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COMMENTARY

Emerging biological roles of Cl^- intracellular channel proteins

Elisabetta Argenzio* and Wouter H. Moolenaar*

ABSTRACT

Cl^- intracellular channels (CLICs) are a family of six evolutionary conserved cytosolic proteins that exist in both soluble and membrane-associated forms; however, their functions have long been elusive. Soluble CLICs adopt a glutathione S-transferase (GST)-fold, can induce ion currents in artificial membranes and show oxidoreductase activity *in vitro*, but there is no convincing evidence of CLICs having such activities *in vivo*. Recent studies have revealed a role for CLIC proteins in Rho-regulated cortical actin dynamics as well as vesicular trafficking and integrin recycling, the latter of which are under the control of Rab GTPases. In this Commentary, we discuss the emerging roles of CLIC proteins in these processes and the lessons learned from gene-targeting studies. We also highlight outstanding questions regarding the molecular function(s) of these important but still poorly understood proteins.

KEY WORDS: Actin dynamics, Membrane trafficking, Signaling, CLIC proteins

Introduction

The Cl^- intracellular channel (CLIC) protein family consists of six members (CLIC1–6) that are highly conserved and exist in both soluble and membrane-associated forms. CLICs are small globular proteins (~28 kDa) that are structurally related to the omega-class of glutathione S-transferases (GSTs) but appear to have distinct, but still poorly understood, cellular functions. Contrary to their original name, CLIC proteins do not function as conventional Cl^- channels but, instead, have roles in such diverse biological processes as tubulogenesis (Berry et al., 2003; Ulmasov et al., 2009), actin-dependent membrane remodeling (Berryman and Bretscher, 2000; Berryman et al., 2004; Bohman et al., 2005; Singh et al., 2007), endosomal trafficking (Argenzio et al., 2014; Chou et al., 2016; Dozynkiewicz et al., 2012), vacuole formation and fusion (Berry et al., 2003), as well as intravesicular pH regulation (Berry et al., 2003; Jiang et al., 2012; Ulmasov et al., 2009), among others. Moreover, they show intrinsic glutaredoxin-like activity *in vitro* (Al Khamici et al., 2015). Although gene-targeting studies have begun to reveal the essential, non-redundant physiological roles of the CLIC proteins, their inner workings and biochemical functions still remain enigmatic (Jiang et al., 2014; Littler et al., 2010).

Recombinant CLIC proteins can associate with artificial membranes and induce rather unselective currents under non-reducing and low pH conditions, which has led to the hypothesis that soluble CLICs can adopt an integral membrane conformation to form ion channels under certain conditions (Littler et al., 2005, 2004). However, the ion channel hypothesis remains speculative because there is no convincing evidence of CLICs having ion channel activity under physiological conditions (see Box 1). In terms of cellular function, CLICs are often found associated with the cortical actin cytoskeleton (Berryman and Bretscher, 2000; Berryman et al., 2004; Jiang et al., 2014) and are detected on intracellular membranes, where they may participate in the formation and maintenance of vesicular compartments; but it is still unclear as to how this function is achieved mechanistically. Growing evidence indicates that CLIC proteins have roles in dynamic actin-dependent trafficking events, during which they can undergo rapid redistribution between subcellular locations in response to agonist stimulation (Ponsioen et al., 2009; Shukla et al., 2009).

In this Commentary, we briefly summarize the structural features of the CLIC proteins and then review their emerging biological roles in vesicular trafficking and integrin recycling. We also discuss the outstanding questions and challenges that need to be addressed in order to better understand the molecular and cellular functions of this intriguing protein family.

The CLIC protein family

The first CLIC protein was named p64 (now known as atypical long isoform CLIC5B) and was identified more than 25 years ago. It was purified from bovine kidney microsomes as a protein that binds to the putative Cl^- channel inhibitor indaloxyacetic acid-94 (IAA94) (Landry et al., 1989), but sequence analysis revealed that p64 was not a conventional Cl^- channel (see Box 1). Over the years, other p64-related mammalian proteins were identified that now make up the CLIC family, which consists of six distinct paralogues, termed CLIC1 through to CLIC6 (Dulhunty et al., 2001; Harrop et al., 2001; Heiss and Poustka, 1997; Jiang et al., 2014; Littler et al., 2005, 2010) (Fig. 1A).

CLIC proteins were identified by either homology screening (Chuang et al., 1999; Edwards, 1999; Heiss and Poustka, 1997) or, in case of CLIC3 and CLIC5, isolated from macromolecular complexes (Berryman and Bretscher, 2000; Qian et al., 1999). Two splice variants of CLIC5, termed CLIC5A and CLIC5B (differing in length with 251 vs 410 aa, respectively), were identified in complex with cytoskeletal components (Berryman and Bretscher, 2000; Shanks et al., 2002), with CLIC5A being the best-studied isoform. The latest addition to the list was CLIC6 (Friedli et al., 2003), but its high molecular mass (~72 kDa) makes it an atypical member whose function remains unknown. Rarely occurring mutations in *CLIC2* and *CLIC5* have been associated with heart disease and deafness, respectively (Seco et al., 2015; Takano et al., 2012).

CLIC proteins display tissue- and cell-type-specific expression patterns (Table 1). CLIC1 and CLIC4 are the most widely expressed and, arguably, the best-studied family members. Most, if not all, cell types coexpress multiple CLICs, which unfortunately hampers the functional analysis of individual family members.

CLIC structure

Structurally, soluble CLICs show a three-dimensional fold that is similar to the omega-class of glutathione-S-transferases (GSTs)

Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam 1066CX, The Netherlands.

*Authors for correspondence (e.argenzio@nki.nl; w.moolenaar@nki.nl)

W.H.M., 0000-0001-7545-198X

Journal of Cell Science (2016) 129, 4165-4174 doi:10.1242/jcs.189795

Box 1. The CLIC ion channel hypothesis

The notion that CLIC proteins – not to be confused with the CLC family of intracellular Cl^- transporters (Stauber and Jentsch, 2013) – might function as intracellular Cl^- channels has a long, and somewhat peculiar, history (Littler et al., 2010; Singh, 2010). Its founding member, the atypical p64/CLC5B protein, promotes Cl^- flux in liposomes (Landry et al., 1993, 1989). Subsequently, recombinant CLIC1, CLIC2 and CLIC4 were found to promote poorly selective ion fluxes across artificial membranes (Littler et al., 2010; Singh, 2010), particularly under oxidative conditions and at low pH values, but the mechanism of membrane insertion remained obscure. Electrophysiological experiments in cells that overexpress CLIC1 or CLIC4 suggested that CLICs possess ion channel activity *in vivo*. However, CLIC overexpression might as well activate endogenous ion channels, thus obscuring any secondary effects. It has also been suggested that that CLIC proteins associate with and somehow regulate the activity of other ion channels (Littler et al., 2010), but there is no immediate reason to invoke such role. In general, extreme caution is needed to interpret ion flux results obtained in often ill-defined artificial membranes, as these systems are prone to artefacts. In fact, there is ample evidence for purified proteins to induce non-specific ion currents in artificial membranes. For example, proteins as diverse as amyloid peptides, tau and transferrin can all induce transmembrane currents in artificial bilayers, without any evidence of physiological relevance (Aguilera et al., 2003; de Planque et al., 2007; Kagan et al., 2004; Patel et al., 2015). Thus, the CLIC 'Cl^- channel hypothesis' appears highly questionable, especially as convincing *in vivo* validation is still absent after more than two decades, and implies that the 'Cl^- intracellular channel' terminology might need to be revised.

(Dulhunty et al., 2001) and consist of an N-terminal thioredoxin-like domain followed by an all α-helical C-terminal domain (Fig. 1B,C). A putative transmembrane region (PTM) and a nuclear localization signal (NLS) are also present in the N- and C-terminal domain, respectively (Fig. 1B). In common with the omega-class of GST proteins, mammalian CLICs contain a conserved glutaredoxin-like site (Harrop et al., 2001; Littler et al., 2005) and a reactive cysteine residue (Cys24 and Cys35 in CLIC1 and CLIC4, respectively), which led to the suggestion that CLIC function can be regulated by redox-dependent processes (Harrop et al., 2001; Littler et al., 2004; Singh and Ashley, 2006).

Under oxidative conditions, CLIC1 can undergo a reversible rearrangement of the GST-like fold and the formation of an intramolecular disulphide bond (Cys24–Cys59) in the N-terminal domain. This conformational switch exposes a large hydrophobic surface within the monomer, favours the transition to a dimeric state and enhances the interaction of CLIC1 with artificial lipid bilayers (Goodchild et al., 2009; Littler et al., 2004). Biophysical studies indicate that, upon oxidation, CLIC1 forms large oligomeric complexes that consist of six to eight subunits, which might serve as a docking interface for membrane association (Goodchild et al., 2011; Hare et al., 2016). However, in the absence of structural studies and *in vivo* evidence, it remains unclear if and how CLIC1 inserts into and spans a lipid bilayer. Although CLIC1 can undergo a redox-controlled structural transition, thus far there is little or no evidence that cytosolic CLICs can act as redox sensors. In this respect it is worth noting that the reactive Cys residue is not conserved among invertebrate CLICs (Littler et al., 2008).

Between the N- and C-terminal domains, the CLICs structure shows an elongated cleft (or groove) similar to that observed in the omega GSTs, where it is involved in glutathione binding (Harrop et al., 2001). Unlike GSTs, however, CLIC proteins exhibit only very low affinity for glutathione. Yet, it is conceivable that CLICs use the GSH-binding site for targeting the CLIC to a particular

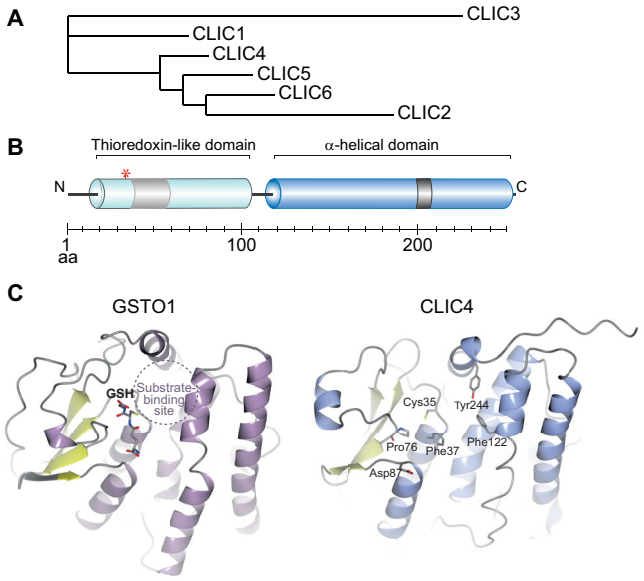

Fig. 1. The mammalian CLIC family and structural relationship with omega-class GST (GSTO-1). (A) Phylogenetic analysis of the mammalian CLIC proteins based on sequence homology. The branch length is proportional to the number of substitutions per site. CLIC proteins are 236–253 aa in length. CLIC5 exists as isoform A and B, with CLIC5B (410 aa) having an extended N-terminal region. CLIC6 is atypical in that it is much larger (704 aa), and has a unique extended N-terminal region without sequence similarity to other proteins. (B) CLIC domain structure. The N-terminal thioredoxin-like and C-terminal α-helical domains of human CLIC4 are shown in light and dark blue, respectively. The thioredoxin-like domain has a hypothetical transmembrane region (light gray), the α-helical domain has a nuclear localization signal (dark gray). The red asterisk indicates the reactive Cys residue (Cys35 in CLIC4). The horizontal bar indicates the number of amino acids. (C) Tertiary structure of GSTO1 (left) and CLIC4 (right). Glutathione (GSH) is depicted in gray. The indicated residues in CLIC4 are essential for agonist-induced, RhoA-dependent translocation to the plasma membrane. See Littler et al. (2005, 2010) and Ponsioen et al. (2009) for further details. Molecular models were created by using the CCP4MG software (Potterton et al., 2004) with optimized models for the structures PDB:1EEM for GSTO1 and PDB:2AHE for CLIC4 downloaded from PDB_REDO (Joosten et al., 2012).

subcellular location (Harrop et al., 2001). As this cleft is more open and elongated compared with that of GSTs, CLIC proteins might interact with partners or factors that are distinct from GST substrates. As such, this groove might serve as a binding site for an extended macromolecular chain, notably a polypeptide or a post-translationally modified protein, or – as the slot is basic – perhaps even for acidic phospholipids. Of note, the crystal structures of CLIC4 and CLIC2 show internal peptide loops in the vicinity of this slot, suggesting that this region can, indeed, incorporate such molecules (Littler et al., 2005; Mi et al., 2008).

In CLIC4, agonist-induced recruitment to the plasma membrane is abolished by mutation of those residues, which – in GSTs – are crucial for substrate binding (Ponsioen et al., 2009). These results strongly suggest that the substrate-binding features of the omega-class of GSTs have been conserved in CLICs – together with the fold itself – and that binding of an as-yet-unknown partner is essential for CLIC function (i.e. acute translocation upon receptor stimulation; see below).

Enzymatic activity of CLICs

Given their similarity in sequence and structure to GSTOs, CLICs have long been thought to possess intrinsic GST-like

Journal of Cell Science (2016) 129, 4165-4174 doi:10.1242/jcs.189795

Table 1. Expression and functions of CLIC proteins

CLIC	Expression*	Biological function	Somatic mutation	Gene knockout phenotype	References
C. elegans					
EXC-4	–	Tubulogenesis	–	Defective formation of excretory canal	See text
Mammals					
CLIC1	Wide (not in brain)	Ion channel activity, phagosomal acidification	–	Mild platelet dysfunction, less phagosomal acidification, resistance to rheumatoid arthritis, reduced autoimmune encephalomyelitis	See text and (Ashley, 2003; Ponnalagu et al., 2016)
CLIC2	Heart and liver	Ryanodine receptor (RyR) activity modulation	H101Q Implicated in RyR activity	–	See text and (Ashley, 2003; Dulhunty et al., 2011, 2005; Molina-Navarro et al., 2013)
CLIC3	Skeletal muscle, heart, kidney, lung and placenta	Endosomal trafficking	–	Slightly reduced phagosomal acidification in macrophages	See text and (Ashley, 2003; Kim et al., 2013; Money et al., 2007)
CLIC4	Wide (not in T cells)	Angiogenesis, vacuole formation, vesicle acidification, integrin trafficking	–	Reduced body weight at birth. More still births, impaired endothelial and renal tubulogenesis. Impaired vacuolar acidification and fusion, defective wound healing and cornea. Mice protected from LPS-induced death	See text and (Ashley, 2003; Duncan et al., 1997; Fernandez-Salas et al., 1999; He et al., 2011; Padmakumar et al., 2014; Ponnalagu et al., 2016)
CLIC5A	Kidney, heart, inner ear, lung, colon and placenta	Membrane-ERM interaction in cilia. Maintenance of podocyte and glomerular architecture. Actin-dependent membrane remodeling	c.96T>A (p.Cys32Ter). Loss of CLIC5 segregates with hearing loss.	Also known as jitterbug mouse. Progressive hearing loss and vestibular dysfunction, altered podocyte morphology, renal dysfunction, proteinuria	See text and (Pierchala et al., 2010; Ponnalagu et al., 2016; Tavasoli et al., 2016b)
CLIC5B	Kidney, heart, bone morrow and skeletal muscle	Actin-dependent membrane remodeling, osteoclast differentiation			See text and (Edwards et al., 2006; Redhead et al., 1997)
CLIC4 and CLIC5	–	–	–	Development of spontaneous proteinuria, glomerular cell proliferation and matrix deposition.	(Tavasoli et al., 2016a)
CLIC6	Bronchial epithelial cells, kidney, brain, gastric mucosa and choroid plexus*	Unknown	–	–	(Ashley, 2003; Nishizawa et al., 2000)

*, mRNA expression details in normal tissues and primary cells can be obtained from the BioGPS microarray database (http://biogps.org) (Wu et al., 2016).

activity, but experimental evidence for this notion has been elusive. It is only recent that purified CLIC proteins (CLIC1, CLIC2 and CLIC4) were shown to display glutaredoxin-like activity *in vitro* (Al Khamici et al., 2015), with the reactive Cys serving as the key catalytic residue. Currently it is still unclear (and will be challenging to assess) how this *in vitro* finding translates into the *in vivo* situation. Intriguingly, the enzymatic activity of CLIC1 is blocked by the indanyloxyacetic acid inhibitor of epithelial chloride channels, IAA-94, which was originally used to identify and purify the first CLIC protein, p64/CLIC5B (Landry et al., 1989). IAA-94 is thought to bind with in the long cleft between the N- and C-terminal domains, thereby interfering with substrate binding and catalysis (Al Khamici et al., 2015).

Insights from genetic studies

Gene-targeting studies in the nematode *Caenorhabditis elegans* and in mice have yielded important clues with regard to the normal physiological functions of CLIC proteins. Yet, we are still far from a detailed molecular understanding of the CLIC family. In mammals, analysis of CLIC protein function is hampered by the fact that most, if not all, cells co-express multiple family members that can have overlapping and redundant activities, thus confusing the interpretation of the findings of single-gene targeting.

Caernorhabditis elegans

The most revealing CLIC-knockout phenotype is observed in *C. elegans*, which expresses only two CLIC family members (EXC-4 and EXL-1) and, thus, makes it a convenient system for

functional investigations. EXC-4 localizes to the luminal membrane of the excretory canal, a renal-like system that is required for fluid-waste expulsion (Nelson et al., 1983). During the first steps of the formation of the excretory canal, large pinocytotic vacuoles fuse into an elongated tube (tubulogenesis) (Berry et al., 2003) that develops into the mature excretory canal.

A seminal study showed that disruption of the *exc-4* gene results in a dramatic failure of the animal to develop and maintain the intracellular excretory canal owing to defective tubulogenesis, as evidenced by the appearance of cystic enlargements in the canal (Berry et al., 2003). Expression of GFP–EXC-4 rescued the phenotype and the protein localized to the apical membrane at the canal lumen. The cyst phenotype could be rescued by human CLIC1, but only when it was fused to the 66 residue long N-terminal membrane-targeting sequence (amphipathic putative transmembrane helix, PTM) region of EXC-4 (Berry and Hobert, 2006). Intriguingly, omega- and sigma-class GSTs that contain the EXC-4 PTM region were also able to rescue the cyst phenotype. It thus appears that the main domains of EXC-4, human CLIC1 and *C. elegans* GSTs are functionally interchangeable. The C-terminal part is, therefore, not specific for the omega class of GSTs and the CLIC protein family, whereas the N-terminal PTM region of EXC-4 directs its targeting to specific membrane regions and can also provide functional specificity.

As mentioned above, the reactive Cys residues in vertebrate CLICs are not conserved within invertebrate CLICs (Berry and Hobert, 2006), which argues against a role for redox regulation in tubulogenesis. Taken together, this suggests that the Cys-dependent enzymatic activity of CLICs that is measured *in vitro* is not a main determinant of CLIC protein function *in vivo*.

As intracellular tube formation generally depends on acidification and fusion of intracellular vesicles or vacuoles, EXC-4 might direct vesicle fusion by promoting intravesicular acidification. Recent studies on mammalian CLIC1, CLIC3 and CLIC4 lend some support for this notion (Jiang et al., 2012; Kim et al., 2013; Salao et al., 2016; Ulmasov et al., 2009) (see below). However, the observed effects on intravesicular pH are relatively small and the possible underlying mechanism remains obscure. Alternatively, EXC-4 might be required for the maintenance of the tubule architecture by regulating H_2O transport across the membrane or directing the addition of new membranes to the mature tubes from a pool of intracellular vesicles, as discussed by Berry et al., (2003).

Interestingly, the phenotype of ERM-1-depleted worms strongly resembles that of *exc-4* null animals (van Furden et al., 2004). ERM-1 is a member of the actin regulatory ezrin-radixin-moesin (ERM) family and the only one expressed in *C. elegans*. Erm-1-deficient embryos show intestinal luminal constrictions and obstructions due to cytoskeletal abnormalities (van Furden et al., 2004). This similarity suggests that CLIC and ERM proteins have overlapping functions or cooperate during tubulogenesis.

Mouse models

In mice, gene disruption studies have been performed for *Clic1*, *Clic3*, *Clic4* and *Clic5* (Table 1), and are discussed below.

Clic1

In mice, disruption of the ubiquitous *Clic*1 gene results in mild platelet dysfunction (Qiu et al., 2010) and compromised phagosome acidification in macrophages and dendritic cells (Jiang et al., 2012; Salao et al., 2016). In macrophages that undergo phagocytosis, CLIC1 is recruited to phagosomal membranes where it colocalizes with ERM proteins, as well as RhoA and Rac2 (Jiang et al., 2012). Macrophages from Clic1$^{-/-}$ mice show reduced phagosome acidification by ∼0.2 pH units, but it is unclear whether CLIC1 is incorporated into the phagosomal membrane.

Consistently, *Clic1* knockout protects from induced arthritis (Jiang et al., 2012) where macrophages also play a key role. Furthermore, in dendritic cells, phagocytosis triggered translocation of CLIC1 to the phagosomal membrane concomitantly with altered phagosomal pH and proteolysis, as well as *in vitro* antigen processing, suggested a role for CLIC1 in the regulation of immune cell function (Salao et al., 2016).

Clic4

CLIC1 and CLIC4 show overlapping functions in that they promote angiogenesis in endothelial cell culture (Tung et al., 2009; Tung and Kitajewski, 2010), but their respective knockout phenotypes in mice are distinct. *Clic4*-null mice are smaller and show more still-births than wild-type animals (Ulmasov et al., 2009). CLIC4 deficiency results in impairment of tubulogenesis and vacuole formation in both endothelial and renal epithelial cells (Chou et al., 2016; Ulmasov et al., 2009), which is reminiscent of the *exc-4* knockout phenotype in *C. elegans*. Similar to the excretory canal in the worm, tubulogenesis in endothelial cells requires the formation and fusion of vacuoles within the cell body. Then, the newly formed lumen fuses with those from the neighboring cells, originating from the extracellular lumen of a multicellular tube.

Clic4$^{-/-}$ mice show defective development of blood vessels in the retina and impaired acidification (by ∼0.2 pH units) of large vacuoles but not endosomes or lysosomes (Ulmasov et al., 2009). These observations and the presence of CLIC4 at the vacuolar membranes cells suggest that CLIC4 regulates endothelial tubulogenesis through intracellular vacuolar acidification. CLIC4 might also contribute to tubulogenesis by modulating the osmotic swelling and trafficking of vacuoles or/and influencing the generation of pinocytotic vesicles that form the vacuoles. In addition to showing angiogenic defects, *Clic4*$^{-/-}$ mice suffer from spontaneous skin erosions, and delayed wound healing in the skin and cornea (Padmakumar et al., 2012). CLIC4-deficient keratinocytes showed impaired migration and reduced adhesion to the extracellular matrix, which may explain, at least in part, the observed skin healing defects.

A role of CLIC4 in the regulation of epithelial cell adhesion and morphology has also emerged from a rat retinal detachment model. Here, specific knockdown of Clic4 in pigment epithelium cells *in situ* led to reduced retinal adhesion, dramatic F-actin redistribution, and a loss of apical microvilli and basal infoldings, which is somewhat reminiscent of an epithelial-mesenchymal transition (EMT) phenotype (Chuang et al., 2010). These effects of *Clic4* knockdown were tentatively attributed to altered transmembrane transport mechanisms leading to subretinal fluid accumulation.

In the mouse kidney, renal proximal tubules of *Clic4*$^{-/-}$ embryos display a closed lumen and a complete loss of microvilli (Chou et al., 2016). The urinary system in mammals is the equivalent of the excretory canal of *C. elegans*. Kidney lumen formation occurs through coalescence of vesicles and subsequent vesicle exocytosis mediated by Rab- and Rho-family GTPases (Apodaca et al., 2012; Bryant et al., 2010). CLIC4-deficient tubule cells show reduced apical coalescence, decreased number of early and recycling endosomes, increased lysosome and/or vacuoles and aberrant dilation. As CLIC4 is located at early and recycling endosomes as well as other apical vesicles in wild-type animals, CLIC4 has been suggested to promote kidney luminogenesis by regulating the trafficking of the apical vesicles in an actin-dependent fashion (Chou et al., 2016).

Journal of Cell Science

Overall, the *Clic4*-null phenotype is consistent with a role for CLIC4 in the acidification, fusion and trafficking of vesicular compartments in order to generate vacuoles or cell body cavities. Vesicular acidification is regulated by the vacuolar (V)-ATPase-driven proton pump in conjunction with Cl^-/H^+ exchangers of the CLC family, which are unrelated to the CLIC family (Stauber and Jentsch, 2013). The possibility that CLIC1 and CLIC4 modulate, either directly or indirectly, V-ATPase activity or CLC antiporter function in distinct vesicular compartments is intriguing and warrants further study.

Clic5

CLIC5-deficient mice show unique phenotypes characterized by defects of the inner-ear and kidney, tissues in which CLIC5 is predominantly expressed. In the *Clic5*$^{-/-}$ mouse mutant, also known as jitterbug (jbg), *Clic5* was found to be inactivated due to a frame shift and premature stop codon (Gagnon et al., 2006). CLIC5-deficient mice show a lack of coordination and become deaf owing to progressive hair cell degeneration. Mechanistically, CLIC5 associates with ERM proteins, such as radixin, and with taperin in hair cell stereocilia and, thereby, helps to stabilize the linkages between the plasma membrane and the actin core (Gagnon et al., 2006; Salles et al., 2014). Consistent with this, the jbg phenotype resembles the radixin-knockout mouse (Kitajiri et al., 2004).

Mutation analysis in humans has shown that CLIC5 is involved in progressive hearing impairment and vestibular dysfunction (Seco et al., 2015). In mouse, renal glomeruli in CLIC5-deficient mice exhibit reduced ezrin levels, broadened podocyte foot processes, as well as large vacuoles in glomerular endothelial cells that can lead to renal dysfunction under unfavorable genetic or environmental conditions (Wegner et al., 2010). These data thus suggest that CLIC5 is required for the development and/or maintenance of glomerular endothelial cells and podocyte architecture. Consistent with this, a recent study has shown that a patient with a homozygous nonsense mutation in *CLIC5* has mild renal dysfunction in addition to hearing loss (Seco et al., 2015).

CLIC interactors

For a better understanding of CLIC function it is essential to identify and characterize the physiological binding partners of these proteins. Many efforts have been undertaken to identify specific interactors of CLICs, but progress in this area has been frustratingly slow. One reason could be that direct interactions are short-lived and/or of low affinity and, thereby, escape detection by classic biochemical methods. Various approaches ranging from yeast-2-hybrid (Y2H) screening to mass spectrometry (MS)-based proteomics have been exploited to identify CLIC-associated proteins in distinct tissues and subcellular compartments.

For CLIC4, candidate binding partners include cytoskeletal components (Berryman and Goldenring, 2003; Ponsioen et al., 2009; Suginta et al., 2001) but, as yet, there is no convincing evidence for a strong direct binding between CLIC4 and other proteins. Thus, a comprehensive inventory of CLIC binding partners has not been forthcoming to date. The most commonly found binding partners of CLICs are actin, actin regulators and signaling proteins. Through Y2H screening that used phosphatidylinositol-4-phosphate 5-kinase (PI4P5Kβ) as a bait (aa 350-500), CLIC1 and CLIC4 were found to interact with PI4P5K, as documented in a publicly available database (http://www.signaling-gateway.org/molecule).

In a COS7-cell overexpression system, CLIC5A promotes the clustering of phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] at the plasma membrane through its interaction with PI5P4K isoforms, which is thought to facilitate actin-dependent membrane remodeling (Al-Momany et al., 2014). Whether these interactions are direct and whether and how CLIC4 and CLIC5 are capable of regulating PIPK activity remains unclear.

Biochemical and colocalization studies suggest that, upon recruitment to the plasma membrane, both CLIC4 and CLIC5 function in a complex with ERM and/or actin-binding scaffold proteins, such as Na^+/H^+ exchange regulatory factor 2 (NHERF2, officially known as SLC9A3R2) and the above-mentioned ERM proteins (Al-Momany et al., 2014; Berryman and Bretscher, 2000; Berryman et al., 2004; Ponsioen et al., 2009; Salles et al., 2014; Tavasoli et al., 2016a; Viswanatha et al., 2013). The association of CLIC4 and CLIC5 with the actin cytoskeleton and the finding that CLICs traffic between distinct subcellular compartments upon receptor stimulation (see below) strongly suggest a role for CLIC proteins in actin-dependent membrane trafficking.

As CLIC interactions are likely to be weak and/or highly dynamic, they might allow CLICs to transiently interact with distinct partners along a given trafficking route, thus facilitating signal integration when multiple inputs are required for biological outcome (Jiang et al., 2014).

CLICs in membrane trafficking and endosomal sorting

CLIC translocation

One common feature of CLIC proteins, at least of CLIC1, CLIC2 and CLIC4, is their responsiveness to agonist stimulation, namely their rapid translocation between cellular compartments. Several studies have shown that cytosolic CLICs are rapidly recruited to the plasma membrane and to vesicular membranes upon receptor stimulation. Stimulation of microglial cell with amyloid-β (Aβ) peptides was found to promote transmembrane Cl^- currents (Novarino et al., 2004), possibly by inducing the translocation of cytosolic CLIC1 to the plasma membrane (Milton et al., 2008) (Fig. 2). Aβ-peptide-induced CLIC1 translocation was also associated to a NADPH oxidase-mediated production of reactive oxygen species (ROS), which is thought to contribute to neurodegeneration (Milton et al., 2008). It should be noted, however, that Aβ peptides can induce ion channel activity in artificial membranes (Box 1) and, furthermore, might activate ion channels that are endogenous to the host cell.

The translocation of cytoplasmic CLIC4 upon different means of stimulation has been extensively documented (Fig. 2). CLIC4 has been reported to translocate to the nucleus of keratinocytes that undergo enforced apoptosis (Suh et al., 2004). This effect is transient and requires both the putative NLS of CLIC4 and the nuclear import machinery. Nuclear translocation of CLIC4 has also been observed in macrophages that are exposed to lipopolysaccharide (LPS) and interferon γ (IFNγ) (Malik et al., 2012). It was shown previously that transforming growth factor β (TGFβ) induces nuclear translocation of CLIC4 (Shukla et al., 2009). Nuclear CLIC4 was proposed to protect phosphorylated SMAD proteins (SMAD2 and SMAD3) from dephosphorylation, thereby enhancing TGF-β signaling (Shukla et al., 2009). Furthermore, it has been suggested that the regulation of wound healing in mice by CLIC4 involves TGFβ-induced epithelial cell migration (Padmakumar et al., 2012). These findings notwithstanding, more recent analysis of CLIC4-deficient mice did not immediately point to a major role for CLIC4 in TGFβ signaling (Edwards et al., 2014).

CLIC4 has been shown to translocate rapidly, i.e. within 1 min, from the cytosol to the plasma membrane upon addition of serum or

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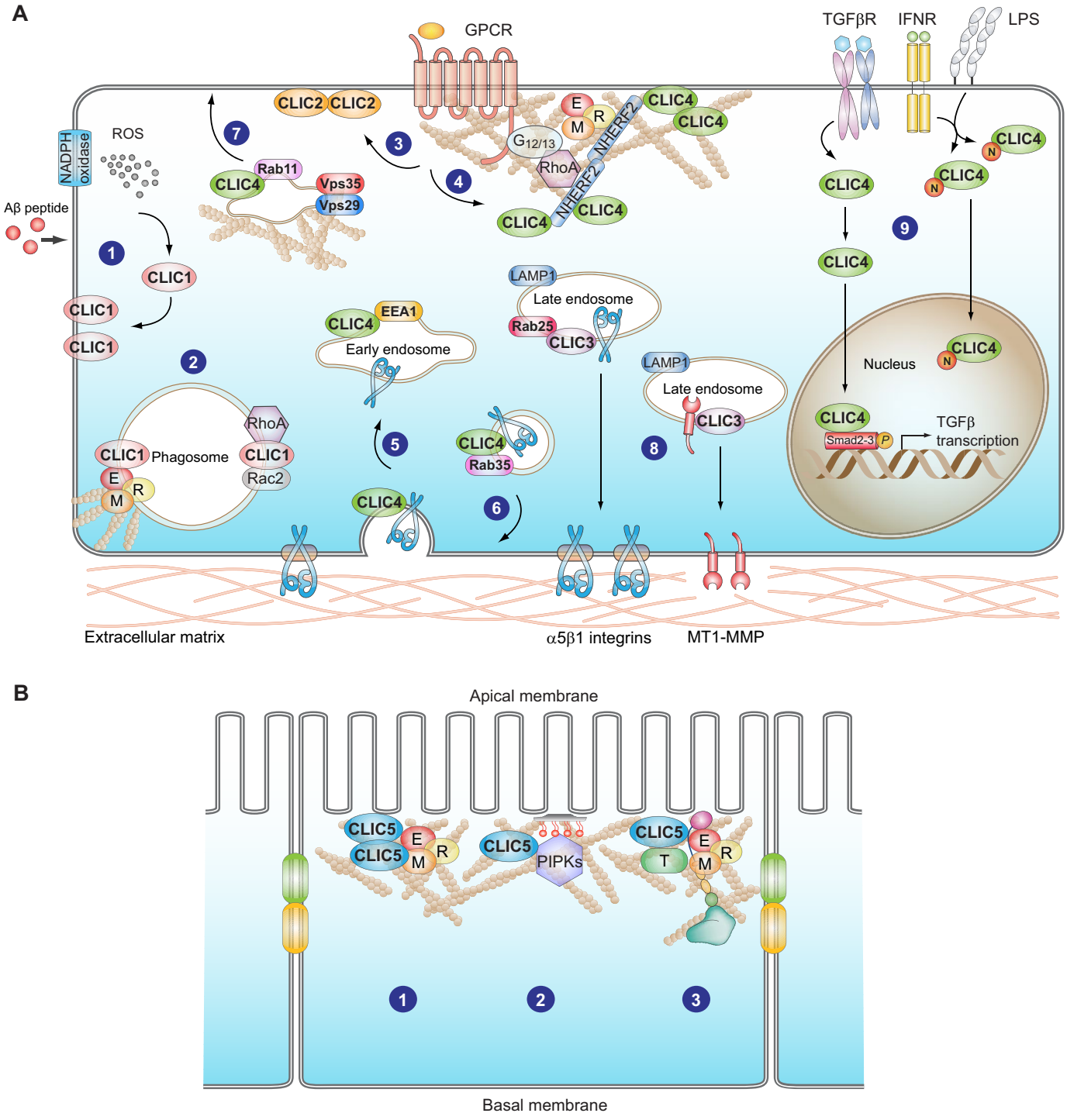

Fig. 2. Intracellular activities of CLIC proteins. (A) Overview of the cellular roles of CLIC1 to CLIC4. (1) Amyloid β (Aβ) peptide induces CLIC1 translocation and insertion into the plasma membrane, which is associated with NADPH oxidase-mediated oxygen species (ROS) production (Milton et al., 2008). (2) CLIC1 is detected on phagosomal membranes in macrophages undergoing phagocytosis and colocalizes with ERM proteins, RhoA and Rac2 (Jiang et al., 2012). (3, 4) CLIC2 and CLIC4 undergo rapid translocation to the plasma membrane upon GPCR stimulation (Lecat et al., 2015). (4) Upon stimulation with LPA, CLIC4 colocalizes with NHERF2 and β1 integrin at the plasma membrane and in a subset of Rab35-positive recycling endosome (Argenzio et al., 2014; Ponsioen et al., 2009). CLIC4 contributes to the internalization of β1 integrin (5) and its LPA-induced recycling back to the plasma membrane (Argenzio et al., 2014) (6). (7) CLIC4 stimulates retromer-mediated endosome trafficking in an actin- and Rab11-dependent manner (Chou et al., 2016). (8) CLIC3 colocalizes with LAMP1 in late endosomes from where it controls the recycling of α5β1 integrins in a Rab25-dependent manner (Dozynkiewicz et al., 2012). CLIC3 also regulates the recycling of matrix metallopeptidase 14 (MT1-MMP) in a Rab25-independent fashion (Macpherson et al., 2014). (9) TGFβ induces translocation of CLIC4 into the nucleus were it prevents the dephosphorylation of SMAD proteins to enhance transcription (Shukla et al., 2009). INFγ and LPS promote CLIC4 S-nitrosylation (N) and nuclear translocation (Malik et al., 2012). TGFβR, transforming growth factor β receptor, IFNR, interferon receptor; GPCR, G-protein-coupled receptor. (B) Activity of CLIC5. (1) CLIC5 colocalizes with ERM proteins in microvilli of polarized cells (Berryman and Goldenring, 2003). (2) CLIC5 recruits PIP kinases (PIPKs) to influence local phosphoinositide levels at the plasma membrane (Al-Momany et al., 2014). (3) CLIC5 stabilizes membrane-actin linkages at hair cell stereocilia in complex with ERM proteins and taperin (T) (Salles et al., 2014). See text for further details. E, ezrin; R, radixin; M, moesin.

G-protein-coupled receptor (GPCR) agonists, such as lysophosphatidic acid (LPA), in diverse cell types (Argenzio et al., 2014; Lecat et al., 2015; Ponsioen et al., 2009). CLIC2 shows a similarly rapid translocation upon GPCR stimulation (Lecat et al., 2015) (Fig. 2). LPA-induced membrane recruitment of CLIC4 requires activation of the $G\alpha_{13}$-mediated RhoA pathway and depends on actin polymerization but not on activity of Rho kinase (ROCK) (Ponsioen et al., 2009). At the plasma membrane, CLIC4 colocalizes with NHERF2, a scaffold protein that connects transmembrane proteins with the actin cytoskeleton to assemble signaling complexes (Ponsioen et al., 2009).

However, the exact mechanisms of CLIC translocation remain unclear. In theory, CLIC translocation could be regulated by post-translational modifications. For instance, LPS- and IFNγ-induced nuclear translocation of CLIC4 in macrophages depends on *S*-nitrosylation, which can induce a conformational change that promotes the association of CLIC4 with the nuclear import machinery (Malik et al., 2012). Other post-translational modifications found or predicted for the CLIC proteins include phosphorylation (Suh et al., 2007), ubiquitylation (Wagner et al., 2012) and palmitoylation (Fang et al., 2016); but biochemical details and functional outcomes remain to be determined.

CLICs in endosomal sorting

Endo- and exocytic membrane trafficking is an important regulatory mechanisms of receptor expression and signal transduction. CLIC proteins have been shown to participate and regulate the trafficking of diverse membrane proteins in different model systems. In particular, CLICs have been implicated in regulating the trafficking of integrin adhesion receptors. Integrins mediate cell-extracellular matrix (ECM) adhesion and consist of two non-covalently associated transmembrane subunits (α and β). Ligand-binding to the extracellular domain of integrins changes their conformation and induces clustering at the plasma membrane, which results in linkage to the actin cytoskeleton and initiation of the so-called 'outside-in signal transduction cascade' (Geiger and Yamada, 2011). Endocytic and exocytic cycling of integrin subunits is a key step in the turnover of adhesion complexes and, thus, has profound effects on signaling, cell–matrix interaction, as well as cell migration and invasion (Caswell and Norman, 2006). Integrin endocytosis is a constitutive process that is accelerated upon cell–matrix adhesion. By contrast, recycling of integrins back to the plasma membrane is induced by a variety of ligands and growth factors (White et al., 2007). Once activated, integrins are rapidly internalized and spatially confined in endosomes from where they can either recycle back to the cell surface (Caswell et al., 2009) or undergo lysosomal degradation (Lobert et al., 2010). Rab GTPases are key regulators of endosomal trafficking and sorting, including that of integrins.

CLIC1, CLIC3 and CLIC4 colocalize with integrin subunits α5, β1 and β3, and with distinct Rab GTPases upon growth factor stimulation or integrin engagement (Argenzio et al., 2014; Dozynkiewicz et al., 2012; Gurski et al., 2015). Both CLIC3 and CLIC4 regulate the trafficking of α5β1 integrin and promote the recycling of activated α5β1 integrin from different cellular compartments back to plasma membrane (Argenzio et al., 2014; Dozynkiewicz et al., 2012) (Fig. 2). In ovarian cancer cells, CLIC3 localizes to late endosomes and/or lysosomes where Rab25 regulates the transport of activated α5β1 integrins. From there, CLIC3 promotes integrin recycling back to the plasma membrane (Dozynkiewicz et al., 2012).

As such, CLIC3 and Rab25 regulate cell adhesion dynamics to promote cancer cell motility and invasiveness. In breast cancer cells, CLIC3 has only little influence on integrin recycling but controls the trafficking of the pro-invasive matrix metallopeptidase 14 (MMP14, hereafter referred to as MT1-MMP). In these cells, CLIC3 regulates the recycling of MT1-MMP from late endosomes and/or lysosomes back to the plasma membrane independently of Rab25 (Macpherson et al., 2014), thereby promoting MT1-MMP-dependent invasiveness. Thus, these data suggest that CLIC3 is involved in late endosomal trafficking to drive tumor cell invasion; however, its specific cargo (active integrin or MT1-MMP) depends on cellular context and the presence of Rab25 (Fig. 2).

Similar to CLIC3, CLIC4 also has a role in integrin trafficking and recycling but there are clear mechanistic differences. In HeLa and MDA-MB-231carcinoma cells, cytosolic CLIC4 is homogeneously distributed and colocalizes with a subset of early endosome antigen 1 (EEA1)- and Rab35-positive endosomes (Argenzio et al., 2014). Rab35 is a central regulator of endocytic recycling and shuttles between the plasma membrane and endosomes (Allaire et al., 2013; Klinkert and Echard, 2016; Kouranti et al., 2006). Consistently, knockdown of CLIC4 in HeLa and MDA-MB-231 cells results in impaired endocytosis of α5β1 integrin and a reduction of integrin recycling upon stimulation with serum or LPA (Argenzio et al., 2014). Here, LPA stimulation leads to rapid colocalization of CLIC4 with β1 integrin in Rab35-positive endosomes and at the plasma membrane. We also observed CLIC4 colocalization with β1 integrin in a small subset of EEA1-positive endosomes, which is consistent with the notion that the interaction between CLIC4 and β1 integrin might be short-lived and occurs at distinct steps along the integrin trafficking pathway (Argenzio et al., 2014). Furthermore, CLIC4 silencing leads to increased levels of active GTP-bound Rab35, suggesting that CLIC4 regulates Rab35 GTPase activity. CLIC4 knockdown also decreases cell–matrix adhesion, cell spreading and integrin signaling, whereas it increases cell motility, which is important for tissue morphogenesis as well as tumor cell invasion (Argenzio et al., 2014).

Taken together, these results suggest that CLIC3 and CLIC4 function at distinct steps along the integrin trafficking route and, possibly, regulate the activity of small Rab GTPases (Fig. 2).

Finally, a recent study on renal tubulogenesis confirmed that CLIC4 regulates intracellular trafficking (Chou et al., 2016). Here, in 3D cysts and monolayer cultures of MDCK cells, CLIC4 was found to colocalize with the retromer complex subunits Vps35 and Vps29, and with Rab11 in recycling endosomes. The retromer complex is a key component of the endosomal sorting machinery that recognizes and sorts cargos to their final destination, such as endosomal compartments, plasma membrane or trans-Golgi network (Seaman, 2012). CLIC4 depletion resulted in the formation of cysts with multiple lumima and also impaired the delivery of Rab11 to the apical membrane of MDCK cells (Chou et al., 2016). These cells phenocopy those obtained through interference with the components of the retromer complex and could be rescued by overexpressing other exocytic proteins (e.g. Cdc42 and Rab8). Furthermore, branched actin was enriched at early endosomes within CLIC4-null cells, suggesting that CLIC4 regulates retromer-mediated early endosome trafficking in an actin-dependent manner (Chou et al., 2016). These findings, thus, confirm CLIC4 as a trafficking regulator that acts in concert with the actin cytoskeleton (Fig. 2).

Conclusions and perspectives

It has been more than 25 years since the first CLIC protein was identified, but many questions about their functions still remain. Fig. 2 summarizes the main cellular processes in which distinct

CLIC proteins play a role. The CLICs appear to be associated with intracellular vesicular membranes and membrane trafficking. A common finding is that CLIC proteins are involved in signaling and actin-dependent membrane remodeling processes that are regulated by Rho-family GTPases. Some of these processes involve specialized plasma membrane structures, such as microvilli, stereocilia and podocytes, whereas others involve the formation of membrane vesicles (phagocytosis) or vesicle trafficking (phagosome–lysosome fusion, endosome recycling). Here, the underlying mechanism might involve the endosomal trafficking from the cytoplasm to the plasma membrane but it is still unclear how exactly CLICs function in these events, and to establish their mode of action remains a main challenge. One could envisage that CLICs somehow contribute to the maintenance of the intravesicular pH balance, or stabilize interactions at intracellular membranes or, perhaps, both. In light of the recent findings discussed here, it may also be possible that CLIC proteins regulate trafficking by modulating the activity of Rab GTPases.

One of the main remaining challenges is the identification of specific binding partners; this should help to clarify how CLICs act or associate to vesicular compartments. Under physiological conditions, post-translational modification of CLIC proteins and/or their binding partners might be required for the interaction to occur. Given the role of CLIC1 and CLIC4 in both angiogenesis, and RhoA signaling mediated by $G\alpha_{12}$ or $G\alpha_{13}$ (Ponsioen et al., 2009), it will be interesting to determine whether the severe angiogenic and cell-migration defects observed in $G\alpha_{13}$-knockout mice (Offermanns et al., 1997) are attributable, at least in part, to impaired CLIC1 and/or CLIC4 function. The generation of new knockout mouse models, together with further molecular studies, should help to uncover new biological functions and yield new insights into the biochemical and inner workings of CLIC proteins, and what makes each of these – important but still enigmatic – proteins unique.

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References

Aguilera, O., Quiros, L. M. and Fierro, J. F. (2003). Transferrins selectively cause ion efflux through bacterial and artificial membranes. *FEBS Lett.* **548**, 5-10.

Al Khamici, H., Brown, L. J., Hossain, K. R., Hudson, A. L., Sinclair-Burton, A. A., Ng, J. P. M., Daniel, E. L., Hare, J. E., Cornell, B. A., Curmi, P. M. G. et al. (2015). Members of the chloride intracellular ion channel protein family demonstrate glutaredoxin-like enzymatic activity. *PLoS ONE* **10**, e115699.

Allaire, P. D., Seyed Sadr, M., Chaineau, M., Seyed Sadr, E., Konefal, S., Fotouhi, M., Maret, D., Ritter, B., Del Maestro, R. F. and McPherson, P. S. (2013). Interplay between Rab35 and Arf6 controls cargo recycling to coordinate cell adhesion and migration. *J. Cell Sci.* **126**, 722-731.

Al-Momany, A., Li, L., Alexander, R. T. and Ballermann, B. J. (2014). Clustered PI(4,5)P(2) accumulation and ezrin phosphorylation in response to CLIC5A. *J. Cell Sci.* **127**, 5164-5178.

Apodaca, G., Gallo, L. I. and Bryant, D. M. (2012). Role of membrane traffic in the generation of epithelial cell asymmetry. *Nat. Cell Biol.* **14**, 1235-1243.

Argenzio, E., Margadant, C., Leyton-Puig, D., Janssen, H., Jalink, K., Sonnenberg, A. and Moolenaar, W. H. (2014). CLIC4 regulates cell adhesion and beta1 integrin trafficking. *J. Cell Sci.* **127**, 5189-5203.

Ashley, R. H. (2003). Challenging accepted ion channel biology: p64 and the CLIC family of putative intracellular anion channel proteins (Review). *Mol. Membr. Biol.* **20**, 1-11.

Berry, K. L. and Hobert, O. (2006). Mapping functional domains of chloride intracellular channel (CLIC) proteins in vivo. *J. Mol. Biol.* **359**, 1316-1333.

Berry, K. L., Bulow, H. E., Hall, D. H. and Hobert, O. (2003). A C. elegans CLIC-like protein required for intracellular tube formation and maintenance. *Science* **302**, 2134-2137.

Berryman, M. and Bretscher, A. (2000). Identification of a novel member of the chloride intracellular channel gene family (CLIC5) that associates with the actin cytoskeleton of placental microvilli. *Mol. Biol. Cell* **11**, 1509-1521.

Berryman, M. A. and Goldenring, J. R. (2003). CLIC4 is enriched at cell-cell junctions and colocalizes with AKAP350 at the centrosome and midbody of cultured mammalian cells. *Cell Motil. Cytoskeleton* **56**, 159-172.

Berryman, M., Bruno, J., Price, J. and Edwards, J. C. (2004). CLIC-5A functions as a chloride channel in vitro and associates with the cortical actin cytoskeleton in vitro and in vivo. *J. Biol. Chem.* **279**, 34794-34801.

Bohman, S., Matsumoto, T., Suh, K., Dimberg, A., Jakobsson, L., Yuspa, S. and Claesson-Welsh, L. (2005). Proteomic analysis of vascular endothelial growth factor-induced endothelial cell differentiation reveals a role for chloride intracellular channel 4 (CLIC4) in tubular morphogenesis. *J. Biol. Chem.* **280**, 42397-42404.

Bryant, D. M., Datta, A., Rodríguez-Fraticelli, A. E., Peränen, J., Martín-Belmonte, F. and Mostov, K. E. (2010). A molecular network for de novo generation of the apical surface and lumen. *Nat. Cell Biol.* **12**, 1035-1045.

Caswell, P. T. and Norman, J. C. (2006). Integrin trafficking and the control of cell migration. *Traffic* **7**, 14-21.

Caswell, P. T., Vadrevu, S. and Norman, J. C. (2009). Integrins: masters and slaves of endocytic transport. *Nat. Rev. Mol. Cell Biol.* **10**, 843-853.

Chou, S.-Y., Hsu, K.-S., Otsu, W., Hsu, Y.-C., Luo, Y.-C., Yeh, C., Shehab, S. S., Chen, J., Shieh, V., He, G.-A. et al. (2016). CLIC4 regulates apical exocytosis and renal tube luminogenesis through retromer- and actin-mediated endocytic trafficking. *Nat. Commun.* **7**, 10412.

Chuang, J. Z., Milner, T. A., Zhu, M. and Sung, C. H. (1999). A 29 kDa intracellular chloride channel p64H1 is associated with large dense-core vesicles in rat hippocampal neurons. *J. Neurosci.* **19**, 2919-2928.

Chuang, J.-Z., Chou, S.-Y. and Sung, C.-H. (2010). Chloride intracellular channel 4 is critical for the epithelial morphogenesis of RPE cells and retinal attachment. *Mol. Biol. Cell* **21**, 3017-3028.

de Planque, M. R. R., Raussens, V., Contera, S. A., Rijkers, D. T. S., Liskamp, R. M. J., Ruysschaert, J.-M., Ryan, J. F., Separovic, F. and Watts, A. (2007). beta-Sheet structured beta-amyloid(1-40) perturbs phosphatidylcholine model membranes. *J. Mol. Biol.* **368**, 982-997.

Dozynkiewicz, M. A., Jamieson, N. B., MacPherson, I., Grindlay, J., van den Berghe, P. V. E., von Thun, A., Morton, J. P., Gourley, C., Timpson, P., Nixon, C. et al. (2012). Rab25 and CLIC3 collaborate to promote integrin recycling from late endosomes/lysosomes and drive cancer progression. *Dev. Cell* **22**, 131-145.

Dulhunty, A., Gage, P., Curtis, S., Chelvanayagam, G. and Board, P. (2001). The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator. *J. Biol. Chem.* **276**, 3319-3323.

Dulhunty, A. F., Pouliquin, P., Coggan, M., Gage, P. W. and Board, P. G. (2005). A recently identified member of the glutathione transferase structural family modifies cardiac RyR2 substate activity, coupled gating and activation by Ca2+ and ATP. *Biochem. J.* **390**, 333-343.

Dulhunty, A. F., Hewawasam, R., Liu, D., Casarotto, M. G. and Board, P. G. (2011). Regulation of the cardiac muscle ryanodine receptor by glutathione transferases. *Drug Metab. Rev.* **43**, 236-252.

Duncan, R. R., Westwood, P. K., Boyd, A. and Ashley, R. H. (1997). Rat brain p64H1, expression of a new member of the p64 chloride channel protein family in endoplasmic reticulum. *J. Biol. Chem.* **272**, 23880-23886.

Edwards, J. C. (1999). A novel p64-related Cl- channel: subcellular distribution and nephron segment-specific expression. *Am. J. Physiol.* **276**, F398-F408.

Edwards, J. C., Cohen, C., Xu, W. and Schlesinger, P. H. (2006). c-Src control of chloride channel support for osteoclast HCl transport and bone resorption. *J. Biol. Chem.* **281**, 28011-28022.

Edwards, J. C., Bruno, J., Key, P. and Cheng, Y.-W. (2014). Absence of chloride intracellular channel 4 (CLIC4) predisposes to acute kidney injury but has minimal impact on recovery. *BMC Nephrol.* **15**, 54.

Fang, C., Zhang, X., Zhang, L., Gao, X., Yang, P. and Lu, H. (2016). Identification of palmitoylated transitional endoplasmic reticulum ATPase by proteomic technique and pan anti-palmitoylation antibody. *J. Proteome Res.* **15**, 956-962.

Fernandez-Salas, E., Sagar, M., Cheng, C., Yuspa, S. H. and Weinberg, W. C. (1999). p53 and tumor necrosis factor alpha regulate the expression of a mitochondrial chloride channel protein. *J. Biol. Chem.* **274**, 36488-36497.

Friedli, M., Guipponi, M., Bertrand, S., Bertrand, D., Neerman-Arbez, M., Scott, H. S., Antonarakis, S. E. and Reymond, A. (2003). Identification of a novel member of the CLIC family, CLIC6, mapping to 21q22.12. *Gene* **320**, 31-40.

Gagnon, L. H., Longo-Guess, C. M., Berryman, M., Shin, J.-B., Saylor, K. W., Yu, H., Gillespie, P. G. and Johnson, K. R. (2006). The chloride intracellular channel

protein CLIC5 is expressed at high levels in hair cell stereocilia and is essential for normal inner ear function. *J. Neurosci.* **26**, 10188-10198.
Geiger, B. and Yamada, K. M. (2011). Molecular architecture and function of matrix adhesions. *Cold Spring Harb. Perspect. Biol.* **3**.
Goodchild, S. C., Howell, M. W., Cordina, N. M., Littler, D. R., Breit, S. N., Curmi, P. M. G. and Brown, L. J. (2009). Oxidation promotes insertion of the CLIC1 chloride intracellular channel into the membrane. *Eur. Biophys. J.* **39**, 129-138.
Goodchild, S. C., Angstmann, C. N., Breit, S. N., Curmi, P. M. G. and Brown, L. J. (2011). Transmembrane extension and oligomerization of the CLIC1 chloride intracellular channel protein upon membrane interaction. *Biochemistry* **50**, 10887-10897.
Gurski, L. A., Knowles, L. M., Basse, P. H., Maranchie, J. K., Watkins, S. C. and Pilch, J. (2015). Relocation of CLIC1 promotes tumor cell invasion and colonization of fibrin. *Mol. Cancer Res.* **13**, 273-280.
Hare, J. E., Goodchild, S. C., Breit, S. N., Curmi, P. M. G. and Brown, L. J. (2016). Interaction of human Chloride Intracellular Channel Protein 1 (CLIC1) with lipid bilayers: a fluorescence study. *Biochemistry* **55**, 3825-3833.
Harrop, S. J., DeMaere, M. Z., Fairlie, W. D., Reztsova, T., Valenzuela, S. M., Mazzanti, M., Tonini, R., Qiu, M. R., Jankova, L., Warton, K. et al. (2001). Crystal structure of a soluble form of the intracellular chloride ion channel CLIC1 (NCC27) at 1.4-A resolution. *J. Biol. Chem.* **276**, 44993-45000.
He, G., Ma, Y., Chou, S.-Y., Li, H., Yang, C., Chuang, J.-Z., Sung, C.-H. and Ding, A. (2011). Role of CLIC4 in the host innate responses to bacterial lipopolysaccharide. *Eur. J. Immunol.* **41**, 1221-1230.
Heiss, N. S. and Poustka, A. (1997). Genomic structure of a novel chloride channel gene, CLIC2, in Xq28. *Genomics* **45**, 224-228.
Jiang, L., Salao, K., Li, H., Rybicka, J. M., Yates, R. M., Luo, X. W., Shi, X. X., Kuffner, T., Tsai, V. W.-W., Husaini, Y. et al. (2012). Intracellular chloride channel protein CLIC1 regulates macrophage function through modulation of phagosomal acidification. *J. Cell Sci.* **125**, 5479-5488.
Jiang, L., Phang, J. M., Yu, J., Harrop, S. J., Sokolova, A. V., Duff, A. P., Wilk, K. E., Alkhamici, H., Breit, S. N., Valenzuela, S. M. et al. (2014). CLIC proteins, ezrin, radixin, moesin and the coupling of membranes to the actin cytoskeleton: a smoking gun? *Biochim. Biophys. Acta* **1838**, 643-657.
Joosten, R. P., Joosten, K., Murshudov, G. N. and Perrakis, A. (2012). PDB_REDO: constructive validation, more than just looking for errors. *Acta Crystallogr. D Biol. Crystallogr.* **68**, 484-496.
Kagan, B. L., Azimov, R. and Azimova, R. (2004). Amyloid peptide channels. *J. Membr. Biol.* **202**, 1-10.
Kim, K. H., Choi, B. K., Song, K. M., Cha, K. W., Kim, Y. H., Lee, H., Han, I.-S. and Kwon, B. S. (2013). CRIg signals induce anti-intracellular bacterial phagosome activity in a chloride intracellular channel 3-dependent manner. *Eur. J. Immunol.* **43**, 667-678.
Kitajiri, S., Fukumoto, K., Hata, M., Sasaki, H., Katsuno, T., Nakagawa, T., Ito, J., Tsukita, S. and Tsukita, S. (2004). Radixin deficiency causes deafness associated with progressive degeneration of cochlear stereocilia. *J. Cell Biol.* **166**, 559-570.
Klinkert, K. and Echard, A. (2016). Rab35 GTPase: a central regulator of phosphoinositides and F-actin in endocytic recycling and beyond. *Traffic* **17**, 1063-1077.
Kouranti, I., Sachse, M., Arouche, N., Goud, B. and Echard, A. (2006). Rab35 regulates an endocytic recycling pathway essential for the terminal steps of cytokinesis. *Curr. Biol.* **16**, 1719-1725.
Landry, D. W., Akabas, M. H., Redhead, C., Edelman, A., Cragoe, E. J., Jr. and Al-Awqati, Q. (1989). Purification and reconstitution of chloride channels from kidney and trachea. *Science* **244**, 1469-1472.
Landry, D., Sullivan, S., Nicolaides, M., Redhead, C., Edelman, A., Field, M., al-Awqati, Q. and Edwards, J. (1993). Molecular cloning and characterization of p64, a chloride channel protein from kidney microsomes. *J. Biol. Chem.* **268**, 14948-14955.
Lecat, S., Matthes, H. W. D., Pepperkok, R., Simpson, J. C. and Galzi, J.-L. (2015). A fluorescent live imaging screening assay based on translocation criteria identifies novel cytoplasmic proteins implicated in G protein-coupled receptor signaling pathways. *Mol. Cell. Proteomics* **14**, 1385-1399.
Littler, D. R., Harrop, S. J., Fairlie, W. D., Brown, L. J., Pankhurst, G. J., Pankhurst, S., DeMaere, M. Z., Campbell, T. J., Bauskin, A. R., Tonini, R. et al. (2004). The intracellular chloride ion channel protein CLIC1 undergoes a redox-controlled structural transition. *J. Biol. Chem.* **279**, 9298-9305.
Littler, D. R., Assaad, N. N., Harrop, S. J., Brown, L. J., Pankhurst, G. J., Luciani, P., Aguilar, M.-I., Mazzanti, M., Berryman, M. A., Breit, S. N. et al. (2005). Crystal structure of the soluble form of the redox-regulated chloride ion channel protein CLIC4. *FEBS J.* **272**, 4996-5007.
Littler, D. R., Harrop, S. J., Brown, L. J., Pankhurst, G. J., Mynott, A. V., Luciani, P., Mandyam, R. A., Mazzanti, M., Tanda, S., Berryman, M. A. et al. (2008). Comparison of vertebrate and invertebrate CLIC proteins: the crystal structures of Caenorhabditis elegans EXC-4 and Drosophila melanogaster DmCLIC. *Proteins* **71**, 364-378.
Littler, D. R., Harrop, S. J., Goodchild, S. C., Phang, J. M., Mynott, A. V., Jiang, L., Valenzuela, S. M., Mazzanti, M., Brown, L. J., Breit, S. N. et al. (2010). The enigma of the CLIC proteins: Ion channels, redox proteins, enzymes, scaffolding proteins? *FEBS Lett.* **584**, 2093-2101.
Lobert, V. H., Brech, A., Pedersen, N. M., Wesche, J., Oppelt, A., Malerød, L. and Stenmark, H. (2010). Ubiquitination of alpha 5 beta 1 integrin controls fibroblast migration through lysosomal degradation of fibronectin-integrin complexes. *Dev. Cell* **19**, 148-159.
Macpherson, I. R., Rainero, E., Mitchell, L. E., van den Berghe, P. V., Speirs, C., Dozynkiewicz, M. A., Chaudhary, S., Kalna, G., Edwards, J., Timpson, P. et al. (2014). CLIC3 controls recycling of late endosomal MT1-MMP and dictates invasion and metastasis in breast cancer. *J. Cell Sci.* **127**, 3893-3901.
Malik, M., Jividen, K., Padmakumar, V. C., Cataisson, C., Li, L., Lee, J., Howard, O. M. Z. and Yuspa, S. H. (2012). Inducible NOS-induced chloride intracellular channel 4 (CLIC4) nuclear translocation regulates macrophage deactivation. *Proc. Natl. Acad. Sci. USA* **109**, 6130-6135.
Mi, W., Liang, Y.-H., Li, L. and Su, X.-D. (2008). The crystal structure of human chloride intracellular channel protein 2: a disulfide bond with functional implications. *Proteins* **71**, 509-513.
Milton, R. H., Abeti, R., Averaimo, S., DeBiasi, S., Vitellaro, L., Jiang, L., Curmi, P. M. G., Breit, S. N., Duchen, M. R. and Mazzanti, M. (2008). CLIC1 function is required for beta-amyloid-induced generation of reactive oxygen species by microglia. *J. Neurosci.* **28**, 11488-11499.
Molina-Navarro, M. M., Roselló-Lletí, E., Ortega, A., Tarazón, E., Otero, M., Martínez-Dolz, L., Lago, F., González-Juanatey, J. R., España, F., García-Pavía, P. et al. (2013). Differential gene expression of cardiac ion channels in human dilated cardiomyopathy. *PLoS ONE* **8**, e79792.
Money, T. T., King, R. G., Wong, M. H., Stevenson, J. L., Kalionis, B., Erwich, J. J. H. M., Huisman, M. A., Timmer, A., Hiden, U., Desoye, G. et al. (2007). Expression and cellular localisation of chloride intracellular channel 3 in human placenta and fetal membranes. *Placenta* **28**, 429-436.
Nelson, F. K., Albert, P. S. and Riddle, D. L. (1983). Fine structure of the Caenorhabditis elegans secretory—excretory system. *J. Ultrastruct. Res.* **82**, 156-171.
Nishizawa, T., Nagao, T., Iwatsubo, T., Forte, J. G. and Urushidani, T. (2000). Molecular cloning and characterization of a novel chloride intracellular channel-related protein, parchorin, expressed in water-secreting cells. *J. Biol. Chem.* **275**, 11164-11173.
Novarino, G., Fabrizi, C., Tonini, R., Denti, M. A., Malchiodi-Albedi, F., Lauro, G. M., Sacchetti, B., Paradisi, S., Ferroni, A., Curmi, P. M. et al. (2004). Involvement of the intracellular ion channel CLIC1 in microglia-mediated beta-amyloid-induced neurotoxicity. *J. Neurosci.* **24**, 5322-5330.
Offermanns, S., Mancino, V., Revel, J.-P. and Simon, M. I. (1997). Vascular system defects and impaired cell chemokinesis as a result of Galpha13 deficiency. *Science* **275**, 533-536.
Padmakumar, V. C., Speer, K., Pal-Ghosh, S., Masiuk, K. E., Ryscavage, A., Dengler, S. L., Hwang, S., Edwards, J. C., Coppola, V., Tessarollo, L. et al. (2012). Spontaneous skin erosions and reduced skin and corneal wound healing characterize CLIC4(NULL) mice. *Am. J. Pathol.* **181**, 74-84.
Padmakumar, V., Masiuk, K. E., Luger, D., Lee, C., Coppola, V., Tessarollo, L., Hoover, S. B., Karavanova, I., Buonanno, A., Simpson, R. M. et al. (2014). Detection of differential fetal and adult expression of chloride intracellular channel 4 (CLIC4) protein by analysis of a green fluorescent protein knock-in mouse line. *BMC Dev. Biol.* **14**, 24.
Patel, N., Ramachandran, S., Azimov, R., Kagan, B. L. and Lal, R. (2015). Ion channel formation by tau protein: implications for Alzheimer's disease and tauopathies. *Biochemistry* **54**, 7320-7325.
Pierchala, B. A., Muñoz, M. R. and Tsui, C. C. (2010). Proteomic analysis of the slit diaphragm complex: CLIC5 is a protein critical for podocyte morphology and function. *Kidney Int.* **78**, 868-882.
Ponnalagu, D., Rao, S. G., Farber, J., Xin, W., Hussain, A. T., Shah, K., Tanda, S., Berryman, M. A., Edwards, J. C. and Singh, H. (2016). Data supporting characterization of CLIC1, CLIC4, CLIC5 and DmCLIC antibodies and localization of CLICs in endoplasmic reticulum of cardiomyocytes. *Data Brief* **7**, 1038-1044.
Ponsioen, B., van Zeijl, L., Langeslag, M., Berryman, M., Littler, D., Jalink, K. and Moolenaar, W. H. (2009). Spatiotemporal regulation of chloride intracellular channel protein CLIC4 by RhoA. *Mol. Biol. Cell* **20**, 4664-4672.
Potterton, L., McNicholas, S., Krissinel, E., Gruber, J., Cowtan, K., Emsley, P., Murshudov, G. N., Cohen, S., Perrakis, A. and Noble, M. (2004). Developments in the CCP4 molecular-graphics project. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2288-2294.
Qian, Z., Okuhara, D., Abe, M. K. and Rosner, M. R. (1999). Molecular cloning and characterization of a mitogen-activated protein kinase-associated intracellular chloride channel. *J. Biol. Chem.* **274**, 1621-1627.
Qiu, M. R., Jiang, L., Matthaei, K. I., Schoenwaelder, S. M., Kuffner, T., Mangin, P., Joseph, J. E., Low, J., Connor, D., Valenzuela, S. M. et al. (2010). Generation and characterization of mice with null mutation of the chloride intracellular channel 1 gene. *Genesis* **48**, 127-136.
Redhead, C., Sullivan, S. K., Koseki, C., Fujiwara, K. and Edwards, J. C. (1997). Subcellular distribution and targeting of the intracellular chloride channel p64. *Mol. Biol. Cell* **8**, 691-704.

Salao, K., Jiang, L., Li, H., Tsai, V. W.-W., Husaini, Y., Curmi, P. M. G., Brown, L. J., Brown, D. A. and Breit, S. N. (2016). CLIC1 regulates dendritic cell antigen processing and presentation by modulating phagosome acidification and proteolysis. *Biol. Open* **5**, 620-630.

Salles, F. T., Andrade, L. R., Tanda, S., Grati, M., Plona, K. L., Gagnon, L. H., Johnson, K. R., Kachar, B. and Berryman, M. A. (2014). CLIC5 stabilizes membrane-actin filament linkages at the base of hair cell stereocilia in a molecular complex with radixin, taperin, and myosin VI. *Cytoskeleton* **71**, 61-78.

Seaman, M. N. J. (2012). The retromer complex - endosomal protein recycling and beyond. *J. Cell Sci.* **125**, 4693-4702.

Seco, C. Z., Oonk, A. M. M., Domínguez-Ruiz, M., Draaisma, J. M., Gandía, M., Oostrik, J., Neveling, K., Kunst, H. P. M., Hoefsloot, L. H., del Castillo, I. et al. (2015). Progressive hearing loss and vestibular dysfunction caused by a homozygous nonsense mutation in CLIC5. *Eur. J. Hum. Genet.* **23**, 189-194.

Shanks, R. A., Larocca, M. C., Berryman, M., Edwards, J. C., Urushidani, T., Navarre, J. and Goldenring, J. R. (2002). AKAP350 at the Golgi apparatus. II. Association of AKAP350 with a novel chloride intracellular channel (CLIC) family member. *J. Biol. Chem.* **277**, 40973-40980.

Shukla, A., Malik, M., Cataisson, C., Ho, Y., Friesen, T., Suh, K. S. and Yuspa, S. H. (2009). TGF-beta signalling is regulated by Schnurri-2-dependent nuclear translocation of CLIC4 and consequent stabilization of phospho-Smad2 and 3. *Nat. Cell Biol.* **11**, 777-784.

Singh, H. (2010). Two decades with dimorphic Chloride Intracellular Channels (CLICs). *FEBS Lett.* **584**, 2112-2121.

Singh, H. and Ashley, R. H. (2006). Redox regulation of CLIC1 by cysteine residues associated with the putative channel pore. *Biophys. J.* **90**, 1628-1638.

Singh, H., Cousin, M. A. and Ashley, R. H. (2007). Functional reconstitution of mammalian 'chloride intracellular channels' CLIC1, CLIC4 and CLIC5 reveals differential regulation by cytoskeletal actin. *FEBS J.* **274**, 6306-6316.

Stauber, T. and Jentsch, T. J. (2013). Chloride in vesicular trafficking and function. *Annu. Rev. Physiol.* **75**, 453-477.

Suginta, W., Karoulias, N., Aitken, A. and Ashley, R. H. (2001). Chloride intracellular channel protein CLIC4 (p64H1) binds directly to brain dynamin I in a complex containing actin, tubulin and 14-3-3 isoforms. *Biochem. J.* **359**, 55-64.

Suh, K. S., Mutoh, M., Nagashima, K., Fernandez-Salas, E., Edwards, L. E., Hayes, D. D., Crutchley, J. M., Marin, K. G., Dumont, R. A., Levy, J. M. et al. (2004). The organellular chloride channel protein CLIC4/mtCLIC translocates to the nucleus in response to cellular stress and accelerates apoptosis. *J. Biol. Chem.* **279**, 4632-4641.

Suh, K. S., Malik, M., Shukla, A. and Yuspa, S. H. (2007). CLIC4, skin homeostasis and cutaneous cancer: surprising connections. *Mol. Carcinog.* **46**, 599-604.

Takano, K., Liu, D., Tarpey, P., Gallant, E., Lam, A., Witham, S., Alexov, E., Chaubey, A., Stevenson, R. E., Schwartz, C. E. et al. (2012). An X-linked channelopathy with cardiomegaly due to a CLIC2 mutation enhancing ryanodine receptor channel activity. *Hum. Mol. Genet.* **21**, 4497-4507.

Tavasoli, M., Al-Momany, A., Wang, X., Li, L., Edwards, J. C. and Ballermann, B. J. (2016a). Both CLIC4 and CLIC5A activate ERM proteins in glomerular endothelium. *Am. J. Physiol. Renal Physiol.*

Tavasoli, M., Li, L., Al-Momany, A., Zhu, L.-F., Adam, B. A., Wang, Z. and Ballermann, B. J. (2016b). The chloride intracellular channel 5A stimulates podocyte Rac1, protecting against hypertension-induced glomerular injury. *Kidney Int.* **89**, 833-847.

Tung, J. J. and Kitajewski, J. (2010). Chloride intracellular channel 1 functions in endothelial cell growth and migration. *J. Angiogenes Res.* **2**, 23.

Tung, J. J., Hobert, O., Berryman, M. and Kitajewski, J. (2009). Chloride intracellular channel 4 is involved in endothelial proliferation and morphogenesis in vitro. *Angiogenesis* **12**, 209-220.

Ulmasov, B., Bruno, J., Gordon, N., Hartnett, M. E. and Edwards, J. C. (2009). Chloride intracellular channel protein-4 functions in angiogenesis by supporting acidification of vacuoles along the intracellular tubulogenic pathway. *Am. J. Pathol.* **174**, 1084-1096.

van Furden, D., Johnson, K., Segbert, C. and Bossinger, O. (2004). The C. elegans ezrin-radixin-moesin protein ERM-1 is necessary for apical junction remodelling and tubulogenesis in the intestine. *Dev. Biol.* **272**, 262-276.

Viswanatha, R., Wayt, J., Ohouo, P. Y., Smolka, M. B. and Bretscher, A. (2013). Interactome analysis reveals ezrin can adopt multiple conformational states. *J. Biol. Chem.* **288**, 35437-35451.

Wagner, S. A., Beli, P., Weinert, B. T., Scholz, C., Kelstrup, C. D., Young, C., Nielsen, M. L., Olsen, J. V., Brakebusch, C. and Choudhary, C. (2012). Proteomic analyses reveal divergent ubiquitylation site patterns in murine tissues. *Mol. Cell. Proteomics* **11**, 1578-1585.

Wegner, B., Al-Momany, A., Kulak, S. C., Kozlowski, K., Obeidat, M., Jahroudi, N., Paes, J., Berryman, M. and Ballermann, B. J. (2010). CLIC5A, a component of the ezrin-podocalyxin complex in glomeruli, is a determinant of podocyte integrity. *Am. J. Physiol. Renal. Physiol.* **298**, F1492-F1503.

White, D. P., Caswell, P. T. and Norman, J. C. (2007). alpha v beta3 and alpha5beta1 integrin recycling pathways dictate downstream Rho kinase signaling to regulate persistent cell migration. *J. Cell Biol.* **177**, 515-525.

Wu, C., Jin, X., Tsueng, G., Afrasiabi, C. and Su, A. I. (2016). BioGPS: building your own mash-up of gene annotations and expression profiles. *Nucleic Acids Res.* **44**, D313-D316.