



VILNIUS GEDIMINAS TECHNICAL UNIVERSITY
FACULTY OF FUNDAMENTAL SCIENCES
DEPARTMENT OF CHEMISTRY AND BIOENGINEERING

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PATHOGENICITY ASSESSMENT OF PENDRIN (SLC26A4) VARIANTS
PENDRINO (SLC26A4) VARIANTŲ PATOGENIŠKUMO NUSTATYMAS

Master's degree final work

Bioengineering study programme, state code 6211FX017

Biotechnology study field

Salzburg, 2019

VILNIUS GEDIMINAS TECHNICAL UNIVERSITY
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For student **Rapolas Jamontas**

Final work title: Pathogenicity assessment of pendrin (SLC26A4) variants

Approved on 19.03.2019 by Dean's decree No. 154fm
The final work has to be completed by 25 May, 2019

THE AIM OF THE FINAL WORK:

To establish whether 5 variants of the pendrin (SLC26A4) protein may account for disease in 4 patients from the Austrian cohort with EVA

THE OBJECTIVES:

1. Functional characterization of five pendrin (SLC26A4) variants by measurement of the ion transport activity
2. Assessment of the cellular expression levels of these variants
3. Discrimination between potentially pathogenic and benign variants

Consultant of the final master degree work:

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Antrosios pakopos studijų Bioinžinerijos programos magistro baigiamasis darbas Pavadinimas Pendrino (SLC26A4) variantų patogeniškumo nustatymas Autorius Rapolas Jamontas Vadovas Jaunius Urbonavičius	
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Kalba: anglų

Anotacija Žmogaus SLC26A4 geno koduojamas baltymas pendrinas (SLC26A4) yra anijonų keitiklis būtinas taisyklingam vidinės ausies vystymuisi ir funkcionavimui. SLC26A4 geno sekos pakitimai sukelia dviejų tipų klausos praradimus – Pendred sindromą ir autosominį recesyvinį kurtumą (DFNB4). Šie genetiniai sutrikimai pasižymi specifiniu vidinės ausies vystymosi sutrikimu – prieangio latako išsiplėtimu (PLI). Kadangi PLI gali būti sukeltas ir kitų faktorių, nesusijusių su SLC26A4, galutinė Pendred sindromo ir DFNB4 diagnozė gali būti suteikta tik atlikus SLC26A4 geno sekoskaitą ir nustatčius baltymo variantų, turinčių sekos pakitimų, patogeninį potencialą. Šiame darbe tiriami penki mutantinio SLC26A4 baltymo variantai (p.Q101R, p.I136N, p.L597S, p.A664V ir p.G740V) rasti keturiuose pacientuose pirmoje Austrijos kohortoje (pacientų grupėje) su klausos praradimu ir PLI. Visų darbe tirtų baltymo variantų patogeniškumas nebuvo visiškai nustatytas ankstesniuose tyrimuose, ir du iš jų (p.Q101R ir p.I136N) šiame darbe paminėti pirmą kartą. Šių variantų patogeniškumas buvo nustatytas tiriant baltymo jonų pernašos aktyvumą naudojant fluorimetrinį metodą ir nustatant baltymo raiškos lygį ląstelėse naudojant konfokalinę mikroskopiją. Keturi baltymo variantai (p.Q101R, p.I136N, p.L597S ir p.A664V) pasižymėjo sutrikusia funkcija, sumažėjusiu raiškos lygiu ląstelėse ir atitiko patogeniškumo kriterijus. Vieno varianto (p.G740V) savybės, lyginant su laukinio tipo baltymu, buvo nepakitusios. Šio tyrimo rezultatai parodė, kad SLC26A4 genas yra atsakingas už dviejų pacientų klausos praradimą. Tyrimo metu stebėta teigiama koreliacija tarp baltymo jonų pernašos ir baltymo raiškos lygio ląstelėse parodė, kad baltymo raiškos sumažėjimas yra pagrindinis veiksnys sąlygojantis SLC26A4 baltymo variantų funkcijos praradimą. Darbą sudaro 6 dalys: įvadas, literatūros apžvalga, tyrimo priemonės ir metodai, tyrimo rezultatai ir jų aptarimas, išvados, literatūros sąrašas. Darbo apimtis 68 p. teksto, 19 iliustr., 16 lent., 67 bibliografiniai šaltiniai.
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Prasminiai žodžiai: pendrinas, SLC26A4, anijonų keitiklis, baltymo variantai, paveldimas klausos praradimas, Pendred sindromas
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Author **Rapolas Jamontas**

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Thesis language: English

Annotation

The SLC26A4 gene encodes a protein (pendrin, SLC26A4), which is essential for the proper development and function of the inner ear. SLC26A4 sequence alterations give rise to two forms of inherited hearing loss that are the Pendred syndrome and the autosomal recessive deafness DFNB4. These clinical entities are characterized by hearing loss associated with a specific malformation of the inner ear, the enlarged vestibular aqueduct (EVA). However, EVA can be caused by factors unrelated to SLC26A4. Therefore, sequencing of the SLC26A4 gene and assessment of the potential pathogenicity of the protein variants found, are necessary to give a conclusive diagnosis of Pendred syndrome or DFNB4. This study focused on five SLC26A4 protein variants (p.Q101R, p.I136N, p.L597S, p.A664V and p.G740V) found in four patients from the first cohort with hearing loss and EVA recruited in Austria. Of these variants, two (p.Q101R and p.I136N) were novel and for all of them the pathogenicity was not unequivocally established in former studies. The possible pathogenicity of these variants was assessed by measurement of their ion transport activities using a fluorometric method and determination of the cellular expression levels by confocal microscopy. Four of these variants (p.Q101R, p.I136N, p.L597S and p.A664V) demonstrated impaired function and reduced expression levels, consistent with a pathogenic potential, whereas one (p.G740V) showed unaltered features compared to the wild type protein. These findings led to a conclusive identification of SLC26A4 being the causative gene in two patients with hearing loss. The strong positive correlation between ion transport and expression levels indicates that reduction of expression is the main feature leading to loss of function of SLC26A4 variants.

Structure: Introduction, Literature review, Materials and methods, Results and discussion, Conclusions, References.
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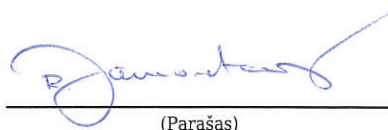
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Patvirtinu, kad mano baigiamasis darbas tema „Pendrinio (SLC26A4) variantų patogeniškumo nustatymas“ patvirtintas 2019 m. kovo 19 d. dekanų potvarkiu Nr. 154fm, yra savarankiškai parašytas. Šiame darbe pateikta medžiaga nėra plagijuota. Tiesiogiai ar netiesiogiai panaudotos kitų šaltinių citatos pažymėtos literatūros nuorodose.

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Kitų asmenų indėlio į parengtą baigiamąjį darbą nėra. Jokių įstatymų nenumatytų piniginių sumų už šį darbą niekam nesu mokėjęs (-usi).


(Parašas)

Rapolas Jamontas
(Vardas ir pavardė)

DISCLAIMER

This thesis contains confidential unpublished data.

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ABBREVIATIONS

2D	Two dimensional
a.a.	Amino acid
AcOH	Acetic acid
ANOVA	Analysis of variance
bp	Base pair
DAPI	4',6-diamidino-2-phenylindole
dbSNP	The Single Nucleotide Polymorphism Database
DFNB4	Autosomal recessive deafness 4
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
EVA	Enlarged vestibular aqueduct
EYFP	Enhanced yellow fluorescent protein
FBS	Fetal bovine serum
HBS	HEPES buffered saline
HBSS	Hank's Balanced Salt Solution
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KOAc	Potassium acetate
LB	Luria Bertani broth
MEM	Minimum essential medium
MOPS	3-Morpholinopropane-1-sulfonic acid
NIS	Sodium and iodide symporter
OMIM	Online Mendelian Inheritance in Man
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RCF	Relative centrifugal force
RPM	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
STAS	Sulphate Transporter and Anti-Sigma factor antagonist
TRIS	Tris(hydroxymethyl)aminomethane
WT	Wild type

Variations in protein amino acid sequence and nucleotide sequence in cDNA in the text are described according to Recommendations for a Nomenclature System for Human Gene Mutations (Antonarakis et al., 1998). Description of a sequence change begins with a letter indicating the type of reference sequence used, where “c.” corresponds to cDNA sequence and “p.” corresponds to protein sequence. Nucleotide substitutions in cDNA sequence are designated by a “>” character e.g.: c.2219G>T denotes that at nucleotide 2219 (Guanine, G) is changed to a thymine (T). Amino acid substitutions in the protein are designated using the one letter amino acid code e.g.: p.G740V denotes that amino acid 740 (Glycine, G) is changed to a Valine (V).

IUPAC one letter amino acid code used in the text:

A	Alanine	L	Lysine
C	Cysteine	N	Asparagine
D	Aspartic Acid	P	Proline
E	Glutamic Acid	Q	Glutamine
F	Phenylalanine	R	Arginine
G	Glycine	S	Serine
H	Histidine	T	Threonine
I	Isoleucine	V	Valine

IUPAC one letter nucleotide code used in the text:

A	Adenine	G	Guanine
C	Cytosine	T	Thymine

INTRODUCTION

The *SLC26A4* gene encodes for a protein called pendrin (SLC26A4), which is extremely important for the correct development of the inner ear and the onset and maintenance of a proper auditory function. Sequence alterations in the *SLC26A4* gene leading to aberrant protein products give rise to two forms of inherited hearing loss in humans, that are the Pendred syndrome (OMIM ID #274600) and the autosomal recessive hearing loss DFNB4 (OMIM ID #600791) (Hamosh, Scott, Amberger, Bocchini, & McKusick, 2005). Both these clinical entities are characterized by hearing loss associated with a specific malformation of the inner ear, which is the enlarged vestibular aqueduct (EVA). In Pendred syndrome, a partial iodide organification defect in the thyroid, occasionally leading to goiter, is also found. The clinical diagnosis of Pendred syndrome and DFNB4 is challenging. The audiological examination and the computed tomography of the temporal bone are precious tools to identify cases of hearing loss associated with EVA. In addition, the association of EVA and a positive perchlorate discharge test is strongly suggestive of Pendred syndrome. However, non-syndromic hearing loss can be caused by factors unrelated to *SLC26A4* mutations, and approximately 50% of patients of Caucasian ethnicity who have EVA do not have sequence alterations in *SLC26A4* (Wangemann & Griffith, 2017). On the other hand, several *SLC26A4* sequence alterations found in the general population as well as in deaf individuals are not pathogenic. The existence of the pseudo-Pendred syndrome (the association between deafness and thyroid disease of unrelated origin) represents an additional confounding factor in this complex scenario. These considerations led to the conclusion that sequencing of the *SLC26A4* gene and the pathogenicity assessment of the protein variants found, are necessary to give a conclusive diagnosis of Pendred syndrome or DFNB4 (Dossena, Nofziger, et al., 2011). The diagnosis is not only important to plan an appropriate intervention and lifestyle changes (scuba-diving and sport contact should be avoided by patients with residual hearing), but it also helps to establish a reliable prognosis for the patients. Disorders caused by the mutations in *SLC26A4* gene, such as hearing loss, can be progressive (residual hearing will most likely be lost later in life) or even appear during the adolescence or early adulthood, as goiter. Therefore, monitoring of the thyroid function in patients with *SLC26A4* sequence alterations is important. In addition, information obtained during these studies can be used for the genetic counselling. When the family-specific pathogenic variants are known, carrier testing for at-risk family members and prenatal testing for pregnancies at risk are possible. This helps increasing awareness of a potential problem for health care providers and family members and is a valuable tool to better assist the patient.

The prevalence and types of *SLC26A4* mutations are population-specific and, for some European countries have been already described; however, there was no conclusive information about the prevalence and types of mutations in Austria. This void was filled by the recruitment of the first cohort of patients with hearing loss and EVA in Salzburg. The first results of the *SLC26A4* analysis in 18 patients of this cohort were reported in 2018. The study identified 4 protein variants, for which functional and molecular features were investigated and the pathogenicity was assessed (Roesch et al., 2018). In addition, 10 new patients were recruited in this cohort recently, new *SLC26A4* variants were found and this thesis is focused on assessment of their potential pathogenicity. The aim of this thesis is:

to establish whether 5 variants of the pendrin (SLC26A4) protein may account for disease in 4 patients from the Austrian cohort with enlarged vestibular aqueduct.

The objectives to reach this aim are:

1. To functionally characterize five pendrin (SLC26A4) variants by measurement of the ion transport activities.
2. To assess the cellular expression levels of these variants.
3. To discriminate between potentially pathogenic and benign variants.

1 LITERATURE REVIEW

1.1 Inherited hearing loss

Hearing loss is the most common sensory deficit. The cause of hearing loss can be classified as genetic or non-genetic, which can occur either prelingually or post linguallly. The prelingual hearing loss affects 1 over 1,000 new-borns worldwide. In the developed countries, at least 50% of these cases are caused by genetic alterations and the other half is caused by other factors such as infantile infections or trauma (Morton, 1991). Depending on the anatomical site of the impaired function, hearing loss is defined as conductive or sensorineural. Any malfunction within the external and the middle ear results in conductive hearing loss, whereas the malfunction of the inner ear and central nervous system results in sensorineural hearing loss. Usually, inherited hearing loss is sensorineural and is often – although not always – a monogenetic disorder (caused by single-gene mutations). In these cases, hearing loss is derived by autosomal dominant, autosomal recessive, X-linked, or mitochondrial mode of inheritance. These genetic forms of hearing loss can be syndromic (hearing loss is found in combination with other abnormalities) or non-syndromic (hearing loss is the only relevant clinical finding) and they account for 30% and 70% of cases, respectively (da Silva Costa, Ramos, Martins, & Sartorato, 2017).

The autosomal recessive trait is the most common inheritance pattern of hearing loss, it accounts for about 77-88% of all cases. Dominant, X-linked and mitochondrial traits are observed less frequently, accounting respectively for 10-20%, 1-2% and 1% of all cases (Nance, 2003). Approximately 300 of human genes are known to be involved in inherited hearing loss, with *GJB2* (encoding connexin 26 protein) being the most common. The *GJB2* gene is involved in up to 50% of cases of autosomal recessive non-syndromic sensorineural hearing loss in various populations (Kenneson, Van Naarden Braun, & Boyle, 2002). *SLC26A4* is the second most common gene associated with autosomal recessive non-syndromic hearing loss. The characteristic feature caused by mutations in *SLC26A4* is an enlarged vestibular aqueduct (EVA). It is the most common form of inner ear abnormality that causes sensorineural hearing loss and can simultaneously appear with other malformations, such as Mondini dysplasia. A non-syndromic hearing disorder with the presence of EVA and *SLC26A4* sequence alterations is defined as autosomal recessive deafness 4 (DFNB4). Furthermore, mutations in *SLC26A4* are responsible for Pendred syndrome, with EVA and Mondini dysplasia apparent as well. It is the second most prevalent autosomal recessive syndromic hearing disorder after Usher syndrome, which is caused by mutations in 10 different genes (da Silva Costa et al., 2017).

1.2 Anatomy of the inner ear

The inner ear is the innermost part of the mammalian as well as the human ear. The two main functions of this organ are the detection of sound and the sense of equilibrium. It is situated in the hollow cavity of the temporal bone of the skull. The essential part of the hearing organ consists of two labyrinthine compartments: the bony labyrinth and the membranous labyrinth. The membranous labyrinth is an endolymph-filled structure of connected membranous sacs and ducts within the bony labyrinth. The bony labyrinth, a cavity in the temporal bone shown in Figure 1, is divided into three sections: the vestibule, three semi-circular canals and the cochlea. The vestibule is the central part of the bony labyrinth. It contains two membrane-covered openings connected to the middle ear: the oval window (vestibular *fenestra*) that receives sound vibrations, and the round window (cochlear *fenestra*) that vibrates in opposite phase of vibrations entering the inner ear through the oval window, which allows the fluid to move within the cochlea. There are three semi-circular canals: superior, posterior and lateral. Each of them begins at the common crus and ends with a dilated sac – the ampulla. The cochlea is a spiral shape cavity with two and three quarters of turns. The vestibular aqueduct which is relevant in this study is a duct located in the hinder part of the bony labyrinth, it extends from the vestibule through the temporal bone (Gray, 1918).

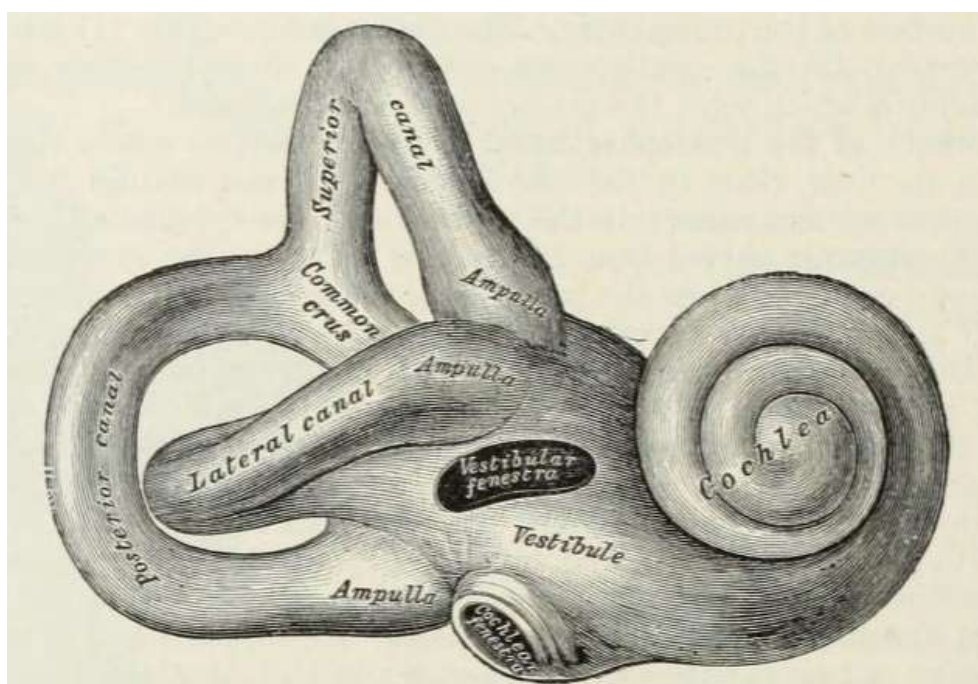


Figure 1. Structure of the bony labyrinth (Gray, 1918).

Apart from the bony structure, the inner ear comprises membranous labyrinth - a system of membranous ducts containing receptor structures for hearing and balance. The two main parts of

it are: the cochlea, responsible for hearing, and the vestibular system, dedicated to balance. The mature inner ear consists of seven interconnected fluid spaces (cochlea, saccule, utricle, three ampullae with semi-circular canals and the endolymphatic sac and duct) lined with epithelial cells and filled with endolymph that house six sensory organs (the Corti organ within the cochlea, the maculae within the saccule and utricle and three cupulae within the ampullae of the semi-circular canals). The structure of the membranous labyrinth is shown in Figure 2.

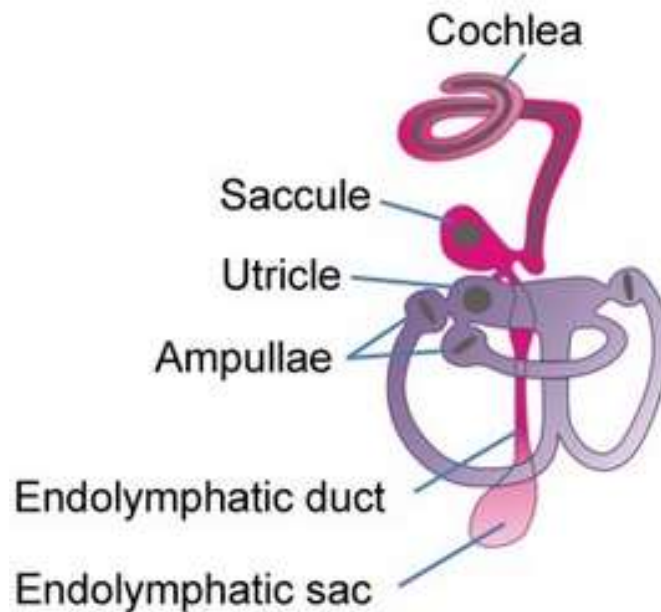


Figure 2. Schematic view of the membranous labyrinth of the inner ear. The inner ear consists of the cochlea, saccule, utricle, three ampullae with semi-circular canals and the endolymphatic sac and duct. All compartments are lined with epithelial cells and filled with endolymph (Li et al., 2013).

The sensory hair cells contained in the cochlea are responsible for hearing. They function by converting sound pressure patterns from the outer ear into electrochemical impulses, which are passed to the brain via the auditory nerve. The utricle and saccule are responsible for sensing linear acceleration, including gravity. The three ampullae with semi-circular canals are for sensing angular acceleration in three spatial axes. These functions are mediated by sensory vestibular hair cells. The seventh fluid compartment is the endolymphatic duct situated within the vestibular aqueduct ending with a blind pouch – the endolymphatic sac. It is in contact with the *dura mater* – the connective tissue surrounding the brain. The endolymphatic sac and duct do not have any sensory functions and are suspected to take part in fluid homeostasis (H. M. Kim & Wangemann, 2010).

1.3 Developement of the mammalian inner ear

Plentiful knowledge about the mammalian inner ear development was obtained by experiments in mice. In these animals, the morphogenesis of this part of the hearing organ starts at the embryonic day 9.5 with the formation the otocyst – a precursor of the inner ear. The formation occurs by thickening of the ectoderm known as the otic placode, which encloses amniotic fluid that has high Na^+ concentration (Mansour & Schoenwolf, 2006; Morsli, et al., 1998). At the embryonic day 10.5, two tube-like protrusions start to extend from the otocyst: these later form the endolymphatic sac and the cochlea. During the elongation, the central part of the otocyst starts to organize itself into the vestibular labyrinth. At the embryonic day 12 the cochlea acquires a more elaborate shape and the semi-circular canals of the vestibular labyrinth start to delineate. Between the embryonic day 13 and 17, the membranous labyrinth attains its mature shape and the coiling process of the cochlea reaches one and three-quarters of turns (Morsli et al., 1998).

The formation of all the lumina in the inner ear development is controlled by fluid secretion and absorption. Fluid secretion occurs in the vestibular labyrinth and fluid absorption occurs in the endolymphatic sac (H. M. Kim & Wangemann, 2010). One hypothesis says that luminal volume control in this phase of development is dependent on the secretion and absorption of NaCl . This conclusion was reached in reference to the composition of the luminal fluid in the epithelial lumen of the cochlea and the endolymphatic sac. Until embryonic day 17.5, these are filled with a solution that contains $\sim 140 \text{ mM Na}^+$, $\sim 126 \text{ mM Cl}^-$, $\sim 10 \text{ mM K}^+$, and $\sim 25 \text{ mM HCO}_3^-$ (Li et al., 2013). With the end of the cochlear development, the main cation of the luminal fluid endolymph changes from Na^+ to K^+ . K^+ secretion begins on the embryonic day 19.5, and two days after birth the endolymph already contains $\sim 70 \text{ mM}$ of K^+ . The concentration of K^+ keeps increasing during the maturation until it almost replaces all Na^+ cations (concentrations 150 mM and 1 mM , respectively) in the mature inner ear (Li, Zhou, et al., 2013).

1.4 The impact of SLC26A4 on the development of the inner ear

Most of the research on the physiological role of SLC26A4 in the inner ear was carried out in mice models. SLC26A4 expression in the mature inner ear is found in the apical membrane of epithelial cells in the cochlea, transitional epithelium of the vestibular labyrinth and the apical membrane of mitochondria-rich cells in the endolymphatic sac (Ines E. Royaux et al., 2003). The graphical representation of the expression locations is shown in Figure 3.

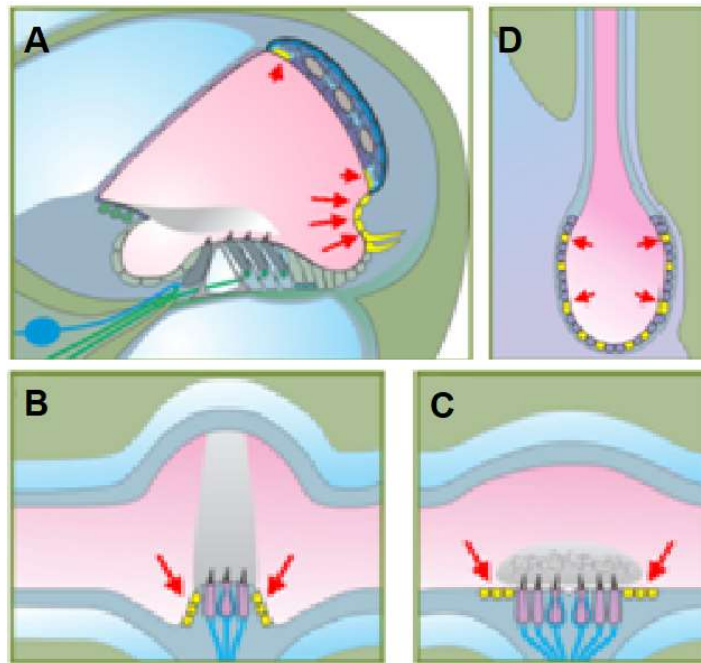


Figure 3. SLC26A4 expression in the inner ear is shown with red arrows. (A) Cross section of one turn of the mature cochlea. SLC26A4 is expressed in epithelial cells of the spiral prominence (long arrows) and in spindle cells of *stria vascularis* (short arrows). (B) Cross section of the ampulla. SLC26A4 is expressed in transitional cells, which are epithelial cells surrounding the cupulae – a structure embedded with sensory hair cells in the ampullae. (C) Cross sections of the saccule or utricle. SLC26A4 is expressed in transitional cells of the maculae - the sensory organs located within the saccule and utricle. (D) Cross sections of the endolymphatic duct and sac. The endolymphatic duct runs through a bony canal - the vestibular aqueduct. SLC26A4 is expressed in mitochondria-rich cells in the endolymphatic sac (Adapted from Wangemann et al. 2017).

The two main pathological alterations that occur in the inner ear lacking SLC26A4 expression are: abnormal enlargement of luminal volume, which starts at the embryonic stage and persists in the adulthood, and the acidification of luminal fluid.

According to the hypothesis mentioned previously, the formation of all the lumina in the developing inner ear is controlled by fluid secretion and absorption and, as the mouse models have shown, SLC26A4 is most likely involved in this process. The *Slc26a4* knock-out mice have shown about 10-fold enlargement of *scala media*. This is an endolymph-filled luminal space in the cochlea, which includes both the sensory hair cells and *stria vascularis* – a structure that forms the outer wall of the cochlear duct. In addition, *Slc26a4* knock-out mice develop EVA and a Mondini dysplasia of the cochlea, fail to develop hearing and display vestibular deficits (L. A. Everett, 2002). Theoretically, the enlargement may result from an overall increase in NaCl concentration in endolymph, resulting in heavy fluid secretion, or from a decrease in NaCl and fluid absorption. Recent investigations have shown that the main location where fluid absorption occurs in SLC26A4-dependent fashion, is in the endolymphatic sac (Honda et al., 2017). Therefore, the lack of SLC26A4 function is more likely leading to a failure of NaCl and fluid absorption. However, the lack of SLC26A4 alone cannot result in such a defect, since SLC26A4 is an anion exchanger

that has the stoichiometry of 1:1 (Dossena et al., 2006; Shcheynikov et al., 2008). Therefore, it is conceivable that SLC26A4 drives Cl⁻ reabsorption and other currently unknown Na⁺ permeation pathways are likely to be involved.

The SLC26A4 protein in the inner ear functions as a chloride-bicarbonate exchanger, it contributes to the transport of bicarbonate in the endolymph, thus causing an increased endolymphatic pH (H. M. Kim & Wangemann, 2010). In case of loss of function of SLC26A4, the consequent luminal acidification alters pH-sensitive processes, including the formation of an endocochlear potential and the reabsorption of Ca²⁺ (Wangemann et al., 2007). In addition, the enlargement of *scala media* resulting from failure of fluid reabsorption in the endolymphatic sac, disturbs cell-to-cell communication, or interferes with the activity of diffusible factors transmitted via lumen. Overall, these alterations disrupt the development of hearing (H. M. Kim & Wangemann, 2011).

1.5 The SLC26A4 protein

SLC26A4, also known as pendrin, is a transmembrane protein found on the apical membrane of epithelial cells. It belongs to the multifunctional anion exchanger family 26 in the solute carrier group (SLC26) (Mount & Romero, 2004). In humans, this protein is encoded by *SLC26A4* gene, which is composed of 21 exons and located on the chromosome 7. The protein itself is 86 kDa in mass, contains 780 amino acids and is produced using approximately 5 kb of length mRNA transcript (Lorraine A. Everett et al., 1997). Originally it was believed that the protein is responsible for sulphate transport due its homology to the proteins having such function, but this theory soon was denied proving that it is a chloride, iodide, bicarbonate and formate transporter (Scott et al., 1999, Soleimani et al., 2001). Studies have shown that SLC26A4 functions as a coupled, electroneutral iodide/chloride, iodide/bicarbonate and chloride/bicarbonate exchanger, having 1:1 stoichiometry, with the preference of iodide anion even in high chloride concentrations (Dossena, Vezzoli, et al., 2006; Shcheynikov et al., 2008).

Although substantial effort was devoted to the elucidation of the function of SLC26A4, the crystal structure of the protein was not determined, neither by X-ray crystallography nor using the other techniques. However, the three-dimensional model of the human SLC26A4 structure exists, and is based on the known crystal structures of its homologues: a rat anion exchanger - prestin (Slc26a5) and a fumarate transporter from *Deinococcus geothermalis* – SLC26Dg. The most recent model suggests that the protein contains a cytoplasmic N-terminal domain, a transmembrane domain, and a cytoplasmic C-terminal Sulphate Transporter and Anti-Sigma factor antagonist (STAS) domain.

The polypeptide begins with a 79 a.a. (amino acid) long N-terminal domain containing 2-3 predicted α -helices. To this date, the functional role of this domain remains unknown. The transmembrane domain encompasses a.a. 88-511 and contains 14 membrane spanning α -helices, which are responsible for substrate binding and transport activity. The two-dimensional (2D) representation of the recent model is shown in Figure 4.

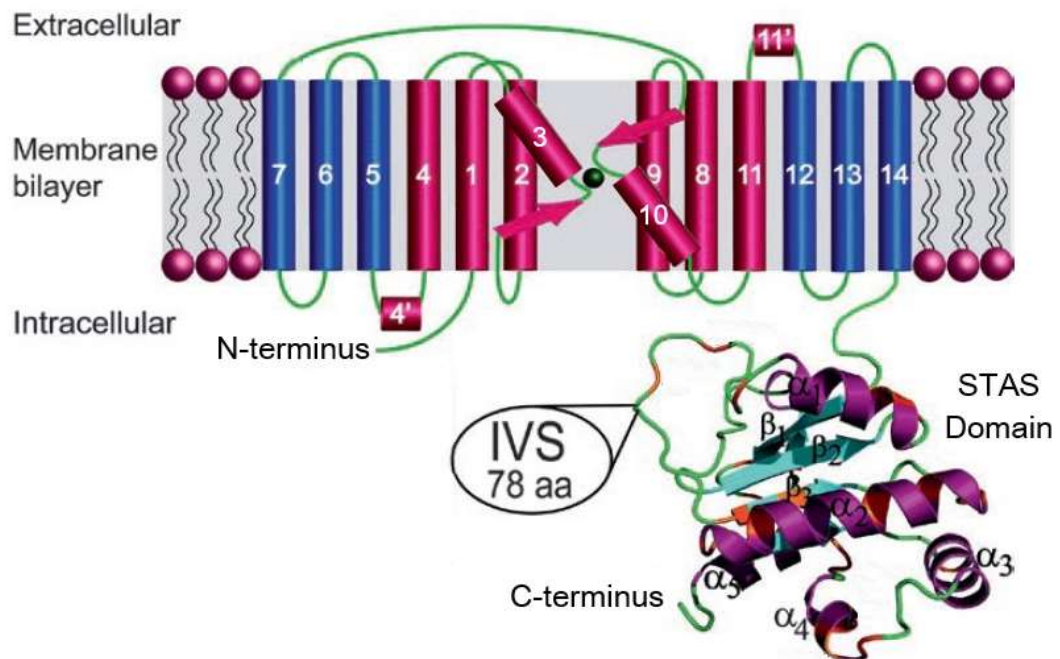


Figure 4. 2D structure representation of the recent human SLC26A4 transmembrane and STAS domain model. α -helices spanning the membrane are numbered 1-14. IVS - intervening sequence of the STAS domain (Adapted from Dossena et al. 2017).

Homology modelling on the SLC26Dg template predicted that the substrate binding site can be located at amino acids Q101, I363 and R409, which are located respectively in the 1st, 8th and 10th alpha helices of the transmembrane domain (Dossena et al., 2017). Interestingly, the p.R409H mutation in human SLC26A4 is already reported as disease-causing (M.P. Gillam, Bartolone, Kopp, & Benvenga, 2009). The polypeptide sequence is terminated by the STAS domain continuing from a.a. 515 to a.a. 734, excluding the disordered intervening sequence of a.a. 574–652. The STAS domain is located in the cytosol and is hypothesized to serve as protein-protein interaction module as it does in bacterial homologues. Mutations in STAS domains of mammalian SLC26 transporters impair function and/or surface expression of these transporters (Dossena et al., 2017).

1.6 SLC26A4-related disorders

Pendred syndrome is a genetic disorder which is inherited in an autosomal recessive manner, meaning that an abnormal allele has to be inherited from each parent to develop the condition. It was described for the first time in 1896 by Dr. Vaughan Pendred after observing a combination of deaf-mutism and goiter in two members of a large Irish family (Pendred, 1896). The reason for the association of these two symptoms remained unknown until 1997, when the *SLC26A4* (originally called *PDS*) gene was discovered and shown to be involved in this disease. The *SLC26A4* gene was discovered using a positional cloning strategy, and several sequence alterations responsible for the disease were described. This disease involves two organs: the ear and the thyroid gland. It is characterized by sensorineural hearing loss, a type of hearing loss which is caused by structural or sensory abnormalities in the inner ear; and thyroid dysfunction, such as an iodide organification defect, goiter or hypothyroidism (Everett et al., 1997). The exact incidence of Pendred's syndrome is difficult to evaluate because of its phenotypic variability. While the hearing loss is a constant feature of Pendred syndrome, the goiter has been reported to vary significantly in size within families and may even be absent (Fraser, 1964). An investigation was performed in a 52 Pendred syndrome patient cohort, and showed that the perchlorate discharge test resulted to be positive in the great majority of patients, of which 83% had goiter, generally developing after the age of 10 years, 56% had a functional thyroid (euthyroid), and 44%, all of whom had goiter, had objective evidence of hypothyroidism. This example shows that Pendred syndrome does not have a defined pattern of thyroid dysfunction (Reardon et al., 1999), and that a positive perchlorate discharge test, and not the presence of an overt goiter, should be considered the diagnostic sign.

In a normally functioning thyroid gland, the inorganic iodide, having been trapped, is immediately organified by binding to thyroglobulin – a precursor of thyroid hormones triiodothyronine and thyroxine. The main Pendred syndrome diagnostic tool is a perchlorate challenge, a test which is widely used in the detection of thyroid inability to organify the iodide (Reardon, 1997). In this test, the radioactive iodine first is administered, sufficient time is allowed to pass so that the radioactive iodine is captured by the thyroid, and then, perchlorate is administered orally. The perchlorate displaces non-organified iodide from the thyroid and the perchlorate discharge test is considered positive if there is an abnormally rapid loss of radioactive iodine from the thyroid (Morgans & Trotter, 1958).

In contrast to the thyroid dysfunction, the hearing loss is a constant feature of Pendred syndrome. Mondini dysplasia – an abnormality of the cochlea having one and a half instead of two and three quarters turns around its axis - is a common but probably not a constant feature of Pendred syndrome. However, enlargement of the endolymphatic sac and duct in association with

a large vestibular aqueduct connected with temporal bone abnormalities is present in all the patients (Phelps et al., 1998). The graphical representation of these abnormalities is visible in Figure 5.

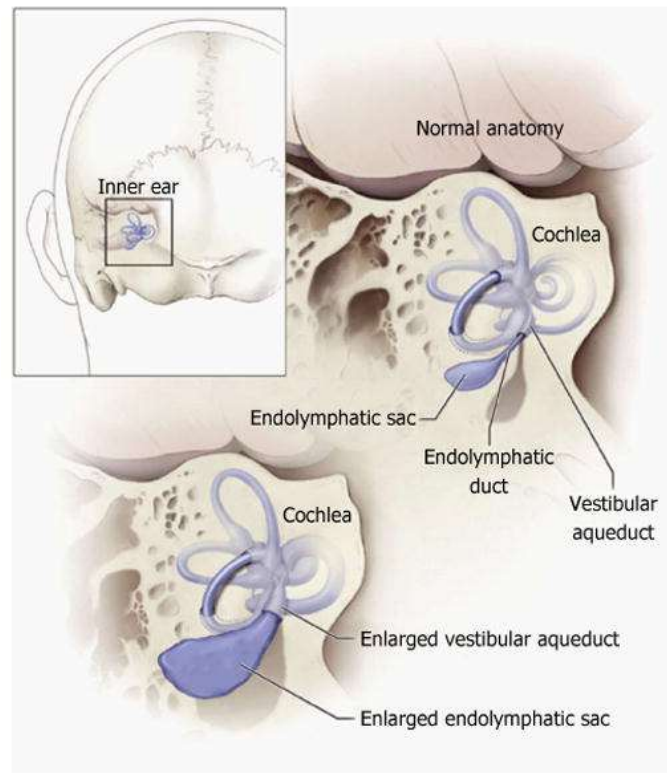


Figure 5. Schematic illustration of the abnormalities related to *SLC26A4* mutations. Normal anatomy of the inner ear structures is shown above. Pathologic enlargement of the endolymphatic sac, abnormal enlargement of the vestibular aqueduct and Mondini cochlea are shown below (Ito, 2013).

In some cases, hearing loss is congenital, but typically it is progressive and fluctuating, occurring at prelingual age (1-3 years) or at young age (4-14 years) (Miyagawa et al., 2014). Apart from the hearing impairment, a vestibular dysfunction in Pendred syndrome is also common, but not constant. This might be caused by the close proximity of the auditory and vestibular organs. It includes: vertigo, clumsiness, head tilting with vomiting at a prelingual age, and/or motor developmental delay (Zalewski et al., 2015).

Non-syndromic deafness DFNB4 (autosomal recessive deafness 4, with enlarged vestibular aqueduct) is another autosomal recessive disorder that is caused by mutations in *SLC26A4* gene. This type of hearing loss is associated with temporal bone abnormalities, which result in the enlargement of the vestibular aqueduct and can be both bilateral and unilateral. It can also include Mondini dysplasia, as in Pendred syndrome (Campbell et al., 2001).

1.7 SLC26A4 expression in other tissues

It is evident that SLC26A4 has a certain function in the thyroid gland, since Pendred syndrome patients develop thyroid abnormalities such as hypothyroidism and goiter (Everett et al., 1997). In addition, it was confirmed by mRNA quantification that SLC26A4 is expressed abundantly in the thyrocytes (I. E. Royaux et al., 2000). However, *in vivo* studies suggest that SLC26A4 does not play an essential role in the thyroid, since knock-out mice lacking both *Slc26a4* alleles do not develop a thyroid phenotype, even on a low iodine diet (Wangemann et al., 2009). The hormone synthesis in the thyroid gland involves several steps: firstly, the iodide is uptaken from the blood through the sodium and iodide symporter NIS located in the basolateral membrane of the thyroid epithelial (follicular) cell. After that, it is released to the follicular lumen of the thyroid gland through the apical membrane of the same cell, where it is oxidized by oxidases and peroxidases (DUOX and TPO) and organified by covalent binding to the thyroglobulin – a precursor of thyroid hormones. These steps are graphically represented in Figure 6 (Twyffels et al., 2011). Although the mechanism mediating iodide uptake to the thyrocytes through the basolateral membrane is well understood, less is known about the efflux of the iodide through the apical membrane. Supposedly, SLC26A4 takes part in this process (Kopp et al. 2008). Current investigations have shown that SLC26A4 is not the only protein which might participate in this process – anoctamin 1 (TMEM16A) is another plausible candidate for this function. However, the exact physiological roles of these transporters are still awaiting further characterization (Silveira & Kopp, 2015).

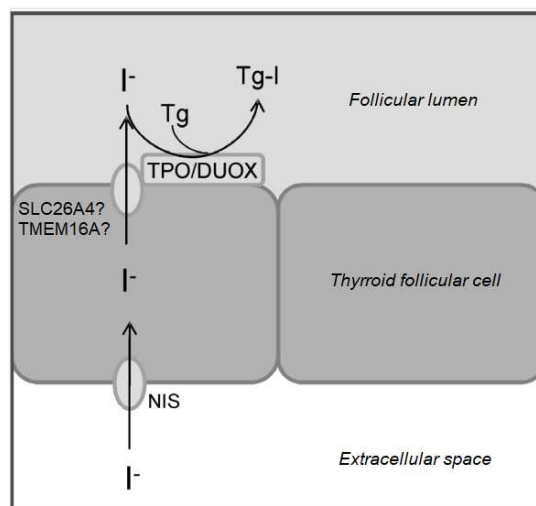


Figure 6. Scheme of iodide transport through a thyroid follicular cell. Iodide is transported to the follicular cell via NIS (sodium iodide symporter) and delivered to the follicular lumen likely by SLC26A4 and TMEM16A (adapted from Twyffels et al. 2011).

SLC26A4 is found abundantly in the kidneys, where it is expressed in the apical regions of type B and non-A and non-B intercalated cells in the connecting tubule (CNT) and the cortical

collecting duct (CCD). SLC26A4 takes a major role in Cl^- reabsorption and HCO_3^- secretion into the urine through apical $\text{Cl}^-/\text{HCO}_3^-$ exchange, thus regulating the salt balance and the pH of the blood (Wall et al., 2004). Knock-out mice models have shown that the absence of *Slc26a4* does not cause any significant salt wasting, as well as Pendred syndrome patients showed a normal renal function, demonstrating that the SLC26A4 role in kidneys can be compensated by other chloride-base exchangers (Amlal et al., 2010; Bizhanova & Kopp, 2010). Although SLC26A4 is usually not essential to maintain acid–base homeostasis in normal conditions, loss of renal bicarbonate excretion by SLC26A4 during a metabolic alkalotic challenge, caused by intercurrent illness or pharmacological therapy, may contribute to life-threatening acid–base disturbances in patients with Pendred syndrome (Kandasamy et al. 2011). Apart from $\text{Cl}^-/\text{HCO}_3^-$ exchange function in the kidneys, SLC26A4 is reported to act like a Cl^-/I^- exchanger as well, and participate in the maintenance of the iodide balance in the organism (Y. H. Kim et al., 2009).

1.8 Epidemiology of SLC26A4 variants

A considerable number of studies have shown that the prevalence and type of *SLC26A4* mutations are population-specific, showing distinct characteristics between different ethnicities. For example, the highest prevalence of *SLC26A4* mutations has been reported in East Asian populations: in China, Taiwan, Japan, and Korea between 82 and 98% of patients with EVA carry mutations of *SLC26A4*. The prevalence of *SLC26A4* mutations in European and North American Caucasians is much lower: about 50% of patients with EVA carry mutations of *SLC26A4* and of these patients, about one-half carries only one detectable mutation in the coding regions and splice sites of *SLC26A4* (Wangemann & Griffith, 2017). The prevalence and types of mutations were already reported in some European countries, for example: Czech Republic, Denmark, France, Germany, Spain, Turkey and the United Kingdom (Tsukada, Nishio, Hattori, & Usami, 2015).

As the prevalence of mutations varies among different ethnicities, the type of mutations varies as well. Some mutations are frequent among multiple populations and some of them are restricted to specific geographical regions. Mutations found in European populations are represented in Figure 7.

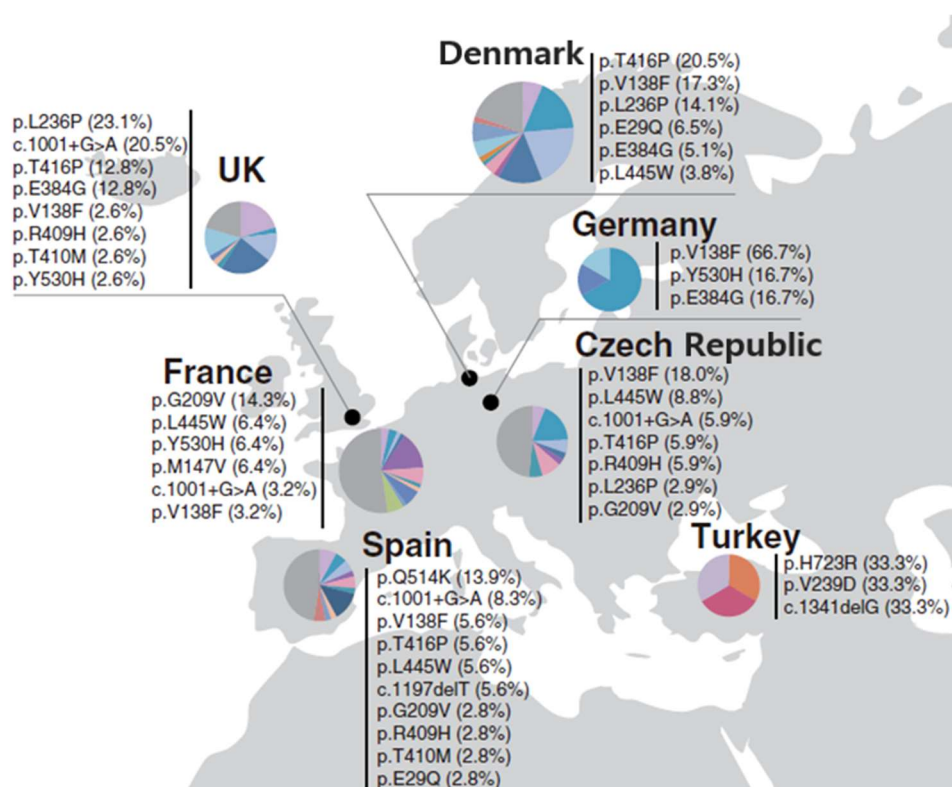


Figure 7. Types and frequencies of *SLC26A4* gene mutations reported in European countries (adapted from Tsukada et al. 2015).

The most prevalent mutations in the Caucasian population are: c.1001+1G>A (splicing donor mutation), p.V138F, p.T416P, p.L236P, and p.G209V. The c.1001+1G>A mutation is the most or second most prevalent mutation in 4 of 7 European countries and the United States. The p.V138F mutation is the most prevalent in countries with Caucasian populations and is predominant in Germany, Czech Republic and Denmark. The p.T416P, p.L236P, and p.G209V mutations are mainly found in Denmark, the United Kingdom and France respectively. In addition, these mutations are found at a moderate frequency in Europe and the United States. Most of the mutations found in the Caucasian population are not found in the Asian populations. The p.H723R and c.919-2A>G (splicing acceptor mutation) are the most common in the Asian population. p.H723R is predominant in Japan and Korea and the c.919-2A>G is predominant in China, Taiwan, and Mongolia (Tsukada et al., 2015).

1.9 Functional properties of *SLC26A4* variants

The cause of the Pendred syndrome, a combination of sensorineural hearing loss and goiter, is defined by the presence of biallelic mutations in *SLC26A4* gene. However, similar conditions can be caused by other factors. A pseudo-Pendred syndrome, an association of deafness and goiter, can occur in patients having no *SLC26A4* mutations, but as a result of environmental factors,

autoimmune thyroid disease, and mutations in deafness genes not related to *SLC26A4* (Fugazzola et al., 2002). In addition, malformations of the inner ear such as an enlarged vestibular aqueduct can be found in patients with mutations either in one *SLC26A4* allele or in both alleles. However, this malformation can occur in patients having both wild type alleles of the *SLC26A4* gene, or patients having a mutation which is not leading to amino acid substitution or predicted exonic splicing enhancer disruption (Pryor et al., 2005). Some of *SLC26A4* variants are reported to be found both in healthy and affected individuals, thus suggesting their low pathogenic potential (Pera et al., 2008). Taking these facts into consideration, it is clear, that the association of deafness and goiter in a given patient, or detection of a sequence alteration in the *SLC26A4* gene do not necessarily imply a functional defect in the protein, and are insufficient to obtain a conclusive diagnosis of Pendred syndrome or DFNB4.

As extensive functional testing of *SLC26A4* variants has shown, it is not easy to predict functional consequences of a mutation by its location within the polypeptide (Dossena et al., 2017). However, some mutations can immediately signal to loss of function. These are nonsense mutations leading to the polypeptide truncation and missense mutations introducing a substitution of a proline, which is disrupting the secondary structure of a transmembrane domain organized in alpha helix (Dossena, Nofziger, et al., 2011). In contrast, the pathogenicity of other mutations can hardly be predicted, for example: a missense sequence alteration leading to the substitution of highly conserved amino acid p.F354S was found not to affect anion transport function (Dossena, Bizhanova, et al., 2011). Moreover, the p.R776C variant, which has a wild-type anion transport functionality, is found at the same frequency in the hearing-impaired cohort and in the control cohort. The same applies to functionally impaired variants, such as p.E29Q, p.V609G, p.D724G (Pera et al., 2008). Therefore, amino acid conservation and prevalence are unreliable predictors of possible functional alteration of a protein variant. As of 2019, nearly 500 sequence alterations of *SLC26A4* gene have been reported, 67% of which are missense or nonsense mutations in the polypeptide sequence. Only 17% of them had anion transport function or subcellular trafficking reported, most of them led to the loss of *SLC26A4* functionality and was linked to Pendred syndrome or non-syndromic deafness. However, ~9% of reported variants maintain the wild-type protein function and two variants are showing a moderate gain of function (Dossena et al., 2017; Dossena, Bizhanova, et al., 2011). In conclusion, it is clear that functional and molecular characterization has to be made in addition to sequence analysis to assign or exclude the pathogenic potential of a *SLC26A4* protein variant.

1.10 Functional characterization of SLC26A4 variants

The first published method for the investigation of transport activity of SLC26A4 with altered sequence was performed in oocytes of *Xenopus laevis*. Various concentrations of mRNA samples coding wild type and mutant SLC26A4 were delivered into cells using microinjection. The transport ability of the protein was determined by observation of radioactive iodine-125 and chlorine-36 uptake into cells using scintillation spectroscopy (D A Scott et al., 2000). However, the physiological environment of a non-mammalian model system was not the most suited to investigate SLC26A4, and the experimentations gradually moved to the mammalian systems. The first functional testing of SLC26A4 in human embryonic kidney (HEK) 293 cells was reported in 2002. The previously mentioned sodium and iodide symporter NIS (see chapter 1.7) was introduced into cells together with wild type and mutant SLC26A4 by co-transfection. The presence of sodium iodide symporter provided a mechanism by which cells were loaded with the ions of radioactive iodide-125 from the extracellular environment. An SLC26A4-dependent radioactive iodide efflux back to the extracellular environment was measured afterwards (Taylor et al., 2002). As similar functional test based on the measurement of the iodide efflux was developed using the polarized Madin-Darby canine kidney (MDCK) cell line and viral DNA introduction techniques for creating a NIS and SLC26A4 heterologous co-expression system (Mary P. Gillam et al., 2004). A functional test using HEK 293 cells was developed for the measurement of chloride uptake and its competition with iodide using the same radioactive isotopes as in the studies mentioned above. The iodide and chloride uptake was determined by quantification of radioactive isotopes in the cell lysate after cell incubation in chloride-36 or iodide and chloride-36 containing buffer (Dossena et al., 2006). Beside the radioactive isotope-based methods, the $\text{Cl}^-/\text{HCO}_3^-$ exchange can be measured by using the pH sensitive fluorescent probe BCECF. However, there are very few such studies compared to I^-/Cl^- exchange (Dossena, Nofziger, et al., 2011; Yoon et al., 2008).

Most of the early methods used for determination of SLC26A4 activity were based on the transport of radioisotopes and their detection, but using radioactive substances was making the whole procedure quite complicated. A milestone in SLC26A4 functionality determination was reached with the development of a non-radioactive method based on the fluorescence of an enhanced yellow fluorescent protein isoform (EYFP) (Dossena et al., 2006). The method is based on the EYFP p.H148Q p.I152L isoform whose fluorescence is quenched when exposed to certain concentrations of halides. Moreover, this isoform has an increased affinity to iodide ($K_i=2.3$ mM at pH=7.35) compared to chloride ($K_{Cl}=85$ mM at pH=7.35), thus making it suitable for measuring the iodide flux across the cell membrane (Galiotta et al. 2001). When cells expressing both the

EYFP isoform and SLC26A4 are exposed to an extracellular environment containing high iodide concentration, SLC26A4 (in the case it is functional) leads to the iodide flux through the cell membrane towards the cytosol, where the fluorescence of the EYFP isoform is quenched. The schematic principle of the method is represented in Figure 8.

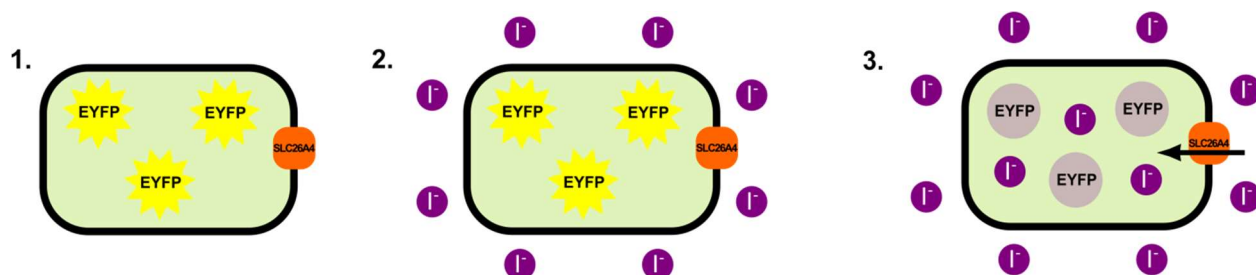


Figure 8. Schematic principle of functional assay of iodide exchange. (1) Cells co-expressing SLC26A4 (orange) and the iodide sensitive EYFP isoform (yellow). (2) Iodide addition to the extracellular environment. (3) Flux of iodide toward cytosol through the SLC26A4 in exchange for chloride, resulting in fluorescence decrease of the iodide-sensitive EYFP isoform.

The change in fluorescence can be measured using multiple techniques. The measurements can be carried out using a fluorescence microscope equipped with a camera and the cells placed in a perfusion chamber. In this case, both efflux and influx of iodide and chloride can be measured on a single-cell level by manipulating buffers in the perfusion chamber (Dossena et al., 2006). Also, fluorescence measurements can be performed using a multi-plate reader equipped with a liquid dispensing system. The liquid dispensing system is injecting high iodide concentration buffer to each well of a 96-well plate, and fluorescence intensity is measured before and after injection. The fluorescence change reflecting the SLC26A4 activity can be then calculated. The output of this technique is higher compared to the single-cell assay concerning the number of variants investigated in one measuring series. Although only the influx of iodide is measured, in contrast to both efflux and influx measured in a perfusion chamber, this test provides sufficient data to determine whether the SLC26A4 variant is functional (de Moraes, Bernardinelli et al., 2016).

2 MATERIALS AND METHODS

2.1 Instruments

Table 1. List of instruments.

Manufacturer	Instrument
Bioer, China	Thermal cycler GeneTouch
General Electric, USA	Spectrophotometer NanoVue Plus
Leica Microsystems, Germany	Confocal laser scanning microscope Leica TCS SP5 II
PerkinElmer, USA	Liquid dispenser for microplate reader 1420-2550 Multilabel Plate Reader VICTOR X3
Sartorius, Germany	Balance Acculab ALC-810.2 Incubator shaker Certomat IS Precision balance LE225D-OCE
Thermo Fisher Scientific, USA	BSL 2 laminar flow hood Herasafe™ KS Centrifuge Heraeus Multifuge 3SR Plus Centrifuge Heraeus Fresco 17 Centrifuge Heraeus Megafuge 40R Clean bench Heraguard ECO CO ₂ Incubator Heracell 150 Spectrophotometer GENESYS 10S Bio Superspeed centrifuge Sorvall RC 6 Plus
VWR, USA	Digital pH-meter SympHony SB70P Mini centrifuge MiniStar Vortex mixer 444-1372
Wescor, USA	Vapor pressure osmometer Vapro 5520

2.2 Reagents and kits

Table 2. List of reagents.

Manufacturer	Reagents (catalog number)
Agilent Technologies, USA	Pfu DNA polymerase (600252-52), Pfu reaction buffer (600153-82)
Alfa Aesar, USA	Manitol (A14030), NaI (40196), RbCl (12892)
AppliChem, Germany	Ampicillin (A0839), HEPES (A3268), Kanamycin (A1493), MgCl ₂ (A4998)
Biontex, Germany	Metafectane PRO (T040-2.0)
Biozym, Germany	dNTP (331520)
Macherey-Nagel, Germany	NucleoBond PC 500 kit (740574.25)
Merck, Germany	KH ₂ PO ₄ (529568), KOAc (529543), CaCl ₂ (1725701000), EtOH absolute (1009862500)
Qiagen, Germany	MinElute PCR Purification Kit (28004)
Sigma-Aldrich, USA	AcOH (45740), DAPI (D9542), Glucose (49139), Glycerol (G5516), HBSS (H1387), Isopropanol (I9516), KCl (P5405), LB Agar (L2897), LB broth (L3022), Manitol (65550), Minimum Essential Medium Eagle (M5650), Minimum Essential Medium Eagle (M0643), Mineral oil (M5904), MnCl ₂ (M8054), MOPS (69947), Sodium pyruvate (P2256), Na ₂ HPO ₄ (71644), NaCl (S3014), NaHCO ₃ (S8761), NaOH (S8045), Paraformaldehyde (76240), Penicillin-streptomycin solution (P0781), TRIS (T1503), Trypsin-EDTA solution (T3924)
Thermo Fisher Scientific, USA	DpnI restriction endonuclease (FD1704), Nuclease-free H ₂ O (Am9930), FBS (10270106)
VWR, USA	EDTA (443882G), EtOH 70% (83.801.410), SDS (444464T)

2.3 Solutions and medias

Table 3. List of medias

Medium	Composition
LB Broth	Prepared according to the recommendations of manufacturer
LB Agar	Prepared according to the recommendations of manufacturer
LB+	LB Broth, Glucose 20 mM
MEM complete	Minimum Essential Medium Eagle (M0643), FBS 10%, Sodium pyruvate 1 mM, Penicillin-streptomycin solution (P0781), NaHCO ₃ 2.2g/L
MEM	Minimum Essential Medium Eagle (M5650) as purchased from manufacturer

Table 4. List of solutions.

Solution	Composition
RF1 Solution	RbCl 100 mM, 4H ₂ O•MnCl ₂ 50 mM, KOAc 30 mM, 2H ₂ O•CaCl ₂ 10 mM, Glycerol 15% (v/v), pH=5.8
RF2 Solution	RbCl 10 mM, MOPS 10 mM, 2H ₂ O•CaCl ₂ 75 mM, Glycerol 15% (v/v), pH=6.8
Mini-Prep Solution 1	Glucose 50 mM, EDTA 10 mM, TRIS 25 mM pH=8, RNase 0.1 mg/mL
Mini-Prep Solution 2	NaOH 0.2 M, SDS 0.1%
Mini-Prep Solution 3	KOAc 3 M, AcOH 1.5%, pH=4.8
Phosphate buffered solution (PBS)	NaCl 136.7 mM, KCl 2.7 mM, Na ₂ HPO ₄ •2H ₂ O 3.2 mM, KH ₂ PO ₄ 1.5 mM
HBS solution	NaCl 140 mM, 2H ₂ O•Na ₂ HPO ₄ 1.5 mM, HEPES 50 mM, pH=7.05
Isotonic high chloride solution	NaCl 135 mM, KCl 2 mM, HEPES 20 mM, CaCl ₂ 1 mM, MgCl ₂ 1 mM, Glucose 10 mM, pH=7.4, 308 mOsm/Kg (adjusted with manitol)
Isotonic high iodide solution	NaI 135 mM, KCl 2 mM, HEPES 20 mM, CaCl ₂ 1 mM, MgCl ₂ 1 mM, Glucose 10 mM, pH=7.4, 308 mOsm/Kg (adjusted with manitol)

2.4 Oligonucleotide primers

Mutagenesis primers:

1. SLC26A4 p.G740V

Sense: 5'-CTCAAGAGGGTCAAGTTTCCATTTTAGAAACG-3'

Antisense: 5'-CGTTTCTAAAATGGAAACTTGACCCTCTTGAG-3'

2. SLC26A4 p.L597S

Sense: 5'-CTAATAAAAAGTGGACAATCAAGAGCAACAAAG-3'

Antisense: 5'-CTTTGTTGCTCTTGATTGTCCACTTTTATTAG-3'

3. SLC26A4 p.I136N

Sense: 5'-CATCAAGACATAACTCAGTTGGACCTTTTC-3'

Antisense: 5'-GAAAAGGTCCAACCTGAGTTATGTCTTGATG-3'

4. SLC26A4 p.A664V

Sense: 5'-CTGCTTGACTGTGGAGTTATATCTTTCCTG-3'

Antisense: 5'-CAGGAAAGATATAACTCCACAGTCAAGCAG-3'

5. SLC26A4 p.Q101R

Sense: 5'-CTAGTGGCCACGCTGCGAGGGATGGCATATG-3'

Antisense: 5'-CATATGCCATCCCTCGCAGCGTGGCCACTAG-3'

Sequencing primers:

1. 1R

Antisense: 5'-TTCCATTGCTGCTGGATACG-3'

2. 2R

Antisense: 5'-GGAAAATGATGCAGCCAGC-3'

3. 2F

Sense: 5'-TGGGATCTGTTGTTCTGAGC-3'

4. 3R

Antisense: 5'-TTGCCATAGAAAATAGGACTGG-3'

5. 4F

Sense: 5'-GAAGCATCCCTAGCACAGATA-3'

6. 981-98F

Sense: 5'-TGTTAAATCCATCCCAAG-3'

7. pEYFP 2192R

Antisense: 5'-AACCCAACTAGAATGCAG-3'

2.5 Bacterial strains and cell lines

Table 5. Bacterial strains

Strain	Genotype	Source
<i>E. coli</i> XL-1 Blue	<i>endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+)</i>	Agilent Technologies, USA

Table 6. Cell lines

Cell line	Description	Source
HEK 293 Phoenix	Virus modified human embryonic kidney cells having high transfection efficiency (DiCiommo, Duckett, Burcescu, Bremner, & Gallie, 2004).	Gift from Prof. D. Di Francesco, University of Milan, Italy
HeLa (ATCC® CCL-2™)	Human adenocarcinoma cell line.	ATCC, USA

2.6 Plasmid vectors

1. **pTARGET SLC26A4 WT** – a mammalian expression vector pTARGET (Promega Corporation, USA) containing cDNA of the wild type SLC26A4 from the human thyroid tissue with an exahistidine tag (his-tag) fused to the C-terminus of the protein.
2. **pEYFP-N1 SLC26A4 WT** – a mammalian expression vector pEYFP-N1 (Clontech, USA) containing cDNA of the wild type SLC26A4 from the human thyroid tissue producing a protein with an enhanced yellow fluorescent protein (EYFP) fused to its C-terminus.
3. **pEYFP-N1 EYFP-H148Q-I152L** - a mammalian expression vector bearing two point mutations in EYFP coding sequence that lead to two amino acid substitutions (p.H148Q and p.I152L). This variant of the protein has an increased sensitivity for iodide, which is quenching its fluorescence. The vector was already available in the institute and was obtained using site directed mutagenesis from the parent pEYFP-N1 vector (Clontech, USA) (Dossena, Bizhanova, et al., 2011).

2.7 Methods

2.7.1 Site directed mutagenesis

Site directed mutagenesis was performed by PCR using specifically designed oligonucleotide primers and parental plasmid vectors bearing the wild type SLC24A4 cDNA. All mutagenic oligonucleotide primers were designed individually according to the desired mutation to obtain in the SLC26A4 cDNA sequence. All mutagenesis reactions performed in the experiments were non-conservative missense point mutations leading to an amino acid substitution. Both of sense and antisense mutagenic primers contained the desired mutation and annealed on opposite strands of the target sequence. At the initial step of the primer design, primer region covered a desired point mutation and ~15 bases of correct sequence on both sides terminating with one or two C or G bases. Designed sequences were analyzed with MacVector software (MacVector inc., USA) to ascertain likelihood of secondary structure formation (dimerisation, hairpin structures) and calculate melting temperature (T_m). If necessary, primer length was modified to decrease the chance of secondary structure formation and adjust T_m to ~60 °C. The reaction mixture for site directed mutagenesis using PCR was prepared as indicated in Table 7.

Table 7. Composition of site directed mutagenesis reaction mixture

Ingredient	Volume (μL)
Nuclease free H ₂ O	40
10× Pfu buffer	5
Antisense mutagenesis primer 10 μM	1.5
Sense mutagenesis primer 10 μM	1.5
dNTP 10 μM	1
Wild type SLC24A4 cDNA template 0.5 μg/μL	1
Pfu Turbo DNA polymerase (2.5 U/μL)	1

The reaction mixture was overlaid with 10 μL of mineral oil to prevent evaporation. Mutagenesis reactions were carried out in a thermocycler following the protocol given in Table 8.

Table 8. Protocol of site directed mutagenesis reaction

Step	Temperature	Duration	Number of cycles
Initial denaturation	95 °C	3 min	1
Denaturation	95 °C	30 sec	18
Primer annealing	55 °C	1 min	
Elongation	68 °C	10 min	
Final hold	4 °C	∞	1

Following the mutagenesis reaction, 1 μ L of *DpnI* (10 U/ μ L) restriction endonuclease was added to the reaction mixture below the mineral oil layer to digest methylated parental plasmid DNA. The incubation of the digestion reaction was carried out at 37 °C for one hour. After digestion, the plasmid DNA was purified from the reaction mixture by MinElute PCR Purification Kit following manufacturer's instructions. Purified plasmid DNA was immediately used for *E. coli* XL-1 Blue competent cell transformation.

2.7.2 Preparation of chemically competent *E. coli* cells

E. coli XL-1 Blue cells were inoculated in 3 mL of antibiotic-free LB medium and incubated in an incubator shaker overnight at 37 °C, 240 RPM. Next day the inoculum was transferred to 200 mL antibiotic-free LB medium and incubated in incubator shaker at 37 °C, 240 RPM until OD₆₀₀ reached 0.7. Whole culture was divided into 4 ice-cold 50 mL conical tubes and incubated on ice for 30 minutes. Later on, cells were pelleted using a centrifuge pre-cooled to 4 °C at 1,942 RCF for 15 minutes. The supernatant was discarded and the pellet of each tube was resuspended in 2 mL of ice-cold RF1 solution (composition in chapter 2.3). The entire suspension (8 mL) was transferred into a single ice-cold 15 mL conical tube and incubated on ice for 30 minutes. After incubation, the suspension was pelleted using a centrifuge pre-cooled to 4 °C at 1,942 RCF for 10 minutes. The supernatant was discarded and cells were resuspended in 2 mL of ice-cold RF2 solution (composition in chapter 2.3). Resuspended cells were aliquoted into ice-cold 1.5 mL microcentrifuge tubes in 50 μ L portions and stored at -80°C.

2.7.3 Transformation of chemically competent *E. coli* cells

Fifty to 100 ng of plasmid DNA was added to competent *E. coli* XL-1 Blue cells thawed on ice, gently mixed and incubated on ice for 10 min. Following the incubation, heat shock was performed by incubating cell suspension and plasmid DNA mix in a 42 °C water bath for 45 seconds. Immediately after heat shock cells were incubated on ice for 2 minutes. Hereafter, 250 μ L of warm (37 °C) LB+ medium (composition in chapter 2.3) was added to the cell

suspension and the mixture was incubated in a 37 °C water bath for at least 30 minutes and up to 60 minutes. All the cells from the single tube were spread on LB agar plates with proper antibiotic in the presence of flame. Plates were incubated at 37 °C overnight.

2.7.4 Small scale plasmid DNA purification from *E. coli* cells

Small scale plasmid DNA purification was carried out using a 3 mL *E. coli* inoculum in LB medium containing a selective antibiotic and grown in incubator shaker at 37 °C, 240 RPM overnight. The inoculum was set up using a single colony grown on an LB-agar plate containing a selective antibiotic. Two mL of the inoculum were transferred into an Eppendorf tube and centrifuged at 1,500 RCF for 10 minutes followed by the removal of supernatant. Pellet was gently resuspended by pipetting in 100 µL of cold Mini-Prep Solution I (resuspension solution; composition in chapter 2.3) and incubated at room temperature (RT) for 5 minutes. Hereafter, 200 µL of Mini-Prep Solution II (lysis solution; composition in chapter 2.3) was added, the suspension was mixed by 8-10 top-to-bottom inversions and incubated on ice for 5 minutes. After the incubation, 150 µL of cold Mini-Prep Solution III (neutralization solution; composition in chapter 2.3) was added, the suspension was mixed by 8-10 top-to-bottom inversions and incubated on ice for 5 more minutes. The lysate was centrifuged for 15 minutes at 4 °C, 17,000 RCF. For the alcohol precipitation step, 400 µL of the supernatant was transferred to a 1.5 mL Eppendorf tube followed by addition of 1 mL of 100 % EtOH and vigorous vortexing. The mixture was incubated at -80 °C for 5 minutes and centrifuged at 4 °C, 13,800 RCF for 15 minutes. The supernatant was immediately discarded and the pellet washed with 750 µL of 70% EtOH. Tubes were centrifuged at 4 °C, 13,800 RCF for 15 minutes, the supernatant was poured off immediately and the tubes were placed upside-down for at least 10 minutes in order for EtOH to evaporate. The dried DNA pellet was resuspended in 10 µL of nuclease-free water and stored at -20 °C.

2.7.5 Large scale plasmid DNA purification from *E. coli* cells

An unquantified plasmid DNA sample obtained by small scale DNA purification as described above and having a confirmed sequence was diluted 100 times and chemically competent *E. coli* XL-1 Blue cells were transformed using 1 µL of the diluted sample. Cells were spread on a LB agar plate with a proper antibiotic and incubated overnight at 37 °C. Next day a single colony was picked, inoculated in 3 mL of LB medium with a proper antibiotic and incubated in the incubator shaker at 37 °C, 240 RPM for 8 hours. In the evening, the whole inoculum was transferred to 400 mL of LB medium with a proper antibiotic and grown overnight in the incubator shaker at 37 °C, 240 RPM. Following morning, the whole inoculum was centrifuged at 4 °C, 3,452 RCF for 30 minutes, the supernatant was discarded and the harvested bacteria were used for plasmid DNA

purification using NucleoBond® PC 500 kit following the manufacturer's instructions. The whole DNA obtained in this process was resuspended in 400 µL of nuclease-free water, quantified using a spectrophotometer and stored at -20 °C.

2.7.6 Sample preparation for sequencing

Sanger sequencing was used for analysing both small and large scale plasmid DNA purification products by using 6 different sequencing primers covering various regions of the whole SLC26A4 cDNA insert (2,340 bp). Regions covered by sequencing reactions with each individual primer are listed in Table 9.

Table 9. Approximate positions in SLC26A4 cDNA insert and corresponding amino acids covered by sequencing reactions using various sequencing primers.

Primer name	Position in SLC26A4 cDNA (bases)	Position in translated SLC26A4 (amino acids)
1R	1-450	1-150
2R	180-990	60-330
2F	510-1,260	170-420
3R	780-1,620	260-540
981-98F	1,020-1,800	340-600
4F	1,650-2,340	550-780
pEYFP 2192R	Covers EYFP sequence	

Sequence analysis of small scale plasmid DNA purification products was performed only with a single primer covering only a fragment of the insert containing the mutated nucleotide. The reaction mixture contained 1.5 µL of purified and unquantified plasmid DNA, 6 µL of 5 µM sequencing primer capable to cover the mutated nucleotide and 7.5 µL of nuclease-free water.

Sequence analysis of large scale plasmid DNA purification products was performed using 6 different reactions with each individual sequencing primer covering the whole insert, an additional sequencing reaction covering EYFP sequence was performed if necessary. The reaction mixture contained 2.4 µL of plasmid DNA (0.5 µg/µL), 6 µL of each single 5 µM sequencing primer per reaction and 6.6 µL of nuclease-free water.

2.7.7 Maintenance of the mammalian cell lines

All the procedures used for the maintenance of the cell lines were performed using sterile consumables and standard sterile techniques under the biosafety level 2 laminar flow hood. All the procedures were performed using medium prewarmed to 37 °C and the other consumables being

RT. Cells were cultivated in a CO₂ incubator at 37°C, 5% CO₂ and 95% relative humidity if it is not indicated differently.

Both HeLa and HEK 293 Phoenix cell lines used for experiments were cultured using MEM complete medium (composition in chapter 2.3) in 10 cm diameter tissue culture plates. After the confluence of cells in the plate reached 80% (for HEK 293 Phoenix) or 70% (for HeLa), medium was removed from the plate and cells were washed by adding 10 mL of PBS buffer (composition in chapter 2.3) and rocking the vessel back and forth several times. Next, the washing solution was removed, and the cell layer was covered with 1 mL of pre-warmed trypsin-EDTA solution in order to dissociate cells. Tissue culture plates containing HEK 293 Phoenix or HeLa cells were incubated for approximately 2 minutes at RT or for approximately 5 minutes in a CO₂ incubator respectively, till more than 90% of the cells were detached from the plate (the exact duration of incubation were dependent on cell dissociation level observed visually or through an optical microscope). The dissociation agent was blocked by adding 9 mL of MEM complete media. The whole 10 mL of cell suspension were transferred to a 15 mL conical tube and centrifuged for 5 minutes at 218 RCF, the supernatant was discarded and cells were resuspended in 10 mL of MEM complete media. For the inoculation of a subculture, cell suspension was diluted 1:5 using MEM complete medium (final volume 10 mL) and seeded in 10 cm diameter tissue culture plate. The same suspension was used for inoculation of cultures used for following experiments.

2.7.8 Transient calcium phosphate co-precipitation transfection

HEK 293 Phoenix cells used for functional testing were transfected using calcium phosphate co-precipitation method and were cultured in a black standard 96 well plate for fluorescence reading with minimum back-scattered light and background fluorescence (Thermo Scientific, USA). Cells used for the transfection were cultivated by seeding 22 µL of cell suspension (obtained as described in section 2.7.7) in 150 µL of MEM complete medium per well and grown for 16 h.

Each transfection reaction mixture was prepared in a 1.5 mL microcentrifuge tube with the final volume of 150 µL and contained a total amount of 3 µg plasmid DNA. Plasmid DNA (from 0.5 µg/µL working stocks) were diluted in water (final volume 67.5 µL) and 7.5 µL of 2.5 M CaCl₂ solution were added followed by addition of 75 µL of HBS solution (composition in chapter 2.3). The reaction mixture was mixed by injecting air bubbles to the bottom of the tube 10-15 times using a 200 µL pipette. Hereafter, the transfection mixture was incubated for 10 minutes at RT. Following the incubation, 12 µL of transfection mixture were added to each well containing cells to be transfected (0.24 µg of DNA per well). Transfected cells were incubated for 48 hours before carrying out the functional testing.

2.7.9 Transfection using lipofection technique

Cells used for the determination of total protein expression levels were transfected using the lipofection technique. HeLa cells were cultivated in a standard 6 well plate by seeding 500 μ L of cell suspension (obtained as described in section 2.7.7) in 2 mL of MEM complete medium per well. After 16 h, cells were transfected by using Metafectane PRO transfection reagent. The transfection was carried out using 1.5 μ g of plasmid DNA and 3 μ L of Metafectane PRO following the manufacturer instructions. Medium was changed 6 hours after transfection.

2.7.10 Functional assay of iodide exchange

The SLC26A4 ability to transport iodide anions was determined by measurement of the iodide influx in the intracellular environment. This was performed following transient co-transfection of pTARGET vector carrying cDNA of the wild type or one of the five SLC26A4 variants and pEYFP-N1 vector carrying the cDNA of EYFP-H148Q-I152L in HEK 293 Phoenix cells. The EYFP-H148Q-I152L isoform of EYFP bears two point mutations that are resulting in two amino acid substitutions; these mutations confer to EYFP an increased sensitivity to intracellular iodide, which is quenching its fluorescence (Galiotta et al., 2001). Being an iodide/chloride exchanger, SLC26A4 can lead to an iodide influx to the cell in exchange for chloride, in the presence of iodide in the extracellular solution. Following this influx, the fluorescence of EYFP-H148Q-I152L, which is abundant in the intracellular environment, is quenched. The efficiency of EYFP-H148Q-I152L fluorescence quenching is an indication of protein activity. The comparison of the maximal fluorescence decrease in % of wild type protein, control and variants can give us an idea about the possible functional impairment, hence pathogenicity, of the mutation carried by the variants. The fluorometric method is based on the method developed by Dossena et al. (Dossena, Rodighiero, et al., 2006) and is performed in a multiplate reader measuring the fluorescence intensity of co-transfected cells before and after injection of an isotonic high iodide solution to the extracellular medium (de Moraes, Bernardinelli et al., 2016).

To generate a heterologous overexpression system, calcium chloride co-precipitation method was used to transfect HEK 293 Phoenix cells with the pTARGET vector carrying one of the five SLC26A4 variants and the pEYFP-H148Q-I152L-N1 vector. In addition, cells were co-transfected with pEYFP-H148Q-I152L-N1 together with the pTARGET vector carrying wild type SLC26A4 cDNA, or pEYFP-H148Q-I152L-N1 together with the empty pTARGET vector as a positive and negative control, respectively. Cells transfected with a mixture of non-fluorescent pTARGET SLC26A4 and pTARGET vectors were used to determine the background autofluorescence (Table 10).

Table 10. A table showing the transfections performed. Sample No. corresponds to a single co-cotransfection series and Vector I and Vector II to vectors used in that process.

Sample No.	Vector I	Amount of Vector I (µg)	Vector II	Amount of vector II (µg)	Overall amount of DNA (µg)
1.	pTARGET SLC26A4 G740V	1.5	pEYFP-H148Q-I152L-N1	1.5	3
2.	pTARGET SLC26A4 L597S				
3.	pTARGET SLC26A4 I136N				
4.	pTARGET SLC26A4 A664V				
5.	pTARGET SLC26A4 Q101R				
6.	pTARGET SLC26A4				
7.	pTARGET				
8.	pTARGET		pTARGET SLC26A4	1.5	

The co-transfections were carried out in cells seeded on a black 96 well plate for fluorescence reading. Each of the transfection reactions was made for 6 independent replicates, each corresponding to an individual well of the plate. The functional assay was performed 48 hours after co-transfection of cells. All the following procedures were performed in dusk environment in order to not affect fluorescent properties of EYFP. Part of the growth medium was removed from the wells and cells were washed 3 times with 150 µL of isotonic high chloride solution (composition in chapter 2.3). Care was taken not to perturb the cells or let them dry. After the washing steps, solution was removed completely, and cells were bathed in 70 µL of isotonic high chloride solution. Measurements were performed in a multilabel plate reader equipped with a liquid dispenser and the following filters: excitation: F485 (excitation centre wavelength: 485 nm, bandwidth: 14 nm), emission: F535 (emission: 535 nm, bandwidth: 25 nm). Procedure was performed as following: 3 measurements of the baseline fluorescence intensity (1 measurement per second), injection of 140 µL of isotonic high iodide solution (composition in chapter 2.3), 16 measurements of the fluorescence intensity after injection (1 measurement per second).

The arithmetical mean of the background fluorescence before and after injection was calculated and subtracted from all the values used in calculations. Data indicating functionality of protein were expressed as percentage of fluorescence variation ($\Delta F\%$) between arithmetical mean of three measurements before the injection of isotonic high iodide solution and the arithmetical mean of the last three measurements after the injection of isotonic high iodide solution to the extracellular milieu. A negative $\Delta F\%$ indicates an influx of iodide to the intracellular milieu.

2.7.11 Determination of total protein expression levels by imaging

Determination of total SLC26A4 expression levels was performed by confocal imaging of HeLa cells expressing a fluorescent SLC26A4-EYFP fusion protein encoded by pEYFP-N1 vector containing a coding sequence of wild type or one of the five SLC26A4 variants.

Forty-eight hours after transfection medium was removed, cells were washed with 2 mL of PBS buffer and 200 μ L of trypsin-EDTA solution were added to each well. Cells were incubated for approximately 5 minutes in a 37 °C, 5% CO₂ incubator in order for them to dissociate. After incubation cells were resuspended in 2 mL of MEM complete medium and transferred to a new standard 6 well plate containing a 3 cm diameter borosilicate microscope slide at the bottom of the well. Twenty-four hours after the transfer, cells situated on microscope slides cells were fixed using 4% paraformaldehyde solution in HBSS. All the following procedures were performed in dark environment in order not to affect fluorescent properties of EYFP. Cell culture medium was removed and the cells were washed 3 times using 2 mL of HBSS solution. Next, 1.5 mL of 4% paraformaldehyde solution was added to each well, plates were covered with aluminium foil and incubated for 30 minutes at RT. After the incubation, paraformaldehyde was removed and cells were washed 3 times with 2 mL of HBSS solution. Washed cells were bathed in 2 mL of HBSS solution before the staining of nuclei.

Nuclear staining was performed in order to obtain an indication of the cell density in the imaged field. Later on, while calculating SLC26A4 expression levels, the fluorescence intensity of the fusion proteins in the imaging field produced by EYFP will be normalized for the fluorescence of nuclei representing the overall cell density. In order to stain nuclei, the HBSS solution in the well was replaced by 1 mL of 0.1 μ g/mL DAPI solution in HBSS and cells were incubated at RT for 10 minutes. After the incubation, the staining solution was removed and cells were washed 3 times with 2 mL of HBSS solution. Washed cells were bathed in 2 mL of HBSS solution before the imaging.

Imaging was performed using a Leica TCS SP5II AOBS confocal laser scanning microscope equipped with HCX PL APO 63 $\times$ Lambda blue water immersion objective (numerical aperture 1.20), an Argon laser and a 405 nm diode laser. All the equipment was controlled by the LAS AF SP 5 software (Leica Microsystem, Germany). EYFP was excited using the 514 nm line of the Argon laser and emission was detected in a wavelength from 525 to 580 nm, DAPI was excited using a 405 nm diode laser and emission was detected in a wavelength from 450 to 490 nm.

For determination of total SLC26A4 expression levels, fluorescence intensity of SLC26A4-EYFP and DAPI in the same imaging area of the sample were detected and subtracted for the background fluorescence determined in an area of the imaging field with no cells. Next, the

EYFP fluorescence was normalized for the DAPI fluorescence. Imaging parameters (laser power, photomultiplier gain, pixel size and zoom factor) were kept rigorously constant for all the acquisitions.

2.7.12 Statistical data analysis

All the data are expressed as arithmetical means $\pm$ standard error of the mean. Statistical analysis was performed using Prism 5.0c software for MAC OS (GraphPad Software, USA). Significance of differences between the datasets was tested by one-way analysis of variance (ANOVA). Dunnett's multiple comparison post-test was used for functionality comparison of each individual SLC26A4 variant versus wild type protein and empty vector after the functional assay. Bonferroni's multiple comparison post-test was used for comparison of each single sample versus all the remaining samples after the determination of expression levels. Differences were considered statistically significant with the p -value being lower 0.05. A correlation between functionality of variants and their expression levels was calculated by applying linear regression, calculating a coefficient of correlation (r) and calculating a p -value for confirmation of model conformity.

3 RESULTS AND DISCUSSION

This work focused on 10 patients recruited between 2018 and 2019 and included in the Austrian cohort having hearing loss and enlarged vestibular aqueduct. Results of the Sanger sequencing analysis performed on genomic DNA prior to this work revealed 4 out of 10 patients having 5 uncharacterized and 1 characterized alterations in the protein coding sequence of *SLC26A4* gene, leading to six protein variants. List of patients and their mutations in *SLC26A4* gene are represented in Table 11.

Table 11. List of 4 patients from deaf Austrian cohort whose mutations found in *SLC26A4* gene were chosen for further investigation.

Patient ID	Nucleotide change		Amino acid change		NCBI dbSNP		Clinical significance according to the NCBI dbSNP	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
#653	c.2219G>T	WT	p.G740V	WT	Reported	-	Uncertain	-
#654	c.1790T>C	WT	p.L597S	WT	Reported	-	Likely benign	-
#659	c.407T>A	c.1991C>T	p.I136N	p.A664V	Novel	Not reported	Unknown	Unknown
#660	c.302A>G	c.1226G>A	p.Q101R	p.R409H	Novel	Reported	Unknown	Pathogenic

Two patients (#653 and #654) had an uncharacterized monoallelic mutation and two patients (#659 and #660) had a mutation in both alleles of which three were uncharacterized and one (p.R409H) was characterized previously and found to be pathogenic. Of the five uncharacterized mutations, two variants (p.I136N and p.Q101R) are completely novel, two were previously mentioned in the scientific literature and are reported in the NCBI Single Nucleotide Polymorphism Database (dbSNP), but are of uncertain (p.G740V) or likely benign (p.L597S) clinical significance (Sherry et al., 2001). One of them (p.A664V) was reported to have a reduced formate transport ability (Yuan et al., 2012), but it is not reported in the NCBI dbSNP, and therefore has no dbSNP ID assigned, with no conclusive statement about its possible pathogenicity. The novel variants (p.I136N and p.Q101R) and variant p.G740V have never been characterized previously, neither on a functional nor on a molecular level.

3.1 Generation of plasmid vectors

Characterization of both functionality and molecular features of SLC26A4 required a creation of heterologous overexpression systems *in vitro*. To achieve that, mammalian expression vectors coding p.G740V, p.L597S, p.I136N, p.A664V and p.Q101R variants of SLC26A4 were generated.

3.1.1 Site directed mutagenesis

All of the sequence alterations described were introduced into plasmid vectors bearing the cDNA of wild type SLC26A4 by site directed mutagenesis using mutagenic oligonucleotide primers designed individually according to the desired mutation (as described in chapter 2.7.1). Plasmids obtained by site directed mutagenesis were used for the transformation of *E. coli* XL-1 Blue competent cells (as described in chapter 2.7.2 and 2.7.3), which were inoculated on LB agar plates. Mature colonies were individually cultivated in LB media, followed by small-scale DNA purification (as described in chapter 2.7.4). The region covering a desired mutation in the purified plasmid DNA samples was sequenced (Microsynth AG, Switzerland) and analysed (as described in chapter 2.7.6). A plasmid DNA sample having a confirmed mutation was used for the transformation of *E. coli* XL-1 Blue competent cells, followed by large-scale DNA purification (as described in chapter 2.7.5). All bacteria cultures were grown under the selective pressure of kanamycin (30 µg/mL) for pEYFP-N1 transformed cells or ampicillin (100 µg/mL) for pTARGET transformed cells.

Each mutation was introduced individually to two different plasmid vectors containing the cDNA of wild type SLC26A4. The list of vectors produced is presented in Table 12.

Table 12. List of plasmid vectors obtained by site directed mutagenesis.

Vectors used for functional testing	Vectors used for determination of expression levels
pTARGET SLC26A4 p.G740V	pEYFP-N1 SLC26A4 p.G740V
pTARGET SLC26A4 p.L597S	pEYFP-N1 SLC26A4 p.L597S
pTARGET SLC26A4 p.I136N	pEYFP-N1 SLC26A4 p.I136N
pTARGET SLC26A4 p.A664V	pEYFP-N1 SLC26A4 p.A664V
pTARGET SLC26A4 p.Q101R	pEYFP-N1 SLC26A4 p.Q101R

The pTARGET vector containing the cDNA of wild type or mutated SLC26A4 was used for functional tests. The structure of the vector is given in Figure 9.

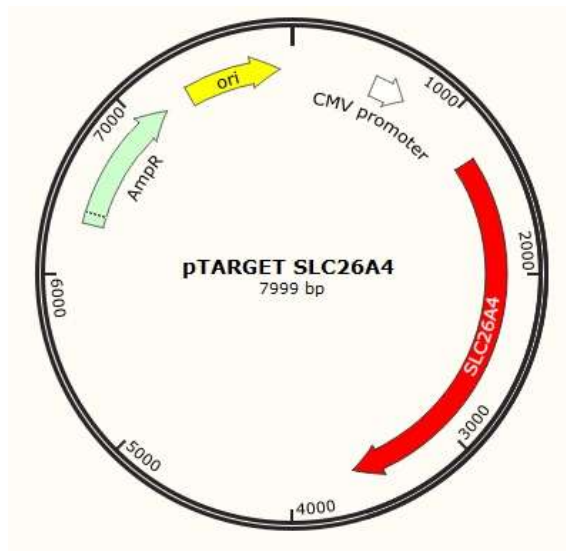


Figure 9. pTARGET vector containing a SLC26A4 sequence used for functional testing.

The pEYFP-N1 vector containing the cDNA of wild type or mutated SLC26A4 was used for determination of SLC26A4 expression levels using confocal microscopy. After the transfection with this construct, mammalian cells produce a fusion protein: the enhanced yellow fluorescent protein (EYFP) is fused to SLC26A4 C-terminus. The structure of the vector is given in Figure 10.

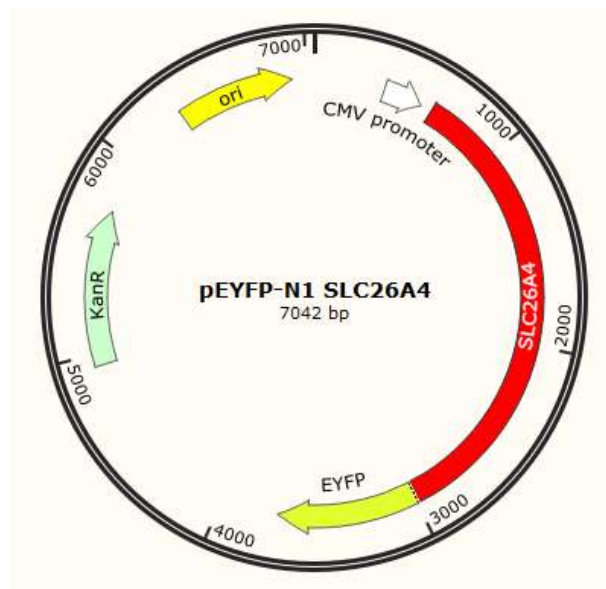


Figure 10. pEYFP-N1 vector containing a SLC26A4 sequence and enhanced yellow fluorescent protein (EYFP) used for determination of protein expression levels using confocal microscopy.

3.1.2 Verification of the protein sequence integrity

Taking in consideration the clinical importance of this study, the integrity of the whole protein coding sequence in obtained vectors was verified by Sanger sequencing (Microsynth AG, Switzerland). It was essential to confirm the presence of the desired mutation and the absence of unwanted mutations that may have arisen during amplification in bacteria in the final constructs used for functional and molecular testing. A fragment from the investigated chromatogram is shown in Figure 11.

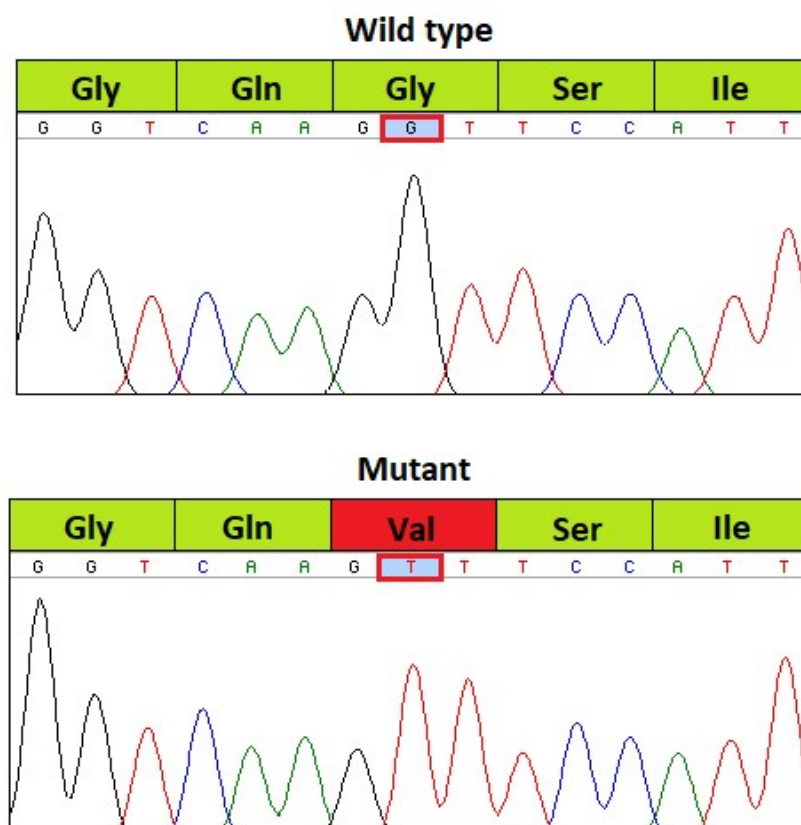


Figure 11. Fragment of investigated chromatogram. A highlighted T seen in chromatogram is a non-conservative missense point mutation introduced to SLC26A4 coding sequence and leading to substitution of glycine for valine in position 740 of translated protein.

Sequences of the whole mutated protein coding DNA (2,343 bp including the STOP codon) in pTARGET were covered by 6 partially overlapping sequencing reactions. Sequences of the whole mutated protein coding DNA (2,343 bp) and EYFP coding DNA (720 bp) in pEYFP-N1 vector were covered by 7 partially overlapping sequencing reactions. All the sequences were aligned with the SLC26A4 NCBI reference sequence NM_000441 using MacVector software (MacVector, USA) (Benson et al., 2017). A vector map was constructed for each individual plasmid.

3.1.3 Generation of heterologous overexpression systems

The generation of heterologous overexpression systems was performed using HEK 293 Phoenix and HeLa host cell lines. The introduction of plasmid DNA vectors containing a SLC26A4 variant to be expressed was performed using transient transfection. The result of a successful transfection can be seen in Figure 12.

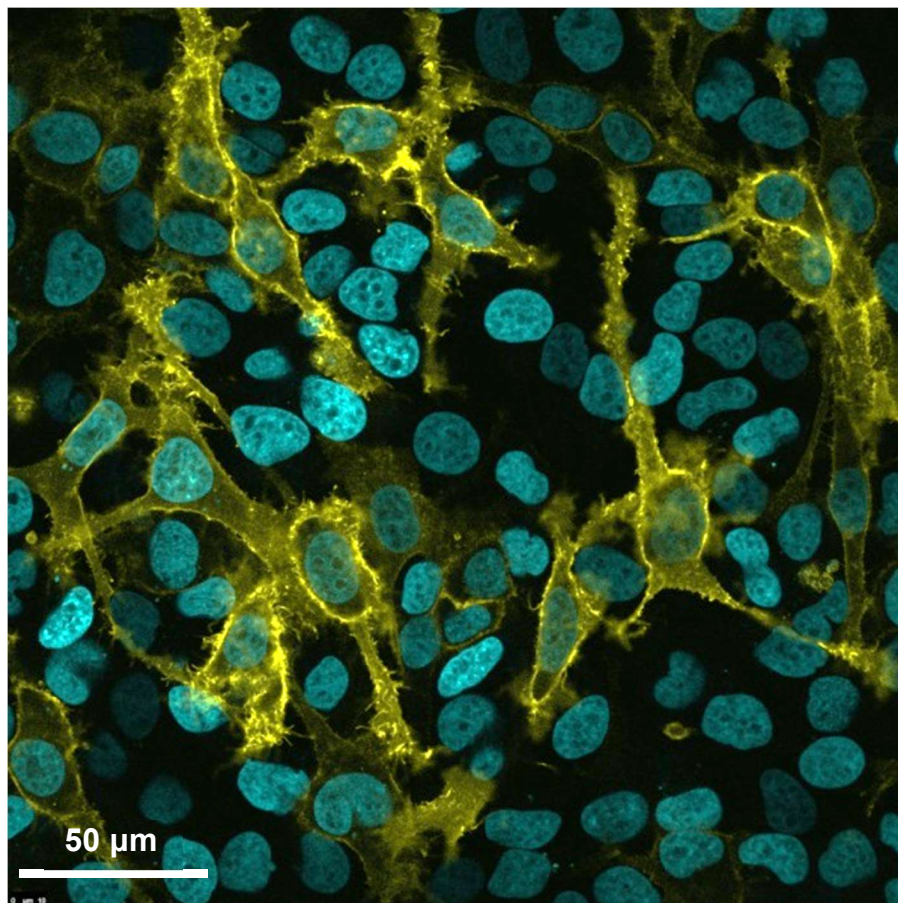


Figure 12. A confocal laser scanning microscope image of successfully transfected HeLa cells, showing fluorescence of the wild type SLC26A4-EYFP fusion protein. It can be noticed that SLC26A4 is located mostly in plasma membrane areas (yellow). For reference, the nuclei were stained with DAPI (cyan). The image is represented in false colors.

Two different transfection methods were used due to the different DNA uptake abilities of host cell lines. The HEK 293 Phoenix line cells were transfected using the calcium phosphate co-precipitation method (as described in chapter 2.7.8) and HeLa cells were transfected using the more efficient lipofection technique (as described in chapter 2.7.9). Transfection efficiency in both cases was equal to or greater than 50%.

3.2 Functional assay of iodide exchange

Following the successful transfection, the capability of iodide intake by HEK 293 Phoenix cells expressing SLC26A4 variants was measured (as described in chapter 2.7.10). HEK 293 Phoenix cells used for the intracellular fluorescence intensity measurements were transfected using wild type (WT) SLC26A4 together with the iodide sensor EYFP-H148Q-I152L coding vectors as a positive control, mutated SLC26A4 together with the iodide sensor EYFP-H148Q-I152L coding vectors, and an empty pTARGET backbone together with the iodide sensor EYFP-H148Q-I152L coding vector as a negative control. Overall, 3 independent experiments were performed and for each of them the background fluorescence was measured, calculated and subtracted. The number of samples measured in each independent experiment is represented in Table 13. Independent experiments were performed on independent cell subcultures; the number of samples represents the number of single wells of a 96-well plate used in the assay.

Table 13. Number of samples measured in 3 independent functional assay of iodide exchange experiments.

Experiment	WT	p.Q101R	p.I136N	p.L597S	p.A664V	p.G740V	Empty vector	Background
Experiment 1	5	6	6	6	6	6	6	6
Experiment 2	6	12	12	12	12	12	6	12
Experiment 3	6	12	12	12	12	12	6	12
Overall	17	30	30	30	30	30	18	

The fluorescence intensity behaviour in time before and after injection of the iodide-containing solution in the bath is represented in two graphs given in Figure 13 and 14. Fluorescence intensity was normalized for the arithmetical mean of fluorescence intensity in the chloride-containing solution acquired in the first three measurements before injection of iodide solution. The graph representing the fluorescence intensity behaviour over time in control cells expressing the wild type protein (positive control) and empty vector carrying cells (negative control) is shown in Figure 13. It is clearly visible that fluorescence of wild type SLC26A4 protein expressing cells decreased rapidly immediately after the injection of iodide to the extracellular solution, thus illustrating an iodide influx into the cell, which is the result of SLC26A4 function. In contrast, the fluorescence of negative control cells expressing no exogenous SLC26A4 showed a less pronounced decrease, which might be associated with poorly expressed endogenous anion exchangers or ion channels permeable to iodide.

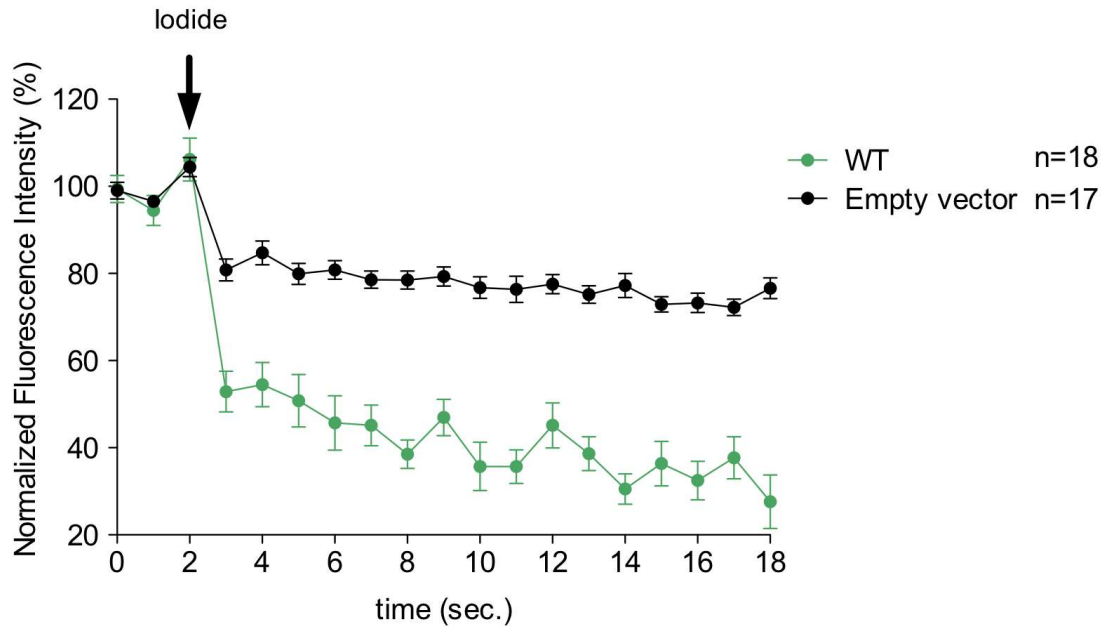


Figure 13. Fluorescence behaviour in time in cells co-transfected with vectors coding the iodide sensor EYFP-H148Q-I152L and wild type (WT) SLC26A4 (positive control) or the iodide sensor and an empty vector (negative control). The arrow indicates the addition of iodide to the extracellular solution. The values represent arithmetical mean of the fluorescence intensity in all the measurements after background fluorescence subtraction and normalization for the fluorescence measured before the injection of iodide to the extracellular medium. Error bars represent standard error of the mean.

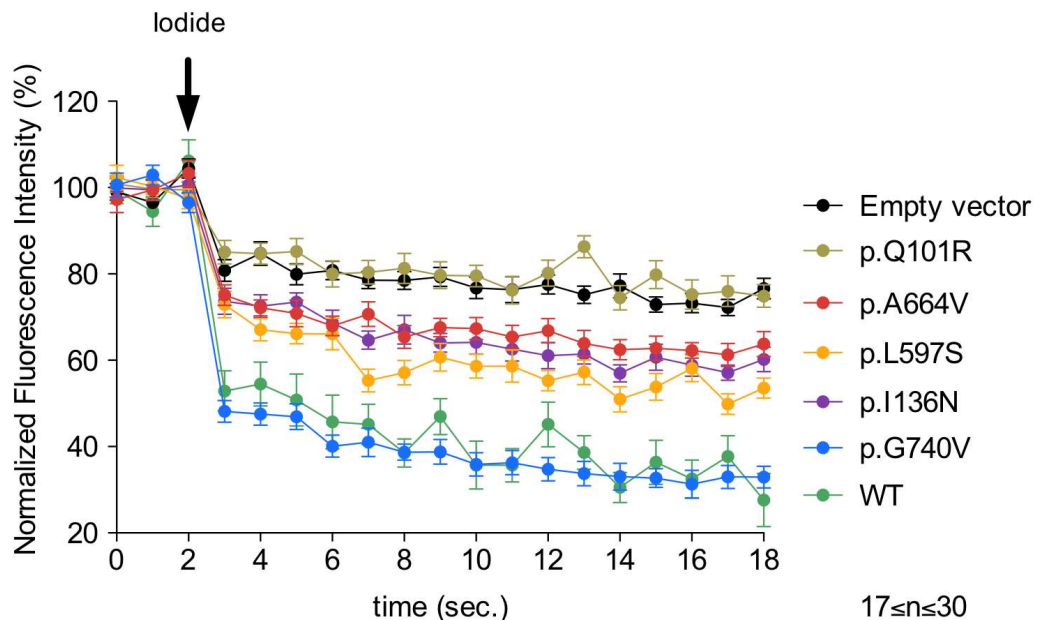


Figure 14. Fluorescence behaviour in time in cells co-transfected with vectors coding the iodide sensor EYFP-H148Q-I152L and wild type (WT) (positive control) or mutated SLC26A4, or the iodide sensor and an empty vector (negative control). The arrow indicates the addition of iodide to the extracellular solution. The values represent arithmetical mean fluorescence intensity after background fluorescence subtraction and normalization for the fluorescence measured before the injection of iodide to the extracellular medium. Error bars represent standard error of the mean.

In the second graph shown in Figure 14, the fluorescence behaviour of cells expressing the investigated SLC26A4 variants is shown, in addition to positive and negative controls. It is visible that the behaviour of two of the variants is similar to one of the controls. The p.Q101R variant curve is situated on the very top of the graph, close to the negative control (empty vector), thus showing that the behaviour of cells transfected with this variant is similar to that of the negative control cells expressing no SLC26A4. The curve of p.G740V variant is situated at the bottom, very close to the wild type SLC26A4 curve, thus suggesting its behaviour to be similar to the wild type SLC26A4. Three other curves representing variants p.A664V, p.L597S and p.I136N are located in intermediate positions, what might suggest a function that is compromised but not completely annihilated.

The functionality of SLC26A4 variants was determined by calculating the fluorescence decrease over the experimental period (19 sec.). The fluorescence variation between arithmetical mean of the first 3 (before the iodide injection) and the last 3 measurements was represented as percentage of fluorescence decrease ($\Delta F\%$). The histogram of the % decrease in fluorescence corresponding to SLC26A4 iodide transport activity is represented in Figure 15.

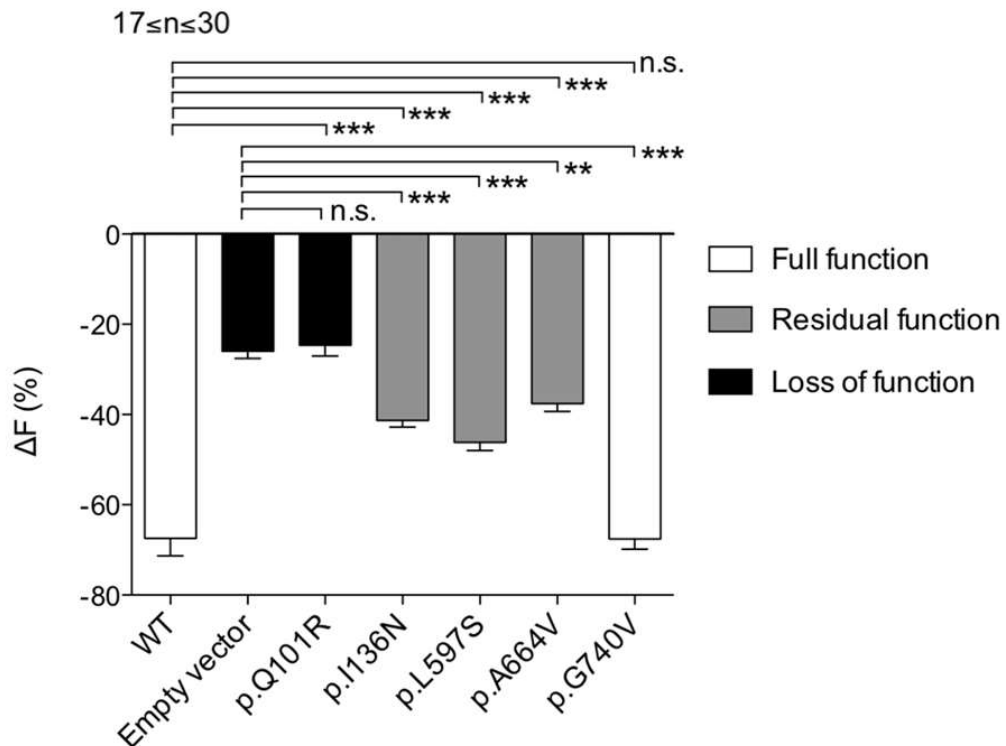


Figure 15. Percentage of the fluorescence decrease ($\Delta F\%$) over the experimental period (19 sec.). A negative fluorescence variation indicates an iodide flux from the extracellular environment to the cytosol of the transfected cells. Error bars represent standard error of the mean. Statistical differences between data sets were verified by the one-way analysis of variance (ANOVA) followed by Dunnett's post-test. Statistically significant differences between data sets are represented with asterisks, where *** represents $p < 0.001$, ** represents $p < 0.01$ and n.s. represents $p > 0.05$.

To ascertain whether a given variant has full function, residual function or loss of function, one-way analysis of variance (ANOVA) with Dunnett's multiple comparison post-test was applied to the results and possible statistically significant differences between each SLC26A4 variant and the wild type protein and each SLC26A4 variant and the negative control were verified. As seen in figure 15, the activity of SLC26A4 p.Q101R was substantially reduced compared to the wild type protein, and it was similar to the negative control. The statistical analysis has shown that cells transfected with this variant are indistinguishable from the negative control cells; thus, the p.Q101R variant shows a complete loss of iodide transport function. The iodide influx measured in cells expressing SLC26A4 p.G740V was statistically indistinguishable from that measured in cells expressing the wild type protein. Thus, the p.G740V is fully functional. The iodide influx measured in cells expressing SLC26A4 variants p.I136N, p.L597S and p.A664V, was significantly reduced compared to the wild type protein, but significantly higher compared to the negative control. This shows that these protein variants were functionally impaired, but had residual functions.

3.3 Determination of total SLC26A4 expression levels

Determination of total expression levels of SLC26A4 variants was performed using confocal microscopy following transfection of HeLa cells using pEYFP-N1 plasmid vectors coding for fluorescent wild-type or variant SLC26A4-EYFP fusion proteins (as described in chapter 2.7.11). Four variants (p.I136N, p.L597S, p.A664V, p.Q101R) and the wild type protein were investigated in three and p.G740V variant was investigated in two independent experiments. In each experiment, 6 different imaging areas of the investigated sample were randomly chosen and acquisitions made for both SLC26A4-EYFP and nuclei stained with DAPI (a summary of overall number of imaging areas is shown in Table 14). Fluorescence signals detected both in EYFP and DAPI emission channels were expressed in average levels of grey, which were calculated by microscope controlling software.

Table 14. Number of imaging areas in 2 or 3 individual experiments, where acquisitions both in EYFP and DAPI emission channels were made.

Experiment	WT	p.Q101R	p.I136N	p.L597S	p.A664V	p.G740V	Background
Experiment 1	6	6	6	6	6	6	6
Experiment 2	6	6	6	6	6	6	6
Experiment 3	6	6	6	6	6	0	6
Overall	18	18	18	18	18	12	

The fluorescence signal intensity emitted by SLC26A4-EYFP in the entire imaging area was normalized for the fluorescence intensity emitted by DAPI in the same field, thus giving the SLC26A4-EYFP fluorescence intensity normalized for the cell density in the investigated area. Background fluorescence in EYFP and DAPI emission channels was calculated for each individual experiment and subtracted from all the calculations prior normalization.

Representative images of cells expressing the different SLC26A4 variants are shown below in Figure 16. In this illustration, each pair of pictures represents a single imaging area.

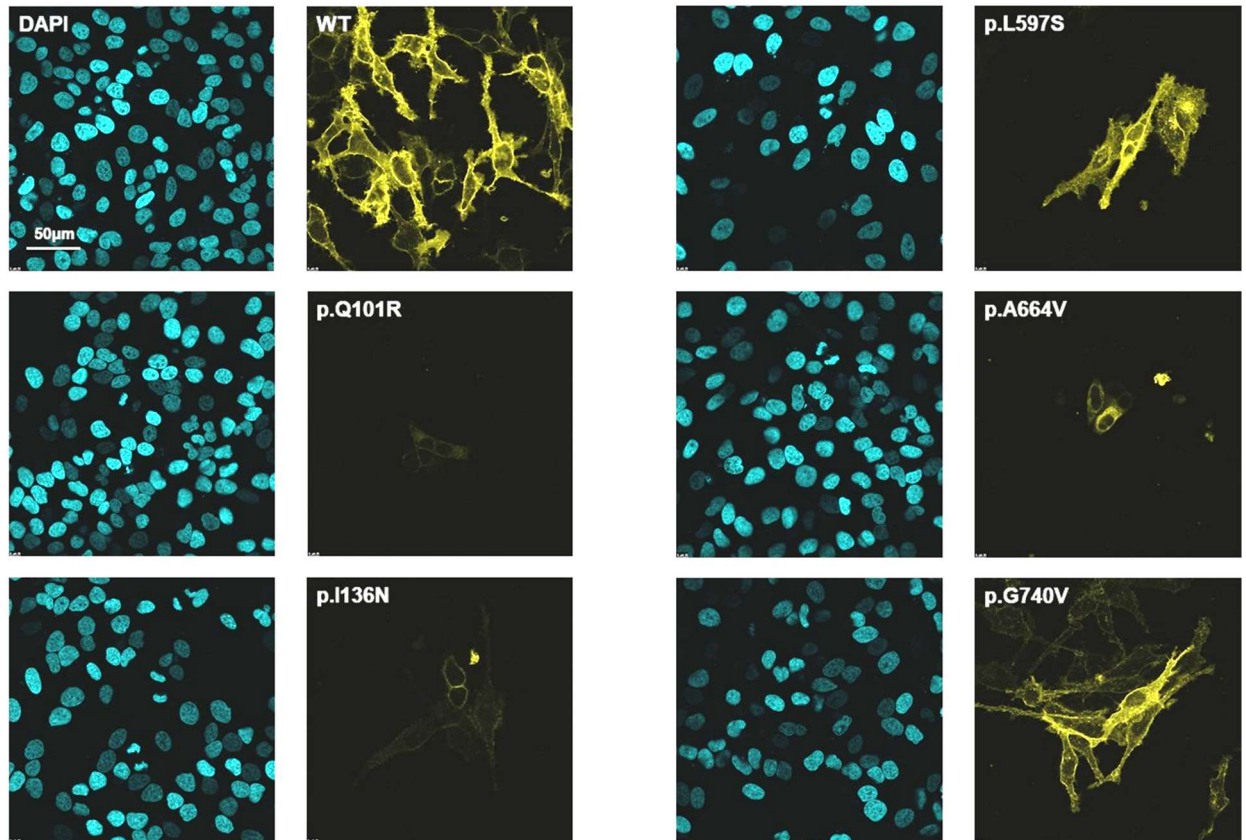


Figure 16. Confocal microscope images showing expression of wild type or mutant SLC26A4-EYFP fusion protein in transfected HeLa cells in the right columns. Cell nuclei counterstained with DAPI are represented in the left columns.

A statistically significant reduction of expression levels was observed for all the investigated SLC26A4 variants, with the exception of one. A histogram showing the expression levels normalized for the wild type is represented in Figure 17. The expression levels of the p.G740V variant were statistically indistinguishable from those of the wild type protein ($p>0.05$, one-way ANOVA with Bonferroni's post-test). The other 4 variants have shown a large reduction of expression levels with respect to the wild type protein. Variants p.I136N, p.L597S and p.A664V have shown a substantial reduction, and p.Q101R variant has shown almost no expression. All of these 4 variants showed highly significant differences ($p<0.001$) compared to the wild type protein

expression levels. Also, the same statistically significant differences were observed when the same variants were compared to p.G740V variant. In addition, a difference among the variants with reduced expression was observed as well. Expression of both p.Q101R and p.I136N variants was found to be significantly reduced compared to p.L597S variant. While the p.L597S variant showed the highest expression levels, the p.A664V was statistically indistinguishable from it.

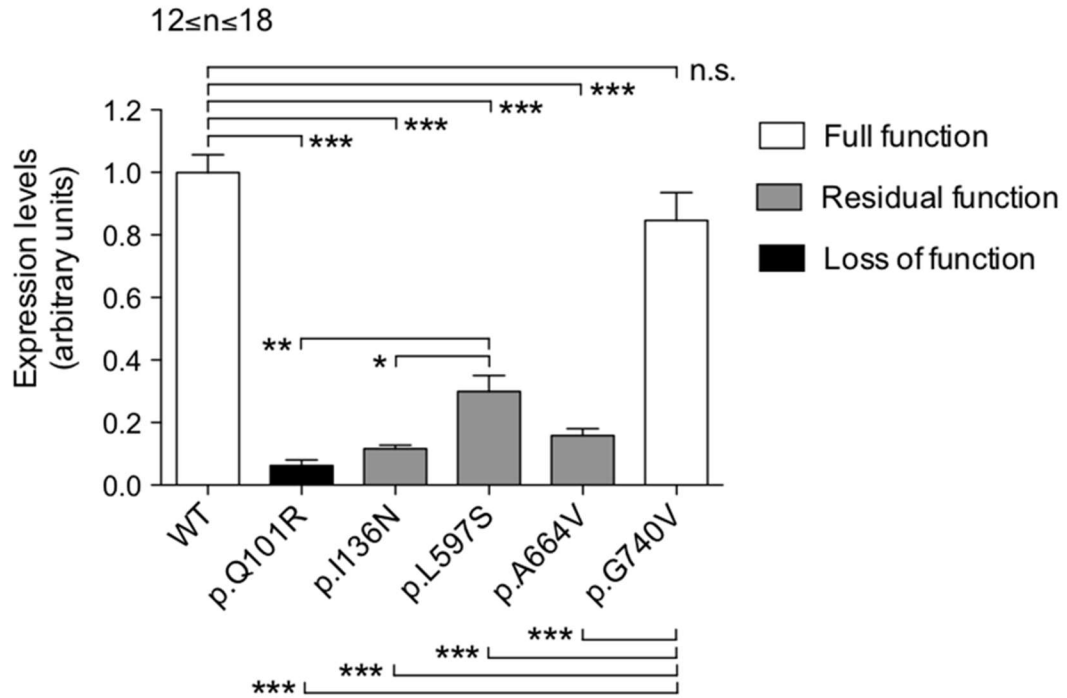


Figure 17. Total expression levels of the wild type (WT) SLC26A4 and its variants. Values are expressed as the ratio in relation to the wild type. Error bars represent standard error of the mean. Statistical differences between data sets were verified by the one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and are represented by asterisks, as follows: *** represents $p < 0.001$, ** represents $p < 0.01$ and * represents $p < 0.05$; n.s. indicates that the difference is not significant.

It is worth taking notice that all the variants having a reduced or loss of function had expression levels decreased as well. Moreover, the p.Q101R variant having complete loss of function had the lowest expression levels. Concerning that, a correlation between the SLC26A4 functionality and expression levels can be perceived.

3.4 Correlation between SLC26A4 functionality and expression levels

To confirm a correlation between the functionality of SLC26A4 variants and their expression levels, a linear regression model was applied. The correlation graph is represented in Figure 18.

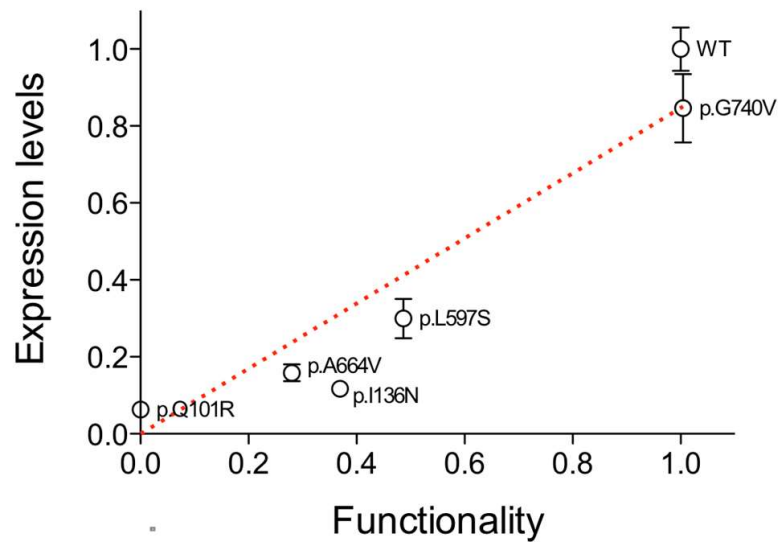


Figure 18. Graph representing a correlation between functionality and expression levels of SLC26A4 protein variants. Red line - linear regression model, error bars represents standard error of the mean. Coefficient of correlation $r=0.9568$, $p=0.0028$.

The functionality of the variants was calculated by subtracting the fluorescence decrease of the negative control (empty vector, see fig. 15) from all measurements and normalizing values for the wild type. Coefficient of correlation was calculated with the value of $r=0.9568$, what shows that the linear regression fits the data well. The significance level of the correlation was calculated and gave a value of $p=0.0028$. These parameters show a strong correlation between functionality of SLC26A4 variants and their expression levels.

3.5 Discussion

This work focused on assessing the functional and molecular features of five pendrin (SLC26A4) variants found in 4 patients with hearing loss and enlarged vestibular aqueduct. Based on the results of the functional assay and the protein expression level determination, a statement about the pathogenicity of these SLC26A4 variants can be made. The pathogenicity assignment according to the nomenclature of the American College of Medical Genetics and Genomic Standards and Guidelines (Richards et al., 2015) is summarized in Table 15.

Table 15. Summary of the SLC26A4 variant features

Variant	Functionality	Expression levels	Significance
p.Q101R	Loss of function	Highly reduced expression	Pathogenic
p.I136N	Reduced function	Highly reduced expression	Pathogenic
p.L597S	Reduced function	Reduced expression	Pathogenic
p.A664V	Reduced function	Reduced expression	Pathogenic
p.G740V	Unchanged function	No significant change in expression	Non pathogenic

The p.G740V SLC26A4 variant was previously reported in dbSNP (ID# rs111033310), but its clinical significance was uncertain. This study indicates that p.G740V may be non-pathogenic because of its full function and normal expression levels.

Another undetermined variant, p.L597S, also previously reported in dbSNP (ID# rs55638457) and thought to be likely benign (see Table 11), had shown both functionality and expression levels reduced, thus indicating a pathogenic potential.

Variant p.A664V has already been described in a study where its ability to transport formate was evaluated, and this resulted to be reduced compared to the wild type protein (Yuan et al., 2012). However, no dbSNP ID was assigned to this variant, and no conclusive statement about its possible pathogenicity was made. The assays performed in this study had confirmed this variant as being pathogenic, since it had both functionality and expression levels reduced.

In addition to the three previously mentioned variants, two novel variants were investigated as well. Both p.Q101R and p.I136N SLC26A4 variants had an impaired function and highly decreased protein expression levels, which were significantly lower than those of p.L597S variant. Functional testing revealed that variants p.Q101R and p.I136N have respectively a complete loss of function and a reduced function. Based on these findings, both of these variants have to be considered as pathogenic.

Overall, four variants of *SLC26A4* (p.Q101R, p.I136I, p.L597S and p.A664V) were found to be pathogenic and may account for the disease. After this investigation, the acceptance or the exclusion of *SLC26A4* as a causative gene for hearing loss can be made for four patients. Data of patients carrying the investigated *SLC26A4* mutations is summarized in Table 16.

Table 16. A list of 4 patients from the deaf Austrian cohort with evaluation of *SLC26A4* as a hearing loss causative gene according to this study.

Patient ID	Amino acid change		Causative gene
	Allele 1	Allele 2	
#653	p.G740V	WT	<i>SLC26A4</i> excluded
#654	p.L597S	WT	Likely <i>SLC26A4</i>
#659	p.I136N	p.A664V	<i>SLC26A4</i>
#660	p.Q101R	p.R409H	<i>SLC26A4</i>

This study suggests that *SLC26A4* is the gene causative of EVA and deafness in patients #659 and #660. These patients carry pathogenic sequence alterations in compound heterozygosis, what is leading to the complete absence of functional *SLC26A4* protein in the organism. *SLC26A4* is likely a causative gene of EVA and deafness in patient #654, because this variant of *SLC26A4* is pathogenic. However, only one allele of this patient is carrying a mutation and whether monoallelic *SLC26A4* mutations are sufficient to determine a pathological phenotype is currently unclear. Scientists suggest that in such a case, the second allele should bear another undetected *SLC26A4* mutation within the promoter or enhancer regions of this gene, leading to reduced transcription of *SLC26A4*. Alterations of *SLC26A4* gene in one allele are most likely insufficient to determine a pathological phenotype, but can be pathogenic in combination with another dysfunctional allele. Sequence alterations may reside in upstream regions distant from *SLC26A4*, in the intergenic regions (Chattaraj et al., 2017) or even in other genes, for example in those encoding the transcription factors (Azaiez et al., 2007).

As mentioned in the Introduction, some *SLC26A4* variants can be found both in healthy and in affected cohorts with the same frequency, thus showing their low pathogenic potential (Pera et al., 2008). Patient #653 carries a *SLC26A4* variant only in one of the alleles. This study has shown that this variant (p.G740V) most likely has no pathogenic potential. Accordingly, a variant involving the same amino acid (p.G740S) was described, and its function was not reduced compared to the wild type (Dossena, Bizhanova, et al., 2011). Indeed, this variant showed a modest gain of function. Based on these observations, the genetic cause of EVA and deafness for patient #653 remains undetermined.

Beside the clinical significance of this research, the biochemical aspects can suggest some interesting information as well. The amino acid substitution investigated are scattered within the SLC26A4 polypeptide. According to a recent SLC26A4 model, two of them (p.Q101R and p.I136N) lie within the transmembrane domain, p.L597S is located in the intervening sequence of STAS domain, p.A664V resides within the STAS domain and p.G740V is located outside the STAS domain, close to the C-terminus. The protein model with previously reported mutations and mutations investigated during this study is represented in Figure 18.

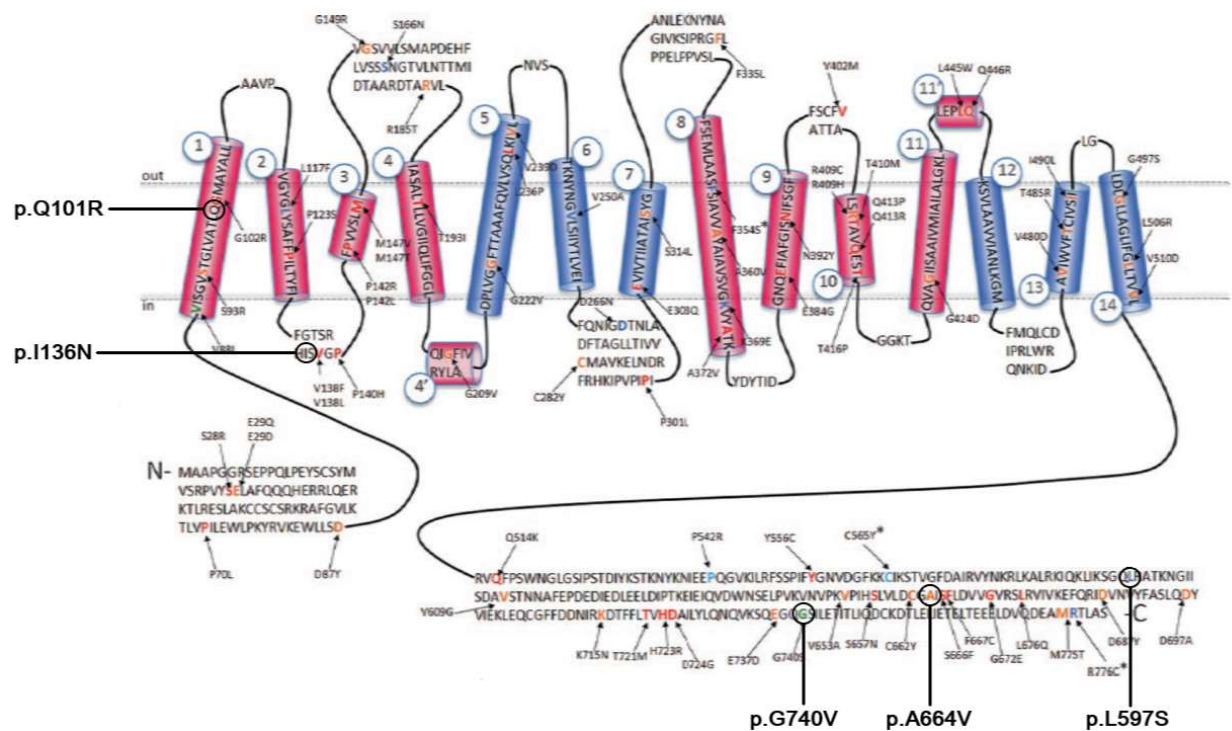


Figure 19. 2D representation of a recent SLC26A4 model. Mutations reported previously are marked with small arrows, amino acids bearing mutations investigated in this work are circled. Red and blue columns represent α -helices of transmembrane domain (adapted from Dossena et al. 2017).

As reviewed in the first part of this work (see chapter 1.5), the homology modelling on the SLC26Dg template predicted that the substrate binding site of SLC26A4 can be formed by Q101, I363 and R409 amino acid residues, which are situated within the transmembrane domain (Dossena et al., 2017). An amino acid substitution involving one of these residues (p.R409H, which was found in one of the patients involved in this study as well) has already been reported to cause an impaired function of the protein (M.P. Gillam et al., 2009). The assessment of pathogenicity of p.Q101R variant was performed during this study. It was an interesting piece of information to obtain, both because of the location and the fully impaired function of this mutation,

which corresponds to a possible disruption of a predicted substrate binding site. Interesting notice can be made about the p.L597S variant as well. This is one of the variants located within the intervening sequence of the STAS domain. Multiple variants having an amino acid substitution within this fragment are reported to be disease-associated (Dossena et al., 2017). As no function for this part of the protein is yet reported, our findings strongly indicate it having one. The STAS domain is hypothesized to serve as a protein-protein interaction module as it does in bacterial homologues (Dossena et al., 2017). The reduced function of p.A664V variant, which has a mutation within the STAS domain, can potentially be related to an impairment of an essential protein-protein interaction.

Reduction of the protein expression levels was identified as a common molecular feature of SLC26A4 variants with affected function in previous works (de Moraes, Bernardinelli et al., 2016; Roesch et al. 2018). The same phenomenon was observed in this study as well. The strong linear correlation between the functionality and the expression levels of SLC26A4 protein variants indicates that the reduction of expression is the main feature leading to loss of function, although additional phenomena including interference with ligand binding or disruption of regulatory protein-protein interactions may also contribute to alter ion transport activity. Our findings give us hints towards possible future strategies for transport activity restoration of dysfunctional SLC26A4 variants. As reduction of expression may arise from a premature degradation of dysfunctional SLC26A4 variants, we suggest that the inhibition of the protein degradation pathway could be used as a potential therapeutic intervention.

CONCLUSIONS

- Two novel (p.Q101R, p.I136N) and two undetermined (p.L597S, p.G740V) SLC26A4 variants were characterized;
- Three of these variants (p.Q101R, p.I136N and p.L597S) were found to be potentially pathogenic and one (p.G740V) non-pathogenic;
- The pathogenicity of a fifth variant (p.A664V) was confirmed;
- These findings led to a conclusive identification of SLC26A4 being the causative gene in 2 over 4 patients with hearing loss;
- Reduction of expression levels was identified as the main feature leading to loss of function of these variants, although altered ligand binding and/or disruption of protein-protein interaction may contribute to impair ion transport.

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