Evolution of the Cation Chloride Cotransporter Family: Ancient Origins, Gene Losses, and Subfunctionalization through Duplication

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Associate editor: Naoko Takezaki

Abstract
The cation chloride cotransporter (CCCs) family comprises of four subfamilies—K⁺-Cl⁻ cotransporters (KCCs), Na⁺-K⁺-2Cl⁻ cotransporters (NKCCs), and Na⁺-Cl⁻ cotransporters (NCCs)—and possibly two additional members—CCC interacting protein (CIP1) and polyamine transporters (CCC9)—as well. Altogether, CCCs can play essential physiological roles in transepithelial ion reabsorption and secretion, cell volume regulation, and inhibitory neurotransmission and so are present across all domains of life. To gain insight into the evolution of this family, we performed a comprehensive phylogenetic analysis using publically available genomic information. Our results clearly support CIP1 as being a true CCC based on shared evolutionary history. By contrast, the status of CCC9 in this regard remains equivocal. We also reveal the existence of a single ancestral CCC gene present in Archaea, from which numerous duplication events at the base of archaeans and eukaryotes lead to the divergence and subsequent neofunctionalization of the paralogous CCC subfamilies. A diversity of ensuing gene-loss events resulted in the complex distribution of CCCs present across the different taxa. Importantly, the occurrence of KCCs in “basal” metazoan taxa like sponges would allow an early formation of fast hyperpolarizing neurotransmission in metazoans. Gene duplications within the CCC subfamilies in vertebrates (in particular, KCCs, NKCCs, and NCCs) lend further evidence to the 2R hypothesis of two rounds of genome duplication at the base of the vertebrate lineage, especially in concert with our syntenic cluster analyses. This increased number of KCCs, NKCCs, and NCCs isoforms facilitates their further, important subfunctionalization in the vertebrate lineage.

Key words: cation chloride cotransporter, whole genome duplication, subfunctionalization, neofunctionalization.

Introduction
The cation chloride cotransporter (CCC) protein family belongs to the solute carrier (SLC) gene series that encodes passive transporters, ion transporters, and exchangers. Although the Human Genome Organisation (HUGO) Nomenclature Committee recognizes 52 distinct SLC gene families based on shared amino-acid identity of 20–25% among family members (Hediger et al. 2004, 2013), the protein family database (Pfam; http://pfam.sanger.ac.uk, last accessed December 4, 2013) divides SLCs in three major superfamilies based on the concept of clans of shared protein domains: the major facilitator, the amino-acid-polyamine-organocation (APC), and the monovalent cation:proton antiporter/anion transporter superfamilies (Shaffer et al. 2009; Höglund et al. 2010; Punta et al. 2012). CCCs have been assigned to the SLC12 gene family and are a member of the APC superfamily (Hediger et al. 2004; Höglund et al. 2010; Arroyo et al. 2013; Hediger et al. 2013).

The CCC family itself comprises up to four subfamilies: Na⁺-K⁺-2Cl⁻ cotransporters (NKCCs, SLC12A1–A2) and Na⁺-Cl⁻ cotransporters (NCC, SLC12A3), K⁺-Cl⁻ cotransporters (KCCs, SLC12A4–A7), a polyamine transporter (CCC9, SLC12A8), and a CCC interacting protein (CIP1, SLC12A9) (Gamba 2005; Di Fulvio and Alvarez-Leefmans 2009; Arroyo et al. 2013; Gagnon and Delpire 2013). However, because of the lack of CCC activity in CIP1 and CCC9 and the insufficient sequence similarity to other family members, their status as a CCC member is uncertain (Di Fulvio and Alvarez-Leefmans 2009). Several of these plasma membrane proteins play a crucial role in various elementary physiological processes, including ion transport across epithelial cells, the secretion of potassium, and regulation of cell volume (Payne et al. 2003; Adragna et al. 2004; Gamba 2005; Kahle et al. 2008; Blesse et al. 2009; Di Fulvio and Alvarez-Leefmans 2009). Furthermore, they are also known to regulate the intracellular chloride concentration in neurons and therefore affect neuronal excitability (Blesse et al. 2009). Loss of function of CCCs is associated with severe human disorders, including Anderman’s, Gitelman’s, or Bartter’s diseases (Howard et al. 2002; Gamba et al. 2009).
2005; Di Fulvio and Alvarez-Leefmans 2009; Gagnon and Delpierre 2013); epilepsy (Aronica et al. 2006); chronic pain (Coull et al. 2003); and deafness (Delpire et al. 1999; Flagella et al. 1999; Boettiger et al. 2002, 2003). Although the aforementioned studies all are derived from vertebrate models, the generality of the findings has been verified by functional analyses in plants (Colmenero-Flores et al. 2007), yeast (Park and Saier 1996; De Hertogh et al. 2002; Jennings and Cui 2008), nematodes (Holtzman et al. 1998), and insects (Hekmat-Scafe et al. 2006). CCCs in some form are probably found across life, ranging from bacteria (Kaplan et al. 1996) and archaea (Warmuth et al. 2009) to mammals (Gamba et al. 1993; Payne 1997; Holtzman et al. 1998; Caron et al. 2000; Daigle et al. 2009).

To date, phylogenetic analyses of CCCs have focused mainly on those occurring in mammals (Gamba 2005; Di Fulvio and Alvarez-Leefmans 2009), teleost fish (Hiroi et al. 2008; Wang et al. 2009), plants (Colmenero-Flores et al. 2007), insects, and nematodes (Tanis et al. 2009; Sun et al. 2010). These data reveal that the family shows a complicated history of gene duplication as well as gene-loss events, even within the individual subfamilies. For instance, four paralogous KCCs (KCC1–KCC4) and three paralogous NKCC1, NKCC2, and NCC exist in mammals (Gamba 2005; Di Fulvio and Alvarez-Leefmans 2009), with teleost fish possessing an additional, paralogous NCC (Hiroi et al. 2008; Wang et al. 2009). Gene-duplication events leading to such patterns could arise from numerous processes, including any of unequal crossing over leading to tandem duplication, retroposition, segmental duplication, and chromosomal (or whole genome) duplication (Zhang 2003; Hurles 2004). Especially important in an evolutionary context is that duplication events lead to a functionally redundant gene copy that is potentially released of selective pressure. The vast majority of these gene copies will be silenced during evolution, resulting in nonfunctionalization (Lynch and Conery 2000). However, some paralogous copies can also be maintained in the genome, either to acquire a new function entirely (neofunctionalization) or to adopt part of the function of the ancestral gene in combination with the other paralog (subfunctionalization), often in association with differences in temporal and spatial expression patterns (Ohno 1970; Force et al. 1999; Lynch and Conery 2000; Lynch and Force 2000; Hurles 2004). In this context, the emergence of both KCCs and NKCCs represent cogent examples of neofunctionalization within CCCs. Within this context, it is interesting to note that the KCC of Arabidopsis thaliana is functionally a NKCC (Colmenero-Flores et al. 2007), showing the potential within the CCC family to acquire new, albeit-related functions.

In this article, we seek to perform a global phylogenetic analysis of the CCC family (including CCC9 and CIP1) across the entire tree of life using currently available genomic data. In doing so, we hope to gain new insights into the evolution of this important gene family, particularly with respect to the potential for neo- versus subfunctionalization of gene copies and the emergence of physiological functions within different taxonomic groups.

Results and Discussion

Paralogous Evolution of CCCs at the Base of the Eukaryotes

Database analyses identified CCCs (SLC12s) in selected model genomic species of Archaea and Eukaryota, with all examined genomes containing members from at least one CCC subfamily (table 1). A combined phylogenetic analysis of all CCC subfamilies rooted with the human CAT1 (high-affinity cationic amino acid transporter 1, SLC7A1) sequence, which represents the closest known relative of the SLC12 gene family (Fredriksson et al. 2008), shows a clear and strongly supported clustering of each CCC subfamily (fig. 1 and supplementary file S1, Supplementary Material online). The tree shows a weakly supported sister-group relationship between KCCs and CIP1 (bootstrap value: 53%), with NKCC/NCCs forming the sister group to this clade (fig. 1; 35% (supplementary file S1, Supplementary Material online). Noteworthy is that the archaean Methanosarcina acetivorans N(K)CC sequence falls outside of the otherwise well-supported main NKCC/NCC clade to form the sister group to the clade of undisputed CCCs (NKCC, NCCs, and KCCs; bootstrap value: 66.9%). This result hints at a single ancestral CCC gene of which the M. acetivorans N(K)CC sequence appears to be a direct descendant.

The positioning of CIP1 within the clade of undisputed CCCs also would indicate it to be a legitimate member of this family (via shared evolutionary history) despite its insufficient protein identity to other members. For the latter criterion, a boundary of 30% is typically used (Murzin et al. 1995), which is the case for the highly conserved central hydrophobic domain of KCCs and NKCCs (Gamba 2005). However, proteins with identities of < 30% can still belong to a given protein family if they possess a similar structure and function (Murzin et al. 1995). Here, CIP1 shares the 12 transmembrane domains (TMDs) and intracellular termini of KCCs, NKCCs/NCCs, and the archaean N(K)CC and also has similar structure generally (Payne et al. 1996; Caron et al. 2000; Gerelsaikhan and Turner 2000; Adragna et al. 2004; Gamba 2005; Di Fulvio and Alvarez-Leefmans 2009; Warmuth et al. 2009). Together, our phylogenetic analyses in combination with other criteria clearly support the subfamilies KCC, CIP1, and NKCC/NCC as well as the ancestral archaean M. acetivorans N(K)CC belonging to a common CCC family.

The case for CCC9 as a potential CCC member, unfortunately, is ambiguous. It falls outside the clade of undisputed CCCs (including CIP1), demonstrates a low protein identity to the CCCs (i.e., < 30%), and its predicted structure of 11 TMDs, intracellular N-termini and extracellular C-termini (Gamba 2005), also differs from the remaining CCCs. This structure, however, also differs from that of the cationic amino acid transporters (CATs) and glycoprotein-associated amino acid transporters (gpaATs) family (SLC7). In the latter families, CAT proteins (SLC7A1–A4) possess 14 TMDs and gpaAT proteins (SLC7A5–A11) possess 12 TMDs (Verrey et al. 2004; Closs et al. 2006; Hansen et al. 2011). However, further structural analyses are needed here to verify the predicted
The different CCC subfamilies show complex taxonomic distributions. Although KCCs occur across Eukaryota (figs. 1 and 2), the remaining subfamilies are more restricted: CIP1s to Ophistokonta (animals, fungi, and some protists; fig. 3), NKCC/NCCs to Metazoa (excluding the M. acetivorans sequence; figs. 1 and 4), and CCC9s to Epitheliozoa (animals excluding sponges; figs. 1 and 5). Based on reconciled trees, the optimal evolutionary scenario indicates three gene duplication events—one at the base of archaeans and two at the base of eukaryotes (fig. 6)—that essentially gave rise sequentially to the CCC9, NKCC/NCC, and finally the KCC and CIP1 subfamilies. Therefore, several losses of entire subfamilies are required to explain the current taxonomic distribution: NKCC/NCC genes were lost independently in plants, fungi, and choanoflagellates; CCC9 in plants, fungi, choanoflagellates, and sponges; CIP1 in plants; and KCC in fungi. Although it cannot be excluded that the gene losses represent incomplete database information, gene-loss events are not unusual because nonfunctionalization represents the major fate of gene copies after duplication (Lynch and Conery 2000).

In addition, we also focused on species with more or less complete genomic information and even here our genomic database searches could not identify the missing CCCs, suggesting their true absence in the various species of the respective taxa. Hence, we propose that the initial divergence of the CCC subfamilies that arose from three gene duplication events at the base of archaeans and eukaryotes was followed by numerous instances of the loss of entire subfamilies in different taxa.

### Table 1. Distribution of CCCs among the Selected Model Genomic Species Used in This Study.

<table>
<thead>
<tr>
<th>Species</th>
<th>KCCs</th>
<th>NKCC/NCCs</th>
<th>CIP1</th>
<th>CCC9</th>
</tr>
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<td>Homo sapiens</td>
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<td>3</td>
<td>1</td>
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<tr>
<td>Rattus norvegicus</td>
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<td>3</td>
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<td>1</td>
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<td>1</td>
<td>1</td>
</tr>
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<td>3</td>
<td>1</td>
<td>1</td>
</tr>
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<td>3</td>
<td>1</td>
<td>1</td>
</tr>
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<td>1</td>
<td>1</td>
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<td>1</td>
<td>1</td>
</tr>
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</tr>
<tr>
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<td>1</td>
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<tr>
<td>Saccharomyces cerevisae</td>
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<tr>
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<tr>
<td>Methanosarcina acetivorans</td>
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The Role of Historical Gene Duplication Events for Paralogous Evolution within the CCC Subfamilies

A comparison between the CCC protein sequences of the urochordate Ciona intestinalis and the vertebrates (table 1) shows the striking emergence of four paralogs in each of the CCC and NKCC/NCC subfamilies in vertebrates, with KCC1 + KCC3 and KCC2 + KCC4 as well as NKCC1 + NKCC2 and NCC1 + NCC2 (NCC3) forming sister groups (figs. 2 and 4). These pattern of duplication events would be one possible expected outcome based on the hypothesis that two rounds of whole genome duplication occurred at the base of the vertebrate lineage (the 2R hypothesis), as inferred initially from duplications within the Hox transcription factor gene family (Ohno 1970, 1999). Similar patterns are also known in other genes including phosphofructokinase (Steinke et al. 2006) and the α-actinin protein family (Virel and Backman 2004).

Further support for the role of whole genome duplication in this instance (as opposed to simple tandem duplications) is provided by our syntenic cluster analyses, which shows that all human KCCs and NKCCs are localized on different chromosomes (supplementary table S2, Supplementary Material online) and organized in paralogs (supplementary figs. S1 and S2, Supplementary Material online). For instance, the human KCC1 (SLC12A4) that is located on chromosome 16 forms a paralogous syntenic cluster to KCC2 (SLC12A5, chromosome 20), KCC3 (SLC12A6, chromosome 15), and KCC4...
Fig. 1. Phylogenetic relationships of the CCC family. Maximum-likelihood analyses with 1,000 nonparametric bootstrap replicates were performed for all CCC subfamily members from selected genomic species and rooted with the human hsCAT1 (SLC7A1, NP_003036.1) sequence. (A) Full tree with support for each branch was color coded according to its bootstrap support. (B) Simplified tree highlighting the relationships among the major subclades; only bootstrap scores >50% are indicated. The accession numbers of all protein sequences are listed in supplementary table S3, Supplementary Material online.
with these clusters sharing 38, 14, and 25 gene pairs, respectively, with that of KCC1 within a 100-gene sliding window. These values are significantly above background levels determined from a randomized distribution (Catchen et al. 2009). Similarly, human NKCC2 (SLC12A1) that is located on chromosome 15 forms a paralogous synteny cluster with NKCC1 (SLC12A2, chromosome 5; 54 gene pairs) and NCC (SLC12A3, chromosome 16; 14 gene pairs, supplementary fig. S2, Supplementary Material online). The expected, second paralogous copy of NCC (which is present additionally in fish, amphibians, and squamates [fig. 4]) is probably missing in humans because of gene-loss events in mammals (see later). Similar events likely explain the independent losses of KCC3 in birds and KCC4 in amphibians (fig. 2).

Admittedly, the pattern for the NKCC/NCC subfamily is not as clear-cut as that for KCC, especially regarding the paralogous copies of NCC, where the maximum likelihood analyses reveal three, not two, paralogous NCC clusters. Here, the clade comprising NCC (all vertebrates) and NCC3 (amphibians and squamates [fig. 4]) is probably missing in humans because of gene-loss events in mammals (see later). Similar events likely explain the independent losses of KCC3 in birds and KCC4 in amphibians (fig. 2).

Fig. 2. Phylogenetic relationships among potassium chloride cotransporters (KCCs). Maximum-likelihood analyses with 1,000 nonparametric bootstrap replicates were performed for selected genomic species and rooted with a monophyletic clade of the plant KCCs (Arabidopsis thaliana and Oryza sativa). Only bootstrap scores >50% are indicated and clades corresponding to orthologous human KCC1-4 members are labeled on the right side. The accession numbers of all protein sequences are listed in supplementary table S3, Supplementary Material online.
Fig. 3. Phylogenetic relationships among CCC interacting proteins (CIP1s). Maximum-likelihood analyses with 1,000 nonparametric bootstrap replicates were performed for CIP1 for selected genomic species and rooted with a monophyletic clade of CIP1s from the fungi *Aspergillus niger* and *Saccharomyces cerevisiae*, and the choanoflagellate *Monosiga brevicollis*. Only bootstrap scores >50% are indicated. The accession numbers of all protein sequences are listed in supplementary table S3, Supplementary Material online.

Fig. 4. Phylogenetic relationships among sodium potassium chloride cotransporters (NKCCs/NCCs). Maximum-likelihood analyses with 1,000 nonparametric bootstrap replicates were performed for selected genomic species and rooted with the N(K)CC of the sponge *Amphimedon queenslandica*. Only bootstrap scores >50% are indicated, and clades corresponding to orthologous human NKCC1, NKCC2, and NCC members as well as additional paralogous NCC2 (exclusive to fish) and NCC3 (exclusive to amphibians and squamates) are labeled on the right side. The accession numbers of all protein sequences are listed in supplementary table S3, Supplementary Material online.
FIG. 5. Phylogenetic relationships among polyamine cotransporters (CCC9s). Maximum-likelihood analyses with 1,000 nonparametric bootstrap replicates were performed for selected genomic species. The analysis was left unrooted because of the lack of a decisive outgroup. Only bootstrap scores > 50% are indicated. The accession numbers of all protein sequences are listed in supplementary table S3, Supplementary Material online.

FIG. 6. Reconciled tree of CCCs indicate three gene duplication (filled circles) events—one at the base of archaeans and two at the base of eukaryotes—that sequentially gave rise to CCC9, NKCC/NCC, KCC, and CIP1. Several subsequent gene-loss events (dashed lines) lead to the current taxonomic distribution: NKCC/NCC genes were lost independently in plants and fungi; CCC9 in plants, fungi, and sponges; CIP1 in plants; and KCC in fungi.
(and not the hypothesized additional round of FSGD in telost fish; Meyer and Schartl 1999; Christoffels et al. 2004; Vandepoele et al. 2004; Meyer and Van de Peer 2005) because these gene copies also do not form syntenic clusters in the D. rerio genome to KCC2 or KCC4 (data not shown).

Contrary to the pattern of up to four gene copies of vertebrate KCCs and NKCCs/NCCs is that only single copies of each of CIP1 and CCC9 exist (figs. 3 and 5). If we accept the existence of the 2R hypothesis and its effects on CCC subfamilies, then the most parsimonious explanation for this latter pattern is multiple gene-loss events in each of CIP1 and CCC9 at the base of the vertebrate lineage. Depending on which copies were lost, it might be that the vertebrate copies are actually paralogs of those found in nonvertebrates.

Finally, in addition to these duplication events within vertebrates, many additional, apparently isolated gene duplications have occurred throughout eukaryotes: KCCs in *Oryza sativa* and Nemotada (fig. 2); N(K)CCs in Placozoa, Cnidaria, Nematota, and Insecta (fig. 4); and CIP1 in Choanoflagellata (fig. 3). Interestingly, some of these duplicated genes reveal no apparent cotransport function, such as *Caenorhabditis elegans_KCC isoform 3* (Acc. No.: NP495555.2, equivalent to U23171 from Holtzman et al. [1998]) and *Drosophila melanogaster_N(K)CC isoform 2* (Acc. No.: NP_730928.1, equivalent to CG31547-PB from Sun et al. [2010]), although the paralogous genes are functional (Tanis et al. 2009; Sun et al. 2010), hinting at the nonfunctionalization (Lynch and Conery 2000) of the one gene copy.

**CCC Neofunctionalization and Functional Implications of CCC Subfamily-Specific Conservation**

The three gene duplication events within CCCs at the base of archaeans and eukaryotes facilitated the apparent neofunctionalization (Ohno 1970; Force et al. 1999; Lynch and Conery 2000) among the new paralogous CCC subfamily members, as exemplified by their different functional properties in vertebrates. Both the KCC subfamily and the NKCC and NCC subfamily are ion cotransporters, whereby KCCs are transport-active \( K^+ \) and \( Cl^- \) transporters and NKCCs and NCCs are transport-active \( Na^+ \)-\( K^+ 
-2Cl^- \) or \( Na^+ \) and \( Cl^- \) transporters (Gamba 2005). By contrast, CIP1 exhibits no known ion cotransporter function (Caron et al. 2000; Jennings and Cui 2008; Wenz et al. 2009), although it does regulate the transport activities of the similarly widespread NKCC1 (Caron et al. 2000) and of KCC2 (Wenz et al. 2009). The fact that CIP1 is also the only CCC family member known for fungi (figs. 1 and 3) indicates that it likely has an additional unknown function. Important is that this different functionality for CIP1 represents an extreme case of neofunctionalization and does not necessarily preclude its membership as a CCC. An analogous case is presented by TASKS, which is a member of the tandem pore \( K^+ \) channel (TASK) family although it does not generate any ion currents (Ashmole et al. 2001).

In addition to this apparent neofunctionalization, subsequent taxon-specific losses of various CCC subfamilies (see earlier) have led to their differential distribution among taxa, with KCCs and CIP1 being more widespread than NKCC, NCC, and CCC9 (table 1). Beginning at least with Metazoa, both KCC and N(K)CC genes are established in the genome. Although functional analyses of KCCs and N(K)CCs in “early” metazoans are missing so far, it is known that one of their major roles is to determine the action of the inhibitory neurotransmitters \( \gamma \)-aminobutyric acid (GABA) and glycine in vertebrates (Delpire 2000; Blaesse et al. 2009). For instance, KCC2 generates a low intracellular chloride concentration \( [Cl^-]_i \) in most mature neurons. This low \( [Cl^-]_i \) in turn, is required for the hyperpolarizing action of these two neurotransmitters via ionotropic GABA\(_A_\) and glycine receptors, which are ligand-gated \( Cl^- \) channels (Rivera et al. 1999; Hübner et al. 2001; Balakrishnan et al. 2003; Zhu et al. 2004). By contrast, NKCC1 activity is more prevalent in immature neurons, causing a high \( [Cl^-]_i \) and thereby causing GABA and glycine to elicit a depolarizing action (Yamada et al. 2004; Achilles et al. 2007; Sipilä et al. 2009) that opens L-type voltage-gated \( Ca^{2+} \) channels (Yuste and Katz 1991; Reichling et al. 1994; Owens et al. 1996). The resulting increase in \( Ca^{2+} \), in turn, activates intracellular signaling pathways that are necessary for proper synapse maturation (Owens et al. 1996; Kullmann et al. 2002; Redmond and Ghosh 2005) and neuronal survival (Satheesh et al. 2012).

Our phylogenetic data therefore touch on the emergence of fast ionotropic inhibitory neurotransmission, a fundamental question in evolutionary neuroscience. Physiological analyses have identified slow metabotropic G-protein coupled GABA\(_A_\) and glutamate receptors just before the emergence of Porifera (sponge; Fountain 2010), whereas fast functional ionotropic AMPA (\( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid) and NMDA (N-Methyl-D-Aspartat) receptors appear to originate later around the divergence of Eumetazoa (Sakarya et al. 2007; Kay and Kass-Simon 2009; Ryan and Grant 2009). This distribution raises the question as to the exact evolutionary timing of the emergence of fast ionotropic inhibitory neurotransmission to balance fast excitatory synaptic transmission. Interestingly, our data reveal that Porifera already contain both KCC and N(K)CC cotransporters. CCNs hence complement other synaptic components that are present before the emergence of synapses in Porifera. These so-called protosynaptic proteins include \( Ca^{2+} \)-inward rectifier \( K^+ \) channels, voltage-gated channels, and PDZ-binding proteins (Sakarya et al. 2007; Ryan and Grant 2009; Srivastava et al. 2010; Liebeskind et al. 2011). Although Porifera lack neuronal cells, they are nevertheless able to react to external stimuli and display spontaneous movements (Carroll 2001; Renard et al. 2009). Furthermore, physiological analyses indicate the emergence of a fast electrochemical system in the group in addition to the already existing paracrine-dominated prenervous system (Nickel 2010). Notable diversity is present here as well. Thus, desmosponges, calcisponges, and homoscleromorph sponges use neuroactive molecules, such as glutamate, GABA, and glycine to coordinate distinct types of contraction (Ellwanger et al. 2007; Elliott and Leys 2010; Nickel 2010), whereas hexactenellid sponges generate \( Ca^{2+} \)-mediated action potentials that control water flow through the organism (Leys et al. 1999). Although our knowledge of the phylogenetic distribution of ionotropic GABA\(_A_\) and
glycine receptors among “basal” metazoan species remains highly incomplete (Kehoe et al. 2009; Gou et al. 2012), pharmacological analyses do reveal a GABA_A-like receptor function in ciliate Paramecium primaurelia (Bucci et al. 2005) and the cnidarian Hydra vulgaris (Concas et al. 1998), suggesting a simultaneous emergence of ionotropic GABA_A receptors and CCCs. Altogether, further phylogenetic and physiological analyses are required to reveal how closely KCNs, N(K)CCs, and ionotropic GABA_A receptors have coevolved and whether fast inhibitory neurotransmission preceded or emerged in parallel with fast excitatory neurotransmission. In examining this question, it must also be kept in mind that KCNs, NKCCs, and NCCs might also be active in cell volume regulation and transepithelial salt transport in “early” metazoan lineages, two functions that have been documented for them in vertebrates (Payne et al. 2003; Gamba 2005; Kahle et al. 2008; Blaesse et al. 2009).

Subfunctionalization of Paralogous CCC Subfamilies in Vertebrates

Gene duplications at the base of vertebrate lineage are assumed to have played a pivotal role in the evolution of the group, particularly with respect to their increased complexity over nonvertebrates (Donoghue and Purnell 2005; Blomme et al. 2006). Another contribution in this regard might arise from the associated duplications within the different CCC subfamilies. Thus, although the paralogous members of each of the NKCC and NCC or KCC subfamilies maintain overall similar functions, all Na^+-driven NKCCs and NCCs are Cl^- inward transporters while the K^+-driven KCCs represent Cl^- transporters (Gamba 2005). Their subfunctionalization (Lynch and Force 2000; Hurles 2004) has resulted in different biochemical properties and expression patterns (see references in Gamba [2005], Di Fulvio and Alvarez-Leefmans [2009], Arroyo et al. [2013], and Gagnon and Delpire [2013]). This, in turn, has led to a highly fine-tuned functionality of CCCs in different organs and tissues.

A good example in this context is provided by the mammalian NKCCs and NCCs, where the subfamily members show clear differences in function and expression. First, in contrast to the thiazide-sensitive NCC, the bumetanide-sensitive NKCCs also transport K^+ across the cell membranes (Gamba et al. 1994). Second, the different cellular localizations of some members also result in different functions. For instance, NKCC1, which is mainly trafficked to the basolateral membrane (Gamba 2005; Carmosino et al. 2008; Nezu et al. 2009; Arroyo et al. 2013; Gagnon and Delpire 2013), is involved in salt secretion (Flagella et al. 1999; Grubb et al. 2000), whereas NKCC2, which is restricted to the apical membrane (Gamba 2005; Carmosino et al. 2008; Arroyo et al. 2013), participates in salt reabsorption (Hebert et al. 1981; Gamba et al. 1994; Payne and Forbush 1994). Furthermore, although NKCC2 and NCC both play key roles in the major salt transport pathways in the kidney, NKCC2 is restricted to the thick ascending limb of Henle, whereas NCC is expressed in the distal convoluted tubule (Gamba 2005; Arroyo et al. 2013; Gagnon and Delpire 2013). Finally, knockout experiments as well as direct disease associations with NKCC1, NKCC2, and NCC clearly reveal that their individual functionalities cannot be compensated by the remaining members. For example, inactivation of NKCC2 leads to the disease-associated Type 1 Bartter Type syndrome, whereas functional loss of NCC results in Gitelman syndrome (Gamba 2005; Di Fulvio and Alvarez-Leefmans 2009; Arroyo et al. 2013; Gagnon and Delpire 2013). Similarly, knockout experiments of the broadly expressed NKCC1 reveal multiple phenotypes such as sensorineural deafness, alteration in endolymph secretion, reduced saliva production, sensory perception abnormalities, male infertility, imbalance, and altered nociception (Delpire et al. 1999; Di Fulvio and Alvarez-Leefmans 2009; Arroyo et al. 2013). Moreover, the trend to subfunctionalization in the NKCC/NCC subfamily extends beyond mammals, where the additional paralogs of NCC in freshwater fish (NCC2) and amphibians (NCC3) probably reflect the increased need for osmoregulation in these taxa in the hypotonic environments in which they live. Thus, the NCC2 of D. rerio (Danio_rerio_NCC2 isoform 2), for example, is responsible for the chloride uptake mechanism in the skin and gills, whereas NCC, like its mammalian homolog, is mainly expressed in the kidney (Hiroi et al. 2008; Wang et al. 2009).

Another cogent example is provided by the different biochemical characteristics within the KCC subfamily. For example, KCC2, which is mainly expressed in neurons, exhibits the highest affinity for [K^+]_o and [Cl^-]_i levels to facilitate hyperpolarization in mature neurons (Payne 1997; Song et al. 2002; Gamba 2005), with the K_m values being in the physiological range for [K^+]_o and [Cl^-]_i. This fact suggests that the transporter acts close to the equilibrium and is therefore directly affected by the driving force of the substrate ions (Payne 1997; Song et al. 2002; Blaesse et al. 2009). Additionally, cell culture analyses indicate that KCC2 has a higher activity under isotonic conditions compared with the remaining KCCs that are activated under hypotonic conditions (Race et al. 1999; Mercado et al. 2000; Strange et al. 2000; Song et al. 2002; Bergeron et al. 2006; Mercado et al. 2006; Rinehart et al. 2009). Thus, these characteristics enable KCC2 to play a crucial role in lowering the [Cl^-]_i levels to facilitate hyperpolarization in mature neurons (Blaesse et al. 2009). Accordingly, KCC1 and KCC3 are involved in cell volume regulation in erythrocyte cells (Adragna and Lauf 2007; Rust et al. 2007), and KCC3 additionally regulates the cell volume in both neurons (Boettger et al. 2003; Kahle et al. 2008; Blaesse et al. 2009) and epithelial cells of the proximal tubule (Boettger et al. 2003). These examples, which probably are by no means exhaustive, support that the subfunctionalization among the KCC and NKCC and NCC isoforms, together with their functional specialization in different organs and tissues probably helped in contributing to the increased complexity that characterizes vertebrates.

Materials and Methods
Nomenclature of the CCC/SLC12 Isoforms
For the purpose of this article, the nomenclature we use for the CCC genes takes its lead from the protein gene names
recognized by the HUGO Gene Nomenclature Committee (HGNC) for the SLC12 gene family to which the CCCs have been assigned (Hediger et al. 2004; Arroyo et al. 2013; Hediger et al. 2013). This nomenclature most closely matches that used in the literature and also is relevant to that we are working with many nonhuman organisms that can possess paralogous gene copies for which no HGNC names can exist by definition. Thus, all CCCs except NCC were named in the first instance after their orthologous human SLC12/CCC protein members (table 2). For the NCCs for which paralogous copies exist in fish or amphibian and squamates that are not present in humans, we use NCC for the human ortholog (SLC12A3) and NCC2 and NCC3 for the paralogs found exclusively in fish or in amphibian and squamates, respectively (fig. 4 and table 2, supplementary fig. S3, Supplementary Material online). A full list of synonyms for all genes examined in this study can be found in supplementary table S3, Supplementary Material online. Finally, we use the term N(K)CC to refer generally to the grouping of NKCCs and NCCs, recognizing both the shared evolutionary history of the latter two groups as well as that the different names derive from a subfunctionalization likely first arises with the emergence of vertebrates.

Database Searches
CCC protein sequences for a diverse selection of organisms for which more or less complete genomic information is available (table 1) were obtained from a combination of Blast searches against GenBank (Altschul et al. 1990) and data mining of the Ensembl database (Hubbard et al. 2005), the Joint Genome Institute (http://www.jgi.doe.gov/, last accessed December 4, 2013), the yeast genome database (http://www.yeastgenome.org/, last accessed December 4, 2013), and the TAIR-homepage (http://www.arabidopsis.org/, last accessed December 4, 2013).

As a query for database analyses of the protein sequences from different species, we used the protein sequences of human KCC1 (NP_005063.1), KCC2 (NP_065759.1), KCC3 (NP_598408.1), KCC4 (NP_006589.2), NKCC1 (NP_001037.1), NKCC2 (NP_001171761.1), NCC (NP_000330.2), CIP1 (NP_064631.2), and CCC9 (NP_078904.3). For each protein in each target species, we saved all sequences with an e-value of at least $10^{-2}$. These sequences were then reverse blasted (BlastP or translated Blast) against the *Homo sapiens* protein database, and only those protein sequences that showed the same CCC protein sequence of *H. sapiens* were retained and used as a query sequence as the best hit (e-value of at least $10^{-2}$). All accession numbers are listed in the supplementary data (supplementary table S3, Supplementary Material online). KCC, NKCC, and NCC, CIP1, or CCC9 protein sequences of each species were then preliminary aligned by MUSCLE (Edgar 2004) to identify identical sequences. For each species, only the longest sequences were kept and used for subsequent analyses; apparent splice variants were also deleted. Paralogous isoforms that are orthologous to the same CCC subfamily members and differ by several amino acid residues along the entire sequence were also retained. We named these isoforms according to their main (human) ortholog supplemented by the suffix “isoform n” (e.g., *Danio rerio_KCC2_isoform_2*). An additional example involves the three paralogous NCC2 isoforms of *D. rerio* that form a clade with other teleost fish sequences that is distinct from both the NCC and NCC3 clades.

Sequence Analyses and Tree Building
For each CCC subfamily, sequences were aligned at the amino-acid level using the default settings in MUSCLE (Edgar 2004) as implemented in SeaView v.4.4.2 (Gouy et al. 2010) and manually improved by eye thereafter. Areas of questionable homology were removed. For each species, only the longest sequences were kept and used for subsequent analyses; apparent splice variants were also deleted. Paralogous isoforms that are orthologous to the same CCC subfamily members and differ by several amino acid residues along the entire sequence were also retained. We named these isoforms according to their main (human) ortholog supplemented by the suffix “isoform n” (e.g., *Danio rerio_KCC2_isoform_2*). An additional example involves the three paralogous NCC2 isoforms of *D. rerio* that form a clade with other teleost fish sequences that is distinct from both the NCC and NCC3 clades.

### Table 2. Used Nomenclature Compared with the HGNC Nomenclature and Further Descriptions.

<table>
<thead>
<tr>
<th>Protein Name as Used in This Article</th>
<th>HGNC Protein Name</th>
<th>HGNC Gene Name</th>
<th>Further Protein Names</th>
<th>Further Gene Names</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKCC2</td>
<td>NKCC2</td>
<td>SLC12A1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NKCC1</td>
<td>NKCC1</td>
<td>SLC12A2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NCC</td>
<td>NCC</td>
<td>SLC12A3</td>
<td>Conventional NCCs (Hiroi et al. 2008)</td>
<td>—</td>
<td>Ortholog to human NCC</td>
</tr>
<tr>
<td>NCC2</td>
<td>—</td>
<td>—</td>
<td>Fish-specific NCCs (Hiroi et al. 2008); NCC-like (Wang et al. 2009)</td>
<td>SLC12A10 (Wang et al. 2009)</td>
<td>Principally fish specific</td>
</tr>
<tr>
<td>NCC3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Additional isoform in amphibians and squamates</td>
</tr>
<tr>
<td>KCC1</td>
<td>KCC1</td>
<td>SLC12A4</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
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<td>—</td>
<td>—</td>
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</tr>
<tr>
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<td>SLC12A6</td>
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<td>—</td>
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<td>CCC9</td>
<td>SLC12A8</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CIP1</td>
<td>CIP1</td>
<td>SLC12A9</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
Outgroups for the individual data sets were determined according to current phylogenetic opinion for the taxa present in them: the flowering plants *A. thaliana* and *O. sativa* for KCCs; the sponge *Amphimedon queenslandica* for N(K)CCs; and the fungi *Aspergillus niger*, *Saccharomyces cerevisiae*, and the choanoflagellate *Monosiga brevicollis* for CIP1. The CCCC analysis was left unrooted because of the lack of a decisive outgroup, given the uncertain phylogenetic placement of *Trichoplax* with respect to the two cnidarians (Srivastava et al. 2008). For display purposes, however, we subjectively rooted the CCCC gene tree between these three taxa and the remaining ones, a position that is arguably close to the real root of this taxon set. Although the *M. acetivorans* CCC sequence was inferred to be an N(K)CC based on our Blast analyses, it also shows high similarity to the remaining CCC families. As such, and given that the precise transport activity of this CCC remains uncharacterized (Warmuth et al. 2009), we only included this sequence in the combined analysis of all gene sequences. This latter analysis was rooted using human hsCAT1 (SLC7A1, NP_003036.1), given that phylogenetic analyses of the human SLC series indicate that SLC7 (CAT/gpaAT family) and SLC12s (CCCs) are sister groups (Fredriksson et al. 2008).

The amino-acid sequence data were analyzed in a maximum-likelihood framework using RAxML v7.2.8 (Stamatakis et al. 2004). A general time reversible model was selected as the optimal protein substitution model for all data sets using the Perl subroutine ProteinModelSelection.pl (http://sco.h-its.org/exelixis/software.html, last accessed December 4, 2013) as implemented in the Perl script batchRAXML.pl v1.2 (http://www.molekularesystematik.uni-oldenburg.de/33997.html, last accessed December 4, 2013). The latter script also performed the main RAxML analyses, consisting of a fast nonparametric bootstrap analysis of 1,000 replicates followed by a maximum likelihood search for the optimal tree (Felsenstein 1985; Stamatakis et al. 2004; Stamatakis et al. 2008). For a better resolution of the terminal branches of the phylogenetical relationship of the CCCs (fig. 1A), we included the RAxML analyses (supplementary file S1, Supplementary Material online) that can be opened by programs such as FigTree (Rambaut and Drummond 2009).

To infer the pattern of gene duplication versus loss, we used the reconciled-tree method as implemented in Jane v2.0 (Conow et al. 2010) and used simplified versions of both the gene and species trees. In doing so, we deliberately excluded sequences from *M. brevicollis* (representing choanoflagellates) because their positions in the gene trees could not easily be accommodated in the simplified structure we used. Gene duplication and loss were each assessed by a cost of 1 relative to codivergence (no cost), with host switching given a cost of 100 to effectively prevent this highly unlikely scenario (i.e., horizontal gene transfer) from occurring in this case.

Syntenic Cluster Analysis

Finally, we used syntenic cluster analysis to distinguish between gene duplications that occurred via tandem duplication versus whole genome duplication events. In the latter case, duplicated genes (either between or within the CCC families) should exist as part of large gene blocks (paralogs) on different chromosomes. To identify paralogous or even orthologous syntenic clusters, we used the Synteny Database (http://syntenyleduoregon.edu/synteny_db/, last accessed December 4, 2013; Catchen et al. 2009) with the reciprocal best hit analysis (mRBH pipeline) and the database itself as tools (Catchen et al. 2009). The mRBH analysis pipeline, which relies on WU-Blast (Washington University-Blast; Altschul et al. 1997) in combination with BLOSUM62 (Henikoff and Henikoff 1992), uses each protein from the primary genome as a query against the outgroup genome. All significant hits (i.e., those with e-values of at least $10^{-5}$) are then used as a query for a modified reciprocal algorithm (Wall et al. 2003). The Synteny Database uses a set of paralogy groups together with their corresponding outgroup genes to search for conserved syntenic areas between the primary and outgroup genomes using a predetermined sliding-window size (number of contiguous genes; Catchen et al. 2009). Here, we used *C. intestinalis* as the outgroup for analyses involving *H. sapiens*, in turn, as the outgroup for *D. rerio*. Both sets of analyses used a sliding window size of 100 genes and the variant Ensembl version 61 (Ens61).

Supplementary Material

Supplementary files S1–S3 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

We gratefully acknowledge Eduard Schwarzkopf for his help with some of the initial data mining. This work was supported by the Deutsche Forschungsgemeinschaft (grants HA 6338/2-1 to A.-M.H. and 428/4-2 to H.G.N.).

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