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Storage pool diseases illuminate platelet dense granule biogenesis

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Abstract

Platelet dense granules are membrane bound compartments that store polyphosphate and small molecules such as ADP, ATP, Ca^{2+} and serotonin. The release of dense granule contents plays a central role in platelet aggregation to form a hemostatic plug. Accordingly, congenital deficiencies in the biogenesis of platelet dense granules underlie human genetic disorders that cause storage pool disease and manifest with prolonged bleeding. Dense granules belong to a family of lysosome-related organelles, which also includes melanosomes, the compartments where the melanin pigments are synthesized. These organelles share several characteristics including an acidic lumen and, at least in part, the molecular machinery involved in their biogenesis. As a result, many genes affect both dense granule and melanosome biogenesis and the corresponding patients present not only with bleeding but also with oculocutaneous albinism. The identification and characterization of such genes has been instrumental in dissecting the pathways responsible for organelle biogenesis. Because the study of melanosome biogenesis has advanced more rapidly, this knowledge has been extrapolat ed to explain how dense granules are produced. However, some progress has recently been made in studying platelet dense granule biogenesis directly in megakaryocytes and megakaryocytoid cells. Dense granules originate from an endosomal intermediate compartment, the multivesicular body. Maturation and differentiation into a dense granule begins when newly synthesized dense granule specific proteins are delivered from early/recycling endosomal compartments. The machinery that orchestrates this vesicular trafficking is composed of a combination of both ubiquitous and cell type specific proteins. Here we review the current knowledge on dense granule biogenesis. In particular, we focus on the individual human and murine genes encoding the molecular machinery involved in this process and how their deficiencies result in disease.

Keywords

AP-3 complex; BLOC; HOPS; Rab38; protein traffic; Hermansky-Pudlak syndrome

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Conflict of interest

The authors declare that they have no conflict of interest.

Introduction

Circulating platelets are anucleate cells that originate from bone marrow megakaryocytes and have fundamental functions in preventing bleeding and minimizing blood vessel damage. Many of the platelets functions are mediated by secreted molecules, which in the resting platelet are stored in membrane-bound compartments known as granules. The contents of these granules are released during platelet activation as a result of granule fusion with the plasma membrane. Platelets contain three types of well-known granules: dense granules (DGs, also known as dense bodies and δ-granules), α-granules and lysosomes [1–4]. A forth type of platelet granule, the T granule, was discovered more recently [5].

Normal human platelets contain three to eight DGs typically measuring 200–300 nm in diameter although both smaller and larger DGs can also be observed [6]. Underscoring DG function in hemostasis, patients with inherited DG deficiency have bleeding problems of variable severity [7–17]. In comparison to α-granules that package hundreds of proteins, DGs contain relatively few small molecules at high concentrations (high mM range): serotonin, ADP, ATP, Ca^{2+}, pyrophosphate (PPi) and polyphosphate of 70–75 phosphate units (polyP) as well as Mg^{2+} and K^{+} [18–21]. These small molecules are typically transported from the megakaryocyte and platelet cytosol into the DG lumen. Our understanding of the integral membrane proteins that mediate small molecule cargo transport across the DG membrane is limited [22]. Likewise, the intracellular trafficking mechanisms that deliver newly synthesized transporters and other proteins to the limiting membrane of maturing DGs are also partially understood.

Platelet dense granules belong to a family of acidic compartments known as lysosome-related organelles (LROs), which also include melanosomes in melanocytes and retinal-pigmented epithelial cells, lytic granules in cytotoxic T lymphocytes and natural killer cells, and many other cell type specific organelles [11, 23–27]. DG biogenesis has been assumed to use similar mechanisms as other LROs. Supporting this idea, several diseases manifest with prolonged bleeding due to DG deficiency together with other manifestations such as oculocutaneous albinism or immune deficiency, which are caused by defects in melanosomes and lytic granules, respectively [11, 23]. The melanosome in particular has served as the prototype LRO whose biogenesis mechanisms are best understood [25, 26]. The relative paucity in direct DG biogenesis knowledge is in part due to the difficulty of conducting cell biology studies with megakaryocytes [1, 28]. Recently, some progress has been made in studying and understanding DG biogenesis using primary megakaryocytes and appropriate models. Initial results are consistent with the concept that DG biogenesis follows analogous pathways to those described for the synthesis of melanosomes and other LROs. Here we review the current knowledge on the mechanisms of DG biogenesis and associated diseases.

Diagnosis of dense granule disorders

Dense granule disorders typically result in defective platelet aggregation of variable severity [9, 11]. Specifically, the secondary aggregation response to exogenous stimuli is absent. A significant reduction in the content and ratio of ADP to ATP or a reduced ATP release

measured by lumi-aggregometry is diagnostic of a DG disorder [9]. Serotonin accumulation deficiency can also be used to diagnose DG disorders [29–31]. The fluorescent dyes mepacrine and DAPI are useful to label and observe the presence of DGs by fluorescence microscopy [20, 32]. Mepacrine uptake and anti-serotonin staining have also been applied to flow cytometric assays as a measure of DG disorder [29, 33]. Super-resolution fluorescence microscopy using anti-CD63 staining as a marker has recently been proposed as a potential approach to diagnose DG deficiency [34]. However, many of these assays do not distinguish between a defect in DG biogenesis and DG release or may not be fully specific for detection of DG deficiency. Dense granules are inherently opaque and can be identified and quantified by electron microscopy using the whole mount technique with unfixed, unstained human platelets [6]. Dense granules are also readily visualized by electron microscopy in thin sections of platelets and megakaryocytes fixed and subsequently treated with osmic acid [6]. Consequently, electron microscopy continues to be the best method to determine the presence or absence of DGs [6, 11].

Dense granule deficiency can occur in isolation (δ-storage pool disease, δ-SPD), combined with α-granule deficiency (α/δ-SPD) and as part of a syndrome [7, 10, 11, 18]. Hermansky-Pudlak Syndrome (HPS) patients exhibit bleeding diathesis caused by DG deficiency and hypopigmentation of skin, hair and eyes due to melanosome defects [11, 12, 35–37]. Some HPS patients present additional manifestations such as lung fibrosis and immune deficiency because the specific gene that is mutated also affects other LROs and cell types [11, 12]. There are 9 well-established HPS types labeled according to the gene that is mutated and numbered chronologically with their discovery (Table 1). Recently a new mutation was described in a patient likely representing HPS10 [38]. As discussed below, elucidation and study of the genes and proteins mutated in HPS has begun to illuminate the mechanisms involved in synthesizing DGs. Individuals suffering from Chediak-Higashi Syndrome (CHS) have bleeding diathesis due to DG deficiency as well as decreased pigmentation and severe immune deficiency due to malformation of additional LROs [39]. CHS patients have giant intracellular organelles that are pathognomonic of the disease [39, 40]. Chediak-Higashi Syndrome is caused by mutation of the *CHS1* gene [39]. HPS and CHS are uncommon, autosomal recessive diseases.

Mouse models

The availability of mouse models of HPS, CHS, α/δ-SPD and δ-SPD has been especially useful in understanding the mechanisms of DG biogenesis (Table 1). Moreover, the discovery of most of the genes known to be involved in DG biogenesis in humans was preceded and facilitated by the identification of the corresponding gene in mouse disease models [17]. Existing mouse disease models without patient counterparts yet described predict that additional human diseases will be defined at a molecular level in the future (Table 1). Conversely, the existence of patients presenting with HPS, α/δ-SPD and δ-SPD that do not have a mutation in any of the currently known disease-associated genes implies new forms of these diseases must exist [11, 16].

Multivesicular bodies and the dense granule origin

Unlike most secretory granules produced in other cell types, DGs do not originate directly from the *trans*-Golgi network. The biogenesis of DGs involves a specialized biosynthetic mechanism that connects the secretory and endocytic pathways (Figure 1). First, vacuolar domains of the endosomal system give rise to multivesicular bodies (MVBs) [41]. The MVB has been shown to constitute the precursor to DGs, α-granules and conventional lysosomes (Figure 1) [27, 41–43]. Consequently, all three organelles can be accessed by material internalized from the plasma membrane by endocytosis [43, 44]. The fact that MVBs are precursors to the platelet granules is also evidenced by the fact that young megakaryocytes have large numbers of MVBs but mature megakaryocytes have less MVBs and more α-granules and DGs. It is unclear whether the development from MVB into DG and differentiation from α-granules and lysosomes occurs through a continuous process of maturation and remodeling of the same compartment or the budding off of an immature DG from the MVB. Nevertheless, development into a mature DG requires delivery of newly synthesized proteins specific to DGs such as serotonin and ADP transporters (Figure 1). In the next sections we will cover the machinery and intermediate compartments that mediate this intracellular trafficking.

Protein machinery

The AP-3 complex

The proteins encoded by most HPS-associated genes indicated in Table 1 assemble into heteromeric complexes (Figure 2). The Adaptor Protein (AP)-3 complex is a tetramer composed of one molecule each of δ, β3, μ3, and σ3 subunits [45]. AP-3 was originally identified on the basis of its homology with the clathrin-associated adaptor protein complexes AP-1 and AP-2, which work in vesicle mediated trafficking [46, 47]. It should be noted that there are two forms of the AP-3 complex, one that is ubiquitously expressed and composed of δ, β3A, μ3A, and σ3A/σ3B subunits and a neuronal form composed of δ, β3B, μ3B, and σ3A/σ3B subunits. The δ subunit is common to both forms of AP-3 and the σ3A σ3B subunits can be in either complex. The importance of AP-3 for the biogenesis of DGs in particular and LROs in general became clear when the mutations carried by the mocha and pearl mouse models of HPS were revealed to impact the genes encoding δ and β3A, respectively [48, 49]. Mutations in β3A were also found in HPS patients, thus defining HPS type 2 (Table 1) [50]. At the time, the discovery that mutation of AP-3 causes HPS had conceptual significance because it demonstrated that Hermansky-Pudlak syndrome is a disease of protein trafficking and organelle biogenesis.

AP-3 mediates transport of integral membrane proteins from tubular domains of early/recycling endosomes to lysosomes and LROs [51–53]. AP-3 engages dileucine- and tyrosine-based sorting signals in the cytosolic tail of integral membrane protein cargos and packages them into transport vesicles destined for the LRO (Figure 1) [44, 52, 54–58]. AP-3 also has the ability to bind other components of the trafficking machinery including the terminal domain of the clathrin heavy chain to orchestrate formation of the transport vesicle [59, 60]. Several likely DG integral membrane protein components have cytosolic tails harboring sequences that conform to the dileucine- and tyrosine-based sorting signal

consensus. One example is the protein SLC35D3, a member of the nucleotide sugar transporter family, whose deficiency causes δ-SPD in mice [31, 61]. SLC35D3 steady state levels in platelets from AP-3 deficient mice are decreased compared to wild type mice, consistent with a defect in transport to DGs during organelle biogenesis [62]. Moreover, SLC35D3 populates early/recycling endosomal tubules labeled with syntaxin 13 and transferrin receptor in megakaryocytes, suggesting these compartments are intermediate stations in the pathway to DGs (Figure 1) [62]. Other examples are LAMP2 and the serotonin transporter VMAT2, which are expressed in megakaryocytes/platelets and reside on the DG membrane [63–66]. The corresponding tyrosine-based and dileucine-based sorting signal mutants of LAMP2 and VMAT2 were mislocalized to the plasma membrane [44]. These observations suggest AP-3 and the sorting signals it binds function to mediate transport of newly synthesized integral membrane proteins from early/recycling endosomal tubules to DGs (Figure 1). However, AP-3 binding to the sorting signal consensus sequences in cargo such as SLC35D3 and VMAT2 has yet to be confirmed experimentally. Interestingly, the SLC35D3, LAMP2 and VMAT2 phenotypes described above were not complete, suggesting a partial defect in transport of cargo to DGs. An analogous scenario has been observed in the study of melanosomal proteins and documented to reflect the existence of multiple pathways that cargo may follow to target this LRO [26, 52, 53, 67–69]. Therefore, it is likely that a similar set of parallel pathways deliver cargo to DGs (Figure 1).

As mentioned above, there are two forms of the AP-3 complex, the ubiquitous AP-3A and the neuronal form, AP-3B. Consistently, the mocha mice – with a mutation in the δ subunit common to both AP-3 forms – present neurological phenotypes in addition to bleeding and oculocutaneous albinism that define HPS [48]. Mutation of AP-3 subunits also causes immunodeficiency in mice and patients. Recently, a patient was reported with a mutation in the δ subunit of AP-3 and showing albinism, neurological manifestations and immunodeficiency [38]. It was not confirmed, as of yet, if the patient has DG deficiency but it likely represents HPS10.

BLOC-1

The Biogenesis of Lysosome-related Organelles Complex-1 (BLOC-1) is composed of one copy each of 8 subunits: BLOS1 (BLOC1S1), BLOS2 (BLOC1S2), BLOS3 (BLOC1S3), Cappuccino (BLOS4 or BLOC1S4), Muted (BLOS5 or BLOC1S5), Pallidin (BLOS6 or BLOC1S6), Snapin (BLOS7 or BLOC1S7), and Dysbindin (BLOC1S8 or DTNBP1) (Figure 2) [70–74]. Five of them – Dysbindin, BLOS3, Pallidin, Cappuccino, and Muted – are encoded by genes mutated in HPS mouse models (Table 1). Of those, the first three were also found to be mutated in HPS patients thus defining HPS-7, HPS-8 and HPS-9, respectively (Table 1) [75–77]. It is therefore likely that uncharacterized HPS patients carry mutations in the genes encoding Cappuccino and Muted (Table 1). Interestingly, the Snapin, BLOS1 and BLOS2 subunits of BLOC-1 are also components of a separate complex known as BORC (BLOC-one-related complex) [78]. BORC functions in lysosome positioning and appears to be fundamental for life since Snapin-KO mice are perinatally inviable and BLOS1-KO mice are embryonic lethal [78]. Alternatively, BLOC-1 and BORC may have partially overlapping functions needed for viability. Consequently, mutations in the genes encoding for Snapin, BLOS1 and BLOS2 are not predicted to be found among HPS patients.

Clinical data on patients with BLOC-1 deficiency is limited but the severity appears to be variable [77].

BLOC-1 function at the molecular level could not be deduced from sequence homology with previously known proteins. BLOC-1 was obtained in recombinant form allowing an initial structural characterization [72]. The complex consists of a linear chain of globular domains spanning 30 nm in length and 3 nm in diameter that can bend by as much as 45° (Figure 2). In melanocytes, BLOC-1 localizes to early/recycling endosome tubules and functions in tubule formation by coordinating the kinesin KIF13A-dependent pulling of the tubules along microtubules with the actin-dependent tubule stabilization [53, 79, 80]. BLOC-1 activity is key to deliver integral membrane proteins to maturing melanosomes through both AP-3-dependent and AP-3-independent pathways [53, 81–86]. Furthermore, BLOC-1 interacts physically with AP-3 and the endosomal SNARE syntaxin 13 [53, 87]. It is likely that BLOC-1 performs similar functions in the transport of DG proteins during organelle biogenesis (Figure 1). Supporting this idea, the phenotype of decreased SLC35D3 steady state levels in platelets from AP-3 mice described above was also observed with platelets from BLOC-1 deficient mice [62].

BLOC-2

The Biogenesis of Lysosome-related Organelles Complex-2 (BLOC-2) is composed of the HPS3, HPS5, and HPS6 proteins (Figure 2) encoded by the genes mutated in HPS-3, HPS-5 and HPS-6 disease patients (table 1) [88, 89]. While the sum of the theoretical molecular weights of the three subunits approximates the mass estimated for the native complex, the existence of additional small subunits cannot be ruled out [88]. The corresponding mouse mutant strains are cocoa, ruby-eye 2 and ruby-eye (Table 1) [89]. Patients with deficiency in the BLOC-2 subunits have similarly mild forms of the disease and do not appear to have manifestations other than bleeding and hypopigmentation [11, 12].

BLOC-2 function in DG biogenesis is very poorly characterized but it is starting to be understood in the context of melanosome biogenesis. In melanocytes, BLOC-2 localizes to early/recycling endosomal compartments and functions in the transport of melanosomal cargo, at least in part in a pathway separate from the one defined by AP-3 [53, 90]. BLOC2 interacts physically with BLOC-1 but appears to have functions downstream of BLOC-1 [53]. BLOC-2 also interacts physically with Rab38 and Rab32, which are two small GTPases that function in melanosome and DG biogenesis (see below) [44, 67, 91]. In melanocytes BLOC-2 and Rab38 regulate the membrane-associated pool of each other [67]. Furthermore, a recent study suggested BLOC-2 works in tethering of transport carriers originating from early/recycling endosomal tubules with the maturing melanosome [92]. BLOC-2 probably performs a similar function in the context of DG biogenesis (Figure 1).

BLOC-3

The Biogenesis of Lysosome-related Organelles Complex-3 (BLOC-3) is composed of the HPS1 and HPS4 proteins (Figure 2) encoded by the genes mutated in patients with HPS-1 and HPS-4 disease (table 1) [93]. Based on the characterization of the recombinant HPS1–HPS4 complex, BLOC-3 does not contain additional subunits [94]. The corresponding

mouse mutant strains are pale ear and light ear, respectively (Table 1) [17]. Patients with deficiency in the BLOC-3 subunits have similarly severe forms of HPS presenting with additional manifestations besides bleeding and hypopigmentation. HPS-1 and HPS-4 patients frequently develop granulomatous colitis and suffer adult-onset fatal pulmonary fibrosis [11, 12]. Mutations in HPS1 are the most common cause of HPS [95, 96].

We are only beginning to understand BLOC-3 function in DG biogenesis. The MRP4 protein (also known as ABCC4) was initially localized to DGs in normal platelets by biochemical approaches and immunofluorescence microscopy analysis [97]. However, in platelets from an HPS-4 patient, MRP4 staining was reduced and mostly found on the plasma membrane suggesting BLOC-3 may function in transport of MRP4 to DGs [97]. Nevertheless, there is a controversy regarding normal MRP4 localization and function (as we discuss below) potentially invalidating the conclusion that BLOC-3 mediates its transport.

Molecular level functional information on BLOC-3 can be extrapolated from other systems. Based on low level sequence homology between HPS4 and the Ccz1 subunit of the yeast Ypt7/Rab7 guanine nucleotide exchange factor (GEF), it was suspected that BLOC-3 could work as a Rab-GEF [98]. In order to function in membrane trafficking, Rab GTPases become activated by the action of GEFs, which catalyze the release of GDP turning them into active, GTP-bound Rabs. Recombinant BLOC-3 demonstrated GEF activity specifically towards Rab32 and Rab38, thus linking BLOC-3 to the trafficking machinery that transports cargo to DGs and melanosomes [98]. Additionally, BLOC-3 binds with high affinity to Rab9a, another Rab protein recently reported to function in melanosome biogenesis [94, 99]. Therefore, BLOC-3 may function in a Rab cascade to regulate biogenesis of LROs [100]. The expectation is that BLOC-3 performs similar functions along the pathways to DGs (Figure 1).

HOPS and CORVET

The buff mouse model of HPS carries a mutation in the *VPS33A* gene encoding the VPS33A protein (Table 1) [101]. VPS33A is a subunit of the homotypic fusion and vacuole protein sorting (HOPS) complex. HOPS is composed of one copy each of six subunits: VPS33A, VPS11, VPS16, VPS18, VPS39, and VPS41 [102, 103]. Figure 2 shows the organization of the HOPS subunits, which is based on significant available structural information [102]. Four subunits of HOPS (VPS33A, VPS11, VPS16, and VPS18) are shared with a related complex known as class C core vacuole/endosome tethering (CORVET). The VPS39 and VPS41 subunits of HOPS are replaced by TGFBRAP1 and VPS8 in CORVET (Figure 2) [103]. No HPS patient has yet been confirmed to have a deficiency in VPS33A or the other complex subunits.

HOPS and CORVET function in membrane trafficking as tethering complexes bringing compartments in close proximity and facilitating SNARE-mediated membrane fusion [102, 103]. CORVET functions at early endosomes and HOPS at late endosomes/MVBs. This specificity is based on interaction with Rab5 and Rab7, master regulators of early and late endosomes/MVBs, respectively. Importantly, the crystal structure of VPS33A confirmed it belongs to the Sec1/Munc18 family of proteins that regulate the formation of cognate

SNARE complexes driving membrane fusion [104]. Even though HOPS and CORVET have not been directly studied in DG biogenesis, it is logical to envision their function in generating the precursor MVB compartment and/or mediating tethering and fusion of DG cargo-containing vesicles with MVBs.

It is worthwhile noting that VPS33A is related to VPS33B. Mutation of VPS33B causes Arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome, which shows α-granule deficiency among other manifestations [105–107]. It is tempting to speculate that two different forms of HOPS/CORVET, one containing VPS33A and another containing VPS33B, would mediate transport to DGs and α-granules, respectively. However, VPS33B may not form a complex with HOPS or CORVET and instead form a separate complex with VPS16B (also known VIPAS39 or VIPAR) [103, 108, 109].

Rab32 and Rab38

The fawn hooded rat carries a mutation in the *RAB38* gene and is one of the earliest known rodent models of storage pool disease and HPS [18, 110–112]. Unlike the fawn hooded rat, which is a null, the chocolate mouse strain carries a hypomorphic mutation in the *RAB38* gene and displays pigmentation dilution but does not appear to have bleeding problems [113, 114]. Rab32 is a very close homolog of Rab38 with partially overlapping functions in LRO biogenesis [67, 91]. Illustrating they perform at least some independent functions, Rab32 deficiency in melanocytes, but not Rab38 deficiency, causes a loss of the melanosomal protein Tyrp2 [67].

Rab32 and Rab38 work in transport of cargo from early/recycling endosomes to maturing DGs in megakaryocytes (Figure 1) and to maturing melanosomes in melanocytes [44, 67, 69, 91]. In melanocytes, Rab32 and Rab38 were shown to interact physically and colocalize partially with AP-3 and BLOC-2 [67]. Rab32 and Rab38 also localize in part to vesicles and melanosomes [67, 69, 91]. Deficiency of Rab32 and Rab38 causes mistrafficking of melanosomal resident proteins, melanosome malformation and lower melanin pigment synthesis [67, 91, 111, 113]. In megakaryocytes, Rab32 and Rab38 localize to AP-3 and clathrin-labeled structures as well as to transport vesicles and DGs (Figure 1) [44]. Rab32 and Rab38 were also shown to function either in tethering or fusion of cargo-containing vesicles with the maturing DG or both [44]. This function of Rab32 and Rab38 is in line with what we know about Rab proteins as key regulators of vesicular trafficking that confer target specificity to vesicle motility, tethering and fusion [100].

Most of the components of the DG biogenesis machinery such as AP-3 and the BLOCs are ubiquitously expressed. This is consistent with their function in the biogenesis of lysosomes, which are ubiquitous organelles. A long-standing question in the field was how the same machinery could mediate transport to two separate organelles, lysosomes and DGs (or lysosomes and melanosomes) that are produced and co-exist in the same cell. This question was answered with the discovery that Rab38 and to large extent Rab32 work as cell type specific factors that re-direct the ubiquitous machinery towards DGs (or melanosomes) [67, 68, 91, 100, 113, 115].

Rab27b

Deficiency of Rab27a elicits Griscelli syndrome, an autosomal recessive disorder similar to HPS but without DG deficiency and bleeding [11]. However, a mouse knock out for the closely related *RAB27B* gene was created and found to have prolonged bleeding and impaired platelet aggregation due to DG deficiency (Table 1) [116]. This mouse does not have hypopigmentation or any of the other manifestation observed in HPS or Griscelli syndrome. Consequently, Rab27b deficiency is a model for δ-SPD [116]. Significantly, a patient displaying Rab27b deficiency presents with absent dense granules and bleeding [14]. Additional work is needed to confirm Rab27b deficiency causes δ-SPD in humans.

RABGGTA

The gunmetal mouse (Table 1) has a mutation in the gene encoding the α-subunit of the Rab geranylgeranyl transferase (RabGGTase, Figure 2) and displays a defect in both DGs and α-granules thus serving as a model of α/δ-SPD [117]. Gunmetal mice also have hypopigmentation [117]. The RabGGTase enzyme adds two geranylgeranyl 20-carbon isoprenoid residues to each of two cysteine residues at the C-terminus of Rab proteins. This modification is needed for normal Rab association with membranes and function. The gunmetal mouse is a hypomorph with RabGGTase activity reduced four-fold but not absent [117]. As a consequence, the modification of Rab proteins and their association with membranes is compromised but not totally abolished. This defect becomes more exacerbated in cell types that express higher levels of Rabs such as megakaryocytes/platelets and melanocytes [118]. The DG and melanosome biogenesis defect can be explained by reduced function of Rab32, Rab38, and/or Rab27b. This observation suggests that another Rab or Rabs are likely involved in α-granule biogenesis.

CHS1/LYST

The *CHS1* gene is mutated in patients suffering Chediak-Higashi Syndrome and the beige mouse model of the disease [39]. The function of CHS1/LYST protein remains unclear, but its ability to interact with SNARE proteins and the presence of huge organelles typical of CHS suggest a function in membrane trafficking [11, 39]. CHS1 has a BEACH domain, similar to NBEAL2 whose mutation causes α-granule deficiency (Gray Platelet syndrome) [119–121].

Cargos of the dense granule biogenesis pathways

There is a remarkable scarcity of proteins truly confirmed as components of the DG membrane. As indicated above, SLC35D3 is believed to reside on DGs and the corresponding KO mouse is considered a model of δ-SPD showing drastically reduced platelet serotonin levels [31]. In megakaryocytoid cells SLC35D3 mostly populates early/recycling endosomes, probably because its transport to DGs takes place at later stages during megakaryocyte maturation [62]. While SLC35D3 was found in platelets by immunoblotting, its localization to DGs should be confirmed.

Multidrug resistance protein 4 (MRP4) was initially reported to reside on DGs in normal platelets but mislocalized to the plasma membrane in platelets from an HPS-4 patient [97].

Furthermore, MRP4 was suggested as the ADP transporter in DGs and two patients with δ-SPD-like phenotype (reduced adenine nucleotides but normal serotonin levels) show undetectable platelet MRP4 [22, 97, 122]. Nevertheless, no mutation in the MRP4 gene was found in these patients and direct evidence that MRP4 actually transports ADP is lacking. Two recent papers using MRP4-KO mice confirmed bleeding problems but reported results that contradict the earlier studies. Both papers implicate MRP4 in cAMP rather than ADP transport [123, 124] and one of them localized MRP4 to the plasma membrane by both structured illumination microscopy and biochemical approaches [124]. The emerging picture is one in which MRP4 is needed for normal platelet function probably by transporting cAMP. However it is not clear if MRP4 functions at DGs, the plasma membrane, or in both locations.

Platelets take up serotonin by the action of the plasma membrane transporter SERT and then package it in DGs by the work of vesicular monoamine transporter 2 (VMAT2) [64–66]. Serotonin transport into DGs by VMAT2 relies on the proton electrochemical gradient generated by a vacuolar H^+-ATPase [125].

Two-pore channel 2 (TPC2) is a component of the DG membrane that regulates the organelle luminal pH and the pool of releasable Ca^{2+} [57]. Release of Ca^{2+} regulated by TPC2 marks DG “kiss-and-run” events where two DGs make transient physical contact and then move away from each other (Figure 1) [57]. During kiss-and-run events DGs exchange contents, therefore this mechanism may play a role in DG maturation. TPC2 also regulates the formation of membrane tubules connected to DGs [57]. These tubules may be involved in DG biogenesis as they exchange both soluble and membrane material with the DG. Tubular extensions of DGs may also correspond to mature DGs with tail-like extensions occasionally observed in platelets by electron microscopy [6]. Consequently, TPC2 has dual properties as both cargo and machinery component of the DG biogenesis pathways. The discovery that TPC2 also regulates melanosome biogenesis and pigmentation suggests TPC2 may define an HPS gene [126–128].

LAMP2 is another integral membrane protein which presence is confirmed in the DG membrane [63, 129]. SNARE proteins needed for DG fusion with the platelet plasma membrane during activation must also be present [130]. A number of glycoproteins and 14-3-3ζ have also been reported in DGs [129]. Establishing *bona fide* DG markers is important to better understand DG function and the pathways and machinery involved in biogenesis.

Perspective

Although some progress has been made in understanding DG biogenesis, many aspects remain poorly defined. A major issue is the paucity of membrane proteins properly verified as DG components, particularly those specific to DGs. Given the importance of ADP for dense granule function, unequivocal identification and characterization of the corresponding transporter would be helpful. The same concept applies to transporters for most of the other molecules stored in the DG lumen. In turn, study of DG membrane protein transport will refine our understanding of the pathways, mechanisms, and molecular machinery involved in

biogenesis. Are these pathways as complex and analogous to the melanosome biogenesis pathways? Do different cargos use separate pathways to DGs? How does a seemingly uniform MVB give rise to three distinct organelles, DGs, α-granules and lysosomes?

The cross talk between storage pool disease and basic biology and between animal models and patients will likely continue to be fruitful. On the one hand, elucidation of the normal function of the genes and proteins mutated in patients with storage pool disease will be needed to achieve a detailed understanding of DG biogenesis. On the other hand, the characterization of genes mutated in animal models with dense granule deficiency predicts the corresponding human disease must exist. Finally, from a clinical point of view, the well-established function of DGs in hemostasis and thrombosis raises the question of whether their biogenesis could be targeted for therapeutic purposes. Therefore, understanding DG biogenesis is a prerequisite to rationally design such approaches.

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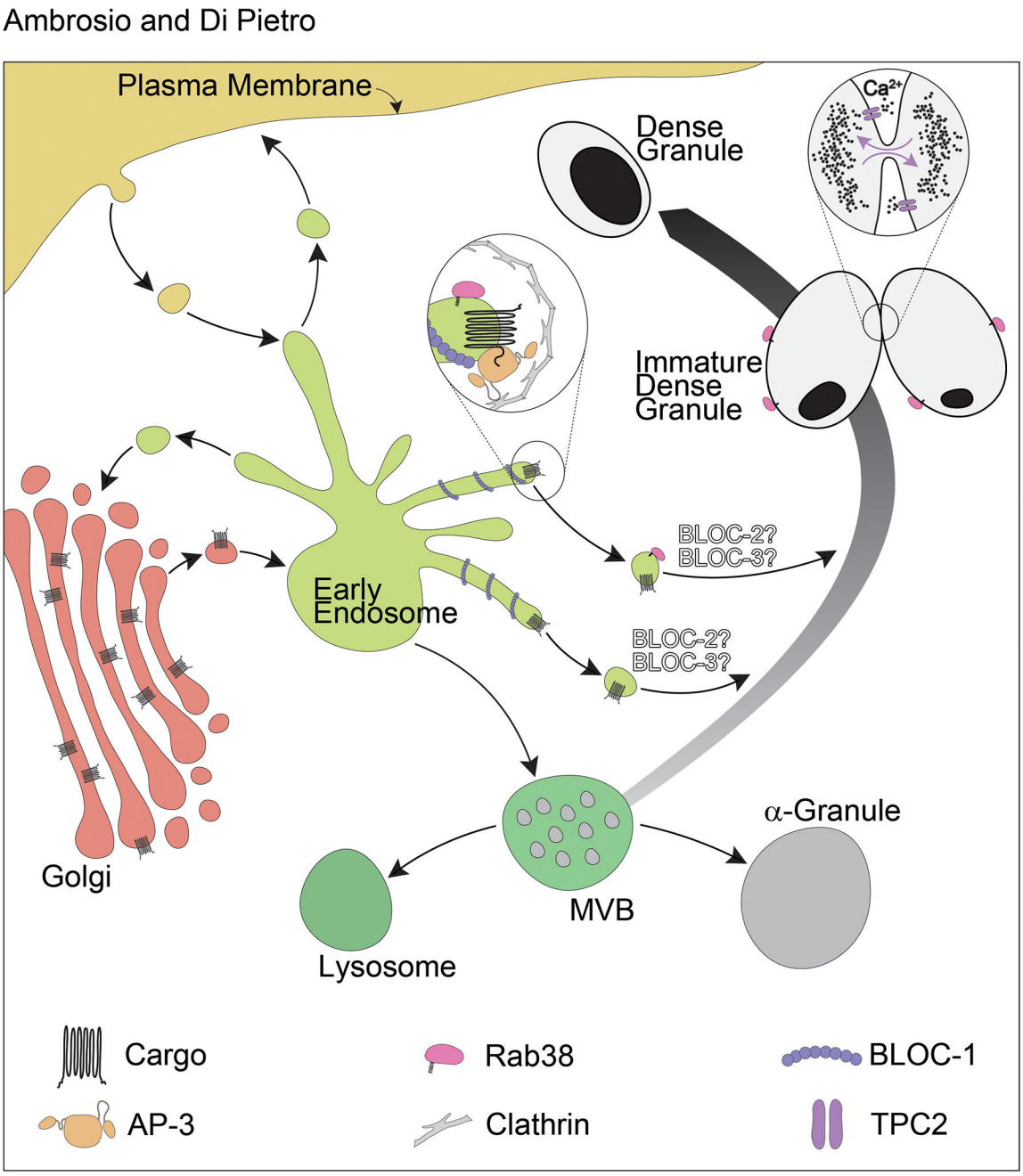

Figure 1. Model for the biogenesis of platelet dense granules

In megakaryocytes, multivesicular bodies (MVBs) mature into dense granules upon receiving newly synthesized specific transmembrane proteins through multiple vesicular trafficking pathways. Dense granule cargo is sorted by adaptor protein (AP) complexes at the level of the early/recycling endosome tubules that are likely stabilized by BLOC-1. The best understood pathway to dense granules is defined by AP-3, which binds sorting signals present in the cytosolic tails of cargo proteins and recruits clathrin, facilitating the formation of the coated vesicle (see inset). Rab38 is present on to the transport vesicle that mediates targeting of the vesicle to the maturing dense granule. Very little is known regarding

BLOC-2 and BLOC-3 function in DG biogenesis. Based on melanosome research they are tentatively placed downstream BLOC-1 and AP-3, and may also work independently of AP-3. TPC2 regulates dense granule pH, the pool of releasable Ca^{2+}, and a “kiss-and-run” mechanism of dense granule membrane dynamics and content exchange (see inset).

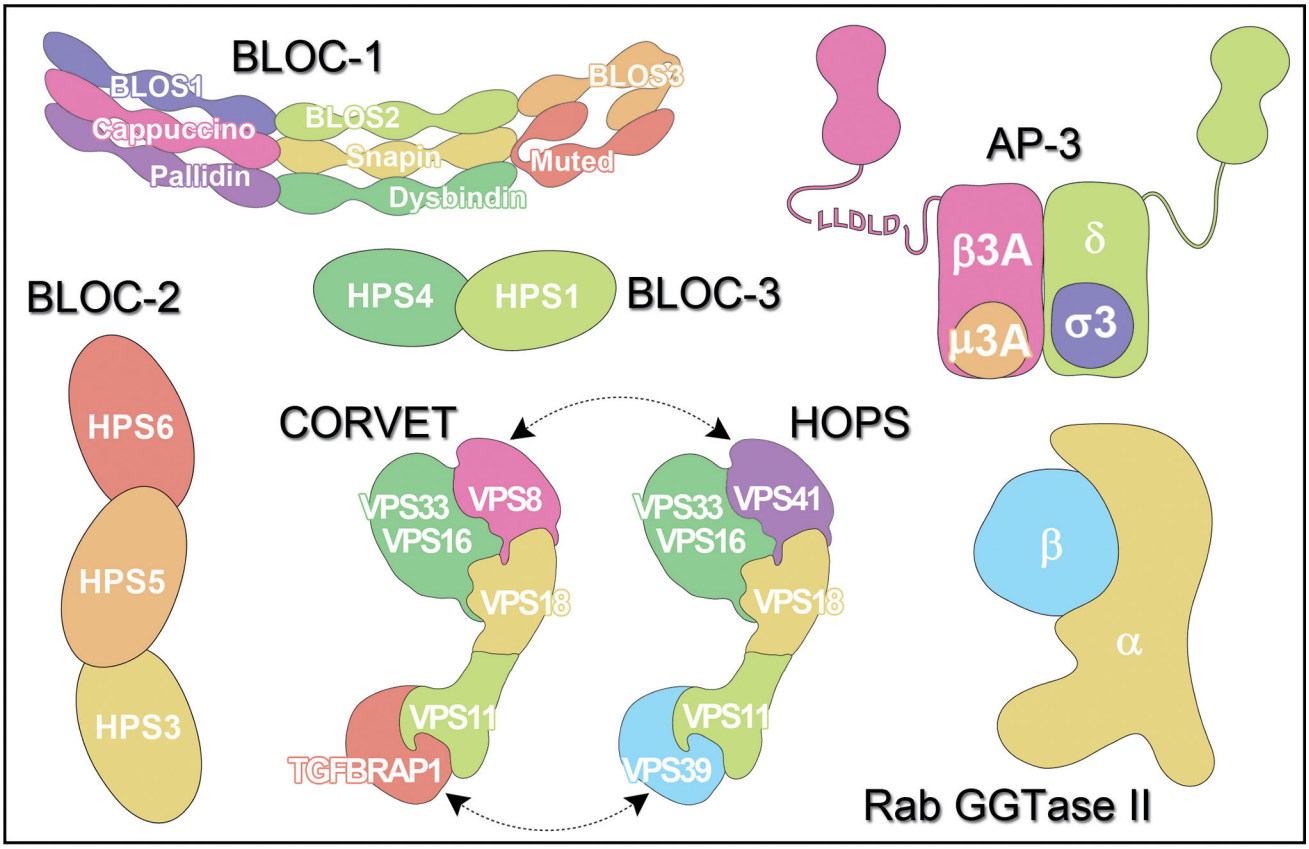

Figure 2. Schematic representation of protein complexes involved in dense granule biogenesis

The cartoon representation of BLOC-1, AP-3, HOPS/CORVET and Rab GGTase II complexes is based on structural information while BLOC-2 and BLOC-3 simply represent their subunit composition.

Table 1

Genes involved in dense granule biogenesis in humans and mice.

Gene Symbol	Human Disease	Rodent Mutation	Protein	Function
HPS1	HPS-1	pale ear	HPS1	BLOC-3 subunit
AP3B1	HPS-2	pearl	AP-3 β3A	AP-3 subunit
HPS3	HPS-3	cocoa	HPS3	BLOC-2 subunit
HPS4	HPS-4	light ear	HPS4	BLOC-3 subunit
HPS5	HPS-5	ruby-eye 2	HPS5	BLOC-2 subunit
HPS6	HPS-6	ruby-eye	HPS6	BLOC-2 subunit
DTNBP1	HPS-7	sandy	Dysbindin	BLOC-1 subunit
BLOC1S3	HPS-8	reduced pigmentation	BLOS3	BLOC-1 subunit
PLDN	HPS-9	pallid	Pallidin	BLOC-1 subunit
AP3D1	HPS-10	mocha	AP-3 δ	AP-3 subunit
CNO	-	cappuccino	Cappuccino	BLOC-1 subunit
MUTED	-	muted	Muted	BLOC-1 subunit
CHS1/LYST	CHS	beige	CHS1/LYST	?
VPS33A	-	buff	VPS33A	HOPS/CORVET subunit
RAB38	-	chocolate	Rab38	small GTPase
RAB27B	-	Rab27b*	Rab27b	small GTPase
RABGGTA	-	gunmetal	RABGGTA	Rab geranylgeranyl transferase α subunit

* All mice described in this table, with the exception of Rab27b, were spontaneous mutants.