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Received 27 Jul 2015 | Accepted 12 Apr 2016 | Published 27 May 2016

DOI: 10.1038/ncomms11600

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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.

1 Department of Molecular Animal Physiology, Donders Institute for Brain, Cognition and Behaviour, Centre for Neuroscience and Radboud Institute for Molecular Life Sciences, Faculty of Science, Radboud University, 6525 GA Nijmegen, The Netherlands. 2 Department of Neurology, Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, 6525 GA Nijmegen, the Netherlands. 3 Department of Laboratory Medicine, Translational Metabolic Laboratory, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, 6525 GA Nijmegen, The Netherlands. 4 Department of Chemistry and Biochemistry, Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403, USA. 5 Metabolic Unit, Ramah Health Care Center, Rappaport School of Medicine, Technion, 3190601 Haifa, Israel. 6 Department of Human Genetics, Radboud Institute for Molecular Life Sciences and Donders Centre for Neuroscience, Radboud University Medical Center, 6525 GA Nijmegen, The Netherlands. 7 The Milman-David Biomedical Research Unit, 24 Hazevi Avenue, 34355 Haifa, Israel. 8 Department of Laboratory Medicine and Pathology, Mayo College of Medicine, Rochester, Minnesota 55905, USA. 9 Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan. 10 Department of Pediatrics, Nijmegen Centre for Mitochondrial Disorders (NCMD), Radboud university medical center, 6525 GA Nijmegen, The Netherlands. 11 Protein Laboratory, Children’s Memorial Health Institute, 04730 Warsaw, Poland. 12 Temple Street Children’s University Hospital, Temple Street, Dublin 1, DC01 YC67, Ireland. 13 Department of Laboratory Medicine, Medical Immunology, Radboud University Medical Center, 6525 GA Nijmegen, The Netherlands. 14 Personalized Genomic Medicine Pediatric Genetics and Metabolism Goryeb Children’s Hospital, Morristown, New Jersey 07960, USA. 15 Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, 1090 Vienna, Austria. 16 Department of Pediatrics I, Medical University of Innsbruck, 6020 Innsbruck, Austria. 17 HELIOS Kliniken Krefeld, Children’s Hospital, Lutherplatz 40, 47805 Krefeld, Germany. 18 Department of Pediatrics, Tulane University Medical School, New Orleans, Los Angeles 70112, USA. 19 Department of Pediatrics, University Medical School of Leuven, 3000 Leuven, Belgium. 20 Department of Pediatrics, Radboudumc, 6525GA, Nijmegen, The Netherlands. 21 Screening Department, Institute of Mother and Child, 01-211 Warsaw, Poland. 22 Centre for Molecular and Biomolecular Informatics, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, 6525GA Nijmegen, The Netherlands. 23 Department of Clinical Genetics, Maastricht University Medical Centre, 6229HX Maastricht, The Netherlands. * These authors contributed equally to this work. Correspondence and requests for materials should be addressed to T.H.S. (email: tstevens@uoregon.edu) or to D.J.L. (email: Dirk.Lefeber@radboudumc.nl).
The vacuolar H^+-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. The V-ATPase consists of two multi-protein domains, V_1 and V_o. The peripheral V_1 domain comprises eight subunits (a–h), localized in the cytoplasm and hydrolyses ATP. The V_o domain is embedded in the organelle membrane, consists of five subunits (a, d, e, c and c'), and harbours the rotary mechanism for proton translocation. 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 ATP6V1B1 (MIM 267300) or ATP6V0A4 (MIM 602722) (refs 3–5). In 2000, osteopetrosis (MIM 259700) was linked to the V-ATPase by identification of mutations in TCIRG1, encoding the osteoclast-specific a3 subunit. In 2008, mutations were found in ATP6V0A2 in a subgroup of cutis laxa syndromes with abnormal protein glycosylation, autosomal recessive cutis laxa type II (MIM 219200&278250). ATP6V0A2 encodes the a2 subunit, which localizes the V-ATPase complex to the Golgi apparatus.

In addition to the core V-ATPase subunits, two accessory proteins are known in vertebrates, that is, ATP6AP1 (also known as Ac45) and ATP6AP2 (also known as (Pro-)) renin receptor). In vertebrates, Ac45 is ubiquitously expressed with the highest levels in neuronal and (endo-) endocrine cells and osteoclasts. This accessory subunit of the proton pump guides the V-ATPase into specialized subcellular compartments such as neuroendocrine regulated secretory vesicles or the ruffled border of the osteoclast thereby regulating its activity. Moreover, the Ac45 protein is involved in membrane trafficking and Ca^2+ dependent membrane fusion. V-ATPase assembly has been extensively studied in yeast, whereas Ac45 has been shown to be a component of the V_o domain in the endoplasmic reticulum (ER) membrane. Additionally, yeast Voa1 has been established as an ER-localized V_o assembly factor. In human, V-ATPase assembly is studied, and no yeast orthologue of human Ac45 has thus far been identified.

In this study, we describe a novel ATP6AP1-linked immunodeficiency and identified disease mutations in 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 organellar acidification.

**Results**

Identification of mutations in X-linked 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. 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 ATP6AP1 with hemizygous variant and KPRP on Chr1 with compound heterozygous variants. Of these variants, the c.1284G>A variant in ATP6AP1 on chrXq28 showed the highest level of conservation (PhyloP 46-way, 5.1) and was predicted to be pathogenic by Sift, Polyphe2 and MutationTaster. Moreover, no potentially pathogenic variants were identified in KPRP in the WES data of patients 2.1 and 6.1. ATP6AP1 encodes the accessory subunit Ac45 of the V-ATPase complex and the proton pump that has been linked with abnormal glycan processing in the Golgi via mutations in its core subunit ATP6V0A2 (ref. 8). Sanger sequencing confirmed the hemizygous missense mutation (c.1284G>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>A mutation with disease in agreement with X-linked inheritance. Exome and Sanger sequencing of 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>G (p.L144P) missense mutation, heterozygous in the mother and absent from the father and a healthy sister. An additional hemizygous missense mutation (c.1036G>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>A variant, in agreement with X-linked inheritance.

Exome sequencing of patient 6.1 revealed a c. 938A>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 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+/CD27− naïve B cells and lower levels of IgD+/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 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 hyperopia. The grandmother also presented deafness at older age. The vision abnormality was documented also in patients 3.1 and 4.1 (E346K substitution). The oldest elder presented deafness at older age. The vision abnormality was documented also in patients 3.1 and 4.1 (E346K substitution). The oldest elder presented deafness at older age. The vision abnormality was documented also in patients 3.1 and 4.1 (E346K substitution). The oldest elder presented deafness at older age. The vision abnormality was documented also in patients 3.1 and 4.1 (E346K substitution). The oldest elder presented deafness at older age. The vision abnormality was documented also in patients 3.1 and 4.1 (E346K substitution).

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.L144F, 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 transudency 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 mature outline with vacuoles (autophagosomes/lysosomes) and atypical lipofuscin bodies. The latter showed a mature outline with vacuoles (autophagosomes/lysosomes) and atypical lipofuscin bodies. The latter showed a mature outline with vacuoles (autophagosomes/lysosomes) and atypical lipofuscin bodies.
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.
Human Ac45 is orthologous to yeast Voa1 and Big1. Orthologs of Ac45 were readily identified by BLAST among the metazoan, 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 organisms. 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 profiles, 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 furin. 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-ATPase while Big1 is essential for beta 1,6 glucan synthesis. Phylogenetic analysis shows that BIG1 and VOA1 appear the result of a gene duplication in the Saccharomycotina 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 searches, we confirmed the homology between the Ac45 and Voa1, Big1, and also detected homologues of Ac45 in major taxa of the eukaryotes, amoeboid organisms (*Dictyostelium discoideum*), brown algae (*Ectocarpus siliculosus*) and plants (Supplementary Fig. 7C). Ac45 orthologs in the model species *Arabidopsis thaliana* (AT3G13410) and *Schizosacharomyces pombe* (*S. pombe*) (SPCC306.06c) have, like Ac45, Voa1 and Big1, been observed in the ER, 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 d lysine 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 cells.

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 stressors. 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 d lysine motif (KKNN) appended to the N-terminal were expressed in a *voa1::H* vma21QQ strain. Cells expressing full-length Ac45 grew poorly, comparable to cells...
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 glycans62. (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. Staining for Sec31 is shown as example, other organelle markers are shown in Supplementary Fig. 5.
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.
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 (TICIRG1 or ATP6V0A3 (MIM 259700) (refs 3,6,7)) and cutis laxa with mild abnormalities in copper metabolism and various degrees of liver disease, elevated alkaline phosphatase and cholesterol, respectively, were identified in patients as predicted orthologs of yeast V-ATPase assembly factors (ref. 29). Very recently, mutations in CCDC115 and TMEM199 (refs 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 fusion18, 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 function42–44 but also to Ac45 (refs 18,43). These events are reported to be required for B-cell differentiation45, antigen processing46,47 and antibody production45. 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-BCR48. 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 trafficking10,14–16,30. 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 dilysine motif, other mechanisms might account for retention of Ac45 to the ER of specific cell types. The functional role of Ac45 in antibody production and its involvement in the 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 the 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. Blood samples were received from patients and their reference genome physicians. Isoelectric focusing of serum transferrin for analysis of protein N-glycosylation defects and of serum apolipoprotein CIII for analysis of mucin type O-glycosylation defects were carried out as described before9. Plasma N-glycan profiling was performed by MALDI linear ion trap mass spectrometry as filter aid42 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 transferrin serum transferrin was performed as described4. Briefly, transferrin was immunopurified from 10 μl of serum using anti-transferrin Sepharose beads. 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-ESI-MS/MS plate 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 were deconvoluted by Mass Hunter software to reconstructed mass spectra.

**Next-generation sequencing.** Genomic DNA was extracted from patient fibroblast according to the manufacturer’s protocol using a QIAamp Minikit (Qiagen, Hilden, Germany), and was checked for DNA integrity on agarose gels. Next-generation sequencing and analysis was performed as described4. In brief, exon 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 human genome with the SOLiD LifeScope software version 2.1. Called variants and indels were annotated using an in-house annotation pipeline 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 calling with <5 variants and <20% variation. Furthermore, synonymous variants, downstream intrinsic, intragenic 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. 54) (http://www.broadinstitute.org/igv/download). The putative consequences of the mutations found in the Ac45 protein were predicted using Sift (2009) (http://sift.jcvi.org/www/SIFT Alignment_segsSubmit.html), Mutation taster, (2014) (http://www.mutationtaster.org/) and Polyphen-2 (2012) (http://genetics.gwu.edu/ hp2/) prediction programs.

**Sanger sequencing.** Genomic DNA was extracted from fibroblast pellets or white blood cells from 10 patients and available family members. Primers (Supplementary Table 4) were designed to amplify the 10 exons of ATP6AP1 (GenBank accession number NM_0018345), including at least 50 bp of the 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 by 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 (http://sift.jcvi.org/www/SIFT Aligned_seqs_submit.html), Mutation taster, (2014) to filter out variants with >0.3% in our in-house database of >1,300 exomes. Quality criteria were applied to filter variant calling with <5 variants and <20% variation. Furthermore, synonymous variants, downstream intrinsic, intragenic 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. 54) (http://www.broadinstitute.org/igv/download). The putative consequences of the mutations found in the Ac45 protein were predicted using Sift (2009) (http://sift.jcvi.org/www/SIFT Alignment_segsSubmit.html), Mutation taster, (2014) (http://www.mutationtaster.org/) and Polyphen-2 (2012) (http://genetics.gwu.edu/hp2/) prediction programs.

**Biochemical studies in immortalized human hepatocytes.** iHH3 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 dexamethasone. Culturing was done at 37 °C under an atmosphere of 3% CO2. iHH cell cultures were tested negative for mycoplasma. For Ac45 expression, iHHC cells were transfected with a hAc45/pECDNA construct using Lipofectamine LTX (Invitrogen) according to manufacture's guidelines.

For immunofluorescence assays, cells were cultured on gelatin-coated cover slips for 3 days and fixed for 1 h by 4% paraformaldehyde in PBS at room temperature. After blocking of residual parafomaldehyde with 50 μM 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-EEA1 (BD Biosciences, 1:200), mouse-anti-MMPr (Abcam, 1:200), mouse-anti-sec31a (Santa Cruz, 1:200), mouse-anti-gm130 (BD Transduction Laboratory, 1:400) and mouse-anti-pdi (Stressgen, 1:500), mouse-anti-rab11 (BD Biosciences, 1:100), mouse-anti-ergic33 (Santa Cruz, 1:100) and goat-anti-tgng38 (Santa Cruz, 1:100) antibodies in 1% BSA in PBS-T (blocking buffer) for 1 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. Hereafter, cells were washed with PBS-T, PBS, MilliQ water and dehydrated using methanol and subsequently mounted in Mowiol containing 2.5% 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 by using the Nucleospin extract II kit (Macherey-Nagel) according to the manufacturer’s protocol.
Newly synthesized protein labeling experiments. Two days prior to labeling, HIS3 cells were seeded into gelatin-coated 12-well plates at a density of $2 \times 10^5$ cells per well. Cells were washed using PBS and starved in starvation medium (DMEM without methione and cysteine, supplemented with 10% dialysed FCS, 0.022 U/ml insulin and 0.045 μg/ml dexametasones) for 1 h. Then, cells were incubated for a 30-min pulse period in starvation medium containing 0.1 mCi/ml $^{35}$S EasyTag EXPRESS S/50 protein labeling Mix (PerkinElmer). For analysis of the N-glycosylation of Ac45, the 30-min pulse was carried out in the presence of tunicamycin. Following the pulse period, cells were washed with PBS and lysed in lysis buffer. For immunoprecipitation, lysates were incubated with anti-Ac45 antibodies (1:500) in lysis buffer supplemented with 0.8% SDS for 12 h at 4 °C. The immune complexes were precipitated by Protein A-Sepharose (GE Healthcare Life Sciences) and resolved on 10% SDS-PAGE. EndoH (New England Biolabs) and PNGaseF (New England Biolabs) treatment was performed on immunoprecipitated Ac45 protein according to manufacturer’s guidelines. Radiolabeled proteins were visualized by fluorography.

Transmission electron microscopy. The tissue cylinder was immediately immersed in cold 2.5% glutaraldehyde in 0.1 M Mocodylate 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) were obtained with a Leica Ultracut and used for orientation, cell identification and gross histopathological changes (that is, lipid droplets). Ultra-thin sections (60 nm) were cut with diamond knives and either left unstained or stained with uranyl acetate and lead citrate. Sections from three blocks were placed on 300-mesh copper grids and viewed and photographed with JEOL JEM 100SX and 100CX electron microscopes, operated at 80 kV.

Bio-informatics studies. For sequence-profile-based homology searching, we used HmPred with 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 followed. The voa1::HYG strain used in this study is isogenic with SF838-1D (ref. 58) and additionally has in common the pRS316 vector (218 bp of UTR flanking sequence). A strain with Voa1 S26–S217 with human Ac45 Q43–S420, PCR amplified from pMR092 template produced pMR1312. pMR1303 and K263Q mutations and pMR1303 template produced pMR1303E346K and pMR1303M428I, respectively. Coding GFP immediately after the HA tag. E346K and M428I mutations were made in pMR1303, resulting in pMR1303E346K and pMR1303M428I, respectively. The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files.

Western blot analysis of yeast proteins. Yeast membrane proteins were prepared from glass bead lysates by centrifugation 13,000g as described (44). The purified membrane fraction was solubilized in sample buffer (8 M urea, 5% SDS, 40 mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 0.01% bromophenol blue) to 100 OD0.6 equivalents per ml, and 0.5 OD equivalents separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and analysed by western blot using a 1:300 dilution of monoclonal anti-HA primary antibody (Biologen) and a 1:15,000 dilution of goat anti-mouse secondary antibody labelled with IRDye 800CW (LI-COR). Blocking of non-specific binding and incubation with antibody was done in 5% non-fat dry milk, 0.1% TWEEN 20, 0.5 M NaCl, 20 mM Tris-HCl pH 7.5. Wash steps after antibody incubation were in the same buffer but without milk. Before imaging (Odyssey F, Image, LI-COR), a final wash was done in 10 mM Tris-HCl pH 7.5, 150 mM NaCl.

Live cell imaging of yeast cells. Yeast cells expressing GFP-tagged proteins were grown overnight in SD-Ura, diluted to 0.2 OD0.6 per ml in SD-Ura containing 2.5 μg/ml −1 DAPI (Sigma) and grown until OD0.6 per ml. Cells were collected by centrifugation, and 1.5 μl of cell pellet was added to an equal volume of molten 1% agarose on a glass slide for live cell imaging. Images were acquired with an Axioplan 2 fluorescence microscope (Carl Zeiss) using a ×100 objective. Axiovision (Carl Zeiss) and ImageJ (http://image.nih.gov/ij) software were used for image acquisition, and Photoshop CS4 for image manipulation.

Yeast strain and plasmids. Standard molecular biology protocols for E. coli and yeast manipulations were followed. The voa1::HYG yeast strain used in this study is isogenic with SF838-1D (MATa ura3–52 leu2–131 his3–110 yeast manipulations were followed. The voa1::HYG strain used in this study is isogenic with SF838-1D (MATa ura3–52 leu2–131 his3–110

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Nature Communications | DOI: 10.1038/ncomms11600 | www.nature.com/naturecommunications


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How to cite this article: Jansen, E. J. R. et al. ATP6AP1 deficiency causes immunodeficiency with hepatopathy, cognitive impairment and abnormal protein glycosylation. Nat. Commun. 7:11600 doi: 10.1038/ncomms11600 (2016).

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