Institute of Veterinary Pathology

Department of Veterinary Medicine

Freie Universität Berlin

Expression Analyses of CLCA Members in the Feline Respiratory Tract – Biomolecules in Feline Asthma?

Thesis submitted for the fulfillment of a
doctoral degree in Veterinary Medicine
(Dr. med. vet.)
at the
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submitted by
Nancy Ann Erickson, PhD
Veterinarian from Papenburg

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I would rather have questions that can´t be answered

than answers that can´t be questioned.

-   Richard P. Feynman

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List of Abbreviations

aa	**a**mino **a**cid
ACVIM	**A**merican **C**ollege of **V**eterinary **I**nternal **M**edicine
BAL	**B**roncho**a**lveolar **L**avage
BALF	**B**roncho**a**lveolar **L**avage **F**luid
BPIFA1	**B**acterial/**P**ermeability **I**ncreasing **F**old-Containing protein family **A1**
BSA	**B**ovine **S**erum **A**lbumin
CaCC	**C**alcium-**a**ctivated **C**hloride **C**hannel
cAMP	**c**yclic **A**denosine **M**ono**p**hosphate
CCL2	**CC**-chemokine **l**igand **2**
CD4+	**C**luster of **D**ifferentiation **4**-positive cells
CF	**C**ystic **F**ibrosis
CFTR	**C**ystic **F**ibrosis **T**ransmembrane **C**onductance **R**egulator
CLCA	**Ch**loride channel regulator, **C**alcium-**a**ctivated (formerly known as Chloride channel, Calcium-activated)
Clca1$^{-/-}$	***Clca1***-deficient
COPD	**C**hronic **O**bstructive **P**ulmonary **D**isease
CXCL	Chemokine (**C**-**X**-**C** motif) **L**igand
CXCL-1	Chemokine (**C**-**X**-**C** motif) **L**igand-**1**, also termed Keratinocyte Chemoattractant (KC)
CXCL-2	Chemokine (**C**-**X**-**C** motif) **L**igand-**2**, also termed Macrophage Inflammatory Protein 2-alpha (MIP-2α)
DAB	**Di**amino**b**enzidine
°C	degree **C**elsius
DIOS	**D**istal **I**ntestinal **O**bstruction **S**yndrome
DOG1	**D**iscovered **O**n **G**astrointestinal Stromal Tumors Protein-**1**
DSS	**D**extran **S**odium **S**ulfate
e.g.	**e**xample **g**iven
ELISA	**E**nzyme-**L**inked **I**mmunosorbent **A**ssay

EPR-3	**E**xpert **P**anel **R**eport **3**
E157Q	Glutamic acid (**E**) replaced by Glutamine (**Q**) at position **157**
F	Single-letter amino acid code for phenylalanine (three-letter code "Phe")
FELASA	**Fe**deration of **L**aboratory **A**nimal **S**cience **A**ssociations
FFPE	**F**ormalin-**f**ixed, **p**araffin-**e**mbedded
FnIII	**F**ibro**n**ectin type **III**
Glu	**Glu**tamine
gob-5	**gob**let cell protein-**5**
GPI	**G**lycosyl**p**hosphatidyl**i**nositol
HEK	**H**uman **E**mbryonal **K**idney
HEXXH	Abbreviation for the amino acids His-Glu-Xaa-Xaa-His (Histidine, Glutamic acid, 2 unspecified amino acids, Histidine)
His	**His**tidine
HUGO	**Hu**man **G**ene **No**menclature Committee
IAD	**I**nflammatory **A**irway **D**isease
i.e.	*id est* (Latin for "that is to say")
Ig	**I**mmuno**g**lobulin
IHC	**I**mmuno**h**isto**c**hemistry
IL	**I**nter**l**eukin
ISH	*In Situ* **H**ybridization
KC	**K**eratinocyte **C**hemoattractant, also termed Chemokine (C-X-C motif) Ligand-1 (CXCL-1)
kDa	**k**ilo **Da**lton
LPS	**L**ipo**p**oly**s**accharide
Lu-ECAM-1	**Lu**ng **E**ndothelial **C**ell **A**dhesion **M**olecule-**1** (also termed bCLCA2)
MALDI-TOF	**M**atrix **A**ssisted **L**aser **D**esorption **I**onization-**T**ime **of** **F**light
MAPK	**M**itogen-**A**ctivated **P**rotein **K**inase
MGNC	**M**ouse **G**ene **N**omenclature **C**ommittee
MIDAS	**M**etal **I**on–**D**ependent **A**dhesion **S**ite

X

min	**min**ute
MIP-2α	**M**acrophage **I**nflammatory **P**rotein **2**-**a**lpha, also termed Chemokine (C-X-C motif) Ligand-2 (CXCL-2)
mRNA	**m**essenger **R**ibo**n**ucleic **A**cid
MSA	**M**ultiple **S**equence **A**lignment
Muc	**Muc**in protein
MW	**M**icro**w**ave
n-CLCA	ami**n**o-terminal **CLCA** domain
ODF2L	**O**uter **D**ense **F**iber of Sperm Tails **2**-**L**ike
OVA	**Ova**lbumin
P	**P**seudogene
PAS	**P**eriodic **A**cid-**S**chiff
PGA	**P**auci**g**ranulocytic **A**sthma
PBS	**P**hosphate-**B**uffered **S**aline
RAO	**R**ecurrent **A**irway **O**bstruction (currently known as SEA)
RGD	**R**at **G**enome **D**atabase
RT-qPCR	**R**everse **T**ranscriptase-**q**uantitative **P**olymerase **C**hain **R**eaction
S. aureus	*Staphylococcus aureus*
SEA	**S**evere **E**quine **A**sthma (formerly known as Recurrent Airway Obstruction, RAO)
SH3GLB1	**SH3**-Domain **G**RB2-**L**ike Endophilin **B1**
SMG	**S**ub**m**ucosal **G**land
SPLUNC1	**S**hort **P**alate, **Lu**ng, and **N**asal Epithelium **C**lone **1**
SS	**S**ignal **S**equence
STAT6	**S**ignal **T**ransducer and **A**ctivator of **T**ranscription **6**
Th	**T h**elper
TM	**T**rans**m**embrane domain
TMEM16A	**T**rans**mem**brane protein **16A** (also known as Anoctamin1 or Discovered On Gastrointestinal Stromal Tumors Protein-1, DOG1)
TGFβ	**T**ransforming **G**rowth **F**actor-**β**

Tris-EDTA	**Tris**(hydroxymethyl)amino-methane-**e**thylene**d**iamine**t**etraacetic **a**cid
vs.	*versus* (Latin for "against")
vWA	**v**on **W**illebrand factor type **A**
W	**W**att (unit of power)
WHO	**W**orld **H**ealth **O**rganization
WT	**W**ild **T**ype

Tris-EDTA   **Tris**(hydroxymethyl)amino-methane-**e**thylene**d**iamine**t**etraacetic **a**cid

versus (Latin for "against")

1   Introduction

Certain members of the highly conserved chloride channel regulator, calcium-activated (CLCA) family have been considered to modulate mucus-based inflammatory conditions of the respiratory tract, such as asthma, chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF, also termed mucoviscidosis). This potential modulatory function pertains particularly to CLCA1, the most intensively investigated family member to date. The CLCA1 protein is highly expressed in these respiratory diseases and has been discussed as a therapeutic target.

Previous work has mainly focused on human, murine, and, to a lesser extent, porcine CLCA members. It not only revealed certain similarities in the gene locus organization and expression pattern – particularly of CLCA1 – but also distinct species-specific differences in numbers, expression patterns or functions of other CLCA orthologs. Furthermore, based on mouse models, putative redundancies or compensatory effects of homologous CLCA members have been hypothesized. Therefore, the interspecies diversity must be carefully considered regarding the translation of results from one species to another.

The CLCA family has been systematically characterized in humans, mice, and pigs. Mucus-based diseases such as asthma, COPD or CF either occur naturally in these mammals or may be experimentally induced in animal species to model the respective human disease. Nevertheless, asthma is a highly relevant and naturally occurring condition also in cats with many parallels to the human disease. The feline CLCA family is, however, unknown to date. Hence, this study aimed at characterizing the expression profile of the CLCA family in healthy cats for the first time. It particularly focused on the airways regarding a potential role of CLCA1 in feline asthma.

1.1 <u>CLCA History and Nomenclature</u>

The first two members of the CLCA family were discovered simultaneously and independently by two working groups in 1991 (Ran and Benos 1991; Zhu et al. 1991). The first founding member, which was isolated from bovine apical tracheal membranes, mediated calcium-dependent chloride currents *in vitro* (Ran and Benos 1991; 1992) and was, hence, termed bovine tracheal calcium-activated chloride channel (CaCC) (Cunningham et al. 1995). The second founding member was discovered in bovine endothelial cells of distinct pulmonary blood vessels, which was shown to promote selective attachment of lung-metastatic melanoma cells *in vitro* and *in vivo* (Zhu et al. 1991). It was therefore termed bovine lung endothelial cell adhesion molecule-1 (Lu-ECAM-1) (Zhu et al. 1991). The nucleotide and amino acid (aa)

sequence conformity of both members was of 92 % and consecutive molecular cloning confirmed their homology (Elble et al. 1997) by which a novel protein family was established.

The initial observation of bovine tracheal CaCC evoking calcium-dependent chloride currents *in vitro* (Ran and Benos 1991; 1992) was also shown for other CLCA members across different species (Gruber et al. 1998a; Loewen and Forsyth 2005; Winpenny et al. 2002; Yamazaki et al. 2005). A first attempt towards nomenclature standardization defined the family members as **chloride channels, calcium activated**, termed these according to the respective species (e.g. h = human, m = murine, p = porcine), and numbered them in chronological order of discovery. Hence, bovine CaCC and bovine Lu-ECAM-1 were designated bCLCA1 and bCLCA2, respectively (Gruber et al. 2000).

Subsequently, state of the art *in silico* analyses revealed that CLCA proteins cannot form chloride channels *per se* since they lack multiple transmembrane domains (Gibson et al. 2005) and are either entirely or partially secreted into the extracellular environment (Anton et al. 2005; Bothe et al. 2008; Elble et al. 2006; Gibson et al. 2005; Huan et al. 2008; Mundhenk et al. 2006). Nevertheless, human CLCA1 may modulate calcium-dependent chloride currents (Hamann et al. 2009). Therefore, the abbreviation was changed to **chloride channel regulators, calcium-activated**, which is now the present denotation.

As up to eight members have been discovered in over 30 species with a species- and tissue-specific expression pattern to date, the nomenclature became increasingly confusing and was not readily associable. Therefore, a third step towards harmonization was performed by the Mouse Gene Nomenclature Committee (MGNC) in accordance with the Human Gene Nomenclature Committee (HUGO) and the Rat Genome Database (RGD) (Erickson et al. 2015). Herein, the individual members were renamed, allowing for systematic ortholog comparison. For example, the murine mucus cell protein gob-5, which had first been renamed to mCLCA3 (Leverkoehne and Gruber 2002), is now termed murine CLCA1 (Erickson et al. 2015) due to its direct orthology to human CLCA1. Regardless, the database nomenclature is still complex and partially inconsistent.

1.2 Genomic Organization of the *CLCA*-Family

The *CLCA* genes are located in a single locus between the two flanking genes, *outer dense fiber of sperm tails 2-like* (*ODF2L*) and *SH3-domain GRB2-like endophilin B1* (*SH3GLB1*) in all mammalian species and also chickens (see 2.1). This genomic organization preserves the same ordering of CLCA family members (Patel et al. 2009) which are grouped in four different clusters based on sequence homology (Plog et al. 2009). Despite this high conservation of the genomic *CLCA* locus organization (Patel et al. 2009), the CLCA family shows a high genetic interspecies diversity. Firstly, the numbers of *CLCA* genes differ between species, e.g. four

CLCA genes in man and horse (Mundhenk et al. 2018; Patel et al. 2009) but eight in mice (Patel et al. 2009), as shown in Table 1. Secondly, pseudogene formation and expressional silencing has occurred only in certain species (Gruber and Pauli 1999; Plog et al. 2009).

Table 1. CLCA clusters and the according species-specific members

CLUSTER	HUMAN[1]	PIG[2]	MOUSE[3] (former nomenclature)	HORSE[4]
Cluster 1	CLCA1	CLCA1	Clca1 (mClca3, gob-5)	CLCA1
Cluster 2	CLCA2	CLCA2	Clca2 (mClca5)	CLCA2
Cluster 3	CLCA3P	CLCA3P	Clca3a1 (mClca1)	CLCA3
			Clca3a2 (mClca2)	
			Clca3b (mClca4)	
Cluster 4	CLCA4	CLCA4a	Clca4a (mClca6)	CLCA4
			Clca4b (mClca7)	
		CLCA4b	Clca4c (mClca8)	

Source: (Patel et al. 2009), [1](Agnel et al. 1999; Gruber et al. 1998a; Gruber and Pauli 1999; Gruber et al. 1999), [2](Plog et al. 2009; 2012a; 2012b), [3](Al-Jumaily et al. 2007; Elble et al. 2002; Erickson et al. 2015; Evans et al. 2004; Gandhi et al. 1998; Komiya et al. 1999; Lee et al. 1999; Romio et al. 1999), [4](Mundhenk et al. 2018; Patel et al. 2009), P = pseudogene.

In detail, only one member exists for clusters 1 and 2 in all mammalian species – including humans, mice, pigs, and horses – investigated so far, e.g. murine *Clca1* (formerly *mClca3* or *gob-5*) and *Clca2* (formerly *mClca5*). For clusters 3 and 4, however, the number of *CLCA* members differs species-specifically.

Mice exhibit three members each for clusters 3 and 4. In murine cluster 3, these three genes furthermore appear to be closely related as a product of two subsequent duplication events. Thereby, murine *Clca3* is subdivided into *Clca3a* and *-3b* (formerly *mClca4*), whilst *Clca3a* is further subdivided into *Clca3a1* and *-3a2* (formerly *mClca1* and *-2*, respectively), all of which are highly likely of expressing fully functional proteins (Patel et al. 2009). Similarly, murine cluster 4 also contains three homologs, *Clca4a*, *-4b*, and *-4c* (formerly *mClca6*, *-7*, and *-8*,

respectively). Nevertheless, latter also exhibits a premature stop codon (Patel et al. 2009), rendering the possibility of pseudogene formation also in the murine CLCA family. Porcine cluster 4 consists of two genes, *CLCA4a* and *-4b*, which are due to a single duplication event (Plog et al. 2012a). In contrast, in humans, both clusters are comprised of only one *CLCA* member, of which *CLCA3* represents a silenced pseudogene due to the presence of premature stop codons (Gruber and Pauli 1999) which are also present in porcine *CLCA3* (Plog et al. 2009). Of note, in the horse, CLCA1 is the only family member experimentally characterized to date (Anton et al. 2005; Range et al. 2007).

On the genomic level, clusters 1 and 2 are highly conserved, whereas clusters 3 and 4 feature conspicuous interspecies diversity. Species-specific homologies and differences are, however, not only genomically present but have also been investigated in detail on the protein level.

1.3   CLCA Expression Patterns – An Interspecies Comparison

Previous work has mainly focused on expression pattern and functional analysis of CLCA members relevant to respiratory diseases such as asthma, COPD, and CF in humans (Agnel et al. 1999; Brouillard et al. 2005; Gibson et al. 2005; Gruber et al. 1998a; Gruber and Pauli 1999; Gruber et al. 1999; Hauber et al. 2003; 2004; 2010; Hoshino et al. 2002; Kamada et al. 2004; Mall et al. 2003; Patel et al. 2009; Toda et al. 2002; Wang et al. 2007; Woodruff et al. 2007; Zhou et al. 2001) and in model animal species, i.e. predominantly the mouse (Brouillard et al. 2005; Dietert et al. 2014; Long et al. 2006; Mei et al. 2013; Nakanishi et al. 2001; Patel et al. 2006; Robichaud et al. 2005; Song et al. 2013; Thai et al. 2005; Young et al. 2007; Zhang and He 2010). Herein, CLCA1 is the CLCA family member studied most intensively to date due to its potential as biomarker or therapeutic target in these respiratory diseases (Patel et al. 2009; Sala-Rabanal et al. 2017).

Beside man and mouse, the expression pattern of all CLCA family members has also been systematically characterized in the pig (Plog et al. 2009; 2010; 2012a; 2012b; 2015) due to its high translational potential regarding human anatomy and physiology (Rogers et al. 2008a) and the recent establishment of porcine CF models (Meyerholz et al. 2010; Rogers et al. 2008b; 2008c; Welsh et al. 2009). In horses, in which Severe Equine Asthma (SEA), formerly known as Recurrent Airway Obstruction (RAO) (Couetil et al. 2016), is considered a model of human asthma and COPD (Bice et al. 2000; Snapper 1986), the first equine member of the CLCA gene family, CLCA1, has also been investigated (Anton et al. 2005; Range et al. 2007).

Strikingly, CLCA1 shows an almost identical expression pattern in all aforementioned species whilst the cellular expression patterns of the CLCA2 and CLCA4 proteins differ between humans, mice, and pigs (Braun et al. 2010a; Connon et al. 2004; Dietert et al. 2015; Plog et

al. 2012b) and CLCA members of cluster 3 show the most complex and striking interspecies differences (Patel et al. 2009).

1.3.1   CLCA1 – A Common Expression Pattern Not Only in the Respiratory Tract

For every species investigated so far, the only member present in cluster 1 is CLCA1. It is expressed in mucus-producing cells predominantly of the respiratory, gastrointestinal, and reproductive tract with a virtually identical expression pattern of its human (Gruber et al. 1998a), murine (Leverkoehne and Gruber 2002), porcine (Plog et al. 2009), and equine orthologs (Anton et al. 2005).

Its most abundant expression is located in the colon (Anton et al. 2005; Gruber et al. 1998a; Johansson et al. 2008; Leverkoehne and Gruber 2002; Plog et al. 2009), in which CLCA1 is secreted by mucus cells into the mucus layer lining the intestinal epithelium (Gibson et al. 2005; Leverkoehne and Gruber 2002; Mundhenk et al. 2006; Plog et al. 2009; Range et al. 2007).

In the respiratory tract, CLCA1 is also exclusively expressed in mucus cells of the trachea and major bronchi, and to a lesser degree, in mucin-producing submucosal glands (SMGs) of the upper trachea and secreted into the extracellular mucus (Anton et al. 2005; Gibson et al. 2005; Hoshino et al. 2002; Leverkoehne and Gruber 2002; Mundhenk et al. 2006; Plog et al. 2009) (also see 2.2, Figure 7). Smaller non-diseased airways or the lung parenchyma show less to no CLCA1 expression, corresponding to the decline of mucus cell numbers or their entire absence, respectively (Anton et al. 2005; Gruber et al. 1998a; Leverkoehne and Gruber 2002; Plog et al. 2009).

Apart from this coinciding expression pattern for CLCA1, the pig and the horse show distinct species-specific differences. Firstly, porcine CLCA1 exhibits strong expression in mucus-producing cells of the pancreatic and salivary ducts as well as the gall bladder and common bile duct (Plog et al. 2009), which are devoid of CLCA1 in man and mouse (Gruber et al. 1998a; Leverkoehne and Gruber 2002; Loewen and Forsyth 2005). In the horse, the pancreas and parotis gland were entirely negative for CLCA1 expression (Anton et al. 2005), whilst biliary ducts – which the horse does possess despite its lack of a gall bladder – have not been investigated (Anton et al. 2005). Secondly, human *CLCA1* mRNA and murine CLCA1 protein have been detected in the uterus (Agnel et al. 1999; Leverkoehne and Gruber 2002). In contrast, porcine and equine CLCA1 have not been discovered in the genital tract (Anton et al. 2005; Plog et al. 2009).

Furthermore, expression of equine CLCA1 was located in tubular non-mucinous sweat glands of the skin – in contrast to mouse (Leverkoehne and Gruber 2002) and pig (Plog et al. 2009) – and – in contrast also to humans – in mucus glands of the renal papilla (Anton et al. 2005).

This particular expression profile can be explained by species-specific anatomical differences. On the one hand, horses exhibit tubular sweat glands throughout the entire skin like humans – in which the expression of CLCA1 has not been investigated to date – but in contrast to any other domestic or laboratory animal species (Banks 1993; Talukdar et al. 1972). On the other hand, renal mucus glands are exclusively found in equids but not in mice, humans or pigs (Dellmann 1998).

In summary, concerning the respiratory tract, CLCA1 is expressed by mucin-producing cells and is secreted into the mucus lining of the respiratory epithelial in all aforementioned species (Anton et al. 2005; Gibson et al. 2005; Hoshino et al. 2002; Leverkoehne and Gruber 2002; Plog et al. 2009).

1.3.2 CLCA2 – A Common Expression Pattern in Respiratory SMGs

Similar to cluster 1, cluster 2 also consists of only one CLCA family member in all of the aforementioned species (Patel et al. 2009), of which the expression of CLCA2 has not been investigated in the horse to date. CLCA2 is primarily expressed in the respiratory tract and squamous epithelia (Braun et al. 2010a; Dietert et al. 2015; Gruber et al. 1999; Plog et al. 2012b). The cellular expression pattern in these tissues, however, differs species-specifically. In the human and porcine respiratory tract, the CLCA2 protein is exclusively expressed in few SMGs whilst bronchial epithelial cells are devoid of CLCA2 expression (Dietert et al. 2015) (also see 2.2, Figure 7). In the mouse, CLCA2 expression was also localized to SMGs – but to a larger number of these cells than in humans and pigs – and occasionally co-localized with CLCA1 in mucus cells (Dietert et al. 2015). Of note, the expression of CLCA2 in airway epithelial cells may be enhanced by interleukin (IL)-13-induced airway inflammation (Mundhenk et al. 2012) similarly to CLCA1. Murine CLCA2 was furthermore uniquely localized to epithelial cells at the bronchial bifurcation (Dietert et al. 2015), consistent with locations of progenitor cell niches and, hence, indicative of fundamental anatomical species-specific differences (Dietert et al. 2015).

Outside the respiratory tract, human CLCA2 was detected in basement membranes of basal epithelial cells (Carter et al. 1990; Connon et al. 2004; 2005), whereas the porcine CLCA2 protein was expressed in mature keratinocytes of the epidermis and in the inner root sheath of hair follicles (Plog et al. 2012b). Similar to the pig, murine CLCA2 was solely expressed in differentiated keratinocytes, i.e. keratohyalin granules of all stratified squamous epithelia (Braun et al. 2010a).

Concluding, the respiratory tract expression pattern of CLCA2 is restricted to SMGs in humans, pigs, and mice (Dietert et al. 2015). Exclusively in latter species, it is expanded to a distinct

niche of airway epithelial cells at the bronchial bifurcation with its cellular expression partially overlapping with that of CLCA1 (Dietert et al. 2015).

1.3.3 CLCA3 – Functionally Relevant Respiratory Expression in the Mouse

The *CLCA3* gene locus of humans and pigs consists of only one member each, *CLCA3*, which represents a silenced pseudogene with no functional protein being expressed (Gruber and Pauli 1999; Plog et al. 2009) whilst in horses, its expression has not been investigated to date.

In the mouse, *Clca3* underwent multiple duplication events, giving rise to three homologs (Patel et al. 2009), *Clca3a1*, *-3a2*, and *-3b*, all of which are seemingly functional proteins (Elble et al. 2002; Gandhi et al. 1998; Gruber et al. 1998b; Patel et al. 2009). CLCA3a1 is expressed in tracheal and bronchial epithelial cells and also in SMGs (Gandhi et al. 1998) (also see 2.2, Figure 7). It is furthermore expressed in the intestinal tract, i.e. basal crypt epithelia of the small and large intestine (Bothe et al. 2008; Gruber et al. 1998b; Leverkoehne et al. 2006) and other secretory tissues such as gall bladder, pancreas, kidney (Gandhi et al. 1998), and mammary gland (Gruber et al. 1998b).

Surprisingly, CLCA3a1 is also expressed in endothelial cells of lymphatic vessels as well as pleural and subpleural blood vessels (Abdel-Ghany et al. 2002; Furuya et al. 2010; Gruber et al. 1998b), in latter of which CLCA3b is also expressed (Elble et al. 2002). Of note, CLCA3b is furthermore expressed in smooth muscle cells (Elble et al. 2002; Patel et al. 2009). Strikingly, this expression of CLCA3a1 or -3b in mesenchymal cells – endothelial cells or both endothelial and smooth muscle cells, respectively – seems to be unique to this cluster since members of other clusters are exclusively expressed in cells of epithelial origin (Patel et al. 2009).

Concerning the expression pattern of CLCA3a2, it is of special mention that CLCA3a1 and -3a2 share high nucleic and aa sequence identity (Gandhi et al. 1998; Lee et al. 1999) and, hence, cross-reactivity of probes and antibodies cannot be fully excluded in literature data (Roussa et al. 2010) (see 2.1). Reliable discrimination between the duplication products is only possible via Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR), which, however, does not aid in elucidating the cellular expression pattern due to the common use of whole tissue lysate in the aforementioned literature. Nevertheless, RT-qPCR analyses showed that both *Clca3a1* and *-3a2* are expressed to similar levels in the respiratory tract and the intestine (Leverkoehne and Gruber 2002).

In more detail, RT-qPCR analyses revealed that *Clca3a1* is predominantly expressed in spleen and bone marrow, *Clca3a2* in mammary gland (Leverkoehne and Gruber 2002), and *Clca3b* in smooth muscle cells of blood vessels in various organs such as the heart, gastrointestinal

tract, bronchioles, and in aortic and pulmonary endothelial cells (Elble et al. 2002). Due to the expression of the three murine *Clca3* homologs each in specific cell types, it is possible that these occupy distinct functional niches (Bothe et al. 2008). The functions of these proteins are, however, entirely unknown to date and caution is warranted in extrapolating results to other species due to the aforementioned duplications.

Summing up, *CLCA3* represents a pseudogene with no known protein expression in man and pigs (Gruber and Pauli 1999; Plog et al. 2009), whereas in the mouse, all three homologs seem to be expressed in endothelial cells of blood vessels of the respiratory tract (Abdel-Ghany et al. 2002; Elble et al. 2002; Furuya et al. 2010; Gruber et al. 1998b). CLCA3a1 and -3a2 are furthermore expressed in respiratory epithelial cells and SMGs (Gandhi et al. 1998; Gruber et al. 1998b), whilst CLCA3b is expressed in smooth muscle cells (Elble et al. 2002; Patel et al. 2006) of blood vessels in the respiratory tract.

1.3.4 CLCA4 – Functionally Relevant Respiratory Expression in Man and Pig

Cluster 4 also consists of only one member in humans, whereas pigs and mice exhibit two or three homologs, respectively (Patel et al. 2009). Whilst the expression pattern of CLCA4 has not been investigated in the horse, its human ortholog is predominantly expressed in the intestinal tract (Agnel et al. 1999; Mall et al. 2003), in various secretory glands, and several other epithelial tissues (Agnel et al. 1999). In the human respiratory tract, *CLCA4* expression has only been found on messenger ribonucleic acid (mRNA) level in nasal mucosa and trachea, however, of unknown cellular localization (Agnel et al. 1999; Mall et al. 2003) (also see 2.2, Figure 7).

In the pig, in which *CLCA4* is duplicated into *CLCA4a* and *-4b* (Plog et al. 2012a; 2015), exclusively CLCA4a was immunohistochemically located to the respiratory tract. More specifically, it was localized to apical membranes of virtually all tracheal and bronchial epithelial cells whilst the distal lung parenchyma and SMGs remained devoid (Plog et al. 2012a). Porcine CLCA4a was also expressed in the intestinal tract, i.e. at the apical membranes of the small intestinal villous epithelial cells (Plog et al. 2012a). Interestingly, its porcine-specific duplication product, CLCA4b, was selectively expressed in intestinal crypt epithelial cells and has thus adopted a novel cellular expression pattern unlike any other known CLCA protein in other species (Plog et al. 2015).

In the mouse, in which the *Clca4* gene underwent several duplication events resulting in three homologous members, *Clca4a*, *-4b*, and *-4c* (Patel et al. 2009), the respiratory tract does not show any expression of these homologs. Clca4a and -4b are exclusively expressed in non-

mucus cell enterocytes (Bothe et al. 2008; Evans et al. 2004; Teske et al. 2020), similar to their porcine ortholog CLCA4a (Plog et al. 2012a). Here, *Clca4a* was localized to the jejunal villi and apical colonic epithelium, whilst *Clca4b* was also found in intestinal crypts, pointing towards different functional roles of these homologs (Teske et al. 2020). The intestine remained devoid of CLCA4c expression (Teske et al. 2020) as did any other organ analyzed as described before (Al-Jumaily et al. 2007).

Hence, the only species for which CLCA4 protein expression has been shown in the respiratory tract is the pig, in which only one of the two duplication products, CLCA4a, is expressed in respiratory ciliated epithelial cells (Plog et al. 2012a).

In summary, regarding the expression in the airways, CLCA1 is predominantly expressed in mucus cells and SMGs of all aforementioned species (Anton et al. 2005; Gibson et al. 2005; Hoshino et al. 2002; Leverkoehne and Gruber 2002; Plog et al. 2009), whereas CLCA2 is exclusively expressed in SMGs of man, pig, and mouse, and in latter also in distinct bronchial epithelial cell niches (Dietert et al. 2015). For CLCA3, the mouse is the only species to express functional proteins, duplications of which – Clca3a1 and -3a2 – are expressed in secretory epithelial and endothelial cells (Gruber et al. 1998b), whilst Clca3b is expressed in endothelial and smooth muscle cells of the respiratory tract (Elble et al. 2002). In contrast, the murine CLCA4 homologs Clca4a, -4b, and -4c are not present in the respiratory tract, whereas one of the two porcine duplication products, CLCA4a, is expressed in the respiratory tract, i.e. in airway epithelial cells (Plog et al. 2012a), in which the human ortholog is expressed at least on mRNA level (Agnel et al. 1999; Mall et al. 2003).

Apart from this partially identical, but also partially diverse expression pattern of the CLCA family members, they show a common, systematic, and highly conserved protein structure and biochemistry.

1.4   Protein Structure and Biochemistry of CLCA Proteins

A common feature of all CLCA proteins biochemically analyzed to date is the posttranslational cleavage of the primary glycosylated translation product of ~ 120 kilo Dalton (kDa) into a larger ~ 80 kDa amino- and a smaller ~ 40 kDa carboxy-terminal subunit (Gruber et al. 1998a; 1999; 2000; Mundhenk et al. 2006; Patel et al. 2009). The aa motif of the posttranslational cleavage site has been shown to be present in all CLCA proteins investigated to date and is located in the carboxy-terminal region (Bothe et al. 2011; Elble et al. 1997), e.g. between aa 695 and 696

for murine CLCA1 as shown by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) and Edman-degradation analysis (Bothe et al. 2011).

The amino-terminal subunit of all CLCA proteins initially consists of a signal sequence which directs the proteins to the secretory pathway (Bendtsen et al. 2004; Patel et al. 2009). The signal sequence is followed by a uniquely conserved, cysteine-rich amino-terminal (n)-CLCA domain (Gruber et al. 2000) which contains a His-Glu-Xaa-Xaa-His (HEXXH) aa sequence discovered *in silico* (Pawlowski et al. 2006). This particular sequence is also known as zinc-binding aa motif suggestive of metalloprotease function (Pawlowski et al. 2006) which typically confers zinc-dependent cleavage (Hooper 1994).

Downstream to the HEXXH aa sequence, a central von Willebrand factor type A (vWA) domain is located, which is present in all CLCA proteins, except for *CLCA3* pseudogenes (Berry and Brett 2020) (see 2.1). This domain contains a Metal Ion-Dependent Adhesion Site (MIDAS), generally known to mediate protein-protein interactions (Whittaker and Hynes 2002). In contrast to other known CLCAs, CLCA2 lacks an intact MIDAS motif due to one or two missing key residues (Berry and Brett 2020) and contains a β4-integrin binding motif instead (Abdel-Ghany et al. 2001; 2002; 2003). This β4-integrin binding motif can be found additionally in other CLCA members. Since members of the CLCA2 family are known to potentiate calcium-dependent chloride currents (Gruber et al. 1999) despite lacking an intact MIDAS, it has been assumed that residues neighboring the β4-integrin binding motif may create a non-standard MIDAS or that these proteins use a different mode of engagement (Berry and Brett 2020). The vWA domain itself has furthermore very recently been proposed to be the domain functionally sufficient for macrophage activation for human CLCA1 *in vitro* (Keith et al. 2019).

The carboxy-terminal CLCA domain includes a Fibronectin type III (FnIII) domain which represents a protein-protein interaction module of the immunoglobulin (Ig) superfamily (Patel et al. 2009).

For CLCA1, the cleavage site was furthermore shown to be recognized by its own metalloprotease domain, pointing toward a self-cleavage mechanism (Bothe et al. 2011) which was proven by cleavage abrogation after introduction of a E157Q mutation into the HEXXH motif of human, murine, and porcine CLCA1 (Bothe et al. 2011; Pawlowski et al. 2006). Furthermore, cross-proteolytic capability was demonstrated by maintained cleavage of this cleavage-deficient mutant after co-transfection with wild type (WT) CLCA1 protein in Human Embryonal Kidney (HEK) 293 cells (Bothe et al. 2011). The zinc-dependency of the cleavage event was shown by cleavage abrogation using cation-chelating metalloproteinase inhibitors (Bothe et al. 2011). Nevertheless, the functional relevance of autocatalytic self-cleavage is unclear to date. On the one hand, self-cleavage does not seem necessary for its secretion

(Bothe et al. 2011; Nystrom et al. 2019; Yurtsever et al. 2012) or proteolytic activity (Nystrom et al. 2018; 2019), as uncleaved CLCA1 is also secreted *in vitro* (Bothe et al. 2011; Nystrom et al. 2019; Yurtsever et al. 2012) and proteolytically active (Nystrom et al. 2018) (see 1.5.3). On the other hand, self-cleavage seems necessary for its activity as chloride channel regulator (Sala-Rabanal et al. 2015; 2017) (see 1.5.1). The underlying mechanisms of this seemingly function-specific relevance to self-cleavage needs to be investigated in future.

Hence, the CLCA family can be biochemically subdivided into two groups. The cleaved subunits are either fully secreted as a soluble heterodimer – proteins of cluster 1 and 3 (Gibson et al. 2005; Gruber et al. 1998a; Gruber and Pauli 1999; Mundhenk et al. 2006; Patel et al. 2009; Plog et al. 2009; Range et al. 2007) – or only the amino-terminal subunit is shed, whilst the single carboxy-terminal subunit remains anchored in the plasma membrane (Bothe et al. 2008; Elble et al. 2006) – proteins of clusters 2 and 4 (Bothe et al. 2008; 2012; Braun et al. 2010a; Elble et al. 2006; Plog et al. 2012a; 2012b). This anchoring region can be comprised of either a transmembrane α-helix or, as proposed for human CLCA4, a glycosylphosphatidyl-inositol (GPI) anchor (Patel et al. 2009).

As an exception, the CLCA members human CLCA3 and murine CLCA4c were shown to be merely truncated *in silico* due to premature stop codons and the protein may not be expressed *in vivo*, as they represent pseudogenes (Patel et al. 2009) (see also 1.2, 1.3.3, and 1.3.4).

1.5   Alleged Functions of CLCA1 and its Homologs in the Respiratory Tract

Upon their discovery, CLCA proteins were thought to act as chloride channels, since certain CLCA members mediated calcium-dependent chloride currents *in vitro* (Gruber et al. 1998a; Loewen and Forsyth 2005; Ran and Benos 1991; 1992; Winpenny et al. 2002; Yamazaki et al. 2005; Zhu et al. 1991). Nonetheless, consecutive *in silico* analyses showed that CLCA proteins lack multiple transmembrane domains (Gibson et al. 2005) and are partially or fully secreted into the extracellular environment (Anton et al. 2005; Bothe et al. 2008; Braun et al. 2010b; Elble et al. 2006; Gibson et al. 2005; Huan et al. 2008; Mundhenk et al. 2006). Therefore, CLCA proteins cannot form anion channels *per se* but may possess various other functions hypothesized to date, including those of soluble signaling molecules (Gibson et al. 2005; Mundhenk et al. 2006) which activate macrophages (Ching et al. 2013; Dietert et al. 2014; Erickson et al. 2018; Keith et al. 2019) or modulate transepithelial anion conductance (Sala-Rabanal et al. 2015), cell-cell adhesion (Abdel-Ghany et al. 2001; 2002; 2003), and cell differentiation (Yu et al. 2013), i.e. in terms of mucus metaplasia (Nakanishi et al. 2001).

These functional roles have mainly been investigated for CLCA1 in humans and murine models of mucus-based inflammatory airway conditions such as asthma, COPD, and CF (Patel et al. 2009) with partially contradictory results (Alevy et al. 2012; Dietert et al. 2014; Long et al. 2006; Mundhenk et al. 2012; Nakanishi et al. 2001; Patel et al. 2006; Song et al. 2013; Young et al. 2007; Zhang and He 2010).

1.5.1 Mucus-Modulating Function – Chloride Channel Regulator

Chloride secretion is central to disease pathogenesis in mucus-based respiratory diseases (see 1.6), particularly to CF (see 1.6.5). Despite CLCA1 not being able to form a chloride channel *per se* (Gibson et al. 2005; Mundhenk et al. 2006), it had been postulated to act as chloride channel regulator, which would also be consistent with the original observation of evoking chloride secretion *in vitro* after transfection (Ran and Benos 1991).

Recently, the Transmembrane protein 16A (TMEM16A, also known as Anoctamin1 or Discovered On Gastrointestinal Stromal Tumors Protein-1, DOG1) was discovered as the first genuine CaCC in mammals (Caputo et al. 2008; Schroeder et al. 2008; Yang et al. 2008) and the potentially first downstream target of CLCA1 (Sala-Rabanal et al. 2015). Self-cleavage of CLCA1 is thought to unmask its amino-terminal (Sala-Rabanal et al. 2015), which enables the vWA domain to bind to and potentiate TMEM16A surface expression (Berry and Brett 2020; Sala-Rabanal et al. 2015), thereby increasing calcium-activated chloride currents (Sala-Rabanal et al. 2015; 2017). Furthermore, both CLCA1 and TMEM16A have been suggested to compensate for the dysfunctional Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in CF or to stimulate secretion in asthma and COPD, thereby solubilizing the obstructive mucus (Mall et al. 2018). A similar role in transepithelial ion conduction had also been proposed for several other CLCA members (Evans et al. 2004; Hamann et al. 2009). Besides CLCA1, human CLCA4 is considered a potential modifier of disease severity in CF (Ritzka et al. 2004) in which it may upregulate the transmembrane potential and mediate secretion of electrolytes and water (Eggermont 2004). The porcine homolog CLCA4a is located to apical membranes of respiratory and intestinal epithelia (Plog et al. 2012a) and colocalizes with the porcine CFTR protein (Plog et al. 2010), which is also suggestive of such a modulatory role in respiratory tract diseases, particularly in CF (Plog et al. 2012a).

Furthermore, not only human CLCA1 but also TMEM16A expression was significantly increased by IL-13 *in vitro* (Alevy et al. 2012; Caputo et al. 2008). In the mouse, however, these findings could not be mirrored (see also 1.5.2). In contrast to human airways (Alevy et al. 2012), IL-13-induced CLCA1 overexpression failed to induce CaCC activity in the murine respiratory tract *in vivo* and lack of murine CLCA1 did not reveal any bioelectric differences in naive or IL-13-challenged conditions (Mundhenk et al. 2012). Additionally, no changes

regarding IL-13-induced mucus cell metaplasia were observed in any of the two genotypes (Mundhenk et al. 2012). Furthermore, restoration of decreased CLCA1 protein in a mouse model of CF ameliorated intestinal mucus plugging, most likely by mucus property alteration and not by CaCC activation or rectification of the CF electrophysiology defect (Young et al. 2007). Hence, in the mouse, CLCA1 may not contribute to CaCC-mediated chloride secretion or its lack may be compensated by other proteins. Interestingly, the mouse shows three seemingly functional homologous proteins of CLCA3 (Patel et al. 2009), which is a single pseudogene in humans and pigs (Gruber and Pauli 1999; Plog et al. 2009). Additionally, the CLCA2 protein is expressed in mucin-producing airway epithelial cells and co-localized with CLCA1 only in the mouse but not in humans (Dietert et al. 2015)

Although a differential upregulation of these murine CLCA homologs was not observed in the $Clca1^{-/-}$ model (Mundhenk et al. 2012), it should be analyzed further if these homologs are candidates for putative compensation for the lack of CLCA1.

However, numerous previous studies linking human CLCA1 to chloride transport had been performed exclusively *in vitro* (Hamann et al. 2009; Ran and Benos 1991; Sala-Rabanal et al. 2015; 2017; Yurtsever et al. 2012). Therefore, it may be assumed that this allegedly species-specific discrepancy – CLCA1 being capable of evoking a chloride current – could perhaps be observed exclusively *in vitro*.

1.5.2   Mucus-Modulating Function – Mucus Cell Metaplasia

Mucus cell metaplasia and consecutive mucus overproduction are key features of mucus-based respiratory diseases, particularly of asthma which CLCA1 overexpression has been associated with not only in humans and mice (Hoshino et al. 2002; Mei et al. 2013; Nakanishi et al. 2001) (see 1.6.1) but also in horses (Patel et al. 2009) (see 1.6.2).

CLCA1 is one of the most strongly upregulated proteins in respiratory challenged conditions (Fernandez-Blanco et al. 2018; Hauber et al. 2010; Zhou et al. 2001). *In vitro* and *in vivo* overexpression of human or murine CLCA1 (Nakanishi et al. 2001; Zhou et al. 2001) led to a significant increase in mucin expression (Nakanishi et al. 2001; Zhou et al. 2001) and aggravation of the asthma phenotype *in vivo* (Nakanishi et al. 2001; Song et al. 2013). This was exclusively associated with mucus cell metaplasia but not airway hyperreactivity (Nakanishi et al. 2001; Patel et al. 2006) and could be ameliorated by CLCA1 suppression (Nakanishi et al. 2001; Song et al. 2013).

An alleged underlying pathomechanism was shown *in vitro* and *ex vivo* for human CLCA1 which regulates IL-13-dependent mucus cell metaplasia (Alevy et al. 2012) in inflammatory airway disease by transforming mucus precursor to mature mucus cells (Patel et al. 2009).

Specifically, mucin gene induction in human airway epithelial cells may be initiated by a virus or, such as in asthma, an environmental stimulus. This triggers the production of IL-13 (Alevy et al. 2012), a member of the T helper (Th) 2 cytokine family. IL-13 then activates a Signal Transducer and Activator of Transcription 6 (STAT6) pathway (Nakano et al. 2006; Yasuo et al. 2006; Zhou et al. 2001; 2002) by which CLCA1 expression is increased. CLCA1, in turn, activates a Mitogen-Activated Protein Kinase (MAPK) 13, leading to increased mucus production, particularly of human mucin protein (MUC) 5AC (Alevy et al. 2012), the main respiratory mucin protein (Hovenberg et al. 1996). Nevertheless, the receptor for CLCA1 is unknown to date.

These findings for human CLCA1, specifically the IL-13-dependency of CLCA1-mediated airway mucus production (Alevy et al. 2012), could not be mirrored in the mouse. Murine *in vivo* models showed no difference in mucus cell metaplasia, neither in naive nor in IL-13-challenged *Clca1*-deficient (*Clca1$^{-/-}$*) *versus* (vs.) WT mice (Mundhenk et al. 2012; Patel et al. 2006). Again, compensatory effects cannot be excluded in the mouse in which the homologs present in the murine respiratory tract, CLCA2 and -4a, are sufficient to produce mucus cell metaplasia (Patel et al. 2006), but not in humans (Alevy et al. 2012).

More specifically, CLCA2, which is expressed in airway epithelial cells exclusively in the mouse (Dietert et al. 2015), is increased during mucus cell metaplasia and airway hyperreactivity after viral infection (Patel et al. 2006) or IL-13 stimulation (Mundhenk et al. 2012). Furthermore, *Clca2* is sufficient to induce mucus cell metaplasia but not airway hyperreactivity (Patel et al. 2006) and is considered, together with CLCA4a, a potential candidate in compensating for loss of CLCA1 (Patel et al. 2009). Hence, species-specific compensatory effects could perhaps explain this discrepancy observed and require further investigation.

1.5.3 Mucus-Modulating Function – Metalloprotease

Although it has been shown that CLCA1 does not seem to be an essential mucus structure-associated component (Erickson et al. 2015), the protein is capable of enzymatically modulating mucus processing.

Due to its colocalization with mucins in granules and its secretion into the mucus, it had been hypothesized that CLCA1 may change mucus properties in terms of viscosity or rheology by altering protein processing (Young et al. 2007). Very recently, CLCA1 was shown to increase mucus expansion and penetrability in colon biopsies *ex vivo* by mucus proteolysis whilst lack of CLCA1 led to increased oligomer density of the murine Muc2 protein, the main intestinal mucus protein (Nystrom et al. 2018). In addition to the known amino-terminal cleavage product of 80 kDa, further studies demonstrated the existence of a second, smaller and highly instable

amino-terminal cleavage product, both of which are capable of cleaving Muc2 – the smaller one with higher efficiency (Nystrom et al. 2019).

Nonetheless, *Clca1*$^{-/-}$ mice did not show any phenotype, neither in homeostatic conditions (Erickson et al. 2015; Mundhenk et al. 2012; Patel et al. 2006) nor during dextran sodium sulfate (DSS)-induced mucus barrier disruption (Erickson et al. 2015). Compensatory mechanisms in terms of differential mucin or CLCA1 homolog expression or changes in microbiota had been ruled out. However, a compensation by CLCA homologs at physiologic levels or by other proteins could not be excluded (Erickson et al. 2015). Latter notion was later confirmed as compensation by a cysteine protease in the mouse (Nystrom et al. 2018).

Therefore, CLCA1 may play a key role in mucus homeostasis and clearance by facilitating its processing and removal, at least in the intestine (Nystrom et al. 2018). However, the exact regulatory mechanisms of these complex processes remain unknown to date, as do putative mucus processing-properties of CLCA1 in the airways. Of note, the major mucus component of the intestinal tract, MUC2, is not expressed in the respiratory tract (Hovenberg et al. 1996). It would therefore be interesting to investigate if MUC5AC, the main respiratory mucus component (Hovenberg et al. 1996), is also a substrate of CLCA1 similar to MUC2 in the intestinal tract.

1.5.4   Functions as a Signaling Molecule in Innate Immunity

Apart from a mucus-related function, CLCA1 has also been hypothesized to act as a signaling molecule in macrophage activation since several *in vitro* and *in vivo* studies showed a CLCA1-dependent differential cytokine expression pattern (Ching et al. 2013; Dietert et al. 2014; Erickson et al. 2018; Long et al. 2006).

First, ovalbumin (OVA)- or Lipopolysaccharide (LPS)-challenge led to a significant increase of murine neutrophil chemoattractant, Chemokine (C-X-C motif) Ligand CXCL-1 (also termed Keratinocyte Chemoattractant, KC), and consecutive increase of neutrophilic bronchoalveolar lavage (BAL) inflammation in *Clca1*$^{-/-}$ vs. WT mice (Long et al. 2006). Moreover, in a mouse model of asthma, CLCA1-antibody or -antisense treatment showed a remarkable amelioration of airway inflammation (Nakanishi et al. 2001; Song et al. 2013). In contrast, acute *Staphylococcus* (S.) *aureus* pneumonia led to decreased CXCL-1 and IL-17 mRNA and protein expression and decreased neutrophil recruitment in *Clca1*$^{-/-}$ vs. WT mice (Dietert et al. 2014).

These seemingly contradictory results may be due to CLCA1-mediated cytokine modulation being dependent on the stimulus – intact, Gram-positive bacteria vs. partial virulence factors of Gram-negative bacteria – or different mouse strains used in aforementioned studies (Dietert et al. 2014).

Isolated investigation of the CLCA1-mediated effect on macrophages *in vitro* showed that human CLCA1 induced an upregulation of IL-8, the human ortholog to the murine CXCL-1 and CXCL-2 (also termed macrophage inflammatory protein 2-alpha, MIP-2α) in primary porcine alveolar macrophages and in a human monocyte-macrophage cell line (Ching et al. 2013). Eliminating possible cross-species effects, an exclusively murine study also demonstrated the CLCA1 capability of activating murine alveolar macrophages *in vitro* and *ex vivo*, furthermore confirming the translatability of CLCA1-dependent macrophage activation (Erickson et al. 2018). Additionally, it was shown that CLCA1 may exert this effect by modulating the expression of bacterial/permeability increasing fold-containing protein family A1 (BPIFA1, also termed short palate, lung, and nasal epithelium clone 1 (SPLUNC1) in humans) protein (Erickson et al. 2018), a known host-protective, immunomodulatory, and liquid homeostasis-associated airway mucus component (Britto and Cohn 2015). Further analysis of recombinantly expressed human CLCA1 protein fragments, each containing a potentially active domain, pinpointed to the vWA domain being responsible for macrophage activation which was further associated with the MAPK and NF-κB (Nuclear Factor 'κ-light-chain-enhancer' of activated B-cells) (Sen and Baltimore 2006) pathway (Keith et al. 2019).

These findings suggest that CLCA1 may directly act on macrophages as a signaling molecule to induce a pro-inflammatory cytokine response and also expand the role of CLCA1 in airway disease to even more complex downstream pathways, including liquid homeostasis, airway protection, and antimicrobial defense (Erickson et al. 2018), which still need to be further elucidated.

1.6   CLCA Proteins in Respiratory Diseases

In numerous respiratory diseases, such as asthma, CF and COPD, mucus stagnates and accumulates in the airways, leading to bacterial colonization, airway inflammation, tissue destruction, and ultimately respiratory failure (Anthonisen 1988; Boucher 2007; Brouillard et al. 2005; Daser et al. 2001; Ratjen and Döring 2003; Rouze et al. 2014; Vankeerberghen et al. 2002; Vestbo et al. 2013; Yoon et al. 2002). Certain members of the CLCA family, especially CLCA1, have been linked to these particular inflammatory mucus-based airway diseases (Gibson et al. 2005; Hauber et al. 2003; 2004; Hoshino et al. 2002; Patel et al. 2006; Patel et al. 2009; Toda et al. 2002; Wang et al. 2007; Woodruff et al. 2007). These share numerous hallmark characteristics such as mucus cell meta- and hyperplasia, mucus overproduction, disturbed clearance, and, interestingly, strong expressional regulation of CLCA1 in human patients (Brouillard et al. 2005; Hauber et al. 2010; Hegab et al. 2004; Kamada et al. 2004; Patel et al. 2009; Zhou et al. 2001) and the corresponding murine disease models (Hauber et

al. 2004; Hegab et al. 2004; Patel et al. 2009; Toda et al. 2002). Furthermore, single nucleotide polymorphisms with certain haplotypes in the human CLCA1 gene were found to be associated with increased susceptibility to these diseases (Hegab et al. 2004; Kamada et al. 2004; van der Doef et al. 2010). Therefore, CLCA1 was thought to act as a biomarker and potential therapeutic target (Patel et al. 2009).

1.6.1   Asthma in Humans

The Expert Panel Report 3 (EPR-3), coordinated by the US National Heart Lung and Blood Institute, defines asthma as a chronic, variable, and recurring airway disorder characterized by airflow obstruction, bronchial hyperresponsiveness, and underlying inflammation (EPR-3 2007). More specifically, further features particularly include bronchial eosinophilic airway inflammation, mucus overproduction or airway remodeling (Daser et al. 2001; McFadden and Gilbert 1992). According to the World Health Organization (WHO), asthma has a high prevalence with about 339 million people affected worldwide (WHO 2020).

In humans, asthma arises from multiple genetic and environmental factors (Barnes and Marsh 1998; Steinke et al. 2003). Latter may include but are not limited to pharmacological products (e.g. aspirin), environmental substances or air pollutants (e.g. ozone, tobacco smoke), infectious agents (in particular certain viruses) or allergens (Cockcroft 2018). Although several types of asthma have been recognized clinically, allergic asthma is the most common form of the disease (Kim et al. 2010), i.e. the inherited predisposition to form immunoglobulin (Ig) E (EPR-3 2007).

As generally accepted pathogenesis, sensitization occurs when inhaled environmental allergens are taken up by dendritic cells at the mucosal surface, processed, and presented to naive cluster of differentiation 4-positive (CD4$^+$) Th cells and B lymphocytes. Th2 cells secrete numerous cytokines (IL-4, -5, -10, -13), which promote the differentiation of B lymphocytes to plasma cells which, in turn, synthesize allergen-specific IgE (Barnes 2008; Mukherjee and Zhang 2011). IgE then binds to its corresponding receptors on mast cells and basophil granulocytes. The effector phase occurs upon re-exposure to the allergen, which binds to IgE and leads to cross-linking and mast cell degranulation, liberation of numerous cytokines (IL-3, -5) and mediators such as histamine or phospholipids, which, in turn, promote eosinophilic granulocyte attraction, smooth muscle cell contraction and consecutive bronchoconstriction, as well as mucus production, vasodilation, and extravasation of neutrophilic granulocytes (Janeway 2001; Stone et al. 2010).

In recent years, human asthma has been divided into four phenotypes based on the predominant inflammatory cell type: eosinophilic, neutrophilic, mixed granulocytic, and

paucigranulocytic asthma (PGA) (Gao et al. 2017). Whilst eosinophilic asthma correlates with Th2-associated inflammation (Gao et al. 2017), neutrophilic asthma is more strongly associated with the presence of one of the other subtypes of CD4$^+$ T lymphocytes, Th17 cells (Newcomb and Peebles 2013). In contrast, PGA shows no evidence of increased eosinophil or neutrophil numbers (Tliba and Panettieri 2019).

Beside the numerous factors mentioned above, CLCA1 had also been identified as an important modulatory factor in the pathogenesis of asthma. Firstly, several genetic studies have linked gene variations of the human *CLCA1* gene to severity modulation in human asthma (Kamada et al. 2004). More specifically, as in COPD, certain single-nucleotide polymorphisms were linked to asthma susceptibility in a Japanese population (Hegab et al. 2004; Kamada et al. 2004).

On protein level, human CLCA1 and its murine ortholog are strongly upregulated in mucin-producing cells and secreted into the BAL fluid (BALF) of human asthma patients (Hoshino et al. 2002; Toda et al. 2002; Wang et al. 2007; Woodruff et al. 2007) and murine asthma models (Gibson et al. 2005; Hoshino et al. 2002; Toda et al. 2002; Zhou et al. 2001). Furthermore, IL-9 antibody or glucocorticoid treatment suppressed *CLCA1* mRNA expression (Woodruff et al. 2007; Zhou et al. 2001) whilst the asthma phenotype was ameliorated by CLCA1-antisense- (Nakanishi et al. 2001) or -antibody-treatment (Song et al. 2013). Experimental overexpression of human (Hoshino et al. 2002) and murine CLCA1 (Mei et al. 2013; Nakanishi et al. 2001) resulted in asthmatic phenotype exacerbation in terms of mucus cell metaplasia, mucin overproduction, and airway inflammation (Hoshino et al. 2002; Mei et al. 2013; Nakanishi et al. 2001) independent of an allergen exposure (Mei et al. 2013).

In veterinary medicine, asthma is known as a highly relevant naturally occurring disease in horses and cats, in latter of which it shares striking similarities with the human disease concerning etiology, clinical presentation, lung histology, and response to therapy (see 1.6.3).

1.6.2 Equine Asthma

Since a variety of terms has been used synonymously for the medical term "equine asthma", which led to great confusion in scientific and lay communities (Lavoie 2020), it was recently defined more precisely. According to the American College of Veterinary Internal Medicine (ACVIM) Consensus Statement (Couetil et al. 2016) and based on the degree of severity, "severe equine asthma" (SEA) sums up all conditions previously known as RAO, "heaves", equine COPD, chronic obstructive bronchitis or equine emphysema, whilst "mild or moderate equine asthma" is used to describe what was previously termed "inflammatory airway disease" (IAD) (Lavoie 2020).

Equine asthma occurs naturally in response to inhaled antigens such as hay, mold, dust (Couetil et al. 2016; Lowell 1964) and may also be induced experimentally by environmental challenge under standardized conditions (Gerber et al. 2003). It is mainly characterized by acute inflammation of the respiratory mucosa with neutrophilic granulocytes, sharing marked similarities with human neutrophilic asthma (Leclere et al. 2011). Specifically, airways of severely asthmatic horses show remodeling, i.e. increased collagen and elastic fiber deposition (Setlakwe et al. 2014) and hyperplasia of airway smooth muscle cells, which is clinically mirrored by the degree of bronchospasm severity (Herszberg et al. 2006; Vargas et al. 2016). SEA, to which milder forms may progress to based on inflammation-induced airway remodeling, is incurable to date (Couetil et al. 2016).

Furthermore, equine asthma shows similarities to human asthma and COPD (Bice et al. 2000; Patel et al. 2009; Snapper 1986), especially concerning the hallmark features of airway hyperreactivity and mucus cell metaplasia (Davis and Rush 2002; Leguillette 2003; Lowell 1964; Patel et al. 2009) with consecutive massive mucus overproduction (Davis and Rush 2002; Leguillette 2003; Patel et al. 2009).

Identical to human asthma patients and murine models of asthma (Hoshino et al. 2002; Toda et al. 2002; Zhou et al. 2001), CLCA1 mRNA and protein expression were highly upregulated in tracheal and lung tissue of SEA-affected horses, primarily in the bronchioles, which were devoid of CLCA1 expression during homeostatic conditions (Anton et al. 2005). This overexpression was, however, exclusively linked to increased mucus cell numbers due to hyper- and metaplasia and not due to a cellular transcriptional *CLCA1* upregulation (Range et al. 2007). Hence, CLCA1 expression in horses is identical to man and mouse not only during steady-state (Anton et al. 2005) – as described in chapter 1.3.1 – but also during chronic inflammatory airway conditions (Range et al. 2007).

Thus, spontaneous or experimentally induced SEA may serve as a valuable model for human asthma and COPD as it shares characteristic features not only concerning clinicopathological findings but also concerning differential CLCA1 expression (Anton et al. 2005; Bice et al. 2000; Snapper 1986).

1.6.3 Feline Asthma

Similar to equine asthma, the nomenclature of the feline condition includes approximately 15 different terms used synonymously (Reinero 2011), ranging from clinical terminology such as "feline lower airway disease" (Adamama-Moraitou et al. 2004), "feline asthma syndrome" (Corcoran et al. 1995), "feline bronchial disease" (Foster et al. 2004) or "idiopathic small-airway disease" (Moriello et al. 2007) to pathologically-descriptive terms such as "chronic non-allergic bronchitis" or "chronic bronchitis with emphysema" (Reinero 2011). Hence, its true prevalence

is not readily assessable, which warrants precise distinction from other respiratory diseases, particularly from non-allergic inflammatory lower airway diseases (Reinero 2011).

Feline asthma is, beside chronic bronchitis, the most common lower airway disorder in cats (Reinero 2011) and shares numerous key aspects with human asthma. First, due to the close and frequent proximity of cats to humans, environmental factors causative of human asthma possibly also contribute to disease initiation and progression of the feline disease (Schäfer et al. 2008). These shared epidemiological factors have furthermore been discussed to be causative also of an increasing prevalence, incidence, and severity not only in humans but also in cats (Ranivand and Otto 2008; Redd 2002; Reinero et al. 2009).

Second, as in humans, the condition emerges naturally in the cat (Norris Reinero et al. 2004) and is most likely induced by allergens (Reinero 2011) similar to those causative of the human condition (Schäfer et al. 2008). Specifically, the house dust mite or Bermuda grass allergen were shown to evoke an asthmatic airway response (Adler et al. 1985; EPR-3 2007; Kurata et al. 2002; Norris Reinero et al. 2004) and may also be used to induce this condition experimentally under standardized conditions (Norris Reinero et al. 2004). In contrast, chronic bronchitis etiologically occurs secondary to an insult (Reinero 2011), leading to permanent airway damage and clinicopathological findings similar or even non-differentiable to those of asthma (Grotheer et al. 2020; Reinero 2011).

The feline condition seems to be mainly allergen-induced in cats, whereas in humans, numerous other factors possibly contributing to disease initiation and progression have been identified. Beside allergens, human asthma arises from genetic, environmental or other factors as discussed above (see 1.6.1). In contrast, no clear evidence points towards factors other than allergens inducing asthma in cats which, however, may be due to lack of respective studies clearly segregating these (Reinero 2011). Furthermore, an inverse correlation of viral infection – particularly of the upper respiratory tract in early development – with asthma has been suggested for humans (Illi et al. 2001; von Mutius 2007). This virally induced enhancement of the Th1 response may be considered protective against Th2-mediated inflammation of asthma in children (Björkstén 2009; Illi et al. 2001). This correlation concerning a possible protective effect has not been investigated comprehensively in cats, although early upper respiratory infections with feline calici- and herpesviruses are common (Reinero 2011). Hence, the differentiation regarding other possibly contributing factors warrants further studies in cats.

Third, numerous characteristics of human asthma, such as recurrent and variable clinical features – bronchial hyperresponsiveness and consecutive airflow obstruction, the hallmark features of human asthma (EPR-3 2007), as well as a Th2 cell cytokine profile in blood and

BALF and induction of allergen-specific IgE – and pathological features – bronchial eosinophilic airway inflammation, mucus overproduction or airway remodeling (Daser et al. 2001; McFadden and Gilbert 1992) – are identically present in the feline condition (Reinero 2011). Cats with feline asthma show intermittent spontaneous expiratory distress due to bronchoconstriction, which is permanent in cats affected by chronic bronchitis (Reinero 2011). Therefore, latter will not show a positive response to bronchodilators in contrast to asthmatic cats (Reinero 2011).

Fourth, feline asthma also shares several important pathohistological features with the human disease, including mucus plugging of small airways, epithelial hyperplasia, eosinophilic inflammation, smooth muscle hypertrophy, and Th2-mediated airway remodeling (Cohn et al. 2010; Corcoran et al. 1995; Dye et al. 1996; Trzil and Reinero 2014). The pathohistological diagnostic criteria of spontaneous feline asthma were determined by literature, as also in this study, as perivascular and peribronchiolar inflammation with eosinophils, chronic emphysema, smooth muscle hypertrophy of the arteries and bronchioles, and excessive mucus secretion and accumulation in the airways (Shibly et al. 2014; Williams and Roman 2016). BALF cytology is still considered the diagnostic gold standard for asthma diagnosis in cats and a crucial diagnostic tool in differentiating asthma from chronic bronchitis (Reinero 2011). Whilst eosinophils are increased in asthma, feline chronic bronchitis shows predominantly neutrophilic instead of eosinophilic inflammation, particularly with increased non-degenerate neutrophils in BALF (Reinero 2011; 2019). However, it must be considered that chronic airway inflammation resulting from feline asthma may lead to mixed eosinophilic and neutrophilic inflammation known as "chronic asthmatic bronchitis" (Moise et al. 1989).

Due to these numerous parallels to human asthma, the feline condition has been discussed as a valuable model in studying and understanding the human disease (Williams and Roman 2016). Since CLCA1 not only exhibits a highly conserved expression pattern in the respiratory tract of various species (see 1.3.1), but also differential expression and various putatively functional roles in diseased airways (see 1.6) alongside several other, perhaps compensatory orthologs, a detailed assessment of CLCA family in the cat is warranted.

1.6.4 Chronic Obstructive Pulmonary Disease

According to the WHO, COPD prevalence was estimated to be at 251 million cases globally with 3.17 million COPD-associated deaths for 2016 and 2015, respectively (WHO 2017), and was predicted to become the third leading cause of death worldwide by 2030 (WHO 2008). COPD is clinically characterized by airflow limitation, mucus overproduction, and enhanced

chronic inflammation in response to irritants such as noxious particles or gases (Anthonisen 1988; Rouze et al. 2014; Vestbo et al. 2013), mainly originating from tobacco smoke and air pollution (WHO 2017). In short, activated epithelial cells and macrophages, which are present in a much larger number than in asthma (Barnes 2008), secrete cytokines such as IL-6 and tumor-necrosis factor and chemokines such as CXCL1 and -8. Latter attract neutrophilic granulocytes and, together with CC-chemokine ligand 2 (CCL2), also monocytes, whilst CXCL9-11 recruit Th1 and type 1 cytotoxic T cells (Barnes 2008). All abovementioned cell types release proteases – leading to emphysema by elastin degradation in the alveolar walls (Barnes 2008; Majo et al. 2001; Taraseviciene-Stewart et al. 2006) – and transforming growth factor-β (TGFβ), which stimulates fibroblast proliferation (Barnes 2008), and hence, fibrosis, causing the characteristic irreversibility of this disease (Hogg et al. 2004).

In contrast to the proximal inflammation pattern in asthma (Barnes 2008), inflammation in COPD occurs predominantly in peripheral airways and lung parenchyma (Barnes 2000; Jeffery 2000), whilst mucus cell hyperplasia and increased mucin gene expression are present in both (Caramori et al. 2004). Chronic airway pathology includes massive mucus overproduction (Davis and Rush 2002; Leguillette 2003) due to mucus cell metaplasia in small bronchioles in which MUC5AC is the predominant airway mucin (Hovenberg et al. 1996).

Several studies have associated CLCA family members with the modulation of COPD disease severity. Genome analyses of COPD patients linked the degree of airflow obstruction to alterations in the *CLCA* family-containing region of human chromosome 1 (Silverman et al. 2002). More specifically, in the Japanese population, susceptibility to COPD was associated with *CLCA1* gene single-nucleotide polymorphisms (Hegab et al. 2004; Kamada et al. 2004). Furthermore, human CLCA1, beside CLCA2 and -4, was predominantly overexpressed in COPD lung samples (Patel et al. 2009; Wang et al. 2007). In order to segregate the influence of CLCA proteins on mucus cell metaplasia and airway hyperreactivity, the main disease characteristics COPD and asthma, a mouse model of Sendai virus-induced bronchiolitis was established (Patel et al. 2006) which linked CLCA1 exclusively to mucus cell metaplasia but not to airway hyperreactivity (Patel et al. 2006).

1.6.5 Cystic Fibrosis

In contrast to the other diseases, CF is an autosomal recessive disorder in which genetic mutations of the *CFTR* render its gene product, the cyclic Adenosine Monophosphate (cAMP)-activated chloride and bicarbonate channel (Choi et al. 2001; Quinton 1999; Welsh 2001), defective.

It is considered the most common lethal genetic disease (Dodge et al. 1997) with more than 2000 CF-causing mutations known to date (De Boeck 2020) and a disease carrier rate of ~ 5 % in Caucasians (Rogers et al. 2008c). Clinical manifestations are primarily due to lack of bicarbonate secretion leading to mucus dehydration and attachment (Ambort et al. 2012; Gustafsson et al. 2012; Johansson et al. 2008; Schütte et al. 2014) and range from meconium ileus and intestinal obstruction shortly after birth (Oppenheimer and Esterly 1975; Quinton 1999; Welsh 2001; Wilschanski and Durie 1998) to pancreatic insufficiency, Distal Intestinal Obstruction Syndrome (DIOS), liver disease, and infertility later in life (Oppenheimer and Esterly 1975; Quinton 1999; Wilschanski and Durie 1998). Respiratory disease, the hallmark feature and leading cause of CF morbidity and mortality, may occur months to years after birth (McAuley and Elborn 2000; Quinton 1999; Rowe et al. 2005; Schwiebert et al. 1998; Stoltz et al. 2015; Vankeerberghen et al. 2002; Welsh 2001). Mucus plugging leads to impaired clearance and secondary bacterial infection with consecutive chronic airway inflammation (Brouillard et al. 2005; McAuley and Elborn 2000; Quinton 1999; Rowe et al. 2005; Schwiebert et al. 1998; Vankeerberghen et al. 2002; Welsh 2001).

Besides the CFTR genotype and environmental factors, modifier genes are known determinants of the CF phenotype (Clarke et al. 1994; Collaco et al. 2008; Gray et al. 1994; Rozmahel et al. 1996; Wilschanski et al. 1996). Certain *CLCA* gene family members, *CLCA1* and *CLCA4*, seem to modulate the basic intestinal chloride secretory defect in CF (Ritzka et al. 2004). Specifically, certain *CLCA1* and *CLCA4* allelic variants had been associated with meconium ileus in CF patients (van der Doef et al. 2010) and discussed to modulate residual colonic chloride secretion (Kolbe et al. 2013), respectively. In detail, the S357N mutation of *CLCA1* had been shown to decrease its expression levels and, consecutively, its ability to potentiate TMEM16A (Berry and Brett 2020). A murine ortholog of CLCA4 was immunohistochemically colocalized with the CFTR protein in non–mucus cell enterocytes, which is suggestive of common pathways in transepithelial electrolyte secretion and/or absorption (Bothe et al. 2008; Leverkoehne et al. 2006; Loewen and Forsyth 2005; Ritzka et al. 2004).

On the one hand, bronchial mucosa and BALF of CF patients showed high amounts of CLCA1 (Hauber et al. 2003; 2004; Hoshino et al. 2002; Toda et al. 2002; Wang et al. 2007; Woodruff et al. 2007) associated with mucus overproduction (Hauber et al. 2003; 2004). On the other hand, severe CF-associated mucus overproduction and accumulation was associated with strongly reduced CLCA1 protein expression in the intestine of a CF-mouse model (Brouillard et al. 2005), whilst restoration of murine CLCA1 protein led to phenotype amelioration (Young et al. 2007). Taken together, these findings suggest that a physiological level of CLCA activity is necessary for normal mucus cell function and homeostasis (Patel et al. 2006).

The pig has recently emerged as a more suitable animal model for human CF than the mouse. Humans and pigs not only share many anatomic and genomic organizational aspects (Rogers et al. 2008a). Systematic characterization of recently generated porcine CF models (Meyerholz et al. 2010; Rogers et al. 2008a; 2008b; 2008c; Welsh et al. 2009) show hallmark features of human CF, including lung disease (Meyerholz et al. 2010; Stoltz et al. 2010). In the light of these findings, systematic characterization of naive pigs concerning CLCA expression showed that porcine CLCA4a is expressed in tracheal and bronchial epithelial cells virtually identical to humans (Agnel et al. 1999; Mall et al. 2003; Plog et al. 2012a) and is furthermore co-expressed with porcine CFTR (Plog et al. 2010). Therefore, a modulatory role of porcine CLCA4a in CF pathogenesis may be likely (Plog et al. 2012a) and the porcine CF model may also be considered more suitable to study CLCA molecules in CF pathogenesis than the mouse (Plog et al. 2012a).

1.7   Hypotheses and Study Setup

Hypothesis 1: The cat possesses CLCA orthologs which are grouped into four clusters.
Each of the species investigated to date possesses members of the CLCA family which cluster in four distinct groups (Patel et al. 2009). Hence, the first aim was to investigate if CLCAs are also present in the cat and if these are also grouped into these four clusters. Therefore, the presence of feline CLCAs was analyzed *in silico.* Feline CLCA protein sequences were derived from the NCBI (http://www.ncbi.nlm.nih.gov) or Ensembl database (www.ensembl.org). The aa sequences of feline CLCA1 and CLCA2 (see 2.2, 2.3, and Supplemental Material S1 and S2, respectively), feline CLCA3 (see 2.1, 2.3, and Supplemental Material S3), and feline CLCA4 (see 2.2, 2.3, and Supplemental Material S4) were investigated using several databases and online prediction softwares to uncover potential structural and expressional species-specific homologies or differences to orthologs of other well-investigated species, i.e. humans, mice, and pigs.

Hypothesis 2. Feline CLCA1 shows a similar, the other feline CLCA members a species-specific respiratory expression pattern in comparison to other species.

CLCA1 shows a similar expression pattern (see 1.3.1), whilst the other family members are prone to species-specific evolvements and show overlapping or distinct expression patterns (see 1.3.2 to 1.3.4). Hence, this study secondly aimed at elucidating the mRNA and protein expression profile of the feline CLCA family members, particularly in the respiratory tract, tissues of which showed no signs of gross or histological changes as reference value for subsequent studies.

Hypothesis 3. CLCA1 expression is increased during asthmatic vs. homeostatic conditions, suggestive of a role in feline asthma.

CLCA1 has been implicated in chronic inflammatory, mucus-based respiratory conditions, i.e. asthma, CF and COPD, across different species. Beside the horse, the cat is one of the relevant species in veterinary medicine in which asthma occurs naturally and, moreover, shares many characteristics with the human disease. In accordance with previous findings in other species (see 1.6.1 and 1.6.2), CLCA1 may also be differentially expressed in feline asthma. Therefore, this study thirdly aimed at elucidating first evidence of differential expressional regulation of CLCA1 in feline asthma by immunohistochemistry (IHC) of respiratory tract tissues with histological patterns suggestive of asthma, perhaps pointing toward a role of CLCA1 in the feline condition.

2 Research Publications in Journals with Peer-Review

2.1 Interspecies Diversity of Chloride Channel Regulators, Calcium-Activated 3 Genes

Authors: *Mundhenk L, *Erickson NA, Klymiuk N, Gruber AD

Year: 2018

Journal: PLoS ONE, doi: 10.1371/journal.pone.0191512

*These authors shared first authorship.

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Bibliographic Source: *Mundhenk L, Erickson NA, Klymiuk N, Gruber AD. Interspecies diversity of chloride channel regulators, calcium-activated 3 genes. PLoS ONE 13(1): e0191512.*

Declaration of own portion of work in this research publication:
Contributions by Nancy Ann Erickson: dissection of cats, preparation and processing of tissue samples, RNA isolation, RT-qPCR gene expression, *in silico* and statistical analyses, interpretation, immunoblot analyses, and graphical depiction. Subsequent preparation of the manuscript. For *in silico* analyses of CLCA3, also see Supplemental Material S3.

This paper was part of the habilitation treatise of Lars Mundhenk.

All co-authors participated considerably to the study design, evaluation of experimental results, and the creation of the manuscript.

Declaration on ethics: The animals had been autopsied in routine pathological diagnostics or were originally used in a separate study for other purposes (State Office of Health and Social Affairs Berlin, approval number A 0274/14). The animal study was conducted in strict accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals (Guillen 2012) and all efforts were made to minimize animal discomfort and suffering.

PLOS ONE

RESEARCH ARTICLE

Interspecies diversity of chloride channel regulators, calcium-activated 3 genes

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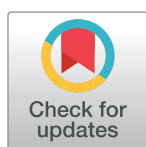
Abstract

Members of the chloride channel regulators, calcium-activated (CLCA) family, have been implicated in diverse biomedical conditions, including chronic inflammatory airway diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis, the activation of macrophages, and the growth and metastatic spread of tumor cells. Several observations, however, could not be repeated across species boundaries and increasing evidence suggests that select *CLCA* genes are particularly prone to dynamic species-specific evolvements. Here, we systematically characterized structural and expressional differences of the *CLCA3* gene across mammalian species, revealing a spectrum of gene duplications, e.g., in mice and cows, and of gene silencing via diverse chromosomal modifications in pigs and many primates, including humans. In contrast, expression of a canonical CLCA3 protein from a single functional gene seems to be evolutionarily retained in carnivores, rabbits, guinea pigs, and horses. As an accepted asthma model, we chose the cat to establish the tissue and cellular expression pattern of the CLCA3 protein which was primarily found in mucin-producing cells of the respiratory tract and in stratified epithelia of the esophagus. Our results suggest that, among developmental differences in other *CLCA* genes, the *CLCA3* gene possesses a particularly high dynamic evolutionary diversity with pivotal consequences for humans and other primates that seem to lack a CLCA3 protein. Our data also help to explain previous contradictory results on CLCA3 obtained from different species and warrant caution in extrapolating data from animal models in conditions where CLCA3 may be involved.

Introduction

Members of the family of chloride channel regulators, calcium-activated (CLCA) have been implicated in diverse physiological and pathophysiological cellular functions, including the modulation of transepithelial anion conductance [1], activation of macrophages [2, 3], cell-cell adhesion [4], and cell differentiation [5], including mucus metaplasia [6]. Strong expressional regulations of CLCA proteins have been reported in distinct disease conditions, including

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chronic airway disease such as asthma and cystic fibrosis (CF) [7] as well as tumor progression and metastasis [4, 5]. Moreover, select CLCA molecules have been suggested as diagnostic markers [5, 8] or even targets in novel therapeutic approaches, particularly in chronic respiratory diseases with mucus overproduction, such as chronic obstructive pulmonary disease (COPD) asthma, and CF [7]. The previous historically-based and partially confusing nomenclature of *CLCA* genes and proteins was recently harmonized to eventually allow for systematic comparisons of orthologs between humans and mice [9]. For example, the originally termed gob-5 mouse protein, later termed mCLCA3, is now designated CLCA1 in the mouse due to its direct orthology to the human CLCA1. Similarly, the previous murine mCLCA1, mCLCA2, and mCLCA4 are now referred to as CLCA3a1, CLCA3a2, and CLCA3b, respectively, in accordance to their orthology to CLCA3 in man. Still, the nomenclature currently used in the databases is complex, in part inconsistent and mostly historically based, especially for duplicated *CLCA3* genes in different species (S1 Table).

One of the most intriguing properties of the CLCA family appears to be its genetic diversity between different mammalian species. First, different numbers of *CLCA* genes have seemingly evolved, for example, with only four *CLCA* genes in man but eight in mice [7]. Second, pseudogene formation and silencing of expression has occurred in some species for members that are fully expressed in other species [10, 11]. Third, contradictory data on alleged functions and regulatory pathways have been found between the human and mouse CLCA1 proteins [12]. Fourth, different cellular expression patterns have been established for the CLCA2 protein between humans, mice, and the pig [13–16] as well as for the CLCA4 protein between humans and mice [17]. We hypothesize that at least some of these differences may result from species-specific evolution of separate *CLCA* genes which would imply restrictions on the translatability of data between different species. In particular, the value of animal models in studying the role of CLCA molecules in disease would be limited and depend on a comprehensive understanding of all relevant differences.

For example, we have recently identified a duplication of the *CLCA4* gene that appears to be unique to the pig [11] as a model for CF research. In contrast to the human CLCA4 protein which is designated CLCA4a in the pig and expressed at the apical membrane of enterocytes at the villus tips and respiratory epithelial cells [17], the porcine-specific duplication product CLCA4b is selectively expressed in intestinal crypt epithelial cells and has thus adopted a novel cellular expression pattern unlike any other known CLCA protein in other species [18].

Among the four *CLCA* gene clusters in mammals [7, 11], the most striking interspecies differences are evident in the *CLCA3* cluster. In humans and pigs, this cluster contains only a single gene, *CLCA3*, which is thought to be a pseudogene with no functional protein being expressed [10, 11]. In contrast, the murine *Clca3* cluster comprises three closely related genes that appear to be the product of two subsequent duplication events. All three mouse genes, *Clca3a1*, *Clca3a2*, and *Clca3b* are thought to express fully functional proteins [7]. For the CLCA3a1 protein, a strong expression was reported in bronchial and tracheal epithelia as well as in submucosal airway glands and in other tissues with secretory functions, including the mammary gland, intestinal tract, gall bladder, pancreas, and kidney [19]. In addition, germinal centers of lymphatic tissues, spermatids, and keratinocytes of the skin, esophagus, and cornea were found to express the *Clca3a1* transcript [20]. Moreover, the CLCA3a1 protein was also detected in endothelial cells of pleural and subpleural blood vessels and in lymphatic endothelium [21, 22]. Of note, CLCA3a1 and CLCA3a2 share 96% nucleic acid sequence identity on the cDNA level and 92% on the amino acid level [19, 23], raising the question of whether some of the expression data may have resulted from unintended cross-reactivity of the probes used. Not surprisingly, available antibodies failed to discriminate between these two homologs [24]. Only a real–time RT-PCR approach using taqman-probes reliably discriminated between

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CLCA3a1 which is predominantly expressed in spleen and bone marrow and CLCA3a2 which was found in the lactating and involuting mammary gland [25]. Intestine and trachea expressed similar levels of both homologues [25]. Transcripts of the third murine CLCA3 homolog, CLCA3b, formerly known as mCLCA4, were detected in smooth muscle cells of major blood vessels in the heart, gastrointestinal tract, bronchioles, and in aortic and pulmonary endothelial cells [26]. It therefore seems as if the two gene duplications in the mouse have resulted in three separate murine CLCA3 homologs which are expressed in quite distinct cell types, including epithelial, endothelial, and smooth muscle cells, possibly occupying distinct functional niches that are almost entirely unknown [27]. Clearly, this scenario would widely limit the value of mouse models for studies on CLCA3.

Further unique peculiarities have been identified for the bovine *CLCA3* gene products. CLCA3, originally termed lung-endothelial cell adhesion molecule-1, Lu-ECAM-1, was the first CLCA family member to be discovered on the molecular level [28]. It was cloned in 1997 from bovine aortic endothelial cells and found strongly expressed in endothelial cells of the lung. Functional investigations had identified a role in mediating adhesion of metastatic melanoma cells to lung endothelium [29, 30]. In contrast, its closely related bovine homolog CLCAx, originally termed calcium activated chloride channel, CaCC, with 88% amino acid identity to CLCA3, was isolated from tracheal respiratory epithelium with no expression in the lung [31]. Furthermore, CLCAx had been cloned as putative anion channel with no known link to cell-cell adhesion. Thus, all data available on the two bovine CLCA3 homologs show major discrepancies to their relatives in humans, mice, and pigs, suggesting another unique picture in *bos taurus*.

The origin and evolutionary background of this striking diversity among mammals in the *CLCA3* gene cluster have not yet been addressed and data on *CLCA3* variations in other mammals are lacking. Here, we aimed at a systematic genomic comparison of the *CLCA3* gene cluster among mammals. We hypothesized that a more comprehensive understanding of the evolutionary divergences may help to tackle the widely contradictory data available to date and aid in the interpretation of animal models for relevant human diseases. As we identified the cat as one of the few mammalian species with only a single genuine and apparently fully expressed and functional *CLCA3* gene, the tissue and cellular expression patterns as well as the predicted protein structure of the feline CLCA3 were established in detail and compared to the scenarios in other mammals, including man.

Materials and methods

In silico analyses of mammalian *CLCA* genes and proteins

A total of 85 CLCA protein sequences was extracted from the GenBank (www.ncbi.nlm.nih.gov) or ensembl (www.ensembl.org) databases or translated *in silico* from pseudogene sequences. The phylogenetic analyses were performed according to Plog et al. [11] by calculating genetic distance and most parsimony trees upon an alignment which comprise the amino acid sequences of all CLCA variants. Cryptic splice acceptor and donor sites within intron 8 were identified using the human splicing finder (http://www.umd.be/HSF3/) and confirmed with the Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html). The structures of the feline CLCA genes were confirmed manually by comparing the entire locus between feline outer dense fiber of sperm tails 2-like, *ODF2L*, and SH3-domain GRB2-like endophilin B1, *SH3GBL1* genes to the genomic organization in the orthologous human locus and to the respective human cDNA sequences. The feline *CLCA* genes were classified as presumably intact if they revealed an open reading frame as well as intact splice sites.

In silico amino acid sequence analyses and generation of antibodies

The amino acid sequences of the feline CLCA3 were analyzed *in silico* using the SignalP 4.1 [32], Kyte–Doolittle [33], SOSUI [34], HMMtop [35], DAS [36], PSORT II [37] and NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc) software. Putative proteolytic cleavage sites were identified by interspecies comparison of the amino acid sequences. The location of the protein domains was depicted from the Genbank database (www.ncbi.nlm.nih.gov). For the generation of antibodies against feline CLCA3, two oligopeptides were synthesized based on immunogenicity prediction, named fe3-1, corresponding to amino acids (aa) 224 to 238 (AFIPEKS QTAKDSI) and fe3-2, corresponding to aa 531 to 545 (LLQDPKGTRYETSD). The only immunogenic epitopes which were not found in other CLCA homologs were located in the predicted amino-terminal cleavage product of CLCA3. Oligopeptides were coupled to keyhole limpet hemocyanin (KLH) and used for standard immunization of two rabbits each as described earlier [11]. The immune sera were designated feCLCA3-1a, feCLCA3-1b, feCLCA3-2a, and feCLCA3-2b. The antiserum feCLCA3-2b was affinity immunopurified as described [11]. The resulting immunopurified polyclonal antibodies were named feCLCA3-2b-ap.

Animals and tissue processing

The following tissues (n = 3 to 5, except for the respiratory bifurcation n = 2) from a total of 11 adult European shorthair cats, *Felis catus*, were shock-frozen on dry ice or immersion-fixed in 4% neutral-buffered formalin: nasal cavity, trachea (dissected into cranial, medial, and caudal parts), respiratory bifurcation, main tracheal bronchus, lung parenchyma, esophagus, stomach, small intestine, caecum, large intestine, skin, mammary gland, heart, femoral skeletal muscle, cerebellum, cerebrum, liver, gall bladder, pancreas, spleen, bone marrow, cornea, ovaries, uterus, heart base aorta, dorsal root ganglion, mandibular glands, parotid salivary gland, mandibular lymph node, thymus, kidney, adrenal gland, urinary bladder, prostate, and vesicular gland. The animals had been autopsied in routine pathological diagnostics or were originally used in a separate study for other purposes (State Office of Health and Social Affairs Berlin, approval number A0274/14). All tissues included here had no histopathological evidence of disease.

All animal studies were conducted in strict accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals, and all efforts were made to minimize animal discomfort and suffering. The cats were anesthetized and euthanized by an intravenous overdose of pentobarbital (Narcoren®).

Tissue expression pattern of feline CLCA3 on mRNA level

Total RNA from tissues listed above was isolated using the NucleoSpin® RNA isolation Kit (Macherey-Nagel, Düren, Germany) according to manufacturer´s instructions. RNA was reversely transcribed as described [3]. Specific primers and probes were designed for feline *CLCA3* and the reference gene *ß-2 microglobulin* (*ß2M*) using Primer3 software (primertool, Whitehead Institute of Biomedical Research; Table 1), encompassing an intron to exclude amplification of contaminating genomic DNA. Quantitative RT-PCR was performed as described [3]. Tissues were defined to express CLCA3 when at least two samples out of 3 to 5 showed cycle threshold (Ct)-values substantially lower than 40 (n = 3 to 5, respiratory bifurcation n = 2).

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Table 1. Primers, probes, and amplicon sizes of feline *CLCA3* and the reference gene.

Gene	GenBank accession no.	Oligonucleotide	Nucleotide Sequence (5'-3')	Amplicon size, bp
CLCA3	XM_003990321	upstream primer	TCCCAGACTGCAAAGGACTC	179 bp
		downstream primer	TGTCATGGGAGGTGCATTCT	
		TaqMan Probe	GCAGAAGCACATGGGATGTA	
ß2M	NM_001009876	Upstream primer	CGTTTTGTGGTCTTGGTCCT	115 bp
		downstream primer	TTGGCTTTCCATTCTCTGCT	
		TaqMan Probe	TCAGGTTTACTCCCGTCACC	

https://doi.org/10.1371/journal.pone.0191512.t001

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues of trachea and esophagus (n = 3 to 5) were cut at 3 μm thickness, mounted on adhesive glass slides, and dewaxed in xylene, followed by rehydration in decreasing ethanol concentrations and blocking of endogenous peroxidase. Each anti-CLCA3 antibody was tested using the following procedural variations: (a) without antigen retrieval, (b) 12 min microwave heating (600 W) in 200 ml 10 mM citric acid, pH 6.0, or (c) 10 min protease treatment at 37˚C. Respective pre-immune sera served as controls. Only the feCLCA3-2b antibody showed a specific staining pattern using procedure (a). To further test for specificity, the feCLCA3-2b antibody was pre-absorbed for approximately 2 h with its peptide (50 μg/ml) used for immunization or with an irrelevant peptide. Slides were blocked using Roti-Immunoblock in goat normal serum at a dilution of 1:5 for 30 min at room temperature, followed by incubation with feCLCA3-2b diluted at 1:2,000, with the pre-absorbed antibody or with the pre-immune serum overnight at 4˚C. After washing, the slides were incubated with secondary goat anti-rabbit antibodies diluted at 1:200 for 30 min at room temperature. ABC solution (Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, CA) was added for 30 min at room temperature. Diaminobenzidine (DAB) was used for color development. Sections were either counterstained with hematoxylin or treated with the periodic acid-Schiff (PAS) reaction.

Immunoblot analyses

Tissue samples including cellular and extracellular proteins of trachea, esophagus, and stomach were homogenized in standard lysis buffer, boiled, and immunoblotted as described [11]. Membranes were probed with anti-feCLCA3-2b-ap diluted at 1:1,000, an irrelevant immunopurified antibody diluted 1:1,000 or anti-beta-actin antibody (Sigma-Aldrich, Munich, Germany) diluted to 1:1,000 as loading control in blocking buffer. Membranes were incubated with secondary horseradish peroxidase-conjugated anti-rabbit diluted 1: 2,000 or anti-mouse IgG diluted 1:10,000, respectively, for 1 hour and protein labelling was visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Rockford, IL).

Results

High evolutionary dynamics in the mammalian *CLCA*3 gene cluster

The *CLCA* gene family can be separated into four distinct clusters in mammals, with designations recently renamed [9] according to the four human representatives, *CLCA1 CLCA2* , ,

CLCA3, and *CLCA4* (Fig 1). In contrast to mammals, only two *CLCA* genes were identified in chickens (Fig 1) which are organized identically to mammals in a single locus flanked by the genes *ODF2L* and *SH3GBL1* (Fig 2). A single copy of the *CLCA2* gene was consistently found throughout all mammals investigated as well as in chickens, pointing towards its evolutionary conservation (Fig 2). In contrast, the clustering of chicken *CLCA1* with all other mammalian *CLCA* genes suggests that their common ancestor has apparently undergone independent duplications in early mammalian evolution, resulting in the three mammalian genes now termed *CLCA1*, *CLCA3* and *CLCA4* (Fig 2). Similar to *CLCA2*, the *CLCA1* gene also appears to be present as one single intact copy in all mammals investigated here (Fig 2).

In contrast, marked differences were observed for *CLCA3* and *CLCA4* between distinct mammalian species. Gene duplications had obviously resulted in two or three distinct *CLCA3* gene variants in cows and mice (Fig 2), respectively, and further distinct *CLCA4* gene variants in pigs and mice (Fig 2), supporting previous observations by others [7, 11]. In addition, several kinds of gene inactivation were found in the *CLCA3* cluster in different species (Fig 2). Frame shift mutations and/or one or several premature stop codons are present in the *CLCA3* gene of certain primates, pigs, and one ovine duplicate (Fig 3). *CLCA3* of all dry-nosed primates investigated here, including chimpanzee, human, gorilla, orangutan, macaque, baboon, marmoset, and tarsier, possess an additional exon resulting from an active cryptic splice site within intron 8. This cryptic exon would not change the frame of the *CLCA3* gene but it contains one or two premature stop codons (Fig 3). The *CLCA3* gene of hominids has several additional premature stop codons and frame-shift mutations with rather unique patterns in each species (Fig 3). Among the primate species investigated, the bushbaby, belonging to the suborder of wet-nosed primates, appears to be the only one with an intact and likely functional *CLCA3* gene (Fig 3), as it does not contain an intact splice acceptor site of the cryptic exon. The porcine *CLCA3* also seems to be inactivated by several premature stop codons and frame-shift mutations but, in contrast to primates, the inactivation does not result from the cryptic exon, as there is no evidence of intact splice sites at this exon (Fig 3). As an additional variance, a unique early frame shift mutation was identified in one ovine duplicate (Fig 3). Of note, none of the species with premature stop codons or frame-shift mutations appears to possess a *CLCA3* gene structure that would suggest the expression of a functional protein. Thus, in contrast to carnivores, guinea pigs, rabbits, and horses which all possess a single and seemingly functional *CLCA3* gene (Fig 3), distinct and rather species- specific mechanisms of silencing of the *CLCA3* gene have evolved in most primates, including man, and in pigs and sheep.

Expression of feline CLCA3 in ciliated epithelial and submucosal cells of airways and keratinocytes of esophagus

We chose the cat to characterize the expression of CLCA3 due to the potential medical relevance of cats as a model for asthma, a condition to which several CLCA members have been linked to in addition to other chronic respiratory diseases including COPD and CF [7]. Feline asthma is a naturally occurring and common condition and can also be induced experimentally, making the cat an accepted animal model that expands the spectrum of models for chronic respiratory disorders [38].

Feline CLCA3 mRNA was detected in the upper respiratory tract from the nasal cavity down to the bronchial bifurcation (Fig 4A) with the following Ct ranges: nasal cavity: 28.48 to 33.21, trachea–cranial part: 28.11 to 31.98,—medial part: 28.52 to 30.89,—caudal part: 29.26 to 35.94, bifurcation: 32.57 to 33.07 but not in main bronchi or lung parenchyma. It was also found to be highly expressed in the esophagus with a Ct range between 26.30 and 31.13. No

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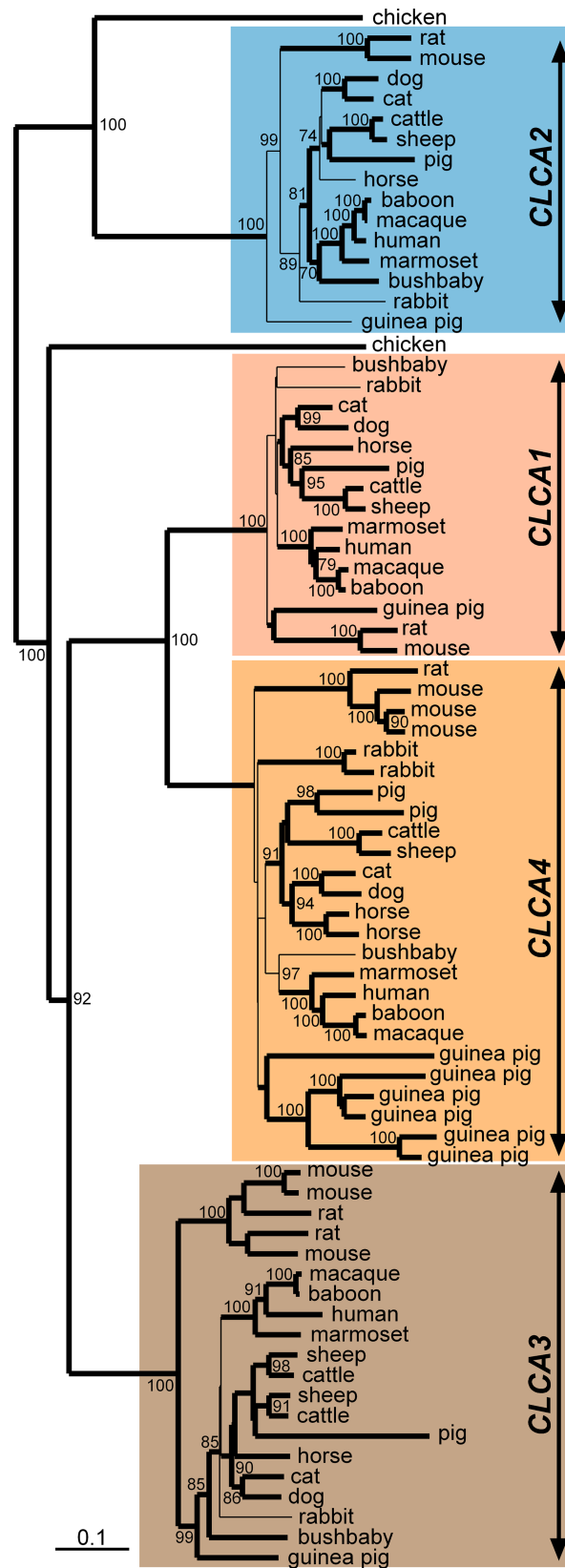
Fig 1. Phylogenetic relationship of mammalian *CLCA* genes. *In silico* analysis revealed four distinct *CLCA* clusters in mammals, named after their human representatives *CLCA1*, *CLCA2*, *CLCA3*, and *CLCA4*. In contrast to mammals, only two *CLCA* genes were identified in chickens. A genetic distance tree was calculated upon the alignment of 77 CLCA protein sequences. Branch nodes that appeared more than 70 times in 100 bootstrapped genetic distance trees are indicated by their bootstrap value and branches that were consistently abundant also in a most-parsimonious tree are shown in bold lines.

https://doi.org/10.1371/journal.pone.0191512.g001

expression was detected in other tissues, including those in which CLCA3 variants had been reported in other species, such as the mammary gland, spleen, lung, and aorta [10, 25, 26, 28].

To identify the expressing cell types in tissues where CLCA3 mRNA was found, these tissues were analyzed by immunohistochemistry using specifically generated anti-feline CLCA3 antibodies. In the respiratory tract, the CLCA3 protein was localized to cells of submucosal glands throughout the airways and in ciliated epithelial cells (Fig 4B, 4E and 4H). Here, the signals were most prominent adjacent to the nucleus, sometimes with a tendency to a more diffuse cytoplasmic staining. In the esophagus, CLCA3 protein was identified in keratinocytes of stratified epithelia, predominantly located in the stratum spinosum (Fig 4G) where the signals appeared to be membrane-associated. In deeper layers, a more punctate, vesicular expression
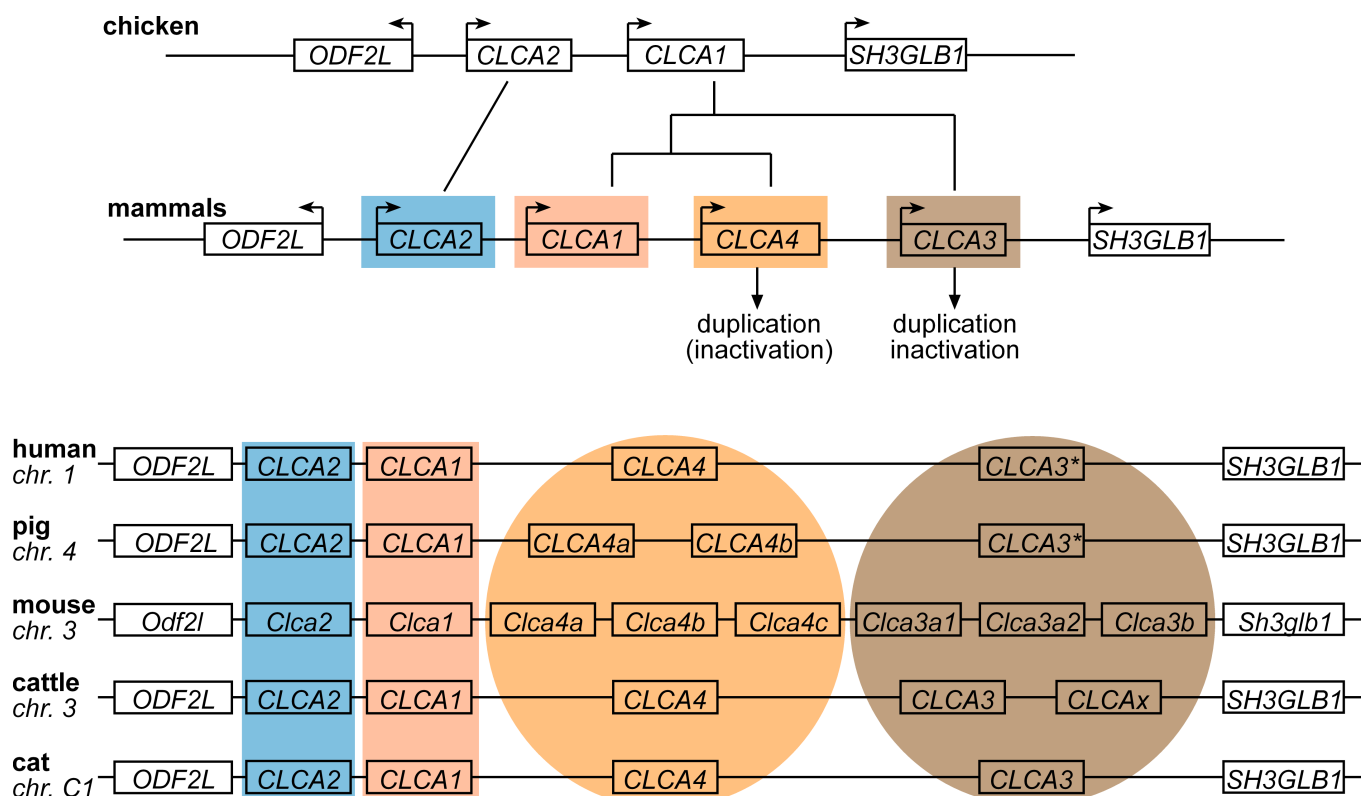
Fig 2. Diverse evolutionary dynamics of *CLCA* genes. In chicken and mammals, all *CLCA* genes are conserved in a single locus, flanked by the *ODF2L* and *SH3GBL1* genes (upper panel). In chickens, only two *CLCA* genes, *CLCA1* and *CLCA2*, are found within this locus with no other *CLCA*-related genes throughout the genome. According to the phylogenetic pattern, *CLCA2* seems highly conserved, with a single intact representative in each mammal examined. In contrast, the ancestor of another vertebrate *CLCA* gene has apparently undergone two independent duplications in early mammalian evolution, resulting in *CLCA1*, *CLCA3*, and *CLCA4*, but it remained as a single copy in chicken. While *CLCA1* is present as a single intact copy in all mammalian species examined and chicken, *CLCA3* and *CLCA4* underwent further duplication or inactivation (*) events in select mammalian branches (lower panel). As the nomenclature of *CLCA* genes is still inconsistent, in particular for duplicated genes, we followed the current designations used in the GenBank database with the exception of bovine unidentified gene *LOC784768* which we here termed *CLCAx* for the ease of understanding (S1 Table).

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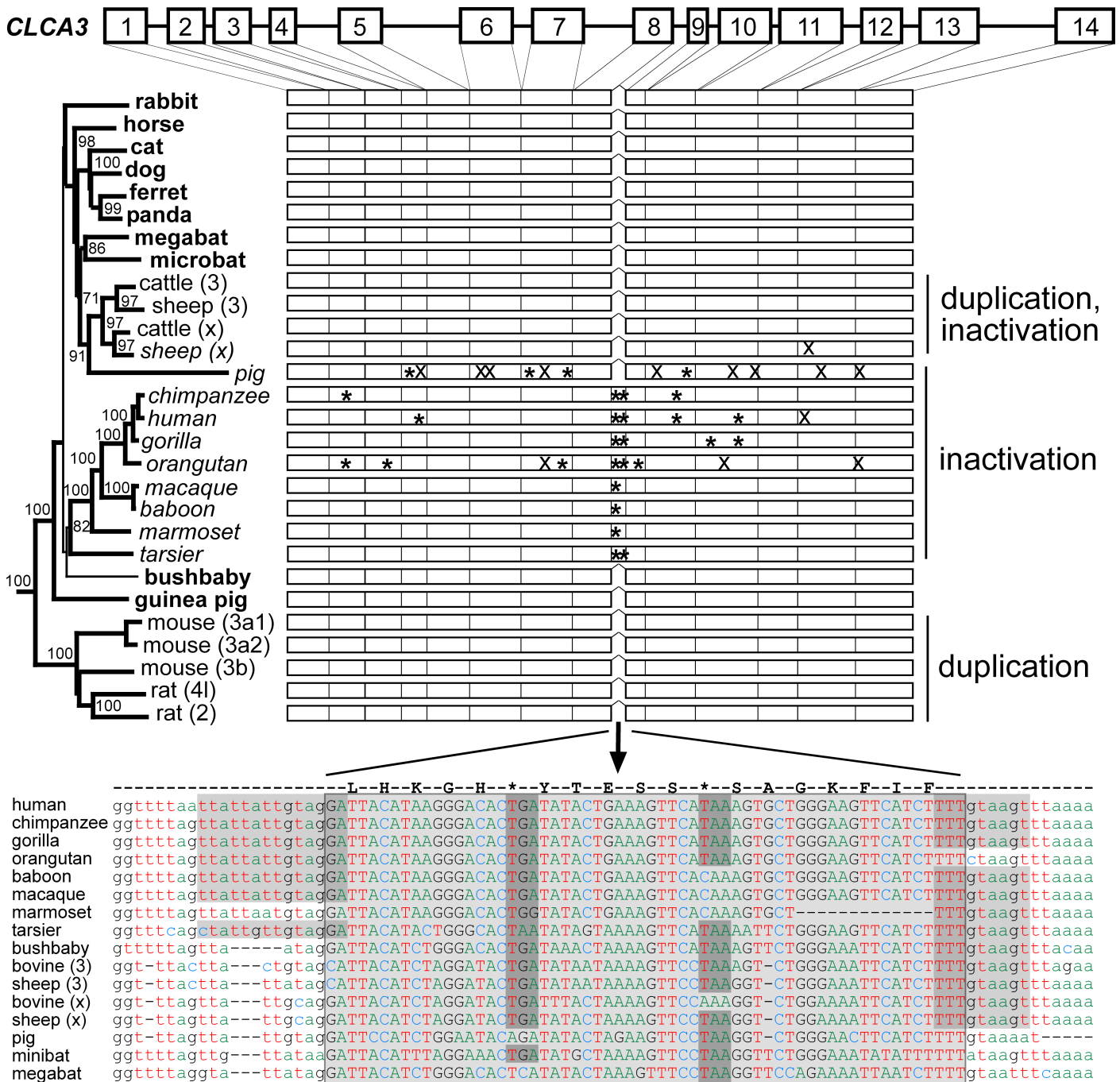
Fig 3. Mechanism of *CLCA3* inactivation in select mammalian species. Duplications of *CLCA3* were found in rodents and ungulates, mutational inactivations in ungulates and in primates (italics), and a single, apparently functional *CLCA3* copy in other mammalian species (bold). The *CLCA3* genes in dry-nosed primates possess an additional exon resulting from cryptic splice sites within intron 8. This genomic segment is also found in other species, including pigs. Each of these species carries at least one stop codon within the predicted exon (shaded within the boxed exon) but cryptic splice acceptor and donor functions are only predicted for dry-nosed primates (gray background). The distinct *CLCA3* gene variants in certain primates displayed further inactivating mutations that were apparently unique for each species. Similar mutational events were found in the porcine *CLCA3* gene and one ovine variant. The nomenclature of the *CLCA3* variants is used according to the GenBank database, except for the bovine unidentified gene *LOC784768* which we termed cattle (x) as well as the ovine unidentified genes *LOC101116002* and *LOC101116267* which we termed sheep (3) and sheep (x), respectively (S1 Table). Box = exons No. 1 to 14 of the *CLCA3* gene, black lines = introns, cross = frame-shift mutations, asterisk = nonsense mutation.

https://doi.org/10.1371/journal.pone.0191512.g003

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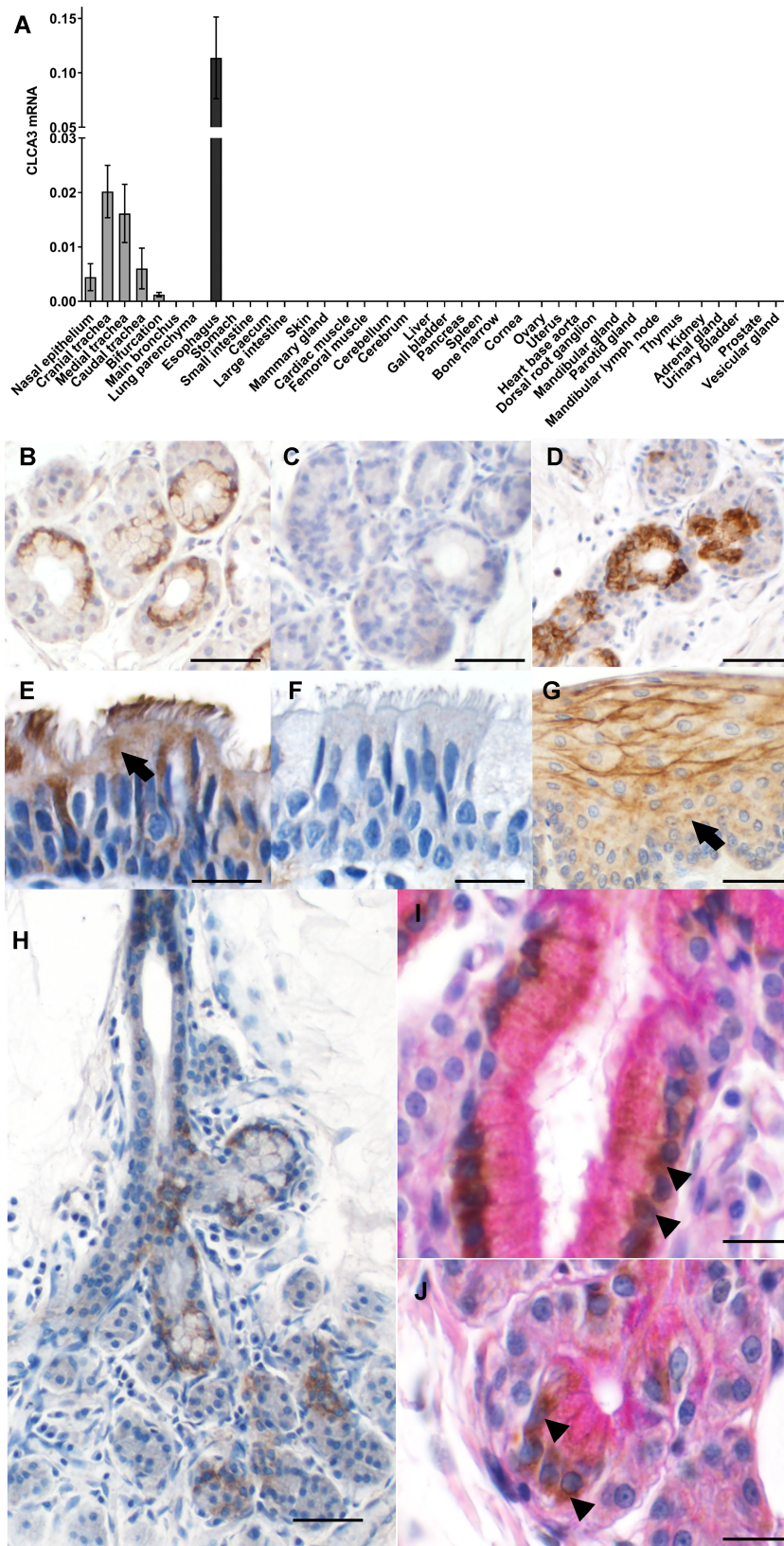
Fig 4. Expression of feline CLCA3 in respiratory epithelial cells and esophageal stratified epithelia. (A) CLCA3 mRNA was detected in the upper respiratory tract. Very high expression levels were found in the esophagus. Data are expressed as mean ± SEM and expression levels are given in ratios of CLCA mRNA copy numbers relative to the copy number of the reference gene ß2M and calculated as $2^{Ctß2m-CtCLCA3} = 2^{\Delta Ct}$ (n = 3 to 5, respiratory bifurcation n = 2) (B) Immunohistochemically, the CLCA3 protein was localized to submucosal glands of the respiratory tract. (C) Slides incubated with the pre-absorbed antibodies failed to show the specific staining, whereas (D) preincubation with an irrelevant peptide failed to abolish the specific signal. (E) Single ciliated epithelial cells lining the airways were also positive for CLCA3. The protein was localized in a perinuclear pattern, a vesicular pattern (arrow), or associated with cilia. (F) Sections incubated with the pre-immune serum at the same dilution failed to yield any staining. (G) CLCA3 was also detected in the stratum spinosum of the esophagus where it seemed to be associated with the plasma membrane. A punctate cytoplasmic pattern was observed in deeper cell layers (arrow). (H) The CLCA3 protein was also detected in select cells of submucosal airway glands. PAS reaction counterstain revealed colocalization of CLCA3 (brown color, arrowheads) with (I) mucin-producing tubular and (J) acinar cells (pink color, PAS-positive). Color was developed using DAB as substrate (brown) with hematoxylin (blue, B-H) or PAS (pink, I, J) as counterstain. Bars: B, C, D = 50 μm; E, F = 30 μm; G = 40 μm; H = 60 μm; I, J = 15 μm.

https://doi.org/10.1371/journal.pone.0191512.g004

pattern was identified. A colocalization using the PAS reaction clearly identified CLCA3-positive cells in the respiratory mucosa as mucin-producing cells of the acini and the duct system of submucosal glands (Fig 4I and 4J). In addition the prominent perinuclear staining, a more punctate intracellular pattern was also observed.

To characterize structural elements of the feline CLCA3 protein, its amino acid sequence was analyzed *in silico*. It starts with a cleavable signal peptide sequence, suggestive of its early translocation into the endoplasmic reticulum (Fig 5A). CLCA3 is predicted to be a fully secreted and soluble protein by the transmembrane prediction softwares used. It contains a hydrophobic region between amino acids 893 and 900 which seems, however, to be too short to represent a transmembrane α-helix that usually contains about 20 amino acids. As reported in the pig and mouse [11, 17, 39], sites of predicted N-linked glycosylation were also found in feline CLCA3 at aa positions 39, 75, 364, 454, 505, 516, 633, 844, and 859 (Fig 5A). Also similar to several previously described CLCA proteins in other species [7, 40], a cysteine-rich domain was found downstream to the signal sequence, designated as n-CLCA (Fig 5A). Adjacent to the n-CLCA domain, a von Willebrand factor type A (vWA) domain was identified (Fig 5A) which is also present in several other CLCA proteins [7]. A unique feature of all known CLCA proteins is their posttranslational cleavage of a 120-kDa precursor protein into a larger, approximately 80-kDa amino terminal and a smaller, approximately 40-kDa carboxy terminal subunit [7]. The exact cleavage site has been experimentally identified only in the murine CLCA1 and the bovine CLCA3 (alias Lu-ECAM-1) [28, 41]. Here, our interspecies protein sequence comparisons revealed a similar site of predicted protein cleavage in the feline CLCA3 protein between amino acids 702 and 703 (Fig 5A). Thus, the feline CLCA3 protein contains several characteristic features that are highly conserved within the CLCA family and thought to be of functional relevance which is still, however, largely elusive [7]. Moreover, it seems to be a soluble protein, possibly secreted similarly to CLCA1 [42] which stands in contrast to CLCA2 and CLCA4 which are thought to be membrane-bound [13, 27].

To characterize the size of the feline CLCA3 protein in tissues, immunoblot analyses were performed on extracts from relevant organs using the immunopurified antibody feCLCA3-2b-ap. An approximately 90 kDa CLCA3 protein was detected in the feline trachea (Fig 5B), consistent with the data obtained by RT-qPCR and immunohistochemistry. The same protein size was found in the esophagus, however, with stronger band intensity (Fig 5B). The size of 90 kDa corresponds well with the predicted size of the amino-glycosylated amino-terminal cleavage product, indicating that this feline variant is posttranslationally cleaved similarly to most other CLCA members investigated so far [7].

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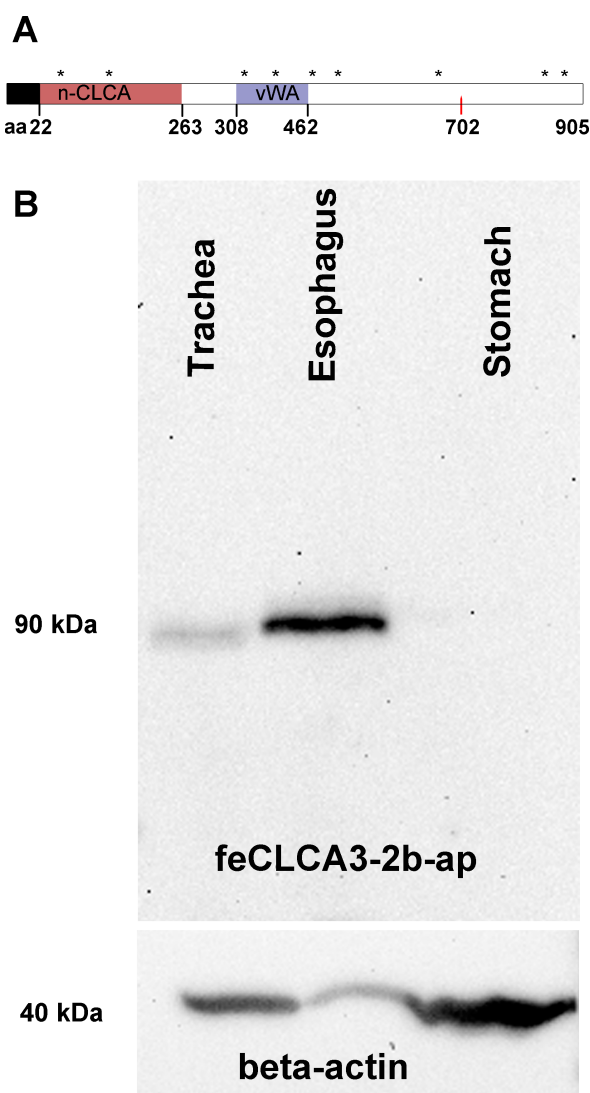
Fig 5. Predicted protein structure of feline CLCA3 and detection of its amino-terminal cleavage product in the respiratory tract and esophagus. (A) The amino acid sequence of feline CLCA3 contains a cleavable signal sequence (black box), an amino-terminal CLCA domain (n-CLCA) followed by a von Willebrand factor type A domain (vWA). No potential transmembrane domain was predicted. Several sites for N-linked glycosylation were predicted (asterisks). The arrow denotes the putative proteolytic cleavage site at 702 aa. aa, amino acids. (B) A 90-kDa protein was detected with the anti-feline CLCA3 antibodies by immunoblotting in the trachea. Much stronger CLCA3 expression was found in the esophagus. The protein was undetectable in the stomach, here shown as tissue without CLCA3 protein expression, as expected from mRNA results. The antibody also detected an approximately 40 kDa band in the tissue lysate of trachea and esophagus, however, these bands were also detected by the irrelevant antibody (S1 Fig). An antibody to beta-actin was used as a loading control (lower panel).

https://doi.org/10.1371/journal.pone.0191512.g005

Discussion

We identified a pronounced interspecies diversity of CLCA3 among mammals: some species, including man and pig, appear to live without a CLCA3 protein, whereas others, including the cat, seem to express a single apparently functional protein with several canonical elements of other CLCA proteins. Furthermore, the *CLCA3* gene is duplicated in other mammals, including mice and cows.

The *CLCA3* Cluster possesses the highest evolutionary dynamics

The *CLCA3* cluster seems to possess the highest evolutionary dynamics compared to clusters 1, 2, and 4. This is reflected by 1) the obviously functional expression of a single CLCA3 protein in species such as carnivores, the horse, rabbit, guinea pig, and bush baby, 2) the duplication of the *CLCA3* gene in ruminants and rodents resulting in two or even three apparently functional homologs, and 3) the inactivation of this gene in several species including most primates, pigs, and sheep (Fig 6). In contrast, the *CLCA* cluster 2 seemed to be highly conserved as only a single copy of this gene is present in each of the mammalian species investigated and even in chickens without any evidence of gene duplication or inactivation. Consistently, only a single, apparently functional *CLCA1* gene exists in mammals. Similar to cluster 3, the *CLCA* cluster 4 seems quite diverse with gene duplications in several species, however, mutational genetic inactivation seems to be uncommon.

Life without a CLCA3 protein

The pseudogene formation of *CLCA3* in humans and pigs had already been described by our group [10, 11]. Here, our extended analysis showed that this formation is not limited to
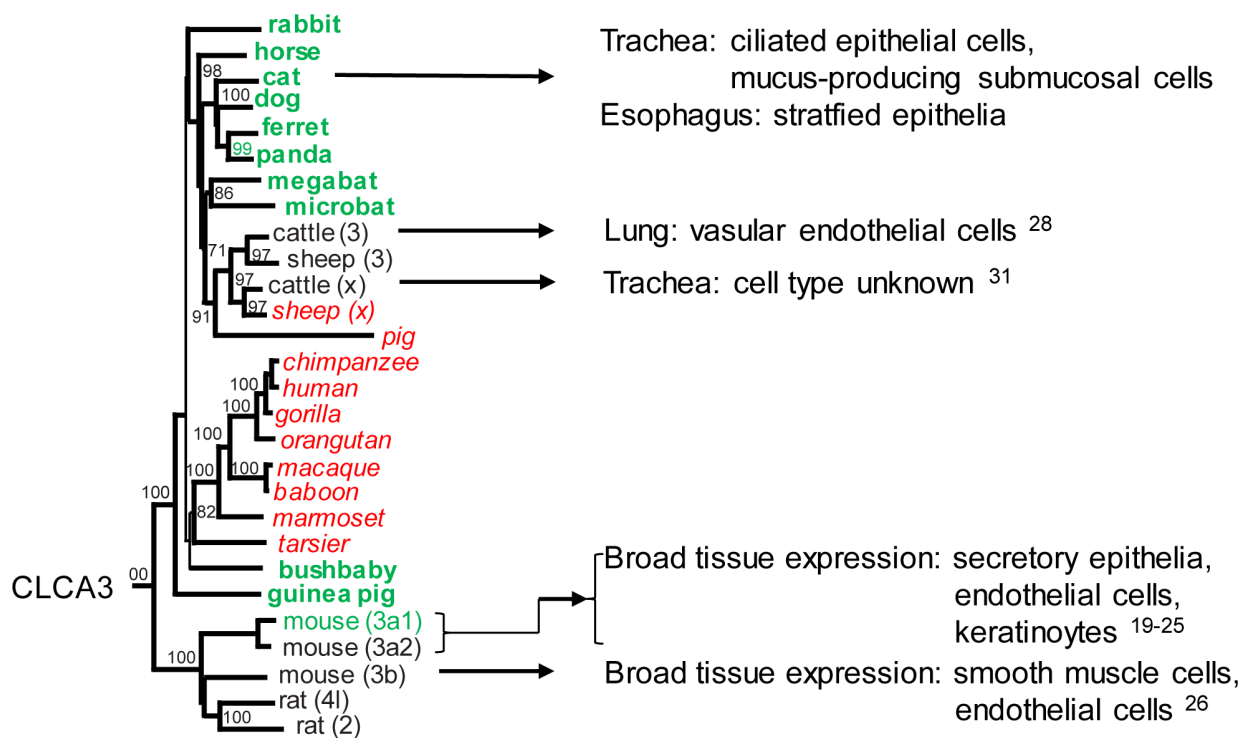
Fig 6. Diversity of CLCA3 protein expression among mammalian species. The CLCA3 protein is expressed as a single version (species colored in green), in duplicated versions (black), or is putatively not expressed at all (red). The duplication events seem to broaden its cellular expression pattern. In the cat, the single CLCA3 protein is found in ciliated epithelial and mucus-producing submucosal cells of the respiratory tract as well as in certain stratified esophageal epithelia. Some orthologs had been detected in the same cell types but further variants had been found in other cell types including endothelial and smooth muscle cells (numbers refer to cited references).

https://doi.org/10.1371/journal.pone.0191512.g006

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humans in the order of primates. Instead, CLCA3 inactivation was found in almost all primates investigated. Additionally, events of mutational inactivation in ungulates seemed not to be limited to pigs but also occur in sheep.

Inactivation of *CLCA3* in primates seems to be initiated by the insertion of a cryptic exon containing premature stop codons due to an active splice acceptor and donor sites in intron 8. In contrast, the gene was inactivated by an accumulation of premature stop codons and/or frame shift mutations in the pig. This mechanistic difference clearly points towards independent pseudogene formations in primates and ungulates. It will be interesting to explore the evolutionary forces that drove the gene inactivation in these species in contrast to other species, making life without a CLCA3 protein possible.

It still remains questionable whether the *CLCA3* gene has no biological function in primates and pigs at all. It is well established that transcripts of certain pseudogenes may play a role in other cellular mechanisms such as the regulation of gene expression, generation of genetic diversity, or have an impact on gene conversion and recombination [43]. Fragments of *CLCA3* cDNA had previously been amplified from different tissues in humans [10] and further studies need to clarify whether *CLCA3* may be associated with functions other than the formation of a functional protein in primates and pigs.

Interestingly, our *in silico* analyses failed to identify mutations leading to an inactivation of *CLCA3* in the bushbaby. In contrast to all other primates investigated here who are members of dry-nosed primates, the bushbaby belongs to the wet-nosed primates. It thus seems plausible to assume that the pseudogene formation of *CLCA3* had occurred in a common ancestor of dry-nosed but not in wet-nosed primates.

Expression of a single putatively functional CLCA3 protein in the cat

In addition to the bushbaby, carnivores, horses, rabbits, and guinea pigs were identified as the species with only a single and apparently functional *CLCA3* gene. As an established model of research on chronic airway inflammation [38], the cat was chosen as a candidate for first detailed characterization of the CLCA3 expression pattern and protein structure.

The feline CLCA3 was identified as a protein with no transmembrane domain and several canonical characteristics of other CLCA proteins including an n-CLCA and vWA domain as well as putative posttranslational cleavage [7]. The expression pattern in respiratory ciliated epithelial cells and mucus-producing submucosal gland cells would be in line with proposed cellular functions of CLCA proteins such as activation of airway macrophages or driving mucus metaplasia [2, 3, 12]. Selected CLCA members have also been linked to a functional role in the maturation and differentiation of epithelial cells [5, 13]. The expression of feline CLCA3 in subsets of keratinocytes of esophageal stratified epithelia might point towards such a function. Although our *in silico* analyses predicted CLCA3 to be a soluble protein, an association with the plasma membrane of esophageal keratinocytes and with cilia of respiratory epithelial cells was observed. It remains to be shown whether CLCA3 may interact with integral membrane proteins of these cells. Further studies are clearly needed to elucidate the functions of the CLCA3 protein in the cat and other species in which this protein is evolutionarily retained.

Species-specific developments and cellular expression patterns of CLCA3

The comparison of the expression pattern of CLCA3 in cats with its orthologs in other species identified some similarities but also marked differences (Fig 6). In humans, no CLCA3 protein expression had been reported, however, cDNA had been detected at low amounts in numerous tissues including the lung, trachea, spleen, thymus, and mammary gland [10]. The murine

orthologs CLCA3a1 and 3a2 had been found in tissues with secretory epithelia, including mammary gland, respiratory, and intestinal tract, and other tissues, such as the spleen [20]. On the cellular level, CLCA3a1 had been detected in murine respiratory epithelia of bronchi and trachea as well as in submucosal epithelial cells [19] identical to feline CLCA3 in this study. Additionally, this murine ortholog had been detected in vascular endothelial cells of the lung and in lymphatic endothelial cells [21, 22]. The third murine CLCA3 ortholog, CLCA3b, had been found to be primarily expressed in smooth muscle cells of the cardiac vessels but also gastrointestinal tract, bronchioles, and in aortic and lung endothelial cells [26]. In the cow, the cellular expression pattern is still incompletely known, although the two bovine CLCA3 variants had been one of the first CLCA members to be identified [28, 31]. The bovine CLCAx, previously termed CaCC, was found to be expressed in the trachea but not in the lung [31], whereas CLCA3, the previous LuECAM-1, was clearly expressed in vascular endothelial cells of the lung [28]. The expression of a CLCA variant in mesenchymal cells such as endothelial or smooth muscle cells is unique since CLCA members of other clusters are generally expressed in epithelial cells [7]. The expression pattern of murine and bovine CLCA3 variants in vascular endothelial cells in the lung had originally led to the assumption of them playing a role in the spread of metastatic tumor cells [30], whereas the members found in epithelial cells had been linked to transepithelial anion conductance [31]. It appears likely that genetic duplications of *CLCA3* have broadened the expression to distinct microenvironments with likely even different functions.

In contrast to *CLCA3*, the *CLCA1* cluster is highly conserved with only a single functional member in each species investigated. It is the best investigated cluster in terms of its cellular expression pattern with localization of CLCA1 to goblet and other mucin-producing cells throughout the body in all investigated species so far, including humans [44], pigs [11], mice [45], and horses [46]. Thus, in contrast to the highly conserved *CLCA1* cluster, CLCA3 has undergone a species-specific development with a distinct cellular expression pattern in epithelial cellular niches and, in select species, even in cells of mesenchymal origin (Fig 6).

Of note, the *CLCA4* cluster also showed a high evolutionary dynamic with several events of gene duplication in certain species. The tissue expression pattern showed similarities but also marked differences between the species [17] with duplicates occupying different cellular niches which had been shown for the porcine CLCA4a and 4b [18]. In contrast to *CLCA3*, pseudogene formation seems not to be a common event in the cluster 4 as the murine *CLCA4c* is described to be the only putative pseudogene in the mouse database. Interestingly, our group had identified a naturally occurring deletion mutant of porcine *CLCA4b* among contemporary and ancient pig breeds in high percentages of animals [18]. This protein silencing did not result in any obvious phenotype. Thus, life without pCLCA4b seems possible and it is tempting to speculate that the *CLCA4* cluster will show similar evolutionary dynamics with gene inactivation in the future as described here for *CLCA3*.

Interspecies diversity–an indicator for possible biological functions?

The tremendous variations of *CLCA3* gene products between species may even add to speculations in terms of their possible functions. For example, a similar interspecies diversity has been established for the bactericidal/permeability-increasing fold-containing (BPIF) protein family, also known as palate, lung and nasal epithelium clone, PLUNC, proteins [47, 48]. Here, 11 genes exist in humans and 14 in the mouse. Both human and mouse gene loci contain lineage-restricted paralogues and pseudogenes [47, 48]. For example, *BPIFA4* had been identified in primates as well as cows and cats, however, it seems to be absent from rodents. Interestingly, in contrast to chimpanzees, gorillas, and rhesus monkeys, a mutational deletion in exon 6 of

the human *BPIFA4* gene results in a frame shift with the introduction of a premature stop codon. The human gene does not encode for a functional protein and it had been described as a 'dying gene' [47, 48]. Also similar to the CLCA family, *BPIF* genes are encoded in a single, conserved locus in all mammals, including man [47]. Again with strong similarities to several CLCA proteins, BPIF proteins are predominantly expressed in the respiratory passage [49] but may also occur in other tissues such as the thymus [50]. The conspicuous parallels between the BPIF family and the CLCA family in terms of their striking interspecies diversities and their expression in a similar microenvironment, in which innate defense is crucial, may give rise to speculations on related or overlapping functions. Specifically, certain BPIF proteins are thought to play a role in innate host defense and liquid homeostasis in the respiratory tract [51]. The high interspecies diversity and rapid evolution of the BPIF family is comparable and even higher than that of other proteins known to be involved in innate immunity [48], further underscoring speculations on its role in innate host defense. Further work will have to address whether a similar scenario may also be relevant for the CLCA family.

Consequences for the interpretation of animal models

Members of the CLCA family, especially CLCA1, are known to modulate inflammatory pulmonary diseases such as asthma, COPD, and CF via an as yet unknown mechanism [7]. Mouse and other animal models are widely used to unravel the biomedical significance of these proteins. However, in contrast to humans, certain members of *CLCA* clusters 2 and 4 in the mouse seem to have a redundant function which compensates for the loss of function of CLCA1 [12] and impedes straight interspecies translatability. The tremendous species-specific differences identified here for the *CLCA3* cluster with either no protein expression, expression of a single CLCA3 protein or the expression of several duplicates will certainly have to be considered when interpreting data obtained from animal models. In particular, our data suggest that species-specific variations of CLCA3 may be relevant for comparing different asthma models in animals, including spontaneously asthmatic cats, horses with chronic obstructive bronchitis, and experimental mouse models [38]. In that regard, it will be interesting to explore the functional consequences of the lack of CLCA3 in humans, in contrast to its presence as a single copy gene in cats and horses and multiple duplications in mice.

Supporting information

S1 Table. Nomenclature of duplicated *CLCA3* genes in different species.
(PDF)

S1 Fig. Original immunoblot of Fig 5.
(TIF)

Acknowledgments

Author Contributions

Conceptualization: Lars Mundhenk, Nikolai Klymiuk, Achim D. Gruber.

Data curation: Nancy A. Erickson, Nikolai Klymiuk.

Formal analysis: Nancy A. Erickson, Nikolai Klymiuk.

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References

1. Sala-Rabanal M, Yurtsever Z, Nichols CG, Brett TJ. Secreted CLCA1 modulates TMEM16A to activate Ca(2+)-dependent chloride currents in human cells. Elife. 2015; 4. https://doi.org/10.7554/eLife.05875 PMID: 25781344; PubMed Central PMCID: PMCPMC4360653.

2. Ching JC, Lobanova L, Loewen ME. Secreted hCLCA1 is a signaling molecule that activates airway macrophages. PLoS One. 2013; 8(12):e83130. https://doi.org/10.1371/journal.pone.0083130 PMID: 24349445; PubMed Central PMCID: PMCPMC3861482.

3. Dietert K, Reppe K, Mundhenk L, Witzenrath M, Gruber AD. mCLCA3 modulates IL-17 and CXCL-1 induction and leukocyte recruitment in murine Staphylococcus aureus pneumonia. PLoS One. 2014; 9 (7):e102606. https://doi.org/10.1371/journal.pone.0102606 PMID: 25033194; PubMed Central PMCID: PMCPMC4102496.

4. Abdel-Ghany M, Cheng HC, Elble RC, Lin H, DiBiasio J, Pauli BU. The interacting binding domains of the beta(4) integrin and calcium-activated chloride channels (CLCAs) in metastasis. J Biol Chem. 2003; 278(49):49406–16. https://doi.org/10.1074/jbc.M309086200 PMID: 14512419.

5. Yu Y, Walia V, Elble RC. Loss of CLCA4 promotes epithelial-to-mesenchymal transition in breast cancer cells. PLoS One. 2013; 8(12):e83943. https://doi.org/10.1371/journal.pone.0083943 PMID: 24386311; PubMed Central PMCID: PMCPMC3873418.

6. Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, et al. Role of gob-5 in mucus over-production and airway hyperresponsiveness in asthma. Proc Natl Acad Sci U S A. 2001; 98(9):5175–80. https://doi.org/10.1073/pnas.081510898 PMID: 11296262; PubMed Central PMCID: PMCPMC33183.

7. Patel AC, Brett TJ, Holtzman MJ. The role of CLCA proteins in inflammatory airway disease. Annu Rev Physiol. 2009; 71:425–49. https://doi.org/10.1146/annurev.physiol.010908.163253 PMID: 18954282; PubMed Central PMCID: PMCPMC4017675.

8. Yang B, Cao L, Liu J, Xu Y, Milne G, Chan W, et al. Low expression of chloride channel accessory 1 predicts a poor prognosis in colorectal cancer. Cancer. 2015; 121(10):1570–80. https://doi.org/10.1002/cncr.29235 PMID: 25603912; PubMed Central PMCID: PMCPMC4654332.

9. Erickson NA, Nystrom EE, Mundhenk L, Arike L, Glauben R, Heimesaat MM, et al. The Goblet Cell Protein Clca1 (Alias mClca3 or Gob-5) Is Not Required for Intestinal Mucus Synthesis, Structure and Barrier Function in Naive or DSS-Challenged Mice. PLoS One. 2015; 10(7):e0131991. https://doi.org/10.1371/journal.pone.0131991 PMID: 26162072; PubMed Central PMCID: PMCPMC4498832.

10. Gruber AD, Pauli BU. Molecular cloning and biochemical characterization of a truncated, secreted member of the human family of Ca2+-activated Cl- channels. Biochim Biophys Acta. 1999; 1444 (3):418–23. PMID: 10095065.

11. Plog S, Mundhenk L, Klymiuk N, Gruber AD. Genomic, tissue expression, and protein characterization of pCLCA1, a putative modulator of cystic fibrosis in the pig. J Histochem Cytochem. 2009; 57 (12):1169–81. https://doi.org/10.1369/jhc.2009.954594 PMID: 19755716; PubMed Central PMCID: PMCPMC2778090.

12. Alevy YG, Patel AC, Romero AG, Patel DA, Tucker J, Roswit WT, et al. IL-13-induced airway mucus production is attenuated by MAPK13 inhibition. J Clin Invest. 2012; 122(12):4555–68. https://doi.org/10.1172/JCI64896 PMID: 23187130; PubMed Central PMCID: PMCPMC3533556.

13. Braun J, Bothe MK, Mundhenk L, Beck CL, Gruber AD. Murine mCLCA5 is expressed in granular layer keratinocytes of stratified epithelia. Histochem Cell Biol. 2010; 133(3):285–99. https://doi.org/10.1007/s00418-009-0667-0 PMID: 20012443.

43

14. Connon CJ, Yamasaki K, Kawasaki S, Quantock AJ, Koizumi N, Kinoshita S. Calcium-activated chloride channel-2 in human epithelia. J Histochem Cytochem. 2004; 52(3):415–8. https://doi.org/10.1177/002215540405200313 PMID: 14966209.

15. Dietert K, Mundhenk L, Erickson NA, Reppe K, Hocke AC, Kummer W, et al. Murine CLCA5 is uniquely expressed in distinct niches of airway epithelial cells. Histochem Cell Biol. 2015; 143(3):277–87. https://doi.org/10.1007/s00418-014-1279-x PMID: 25212661; PubMed Central PMCID: PMCPMC4317516.

16. Plog S, Mundhenk L, Langbein L, Gruber AD. Synthesis of porcine pCLCA2 protein during late differentiation of keratinocytes of epidermis and hair follicle inner root sheath. Cell Tissue Res. 2012; 350 (3):445–53. https://doi.org/10.1007/s00441-012-1482-9 PMID: 22968961.

17. Plog S, Grotzsch T, Klymiuk N, Kobalz U, Gruber AD, Mundhenk L. The porcine chloride channel calcium-activated family member pCLCA4a mirrors lung expression of the human hCLCA4. J Histochem Cytochem. 2012; 60(1):45–56. https://doi.org/10.1369/0022155411426455 PMID: 22205680; PubMed Central PMCID: PMCPMC3283134.

18. Plog S, Klymiuk N, Binder S, Van Hook MJ, Thoreson WB, Gruber AD, et al. Naturally Occurring Deletion Mutants of the Pig-Specific, Intestinal Crypt Epithelial Cell Protein CLCA4b without Apparent Phenotype. PLoS One. 2015; 10(10):e0140050. https://doi.org/10.1371/journal.pone.0140050 PMID: 26474299; PubMed Central PMCID: PMCPMC4608703.

19. Gandhi R, Elble RC, Gruber AD, Schreur KD, Ji HL, Fuller CM, et al. Molecular and functional characterization of a calcium-sensitive chloride channel from mouse lung. J Biol Chem. 1998; 273(48):32096–101. PMID: 9822685.

20. Gruber AD, Gandhi R, Pauli BU. The murine calcium-sensitive chloride channel (mCaCC) is widely expressed in secretory epithelia and in other select tissues. Histochem Cell Biol. 1998; 110(1):43–9. PMID: 9681688.

21. Abdel-Ghany M, Cheng HC, Elble RC, Pauli BU. Focal adhesion kinase activated by beta(4) integrin ligation to mCLCA1 mediates early metastatic growth. J Biol Chem. 2002; 277(37):34391–400. https://doi.org/10.1074/jbc.M205307200 PMID: 12110680.

22. Furuya M, Kirschbaum SB, Paulovich A, Pauli BU, Zhang H, Alexander JS, et al. Lymphatic endothelial murine chloride channel calcium-activated 1 is a ligand for leukocyte LFA-1 and Mac-1. J Immunol. 2010; 185(10):5769–77. https://doi.org/10.4049/jimmunol.1002226 PMID: 20937843; PubMed Central PMCID: PMCPMC3367505.

23. Lee D, Ha S, Kho Y, Kim J, Cho K, Baik M, et al. Induction of mouse Ca(2+)-sensitive chloride channel 2 gene during involution of mammary gland. Biochem Biophys Res Commun. 1999; 264(3):933–7. https://doi.org/10.1006/bbrc.1999.1583 PMID: 10544033.

24. Roussa E, Wittschen P, Wolff NA, Torchalski B, Gruber AD, Thevenod F. Cellular distribution and subcellular localization of mCLCA1/2 in murine gastrointestinal epithelia. J Histochem Cytochem. 2010; 58 (7):653–68. https://doi.org/10.1369/jhc.2010.955211 PMID: 20385786; PubMed Central PMCID: PMCPMC2889406.

25. Leverkoehne I, Horstmeier BA, von Samson-Himmelstjerna G, Scholte BJ, Gruber AD. Real-time RT-PCR quantitation of mCLCA1 and mCLCA2 reveals differentially regulated expression in pre- and postnatal murine tissues. Histochem Cell Biol. 2002; 118(1):11–7. https://doi.org/10.1007/s00418-002-0420-4 PMID: 12122442.

26. Elble RC, Ji G, Nehrke K, DeBiasio J, Kingsley PD, Kotlikoff MI, et al. Molecular and functional characterization of a murine calcium-activated chloride channel expressed in smooth muscle. J Biol Chem. 2002; 277(21):18586–91. https://doi.org/10.1074/jbc.M200829200 PMID: 11896056.

27. Bothe MK, Braun J, Mundhenk L, Gruber AD. Murine mCLCA6 is an integral apical membrane protein of non-goblet cell enterocytes and co-localizes with the cystic fibrosis transmembrane conductance regulator. J Histochem Cytochem. 2008; 56(5):495–509. https://doi.org/10.1369/jhc.2008.950592 PMID: 18285349; PubMed Central PMCID: PMCPMC2324190.

28. Elble RC, Widom J, Gruber AD, Abdel-Ghany M, Levine R, Goodwin A, et al. Cloning and characterization of lung-endothelial cell adhesion molecule-1 suggest it is an endothelial chloride channel. J Biol Chem. 1997; 272(44):27853–61. PMID: 9346932.

29. Zhu D, Cheng CF, Pauli BU. Blocking of lung endothelial cell adhesion molecule-1 (Lu-ECAM-1) inhibits murine melanoma lung metastasis. J Clin Invest. 1992; 89(6):1718–24. https://doi.org/10.1172/JCI115773 PMID: 1601982; PubMed Central PMCID: PMCPMC295857.

30. Zhu DZ, Cheng CF, Pauli BU. Mediation of lung metastasis of murine melanomas by a lung-specific endothelial cell adhesion molecule. Proc Natl Acad Sci U S A. 1991; 88(21):9568–72. PMID: 1946371; PubMed Central PMCID: PMCPMC52759.

31. Cunningham SA, Awayda MS, Bubien JK, Ismailov II, Arrate MP, Berdiev BK, et al. Cloning of an epithelial chloride channel from bovine trachea. J Biol Chem. 1995; 270(52):31016–26. PMID: 8537359.

44

32. Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from trans-membrane regions. Nat Methods. 2011; 8(10):785–6. https://doi.org/10.1038/nmeth.1701 PMID: 21959131.

33. Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. J Mol Biol. 1982; 157(1):105–32. PMID: 7108955.

34. Hirokawa T, Boon-Chieng S, Mitaku S. SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics. 1998; 14(4):378–9. PMID: 9632836.

35. Tusnady GE, Simon I. The HMMTOP transmembrane topology prediction server. Bioinformatics. 2001; 17(9):849–50. PMID: 11590105.

36. Cserzo M, Wallin E, Simon I, von Heijne G, Elofsson A. Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. Protein Eng. 1997; 10(6):673–6. PMID: 9278280.

37. Nakai K, Horton P. PSORT: a program for detecting sorting signals in proteins and predicting their sub-cellular localization. Trends Biochem Sci. 1999; 24(1):34–6. PMID: 10087920.

38. Williams K, Roman J. Studying human respiratory disease in animals—role of induced and naturally occurring models. J Pathol. 2016; 238(2):220–32. https://doi.org/10.1002/path.4658 PMID: 26467890.

39. Mundhenk L, Alfalah M, Elble RC, Pauli BU, Naim HY, Gruber AD. Both cleavage products of the mCLCA3 protein are secreted soluble proteins. J Biol Chem. 2006; 281(40):30072–80. https://doi.org/10.1074/jbc.M606489200 PMID: 16895902.

40. Pawlowski K, Lepisto M, Meinander N, Sivars U, Varga M, Wieslander E. Novel conserved hydrolase domain in the CLCA family of alleged calcium-activated chloride channels. Proteins. 2006; 63(3):424–39. https://doi.org/10.1002/prot.20887 PMID: 16470849.

41. Bothe MK, Mundhenk L, Kaup M, Weise C, Gruber AD. The murine goblet cell protein mCLCA3 is a zinc-dependent metalloprotease with autoproteolytic activity. Mol Cells. 2011; 32(6):535–41. https://doi.org/10.1007/s10059-011-0158-8 PMID: 22080371; PubMed Central PMCID: PMCPMC3887686.

42. Gibson A, Lewis AP, Affleck K, Aitken AJ, Meldrum E, Thompson N. hCLCA1 and mCLCA3 are secreted non-integral membrane proteins and therefore are not ion channels. J Biol Chem. 2005; 280(29):27205–12. https://doi.org/10.1074/jbc.M504654200 PMID: 15919655.

43. Balakirev ES, Ayala FJ. Pseudogenes: are they "junk" or functional DNA? Annu Rev Genet. 2003; 37:123–51. https://doi.org/10.1146/annurev.genet.37.040103.103949 PMID: 14616058.

44. Gruber AD, Elble RC, Ji HL, Schreur KD, Fuller CM, Pauli BU. Genomic cloning, molecular characterization, and functional analysis of human CLCA1, the first human member of the family of Ca2+-activated Cl- channel proteins. Genomics. 1998; 54(2):200–14. https://doi.org/10.1006/geno.1998.5562 PMID: 9828122.

45. Leverkoehne I, Gruber AD. The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells. J Histochem Cytochem. 2002; 50(6):829–38. https://doi.org/10.1177/002215540205000609 PMID: 12019299.

46. Anton F, Leverkoehne I, Mundhenk L, Thoreson WB, Gruber AD. Overexpression of eCLCA1 in small airways of horses with recurrent airway obstruction. J Histochem Cytochem. 2005; 53(8):1011–21. https://doi.org/10.1369/jhc.4A6599.2005 PMID: 15879574; PubMed Central PMCID: PMCPMC1383431.

47. Bingle CD, Bingle L, Craven CJ. Distant cousins: genomic and sequence diversity within the BPI fold-containing (BPIF)/PLUNC protein family. Biochem Soc Trans. 2011; 39(4):961–5. https://doi.org/10.1042/BST0390961 PMID: 21787330.

48. Bingle CD, LeClair EE, Havard S, Bingle L, Gillingham P, Craven CJ. Phylogenetic and evolutionary analysis of the PLUNC gene family. Protein Sci. 2004; 13(2):422–30. https://doi.org/10.1110/ps.03332704 PMID: 14739326; PubMed Central PMCID: PMCPMC2286710.

49. Bingle L, Bingle CD. Distribution of human PLUNC/BPI fold-containing (BPIF) proteins. Biochem Soc Trans. 2011; 39(4):1023–7. https://doi.org/10.1042/BST0391023 PMID: 21787341.

50. LeClair EE, Nguyen L, Bingle L, MacGowan A, Singleton V, Ward SJ, et al. Genomic organization of the mouse plunc gene and expression in the developing airways and thymus. Biochem Biophys Res Commun. 2001; 284(3):792–7. https://doi.org/10.1006/bbrc.2001.5024 PMID: 11396972.

51. Britto CJ, Cohn L. Bactericidal/Permeability-increasing protein fold-containing family member A1 in airway host protection and respiratory disease. Am J Respir Cell Mol Biol. 2015; 52(5):525–34. https://doi.org/10.1165/rcmb.2014-0297RT PMID: 25265466; PubMed Central PMCID: PMCPMC4491141.

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S1 Table: Nomenclature of duplicated *CLCA3* genes in different species

Species	Nomenclature used in this report	Nomenclature according to the Genbank database	Genbank accession number	Previously used names and references
cattle	*CLCA3* or (*3*)	*CLCA3*	NM_181018	*Lu-ECAM-1* [1] *bCLCA2* [2]
	CLCAx or (*x*)	*LOC784768*	NM_001242583	*CaCC* [3] *bCLCA1* [2]
sheep	*CLCA3* or (*3*)	*LOC101116002*	XM_004002159	-
	CLCAx or (*x*)	*LOC101116267*	XM_012134623	-
mouse	*CLCA3a1* or (*3a1*)	*Clca3a1*	XM_006500968	*mCLCA1* [2] *mCaCC* [4]
	CLCA3a2 or (*3a2*)	*Clca3a2*	XM_006502346	*mCLCA2* [5]
	CLCA3b or (*3b*)	*Clca3b*	NM_139148	*mCLCA4* [6]
rat	*CLCA4l* or (*4l*)	*Clca4l*	NM_001077356	-
	CLCA2 or (*2*)	*Clca2*	NM_001013202	-

1.   Elble RC, Widom J, Gruber AD, Abdel-Ghany M, Levine R, Goodwin A, et al. Cloning and characterization of lung-endothelial cell adhesion molecule-1 suggest it is an endothelial chloride channel. J Biol Chem. 1997;272(44):27853-61.   PubMed PMID: 9346932.

2.   Gruber AD, Fuller CM, Elble RC, Benos DJ, Pauli BU. The CLCA gene family: a novel family of putative chloride channels. Current Genomics. 2000;1(2):201-22.

3.   Cunningham SA, Awayda MS, Bubien JK, Ismailov, II, Arrate MP, Berdiev BK, et al. Cloning of an epithelial chloride channel from bovine trachea. J Biol Chem. 1995;270(52):31016-26. PubMed PMID: 8537359.

4.   Gandhi R, Elble RC, Gruber AD, Schreur KD, Ji HL, Fuller CM, et al. Molecular and functional characterization of a calcium-sensitive chloride channel from mouse lung. J Biol Chem. 1998;273(48):32096-101.   PubMed PMID: 9822685.

5.   Lee D, Ha S, Kho Y, Kim J, Cho K, Baik M, et al. Induction of mouse Ca(2+)-sensitive chloride channel 2 gene during involution of mammary gland. Biochem Biophys Res Commun. 1999;264(3):933-7.   doi: 10.1006/bbrc.1999.1583.   PubMed PMID: 10544033.

6.   Elble RC, Ji G, Nehrke K, DeBiasio J, Kingsley PD, Kotlikoff MI, et al. Molecular and functional characterization of a murine calcium-activated chloride channel expressed in smooth muscle. J Biol Chem. 2002;277(21):18586-91.   doi: 10.1074/jbc.M200829200.   PubMed PMID: 11896056.

S1 Fig. Original immunoblot of Fig 5.
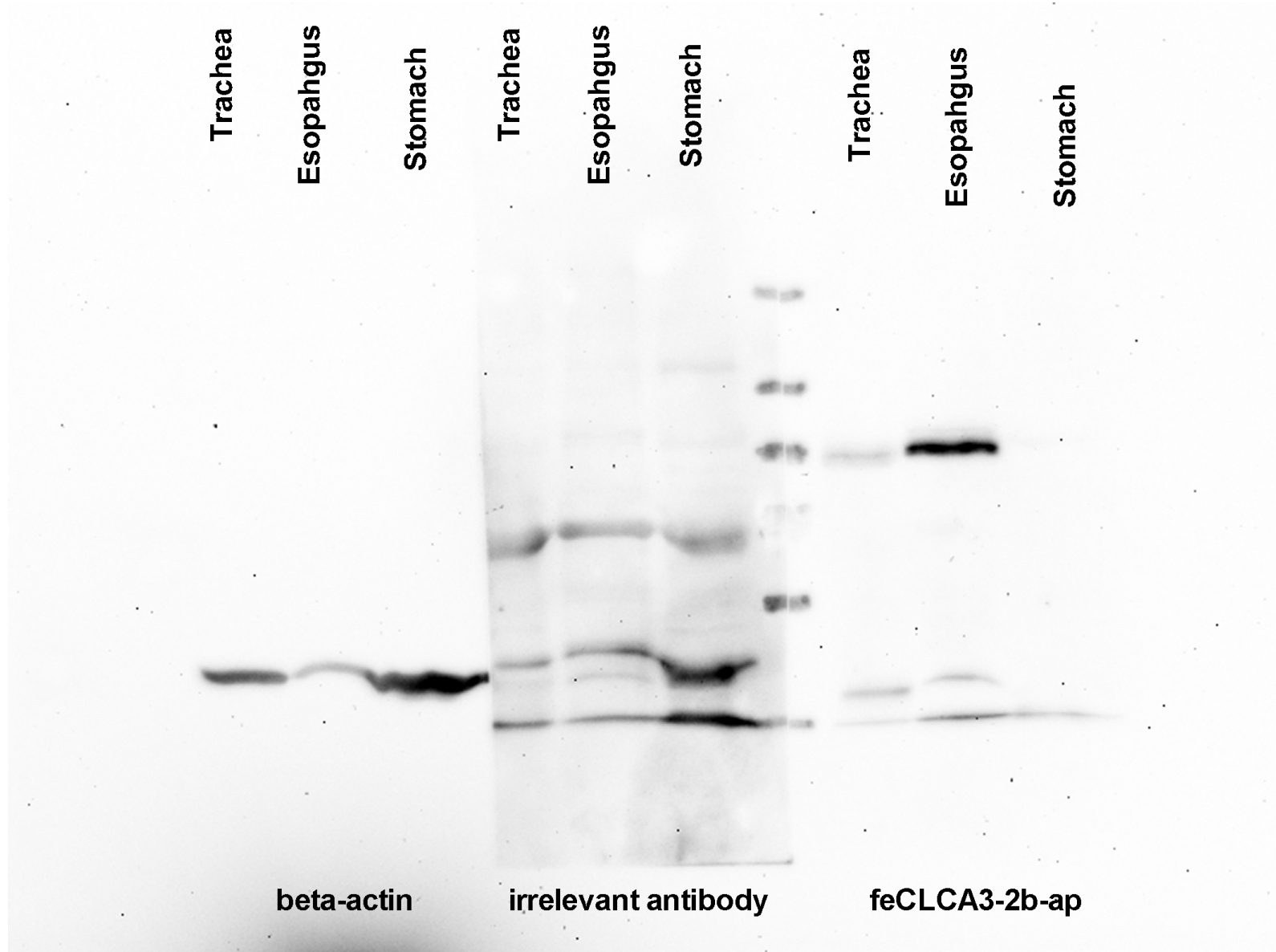
2.2 The Family of Chloride Channel Regulator, Calcium-Activated Proteins in the Feline Respiratory Tract – A Comparative Perspective on Airway Diseases in Man and Animal Models

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Year: 2020

Journal: Journal of Comparative Pathology, doi: 10.1016/j.jcpa.2019.10.193

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Declaration of own portion of work in this research publication:
Contributions by Nancy Ann Erickson: dissection of cats, preparation and processing of tissue samples, RNA isolation, RT-qPCR gene expression, *in silico* and statistical analyses, interpretation, immunoblot analyses, and graphical depiction as well as *in situ* hybridization (ISH) of feline *CLCA2*, IHC, and CLCA1 transfection. Subsequent preparation of the manuscript. For *in silico* analyses of CLCA1 and -2, also see Supplemental Material S1 and S2.

This paper was part of the habilitation treatise of Lars Mundhenk.

All co-authors participated considerably to the study design, evaluation of experimental results, and the creation of the manuscript.

Declaration on ethics: The animals had been involved in a separate animal study (State Office of Health and Social Affairs of Berlin, approval number A 0274/14) – tissues of which had also been used for the previous publication (Mundhenk et al. 2018) (see 2.1) – or had been autopsied in routine pathological diagnostics. The animal study was conducted in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals (Guillen 2012) and all efforts were made to minimize animal discomfort and suffering.

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3   Concluding Discussion

Members of the CLCA family have been systematically characterized in various species, particularly in humans and mice, but also in pigs and horses, in which species-specific homologies but also differences in ortholog numbers, expression patterns, and potential functions have been discovered. Particularly the respective CLCA1 orthologs have been shown to be differentially regulated in respiratory diseases such as in human and equine asthma.

Since asthma is a significant and naturally occurring condition also in cats with many parallels to the human disease, this study aimed at characterizing the CLCA family in the cat for the first time with particular focus on the feline respiratory tract and CLCA1 expression regarding a potential role in feline asthma. More specifically, gene and protein structure, expression pattern in feline tissues and comparative analysis of CLCA expression in the respiratory tract of healthy and asthmatic cats were carried out.

3.1 Comparative Interspecies Analysis of Feline CLCAs

3.1.1 Comparative Genomic Organization

The CLCA family members are located on one single locus in preserved order (Patel et al. 2009) and grouped into four different clusters based on homology (Plog et al. 2009). Of these, clusters 1 and 2 seem to be highly conserved in every species with only one single and apparently functional member each, CLCA1 and -2, respectively (Patel et al. 2009). Also in the cat, the *Clca1* and *-2* genes appear to be present as one single intact copy as investigated by clustal analyses (see 2.1, 2.3, and Supplemental Material S1 and S2).

In contrast, the number of CLCA members differs species-specifically in clusters 3 and 4, of which cluster 3 shows the highest interspecies diversity. It consists of either (1) multiple homologs due to gene duplications, such as in mice in which two subsequent duplication events led to the presence of three closely related and apparently functional homologs (Patel et al. 2009), (2) gene silencing, leading to pseudogene formation in humans and pigs (Gruber and Pauli 1999; Plog et al. 2009) by two distinct, also species-specific mechanisms (see 2.1) or (3) one single functional gene with several canonical elements of other CLCA proteins, such as in the horse (see 2.1). Interestingly, this study showed that the cat also exhibits a single and fully functional gene, CLCA3, in this cluster (see 2.1, 2.3, and Supplemental Material S3). It is therefore likely that feline CLCA3 may represent the archetype of this gene, while it is silenced or duplicated in other species.

Similar to cluster 3, cluster 4 also bears species-specific diversity. Gene duplication led to two or three homologous genes in the pig (Plog et al. 2012a; 2015) or the mouse (Bothe et al. 2008), respectively, each with distinct expression pattern differences, except for *Clca4c* in the mouse which is considered a pseudogene (Patel et al. 2009). In contrast, the cat, like humans (Agnel et al. 1999), exhibits only one functional homolog of this cluster (see 2.2, 2.3, and Supplemental Material S4)

Concluding, the cat also shows the typical conservation of *CLCA1* and *CLCA2* on genomic level (see 2.2) as seen in other species. In clusters 3 and 4, however, the cat is the only species investigated so far which shows one discrete and seemingly fully functional CLCA ortholog each (see 2.1, 2.3 and Supplemental Material S3 and S4).

3.1.2   Comparative Protein Structure and Processing

Concerning protein structure and processing, feline CLCA proteins bear great similarities, i.e. canonical structural elements, in comparison to their orthologs in other species (Gibson et al. 2005; Mundhenk et al. 2006; Plog et al. 2009; Range et al. 2007) (see 2.1 to 2.3 and Supplemental Material S1 to S4).

All feline CLCA proteins show an amino-terminal signal sequence which directs the proteins into the endoplasmic reticulum (Bendtsen et al. 2004; Patel et al. 2009), consistent with the protein entering the secretory pathway as already shown in detail, e.g. for murine CLCA1 (Mundhenk et al. 2006). It is followed by the conserved n-CLCA and vWA domains (see 2.2 for CLCA1 and 2, 2.1 for CLCA3, and 2.3 for CLCA4, and Supplemental Material S1 to S4), structural elements which have also been previously shown for other CLCA proteins and species (Patel et al. 2009). Hence, a similar function for CLCA proteins may be assumed across these species.

The posttranslational cleavage site is also a common feature of all CLCA proteins analyzed to date (Bothe et al. 2011; Elble et al. 1997; Patel et al. 2009) (see 2.3 and Supplemental Material S1 to S4). It was identified between aa 694 and 695 for feline CLCA1, 708 and 709 for feline CLCA2 (see 2.2, 2.3 and Supplemental Material S1 and S2, respectively), 702 and 703 for feline CLCA3 (see 2.1, 2.3 and Supplemental Material S3), and 696 and 697 for feline CLCA4 (see 2.3 and Supplemental Material S4), and, hence, at a similar position as in all other CLCA proteins as determined by comparative analysis. Furthermore, in accordance with the orthologs of all other species investigated, all feline CLCA members show asparagine-linked glycosylation sites, of which particularly CLCA2 but also CLCA3 and CLCA4 are more heavily glycosylated than CLCA1 (see Supplemental Material S1 to S4).

Transmembrane prediction analyses suggest that feline CLCA1 and 3 are soluble and fully secreted proteins (see 2.2 and 2.1 or Supplemental Material S1 and S3, respectively), identical to the corresponding CLCA1 orthologs in humans, mice, and pigs (Gibson et al. 2005; Gruber et al. 1998a; Gruber and Pauli 1999; Mundhenk et al. 2006; Patel et al. 2009; Plog et al. 2009; Range et al. 2007) or the three murine CLCA3 orthologs (Patel et al. 2009), respectively. On the contrary, feline CLCA2 bears a transmembrane domain in the carboxy-terminal region (see 2.3 and Supplemental Material S2), also seen for CLCA2 in aforementioned species (Braun et al. 2010a; Elble et al. 2006; Patel et al. 2009; Plog et al. 2012b). Whilst HMMtop, Phobius, and SOSUI also predicted a carboxy-terminal transmembrane domain for feline CLCA4 (see Supplemental Material S4), the presence of a carboxy-terminal GPI anchor (see 2.3 and Supplemental Material S4), as had been assumed for its human counterpart (Patel et al. 2009), cannot be excluded. Further experimental data needs to clarify the nature of the assumed CLCA4 anchoring in the plasma membrane.

Hence, feline CLCA proteins can also be biochemically subdivided into two groups of CLCAs – the fully secreted members of clusters 1 and 3 lacking a transmembrane domain (Anton et al. 2005; Gibson et al. 2005; Gruber et al. 1998a; Gruber and Pauli 1999; Mundhenk et al. 2006; Patel et al. 2009; Plog et al. 2009) and the members of clusters 2 and 4 secreted only amino-terminally with the carboxy-terminal remaining anchored to the plasma membrane (Braun et al. 2010a; Elble et al. 2006; Gruber et al. 1998b; 1999; Patel et al. 2009; Plog et al. 2012a).

Further processing analyses – specifically of CLCA1 as the most intriguing potential target in respiratory conditions – support the notion of it being a soluble protein. It was expressed in feline mucus cells and partially secreted extracellularly into the mucus layer as seen by CLCA1 immunohistochemical staining (see 2.2). Furthermore, lysate analysis of feline CLCA1-transfected HEK 293 via Western Blot cells showed an approximately 75 kDa protein (see 2.2), identical to its porcine ortholog (Plog et al. 2009) whilst a slightly larger and more pronounced protein band was present in the supernatant, pointing toward abundant secretion of the posttranslationally glycosylated protein (see 2.2 and Supplemental Material S1).

Nevertheless, two findings deviate between the respective orthologs of these two species. First, two proteins of 120 kDa and 130 kDa in size were identified in the cell lysate and supernatant for porcine CLCA1, respectively (see 2.2), likely representing the uncleaved primary translation products as had been found previously (Plog et al. 2009). However, both of these proteins were not found for feline CLCA1 (see 2.2), possibly due to a more effective post-translational cleavage of the feline CLCA1 primary translation product (see 2.2). Secondly, a weak protein band was additionally present at approximately 270 kDa not only in

the supernatant of feline but also of porcine CLCA1-transfected cells (see 2.2), latter of which, however, had not been reported previously (Plog et al. 2009). Here, it may be assumed that CLCA1 is bound to another protein but its nature and origin remain speculative and should be analyzed in further experiments (see 2.2).

CLCA3 immunoblot analyses of feline trachea and oesophagus revealed an approximately 90 kDa protein, which is in accordance with the predicted size of the amino-glycosylated amino-terminal cleavage product (see 2.1), indicating that this feline variant may be posttranslationally cleaved similar to most other CLCA members investigated so far (Patel et al. 2009).

In summary, feline CLCAs exclusively show strong homologies to their orthologs in other species on protein structure and processing level. Therefore, based on the *in silico* analyses, it may be assumed that the feline CLCAs possess similar functions as compared to their respective orthologs (see 1.5), which needs to be investigated in future. In this respect, as a further step, the tissue expression pattern of feline CLCAs was investigated in comparison to the other species (see 1.3), in order to more closely determine the tissue and cellular microenvironment in which feline CLCAs may act.

3.1.3   Comparative CLCA Tissue Expression Profile

In all species investigated so far, CLCA1 shares a virtually identical interspecies expression pattern – predominantly in mucus-producing cells of the gastrointestinal tract, i.e. the colon, and also of the respiratory and reproductive tract (Anton et al. 2005; Gruber et al. 1998b; Hoshino et al. 2002; Leverkoehne and Gruber 2002; Plog et al. 2009).

CLCA1 is most strongly expressed on mRNA level in the healthy feline respiratory tract and, to a lesser degree, in the gastrointestinal tract (see 2.2). Of all further tissues investigated, exclusively the conjunctiva and dorsal root ganglion show traces of *CLCA1* mRNA expression whilst all other tissues remained devoid (see 2.2). Interestingly, this also pertains to the reproductive tract which, in contrast, shows CLCA1 mRNA or protein expression in all other species analyzed to date (Anton et al. 2005; Gruber et al. 1998b; Hoshino et al. 2002; Komiya et al. 1999; Leverkoehne and Gruber 2002; Plog et al. 2009). However, it cannot be excluded that the reproductive cycle may influence uterine CLCA1 expression which needs to be investigated.

On protein level, feline CLCA1 was identified as the most abundantly expressed CLCA family member and was immunohistochemically localized exclusively to mucus cells from the nasal cavity down to the main bronchi (see 2.2). Specifically, CLCA1 was associated with multiple cytoplasmic mucus cell granules and occasionally with the PAS-positive mucus layer lining the

apical surface, which points towards a secretory mechanism (see 2.2). These findings are entirely identical to those of its human, murine, porcine, and equine orthologs (Anton et al. 2005; Gruber et al. 1998b; Hoshino et al. 2002; Leverkoehne and Gruber 2002; Plog et al. 2009). Hence, due to this coinciding microenvironment, a similar function may be speculated for feline CLCA1 as reported for CLCA1 of other species (see 1.5). In contrast to the expression of these CLCA1 orthologs of all aforementioned species (Anton et al. 2005; Leverkoehne and Gruber 2002; Plog et al. 2009), feline CLCA1 was not detected in SMGs (see 2.2). This niche seems to be occupied by a different feline CLCA member, CLCA3 (see below).

In the respiratory tract, interspecies differences exist for CLCA2, which is expressed in few or numerous SMGs of humans and pigs or mice (Dietert et al. 2015), respectively. In latter species, CLCA2 is also uniquely expressed in distinct bronchial epithelial cells (Dietert et al. 2015). In the cat, however, only RT-qPCR revealed low level expression of *CLCA2* mRNA in whole tissue lysates of trachea and bronchi, whilst neither ISH nor IHC localized CLCA2 mRNA or protein, respectively, in the respiratory tract, possibly due to very minute mRNA expression levels only detectable with this highly sensitive method (see 2.2). Therefore, surprisingly and in contrast to all aforementioned species, feline CLCA2 – like feline CLCA1 – was not found in SMGs and, moreover, in any other cell type of the feline respiratory tract (see 2.2.). Hence, a possible function, e.g. in terms of compensatory or synergistic effects regarding CLCA1 in the respiratory tract, as had been proposed for CLCA2 in the mouse (Patel et al. 2006; 2009), seems very unlikely in the cat, at least during homeostatic conditions.

The most striking differences were present in cluster 3, which seemingly underwent species-specific evolvements. Whilst CLCA3 is a silenced pseudogene in humans and pigs (Gruber and Pauli 1999; Plog et al. 2009), the mouse bears three closely related homologs due to duplication events (Bothe et al. 2008). These are not only located to epithelial cells of the respiratory tract (Abdel-Ghany et al. 2002; Elble et al. 2002; Furuya et al. 2010; Gandhi et al. 1998; Gruber et al. 1998b) but also to cells of mesenchymal origin (Elble et al. 2002; Patel et al. 2006). In contrast, the cat is the only species in which a single CLCA3 ortholog has been shown to be expressed on protein level to date (see 2.1) and may therefore become particularly valuable to discretely study this particular CLCA member.

Identical to CLCA1, feline CLCA3 was predicted to be a soluble protein and detected on mRNA level in the upper respiratory tract from the nasal cavity down to the bronchial bifurcation but not in main bronchi or lung parenchyma (see 2.1). Feline CLCA3 protein was expressed in respiratory ciliated epithelial cells and in mucus-producing SMG cells (see 2.1), similar to its murine orthologs CLCA3a1 and 3a2 (Gruber et al. 1998b). Whilst murine CLCA1 had been proposed to function in airway macrophage activation (Ching et al. 2013; Dietert et al. 2014;

Erickson et al. 2018) or mucus cell metaplasia (Alevy et al. 2012), feline CLCA3 shows certain similarities with CLCA1 in terms of structure and processing as a soluble protein (see 2.1 to 2.3 and Supplemental Material S1 and S3, respectively) and is shed into the extracellular environment (see 2.1). It therefore appears that CLCA3 may functionally substitute for the lack of CLCA1 specifically in SMGs, with possible compensatory functions in that microenvironment, at least in cats.

Nonetheless, unlike CLCA1, feline CLCA3 is not expressed in mucus cells of the airway epithelial lining (see 2.1). Furthermore, other tissues, except for the oesophagus, were devoid of *CLCA3* mRNA expression (see 2.1), including those in which its orthologs are known to be expressed in other species, e.g. mammary gland, spleen, lung, and aorta (Elble et al. 1997; Elble et al. 2002; Gruber and Pauli 1999; Leverkoehne and Gruber 2002).

Interestingly, feline CLCA3 is also expressed in certain subsets of keratinocytes of esophageal stratified epithelia (see 2.1), similar to CLCA2, which may point toward a putative function in epithelial maturation and differentiation (Braun et al. 2010a; Yu et al. 2013). More specifically, despite it being a soluble protein, it was shown to be associated with the plasma membrane of esophageal keratinocytes and cilia of respiratory epithelial cells (see 2.1), in which it may interact with integral membrane proteins of these cells (see 2.1). However, the exact nature and function of CLCA3 and its association needs yet to be elucidated and comparatively analyzed in other species in which this protein is also evolutionarily retained and singularly present, such as perhaps in the horse (Patel et al. 2009).

Similar to cluster 3, cluster 4 also shows several gene duplication events, at least in mice and pigs, as well as similarities and differences in the interspecies expression pattern. Regarding the respiratory tract, porcine CLCA4a protein is expressed in respiratory epithelial cells from the nasal cavity to the small bronchi (Plog et al. 2012a) whilst human *CLCA4* is expressed in nasal mucosa and trachea only on mRNA level (Agnel et al. 1999; Mall et al. 2003). In contrast to its human and porcine orthologs but identical to the mouse (Bothe et al. 2008), the healthy feline respiratory tract is completely devoid of *CLCA4* (see 2.2). In this respect, the cat and mouse appear to contrast with pig and man.

Feline *CLCA4* mRNA was exclusively identified in the intestinal tract (see 2.2), consistent with its orthologs in other species, i.e. humans (Agnel et al. 1999), mice (Bothe et al. 2008), and pigs (Plog et al. 2012a; 2015). Unfortunately, the intestinal cell type expressing feline CLCA4 protein could not be identified in this study since no antibody was available for feline CLCA4 (see 2.3). Nonetheless, it is tempting to speculate that CLCA4 is also expressed by non-mucus cell enterocytes as had been shown for the murine and porcine orthologs (Bothe et al. 2008; Evans et al. 2004; Plog et al. 2012a; 2015; Teske et al. 2020). The cat is the only species

investigated to date with a single CLCA4 which, thus, may represent the ortholog archetype. Therefore, the cat seems to be a suitable species in studying this protein. Other species, such as mice and pigs, possess CLCA4 duplications. In the mouse, *Clca4a* and *-b* were localized to the jejunal villi and apical colonic epithelium, whilst *Clca4b* was also found in intestinal crypts, which may be indicative of different functions (Teske et al. 2020). A distinct cellular expression pattern had also been reported for the duplicated CLCA4 genes in pigs. The porcine CLCA4a was expressed in apical membranes of villous epithelial cells (Plog et al. 2012a), whereas the porcine CLCA4b was expressed in crypt epithelial cells (Plog et al. 2015). Hence, further studies need to show if feline CLCA4 is expressed in one distinct compartment or in both of these compartments.

Taken together, feline CLCA1 shows a similar expression pattern in the respiratory tract compared to all species previously investigated (Anton et al. 2005; Gibson et al. 2005; Hoshino et al. 2002; Leverkoehne and Gruber 2002; Mundhenk et al. 2006; Plog et al. 2009) (also see 2.2, Figure 7). However, fundamental species-specific differences of the other CLCA family members on genomic and expressional level may also imply functional diversity in homeostatic and challenged airways. Consequentially, the CLCA orthologs of respective species may modulate the respiratory conditions differently, thereby impeding the translatability of animal models. Therefore, an in-depth understanding of the heterogeneity of this gene family is required, especially concerning the respiratory tract.

3.2   Role in Feline Asthma

Due to the consistent protein structure and expression pattern of CLCA1 in respiratory mucus cells across mammalian species (Anton et al. 2005; Gruber et al. 1998b; Leverkoehne and Gruber 2002; Plog et al. 2009) (see 2.2) as well as its differential regulation in asthma patients (Hoshino et al. 2002; Toda et al. 2002; Wang et al. 2007; Woodruff et al. 2007) and corresponding animal models (Anton et al. 2005; Gibson et al. 2005; Hoshino et al. 2002; Mei et al. 2013; Nakanishi et al. 2001; Song et al. 2013; Toda et al. 2002; Woodruff et al. 2007; Zhou et al. 2001), a similar regulation of CLCA1 in the airways can be suggested in cats. However, caution is warranted concerning fundamental species-specific differences seen in the other CLCA family members which may have modulatory functions impeding translatability (see 3.1). The only other CLCA member expressed in the feline respiratory tract beside CLCA1 is CLCA3 (see 2.1), for which IHC revealed an identical staining pattern in asthmatic as compared to healthy cats (see 2.1). Hence, a functional relevance may be speculative and needs further investigation.

In detail, CLCA1 had been shown to be strongly upregulated in mucus cells and BALF in asthma patients (Hoshino et al. 2002; Toda et al. 2002; Wang et al. 2007; Woodruff et al. 2007) and murine models of asthma (Gibson et al. 2005; Hoshino et al. 2002; Toda et al. 2002; Zhou et al. 2001). Coinciding, CLCA1 expression in murine asthma models decreased after glucocorticoid (Woodruff et al. 2007) or Il-9 (Zhou et al. 2001) treatment whilst the asthma phenotype was ameliorated or aggravated by CLCA1-antisense and -antibody treatment (Nakanishi et al. 2001; Song et al. 2013) or experimental CLCA1 overexpression (Hoshino et al. 2002; Mei et al. 2013; Nakanishi et al. 2001), respectively. Similarly, in horses affected by SEA, CLCA1 expression is also highly increased due to mucus cell metaplasia primarily in the bronchioles (Range et al. 2007), which were devoid of CLCA1 expression during homeostatic conditions (Anton et al. 2005). Due to these similarities across numerous species, a similarly differential expression of CLCA1 could also be speculated for feline asthmatic airways.

In healthy cats, the mucus covering the respiratory epithelial lining only occasionally stained positively for CLCA1 in IHC (see 2.2). In the airways of asthmatic cats, however, excessive mucus secretion and accumulation in the airways became evident by PAS reaction and the entire bronchial mucus layer labelled intensely positive for CLCA1 (see 2.2). Hence, CLCA1 seems to be strongly secreted together with the PAS-positive mucus during asthmatic conditions, identical to all species analyzed to date (Anton et al. 2005; Gibson et al. 2005; Hoshino et al. 2002; Leverkoehne et al. 2006; Nakanishi et al. 2001; Range et al. 2007; Song et al. 2013; Toda et al. 2002; Wang et al. 2007; Woodruff et al. 2007; Zhou et al. 2001). These coinciding results also suggest a similar function of CLCA1 in the pathogenesis of feline asthma as in the corresponding disease of other animal species (see 1.5 and below).

Several modes of action have been hypothesized for CLCA1 under various challenged conditions of acute or chronic, inflammatory mucus-based diseases, either as a modulator of mucus expression and structure (see 1.5.1 to 1.5.3) or as a signaling molecule of innate immunity (see 1.5.4). The human and murine CLCA1 orthologs appear to modulate pathways of innate immunity in the respiratory tract by regulating airway macrophage cytokine expression (Ching et al. 2013; Dietert et al. 2014; Erickson et al. 2018; Keith et al. 2019). Additionally, murine CLCA1 was recently associated with mucus-processing or -altering properties (Nystrom et al. 2018; Nystrom et al. 2019), i.e. with the enzymatic control of intestinal mucus expansion (Nystrom et al. 2018) which could also be relevant to the respiratory tract. Hence, due to its identical protein structure and cellular expression pattern in healthy and diseased tissues, a similar function could also be hypothesized for cats. In this context, it would be interesting to investigate if feline CLCA1 may also serve as a biomarker particularly in asthmatic conditions, during which its ortholog is increased in humans (Hoshino et al. 2002; Toda et al. 2002; Wang et al. 2007; Woodruff et al. 2007), horses (Anton et al.

2005), and murine models (Gibson et al. 2005; Hoshino et al. 2002; Toda et al. 2002; Zhou et al. 2001).

Nonetheless, despite absence of apparent differential regulation of CLCA3 in the asthmatic cat, the species-specific CLCA3 variations – single gene copy in cats and multiple duplications in mice – and their potential functional consequences need to be considered regarding future studies of naturally or experimentally induced asthma models in animals with respect to its lack in humans (see 2.2).

Taken together, this first and still limited data on differential regulation and, hence, regarding possible role of CLCA1 in feline asthma may indicate a similar function as considered for human asthma patients and respective animal models, which yet needs to be investigated in detail.

3.3 Conclusions and Outlook

Members of the CLCA family have been systematically characterized in various species and show species-specific homologies but also differences on genetic, expressional, and functional levels. Particularly CLCA1 seems to be, on the one hand, highly conserved on genetic and expressional levels, also concerning its increased expression in respiratory diseases of humans and respective animal models. On the other hand, functional discrepancies may lead to limited translatability of data between species.

Since feline asthma shows many parallels to the human disease, this study aimed at characterizing the expression profile of the CLCA family in the cat with particular focus on the healthy feline respiratory tract and CLCA1 regarding a potential role in feline asthma.

Here, it could be shown for the first time that the cat possesses CLCA orthologs which are grouped into four clusters, identical to all other species investigated to date. Furthermore, but in contrast to all other species, the cat possesses a single and fully functional CLCA member in each cluster which, hence, makes the cat particularly relevant in studying individual CLCA members. This pertains particularly to CLCA4 as an alleged archetype of the orthologs of other species which are exclusively present as duplication products with distinct expression patterns (Bothe et al. 2008; Evans et al. 2004; Plog et al. 2012a; 2015; Teske et al. 2020).

In the naive respiratory tract, CLCA1 shows a similar expression pattern as compared to all other species analyzed to date. Furthermore, feline CLCA1 expression and its secretion into the mucus of the respiratory tract seems to be highly increased during asthmatic conditions, identical to all other species exhibiting chronic respiratory conditions with increased mucus production, which is suggestive of a role in feline asthma. Furthermore, particularly in the feline respiratory tract, compensatory or synergistic effects, as had been proposed for CLCA2 in

other species (Patel et al. 2006; 2009), seem unlikely, at least during homeostatic conditions (see 2.2). This also relates to feline CLCA4, which the healthy feline respiratory tract is also completely devoid of (see 2.2). Hence, putative compensatory or synergistic effects may be exclusively exerted by CLCA3 which, however, did not show any obvious differential regulation during asthmatic conditions (see 2.2). Nevertheless, a function on physiologic levels or *de novo* upregulation of other CLCA members cannot be excluded, which warrants further investigation.

In this regard, it will be interesting to explore a larger number of cats, which will perhaps also offer the possibility of investigating CLCA1 expression at different stages or conditions of the disease and potential effects of age, breed, and genetic variations, as well as comorbidities and patient environments (see 2.2).

One drawback of the present study was the limited number of asthmatic cats included as well as the lack of additional RT-qPCR-based expression level quantification of all CLCA members from FFPE archival tissues. However, lack of standardization of tissue processing, fixation, and storage of archival tissue was highly likely of hampering the accuracy in any mRNA data obtained (see 2.2).

In humans, mice, and horses, CLCA1 protein content is increased in BALF (Anton et al. 2005; Gibson et al. 2005; Hoshino et al. 2002; Range et al. 2007; Toda et al. 2002; Wang et al. 2007; Woodruff et al. 2007; Zhou et al. 2001) and had therefore been discussed as potential biomarker (Patel et al. 2009). Hence, the preliminary findings of increased CLCA1 expression and secretion also in feline asthmatics may point towards a potential diagnostic value regarding feline asthma or at least regarding diseases generally associated with increased mucus production such as feline chronic bronchitis. Hence, additional techniques, e.g. enzyme-linked immunosorbent assay (ELISA) could be used on BALF collected from healthy cats, asthmatic cats, and cats with other respiratory diseases with a subsequent comparative analysis of CLCA1 protein content.

Last but not least, feline CLCA1 could also be considered a therapeutic target. Since CLCA1-antisense or -antibody treatment (Nakanishi et al. 2001; Song et al. 2013) led to a striking amelioration of the asthma phenotype in the mouse, it would be interesting to investigate if such a procedure performed analogously in the asthmatic cat would also lead to phenotype amelioration.

4   Summary

Expression Analyses of CLCA Members in the Feline Respiratory Tract – Biomolecules in Feline Asthma?

Nancy Ann Erickson, PhD

Certain members of the highly conserved chloride channel regulator, calcium-activated (CLCA) family have been implicated in inflammatory mucus-based respiratory conditions such as asthma, chronic obstructive pulmonary disease or cystic fibrosis which may either occur naturally or may be experimentally induced in animal species to model the respective human disease. CLCA1, the most intensively investigated member of this family to date, was shown to be highly expressed in these respiratory diseases and had been discussed as a therapeutic target.

The CLCA family has been systematically characterized in naive and diseased conditions in humans and mice, and, to a lesser extent, in horses and naive pigs. It has not only revealed strong similarities but also distinct species-specific differences in genetic organization or protein structure and expression pattern, with partially functional contradictory results, particularly between human and murine CLCA orthologs. These interspecies differences are further complicated by possible redundancies or compensatory effects of homologous CLCA members and may limit the value of mouse models. Hence, caution is warranted in translating results from one species to another.

Asthma is a highly relevant and naturally occurring disease not only in horses but also in cats, in which it shares many characteristics with the human disease. The feline CLCA family is, however, unknown to date. Hence, this study aimed at characterizing the gene and protein structure of the CLCA family in comparison to the orthologs of other well-investigated species, i.e. humans, mice, and pigs. Furthermore, it aspired at elucidating the mRNA and protein expression profile of feline CLCA members in healthy cats for the first time, particularly in the respiratory tract. Last but not least, it aimed gathering first evidence of allegedly differential expressional regulation of CLCA1 in feline asthma.

It could be shown that the feline CLCA orthologs are grouped into four clusters, identical to all other species investigated to date with typical conservation of *CLCA1* and *CLCA2* on the genomic level. However, the cat possesses single and fully functional CLCA members not only in clusters 1 and 2 but, in contrast to all other species, both in clusters 3 and 4, which, hence, makes the cat particularly relevant in studying these individual CLCA members.

Regarding CLCA1, the feline ortholog not only shows an identical genetic and protein structure as compared to the other species investigated, but also concerning its tissue expression pattern.

In the naive respiratory tract, CLCA1 immunohistochemically localized to virtually all mucus cells and was found to be secreted extracellularly mucus. This expression pattern identical to all other species analyzed to date may point toward a conserved function of CLCA1 in this microenvironment. However, feline CLCAA1 was not detected in submucosal glands in which it is expressed in all aforementioned species. This niche seems to be occupied by a different CLCA member, CLCA3, in the cat. Surprisingly and in contrast to all aforementioned species, feline CLCA2 was neither found in SMGs nor in any other cell type of the feline respiratory tract, hence, excluding any possible compensatory or synergistic effect of CLCA2 in the cat, as had been proposed for CLCA2 in other species, at least during homeostatic conditions. The allegedly soluble protein CLCA3 was primarily found in ciliated respiratory epithelial cells and, importantly, also in mucus-producing SMG cells of the upper respiratory tract, similar to its murine orthologs CLCA3a1 and -3a2. It therefore appears that in cats, CLCA3 may substitute for the lack of CLCA1 and CLCA2 specifically in SMGs, with possible overlapping functions in that microenvironment. Similar to mice, but in contrast to man and pigs, the feline respiratory tract was devoid of CLCA4 expression.

In the airways of asthmatic cats, CLCA1 intensely labelled the entire bronchial mucus layer, identical to all other species exhibiting chronic respiratory conditions with increased mucus production. This seemingly increased CLCA1 secretion cat may indicate a similar function as considered for human asthma patients and respective animal models. It is suggestive of a role in feline asthma in which it may serve as a biomarker and/or therapeutic target and needs to be investigated in future studies in detail.

However, fundamental species-specific differences of other CLCA family members on the genomic and protein level may also imply functional diversity in homeostatic and challenged airways and, hence, may modulate the respiratory conditions differently and thereby impede the translatability of animal models. Therefore, an in-depth understanding of the heterogeneity of this gene family is required, especially concerning the respiratory tract.

5   Zusammenfassung

Expressionsanalyse der CLCA Familie im Felinen Respirationstrakt – Biomoleküle bei Felinem Asthma?

Nancy Ann Erickson, PhD

Mitglieder der hochkonservierten Chloridkanal-Regulator, Calcium-aktiviert (CLCA)-Familie werden mit entzündlichen, Mukus-basierten Atemwegserkrankungen wie Asthma, chronisch obstruktiver Lungenerkrankung oder zystischer Fibrose assoziiert, die in verschiedenen Tierspezies entweder natürlicherweise vorkommen oder experimentell induziert werden können, um die humane Erkrankung zu modellieren. CLCA1, das bislang am intensivsten untersuchte Mitglied dieser Familie, ist bei diesen Atemwegserkrankungen stark exprimiert und wurde daher als therapeutisches Target diskutiert.

Die CLCA-Familie wurde bereits systematisch in gesunden und erkrankten Menschen und Mäusen, und in geringerem Umfang auch in Pferden und gesunden Schweinen charakterisiert. Dabei wurden nicht nur deutliche Ähnlichkeiten, sondern auch speziesspezifische Unterschiede in genetischer Organisation, Proteinstruktur und Expressionsmuster festgestellt, teils mit funktionell widersprüchlichen Ergebnissen, insbesondere zwischen menschlichen und murinen CLCA-Orthologen. Diese speziesspezifischen Unterschiede werden vermutlich durch Redundanzen oder kompensatorische Effekte homologer CLCA-Vertreter weiter erschwert und können die Aussagekraft von Mausmodellen einschränken. Daher ist bei der Übertragung von Ergebnissen von einer Spezies auf eine andere Vorsicht geboten.

Asthma ist eine hochrelevante und natürlicherweise vorkommende Erkrankung nicht nur bei Pferden, sondern auch bei Katzen, die viele Merkmale mit der humanen Erkrankung teilt. Die feline CLCA-Familie ist jedoch bislang unbekannt. Ziel dieser Arbeit war daher die Charakterisierung der Gen- und Proteinstruktur im Vergleich zu den Orthologen anderer gut untersuchter Spezies Mensch, Maus und Schwein. Darüber hinaus sollte erstmals das mRNA- und Proteinexpressionsprofil der felinen CLCA-Mitglieder in gesunden Katzen, insbesondere im Respirationstrakt, untersucht werden. Zudem wurde angestrebt, erste Hinweise auf eine mögliche differentielle Expressionsregulation von CLCA1 bei felinem Asthma zu sammeln.

Es konnte gezeigt werden, dass sich die felinen CLCA-Ortholge, wie in allen anderen bisher untersuchten Spezies, mit typischer genomischer Konservierung von CLCA1 und CLCA2 in vier Cluster gruppieren. Allerdings besitzt die Katze jeweils ein einzelnes und voll funktionsfähiges CLCA-Mitglied nicht nur in den Clustern 1 und 2, sondern, im Gegensatz zu

allen anderen bislang untersuchten Spezies, auch in den beiden anderen Clustern, wodurch die Katze besonders relevant für die Untersuchung einzelner CLCA-Mitglieder erscheint.

Bezüglich CLCA1 zeigt das feline Ortholog nicht nur eine nahezu identische Gen- und Protein-Struktur im Vergleich zu den anderen untersuchten Spezies, sondern auch bezüglich seines Gewebeexpressionsmusters.

Im gesunden Respirationstrakt ist CLCA1 immunhistochemisch in nahezu allen Becherzellen lokalisiert und wird extrazellulär in den Schleim sezerniert. Dieses zu allen anderen bisher untersuchten Spezies identische Expressionsmuster weist auf eine Konservierung auch der Funktion von CLCA1 in dieser Mikroumgebung hin. Felines CLCA1 wurde jedoch nicht in den submukösen Drüsen, in denen der jeweilige Orthologe der vorgenannten Spezies exprimiert wird, nachgewiesen. Diese Nische scheint von einem anderen felinen CLCA-Mitglied, CLCA3, besetzt. Darüber hinaus wurde überraschenderweise und im Gegensatz zu allen genannten Spezies felines CLCA2 weder in submukösen Drüsen noch in einem anderen Zelltyp des felinen Respirationstraktes gefunden, was einen kompensatorischen oder synergistischen Effekt von CLCA2, wie bereits für andere Spezies vermutet, bei der Katze zumindest unter homöostatischen Bedingungen ausschließt. Das vermutlich lösliche Protein CLCA3 wurde vorwiegend in respiratorischen Flimmerepithelzellen und schleimproduzierenden submukösen Drüsen des oberen Respirationstraktes nachgewiesen, ähnlich seiner murinen Orthologen CLCA3a1 und 3a2. Somit scheint CLCA3 das Fehlen von CLCA1 und CLCA2 speziell in sub-mukösen Drüsen der Katze zu kompensieren, mit entsprechend denkbaren, überlappenden Funktionen in dieser Mikroumgebung. Ähnlich wie bei Mäusen, aber im Gegensatz zu Menschen und Schweinen, ist CLCA4 im felinen Respirationstrakt nicht exprimiert.

In den Atemwegen asthmatischer Katzen war CLCA1 in der extrazellulären Mukusschicht stark exprimiert, wie bei allen anderen Spezies mit chronischen Atemwegserkrankungen mit erhöhter Schleimproduktion. Diese scheinbar erhöhte CLCA1-Sekretion bei asthmatischen Katzen könnte auf eine ähnliche Funktion bei felinem Asthma wie für Asthmapatienten und entsprechende Tiermodelle bereits vermutet hindeuten, die als Biomarker und/oder therapeutisches Target genutzt werden könnte und in zukünftigen Studien en détail untersucht werden muss.

Es ist jedoch zu beachten, dass grundlegende speziesspezifische Unterschiede anderer Mitglieder der CLCA-Familie auf Genom- und Proteinebene eine funktionelle Diversität und somit Modulation in gesunden und erkrankten Atemwegen hervorrufen und dadurch die Übertragbarkeit von Tiermodellen erschweren können. Daher ist ein tieferes Verständnis der Heterogenität dieser Genfamilie, insbesondere in Bezug auf den Respirationstrakt, erforderlich.

6   References

Abdel-Ghany M, Cheng HC, Elble RC, Pauli BU (2001): The breast cancer beta 4 integrin and endothelial human CLCA2 mediate lung metastasis. J Biol Chem 276:25438-25446. doi:10.1074/jbc.M100478200

Abdel-Ghany M, Cheng HC, Elble RC, Pauli BU (2002): Focal adhesion kinase activated by beta(4) integrin ligation to mCLCA1 mediates early metastatic growth. J Biol Chem 277:34391-34400. doi:10.1074/jbc.M205307200

Abdel-Ghany M, Cheng HC, Elble RC, Lin H, DiBiasio J, Pauli BU (2003): The interacting binding domains of the beta(4) integrin and calcium-activated chloride channels (CLCAs) in metastasis. J Biol Chem 278:49406-49416. doi:10.1074/jbc.M309086200

Adamama-Moraitou KK, Patsikas MN, Koutinas AF (2004): Feline lower airway disease: a retrospective study of 22 naturally occurring cases from Greece. J Feline Med Surg 6:227-233. doi:10.1016/j.jfms.2003.09.004

Adler TR, Beall GN, Heiner DC, Sabharwal UK, Swanson K (1985): Immunologic and clinical correlates of bronchial challenge responses to Bermuda grass pollen extracts. J Allergy Clin Immunol 75:31-36. doi:10.1016/0091-6749(85)90008-9

Agnel M, Vermat T, Culouscou JM (1999): Identification of three novel members of the calcium-dependent chloride channel (CaCC) family predominantly expressed in the digestive tract and trachea. FEBS Lett 455:295-301. doi:10.1016/s0014-5793(99)00891-1

Al-Jumaily M, Kozlenkov A, Mechaly I, Fichard A, Matha V, Scamps F, Valmier J, Carroll P (2007): Expression of three distinct families of calcium-activated chloride channel genes in the mouse dorsal root ganglion. Neurosci Bull 23:293-299. doi:10.1007/s12264-007-0044-8

Alevy YG, Patel AC, Romero AG, Patel DA, Tucker J, Roswit WT, Miller CA, Heier RF, Byers DE, Brett TJ, Holtzman MJ (2012): IL-13-induced airway mucus production is attenuated by MAPK13 inhibition. J Clin Invest 122:4555-4568. doi:10.1172/JCI64896

Almagro Armenteros JJ, Tsirigos KD, Sønderby CK, Petersen TN, Winther O, Brunak S, von Heijne G, Nielsen H (2019): SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol 37:420-423. doi:10.1038/s41587-019-0036-z

Ambort D, Johansson ME, Gustafsson JK, Nilsson HE, Ermund A, Johansson BR, Koeck PJ, Hebert H, Hansson GC (2012): Calcium and pH-dependent packing and release of the gel-forming MUC2 mucin. Proc Natl Acad Sci U S A 109:5645-5650. doi:10.1073/pnas.1120269109

Anthonisen NR (1988): Chronic obstructive pulmonary disease. Cmaj 138:503-510

REFERENCES

Anton F, Leverkoehne I, Mundhenk L, Thoreson WB, Gruber AD (2005): Overexpression of eCLCA1 in small airways of horses with recurrent airway obstruction. J Histochem Cytochem 53:1011-1021. doi:10.1369/jhc.4A6599.2005

Banks W (1993): Applied Veterinary Histology. 3 edn, St. Louis: Mosby – ISBN: 978-0801666100

Barnes KC, Marsh DG (1998): The genetics and complexity of allergy and asthma. Immunol Today 19:325-332

Barnes PJ (2000): Mechanisms in COPD: differences from asthma. Chest 117:10s-14s. doi:10.1378/chest.117.2_suppl.10s

Barnes PJ (2008): Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol 8:183-192. doi:10.1038/nri2254

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004): Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 340:783-795. doi:10.1016/j.jmb.2004.05.028

Berry KN, Brett TJ (2020): Structural and Biophysical Analysis of the CLCA1 VWA Domain Suggests Mode of TMEM16A Engagement. Cell Rep 30:1141-1151.e1143. doi:10.1016/j.celrep.2019.12.059

Bice DE, Seagrave J, Green FH (2000): Animal models of asthma: potential usefulness for studying health effects of inhaled particles. Inhal Toxicol 12:829-862. doi:10.1080/08958370050123207

Björkstén B (2009): The hygiene hypothesis: do we still believe in it? Nestle Nutr Workshop Ser Pediatr Program 64:11-18; discussion 18-22, 251-257. doi:10.1159/000235780

Bothe MK, Braun J, Mundhenk L, Gruber AD (2008): Murine mCLCA6 is an integral apical membrane protein of non-goblet cell enterocytes and co-localizes with the cystic fibrosis transmembrane conductance regulator. J Histochem Cytochem 56:495-509. doi:10.1369/jhc.2008.950592

Bothe MK, Mundhenk L, Kaup M, Weise C, Gruber AD (2011): The murine goblet cell protein mCLCA3 is a zinc-dependent metalloprotease with autoproteolytic activity. Mol Cells 32:535-541. doi:10.1007/s10059-011-0158-8

Bothe MK, Mundhenk L, Beck CL, Kaup M, Gruber AD (2012): Impaired autoproteolytic cleavage of mCLCA6, a murine integral membrane protein expressed in enterocytes, leads to cleavage at the plasma membrane instead of the endoplasmic reticulum. Mol Cells 33:251-257. doi:10.1007/s10059-012-2217-1

Boucher RC (2007): Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. Annu Rev Med 58:157-170. doi:10.1146/annurev.med.58.071905.105316

Braun J, Bothe MK, Mundhenk L, Beck CL, Gruber AD (2010a): Murine mCLCA5 is expressed in granular layer keratinocytes of stratified epithelia. Histochem Cell Biol 133:285-299. doi:10.1007/s00418-009-0667-0

Braun J, Mundhenk L, Range F, Gruber AD (2010b): Quantitative expression analyses of candidates for alternative anion conductance in cystic fibrosis mouse models. J Cyst Fibros 9:351-364. doi:10.1016/j.jcf.2010.06.003

Britto CJ, Cohn L (2015): Bactericidal/Permeability-increasing protein fold-containing family member A1 in airway host protection and respiratory disease. Am J Respir Cell Mol Biol 52:525-534. doi:10.1165/rcmb.2014-0297RT

Brouillard F, Bensalem N, Hinzpeter A, Tondelier D, Trudel S, Gruber AD, Ollero M, Edelman A (2005): Blue native/SDS-PAGE analysis reveals reduced expression of the mClCA3 protein in cystic fibrosis knock-out mice. Mol Cell Proteomics 4:1762-1775. doi:10.1074/mcp.M500098-MCP200

Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E, Pfeffer U, Ravazzolo R, Zegarra-Moran O, Galietta LJ (2008): TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. Science 322:590-594. doi:10.1126/science.1163518

Caramori G, Di Gregorio C, Carlstedt I, Casolari P, Guzzinati I, Adcock IM, Barnes PJ, Ciaccia A, Cavallesco G, Chung KF, Papi A (2004): Mucin expression in peripheral airways of patients with chronic obstructive pulmonary disease. Histopathology 45:477-484. doi:10.1111/j.1365-2559.2004.01952.x

Carter WG, Kaur P, Gil SG, Gahr PJ, Wayner EA (1990): Distinct functions for integrins alpha 3 beta 1 in focal adhesions and alpha 6 beta 4/bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. J Cell Biol 111:3141-3154. doi:10.1083/jcb.111.6.3141

Ching JC, Lobanova L, Loewen ME (2013): Secreted hCLCA1 is a signaling molecule that activates airway macrophages. PLoS One 8:e83130. doi:10.1371/journal.pone.0083130

Choi JY, Muallem D, Kiselyov K, Lee MG, Thomas PJ, Muallem S (2001): Aberrant CFTR-dependent HCO3- transport in mutations associated with cystic fibrosis. Nature 410:94-97. doi:10.1038/35065099

Chojnacki S, Cowley A, Lee J, Foix A, Lopez R (2017): Programmatic access to bioinformatics tools from EMBL-EBI update: 2017. Nucleic Acids Res 45:W550-w553. doi:10.1093/nar/gkx273

Clarke LL, Grubb BR, Yankaskas JR, Cotton CU, McKenzie A, Boucher RC (1994): Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride conductance to organ-level disease in Cftr(-/-) mice. Proc Natl Acad Sci U S A 91:479-483

Cockcroft DW (2018): Environmental Causes of Asthma. Semin Respir Crit Care Med 39:12-18. doi:10.1055/s-0037-1606219

REFERENCES

Cohn LA, DeClue AE, Cohen RL, Reinero CR (2010): Effects of fluticasone propionate dosage in an experimental model of feline asthma. J Feline Med Surg 12:91-96. doi:10.1016/j.jfms.2009.05.024

Collaco JM, Vanscoy L, Bremer L, McDougal K, Blackman SM, Bowers A, Naughton K, Jennings J, Ellen J, Cutting GR (2008): Interactions between secondhand smoke and genes that affect cystic fibrosis lung disease. Jama 299:417-424. doi:10.1001/jama.299.4.417

Connon CJ, Yamasaki K, Kawasaki S, Quantock AJ, Koizumi N, Kinoshita S (2004): Calcium-activated chloride channel-2 in human epithelia. J Histochem Cytochem 52:415-418. doi:10.1177/002215540405200313

Connon CJ, Kawasaki S, Yamasaki K, Quantock AJ, Kinoshita S (2005): The quantification of hCLCA2 and colocalisation with integrin beta4 in stratified human epithelia. Acta Histochem 106:421-425. doi:10.1016/j.acthis.2004.08.003

Corcoran BM, Foster DJ, Fuentes VL (1995): Feline asthma syndrome: a retrospective study of the clinical presentation in 29 cats. J Small Anim Pract 36:481-488. doi:10.1111/j.1748-5827.1995.tb02787.x

Couetil LL, Cardwell JM, Gerber V, Lavoie JP, Leguillette R, Richard EA (2016): Inflammatory Airway Disease of Horses--Revised Consensus Statement. J Vet Intern Med 30:503-515. doi:10.1111/jvim.13824

Cunningham SA, Awayda MS, Bubien JK, Ismailov, II, Arrate MP, Berdiev BK, Benos DJ, Fuller CM (1995): Cloning of an epithelial chloride channel from bovine trachea. J Biol Chem 270:31016-31026

Daser A, Daheshia M, De Sanctis GT (2001): Genetics of allergen-induced asthma. J Allergy Clin Immunol 108:167-174. doi:10.1067/mai.2001.116987

Davis E, Rush BR (2002): Equine recurrent airway obstruction: pathogenesis, diagnosis, and patient management. Vet Clin North Am Equine Pract 18:453-467, vi. doi:10.1016/s0749-0739(02)00026-3

DDBJ (2015): Bioinformation and DDBJ Center. Center for Information Biology and DNA Data Bank of Japan. Accessed 01/31/2021 from https://www.ddbj.nig.ac.jp/faq/en/explain-three-symbols-e.html

De Boeck K (2020): Cystic fibrosis in the year 2020: A disease with a new face. Acta Paediatr 109:893-899. doi:10.1111/apa.15155

de Sousa Abreu R, Penalva LO, Marcotte EM, Vogel C (2009): Global signatures of protein and mRNA expression levels. Mol Biosyst 5:1512-1526. doi:10.1039/b908315d

Dellmann HE, J (1998): Textbook of Veterinary Histology. 5 edn, Philadelphia: Lippincott Williams & Wilkins – ISBN: 978-0683301687

Dietert K, Reppe K, Mundhenk L, Witzenrath M, Gruber AD (2014): mCLCA3 modulates IL-17 and CXCL-1 induction and leukocyte recruitment in murine Staphylococcus aureus pneumonia. PLoS One 9:e102606. doi:10.1371/journal.pone.0102606

Dietert K, Mundhenk L, Erickson NA, Reppe K, Hocke AC, Kummer W, Witzenrath M, Gruber AD (2015): Murine CLCA5 is uniquely expressed in distinct niches of airway epithelial cells. Histochem Cell Biol 143:277-287. doi:10.1007/s00418-014-1279-x

Dodge JA, Morison S, Lewis PA, Coles EC, Geddes D, Russell G, Littlewood JM, Scott MT (1997): Incidence, population, and survival of cystic fibrosis in the UK, 1968-95. UK Cystic Fibrosis Survey Management Committee. Arch Dis Child 77:493-496

Dye JA, McKiernan BC, Rozanski EA, Hoffmann WE, Losonsky JM, Homco LD, Weisiger RM, Kakoma I (1996): Bronchopulmonary disease in the cat: historical, physical, radiographic, clinicopathologic, and pulmonary functional evaluation of 24 affected and 15 healthy cats. J Vet Intern Med 10:385-400. doi:10.1111/j.1939-1676.1996.tb02086.x

Eggermont J (2004): Calcium-activated chloride channels: (un)known, (un)loved? Proc Am Thorac Soc 1:22-27. doi:10.1513/pats.2306010

Elble RC, Widom J, Gruber AD, Abdel-Ghany M, Levine R, Goodwin A, Cheng HC, Pauli BU (1997): Cloning and characterization of lung-endothelial cell adhesion molecule-1 suggest it is an endothelial chloride channel. J Biol Chem 272:27853-27861

Elble RC, Ji G, Nehrke K, DeBiasio J, Kingsley PD, Kotlikoff MI, Pauli BU (2002): Molecular and functional characterization of a murine calcium-activated chloride channel expressed in smooth muscle. J Biol Chem 277:18586-18591. doi:10.1074/jbc.M200829200

Elble RC, Walia V, Cheng HC, Connon CJ, Mundhenk L, Gruber AD, Pauli BU (2006): The putative chloride channel hCLCA2 has a single C-terminal transmembrane segment. J Biol Chem 281:29448-29454. doi:10.1074/jbc.M605919200

EPR-3 (2007): Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report 2007. J Allergy Clin Immunol 120:S94-138. doi:10.1016/j.jaci.2007.09.043

Erickson NA, Nystrom EE, Mundhenk L, Arike L, Glauben R, Heimesaat MM, Fischer A, Bereswill S, Birchenough GM, Gruber AD, Johansson ME (2015): The Goblet Cell Protein Clca1 (Alias mClca3 or Gob-5) Is Not Required for Intestinal Mucus Synthesis, Structure and Barrier Function in Naive or DSS-Challenged Mice. PLoS One 10:e0131991. doi:10.1371/journal.pone.0131991

Erickson NA, Dietert K, Enders J, Glauben R, Nouailles G, Gruber AD, Mundhenk L (2018): Soluble mucus component CLCA1 modulates expression of leukotactic cytokines and BPIFA1 in murine alveolar macrophages but not in bone marrow-derived macrophages. Histochem Cell Biol 149:619-633. doi:10.1007/s00418-018-1664-y

REFERENCES

Evans SR, Thoreson WB, Beck CL (2004): Molecular and functional analyses of two new calcium-activated chloride channel family members from mouse eye and intestine. J Biol Chem 279:41792-41800. doi:10.1074/jbc.M408354200

Fernandez-Blanco JA, Fakih D, Arike L, Rodriguez-Pineiro AM, Martinez-Abad B, Skansebo E, Jackson S, Root J, Singh D, McCrae C, Evans CM, Astrand A, Ermund A, Hansson GC (2018): Attached stratified mucus separates bacteria from the epithelial cells in COPD lungs. JCI Insight 3. doi:10.1172/jci.insight.120994

Foster SF, Allan GS, Martin P, Robertson ID, Malik R (2004): Twenty-five cases of feline bronchial disease (1995-2000). J Feline Med Surg 6:181-188. doi:10.1016/j.jfms.2003.12.008

Furuya M, Kirschbaum SB, Paulovich A, Pauli BU, Zhang H, Alexander JS, Farr AG, Ruddell A (2010): Lymphatic endothelial murine chloride channel calcium-activated 1 is a ligand for leukocyte LFA-1 and Mac-1. J Immunol 185:5769-5777. doi:10.4049/jimmunol.1002226

Gandhi R, Elble RC, Gruber AD, Schreur KD, Ji HL, Fuller CM, Pauli BU (1998): Molecular and functional characterization of a calcium-sensitive chloride channel from mouse lung. J Biol Chem 273:32096-32101. doi:10.1074/jbc.273.48.32096

Gao H, Ying S, Dai Y (2017): Pathological Roles of Neutrophil-Mediated Inflammation in Asthma and Its Potential for Therapy as a Target. J Immunol Res 2017:3743048. doi:10.1155/2017/3743048

Gerber V, Robinson NE, Venta RJ, Rawson J, Jefcoat AM, Hotchkiss JA (2003): Mucin genes in horse airways: MUC5AC, but not MUC2, may play a role in recurrent airway obstruction. Equine Vet J 35:252-257. doi:10.2746/042516403776148291

Gibson A, Lewis AP, Affleck K, Aitken AJ, Meldrum E, Thompson N (2005): hCLCA1 and mCLCA3 are secreted non-integral membrane proteins and therefore are not ion channels. J Biol Chem 280:27205-27212. doi:10.1074/jbc.M504654200

Gray MA, Winpenny JP, Porteous DJ, Dorin JR, Argent BE (1994): CFTR and calcium-activated chloride currents in pancreatic duct cells of a transgenic CF mouse. Am J Physiol 266:C213-221. doi:10.1152/ajpcell.1994.266.1.C213

Grotheer M, Hirschberger J, Hartmann K, Castelletti N, Schulz B (2020): Comparison of signalment, clinical, laboratory and radiographic parameters in cats with feline asthma and chronic bronchitis. J Feline Med Surg 22:649-655. doi:10.1177/1098612x19872428

Gruber AD, Elble RC, Ji HL, Schreur KD, Fuller CM, Pauli BU (1998a): Genomic cloning, molecular characterization, and functional analysis of human CLCA1, the first human member of the family of Ca2+-activated Cl- channel proteins. Genomics 54:200-214. doi:10.1006/geno.1998.5562

Gruber AD, Gandhi R, Pauli BU (1998b): The murine calcium-sensitive chloride channel (mCaCC) is widely expressed in secretory epithelia and in other select tissues. Histochem Cell Biol 110:43-49. doi:10.1007/s004180050263

Gruber AD, Pauli BU (1999): Molecular cloning and biochemical characterization of a truncated, secreted member of the human family of Ca2+-activated Cl- channels. Biochim Biophys Acta 1444:418-423. doi:10.1016/s0167-4781(99)00008-1

Gruber AD, Schreur KD, Ji HL, Fuller CM, Pauli BU (1999): Molecular cloning and transmembrane structure of hCLCA2 from human lung, trachea, and mammary gland. Am J Physiol 276:C1261-1270

Gruber AD, Fuller CM, Elble RC, Benos DJ, Pauli BU (2000): The CLCA Gene Family A Novel Family of Putative Chloride Channels. Current Genomics 1:201 - 222. doi:10.2174/1389202003351526

Guillen J (2012): FELASA guidelines and recommendations. J Am Assoc Lab Anim Sci 51:311-321

Gustafsson JK, Ermund A, Ambort D, Johansson ME, Nilsson HE, Thorell K, Hebert H, Sjovall H, Hansson GC (2012): Bicarbonate and functional CFTR channel are required for proper mucin secretion and link cystic fibrosis with its mucus phenotype. J Exp Med 209:1263-1272. doi:10.1084/jem.20120562

Hamann M, Gibson A, Davies N, Jowett A, Walhin JP, Partington L, Affleck K, Trezise D, Main M (2009): Human ClCa1 modulates anionic conduction of calcium-dependent chloride currents. J Physiol 587:2255-2274. doi:10.1113/jphysiol.2009.170159

Hauber HP, Manoukian JJ, Nguyen LH, Sobol SE, Levitt RC, Holroyd KJ, McElvaney NG, Griffin S, Hamid Q (2003): Increased expression of interleukin-9, interleukin-9 receptor, and the calcium-activated chloride channel hCLCA1 in the upper airways of patients with cystic fibrosis. Laryngoscope 113:1037-1042. doi:10.1097/00005537-200306000-00022

Hauber HP, Tsicopoulos A, Wallaert B, Griffin S, McElvaney NG, Daigneault P, Mueller Z, Olivenstein R, Holroyd KJ, Levitt RC, Hamid Q (2004): Expression of HCLCA1 in cystic fibrosis lungs is associated with mucus overproduction. Eur Respir J 23:846-850

Hauber HP, Lavigne F, Hung HL, Levitt RC, Hamid Q (2010): Effect of Th2 type cytokines on hCLCA1 and mucus expression in cystic fibrosis airways. J Cyst Fibros 9:277-279. doi:10.1016/j.jcf.2010.05.002

Hegab AE, Sakamoto T, Uchida Y, Nomura A, Ishii Y, Morishima Y, Mochizuki M, Kimura T, Saitoh W, Massoud HH, Massoud HM, Hassanein KM, Sekizawa K (2004): CLCA1 gene polymorphisms in chronic obstructive pulmonary disease. J Med Genet 41:e27

REFERENCES

Herszberg B, Ramos-Barbón D, Tamaoka M, Martin JG, Lavoie JP (2006): Heaves, an asthma-like equine disease, involves airway smooth muscle remodeling. J Allergy Clin Immunol 118:382-388. doi:10.1016/j.jaci.2006.03.044

Hirokawa T, Boon-Chieng S, Mitaku S (1998): SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics 14:378-379. doi:10.1093/bioinformatics/14.4.378

Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, Paré PD (2004): The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med 350:2645-2653. doi:10.1056/NEJMoa032158

Hooper NM (1994): Families of zinc metalloproteases. FEBS Lett 354:1-6

Hoshino M, Morita S, Iwashita H, Sagiya Y, Nagi T, Nakanishi A, Ashida Y, Nishimura O, Fujisawa Y, Fujino M (2002): Increased expression of the human Ca2+-activated Cl-channel 1 (CaCC1) gene in the asthmatic airway. Am J Respir Crit Care Med 165:1132-1136. doi:10.1164/ajrccm.165.8.2107068

Hovenberg HW, Davies JR, Herrmann A, Lindén CJ, Carlstedt I (1996): MUC5AC, but not MUC2, is a prominent mucin in respiratory secretions. Glycoconj J 13:839-847. doi:10.1007/bf00702348

Huan C, Greene KS, Shui B, Spizz G, Sun H, Doran RM, Fisher PJ, Roberson MS, Elble RC, Kotlikoff MI (2008): mCLCA4 ER processing and secretion requires luminal sorting motifs. Am J Physiol Cell Physiol 295:C279-287. doi:10.1152/ajpcell.00060.2008

Illi S, von Mutius E, Lau S, Bergmann R, Niggemann B, Sommerfeld C, Wahn U (2001): Early childhood infectious diseases and the development of asthma up to school age: a birth cohort study. Bmj 322:390-395. doi:10.1136/bmj.322.7283.390

Janeway CJT, P; Walport, M (2001): Immunobiology: The Immune System in Health and Disease. 5 edn, New York: Garland Science – ISBN: 978-0-8153-3642-6

Jeffery PK (2000): Comparison of the structural and inflammatory features of COPD and asthma. Giles F. Filley Lecture. Chest 117:251s-260s. doi:10.1378/chest.117.5_suppl_1.251s

Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC (2008): The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A 105:15064-15069. doi:10.1073/pnas.0803124105

Käll L, Krogh A, Sonnhammer EL (2004): A combined transmembrane topology and signal peptide prediction method. J Mol Biol 338:1027-1036. doi:10.1016/j.jmb.2004.03.016

Käll L, Krogh A, Sonnhammer EL (2007): Advantages of combined transmembrane topology and signal peptide prediction--the Phobius web server. Nucleic Acids Res 35:W429-432. doi:10.1093/nar/gkm256

Kamada F, Suzuki Y, Shao C, Tamari M, Hasegawa K, Hirota T, Shimizu M, Takahashi N, Mao XQ, Doi S, Fujiwara H, Miyatake A, Fujita K, Chiba Y, Aoki Y, Kure S, Tamura G, Shirakawa T, Matsubara Y (2004): Association of the hCLCA1 gene with childhood and adult asthma. Genes Immun 5:540-547. doi:10.1038/sj.gene.6364124

Keith BA, Ching JCH, Loewen ME (2019): Von Willebrand Factor Type A domain of hCLCA1 is sufficient for U-937 macrophage activation. Biochem Biophys Rep 18:100630. doi:10.1016/j.bbrep.2019.100630

Kim HY, DeKruyff RH, Umetsu DT (2010): The many paths to asthma: phenotype shaped by innate and adaptive immunity. Nat Immunol 11:577-584. doi:10.1038/ni.1892

Kolbe EW, Tamm S, Hedtfeld S, Becker T, Tummler B, Stanke F (2013): CLCA4 variants determine the manifestation of the cystic fibrosis basic defect in the intestine. Eur J Hum Genet 21:691-694. doi:10.1038/ejhg.2012.234

Komiya T, Tanigawa Y, Hirohashi S (1999): Cloning and identification of the gene gob-5, which is expressed in intestinal goblet cells in mice. Biochem Biophys Res Commun 255:347-351. doi:10.1006/bbrc.1999.0168

Kurata K, Masuda K, Sakaguchi M, Ohno K, Tsujimoto H (2002): Reactivity to common allergens in spontaneous feline asthma. Journal of Allergy and Clinical Immunology 109:26. doi:10.1016/S0091-6749(02)81156-3

Lavoie JP (2020): Equine Asthma, review and novel findings on inflammation and anti-inflammatory therapy Tagungsband 2 / Rackwitz R, OPees M, Aschenbach JR et al. (eds): 10 Leipziger Tierärztekongress, Leipzig, Germany, 2020. vol Tagungsband 2. Leipziger Blaue Hefte (LBH), pp 163-165

Leclere M, Lavoie-Lamoureux A, Lavoie JP (2011): Heaves, an asthma-like disease of horses. Respirology 16:1027-1046. doi:10.1111/j.1440-1843.2011.02033.x

Lee D, Ha S, Kho Y, Kim J, Cho K, Baik M, Choi Y (1999): Induction of mouse Ca(2+)-sensitive chloride channel 2 gene during involution of mammary gland. Biochem Biophys Res Commun 264:933-937. doi:10.1006/bbrc.1999.1583

Leguillette R (2003): Recurrent airway obstruction – heaves. Vet Clin North Am Equine Pract 19:63-86, vi. doi:10.1016/s0749-0739(02)00067-6

Leverkoehne I, Gruber AD (2002): The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells. J Histochem Cytochem 50:829-838

Leverkoehne I, Holle H, Anton F, Gruber AD (2006): Differential expression of calcium-activated chloride channels (CLCA) gene family members in the small intestine of cystic fibrosis mouse models. Histochem Cell Biol 126:239-250. doi:10.1007/s00418-006-0164-7

REFERENCES

Loewen ME, Forsyth GW (2005): Structure and function of CLCA proteins. Physiol Rev 85:1061-1092. doi:10.1152/physrev.00016.2004

Long AJ, Sypek JP, Askew R, Fish SC, Mason LE, Williams CM, Goldman SJ (2006): Gob-5 contributes to goblet cell hyperplasia and modulates pulmonary tissue inflammation. Am J Respir Cell Mol Biol 35:357-365. doi:10.1165/rcmb.2005-0451OC

Lowell FC (1964): Observations on heaves. An Asthma-like sydrome in the horse. J Allergy 35:322-330. doi:10.1016/0021-8707(64)90095-4

Maier T, Güell M, Serrano L (2009): Correlation of mRNA and protein in complex biological samples. FEBS Lett 583:3966-3973. doi:10.1016/j.febslet.2009.10.036

Majo J, Ghezzo H, Cosio MG (2001): Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. Eur Respir J 17:946-953. doi:10.1183/09031936.01.17509460

Mall M, Gonska T, Thomas J, Schreiber R, Seydewitz HH, Kuehr J, Brandis M, Kunzelmann K (2003): Modulation of Ca2+-activated Cl- secretion by basolateral K+ channels in human normal and cystic fibrosis airway epithelia. Pediatr Res 53:608-618. doi:10.1203/01.Pdr.0000057204.51420.Dc

Mall MA, Danahay H, Boucher RC (2018): Emerging Concepts and Therapies for Mucoobstructive Lung Disease. Ann Am Thorac Soc 15:S216-s226. doi:10.1513/AnnalsATS.201806-368AW

McAuley DF, Elborn JS (2000): Cystic fibrosis: basic science. Paediatr Respir Rev 1:93-100. doi:10.1053/prrv.2000.0029

McClain S (2017): Bioinformatic screening and detection of allergen cross-reactive IgE-binding epitopes. Mol Nutr Food Res 61. doi:10.1002/mnfr.201600676

McFadden ER, Jr., Gilbert IA (1992): Asthma. N Engl J Med 327:1928-1937. doi:10.1056/nejm199212313272708

Mei L, He L, Wu SS, Zhang B, Xu YJ, Zhang ZX, Zhao JP, Zhang HL (2013): Murine calcium-activated chloride channel family member 3 induces asthmatic airway inflammation independently of allergen exposure. Chin Med J (Engl) 126:3283-3288

Meyerholz DK, Stoltz DA, Pezzulo AA, Welsh MJ (2010): Pathology of gastrointestinal organs in a porcine model of cystic fibrosis. Am J Pathol 176:1377-1389. doi:10.2353/ajpath.2010.090849

Mitaku S, Hirokawa T (1999): Physicochemical factors for discriminating between soluble and membrane proteins: hydrophobicity of helical segments and protein length. Protein Eng 12:953-957. doi:10.1093/protein/12.11.953

Mitaku S, Hirokawa T, Tsuji T (2002): Amphiphilicity index of polar amino acids as an aid in the characterization of amino acid preference at membrane-water interfaces. Bioinformatics 18:608-616. doi:10.1093/bioinformatics/18.4.608

Moise NS, Wiedenkeller D, Yeager AE, Blue JT, Scarlett J (1989): Clinical, radiographic, and bronchial cytologic features of cats with bronchial disease: 65 cases (1980-1986). J Am Vet Med Assoc 194:1467-1473

Moriello KA, Stepien RL, Henik RA, Wenholz LJ (2007): Pilot study: prevalence of positive aeroallergen reactions in 10 cats with small-airway disease without concurrent skin disease. Vet Dermatol 18:94-100. doi:10.1111/j.1365-3164.2007.00573.x

Mukherjee AB, Zhang Z (2011): Allergic asthma: influence of genetic and environmental factors. J Biol Chem 286:32883-32889. doi:10.1074/jbc.R110.197046

Mundhenk L, Alfalah M, Elble RC, Pauli BU, Naim HY, Gruber AD (2006): Both cleavage products of the mCLCA3 protein are secreted soluble proteins. J Biol Chem 281:30072-30080. doi:10.1074/jbc.M606489200

Mundhenk L, Johannesson B, Anagnostopoulou P, Braun J, Bothe MK, Schultz C, Mall MA, Gruber AD (2012): mCLCA3 does not contribute to calcium-activated chloride conductance in murine airways. Am J Respir Cell Mol Biol 47:87-93. doi:10.1165/rcmb.2010-0508OC

Mundhenk L, Erickson NA, Klymiuk N, Gruber AD (2018): Interspecies diversity of chloride channel regulators, calcium-activated 3 genes. PLoS One 13:e0191512. doi:10.1371/journal.pone.0191512

Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, Fujisawa Y, Nishimura O, Fujino M (2001): Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. Proc Natl Acad Sci U S A 98:5175-5180. doi:10.1073/pnas.081510898

Nakano T, Inoue H, Fukuyama S, Matsumoto K, Matsumura M, Tsuda M, Matsumoto T, Aizawa H, Nakanishi Y (2006): Niflumic acid suppresses interleukin-13-induced asthma phenotypes. Am J Respir Crit Care Med 173:1216-1221. doi:10.1164/rccm.200410-1420OC

NCBI (2017): calcium-activated chloride channel regulator 4, partial [Felis catus]. National Center for Biotechnology Information, U.S. National Library of Medicine. Accessed 05/03/2020 from https://www.ncbi.nlm.nih.gov/protein/1304916858

Newcomb DC, Peebles RS, Jr. (2013): Th17-mediated inflammation in asthma. Curr Opin Immunol 25:755-760. doi:10.1016/j.coi.2013.08.002

Nielsen H, Engelbrecht J, Brunak S, von Heijne G (1997): Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng 10:1-6. doi:10.1093/protein/10.1.1

Norris Reinero CR, Decile KC, Berghaus RD, Williams KJ, Leutenegger CM, Walby WF, Schelegle ES, Hyde DM, Gershwin LJ (2004): An experimental model of allergic

asthma in cats sensitized to house dust mite or bermuda grass allergen. Int Arch Allergy Immunol 135:117-131. doi:10.1159/000080654

Nystrom EEL, Birchenough GMH, van der Post S, Arike L, Gruber AD, Hansson GC, Johansson MEV (2018): Calcium-activated Chloride Channel Regulator 1 (CLCA1) Controls Mucus Expansion in Colon by Proteolytic Activity. EBioMedicine 33:134-143. doi:10.1016/j.ebiom.2018.05.031

Nystrom EEL, Arike L, Ehrencrona E, Hansson GC, Johansson MEV (2019): Calcium-activated chloride channel regulator 1 (CLCA1) forms non-covalent oligomers in colonic mucus and has mucin 2-processing properties. J Biol Chem 294:17075-17089. doi:10.1074/jbc.RA119.009940

Oppenheimer EH, Esterly JR (1975): Pathology of cystic fibrosis review of the literature and comparison with 146 autopsied cases. Perspect Pediatr Pathol 2:241-278

Patel AC, Morton JD, Kim EY, Alevy Y, Swanson S, Tucker J, Huang G, Agapov E, Phillips TE, Fuentes ME, Iglesias A, Aud D, Allard JD, Dabbagh K, Peltz G, Holtzman MJ (2006): Genetic segregation of airway disease traits despite redundancy of calcium-activated chloride channel family members. Physiol Genomics 25:502-513. doi:10.1152/physiolgenomics.00321.2005

Patel AC, Brett TJ, Holtzman MJ (2009): The role of CLCA proteins in inflammatory airway disease. Annu Rev Physiol 71:425-449. doi:10.1146/annurev.physiol.010908.163253

Pawlowski K, Lepisto M, Meinander N, Sivars U, Varga M, Wieslander E (2006): Novel conserved hydrolase domain in the CLCA family of alleged calcium-activated chloride channels. Proteins 63:424-439. doi:10.1002/prot.20887

Pierleoni A, Martelli PL, Casadio R (2008): PredGPI: a GPI-anchor predictor. BMC Bioinformatics 9:392. doi:10.1186/1471-2105-9-392

Plog S, Mundhenk L, Klymiuk N, Gruber AD (2009): Genomic, tissue expression, and protein characterization of pCLCA1, a putative modulator of cystic fibrosis in the pig. J Histochem Cytochem 57:1169-1181. doi:10.1369/jhc.2009.954594

Plog S, Mundhenk L, Bothe MK, Klymiuk N, Gruber AD (2010): Tissue and cellular expression patterns of porcine CFTR: similarities to and differences from human CFTR. J Histochem Cytochem 58:785-797. doi:10.1369/jhc.2010.955377

Plog S, Grotzsch T, Klymiuk N, Kobalz U, Gruber AD, Mundhenk L (2012a): The porcine chloride channel calcium-activated family member pCLCA4a mirrors lung expression of the human hCLCA4. J Histochem Cytochem 60:45-56. doi:10.1369/0022155411426455

Plog S, Mundhenk L, Langbein L, Gruber AD (2012b): Synthesis of porcine pCLCA2 protein during late differentiation of keratinocytes of epidermis and hair follicle inner root sheath. Cell Tissue Res 350:445-453. doi:10.1007/s00441-012-1482-9

Plog S, Klymiuk N, Binder S, Van Hook MJ, Thoreson WB, Gruber AD, Mundhenk L (2015): Naturally Occurring Deletion Mutants of the Pig-Specific, Intestinal Crypt Epithelial Cell Protein CLCA4b without Apparent Phenotype. PLoS One 10:e0140050. doi:10.1371/journal.pone.0140050

Quinton PM (1999): Physiological basis of cystic fibrosis: a historical perspective. Physiol Rev 79:S3-s22

Ran S, Benos DJ (1991): Isolation and functional reconstitution of a 38-kDa chloride channel protein from bovine tracheal membranes. J Biol Chem 266:4782-4788

Ran S, Benos DJ (1992): Immunopurification and structural analysis of a putative epithelial Cl-channel protein isolated from bovine trachea. J Biol Chem 267:3618-3625

Range F, Mundhenk L, Gruber AD (2007): A soluble secreted glycoprotein (eCLCA1) is overexpressed due to goblet cell hyperplasia and metaplasia in horses with recurrent airway obstruction. Vet Pathol 44:901-911. doi:10.1354/vp.44-6-901

Ranivand L, Otto C Feline asthma trends in Philadelphia, Pennsylvania 1896–2007. / 26th Annual Symposium of the Veterinary Comparative Respiratory Society, Oklahoma City, OK, 2008.

Ratjen F, Döring G (2003): Cystic fibrosis. Lancet 361:681-689. doi:10.1016/s0140-6736(03)12567-6

Redd SC (2002): Asthma in the United States: burden and current theories. Environ Health Perspect 110 Suppl 4:557-560. doi:10.1289/ehp.02110s4557

Reinero CR, DeClue AE, Rabinowitz P (2009): Asthma in humans and cats: is there a common sensitivity to aeroallegens in shared environments? Environ Res 109:634-640. doi:10.1016/j.envres.2009.02.001

Reinero CR (2011): Advances in the understanding of pathogenesis, and diagnostics and therapeutics for feline allergic asthma. Vet J 190:28-33. doi:10.1016/j.tvjl.2010.09.022

Reinero CR, Masseau I, Grobman M, Vientos-Plotts A, Williams K (2019): Perspectives in veterinary medicine: Description and classification of bronchiolar disorders in cats. J Vet Intern Med 33:1201-1221. doi:10.1111/jvim.15473

Ritzka M, Stanke F, Jansen S, Gruber AD, Pusch L, Woelfl S, Veeze HJ, Halley DJ, Tummler B (2004): The CLCA gene locus as a modulator of the gastrointestinal basic defect in cystic fibrosis. Hum Genet 115:483-491. doi:10.1007/s00439-004-1190-y

Robichaud A, Tuck SA, Kargman S, Tam J, Wong E, Abramovitz M, Mortimer JR, Burston HE, Masson P, Hirota J, Slipetz D, Kennedy B, O'Neill G, Xanthoudakis S (2005): Gob-5 is not essential for mucus overproduction in preclinical murine models of allergic asthma. Am J Respir Cell Mol Biol 33:303-314. doi:10.1165/rcmb.2004-0372OC

Rogers CS, Abraham WM, Brogden KA, Engelhardt JF, Fisher JT, McCray PB, Jr., McLennan G, Meyerholz DK, Namati E, Ostedgaard LS, Prather RS, Sabater JR, Stoltz DA,

REFERENCES

Zabner J, Welsh MJ (2008a): The porcine lung as a potential model for cystic fibrosis. Am J Physiol Lung Cell Mol Physiol 295:L240-263. doi:10.1152/ajplung.90203.2008

Rogers CS, Hao Y, Rokhlina T, Samuel M, Stoltz DA, Li Y, Petroff E, Vermeer DW, Kabel AC, Yan Z, Spate L, Wax D, Murphy CN, Rieke A, Whitworth K, Linville ML, Korte SW, Engelhardt JF, Welsh MJ, Prather RS (2008b): Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. J Clin Invest 118:1571-1577. doi:10.1172/jci34773

Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Uc A, Starner TD, Brogden KA, Shilyansky J, McCray PB, Jr., Zabner J, Prather RS, Welsh MJ (2008c): Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science 321:1837-1841. doi:10.1126/science.1163600

Romio L, Musante L, Cinti R, Seri M, Moran O, Zegarra-Moran O, Galietta LJ (1999): Characterization of a murine gene homologous to the bovine CaCC chloride channel. Gene 228:181-188. doi:10.1016/s0378-1119(98)00620-9

Roussa E, Wittschen P, Wolff NA, Torchalski B, Gruber AD, Thévenod F (2010): Cellular distribution and subcellular localization of mCLCA1/2 in murine gastrointestinal epithelia. J Histochem Cytochem 58:653-668. doi:10.1369/jhc.2010.955211

Rouze A, Cottereau A, Nseir S (2014): Chronic obstructive pulmonary disease and the risk for ventilator-associated pneumonia. Curr Opin Crit Care 20:525-531. doi:10.1097/mcc.0000000000000123

Rowe SM, Miller S, Sorscher EJ (2005): Cystic fibrosis. N Engl J Med 352:1992-2001. doi:10.1056/NEJMra043184

Rozmahel R, Wilschanski M, Matin A, Plyte S, Oliver M, Auerbach W, Moore A, Forstner J, Durie P, Nadeau J, Bear C, Tsui LC (1996): Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. Nat Genet 12:280-287. doi:10.1038/ng0396-280

Sala-Rabanal M, Yurtsever Z, Nichols CG, Brett TJ (2015): Secreted CLCA1 modulates TMEM16A to activate Ca(2+)-dependent chloride currents in human cells. Elife 4. doi:10.7554/eLife.05875

Sala-Rabanal M, Yurtsever Z, Berry KN, Nichols CG, Brett TJ (2017): Modulation of TMEM16A channel activity by the von Willebrand factor type A (VWA) domain of the calcium-activated chloride channel regulator 1 (CLCA1). J Biol Chem 292:9164-9174. doi:10.1074/jbc.M117.788232

Schäfer T, Merkl J, Klemm E, Wichmann HE, Ring J (2008): We and our pets: allergic together? Acta Vet Hung 56:153-161. doi:10.1556/AVet.56.2008.2.2

Schroeder BC, Cheng T, Jan YN, Jan LY (2008): Expression cloning of TMEM16A as a calcium-activated chloride channel subunit. Cell 134:1019-1029. doi:10.1016/j.cell.2008.09.003

Schütte A, Ermund A, Becker-Pauly C, Johansson ME, Rodriguez-Pineiro AM, Bäckhed F, Müller S, Lottaz D, Bond JS, Hansson GC (2014): Microbial-induced meprin β cleavage in MUC2 mucin and a functional CFTR channel are required to release anchored small intestinal mucus. Proc Natl Acad Sci U S A 111:12396-12401. doi:10.1073/pnas.1407597111

Schwiebert EM, Benos DJ, Fuller CM (1998): Cystic fibrosis: a multiple exocrinopathy caused by dysfunctions in a multifunctional transport protein. Am J Med 104:576-590

Sen R, Baltimore D (2006): Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 1986. 46: 705-716. J Immunol 177:7485-7496

Setlakwe EL, Lemos KR, Lavoie-Lamoureux A, Duguay JD, Lavoie JP (2014): Airway collagen and elastic fiber content correlates with lung function in equine heaves. Am J Physiol Lung Cell Mol Physiol 307:L252-260. doi:10.1152/ajplung.00019.2014

Shibly S, Klang A, Galler A, Schwendenwein I, Christian M, Guija A, Tichy A, Hirt RA (2014): Architecture and inflammatory cell composition of the feline lung with special consideration of eosinophil counts. J Comp Pathol 150:408-415. doi:10.1016/j.jcpa.2013.12.007

Silverman EK, Palmer LJ, Mosley JD, Barth M, Senter JM, Brown A, Drazen JM, Kwiatkowski DJ, Chapman HA, Campbell EJ, Province MA, Rao DC, Reilly JJ, Ginns LC, Speizer FE, Weiss ST (2002): Genomewide linkage analysis of quantitative spirometric phenotypes in severe early-onset chronic obstructive pulmonary disease. Am J Hum Genet 70:1229-1239. doi:10.1086/340316

Snapper JR (1986): Large animal models of asthma. Am Rev Respir Dis 133:351-352. doi:10.1164/arrd.1986.133.3.351

Song L, Liu D, Wu C, Wu S, Yang J, Ren F, Li Y (2013): Antibody to mCLCA3 suppresses symptoms in a mouse model of asthma. PLoS One 8:e82367. doi:10.1371/journal.pone.0082367

Steinke JW, Borish L, Rosenwasser LJ (2003): 5. Genetics of hypersensitivity. J Allergy Clin Immunol 111:S495-501

Stoltz DA, Meyerholz DK, Pezzulo AA, Ramachandran S, Rogan MP, Davis GJ, Hanfland RA, Wohlford-Lenane C, Dohrn CL, Bartlett JA, Nelson GAt, Chang EH, Taft PJ, Ludwig PS, Estin M, Hornick EE, Launspach JL, Samuel M, Rokhlina T, Karp PH, Ostedgaard LS, Uc A, Starner TD, Horswill AR, Brogden KA, Prather RS, Richter SS, Shilyansky J, McCray PB, Jr., Zabner J, Welsh MJ (2010): Cystic fibrosis pigs develop lung disease

and exhibit defective bacterial eradication at birth. Sci Transl Med 2:29ra31. doi:10.1126/scitranslmed.3000928

Stoltz DA, Meyerholz DK, Welsh MJ (2015): Origins of cystic fibrosis lung disease. N Engl J Med 372:351-362. doi:10.1056/NEJMra1300109

Stone KD, Prussin C, Metcalfe DD (2010): IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol 125:S73-80. doi:10.1016/j.jaci.2009.11.017

Talukdar AH, Calhoun ML, Stinson AW (1972): Microscopic anatomy of the skin of the horse. Am J Vet Res 33:2365-2390

Taraseviciene-Stewart L, Douglas IS, Nana-Sinkam PS, Lee JD, Tuder RM, Nicolls MR, Voelkel NF (2006): Is alveolar destruction and emphysema in chronic obstructive pulmonary disease an immune disease? Proc Am Thorac Soc 3:687-690. doi:10.1513/pats.200605-105SF

Teske K, Erickson NA, Mundhenk L, Glauben R, Dzamukova M, Löhning M, Gruber AD (2020): Murine CLCA4A and CLCA4B are expressed in partially different cellular niches of the intestinal epithelium. Journal of Comparative Pathology 174:172. doi:10.1016/j.jcpa.2019.10.105

Thai P, Chen Y, Dolganov G, Wu R (2005): Differential regulation of MUC5AC/Muc5ac and hCLCA-1/mGob-5 expression in airway epithelium. Am J Respir Cell Mol Biol 33:523-530. doi:10.1165/rcmb.2004-0220RC

Tliba O, Panettieri RA, Jr. (2019): Paucigranulocytic asthma: Uncoupling of airway obstruction from inflammation. J Allergy Clin Immunol 143:1287-1294. doi:10.1016/j.jaci.2018.06.008

Toda M, Tulic MK, Levitt RC, Hamid Q (2002): A calcium-activated chloride channel (HCLCA1) is strongly related to IL-9 expression and mucus production in bronchial epithelium of patients with asthma. J Allergy Clin Immunol 109:246-250

Trzil JE, Reinero CR (2014): Update on feline asthma. Vet Clin North Am Small Anim Pract 44:91-105. doi:10.1016/j.cvsm.2013.08.006

Tusnady GE, Simon I (1998): Principles governing amino acid composition of integral membrane proteins: application to topology prediction. J Mol Biol 283:489-506. doi:10.1006/jmbi.1998.2107

Tusnady GE, Simon I (2001): The HMMTOP transmembrane topology prediction server. Bioinformatics 17:849-850. doi:10.1093/bioinformatics/17.9.849

van der Doef HP, Slieker MG, Staab D, Alizadeh BZ, Seia M, Colombo C, van der Ent CK, Nickel R, Witt H, Houwen RH (2010): Association of the CLCA1 p.S357N variant with meconium ileus in European patients with cystic fibrosis. J Pediatr Gastroenterol Nutr 50:347-349. doi:10.1097/MPG.0b013e3181afce6c

Vankeerberghen A, Cuppens H, Cassiman JJ (2002): The cystic fibrosis transmembrane conductance regulator: an intriguing protein with pleiotropic functions. J Cyst Fibros 1:13-29

Vargas A, Roux-Dalvai F, Droit A, Lavoie JP (2016): Neutrophil-Derived Exosomes: A New Mechanism Contributing to Airway Smooth Muscle Remodeling. Am J Respir Cell Mol Biol 55:450-461. doi:10.1165/rcmb.2016-0033OC

Vestbo J, Anderson J, Brook RD, Calverley PM, Celli BR, Crim C, Haumann B, Martinez FJ, Yates J, Newby DE (2013): The Study to Understand Mortality and Morbidity in COPD (SUMMIT) study protocol. Eur Respir J 41:1017-1022. doi:10.1183/09031936.00087312

Vogel C, Marcotte EM (2012): Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat Rev Genet 13:227-232. doi:10.1038/nrg3185

von Mutius E (2007): Allergies, infections and the hygiene hypothesis--the epidemiological evidence. Immunobiology 212:433-439. doi:10.1016/j.imbio.2007.03.002

Wang K, Wen F-Q, Feng Y-L, Ou X-M, Xu D, Yang J, Deng Z-P (2007): Increased expression of human calcium-activated chloride channel 1 gene is correlated with mucus overproduction in Chinese asthmatic airway. Cell Biology International 31:1388-1395. doi:10.1016/j.cellbi.2007.06.004

Welsh MJ, Rogers CS, Stoltz DA, Meyerholz DK, Prather RS (2009): Development of a porcine model of cystic fibrosis. Trans Am Clin Climatol Assoc 120:149-162

Welsh MJ, Ramsey B.W., Accurso F., Cutting G.R. (2001) Cystic Fibrosis. In: The Metabolic and Molecular Bases of Inherited Disease,Valle D AS, Ballabio A, Beaudet AL, Mitchell, GA (ed), USA: McGraw-Hill – ISBN: 9780071459969

Whittaker CA, Hynes RO (2002): Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Mol Biol Cell 13:3369-3387. doi:10.1091/mbc.E02-05-0259

WHO (2008): COPD predicted to be third leading cause of death in 2030. World Health Organization. Accessed 25/06/2020 from https://www.who.int/respiratory/copd/World_Health_Statistics_2008/en/

WHO (2017): Chronic obstructive pulmonary disease (COPD). World Health Organization. Accessed 06/25/2020 from https://www.who.int/news-room/fact-sheets/detail/chronic-obstructive-pulmonary-disease-(copd)

WHO (2020): Asthma. World Health Organization. Accessed 06/25/2020 from https://www.who.int/news-room/q-a-detail/asthma

Williams K, Roman J (2016): Studying human respiratory disease in animals--role of induced and naturally occurring models. J Pathol 238:220-232. doi:10.1002/path.4658

REFERENCES

Wilschanski M, Corey M, Durie P, Tullis E, Bain J, Asch M, Ginzburg B, Jarvi K, Buckspan M, Hartwick W (1996): Diversity of reproductive tract abnormalities in men with cystic fibrosis. Jama 276:607-608

Wilschanski M, Durie PR (1998): Pathology of pancreatic and intestinal disorders in cystic fibrosis. J R Soc Med 91 Suppl 34:40-49

Winpenny JP, Lavery WL, Watson N, Chazot PL (2002): Biochemical and electrophysiological characterisation of the GOB5 (mCLCA3) chloride ion channel protein after expression in HEK293 cells. J Physiol 539

Woodruff PG, Boushey HA, Dolganov GM, Barker CS, Yang YH, Donnelly S, Ellwanger A, Sidhu SS, Dao-Pick TP, Pantoja C, Erle DJ, Yamamoto KR, Fahy JV (2007): Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. Proc Natl Acad Sci U S A 104:15858-15863. doi:10.1073/pnas.0707413104

Yamazaki J, Okamura K, Ishibashi K, Kitamura K (2005): Characterization of CLCA protein expressed in ductal cells of rat salivary glands. Biochim Biophys Acta 1715:132-144. doi:10.1016/j.bbamem.2005.08.001

Yang YD, Cho H, Koo JY, Tak MH, Cho Y, Shim WS, Park SP, Lee J, Lee B, Kim BM, Raouf R, Shin YK, Oh U (2008): TMEM16A confers receptor-activated calcium-dependent chloride conductance. Nature 455:1210-1215. doi:10.1038/nature07313

Yasuo M, Fujimoto K, Tanabe T, Yaegashi H, Tsushima K, Takasuna K, Koike T, Yamaya M, Nikaido T (2006): Relationship between calcium-activated chloride channel 1 and MUC5AC in goblet cell hyperplasia induced by interleukin-13 in human bronchial epithelial cells. Respiration 73:347-359. doi:10.1159/000091391

Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, Kamani MC, Allen HL, DeKievit TR, Gardner PR, Schwab U, Rowe JJ, Iglewski BH, McDermott TR, Mason RP, Wozniak DJ, Hancock RE, Parsek MR, Noah TL, Boucher RC, Hassett DJ (2002): Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev Cell 3:593-603. doi:10.1016/s1534-5807(02)00295-2

Young FD, Newbigging S, Choi C, Keet M, Kent G, Rozmahel RF (2007): Amelioration of cystic fibrosis intestinal mucous disease in mice by restoration of mCLCA3. Gastroenterology 133:1928-1937. doi:10.1053/j.gastro.2007.10.007

Yu Y, Walia V, Elble RC (2013): Loss of CLCA4 promotes epithelial-to-mesenchymal transition in breast cancer cells. PLoS One 8:e83943. doi:10.1371/journal.pone.0083943

Yurtsever Z, Sala-Rabanal M, Randolph DT, Scheaffer SM, Roswit WT, Alevy YG, Patel AC, Heier RF, Romero AG, Nichols CG, Holtzman MJ, Brett TJ (2012): Self-cleavage of human CLCA1 protein by a novel internal metalloprotease domain controls calcium-

activated chloride channel activation. J Biol Chem 287:42138-42149. doi:10.1074/jbc.M112.410282

Zhang HL, He L (2010): Overexpression of mclca3 in airway epithelium of asthmatic murine models with airway inflammation. Chin Med J (Engl) 123:1603-1606

Zhou Y, Dong Q, Louahed J, Dragwa C, Savio D, Huang M, Weiss C, Tomer Y, McLane MP, Nicolaides NC, Levitt RC (2001): Characterization of a calcium-activated chloride channel as a shared target of Th2 cytokine pathways and its potential involvement in asthma. Am J Respir Cell Mol Biol 25:486-491. doi:10.1165/ajrcmb.25.4.4578

Zhou Y, Shapiro M, Dong Q, Louahed J, Weiss C, Wan S, Chen Q, Dragwa C, Savio D, Huang M, Fuller C, Tomer Y, Nicolaides NC, McLane M, Levitt RC (2002): A calcium-activated chloride channel blocker inhibits goblet cell metaplasia and mucus overproduction. Novartis Found Symp 248:150-165; discussion 165-170, 277-182

Zhu DZ, Cheng CF, Pauli BU (1991): Mediation of lung metastasis of murine melanomas by a lung-specific endothelial cell adhesion molecule. Proc Natl Acad Sci U S A 88:9568-9572

7   Supplemental Material

Supplemental Material S1. Protein structure analysis and interspecies comparison of CLCA1

A) Multiple Sequence Alignment

```
mouse CLCA1    MESLKSPVFLLILHLLEGVLSESLIQLNNNGYEGIVIAIDHDVPEDEALIQHIKDMVTQA 60
pig   CLCA1    MRSFRSSLFILVLHLLEGARSNSLIQLNGNGYEGIVIAIDPNVPEDETLIQNIKDMVTKA 60
human CLCA1    MGPFKSSVFILILHLLEGALSNSLIQLNNNGYEGIVVAIDPNVPEDETLIQQIKDMVTQA 60
cat   CLCA1    MGSFKCSVFILVLHLLEGAVSKSLIQLNNNGYEGIVIAIDPNVPEDETLIQQIKDMVTQA 60
               *  ::. :*:*:*******. *:******.*******:*** :*****:***:******.*

mouse CLCA1    SPYLFEATGKRFYFKNVAILIPESWKAKPEYTRPKLETFKNADVLVSTTSPLGNDEPYTE 120
pig   CLCA1    SPYLFEATEKRFYFKNVAILIPASWKAKPEYVKPKLETYKNADVVVTEPNPPENDGPYTE 120
human CLCA1    SLYLLEATGKRFYFKNVAILIPETWKTKADYVRPKLETYKNADVLVAESTPPGNDEPYTE 120
cat   CLCA1    SPYLFEATERRFYFKKVAILIPEKWKTKPEYVRPKLETYKNADVLVAEPTAPGNDEPYTE 120
               * **:*** :*****:****** .**:* :*.:*****:*****:*:  .   ** ****

mouse CLCA1    HIGACGEKGIRIHLTPDFLAGKKLTQYGPQDRTFVHEWAHFRWGVFNEYNNDEKFYLSKG 180
pig   CLCA1    QMGNCGEKGEKIYFTPDFVAGKKVLQYGPQGRVFVHEWAHLRWGVFNEYNNEQKFYLSNK 180
human CLCA1    QMGNCGEKGERIHLTPDFIAGKKLAEYGPQGRAFVHEWAHLRWGVFDEYNNDEKFYLSNG 180
cat   CLCA1    QMGNCGEKGERIHFTPDFLAGKKLAQYGPQGRVFVHEWAHLRWGVYDEYNNNQKFYFSNG 180
               ::* ***** :*::****:****: :****.*.*******:****::****::***:*:

mouse CLCA1    KPQAVRCSAAITGKNQVRRCQGGSCITNGKCVIDRVTGLYKDNCVFVPDPHQNEKASIMF 240
pig   CLCA1    KNKPVICSAAIRGTNVLPQCQGGSCVTK-PCRADRVTGLFQKECEFIPDPQQSEKASIMY 239
human CLCA1    RIQAVRCSAGITGTNVVKKCQGGSCYTK-RCTFNKVTGLYEKGCEFVLQSRQTEKASIMF 239
cat   CLCA1    KRQAVRCSADITGKNVIKKCQGGSCVTK-SCKLDKVTGLYEEGCEFIPHGVQTVKASIMF 239
               : : * *** * *.* : :****** *:   *   ::.***::. * *: .   *. *****:

mouse CLCA1    NQNINSVVEFCTEKNHNQEAPNDQNQRCNLRSTWEVIQESEDFKQTTPMTAQPPAPTFSL 300
pig   CLCA1    AQSIESVVEFCKEKNHNKEAPNDQNQKCNLRSTWEVIQDSEDFKKTTPMTTQPPAPTFSL 299
human CLCA1    AQHVDSIVEFCTEQNHNKEAPNKQNQKCNLRSTWEVIRDSEDFKKTTPMTTQPPNPTFSL 299
cat   CLCA1    AQSIDSVVEFCTTEKNHNKEAPNLQNQKCNLRSTWEVISDSEDFKKTTPMTTQPPTPTFSL 299
               * ::*:**** .*:***.**** ***:********** :*****:*****:*** *****

mouse CLCA1    LQIGQRIVCLVLDKSGSMLNDDRLNRMNQASRLFLLQTVEQGSWVGMVTFDSAAYVQSEL 360
pig   CLCA1    LQIGQRIVCLVLDKSGSMTVGGRLKRLNQAGKLFLLQTVEQGAWVGMVAFDSAAYVKSEL 359
human CLCA1    LQIGQRIVCLVLDKSGSMATGNRLNRLNQAGQLFLLQTVELGSWVGMVTFDSAAHVQNEL 359
cat   CLCA1    LQIGQRIVCLVLDKSGSMANGDRLNRLNQAGKLFLLQTVEQGSWVGMVTFDSVAHVQSEL 359
               *****************  ..**:*:***.:******** *:*****:***.*:*:.**

mouse CLCA1    KQLNSGADRDLLIKHLPTVSAGGTSICSGLRTAFTVIKKKYPTDGSEIVLLTDGEDNTIS 420
pig   CLCA1    VQINSAAERDALARSLPTAASGGTSICSGLRSAFTVIKKKYPTDGSEIVLLTDGEDNTIS 419
human CLCA1    IQINSGSDRDTLAKRLPAAASGGTSICSGLRSAFTVIRKKYPTDGSEIVLLTDGEDNTIS 419
cat   CLCA1    IQINSDVERNALTRSLPTVAAGGTSICSGLRSAFAVIRKKYSTDGSEIVLLTDGEDNTIS 419
               *:**   :*: * : **:.::**********:**:**:*** *****************

mouse CLCA1    SCFDLVKQSGAIIHTVALGPAAAKELEQLSKMTGGLQTYSSDQVQNNGLVDAFAALSSGN 480
pig   CLCA1    ACFAEVKQSGAIIHTVALGPSAAKELEELSQMTGGLQTYASDQAENNGLIDAFGALSSGN 479
human CLCA1    GCFNEVKQSGAIIHTVALGPSAAQELEELSKMTGGLQTYASDQVQNNGLIDAFGALSSGN 479
cat           SCFNEVKQSGAIIHTVALGPSAAKELEELSKMTGGLQTYASDQAQNNGLIDAFGALSSGN 479
               .**  *************.**:***.**:**:*********:***.:****:***.******

mouse CLCA1    AAIAQHSIQLESRGVNLQNNQWMNGSVIVDSSVGKDTLFLITWTTHPPTIFIWDPSGVEQ 540
pig   CLCA1    GAVSQRSIQLESRGLTLQNNEWMNGTVVVDSTVGKDTLFLITWTDVPPQILLWDPSGTKQ 539
human CLCA1    GAVSQRSIQLESKGLTLQNSQWMNGTVIVDSTVGKDTLFLITWTMQPPQILLWDPSGQKQ 539
cat   CLCA1    GADSQRSIQLESKGLTLQNNQWMNGTVLVDSTVGKDTLFLITWTKQPPKILLWDPSKKKQ 539
               .* :*:*******:*:.***.:****:*:***:***.************  ** *::*****  :*

mouse CLCA1    NGFILDTTTKVAYLQVPGTAKVGFWKYSIQASSQTLTLTVTSRAASATLPPITVTPVVNK 600
pig   CLCA1    DSFLVDTHNKMAYLQVPGTAKVGMWKYSLQASSQTLTLTVSSRASSATLPPVTVTSKMNK 599
human CLCA1    GGFVVDKNTKMAYLQIPGIAKVGTWKYSLQASSQTLTLTVTSRASNATLPPITVTSKTNK 599
cat   CLCA1    DGFVVDTNTKMAYLQIPGTAKVGIWSYSLQASSQTLTLTVTSRPSSATVPPITVTSKINK 599
               ..*::*. .*:****:** **** *.**:*************:** :.**:**:*** **
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```
mouse CLCA1    NTGKFPSPVTVYASIRQGASPILRASVTALIESVNGKTVTLELLDNGAGADATKNDGVYS  660
pig   CLCA1    DTGKFPSPMVVYAKIHQGTLPILRAKVTALIESENGKTVTLELLDNGAGADATKNDGIYS  659
human CLCA1    DTSKFPSPLVVYANIRQGASPILRASVTALIESVNGKTVTLELLDNGAGADATKDDGVYS  659
cat   CLCA1    NTGKFPSAMTVYAKIHQGASPILRATVTAIIESVNGKTVTLELLDNGAGADATKDDGVYS  659
               :*.**** :.***.*:**: *****.***:*** ********************.**:**

mouse CLCA1    RFFTAFDANGRYSVKIWALGGVTSDRQRAAPPKN[RA]MYIDGWIEDGEVRMNPPRPETS--  718
pig   CLCA1    RYFTAYDANGRYSVKVWALGGVNTATQKATPPRS[GA]MYIRGWAENGEIKWNPPRPDINKD  719
human CLCA1    RYFTTYDTNGRYSVKVRALGGVNAARRRVIPQQS[GA]LYIPGWIENDEIQWNPPRPEINKD  719
cat   CLCA1    RYFTAYNANGRYSVKVWALGGINAATQRLIPQQN[GA]MYIPGWIENGEVNWNPPRPEINKD  719
               *:**:::*******: ****:.:  ::  * :. *:** ** *:.*:. *****: .

mouse CLCA1    YVQDKQLCFSRTSSGGSFVATNVPAAAPIPDLFPPCQITDLKASIQGQNLV**N**LTWTAPGD  778
pig   CLCA1    DLQGKQVCFSRTASGGSFVASDVPK-SPVPDLFPPCKITDLKAGIQGDNLI**N**LTWTAPGD  778
human CLCA1    DVQHKQVCFSRTSSGGSFVASDVPN-APIPDLFPPGQITDLKAEIHGGSLI**N**LTWTAPGD  778
cat   CLCA1    DPQSKQVCFSRTSSGGSFVASGVPQ-APIPDLFPPCQITDLKAKIHGYKFI**N**LTWTAPGD  778
               * **:*****:*******.**  :*:****** :****** *:* .::*********

mouse CLCA1    DYDHGRASNYIIRMSTSIVDLRDHFNTSLQVNTTGLIPKEASSEEIFEFELGGNTFG**N**GT  838
pig   CLCA1    DYDHGRADRYIIRISTNILDLRDKFNDSVQVNTTDLIPKEANSEEVFVFKPEGIPFT**N**GT  838
human CLCA1    DYDHGTAHKYIIRISTSILDLRDKFNESLQVNTTALIPKEANSEEVFLFKPE**N**ITFENGT  838
cat   CLCA1    DYDHGRAHKYIIRISANILDLRDKFNDSIQVNTSDLIPKEANSEEVFVFTPE**N**ITFENGT  838
               ***** * .****:*:.*:****:** *:****: ******.***:* *   * ***

mouse CLCA1    DIFIAIQAVDKSNLKSEISNIARVSVFIPAQEPPIPE----DSTPPCPDISI**N**STIPGIH  894
pig   CLCA1    DLFIAVQAVDKTNLKSEISNIAQVSLFLPPEAPPETPPETPAPSLPCPEIQV**N**STIPGIH  898
human CLCA1    DLFIAIQAVDKVDLKSEISNIARVSLFIPPQTPPETPSP-DETSAPCPNIHINSTIPGIH  897
cat   CLCA1    DLFIAVQAVDKANLKSEISNIARVSLFIPPEIPPEVPSP----SLPCPEINV**N**STIPGLH  894
               *:***:***** :*********:**:*:* : **       : ***:* :******:*

mouse CLCA1    VLKIMWKWLGEMQVTLGLH        913
pig   CLCA1    ILKIMWKWLGELQLSIA--        915
human CLCA1    ILKIMWKWIGELQLSIA--        914
cat   CLCA1    ILKIMWKWLGELQLSVALG        913
               :*******:**:*:::.
```

Figure S1A. Multiple Sequence Alignment (MSA) and interspecies comparison of the human, murine, porcine, and feline CLCA1 orthologs via Clustal Omega (1.2.4) (Chojnacki et al. 2017) (03.03.2019). Asterisks indicate perfect alignment, colons or semicolons indicate a site belonging to a group exhibiting strong or weak similarity, respectively (DDBJ 2015). For signal sequence (blue), conserved amino-terminal CLCA domain (n-CLCA, yellow), von Willebrand factor type A (vWA) domain (green), putative proteolytic cleavage site (red), and homologue-specific asparagine-linked glycosylation sites (bold, grey), also refer to S1B. Binding site of the porcine CLCA1 antibody p1-N-1ab-p, corresponding to aa CKEKNHNKEAPNDQN (green lettering) (Plog et al. 2009) and cross-reacting with the feline epitope CTEKNHNKEAPNLQN (green lettering, aa mismatches in red) (see 2.2).

B) CLCA1 *in silico* structure analysis and comparison

ortholog (accession number)	length	signal sequence	n-CLCA	vWA domain	putative proteolytic cleavage site, between aa	aa positions of glycosylation sites	transmembrane domains
mouse CLCA1 ENSMUST00000029919.6	913	1-21	25-290	309-415	695 and 696	504, 770, 836	none[1]
							none[2]
							soluble[3]
pig CLCA1 ENSSSCT00030015958.1	915	1-21	25-289	308-414	694 and 695	503, 770, 836, 891	none[1]
							none[2]
							soluble[3]
human CLCA1 ENST00000394711.2	914	1-21	25-289	308-414	694 and 695	294*, 503, 585, 770, 831, 890	8-25[1]
							none[2]
							soluble[3]
cat CLCA1 ENSFCAT00000023191.4	913	1-21	25-289	308-414	694 and 695	503, 770, 831, 887	6-25[1]
							none[2]
							soluble[3]

Table S1B. The respective sequence lengths were determined using NCBI Genbank (http://www.ncbi.nlm.nih.gov) (17.03.2019), the signal sequence according to SignalP 5.0 (Almagro Armenteros et al. 2019; Nielsen et al. 1997), the n-CLCA and vWA domains according to Ensembl (Pfam) (www.ensembl.org) (19.04.2020). The putative proteolytic cleavage site was identified by interspecies comparison of the aa sequences and homologue-specific asparagine-linked glycosylation sites were predicted via NetNglyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) (17.03.2019). *Proline occurs just after the asparagine residue which makes it highly unlikely that the Asparagine is glycosylated, presumably due to conformational constraints (http://www.cbs.dtu.dk/services/NetNGlyc/output.php). Potential transmembrane domains were excluded via [1]HMMtop (Tusnady and Simon 1998; 2001) (18.03.2019), [2]Phobius (Käll et al. 2004; 2007) (03.05.2020), and [3]SOSUI (Hirokawa et al. 1998; Mitaku and Hirokawa 1999; Mitaku et al. 2002) (03.05.2020) *in silico* tools.

Supplemental Figure S2. Protein structure analysis and interspecies comparison of CLCA2

A) Multiple Sequence Alignment

```
mouse CLCA2   MTHRDSTGPVIGLKLVTLLFTLSPELLFLGAGLKLKENGYDGLLVAINPRVPEDLKLITN 60
pig   CLCA2   MTPRDSAGPVRSLKFVTLLVALSPDLLRWGAGLQLRDNGYEELLVALHPQVSEDPSLIPH 60
human CLCA2   MTQRSIAGPICNLKFVTLLVALSSELPFLGAGVQLQDNGYNGLLIAINPQVPENQNLISN 60
cat   CLCA2   MTRRDNVGPVCSLKFVTLLVALIPELLFLGAGVQLQDNGYDGLLIAINPQVSEDQNLIPN 60
              ** *. .**: .**:****.:*  :*   ***::*::***: **:*::*:* *: .** :

mouse CLCA2   IKEMITEASFYLFNATKRRVFFRNVQILVPATWTDHNYSRVRQESYDKANVIVAEQSEEH 120
pig   CLCA2   IKEMITEASFYLFNATKRRVFFRSIKILIPATWTANNYSKVKQESYEKAQVLVTDWHAAP 120
human CLCA2   IKEMITEASFYLFNATKRRVFFRNIKILIPATWKANNNSKIKQESYEKANVIVTDWYGAH 120
cat   CLCA2   IKEMITEASFYLFNATKRRVFFRNIKILIPTTWKANNYSKVKQELYEKANVIVTDWYGAH 120
              *********************.::**:*:**. :* *:::** *:**:*:*::

mouse CLCA2   GDDPYTLQHRGCGQEGRYIHFTPSFLLNDELAAGYGARGRVFVHEWAHLRWGVFDEYNND 180
pig   CLCA2   GDSPHTLQYRGCGKEGKYIHFTPDFLLNDALTAGYGSRGRVFVHEWAHLRWGVFDEYNRE 180
human CLCA2   GDDPYTLQYRGCGKEGKYIHFTPNFLLNDNLTAGYGSRGRVFVHEWAHLRWGVFDEYNND 180
cat   CLCA2   GDDPYTLQYRGCGKEGKYIHFTSNFLLNDDLTAGYGPRGRVFVHEWAHLRWGVFDEYNNE 180
              **.*:***:****:**:***** .***** *:**** ********************.:

mouse CLCA2   KPFYVNGRNEIQVTRCSSDITGVFVCEKGLCPHEDCIISKIFREGCTFLYNSTQNATGSI 240
pig   CLCA2   KPFYINGQNQVKVTRCSSDITGIFVCEKGPCPQENCIISKLFKEGCMFLFNSTQHATASI 240
human CLCA2   KPFYINGQNQIKVTRCSSDITGIFVCEKGPCPQENCIISKLFKEGCTFIYNSTQNATASI 240
cat   CLCA2   KPFYINGQNQIKVTRCSSDITGIFVCEKGPCPQENCIISKLFKEGCMFIYNSTQNATASI 240
              ****:**:*::.:*******.:****** **.*:*****:*:*** *::*****:** **

mouse CLCA2   MFMPSLPSVVEFCNESTHNQEAPNLQNQVCSLRSTWDVITASSDLNHSLPVHGVGLPAPP 300
pig   CLCA2   MFMQSLPSVVEFCNASTHNQEAPNLQNQLCSLRSAWDVIRASGDFQHSEPMKGTELPPPP 300
human CLCA2   MFMQSLSSVVEFCNASTHNQEAPNLQNQMCSLRSAWDVITDSADFHHSFPMNGTELPPPP 300
cat   CLCA2   MFMQSLSSVVEFCNARTHNQEAPNLQNQMCSLRSAWDVITDSADFNHSFPMNRTGLPPPP 300
              *** ** ******* *************:*****:****  *.*::** *:: . ** **

mouse CLCA2   TFSLLQAGDRVVCLVIDVSRKMAEGDRLLRLQQAAELYLMQVVEAHTFVGIVTFDSKGEI 360
pig   CLCA2   TFTLLQAGDRVVCLVLDVSSRMAEADRLLRLQQAAAFYLTHMVESHTFVGIASFNGQGEI 360
human CLCA2   TFSLVQAGDKVVCLVLDVSSKMAEADRLLQLQQAAEFYLMQIVEIHTFVGIASFDSKGEI 360
cat   CLCA2   VFSLIQAGDKVVCLVMDVSSKMEEADRLLRLKQAAEFYLMQIVEIHTFVGIVSFNSKGEI 360
              .*:*:****:*****:*** :*  :* *.****:*:*** :** ::** ******.:*:.:***

mouse CLCA2   RASLQQIYSDDDRKLLVSYLPTAVSTDAETNICAGVKKGFEVVEERNGRADGSVLILVTS 420
pig   CLCA2   RAQLHQINSDDDRKLLVSYLPAAVSAEAEASVCSGLKKGFEVVEKLKGKAFGSVMILAAS 420
human CLCA2   RAQLHQINSNDDRKLLVSYLPTTVSAKTDISICSGLKKGFEVVEKLNGKAYGSVMILVTS 420
cat   CLCA2   RAQLHQINSDDDRKLLVSHLPLTVSAEAQTSVCSGLKKGFEVVEKLNGKAHGSVMILVTS 420
              **.*:** *:*********:** :**:.:: .:*:*:*********: :*:* ***:**.:*

mouse CLCA2   GADEHIANCLLTSMNSGSTIHSMALGSSAARKVGELSRLTGGLKFFIPDKFTSNGMTEAF 480
pig   CLCA2   GGDQRISDCFLAALSSGSTVHTLALGSSAVDSLEELARLTGGLKFFVPDKSDSTSMIDAF 480
human CLCA2   GDDKLLGNCLPTVLSSGSTIHSIALGSSAAPNLEELSRLTGGLKFFVPDISNSNSMIDAF 480
cat   CLCA2   GGDEHIGNCLLTVLSSGSTIHSIALGSSVVENLEELSHHTGGVKFFVPDKSNSNSMIDAF 480
              * *: ::*: : :.****:*::****.. .: **:: ***:***:***:**   *..* :**

mouse CLCA2   VRISSGTGDIFQQSLQVESVCETVQPQHQLADTMTVDSAVGNDTLFLVTWQTGGPPEIAL 540
pig   CLCA2   SRISSGTGDVFQQHIQLESTGETVKPHHQLTNSVTVDDGVGNDTTFLVTWQTGGPPEMVL 540
human CLCA2   SRISSGTGDIFQQHIQLESTGENVKPHHQLKNTVTVDNTVGNDTMFLVTWQASGPPEIIL 540
cat   CLCA2   SRIPSGTGDIFQQRIQLESTGENVKPHHQLKNTVTVDNSVGNDTTFLVTWQTSGPPEIAL 540
              ** *****:*** :*:**. *.*:*:*** :::***. ***** ******:.****: *

mouse CLCA2   LDPSGRKYNTGDFIINLAFRTASLKIPGTAKHGHWTYTLNNTHHSPQALKVTVASRASSL 600
pig   CLCA2   RDPNGRKYHTDDFVTSLALQTARLWIPGTAQPGLWTYTLNNSHHSPQALKVTVTSRASLP 600
human CLCA2   FDPDGRKYYTNNFITNLTFRTASLWIPGTAKPGHWTYTLNNTHHSLQALKVTVTSRASNS 600
cat   CLCA2   VDPNGRKYYTSDFITNPALRTARLCIPGTAEPGLWTYTLNNTHHSLQALKVTVTSRASSS 600
              **.**** *.:*: . :::** * *****: * ********:*** *******:****:****
```

```
mouse CLCA2    AMSPATLEAFVERDSTYFPQPVIIYANVRKGLHPILNATVVATVEPEAGDPVVLQLLDGG 660
pig   CLCA2    AVPPATVEAFVERDSTRFPHPMMIYAIVRRGFYPILNATVTATVEPEAADPVTLRLLDDG 660
human CLCA2    AVPPATVEAFVERDSLHFPHPVMIYANVKQGFYPILNATVTATVEPETGDPVTLRLLDDG 660
cat   CLCA2    AMPPATVEVFVERDSPRFPHPVMIYANVRKGIYPILNATVTATIEPEAADPVVLKLFDDG 660
               *: ***:*.****** **:*::*** *::*:*:*******.**:***:.***.*:*:*.*

mouse CLCA2    AGADVIRNDGIYSRYFSSFAVSGSYSLTVHVRHSPSTSTLALPVPGNHAMYVPGYITNDN 720
pig   CLCA2    AGADVIKNDGIYSRYFFSFAGNGRYSLKVHVSHPPSVSAATRSGPGSHAMYVPGYITNGN 720
human CLCA2    AGADVIKNDGIYSRYFFSFAANGRYSLKVHVNHSPSISTPAHSIPGSHAMYVPGYTANGN 720
cat   CLCA2    AGADIIKNDGIYSRYFFSFAVNGSYSLKVHVSYPSSISGLAHSVPGNRAVYVPGYIVNGN 720
               ****:*:********* *** .* ***.*** :  * *  :   **.:*:***** .*.*

mouse CLCA2    IQMNAPKN-LGHRPVKERWGFSRVSSGGSFSVLGVPDGPHPDMFPPCKITDLEAMKVEDD 779
pig   CLCA2    IQMNAPRKPVGRSEEEQKWGLSRATSGGSFSVLGVPAGPHPDMFPPCKIIDLEAMKVEEE 780
human CLCA2    IQMNAPRKSVGRNEEERKWGFSRVSSGGSFSVLGVPAGPHPDVFPPCKIIDLEAVKVEEE 780
cat   CLCA2    IQMNAPRKSAGRHEEEQKWGFSRVSSGGSFSVLGVPAGPHPDVFPPCKIIDLEAVKVEEE 780
               ******:: *: :.:**:**.:*********** *****:****** ****:***::

mouse CLCA2    VVLSWTAPGEDFDQGQTTSYEIRMSRSLWNIRDDFDNAILNSSE-LVPQHAGTRETFTF 838
pig   CLCA2    VALSWTAPGEDFDQGPAKSYEIRISKSLQNIQDDFNSAILVNSSK-LKPQQAGTKETFTF 839
human CLCA2    LTLSWTAPGEDFDQGQATSYEIRMSKSLQNIQDDFNNAILVNTSK-RNPQQAGIREIFTF 839
cat   CLCA2    VTLSWTAPGEDYDQGQANSYEIRMSQSLQKIQDDFNNAILVNISKLLSPQQAGTKEIFTF 840
               :.**********:*** :.*****:*:** :*:***:.***** *:   **:** :* ***

mouse CLCA2    SPKLVTHELDHELAEDAQEPYIVYVALRAMDRSSLRSAVSNIALVSMSLPPNSSPVVSRD 898
pig   CLCA2    SPELFTNGPEHQADGETQRSHRIYVAIRAVDRNSLRSAVSNVAQASLSVPPNSTPVLARD 899
human CLCA2    SPQISTNGPEHQPNGETHESHRIYVAIRAMDRNSLQSAVSNIAQAPLFIPPNSDPVPARD 899
cat   CLCA2    SPKLFTNGPDHQPDGETQESHRIYVAIRAIDKNSLKSAVSNIAQVSLFIPPNSAPVLARD 900
               **:: *: :*:   ::. : :***:**:*:.**:*****:* . : :**** ** :**

mouse CLCA2    DLILKGVLTTVGLIAILCLIMVVAHCIFNRKKRPSRKENETKFL      942
pig   CLCA2    DLILKGVLTAISFIGVICLTIVIIHCTLNRKKRADKRGNETKLL      943
human CLCA2    YLILKGVLTAMGLIGIICLIIVVTHHTLSRKKRADKKENGTKLL      943
cat   CLCA2    HLILKGVLTAVSSIGIICLTIAVTHYTLNRKKKADKKENGTKLI      944
               *******::. *.::** :.: *  :.***: .:: * **::
```

Figure S2A. Multiple Sequence Alignment (MSA) and interspecies comparison of the human, murine, porcine, and feline CLCA2 orthologs via Clustal Omega (1.2.4) (Chojnacki et al. 2017) (03.03.2019). Asterisks indicate perfect alignment, colons or semicolons indicate a site belonging to a group exhibiting strong or weak similarity, respectively (DDBJ 2015). For signal sequence (blue), conserved amino-terminal CLCA domain (n-CLCA, yellow), von Willebrand factor type A (vWA) domain (green), putative proteolytic cleavage site (red), and homologue-specific asparagine-linked glycosylation sites (bold, grey), also refer to S2B. The primary carboxy-terminal transmembrane domains (purple) are annotated according to SOSUI which rendered identical values as HMMtop for human, porcine, and feline CLCA2 (see Table S2B). Binding site of the murine CLCA2 antibody αm5-C1-a, corresponding to aa WTAPGEDFDQGQTT (green lettering) (Braun et al. 2010a) and cross-reacting with the feline epitope WTAPGEDYDQGQAN (green lettering, aa mismatches in red) (see 2.2).

B) CLCA2 *in silico* structure analysis and comparison

ortholog (accession number)	length	signal sequence	n-CLCA	vWA domain	putative proteolytic cleavage site, between aa	aa positions of glycosylation sites	transmembrane domains		
							primary (C-terminal)	secondary (C-terminal)	secondary (N-terminal)
mouse CLCA2 ENSMUST00000040465.10	942	1-32	34-291	311-483	708 and 709	74, 97, 231, 235, 286, 522, 580, 637, 821, 937	904-926[1]	-	15-33[1]
							900-926[2]	-	-
							901-923[3]	874-895[3]	10-23[3]
pig CLCA2 ENSSSCT00000007598.4	943	1-32	34-291	311-483	708 and 709	74, 97, 231, 522, 637, 938	901-923[1]	-	-
							901-927[2]	-	-
							901-923[3]	-	7-29[3]
human CLCA2 ENST00000370565.5	943	1-31	33-292	311-483	708 and 709	74, 97, 150, 231, 235, 292, 522, 556, 580, 637, 822, 938	901-923[1]	-	15-33[1]
							900-922[2]	-	-
							900-922[3]	-	9-31[3]
cat CLCA2 ENSFCAT00000068708.1	944	1-32	33-292	311-483	708 and 709	74, 97, 231, 235, 292, 522, 637, 682, 822, 939	902-924[1]	-	15-33[1]
							902-928[2]	-	-
							902-924[3]	877-899[3]	10-32[3]

Table S2B. The respective sequence lengths were determined using NCBI Genbank (http://www.ncbi.nlm.nih.gov) (17.03.2019), the signal sequence according to SignalP 3.0 (Bendtsen et al. 2004; Nielsen et al. 1997), the n-CLCA and vWA domains according to Ensembl (Pfam) (www.ensembl.org) (21.03.2019). The putative proteolytic cleavage site was identified by interspecies comparison of the aa sequences and homologue-specific asparagine-linked glycosylation sites were predicted via NetNglyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) (17.03.2019). Potential transmembrane domains were identified via [1]HMMtop (Tusnady and Simon 1998; 2001) (18.03.2019), [2]Phobius (Käll et al. 2004; 2007) (03.05.2020), and [3]SOSUI (Hirokawa et al. 1998; Mitaku and Hirokawa 1999; Mitaku et al. 2002) (03.05.2020) *in silico* tools.

Supplemental Material S3. Protein structure analysis and interspecies comparison of CLCA3

A) Multiple Sequence Alignment

```
mouse CLCA3a1   MESLKSPVFLLILHLLEGVLSESLIQLNNNGYEGIVIAIDHDVPEDEALIQHIKDMVTQA 60
cat   CLCA3     MVLSLYAILFLALHLLPGM-KSSMVHLNKNGYDGVVIAINPSVPEDEKLIQNIKEMVTEA 59
mouse CLCA3a2   MVPGLQVLLFLTLHLLQNT-ESSMVHLNSNGYEGVVIAINPSVPEDERLIPSIKEMVTQA 59
mouse CLCA3b    MVPGLQVLLFLTLHLLQNT-ESSMVHLNSNGYEGVVIAINPSVPEDERLIPSIKEMVTQA 59
                *      ::* ****  .  ..*::**.***:*:****: .***** **  **:***:*

mouse CLCA3a1   SPYLFEATGKRFYFKNVAILIPESWKAKPEYTRPKLETFKNADVLVSTTSPLGNDEPYTE 120
cat   CLCA3     STYLFHATKRRVYFRNVSILIPMTWKSKSEYLMPKQESYDQADVIVANPYLKYGDDPYTL 119
mouse CLCA3a2   STYLFEASQGRVYFRNISILVPMTWKSKSEYLMPKRESYDKADVIVADPHLQHGDDPYTL 119
mouse CLCA3b    STYLFEATERRFYFRNVSILVPITWKSKTEYLTPKQESYDQADVIVADPHLQHGDDPYTL 119
                * ***.*:  *.**:*::**:* :**:* **  ** *:::.***:*:    .*:***

mouse CLCA3a1   HIGACGEKGIRIHLTPDFLAGKKLTQYGPQDRTFVHEWAHFRWGVFNEYNNDEKFYLSKG 180
cat   CLCA3     QYGQCGEKGKYIHFTPNFLLTNNLPIYGSRGRVFVHEWAHLRWGIFDEYNVDQPFYISRR 179
mouse CLCA3a2   QYGQCGDRGQYIHFTPNFLLTDNLRIYGPRGRVFVHEWAHLRWGVFDEYNVDRPFYISRK 179
mouse CLCA3b    QYGQCGDRGQYIHFTPNFLLTDNLGIYGPRGRVFVHEWAHLRWGVFDEYNMDRPFYMSRK 179
                :  * **::*  **:**:**  .:*  **  :.*.********:***:*:*** *. **:*:

mouse CLCA3a1   -KPQAVRCSAAITGKNQ-VRRCQGGSCITNGKCVIDRVTGLYKDNCVFVPDPHQNEKASI 238
cat   CLCA3     NTIEATRCSTHITGINVVFKECQGGSCITR-PCRRDSLTGLYEAKCAFIPEKSQTAKDSI 238
mouse CLCA3a2   NTIEATRCSASITGKKV-VHECQRGSCVTR-ACRRDSKTRLYEPKCTFIPDKIQTAGASI 237
mouse CLCA3b    NTVEATRCSTDITGTSV-VRECQGGSCVSR-RCRRDAKTGMQEAKCTFIPNKSQTARGSI 237
                .  :*.***: ***  .  .:.** ***::   *   *   * : : :*.*:*: *.   **

mouse CLCA3a1   MFNQNINSVVEFCTEKNHNQEAPNDQNQRCNLRSTWEVIQESEDFKQTTPMTA--QPPAP 296
cat   CLCA3     MFMQSLSSVTEFCTAETHNMEAPNLQNKMCNCRSTWDVIKDSDDFQNAPPMTGTDSPPRP 298
mouse CLCA3a2   MFMQNLNSVVEFCTENNHNAEAPNLQNKMCNRRSTWDVIKASADFQNSPPMRGTEAPPPP 297
mouse CLCA3b    MFMQSLDSVVEFCTEKTHNVEAPNLQNKMCNLRSTWDVIKASADFQNASPMTGTEAPPLP 297
                ** *.::.**.****  :.** **** **: ** ****:**: * **:: **  . ** *

mouse CLCA3a1   TFSLLQIGQRIVCLVLDKSGSMLNDD---RLNRMNQASRLFLLQTVEQGSWVGMVTFDSA 353
cat   CLCA3     TFSLLKAKQRVVCLVLDKSGSMDSED---RLFRMNQAVELYLIQIIEKGSLVGMVTFESY 355
mouse CLCA3a2   TFSLLKSRRRVVCLVLDKSGSMDKED---RLIRMNQAAELYLTQIVEKESMVGLVTFDSA 354
mouse CLCA3b    TFSLLKSRQRVVCLVLDKSGSMRLGSPITRLTLMNQAAELYLIQIIEKESLVGLVTFDST 357
                *****:  :*:*:********** .       **  **** .*:* * :*: * **:***:*

mouse CLCA3a1   AYVQSELKQLNSGADRDLLIKHLPTVSAGGTSICSGLRTAFTVIK-KKYPTDGSEIVLLT 412
cat   CLCA3     ATIQNYLTNITDENAYEKITANLPQAADGGTSICSGLSAGFQAIIQSNQSTSGSEIVLLT 415
mouse CLCA3a2   AHIQNYLIKITSSSDYQKITANLPQQATGGTSICHGLQAGFQAITSSDQSTSGSEIVLLT 414
mouse CLCA3b    ATIQTNLIRIINDSSYLAISTKLPQYPNGGTSICNGLKKGFEAITSSDQSTSGSEIVLLT 417
                * :*. * .:.       : :**      ****** **  .* .*  .. *.********

mouse CLCA3a1   DGEDNTISSCFDLVKQSGAIIHTVALGPAAAKELEQLSKMTGGLQTYSSDQVQNNGLVDA 472
cat   CLCA3     DGEDNQISLCFEEVKQSGSIIHTIALGPAAAKELETLSNMTGGHRFYANKDI--NGLTDA 473
mouse CLCA3a2   DGEDNGISSCFEAVSRSGAIIHTIALGPSAARELETLSDMTGGLRFYANKHV--SSLIDA 472
mouse CLCA3b    DGEDNRISSCFQEVKHSGAIIHTIALGPSAARELETLSDMTGGLRFYAKEDV--NGLIDA 475
                ***** ** **: *.:**:****:****:** ** ***:*** **.****  : *:....: ..* **

mouse CLCA3a1   FAALSSGNAAIAQHSIQLESRGVNLQNNQWMNGSVIVDSSVGKDTLFLITWTTHPPTIFI 532
cat   CLCA3     FSRISSRSGSITQQAIQLESKSLTITGKKWINGTVPVDSTIGNDTFFVVTWTIQKPEILL 533
mouse CLCA3a2   FSRISSTSGSVSQQALQLESKAFNVRAGAWINSTVPVDSTVGNDTFFVITWTVQKPEIIL 532
mouse CLCA3b    FSGISSKSGSISQQALQLESKAFNVGAGAWINSTVPVDSTVGNDTFFVITWTVRKPEIIL 535
                *: :** ..::*::.****:.::   *:.:* ***:**:*:**:::*** : * *::

mouse CLCA3a1   WDPSGVEQNGFIL--D-TTTKVAYLQVPGTAKVGFWKYSIQ---ASSQTLTLTVTSRAAS 586
cat   CLCA3     QDPKGTRYETSDFKEDKLNIRSARLRIPGIAETGTWTYHLLNKQAKSQVLTVTMTTRARS 593
mouse CLCA3a2   QDPKGKKYITSDFQDDELNIRSARLQIPGTAETGTWTYSIT--GTKSQLITMTVTTRARS 590
mouse CLCA3b    QDPKGKNYTTSDFQEDKLNIFSVRLRIPGIAETGTWTYSLLNKGATSQLLTVTVTTRARS 595
                **.* .     :  * .   . *::** *:.* *.* :   :.** :*:*:*:** *
```

```
mouse CLCA3a1    ATLPPITVTPVVNKNTGKFPSPVTVYASIRQGASPILRASVTALIESVNGKTVTLELLDN 646
cat   CLCA3      PTTLPVIATAHMSQSTTRYPSPMIVYAQVSQGFLPVLGINVTAIIESEDGHQVILELWDN 653
mouse CLCA3a2    PTMEPLLATAHMSQSTAQYPSRMIVYVRVSQGFLPVLGANVTAIIEAEHGHQVTLELWDN 650
mouse CLCA3b     PTTLPVIATAHMSQSTAQYPSRMIVYARVSQGFLPVLGANVTAVIEAESGNQVTLELWDN 655
                 *  *: .*  :.::.* ::** : **. : **  *:*  .***:**:  *: * *** **

mouse CLCA3a1    GAGADATKNDGVYSRFFTAFDANGRYSVKIWALGGVTSDRQRAAPPKNRAMYIDGWIEDG 706
cat   CLCA3      GAGADTVKNDGIYSRYFTDFHGNGRYSLKVHAQARKNTARLSLRQKQNRALYMPGYLENG 713
mouse CLCA3a2    GAGADTVKNDGIYTRYFTDYHGNGRYSLKVRVQARKNKARLSLRQK-NKSLYIPGYVENG 709
mouse CLCA3b     GAGADTLKNDGIYSRYFTDFHGNGRYSLKVNAQARKNMAKLNLKQK-NKSLYIPGYVEND 714
                 *****: ****:*:*:** :..*****:*: . .  . :   *:::*: *::*:.

mouse CLCA3a1    EVRMNPPRPETSY--VQDKQLCFSRTSSGGSFVATNVPAAAPIPDLFPPCQITDLKASIQ 764
cat   CLCA3      KIVLNPPRPDVNDDMAEAEVEDFSRLSSGGSFIVSGAPPAGSHTHVFPPGKITDLEAKFK 773
mouse CLCA3a2    KIVLNPPRPDVQEEAIEATVEDFNRVTSGGSFTVSGAPPDGDHARVFPPSKVTDLEAEFI 769
mouse CLCA3b     QIVLNPPRPEIPE-ATEATVEDFSRLTSGGSFTVSGAPPDGDHARVFPPSKVTDLEAEFI 773
                 ::  :*****:        :       *.* :***** .::.*  .   :*** ::***:*.:

mouse CLCA3a1    GQNLVNLTWTAPGDDYDHGRASNYIIRMSTSIVDLRDHFNTSLQVNTTGLIPKEASSEEI 824
cat   CLCA3      GDQ-IQLSWTAPGNVLDKGKANSYIIRISEHFQDLQEDFDNAALVNTSSLTPKEAGSIEV 832
mouse CLCA3a2    GDY-IQLTWTAPGKVLDKGRAHRYIIRVSQHPLGLQEDFNNATLVNASSLIPKEAGSKET 828
mouse CLCA3b     GDH-IHLTWTAPGKVLDKGRAYRYVIRMSGHSLALQEDFSNSTLVNTSSVMPKEAGSKET 832
                 *:  ::*:*****.  *:*:*  *:**:*       *::.*.:  **:::.: ****.* *

mouse CLCA3a1    FEFELGGNTFGNGTDIFIAIQAVDKSNLKSEISNIARVSVFIPAQEPPIPEDSTPPCPDI 884
cat   CLCA3      FEFKPEPFKIENGTKIYFAIQAIHEANLTSEVSNIAQAIKFIPPQESSVPALGTKT---S 889
mouse CLCA3a2    FKFKPETFKIANDTQLYIAIQAYNEAGLTSEVSNIAQAVKFTSLEDSISA-LGADI---S 884
mouse CLCA3b     FKFKPETFKIENGTQVYIAIQADNEARLSSEVSNIAQAVKFIPPQVYLTP-STPPG---L 888
                 *:*:     .: *.*.::::**** .:: *.**:****:.  *    :

mouse CLCA3a1    SINSTIPGIHVLKIMWKWLGEMQVTLGLH-------------------------------- 913
cat   CLCA3      ATSLAILGLAVVLSIF-------------------------------------------- 905
mouse CLCA3a2    AISMTVWGLAVIFN-SI-LN---------------------------------------- 902
mouse CLCA3b     STPSTPPGLSTPSTPPG-LSTPSTPPGLSTPSTPPGLSTPSTPPGLSTPSTPPGLSTPST 947
                 :   :  *:. .

mouse CLCA3a1    -------------------------------------------------------- 913
cat   CLCA3      -------------------------------------------------------- 905
mouse CLCA3a2    -------------------------------------------------------- 902
mouse CLCA3b     PPGLSTPSTPPGLSTPSTPPGLSTPSTPPGLSTPSTPPGLSTPSTPPGLSTPSTPPGLST 1007


mouse CLCA3a1    ------------------------------------        913
cat   CLCA3      ------------------------------------        905
mouse CLCA3a2    ------------------------------------        902
mouse CLCA3b     PSTPPGLSTPSTPPGLGTKVSVPSLTVFVLVATLFIF       1044
```

Figure S3A. Multiple Sequence Alignment (MSA) and interspecies comparison of the murine and feline CLCA3 orthologs via Clustal Omega (1.2.4) (Chojnacki et al. 2017) (26.03.2020). Asterisks indicate perfect alignment, colons or semicolons indicate a site belonging to a group exhibiting strong or weak similarity, respectively (DDBJ 2015). For signal sequence (blue), conserved amino-terminal CLCA domain (n-CLCA, yellow), von Willebrand factor type A (vWA) domain (green), putative proteolytic cleavage site (red), and homologue-specific asparagine-linked glycosylation sites (bold, grey), also refer to S3B. Binding site of the feCLCA3-2b-specific antibody, LLQDPKGTRYETSD (green lettering) (see 2.1).

B) CLCA3 *in silico* structure analysis and comparison

ortholog (accession number)	length	signal sequence	n-CLCA	vWA domain	putative proteolytic cleavage site, between aa	aa positions of glycosylation sites	transmembrane domains primary (C-terminal)	secondary (N- or C-terminal)
mouse CLCA3a1 ENSMUST00000059091.5	913	1-21	24-288	309-473	695 and 696	504, 770, 836	none[1]	
							none[2]	
							soluble[3]	
mouse CLCA3a2 ENSMUST00000029929.11	902	1-21	24-288	310-417	698 and 699	39*, 75, 504, 630, 840	none[1]	
							none[2]	
							878-900[3]	1-22[3]
mouse CLCA3b ENSMUST00000159989.1	1044	1-21	24-289	309-472	703 and 704	39*, 75, 228, 507, 542, 635, 702, 813, 844	772-788[1]	1027-1043[1]
							none[2]	
							soluble[3]	
pig CLCA3	pseudogene							
human CLCA3	pseudogene							
cat CLCA3 ENSFCAT00000031309.4	905	1-21	24-290	310-475	702 and 703	39*, 75, 364, 454, 505, 516, 633, 844, 859	none[1]	
							none[2]	
							soluble[3]	

Table S3B. The respective sequence lengths were determined using NCBI Genbank (http://www.ncbi.nlm.nih.gov) (17.032019), the signal sequence according to SignalP 5.0 (Almagro Armenteros et al. 2019; Nielsen et al. 1997), the n-CLCA and vWA domains according to Ensembl (Pfam) (www.ensembl.org) (19.04.2020). The putative proteolytic cleavage site was identified by interspecies comparison of the aa sequences and homologue-specific asparagine-linked glycosylation sites were predicted via NetNglyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) (27.03., 22.06.2020). *Proline occurs just after the asparagine residue which makes it highly unlikely that the Asparagine is glycosylated, presumably due to conformational constraints (www.cbs.dtu.dk/services/NetNGlyc/output.php). Potential transmembrane domains were excluded via [1]HMMtop (Tusnady and Simon 1998; 2001), [2]Phobius (Käll et al. 2004; 2007), and [3]SOSUI (Hirokawa et al. 1998; Mitaku and Hirokawa 1999; Mitaku et al. 2002) (27.03., 22.06.2020) *in silico* tools.

Supplemental Material S4. Protein structure analysis and interspecies comparison of CLCA4

A) Multiple Sequence Alignment

```
murine   CLCA4a   MMAFSRGPVFLLLLLYLLWGSDTSLIRLNENGYEDIIIAIDPAVPEDTTIIEHIKGMVTK 60
murine   CLCA4b   MMAFSRGPVFLLFLLYLLWGSDTSLIKLNGNGYEDIIIAIDPAVPEDTTIIEHIKGMVTK 60
porcine  CLCA4a   -MGFFS-SVL-LLVLHLLQGSRTSLVQLNANGYEGVLIAIDPAVPEDETLITEIKDMVTA 57
porcine  CLCA4b   -MGFFS-SVL-LLVLHLLQGSRTSLVQLNANGYEGVLIAIDPAVPEDETLITEIKDMVTT 57
human    CLCA4    -MGLFRGFVF-LLVLCLLHQSNTSFIKLNNNGFEDIVIVIDPSVPEDEKIIEQIEDMVTT 58
cat4     CLCA4    -MGL-LDFVF-LLVLYLLQGSNTSLVQLNNNGYEGIIIAIDPHVPEDGKIIEEIKGMVTT 57
                   *.:      *: *::* **   * **::**  **:*.::*.*** **** ..* .*:.***

murine   CLCA4a   ASTYLFEATEKRFFFKNVSILIPESWKDSPQYRRPKQESYKHADIKVAPPTVEGRDEPYT 120
murine   CLCA4b   ASTYLFEATEKRFFFKNVSILIPESWKNSSQYRRPKQESYKHADIKVAPPAFEGRDEPYT 120
porcine  CLCA4a   ASTYLFEATEKRFYFKNVSILIPESWKNITQYKRPKQESYKHADVIVAPPTVPGRDEPYT 117
porcine  CLCA4b   ASTFLFEATEKRFFFKNVSILIPESWKN-TQYKRPKHESYKHADVIVAPPTVPGRDEPYT 116
human    CLCA4    ASTYLFEATEKRFFFKNVSILIPENWKENPQYKRPKHENHKHADVIVAPPTLPGRDEPYT 118
cat4     CLCA4    ASTYLFEATEKRFFFKNVSILIPNSWKENSQYKRPKHESYKHADVLVAPPTLPGRDEPYT 117
                   ***:*********:*********:.**:  **:***:*.:****: ****.. *******

murine   CLCA4a   RQFTQCEEKAEYIHFTPDFVLGRKQDEYGDSGKVLVHEWAHLRWGVFDEYNEDQPFYSAS 180
murine   CLCA4b   RQFTQCEEKAEYIHFTPDFVLGRKQVEYGDSGKLLVHEWAHLRWGVFDEYNEDQPFYSAS 180
porcine  CLCA4a   KQFTECGEKAEYIHFTPDFVLGKKQNEFGPSGKALVHEWAHLRWGVFDEYNDDEPFYSAK 177
porcine  CLCA4b   KQFTECGEKAEYIHFTPDFVLGKKQNEFGPPGRALVHEWAHLRWGVFDEYNDDEPFYIAK 176
human    CLCA4    KQFTECGEKGEYIHFTPDLLLGKKQNEYGPPGKLFVHEWAHLRWGVFDEYNEDQPFYRAK 178
cat4     CLCA4    KQFTACEEKGEYIHFTPDFVLGKKQNEYGPSDRLLVHEWAHLRWGVFDEYNEDEPFYSAK 177
                   :*** * **.*********::**:** *:*   .: :*****************:*:*** *.

murine   CLCA4a   SKKIEATRCSTGITGTNRVYACQGGSCAMRRCRTNSTTKLYEKDCQFFPDKVQSEKASIM 240
murine   CLCA4b   SKKIEATRCSTGITGMNRVHTCQGGSCITRRCRTNSTTKLYEKDCQFFPDKVQSEKASIM 240
porcine  CLCA4a   SKKIEATRCSTGITGINRVHKCQGGSCATRPCRTDANTKLYEKDCQFFPDKYQIEKTSIM 237
porcine  CLCA4b   SKKIEATRCSIDITGINRVYKCQENNCVTRTCRVDANTKLYEKDCQFFPDKHQTEKTSIM 236
human    CLCA4    SKKIEATRCSAGISGRNRVYKCQGGSCLSRACRIDSTTKLYGKDCQFFPDKVQTEKASIM 238
cat4     CLCA4    SKKIEATRCSTGITGINRVYKCQGNSCTTRGCRIDSKTKLYEKDCQFFPDKDQTEKASIM 237
                   ********** .*:* ***: ** ..*  * * ** ::.**** ********* * **:***

murine   CLCA4a   FMQSIDSVTEFCKKENHNREAPTLHNKKCNYRSTWEVISTSEDFNSSTPMETSPSPPFFS 300
murine   CLCA4b   FMQSIDSVTEFCKKENHNREAPTLHNEKCQWRSTWEVISSSEDFNSSTPMETPPAPPFFS 300
porcine  CLCA4a   FMQGIDSVAEFCNEKNHNREAPSLQNKKCDSRSTWEVISSSEDFNITEVMVAPPPAPVFS 297
porcine  CLCA4b   FMQGIDSITRFCNEKNHNREAPSLQNKKCDSRSTWEVISNSEDFKGTVPIAAPPPPPVFS 296
human    CLCA4    FMQSIDSVVEFCNEKTHNQEAPSLQNIKCNFRSTWEVISNSEDFKNTIPMVTPPPPPVFS 298
cat4     CLCA4    FMQGINSVVEFCNKKNHNQEAPSLQNKMCNSRSTWEVISNSEDFKNTTSMEALPPPPVFS 297
                   ***.*:*:..**::.. **:***:*:*  *: ******** .**** : :  : * *.**

murine   CLCA4a   LLRISERIMCLVLDVSGSMTSYDRLNRMNQAAKYFLSQIIENRSWVGMVHFSSQATIVHE 360
murine   CLCA4b   LLRISERIVCLVLDVSGSMSSSDRLNRMNQAAKYFLSQIIENRSWVGMVHFSSQATIVHE 360
porcine  CLCA4a   LLKISERIVCLVLDKSGSMSSSNRLNRMNQAAKYFLMQIVENGSWVGMVHFDGTASIRSD 357
porcine  CLCA4b   LLKISERIVCLVLDKSESMGNHNRLNRMNQAVKYFLLQTIENGSWVGVVDFDTTAHIKSK 356
human    CLCA4    LLKISQRIVCLVLDKSGSMGGKDRLNRMNQAAKHFLLQTVENGSWVGMVHFDSTATIVNK 358
cat4     CLCA4    LLKIRERIACLVLDKSGSMNSFNRLNRMNQAAKHFLLQTIENGSWVGMVHFDSTANVKSN 357
                   **:* :** ***** * **  . :*********.*:** * :** ****:*.*.  * :  .

murine   CLCA4a   LIQINSDIERNQLLQTLPTSANGGTSICSGIKAAFQVFKNGEYQTDGTEILLLSDGEDST 420
murine   CLCA4b   LIQMNSDIERNKLLQTLPTSAIGGTSICSGIKTAFQVFKNGEYQTDGTEILLLSDGEDST 420
porcine  CLCA4a   LIQITGSNERDKLLGSLPTTANGGTSICSGIQTAFEVVRKLYSHTDGSEIVLLTDGEDNT 417
porcine  CLCA4b   LIQIKSNNERRKLLESLPTEASGGISICSGIESAFQVIKEIYPQVDGSEIILVVAGEDKN 416
human    CLCA4    LIQIKSSDERNTLMAGLPTYPLGGTSICSGIKYAFQVIGELHSQLDGSEVLLLTDGEDNT 418
cat4     CLCA4    LIQIISSKERNKLLESLPTAANGGTSICAGIKSAFQVVREIHPQIDGSEIVLLTDGEDNS 417
                   ***: .. ** *: *** ** ***:**: **:*. :   : **:*::*: ***..

murine   CLCA4a   AKDCIDEVKDSGSIVHFIALGPSADLAVTNMSILTGGNHKLATDEAQNNGLIDAFGALAS 480
murine   CLCA4b   AKDCIDEVKDSGSIVHFIALGPSADLAVTNMSILTGGNHKLATDEAQNNGLIDAFGALAS 480
porcine  CLCA4a   AGACVNEVKQSGAIIHFIALGPSADKAVIEMSTATGGVHFYATDEAENNGLIDAFGALAS 477
porcine  CLCA4b   IRNCMDRVKQSGAIIHSIALGPNADPAVTEMSAVTGGMHFYTTDQSESRGLTDALWTFGS 476
human    CLCA4    ASSCIDEVKQSGAIVHFIALGRAADEAVIEMSKITGGSHFYVSDEAQNNGLIDAFGALTS 478
cat4     CLCA4    AKNCIDEVKQSGAIIHLIALGPSADQAVIEMSTLTGGNHFFASDEAQNNGLIDAFGALAS 477
                   *:: .**:**:*:* **** ** ** :** *** *  .:*::::..** **: :: *
```

```
murine    CLCA4a    ENADITQKSLQLESKGAILNNSLWLNDTVVIDSTLGRDTFFLVTWSKQAPAIYLRDPKGT  540
murine    CLCA4b    ENTDITQKSLQLESKGAILNNSLWLNDTVVIDSTVGRDTFFLVTWSKQAPAIYLRDPKGT  540
porcine   CLCA4a    GNTDISQQSLQLESKGLKLNSNEWLNGTVIIDSTVGKDTFFLVTWVQQRPDISLLDPNGT  537
porcine   CLCA4b    GNTNSSQHSLQLESKGLVLSSNPWMNGTVTIDSTVGKDTFCLVTWDKQPPGISLWDPSGT  536
human     CLCA4     GNTDLSQKSLQLESKGLTNSNAWMNDTVIIDSTVGKDTFFLITWNSLPPSISLWDPSGT  538
cat4      CLCA4     GNTDISQQPLQLESKGLTLNNNPWMNGTVIIDSTVGKDTFFLITWARQSPIISLWDPSGT  537
                    *:: :*: *******  *... *:*.** ****:*:*** *:**    * * * **.**

murine    CLCA4a    QTTNFTMDSASKMAYLSIPGTAQVGVWTYNLEAKENSEILTITVTSRAANSSVPPITVNA  600
murine    CLCA4b    QTTNFTMDFVSKMAYLSIPGTAEVGVWTYNLEAKENSEILTITVTSRAANSSVPPISVNA  600
porcine   CLCA4a    LMGSFTVDAVSKMAHLSIPGTAKVGVWTYSLQAKADAETLTITVNSQASNSAVPPITVNA  597
porcine   CLCA4b    PRGNFTVDEDSKMAYLSIPGTAKVGVWTYSLQAKANPETLTITVNSRAANSAVPPITVNA  596
human     CLCA4     IMENFTVDATSKMAYLSIPGTAKVGTWAYNLQAKANPETLTITVTSRAANSSVPPITVNA  598
cat4      CLCA4     PMRNFTVDTVSKMAYLSIPGTAKVGVWTYSLQAKANPETLMTVNSQAANSSVPPITVNA  597
                    .**:*  ****:*******:**.*:*.*:** : * **:**.*:*:**:****:***

murine    CLCA4a    KVNTDTNTFPSPMIVYAEVLQGYTPIIGARVTATIESNSGKTEELVLLDNGAGADAFKDD  660
murine    CLCA4b    KVNTDTNTFPSPMIVYAEVLQGYTPIIGARVTATIESNSGKTEELVLLDNGAGADAFKDD  660
porcine   CLCA4a    KMNKDTSSFPSPMIVYAEILQGYIPILGAGVTAFIESNTGKREVLELLDNGAGADSIKND  657
porcine   CLCA4b    KMNKDTSSFPSPMIVYAEILQGYIPILGASVTAFIESDNGKTEVLELLDNGAGADSFKND  656
human     CLCA4     KMNKDVNSFPSPMIVYAEILQGYVPVLGANVTAFIESQNGHTEVLELLDNGAGADSFKND  658
cat4      CLCA4     KMNKDTNSFPSPMVVYAEVLQGHVPILGANVTAFIESSNGNIEVLELLDNGAGADSFKND  657
                    *:*.*..:*****:****:***: *:.:** *** ***..*: * * *********::*:*

murine    CLCA4a    GVYSRFFTAYSVNGRYSLKVRADGGRNSARRSLRHPSSRAAYIPGWVVDGEIQGNPPRPE  720
murine    CLCA4b    GVYSRFFTAYSVNGRYSLKVRADGGTNSARRSLRHPSSRAAYIPGWVVDGEIQGNPPRPE  720
porcine   CLCA4a    GVYSRYFTAYSENGRYSLKVRALGGASAVTRNLRHPLNRAAYIPGWVVNGEIEENPPRPE  717
porcine   CLCA4b    GVYSRYFTTYQENGKYSLKVQAAGKAKTVMRSLRHLPNRGTYIPGWAVSGEIEGNPPRPE  716
human     CLCA4     GVYSRYFTAYTENGRYSLKVRAHGGANTARLKLRPPLNRAAYIPGWVVNGEIEANPPRPE  718
cat4      CLCA4     GVYSRYFIAYSENGRYSLKVRAYRGANVTTQNLRRPPNRAAHIPGWVVDGKIEGNPPRVE  717
                    *****:* :*  **:*****:*  .   .**  .*.::****.*.*:*: **** *

murine    CLCA4a    TTEATQPVLEDFSRTASGGAFVMSNVPIGPLPDVYPPNRITDLQATLDGEEISLTWTAPG  780
murine    CLCA4b    MTEATQPVLENFSRTASGGAFVMSNVPIGPLPDQYPPNRITDLQATLDGEEISLTWTAPG  780
porcine   CLCA4a    IDENTQTNLESFTRTAIGGAFVVSNVPNGPLPDLYPPSQITDLEATSDEDEIRITWTAPG  777
porcine   CLCA4b    SDEDTQTDLESFTRIASGGAFVISNVSKLPLPDLYPPSQITDLEATSDEDEIKITWTAPG  776
human     CLCA4     IDEDTQTTLEDFSRTASGGAFVVSQVPSLPLPDQYPPSQITDLDATVHEDKIILTWTAPG  778
cat4      CLCA4     IDEDTHTTLESFTRTASGGAFVVSNISKLPLPDLYPPSQITDLEATLNGDEINLTWTAPG  777
                    * *:  **.*:* * *****:*::   **** ***.:****:** . ::* :******

murine    CLCA4a    DDYDVGRVQQYIIRTSKNIIELRDNFNNSPRVDTTNLTPKEANSEETFAFKPENITEENA  840
murine    CLCA4b    DDYDVGRVQQYIIRTSENIIDLRDNFNNSLRVDTTKLTPKEANSKETFAFKPENISEENA  840
porcine   CLCA4a    DDFDVGTVEQYIIRISGSLLDLRDNFDDALQINTSALLPSEANTKESFAFKPGNLSEENA  837
porcine   CLCA4b    DDFDVGTVEQYIIRISGSLLDLRDNFDDALQINTSALLPSEANTKESFAFKPGNLSEENA  836
human     CLCA4     DNFDVGKVQRYIIRISASILDLRDSFDDALQVNTTDLSPKEANSKESFAFKPENISEENA  838
cat4      CLCA4     DNFDVGKVQRYIIRISGSILDLRDNFDNALQVNTTDLLPNEANSKETFAFKQGNISEENA  837
                    *::*** *::**** * .:::***.*::: :::*: * *.***::*:**** *::****

murine    CLCA4a    TYIFIAIESVDKSSLSSGPSNIAQVALFTPQAEPDPDESPS-----------LSGVSVA  888
murine    CLCA4b    TYIFIAIESVDKNNLSSGPSNIAQVAMFTPQAEPVPDESPR-----------SSGVSIS  888
porcine   CLCA4a    THIFIAIRSVDKSNLTSKVSNIAQVALFTPEADYTPDDSHPDPGPA-------KSGVSIS  890
porcine   CLCA4b    THIFIAIQSVDKSNLTSKVSNIAQVALFTPEADPSPGKSHPNPR-----------INIS  884
human     CLCA4     THIFIAIKSIDKSNLTSKVSNIAQVTLFIPQANPDDIDPTPTPTPTPTPDKSHNSGVNIS  898
cat4      CLCA4     THIFIAIQSVDKSNLTSKLSNIAQVALFIPQAEPGPDESPPNPSPDE---NQPNSRVNIV  894
                    *:****.*:**..*:*  ******:* *:*:    .        :.:

murine    CLCA4a    TIVLSVLGAL-VVCIIVGTTICILKNKRSSSAAITKF       924
murine    CLCA4b    TIVLSVVGSVVLVCIIVSTTICILKNKRSSSGAATTF       925
porcine   CLCA4a    TLVLIVVGSVVIVSLILSVTICILNKNRNTRRPRTGF       927
porcine   CLCA4b    ALVLLVVGSVAVVSAILSATICILKKKRNAIRSKTGF       921
human     CLCA4     TLVLSVIGSVVIVNFILSTTI----------------       919
cat4      CLCA4     ILVLLVVGSVAIVSTIIGATI----------------       915
                    :** *:*:: :*  *:..**
```

Figure S4A. Multiple Sequence Alignment (MSA) and interspecies comparison of the human, murine, porcine, and feline CLCA4 orthologs via Clustal Omega (1.2.4) (Chojnacki et al. 2017) (26.03.2020). Asterisks indicate perfect alignment, colons or semicolons indicate a site belonging to a group exhibiting strong or weak similarity, respectively (DDBJ 2015). For signal sequence (blue), conserved amino-terminal CLCA domain (n-CLCA, yellow), von Willebrand factor type A (vWA) domain (green), putative proteolytic cleavage site (red), and homologue-specific asparagine-linked glycosylation sites (bold, grey), also refer to S4B. The primary carboxy-terminal transmembrane domains (purple) are annotated according to Phobius which rendered identical values as HMMtop for murine and porcine CLCA4 (see Table S4B). According to PredGPI, human and cat CLCA4 are highly likely of possessing a GPI anchor (dark purple, white lettering).

B) CLCA4 *in silico* structure analysis and comparison

ortholog (accession number)	length	signal sequence	n-CLCA	vWA domain	putative proteolytic cleavage site, between aa	aa positions of glycosylation sites	transmembrane domains primary (C-terminal)	secondary (N-terminal)	GPI probability
mouse CLCA4a ENSMUST00000029923.9	924	1-23	25-289	309-446	699 and 700	77, 342, 500, 506, 544, 590, 834	887-911[1]	7-26[1]	42.5 %
							887-911[2]	-	
							886-908[3]	1-23[3]	
mouse CLCA4b ENSMUST00000098549.3	925	1-23	26-290	309-447	699 and 700	77, 88, 342, 500, 506, 544, 590, 731, 834, 839	890-912[1]	7-26[1]	22.7 %
							890-912[2]	-	
							888-910[3]	1-23[3]	
mouse CLCA4c	pseudogene								
pig CLCA4a ENSSSCT00000059410.2	927	1-20	23-287	306-464	696 and 697	74, 85, 282, 339, 503, 535, 831, 836	892-914[1]	-	84.6 %
							892-914[2]	-	
							888-910[3]	1-23[3]	
pig CLCA4b ENSSSCT00045022111.1	921	1-20	23-286	305-463	695 and 696	74, 338, 480, 502, 540, 741, 830, 835, 882	885-908[1]	-	94.0 %
							883-908[2]	-	
							884-906[3]	1-23[3]	
human CLCA4 ENST00000370563.3	919	1-21	24-288	307-465	697 and 698	20, 75, 504, 542, 588, 628, 811, 832	-	6-24[1]	100 %
							895-915[2]	-	
							895-917[3]	1-23[3]	
cat CLCA4 ENSFCAT00000019194.4	915	1-20	23-287	308-464	696 and 697	19, 74, 283, 339, 503, 541, 587, 627, 684	893-912[1]	6-23[1]	99.9 %
							893-913[2]	-	
							891-913[3]	1-23[3]	

Table S4B. The respective sequence lengths were determined using NCBI Genbank (http://www.ncbi.nlm.nih.gov) (17.03.2019), the signal sequence according to SignalP 5.0 (Almagro Armenteros et al. 2019; Nielsen et al. 1997), the n-CLCA and vWA domains according to Ensembl (Pfam) (www.ensembl.org) (19.04.2020). The putative proteolytic cleavage site was identified by interspecies comparison of the aa sequences and homologue-specific asparagine-linked glycosylation sites were predicted via NetNglyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) (27.03., 22.06.2020). Potential transmembrane domains were identified via [1]HMMtop (Tusnady and Simon 1998; 2001), [2]Phobius (Käll et al. 2004; 2007), and [3]SOSUI (Hirokawa et al. 1998; Mitaku and Hirokawa 1999; Mitaku et al. 2002) (27.03., 22.06.2020) *in silico* tools. According to PredGPI (Pierleoni et al. 2008) (22.06.2020), human and cat CLCA4 are highly likely of possessing a GPI anchor.

8   Publications Related to this Research Thesis

Research publications in scientific journals:

- **Erickson NA**, Gruber AD, Mundhenk L: The Family of Chloride Channel Regulator, Calcium-activated Proteins in the Feline Respiratory Tract: A Comparative Perspective on Airway Diseases in Man and Animal Models.[#] J. Comp. Path. (2020) DOI: 10.1016/j.jcpa.2019.10.193A

  [#]cover page article

- *Mundhenk L, ***Erickson NA**, Klymiuk N, Gruber AD: Interspecies Diversity of Chloride Channel Regulators, Calcium-Activated 3 Genes. PLoS One (2018) DOI: 10.1371/journal.pone.0191512

  *shared first authorship

Oral presentations:

- **Erickson NA**, Klymiuk N, Gruber AD, Mundhenk L: Molekulare speziesspezifische Unterschiede und deren Einfluss auf die translationale Forschung - Beispiel der Evolution der CLCA-Genfamilie. 60th Annual Conference of the German Veterinary Medical Society, Section Veterinary Pathology, Fulda, Germany (2017). Tierarztl Prax Ausg K 2017; 45(3), S. A 20–A 21

Poster presentations:

- **Erickson NA**, Gruber AD, Mundhenk L. Auffällige Speziesunterschiede in der Expression feliner CLCA-Proteine zu anderen Spezies im Respirationstrakt. 62nd Annual Conference of the German Veterinary Medical Society, Section Veterinary Pathology, Fulda, Germany (2019). Tierarztl Prax Ausg K 2019; 47(03): 212-213

- Mundhenk L, **Erickson NA**, Klymiuk N, Gruber AD. Genomic and Molecular Basis for Suitability Assessment of Animal Models: Differences in CLCA Genes and Protein Expression Between Humans, Mice and Cats.[§] 3rd Joint European Congress of the ESVP, ESTP and ECVP, Lyon, France (2017). J. Comp. Path. (2018) Vol. 158, 93e149

  [§]poster prize

9   Funding

10 Conflict of Interest

The author of this thesis declares no conflict of interest.

11 Acknowledgements / Danksagung

Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, 28.06.2021   Nancy Ann Erickson, PhD