Endosomal SNX Proteins are Required for Recycling of the Epithelial Sodium Channel.

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Abstract

The epithelial sodium channel (ENaC) facilitates Na⁺ absorption of polarised epithelia and is required for regulation of salt and water homeostasis. ENaC’s apical membrane population is strictly controlled, and loss of this control can lead to Liddle’s Syndrome, a form of hypertension. The retromer and retriever complexes are conserved endosome-localised protein complexes that mediate recycling of membrane proteins back to the cell surface. SNX3 and SNX17, sorting nexin proteins, are cargo-binding proteins that interact with retromer and retriever respectively, to facilitate cargo recycling. The aim of this study was to investigate whether the retromer/retriever associated SNX3 or SNX17 proteins are required for the recycling of ENaC.

Fischer rat thyroid (FRT) and mouse cortical collecting duct (mCCD) cells were transiently transfected with control, SNX3 or SNX17 siRNA. Subsequently, Western blot analysis was carried out to confirm knockdown; ‘Ussing’ chamber experiments were conducted to measure changes in ENaC’s amiloride-sensitive short circuit current (I_{sc-Amil}), and cell surface biotinylation assays were used to measure ENaC cell surface population. Finally, co-immunoprecipitation (Co-IP) experiments were performed to detect if protein-protein interactions exist between ENaC and SNX3 or SNX17. Results were analysed using a One-sample T-test.

Significant protein knockdown was observed for both SNX proteins in the two cell lines (n = 3, \( P < 0.05 \)). A significant reduction in I_{sc-Amil} of ENaC was observed in both cell lines with a SNX3 or SNX17 siRNA knockdown (n = 9, \( P < 0.001 \)). Furthermore, a significant
reduction in ENaC cell surface population with either a SNX3 or SNX17 siRNA knockdown was observed (n = 3, \( P < 0.05 \)), demonstrating the reduction in \( I_{sc} \)-Amil was due to reduced ENaC cell surface population. Finally, Co-IP experiments demonstrated a protein-protein interaction between SNX17 and ENaC, however, not SNX3 and ENaC.

These results suggest that knockdown of either SNX3 and SNX17 can facilitate ENaC recycling and this may occur through different pathways. Additionally, SNX17 may specifically interact with ENaC to localise ENaC in recycling structures in the endosome. This investigation adds to our understanding of the mechanism involved in ENaC recycling, contributing to the understanding of and prevention of hypertension.
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\[ \text{NaHCO}_3 \quad \text{Sodium Bicarbonate} \]
\[ \text{Na}^+ / \text{K}^+ - \text{ATPase} \quad \text{Sodium-Potassium Adenosine Triphosphatase Pump} \]
\[ \text{O}_2 \quad \text{Oxygen} \]
\[ \text{HEPES} \quad \text{4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid} \]
\[ \text{hr} \quad \text{Hour(s)} \]
\[ \text{min} \quad \text{Minutes} \]
\[ \text{sec} \quad \text{Seconds} \]
\[ \text{cm} \quad \text{Centimetre} \]
\[ \text{dpi} \quad \text{Dots Per Inch} \]
\[ \text{kDa} \quad \text{Kilodalton} \]
\[ \text{mA} \quad \text{Milliampere} \]
\[ \text{mA/cm}^2 \quad \text{Milliampere Per Square Centimetre} \]
\[ \text{mg} \quad \text{Milligram} \]
\[ \text{mL} \quad \text{Milliliter} \]
\[ \text{mm} \quad \text{Millimeter} \]
\[ \text{mm}^2 \quad \text{Square Millimetre} \]
\[ \text{nm} \quad \text{Nanometre} \]
\[ \text{pS} \quad \text{Picosiemens} \]
\[ \text{pM} \quad \text{picoMolar} \]
\[ \text{r.p.m} \quad \text{Revolutions Per Minute} \]
\[ \text{V} \quad \text{Volt} \]
\[ \text{μg} \quad \text{Microgram} \]
\[ \text{μL} \quad \text{Microliter} \]
\[ \text{μM} \quad \text{MicroMolar} \]
\[ \text{Ω.cm}^2 \quad \text{Ohm-Per Square Centimetre} \]
α  Alpha
β  Beta
γ  Gamma
αβ1  Alpha-5 Beta-1
αENaC  Alpha ENaC Subunit
β1 Integrin  Beta-1 Integrin
βENaC  Beta ENaC Subunit
β-HA ENaC  β HA-epitope-tagged ENaC Subunit
γENaC  Gamma ENaC Subunit
μ2  AP-2 Adaptor Complex Medium Subunit
A6  Amphibian Epithelial Cells.
ACE  Angiotensin-Converting Enzyme
adPHA1  Autosomal Dominant Pseudohypoaldosteronism Type 1
ANGI  Angiotensin I
ANGII  Angiotensin II
AP-2  Adaptor Protein-2
APP  Amyloid Precursor Protein
APS  Ammonium Persulphate
Arp2/3  Actin-Related Proteins 2 and 3 Complex
arPHA1  Autosomal Recessive Pseudohypoaldosteronism Type 1
ASL  Airway Surface Layer
ATP7A  Copper-transporting ATPase 1
BAR  Bin/Amphiphysin/Rvs Domain
BFA  Brefeldin A
BSA  Bovine Serum Albumin
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<td>DMT1</td>
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<tr>
<td>DNA</td>
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<td>ECF</td>
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<td>EGF</td>
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<td>ENaC</td>
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<td>Description</td>
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<td>Penicillin/Streptomycin Sulphate</td>
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<td>Pseudohypoaldosteronism Type 1</td>
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<tr>
<td>PMSF</td>
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<td>RAAS</td>
<td>Renin-Angiotensin Aldosterone System</td>
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<tr>
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<td>Ribonucleic Acid</td>
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RT  Transepithelial Resistance

*S. cerevisiae*  *Saccharomyces cerevisiae*

SCNN1A  Sodium Channel Epithelial 1 Alpha Subunit

SCNN1B  Sodium Channel Epithelial 1 Beta Subunit

SCNN1G  Sodium Channel Epithelial 1 Gamma Subunit

SD  Standard Deviation

SDS  Sodium Dodecyl Sulphate

SDS-PAGE  SDS Polyacrylamide Gel Electrophoresis

SF  Serum Free Media

SGK1  Serum and Glucocorticoid-Regulated Kinase 1

siRNA  Small Interfering RNA

SNAP23  Synaptosomal-Associated Protein 23

SNARE  Soluble N-Ethylmaleimide-Sensitive Attachment Receptor

SNX (1/2/3/5/6/17/27)  Sorting Nexin Protein (1/2/3/5/6/17/27)

SNX-BAR  Sorting Nexin-Bin/Amphiphysin/Rvs Domain

SNX-MIT  Sorting Nexin-Microtubule Interacting and Transporter

SNX-PX  Sorting Nexin-Phox Homology Domain

SORL1  Sortilin-Related Receptor 1

SWIP  Wash Complex Subunit 4

T3  3,3',5-Triiodo-L-Thyronine Sodium Salt

t-SNARE  Target SNARE

TBC1D5  TBC1 Domain Family Member 5

TBS  Tris-Buffered Saline

TBS-T  Tris-Buffered Saline Tween

TEMED  Tetramethylethylenediamine
Tf  Transferrin
TGN  Trans-Golgi Network
Ub  Ubiquitin
Usp (10/45/L3)  Ubiquitin-Specific Protease (10/45/L3)
v-SNARE  Vesicle SNARE
VAMP (7/8)  Vesicle-Associated Membrane Protein (7/8)
VPS (5/10/17/26/29/35)  Vacuolar Sorting Protein (5/10/17/26/29/35)
VPS26C  VPS26 Endosomal Protein Sorting Factor C
VPS35L  VPS35 Endosomal Protein Sorting Factor Like
WASH  Wiskott-Aldrich Syndrome Protein and SCAR Homologue
WLS  Wnt Ligand Secretion Mediator
Wnt  Wntless
WW  WWP Repeating Motif
X. laevis  *Xenopus laevis*
1. Introduction
1.1 Overview

The epithelial sodium channel (ENaC) is an integral membrane protein found in various epithelia where it acts to facilitate Na⁺ ion movement across the apical membrane into the cell as the first step in a two-step process of transepithelial sodium transport, subsequently influencing water absorption and salt homeostasis. It is important to understand the trafficking and recycling of ENaC to alleviate symptoms of ENaC associated diseases including; Liddle’s syndrome (Section 1.7.1), pseudohypoaldosteronism type 1 (PHA1) (Section 1.7.2) and Cystic Fibrosis (CF) (Section 1.7.3).

1.2 The Epithelial Sodium Channel (ENaC)

ENaC, a member of the ENaC/Degenerin superfamily (Hanukoglu & Hanukoglu, 2016; Hanukoglu, 2017), is a trimeric protein comprising the subunits; α- (SCNN1A), β- (SCNN1B) and γ- (SCNN1G) (Figure 1.1) (Noreng & Bharadwaj, 2018). Functional properties of ENaC were characterised long before its cloning when Ussing was investigating sodium movement across frog skin (Ussing & Zerahn, 1951). Ussing designed an experimental apparatus to measure epithelial short-circuit current carrying sodium ions across frog skin epithelia, allowing for the first functional measurements of what would later be known as the amiloride-sensitive sodium current (Ussing & Zerahn, 1951). Furthermore, measurements of this sodium current led to the proposal of the Koefoed-Johnsen-Ussing two-membrane model for epithelial transport (Section 1.5) (Koefoed-Johnsen & Ussing, 1958). Subsequently, Lindemann and colleagues also described an amiloride inhibited conductive Na⁺ entry pathway found in electrically high resistance frog skin epithelia (Lindemann & Van Driessche, 1977). However, the presence of an amiloride-sensitive apical sodium channel was not established until Hamilton and Eaton, using amphibian (A6) kidney epithelia, provided the first experimental
evidence of an amiloride-inhibitable epithelial Na\(^+\) channel through patch clamp studies (Hamilton & Eaton, 1985).

Figure 1.1. Schematic Representation of the ENaC Subunit Structure. The fully functional ENaC channel is comprised of three homologous subunits, αβγ, with each containing four distinct domains: cytoplasmic N-termini, large extracellular loops, two membrane spanning segments that contribute to Na\(^+\) selective pore formation, and cytoplasmic C-terminal domains containing a 'PY' motif (Section 1.3) (Noreng & Bharadwaj, 2018).

Canessa et al., (1993) were the first to clone an αENaC subunit from distal rat colon (rαENaC), after an amiloride-sensitive current was observed in epithelia of the colon (Will et al., 1981; Schultz, 1984; Canessa et al., 1993; Lingueglia et al., 1993). This allowed McDonald et al., (1994) to use a probe from that sequence to clone an αENaC subunit from human kidney. When rαENaC was expressed in Xenopus laevis (X. laevis) oocytes, the amiloride-sensitive current generated was observed to be considerably lower than that observed in vivo, leading to the proposal that αENaC is the pore forming subunit of the channel, but other components must be involved in vivo (Canessa et al., 1993).

The cloning of β- and γ- ENaC subunits from both rat distal colon (Canessa et al., 1994b) and human kidney (McDonald et al., 1995) soon followed. Expression of β- and γ- ENaC in X. laevis oocytes revealed that unlike αENaC, these subunits were unable to generate an amiloride
sensitive current alone, however, if one was co-injected alongside αENaC this enhanced current. If all three subunits were co-expressed together, this generated a 100-fold increase in amiloride-sensitive current resembling that of a native ENaC channel. Therefore, it was concluded that all three subunits are required to form a fully functional ENaC channel, with the β- and γ- subunits acting to enhance αENaC activity (Canessa et al., 1994b).

Notably, it is hypothesised that all three α-, β- and γ- subunits are required for correct protein folding and formation of a functional channel (Valentijn et al., 1998; Buck et al., 2010). In the kidney, β- and γ- subunits are observed to be expressed at a higher level than the α- subunit, yet, due to the lack of αENaC both β- and γ- subunits are degraded by proteasomes (Staub et al., 1997). However, in the presence of aldosterone αENaC expression is induced (Section 1.6) allowing formation of functional ENaC channels (Asher et al., 1996; Masilamani et al., 1999), therefore suggesting that the formation of a heteromeric complex is a limiting factor for the expression of functional ENaC (Valentijn et al., 1998).

ENaC has a wide expression pattern throughout the body, as immunohistochemical studies have shown epithelial tissue localisation of the α-, β- and γ- subunits in kidney, lung, salivary glands, skin, placenta and the colon (O'Brodovich et al., 1993; Canessa et al., 1994a; Hanukoglu & Hanukoglu, 2016). There is also now evidence for the presence of ENaC subunits in non-epithelial locations including the vascular endothelium (Kusche-Vihrog et al., 2014), smooth muscle cells (Drummond et al., 2008) and the brain (Miller & Loewy, 2013).

1.3 Structural Characteristics of ENaC

ENaC channel subunits possess two membrane-spanning domains, with each subunit being defined in terms of four distinct domains; the cytoplasmic N-terminal domain, the large
extracellular loop, the two short hydrophobic segments, and the cytoplasmic C-terminal domain (Figure 1.2) (Canessa et al., 1994a; Schild et al., 1997). The extracellular loop contains conserved cysteine-rich sequences (Figure 1.2), which are required to establish the ENaC tertiary structure and to ensure a proper Na\(^+\) self-inhibition response, likely by formation of multiple intrasubunit disulfide bonds (Firsov et al., 1998). A unique conserved motif; the PY motif, is located within the cytoplasmic C-terminal tail of all three ENaC subunits (Figure 1.2) (Snyder et al., 1994; Schild et al., 1996). This PY motif is recognised by WW domains of the E3 ubiquitin-protein ligase; NEDD4-2 (neural precursor cell expressed developmentally down-regulated gene 4-2), initiating ubiquitination, and therefore, internalisation and degradation of ENaC (Section 1.8.2) (Rotin & Staub, 2011). Mutations or deletions of the PY motif have been demonstrated to reduce ENaC ubiquitination thus leading to an accumulation of cell surface ENaC, in turn enhancing Na\(^+\) absorption and consequently increasing blood pressure (Section 1.5), leading to disorders such as Liddle’s syndrome (Section 1.7.1) (Lu et al., 2007; Rotin & Schild, 2008).

As ENaC transits the Golgi it undergoes maturation through proteolytic cleavage (Hughey et al., 2003; Hughey et al., 2004b; Ergonul et al., 2006). There are a number of serine proteases that have been reported to cleave and thus activate α- and γ- ENaC, with one of the most characterised being furin (Hughey et al., 2004a; Hughey et al., 2004b; Kleyman et al., 2009). Furin is predominantly expressed in the trans-Golgi network (TGN), where it cleaves both α- and γ- subunits at furin cleavage consensus sequences (RXXR) (Figure 1.2) (Misumi et al., 1991; Molloy et al., 1992; Hughey et al., 2004a). Furin cleavage has been demonstrated to activate ENaC and increase channel open probability (P\(_o\)) in various cell types including X. laevis oocytes and Madin-Darby canine epithelial (MDCK) cells (Chraibi et al., 1998; Caldwell et al., 2004; Hughey et al., 2004a; Sheng et al., 2006).
As mentioned previously, it is widely accepted that each of the three α-, β- and γ- subunits contribute to formation of a functional ENaC channel (Canessa et al., 1994a; Firsov et al., 1998), however, the actual subunit stoichiometry remained controversial for many years, with evidence for several models demonstrated. Proposed models included a fully oligomerised channel complex within the plasma membrane containing 2α:1β:1γ (Firsov et al., 1998; Kosari et al., 1998), and that ENaC contains several copies of each subunit in a higher order structure (Snyder et al., 1998; Eskandari et al., 1999; Staruschenko et al., 2004). Staruschenko et al., (2005) determined that functional ENaC within the membrane contains equal numbers of α-, β- and γ- subunits while also demonstrating that heteromeric channels are preferentially

Figure 1.2. Schematic Representation of the Common Features and Membrane Topology of ENaC Subunits. Each ENaC subunit has two membrane-spanning domains (MSD), one extracellular loop with two glycosylated cysteine rich domains (CRD) and intracellular N- and C-terminal domains. Each C-terminal domain contains a PY motif unique to ENaC subunits. Furin has been demonstrated to cleave the α- and γ- subunits at RXXR furin cleavage consensus sites indicated with black lines.
formed over homomeric channels when all three subunits are co-expressed. Supporting this model, Noreng et al., (2018), recently demonstrated the structure of ENaC using cryo-electron microscopy, revealing that human ENaC assembles with a 1:1:1 stoichiometry of α:β:γ subunits arranged in a counter-clockwise manner.

1.4 Biophysical Properties of ENaC

ENaC has several defining biophysical characteristics, including its cation selectivity. Palmer, (1982), using toad urinary bladder, established that ENaC is highly selective for Na\textsuperscript{+} over K\textsuperscript{+}, which has now been described in other tissues as a ratio of 10:1 (Palmer & Frindt, 1988; Canessa et al., 1993; Puoti et al., 1995). A three-residue selectivity filter, consisting of a G/S–X–S motif, is present in the MSD2 (transmembrane-spanning domain two) of ENaC subunits, with introduction of specific mutations to this motif resulting in channels with increased K\textsuperscript{+} permeation (Kellenberger et al., 1999; Snyder et al., 1999). In regard to single channel conductance, ENaC has a mean conductance of between 5 and 8 pS (picoSiemens) (Hamilton & Eaton, 1985; Palmer & Frindt, 1986).

Another defining characteristic of ENaC involves its slow gating kinetics where mean open and closed times have been exhibited to be in the range of 0.5-5 sec for A6 and rat renal cortical collecting tubule (CCT) cells (Hamilton & Eaton, 1986; Palmer & Frindt, 1986). Similar kinetics were observed with cloned ENaC channels expressed in X. laevis oocytes (Canessa et al., 1994a). Several factors have been shown to influence the P\textsubscript{o} of ENaC, including: changes in membrane voltage, intracellular pH, aldosterone, intracellular and extracellular Na\textsuperscript{+} concentrations and proteolytic cleavage (Palmer & Frindt, 1986; Firsov et al., 1996; Krappitz et al., 2014).
1.5 Physiological Role of ENaC in Kidney Epithelia

ENaC functions to mediate sodium absorption in tight epithelia throughout the body, thus making ENaC crucial in the control of salt and water homeostasis, and therefore, the regulation of blood pressure and total blood volume (Reviewed in Rossier et al., 2002). The kidney is the main organ involved in the regulation of salt and water homeostasis, and here, ENaC is responsible for fine-tuning \( \text{Na}^+ \) reabsorption in the distal kidney (the late distal convoluted tubule (DCT), the connecting tubule (CT) and the cortical collecting duct (CCD)) (Lifton et al., 2001; Loffing et al., 2001; Mironova et al., 2017).

Koefoed-Johnsen and Ussing were the first to predict how exactly this sodium absorption occurs through their two-membrane hypothesis, suggesting \( \text{Na}^+ \) moved across the apical membrane passively via a conductance pathway and that the active transport of \( \text{Na}^+ \) was located at the basolateral membrane of an epithelial cell (Koefoed-Johnsen & Ussing, 1958; Hamilton, 2011). ENaC is located at the apical membrane of epithelial cells where \( \text{Na}^+ \) ions can move down their electrochemical gradient via ENaC into the cell, due to the \( \text{Na}^+/\text{K}^+\)-ATPase pump actively transporting \( \text{Na}^+ \) ions out of the cell across the basolateral membrane (Figure 1.3) (Benos, 1982). \( \text{K}^+ \) channels maintain the electrochemical gradient for the \( \text{Na}^+/\text{K}^+\)-ATPase pump with the recycling of \( \text{K}^+ \) ions back across the apical and basolateral membranes via \( \text{K}^+ \) specific channels (Figure 1.3). Absorption of \( \text{Na}^+ \) across the epithelium drives water to follow this \( \text{Na}^+ \) movement resulting in expansion of the extracellular fluid (ECF).
The importance of fine-tuning sodium reabsorption by ENaC in the kidney has been highlighted in a mouse model possessing a knockout for the mineralocorticoid receptor (Section 1.6) (Hummler et al., 1997), which led to depleted ECF and the development of PHA1 (Section 1.7.2). In addition, heterozygous mouse models (αENaC +/−) were observed to display reduced Na⁺ transport and exhibited a PHA1 phenotype (Berger et al., 1998). Furthermore, β-

Figure 1.3. Cell Model of Na⁺ Absorption in an Epithelial Cortical Collecting Duct Cell. Luminal Na⁺ ions move down their electrochemical gradient into the cell via ENaC. Na⁺ ions are then actively pumped out of the cell across the basolateral membrane via the Na⁺/K⁺-ATPase. K⁺ channels maintain the electrochemical gradient for the Na⁺/K⁺-ATPase pump by recycling K⁺ ions back out of the cell across the apical and basolateral membranes. The movement of Na⁺ ions creates a positive potential on the ECF side of the epithelium, which in turn drives the movement of H₂O across the transcellular pathway resulting in expansion of the ECF.
and γ-ENaC null mutants exhibit early renal dysfunction leading to death within 48 hr, therefore, reflecting the renal phenotype found in patients with PHA1 (Barker et al., 1998; McDonald et al., 1999). Furthermore, ENaC function is highlighted with the hypertensive disorder Liddle’s syndrome (Section 1.7.1). Liddle’s syndrome is the consequence of a gain of function ENaC phenotype where ENaC accumulates at the cell surface in turn increasing Na⁺ and water absorption, thus leading to hypertension (Firsov et al., 1996; Abriel et al., 1999).

1.6 The Renin-Angiotensin Aldosterone System (RAAS)

The mineralocorticoid hormone aldosterone functions in regulation of salt and water balance, acting primarily by increasing apical Na⁺ permeability through activation of ENaC, using a hormonal cascade known as RAAS. Activation of RAAS begins with a decrease in the filtrate sodium chloride concentration or a decreased filtrate flow rate stimulating the release of the enzyme renin from juxtaglomerular cells (Zhou et al., 2010; Wu et al., 2011). Renin cleaves angiotensinogen into the peptide angiotensin I (ANGI) followed by conversion to angiotensin II (ANGII) by angiotensin-converting enzyme (ACE) (Zhou et al., 2010; Wu et al., 2011). ANGII stimulates the adrenal cortical juxtaglomerular cells to synthesise and release the mineralocorticoid hormone aldosterone (Cozza et al., 1989) which then binds to intracellular mineralocorticoid receptors (MR) with the translocation of this transcription factor complex to the nucleus to modulate gene expression.

The response of the kidney to aldosterone can be divided into two phases: an early and late response phase (Asher et al., 1996; Chen et al., 1999; Snyder et al., 2002). The early response phase takes place over the first 0.5-4 hr and involves an increase in distal kidney ENaC-dependent Na⁺ transport. This is primarily facilitated by the rapid stimulation of genes encoding ENaC regulatory proteins such as the serum glucocorticoid-regulated kinase (SGK1)
SGK1 acts to increase Na$^+$ absorption through increasing the number of active channels at the cell surface, as SGK1 phosphorylates the E3 ubiquitin ligase NEDD4-2, thus preventing its ability to ubiquitinate ENaC, in turn preventing endocytosis and degradation of ENaC (Section 1.8.2) (Chen et al., 1999; Bhargava et al., 2001; Robert-Nicoud et al., 2001; Shi et al., 2002; Snyder et al., 2002; Snyder et al., 2004). Recently it has been demonstrated that in NEDD4-2 knockout mouse models on a normal diet, there is low aldosterone levels indicating increased Na$^+$ and reduced K$^+$ retention, signifying the critical role of NEDD4-2 in regulating Na$^+$ and K$^+$ levels in the blood (Henshall et al., 2017). An additional regulator of ENaC ubiquitination, the deubiquitinating enzyme USP-45, has also been identified as an early aldosterone-induced gene product allowing for increased recycling of the channel back to the plasma membrane (Section 1.8.3) (Fakitsas et al., 2007).

The aldosterone late response phase is initiated 4-24 hr after aldosterone release, where there is an increase in transcription of αENaC accompanied by redistribution of β- and γ-subunits from intracellular compartments to the apical membrane (Asher et al., 1996; Masilamani et al., 1999; Loffing et al., 2000). Proteolytic cleavage of the α- and γ-subunits correlating with an increase in chronic activity has also been observed as a response to aldosterone (Masilamani et al., 1999; Henshall et al., 2017).

1.7 ENaC Related Disorders

1.7.1 Liddle’s Syndrome

Liddle’s syndrome is a rare autosomal dominant disorder characterised by a severe form of early onset hypertension (Liddle, 1963). Liddle’s syndrome results from a gain of function ENaC phenotype as the consequence of deletions or mutations in the PY motif of β- or γ-ENaC subunits, in turn inhibiting association between ENaC and the ubiquitin ligase NEDD4-2.
(Section 1.8.2) (Shimkets et al., 1994). Accumulation of ENaC at the cell surface results from a reduction in ENaC endocytosis, in turn increasing Na\(^+\) and water absorption, thus leading to hypertension (Firsov et al., 1996; Abriel et al., 1999). In addition, Liddle’s syndrome has been observed to increase the fraction of ENaC at the cell surface that is cleaved hence increasing channel P\(_o\) (Knight et al., 2006; Boiko et al., 2015; Salih et al., 2017). The increased cell surface population along with the increased Na\(^+\) current passing through the channels leads to prolonged hypertension, low plasma renin, hypoaldosteronism and hypokalaemia (Botero-Velez et al., 1994; Shimkets et al., 1994). Treatment involves following a low sodium diet and taking potassium-sparing diuretics which reduces blood pressure and corrects hypokalaemia.

### 1.7.2 PHA

Pseudohypoaldosteronism type 1 (PHA1) is an inherited form of salt wasting that exists in a dominant (adPHA1) and recessive (arPHA1) form. arPHA1 is classed as the more severe form, and is associated with organ resistance to aldosterone, including the kidneys, colon and lungs (Kerem et al., 1999; Riepe, 2013). arPHA1 is characterised by severe neonatal salt wasting, hypotension, hyperkalaemia, metabolic acidosis and failure to thrive (Cheek & Perry, 1958). The genetic cause for arPHA1 involves autosomal recessive mutations in genes encoding for the ENaC subunits. Chang et al. (1996), identified homozygous PHA1-causing mutations in the α- subunit gene (SCNN1A) and the β- subunit gene (SCNN1B). These mutations introduced frameshift, premature termination, or missense mutations resulting in loss of channel activity. Strautnieks et al., (1996) subsequently showed that a homozygous splice site mutation of the γ- subunit (SCNN1G) gene can also cause arPHA1.
1.7.3 Cystic Fibrosis

Cystic Fibrosis (CF), a hereditary recessive disease that is the consequence of mutations to the Cl⁻ channel ‘Cystic fibrosis transmembrane conductance regulator’ (CFTR). CF leads to abnormal electrolyte transport in epithelia of the lungs, kidney, liver and pancreas (Riordan et al., 1989). One of CFTR’s primary functions is in the lungs where it maintains homeostasis of the airway surface fluid layer (ASL) to trap and remove incoming debris from the airway. CF disrupts this process by decreasing the efficiency of CFTR to secrete Cl⁻ across the epithelia. Na⁺ absorption remains active leading to water retention which in turn further decreases the ASL eliminating the ability to remove trapped debris, therefore, resulting in airway inflammation and respiratory issues (Khan et al., 1995; Rosenstein & Cutting, 1998).

In CF, enhanced Na⁺ absorption is observed with a link between CF and increased ENaC activity proposed. One hypothesis explaining this enhanced Na⁺ absorption involves downregulation of ENaC activity by CFTR in normal non-CF patients. This hypothesis is supported by studies where measurement of ENaC current increased across CF-airway epithelia (Gowen et al., 1986; Stutts et al., 1995; Mall et al., 1996; Rowe et al., 2005). Furthermore, a transgenic mouse model developed to overexpress βENaC exhibited a lung phenotype characteristic of human CF airways. Mucociliary clearance was decreased due to volume depletion of the ASL, alongside dehydrated mucus, neutrophilic inflammation and poor bacterial clearance (Mall et al., 2004).

Despite a large number of studies demonstrating evidence supporting CFTR regulation of ENaC, the mechanisms involved remain unclear due to contradictory results reported by other groups. Lazrak and colleagues reported that CFTR regulates ENaC activity in isolated type II alveolar cells, even when CFTR protein levels were minimal (Lazrak et al., 2011). However, CF
pig models have not been reported to exhibit increased Na\textsuperscript{+} or water absorption compared to wild-type animals (Itani et al., 2011), in spite of an increased amiloride-sensitive sodium current observed in airway epithelial cultures from these models (Itani et al., 2011). A regulatory effect of CFTR on ENaC is yet to be confirmed, however, several groups are pursuing ENaC inhibitors as a therapeutic approach to CF (Murphy & Caraher, 2016).

1.8 Trafficking of ENaC

Anterograde and retrograde trafficking of plasma membrane proteins involves a pathway to (exocytosis) and from (endocytosis) the plasma membrane. As plasma membrane proteins are removed from the cell surface, the plasma membrane must be replenished for the cell to function correctly, which is why coordination of these events is essential in maintaining cell homeostasis. Epithelial cells are polarised and require specific proteins to be trafficked to particular membrane domains in order for the epithelia to transport certain ions and solutes. Therefore, in polarised epithelia precise trafficking events are required for plasma membrane proteins to not only reach the correct membrane, but to also determine their cell surface residency and internalisation.

1.8.1 Synthesis and Exocytotic Trafficking of ENaC

Transmembrane protein trafficking begins with the synthesis of the protein by endoplasmic reticulum (ER)-associated ribosomes, followed by post-translational modifications as proteins move through the ER and Golgi, where at the TGN, proteins are sorted and directed to the appropriate membrane domain (Rodriguez-Boulan et al., 2004) (Figure 1.4).
ENaC mRNA is translated by ribosomes before translocation into the ER (Masilamani et al., 1999; Ergonul et al., 2006; Kota et al., 2014). N-glycan addition to the ENaC subunits facilitates the interaction between immature subunits and protein chaperones, which induces protein folding (Hughey et al., 2003). ENaC exits the ER and is trafficked to the Golgi, however, it has also been demonstrated that ENaC is able to bypass the Golgi and traffic directly to the plasma membrane in an immature form (Hughey et al., 2004a; Hughey et al., 2004b; Frindt et al., 2016). ENaC processing continues as ENaC transits through the Golgi as the channel undergoes maturation through proteolytic cleavage by serine proteases such as furin (Section 1.3) and alterations in N-linked glycosylation (Rotin et al., 2001; Ergonul et al., 2006; Heidrich et al., 2015).

Proteins contain signals that determine the route they will follow in order to reach their desired destination; these signals can take several forms and may be encoded on ectodomains or transmembrane domains. Examples for ENaC include N- and O-linked glycosylation for apical membrane-embedded signals (Fiedler & Simons, 1995; Yeaman et al., 1997). Along with these specific signals encoded on the proteins themselves, several apical proteins, including ENaC, are able to be trafficked via glycolipoprotein membrane domains otherwise known as lipid rafts, which act as specialised micro-domains serving as organising centres to aid in membrane trafficking (van Meer et al., 1987; Hill et al., 2007).

Kinesins are known to bind to a diverse range of vesicle cargo including membrane proteins allowing for the movement of vesicles towards the plasma membrane (Noda et al., 2001; Delevoye & Goud, 2015). Targeting of ENaC to the apical membrane is believed to involve use of cytoskeletal and molecular motor proteins to move ENaC-containing vesicles from the TGN towards the apical membrane. Evidence supporting this model was demonstrated with
disruption of the cytoskeletal components; actin and tubulin leading to failure in ENaC apical membrane delivery (Butterworth et al., 2005).

Figure 1.4. ENaC’s Trafficking Pathway. ENaC is synthesised at the ER and processed through the Golgi before being exocytosed from the TGN to the apical membrane to function in Na⁺ absorption. ENaC endocytosis involves the channel being tagged with ubiquitin and endocytosed into clathrin-coated vesicles where it is trafficked to early endosomes. The channel can then be degraded by lysosomes or recycled back to the cell surface. The retromer complex is hypothesised to be involved in this recycling process.

As the cargo containing vesicles reach the apical surface, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNAREs) proteins such as Syntaxin 3, VAMP7 and 8, and SNAP-23 facilitate the docking and fusion of the vesicle with the epithelial membrane (Kawanishi et al., 2000; Rodriguez-Boulan et al., 2005). In short, target SNAREs (t-SNAREs) and vesicle SNAREs (v-SNAREs) bring vesicles and the plasma membranes close together initiating their fusion (Ramakrishnan et al., 2012). The t-SNARE Syntaxin 1A has been reported to play
a key role in ENaC exocytosis with Syntaxin 1A influencing ENaC membrane population and channel $P_o$ (Qi et al., 1999; Condliffe et al., 2003; Condliffe et al., 2004).

### 1.8.2 ENaC Endocytosis

At the apical surface ENaC functions as the rate-limiting step for Na$^+$ reabsorption across epithelia. Turnover of ENaC has been demonstrated to occur rapidly with a reported half-life of $\sim 40 – 120$ min in cultured cells (Shimkets et al., 1997; Staub et al., 1997; Weisz et al., 2000). The activity of ENaC can be further regulated by channel activating proteases, which function in cleaving ENaC subunit extracellular loops, increasing channel $P_o$, thus increasing Na$^+$ transport (Vuagniaux et al., 2002). To down regulate Na$^+$ transport, ENaC needs to either be inactivated (e.g., gated by inhibitors) or active channels must be internalised from the apical membrane.

ENaC endocytosis involves interaction with the E3 ubiquitin ligase NEDD4-2, where NEDD4-2 WW domains bind to proline rich PY motifs in the C-terminal domains of ENaC subunits, facilitating the multi-ubiquitination of lysine residues in the N-terminal domain of the subunits (Figure 1.5) (Staub et al., 1997; Wiemuth et al., 2007; Kabra et al., 2008). Evidence supporting this interaction was given when mutated lysines in N-terminal $\alpha$- and $\gamma$- ENaC subunits prolonged ENaC half-life due to apical retention of the channels (Hansson et al., 1995; Debonneville et al., 2001). Additionally, overexpression of NEDD4-2 in cellular expression systems as well as mutagenesis studies within the PY motif, led to prolonged ENaC surface half-life and increased Na$^+$ transport (Goulet et al., 1998; Malik et al., 2005; Zhou et al., 2007).

ENaC endocytosis involves internalisation into clathrin-coated vesicles where ENaC ubiquitination allows for the recruitment of epsin, which binds to ubiquitinated ENaC (Wang
et al., 2006). In parallel or sequentially, an adaptor complex can bind to an ENaC endocytosis motif, YXXL, together triggering the formation of clathrin-coated vesicles (Shimkets et al., 1997; Conner & Schmid, 2003). Once endocytosed, vesicles are transported to early endosomes where the fate of the protein is decided (Figure 1.4).

**Figure 1.5. Ubiquitination of ENaC by NEDD4-2.** Removal of ENaC from the plasma membrane requires the channel to be ‘tagged’ with ubiquitin (Ub). WW domains of the E3 ubiquitin-protein ligase NEDD4-2 bind to C-terminal PY motifs of ENaC subunits, allowing for the multi-ubiquitination of lysine residues on the subunit N-terminal domains.
1.8.3 ENaC Recycling

As the endocytosed vesicles reach the early endosomes the fate of the protein is decided where the endocytosed protein can be sent to late endosomes and then onto lysosomes for degradation (Figure 1.4) (Raiborg et al., 2003), or proteins can also be recycled back to the apical membrane either directly, through recycling compartments or via the TGN (Grant & Donaldson, 2009). In order for ENaC to divert from a degradative fate, deubiquitination of ENaC via deubiquitinating enzymes such as UCH-L3, and USP-45 is required (Butterworth et al., 2007; Fakitsas et al., 2007). The removal of ubiquitin from ENaC allows for the channel to enter a recycling pathway, however the exact mechanism and pathway for ENaC’s recycling are not yet fully understood as not all proteins involved have been defined.

Despite ENaC recycling not yet being entirely understood, it is hypothesised this process is facilitated by interactions with members of the small GTP binding Rab protein family. A number of epithelial channels including transient receptor potential channels and CFTR, have previously been shown to be regulated by Rab proteins (Cayouette & Boulay, 2007; Swiatecka-Urban et al., 2007). Several studies have also demonstrated involvement of Rab 4, 11, and 27 in fast and slow ENaC regulation (Saxena et al., 2006a; Saxena et al., 2006b; Karpushev et al., 2008; Butterworth et al., 2012). Another relatively recently described protein complex implicated in endosomal sorting and recycling is the retromer complex which binds specific cargos directing them into membrane bound tubules to direct back towards the cell surface. The retromer complex and its associated proteins will be discussed further in the following sections.
1.9 The Retromer Recycling Complex

The retromer complex is a conserved protein complex that localises to endosomes where it acts in mediating protein sorting and trafficking, along with rescuing proteins from a degradative fate through recycling back to the TGN or plasma membrane (Reviewed in Seaman, 2012). A wide variety of retromer cargoes have been identified, including the cation independent mannose 6-phosphate receptor (CI-MPR) (Seaman, 2004), the iron transporter DMT1 (Tabuchi et al., 2010), the Wnt (Wingless/Integrated) transport protein Wntless (Belenkaya et al., 2008), Crumbs (Pocha et al., 2011), and the sortilin-related receptor (SORL1) (Fjorback et al., 2012). The requirement of retromer-mediated delivery and rescue of various cargo proteins links retromer to fundamental cellular processes with reduced retromer function leading to several pathologies such as Alzheimer’s disease and Parkinson’s diseases (Small et al., 2005; Follett et al., 2014; Small & Petsko, 2015).

Retromer was first identified in the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) where it was recognised as being vital for the endosome-to-Golgi retrieval of the receptor vacuolar protein sorting 10 protein (VPS10); a transmembrane sorting receptor that shuttles between the early endosomes and the TGN (Seaman et al., 1997). Retromer was originally thought to be involved with endosome to Golgi retrieval of proteins; however, it is now recognised to play a larger role beyond this including endosome-to-plasma membrane sorting and regulation of trafficking events. A close parallel between yeast retromer subunits and its human orthologs has been identified, suggesting retromer has a conserved function (Haft et al., 2000).

Since retromer was identified as a regulator of endosomal protein sorting and trafficking, understanding of the molecular mechanisms underlying retromer-mediated trafficking has
progressed significantly (Reviewed in McNally & Cullen, 2018). Cargo sorting by retromer is hypothesised to occur in four stages, first beginning with the endosomal membrane recruitment of retromer, followed by cargo binding by retromer. Endosomal membrane deformation and tubulation then follows, driven by the SNX-BAR heterodimer (Section 1.9.5) and actin network polymerisation triggered by the WASH complex (Section 1.9.8). Finally, via actin filaments the resulting cargo-enriched tubules bud from the endosomes and are trafficked to either the plasma membrane or the TGN. The core retromer complex along with its various associated proteins will be discussed further in the following sections.

1.9.1 Yeast Retromer Structure

As mentioned previously, the retromer complex was first identified in _S. cerevisiae_ through genetic screens for genes involved in endosome-to-Golgi retrieval of VPS10 (Seaman et al., 1997). It was characterised as an endosomal coat complex that serves both cargo recognition and membrane deformation functions essential for sorting and trafficking of transmembrane proteins (Seaman et al., 1998). The complex, as characterised in yeast, comprises five proteins that are all encoded by vacuole protein sorting (VPS) genes (Seaman et al., 1997; Seaman et al., 1998). Yeast retromer is described to have a heteropentameric assembly that can be biochemically and phenotypically divided into two subcomplexes: a trimer of VPS35, VPS29 and VPS26 and a dimer of VPS5 with VPS17 (Horazdovsky et al., 1997; Seaman et al., 1998). The VPS5 and VPS17 proteins are members of the sorting nexin (SNX) family and will be referred to as the SNX-BAR heterodimer.

The VPS35-VPS29-VPS26 heterotrimer, is conserved across all eukaryotes and has been shown to interact with various cargo proteins and was hence termed the cargo-recognition complex (CRC) (Nothwehr et al., 1999; Haft et al., 2000; Arighi et al., 2004; Seaman, 2004; Fjorback et al., 2012). A loss of any one of these CRC components can eliminate retromer function, which
is why the CRC is considered as the ‘core’ element of the retromer complex (Seaman, 2004).
In yeast, the SNX heterodimer was discovered to tightly associate with the CRC to form a stable, biochemically isolatable complex (Seaman et al., 1998; Seaman & Williams, 2002), however, in higher eukaryotes, the interaction between the CRC and SNX proteins is observed to be much weaker and more transient (Section 1.9.2) (Harbour & Seaman, 2011; Swarbrick et al., 2011).

1.9.2 Mammalian Retromer Structure

Mammalian retromer displays more variability in composition when compared to the yeast structure. The yeast VPS5 and VPS17 genes have diversified where SNX1 and SNX2 are the mammalian homologues of the VPS5 gene, and, SNX5 and SNX6 are the mammalian homologues of the VPS17 gene. Any combination of a SNX1 or SNX2 protein associating with a SNX5 or SNX6 protein can form the heterodimeric SNX-BAR subcomplex (Figure 1.6) (Rojas et al., 2007; Wassmer et al., 2007). Variability between yeast and mammalian retromer is also observed with the VPS26 protein; mammalian cells contain two paralogues of the protein; VPS26A and VPS26B. VPS26A and VPS26B are both similar in size, sequence (~80% homology), and functionality; however, they exhibit specificity as they bind to distinct retromer cargo (Kerr et al., 2005; Bugarcic et al., 2011). Interestingly, it has been reported that in mammalian cells another member of the SNX protein family, SNX3, is also involved in mammalian retromer function where a stronger CRC-binding ability with SNX3 over SNX5 has been observed, suggesting that the interaction between the CRC and SNX proteins may vary to regulate different biological processes (Section 1.9.6) (Harterink et al., 2011).
Figure 1.6. Structure of the Retromer Complex. Retromer is composed of the VPS26-VPS29-VPS35 heterotrimer and the SNX-BAR heterodimer. Retromer also interacts with other protein complexes, including the Wiskott-Aldrich syndrome protein and scar homologue (WASH) protein complex and the CCC complex composed of COMM Domain-containing Protein 1-10 (COMMD1-10), Coiled-Coil Domain Containing 22 (CCDCC22) and Coiled-Coil Domain Containing 93 (CCDCC93).

1.9.3 The Cargo-recognition Complex (CRC)

As mentioned previously, the VPS35-VPS29-VPS26 CRC; a conserved protein complex making up the core of retromer (Norwood et al., 2011), has a primary role in rescuing cargo from lysosome-mediated degradation (Kerr et al., 2005; Bugarcic et al., 2011). It is currently hypothesised that this process involves the capture of specific cargo by the CRC, however, the
exact mechanisms of how this process occurs are currently unknown. One theory involves the capture and concentration of cargos via a multivalent binding site on CRC proteins. After selection by the CRC, the cargo is directed into tubules of high relative membrane curvature driven by interactions involving the Bin/amphiphysin/Rvs (BAR) domain of the SNX-BAR heterodimer proteins (Section 1.9.5) (Carlton et al., 2004).

1.9.4 Sorting Nexin (SNX) Proteins

The retromer CRC subcomplex is described as essential to retromer function, however, SNX proteins have also been demonstrated to play an important role in both selection of specific cargoes and overall retromer function. Retromer associated SNX proteins are primarily involved in the formation of a ‘tubulation complex’ that is responsible for the remodeling of the endosomal membrane leading to the formation of tubule-shaped extensions which contain the cargo selected for recycling (Peter et al., 2004; Pylypenko et al., 2007; Frost et al., 2009).

Currently there are 33 mammalian SNX proteins identified, with the hallmark of this family of proteins being a conserved specific Phox homology (PX) domain; the SNX–PX domain (Figure 1.7) (Teasdale & Collins, 2012). The SNX–PX domain associates with phosphatidylinositol-enriched early endosomes, with the most common phosphatidylinositol being phosphatidylinositol 3-phosphate (PI3P) (Gillooly et al., 2000). This binding of the SNX-PX domain to PI3P acts as an ‘anchor’ to the endosomal membrane allowing for curvature of the membrane (Seaman & Williams, 2002; Griffin et al., 2005). The interaction between PI3P and the endosomal membrane is essential to the function of SNX proteins, this is evident as N-terminal truncations of either VPS5 or VPS17 (Yeast homologues of mammalian SNX1/2 and
SNX5/6 proteins) was observed to lead to mis-localisation of vacuolar proteases such as carboxypeptidase Y (CPY) (Song et al., 2001; Seaman & Williams, 2002).

In addition to the SNX-PX domain, SNX proteins often possess other domains, including BAR and FERM (protein 4.1/ezrin/radixin/moesin) domains (Teasdale & Collins, 2012). The presence of these additional domains allow SNX proteins to be divided into five subfamilies: SNX-PX, SNX-BAR, SNX-FERM, SNX-PXA-RGS-PXC and SNX-MIT (Teasdale & Collins, 2012) (Figure 1.7). Several members from three of these subfamilies including: SNX3 of the SNX-PX subfamily, SNX1/2/5/6 of the SNX-BAR subfamily, and SNX27/SNX17 of the SNX-FERM subfamily, have been identified to associate with retromer or the recently described retriever complex (Section 1.9.7), to mediate distinct endosomal recycling pathways.

**Figure 1.7. Domain Organisation of Retromer/Retriever-Associated SNX Proteins.** All SNX proteins contain a conserved PX domain. Members of the SNX-BAR subfamily also possess a BAR domain, whereas SNX-FERM family members possess a FERM domain. SNX27 of the SNX-FERM subfamily also possesses a unique N-terminal PDZ domain, and in comparison, SNX17 has a unique C-terminal tail (Represented in Purple). Figure modified from Wang et al., (2018).
1.9.5 SNX-BAR Subfamily

There is a subfamily of SNX proteins known as the SNX-BAR subfamily that in addition to the SNX-PX domain, encompass a C-terminal BAR domain (Figure 1.7). This SNX-BAR domain is responsible for driving two corresponding processes in the retromer complex. The first process involves the dimerization between SNX proteins where a SNX-BAR domain containing three α-helices, dimerises with another SNX-BAR domain leading to the formation of a positively charged rigid crescent-shaped structure known as a SNX-BAR heterodimer (Peter et al., 2004). In mammalian retromer this SNX-BAR heterodimer is comprised of a SNX1 or SNX2 protein associating with a SNX5 or SNX6 protein (Figure 1.8 A). The concave surface generated by the SNX-BAR dimer bears basic residues that associate with curved membranes through electrostatic interactions essentially ‘sensing’ membrane curvature (Peter et al., 2004).

Secondly, the SNX-BAR heterodimer is also capable of inducing endosomal membrane remodelling, leading to the formation of membrane tubules. Both SNX1 and SNX2 proteins contain amphipathic helices amino-terminal to their BAR domains that promote formation of surface tension between leaflets of the bilayer which the membrane accommodates by generating positive curvature (Peter et al., 2004). Therefore, by forming higher order helical arrays, BAR domains are believed to drive membrane curvature and stabilise high curvature membranes (Frost et al., 2008).

Furthermore, the SNX5 and SNX6 proteins have been identified in linking retromer carriers to the microtubule cytoskeleton allowing for the transport of carriers containing retromer cargo along microtubules (Hong et al., 2009; Wassmer et al., 2009). The CRC is capable of binding to the N-terminal sequence of SNX-BAR proteins, however, outside of its interaction with the SNX heterodimer, the CRC lacks the ability to influence membrane curvature. This
demonstrates that although the CRC is viewed as the central element of the retromer complex, the SNX heterodimer is also necessary to retromer function (Seaman et al., 1998).

However, as mentioned previously (Section 1.9.1), the yeast SNX–BAR heterodimer interacts strongly with the CRC heterotrimeric subcomplex, although in mammals this interaction appears to be less robust. It has been reported that mammalian SNX1 binds to VPS35 and to VPS29 (Seaman, 2004; Temkin et al., 2011), but these interactions have not been

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**Figure 1.8. SNX Protein Retromer and Retriever Models.** (A) The SNX1/2-5/6 SNX-BAR proteins come together to form the SNX-BAR heterodimer. (B,C) Both SNX3 and SNX27 bind to the VPS heterotrimer (VPS26-VPS35-VPS29). (D) SNX17 associates with retriever, compromised of the VPS26C-VPS35L-VPS29 subcomplex. All SNX-retromer/retriever models are able to bind cargo. Figure modified from Wang et al., (2018).
demonstrated with co-immunoprecipitation experiments, but have been suggested by mass spectrometric data. Given the apparently weak association of the SNX–BAR heterodimer with the mammalian CRC subcomplex and the large number of additional proteins that interact with mammalian retromer compared with yeast retromer, the SNX–BAR subcomplex is not regarded as a ‘core’ component of the mammalian retromer complex, despite its necessity in membrane curvature.

1.9.6 SNX3

Though the SNX–BAR heterodimer can bind PI3P in the endosome membrane, retromer itself does not have intrinsic membrane-binding ability, rather, targeting of retromer to early endosomes occurs, in part, through its interaction with SNX3, another member of the SNX–PX protein family (Xu et al., 2001; Pons et al., 2008). SNX3, a small protein of roughly 100 amino acids, does not contain a BAR domain rather only bearing a single characteristic SNX–PX domain (Figure 1.7). The cellular localisation of SNX3 is principally determined by the specific interaction of its SNX-PX domain with PI3P, which was demonstrated when mutations within the conserved PI3P pocket of SNX3 abolished membrane binding and compromised its endosomal functions (Xu et al., 2001; Pons et al., 2008).

It was recently identified that SNX3 binds directly to retromer (Figure 1.8 B), where a crystal structure of the VPS35-VPS26-SNX3-DMT1 tail complex revealed that SNX3 binds at the interface of VPS35 and VPS26 (Harrison et al., 2014; Lucas et al., 2016). Upon binding to SNX3, VPS26 undergoes a conformational change in its cargo-binding motif, allowing for the recognition of the Divalent metal transporter 1 (DMT1) tail by both VPS26 and SNX3 (Harrison et al., 2014; Lucas et al., 2016). Therefore, this provides evidence for the SNX3-retromer model in integrating membrane binding and cargo recognition.
In addition to recruitment of retromer to the endosomal membrane, there is also evidence supporting a role for SNX3 in endosomal recycling of WLS (Wnt ligand secretion mediator) to the TGN (Harterink et al., 2011). Wnts are delivered to the plasma membrane bound to the transmembrane G-protein coupled receptor WLS, which then releases Wnt into the extracellular environment. Unbound WLS is trafficked to endosomes and recycled back to the TGN to bind more Wnt for a further round of delivery to the plasma membrane (Banziger et al., 2006; Bartscherer et al., 2006). It has been demonstrated that retrograde trafficking of WLS depends on both retromer and SNX3, with this process not being dependent on the SNX-BAR subcomplex (Belenkaya et al., 2008; Franch-Marro et al., 2008; Harterink et al., 2011). This supports the hypothesis of different retromer models mediating distinct trafficking pathways from endosomes to the TGN; e.g., the SNX3–retromer model transporting WLS and the SNX–BAR–retromer pathway transporting, for example CI-MPR (Kvainickas et al., 2017). It is important to note that SNX3 does not physically link WLS to retromer which is why SNX3 is not regarded as a core retromer cargo binding protein. Nonetheless, the difference in trafficking of WLS via SNX3 compared to the mammalian SNX-BAR retromer supports the concept of the SNX–BAR heterodimer as just one of a number of accessory proteins to mammalian retromer, rather than a core component of the complex.

1.9.7 The SNX-FERM Subfamily and the Retriever Recycling Complex

The SNX-FERM subfamily, of the SNX protein family, contains two SNX proteins, SNX17 and SNX27 which have been shown to be significant in the recycling of a number of cell surface proteins (Temkin et al., 2011; Yin et al., 2012; Lin & Lai, 2015). SNX-FERM subfamily members contain the SNX-PX domain, along with a FERM domain (Figure 1.7), which recognises NPXY/NXXY motifs found in a variety of proteins including growth factor receptors, solute carriers and integrins (Ghai et al., 2013).
SNX27 contains a unique postsynaptic density 95/disc large/zona occludens (PDZ) domain at its N-terminus (Figure 1.7) which binds directly to the retromer protein; VPS26 (Figure 1.8 C), along with cargo proteins containing PDZ-binding motifs such as the glucose transporter GLUT1 (Temkin et al., 2011; Steinberg et al., 2013; Gallon et al., 2014). Therefore SNX27 is described as mediating cargo recycling through a retromer-dependent manner. It was hypothesised that ENaC may be a SNX27 cargo, however, in house studies have shown no effect on ENaC current in SNX27 siRNA knockdown studies (Cheung and McDonald, unpublished).

In comparison to SNX27, SNX17 proteins do not contain a PDZ domain, but instead possess a unique C-terminal polypeptide sequence (Figure 1.7). It has been known for many years that SNX17 mediates cargo retrieval from the lysosomal degradation pathway in a retromer-independent manner (Stockinger et al., 2002; Steinberg et al., 2012); however, the exact proteins that functions alongside SNX17 in this process were largely unknown. Through a series of proteomic and functional studies McNally et al., (2017), uncovered that SNX17 utilises its C-terminal polypeptide sequence to interact with DSCR3, along with C16orf62, and VPS29, among other proteins. C16orf62 contains α-helical repeats, a structural feature present in its distant homolog VPS35, and has thus been termed VPS35L (Hierro et al., 2007; Lucas et al., 2016; McNally et al., 2017). DSCR3, is predicted to have an arrestin-like fold analogous to VPS26, and has, therefore, been named VPS26C (Hierro et al., 2007; McNally et al., 2017). VPS35L, VPS26C and VPS29 come together to form a stable trimeric complex similar to that of the retromer VPS35-VPS29-VPS26 heterotrimer (McNally et al., 2017) (Figure 1.8 D). Interestingly, knockdown or deletion of the retromer VPS35 protein has been observed to result in a substantial loss of VPS26, while in comparison, retriever VPS35L knockdown results in depletion of VPS26C (McNally et al., 2017). This new complex has been named ‘retriever’,
and similar to retromer, retriever has also been demonstrated to associate with endosomes and is essential for the recycling of multiple cargoes (McNally et al., 2017). Notably, the loss of the retriever components VPS35L and VPS26C resulted in defective retrieval of SNX17 cargo from the endosome (Phillips-Krawczak et al., 2015; McNally et al., 2017).

The interaction of retriever with SNX17 is essential for cargo selection, specifically, the C-terminal domain of SNX17 is necessary and sufficient for association with retriever. However, interestingly this interaction is not required for retriever-endosomal association as depletion of SNX17 did not affect recruitment of retriever to the membrane (McNally et al., 2017). Instead, endosomal recruitment of retriever requires a functional CCC complex (Section 1.9.9).

1.9.8 WASH Complex

Both retromer and retriever interact with accessory proteins such as the Wiskott-Aldrich syndrome protein and scar homologue (WASH) protein complex. WASH is formed from the assembly of five proteins: strumpellin, FAM21, SWIP, ccdc53, and WASH (Harbour et al., 2012). On the endosomal membrane, the WASH complex activates the Arp2/3 complex, facilitating the nucleation of actin branching to a pre-existing actin filament (Rotty et al., 2013). Sorting of proteins into the correct transport pathways from endosomes requires actin, as inhibition of actin polymerisation was demonstrated to lead to defective segregation of epidermal growth factor (EGF) and transferrin (Tf) receptors into both the recycling and degradation pathways (Ohashi et al., 2011). It has also been determined that the WASH complex is required for retromer-mediated trafficking from the endosome to both the TGN and plasma membrane (Gomez & Billadeau, 2009; Temkin et al., 2011).
Retromer recruits the WASH complex to endosomes through an interaction between the FAM21 subunit with VPS35 (Figure 1.6) (Harbour et al., 2010). The C-terminal region of FAM21 possesses 21 copies of a motif distributed over almost 1000 unstructured amino acids that is recognised by VPS35 (Jia et al., 2012). This interaction was found to occur in vivo only when VPS35 was associated with VPS29, indicating that the WASH complex only associates with a functional retromer complex (Helfer et al., 2013).

As stated previously, the retromer subunit VPS35 binds to FAM21, with this interaction being regarded as essential in recruitment of the WASH complex to endosomes (Harbour et al., 2012; Jia et al., 2012). However, in a VPS35-KO cell line, it has been shown that a significant proportion of FAM21 remains associated with the endosomal network, suggesting that the endosomal association of FAM21–WASH could be mediated through retromer-dependent and retromer-independent mechanisms (McNally et al., 2017). This conclusion is supported with work in Dictyostelium describing the retromer-independent recruitment of the WASH complex (Yin et al., 2012).

The CCC complex (Section 1.9.9), another retromer associated protein complex, does not associate directly with retromer or endosomes, instead interacting with the FAM21 subunit of the WASH. Now with the identification of retriever, and data demonstrating the interaction of retriever with FAM21 through the CCC complex, this retromer-independent recruitment of the WASH complex is hypothesised to occur through retriever (Phillips-Krawczak et al., 2015). The WASH-FAM21-CCC affiliation is essential for the localisation of retriever to endosomes, and importantly, identification of the WASH-FAM21-CCC-retriever-SNX17 pathway provides a potential mechanism to account for several studies which have demonstrated a role for the WASH complex in recycling cargoes known to be retromer-independent (Bergant Marusic et
al., 2012; Steinberg et al., 2012; Yin et al., 2012). The establishment of functionally distinct retriever-WASH and retromer-WASH pathways serves to further highlight the diversity that retromer/retriever and their associated accessory proteins play in the regulation and trafficking of proteins within the endosomal network.

1.9.9 The CCC Complex and COMMD Proteins

The COMMD/CCDC22/CCDC93 (CCC) complex has been identified to interact and colocalise with both retromer/retriever and the WASH complex at the endosomal membrane (Harbour et al., 2012; Freeman et al., 2014; Phillips-Krawczak et al., 2015). The CCC complex consists of copper metabolism MURR1 domain-containing (COMMD) proteins, coiled-coil domain-containing protein 22 (CCDC22) and coiled-coil domain-containing protein 93 (CCDC93) (Figure 1.6) (Phillips-Krawczak et al., 2015).

Among the 10 COMMD proteins (Burstein et al., 2005), COMMD1 (COMM Domain-containing Protein 1), a gene product that is mutated in Bedlington terriers affected by a hepatic copper storage disorder resulting in copper toxicosis (van De Sluis et al., 2002), has been shown to regulate the recycling of the copper transporter ATP7A (Phillips-Krawczak et al., 2015). COMMD1 is able to interact with the retromer/retriever associated CCC complex and thus this suggests a role for COMMD1 in trafficking of cargo to and from the plasma membrane (Phillips-Krawczak et al., 2015; Bartuzi et al., 2016).

Importantly for this project, COMMD1 binds to α-, β- and γ- ENaC subunits and co-expression of COMMD1 with ENaC results in reduced sodium current, suggesting a reduction in ENaC in the plasma membrane (Biasio et al., 2004; Ke et al., 2010). Subsequent studies have shown that COMMD1 decreases ENaC numbers at the plasma membrane and increases the level of
ENaC ubiquitination (Ke et al., 2010; Chang et al., 2011), and that all COMMD family members are able to co-immunoprecipitate with ENaC (Liu et al., 2013). Whether ENaC recycling is determined through COMMD1-mediated retromer/retriever is currently unknown and requires further investigation.

1.10 Aim, Objectives and Hypothesis

The primary aim of my 400-level research project investigated whether there was a role for retromer in ENaC recycling, focusing specifically on the SNX-BAR proteins; SNX1 and SNX2, which form one half of the SNX-BAR heterodimer. Using a siRNA knockdown approach in FRT epithelia, an individual knockdown of SNX1 and SNX2 reduced ENaC current, suggesting both of these proteins, and therefore, retromer are important for ENaC recycling. However, when SNX1 and SNX2 were knocked down together preventing formation of the SNX-BAR heterodimer, ENaC current reduced to a similar value of the individual SNX1 and SNX2 protein knockdowns. This indicated the involvement of other SNX proteins in ENaC recycling, whether it be compensation for compromised SNX-BAR proteins or another pathway working alongside SNX-BAR mediated recycling. When searching for retromer associated SNX proteins which associate with retromer indirectly to the SNX-BAR heterodimer, SNX3 and SNX17 stood out due to their role in the recycling of a wide variety of membrane proteins, providing the rationale for this project.

1.10.1 Aims

The aim of my project was to determine whether SNX3-retromer or SNX17-retriever play a role in the ENaC recycling pathway.
1.10.2 Hypothesis

Based on the previous studies I have conducted as part of my 400-level project and the evidence surrounding the importance of SNX3 and SNX17 in the trafficking of a wide variety of membrane proteins, I hypothesised that a siRNA knockdown of SNX3 and/or SNX17 would reduce ENaC current and cell surface population, therefore, highlighting the importance of retromer and/or retriever in ENaC recycling.

1.10.3 Objectives

1. **Knockdown SNX3 and SNX17 protein level in FRT and mCCD cells.**
   To examine if SNX3 or SNX17 and their associated recycling complexes have a role in ENaC recycling, siRNA knockdown of both SNX proteins was required. Therefore, FRT and mCCD cells were transiently transfected with SNX3 or SNX17 siRNA to reduce protein level. Successful protein knockdown of SNX3 and SNX17 was examined using Western blot.

2. **Determine the functional effect of a SNX3 and SNX17 siRNA knockdown on ENaC recycling in FRT and mCCD epithelia through electrophysiological measurements.**
   Ussing Chamber assays were conducted using both FRT and mCCD epithelial cell lines in order to measure if there was an effect on ENaC current in the presence of a SNX3 or SNX17 siRNA knockdown.

3. **Cell surface protein biotinylation assays to determine if SNX3 and SNX17 knockdown decreases cell surface population of ENaC.**
   Cell surface protein biotinylation assays were used to quantify the ENaC cell surface population in the presence of a SNX3 or SNX17 siRNA knockdown, to determine if changes in ENaC current were the result of changes in ENaC cell surface population.
4. Investigate whether SNX3 and SNX17 proteins can act as cargo binding proteins for ENaC using co-immunoprecipitation experiments.

Co-immunoprecipitation experiments between SNX3 or SNX17 and ENaC were performed to determine whether protein-protein interactions exist between these SNX proteins and ENaC.
2. Methods
2.1 Mammalian Cell-Culture

All techniques involving cell maintenance and cell culture, including seeding cells for experimental set up, were completed in a Physical Containment Level 2 (PC2) laboratory using a laminar flow hood (Labconco Purifier Biological Safety cabinet) to maintain a sterile environment, in order to prevent microbial growth and infection. Three mammalian cell lines have been used in this study; FRT, HEK-293 and mCCDcl1 (described further below). All cell lines were grown at 37°C with 5% CO\textsubscript{2} in a humidified cell culture incubator (Forma Series II Water Jacketed CO\textsubscript{2} Incubator, Thermal Electric Co.).

2.1.1 Fischer Rat Thyroid (FRT)

Fischer Rat Thyroid (FRT) is a mammalian epithelial cell line derived from the Fischer rat thyroid gland cell line (Zurzolo et al., 1991; Ambesi-Impiombato et al., 1993). When grown on permeable filter supports, FRT cells form polarised epithelia with a high transepithelial resistance (Sheppard et al., 1994). For this study FRT epithelia was used to determine the effects of a siRNA knockdown of SNX3 and SNX17 proteins on ENaC cell surface population and current.

FRT cells were maintained in full Ham’s F12 nutrient mixture (F-12 media) (Sigma Aldrich, Cat No. F6636-IL, Auckland, New Zealand) supplemented with 10% Foetal Bovine Serum (FBS) (Gibco, Cat No. 16091-148, USA) and 1% penicillin-streptomycin sulphate (Pen/Strep) (final concentration 100 μg/mL) (Gibco, Cat No. 15140-122).
2.1.2 Mouse Cortical Collecting Duct (mCCDcl1)

mCCDcl1 (mCCD) is a mouse cortical collecting duct cell line that exhibits many phenotypic properties of the kidney cortical collecting duct. These properties include being an example of tight absorbing epithelia along with endogenous expression of ENaC, and therefore, the mCCD cell line can be used as a model to study mechanisms of kidney ion transport. This mCCDcl1 cell line was kindly provided by E. Hummler, Universite de Lausanna, Switzerland (Gaeggeler et al., 2005). In this study the mCCD cell line was used to examine ENaC cell surface population and current measurements in the presence of a SNX3 and SNX17 siRNA knockdown.

mCCD cells were maintained in DMEM:F12 (Sigma Aldrich, Cat No. D2906-10L) supplemented with 10% FBS, Insulin-Transferrin-Selenium (ITS-G) (Gibco, Cat No. 100x 41400045), dexamethasone (Sigma Aldrich, New Zealand), T3 (3,3',5-Triiodo-L-thyronine sodium salt) (Sigma Aldrich, Cat No. T5516-1MG), Epidermal Growth Factor (EGF recombinant mouse protein) (Life Technologies, Cat No. PMG8043, New Zealand), Glutamax (100x) (Gibco, New Zealand) and 1% Pen/Strep (final concentration 100 μg/mL).

2.1.3 Human Embryo Kidney 293 (HEK-293)

HEK-293 are a cell line derived from human embryonic kidney (Shaw et al., 2002) and were maintained in DMEM (Dulbecco’s Modified Eagle Medium) (Gibco, Cat No. 12800-017, Australia) supplemented with 3.7 g/L sodium bicarbonate (NaHCO₃), 10% FBS and 1% Pen/Strep (final concentration 100 μg/mL). In this project, HEK-293 cells were used to perform co-immunoprecipitation experiments to detect the presence of protein-protein interactions between ENaC and either the SNX3 or SNX17 proteins.
2.1.4 Maintenance and Passaging of Cell Lines.

All cell stocks were grown in 60 mm² diameter culture dishes and were passaged when they reached ~90-95% confluency. Old growth media was removed by aspiration and cells were washed with sterile pre-warmed phosphate buffered saline (PBS) (Sigma, Cat No. P4417). PBS was removed via aspiration before the cells were incubated at 37°C with 2 mL trypsin-EDTA (0.25% trypsin, 1mM EDTA) (Gibco, Cat No. 25206-56) to dislodge the cells from the bottom of the plate. Incubation time with trypsin varied from 5-15 min depending on the cell line (~5 min for HEK-293 cells, ~15 min for FRT and mCCD cells). The appropriate culture medium (4 mL) was then added to the dish to neutralise the trypsin activity. Cells in suspension were centrifuged in a 1.5 mL microcentrifuge tube (Multimix, BioExpress, Cat No. 405757-X23018) for 5 min at 2000 r.p.m, in a bench top centrifuge (Eppendorf centrifuge 5424, Germany), to recover the cells. Cells were resuspended in culture medium and seeded at a ratio of 1:4 (FRT, HEK-293) to 1:10 (mCCD) depending on the cell line being passaged. The new stock plate was gently swirled to ensure cells were dispersed evenly before being placed back in the 37°C (5% CO₂, 95% O₂) incubator.

2.1.5 Freeze and Thaw Liquid Nitrogen Stocks of Cells

For long term storage of cell stocks, they were kept in a -80°C freezer or a liquid nitrogen reservoir. Cells on culture plates to be processed for freezing were washed with PBS and trypsinised as with normal passaging of cells (Section 2.1.4) in order to detach cells from the culture plate. Cells were then centrifuged in a bench top centrifuge for 3 min at 2000 r.p.m, with the cell pellet being resuspended in freezing medium (full growth medium plus 10% DMSO (dimethylsulfoxide)). Cells were aliquoted into cryo-vials and placed in a freezing box filled with isopropanol at -80°C to keep the cells from freezing too fast. Cryo-vials of cells were
placed in boxes and stored in a freezer box in the -80°C freezer or a liquid nitrogen reservoir for long-term storage.

When retrieving cells from the -80°C freezer or liquid nitrogen, the cryo-vial of cells was removed and placed in a water bath (37°C) for rapid thawing. The cells were gently pipetted into 8 mL of warm growth medium in a 15 mL centrifuge tube. Cells were then centrifuged in a benchtop centrifuge for 3 min at 2000 r.p.m and the supernatant (media/DMSO) was removed by aspiration. The pellet of cells was resuspended with 1 mL fresh growth medium and transferred to a 35 mm² plate containing 1 mL warm full growth media and kept in a 37°C (5% CO₂, 95% O₂) cell culture incubator. The following day if many dead cells were present, media was aspirated and changed to fresh growth medium. Cells underwent two passages before they were used for any experiments involving transfection.

2.2 siRNA Interference and Transient Transfection

RNA interference is a post-transcriptional, highly conserved process in eukaryotes that leads to specific gene silencing through degradation of the target mRNA (Reviewed in Stanislawska & Olszewski, 2005). This project utilised RNA interference where silencing RNA sequences (siRNA) were introduced into FRT and mCCD cell lines in order to decrease target protein gene level. The utilisation of siRNA allows for inhibition of mRNA translation through degrading the target gene mRNA after transcription; however, it also requires multiple transfections along with the risk of poor or incomplete transfections.

Transfection is a process whereby foreign DNA/RNA is introduced into cells to produce genetically modified cells. Transient transfection involves introducing DNA/RNA for a brief period of time as the introduced nucleic acids are lost after cell division as the DNA/RNA does
not integrate into the cell’s chromosomes. In this study Lipofectamine™ 3000 (Life Technologies, Invitrogen 3000 Transfection Kit, Cat No. L3000-015, USA) was used to transiently transfect the FRT, HEK-293 and mCCD cell lines with siRNA and/or plasmids.

siRNAs used in knockdown experiments along with the specific mRNA sequence they are designed to target are described in Table 1.

Table 1. siRNA Used in Knockdown Experiments.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Batch #</th>
<th>Manufacturer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat siControl</td>
<td>WD06160117</td>
<td>Sigma-Aldrich</td>
<td>UGGAGUGAAUACCACGACGAU</td>
</tr>
<tr>
<td>Rat siSNX3</td>
<td>WD06704264</td>
<td>Sigma-Aldrich</td>
<td>GACUUUGAGUGCUUCCGAA</td>
</tr>
<tr>
<td>Rat siSNX17</td>
<td>WD06269620</td>
<td>Sigma-Aldrich</td>
<td>CAUGCAACGUGUUCGGCAA</td>
</tr>
</tbody>
</table>

Before transfection FRT, mCCD and HEK-293 cells were seeded at a density of 4.5 x 10⁵ cells onto either 35 mm² culture plates (Western blot, Section 2.3) or onto the apical side of the membrane of Snapwells™ (Costar Snapwells, Cat No. 3801) (Ussing chamber, Section 2.4), or 5.5 x 10⁵ cells onto 60 mm² culture plates (cell surface protein biotinylation assay, Section 2.5 and Co-IP, Section 2.6) and incubated overnight at 37°C (5% CO₂, 95% O₂) in order for the cells to reach approximately 50% confluency at the time of transfection.

The following day, for one 35 mm² culture plate or Snapwell™, 3 µL Lipofectamine™ 3000 was added to 50 µL serum free (SF) media (F12 + 1% Pen/Strep for FRT or DMEM:F12 + 1% Pen/Strep for mCCD/HEK-293) (Tube A) and incubated for 5 min. Simultaneously, in a second tube (Tube B), 20 pM Control siRNA, siSNX3 or siSNX17 siRNA was added to 50 µL SF media along with 2 µL p3000 reagent (Life Technologies, Invitrogen 3000 Transfection Kit, Cat No.
L3000-015). When 60 mm² culture plates were required (Section 2.5, 2.6), the transfection mix prepared for a 35 mm² culture plate was doubled.

Tube A and Tube B were subsequently added together, mixed thoroughly by pipetting and incubated for 15 min at room temperature to allow the formation of Lipofectamine-siRNA complexes. SF media replaced full media on cells and the transfection mix was added to this in a dropwise fashion before incubation at 37°C (5% CO₂, 95% O₂). At 4-6 hr post transfection, SF media was replaced with full growth media before once again being returned to the incubator for 24-96 hr (depending on cell line) awaiting cell lysis.

Ussing chamber (Section 2.4), cell surface biotinylation assay (Section 2.5) and Co-IP (Section 2.6) experiments also required the transfection of plasmids encoding human α-, β- or β-HA tagged and γ- ENaC. 0.067 μg of each ENaC subunit was added to Tube B transfection mix, alongside the siRNA.

2.3 Western Blot

Western blot refers to a technique which uses three elements in order to detect levels of a specific protein of interest. Western blot begins with separation of proteins by size using gel electrophoresis followed by transfer of the proteins to a solid support and finally, the protein of interest is targeted using a primary and secondary antibody to visualise protein levels (Mahmood & Yang, 2012).
2.3.1 Cell Lysis

Cells to be lysed were removed from the incubator and placed onto ice where they were washed twice in cold PBS before incubation with cold lysis buffer (1 x TBS (Tris-buffered saline) (50 mM Tris (pH6.8), 150 mM NaCl)) (Lab Supply, Cat No. APPA2264,1000, APPA1371,100, Dunedin, New Zealand) +1% Triton X-100 (BDH, Cat No. 30632, UK) with protease inhibitors (10µg/mL PMSF (phenylmethylsulfonyl fluoride) (Gibco, Cat No. 15521-016), 2µg/mL leupeptin (Sigma, Cat No. L2884), 2µg/mL aprotinin (Sigma, Cat No. A 6103) and 1µg/mL pepstatin (Sigma, Cat No. P4265)) for 10-15 min on a platform rocker. Lysed cells were scraped off the culture plate and the lysate was collected and pipetted into a 1.5 mL microcentrifuge tube that was centrifuged in a bench top centrifuge for 10 min at 13,200 r.p.m at a temperature of 4°C to pellet cell debris and unbroken membranes. The supernatant was then transferred to a fresh microcentrifuge tube ready for the protein concentration to be determined (Section 2.3.2).

2.3.2 DC™ (Detergent Compatible) Protein Assay

The DC™ Protein assay kit (Bio-Rad, Cat No. 500-0116, California, USA) was used to determine the protein concentration of cell lysates ensuring that an equal input of proteins was used in Western blot experiments. Purified BSA (Bovine Serum Albumin) (Gibco, Cat No. 30063-572) was diluted to concentrations between 0 and 1.2 mg/mL to create standards in order to generate a calibration curve for the assay. Reagent A’ was prepared by adding 20 µL reagent S (supplied by manufacturer) for every 1 mL of reagent A (supplied by manufacturer). Cell lysate samples and BSA standards (5 µL of each) were added, in triplicate, into a 96-well plate and combined with 25 µL reagent A’ in each well. Reagent B (supplied by manufacturer) (200 µL) was added to each well and the plate was incubated at room temperature for 15 min. The
absorbance of each well at a wavelength of 750 nm was determined on a microplate reader (Synergy 2, BioTek Instrument, USA). Using excel (Microsoft Corporation, Washington, USA) a calibration curve was generated using the recorded absorbance of the BSA standards, thereby allowing the calculation of the protein sample concentrations.

2.3.3 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis is a method used for the separation and analysis of DNA, RNA, and proteins based on their molecular weight. Proteins are run in a sodium dodecyl sulphate (SDS) polyacrylamide gel, and when subjected to a voltage, the negatively charged protein molecules migrate through the gel towards to positively charged anode. The gel acts like a sieve with smaller proteins able to migrate the mesh of the gel easier than larger proteins, therefore, allowing for the separation of proteins based on molecular weight (Meyers et al., 1976).

Protein samples containing equivalent amounts of proteins (30 µg) (determined from the DC™ Protein assay) were mixed with 5x Laemmli sample buffer (10% Tris (pH6.8), 5% SDS, 26% glycerol, 0.8% bromophenol blue) with 1/100 volume of 2-mercaptoethanol (Sigma, Cat No. 200-464-6) and denatured by heating at 95°C for 10 min in a heat block before being centrifuged in a benchtop centrifuge for 30 sec at 13,200 r.p.m at room temperature.

Polyacrylamide gels consisting of a separating gel and a stacking gel were prepared using a 30% Acrylamide/Bis solution (Bio-Rad, Cat No. 161-0156) (Table 2). As these samples were being analysed by Western blot, gels of 1.5 mm thickness were used to allow a maximum of
50 μL of sample to be loaded. Once the gels had set, they were placed in a Hoefer Mini-Vertical system (Hoefer, Cat No. 80-6149-35, USA) that was then filled with SDS running buffer (25mM Tris (pH6.8), 192 mM glycine, 0.1% (w/v) SDS, H₂O). Pre-stained protein marker (7.5 μL) (Periscope pre-stained protein marker, Bio-Rad, Cat No. 161-0374) was loaded onto the gel to allow the estimation of protein molecular weight, followed by the samples in subsequent lanes. Tank electrodes were attached to a Hoefer EPS2A200 power pack and the gel was run at 150 V until the bromophenol blue dye reached the bottom of the gel (~120 min).

Table 2. Composition of Separating and Stacking Gels for the Preparation of SDS-Polyacrylamide Gels.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>8% Separating Gel</th>
<th>10% Separating Gel</th>
<th>15% Separating Gel</th>
<th>4% Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ H₂O</td>
<td>4.7 mL</td>
<td>4 mL</td>
<td>2.35 mL</td>
<td>3 mL</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
<td>-</td>
</tr>
<tr>
<td>0.5 M Tris pH 6.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 μL</td>
<td>100 μL</td>
<td>100 μL</td>
<td>50 μL</td>
</tr>
<tr>
<td>30% acrylamide (BioRad)</td>
<td>2.66 mL</td>
<td>3.34 mL</td>
<td>5 mL</td>
<td>665 μL</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>50 μL</td>
<td>50 μL</td>
<td>50 μL</td>
<td>25 μL</td>
</tr>
<tr>
<td>Tetramethylethlenediamine (TEMED)</td>
<td>5 μL</td>
<td>5 μL</td>
<td>5 μL</td>
<td>5 μL</td>
</tr>
<tr>
<td>Total</td>
<td>10 mL</td>
<td>10 mL</td>
<td>10 mL</td>
<td>5 mL</td>
</tr>
</tbody>
</table>

2.3.4 Semi-Dry Protein Transfer

Semi-dry protein transfer involves the transfer of proteins separated by electrophoresis from the polyacrylamide gel onto a polyvinylidene difluoride (PVDF) membrane (Roche, Cat No. 30-010-040-001, Germany). This protein transfer process allows for the covalent binding of proteins to the PVDF membrane in order to stop proteins being washed off during the antibody binding/wash steps (Section 2.3.5). A transfer buffer was prepared (20% methanol (MeOH), 25 mM Tris-base, 192 mM glycine, H₂O), where 50 mL per membrane was required.
For each polyacrylamide gel, one PVDF membrane (7 mm x 8 mm) was cut and soaked in MeOH for 30 sec to activate it before being washed with mQH\(_2\)O (milli Q H\(_2\)O) and placed into transfer buffer.

The electrotransferring of proteins was performed using a Hoefer semi-dry transfer unit (Hoefer Semiphor, Semi-dry transfer unit, Cat No. 80-6211-86). The anode of the semi-dry transfer unit was rinsed with distilled water (dH\(_2\)O) and a plastic overhead projector (OHP) sheet with the appropriate number of cut outs for number of transfers was positioned onto the anode. The transfer sandwich stack was then prepared by saturating six pieces of 7 mm x 8 mm blotting paper (Whatman, Cat No. 3030 917, Germany) with transfer buffer and placing them one by one over the cut out on the OHP sheet. The activated PVDF membrane was then placed on top of this filter paper stack (Figure 2.1).

**Figure 2.1. Semi Dry Protein Transfer Stack.** Current is run from the negatively charged electrode to the positively charged electrode, inducing the proteins on the polyacrylamide gel to transfer onto the PVDF membrane.
Following completion of the gel electrophoresis, the gel was removed from the gel plate, the stacking gel was cut off and the gel was placed on top on the PVDF membrane (Figure 2.1). Another six pieces of 7 mm x 8 mm filter paper soaked in transfer buffer were placed on top of the gel, with air bubbles removed by using a roller after addition of each piece of filter paper (Figure 2.1). The cathode of the semi-dry transfer unit was then rinsed with dH$_2$O and placed on top of the stack. The cathode was connected to the anode and the transfer unit was connected to the power supply. The power supply voltage limit was set at 50 V and the current to 0.8 mA/cm$^2$ of gel surface, i.e., 45 mA for one 7 x 8 cm gel, the transfer ran for 2 hr.

2.3.5 Membrane Blocking and Antibody Detection

Following completion of the transfer, the PVDF membrane was carefully removed from the stack and placed in blocking buffer (5% non-fat milk powder in 1 x TBS with 0.1% Tween-20) before incubation for 1 hr at room temperature. Blocking with milk powder is important as proteins within the milk powder bind to the empty binding sites on the PVDF membrane, therefore, helping to prevent nonspecific binding of antibodies to the PVDF.

Specific antibodies were used to immunostain the proteins on the PVDF membrane in order to visualise the proteins of interest. Primary antibodies, at a ratio defined in Table 3, were diluted in TBS-Tween (TBS-T) (20 mM Tris (pH6.8), 150mM NaCl, 0.1% Tween-20, pH7). The blocking buffer was poured off the membrane which was then washed with TBS-T before being incubated with primary antibody overnight at 4°C. Following incubation, membranes were washed for 3 x 10 min in TBS-T and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody diluted in TBS-T (Table 3) for 1 hr at room temperature. Following incubation with the secondary antibody the membranes were again washed for 3 x 10 min with TBS-T before chemiluminescent detection.
### Table 3. List of Antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Epitope/Antigen</th>
<th>Dilution for Immunoblotting</th>
<th>CO-IP</th>
<th>Type</th>
<th>Host</th>
<th>Source: Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-SNX3</td>
<td>SNX3</td>
<td>1:1000</td>
<td>-</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>Abcam Ab174123</td>
</tr>
<tr>
<td>Anti-SNX17</td>
<td>SNX17</td>
<td>1:1000</td>
<td>-</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>Protein Tech 10275-1-AP</td>
</tr>
<tr>
<td>Anti-Beta-actin</td>
<td>Beta-actin</td>
<td>1:2000</td>
<td>-</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>Sigma Aldrich A5441</td>
</tr>
<tr>
<td>Anti-HA</td>
<td>YPYDVPDYA</td>
<td>1:1000</td>
<td>2 μL</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>Sigma Aldrich H6908</td>
</tr>
<tr>
<td>Anti-COMMD1</td>
<td>GST-COMMD1</td>
<td>1:2500</td>
<td>-</td>
<td>Polyclonal</td>
<td>Rat</td>
<td>Raised in McDonald lab and Hercus-Taieri Resource Unit (HTRU), University of Otago, Dunedin, NZ</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Primary Antibodies</th>
<th>Secondary Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Rabbit-HRP</td>
<td>Rabbit IgG</td>
<td>Goat IgG</td>
</tr>
<tr>
<td>1:10,000</td>
<td>-</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Anti-Mouse-HRP</td>
<td>Mouse IgG</td>
<td>Goat IgG</td>
</tr>
<tr>
<td>1:10,000</td>
<td>-</td>
<td>2 μL</td>
</tr>
<tr>
<td>Anti-Goat-HRP</td>
<td>Goat IgG</td>
<td>Donkey</td>
</tr>
<tr>
<td>1:10,000</td>
<td>2 μL</td>
<td></td>
</tr>
<tr>
<td>Anti-Rat-HRP</td>
<td>Rat IgG</td>
<td>Goat IgG</td>
</tr>
<tr>
<td>1:10,000</td>
<td>-</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>

#### 2.3.6 Membrane Development

Once the membrane wash steps were complete, the membranes were taken to a dark room for membrane development. The chemiluminescent bands on the PVDF membrane were detected using an enhanced chemiluminescent (ECL) (GE Healthcare, Amersham ECL Prime Western blot Reaction Reagent, Cat No. RPN2236, UK) substrate to enable detection of HRP enzyme activity. Membranes were incubated with the ECL as per manufacturer’s instructions.
for 5 min at room temperature before the reagents were poured off and the membrane was
placed in a developing cassette between two plastic OHP sheets. Under infra-red light a piece
of X-ray film (Carestream Biomax ZAR film, Cat No. CAT1651454, Canada) was placed over the
membrane before the cassette was closed to allow exposure to the light emitted by the
chemiluminescent reagents. Exposure times varied from 5 sec to overnight depending on the
intensity of the signal as well as which primary antibody had been used. Film was subsequently
developed in developer solution (Carestream, Cat No. 4037180) for 3-5 min before being
rinsed in running water and fixed in fixer solution (Carestream, Cat No. 4037214) for another
3-5 min before finally being rinsed again and air dried.

2.3.7 Densitometry and Statistical Analysis

To quantify the intensity of signals obtained from Western blot, the X-ray films were scanned
at 1200 dpi and subsequently analysed. The computer software ImageJ (Version 7, NIH, USA)
was used to measure the density of protein bands. Each protein band of interest was selected
carefully to ensure all consistent band area was included, along with minimal background
interference. The abundance of protein was measured as the mean optical density. Raw data
was exported and protein level (relative to loading control, β-actin) was determined using
Excel. The control mean optical density (control siRNA cells) was normalised to the value of 1
and protein bands were compared to this normalised control. Data is presented as mean ±
standard deviation (SD). Statistical analysis was conducted using the computer software
programme GraphPad Prism (GraphPad software, California, USA). A One-sample T-test was
used to analyse changes in protein levels between control and siRNA knockdown cells and
were regarded as significant if \( P < 0.05 \).
2.4 Measurement of Amiloride-Sensitive Short-Circuit ENaC Current ($I_{sc-Amil}$) in FRT and mCCD Epithelia.

The electrophysiological Ussing chamber is an apparatus used for measurement of epithelial membrane properties, where an epithelium is clamped under short circuit conditions allowing for measurement of net ion transport across an epithelial monolayer (Ussing & Zerahn, 1951). For this project, this apparatus was utilised to examine changes in ENaC amiloride sensitive current in the presence of a siRNA SNX protein knockdown.

2.4.1 Measuring Amiloride-Sensitive Short-Circuit Current ($I_{sc-Amil}$)

$\text{Na}^+$ current across the epithelium was measured using modified Ussing chambers connected to a multichannel V/A clamp (Physiologic Instruments, San Diego, CA, USA) via a DI-720 data acquisition system (Data Q instruments, Ohio, USA) and was recorded using the Acquire and Analyse 2.3 program (Physiologic Instruments) running on a PC. To prepare the voltage and current electrodes, the tips of the electrodes were filled and sealed with 3% molten agar in 3 M KCl before being backfilled with fresh 3 M KCl and screwed tightly onto the electrodes making sure no air bubbles were present. The epithelium, growing on the Snapwells™, was removed from the incubator, washed in warmed 1 x Ringers (in mM, 135 NaCl, 2.4 K$_2$HPO$_4$, 10 HEPES, 1.2 CaCl$_2$, 1.2 MgCl$_2$, pH 7.4) to remove media, and were mounted into the Ussing chamber set-up (Figure 2.2 A). The apical and basolateral surfaces were bathed in 1 x Ringer’s solution, kept at 37°C and bubbled with O$_2$. 
The epithelia were clamped under short-circuit conditions (short-circuit current is an indicator of net ion transport taking place across an epithelium). Once the current had reached a plateau, amiloride 5 μM amiloride (final concentration = 5 nM) was added to the apical side of the epithelium to inhibit ENaC. The rapid drop in current caused by amiloride is referred to as the $I_{sc}$-Amil (Figure 2.2 B) and provides a representative measurement of ENaC function at

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**Figure 2.2. Ussing Chamber Set-Up and Representative Trace.** A. Apical and basolateral sides of the chamber were bathed in 1 x Ringer’s solution with O$_2$ to ensure circulation of the Ringers. The epithelia was clamped under short-circuit conditions and 5 μM Amiloride was added to the apical side allowing for measurement of $I_{sc}$-Amil. B. A representative Ussing chamber recording trace. The $I_{sc}$-Amil was determined as the difference in current recorded prior to and after the addition amiloride into the apical bathing solution, black bar indicates the presence of amiloride.
the apical membrane. Therefore, an increase in $I_{sc}$-Amil indicates either an increased ENaC cell surface population or higher ENaC $P_0$.

Alongside the measurement of short-circuit current, the transepithelial resistance (RT) was also monitored throughout the Ussing chamber measurements, in order to provide an indication of the integrity of the epithelia (i.e., ‘tightness’ of tight junctions between cells, or flow of ions through the tight junctions). The higher the RT, the more likely that the epithelia has formed a confluent monolayer and there is less chance for the paracellular flow of ions. This is important as it means the $I_{sc}$-Amil measured is due to ion transport moving across the epithelium through channels and transport proteins in the apical and basolateral membranes and not due to “leaky” tight junctions. Therefore, epithelia with resistances lower than 300 $\Omega \cdot \text{cm}^2$ were omitted from analyses so as to ensure that $I_{sc}$-Amil recordings were due to ion transport across the epithelia, not due to any paracellular ion movement.

2.4.2 Statistical analysis

For Ussing chamber data analysis, $I_{sc}$-Amil was calculated using Microsoft Excel. Control siRNA knockdown $I_{sc}$-Amil was normalised to the value of 1 and $I_{sc}$-Amil for other conditions, SNX3 and SNX17 siRNA knockdown, were compared to the normalised control. Data are presented as mean ± SD. Statistical analysis was conducted using the computer software programme GraphPad Prism (GraphPad software). A One-sample T-test was used to compare control against siRNA knockdown conditions, where results were regarded as significant if $P < 0.05$. 
2.5 Biotinylation Assay

In this study a biotinylation assay has been used to determine, and compare, the levels of ENaC found in the apical membrane in FRT control and SNX3 or SNX17 siRNA knockdown cells. Biotin is a small, water-soluble vitamin that binds with high affinity to avidin. A modified version of biotin, EZ-Link® Sulfo-NHS-LC biotin (Thermo Fisher Cat No.21335), is commonly used for the labelling of cell surface proteins, as it is able to react with amine groups in the side chains of lysine residues (Inouye & Nakamura, 2003), which are present in the extracellular loop of all three ENaC subunits (McDonald et al., 1994; McDonald et al., 1995).

The protocol used in this study was adapted from Silvis et al., (2009). Firstly, FRT cells were seeded at a density of 5 x 10^5 cells per 60 mm² plate and were incubated overnight before transfection with plasmids encoding α-, epitope-tagged β-HA and γ-ENaC, along with control, SNX3 or SNX17 siRNA (Section 2.2). After the 24 hr incubation period, cells were washed with cold PBS 5x and incubated with biotin buffer consisting of 1mg/mL Pierce-Sulfo-NHS-LC-biotin in borate buffer (85 mM NaCl + 4mM KCL + 1mM Na₂B₄O₇) for 20 min on a platform shaker at 4°C. Biotin Buffer was then aspirated and a FBS quenching solution (10% FBS + PBS) was added to inactivate residual biotin, before being removed and the cells were washed once again with cold PBS 5x. Cells were subsequently lysed in lysis buffer (50mM EDTA + 10 mM Tris pH7.4 + 1% NP-40 + 0.4% sodium deoxycholate) for 10 min, scraped off the plates and transferred to a microcentrifuge tube where they were then centrifuged at 13,200 r.p.m at 4°C in a benchtop centrifuge, the lysate was transferred to a fresh microcentrifuge tube and the pellet discarded. Lysate protein concentrations were determined using the DC™ protein assay kit (Section 2.3.2), and equal aliquots of lysate were incubated with 100 μL Neutravidin™ Ultra Link™ beads (Thermo Fisher, Cat No. 53150) overnight at 4°C to separate biotin-labelled surface proteins from cytosolic fractions.
The following day the mixtures were centrifuged at 2,000 r.p.m at 4°C in a benchtop centrifuge for 1 min to pellet beads and biotinylated proteins. The cytosolic portion was transferred to a fresh microcentrifuge tube where 50 μL of supernatant was retained in a new tube to analyse cytosolic proteins by Western blot. Beads were washed with lysis buffer 4x before being incubated with Laemmli sample buffer at 95°C for 5 min to elute biotinylated proteins from the beads. Surface protein and cytosolic samples were subsequently analysed through Western blot (Section 2.3).

2.5.1 Biotinylation Assay Statistical Analysis

Western blot densitometry was analysed as described in Section 2.3.8. The surface ENaC protein level was made relative to cytosolic ENaC protein level (which had been made relative to the loading control; β-actin). This control was then normalised to the value of 1 with the SNX3 and SNX17 siRNA knockdown ENaC protein bands being compared to the normalised control. Data is presented as mean ± SD. Statistical analysis was conducted using the computer software programme GraphPad Prism (GraphPad software, California, USA). A One-sample T-test was used to analyse changes in protein levels between control and siRNA knockdown cells and were regarded as significant if P < 0.05.

2.6 Co-immunoprecipitation (Co-IP)

Co-IP is a technique used to identify protein-protein interactions through the use of target specific antibodies, which indirectly capture proteins bound to the target protein (Free et al., 2009). In this study Co-IP experiments were carried out using HEK-293 cells, in order to determine, and compare, if protein-protein interactions between ENaC and SNX3 or SNX17 proteins exist.
Firstly, HEK-293 cells were seeded at a density of 5.5 x 10^5 cells per 60 mm² plate and were incubated overnight before being transfected with plasmids encoding α-, β-HA tagged and γ-ENaC, (Section 2.2). Following the 24 hr incubation, cells were washed with cold TBS 4x before being lysed in lysis buffer (1% Triton + 1xTBS + Protease Inhibitors (10µg/mL PMSF, 2µg/mL leupeptin, 2µg/mL aprotinin and 1µg/mL pepstatin) for 10 min. Lysed cells were scraped off the plates, transferred to a 1.5 mL microcentrifuge tube and centrifuged at 13,200 r.p.m at 4°C in a benchtop centrifuge, after which the lysate was subsequently transferred to a fresh tube and the pellet discarded. Lysate protein concentrations were determined using the DC™ protein assay kit (Section 2.3.2). 30 µg of protein was then removed for lysate/input controls – where Laemmli sample buffer was added, and these samples were incubated for 10 min at 95°C. For the remaining lysate, 2 µL of the appropriate antibody (donkey anti-Goat for negative control, anti-HA for positive control and other experimental conditions) (Table 3) was added to equal amounts of lysate. The lysate/antibody mix was then incubated for 3 hr at 4°C. Following this incubation, 30 µL of Protein G-Sepharose beads were added to each lysate sample, which was then incubated for another hour at 4°C. After the second incubation period, samples were centrifuged to pellet the beads and immunoprecipitated proteins, this was followed by 4x washes with 1% TBS + 1% Triton X-100, using a benchtop centrifuge, at a low spinning speed of 2000 r.p.m at 4°C. Finally, 20 µL of Laemmli sample buffer was added to each bead pellet and was subsequently incubated for 10 min at 95°C. Input controls and immunoprecipitation samples were then analysed using SDS-PAGE and Western blot (Section 2.3).
3. Results
3.1 siRNA SNX3 Knockdown Reduces ENaC Recycling in FRT and mCCD Epithelia.

3.1.1 Detection of Endogenous SNX3 in FRT Cells.

The aim of this project was to investigate retromer and retriever-mediated ENaC recycling through determining whether SNX3 and/or SNX17 play a role in these pathways. Therefore, an important first step for this project was to locate antibodies able to detect the target SNX protein; SNX3, in the chosen cell lines. For this project, I used two epithelial cell lines, with the first of these being an FRT cell line, a model mammalian epithelium (Zurzolo et al., 1991) able to form a polarised epithelium when grown on Snapwell™ filters. Therefore, an antibody able to detect SNX3 in rat epithelia was required, with a commercial Sigma Aldrich antibody (Table 3) selective for rat being chosen. Prior to this project, it was not established whether SNX3 is endogenously expressed and able to be detected in FRT cells. However, as retromer is a conserved protein complex from yeast through to mammals, and with bioinformatics suggesting SNX3 expression in thyroid epithelia (Bastian et al., 2008), it was hypothesised that SNX3 would be endogenously expressed in FRTs. In order to examine this hypothesis, untransfected FRTs were lysed and using Western blot along with the selected SNX3 antibody, a protein band was detected at a molecular weight of approximately 19 kDa (Figure 3.1 A). The predicted molecular weight for SNX3 is 19 kDa (Akman et al., 2015), supporting the assumption that the protein detected was endogenous SNX3.
The transient knockdown of SNX3 in FRT epithelia via siRNA was used to provide evidence of a role for SNX3 and thus retromer in ENaC protein recycling. To determine the efficiency of the SNX3 siRNA knockdown, FRT cells were transfected with either control or SNX3 siRNA and incubated for 72 hr before being lysed, followed by SNX3 protein detection using Western

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**Figure 3.1. siRNA Knockdown of Endogenous SNX3 Protein in FRT and mCCD Cells.** FRT and mCCD cells were transiently transfected with control siRNA or SNX3 siRNA, with Lipofectamine 3000™. Cells were lysed 72 hr (FRTs) or 5 days (mCCDs) post transfection and the level of SNX3 and β-actin (an internal control) was detected by using Western blot with anti-SNX3 or anti-β-actin respectively. **A, B.** The level of endogenous SNX3 in the presence of control siRNA or SNX3 siRNA in a representative experiment for each cell line are shown; protein marker molecular weights are indicated. (Band below 19 kDa is nonspecific, only the SNX3 19 kDa band was used for densitometry) **C, D.** SNX3 and β-actin bands were scanned and quantified by densitometry using Image J™ software, and statistically analysed using GraphPad Prism. Each SNX3 band was normalised against the corresponding β-actin band and to the control (siControl), and plotted as mean ± SD. N = 3, One-sample T-test, * indicates P < 0.05.
blot. It was observed that the siRNA knockdown of SNX3 in the FRT cell line, resulted in an average of 62% ± 16% reduction in SNX3 protein levels compared with control cells ($P < 0.05$, $N = 3$) (Figure 3.1 A, C), suggesting a significant knockdown of endogenous SNX3 in FRTs with this siRNA.

### 3.1.2 Detection of Endogenous SNX3 in mCCD Cells.

FRTs are a well-established model mammalian epithelium, however, they do not endogenously express the protein of interest for this project; ENaC. Therefore, in order to support and extend observations from the FRT cell line in relation to the kidney, a second epithelial cell line, mCCDs, a kidney cell line endogenously expressing ENaC was incorporated into the project. As with the FRTs, an antibody able to detect endogenous SNX3 in the mCCDs was required. The antibody previously used with the FRT cell line was also predicted to work with mouse cell lines and therefore the commercial Sigma Aldrich antibody (Table 3) was tested with the mCCDs. Unlike the FRTs, SNX3 expression in the mCCDcl1 cell line has previously been reported (Boulkroun et al., 2008). To confirm SNX3 detection in the mCCDcl1 cell line, mCCD cells were lysed, and using Western blot with the selected rat anti-SNX3 antibody, a protein band at a molecular weight of approximately 19 kDa was detected, indicating detection of endogenous SNX3 (Figure 3.1 B).

As with the FRT epithelia, the transient knockdown of SNX3 in mCCD epithelia via siRNA was used to further provide evidence of a role for SNX3 and retromer in ENaC recycling. To determine the efficiency of the SNX3 knockdown, mCCDs cells were transfected with either control or SNX3 siRNA and incubated for 5 days before being lysed, followed by detection of the SNX3 protein using Western blot. It was observed that the knockdown of SNX3 via siRNA in the mCCD cell line, resulted in an average of 72% ± 20% reduction of SNX3 protein compared
with control cells ($P < 0.05$, $N = 3$) (Figure 3.1 B, D), suggesting a significant knockdown of endogenous SNX3. Therefore, in brief using siRNA, SNX3 protein expression was significantly reduced in two epithelial cell lines indicating the successful knockdown of SNX3.

### 3.1.3 Measurement of $I_{sc}$-Amil in FRT Epithelia with siRNA Knockdown of SNX3.

Once the transient SNX3 siRNA knockdown in both cell lines was confirmed, the cells were able to be utilised for electrophysiological studies. The FRT cell line, when grown on Snapwell™ filters, FRT cells form a polarised epithelial monolayer and demonstrate a morphology of tight epithelia making the FRT cell line a suitable model to study ENaC abundance and activity at the apical membrane (Zurzolo et al., 1991). FRT cells do not endogenously express ENaC, and therefore, plasmids encoding the $\alpha$, $\beta$- and $\gamma$- subunits were transfected into the epithelium in order to generate an amiloride-sensitive ENaC current that could be utilised for measurement in an Ussing chamber when clamped under short-circuit conditions (Section 2.4). Initially, to serve as a negative control, experiments were performed on untransfected FRT wild type epithelia to demonstrate that there was no amiloride-sensitive current in the absence of ENaC within the epithelia. It was observed that there were no changes in $I_{sc}$-Amil following the addition of 5 µM amiloride, highlighting that the effects observed in Ussing chamber experiments are due to amiloride’s inhibitory effect on ENaC ($N = 1$) (Figure 3.2).
To investigate the effect of a transient siRNA knockdown of SNX3 on ENaC current, FRT cells were co-transfected with control or SNX3 siRNA alongside plasmids encoding α-, β- and γ-subunits before being left to grow on Snapwell™ filters for 72 hr to allow the formation of a confluent, polarised, epithelial monolayer. FRT control epithelia averaged a basal $I_{sc-Amil}$ of $0.28 \pm 0.05 \, \mu A/cm^2$ ($N = 9$). This basal $I_{sc-Amil}$ was relatively low and is likely attributed to the high passage number of cells used for these experiments, as it has been demonstrated that increasing cell passage number reduces the efficiency of transfection (Madeira et al., 2010; Aydin et al., 2012; de Los Milagros Bassani Molinas et al., 2014). In-house studies have demonstrated a similar reduction in $I_{sc-Amil}$ as cell passage number increases, thus indicating a reduction in ENaC numbers at the plasma membrane when using FRT epithelia (McDonald, unpublished work). Therefore, due to variation in passage numbers, slight changes in number of cells seeded, transfection efficiency and monolayer confluence, each experiment was normalised to the control knockdown FRT epithelia in order for the measured $I_{sc-Amil}$ of the SNX3 siRNA knockdown FRT epithelia to be compared on the same day with control knockdown epithelia from the same passage number. This allowed for any changes in $I_{sc-Amil}$ to be analysed over a number of passages.

The observed $I_{sc-Amil}$ produced by the siRNA SNX3 knockdown FRT epithelia decreased significantly by $47 \pm 0.05 \%$ compared with the control epithelia ($1$ for control epithelia vs.
0.52 ± 0.17 for SNX3 siRNA knockdown epithelia; \( P < 0.0001, N = 9 \) (Figure 3.3 A-B). This significant decrease in \( I_{sc} \)-Amil indicates that a reduction of SNX3 protein expression decreases the measured ENaC current likely through reducing ENaC cell surface population, and therefore, suggesting a role for retromer in ENaC recycling.

**Figure 3.3. SNX3 siRNA Knockdown Decreases \( I_{sc} \)-Amil in FRT Epithelia.** FRT cells co-transfected with plasmids encoding \( \alpha \), \( \beta \), and \( \gamma \)-ENaC (0.067 \( \mu \)g of each plasmid) and either control or SNX3 siRNA were grown on Snapwell\textsuperscript{TM} filters for 72 hr before being placed into a Ussing chamber setup where they were clamped under short circuit conditions. **A.** Representative ENaC current trace for control and SNX3 knockdown FRT epithelia. The addition of amiloride (5 \( \mu \)M) is indicated by the black box and the subsequent change in current was calculated and termed \( I_{sc} \)-Amil. **B.** Collective data for \( I_{sc} \)-Amil control and SNX3 siRNA knockdown epithelia. Data shown as mean ± SD relative to \( I_{sc} \)-Amil in control knockdown epithelia. One-sample T-test, *** Indicates \( P < 0.001, N = 9 \). **C.** Summary of data for RT in control and SNX3 siRNA knockdown epithelia with the 300 \( \Omega \cdot \text{cm}^2 \), RT threshold indicated by dotted line. Data shown as mean ± SD, \( N = 9, P > 0.05 \).

The transient SNX3 knockdown had no significant effect on the RT of the FRT epithelia (1694 \( \Omega \cdot \text{cm}^2 ± 500 \Omega \cdot \text{cm}^2 \) for control epithelia vs. 1709 \( \Omega \cdot \text{cm}^2 ± 473 \Omega \cdot \text{cm}^2 \) for SNX3 siRNA knockdown.)
epithelia, \( P > 0.5, \, N = 9 \) (Figure 3.3 C) suggesting that the knockdown of SNX3 did not affect the integrity of the epithelial monolayer.

3.1.4 Measurement of \( I_{sc}\text{-Amil} \) in mCCD Epithelia with siRNA Knockdown of SNX3.

To further validate observations made from the electrophysiological Ussing chamber measurements with the siRNA SNX3 knockdown in FRT epithelia, \( I_{sc}\text{-Amil} \) was also measured in the second cell line of interest for this project; mCCDs. mCCD cells were transiently transfected with control or SNX3 siRNA, before being left to grow on Snapwell™ filters for 5 days to allow the formation of a confluent, polarised, epithelial monolayer. Similar to the FRT epithelia, due to variation in passage numbers, slight changes in number of cells seeded, transfection efficiency and monolayer confluency, each experiment was normalised to the control knockdown mCCD epithelia in order for the measured \( I_{sc}\text{-Amil} \) of the SNX3 knockdown mCCD epithelia to be analysed over a number of passages.

The observed \( I_{sc}\text{-Amil} \) produced by the SNX3 siRNA knockdown mCCD epithelia, was decreased compared to the control epithelia (1 for control epithelia vs. 0.64 ± 0.14 for SNX3 siRNA knockdown epithelia \( N = 9, \, P < 0.0001 \) (Figure 3.4 A-B). This was a significant reduction of 35 ± 0.04 % in the \( I_{sc}\text{-Amil} \) produced by the SNX3 siRNA knockdown epithelia in comparison with the control epithelia. This significant decrease in \( I_{sc}\text{-Amil} \) supports the observations made from the FRT siRNA SNX3 knockdown functional experiments and further suggests that a reduction in SNX3 protein expression either reduces ENaC \( P_o \) or the ENaC cell surface population, and therefore, ENaC trafficking and recycling.
The transient knockdown of SNX3 had no significant effect on the transepithelial resistance of the mCCD epithelia (503 Ω·cm² ± 53 Ω·cm² for control epithelia vs. 607 Ω·cm² ± 48 Ω·cm² for SNX3 siRNA knockdown epithelia, P > 0.5, N = 9) (Figure 3.4.C), suggesting that the knockdown of SNX3 protein did not affect the integrity of the mCCD epithelial monolayer.

Figure 3.4. SNX3 siRNA Knockdown Decreases \( I_{sc}-Amil \) in mCCD Epithelia. mCCD cells were transfected with either control or SNX3 siRNA and grown on Snapwell™ filters for 5 days before being placed into a Ussing chamber setup where they were clamped under short circuit conditions. A. Representative \( I_{sc} \) trace for control and SNX3 knockdown mCCD epithelia. The addition of amiloride (5 μM) is indicated by the black box and the subsequent changes in current were calculated and termed \( I_{sc}-Amil \). B. Collective data for \( I_{sc}-Amil \) control and SNX3 siRNA knockdown epithelia. Data shown as mean ± SD relative to \( I_{sc}-Amil \) in control knockdown epithelia. One-sample T-test, *** Indicates \( P < 0.001, N = 9 \). C. Summary of data for RT in control and SNX3 siRNA knockdown epithelia with the 300 Ω·cm², RT threshold indicated by dotted line. Data shown as mean ± SD, \( P > 0.05, N = 9 \).
To summarise, siRNA knockdown of the SNX3 protein in two epithelial cell lines led to a significant reduction in ENaC $I_{sc}$-Ami, indicating a reduced ENaC cell surface population due to decreased recycling. In order to support this hypothesis and determine whether ENaC cell surface population or channel $P_o$ was affected, ENaC cell surface protein levels were examined using cell surface protein biotinylation assays (Section 3.1.5).

3.1.5 FRT ENaC Plasma Membrane Levels in the Presence of SNX3 siRNA Knockdown.

Electrophysiological Ussing chamber measurements demonstrated that in both FRT and mCCD epithelia with a siRNA SNX3 knockdown, $I_{sc}$-Ami is significantly reduced in comparison with control epithelia. This functional response indicated a reduced ENaC cell surface population in the SNX3 siRNA knockdown epithelia, as a result of decreased recycling back to the cell surface due to reduced retromer function. However, an Ussing chamber assay does not indicate whether changes in $I_{sc}$-Ami are the result of decreased cell surface population or due to changes in channel $P_o$. Therefore, to analyse ENaC cell surface levels in the presence of a SNX3 siRNA knockdown, cell surface protein biotinylation assays were performed. In brief, FRT cells were transfected with plasmids encoding $\alpha$, $\beta$-HA tagged and $\gamma$- ENaC along with either control or SNX3 siRNA. These transfected cells were subsequently incubated with EZ-link-Sulfo-NHS-LC biotin. Notably, a control plate was not incubated with EZ-link-Sulfo-NHS-LC biotin, in order to serve as a negative control. Cells were then lysed, and lysates were incubated overnight with Neutravidin beads to separate surface from cytosolic proteins. ENaC levels, both cell surface and total were subsequently determined using Western blot (Section 2.3).
Figure 3.5. SNX3 siRNA Knockdown Decreases ENaC Levels at the Plasma Membrane. FRT cells, transfected with control or SNX3 siRNA and plasmids encoding α, β-HA and γ-ENaC, were incubated with EZ-link-Sulfo-NHS-LC biotin before being lysed and incubated with Neutravidin-agarose beads to separate out cytosolic and plasma membrane proteins. Proteins were then separated by SDS-PAGE and ENaC levels, both plasma membrane and total, were determined by Western blot. A. Representative experiment demonstrating cell surface and cytosolic ENaC. Lane 1 shows surface β-HA ENaC without the presence of biotin (Negative Control), Lane 2 shows surface β-HA ENaC in the presence of biotin (Positive Control) and Lane 3 shows a reduction in surface β-HA ENaC in the presence of SNX3 siRNA and biotin. Protein marker molecular weights are indicated. B. β-HA ENaC and β-actin bands were scanned and quantified by densitometry using Image J™ software, and statistically analysed using GraphPad Prism. Each β-HA ENaC cytosolic band was normalised against the corresponding β-actin band, which was then used against the corresponding β-HA ENaC surface band before being normalised to the control (siControl), and plotted as mean ± SD. One-sample T-test, N = 3, * indicates P < 0.05.

Figure 3.5 A shows a representative experiment demonstrating an absence of surface β-HA ENaC without the presence of biotin (negative control), surface β-HA ENaC (positive control) and surface β-HA ENaC in the presence of SNX3 siRNA. siRNA knockdown of SNX3 resulted in
a significant reduction of 68% ± 25% in ENaC cell surface levels compared with control levels ($P < 0.05, N = 3$) (Figure 3.5 B). This indicates that the functional response observed in the FRT cells and suggestive for the mCCD SNX3 siRNA knockdown epithelia was due to decreased ENaC cell surface population rather than changes in ENaC $P_o$. This observation further supports the hypothesis that SNX3 plays a role in ENaC recycling, where a reduction in SNX3 protein levels leads to reduced retromer function, therefore, decreasing ENaC recycling back to the apical surface.

3.1.6 SNX3 Does Not Co-immunoprecipitate with β-HA ENaC

The functional electrophysiological Ussing chamber and cell surface biotinylation assay data gathered as part of this project have both demonstrated that in the presence of a SNX3 siRNA knockdown, ENaC cell surface population is reduced, indicating reduced ENaC channel recycling. SNX3 has been identified as a cargo binding protein for several membrane proteins, bringing these proteins into their retromer mediated recycling pathway (Strochlic et al., 2007; Harterink et al., 2011; Zhang et al., 2011). In order to investigate if a protein-protein interaction exists between SNX3 and ENaC allowing recycling by retromer, Co-IP experiments were performed.

Co-IP experiments were conducted using HEK-293 cells as the protocol used was established to work efficiently in this cell line. HEK-293 cells were transiently transfected with genes encoding α-, β-HA tagged and γ- ENaC, before being incubated for 24 hr and lysed. Lysates were incubated with anti-HA antibody to immunoprecipitate β-HA ENaC, followed by Western blot with anti-SNX3 to detect if endogenous SNX3 was pulled down along with β-HA ENaC. As seen from the representative blot in Figure 3.6, β-HA tagged ENaC and endogenous SNX3 were both present in the cytosolic lysates. However, when one lysate was incubated with donkey
anti-goat antibody, the β-HA tagged ENaC was unable to be immunoprecipitated with this antibody thus serving as a negative control. However, when the second lysate sample was incubated with an anti-HA antibody, β-HA tagged ENaC was able to be immunoprecipitated. To determine if SNX3 was co-immunoprecipitated alongside β-HA ENaC, an anti-SNX3 antibody was used to detect the presence of SNX3. Figure 3.6 (lane 4) demonstrates that no SNX3 protein was pulled down with ENaC indicating that a protein-protein interaction does not exist between the two proteins. As a positive control, in parallel experiments, it was observed that SNX17 interacts with ENaC (see below) therefore the Co-IP technique used is robust.

![Figure 3.6. SNX3 Does Not Interact With ENaC.](image)

**Figure 3.6. SNX3 Does Not Interact With ENaC.** HEK-293 cells were transiently transfected with plasmids encoding α-, β-HA-tagged and γ- ENaC. Cells were lysed 24 hr post transfection, and immunoprecipitation experiments were performed with anti-HA or anti-goat (Negative Control) antibodies to immunoprecipitate ENaC. Lysates (input) and immunoprecipitates (IP) were analysed using Western blot (IB) using anti-HA and anti-SNX3 antibodies. Protein marker molecular weights are indicated. N = 3
3.2 siRNA SNX17 Knockdown Reduces ENaC Recycling in FRT and mCCD Epithelia.

3.2.1 Detection of Endogenous SNX17 in FRT Cells.

With no interaction between SNX3 and ENaC detected, this project shifted to the second protein of interest; SNX17. As discussed previously (Section 1.9.10) SNX17 and its associated recycling complex retriever, have been established in the recycling of various membrane proteins (Stockinger et al., 2002; Steinberg et al., 2012; McNally et al., 2017). Therefore, in order to investigate whether ENaC recycling is mediated by SNX17-retriever, an antibody able to detect SNX17 in the two epithelial cell lines used for this project was required. A commercial Sigma Aldrich antibody selective for rat and predicted to work with mouse was selected (Table 3). SNX17 has not been previously established as endogenously expressed in the FRT cell line. However, due the conserved nature of the retromer complex and its similarities with the retriever complex, along with bioinformatic data suggesting SNX17 expression in thyroid epithelia (Bastian et al., 2008), it was hypothesised that the FRT cell line would endogenously express SNX17. To examine this hypothesis, untransfected FRTs were lysed and using Western blot along with the selected anti-SNX17 antibody, a protein band was detected at a molecular weight of approximately 49 kDa. SNX17 has a predicted molecular weight of 49 kDa (Bottcher et al., 2012), therefore indicating the protein detected was endogenous SNX17 (Figure 3.7 A).
The transient knockdown of SNX17 in FRT epithelia using a SNX17 siRNA was used to provide evidence of a role for SNX17 and thus the retriever complex in ENaC protein recycling. To determine the efficiency of the siRNA knockdown, FRT cells were transfected with either control or the SNX17 siRNA that was followed by a 72 hr incubation period before cell lysis and Western blot. It was observed that the siRNA knockdown of SNX17 reduced SNX17 protein
levels by 72% ± 19% compared with the control (P < 0.05, N = 3), suggesting the significant knockdown of endogenous SNX17 (Figure 3.7 A, C)

3.2.2 Detection of Endogenous SNX17 in mCCD Cells.

As mentioned previously, a second epithelial cell line, mCCD; a kidney epithelial cell line endogenously expressing ENaC, was used for this project in order to further support observations with the FRT cell line. The rat anti-SNX17 antibody previously purchased for use with the FRT cell line was also predicted to detect SNX17 in mouse cell lines and was, therefore, tested with the mCCDs. Endogenous SNX17 expression in the mCCD cell line was examined, where through Western blot, detection of a protein band at a molecular weight of approximately 49 kDa was observed indicating the presence of endogenous SNX17 (Figure 3.7 B).

The transient knockdown of SNX17 in mCCD epithelia via siRNA was used to further provide evidence of a role for SNX17, and thus, retriever in ENaC protein recycling. Similar to the FRTs, in order to determine the efficiency of the SNX17 knockdown, mCCDs cells were transfected with either control or SNX17 siRNA and incubated for 5 days before cell lysis, followed by detection of the SNX17 protein using Western blot. It was observed that the siRNA knockdown of SNX17 resulted in an average of 64% ± 25% reduction of SNX17 protein compared with control cells (P < 0.05, N = 3), indicating a significant knockdown of endogenous SNX17 in the mCCD cell line (Figure 3.7 B, D).
3.2.3 Measurement of $I_{sc}$-Amil in FRT Epithelia with siRNA Knockdown of SNX17.

With a significant siRNA knockdown of SNX17 in the two epithelial cell lines established, the cells were then able to be utilised for electrophysiological studies. As mentioned earlier, ENaC is not endogenously expressed within FRTs and therefore, required plasmids encoding the $\alpha$, $\beta$- and $\gamma$- ENaC subunits to be co-transfected into the FRTs alongside control or SNX17 siRNA. These FRT cells were then left to grow on Snapwell™ filters for 72 hr to allow the formation of a confluent, polarised, epithelial monolayer. The epithelia was clamped under short-circuit conditions, with the control epithelia averaging a basal $I_{sc}$-Amil of $0.23 \pm 0.01 \mu A/cm^2$ ($N = 9$).

This basal $I_{sc}$-Amil was relatively low and as mentioned earlier this is likely attributed to the high passage number of cells used for these experiments. Therefore, once again due to variation in passage numbers, changes in number of cells seeded, transfection efficiency and monolayer confluency, each experiment was normalised to the control epithelia. This allowed for the measured $I_{sc}$-Amil of the SNX17 knockdown FRT epithelia to be compared on the same day with control knockdown epithelia from the same passage number, allowing for any changes in $I_{sc}$-Amil to be analysed over a number of passages.

The recorded $I_{sc}$-Amil produced by the siRNA SNX17 knockdown FRT epithelia significantly decreased by $56 \pm 0.05 \%$ compared with the control epithelia (1 for control vs. $0.44 \pm 0.14$ for SNX17 siRNA knockdown, $P < 0.0001$, $N = 9$) (Figure 3.8 A, B). This significant decrease in $I_{sc}$-Amil suggests that a reduction in SNX17 protein level reduces the ENaC cell surface population, and therefore, indicates a role for retriever in ENaC recycling.

The transient SNX17 siRNA knockdown had no significant effect on RT of the epithelia ($1093 \Omega \cdot cm^2 \pm 202 \Omega \cdot cm^2$ for control epithelia vs. $1179 \Omega \cdot cm^2 \pm 180 \Omega \cdot cm^2$ for SNX17 siRNA
Figure 3.8. The Effect of a SNX17 siRNA Knockdown on I_{sc}-Amil in FRT Epithelia. FRT cells transfected with plasmids encoding α-, β-, and γ- ENaC (0.067 μg of each plasmid) along with either control or SNX17 siRNA and, were grown on Snapwell™ filters for 72 hr before being placed into a Ussing chamber setup where they were clamped under short circuit conditions. A. Representative I_{sc} trace for control and SNX17 knockdown FRT epithelia. The addition of amiloride (5 μM) is indicated and the subsequent change in current was calculated and termed I_{sc} - Amil. B. Collective data for I_{sc} - Amil control and SNX17 siRNA knockdown epithelia. Data shown as mean ± SD relative to I_{sc} - Amil in control knockdown epithelia. One-sample T-test, *** Indicates P < 0.001, N = 9. C. Summary of data for RT in control and SNX17 siRNA knockdown epithelia with the 300 Ω·cm², RT threshold indicated by dotted line. Data shown as mean ± SD, P > 0.05, N = 9.
3.2.4 Measurement of $I_{sc}$-Amil in mCCD Epithelia with siRNA Knockdown of SNX17.

With functional Ussing chamber measurements demonstrating a significant reduction in $I_{sc}$-Amil in SNX17 siRNA knockdown FRT epithelia, the next step of this project was to determine if this response was reflected in a cell line endogenously expressing ENaC. As with the FRT epithelia, the mCCDs were transiently transfected with control or SNX17 siRNA, before being left to grow on Snapwell™ filters for 5 days to allow the formation of a confluent, polarised, epithelial monolayer. Similar to the FRT epithelia, due to variation in passage numbers, changes in number of cells seeded, transfection efficiency and monolayer confluency, each experiment was normalised to the control epithelia in order for the measured $I_{sc}$-Amil of the SNX17 knockdown mCCD epithelia to be analysed over a number of passages.

Figure 3.9 A and 3.9 B demonstrates that the observed $I_{sc}$-Amil produced by the SNX17 siRNA knockdown mCCD epithelia was decreased (1 for control vs. 0.65 ± 0.20 for SNX17 siRNA knockdown, $P < 0.0001$, $N = 9$), with a significant reduction of 35 ± 0.06 % in the $I_{sc}$-Amil produced by the SNX17 siRNA knockdown epithelia in comparison to the control epithelia. This significant decrease in $I_{sc}$-Amil suggests ENaC cell surface population is reduced and supports observations from the FRT cell line, indicating a role for retriever in ENaC recycling.

The transient knockdown of SNX17 had no significant effect on the RT of the epithelia (508 $\Omega \cdot \text{cm}^2 \pm 46 \Omega \cdot \text{cm}^2$ for control epithelia vs. 607 $\Omega \cdot \text{cm}^2 \pm 52 \Omega \cdot \text{cm}^2$ for SNX17 siRNA knockdown epithelia $N = 9$, $P > 0.5$) (Figure 3.9 C), suggesting that the knockdown of SNX17 did not affect the integrity of the epithelial monolayer.
In summary, knockdown of SNX17 via siRNA in two epithelial cell lines resulted in a significant reduction in ENaC $I_{sc}$-Amil. This response indicated that in the SNX17 knockdown epithelia there was reduced ENaC cell surface population as the result of decreased ENaC recycling due to impaired retriever function. To further support this hypothesis, these functional experiments were followed up with measurements of ENaC cell surface population in the presence of SNX17 siRNA. (Section 3.2.5).
3.2.5 SNX17 siRNA Knockdown Decreases ENaC Plasma Membrane Levels in FRT Cells

Functional Ussing chamber measurements demonstrated that a siRNA SNX17 knockdown in two epithelial cell lines led to reduced \( I_{sc} \)-Amil in comparison with control epithelia. This decrease in current indicated that in the presence of SNX17 siRNA, retriever function is impaired therefore decreasing ENaC recycling to the apical membrane. However, as mentioned previously, measuring ENaC current using an Ussing chamber does not indicate whether changes in \( I_{sc} \)-Amil are the result of decreased cell surface population or due to changes in channel \( P_0 \).

Therefore, in order to analyse ENaC levels at the plasma membrane, cell surface protein biotinylation assays were performed. FRTs were transiently transfected with genes encoding \( \alpha, \beta \)-HA tagged and \( \gamma \)-ENaC alongside either control or SNX17 siRNA before these transfected cell culture plates were subsequently incubated with EZ-link-Sulfo-NHS-LC biotin. Notably, an FRT control plate was not incubated with EZ-link-Sulfo-NHS-LC biotin in order to serve as a negative control. Cells were then lysed, and lysates were incubated overnight with Neutravidin beads to separate surface from cytosolic proteins. ENaC levels, both cell surface and total were detected with Western blot.

As seen from the representative experiment displayed in Figure 3.10 A, surface \( \beta \)-HA ENaC in the presence of SNX17 siRNA was decreased compared with control surface \( \beta \)-HA ENaC. This was a significant reduction of 77% ± 17% \((P < 0.05, N = 3)\) (Figure 3.10 B) and indicates that the decrease in \( I_{sc} \)-Amil observed with the SNX17 siRNA knockdown epithelia is not the consequence of changes to ENaC \( P_0 \), rather suggesting ENaC recycling from the early endosome to the cell surface is reduced through decreased retriever activity.
Figure 3.10. SNX17 siRNA KD Decreases ENaC Levels at the Plasma Membrane. FRT cells transfected with either control or SNX17 siRNA along with plasmids encoding α, β-HA and γ-ENaC, were incubated with EZ-link-Sulfo-NHS-LC biotin before being lysed and incubated with Neutravidin-agarose beads to separate out cytosolic and plasma membrane proteins. Proteins were then separated by SDS-PAGE and ENaC levels, both plasma membrane and total, were determined by Western blot. A. Representative experiment demonstrating cell surface and cytosolic ENaC. Lane 1 shows surface β-HA ENaC without the presence of biotin (Negative Control), Lane 2 shows surface β-HA ENaC in the presence of biotin (Positive Control) and Lane 3 shows surface β-HA ENaC in the presence of SNX17 siRNA and biotin. Protein marker molecular weights are indicated. B, β-HA ENaC and β-actin bands were scanned and quantified by densitometry using Image J software, and statistically analysed using GraphPad Prism. Each β-HA ENaC cytosolic band was normalised against the corresponding β-actin band, which was then used against the corresponding β-HA ENaC surface band before being normalised to the control (siControl), and plotted as mean ± SD. One-sample T-test, N =3, * indicates P<0.05.
3.2.6 SNX17 Co-Immunoprecipitates with β-HA ENaC

The data gathered from this project has demonstrated that in the presence of a SNX17 siRNA knockdown, ENaC cell surface population is reduced, indicating reduced ENaC channel recycling. SNX17 is a known cargo binding protein for recruiting several proteins for their retriever mediated recycling pathway (Steinberg et al., 2012; Sotelo et al., 2014; Osborne et al., 2015). In order to investigate whether SNX17 binds ENaC as a cargo protein, Co-IP experiments were performed.

HEK-293 cells were transiently transfected with plasmids encoding α-, β-HA tagged and γ-ENaC, before a 24 hr incubation period followed by cell lysis. Lysates were subsequently incubated with anti-HA antibody to immunoprecipitate β-HA ENaC. Western blot with anti-SNX17 was then performed in order to detect if SNX17 was pulled down alongside β-HA ENaC. As seen in Figure 3.11, β-HA tagged ENaC and endogenous SNX17 were present in the cytosolic lysates. However, when one lysate was incubated with anti-goat antibody, the β-HA tagged ENaC was unable to be immunoprecipitated with this antibody thus serving as a negative control (lane 4). However, when the second lysate sample was incubated with an anti-HA antibody, β-HA tagged ENaC was able to be immunoprecipitated. Furthermore, to determine if SNX17 was pulled down with β-HA ENaC, an anti-SNX17 antibody was used to detect the presence of SNX17, where it was observed that SNX17 was co-immunoprecipitated with ENaC (Figure 3.11) (lane 5). This indicated that a protein-protein interaction exists between SNX17 and ENaC suggesting SNX17 binds ENaC as a cargo protein in a SNX17-retriever mediated recycling pathway.

With an interaction between SNX17 and ENaC detected, the next step in understanding the mechanism of retriever-mediated ENaC recycling, involves locating a binding site between the
two proteins. ENaC binds a number of proteins involved in trafficking as each subunit C-terminal domain is able to bind at least two proteins needed for ENaC trafficking. Namely the PY motif which commonly serves as the binding site for the E3-ubiquitin ligase NEDD4-2, initiating ubiquitination and therefore, internalisation of ENaC (Schild et al., 1996; Staub et al., 1996) and the endocytosis motif (YXXL) that binds the μ2 subunit of the AP-2 complex to promote targeting of ENaC to clathrin coated pits (Wiemuth et al., 2007). Therefore, with evidence demonstrating interactions between ENaC subunits and trafficking proteins such as NEDD4-2 and AP-2, these binding motifs were hypothesised as a site for interaction with SNX17-retriever. An available construct with a PY motif deletion was used to start the process of identifying a potential binding site between SNX17 and ENaC. This available construct was a mutant βY620A ENaC subunit, where a conserved tyrosine, in the PY motif, has been mutated to alanine and prevents binding to NEDD4-2 (Lu et al., 2007), was utilised for Co-IP experiments. HEK-293 cells were transiently transfected with plasmids encoding α-, βY620A-HA tagged and γ- ENaC, before being incubated for 24 hr and lysed. Lysates were incubated with anti-HA antibody to immunoprecipitate βY620A-HA ENaC, followed by Western blot with anti-SNX17 to detect if SNX17 was pulled down alongside βY620A-HA ENaC. As seen in lane 6 of Figure 3.11, SNX17 was demonstrated to co-immunoprecipitate alongside βY620A-HA ENaC. With a protein-protein interaction detected between SNX17 and the mutant βY620A-HA ENaC, this indicates that the two proteins are not interacting at this site.
SNX17 Interacts With β-HA ENaC and β_{Y620A}-ENaC. HEK293 cells were transiently transfected with plasmids encoding α-ENaC and γ-ENaC along with HA-tagged β-ENaC or HA-tagged β_{Y620A}-ENaC. Cells were lysed 24 hours post transfection, and immunoprecipitation experiments were performed using anti-HA or anti-goat (Negative Control) antibodies to immunoprecipitate ENaC. Lysates (input) and immunoprecipitates (IP) were analysed with Western blot (IB) using anti-HA and anti-SNX17 antibodies. Protein marker molecular weights are indicated. N = 3

<table>
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<th>Input</th>
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<tr>
<td>αβ-HAyENα</td>
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To conclude, siRNA knockdown of SNX3 and SNX17 in two epithelial cell lines are demonstrated to decrease ENaC current and cell surface population, thus suggesting a role for both proteins and therefore their associated recycling complexes in the recycling of ENaC. However, while investigating the potential of protein-protein interactions between SNX3 or SNX17 and ENaC using Co-IPs, an interaction between SNX17 and ENaC was detected, further supporting a role for SNX17-retriever in ENaC recycling. The significance of these findings will be discussed in the following section.
4. Discussion
4.1 Preface

ENaC is an integral epithelial membrane protein acting to facilitate Na\(^+\) ion movement across the apical membrane subsequently influencing water absorption and salt homeostasis, and therefore, blood pressure. Hence, tight control over ENaC trafficking events which determine ENaC cell surface population is crucial. It is now understood that ENaC endocytosis requires the E3 ubiquitin ligase NEDD4-2, which interacts with proline rich PY motifs on the C-terminal domains of ENaC subunits, facilitating the ubiquitination of lysine residues on the N-terminal domain of the subunits (Section 1.8.2) (Staub \textit{et al.}, 1997; McDonald \textit{et al.}, 2002; Kabra \textit{et al.}, 2008). This ubiquitination promotes sequestration of ENaC to clathrin coated pits through interaction with AP-2 and epsin, and ENaC containing clathrin vesicles are transported to the early endosome where the fate of the protein is decided (Section 1.8.2) (Shimkets \textit{et al.}, 1997; Conner & Schmid, 2003; Wang \textit{et al.}, 2006). From the endosome ENaC can either be trafficked to the lysosome for degradation or diverted from this degradative fate after deubiquitination by USP-45 or UCH-L3 (Section 1.8.3) (Butterworth \textit{et al.}, 2007; Fakitsas \textit{et al.}, 2007; Oberfeld \textit{et al.}, 2011). The removal of ubiquitin from ENaC allows for the channel to enter its recycling pathway through fast Rab11 dependent or slower Rab4/27 dependent pathways (Section 1.8.3) (Saxena \textit{et al.}, 2006a; Saxena \textit{et al.}, 2006b; Yudowski \textit{et al.}, 2009; Butterworth \textit{et al.}, 2012). The mechanisms involved within ENaC recycling are still yet to be fully understood, however, recently, both the retromer and retriever multiprotein complexes have emerged as key players in the recycling of many cargoes from the early endosome back to the plasma membrane (Strochlic \textit{et al.}, 2007; Harterink \textit{et al.}, 2011; Zhang \textit{et al.}, 2011; Steinberg \textit{et al.}, 2012; Sotelo \textit{et al.}, 2014). In this study, I investigated two SNX cargo binding proteins associated with either retromer or retriever to ask if one or both of these recycling protein complexes have a role in ENaC trafficking and recycling.
Both the retromer and retriever complexes are described as having a core complex comprised of a heterotrimer of proteins that associates with SNX proteins to carry out its function (Griffin et al., 2005; McNally et al., 2017). The SNX-BAR proteins; SNX1 and SNX2, are both able to associate with the retromer core VPS heterotrimer forming the classic model of retromer, allowing for the selected cargo to be directed into SNX-BAR induced endosomal membrane tubule extensions which are subsequently recycled back to the plasma membrane (van Weering et al., 2012). The primary aim of my 400-level project was to investigate whether there is a role for retromer in ENaC recycling, focusing specifically on the SNX1 and SNX2 proteins. Data collected from that project suggested a role for both of these proteins, and therefore, retromer in ENaC recycling (Scott, unpublished). However, the data gathered also suggested involvement of other SNX proteins in ENaC recycling, whether it be compensation for these compromised SNX-BAR proteins or another pathway working alongside SNX-BAR mediated recycling (Scott, unpublished). This led to a search for other SNX proteins which have been identified as associating with retromer and are involved in recycling of other membrane proteins. Two SNX proteins; SNX3 and SNX17, were identified from the literature to associate with retromer and the recently described retriever complex respectively. These proteins are involved in capturing specific cargo directing them into their recycling pathway (Stockinger et al., 2002; Harterink et al., 2011; Steinberg et al., 2012; Harrison et al., 2014; Lucas et al., 2016; McNally et al., 2017). This provided the rationale for this current project, which aimed to expand our understanding of retromer or retriever-mediated ENaC recycling through determining whether SNX3 or SNX17 play a role in this pathway.

Therefore, in order to investigate this aim, several experiments were performed including, the knockdown of SNX3 and SNX17 protein levels in two epithelial cell lines using siRNA. The functional effect of a SNX3 and SNX17 siRNA knockdown on ENaC recycling was examined.
using electrophysiological measurements, which were followed up with cell surface biotinylation assays to examine ENaC cell surface population in the presence of the siRNA knockdown. Finally, Co-IPs were performed to investigate possible protein-protein interactions between SNX3 or SNX17 and ENaC. The significance of the findings from these experiments are discussed in the following sections.

4.2 The Importance of SNX3 in ENaC Recycling.

The aim of this project was to determine whether SNX3-retromer or SNX17-retriever play a role in the ENaC recycling pathway. In-house studies have indicated that a siRNA knockdown of the SNX-BAR proteins; SNX1 or SNX2 reduced ENaC recycling in FRT epithelia (Scott, unpublished). However, when both SNX-BAR proteins were knocked down simultaneously, preventing the formation of the SNX-BAR heterodimer, the effects on ENaC current and recycling were similar to that of the individual SNX1 or SNX2 siRNA knockdowns. This unexpected finding indicated a role for other proteins in the recycling pathway of ENaC to compensate when the pathway is compromised, or another pathway, such as that mediated by retriever, working in conjunction with the retromer pathway.

It has been demonstrated that although the SNX–BAR heterodimer can bind PI3P in the endosomal membrane (Carlton et al., 2004), retromer itself does not have intrinsic membrane-binding ability. Targeting of retromer to the early endosome occurs, in part, through its interaction with SNX3, due to the specific interaction of the SNX3 SNX-PX domain with PI3P (Xu et al., 2001; Pons et al., 2008). In addition to recruiting retromer to the early endosome, there has also been evidence supporting a role for SNX3 in the binding of specific cargo proteins bringing them into their endosomal recycling pathway (Harterink et al., 2011). The retrograde trafficking of several proteins has also been shown to be dependent on both
retromer and SNX3, although independent to the SNX-BAR subcomplex (Belenkaya et al., 2008; Franch-Marro et al., 2008; Harterink et al., 2011). This supports the hypothesis of different retromer models mediating distinct trafficking pathways from endosomes to the TGN that was previously suggested in-house. Therefore, this project began with focusing on the SNX3-retromer model and whether it plays a role the recycling of ENaC.

SNX3 was targeted using a transient siRNA knockdown in two epithelial cell lines, one endogenously expressing ENaC, the other requiring transfection of genes encoding ENaC channel subunits. This knockdown of SNX3 was confirmed using Western blot and with a SNX3 protein knockdown confirmed in both cell lines (Figure 3.1), the functional consequence of this knockdown was investigated through observing changes in ENaC current when blocked with amiloride. The $I_{sc}$-Amil produced by the SNX3 siRNA knockdown epithelia was significantly reduced in comparison with the control epithelia (Figures 3.3, 3.4). This response indicated a reduced ENaC cell surface population as a result of the siRNA knockdown, however, measuring ENaC current using an Ussing chamber is an indirect measurement of ENaC surface population and therefore changes in measured current are unable to be directly attributed to disruptions in exocytosis, endocytosis or recycling. Measurement of $I_{sc}$-Amil also does not indicate altered $P_o$ of membrane bound channels. Therefore, to analyse ENaC levels at the plasma membrane, cell surface biotinylation assays were performed which demonstrated that the siRNA knockdown of SNX3 led to a reduction in ENaC cell surface levels compared with control cells (Figure 3.5). This reduction is consistent with the hypothesis that the reduction in $I_{sc}$-Amil observed in the two epithelial cell lines with SNX3 knockdown was likely due to a decrease in ENaC cell surface population rather than changes in ENaC $P_o$. This observation further supports the hypothesis that SNX3-retromer plays a role in ENaC
recycling, where a reduction in SNX3 protein levels leads to reduced recycling of ENaC back to the apical surface resulting in a decreased ENaC cell surface population.

Notably, Boulkroun and colleagues (2008), have demonstrated that the vasopressin-induced deubiquitinating enzyme Usp-10 regulates ENaC via an indirect mechanism, where instead of deubiquitylating ENaC to rescue it from degradation, Usp-10 deubiquitylates SNX3 to increase the level of SNX3 available for recycling cargo proteins. Boulkroun et al., (2008) also demonstrated that overexpression of both Usp-10 and SNX3 in HEK-293 cells led to an increase in ENaC levels. Interestingly, when this group performed cell surface biotinylation assays with overexpressed SNX3, ENaC levels were increased both in the total lysate and at the cell surface. This elevated total pool of ENaC was attributed to increased recycling of ENaC, which would otherwise be degraded by the lysosomal system. The results from the current investigation confirm the findings of the Boulkroun study, with both projects demonstrating that SNX3 is involved in the recycling of ENaC, across multiple cell lines, to the apical surface, and suggesting that increasing SNX3 promotes ENaC recycling, while reduction of SNX3 inhibits ENaC recycling.

With data indicating a role for SNX3, and therefore, the retromer complex in ENaC recycling, the next step was to investigate whether SNX3 acts as the cargo binding protein facilitating interaction between ENaC and retromer. Co-IP assays were performed to detect if protein-protein interactions exist between SNX3 and ENaC. Therefore, genes encoding α-, β-HA tagged and γ- ENaC subunits were transiently transfected into HEK-293 cells and when ENaC was immunoprecipitated, SNX3 was not observed to interact with ENaC (Figure 3.6), suggesting ENaC is not a cargo of SNX3. As a positive control, in parallel experiments, it was observed that SNX17 interacts with ENaC (see Figure 3.11), therefore the Co-IP technique used is robust.
Interestingly, when Boulkroun and co-workers (2008), performed Co-IP assays using HEK-293 transiently transfected with α-HA tagged, β- and γ- ENaC, a protein-protein interaction was detected between SNX3 and ENaC, therefore, providing conflicting results to the observations from this project. It is important to note that Boulkroun et al., (2008) overexpressed S-tagged SNX3 alongside ENaC in their HEK-293 cells, whereas for this project endogenous SNX3 levels were used. This may provide an explanation as to why an interaction between ENaC and SNX3 was not observed as endogenous levels of SNX3 may have been too low or there may be weak associations between the two proteins that this technique was unable to detect. Repeating these experiments using overexpressed SNX3 would be useful in determining if an interaction between the two proteins exists, and ultimately to perform a Co-IP in either kidney tissue or mCCD cell lysates using antibodies recognising both endogenous ENaC and endogenous SNX3. Immunocytochemistry would also be useful to investigate cellular co-localisation of SNX3 and ENaC in kidney epithelia, such as the mCCD cell line.

4.3 SNX17 and ENaC Recycling to the Cell Surface

With no protein-protein interaction detected between SNX3 and ENaC, the SNX17-retriever complex became the focus for this project. Similar to SNX3, a transient siRNA knockdown of SNX17 in two epithelial cell lines was confirmed (Figure 3.7), with the functional significance of this siRNA knockdown investigated using electrophysiological Ussing chamber assays, where ENaC current was measured. After addition of amiloride to block ENaC, the $I_{sc}$-Amil produced by the siRNA SNX17 knockdown epithelia was significantly reduced in comparison with the control epithelia indicating ENaC cell surface population was reduced (Figures 3.8, 3.9). However, as mentioned previously, changes in measured $I_{sc}$-Amil cannot be directly attributed to disruptions in exocytosis, endocytosis or recycling, as $I_{sc}$-Amil also gives a measure of $P_{o}$ of membrane channels. To support the functional data, cell surface
biotinylation assays were performed in order to directly analyse ENaC levels at the plasma membrane. Here, it was observed that siRNA knockdown of SNX17 significantly reduced ENaC cell surface levels compared with control FRT cells (Figure 3.10), indicating that the reduction in I_{sc}-Amil observed with the Ussing chamber assays was likely due to decreased ENaC cell surface population rather than changes in ENaC P_0. Collectively, the data gathered suggests that SNX17, and thus the retriever complex influences ENaC recycling back to the apical surface. This is the first report of a relationship between SNX17 and ENaC, however, SNX17 siRNA knockdown experiments with other SNX17 regulated proteins including T-cell receptor and integrin were demonstrated to decrease cell surface expression similar to that observed with ENaC (Osborne et al., 2015).

Similar to SNX3, with data indicating a role for SNX17-retriever in ENaC recycling, the next step for this project was to investigate whether SNX17 binds ENaC as a cargo protein. Co-IP assays were performed where HEK-293 cells were transiently transfected with genes encoding α-, β-HA tagged and γ-ENaC subunits with ENaC being immunoprecipitated. Western blot with anti-SNX17 was then performed in order to detect if endogenous SNX17 was co-immunoprecipitated alongside β-HA ENaC. SNX17 was observed to interact with ENaC (Figure 3.11), suggesting that SNX17 may be the cargo binding protein bringing ENaC into a retriever-mediated recycling pathway.

With an interaction between SNX17 and ENaC detected, the next step in understanding the mechanism of retriever-mediated ENaC recycling was to determine a potential binding site between the two proteins. Each ENaC subunit C-terminal domain contains a conserved stretch of amino acids (PPPXYXXL) that is confirmed to bind to at least two proteins needed for ENaC trafficking, namely the PY motif, which commonly serves as the binding site for the E3-
ubiquitin ligase NEDD4-2, initiating ubiquitination and therefore, internalisation of ENaC (Schild et al., 1996; Staub et al., 1996), and the endocytosis motif (YXXL) that binds the μ2 subunit of the AP-2 complex to promote targeting of ENaC to clathrin coated pits (Wiemuth et al., 2007). By the time ENaC is in the endosome NEDD4-2 and μ2 are unlikely to remain binding to this part of ENaC, therefore this region would be available to be used as a recycling motif similar to other tyrosine-based motifs such as NPXY used by SNX17 (Steinberg et al., 2012). To determine if this C-terminal region also serves as an SNX17-ENaC binding site, a mutant βY620A-HA tagged ENaC subunit, where the conserved tyrosine has been mutated to alanine was utilised for the Co-IP experiments. SNX17 was demonstrated to co-immunoprecipitate with βY620A-HA ENaC (Figure 3.11), hence indicating that the two proteins are not interacting at this particular site. Therefore, it can be concluded that although this project suggests a role for SNX17-retriever in ENaC recycling, further investigation needs to be conducted to understand how these two proteins associate.

4.4 Comparing the Retromer and Retriever Recycling Complexes

When endocytosed from the cell surface, proteins are directed to enter early endosomes where they follow a pathway resulting in the degradation of the endosomal contents by lysosomes. However, several proteins are able to be rescued from this degradative fate and can be recycled back to the cell surface either directly, via recycling endosomes or via the TGN (Grant & Donaldson, 2009). This recycling pathway is mediated by multiprotein recycling complexes; with the most established complex being retromer, an ancient endosomal sorting complex, conserved in all eukaryotes (Koumandou et al., 2011). Mammalian retromer consists of the VPS26, VPS35, and VPS29 proteins which exist as a heterotrimer alongside (Norwood et al., 2011), SNX1 or SNX2 proteins which dimerise with SNX5 or SNX6 (van Weering et al., 2012). These SNX proteins all contain a BAR domain that can sense and induce membrane
Several cargo have been established as binding directly to retromer, including TBC1D5 – a member of the Tre2-Bub2-Cdc16 (TBC) family of Rab GTPase-activating proteins, TBC1D5 binds directly to VPS29 (Seaman et al., 2009; Harbour & Seaman, 2011). However, it has also been demonstrated that SNX3, a sorting nexin protein lacking a BAR domain, directly engages cargo such as the yeast iron transporter Fet3p-Ftr1p and Wnt while simultaneously associating with retromer for retrieval of these cargo into their recycling pathway (Strochlic et al., 2007; Harterink et al., 2011). Therefore, retromer is suggested to function by directly binding specific cargo through its VPS26-VPS35-VPS29 heterotrimer or through association with SNX3 (Temkin et al., 2011; Lucas et al., 2016). This captured cargo is then enriched and enclosed into endosomal membrane tubules generated by the retromer SNX-BAR heterodimer with the aid of WASH-dependent actin polymerisation to target the captured cargo to a specific membrane (Gallon & Cullen, 2015). Interestingly, it was originally reported that retromer is able to interact with the CCC complex via the WASH complex FAM21 protein (Figure 1.6). However, more recently it has been recognised that this CCC complex appears to more likely function with retriever (see below). Super-resolution imaging has shown that retromer cargo destined for the plasma membrane and the TGN are sorted into the same tubular carriers, suggesting that targeted recycling to a specific membrane occurs downstream of endosomal retromer (Varandas et al., 2016).

SNX3 contains only one structural domain, the PX domain (Figure 1.7), which binds to PI3P allowing association with the endosomal membrane (Xu et al., 2001). Retromer is able to be recruited to the endosomal membrane through SNX3, with SNX3 binding to the retromer.
VPS26–VPS35 interface. Upon binding to SNX3, VPS26 undergoes a conformational change in its cargo-binding motif, allowing for recognition of cargo by both VPS26 and SNX3 (Lucas et al., 2016). Thus, the SNX3-retromer complex integrates two different activities: membrane binding and cargo recognition. The ability of retromer to directly interact with cargo and via cargo adaptors such as SNX3 increases the number of cargo retromer can retrieve and recycle, signifying the range of cellular functions for which retromer-mediated sorting and recycling is required (Cullen & Korswagen, 2011).

The recently identified retriever complex is another example of an established recycling complex involved in rescuing membrane proteins from their degradative fate (McNally et al., 2017). Similar to retromer, retriever is a heterotrimer consisting of the DSCR3 (VPS26C) and C16orf62 (VPS35L) proteins alongside the VPS29 protein, shared with retromer. Retriever and retromer share similarities in their structural composition, with the most obvious similarity being VPS29, a subunit of both complexes. In addition, the VPS26C protein is a homolog of the retromer component VPS26 (Aubry et al., 2009; Koumandou et al., 2011). Furthermore, VPS3L is predicted to contain \( \alpha \)-helical repeats, a structural feature present in the retromer subunit VPS35 (Hierro et al., 2007; Lucas et al., 2016). Therefore, retriever and retromer are both heterotrimers that contain a VPS29 subunit, a protein with an arrestin-like fold, and a protein containing a series of \( \alpha \)-helical repeats (McNally et al., 2017). It has yet to be established whether the retriever heterotrimer can bind to cargo directly, as has been reported for retromer.

SNX17, a sorting nexin protein that associates with endosomes through a phosphoinositide-binding module, has been shown to mediate recycling of several cargoes in a retromer-independent manner (Stockinger et al., 2002; Steinberg et al., 2012). However, the associated
proteins that function alongside SNX17 in this process were largely unknown until McNally et al., (2017), using comparative proteomic analysis, demonstrated that functional SNX17 is physically linked to the retriever proteins; VPS26C, VPS35L and VPS29. SNX17 possesses a unique C-terminal polypeptide sequence that interacts with the VPS26C protein. This relationship between VPS26C and SNX17 is similar to SNX27 association with the equivalent retromer subunit, VPS26 (Section 1.9.7) (Steinberg et al., 2013; Gallon et al., 2014). In addition to its PX domain, SNX17 contains a FERM domain, which recognizes NPXY/NXXY motifs found in a variety of cargo proteins such as β1-integrin (Bottcher et al., 2012). Notably, siRNA suppression of the retriever subunits VPS26C and VPS35L was demonstrated to result in the lysosomal degradation the SNX17 cargo α5β1-integrin (McNally et al., 2017). In contrast, knockout of the retromer subunit VPS35 did not show this phenotype, demonstrating that retriever-dependent sorting of cargo is independent of retromer (McNally et al., 2017).

Interestingly, unlike retromer association with SNX3, recruitment of retriever to the endosomal membrane does not require an interaction between SNX17 and retriever (Phillips-Krawczak et al., 2015). Rather it has been demonstrated the recruitment of retriever to the endosomal membrane requires a functional CCC complex that is interacting with the FAM21 subunit of the WASH complex present at the surface of endosomes (Phillips-Krawczak et al., 2015). It has been identified that the CCC complex interacts with components of retriever, and more importantly components of the CCC complex have been identified to interact with SNX17 (Phillips-Krawczak et al., 2015; McNally et al., 2017). This interaction between SNX17 and the CCC complex is dependent upon retriever and furthermore, suppression of the CCC complex proteins CCDC22 or CCDC93 results in the lysosomal degradation of the SNX17 cargo α5β1-integrin, indicating that SNX17, retriever, and the CCC complex are functionally linked (McNally et al., 2017). Importantly for this project, in-house studies using siRNA knockdown
of the CCC complex subunit CCDC22, altered ENaC $l_{sc}$-Amil, and reduced β-ENaC at the cell surface, supporting a role for the retriever complex in ENaC recycling (McDonald, unpublished).

In addition to their structural parallels, retromer and retriever also share functional similarities. McNally et al., (2017), demonstrated that the retriever complex is essential for the recycling of previously characterised SNX17 cargoes, such as β1-integrin (Stockinger et al., 2002; Burden et al., 2004; Bottcher et al., 2012; Steinberg et al., 2012). siRNA suppression of retriever components decreased β1-integrin surface abundance and total protein levels. In addition, pharmacological inhibition of lysosomal activity led to the recovery of β1-integrin protein levels suggesting that β1-integrin was being degraded in the lysosome.

To identify new SNX17–retriever cargoes McNally et al., (2017) performed a proteomic screen for proteins enriched on the surface of control versus SNX17 depleted cells. In addition to known SNX17-associated cargoes, a number of new transmembrane proteins containing the SNX17 interaction motif (NXXY) were identified. By comparing the datasets of cargoes enriched on the cell surface after SNX17 depletion with those of cargoes enriched following depletion of the retromer subunits VPS35 or SNX27, it was demonstrated that there are cargoes specific to retromer (e.g., the glucose transporter GLUT1), some that are specific for retriever (e.g., β1-integrin) and some cargoes that are affected by depletion of either retromer or retriever (e.g., CD97). These findings could suggest either that co-operation between the two recycling complexes exists or that some cargoes are able to associate with both recycling complexes.
A key question still remains surrounding how cargo-bound SNX17-retriever exits the endosome. Immunoelectron microscopy studies have shown that overexpressed SNX17 is found on tubular structures indicating a tubular coat and is reminiscent of SNX-BAR/retromer tubules (van Kerkhof et al., 2005). As of yet, no SNX-BAR proteins have been demonstrated to functionally interact with retriever, however, it is possible that the retromer SNX-BAR proteins have a dual role with retriever, or instead perhaps other SNX-BAR or curvature-inducing coat proteins are required for retriever tubule formation. In addition, it is also possible that cargo-bound retriever exits the endosome in tubular structures containing retromer, utilising the SNX-BAR tubules. Future studies are required to determine whether these tubules contain both retromer and retriever or whether two classes of tubules exist.

To summarise, the retromer and retriever recycling complexes are both trimeric complexes that are structurally and functionally analogous to one another. Both complexes localise to the same endosomal domain (Phillips-Krawczak et al., 2015; McNally et al., 2017), associate with sorting nexins for specific cargo selection, rescue transmembrane cargoes from the lysosomal pathway and depend on the WASH complex for cargo sorting. However, several differences exist between the two complexes, including the different modes of recruitment to endosomes. Retromer does not contain any membrane binding domains and instead relies upon interactions with SNX3 for endosomal membrane association (Rojas et al., 2008; Seaman et al., 2009; van Weering et al., 2012), while retriever instead relies upon interactions with an additional endosomal sorting complex, the CCC complex for its recruitment to endosomes (McNally et al., 2017). Another difference between the complexes is the differential involvement of the CCC complex which is essential for retriever, but not necessary for the function of retromer. Future studies will be essential to further dissect the relationship between retromer and retriever.
4.5 A Working Model for ENaC Protein Recycling.

The data collected in this project demonstrated that SNX17 is co-immunoprecipitated alongside ENaC (Figure 3.11), indicating a protein-protein interaction exists between the two proteins and that SNX17 may bind ENaC directing the channel into a retriever mediated recycling pathway. This hypothesis is further supported with functional Ussing chamber measurements where in two epithelial cell lines with a transient SNX17 siRNA knockdown, ENaC current was significantly decreased (Figures 3.8, 3.9). Furthermore, cell surface biotinylation assays demonstrated that in the presence of a SNX17 siRNA knockdown, ENaC cell surface population was significantly reduced (Figure 3.10). These findings together support the hypothesis of ENaC as a cargo of SNX17 with the SNX17-retriever recycling complex mediating ENaC trafficking and recycling.

Figure 4.1 presents a working model for SNX17-retiever mediated ENaC recycling in an epithelial cell. Recycling of ENaC begins with deubiquitination by deubiquitinating enzymes including UCH-L3, and USP-45, rescuing ENaC from a degradative fate and allowing the channel to enter its recycling pathway (Butterworth et al., 2007; Fakitsas et al., 2007). This process occurs firstly with recruitment of the CCC complex to early endosomes by the WASH complex through the interaction of the CCC complex CCDC93 protein with the WASH FAM21 protein (Figure 4.1 A (1)). Retriever is then able to associate with the CCC complex (Figure 4.1 A (2)), where it can engage SNX17 (Figure 4.1 A (3)) and bind ENaC (Figure 4.1 A (4)). With ENaC now bound to retriever via SNX17, endosomal tubular structures containing the retriever bound ENaC bud from endosomes and are directed to the apical membrane or TGN (Figure 4.1 B). As to whether SNX17-reteriver remains bound to ENaC within these tubules, there is some evidence of a SNX17 tubular coat from immunoelectron microscopy studies (van Kerkhof et al., 2005).
The SNX-BAR heterodimer is typically responsible for the formation of the endosomal membrane tubules, yet as mentioned previously no BAR-domain proteins have been demonstrated to functionally interact with the retriever complex. However, it is possible that the SNX-BAR heterodimer associated with retromer could have a dual role with retriever (as suggested in Figure 4.1), or that there are other SNX-BAR or curvature-inducing proteins (Reviewed by Jarsch et al., 2016), required for retriever budding into membrane tubular

**Figure 4.1. Proposed Model for SNX17-Retriever and SNX3-Retromer Recycling of ENaC.**

A. (1) Recruitment of the CCC complex to the endosomal membrane by WASH (2) Retriever association with the CCC complex (3) Interaction between retriever and SNX17 (4) SNX17 directly binds ENaC as a cargo protein. B. ENaC bound to SNX17-retriever or SNX3-retromer is directed into tubular structures that bud off from the endosome forming vesicles which traffic ENaC back to the apical membrane or the TGN. (Original Figure by M Scott, 2019)
carriers. In addition, cargo-bound retriever may be able to exit endosomes in tubules alongside retromer formed tubules.

When Co-IPs were performed between retromer-associated SNX3 and ENaC, no interaction was detected indicating that ENaC is not a cargo of SNX3 (Figure 3.6). However, when SNX3 was transiently knocked down using siRNA in the two epithelial cells lines, a significant reduction in ENaC current was observed (Figures 3.3, 3.4). When this functional response was followed up with cell surface biotinylation assays, a decrease in ENaC cell surface population was observed (Figure 3.5), accounting for the reductions in ENaC current previously measured. Therefore, despite the evidence indicating no apparent protein-protein interaction between SNX3 and ENaC, others have reported an interaction (Boulkroun et al., 2008), therefore, it is hypothesised that ENaC is able to use the SNX3 retromer-mediated recycling pathway whereby SNX3 recruits retromer to the endosomal membrane through binding with the retromer heterotrimer (Figure 4.1 B). SNX3 then specifically binds ENaC before the SNX-BAR heterodimer comprised of the SNX1/2/5/6 proteins initiates endosomal membrane deformation and tubulation formation (Burd & Cullen, 2014). The resulting cargo-enriched tubules bud from endosomes and are trafficked to the plasma membrane of TGN (Gallon & Cullen, 2015).

The literature demonstrates that some cargoes are specific for retromer (e.g., the glucose transporter GLUT1; Steinberg et al., 2013), some are specific for retriever (e.g., β1-integrin; Bottcher et al., 2012) and some cargoes are affected by depletion of either retromer or retriever (e.g., CD97; McNally et al., 2017). The findings from this project suggest that there is some co-operation between retriever and retromer in regard to ENaC recycling, or that ENaC is able to associate with, and therefore, be regulated by both recycling complexes. Due
to the importance of Na\(^+\) transport in fine-turning Na\(^+\) reabsorption to maintain Na\(^+\) homeostasis throughout the body, this serves as a possible explanation as to why two recycling pathways exist for this channel.

Several studies from the McDonald lab have investigated a number of retromer and recycling components to ask if those proteins have a role in ENaC trafficking and recycling. Increasing stability of the core retromer VSP26-VPS29-VPS35 heterotrimer using R55 was demonstrated to increase ENaC channel activity; while in contrast knockdown of the VPS35 protein reduced \(I_{sc}\)-Amil (Cheung and McDonald, unpublished). Both the retromer and retriever complexes interact with the WASH and CCC complexes (Section 1.9). Alterations to WASH, CCDC22 and FAM21 using siRNA or plasmids altered \(I_{sc}\)-Amil, and knockdown of CCDC22 protein reduced \(\beta\)ENaC at the cell surface (Cheung and McDonald, unpublished).

COMMD proteins have emerged as mediators of protein recycling through formation of the CCC complex alongside CCDC22 and CCDC93 (Section 1.9.9) (Phillips-Krawczak et al., 2015; McNally et al., 2017). As described previously, both retromer and retriever interact with the CCC complex where it is recruited to endosomal domains to regulate the recycling of cargo proteins (Phillips-Krawczak et al., 2015; McNally et al., 2017). The McDonald lab isolated COMMD1 as an ENaC binding protein (Biasio et al., 2004), and it was subsequently shown that all COMMDs interact with ENaC, and changes in COMMD protein populations alter ENaC current and cell surface population (Biasio et al., 2004; Ke et al., 2010; Chang et al., 2011; Liu et al., 2013; Ware et al., 2018). Investigation into these retromer and retriever components along with several of their associated proteins has further provided evidence for a role for these recycling complexes in ENaC trafficking and recycling.
With wide expression of retromer and retriever components throughout the body, it is probable that both SNX3 and SNX17 are required for the regulation of a wide variety of plasma membrane proteins including other epithelial channels and transporters, and thus may be involved in a large number of retromer and retriever associated diseases. Such examples include CFTR which is mutated in cystic fibrosis (Section 1.7.3), amyloid precursor protein (APP) that is misprocessed in the early endosome in Alzheimer’s disease (Small et al., 2005; Derivery & Gautreau, 2010; Vardarajan et al., 2012), alpha-synuclein which alters recycling in Parkinson’s disease (Vilarino-Guell et al., 2011; Zimprich et al., 2011; Zavodszyky et al., 2014; Mazzulli et al., 2016) and even ENaC which when trafficking is disrupted, can lead to hypertensive disorders (Section 1.7.1). However, further investigation centred around CFTR, APP, alpha-synuclein and ENaC would be required to analyse whether SNX3 and SNX17 are involved in these clinical manifestations.
4.6 Limitations

There were a number of limitations and set-backs encountered throughout the duration of this project which have either been overcome or adaptations made so that the data gathered was not affected. The first limitation of note was the low basal $I_{sc}$-Amil measured from the FRT epithelia in the Ussing chamber experiments. As discussed previously, this is likely attributable to the high passage number of cells used for these experiments as it has been demonstrated that increasing cell passage number reduces the efficiency of transfection (Madeira et al., 2010; Aydin et al., 2012; de Los Milagros Bassani Molinas et al., 2014). With regards to ENaC, Widdicombe et al., (2005) demonstrated that as the passage number of the epithelium increases the $I_{sc}$-Amil reduces, suggesting a reduction in ENaC numbers in the plasma membrane, this study however was conducted in human tracheal epithelium. In terms of FRT epithelia, as the McDonald and Hamilton labs have used FRT cells for a number of years, the lowest passage number available has increased, and cells must be passaged twice after thawing before they can be transfected increasing the passage number. Experiments are continued as long as ENaC current can be observed with a transepithelial resistance > 300 $\Omega \cdot \text{cm}^2$ thus allowing for increasing passage number over a series of experiments (McDonald, unpublished work).

In terms of the siRNA knockdown of both SNX3 and SNX17 in the two cell lines used, it was difficult at times to confirm a high efficiency knockdown. This may be attributed to the efficiency of the transfection or could be due to the high passage number of cells used in these experiments. Another possibility may involve the incubation period between transfection and cell lysis, FRTs were transfected and then lysed after a 72 hr incubation period in order to match the incubation time of the FRTs used in the electrophysiological experiments, where they were left for 72 hr to form a polarised epithelia. The mCCD cell line required an even
longer incubation period time of five days. This time course may have been too long to observe
the optimal effect of the siRNA knockdown on protein level due to degradation of the siRNA
over time. Although, if the post transfection incubation period was an issue, this is curious as
in the functional Ussing chamber assays, a relatively large response of approximately 35-55%
reduction in $I_{sc}$-Amil was observed for the knockdown compared with the control epithelia
and the siRNA datasheet (Table 2) stated that siRNAs should be active for 7 days.
Concentration dependent siRNA studies or use of a mix of different siRNAs, along with a time
course study or even a stable SNX3 or SNX17 knockdown cell line (using CRISPR-cas or
lentivirus) would be helpful in investigating this issue further.

The SNX3 and SNX17 antibodies purchased for this project also provided several difficulties
when used for Western blot, where it was extremely difficult to determine the siRNA
knockdown efficiency, along with detection of the target proteins in the cell surface
biotinylation assays and Co-IPs. Both antibodies bound non-specifically to a number of
proteins, therefore, giving significant background interference when developed onto film.
Consequently, several changes to the Western blot protocol were made to optimise
conditions for a clear blot. These changes included longer TBS-T wash periods, blocking with
5% milk powder overnight at 4°C instead of one hour at room temperature and using fresh
antibody with each experiment. These changes greatly reduced this background inference
making the film suitable for statistical analysis.
4.7 Future Directions

In order to demonstrate and further investigate the underlying mechanisms by which SNX3 and SNX17 could regulate ENaC recycling, several avenues should be investigated. Firstly, this project primarily utilised a transient siRNA knockdown of the target SNX proteins to determine the effect this had on ENaC recycling, stable SNX3 and SNX17 knockdown cell lines would, therefore, be a useful tool in eliminating issues related to transient transfections.

In order to confirm that the reduction in ENaC cell surface population is the presence of SNX3 and SNX17 knockdown is not due to changes in exocytosis, Brefeldin A (BFA), a fungal metabolite that causes the collapse of the Golgi apparatus, therefore blocking anterograde trafficking between the endoplasmic reticulum and the Golgi, could be employed (Fujiwara et al., 1988). It has been demonstrated that ENaC has a high turnover rate with the rapid occurrence of both endocytosis and recycling (Wang et al., 2006; Lu et al., 2007). Therefore, treatment with BFA should result in a decrease in ENaC cell surface population by preventing the insertion of new channels into the plasma membrane (Ware et al., 2018; Scott, unpublished). Thus, whilst performing electrophysiological Ussing chamber assays, the remaining short circuit current measured would represent current generated from ENaC channels already present in the plasma membrane, or recycled ENaC channels from the endosome to the plasma membrane. When this assay was performed with SNX1 plus SNX2 knockdown there was no effect of BFA on $I_{sc}$-Amil (Scott, unpublished) suggesting at least these SNX proteins do not have a role in exocytosis of ENaC. However, this should be repeated with a siRNA knockdown of SNX3 or SNX17, and if an altered $I_{sc}$-Amil was measured, this could suggest a disruption in ENaC synthesis or exocytosis and that interference in recycling may not be the reason for the reductions in $I_{sc}$-Amil observed previously as part of this project. This may serve as a possible explanation as to why no protein-protein interaction between SNX3
and ENaC was detected, yet a strong functional response was observed with the Ussing chamber measurements. Notably, short term BFA treatments do not affect the recycling of ENaC trafficking from endosomal compartments to the plasma membrane (Butterworth et al., 2005).

Another route to investigate the importance of SNX3 and SNX17 in ENaC recycling is through overexpression experiments using transient transfection of plasmids encoding SNX3 or SNX17 genes. Similar electrophysiological experiments, along with cell surface biotinylation assays could be carried out to determine if the overexpression of SNX3 or SNX17 up regulates ENaC recycling and therefore increases ENaC cell surface population and current.

Immunocytochemistry would also be a useful tool where specific antibodies against transfected or endogenous SNX3 or SNX17 could be used to investigate their intracellular localisation together with ENaC in mCCD cells. Immunostaining specific subcellular organelles such as EEA1, which marks the early endosome, (Wilson et al., 2000), could further identify the subcellular compartments in which SNX3 and SNX17 co-localise with ENaC. A search for suitable SNX3 and 17 antibodies would be needed as the antibodies used in this study bound to multiple non-specific bands on Western blots. Another interesting element could involve investigating whether these SNX proteins are transported back to the plasma membrane with the ENaC containing vesicles or if they remain at the early endosome, and whether both retromer and retriever use the same membrane tubules to rescue their cargo.

The use of SNX3 and SNX17 knockout (KO) mouse models could also provide a suitable tool for studying the physiological effects of SNX3 and SNX17 knockdown on ENaC recycling. The knockdown effect of SNX3 and SNX17 could be examined in tissues that normally express...
ENaC by employing immunostaining and Western blot to look for changes in protein levels of ENaC in wild type, KO or diseased state mice. Similar to the SNX1/SNX2 KO mouse model (Schwarz et al., 2002; Griffin et al., 2005), if a SNX3 or SNX17 KO mouse model was identified to be embryonic lethal, a conditional KO for kidney or in a tissue where ENaC is present, such as the lungs could also be generated if necessary.

Even though the Co-IP assays between ENaC and SNX3 indicated that no interaction exists between SNX3 and ENaC, this interaction may be weak, and with others reporting an interaction between these two proteins (Boulkroun et al., 2008) and with Co-IP being one of the most commonly used methods to determine protein-protein interactions, it would be practical to repeat these experiments. Reversing the precipitated protein from ENaC to SNX3 would further help clarify if an interaction exists between the two. This was not possible for this project as the SNX3 antibody used was not able to precipitate SNX3 along with any interacting proteins. Overexpression of SNX3 or tagging the SNX3 protein similar to Boulkroun et al., (2008), would be a useful tool in overcoming this issue. Where possible, a repeat of several of the experiments from this project, including the cell surface biotinylation assays, and Co-IP assays, in another cell line that endogenously expresses ENaC, such as the kidney epithelial mCCD cell line used with the Ussing chamber assays will provide a more physiologically relevant result compared with transiently transfected cell lines such as the FRTs and HEK-293s.

ENaC is known to undergo Rab4-mediated fast (direct endosome to plasma membrane trafficking) (Saxena et al., 2006b) or Rab27/Rab11-mediated slow (to the plasma membrane via recycling endosomes) (Saxena et al., 2006a; Butterworth et al., 2012) recycling. To investigate whether SNX3-retromer or SNX17-retriever are involved in one or more of these
Rab-mediated recycling pathways, several approaches could be taken. Firstly, comparing ENaC current and cell surface population in the presence of a Rab4, Rab27 or Rab11 siRNA knockdown and a SNX3 or SNX17 siRNA knockdown would be useful to determine if there was a similar effect on ENaC recycling. In addition, knockdown of these Rab-proteins along with retromer/retriever components such as SNX3 or SNX17 could be used to investigate if there is a cumulative effect, indicating a role for retromer/retriever in these Rab-mediated recycling pathways. Finally, inhibition of fast or slow recycling alongside the knockdown of retromer/retriever components to see if there is a cumulative effect, would be useful to further support retromer/retriever involvement in Rab-mediated ENaC recycling.

If further investigations into SNX3 and SNX17 provide additional evidence of a role for these proteins in regulating ENaC recycling, then pharmacologically targeting these two SNX proteins for the treatment of hypertension may be a possibility. Hypertension affects up to one in five New Zealanders and is a significant contributor to the incidence of heart disease and stroke (www.southerncross.co.nz). Current treatments for hypertension include thiazide diuretics inhibiting sodium reabsorption through ion channels such as NCC (sodium-chloride symporter) (McCormick et al., 2011). New treatments for hypotensive and hypertensive disorders are continually being pursued. Targeting recycling of ion channels involved in regulating blood pressure, such as ENaC, may offer new treatment options for these disorders. R55, a compound established to increase the stability of the retromer complex (Mecozzi et al., 2014), has the potential to serve as one of these treatments where hypothetically R55 treatment would increase protein recycling back to the plasma membrane. In regard to hypotension treatment, R55 could be utilised to stabilise retromer, therefore upregulating ENaC recycling. Furthermore, it may be possible to employ a similar tactic to identify compounds that could down-regulate retromer or retriever, possibly through SNX3 or SNX17,
to reduce sodium reabsorption through ENaC. However, both retromer and retriever have been linked to the recycling of many membrane proteins and a drug would be required that could specifically inhibit ENaC recycling.

The findings from this project demonstrate the first evidence for SNX17-retriever mediated recycling for an ion channel in tight-absorbing epithelia. The possibility of determining the effect of SNX17-retriever and SNX3-retromer recycling on other ion channels or membrane proteins is also a possible avenue of investigation (i.e., is this observed effect of siRNA SNX3 and SNX17 protein knockdown ENaC-specific or applicable to other ion channels). There are a number of different epithelial ion channels and proteins that could be investigated, including comparing the effects of a SNX protein knockdown, as well as other retromer and retriever proteins, on proteins, such as CFTR or the potassium channel KCa3.1.

4.8 Conclusion

In conclusion, the aim of this project was to investigate a role for SNX3-retromer and SNX17-retriever in ENaC recycling. This project has shown that a transient siRNA knockdown of both SNX3 and SNX17 significantly reduces ENaC cell surface population and therefore ENaC current in two epithelial cell lines. This outcome was attributed to a reduction in ENaC recycling through inhibition of the formation of functional retromer and retriever. It can therefore be inferred that both SNX3 and SNX17 proteins play an important role in ENaC recycling. Notably, an interaction between SNX17 and ENaC was identified suggesting that ENaC is a cargo of SNX17 indicating how the channel may be brought into its retriever mediated recycling pathway. However, the exact mechanisms of ENaC recycling are yet to be determined. Future studies will be essential to further dissect the relationship between retromer and retriever in ENaC recycling, therefore, the results of this project support a role
for these complexes in this process. Understanding ENaC’s recycling pathway and how ENaC
cell surface populations are regulated could provide the opportunity to develop novel
methods to control ENaC trafficking therefore controlling Na⁺ absorption, and thus the
opportunity to regulate blood pressure and treat ENaC related disorders such as Liddle’s
syndrome and cystic fibrosis.
5. References


Cheung T & McDonald FJ. (unpublished)


Hughey RP, Mueller GM, Bruns JB, Kinlough CL, Poland PA, Harkleroad KL, Carattino MD & Kleyman TR. (2003). Maturation of the epithelial Na\(^{+}\) channel involves proteolytic


Malik B, Yue Q, Yue G, Chen XJ, Price SR, Mitch WE & Eaton DC. (2005). Role of Nedd4-2 and polyubiquitination in epithelial sodium channel degradation in untransfected renal A6


McDonald FJ. (unpublished work).


Scott ML. (unpublished work).


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Vuagniaux G, Vallet V, Jaeger NF, Hummler E & Rossier BC. (2002). Synergistic activation of ENaC by three membrane-bound channel-activating serine proteases (mCAP1, mCAP2, and mCAP3) and serum- and glucocorticoid-regulated kinase (Sgk1) in Xenopus Oocytes. Journal of General Physiology 120, 191-201.


https://www.southerncross.co.nz/group/medical-library/high-blood-pressure-hypertension


