Additional Disruption of the ClC-2 Cl\(^-\) Channel Does Not Exacerbate the Cystic Fibrosis Phenotype of Cystic Fibrosis Transmembrane Conductance Regulator Mouse Models*  

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Cystic fibrosis is a fatal inherited disease that is caused by mutations in the gene encoding a cAMP-activated chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR). It has been suggested that the cystic fibrosis phenotype might be modulated by the presence of other Cl\(^-\) channels that are co-expressed with CFTR in epithelial cells. Because the broadly expressed plasma membrane Cl\(^-\) channel, ClC-2, is present in the tissues whose function is compromised in cystic fibrosis, we generated mice with a disruption of both Cl\(^-\) channel genes. No morphological changes in their intestine, lung, or pancreas, tissues affected by cystic fibrosis, were observed in these mice. The mortality was not increased over that observed with a complete lack of functional CFTR. Surprisingly, mice expressing mutant CFTR (deletion of phenylalanine 508), survived longer when CIC-2 was disrupted Additionally. Currents acrosenic epithelia were investigated in Ussing chamber experiments. The disruption of CIC-2, in addition to CFTR, did not decrease Cl\(^-\) secretion. Colon expressing wild-type CFTR even secreted more Cl\(^-\) when CIC-2 was disrupted, although CFTR transcript levels were unchanged. It is concluded that CIC-2 is unlikely to be a candidate rescue channel in cystic fibrosis. Our data are consistent with a model in which CIC-2 is located in the basolateral membrane.

Cystic fibrosis (CF) is a severe autosomal recessive disease that is associated with pulmonary, pancreatic, and intestinal symptoms. Most patients eventually die in adulthood because of the pulmonary phenotype. In the lung and trachea, very viscous tracheal mucus results in reduced lung clearance and accompanying recurrent bacterial infections. In addition, the pancreas often undergoes cystic and fibrotic changes with aging, resulting in pancreatic insufficiency in some, but not all, patients. Further symptoms include male infertility due to the congenital absence of vas deferens and, in some cases, an intestinal occlusion due to thick feces (meconium ileus) and biliary cirrhosis.

Cystic fibrosis is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (1), the only member of the large ABC transporter family that is known to function as a cAMP-activated chloride channel. The most common CFTR mutation in Caucasians is ΔF508, which deletes a phenylalanine (Phe-508) in-frame. More than one thousand other mutations, many of them leading to a complete loss of function, are known. CFTR mediates chloride transport in the apical membranes of many epithelia. In addition, CFTR was suggested to have several other functions, including the regulation of various transport proteins such as the epithelial Na\(^+\) channel ENaC and certain other Cl\(^-\) channels (2–4). If the pathological changes in cystic fibrosis were predominantly due to defective chloride transport, the pharmacological activation of other Cl\(^-\) channels present in the same membranes would potentially prove beneficial in patients.

Several CFTR mouse models have been generated. They include complete disruptions of the CFTR gene (as in the Cftr\(^{-/-}\) mouse (5), called Cftr\(^{+/+}\) from here on) and mice that carry CFTR point mutations analogous to those found in patients (like the Cfr\(^{A532V}\) mice as a model for the human ΔF508 mutation (6), called Cfr\(^{A532V}\) from here on). These mice, however, failed to show obvious pulmonary and pancreatic phenotypes, but rather exhibited intestinal occlusion (ileus). Without a laxative diet these mice rarely survive beyond the age of 30 days. But even when fed an osmotic laxative, no clear pulmonary and pancreatic phenotypes developed at older ages. The discrepancy between human and mouse pathology may be explained, for example, by differences in airway anatomy or by the expression of other Cl\(^-\) channels that may compensate for the loss of CFTR in mice. Among the several putative modifier genes that co-determine the severity of CF, there may be genes encoding other Cl\(^-\) channels (7, 8).

The CLC gene family encodes nine Cl\(^-\) channels in mammals (9). Many other Cl\(^-\) channels, probably including Ca\(^{2+}\)-activated Cl\(^-\) channels, have not yet been identified at the molecular level. Human mutations in several CLC channel genes underlie diseases as diverse as myotonia (10), the kidney diseases Bartter syndrome (11) and Dent’s disease (12), osteopetrosis (13), and possibly a form of epilepsy (14). Only four CLC channels, i.e. ClC-1, ClC-2, ClC-Ka, and ClC-Kb, reside predominantly in the plasma membrane. ClC-2 is nearly ubiquitously expressed in epithelia and non-epithelial tissues (15). ClC-1 is muscle-specific (16), and ClC-K expression is probably limited to certain renal cells and the stria vascularis (17).
CFTR/CIC-2 Double Knock-out Mice

Immunocytochemical studies have suggested that CIC-2 is expressed in the apical membranes of lung and intestinal epithelia (18, 19), where it may transport Cl\(^-\) in a pathway that is independent of and parallel to CFTR. Thus, CIC-2 appears to be a prime candidate for an alternative pathway for Cl\(^-\) secretion in CF (20–22).

CIC-2 is activated by hyperpolarization, cell swelling, and acidic extracellular pH (15, 20, 23). Many physiological functions have been postulated for CIC-2 (9), but blindness and male infertility were, surprisingly, the only phenotypes noted in CIC-2 knock-out mouse models (24, 25). An impaired transepithelial transport across the retinal pigment epithelium and Sertoli cells was hypothesized as the mechanism underlying the retinal and testicular degeneration, respectively (24).

Indeed, Ussing chamber experiments revealed that transepithelial voltage and resistance were reduced in the pigmented epithelium of Clnce\(^{-/-}\) mice (24).

If CIC-2 transports chloride across the same cell membranes as CFTR, then a disruption of both channels should lead to a more severe phenotype in the respective tissues. This may then also include the lung and the pancreas, tissues that are affected in CF patients but not in CFTR mouse models. We therefore produced mice in which both genes are disrupted. In addition to histomorphological analysis, we performed Ussing chamber experiments on colon and trachea, tissues that are affected in CF.

**Experimental Procedures**

**Mice**—CIC-2 knock-out mice, described previously (24) and backcrossed for seven generations into C57Bl6, were mated with heterozygous CFTR \( \Delta F 508 \) mice (6) (CFTR\(^{+/-}\)) and CFP S489x (5) (CFTR\(^{+-}\)), called CFP\(^{+/-}\) (26) (both in C57Bl6 background) obtained from Jackson Laboratories. Both types of mutations lead to a severe phenotype in humans. The resulting offspring were again mated. Genotypes were determined by touch-down PCR (33–37 cycles) on tail DNA, using the following: C2wtF1 (5'-GGGTGACAGTGGACTAGC-3') and PGK (5'-CTAAAGGCCATGCTCGACT-3') for genotyping the CIC-2 locus; primers IMR0260/0270/0271 (5'-TTCAGCCAAGAGCTCGGA-3', 5'-CTCCCTCTCTTCTGCAACCC-3', and 5'-CTGTTATGGAAGCTTCAGAGG-3') for Cftr\(^{+/-}\) and; and primers IMR-1125/1126/1127 (5'-GAAAGCTGGAAGCTCGACGG-3', 5'-TCCAT-CTTGGTCAATGGG-3', and 5'-TCTAGTGATGGTGGTGGAGG-3'), respectively) for Cftr\(^{+/-}\) (as described by Jackson Laboratories). Mice were fed a normal mouse diet (sniff, Soest, Germany), but water was replaced by an osmotic laxative (26) containing 25 mM NaCl, 40 mM Na\(_2\)SO\(_4\), 20 mM NaHCO\(_3\), 18 mM polyethylene glycol 3000, and 10 mM KCl. Mice were housed under virtually standard pathogen-free (SPF) conditions. For some experiments, mice in which only CIC-2 was disrupted by a color CCD camera.

**Real Time PCR**—Mice were genotyped by sex and an osmotic laxative (26) containing 25 mM NaCl, 40 mM Na\(_2\)SO\(_4\), 20 mM NaHCO\(_3\), 18 mM polyethylene glycol 3000, and 10 mM KCl. Mice were housed under virtually standard pathogen-free (SPF) conditions. For some experiments, mice in which only CIC-2 was disrupted by a color CCD camera.

**Results**

To investigate whether the disruption of both CFTR and CIC-2 leads to a more severe phenotype than a disruption of CFTR alone, we crossed CIC-2 knock-out mice (24) with two

**Table I**

| A. Cftr\(^{+/-}\)/Clnce\(^{-/-}\) | B. Cftr\(^{+/-}\)/CIC-2\(^{-/-}\) |
|-------------------------------|-------------------------------|
| **CFTR** | **CIC-2** | **Obtained no.** | **Expected no.** | **Expected fraction** |
| +/+ | +/+ | 11 | 14 | 1/16 |
| +/+ | -/- | 12 | 14 | 1/16 |
| +/+ | +/- | 26 | 29 | 1/8 |
| +/- | +/- | 29 | 29 | 1/8 |
| +/- | +/+ | 79 | 58 | 1/4 |
| -/- | +/- | 34 | 29 | 1/8 |
| -/- | -/- | 29 | 29 | 1/8 |
| -/+ | -/+ | 9 | 14 | 1/16 |
| -/+ | -/+ | 7 | 14 | 1/16 |

**CFTR** CIC-2

| **CFTR** | **CIC-2** | **Obtained no.** | **Expected no.** | **Expected fraction** |
| +/+ | +/+ | 7 | 9 | 1/16 |
| +/+ | -/- | 14 | 9 | 1/16 |
| +/+ | +/- | 17 | 18 | 1/8 |
| +/+ | +/- | 12 | 18 | 1/8 |
| +/+ | +/+ | 43 | 35 | 1/4 |
| +/+ | -/+ | 19 | 18 | 1/8 |
| +/+/+ | +/+ | 11 | 18 | 1/8 |
| +/+/+ | +/- | 5 | 9 | 1/16 |
| +/+/+ | +/- | 14 | 9 | 1/16 |
different CFTR mouse models, Cfr<sup>ΔAF</sup> (6) and Cfr<sup>−/−</sup> (5). Both mice displayed a severe intestinal phenotype that led to occasional intestinal occlusion independent of the CIC-2 genotype. Moribund mice showed decreased food intake, signs of abdominal distress, and weight loss. When an autopsy was performed on such animals, it invariably revealed intestinal obstruction (n = 9)

The lethality of double mutant offspring from Clcn2<sup>−/−</sup>/Cfr<sup>ΔAF</sup> or Clcn2<sup>−/−</sup>/Cfr<sup>−/−</sup> double heterozygous matings was highest in the first week after birth, but some of these mice survived for >6 months (5 of 13 and 3 of 7 animals, respectively). The genotypes of offspring from these matings were determined in mice that had survived until tail biopsies were taken at day 3 ± 1 after birth and conformed roughly to Mendelian inheritance (Table I, A and B). Surprisingly, Cfr<sup>ΔAF</sup>sw/Clcn2<sup>−/−</sup> mice survived better than Cfr<sup>ΔAF</sup> mice (Fig. 1). This difference, significant at a level of p < 0.05, was consistent with an apparent deviation from Mendelian inheritance of the number of mice that survived 3 ± 1 days after birth and resulted in a larger than expected number of Cfr<sup>ΔAF</sup>/Clcn2<sup>−/−</sup> animals (Table I, A and B). Unfortunately, attempts to perform a similar analysis with the Cfr<sup>−/−</sup> line did not yield meaningful results because of poor breeding and high perinatal mortality. Weight development of Cfr<sup>ΔAF</sup>/Clcn2<sup>−/−</sup> double mutant mice was indistinguishable from that of

**Fig. 1. Survival of Cfr<sup>ΔAF</sup> and Clcn2<sup>−/−</sup>/Cfr<sup>ΔAF</sup> mice (A) and weight development (B).** A. A group of 23 Cfr<sup>ΔAF</sup> mice (solid line) was compared with a group of 22 Clcn2<sup>−/−</sup>/Cfr<sup>ΔAF</sup> double mutant mice (dashed line). The number of surviving mice was normalized to those living at day 4, when genotyping had been completed. Lethality was highest during the first week after birth. The Kolmogorov-Smirnov test revealed a significantly better survival rate for double mutant mice over Cfr<sup>ΔAF</sup> mice on a p < 0.05 basis. B, weight development of WT, Cfr<sup>−/−</sup>, Clcn2<sup>−/−</sup>/Cfr<sup>ΔAF</sup> and Clcn2<sup>−/−</sup>/Cfr<sup>−/−</sup> mice. Weights were fitted with a linear function that described the data sufficiently well up to 25 days. Weight development was nearly identical in WT and Clcn2<sup>−/−</sup> mice and in Cfr<sup>ΔAF</sup> and Clcn2<sup>−/−</sup>/Cfr<sup>ΔAF</sup> mice, respectively. n > 5 for each group.

Cfr<sup>ΔAF</sup> mice and lagged behind the weight development of WT and Clcn2<sup>−/−</sup> mice (Fig. 1B).

CFTR mouse models suffer from intestinal occlusion (26), but, in contrast to human patients, the mice rarely develop lung or pancreatic phenotypes. We therefore investigated whether the additional disruption of the CIC-2 Cl<sup>−</sup> channel led to morphological changes in these and other tissues. Comparative histological analysis was performed at 1, 2, 3, 8, and 12 months of age.

Lung sections of 2, 8, and 12-month-old mice were stained with hematoxylin and eosin (staining nucleic acids and proteins, respectively) or with azan, which stains fibrotic tissue in blue. The scale bars in Aa represent 20 μm for Aa–Ac, the bar in Ba represents 20 μm for Ba–Bc, the bar in Ca represents 80 μm for Ca–Cc and 12.5 μm for Da–Ec, and the bar in Ea represents 20 μm for Ea–Ec. Neither fibrosis nor inflammation was observed.

Azan is a mixture of azocarmin, orange G, and aniline blue, the latter of which stains fibrotic tissue, in particular collagen, with an intensive blue. The scale bar in Aa represents 100 μm for Aa–Ac, bar in Ba represents 20 μm for Ba–Bc, bar in Ca represents 80 μm for Ca–Cc and 12.5 μm for Da–Ec, and the bar in Ea represents 20 μm for Ea–Ec. Neither fibrosis nor inflammation was observed.

**Fig. 2. Histological analysis of lung tissue from different genotypes.** Paraffin sections from WT (left column; Aa–Ac), Cfr<sup>ΔAF</sup> (center column; Ab and Bb), Clcn2<sup>−/−</sup>/Cfr<sup>ΔAF</sup> (right column; Ac and Bc), Cfr<sup>−/−</sup> (center column; Dd, Db, and Ee), and Clcn2<sup>−/−</sup>/Cfr<sup>−/−</sup> mice (right column; Ec, Dc, and Ea) are shown. Aa–Ac and Da–Ec are from age-matched, 1-year-old mice, Ca–Cc and Da–Ec are from 8-month-old mice, and all were stained with hematoxylin and eosin. The bronchus walls are shown in higher magnification in Ba–Bc and Da–Ec. Ea–Ec are sections from 2-month-old litter mates and were stained with azan. Azan is a mixture of azocarmin, orange G, and aniline blue, the latter of which stains fibrotic tissue, in particular collagen, with an intensive blue. The scale bar in Aa represents 20 μm for Aa–Ac, the bar in Ba represents 20 μm for Ba–Bc, the bar in Ca represents 80 μm for Ca–Cc and 12.5 μm for Da–Ec, and the bar in Ea represents 20 μm for Ea–Ec. Neither fibrosis nor inflammation was observed.

CFTR/CIC-2 Double Knock-out Mice
Fig. 3. Pancreas and colon morphology of WT, Cftr\(^{-/-}\), and Cftr\(^{-/-}\)/Cln2\(^{-/-}\) mice. A–C, pancreatic sections from 8-month-old WT (A), Cftr\(^{-/-}\) (B), and Cftr\(^{-/-}\)/Cln2\(^{-/-}\) (C) mice. Note the absence of fibrotic or cystic changes, or inflammation that are seen in human CF. Specifically, the pancreas lacked the signs of fibrosis, cystic and other pathological changes. Histological analysis of the pancreas (Fig. 3, A–C) from WT (Fig. 3A), Cftr\(^{-/-}\) (Fig. 3B), and Cln2\(^{-/-}\)/Cftr\(^{-/-}\) animals (Fig. 3C) did not reveal differences up to an age of 8 months either. Specifically, the pancreas lacked the signs of fibrosis, cystic changes, or inflammation that are seen in human CF. Likewise, the colon of Cln2\(^{-/-}\)/Cftr\(^{-/-}\) mice (Fig. 3, D and E) was unremarkable up to 8 months, when mice free of abdominal distress were investigated. The epithelium was normal, without inflammation or mucoid retention in goblet cells or at the base of the crypts. Similar results were obtained in the small intestine. The liver, including the bile duct epithelia, lacked pathological changes.

However, an impairment of physiological function is not necessarily paralleled by morphological alterations. We therefore performed Ussing chamber experiments to assess whether the additional disruption of CIC-2 exacerbated the defects in trans-epithelial chloride transport that are caused by CFTR mutations (Fig. 4). Ion transport across colonic epithelia has three main components, i.e. aldosterone-stimulated Na\(^{+}\) reabsorption, cAMP-stimulated Cl\(^{-}\) secretion, and K\(^{+}\) secretion. These processes involve luminal ENaC Na\(^{+}\) channels, luminal CFTR Cl\(^{-}\) channels, and luminal K\(^{+}\) channels not yet identified at the molecular level, respectively. Na\(^{+}\) reabsorption and Cl\(^{-}\) secretion result in positive I\(_{sc}\) components, whereas K\(^{+}\) secretion is manifested by a negative I\(_{sc}\) component. These different components can be dissected pharmacologically by using stimulatory and inhibitory drugs. To increase the sensitivity of detection for Cl\(^{-}\) currents, ENaC was blocked by adding 10 \(\mu\)M amiloride to the apical perfusate. In experiments on WT colon (Fig. 4A), the subsequent basolateral addition of 10 \(\mu\)M forskolin, an activator of adenylyl cyclase, drastically increased I\(_{sc}\). This positive current component is likely to represent cAMP-activated Cl\(^{-}\) secretion into the lumen via CFTR. This was indirectly confirmed by the inhibitory effect of the subsequent basolateral addition of 100 \(\mu\)M bumetanide, an inhibitor of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport. The latter is responsible for the cytoplasmic accumulation of Cl\(^{-}\) as a prerequisite for a chloride secretory response (see Fig. 6 for transport model). In the experiments of Fig. 4, B and C, which show typical recordings for the Cftr\(^{-/-}\)/Cftr\(^{-/-}\) and the Cln2\(^{-/-}\)/Cftr\(^{-/-}\) colon, respectively, the positive forskolin-induced I\(_{sc}\) component was missing, which is consistent with the absence of an apical cAMP-stimulated CFTR chloride channel. In the absence of functional CFTR, forskolin induced a negative I\(_{sc}\) component. There was no statistically significant difference in forskolin-stimulated currents between the wild-type and the Cln2\(^{-/-}\)/Cftr\(^{-/-}\) colon irrespective of the CFTR genotype (WT or Cftr\(^{-/-}\)/Cftr\(^{-/-}\)) (Fig. 4D).

The negative current component observed under amiloride and forskolin in CFTR mutant mice cannot be due to a stimulation of Cl\(^{-}\) secretion or a stimulation of cation reabsorption. Experiments using 5 mM Ba\(^{2+}\), an inhibitor of certain classes of K\(^{+}\) channels, revealed that this negative I\(_{sc}\) component can be explained by K\(^{+}\) secretion. Ba\(^{2+}\) caused a significant inhibition only from the luminal but not from the basolateral side, which is consistent with a specific effect on luminal K\(^{+}\) channels (Fig. 4B).

Thus, genetic ablation of CFTR function essentially abolished cAMP-dependent K\(^{+}\) secretion and unmasked a cAMP-stimulated K\(^{+}\) secretion. Both the basolateral KCNQ1/KCNE3 K\(^{+}\) channels (28) and the NKCC1 cotransporter (29) are activated by cAMP. This may explain the prominent secretion of K\(^{+}\) during stimulation with cAMP, although we cannot rule out a direct effect on apical K\(^{+}\) channels. In the Ussing chamber experiments described so far, colonic epithelia from Cftr\(^{-/-}\)/Cftr\(^{-/-}\) (Fig. 4C) behaved similarly to tissues in which only CFTR has been mutated (Fig. 4B), as was evaluated statistically in Fig. 4D.

Although there was no obvious influence of CIC-2 on the short circuit current under stimulation in the experiments described so far, we performed additional experiments under more physiological conditions to further investigate this issue. A bicarbonate-buffered bath solution and lower concentrations of forskolin (1 \(\mu\)M instead of 10 \(\mu\)M) were used. These conditions may lead to less than maximal CFTR activation, thereby facilitating the detection of the relative contribution of CIC-2 to anion secretion. We also used mice that were kept on low sodium diet, a procedure known to up-regulate apical ENaC, thus allowing us to test the viability of the tissue. In Ussing chamber experiments, currents across Cln2\(^{-/-}\)/Cftr\(^{-/-}\) control and
Cln2<sup>−/−</sup> colonic tissue were compared (Fig. 5, A and B, respectively). The inhibitory effect of apical amiloride revealed that almost all resting \( I'_w \) was due to Na<sup>+</sup> reabsorption through ENaC. In the continued presence of amiloride, the application of 1 \( \mu \)M forskolin to the apical bath elicited a positive \( I'_w \) component. This result was due to anion secretion, as revealed by the inhibition of this current component with the luminal addition of the chloride channel inhibitor DPC (1 mM) at the end of the experiment. Application of luminal Ba<sup>2+</sup> resulted in a small additional increase in \( I'_w \) in both control and Cln2<sup>−/−</sup> mice, revealing, as in Fig. 4, B and C, a K<sup>-</sup>-secretory component. This component was minor in comparison with the large anion secretory \( I'_w \) component. A statistical evaluation of the \( I'_w \) values is given in Fig. 5C. There was no significant influence of the Cln2 genotype on the Na<sup>+</sup> current (Fig. 5C, first two columns on the left). However, forskolin-induced anion secretory currents were roughly 40% larger in Cln2<sup>−/−</sup> mice than in Cln2<sup>+/−</sup> mice (p < 0.001) (Fig 5C). This result is surprising, as it is incompatible with the postulated secretory role of apical CIC-2 channels in colonic epithelia. One might argue, however, that CFTR may be up-regulated in response to the absence of CIC-2. To address this issue, CFTR mRNA abundance in the WT and Cln2<sup>−/−</sup> colons of three litters pairs was compared by real-time PCR. The K<sup>-</sup> channel, KCNQ1, and the Na<sup>+</sup>/K<sup>-</sup>/2Cl<sup>-</sup> co-transporter, NKCC1, both of which are expressed in colonic epithelia (28), were used as controls. As shown in Table II, there was no significant difference in transcript levels. This, however, does not strictly exclude a difference in the number of active CFTR channels.

Because both CFTR and CIC-2 are also expressed in the heart (15), electrocardiogram recordings were performed on anesthetized Cfr<sup>−/−</sup>, Cln2<sup>−/−</sup>, and Cln2<sup>−/−</sup>/Cfr<sup>−/−</sup> mice. No arrhythmia or other abnormalities were detected (\( n = 2 \) for each genotype; data not shown).

**DISCUSSION**

Several regulatory functions have been proposed for CFTR, the gene product affected in cystic fibrosis (31). However, its main role is in salt and fluid transport, where it serves as a CAMP-activated Cl<sup>-</sup> channel in the apical membrane of several epithelia. In the colon and in pancreatic ducts, CFTR mediates Cl<sup>-</sup> secretion, but the direction of Cl<sup>-</sup> transport in lung epithelia is controversial (32). These three tissues are the main sites of pathological changes in cystic fibrosis. In addition, CFTR is also expressed in several other tissues such as the kidney and the heart, where its function is less clear.

The chloride channels that are expressed in the same epithelial membranes as CFTR are interesting in regard to cystic fibrosis for two reasons. First, the genetic variability in their expression levels or functional properties may influence the severity of cystic fibrosis, as these channels will co-determine the extent of remaining Cl<sup>-</sup> transport in the absence of functional CFTR. Thus, genes encoding such chloride channels may be modifier genes for cystic fibrosis (7, 8). A differential expression in mice and men might explain the fact that CFTR knockout mice do not exhibit the full CF phenotype observed in humans. Second, such channels are potentially interesting targets for pharmacological intervention. Specific openers of these channels might be useful in treating CF.

CIC-2 is almost ubiquitously expressed (15) and is therefore present in the airway, colonic, and pancreatic epithelia, whose function is compromised in cystic fibrosis. Furthermore, several studies suggested that CIC-2 may be present in apical membranes of lung (18) and intestinal (19, 33) epithelia, although others obtained conflicting results (34, 35). Finally, the testicular and retinal degeneration observed in Cln2<sup>−/−</sup> mice was suggested to result from a defect in transepithelial transport across the Sertoli cell and retinal pigment epithelium, respectively (24).

Thus, CIC-2 appeared to be an excellent candidate for a chloride channel that operates in parallel to CFTR and may modulate the CF phenotype. To finally clarify this issue, which has been raised in several reports (21–23), we mated Cln2<sup>−/−</sup> mice (24) with CFTR mouse models. We used a line, Cfr<sup>−/−</sup> (5), which results in a complete loss of CFTR function, as well as
and Clcn2 CFTR inhibitor DPC. In changed short circuit current, which was strongly inhibited by the Clcn2 in the presence of functional CFTR, luminal Ba$^+$ p the otherwise functional CFTR Cl mutations lead to a significant positive $I_{sc}$ absorption from the lumen that could be blocked by the ENaC inhibitor amiloride (10 $\mu$m). In addition, a lower concentration of forskolin (1 $\mu$m) was used. In the presence of functional CFTR, luminal Ba$^+$ only marginally changed short circuit current, which was strongly inhibited by the CFTR inhibitor DPC. In C, the data from Clcn2$^{-/-}$ (n = 30) (black bars) and Clcn2$^{-/-}$ (n = 33) (white bars) were compared. The difference between $I_{sc}$ in the presence of amiloride and forskolin was significant at the $p = 0.001$ level.

$\text{CFTR/CIC-2 Double Knock-out Mice}$

![Comparison of transepithelial current in distal colon from control, Clcn2$^{+/+}$ (A), and Clcn2$^{-/-}$ mice (B). In contrast to the experiments in Fig. 4, mice had been kept on low sodium diet. This led to a significant positive $I_{sc}$ component due to Na$^+$ absorption from the lumen that could be blocked by the ENaC inhibitor amiloride (10 $\mu$m). Additionally, a lower concentration of forskolin (1 $\mu$m) was used. In the presence of functional CFTR, luminal Ba$^+$ only marginally changed short circuit current, which was strongly inhibited by the CFTR inhibitor DPC. In C, the data from Clcn2$^{-/-}$ (n = 30) (black bars) and Clcn2$^{-/-}$ (n = 33) (white bars) were compared. The difference between $I_{sc}$ in the presence of amiloride and forskolin was significant at the $p = 0.001$ level.](image)

$\text{Cftr}^{\Delta F508F}$ mice (6), which carry an in-frame deletion of phenylalanine 508. The latter mice survived longer and therefore allowed us to assess the effect of an additional disruption of Clcn2 in older animals. The deletion of Phe-508 ($\Delta F508$), which is the most common CFTR mutation in Caucasian CF patients, results in a temperature-sensitive trafficking defect in an otherwise functional CFTR Cl$^-$ channel to the cell surface (36).

Contrary to what might have been expected, the additional disruption of the CIC-2 channel was neither lethal nor did it result in a higher mortality. In our study, $\text{Cftr}^{\Delta F508F/\text{Clcn2}^{-/-}}$ mice survived better than $\text{Cftr}^{\Delta F508F}$ mice. Morphological changes could not be detected in double mutant mouse tissues (lung, pancreas, and colon) that are affected in human CF patients, even when long-time surviving animals were investigated.

The inflammatory infiltrates and goblet cell hyperplasia reported to be present occasionally in CFTR mouse colon (26) were not detected in our study. These phenotypes may not have developed because our mice were kept on an oral laxative in a good hygienic and nutritional state. As CIC-2 is expressed in the lung earlier than CFTR, it was postulated to mediate the chloride fluxes deemed to be important for lung development (37). We have shown previously that CIC-2 is not necessary for lung development (24). This work shows that, even in a CFTR-deficient background, lung morphology is normal. A recent paper has again raised this issue using CIC-2 antisense oligonucleotides (38). However, oligonucleotides with identical sequences were used in a study on choroid plexus chloride channels (39), and they later turned out to down-regulate a chloride channel not related to CIC-2 (40). The lack of an overt pulmonary CF-like phenotype in $\text{Cftr}^{-/-}$ mice may also be due to differences in mouse and human lung morphology (41).

Ussing chamber experiments on colon epithelia confirmed the well known impairment of cAMP-stimulated anion current in CF for two CFTR mouse models. The finding that the additional disruption of CIC-2 did not lead to less colonic anion secretion does not support the hypothesis that CIC-2 provides a significant anion current in parallel to that mediated by CFTR. Hence, CIC-2 is unlikely to mitigate the pathophysiological effects in cystic fibrosis. However, these results do not strictly rule out the possibility that CIC-2 may be expressed in the apical membranes that also express CFTR, although it does not mediate significant currents under our experimental conditions.

A different subcellular localization is suggested by the better survival of Clcn2$^{-/-}$/$\text{Cftr}^{\Delta F508F}$ as compared with $\text{Cftr}^{\Delta F508F}$ mice and the second set of Ussing chamber experiments on colonic tissues. When comparing currents from Clcn2$^{-/-}$ and Clcn2$^{-/-}$ (control) mice during moderate apical stimulation with 1 $\mu$m forskolin in the presence of physiological bicarbonate concentrations, cAMP-activated currents were actually increased in mice lacking CIC-2. A less than maximal stimulation of apical CFTR may render the detection of the contribution of Cl$^-$ currents mediated by CIC-2 more sensitive, and the presence of bicarbonate, which can permeate through CFTR (42), may modulate several conductances via changes in pH$_i$. We do not know which of these factors accounts for the fact that we detected a difference in anion secretion between WT and Clcn2$^{-/-}$ mice under these conditions but not under the conditions of the experiments shown in Fig. 4. The increase in anion secretion observed in the CIC-2 knock-out could not be accounted for by an up-regulation of CFTR message. It might instead be explained by a basolateral rather than an apical localization of CIC-2, as depicted in the model of Fig. 6. Any additional anion conductance in the same (apical) membrane as CFTR should increase transepithelial anion secretion, whereas a basolateral chloride efflux would lower the intracellular anion concentration and decrease apical anion exit. Basolateral Cl$^-$ channels would also depolarize the basolateral voltage toward $E_{Cl}$, thereby decreasing $V_{ls}$, and, hence, the short circuit current. With such a localization, CIC-2 disruption should result in an enhanced anion secretion.

In fact, the subcellular localization of CIC-2 in colonic epi-

| Wild type colon | Clcn2$^{-/-}$ colon |
|-----------------|-------------------|
| CFTR            | 30.83 ± 0.29      |
| KCNQ1           | 36.06 ± 0.39      |
| NKCC1           | 30.42 ± 0.17      |
thelia is controversial. On the one hand, antibodies against ClC-2 detected signals in apical membranes of intestinal membranes in a study by Zeitlin and co-workers (33), whereas Bear and co-workers (19), using a different ClC-2 antisera, found immunoreactivity predominantly at the apical junctional complexes of murine intestinal epithelia. By contrast, a third antisera by Lipecka, Fritsch and co-workers (34) stained the basolateral Na\(^+\)-K\(^+\) cotransporter NKCC1 that remains in the apical membranes of tissues. Experiments using these results may reflect nonspecific binding. Our own anti-guinea pig colonocytes. None of these studies included controls. Such localization would predict that inhibition rather than activation of ClC-2 should mitigate the phenotype resulting from CFTR mutations that do not abolish its currents completely.

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Fig. 6. Model for transepithelial transport in the colon. The Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporter NKCC1 to accumulate intracellular Cl\(^-\) above its electrochemical equilibrium. This requires a basolateral K\(^+\) conductance for K\(^+\) recycling that is predominantly mediated by the KCNQ1/KCNE3 K\(^+\) channels that hyperpolarize the basolateral membrane. A minor fraction of K\(^+\) leaves the cell through apical Ba\(^2+\)-inhibitable K\(^+\) channels. Cl\(^-\) leaves the cell apically through DPC-inhibitable CFTR Cl\(^-\) channels. Apical CFTR Cl\(^-\) channels, basolateral KCNQ1/KCNE1 K\(^+\) channels, and NKCC1 co-transporters are all stimulated by cAMP (not shown). The apical membrane also contains the Na\(^+\) channel ENaC, which is up-regulated by aldosterone and mediates Na\(^+\) reabsorption.
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