NMIHBA results from hypomorphic PRUNE1 variants that lack short-chain exopolyphosphatase activity

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Abstract

Neurodevelopmental disorder with microcephaly, hypotonia and variable brain anomalies (NMIHBA) is an autosomal recessive neurodevelopmental and neurodegenerative disorder characterized by global developmental delay and severe intellectual disability. Microcephaly, progressive cortical atrophy, cerebellar hypoplasia and delayed myelination are neurological hallmarks in affected individuals. NMIHBA is caused by biallelic variants in PRUNE1 encoding prune exopolyphosphatase 1. We provide in-depth clinical description of two affected siblings harboring compound heterozygous variant alleles, c.383G>A (p.Arg128Gln), c.520G>T (p.Gly174*) in PRUNE1. To gain insights into disease biology, we biochemically characterized missense variants within the conserved N-terminal aspartic acid-histidine-histidine (DHH) motif and provide evidence that they result in the destabilization of protein structure and/or loss of exopolyphosphatase activity. Genetic ablation of Prune1 results in midgestational lethality in mice, associated with perturbations to embryonic growth and vascular development. Our findings suggest that NMIHBA results from hypomorphic variant alleles in humans and underscore the potential key role of PRUNE1 exopolyphosphatase activity in neurodevelopment.

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Introduction

Prune exopolyphosphatase 1 (PRUNE1) belongs to the DHH (Asp-His-His) superfamily of proteins, which includes phosphoesterases, exopolyphosphatases and pyrophosphatases (1–3). Members of this superfamily are characterized by the DHH (p.D30N, p.P54T, p.D106N and p.R128Q) clustering throughout the conserved N-terminal domain (1–3). Previous studies have implicated PRUNE1 in a complex network of interactions regulating cell cycle and motility, with elevated PRUNE1 expression associated with cancer metastasis (6–8).

Karaca et al. reported five individuals from four unrelated families segregating biallelic predicted pathogenic variants in PRUNE1 with a novel neurodevelopmental disorder characterized by microcephaly, hypotonia and variable brain anomalies (NMIHBA, MIM #617481) (9). The authors reported two different homozygous missense variants (p.D30N and p.D106N) in three consanguineous families and compound heterozygous (p.R128Q; p.G174*) variants in a non-consanguineous European ancestry family. Several groups have since identified other disease associated recessive alleles (p.P54T; p.R297W; c.521-2A > G; IVS4-2A > G) clustering throughout the conserved N-terminal domain (8,10,11). The observed incidence of NMIHBA in geographically distant families establishes this disorder as a pan-ethnic neurodevelopmental disorder. In addition to its nondescript developmental delay (DD)/intellectual disability (ID) nervous system hallmarks, NMIHBA patients may manifest more variable clinical features, including craniofacial anomalies (sloped forehead, large prominent ears, prominent eyes and narrow palate), scoliosis, joint contractures, muscle atrophy, hypereflexia, profound global DD, fronto-temporal atrophy, cortical and cerebellar atrophy, seizures, and spastic quadriplegia.

Although the neurodevelopmental and neurobiological etiology of NMIHBA remains unclear, the severe clinical and developmental aspects of this disorder suggest an important role for PRUNE1 in fundamental cellular functions. Indeed, prior studies have implicated PRUNE1 in cell proliferation and migration, processes mediated in part through interactions with nucleoside diphosphate kinase 1 (NMNAT1), glycogen synthase kinase-3β (GSK-3β) and β-tubulin respectively (4,8,12). Although in silico predictions of likely damaging effects of missense alleles classify them as likely pathogenic, the direction of effect (i.e. loss-of-function versus gain-of-function) and functional consequences of these variants have not been established. The observation of biallelic variants for a recessive disease trait, and the identification of putative loss of function splicing and nonsense variants support the notion of a reduced or loss of PRUNE1 function in NMIHBA pathophysiology. In agreement, patients harboring compound heterozygous mutations p.R128Q and p.G174* (described herein) manifest typical NMIHBA similar to those patients with homozygous missense variants spanning the conserved DHH (p.D30N, p.P54T, p.D106N and p.R128Q) and DHHAI2 domains (p.R297W) (8–10). Heterozygous carriers of reported pathogenic missense and truncating variants in PRUNE1 do not appear to have any phenotypic manifestations in the spectrum of NMIHBA, indicating that PRUNE1 haploinsufficiency in heterozygous carrier parents is permissive for neurodevelopmental failure. To gain further neurodevelopmental and neurobiological insights into NMIHBA, we investigated the functional effects of PRUNE1 variant alleles and generated a knock-out mouse model of Prune1.

We undertook comprehensive clinical phenotyping of two siblings from one of the initially reported families (SZ51) with NMIHBA and summarize the phenotypic spectrum across all NMIHBA patients reported to date. Additionally, we provide experimental evidence that disease-associated missense variant alleles that map within the conserved N-terminal domain can result in destabilization of the protein structure and consequent loss of exopolyphosphatase activity. Furthermore, we demonstrate that homozygous null alleles in Prune1−/− mice results in multiple vascular anomalies including abnormal vascular network within the yolk sac and disrupted cephalic vascular plexus culminating in midgestational lethality (E9.5).

Although embryonic lethality of Prune1−/− mice precluded the molecular exploration of the neurodevelopmental phenotypes, our study provides a potential biochemical basis to NMIHBA pathophysiology and implicitly an avenue for therapeutic exploration.

Results

Clinical description of NMIHBA patients

Family SZ51 is composed of two affected siblings (Patient 1 and Patient 2), an unaffected female sibling and non-consanguineous parents of European ancestry (Fig. 1A). Familial genomic analyses of these two affected patients and available family members through whole exome sequencing identified, as previously reported, shared compound heterozygous variants in PRUNE1 (9). Both affected siblings inherited a missense c.383G > A; p.Arg128Gln variant from their unaffected father and a nonsense c.520G > T; p.Gly174* variant from their unaffected mother (Supplementary Material, Fig. S1). The unaffected sister is a heterozygous carrier for the paternal missense variant only.

Detailed clinical evaluation of both affected siblings was performed through the NIH Undiagnosed Diseases Program (UDP) at ages 4 years and 20 months, respectively. Both patients presented with profound DD, ID, brain atrophy (Fig. 1C–E), seizures and absent language. Additional features not documented in the initial report include optic atrophy, esotropia, scoliosis, gastrointestinal reflux (GERD), hypotonia and spasticity. Onset of seizures in both siblings occurred at 6 months of age; it was observed initially as infantile spasms that developed into gelastic complex partial myoclonic seizures in Patient 1. EEG showed slow waking background and slow spike waves with progression to multifocal epileptiform discharges. Additional detailed clinical information for these patients can be found in supplementary information (including Supplementary Material, Tables S2 and S3).

Additionally, we performed detailed literature and clinical review of all 35 NMIHBA patients reported to date in order to better understand the phenotypic spectrum of NMIHBA (Table 1, Fig. 1B and F) (8–11,13–15). Consistent features across the majority of patients include severe global DD (94.59%), profound ID (91.89%) with absent language (83.78%) and brain abnormalities (86.48%) characterized primarily by cerebral and cerebellar atrophy, thin corpus callosum and white matter changes. Hypotonia and spasticity are features observed in the majority of patients (97.29%), whereas microcephaly and seizures (64.86%) appear to be more variable than initially reported, as more cases are documented. Visual (43.24%) and gastrointestinal (37.83%) problems are additional clinical features in a sizable number of patients. Interestingly, Alhaddad et al. reported about half of their cohort having absent deep tendon reflexes (DTRs) and/or reduced nerve conduction...
Figure 1. Clinical presentation of NMIHBA patients. (A) Immediate family pedigree and genotypes of patient 1 (P1) and patient 2 (P2). (B) Pathogenic variants in PRUNE1 identified in NMIHBA patients reported to date. The majority of pathogenic variants cluster in the DHH and DHHA2 domains. Variants characterized in this study are highlighted in red. (C–E) Sagittal T1-weighted brain MRI images. (C) Image of patient 1 at 4 months of age showing no significant findings. (D) Image of patient 1 at 4 years of age showing severe cortical and cerebellar atrophy and milder corpus callosum and brainstem atrophy. The craniofacial ratio is decreased. The cerebellar vermis decreased in height from 29.5 to 25.6 mm between the time the two images were obtained. (E) Image obtained for patient 2 at 20 months of age demonstrating cerebellar vermis hypoplasia and mild cortical atrophy. (F) Frequency of clinical manifestations ascertained in reported NMIHBA cases.
velocities (NCVs) suggesting peripheral neuropathy as part of the clinical spectrum of NMIHBA (13). Other patients also showed neurogenic findings on electromyography (EMG) studies and spinal motor neuron involvement. However, the majority of patients were not assessed for these clinical phenotypes and the true prevalence of peripheral nervous system involvement in this disorder may still be underappreciated. A few patients including probands described in this report developed scoliosis as well as foot anomalies such as talipes equinovarus or clubfoot, whether these features are secondary to the peripheral neuropathy and spinal muscular atrophy like phenotype or independent skeletal findings remains to be determined. Attempts at establishing a genotype–phenotype correlation for patients with NMIHBA were unsuccessful given that the clinical severity of the phenotype was variable even among individuals harboring the same variant allele (e.g. the recurrent D106N variant). No relationship between severity of the clinical presentation or presence/absence of specific clinical features with the type of mutation or zygosity of variants was evidently observed.

AFFECTED SUBJECTS have been reported to originate from countries around the globe (Supplementary Material, Fig S2). As expected, when homozygous alleles were identified, there was often a history of consanguinity. Of note, most homozygous subjects have missense variant alleles suggesting that NMIHBA results in the majority of cases from hypomorphic alleles. The likely loss of function null allele, c.1759A>T; p.G174∗ as a compound heterozygote with the missense c.383G>A; p.Arg128Gln variant in patients 1 and 2 is suggestive of partial function in these individuals. More recently, a homozygous splicing variant (11) and microdeletions spanning exon 2-8 in PRUNE1 individuals. More recently, a homozygous splicing variant (11) in patients 1 and 2 is suggestive of partial function in these PRUNE1 function.

suggests that NMIHBA results from partial or complete loss of either homozygous or compound heterozygous null alleles possibly result in complete loss of PRUNE1 function have been observed. AFFECTED SUBJECTS have been reported to originate from countries around the globe (Supplementary Material, Fig S2). As expected, when homozygous alleles were identified, there was often a history of consanguinity. Of note, most homozygous subjects have missense variant alleles suggesting that NMIHBA results in the majority of cases from hypomorphic alleles. The likely loss of function null allele, c.1759A>T; p.G174∗ as a compound heterozygote with the missense c.383G>A; p.Arg128Gln variant in patients 1 and 2 is suggestive of partial function in these individuals. More recently, a homozygous splicing variant (11) and microdeletions spanning exon 2-8 in PRUNE1 (13) that possibly result in complete loss of PRUNE1 function have been reported. These patients have similar clinical presentations as those with homozygous missense variants. The neuropathologic phenotype of heterozygous loss of function carrier parents with presumed haploinsufficiency at the PRUNE1 locus and the report of either homozygous or compound heterozygous null alleles suggests that NMIHBA results from partial or complete loss of PRUNE1 function.

Comparative modeling of PRUNE1 mutations

Similar to other members of the DHH superfamily, PRUNE1 contains a common N-terminal DHH domain resulting from four highly conserved motifs, with five invariant aspartases and two conserved histidine residues, one of which is replaced by Arginine (R128). One of the conserved aspartates is thought to act as nuclease forming a charge-relay system with the conserved histidine, whereas the other aspartates bind divalent cations required for the catalytic activity (4). Interestingly, most of the pathogenic variants map to the highly conserved N-terminal DHH domain. In order to understand how NMIHBA-causing PRUNE1 variants drive the disease phenotype, we chose to characterize four variants (p.D30N, p.D106N, p.R128Q and p.G174∗) within the conserved N-terminal domain for which we had patient-derived fibroblasts and/or comprehensive clinical information.

Importance of the D30, D106 and R128 residues to PRUNE1 function is revealed by considering the crystal structure of a homologous enzyme, Saccharomyces cerevisiae cytosolic exopolyphosphatase (PPX1) (PDB ID: 2QB7) (16). PPX1 is 25% identical and 45% similar to human PRUNE1 and has an equivalent enzymatic function (Supplementary Material, Fig S3A) (4,17). In this structure, a series of divalent metal ions (M1 and M2) and bound phosphate ions (P1, P2 and P3) delineate the active site and define residues necessary for binding the polyphosphate substrate as well as the catalytic metal ions. Strikingly, all three of the missense mutations under investigation map to the same region of the PPX1 active site: PPX1 residues D41 and D127 (equivalent to PRUNE1 D30 and D106) coordinate a divalent metal ion, while PPX1 residue H149 (equivalent to PRUNE1 residue R128) forms a charge–charge interaction with the adjacent bound phosphate (Fig 2A).

Substitution of Asn for Asp (as in D30N and D106N) will eliminate that amino acid’s ability to coordinate the divalent metal ion, thereby abolishing catalytic activity and destabilizing the local protein structure that was ordered by interactions with that metal ion. Similarly, substitution of Arg 128 by Gln eliminates the positive charge on that residue, likely preventing the mutated residue from interacting with the negatively charged phosphate group of a bound substrate.

To further verify these structural predictions, we performed circular dichroism (CD) on mammalian cell (Expi293) derived recombinant muteins harboring the D106N and R128Q variants. Whereas D106N and R128Q recombinant proteins expressed and migrated similarly to wild-type, rapid turnover of the D30N mutant precluded protein purification and further analyses (Fig 3A; Supplementary Material, Fig S3B and C). Far and near-UV CD spectra were collected for wild-type PRUNE1 and the two mutants (D106N and R128Q). Qualitative differences in the far and near-UV CD spectra were observed for each mutant compared with wild-type PRUNE1 (Fig 2B and C). Spectral deconvolution of the far-UV CD revealed differences in helical content in the D106N and R128Q muteins (45 and 48.8%, respectively) as compared with wild-type (37.3%). Near-UV CD analyses revealed differences in tertiary structure between D106N and R128Q muteins as compared with wild-type. Furthermore, increased magnitude of CD signal particularly in the region contributed by Phe and Tyr residues indicated change in overall tertiary structure. The increase in CD signal possibly resulted from a more rigid conformation of the aromatic sidechains induced by the D106N and R128Q mutations. Lastly, quantitative assessment of spectral similarity (assessed by TQ similarity match scores) of the far and near-UV CD between the wild-type and muteins demonstrated a low similarity between the spectra of the mutants compared with that of wild-type (Fig 2D and E). Therefore, our preliminary in silico and protein biochemistry analyses predicted that these three missense mutations could result in loss of protein function, by abolishing substrate binding and catalytic activity or by destabilizing the structure of the active site.

Missense mutations result in loss of exopolyphosphatase activity

In order to evaluate protein stability, mammalian expression constructs encoding epitope (HA)-tagged wild-type or missense PRUNE1 muteins (D30N, D106N, R128Q and G174∗) were transfected into HEK293 cells (Supplementary Material, Fig S4A). HEK293 cells have no detectable endogenous PRUNE1 expression. At steady state, immunoblotting analyses of whole cell lysates demonstrated a significant reduction of PRUNE1 levels in cells expressing D30N or D106N variants in comparison to wild-type-tagged protein. Whereas lysates from cells expressing G174∗ showed no discernable protein product, the R128Q variant-expressing cells showed similar expression compared with cells expressing the wild-type protein (Fig 3A). Degradation kinetics visualized by immunoblot
| Table 1. Summary of clinical findings of individuals with biallelic PRUNE1 mutations |
|---------------------------------------------------------------|
| **Age at evaluation** | Patient 1 (SZ51-1) | Patient 2 (SZ51-2) | Karaca et al. (2015) | Zollo et al. (2017) | Costain et al. (2017) | Karakaya et al. (2017) | Iacomino et al. (2018) | Alfadhel et al. (2018) | Alhaddad et al. (2018) | Papuc (2019) | Fujii (2019) |
| **Sex** | F | M | 1F; 2M | 1F; 2M; 1 Saudi; 2 Turkish | M | M | M | M | M | F | M |
| **Ethnicity** | European | European | 1 Saudi; 2 Turkish; 1 Omani, Iranian, Italian and Indian | Turkish | European | (Italian) | | | | Sri Lankan | Japanese |
| **Variant Zygosity** | Comp Het c.383G > A; p.Arg128Gln | Comp Het c.383G > A; p.Arg128Gln | Homozygous c.88G > A; p.Asp30Asn, c.316G > A; p.Asp106Asn | Homozygous c.874_875insA; p.H292Qfs*3 | Homozygous c.521-2A > G | Homozygous c.316G > A; c.383G > A; p.Arg128Gln | Homozygous c.316G > A; p.Asp106Asn | Homozygous c.316G > A; p.Asp106Asn | Homozygous c.316G > A; c.383G > A; p.Arg128Gln | Homozygous c.316G > A; p.Asp106Asn | Homozygous c.316G > A; p.Asp106Asn |
| **Consanguinity/ID/DD** | No +; profound | No +; profound | Yes +; profound | Yes +; profound (3/3) | No +; profound | Yes +; regression | No | Yes +; profound (2/2) | Yes | Yes | No |
| **Brain abnormalities** | + | + | +; (3/3) | +; (3/3) | +; (3/3) | +; (3/3) | +; (3/3) | +; (2/3) | +; (2/2) | +; (2/2) | +; (2/13) | +; (10/12); 2 NA |
| **Microcephaly** | + | + | +; (3/3) | +; (3/3) | +; (3/3) | +; (3/3) | +; (3/3) | +; (2/2) | +; (2/2) | +; (2/2) | +; (3/12) | +; (10/12); 2 NA |
| **Language delay** | +; absent N A | +; absent N A | +; absent N A | +; absent N A | +; absent N A | +; absent N A | +; absent N A | +; absent N A | +; absent N A | +; absent N A | +; absent N A | +; absent N A |
| **Scoliosis/Kyphosis** | +; optic +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic |
| **Vision problems** | +; optic +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic |
| **Muscle tone abnormalities** | +; axial +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic | +; optic |
| **Brain abnormalities** | 0.3–21.0 yo | 9 F; 4 M | Omani, Iranian, Italian and Indian | Turkish | European | Italian | 12 yo | 2.2 yo | 3 yo | 9 mo | 0 m; 12 yo | 2 m; 0 m |
| **Brain abnormalities** | 4 yo | 20 mo | 1.5–5.5 yo | M | 1.5; 5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo |
| **Scoliosis/Kyphosis** | 1.5–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo |
| **Vision problems** | 1.5–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo |
| **Muscle tone abnormalities** | 1.5–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo |
| **Brain abnormalities** | 1.5–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo |
| **Scoliosis/Kyphosis** | 1.5–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo |
| **Vision problems** | 1.5–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo |
| **Muscle tone abnormalities** | 1.5–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo | 0.3–5.5 yo |

Continued
| Patient 1 (SZ51-1) | Patient 2 (SZ51-2) | Karaca et al. (2015) | Zollo et al. (2017) | Costain et al. (2017) | Karakaya et al. (2017) | Iacomino et al. (2018) | Alfadhel et al. (2018) | Alhaddad et al. (2018) | Papuc (2019) | Fujii (2019) |
|-------------------|-------------------|----------------------|--------------------|-----------------------|-----------------------|------------------------|------------------------|------------------------|----------------|---------------|
| **GERD/Dysphagia** |                   | ++; severe GERD      |                    |                       |                       |                       |                        |                        |                |               |
| **Seizure type**  |                   | +                    | NA                 | NA                    | NA                    | NA                     | NA                     | ++; Dysphagia       | NA             |               |
| and onset        |                   | Onset at 6 mo;      | NA                 | NA                    | NA                    | NA                     | (1/2)                  | Onset at 6 mo;     | NA             | Onset at 8 mo;|
|                   |                   | Gelastic,           |                    |                       |                       |                        |                        | upper/lower        |                |               |
|                   |                   | complex partial     |                    |                       |                       |                        |                        | limbs epileptic      |                |               |
|                   |                   | myoclonic seizures, |                    |                       |                       |                        |                        | spasms               |                |               |
|                   |                   | infantile spams     |                    |                       |                       |                        |                        |                       |                |               |
| **Abnormal EEG**  |                   | NA                   | NA                 | Hypsarrhythmia        | NA                    | Slow background,      | Normal EEG            | Focal spasms       | Focal toni,     | Hypsarrhythmia |
| findings          |                   |                      |                    |                      |                       | multifocal epileptic   |                        | (2/12)            | myoclonic       |                |
|                   |                   |                      |                    |                      |                       | abnormalities         |                        |                        | seizures         |                |
| **Abnormal MRI**  | Moderate/severe   | Modified hypsarrhythmia, |                   |                      |                       | Abnormal corpus        | Cerebral atrophy (7/12) | Hyperintense       |                    |                |
| findings          | progressive global brain atrophy; cerebral and cerebellar atrophy |                        |                    |                      |                       | callosum and mild      |                        | brain lesions (4/12) |                    |                |
|                   |                    |                      |                    |                       |                       | cerebral atrophy (6/12) |                        |                        |                    |                |
|                   |                    |                      |                    |                       |                       |                        |                        |                        |                    |                |
| **NCV/EMG**       | NA                 | NA                   | NA                 | NA                    | NA                    | Neurogenic findings    | NA                     | NA                     | NA              |               |
| **findings**      | OFC: 41.5 cm (−5.3 s.d.); Weight: 15.1 kg (24th %ile); Length: 94 cm (−2.0 s.d.) | OFC: 41.0 cm (−5.3 s.d.); Weight: 12.8 kg (70th %ile); Length: 86 cm (77th %ile) | OFC: 49.5 cm (50th %ile); Weight: 16.5 kg (95th %ile) | OFC: 48 cm (−2 s.d.) | Neurogenic findings OfC:48 cm (−2 s.d.) | Neurogenic findings NA | OFC: −2.1 and −2.3 s.d.; Weight: −1.08 to +2.63 s.d. | NA                     | NA              |               |
| **Measurements**  | at evaluation     |                       |                    |                       |                       |                        |                        |                        | OFC < 3rd %ile | OfC: 43.5 cm (−0.82 s.d.); Weight: 8.4 kg (−0.31 s.d.); Length: 73.8 cm (−0.26 s.d.) |
|                   | OfC: 41.5 cm (−5.3 s.d.); Weight: 15.1 kg (24th %ile); Length: 94 cm (−2.0 s.d.) | OfC: 41.0 cm (−5.3 s.d.); Weight: 12.8 kg (70th %ile); Length: 86 cm (77th %ile) | OfC: 49.5 cm (50th %ile); Weight: 16.5 kg (95th %ile) | OfC: 48 cm (−2 s.d.) | Neurogenic findings OfC:48 cm (−2 s.d.) | Neurogenic findings NA | OfC: −2.1 and −2.3 s.d.; Weight: −1.08 to +2.63 s.d. | NA                     | NA              |               |
|                   | OfC: 41.5 cm (−5.3 s.d.); Weight: 15.1 kg (24th %ile); Length: 94 cm (−2.0 s.d.) | OfC: 41.0 cm (−5.3 s.d.); Weight: 12.8 kg (70th %ile); Length: 86 cm (77th %ile) | OfC: 49.5 cm (50th %ile); Weight: 16.5 kg (95th %ile) | OfC: 48 cm (−2 s.d.) | Neurogenic findings OfC:48 cm (−2 s.d.) | Neurogenic findings NA | OfC: −2.1 and −2.3 s.d.; Weight: −1.08 to +2.63 s.d. | NA                     | NA              |               |
|                   | OfC: 41.5 cm (−5.3 s.d.); Weight: 15.1 kg (24th %ile); Length: 94 cm (−2.0 s.d.) | OfC: 41.0 cm (−5.3 s.d.); Weight: 12.8 kg (70th %ile); Length: 86 cm (77th %ile) | OfC: 49.5 cm (50th %ile); Weight: 16.5 kg (95th %ile) | OfC: 48 cm (−2 s.d.) | Neurogenic findings OfC:48 cm (−2 s.d.) | Neurogenic findings NA | OfC: −2.1 and −2.3 s.d.; Weight: −1.08 to +2.63 s.d. | NA                     | NA              |               |
|                   | OfC: 41.5 cm (−5.3 s.d.); Weight: 15.1 kg (24th %ile); Length: 94 cm (−2.0 s.d.) | OfC: 41.0 cm (−5.3 s.d.); Weight: 12.8 kg (70th %ile); Length: 86 cm (77th %mile) | OfC: 49.5 cm (50th %ile); Weight: 16.5 kg (95th %ile) | OfC: 48 cm (−2 s.d.) | Neurogenic findings OfC:48 cm (−2 s.d.) | Neurogenic findings NA | OfC: −2.1 and −2.3 s.d.; Weight: −1.08 to +2.63 s.d. | NA                     | NA              |               |
|                   | OfC: 41.5 cm (−5.3 s.d.); Weight: 15.1 kg (24th %ile); Length: 94 cm (−2.0 s.d.) | OfC: 41.0 cm (−5.3 s.d.); Weight: 12.8 kg (70th %ile); Length: 86 cm (77th %ile) | OfC: 49.5 cm (50th %ile); Weight: 16.5 kg (95th %ile) | OfC: 48 cm (−2 s.d.) | Neurogenic findings OfC:48 cm (−2 s.d.) | Neurogenic findings NA | OfC: −2.1 and −2.3 s.d.; Weight: −1.08 to +2.63 s.d. | NA                     | NA              |               |
|                   | OfC: 41.5 cm (−5.3 s.d.); Weight: 15.1 kg (24th %ile); Length: 94 cm (−2.0 s.d.) | OfC: 41.0 cm (−5.3 s.d.); Weight: 12.8 kg (70th %ile); Length: 86 cm (77th %ile) | OfC: 49.5 cm (50th %ile); Weight: 16.5 kg (95th %ile) | OfC: 48 cm (−2 s.d.) | Neurogenic findings OfC:48 cm (−2 s.d.) | Neurogenic findings NA | OfC: −2.1 and −2.3 s.d.; Weight: −1.08 to +2.63 s.d. | NA                     | NA              |               |
|                   | OfC: 41.5 cm (−5.3 s.d.); Weight: 15.1 kg (24th %ile); Length: 94 cm (−2.0 s.d.) | OfC: 41.0 cm (−5.3 s.d.); Weight: 12.8 kg (70th %ile); Length: 86 cm (77th %ile) | OfC: 49.5 cm (50th %ile); Weight: 16.5 kg (95th %ile) | OfC: 48 cm (−2 s.d.) | Neurogenic findings OfC:48 cm (−2 s.d.) | Neurogenic findings NA | OfC: −2.1 and −2.3 s.d.; Weight: −1.08 to +2.63 s.d. | NA                     | NA              |               |
|                   | OfC: 41.5 cm (−5.3 s.d.); Weight: 15.1 kg (24th %ile); Length: 94 cm (−2.0 s.d.) | OfC: 41.0 cm (−5.3 s.d.); Weight: 12.8 kg (70th %ile); Length: 86 cm (77th %ile) | OfC: 49.5 cm (50th %ile); Weight: 16.5 kg (95th %ile) | OfC: 48 cm (−2 s.d.) | Neurogenic findings OfC:48 cm (−2 s.d.) | Neurogenic findings NA | OfC: −2.1 and −2.3 s.d.; Weight: −1.08 to +2.63 s.d. | NA                     | NA              |               |
| Dysmorphic features | Patient 1 (SZ51-1) | Patient 2 (SZ51-2) | Karaca et al. (2015) | Zollo et al. (2017) | Costa et al. (2017) | Karakaya et al. (2017) | Iacomino et al. (2018) | Alfadhel et al. (2018) | Alhaddad et al. (2018) | Papuc (2019) | Fujii (2019) |
|---------------------|---------------------|---------------------|----------------------|---------------------|---------------------|----------------------|----------------------|----------------------|----------------------|-------------|-------------|
| +; Phalangeal +; (2/3) +; tall forehead, bitempral narrowing, low set ears, flat nasal bridge, narrow high arched palate +; Brachycephaly, large ears +; Epididymis, frontal plagiocephaly, brachydactyly, detached and hypoplastic nipples | NA | NA | +; Plagiocephaly (13/13) | +; Brachycephaly, large ears | +; Plagiocephaly, bitempral narrowing, low set ears, flat nasal bridge, narrow high arched palate | Bilateral talipes equinovarus, large ears | Bilateral talipes equinovarus, large ears | Bilateral talipes equinovarus, large ears | Bilateral talipes equinovarus, large ears | Bilateral talipes equinovarus, large ears | Bilateral talipes equinovarus, large ears |
| Other findings | Exaggerated startle and aversion to various sensory stimuli; Left hip dysplasia | Exaggerated startle; bilateral Babinski signs and sustained ankle clonus | Exaggerated startle; bilateral Babinski signs and sustained ankle clonus | Exaggerated startle; bilateral Babinski signs and sustained ankle clonus | Exaggerated startle; bilateral Babinski signs and sustained ankle clonus | Exaggerated startle; bilateral Babinski signs and sustained ankle clonus | Exaggerated startle; bilateral Babinski signs and sustained ankle clonus | Exaggerated startle; bilateral Babinski signs and sustained ankle clonus | Exaggerated startle; bilateral Babinski signs and sustained ankle clonus | Exaggerated startle; bilateral Babinski signs and sustained ankle clonus | Exaggerated startle; bilateral Babinski signs and sustained ankle clonus |
| Regression. Knee contractures, hip dislocation; respiratory insufficiency (8 mo). Tracheostomy, nasogastric tube dependent | Distal joint arthrogyrosis; respiratory distress. Babinski sign observed. Bilateral talipes equinovarus, bell shaped thorax. Spinal motor neuron involvement | Distal joint arthrogyrosis; respiratory distress. Babinski sign observed. Bilateral talipes equinovarus, bell shaped thorax. Spinal motor neuron involvement | Distal joint arthrogyrosis; respiratory distress. Babinski sign observed. Bilateral talipes equinovarus, bell shaped thorax. Spinal motor neuron involvement | Distal joint arthrogyrosis; respiratory distress. Babinski sign observed. Bilateral talipes equinovarus, bell shaped thorax. Spinal motor neuron involvement | Distal joint arthrogyrosis; respiratory distress. Babinski sign observed. Bilateral talipes equinovarus, bell shaped thorax. Spinal motor neuron involvement | Distal joint arthrogyrosis; respiratory distress. Babinski sign observed. Bilateral talipes equinovarus, bell shaped thorax. Spinal motor neuron involvement | Distal joint arthrogyrosis; respiratory distress. Babinski sign observed. Bilateral talipes equinovarus, bell shaped thorax. Spinal motor neuron involvement | Distal joint arthrogyrosis; respiratory distress. Babinski sign observed. Bilateral talipes equinovarus, bell shaped thorax. Spinal motor neuron involvement | Distal joint arthrogyrosis; respiratory distress. Babinski sign observed. Bilateral talipes equinovarus, bell shaped thorax. Spinal motor neuron involvement | Distal joint arthrogyrosis; respiratory distress. Babinski sign observed. Bilateral talipes equinovarus, bell shaped thorax. Spinal motor neuron involvement |
| Slow NCVs (5/12); Arthrogryposis/contractures (2/12); Hypertrophic cardiomyopathy (2/12); Pectus excavatum (1/12); Clubfoot (1/12); Death (8/12) | Normal CMA, Beckwith–Wiedemann, Prader–Willi, SMARD1, DM1, and CCHS testing. Normal metabolic and biochemical testing | Normal CMA, Beckwith–Wiedemann, Prader–Willi, SMARD1, DM1, and CCHS testing. Normal metabolic and biochemical testing | Normal CMA, Beckwith–Wiedemann, Prader–Willi, SMARD1, DM1, and CCHS testing. Normal metabolic and biochemical testing | Normal CMA, Beckwith–Wiedemann, Prader–Willi, SMARD1, DM1, and CCHS testing. Normal metabolic and biochemical testing | Normal CMA, Beckwith–Wiedemann, Prader–Willi, SMARD1, DM1, and CCHS testing. Normal metabolic and biochemical testing | Normal CMA, Beckwith–Wiedemann, Prader–Willi, SMARD1, DM1, and CCHS testing. Normal metabolic and biochemical testing | Normal CMA, Beckwith–Wiedemann, Prader–Willi, SMARD1, DM1, and CCHS testing. Normal metabolic and biochemical testing | Normal CMA, Beckwith–Wiedemann, Prader–Willi, SMARD1, DM1, and CCHS testing. Normal metabolic and biochemical testing | Normal CMA, Beckwith–Wiedemann, Prader–Willi, SMARD1, DM1, and CCHS testing. Normal metabolic and biochemical testing | Normal CMA, Beckwith–Wiedemann, Prader–Willi, SMARD1, DM1, and CCHS testing. Normal metabolic and biochemical testing |

MR: magnetic resonance
assays following cycloheximide-mediated translational block demonstrated rapid and significant loss of D30N and D106N mutant proteins, which was rescued by the cell-permeable proteasome inhibitor, MG132 (Fig. 3B). In contrast, the R128Q variant and wild-type PRUNE1 levels were stable after synthesis, as they were observed at >80% of baseline (0 h levels) after 24 h of cycloheximide treatment (Fig. 3B). In subsequent experiments, we evaluated PRUNE1 protein levels in patient-derived skin fibroblasts harboring the compound heterozygous R128Q/G174∗ variants or homozygous D106N variants (Supplementary Material, Fig. S4B). PRUNE1 levels were comparable between fibroblasts harboring compound heterozygous variants and the wild-type allele. However, cells homozygous for the D106N variant showed dramatic reduction in PRUNE1 levels at steady state (Supplementary Material, Fig. S4C). Proteasome inhibition by MG132 rescued the loss of the mutant protein in these cells (Fig. 3C). Taken together, these studies suggested that the D30N and D106N variants lead to reduced protein stability and degradation by the proteasome pathway, whereas the R128Q variant results in a stable mutant protein.
Figure 3. D30N and D106N variants result in reduced protein stability and proteosomal degradation, whereas R128Q variant results in stable mutant protein. (A) HEK293 cells were transfected with N-terminal HA-tagged wild-type or mutant PRUNE1 cDNA. Equal amounts of protein from whole cell lysate were used for immunoblotting using antibodies against N-terminal HA-tag, and C-terminal PRUNE1 epitope (a.a. 393-420, dotted line). GAPDH was used as a loading control. G174 mutant showed no expression, whereas D30N and D106N mutants showed significantly reduced expression as compared with wild-type and R128Q levels. (B) Immunoblots of cells overexpressing N-terminal HA-tagged wild-type or mutant PRUNE1 treated with cycloheximide (CHX) at 200 μM for 0, 6 and 24 h (with or without proteasome inhibitor MG132 at 15 μM). Bar graphs in (A) and (B) represent densitometry analyses of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, error bars: SEM. (C) Immunoblotting of endogenous PRUNE1 in patient derived fibroblasts harboring compound heterozygous R128Q; G174∗ variants or homozygous D106N missense variant treated with or without MG132 (15 μM). Densitometry analysis of the representative immunoblot shown in numerical format.

PRUNE1 has been ascribed exopolyphosphatase and phosphodiesterase activity based on sequence homology to yeast exopolyphosphatase PPX1 and bacterial nuclease RecJ, respectively (4). In subsequent experiments, we assessed how missense variants in PRUNE1 influence its enzymatic function. GloSensor assays established no detectable cAMP or cGMP phosphodiesterase activity of wild-type or missense PRUNE1 variants as compared with PDE4A and PDE5A (Supplementary Material, Fig. S5A). However, consistent with the findings of Tammenkoski et al. (17), wild-type PRUNE1 catalyzed polyphosphate hydrolysis of short-chain polyphosphates P₃ (kₐₚ = 6.24 s⁻¹; Kₐₚ = 8.4 μM; Vₘₐₓ = 0.06 μM/s) and P₄ (kₐₚ = 4.2 s⁻¹; Kₐₚ = 16.13 μM; Vₘₐₓ = 0.04 μM/s) using Mg²⁺ as the metal cofactor (Fig. 4A and B) and demonstrated no exopolyphosphatase against long-chain polyphosphates P₄₅, P₆₀ and P₇₀₀ (Supplementary Material, Fig. S5C). D106N and R128Q variants completely abolished short-chain exopolyphosphatase activity compared with wild-type as assessed by BIOMOL® Green phosphate reagent using P₃ (1–50 μM) and P₄ (5–100 μM) as polyphosphate substrates (Fig. 4C). Similarly, Escherichia coli expressed D106N and R128Q variants had no detectable short-chain exopolyphosphatase activity as compared with wild-type (Supplementary Material, Fig. S5B).

Taken together, these results indicated that missense variants D30N, D106N and R128Q result in loss of PRUNE1 function either due to impaired protein stability or due to the loss of enzymatic function. These findings are consistent with the
Figure 4. D106N and R128Q variants result in loss of short chain exopolyphosphatase activity. (A) Lineweaver–Burk plots depicting short-chain exopolyphosphatase kinetics of wild-type PRUNE1 using sodium tripolyphosphate (P3) and sodium tetrapolyphosphate (P4) as substrates. Dotted black lines represent 95% CI. (B) Kinetic parameters for hydrolysis of polyphosphates (P3 and P4) by wild-type PRUNE1 in the presence of Mg2⁺ (2 mM) as the cofactor. Values reported by Tammenkoski et al. are shown within parenthesis (17). (C) Short-chain exopolyphosphatase activity of wild-type, D106N and R128Q mutants on P3 and P4 determined using fixed-time BIOMOL Green phosphate detection assay. Data represented as mean ± SEM over three independent experiments with six technical replicates per sample.

observed phenotypic concordance between patients harboring homozygous missense D30N, D106N variants and the compound heterozygous individuals (p.R128Q; p.G174∗) described herein (patients 1 and 2). Importantly, these studies support the loss of PRUNE1 function as the molecular etiology of NMIHBA.

Loss of Prune1 results in embryonic lethality in mouse
In an effort to model NMIHBA in vivo, we next studied the consequences of Prune1 loss-of-function by generating Prune1−/− mice as shown in Supplementary Material, Figure S6A and B. Here, homologous recombination was employed to replace exon 2 with a lacZ reporter cassette. A stable transcript derived from this allele predicts a non-functional Prune1 protein missing amino acids 15-454. Importantly, this mutant allele provides for a lacZ reporter, enabling spatio-temporal localization of Prune1 expression. Prune1+/− animals (both sexes) are viable and appeared normal as compared with wild-type littermates, with no differences in body weight, body composition, serum chemistry and hematology (Supplementary Material, Table S4). LacZ expression profiling at E12.5 demonstrated widespread expression across multiple tissues including the brain (Supplementary Material, Fig. S6B). In adult Prune1+/− mice (postnatal day 56), strong CNS expression was observed in the
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mutants. First, revealed significant defects in vessel morphology in Prune1 employing endothelial-specific Pecam 1 immunochemistry Prune1 that data/genes/MGI:1925152). These rodent studies demonstrate Phenotyping Consortium (https://www.mousephenotype.org/tent with the phenotype reported by the International Mouse Fig. 5A and B). The observed mid-gestational lethality is consis-

ted cardiac hypoplasia were fully penetrant in all

Material, Fig. S7B). Second, mutant embryos also show severe
defects in the formation and remodeling of the cephalic vascular
plexus, most strikingly revealed in 3D in Optical Projection
Tomography (OPT). In addition, anomalies to the limb bud
vasculature, dorsal vascular plexus (along the spinal cord)
and marked cardiac hypoplasia were fully penetrant in all
observed Prune1−/− embryos (Supplementary Material, Fig. S7A,
Fig. 5A and B). The observed mid-gestational lethality is consist-
tent with the phenotype reported by the International Mouse
Phenotyping Consortium (https://www.mousephenotype.org/
data/genes/MGI:1925152). These rodent studies demonstrate
that Prune1 is essential for mouse embryonic development. Fur-
thermore, if PRUNE1 homozygous loss of function is similarly
lethal in humans, these results suggest that NMIHBA-causing
PRUNE1 variants are likely hypomorphic.

Discussion

Since our initial report of PRUNE1 as a novel candidate gene
for abnormal neurodevelopment and brain malformation, 37
NMIHBA patients have been reported to date by us and others
(8−11,13−15,18,19). Detailed clinical and literature review of these
reported NMIHBA cases allows for a more comprehensive view
of this novel neurodevelopmental disorder (Table 1, Fig. 1). The
main features defining the disorder, namely profound DD, ID
and brain malformations, remain consistent; however, addi-
tional clinical features such as optic nerve atrophy, gastroin-
testinal problems and peripheral nervous system involvement
have emerged with additional reports of patients with NMIHBA.
Subsequent reporting of patients with novel genetic disorders
after the initial report enables a better understanding of the
phenotypic spectrum and often helps in informing newly diag-
nosed patients and their caregivers of the natural history of
disease.

The worldwide distribution of reported patients and disease-
associated variants conforms with the clan genomics hypothesis
where rare alleles of high effect arising in recent ancestors
are more likely to contribute to disease burden versus com-
mon variants of small effect in the population (20). These rare
alleles are more likely to come together in homozygosity in
certain populations due to genetic mechanisms such as drift in
founder populations or inbreeding in consanguineous popula-
tions. For example, the splicing variant allele c.521-2A>G has
now been reported multiple times as a founder allele in the
Cree population from Canada (14,21). Similarly, the D30N allele,
so far reported in Saudi and Omani individuals (8,9), appears
to be a founder allele in Middle Eastern populations, whereas
other pathogenic rare alleles have been reported once so far
in single families from different populations around the world.
Interestingly, the most common pathogenic allele reported in
NMIHBA patients is the D106N variant, observed in individuals
from diverse ethnic backgrounds from Turkey, Italy, Lebanon, Sri
Lanka and Japan. While this variant appears to be a founder
allele in the Turkish population, the diversity of populations
where it has been observed poses the possibility that the D106
residue is a recurrent mutational site and that the allele has arisen multiple times in different population haplotypes.

Furthermore, in this study, we investigated the molecular etiology underlying PRUNE1 dysfunction in NMIHBA. Based on our in vitro and in vivo data, we conclude that the molecular pathomechanism of this rare genetic disorder is reduced or altered function of PRUNE1 due to hypomorphic alleles. The majority of disease-associated alleles including the splice site variant within intron 4 (c.521−2A>G: IVS4−2A>G) cluster in the catalytic DHH domain of PRUNE1, underscoring the critical role of this conserved domain in NMIHBA pathogenesis. Using in silico and biochemical approaches, we explored the functional effects of four reported pathogenic PRUNE1 variants: p.D30N, p.D106N, p.R128Q and p.G174∗ on PRUNE1 function and examined the potential developmental and organisinal functional consequences of homozygous Prune1 ablation.

Protein homology modeling using the S. cerevisiae orthologue, PFX1 demonstrated that the D30, D106 and R128 residues fall within a metal-ion and phosphate binding interface representing the active-site for phosphate hydrolysis. Disruption of these charged residues within the active-site is predicted to impair metal coordination (D30N and D106N) and substrate binding (R128Q). Overexpression of human PRUNE1 cDNA harboring NMIHBA pathogenic variant alleles in HEK293 cells demonstrated a complete loss of protein product for the D30N mutant and a dramatic reduction in the D106N protein. Both isoforms were rescued upon inhibition of the proteasome degradation pathway, suggesting that the loss of metal-binding residues destabilizes local protein structure resulting in protein degradation. In contrast, the R128Q variant was stably expressed at levels comparable to wild-type protein. Patient derived fibroblasts further confirmed these findings, implicating the metal coordinating D106 residue to be critical for maintaining active-site structure and protein stability. It has been proposed that the R128 (H149 in S.-PPX1) along with the bincus aromatic metal cluster (M1 and M2 in Fig. 2A) coordinate binding of one of the phosphate moieties (Pr; Fig. 2A) of the substrate, rendering the P-O bond more susceptible to nucleophilic attack. This model would suggest impaired polyphosphate processivity by the R128Q mutant. Consistent with such a model, in vitro short-chain exopolyphosphatase assays using recombinant proteins derived from mammalian or E. coli overexpression systems demonstrated the loss of exopolyphosphatase activity in R128Q and D106N mutants as compared with the wild-type. Kinetic parameters for hydrolysis of short-chain polyphosphates P4 and P1 (in the presence of 2 mM Mg2+) by wild-type PRUNE1 in our assays were comparable to those reported by Tammenkoski et al. (17). Our data related to the R128Q mutant in conjunction with enhanced kinetics of P1 hydrolysis by R128H mutant (where the positive charge is retained) demonstrated by Tammenkoski et al., further reinforce a model wherein binding of the metal ion at the active site accelerates substrate binding, and the bound substrate in turn enhances the enzyme affinity for the metal ion (17). Therefore, mutations that disrupt metal coordinating residues or substrate binding residues may result in the loss of enzymatic function. Furthermore, in line with previous reports, we demonstrate that wild-type PRUNE1 has no detectable cyclic nucleotide (cAMP or cGMP) phosphodiesterase activity or long-chain exopolyphosphatase activity (17). Taken together, these studies suggest the loss of short-chain exopolyphosphatase activity as a potential mediator of NMIHBA pathophysiology. In light of our biochemical observations on disease associated mutant proteins, it is intriguing to note that Zollo et al. (8) ascribe enhanced short-chain exopolyphosphate activity (based on P1 hydrolysis) for two NMIHBA variants they characterized including the D30N mutant which is demonstrably a loss of function variant in our analyses. Furthermore, their reported $K_{cat}/K_m$ ratio for $P_4$ hydrolysis by wild-type PRUNE1 (0.014 μM⁻¹ s⁻¹) is an order of magnitude different than those observed in the present study (0.26 μM⁻¹ s⁻¹) and reported by Tammenkoski et al. (0.18 μM⁻¹ s⁻¹) (8,17). These discrepant results may be due to differences in cofactor concentration (10 mM Mg2⁺ as opposed to 2 mM Mg2⁺ in our assays and Tammenkoski et al.), substrate purity or other confounding factors specific to the experimental methods used.

At the time of our initial discovery of PRUNE1 as the disease causing gene, no mouse or other model organism data were available to provide further insights into the biological role of PRUNE1 in neurodevelopment and disease pathophysiology. Conservation at the protein level and similar developmental expression suggested preserved function between the mouse and human orthologues. Based on functional analyses of disease causing human alleles, we engineered a mouse model of NMIHBA by knocking out the murine Prune1 gene. Since the original publication of PRUNE1 as a novel disease gene in Karaca et al. (9), a Prune1-null mouse was developed and phenotyped by the International Mouse Phenotyping Consortium (IMPC). (http://www.mousephenotype.org/data generar CV/1925152). Consistent with the IMPC data, we observed fully penetrant embryonic lethality in the null mouse prior to E12.5. Our in depth developmental phenotyping of the Prune1-null embryos demonstrated profound vascular defects manifested by poorly branched vessels within the yolk sac, cardiac hypoplasia and a disrupted cephalic vascular plexus resulting in embryonic lethality between E9 and E10. We were intrigued by the profound cardiovascular defects in the Prune1-null embryos, given the reported absence of such manifestations in NMIHBA patients. Interestingly, we observed robust LacZ reporter expression in embryonic heart (E12.5, Supplementary Material, Fig. S6A), and robust cardiac expression in both adult mouse (median TPM: 13.34; n=6 samples) and human (median TPM: 16.39; n=432 samples) based on in-house mouse transcriptomics and publicly available data from the Genotype-Tissue Expression (GTEx) project portal, respectively. Unfortunately, our current analyses using a germline loss of function model do not address if the cardiovascular anomalies result in lethality. Moreover, it is unclear if these represent cell-autonomous defects or a consequence of altered hemodynamics secondary to impaired vasculogenesis within the yolk sac. Conditional ablation of Prune1 in these tissues would help elucidate this further.

LacZ reporter expression in heterozygous mice demonstrates broad diffuse Prune1 expression across multiple tissues and cell lineages at E12.5 (Supplementary Material, Fig. S6B). Consistently, Prune1 expression in adult mice is observed across multiple tissues with strong expression within the CNS (Supplementary Material, Fig. S6D). Despite this widespread expression profile, the most overt phenotypes arising from loss of Prune1 are vascular deficits most prominent in the cephalic vascular plexus possibly resulting from a local deficit in angiogenic cues or due to altered hemodynamics downstream of defective vascular development. Further analyses are required to discriminate the effect of hemodynamic defects versus the signaling perturbations downstream of Prune1 loss of function, and more importantly as to why the cephalic vessels are most affected.

It is intriguing as to why homozygous deletion results in embryonic lethality in mouse, while probands in this report and others survive past gestation. Whereas two patients have been reported to be homozygous for splice-site and frameshift
variants in PRUNE1 (11,15), comprehensive analyses of protein-coding variation in PRUNE1 across several large-scale human genetic variation databases including ExAC/gnomAD and our internal (DiscoEHR) database identified no homozygous carriers of predicted loss-of-function variants despite the observation that heterozygous loss-of-function variants are well tolerated (Supplementary Material, Fig. S8) (22,23). Residual enzymatic function of the hypomorphic missense alleles beyond the range of detection of our in vitro assays, alternative non-enzymatic developmental function (in mouse) or potential compensation by other phosphatases during neurodevelopment (in human) could potentially explain the observed species-specific difference in phenotype. Future analyses using knock-in mouse models or gyrrified mammals such as ferrets along with human iPSC-derived brain organoids may help discern the allelic architecture of NMIHBA and allow for molecular interrogation of PRUNE1 function in mammalian neurodevelopment.

Polyphosphate has been implicated in diverse physiological processes in higher eukaryotes, including apoptosis, mTOR activation and neuronal signaling (24–26). This pleiotropy is consistent with the essential role of inorganic diphasosphate (PPi) for cellular metabolism across taxa and the potential role of polyphosphate metabolizing enzymes (including PRUNE1) in maintaining phosphate homeostasis. However, mechanistic understanding of how polyphosphate metabolism influences cellular metabolism and in turn organogenesis remains obscure. More recently, Cremers et al. proposed polyphosphate as a conserved modifier of amyloidogenic processes capable of exopolypophosphatase activity may facilitate the exploration of processes. The fact that NMIHBA-causing PRUNE1 variants lack exopolypophosphatase activity may facilitate the exploration of therapeutic avenues that would allow for preemptive rather than symptomatic intervention of NMIHBA in the future.

Materials and Methods

Cell culture

HEK293 and human fibroblast culture medium consisted of DMEM (Gibco; 11995-065) supplemented with 10% fetal bovine serum (Gibco; 16000-038), 1% non-essential amino acids (Gibco; 11140-050) and 1% Penicillin–Streptomycin (Gibco; 15140-122). Cells were dissociated using 0.05% Trypsin-EDTA (Gibco; 25300-054). HEK293 cells were seeded in 96-well plates at a density of 20,000 cells/well and in other formats (12- and 24-well) at a density of 20,000 cells/well and in further plates in the range of detection of our in vitro transfection assay reagents (Promega; E1290) in CO2-independent medium (Gibco; 18 045-088). Analyses of parallel plates demonstrated no differences in cell viability (evaluated using MTT assay, ThermoFisher; V13154) and total protein concentration (evaluated using BCA) across different experimental groups. Upon equilibration at room temperature for 2 h, basal luminescence was added. Whole cell lysates were collected 0, 6 and 24 h of treatment. Total protein concentration was quantified and alternative non-enzymatic developmental function (in mouse) or potential compensation by other phosphatases during neurodevelopment (in human) could potentially explain the observed species-specific difference in phenotype. Future analyses using knock-in mouse models or gyrrified mammals such as ferrets along with human iPSC-derived brain organoids may help discern the allelic architecture of NMIHBA and allow for molecular interrogation of PRUNE1 function in mammalian neurodevelopment.

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**Immunoblotting and protein stability analyses**

N-terminal HA-tagged PRUNE1 overexpression constructs were transfected into PRUNE1 non-expressing HEK293 cells using X-tremeGENE HP DNA (Roche; 06366236001) transfection reagent. As a transfection control, CMV-driven eGFP expression construct was co-transfected at a 1:19 ratio. Two days later, whole cell lysate was collected, and total protein concentration was quantified using bicinchoninic acid (BCA) assay (ThermoFisher; 23225). PRUNE1 levels in overexpressing HEK293 and in human fibroblasts were analyzed by immunoblotting using anti-PRUNE1 (Origene; TA344725) and/or anti-HA (Abcam; 18 181) antibodies at 1:1000 dilution, using GAPDH (CST; 3683S) as a loading control.

Stability of the various PRUNE1 mutesins was evaluated by overexpressing N-terminal HA-tagged PRUNE1 constructs in HEK293 cells. After 48 h, medium containing either cycloheximide (200uM, Sigma 1810) and/or MG132 (15uM, Sigma; M8699) was added. Whole cell lysates were collected 0, 6 and 24 h of cycloheximide treatment. Whole cell lysate from cells treated with both cycloheximide and MG132 were collected after 24 h of treatment. Total protein concentration was quantified and immunoblotted using Li-COR Odyssey CLx imaging system. In complementary immunoblotting experiments, endogenous PRUNE1 levels in Sanger verified (Supplementary Material, Fig. S4b) patient-derived fibroblasts BAB3500 (PRUNE1R128Q/G174K) and UDP760 (PRUNE1D106Q/G174K) was compared with control neonatal dermal fibroblasts (ATCC, PCS-201-010).

**GloSensor cAMP and cGMP assays**

HEK293 cells were plated at a density of 20,000 cells/well in poly-D-lysine-coated 96-well plates (Corning; 354 651). The following day cells were co-transfected with 12.5 ng pcGloSensor-22F or pgloSensor-42F and 10 ng of PRUNE1 overexpression constructs (D30N, D106N, R128Q and 4DD) or control PS100013, PDE4A or PDE5A vectors per well. Total DNA was maintained at 100 ng per well. Two days later, cells were changed into GloSensor equilibration medium (10% fetal bovine serum and 2% GloSensor assay reagent (Promega; E1290) in CO2-independent medium (Gibco; 18 045-088). Analyses of parallel plates demonstrated no differences in cell viability (evaluated using MTT assay, ThermoFisher; V13154) and total protein concentration (evaluated using BCA) across different experimental groups. Upon equilibration at room temperature for 2 h, basal luminescence was acquired using a PerkinElmer 2104 multimode plate reader. Forskolin (Sigma; F3917) or sodium nitroprusside dihydrate (Millipore; S67538) was added to the medium to a final concentration of 10 or 50 μM. cAMP or cGMP phosphodiesterase activity was evaluated by measuring luminescence recorded at 1-min intervals up to 45 or 60 min, respectively.

**Exopolypophosphatase assay**

Exopolypophosphatase activity of PRUNE1 against short-chain polyphosphate (PolyP) substrates, sodium tripolyphosphate (P3, Sigma; 7727-67-5) was determined by a fixed time assay using BIOMOL GREEN phosphate detection kit (Enzo Lifesciences; BML-AK111). Reaction was performed 15–120 s in 50 μL of 100 mM Tris/HCl, 50 μM EGTA, 2 mM MgCl₂, pH 7.2 at substrate concentrations ranging from 0.5 to 100 μM and 1 to 50 μM for P3 and P4, respectively. Reactions were terminated by addition of 100 μL of BIOMOL GREEN reagent; absorbance at 620 nm was measured following a 30-min incubation at 25°C. Kinetic parameters were assessed by plotting Lineweaver–Burk plots using GraphPad PRISM 7 software.

Exopolypophosphatase activity of wild-type PRUNE1 on medium and long-chain PolyP substrates (consisting of 45, 65 and 700 orthophosphate monomers, Sigma S4379, Sigma; S6253, Kerafast; EU002) was assessed by comparing to S. cerevisiae PPX1 (Bioorby; ORB419100). About 50 nM of PRUNE1 or 100 nM PX1 were incubated with polyphosphates at a final concentration of 2 mM (in terms of phosphate residues) in 100 mM Tris–HCl, pH 7.2, 50 μM EGTA, 2 mM MgCl₂ or 50 mM HEPES/KOH, pH 8.0,
1 mM MgCl₂, 125 mM KCl, respectively. Reactions were allowed to proceed at 37°C for 150 min. Reactions were terminated at 15, 30, 60 and 150 min. Negative control reactions, containing no enzyme in both buffer systems, were also prepared as above.

Polyphosphate hydrolysis was tracked by running fractions of the reactions on TBE gels and staining polyphosphates present using toluidine blue O, as previously described (28). Briefly, reactions were diluted with Novex Hi-Density TBE Sample Buffer (Thermo Fisher; LC6678), and run on precast TBE gels, either 20% (EC63155; Novex) for P45 and P65 reactions or 6% (EC62655; Novex) for P70 reactions. Following electrophoresis, the gels were stained in 0.05% toluidine blue O in fixative solution (an aqueous solution of 25% methanol and 5% glycerol) for 15 min, with agitation, followed by 3 h of washing in fixative solution and imaging. In concurrent analyses, released phosphate was quantified using BIOMOL GREEN phosphate detection kit as described above. All experiments were repeated at least three times with a minimum of four technical replicates per sample. Data were analyzed using PRISM 7 (GraphPad) software.

**CD and in silico modeling of PRUNE1 mutations**

PRUNE1 wild-type, D106N and R128Q muteins were analyzed by CD spectroscopy by collecting far-UV (-250 nm) and near-UV (260–320 nm) CD spectra. Prior to CD measurements, all samples were dialyzed twice in a base buffer, 20 mM Tris–HCl, pH 7.8 to eliminate DTT that could potentially interfere with far-UV CD measurements. Following overnight dialysis, concentrations were determined by A280 measurements, using a theoretical extinction coefficient based on amino acid sequence of each protein. Near-UV CD measurements were obtained at 0.75, 0.7 and 0.45 mg/ml for wild-type, D106N and R128Q samples, respectively. All samples were further diluted (2-fold) using 0.02 mM filtered dialysate for far-UV CD measurements. Far-UV CD spectra were evaluated at 0.38, 0.35 and 0.21 mg/ml for wild-type, D106N and R128Q samples, respectively. CD measurements were performed on a Jasco 1500 spectropolarimeter at 25°C using a 1 cm cell (Jasco; Type J3) for near-UV CD and 0.1 cm cell (Jasco; Type J10559) for far-UV CD. The raw data were baseline subtracted and converted to units of molar ellipticity to correct for protein concentration differences. Degree of spectral difference between wild-type and D106N or R128Q muteins was assessed using a spectral classification technique (TQ Analyst®). The reported TQ Analyst® similarity match score indicates how closely the spectrum of each mutant matches that of the wild-type protein. Highly comparable far-UV CD spectra result in scores >92.6, whereas highly comparable near-UV CD give TQ similarity match scores >98.

The RCSB Protein Data Bank was searched for a structure with high sequence homology to human PRUNE1. Saccharomyces cerevisiae cytosolic exopolysphatase (PPX1) (PDB ID: 2QBT), with a Blast e-value of 2.3E−19 and 25% sequence identity (45% similarity), was the best hit. Since the active site residues were conserved, we did not attempt to generate a PRUNE1 homology model. PPX1 structure (shown in Fig. 2A) was rendered by exchanging orthologous active site residues: Asp41 to Asn, Asp127 to Asn, and H149 to Gln. Three bound phosphate moieties P₁, P₃s and P₃, shown in ball-and-stick, and the two catalytic metal ions as M1 and M2 as pink spheres.

**Animal studies**

Prune1−/− mice were generated by replacing exon 2 (ENS-MUSE00001210512) of the Prune1 gene with a β-galactosidase (lacZ) reporter cassette using VelociGene technology (29). Mice carrying the deletion were genotyped by a loss of allele assay as described previously (30). Targeted, cassette-deleted heterozygous mice were bred to obtain desired genotypes. Homozygous and heterozygous knockout mice cohorts derived from F1 breeding were used for phenotypic evaluation. All mice used in this study were in a 75% C57Bl/6NTac, 25% 129S6/SvEvTac genetic background, and housed in a pathogen free environment. Autoclaved water and sterile mouse chow were provided ad libitum.

**Ethics statement**

All experimental protocols in mice, including anesthesia, imaging procedures and tissue sampling procedures performed in this study, were approved by the Regeneron Pharmaceuticals Inc. Institutional Animal Care and Use Committee (IACUC) under protocol number 430 and the US Animal Welfare Act.

**Anti-PECAM1 staining and imaging**

Embryo processing, immunostaining and imaging were performed as described previously (31). Briefly, embryos were collected at embryonic day (E) 9.5 and fixed for 3 h in 4% paraformaldehyde at 4°C. Prior to staining, endogenous peroxidase activity was quenched with 3% H₂O₂. Non-specific antibody binding was blocked by preincubating embryos in 10% normal goat serum (Vector Labs; S-1000) and 0.5% Bovine Serum Albumin (Fisher Scientific; BP1600). Embryos were stained overnight at 4°C with rat anti-PECAM1 antibody at 1:300 dilution (Pharmingen; 553370). After primary antibody incubation, embryos were incubated overnight with donkey anti-rat HRP secondary antibody at 1:1000 dilution (Jackson Immunoresearch; 712-036-153) followed by incubation with 488-tyramide reagent at 1:25 dilution made using Dylight 488 NHS ester (Thermo Scientific; 46402). Images were acquired through optical sectioning with an ApoTome.2 on a Zeiss Axiolum8. Extended Depth of Focus images were generated by Zen software. Imaging was performed using Leica MZFLIII stereomicroscope quipped with a 0.5x objective and 1.0x camera lens, with a zoom between 4.0x and 6.3x. Alternatively, stained embryos were imaged using OPT as described previously (31). For OPT, samples were embedded in low melting agarose and subsequently cleared using a mixture of benzyl alcohol and benzyl benzoate (1:2 ratio). Sample was rotated stepwise (with a 0.9° step size) for one revolution, and a GFP1 filtered autofluorescence view and a Cy3 fluorescence view (captured using a Cy3 filter) were acquired using Retiga Exi CCD camera. Reconstruction of all image slices yielded a 3D volumetric rendering of the specimen as described previously (32).

**Statistical analysis**

Statistical analyses were conducted using Prism 7 (GraphPad) software. Unpaired Student’s t-test was used unless indicated otherwise. Statistical significance reported when P < 0.05. Where appropriate sample sizes, the statistical test used and significance are indicated in each figure legend.

**Supplementary Material**

Supplementary Material is available at HMG online.
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Conflict of Interest statement. H.N., C.G.-J., S.M.C., S.R., S.N., D.D., M.C.F., P.S., M.P., Y.T., M.G.D., R.A.D., C.J.S., B.Z., N.W.C. and A.N.E. are full-time employees of the Regeneron Genetics Center or Regeneron Pharmaceuticals Inc. and receive stock options/restricted stock as part of compensation. J.R.L. is a paid consultant for Regeneron Pharmaceuticals, Inc., has stock ownership in 23andMe and is a co-inventor on multiple USA and European patents related to molecular diagnostics for inherited neuropathies, eye diseases and bacterial genomic fingerprinting. The remaining authors declare no conflict of interest.

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