Abnormal megakaryocyte development and platelet function in Nbeal2−/− mice

Running title: Mouse model of gray platelet syndrome

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Key Points

*Nbeal2*−/− mice are a model of human GPS, characterized by macrothrombocytopenia and α-granule deficient platelets.

*Nbeal2* is required for normal platelet function and megakaryocyte development.

Abstract

Gray platelet syndrome (GPS) is an inherited bleeding disorder associated with macrothrombocytopenia and α-granule-deficient platelets. GPS has been linked to loss of function mutations in *NBEAL2* (neurobeachin-like 2), and we describe here a murine GPS model, the *Nbeal2*−/− mouse. As in GPS, *Nbeal2*−/− mice exhibit splenomegaly, macrothrombocytopenia and a deficiency of platelet α-granules and their cargo, including von Willebrand factor (VWF), thrombospondin-1 and platelet factor 4. The platelet α-granule membrane protein P-selectin is expressed at 48% of wild type levels and externalized upon platelet activation. The presence of P-selectin and normal levels of Vps33B and Vps16B in *Nbeal2*−/− platelets suggests that Nbeal2 acts independently of Vps33B/Vps16B at a later stage of α-granule biogenesis. Impaired *Nbeal2*−/− platelet function was shown by flow cytometry, platelet aggregometry, bleeding assays and intravital imaging of laser-induced arterial thrombus formation. Microscopic analysis detected marked abnormalities in *Nbeal2*−/− bone marrow megakaryocytes, which when cultured showed delayed maturation, decreased survival, decreased ploidy and developmental abnormalities, including abnormal extracellular distribution of VWF. Our results confirm that α-granule secretion plays a significant role in platelet function and they also indicate that abnormal α-granule formation in *Nbeal2*−/− mice has deleterious effects on megakaryocyte survival, development and platelet production.
Introduction

As bone marrow megakaryocytes (MKs) develop they undergo increases in ploidy, nuclear lobulation and cytoplasmic mass prior to the emergence of the demarcation membrane system. Their development culminates in the formation of proplatelets, microtubule-containing cell extensions\(^1,2\) that protrude through the vascular endothelium and are released into the bloodstream as nascent platelets.\(^3\) Mature platelets contain abundant stores of secretory vesicles that include dense (δ-) granules, lysosomes and α-granules (50-80/platelet), which carry endogenously synthesized or endocytosed cargo\(^4,5\) and arise from budding vesicles in the MK trans-Golgi network that mature into multivesicular bodies (MVBs) and nascent α-granules\(^5,6\) that are transported into proplatelets.\(^7\)

Insights into platelet granule formation and function have come from investigating patients and mutant mice with secretory granule deficiencies. Studies of conditions associated with a lack of δ-granules: Hermansky-Pudlak syndrome and Chediak-Higashi syndrome (MIM214500), have implicated several vesicular trafficking molecules in δ-granule formation.\(^8-11\) Studies of ARC (Arthrogryposis, renal dysfunction, and cholestasis) syndrome, where platelets lack α-granules, have shown two proteins to be required for α-granule development: VPS33B and its binding partner VPS16B.\(^12,13\) Recently, we and others have identified loss of function mutation of NBEAL2 (neurobeachin-like 2) as the cause of gray platelet syndrome (GPS; MIM139090), an autosomal recessive bleeding disorder characterized by macrothrombocytopenia and gray-appearing platelets with decreased/absent α-granules\(^14-18\) and α-granule proteins.\(^19,20\) Patients with GPS have moderate bleeding symptoms, splenomegaly and progressive bone marrow fibrosis characterized by abnormal reticulin deposition in the bone marrow.\(^16,19\)

Although studies using mouse models of δ-granule deficiency have shown the importance of platelet δ-granule secretion during in vivo thrombus formation,\(^21\) the importance of isolated α-granule secretion has not been investigated due to lack of an appropriate model. Here we report the results of studies using the Nbeal2\(^{-/-}\) mouse as a model of GPS. Compared to wild type (WT), Nbeal2\(^{-/-}\) mice show splenomegaly, low platelet counts and a profound deficiency of platelet α-granules and their soluble cargo proteins. Expression of the α-granule membrane protein P-selectin was decreased in Nbeal2\(^{-/-}\) platelets but it was observed to mobilize to the surface of activated cells. Nbeal2\(^{-/-}\) platelets showed decreased response to agonists in vitro, while in vivo assays showed increased bleeding in Nbeal2\(^{-/-}\) mice and impaired platelet thrombus formation in response to laser-induced arterial injury. These results confirm the importance of α-granule secretion for platelet function. Studies of bone marrow and cultured MKs also revealed significant abnormalities in Nbeal2\(^{-/-}\) mice, providing the first indication that impaired α-granule formation due to the absence of Nbeal2 can have major consequences for MK development and platelet production.
Methods

Animals

*Nbeal2*−/− mice were generated from cryo-preserved spermatozoa obtained from the Mutant Mouse Regional Resource Center (MMRRC) at UC Davis from strain B6;129S5-*Nbeal2*^tm1Lex^/Mmucd, where exons 4-11 from the 54 exon *Nbeal2* gene were deleted by homologous recombination. Spermatozoa were used for *in vitro* fertilization of wild type C57BL/6J mouse oocytes to generate embryos that where transferred into pseudopregnant C57BL/6J females by the Toronto Centre for Phenogenomics (TCP) to generate heterozygous litters, which were crossed to generate homozygous *Nbeal2*−/− mice. Sequencing of the targeted deletion region (exons 4-11 of *Nbeal2*) of ear punch samples was done via PCR using one 5' primer: 5'-GTCCTGCTTGACCTACCGTC-3' and two 3' primers: 5'-CAGGGAGGATAACGAGATAGTCTT-3' and 5'-CCTAGGAATGCTCGTCAA GA-3'. Two PCR reactions with the same 5' primer and one of each 3' primer results in predicted 223 bp + no product for WT and no product + 401 bp for the homozygous deletion respectively. These confirmed *Nbeal2*+/+, *Nbeal2*+/− or *Nbeal2*−/− status. Age and sex matched WT mice were C57BL/6J obtained from the TCP. This study was approved by the Toronto Centre for Phenogenomics (TCP) Animal Use Protocols (AUP): AUP #0215-H.

Platelet enumeration, bone marrow and spleen analysis, bright field and electron microscopy, immunoblotting, platelet preparations for immunofluorescence microscopy, megakaryocyte culture, immunostaining, high resolution confocal laser immunofluorescence microscopy, in vitro assessments of platelet function, tail bleeding assay, intravital analysis of platelet accumulation and activation after laser-induced injury to cremaster muscle arterioles

The experimental details are described in Supplemental Methods and Figures.

Results

Mice

*Nbeal2*−/− mice were fertile, born with expected Mendelian frequency and did not reveal morphological, viability or behavioral abnormalities. Comparison of spleen weights with age-matched (~4 month old) WT (C57BL/6J) mice revealed significant splenomegaly (P=0.0001, Mann Whitney 2-tailed test) in *Nbeal2*−/− mice (WT: 0.09 g SD 0.02, n=13; *Nbeal2*−/−: 0.16 g SD 0.05, n=13). Hematoxylin/eosin stained *Nbeal2*−/− spleens contained significantly more MKs (WT spleen slice: 67 SD 19, n=3; *Nbeal2*−/− spleen slice: 330 SD 12, n=3; P<0.0001, two-tailed T-test). There was no increased reticulin staining in *Nbeal2*−/− spleens. Bone marrow morphology assessed by hematoxylin/eosin and reticulin staining was indistinguishable from WT (Supplemental Figure S1), confirming the absence of myelofibrosis.
Platelet number and morphology
Automated blood analysis showed mean platelet counts for Nbeal2<sup>−/−</sup> mice lower than WT (WT: 848 x 10<sup>9</sup>/L SD 202, n=3; Nbeal2<sup>−/−</sup>: 519 x 10<sup>9</sup>/L SD 55, n=3), while mean platelet volume was greater (Nbeal2<sup>−/−</sup>: 6.69 fL SD 0.35, n=3 vs. WT: 5.10 fL SD 0.85, n=3). On blood films platelets from WT (Figure 1A) and Nbeal2<sup>−/−</sup> heterozygous mice (Figure 1B) show typical punctate staining, while Nbeal2<sup>−/−</sup> platelets appear pale (Figure 1C). These observations are consistent with GPS.

Platelet ultrastructure was examined via thin section TEM, where Nbeal2<sup>−/−</sup> α-granule deficiency was clearly evident (Figure 1D,E, Supplemental Figure S2). Morphometric analysis revealed a virtual absence of α-granules in Nbeal2<sup>−/−</sup> platelets, with an average of 0.25 α-granules per platelet thin section compared to 4.8 in WT (Nbeal2<sup>−/−</sup> n=100 platelets; WT n=25 - human platelets have approximately 5.5 α-granules per platelet thin section<sup>12</sup>). Whole mount electron microscopy revealed a dense granule count of 2.6/platelet in Nbeal2<sup>−/−</sup> mice compared to 4.9/platelet in WT mice (n=50 platelets).

Platelet proteins
Platelet lysates were examined for protein content via immunoblot analysis; equivalent sample loading was achieved by loading lysates from similar numbers of platelets, measuring total protein concentrations and probing for actin or GAPDH. We observed (Figure 2) that the MK-synthesized α-granule cargo proteins von Willebrand factor (VWF), platelet factor 4 (PF4) and thrombospondin-1 (TSP1) were greatly reduced or absent in Nbeal2<sup>−/−</sup> platelet lysates (Figure 2A-C). Normal levels of endothelium-derived VWF were present in Nbeal2<sup>−/−</sup> mouse plasma (Figure 2F). Reduced amounts of plasma-derived fibrinogen were present in Nbeal2<sup>−/−</sup> platelets, while plasma levels were normal (Figure 2D,F). The α-granule membrane protein P-selectin is present at approximately 48% of WT levels (Figure 2G; 48.4 SD 5.3, n=3 determinations), indicating that despite lacking mature granules and their soluble cargo Nbeal2<sup>−/−</sup> platelets appear to contain the membrane constituents of α-granules. This contrasts with the effects of loss of function mutations in VPS33B and/or VPS16B, which result in human platelets lacking both α-granule cargo proteins and membrane constituents including P-selectin, suggesting that VPS33B/VPS16B are required for the formation of precursor α-granules.<sup>12,13</sup> Vps33b and Vps16b are both present in approximately equivalent amounts in Nbeal2<sup>−/−</sup> and WT platelets (Figure 2H,I), suggesting that Nbeal2 affects a later stage of α-granule biogenesis than these proteins.

The intracellular distribution of platelet proteins was examined via high resolution immunofluorescence confocal microscopy. Staining for α-granule cargo proteins was negative in Nbeal2<sup>−/−</sup> platelets (not shown), which showed normal distributions (Figure 3A,B) of surface membrane CD41/α<sub>IIb</sub> and circumferential ring cytoskeletal α-tubulin. Consistent with blood cell analysis, morphometric measurements of the long axis of microtubule rings showed Nbeal2<sup>−/−</sup> platelets to be significantly larger than wild type (mean values for WT: 2.91 µm SD 0.69,
Platelet function in vitro
Flow cytometry was used to measure platelet activation via surface exposure of activated α\textsubscript{II}β\textsubscript{3} (detected via binding of JON/A-PE antibody; Figure 4A,B) and P-selectin (detected by binding of anti-P-selectin-PE antibody; Figure 4C). In both assays platelets from Nbeal2\textsuperscript{−/−} mice had impaired activation induced by thrombin relative to WT (Figure 4A,C), and their α\textsubscript{II}β\textsubscript{3} activation response to ADP was also weaker (Figure 4B). Nbeal2\textsuperscript{−/−} maximal P-selectin exposure at high thrombin concentrations was one third that of WT (Figure 4C), thus while Nbeal2\textsuperscript{−/−} platelets have half the P-selectin of WT (Figure 2G) its externalization appears to be impaired. The response of washed platelets to agonists was measured via light transmission aggregometry (Figure 4D-F), where activation by thrombin, collagen related peptide (CRP) and the thromboxane-prostanoid receptor agonist U46619 produced weaker aggregation in Nbeal2\textsuperscript{−/−} platelets at all concentrations. At 0.05 U/mL thrombin Nbeal2\textsuperscript{−/−} platelets showed no aggregation; addition of 200 µg/mL of human fibrinogen yielded 19% (SD 7.1) aggregation, compared to 35.7% (SD 8.5) for WT (not shown). Impedance aggregometry in the Multiplate® analyzer was used to measure platelet response to collagen in citrated whole blood, where platelets from Nbeal2\textsuperscript{−/−} mice had significantly reduced (P=0.04) area-under-the-curve (AUC) aggregation compared with WT (Figure 4G). Lumiaggregometry was used to assess dense granule ATP release. For thrombin (1U), peak ATP release was 4.88 (SD 0.81) nmoles for WT and 4.07 (SD 0.30) for Nbeal2\textsuperscript{−/−} platelets; for U46619 (2 µM) the values were 3.75 (SD 0.28) and 1.52 (SD 0.20) nmoles respectively. Taken together these results indicate that Nbeal2\textsuperscript{−/−} platelets have impaired responses to activating stimuli.

Bleeding phenotype
In a tail transection assay (Figure 4H) Nbeal2\textsuperscript{−/−} mice showed increased rate of blood loss compared with WT and a 5-fold higher cumulative blood loss after 40 minutes (Nbeal2\textsuperscript{−/−} 322 ± 125 µL vs. WT 71 ± 30 µL, n=11 for both, P=0.03, unpaired t-test). These results point to a hemostatic deficiency in Nbeal2\textsuperscript{−/−} mice.

Platelet function in vivo
Platelet accumulation in laser-injured cremaster arterioles was assessed using established methods. Platelet accumulation in thrombi (measured by Dylight-tagged anti-GPIbβ, X488) expressed as sum of accumulation three minutes after injury and maximal accumulation was lower in Nbeal2\textsuperscript{−/−} mice (Figure 4I, P=0.03, Figure 4J, P=0.04) compared to WT. These results confirm that Nbeal2\textsuperscript{−/−} mice

n=50 platelets; for Nbeal2\textsuperscript{−/−}: 3.21 µm 0.57 SD, n=50 platelets; p<0.01, Mann Whitney 2-tailed test). The distribution of P-selectin within resting Nbeal2\textsuperscript{−/−} platelets appeared less well-defined than in WT cells (Figure 3A,B; middle panels), while both showed similar patterns of shape change and surface mobilization of P-selectin after thrombin activation (Figure 3C,D). The amount and distribution of the membrane protein LAMP1 did not differ between resting Nbeal2\textsuperscript{−/−} and WT platelets (Supplemental Figure S2B,C), indicating their lysosomal/late endosomal compartments are similar.
have impaired platelet accumulation in thrombi \textit{in vivo}. As a measure of platelet activation at the site of injury, WT and \textit{Nbeal2}\(^{-/-}\) mice had similar time to half-maximal activation ratio of CD41 and of P-selectin (Figure 4K,L). However, further inspection of the P-selectin activation reveals the maximal anti-P-selectin to anti-GP\(\text{Ib}\beta\) ratio is lower in \textit{Nbeal2}\(^{-/-}\) mice compared to WT mice (Figure 4M). Thus, although platelet accumulation in thrombi that form at sites of laser injury in \textit{Nbeal2}\(^{-/-}\) mice is impaired, the rate of activation of the platelets within the thrombi is not different to WT. The amount of P-selectin that \textit{Nbeal2}\(^{-/-}\) platelets can express in thrombi appears to be limited. These results show that the platelet activation time in thrombi is not altered in \textit{Nbeal2}\(^{-/-}\) mice, but their maximal P-selectin expression is impaired.

\textbf{Abnormalities in megakaryocyte structure and development}

The \(\alpha\)-granule deficiency observed in platelets was also noted in bone marrow MKs from \textit{Nbeal2}\(^{-/-}\) mice analyzed by TEM (Figure 5). Morphologically distinct \(\alpha\)-granules were numerous in WT MKs (Figure 5A,C n=50 MK) and greatly reduced in \textit{Nbeal2}\(^{-/-}\) MKs (Figure 5B,D n=50). WT MKs also contain numerous “platelet territories”\(^{23}\) (Figure 5A) that were clearly deficient in \textit{Nbeal2}\(^{-/-}\) MKs (Figure 5B), where emperipolesis (the presence of an intact cell within the cytoplasm of another cell) was observed much more frequently (Figure 5E). Our observations of ultrastructural abnormalities in marrow resident \textit{Nbeal2}\(^{-/-}\) MKs point to developmental abnormalities that may contribute to the lower platelet counts observed in these mice. We investigated this possibility by culturing MKs from primary bone marrow cells in the presence of TPO. Initial counts showed similar proportions of CD41\(^+\) cells among all cells recovered from marrow (WT: 0.228\% \pm 0.094; \textit{Nbeal2}\(^{-/-}\): 0.231\% \pm 0.051; n=3 mice and 7500 cells for each). Examination of bone marrow MKs revealed reduced ploidy (16N, 32N) in \textit{Nbeal2}\(^{-/-}\) compared to WT (n=3, not shown). MKs were observed to undergo differentiation leading to terminal proplatelet and platelet formation after 5 days (Figure 6), and while cells in this final stage of development appeared similar, comparisons of all stages (Figures 6, 8A-F, Supplemental Figure S3) detected by CD41 expression, size and nuclear morphology within MK populations indicated developmental abnormalities. The relatively higher proportion of \textit{Nbeal2}\(^{-/-}\) MKs found in the earliest stages (Figure 6G) indicates their development is delayed relative to WT, while the high proportion of \textit{Nbeal2}\(^{-/-}\) cells that appeared to be stalled in the spread phase (Figure 6D) - which we presume precedes terminal proplatelet development (Figure 6E) - indicates later stages may also be affected. Ploidy analysis of 5-day MK cultures revealed increased 2N but reduced 8N and >16N \textit{Nbeal2}\(^{-/-}\) MKs compared to WT (Figure 6H). In addition, we observed that while the proportion of MK-lineage cells present in 5-day WT cultures was 14.0\% \pm 0.62, consistent with other reports,\(^{24}\) the proportion present in \textit{Nbeal2}\(^{-/-}\) cultures was significantly lower at 7.67\% \pm 0.12 (p<0.01, two-tailed t-test; n=3 cultures and 900 cells for each). This indicates that \textit{Nbeal2}\(^{-/-}\) MKs may be overall less viable than WT.
Developmental differences between WT and Nbeal2−/− MKs were explored in detail by examining expression of VWF, a large protein normally packaged in distinct regions of α-granules. We observed that while VWF is present in early Nbeal2−/− and WT MKs, by the mature stages there is a marked difference in intracellular VWF distribution (Figure 7, Supplemental Figure 3A,B). In permeabilized WT MKs VWF is distributed throughout the nascent platelet territories delineated by CD41 (Figure 7A), while in Nbeal2−/− MKs VWF is concentrated at the cell periphery (Figure 7B). This pattern of VWF distribution was confirmed by examining unpermeabilized MKs (Figure 7C), where the absence of VWF staining in WT cells is contrasted by the strong colocalization of VWF and CD41 at the outer membrane of mature Nbeal2−/− MKs. In some cases we also saw evidence that VWF may accumulate in inclusions or be released from Nbeal2−/− cells. The abnormal presence of VWF outside of Nbeal2−/− MKs was also observed via TEM of pre-embed immunogold labeled sections (Figure 7D).

Discussion

As with human heterozygous carriers of GPS-associated NBEAL2 mutations, we observed no obvious differences between WT and Nbeal2−/− mice or their platelets, so we focused our efforts on comparisons of Nbeal2−/− and WT. As with GPS patients, Nbeal2−/− mice have a low platelet count (approximately 60% of WT) and their platelets are enlarged as assessed by both mean platelet volume and diameter of the cytoskeletal microtubule ring (see Results). Nbeal2−/− mice also have pale gray-appearing platelets compared to WT and Nbeal2+/− mice, and TEM examination confirmed the virtual absence of α-granules (Figure 1, Supplemental Figure S2A). On average 4.8 α-granules are seen in WT mouse platelet thin sections; this is slightly less than the 5.5 reported for human platelets, which may reflect the relatively smaller size of mouse platelets. These observations confirm that as with human NBEAL2, the absence of functional Nbeal2 protein in mice results in macrothrombocytopenia with platelets that are profoundly α-granule deficient. In contrast to their α-granule deficiency Nbeal2−/− mouse platelets had normal lysosome constituents (LAMP1, Supplemental Figure S2B,C). However, Nbeal2−/− mouse platelets had fewer dense granules per whole mount EM than WT and lower ATP secretion in response to both thrombin (Nbeal2−/−/WT = 0.83) and U66619 (Nbeal2−/−/WT = 0.41). By comparison, human GPS platelets contain normal to increased dense granules as measured by whole mount EM.

Splenomegaly is observed in GPS patients, and was also evident in 4-month old Nbeal2−/− mice, where the average spleen weight was 1.8x that of WT, containing increased numbers of MKs suggesting augmented extramedullary megakaryopoiesis. Another feature of GPS is the prevalence of myelofibrosis in the bone marrow, indicated by increased reticulin staining. We did not detect differences in reticulin staining between Nbeal2−/− and WT mice in either the bone
marrow (Supplemental Figure S1) or spleen, however, myelofibrosis progresses with age in GPS\textsuperscript{16} and it may be that 4 month old mice are not old enough for this condition to become evident. We will continue to monitor our Nbeal2\textsuperscript{−/−} mice as they age.

As in GPS, the absence of α-granules in Nbeal2\textsuperscript{−/−} platelets is expected to be accompanied by a lack of cargo proteins. Immunoblot analysis of platelet lysates failed to detect the megakaryocyte-synthesized soluble cargo proteins platelet factor 4, thrombospondin-1 and VWF (Figure 2A-C), whereas plasma-derived fibrinogen was greatly reduced (Figure 2D). VWF and fibrinogen were present at normal levels in Nbeal2\textsuperscript{−/−} plasma (Figure 2E,F). These cargo protein deficiencies have also been observed in GPS.\textsuperscript{16,19} The membrane-spanning α-granule protein P-selectin was present in Nbeal2\textsuperscript{−/−} platelets, albeit at approximately half of the normal amount (Figure 2G), and granule membranes and membrane-associated proteins such as P-selectin have been observed in GPS platelets by us\textsuperscript{15} and others.\textsuperscript{16,19,20,25,28-31}

The presence of P-selectin in platelets from Nbeal2\textsuperscript{−/−} mice and GPS patients suggests that despite the lack of cargo some of the membrane constituents and/or vesicular precursors of α-granules may be present. This contrasts with the situation in ARC syndrome, where loss of function of VPS33B or VPS16B leads to platelets lacking P-selectin\textsuperscript{12,13} and presumably α-granule precursors. Loss of VPS16B expression results in decreased levels of VPS33B, suggesting that these proteins interact during α-granule biogenesis.\textsuperscript{13} We examined their possible interaction with Nbeal2 by assessing levels of VPS33B and VPS16B in Nbeal2\textsuperscript{−/−} platelets (Figure 2H,I), which proved to be the same as WT. Since loss of Nbeal2 function appears to have no effect on Vps33b and Vps16b, we reason that these proteins and their human homologs likely act independently of Nbeal2 and are involved in an earlier stage of α-granule formation. NBEAL2 encodes a polypeptide of 2754 amino acids containing multiple domains\textsuperscript{15,17,18} (Supplemental Figure S4) which like other BEACH domain containing proteins has been implicated in cellular vesicular trafficking.\textsuperscript{15,32-35}

High resolution confocal immunofluorescence microscopy\textsuperscript{15} revealed that while P-selectin is present in the central region of both WT and Nbeal2\textsuperscript{−/−} platelets, the latter show a less orderly and more diffuse P-selectin distribution (Figure 3B) relative to the tightly-packed loops visible in WT platelets (which presumably correlate with mature α-granules). These differences, however, do not prevent P-selectin from being externalized when Nbeal2\textsuperscript{−/−} platelets are activated with thrombin (Figure 4C,L, M, Supplemental Figure S2), although at lower amounts, as has also been observed in GPS platelets.\textsuperscript{28} This may relate to the decreased total levels of P-selectin in Nbeal2\textsuperscript{−/−} platelets and abnormal intracellular distribution, possibly indicating altered precursor α-granule structures that cannot fuse as effectively with the plasma membrane as normal α-granules do in activated platelets.
Optical aggregometry revealed a decreased response of washed \(N\text{beal2}^{-/-}\) platelets to collagen-related peptide (CRP), thrombin and the thromboxane analog U46619 (Figure 4D-F). Unlike WT, \(N\text{beal2}^{-/-}\) platelets did not aggregate in the presence of 0.05 U/mL thrombin; weak aggregation was seen when exogenous fibrinogen was added. This indicates that lack of \(\alpha\)-granule borne fibrinogen may partially account for the relatively weak aggregation of \(N\text{beal2}^{-/-}\) platelets. Since flow cytometry showed decreased thrombin and ADP induced \(\alpha_{IIb}\beta_3\) activation (Figure 4A,B), and impedance aggregometry showed a decreased collagen response (Figure 4G), impaired platelet activation independent of \(\alpha\)-granule borne fibrinogen is also evident. Taken together these results show that \(N\text{beal2}^{-/-}\) platelets have a lowered response relative to WT to agonist-mediated activation. For comparison, \textit{in vitro} platelet aggregation studies summarized by the Nurden group (Table I)\(^{19}\) and Gunay-Aygun et al. (Table 2)\(^{16}\) show that decreased responses to varying agonists, including collagen, thrombin and ADP, have been observed in many GPS patients.

Several proteins secreted from platelet \(\alpha\)-granules, including VWF and coagulation factor V, are known to be important for platelet hemostatic function at sites of blood vessel injury, where an absence \(\alpha\)-granules in GPS platelets is thought to contribute to a bleeding phenotype that is characteristically mild to moderate.\(^{16,36,37}\) The results of tail bleeding assays (Figure 4H) clearly show increased blood loss in \(N\text{beal2}^{-/-}\) mice compared to WT, suggesting that their platelets do not function normally \textit{in vivo}. Increased bleeding of \(N\text{beal2}^{-/-}\) mice is not due to abnormalities in endothelial cell derived VWF, since plasma levels are normal (Figure 2E). Nor are their decreased platelet counts likely to be the cause since reductions up to 97.5% have been shown to have little influence on bleeding in mice.\(^{38}\)

The \textit{in vivo} function of \(N\text{beal2}^{-/-}\) mouse platelets was directly examined using high-speed confocal immunofluorescence microscopy to monitor response to laser-induced damage in cremaster muscle arterioles. We observed that \(N\text{beal2}^{-/-}\) mice show relatively decreased platelet accumulation at injury sites (Figure 4I,J), while their rate of platelet activation within the thrombi is not significantly different from WT (Figure 4K,L). The future availability of an appropriate Nbeal2 antibody will allow testing the possibility that absent Nbeal2 in the vasculature contributes to the decreased platelet accumulation at injury sites.

Splenectomy increases platelet counts in GPS patients,\(^{16}\) and a shortened mean platelet half life has been described using In\(^{111}\) labeling,\(^{19}\) suggesting that splenic sequestration and increased platelet destruction are causes of GPS-associated thrombocytopenia. Since this thrombocytopenia is noted to be progressive with age and accompanied by myelofibrosis,\(^{16}\) the more severe thrombocytopenia seen in older GPS patients likely indicates that fibrotic bone marrow is a poor environment for MK maturation and platelet production. The overall bone marrow morphology of our 4-month old \(N\text{beal2}^{-/-}\) mice was normal (Supplemental Figure S1), indicating that their thrombocytopenia may be related to abnormal MK
development. The presence of ultrastructural abnormalities in Nbeal2−/− MKs was confirmed by transmission electron microscopy using direct fixation of bone marrow cells (Figure 5). Nbeal2−/− MKs revealed a marked reduction of α-granules and platelet territories, which is likely associated with abnormalities in the demarcation membrane system (Figure 5B,D). Vesicles within Nbeal2−/− MKs appeared empty with a vacuolated appearance (compare Figure 5C WT with 5D Nbeal2−/−). Ultrastructural examination of GPS MKs has also revealed highly vacuolated cells with decreased α-granules.19 A striking feature noted in Nbeal2−/− MKs was the frequent observation of emperipolesis (the presence of an intact cell within the cytoplasm of another cell); for example Figure 5E shows 3 cells within the same MK and an Nbeal2−/− MK containing a nucleated cell is also shown in Figure 7B (DAPI+merge panel). MK emperipolesis has also been observed in GPS,31 MYH9-related disease,39 thrombocytosis, myelofibrosis and other conditions.39 While the mechanism is unclear, the involvement of aberrant P-selectin expression has been proposed to mediate emperipolesis.19

The presence of marked ultrastructural abnormalities in bone marrow Nbeal2−/− MKs suggests that their differentiation into platelets may be impaired, which is also indicated by their decreased ploidy. In a population analysis of bone marrow MKs using CD41 as a lineage-specific marker, we determined that the proportion of MK-lineage cells relative to all nucleated marrow cells (i.e. DAPI staining) was the same (approximately 0.23%) in WT and Nbeal2−/− mice. However, when these cells were cultured in the presence of TPO under conditions that promoted MK maturation, after five days the proportion of nucleated cells scored as MKs was 14% in WT (comparable to other studies24) and 7.8% in Nbeal2−/− cultures. Both WT and Nbeal2−/− MK cultures contained large cells showing extension of microtubule-containing proplatelets that were capable of producing platelets (Figure 6E,F). We evaluated their development by classifying populations (n=600) of cells into visually distinguishable stages: Early, Mature, Extending, Spread and Proplatelet MKs (Figures 6A-E). The resulting population distributions (Figure 6G) show that relative to WT a significantly larger proportion of Nbeal2−/− MKs were in the Early stage and fewer were in the Mature, Extending and Proplatelet stages, indicating a developmental delay in Nbeal2−/− MKs. In addition, the proportion of Spread MKs was significantly higher in the Nbeal2−/− MK population, which may indicate that Spread cells represent an abnormal, stalled or abortive stage of MK development. The abnormal development of Nbeal2−/− MKs was also indicated by the presence of increased 2N and greatly reduced 8N and >16N MKs in 5-day cultures (Figure 6H).

Several mechanisms can be proposed to account for abnormal Nbeal2−/− MK development. One possibility is that in addition to being important for α-granule formation, Nbeal2 may also be involved in the development of other membrane compartments such as the demarcation membrane, since Nbeal2−/− MKs appear to be more vacuolated than WT (Figure 5D). Another possibility is that Nbeal2 is involved in formation of the cytoskeletal organization of MKs, proplatelets and platelets, which influences platelet size and production.40 Platelets from GPS
patients are larger and rounder than normal\textsuperscript{15,20,40} and contain thicker peripheral microtubule coils.\textsuperscript{40,41} NBEAL2 contains multiple domains (Supplemental Figure S3) which may interact with components of the MK and platelet cytoskeleton in unknown ways. Atypical \textit{Nbeal2}\textsuperscript{-/-} MK development could also be associated with abnormal intracellular protein distribution arising from the failure to incorporate cargo proteins into maturing \(\alpha\)-granules, as we observed with VWF (Figure 7). In mature WT MKs, VWF is dispersed throughout the cytoplasm (Figure 7A) while in mature \textit{Nbeal2}\textsuperscript{-/-} MKs VWF protein is concentrated at the periphery (Figure 7B), and in some cells was observed in concentrated structures that sometimes extended beyond the outer membrane delineated by CD41 labeling and is observed outside of native (not cultured) \textit{Nbeal2}\textsuperscript{-/-} MKs labeled with immunogold (Figure 7D). VWF forms multimeric complexes that are organized into highly structured tubules that are either stored in \(\alpha\)-granules in MKs or Weibel-Palade bodies in endothelial cells in an acidic environment \textsuperscript{42,43}. The failure of VWF to enter \(\alpha\)-granules in \textit{Nbeal2}\textsuperscript{-/-} MKs may lead to its deposition in other membrane compartments or the cytoplasm, where a neutral pH may cause VWF to degrade and/or form abnormal structures (e.g. inclusions) that physically interfere with terminal MK differentiation.

In summary, we propose that \textit{Nbeal2}\textsuperscript{-/-} mice represent a plausible animal model of human GPS caused by loss of Nbeal2 expression, since they show many GPS-specific aspects of platelet and megakaryocyte structure, function and development both \textit{in vitro} and \textit{in vivo}. Our observations of protein expression in \textit{Nbeal2}\textsuperscript{-/-} mouse platelets indicate that Nbeal2 affects a later stage of \(\alpha\)-granule biogenesis than the mouse homologs of two other proteins identified as essential for this process in humans: VPS33B and VPS16B. Finally, exploiting the potential of our mouse model we have observed the development of \textit{Nbeal2}\textsuperscript{-/-} mouse MKs in culture and noted abnormalities which indicate that GPS-associated thrombocytopenia likely arises from delayed and aberrant platelet development that may be directly linked to the failure of MKs to form mature \(\alpha\)-granules and properly package their cargo.
Acknowledgments

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Authorship

Contribution: W.H.A.K., C.L.M., and P.L.G. designed experiments, performed research, wrote and edited manuscript; R.W.L. performed and analyzed mouse bone marrow cultures; L.L. performed sequencing, immunoblotting, mouse work, and analyzed data; F.G.P. performed confocal immunofluorescence microscopy, analyzed data, wrote and edited manuscript; H.C. performed transmission electron microscopy; R.N. performed intravital microscopy experiments; N.V. performed tail bleeding assays; C.E.H. performed bone marrow pathology analysis; A.S.W., and J.D.P., wrote and edited manuscript.

Conflicts-of-interest disclosure

The authors declare no competing financial interests.
References


Figure legends

Figure 1. Blood film and ultrastructure abnormalities of Nbeal2^-/- mouse platelets. Blood films were prepared from WT (A), Nbeal2^+/- (B) and Nbeal2^-/- (C) mice and stained with Wright-Giemsa stain prior to light microscopy. Wild type (A) and Nbeal2^+/- (B) blood films show typical platelet (black arrowheads) size and morphology with discernible granulation against light cytoplasmic staining, while the Nbeal2^-/- film (C) shows gray-appearing platelets with indistinct granulation and visible vacuoles. Thin section transmission electron micrographs of representative platelets from: (D) WT and (E) Nbeal2^-/- mice. Multiple α-granules (white arrowheads) were evident in WT platelets and absent in Nbeal2^-/- platelets, which are larger on average. Magnification 40000x; black bars represent 500 nm.

Figure 2. Protein content in Nbeal2^-/- mouse platelets and plasma. Immunoblots comparing platelet (PLT) whole-cell lysates or plasma from WT and Nbeal2^-/- mice. MK-derived thrombospondin-1 (TSP1, A), platelet factor 4 (PF4, B) and von Willebrand factor (VWF, C) were undetectable or significantly reduced in Nbeal2^-/- compared to WT platelet lysates, while plasma VWF (E) and fibrinogen (F) levels were normal. Plasma-derived fibrinogen (Fgn) was present in decreased amounts in Nbeal2^-/- platelets (D). P-selectin in Nbeal2^-/- platelets was present at approximately 48% of WT levels (G). VPS33B (H) and VPS16B (I) were present at similar levels in WT and Nbeal2^-/- platelets. Lysate from equivalent numbers of platelets was loaded in each lane and protein loading is indicated by probing for actin or GAPDH. See Methods for antibody details.

Figure 3. P-selectin in resting and thrombin activated Nbeal2^-/- platelets. High-resolution confocal laser immunofluorescence microscopy imaging of intracellular P-selectin membrane proteins in fixed resting platelets. Permeabilized cells were stained for α-tubulin (violet), P-selectin (red) and CD41/Integrin α_IIb (green) and imaged (final magnification = 150x, Z-stepping = 250 nm). Single channel and merged mid-cell ZY/XY slices of representative individual platelets are shown (A,B). Resting platelets from WT (A) and Nbeal2^-/- (B) mice have similar flat, discoid morphology with a well-defined circumferential tubulin ring cytoskeleton. Both contain P-selectin, which defines compact looping structures of the α-granule secretory matrix typical of wild type platelets that generally appear to be less orderly in Nbeal2^-/- platelets. Thrombin activated WT (C) and Nbeal2^-/- (D) platelets show characteristic activation-triggered changes in shape and surface mobilization of P-selectin.

Figure 4. Impaired Nbeal2^-/- mouse platelet function in vitro and in vivo. Measurements of activation of platelets from WT (solid symbols) and Nbeal2^-/- mice (open symbols). (A-C) Washed platelets were assessed by flow cytometry for activation in response to varying concentrations of agonists (X-axis) by measuring antibody binding (JON/A) to activated α_IIbβ_3 (A,B) or exposed P-selectin (C). Graphs show mean±SEM (n=6 mice per group) for mean
fluorescence intensity (MFI) after activation by thrombin (A,C) or ADP (B). (D-F) Optical aggregometry in physiological buffer of washed platelets exposed to varying concentrations of collagen-related peptide (CRP, D), thrombin (E) or the thromboxane analog U46619 (F). The agonist concentration is plotted against the percentage of aggregation (see Methods). (G) Impedance aggregometry measurement of platelets in citrated blood exposed to collagen (7 μg/mL, n=6 mice per group); horizontal lines represent mean±SEM for area under the curve (AUC) of Multiplate aggregation. (H) Nbeal2−/− mice showed greater cumulative blood loss relative to WT in a tail transection bleeding assay; mean ± SEM is shown for WT (closed squares) and Nbeal2−/− mice (open circles; n=11 for each). (I-M) Results of intravital videomicroscopy monitoring of platelet accumulation and activation in thrombi formed in response to laser injury of cremaster muscle arterioles. (I,J) Sum and maximal platelet accumulation in thrombi formed in response to injury were determined using a fluorescent anti-mouse GPIbβ antibody (X488); values shown are mean ± SEM for 170 thrombi in 12 WT mice and 155 thrombi in 12 Nbeal2−/− mice (*p < 0.05, unpaired t-test). (K,L) The time to half-maximal activation ratio for CD41 was determined using an anti-mouse CD41 Fab fragment (K), and for P-selectin (L) using an anti-mouse P-selectin antibody. Values shown are mean ± SEM; for CD41 n=119 thrombi in 6 WT mice and 93 thrombi in 6 Nbeal2−/− mice, for P-selectin n=69 thrombi in 6 wild type mice and 60 thrombi in 6 Nbeal2−/− mice. (M) The time course of P-selectin activation after injury. Mean ± SEM for wild type mice (closed squares) and Nbeal2−/− mice (open circles) are shown.

Figure 5. Abnormal ultrastructure of Nbeal2−/− bone marrow megakaryocytes. (A) Thin-section transmission electron micrographs of representative WT MKs show typical platelet territories (white arrowheads) and platelet-like structures containing α-granules. (B) In contrast, Nbeal2−/− MK are deficient in both α-granules and platelet territories (magnification 4000x, scale bars = 2 µm. (C) Higher magnification (15000x) images of WT MK reveal multiple α-granules (white arrowheads) and platelet territories, while (D) α-granules (white arrowheads) are rare in Nbeal2−/− MK which show poorly-defined platelet territories (scale bars = 500 nm). (E) Example of the emperipolesis that was frequently observed in Nbeal2−/− MK; here 3 exogenous cells (white arrowheads) are present in the MK (magnification 3000x, scale bar = 10 µm).

Figure 6. Abnormal Nbeal2−/− megakaryocyte development in populations of cultured bone marrow cells. Immunofluorescence images of megakaryocytes cultured to the terminal proplatelet stage. (A-E) Cells present in fixed 5-day cultures (n=5 mice and 600 cells for both Nbeal2−/− and WT) were identified by immunofluorescence microscopy as MKs by size (>10 μm diameter), large/lobulated nuclei (light blue) and expression of lineage-specific CD41 (green) and VWF (red). Individual cells were classified by apparent developmental stage as: (A) early, <20 μm in diameter (ZY sections show these cells to be spheroidal); (B) mature, >20 μm without projections (ZY sections show flattening with increased size); (C) extending, round cells with membrane...
projection; (D) spread, large cells with extensive membrane and tubulin (violet) projections but not showing clearly defined proplatelets; and (E) terminal proplatelets emanate from these very large megakaryocytes. (E) A distinctive pattern of nuclear retraction (light blue) and elaboration of nascent platelets defined by extensions of membrane (visualized by CD41, green) and cytoskeletal α-tubulin strands and loops (violet). Insets (F) show higher magnification views of nascent free platelets (1,2) and a proplatelet bud (3) from the WT cell having the distinctive platelet tubulin cytoskeletal ring and the presence of VWF (red, not shown E), which is absent in Nbeal2−/− platelets (Figure 2C). (A-C) show left to right: YZ (bars = 5 µm) and XY confocal mid-cell sections and extended focus images (bars = 10 µm); (D,E) show extended focus images (D, scale bar = 10 µm; E scale bar = 20 µm; (F, scale bars = 1 µm). (G) Population distributions of mean proportions of cells in each stage showed significant differences between Nbeal2−/− and WT (* P<0.05; ** P<0.01, 2-tailed t-test), with Nbeal2−/− cultures showing a markedly higher proportion of cells in both the early and spread stages and fewer in the mature, extending and proplatelet stages. (H) Distribution of Nbeal2−/− and WT CD41+ MK cells and ploidy (n=3 per group) after 5-day cultured cells, revealing increased 2N and greatly reduced 8N and >16N Nbeal2−/− MKs.

Figure 7. Abnormal VWF distribution in Nbeal2−/− megakaryocytes. (A) Immunofluorescence imaging of a representative permeabilized mature extending stage WT megakaryocyte shows elaboration of demarcation membranes stained with CD41 (green), dispersed VWF expression (red) and a peripheral tubulin cytoskeletal meshwork (violet). (B) A representative Nbeal2−/− MK at the same stage shows a strong peripheral distribution of VWF, which is absent in Nbeal2−/− platelets; this MK also contains the nucleus of an exogenous cell in its cytoplasm visible in the XZ view of the merged confocal panel. Comparisons of non-permeabilized cells (C) confirm that VWF is abnormally concentrated near and/or on the surface of mature Nbeal2−/− megakaryocytes (scale bars in ZY panels = 5 µm, bars in XY, XZ and extended focus panels = 10 µm). (D) Transmission electron micrograph of immunogold labeled VWF present on the surface of a Nbeal2−/− megakaryocyte. Magnification 50000x; black bar represents 500 nm.
Figure 1

A  WT  B  Nbeal2+/-  C  Nbeal2/-

D  WT  E  Nbeal2/-

[Image of cellular structures and annotations]

Scale bars: 500 nm
Figure 3

A WT CD41 P-selectin Tubulin + merge

B Nbeal2/- CD41 P-selectin Tubulin + merge

C WT CD41 P-selectin Tubulin merge

D Nbeal2/- CD41 P-selectin Tubulin merge
Figure 5

A  WT  B  Nbeal2-/-

C  WT  D  Nbeal2-/-  E  Nbeal2-/-
Figure 6

**A** Early  

**B** Mature  

**C** Extending  

**E** Proplatelet  

**D** Spread  

**F** CD41 VWF Tubulin DAPI  

**G**

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Figure 7

A WT CD41 VWF Tubulin DAPI + merge extended focus
B Nbeal2/− CD41 VWF Tubulin DAPI + merge extended focus
C WT Nbeal2/−
D Nbeal2/−
Abnormal megakaryocyte development and platelet function in Nbeal2−/− mice