Transport activity and presence of CIC-7/Ostm1 complex account for different cellular functions

Stefanie Weinert¹,², Sabrina Jabs¹,², Svea Hohensee¹, Wing Lee Chan³,⁴, Uwe Kornak³,⁴ & Thomas J Jentsch¹,²,⁵,*

Abstract

Loss of the lysosomal CIC-7/Ostm1 2Cl⁻/H⁺ exchanger causes lysosomal storage disease and osteopetrosis in humans and additionally changes fur colour in mice. Its conversion into a Cl⁻ conductance in Clcn7⁺/⁺ unc mice entails similarly severe lysosomal storage, but less severe osteopetrosis and no change in fur colour. To elucidate the basis for these phenotypical differences, we generated Clcn7⁺/⁺ mut mice expressing an ion transport-deficient mutant. Their osteopetrosis was as severe as in Clcn7⁻/⁻ mice, suggesting that the electric shunt provided by CIC-7⁺/⁺ unc can partially rescue osteoclast function. The normal coat colour of Clcn7⁺/⁺ mut mice and their less severe neurodegeneration suggested that the CIC-7 protein, even when lacking measurable ion transport activity, is sufficient for hair pigmentation and that the conductance of CIC-7⁺/⁺ unc is harmful for neurons. Our in vivo structure-function analysis of CIC-7 reveals that both protein-protein interactions and ion transport must be considered in the pathogenesis of CIC-7-related diseases.

Keywords: acidification; anion transport; grey-lethal; lysosome; Wnt signalling

Introduction

Acidic luminal pH in endosomes and lysosomes influences their trafficking, enzymatic activities and transport of substances across their limiting membranes. Luminal acidification is accomplished by electrogenic vacuolar H⁺-ATPases that require an electric shunt, which in the classical model was thought to be mediated by chloride channels. Members of the CLC anion transporter gene family [1,2], five of which reside in endosomes or lysosomes, were thought to represent these channels. However, CIC-4 through CIC-7 are rather exchangers that couple Cl⁻ influx to H⁺ efflux [3–7]. Electrogenic Cl⁻/H⁺ exchange can support proton pumping [8,9] and might be even more efficient than Cl⁻ channels in supporting vesicular acidification [6]. However, lysosomal pH is normal in mice lacking CIC-7 [6,10,11], and the strict coupling of Cl⁻ flux to H⁺ countertransport suggested that vesicular CLCs accumulate Cl⁻ into acidic compartments [12] as shown for lysosomes [6]. To clarify the relative contributions of shunt conductance and proton coupling to their biological roles, we had generated Clcn5⁺/⁺ unc and Clcn7⁺/⁺ unc mice in which Cl⁻ transport was uncoupled from H⁺ transport by single point mutations [6,8]. Surprisingly, these unc mice [6,8] displayed grosso modo the same phenotypes as the respective null mice [10,13,14], that is, impaired renal endocytosis in Clcn5⁺/⁺ unc mice and osteopetrosis associated with a lysosomal storage disorder and neurodegeneration in Clcn7⁺/⁺ unc mice (Supplementary Table S1). Hence, a Cl⁻ conductance cannot replace electrogenic Cl⁻/H⁺ exchange in many cellular functions.

CIC-7, together with its obligate β-subunit Ostm1 [11], is expressed in virtually all tissues [14,15]. It localizes to late endosomes and lysosomes and is inserted into the acid-secreting ruffled border of bone-resorbing osteoclasts [10,14]. Loss of CIC-7 function causes osteopetrosis in mice [14], humans [14,16] and cattle [17] and entails lysosomal storage and neurodegeneration in mice [10]. Decreased proteolytic capacity of lysosomes was demonstrated in Clcn7⁻/⁻ proximal tubules [18]. The unchanged steady-state pH of Clcn7⁻/⁻ lysosomes [10,11] was explained by a lysosomal cation conductance that shunts H⁺-ATPase currents in parallel to CIC-7 [6,19]. By contrast, the osteopetrosis of Clcn7⁻/⁻ mice was attributed to impaired acidification of the osteoclast resorption lacuna [14]. Together with the H⁺-ATPase, CIC-7 is inserted by lysosomal exocytosis into the ruffled border of osteoclasts where it may shunt H⁺-ATPase currents [14]. Finally, Clcn7⁻/⁻ mice display grey fur in an agouti background. This phenotype might be linked to melanosomes, a lysosome-related compartment. Phenotypes virtually identical to those of Clcn7⁻/⁻ mice are found in grey-lethal mice [11,20] which carry a mutation in the gene encoding Ostm1.

Whereas the phenotypes of Clcn5⁻/⁻ and Clcn7⁺/⁺ unc mice are nearly identical, some of the phenotypes of Clcn7⁻/⁻ and Clcn7⁺/⁺ unc

1 Leibniz-Institut für Molekulare Pharmakologie (FMP), Berlin, Germany
2 Max-Delbrück-Centrum für Molekulare Medizin (MDC), Berlin, Germany
3 Institut für Humangenetik, Charité Universitätsmedizin Berlin, Berlin, Germany
4 Max-Planck-Institut für Molekulare Genetik, Berlin, Germany
5 Neurocure Cluster of Excellence, Charité Universitätsmedizin Berlin, Berlin, Germany
*Corresponding author. Tel: +49 30 9406 2961, Fax: +49 30 9406 2960, E-mail: jentsch@fmp-berlin.de

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mice differ in severity [6], that is, Clcn7

mutant protein neither transports Cl\(^-\)/H\(^+\) to a measurable degree [7], but, like Clcn7

unc, is expected to be fully interaction competent. Comparative analyses of these mice suggest that a pure Cl\(^-\) conductance partially rescues the lack of Cl\(^-\)/H\(^+\) exchange in osteoclasts, whereas normal pigmentation requires ClC-7 protein interactions, but not ClC-7 ion transport activity. Surprisingly, our study also shows that the Cl\(^-\) conductance of Clcn7

mutant may have detrimental effects on CNS neurons.

**Results**

**Transport-deficient ClC-7 mutant and Ostm1 are expressed normally**

We generated mice in which the ‘proton glutamate’ E312 of ClC-7 was mutated to alanine (Supplementary Fig S1) abolishing both Cl\(^-\) and H\(^+\) transport of Clcn7/Ostm1 [7], hence our designation of this allele as ‘transport deficient’ (td). Homozygous Clcn7

\(^{td/td}\) mice were born at Mendelian ratio. Like Clcn7

\(^{unc/unc}\) and Clcn7

\(^{−/−}\) mice, they were growth retarded and most of them died within 6 weeks after birth. Surprisingly, a few Clcn7

\(^{td/td}\) mice survived more than 1 year (Supplementary Fig S2). The genetic background of Clcn7

\(^{td/td}\) mice cannot account for this difference as these mice were studied in comparable mixed genetic backgrounds. Clcn7

\(^{−/−}\) mice lacked an obvious phenotype. Clcn7

\(^{td}\) protein levels were undistinguishable from Clcn7

\(^{−/−}\) levels in wild-type (WT) mice (Fig 1A and B). Like in Clcn7

\(^{unc/unc}\) mice [6], neither the abundance of Ostm1 [11] nor its processing by lysosomal proteases was changed in Clcn7

\(^{−/−}\), but not in Clcn7

\(^{unc/unc}\) mice which express a correctly targeted ClC-7 mutant at normal levels [6].

Here, we generated a novel Clcn7

\(^{td/td}\) mouse model that expresses a transport-deficient point mutant of ClC-7. The Clcn7

\(^{td}\) mutant protein neither transports Cl\(^-\)/H\(^+\) exchange in osteoclasts, whereas normal pigmentation requires ClC-7 protein interactions, but not ClC-7 ion transport activity. Surprisingly, our study also shows that the Cl\(^-\) conductance of Clcn7

\(^{td}\) may have detrimental effects on CNS neurons.

**Lyosomal ion homeostasis in Clcn7

\(^{td/td}\) mice**

Because ClC-7 may contribute to a countercurrent for the vacuolar H\(^+\)-ATPase [7,14], we measured lysosomal pH of Clcn7

\(^{td/td}\) fibroblasts and found it to be unchanged (Supplementary Fig S3A). Measurements of lysosomal Cl\(^-\) concentration with a dextran-coupled Cl\(^-\)-sensitive ratiometric dye [6] revealed reduced lysosomal Cl\(^-\) accumulation (Supplementary Fig S3B). Both results resemble those made with Clcn7

\(^{−/−}\) and Clcn7

\(^{unc/unc}\) mice [6,10].

**Delayed neurodegeneration in Clcn7

\(^{td/td}\) mice**

Like Clcn7

\(^{−/−}\) and Clcn7

\(^{unc/unc}\) mice, Clcn7

\(^{td/td}\) mice displayed progressive degeneration in the hippocampus (Fig 2). However, it appeared much later and was only detectable in the few surviving older mice. Neuronal cell loss was observed within the CA3 region and progressed to an almost complete loss of CA3 pyramidal cells at 10 months of age (Fig 2D and E). There was no detectable hippocampal cell loss in 4-week-old Clcn7

\(^{td/td}\) mice (Fig 2C).
Nevertheless, pathological changes were observed, in particular in CA3 neurons and in some parts of the cortex. In those regions, lysosomal membrane proteins like Lamp-1 and ClC-7td itself were more intensely labelled and showed a broad distribution in neuronal somata rather than being stained in scattered puncta as in the WT (Fig 3A and B and Supplementary Fig S4). A similar observation was made for ClC-7unc (Fig 3A). Lysosomal storage was apparent 4 weeks after birth, including intracellular carbohydrate accumulation (Fig 2F) and increased levels of lysosomal acid phosphatase (Fig 2G). At P21, electron-dense osmiophilic material accumulated in lysosomal

Figure 2. Brain pathology of Clcn7td/td mice.
A–C Neuronal cell loss (arrowheads) in hippocampal CA3 region of Clcn7−/− but not of Clcn7td/td mice (Nissl staining).
D, E Incipient (D) and complete (E) CA3 neurodegeneration (arrowheads) of 5- and 10-month-old Clcn7td/td mice, respectively. Right panels: higher magnification not necessarily of section shown at left (scale bars: left, 400 μm; right, 100 μm).
F Strong PAS staining (arrowheads) in P28 CA3 neurons of Clcn7td/td but not of WT mice (scale bar: 100 μm).
G Increased lysosomal acid phosphatase activity in the cortex of P28 Clcn7td/td mice compared to WT (scale bar: 100 μm).
H Lysosomal storage material in CA3 pyramidal neuron somata shown by electron microscopy (N, nucleus; dotted line around soma). Right: higher magnification of a soma different from that at left (scale bars: left: 2 μm; right: 1 μm).
I Immunobots showing an increase in the autophagic marker LC3-II in the brain of 3-week-old Clcn7−/− and Clcn7unc/unc versus Clcn7td/td and WT mice. Actin, loading control. Right panel: Quantification of immunobots normalized to actin and LC3-I. Error bars denote s.d.
density was similarly increased in $\text{Clcn7}^{+/0}$ and $\text{Clcn7}^{-/-}$ mice (Fig 4C). As observed for the other CIC-7 mouse models, teeth were formed in $\text{Clcn7}^{+/0}$ mice, but did not erupt (Fig 4B). Electron micrographs showed a partially deranged ruffled border membrane in $\text{Clcn7}^{+/0}$ mice (Fig 4D). Using the sealing zone, which laterally delimits the resorption lacuna between osteoclasts and bone matrix, as localization marker, we categorized ruffled borders in situ as absent, immature or mature (Fig 4D and E). 20% of osteoclasts from $\text{Clcn7}^{-/-}$ and $\text{Clcn7}^{+/0}$ mice totally lacked a ruffled border, and only about 40% showed a mature ruffled border (Fig 4E). All osteoclasts from $\text{Clcn7}^{+/-}$ mice formed ruffled border membranes, of which 70% appeared mature. Hence, the severity of osteopetrosis correlates with an impairment of ruffled border formation.

**Coat colour phenotype is absent in CIC-7$^{td}$ mice**

The pigments of hair and skin are synthesized in melanosomes, a lysosome-related compartment of melanocytes, and are then transferred to keratinocytes. The grey fur of $\text{Clcn7}^{-/-}$ or $\text{Ostm1}^{-/-}$ mice [14,20] thus agrees with the lysosomal localization of CIC-7/Ostm1. Surprisingly, the fur colour was changed neither in $\text{Clcn7}^{zinc/unc}$ mice [6] nor in $\text{Clcn7}^{+/0}$ mice (Fig 5A) which express mutant full-length CIC-7 proteins that display or lack, respectively, a Cl$^+$ conductance. The agouti gene modulates the colour of the hair shaft, resulting in a band of yellow (owed to pheomelanin granules) in the otherwise dark (eumelanin) pigmented hair shaft. The pigment in the yellow band was clamped and reduced in $\text{Clcn7}^{-/-}$ and $\text{Ostm1}^{-/-}$ (gl) mice, whereas eumelanin granules were unchanged in their dark hair shafts (Fig 5B). Hair shaft pigmentation of $\text{Clcn7}^{unc/unc}$ and $\text{Clcn7}^{td/td}$ mice was unchanged compared to WT (Fig 5B).

**Activation of Wnt signalling in primary fibroblasts and melanocytes**

Melanocyte differentiation depends on Wnt signalling [21] and Ostm1 has been proposed to play a role in the canonical Wnt pathway [22] although the molecular mechanism remains obscure. Because the Ostm1 protein is absent or severely reduced in $\text{Clcn7}^{-/-}$ and $\text{Clcn7}^{+/0}$ mice, respectively [11], but unchanged in $\text{Clcn7}^{zinc/unc}$ [6] and $\text{Clcn7}^{+/0}$ mice, we asked whether the difference in coat colour might be due to differences in Wnt signalling. Primary fibroblasts from WT, $\text{Clcn7}^{-/-}$ and $\text{Ostm1}^{-/-}$ mice were exposed to Wnt3a to activate canonical Wnt signalling and mRNA levels of the target gene axin2 [23] were determined. Basal and Wnt3-stimulated axin2 expression was unchanged in $\text{Clcn7}^{-/-}$ and gl ($\text{Ostm1}^{-/-}$) fibroblasts (Supplementary Fig S7A and B) and in $\text{Clcn7}^{-/-}$ melanocytes (Supplementary Fig S7C) compared to WT. Hence, differential activation of the Wnt signalling pathway is unlikely to contribute to the phenotypical differences of the present $\text{Clcn7}$ mouse models.

**Discussion**

Our analysis of $\text{Clcn7}^{+/0}$, $\text{Clcn7}^{-/-}$ and $\text{Clcn7}^{zinc/unc}$ mice [6,14] represents a novel in vivo structure-function analysis of CIC-7/Ostm1 that complements similar in vitro studies focusing on biophysical properties [7,24]. Comparison of the pathologies of these mice

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**Figure 3. Abnormal lysosomal morphology in CIC-7 mouse models.**

A CIC-7 immunolabelling in the somata of CA1 and CA3 pyramidal and of cortical neurons in $\text{Clcn7}^{+/0}$, $\text{Clcn7}^{+/0}$ and WT mice. CIC-7$^{td}$ is abnormal in the CA3 region and partially in the cortex. Increased labelling intensity suggests larger CIC-7 amounts in $\text{Clcn7}^{+/0}$ CA3, and in $\text{Clcn7}^{+/0}$ CA3, CA1, and cortex (scale bar: 10 μm; PC, Purkinje cells). B Abnormal Lamp-1 distribution in cortical and CA3 (arrowheads), but not CA1 neurons of 10-month-old $\text{Clcn7}^{+/0}$ mice. DNA stained with DAPI (scale bar: 10 μm).

Osteopetrosis of CIC-7$^{td}$ mice as severe as in CIC-7 KO

Immunolabelling of tibiae revealed that both CIC-7$^{td}$ and its β-subunit Ostm1 were normally expressed in $\text{Clcn7}^{+/0}$ osteoclasts (Supplementary Fig S6A). CIC-7 and CIC-7$^{td}$ similarly co-localized with the a3 subunit of the V-type H$^+$-ATPase at the ruffled border (Supplementary Fig S6B). Unlike the milder osteopetrosis of $\text{Clcn7}^{zinc/unc}$ mice [6], the osteopetrosis of $\text{Clcn7}^{td/td}$ (Fig 4A) was as severe as in $\text{Clcn7}^{-/-}$ [14] or grey-lethal ($\text{Ostm1}^{-/-}$) mice [20]. Bone

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(Supplementary Table S1) yielded a surprisingly complex picture of the roles of ClC-7 in lysosome, osteoclast and melanocyte biology. Phenotypes resulting from Clcn7 mutations cannot be assigned exclusively to a loss of ion transport activity.

As vesicular CLCs may shunt proton pump currents in the endolysosomal system and at the osteoclast resorption lacuna [2,8,9,14], we assumed that a similar shunt by the ClC-7unc Cl− conductance may rescue some of the pathologies of Clcn7−/− mice. Comparison of the present Clcn7−/−/− with Clcn7−/− and Clcn7unc/unc mice showed that this holds true for osteopetrosis, but not for the changed fur colour or neurodegeneration. The severity of osteopetrosis, which is less severe in Clcn7unc/unc mice than in the other two

Figure 4. Osteopetrosis in Clcn7−/−/− mice.
A Micro-CT revealed similar osteopetrosis of tibiae of 3-week-old Clcn7−/− and Clcn7−/−/− mice (scale bar: 1 mm).
B Micro-CT image of skull from P22 Clcn7−/−/− mouse showed impaired tooth eruption (scale bar: 10 mm).
C Similarly, increased bone volume fraction of proximal tibia metaphyseal trabecular bone in Clcn7−/−/− mice and Clcn7−/− mice. BV, bone volume; TV, tissue volume. Student’s t-test was applied; n.s., not significant. Error bars denote s.e.m.
D Electron microscopy showed mature, immature and lacking ruffled borders of Clcn7−/−/− osteoclasts despite the presence of a sealing zone (SZ). Arrows point at ruffled borders (scale bar: 5 μm) (Bo = bone; N = nucleus).
E Percentage of WT, Clcn7unc/unc, Clcn7−/−/−, Clcn7−/−− osteoclasts exhibiting absent, immature or mature ruffled borders. Error bars denote s.e.m.
mouse models, correlated with the malformation of the ruffled border. Hence, the formation of this acid-secreting membrane does not only require the presence of the ClC-7/Ostm1 protein complex, but also its ion transport activity. A Cl\(^-\) conductance can only partially substitute for 2Cl\(^-\)/H\(^+\) exchange in osteoclast function. However, there may be differential effects on lysosomal voltage that may influence transmembrane transport processes and possibly membrane budding and fusion. Reductionist model calculations predict a lumen-positive potential (~20 mV) with a Cl\(^-\) channel, but a lumen-negative potential with a 2Cl\(^-\)/H\(^+\) exchanger [6]. Moreover, the ClC-7 unc mutation (E245A) [7] not only uncouples Cl\(^-\) transport from H\(^+\) transport, but also abolishes voltage- and time-dependent gating [3–5,7,24–26]. WT ClC-7 almost lacks transport activity at cytoplasmic negative (i.e. lumen-positive) potentials. Currents increase steeply when cytoplasmic voltage exceeds approximately ~20 mV [7]. Hence, the unc mutation will robustly increase steady-state ClC-7 currents in lysosomes. Moreover, the slow gating of WT ClC-7/Ostm1 [7] would prevent a full activation of ClC-7 during transient inside-negative voltage excursions that may occur, for example, upon NAADP-induced Ca\(^2+\) release [27,28]. Intriguingly, many pathogenic CLCN7 mutations [7,17,29] accelerate ClC-7/Ostm1 gating, suggesting that early exchange currents may be pathogenic. ClC-7\(^{unc}\) currents respond instantaneously to voltage and may thus be more harmful than those from accelerating mutants expressed in patients.

The beneficial effect of ClC-7\(^{td}\)/Ostm1 on melanocytes and neurons raises the question whether it is totally transport deficient as assumed above. We cannot exclude that the mutant mediates currents below our detection limit of about 3% of WT. If small currents remain in ClC-7\(^{nd}\) mutants, they likely resemble ClC-7\(^{unc}\) currents because similar mutations in EcClC-1 convert this bacterial 2Cl\(^-\)/H\(^+\) exchanger into a pure Cl\(^-\) conductance [30]. As ClC-7\(^{unc}\) currents are detrimental for neurons, we conclude that indeed the ClC-7\(^{td}\)/Ostm1 complex itself, and not a putative ion transport activity, is beneficial for neurons and by extension for melanocytes. Identifying novel binding partners for ClC-7/Ostm1 that may explain these beneficial effects is a daunting task for future investigations.

Materials and Methods

Detailed methods can be found in Supplementary Materials and Methods. See Supplementary Table S3 for number of animals/cell lines used for experiments.

Mice

Clcn7\(^{-/-}\) [14] and Clcn7\(^{unc/unc}\) mice [6] have been described. Grey-lethal (Ostm1\(^{-/-}\)) mice [20] were from Jackson Laboratories. Clcn7\(^{nd/nd}\) mice were generated by homologous recombination using a construct in which the E312A mutation was inserted into exon 11 of Clcn7. Animals were housed under standard conditions in the MDC animal facility according to institutional guidelines and kept on a 12-h light/dark cycle. LAGEso, Berlin, Germany, approved all experimental procedures.
Antibodies

Primary antibodies used can be found in Supplementary Table S2. Secondary antibodies were coupled to Alexa Fluor 488, 546 (Invitrogen) or HRP (Jackson Immunoresearch).

Membrane preparation, tissue homogenates and immunoblot

Brain extracts were prepared from adult mice, blotted on PVDF membrane and probed according to standard procedures.

Histology and electron microscopy

Sections were stained with H&E, Nissl, periodic acid Schiff reagent (PAS), indicated antibodies and for lysosomal acid phosphatase activity. For EM, mice were perfused with 4% (w/v) PFA and 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). 150-μm sagittal sections were prepared with a vibratome. Slices were postfixed in 2% (v/v) OsO4, dehydrated and embedded in epon. Semi-thin sections (0.5 μm) were labelled with toluidine blue. Ultrathin sections (60 nm) were stained with uranyl acetate and lead citrate.

Microcomputed tomography (CT)

PFA-fixed tibiae were analysed with a SkyScan 1172 μCT (Bruker-MicroCT) at 7 μm resolution. A ROI of 350 μm situated 200 μm below the growth plate comprising the secondary spongiosa was evaluated using the CTA n software with a lower grey threshold of 30 (Bruker-MicroCT). 3D reconstruction was done by the AMIRA software package (Visualization Sciences Group).

WNT stimulation of primary cells

Primary fibroblasts were starved > 6 h in DMEM containing 0.1% (w/v) BSA and stimulated with 80 ng/ml recombinant murine (rm) Wnt-3A (CF, R&D Systems) overnight in growth medium. Melanocytes were starved overnight in MEM Eagle containing 0.1% BSA and stimulated overnight with 80 ng/ml rmWnt-3A in MEM Eagle containing 10% (v/v) FBS (all Pan-Biotech) and 200 nM TPA.

Determination of lysosomal pH

Lysosomal pH was measured by ratiometric fluorescence imaging of the pH sensor Oregon Green dextran 488 (Invitrogen) as described [6].

Determination of relative lysosomal chloride concentrations

Lysosomal chloride was measured by ratiometric fluorescence live cell imaging of MEQ/TMR-dextran [6].

Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions

SW generated knock-in mice, performed biochemical experiments, immunohistochemistry and measured lysosomal pH; SJ measured lysosomal [Cl−] and investigated Wnt signalling; SH performed electron microscopy, UK, and WLC performed μ-CT and bone characterization; TJ planned the study; and TJ, SW and SJ wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

References


