The adhesion G-protein coupled receptor VLGR1/ADGRV1 controls autophagy

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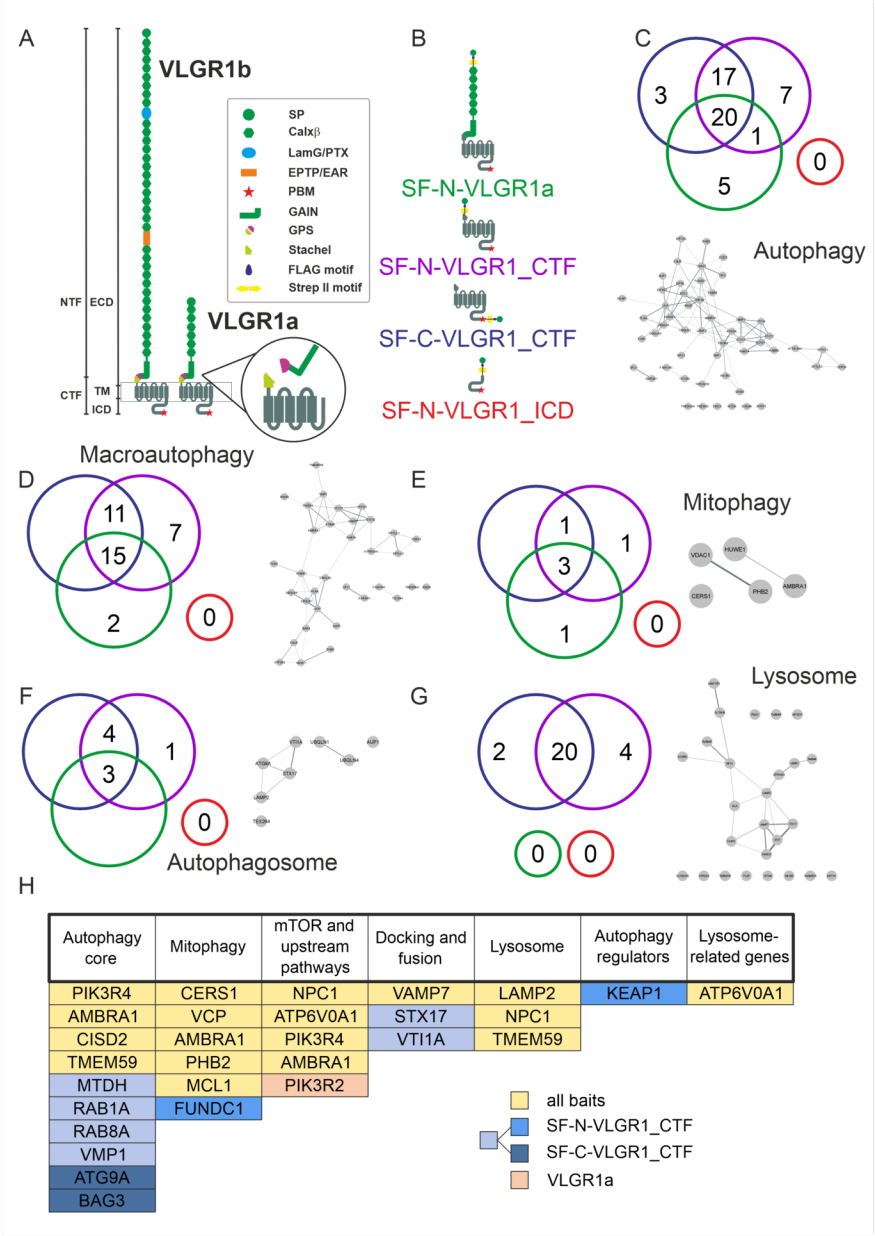
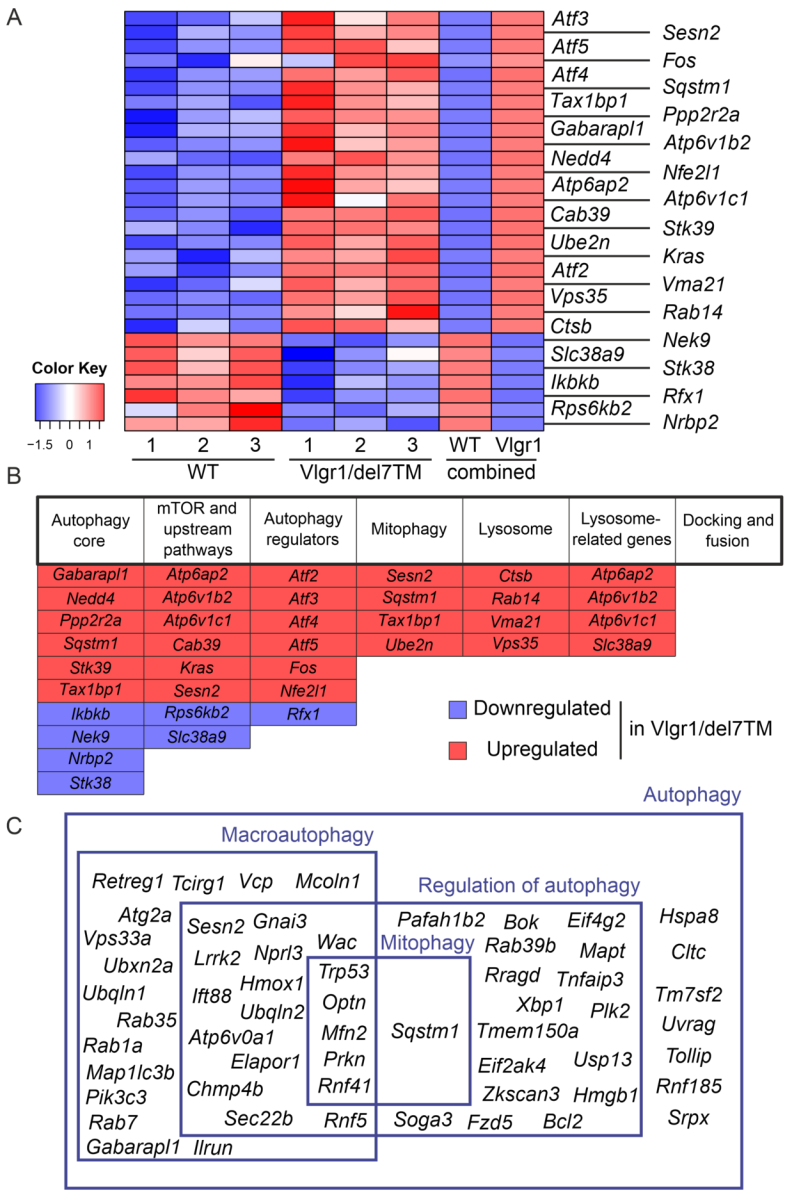
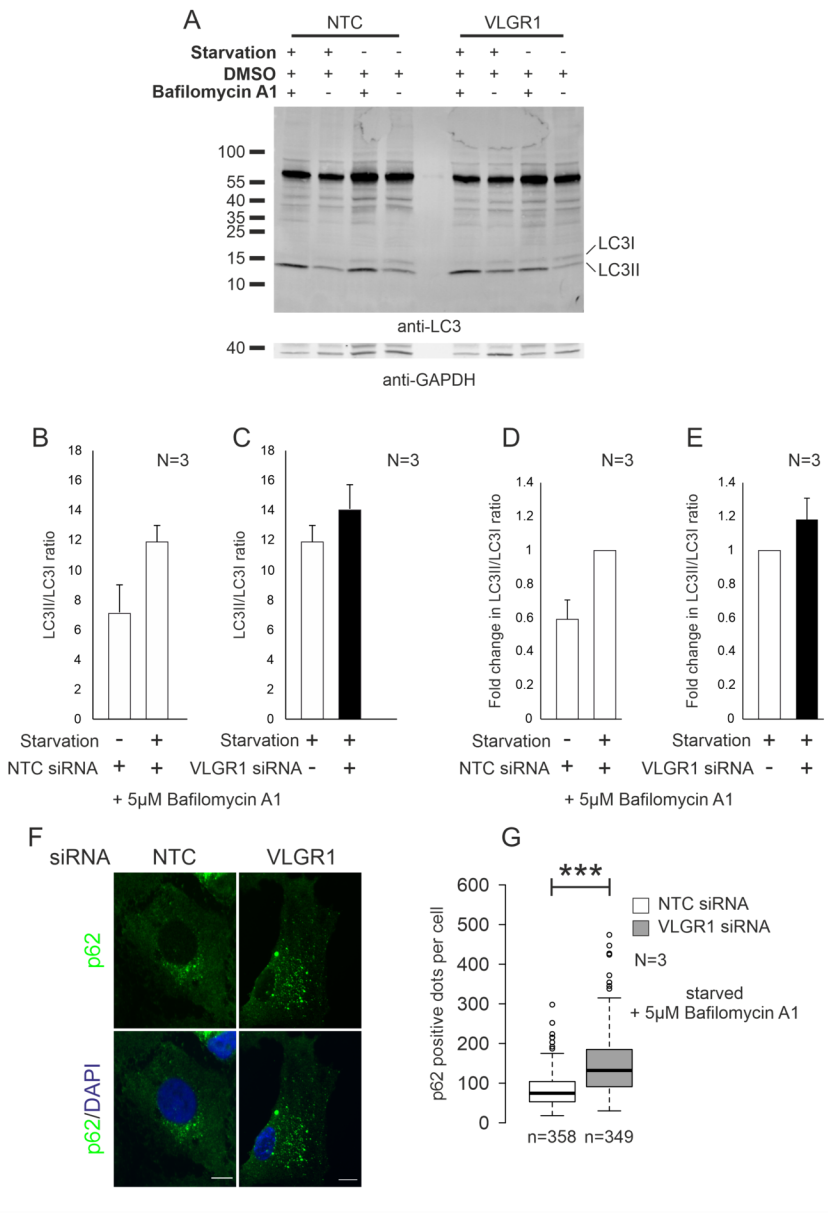
Abstract

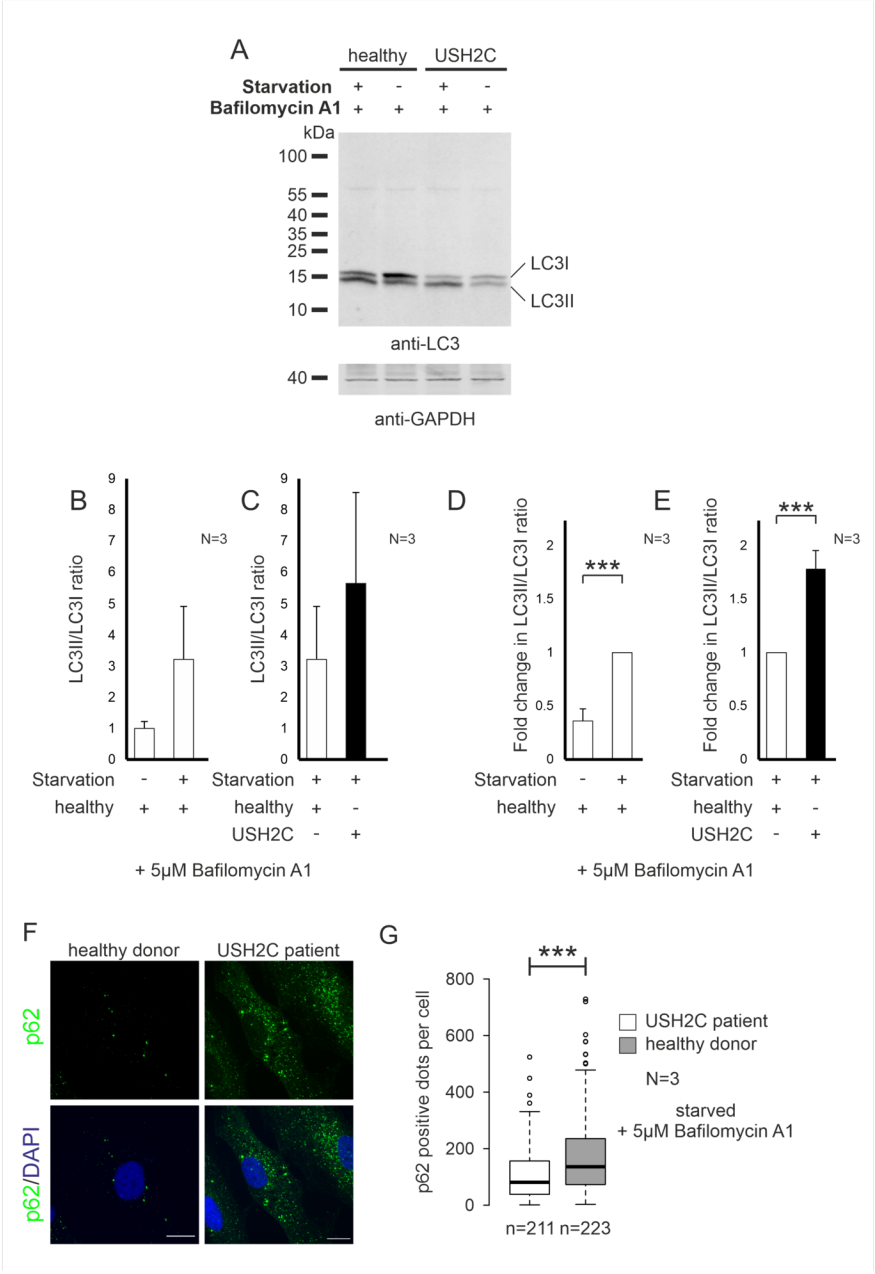
VLGR1/ADGRV1 (very large G protein-coupled receptor-1) is the largest known adhesion G protein-coupled receptor. Mutations in VLGR1/ADGRV1 cause Usher syndrome (USH), the most common form of hereditary deaf-blindness, and have been additionally linked to epilepsy. Although VLGR1/ADGRV1 is almost ubiquitously expressed, little is known about the subcellular function and signalling of the VLGR1 protein and thus about mechanisms underlying the development of diseases. Using affinity proteomics, we have identified key components of autophagosomes as putative interacting proteins of VLGR1. In addition, whole transcriptome sequencing of the retinae of the Vlgr1/del7TM mouse model revealed altered expression profiles of gene-related autophagy. Monitoring autophagy by immunoblotting and immunocytochemistry of the LC3 and p62 as autophagy marker proteins revealed evoked autophagy in VLGR1-deficient hTERT-RPE1 cells and USH2C patient-derived fibroblasts. Our data demonstrate the molecular and functional interaction of VLGR1 with key components of the autophagy process and point to an essential role of VLGR1 in the regulation of autophagy at internal membranes. The close association of VLGR1 with autophagy helps to explain the pathomechanisms underlying human USH and epilepsy-related to VLGR1 defects.



Autophagy core	Mitophagy	mTOR and upstream pathways	Docking and fusion	Lysosome	Autophagy regulators	Lysosome-related genes
PIK3R4	CERS1	NPC1	VAMP7	LAMP2	KEAP1	ATP6V0A1
AMBRA1	VCP	ATP6V0A1	STX17	NPC1		
CISD2	AMBRA1	PIK3R4	VTI1A	TMEM59		
TMEM59	PHB2	AMBRA1				
MTDH	MCL1	PIK3R2				
RAB1A	FUNDC1					
RAB8A						
VMP1						
ATG9A						
BAG3						







The adhesion G-protein coupled receptor VLGR1/ADGRV1 controls autophagy

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Abstract

VLGR1/ADGRV1 (very large G protein-coupled receptor-1) is the largest known adhesion G protein-coupled receptor. Mutations in *VLGR1*/*ADGRV1* cause Usher syndrome (USH), the most common form of hereditary deaf-blindness, and have been additionally linked to epilepsy. Although VLGR1/ADGRV1 is almost ubiquitously expressed, little is known about the subcellular function and signalling of the VLGR1 protein and thus about mechanisms underlying the development of diseases.

Using affinity proteomics, we have identified key components of autophagosomes as putative interacting proteins of VLGR1. In addition, whole transcriptome sequencing of the retinae of the Vlgr1/del7TM mouse model revealed altered expression profiles of gene-related autophagy. Monitoring autophagy by immunoblotting and immunocytochemistry of the LC3 and p62 as autophagy marker proteins revealed evoked autophagy in VLGR1-deficient hTERT-RPE1 cells and USH2C patient-derived fibroblasts.

Our data demonstrate the molecular and functional interaction of VLGR1 with key components of the autophagy process and point to an essential role of VLGR1 in the regulation of autophagy at internal membranes. The close association of VLGR1 with autophagy helps to explain the pathomechanisms underlying human USH and epilepsy-related to VLGR1 defects.

1. Introduction

G protein-coupled receptors (GPCRs) are the most important receptors of our body as they respond to almost all external stimuli and therefore prime targets for pharmacological interventions. Although adhesion GPCRs (ADGRs) are the second largest subclass of GPCRs, their function is the least understood of all GPCR classes, so their pharmacological significance has also had to remain fairly unexplored. ADGRs are characterized by signature domains of serpentine (7TM) and adhesion proteins (Figure 1A). Among ADGRs the very large G protein-coupled receptor 1 (VLGR1), also named ADGRV1, GPR98 or MASS1 is the largest [1,2]. As other ADGRs, VLGR1 is composed of an extracellular N-terminal fragment (adhesion part), which is extremely long in VLGR1, fused by a GAIN domain, which includes the GPCR autoproteolytic cleavage site (GPS) to a C-terminal fragment defined by 7TM domain (receptor part) (Figure 1A). Evidence suggests that autocleavage at GPS exposes the short so-called "spike" sequence at the N-terminal end of CTF, which serves as a bound agonist to activate aGPCRs [3,4]. In VLGR1, we have recently identified 11 amino acids that act as the “Stachel” peptide[5]. Furthermore, we also found evidence that this activation induces a switch from Gs- to Gi-mediated signalling of VLGR1.

In mammals, VLGR1 is almost ubiquitously expressed, with high expression in the nervous system, especially in the neural cells of the developing brain and the sensory cells of the eye and inner ear [1,2,6] (Protein Atlas: https://www.proteinatlas.org/). Mutations in the *VLGR1/ADGRV1* gene cause Usher syndrome type 2C (USH2C) which is characterized by congenital sensorineural hearing loss and retinitis pigmentosa (RP) [7]. Additionally, mutations, even haploinsufficiency of *VLGR1/ADGRV1* have been associated with different forms of epilepsy in humans and audiogenic seizures in mice (Dahawi et al. 2021; Myers et al. 2018; Zhou et al. 2022). Almost nothing is known to date about the pathomechanisms underlying epileptogenesis that lead to the imbalance between excitatory and inhibitory neurotransmission described in epilepsy patients. In the two sensory cell types affected in USH type 2, retinal photoreceptor cells and cochlear hair cells, VLGR1 is essential for the formation of filamentous connections between membranes, namely the membranes of the connecting cilium and the inner segment in photoreceptor cells and ankle-links connecting adjacent stereocilia in differentiating hair bundles of the cochlear hair cells [1,6,10,11]. The absence of VLGR1 leads to a disturbance of membrane-membrane adhesion, which is manifested by the conspicuous disorganization of the stereotypic arrangement of stereocilia in the hair bundles in the hair cells. However, it remains

unknown whether, in addition to these apparent defects in adhesion, altered G protein-coupled signalling contributes to the pathophysiology of sensory cells in USH2C.

Because knowledge of potential interaction partners often provides reliable insights into the function of proteins, we have searched for potential partners of VLGR1 by an affinity proteomics capture approach to provide insights into its cellular functions [5,12]. This strategy has recently enabled us to unravel valuable new insights into the downstream receptor signalling of VLGR1 [5], its participation as a metabotropic membrane mechanoreceptor in the regulation of focal adhesion during cell migration [13,14], and its role in the function of internal membrane compartments, such as the mitochondria-associated membranes (MAMs) of the endoplasmic reticulum (ER) [15]. The absence of VLGR1 results in a disturbance in the MAM architecture and the dysregulation of the Ca^{2+} transient from ER to mitochondria [15] MAMs are nuclei for autophagosomes in the autophagy process [16], the intracellular degradation system for cytoplasmic contents, e.g., for defective intracellular proteins, excess or damaged organelles, or invaded microorganisms [17–20]. Classical autophagy also named as macroautophagy is characterized be sequentially steps, such as the formation of autophagosomes from the phagophore and the fusion with lysosomes leading to digestive autolysosomes [17,21]. There are also several subtypes of autophagy, for instance chaperone-mediated autophagy is mainly based on the interaction of heat shock proteins with proteins determined for degradation[17,18]. Also, autophagy of whole organelles such as mitochondria or the ER are defined as mitophagy or ER-phagy, respectively[22–24]. Defects in autophagy can evoke or exacerbate diseases namely neurogenerative diseases such as Huntington, Alzheimer's or retinal degeneration [25–27].

Here, we show that the adhesion GPCR VLGR1 interacts with core components of autophagy and that in the absence of VLGR1, autophagy activities increase, leading to differential expression of autophagy-related genes. This close association of VLGR1 with the autophagy process may help to explain the pathomechanisms underlying the diseases related to VLGR1, namely the human Usher syndrome type 2C and epilepsy.

2. Material and Methods

2.1 Animals

All experiments were performed in compliance with guidelines established by the Association for Research in Vision and Ophthalmology. Mice were kept under 12/12 hours light/dark cycles, food and water *ad libitum*. Vlgr1/del7TM mice carry a premature STOP codon at the exon 82 of *Vlgr1* which leads to the deletion of the entire 7TM domain and only the expression

of the extracellular domain [27]. The breeding background of Vlgr1del7TM mice was the C57BL/6 strain which were also used as wild type (WT) controls.

2.2 Antibodies

Primary antibodies used in this study were the following: rabbit anti-p62 (Proteintech, 18420-1-AP), rabbit anti-LC3 (Proteintech, 14600-1-AP), mouse anti GAPDH (Abcam, ab9484), mouse anti-actin (Thermo Fisher Scientific, MA5-11869). Secondary antibodies used in this study were conjugated to Alexa 488, Alexa 555, or Alexa568, purchased from Invitrogen or Rockland Immunochemicals. Nuclear DNA was stained with DAPI (4',6-diamidino-2-phenylindole) (1 mg/ml, diluted 1:12000) (Sigma-Aldrich).

2.3 Cell culture

hTERT-RPE1 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Thermo Fisher Scientific) containing 10% heat-inactivated fetal calf serum (FCS). Cells were transfected with GeneJuice® (Merck Millipore) according to the manufacturer's instructions.

2.4 Human primary fibroblast cultures

Healthy dermal primary fibroblast lines were expanded from skin biopsies of human subjects (ethics vote: Landesärztekammer Rhineland-Palatinate to KNW). Primary fibroblast lines were mycoplasma negative and cultured in DMEM, 10% FCS and 1% penicillin-streptomycin at 37°C and 5% CO_2. USH2C *VLGR1/ADGRV1* R2959* patient-derived fibroblasts were a kind gift from Dr Erwin van Wijk (Radboud University Medical Center, Nijmegen) and were derived from skin biopsies of a 57-year-old male USH2C patient who carrys a nonsense mutation in the *VLGR1/ADGRV1* gene (g.[90006848C>T])[28].

2.5 DNA constructs

VLGR1_CTF (Uniprot ID Q8WXG9-1, aa 5891-6306) sequence was used for VLGR1 constructs. For tandem affinity purifications, Strep II-FLAG (SF)-tagged human VLGR1_CTF was used. The SF-tag was N-terminally and C-terminally fused to VLGR1_CTF.

2.6 Tandem affinity purification (TAP) and mass spectrometry

Tandem affinity purification and mass spectrometry analysis were performed as previously described[5,15,29]. The constructs illustrated in Figure 1A were expressed in HEK293T cells. After 48 h incubation, cells were lysed, cleared by centrifugation and supernatants were subsequently purified by using Strep-Tactin® Superflow® beads (IBA) and anti-Flag M2 agarose beads (Merck). Precipitation of eluates was performed with Methanol-chloroform. These eluates were

then used for liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). To validate the MS/MS-based peptide and polypeptide identifications, raw MS spectra were searched against the human SwissProt database using Mascot. The obtained results were additionally verified by Scaffold (version 4.02.01, Proteome Software Inc). Results were compared to mock-transfected cells and common RAF1 control TAPs. Proteins evident in mock and RAF1 Taps were excluded from the analysis. VLGR1 preys were used as input for the Cytoscape plugins STRING and ClueGO according to their gene names based on HGNC. Gene Ontology (GO) term enrichment analysis was performed by ClueGO v2.3.3.

2.7 siRNA-mediated knockdown in hTERT-RPE1 cells

Human hTERT-RPE1 cells were transfected with siRNAs specific for human *VLGR1* (L-005656-00-0005) and non-targeting control (NTC) siRNAs (D-001810-10-05), previously validated[13]. siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

2.8 Arrest of autophagy in hTERT-RPE1 cells and primary dermal fibroblasts

To induce starvation of hTERT-RPE1 and primary dermal fibroblasts normal growth media were changed to Earle's Balanced Salt Solution (EBSS) and control cells were continually kept in normal growth media for 2 h. To arrest of autophagy cells were additionally treated with 5 µM Bafilomycin A1 (BA1) (Sigma-Aldrich). Subsequently cells process

2.9 Immunocytochemistry

Cells were cultured on glass coverslips and fixed with 4% paraformaldehyde for 10 min at room temperature (RT), washed with PBS three times, permeabilized with PBST (0.2% Triton-X100 (Roth)) 10 min at RT, washed once with PBS and blocked with 0.1% ovalbumin, 0.5% fish gelatin in PBS for 1 h at RT. Primary antibodies were incubated overnight at 4°C, followed by washing three times with PBS and secondary antibody incubations for 1 h at RT. After another three times of washing with PBS, cells were mounted with Mowiol 4.88 (Hoechst) and analyzed with a Leica DM6000B microscope (Leica). Fiji/ImageJ software (NIH) was used for image processing and quantifications. For statistical analysis, R-Studio was used [30].

2.10 Data processing

For the analysis of p62/SQSTM1 positive dots during immunostaining, quantification was done using the Fiji/ImageJ software (https://fiji.sc). Images were loaded into Fiji using the Bio-Formats plugin. Cells were manually encircled, and the threshold was adjusted to Intermodes.

The selected cells were analyzed for dot number with the Fiji function analyze particles. The numbers were summarized in Excel and an average number per cell was calculated.

2.11 Western blot analyses

Protein lysates were prepared using Triton-X-100 lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 0.5% Triton-X-100, pH 7.4) containing complete protease inhibitor cocktail (04693132001, Roche Diagnostics) and sonicated. Protein content was quantified using a BCA protein assay (Merck Millipore) and subjected to SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore). After blotting, membranes were blocked in AppliChem blocking reagent (AppliChem) for 1 h and subsequently incubated with primary antibodies overnight at 4°C followed by appropriate secondary antibodies Alexa Flour 680 (Invitrogen) or IR Dye 800 (Rockland). Scans of the blots were made employing the Odyssey infrared imaging system (LI-COR Biosciences). For densitometry analysis, the LI-COR software Empiria Studio was used, and for statistical analysis, R-Studio was applied [30].

2.12 RNA isolation and transcriptome sequencing

Adult (pn 40) WT and Vlgr1/del7TM mice were euthanized using cervical dislocation. Directly following the skull was opened and the brain was dissected to obtain the hippocampus, the cerebellum, and the cortex. The retina was extracted from the eyes. Tissues were flash-frozen using liquid nitrogen. Tissues were homogenized in RNeasy lysis buffer using 27 gauge needles, following RNA was isolated according to the instructions of the Qiagen RNeasy Mini Kit. RNA quality was determined using a Nanodrop (Thermo Fisher Scientific), and RNA was subsequently stored at -80°C. Whole transcriptome sequencing was performed by the company Novogene. mRNA sequencing was performed using the Illumina platform. Pair end reads were mapped and quantified. Following, differential gene expression analysis and Gene Ontology (GO) enrichment analysis was performed (https://en.novogene.com/services/research-services/transcriptome-sequencing/mrna-sequencing/).

2.13 Policy for experimental and clinical studies

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies [31].

3. Results

3.1 Analysis of TAP data sets identified interactions of VLGR1/ADGRV1 with core components of the autophagy process.

We have recently identified more than 1000 putative interacting partners of VLGR1 in tandem purifications (TAPs) with VLGR1_ICD, VLGR1_CTFs, and full-length VLGR1a as baits [5]. GO term analysis of the TAP datasets using the Cytoscape plugin ClueGO (accessed September 28, 2022) revealed numerous associations of VLGR1 with autophagy in the different GO categories (Figure 1C-G). In TAPs with full-length VLGR1a and both, the N- or C-terminal SF-tagged VLGR1_CTFs, 53 proteins associated with autophagy were enriched (Table 1). In contrast, no proteins associated with autophagy were found in the VLGR1_ICD TAP.

We used the GO aspect "Biological Process" and categorized the TAP hits into the three GO terms "autophagy", "macroautophagy", and "mitophagy". For the term "autophagy", we found 48 proteins in total for VLGR1a for both VLGR1_CTF. Approx. 80% of the proteins identified for the two VLGR1_CTFs were overlapping. For all three constructs together, the overlap was 40% of the identified proteins. Interestingly the autophagy core component ATG9a is only associated with SF-VLGR1_CTF, similarly, AMBRA1 is only present in the VLGR1a TAP. A STRING network analysis revealed multiple interactions between prey proteins of the GO term "autophagy" (Figure 1C).

For the downstream GO terms "macroautophagy" and "mitophagy" we identified 35 proteins and 6 proteins, respectively (Figure 1D, E; right hand). For both terms, the biggest overlap was between all three baits and between the two CTFs. Again, autophagy core components were identified for VLGR1a (AMBRA1, PIK3R2) and the two VLGR1_CTFs (TMEM59). In the String network, several of the identified proteins cluster in specific subgroups (Figure 1D, E; left hand).

In the category *Cellular component* TAP hits could be categorized by ClueGO into the three GO terms "autophagosome" and "lysosome", 8 proteins and 26 proteins, respectively (Figure 1F, G). Most of the proteins identified for "autophagosomes" were present in TAP data of full-length VLGR1a and both VLGR1_CTFs or were found in TAPs of both VLGR1_CTFs. For "lysosome", only in the two CTF TAP data sets, associated proteins were identified. As in the category *Biological process,* we found in the category *Cellular component* the autophagy core components ATG9a, AMBRA1, STX17, TM9SF1, and PIK3R4. The String network for the "autophagosome" clustered into two groups, autophagy core components and the group of ubiquillins and ubiquitin-associated proteins (Figure 1F). Several of the proteins associated with lysosomes, group together in the String network (Figure 1G).

We confirmed the GO term analyses by comparison with the data sets recently published gene toolbox for monitoring autophagy transcription [21]. All prey proteins identified in our VLGR1-TAP categorized in the autophagy-related GO terms in the categories *Biological*

process and *Cellular component* were found in the subcategories of the autophagy process defined in this autophagy toolbox (Figure 1 H).

In summary, the identification of proteins participating in the autophagy process as potential interaction partners of VLGR1 indicated a close association of VLGR1 with autophagy.

3.2 RNA sequencing of *Vlgr1*-deficient retinae revealed multiple associations of VLGR1 to autophagy molecules

We next explored whether there were any differences in the expression of genes related to autophagy in the absence of regular *VLGR1* expression. To this end, we performed genome-wide mRNA sequencing of retinas from *Vlgr1*-mutated and deficient Vlgr1/del7TM mice compared to wild-type retinae samples. Total RNA was extracted from three biological replicates each of adult (pn 40) wild type and Vlgr1/del7TM retinae, followed by RNA sequencing of the samples using the Illumina platform. Pair end reads were mapped and quantified, followed by differential gene expression analysis and Gene Ontology (GO) enrichment analysis (accessed on 18.05.2022). In total 2,824 genes were differentially expressed in Vlgr1/del7TM retinae compared to WT retinae: 1,671 of those were upregulated, and 1,153 were downregulated (Table S1, S2).

Using the recently defined autophagy gene toolbox with defined categories of functional classes [21] we identified 30 genes that were differentially expressed in Vlgr1/del7TM retinae (Figure 2A, B). From these genes 7 genes were down-regulated and 23 up-regulated with simple to high significance in Vlgr1/del7TM retinae. The downregulated genes could be categorized into the terms “mTOR and upstream pathways”, “autophagy core machinery”, and “autophagy regulators” (Figure 2B). Among those genes, *Rps6kb2* and *Slc38a9* are core components of the mTOR pathway, and *Nek9* and *Stk38* play important roles in selective and chaperone-mediated autophagy.

The downregulated genes spanned all categories, except for docking and fusion. For example, a group of ATPase genes (*Atp6ap2*, *Atp6v1b2*, *Atp6v1c1*), important for lysosome function, four activating transcription factors (*Atf2, Atf3, Atf4, Atf5*), which regulate autophagy in response to various stresses, and the important autophagy adapter protein *p62/sqstm1* were downregulated in *Vlgr1* deficient retinae. Additional GO-Term analysis revealed that genes differentially expressed in the Vlgr1/del7TM retinae associate with the following *Biological process* subcategorizes: 58 genes with “autophagy”, 33 genes with “macroautophagy”, 6 genes with “mitoautophagy”, and 36 genes with “regulation of autophagy” (Figure 2C).

Taken together, the deficiency of *Vlgr1* leads to the dysregulation of the expression of genes related to autophagy in the mouse retina. Furthermore, this confirms the close relation of VLGR1 to autophagy processes indicated by the potential interacting proteins of the VLGR1 protein identified by TAP-based affinity proteomics.

3.3 Depletion of *VLGR1* increases autophagy in hTERT-RPE1 cells

Next, we investigated the consequences of *VLGR1* deficiency on autophagy. For this, we depleted *VLGR1* in hTERT-RPE1 cells by siRNA-mediated knockdown and then activated autophagy by starvation. To evaluate the levels of the key autophagy markers LC3 and p62 we arrested the autophagic flux. Autophagic flux is the formation of autophagosomes at compartments like the MAMs and then eventually the fusion of these with lysosomes. In the formed autolysosomes proteins or organelles get degraded. To establish an autophagy arrest, we blocked the fusion of autophagosomes with lysosomes using Bafilomycin A1 to prevent lysosome acidification and protein degradation (Figure 3).

In Western blots of cell lysates, we determined the protein content of the autophagy marker LC3, which is converted from the cytoplasmic form LC3I to the autophagosome membrane-bound form LC3II during autophagy (Figure 3A). The increased ratio of LC3II/LC3I observed in starved cells compared to unstarved cells confirmed that starvation increases autophagy in hTERT-RPE1 cells (Figure 3 A, B, D). In addition, siRNA-mediated knockdown of VLGR1 increased the LC3II/LC3I ratio in hTERT-RPE1 cells when compared to non-targeting control (NTC) siRNA-treated cells (Figure 3 A, C, E).

In the cytoplasm, autophagic cargos (liquid droplets, damaged organelles and aggregated proteins) are tagged with ubiquitin chains to which the autophagy adaptor protein p62 can bind[32]. This allows p62 antibodies to serve as a common marker for autophagosomes in immunocytochemistry. Immunocytochemical staining revealed an accumulation of anti-p62-positive dot-like structures representing autophagosomes in starved, Bafilomycin A1-treated hTERT-RPE1 cells (Figure 3F). Quantification revealed a highly significant increase of anti-p62-positive autophagosomes after *VLGR1*-depletion when compared to the NTC-treated hTERT-RPE1 cells (Figure 3 G).

Our combined results using the autophagy marker LC3 or p62 showed significant increases in autophagy in hTERT-RPE1 cells depleted for *VLGR1*.

3.4 Autophagic activity is greatly increased in USH2C patient-derived dermal fibroblasts

Next, we analysed the activity of autophagy in dermal fibroblasts derived from skin biopsies of a clinically characterized USH2C patient with a pathogenic mutation in *VLGR1/ADGRV1*. The *VLGR1/ADGRV1*^{Arg2959*} nonsense mutation leads to premature termination of translation and should result in the expression of a very truncated non-functional VLGR1 protein or due to nonsense-mediated mRNA decay no VLGR1 protein expression at all.

We starved USH2C fibroblast and control fibroblasts derived from a healthy individual, treated both with Bafilomycin A1 and analysed the autophagy activity in Western blots and by immunocytochemistry (Figure 4A). The LC3II/LC3I ratio of protein levels determined in Western blots revealed a significant increase of autophagy in USH2C fibroblasts when compared to control fibroblasts derived from a healthy individual (Figure 4A-E). Immunohistochemical analysis showed a significant increase of anti-p62-positive autophagosomes in USH2C patient-derived fibroblast compared to healthy controls (Figure 4F, G).

Taken together, our complementary assays revealed that the autophagy activity is significantly increased in both the knockdown of *VLGR1* in hTERT-RPE1 cells and the absence of functional VLGR1 protein in USH2C patient-derived fibroblast.

4. Discussion

In the present study, we identified close associations of the adhesion GPCR VLGR1/ADGRV1 with autophagy (macro-autophagy), a conserved catabolic process of the cell proceeding the clearance of dysfunctional proteins, protein aggregates, and organelles by "self-digestion" [24,33] Autophagy is highly dynamic, characterized by sequential steps of the formation of autophagosomes from the phagophore and the fusion with lysosomes leading to digestive autolysosomes [17,21].

Applying an affinity capture approach based on tandem affinity purifications (TAPs), we identified several autophagy core proteins as putative interaction partners of VLGR1 (Figure 1). The absence of any autophagy-related proteins in TAPs with the cytoplasmic *C*-terminal domain (ICD) and the high number of preys found in both VLGR1 CTFs indicate that VLGR1 likely interacts with components of the autophagy machinery through the seven transmembrane membrane domain of VLGR1. The recently published autophagy monitoring toolbox [21], allowed us to assign these autophagy proteins to diverse, almost all stages of the autophagy

process from the initial phagophore to the digestive autolysosome, suggesting that VLGR1 is present almost throughout the entire autophagy process. One explanation for this is that VLGR1 polypeptides tagged and overexpressed for TAPs are recognized as defective in the cell and degraded via autophagy, thereby interacting with the autophagy molecules. This should then be true for other adhesion GPCRs that we have recently studied by TAPs [12]. However, we did not identify any autophagy molecules or much less prey in TAPs with adhesion GPCRs other than VLGR1. The potential physical interaction with key autophagy proteins may therefore indicate a role of VLGR1 in the control of the autophagy process. This is supported by our finding that the deficiency of VLGR1 in the Vlgr1/del7TM mouse model leads to alterations in the expression of numerous autophagy-related genes. Elevated autophagy activity observed after silencing of *VLGR1* in hTERT-RPE1 cells and VLGR1-deficient fibroblasts derived from USH1C patients further confirms a regulatory role of VLGR1 in the autophagy process, which is also in line with the putative interaction of VLGR1 with KEAP1 categorized as "regulation of autophagy" [21].

The core autophagy proteins ATG9a, AMBRA1, RAB1A, or PIK3R4, found as prey in VLGR1 TAPs, are known to be essential in the initiation steps of the autophagy which may be indicative for VLGR1´s participation there [34–37]. The role of VLGR1 in the initiation of the autophagy processes is also consistent with the localization of VLGR1 as a specific site of the ER membrane, the mitochondria-associated ER membranes (MAMs) as we have recently demonstrated [15]Indeed, MAMs have been identified as a compartment of initiation for autophagy at which autophagosome formation starts [16]. The absence of VLGR1 resulted in a disturbance in the MAM architecture and the dysregulation of the Ca^{2+} transient from ER to mitochondria[15]. The disruption of Ca^{2+} signalling between the ER and mitochondria and the resulting imbalance of Ca^{2+} homeostasis induces mitophagy [38,39], a specific form of autophagy which selectively removes defective mitochondria [22]. We additionally identified several proteins related to mitophagy, as potential interactors of VLGR1 (see Figure 1E, H) supporting the association of VLGR1 with this form of autophagy.

Another set of proteins found in VLGR1 TAPs was grouped into categories related to the process of autophagosome-lysosome fusion and lysosomal digestion. Deficiencies in those proteins, namely MCL1, VCP, NPC1, STX17, or LAMP2 lead to the increase of autophagic fluxes or extensive accumulation of autophagic aggregates [40–44]. This is exactly what we observed in the present study in the VLGR1 deficient hTERT-RPE1 cells and USH2C patient-derived fibroblasts evidencing a potential role of VLGR1 in the conversion of the autophagosome to the digestive autolysosome.

Multiple signals downstream of GPCRs regulate autophagy [45]. A variety of GPCRs, such as the muscarinic, the glucagon-like peptide-1 (GLP-1), the β-adrenergic, or the purinergic GPCRs couple through Gαi, Gαs or Gαq and the liberation of Gβγ mostly promote autophagy via second messenger cascades, e.g. cAMP or Ca^{2+}. As with other adhesion GPCRs, self-cleavage of VLGR1 at the GPS in the GAIN domain results in the separation of the extracellular N-terminal fragment (NTF) and the C-terminal fragment (CTF). This leads also to the activation of the receptor by binding of a tethered agonist, a short peptide of the very N-terminal part of the CTF called "Stachel" (Figure 1A), to the exoplasmic face of the receptor [5,46]. There is growing evidence that the resulting conformation change in the VLGR1 also leads to the switch in the G protein coupling from Gαs constitutively coupled to the full-length uncleaved VLGR1 to $G\alpha_i$-mediated signalling by the "activated" VLGR1-CTF [5,47,48]. It has been previously shown that both, $G\alpha_s$- and $G\alpha_i$ signalling cascades can context-dependently regulate autophagy [45]. $G\alpha_s$ interacts with the adenylate cyclase and cAMP to induce autophagy whereas Gαi can activate autophagy through the LKB1/AMPK axis [49,50]. Both downstream pathways have been linked to VLGR1 [5].

We have recently shown that VLGR1 functions as a metabotropic mechanoreceptor in focal adhesions by shear stress experiments [13]. Recent findings indicate that the sensing of mechanical stresses also contributes directly to the activation of autophagy [26]. By physical interaction of core proteins of both autophagy and focal adhesion, paxillin promotes the disassembly of focal adhesions and cell motility [51]. As we have recently demonstrated VLGR1 is also key in the regulation of focal adhesion dynamics and cell migration [13,14]. Taken together, our data provide evidence that VLGR1 regulates the two interrelated processes of autophagy and cell migration by sensing mechanical signals at focal adhesions.

Mutations in *VLGR1/ADGRV1* are the cause of Usher syndrome type 2, characterized by congenital sensorineural hearing loss and retinitis pigmentosa (RP) [7]. In the present study, we demonstrated that in cellular models, namely after *VLGR1/ADGRV1* silencing in hTERT-RPE1 cells and fibroblasts from USH1C patients, autophagy activity is significantly increased. This was confirmed by our transcriptome data obtained from the retina of the Vlgr1/del7TM mouse model demonstrating the upregulation of key autophagy genes compared to the wild type. Among these genes, *Nek9* was upregulated in the *Vlgr1*-deficient mouse retina. The encoded NIMA-related kinase 9 (NEK9) is a selective autophagy adaptor essential for the formation of primary cilia [52]. We have recently that VLGR1 participates also in ciliogenesis [5] and a mutual pathway between NEK9 and VLGR1, related autophagy for the promotion of ciliogenesis seems reasonable.

In the sensory cells of the eye and ear, USH type 2 proteins physically interact in membrane-membrane adhesion complexes and therefore defects in both molecules are thought to result in the same pathomechanisms leading to the disease [7,53,54]. Indeed, an increase in autophagy has been recently reported in a *USH2A* zebrafish model associated with retinal degeneration [55]. Because the USH2 proteins VLGR1 and USH2A physically interact in membrane-membrane adhesion complexes of photoreceptor and hair cells, the sensory cells affected by USH disease in the eye and ear, defects in both molecules most likely result in the same pathomechanisms leading to USH. This finding is confirmed by our transcriptome data obtained from the retina of the Vlgr1/del7TM mouse model, which demonstrates the upregulation of key autophagy genes compared to the wild type.

Besides USH2C, defects, namely haploinsufficiency of *VLGR1/ADGRV1* can also lead to the development of epilepsy in humans [9,56] and audiogenic epilepsy in mice [2]. There is increasing evidence that alterations in autophagy are present in epileptogenesis, leading to imbalanced excitatory-inhibitory neurotransmission and epilepsy-induced neuronal damage [57,58]. It is notable, the application of the inhibitor rapamycin of the mTOR pathway, which induces autophagy reduces the seizure frequency *in vivo*. Interestingly, RNAseq data of the retina of Vlgr1/del7TM mouse, a validated audiogenic seizure model, indicated differential expression, mainly up-regulation, of genes related to the mTOR pathway (Figure 2). A link of VLGR1 to the mTOR pathway is further supported by the identifications of mTOR pathway components as potential interacting partners of VLGR1 by the present TAPs (Figure 1). Collectively, *VLGR1/ADGRV1*-associated epilepsy may be associated with disruption of the mTOR pathway and altered autophagy, opening possible treatment options with rapamycin.

5. Conclusions

In conclusion, we provide evidence that the USH2C protein VLGR1 interacts with autophagy core proteins imitating autophagy and with molecules related to autolysosome formation, indicating a close association of VLGR1 with autophagy. In the absence of VLGR1 autophagy activities increases and leads to differential expression of genes related to autophagy. Our findings support the role of VLGR1 in the control of autophagy in a multifaceted way at internal membranes of the ER, mitochondria and focal adhesions and provide evidence of the role of autophagy in the pathophysiology of VLGR1-related diseases, such as human Usher syndrome and epilepsy.

Author Contributions: J.L. conducted the majority of the experiments, analysis of data, and figure preparation. B.E.G. helped isolate mice retinae for RNA sequencing. J.K. performed the

TAP assays and assisted during data processing. U.W. and J.L. designed the studies. U.W. and J.L. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Figure legends

Figure 1. GO term analysis of TAP data from the adhesion GPCR VLGR1. (A) VLGR1 isoform structure for VLGR1a and VLGR1b. (B) Illustration of VLGR1 baits used for tandem affinity purifications (TAPs): VLGR1a, the C-terminal fragment (CTF), or the intracellular domain (ICD) of VLGR1 were either N- or C-terminal tagged with Strep-FLAG. (C) Venn diagram of VLGR1 preys assigned to the GO term autophagy in the category Biological Process. The interaction of these prey is visualised in a STRING network. (D) Venn diagram and STRING network of VLGR1 prey assigned to the GO term macroautophagy. (E) Venn diagram and STRING network of VLGR1 prey associated with the GO term mitophagy. (F) Venn diagram and STRING network of VLGR1 prey associated with the GO term autophagosome in the category Cellular Component. (G) Venn diagram and STRING network of VLGR1 preys assigned to the GO term Lysosome. (H) Categorisation of the VLGR1 interactors associated with autophagy, identified by GO-term analysis, into subcategories of the autophagy process previously defined by Bordi and co-workers [21].

Figure 2: Whole transcriptome sequencing of VLGR1 deficient mice retinae. (A) Heatmap of dysregulated genes associated with autophagy. Genes were chosen based on the autophagy gene toolbox created by Bordi and co-workers [21]. The dysregulation of these genes was highly significant. (B) Genes were again subcategorised into functional classes defined in the autophagy gene toolbox. (C) GO-term analysis of dysregulated genes. Venn-diagram shows results for the terms autophagy, macroautophagy, regulation of autophagy and mitophagy in the category biological process.

Figure 3: VLGR1 depletion increases the abundance of autophagy markers LC3 and p62 in RPE1 cells. (A) Western blot analysis of autophagy marker LC3 in VLGR1 and NTC-depleted RPE1 cells. Quantification (B, D) revealed an increase in the ratio of LC3II to LC3I already in starved control-depleted cells. (C, E) After VLGR1 depletion the ratio of LC3II to LC3I increased even greater when compared to the control. Ratios were normalized to loading control. (F) Immunolabelling of the autophagy marker p62 revealed an increase of p62 accumulations in VLGR1-depleted cells compared to control cells. Cells were treated for 2 h with 5 µM Bafilomycin A1 and were cultured in EBSS to induce starvation. (G) Quantification confirmed that VLGR1-depleted cells show significantly more p62-positive accumulations than

control cells. Scale in F = 10 µm. Statistical significance was determined by Mann–Whitney U test *=p<0.05, **=p<0.01, ***=p<0.005.

Figure 4: VLGR1 deficiency in patient-derived fibroblasts increases autophagic activity. (A) Western Blot analysis of patient-derived USH2C fibroblasts and healthy control cells. (B, D) Quantification revealed a significant increase in the ratio of LC3II to LC3I in starved healthy cells compared to unstarved cells. (C, E) VLGR1 deficient fibroblasts showed an even greater increase in the ratio of LC3II to LC3I compared to starved healthy cells. Ratios were normalized to loading control. (F) Immunolabeling of p62 revealed an increase of accumulations in patient-derived USH2C fibroblasts compared to healthy control cells. Cells were treated for 2h with 5µM Bafilomycin A1 and were cultured in EBSS to induce starvation. (G) Quantification revealed a significant increase of p62 accumulations in patient-derived USH2C cells compared to healthy control cells. Scale in F = 10 µM Statistical significance was determined by Mann–Whitney U test *=p<0.05, **=p<0.01, ***=p<0.005.

Tables

Table 1. Autophagy related proteins identified by VLGR1 TAPs

Gene	Protein	Autophagy related protein function	Reference
RAB1A	RAB1A, member RAS oncogene family	Autophagosome formation	36
RAB8A	RAB8A, member RAS oncogene family	Autophagosome formation	36
STX12	Syntaxin 12	Autophagosome maturation	59
TOM1	target of myb1 membrane trafficking protein	Autophagosome maturation	60
UBQLN1	Ubiquilin 1	Autophagosome maturation	61
UBQLN2	Ubiquilin 2	Autophagosome maturation	61
UBQLN4	Ubiquilin 4	Autophagosome maturation	62
VTI1A	Vesicle transport through interaction with t-SNAREs 1A	Autophagosome maturation	63
HSPA8	Heat shock protein family A (Hsp70) member 8	Chaperone-mediated autophagy	64
ATP2A2	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2	ER/mitophagy	65
RETREG3	Reticulophagy regulator family member 3	ER-phagy	66
TEX264	Testis expressed 264, ER-phagy receptor	ER-phagy	67
UFL1	UFM1 specific ligase 1	ER-phagy	23
ATG9A	Autophagy related 9A	Induction	34
AUP1	AUP1 lipid droplet regulating VLDL assembly factor	Induction	68
CALR	Calreticulin	Induction	69
EIF4E	Eukaryotic translation initiation factor 4E	Induction	70
VMP1	Vacuole membrane protein 1	Induction	71
SPTLC1	Serine palmitoyltransferase long chain base subunit 1	Induction/ER-phagy	72
SPTLC2	Serine palmitoyltransferase long chain base subunit 2	Induction/ER-phagy	72
DDRGK1	DDRGK domain containing 1	Lysosome	73
LAMP2	Lysosomal associated membrane protein 2	Lysosome	74
PLAA	Phospholipase A2 activating protein	Lysosome	75
RIMOC1	RAB7A interacting MON1-CCZ1 complex subunit 1	Lysosome fusion	76
STX17	Syntaxin 17	Lysosome fusion	77
VAMP7	Vesicle associated membrane protein 7	Lysosome fusion	78
ATP5IF1	ATP synthase inhibitory factor subunit 1	Mitophagy	79
CERS1	Ceramide synthase 1	Mitophagy	80
FKBP8	FKBP prolyl isomerase 8	Mitophagy	81
FUNDC1	FUN14 domain containing 1	Mitophagy	61
PHB2	Prohibitin 2	Mitophagy	82
VCP	Valosin containing protein	Mitophagy	83
VDAC1	Voltage dependent anion channel 1	Mitophagy	84
HUWE1	HECT, UBA and WWE domain containing E3 ubiquitin protein ligase 1	Mitophagy/mTOR	85
GNAI3	G protein subunit alpha i3	mTOR	86
HK2	Hexokinase 2	mTOR	87
SCFD1	Sec1 family domain containing 1	mTOR	88
ATP6V0A1	ATPase H+ transporting V0 subunit a1	mTOR/Lysosome	89
NPC1	NPC intracellular cholesterol transporter 1	mTOR/Lysosome	90
AMBRA1	Autophagy and beclin 1 regulator 1	Regulation of autophagy	35
BAG3	BAG cochaperone 3	Regulation of autophagy	91
CISD2	CDGSH iron sulfur domain 2	Regulation of autophagy	92
EIF4G1	Eukaryotic translation initiation factor 4 gamma 1	Regulation of autophagy	93
EMC6	ER membrane protein complex subunit 6	Regulation of autophagy	94
KEAP1	Kelch like ECH associated protein 1	Regulation of autophagy	95
MCL1	MCL1 apoptosis regulator, BCL2 family member	Regulation of autophagy	96
MTDH	Metadherin	Regulation of autophagy	97
S100A8	S100 calcium binding protein A8	Regulation of autophagy	98
TMEM39A	Transmembrane protein 39A	Regulation of autophagy	99
TMEM41B	Transmembrane protein 41B	Regulation of autophagy	100
TMEM59	Transmembrane protein 59	Regulation of autophagy	101
PIK3R2	Phosphoinositide-3-kinase regulatory subunit 2	Upstream pathways	37
PIK3R4	Phosphoinositide-3-kinase regulatory subunit 4	Upstream pathways	37

Supplemental data

Supplemental Table S1. Upregulated genes identified by whole transcriptome sequencing of Vlgr1/del7TM retinae compared to WT retinae.

Supplemental Table S2. Downregulated genes identified by whole transcriptome sequencing of Vlgr1/del7TM retinae compared to WT retinae.