ATP6AP1 deficiency causes an immunodeficiency with hepatopathy, cognitive impairment and abnormal protein glycosylation


The V-ATPase is the main regulator of intra-organellar acidification. Assembly of this complex has extensively been studied in yeast, while limited knowledge exists for man. We identified 11 male patients with hemizygous missense mutations in ATP6AP1, encoding accessory protein Ac45 of the V-ATPase. Homology detection at the level of sequence profiles indicated Ac45 as the long-sought human homologue of yeast V-ATPase assembly factor Voa1. Processed wild-type Ac45, but not its disease mutants, restored V-ATPase-dependent growth in Voa1 mutant yeast. Patients display an immunodeficiency phenotype associated with hypogammaglobulinemia, hepatopathy and a spectrum of neurocognitive abnormalities. Ac45 in human brain is present as the common, processed 40-kDa form, while liver shows a 62-kDa intact protein, and B-cells a 50-kDa isoform. Our work unmasks Ac45 as the functional ortholog of yeast V-ATPase assembly factor Voa1 and reveals a novel link of tissue-specific V-ATPase assembly with immunoglobulin production and cognitive function.
The vacuolar $\text{H}^+\text{-ATPase}$ (V-ATPase) is a ubiquitously expressed protein complex, required for luminal acidification of secretory vesicles to acidify the extracellular milieu, compartments of the endocytic pathway including lysosomes, and of the Golgi apparatus\textsuperscript{12,13}. The V-ATPase consists of two multi-protein domains, $V_1$ and $V_0$. The peripheral $V_1$ domain comprises eight subunits (A–H), is localized in the cytoplasm and hydrolyses ATP. The $V_0$ domain is embedded in the organellar membrane, consists of five subunits (a, d, e, c and c') and harbours the rotary mechanism for proton translocation\textsuperscript{14}. Human disease mutations in V-ATPase core subunits result in distinct clinical syndromes. In 1999, renal tubular acidosis with deafness was the first phenotype linked to the V-ATPase with mutations in the kidney-specific isoforms $\text{ATP6V1B1}$ (MIM 267300) or $\text{ATP6V0A4}$ (MIM 602722) (refs 3–5). In 2000, osteopetrosis (MIM 259700) was linked to the V-ATPase by identification of mutations in $\text{TCIRG1}$, encoding the osteoclast-specific $a3$ subunit\textsuperscript{6,7}. In 2008, mutations were found in $\text{ATP6V0A2}$ in a subgroup of cutis laxa syndromes with abnormal protein glycosylation, autosomal recessive cutis laxa type II (MIM 219200&278250), $\text{ATP6V0A2}$ encodes the a2 subunit, which localizes the V-ATPase complex to the Golgi apparatus\textsuperscript{8}.

In addition to the core V-ATPase subunits, two accessory proteins are known in vertebrates, that is, $\text{ATP6AP1}$ (also known as Ac45) and $\text{ATP6AP2}$ (also known as (Pro-) renin receptor)\textsuperscript{9}. In vertebrates, Ac45 is ubiquitously expressed with the highest levels in neuronal and (neuro-) endocrine cells and osteoectasts\textsuperscript{10–13}. This accessory subunit of the proton pump guides the V-ATPase into specialized subcellular compartments such as neuroendocrine regulated secretory vesicles\textsuperscript{14} or the ruffled border of the osteoclast\textsuperscript{10,16,17} thereby regulating its activity. Moreover, the Ac45 protein is involved in membrane trafficking and Ca\textsuperscript{2+}-dependent membrane fusion\textsuperscript{18}. V-ATPase assembly has been extensively studied in yeast, where Vma21, Vma12 and Vma22 cooperate in the assembly of the $V_0$ domain in the endoplasmic reticulum (ER) membrane\textsuperscript{19–23}. Additionally, yeast Voa1 has been established as an ER-localized $V_0$-assembly factor in 2008. However, no human orthologue has been identified so far\textsuperscript{24}. In human, V-ATPase assembly is hardly studied, and no yeast orthologue of human Ac45 has thus far been identified\textsuperscript{25–27}.

In this study, we describe a novel $\text{ATP6AP1}$-linked immuno-deficiency and identified disease mutations in $\text{ATP6AP1}$ in 11 male patients with abnormal protein glycosylation. Yeast V-ATPase assembly factor Voa1 was predicted to be homologous to Ac45, which was confirmed by functional complementation of Voa1 mutant yeast with the processed C-terminal domain of Ac45. Identification of different Ac45 protein isoforms in human brain, liver and B cells indicated the presence of tissue-specific regulation of organelle acidification.

Results

Identification of mutations in X-linked $\text{ATP6AP1}$. In our cohort of unsolved patients with deficient glycosylation of proteins, we performed exome sequencing to identify the causative gene defect. Exome sequencing of a male patient (individual 1.1, Table 1) was performed as previously described\textsuperscript{28}. After filtering out poor-quality variants as well as common and synonymous variants (see Methods section), 131 rare missense variants were selected. Based on a recessive inheritance model, two candidate genes remained (Supplementary Table 1): X-linked $\text{ATP6AP1}$ with hemizygous variant and $\text{KPRP}$ on Chr1 with compound heterozygous variants. Of these variants, the c.1284G $\rightarrow$ A variant in $\text{ATP6AP1}$ on chrXq28 showed the highest level of conservation (PhyloP 46-way, 5.1) and was predicted to be pathogenic by Sift, Polyphen-2 and MutationTaster. Moreover, no potentially pathogenic variants were identified in $\text{KPRP}$ in the WES data of patients 2.1 and 6.1. $\text{ATP6AP1}$ encodes the accessory subunit Ac45 of the V-ATPase complex\textsuperscript{13}, the proton pump that has been linked with abnormal glycan processing in the Golgi via mutations in its core subunit $\text{ATP6V0A2}$ (ref. 8). Sanger sequencing confirmed the hemizygous missense mutation (c.1284G $\rightarrow$ A, p.M428I) in the patient as well as in two affected male family members that became known during the sequencing process (Fig. 1). All maternal alleles showed heterozygosity and healthy males were hemizygous wild type (Supplementary Fig. 1A), confirming complete segregation of the c.1284G $\rightarrow$ A mutation with disease in agreement with X-linked inheritance. Exome and Sanger sequencing of $\text{ATP6AP1}$ in a cohort of unsolved male patients with abnormal protein glycosylation revealed additional mutations in eight patients from five families (Table 1, Supplementary Fig. 1B). Patient 2.1 showed a c.431T $\rightarrow$ C (p.L144P) missense mutation, heterozygous in the mother and absent from the father and a healthy sister. An additional hemizygous missense mutation (c.1036G $\rightarrow$ A, p.E346K) was identified by Sanger sequencing in three non-related male sib pairs (families 3–5). Fathers carried wild-type alleles and mothers were heterozygous for the c.1036G $\rightarrow$ A variant, in agreement with X-linked inheritance. Exome sequencing of patient 6.1 revealed a c. 938A $\rightarrow$ G (p.Y313C) missense mutation.

All four missense mutations (L144P, Y313C, E346K and M428I) affect amino acids that are highly conserved down to fruitfly, tetraodon and frog (Supplementary Fig. 1C). Ac45 homologues in more distantly related species could not readily be retrieved by use of standard BLAST searches. L144P is located in the N-terminal domain, while Y313C, E346K and M428I are located in the processed C-terminal domain of Ac45 (Fig. 1b).

Clinical phenotype of Ac45 deficiency. The dominating clinical symptoms displayed by the present cohort of patients with various $\text{ATP6AP1}$ mutations (Table 1) include hepatopathy and immune abnormalities. Recurrent bacterial infections were associated with hypogammaglobulinemia, ranging from plantar abscesses and gastrointestinal infections in family 1 to multiple episodes of childhood pneumonia and purulent otitis media in families 2–6. Several patients were successfully treated with intravenous immunoglobulins. Of note, some of the patients responded very poorly to childhood vaccinations. Hepatopathy ranged from mild hypertransaminasemia to cirrhosis and end-stage liver failure. In addition, gastric problems were noted in the majority of patients and laboratory abnormalities included leukopenia, slightly elevated serum transaminases, low serum copper and ceruloplasmin, and high alkaline phosphatase. The two brothers from family 4 displayed high-normal levels of IgD $\div$/CD27 − naïve B cells and lowered levels of IgD $\div$/CD27 + intermediate and switched memory B cells, suggesting a problem in B-cell differentiation.

Patients with the p.E346K substitution (families 3–5) showed in addition splenomegaly, abnormal hepatic histology and neurological symptoms such as epilepsy, mild intellectual disability and behaviour abnormalities. Muscle weakness with mildly elevated serum creatine kinase (CK) was demonstrated in a few patients, including patient 6.1. Of note, the presence of the p.E346K mutation predicted a more severe phenotype within the $\text{ATP6AP1}$ disease spectrum, which is in accordance with the early death of two patients (3.2 and 5.1) due to liver failure. Patients from families 1, 2 and 6 display a milder disease course and are currently doing well without cognitive impairment.
The index family (substitution p.M428I) presented with a milder disease course (oldest patient 34 years of age). All three patients in this kindred presented with sensorineural hearing loss to various extents and hypertonia. The grandmother also presented deafness at older age. The vision abnormality was documented also in patients 3.1 and 4.1 (E346K substitution). Female carriers did not show any obvious clinical symptoms. In the total cohort of patients, only minor clinical symptoms were observed that overlap with symptoms from known genetic defects in the V-ATPase, including deafness (ATP6V1B1 and ATP6V0A4) and muscle weakness, as reported for X-linked Myopathy with Excessive Autophagy (XMEA, MIM 310440) due to ATP6V0A4 defects in the V-ATPase, including deafness (ATP6V1B1 and ATP6V0A4) and muscle weakness, as reported for X-linked Myopathy with Excessive Autophagy (XMEA, MIM 310440).

Liver biopsy findings. Liver biopsy was performed in six patients and was (near)-normal for patients 1.3 and 2 (with the substitutions p.M428I and p.L144P, respectively), but revealed steatosis, fibrosis and even micronodular cirrhosis in patients with the p.E346K mutation (Table 1, Supplementary Fig. 2). Electron microscopy was performed in a liver tissue specimen of patient 3.1 (Fig. 2). Hepatocytes showed variable transcellularity due to the presence of noticeable proliferating smooth endoplasmic reticulum, and of numerous alpha-glycogen monoparticles, accumulated within the cell centre. At the periphery, mitochondria were disposed along the plasma membrane (in a mode similar to that observed in glycogen storage diseases). Mitochondria were of usual size but frequently showed cristolysis and occasional absence of cristae. The dense-matrical bodies were preserved. No intra-mitochondrial crystals were noticed. Within hepatocytes, fat globules with the typical shape and size of triglycerides were seen, in variable amounts (Fig. 2a, Supplementary Fig. 2). Their sizes varied from small (1.5–6.0 nm) to medium (8.0–20 nm) and rarely large (21–40 nm). Lysosomes (single limit membrane) were identified as typical (Fig. 2b, arrows) and atypical lipofuscin bodies. The latter showed a central electron-lucent accumulation with a reticular network (Fig. 2c, arrows). Mitochondria were seen occasionally within phagosomes (autophagocytosis, Fig. 2d, arrow). No dilated or enlarged Golgi apparatus was detected in hepatocytes.

Ac45 deficiency alters Golgi processing of protein glycans. Analysis of protein N-glycosylation in serum showed abnormal profiles of transferrin in all studied patients (Fig. 3a, Supplementary Table 3). In addition, mucin type O-glycosylation of serum apolipoprotein CIII was abnormal in most patients, showing decreased sialylation. This combination of abnormal profiles is comparable with other defects of Golgi homeostasis, such as ATP6V0A2-CDG (ref. 8). In patients 3.2 and 5.1 normal mucin type O-glycosylation was observed with even increased sialylation in patient 5.1. This might complicate recognition of Ac45-deficient patients as a genetically determined glycosylation disorder, since highly similar profiles are observed in patients with non-specific liver disease. Considerable variation in glycosylation abnormalities was observed in some of the

### Table 1 | ATP6AP1-deficient patients: genetic, clinical and laboratory data.

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<th>Individual</th>
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<th>Leucocytosis</th>
<th>Increased transaminases</th>
<th>Liver serum copper</th>
<th>N</th>
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IVIG, intravenous immunoglobulin; ND, no data; m, months; wks, weeks; y, year; N ~ O, borderline abnormal O-glycosylation.
patients tested (3.1 and 3.2). Mass spectrometry of total serum N-glycans revealed minor accumulations of truncated glycans (Fig. 3b) and no overlapping glycan signature could be regarded as specific for Ac45 deficiency. Mass spectrometric analysis of isolated transferrin revealed a clear accumulation of similar types of truncated glycans lacking galactose and sialic acid in all patients (Fig. 3c, Supplementary Fig. 3).

Differential processing of Ac45 in liver, brain and B cells. We studied ATP6AP1 expression in human fetal and adult tissues. Both in fetal (data not shown) and adult tissues, the highest Ac45 mRNA expression was found in brain and the lowest expression level in liver and duodenum (Supplementary Fig. 4). To study Ac45 expression at the protein level, we performed western blot analysis of mouse cortex, human brain and liver, and human B cells using an Ac45 antibody directed to the C-terminal half of mouse Ac45. Ac45 is synthesized as a 62-kDa precursor protein (intact-Ac45) that in neuronal and neuroendocrine cells is subsequently processed to its ~40-kDa cleaved form (cleaved-Ac45) (refs 12,13,15,30, Fig. 4a). In mouse and human brain, most Ac45 protein was present in its cleaved ~40-kDa form, with human Ac45 migrating slightly faster than its mouse counterpart (Fig. 4b, lanes 1 and 3). Furthermore, and in-line with earlier studies in Xenopus neuroendocrine cells31, these proteins were N-glycosylated as shown by their sensitivity towards endoglycosidase PNGaseF (Fig. 4b, lanes 2 and 4). These results are in agreement with the slightly lower molecular mass of human Ac45 as compared with mouse Ac45 and the presence of one extra N-glycan on mouse Ac45. In addition, in human brain a thus far unknown 50-kDa form was observed. In human and mouse liver, considerable Ac45 protein expression was observed, predominantly as the 62-kDa intact proteoform. Under the conditions used, this band was insensitive to PNGaseF treatment (Fig. 4b, lanes 5 and 6). Western blot analysis of primary B-cell isolates as well as B-cell lines (data not shown) revealed Ac45 as an ~50-kDa protein isoform (Fig. 4c). Analysis of Ac45 protein expression in patient liver biopsy material revealed a strong reduction in the expression of the ~62- and ~40-kDa Ac45 variants and an additional ~50-kDa protein was observed (Fig. 4d).

Subsequently, we performed newly synthesized protein labeling with 35S methionine in immortalized human hepatocytes.

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**Figure 1 | Overview of identified ATP6AP1 mutations.** (a) Pedigree of index family 1. Black arrow (†) indicates the index patient P1.1 with the mutation c.1284G>A (p.Met428Ile). (b) ATP6AP1 gene, located on chromosome Xq28, and its gene structure consisting of 10 exons. All mutations identified in the six families are indicated in boxes. Domain structure of Ac45 as published on uniprot.org for human Ac45 (http://www.uniprot.org/uniprot/Q15904; Entry version 146, 07 January 2015). CS, furin proteolytic cleavage site30; SP, signal peptide; TM, transmembrane region. Stars (★) indicate the location of the mutations at the protein level. See also Supplementary Fig. 1.
(IHII, after transfection with GFP (mock) and wild-type human Ac45 complementary DNA. Immunoprecipitation with the Ac45 antibody revealed a dominant ~62-kDa newly synthesized endogenous as well as exogenous Ac45 form (Supplementary Fig. 5A). Tunicamycin treatment of the IHII cells as well as PNGaseF digestion after labelling and immunoprecipitation revealed an ~50-kDa Ac45 form, confirming N-glycosylation of the 62-kDa Ac45 isoform. In addition, EndoH sensitivity indicates the presence of high mannose glycans (Fig. 4e). Transfection of IHII cells with clinically relevant mutant Ac45 constructs showed similar expression as wild type (Supplementary Fig. 5B).

The subcellular localization of Ac45 in hepatocytes was studied by immunostaining of IHII cells. Ac45 was found to be mainly localized to the ER, and ER-to-Golgi Intermediate Compartment (ERGIC), but not to the trans-Golgi network (TGN) or components of the endosomal system (Fig. 4f, Supplementary Fig. 6).

**Figure 2 | Ultrastructural studies of a liver biopsy of patient 3.1.** (a) A hepatocyte is surrounded by fat globules of variable size having the typical aspect of triglycerides, × 4,000. (b) Hepatocyte showing relative translucency due to proliferated SER. Arrows point to lipofuscin bodies (lysosomes), × 6,000. (c) Higher magnification showing an atypical lipofuscin body with a central reticulate content (black arrow). White arrow: section through a Golgi apparatus, × 10,000. (d) A mitochondrion is engulfed in an autophagosome (arrow). BC, Bile canaliculus, × 12,000.

**Human Ac45 is orthologous to yeast Voa1 and Big1.** Orthologs of Ac45 were readily identified by BLAST among the metazoans, including nematodes like *Caenorhabditis elegans*, but not outside of that taxon, leading to speculations about a role of Ac45 in specialized and complex vacuolar systems in multicellular eukaryotes25. Nevertheless, the degree of sequence identity between vertebrate and invertebrate members of the protein family is relatively low, suggesting a high rate of sequence evolution as an alternative explanation for the inability to detect non-metazoan homologues. Using orthology prediction at the level of sequence profiles32, we detected two *S. cerevisiae* Ac45 homologues: Voa1 and Big1. Voa1’s C-terminal transmembrane helix is significantly similar to the C terminus of Ac45 (E = 9.1e – 5) (Fig. 5a), while the sequence similarity of Ac45 to Big1 (E = 3.6e – 10) is mainly restricted to the N-terminal ~250 amino-acid residues of Ac45 and therewith coincides with the part of Ac45 that is proteolytically cleaved by furin33. No significant sequence similarity could be detected in the dotted lines, or for the comparison of the C-terminal helix of Big1 with Ac45. Both proteins are located in the ER membrane, where Voa1 has been implicated in assembly of the V0 domain of the V-ATPase24 while Big1 is essential for beta 1,6 glucan synthesis33. Phylogenetic analysis shows that BIG1 and VOAI appear the result of a gene duplication in the Saccharomyces phylum of the Fungi (Supplementary Fig. 7B), indicating that both are orthologous to Ac45. Interestingly, the evolutionarily conserved residues between Voa1 and Ac45 are at a distance of 3-4 amino acids from each other (Fig. 5a) and concentrate on one side of the predicted transmembrane helix (Supplementary Fig. 7A), potentially forming a conserved interaction interface. Using iterative profile-based homology searches34, we confirmed the homology between the Ac45 and Voa1, Big1, and also detected homologues of Ac45 in major taxa of the eukaryotes, amoebozoa (*Dictostelium discoideum*), brown algae (*Ectocarpus siliculosis*) and plants (Supplementary Fig. 7C). Ac45 orthologs in the model species *Arabidopsis thaliana* (AT3G13410) and *Schizosaccharomyces pombe* (*S. pombe*) (SPCC306.06c) have, like Ac45, Voa1 and Big1, been observed in the ER35,36, indicating that the evolutionary origin of Ac45 lies at the root of the eukaryotes.

**Processed Ac45 functions in place of Voa1 in *S. cerevisiae*.** In yeast, Vma21 and Voa1 are assembly factors of the V-ATPase V0 domain in the ER membrane. Both are retained in the ER via a C-terminal dilysine motif. When this motif is mutated to diglutamine, the resulting Vma21QQ or Voa1QQ protein is mislocalized to the vacuole with concomitant reduction in V-ATPase assembly and activity. The effect on V-ATPase assembly is cumulative, becoming most apparent when Voa1 is absent (voa1::H) or Voa1QQ is expressed in vma21QQ cells24. Yeast lacking functional V-ATPase have a characteristic growth phenotype: they are unable to grow on medium buffered to pH 7.5, or medium containing elevated levels of calcium, or a combination of the two stresses24,37,38. Reduced V-ATPase function can be detected by reduced growth under any of these conditions. A growth assay on rich medium supplemented with 100 mM CaCl2 was used to assess the ability of human Ac45 to substitute for Voa1 (Fig. 5b,c). Full-length or processed Ac45 proteins with or without a dilysine motif (KKNN) appended to the C terminus were expressed in *S. cerevisiae* (Supplementary Table 3), and *voa1::H vma21QQ* cells24. When this strain was grown in minimal medium containing 0.5 µM CaCl2, ~50% of the cells produced colony-forming units (CFUs), indicating that processed Ac45 has a partial substitutive activity in place of Voa1 (Fig. 5d).

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transformed with empty vector or cells expressing Voa1QQ. Adding a dilysine motif to full-length Ac45 did not significantly improve growth. Conceivably, Ac45 function is dependent on proper processing of the protein, which might not be accomplished in yeast. Therefore, simulating a processed Ac45 protein\textsuperscript{12,14,15}, the C-terminal half of Ac45 was expressed. This processed Ac45 was able to function in place of Voa1, but only when expressed with a dilysine motif (cleaved-Ac45-KKNN in Fig. 5c). By complementation, cleaved-Ac45-KKNN function is comparable to that of Voa1.

Figure 3 | Glycosylation studies. (a) Routine screening for N-glycosylation by isofocusing of serum transferrin and for mucin type O-glycosylation by isofocusing of apolipoprotein CIII. The numbers on the y axis mark the number of sialic acids, each column shows the profile of the respective control (C) or patient. (b) Analysis of total serum protein N-glycans by MALDI mass spectrometry. (c) Analysis of intact serum transferrin by nanoLC-chip-QTOF mass spectrometry. Glycans are synthesized from individual monosaccharide building blocks: a purple diamond represents one sialic acid, a yellow circle one galactose, a green circle one mannose, a blue square one N-acetylglucosamine and a red triangle one fucose. See also Supplementary Fig. 3 and Supplementary Tables 2 and 3.
**Figure 4 | Differential expression of the Ac45 protein in human brain, liver and B cells.**  
(a) Schematic representation of the human Ac45 protein. CS, furin proteolytic cleavage site; SP, signal peptide; TM, transmembrane domain; † represent predicted N-glycan structures, whereas the structures shown in black (†) are the experimentally confirmed glycans. (b) Western blot analysis of Ac45 in mouse cortex and in human brain and liver. Asterisk (*) indicates the deglycosylated form of cleaved-Ac45. Hash tags (#) indicate non-specific antibody reaction with PNGaseF present in the samples. (c) Western blot analysis of Ac45 in primary B cells from healthy controls in comparison with human liver. One of the two representative analyses is shown. (d) Western blot analysis of Ac45 in liver tissue homogenates of control and patient 4.2. GapdH was used as loading control. (e) Analysis of newly synthesized Ac45 in immortalized human hepatocytes (IHH). Cells were transfected with Ac45 construct, pulsed for a 30-min period with 35S, and Ac45 was immunoprecipitated and analysed by SDS-PAGE. Cells were treated with or without tunicamycin during the 30-min pulse (left panel). Immunoprecipitated Ac45 protein was treated with or without Endo H or PNGaseF (right panel). Note during the 30-min pulse period, the presence of a minor portion of newly synthesized pre-intact-Ac45 protein is still in its unglycosylated proform and containing the signal peptide for translocation over the ER membrane. (f) IHH cells were stained with anti-Ac45 antibody (green) and antibodies against various organelle markers (magenta). Nuclear staining is shown in blue (DAPI). Co-localization is indicated by a white colour in the merged channel. The graph shows the fluorescent intensity profile along the cross-section indicated. Scale bar represents 10 µm.
Figure 5 | Identification of Voa1 as the yeast ortholog of human Ac45. (a) Overview of the regions of Ac45 that are homologous to the yeast proteins Voa1 and Big1 and an alignment of Ac45’s and Voa1’s C-terminal transmembrane helices (in blue, based on TMHMM63) and their flanking amino acids. Ac45 and Voa1 are separated by a sequence logo representation of this region among all the homologs that could be detected using JACKHMMER64. A pattern in which the level of sequence conservation in the transmembrane helix peaks every 3–4 amino acids is indicated with arrows. (b) Schematic of Voa1 and Ac45 proteins expressed from centromere plasmids in voa1::H vma21QQ yeast24. Ac45 proteins are either full length (intact-Ac45) or processed (cleaved-Ac45), with (shown) or without KKNN appended to the natural C terminus. Numbers indicate amino-acid residues. Residues mutated in Ac45 are shown. (c) Cleaved-Ac45 can substitute for Voa1 when a C-terminal dilysine motif is present. The voa1::H vma21QQ strain was transformed with plasmids coding for the indicated proteins (HA-tagged, diagrammed in (b)). Voa1QQ denotes Voa1 with K262Q and K263Q mutations. (d) The Y313C or E346K mutation in cleaved-Ac45-KKNN reduces V-ATPase function while protein levels are unaffected. Serial dilution growth test of voa1::H vma21QQ yeast expressing the indicated proteins tagged with HA. Restrictive growth is on rich medium adjusted to pH 7.5 and supplemented with 60 mM CaCl2. Membrane proteins prepared from the same cultures used in the growth test were analysed by western blot using anti-HA antibody to detect Voa1, cleaved-Ac45-KKNN and its mutant forms (band locations marked on the right, molecular mass (kDa) is indicated on the left). (e) Voa1 and cleaved-Ac45 require a C-terminal dilysine motif for ER localization. Fluorescent microscopy of live yeast cells showing DAPI stained DNA, GFP, the merged image of both, and cells viewed by differential interference contrast (DIC) to locate the vacuole as apparent indentation. The indicated proteins are N-terminally tagged with HA-GFP and expressed in voa1::H vma21QQ yeast cells. Exposure times for GFP images of cleaved-Ac45 were 10× longer than for Voa1 or Voa1QQ. Perinuclear GFP fluorescence indicates ER localization. Mutated and non-mutated cleaved-Ac45-KKNN show the same localization. See also Supplementary Fig. 7.
Growth assays were used next to measure the effect of three pathogenic Ac45 substitutions, Y313C, E436K or M428I (Fig. 5d, left panels). The mutations were introduced into cleaved-Ac45-KKNN and expressed in *voa1::Hvma21QQ* yeast. Growth was tested on rich medium supplemented with 60 mM CaCl2 and buffered to pH 7.5. Cells expressing Y313C or E436K mutant protein showed a growth defect, with the E436K mutant most severely compromised, exhibiting reduced growth nearing that of yeast having no Voa1. The effect of the M428I substitution was less disruptive and appeared indistinguishable from non-mutated Ac45. To ascertain that the growth defect observed for the Y313C or E436K substitution was not the result of protein instability, membrane proteins from the cells used in the growth assay were examined by western blot (Fig. 5d, right panel). While Ac45 protein levels were lower than Voa1, levels for mutated Ac45 proteins were unchanged compared with non-mutated Ac45 protein. Therefore, reduced V-ATPase function observed for the Y313C or E436K mutation cannot be ascribed to decreased protein abundance.

Since both Voa1 and processed Ac45 require a dilysoine motif for function, it is expected that, like Voa1, processed Ac45-KKNN is retained in the ER membrane, while absence of the motif would result in mislocalization to the vacuole. This was tested using GFP-tagged proteins (Fig. 5e, Supplementary Fig. 8). Though GFP-tagging slightly reduced fitness of processed Ac45-KKNN on restrictive medium (data not shown), dilysoine-dependent ER localization was verified. Together with growth assay results, these results indicate that the human and yeast proteins function about equally well in V0 V-ATPase assembly in the ER.

**Discussion**

Much has been learned about V-ATPase function and assembly by studies in yeast, however, studies in human have been very limited. The clinical symptoms resulting from Ac45 deficiency, mostly affecting the liver, immune system and brain, significantly differ from other known human genetic defects in various V-ATPase subunits. Disease mutations in subunits of the V0 domain result in, respectively, renal tubular acidosis with deafness (*ATP6V0A4* (MIM 602722) (refs 3–5)), osteopetrosis (*TCIRG1* or *ATP6V0A3* (MIM 259700) (refs 3,6,7)) and cutis laxa with abnormal glycosylation (*ATP6V0A3* (MIM 219200&278250) (ref. 8)). One subunit of the V0 domain is linked to human genetic disease (*ATP6V1B1* (MIM 267300)) resulting in renal tubular acidosis with deafness, while previously one of the V-ATPase assembly factors, VMA21, has been linked to XMEA (X-linked myopathy with excessive autophagy (MIM 310440) (ref. 29)). Very recently, mutations in *CCDC115* and *TMEM199* have been observed in patients with XMEA (X-linked myopathy with excessive autophagy (MIM 310440) (ref. 29)). As predicted orthologs of yeast V-ATPase assembly factors Vma22p and Vma12p, respectively, were identified in patients with liver disease, elevated alkaline phosphatase and cholesterol, mild abnormalities in copper metabolism and various degrees of cognitive impairment (refs 39,40). No immune dysfunction was noticed. The combination of clinical symptoms as observed in Ac45-deficient patients is currently poorly understood since research on the functional roles of Ac45 has mainly been focused on neuroendocrine cells and osteoclasts. Certain specific symptoms could be related to other V-ATPase defects or known roles of the V-ATPase complex. These include deafness as reported in family 1, and also reported for *ATP6V0A4* and *ATP6V1B1* defects. In addition, electron microscopy of a liver biopsy of patient 3.1 suggested evidence for enhanced mitochondrial autophagy and muscle weakness with mildly elevated creatine kinase was found in some patients. This could suggest a partially overlapping disease mechanism with VMA21-deficient XMEA patients. Finally, in two of the patients, decreased enamelization of the teeth was reported, which could correspond with a recently reported role of V-ATPase-mediated acidification in enamelization (ref. 41). Thus, likely at least some of the symptoms are related to V-ATPase dysfunction, which is supported by our studies on V-ATPase restricting growth conditions in yeast.

Immune and liver dysfunction have not yet been reported in genetic defects of the V-ATPase, although liver disease was recently described for defects in V-ATPase assembly factors TMEM199 and CCDC115 (ref. 39,40). The question is why these systems are affected. Possible explanations could include the tissue-specific processing of Ac45 or the existence of additional functions of Ac45 beyond pH regulation via its effect on the V-ATPase. Our studies in human hepatocytes show that Ac45, in contrast to what was observed in neuroendocrine cells, mostly localizes to the early secretory pathway. This is in agreement with the presence of mostly intact-Ac45 carrying non-processed high-mannose glycans. Further studies are needed to elucidate the mechanisms driving differential Ac45 glycosylation and processing in brain, liver and immune cells, since tissue-specific forms of Ac45 could suggest a possible mechanism for the tissue-restricted disease symptoms in Ac45-deficient patients.

Thus far, the relationship of Ac45 with immune deficiency has remained unnoticed. In view of the reported multiple functions of Ac45 in, for example, pH regulation and membrane trafficking and fusion (ref. 18), many possible links exist. Acidification of phagolysosomes in, for example, macrophages is important for killing of internalized microorganisms, while antigen processing is also dependent on acidic pH. Our growth assay in yeast under conditions that are dependent on V-ATPase activity support the notion that the patients’ phenotypes could be related to aberrant acidification due to dysfunction of the V-ATPase. Membrane trafficking and fusion events have not only been linked to V-ATPase function (ref. 42–44) but also to Ac45 (refs 18,43). These events are reported to be required for B-cell differentiation (ref. 45), antigen processing (ref. 46,47) and antibody production (ref. 45). Thus, pathogenic mutations in *ATP6AP1* might affect B-cell function at all these levels, resulting in decreased levels of immunoglobulins and recurrent infections in our patients. The observed hypoglycosylation on serum transferrin in our patients might indicate hypoglycosylation on other proteins as well. Several membrane-bound proteins such as CD19 and CD40 that are involved in B-cell activation are glycosylated, and antigen recognition and antibody production by B cells require fucosylated IgG-BCR (ref. 48). A glycosylation defect therefore may affect B-cell activation and thus antibody production. To find out which processes, that is, glycosylation, vesicular trafficking and fusion, or pH regulation are mainly affecting antibody production by a defective accessory subunit of the V-ATPase, further studies are required.

Previous studies have described an important role for Ac45 in intraorganellar pH regulation and membrane trafficking (ref. 10,14–16,30). Identification of processed Ac45 as the functional ortholog of yeast V-ATPase assembly factor Voa1 only when the KKNN ER retention signal is present, indicates the importance of ER localization for its function in yeast, and provides a valuable model to further dissect the different functions and functional domains of Ac45. As the human ortholog lacks this dilysoine motif, other mechanisms might account for retention of Ac45 to the ER of specific cell types such as liver cells. Our observation in liver cells that the Ac45 protein is mostly present in its unprocessed form, which in neuroendocrine cells appears to implicate ER localization (ref. 12,15,18), combined with its observed steady-state localization in the early secretory pathway (ER, ERGIC) in hepatocytes, suggests that differential proteolytic processing might represent such a mechanism.
In summary, the identification of tissue-specific proteolytic processing of Ac45, and the availability of Voα1 mutant yeast as valuable model to further dissect the individual functions of Ac45 will facilitate future research to understand the functional roles and isoforms of Ac45 in the immune system, liver, muscle and brain and its relation to V-ATPase in human. Screening for abnormal protein glycosylation in plasma of patients with hepatopathy and immune dysfunction with or without neurological symptoms provides a rapid way to identify additional individuals with ATP6AP1 deficiency.

Methods

Patients and glycosylation studies. Blood and fibroblasts of patients (clinical information in Table 1) were obtained for diagnostics of inborn errors of metabolism, space biopsies were obtained from patients and reference genome physicians. Isoelectric focusing of serum transferrin for analysis of protein N-glycosylation defects and of serum apolipoprotein CII for analysis of mucin type O-glycosylation defects were carried out as described before53. Plasma N-glycan profiling was performed by MALDI linear ion trap mass spectrometry as filter rod54 using 10 μl of plasma. Briefly, serum was treated with PNGaseF, free N-glycans were permethylated, extracted and dried, purified and spotted onto a MALDI plate. Samples were dried and measured on a linear ion trap mass spectrometer. High resolution mass spectrometry of intact serum transferrin was performed as described55. Briefly, transferrin was immuno-purified from 1 ml serum with an anti-transferrin Sepharose bead. The elution with glycine-HCl pH 2.7 was neutralized by Tris-HCl pH 9.0 and was directly available for injection onto the nanoLC-MS/MS chip of the QTOF. Transferrin was eluted from the chip in a 10 min gradient of H2O and Acetonitrile, 0.1% formic acid. Charge distribution raw data was deconvoluted by Mass Hunter software to reconstructed mass spectra55.

Next-generation sequencing. Genomic DNA was extracted from patient fibroblasts according to the manufacturer’s protocol using a Qiagen Mini kit (Qiagen, Hilden, Germany), and was checked for DNA integrity on agarose gels. Next-generation sequencing and analysis was performed as described56. In brief, exome enrichment was performed using the SureSelect Human All Exon 50 Mb Kit (Agilent, Santa Clara, CA, USA). Colour space reads were iteratively mapped to the hg19 genome with the SOLID LifeScope software version 2.1. Called variants and indels were annotated using an in-house annotation pipeline57,58 and common variants were filtered out based on a frequency of >0.5% in dbSNP (137) and a frequency of >0.3% in our in-house database of >1,300 exomes. Quality criteria were applied to filter variant calls with <5 variant reads and <2% variant read. Furthermore, synonymous variants, deep intronic, intergenic and UTR variants were excluded. Raw data of candidate variants were inspected using the Integrative Genomic Viewer software (IGV browser) version 2.3.14 (2013) (ref. 59) (http://www.broadinstitute.org/igv/download). The putative consequences of the mutations were predicted using the SIFT program (http://sift.jcvi.org/www/SIFT_aligned_seqs_submit.html) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) prediction programs.

Sanger sequencing. Genomic DNA was extracted from fibroblast pellets or white blood cells from 10 patients and available family members. Primers flanking intronic regions. Standard PCR reactions were based on 1 μl DNA and 0.2 μl Platinum Taq polymerase (Invitrogen) in a total volume of 25 μl. Standard reaction conditions were 10 min at 95°C, then 35 cycles of 30 s at 95°C, 30 s at 60°C and 1 min at 72°C. The reaction was completed with a final elongation of 7 min at 72°C. PCR products were sequenced using Sanger sequencing. Terminator Ready reaction cycle sequencing kit v.3.1 (Applied Biosystems) was used. Analysis of the results was performed on an ABI3100 Avant (Applied Biosystems).

ATP6AP1 gene expression profiling in human tissues by qPCR. Total RNA from different human adult and fetal tissues was ordered from Stratagene Europe (Amsterdam, The Netherlands). All fetal tissues are from 20- or 21-week-old embryosExpired embryo. RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocols. To remove residual traces of genomic DNA, the RNA was treated with DNase I (Invitrogen, Leek, The Netherlands) while bound to the RNA binding column. The integrity, concentration and purity of the RNA were assessed using agarose gel electrophoresis and spectrophotometry. Of all tissues, 5 μg of total RNA was transcribed into cDNA by using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s protocol. cDNA was purified by using the NucleoSpin extract II kit (Macherey-Nagel) according to the manufacturer’s instructions. Quantitative PCR quantifications were performed in on the equivalent of 12.5 ng total RNA input using the Sensifast SYBR no ROX qPCR kit (Bioneer) and a Rotor-GeneTM 6000 real-time analyzer (Qiagen). qPCR program used was 2 (min 95°C (5 × 95°C, 10 s at 60°C or 65°C and 15 × 72°C) × 40 cycles). Two pairs of intron-spanning ATP6AP1 primers were used. Primers used are listed in Supplementary Table 4. As reference transcripts, GUSB and PPIB were used. qPCR data were analysed by using comparative quantitation and the relative q-values of the genes of interest calculated by equalizing the lowest Ct value to 1. The normalization factor for the reference genes was determined using the GeNorm program (medgen.ugent.be/genorm) and used to normalize the q-values. Individual experiments were performed in triplicate.

Western blot analysis of Ac45 in cells and tissues. B-cell isolation. Buffy coats from two healthy donors, who gave written informed consent for scientific use of the buffy coats, were purchased from Sanquin Blood Bank, Nijmegen, The Netherlands. Peripheral blood mononuclear cells were isolated by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Roskilde, Denmark). CD19+ and B-cells were positively selected using an anti-CD19 antibody (Becton-Dickinson, Lier, Belgium), Utrecht, The Netherlands. The isolated human donor B cells were lysed in cold lysis buffer (50 mM Hepes pH 7.4, 140 mM NaCl, 0.1% Triton-X100, 1% Tween-20, 0.1% deoxycholate supplemented with complete protease inhibitor mix (Roche Diagnostics)) to a final concentration of around nine million B cells per 20 μl lysisbuffer.

Tissue lysates. Tissue samples were powdered using a vessel and liquid nitrogen and subsequently lysed with cold lysis buffer to a final concentration of 100 mg tissue sample per 500 μl lysis buffer. All samples were incubated on ice for 15 min and repeatedly vortexed during the incubation. Then, the homogenates were centrifuged at 14,000 g for 10 min, after which the supernatants were collected. Subsequent PNGase F treatment was done as follows: supernatant containing 21 μg of protein was incubated for 4–6 h at 37°C with 2.5 μl of 500,000 U ml–1 of PNGase F (New England Biolabs) in a final volume of 34 μl containing 50 mM sodium phosphate buffer pH 7.5 (7 g buffer from PNGase F kit, GIBCO), 3 mg of deoxycholate and 2 μl of protease inhibitor cocktail (Roche Diagnostics). A second incubation was performed overnight at 37°C after adding an additional 1 μl of PNGase F, 0.02 μl of G7 buffer, and 1 μl of protease inhibitor. The next day, the tubes were shortly centrifuged, SDS sample buffer was added, and the samples were boiled for 5 min at 99°C. Samples were separated on 10% SDS–PAGE (7 μg of total protein sample per well) and subsequently transferred to a 0.2-μm polyvinylidene difluoride membrane. After blocking in 5% milk in PBS + 1% Tween-20, the membrane was incubated overnight at 4°C with primary antibody rabbit anti-mouse Ac45 polyclonal #49 antiserum (directed towards A271-T283 and L443-L457 of mouse Ac45, kindly provided by Dr. C. Cremer, Catholic University, Leuven, Belgium) at a dilution of 1:5,000 in blocking buffer. Goat-antibody-HRP secondary antibody (Dako, P0448) at a dilution of 1:5,000 in 2.5% milk in PBS-1% Tween-20 were used for ECL detection. To check for protein loading, the membrane was incubated with mouse anti-GapDH monoclonal antibody (Ab8245-100, Abcam) at a dilution of 1:12,000 in 3% BSA in PBS/0.1% Tween-20 for 1 h at room temperature. Incubation with the antibody (Goat-antibody-mouse-HRP, Dako P0447) was for 1 h at room temperature at a dilution of 1:5,000 in 2.5% milk in PBS-1% Tween-20. Chemiluminescent signals was detected using ECL (Pierce).

Biochemical studies in immortalized human hepatocytes. IHH32 were cultured in gelatin-coated culture flasks in Williams medium E supplemented with 10% FCS, 0.022 U ml–1 insulin and 0.045 μg ml–1 deoxetametasone. Culturing was done at 37°C under an atmosphere of 3% CO2. IHH cell cultures were tested negative for mycoplasma. For Ac45 expression, IHH cells were transfected with a hAc45/pC DNA construct using Lipofectamine LTX (Invitrogen) according to manufacturers’ guidelines.

For immunofluorescence assays, cells were cultured on gelatin-coated coverslips for 3 days and fixed for 1 h by 4% paraformaldehyde in PBS at room temperature. After blocking of residual parafomaldehyde with 50 mM NH4Cl in PBS, cells were permeabilized using 0.1% Triton-X100 in PBS (PBS-T) and incubated with anti-Ac45 antibody (1:1,000) and mouse-anti-EAA1 (BD Biosciences, 1:200), mouse-anti-MEPR (Abcam, 1:200), mouse-anti-sec31a (Santa Cruz, 1:200), mouse-anti-GM130 (BD Transduction Laboratory, 1:200), mouse-anti-PDI (Stressgen, 1:500), mouse-anti-Rab11 (BD Biosciences, 1:200), mouse-anti-ERGIC53 (Santa Cruz, 1:100) and goat-anti-TGN38 (Santa Cruz, 1:100) antibodies in 1% BSA in PBS-T (blocking buffer) for 12 h at 4°C. After washing with PBS-T, cells were incubated for 45 min at room temperature with secondary antibodies: Goat-anti-rabbit-Alexa488 and Goat-anti-mouse-Alexa568 or Donkey-anti-rabbit-Alexa488 combined with Donkey-anti-goat-Alexa568 at a dilution of 1:200 in blocking buffer. After fixation, cells were washed with PBS-T, PBS, MilliQ water and dehydrated using methanol and subsequently mounted in Mowiol containing 2.5 μm DAPI. Imaging was performed using an Olympus FV1000 confocal laser scanning microscope × 63 oil objective. Images were captured with an aspect ratio of 1.200 × 1,200 using the Fluoview version 4.1 software at a scanning speed of 12.5 μs per pixel. Image analysis was performed
Newly synthesized protein labelling experiments using Fiji software. Relative fluorescence intensities were calculated over a 10-μm cross section by setting the highest measured value to 100.

Transmission electron microscopy. The tissue cylinder was immediately immersed in cold 2.5% glutaraldehyde in 0.1 M Mocadode buffer (pH 7.4) for 2 h, post fixed in 1% osmium tetroxide for 1 h, dehydrated through ethanol series and embedded in semi-thin sections [1 μm with 10% gelatin]. Immunolabelling was performed using a biotin Streptavidin technique. The tissue blocks were rehydrated before being deparaffinized and mounted on a 1% agarose on a glass slide for live cell imaging. Images were acquired with a Zeiss LSM 510 confocal microscope (Carl Zeiss) using a 10×0.3 NA lens.

Bio-informatics studies. For sequence-profile-based homology searching, we used HHpred39 with three PSI-BLAST iterations to create the initial sequence profiles. The initial profiles were based either on Ac45 after which that profile was compared to the profiles of S. cerevisiae and S. pombe, on Voa1 after which that profile was compared to the profiles of H. sapiens, or with Big1 after which that profile was compared with the profiles of H. sapiens. The expectation values (E-values) obtained via homology detection using HHpred with the sequence profile based on Ac45 (arrows from Ac45 outwards) and for the reciprocal searches with Voa1/Big1 (arrows towards Ac45) are indicated in Fig. 5a.

Yeast strain and plasmids. Standard molecular biology protocols for E. coli and yeast manipulations were followed37. The vma1::H VMA21QQ yeast strain used in this study is isogenic with SF838-1D 

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