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REVIEW ARTICLE

# ENaC activation by proteases

**Deepika Anand**[1,2] | **Edith Hummler**[1,2] | **Olivia J. Rickman**[1,2]

[1]Department of Biomedical Sciences, University of Lausanne, Lausanne, Switzerland

[2]National Center of Competence in Research, Kidney.CH, Lausanne, Switzerland

**Correspondence**
Edith Hummler, University of Lausanne, Department of Biomedical Sciences, Rue du Bugnon 27, CH-1011 Lausanne, Switzerland.
Email: edith.hummler@unil.ch

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**Abstract**

Proteases are fundamental for a plethora of biological processes, including signalling and tissue remodelling, and dysregulated proteolytic activity can result in pathogenesis. In this review, we focus on a subclass of membrane-bound and soluble proteases that are defined as channel-activating proteases (CAPs), since they induce $Na^+$ ion transport through an autocrine mechanism when co-expressed with the highly amiloride-sensitive epithelial sodium channel (ENaC) in *Xenopus* oocytes. These experiments first identified CAP1 (channel-activating protease 1, prostasin) followed by CAP2 (channel-activating protease 2, TMPRSS4) and CAP3 (channel-activating protease 3, matriptase) as in vitro mediators of ENaC current. Since then, more serine-, cysteine- and metalloproteases were confirmed as in vitro CAPs that potentially cleave and regulate ENaC, and thus this nomenclature was not further followed, but is accepted as functional term or alias. The precise mechanism of ENaC modulation by proteases has not been fully elucidated. Studies in organ-specific protease knockout models revealed evidence for their role in increasing ENaC activity, although the proteases responsible for ENaC activation are yet to be identified. We summarize recent findings in animal models of these CAPs with respect to their implication in ENaC activation. We discuss the consequences of dysregulated CAPs underlying epithelial phenotypes in pathophysiological conditions, and the role of selected protease inhibitors. We believe that these proteases may present interesting therapeutic targets for diseases with aberrant sodium homoeostasis.

**KEYWORDS**

epithelial phenotype, epithelial sodium channel, homoeostasis, kidney disease

## 1 | INTRODUCTION

Epithelia form barriers that are essential for life by lining surfaces of organs and body cavities to maintain homoeostasis. At the same time, they need to restrict free passage of water, ions, and larger solutes. In an intact epithelium, specialized cell junctions that confer strength and selective permeability determine the epithelial barrier function and its integrity, although multiple regulatory proteins as matrix components, adhesion molecules and/or proteases are implicated and their composition may change during development. Within the last years, the list of factors that change epithelial barrier function and its integrity is steadily increasing, and the role of proteases here just

starts to emerge. Amongst those, serine proteases belong to the largest class of proteolytic enzymes, and, here, the catalytic triad of the amino acids histidine (H), aspartate (D) and serine (S) functions as a site for enzymatic activity. A manually curated proteolytic enzyme database called MEROPS offers detailed information on serine protease families and subfamilies in the human degradome.[1] These proteases are further classified either as membrane-anchored (type-I, type-II or glycosylphosphatidyinositol-anchored), secreted or as intracellular serine proteases (Figure 1). In the *Xenopus* oocyte co-expression assay where cRNAs of all ENaC subunits were co-injected with candidate proteases, an increased inward amiloride-sensitive $Na^+$ current was observed. Using this approach or whole-cell patch-clamp techniques on cellular systems, several channel activating proteases have been identified.[2,3] This includes the membrane-bound channel activating proteases, the soluble serine proteases, the cysteine proteases and the metalloproteases (Figure 1).

Activation of ENaC is needed for $Na^+$ reabsorption across epithelia such as kidney, lung and intestine, and thus, any dysregulation may result in disease, eg, cystic fibrosis, salt-sensitive hypertension, liver cirrhosis[28] or nephrotic syndrome.[29] In this review, we explore several

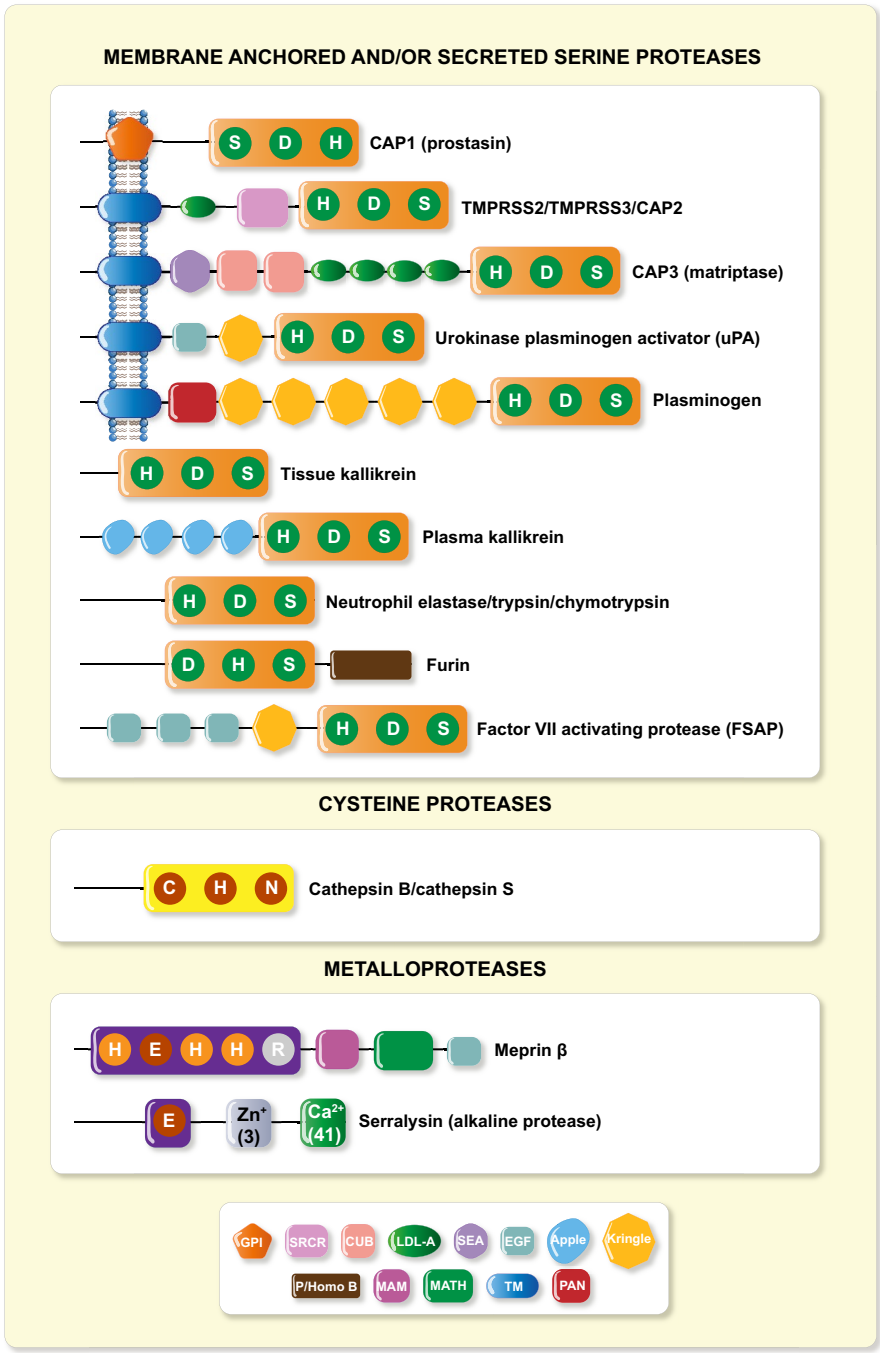



**FIGURE 1** Structural schematic of identified ENaC CAPs. CAP1 (Prss8, prostasin),[4,5] CAP2 (Tmprss4),[6] CAP3 (St14/Matriptase),[6] Tmprss3,[7] Tmprss2,[8,9] uPA (urokinase-type plasminogen activator),[10,11] plasminogen,[12-15] trypsin,[16] chymotrypsin,[14] tissue[17,18] and plasma kallikrein,[19] elastase,[20] furin,[21] factor VII activating protease,[22] cathepsin B,[23,24] cathepsin S,[14] meprin β[25] and serralysin.[26] Predicted structural domains of mouse proteases are indicated[27]: CUB, complement C1r/C2s, urchin embryonic growth factor, bone morphogenic protein 1; EGF, epidermal growth factor-like; apple; kringle; GPI, glycophosphatidylinositol anchor; LDL-A, low density lipoprotein A; MAM, meprin, A5 protein, receptor protein phosphatase μ; MATH, meprin and TRAF-C homology; P/Homo B, paired basic amino acid residue-cleaving enzyme/homo sapiens B; PAN, PAN/apple; SEA, sperm protein, enterokinase and agrin; SRCR, scavenger receptor cysteine-rich; TM, transmembrane

questions: how these proteases modulate ENaC function mechanistically, whether ENaC activation is dependent on its proteolytic cleavage, whether this proteolytic activity is essential in vivo and to which extent a loss- or gain-of-function of proteases in mice determines ENaC-mediated $Na^+$ losing or retaining phenotypes.

## 2 | ENAC REGULATION BY PROTEASES

ENaC is expressed in the tight epithelia of various organs and plays an essential role in regulating fluid volume and sodium homoeostasis. It belongs to the ENaC/degenerin family of ion channels and is highly $Na^+$-selective and voltage-insensitive.[2] ENaC subunits are encoded by different genes, *SCNN1A* (alpha/α), *SCNN1B* (beta/β), *SCNN1G* (gamma/γ), and, in human, there is a less expressed fourth subunit *SCNN1D* (delta/δ). The functional unit of ENaC consists of a trimeric assembly of α, β and γ subunits.[30,31] ENaC is expressed in the epithelia such as the distal colon, airways, sweat and salivary glands, skin, placenta and female reproductive tract (Figure 2A).[32] In the kidney, ENaC is expressed in the aldosterone-sensitive distal part of the nephron (ASDN) that includes the distal convoluted tubule (DCT2), the connecting tubule (CNT) and the collecting duct (CCD) (Figure 2B).[33] Other extracellular factors, such as ions, mechanical forces and proteases may affect single-channel open probability. The latter might occur by sequential cleavage steps carried out by at least two proteases targeting unique regions termed 'inhibitory tract' in the extracellular domains. Furin cuts twice on the α- and once on the γ-ENaC subunit. This is followed by an additional proteolytic cleavage on the γ-ENaC subunit.[33] Up to now, several potential candidate proteases were identified in mouse and tested in vitro, and their corresponding predicted consensus cleavage sites were determined (Figure 3). Many proteases have been identified to cleave human and rat ENaC in vitro, and given the high degree of sequence homology between human, rat and mouse, those proteases would be predicted to also cleave mouse ENaC (Figure 4).[3] Nevertheless, the responsible protease(s) for proteolytic activation of ENaC in vivo is yet to be identified. To summarize the current in vivo findings exploring ENaC activation by CAPs, different aspects are detailed below based on data obtained from genetically modified mouse models (Table 1).

### 2.1 | CAP1/Prss8 (prostasin)

CAP1/Prss8 belongs to the GPI (glycophosphatidylinositol)-anchored class of serine proteases,[4] and it was the first identified CAP shown to activate ENaC in vitro.[4,5] In the mouse, the constitutive knockout of CAP1 caused embryonic lethality due to placental failure.[40] Epiblast-specific CAP1 knockout mice survived until birth, but then died due to rapid and severe dehydration caused by ichthyosis indicating a severe impairment of the epidermal barrier function.[40] Surprisingly, zymogen-locked CAP1/Prss8 knock-in ($KI^{R44Q}$) mice, in which the activation site was rendered cleavage-resistant, developed only a minor epidermal phenotype comparable to mice carrying the CAP1/Prss8 mutant frizzy (fr/fr).[47,49] Mice carrying a mutation at the CAP1 catalytic site (S238A) displayed normal tissue development and homoeostasis.[51] Sodium homoeostasis was preserved in these mice during $Na^+$-deprivation, while zymogen-locked CAP1 mice developed a compromised triamterene tolerance. Interestingly, in both models, proteolysis of α- and γ-ENaC subunits as well as their subcellular localization were conserved in kidney (Table 1).[50]

The adult epithelial phenotype caused by tissue-specific gene deletion clearly confirmed an implication of CAP1 in ENaC-mediated sodium transport in lung and colon[43,45,47] but not in skin.[42,47,53,54] Alveolar-specific CAP1 knockout mice displayed a 40% reduction in the ENaC-mediated $Na^+$ current and impairment in alveolar fluid clearance. However, alveolar oedema, change in lung morphology, or tight junction protein abundance was not observed in unchallenged mice, but only under increased hydrostatic pressure.[45] Likewise, reduced ENaC activity was observed in colon-specific CAP1 knockout mice.[43,47] Using an experimental rat colitis model, CAP1 was found to preserve colonic integrity, and to protect against dextran sodium sulphate (DSS)-induced inflammation, and likely against tissue remodelling.[48] In skin, the tight junction protein occludin was completely missing in epidermis-specific CAP1 knockout mice resulting in an impaired barrier function leading to fatal dehydration.[42] In this context, it is interesting to note that Gong and coworkers revealed an effect of CAP1 on paracellular chloride permeation that is regulated through tight junctions in renal epithelia and therefore may participate in blood pressure regulation.[69] Interestingly, adenovirus-induced CAP1 overexpression was linked to increased aldosterone production and hypertension in rats[52] (Table 1). Overall, in vivo studies suggest a link between CAP1, epithelial $Na^+$ transport and barrier function, although the direct interaction and the mechanism of proteolytic ENaC activation are still not completely determined.

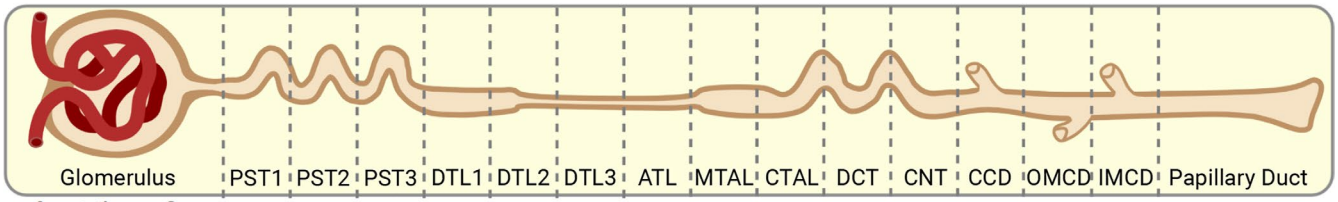
### 2.2 | CAP2/Tmprss4

In *Xenopus* oocytes, CAP2/Tmprss4 increased ENaC activity via its open probability.[70] It cleaves at the identified

**(A)**

| Gene ID | Gene | Brain | Colon | Heart | Kidney | Liver | Lung | Skeletal muscle | Spleen | Testis | Thymus | Skin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ENSMUSG00000030340 | Scnn1a | 4 | 7 | 1 | 47 | 18 | 346 | 0.2 | 1 | 0.2 | 34 | 26 |
| ENSMUSG00000030873 | Scnn1b | 0.3 | 0.2 | 0.1 | 32 | 0 | 189 | 0.3 | 0 | 0.1 | 3 | 5 |
| ENSMUSG00000000216 | Scnn1g | 0.5 | 0 | 0.1 | 36 | 0 | 191 | 0 | 0 | 0 | 4 | 4 |
| ENSMUSG00000030800 | Prss8 | 0.3 | 25 | 0 | 406 | 4 | 120 | 0.1 | 0.1 | 0.3 | 5 | 49 |
| ENSMUSG00000000385 | Tmprss2 | 0.1 | 67 | 0.2 | 70 | 1 | 46 | 0 | 0 | 0.4 | 2 | 0.8 |
| ENSMUSG00000024034 | Tmprss3 | 0.1 | 0 | 0 | 0 | 0 | 0 | 0 | 0.1 | 0.1 | 0.5 | 0 |
| ENSMUSG00000032091 | Tmprss4 | 0.2 | 75 | 0.1 | 0.8 | 0.1 | 24 | 0 | 0.1 | 0.1 | 6 | 67 |
| ENSMUSG00000031995 | St14 | 0.4 | 93 | 0.3 | 20 | 0.4 | 30 | 0 | 14 | 0.1 | 9 | 29 |
| ENSMUSG00000021822 | Plau | 5 | 11 | 5 | 679 | 1 | 15 | 63 | 16 | 2 | 71 | 92 |
| ENSMUSG00000059481 | Plg | 0.1 | 0 | 0 | 0.2 | 1833 | 0 | 0 | 0 | 0.1 | 0.2 | 0.1 |
| ENSMUSG00000063903 | Klk1 | 0.1 | 1818 | 0 | 2915 | 0.4 | 2 | 0 | 14 | 6 | 17 | 3 |
| ENSMUSG00000109764 | Klkb1 | 0.1 | 0.5 | 0 | 0.7 | 120 | 0.6 | 0 | 0.1 | 1 | 0.1 | 0 |
| ENSMUSG00000020125 | Elane | 0 | 0 | 0 | 0 | 0.1 | 2 | 0.2 | 50 | 0.1 | 3 | 0.5 |
| ENSMUSG00000062751 | Prss1 | 0 | 0.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.7 | 0 |
| ENSMUSG00000023031 | Cela1 | 2 | 13947 | 0.4 | 131 | 10 | 1 | 0.1 | 17 | 0.2 | 6 | 3 |
| ENSMUSG00000030530 | Furin | 21 | 23 | 37 | 44 | 79 | 56 | 14 | 24 | 8 | 24 | 30 |
| ENSMUSG00000025075 | Habp2 | 0.1 | 6 | 1 | 109 | 137 | 0.1 | 4 | 0.1 | 0 | 0.1 | 0.9 |
| ENSMUSG00000021939 | Ctsb | 360 | 230 | 225 | 410 | 262 | 279 | 107 | 597 | 40 | 417 | 487 |
| ENSMUSG00000038642 | Ctss | 110 | 286 | 28 | 22 | 40 | 289 | 10 | 1365 | 10 | 390 | 206 |
| ENSMUSG00000024313 | Mep1b | 0 | 884 | 0 | 445 | 0.1 | 0.1 | 0 | 0 | 0.5 | 0.4 | 0 |

**(B)**




| Annotation | Gene | PST1 | PST2 | PST3 | DTL1 | DTL2 | DTL3 | ATL | MTAL | CTAL | DCT | CNT | CCD | OMCD | IMCD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| α-ENaC | Scnn1a | 0.3 | 10 | 9 | 4 | 3 | 0.6 | 1 | 70 | 155 | 220 | 383 | 429 | 195 | 0.5 |
| β-ENaC | Scnn1b | 0.3 | 0.6 | 0 | 2 | 0.2 | 0.9 | 0.2 | 0 | 0.6 | 307 | 1149 | 1318 | 694 | 0.2 |
| γ-ENaC | Scnn1g | 0 | 0.4 | 0 | 0.8 | 0.2 | 0.2 | 0.3 | 0 | 0.3 | 344 | 951 | 1048 | 623 | 0.4 |
| CAP1/prostasin | Prss8 | 393 | 567 | 439 | 155 | 179 | 126 | 30 | 126 | 104 | 63 | 115 | 144 | 171 | 43 |
| TMPRSS2 | Tmprss2 | 42 | 78 | 31 | 35 | 27 | 54 | 63 | 127 | 296 | 468 | 669 | 546 | 674 | 520 |
| TMPRSS3 | Tmprss3 | 0 | 0 | 0 | 0.2 | 0.1 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0.1 | 0.6 | 0.1 |
| CAP2 | Tmprss4 | 4 | 0 | 0 | 0.7 | 0.2 | 0.7 | 0.3 | 0 | 0.4 | 0.8 | 1 | 1 | 0.2 | 1 |
| CAP3/matripase | St14 | 1 | 12 | 15 | 205 | 129 | 179 | 232 | 207 | 200 | 176 | 311 | 332 | 342 | 203 |
| uPA | Plau | 3 | 21 | 17 | 162 | 23 | 31 | 0 | 43 | 41 | 0.3 | 1 | 2 | 2 | 0 |
| Plasminogen | Plg | 0.1 | 0.7 | 0.4 | 0.3 | 0.2 | 0.2 | 0.6 | 0 | 0 | 0 | 0 | 0 | 0.2 | 0 |
| Tissue kallirein | Klk1 | 0.8 | 4 | 0.9 | 0.5 | 0.6 | 0.4 | 2 | 1054 | 9422 | 48967 | 4226 | 147 | 0.4 | 0 |
| Plasma kallikrein | Klkb1 | 0.2 | 0 | 0 | 1 | 0.3 | 0.8 | 0.9 | 0 | 0 | 3 | 14 | 11 | 1 | 0.1 |
| Neutrophil elastase | Elane | 0.1 | 0 | 0 | 1 | 0.2 | 0.5 | 0 | 0 | 0 | 0 | 0 | 0 | 0.2 | 0 |
| Trypsin | Prss1 | 0 | 0 | 0 | 0.5 | 0.1 | 0.1 | 0 | 0 | 0 | 0 | 0 | 0 | 0.1 | 0 |
| Chymotrypsin | Cela1 | 885 | 76 | 25 | 19 | 28 | 52 | 9 | 44 | 33 | 45 | 14 | 12 | 14 | 40 |
| Furin | Furin | 42 | 85 | 44 | 39 | 48 | 59 | 52 | 38 | 50 | 41 | 68 | 63 | 82 | 74 |
| FSAP | Habp2 | 323 | 65 | 1 | 2 | 1 | 1 | 1 | 0 | 0 | 61 | 166 | 102 | 89 | 0 |
| Cathepsin B | Ctsb | 1199 | 584 | 267 | 392 | 463 | 283 | 202 | 769 | 880 | 965 | 742 | 639 | 360 | 237 |
| Cathepsin S | Ctss | 3 | 2 | 0.9 | 3 | 5 | 0.5 | 4 | 10 | 32 | 13 | 16 | 11 | 11 | 2 |
| Meprin 1 β | Mep1b | 1 | 708 | 617 | 42 | 7 | 0.2 | 0.8 | 0 | 1 | 0.1 | 0.3 | 1 | 0.2 | 0.1 |

**FIGURE 2** ENaC and CAP transcriptional expression in male C57BL/6 mouse organs and nephron segments shown as transcripts per million. A, Data according to the EMBL-EBI expression atlas data.[34,35] B, RNA expression data across 14 mouse renal tubule segments from 6- to 8-week-old mouse microdissected tubules.[36] ATL, thin ascending limb of the loop of Henle; CCD, cortical collecting duct; CNT, connecting tubule; CTAL, cortical thick ascending limb of the loop of Henle; DCT, distal convoluted tubule; DTL1, short descending limb of the loop of Henle; DTL2, long descending limb of the loop of Henle in the outer medulla; DTL3, long descending limb of the loop of Henle in the inner medulla; IMCD, inner medullary collecting duct; MTAL, medullary thick ascending limb of the loop of Henle; OMCD, outer medullary collecting duct; PST1, initial segment of the proximal tubule; PST2, proximal straight tubule in cortical-medullary rays; PST3, last segment of the proximal straight tubule in the outer stripe of outer medulla

furin consensus cleavage site of the γ-ENaC subunit which, when mutated, completely abolished $Na^+$ current therefore strongly supporting proteolytic activation of ENaC by CAP2.[37] The epithelial phenotype in CAP2/Tmprss4 constitutive knockout mice, however, did not reveal any impairment of ENaC-mediated $Na^+$ transport even under $Na^+$-deprived diet. No difference was observed in the protein abundance of full-length and cleaved γ-ENaC subunit (Table 1).[55] However, we identified a dysregulation of renal water handling upon dietary potassium depletion with upregulation of adenylate cyclase 6, cAMP overproduction and increased protein expression of

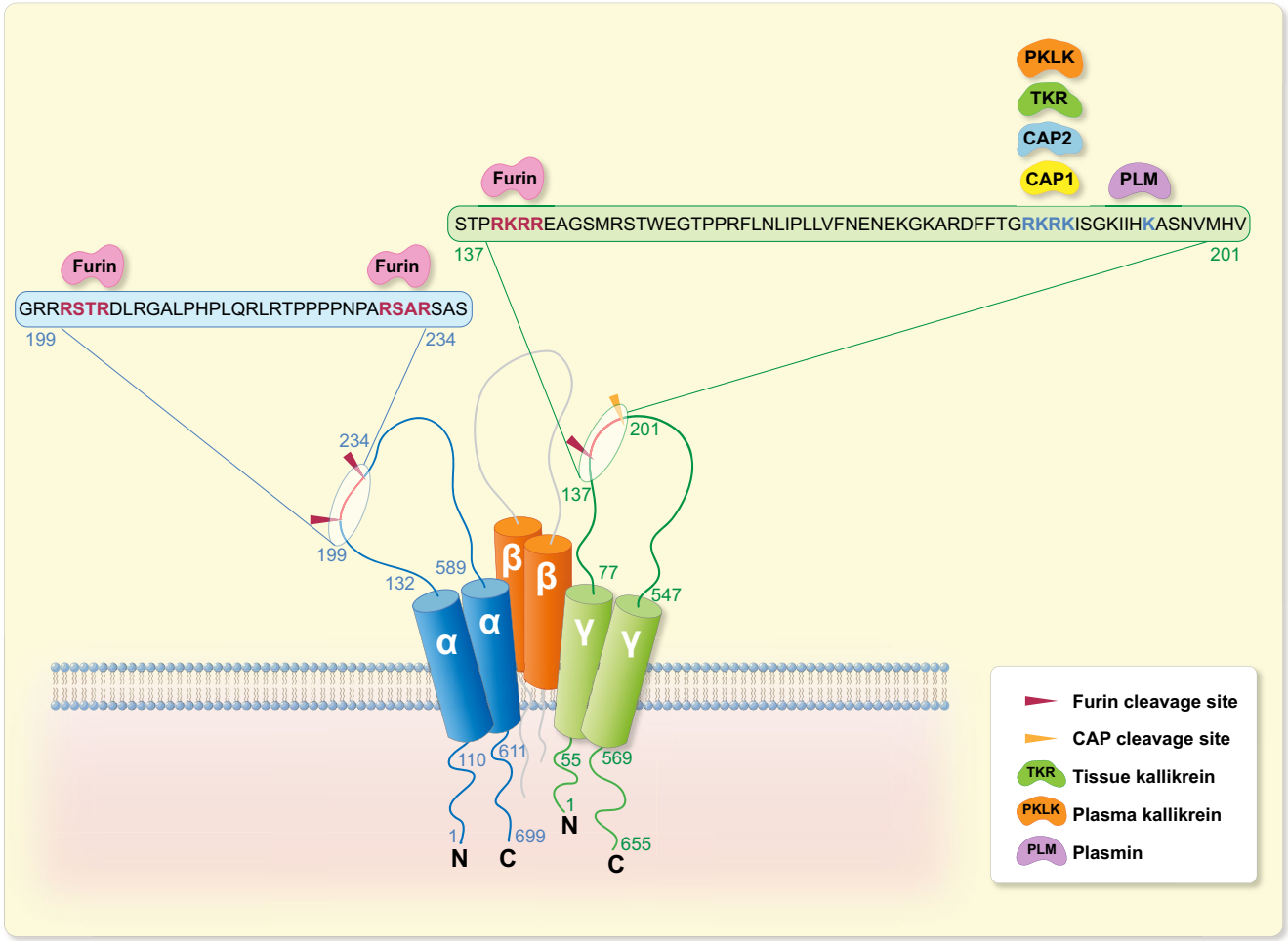



**FIGURE 3** Identified consensus sites for proteolytic ENaC cleavage by CAPs in mouse. Illustration of the mouse α-, β- and γ-ENaC subunits depicting in vitro identified mouse CAP cleavage sites. α-ENaC amino acid residues numbered in blue and γ-ENaC in green. Furin cleaves at two sites in the α subunit and at one site in the γ subunit.[21] CAP1,[75] CAP2,[76] tissue kallikrein,[17] plasma kallikrein[19] and plasmin[15] cleave mouse γ-ENaC distal to the furin cleavage site

aquaporin 2 (AQP2) and the $Na^+$-$K^+$-$2Cl^-$ cotransporter 2 (NKCC2).[56] Interestingly, nephron-specific deletion of the glucocorticoid receptor (GR) in mice led to the "mirrored" phenotype including increased water intake and urine output, urinary alkalinization and downregulation of HKA2, AQP2 and NKCC2 therefore unveiling a novel role of this serine protease and the GR in renal water handling. Under $Na^+$ restriction, amiloride-sensitive renal potential difference and ENaC-mediated sodium balance remained unchanged in vivo.[56] Overall, CAP2/Tmprss4 knockout mice did not seem to be directly implicated in the regulation and/or proteolytic ENaC activation.

## 2.3 | CAP3/St14 (matriptase)

CAP3/St14 is known to induce a 6.8-fold higher $I_{Na+}$ when co-expressed with rat α-, β- and γ-ENaC subunits in *Xenopus* oocytes.[70] In vitro analyses by Kota and coworkers identified a region near the rat γ-ENaC furin site as a critical cleavage site for CAP3-mediated ENaC stimulation.[38] Contrary to the constitutive CAP1/Prss8 knockout, mice with constitutive lack of CAP3/St14 survived until birth, but then died due to severe ichthyosis. All epidermal surfaces were grossly abnormal which consequently compromised the epidermal barrier function (Table 1).[57] Deficiency of matriptase in adult mice (using tamoxifen-induced deletion) predominantly resulted in impaired epithelial barrier function in the salivary gland epithelium with a near-complete loss of saliva production[59] and led to a Sjögren's syndrome-like disease.[60] In the large intestine, a disruption of normal tissue architecture was documented and accompanied by an increased intestinal permeability and oedema of crypt and submucosa cells.[59] Surprisingly, further matriptase-expressing epithelia eg the upper digestive tract, small intestine, kidney, liver, lungs, spleen and pancreas showed normal macroscopic appearance.[59]

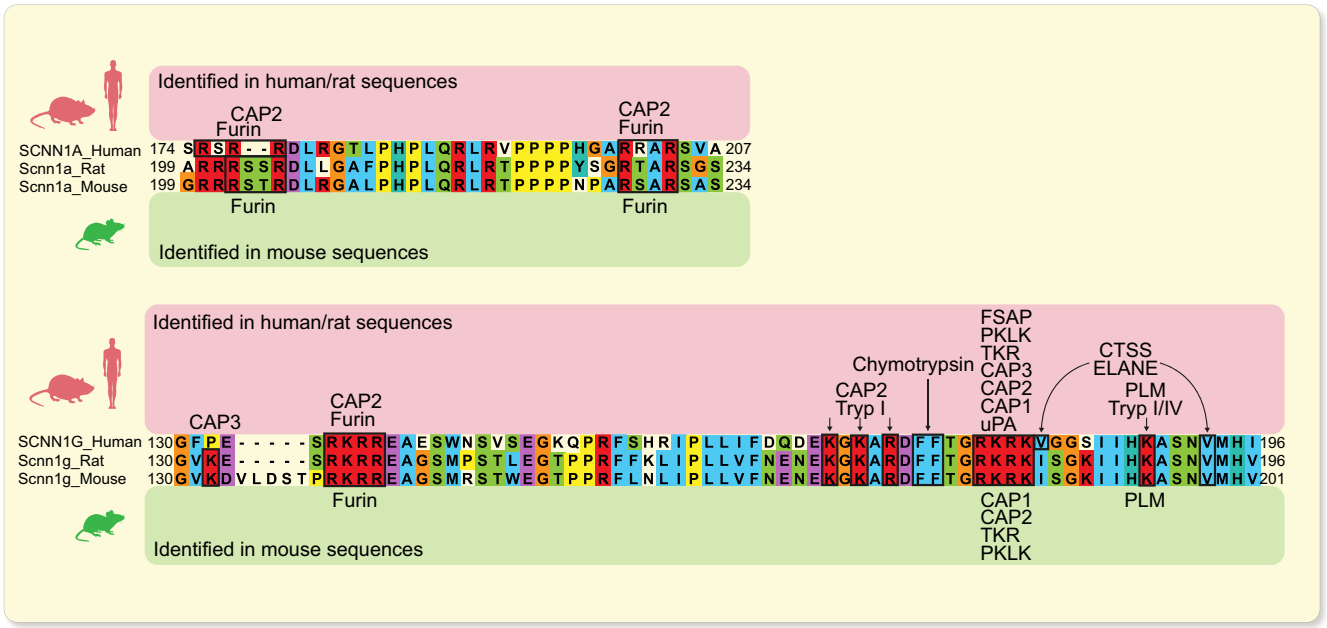



**FIGURE 4** In vitro identified consensus sites for proteolytic ENaC cleavage by CAPs. Alignment of human, rat and mouse α- and γ-ENaC subunits showing a high degree of conservation between species at cleavage sites. Amino acid numbering at the end of transcripts indicates the portion of sequence analysed. CAPs shown to cleave human/rat (indicated above) and mouse (indicated below) ENaC consensus sites in vitro are specified.[3,37-39] CTSS, cathepsin S; ELANE, neutrophil elastase; FSAP, factor VII-activating protease; PKLK, plasma kallikrein; PLM, plasmin; TKR, tissue kallikrein; Tryp, trypsin; uPA, urokinase-type plasminogen

CAP3 hypomorphic mice displayed a ~30% reduction in intestinal transepithelial electrical resistance likely via dysregulated claudin-2 expression at intercellular junctions.[58] Intestine-specific matriptase deficiency induced malignant transformation of colonic epithelium.[71] Overall, although a role of CAP3 in epidermal barrier function was confirmed, its implication in the proteolytic activation of ENaC has not yet been demonstrated.

## 2.4 | Urokinase-type plasminogen activator

In human, nephrotic syndrome characterized by severe peripheral oedema and ENaC-mediated $Na^+$ retention is thought to be due to aberrantly filtered plasminogen which is cleaved into plasmin.[63,72] In *Xenopus* oocytes, preincubation with plasminogen and uPA increased ENaC current, whereas uPA alone showed only a marginal effect.[12] Experimentally induced nephrotic mouse models with chronic versus acute inhibition of uPA resulted in opposite conclusions.[72] Indeed, constitutive uPA knockout mice in which the nephrotic syndrome was induced by doxorubicin showed no phenotypic difference when compared with controls, ie no implication in ENaC-mediated $Na^+$ retention,[64] whereas an antagonistic uPA treatment of podocin knockout mice led to a marked attenuation of $Na^+$ retention.[63] Furthermore, plasminogen deficiency did not prevent ENaC-mediated sodium retention in an induced experimental nephrotic mouse model, and thus, the uPA/plasmin-mediated pathway might not be the only player for ENaC activation (Table 1).[65] uPA, like tissue-type plasminogen activator, has a divergent role in fibrinolysis and macrophage function and further affects complex biological processes.[73]

## 2.5 | Tissue and plasma kallikrein

Tissue kallikrein has been proposed to be the physiologically relevant protease modulating ENaC activity.[17] It is highly expressed in the colon and kidney (Figure 2A), and in the kidney, it localizes to the distal portion of the nephron including the principal cells of the connecting tubule (CNT) where it is also secreted into the urine. It cleaves kininogen to bradykinin and activates the B2 bradykinin receptor to increase sodium excretion. This is facilitated by the inhibition of sodium reabsorption in the collecting duct.[74] Tissue kallikrein-deficient mice adapt normally to dietary sodium restriction despite defective γ-ENaC activity and reduced renal ENaC activity (Table 1).[18] However, the authors did not exclude that this effect on ENaC may be indirect. Absence of plasma kallikrein (PKLK), an aprotinin-sensitive serine protease, did not protect nephrotic mice from oedema formation,

**TABLE 1** ENaC channel-activating proteases tested in animal models and their associated epithelial phenotypes

| Serine proteases | Rodent model & study condition | Phenotype (effect) | Identified in vivo substrate(s) | Ref. |
|---|---|---|---|---|
| CAP1/Prss8 (prostasin) | Constitutive KO, unchallenged | Placenta – syncytialization defect (impaired differentiation and signal transduction) | Not reported; CAP3/St14 | 40,41 |
| | Epidermal-specific KO, unchallenged | Skin – orthokeratotic hyperkeratosis, hair follicle dysmaturation, tight junction leakiness (impaired barrier function/integrity) | Profilaggrin, occludin | 42 |
| | Colon-specific KO, unchallenged, low $Na^+$ diet | Colon – colonic pseudohypoaldosteronism type 1 (impaired ion transport) | ENaC | 43 |
| | Colon-specific KO, DSS-induced colitis | Colon – inflammation (altered signal transduction) | TLR4 | 44 |
| | Alveolar-specific KO, unchallenged, acute volume overload | Lung – decreased alveolar fluid clearance hydrostatic oedema (impaired ion transport) | ENaC | 45 |
| | Liver-specific KO, high fat diet | Liver – insulin resistance | TLR4 | 46 |
| | Spontaneous mutation $fr^{V170D}$, unchallenged | Reduced embryonic vitality; skin – dehydration, hyperkeratosis; colon – reduced ENaC activity (impaired ion transport) | ENaC (colon) | 47 |
| | Spontaneous mutation $fr^{CR}$ (rats), unchallenged | Reduced embryonic vitality; skin – baldness, dehydration, hyperkeratosis; colon – reduced ENaC activity, diarrhea (impaired ion transport) | ENaC (colon) | 47 |
| | Spontaneous mutation $fr^{CR}$ (rats), DSS-induced colitis | Colon – epithelial remodelling; intestinal inflammation (impaired signal transduction and differentiation) | ENaC not confirmed | 48 |
| | Knockin $Prss8^{R44Q}$ (zymogen-locked), unchallenged | Skin – impaired/delayed whisker and pelage hair formation (altered signal transduction) | CAP3/St14 suspected | 49 |
| | Knockin $Prss8^{R44Q}$ (zymogen-locked), low $Na^+$ (high $K^+$) diet, triamterene | Kidney – normal $Na^+$ conservation; hypokalaemia; hyperaldosteronism<br>$Na^+$ wasting, weight loss (impaired ion transport) | ENaC not confirmed, ENaC suspected | 50 |
| | Knockin $Prss8^{S238A}$ (catalytically inactive), unchallenged | Skin – delayed whisker and pelage hair formation (altered signal transduction) | Not reported | 51 |
| | Knockin $Prss8^{S238A}$ (catalytically inactive), low $Na^+$ (high $K^+$) diet, triamterene | Kidney – normal $Na^+$ conservation; no obvious phenotype | ENaC not confirmed | 50 |
| | Adenovirus-induced $Prss8^{wt}$ overexpression, unchallenged | Kidney – induced mineralocorticoid production (hypertension, impaired electrolyte homoeostasis) | Kallikrein | 52 |
| | Epidermal-specific transgenic mice ($Prss8^{wt}$), unchallenged | Skin – hyperkeratosis, dehydration, inflammation (altered signal transduction) | PAR2; nexin-1 | 53,54 |
| | Epidermal-specific transgenic mice ($Prss8^{S238A}$), unchallenged | Skin – hyperkeratosis, dehydration, inflammation (altered signal transduction) | PAR2, nexin-1 | 53 |

(Continues)

**TABLE 1** (Continued)

| Serine proteases | Rodent model & study condition | Phenotype (effect) | Identified in vivo substrate(s) | Ref. |
|---|---|---|---|---|
| CAP2/ TMPRSS4 | Constitutive KO, low $Na^+$ diet | No obvious phenotype; kidney | ENaC excluded | 55 |
| | Constitutive KO, low $K^+$ diet | Skin – ichthyosis; impaired water handling | HKA2, Nr3c1, AC6 | 56 |
| CAP3/ St14 (matriptase) | Constitutive KO, unchallenged | Skin, thymus – postnatal lethality, ichthyosis, thymocyte apoptosis (impaired epithelial barrier function and thymus development) | Not reported | 57,58 |
| | Tamoxifen-induced | Skin, intestine – loss of tight junction, ichthyosis, enlarged colon (impaired integrity of tight junctions) | Occludin, ZO-1, claudin-1 | 59 |
| | Adenoviral-induced salivary gland KO, virus-induced | Salivary glands – altered tight junction distribution (Sjögren's syndrome-like disease) | Claudin-3 | 60 |
| | Salivary-gland KO, unchallenged | (Impaired gland function) | Not reported | 59 |
| | Intestinal-specific KO, unchallenged | Colon – failed terminal differentiation, colitis, adenocarcinoma, (altered signal transduction, impaired epithelial integrity) | E-cadherin, ZO-1, occluding, β-catenin and laminin suspected | 59 |
| | Hypomorphic mice, unchallenged | Skin – ichthyosis with hypotrichosis-like syndrome (impaired epidermal barrier) | CAP1/Prss8, profilaggrin, claudin-2 | 61 |
| | Epidermal-specific transgenic mice, unchallenged, DMBA-induced | Skin – carcinogenesis (malignant transformation, altered differentiation) | Ras | 62 |
| uPA (uro-kinase-type plasminogen activator) | Anti-uPA targeting antibody; induced podocin KO, tamoxifen-induced, ± amiloride | Kidney – attenuation of sodium retention (impaired ion transport) | ENaC | 63 |
| | Constitutive KO, amiloride, doxorubicin-induced nephrotic syndrome | Kidney – phenotype not different from control | ENaC suspected | 64 |
| Plasminogen | Constitutive KO; inducible podocin KO, doxycycline-induced nephrotic syndrome | Kidney – phenotype not different from control | ENaC not confirmed | 65 |
| Tissue Kallikrein | KO, aldosterone infusion or low $Na^+$ diet | Kidney, colon, lung – decreased ENaC activity in kidney and colon, but not in lung (partly impaired ion transport) | ENaC | 18 |
| Plasma kallikrein | KO, doxorubicin-induced nephrotic syndrome | Kidney – phenotype not different from control | ENaC not confirmed | 19 |
| Tmprss3 | Constitutive KO, unchallenged | Ear – organ of Corti and hair cell degeneration, deafness (impaired ion transport) | ENaC not confirmed | 66,67 |
| Tmprss2 | Constitutive KO, unchallenged | No obvious phenotype | Not reported | 68 |
| FSAP | Constitutive KO, doxorubicin-induced nephrotic syndrome | Kidney – no obvious phenotype | ENaC not confirmed | 22 |

*Note:* Unchallenged, no specific pretreatment.

Abbreviations: AC6, adenylate cyclase 6; FSAP, factor VII-activating protease.; HKA2, $H^+$, $K^+$-ATPase type 2; KO, knockout; Nr3c1, nuclear receptor subfamily 3 group C member 1; TLR4, toll-like receptor 4; ZO-1, zona occludens 1.

suggesting that ENaC-mediated $Na^+$ retention is independent of this protease (Table 1).[19]

### 2.6 | Tmprss3

Proteolytic processing of ENaC by Tmprss3 was associated with increased ENaC-mediated current in vitro, and Tmprss3 mutants causing human deafness failed to proteolytically cleave and activate ENaC in the *Xenopus* oocyte expression system.[7] Constitutive Tmprss3 knockout mice and ENU (ethyl-nitrosourea)-induced mutant Tmprss3 mice carrying a protein-truncating nonsense mutation both exhibited a cochlear hair cell degeneration,[66,67] although direct experimental evidence of in vivo ENaC activation by Tmprss3 is still missing (Table 1). Further epithelial phenotypes of the Tmprss3 knockout mice were not yet reported.

### 2.7 | Tmprss2

Early functional experiments investigating the impact of TMPRSS2 on ENaC in *Xenopus* oocytes showed a significant decrease in ENaC current and protein levels which was not prevented by the addition of aprotinin.[8] A later study reported the opposite effect when both TMPRSS2 and ENaC cRNAs were injected into *Xenopus* oocytes, since an increase in ENaC current was observed similar to that of other serine proteases.[9] RNA sequencing data revealed that TMPRSS2 is highly expressed in the distal portion of the kidney (Figure 2B), but little is known about a physiological role, since Tmprss2 KO mice did not present with an observable phenotype (Table 1).[68]

### 2.8 | FSAP-SPD

Active factor VII activating protease (FSAP) is excreted in urine of nephrotic patients and doxorubicin-induced nephrotic mice, and found to activate ENaC in vitro in the *Xenopus* oocyte expression system.[22] However, in nephrotic FSAP-deficient mice, the proteolytic cleavage pattern of α- and γ-ENaC was similar to untreated animals and these mice were not protected from $Na^+$ retention, rendering it unlikely that this protease is responsible for proteolytic ENaC activation.[3,22]

## 3 | SERINE PROTEASE INHIBITORS OF ENAC-MEDIATED $NA^+$ ABSORPTION

Serpins or serine protease inhibitors belong to a family of proteins that were first identified for their protease inhibition activity.[77] Most serpins are substrates and suicide inhibitors of serine proteases. They irreversibly inhibit their target protease by a conformational change that disrupts and blocks access to its active site. Some proteins with serpin function lack the enzyme inhibitory function.[78] The serine protease inhibitor nexin-1 (PN-1) antagonized CAP1/Prss8 (prostasin) activity in mice overexpressing CAP1/Prss8 in the epidermis independent of its catalytic site.[53] Thereby, co-expression of either the wildtype or a catalytic triad-mutant CAP1/Prss8 with nexin significantly reduced protease-induced ENaC current in *Xenopus* oocytes.[53] In the kidney, a reciprocal regulation of the expression of nexin-1 by TGF βand of CAP1/Prss8 by aldosterone has been proposed that might result in $Na^+$ retention or natriuresis, respectively,[79] although the experimental in vivo proof of such an interaction is still missing. Experiments in mice revealed, that embryonic lethality of either hepatocyte growth factor activator inhibitor (HAI)-1 or -2 was rescued by simultaneous inactivation of CAP3/St14 (matriptase).[80] Hypomorphic CAP1/Prss8 (*frizzy*; *fr/fr*) mice restored placentation and development in HAI-1 (*Spint1*)-deficient embryos. However, these defects seemed not to be caused by aberrant activity of ENaC, since neither the pharmacological block by ENaC inhibitor amiloride, nor ENaC inactivation did rescue the *Spint1*-deficient mice.[80] Indeed, depending on the concentration used, amiloride can block u-PA.[81] In human, mutations in the serine protease inhibitor *SPINT2* were associated with congenital tufting enteropathy characterized by severe intestinal dysfunction.[82] Organoid crypt cultures indicated that *Spint2* ablation induced decreased claudin-7 expression in tight junctions that resulted in organoid rupture.[82] These clinical features could be prevented by intestinal-specific inactivation of CAP3/St14 encoding matriptase.[83] The authors proposed that excessive matriptase activity might be causative for this genetic disorder.[20]

In mouse M-1 cortical collecting duct cells, aprotinin inhibited the amiloride sensitive current and transepithelial resistance.[84] A mathematical model using renal epithelial A6 cells from *Xenopus laevis* predicted that the serine protease inhibitors might affect intracellular trafficking and reduce the residency time of ENaC at the apical membrane.[85] Treatment of doxorubicin-induced nephrotic mice with the serine protease inhibitor aprotinin normalized urinary serine protease activity and prevented $Na^+$ retention,[86] and γ-ENaC cleavage was reduced.[87] Hence, inhibition of urinary serine protease activity was proposed as treatment for proteinuria and volume retention. In *Xenopus* oocytes, aprotinin had no direct inhibitory effect on channel activity.[86] In a venom-induced acute kidney injury (AKI) rat model, aprotinin

prevented glomerular injury and a decrease in glomerular filtration rate, thereby restoring fluid and electrolyte homoeostasis by inhibition of augmented serine protease kallikrein levels. Pretreatment with aprotinin restored fluid homoeostasis and protected from kidney injury.[88] However, inhibition of ENaC activity was not reported here. Increased levels of proteases in the tubular fluid contributed to enhanced ENaC activity and thus $Na^+$ retention as commonly seen in patients with chronic heart failure.[89] Two weeks of aprotinin treatment of a myocardial infarct rat model abrogated the enhanced diuretic and natriuretic responses to ENaC inhibitor benzamil.[89] In this context, it is interesting to note that aprotinin exerted nephrotoxic effects in healthy mice by inhibiting proximal tubular function and unexpectedly led to increased proteolytic ENaC activation. This was explained by a counterregulatory stimulation of ENaC-mediated sodium transport.[90] Further studies are needed to unveil the in vivo effect of aprotinin on sodium transport independent of the proteolytic ENaC activation.

Amongst the chemical inhibitors, camostat mesylate (CM) inhibited several serine proteases and was used as an inhibitor of progressive chronic renal failure.[91,92] Use of CM resulted in reduced proteinuria and oedema, showing a beneficial effect on diabetic nephropathy that is associated with progressive loss in kidney function due to the high blood glucose levels.[93] In Dahl salt-sensitive rats fed a high $Na^+$ diet, systolic blood pressure and urinary protein excretion were reduced by oral administration of CM.[94] However, systemic treatment of ENaC inhibitors has not been reported in this experiment. Treatment of CM in nephrectomy-induced kidney disease inhibited the progression of chronic renal failure by decreasing serum creatinine and proteinuria.[91] Similarly, in rats that developed an adenine-induced chronic kidney disease, CM attenuated the progression of the disease through its antioxidant effects and decreased blood pressure, serum creatinine and fibrotic markers.[92] A water-soluble irreversible serine protease inhibitor, AEBSF (4-(2-aminoethyl) benzenesulphonyl fluoride), has been recently shown to inhibit serine protease activity in nephrotic mice and also in nephrotic patients.[95]

## 4 | CONCLUDING REMARKS

Proteases might be the important therapeutic targets for epithelial disorders due to their pivotal role in the maintenance of epithelial homoeostasis in a variety of tissues. The mechanism by which these proteases modulate ENaC function is still not completely understood. The proteolytic activation of ENaC was well demonstrated in vitro, although less clear in vivo. In this context, loss- or gain-of-function mutations of these proteases in rodent models had nearly no effect or resulted in only mild ENaC-mediated $Na^+$ losing or retaining phenotypes. CAP1/Prss8 (prostasin)-deficient mice exhibited a reduced ENaC-mediated $Na^+$ transport when exposed to challenging conditions in lung and colon, but this was not associated with reduced ENaC cleavage. Additionally, during $Na^+$ deprivation, mice with catalytically inactive prostasin showed similar proteolytic ENaC activation as controls and, overall, a normal sodium balance. Tissue kallikrein-deficiency, on the other hand, leads to a defective ENaC processing and function, but mice adapted normally to dietary changes. This could mean that the responsible protease for proteolytic ENaC activation is not yet identified and/or there is functional redundancy amongst these proteases in activating ENaC. The detailed analysis of these knockout models revealed additional substrates, which may explain the described phenotype(s) and thus elucidating pathways that may directly and/or indirectly affect ENaC activation.

Many of the findings discussed in this review were obtained from animal studies which have been proven instrumental for our understanding of the role of proteases in physiology and pathophysiology. The specific origin and the developmental timing of the mutation (partial vs complete, spontaneous vs genetically engineered, constitutive vs tissue-specific vs induced) might influence the severity of the disease, and finally explain discrepancies between findings across different studies. Further limitations of previous studies include changes of protease composition and/or activity upon physiological and pathophysiological conditions, and, therefore, their redundancy may not be reflected in single knockout mouse models. Regardless of whether altered protease expression is a cause or a consequence of the epithelial phenotype, the tight regulation of serine proteases and/or potential interaction with other proteins may be central for the maintenance of tissue/organ homoeostasis. To dissect the fine-tuning of the regulation and downstream effects of those proteases, the development of more specific serine protease inhibitors is necessary. Their functional equilibrium is integral to many biological processes, and hence, a disturbed balance results in a wide range of epithelial pathologies.

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## CONFLICT OF INTEREST

The authors declare no competing interest.

## ORCID

*Deepika Anand* https://orcid.org/0000-0002-7844-7051
*Edith Hummler* https://orcid.org/0000-0002-3887-2715
*Olivia J. Rickman* https://orcid.org/0000-0002-7842-1150

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