Localisation of the COMMD1 and COMMD3 proteins in the kidney and mammalian cells

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Abstract
The amiloride-sensitive epithelial sodium channel (ENaC), which is a key regulator of sodium (Na\(^+\)) homeostasis, is expressed as a protein complex on the apical cell surface of many epithelial cells. ENaC is composed of three similar subunits named α-, β- and γENaC. Together these three subunits provide a regulated pathway for Na\(^+\) ions to enter epithelial cells from the lumen thus its activity at the cell surface requires tight regulation.

In the distal nephron segments of the kidney this mode of regulation is especially important for maintaining total body Na\(^+\) and therefore extracellular fluid balance and arterial blood pressure. A number of factors have been identified as regulators of ENaC including COMMD1 (copper metabolism Murr1 domain 1). This protein factor, which belongs to a family of ten ubiquitously expressed proteins, is involved in a number of distinct cellular processes including inhibition of nuclear factor (NF-)κB. Previous work in our laboratory has shown that both COMMD1 and COMMD3 bind to all three ENaC subunits and subsequently mediate an inhibitory effect on the amiloride sensitive Na\(^+\) current generated by αβγENaC. Based on this knowledge it was hypothesised that in order for the COMMD proteins to have an effect on ENaC, colocalisation and co-expression of these two proteins to the same intracellular compartments and cell types are required in vivo. This was investigated using different mammalian cell lines that are derived from tissues in which COMMD proteins and/or mRNA have previously been identified, as well as in rat kidney. The specific objectives were addressed using Western blot analysis, immunocytochemistry (ICC) and immunohistochemistry (IHC).

Here double label indirect IHC studies have shown for the first time that endogenously expressed COMMD1 and COMMD3 proteins colocalise with αENaC in the principal cells of the cortical and inner and outer medullary collecting ducts. This colocalisation was also shown to hold significance at the intracellular level. ENaC has previously been shown to localise to intracellular vesicular compartments that form part of the endosomal and recycling pathways. Here indirect ICC studies have provided evidence to show that endogenously expressed COMMD1 and COMMD3 proteins localise to the early endosomes. The absence of both COMMD1 and COMMD3 in the Golgi apparatus, which forms part of the secretory pathway, suggests that the COMMD proteins mediate
their effects on ENaC in a post-Golgi compartment, possibly by initiating or promoting endocytosis of ENaC from the apical cell surface.

In summary, these results provide strong evidence to suggest that the interaction between the COMMD proteins and ENaC is not only biochemically significant but also physiologically relevant thus implicating a possible regulatory role for the COMMD proteins on ENaC activity and therefore indirectly the regulation of Na\(^+\) homeostasis.
Acknowledgements
There are a multitude of people I would like to thank for making my brief re-visit to New Zealand not only memorable but also a great success!

My first thanks goes out to Dr Fiona McDonald. Thank you once again for taking me on as your student. You are an exceptional supervisor, teacher and role model and naturally the number one on my list of ‘recommended supervisors’!

To my friends and fellow students within the Department of Physiology. Thank you for your help, support, advice and social company. I would like to especially thank Ray Bartolo (immunochemistry), Mike Gill (Western blots and tissue retrieval), Toni Alsop (confocal microscope) and YongFeng Liu (general lab work). To my MSc advisory committee members: Kirk Hamilton (chair) and Rebecca Campbell – thank you for your advice, praise and words of encouragement. To Andrew McNaughton (Otago Centre for Confocal Microscopy) and Mandy Fisher (histology) for their technical expertise. To Kieran Garbutt and Paul Treadwell for their computer support especially while I was writing up. Thanks to Jenny Bedford for the AQP2 antibodies, Sharad Kumar for the ENaC antibodies and Alex McLellan for the Raji cells.

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<thead>
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<tbody>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>mm</td>
<td>millimeters</td>
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<tr>
<td>μm</td>
<td>micrometer</td>
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<td>adaptor protein 2</td>
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<td>ammonium persulfate</td>
</tr>
<tr>
<td>AQP2</td>
<td>aquaporin 2</td>
</tr>
<tr>
<td>ASDN</td>
<td>aldosterone sensitive distal nephron</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>C</td>
<td>Celsius</td>
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<td>Ca^{2+}</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>channel activating proteases</td>
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<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
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<td>carboxy terminal</td>
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<tr>
<td>Cl^{-}</td>
<td>chloride</td>
</tr>
<tr>
<td>CO_{2}</td>
<td>carbon dioxide</td>
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<td>COMMD</td>
<td>copper metabolism \textit{Murr1} domain</td>
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<tr>
<td>COS-7</td>
<td>CV-1 in origin, and carrying the SV40 genetic material 7</td>
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DAB  3,3’-diaminobenzidine tetrahydrochloride
DAPI  4’-6-diamidino-2-phenylindole
DCT  distal convoluted tubule
DMEM  Dulbecco’s modified Eagle’s medium
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
dpi  dots per inch
DPX  di-n-butylPhthalate in Xylene
EDTA  ethylenediaminetetraacetic acid
EEA1  early endosomal antigen 1
ENaC  epithelial sodium (Na+) channel
ER  endoplasmic reticulum
FCS  fetal calf serum
FRET  fluorescence resonant energy transfer
g  gravity force
GST  glutathione S-transferase
H+  hydrogen ion
h  hour
H2O  water
HECT  homologous to E6-associated protein C terminus
HEK293  human embryonic kidney 293
HIER  heat-induced epitope retrieval
HIF-1  hypoxia inducible factor 1
HIV-1  human immunodeficiency virus 1
HRP  horseradish peroxidase
ICC  immunocytochemistry
IgG  immunoglobulin G
IHC  immunohistochemistry
IκB  inhibitor of κappa B
IPTG  isopropyl β-D-1-thiogalactopyranoside
kDa  kilo Dalton
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<tr>
<th>Term</th>
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<tr>
<td>K⁺</td>
<td>potassium ion</td>
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<tr>
<td>LAMP1</td>
<td>lysosomal-associate membrane protein 1</td>
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<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>mA</td>
<td>milliamperes</td>
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<tr>
<td>mQH₂O</td>
<td>milli-Q H₂O</td>
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<td>NGS</td>
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<td>O₂</td>
<td>oxygen</td>
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<td>OD</td>
<td>optical density</td>
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<td>phosphate-buffered saline</td>
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<td>proximal convoluted tubule</td>
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</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethyl-sulfonyl fluoride</td>
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<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<td>proline-tyrosine</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide-sensitive factor attachment receptor</td>
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<td>TBS</td>
<td>Tris-buffered saline</td>
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xiii
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>TBST</td>
<td>Tris-buffered saline containing Tween-20</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethlenediamine</td>
</tr>
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<td>UV</td>
<td>ultra violet</td>
</tr>
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<td>V</td>
<td>volts</td>
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<td>v/v</td>
<td>volume per volume</td>
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<td>WGA</td>
<td>wheat germ agglutinin</td>
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<tr>
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<td>weight per volume</td>
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<tr>
<td>WW</td>
<td>tryptophan-tryptophan</td>
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<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
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1. Introduction

1.1. The Epithelial Sodium Channel - ENaC

1.1.1. ENaC: A member of the ENaC/Degenerin (DEG) gene family

The epithelial sodium channel (ENaC) belongs to the ENaC/Degenerin (DEG) gene family that was discovered in the early 1990s (Canessa et al., 1993; Canessa et al., 1994). Protein products of the ENaC/DEG genes range from approximately 530 to 740 amino acids each and all encode non-voltage gated amiloride-sensitive sodium (Na\(^+\)) selective channels that are involved in a number of distinct physiological functions, including Na\(^+\) transport, neurotransmission, mechanotransduction and nociception across invertebrates and mammals (Canessa et al., 1993; Canessa et al., 1994; Mano and Driscoll, 1999). The wide range of tissues in which members are expressed, including transporting epithelia and neuronal excitable tissues, reflects this functional diversity.

Despite being activated by a variety of stimuli, ENaC/DEG family members share conserved functional domains that are involved in the control of channel activity and in the formation of the pore. Sequence similarity between the different ENaC/DEG subfamilies is estimated to be between 15 - 20% whereas within a specific subfamily sequence conservation is slightly higher at approximately 30 - 60% (Kellenberger and Schild, 2002).

The membrane topology of the ENaC/DEG family members has been determined using ENaC as a model (Canessa et al., 1993; Renard et al., 1994; Snyder et al., 1994; Voillley et al., 1994). Based on the overall sequence homology and hydrophobicity profiles of the ENaC/DEG family members it is thought that all members share a common predicted membrane organisation. There appears to be two membrane-spanning motifs that are separated by a large extracellular loop comprising more than half of the protein with cytosolic amino (N) and carboxy (C) termini (Figure 1.1.). The N and C terminal domains are targets for protein-protein interactions whereas the large extracellular domain contains several conserved cysteine proteolytic cleavage and glycosylation sites however the functional significance of the latter remains unknown (Kellenberger and Schild, 2002; Rotin et al., 2001).
Figure 1.1. Predicted membrane topology of the ENaC/DEG family members

Based on the overall sequence homology and hydrophobicity profiles of the ENaC/DEG members it is reasonable to assume a common membrane organisation. There are two membrane-spanning domains (represented by the two green cylinders) that are separated by a large extracellular loop and cytoplasmic N and C termini. ‘Out’ indicates the lumen or extracellular compartment whereas ‘in’ refers to the intracellular compartment.

As the focus of this project is based around the regulation of ENaC more detail related to ENaC structure, function, regulation and in particular cellular and intracellular localisation will be discussed.

1.1.2. A brief introduction to ENaC

ENaC is a membrane constituent of many polarised epithelial cells throughout the body including those in the kidney, lung and colon (Garty and Palmer, 1997). ENaC is expressed as a protein complex on the apical cell surface where it provides a regulated pathway for Na\(^+\) to enter the cell from the lumen. Four similar ENaC subunits have been identified in mammals to date and are designated as α, β, γ and δENaC. α, β and γENaC are typically referred to as the ‘classic’ ENaC subunits and are preferentially expressed as heteromultimers around a central pore (Firsov et al., 1998; Staruschenko et al., 2005). ENaC exhibits a number of properties that allow it to be biophysically characterised (Palmer, 1992; Palmer and Frindt, 1986a). These include a single-channel conductance of 4-5 pS for Na\(^+\) ions (Frings et al., 1988; Hamilton and Eaton, 1985; Palmer and Frindt, 1986a), a high selectivity for Na\(^+\) over K\(^+\) (greater than 10:1) (Ling et al., 1991; Palmer and Frindt, 1986b) and is blocked by luminal addition of amiloride (K\(_i\) = 0.1 – 0.5 μM) (Palmer, 1992).
The formation of a functional $\alpha\beta\gamma$ENaC pore is dependent on the presence of $\alpha$ENaC (Canessa et al., 1993; Lingueglia et al., 1993; McDonald et al., 1994). This finding has been supported by work conducted by Hummler et al. (1996). Transgenic mice in which the $\alpha$ subunit of ENaC was knocked out provided evidence to show that proper assembly and expression of a functional ENaC is dependent on $\alpha$ENaC (Hummler et al., 1996). Further work performed on ENaC showed that the biophysical properties of the native ENaC channel could be reconstituted when $\alpha\beta\gamma$ENaC are co-expressed in Xenopus oocytes (Canessa et al., 1994; McDonald et al., 1995; Palmer and Frindt, 1986a). Based on these data it is reasonable to propose that $\alpha\beta\gamma$ENaC co-assemble in vivo to form a functional channel at the apical cell surface.

The composition and stoichiometry of ENaC remained a matter of controversy until just recently when Gonzales et al. demonstrated chicken ASIC1, which is related to ENaC in amino acid sequence, to exist as trimers using crystal structure analysis (Gonzales et al., 2009). This finding has been supported by both atomic force microscopy (Carnally et al., 2008) and biochemical analysis of ASIC1 (Zha et al., 2009). This work, by extension, provides strong evidence that the related ENaC and DEG family members also exist as trimers, thus ending the stoichiometry controversy.

Despite sharing 37% sequence identity with $\alpha$ENaC, the fourth ENaC subunit, called $\delta$ENaC, exhibits a tissue distribution that is distinct from that of the classic ENaC subunits (Waldmann et al., 1995; Yamamura et al., 2004). Instead, $\delta$ENaC shows a similar tissue distribution to the mammalian acid sensing ion channels (ASICs); it is predominantly expressed in neuronal tissues, in particular the brain however lower levels of mRNA have also been detected in the testis, ovary, heart, kidney and pancreas of humans. This is indicative of a role for $\delta$ENaC that is principally non-epithelial. Interestingly, when expressed in Xenopus oocytes and Chinese hamster ovary cells, a reduction in extracellular pH constitutively activates $\delta$ENaC. The activity of this response is distinct from that of any of the ASICs expressed in the brain and is abolished by submicromolar concentrations of amiloride, a response that is also a characteristic feature of the classic ENaC subunits. The acid-evoked response has only been observed in
channels formed by the δ subunit of ENaC. This is suggestive of a physiological function that is unique from that of the classic ENaC subunits.

1.1.3. Mechanism of transepithelial Na\(^+\) transport

The basic model for transepithelial Na\(^+\) transport was formulated by Koefoed-Johnsen and Ussing in the 1950s (Koefoed-Johnsen and Ussing, 1958). This ‘two-membrane hypothesis’ was developed to help explain the mechanism by which Na\(^+\) transport in the frog skin epithelium occurred. This model has since been successfully applied to a number of other transporting epithelia (Reuss, 1997). The basic mechanism of ENaC-mediated transepithelial Na\(^+\) transport in the lung, kidney, colon, sweat and salivary ducts is a variation on this classic ‘two-membrane’ model of epithelial transport.

The process of Na\(^+\) absorption via ENaC involves a two-phase reaction that requires the assistance of the basolateral Na\(^+\)-K\(^+\)-ATPase pump (Figure 1.2.). Together these two protein complexes mediate electrogenic transepithelial Na\(^+\) transport from the lumen to the blood. The initial phase of Na\(^+\) absorption involves luminal Na\(^+\) ions entering the cell via the pore of ENaC by means of electrodiffusion. Intracellular Na\(^+\) is actively pumped out across the basolateral membrane via the Na\(^+\)-K\(^+\)-ATPase in exchange for K\(^+\). The stoichiometry of this process is 3 Na\(^+\) exchanged for 2 K\(^+\) per ATP hydrolysed. This process maintains intracellular Na\(^+\) concentrations low (approximately 15 mM) and intracellular K\(^+\) concentrations high (approximately 120 mM) thereby creating an electrochemical gradient across the apical membrane that allow Na\(^+\) ions in the lumen to passively diffuse across the apical membrane via ENaC. Depending on the physiological setting, K\(^+\) ions actively entering the cell via the Na\(^+\)-K\(^+\)-ATPase can be either recycled back across the basolateral membrane by K\(^+\) selective channels to conserve extracellular K\(^+\) (Lachheb et al., 2008), or conversely, excess K\(^+\) can be excreted into the lumen via the apical renal outer medullary potassium 1 (ROMK1) channel (Gray et al., 2005). Electrogenic Na\(^+\) reabsorption creates a lumen negative electrical potential and thereby a passive driving force that results in chloride (Cl\(^-\)) reabsorption via the transcellular pathway and the osmotic uptake of water (H\(_2\)O) via the paracellular path or through aquaporins (i.e. water channels). Consequently, ENaC plays a critical role in the
movement of electrolytes and H$_2$O across epithelia. Impaired function and regulation of ENaC leads to a number of diseased states including respiratory syndromes that are characterised by excessively wet (Hummler et al., 1996) or dry (Mall et al., 2004) airway spaces as well as inheritable forms of hyper- and hypotension that are caused by gain and loss of ENaC function, respectively (Chang et al., 1996; Kuhnle, 1997; Shimkets et al., 1994; Strautnieks et al., 1996).

**Figure 1.2. Transepithelial Na$^+$ transport in cortical CD principal cells**

ENaC provides a regulated pathway for Na$^+$ entry into the cell across the apical membrane and is actively pumped out across the basolateral membrane into the blood via the Na$^+$-K$^+$-ATPase. K$^+$ channels resident on the apical and basolateral membranes mediate K$^+$ secretion and recycling, respectively. Together these channels set up the necessary electrochemical gradients required for Na$^+$ ions to passively enter the cells via ENaC.

### 1.1.4. Physiological functions of ENaC

As alluded to previously, ENaC performs different functional roles in the various tissues in which it is expressed. Its function is particularly important for reabsorption of fluid and salt in the kidney and colon and for clearing airspace fluid in the lung at the time of birth (Garty, 1994; Garty and Palmer, 1997). Along with these functions, the role of ENaC in excretory ducts of salivary and sweat glands as well as non-epithelial tissues are discussed in more detail below.

#### 1.1.4.1. Kidney

The kidney is the most important site of ENaC expression in terms of regulating extracellular fluid balance and therefore blood pressure (Garty and Palmer, 1997). The distal tubule and the collecting duct system are sites for the final adjustment of Na$^+$ and
H₂O content of the body. These segments are sensitive to a number of hormones, including aldosterone. Aldosterone acts to upregulate the activity of ENaC in these nephron segments and thus, the rate of Na⁺ reabsorption. Na⁺ ions constitute the major extracellular osmotic component and its transport is coupled with fluid movement; hence, where Na⁺ goes, H₂O follows (Boron and Boulpaep, 2005). Because extracellular fluid volume is directly linked to blood pressure, ENaC plays an important role in regulating overall arterial blood pressure.

This project partly aimed to investigate endogenous colocalisation of ENaC and the COMMD proteins in native tissue, in particular the kidney, thus it is relevant to briefly introduce the kidney’s basic functions and anatomy. More detail relating to the COMMD proteins are discussed elsewhere (refer to section 1.2.).

**Function of the kidney**

The kidneys are the principal organs of the urinary system, which consists of paired kidneys and their associated ureters and a single urinary bladder and urethra (Kierszenbaum, 2007) (figure 1.3.).

![Figure 1.3. The urinary system](http://www.kidney.niddk.nih)

The urinary system is composed of paired kidneys and their associated ureters, a single urinary bladder and urethra. This image is not copyrighted. Retrieved from: [http://www.kidney.niddk.nih](http://www.kidney.niddk.nih)

The urinary system is critical for a number of physiological functions including removal of metabolic waste products from the plasma, regulation of plasma hydrogen (H⁺) ion concentration (i.e. pH), plasma ionic composition, osmolarity and volume. The latter has a direct effect on total blood volume and therefore, arterial blood pressure. These
functions are basic requirements for animal life and are carried out in the kidneys by means of filtration, reabsorption and secretion. Consequently the kidney is one of the major homeostatic devices of the body. The importance of proper kidney function becomes apparent during diseased states of the kidney, such as renal failure, which results in impaired electrolyte and acid-base homeostasis, derangement of extracellular fluid volume and retention of nitrogenous waste. In addition to this, the kidneys also produce a number of humoral agents thereby acting as endocrine organs (Bray et al., 1999). Aldosterone release from the adrenal cortex is limited by the enzyme renin, which in turn is released from juxtaglomerular cells in the kidney in response to low arterial blood pressure or renal perfusion pressure (Laragh and Sealey, 1992). This mechanism is known as the renin-angiotensin mechanism and is discussed in more detail in section 1.1.5.1. The kidneys also secrete erythropoietin, a stimulant of red blood cell production, and activate 1,25-hydroxycholecalciferol, a vitamin D derivative involved in promoting calcium (Ca\(^{2+}\)) absorption from the digestive tract (Kierszenbaum, 2007).

Anatomy of the kidney
The kidneys are divided into two major internal structures: the renal cortex (subdivided into outer cortex and juxtamedullary cortex) and medulla (subdivided into the outer and inner medulla) (Thibodeau and Patton, 2003). These structures refer to the outer and inner regions of the kidney, respectively, and are best viewed when sectioned in the coronal plane (figure 1.4.).
The cortex and medulla represent the inner and outer regions of the kidney, respectively. The medulla consists primarily of the renal pyramids. The areas in between the pyramids are projections of the cortex that are referred to as the renal columns (Gray, 2000). This image is in the public domain because its copyright has expired.

Each kidney is made up of approximately one million nephrons, which in turn constitutes the basic functional unit of the kidney (Boron and Boulpaep, 2005). A nephron consists of a glomerulus and a tubule (Figure 1.5.). The former is a cluster of blood vessels from which the nephric filtrate forms whereas the latter is an epithelial structure that contains segments of diverse structure and transport characteristics through which the filtrate passes. The tubules are divided into the proximal convoluted tubule (PCT), thin descending and thin ascending limbs of the loop of Henle (‘thin’ segments), thick ascending limb of the loop of Henle (‘thick’ segments) and the distal convoluted tubule (DCT). Strictly speaking this is the last segment of the nephron proper. Beyond this lies the branched collecting duct system that is made up of connecting tubules and collecting ducts (CD). Several nephrons converge onto a single CD to accumulate the remaining filtrate (i.e. the urine) into larger CD referred to as the ducts of Bellini. The CDs begin in the renal cortex and descend through the cortex in medullary rays. The urine is then carried through the renal pyramids where they become medullary CD. The ducts traverse the outer medulla as unbranched tubes but merge within the inner medulla, increase in size and terminate into the ducts of Bellini where urine flows to the bladder via the ureters (Bray et al., 1999).
A nephron consists of a glomerulus and tubule. The latter is divided into different segments based on morphology and transporting properties. Following the glomerulus, these segments are the proximal (convoluted) tubule (PCT), thin descending and thin ascending limbs of the loop of Henle (thin), thick ascending limb of the loop of Henle (thick) and the distal (convoluted) tubule (DCT). Beyond this lies the branched collecting duct system that is made up of connecting tubules and collecting ducts (CD).

Nephric filtrate filters through capillary walls and collects in the glomerular space (or Bowman’s space) within the glomerular capsule. The filtrate consists primarily of water, ions, glucose, organic acids, amino acids and urea. The filtrate contained within Bowman’s space drains into the first part of the renal tubule located within the renal cortex, the proximal convoluted tubule (PCT). This segment is a ‘leaky’ epithelium that is specialised for bulk reabsorption of the filtered fluid back into the circulation. An extensive brush border covers the luminal surface of the PCT. This provides a large surface area to volume ratio that allows bulk reabsorption of the filtrate. All the filtered glucose, most of the amino acids and approximately two thirds of the filtered H$_2$O, Na$^+$ and Cl$^-$ is reabsorbed by the PCT by means of co- and counter-transporting mechanisms (Bray et al., 1999). As these electrolytes are reabsorbed from the filtrate a large volume of H$_2$O follows by osmosis. ENaC does not appear to be involved in transepithelial Na$^+$ transport in the PCT (Duc et al., 1994; Garty and Palmer, 1997).
Following the PCT the nephric filtrate flows into the descending and then the (thin and thick) ascending segments of the loop of Henle. As a whole the loop of Henle participates in the formation of concentrated or dilute urine. However, the mechanism by which this is achieved will not be discussed here. The descending loop of Henle descends down the medulla where it forms a hairpin turn in the inner medulla. The ascending segment projects back into the cortex. The squamous cells contained within the descending loop of Henle are freely permeable to water, but much less so to \( \text{Na}^+ \), \( \text{Cl}^- \) and urea. The cuboidal cells in the ascending segment are impermeable to \( \text{H}_2\text{O} \), somewhat permeable to urea and highly permeable to \( \text{Na}^+ \) and \( \text{Cl}^- \) (Bray et al., 1999). Following the thick ascending loop of Henle the nephron continues as the DCT.

The DCT begins in a region where specialised epithelial cells of the ascending segment of the loop of Henle contact its glomerulus. This region is referred to as the macula densa. The DCT terminates at the transition to the connecting tubule, which in turn ends at the transition to the CD. These segments are representative of ‘tight’ epithelia. Several hormones, such as aldosterone and vasopressin, act on these segments to deliver their actions. These include up-regulation of ENaC and aquaporin-2 (AQP2) activity thereby regulating \( \text{Na}^+ \) and \( \text{H}_2\text{O} \) balance, respectively (Laragh and Sealey, 1992; Nielsen et al., 1999; Verrey et al., 2008). Of particular interest is the expression of ENaC in the aldosterone sensitive regions of the distal nephron (ASDN): the connecting tubule, cortical CD and outer and inner medullary CD (Duc et al., 1994; Hager et al., 2001). These segments are responsible for the final adjustment of total body \( \text{Na}^+ \) and \( \text{H}_2\text{O} \) via ENaC and AQP2. In the CD (or inner medullary CD for AQP2 specifically), these membrane proteins are confined to the apical regions of kidney principal cells, which in turn make up approximately two thirds of the cells in the distal renal tubules. Some of the first evidence for this was demonstrated in an experiment conducted by O’Neil and Hayhurst (O’Neil and Hayhurst, 1985). Principal and intercalated cells in isolated perfused rabbit cortical CD were differentiated by fluorescent microscopy. Subsequent current-induced swelling was only observed in principal cells. Luminal addition of amiloride inhibited this current-induced swelling and this demonstrated that ENaC is involved in the \( \text{Na}^+ \) absorption process.
It is important to highlight here that AQP2 is confined to some of the same nephron segments as ENaC in the kidney: the connecting tubules and CD. However, the polarisation of AQP2 differs in some of these segments when compared with ENaC, which is present in the apical cell surface and/or subapical intracellular vesicles. AQP2 is present in the apical cell surface and in subapical vesicles along the length of the connecting tubule to the inner medullary CD (i.e. this includes the cortex and outer medullary CD principal cells), and in the basolateral cell surface of the connecting tubule and inner medullary CD (i.e. this excludes the cortex and outer medullary CD principal cells) (Nejsum, 2005). Despite the difference in polarisation of ENaC and AQP2 in some nephron segments, localisation of these two proteins to some of the same nephron segments is advantageous in the sense that it allows AQP2 antibodies to be utilised as markers for ENaC specific segments when performing immunohistochemistry (IHC) on the kidney.

1.1.4.2. Lung
In contrast to the kidney, Na\(^{+}\) transport in the lung is not involved in overall Na\(^{+}\) balance. Instead ENaC, which appears to be the major Na\(^{+}\) channel in the lung, plays a critical role in clearing liquid secreted during fetal life at birth. Along with the cystic fibrosis transmembrane conductance regulator (CFTR), ENaC is also involved in maintaining the optimum hydration levels and composition of the fluid lining the airway (Garty and Palmer, 1997). The physiological importance of ENaC in the lung becomes especially apparent during certain pathophysiological states that arise from either abnormal or diseased conditions.

The role of ENaC in fetal lung clearance has been demonstrated in both heterologous expression systems and animal studies. Previously it has been shown that α-, β- and γENaC are highly expressed in the adult lung (McDonald et al., 1994; McDonald et al., 1995). However, α-, β- and γENaC mRNA appear to be differentially regulated during fetal development (Talbot et al., 1999; Tchepichev et al., 1995). In particular, αENaC mRNA expression has been shown to be upregulated towards late gestation and at birth, a response that is not observed in the kidney therefore modulation of the Na\(^{+}\) transport
system around the time of birth is specific to the lung (Dagenais et al., 1997). The importance of fetal lung liquid in the regulation of pulmonary development has been demonstrated previously and it has also been shown that the degree of lung maturity affects its ability to clear the fetal lung of liquid around the time of birth (Alcorn et al., 1977; Brown et al., 1983). Previous in vitro studies have demonstrated that proper assembly and expression of functional ENaC is dependent on αENaC (Canessa et al., 1994; Hummler et al., 1996). Along with the observation that luminal addition of amiloride to the lungs delays lung liquid clearance in newborn guinea pigs (O'Brodovich et al., 1990), these data is consistent with a role for Na\(^{+}\) transport in liquid clearance of the perinatal lung. In order to assess the role of ENaC-mediated Na\(^{+}\) transport in the lung, Hummler et al. (1996) produced αENaC-deficient mice by gene targeting (Hummler et al., 1996). No measureable amiloride sensitive Na\(^{+}\) current could be detected in the airway epithelia of these mice and neonates developed respiratory distress that resulted in death within 40 h of birth. This is consistent with previous findings that suggest loss of ENaC function is associated with respiratory distress syndrome (Keszler and Sivasubramanian, 1983; Malagon-Rogers, 1999). Hummler et al. (1996) concluded that death resulted from an impaired ability to absorb fetal lung liquid (Hummler et al., 1996). Combined, these data provides and supports strong evidence that αENaC is required for proper assembly and expression of functional ENaC, that the developmental expression of αENaC is critical for neonatal adaptation and that proper ENaC function and lung maturity is critical in the transition of the fluid-filled fetal lung to an air-filled absorptive organ around the time of birth.

Conversely, gain of ENaC function due to impaired regulation by CFTR is thought to be a major contributing factor leading to the clinical manifestations of cystic fibrosis (CF): depletion of airway surface liquid volume, impaired mechanical clearance of mucus, and infection (Boucher et al., 1986; Knowles and Boucher, 2002; Matsui et al., 1998). It is noteworthy to mention that CFTR is an epithelial cAMP-activated Cl\(^{-}\) channel that is expressed in the lung, colonic epithelium and sweat ducts, as well as a number of other tissues not mentioned here. An interaction between CFTR and ENaC has been functionally demonstrated by cAMP-dependent stimulation of wild type CFTR, which
leads to an inhibition of ENaC activity (Boucher et al., 1989; Briel et al., 1998; Chabot et al., 1999; Mall et al., 1996; Stutts et al., 1995). This suggests that in CF affected lungs, dysfunctional CFTR results in enhanced Na\(^+\) absorption and impaired cAMP-dependent Cl\(^-\) secretion. Indeed, overexpression of ENaC in a βENaC transgenic lung mouse model has been shown to generate the CF phenotype thus providing further evidence of CFTR-mediated inhibition of ENaC (Mall et al., 2004).

1.1.4.3. Colon

αENaC was initially cloned from the distal colon of Na\(^+\) deprived rats (Canessa et al., 1993). The mRNA encoding all three ENaC subunits as well as protein were subsequently shown to be confined to the aldosterone-responsive surface epithelial cells where ENaC is thought to prevent excessive Na\(^+\) loss in the stools (Duc et al., 1994; Garty and Palmer, 1997; Lingueglia et al., 1994; Renard et al., 1995). As mentioned previously, CFTR has an inhibitory effect on ENaC activity. However during bouts of secretory diarrhea there is pronounced activation of CFTR which in turn leads to enhanced inhibition of ENaC (Kunzelmann and Mall, 2002). This creates an imbalance between secretion and absorption of electrolytes. A compensatory up-regulation of ENaC has been reported in the colon under these circumstances (Schultheis et al., 1998). This mechanism is impaired in inflammatory bowel disease and the subsequent defective electrogenic Na\(^+\) absorption has been identified to contribute to diarrhea in both Crohn’s disease and ulcerative colitis (Bergann et al., 2009; Greig et al., 2004; Sandle et al., 1990).

1.1.4.4. Sweat and salivary ducts

High levels of ENaC mRNA and protein have been detected in sweat and salivary ducts (Duc et al., 1994). Similar to the lung and colon, ENaC works in conjunction with CFTR in these tissues. The predominant function of ENaC here is to retrieve salt from the primary secretions (Garty and Palmer, 1997).
1.1.4.5. Non-epithelial tissues
ENaC has been implicated as playing a major role in vascular smooth muscle cell (VSMC) mechanotransduction, which in turn mediates myogenic vasoconstriction (Jernigan and Drummond, 2005). Golestaneh et al. (Golestaneh et al., 2001) established the presence of ENaC in vascular endothelial cells and subsequent work performed by Drummond et al. (Drummond et al., 2004) conclusively demonstrated the presence of β- and γENaC mRNA and protein in rat VSMC. The dominant non-epithelial-like tissue distribution of δENaC and its ability to functionally substitute for αENaC all implicate a likely role for δENaC in mechanotransduction, possibly together with β- and γENaC. In addition to this, the activity of δENaC appears to be regulated by protons thus a role in proton-activated currents in the human brain has also been proposed for δENaC (Waldmann et al., 1995; Yamamura et al., 2004). Similar to ASIC1, which also belongs to the ENaC/DEG gene family and responds to changes in pH, this may implicate a role for δENaC in long-term potentiation in the brain, whereas in the heart its response to acidification suggests a role in ischemic pain (Bianchi and Driscoll, 2002; Reeh and Steen, 1996).

1.1.5. Regulation of ENaC
ENaC provides the rate-limiting step for Na\(^+\) reabsorption from the lumen to the blood therefore the rate at which epithelial Na\(^+\) reabsorption occurs is largely determined by mechanisms that control the activity and expression of ENaC at the apical cell surface. This contrasts to voltage and ligand-gated ion channels, which are regulated by changes in gating mechanics (Snyder, 2005). The regulation of ENaC may be accomplished at multiple levels and its activity is influenced by both extrinsic factors, such as aldosterone, and intrinsic factors, such as ENaC trafficking (Bhalla and Hallows, 2008).

The rate at which epithelial Na\(^+\) reabsorption occurs via ENaC can be translated into the amount of current generated per unit area of apical membrane (Kemendy et al., 1992). This current is the result of Na\(^+\) ions moving across the membrane and is referred to as $I_{Na}$. $I_{Na}$ is largely determined by variables that control the activity and expression of ENaC at the apical cell surface. These include the single channel conductance ($g$), the
number of channels present on the apical cell surface ($N$) and the single channel open probability ($P_o$). The relationship between these variable can be summarised by the following equation:

$$I_{Na} = g_N P_o$$

Altering any one of the above variables provides a mechanism for regulating the amount of $Na^+$ ions that are being reabsorbed, or alternatively, the value of $I_{Na}$.

At the protein level, ENaC is primarily regulated by mechanisms that alter either the rate of insertion/retrieval of the channel from the apical membrane (i.e. $N$) or the $P_o$ of the channel (Butterworth et al., 2009). Increased expression of ENaC at the apical cell surface can be achieved by increasing protein synthesis and delivery (or exocytosis), by decreasing the rate of retrieval (or endocytosis) of ENaC from the apical membrane or by increasing the rate of delivery of existing channels from subapical intracellular pools (Butterworth et al., 2005). In contrast to this, decreased ENaC surface expression is achieved by increasing the rate of retrieval of ENaC from the apical surface, a process that is mediated by the ubiquitin protein ligase, Nedd4-2 (neural precursor cell expressed developmentally down-regulated gene 4) (Kamynina and Staub, 2002).

A number of extrinsic and intrinsic factors, including several hormones and enzymes, have been identified as being involved in the regulation of ENaC. Some of these factors and their associated effects on ENaC are discussed in more detail below.

### 1.1.5.1. Extrinsic factors

#### Hormones

- **Aldosterone**

  Aldosterone is the primary hormone involved in the maintenance of $Na^+$ balance, extracellular fluid volume and thereby arterial blood pressure (Rossier, 2002; Rossier et al., 1994). Aldosterone secretion is controlled mainly by the renin-angiotensin mechanism and is a long-term regulator of $Na^+$ balance and extracellular fluid volume, $K^+$ balance and effective arterial blood pressure (Laragh and Sealey, 1992). As previously mentioned, the kidneys also act as endocrine organs partly by stimulating the release of
aldosterone from the adrenal cortex. More specifically, the enzyme renin is secreted from the juxtaglomerular cells of the renal afferent arterioles in response to a reduction in arterial blood pressure or renal perfusion pressure. The first reaction in the renin-angiotensin cascade is the cleavage of angiotensinogen (from the liver) by renin with the release of angiotensin I. Angiotensin I circulates to the lungs, the most physiologically important site for the conversion of circulating angiotensin I. Angiotensin-converting enzymes (ACE) resident at the luminal surface of the lung endothelium catalyse the cleavage of angiotensin I to produce angiotensin II. Angiotensin II acts to increase blood pressure in a number of ways. First it induces vasoconstriction of the arteriolar bed in a direct manner and then, on a somewhat slower schedule, it stimulates the adrenal cortex to synthesise aldosterone and release it into the blood (Laragh and Sealey, 1992). Once released into the blood, aldosterone binds to mineralcorticoid receptors that are present in aldosterone sensitive epithelia, including the distal nephron and colon (Garty and Palmer, 1997). Here aldosterone acts to stimulate apical Na$^+$ reabsorption by increasing the apical and basolateral expression of ENaC and Na$^+$/K$^+$-ATPase, respectively. The subsequent increase in blood volume has an inhibitory effect on the renin-angiotensin mechanism thereby completing the feedback loop. This mechanism is only activated again once blood pressure or renal perfusion pressure falls below its set point. The renin-angiotensin system is summarised in figure 1.6.
Circulating angiotensinogen is converted to angiotensin I by the hormone renin, which in turn is released from the kidney in response to low blood pressure or renal perfusion pressure. Angiotensin I circulates to the lung where it is cleaved by angiotensin converting enzymes (ACE) to release angiotensin II. Angiotensin II acts on the adrenal cortex to stimulate the release of aldosterone, which then acts on the distal renal tubules to up-regulate the expression of ENaC. The subsequent increase in Na\(^+\) reabsorption restores blood pressure to set point. This inhibits the release of renin thereby completing the feedback loop.

Aldosterone-mediated effects on ENaC have been extensively studied and it is generally accepted that its natriuretic effects involve two phases. During the initial thirty minutes (or the early phase), aldosterone upregulates the abundance of both ENaC and Na\(^+\)-K\(^+\)-ATPase at the apical cell surface without changing the total cellular expression of these two transporters (Verrey et al., 2008). This effect is thought to be due to the translocation of pre-existing ENaC to or a reduction in the rate of retrieval of ENaC from the apical cell surface thus pointing to trafficking mechanisms that regulate ENaC surface density. The early response has also been shown to depend on the actions of a number of early aldosterone-induced gene products, including serum and glucocorticoid-induced kinase 1 (SGK1) (Chen et al., 1999; Flores et al., 2005; Naray-Fejes-Toth et al., 1999). SGK1 is a serine/threonine protein kinase that is activated by phosphorylation and has previously been shown to modulate the activity of the ubiquitin protein ligase Nedd4-2 and the
interaction between Nedd4-2 and ENaC to promote apical localisation of ENaC in the distal renal tubules (refer to ubiquitination of ENaC under section 1.1.5.2.). A late phase is observed 3-6 hours following aldosterone stimulation in which Na\(^+\) absorption slowly but steadily increases over a matter of days. This response appears to be sustained and is paralleled by a concomitant biosynthetic increase of both ENaC and Na\(^+\)-K\(^+\)-ATPase. Aldosterone preferentially increases αENaC transcription (Masilamani et al., 1999; McTavish et al., 2009), which in turn enhances proper channel assembly and therefore trafficking of ENaC from the endoplasmic reticulum (ER) to the apical cell surface. More specifically, aldosterone binds to mineralcorticoid receptors, which belong to the family of nuclear receptors, present in the cytosol. Upon binding, the ligand-receptor complex translocates to the nucleus where it binds to the promoter regions of a large number of genes and subsequently induces their transcription. Corticosteroids, via glucocorticoid receptors, increase Na\(^+\) transport in a similar manner.

- **Vasopressin**

Vasopressin has been shown to up-regulate both ENaC and AQP2 to increase Na\(^+\) and H\(_2\)O absorption, respectively (Djelidi et al., 1997; Ecelbarger et al., 2000; Nicco et al., 2001; Nielsen et al., 2002). Vasopressin is released from the hypothalamus in response to increased extracellular fluid osmolality (Verney, 1947). Similar to aldosterone, vasopressin has both long- and short-term actions in the kidney that are primarily mediated by intracellular cyclic adenosine monophosphate (cAMP) levels. Vasopressin mediates its effects by binding to vasopressin V\(_2\) receptors at the basolateral membrane, which is coupled to adenylyl cyclase via G proteins. Once activated, adenylyl cyclase converts adenosine triphosphate (ATP) to cAMP, which in turn activates protein kinase A (PKA). Over the short-term, vasopressin increases Na\(^+\) absorption by increasing the apical membrane density of ENaC. This has been shown to be the result of ENaC trafficking to the apical membrane from a recycling subapical channel pool (Butterworth et al., 2005; Lu et al., 2007; Snyder, 2000). It is thought that PKA and SGK1 regulate ENaC through convergent phosphorylation of Nedd4-2. Thus, vasopressin and aldosterone act synergistically to stimulate Na\(^+\) absorption in the renal distal tubules (Snyder et al., 2004b). Over a longer time frame, vasopressin has been shown to increase
Na$^+$ transport through transcriptional control of ENaC, in particular that of β- and γENaC (Butterworth et al., 2005; Djelidi et al., 1997; Ecelbarger et al., 2000; Morris and Schafer, 2002).

- Insulin

Similar to aldosterone and vasopressin, insulin has been shown to enhance Na$^+$ transport partly by promoting translocation of ENaC to the apical cell surface (Song et al., 2006; Tiwari et al., 2007). The phosphoinositide 3-kinases (PI3K) are key mediators of insulin signaling. The mechanism thought to be responsible for the increase in Na$^+$ reabsorption in response to insulin is considered to be due to PI3K-dependent activation of SGK (Wang et al., 2001).

1.1.5.2. Intrinsic factors

Trafficking of ENaC to the cell surface

Proper assembly of α-, β- and γENaC into a complex is a prerequisite for successful trafficking of ENaC from the ER to the apical cell surface (Butterworth et al., 2008; Snyder, 2005; Valentijn et al., 1998; Weisz et al., 2000). Assembly of this complex, which occurs early during its processing in the ER, is dependent on αENaC. Under a normal salt diet and water repletion (i.e. basal conditions) in the kidney transcription of αENaC is lower than that of β- and γENaC therefore trafficking of ENaC from the ER to the cell surface appears to be an inefficient process. Thus, the presence of ENaC at the apical cell surface is almost undetectable under these conditions. Unassembled or misfolded ENaC subunits are targeted to the proteasomes for degradation whereas properly assembled and mature ENaC complexes are targeted to the lysosome for subsequent degradation by means of specific ubiquitination signals (Staub et al., 1997b). Inactive ENaC is transported from the ER to the Golgi where it undergoes further processing in the form of proteolytic cleavage that converts ENaC to an active channel (Hughey et al., 2004). However, some ENaC appear to bypass this route (i.e. the Golgi) and hence exist as inactive channels on the apical cell surface. This inactive pool can undergo subsequent cleavage and activation by a number of extracellular or membrane-tethered serine proteases including trypsin and channel activating proteases (CAPs).
(Chraibi et al., 1998; Vallet et al., 1997; Vuagniaux et al., 2002). The mechanism responsible for targeting ENaC to the apical cell surface remains unclear but recent work has identified the membrane-localised SNARE (soluble N-ethylmaleimide-sensitive factor attachment receptor) components, including syntaxin 1A and 3, as candidates that mediate fusion of ENaC-containing vesicles with the apical cell surface (Butterworth et al., 2009).

**Ubiquitination of ENaC**

α-, β- and γENaC all contain a conserved proline-tyrosine (PY) motif in their C terminal domains that is thought to be involved in the ubiquitination and subsequent internalisation of ENaC from the apical membrane. This provides a means by which the residency status of ENaC is regulated at the apical cell surface (Goulet et al., 1998; Hansson et al., 1995; Lu et al., 2007; Volk et al., 2001) (Figure 1.7.). The conserved PY motif is absent in the ENaC sequence. This is suggestive of a regulatory mechanism for ENaC that is distinct from that of the classic ENaC subunits (Waldmann et al., 1995). One such mechanism may potentially involve the COMMD {COMM [copper metabolism Murr1 (mouse U2af1-rs1 region 1)] domain} family of proteins (Biasio et al., 2004; Burkhead et al., 2009), which will be discussed in more detail under section 1.7.

**Figure 1.7. Protein sequence alignment of the C-terminal domains of human α, β and γENaC subunits**

The PY motif (indicated in bold green letters) is conserved across -α, -β and -γENaC C termini but not ENaC. Missense mutations or deletion of this PY motif results in over-expression of ENaC at the cell surface therefore this motif is thought to be involved in the regulation of ENaC. The multiple sequence alignment was performed using the T-Coffee::Regular alignment tool available at www.ebi.co.uk. The accession numbers corresponding to each of the mRNA sequences for -α, -β and ENaC are CAB07505, NP_000327, AAK50910 and ABI64068, respectively. Sequences were retrieved from www.ncbi.nlm.nih.gov.
The stability of ENaC at the apical cell surface has been estimated to be relatively short-lived (Butterworth et al., 2009). The ubiquitin protein ligase Nedd4 and its homologue Nedd4-2 have previously been shown to interact with and thereby regulate ENaC surface stability (Abriel et al., 1999; Goulet et al., 1998; Lott et al., 2002; Snyder et al., 2004a; Staub et al., 1996). Nedd4-2 is expressed in the kidney CD and it appears to be the most important Nedd4 family member involved in the \textit{in vivo} regulation of ENaC (Itani et al., 2005; Snyder et al., 2004a).

The process of ubiquitination essentially involves three steps. First the ubiquitin-activating enzyme E1 activates the ubiquitin molecule via an ATP dependent process. The activated ubiquitin molecule is then transferred to the ubiquitin-conjugating enzyme E2 before reaching the third and final step in the ubiquitination cascade, E3. E3 is a ubiquitin protein ligase that recognises and binds to specific target substrates and subsequently labels the substrate with ubiquitin. Nedd4-2 is an E3 ubiquitin protein ligase that is composed of an N terminal C2 domain, three to four WW domains (depending on the species) and a catalytic HECT (homologous to \textit{E6}-associated protein C terminus) domain (Kumar et al., 1992) (Figure 1.8.).

![Figure 1.8. Schematic representation of Nedd4 and its homologue Nedd4-2](image)

The N-terminal C2 domain is thought to localise ENaC to the apical membrane in a Ca\textsuperscript{2+}-dependent manner. Each Nedd4 and Nedd4-2 contain three to four WW domains that mediate protein-protein interactions and a C terminal E3 ubiquitin protein ligase HECT domain.

The C2 domain, which has previously been shown to bind membrane and phospholipids in a Ca\textsuperscript{2+} dependent manner in other proteins, is thought to associate with annexin XIIIb in response to elevated intracellular Ca\textsuperscript{2+} levels thus localising Nedd4-2 to the apical cell surface (Fiedler et al., 1995; Plant et al., 1997; Plant et al., 2000). Nedd4-2, via its WW
interaction motifs, provides substrate recognition capabilities by binding to PY motifs present in the cytoplasmic C terminal domains of ENaC (Hansson et al., 1995; Schild et al., 1996; Snyder et al., 2001; Staub et al., 1996) (Figure 1.9).

Figure 1.9. Ubiquitination of ENaC by Nedd4/Nedd4-2

Inhibition of ENaC by Nedd4/Nedd4-2 is mediated by an interaction between the WW domains of Nedd4/Nedd4-2 and the cytoplasmic C terminal PY motifs in ENaC. Upon binding, the HECT domain of Nedd4/Nedd4-2 transfers ubiquitin (ub, indicated by green circles present on the N terminal tail of the above ENaC subunit) to lysine residues present in the cytoplasmic N terminal tails of the different ENaC subunits. This serves as a signal for ENaC to be internalised thus Nedd4-2 plays an important role in the regulation of ENaC cell surface stability.

Under basal conditions Nedd4-2 primarily exists in an unphosphorylated form. Unphosphorylated Nedd4-2 has a higher affinity for ENaC and upon binding promotes the transfer of ubiquitin, which is bound to its HECT domain, to lysine residues present in the N terminal domain of α- and γENaC (Debonneville et al., 2001; Rotin et al., 2000; Snyder et al., 2002; Staub et al., 1997b). Nedd4-2 can both mono- and multi-ubiquitinate its substrate proteins, including ENaC (Butterworth et al., 2007; Wiemuth et al., 2007). Ubiquitination targets ENaC for endocytosis and/or degradation by the lysosomes. Specific mutations of the β- or γENaC PY motifs disrupt the interaction between Nedd4-2 and ENaC, which results in increased ENaC activity and leads to a hereditary form of hypertension known as Liddle’s syndrome (Schild et al., 1996; Shimkets et al., 1994; Snyder et al., 1995). Moreover, inhibition of the catalytic activity of Nedd4-2 affects its ability to downregulate surface ENaC (Hicke, 1997; Snyder et al., 2004a; Staub et al., 1996).
The presence of ENaC in the apical membranes of distal nephron segments is undetectable by classical immunohistochemical techniques under basal conditions and is only seen when the animal is salt deprived, which in turn provides a physiological stimulus for aldosterone secretion (Loffing et al., 2000; Masilamani et al., 1999; Pacha et al., 1993). The previously mentioned aldosterone-induced protein SGK1 phosphorylates Nedd4-2 under these conditions and subsequently reduces the affinity between Nedd4-2 and ENaC thus it can be concluded that Nedd4-2 negatively regulates the abundance of surface ENaC (Alvarez de la Rosa et al., 1999; Debonneville et al., 2001; Flores et al., 2005; Loffing et al., 2001; Snyder et al., 2002).

**Clathrin-dependent endocytosis**

Ubiquitinated ENaC undergoes clathrin-mediated endocytosis by indirectly binding to the ubiquitin-binding protein epsin, which in turn binds to the medium (μ2) subunit of clathrin-adaptor protein 2 (AP-2) thereby linking ENaC to the clathrin-based endocytic machinery (Wang et al., 2006; Wiemuth et al., 2007). Once internalised ENaC can be either degraded or sorted for recycling back to the apical cell surface (Lu et al., 2007). Using live-cell imaging and immunofluorescence Lu et al. (2007) demonstrated the presence of ENaC in sorting/recycling endosomes as well as late endosomes/lysosomes. Interestingly, cAMP was shown to divert ENaC from the lysosomal to the recycling pathway thereby augmenting the activity of ENaC by mobilising synthesised intact ENaC present in recycling endosomes in the subapical compartment.

**Summary of ENaC trafficking**

Trafficking of ENaC appears to be an important mode of ENaC regulation and thereby Na⁺ homeostasis. By identifying other molecules and pathways involved in the physiological regulation of ENaC more insight can be gained into pathophysiological conditions that arise from ENaC dysregulation. Figure 1.10 summarises the current model on ENaC trafficking.
Figure 1.10. Schematic diagram of ENaC trafficking
Following biosynthesis in the nucleus and endoplasmic reticulum (ER) ENaC is assembled and modified in the Golgi apparatus. From here ENaC is targeted to the apical cell surface via specially targeted vesicles (depicted by green circles). ENaC resident on the apical cell surface is regulated via ubiquitination, which targets ENaC for clathrin-dependent endocytosis via endosomes. Once internalised, ENaC can be either targeted for lysosomal degradation via the late endosomes or it could be recycled back to the apical cell surface via recycling endosomes. The Na\(^+\)-K\(^+\)-ATPase resident on the basolateral cell surface (depicted in grey) is essential in maintaining an electrochemical gradient across the apical cell surface. This is important for allowing passive diffusion of luminal Na\(^+\) ions via ENaC into the cell.

1.1.6. Cellular and intracellular localisation of ENaC
α, β and γENaC subunit proteins are not only expressed in the apical membranes of specific epithelial target cells but have also been identified intracellularly (Duc et al., 1994). The cellular and intracellular investigations regarding ENaC have mainly been performed on the α-, β- and γ-subunits and not δ. The cellular localisation of ENaC has been studied in a number of native tissues including the kidney, lung and colon. This has been investigated at the mRNA and protein level using Northern blot analysis and/or in situ hybridisation and IHC, respectively (Duc et al., 1994; Farman et al., 1997; Loffing et al., 2000; Talbot et al., 1999). In contrast to this, the intracellular localisation of ENaC
has mainly been determined in studies aimed at characterising its processing and trafficking (Rotin et al., 2001). This has primarily been investigated using *Xenopus* A6 CD cells, which expresses ENaC endogenously, and heterologous expression systems such as *Xenopus* oocytes, COS [CV-1 (simian) in origin, and carrying the SV40 genetic material)-7, human embryonic kidney 293 (HEK293) and Madin-Darby canine kidney (MDCK) epithelial cells.

Cell specific localisation of ENaC was initially studied at the mRNA and protein level using *in situ* hybridisation and immunoperoxidase labeling, respectively. Employing these techniques Duc et al. demonstrated that - , - and ENaC were co-expressed in the apical membranes of the renal distal tubule as well as intracellularly. These findings have subsequently been confirmed using more powerful techniques such as those employed by Hager et al. (Hager et al., 2001). Using IHC in combination with laser confocal microscopy all three ENaC subunits were shown to localise to the principal cells of the connecting tubules, cortical CD, and outer and inner medullary CD in kidneys from control rats. In addition to this, all three ENaC subunits were also shown to be present in urothelial cells covering the renal pelvis. Laser confocal microscopy also revealed differential intracellular localisation of the ENaC subunits in both the cortex and outer medulla as well as along the axis of the CD. In the cortical CD and outer medullary CD, ENaC was mostly present in the extreme apical domains including the plasma membrane whereas - and ENaC were associated with intracellular vesicles dispersed throughout the entire cytoplasm. Based on this observation it was concluded that ENaC trafficking is a potential mechanism for the regulation of Na\(^+\) reabsorption (refer to section 1.1.5.). In the inner medullary CD all three subunits were confined to the cytoplasm and labeling for - , - and ENaC gradually decreased along the axis of the CD. The presence of ENaC in both the apical membrane and cytoplasm was confirmed with immunoelectron microscopy.

The segmental distribution of ENaC remains uniform despite variations in dietary Na\(^+\) intake (Loffing et al., 2000). However, high and low dietary Na\(^+\) intake have been shown to influence the intracellular distribution pattern of - , - and ENaC differentially. Staining performed on the kidneys of mice on standard diets (3 weeks) revealed weak ENaC-
related staining in the apical membranes of the DCT and connecting tubule whereas further downstream immunostaining appeared fine and granular throughout the cytoplasm (Loffing et al., 2000). Apical staining in these very distal regions were absent. In response to high dietary Na\(^+\) intake for 3 weeks, no apical staining was detected for ENaC. ENaC was undetectable whereas \(\alpha\) and ENaC were exclusively present in the cytoplasm. In response to low dietary Na\(^+\) intake for 3 weeks, which provides a physiological stimulus for aldosterone secretion by the adrenals, strong apical staining for all three ENaC subunits was detected. Apical staining was most marked in the connecting tubule and from here staining gradually decreased along the length of the distal renal tubules. The cytoplasmic abundance of \(\alpha\) and ENaC gradually increased along the length of the renal distal tubule. It is important to note that induction of ENaC subunits by aldosterone is highly tissue specific (Nakamura et al., 2002).

In the lung, there is significant variation in the timing and distribution of the three ENaC subunits (Nakamura et al., 2002; Talbot et al., 1999; Tchepichev et al., 1995). Northern blot analysis (or mRNA analysis) demonstrated that \(\alpha\) and ENaC expression levels surge in late fetal gestation and reach near adult levels on the first day of postnatal life. In contrast to this, ENaC mRNA expression levels gradually increases through late fetal and early postnatal life and progressively increases until adulthood. Once adulthood is reached, \(\beta\), \(\gamma\) and ENaC mRNA are intensely expressed in small and medium-sized airways whereas in the alveolar region, both \(\alpha\) and ENaC mRNA exhibit a punctate distribution that is consistent with an alveolar type II cell distribution (Farman et al., 1997).

ENaC localises to the surface epithelial cells of the distal colon but not crypts (Duc et al., 1994; Lingueglia et al., 1994). These data is consistent with electrophysiological data previously obtained in which an amiloride sensitive Na\(^+\) current was demonstrated to be confined to the surface epithelium but not the crypts (Kockerling et al., 1993). The distal colon falls into the category of tight epithelia, which are typically responsive to steroid hormones. Thus, similar to the kidney, the localisation of ENaC in the distal colon is influenced by dietary Na\(^+\) intake and steroid hormones. For instance, dexamethasone,
which is a potent synthetic steroid hormone, has been shown to control ENaC activity in the distal colon by stimulating transcription of - and - but not ENaC (Bergann et al., 2009). Under Na⁺- replete conditions, no ENaC signal could be detected by IHC whereas strong apical labeling was detected under Na⁺-deplete conditions.

On a more intracellular level, the localisation of ENaC has been determined using co-localisation with markers for specific intracellular compartments (Butterworth et al., 2009). ENaC has been shown to traffic through the endosomal pathway. Studies investigating ENaC trafficking in endogenously expressing cortical CD cells revealed its presence in early endosomes within minutes of being endocytosed through clathrin-coated pits (Wang et al., 2006). Heterologously expressed ENaC has been localised separately to both recycling and late endosomes as well as lysosomes for degradation (Lu et al., 2007; Saxena et al., 2005; Staub et al., 1997b). Co-localisation studies performed in our lab have demonstrated that δENaC co-localises with COMMD1 in both early and recycling endosomes (Chang, 2007). This suggests that the COMMD proteins influence the endocytic/recycling pathway of ENaC.
1.2. The COMMD Gene Family

The COMMD family of proteins is a group of factors that contain a highly conserved and unique C terminal motif known as the COMM domain (Burstein et al., 2005). This defining motif contains several conserved tryptophan, proline and leucine residues and in total consists of approximately 85 amino acids (Figure 1.11.). A number of fundamentally distinct functions have been ascribed to these factors, which is consistent with their ubiquitous expression pattern. These are discussed in more detail below.

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**Figure 1.11. Alignment of the COMM domains of the human COMMD proteins**

The C terminal domain is highly conserved across all members of the COMMD family and is known as the COMM domain. Highlighted amino acid residues represent those that are conserved between specific COMMD sequences. Light grey, 50-60% conserved; dark grey, 70-80% conserved; green, 90-100% conserved. The mRNA sequences were retrieved from www.ncbi.nlm.nih.gov. The accession numbers corresponding to each of COMMD1-10 are AAH09266, AAS22240, AAS22241, AAS22242, NP_054785, AAS22243, AAS22244, AAS22245, AAS22246 and AAS22247. The multiple sequence alignment was performed using the T-Coffee::Regular alignment tool available at www.ebi.co.uk.

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1.2.1. Identification of Murr1/COMMD1

COMMD1 (previously *Murr1*) was initially identified as a gene located around the imprinted mouse gene *U2af1-rs1* and was therefore designated as *Murr1* (mouse *U2af1-rs1* region) (Nabetani et al., 1997). More specifically, *U2af1-rs1* is located within the first intron of the *Murr1* gene. These two genes essentially compose two independent genes that are transcribed in opposite directions. This was the first reported example of an endogenous imprinted gene found in the intron of another gene. The genomic organisation of the *U2af1-rs1* and *Murr1* loci is unique to the mouse and has not been
observed in other species, including human. *U2af1-rs1* and *Murr1* are located on chromosome 11 in the mouse whereas the human homolog of *Murr1* has been mapped to chromosome 2 and there are no corresponding counterparts of mouse imprinted gene *U2af1-rs1* in human.

Subsequent to its initial designation, the *Murr1* gene was identified as the disease-causing gene in copper toxicosis in Bedlington terriers (refer to section 1.2.6.) hence it was designated as the copper metabolism *Murr1* gene (van De Sluis et al., 2002). Additional homologs of *Murr1* have since been identified and are now collectively referred to as the COMMD family (Burstein et al., 2005). All ten family members are present in vertebrates with orthologs in both mammals and fish being significantly conserved. All ten proteins are also present in *Dictyostelium discoideum* (a soil-living amoeba), which contains 8,000 – 10,000 genes in its genome with many showing a high degree of sequence similarity to those in vertebrate species. Several COMMD genes have also been identified in *Drosophila*, *Caenorhabditis* and several unicellular protozoa (Eichinger et al., 2005; Maine and Burstein, 2007b). This suggests that the COMMD proteins are likely to perform a common function across species and have therefore been widely conserved throughout evolution.

1.2.2. Discovery of the COMMD family

Members of the COMMD family were first identified in a biochemical screen for COMMD1 (or *Murr1*) – associated factors (Burstein et al., 2005). Three protein factors, which contained a region with close homology to COMMD1 in their C terminal domain, were co-purified (these were later designated as COMMD3, -4, and -6). This region of homology had not been previously recognised. Additional homologs were identified through searches in sequence databases. This approach led to the identification of all ten family members in humans, which were subsequently named COMMD1 through 10 because the name *Murr2* was given to a gene unrelated to *Murr1*. The name ‘COMMD’ was proposed in order to specify these newly identified factors based on the shared structural domain that is the defining characteristic of this family of proteins. The majority of the COMMD proteins had not been previously characterised and were only
known as open reading frames. The COMMD proteins show no homology to any known protein and contain no previously characterised motifs or functional domains (Sommerhalter et al., 2007).

1.2.3. Pre-COMMD era: Ascribed functions of COMMD proteins

Prior to the classification of the COMMD family of proteins none of the COMMD factors had been described in any detail.

COMMD5, previously known as HCaRG (hypertension-related, calcium-regulated gene), has been shown to be overexpressed in spontaneously hypertensive rats (SHR) (Solban et al., 2000). This effect is thought to be due, at least in part, by low extracellular Ca\(^{2+}\) concentrations that have previously been described in SHR (Erne and Hermsmeyer, 1989). Therefore it is thought that extracellular Ca\(^{2+}\) negatively regulates COMMD5. Moreover, a comparison of fetal versus adult organs and normal versus tumour cells revealed that COMMD5 mRNA is significantly more expressed in normal adult cells/tissues. These results suggest that COMMD5 is developmentally regulated and implicate a possible role for COMMD5 in the control of both cell proliferation and differentiation. COMMD5 has also been shown to be involved in kidney repair after injury (Devlin et al., 2003; El Hader et al., 2005). More specifically, COMMD5 increases renal cell migration by a transforming growth factor (TGF) - autocrine loop mechanism. The nuclear localisation of COMMD5 makes it a potential regulator of the above processes (Solban et al., 2000).

The COMMD6 mouse ortholog Uch-L3 was previously mapped to a region that is essential for normal embryonic development. Homozygous deletions in this gene results in embryonic lethality however it is not known if COMMD6 is required for normal embryogenesis in humans (Kurihara et al., 2000; Semenova et al., 2003).

The COMMD3 locus was previously identified as being in close proximity to the Polycomb group gene bmi-1 (Haupt et al., 1992). An expressed sequence corresponding to COMMD7 had been found to be consistently downregulated in an experimental system
specifically designed to screen for proteins involved in the development of leukemia (Roperch et al., 1999).

Since the identification of COMMD1 and the subsequent discovery of the COMMD family of proteins, members have been shown to be involved in a number of distinct biological processes including the regulation of copper homeostasis (de Bie et al., 2005; van De Sluis et al., 2002) (refer to section 1.2.6.), intracellular Na\(^+\) regulation (Biasio et al., 2004) (refer to section 1.2.9.), the inhibition of transcription factor NF (nuclear factor)-\(\kappa B\) activity (Burstein et al., 2005; Ganesh et al., 2003; Greene, 2004) (refer to section 1.2.8.) and most recently, regulation of hypoxia-inducible factor 1 (HIF-1) activity (van de Sluis et al., 2007) (refer to section 1.2.11).

1.2.4. COMMD genes are ubiquitously expressed
COMMD mRNAs are ubiquitously expressed in human tissues with preferential expression demonstrated for any given COMMD protein. For example, COMMD1 expression is highest in the testis and heart; COMMD3 is highly expressed in the thymus, followed by the testis, pancreas and kidney whereas COMMD9 is highest expressed in the testis, thymus, lung and kidney (Burstein et al., 2005). COMMD1 protein has also been shown to be widely expressed in murine tissues and underscoring this ubiquitous pattern of expression is the presence of steady-state COMMD1 in several human cell lines including HEK293 (human kidney), HeLa (cervical carcinoma), Caco2 (colon carcinoma), A549 and H441 (both lung carcinoma cell lines) (Klomp et al., 2003).

1.2.5. COMMD protein structure and function
With the exception of COMMD6, which consists primarily of the COMM domain, all COMMD proteins are composed of approximately 200 amino acids each (Burstein et al., 2005). No membrane-spanning motifs have been identified in COMMD protein sequences and the absence of a putative signal peptide sequence suggests that these proteins reside within the cell (Solban et al., 2000) (refer to section 1.2.12.). The N terminal domains of the ten COMMD proteins vary in sequence and in length, ranging
from 18 to 151 residues for COMMD6 and COMMD5, respectively (Figure 1.12.).

**Figure 1.12. Protein organisation of the human COMMD family of proteins**

The highly conserved COMM domain, which consists of approximately 85 amino acids each, is indicated in green rectangles. The specific amino acid length for each of the human COMMD proteins is indicated to the right of each protein. (Adapted from Maine and Burstein, 2007).

The N terminal sequence, which makes up a significant proportion of the sequence of each COMMD protein, is composed of unique regions that are divergent across members of the same family but are highly conserved among orthologs in other species. For example, full length human COMMD1 and COMMD10 are only 38.6% conserved whereas full length human and mouse COMMD1 share an 88.7% sequence similarity. The COMMD1 N terminal domain adopts a compact -helical structure and it is predicted that this domain confers distinct functions on each COMMD protein (Solban et al., 2000; Sommerhalter et al., 2007). Unlike COMMD-COMMD protein interactions, which seem to be exclusively mediated by the COMM domain, binding of COMMD1 to NF-B requires full length COMMD1 (Burstein et al., 2005) (refer to section 1.2.8.). Several of the other COMMD proteins also associate with NF-B but to varying degrees. This implicates a unique and non-redundant function for each of the COMMD proteins that are likely due, at least in part, by the variable N terminal domains.

In contrast to this, the highly conserved C terminal COMM domain is composed of
approximately 70 to 85 residues in all COMMD proteins and is predicted to form a pleated sheet (Burstein et al., 2005). The COMM domain functions as a site for protein-protein interactions. More specifically, the COMM domain mediates the formation of COMMD protein hetero- and/or homodimerisation. Evidence for this was initially observed by Solban and colleagues (Solban et al., 2000). In the absence of the reducing agent -mercaptoethanol in rabbit reticulocyte lysate containing the translational product from COMMD5 (then known as HCaRG), polyacrylamide gel electrophoresis analysis revealed the presence of two protein bands, one of 27 kDa and the other 43 kDa. These data suggest possible intra- and/or intermolecular disulfide bridge formation via the cysteine residues present in the COMMD proteins and/or homo-/heterodimerisation with other proteins present in the lysates analysed, likely via the COMM domains. In a series of GST (glutathione S-transferase) pull down assays utilising a variety of COMMD1 deletion constructs it was demonstrated that COMMD – COMMD protein interactions are specifically mediated by the COMM domain however the composition of these complexes in vivo is still to be determined (Burstein et al., 2005; de Bie et al., 2006).

1.2.6. COMMD1 is a copper metabolism gene
In a study aimed at isolating the canine copper toxicosis gene van de Sluis et al. (2002) identified COMMD1 as the disease-causing gene (van De Sluis et al., 2002). Murr1, as it was known then, was subsequently denoted the copper metabolism Murr1 gene. Mutation analysis revealed that exon 2 of the COMMD1 gene was deleted in both alleles of all affected canines. This translated to a predicted truncated protein of 94 amino acids (out of the approximately 190), which suggested a possible loss-of-function mutation. In a subsequent study conducted by Klomp et al. (2003) no detectable full-length or truncated COMMD1 proteins were reported in affected livers thus this provided biochemical data in support of the previous suggestion of a complete loss-of-function mutation of COMMD1 (Klomp et al., 2003). Impaired biliary excretion is well documented in canines affected with copper toxicosis and this can be seen as electron-dense granules in the lysosomes mainly present centrilobularly (Hultgren et al., 1986; Owen and Ludwig, 1982). The copper-transporter ATP7B is responsible for the excretion of excess copper from the liver into the bile (Tao et al., 2003). Interestingly, a direct interaction between
ATP7B and COMMD1 has been shown and it has been suggested that COMMD1 somehow facilitates ATP7B-mediated copper excretion. Knockdown of endogenous COMMD1 with small interference RNA in cultured cells results in increased copper retention (Burstein et al., 2004; Spee et al., 2007). Copper transport to the trans-Golgi network (TGN) remains unchanged in canine copper toxicosis therefore COMMD1 is likely to mediate its effects on ATP7B downstream of the TGN possibly by regulating the intracellular trafficking of ATP7B and thereby facilitating degranulation of lysosomal contents into the bile (Klomp et al., 2003). The mechanism by which COMMD1 is likely to be recruited to intracellular vesicular compartments has recently been reported as well as its proposed intracellular localisation, which places COMMD1 in a post-Golgi vesicular compartment (Burkhead et al., 2009). This fits well with the proposed role of COMMD1, which is to facilitate ATP7B-containing lysosomal vesicles to transport to the bile canicular membrane.

1.2.7. COMMD1 interacts with XIAP
The interaction between COMMD1 and X-linked inhibitor of apoptosis protein (XIAP) was first reported following a yeast two-hybrid screen aimed at identifying novel interacting partners of XIAP (Burstein et al., 2004). XIAP exhibits potent anti-apoptotic properties that enable it to suppress specific members of the caspase family of cysteine proteases (Chai et al., 2001; Holcik and Korneluk, 2001). Interestingly, the interaction between COMMD1 and XIAP did not have any noticeable effects on the anti-apoptotic activity of XIAP. Instead, XIAP was shown to regulate COMMD1 levels by acting as an E3 ubiquitin ligase thus promoting ubiquitination and subsequent proteosomal degradation of COMMD1. A subsequent role for XIAP in copper metabolism was suggested and this was followed up in a number of in vitro studies that have shown XIAP to increase intracellular copper levels effectively by regulating the activity of COMMD1 (Burstein et al., 2004). This is consistent with the impaired ability of animals carrying mutations in the gene encoding COMMD1 to regulate copper levels. In contrast to this, copper levels were reduced in transformed fibroblasts derived from Xiap-deficient mice. The ability of XIAP to bind copper allows it to be negatively regulated by intracellular copper levels (Mufti et al., 2006). These data implicate a role for XIAP in the regulation
of copper homeostasis by functioning through COMMD1.

1.2.8. COMMD1 inhibits NF-κB

NF-κB is a dimeric transcriptional activator that regulates the expression of genes involved in a number of physiological processes including inflammation, proliferation, immunity and apoptosis (Ghosh et al., 1998; Karin and Lin, 2002). NF-κB also induces transcription of a number of viral genomes such as human immunodeficiency virus 1 (HIV-1) (Perkins et al., 1993). NF-κB subunits, which are encoded by five genes, normally reside in the cytoplasm and are retained there by inhibitor of kappa B (IκB) in an inactive form (Baldwin, 1996). Upon activation NF-κB translocates to the nucleus to initiate transcription of its target genes. COMMD1 (and later several of the other COMMD factors) have previously been identified as negative regulators of NF-κB-mediated transcription (Burstein et al., 2005; Ganesh et al., 2003; Greene, 2004). More recently COMMD1 has been shown to contain two highly conserved nuclear export signals required for nuclear export (Muller et al., 2009). COMMD1-mediated regulation of NF-κB has been shown to take place once NF-κB has translocated and entered the nucleus (Burstein et al., 2005). Here COMMD1 has been shown to associate with the RelA subunit of NF-κB. Through this action COMMD1 negatively regulates the association of RelA to chromatin. Downregulation of COMMD1 by small interference RNA increases the duration of RelA association to the promoter site. There is some evidence to support COMMD1-mediated ubiquitination of NF-κB subunits and therefore a mechanism by which the COMMD proteins inhibit the activity of NF-κB (Maine et al., 2007). Previous work has demonstrated that transient transfection of COMMD1 in cultured cells increases the amount of ubiquitinated RelA whereas downregulation of endogenous COMMD1 with small interference RNA decreases the recovered amount of ubiquitinated RelA. Similarly, downregulation of COMMD1 stabilises NF-κB and results in nuclear accumulation of RelA following activation of NF-κB. These studies have shed new light on the pathways involved in the regulation of κB-mediated transcription and have defined yet another in vivo role for the COMMD family of proteins.
1.2.9. COMMD1 regulates ENaC

Previously our lab identified COMMD1 as a novel δENaC interacting protein (Biasio et al., 2004). COMMD1 was shown to bind to the C terminal domain of δENaC, as well as to β- and γENaC and more recently, αENaC (Ke et al., 2008). Functional analysis revealed COMMD1-mediated inhibition of the amiloride-sensitive Na$^+$ current in a dose-dependent manner when coexpressed with either ENaC or ENaC in Xenopus oocytes. The interaction of COMMD1 and ENaC has been confirmed by glutathione S-transferase pulldown assay and co-immunoprecipitation. In addition to this, our lab has shown, using both functional and biochemical assays, that ENaC interacts with several of the other COMMD family members too (Ke, 2008; Swart, 2006). These results implicate a possible regulatory role for the COMMD proteins on ENaC and therefore, intracellular Na$^+$ regulation.

The COMMD proteins interact with both cytosolic and intrinsic membrane proteins however, in the case of COMMD1, it is largely seen as being targeted to cellular membranes. In a study aimed at investigating the mechanism of COMMD1 recruitment to cell membranes Burkhead et al. demonstrated that in the absence of other proteins, COMMD1 specifically and preferentially binds to the phosphorylated form of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2], an important signaling and regulatory lipid that has been shown to be present in the plasma membrane, nucleus and Golgi (Burkhead et al., 2009; Halstead et al., 2005). The COMM domain of COMMD1 was shown to be sufficient for this interaction therefore it was suggested that COMMD1 recruits to cellular membranes by means of binding to PI(4,5)P2. This may also be true for the other COMMD proteins as they all share the conserved COMM domain necessary for binding PI(4,5)P2. These data also provide further evidence that the COMM domain mediates protein-protein interactions (Burstein et al., 2005). Interestingly, the phosphorylated inositol group of PI(4,5)P2 has previously been shown to sequester PI(4,5)P2 effectors to specific cellular locations (Halstead et al., 2005). Examples of PI(4,5)P2 effectors include components of the endocytic machinery including adaptor protein complex 2 (AP-2), AP-180 and epsin. In the case of AP-2, PI(4,5)P2 has been shown to regulate its recruitment to specific transmembrane proteins and interaction with
clathrin-dependent vesicular sorting machinery (Balla, 2005). A similar regulatory mechanism may be responsible for COMMD-mediated inhibition of ENaC. COMMD1 interacts with δENaC (Biasio et al., 2004), PI(4,5)P2 (Burkhead et al., 2009), the μ2 subunit of AP-2 (Ke, 2008) and ubiquitin ligases (Maine et al., 2007), which in turn regulates the activity of ENaC at the apical plasma membrane. Ubiquitin ligases, in particular Nedd4-2, do not directly interact with δENaC (Waldmann et al., 1995). Thus it is suggested that COMMD1 acts as an accessory protein for recruitment of δENaC regulatory proteins. This proposed mechanism of recruiting δENaC regulators may also extend to the other ENaC subunits as functional and biochemical data have shown α-, β-, γENaC to also interact with a number of COMMD proteins, including COMMD1. In addition to this, Ke, Y. also demonstrated that COMMD1 interacts with both Nedd4-2 and SGK. It is likely that, via a simultaneous interaction with Nedd4-2 and SGK, COMMD1 is likely to prevent SGK from phosphorylating Nedd4-2. The interaction between COMMD1 and SGK in effect is likely to result in a continued interaction between Nedd4-2 and ENaC and thus lead to COMMD1-mediated inhibition of ENaC.

1.2.10. COMMD5 affects cell proliferation
COMMD5 has previously been shown to negatively regulate cell growth (Solban et al., 2000) (section 1.2.3.). More specifically, COMMD5 protein levels were shown to be lower in tumors and cancerous cell lines, including lymphocytes and leukocytes, and stable expression of this protein in HEK293 cells generated an inhibitory effect not only on DNA synthesis but also on HEK293 cell numbers. Based on these results it has been suggested that COMMD5 has a profound inhibitory effect on cell proliferation however the mechanism by which COMMD5 mediates these effects remains to be elucidated.

1.2.11. COMMD1 regulates HIF-1 activity
In a recent study aimed at exploring the in vivo function of COMMD1, van de Sluis et al. (2007) generated and characterised Commd1 knockout mice. It was found that Commd1 deficient embryos died in utero between 9.5 and 10.5 days postcoitum. Vascularisation of the placenta in all Commd1 knockout embryos was absent thus creating hypoxic embryos. A subsequent screen for genes that were differentially regulated by Commd1
knockout embryos compared to wild type and heterozygous embryos identified a number of genes that were either up- or down-regulated. Half of the genes that were shown to be upregulated in Commd1 knockout embryos appeared to be targets of hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor that is rapidly degraded under normoxic conditions. In vitro results demonstrated that under both hypoxic and normoxic conditions, downregulation of COMMD1 in cultured cells induced HIF-1 target gene activity whereas overexpression of COMMD1 inhibited HIF-1 target gene transcription (van de Sluis et al., 2007). Elevated HIF-1α protein levels are associated with transcriptional induction of HIF-1 target genes. This study demonstrated that HIF-1α activity was increased in Commd1 knockout embryos therefore it was concluded that COMMD1 plays an important role during hypoxia by inhibiting HIF-1 mediated gene expression. These results have been complimented by recent data obtained by Muller et al. (2009) whom have demonstrated nuclear export of COMMD1 to be an important event associated with the regulation of HIF-1 activity (Muller et al., 2009).

1.2.12. Intracellular localisation of the COMMD proteins

COMMD1 is the best-studied member amongst all the COMMD proteins. Most of the work investigating the cellular and intracellular localisation of the COMMD proteins has been carried out using COMMD1 as a model. Evidence to date suggests that COMMD1 localises to both nuclear and cytosolic fractions (Burstein et al., 2004; Burstein et al., 2005; Klomp et al., 2003). However the predominant localisation appears to be in the cytoplasm. This has been demonstrated using HeLa (human cervical cancer), Caco2 (human colon adenocarcinoma), H441 and A549 (both lung carcinoma), HEK293 (human kidney) and HepG2 (hepatic) cells where COMMD1 accumulation appears to be marked in the vesicles of the endocytic pathway including early endosomes and lysosomes (Burkhead et al., 2009; Klomp et al., 2003). The TGN and mitochondria do not appear to be major sites for COMMD1 localisation. Similar data have been obtained in our laboratory regarding the intracellular localisation of COMMD1. It was shown that COMMD1 is confined to intracellular vesicular compartments within the cytosol (Chang, 2007). COMMD1 predominantly co-localised with δENaC in early endosomes and recycling endosomes whereas its presence in late endosomes/lysosomes have been
inconclusive. Nonetheless, these results imply that the COMMD proteins are potential
regulators of ENaC trafficking, possibly by promoting internalisation of ENaC from the
apical cell surface. Moreover, COMMD1 has been shown to be recruited to membranes
via interaction with PI(4,5)P2 (Burkhead et al., 2009). This lipid has a well-described role
in vesicular trafficking (Yin and Janmey, 2003). Therefore these data provides a
mechanism by which COMMD1-dependent effects involve membrane-proteins, such as
ENaC, or membrane dependent events, including HIV1 infection. In contrast to this,
COMMD5 localises primarily to the nucleus when transiently expressed in COS-7 cells
(Solban et al., 2000).
2. Hypotheses and Objectives

COMMD-mediated regulation of ENaC is a continued area of research in our laboratory. Based on the literature reviewed above it was hypothesised that, in order for COMMD1 and COMMD3 to inhibit ENaC:

- The COMMD proteins localise to specific intracellular vesicular compartments that overlap with the trafficking pathways of ENaC and that
- The COMMD proteins localise to the same cell types as ENaC in vivo.

In order to gain a better understanding of the physiological functions of the COMMD proteins in vivo this project aimed to investigate the cellular and intracellular localisation of endogenously expressed COMMD proteins, in particular that of COMMD1 and COMMD3, in different cell lines derived from tissues in which the COMMD proteins are expressed, as well as in native tissue. This was investigated by first screening different cell lines for endogenously expressed COMMD1 and COMMD3 using Western blot analysis. The cellular localisation of COMMD1 and COMMD3 was determined using IHC on native tissue in which these proteins are expressed whereas immunocytochemistry (ICC) was employed to determine the intracellular location of these two proteins in mammalian cell lines. Furthermore, as the focus of our research is based around the regulation of ENaC, which in turn is partly dependent on the trafficking of ENaC, IHC was used to determine if COMMD1 and COMMD3 independently co-localise with ENaC in native tissue in which both proteins are expressed. This approach would likely provide further evidence that the interaction between the COMMD proteins and ENaC is not only functionally significant, but also physiologically relevant.

This project will be novel in the sense that it aims to investigate the cellular and intracellular localisation of endogenously expressed COMMD proteins in different cell lines and native tissue, as well as co-localisation of the COMMD proteins and ENaC in native tissue. Previously our lab investigated the intracellular localisation of transiently transfected δENaC and COMMD1 in three different kidney cell lines (Chang, 2007). The expression plasmids for these two proteins encoded tagged proteins. In general, tagging of proteins and their over-expression could potentially alter the intracellular localisation
or the function of the target protein and subsequently lead to a false representation of the true *in vivo* localisation and therefore function of these proteins. Our laboratory has now generated rat polyclonal antibodies against COMMD1 and COMMD3, which were available for this project to investigate the co-localisation of the COMMD proteins and ENaC in native kidney tissues. This has not been previously investigated therefore, in order to add a dimension of power to this study, the objectives were as follows:

- To screen different mammalian cell lines and native kidney tissue for endogenous protein expression of COMMD1 and COMMD3 using Western blot analysis.
- To investigate the intracellular distribution and location of endogenous COMMD1 and COMMD3 using ICC in mammalian cell lines.
- To investigate the cellular location of both COMMD1 and COMMD3 in native kidney tissue using IHC.
- To determine if COMMD1 and COMMD3 localise to the same cell types as ENaC in native kidney tissue.

More detail concerning the techniques employed to achieve the objectives listed are described under section 3.
3. Methods

3.1. Isolation and preparation of plasmid DNA

3.1.1. HiSpeed® plasmid midi preparation

Plasmid DNA used for transfections of mammalian cells was prepared using the QIAgen HiSpeed® Plasmid Midi Kit. This kit allows the isolation of up to 200μg high-copy plasmid DNA, which was prepared according to the manufacturer’s protocol. This protocol is based on a modified alkaline lysis procedure. Briefly, 50 mL overnight bacteria cultures were grown in standard Luria Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl) containing ampicillin (75 μg/mL) in a 37°C shaking incubator at 225 revolutions per minute (rpm). Vectors containing the different DNA constructs used during this project were previously transformed into the DH5α strain of Escherichia coli (E. coli) and were stored as laboratory glycerol stocks at -80°C. Bacteria cells were harvested by centrifugation in a multifuge (Heraeus Multifuge 3S-R, Kendro Laboratory Products, Hanau, Germany) at 6000 x g for 15 min at 4°C. The pelleted bacteria cells were re-suspended in 6 ml buffer A [50 mM Tris-chloride (Cl), pH 8.0, 10 mM EDTA (disodium ethylenediaminetetraacetic acid), 100 μg/mL RNase A] and were then lysed by adding 6 ml buffer B [200mM sodium hydroxide (NaOH), 1% SDS (sodium dodecyl sulfate) (w/v)]. DNA was precipitated by the addition of 6 mL buffer C (3 M potassium acetate, pH 5.5), which was immediately removed by filtration. Binding to an anion-exchange resin further purified the lysate containing the plasmid DNA, which was eluted from the resin with 5 mL buffer D [1.25 M NaCl, 50 mM Tris-Cl pH 8.5, 15% isopropanol (v/v)] and precipitated with 3.5 mL isopropanol. The precipitated plasmid DNA was retrieved using a special filtering device. The DNA was washed with 70% ethanol (2 mL) and eluted from the filtering device with 1 mL buffer TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA). DNA yield was determined as outlined under section 3.1.2.

3.1.2. DNA yield determination

DNA yield was quantified using ultra-violet (UV) spectrophotometry (GeneQuant, Pharmacia). DNA absorbs light most strongly at 260 nm thus the absorbance value or optical density (OD) value at this wavelength (A_{260}) was used to estimate the DNA concentration and hence the yield. Contamination of the DNA preparations was
negligible. DNA samples were diluted 1 μL in 100 μL to give a dilution factor of 100. An OD value of 1 corresponds to approximately 50 ng/μL of DNA thus the following equation was used to calculate the DNA concentrations:

$$\text{OD}_{260} \times 50 \text{ ng/μL} \times \text{dilution factor}$$

DNA concentrations were converted to μg/μL and working stock solutions of 0.1 μg/μL were prepared using buffer TE from the QIAgen HiSpeed® Plasmid Midi kit. All DNA samples were labeled and stored at -20°C.

### 3.2. Cell cultures
A number of different mammalian cell lines derived from tissues in which the COMMD messenger ribonucleic acid (mRNA) and/or proteins have previously been identified were selected for this project. Their origins, sources and morphology are described in more detail below.

#### 3.2.1. Mammalian cell lines

##### 3.2.1.1. COS-7
The COS-7 cell line (a gift from Professor Robin Olds) was derived from CV-1 simian cells and was developed via transformation with an origin-defective mutant of the simian virus 40 (SV40) (Gluzman, 1981). The CV-1 cell line originated from the kidney of a male African green monkey (*Cercopithecus aethiops*) thus COS [CV-1 (simian) in Origin, and carrying the SV40 genetic material)]-7 cells are often described as being transformed African green monkey kidney fibroblast cells. These cells grow as adherent monolayers and are often used as hosts in which foreign plasmid DNA is transfected to produce recombinant proteins for use in scientific experiments.

##### 3.2.1.2. HEK293
The human embryonic kidney 293 cell line (HEK293 cells were a gift from Dr Paul Hessian) was developed via transformation with DNA from human adenovirus type 5 (Graham et al., 1977). Like many other transformed cells, HEK293 cells exhibit elaboration of a virus-specific tumour antigen. HEK293 cells grow as adherent cells in
culture at 37°C and exhibit epithelial-like morphology.

3.2.1.3. SH-SY5Y

They SH-SY5Y cell line (a gift from Dr Mark Grimes) is a third generation human neuroblastoma cell line derived from the neuroepithelioma SK-N-SH cell line that was established in 1970 from the bone marrow biopsy of a 4-year-old girl with metastatic neuroblastoma (Biedler, 1973). Morphologically SH-SY5Y cells are epithelial-/neuronal-like elongated cells that exhibit very different growth phases. Dividing cells can form clusters of neuroblastic cells containing multiple neurites that aggregate, form clumps and float therefore SH-SY5Y cells grow as a mixture of floating and adherent cells.

3.2.1.4. Raji

The Raji cell line (a gift from Dr. Alex McLellan) was established in 1963 from the maxilla of an African boy with Burkitt’s lymphoma (Pulvertaft, 1964). Raji cells exhibit lymphoblast-like morphology and grow in suspension in culture at 37°C.

3.2.2. Maintenance of mammalian cell lines

All cell lines were cultured in low bicarbonate (1.5 g/L) Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen) and 1% penicillin/streptomycin (10 units/ml penicillin, 10 mg/ml streptomycin) (Invitrogen). All cell lines were cultured in the same medium to exclude any influence that different media may have had as a variable. The cells were maintained at 37°C under sterile conditions in a humidified incubator gassed with 5% carbon dioxide (CO₂) and 95% oxygen (O₂). Cells were passaged every 3 - 4 days once they had reached 80 – 90% confluency. This was required in order to maintain cells in exponential growth.

COS-7 and HEK293 cells were passaged as follows. Spent medium was removed by aspiration and cells were rinsed twice with 2 mL sterile phosphate buffered saline (PBS) (Sigma). Cells were detached from the surface of the cell culture dish (BD Biosciences, Durham, NC, USA) by the addition of 1 mL trypsin (0.25% w/v) in 1 mM EDTA-diNa⁺. COS-7 cells were treated with trypsin for 5 min and HEK293 for 1 min, both at 37°C.
Following this incubation period, 5 mL growth medium was added to neutralise the trypsin and the detached cells were recovered by centrifugation for 5 min at 1000 rpm in a bench-top centrifuge (Eppendorf, Hamburg, Germany). The supernatant was removed and the cell pellet was resuspended in 1 mL fresh growth medium. The split ratio for COS-7 and HEK293 cells was approximately 1:14. This was seeded into new 10 cm culture dishes containing 10 mL fresh growth medium. Cells were evenly distributed in the culture dish by gently swirling it in a figure ‘8’.

SH-SY5Y cells adhere lightly to the surface of culture dishes thus passaging involved aspirating the spent media and dislodging the cells from the surface by mechanical dissociation. This involved gently pipetting 1 mL of fresh growth medium up and down until all cells detached from the surface of the culture dish. SH-SY5Y cells were seeded into new 35 mm cell culture dishes at a split ratio of 1:3 containing 2 mL fresh growth medium. The dish was gently swirled to ensure even distribution of the cells. Raji cells were cultured in Nunc™ T25 EasY cell culture flasks (Biolab, Auckland, NZ) and were passaged by pipetting 1 mL of the 80 – 90% confluent culture into 6 mL fresh growth medium contained in a new culture flask.

3.2.3. Cryopreservation, storage and thawing
COS-7 and HEK293 cells were prepared for cryopreservation by pelleting cells from an 80 – 90% confluent cell culture as described in section 3.2.2. The supernatant growth medium was removed and the cell pellet was gently resuspended in 1 mL freezing medium [complete growth medium supplemented with 5% dimethyl sulfoxide (DMSO)]. SH-SY5Y cells were dislodged by mechanical dissociation for preservation purposes using 1 mL freezing medium whereas 1.5 mL of an 80 – 90% confluent Raji cell culture was recovered by centrifugation for 1 min at 1000 rpm in a bench-top centrifuge. The Raji cell pellet was resuspended in 1 mL freezing medium. The resuspended cell pellets were aliquoted at 1 mL/cryogenic vial (Corning Life Sciences, Canada). Vials were labeled, bubble wrapped and frozen upright in a Styrofoam box overnight at -80°C. The vials were transferred to liquid nitrogen storage the following day. This two-step freezing process mimics the ideal freezing rate for cells.
Cells were rapidly thawed on an as required basis. This was done by removing cryogenic vials from liquid nitrogen storage and rapidly thawing the cells in a 37°C water bath. To reduce the risk of contamination care was taken not to get water around the thread of the vial. Once thawed, the cells were added to 10 mL of pre-warmed complete growth medium in a 15 mL tube. This was gently mixed and pelleted by centrifugation for 5 min at 1000 rpm. The supernatant was removed by aspiration and the cell pellet was resuspended in 1 mL fresh growth medium. The approximate dilution was initially added to a new 6 cm cell culture dish containing 5 mL fresh growth medium. This was incubated and maintained as described under section 3.2.2. Growth medium was aspirated and replaced with fresh, pre-warmed growth medium the next day. The cells were transferred to and cultured in a 10 cm culture dish following the first passage after thawing.

3.2.4. Transient transfection

COS-7 and HEK293 cells were transiently transfected with plasmid DNA using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) in order to generate suitable controls for use in Western blotting and immunoperoxidase experiments. This was performed according to the manufacturer’s guidelines. Cells were seeded at a density of 3 - 4 x 10⁵ cells per 35 mm culture dish containing 2 mL full growth medium a day prior to transfection. Cell concentration was determined with the use of a hemacytometer. Depending on the cell line used, seeding at this concentration provided a 60 – 80% confluent cell culture at the time of transfection. For each transfection sample (i.e. each 35 mm dish) complexes were prepared as follows: 1 μg plasmid DNA was diluted in 50 μL serum and antibiotic free low bicarbonate DMEM. Similarly, 2 μL of Lipofectamine™ 2000 was diluted in 50 μL serum and antibiotic free low bicarbonate DMEM. The two diluted samples were gently mixed and incubated for 5 min at room temperature. Following the incubation period the diluted plasmid DNA and transfection reagent were combined, gently mixed and incubated for a further 20 min at room temperature. During this incubation period, the full growth medium of each of the transfection samples was replaced with 2 mL serum and antibiotic free low bicarbonate DMEM. Plasmid DNA and transfection reagent complexes (100 μL) were added to each transfection sample in a
drop-wise manner and mixed. The cells were returned to the cell incubator where they were left to express protein for 24 – 48 h. Whole-cell proteins were extracted by means of lysis (refer to section 3.2.5.) and for immunoperoxidase labeling cells were processed as outlined under section 3.8.5.

3.2.5. Cell lysis

Whole-cell protein extraction in this project was carried out to screen different cell lines for the endogenous expression of COMMD1 and COMMD3 proteins. This was achieved using either one of two lysis buffers: 1X SDS sample buffer [50 mM Tris, 1% SDS, 5% glycerol, 0.1% (w/v) bromophenol blue] or 1X Tris buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.5) containing 1% Triton® X-100 and protease inhibitors [10 μg/mL phenylmethyl-sulfonyl fluoride (PMSF), 2 μg/mL aprotinin, 2 μg/mL leupeptin and 1 μg/mL pepstatin]. 1X SDS sample buffer had a tendency to generate a viscous lysate that was difficult to handle therefore 1X TBS containing 1% Triton® X-100 was used as an alternative lysis buffer.

Cells were seeded at a density of 3 - 4 x 10^5 cells per 35 mm culture dish. Cells used as positive controls were transfected the following day as described under section 3.2.4. Untransfected cells that were used to screen for endogenous COMMD1 and COMMD3 protein expression were treated the same as the transfected cells except that they were either transfected with an empty pMT3 vector (i.e. pMT3 containing no recombinant DNA) as described under section 3.2.4. or no transfection complexes were added.

Adherent cell cultures were lysed as follows. Spent media was removed by aspiration and the cells were rinsed twice with 1 mL cold PBS. Following the removal of the last PBS 100 – 200 μL of lysis buffer (either 1X SDS sample buffer or 1X TBS containing 1% Triton® X-100) was added to the cells. The culture dish was incubated on ice (~4°C) for 1 h at 50 rpm on a shaking platform. Cells in suspension (i.e. Raji cells) were first pelleted by centrifugation for 3 min at 13200 rpm. This was followed by removal of the supernatant and the addition of lysis buffer to the cells which were then incubated at 4°C on a rotator. Lysed adherent cells were scraped from the surface of the cell culture dish.
following the 1 h incubation period and were transferred into pre-cooled 1.5 mL tubes. Cell membrane fractions for both adherent and suspension cells were pelleted by centrifugation in a bench-top microcentrifuge (Eppendorf, Hamburg, Germany) for 5 min at 13200 rpm and the supernatant was transferred into fresh 1.5 mL tubes. At this stage, whole-cell protein concentration was determined as described under section 3.4. A 1:10 volume of 5X SDS sample buffer (250 mM Tris, 5% SDS, 25% glycerol, 0.1% (w/v) bromophenol blue, pH 6.8) containing 10% β-mercaptoethanol was added to the remaining cell lysates. This was boiled at 100°C for 3 min prior to being subjected to SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot analysis (refer to section 3.7.) to determine whole-cell protein expression of either endogenous or transfected COMMD1 and COMMD3. Un-used cell lysates containing 5X SDS sample buffer with 10% β-mercaptoethanol were stored at -20°C for later use.

3.3. Animal tissues

3.3.1. Ethical approval, animal and treatment details
Ethical approval was obtained through the University of Otago’s animal ethics committee for the retrieval of tissues (either kidney, brain or thymus) from animals previously euthanised for another approved research/teaching protocol. Whole mouse kidneys for this project were retrieved from approximately 6-week-old untreated/control male mice killed by cervical dislocation. Rat kidneys were from either ~2 month old untreated/control or dexamethasone treated female rats euthanised with CO2. Dexamethasone treated rats were subcutaneously injected with 6 mg/kg dexamethasone suspended in 300 μL of corn oil on three separate occasions: 66 h, 42 h and 18 h before the experiment, respectively. This dosage regime ensured maximum stimulation of amiloride-sensitive Na+ transport in the colon and it was thought to be beneficial for detecting ENaC by IHC and therefore to investigate co-localisation of the COMMD proteins and ENaC in vivo.

3.3.2. Whole tissue lysis
Whole-tissue protein extraction was performed to identify endogenous COMMD1 and COMMD3 proteins in native tissue by SDS-PAGE followed by Western blot analysis.
Control mouse and rat kidneys were collected immediately after the animals were killed by cervical dislocation (mice) or euthanasia (rats). The kidneys were frozen with liquid nitrogen and were pulverized with a mortar and pestle. Homogenisation buffer (1 mL) [250 mM sucrose, 100 mM NaCl, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2 mM EDTA-diNa\(^{+}\), PMSF (10 μg/mL), pepstatin (1 μg/mL) aprotonin (2 μg/mL), leupeptin (2 μg/mL)] with a pH of 7.4 was added for every whole kidney used and the already pulverized kidneys were sufficiently ground by hand. The samples were transferred into 1.5 mL tubes and the cell membrane fraction was pelleted by centrifugation for 5 min at 3400 rpm. The supernatant, which now contained whole-tissue protein (i.e. the tissue lysate), was carefully removed and transferred into a fresh 1.5 mL tube. The protein concentration of the whole-tissue lysate was determined as outlined under section 3.4. The tubes were labeled and stored at -80°C. Prior to being separated by SDS-PAGE, an equal volume of 5X SDS sample buffer containing 10% β-mercaptoethanol was added to the samples, which were then heated for 3 min at 60°C.

3.4. Protein quantitation

The Bio-Rad RC DC colorimetric protein assay (Hercules, CA, USA) was employed to quantitatively determine the protein concentrations in both whole-cell (section 3.2.5.) and whole-tissue (section 3.3.2.) lysates. This was performed according to the manufacturer’s protocol. Briefly, a working reagent A’ was prepared by adding 20 μL of reagent S to 1mL of reagent A. Between 3 and 5 dilutions of a protein standard ranging from 0.2 mg/mL to 1.5 mg/mL was prepared. Equal volumes (5 μL) of each protein standard and sample was pipetted into a clean 96 well microplate. Reagent A’ (25 μL) was added to each well and was incubated for 5 min at room temperature. This was followed by the addition of 200 μL of reagent B to each well and an incubation period of 15 min at room temperature. The OD value for each of the samples was obtained by reading the absorbances at 750 nm in a microplate plate reader (Synergy2, BioTek). A standard curve was constructed using the OD\(_{750}\) values obtained from the protein standard. Protein concentrations of the unknown samples were determined using the standard curve.
3.5. GST fusion proteins

3.5.1. Production and purification of GST fusion proteins in *E.coli*

Glutathione S-transferase (GST) fusion proteins were produced to investigate the specificity of the antisera used in this project. GST fusion proteins were prepared by innoculating 10 mL LB medium containing ampicillin (75 μg/mL) (LBamp) with the BL21 strain of *E.coli* transformed with either pDEST™15 vector, which is a GST gene fusion system, alone or pDEST™15 containing full-length human COMMD1 or COMMD3 from the appropriate glycerol stocks. These glycerol stocks were prepared previously and were stored at -80°C (Swart, 2006). The innoculated culture was incubated overnight at 37°C in a shaking incubator (225 rpm) and was added to 90 mL LBamp the next day. This expression culture was incubated as before for a further 1.5 h until an OD$_{600}$ of 0.6 was reached. Expression of the GST fusion proteins was induced by the addition of 30 μL of 1 M IPTG (isopropyl β-D-1-thiogalactopyranoside) per 100 mL expression culture and a 3 h incubation period at 30°C in a shaking incubator. Bacteria cells were harvested by centrifugation at 5000 x g for 10 min. The supernatant was discarded and the remaining cell pellet was resuspended in 2 mL cold PBS containing PMSF inhibitor (10 μg/mL). Bacteria cells were lysed by sonication for 1 x 20 sec and then 3 x 10 sec. The solution was held on ice for 1 min in between sonication periods and appeared brown/green upon lysis. Triton® X-100 (10%) in PBS was added to a volume of 1:10 and the lysate was transferred into 1.5 mL tubes. Membranes and unbroken cells were pelleted by a 5 min centrifugation at 13200 rpm. The glutathione-agarose beads (Sigma) were prepared at this stage by washing 500 μL of 50% slurry for every 10 mL overnight culture 3 times with 200 μL of cold PBS containing 1% Triton® X-100. Following removal of the last PBS the remaining beads were resuspended in an equal volume of PBS containing PMSF inhibitor. After centrifugation the supernatant was added to the appropriate volume of 50% glutathione agarose bead slurry and was incubated for 30 min at 4°C on a rotator. The beads, which were now bound to the GST fusion proteins, were spun down for 30 sec at 13200 rpm. The supernatant was removed and discarded. The remaining fusion proteins suspended on the beads were washed 3 times with 1 mL cold PBS containing 1% Triton® X-100. Following removal of the last washing solution the GST fusion proteins and bead complexes were resuspended in an
equal volume of PBS containing PMSF.

Fusion protein concentration was determined by resolving equal volumes of GST, GST-COMMD1 and GST-COMMD3 alongside known bovine serum albumin (BSA) standards on a 0.75 mm, 12% SDS-PAGE gel (refer to section 3.7.1.) that was subsequently stained with Coomassie™ Blue as described under section 3.7.2. Once the fusion protein concentrations were known, 3 sets of equal concentrations (usually 5 μg) of GST and the relevant antigen, being either GST-COMMD1 or GST-COMMD3, were separated alongside prestained protein standards on a 1.5 mm 12% SDS-PAGE gel. GST-precleared, antigen-cleared and non-precleared antiserum was subsequently used in Western blot analysis to determine the specificity of the relevant antisera (refer to section 3.6.2.). GST fusion proteins were stored at 4°C and could be used for up to 2 weeks.

3.6. Antiserum
Antisera against COMMD1 and COMMD3 was used in this project to first detect the presence of endogenously expressed COMMD1 and COMMD3 proteins in different cell lines and native tissue using Western blot analysis followed by identifying their intracellular and cellular location by ICC and IHC, respectively.

3.6.1. Antiserum generation
Polyclonal antiserum to the human COMMD1 and COMMD3 proteins were raised by serial immunisation of rats with an approximately 50 kDa purified recombinant GST-COMMD1 or GST-COMMD3 protein (refer to section 3.5.1.). COMMD1 antiserum had previously been characterised by Ke, Y. (Ke, 2008) and COMMD3 antiserum has been partially characterised by Liu, Y. (personal communication). For the purpose of this project both COMMD1 and COMMD3 antisera were partially characterised (refer to section 4.1.).

3.6.2. Pre-cleared antiserum
GST pre-cleared polyclonal antiserum was prepared by diluting the desired amount of antiserum in the appropriate blocking solution. BSA (4%) and normal goat serum (NGS)
(2%) in PBS was used as a blocking solution for immunocyto/-histochemistry whereas for Western blot analysis 1X TBST (0.05% Tween-20 in 50 mM Tris, 150 mM NaCl, pH 7.5) was used. For every 1 μL of antiserum used 100 μg of purified GST protein was added to the dilution. The diluted antiserum and purified GST protein mix was incubated on a rotator for 48 h at 4°C. This was centrifuged for 5 min at 13200 rpm following the incubation period. The supernatant was removed and placed into a fresh 1.5 mL centrifuge tube. The supernatant containing the precleared diluted antiserum was spun down again as before and was then transferred into a fresh 1.5 mL tube, labeled and ready for use. For competition experiments (refer to section 4.1.) purified recombinant antigen (i.e. GST-COMMD1 or GST-COMMD3) or GST itself was used as described above to examine the specificity of the antisera. All non-precleared controls were treated the same as the GST-precleared or antigen-cleared samples.

3.7. Protein Analysis

3.7.1. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a technique commonly used to separate proteins according to their molecular weight. The size of the resolved protein is determined by comparison to a series of known prestained protein standards that are run on the same gel. The acrylamide concentration of the gel can be varied. Low acrylamide percentage gels are required to separate high molecular weight proteins whereas higher acrylamide concentrations are required to resolve smaller proteins. For this project whole- tissue and whole-cell proteins were separated on 1.5 mm 15% and 12% SDS-PAGE gels, respectively, and were analysed by Western blotting (section 3.7.3.). GST fusion proteins were separated on either 0.75 mm 12% SDS-PAGE gels for Coomassie™ Blue staining (refer to section 3.7.2.) or 1.5 mm 12% SDS-PAGE gels for subsequent Western blot analysis (section 3.7.3.).

SDS-PAGE was performed using a Hoefer PAGE system (San Francisco, USA). Gel moulds were prepared by assembling glass and aluminum plates separated by two spacers, either 0.75 mm or 1.5 mm thick, at either end. Resolving gels of the desired acrylamide concentration were prepared as outlined in table 3.1. For every 0.75 mm and
1.5 mm resolving gel that was prepared, 5 mL and 10 mL of gel solution was required, respectively. Resolving gels were poured between the glass and aluminum plates and isobutanol was added to prevent the formation of air bubbles on the surface of the gels. The resolving gels were left to polymerize for approximately 45 min. The isobutanol was washed off with H₂O and enough 4% stacking gel (refer to Table 3.1.) was poured on top of the resolving gel to fill the area between the plates to the top. A ten-well comb was put in place to allow the formation of the loading wells. Approximately 30 min later, once the stacking gel had polymerised, the ten-well comb was carefully removed. The plates, which now sandwiched the resolving and stacking gel, were removed from the mould and were transferred to the Hoefer electrophoresis unit.

Table 3.1. Ingredients required for 10mL SDS-PAGE solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15%</td>
<td>12%</td>
</tr>
<tr>
<td>H₂O (mL)</td>
<td>2.35</td>
<td>3.35</td>
</tr>
<tr>
<td>1.5M Tris, pH8.8 (mL) (Invitrogen)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>0.5M Tris, pH6.8 (mL) (Invitrogen)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10% sodium dodecyl sulfate (SDS) (w/v) (μL)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(Invitrogen)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30% Acrylamide/bisacrylamide (mL)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>(BioRad)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% Ammonium persulfate (APS) (w/v) (μL)</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>(BioRad)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED) (μL)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>(BioRad)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (mL)</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

1X SDS running buffer [24 mM Tris, 0.5% SDS (w/v), 192 mM glycine] was prepared and added to both the anode and cathode chambers of the electrophoresis unit. The protein samples were denatured to their primary structures by the addition of a 1:10 volume of 5X SDS sample buffer, which is a strong reducing agent, containing 10% β-mercaptoethanol for whole-cell protein lysates and an equal volume for whole-tissue protein lysates. This treatment also render proteins negatively charged, a property that allows them to migrate through the acrylamide mesh towards the positively charged
electrode. Because small molecular weight proteins migrate faster than larger molecular weight proteins, this technique allow the proteins to become separated based on their molecular weights. Whole-cell protein lysates were boiled for 3 min at 100°C prior to being used for SDS-PAGE whereas whole-tissue protein lysates were heated for 3 min at 60°C. Equal volumes or concentrations of protein samples were loaded into the wells alongside one of two standards: a series of known BSA standards for gels that were to be analysed by Coomassie™ Blue staining or for subsequent Western blot analysis, a prestained Kaleidoscope protein ladder (2 μL) (BioRad). A constant voltage ranging between 150 V and 180 V was applied for approximately 1.5 h to separate the proteins.

Following completion of the gel run the gels were subjected to either Coomassie™ Blue staining (refer to section 3.7.2.) or Western blot transfer (refer to section 3.7.3.).

3.7.2. Coomassie™ Blue Staining
GST fusion proteins were resolved by 12% SDS-PAGE (0.75 mm) and protein bands were visualised by staining with Coomassie™ Blue, a dye that binds nonspecifically to virtually all proteins. This method allowed GST fusion protein concentrations to be visually determined against known standards, in this case BSA. SDS-PAGE gels were incubated overnight in Coomassie™ Blue stain solution [40% methanol, 10% acetic acid and 0.1% (w/v) Coomassie™ Brilliant Blue R-250 (Life Technologies, NY, USA)]. Polyacrylamide gel avidly absorbs Coomassie™ Blue stain and as a result destaining was necessary before the protein bands could be seen. Background staining was removed by soaking the gels in a destaining solution containing 40% methanol and 10% acetic acid in mQH₂O for 2 – 3 h. Two small squares of paper towel were added to the gel and destaining solution to help absorb some of the stain. The gels were scanned at 300 dpi (dots per inch) and the digital images were saved in JPEG format. These images were then formatted in Adobe Illustrator CS4.
3.7.3. Western blot transfer

Proteins separated by SDS-PAGE were made accessible to antibody detection by transferring them onto a polyvinylidene difluoride (PVDF) membrane. This technique utilises an electric field to pass the proteins within the gel onto the membrane whilst preserving the organisation of the separated proteins.

Prior to completion of the SDS-PAGE gel run, 100 mL of Western transfer buffer [25 mM Tris, 192 mM glycine, 20% methanol (v/v)] was prepared for every 2 SDS-PAGE gels subjected to Western blot transfer, which was performed using the semi-dry Western blotting system from Hoefer. Filter paper (3 mm) and PVDF membranes were cut into 7.5 cm x 8 cm pieces. For each transfer, 8 pieces of filter paper and 1 PVDF membrane was required. The filter paper was saturated in Western transfer buffer and the PVDF membranes were incubated in pure methanol for approximately 10 sec. PVDF membranes were briefly rinsed in mQH$_2$O prior to being incubated in Western transfer buffer for a further 5 min. SDS-PAGE gels were carefully removed from the plates and the stacking gels were cut off and disposed of. A stack consisting of 4 x 3 mm pieces of saturated filter paper followed by a PVDF membrane, SDS-PAGE gel and another 4 x 3 mm pieces of saturated filter paper were compiled between the positive and negative electrodes. A current of 50 mA was applied per SDS-PAGE gel and the electro-transfer was carried out for 3 – 4 h.

Following completion of the Western blot transfer, the PVDF membranes were incubated overnight at 4°C or on a shaker (50 rpm) for 1 h at room temperature in TBST containing 5% non-fat milk powder. This prevented or minimized non-specific binding of the antibodies during immunostaining (section 3.7.4.).

3.7.4. Immunostaining and chemiluminescence

Following the blocking procedure for non-specific antibody binding, the blocking solution was discarded and the PVDF membranes were probed with a 1:5000 dilution of primary antibody specific to the antigen of interest for 2 h at room temperature on a shaking platform at 50 rpm. All antibodies used for immunostaining following Western
blot transfer were diluted in TBST. The primary antibody was discarded and the membranes were washed 3 x 5 min in TBST. Horseradish peroxidase (HRP) conjugated goat anti-rat secondary antibody was applied (1:10000) and incubated for 1 h at room temperature under constant shaking (50 rpm). The secondary antibody was discarded and the membranes were again washed for 3 x 5 min in TBST. During this last 5 min washing period Lumi-Light Western blotting substrate (Roche) was prepared by mixing equal volumes of Lumi-Light enhancer and Lumi-Light stable peroxide solution. A total volume of 1.5 mL Lumi-Light substrate per PVDF membrane was prepared in this project. Lumi-Light substrate was incubated with the membranes for 5 min in order allow the HRP to convert the substrate, which results in light emission that is detectable on X-ray film. The membranes were placed onto transparent film facing protein side up and were covered with a second transparency film. Air bubbles were removed prior to exposing the membranes on X-ray film (Kodak). Following completion of the desired exposure period, the film was submerged in Kodak developer solution for approximately 3 min and was briefly rinsed with H2O prior to being submerged in Kodak fixer for another 3 min. The film was rinsed in H2O and was allowed to dry prior to being scanned at 300 dpi. The digital images were saved in JPEG format and were formatted in Adobe Illustrator.

3.8. Immunochemistry

Immunochemistry is the technique used to identify a certain antigen in a cytological preparation (referred to as ICC) or a histological tissue section (referred to as IHC) by a specific antibody-antigen interaction. Both techniques can be performed using either direct or indirect methods. For this project the latter method was employed and involved an unlabeled primary antibody to recognise an antigen of interest, and a labeled secondary antibody, which reacted with the unlabeled primary antibody. A number of detection systems, which include the use of enzymatic or fluorescent labels in combination with light or epifluorescent and confocal microscopy, respectively, can be employed to visualise and subsequently localise the antigen of interest. The protocols used to perform both immunoperoxidase (or enzymatic) and fluorescent labeling on cells
and tissues are described in more detail below.

3.8.1. Solutions and materials

3.8.1.1. Preparation of 4% paraformaldehyde

Paraformaldehyde (PFA) (4%), used to fix both cytological and histological specimens, was prepared in a fume cupboard by dissolving 8 g of PFA powder in 200 mL 1X PBS on a 60°C heated stirring plate. Sodium hydroxide (NaOH) was added to help dissolve the PFA particles. Once the particles were dissolved the solution was filtered into a clean 200mL glass bottle. The solution was allowed to cool and the pH was adjusted to physiological pH (pH 7.4). This was labeled and stored at 4°C. Fixative solution was freshly prepared every 2 weeks or as required.

3.8.1.2. Preparation of microscope glass slides

Microscope glass slides used for IHC were coated with APES (3-aminopropyl-triethoxy-silane) (Sigma, cat#: A3648) prior to use. This product is commonly used in solution to prepare positively charged glass which promotes binding of histological sections to glass slides thus preventing separation during staining. Slides were prepared according to the manufacturer’s recommended protocol. Prior to coating the slides were immersed in pure acetone for approximately 2 min and air-dried to free them of any traces of oil or water. A fresh 2% solution of APES in pure acetone was prepared and the clean dry slides were dipped in this solution for approximately 30 sec. The slides were washed twice in distilled H₂O and were dried overnight at 37°C. APES treated slides were stored in boxes at room temperature and were able to be used for up to 6 months.

3.8.1.3. Preparation of paraffin embedded tissue sections and slides

Mouse and rat kidneys were removed following euthanasia or cervical dislocation and were immediately fixed with cold 4% PFA (refer to section 3.8.1.1.) in PBS in 100 mL lidded plastic containers for approximately 24 h at room temperature. Immediate fixation was required to prevent any deleterious effects to antigen preservation such as hypoxia. Rat kidneys were sliced into coronal hemisections to ensure proper penetration of the fixative solution. The kidneys were transferred to specimen jars containing 70% ethanol
the following day and were labeled appropriately. Fixed kidneys were stored in this solution indefinitely at room temperature and were paraffin embedded as required. Kidneys processed to paraffin were cut into 4 μm sections using a Jung RM 2025 microtome (Leica, Nussloch, Germany). The sections were floated in a 37°C water bath and were collected onto clean APES treated glass microscope slides (refer to section 3.8.2.). The sections were dried overnight at 37°C and were stored at 4°C for up to 6 months.

A labeling system was adopted to ensure that serial tissue sections were used for each new experiment e.g. for every positively stained tissue section (either single or double stained) the appropriate negative controls were included in series to ensure that the sections used for each new experiment were from approximately the same area within the kidney. This meant that for every new single or double labeled positive sample, positive samples were spaced 8 μm or 12 μm apart. This allowed for either one (single staining) or two (double staining) negative controls to be included in series with every positive sample. It is noteworthy to mention however that two positively labeled sections were sometimes included in a set of positive and negative samples thus altering the thickness between each new set of experiments.

3.8.2. Immunoperoxidase labeling

DAB (3,3’-diaminobenzidine tetrahydrochloride) is a commonly used peroxidase chromogen that reacts with the enzyme horseradish peroxidase (HRP) that is isolated from the root of the horseradish plant. A chemical reaction occurs in the presence of DAB, which acts as an electron donor, whereby HRP forms a complex with hydrogen peroxide and catalyses its breakdown into H₂O and O₂. The oxidation of DAB produces a brown precipitate at the site of the reaction (Renshaw, 2007). Thus, when HRP is reacted with DAB precipitate production occurs at the site where secondary antibody and therefore indirectly the primary antibody and the target antigen is present. This technique is referred to as indirect immunoperoxidase labeling. For this project, pilot studies were performed using HRP conjugated to secondary antibodies. Indirect immunoperoxidase protocols employed in this project, using both cells and tissues, were similar to those
described for indirect fluorescent ICC (section 3.8.3.) and IHC (section 3.8.4.), respectively. These protocols are described in more detail under section 3.8.3 and section 3.8.4. Where appropriate, differences between immunoperoxidase and fluorescent labeling are indicated.

3.8.3. Immunocytochemistry (ICC)

Cells were seeded onto 10 mm circular glass coverslips at a density of 4 x 10^5 cells per 35 mm 24 h prior to being fixed with 4% PFA (refer to section 3.8.1.1.). Prior to cell fixation the spent growth medium was removed from the cells by aspiration and cells were briefly rinsed with PBS. Cells were fixed in a fume cupboard with 4% PFA 2 x 1 min and then 1 x 10 min. This step was required to rapidly terminate all enzymatic and other metabolic activities in order to preserve the structural properties of the specimen in question (Fischer et al., 2008). Following removal of the last fixative solution the cells were rinsed 3 times with PBS and were then permeabilised with 0.2% Triton® X-100 in PBS for 15min to allow free and efficient access of the antibodies to the antigen of interest in subsequent steps. Cells were rinsed 3 times with PBS following the permeabilisation step. At this stage during the protocol immunoperoxidase labeled cells were treated with 1% hydrogen peroxide (H_2O_2) in 0.2% Triton® X-100 in PBS for 30min to quench any endogenous peroxidase activity. This protocol was followed for standard ICC and immunoperoxidase labeling. Variations in the order in which cells were fixed and permeabilised were employed in some double labeling ICC experiments (refer to Figure 3.1. for a flow chart summarising standard, immunoperoxidase and double labeling protocols). This was dependent on the specific organelle marker used. More details regarding these variations are described under section 3.8.6.

Following permeabilisation or H_2O_2 treatment cells were blocked with 4% BSA and 2% NGS in PBS for 30 min. During this incubation period the appropriate primary antibody was prepared by diluting to the desired concentration in blocking solution (refer to table 3.2. for antibody dilutions). Prior to applying the primary antibodies to the cells, one coverslip for each antibody combination used was transferred into a new 35 mm cell culture dish and a circle was traced around each coverslip with an Imm Edge™ wax pen
(Vector Laboratories, Cat. # H-4000). By doing this only 200 μL of diluted antibody was required for each coverslip thus greatly reducing the amount of antibody and blocking solution used. Cells were incubated with primary antibody for 2 h at room temperature or overnight at 4°C. Cells were rinsed 3 times with PBS prior to the addition of the secondary antibodies, which was also diluted in blocking solution. HRP or fluorochrome conjugated secondary antibodies were used for immunoperoxidase or fluorescent labeling, respectively (refer to Table 3.2. for antibody dilutions). Cells were incubated with secondary antibody for 45 min and were then rinsed 3 times with PBS.

Immunoperoxidase labeled cells were incubated with DAB substrate (1 mg/mL DAB, 0.02% H₂O₂ in PBS) for 2 – 30 min at this stage. The staining intensity was monitored with a light microscope and was subsequently terminated by rinsing the cells several times in PBS. The cells were dehydrated prior to being mounted onto glass microscope slides. This involved submerging the coverslips in a series of ethanol concentrations: 1 x 2 min in 70% ethanol, 2 x 2 min in 100% ethanol and 1 x 2 min in toluene. The coverslips were mounted with di-n-butylphthalate in Xylene resin (DPX) and allowed to dry overnight. Immunoperoxidase labeling does not fade with time and samples were stored indefinitely at room temperature.

The dehydration step employed for immunoperoxidase labeling was omitted prior to mounting fluorochrome labeled cells. Instead, these cells were mounted with one of two aqueous mounting media: VECTASHIELD® Hard Set mounting medium with 4’-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) or 90% glycerol in PBS. Samples mounted with 90% glycerol in PBS were sealed around the coverslip edges with clear nail polish. The latter also did not contain an anti-fading agent thus samples mounted using this medium only lasted for approximately 1 week as opposed to approximately 4 weeks. Fluorescent labeled samples were stored in the dark wrapped in foil at 4°C.
Table 3.2. Cellular immunoperoxidase labeling (peroxidase) and ICC antibodies

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilution factor</th>
<th>Incubation period</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peroxidase</td>
<td>ICC</td>
<td></td>
</tr>
<tr>
<td><strong>Primary</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat anti-GST-COMMD1/COMMD3 antiserum</td>
<td>1:1000</td>
<td>1:50</td>
<td>2h, RT</td>
</tr>
<tr>
<td>AF®546 conjugated transferrin</td>
<td>-</td>
<td>50μg/mL</td>
<td>10min, 37°C</td>
</tr>
<tr>
<td>Mouse anti-lysosomal associated membrane protein 1 (LAMP1)</td>
<td>1:100/50</td>
<td>2h, RT</td>
<td>Lysosomal marker</td>
</tr>
<tr>
<td>Mouse anti early endosome antigen 1 (EEA1)</td>
<td>-</td>
<td>1:50</td>
<td>2h, RT</td>
</tr>
<tr>
<td>Wheat Germ Agglutinin (WGA) AF®488 conjugate</td>
<td>-</td>
<td>1:50</td>
<td>10min, RT</td>
</tr>
<tr>
<td><strong>Secondary</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat anti-rat HRP</td>
<td>1:500</td>
<td>-</td>
<td>45min, RT</td>
</tr>
<tr>
<td>AF®488/633 goat anti-rat</td>
<td>-</td>
<td>1:200</td>
<td>45min, RT</td>
</tr>
<tr>
<td>AF®546 goat anti-mouse</td>
<td>-</td>
<td>1:200</td>
<td>45min, RT</td>
</tr>
</tbody>
</table>

All steps were performed at room temperature unless stated otherwise and care was taken not to allow the cells to dry during the procedure or to dislodge them from the glass coverslips.

3.8.4. Immunohistochemistry (IHC)

Conventional deparaffinisation and rehydration steps were employed prior to staining. This involved submerging the tissue sections in toluene and a series of ethanol concentrations as follows: 3 x 2 min in absolute toluene followed by 2 x 1 min in each of 100% ethanol, 95% ethanol, 90% ethanol and 70% ethanol. These solutions were changed on a weekly basis. Slides were washed 2 x 2 min in PBS and were transferred
into milliQ H₂O (mQH₂O) prior to proceeding to the subsequent step. Formaldehyde fixation induces antigen-masking effects which could potentially diminish specific staining however these effects are reversible to varying degrees by a process known as antigen retrieval. For this project, heat-induced epitope retrieval (HIER) was carried out prior to commencing IHC staining procedures to reveal antigens. This involved immersing the tissue sections in a buffered solution, in this case 1 mM di-sodium ethylenediaminetetraacetic acid (EDTA-diNa⁺; 10 mM stock solution 0.96 g EDTA diNa⁺, 250 mL H₂O, pH 8.0), and bringing the solution to boil in a microwave followed by maintaining heat at 10% power for 8 min. The solution was allowed to cool to room temperature before washing the tissue sections 2 x 2 min in PBS.

For immunoperoxidase labeling, endogenous peroxidase was blocked at this stage during the procedure with fresh 3% H₂O₂ in PBS for 30 min and washed 1 x 2 min in PBS. This step was omitted for fluorochrome labeled tissue sections. Tissue sections were then blocked for 30 min at room temperature with 4% BSA and 2% NGS in PBS to prevent any non-specific staining. During this incubation period, primary antibody dilutions were prepared in blocking solution (refer to table 3.3. for antibody dilutions) and the tissue sections were incubated with primary antibody for 2 h at room temperature or overnight at 4°C. Prior to applying the appropriate HRP or fluorochrome conjugated secondary antibody diluted in blocking solution (refer to Table 3.3. for dilutions) tissue sections were washed 2 x 5 min in PBS. HRP or fluorochrome conjugated secondary antibodies were applied for 45 min at room temperature and the sections were washed 2 x 5 min in PBS. Following this washing step fluorochrome labeled sections were mounted using one of the two aqueous mounting media as described under section 3.8.5.: VECTASHIELD® Hard Set mounting medium with DAPI or 90% glycerol in PBS.

Immunoperoxidase labeled tissue sections were incubated with DAB substrate (1 mg/mL DAB, 0.02% H₂O₂ in PBS) for 1 – 2 min. The tissue sections were rinsed in mQH₂O to terminate immunoperoxidase staining and were counterstained with hematoxylin and/or eosin if desired (refer to section 3.8.5.). Immunoperoxidase labeled tissue sections were dehydrated 3 x 1 min in 100% ethanol then 2 x 5 min in fresh absolute toluene prior to
being mounted with DPX resin.

Table 3.3. Histological immunoperoxidase labeling (peroxidase) and IHC antibodies

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilution factor</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immunoperoxidase</td>
<td>IHC</td>
</tr>
<tr>
<td>Primary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat anti-GST-COMMD1/COMMD3 antiserum</td>
<td>1:50</td>
<td>1:50</td>
</tr>
<tr>
<td>Chicken anti-α, β or γENaC</td>
<td>1:50</td>
<td>1:50</td>
</tr>
<tr>
<td>Rabbit anti-aquaporin 2 (AQP2)</td>
<td>1:50</td>
<td>1:50</td>
</tr>
<tr>
<td>Wheat Germ Agglutinin (WGA) AF®-488 conjugate</td>
<td>-</td>
<td>1:50</td>
</tr>
<tr>
<td>Secondary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat anti-rat HRP</td>
<td>1:200</td>
<td>-</td>
</tr>
<tr>
<td>Goat anti-chicken HRP</td>
<td>1:200</td>
<td>-</td>
</tr>
<tr>
<td>Goat anti-rabbit HRP</td>
<td>1:200</td>
<td>-</td>
</tr>
<tr>
<td>AF® 488/633 goat anti-rat</td>
<td>-</td>
<td>1:200</td>
</tr>
<tr>
<td>AF® 488/633 goat anti-chicken</td>
<td>-</td>
<td>1:200</td>
</tr>
<tr>
<td>AF® 546 goat anti-rabbit</td>
<td>-</td>
<td>1:200</td>
</tr>
</tbody>
</table>

3.8.5. Hematoxylin and eosin staining

Following immunoperoxidase labeling, tissue sections were counterstained with Mayer’s hematoxylin and/or eosin (H&E) if desired. Hematoxylin stains cell nuclei blue-purple whereas eosin stains the cytoplasm bright pink therefore it helps to define histological samples anatomically. For this project, kidney sections were stained with Mayer’s hematoxylin 3 x 1 min. Sections were rinsed under running tap water for approximately 5 min to allow the stain to develop. Hematoxylin stained cells and tissue sections were briefly submerged in 70% ethanol containing 0.25% hydrogen chloride (HCl) to destain them to the desired intensity. Kidney sections were briefly rinsed under tap water prior to proceeding to eosin staining. The kidney sections were submerged 1 x 30 sec in eosin

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followed by destaining for 3 x 5 min in 95% ethanol. Following this step, immunoperoxidase labeled kidney sections were dehydrated and mounted as described under section 3.8.4.

3.8.6. Organelle markers
To investigate the specific intracellular location of endogenously expressed COMMD1 and COMMD3 proteins a number of different organelle markers were used in combination with antisera against COMMD1 and COMMD3 in a series of double label ICC and IHC experiments. Details regarding the staining protocols employed for these organelle markers are described below.

3.8.6.1. DAPI
DAPI preferentially binds to double-stranded (ds) DNA and is most commonly used as a nuclear counterstain in both ICC and IHC. The DAPI/dsDNA complex is excited by UV light (~360 nm) and emits light at about 460 nm, producing a blue fluorescence. For this project DAPI was used in the form of VECTASHIELD® Hard Set mounting medium with DAPI (Vector Laboratories) for both ICC and IHC experiments. A sufficient amount of VECTASHIELD® Hard Set mounting medium with DAPI was dispensed onto glass microscope slides for cytological samples or directly onto the histological sections. The samples were coverslipped/mounted and the mounting medium was allowed to disperse over the entire sample and dried overnight at room temperature in the dark prior to being viewed using a microscope (refer to section 3.8.7.).

3.8.6.2. Transferrin
Transferrin is an approximately 80 kDa iron-transporting glycoprotein that binds to iron in a reversible manner. Iron is taken up into the cell via transferrin receptor-mediated endocytosis of the iron-transferrin complex (Dautry-Varsat, 1986; Rothenberger et al., 1987). A subsequent decrease in the pH of the endosomes facilitates removal of iron from transferrin and receptor-bound apotransferrin recycles back to the cell surface. From here apotransferrin enters the blood circulation where it is free to bind iron once again. Transferrin is often used a marker for recycling/early endosomes because of its recycling
between the endosomal compartments. For double label ICC experiments, cells were prepared for ICC as described under section 3.8.3. Spent growth medium was removed by aspiration and the cells were incubated with 50 μg/mL Alexa Fluor® 546 conjugated transferrin (Molecular Probes) diluted in low-bicarbonate DMEM for 10 min at 37°C (refer to table 3.2.). Cells were fixed and processed as per the standard protocol (refer to section 3.8.3. and figure 3.1.).

3.8.6.3. Lysosomal Associated Membrane Protein (LAMP1)
LAMP1 (lysosomal associated membrane protein 1) is a highly glycosylated membrane-associated protein that predominantly localises to the limiting membrane of late endosomes-lysosomes, but can also be found on the plasma membrane (Fukuda, 1991; Kornfeld and Mellman, 1989; Parkinson-Lawrence et al., 2005). Being a membrane-associated protein it contains a putative signal peptide, 18 sites for N-linked glycosylation, one membrane spanning domain and a short cytosolic tail (Fukuda et al., 1988). Antibodies generated against LAMP1 are often used as lysosomal markers for use in ICC. For this project, anti-mouse LAMP1 antibody (BD Biosciences) was simultaneously incubated with antiserum against COMMD1 or COMMD3 for 2 h at room temperature for double label ICC experiments (refer to section 3.8.3.) and was visualised with Alexa Fluor® 546 conjugated goat anti-mouse IgG (refer to table 3.2. for antibody dilution factors).

3.8.6.4. Early Endosomal Antigen 1 (EEA1)
EEA1 (early endosomal antigen 1) is a 180 kDa membrane bound protein component that is essential for fusion between early endocytic vesicles (Mu et al., 1995). Because EEA1 is specific to early endosomes, antibodies generated against this antigen are often used as a marker for early endosomes in ICC experiments. Monoclonal mouse anti-EEA1 (1:100) was used for double labeling experiments to investigate if COMMD1 and COMMD3 localise to early endosomes. Mouse anti-EEA1 was simultaneously incubated with antiserum against COMMD1 or COMMD3 and was visualised with Alexa Fluor® 546 goat anti-mouse IgG (refer to table 3.2. for antibody dilutions).
3.8.6.5. Wheat Germ Agglutinin (WGA)

WGA (wheat germ agglutinin) is an approximately 36 kDa carbohydrate-binding protein (i.e. a lectin) that selectively binds to sialic acid and N-acetylglucosaminyl sugar residues (Wright, 1984). These sugar residues are most abundant in the plasma membrane but are also associated with Golgi bodies. For this project, Alexa Fluor® 488 conjugated WGA was used as a marker for the Golgi apparatus in both ICC and IHC double labeling experiments. For ICC the samples were prepared as outlined under section 3.8.3. The spent growth medium was removed by aspiration and cells were fixed with 4% paraformaldehyde for 15 min at 37°C. The cells were washed twice with PBS and a 1:50 dilution of Alexa Fluor® 488 conjugated WGA (stock solution was 1 mg/mL) was prepared in PBS. A sufficient amount of the diluted Alexa Fluor® 488 conjugated WGA was applied to the cells. This was incubated for 10min at room temperature (refer to table 3.2.). The labeling solution was removed and the cells were washed, permeabilised, blocked, labeled with antiserum against COMMD1 or COMMD3 and mounted as described under section 3.8.3. (refer to figure 3.1. for protocol summary) For IHC experiments using Alexa Fluor® 488 conjugated WGA, samples were processed as outlined under section 3.8.4. Alexa Fluor® 488 conjugated WGA (1:50) was simultaneously incubated with COMMD1 or COMMD3 antiserum for 2 h at room temperature. The sections were then immunoblotted with Alexa Fluor® 633 goat anti-rat secondary antibody to visualise COMMD1 and COMMD3 and were then further processed (i.e. washed and mounted) as described under section 3.8.4.
Variations of the standard ICC protocol (in black) were employed for immunoperoxidase (in blue) and double labeling experiments using different organelle markers. Cells were incubated with transferrin prior to being fixed (in red) whereas labeling with WGA required cell fixation at 37°C for 15 min followed by a 10 min incubation with WGA at room temperature (RT) (in green). Cells were washed in between each step (indicated by arrows). All other organelle markers not specified here were simultaneously incubated with antiserum against either COMMD1 or COMMD3 using the standard ICC protocol and were visualised with fluorochrome conjugated secondary antibodies (i.e. indirect ICC).

3.8.7. Microscopy

Confocal microscopy is a powerful tool for visualising fluorescent specimens and offers many advantages over conventional wide-field microscopy (Smith, 2008). The principle of confocal microscopy incorporates the ideas of point-by-point illumination and spatial filtering. Point-by-point illumination enables a confocal microscope to selectively collect light from a thin optical section (<1 μm) at the plane of focus in the specimen. This allows for a series of images, which are referred to as Z sections, to be captured at fixed intervals throughout the entire depth of the specimen. Images acquired by optical sectioning can be used to reconstruct a three-dimensional view of the specimen. This contrasts with conventional wide-field fluorescent microscopes which collect fluorescent signals not only from the plane of focus but also from areas above and below thus
producing relatively out-of-focus images that lack contrast. Spatial filtering eliminates out-of-focus light in specimens that are thicker than the plane of focus thus allowing structures within the focal plane to appear more sharply defined than with conventional wide-field microscopy. Using confocal microscopy, out-of-focus light can be limited by adjusting the diameter of the pinhole. Increasing the diameter of the pinhole produces a thicker optical section and reduced resolution. Decreasing the pinhole diameter creates the opposite effect: a decrease in the thickness of the optical section and brightness. Optimum resolution is reached at a certain minimum pinhole diameter. These features make confocal microscopy well suited for studying the structure and function of cells and/or proteins using immunofluorescence reagents.

For this project all immunoperoxidase labeled samples were examined using an Olympus AX70 Provis light microscope (Tokyo, Japan). Representative digital images were acquired using dedicated software and digital images were saved in TIFF format.

The OlymousAX70 microscope can also function as a reflected fluorescence light (epi-fluorescence) microscope. It is equipped with a mercury arc lamp that provides a mixture of wavelengths from UV to red. The costs associated with running this microscope is much less compared to that of the confocal microscope therefore the epifluorescent microscope was occasionally used to determine if fluorescent staining was successful prior to viewing the specimens with the more advanced laser scanning confocal microscope.

Fluorochrome conjugated ICC and IHC preparations were examined with a Zeiss LSM (laser scanning microscope) 510 upright confocal microscope system (Jena, Germany) equipped with combined argon and helium-neon (HeNe) lasers, which provide 488 nm and 543 nm laser lines, respectively. Appropriate emission filter settings were used to avoid spectral crossover between different fluorochromes. A 488/543 nm primary dichroic mirror and a band pass filter of 505 – 550 nm was used to collect signals that were excited at a wavelength of 488 nm. Signals that were excited at a wavelength of 543 nm were collected using a 488/543 nm primary dichroic mirror and a 560 nm long pass
filter. Confocal images were captured using either the 40x or 63x oil objective lenses and Z series were collected at 0.36 μm intervals. Some fluorochrome conjugated ICC and IHC preparations were examined using the 100x oil objective lens of an Olympus AX70 microscope (also described above). Both microscopes were equipped with mercury burners to visualise the nuclear stain DAPI. The mercury burner equipped on the confocal microscope had a peak excitation at 365 nm and a 395 nm long pass dichroic filter. All settings were kept constant for each independent experiment and representative images were acquired sequentially. Digital images that were acquired using the LSM510 control software on the confocal microscope were saved in LSM format and were processed using ImageJ freeware software available online from NIH (Collins, 2007). All microscope images were formatted for presentation using Adobe Illustrator CS4.
4. Results

4.1. Characterisation of COMMD1 and COMMD3 antisera

Other members of our laboratory produced the COMMD1 and COMMD3 antisera used in this project (refer to section 3.6.1.). This was generated by serial immunisation of rats with purified recombinant GST-COMMD1 and GST-COMMD3 protein, respectively. In theory, use of the purified recombinant GST-COMMD1 and GST-COMMD3 protein in the generation of COMMD1 and COMMD3 polyclonal antibodies would have resulted in the production of antiserum that contained a mix of anti-GST and anti-COMMD1 or anti-COMMD3 antibodies therefore the specificity of the produced antiserum was partially characterised in this project. This was done by preincubating the COMMD1 and COMMD3 antiserum with 100 μg of either GST or the relevant immunising antigen, being GST-COMMD1 and GST-COMMD3, for every 1 μL of antiserum cleared (refer to section 3.6.2.). The different antisera were used on Western blots of GST or GST-COMMD1/GST-COMMD3.

GST fusion proteins were prepared (section 3.5.1.) and then the protein concentration was determined by loading equal volumes (10 μL) of each fusion protein alongside a series of known BSA standards. This was separated on a 0.75 mm 12% SDS-PAGE gel (refer to section 3.7.). Following separation of the fusion proteins, the gel was stained with Coomassie™ Blue as described under section 3.7.2. A representative Coomassie™ Blue stained gel is shown in figure 4.1. Typical fusion protein concentrations based on a 20 mL overnight culture ranged from 4 – 5 μg/μL for GST, 0.5 – 2.5 μg/μL for GST-COMMD1 and 0.2 – 1 μg/μL for GST-COMMD3.
Figure 4.1. Expression of GST fusion proteins

This figure represents a typical Coomassie Blue stained gel that was used to determine GST fusion protein concentrations in this project. GST fusion protein concentrations were determined by resolving equal volumes (10 μL) of fusion proteins on a 0.75 mm 12% SDS-PAGE gel alongside a series of known BSA standards. Lanes 1, 2 and 3 show staining for 2.5 μg, 5 μg and 10 μg of BSA, respectively. A gap (lane 4) was allowed between the standards and fusion proteins. Staining for 10 μl of each of GST, GST-COMMD1 and GST-COMMD3 is shown in lanes 5, 6, and 7, respectively.

Subsequent to the determination of the GST fusion protein concentrations, equal protein concentrations (5 μg) of each of GST, GST-COMMD1 and GST-COMMD3 fusion protein was resolved on a 1.5 mm 12% SDS-PAGE gel and subjected to Western blot analysis with antiserum against COMMD1 and COMMD3 that was either 1) non-precleared, 2) GST pre-cleared or 3) antigen cleared (either GST-COMMD1 or GST-COMMD3) (n = 4). This was performed as outlined previously (refer to the previous page and section 3.6.2.).

The specificity of the antiserum to their respective antigens was visualised using chemiluminescence (Figure 4.2.). GST has a molecular weight of approximately 26 kDa and when expressed in frame with either full-length human COMMD1 or COMMD3 it has a combined molecular weight of approximately 50 kDa. Immunoreactivity against GST and GST-COMMD1 or GST-COMMD3 was present when the appropriate non-precleared antiserum was used for immunoblotting. Preincubation of the antiserum with GST abolished immunoreactivity against the GST fusion protein but not GST-COMMD1 or GST-COMMD3 fusion proteins, whereas immunoreactivity against both GST and GST-COMMD1 or GST-COMMD3 was completely abolished when the antiserum was
preincubated with the appropriate antigen. The non-specific bands apparent on the immunoblots are likely products of proteolysis. This could have been minimised by decreasing the amounts of antigen resolved by SDS-PAGE. In addition to the results presented here, Liu, Y. has demonstrated that the COMMD1 antiserum used in this project does not cross-react with the COMMD3 antigen, and *vice versa* (personal communication). Based on these data and the results presented here, it is evident that the COMMD1 and COMMD3 antiserum used in this project contained antibodies that specifically recognised COMMD1 and COMMD3, respectively.

**Figure 4.2. Specificity of COMMD1 and COMMD3 antiserum**

Antiserum against COMMD1 or COMMD3 was either non-precleared or precleared with GST or the respective antigen, being GST-COMMD1 or GST-COMMD3. The different COMMD1 and COMMD3 antiserum samples were used in subsequent Western blot analysis to detect either GST (approximately 25 kDa) or GST-COMMD1/GST-COMMD3 (approximately 50 kDa). Non-precleared COMMD1 antiserum recognised both GST (lane 1) and GST-COMMD1 (lane 2) fusion proteins. COMMD1 antiserum precleared with GST only recognised GST-COMMD1 (lane 4) and not GST (lane 3) fusion proteins whereas COMMD1 antiserum precleared with GST-COMMD1 did not recognise GST (lane 5) or GST-COMMD1 (lane 6). Non-precleared COMMD3 antiserum recognised both GST (lane 7) and GST-COMMD3 (lane 8) fusion proteins. COMMD3 antiserum precleared with GST only recognised its antigen (lane 10) and not GST (lane 9) whereas antigen cleared COMMD3 antiserum did not recognise GST (lane 11) or GST-COMMD3 (lane 12) fusion proteins. These experiments (n = 4) confirmed that antiserum against COMMD1 and COMMD3 contained antibodies that specifically recognised their respective antigens.
Non-precleared antisera was used for immunoblotting in all Western blots, ICC and IHC (refer to section 4.4.1. for antiserum characterisation in native tissue) experiments performed during this project.

4.2. Endogenous protein expression of COMMD1 and COMMD3
To address the first hypothesis of this project, which was that in order for COMMD1 and COMMD3 to inhibit ENaC the COMMD proteins localise to specific intracellular vesicular compartments that overlap with the trafficking pathways of ENaC, two objectives were developed (refer to section 2 for aims and hypotheses). The first of these objectives was to screen different cell lines and native tissue for the expression of endogenous COMMD1 and COMMD3 proteins. This was achieved by performing Western blot analysis on both whole-cell and whole-tissue lysates. The results obtained from these experiments are presented below in sections 4.2.1. and 4.2.2. and are summarised in section 4.2.3.

4.2.1. Expression of COMMD1 and COMMD3 in selected mammalian cell lines
Western blot analysis was employed to investigate the presence of endogenously expressed COMMD1 and COMMD3 proteins in four different mammalian cell lines. These experiments were performed to identify cell lines that were suitable for use in determining the intracellular distribution and specific location of endogenous COMMD1 and COMMD3 proteins using ICC. The cell lines selected for this project are all derived from tissues in which COMMD mRNA and/or proteins have previously been identified: HEK293 (human kidney), COS-7 (monkey kidney), SH-SY5Y (human neuroblastoma) and Raji (human thymus) cells. Employing reverse transcriptase polymerase chain reaction (RT-PCR), Burstein et al. (Burstein et al., 2005) identified all ten COMMD genes in HEK293 cells. The presence of COMMD genes, mRNA or proteins had not been previously reported in COS-7, SH-SY5Y or Raji cells.

Endogenous COMMD1 protein was easily detected in all investigated cell lines (section 4.2.1.1.) when cells were lysed with either 1X SDS sample buffer or 1X TBS containing 1% Triton® X-100 lysis buffer. Detection of endogenous COMMD3 proteins proved to
be more challenging and a number of optimisation steps were employed in an attempt to solve this issue. The optimisation steps were carried out using 1X TBS containing 1% Triton X-100 as the lysis buffer as opposed to 1X SDS sample buffer as the latter had a tendency to produce a very viscous lysate that was difficult to handle and load onto SDS-PAGE gels. Decreasing the volume of lysis buffer that was used was the first optimisation step that was employed in an attempt to increase the yield of whole-cell protein concentration for any volume. This was required to increase the amount of whole-cell protein loaded onto the SDS-PAGE gels and in turn to increase the amount of COMMD3 proteins available for detection. This step did not prove to be very successful and an alternative measure therefore was to decrease the boiling temperature of the lysate and to increase the duration of this step in an attempt to minimise the risk of protein degradation. Detection of endogenously expressed COMMD3 protein was inconsistent throughout the duration of this project and was therefore only detected in HEK293 and SH-SY5Y cells on two separate occasions (n = 2) (section 4.2.1.2.).

4.2.1.1. COMMD1 is ubiquitously expressed

During the initial stages of this project equal volumes (40 μL) of the four different whole-cell lysates were separated alongside one another by SDS-PAGE as opposed to equal protein concentrations. This was later changed to loading equal protein concentrations (10 – 40 μg). Following separation of the whole-cell proteins by 12% SDS-PAGE (1.5 mm), the proteins were electro transferred onto PVDF membranes and immunoblotted with COMMD1 antiserum. This was followed by a 1 h incubation period with HRP conjugated goat anti-rat secondary antibody and the product was detected using chemiluminescence. The results are shown in figure 4.3. For each of the four different whole-cell lysate samples analysed a single band corresponding to approximately 25 kDa was detected for COMMD1. Lanes 1 to 4 correspond to HEK293, COS-7, Raji and SH-SY5Y whole-cell lysates, respectively (Figure 4.3.).
4.2.1.2. Endogenous expression of COMMD3

The presence of endogenously expressed COMMD3 protein in HEK293, COS-7, SH-SY5Y and Raji cells was investigated by Western blot analysis of whole-cell lysates. Equal volumes (40 μL) of the four different whole-cell lysates were separated by 12% SDS-PAGE (1.5 mm) and transferred onto PVDF membranes. The membranes were immunoblotted with COMMD3 antiserum followed by a 1 h incubation with HRP conjugated goat anti-rat secondary antibody. The product was detected using chemiluminescence and the results are shown in figure 4.4. Endogenous COMMD3 protein was detected in HEK293 (Figure 4.4. lane 1) following a 5 min exposure but not in SH-SY5Y (Figure 4.4. lane 8), COS-7 (Figure 4.4 lane 2) and Raji (Figure 4.4. lane 7) whole-cell lysates. Following a longer exposure period of 30 min, endogenous COMMD3 protein was also detected in SH-SY5Y whole-cell lysate (Figure 4.4. lane 9) but not in COS-7 and Raji whole-cell lysate (result not shown). Whole-cell lysates from HEK293 (Figure 4.4. lane 4) and COS-7 cells (Figure 4.4. lane 5) transiently transfected with FLAG tagged COMMD3 were used as positive controls in these experiments. Tagged proteins have a higher molecular weight than their endogenous counterparts therefore the protein band corresponding to transfected FLAG (molecular weight of ~1 kDa) tagged COMMD3 (~24 kDa) in HEK293 and COS-7 cells (Figure 4.4. lanes 4 and 5) appeared higher on the Western blot compared to endogenous COMMD3.
Figure 4.4. Endogenous protein expression of COMMD3 in different mammalian cell lines

Equal volumes (40 μl) of whole-cell lysates were loaded onto a 12% SDS-PAGE gel. The Western blot was probed with COMMD3 antiserum (1:5000) followed by HRP conjugated goat anti-rat secondary antibody (1:10000). FLAG tagged COMMD3 transiently transfected in HEK293 (higher molecular weight band in lane 4) and COS-7 cells (lane 5) were used as positive controls. Following a 5 min exposure, endogenous COMMD3 was detected by Western blot analysis in HEK293 (lane 1 and also the lower molecular weight band in lane 4) but not in COS-7 (lane 2), Raji (lane 7) or SH-SY5Y whole-cell lysates (lane 8). Endogenous COMMD3 was detected in SH-SY5Y whole-cell lysate (lane 9) following a longer exposure of 30 min. (n = 2)

4.2.2. Expression of COMMD1 and COMMD3 in the rat kidney

Mouse and/or rat whole-kidney lysates, which were prepared using kidneys from control animals, were investigated for endogenous protein expression of COMMD1 and COMMD3 using Western blot analysis. These experiments were performed to identify the presence of COMMD1 and COMMD3 in the kidney, which was subsequently used for IHC to investigate the cellular localisation and possible colocalisation of these two proteins with ENaC and AQP2 in vivo. The latter was used as a protein marker for the principal cells of the collecting ducts.

Kidneys from control mice and/or rats were homogenised as described under section 3.3.2. and the protein concentration of the whole-kidney lysates was quantified as outlined under section 3.4. Mouse kidneys, which were harvested during the initial stages of this project, were homogenised using a standard RIPA (radio-immunoprecipitation assay) buffer solution (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.25%
sodium deoxycholate, 1 mM EDTA, 1 mM PMSF and 1 μg/mL of each of aprotinin, leupeptin and pepstatin). Detection of endogenous COMMD1 and COMMD3 proteins using Western blot analysis was largely unsuccessful when the RIPA buffer solution was used. The immunoblots appeared very messy despite experimenting with different blocking solutions (5% low-fat milk powder and 1% BSA, respectively), chemiluminescence detection systems (Lumi-Light and Lumigen), boiling temperatures and duration as well as SDS-PAGE gel concentrations (from 12% to 15%). Whole-kidney lysis was later performed using rat kidneys and the homogenisation buffer detailed under section 3.3.2. This, in combination with a decrease in ‘boiling’ temperature and duration (from 3 min at 100°C to 15 min at 60°C) as well as a SDS-PAGE gel concentration of 15% resulted in detection of endogenous COMMD1 and COMMD3 proteins being successful.

For these experiments, equal protein concentrations (ranging between 30 – 50 μg) of whole-kidney lysate were separated on 15% SDS-PAGE gels (1.5 mm) followed by Western blot analysis. COMMD1 and COMMD3 were immunoblotted with antiserum against COMMD1 and COMMD3, respectively. This was followed by a 1 h incubation period with HRP conjugated goat anti-rat secondary antibody and the product was detected by chemiluminescence.

Endogenously expressed COMMD1 protein was detected in whole-kidney lysates from mice (results not shown) and rats (Figure 4.5. panel A; 30 μg and 50 μg of whole-kidney protein separated in lanes 2 and 3, respectively). Endogenously expressed COMMD3 protein was only detected in whole-kidney lysates from rats (Figure 4.6. panel B; 30 μg of whole-kidney protein separated in lanes 2 and 5 and 50 μg of protein in lanes 3 and 6). All subsequent IHC was performed on kidney sections from dexamethasone treated rats (refer to section 4.5.).
Equal protein concentrations (20 μg, 30 μg or 50 μg) were loaded and separated on 15% SDS-PAGE gels followed by Western blot analysis with (A) antiserum against COMMD1 (1:5000) and (B) antiserum against COMMD3 (1:5000). Both Western blots were then probed with HRP conjugated goat anti-rat secondary antibody followed by chemiluminescence. (A) COMMD1 was shown to be endogenously expressed in COS-7 cells (refer to section 4.2.1.1.) therefore untransfected COS-7 whole-cell protein (20 μg) was used as a positive control for the detection of COMMD1 (lane 1). Whole-kidney protein (30 μg in lane 2 and 50 μg in lane 3) was loaded alongside the positive control for COMMD1 (lane 1). COMMD1 was detected following a 1 min exposure in both the 30 μg and 50 μg samples. (B) FLAG tagged COMMD3 transiently transfected in COS-7 cells was used as a positive control for the detection of COMMD3 and whole-cell protein (20 μg) was loaded into lanes 1 and 4 (positive controls). Whole rat kidney lysate (30 μg of protein in lanes 2 and 5; 50 μg protein in lanes 3 and 6) was used to detect endogenous COMMD3. Some endogenously expressed COMMD3 protein was detected following a 1 min exposure but this was more apparent after a 5 min exposure.

4.2.3. Summary

Whole-cell and whole-kidney lysate samples were screened for endogenous protein expression of COMMD1 and COMMD3 using Western blot analysis. Results obtained from these experiments are summarised in Table 4.1. Endogenous protein expression of COMMD1 was detected in all cell lines screened (HEK293, COS-7, SH-SY5Y and Raji.
cells) as well as in mouse and rat whole-kidney lysate. The expression of COMMD3 was more selective and was only detected in HEK293 and SH-SY5Y cells and rat whole-kidney lysate. No endogenous COMMD3 protein was detected in mouse whole-kidney lysate or COS-7 and Raji whole-cell lysates.

**Table 4.1. Summary of results: endogenous protein expression of COMMD1 and COMMD3**

*n* = number of experiments performed; ✓ = endogenous expression detected; ✗ = endogenous expression not detected

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>COMMD1</th>
<th>COMMD3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293</td>
<td>✓ (n = 4)</td>
<td>✓ (n = 2)</td>
</tr>
<tr>
<td>COS-7</td>
<td>✓ (n = 4)</td>
<td>✗ (n = 2)</td>
</tr>
<tr>
<td>SH-SY5Y</td>
<td>✓ (n = 4)</td>
<td>✓ (n = 2)</td>
</tr>
<tr>
<td>Raji</td>
<td>✓ (n = 4)</td>
<td>✗ (n = 2)</td>
</tr>
<tr>
<td>Native tissue Mouse kidney</td>
<td>✓ (n = 1)</td>
<td>✗ (n = 1)</td>
</tr>
<tr>
<td></td>
<td>✓ (n = 3)</td>
<td>✓ (n = 2)</td>
</tr>
</tbody>
</table>

4.3. *Intracellular distribution of the COMMD proteins*

With the exception of COMMD1, the intracellular distribution of endogenously expressed COMMD proteins remain largely undefined. This, in combination with previous work performed in our laboratory, which have demonstrated a COMMD-mediated inhibitory effect on ENaC, provided the motivation behind the next series of experiments.

Identification of an endogenously expressed protein’s intracellular distribution and specific location can provide significant insight into its true *in vivo* function and thereby the way in which it mediates its effect on interacting proteins. With an aim to investigate the mode by which COMMD1 and COMMD3 mediate its effects on ENaC, the objective was to investigate the intracellular distribution and specific location of endogenously expressed COMMD1 and COMMD3 proteins using single and double label ICC. This was the second objective (refer to section 2 for aims and hypotheses) that was developed to help address the first hypothesis of this project further, which was that, in order for
COMMD1 and COMMD3 to inhibit ENaC, COMMD1 and COMMD3 localise to specific intracellular vesicular compartments that overlap with the trafficking pathways of ENaC.

4.3.1. Pilot experiments – immunoperoxidase labeling for COMMD1 and COMMD3

The presence of endogenously expressed COMMD1 and COMMD3 protein was investigated in four different mammalian cell lines using Western blot analysis (refer to section 4.2.1.). COMMD1 was identified in all investigated cell lines (HEK293, COS-7, SH-SY5Y and Raji cells) whereas COMMD3 was only detected in HEK293 and SH-SY5Y cells. Based on these results, indirect ICC was only performed on HEK293 and SH-SY5Y cells to investigate the intracellular distribution and specific location of endogenously expressed COMMD1 and COMMD3 proteins.

Prior to commencing indirect ICC, immunoperoxidase labeling was employed to optimise the protocol for the detection of endogenous COMMD1 in SH-SY5Y cells and COMMD3 in HEK293 and SH-SY5Y cells. Protocols described by Chang, T. (Chang, 2007) were employed during the initial stages of the immunoperoxidase and ICC experiments performed in this project however these were suitably adapted. Optimisation steps included changing the fixative solution from a mixture of buffered 4% paraformaldehyde and 0.5% glutaraldehyde, which Chang, T. used for both confocal and electron microscopy, to 4% paraformaldehyde in PBS. Buffered 4% paraformaldehyde is a standard fixative for fluorescence microscopy (Smith, 2008) that has been used for preparing both cytological (Burkhead et al., 2009) and histological (Bedford et al., 2003) specimens. Antibody dilutions were also experimented with and were typically much lower for ICC experiments compared to immunoperoxidase labeling. For example, cellular immunoperoxidase labeling for endogenous COMMD3 protein was performed using a 1:500 - 2000 dilution of COMMD3 antiserum followed by a 1:100 dilution of HRP conjugated goat-anti-rat secondary antibody. For ICC experiments, a 1:50 dilution of COMMD1 and COMMD3 antiserum was used followed by a 1:200 dilution of the appropriate fluorochrome conjugated (either Alexa Fluor® 488 or 633) goat anti-rat
secondary antibody.

During the initial stages of this project staining for only COMMD3 was to be investigated. However as the project evolved it was later decided to also include staining for COMMD1 to use as a comparison therefore no immunoperoxidase staining was carried out for COMMD1 in HEK293 cells. Immunoperoxidase staining was performed as outlined under section 3.8.3. Briefly, cells were fixed, permeabilised, quenched and blocked prior to being immunoblotted with COMMD1 or COMMD3 antiserum. This was followed by a 1 h incubation period with HRP conjugated goat anti-rat secondary antibody. DAB substrate was applied and the staining intensity was monitored using a light microscope. The samples were rinsed, mounted and analysed using an Olympus AX70 light microscope. Primary antibodies were omitted for use as negative controls in all immunoperoxidase labeling and subsequent ICC experiments. These were included for each independent experiment and non-specific staining was consistently absent. Positive controls were also included for immunoperoxidase experiments by labeling transiently transfected FLAG tagged COMMD1 and FLAG tagged COMMD3 complementary deoxyribonucleic acid (cDNA) in COS-7 and HEK293 cells. The intracellular distribution pattern of the COMMD1 and COMMD3 positive controls appeared similar to that described for endogenous COMMD1 and COMMD3 proteins (Figure 4.6.). COS-7 cells were also used as negative controls for COMMD3 immunoperoxidase labeling experiments. These controls appeared clear of any COMMD3 specific staining thus supporting the Western blot results showing that endogenously expressed COMMD3 protein was undetectable in COS-7 cells (negative result not shown).

Preliminary results from immunoperoxidase labeling experiments revealed that COMMD1 exhibited a punctate cytoplasmic distribution that was denser around the nuclei (i.e. perinuclear) in SH-SY5Y cells whereas COMMD3 appeared more diffuse throughout the cytosol in both SH-SY5Y and HEK293 cells (Figure 4.6.). Some vesicular staining for COMMD3 cannot be excluded.
Figure 4.6. Intracellular distribution of COMMD1 and COMMD3 as determined by immunoperoxidase labeling in HEK293 and SH-SY5Y cells

Staining for COMMD1 in SH-SY5Y cells (A) revealed a cytoplasmic vesicular distribution (indicated by white arrows). COMMD3 exhibited a more diffuse cytosolic distribution (indicated by white arrows) in both SH-SY5Y (B) and HEK293 cells (C) when compared with COMMD1. These distribution patterns for COMMD1 and COMMD3 were observed in approximately 85 - 95% of the cells investigated. Primary antibodies were omitted for use as negative controls in both SH-SY5Y (D) and HEK293 cells (E) and were consistently clear of any non-specific staining. Samples were analysed using an Olympus AX70 light microscope and the above images were captured using a 100x oil objective lens. Depicted scale bars (lower right corners) represent 10 μm.
Immunoperoxidase labeling is widely used in basic research to understand the distribution and localisation of a particular protein however it is not particularly good for studying colocalisation between two or more proteins. One major objective of this project was to investigate colocalisation of the COMMD proteins with specific organelle markers as well as with ENaC, therefore immunofluorescent ICC and IHC in combination with laser scanning confocal microscopy was employed specifically for this purpose.

4.3.2. Intracellular distribution of endogenously expressed COMMD1 and COMMD3 proteins as determined by indirect immunocytochemistry

Using the optimised staining protocol as determined by immunoperoxidase labeling, the intracellular distribution and specific location of endogenously expressed COMMD1 and COMMD3 proteins were further investigated by indirect ICC. Further optimisation of the COMMD1 and COMMD3 antiserum dilutions was required during the initial ICC experiments. All other steps in the staining procedure remained as was determined and optimised during the immunoperoxidase experiments.

Indirect ICC was performed as outlined under section 3.8.3. Cells were fixed, permeabilised, blocked and immunoblotted with antiserum against either COMMD1 (1:50) or COMMD3 (1:50). Labeling for COMMD1 and COMMD3 was visualised with Alexa Fluor®488/633 goat anti-rat IgG and analysed using laser scanning confocal microscopy.

Endogenously expressed COMMD1 protein was shown to predominantly exhibit a perinuclear but cytoplasmic vesicular distribution with some diffuse cytosolic staining present in both HEK293 and SH-SY5Y cells (Figure 4.7.). The nuclear counterstain DAPI was used in all ICC experiments. Some overlap in staining patterns between COMMD1 and DAPI was observed in this project. The detectable presence of some COMMD1 in the nucleus supports a role for this protein in the regulation of NF-κB activity (Ganesh et al., 2003) and HIF-1 activity during hypoxia (van de Sluis et al., 2007). Both processes would require COMMD1 to shuttle between the cytosol and nucleus. However, COMMD1 was predominantly localised to cytoplasmic vesicles that
exhibited a perinuclear distribution in both HEK293 and SH-SY5Y cells in this project therefore it is unlikely that, under the conditions employed, COMMD1 plays a major role in regulating NF-κB and HIF-1 activity in these two cell types. In contrast to this, endogenously expressed COMMD3 proteins were shown to exhibit a more diffuse cytosolic pattern of distribution in both HEK293 and SH-SY5Y cells (Figure 4.7.) and no overlap with DAPI. The ICC results obtained for the intracellular distribution of endogenously expressed COMMD1 and COMMD3 proteins are similar than those observed and described for the pilot experiments using immunoperoxidase labeling (refer to section 4.3.1.).
Figure 4.7. Intracellular distribution of endogenously expressed COMMD1 and COMMD3 in HEK293 and SH-SY5Y cells

(A) Staining for COMMD1 (shown in green) appeared perinuclear and vesicular (indicated by white arrows) in both HEK293 and SH-SY5Y cells. Cell nuclei were counterstained with DAPI (blue). Little staining for COMMD1 was observed in the cell nuclei. This is demonstrated by the absence of overlap in
the merged images. (B) COMMD3 (shown in green) exhibited a diffuse cytosolic distribution (indicated by white arrows) with no staining present in the cell nuclei. Likewise, this was demonstrated by the absence of overlap in staining patterns between COMMD3 and DAPI. This pattern of distribution was similar for HEK293 and SH-SY5Y cells. (C) Primary antibody was omitted for use as a negative control in both HEK293 and SH-SY5Y cells. These cells were consistently clear of any non-specific staining and only the cell nuclei (shown in blue) were present in these images. The above images were captured sequentially using either the 40x oil objective lens with a zoom or the 63x oil objective lens. The depicted scale bars (lower right corner) represent 10 μm.

4.3.3. Double labeling experiments using indirect immunocytochemistry
To further define the specific intracellular location of endogenously expressed COMMD1 and COMMD3 proteins and to identify at which point in the ENaC trafficking pathway COMMD1 and COMMD3 mediate their effects on ENaC in vivo a series of double labeling experiments were performed in HEK293 and SH-SY5Y cells. These experiments were performed as outlined under section 3.8.3. Briefly, COMMD1 and COMMD3 were labeled with antiserum against COMMD1 or COMMD3 in combination with a number of specific organelle markers (refer to section 3.8.6). Not all double labeling experiments performed during this project were successful however all are discussed in more detail below and images were included where available.

4.3.3.1. COMMD1 and COMMD3 do not localise to the Golgi apparatus
The Golgi apparatus forms part of the secretory pathway. It is the organelle responsible for processing proteins that are synthesised in the ER, including ENaC (refer to section 1.1.5.2.). Double labeling experiments using antiserum against COMMD1 or COMMD3 in combination with the Golgi marker WGA was performed to determine if endogenously expressed COMMD1 and COMMD3 proteins localise to the Golgi apparatus. The presence of the COMMD proteins in the Golgi apparatus may implicate a possible role for COMMD1 and COMMD3 in post-translational modifications and/or proteolytic cleavage of ENaC or regulation of its trafficking through the secretory pathway. This may potentially alter the normal mode of ENaC trafficking to the apical cell surface and therefore may explain the inhibitory effect that COMMD1 and COMMD3 have on ENaC. Golgi were stained with Alexa Fluor®488 conjugated WGA as described under
Endogenous COMMD1 and COMMD3 proteins were detected with antiserum against COMMD1 or COMMD3, respectively, and were visualised with Alexa Fluor® 633 conjugated goat anti-rat IgG (refer to section 3.8.3.). Samples were viewed with a laser scanning confocal microscope and Z series were captured at an interval of 0.36 μm.

No overlap in staining pattern was observed for COMMD1 or COMMD3 with the Golgi apparatus marker WGA in SH-SY5Y cells (Figure 4.8.). A series of Z sections were captured to confirm the absence of overlap in staining pattern between COMMD1 (Figure 4.9.) or COMMD3 (Figure 4.10.) with WGA in SH-SY5Y cells. The images were captured from the top (Figures 4.9. and 4.10. A) of the cell and successive images (Figure 4.9. B – I; Figure 4.10. B - K) gradually moved down the depth of the cell in increments of 0.36 μm. The results presented here are based on a single double labeling experiment (n = 1) performed for COMMD1 or COMMD3 with WGA. Absence of overlap in staining patterns for COMMD1 or COMMD3 with WGA was observed in the majority of the cells in the sample. WGA selectively binds to sialic acid and N-acetylglucosaminyl sugar residues, which are most abundant in the plasma membrane. However, no detectable plasma membrane staining as a result of WGA binding to these carbohydrates were observed in the double label ICC experiments presented here. Double labeling experiments in which HEK293 cells were used were unsuccessful (results not shown). The mounting media used (VECTASHIELD® Hard Set mounting medium with DAPI) appeared to have formed bubbles around groups of cells that made focusing in on the cells challenging. This effect may have resulted in spherical aberration that may potentially have lead to incorrect depth discrimination and therefore deterioration of the image.
Colocalisation of endogenous COMMD1 and COMMD3 with the Golgi apparatus marker WGA was investigated using indirect ICC and confocal microscopy. COMMD1 and COMMD3 were detected using antiserum against COMMD1 and COMMD3, respectively, and were visualised with Alexa Fluor®633 conjugated goat anti-rat IgG. Cell nuclei were counterstained with DAPI (shown in blue in both A and B). Each set of the images were taken from the same level in a Z series stack. (A) Staining for COMMD1 appeared perinuclear and vesicular (shown in red). No overlap between COMMD1 and WGA (shown in green; indicated by white arrows) was observed. This is demonstrated by the absence of any yellow staining in the merged image for COMMD1 and WGA. (B) COMMD3 (shown in red) exhibited a diffuse pattern of distribution in this image. No overlap in staining pattern was observed between COMMD3 and WGA (shown in green; indicated by white arrows). Again, this was demonstrated by the absence of any yellow staining in the merged image for COMMD1 and WGA. The above images were captured sequentially using the 40x oil objective lens and depicted scale bars (lower right corner) represent 10 μm. Negative controls were included and were prepared as described in section 4.3.1. The results of the negative controls (not shown) were comparable to that observed for figure 4.7.
Figure 4.9. Double labeling for endogenous COMMD1 and the Golgi apparatus in SH-SY5Y cells

Double label indirect ICC was employed to investigate colocalisation of COMMD1 (red) and the Golgi marker WGA (green). Cell nuclei were counterstained with DAPI (blue). The absence of overlap (or yellow staining) and therefore colocalisation between these two proteins was confirmed by serial images taken in the Z plane with an optimal interval of 0.36 μm. Images were taken sequentially starting from the top of the cell and ending at the bottom of the cell (A – I). Negative controls were included and appeared similar to that shown in figure 4.7. The above images were captured using the 40x oil objective lens and the depicted scale bars (lower right corner) represent 10 μm.
Double label indirect ICC was employed to investigate colocalisation of COMMD3 (red) and the Golgi marker WGA (green). Cell nuclei were counterstained with DAPI (blue). The absence of colocalisation (or yellow staining) between these two proteins was confirmed by serial images taken in the Z plane with an optimal interval of 0.36 μm. The images were taken sequentially starting from the top of the cell and ending at the bottom of the cell (A – K). Box ‘L’ contains no image. Negative controls were included (not shown here) and were clear of any non-specific staining. The images were captured using the 40x oil objective lens and depicted scale bars (lower right corner) represent 10 μm.

4.3.3.2. Double labeling for COMMD1 and COMMD3 with the lysosomal marker LAMP1

Lysosomes are responsible for the degradation of microorganisms, misfolded and improperly assembled proteins as well as mature and properly assembled transmembrane proteins, including ENaC, that are targeted for degradation via ubiquitination (Staub et al., 1997b; Wiemuth et al., 2007). To determine if endogenous COMMD1 and/or COMMD3 proteins localise to lysosomes in HEK293 and SH-SY5Y cells and thereby play a role in the degradation of ENaC, a series of double label indirect ICC experiments were performed using COMMD1 or COMMD3 antiserum in combination with LAMP1, which is a lysosomal marker (Kornfeld and Mellman, 1989; Parkinson-Lawrence et al., 2005) (refer to section 3.8.6.3.). This set of double label ICC experiments was performed.
on two separate occasions (n = 2) in both SH-SY5Y and HEK293 cells, once using LAMP1 at a 1:100 dilution factor and the other using a 1:50 dilution factor. Mouse anti-LAMP1 (refer to section 3.8.6.3.) was simultaneously incubated with antiserum against COMMD1 or COMMD3. Mouse anti-LAMP1 was visualised with Alexa Fluor® 546 conjugated goat anti-mouse IgG and staining using COMMD1 and COMMD3 antiserum was visualised with Alexa Fluor® 488 conjugated goat anti-rat IgG.

Staining for LAMP1 was undetectable on both occasions and thereby inconclusive. Due to time constraints double labeling experiments were continued using different organelle markers to further investigate the intracellular location of COMMD1 and COMMD3. Subsequent personal communication (Dr D. Devor, University of Pittsburgh School of Medicine) suggested LAMP1 was a difficult antibody to work with and that LAMP2 (Kornfeld and Mellman, 1989) might have been a better lysosomal marker to use in these experiments.

4.3.3.3. Double labeling for COMMD1 and COMMD3 with the early/recycling endosome marker transferrin

ENaC has previously been shown to localise to early/recycling endosomes (Chang, 2007; Lu et al., 2007) therefore a series of double label ICC experiments were performed to investigate if endogenous COMMD1 and/or COMMD3 proteins localise to the recycling pathway. Localisation of the COMMD proteins to early/recycling endosomes may implicate a potential role for these proteins in initialising or promoting and thereby regulating endocytosis and/or recycling of ENaC from and to the apical cell surface. This was investigated by labeling for COMMD1 or COMMD3 in combination with the early/recycling endosome marker transferrin. The early/recycling endosomes were labeled with transferrin as described under section 3.8.6.2. in both HEK293 (n = 3) and SH-SY5Y cells (n = 1). The COMMD proteins and transferrin were visualised with Alexa Fluor® 488 conjugated goat anti-rat IgG and Alexa Fluor® 546 conjugated goat anti-mouse IgG, respectively.

Staining for COMMD1 and COMMD3 were present on all occasions and appeared
similar to that described earlier (refer to sections 4.3.1. and 4.3.2.). In contrast to this, staining for transferrin was consistently undetectable. This may have been the result of the age of the antibody and/or storage conditions (refer to section 5.4.2.). Based on these results, localisation of COMMD1 and COMMD3 proteins to the early/recycling endosomes was inconclusive however a second early endosome marker named EEA1 was subsequently used for similar investigations (refer to section 4.3.3.4.).

4.3.3.4. COMMD1 and COMMD3 co-localise with the early endosome marker EEA1

EEA1 (refer to section 3.8.6.4.) was used as an alternative early endosome marker to determine if endogenously expressed COMMD1 and COMMD3 localise to the early endosomes. This was investigated by labeling COMMD1 or COMMD3 in combination with EEA1 in HEK293 \((n = 4)\) and SH-SY5Y cells \((n = 4)\). Staining for the COMMD proteins was visualised with Alexa Fluor®488 conjugated goat anti-rat IgG and staining for EEA1 was visualised with Alexa Fluor®546 conjugated goat anti-mouse IgG. Cell nuclei were counterstained with DAPI on all occasions.

Images were unable to be obtained for experiments in which SH-SY5Y cells were double labeled with COMMD1 or COMMD3 and EEA1. The VECTASHIELD® Hard Set mounting medium with DAPI mounting media that was used formed bubbles around large groups of cells that covered most of the area under the coverslip. The presence of these bubbles may potentially have lead to incorrect depth discrimination that made focusing in on the cells challenging therefore no images were acquired.

Staining for COMMD1 (green) exhibited a perinuclear, cytoplasmic vesicular distribution (Figure 4.11. A). Staining for COMMD3 (green) appeared diffuse and cytosolic (Figure 4.11. B). The staining patterns observed for COMMD1 and COMMD3 in this series of experiments are consistent with the previous results obtained in this project for the distribution of COMMD1 and COMMD3 using ICC. As was expected, staining for EEA1 (red) appeared punctate throughout the cytosol on all occasions (Figure 4.11. A and B). Partial overlap in staining patterns between EEA1 and COMMD1 (yellow, Figure 4.11. A).
A) or COMMD3 (yellow, Figure 4.11. B) was observed in HEK293 cells. Yellow staining in the images combined for DAPI, EEA1 and COMMD1 or COMMD3 indicates this overlap in staining pattern. Some nuclear staining for COMMD1 was also present and is indicated by partial overlap in staining pattern (shown in light blue) in the merged image between DAPI (blue) and COMMD1 (green). These results are indicative of a possible regulatory role for COMMD1 and COMMD3 in ENaC endocytosis (refer to discussion).
Figure 4.11. Colocalisation of COMMD1 and COMMD3 with the early endosome marker EEA1 in HEK293 cells

Cells were simultaneously immunoblotted with antiserum against COMMD1 (A) or COMMD3 (B) and mouse anti-EEA1. Staining for COMMD1 or COMMD3 was visualised with Alexa Fluor®488 conjugated goat anti-rat IgG (green) and staining for anti-EEA1 was visualised with Alexa Fluor®546 conjugated goat anti-mouse IgG (red). Cell nuclei were counterstained with DAPI (shown in blue in both A and B). (A) COMMD1 (shown in green) exhibited a perinuclear, cytoplasmic vesicular distribution with some nuclear
staining also seen (shown in light blue in the merged image). As expected, staining for EEA1 (in red) appeared vesicular within the cytoplasm. Overlap in staining patterns between COMMD1 and EEA1 is depicted in yellow in the merged image (indicated by white arrows). (B) COMMD3 (in green) appeared diffuse and cytosolic whereas EEA1 (in red) appeared vesicular within the cytoplasm. Some overlap in staining patterns between COMMD3 and EEA1 was observed in the merged image and is depicted in yellow (indicated by white arrows). Little to no overlap (i.e. light blue) was seen between COMMD3 and the nuclear stain DAPI in the merged image. Negative controls were included as described in section 4.3.1. and appeared similar to that observed in figure 4.7. The above images were taken sequentially using the 40x oil objective lens with (B) or without (A) a zoom factor of 3. The depicted scale bars (lower right corner) represent 20 μm (A) and 10 μm (B).

4.3.4. Summary of immunocytochemistry results
The ICC results presented above in section 4.3. are summarised below in Table 4.2. and provide some of the first evidence regarding the intracellular distribution and specific location of endogenously expressed COMMD1 and COMMD3 proteins. COMMD1 was shown to predominantly exhibit a perinuclear, cytoplasmic vesicular distribution with some nuclear staining present whereas COMMD3 appeared more diffuse throughout the cytosol. The specific intracellular location of COMMD1 and COMMD3 was investigated in a series of double label ICC experiments whereby COMMD1 or COMMD3 was labeled in combination with one of several specific organelle markers, including DAPI, transferrin, LAMP1, EEA1 and WGA. Overlap in staining patterns between EEA1 and COMMD1 and COMMD3 was observed thus indicating that both COMMD1 and COMMD3 play a (regulatory) role in the endocytic pathway of ENaC. No overlap in staining patterns between WGA and COMMD1 and COMMD3 was observed. This suggests that neither COMMD1 nor COMMD3 play a role in the regulation of ENaC trafficking through the Golgi, its posttranslational modifications and/or proteolytic cleavage. Localisation of COMMD1 and COMMD3 to the recycling endosomes remains inconclusive.
Table 4.2. Summary of results: double label ICC

\( n \) = number of experiments performed; \( \checkmark \) = positive for colocalisation; \( \times \) = negative for colocalisation

<table>
<thead>
<tr>
<th>Organelle marker</th>
<th>COMMD1</th>
<th>HEK293</th>
<th>SH-SY5Y</th>
<th>COMMD3</th>
<th>HEK293</th>
<th>SH-SY5Y</th>
</tr>
</thead>
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<tr>
<td>DAPI</td>
<td>✔</td>
<td>n = 4</td>
<td>n = 4</td>
<td>❌</td>
<td>n = 4</td>
<td>n = 4</td>
</tr>
<tr>
<td>Transferrin</td>
<td>inconclusive</td>
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<td>n = 1</td>
<td>inconclusive</td>
<td>n = 3</td>
<td>n = 1</td>
</tr>
<tr>
<td>LAMP1</td>
<td>inconclusive</td>
<td>n = 2</td>
<td>n = 2</td>
<td>inconclusive</td>
<td>n = 2</td>
<td>n = 2</td>
</tr>
<tr>
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<td>n = 1</td>
<td>n = 1</td>
<td>❌</td>
<td>n = 1</td>
<td>n = 1</td>
</tr>
</tbody>
</table>

4.4. Antiserum specificity in native tissue

4.4.1. GST precleared versus non-precleared antiserum

Prior to commencing IHC, the specificity of the COMMD1 and COMMD3 rat antiserum, which was generated using purified recombinant GST-COMMD1 and GST-COMMD3 proteins, was examined to determine if the anti-GST antibodies would cross-react with those natively present in the rat kidney. The GST superfamly represents a group of detoxification enzymes that utilise glutathione to transform a wide range of compounds (Hayes and Pulford, 1995). GSTs are found in plants, animals, fungi and some bacteria and all share a significant degree of sequence conservation (Sheehan et al., 2001). Polyclonal antibodies raised against a particular GST class have been shown to often cross-react with the same class from other species. However, no cross-reactivity is generally noted between GST classes, even within the species from which the original antigen was derived (Hayes and Mantle, 1986).

Serial kidney sections were prepared (refer to section 3.8.) and immunoblotted with COMMD1 or COMMD3 antiserum that had been 1) non-precleared or 2) GST-precleared (refer to section 3.6.2.). Negative controls were included by immunoblottting kidney sections with antigen precleared antiserum (i.e. GST-COMMD1 or GST-COMMD3). Results obtained from this experiment (\( n = 1 \)) revealed that the staining pattern between kidney sections immunoblotted with GST-precleared and non-precleared COMMD1 and COMMD3 antiserum appeared very similar indicating that the rat antiserum was not binding to endogenous GST proteins (Figure 4.12.). There was an
absence of staining when antiserum was precleared with antigen therefore non-precleared antiserum was used in subsequent IHC experiments.

**Figure 4.12. Antiserum specificity in the rat kidney**

Epifluorescent images of rat kidney sections (cortex) were captured using the 40x objective lens. Sections were immunoblotted with antiserum against COMMD1 (top three images) and COMMD3 (bottom three images) that was either non-precleared (first column), GST precleared (second column) or antigen precleared (third column). Staining was visualised with Alexa Fluor®488 conjugated goat anti-rat IgG (green). The staining patterns observed for non-precleared and GST precleared COMMD1 and COMMD3 antiserum appeared identical. Antigen precleared antisera was used as negative controls and were clear of any non-specific staining.
4.5. Cellular location of the COMMD proteins in native kidney tissue

Two objectives were developed to address the second hypothesis, which was that, in order for COMMD1 and COMMD3 to inhibit ENaC, the COMMD proteins localise to the same cell types as ENaC *in vivo*. The first of these objectives was to investigate the location of both COMMD1 and COMMD3 in native tissue using IHC. The distribution pattern of COMMD1 and COMMD3 in the kidney has not been previously investigated. In contrast to this, the distribution of ENaC and AQP2 in the kidney is well defined. The anti-AQP2 antibody was used as marker for the principal cells of the collecting ducts in this project. ENaC and AQP2 are expressed in the distal nephron segments including the distal convoluted tubules (only ENaC) and connecting tubules and cortical and medullary (both inner and outer) collecting ducts (Figure 4.13.).

![Figure 4.13. Location of ENaC and AQP2 in the nephron](image)

ENaC and AQP2 proteins are expressed in the distal nephron segments. These include the distal convoluted tubules (DCT), connecting tubules and cortical and medullary (inner and outer) collecting ducts (CD).

Dexamethasone is a potent synthetic glucocorticoid that has been shown to upregulate ENaC *in vivo* (Bridges et al., 1987) and *in vitro* (Dagenais et al., 2001; Nakamura et al., 2002). Interestingly, the latter two studies have shown that dexamethasone preferentially upregulates αENaC. Because of this effect, kidneys from dexamethasone treated rats
were used for the remaining IHC experiments to detect αENaC by IHC. A comparison in the location of COMMD1 and COMMD3 in kidneys from control and dexamethasone treated rats was not performed as part of this project.

Kidney sections from dexamethasone treated rats were immunoblotted with antiserum against COMMD1 or COMMD3 (refer to table 3.3. for antibody dilution factors) and were visualised with Alexa Fluor®488 conjugated goat anti-rat IgG and HRP conjugated goat anti-rat secondary antibody for IHC and immunoperoxidase labeling, respectively (refer to section 3.8.4.). Negative controls whereby primary antibodies were omitted were included for each independent experiment. Throughout this project, negative controls were consistently clear of any non-specific staining and appeared similar to that shown in figure 4.12. Some autofluorescence was present in the negative controls however this is a problem that is often encountered when using a post-fixation method and/or an aldehyde fixative solution (Del Castillo et al., 1989; Schnell et al., 1999).

Specific nephron segments were identified in this project based on their structural features that had previously been described. These features are summarised in Table 4.3. and were compiled using several references as a guide (Ovalle and Nahirney, 2008; Ross and Pawlina, 2006; Ross et al., 1995).

<table>
<thead>
<tr>
<th>Nephron segment</th>
<th>Structural features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal convoluted tubule (PCT)</td>
<td>Brush border; star-shaped lumen often seen; fewer nuclei, larger outside diameter and more common than DCT</td>
</tr>
<tr>
<td>Thin segment of the loop of Henle (thin)</td>
<td>Simple squamous cells; thinnest walls of the renal tubules seen in medulla</td>
</tr>
<tr>
<td>Thick segment of the loop of Henle (thick)</td>
<td>Cuboidal (boxy) cells; lumen often star-shaped</td>
</tr>
<tr>
<td>Distal convoluted tubule (DCT)</td>
<td>No brush border; clean sharp luminal surface; more nuclei than PCT</td>
</tr>
<tr>
<td>Collecting ducts (CD)</td>
<td>Cuboidal cells with distinct cell boundaries; large lumen</td>
</tr>
</tbody>
</table>
4.5.1. Localisation of COMMD1 in the kidney
Indirect IHC performed on serial kidney sections from dexamethasone treated rats \((n = 3)\) revealed that COMMD1 is differentially expressed throughout the kidney with expression being particularly strong in the thick segments of the loop of Henle (thick), distal convoluted tubules (DCT) and inner and outer medullary collecting ducts (CD) (Figure 4.14.). Staining for COMMD1 in the proximal convoluted tubules (PCT) and thin segments of the loop of Henle (thin) were also present however this appeared much less intense compared to the previously mentioned segments. Interestingly, COMMD1 was not expressed in the cell nuclei of these nephron segments indicating that the predominant function of COMMD1 in these cells occurs in the cytosol.
Figure 4.14. Localisation of COMMD1 in the kidney using indirect IHC

Indirect IHC and laser scanning confocal microscopy revealed that COMMD1 is differentially expressed throughout the rat kidney. COMMD1 was immunoblotted with antiserum against COMMD1 and staining was visualised with Alexa Fluor®488 conjugated goat anti-rat IgG. Staining for COMMD1 (green) was particularly strong in the DCT within the cortex, the thick segments of the loop of Henle (thick) in the outer medulla and CD of the cortex and outer and inner medulla. Cell nuclei were counterstained with DAPI (blue) in the above images (middle column). Images were captured sequentially. Merged images for COMMD1 and the cell nuclei are shown in the right column. Images were taken sequentially. Depicted scale bars represent 20 μm.
Immunoperoxidase labeling for COMMD1 was carried out to assist in identifying the specific nephron segments in which COMMD1 is expressed. COMMD1 labeled kidney sections were anatomically defined by counterstaining with hematoxylin and eosin as described under section 3.8.5. Hematoxylin stains basophilic structures such as cell nuclei blue-purple whereas eosin stains eosinophilic structures, including the cytoplasm, extracellular proteins (e.g. collagen) and red blood cells (RBC) bright pink. The results from the immunoperoxidase experiments revealed apical and subapical labeling for COMMD1 in the DCT and cortical and medullary CD (Figure 4.15.). These results are consistent with those reported for indirect IHC however in contrast to the results reported for indirect IHC, no labeling for COMMD1 was seen in the PCT and thin segments of the loop of Henle using immunoperoxidase labeling. This may have been due to the lower sensitivity of this technique (immunoperoxidase labeling) compared to ICC.
Figure 4.15. Localisation of COMMD1 in the kidney using immunoperoxidase labeling

Immunoperoxidase labeling revealed staining for COMMD1 (light brown; indicated by asterisks, *) in the DCT (A), cortical CD (B) and outer medullary CD (C). No staining for COMMD1 was observed in the PCT (A). All sections were counterstained with hematoxylin (purple-blue) and eosin (pink) to anatomically define the structures. Hematoxylin stains basophilic structures, such as the cell nuclei (indicated by a white arrow in a), a purple-blue hue whereas eosin stains eosinophilic structures including the cytoplasm and red blood cell (RBC; indicated by a white arrow in b) pink. The above images were captured using the 100x oil objective lens of an Olympus AX70 light microscope. The depicted scale bars (lower right corners) represent 10 μm.
4.5.2. Localisation of COMMD3 in the kidney

COMMD3 was shown to exhibit a very distinct distribution pattern throughout the rat kidney (Figure 4.16.). Expression for COMMD3 appeared strongest in the PCT, thick segments of the loop of Henle (thick), CT and CD. Staining for COMMD3 appeared to be less intense in the DCT and was virtually absent in the thin segments of the loop of Henle (thin). Upon closer inspection COMMD3 exhibited a relatively even cytosolic distribution in the proximal convoluted tubules (Figure 4.16. B) whereas in the thick segments of the loop of Henle in the outer medulla (Figure 4.16. C) COMMD3 appeared to exhibit a more uneven pattern of expression that was also diffuse and cytosolic. The relevance of this cell-type/segment specific difference remains to be determined.
Figure 4.16. Localisation of COMMD3 in the rat kidney

The location of COMMD3 in the rat kidney was investigated using indirect IHC and laser scanning confocal microscopy. COMMD3 was immunoblotted with antiserum against COMMD3 and staining was visualised with Alexa Fluor®488 conjugated goat anti-rat IgG. (A) Significant labeling for COMMD3 (shown in green) was seen in the PCT in the cortex, thick segments of the loop of Henle (thick), cortical CD and outer and inner medullary CD. Staining for COMMD3 appeared much less intense in the DCT and was near absent in the thin segments of the loop of Henle (thin). (B) Close inspection revealed that COMMD3 exhibited a diffuse and even cytosolic distribution throughout the cortical nephron segments in which it is expressed. (C) COMMD3 appeared diffuse but unevenly distributed throughout the cytosol of the thick segments of the loops of Henle. The depicted scale represents 20 μm.
Immunoperoxidase labeling for COMMD3 in the rat kidney was carried out to assist in identifying the location of COMMD3 expression. Following immunoperoxidase labeling the sections were counterstained with hematoxylin and eosin to help identify the structures anatomically. Briefly, hematoxylin stains basophilic structures such as the cell nuclei blue-purple whereas eosin stains eosinophilic structures (e.g. cytoplasm and RBC) pink. Labeling for COMMD3 was identified in the PCT, DCT, thick segment of the loop of Henle (thick) and CD (Figure 4.17.). This immunoperoxidase labeling pattern was consistent with the confocal microscopic observations (refer to Figure 4.16.).

Figure 4.17. Localisation of COMMD3 in the kidney using immunoperoxidase labeling
Immunoperoxidase labeling for COMMD3 (light brown) was significant in the PCT (A, B and C), DCT (A), thick segments of the loop of Henle (thick) (C) and CD (D). All of the above images were captured in the cortex using the 100x oil objective lens of an Olympus AX70 microscope. The depicted scale bars (yellow; lower right corners) represent 10 μm.
4.6. *In vivo* colocalisation of the COMMD proteins with ENaC and AQP2

The second objective that was developed to address the second hypothesis of this project (refer to sections 2 and 4.5.) was to determine if COMMD1 and COMMD3 localise to the same cell types as ENaC in native tissue. This was approached by carrying out a series of double label indirect IHC experiments with COMMD1 or COMMD3 in combination with αENaC or AQP2 on dexamethasone treated rat kidney sections.

ENaC and AQP2 exhibit some overlap in their expression in the distal nephron segments. Both ENaC and AQP2 are expressed in the connecting tubules and principal cells of the collecting ducts. Apical and/or subapical intracellular vesicular staining has previously been observed for ENaC in the DCT, connecting tubules and CD. The polarisation of AQP2 differs in some of the segments in which both ENaC and AQP2 are expressed. For example, AQP2 is present in the apical cell surface and subapical vesicles along the length of the connecting tubule to the inner medullary CD as well as on the basolateral cell surface of the connecting tubule and inner medullary CD (Nejsum, 2005).

Double labeling for COMMD1 and COMMD3 with AQP2 was performed as an alternative measure to help identify the location of the COMMD proteins in the rat kidney. The COMMD proteins were immunblotted with antiserum against their respective antigens and were visualised with Alexa Fluor® 488 conjugated goat anti-rat IgG. ENaC and AQP2 were immunblotted with chicken anti-αENaC or rabbit anti-AQP2. Staining for αENaC was visualised with Alexa Fluor® 546 conjugated goat anti-chicken and AQP2 with Alexa Fluor® 546 conjugated goat anti-rabbit IgG.

4.6.1. Colocalisation of COMMD1 and ENaC in the kidney

Double label indirect IHC revealed that colocalisation between COMMD1 and αENaC (n = 3) was most pronounced in the CD of the inner (Figure 4.18.) and outer medullary regions of the rat kidney (Figure 4.19.).

COMMD1 and αENaC exhibited diffuse cytosolic patterns of distribution in the outer medullary CD (Figure 4.18.) Staining for ENaC has been reported to be present in both
the apical cell surface and subapical intracellular vesicles (Duc et al., 1994; Hager et al., 2001). ICC results obtained during this project revealed that the predominant location of αENaC appears to be diffuse and cytosolic with no apical or apparent intracellular punctate staining present in the outer medulla. Overlap in staining patterns between COMMD1 and αENaC in the outer medulla is best seen in the high magnification image. The area of magnification is indicated by a white square (Figure 4.18. A) and the image is presented in figure 4.18. B. There appeared to be a high degree of overlap between COMMD1 and αENaC, which is seen as yellow staining in the merged images (Figure 4.18. A and B). As a result of using a post-fixation and/or aldehyde fixation method (Del Castillo et al., 1989; Schnell et al., 1999), some autofluorescence was also evident in these images and are indicated by asterisks (*) (Figures 4.18 A and Figure 4.19.).
Figure 4.18. Colocalisation of COMMD1 and αENaC in the kidney outer medulla

Staining for COMMD1 (green) and αENaC (red) appeared largely diffuse and cytosolic with little to no staining observed in the cell nuclei. Strong overlap in staining patterns between COMMD1 (green) and αENaC (red) was observed in the collecting ducts (CD) (indicated by white arrows) of the kidney outer medulla. Overlap in staining patterns appeared as yellow staining (merge images, A and B). Cell nuclei were counterstained with DAPI (blue; A and B). Autofluorescence (indicated by asterisks, *) was evident and was likely the result of using a post-fixation and/or aldehyde fixation method. The above images were taken sequentially using the 40x oil objective lens and the higher magnification image (B) was captured using the zoom function of the LSM510 control software. The depicted scale bars (lower right corners) represent 20 μm.
COMMD1 and αENaC largely co-localised in the CD of the inner medulla (Figure 4.19.). Staining for COMMD1 appeared mostly diffuse and cytosolic and absent from the cell nuclei. Contrary to what was expected (Hager et al., 2001), staining for αENaC appeared mostly diffuse and cytosolic with some apical cell surface labeling present in the CD principal cells. The presence of ENaC in the inner medullary CD principal cells (indicated by white arrowheads) is consistent with the distribution pattern described for ENaC in the literature (Hager et al., 2001). Little to no αENaC staining was present in the cell nuclei. Overlap in staining patterns between COMMD1 and αENaC revealed a significant degree of colocalisation in the inner medullary CD cells, including the principal cells. This is seen as yellow staining in the merged image (Figure 4.19.). Labeling for αENaC in the kidney cortex, which would correspond to either the DCT or cortical CD, was undetectable and/or absent.
Figure 4.19. Colocalisation of COMMD1 and αENaC in the kidney inner medulla

Staining for COMMD1 (green) and αENaC (red) appeared diffuse and cytosolic with some apical cell surface staining present on the principal cells of the CD. CD are indicated by white arrows in the COMMD1 image and the apical cell surface labeling in the principal cells are indicated by white arrowheads in the αENaC image. Some autofluorescence was evident in these images (indicated by asterisks, *). A significant degree in overlap in staining patterns between COMMD1 and αENaC was present. This appeared as yellow staining in the merged image. Cell nuclei were counterstained with DAPI (blue). The above images were captured sequentially using the 40x oil objective lens and the zoom function of the LSM510 control software. The depicted scale bars (lower right corners) represent 20 μm.
A number of optimisation steps were employed to improve the staining intensity observed for ENaC labeling, which, according to previous reports, should at least have been detectable in intracellular vesicles (Duc et al., 1994). Masilamani et al. (Masilamani et al., 1999) have previously reported that aldosterone preferentially increases αENaC transcription therefore it was expected, following dexamethasone administration, that the anti-αENaC antibody would produce the highest intensity of staining for endogenous ENaC in this project. A comparison study (n = 1) between the different ENaC antibodies was performed to investigate which antibody produced the best staining in rat kidney sections (results not shown). Staining with anti-βENaC antibody produced no visible staining whereas staining with anti-γENaC antibody appeared similar to that seen in the negative controls and therefore was classified as being non-specific. As was expected, staining with anti-αENaC produced the best results however the staining intensity remained suboptimal for the duration of this project. All double label IHC experiments where co-expression of COMMD1 or COMMD3 with ENaC was investigated subsequently utilised anti-αENaC as the preferred antibody. A comparison between histological sections that underwent heat-induced epitope retrieval (HIER) and no HIER was also performed (results not shown) however no obvious difference in the staining intensity for αENaC was observed.

4.6.2. COMMD1 and AQP2 in the kidney

The location of COMMD1 protein expression in the rat kidney was further explored in a series of double label indirect IHC experiments using antiserum against COMMD1 and rabbit anti-AQP2 antibody (n = 3). AQP2 is expressed in some of the same distal nephron segments as ENaC therefore it was utilised in this project as an alternative marker for the connecting tubules, cortical CD and outer and inner medullary CD. The polarisation between ENaC and AQP2 differ in these segments. ENaC is expressed on the apical cell surfaces of the DCT, connecting tubule, cortical CD and outer and inner medullary CD. AQP2 is present on the apical cell surface and in subapical vesicles along the length of the connecting tubule to the inner medullary CD as well as on the basolateral cell surface of the connecting tubule and inner medullary CD, but not the cortical of outer medullary CD.
Colocalisation between COMMD1 and AQP2 was observed in the cortical CD (Figure 4.20.) and the inner medullary CD (Figure 4.21.). Upon first inspection, staining for COMMD1 was most apparent in the DCT with some low intensity staining also observed in structures that corresponded to the PCT and cortical CD (Figure 4.20.). Staining for AQP2 was mostly present on the apical cell surfaces of the cortical CD principal cells. Cell nuclei were counterstained with DAPI (Figure 4.20. A). Little overlap in staining patterns between COMMD1 and AQP2 was seen on the lower magnification image (Figure 4.20. A). However upon closer inspection overlap in staining patterns between COMMD1 and AQP2 was evident. The enlarged image is indicated by a white square (Figure 4.20. A) and the image is presented in figure 4.20. B. It is apparent from this image that COMMD1 and AQP2 co-exist on the apical cell surface and subapical regions of some cortical CD principal cells (indicated by white arrowheads). Overlap in staining patterns between COMMD1 and AQP2 is shown as yellow staining in this image (Figure 4.20. B).
Figure 4.20. Colocalisation of COMMD1 and AQP2 in the kidney cortex

(A) Staining for COMMD1 (green) was most apparent in the DCT in the cortex whereas AQP2 (red) was present on the apical cell surface of the cortical CD principal cells (indicated by a white arrow). Cell nuclei were counterstained with DAPI (blue, A and B). Little overlap in staining patterns between COMMD1 and AQP2 was evident in the lower magnification merged image (A). (B) Upon closer inspection overlap in staining patterns between COMMD1 and AQP2 was present on the apical cell surfaces and subapical regions in some cortical CD principal cells (indicated by white arrowheads). Overlap in staining patterns between COMMD1 and AQP2 appeared as yellow staining. Images were captured sequentially. Images in (A) were taken using the 40x oil objective lens and the high magnification image in (B) was captured using the zoom function of the LSM510 control software. Depicted scale bars (lower right corners) represent 20 μm.
Overlap in staining patterns between COMMD1 and AQP2 was most pronounced in the inner medullary CD (Figure 4.21.). Staining for COMMD1 appeared diffuse and cytosolic and absent from the cell nuclei (Figure 4.21. A). Some autofluorescence was also evident in the images for COMMD1 and is indicated by asterisks. Strong staining for AQP2 was seen in both the apical (indicated by white arrowheads) and basolateral membranes (indicated by yellow arrowheads) of the inner medullary CD principal cells (Figure 4.21. A and B). The polarised distribution observed for AQP2 in these images is consistent with that described in the literature (Nejsum, 2005). Little to no autofluorescence was seen in the images for AQP2 however this might have been masked by the strong signal obtained after staining with rabbit anti-AQP2 antibody. Cell nuclei were counterstained with DAPI (Figure 4.21. A and B). Overlap in staining patterns between COMMD1 and AQP2 was evident in the lower magnification image (Figure 4.21. A) and upon closer inspection (Figure 4.21. B) it was apparent that these two proteins co-exist on the apical (indicated by a white arrowhead) and basolateral membranes (indicated by a yellow arrowhead) as well as subapically (not indicated but evident as yellow staining). The area of magnification is indicated by a white square (Figure 4.21. A) and is presented in figure 4.21. B.
Figure 4.21. Colocalisation of COMMD1 and AQP2 in the kidney inner medulla

(A) COMMD1 (green) exhibited a diffuse cytosolic pattern of distribution in the inner medullary CD. Some autofluorescence (indicated by asterisks, *) was evident in the image for COMMD1 but not AQP2. Staining for AQP2 (red) was present on the apical (indicated by white arrowheads) and basolateral cell surfaces (indicated by yellow arrowheads) of the CD principal cells. Cell nuclei were counterstained with DAPI (blue) (A and B). Overlap in staining patterns between COMMD1 and AQP2 was apparent in the merged image and is shown as yellow staining. An enlarged view, which is indicated by the white square, of the merged image (A) is shown in B. It is clear from this image (B) that overlap in staining patterns between COMMD1 and AQP2 was present in the apical (indicated by a white arrowhead) and basolateral membranes (indicated by a yellow arrowhead) as well as subapically (not indicated). Autofluorescence is indicated by *. The above images were captured sequentially using the 40x oil objective lens and the magnified image was captured using the zoom function of the confocal microscope. Depicted scale bars (lower right corner) represent 20 μm.
4.6.3. Colocalisation of COMMD3 and ENaC in the kidney

Previous data obtained from our laboratory have shown that COMMD3 binds to all three ENaC subunits. Upon coexpression with αβγENaC in FRT cells, COMMD3 has also shown to mediate an inhibitory effect on the amiloride-sensitive Na\(^+\) current generated by αβγENaC. Leading on from these experiments, results from this project have shown COMMD3 proteins to be endogenously expressed in rat and mouse kidney (refer to section 4.2.2.1.). However, in vivo colocalisation of the COMMD3 and ENaC proteins had not been previously reported. Therefore, to determine if the COMMD3 and ENaC proteins co-localise in vivo, a series of double label IHC experiments were performed. COMMD3 was labeled with antiserum generated against COMMD3 and αENaC with chicken anti-αENaC antibody. Staining for COMMD3 was visualised with Alexa Fluor®488 conjugated goat anti-rat IgG and staining for αENaC was visualised with Alexa Fluor®546 conjugated goat anti-chicken IgG.

The results from this series of double label IHC (n =3) revealed that colocalisation between COMMD3 and αENaC was most apparent in the outer medullary CD of the rat kidney (Figure 4.22.). Unfortunately, staining for αENaC in the cortical regions of the kidney appeared absent and/or undetectable therefore the relationship between COMMD3 and αENaC in the kidney cortex remain inconclusive (results not shown). COMMD3 appeared diffuse and cytosolic in the outer medullary CD and was differentially expressed within these segments. The diffuse cytosolic distribution exhibited by COMMD3 in these segments is consistent with the distribution pattern reported for COMMD3 in the rat kidney under section 4.5.2. This pattern of distribution is also in agreement with that observed for ICC experiments (section 4.3.2.). Strong apical cell surface staining for αENaC was expected in the DCT, connecting tubules, cortical CD and inner and outer medullary CD principal cells. However, this was not the case. Labeling for αENaC in the outer medullary CD appeared cytoplasmic and vesicular with no obvious apical membrane staining present (Figure 4.22.). This pattern of distribution observed for αENaC is similar to that reported by Hager et al. (Hager et al., 2001) and Pacha et al. (Pacha et al., 1993) and it reflects a physiological state in which the animal was replete of Na\(^+\) ions and therefore was no longer influenced by the
dexamethasone, which was administered 18 h prior to the animal being sacrificed.

**Figure 4.22. Colocalisation of COMMD3 and ENaC in the rat kidney medulla**

COMMD3 (green) exhibited a diffuse pattern of distribution throughout the cytosol. Labeling for αENaC (red) appeared cytoplasmic and vesicular and contrary to what was expected was absent from the apical cell surface of the CD principal cells. Cell nuclei were counterstained with DAPI (blue). Overlap in staining patterns (apparent as yellow staining in the merged image that is indicated by white arrowheads) between COMMD3 and αENaC was strongest in these segments when compared with other regions of the kidney. The above images were captured sequentially using the 40x oil objective lens and the depicted scale bars (bottom right corners) represent 20 μm.

4.6.4. COMMD3 and AQP2 in the kidney

The distribution of COMMD3 in the rat kidney was further explored by a series of double label IHC experiments with AQP2 (n = 3), which exhibits a similar pattern of distribution to ENaC (refer to section 4.6.). COMMD3 was shown to co-localise with AQP2 in both the cortical CD (Figure 4.23.) and medullary CD (Figure 4.24.).
COMMD3 exhibited a predominantly diffuse cytosolic distribution in the cortex however some punctate staining cannot be excluded (Figure 4.23.). Staining for COMMD3 appeared absent from the nucleus indicating that it functions in the cytosol and possibly associates with intracellular vesicles in these cells. The segments in which COMMD3 was shown to localise to in these cortical images are consistent with those described earlier: PCT (indicated by white arrows), thick segments of the loop of Henle (thick) (not indicated) and cortical CD (indicated by a white arrow). Strong apical cell surface labeling was observed for AQP2 in the cortical CD (indicated by a white arrow) (Figure 4.23. A). Cell nuclei were counterstained with DAPI in these experiments. Overlap in staining patterns between COMMD3 and AQP2 was apparent in the merged image (Figure 4.23. A) and is seen as yellow staining. Closer inspection of this area of overlap, which is indicated by a white square in figure 4.23. A and is presented in full scale in figure 4.23. B, revealed that COMMD3 and AQP2 show some degree of overlap in the apical cell surface and cytosol of cortical CD principal cells (indicated by white arrowheads). Interestingly, COMMD3 appeared to be expressed in both the principal (positive for AQP2 staining) and intercalated cells (negative for AQP2 staining) in the cortical CD (Figure 4.23.).
Figure 4.23. Colocalisation of COMMD3 and AQP2 in the kidney cortex
Overlap in staining patterns between COMMD3 (green) and AQP2 (red) was observed in the apical cell surface and cytosol of the cortical CD principal cells (A). This colocalisation is best seen in the high magnification image (B). The magnified area (B) is indicated by a white square in the merged image (A). Overlap in staining patterns between COMMD3 and AQP2 are indicated (B, white arrowheads) and was present on the apical cell surface of the CD principal cells as well as intracellularly. Cell nuclei were counterstained with DAPI (blue) in these images (A and B). The above images were captured sequentially using the 40x oil objective lens and the high magnification images were captured using the zoom function of the confocal microscope. The depicted scale bars (bottom right corners) represent 20 μm.
Staining for COMMD3 appeared diffuse and cytosolic in the inner medullary CD (Figure 4.24. A). Staining AQP2 was present on both the apical (indicated by white arrowheads in the AQP2 image, Figure 4.24. A) and basolateral cell surfaces (indicated by yellow arrowheads in the AQP2 image, Figure 4.24. A). This polarised distribution is consistent with that described in the literature and seen during double labeling experiments performed with COMMD1 (section 4.6.2.). Cell nuclei were counterstained with DAPI in these images and overlap in staining patterns with COMMD3 or AQP2 was absent (i.e. COMMD3 and AQP2 is not present in the cell nuclei). Overlap in staining patterns between COMMD3 and AQP2 was evident in the merged images (Figure 4.24. A and B). The magnified area shown in B was taken from the same field of view shown in A (indicated by a white box in the merged image in A). It is apparent from the high magnification image (B) that COMMD3 and AQP2 co-localise on the apical cell surface and in the cytosol of the inner medullary CD principal cells. No overlap in staining patterns between these two proteins was seen on the basolateral cell surfaces and COMMD3 also appeared to be present in the intercalated cells in the inner medulla CD (cells which are negative for AQP2 staining in the CD).
Figure 4.24. Colocalisation of COMMD3 and AQP2 in the kidney inner medulla

Staining for COMMD3 (green) appeared diffuse and cytosolic in the inner medullary collecting ducts (CD) (A). AQP2 was present on the apical (indicated by white arrowheads) and basolateral cell surfaces (indicated by yellow arrowheads) of the CD principal cells in this same area (A). Cell nuclei were counterstained with DAPI (blue). COMMD3 was shown to co-localise with AQP2 in the CD in this region (A). Closer inspection revealed that COMMD3 and AQP2 co-localise to the apical cell surface (indicated by a white arrowhead in the merged image in B) and in the cytosol (not indicated but shown as yellow in the cytosol) of CD principal cells. The above images were captured sequentially using the 40x oil objective lens and the high magnification image in B was captured using the same objective and zoom function of the confocal microscope. Depicted scale bars (lower right corners) represent 20 μm.
4.6.5. Summary of the distribution and colocalisation of the COMMD proteins, ENaC and AQP2 in the kidney

Single and double label indirect IHC was employed to investigate the location of COMMD1 and COMMD3 in the rat kidney and possible colocalisation between COMMD1 and COMMD3 with both αENaC and AQP2, respectively. The latter was used as an alternative marker for identifying specific segments of the distal nephron and thereby assisted in identifying the location of the COMMD proteins in the kidney. Results based on these experiments revealed that COMMD1 and COMMD3 are differentially expressed in the kidney (Figure 4.25.). COMMD1 was shown to exhibit strong expression in the thick segments of the loop of Henle, DCT and CD. Staining for COMMD1 was less intense in the PCT and thin segments of the loop of Henle. In contrast to this, COMMD3 exhibited a very distinct pattern of distribution throughout the kidney with staining being particularly strong in the PCT, thick segments of the loop of Henle and CD. Staining for COMMD3 was less intense in the DCT and appeared near absent in the thin segments of the loop of Henle. Double labeling experiments revealed some degree of overlap in staining patterns between COMMD1 and COMMD3 with αENaC. This colocalisation was most pronounced in the inner medullary CD for COMMD1 and in the CD for COMMD3. Colocalisation between COMMD1 and COMMD3 with AQP2 was most significant in the principal cells of the collecting ducts however the polarisation of overlap between COMMD1 and COMMD3 with AQP2 differed.

**Figure 4.25. Distribution patterns of COMMD1, COMMD3, ENaC and AQP2 in the rat kidney**

This figure shows the proximal convoluted tubules (PCT), thin and thick segments of the loop of Henle, the distal convoluted tubule (DCT), collecting tubule (CT) and collecting ducts (CD). The location of COMMD1 (green), COMMD3 (red), ENaC (blue) and AQP2 (orange) along the different segments of the nephron is shown.
5. Discussion

5.1. A role for the COMMD proteins in the trafficking of ENaC

ENaC provides the rate-limiting step for Na\(^+\) reabsorption in all the tissues in which it is expressed thus its activity at the apical cell surface requires tight regulation (Garty and Palmer, 1997). Previous studies have identified trafficking of ENaC to and from the apical cell surface to be an important mode by which ENaC is regulated. For example, an increase in the rate of retrieval of ENaC from the apical cell surface results in a concomitant decrease in the rate of Na\(^+\) ions being reabsorbed (refer to section 1.1.5.). This mode of regulation is crucial in maintaining proper Na\(^+\) balance in the various tissues in which ENaC is expressed. Data obtained from experiments performed in our laboratory have demonstrated that several members of the COMMD family of proteins, which are ubiquitously expressed (Burstein et al., 2005; Klomp et al., 2003), interact with ENaC and subsequently mediate an inhibitory effect on the amiloride sensitive Na\(^+\) current generated by αβγENaC (Biasio et al., 2004; Swart, 2006) (Liu, Y., unpublished data). In a recent study conducted by Burkhead et al. (Burkhead et al., 2009), COMMD1 was shown to localise to vesicles of the endocytic pathway possibly via an interaction with the membrane lipid PI(4,5)P2 thus recruitment of COMMD1 to the cell membrane appears to be via an interaction with membrane lipids. Burkhead et al. provided evidence to show that this interaction is mediated via the C terminal COMM domain of COMMD1. Interestingly, this C terminal COMM domain is highly conserved across all members of the COMMD family (Burstein et al., 2005) therefore this interaction is likely to extend to other members of this family although this remains to be investigated. Combined with the punctate cytoplasmic staining previously reported for COMMD1 (Klomp et al., 2003; Muller et al., 2009), these data implies that the COMMD proteins are likely to play a role in the regulation of ENaC trafficking and therefore to affect the number of ENaC present on the apical cell surface. However, the intracellular and cellular location of COMMD1, COMMD3 and other members of the COMMD family of proteins remain largely undefined.

This project was developed with the aim to provide evidence that ENaC and the COMMD proteins co-localise in vivo. The specific objectives were to determine the
cellular location of COMMD1 and COMMD3 in relation to ENaC in a physiologically relevant tissue, i.e. one in which both the COMMD proteins and ENaC have been identified, and also to determine the specific intracellular locations of the COMMD1 and COMMD3 proteins. The latter was investigated to determine if COMMD1 and COMMD3 localise to the same trafficking pathways in which ENaC have previously been identified. These objectives were investigated using Western blot analysis followed by single and double label indirect IHC and ICC, respectively.

5.2. Detection of endogenously expressed COMMD1 and COMMD3 proteins

5.2.1. Detection of COMMD1 and COMMD3 in the rat kidney

ENaC provides a regulated pathway for Na\(^+\) reabsorption in all the tissues in which it is expressed. In the kidney, proper ENaC function is crucial for maintaining extracellular fluid balance and therefore arterial blood pressure (Garty and Palmer, 1997). ENaC (Canessa et al., 1993; Duc et al., 1994; McDonald et al., 1994; McDonald et al., 1995) and COMMD mRNA and/or proteins (Burstein et al., 2005; Klomp et al., 2003) had previously been identified in the kidney therefore it was used as an in vivo model to address the hypothesis that, in order for COMMD1 and COMMD3 to inhibit ENaC, the COMMD proteins localise to the same cell types as ENaC in vivo. Colocalisation of ENaC and COMMD1 or COMMD3 may potentially implicate a possible physiologically relevant regulatory role for the COMMD proteins on ENaC and therefore indirectly on arterial blood pressure.

For this project, both mice and rat kidneys were available for harvesting and subsequent Western blot analysis and IHC. The results obtained from Western blot analysis in this project have shown for the first time the detectable presence of endogenously expressed COMMD1 and COMMD3 proteins in the rat kidney (refer to section 4.2.). In addition to this and in agreement with results published by Klomp et al. (Klomp et al., 2003), endogenously expressed COMMD1 protein was also detected in the mouse kidney. These results confirmed that the rat COMMD1 antiserum specifically recognised mouse and rat COMMD1 proteins. Likewise, rat COMMD3 antiserum specifically recognised rat COMMD3 proteins.
All ten COMMD family members have been identified at the mRNA level in several human tissues, including the kidney (Burstein et al., 2005). However, with the exception of COMMD1, none of the remaining COMMD family members have been identified at the protein level in native tissue. Future studies that are aimed at investigating the presence of endogenously expressed COMMD proteins in physiologically relevant tissues, such as the kidney and lung, will provide the basis for ongoing investigations into identifying the \textit{in vivo} functions of the COMMD proteins. For example, the tissue specific distribution of the different COMMD proteins could provide information regarding their sites of action and using techniques such as IHC, colocalisation of these proteins with their potential interacting partners could be visualised and confirmed \textit{in vivo} however at this time antibodies are not available to all the COMMD family members.

5.2.2. Detection of COMMD1 and COMMD3 in different mammalian cell lines
HEK293, COS-7, SH-SY5Y and Raji cells were investigated for endogenous COMMD1 and COMMD3 proteins in this project using Western blot analysis. The investigated cell lines are all derived from tissues in which COMMD mRNA and/or proteins have previously been identified. Results from these experiments have shown for the first time that endogenously expressed COMMD1 proteins are present in COS-7, SH-SY5Y and Raji cells and endogenously expressed COMMD3 proteins are present in HEK293 and SH-SY5Y cells (refer to section 4.2.).

Burstein et al. (Burstein et al., 2005) reported the presence of all ten COMMD mRNAs in HEK293 cells whereas Klomp et al. (Klomp et al., 2003) was the first to report the presence of endogenously expressed COMMD1 proteins in HEK293 as well as several other cell lines. However, COS-7, SH-SY5Y and Raji cells had not been previously investigated for endogenous protein expression of the COMMD family members. Nonetheless, the presence of COMMD1 in all cell lines investigated to date, including those reported in this study, reflects its ubiquitous pattern of expression. The detectable presence of endogenously expressed COMMD3 protein in HEK293 cells was partly expected as Burstein et al. (Burstein et al., 2005) detected COMMD3 at the mRNA level in HEK293 cells. However, no other reports have previously confirmed the presence of
COMMD3 mRNA and/or proteins in any other cell lines. Despite the ubiquitous expression of COMMD3 mRNA (Burstein et al., 2005), the apparent absence of endogenously expressed COMMD3 protein in COS-7 and Raji cells may be explained by COMMD3 mRNA not being translated into protein products or being translated into low levels of protein. Alternatively the sensitivity of the rat COMMD3 antiserum may not have been high enough to detect endogenous COMMD3 proteins.

Future work will be required to identify the endogenous presence of the remaining COMMD family of proteins in multiple cell lines. Similar to what has been done in this project, an investigation into the remaining COMMD proteins’ intracellular distribution using single and double label ICC could potentially identify their specific intracellular location and thus provide great insight into their likely in vivo function(s).

Based on the results obtained from this project, which have confirmed the presence of endogenously expressed COMMD1 and COMMD3 proteins in HEK293 and SH-SY5Y cell lines, these two cell lines were subsequently used in double label ICC experiments to address the hypothesis that the COMMD proteins localise to specific intracellular vesicular compartments that overlap with the trafficking pathways of ENaC (refer to section 4.4.).

5.3. Potential roles for the COMMD1 and COMMD3 proteins

5.3.1. A potential role for COMMD1 in the endocytic/recycling pathway of ENaC

Single and double label ICC experiments performed during this project revealed that endogenous COMMD1 proteins are present in punctate vesicles throughout the cytoplasm in HEK293 and SH-SY5Y cells (refer to section 4.3.2.). Some overlap in staining patterns between COMMD1 and the early endosome marker EEA1 was observed (refer to section 4.3.3.4.). No overlap in staining pattern was observed for COMMD1 or COMMD3 with the Golgi apparatus marker WGA in SH-SY5Y cells (refer to section 5.3.1.). Using the Golgi apparatus marker WGA and the trans-Golgi marker anti-Golgin97 Chang, T. (Chang, 2007) reported that overexpressed COMMD1 partially co-localised with both of these Golgi markers and suggested, based on triple labeling
experiments, that the Golgi is a major intracellular compartment where COMMD1 interacts with δENaC. In contrast to this, other groups have reported little to no overlap between endogenous COMMD1 and the Golgi markers p230, p58 (Klomp et al., 2003) and anti-Golgin97 (Burkhead et al., 2009). COMMD1 was also shown to exhibit a discrete perinuclear focus in the majority of the cells investigated and some nuclear and cytosolic staining could not be excluded.

The intracellular distribution of endogenously expressed COMMD1 has previously been investigated in multiple cell lines, including HEK293 but not SH-SY5Y cells (Burkhead et al., 2009; Klomp et al., 2003). Subcellular fractionation experiments were not performed as part of this project, however it is interesting to note that results obtained from these type of experiments have shown endogenously expressed COMMD1 proteins to be present in both the soluble (i.e. cytosolic) and membranous fractions in both Caco2 (Klomp et al., 2003) and HepG2 (Burkhead et al., 2009) cells. These data supports the idea that some COMMD1 associates with membrane lipids, such as PI(4,5)P2 (Burkhead et al., 2009). This contrasts slightly to that reported for overexpressed COMMD1 which has been detected in nuclear and cytosolic fractions in HEK293 cells (Burstein et al., 2005). The above findings have been complimented by studies that have investigated the intracellular distribution of both endogenous and overexpressed COMMD1 proteins.

There is a degree of consistency in the intracellular location reported for endogenously expressed versus overexpressed COMMD1 proteins across multiple cell types. Employing techniques such as ICC (Burkhead et al., 2009; Burstein et al., 2004; Klomp et al., 2003), live cell imaging (Muller et al., 2009) and bimolecular fluorescence (de Bie et al., 2006), COMMD1 has largely been localised to cytoplasmic vesicular compartments that overlap with markers for the early/recycling endosomes and late endosomes/lysosomes. Perinuclear aggregation of COMMD1-associated punctate vesicles has also been reported and several groups have described the presence of some COMMD1 in the nucleus (Burstein et al., 2004; Burstein et al., 2005; de Bie et al., 2006; Klomp et al., 2003). This intracellular distribution contrasts somewhat to that reported by a previous student in our laboratory. Chang, T. (Chang, 2007) reported overexpressed
tagged COMMD1 proteins to exhibit a predominantly diffuse cytosolic expression pattern in COS-7, HEK293 and MDCK cells that was modified to a more cytoplasmic vesicular pattern upon co-expression with δENaC. In addition to this and contrary to previous reports (Burkhead et al., 2009; Klomp et al., 2003), Chang, T. also localised COMMD1 to the Golgi bodies using anti-Golgin97 and WGA. Cell-type specific differences or overexpression of the COMMD1 protein may have accounted for this discrepancy in localisation. The latter in particular has the potential to alter the intracellular location or function of the protein concerned therefore a misrepresentation of its true \textit{in vivo} function may have been likely. It is important to highlight here that an investigation based on an endogenous protein’s intracellular location adds a dimension of power to any study therefore this project was superior to those investigating the intracellular distribution of exogenously expressed COMMD1 proteins.

With the exception of the latter results reported by Chang, T., all of the above reports are comparable to that described in this project however no late endosome/lysosomal staining could be confirmed. Association of the COMMD1 proteins with PI(4,5)P2 present in the membranes of the early/recycling (Burkhead et al., 2009) and/or late endosomes/lysosomes is likely to give COMMD1 its punctate appearance. At the same time, this association may also explain the presence of COMMD1 in both the cytosolic and membranous fractions that had previously been reported (Burkhead et al., 2009; Klomp et al., 2003). The detectable presence of some COMMD1 in the nucleus is in agreement with this protein having an effect on NF-κB signaling (Ganesh et al., 2003) and HIF-1 activity during hypoxia (van de Sluis et al., 2007), which would require COMMD1 to shuttle between the cytosol and nucleus. However, COMMD1 predominantly localised to the cytoplasm therefore it is unlikely that its main function in HEK293 and SH-SY5Y cells are associated with regulating NF-κB and/or HIF-1 activity under the culture conditions employed. This localisation could be experimented with by employing different culture conditions. For example, the intracellular distribution of endogenously expressed COMMD1 and COMMD3 proteins could be investigated at different time points in HEK293 and SH-SY5Y cells that have been maintained under hypoxic conditions. This could potentially provide some insight into the temporal
response of the distribution exhibited by the COMMD1 and COMMD3 proteins under these conditions.

Future studies investigating colocalisation between overexpressed ENaC subunits and endogenously expressed COMMD1 and/or COMMD3 proteins could provide stronger evidence in support of a role for the COMMD proteins in the trafficking of ENaC. Nonetheless, localisation of COMMD1 in a post Golgi vesicular compartment in this study supports a role of COMMD1 in the regulation of ENaC, the copper transport protein ATP7B and possibly other membrane protein trafficking. Maine and colleagues (Maine and Burstein, 2007a; Maine et al., 2007) defined a novel pathway by which COMMD1 promotes the ubiquitination of NF-κB subunits and thereby regulate κB-mediated transcription. Ke et al. (2010) demonstrated that COMMD1 down-regulates ENaC by increasing ubiquitination and endocytosis of ENaC, possibly via a simultaneous interaction with the ubiquitin protein ligase Nedd4-2 and the μ2 subunit of the clathrin-adaptor protein 2. COMMD1 was also shown to be capable of forming a complex with both SGK and Nedd4-2 (Ke et al., 2010). The interaction between SGK and COMMD1 may potentially prevent SGK from phosphorylating Nedd4-2 and thus allowing a continued interaction between Nedd4-2 and ENaC to occur. These results in combination with the localisation of COMMD1 to a post-Golgi vesicular compartment support a generalised role for COMMD1 in promoting or initiating binding of membrane bound protein substrates to enzymes, such as Nedd4-2, and thereby regulating endocytosis and/or recycling of its target substrates.

5.3.2. COMMD3: A potential scaffolding protein

COMMD3 was initially identified biochemically by its ability to interact with COMMD1 (Burstein et al., 2005). In a series of GST pull-down assays using a variety of COMMD1 deletion constructs, the COMM domain of COMMD1 was shown to be sufficient for COMMD multimer formation to occur. It is intriguing to note that unlike COMMD1, which is a strong inhibitor of NF-κB activity, COMMD3 inhibits NF-κB only weakly (Burstein et al., 2005). The interaction between COMMD1 and the RelA subunit of NF-κB is only detectable with full-length COMMD1 thus the presence of other elements
possibly present in the N terminal region of COMMD1 are likely to mediate this strong interaction. Variation in the N terminal sequences between COMMD1 and COMMD3 may well account for the diffuse cytosolic distribution of COMMD3 and absence of COMMD3 in the nucleus, which have been shown for the first time in this project using ICC (refer to section 4.3.2.). For example, COMMD3 did not appear to associate with any intracellular vesicular compartments as such, i.e. little to no punctate staining was observed for COMMD3, which may suggest that it functions as an accessory protein, possibly via an interaction with the COMM domain of COMMD1 or with one, or in combination with more than one, of the other COMMD family members. An interaction between COMMD3 and membrane lipids, such as PI(4,5)P2 remains to be investigated to determine if COMMD3 interacts with membrane associated lipids. In addition to this, COMMD3 may also play a role in regulating trafficking of ENaC to the cell surface however this requires further investigation.

The results of the double label ICC experiments revealed some partial overlap in staining patterns between COMMD3 and EEA1 (refer to section 4.3.3.4.), but not with WGA (refer to section 4.3.3.1.). This pattern of colocalisation is similar to that observed for COMMD1 however it could be argued that the cytosolic distribution of COMMD3 may reveal overlap with most organelle markers. In contrast to this, localisation of both COMMD1 and COMMD3 to the early endosomes but not Golgi bodies may further support a role for COMMD3 as an accessory/scaffolding protein involved in promoting or initiating endocytosis/recycling of ENaC and thereby its regulation. An alternative method, such as subcellular fractionation using differential centrifugation, may be required to define the localisation of COMMD3 further.

In addition to the ICC experiments performed during this project, double label IHC experiments whereby COMMD1 or COMMD3 were labeled in combination with EEA1 (n = 1) and WGA (n = 1) were performed as an alternative measure to further determine and confirm the intracellular location of endogenously expressed COMMD1 and COMMD3 proteins. Successful staining was observed for the COMMD proteins and their distribution pattern across the kidney appeared similar to that described earlier (refer to
section 4.5.1. and 4.5.2.). No staining for EEA1 was detected (results not shown) in this series of experiments. Where WGA was used, strong plasma membrane staining was observed which appeared to have masked any specific Golgi apparatus staining (results not shown). This was partly expected as WGA selectively binds to sialic acid and N-acetylglucosaminyl sugar residues, which are most abundant in the plasma membrane (refer to section 3.8.6.5.). Due to time constraints, optimisation of these protocols was not carried out.

Repeat double label ICC experiments using anti-LAMP1 or preferably LAMP2 (lysosomal markers) and transferrin (early/recycling endosome marker) may also be required. The anti-LAMP1 antibody used in this project was stored undiluted at 4°C, as was recommended by the manufacturer. However, this antibody was at least 3 years old at the time it was utilised for this project. The recommended stability period for transferrin is 1 year when stored at less than -20°C. The transferrin conjugate used in this project was more than a year old and stock solutions were stored at approximately -18°C. These factors may have contributed to the low signals obtained for these antibodies during the course of this project therefore further investigations will be required to confirm the intracellular location of COMMD1 and COMMD3 proteins.

To further investigate the in vivo function of COMMD1 and COMMD3 proteins double label ICC with markers specific to secretory and endocytic pathway organelles could be employed. For example, antibodies generated against the different Rab proteins, which are small GTP-binding proteins exposed on the cytoplasmic face of different membranes and are involved in orchestrating consecutive stages of intracellular vesicle transport (Deneka et al., 2003) could be utilised to characterise the intracellular location of COMMD1 and COMMD3 proteins further. For instance Rab1, in particular, is involved in regulating vesicular transport between the ER and cis-Golgi and thus antibodies generated against this protein could be used in double label ICC experiments to determine if COMMD1 or COMMD3 are associated with or localise to ER-Golgi bound vesicles and therefore localise to the secretory pathways. Alternative organelle markers, such as Golgin-97, p58 and p230 for the Golgi bodies, CD63 for the late
endosomes/lysosomes and Rab5 or Rab4 for early or recycling endosomes could also be used in double label ICC experiments to further characterise the intracellular location of COMMD1 and COMMD3.

5.4. Distribution of COMMD1 and COMMD3 in the rat kidney

5.4.1. Antibody affinity

Achieving successful IHC staining with the chicken anti-α-, -β and γENaC subunit antibodies was one of the major challenges encountered during this project despite using kidneys from dexamethasone treated rats.

For this project, IHC staining using the chicken anti-αENaC antibody consistently produced low intensity staining that appeared punctate and vesicular but absent from the apical cell surfaces of the distal nephron segments. αENaC positive staining was best seen in the outer and inner medullary CD and was virtually undetectable in the cortical segments in which ENaC is expressed: the DCT and cortical CD. Autofluorescence was present in the majority of histological sections that were viewed however this did not interfere with the visual analysis performed during this project. With the exception of the absence of any cortical and apical cell surface staining, the pattern of distribution observed for αENaC during this project is similar to that originally described by Duc et al. (Duc et al., 1994).

Dexamethasone is a synthetic steroid hormone that mediates in vivo effects similar to that described for native aldosterone (section 1.1.5.1.). For this project, dexamethasone was expected to upregulate the abundance of αENaC at the apical cell surface during the initial 30 min post administration in the animals used for this project. This aldosterone-mediated response has been extensively studied in the past (Verrey et al., 2008) and a late response, which is initiated 3 – 6 h post aldosterone release, has also been described. However, the duration of the early and delayed responses in the absence of any further physiological stimuli, i.e. aldosterone release and/or dexamethasone administration, has not been well defined. For instance, several groups have studied dexamethasone-mediated effects on ENaC in several cell lines including the human distal airway
epithelial cell line H441 (McTavish et al., 2009). Constant exposure to dexamethasone was shown to activate the αENaC gene promoter after approximately 6 h and reached a plateau at approximately 18 h. These results are consistent with data obtained by Nakamura et al. (Nakamura et al., 2002), which have shown dexamethasone to increase αENaC mRNA expression in fetal lung and kidney explants within 24 h of exposure. Masilamani et al. (Masilamani et al., 1999) induced an increase in circulating aldosterone levels by either restricting NaCl in the diets of the experimental animals for ten days or infusing a constant amount of aldosterone once a day for ten days. The animals were sacrificed on the tenth day and IHC results using rabbit polyclonal rat anti-ENaC antibodies revealed a marked and preferential increase in the strength of αENaC labeling in the principal cells of the CD. Muller et al. (Muller et al., 2003) on the other hand employed a similar dexamethasone treatment protocol to that described for this project, i.e. they administered dexamethasone to the animals at three different time points 12 h apart, however animals were sacrificed 2 h following the last dexamethasone injection, whereas for this project, animals were sacrificed 18 h after the last dexamethasone injection. αENaC mRNA expression levels increased following the treatment protocol employed by Muller et al. but unfortunately no IHC was performed to visualise the effect on ENaC. Sacrificing of the animals 18 h post dexamethasone treatment, in combination with the effects that a possible standard chow diet/free access to food and water may have had, may have concealed the anticipated dexamethasone-mediated effects on the distribution pattern of αENaC 18 h post administration of dexamethasone. Future studies may be required to define the temporal effects that dexamethasone mediate on ENaC, i.e. across approximately 36 h. This could potentially be investigated by sacrificing dexamethasone treated animals at different time points. The kidneys from these animals could subsequently be used to investigate the level of αENaC mRNA expression using RT-PCR and/or be used for αENaC specific staining using IHC.

It is also important to consider tissue specific differences that the dexamethasone-mediated response may have had. Nakamura et al. (Nakamura et al., 2002) showed that exposure of dexamethasone on fetal lung and kidney explants produced a significant increase in αENaC mRNA levels within 24 h. However, dexamethasone exposure only
increased β- and γENaC mRNA levels in the lung but not the kidney after more than 48 h. The dexamethasone treatment protocol employed in this project was designed with the aim to measure an amiloride-sensitive response in rat colons, and not kidneys. It may well be that for colon epithelium the effects of dexamethasone exposure is longer lasting than that experienced by the kidney. However at the time of writing, no known literature was available to support this statement. Likewise, this tissue specific difference will require further investigation. Future studies aimed at comparing the expression levels of ENaC mRNA in different tissues and at different time points from dexamethasone treated animals could potentially provide some insight into this matter.

The low staining intensity observed for αENaC in this project may be further explained by the absence of any significant difference in amiloride-sensitive short circuit current (I_{sc}) measurements obtained between the colons from control and dexamethasone treated rats (personal communication with Dr. K Hamilton and Mike Gill). The kidneys used in this project were harvested from these same dexamethasone treated rats and therefore an absence in this response may account for the absence of any αENaC staining in the apical membrane. It is noteworthy to mention however that this protocol normally produces an increase in the amiloride-sensitive I_{sc} in the colons of these animals (personal communication with Dr K Hamilton). This project provided the first opportunity to investigate the kidneys from these animals thus the dexamethasone-mediated response on ENaC in the kidney using this treatment protocol was not known. The dexamethasone-mediated response on ENaC could potentially be improved in future studies by either sacrificing the animals within a shorter time period following the last dexamethasone injection and/or putting the animals on a salt-restricted diet.

Strain-related variations in the specificity of antibodies to a variety of antigens in mice, guinea pigs and rats have been recognised previously and there is some evidence to suggest genetic control of antibody affinity (Steward, 1979). The anti-chicken α-, β- and γENaC polyclonal antibodies used in this project may have been raised in a low-affinity strain of chickens, which in turn may have produced low-affinity antibodies to the α-, β- and γENaC subunits. In general, staining for anti-αENaC was difficult to detect in the
IHC experiments performed in this project. The most vivid αENaC related staining was observed in the medullary CD and appeared absent or undetectable in the cortical regions in which ENaC is expressed: the DCT and cortical CD. Although this segment specific difference remains to be determined the low staining intensities may also have been accounted for the antigen used to generate the antibodies. For instance if the antigen was similar in sequence, i.e. highly conserved, to that natively found in the animal used to produce the antibodies, antibody production may have been less compared when an antigen that was poorly conserved across species was used for antibody production. Masilamani et al. (Masilamani et al., 1999) successfully detected abundant amounts of αENaC in rat kidney using their in-house rabbit polyclonal antibodies that were generated against rat αENaC antigen. Similar to this project, this was detected following aldosterone treatment. Therefore, ENaC antibodies raised in different animals and/or tissues obtained from animals other than mice or rats could be experimented with in future IHC studies.

An additional and alternative optimisation step for the detection of endogenous ENaC in rat tissues may involve comparing different specimen fixation (post-fixation versus perfusion fixed) and tissue processing techniques (paraffin embedded sections versus cryosections). Experimenting with the former may provide a solution to the natural fluorescence observed during this project, which may have been a result of lipofuscins (Schnell et al., 1999) or the presence of ENaC in the rat kidney vasculature (Jernigan and Drummond, 2005).

5.4.2. Distribution of COMMD1 and COMMD3 in the rat kidney in relation to ENaC
It was hypothesised that the COMMD1 and COMMD3 proteins localise to the same cell types as ENaC in vivo. This was investigated through a series of single and double label IHC experiments. Western blot analysis performed in this project demonstrated the presence of endogenously expressed COMMD1 and COMMD3 proteins in rat kidney (refer to section 4.2.) thus the distribution of COMMD1 and COMMD3 in the rat kidney was investigated (refer to section 4.5.). Staining for COMMD1 and COMMD3 partially overlapped with that of αENaC in the medullary CD, however, this was not exclusive.
Staining for COMMD1 also appeared in the thin and thick segments of the loop of Henle whereas staining for COMMD3 was also detected in PCT and thick segment of the loop of Henle. The distribution patterns for COMMD1 or COMMD3 in relation to αENaC suggests that the regulatory functions described for the COMMD proteins are likely to extend to other membrane-bound proteins such as ion or water channels and not just ENaC.

Double label IHC was also employed to investigate if COMMD1 or COMMD3 co-localise with AQP2. Double label IHC experiments in which AQP2 antiserum was used in combination with COMMD1 or COMMD3 antiserum were performed to assist in identifying specific nephron segments and cell types (i.e. principal cells) however partial overlap in staining patterns between the COMMD proteins and AQP2 was also observed in the kidney cortical CD and outer and inner medullary CD. This implies that the COMMD proteins might also be involved in the trafficking of AQP2. Similar to ENaC, trafficking of AQP2 is also an important mechanism by which this protein’s activity is regulated (Tajika et al., 2005; Takata et al., 2004). AQP2 is a vasopressin regulated water channel that resides on the apical cell surface of collecting duct principal cells. In response to increased extracellular fluid osmolality an acute increase in the permeability of H$_2$O in the CD is mediated by trafficking of AQP2 to the apical cell surface from intracellular vesicles. Unlike the aldosterone-mediated pathway responsible for regulating apical cell surface ENaC (section 1.1.5.1.), this event is triggered by vasopressin binding to basolateral V$_2$ receptors. This interaction induces an increase in cAMP and subsequent activation of protein kinase A (PKA), which results in translocation of AQP2-bearing vesicles from the intracellular pool to the apical cell surface (Nejsum, 2005). In the absence of vasopressin-mediated stimulation AQP2 is retrieved from the apical cell surface and transported to intracellular storage compartments/vesicles. The trafficking pathways of AQP2 are not fully deciphered therefore a potential role for the COMMD proteins on AQP2 trafficking will require further investigations. Protein-protein interaction assays, such as GST pulldown or co-immunoprecipitation experiments, could be employed as a starting point in future studies to determine if the different COMMD proteins interact with AQP2 or other membrane proteins. Such an interaction could
potentially define a more generalised role for the COMMD proteins in the regulation of membrane-bound proteins such as ion or water channels.

IHC results obtained during this project showed that COMMD1 is predominantly expressed in the thick segment of the loop of Henle, DCT and CD (refer to section 4.5.1.) and COMMD3 in the PCT, thick segments of the loop of Henle and CD (refer to section 4.5.2.). The differential expression observed between COMMD1 and COMMD3 in the kidney implies that these two proteins are likely to form complexes that do not always involve one or the other. For example, COMMD1 did not appear to be highly expressed in the PCT therefore COMMD3 may potentially form a regulatory complex with other members of the COMMD family and/or other proteins. COMMD-COMMD protein interactions have previously been shown to be mediated via the COMM domain (Burstein et al., 2005) and COMMD1 in particular has been shown to interact with several of its binding partners, including PI(4,5)P2 (Burkhead et al., 2009) and δENaC (Biasio et al., 2004), via its COMM domain. Further investigations into the cellular and intracellular locations of the remaining COMMD family members will be required to provide evidence in support of the suggestion that COMMD3 is likely to form complexes with other COMMD family members where COMMD1 is not expressed, and vice versa. In addition to this, the relative proximity between ENaC and COMMD1 or COMMD3 interactions could be determined more precisely using fluorescence resonance energy transfer (FRET) techniques.

The differential expression observed between COMMD1 and COMMD3 could be compared with that for Nedd4-2 and SGK. Nedd4-2 is a ubiquitin ligase protein that has been shown to interact with and thereby regulate apical cell surface ENaC (Abriel et al., 1999; Wiemuth et al., 2007). Nedd4-2 and ENaC are both expressed in principal cells of the cortical and medullary CD as well as airway and distal lung epithelia (Staub et al., 1997a). Contrary to this, the distribution of SGK does not exclusively converge to classical aldosterone-responsive tubule segments. Instead SGK has been shown to be expressed in the thick ascending limb of the loop of Henle as well as the aldosterone-responsive tubule segments: the DCT and cortical CD (Alvarez de la Rosa et al., 2003).
SGK, which is a downstream mediator of aldosterone, has been shown to increase the expression of ENaC at the apical cell surface by modulating the activity of Nedd4-2 (Snyder et al., 2002). However, the fact that these two proteins are not always co-expressed in vivo implicates a likely role for other proteins, such as the COMMD family of proteins, in their regulation and/or mode of action.

The assigned locations of the COMMD1 and COMMD3 proteins to specific nephron segments and cell types reported in this project require confirmation. Several references were utilised to assist in identifying specific nephron segments in this project and therefore to define the location of COMMD1 and COMMD3 in the rat kidney. Antibodies against specific protein markers of nephron segments and cell types can be utilised in future double label IHC experiments to assist in this task. Examples of specific protein markers of nephron segments and cell types include AQP1 for the proximal tubules and thin descending limbs of the loop of Henle and alkaline phosphatase for the proximal tubules (Ross et al., 2001).

The distribution pattern observed for αENaC in the rat kidney during this project is similar to that reported previously. Using kidneys from normal/control rats, Hager et al. (Hager et al., 2001) demonstrated that α-, β- and γENaC localise to the principal cells of the cortical collecting ducts and outer and inner medullary collecting ducts in the rat kidney. Labeling for αENaC in the inner medullary collecting duct cells was distributed throughout the cytoplasm. This is consistent with results obtained during this project, which have demonstrated staining for αENaC to be confined to the cytoplasm in the principal cells of the outer and inner medullary collecting ducts. Punctate cytoplasmic and apical staining for αENaC was not observed in this project. Loffing et al. (Loffing et al., 2000) reported weak staining for ENaC in the apical membranes of the DCT and CNT with staining further downstream appearing fine and granular throughout the cytoplasm. It may be possible that the anti-αENaC antibody used during this project exhibited low-affinity for its antigen in the rat (refer to section 4.2.) or alternatively, was a low-titer antibody. These factors may have accounted for the absence of any detectable
punctate staining.
6. Summary and Conclusion

The regulation of ENaC is crucial in the maintenance of Na⁺ homeostasis in the various tissues in which it is expressed. A number of ENaC regulators have previously been identified and studied in depth in order to gain a better understanding of the mechanisms involved in maintaining proper Na⁺ balance. Our laboratory identified several members of the COMMD family to be novel regulators of ENaC. In addition to this, these proteins have been shown to be involved in a number of other distinct biological processes including inhibition of NF-κB activity (Ganesh et al., 2003), regulation of copper homeostasis (van De Sluis et al., 2002) and inhibition of HIF-1 mediated gene expression during hypoxia (van de Sluis et al., 2007). The mechanism(s) by which the COMMD family of proteins mediates their effects on other proteins, as well as their cellular and intracellular locations, remain largely undefined.

The in vivo localisation of endogenously expressed COMMD1 and COMMD3 proteins were studied at both the cellular and intracellular level in this project using physiologically relevant tissues and cell lines. The ICC experiments presented in this thesis showed that, despite their high degree of sequence similarity, endogenous COMMD1 and COMMD3 proteins exhibit distinct patterns of distribution that may reflect functions that are unique to each of these proteins. COMMD1 and COMMD3 were shown to both localise to early/recycling endosomes. However to confirm this localisation and to further explore the intracellular location of the COMMD proteins, alternative early and recycling endosomes markers as well as other specific organelle markers could be utilised in double label ICC experiments in the future.

The IHC results obtained during this project provide the first evidence to show that the COMMD1 and COMMD3 proteins localise to the same cell types as ENaC in a physiologically relevant tissue (i.e. the kidney). To determine if the interaction between ENaC and the other COMMD proteins are also physiologically relevant, double label IHC experiments could be a task for the future. Triple label IHC experiments would be a useful extension to this investigation to determine if COMMD1, COMMD3 and αENaC co-localise. This could be repeated for the other COMMD proteins in different
combinations with ENaC in triple label IHC experiments.

From the results obtained during this project and that reported in the literature to date it is reasonable to propose that COMMD1 and COMMD3 are largely associated with early/recycling endosomes via a direct interaction with membrane lipids such as PI(4,5)P2 (Burkhead et al., 2009), which has a well described role in vesicular trafficking in polarised cells (Yin and Janmey, 2003). COMMD1 is likely to form a complex with COMMD3 and similar to the pathways proposed by Maine et al. (Maine et al., 2007) and Ke, Y. (Ke, 2008), COMMD1 is likely to promote binding of the substrate protein (e.g. ENaC) via its COMM domain to the regulatory complex, which is likely to contain Nedd4-2. Through this interaction and proposed mechanism, COMMD1 and COMMD3 are likely to be involved in initiating and/or promoting endocytosis and/or recycling of ENaC to and from the apical cell surface.
7. References


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8. Appendix

8.1. Nucleotide and amino acid sequences

The following nucleotide and amino acid sequences were retrieved from the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov).

8.1.1. human COMMD1

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