DHHC7-mediated palmitoylation of the accessory protein barttin critically regulates the functions of ClC-K chloride channels

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ABSTRACT

Barttin is the accessory subunit of the human ClC-K chloride channels, which are expressed in both the kidney and inner ear. Barttin promotes trafficking of the complex it forms with ClC-K to the plasma membrane and is involved in activating this channel. Barttin undergoes post-translational palmitoylation that is essential for its functions, but the enzyme(s) catalyzing this posttranslational modification is unknown. Here, we identified zinc finger DHHC-type containing 7 (DHHC7) protein as an important barttin palmitoyl-acyltransferase, whose depletion affected barttin palmitoylation and ClC-K–barttin channel activation. We investigated the functional role of barttin palmitoylation in vivo in Zdhhc7−/− mice. Although palmitoylation of barttin in kidneys of Zdhhc7−/− animals was significantly decreased, it did not pathologically alter kidney structure and functions under physiological conditions. However, when Zdhhc7−/− mice were fed a low-salt diet, they developed hyponatremia and mild metabolic alkalosis, symptoms characteristic of human Bartter syndrome (BS).
type IV. Of note, we also observed decreased palmitoylation of the disease-causing R8L barttin variant associated with human BS type IV. Our results indicate that dysregulated DHHC7-mediated barttin palmitoylation appears to play an important role in chloride channel dysfunction in certain BS variants, suggesting that targeting DHHC7 activity may offer a potential therapeutic strategy for reducing hypertension.

INTRODUCTION

Bartter syndrome (BS) is a group of hereditary autosomal recessive disorders characterized by impaired ion transport across renal epithelia in the ascending limb of Henle’s loop. This leads to renal salt-losing nephropathies associated with hypokalemic metabolic alkalosis. BS can generally be divided into five subfamilies. Type I is associated with mutations in the NKCC2 transporter, Type II with mutations in the potassium ROMK channel, and Type III with mutations in the chloride CIC-Kb channel. BS Type V is characterized by mutations within the calcium-sensing receptor (CASR) (1), whereas mutations within the barttin protein lead to BS Type IV, a human syndrome that combines sensorineural deafness and a deficit in urinary concentration (2), (3).

Barttin is the accessory subunit of the human CIC-Ka and CIC-Kb chloride channels expressed in both the kidney and the inner ear. Barttin promotes trafficking of the CIC-K/barttin complex to the plasma membrane, increases channel stability, and switches CIC-K/barttin channels into an active state (4), (5). Chloride permeability in the thin and thick ascending loops of Henle is a crucial component in establishing the cortico-medullary osmotic gradient that is a central component of urine concentration (6), (7). Thus, functional regulation of CIC-K channels via barttin is critically involved in adjusting water excretion and intake.

We recently showed that barttin is palmitoylated both in vivo and in vitro, and identified cysteine residues Cys54 and Cys56 as the palmitoylation sites (8). Expression of palmitoylation-deficient barttin mutants reduced the macroscopic current amplitudes of CIC-K/barttin channels without affecting CIC-K/barttin expression and plasma membrane insertion, demonstrating that barttin palmitoylation is necessary for the activation of plasma membrane-inserted CIC-K/barttin channels (8).

Palmitoylation is the post-translational attachment of palmitate to cysteine residue(s) within the protein via a labile thioester linkage. As palmitoylation can be dynamically regulated, it is now widely accepted that repeated cycles of palmitoylation and depalmitoylation could have important consequences for protein functions (9), (10). Palmitoylation is catalyzed by a family of enzymes containing a DHHC (Asp-His-His-Cys) cysteine-rich domain, which is directly involved in the palmitoyl transfer reaction. A multitude of DHHC proteins exists in eukaryotic cells, including 8 in yeast (11), (12) and 23 in humans (13). Various studies, including our own, indicate that DHHC proteins possess distinct but overlapping substrate specificities (14), (15), (16). As palmitoylation controls a variety of important cellular processes, it is not surprising that several DHHC proteins are implicated in human disease (17), (18), (19), (20), (21). The importance of palmitoylation has also been demonstrated for multiple renal proteins, including ADP-ribosylation factor-like GTPase 13b (ARL13b) (22), DHHC2-mediated palmitoylation of the γ-subunit of epithelial sodium channels (ENaCs) (23), and the type II Na/phosphate co-transporter (NaPi-II) (24).

In the present study, we identified DHHC7 as the cognate palmitoylating enzyme for barttin and demonstrated the importance of DHHC7-mediated barttin palmitoylation for the activation of CIC-K channels. To investigate the functional role of barttin palmitoylation in vivo, we established the Zdhhc7−/− mouse line. Palmitoylation of barttin in the kidneys of Zdhhc7−/− mice was
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RESULTS

DHHC7 is a palmitoyl acyltransferase for barttin - To identify the enzyme(s) palmitoylating barttin, we co-expressed barttin-CFP with each of the 23 HA-tagged mouse DHHC palmitoyl acyltransferases (PATs) in HEK293T cells, followed by metabolic labelling with [9,10(n)H]-palmitic acid. Substantially increased barttin palmitoylation was observed after co-expression of DHHC3, DHHC6, DHHC7, and DHHC20, whereas barttin expression was comparable to the amount of barttin in cells with endogenously expressed DHHCs (Fig. S1). The role of these DHHCs was further evaluated by the ABE approach, with DHHC3 and DHHC7 as the only PATs, increasing barttin palmitoylation significantly (Fig. 1A). Notably, both candidate DHHCs belong to the same subfamily of DHHC proteins, with relatively high sequence homology. The sequence homology between human DHHC3 and DHHC7 is 52%, and even higher between mouse and rat (25). Moreover, each of these DHHCs is highly conserved between different mammalian species, with approximately 80% homology between mouse, rat, and human isoforms. These two candidate DHHCs are distributed within Golgi compartments. It is notable that DHHC7 is expressed in all parts of Golgi apparatus (i.e. trans-, medial- and cis-Golgi), while DHHC3 only in the cis-Golgi compartment (26). More importantly, the relative expression of the DHHC7 in kidney is higher than that for the DHHC3 (12).

To evaluate the role of DHHC7 in barttin palmitoylation, we generated a specific short hairpin RNA (shRNA) directed against a non-coding region of DHHC7 mRNA (Fig. S2A). Expression of this shRNA in HEK293T cells significantly reduced the amount of DHHC7 transcript compared to cells transfected with scrambled shRNA (Fig. 1B). Knockdown of endogenous DHHC7 by the shRNA significantly decreased palmitoylation of barttin as assessed by both ABE assay (Fig. 1C) and radioactive labelling (Fig. S2B). Notably, knockdown of DHHC7 did not affect barttin expression (Fig. 1C, Fig. S2B).

To verify a possible involvement of DHHC3 in barttin palmitoylation, we knocked-down this acyltransferase using specific shRNA. Although expression of this shRNA resulted in significant reduction of the amount of DHHC3 transcript (Fig. 1D), we did not observe significant changes in barttin palmitoylation, even though the tendency to palmitoylation decrease was visible (Fig. 1E). When DHHC3 and DHHC7 were knocked-down simultaneously, we obtained significant decrease of barttin palmitoylation (Fig. 1E), which was higher than in the case of the single DHHC7 knockdown (Fig. 1C).

Based on these results in combination with the results of radioactive labelling, which revealed the highest increase of barttin palmitoylation after co-expression with DHHC7 (Fig. S1), we decided to focus on DHHC7 as the presumably more potent PAT for barttin.

One of the central barttin functions is the proper localization of CIC-K channel proteins in the surface membrane of the nephron epithelium (5), (4). Therefore, we analyzed whether the overexpression or knockdown of DHHC7 can influence the subcellular distribution of barttin and CIC-K in MDCK II cells. YFP-CIC-Ka was expressed in MDCK II cells either alone (Fig. S3A) or together with barttin-CFP (Fig. S3B). In another set of experiments, barttin-CFP and YFP-CIC-Ka were co-expressed with DHHC7-GFP (Fig. S3C) or the shRNA against DHHC7 (Fig. S3D). The results confirmed the well-known barttin function of promoting CIC-Ka insertion into the plasma membrane (8). More importantly, manipulation of barttin palmitoylation via DHHC7 overexpression or knockdown did not result in any visible
changes in the distribution of the CIC-Ka/barttin complex in MDCKII cells (Figs S3C,D).

**Knockdown of DHHC7 reduces activation of hCIC-Ka/barttin channels** - Our previous results demonstrated that palmitoylation of barttin is mandatory for activation of the hCIC-K/barttin channel complex, whereas the voltage dependence of activation and plasma membrane integration remained unchanged (8). Therefore, we hypothesized that modulation of barttin palmitoylation by overexpression or knockdown of DHHC7 influences the whole cell current amplitudes of HEK293T cells expressing hCIC-Ka/barttin.

Though the addition of scrambled shRNA or overexpression of DHHC7 did not substantially affect the current amplitudes, patch clamp experiments revealed a significant decrease in current amplitudes (>80% decrease) when shRNA against DHHC7 was co-expressed together with barttin-CFP and mCherry-hCIC-Ka (Fig. 2A,B,C). As barttin expression levels were not affected by co-expression of DHHCs or shRNA (Fig. 1, Figs S1, S2), the reduced current amplitudes could be the result of decreased single channel conductance, reduced open probabilities or a reduced number of active channels in the cell membrane. Stationary noise analysis was performed to define these parameters based on the variance of whole cell currents. The steady state current variance ($\sigma^2$) was measured for voltages between -5 mV and -245 mV, normalized by the product of the steady-state current amplitude ($I$) and the electrical driving force $V-V_{rev}$ and plotted against the whole cell conductance $I/(V-V_{rev})$ (Fig. 2D). A linear regression line revealed the single pore conductance $gCl$ ($= i/(V-V_{rev})$) as the y-axis intercept (Fig. 2E), and the number of active channels in the cell membrane as negative inverse slope ($N = -1/slope$). Furthermore, these values allow calculation of the absolute open probabilities of active channels (Fig. 2F). The reduced barttin palmitoylation mediated by the knockdown of DHHC7 affected neither the single channel conductance nor the voltage dependence of absolute open probabilities. The maximum and minimum open probabilities and mid-voltage of activation ($V_{mid}$) were comparable for controls, over-expressed DHHC7, and diminished DHHC7 (Figs 2F,G). Since single channel conductance and open probabilities were seemingly not altered, these results suggest that the current reduction obtained after DHHC7 knockdown most likely resulted from a reduced number of active channels ($N$) in the plasma membrane, as also indicated by the steep regression line for shDHHC7 in the representative noise analysis (Fig. 2D, red symbols).

To verify whether the decreased channel activation after DHHC7 knockdown can be mediated by decreased surface expression of CIC-K/barttin complexes, we performed surface biotinylation experiments (Fig. 3). Results of this analysis revealed that the amount of CIC-K channels as well as barttin in the plasma membrane were not affected after selective knockdown of DHHC7, when compared to cells transfected with pcDNA3.1 or scrambled shRNA.

Altogether, the above-mentioned results (i.e., Figs 2, 3 and S3) are in line with our previous observation that non-palmitoylated barttin did not reduce surface membrane insertion of CIC-K/barttin, but selectively impaired activation of the channel complex (8).

**Characterization of the kidney morphology of Zdhhc7−/− mice** - To investigate the impact of DHHC7 on barttin palmitoylation in vivo, we established a Zdhhc7−/− mouse line (27). We previously demonstrated that Zdhhc7−/− mice possess a normal range of sensory and motor activity and reproduction. We did not observe any signs of burden or stress under standard housing conditions. Kidney slices from Zdhhc7−/− and WT mice (P90, males, littermates) were analyzed for possible pathological changes, including interstitial fibrosis, sclerotic changes in glomeruli, hyperplasia of the juxtaglomerular apparatus, and dilated or atrophic tubules. As shown in Fig. S4, no
apparent morphological differences were detected in the renal cortex or outer or inner medulla of Zdhhc7−/− mice.

Next, we compared the distribution of CIC-K/barttin channels within a nephron. For detection of the CIC-K channels, we applied rabbit polyclonal antibody from Alomone Labs, which has long been accepted as a highly specific and fairly unambiguous tool for visualization of the CIC-K channel both in vitro and in vivo (28), (29), (30). For visualization of barttin, we used the goat polyclonal antibody sc-49611 from Santa Cruz. This antibody was characterized in detail in our previous study (8). The specificity of both antibodies was verified using corresponding blocking peptides, which are available from the manufacturers. Under physiological conditions, the CIC-K/barttin complex is highly expressed in the distal convolute tubule (DCT), medullar thick (mTAL) and thin ascending limb (mTL) of the loop of Henle, and in intercalated cells of the collecting duct (CD) (31), (32). Immunofluorescence analysis of kidney slices from adult mice (P90, males, littermates) revealed similar basolateral localization of barttin in DCT, mTAL, and a-intercalated cells of the CD in both, WT and Zdhhc7−/− mice (Fig. S5A,B). In line with previous observations (31), barttin and CIC-K channels were co-localized on both the apical and basolateral sites of the mTL in WT and Zdhhc7−/−mice (Fig. S5A).

**Barttin is a DHHC7 substrate in mouse kidney**

To further verify the role of DHHC7 in barttin palmitoylation in the mouse kidney, we applied a high-throughput PANIMoni proteomics technique that enables identification of S-palmitoylated cysteine residues in complex biological mixtures (33). Using this approach in combination with mass spectrometry protein identification, we confirmed barttin palmitoylation in the mouse kidney of WT mice (Figs 5A,B; Supplemental Table 1). Quantitative analysis of the palmitoylomics data from the kidneys of WT and Zdhhc7−/− mice demonstrated that knocking out DHHC7 led to a significant decrease in barttin palmitoylation to 68 ± 25% (Fig. 5A). This is similar to the results obtained by ABE (Fig. 4) and confirms that barttin is a physiological substrate for DHHC7 in mouse kidney.

In addition, mass spectrometry-based PANIMoni analysis of palmitoylated peptides revealed two differently palmitoylated barttin populations: one containing barttin palmitoylated on both Cys54 and Cys56, and one in which only Cys54 undergoes palmitoylation (Figs 5B,C). Notably, we were not able to detect peptides with a single palmitoylated Cys56, suggesting that this cysteine residue can only be palmitoylated in combination with Cys54. The existence of two different populations of palmitoylated barttin was confirmed by the acyl-PEGyl exchange gel shift approach (Fig. 5D).

**Zdhhc7−/− mice develop a Bartter-like phenotype with a low-salt diet**

- Having demonstrated the importance of barttin palmitoylation for CIC-K channel activation (Fig. 2) and decreased barttin palmitoylation in the kidneys of Zdhhc7−/− mice (Figs 4, 5), we investigated whether Zdhhc7−/− animals develop a Bartter-like phenotype. When fed a normal diet, Zdhhc7−/− mice did not present any apparent phenotype in gross physical appearance, body weight, kidney morphology, or laboratory measurements in urine or blood (Table 1, Figs 6A,B). However, when the mice were fed a low-salt diet for 12 days, they
developed a salt-losing phenotype characterized by hyponatremia and mild metabolic alkalosis (Table 1, Figs 6C,D). Interestingly, on the low-salt diet, the relative decrease in barttin palmitoylation was more pronounced in Zdhhc7−/− mice than the animals fed a normal diet (38% and 24%, respectively; Figs 7A, 4B). Importantly, neither the level of barttin expression nor the global palmitoylation profile was affected by the low-salt diet (Fig. 7). More detailed analysis of barttin palmitoylation revealed that a low-salt diet boosts barttin palmitoylation, which is needed to facilitate the compensatory renal salt reabsorption by switching CIC-K channels into an active state, in the WT mice but not in Zdhhc7−/− mice (Fig. 7B).

Thus, the decreased barttin palmitoylation results in hyponatremia and metabolic alkalosis in Zdhhc7−/− mice fed a low-salt diet.

**Disease-causing R8L mutation impairs barttin palmitoylation** - Similar systemic effects of a low-salt diet were previously reported for R8L barttin knock-in mice (34). The naturally occurring R8L mutation in BSND has been shown to be associated with BS Type IV in humans (2). Though this mutation does not affect the subcellular transport and plasma membrane localization of the CIC-K/barttin complex, the current amplitude of hCIC-Ka channels was significantly decreased after co-expression of barttin R8L mutant compared to WT barttin (3). As co-expression of the palmitoylation-deficient barttin mutant (8) or knockdown of endogenous DHHC7 (Fig. 3) result in similar abrogation of CIC-Ka channel functions, we hypothesized that altered palmitoylation of the R8L barttin mutant may be the reason for its functional defect. Supporting this view, palmitoylation of R8L barttin heterologously expressed in HEK293T cells was significantly reduced to 43.4 ± 14.9% (Fig. 8A,B).

We previously showed that barttin is palmitoylated at Cys54 and Cys56, and palmitoylation is essential for activation of the CIC-K/barttin channel complex (8). In the present study, we identified DHHC3 and DHHC7 as the cognate palmitoylating enzymes and found that reduced DHHC7 expression leads to attenuated barttin palmitoylation status. Because DHHC3 and DHHC7 belong to the same subfamily of PATs with high sequence homology within the CRD (25), it is not surprising that these enzymes may have overlapping substrates. On the other hand, in vivo studies have revealed distinct substrate specificity for these PATs in the mouse brain (35). Knockdown experiments in cultured cells demonstrated that the presence of endogenous DHHC3 is not enough to fully compensate for DHHC7 deficiency. These findings suggest that DHHC7 is an important regulator of barttin palmitoylation.

Our electrophysiological experiments revealed that knocking down endogenous DHHC7 leads to impaired barttin palmitoylation, which significantly reduces macroscopic CIC-K current amplitudes. In combination with the observation that DHHC7 knockdown does not alter the subcellular distribution of the CIC-K/barttin complex, this finding suggests that hCIC-K channels remain inactive in complex with non-palmitoylated barttin, whereas barttin palmitoylation can switch channels to an active state. Decreased barttin palmitoylation mediated by the DHHC7 knockdown appears to promote long-lasting closure of CIC-K channels. When dwell-times for being trapped in such closed states are longer than applied voltage steps in noise analyses, an apparent reduction of counted channels (N) is observed. Active channels, however, have unaltered absolute open probabilities. This notion is in agreement with previous observations of transiently transfected HEK293T cells (8) and native renal epithelia (36) that indicated the presence of large numbers of well-integrated but inactive CIC-K channels in the plasma membrane.
The presence of palmitate groups on barttin might have several functional consequences. Palmitoylation can be involved in the creation of a direct hydrophobic interface between barttin and the plasma membrane, which can adjust the orientation of barttin within the lipid bilayer and facilitate specific interaction and conformational changes within the CIC-K channel itself, particularly within its B- and J-helices (37). A similar mechanism has been proposed for human voltage-dependent K+ channels (38). In addition, through direct interaction with the membrane lipids, the palmitate groups of barttin can change the lipid membrane environment surrounding the CIC-K channel and/or create membrane dipoles, which in turn influence the functional activity of the CIC-K/barttin channels (39).

Intriguingly, though reduction of barttin palmitoylation strongly affects CIC-K channel activity, increased barttin palmitoylation evoked by the overexpression of DHHC7 did not influence the CIC-K/barttin surface expression in MDCKII cells and channel properties. One possible explanation is that, because of overexpression, a supra-optimal amount of palmitoylated barttin with a higher affinity for the CIC-K channel participates in CIC-K/barttin complex formation, leading to functional “saturation”. Conceivable for the heterologous system, this scenario seems not to be relevant for the in vivo conditions. In our previous study, we measured a significant increase in barttin palmitoylation in mouse kidney upon water deprivation (8). Similar effects were observed in the present study in the kidneys of mice subjected to a low-salt diet. In both cases, increased barttin palmitoylation in the CIC-K/barttin channel may modify chloride conductance in the thin and thick ascending limb of the loop of Henle and facilitate urinary concentration and reduce water excretion. Thus, dynamic palmitoylation of barttin in the kidney could represent a mechanism for fine-tuning CIC-K/barttin channel function.

To investigate the physiological importance of barttin palmitoylation in CIC-K/barttin function in vivo, we generated Zdhhc7−/− mice. In these animals, palmitoylation of barttin was significantly decreased throughout their entire life span, further confirming DHHC7 as an important barttin palmitoyl acyltransferase. On the other hand, Zdhhc7−/− mice were indistinguishable from WT mice in terms of general health, motor and sensory activity, reflexes, and reproduction. We also did not find any morphological and histological differences in the renal cortex or outer or inner medulla. Moreover, barttin and CIC-K channel expression, distribution, and colocalization within the mTAL and DCT were not affected. These data suggest that knocking out Zdhhc7 (consequently decreasing barttin palmitoylation) has no critical consequences for normal kidney function under physiological conditions. However, when Zdhhc7−/− animals were fed a low-salt diet, they developed hyponatremia and mild metabolic alkalosis, symptoms characteristic of human BS Type IV with late onset (40). Our experimental data suggest that decreased barttin palmitoylation can be the main reason for a missing compensatory effect of the salt-losing phenotype in Zdhhc7−/− mice: As part of the compensatory facilitation of CIC-K channel activity, we achieved increased barttin palmitoylation in WT mice, and it was completely absent in Zdhhc7−/− mice.

Interestingly, a similar phenotype (i.e., hyponatremia and mild metabolic alkalosis manifested with a low-salt diet) was obtained in R8L knock-in mice (34). The R8L mutation was identified in human BS Type IV (2) and was previously shown to eliminate the function of CIC-K/barttin channels in MDCK II cells (3). In the latter study, authors have shown that R8L modifies intracellular distribution of barttin and CIC-Kb, but does not prevent their surface membrane insertion. Moreover, the authors used concatamers of hClC-1 and hClC-Kb, which can be transported to the plasma membrane without barttin, to explicitly show that the CIC-K subunit could not be switched to an active conformation by the R8L barttin mutant (3). Results obtained from the knock-in mouse model of Nomura and co-workers (34)
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tend in the same direction: Authors show reduced or unclear surface membrane staining of R8L barttin in renal epithelia of the mice. However, in contrast to their Neo/Neo mouse that lacks barttin expression, the R8L knock-in mouse displays no phenotype under normal diet and only a mild phenotype with affected urine or blood data under low-salt diet. These results clearly indicate that CIC-K and R8L barttin must be inserted in the surface membrane of renal epithelia of these mice.

Lack of proper activation of the CIC-K channels has also been described for R8W, another naturally occurring mutation in BSND that is associated with BS Type IV in humans (2). These data suggest that the major pathogenic consequence of R8 mutations is their inability to functionally activate CIC-K. Searching for underlying mechanisms, we found that palmitoylation of both R8L (this study) and R8W (8) mutants is significantly reduced. These combined results suggest that the negative effects of R8L and R8W mutations on barttin functions may be explained, at least in part, by changes in their palmitoylation. Arginine at position 8 is proposed to be localized within the first intracellular barttin domain very close to the cytosolic membrane border (2) (Fig. 8C) and can thus be part of the structural consensus recognized by the acyl-enzyme intermediate of DHHC7 (41).

Taken together, in the present study we identified DHHC7 as a main palmitoyl acyltransferase for barttin and demonstrated an important role of DHHC7-mediated barttin palmitoylation in the renal adaptation to salt-deprivation in mice. Thus, Zdhhc7/- mice may be a suitable model for investigating chronic hyponatremia caused by the renal failure associated with BS Type IV. Moreover, targeting DHHC7 activity may allow the development of novel therapeutic strategies for anti-hypertensive treatment.

EXPERIMENTAL PROCEDURES

Ethical statement - This study was conducted in accordance with the German animal protection law and with the European Directive 2010/63/EU. All experiments were approved by the Local Institutional Animal Care and Research Advisory Committee and permitted by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES; file number: 16/2230).

Cell culturing and transfection - For experiments in vitro, human embryonic kidney cells (HEK293T) and Madin-Darby canine kidney cells (MDCK II) were handled as described previously (8). For electrophysiological experiments, HEK293T cells were first transfected with cDNA encoding DHHC7, shRNA against DHHC7 or scrambled, or with an empty pcDNA3.1(+) vector. The second transfection step with cDNA encoding mCherry-hClC-Ka and barttin-CFP followed after 24 h. Twelve hours after the second transfection, cells expressing barttin-CFP and mCherry-hClC-Ka were analyzed as previously described (8).

Cloning and Constructs - Hemagglutinin-tagged (HA)-DHHCs (DHHC1 to 23) and DHHC7-GFP subcloned into pEF-BOS-HA and pEGFP-C1 plasmids, respectively, were a kind gift from Masaki Fukata. To knockdown the endogenous DHHC7 or DHHC3 in HEK293T cells, shRNA against human DHHC7 (5'-GGATATCAACAGCTCATCTC) binding to the non-coding region or shRNA against human DHHC3 (5'-GTACCTCGTCAGGTGTTACATA) binding to the coding region as well as scrambled shRNA (5'-AACAGTCCGTTTGCAGCTGG) were cloned into pSuper.gfp/neochrome vector (Oligoengine). The shRNA efficacy against DHHC7 or DHHC3 was tested in HEK293T cells by qPCR using primer probe Hs00938102_m1 or Hs00213209_m1 from TaqMan Gene Expression Assay, respectively. For quantification, ΔΔCT method was used (Applied Biosystems). Clones for YFP-hClC-Ka and R8L barttin were described before (8), (3).

Antibodies - For immunodetection, the following primary antibodies were used: anti-GFP (HRP) (LifeSpan BioScience, #LS-
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C50850), anti-GFP (GeneTex, #GTX26556), anti-Biotin (HRP) (SIGMA, #A4541), anti-HA (HRP) (Roche, #12013819001), and anti-Barttin (SantaCruz Biotechnology, #sc-49611). Secondary antibodies donkey-anti-goat IgG-HRP and goat-anti-rabbit IgG-HRP were from SantaCruz Biotechnology and ThermoScientific, respectively. The intensity of protein bands after Western blots was quantified using ImageQuant™ software (GE Healthcare).

**Radioactive labelling with [9,10(n)3H] palmitic acid -** HEK293T cells were transfected with plasmid encoding human barttin-CFP together with individual HA-tagged palmitoyl acyltransferases (DHHC1 to 23). Twenty-four hours after transfection, cells were labelled with 300 μCi/mL [9,10(n)3H] palmitic acid (American Radiolabeled Chemicals, Inc) for 2 h. Expression of barttin-CFP was detected by fluorescence scan of SDS-PAGE gels. Amount of radioactive labelled barttin-CFP was visualized by autoradiography (exposures for one week). Expression of HA-DHHCs was visualized by Western blot using anti-HA (HRP) antibody.

**Acyl-Biotinylation Exchange (ABE) and APEGs assays -** ABE method was used as described previously (8). Palmitoylation level of barttin was calculated by sum of replicates as described previously (42). To address the stoichiometry of barttin palmitoylation, we made use of a recently developed method called acyl-PEGyl exchange gel shift (APEGS) (43). Briefly, HEK293T cells transfected with barttin-CFP were lysed, free thiol groups were blocked using 40 mM N-Ethylmaleimide (NEM). After treatment with 1.3 M hydroxylamine (NH2OH) or 1.3 M Tris (pH 7.0), samples were incubated with 20 mM Poly(ethylene glycol) methyl ether maleimide (5 kDa) or 20 mM NEM. Finally, lysates were subjected to SDS-PAGE and western blotting.

**Electrophysiology -** The whole cell patch clamp recordings of HEK293T cells were performed from transiently transfected HEK293T cells as previously described (8). We performed stationary noise analyses as described before (8) to determine absolute open probabilities, single channel conductance and the number of active channels from the variance ($\sigma^2$) of whole cell currents. Plotting the variance normalized by the product of the steady-state current amplitude ($I$) and the electrical driving force ($V_{\text{rev}}$) versus the whole cell conductance $I/(V_{\text{rev}})$ reveals a linear relation with the single channel conductance ($i(V_{\text{rev}})$) as y axis intercept and the number of active channels as negative inverse slope of the regression line:

$$\frac{\sigma^2}{I \cdot (V - V_{\text{rev}})} = \frac{i}{(V - V_{\text{rev}})} - \frac{1}{N} \cdot \frac{I}{V - V_{\text{rev}}}$$

The absolute channel open probability ($P$) was subsequently determined by $P = I/i \cdot N$.

**Biochemical analysis of CIC-Ka/barttin surface membrane insertion -** Plasma membrane expression of CIC-Ka/barttin complexes in presence of empty pcDNA3.1(+) vector, scrambled shRNA or shRNA against DHHC7 was investigated using HEK293T cells. Twenty-four hours after transfection, cells were exposed to 0.375 mg of biotin (EZ-link sulfo-NHS-SS-biotin, Thermo Fisher Scientific) for 30 min. Biotinylation process was terminated by washing the cells with 10 mM glycine/PBS for 20 min followed by cell lysis with radioimmune precipitation assay buffer (100 mM NaCl, 20 mM HEPES, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM EDTA, 1% SDS, 1% deoxycholate, 1% protease inhibitor mix, pH 7.4). After purification via NeutrAvidin affinity chromatography (High Capacity NeutrAvidin Agarose, Thermo Fisher Scientific) biotinylated proteins were eluted by 2x Laemmli sample buffer. Subsequently, samples of total cell lysate as well as biotinylated protein fraction were electrophoresed on 12% SDS-polyacrylamide gels and scanned for fluorescent bands of mCherry-CIC-Ka, barttin-CFP and GFP(+ shRNA scrambled/DHHC7). In order to reassure exclusive biotinylation of surface membrane proteins Western blots were performed with antibodies detecting GAPDH
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(palmitoylated by DHHC7) (primary antibody, GAPDH (A-3), sc-137179 (Santa Cruz Biotechnology, Heidelberg, Germany); secondary antibody, rabbit anti-mouse IgG Fc, horseradish peroxidase conjugate (Thermo Fisher Scientific)).

Palmitoylomics - To identify specific sites of palmitoylated proteins in mouse kidneys, PANIMoni method was used (33). Obtained after ABE biotinylated protein lysates were digested using sequencing-grade modified trypsin (Promega V 5111) for 16 h at 37°C. Digestion was terminated using protease inhibitor cocktail (Complete, Roche). The tryptic peptide mixture was incubated with 100 μl of neutravidin beads (Amersham) at room temperature for 1 h. The neutravidin beads were washed five times in 1ml of wash buffer. Neutravidin-bound peptides were eluted with 150 μl of elution buffer (25 mM NH₄CO₃ [pH 8.2] and 5 mM TCEP (Sigma Aldrich) and concentrated in a SpeedVac. Trifluoroacetic acid was added to the peptide solution to (liquid chromatography coupled to tandem mass spectrometry) using a Nano-Acquity (Waters) LC system and an Orbitrap Velos mass spectrometer (Thermo Electron Corp).

Peptides in 0.1% formic acid/water were loaded from a cooled (10 ºC) autosampler tray to a pre-column (Symmetry C18, 180 μm x 20 mm, 5 μm; Waters) and resolved on a BEH130 column (C18, 75 mm x 250 mm, 1.7 mm; Waters) in a gradient of 5-30% acetonitrile/water for 70 min at a flow rate of 0.3 μl/min. The ultra-performance LC system was directly connected to the ion source of the mass spectrometer. All MS runs were separated by blank runs to reduce the carry-over of peptides from previous samples. The mass spectrometer resolution was set to 50,000 for MS acquisitions, with an m/z measurement range of 300-2000 Th and up to 10 fragmentation events were allowed for each parent ion. Datasets of parent and daughter ions were processed using MascotDistiller 2.6.1 software (MatrixScience). The Mascot search engine (version 2.5.1) was used to survey data against the UniProtKB/Swiss-Prot database (Swissprot 2016_02; 552,259 sequences; 197,423,140 residues). The Mascot search parameters were set to the following: taxonomy (Mus musculus), variable modifications (cysteine carbamidomethylation or N-ethylmaleimide, methionine oxidation, peptide tolerance (5 ppm), fragment mass tolerance (5 ppm). Enzyme specificity was set to trypsin with one missed or non-specific cleavages permitted. All obtained data were merged into one selected peptide list (SPL) using MascotScan software (http://proteom.ibb.waw.pl/mscan/). The SPL consists of sequences of peptides with Mascot scores exceeding the threshold value corresponding to < 5% expectation value and FDR < 1%. The lists of the peptide sequences that were identified in all of the LC-MS/MS runs from Zdhhc7+/+ (control; n = 3) and Zdhhc7−/− (KO; n = 3) mice are presented in Supplemental Table 2. Subsequently, probable contaminants (keratin, albumin) were removed from the analysis. For evaluation of the relative protein abundance in each sample, spectral count values determined using exponentially modified protein abundance index (emPAI) scores were used. Only proteins that met the acceptance criteria: FDR < 1%, at least two unique peptides, Mascot score over 30, non-redundant proteins, were taken for further analysis.

Immunofluorescence analysis and imaging - Mouse kidneys were perfused with 4% paraformaldehyde, immersed in sucrose and stored at -80°C. Kidney cryosections (10 μm slices) were cut on cryotome from ThermoScientific (Microm HM560) and subjected to immunofluorescence analysis. The following primary antibodies were used: anti-Barttin (#sc-49611), anti-AQP2 (H40) (#sc-28629) and anti-V-ATPase B1/2 (F-6) (#sc-55544) from SantaCruz Biotechnology, anti-CLC-K from Alomone Lab (#ACL-004). AlexaFluor secondary antibodies were from Jackson Immunoresearch. Slices were mounted in anti-quenching medium (Fluoromount G, Southern Biotechnology Associates, Biozol, Eching, Germany). Imaging was performed by a confocal laser scanning microscope Zeiss 710.
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(Carl Zeiss, Germany). Subcellular distribution of transiently expressed constructs in living MDCK II cells was analyzed by a confocal laser scanning microscope Zeiss 780 (Carl Zeiss, Germany) using 40x water immersion objective. The cells were imaged using online fingerprint mode enabling for the spectral unmixing.

**Histology and imaging** – CO₂-narcotized animals were sacrificed by intracardiac perfusion with 4% paraformaldehyde in PBS (pH 7.4). After preparation, the kidneys were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections (2-4 μm thick) were deparaffinized in xylene and stained according to standard protocols. For detailed morphological investigations haematoxylin and eosin stain (H&E), Periodic acid-Schiff reaction (PAS) and a silver stain (Jones stain) were performed on serial sections. Morphologic evaluation (Axioskop 40, Zeiss microscope) was performed by a trained pathologist and representative microphotographs were taken (AxioCam MRc, Zeiss).

**Low-salt diet experiment and blood measurements** - For in vivo experiments, three months old male Zdhhc7+/+ (WT) and Zdhhc7-/- (KO) mice (27) were subjected to standard diet (Na 2181.580 mg/kg; Cl 3628.760 mg/kg; K 6961.760 mg/kg) or to low-salt diet (Na 130.590 mg/kg; Cl 113.548 mg/kg; K 7088.293 mg/kg) for 12 days with water ad libitum. Both diets were purchased from Altromin (Germany). Volumes of remaining water in the drinking bottles were noted every day. For blood collection, mice were anesthetized by 4.5% isoflurane and blood was collected from the retro-orbital sinus via a glass capillary coated with lithium EDTA and collected in a likewise coated vial to prevent coagulation. Blood measurements were prepared using blood gas analyzer ABL800FlX (Radiometer, Denmark). After blood collection animals were euthanized by cervical dislocation, and dissected kidneys were shock frozen in liquid nitrogen and stored at -80°C before the ABE analysis.

**Statistical analysis** - For statistical analyses, GraphPad Prism 7.0 was used. Data are represented as means ± S.D. or + S.D. from at least three independent experiments. Significance was calculated by two-tailed unpaired Student’s t-test or One-way ANOVA (Dunnett’s multiple comparisons test; for qPCR Tukey’s multiple comparisons test was used). *, P < 0.05; **, P < 0.01; ***, P < 0.001. Electrophysiological data analyses were performed using a software combination of Clampfit, SigmaPlot and GraphPad Prism. Data are shown as mean values ± S.E.M. or + S.D. Student’s t-test was performed to determine significant changes of mean current amplitudes.

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**DATA AVAILABILITY**
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017231.

All data supporting the findings of this study are available within the article and its supplementary information file or are available from the corresponding author upon reasonable request.

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CONFLICT OF INTEREST
None

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ABBREVIATIONS

ABE acyl-biotinyl exchange
APEGs acyl-PEGyl-exchange gel shift
AQP2 aquaporin 2
ARL13b ADP-ribosylation factor-like GTPase 13b
BS Bartter syndrome
CASR calcium-sensing receptor
CD collecting duct
CFP cyan fluorescent protein
CRD cysteine-rich domain
CSP cysteine-string protein
Cys cysteine
dCT distal convolute tubule
ENaC epithelial sodium channel
HA hemagglutinin
HEK human embryonic kidney
H&E haematoxylin and eosin stain
MDCK Madin-Darby canine kidney
NaPi-II type II Na/phosphate cotransporter
NEM N-Ethylmaleimide
NKCC Na-K-Cl cotransporter
PAS Periodic acid-Schiff reaction
PAT palmitoyl acyltransferase
ROMK renal outer medullary potassium channel
mTAL medullar thick ascending limb
TCEP tris(2-carboxyethyl)phosphine
mTL medullar thin ascending limb
YFP yellow fluorescent protein
Table 1. Weights and blood parameters from Zdhhc7<sup>+/−</sup> (WT) and Zdhhc7<sup>−/−</sup> (KO) mice fed at a normal diet or subjected to a low-salt diet

| Parameters | Standard Diet | Low-Salt Diet |
|------------|---------------|---------------|
|            | WT            | KO            | WT            | KO            |
| Weight, g  | 29.06 ± 0.37 (10) | 29.61 ± 0.79 (9) | 30.36 ± 0.38 (10) | 30.66 ± 0.81 (9)<sup>b</sup> |
| Blood:     |               |               |               |               |
| Na, mmol/l | 146.20 ± 0.80 (5) | 145.80 ± 0.45 (5) | 141.60 ± 0.40 (10) | 137.78 ± 1.39 (9)<sup>a</sup> |
| K, mmol/l  | 3.94 ± 0.081 (5) | 3.96 ± 0.075 (5) | 3.72 ± 0.081 (10) | 3.81 ± 0.072 (9)<sup>b</sup> |
| Cl, mmol/l | 112.00 ± 0.84 (5) | 110.80 ± 0.86 (5) | 104.80 ± 1.44 (10) | 104.44 ± 1.13 (9)<sup>b</sup> |
| pH         | 7.247 ± 0.011 (5) | 7.225 ± 0.019 (5) | 7.323 ± 0.012 (10) | 7.363 ± 0.017 (9)<sup>b</sup> |
| HCO₃⁻, mmol/l | 18.14 ± 0.67 (5) | 17.44 ± 0.48 (5) | 23.88 ± 0.62 (10) | 25.28 ± 0.68 (9)<sup>b</sup> |

Data represent ± S.D. n as indicated in parentheses. <sup>a</sup>P < 0.05. Tukey’s multiple comparisons test. <sup>b</sup>NS.
FIGURE LEGENDS

Figure 1: DHHC7 is an important barttin palmitoyl acyltransferase.

(A) HEK293T cells co-expressing barttin and indicated DHHCs were analyzed by ABE. Representative blots are shown on the left, and quantification of barttin palmitoylation after co-expression of DHHC3, DHHC6, DHHC7 and DHHC20 (n = 3) – on the right (see also Fig. S1). **, P < 0.01. One-way ANOVA; Dunnett’s multiple comparisons test. (B, D) Bar graphs indicate expression level of DHHC7 mRNA (n = 4, (B)) or DHHC3 mRNA (n = 6 (D)) in HEK293T cells after transfection with shRNA against DHHC7 or DHHC3, respectively. RT-qPCR analysis was evaluated by ΔΔCt method. ***, P < 0.001. One-way ANOVA, Tukey's multiple comparisons test. (C, E) ABE analysis of barttin palmitoylation in HEK293T cells after knockdown of endogenous DHHC7 (C) or DHHC3 (E) by shRNA (left panels). Quantification is shown on the right (see also Fig. S2A). Western blots are representative of at least three independent experiments. **, P < 0.01. Student's t-test (for (C)). One-way ANOVA, Dunnett’s multiple comparisons test (for (E)). All data are shown as mean ± S.D.

Figure 2: Knockdown of DHHC7 decreases macroscopic ClC-Ka/barttin currents.

(A) Voltage protocol and representative whole cell current recordings from HEK293T cells expressing hClC-Ka/barttin channels under varying conditions of DHHC7 expression: control = presence of endogenous DHHC7; DHHC7 = overexpression of DHHC7; shRNA = partial knockdown of DHHC7 (shRNA against a scrambled target sequence (scr) served as control). (B) Voltage dependence of hClC-Ka/barttin currents under varying conditions of DHHC7 expression. (C) Comparison of mean current amplitudes at 105 mV. (D-G) Stationary noise analysis of hClC-Ka/barttin currents: (D) Representative plot of the current variance $\sigma^2$, normalized to the product of the mean current amplitude ($I$) and the electrical driving force ($V-V_{rev}$), versus the macroscopic conductance ($I/(V-V_{rev})$). A linear regression provides the single channel conductance ($gCl$) as y-axis intercept and the number of active channels as inverse slope. (E) Mean values of single channel conductance ($gCl$). (F) Voltage dependence of absolute open probabilities of hClC-Ka/barttin channels. (G) Mean values of $V_{mid}$, the voltage of half-maximal activation of hClC-Ka/barttin. (B, F: all data are mean ± S.E.M., C, E, G: all data are mean ± S.D., n as indicated in bar graphs, ***, P ≤ 0.001, Student's t-test).
**Figure 3: Knockdown of endogenous DHHC7 induces no changes in ClC-Ka/barttin distribution.**

(A) Fluorescent scans of SDS-polyacrylamide gels depicting mCherry-ClC-Ka, barttin-CFP and eGFP (used as a cytosolic reporter for the shRNA transfection) in whole-cell lysates (t) and the biotinylated protein fractions of the surface membrane (s) of HEK293T cells that additionally co-expressed either no shRNA (empty pcDNA vector) or scrambled shRNA (scr) or shRNA against DHHC7. Lower panel: Western blot analysis using anti-GAPDH antibodies excludes contamination of the surface membrane fraction by cytosolic proteins. (B) Quantification of the relative expression of mCherry-ClC-Ka and barttin-CFP in the surface membrane. Fluorescence intensities in the surface membrane fractions were normalized by the corresponding intensities of proteins in the whole-cell lysates. There was no statistical difference between experimental conditions as tested by one-way ANOVA. Data are presented as means + S.D. (n=3).

**Figure 4: Palmitoylation of barttin is decreased in the kidneys of Zdhhc7−/− mice (ABE).**

(A, B) Kidney tissues isolated from the newborn (P0) and adult (P90) Zdhhc7+/+ (WT) (n = 3) and Zdhhc7−/− (KO) (n = 3) mice were collected for ABE analysis. Representative Western blots are shown in (A) following by quantification (B). *, P < 0.05; **, P < 0.01. Student’s t-test. Bars show means + S.D. (C) Representative silver staining demonstrating a global protein palmitoylation in mouse kidneys from newborn (P0) and adult (P90) Zdhhc7+/+ and Zdhhc7−/− mice.

**Figure 5: Palmitoylation of barttin is decreased in the kidneys of Zdhhc7−/− mice (palmitoylomics), where two differently palmitoylated barttin populations co-exist.**

(A) Kidney tissues isolated from adult (P90) Zdhhc7+/+ (WT) (n = 3) and Zdhhc7−/− (KO) (n = 3) male mice were subjected to quantitative palmitoylomics approach to evaluate the levels of barttin palmitoylation. Bars show means + S.D.; *, P < 0.05, Student’s t-test. (B) PANIMoni mass spectrometry-based analysis of palmitoylated peptides isolated from the mouse kidney. Red-labelled peaks in the spectrum correspond to matched y-ions and blue-labelled peaks correspond to matched b-ions which differentiate peptides with one and two modified cysteines. The number paired with each ion identification (i.e., b3, y7, etc.) indicates the number of amino acids present on N-terminal fragments for b-ions and C-terminal fragments for y-ions. Modified cysteines in the sequence are marked red and indicated by asterisks. (C) Schematic representation of two palmitoylated barttin forms. Protein sequence is shown with a single letter code. Palmitoylation
Barttin is palmitoylated by DHHC7

sites (C54 and C56) are depicted in red. TMD, transmembrane domain. (D) HEK293T cells transfected with barttin-CFP wild-type were lysed, subjected acyl-PEGyl exchange gel shift (APEGS), separated by SDS-PAGE, and analyzed by Western blot. The number of PEGylation events is indicated by asterisks (*).

Figure 6: Zdhhc7−/− mice develop a salt-losing phenotype under a low-salt diet.
(A) Schematic diagram of experimental design for the low-salt diet experiments. (B) Water intake in WT and Zdhhc7−/− mice during experiment. Water intake was normalized to the body weight (WT: standard diet n = 9, low-salt diet n = 11 and Zdhhc7−/−: standard diet n = 7, low-salt diet n = 10). All data are mean ± S.D. (C) Blood sodium concentration in Zdhhc7+/+ (WT) and Zdhhc7−/− (KO) mice was measured after feeding a normal (standard diet; SD) and low-salt diet (LSD) for 12 days. Data represent means ± S.D. (n as indicated in dots). *, P < 0.05; ****, P < 0.0001. Tukey’s multiple comparisons test. Upon low-salt diet, Zdhhc7−/− animals developed a salt-losing phenotype characterized by hyponatremia (Table 1). (D) Analysis of blood pH in animals subjected to a standard and low-salt diet. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P <0.0001. One-way ANOVA. All data are mean ± S.D.

Figure 7: Palmitoylation of barttin is affected in salt-losing phenotype under a low-salt diet.
(A) Representative image of barttin palmitoylation in kidneys from WT and Zdhhc7−/− mice subjected to a low-salt diet as assessed by ABE followed by SDS-PAGE and Western blot (left). Relative palmitoylation and expression levels of barttin are shown on the right. Data represent means + S.D. (n = 3). *, P < 0.05. Student’s t-test. (B) Direct comparison of barttin palmitoylation and expression in kidney of WT and Zdhhc7−/− mice under standard diet (SD) and low-salt diet (LSD). Representative Western blot images after ABE are shown on the top. Quantification of relative changes in palmitoylation and expression between SD and LSD for WT and Zdhhc7−/− is shown below in bar graphs Data represent means + S.D. (n = 3). *, P < 0.05. Student’s t-test. (C) Representative silver staining demonstrating a global protein palmitoylation in mouse kidneys from Zdhhc7+/+ (WT) and Zdhhc7−/− (KO) mice after standard diet (SD) and low-salt diet (LSD).

Figure 8: Disease-causing barttin mutation R8L affects palmitoylation of barttin.
(A) Representative image from ABE palmitoylation assay followed by SDS-PAGE and Western blot for WT and R8L barttin mutant. (B) Relative palmitoylation levels of WT and R8L barttin
Barttin is palmitoylated by DHHC7

mutant. Data are shown as means + S.D. (n = 4). *, P < 0.05. Student’s t-test. (C) Putative topology of barttin with positions of R8, C54 and C56.
Figure 1
Figure 2
A Relative surface membrane expression of barttin-CFP and mCherry-ClC-Ka.

- **mCherry-ClC-Ka**
- **barttin-CFP**
- **eGFP**
- **GAPDH**

**t**: total cell lysate
**s**: surface membrane expression (biotinylated fraction)

B Figure 3

- Histogram of relative surface membrane expression of barttin-CFP.

**shRNA**
- control
- scr
- DHHC7

**Graphs**
- mCherry-ClC-Ka
- barttin-CFP

By guest on May 5, 2020
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
DHHC7-mediated palmitoylation of the accessory protein barttin critically regulates the functions of CIC-K chloride channels
Nataliya Gorinski, Daniel Wojciechowski, Daria Guseva, Dalia Abdel Galil, Franziska E. Mueller, Alexander Wirth, Stefan Thiemann, Andre Zeug, Silke Schmidt, Monika Zareba-Koziol, Jakub Wlodarczyk, Boris V. Skryabin, Silke Glage, Martin Fischer, Samer Al-Samir, Nicole Kerkenberg, Christa Hohoff, Weiqi Zhang, Volker Endeward and Evgeni Ponimaskin

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