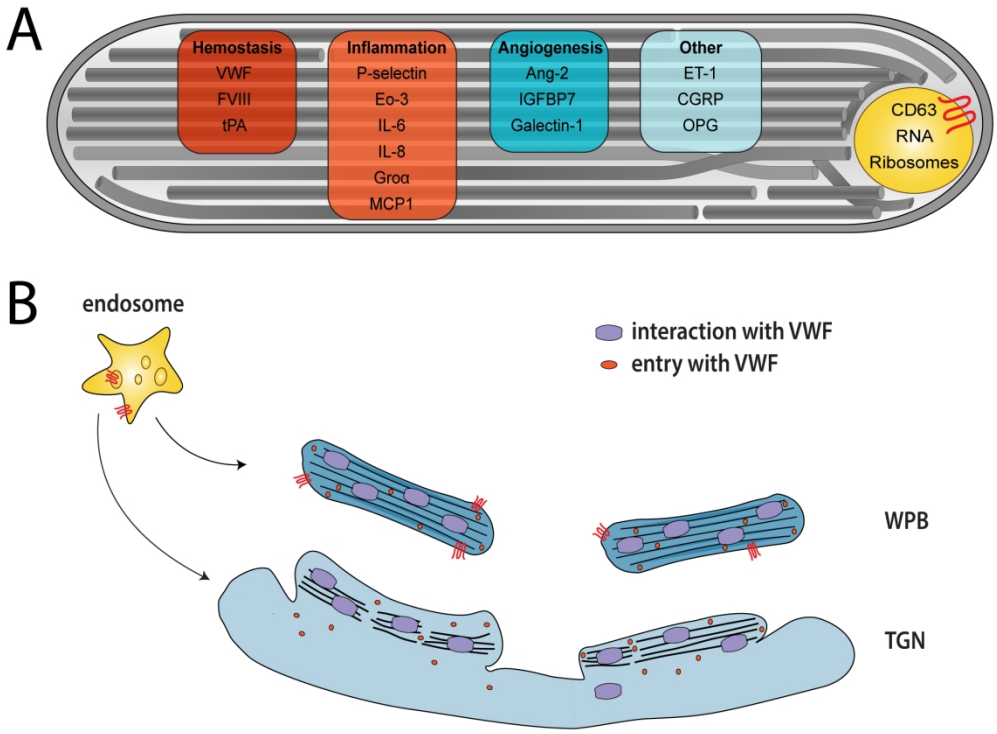

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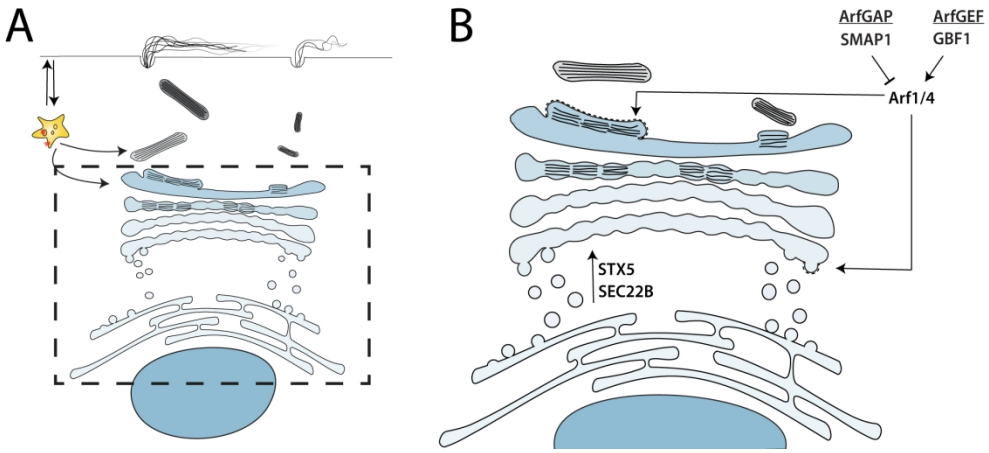

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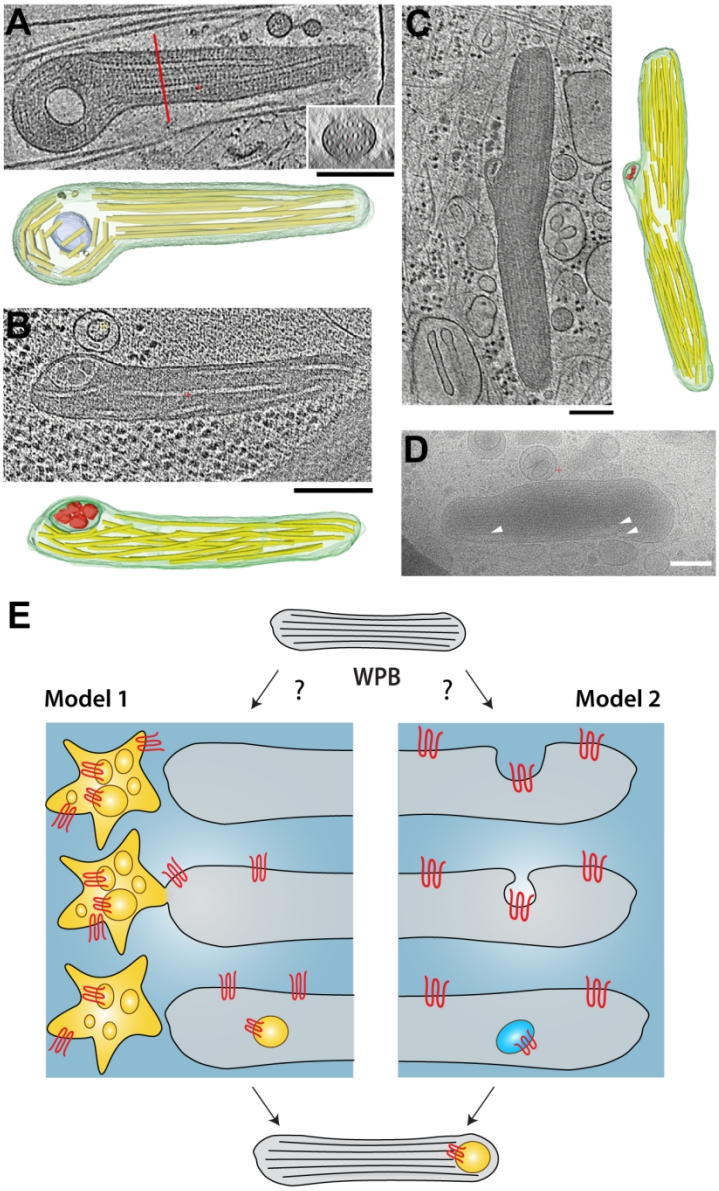

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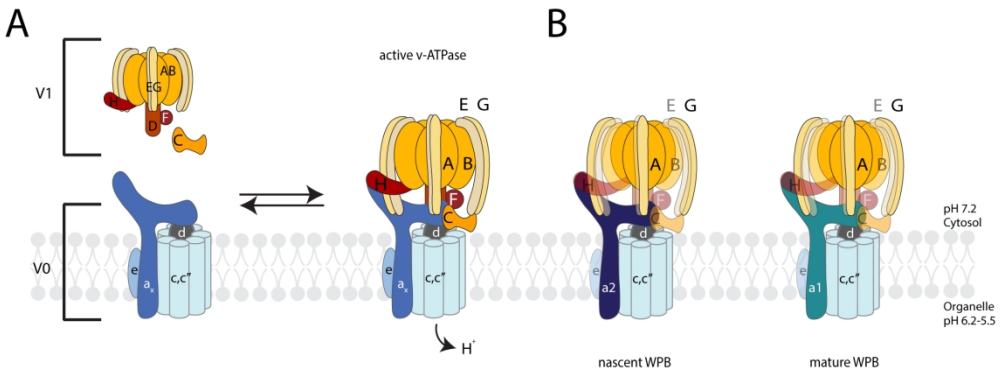

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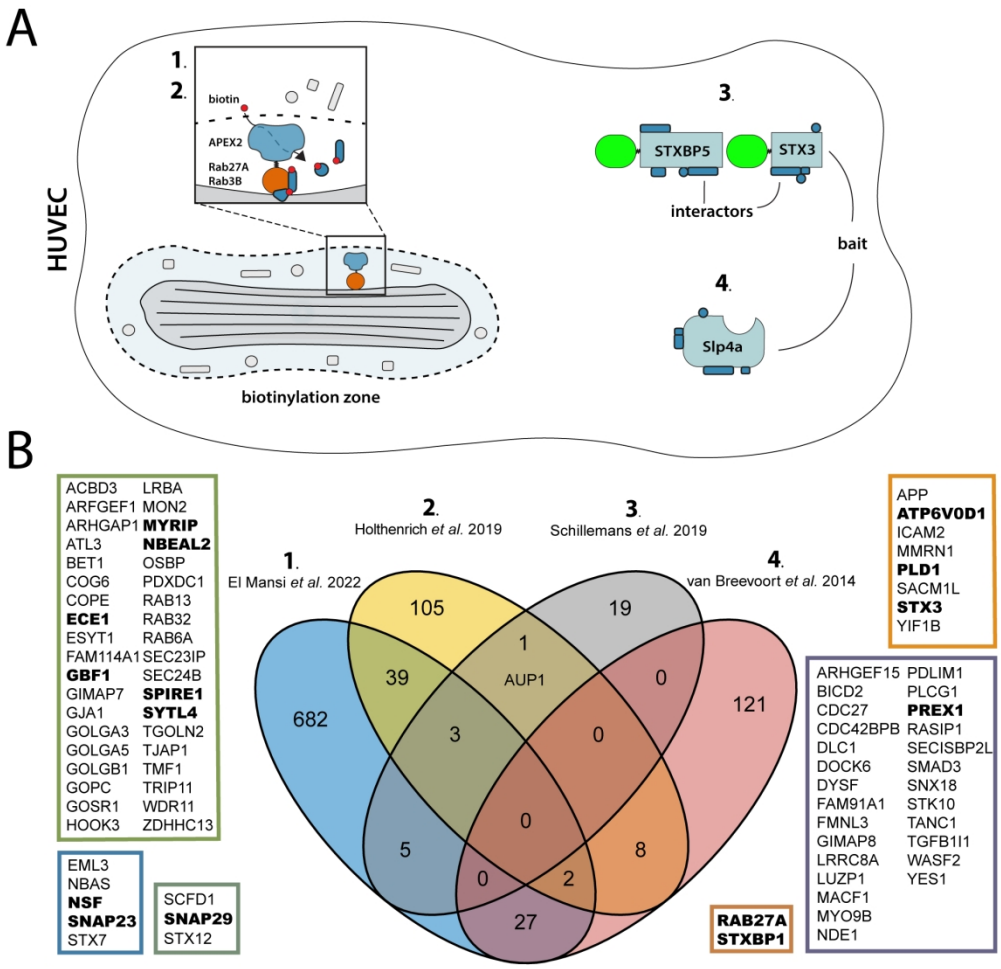

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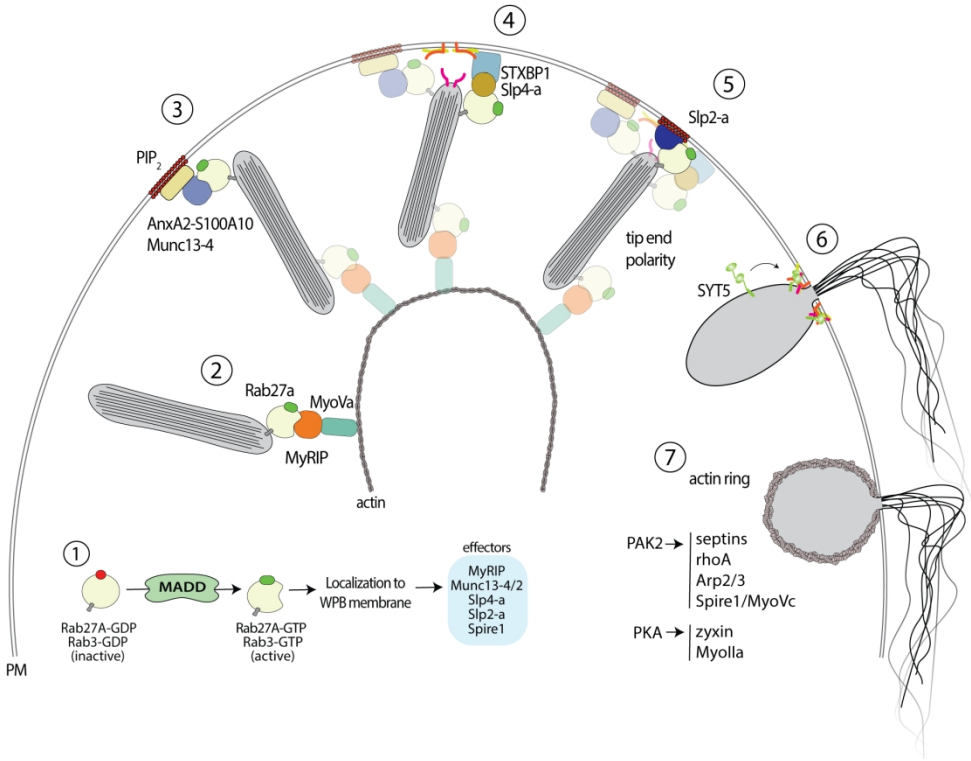

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