Deficiency in the endocytic adaptor proteins PHETA1/2 impairs renal and craniofacial development

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ABSTRACT

A critical barrier in the treatment of endosomal and lysosomal diseases is the lack of understanding of the in vivo functions of the putative causative genes. We addressed this by investigating a key pair of endocytic adaptor proteins, PH domain-containing endocytic trafficking adaptor 1 and 2 (PHETA1/2; also known as FAM109A/B, Sess1/2, IPIP27A/B), which interact with the protein product of OCRL, the causative gene for Lowe syndrome. Here, we conducted the first study of PHETA1/2 in vivo, utilizing the zebrafish system. We found that impairment of both zebrafish orthologs, pheta1 and pheta2, disrupted endocytosis and ciliogenesis in renal tissues. In addition, pheta1/2 mutant animals exhibited reduced jaw size and delayed chondrocyte differentiation, indicating a role in craniofacial development. Deficiency of pheta1/2 resulted in dysregulation of cathepsin K, which led to an increased abundance of type II collagen in craniofacial cartilages, a marker of immature cartilage extracellular matrix. Cathepsin K inhibition rescued the craniofacial phenotypes in the pheta1/2 double mutants. The abnormal renal and craniofacial phenotypes in the pheta1/2 mutant animals were consistent with the clinical presentation of a patient with a de novo arginine (R) to cysteine (C) variant (R6C) of PHETA1. Expressing the patient-specific variant in zebrafish exacerbated craniofacial deficits, suggesting that the R6C allele acts in a dominant-negative manner.

INTRODUCTION

Endocytic trafficking is essential for a variety of biological processes, including nutrient uptake, cell signaling and cellular morphogenesis (Doherty and McMahon, 2009). This diversity in cellular functions is reflected in the broad range of pathologies associated with deficiencies in endocytic factors. For example, mutations in endocytic factors dynamin 2 (DNM2) and RAB7 result in Charcot-Marie-Tooth disease, a clinically and genetically heterogeneous group of peripheral neuropathies (Verhoeven et al., 2003; Züchner et al., 2005). Disruptions in endocytosis have been identified in autosomal recessive hypercholesterolemia (Garuti et al., 2005) and autosomal dominant polycystic kidney disease (Obermuller et al., 2001). These disparate clinical outcomes resulting from endocytic protein deficiency underscore the importance of investigations in the organismal context. Currently, endocytic pathways have been identified and defined through their differential interactions with specific phosphoinositides and proteins (e.g. clathrin, actin and dynamin), but most components of the endocytic machinery have only been examined in cell lines (Doherty and McMahon, 2009). In this study, our goal was to use an in vivo experimental system to investigate two important regulators of endocytosis, PH domain-containing endocytic trafficking adaptor 1 and 2 (PHETA1/2).

PHETA1/2 (also known as FAM109A/B, Sess1/2, IPIP27A/B) were identified in vitro as regulators of endosomal trafficking, specifically for receptor recycling to endosomes and for cargo sorting to lysosomes (Noakes et al., 2011; Swan et al., 2010). Both PHETA1 and PHETA2 have a C-terminal phenylalanine-histidine motif (F&H motif) that serves as a binding site for OCRL, encoded by a gene that is mutated in Lowe syndrome (MIM #309000) (Pirruccello et al., 2011). OCRL is an inositol 5-phosphatase, with phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] as the preferred substrate (Attree et al., 1992; Noakes et al., 2011). Binding to PI(4,5)P2 occurs at the pleckstrin homology (PH) domain in OCRL, which also contains a loop outside the domain fold with a clathrin-binding motif. This motif directs OCRL specifically to clathrin-coated endocytic pits on the plasma membrane (Choudhury et al., 2009; Mao et al., 2009). PI(4,5)P2 is abundant at the plasma membrane.
membrane and is involved in a wide variety of processes, including actin dynamics and endocytosis (Sasaki et al., 2009). Disrupting the phosphatase activity of OCRL interferes with PI(4,5)P₂ homeostasis, which is thought to contribute to the disease manifestations of Lowe syndrome (De Leo et al., 2016; Lowe, 2005; Vicinanza et al., 2011).

Several studies have shown that PHETA1 is crucial in maintaining optimal OCRL function. Specifically, the 5-phosphatase activity of OCRL relies upon PHETA1-mediated interaction with protein kinase C and casein kinase substrate in neurons 2 (PACSIN2), a protein that interacts with the actin cytoskeleton. A proline-rich PPPxPPPR motif in PHETA1 located upstream of the F&H motif serves as the necessary PACSIN2 binding site (Billcliff et al., 2016). PHETA2 lacks the PPPxPPPR motif. OCRL also promotes ciliogenesis by way of endosomal trafficking in a Rab8 (also known as RAB8A)/PHETA1-dependent manner (Coon et al., 2012). These findings suggest that PHETA1 and OCRL functionally interact to mediate both endocytosis and ciliogenesis.

Besides endocytosis and ciliogenesis, PHETA1 and PHETA2 are also involved in the transport of newly synthesized lysosomal hydrolases from the trans-Golgi network (TGN) to the endosomes (Noakes et al., 2011). Thus, loss of PHETA1/2 could result in improper sorting of lysosomal hydrolases. Consistent with this idea, loss of PHETA2 results in hypersecretion of pro-cathepsin D (Noakes et al., 2011). Similar disruptions in lysosomal proteins have also been found in mucolipidosis type II (MLII), where the loss of mannose 6-phosphate-dependent targeting results in hypersecretion of multiple lysosomal enzymes (Kollmann et al., 2010; Kornfeld, 1986; Kudo et al., 2006). Dysregulation of cathepsins in MLII zebrafish models results in craniofacial and skeletal deformations, recapitulating the clinical features of MLII patients (Flanagan-Steeet et al., 2009; Petrey et al., 2012). Thus, PHETA1/2-dependent regulation of protease transport may be important for craniofacial development.

The involvement of PHETA1 during development is further supported by recent findings in a human patient with a de novo arginine to cysteine (R6C) variant in PHETA1, identified through the National Institutes of Health (NIH) Undiagnosed Diseases Program (UDP) (Gahl et al., 2012, 2016, 2015). The UDP patient presented with global developmental delay, coarse facial features, renal abnormalities and other developmental deficits (Table 1). Despite the known roles of PHETA1 in facilitating OCRL function, the UDP patient did not present with the typical manifestations of Lowe syndrome, i.e. congenital cataracts, cognitive impairment, and renal tubular and glomerular dysfunction (Mehta et al., 2014). These findings suggest that PHETA1 may have OCRL-independent functions in vivo.

To investigate the in vivo functions of PHETA1 and its close homolog PHETA2, we utilize zebrafish, an informative small vertebrate model organism for validating the pathogenicity of genes or alleles in human patients. This approach has offered valuable insight for clinicians into a broad range of genetic disorders, including neurodevelopmental disorders, ciliopathies and Lowe syndrome (Coon et al., 2012; Oltibella et al., 2015; Phillips and Westerfield, 2014; Ramirez et al., 2012; Sakai et al., 2018; Song et al., 2016). Using histological, physiological and behavioral analyses, we found that zebrafish pheta1 and pheta2 are required for endocytosis, ciliogenesis and craniofacial development. Consistent with a role in trafficking lysosomal enzymes, disruption in craniofacial development in the pheta1/2 mutants was associated with dysregulated cathepsin K activity. The abnormal craniofacial development is exacerbated further in the presence of the R6C variant, suggesting a dominant-negative mode of action in human disease.

## RESULTS

### Identification of a de novo PHETA1 variant in undiagnosed human disease

The UDP enrolled a 6-year-old female patient with craniofacial dysmorphic features, scoliosis, clefting defects, global developmental delay, vision and auditory impairments, and renal tubular or glomerular dysfunction (Fig. 1A,B and Table 1). Whole-exome sequencing and Sanger sequencing of the patient, unaffected fraternal twin and unaffected parents identified a heterozygous de novo arginine (R) to cysteine (C) mutation in PHETA1, present only in the patient (NM_001177997.2:c.55C>T; p.R6C in the short isoform; p.R19C in the long isoform, NM_001177996.1) (Fig. 1C). The R6 residue in PHETA1 is highly conserved across species (Fig. 1D) (Papadopoulos and Agarwala, 2007), and the R6C mutation was predicted to be damaging with the use of Polyphen (Probably damaging, HumDiv: 1; HumVar: 0.995), SIFT (Deleterious, Score 0.01), and MutationTaster (Disease causing, Prob: 1) (Adzhubei et al., 2010; Schwarz et al., 2014; Sim et al., 2012).

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**Table 1. Clinical features of a UDP patient affected with a de novo arginine (R) to cysteine (C) mutation in PHETA1**

| Feature                | Description                                                                 |
|------------------------|-----------------------------------------------------------------------------|
| Presentation at birth  | Abnormality of the placenta, breech presentation (C-section performed), gestational diabetes, congenital bilateral hip dislocation, congenital muscular torticollis, decreased body weight, birth length less than 3rd percentile, episodic vomiting, feeding difficulties in infancy, self-injurious behavior, small for gestational age. |
| Facial features        | Facial asymmetry, coarse facial features, concave nasal ridge, flat occiput, malar flattening, narrow mouth, sparse scalp hair, relative macrocephaly. |
| Brain                  | Subdural hemorrhage, absence of acoustic reflex, ventriculomegaly, elevated brain lactate level by magnetic resonance spectroscopy (MRS), reduced brain N-acetyl aspartate level by MRS, cavum septum pellucidum, widened subarachnoid space, decreased sensation to painful stimuli. |
| Eyes                   | Congenital cataract, ambylophia, bilateral ptosis, hypertelorism, nystagmus, optic nerve dysplasia/hypoplasia, short palpebral fissure, telecanthus. Patient had corrective surgery and vision is now normal. |
| Kidney                 | Horsehoe kidney, oligosacchariduria. |
| Motor                  | Broad-based gait, delayed fine and gross motor development, generalized hypotonia, oral motor hypotonia. |
| Hands/feet             | Clindactyly of 4th and 5th finger, multiple palmar and plantar creases, pes planus, short foot and palm, tapered fingers, slow-growing nails, metatarsus adductus. |
| Dental Other           | Abnormality of dental morphology, dental malocclusion, difficulty in tongue movements, widely spaced teeth. Moderate receptive language delay, severe global developmental delay, hearing impairment, thoracolumbar kyphoscoliosis, chronic constipation, mitral valve prolapse, freckled genitilia, hyperpigmented streaks, prominent crus of helix, upper airway obstruction, hip dysplasia, seasonal allergy. |
This variant has been reported in the ExAC browser, with a minor allele frequency of 0.000009398 (1/106410). Using patient-derived fibroblasts and quantitative real-time PCR (qRT-PCR), we found that the R6C mutation does not affect the mRNA expression of PHETA1 (Fig. 1E).

Protein modeling was performed using the I-TASSER, MUSTER and PHYRE2 servers (Fig. 1F) (Adzhubei et al., 2010; Kelley et al., 2015; Roy et al., 2010; Wu and Zhang, 2008). Based on the homology model, the arginine residue is highlighted in red, and amino acid residues that differ from the sequence of the human PHETA1 protein are highlighted in green. The arginine residue is highly conserved across multiple species. (E) Relative quantification of mRNA expression in the patient cells showing that the expression of PHETA1 is not significantly different from that in controls. Error bar represents s.d. from six replicates. (F) 3-D structure of the human PHETA1 protein showing the PH domain (green) with a four-stranded N-terminal and three-stranded C-terminal β-sheet with a helix (orange). The conserved arginine amino acid (Arg19 in the PHETA1 long isoform, yellow) is far from the F&H motif (magenta); however, it stabilizes the folded domain around the C-terminal helix. (G) GFP-tagged full-length WT PHETA1 or PHETA1R6C were expressed in HeLa cells and tested for interaction with full-length HA-tagged OCRL1. Bound proteins detected by western blotting with the indicated antibodies. 'IP: anti-GFP' refers to the anti-GFP antibody-bound fraction; 'Total' represents the total cell lysate.

Identification and CRISPR-Cas9 targeting of zebrafish pheta1 and pheta2

We identified two PHETA1/2 homologs in zebrafish using in silico sequence searches (see Materials and Methods). We refer to them as Pheta1 (encoded by si:ch211-193c2.2) and Pheta2 (encoded by zgc:153733). All known protein domain structures are conserved between human and zebrafish proteins, including the F&H motif (site of OCRL binding) (Fig. 2A). Like human PHETA1, Pheta1 (but not Pheta2) contains the PPPxPPRR motif for PACSIN2 binding. The neighboring genes of the human PHETA1 (SH2B3) and zebrafish pheta1 (sh2b3) are also homologous, indicating that the loci are syntenic and suggesting that zebrafish pheta1 is the most likely ortholog of PHETA1 (Fig. 2B). The zebrafish pheta2 locus

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Protein modeling was performed using the I-TASSER, MUSTER and PHYRE2 servers (Fig. 1F) (Adzhubei et al., 2010; Kelley et al., 2015; Roy et al., 2010; Wu and Zhang, 2008). Based on the homology model, the arginine residue (highlighted in yellow) is far from the OCRL binding site (highlighted in magenta). However, it stabilizes the folded domain around the C-terminal helix, close to the F&H motif, such that the mutation is predicted to disrupt the folded domain and thus may interfere with OCRL binding to PHETA1. To test this, we expressed wild-type (GFP-PHETA1WT) and mutant (GFP-PHETA1R6C) GFP-tagged PHETA1 in HeLa cells, along with hemagglutinin (HA)-tagged OCRL. Surprisingly, we found that HA-OCRL was co-immunoprecipitated by both wild-type and mutant PHETA1 (Fig. 1G). This suggests that the R6C mutation might disrupt PHETA1 protein function in a manner that does not affect OCRL binding. Since the in vivo functions of PHETA1 and its close homolog, PHETA2, were still unknown, we used zebrafish as the experimental system to determine the roles of PHETA1/2 in the context of a vertebrate organism.
lacked obvious synten with either the human PHETA1 or PHETA2 loci (three neighboring genes examined; Fig. 2C). Based on overall amino acid sequence similarity and phylogenetic distance (see Materials and Methods), zebrafish Pheta1 is more similar to mammalian PHETA1/2 (57.3% and 48.9% similarity to human PHETA1 and PHETA2, respectively), whereas Pheta2 is more divergent (44.2% and 39.2% similarity, respectively) (Fig. 2D).

To determine the functions of pheta1 and pheta2 in vivo, we generated mutant alleles of pheta1 and pheta2 utilizing CRISPR-Cas9 genome engineering (Gagnon et al., 2014; Jao et al., 2013) (Fig. 2E). Genomic DNA sequencing showed that the pheta11/2 allele contains a 38 bp deletion after the start codon of pheta1, resulting in frameshift and predicted premature translational termination, suggesting that this is likely a null allele (Fig. 2E,G). The pheta21/2 allele contains an 11 bp deletion in exon 3 of pheta2, also resulting in a frameshift and predicted premature translational termination, suggesting that this is also a null allele (Fig. 2F,H). Sequencing of purified cDNA from pheta11/2 and pheta21/2 homozygous animals (pheta11/2 and pheta21/2) confirmed the incorporation of the 38 bp and 11 bp deletion in the pheta1 and pheta2 transcript, respectively. Using RNA sequencing (RNA-seq), we found no significant differences in the amount of pheta1 or pheta2 transcripts among wild-type (WT), pheta1−/− or double mutant (dKO) animals, suggesting that the CRISPR-induced mutations do not induce nonsense-mediated decay of pheta1/2 (Fig. 2I). RT-PCR with primers flanking the CRISPR-Cas9 target site showed that pheta1 and pheta2 mRNA were spliced correctly in the respective mutants, and no splice variants were detected (Fig. 2J,K).

Zygotic pheta1−/− and pheta2−/− animals were viable and fertile, with no external abnormalities during development (not shown). pheta1 and pheta2 transcripts were detected from before zygotic genome activation (one-cell and 512-cell stages) and were present during early development (1-3 days post-fertilization (dpf)) (Fig. S1). The early expression indicates that pheta1 and pheta2 are maternally inherited, and the maternal transcript might compensate for the loss of zygotic transcripts during the early stages of development. We, therefore, focused on the maternal-zygotic pheta1−/−, pheta2−/− and dKO animals (progeny from homozygous mutant mothers), which lacked functional maternal and zygotic transcript during development. We found that maternal-zygotic pheta1−/−, pheta2−/− and dKO animals were viable and fertile as well, with no external abnormalities during development. Therefore, pheta1 and pheta2 are not required for viability and fertility in zebrafish. Maternal-zygotic mutant animals were used for all subsequent studies except for oculomotor function assays, in which zygotic mutants were tested.

**Loss of pheta1 and pheta2 impairs fluid-phase endocytosis**

Previous findings in ocrl-deficient zebrafish and the UDP patient phenotypes suggested that pheta1/2 might regulate endocytosis in the renal system (Oltrabella et al., 2015). We examined renal
endocytosis in the zebrafish, utilizing an established assay in which fluorescent tracers were injected into the common cardinal vein (CCV), followed by filtration and reabsorption into the renal tubular cells lining the pronephric kidney, commonly referred to as the pronephros (Anzenberger et al., 2006; Drummond et al., 1998; Elmonem et al., 2017; Oltrabella et al., 2015). Endocytic uptake into the renal tubular cells can then be analyzed using fluorescent microscopy. We first tested fluid-phase endocytosis and micropinocytosis, using 10 kDa fluorescent dextran as the tracer (Li et al., 2015). Animals were injected at 3 dpf and then categorized into three groups: high uptake, low uptake or no uptake (Fig. 3A). The pheta1 heterozygous (pheta1+/−) and homozygous (pheta1−/−) animals showed a trend of reduced tracer uptake, compared to the WT control animals, but the difference did not reach statistical significance (Fig. 3B) (Chi-square test; sample sizes are shown in the figure). The dKO animals, however, exhibited a significant reduction in tracer uptake compared to WT controls (P=0.0085). This suggests that pheta1 and pheta2 acted redundantly in fluid-phase endocytosis, such that endocytic deficit was only observed when both proteins were depleted.

To verify that the reduction of 10 kDa dextran uptake in the dKO animals was a fluid-phase endocytosis-specific defect, and not due to the disruption of the glomerular filtration barrier, we tested glomerular filtration in the pheta1−/− and dKO animals by injecting 500 kDa dextran. The 500 kDa dextran is too large to pass through a normally functioning glomerular filtration barrier. Therefore, it is expected to remain in the bloodstream. As shown in Fig. 3C, the 500 kDa dextran was retained in the bloodstream in both the pheta1−/− and dKO animals at 24 h post-injection (hpi), like in the WT controls. Together, these results show that pheta1/2 are required specifically for fluid-phase endocytosis in the renal organ.

Consistent with the in vitro findings that OCRL and PHETA1/2 physically and functionally interact during endosomal trafficking, we found that reduction of zebrafish ocrl gene function significantly exacerbated the fluid-phase endocytosis deficits in dKO animals. To inhibit ocrl function, we used an ocrl anti-sense morpholino (MO) that had been validated by ocrl mRNA rescue and phenotypic similarity to ocrl germline mutants (Coon et al., 2012). To test fluid-phase endocytosis, ocrl MO was injected at the one-cell embryonic stage, and then 10 kDa dextran was injected into the CCV at 3 dpf. Sample sizes are as indicated in Fig. 3D. Injection of 5 ng/ml ocrl MO in the WT animals resulted in severe reduction in dextran uptake, consistent with the previous finding that ocrl deficiency resulted in impaired fluid-phase endocytosis (Oltrabella et al., 2015). Injection of 4 ng/ml ocrl MO in WT animals resulted in a partial reduction in dextran uptake. The same ocrl MO concentration, however, resulted in a more severe reduction in dextran uptake in dKO animals (Chi-square test, P=0.0001) (Fig. 3D). This suggests that ocrl and pheta1/2 functionally interact in zebrafish to enable fluid-phase endocytosis. Interestingly, although ocrl is required for receptor-mediated uptake of the receptor-associated protein (RAP) (Anzenberger et al., 2006; Oltrabella et al., 2015), we found no significant differences among WT, pheta1−/− and dKO animals in RAP endocytic uptake (Chi-square test; sample size shown in figure) (Fig. 3E). Together, these results indicate that pheta1/2 are only involved in a subset of the endocytic functions of ocrl, specifically fluid-phase endocytosis.

**Loss of pheta1/2 disrupts ciliogenesis in the pronephros**

Several in vitro studies have described defects in ciliogenesis after OCRL depletion, and it has been suggested that OCRL regulates protein trafficking to the cilia in a Rab8/PHETA1-dependent manner (Coon et al., 2012; Luo et al., 2012; Rbaibi et al., 2012). To determine if depletion of pheta1 and/or pheta2 affect ciliogenesis or cilia maintenance in vivo, we analyzed the cilia in the pronephros of 3 dpf larvae (Fig. 3F-K). We found that dKO animals had shorter and fewer cilia, similar to the phenotype seen in ocrl-deficient fish (Fig. 3L-O) (Oltrabella et al., 2015). In the dKO, cilia length was reduced in the anterior pronephros (one-way ANOVA with Holm-Sidak post-test; WT, n=15; pheta1−/−, n=11; dKO, n=10; P=0.0058) (Fig. 3F-H), and cilia number and length were both reduced in the posterior pronephros (one-way ANOVA with Holm-Sidak post-test; WT, n=20; pheta1−/−, n=11; dKO, n=12; P=0.0213 for cilia number, P=0.0001 for cilia length) (Fig. 3I-K) (Oltrabella et al., 2015). Cilia diameter was not analyzed (due to the resolution limitations of light microscopy). Since dKO animals exhibited a similar cilia phenotype to that of the ocrl mutants, this further supports the hypothesis that OCRL and PHETA proteins are involved in the same pathway.

We next examined if there was a potential ciliary deficit in other ciliated organs, including the inner ear (macula and cristae), the olfactory placode and the lateral line. We found no disruption of ciliogenesis or cilia maintenance in these tissues analyzed in pheta1−/− and dKO animals (Fig. S2). We also examined the outer segments of photoreceptors, which are specialized cilia that originate from the apical-most region of the inner segment. Staining with rod and cone photoreceptor markers did not reveal any differences among WT, pheta1−/−, pheta2−/− and dKO animals (Fig. S2). This suggests that the role of pheta1/2 in ciliogenesis is restricted to the pronephros.

**pheta1/2 is not required for oculomotor function**

The UDP patient was born with multiple visual complications, including deficits with oculomotor function (e.g. congenital exotropia, amblyopia, nystagmus). To test whether a deficiency in PHETA1/2 contributes to oculomotor deficits, we analyzed the optokinetic response (OKR) in pheta1/2 mutants. Zygotic mutants were used to allow for direct comparisons between siblings, which minimized the confounding effects of genetic background-associated variability in behavioral assays (Gorissen et al., 2015; Lange et al., 2013). OKR is a gaze stabilization response that utilizes the extraocular muscles to stabilize an image on the retina in response to visual motion. It is necessary for maintaining optimal visual acuity and is conserved in all vertebrates (Huang and Neuhauss, 2008). In zebrafish, OKR matures at 3-4 dpf but is commonly tested at 5-6 dpf to ensure the complete development of involving neural and extraocular systems (Easter and Nicola, 1997; Huang and Neuhauss, 2008). We tested 5-6 dpf animals inside a circular arena with projections of moving black and white gratings, the grating directions alternated clockwise and counter-clockwise at different contrasts at 3-s periodicity. We tested siblings in the progeny of pheta1/2 double heterozygous animals (pheta1+/−:pheta2+/−), and the eye velocity in response to the moving gradients was analyzed. No significant differences in eye velocity were observed among WT, pheta1−/−, pheta2−/− or dKO siblings (two-way ANOVA with Holm-Sidak post-test; WT, n=34; pheta1−/−, n=18; pheta2−/−, n=8; dKO, n=10). We then investigated the correlation of velocity and angle between the left and right eyes (Fig. S3B, C). A reduced correlation would suggest strabismus, in which the eyes do not properly align with each other. No significant differences in angle or velocity correlation were observed among WT, pheta1−/−, pheta2−/− or dKO siblings (two-way ANOVA with Holm-Sidak post-test; WT, n=34; pheta1−/−, n=18; pheta2−/−, n=8; dKO, n=10). Thus, the deficiency in pheta1/2 did not affect oculomotor function.
Loss of pheta1/2 disrupts craniofacial morphogenesis

As mentioned previously, PHETA1 and PHETA2 were previously found to be involved in the sorting of lysosomal hydrolases in vitro. Disruption of this pathway in vivo could give rise to craniofacial abnormalities, as seen in lysosomal storage disorders such as MLII (Cathey et al., 2010; Koehne et al., 2016; Kudo et al., 2006). Therefore, we investigated whether craniofacial development was affected by the loss of pheta1/2.
WT, pheta1<sup>−/−</sup>, pheta2<sup>−/−</sup> and dKO animals at 6 dpf were stained with Alcian Blue and Alizarin Red to label the cartilage and bone, respectively. Representative lower jaw images of the WT and mutants are shown (Fig. 4A,B). We did not observe any loss of cartilage or bone structures. There is a reduction in ceratohyal ossification (arrowheads in Fig. 4B), with pheta2<sup>−/−</sup> and dKO

![Fig. 4. Loss of pheta1/2 disrupts craniofacial development.](image)
animals lacking the ceratohyal bone collar at 7 dpf (Fig. S4A). This suggests a developmental deficit in the ceratohyal (explored further in the next section).

Overall, the mutants exhibited shorter and narrower jaws. To quantitate this difference, we used a set of morphological parameters previously utilized to describe craniofacial phenotypes in zebrafish larvae, namely cranial distance, ceratohyal distance, ceratohyal length, Meckel’s area, jaw width and jaw length (Fig. 4C-H) (de Penita et al., 2016). Together, these measurements informed us about the overall length and proportions of the head, as well as the growth of an individual cartilage structure (ceratohyal). In the pheta2−/− and dKO animals, we found a significant reduction in all of the above parameters. In the pheta1−/− animals, only the Meckel’s area, jaw width and jaw length were reduced (one-way ANOVA with Holm-Sidak post-test; WT, n=11; pheta1−/−, n=16; pheta2−/−, n=16; dKO, n=16; cranial distance: P<0.0001 for WT versus pheta2−/− and WT versus dKO; ceratohyal distance: P<0.0001 for WT versus pheta2−/−, P=0.0006 for WT versus dKO; ceratohyal length: P=0.0066 for WT versus pheta2−/−, P=0.0001 for WT versus dKO; Meckel’s area: P=0.0496 for WT versus pheta1−/−, P=0.0001 for WT versus pheta2−/−, P=0.0004 for WT versus dKO; jaw width: P=0.0084 for WT versus pheta1−/−, P=0.0001 for WT versus pheta2−/− and WT versus dKO; jaw length: P=0.0132 for WT versus pheta1−/−, P=0.0057 for WT versus pheta2−/−, P=0.0003 for WT versus dKO) (Fig. 4C-H). Depletion of both pheta1 and pheta2 had an additive effect, indicating functional redundancy during craniofacial development.

**Loss of pheta1/2 disrupts chondrocyte differentiation**

The craniofacial morphogenesis defects observed in pheta1/2 mutants suggest an underlying developmental abnormality. To test this, we first examined the morphology of chondrocytes, which assume a more elongated shape during convergent extension (Kimmel et al., 1998; Le Pabic et al., 2014). If there is a delay in early chondrocyte differentiation, the chondrocytes can persist with more rounded morphology, with a long-axis/short-axis ratio closer to one (Flanagan-Steet et al., 2009; Le Pabic et al., 2014; LeClair et al., 2009). To test this, we measured the long-axis/short-axis ratio of individual cells in flat-mount preparations of the lower jaw (Figs 4B and 5A). One ceratohyal cartilage per animal was imaged, and all cells within the ceratohyal cartilage were measured manually in the Fiji software. We found significant reduction in the elongation of chondrocytes (lower ratio) in the pheta1−/−, pheta2−/− and dKO animals, suggesting a delay in chondrocyte differentiation (Fig. 5B) (one-way ANOVA with Holm-Sidak post-test; WT, n=106 cells from three animals; pheta1−/−, n=137 cells from four animals; pheta2−/−, n=127 cells from three animals; dKO, n=152 cells from four animals; P=0.0439 for WT versus pheta1−/−, P=0.0178 for WT versus pheta2−/−, P=0.0167 for WT versus dKO).

Next, we examined the expression of various markers that characterize the sequential stages of development. During chondrocyte differentiation, there is a decline in TGF-β-signaling, and thus a decrease in Smad2/3 and Sox9 transcriptional regulators. At the same time, there is a coordinated change in the extracellular matrix protein composition as the chondrocytes mature (Flanagan-Steet et al., 2016; Goldring et al., 2006). Type II collagen (Col2, encoded by col2a1a) is one of the earliest markers of chondrocyte differentiation (Goldring et al., 2006). Col2 expression decreases as chondrocytes become more mature at 4 dpf (Flanagan-Steet et al., 2016). Immunostaining for Col2 at 4 dpf showed that pheta2−/− and dKO animals exhibited a striking increase in Col2 expression compared to WT controls, while the pheta1−/− animals had a modest increase (Fig. 5C-E) (one-way ANOVA with Holm-Sidak post-test; n=10 animals per genotype; ceratohyal: P=0.0498 for WT versus pheta1−/−; P<0.0001 for WT versus pheta2−/− and WT versus dKO; Meckel’s: P=0.0001 for WT versus pheta1−/−, P<0.0001 for WT versus pheta2−/− and WT versus dKO). This is consistent with our morphological measurements, in which pheta2−/− and dKO animals exhibited a more severe deficit in overall craniofacial development compared to pheta1−/− animals.

Given the upregulation of Col2, we asked whether other markers of chondrocyte differentiation were affected by the loss of pheta1/2 (Flanagan-Steet et al., 2016). We examined two later-stage markers, acana and dcn, which encode the protein cores (Aggrekan and Decorin) of the chondroitin sulfate proteoglycan extracellular matrix. For earlier-stage markers, we looked at col2a1a (encodes Col2) and sox9a (encodes Sox9). The Sox9 transcription factor is required for early chondrocyte differentiation, but sustained Sox9 expression can inhibit later stages of development (Akiyama et al., 2004; Furumatsu et al., 2005; Yan et al., 2002). Using RNA-seq, WT transcript levels were compared to those from pheta1−/− and dKO (5 dpf). Two-way ANOVA with Holm-Sidak post-test was used for statistical analysis (n=3 pools of animals for each genotype). If the pheta1/2 mutants have more immature chondrocytes, then they would have lower expression of acana and dcn and higher levels of col2a1a and sox9a. We found that the expression of acana and dcn was not significantly changed in the pheta1−/− and dKO groups, compared to WT (Fig. S4B,C). There was a significant increase in col2a1a expression in pheta1−/− and dKO groups, consistent with our Col2 immunostaining findings (Fig. S4D) (P=0.01 for WT versus pheta1−/− and WT versus dKO). This indicates that increased levels of Col2 may be due to increased col2a1a mRNA levels. We found no significant changes in sox9a transcript levels (Fig. S4E). *In situ* hybridization also revealed no significant differences in sox9a expression in the lower jaw among 4 dpf WT, pheta1−/−, pheta2−/− and dKO animals (Fig. S4F).

Together, we find evidence of delayed chondrocyte differentiation, as evidenced by rounded cell morphology and increased expression of Col2/col2a1a. The fact that other markers of chondrocyte differentiation (acana, dcn and sox9a) were unaffected suggests that loss of pheta1/2 does not globally alter the differentiation program.

**pheta1/2 regulates Col2 expression through cathepsin K**

Extracellular matrix remodeling and homeostasis during chondrogenesis and osteogenesis are dependent on the function of proteolytic enzymes, including cysteine proteinases (known as cathepsins) and metalloproteinases (Holmbeck and Szabova, 2006; Yashuda et al., 2005). For example, dysregulation of cathepsin K causes craniofacial abnormalities in zebrafish models of the lysosomal storage disorder MLI (Flanagan-Steet et al., 2018; Petrey et al., 2012). Since PHETA1/2 was found to be involved in the transport of lysosomal hydrolases from the TGN to the endosomes, we hypothesized that cathepsin dysregulation caused by a deficiency in pheta1/2 might also cause deficits in craniofacial development. To test this, animals were treated with a cathepsin K-specific inhibitor, odanacatib (Od), at 3 dpf and then collected and immunostained for Col2 at 4 dpf (Flanagan-Steet et al., 2018; Gauthier et al., 2008). Od significantly reduced Col2 in the dKO animals at both 25 nM and 50 nM concentrations (Kruskal–Wallis test with Dunn’s post-test; WT, n=5; WT+50 nM Od, n=3; dKO, n=9; dKO+25 nM Od, n=5; dKO+50 nM Od, n=5; ceratohyal: adjusted P=0.0403 and 0.0323 for 25 nM and 50 nM Od, respectively; Meckel’s: adjusted P=0.0020 and 0.0176 for 25 nM and 50 nM Od, respectively), while not affecting Col2 levels in WT (Mann–Whitney U-test) (Fig. 6A-D).
Interestingly, there was no significant Col2 reduction in *pheta1*−/− and *pheta2*−/−, indicating that *pheta1* and *pheta2* may be able to compensate for one another in regulating cathepsin K activity (Mann–Whitney *U*-test; *pheta1*−/−, *n*=6; *pheta1*−/−+50 nM Od, *n*=5; *pheta2*−/−, *n*=5; *pheta2*−/−+50 nM Od, *n*=5) (Fig. S5).

We then tested whether Od could rescue the skeletal phenotypes caused by *pheta1/2* deficiency. To do this, we performed lower-jaw morphological measurements of 4 dpf WT and *dKO* animals with and without exposure to 50 nM Od from 3 dpf to 4 dpf (Fig. 6E). Statistical analyses were performed with one-way ANOVA and Holm-Sidak post-test (WT, *n*=14; WT+50 nM Od, *n*=15; *dKO*, *n*=14; *dKO*+50 nM, *n*=14). Similar to what was found in 6 dpf animals (Fig. 4C–H), *dKO* animals had significantly smaller craniofacial structures, compared to WT (Fig. 6F–L). Specifically, cranial distance (*P*=0.0190), ceratohyal distance (*P*=0.0165), ceratohyal length (*P*=0.0224), ceratohyal thickness (*P*=0.0017) and Meckel’s area (*P*=0.0025) were all reduced in *dKO* animals, compared to WT. These metrics were all rescued by Od (*P*>0.05 for WT versus *dKO*+50 nM Od). Jaw width was the only metric not rescued by Od (*P*=0.0009 between WT and *dKO*, *P*=0.0001 between WT and *dKO*+50 nM Od). Interestingly, Od increased jaw length in *dKO* but not in WT (*P*=0.0021 for WT versus *dKO* treated with Od). The increase in jaw length is not caused by a substantial whole-specimen soft-tissue defect, which was not observed.
Fig. 6. Craniofacial deficits are rescued by Od-mediated inhibition of cathepsin K. (A–D) Mean fluorescence intensity of Col2 immunostaining in the ceratohyal (WT in A, dKO in B) and Meckel’s cartilage (WT in C, dKO in D) of 4 dpf larvae with and without Od treatment. (E) Representative images of larvae stained with Alcian Blue. Scale bars: 200 µm. (F–L) Craniofacial morphological measurements at 4 dpf. The measured parameters are highlighted in red in the schematics. (M) In-gel analyses of BMV109, showing cathepsin activities in WT and pheta1/2 mutants at 4 dpf. Blue lines indicate the lane boundaries. (N,O) Quantitation of the cathepsin K and cathepsin L bands from four experiments. Error=s.e.m. ch, ceratohyal; Ctsk, cathepsin K; Ctsl, cathepsin L; m, Meckel’s cartilage. *P<0.05, **P<0.01, ****P<0.0001.
Together, these results show that the craniofacial deficits in dKO animals can largely be rescued by reducing cathepsin K activity with Od.

Next, we asked if pheta1/2 affects the level of cathepsin K activity. We utilized a cathepsin-specific activity-based probe (ABP), BMV109, to measure global (whole-animal) cysteine cathepsin activity in both WT and pheta1/2 mutants (Flanagan-Steet et al., 2018; Verdoes et al., 2013). The animals were treated at 3 dpf, a period when certain cathepsin activities (e.g. cathepsin K) typically begin to wane in WT animals (Flanagan-Steet et al., 2018). pheta1/2 deficiency did not significantly impact cathepsin L activity, the most prominent cathepsin activity at this stage (Kruskal–Wallis test with Dunn’s post-test; n=5 for all genotypes) (Fig. 6M,N). Cathepsin K activity was also not significantly changed, but there was a trend toward increased activity in pheta2−/− animals and increased variability in dKO animals (Kruskal–Wallis test with Dunn’s post-test; n=5 for all genotypes) (Fig. 6M,O). Together, these results indicate that pheta1/2 may have a specific role in regulating cathepsin K in the cartilage, rather than a global role in cathepsin K activity.

**pheta1R6C exerts a dominant-negative effect on craniofacial development**

One striking clinical feature identified in the UDP patient is abnormal craniofacial development. The patient presented with coarse facial features and facial asymmetry. She also had shorter feet and palms, as well as abnormal dental morphology and malocclusion (Table 1). These phenotypes could be due to cartilage development deficits caused by PHETA1 haploinsufficiency or dominant-negative effects of the R6C allele.

If pheta1R6C was non-functional or partly functional, then ectopic expression of Pheta1R6C in pheta1−/− or pheta1+/− backgrounds should have no effect or partially improve craniofacial development. However, if pheta1R6C was dominant negative, then ectopic expression of pheta1R6C should worsen craniofacial development in the same backgrounds. Thus, we generated two zebrafish transgenic lines, one that ubiquitously expressed an EGFP-Pheta1R6C fusion protein [Tg(ubb:pheta1_R6C-GFP), referred to as Tg(R6C)], and another that expressed EGFP fused with WT Pheta1 [Tg(ubb:pheta1-GFP), referred to as Tg(WT)] (Fig. 7A). Confocal imaging confirmed the broad expression of Tg(R6C) and Tg(WT) (Fig. 7B).

We then tested whether Tg(R6C) and Tg(WT) affected craniofacial development. To mimic the genetic background of the UDP patient, which is heterozygous for the R6C allele, we analyzed the effects of Tg(R6C) and Tg(WT) in the pheta1 heterozygous (pheta1−/+), pheta1−/− background (Fig. 7C-E). We also analyzed the effects of Tg(R6C) and Tg(WT) in the pheta1−/− background to test whether there could be an effect in the absence of functional pheta1 (Fig. 7D,E). We found that, in both pheta1−/+ and pheta1−/− backgrounds, Tg(R6C) significantly reduced jaw width [two-tailed Student’s t-test; pheta1−/+; n=15; Tg(R6C); pheta1−/+; n=16; pheta1−/−; n=13; Tg(R6C); pheta1−/−; n=15; P<0.0001 for pheta1−/+ background, P=0.0004 for pheta1−/− background]. Tg(WT) had no significant effects on jaw width in the pheta1−/+ background [two-tailed Student’s t-test; pheta1−/+; n=15; Tg(WT); pheta1−/+; n=15; pheta1−/−; n=13; Tg(WT); pheta1−/−; n=15] and a weak negative effect in the pheta1−/− background.

![Fig. 7. Pheta1R6C exerts a dominant-negative effect on craniofacial development in the partial or complete absence of Pheta1.](image)
background ($p=0.0418$). These results indicate that the R6C variant likely acts on craniofacial development in a dominant-negative manner. Interestingly, Pheta1R6C impacted the craniofacial morphological parameters that were affected by the loss of both pheta1 and pheta2 (jaw width, Fig. 4G), but not those that were only affected by the loss of pheta2 (cranial distance and ceratohyal length, Fig. 4C,E; Fig. S6) [two-tailed Student’s $t$-test; pheta1+/−, $n=15$; Tg(R6C);pheta1+/−, $n=16$; pheta1−/−, $n=13$; Tg(R6C); pheta1−/−, $n=15$]. This suggests that Pheta1R6C may have a relatively limited capacity to interfere with Pheta2 function.

**DISCUSSION**

The regulation of endocytic trafficking is essential for the development and function of an organism. In this study, we present the first in vivo investigation of the functions of PHETA proteins, which are membrane adaptor proteins for the Lowe syndrome causative protein, OCRL. Using zebrafish as the experimental system, we found that pheta1 and pheta2 were necessary for renal fluid-phase endocytosis and ciliogenesis. Furthermore, we found that loss of pheta1/2 impaired craniofacial development and altered the composition of the cartilage extracellular matrix. Evidence also indicates that cathepsin K dysregulation contributes to the craniofacial deficits caused by pheta1/2 deficiency.

These findings provide insight into the possible pathophysiology of an individual with a de novo R6C mutation in PHETA1. The patient presented with renal and craniofacial phenotypes that were similar to the observed phenotypes in pheta1/2 mutant zebrafish, suggesting that deficiency in PHETA1 contributes to disease. Using transgenic expression in zebrafish, we found that the R6C allele acted in a dominant-negative manner. Together, our results reveal the essential physiological and developmental roles of PHETA proteins and indicate cathepsin proteases as potential targets for PHETA-associated diseases. A summary model is shown in Fig. 7F.

**The roles of pheta1 and pheta2 in renal fluid-phase endocytosis and ciliogenesis**

Loss of pheta1/2 affected the renal fluid-phase endocytosis (of 10 kDa dextran substrate), but not receptor-mediated endocytosis (of RAP) (Anzenberger et al., 2006). In contrast, loss of ocrl in zebrafish resulted in a strong reduction in both types of endocytosis (Oltrabella et al., 2015). Partial knockdown of ocrl in the dKO animals exacerbated the fluid-phase endocytic deficit, indicating that pheta1/2 and OCRL likely function in a common endocytic pathway. These results suggest that pheta1/2 participates in only a subset of the functions of OCRL in vivo. Likely, other F&H motif-containing OCRL adaptor proteins such as APPL1 can partially compensate for the loss of PHETA1/2 (Noakes et al., 2011; Pirruccello et al., 2011; Swan et al., 2010).

The pronephros of dKO animals had fewer and shorter cilia, similar to what was found in ocrl-deficient zebrafish (Oltrabella et al., 2015). However, the ciliogenesis defect in dKO and ocrl−/− animals likely does not account for the endocytosis deficits. First, unabsorbed fluorescent dextran was normally excreted from the cloaca in the dKO animals, indicating that there was no impairment of cilia-directed fluid flow within the pronephros. Second, we did not see the development of renal cysts in any of our pheta1/2 mutants, which is consistent with normal fluid flow. Lastly, even mutants with severe ciliogenesis deficits (e.g. the double bubble mutant) could endocytose dextran normally (Drummond et al., 1998; Liu et al., 2007; Oltrabella et al., 2015). Thus, pheta1/2 likely contributes to fluid-phase endocytosis independently of its role in ciliogenesis.

**A novel role for pheta1 and pheta2 in craniofacial development**

We identified a novel role for pheta1 and pheta2 in craniofacial morphogenesis. Craniofacial development appeared to rely more on pheta2, but depletion of both pheta1 and pheta2 resulted in an additive effect, indicating that pheta1 plays a role as well. In pheta2−/− and dKO animals, we observed features indicative of abnormal chondrocyte differentiation, including abnormal chondrocyte morphology, reduced ceratohyal ossification, changes in marker gene expression and altered extracellular matrix composition (i.e. increased Col2). As a first foray into the underlying molecular mechanisms, we found that inhibition of cathepsin K using the specific inhibitor Od significantly reduced Col2 protein levels in the dKO animals and rescued most of the lower jaw morphological deficits in the dKO animals. This indicates that overactive cathepsin K activity may be the cause of abnormal craniofacial development.

Interestingly, we did not see a consistent global increase in cathepsin K activity using an in vivo activity probe (BMV109), indicating that the dysregulation of cathepsin K activity may stem from changes in a subset of cells within craniofacial structures. Alternatively, there may be clutch-to-clutch differences in compensatory mechanisms to control cathepsin K activity. For example, there may be variability in cystatin activity, which inhibits cathepsin K (Vidak et al., 2019). Cathepsins also regulate growth factor activity in the cellular microenvironment, which may result in stochastic changes and more variability during the course of development. Future studies might explore where active cathepsin K resides as development progresses and how Col2 levels are modulated by protease activity in pheta1/2 mutant animals. It was previously shown that TGF-β signaling is enhanced by mislocalized cathepsin K activity (Flanagan-Stee et al., 2016, 2018; Vidak et al., 2019). Thus, the absence of pheta1/2 could lead to altered TGF-β signaling, which may, in turn, mediate the abnormal craniofacial morphogenesis.

**Investigating the pathogenesis of the UDP patient’s disease**

A primary motivation for understanding the in vivo function of PHETA1/2 was the identification of a patient carrying a de novo PHETA1 mutation. To the best of our knowledge, this patient was the first reported case of human disease associated with PHETA1 or PHETA2 mutation. Although the R6C mutation did not affect interaction with OCRL, it did exert a dominant-negative effect on craniofacial development, even in the absence of endogenous pheta1. Since the R6C mutant can interact with OCRL, it may be able to disrupt the function of OCRL complexes, analogous to how the G59S mutation in dynactin subunit 1 (DCTN1) disrupts the function of the dynein/dynactin complex (Lai et al., 2007). Alternatively, since PHETA1 and PHETA2 can form homodimers and heterodimers, the R6C mutant may bind to and interfere with the normal functions of PHETA1 and PHETA2. Our hypothesis that the R6C mutation resulted in a deficiency of PHETA1/2 function is supported by the overlapping phenotypes between the patient and our zebrafish mutants, specifically in craniofacial development and renal function (Table 1).

When drawing comparisons between zebrafish and human craniofacial phenotypes, it is important to note the relationships between zebrafish lower-jaw elements and human jaw anatomy (DeLaurier, 2019; Mork and Crump, 2015). The first pharyngeal
arg (Meckel’s, palatoquadrate)- and second pharyngeal arch (hyosymplectic, ceratohyal)-derived elements become the lower jaw and craniofacial skeleton in both species. The Meckel’s cartilage gives rise to part of the lower jaw and the inner ear, whereas the ceratohyal gives rise to the styloid process and the hyoid. With this in mind, the deficits we observed in Meckel’s and ceratohyal cartilage could provide plausible explanations to some of the UDP patient’s clinical presentations. Specifically, dental abnormalities and hearing impairments may be linked to deficits in the Meckel’s cartilage, whereas difficulty in tongue movements could be caused by deficits in the ceratohyal, which is a ceratohyal-derived structure (Fig. 7F).

Lastly, we note that the UDP patient has three other de novo mutations considered less likely to be contributing to disease. One variant in DnaJ heat shock protein family (Hsp40) member B5 (DNAJB5; NM_001135004; p.R419H) has inconsistent predictions with SIFT and Polyphen, and occurs in a moderately conserved amino acid, so it is unlikely that this causes the UDP patient’s disease. A second variant, in uridine phosphorylase 1 (UPP1; NM_003364;p.1117V), is seen in 12 normal individuals and is predicted benign by SIFT and Polyphen, so it is unlikely to be pathogenic. The third variant, is in plant homeodomain (PHD)-like finger protein 6 (PHF6; NM_001015877.1; p.Leu244del), which has been associated with X-linked Borjeson–Forsmann–Lehmann syndrome (BFLS; MIM #301900); one female patient has been reported with a loss of function allele and X-inactivation (Turner et al., 2004). X-inactivation studies in our patient showed a skewed pattern, but an association with PHF6 was unlikely due to a lack of phenotypic overlap with BFLS. Furthermore, the variant identified in our patient, unlike a clear loss of function mutation reported in BFLS, leads to an in-frame deletion with no splicing defect (Fig. S7). Identification of additional patients carrying deleterious PHETA1 mutation will help to clarify which phenotypes are more closely associated with PHETA1 deficiency in humans.

Conclusions

In conclusion, we have determined novel in vivo functions of the OCRL adaptor proteins PHETA1 and PHETA2. Deficiency in pheta1/2 resulted in impaired renal physiology and craniofacial development in zebrafish, resembling the renal and craniofacial phenotypes in a UDP patient carrying a dominant-negative allele of PHETA1. The craniofacial deficits in zebrafish pheta1/2 mutants were likely caused by a dysregulation of cathepsin K, which altered the extracellular composition of craniofacial cartilages and craniofacial morphogenesis. These results support the hypothesis that PHETA1 mutation was contributory to disease, but further studies with additional patients will be needed to determine the roles of PHETA1/2 in human disease fully.

MATERIALS AND METHODS

Patient enrollment, consent and sample analysis

The patient (UDP.5352) was enrolled in the NIH UDP (Gahl et al., 2012, 2016, 2015) under the protocol 76-HG-0238, ‘Diagnosis and Treatment of Patients with Inborn Errors of Metabolism and Other Genetic Disorders’, which was approved by the Institutional Review Board of the National Human Genome Research Institute. Written informed consent to publish was obtained from the parents of the patient.

Patient-derived fibroblasts were cultured in high-glucose Dulbecco’s modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS), non-essential amino acid solution, and penicillin-streptomycin with L-glutamine (Thermo Fisher Scientific). Normal adult human sex-matched dermal fibroblasts (ATCC PCS-201-012) were used as controls. Cell cultures were checked regularly for contamination. DNA was isolated using an RNeasy Mini Kit (Qiagen), and first-strand cDNA was synthesized by a high-capacity RNA to cDNA kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. For qRT-PCR, primer pairs specific to the three common isoforms (NM_001177996.1, NM_001177997.1 and NM_144671.4) of human PHETA1 (forward primer, 5’-GAAGAGCGAGCTAGGCTGTG-3’; reverse primer, 5’-GTCAAGGGTTGACGAGAGC-3’) and housekeeping gene POLR2A (forward primer, 5’-CATGTCGAGAAGAATGCA-3’; reverse primer, 5’-GCCGAAGAAGACAGAGCAGC-3’) were PCR amplified and monitored using a CFX96 Touch Real-Time PCR detection system (Bio-Rad). Relative expression of PHETA1 transcripts was normalized to the expression of POLR2A and analyzed using standard delta delta Ct method. qRT-PCR experiments were performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). For splice site analysis of the variant in PHF6 (NM_001015877.1: c.732_744del; p.Leu244del), we amplified the patient cDNA using PHF6-specific primers flanking the site of mutation and subcloned into a plasmid vector using TOPO-TA cloning (Thermo Fisher Scientific), and sequenced according to the manufacturer’s instructions. Recombinant colonies were picked up by blue-white screening and extracted plasmids were sequenced using vector-specific M13 primers.

Zebrafish husbandry

Zebrafish of both sexes and all ages were maintained under a standard protocol in accordance with Institutional Animal Care and Use Committee guidelines at Augusta University, Virginia Tech and Greenwood Genetic Center. All zebrafish used in this study were in a mixed background of AB and TL WT lines (Zebrafish International Resource Center). Sex is not a relevant variable for the stages being used (0-7 dpf), as laboratory zebrafish remain sexually undifferentiated until 2 weeks of age (Maack and Segner, 2003; Wilson et al., 2014). To prevent pigment formation for selected experiments, embryos were transferred to embryo medium containing 0.003% 1-phenyl-2-thiourea (PTU; Sigma-Aldrich) between 18 h post-fertilization (hpf) and 24 hpf.

Mutant and transgenic zebrafish lines

pheta1 (sich211-193c2.2, ZFIN ID: ZDB-GENE-041210-163, Chromosome 5: 9,677,305-9,678,075) was identified by a BLAT search using a human PHETA1 coding sequence against the UCSC zebrafish genome database (Kent, 2002). pheta2 (zgc:155373, ZFIN ID: ZDB-GENE-060825-273, Chromosome 3: 32,821,205-32,831,971) was identified as a paralog of pheta1 in the Ensembl database (Zebino et al., 2018). Neighboring genes of pheta1/2 were identified using the UCSC genome browser (Kent et al., 2002). Phylogenetic tree of PHETA proteins was inferred using the Neighbor-Joining method in the MEGA X software (Kumar et al., 2018). The evolutionary distances were computed using the Poisson correction method. An in silico search for pheta1/2 paralogs was performed, utilizing BLAST and the Comparative Genomics tool in the Ensemble website (ensemble.org). No pheta1/2 paralogs were identified. A search was performed on the UCSC genome database for potential syntetic regions between human and zebrafish. In humans, PHETA1 is adjacent to CUX2, SHZB3 and ATXN2, whereas PHETA2 is adjacent to NAGA, SMDT1 and NDUF4. The genomic regions containing the zebrafish homologs of these genes did not contain any additional PH domain-containing genes in these regions. These findings support the idea that pheta1/2 are the only zebrafish homologs of human PHETA1/2. Protein sequence similarity was calculated using the AlignX module from the Vector NTI 9 software suite (Invitrogen).

Mutants were generated using CRISPR-Cas9 genome engineering, as previously described (pheta1 target sequence, GGAGCCTAGAGAGAGAGCTG; pheta2 target sequence, GGTCTCTGACTATCATGGAG) (Gagnon et al., 2014; Montague et al., 2014). The pheta1/2 allele harbored a 38 bp deletion (frameshift), resulting in the deletion of a Mwol restriction site, which was used to distinguish between WT and pheta1/2 alleles. Genomic DNA flanking the deletion was amplified by PCR, followed by Mwol digestion for 2 h at 60°C (primer sequences 5’-CTCTAACAACAACTAGGGCACGGTGTGAGTA-3’ and 5’-CCGCGACAAGGCGTCTACCACCACTCTCAATA-3’). After Mwol digestion, the cut
WT bands were 230 bp and 300 bp in length, whereas the mutant band was 531 bp (uncut). The *phaeta*1 allelic line harbored an 11 bp deletion, resulting in the deletion of an NlAI restriction site, which was used to distinguish between WT and *phaeta*2 allelic lines. Genomic DNA flanking the deletion was amplified by PCR, followed by NlAI digestion for 2 h at 37°C (primer sequences 5′-GGACGTCATGTTGTTTCTTCT′-3′ and 5′-CATGTTAAA-CATACTTCCGTGATGTC-3′). After NlAI digestion, the cut WT bands were 180 bp and 44 bp in length, whereas the mutant band was 213 bp (uncut).

* tg(ubb:phaeta1-GFP)vt4 and * tg(ubb:phaeta1_R6C-GFP)vt5* transgenic zebrafish lines were generated utilizing the Tol2-transgenesis system (Kawakami, 2007). Coding sequence for EGFP was ligated in frame to the 3′ end of the coding sequence of either the WT (*Pheta1-GFP*) or the patient-specific (*Pheta1_R6C-GFP*), Pheta1 protein, and placed into the Tol2 vector, preceded by the zebrafish ubiquitin promoter from Mosimann et al. (2011). The Tol2-ubb:phaeta1-GFP and Tol2-ubb:phaeta1_R6C-GFP vectors were then injected with Tol2 transposase mRNA into WT zebrafish larvae at the one-cell stage. Potential founders were crossed to WT and *ubb:pheta1-GFP* pheta1 and *ubb:pheta1_R6C-GFP* pheta2 and *ubb:pheta2 vt3* pheta2 during early development. 300 ng of total RNA from 512-cell, 1 dpf, and 3 dpf WT zebrafish was used for first-strand cDNA synthesis (SuperScript III, Thermo Fisher Scientific), followed by PCR amplification (GoTaq G2 Master Mix, Promega). Purified PCR products were sequenced by Sanger sequencing at the Virginia Tech Genomic Sequencing Center. Sequencing results confirmed that the CRISPR-induced deletions 38 bp and 11 bp deletions were incorporated into the *phaeta1* and *phaeta2* transcripts, respectively. To determine the expression of *phaeta1* and *phaeta2* during early development, 300 ng of total RNA from 512-cell, 1 dpf, and 3 dpf WT zebrafish was used for first-strand cDNA synthesis (LunaScript RT SuperMix, NEB), followed by PCR amplification. Primers used were as follows: pheta1, 5′-GGAGAGATGAAAGGAAGAAGAAACCTGC-3′ and 5′-TCTGCTGAAAGAAAGACGATGTGC-3′; pheta2, 5′-ACCTTGTCTCCTGACACAG-3′ and 5′-CTAGCCAGATCATATGGAGGCTCTC-3′; rp4, 5′-GTGCCGACGGTTAATC-3′ and 5′-ACCTGCTCGGATACCAAC-3′.

Whole-mount *in situ* hybridization

*In situ* hybridization was performed using protocols described previously (Pan et al., 2012; Prober et al., 2008). Sense and antisense probes were transcribed from linearized plasmid DNA using a DIG RNA Labelling Kit (Roche). *In situ* probes were synthesized using pheta1 5′UTR sequence (primer sequences 5′-TGGATCCGGAAGAATCAAGGGAG-3′ and 5′-CTCGAGAGCAGACATATGGAGGCTCTC-3′). The *sox9a* anti-sense probes have been described previously (Flanagan-Stee et al., 2016).

**Histochemistry and immunohistochemistry**

Alcian Blue/Alizarin Red staining was performed using the 'No acid' protocol (Walker and Kimmel, 2007). Briefly, after fixation with 2% paraformaldehyde and rinse with 50% ethanol, samples were stained overnight in 0.04% Alcian Blue (Anatoch)/0.01% Alizarin Red S (Sigma-Aldrich)/10 mM MgCl2/80% ethanol. Stained samples were rinsed in 80% ethanol/10 mM MgCl2 for several hours, and washed in 50% and 25% ethanol. After washing, samples were bleached in 3% H2O2/0.5% KOH for 10 min with the cap open, followed by rinsing in 15% and 50% glycerol/0.1% KOH and stored in 50% glycerol/0.1% KOH. For Od treatment experiments, Alcian Blue was not included in the staining solution. Flat mount preparation was performed as described by Javadan and Schilling (2004). Live staining of cartilaginous bone collar was performed as described previously (Flanagan-Stee et al., 2016). Fish at 7 dpf were placed in Eppendorf tubes and stained in 0.05% Alizarin Red/10 mM HEPES pH 7.0 (Fisher Scientific)/E3 for 1 h in the dark, rinsed in 10 mM/10 mM HEPES pH 7.0, and anesthetized in 0.013% tricaine (Fisher Scientific)/E3. Anesthetized animals were mounted face down on an uncoated 50 mm glass-bottom Petri dish (MatTek) in 1.7% low melting agarose (Fisher Scientific) in E3 buffer for imaging.

**Immunohistochemistry**

was performed as previously described (Randlett et al., 2015). Primary antibodies were as follows: anti-acetylated α-tubulin (T6793; Sigma-Aldrich; 1:1000), anti-γ-tubulin (T5326; Sigma-Aldrich; 1:100), anti-Znp1 [ANZNP-1; Developmental Studies Hybridoma Bank (DSHB); 1:25], anti-Zpr1 [zpr1; Zebrafish International Resource Center (ZIRC); 1:100], anti-Zpr3 [zpr3; ZIRC; 1:100], anti-Collagen type II ([II]-II6B3; DSHB; 1:100), anti-GFP (ab13970; Abcam; 1:1000). Alexa Fluor-conjugated secondary antibodies (Life Technologies; 1:500), 4′,6-diamidino-2-phenylindole (DAPI; D1306; Life Technologies; 1:1000) and toto-3 (T3604, Life Technologies, 1:2000) were used after primary antibody incubation. To image Alcian Blue/Alizarin Red-stained samples, individual fish was mounted on a glass slide in 50% glycerol/0.1% KOH and imaged using a Nikon SMZ218 fluorescent stereomicroscope with an image capture system. To image cartilaginous chondrocytes, flat-mount specimens were imaged under bright-field illumination using a Zeiss Axios Imager 1 upright compound microscope with a 20x objective. For fluorescent imaging, animals were mounted on an uncoated 50 mm glass-bottom Petri dish (MatTek) in 1.7% low melting agarose (Fisher Scientific) and imaged with a Nikon SMZ218 fluorescent stereomicroscope or a Nikon A1 laser scanning confocal system with a CFI 175 Apochromat LDW 25× water-immersion objective. The same imaging settings were used for all samples in each experiment.

**Injection of endocytic tracers and analysis**

Lysine-fixable 10 kDa dextran (Alexa Fluor 488 conjugated) or 500 kDa dextran [fluorescein isothiocyanate (FITC) conjugated] (Thermo Fisher Scientific) were prepared in PBS at 2 µg/µl final concentration. In addition, recombinant Cy3-conjugated His-tagged RAP (39 kDa), prepared in PBS at 5 µg/µl final concentration, was kindly provided by Dr Martin Lowe (University of Manchester, Manchester, UK). Zebrafish embryos were anesthetized in tricaine (0.013% w/v; Fisher Scientific) diluted in embryo water at 72 hpf. Approximately 0.5–1 nl of dextran or RAP was injected into the common cardinal vein using a glass micropipette and a pneumatic pressure injector (PL190; Harvard Apparatus) and micromanipulator. Uptake in the renal tubular cells of the proximal pronephros was analyzed at 1–2.5 hpi, using a Nikon SMZ218 fluorescent stereomicroscope with an image capture system. High dextran uptake was defined as >20 fluorescent puncta observed along the proximal pronephros. Low dextran uptake was defined as one to 20 fluorescent puncta observed along the proximal pronephros, and no uptake indicated that no fluorescent puncta were seen. Animals injected with 500 kDa dextran were analyzed at 24 hpi.

**MO inhibition of *ocr1* gene expression**

The *ocr1* translation-blocking MO (a gift from Dr Martin Lowe, University of Manchester) has previously been described (sequence AATTCCCA-ATGAAGGTCCCATCATG) (Coon et al., 2012). MO was injected into embryos at the one-cell stage at 1–5 ng/µl.

**Cilia and craniofacial quantification and analysis**

Cilia in the anterior (just anterior to the yolk extension) and posterior (near the cloacal) portion of the pronephros in the zebrafish larvae were imaged and analyzed. The number of cilia within a 100×100 μm2 area were quantified, and the length of five randomly selected cilia was measured within the area. Craniofacial morphological measurements were performed with Fiji (Schindelin et al., 2012). Type II collagen was quantified by mean fluorescence intensity within a 2500 μm2 area in the cartilaginous cartilage and a 1000 μm2 area in Meckel’s cartilage.

**BMV109 delivery and in-gel analyses**

The BMV109 fluorescent ABP was injected into 3 dpf larvae (1 nl at 10 µM) pericardially via microinjection. This equates to a final global concentration of 10 nM. Probe was circulated overnight at 28.8°C and harvested at 15 hpi. Twenty-five larvae per condition were collected and
lysed in citrate buffer (50 mM citrate buffer pH 5.5, 5 mM DTT, 0.5% CHAPS, 0.75% Triton X-100) by brief sonication. Samples were centrifuged for 15 min at 15,000 g and the supernatant collected. Protein concentration was determined via a micro BCA assay (23235; Thermo Fisher Scientific) and samples run on 4-20% precast gradient gels containing the ‘stain free’ tri-halo compound (Bio-Rad). UV light-activated tri-halo covalently binds tryptophan residues. Equivalent protein loads were evaluated on a Bio-Rad Chemidoc MP Imaging System using this stain-free method. BMV109 Cy5 fluorescence was subsequently analyzed in gel. Total protein load per lane and individual ABP-reactive bands were quantitated using Chemidoc MP software. Individual ABP-reactive bands were normalized to total protein load and the fold difference calculated between WT and MLII samples.

Pharmacological inhibition
Cathepsin K activity was inhibited from 3 dpf to 4 dpf in live embryos by introducing 25 nM and 50 nM Od [solubilized in dimethyl sulfoxide (DMSO)] directly into their growth medium. In all cases, WT control larvae were treated with an equivalent amount of DMSO (0.1%).

Cell culture
HeLa cells (ATCC CCL-2) were grown in DMEM supplemented with 5% FBS (Thermo Fisher Scientific) and 1% penicillin-streptomycin (Life Technologies). Cells were transfected using Effectene (Qiagen) according to instructions provided by the manufacturer. The PHET111 cDNA was synthesized by Geneviz and cloned into the pEGFP-C3 vector (Promega). The R6C mutation was introduced into the construct using a Q5 site-directed mutagenesis kit (New England Biolabs). The pcDNA3-HA-human OCRL plasmid was Addgene plasmid #22207 (http://n2t.net/addgene:22207; RRID: Addgene_22207), deposited by Pietro De Camilli.

Protein-protein interaction
Lysates were prepared from transfected HeLa cells by incubating the cell pellet in RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% NP40, 1 mM EDTA). The lysate was clarified by centrifugation at 10,000 g for 5 min at 40°C. Then, 1× Halt protease inhibitor cocktail (Pierce) was added to the lysate, with 800 μg total protein used per immunoprecipitation. Immunoprecipitation was performed using GFP-Trap beads (Chromotek) in binding buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.2 mM EDTA, 0.05% NP40). The lysate was incubated with beads for 75 min at 40°C. Subsequently, the beads were washed four times using binding buffer. The bound proteins were eluted by boiling in Laemmli buffer, run on a gel and analyzed by western blotting. A monoclonal GFP antibody (JL-8; Clontech) and a monoclonal hemagglutinin (HA) antibody (sc-7392; Santa Cruz Biotechnology) were used in the western blot analyses. The HRP signal was acquired on a Chemidoc MP (Bio-Rad) imaging system.

RNA-seq analysis of transcript abundance in zebrafish
Total RNA from WT, pheta1−/− and dKO larvae (5 dpf, head only) was isolated using an RNA Miniprep Kit (Zymo). Three biological replicates for each group were analyzed, each containing RNA from a pool of ten animals. All samples had an RNA integrity number ≥8.0 and were converted into a strand-specific library using Illumina’s TruSeq Stranded mRNA HT Sample Prep Kit (Illumina, RS-122-2103) for subsequent cluster generation and sequencing on Illumina’s NextSeq. The library was enriched by 13 cycles of PCR, validated using Agilent TapeStation and quantitated by qPCR. Individually indexed cDNA libraries were pooled and sequenced on NextSeq 75 SR to obtain a minimum of 30 million reads/sample. Following sequencing, data were trimmed for both adaptor and quality using a combination of ea-utils and Bitrim (Aronesty, 2013; Kong, 2011). Sequencing reads were then aligned to the genome (Ensembl Danio rerio.GRCz11.92 with and without unplaced contigs) using Tophat2/HiSat2 (Kim et al., 2015) and counted via HTSeq (Anders et al., 2015). Read counts for genes annotated on the unplaced contigs were added to the chromosome-only count summary. QC summary statistics were examined to identify any problematic samples (e.g. total read counts, quality and base composition profiles (+/− trimming)), raw fastq-formatted data files, aligned files (bam and text file containing sample alignment statistics) and count files (HTSeq text files). The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO) (Edgar et al., 2002) and are accessible through GEO series accession number GSE142673 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142673). The expression of pheta1, pheta2 and condrogenes genes (col2a1a, acana, dcn, sox9a) was compared using two-way repeated-measures ANOVA with Holm-Sidak correction.

OKR in zebrafish
VisioTracker 302060 (New Behavior TSE) was used for OKR assay. Eye movements of individual fish were recorded at five frames/s by an overhead CCD camera. Zebrafish larvae were placed in the center of an uncoated 50 mm glass-bottom Petri dish (MatTek) and immobilized in 1.5–2% low melting agarose (Fisher Scientific) in E3 buffer. Agarose around the eye was removed with forceps to allow free eye movement. The dish was then filled with water. To test slow-phase performance under short periodicity, the direction of black and white grating switched every 3 s with grating velocity at 7.5°/s. Each experimental run (trial) was 108 s long and included twelve 9-s phases at varying contrast levels (0.99, 1.0, 0.5, 0.2, 0.1, 0.05, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0). Five to six trials were tested for each animal. Contrast sensitivity and eye correlation were calculated using custom MATLAB scripts (available upon request).

Image processing and statistical analyses
Images were quantified with Fiji (Schindelin et al., 2012), and figures were made in Photoshop (Adobe Systems). All statistical analyses were performed in GraphPad Prism (Version 8). Chi-square tests were used to analyze categorical data from endocytosis assays. For normally distributed data, parametric tests (Student’s t-test or ANOVA) were used. For ANOVA tests, the Holm-Sidak post-test was performed to correct for multiple comparisons. For non-normally distributed data, non-parametric tests (Mann–Whitney U, Kruskal–Wallis) were used. Dunn’s correction was used to correct for multiple comparisons after Kruskal–Wallis tests. All values are expressed as mean±s.e.m., unless otherwise noted. The test was considered significant when P≤0.05.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
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Data availability
The data discussed in this publication are available at GEO under accession number GSE142673.
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