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Abstract

The mutations in *CLCNKB* gene encoding the ClC-Kb chloride channel are responsible for Bartter syndrome type 3, one of the 4 variants of Bartter syndrome in the genetically-based nomenclature. All forms of Bartter syndrome are characterized by hypokalaemia, metabolic alkalosis and secondary hyperaldosteronism but Bartter syndrome type 3 has the most heterogeneous presentation, extending from severe to very mild. A relatively large number of *CLCNKB* mutations have been reported, including gene deletions and nonsense or missense mutations. However, only 20 *CLCNKB* mutations have been functionally analyzed, due to technical difficulties regarding ClC-Kb functional expression in heterologous systems. This review provides an overview of recent progress in the functional consequences of *CLCNKB* mutations on ClC-Kb chloride channel activity. It has been observed that: 1) all ClC-Kb mutants have an impaired expression at the membrane; and 2) a minority of the mutants combines reduced membrane expression with altered pH-dependent channel gating. Although further investigation is needed to fully characterize disease pathogenesis, Bartter syndrome type 3 probably belongs to the large family of conformational diseases, in which the mutations destabilize channel structure, inducing ClC-Kb retention in the endoplasmic reticulum and accelerated channel degradation.

Keywords: Bartter syndrome, CLC family of chloride transporters and channels, chloride channel.

Bartter syndrome

Bartter syndrome is an autosomal recessive salt wasting tubulopathy, characterized by hypokalaemia, metabolic alkalosis and secondary hyperaldosteronism with normal-to-low blood pressure. Clinically, two variants are distinguished: antenatal Bartter syndrome (aBS) and classic Bartter syndrome (cBS)(8, 39, 48, 80, 81, 97). The aBS is a severe form leading to polyhydramnios due to polyuria *in utero,* and premature birth. It is often complicated by dehydration episodes in the neonatal period and growth retardation. Nearly all patients present with hypercalciuria and medullary nephrocalcinosis. An elevated PGE2 production contributes to aggravate the course of the disease, a fact which prompted the alternate denomination of hyperprostaglandin E syndrome (81). The aBS derives from mutations of the genes encoding the Na^+-K^+-$2Cl^-$ cotransporter and the Kir1.1 K^+ channel (ROMK), *SLC12A1* and *KCNJ1*, respectively (84-86). In the genetically-based nomenclature they correspond to Bartter syndrome type 1 (OMIM #601678) and 2 (OMIM #241200). The cBS is usually diagnosed in early childhood. Failure to thrive is usually accompanied by low chloremia and severe hypokalemic alkalosis (8, 39, 81), polyuria and hypercalciuria being less frequent. The cBS is due to mutations of the *CLCNKB* gene encoding ClC-Kb Cl^- channel (83). This corresponds to Bartter syndrome type 3 in the genetically-based nomenclature (OMIM #607364). There is a large heterogeneity in Bartter syndrome type 3 presentation, extending from aBS to Gitelman syndrome (8, 38, 48). Gitelman syndrome is classically a defect in distal convoluted tubule (DCT) transport due to mutations in the *SLC12A3* encoding the Na^+-Cl^- cotransporter (37, 97), but some patients carrying *CLCNKB* mutations have quite a similar phenotype, exhibiting hypomagnesemia, hypo- or normo-calciuria, insensitivity to thiazide administration and absence of polyuria (8, 38).

The population of chloride channels in the renal tubule includes two additional players: ClC-Ka, a Cl^- channel α-subunit of the same family as ClC-Kb, which is encoded by the *CLCNKA* gene, and the regulatory β-subunit Barttin encoded by the *BSND* gene. A fourth variant of the Bartter syndrome, the aBS with sensorineural hearing loss (SNHL) is caused either by *BSND* mutations [OMIM

#602522 (7)(see (21) for review)], or by simultaneous mutations in the *CLCNKB* and *CLCNKA* genes [OMIM#613090, (69, 77)].

ClC-K chloride channels

ClC-Ka and ClC-Kb (ClC-K1 and ClC-K2 in rat and mouse) chloride channels were cloned in the 90s by the groups of Uchida and Jentsch (44, 90, 95). They comprise 687 amino acids and are probably present as homodimers at the plasma membrane. Except for the rat ClC-K1 (95), functional expression of the ClC-Ks was unsuccessful until the cloning of Barttin in 2001(7) when functional studies established that Barttin was a ClC-K regulatory subunit (19, 100). The electrophysiological properties of the ClC-Ks remain imperfectly known. ClC-Ka and rat and mouse ClC-K1 have been studied at the single-channel level and show conductances of 25 to 45 pS (3, 24, 43, 74) while it has not been possible until now to measure the unit conductance of ClC-Kb / ClC-K2. The only regulatory properties identified so far are the inhibition at acid extracellular pH and the activation at high extracellular calcium (19, 30, 31, 100). In addition, a block at very basic 9-11 pH has also been reported (32). The physiological impact of these regulations remains uncertain. On the one hand, ClC-Ka and ClC-Kb are relatively insensitive to calcium between 0.5 and 2 mM (3, 33, 43), which makes it unlikely that calcium variations in the physiological range affect channel activity. On the other hand, pH changes such as those experienced during acidosis or alkalosis might affect ClC-Kb (pKa=7.9, (3)) and ClC-Ka (pKa = 7.3, (33)).

At the protein level, the degree of identity between the two isoforms is slightly higher within (about 80-90%) than across (human *vs.* rodent) species (44, 89, 94). This makes it impossible to determine homologs between species on the basis of sequence comparison. However, the diverse available data have led to the consensus that rodent ClC-K1 and human ClC-Ka, on the one hand, and rodent ClC-K2 and human ClC-Kb, on the other hand, are functional orthologs (89, 94).

Collectively, the two ClC-K proteins are present all along the distal nephron, from the thin ascending limb to the collecting duct (in the intercalated cells), but not in the proximal tubule (19). This is confirmed by Barttin antibody staining the same segments of the renal tubule (19). The

expression is restricted to the basolateral membrane (19, 89) except for the thin ascending limb (tAL) where ClC-K is present on both apical and basolateral membranes (96). The differential distribution of the two channels along the renal tubule has been difficult to ascertain in the absence of isoform-specific antibodies. However, immunocytochemistry performed on the kidneys of ClC-K1 $^{-/-}$ mice demonstrated that ClC-K1 was the only isoform present in the tAL (45) and that ClC-K2 was present in the thick ascending limb (TAL), DCT and the intercalated cells of cortical collecting duct (CCD) (45). Patch-clamp experiments have also given some insight into the localization of the two channels: a recent study has demonstrated the correspondence between 45-pS chloride channel in the mouse TAL (70) and recombinant mouse ClC-K1 (50). Thus, a second 10-pS channel, activated by external calcium and at alkaline pH, which is expressed in the DCT and intercalated cells of the CNT and CCD, probably represents ClC-K2 (56, 66, 67, 70, 91). Together with results obtained with RT-PCR on rat renal segments (19, 44-46, 49, 96, 100), a consensus pattern emerges, which places ClC-K1 in the tAL and TAL, and ClC-K2 in the TAL, DCT and the intercalated cells of the CNT and CD (Fig. 1a).

A major insight in ClC-Ka function was provided by Uchida and associates (1, 54, 61) who analyzed the consequences of CLCNK1 deletion in the mouse. Matsumura et al (61) observed no hypokalaemic alkalosis in *Clcnk1*$^{-/-}$ mice, which would have been suggestive of Bartter syndrome, but polyuria (x5 compared to wild type), associated with low urine osmolality (3-fold lower than WT), was dramatic. Furthermore, *Clcnk1*$^{-/-}$ mice failed to concentrate urine after 24-h water deprivation or intraperitoneal injection of dDAVP (~2500 mOsm/kg H_2O in WTmice *vs.* ~850 mOsm/kg H_2O in *Clcnk1*$^{-/-}$ mice) (61). A follow-up study showed that the fractional excretion of sodium, chloride and urea, as well as the total osmolar clearance, were not altered in *Clcnk1*$^{-/-}$ mice (1). Thus, polyuria is due to water diuresis, not osmotic diuresis. In addition, the urea and NaCl contents in the interstitium of the inner medulla were decreased by a factor 2 in *Clcnk1*$^{-/-}$ as compared to *Clcnk1*$^{+/+}$ mice (1). Furthermore, no Cl^- conductance was detected in microperfused tALs of ClC-K1$^{-/-}$ mice (54, 61). Thus, the loss-of-function of ClC-K1 causes nephrogenic diabetes insipidus by impairing the countercurrent system in the inner medulla (1). As pointed out by Matsumura et al (61), it is doubtful

that CLCNKA deletion would cause a similar pathology in humans, who have a less developed inner medulla.

The results of Uchida's group confirm that ClC-K1 is mostly present in the thin ascending limb. Thus, although no *Clcnk2*$^{-/-}$ mouse line is yet available, we may speculate that ClC-K2 has a predominant role in the other parts of the mouse renal tubule. Ion transport models for the TAL and DCT (Fig 1b, c) involve two distinct Na^+-Cl^- cotransporters, NKCC2 and NCC, respectively, at the apical membrane. The exit of chloride on the basolateral side is dependent on Cl^- channels (18, 39, 79). Basolateral Cl^- channels are also important for ion transport in the intercalated cells (18, 79, 88) (Fig1d). Classically, the β intercalated cells participate in bicarbonate secretion but Eladari and Chambrey (11, 18, 52) recently discovered a novel, neutral pathway for NaCl absorption in these cells (Fig 1d), which includes a Cl^- conductance at the basolateral side (18, 79, 88). The α intercalated cells (Fig 1d) participate in acid-base balance by secreting protons *via* the V-type H^+ ATPase at the apical membrane and the AE1 HCO_3^-/Cl^- exchanger at the basolateral membrane. Basolateral Cl^- channels optimize H^+ secretion by recycling Cl^-, thus facilitating the functioning of the AE1 exchanger (88). In summary, ClC-K2 is expected to play a key role in Cl^- absorption in the DCT, CNT/CCD and to a lower extent in the TAL, where ClC-K1 is present (50, 70). There has been yet no patch-clamp study of ClC-K chloride channels in the α intercalated cells, letting open the possibility that the two channels cooperate at this site.

Structure of the ClC-K chloride channels

Renal chloride channels belong to the channel subgroup of the ClC family of chloride channels and transporters, which also includes ClC-1 and ClC-2 (21, 42, 93). The structure of this protein family was revealed in 2002 with the crystallization of two bacterial Cl^-/H^+ exchangers from *S. typhimurium* and the *E. coli (16)*. Each monomer is defined by 18 α-helices (from A to R) with an antiparallel structure, meaning that two structurally related halves (α-helices A to I and J to R, respectively) span the membrane with an opposite orientation (16). The ClC proteins are functional dimers constituted by two independent permeation pathways called protopores (see Fig. 2). The protopore, localized

between the two structurally related halves of the ClC monomer, is formed by an anion-selective filter between two aqueous vestibules comprising three Cl^--binding sites. The binding sites are located close to the extracellular side (S_{ext}), in the central region of the protein (S_{cen}) and close to the intracellular side (S_{in}) (17). These are formed by the N-termini of α-helices D and F in the first half of the monomer and the N-termini of α-helices N and R in the second half of the monomer. In ClC-0, ClC-1 and ClC-2 but not ClC-K, the access to each protopore is regulated *via* one opening / closing mechanism called *fast,* or "glutamate" gate. The structural determinant of this gating consists of an external glutamate (E148 in *E. coli*, ec-ClC), the protonation/deprotonation of which is directly implicated in gating (17, 64). In addition, the two protopores can be opened / closed simultaneously by the so called *common,* or *slow* gate (12, 72). This second mechanism is particularly important for the renal ClC-Ks that lack the characteristic "glutamate" responsible for protopore gating in the others ClC proteins (17).

Eukaryotic ClC proteins, including ClC-Ks, have a large cytoplasmic C-terminus domain constituted by a linker sequence connecting α-helix R to two cystathione-β-syntase domains (CBSs) [see Fig. 2]. The crystal structure of human CBS of ClC-5 (63) and ClC-Ka (58) reveals a dimeric interaction between two ClC protein C-terminal domains (59), likely to be conserved among the eukaryotic ClC (62). Several studies reported a regulatory role of binding of adenine nucleotides to CBS domains in ClC-1, ClC-2 and ClC-5 (65, 92, 105). This part of the protein is known to be involved in the regulation of the common gating (9, 20). A similar role has yet to be demonstrated for the ClC-Ks.

As noted above, the ClC-K currents recorded in over-expression systems are inhibited at acid pH and activated by an increase of $[Ca^{2+}]_{ext}$ (19, 30, 31, 100). A detailed screening of acidic amino-acid residues led to the identification of two critical amino-acids (E261 and D278, localized in the extracellular loop between α-helices I and J), which form the putative extracellular Ca^{2+}-binding site (31). A histidine residue on position 497 at the beginning of α-helix Q is responsible for the H^+-induced block (30) [Fig. 2].

***CLCNKB* mutations in Bartter syndrome**

Since the pioneering work of Simon et al. identifying mutations in the *ClCNKB* gene as a cause of Bartter syndrome type 3 (83), a number of publications have reported pathogenic mutations (3, 8, 26, 43, 48, 51, 76, 83, 102, 103). As a whole, according to the Human Gene Mutation Database (free access HGMD database, Biobase International, www.hgmd.cf.ac.uk), more than 54 mutations have been described, including complex rearrangements, large and small deletions, small insertions, nonsense or missense mutations and splice site mutations. One characteristic feature of the disease is the frequent occurrence of total *CLCNKB* gene deletion (8, 29, 38, 48, 83).

The 50 point mutations (missense and nonsense) illustrated in Fig. 1 and summarized in Table 1 are distributed all along the protein with no clear hotspots. Several mutations are located close to several parts of the selectivity filter, in C-D linker and α-helix D (3 mutations), E-F linker and α-helix F (2mutations) and α-helix N (8 mutations), respectively. By contrast, no mutations were reported in the vicinity of H^+ and Ca^{2+} binding sites. Altogether, 60% mutations are located on α-helices, 20% in helix linkers and 20% in the cytoplasmic part of the protein. About 16 homozygous mutations have been found in patients (A77P, L81P, T115P, P124L, G164C, V170M, A204T, G246R, S297R, R351W, H357Q, G424E, R438H, K560M, R595X, W610X). A few mutations such as A204T (29, 76, 83), P124L (3, 5, 43, 48, 83), R438H (3, 5, 43, 48, 83) and W610X (14, 51, 68) have been reported independently by several authors. In particular, A204T, a founder mutation in Spain, is the prevalent mutation in this country (28). W610X is the most common mutations in Korea (51).

Functional consequences of *CLCNKB* mutations

Only 20 *CLCNKB* mutations have been functionally analyzed. This low figure is mainly due to technical problems regarding ClC-Kb functional expression. Current recording of ClC-Kb in *X. laevis* oocytes or cultured cells has remained difficult even after the discovery of the Barttin regulatory subunit: ClC-Kb levels of current are 5-fold lower than those elicited by ClC-Ka (36, 43). An additional, specific problem concerns Western blotting of the ClC-K protein: over-expressed ClC-K proteins tend

to form stable aggregates so that the band corresponding to the monomeric protein is often hardly visible [(10, 43, 100, 101) but see (36)].

Before the discovery of Barttin, Waldegger et al. (101) succeeded in recording chloride currents from a chimeric human ClC-Kb/rat ClC-K1 construct [ClC-Kb (c)] and analyzed a selection of mutations reported by Simon et al. (83) in *X. laevis* oocytes using two-electrode voltage-clamp: P124L, A204T, A349D and R438C abolished ClC-Kb (c) current while Y432H dramatically reduced it, by at least 4 fold. Obviously, a limit of this interesting study lies in the use of chimeric channels, as it is difficult to anticipate how the presence of rat ClC-K1 stretches in the ClC-Kb protein might influence the observed effects. Soon afterwards, Estevez et al. (19) recorded for the first time ClC-K/Barttin complexes in *X. laevis* oocytes. They tested several of the mutations reported by Simon et al and Konrad et al (48, 83) by co-injecting mRNA for several ClC-Kb mutants with Y98A-mutant Barttin in order to increase surface expression of the channel complex. Overall A349D and R438C mutations totally abolished currents, as in the abovementioned study, while P124L, A204T, R538P and K560M mutations decreased the currents by 60-80%. More recently, Yu et al (102) analyzed R30X, A210V and R351W mutations by the same methods. As expected, R30X mutant generated no current; A210V and R351W currents were reduced by 53 and 39%, respectively. Overall, all these mutations (except A210V and R351W) reduced channel currents by more than 60% (19, 102).

Our group has recently investigated 8 novel *CLCNKB* mutations (L81P, R92W, G120R, V170M, G246R, R351P, G424E and L439P) (3, 43). We also selected some published mutations to gain a deeper understanding of the molecular defect behind the development of Bartter syndrome type III (T115P, P124L, L139P and R438H) (8, 48, 83, 103). In these studies, we co-expressed mutant ClC-Kb proteins with wild-type Barttin in *X. laevis* oocytes or HEK293 cells. The currents carried by the ClC-Kb mutants were reduced to different extents. For nearly half of the mutants (3, 43), no current was detected (T115P, G246R, G424E, R438H and L439P). Four mutants showed 50-70% residual activity (R92W, P124L, V170M, R351P), and two had a low activity around 20-40% (L81P, G120R) (3, 43).

222 Note that in our hands, P124L generated a noticeable current (40%) while lower currents (0-20%)
223 were detected in previous studies (48, 85).

224 Surface expression varied from 0 to 85% when compared to WT ClC-Kb and was proportional
225 to current level, indicating that the primary defect shown by mutants concerns the number of
226 channel units in the membrane (3, 43). For mutants generating sufficient current, we evaluated unit
227 conductance (R92W, R351P), anion selectivity (L81P, R92W, V170M and R351P), and pH- and Ca^{2+}-
228 sensitivity (L80P, R92W, R351P, P124L and V170M) (3, 43). There was no change in conductance,
229 selectivity or regulation except for two mutants (P124L, V170M) that displayed dramatic alteration in
230 pH-sensitivity (see below). These results go along with the hypothesis that the primary defect
231 induced by mutations is generally an alteration in surface expression of the channel.

232 Altogether, among the 20 mutations analyzed for the total current, 40% do not show any
233 detectable activity (T115P, G246R, A349D, G424E, G424R, R438C, R438H and L439P), 40% maintain
234 more than 50% activity (R92W, G120R, P124L, V170M, A210V, R351P, R351W and K560M) and 20%
235 have a smaller activity around 20-30% (L81P, L139P, A204T and R538P). Total protein abundance
236 (considering bands corresponding to monomeric and dimeric proteins) was assayed in HEK293 cells
237 for a minority of mutants (43). Protein abundance was clearly reduced for those mutants that
238 showed no current and no surface expression (G246R, G424E, R438H and L439P). This strongly
239 suggests that the produced transcript is not stable in the expression system. Protein abundance was
240 not reduced for mutants showing more than 50% activity (R92W and R351P) nor for one mutant with
241 20-30% activity (L81P). This suggests that the stability of the protein (for instance, accelerated
242 retrieval from the membrane) in the membrane could be decreased.

243 Mutations that alter regulation/conduction appear to be predominant in myotonia caused by
244 mutations in the *CLCN1* gene: ClC-1 is a voltage-dependent channel, which is activated by
245 depolarization; many mutations profoundly shift voltage dependence towards more positive voltages
246 (71, 73) or even convert ClC-1 to an inwardly-rectifying channel (22, 104). By contrast, mutations
247 altering channel gating are not frequent in Bartter disease type 3. We have evaluated pH- and Ca^{2+}-

sensitivity for 6 ClC-Kb mutants and found alterations in only 2 (3, 43), while studies by others detected 2 additional mutants showing some kind of altered regulation. Maduke et al (59) reported that R538P mutation totally abolished sensitivity to Ca^{2+}. This is associated with a clear reduction in current (19). According to the authors, the arginine 538 being located in the cytoplasm after helix R, the lack of sensitivity to Ca^{2+} implies a long distance effect from the cytoplasmic side of the protein to its extracellular side (59). Similar Ca^{2+}-independence was reported by Yu et al (102) for R351W mutation (at the outer aspect of the protein in K-L linker), which was also associated with 60% decrease in current.

In our hands, two mutations (V170M and P124L) dramatically altered pH and Ca^{2+} sensitivity (3). The curve of H^+-dependent inhibition shifted by about 1.5 pH units towards more acid values for V170M and P124L mutants as compared to WT ClC-Kb (pK_H: 6.0 for V170M and 6.1 for P124L *vs.* 7.6 for WT). Our results suggest that these mutations do not alter directly the binding-site of H^+ but disrupt the gating function of the channel, thus indirectly affecting the sensitivity to pH_{ext} (3). As a direct consequence, the mutant ClC-Kb channels are maximally active at pH 7.4 while the activity of WT ClC-Kb at this pH is only 20% of its maximal activity. In addition, we observed that the mutants were completely independent of Ca^{2+} at pH 7.4, perhaps because they are maximally active at this pH (3). In parallel, there was a clear reduction in current and surface expression in the two mutants (3). Therefore, these mutations result in hyperactive channels, which tend to offset the functional impact of reduced expression at the membrane. The opposite mechanism might operate in the case of T481S ClC-Kb, a *CLCNKB* single-nucleotide polymorphism that has been linked to essential hypertension (41, 82). Jeck et al (40) showed that the 5-20-fold increase in ClC-Kb current induced by T481S mutation was associated with higher sensitivity to pH (lower activity at pH 7.4) and an increase in membrane channel expression, a mirror image of what is observed in Bartter syndrome for ClC-Kb mutants.

Alteration of functional motifs as a cause of Bartter syndrome

Several of the mutations that have been functionally analyzed are located around the selectivity filter. Three mutations in α-helix N (R438C, R438H, L439P) and one close to it (G424E) are associated with reduction in total protein abundance, absence of expression at the membrane and absence of current. Two mutations (P124L and V170M) alter channel regulation. These results strongly suggest that the selectivity filter is of paramount importance for structure stability and gating. The hyperactivity of P124L and V170M is puzzling when considered in the context of disease pathophysiology. As already mentioned, mutations of the *CLCN1* gene causing myotonia often result in gating alterations. However, these alterations always render the channel less active (71). Thus, it is currently difficult to appreciate whether the uncoupling of channel activity from pH- and Ca^{2+}-dependent gating is a co-incidental side-effect of the mutations or whether these mutations alter yet unidentified regulators of channel activity.

By contrast, the available data provides little information about the functional effects of *CLCNKB* mutations in two other regions: α-helices B and J (8 mutations), which interact with Barttin, and CBS domains (7 mutations). The CBS domains might be particularly critical for channel function. They participate in ClC *common* gating (20, 25), likely *by* sensing intracellular metabolites (35). They also participate in channel trafficking and targeting to the plasma membrane. The partial deletion of the cytoplasmic domain leads to a retention of ClC-0 and ClC-1 proteins into the ER (20, 34, 57, 78). The only CBS1-located mutation that has been functionally characterized in over-expression systems (K560M) reduces ClC-Kb current to 25% of control (19).

The interface between the two monomers, mainly composed of α-helices H, I, P and Q, is potentially another critical region. In the case of Dent's disease, an X-linked hereditary disease due to mutations in the *CLCN5* gene encoding the Cl^-/H^+ exchanger ClC-5, about 60% of the mutations are located at the dimer interface (55). These mutations either result in ER retention or channel dysfunction (55). Mutations at the same location are also frequent in *CLCN7* gene causing osteopetrosis, or in *CLCN1* gene, where they modify channel gating (71). Unexpectedly, only 3 mutations have been found at the dimer interface in Bartter disease: P216L (51) and A242E (5),

which have not been functionally investigated and G246R which shows no current, no surface expression and reduced protein abundance (43). The latter result suggests a destabilization of the protein.

Obviously, further experiments focusing on surface channel expression and sub-cellular distribution would be necessary in the future to investigate the functional consequences of mutations in the CBS region, in the α-helices interacting with Barttin and at the dimer interface.

Phenotype-genotype relationship

The first papers reporting mutations in the *CLCNKB* gene causing Bartter syndrome type 3 already mentioned that the severity of the disease was highly variable (38, 48, 83). In particular, whole gene deletion, which is frequent in this disease, may cause aBS or cBS (38, 48, 83). This is also the case for A204T and R438H mutations (29, 76, 103). R438H mutation results in a non-functioning channel (43), A204T reduces current to 0-25% of control (48, 83). In particular, Zelikovic et al (103) showed that patients carrying the mutation R438H within one same large family could present with mild or severe symptoms. Especially in the case of *CLCNKB* gene deletion, the variable severity of the disease suggests that ClC-Ka Cl^- channel, K^+-Cl^- cotransporter (8) or additional Cl^- channels (60) might be expressed in the TAL in a variable fashion and compensate for the loss of function of ClC-Kb in some patients. In the case of point mutations, one alternative possibility would be that the defect being due to conformational destabilization has variable impact depending on the individual.

The 8 patients homozygous for V170M, or compound heterozygous with another mutation (whole gene deletion or mutations resulting in the production of unstable mRNAs or truncated proteins) have a mild phenotype (3). It is likely that these patients mainly express V170M homodimers of this hyperactive channel with reduced expression at the membrane. This is also the case for one patient homozygous for P124L, but not for 2 other patients compound heterozygous for P124L and G465R (3). It will be interesting to observe whether more mutations of this type are detected in the future and whether they are associated with a mild presentation.

Conclusion

Overall, currently available data suggests that *CLCNKB* mutations might be arranged in two main classes: (i) mutations causing fully impaired (about 40% of the mutations) or reduced (about 60% of the mutations) channel expression at the membrane; (ii) mutations combining reduced channel surface expression with altered channel properties (about 20% of the total number of mutations analyzed).

It is clear that further investigation is needed to fully characterize the pathogenesis of Bartter disease type 3, which has been hampered by technical difficulties in the expression of this particular chloride channel. However, Bartter disease type 3 probably belongs to the large family of conformational diseases, in which the mutations destabilize channel structure, inducing retention in the endoplasmic reticulum and accelerated channel degradation. Accordingly, a classical approach to treatment would be to test the efficacy of adapted chaperons for enhancing ClC-Kb surface expression. An approach of this type has been recently performed with some success on W610X ClC-Kb mutants in MDCK cells using an aminoglycoside derivative (14). This long-term strategy might be helpful for those patients who present with a severe form of Bartter syndrome type 3.

At the end of this review dedicated to the pathophysiology of Bartter syndrome, it is worth reminding that one *CLCNKB* single-nucleotide polymorphism (T481S) has been linked to essential hypertension (41, 82). Although the association with hypertension was not replicated by some studies (23, 47, 87), the idea that ClC-Kb might be functionally important for long-term control of blood pressure remains attractive, and keeps in line with recent findings pointing out the importance of chloride in blood pressure regulation (18). In addition, several *CLCNKA* single-nucleotide polymorphisms have been associated with salt-sensitive hypertension (4) or heart failure (10). Altogether, these various reports suggest that the pathophysiological impact of ClC-Kb and ClC-Ka on NaCl balance might be not restricted to Bartter syndrome.

Grants

This work was supported by grants from French Research National Agency (ANR BLAN 2010-111201, ANR-14-CE12-0013-02), French Kidney Foundation and ECOS-sud (C14-S01). The work of R. Briones was supported by a grant from DFG (SFB803).

Acknowledgments

We thank the patients, their families, and the nephrologists from the French tubulopathy network. The English text was edited by Charlotte Sumida. The present address of Mathilde Keck is UMR INSERM 1166 ICAN, 91 boulevard de l'Hôpital, 75013 PARIS, France

Conflict of interest

None

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FIGURE LEGEND

Figure 1. Localization of the two ClC-K channels along the rodent renal tubule and ion transport systems in various parts of the distal nephron. A) Localization of ClC-K1 (blue) and ClC-K2 (orange) along the nephron. The localization is based on RTPCR on rat isolated renal segments (49, 66, 100), immunochemistry (19, 45) and patch-clamp studies on the mouse renal tubule (50, 56, 66, 67, 70). The distribution pattern remains incomplete: 1) in the absence of a dedicated patch-clamp study, it is not known whether ClC-K1 is present in the α intercalated cells together with ClC-K2; 2) Although, ClC-K1 messenger RNA is present in the late distal tubule and the collecting duct, ClC-K1 has not been detected in these two renal segments using the patch-clamp technique (50, 56, 66, 67, 70). ClC-K1 might be active only during specific physiologic conditions. B) NaCl absorption in the thick ascending limb involves NKCC2 Na^+-K^+-$2Cl^-$ cotransport in the apical membrane and ClC-K2 and ClC-K1 in the basolateral membrane. C) NaCl absorption in the distal convoluted tubule involves NCC Na^+-Cl^- cotransport in the basolateral membrane and ClC-K2 in the basolateral membrane. D) The β intercalated cells (β ic) absorb NaCl (18) and the α intercalated cells (α ic) secrete protons. NaCl absorption involves the Pendrin Cl^-/HCO_3^- exchanger and NDCBE Na^+-driven Cl^-/HCO_3^- exchanger in the apical membrane, and AE4 Cl^-/HCO_3^- exchanger and ClC-K2 in the basolateral membrane. The KCC4 K^+-Cl^- cotransporter might also allow Cl^- exit. The α intercalated cells secrete H^+ by the operation of the V-type H^+ ATPase in the apical membrane and AE1 Cl^-/HCO_3^- exchanger in the basolateral membrane. ClC-K2 with or without ClC-K1 is present in the basolateral membrane and might optimize the turnover rate of the exchanger.

Figure 2. Homology model of the ClC-Kb Cl^- channel based on the Eukaryotic ClC of Cyanidioschyzon merolae (PDB id. 3ORG) that includes the cystathione-β-syntase domains. A side-view of the dimeric structure is shown in cartoon representation, where the membrane is delimited by dotted lines. The monomers are colored orange and blue. The permeation pore in the left-hand side monomer is shown as a continuous series of white spheres, and amino acids participating in the

677 selectivity filter are shown in sticks in both monomers. On the extracellular side, green and purple
678 spheres show the putative location of Ca^{2+} and H^{+} binding sites, respectively, as inferred from
679 comparison with prokaryotic *Escherichia coli* (PDB id. 1KPK) and eukaryotic *Cyanidioschyzon merolae*
680 ClCs using using Multalin software for multiple sequence alignments (15), plus manual adjustements.
681 The location of the binding sites slightly depends on the homology model, especially for the Ca^{2+}
682 binding site. As a result, the number of spheres is larger than the number of involved amino acids.
683 Pymol software (The PyMOL Molecular Graphics System, Version 1.7, Schrödinger, LLC) was used to
684 render figure.
685
686 **Figure 3**. Mutations in the ClC-Kb Cl^{-} channel associated with Bartter disease. The location of
687 mutations is illustrated on a topological model of ClC-Kb where rectangles represent α-helices. The
688 plasma membrane is not shown; the lower part of the model is intracellular, the upper part
689 extracellular. The letters indicate the corresponding α-helices and CBS1 and CBS2 the two
690 cystathione-β-syntase domains. The α-helices participating in the selectivity filter, those interacting
691 with Barttin and those located at the dimer interface are colored in green, yellow and purple,
692 respectively. Each symbol gives the position and the type of a single mutation, the number referring
693 to mutations in Table 1.
694

695

Table 1. *CLCNKB* mutations and corresponding changes in amino acid sequence of the ClC-Kb chloride channel.

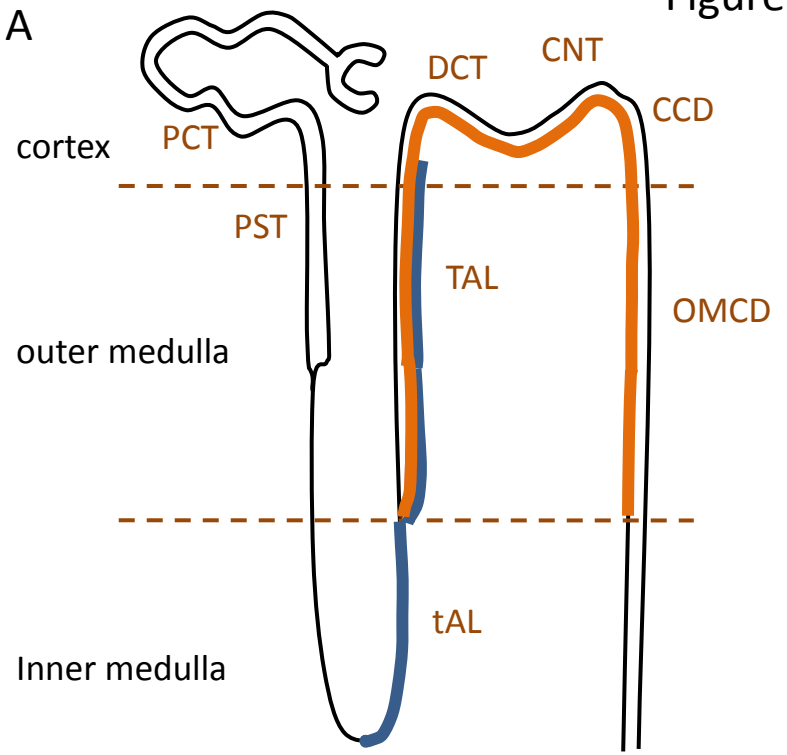
Mutation number	Nucleotide change	Amino acid change	Protein domain	Remaining current	Sensitivity to H^+ and Ca^{2+}	Surface expression	Total ClC-Kb protein

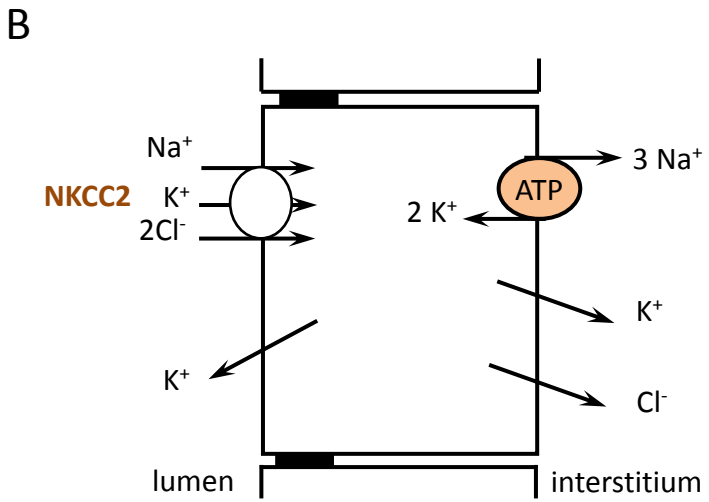
697

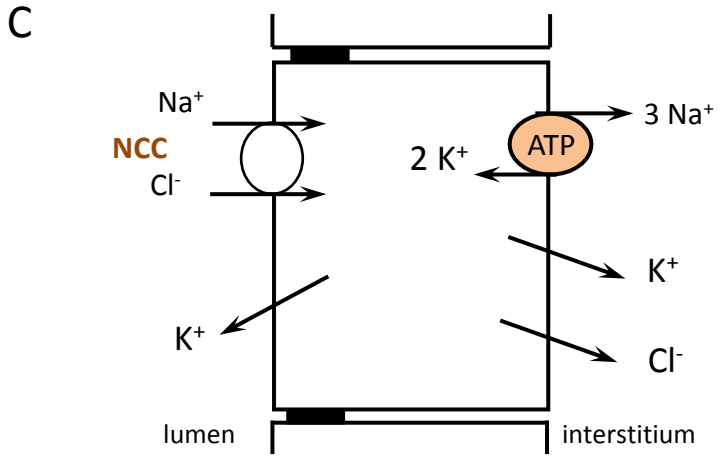
1	c.88C>T (102)	R30X	α helix A				
2	c.216C>A (6)	A61D	α helix B				
3	c.226C>T (68)	R76X	α helix B				
4	c.229G>C (75)	A77P	α helix B				
5	c.229G>A (48)	A77T	α helix B				
6	c.242T>C (36)	L81P	α helix B	35% (43)	= (43)	30% (43)	= (43)
7	c.274C>T (43)	R92W	α helix C	67% (43)	= (43)	85% (43)	= (43)
8	c.343A>C (43)	T115P	α helix C	18% (3)			
9	c.358G>C (3)	G120R	C-D linker	60% (3)	= (3)		
10	c.359G>T (51)	G120V	C-D linker				
11	c.371C>T (48, 83)	P124L	α helix D	0% (101); 45% (19); 73% (3)	Altered (3)	= (101)	= (101)
12	c.451T>C (83)	L139P	D-E linker	27% (3)			
13	c.480T>A (6)	V149E	α helix E				
14	c.490G>T (2)	G164C	E-F linker				
15	c.508G>A (3)	V170M	α helix F	60% (3)	Altered (3)	60% (3)	
16	c.610G>A (76, 83)	A204T	α helix G	25% (19); 0% (101)		= (101)	= (77)
17	c.629C>T (102)	A210V	α helix G	~50% (102)	= (102)		
18	c.647C>T (51)	P216L	G-H linker				
19	c.708C>A (3)	Y236X	H-I linker				
20	c.725C>A(5)	A242E	α helix I				
21	c.736G>C (43)	G246R	α helix I	10%* (43)		None (43)	↓ (43)
22	c.887G>A (99)	G296D	α helix J				
23	c.927G>A (48)	S297R	α helix J				
24	908A>C (98)	Q303P	α helix J				
25	c.1004T>C (13)	L335P	α helix K				
26	c.1045T>C (83)	S337F	α helix K				
27	c.1046G>A (83)	A349D	K-L linker	0% (101); 10% (19)		↓ (101)	↓ (101)
28	c.1052G>C (43)	R351P	K-L linker	63% (43)	= (43)	65%(43)	= (43)
29	c.1051C>T (102)	R351W	K-L linker	~60% (102)	Ca^{2+} altered (102)		
30	c.1107T>A (48)	H357Q	α helix L				
31	c.1172G>A (68)	A391X	L-M linker				
32	c.1270G>A (51)	G424R	M-N linker				
33	c.1270G>A (43)	G424E	M-N linker	10%* (43)		None (43)	↓ (43)
34	c.1294T>C (83)	Y432H	α helix N	~20% (101)		↓ (101)	= (101)
35	c.1309G>A (51)	G437C	α helix N				
36	c.1312C>T (83)	R438C	α helix N	0% (101); 10%* (43)		↓ (101)	= (101)
37	c.1313G>A (48, 103)	R438H	α helix N	18%* (43)		None (43)	↓ (43)
38	c.1316T>C (43)	L439P	α helix N	15%* (43)		None (43)	↓ (43)
39	c.1325A>G (29)	E442G	α helix N				
40	c.1340T>C (99)	I447T	N-O linker				
41	c.1409G>A (53)	G470E	α helix O				
42	c.1537C>T (83)	Q513X	α helix Q				
43	c.1648G>C 31)	R538P	Linker R-CBS1	25% (19)	Ca^{2+} altered (59)		
44	c.1714A>T 31)	K560M	CBS1	50% (19)			
45	c.1685C>T (27)	M562T	CBS1				
46	c.1753T>A (48)	S573Y	CBS1				
47	c.1732G>A (27)	K578E	CBS1				
48	c.1783C>T (6)	R595X	CBS1				
49	c.1830G>A (26, 51)	W610X	CBS1				
50	c.1877G>A (99)	C626Y	CBS2				

*Residual current not statistically different from measured current in noninjected oocytes (36).
Numbers between parentheses indicate references.

Figure 1


A
cortex
PCT
DCT
CNT
CCD
PST
TAL
OMCD
outer medulla
tAL
Inner medulla


B
Na+
NKCC2
K+
2Cl-
ATP
2 K+
3 Na+
K+
K+
Cl-
lumen
interstitium


C
Na+
NCC
Cl-
ATP
2 K+
3 Na+
K+
K+
Cl-
lumen
interstitium

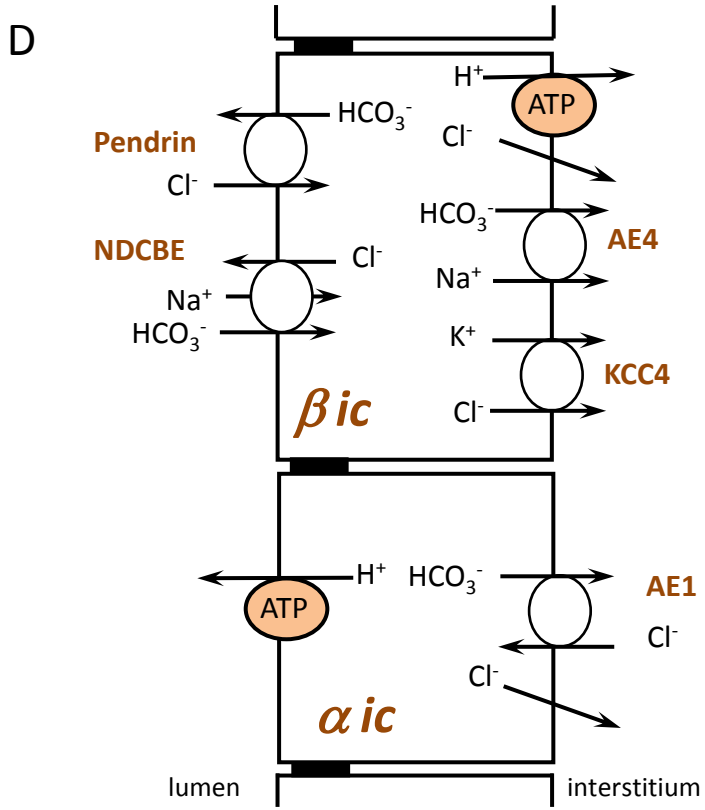

D
Pendrin
Cl-
HCO3-
H+
ATP
Cl-
HCO3-
AE4
NDCBE
Cl-
Na+
HCO3-
Na+
K+
KCC4
Cl-
βic
H+
HCO3-
ATP
AE1
Cl-
Cl-
αic
lumen
interstitium

Figure 2

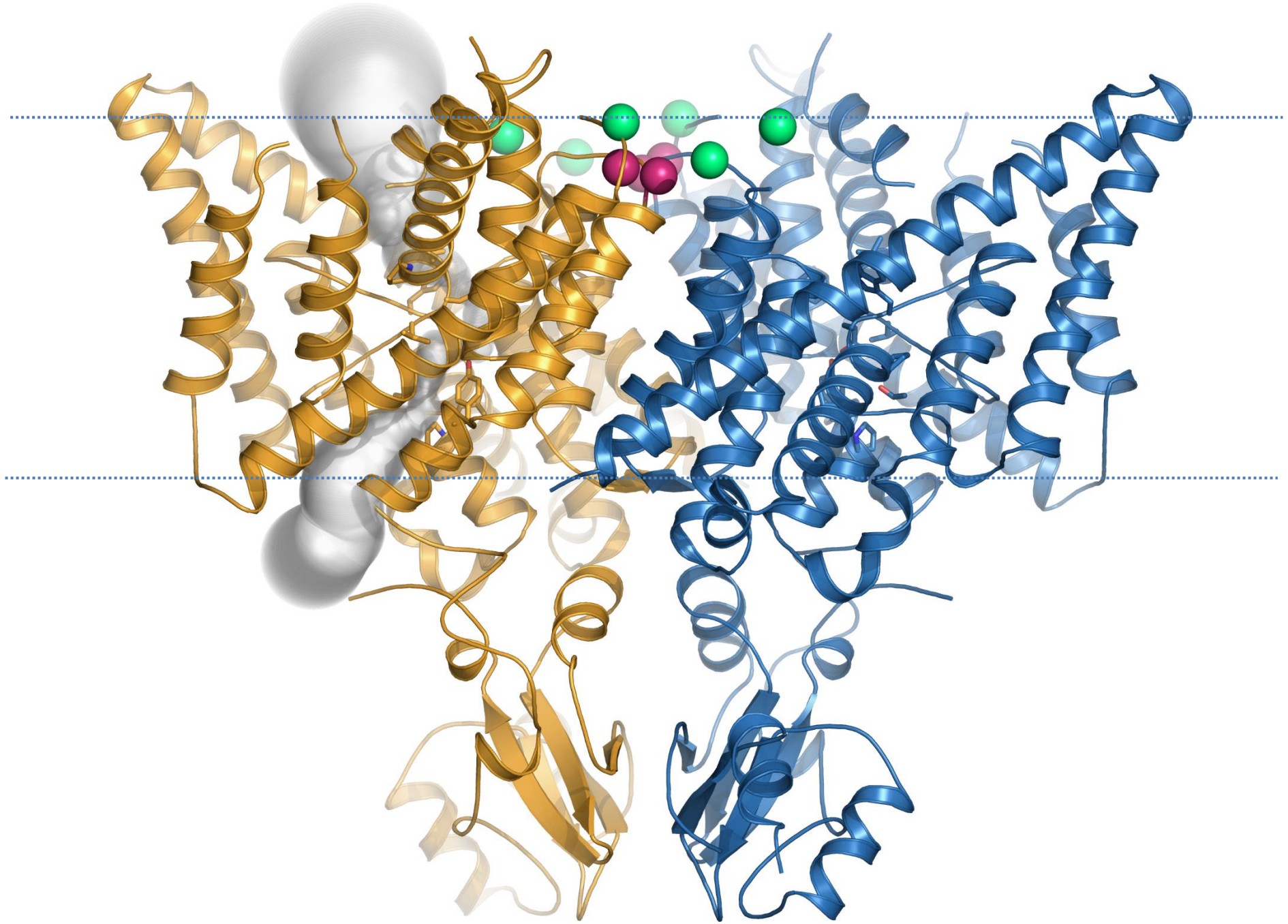
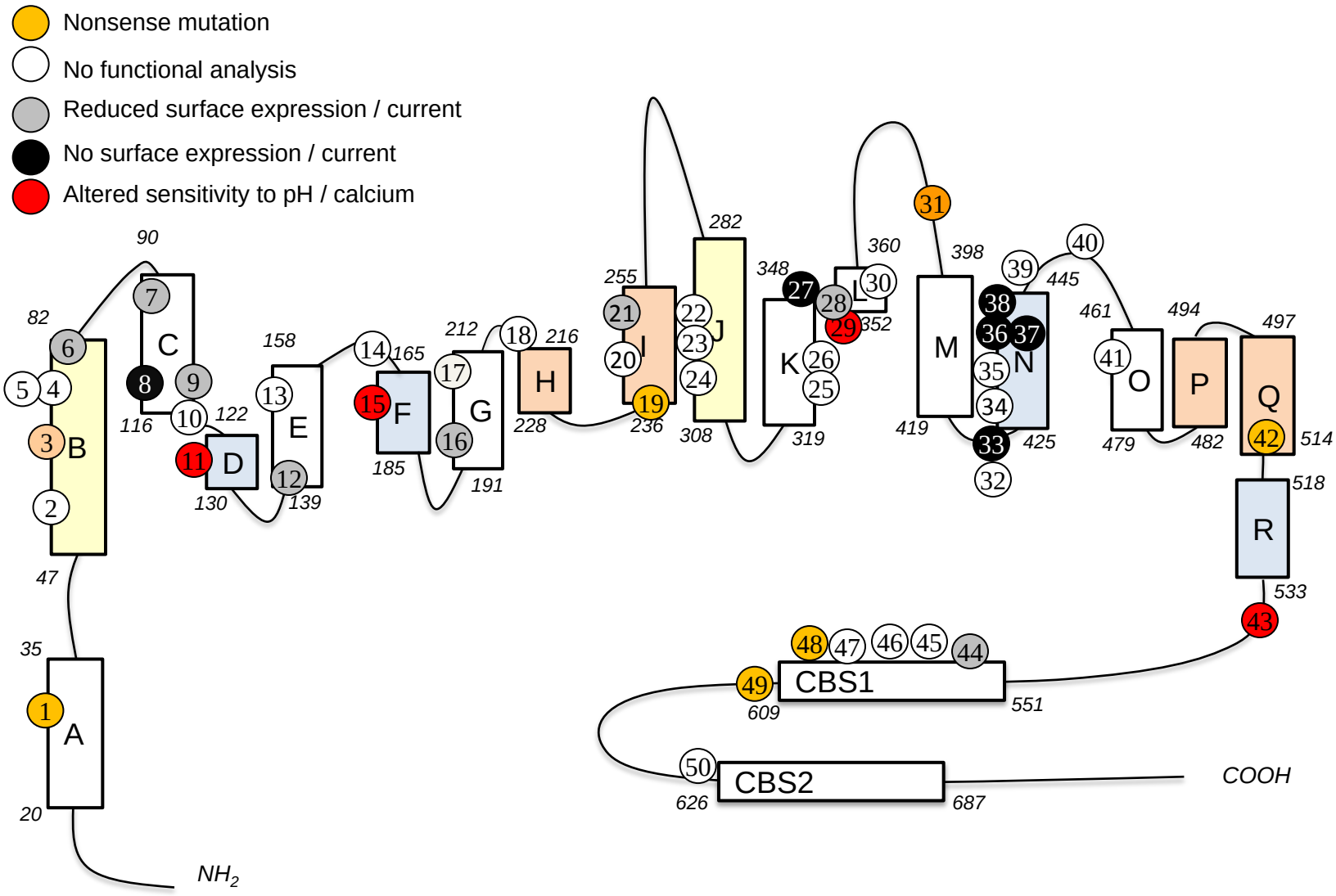


Figure 3


Nonsense mutation
No functional analysis
Reduced surface expression / current
No surface expression / current
Altered sensitivity to pH / calcium
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
CBS1
CBS2
NH2
COOH