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Mutation update: Variants of the *ENPP1* gene in pathologic calcification, hypophosphatemic rickets, and cutaneous hypopigmentation with punctate keratoderma

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Abstract

ENPP1 encodes ENPP1, an ectonucleotidase catalyzing hydrolysis of ATP to AMP and inorganic pyrophosphate (PPi), an endogenous plasma protein physiologically preventing ectopic calcification of connective tissues. Mutations in *ENPP1* have been reported in associated with a range of human genetic diseases. In this mutation update, we provide a comprehensive review of all the pathogenic variants, likely pathogenic variants, and variants of unknown significance in *ENPP1* associated with three autosomal recessive disorders – generalized arterial calcification of infancy (GACI), autosomal recessive hypophosphatemic rickets type 2 (ARHR2), and pseudoxanthoma elasticum (PXE), as well as with a predominantly autosomal dominant Cole disease. The classification of all variants is determined using the latest ACMG guidelines. A total of 140 *ENPP1* variants were curated consisting of 133 previously reported variants and 7 novel variants, with missense variants being the most prevalent (70.0%, 98/140). While the pathogenic variants are widely scattered in *ENPP1* of patients without apparent genotype-phenotype correlation, eight out of nine variants associated with Cole disease are confined to the somatomedin-B-like (SMB) domains critical for homo-dimerization of the ENPP1 protein.

Keywords

Cole disease; ENPP1; generalized arterial calcification of infancy; hypophosphatemic rickets; inorganic pyrophosphate; pathologic calcification; pseudoxanthoma elasticum

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1 BACKGROUND

The ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) belongs to a family of ecto-enzymes consisting of seven single-pass transmembrane proteins implicated in key biological and pathophysiological processes (Borza et al., 2021). ENPP1, the founding member of the family, is widely expressed and is the best characterized and only member of the family associated with several genetic diseases. It was discovered more than 50 years ago as a plasma cell membrane glycoprotein (PC-1) (Takahashi et al., 1970; van Driel & Goding, 1987) but was subsequently classified as NPP1 based on its nucleotide pyrophosphatase and phosphodiesterase (NPP) enzymatic activities (Bollen et al., 2000), and now termed ENPP1 to indicate that its active site is extracellular. The ENPP1 protein consists of an N-terminal cytoplasmic domain (CD), a transmembrane domain (TM), two tandem somatomedin-B-like domains (SMB1-2), followed by the phosphodiesterase catalytic domain (PCD), L1 and L2 linkers, and a C-terminal nuclease-like domain (NLD) (Fig. 1) (Kato et al., 2012). The SMB1-2 domains are tightly compacted by disulfide bridges, which facilitate homo-dimerization of the ENPP1 protein (Gijsbers et al., 2003; Jansen et al., 2012). The PCD domain confers the NPP activity with the catalytic site characterized by two zinc ions. The NLD domain, similar to bacterial non-specific endonucleases consisting of a six-stranded antiparallel β-sheet surrounded by eight α-helices, is catalytically dead, but it interacts with the PCD domain contributing to the stability and enzymatic activity of ENPP1 (Koyama et al., 2012). The L1 and L2 linkers maintain tight interdomain interactions (Jansen et al., 2007). ENPP1 hydrolyses extracellular adenosine triphosphate (ATP) to generate adenosine monophosphate (AMP) and inorganic pyrophosphate (PPi), the latter being a potent physiological inhibitor of mineralization. This activity endows ENPP1 with particular pathophysiological relevance to vascular calcification and skeletal mineralization, and it is the only ENPP family member to date with disease-associated mutations.

The *ENPP1* gene maps to chromosome 6q23.2 and has one transcript, NM_006208.3. It comprises ~87.1 kb of genomic DNA and is composed of 25 exons encoding 925 amino acids. ENPP1 contributes to various human genetic diseases encompassing vascular calcification, hypophosphatemic rickets, and aberrant skin pigmentation with keratoderma. Variants in *ENPP1* were initially associated with generalized arterial calcification of infancy (GACI) and autosomal recessive hypophosphatemic rickets type 2 (ARHR2), and subsequently with a subset of patients with pseudoxanthoma elasticum (PXE); these three autosomal recessive diseases are PPi deficiency syndromes (Ralph et al., 2022a). Variants in *ENPP1* are also associated with autosomal dominant and recessive Cole disease characterized by cutaneous hypopigmentation with punctate keratoderma. To date, most available research on ENPP1 has focused on vascular calcification and skeletal mineralization. This overview highlights known and novel *ENPP1* variants and focuses on animal models and pathomechanisms underlying the distinct vascular, bone, and skin phenotypes.

2 VARIANTS

All *ENPP1* variants are described according to current Human Genome Variation Society mutation nomenclature guidelines based on Genbank accession number NM_006208.3. Previously reported variants and novel variants are interpreted and classified using the latest American College of Medical Genetics and Genomics (ACMG)/Association of Molecular Pathology classification standards and guidelines (Richards et al., 2015).

The number of unique *ENPP1* variants has expanded tremendously since this gene was recognized for its role in regulating soft tissue calcification in a *ttw* mouse model of GACI, and as the gene harboring variants in patients with ectopic calcification of the spine (Nakamura et al., 1999; Okawa et al., 1998). We report a total of 133 unique variants retrieved from the peer-reviewed literature, the Human Gene Mutation Database (HGMD), the Leiden Open Variation Database (LOVD), ClinVar, and Mastermind (Table 1). We added 7 novel variants from our Molecular Diagnostics Center at Thomas Jefferson University, from the Children's Hospital of Philadelphia, and from two commercial genetic companies, GeneDx and Prevention Genetics, leading to identification of a total of 140 distinct variants in *ENPP1* (Table 1). Among these variants, missense variants are the most frequent (70.0%, 98/140), followed by putative splicing variants (12.1%, 17/140), nonsense (8.6%, 12/140), small indel (7.1%, 10/140), large deletion (1.4%, 2/140), and variants located in the promoter region (0.7%, 1/140). VUS (variant of unknown significance), LP (likely pathogenic), and P (pathogenic) variants account for 67.1% (94/140), 24.3% (34/140), and 8.6% (12/140), respectively. Most of the variants are clustered in the PCD and NLD domains (Fig. 1), accounting for 49.3% and 27.1% of all variants, respectively. In addition, we describe an additional 55 variants (VUS, LP, and P) found in either apparently healthy individuals or in individuals without any clinical information (Supp. Table S1).

Variant interpretation remains challenging due to the highly polymorphic nature of *ENPP1* and limited available evidence for pathogenicity for most types of variants. In general, truncating variants are classified as LP or P, and missense variants as VUS unless additional evidence from segregation and/or functional experiments is available. In recent years, the variant databases in large population genetic studies have provided powerful tools that aid in variant interpretation by considering the frequency of the alleles of interest. As such, 20 variants, previously reported as pathogenic in the literature (Supp. Table S2), have now been found to be common in the general population and unlikely to be causative of a rare genetic disease, as their frequencies are greater than the recommended *ENPP1* gene-specific minor allele frequency threshold of 0.1% (https://franklin.genoox.com/clinical-db/home). Consequently, ACMG has now reassigned them as B (benign) or LB (likely benign) variants (Supp. Table S2).

2.1 Generalized arterial calcification of infancy (GACI)

Loss-of-function *ENPP1* variants were first described in 1999 in patients with ossification of the posterior longitudinal ligament of the spine, followed by a report in patients with GACI in 2003 (Rutsch et al., 2003). GACI (MIM# 208000) is an autosomal recessive, life-threatening disorder of early-onset vascular calcification that is often diagnosed by prenatal ultrasound, revealing calcium deposits in the fetal heart and arteries

(Rutsch et al., 2003). GACI is an ultra-rare disease with an estimated prevalence of 1:200,000 (Ferreira, Hackbarth, et al., 2021). Affected individuals with GACI manifest cardiovascular calcification and intimal proliferation, causing arterial stenosis, that when severe is associated with cardiovascular collapse and early death with mortality of ~55% *in utero* or during the first six months of life (Ferreira, Kintzinger, et al., 2021). Extravascular calcification also occurs commonly in the joints, spine, and visceral tissues of GACI survivors, leading to skeletal, renal, gastrointestinal, and pulmonary complications. Survivors typically develop hypophosphatemic rickets or are predicted to develop it by 13.6 years of age (Ferreira, Hackbarth, et al., 2021). GACI-associated *ENPP1* variants are widely scattered throughout the ENPP1 protein, with 62.7% of all variants located in the PCD domain (Fig. 1).

2.2 Hypophosphatemic rickets type 2 (ARHR2)

ARHR2 (MIM# 613312), an ultra-rare form of autosomal recessive hypophosphatemic rickets, is caused by inactivating variants in *ENPP1*. GACI and ARHR2 were initially recognized as two distinct clinical diagnoses based on genetic changes in *ENPP1*. Later, there were reports in individuals with ARHR2 who had arterial stenosis, intimal proliferation, and increased carotid intima-media thickness despite absence of vascular calcification, cardiovascular compromise, or clinical GACI (Hoppner et al., 2021). In addition, a substantial proportion of GACI patients who survive the first critical period of six months of life experience some spontaneous resolution of arterial calcification but nevertheless progress to ARHR2 later in life (Ferreira, Kintzinger, et al., 2021). Regardless of the diagnosis of ARHR2 or GACI, the clinical manifestations of rickets include short stature and bone deformities, hypophosphatemia, elevated alkaline phosphatase, and elevated or high-normal fibroblast growth factor 23 (FGF23). Based on these observations, ARHR2 does not seem to be a condition distinct from GACI but rather represents part of the clinical spectrum of *ENPP1* deficiency (Hoppner et al., 2021). These observations support the concept that *ENPP1* variants can cause GACI and/or ARHR2 (Table 1, Fig. 1).

2.3 Pseudoxanthoma elasticum (PXE)

Variants in *ENPP1* have also been associated with PXE, which in most patients is due to biallelic loss-of-function variants in the *ABCC6* gene (Luo et al., 2021). PXE (MIM# 264800) is an autosomal recessive disorder characterized by late-onset, yet progressive ectopic calcification in the skin, eyes, and arterial blood vessels (Neldner, 1988). The estimated prevalence of PXE is 1: 50,000. In contrast to GACI, the clinical manifestations of PXE are usually not recognized until adolescence or early adulthood. Some GACI patients, initially diagnosed prenatally or neonatally with vascular calcification, were reported to develop skin lesions and/or angioid streaks, similar to those in PXE (Ferreira, Hackbarth, et al., 2021; Ferreira, Kintzinger, et al., 2021). Subsequently, inactivating variants in *ENPP1* were associated with classic PXE in adult patients (Ralph et., 2022b; Jin et al., 2015). The same *ENPP1* variant can cause both PXE and GACI, highlighting the genotypic and phenotypic overlap between these two disorders (Table 1). In addition, uniparental disomy inheritance of the c.1530G>C (p.Leu510Phe) variant in *ENPP1* was reported, for the first time, in a patient with PXE (Ralph et al., 2022b).

2.4 Cole disease

Cole disease (MIM# 615522) is a rare autosomal dominant disorder with coexistence of patchy hypopigmentation, pigmentation with punctate keratoderma, and uncommonly, cutaneous calcification (Schmieder et al., 2011). Cole disease has been identified in less than 40 individuals worldwide. The association of dominant *ENPP1* variants with Cole disease was first reported in 2013 (Eytan et al., 2013). Interestingly, some family members of Cole disease patients carrying the pathogenic *ENPP1* variants present no clinical features of Cole disease (Schlipf et al., 2016). This suggests that *ENPP1* variants alone are not always sufficient for the manifestation of Cole disease. A recent study reported that a novel homozygous *ENPP1* variant, c.358T>C (p.Cys120Arg), was associated with an autosomal recessive and more extensive form of Cole disease (Chourabi et al., 2018). A total of nine *ENPP1* variants are currently reported for Cole disease (Table 1, Fig. 1). Except for c.1391A>G (p.Asp464Gly), the other eight variants are cysteine amino acid substitutions within the somatomedin-B-like (SMB) domains critical for homo-dimerization of the ENPP1 protein.

3 BIOLOGICAL RELEVANCE

3.1 Pathomechanisms of *ENPP1* variants in GACI

ENPP1 protein was first known for regulating pathologic calcification through the hydrolysis of extracellular ATP to PPi and AMP. PPi was known since the early 1960s as a potent physiologic “water-softener” by preventing the formation and growth of calcium hydroxyapatite crystals in soft connective tissues (Orriss et al., 2016). Before ENPP1 variants were identified in patients with GACI, a “tip toe walking” (*ttw*) mouse with spontaneous, homozygous *Enpp1* variant was identified, placing ENPP1 at a pivotal role in the regulation of connective tissue calcification (Okawa et al., 1998). The *ttw* mice had features similar to those in children with GACI, such as periarticular and vascular calcifications, decreased or barely detectable concentrations of plasma PPi, and reduced bone mineral density. While the marked reduction of plasma PPi causes vascular calcification, the arterial stenosis in GACI appear to be the result of intimal proliferation possibly resulting from a lack of AMP generation (Nitschke et al., 2018). Thus, vascular calcification and arterial stenosis in GACI are thought to occur due to the reduction in PPi and AMP, respectively.

Functional studies on 39 *ENPP1* variants, mostly missense, identified in patients with GACI and/or ARHR2, were performed in transfected COS-7, SaOS-2, or HEK293 cells, in comparison to a wild-type *ENPP1* cDNA (Tables 2, 3). The consequences of missense variants were manifold, and include loss-of-function through reduced protein abundance, impaired cellular localization, and reduced enzyme activity, collectively contributing to inability to generate PPi. In addition, the c.241G>T (p.Val81Leu) and c.1441C>T (p.Arg481Trp) variants, initially thought to be missense variants, were found to cause partial skipping of exon 2 and 15, respectively, presumably causing out-of-frame reading and premature termination of translation (Ralph et al., 2022b; Rutsch et al., 2003).

3.2 Pathomechanisms of *ENPP1* variants in ARHR2

In addition to GACI, *ENPP1* variants can also cause ARHR2 without apparent involvement of vascular calcification, a paradox that lacks a biochemical explanation (Levy-Litan et al., 2010; Lorenz-Depiereux et al., 2010; Saito et al., 2011). ENPP1 deficiency, regardless of the diagnosis of ARHR2 or GACI, is associated with elevated FGF23, a bone-derived hormone that regulates renal phosphate reabsorption and vitamin D metabolism. Elevated levels of FGF23 are known to result in phosphate wasting and hypophosphatemic rickets (Beck-Nielsen et al., 2019). The elevated FGF23 levels, inversely correlated with phosphate in the blood, might be an adaptive physiologic response and represent a protective response of the body to counter ectopic calcification by inducing hypophosphatemia in individuals with GACI (Hoppner et al., 2021).

3.3 Pathomechanisms of *ENPP1* variants in PXE

Some GACI survivors with *ENPP1* variants develop PXE-like skin and eye findings (Ferreira, Hackbarth, et al., 2021; Ferreira, Kintzinger, et al., 2021), whereas some adult PXE patients harboring *ENPP1* variants do not manifest with vascular phenotypes (Ralph et al., 2022b; Jin et al., 2015). The reduced plasma PPi concentrations of varying degrees are reported to correlate, in general, with the severity of pathologic calcification in several monogenic disorders of pathologic calcification, including ABCC6-deficient PXE (60-70% reduction) and ENPP1-deficient GACI (90-100% reduction) (Ralph et al., 2022a). However, a poor correlation between the severity of calcification and the amount of plasma PPi was encountered for a global *Abcc6* knockout mouse and a liver-specific *Abcc6* knockout mouse (Ziegler et al., 2017). Our patients with *ENPP1* variants, regardless of the clinical diagnosis of GACI or PXE with drastically different severity, have indistinguishable, equally low PPi plasma levels, challenging the concept that plasma PPi levels correlate with the severity of vascular calcification (Ralph et al., 2022b).

3.4 Pathomechanisms of *ENPP1* variants in Cole disease

Eight out of nine *ENPP1* variants causing Cole disease are cysteine substitutions in the SMB domains (Table 1, Fig. 1). A skin biopsy from hypopigmented areas of patients with Cole disease showed hyperkeratosis and overall reduction of melanin in melanocytes (Schlipf et al., 2016). Further analysis showed that the p.Cys120Arg variant, responsible for recessive Cole disease, retained partial ENPP1 dimer formation, while the p.Cys164Ser variant, responsible for the autosomal dominant Cole disease, severely affected dimerization (Chourabi et al., 2018). RNA sequencing analysis of patient-derived melanocytes revealed that melanocyte development and pigmentation signaling pathways were significantly altered (Chourabi et al., 2018). The resulting impairment of ENPP1's role in melanin transport and keratinocyte development leads to hypopigmentation and keratoderma.

4 ANIMAL MODELS

Several mouse models have been identified to recapitulate the features of human GACI with vascular calcification and hypophosphatemic rickets (Table 4). The first *Enpp1* mouse model was a naturally occurring mutant, "tip toe walking" mouse (*ttw*), initially identified in 1998 in the Jcl:ICR strain in Japan (Okawa et al., 1998). In addition to ossification of the

spine, the *ttw* mouse also calcifies arterial blood vessels (Dedinszki et al., 2017). This mouse harbors a stop codon variant, p.Gly568*, in the *Enpp1* gene, while a mouse with a similar phenotype, $Enpp1^{ttw\text{-}Ham}$, harbors a splice-site variant, c.259+1G>T, in *Enpp1* (Takabayashi et al., 2014). A genetically engineered knock-out mouse, $Enpp1^{tm1Gdg}$ (true null), developed on 129S1/Sv background by the targeted deletion of exon 9 of *Enpp1*, develops vascular calcification and hypo-mineralization of the long bones (Sali A, 1999). These mouse models were created before identifying *ENPP1* variants in patients with GACI. The $Enpp1^{m1Amgn}$ mutant mouse on C57BL/6J background, with a homozygous p.Cys397Ser missense variant induced by mutagenesis using N-ethyl-N-nitrosourea (ENU), was characterized by low bone mineral density, crystal-related arthropathy, and vascular calcification (Babij et al., 2009). Another *ENPP1* mutant mouse originated from C57BL/6J strain at The Jackson Laboratory as a result of ENU treatment and was found to have a missense variant, p.Val246Asp, in the *Enpp1* gene (Li et al., 2013). These mice demonstrated stiff posture, abnormalities in the front legs, and stiffening of the joints; hence, this mutation was named "*ages with stiffened joints"* (*asj*). The $Enpp1^{asj}$ mice develop systemic calcification affecting the arterial vasculature, a number of internal organs, and the dermal sheath of connective tissue capsule of vibrissae, an observation that we had previously made in *Abcc6* knock-out mice, a model for PXE (Klement et al., 2005). Furthermore, the $Enpp1^{asj\text{-}2J}$ mice were identified as part of a deviant phenotypic search in a large-scale production colony of BALB/cJ mice at The Jackson Laboratory, with phenotypes similar to those of the $Enpp1^{asj}$ mice (Li et al., 2014). These mice had a large, 40,035 bp, spontaneous deletion spanning from intron 1 to the 3'-untranslated region of the *Enpp1* gene, coupled with a 74 bp insertion. Similar to human individuals with GACI, the *Enpp1* mutant mice, regardless of the type of variants, show an abnormally low plasma concentration of PPi, and they are widely used as mouse models of GACI (Dedinszki et al., 2017; Lomashvili et al., 2014; Nitschke et al., 2018; Zhao et al., 2017). More recently, an osteoblast-specific conditional knock-out mouse was developed by crossing the exon 9 floxed *Enpp1* mice ($Enpp1^{fl/fl}$) with osteocalcin-cre mice (Roberts et al., 2021).

An *Enpp1* zebrafish (*Danio rerio*) mutant, *dragonfish* (dgf^{hu4581}), was developed to investigate the soft tissue calcification phenotype (Table 4). It was identified after ENU mutagenesis followed by phenotypic screening initially exhibiting ectopic calcification in the craniofacial and axial skeleton (Huitema et al., 2012). This mutant carries a variant in the splice acceptor site, c.993-2A>T, in intron 10 of the *Enpp1* gene, presumably causing a translational frameshift and premature translation termination. Later, this mutant was found to display canonical features of human GACI, such as calcification in the blood vasculature, skin, and eye (Apschner et al., 2014).

5 CLINICAL AND DIAGNOSTIC RELEVANCE

The association between vascular calcification and increased mortality is well recognized and is more commonly observed in elderly patients with atherosclerosis, renal failure, and diabetics. When arterial calcification is seen *in utero*, in infants and children, congenital genetic disorders should be suspected. Thus far, variants in the *ENPP1* gene have been found in approximately 75% of patients with GACI. In comparison to Sanger sequencing of *ENPP1* and gene-targeted hypophosphatemic rickets panels including *ENPP1*, exome- and

genome-wide next generation sequencing approaches are expected to provide an unbiased manner to uncover unrecognized variants in *ENPP1* as well as variants in other genes. Thus, the number of variants is expected to increase in the coming years. Identification of variants in the *ENPP1* gene can confirm the clinical diagnosis, enable carrier detection, facilitate presymptomatic mutation analysis of affected individuals, and prompt genetic counseling for family planning. Implementation of preimplantation genetic diagnosis or noninvasive prenatal genetic testing is crucial given the early onset of the manifestations in GACI during gestation.

6 GENOTYPE-PHENOTYPE CORRELATIONS

The clinical presentation of patients with GACI and/or ARHR2 ranges from extensive vascular calcification with high mortality before six months of age to hypophosphatemic rickets later in childhood or osteomalacia in adulthood without apparent vascular and multiorgan involvement. Significant clinical variability has been observed within sibling pairs in the same family and among families with identical genotypes in *ENPP1*, arguing against a distinctive genotype-phenotype correlation in GACI and/or ARHR2 (Ferreira, Hackbarth, et al., 2021). Specifically, in one family with two affected siblings with the same p.Arg481Trp and c.2312_2313del biallelic compound heterozygous variants in *ENPP1*, the older sister, presented in childhood with bone pain and deformities related to ARHR2, while her younger brother, presented at 7 weeks with severe GACI leading to cardiac arrest, necessitating resuscitation and extracorporeal membrane oxygenation. In another family with two affected siblings with the same p.Cys480Arg and p.Gly805Val biallelic variants in *ENPP1*, the brother presented with periarticular calcifications of both shoulders in the absence of vascular calcification, while his younger sister had cardiovascular calcifications diagnosed *in utero*. The substantial differences in the severity and organ involvement suggest the presence of environmental factors or epigenetic and genetic modifiers that might contribute to the varying phenotypes. In contrast to the apparent lack of clustering of variants in any specific region of the gene in GACI and/or ARHR2, seven out of eight *ENPP1* variants associated with Cole disease are confined to cysteine residues in the SMB domains that affect protein homo-dimerization.

7 FUTURE PROSPECTS

This study provides an up-to-date overview of *ENPP1* variants in genetic diseases. As missense variants account for up to 70% of all variants in *ENPP1*, and many of them are classified as VUS, their functional characterization in appropriate model systems is of paramount importance to improve pathogenicity classification. A better understanding of the molecular alterations due to *ENPP1* variants can also highlight the potential of allele-specific treatments. For example, premature termination codon (PTC) variants are amenable to PTC read-through molecules, possibly resulting in the synthesis of a full-length ENPP1 protein. Most missense variants in *ENPP1* result in subcellular mislocalization of the mutant protein, yet with the synthesis of the full-length protein with intact ATP hydrolysis activity. These missense variants may be amenable to chemical chaperone treatment to correct the trafficking defect. In addition, as *ENPP1* variants are associated with multiple

genetic diseases, multi-disciplinary clinical evaluation across different specialties is critical to establish their association with a clinical condition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

All data associated with this study are presented in the paper. All novel variants have been submitted to the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/).

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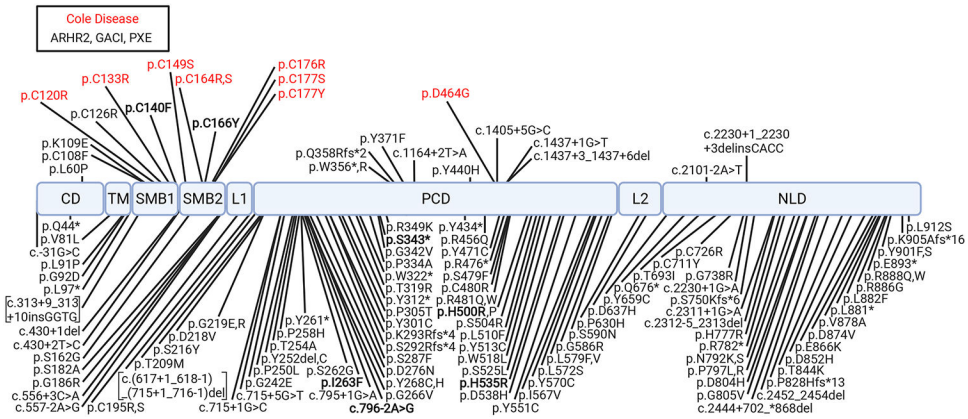

Figure 1.

Schematic representation of the ENPP1 protein domains together with positions of 140 variants identified in patients with ARHR2, Cole disease, GACI, and PXE. The variants associated with Cole disease are shown in red. The other sequence variants are frequently associated with overlapping clinical phenotypes of ARHR2, GACI and/or PXE. The novel variants are in bold. This figure was created with Biorender.com.

Table 1.

ENPP1 variants found in individuals with ARHR2, Cole disease, GACI, and PXE, previously reported and from this study

Region	Protein domain	Nucleotide change	Protein change	Variant Type	Diagnosis	ACMG	Reference
Promoter	-	c.−31G>C	-	-	GACI	VUS	(Weingarten, 2021)
Exon 1	CD	c.130C>T	p.Gln44*	Nonsense	GACI	LP	(Liu et al., 2018)
Exon 1	CD	c.179T>C	p.Leu60Pro	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 2	TM	c.241G>T	p.Val81Leu	Missense	GACI	VUS	(Ralph et al., 2022b)
Exon 2	TM	c.272T>C	p.Leu91Pro	Missense	GACI	VUS	(Nakamura et al., 1999)
Exon 2	TM	c.275G>A	p.Gly92Asp	Missense	ARHR2	VUS	(Steichen-Gersdorf et al., 2015)
Exon 2	TM	c.288del	p.Leu97*	Indel	GACI	P	(Rutsch et al., 2008)
Intron 2	SMB1	c.313+9_313+10insGGTG	-	Splice	ARHR2	VUS	(Rush et al., 2022)
Exon 3	SMB1	c.323G>T	p.Cys108Phe	Missense	ARHR2	LP	(Kotwal et al., 2020)
Exon 3	SMB1	c.325A>G	p.Lys109Glu	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 3	SMB1	c.358T>C	p.Cys120Arg	Missense	Cole Disease	VUS	(Chourabi et al., 2018)
Exon 3	SMB1	c.376T>C	p.Cys126Arg	Missense	GACI	VUS	(Rutsch et al., 2003)
Exon 3	SMB1	c.397T>C	p.Cys133Arg	Missense	Cole Disease	VUS	(Schlipf et al., 2016)
Exon 3	SMB1	c.419G>T	p.Cys140Phe	Missense	ARHR2	VUS	**Novel, this report**
Intron 3	SMB2	c.430+1del	-	Splice	GACI	P	(Brachet et al., 2014)
Intron 3	SMB2	c.430+2T>C	-	Splice	GACI	LP	(Rutsch et al., 2008)
Exon 4	SMB2	c.446G>C	p.Cys149Ser	Missense	Cole Disease	VUS	(Eytan et al., 2013)
Exon 4	SMB2	c.484A>G	p.Ser162Gly	Missense	GACI	VUS	Clinvar, VCV001034254.1
Exon 4	SMB2	c.490T>C	p.Cys164Arg	Missense	Cole Disease	VUS	(Nanda et al., 2022)
Exon 4	SMB2	c.491G>C	p.Cys164Ser	Missense	Cole Disease	VUS	(Eytan et al., 2013)
Exon 4	SMB2	c.497G>A	p.Cys166Tyr	Missense	ARHR2	VUS	**Novel, this report**
Exon 4	SMB2	c.526T>C	p.Cys176Arg	Missense	Cole Disease	VUS	(Gabaton et al., 2020)
Exon 4	SMB2	c.530G>C	p.Cys177Ser	Missense	Cole Disease	VUS	(Schlipf et al., 2016)
Exon 4	SMB2	c.530G>A	p.Cys177Tyr	Missense	Cole Disease	LP	(Eytan et al., 2013)
Exon 4	SMB2	c.544T>G	p.Ser182Ala	Missense	ARHR2	VUS	(Rush et al., 2022)

Region	Protein domain	Nucleotide change	Protein change	Variant Type	Diagnosis	ACMG	Reference
Exon 4	SMB2	c.556G>C	p.Gly186Arg	Missense	GACI	VUS	(Staretz-Chacham et al., 2019)
Intron 4	SMB2	c.556+3C>A	-	Splice	ARHR2	VUS	(Lorenz-Depiereux et al., 2010)
Intron 4	SMB2	c.557-2A>G	-	Splice	GACI	LP	(Rutsch et al., 2008)
Exon 5	L1	c.583T>A	p.Cys195Ser	Missense	GACI	VUS	(Stella et al., 2016)
Exon 5	L1	c.583T>C	p.Cys195Arg	Missense	GACI	LP	(Edouard et al., 2011)
Intron 5 - Intron 6	L1 - PCD	c. (617+1_618-1)_(715+1_716-1)del	-	Large deletion	GACI	LP	(Ferreira, Hackbarth, et al., 2021)
Exon 6	PCD	c.626C>T	p.Thr209Met	Missense	ARHR2	VUS	(Rush et al., 2022)
Exon 6	PCD	c.647C>A	p.Ser216Tyr	Missense	GACI	VUS	(Rutsch et al., 2008)
Exon 6	PCD	c.653A>T	p.Asp218Val	Missense	GACI	VUS	(Galletti et al., 2011)
Exon 6	PCD	c.655G>A	p.Gly219Arg	Missense	ARHR2	VUS	(Steichen-Gersdorf et al., 2015)
Exon 6	PCD	c.656G>A	p.Gly219Glu	Missense	GACI	VUS	(Ralph et al., 2022b)
Intron 6	PCD	c.715+1G>C	-	Splice	ARHR2	LP	(Ferreira, Kavanagh, et al., 2021)
Intron 6	PCD	c.715+5G>T	-	Splice	GACI	VUS	(Ralph et al., 2022b)
Exon 7	PCD	c.725G>A	p.Gly242Glu	Missense	GACI	VUS	(Rutsch et al., 2008)
Exon 7	PCD	c.749C>T	p.Pro250Leu	Missense	GACI	LP	(Ruf et al., 2005)
Exon 7	PCD	c.753_755del	p.Tyr252del	Indel	GACI	VUS	(Ruf et al., 2005)
Exon 7	PCD	c.755A>G	p.Tyr252Cys	Missense	ARHR2	VUS	(Oheim et al., 2020)
Exon 7	PCD	c.760A>G	p.Thr254Ala	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 7	PCD	c.773C>A	p.Pro258His	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 7	PCD	c.783C>G	p.Tyr261*	Nonsense	ARHR2, GACI, PXE	P	(Jin et al., 2015; Liu et al., 2017; Ruf et al., 2005)
Exon 7	PCD	c.784A>G	p.Ser262Gly	Missense	GACI	VUS	(Brunod et al., 2018)
Exon 7	PCD	c.787A>T	p.Ile263Phe	Missense	GACI	VUS	**Novel, this report**
Intron 7	PCD	c.795+1G>A	-	Splice	GACI	P	(Nitschke et al., 2012)
Intron 7	PCD	c.796-2A>G	-	Splice	ARHR2	LP	**Novel, this report (GeneDx)**
Exon 8	PCD	c.797G>T	p.Gly266Val	Missense	ARHR2, GACI	LP	(Lorenz-Depiereux et al., 2010)
Exon 8	PCD	c.802T>C	p.Tyr268His	Missense	GACI	VUS	(Nakamura et al., 1999)

Region	Protein domain	Nucleotide change	Protein change	Variant Type	Diagnosis	ACMG	Reference
Exon 8	PCD	c.803A>G	p.Tyr268Cys	Missense	GACI	VUS	(Ferreira, Hackbarth, et al., 2021)
Exon 8	PCD	c.826G>A	p.D276N	Missense	GACI	VUS	(Rutsch et al., 2008)
Exon 8	PCD	c.860C>T	p.S287F	Missense	GACI	VUS	(Nakamura et al., 1999)
Exon 8	PCD	c.876_880del	p.S292Rfs*4	Indel	GACI	LP	(Ralph et al., 2022b)
Exon 8	PCD	c.878_879del	p.K293Rfs*4	Indel	GACI	LP	(Ruf et al., 2005)
Exon 8	PCD	c.902A>G	p.Tyr301Cys	Missense	GACI	VUS	(Stella et al., 2016)
Exon 8	PCD	c.913C>A	p.Pro305Thr	Missense	GACI	P	(Ruf et al., 2005)
Exon 9	PCD	c.936T>G	p.Tyr312*	Nonsense	GACI	LP	(Rutsch et al., 2003)
Exon 9	PCD	c.956C>G	p.Thr319Arg	Missense	ARHR2	VUS	(Mehta et al., 2012)
Exon 9	PCD	c.966G>A	p.Trp322*	Nonsense	GACI	LP	(Yapicioglu-Yildizdas et al., 2016)
Exon 9	PCD	c.1000C>G	p.Pro334Ala	Missense	GACI	VUS	Clinvar, VCV000634992.2
Exon 9	PCD	c.1025G>T	p.Gly342Val	Missense	GACI	LP	(Ruf et al., 2005)
Exon 10	PCD	c.1028C>A	p.Ser343*	Nonsense	ARHR2	LP	**Novel, this report (GeneDx)**
Exon 10	PCD	c.1046G>A	p.Arg349Lys	Missense	GACI	VUS	(Rutsch et al., 2008)
Exon 10	PCD	c.1066T>C	p.Trp356Arg	Missense	GACI	VUS	Clinvar, VCV000800816.1
Exon 10	PCD	c.1068G>A	p.Trp356*	Nonsense	GACI	P	Clinvar, VCV000692042.1
Exon 10	PCD	c.1072_1082del	p.Gln358Argfs*2	Indel	GACI	P	(Rutsch et al., 2003)
Exon 11	PCD	c.1112A>T	p.Tyr371Phe	Missense	GACI	VUS	(Ruf et al., 2005)
Intron 11	PCD	c.1164+2T>A	-	Splice	GACI	LP	(Ruf et al., 2005)
Exon 13	PCD	c.1302C>A	p.Tyr434*	Nonsense	ARHR2	LP	(Turan et al., 2021)
Exon 13	PCD	c.1318T>C	p.Tyr440His	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 13	PCD	c.1367G>A	p.Arg456Gln	Missense	GACI	VUS	(Rutsch et al., 2003)
Exon 13	PCD	c.1391A>G	p.Asp464Gly	Missense	Cole Disease	VUS	Clinvar, VCV000982597.1
Intron 13	PCD	c.1405+5G>C	-	Splice	GACI	VUS	(Del Castillo Velilla et al., 2021)
Exon 14	PCD	c.1412A>G	p.Tyr471Cys	Missense	GACI, PXE	VUS	(Ralph et al., 2022b; Rutsch et al., 2008; Saeidian et al., 2022)
Exon 14	PCD	c.1426C>T	p.Arg476*	Nonsense	GACI	LP	(Rutsch et al., 2008)

Region	Protein domain	Nucleotide change	Protein change	Variant Type	Diagnosis	ACMG	Reference
Exon 14	PCD	c.1436C>T	p.Ser479Phe	Missense	PXE	VUS	(Jin et al., 2015)
Intron 14	PCD	c.1437+1G>T	-	Splice	ARHR2	P	(Rush et al., 2022)
Intron 14	PCD	c.1437+3_1437+6del	-	Splice	ARHR2	LP	(Rush et al., 2022)
Exon 15	PCD	c.1438T>C	p.Cys480Arg	Missense	GACI	VUS	(Thumbigere-Math et al., 2018)
Exon 15	PCD	c.1441C>T	p.Arg481Trp	Missense	ARHR2, GACI	LP	(Kotwal et al., 2020; Rutsch et al., 2003)
Exon 15	PCD	c.1442G>A	p.Arg481Gln	Missense	GACI	VUS	(Ferreira, Kavanagh, et al., 2021)
Exon 15	PCD	c.1499A>C	p.His500Pro	Missense	GACI	VUS	(Rutsch et al., 2008)
Exon 15	PCD	c.1499A>G	p.His500Arg	Missense	ARHR2	VUS	**Novel, this report**
Exon 15	PCD	c.1510A>C	p.Ser504Arg	Missense	GACI	VUS	(Rutsch et al., 2008)
Exon 15	PCD	c.1530G>C	p.Leu510Phe	Missense	PXE	VUS	(Ralph et al., 2022b)
Exon 15	PCD	c.1538A>G	p.Tyr513Cys	Missense	GACI	VUS	(Rutsch et al., 2008)
Exon 15	PCD	c.1553G>T	p.Trp518Leu	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 16	PCD	c.1574C>T	p.Ser525Leu	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 16	PCD	c.1604A>G	p.His535Arg	Missense	GACI	VUS	**Novel, this report (Prevention Genetics)**
Exon 16	PCD	c.1612G>C	p.Asp538His	Missense	GACI	VUS	(Nitschke et al., 2012)
Exon 17	PCD	c.1652A>G	p.Tyr551Cys	Missense	ARHR2	VUS	(Ferreira, Hackbarth, et al., 2021)
Exon 17	PCD	c.1699A>G	p.Ile567Val	Missense	ARHR2	VUS	(Rush et al., 2022)
Exon 17	PCD	c.1709A>G	p.Tyr570Cys	Missense	GACI	VUS	(Rutsch et al., 2008)
Exon 17	PCD	c.1715T>C	p.Leu572Ser	Missense	GACI	VUS	(Bulfamante et al., 2021)
Exon 18	PCD	c.1735T>G	p.Leu579Val	Missense	ARHR2	VUS	(Rush et al., 2022)
Exon 18	PCD	c.1737G>C	p.Leu579Phe	Missense	GACI	VUS	(Rutsch et al., 2003)
Exon 18	PCD	c.1756G>A	p.Gly586Arg	Missense	ARHR2, GACI	LP	(Nitschke et al., 2012; Rush et al., 2022)
Exon 18	PCD	c.1769G>A	p.Ser590Asn	Missense	GACI	LP	Clinvar, VCV000870410.1
Exon 18	L2	c.1889C>A	p.Pro630His	Missense	ARHR2	VUS	(Rush et al., 2022)
Exon 19	L2	c.1909G>C	p.Asp637His	Missense	ARHR2	VUS	(Rush et al., 2022)
Exon 20	NLD	c.1976A>G	p.Tyr659Cys	Missense	GACI	VUS	(Rutsch et al., 2008)

Region	Protein domain	Nucleotide change	Protein change	Variant Type	Diagnosis	ACMG	Reference
Exon 20	NLD	c.2026C>T	p.Gln676*	Nonsense	ARHR2	LP	(Steichen-Gersdorf et al., 2015)
Exon 20	NLD	c.2078C>T	p.Thr693Ile	Missense	PXE	VUS	(Saeidian et al., 2022)
Intron 20	NLD	c.2101-2A>T	-	Splice	GACI	P	Clinvar, VCV000807411.2
Exon 21	NLD	c.2132G>A	p.Cys711Tyr	Missense	PXE	VUS	(Omarjee et al., 2020)
Exon 21	NLD	c.2176T>C	p.Cys726Arg	Missense	GACI	VUS	(Rutsch et al., 2003)
Exon 21	NLD	c.2212G>A	p.Gly738Arg	Missense	GACI	VUS	(Yapicioglu-Yildizdas et al., 2016)
Intron 21	NLD	c.2230+1_2230+3delinsCACC	-	Splice	ARHR2	LP	(Saito et al., 2011)
Intron 21	NLD	c.2230+1G>A	-	Splice	ARHR2	LP	(Steichen-Gersdorf et al., 2015)
Exon 22	NLD	c.2248dup	p.Ser750Lysfs*6	Indel	ARHR2	P	(Lorenz-Depiereux et al., 2010)
Intron 22	NLD	c.2311+1G>A	-	Splice	ARHR2	VUS	(Capelli et al., 2015)
Intron 22 - Exon 23	NLD	c.2312-5_2313del	-	Indel	GACI	LP	(Ferreira, Hackbarth, et al., 2021)
Exon 23	NLD	c.2330A>G	p.His777Arg	Missense	GACI, PXE	VUS	(Rutsch et al., 2008; Saeidian et al., 2022)
Exon 23	NLD	c.2344C>T	p.Arg782*	Nonsense	ARHR2, GACI	P	(Mehta et al., 2012; Numakura et al., 2006)
Exon 23	NLD	c.2375A>G	p.Asn792Ser	Missense	ARHR2, GACI	LP	(Rutsch et al., 2003; Steichen-Gersdorf et al., 2015)
Exon 23	NLD	c.2376T>A	p.Asn792Lys	Missense	ARHR2, GACI	LP	(Ferreira, Kavanagh, et al., 2021); Clinvar, VCV000870422.1
Exon 23	NLD	c.2390C>G	p.Pro797Arg	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 23	NLD	c.2390C>T	p.Pro797Leu	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 23	NLD	c.2410G>C	p.Asp804His	Missense	GACI	VUS	(Ruf et al., 2005)
Exon 23	NLD	c.2414G>T	p.Gly805Val	Missense	GACI	VUS	(Thumbigere-Math et al., 2018)
Intron 23 - 3' UTR	NLD	c.2444+702_*868del	-	Large deletion	ARHR2	LP	(Lorenz-Depiereux et al., 2010)
Exon 24	NLD	c.2452_2454del	p.Arg818del	Indel	ARHR2	VUS	(Rush et al., 2022)
Exon 24	NLD	c.2479_2482dup	p.Pro828Hisfs*13	Indel	GACI	LP	(Rutsch et al., 2008)
Exon 24	NLD	c.2531C>A	p.Thr844Lys	Missense	PXE	VUS	(Saeidian et al., 2022)

Region	Protein domain	Nucleotide change	Protein change	Variant Type	Diagnosis	ACMG	Reference
Exon 24	NLD	c.2554G>C	p.Asp852His	Missense	ARHR2	VUS	(Coskunpinar et al., 2018)
Exon 24	NLD	c.2596G>A	p.Glu866Lys	Missense	GACI, PXE	VUS	(Ralph et al., 2022b; Ferreira, Hackbarth, et al., 2021; Omarjee et al., 2020)
Exon 25	NLD	c.2621A>T	p.Asp874Val	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 25	NLD	c.2633T>C	p.Val878Ala	Missense	ARHR2	VUS	(Rush et al., 2022)
Exon 25	NLD	c.2642T>A	p.Leu881*	Nonsense	PXE	LP	(Saeidian et al., 2022)
Exon 25	NLD	c.2646A>T	p.Leu882Phe	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 25	NLD	c.2656A>G	p.Arg886Gly	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 25	NLD	c.2662C>T	p.Arg888Trp	Missense	ARHR2, GACI, PXE	VUS	(Ruf et al., 2005; Saeidian et al., 2022; Thiele et al., 2020)
Exon 25	NLD	c.2663G>A	p.Arg888Gln	Missense	ARHR2	VUS	(Thiele et al., 2020)
Exon 25	NLD	c.2677G>T	p.Glu893*	Nonsense	GACI	P	(Rutsch et al., 2003)
Exon 25	NLD	c.2702A>C	p.Tyr901Ser	Missense	ARHR2	LP	(Levy-Litan et al., 2010)
Exon 25	NLD	c.2702A>T	p.Tyr901Phe	Missense	PXE	VUS	(Saeidian et al., 2022)
Exon 25	NLD	c.2735T>C	p.Leu912Ser	Missense	GACI	VUS	(Ferreira, Hackbarth, et al., 2021)
Exon 25	NLD	c.2713_2717del	p.Lys905Alafs*16	Indel	GACI	VUS	(Rutsch et al., 2003)

The human *ENPP1* accession number is NM_006208.3.

Table 2.

ENPP1 missense variants characterized in functional studies

Region	Protein domain	Nucleotide change	Protein change	ENPP1 expression	Cellular localization	ENPP enzyme activity (Relative to the WT protein)	Extracellular PPi generation (Relative to the WT protein)	Experiment used	Reference
Exon 3	SMB1	c.323G>T	p.Cys108Phe	-	-	Significantly reduced	-	Transfection of CHO-K1 cells	(Kotwal et al., 2020)
Exon 3	SMB1	c.376T>C	p.Cys126Arg	-	-	Significantly reduced	-	Transfection of SaOS-2 cells	(Rutsch et al., 2003)
Exon 4	SMB2	c.517A>C	p.Lys173Gln	-	-	ns	-	Transfection of SaOS-2 cells	(Rutsch et al., 2003)
				Yes	Plasma Membrane	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
				Yes	-	ns	-	Transfection of COS-1 cells	(Gijsbers et al., 2003)
Exon 5	L1	c.583T>A	p.Cys195Ser	Yes	Intracellular	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
Exon 5	L1	c.583T>C	p.Cys195Arg	Yes	Intracellular	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
Exon 6	PCD	c.656G>A	p.Gly219Glu	Yes	Intracellular	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Ralph et al., 2022b)
Exon 7	PCD	c.766A>T	p.Thr256Ser	Yes	-	Severely reduced	-	Transfection of MCF-7 cells	(Grupe et al., 1995)
				Yes	-	Severely reduced	-	HEK293F (purified protein)	(Chin et al., 2009)
Exon 7	PCD	c.766A>G	p.Thr256Ala	Yes	-	Severely reduced	-	HEK293F (purified protein)	(Chin et al., 2009)
Exon 8	PCD	c.876_880del	-	No	-	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Ralph et al., 2022b)
Exon 8	PCD	c.902A>G	p.Tyr301Cys	Yes	Intracellular	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
Exon 8	PCD	c.913C>A	p.Pro305Thr	Yes	Plasma Membrane	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
Exon 9	PCD	c.936T>G	p.Tyr312*	-	-	Significantly reduced	-	Transfection of SaOS-2 cells	(Rutsch et al., 2003)
Exon 13	PCD	c.1367G>A	p.Arg456Gln	-	-	Significantly reduced	-	Transfection of SaOS-2 cells	(Rutsch et al., 2003)

Region	Protein domain	Nucleotide change	Protein change	ENPP1 expression	Cellular localization	ENPP enzyme activity (Relative to the WT protein)	Extracellular PPi generation (Relative to the WT protein)	Experiment used	Reference
Exon 14	PCD	c.1412A>G	p.Tyr471Cys	Yes	Plasma Membrane	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
				-	-	Significantly reduced	-	Transfection of CHO-K1 cells	(Oheim et al., 2020)
Exon 15	PCD	c.1441C>T	p.Arg481Trp	-	-	Significantly reduced	-	Transfection of SaOS-2 cells	(Rutsch et al., 2003)
				-	-	Significantly reduced	-	Transfection of CHO-K1 cells	(Kotwal et al., 2020)
Exon 15	PCD	c.1510A>C	p.Ser504Arg	Yes	Plasma Membrane	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
Exon 15	PCD	c.1530G>C	p.Leu510Phe	Yes	Plasma Membrane + Intracellular	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Ralph et al., 2022b)
Exon 15	PCD	c.1538A>G	p.Tyr513Cys	Yes	Plasma Membrane	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
Exon 16	PCD	c.1612G>C	p.Asp538His	Yes	Plasma Membrane	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
Exon 18	PCD	c.1737G>C	p.Leu579Phe	-	-	Significantly reduced	-	Transfection of HEK293 and COS-7 cells	(Rutsch et al., 2003)
Exon 18	PCD	c.1756G>A	p.Gly586Arg	Yes	Intracellular	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
Exon 18	L2	c.1831C>G	p.Leu611Val	-	-	Significantly reduced	-	Transfection of SaOS-2 cells	(Rutsch et al., 2003)
Exon 20	NLD	c.1976A>G	p.Tyr659Cys	Yes	Plasma Membrane	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
Exon 20	NLD	c.2002G>A	p.Glu668Lys	-	-	ns	-	Transfection of SaOS-2 cells	(Rutsch et al., 2003)
				Yes	Plasma Membrane	ns	ns	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
Exon 21	NLD	c.2133T>G	p.Cys711Trp	Yes	Intracellular	Significantly reduced	Significantly reduced	Transfection of HEK293 cells	(Omarjee et al., 2020)
Exon 21	NLD	c.2176T>C	p.Cys726Arg	-	-	Significantly reduced	-	Transfection of SaOS-2 cells	(Rutsch et al., 2003)
Exon 23	NLD	c.2320C>T	p.Arg774Cys	-	-	Significantly reduced	-	Transfection of SaOS-2 cells	(Rutsch et al., 2003)
				Yes	Plasma Membrane	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)

Region	Protein domain	Nucleotide change	Protein change	ENPP1 expression	Cellular localization	ENPP enzyme activity (Relative to the WT protein)	Extracellular PPi generation (Relative to the WT protein)	Experiment used	Reference
Exon 23	NLD	c.2330A>G	p.His777Arg	Yes	Plasma Membrane	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
				-	-	Significantly reduced	-	Transfection of CHO-K1 cells	(Oheim et al., 2020)
Exon 23	NLD	c.2344C>T	p.Arg782*	-	-	Significantly reduced	-	Patient's plasma	(Numakura et al., 2006)
Exon 23	NLD	c.2375A>G	p.Asn792Ser	-	-	Significantly reduced	-	Transfection of SaOS-2 cells	(Rutsch et al., 2003)
Exon 24	NLD	c.2462G>A	p.Arg821His	Yes	Plasma Membrane	ns	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
Exon 24	NLD	c.2596G>A	p.Glu866Lys	Yes	Plasma Membrane	Significantly reduced	Significantly reduced	Transfection of HEK293 cells	(Omarjee et al., 2020)
Exon 25	NLD	c.2662C>T	p.Arg888Trp	Yes	Intracellular	Significantly reduced	Significantly reduced	Transfection of HEK293 and COS-7 cells	(Stella et al., 2016)
Exon 25	NLD	c.2677G>T	p.Glu893*	-	-	Significantly reduced	-	Transfection of SaOS-2 cells	(Rutsch et al., 2003)
Exon 25	NLD	c.2702A>C	p.Tyr901Ser	Yes	Plasma Membrane	Significantly reduced	-	Transfection of COS-7 cells	(Levy-Litan et al., 2010)
Exon 25	NLD	c.2713_2717del	p.Lys905Alafs*16	-	-	Significantly reduced	-	Transfection of SaOS-2 cells	(Rutsch et al., 2003)

The human *ENPP1* accession number is NM_006208.3.

Table 3.

Other *ENPP1* variants characterized in functional studies

Region	Protein domain	Nucleotide change	Protein change	Effect on *ENPP1* pre-mRNA splicing	Effect on ENPP1 protein dimerization	Experiment used	Reference
Exon 2	TM	c.241G>T	p.Val81Leu	Skipping of exon 2	-	Patient's peripheral blood leukocytes	(Ralph et al., 2022b)
Exon 3	SMB1	c.358T>C	p.Cys120Arg	-	Moderately affected	Transfection of HEK293T cells	(Chourabi et al., 2018)
Exon 4	SMB2	c.491G>C	p.Cys164Ser	-	Severely affected	Transfection of HEK293T cells	(Chourabi et al., 2018)
Intron 6	PCD	c.715+5G>T	-	Skipping of exon 6	-	Transfection of HEK293 cells	(Ralph et al., 2022b)
Exon 15	PCD	c.1441C>T	p.Arg481Trp	Skipping of exon 15	-	Patient's fibroblasts or lymphocytes	(Rutsch et al., 2003)
Intron 21	NLD	c.2230+1_2230+3delinsCACC	-	Skipping of exon 21	-	Patient's peripheral blood leukocytes	(Saito et al., 2011)

The human *ENPP1* accession number is NM_006208.3.

Table 4.

ENPP1-associated animal models

Animal model	Mutation	Type of mutation	Strain	Reference
Mouse				
Enpp1^{ttw}	p.Gly568*	Spontaneous	Jcl:ICR	(Okawa et al., 1998)
Enpp1^{tm1Gdg}	Deletion of exon 9	Targeted knock-out	129S1/Sv	(Sali A, 1999)
Enpp1^{m1Amgn}	p.Cys397Ser	Chemically induced (ENU)	C57BL/6J	(Babij et al., 2009)
Enpp1^{asj}	p.Val246Asp	Chemically induced (ENU)	C57BL/6J	(Li et al., 2013)
Enpp1^{asj-2J}	Δ40,035bp, ins74bp	Spontaneous	BALB/cJ	(Li et al., 2014)
Enpp1$^{twt-Ham}$	c.259+1G>T	Spontaneous	Jcl:ICR	(Takabayashi et al., 2014)
Osteoblast-specific *Enpp1* knock-out	Deletion of exon 9 in osteoblasts	Crossing the *Enpp1*$^{fl/fl}$ mouse with osteocalcin-cre mouse	C57BL/6	(Roberts et al., 2021)
Zebrafish				
Dg1^{hu4581}	c.993-2A>T	Chemically induced (ENU)	-	(Apschner et al., 2014; Huitema et al., 2012)

The mouse and zebrafish *ENPP1* accession numbers are NM_008813.4 and NM_001030168.1, respectively.