SHORT PALATE LUNG AND NASAL EPITHELIUM CLONE 1 DISSOCIATES AND ENDOCYTOSES THE EPITHELIAL SODIUM CHANNEL

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ABSTRACT

Christine Seulki Kim: Short Palate Lung and Nasal Epithelium Clone 1 Dissociates and Endocytoses the Epithelial Sodium Channel
(Under the direction of Robert Tarran)

The movement of Na\(^+\) through the epithelial Na\(^+\) channel (ENaC) is the rate-limiting step for Na\(^+\) absorption across the apical plasma membrane of airway epithelia. In cystic fibrosis (CF), increased ENaC activity reduces airway surface liquid (ASL), thickening mucus and increasing the risk of bacterial infection. Increasing the ASL may help restore proper mucociliary clearance in airways of patients with CF. Short Palate Lung and Nasal Epithelium Clone 1 (SPLUNC1) is a multi-functional protein that is secreted in the airways. SPLUNC1 binds to βENaC through its ENaC inhibitory domain and inhibits ENaC’s ability to conduct Na\(^+\). In this dissertation, we report our novel, automated method of measuring ASL height for a potential high-throughput approach to drug evaluation on ASL hydration, and we report our findings regarding SPLUNC1’s mechanism of ENaC inhibition.

Using our automated method for measuring ASL height, we found that SPLUNC1 has a half maximal inhibitory concentration (IC\(_{50}\)) of 6.53 µM. Additionally, our surface biotinylation data revealed that SPLUNC1 selectively internalizes αγENaC while leaving βENaC at the plasma membrane. Immunostaining demonstrated that SPLUNC1’s extracellular binding to βENaC is crucial for the internalization of αγENaC and that internalized αγENaC is degraded via lysosomes. Immunoprecipitation data showed that SPLUNC1 promotes αENaC ubiquitination through the interaction of an ubiquitin ligase, Nedd4-2, and αENaC’s PY-motif. Förster
resonance energy transfer data indicated that SPLUNC1 induces conformational changes in ENaC. Time-dependent experiments revealed the order of these events: (1) SPLUNC1 binds to βENaC, changing αβγENaC’s conformation, leading to (2) the ubiquitination of αENaC and, consequently, (3) the internalization/degradation of αγENaC. This is the first report of heterotrimeric ENaC physically dissociating and leading its subunits to two different fates. These findings suggest that SPLUNC1 can increase ASL levels and lung hydration, improving mucociliary clearance, and may be a promising therapeutic agent for patients with cystic fibrosis.
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“I have learned in whatever situation I am to be content. I know how to be brought low, and I know how to abound. In any and every circumstances, I have learned the secret of facing plenty and hunger, abundance and need. I can do all things through Christ who strengthens me. (Philippians 4:10-13)”
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<td>ASIC</td>
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<td>Airway surface liquid</td>
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<td>BCA</td>
<td>Bicinchoninic acid</td>
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<td>Bcc</td>
<td>Burkholderia cepacia complex</td>
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<td>BPI</td>
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<td>BPIFA1</td>
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<td>CaCC</td>
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<td>cAMP</td>
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<td>CAP1</td>
<td>Channel-activating protease</td>
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<td>CF</td>
<td>Cystic fibrosis</td>
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<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
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<td>CFU</td>
<td>Colony-forming unit</td>
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<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<td>COPII</td>
<td>Coat complex II</td>
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<td>DEG</td>
<td>Degenrin</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>DUB</td>
<td>Deubiquitinating enzymes</td>
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<td>E3</td>
<td>Ubiquitin-protein ligase</td>
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<td>EGTA</td>
<td>Thylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
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<td>ENaC</td>
<td>Epithelial Na⁺ Channel</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ERAD</td>
<td>Endoplasmic reticulum associated degradation</td>
</tr>
<tr>
<td>ERp29</td>
<td>Endoplasmic reticulum protein 29</td>
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<td>FBS</td>
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<td>FRET</td>
<td>Förster Resonance Energy Transfer</td>
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<td>gNa</td>
<td>Channel conductance for Na⁺</td>
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<td>HBEC</td>
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<td>HG</td>
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<td>Hsp70</td>
<td>70 kDa heat shock protein</td>
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<td>I Na</td>
<td>Na⁺ current</td>
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<td>LAMP1</td>
<td>Lysosomal membrane protein 1</td>
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<td>Definition</td>
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<td>LUNX</td>
<td>Lung specific protein-X</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>N</td>
<td>Number of plasma membrane ENaC</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
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<td>NASG</td>
<td>Nasopharyngeal carcinoma-related protein</td>
</tr>
<tr>
<td>Nedd4</td>
<td>Neutral precursor cell expressed developmentally down-regulated protein 4</td>
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<tr>
<td>N-linked</td>
<td>Asparagine-linked</td>
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<tr>
<td>NSCLC</td>
<td>Non-small cell lung carcinoma</td>
</tr>
<tr>
<td>NT</td>
<td>Non-transfected</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS++</td>
<td>Phosphate buffered saline with MgCl2 and CaCl</td>
</tr>
<tr>
<td>PCL</td>
<td>Periciliary Layer</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PHA-I</td>
<td>Type I pseudohypoaldosteronism</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-OH kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PIP3</td>
<td>Phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>Po</td>
<td>Open probability or open state of ENaC</td>
</tr>
<tr>
<td>PY</td>
<td>Proline rich motif</td>
</tr>
<tr>
<td>RANSAC</td>
<td>Random Sample Consensus</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<td>SGK</td>
<td>Serine/threonine-protein kinase</td>
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<td>SLRM</td>
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<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor activating protein receptor</td>
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<td>Δ44SPLUNC1</td>
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<td>SPURT</td>
<td>Secreted protein from upper respiratory tract</td>
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<td>Definition</td>
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<tr>
<td>STIM1</td>
<td>Stromal interaction molecule 1</td>
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<tr>
<td>TBS</td>
<td>TRIS buffered saline</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TRIS</td>
<td>2-Amino-2-hydroxymethyl-propane-1,3-diol</td>
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Chapter 1: Introduction

1.1. The epithelial Na\(^{+}\) channel

According to sequence homology, the epithelial Na\(^{+}\) channel (ENaC) is a member of the ENaC/degenerin (DEG) family. The crystal structure of ENaC has not yet been solved and it has been predicted that ENaC shares a structure similar to the acid-sensing ion channel (ASIC), a closely related member of the DEG family that shares ~15–20% amino acid sequences with ENaC (de la Rosa et al., 2004; Kashlan and Kleyman, 2011; Kellenberger and Schild, 2002). Though in the same family, members of the DEG superfamily differ in their distribution. For example, the ASIC is widely expressed in neurons, while ENaC is expressed at the apical membrane of epithelia in kidneys, sweat glands, intestine, salivary glands, tongue, skin, and lungs.

The function of ENaC is sensitive to corticosteroids. Corticosteroids can be considered in terms of five different types according to the receptors to which they bind. Of these, mineralocorticoids and glucocorticoids regulate ENaC in a tissue-specific manner (Bhalla and Hallows, 2008; Garty and Palmer, 1997). For example, the function of ENaCs expressed in the kidneys, colon, and exocrine glands is under the control of the mineralocorticoids aldosterone and vasopressin (Butterworth, 2010). Similarly, the function of ENaCs expressed in epithelial cells of non-transporting epithelia, such as keratinocytes and hair follicles, is also mineralocorticoid sensitive (Brouard et al., 1999).
On the other hand, the function of ENaCs in the lung is regulated by the glucocorticoid cortisol (Rossier and Stutts, 2009). ENaC is classically characterized by its high selectivity for Na\(^+\) over K\(^+\), a low unitary conductance of 4–5 pS, and its role in Na\(^+\) transport across epithelia. It is well studied that ENaC-mediated transcellular Na\(^+\) transport plays a crucial role in maintaining the entire body’s Na\(^+\) homeostasis and hence plays a role in the regulation of luminal fluid composition and volume, and ultimately blood pressure (Bhalla and Hallows, 2008; Garty and Palmer, 1997). However, researchers have also demonstrated that ENaC has additional functions. For example, ENaCs can function as Na\(^+\) taste receptors in the tongue (Chandrashekar et al., 2010), regulate keratinocyte differentiation (Brouard et al., 1999), modulate neuronal activity and vasomotor sympathetic drive (Miller et al., 2013), and contribute to epithelial wound healing (Justet et al., 2013).

To date, five different ENaC subunits—α, β, γ, δ, and ε—have been identified (Rossier and Stutts, 2009). Of these, the ε subunit is only found in *Xenopus* renal cells (Babini et al., 2003). Furthermore, δENaCs are mostly found in the brain, testis, ovaries, and pancreas, with little to none in airways (Schwagerus et al., 2015). By contrast, α, β, and γ subunits are found in the human lung (Canessa et al., 1994; Eaton et al., 2010) and are responsible for conducting Na\(^+\) in the airways (Eaton et al., 2010). The α, β, and γ subunits share a 30% homology at the protein level with similar primary structures and membrane topologies (Canessa et al., 1994; Kashlan and Kleyman, 2011).

### 1.1.1. Molecular structure and composition of ENaC

The α, β, and γ subunits share the same basic structure of short intracellular N- and C-termini and two transmembrane domains and a large extracellular loop (Figure 1). The two transmembrane domains of all three subunits are α helices (Bonny et al., 1999; Firsov, 1998). All three subunits also share the His-Gly (HG) motif at their N-termini in close proximity to the first
transmembrane domain (Kellenberger and Schild, 2002). This HG motif functions as a link between the hydrophobic transmembrane and the intracellular N-terminus. Another conserved motif shared by α, β, and γ subunits is the proline rich (PY) motif (Figure 1). All three subunits possess the PY motif in their C-termini which serves as a binding site for the cytosolic ubiquitin ligase, neutral precursor cell expressed developmentally down-regulated protein 4 (Nedd4), and its close relative Nedd4-2 (Figure 1) (Snyder, 2002; Staub, 1997). The ENaC subunits also have cysteine-rich domains in their extracellular loops, responsible for forming disulfide bridges to maintain the tertiary structure (Firsov et al., 1999). Additionally, all three subunits possess glycosylation sites in their extracellular loops where they undergo glycosylation during maturation in the endoplasmic reticulum (ER) and trans-Golgi network (TGN) (Figure 1) (Hanwell et al., 2002; Rotin et al., 2001). Moreover, multiple protease cleavage sites have been identified in the extracellular loops of all three subunits (Figure 1). However, due to heavy glycosylation that covers the cleavage sites, the protease cleavage sites of the β subunit are inaccessible (Figure 1) (Rossier and Stutts, 2009). Therefore, only α and γ subunits undergo proteolytic cleavage, which is known to play a role in the channel’s activation (Figure 1-2) (Rossier and Stutts, 2009). A variety of serine proteases and other enzyme classes evolutionally related to serine proteases are known to affect the function of the plasma membrane ENaC through extracellular proteolysis (Rossier and Stutts, 2009). However, previous studies have demonstrated that proteolytic cleavage in the extracellular loops of α and γ subunits can also take place intracellularly with the proprotein convertase family member Furin, as the subunits of ENaC undergo processing in the ER and TGN (Hughey et al., 2004, 2003).

It has also been shown that the expression of single subunits results in inefficient trafficking of the subunit to the plasma membrane (Firsov, 1998; Kellenberger and Schild, 2002). The α, β,
and γ subunits are required for maximal expression of ENaCs on a cell’s surface (Firsov et al., 1996). Additionally, while the αENaC can function alone as a channel, the current is drastically reduced, amounting to ~2% of the maximum current (Bonny et al., 1999). Similarly, the γENaC alone can only generate a small current, ~0.5% of the maximum current. While α-β, α-γ, and β-γ co-expression is able to generate a higher current than that of a single subunit, the level of current still remains low, reaching only ~5% of the maximum current (Canessa et al., 1993; Firsov et al., 1996; McNicholas and Canessa, 1997). Research indicates that maximum ENaC expression and function occur when the ENaC is a heteromultimeric channel comprised of α, β, and γ subunits. While it is well established that the maximum current is produced when ENaC is composed of the three subunits discussed, the exact stoichiometry of ENaC is still controversial, with some studies suggesting that ENaC is a tetramer composed of 2 α, 1 β, and 1 γ subunits (Firsov, 1998; Kosari et al., 1998) and others proposing that ENaC is composed of an equal number of each subunit (Staruschenko et al., 2005, 2004).

1.1.2. ENaC regulation

1.1.2.1 Subunit regulation by genes and proteins

Even though ENaC functions as a heteromultimer comprised of α, β, and γ subunits that share similar molecular structures, each subunit is uniquely controlled in terms of genetics and proteins. In terms of genetics, α, β, and γ proteins are separately encoded by the genes SCNN1α, located on Chromosome 12 p13, SCNN1β, located on Chromosome 16 p12.2, and SCNN1γ, located on Chromosome 16 p12, respectively, suggesting that the transcription of each subunit is independent. It is understood that the mRNA levels of each subunit are regulated in a non-coordinated manner. For example, aldosterone increases the transcription of only the α subunit in the kidney, while the β and γ subunits are unaffected (Escoubet et al., 1997; Volk et al., 1995).
However, in the colon, the reverse can be observed, as aldosterone dramatically affects the transcription of β and γ subunits but not α (Farman et al., 1997). Additionally, the hormone estrogen has been shown to increase the transcription of α and γ subunits but not β in the kidney (Gambling et al., 2004). In the airways, dexamethasone has been shown to have different effects depending on incubation time. Incubation with dexamethasone for 24 h increases the transcription of the α subunit, while incubation for 24–48 h increases the transcription of β and γ subunits (Itani et al., 2002; Lazrak et al., 2000).

Similarly, ENaC subunits are individually regulated at protein levels. Treatment with dexamethasone on airway cells has been shown to result in different protein levels for ENaCs’ subunits, depending on the cell culturing system and length of incubation (Weisz and Johnson, 2003). For example, long term dexamethasone treatment of A549 cells has been shown to increase the protein levels of β and γENaCs but not αENaCs (Lazrak et al., 2000). On the other hand, on H441 cells, 24 h dexamethasone treatment has been shown to increase the protein level of αENaCs but not β and γENaCs (Itani et al., 2002). Furthermore, mRNA levels of ENaC subunits often do not correlate to their respective protein levels. For example, tumor necrosis factor-alpha (TNF-α) decreases the mRNA levels of α, β, and γ subunits, but only decreases the protein level of αENaCs (Dagenais et al., 2004).

1.1.2.2 ENaC regulation at the plasma membrane

ENaC maturation in the ER and the TGN is inefficient and only a small portion of the total ENaC protein eventually reaches the plasma membrane (Hughey et al., 2004; Valentijn et al., 1998). Thus, the fine tuning of ENaC activity at the plasma membrane plays a crucial role in the overall regulation of ENaC function (Valentijn et al., 1998; Weisz et al., 2000). ENaC is a voltage-independent channel and its \( \text{Na}^+ \) current (\( I_{\text{Na}} \)) can be calculated at the macroscopic level with the...
equation $I_{Na} = N \cdot P_{o} \cdot g_{Na}$, where $N$ is the number of channels available at the plasma membrane, $P_{o}$ is the open probability or open state of the channel, and $g$ is the channel conductance for $Na^{+}$ (Gründer et al., 1997). While the conductance of ENaC can vary depending on the location of expression, reports have shown that wild type ENaCs exhibit a consistent, averaged single-channel conductance of ~5 pS (Canessa et al., 1994; Gründer et al., 1997). Therefore, ENaC can rapidly modulate the $Na^{+}$ current at the plasma membrane via two major pathways, changing either $N$ or $P_{o}$ (Figure 1.2). The number of channels available at the apical membrane is primarily regulated by ENaC trafficking to and from the plasma membrane. In this chapter, ENaC trafficking is detailed in section 1.1.3.

Similar to other members of the DEG family, ENaC subunits arrange themselves around a central channel pore (Snyder et al., 1999). The pores of DEG family members can either be closed or open, denoted with $P_{o}$ values ranging from 0 (completely closed) to 1 (completely open). The control of the pore is referred as the gating mechanism. While DEG family members have many different gating mechanisms, unique to each member, they share a basic gating mechanism. Degenerin family members all possess either serine or glycine residue in the extracellular loop, preceding the second transmembrane domain, also known as the DEG site (Kellenberger et al., 2002). The DEG site of ENaC is located close to the outer pore entrance at $\alpha$S576, $\beta$S518, and $\gamma$S530 (Firsov et al., 1999; Rotin and Staub, 2011). Mutation of $\beta$S518 to cysteine allows hydrophilic sulfhydryl reagents to form a covalent bond, altering the channel conformation such that the open state of the channel is prolonged (Firsov et al., 1999; Kellenberger et al., 2002).

Amiloride is a K$^{+}$-sparing diuretic drug that works to inhibit ENaC functioning by binding to the channel pore and preventing $Na^{+}$ permeation through the pore (Schild et al., 1997). The binding site of amiloride in ENaC is located several residues downstream of the DEG site at $\alpha$S583,
βG525, and γG537. Mutating these amiloride binding sites significantly reduces the ability of amiloride to inhibit ENaC (Schild et al., 1997). ENaC is known to be highly sensitive to amiloride with an inhibitory constant of $10^{-7}$ M (Schild et al., 1997). This discovery led to the identification of ENaC current as amiloride sensitive. However, amiloride can also block other ion channels, including ASICs (Ugawa et al., 2002).

While many studies have suggested that proteases may regulate transepithelial Na$^+$ transport (Lewis and Clausen, 1991; Orce et al., 1980), Vallet et al. were the first to directly demonstrate that a protein from the serine protease family, the channel-activating protease (CAP1), increases ENaC activity (Vallet et al., 1997). Identified as prostasin, CAP1 is a unique serine protease as it is attached to the plasma membrane via a glycosylphosphatidylinositol anchor (Chen et al., 2001; Vallet et al., 1997). While CAP1 only cleaves the extracellular loops of α and/or γENaCs at the plasma membrane, other serine proteases and evolutionary related families of proteases such as furin or trypsin act both intracellularly and extracellularly on different proteolytic cleavage sites located in the extracellular loops of α and γENaCs (Figure 1.2) (Bruns et al., 2007; Caldwell et al., 2004; Chraïbi et al., 1998; Hughey et al., 2003; Vallet et al., 1997).

Another important regulator of ENaC are anionic phospholipids, such as phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$), products of phosphoinositide 3-OH kinase (PI3K) (Blazer-Yost et al., 1999). Studies have demonstrated that PIP$_2$ and PIP$_3$ increase the probability that ENaCs will be open through direct interaction with the N-terminal tails of the β and γ subunits (Ma et al., 2002; Tong et al., 2004; Yue et al., 2002). The P2Y receptor, a member of purinergic G protein-coupled receptors, binds nucleotides such as ATP, ADP, UTP, UDP, and DUP-glucose (Abbracchio et al., 2006). Upon binding these ligands, P2Y receptors are stimulated to activate phospholipase C (PLC), resulting
in the hydrolysis of PIP$_2$. Activated P2Y receptors can also activate PI3K, thereby increasing levels of PIP$_2$ and PIP$_3$. As such, P2Y receptors participate in the regulation of ENaCs at the plasma membrane (Ma et al., 2002; Palmer et al., 2012).

1.1.3. ENaC trafficking

1.1.3.1 Trafficking to and from the plasma membrane

As discussed, ENaC subunits move from the ER to the TGN where they are glycosylated, assembled, and delivered to the apical plasma membrane (Butterworth, 2010; Ergonul et al., 2006; Hughey et al., 2003). It is well characterized that ENaC obtains core glycans and is trimmed to a high mannose glycosylated (immature glycans) in the ER and then processed to complex Asn-linked (N-linked) glycosylation (mature glycosylation) in the TGN (Rotin et al., 2001). However, it should be noted that ENaC subunits can reach the plasma membrane with both immature and matured glycans (Rotin et al., 2001). While the details of the ENaC’s delivery route are not fully understood, a number of accessory proteins involved in its trafficking have been identified. For example, disruption of actin or tubulin generates improper ENaC trafficking (Ilatovskaya et al., 2011; Wang et al., 2013). Furthermore, soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins are known to play a role in the final vesicle fusion process (Gormley et al., 2003; Hill et al., 2007). Specifically, syntaxin, a SNARE protein, and the cytoplasmic C-termini of ENaC subunits have been shown to be directly associated (Condliffe et al., 2003; Hill et al., 2007).

Vasopressin is one of the hormones that can regulate ENaC activity by increasing the abundance of ENaCs at the plasma membrane. Vasopressin binds to a vasopressin receptor to activate adenylate cyclase, which produces cyclic adenosine monophosphate (cAMP) and activates protein kinase A (PKA). Next, PKA phosphorylates and inhibits the ubiquitin ligase Nedd4-2 from
binding to ENaCs, thus increasing the time spent by ENaCs at the plasma membrane. Additionally, serine/threonine-protein kinase (SGK) has been found to inhibit Nedd4-2 and thus increase ENaC activity. The details of how ubiquitin ligase regulates ENaC are detailed in the following section. Similar to vasopressin, epinephrine functions in the lungs to increase ENaC activity (Baines, 2013; Olver and Strang, 1974). Epinephrine is one of the ligands that activates β-adrenergic receptors, a class of G protein-coupled receptors (Olver and Strang, 1974). Activated β-adrenergic receptors increase the intracellular cAMP concentration, resulting in the activation of downstream effectors such as PKA (Olver and Strang, 1974).

1.1.3.2 Endocytosis via ubiquitination

Ubiquitin-dependent ENaC regulation is the best-characterized endocytosis pathway (Figure 1.3). However, it should be noted that ENaC can also be endocytosed via caveolae-rich lipid rafts (Butterworth, 2010; Firsov et al., 1999; Hill et al., 2007). Here, we focus on ubiquitin-dependent ENaC endocytosis. Internalization by ubiquitination requires three major actions dictated by different enzymes: first, an ubiquitin-activating enzyme (E1) activates ubiquitin by forming a thioester bond between cysteine in the catalytic site of E1 and the C-terminus of ubiquitin; second, ubiquitin is transferred to a cysteine of an ubiquitin-conjugating enzyme (E2); third, E2 together with ubiquitin interacts with an ubiquitin-protein ligase (E3), which recognizes, binds to, and transfers ubiquitin to the target protein (Rotin and Staub, 2011). Ubiquitin-protein ligases are divided into two classes based on the C-terminus domain. One class of E3 proteins contains the homologous to the E6AP carboxyl terminus (HECT) catalytic domain that binds and transfers ubiquitin to the target protein through transthiolation reactions, while the other class of E3 proteins contain a zinc-binding Really Interesting New Gene (RING) finger domain that
facilitates the direct transfer of ubiquitin to the target protein (Ciechanover, 2005; Rotin and Staub, 2011).

Ubiquitin-protein ligases Nedd4 and Nedd4-2, a Nedd4 paralogue, are key players in the ubiquitin-mediated endocytosis of ENaC (Snyder et al., 2004b; Soundararajan et al., 2012; Wiemuth et al., 2007). Both Nedd4 and Nedd4-2 contain a HECT domain. At the N-termini of Nedd4 and Nedd4-2 are four WW domains, named after the presence of two conserved tryptophans located 20–22 amino acids apart (Figure 1.3) (Bork and Sudol, 1994). The WW domains mediate the interaction with several substrates, including ENaC (Figure 1.3) (Ingham et al., 2004). While both Nedd4 and Nedd4-2 can ubiquitinate ENaC, it is known that Nedd4-2 ubiquitinates ENaC with a higher efficiency than Nedd4 (Rotin and Staub, 2011). Furthermore, it has been demonstrated that ubiquitination of ENaC takes place through the binding of Nedd4-2’s WW domain to the ENaC’s C-terminal PY-motif. Blocking the function of Nedd4-2 by inhibiting its enzymatic activity or binding to ENaC reduces ENaC ubiquitination and thus increases the level of plasma membrane ENaCs (Yang and Kumar, 2010; Zhou et al., 2007). Moreover, Nedd4-2 knock out mice exhibit salt-sensitive hypertension due to increased levels of plasma membrane ENaCs which are substantially reduced in the presence of amiloride (Yang and Kumar, 2010).

1.1.3.3 Degradation and recycling of ENaC

After internalization, ENaCs are either recycled or degraded. To prevent ENaCs from degradation for recycling, rapid deubiquitination must first take place (Figure 1.3) (Butterworth, 2010). Several deubiquitinating enzymes (DUBs) have been identified, such as UCH-L3 and USP2-45 (Rotin and Staub, 2011). The detection of DUBs in early endosomal compartments suggests that DUBs exert their effects near the apical plasma membrane (Butterworth et al., 2007; Ronzaud and Staub, 2014). Following deubiquitination, ENaCs are repackaged and trafficked back
to the apical plasma membrane (Figure 1.3). Nonetheless, the full extent of DUB’s actions on ENaC recycling and the recycling pathway of ENaC is not understood. For degradation, ENaCs take either a proteasome- or lysosome-dependent pathway (Ciechanover, 2005). While it is classically believed that ubiquitinated proteins are degraded via proteasomes (Ciechanover, 2005), it has been demonstrated that ubiquitinated plasma membrane ENaCs are likely to be degraded via lysosomes (Staub, 1997; Wiemuth et al., 2007). It is understood that ENaCs faced with both degradation and recycling are present in the early endosome (Butterworth et al., 2012). However, it is unclear how and where ENaCs are differentiated for different endocytic fates.

1.1.4. Clinical correlations between ENaCs and human diseases

1.1.4.1 Lung

ENaC plays an important role in lung fluid homeostasis. At birth, the fetal lung is filled with fluid which must be reabsorbed for it to mature and develop into a post-fetal lung (Barker et al., 1998; Hummler et al., 1996; Olver and Strang, 1974). Fetal lung epithelium depends on active Cl\(^-\) secretion for lung expansion (Barker and Olver, 2002; Jain and Eaton, 2006). At birth, the lung epithelium switches from secretory to absorption modes where ENaCs increase Na\(^+\) absorption and thereby clear the lung fluid (Barker and Olver, 2002; Jain and Eaton, 2006). A failure to actively absorb fluid and clear neonatal fluid results in transient tachypnea in newborns and neonatal respiratory distress syndrome (Elias and O’Brodovich, 2006; Gibson et al., 2003). This pivotal role for ENaC in the process of fluid absorption has been demonstrated by the creation of αENaC-deficient mice which experienced early death due to defective neonatal lung liquid clearance (Hummler et al., 1996).

The absorption of neonatal fluid results in post-fetal lungs lined with a thin layer of fluid, called airway surface liquid (ASL), that sits on top of the airway epithelium and allows normal gas
exchange to fill the lungs with air (Figure 1.4) (Matsui et al., 1998b; Tarran and Boucher, 2002; Widdicombe and Widdicombe, 1995). The ASL is comprised of two layers: a lower periciliary layer (PCL) and an upper mucus layer (Figure 1.4) (Wanner et al., 1996). The mucus layer is a combination of mucus produced by the airway epithelium and fluid, providing a thin, watery layer that is more viscoelastic than the lower PCL (Kilburn, 1968; Wanner et al., 1996). Such characteristics of the mucus layer protect the lung from inhaled pathogens (Kilburn, 1968; Matsui et al., 1998b). The lower PCL is important in facilitating the movement of mucus by providing enough fluid for cilia to beat, a process called mucociliary clearance (Boucher, 1999; Matsui et al., 1998b). Well-balanced ASL hydration is important to conserve energy, the rate of cilia beating, and mucus clearance. In vitro studies have indicated that normal ASL height is approximately 7 µm (Boucher, 1994; Kilburn, 1968; Matsui et al., 1998a; Tarran and Boucher, 2002).

Airway surface liquid homeostasis is mediated by ion and water transport (Figure 1.4) and ASL regulation is driven by Na⁺/K⁺ ATPase expressed in the basolateral membrane of ciliated cells. Na⁺/K⁺ ATPase pumps Na⁺ from cells and pumps in K⁺ (Figure 1.4). This is a primary active process that consumes ATP (Stutts et al., 1986). Based on the electrochemical gradient generated by Na⁺/K⁺ ATPase, the basolateral Na⁺/K⁺/2Cl⁻ cotransporter takes up Na⁺, K⁺, and 2 Cl⁻ into the cell (Figure 1.4) (Hollenhorst et al., 2011). Intracellular Na⁺ and K⁺ are recycled across the basolateral membrane, resulting in an energy efficient increase in intracellular Cl⁻ (Chambers et al., 2007; Lebowitz et al., 2003). The combined action of Na⁺/K⁺ ATPase and K⁺ channels creates a favorable electrochemical gradient for Na⁺ to enter the cell from the apical membrane. Na⁺ is absorbed into the cell following the electrochemical gradient through apical ENaCs (Figure 1.4) (Boucher, 2007). In turn, the vectorial transport of Na⁺ causes Cl⁻ and water to passively follow the direction of Na⁺ movement through the paracellular pathway by osmosis (Figure 1.4) (Boucher,
Likewise, during Cl⁻ secretion via the cystic fibrosis transmembrane conductance regulator (CFTR), the vectorial transport of Cl⁻ causes Na⁺ and water to passively follow the direction of Cl⁻ movement through the paracellular pathway in the opposite direction (Figure 1.4) (Boucher, 1994; Chambers et al., 2007).

An imbalance of ion/water regulation in the ASL results in harmful lung diseases. Impaired Na⁺ absorption leads to the flooding of ASL and consequently leads to persistent pulmonary edema (Deng et al., 2012; Hollenhorst et al., 2011). In the case of cystic fibrosis (CF), the absence of functioning apical CFTRs results in the impaired secretion of Cl⁻, which consequently leads to an increased level of ENaC activity and hyperabsorption of Na⁺. Cystic fibrosis transmembrane conductance regulators have been shown to directly interact with ENaCs to protect ENaCs from proteolytic cleavages and thereby inhibiting the function of ENaCs (Gentzsch et al., 2010). Thus, lack of CFTRs directly causes the hyperactivity of ENaC. An imbalance of Cl⁻ secretion and Na⁺ absorption leads to the dehydration of ASL (Boucher, 2007; Verkman et al., 2003) (Figure 1.4). As a result, mucociliary clearance is impaired and mucus accumulation results (Figure 1.4) (Boucher, 2007). Additionally, CFTR secretes HCO₃⁻ and therefore CF airways lacking CFTRs have lower levels of HCO₃⁻, leading to a lower pH of ASL (Coakley et al., 2003). This lower pH also contributes to increased incidences of bacterial colonization (Pezzulo et al., 2012). The role of ENaCs in mucociliary clearance has been validated with βENaC overexpressing mice. These mice had CF-like lungs with dehydrated ASL, increased mucus accumulation, and bacterial colonization (Zhou et al., 2011). While CF is predominantly thought of as a lung disease, it is a multi-organ disease, as CFTRs are expressed throughout the body. For example, CF patients exhibit exocrine pancreatic insufficiency, dysregulated sweat glands resulting in hypotonic sweat, and intestinal obstruction (FitzSimmons, 1993; van der Doef et al., 2011).
1.1.4.2 Kidney

In the kidney, ENaCs are expressed throughout distal convoluted tubules, connecting tubules, and collecting ducts where it functions as the rate-limiting step for Na\(^+\) reabsorption (Garty and Palmer, 1997). The importance of ENaC function in the kidney can be seen in patients with Liddle’s syndrome. These patients show blood volume expansion, hypertension, hypokalemia, low aldosterone levels, and metabolic alkalosis (Bhalla and Hallows, 2008; Shimkets et al., 1994). These clinical manifestations are due to the gain-of-function mutation introduced into the PY-motifs of β and/or γENaCs (Lu et al., 2007; Schild et al., 1995; Snyder et al., 1995). Different types of ENaC mutations have been identified in Liddle’s syndrome patients. For example, there is a premature stop codon that truncates the last 45–76 amino acids in the cytoplasmic carboxyl terminus of β and γ subunits and a frameshift mutation in the same C-terminal domain that prevents translation of the PY-motif (Melander et al., 1998). These mutations result in increased ENaC activity and consequently increase renal Na\(^+\) absorption (Shimkets et al., 1994). Interestingly, Liddle’s syndrome patients do not develop lung disease despite the expression of mutated ENaC in the lung (Mall et al., 2010). In the lung, expression of CFTR is sufficient to inhibit ENaC, demonstrating the importance of the microenvironment in the regulation of ENaC (Mall et al., 2010).

Patients with type I pseudohypoaldosteronism (PHA-I) display the opposite symptoms of patients with Liddle’s syndrome and are characterized by volume depletion, hypotension, and hyperkalemia (Bhalla and Hallows, 2008; Furgeson and Linas, 2010). These manifestations are due to reduced Na\(^+\) absorption in the kidney caused by either the homozygous or heterozygous mutations in any ENaC subunits that lead to loss-of-function in ENaCs (Chang et al., 1996; Strautnieks et al., 1996). Four genes that are commonly lost or mutated in PHA-I patients have
been identified, namely NR3C2, SCNN1A, SCNN1B, and SCNN1G, which code for the nuclear receptor subfamily 3 group C member 2, α-, β-, and γ-ENaCs, respectively (Chang et al., 1996; Pujo et al., 2007; Riepe et al., 2006; Strautnieks et al., 1996).

1.1.4.3 Colon

In the distal colon, ENaCs are expressed and function as the rate-limiting step for Na\(^+\) absorption. The channel’s importance can be seen in Crohn’s disease, a chronic inflammatory bowel disease (Palmer et al., 2012; Zeissig et al., 2008). Patients with Crohn’s disease have reduced levels of γENaC transcripts and therefore display decreased ENaC activity and impaired Na\(^+\) transport (Zeissig et al., 2008). Similarly, ulcerative colitis patients display decreased levels of β and γENaCs which also leads to impair Na\(^+\) transport (Dagenais et al., 2006; Picher, 2011). Reducing colonic sodium absorption contributes to increased fluid retention and hence diarrhea, one of the major symptoms of both Crohn’s disease and ulcerative colitis (Bhalla and Hallows, 2008). Conversely, CF patients display intestinal obstruction characterized by distension and constipation due to disruption in normal ENaC regulation in the colon (FitzSimmons, 1993; van der Doef et al., 2011).

1.1.4.4 Salivary glands

ENaCs have been known to play a role in saliva production (Martinez et al., 1966). The composition of saliva can vary among individuals. Level of hydration, smoking history, circadian and circannual cycle, the size of salivary glands, and body weight are a few factors that influence the composition of saliva (de Almeida et al., 2008). However, the mechanism by which saliva is produced and secreted into the oral cavity is conserved throughout mammals (Catalán et al., 2009). There are two stages in the production of saliva. First, acinar cells produce isotonic plasma-like primary saliva which is rich in NaCl. Ion channels and transporters coordinately promote vectorial
ion transport that leads to fluid secretion (Catalán et al., 2009). In the second stage, fluid flows through the ductal epithelium where the majority of NaCl is reabsorbed (Catalán et al., 2009). The salivary ducts are a tight, water impermeable epithelium, which absorb ions but not water, leading to hypotonic saliva (Martinez et al., 1966). Here, ENaCs play a role in the reabsorption of \( \text{Na}^+ \) to produce the final form of saliva (Mangos et al., 1966; Martinez et al., 1966; Young and Schögel, 1966). Xerostomia, also known as dry mouth, is common in patients with autoimmune disorders such as Sjogren’s syndrome and during aging (predominantly in women after menopause) (Li et al., 2013; Thelin et al., 2008). It has been demonstrated that xerostomia is caused by the altered composition and secretion of saliva. While the specific mechanism has not been fully determined, it has been shown that the hyperactivity of ENaCs is one factor in aberrant saliva composition and secretion in xerostomia (Li et al., 2013; Thelin et al., 2008).

1.2. Short palate lung and nasal epithelium clone 1

The short palate, lung and nasal epithelium clone 1 (SPLUNC1) is a member of the larger bacterial permeability-increasing protein (BPI) family and is also known as BPI-family member A1 (BPIFA1), secreted protein from upper respiratory tract (SPURT), lung specific protein-X (LUNX), nasopharyngeal carcinoma-related protein (NASG), and palate, lung and nasal epithelium clone (PLUNC) (Di et al., 2003; Iwao et al., 2001; Zhang et al., 2003). To date, seven related PLUNC genes—short PLUNC (SPLUNC)-1, 2, 3 and long PLUNC (LPLUNC)-1, 2, 3, 4—have been identified on human chromosome 20 (Di et al., 2003; Weston et al., 1999). The major difference between short and long PLUNCs is that SPLUNCs consist of one domain while LPLUNCs consist of two domains. Furthermore, SPLUNCs have 8–9 exons and are translated to ~250 amino acids and LPLUNCs have 15–16 exons and are translated to ~450 amino acids (LeClair, 2003). All of these genes contain 5’ signal sequences, indicating that they are translated
into secreted proteins (LeClair, 2003). A secreted, minimally glycosylated protein, SPLUNC1 has a molecular weight of approximately 25 kDa and is most abundantly found on the surface of the airway at an estimated concentration range of 10–250 μg/mL (Gakhar et al., 2010). The crystal structure of SPLUNC1 has been revealed to show that while the N-terminal half of BPI and SPLUNC1 shared structural similarity at the core, SPLUNC1 is different from BPI with two additional α helices flanking the core structure (Figure 1.5) (Garland et al., 2013).

1.2.1. SPLUNC1 expression

In the proximal trachea, kidney, colon, SPLUNC1 expression is high (Garcia-Caballero et al., 2009; Musa et al., 2012; Zhang et al., 2003). Particularly in the lung, SPLUNC1 expression has been observed in mucus cells of submucosal glands and their ducts, non-ciliated epithelial cells, and airway luminal secretions (Bingle et al., 2005; Campos et al., 2004). It is speculated that SPLUNC1 is produced by mucus cells based on the detection of SPLUNC1 in the mucous cells of the majority of glands, including submandibular and sublingual glands, but not in the serous cells of parotid glands (Campos et al., 2004; Vargas et al., 2008). Additionally, SPLUNC1 has been found in nasal lavage fluid and middle-ear effusion (McGillivary and Bakaletz, 2010), as well as in minor glands of the nose, sinus, posterior tongue, tonsil, and saliva (Musa et al., 2012; Vargas et al., 2008). However, SPLUNC1 is not expressed in the heart, liver, brain, placenta, skeletal muscle, pancreas, spleen, lymph nodes, peripheral lymphocytes, prostate, testis, or ovaries (Britto and Cohn, 2014; Di et al., 2003).

Moreover, SPLUNC1 expression is regulated by the differentiation status of cells; cell cultures withdrawn from growth factors lose the expression of SPLUNC1 (Britto and Cohn, 2015). Additionally, the level of SPLUNC1 secretion in nasal polyepithelial cells is modulated when the differentiation process is triggered by an air/liquid interface culturing system (Ross et al., 2007).
An increased level of SPLUNC1 has also been seen observed in salivary gland tumors (Yang Liu et al., 2013). Furthermore, SPLUNC1 mRNA levels have been shown to increase in patients with progressive idiopathic pulmonary fibrosis compared to patients with stable idiopathic pulmonary fibrosis (Chu et al., 2010). Also, the expression of SPLUNC1 mRNA was found to increase in non-small cell lung carcinoma (NSCLC), suggesting it is a positive marker for circulating NSCLC tumor cells (Barnes et al., 2008; Kim et al., 2007). Furthermore, in the stomach, increased levels of SPLUNC1 have been correlated to hepatoid adenocarcinoma (Sentani et al., 2008). Conversely, SPLUNC1 expression levels have been shown to be significantly decreased by inflammatory cytokines, particularly IL-13 (Chu et al., 2007). Likewise, decreased levels of SPLUNC1 have been detected in the nasal lavage fluid of cigarette smokers and allergic subjects (Britto and Cohn, 2015; Chu et al., 2007). Together, these studies suggest SPLUNC1 can be a useful diagnostic tool.

1.2.2 Functions of SPLUNC1

1.2.2.1 Antimicrobial

As previously mentioned, SPLUNC1 was first thought to have antimicrobial functions since it has structural homology to BPI. Chu et al. reports that SPLUNC1, when exogenously applied to human and mouse bronchial epithelial cells, decreased *Mycoplasma pneumoniae* levels (Chu et al., 2007). Additionally, SPLUNC1’s antimicrobial effects have been shown in mouse models. For example, SPLUNC1 knockout mice display an increased mortality and susceptibility to *Pseudomonas aeruginosa* infection, as well as increased biofilm formation of *P. aeruginosa* (Jiang et al., 2013; Yanyan Liu et al., 2013). While endogenous PLUNC1 mice were able to defend against inhaled *P. aeruginosa*, transgenic mice that produced human SPLUNC1 in the mouse airway epithelium using a Scgb1a1 promoter displayed increased protection against *P. aeruginosa* (Lukinskiene et al., 2011). Additionally, SPLUNC1 has been shown to inhibit the growth of
*Haemophilus influenzae*, *Klebsiella pneumonia*, and *Burkholderia cenocepacia* (Yang Liu et al., 2013; McGillivary and Bakaletz, 2010; Walton et al., 2016). These bacteria are Gram-negative, differentiated from Gram positive bacteria by the presence of an outer membrane that contains a high level of lipopolysaccharide (LPS). This suggests that SPLUNC1’s antimicrobial effects may be selective against Gram-negative bacteria (Di, 2011). Furthermore, SPLUNC1’s mechanism of antimicrobial action against *P. aeruginosa* and *B. cepacia* is bacteriostatic as it inhibits bacterial growth (Sayeed et al., 2013; Walton et al., 2016). This is different from BPI, which is bactericidal wherein it kills bacteria. While it is known that BPI binds to the LPS of Gram-negative bacteria, it is unclear whether SPLUNC1 exerts its bacteriostatic function on Gram-negative bacteria through the binding of LPS.

1.2.2.2 Surfactant

There is a high level of sequence homology between SPLUNC1 and the equine surfactant, latherin (Gakhar et al., 2010). Both latherin and SPLUNC1 are largely hydrophobic with ~45% total hydrophobic residues (Gakhar et al., 2010). While surfactants are known to reduce surface tension, they also play an important role in lung host defense against bacterial infection. For example, surfactants have the ability to disrupt biofilms, which include matrices of extracellular polymeric substances produced by bacterium to create a protective niche to increase survival (Bartlett et al., 2011; Gakhar et al., 2010; Walton et al., 2016). Previous studies have shown that SPLUNC1 can lower surface tension and that this property is critical to SPLUNC1’s antimicrobial activity against Gram-negative bacteria. By lowering surface tension, SPLUNC1 disrupts/inhibits the biofilm formation of *P. aeruginosa* and *K. pneumoniae* (Gakhar et al., 2010; Yang Liu et al., 2013; Walton et al., 2016). Thus, data suggest that SPLUNC1’s surfactant activity may be a way of explaining its bacteriostatic activity against Gram-negative bacteria. Moreover, SPLUNC1’s
surfactant-like abilities play an important role in the middle ear, as shown by McGillivary and Bakaletz with chinchillas (McGillivary and Bakaletz, 2010). Reduced SPLUNC1 expression led to defective middle ear pressure, marked by retraction of the tympanic membrane (McGillivary and Bakaletz, 2010). Additionally, SPLUNC1 knock down in chinchillas resulted in a greater accumulation of mucus, suggesting that SPLUNC1 plays a role in mucociliary clearance.

1.2.2.3 ENaC Inhibition

Tarran et al. demonstrated that ENaC activity is inhibited by soluble mediators present in ASL (Tarran et al., 2006b). One such soluble mediator that inhibits the ENaC was later identified as SPLUNC1 (Garcia-Caballero et al., 2009). Microelectrode studies have revealed that SPLUNC1 reduces amiloride-sensitive voltage in HBECs (Garcia-Caballero et al., 2009). Furthermore, knocking down SPLUNC1 caused ASL dehydration in normal human bronchial epithelial cultures (HBECs), indicating that SPLUNC1 is required for normal ASL hydration (Garcia-Caballero et al., 2009). Additionally, the ENaC inhibitory domain of SPLUNC1 has been identified as 18 amino acids from G22 to A39 (Hobbs et al., 2013). Because this domain consists of 18 amino acids of SPLUNC1, it is named the S18 region (Hobbs et al., 2013). Moreover, it has been shown that S18 inhibits the ENaC by binding to a mature, glycosylated βENaC (Hobbs et al., 2013). Additionally, it has been demonstrated that S18 is able to inhibit ENaCs and hydrates the ASL of CF HBECs for 24 h (Hobbs et al., 2013). By contrast, full length SPLUNC1 has been shown to be a pH-sensitive protein that loses its ability to inhibit the ENaC in pH 6.5 or lower environments, while the ability of S18 to inhibit the ENaC is pH-insensitive (Garland et al., 2013; Hobbs et al., 2013). The surface of SPLUNC1 is electrostatic with charged residues (D112, K138, R152 and D193), leading SPLUNC1 to be pH-sensitive (Garland et al., 2013). Therefore, it has been suggested that SPLUNC1 in CF ASL does not function properly to inhibit the ENaC because the ASL of patients
with CF is slightly acidic with a pH of 6–6.5, resulting in ASL dehydration and ultimately impaired mucociliary clearance (Coakley et al., 2003; Garland et al., 2013).
ENaC exists as a heterotrimer comprised of α, β, and γ subunits at the plasma membrane. Each subunit has two transmembrane domains (TM), a large extracellular loop and short N- and C-termini. There are multiple glycosylation sites in the extracellular loop and one or more protease cleavage sites for each subunit. Each subunit also contains a PY-motif at its C-terminus.

**Figure 1.1. Schematics of ENaC Structure.** ENaC exists as a heterotrimer comprised of α, β, and γ subunits at the plasma membrane. Each subunit has two transmembrane domains (TM), a large extracellular loop and short N- and C-termini. There are multiple glycosylation sites in the extracellular loop and one or more protease cleavage sites for each subunit. Each subunit also contains a PY-motif at its C-terminus.
Figure 1.2. Schematics of trafficking and regulation of ENaC. (A) General schematic of ENaC trafficking to the plasma membrane. ENaC moves from the ER to the TGN where it is assembled and packaged into a vesicle to be delivered to the plasma membrane. (B) At the plasma membrane, the probability of ENaC being in an open position to conduct Na⁺ ($P_0$) is regulated primarily by proteases that act on the extracellular loops of α and γENaCs. Scissors represent protease activity on ENaCs. The density of ENaC in the plasma membrane (N) is primarily regulated by trafficking to/from the plasma membrane.
Figure 1.3. A Schematic of ENaC trafficking by ubiquitination and de-ubiquitination. The ubiquitin ligase Nedd4-2 is comprised of four WW domains that are known to identify the target protein. The fourth WW domain (WW4) binds to the PY-motifs on ENaC subunits to transfer ubiquitin through its catalytic HECT domain. Ubiquitinated ENaCs are subject for endocytosis and lysosomal degradation (Rotin and Staub, 2011). De-ubiquitinating enzymes (DUBs), such as UCHL3, are able to remove ubiquitin from ENaC either at the plasma membrane or in the endosome, which leads to ENaC recycling rather than degradation.
Figure 1.4. Schematics of non-CF and CF airways demonstrating that absence of CFTR leads to imbalance of ion transportations and consequently to ASL dehydration. (A) ASL conditions of non-CF (left) and CF (right). ASL of the CF airways is dehydrated which leads to impaired mucociliary clearance and reduced pH level as indicated with red box. (B) Details of ion transportation in non-CF airway that maintains healthy ASL (upper). Dysregulation of ions in CF airways that dehydrates ASL (lower). CF airways lack functional CFTR that secretes Cl\(^{-}\). This leads to hyperactivity of ENaC resulting in Na\(^{+}\) hyperabsorption into the cell. Consequently, the expression of Na\(^{+}/K^{+}\) ATPase and K\(^{+}\) channels are increased in the basolateral membrane.
Figure 1.5. Crystallographic structure of human SPLUNC1. (A) S18 of SPLUNC1 was not visible on the crystal structure because S18 is disordered without a secondary structure. Therefore, S18 is drawn to the crystal structure in red. (B) Crystal structure of SPLUNC1 (blue) overlapped with that of its close family member BPI (yellow). SPLUNC1’s core structure resembles the N-terminus half of BPI but SPLUNC1 additionally possesses two alpha helices (α4 and α6) that flank the core structure. (Garland et al., 2013)
Chapter 2: Automated Acquisition and Analysis of Airway Surface Liquid Height by Confocal Microscopy

2.1. Introduction

The airway surface liquid (ASL) is a thin layer of salt, water and protein that lines the respiratory tract and facilitates innate immunity in the lung. ASL is comprised of two layers, one that is directly adjacent to the epithelial cells, which is called the periciliary liquid layer (PCL) and the other, called the mucus layer, which lies on top of the PCL (Kunzelmann and Schreiber, 2012; Schmid et al., 2011; Verkman et al., 2003). Maintaining hydrated ASL is important to provide a low viscosity environment for cilia to beat effectively and to propel mucus, pathogens and particles toward the mouth (Boucher, 1999; Kunzelmann and Schreiber, 2012; Verkman et al., 2003; Widdicombe, 2002). Furthermore, dehydrated ASL accelerates the development of mucus plugs, which can obstruct air flow and serve as focal points for bacterial colonization (Boucher, 2007; Cohen-Cymberknok et al., 2013; Kunzelmann and Schreiber, 2012; Verkman et al., 2003; Zhou et al., 2011). ASL hydration is controlled by the transepithelial movement of ions and water (Boucher, 1999; Chambers et al., 2007; Kunzelmann and Schreiber, 2012; Verkman et al., 2003). At the apical membrane of airway epithelia, anions are mostly secreted by the cystic fibrosis transmembrane conductance regulator (CFTR) and Na$^+$ is absorbed by the epithelial sodium channel (ENaC) (Boucher, 1999; Kunzelmann and Schreiber, 2012; Pilewski and Frizzell, 1999; Thibodeau and Butterworth, 2013; Verkman et al., 2003). In the case of cystic fibrosis (CF), where

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CFTR is mutated and has disrupted or diminished function, the balance between anion secretion and Na\(^+\) absorption is altered (Boucher, 1999; Chambers et al., 2007; Kunzelmann and Schreiber, 2012; Pilewski and Frizzell, 1999; Verkman et al., 2003; Widdicombe, 2002). An absence of functioning CFTR leads to reduced anion secretion and triggers hyperactive ENaC that excessively absorbs Na\(^+\), which together leads to dehydrated ASL (Althaus, 2013; Boucher, 1999; Kunzelmann and Schreiber, 2012; Verkman et al., 2003). While calcium-activated chloride channels (CaCCs) are functional in CF airways, CaCC activity is short-lived and therefore cannot compensate fully for the loss of CFTR function in CF airways (Tarran et al., 2006b, 2001a). Consequently, ASL dehydration impairs mucociliary clearance and results in chronic bacterial infection of the lung (Boucher, 2007; Cohen-Cymerknoh et al., 2013; Kunzelmann and Schreiber, 2012; Pilewski and Frizzell, 1999; Verkman et al., 2003; Zhou et al., 2011).

Measuring the ASL height has served as a useful tool to study ASL physiology pertaining to ASL volume, ionic movement and mucus transport. Previously, ASL height measurements have been used to show that PCL transport is dependent on mucus transport (Matsui et al., 1998a, 1998b; Tarran et al., 2001a). Also, studies on ASL absorption rates using ASL measurements revealed the importance of restoring ASL volume to treat dehydrated airway in CF and chronic obstructive pulmonary disease (COPD) (Astrand et al., 2014; Garland et al., 2013; Jayaraman et al., 2001; Tarran et al., 2001b). Moreover, the function of ENaC and CFTR and ASL hydration was studied using ASL measurements (Garcia-Caballero et al., 2009; Jayaraman et al., 2001; Song et al., 2009; Zhang et al., 2009). Different ASL mediators that alter ENaC activity such as channel-activating proteases (CAP), CAP inhibitors, ATP, ADO and SPLUNC1 have all been assessed using ASL height measurements (Garland et al., 2013; Hobbs et al., 2013; Tarran et al., 2006b). ASL
measurements were also used to evaluate the efficacy of pharmacological agents such as drugs from Vertex including Ivacaftor and Lumacaftor for CF lung disease (Van-Goor et al., 2011, 2009).

A variety of methodologies have been proposed and used to measure ASL height. Light and electron microscopy techniques have been used to measure ASL in airways fixed in situ (Wu et al., 1998). A potential drawback of this method is that adding of fixatives during the sample preparation process may alter the ASL. For an alternative model, an in vitro well-differentiated cell culture system has been adopted to measure native ASL in live cells (Fulcher and Randell, 2013; Matsui et al., 1998a). Using this system, several methods have been proposed to measure ASL height: The surface laser reflectance microscopy (SLRM) method takes reflections of laser light to reconstruct an image of the mucosal surface (Thiagarajah et al., 2010). Light refraction microscopy measures refraction of light created by fluid meniscus to determine the volume of the meniscus at the interface between the culture and the wall of the culture insert (Harvey et al., 2011). Optical coherence tomography (OCT) measures the thickness of the ASL based on the reflectance of sample. This method requires micro-OCT instrument that produces cross-sectional images with 1 μm resolution (L. Liu et al., 2013). For another direct and non-invasive method of measuring ASL height, confocal microscopy method is used. This method is previously described in details (Tarran and Boucher, 2002; Worthington and Tarran, 2011). In brief, ASL is labeled with an ASL permeable but epithelium impermeable 10-kDa dextran conjugated with a fluorophore. Images of fluorescent labeled ASL are obtained using XZ scanning with a confocal microscope. Acquired images are analyzed to determine ASL height, which reflects the hydration state of ASL.

Previously, ASL height measurements were performed manually (Garland et al., 2013; Tarran et al., 2006a). That is, for each culture, images were acquired at certain locations by moving the microscope stage by hand. Images were then analyzed manually by selecting the region of
interest around the ASL by eye to obtain ASL height values in microns. However, this method is labor-intensive and time consuming, which together limit the throughput. Here, we propose and outline an automatic ASL measurement method that uses an automated stage on a confocal microscope to acquire images and automatic image analysis technique to make consistent and accurate ASL height measurements in a high-speed manner.

2.2. Methods

2.2.1. Primary human bronchial epithelial culture (HBEC)

HBECs were obtained from freshly excised bronchial specimens from normal and CF subjects (n = 4 donors for each type) and were harvested by enzymatic digestion as previously described under a protocol approved by the University of North Carolina Institutional Review Board (Fulcher and Randell, 2013). HBECs were cultured at an air-liquid interface in a modified bronchial epithelial growth medium with 5% CO₂ at 37°C and were used 3-4 weeks after the seeding on 12-mm T-clear inserts (Corning-Costar, Corning, NY, USA). During image acquisition, HBECs were maintained in a modified Ringer Solution as described previously (Tarran et al., 2001a).

2.2.2. Confocal microscopy measurements of ASL

ASL labeling with fluorescent dextrans was performed as described previously (Tarran and Boucher, 2002; Worthington and Tarran, 2011). Briefly, phosphate buffered saline (PBS) (20 μL) with 0.5 mg/ml of 10 kDa Dextran-tetramethylrhodamine (Thermo Fisher Scientific, D-1817) was added to each culture. Perfluorcarbon (3M™ Fluorient™ FC-770) was added apically to prevent ASL evaporation. In most cases, images were then obtained in XZ-scanning mode using a Leica SP8 confocal microscope with a 63x/1.3 Numerical Aperture (NA) glycerol immersion lens (e.g. for the SPLUNC1 time-course and dose-response experiments). ASL images were acquired using
an automatic stage with the “Mark-and-Find” function, which is available for use in the Leica LAS AF Application Suite. With this function, one can preset multiple XY locations and the automatic stage then moves the culture to each stored location to acquire images. We then used our proposed automatic image acquisition and analysis methods. ASL height values from all the acquired images per culture were averaged to yield an \( n = 1 \). In some cases, a Zeiss LSM 510 confocal microscope and a 40x/1.2 NA water immersion lens was used (e.g. for the ATP stimulation experiments and the comparison between normal and CF HBECs). The Zeiss 510 was not equipped with an automatic stage, and in this case, 5 images per culture were obtained manually, as previously described (Tarran et al., 2006b).

2.2.3. ATP or dexamethasone stimulation and SPLUNC1 time-course and dose-response

HBECs were cultured at the air-liquid interface for 3-4 weeks. 24 h before the experiment, apical mucus was removed by washing the mucosal surface with PBS for 30 min at 37°C. For the SPLUNC1 exposures, endogenous SPLUNC1 was removed 2 h before the experiment by incubating the HBEC’s apical surfaces with PBS at 37°C for 30 min, after which time, all additional PBS was aspirated away from the surface. For the time-course experiments, 10 \( \mu \text{M} \) of SPLUNC1 and dextran-tetramethylrhodamine in PBS were added apically and ASL height was measured immediately and at additional, timed intervals with the cultures being returned to the 37°C incubator between time-points. For the dose-response experiment, 0.1 - 100 \( \mu \text{M} \) of SPLUNC1 in PBS was added apically and the cultures were incubated at 37°C for 4 h and ASL heights were then measured. Recombinant human SPLUNC1 was purified from *E.Coli* as previously described (Garland et al., 2013). For ATP stimulation, cultures were preloaded with dextran-tetramethylrhodamine 24h earlier and then on the day of the experiment, 300 \( \mu \text{m} \) ATP was added as a dry powder suspended in perfluorocarbon as described previously(Tarran et al., 2001b).
For dexamethasone stimulation, 100 nM dexamethasone were incubated basolaterally 12-16 h prior to the apical delivery of dextran-tetramethylrhodamine. ASL measurement was made 4 h after the loading of dextran-tetramethylrhodamine.

2.2.4. Statistical analyses

All ASL height measurements were first subjected to the D’Agostino and Pearson omnibus normality test. Since not all ASL height data sets were normally distributed, we performed the nonparametric Kruskal-Wallis test followed by Dunns test for multiple comparisons among the time points or the dose treatments. SPLUNC1 time-course and dose-response experiments and ATP/dexamethasone stimulation experiments are reported as mean ± Standard Error of the Mean (SEM). Figure for ASL height distribution among different Mark-and-Find designs analyzes the overall performance of the technique and therefore is reported as mean ± Standard Deviation (SD). \( P < 0.05 \) was considered statistically significant.

2.3. Results

2.3.1. Automatic binarizing of images and finding boundaries of image subsections

To test the proposed measuring technique, we gathered 212 fluorescent images of ASL. The image size was 512² pixels, which corresponded to an actual area of 145 μm². This image set included ASL heights that widely varied from very narrow to very wide (Figure 2.1A). Our measurement algorithm consisted of two main image processing techniques. The first one was Otsu’s threshold (Otsu, 1975), which uses an adaptive threshold to binarize an image and obtain a region of interest called the ‘foreground’ with the other region being called the ‘background.’ Here, adaptive means optimal to differentiate foreground from background under various conditions of brightness and contrast. In Otsu’s method, an adaptive threshold is found by exhaustively searching the threshold of maximal inter-class variance or minimal intra-class variance between
background and foreground. In an XZ scanned fluorescent ASL image, the foreground was the red-colored ASL region and the background was in black (Figure 2.1A). So, the red ASL region was selected and applied with Otsu’s threshold. Figure 2.1A shows a binarized image after applying Otsu’s threshold. Since the obtained ASL images are in 8 bit images, the minimum pixel value of 0 and the maximum pixel value of 255 are used to distinctively select the background and the foreground. A binarized image with noisy edges may result in an inaccurate detection of boundaries in the additional processing steps (Figure 2.1B). Therefore, in order to smooth out the ASL boundaries, we selected a series of morphological operations, which consisted of 2 iterations of dilation and 3 iterations of erosion, with dilation and erosion being two basic operations that are commonly used for image processing to detect edges, remove noise and enhance images (Choi and Oh, 2005). In this case, they were used to describe the turning on and off of the pixels along the boundaries of the ASL. By doing so, detected ASL boundaries were smoothed out (Openings et al., 1994; Whittaker and Robinson, 1924). The application of this series of algorithms resulted in the image shown in Figure 2.1C, which has no noisy pixels on its boundaries.

After getting a binarized image to which Otsu’s threshold and the morphological operations were applied, the boundaries of the foreground were found by histogram analysis. To deal with varying boundaries, the foreground region was divided into a number of horizontal subsections. Then, the pixel values of each subsection were horizontally accumulated to give the pixel value index (x-axis) on a histogram profile. Figure 2.1C shows the subsections and Figure 2.1D-G shows the corresponding histogram profiles. The upper and the lower boundaries of a subsection correspond to the increasing and decreasing part of the histogram profile respectively, within a range between 0 and 1 as listed on the y-axis of a histogram profile. The mid-range value of 0.5 was applied as the histogram profile threshold to get the upper and lower boundaries of each
subsection. To prevent processing failures due to noisy bounce, which is a phenomenon where the background is mistakenly read as the foreground and vice versa, we performed a preprocessing step using a 5-point moving average to eliminate the noisy bounce of the profile (Figure 2.2A, B) (Kenney and Keeping, 1954; Openings et al., 1994). The 5-point moving average is a calculation that analyzes each pixel by creating a series of averages of the neighboring 5 pixels (Arce, 2005). Using this calculation, anything less than 5 pixels was regarded as an artifact. Following the noise reduction, the same threshold of 0.5 was applied to obtain a bipolar profile (Figure 2.2C); bipolar profiles assign 1 to the parts above the threshold and -1 to the parts below the threshold.

Filtering is a type of processing used to detect the exact location of the signal. Here, we used a filtering technique to detect the exact location of the ASL boundaries, namely, 7-point filtering which is a type of image analysis methodology based on 7 pixels. 7-point filtering consists of \( A = [-1\ -1\ -1\ -1\ 1\ 1\ 1] \) for the rising edge detection, a location where the ASL region begins, and \( B = [1\ 1\ 1\ -1\ -1\ -1\ -1] \) for the falling edge, a location where the ASL region ends (Figure 2.2D, E). Upon experimental analysis, we found that any filtering below 7 pixels was unable to deal with the noisy peaks produced on the edges of ASL. On the other hand, filtering above 7 pixels was unnecessary because (i) the results did not improve any further and (ii) the increased filtering slowed the analysis speed. Thus, to maximize the speed of our algorithm whilst maintaining accuracy, we decided to use 7-point filtering.

The trace in Figure 2.2F represents a rising edge that was characterized by having the first peak with a value of +7 and the second peak with a value of -7. The value of 7 from Figure 2.2F occurs when the fifth value of filter A (+1), is aligned with the first location from the left where the bipolar profile value is +1. This results in the equation \((-1\cdot-1)+(-1\cdot-1)+(-1\cdot-1)+(-1\cdot-1)+(1\cdot1)+(1\cdot1)+(1\cdot1) = +7\). Differently put, it is where the first location from the left in Figure
2.2C that returns a value of +1 is aligned with the Point Index #5 with a value of +1 in Figure 2.2D. The -7 peak from Figure 2.2F is returned when the fourth integer of filter A (-1), is aligned with the last location from the left where the bipolar profile value is +1 in the equation \((-1 \cdot 1)+(-1 \cdot 1)+(-1 \cdot 1)+(-1 \cdot 1)+1 \cdot (-1)+1 \cdot (-1)+1 \cdot (-1) = -7\). In other words, it is where the first value of +1 from the right in Figure 2.2C is aligned with the Point Index #4 which returns a value of -1 in Figure 2.2D. Conversely, the graph in Figure 2.2G represents a falling edge that is characterized by having its first peak with a value of -7, followed by a second peak with a value of +7. These values are produced using the same logic of finding the rising edge. The first peak with a value of -7 occurs when the fourth value of filter B (-1), is aligned with the first point from the left where the bipolar profile value is 1. This results in the equation \((1 \cdot -1)+(1 \cdot -1)+(1 \cdot -1)+(-1 \cdot 1)+(-1 \cdot 1)+(-1 \cdot 1) = -7\). The second peak with a value of 7 occurs when the third value of the filter B (+1), is aligned with the first point from the right where the bipolar profile value is 1 in the equation \((1 \cdot 1)+(1 \cdot 1)+(1 \cdot 1)+(-1 \cdot -1)+(-1 \cdot -1)+(-1 \cdot -1)+(-1 \cdot -1) = 7\). Therefore, Figure 2.2G represents the alignment between Figure 2.2C and Figure 2.2E.

Occasionally, the boundaries could not be found in some subsections. This typically occurred when the ASL region was either very narrow or unclear. Since the HBECs represent a heterogeneous cell population of varying height and composition, and since the inverted confocal must scan through the cells in order to image the ASL, this sometimes occurs during acquisition. In these cases, the subsections without boundaries were excluded from further processing. Our studies indicated that omitting some subsections did not affect the final height calculation since the total number of remaining subsections was more than sufficient to calculate the mean ASL height.
2.3.2. Finding the optimal number of subsections

Figure 2.3A-E shows the analyzed ASL height images according to the specific number of subsections. The number of subsections directly affects the detected boundaries of the ASL region and hence the measured height. Although the number of subsections can be increased for more accurate boundary detection, the number should not be more than 128, because in such a case the width of a subsection decreases to \( \leq 3 \) pixels, and the analysis of the histogram profiles becomes unreliable. Moreover, increasing the number of subsections decreases the speed of analysis (Table 2.1). The maximum difference of the measured heights between 8 and 16 subsections was 4.48\% and that of 16 and 32 subsections was 3.44\% (Figure 2.3G). However, the maximum difference of the measured heights between 32 and 64 subsections was 1.28 \%, a value small enough to dismiss without any expectation to dramatically increase the accuracy of the result (Figure 2.3G). Since the purpose of the subsections is not to find exact boundaries of the ASL region but to calculate a representative ASL height, no more than 32 subsections is necessary. Therefore, for the optimal number of subsections, we decided to use 32 subsections of 16 pixels width (Figure 2.3F).

2.3.3. Finding valid subsections

There is a possibility that detected boundaries within the subsections may not be real due to computational detection errors. Furthermore, when a cell dies, the space occupied by that cell may fill the fluorescent dextran, which may cause additional artifact. To avoid using these invalid artifactual boundaries, we decided to find an optimal threshold for excluding outlier subsections. All of the subsections were independently considered as a sample of data in 2-dimensional feature space of the relative difference and the absolute difference of height. The absolute difference of height was calculated as the difference between the height of a subsection and the initial average height of the median 60\% of all subsections. Upon experimental analysis, we found that when \( \geq \)
50% of the ASL regions were noisy, the ASL height analysis was unsuccessful and the data was mostly artifactual. However, when 40% or less of the ASL regions contained noise, then the ASL height analysis was consistently successful. Therefore, to maximize the selection of real ASL heights, while cautiously filtering out any artifacts, we used a median 60% threshold. The relative difference of height is calculated as the ratio of the absolute difference to the initial average height.

Figure 2.4 shows the distribution of all subsections of the test image set in the feature space. Blue circles and red asterisks represent the ground truth inliers and outliers respectively. Although inliers and outliers are not completely separable in this feature space as seen in the graph, we can select sub-optimal threshold values for each dimension. The vertical and the horizontal red lines represent the relative difference threshold of 0.3 and the absolute difference threshold of 30 pixel respectively. We selected these values to tightly restrict the inlier boundary in order to minimize the loss of inliers while precisely excluding outliers. In the process of defining inliers and outliers, inaccurate selection of outlier rarely occurs. However, an example of a rare included outlier is shown at point ~(0.12, 40) in Figure 2.4 (see arrow). Moreover, the accuracy of the mean ASL height is not tempered as our algorithm is not dependent on individual data points and rather, is an aggregate of the data set.

Our automatic analysis was performed on ASL images obtained from normal and CF HBECs and the final output of the image analysis and the subsequent ASL height measurements were shown in Figure 2.5 and Table 2.2. Using our method, we were able to detect extremely thin ASL regions from CF HBECs. As such, the minimum required for our algorithm to successfully detect ASL was 4-pixels in height.
2.3.4. Finding the optimal Mark-and-Find design

We next acquired XZ scanned images for each culture using the “Mark-and-Find” function on a Leica SP8 confocal microscope equipped with an automatic stage. The Mark-and-Find function allows the user to save multiple pre-determined locations and then to acquire images. Harvey and colleagues have previously shown that the edges of the cultures do not represent a thin film ASL due to meniscus that exists between the culture and the plastic sidewall of the culture insert (Harvey et al., 2011). Based on their work, anything outside ~0.6mm from the edge can be counted as “thin film” when using 12mm diameter Transwell culture inserts (Harvey et al., 2011). We designed 5 different Mark-and-Find protocols, ranging from 5 to 40 saved locations per protocol (Figure 2.6A) and all locations for our Mark-and-Find functions were designed to avoid the meniscus-containing edges of the cultures. An automatic stage positioned each culture to pre-saved locations to acquire one image per location at a rate of ~1 image/s. Immediately after the loading of the dye, there was an increase in the ASL volume due to the addition of the liquid vehicle (20 μL PBS). We found that ASL absorption to the steady state level of ~7 μm took approximately 4 h. Therefore, we acquired ASL images 4 h after the loading of the dye to look at the distribution of ASL heights. All the images acquired for each of the Mark-and-Find designs were analyzed using our proposed automatic analysis method to obtain ASL heights and the data set was plotted as histograms (Figure 2.6A). From the histograms, it was appeared that the recorded ASL heights were not normally distributed (Figure 2.6A). Further analysis using D’Agostine and Pearson’s omnibus normality test revealed that all of the data sets had P values <0.0001, indicating that they failed to pass the normality test and were skewed.

To obtain a mean ASL height for each culture, ASL heights recorded for the saved locations for each Mark-and-Find design were averaged. The most representative ASL height of a culture is
obtained when the marked locations densely covered the entire culture and the images acquired at those locations were analyzed. Therefore, within our Mark-and-Find designs, the most representative ASL height was obtained using the 40 images/culture design to give an averaged ASL height (Figure 2.6A). Figure 2.6B shows distribution of the averaged ASL heights obtained for each of the Mark-and-Find design. Each data point in Figure 2.6B represents a mean ASL height from each culture. The same cultures were reimaged using each of the 5 different Mark and Find algorithms. The distribution of the average ASL heights was wider when fewer images were taken per culture (Figure 2.6B). The mean values of the averaged ASL heights were 8.32 ± 1.15 μm, 8.17 ± 1.11 μm, 8.13 ± 0.79 μm, 8.09 ± 0.80 μm, and 7.88 ± 0.86 μm for 5, 10, 20, 30, and 40 images/culture design respectively (Figure 2.6B).

2.3.5. Applications of the proposed automated method

We applied our automatic ASL height measurement method to produce a time-course for ASL absorption and a dose-response curve after the treatment of recombinant SPLUNC1 on HBECs using our method. We have previously shown that SPLUNC1 hydrates ASL by inhibiting ENaC (Garland et al., 2013; Hobbs et al., 2013) and that when washed away, it takes ~12 h for endogenous SPLUNC1 to return to the ASL and to regulate ENaC (Hobbs et al., 2013). Here, taking advantage of our automatic ASL measurement method, we measured ASL height over time in normal HBECs after treatment with recombinant SPLUNC1 (all n = 24). To eliminate the effect of endogenously secreted SPLUNC1, we washed each culture apically with 500 μL PBS prior to the loading of 20 μL tetramethylrhodamine-dextram mixed with varying concentrations of SPLUNC1. Immediately after loading of the dye, ASL height was found to be ~25 μm. However, over the 8 h, ASL is absorbed by the ion transport mediated by ENaC. Increasing the concentration of SPLUNC1 in the ASL increased steady state ASL height (Figure 2.7A). However, the first time
point to see the significant difference in the hydration state of ASL was at 4 h. Therefore, we selected this time in order to perform a dose-response of the effect of SPLUNC1 on ASL height. The ASL height with 1 μM SPLUNC1 after 4 h was 9.00 ± 0.48 μm and at this concentration, ASL height was significantly different from all the lower dosages ($P < 0.05$; Figure 2.7B). The average ASL height continued to increase significantly with 10 μM and 25 μM with the measured ASL height of 9.95 ± 0.21 μm and 11.94 ± 0.50 μm respectively (Figure 2.7B). Treatment with 100 μM SPLUNC1 resulted in the measured ASL height of 11.75 ± 0.54 μm (Figure 2.7B). The $IC_{50}$ of SPLUNC1 was 6.53 μM. As a second validation, we also used dexamethasone to increase the activity of ENaC (Tchepichev et al., 1995). To test whether our automated analysis approach could be applied to other confocal systems, we then obtained images from normal and CF HBECs using a Zeiss 510 confocal microscope that was equipped with a manual stage. Analysis of data acquired in this fashion revealed a significant difference in ASL height between normal and CF HBECs (Figure 2.7C). We then activated Cl-/ASL secretion by exposing these HBECs to ATP, which was added as a dry powder in perfluorocarbon (Tarran et al., 2001b). For normal HBECs, addition of 300 μM ATP significantly increased the ASL height from 8.85 ± 0.49 μm to 15.66 ± 1.06 μm. For CF cultures, the same ATP stimulation significantly increased ASL height from 4.82 ± 0.72 μm to 9.09 ± 1.16 μm (Figure 2.7C).

2.4. Discussion

Understanding the physiology which underlies ASL volume regulation may help to develop therapeutic agents for the treatment of obstructive lung diseases such as CF and COPD where ASL/mucus dehydration is an issue. ASL height measurements by confocal microscopy have been widely used to study the regulation of ASL height by airway epithelia and have revealed that maintaining hydrated ASL by correctly modulating ENaC and CFTR activity maintains
efficient mucus transport (Matsui et al., 1998a; Song et al., 2009; Tarran et al., 2001b). Moreover, this technique has been used to investigate the efficacy of therapeutic agents for CF and COPD (Astrand et al., 2014; Tarran et al., 2001b; Van-Goor et al., 2009). To reduce the time-burden of manually acquiring and analyzing ASL images, we have designed a novel automatic image acquisition and analysis method. Using this technique, image acquisition and analysis were relatively easy, quick and unbiased.

2.4.1. Summary of the algorithm

Our algorithm requires 60% of the ASL region to be detected in order to set inlier and outlier boundaries. In our cell culture system, it is not difficult to meet this requirement. For a more robust elimination of outlier subsections, the RANSAC (Random Sample Consensus) technique can be applied instead of using median 60% of subsections (Fischler and Bolles, 1981). RANSAC randomly selects subsections to create a pool of inliers and therefore does not require a large amount of inliers to eliminate outliers. However, this process needs a very long time to search all of the inliers and outliers, especially when inliers are small. So, the computational cost of RANSAC is higher than the median technique of the proposed measuring algorithm. This is a trade-off between computational cost and robustness.

The analysis algorithms were compiled using MATLAB code for fast prototyping. Currently, the average processing time from binarizing a fluorescence image to calculating a representative height of ASL using 128 subsections only takes several tens of millisecond on a laptop with a single thread of Intel i7 2.4Ghz CPU (Table 2.1). The analysis can be performed on any images as long as the ASL is shown in red. For other colors, the threshold for selecting the foreground must be changed. If this program is converted to C code, then the program will run 5 to 10 times faster, bringing the processing time down to several millisecond, owing to C compiler’s
optimal characteristics. This super-fast speed may be advantageous when dealing with even larger quantities of fluorescence images.

2.4.2. Parameter settings

When our proposed automated image analysis with 32 subsections as a parameter was used to measure ASL height, the values repeatedly remained the same and there was no error in computation or no exceptional failure in running the algorithm of the proposed measuring techniques (Table 2.2). Our measurement method accurately found the real boundaries (green lines), average of boundaries (white), and the outlier subsections (blue) in all conditions where the height of ASL, brightness of the image, and the clarity of the boundaries varied from image to image (Figure 2.5). Moreover, our method can read ASL images from different types of microscope, and with different lenses, so long as ASL region is shown using a standard 8 or 16 bit grayscale look-up table. While the threshold parameters are experimentally determined, in the case of ASL images with dimmer fluorescent labeling, those parameters can easily be modified, e.g. by multiplying each image by a suitable integer (e.g. 2) using a program such as Image J (NIH Freeware), which only has a small effect on the background, can enhance the foreground/ASL image.

2.4.3. Mark-and-Find analysis

When comparing different Mark-and-Find designs, the distribution of the raw data set as well as representative averaged ASL heights did not show much difference (Figure 2.6). That is, the mean values of the averaged ASL heights among difference Mark-and-Find designs were not statistically different from one another (Figure 2.6B). Interestingly, 5 and 10 images per culture both returned standard deviations > 1.0 (1.15 and 1.11 respectively), while 20, 30, and 40 images/culture gave standard deviation values < 1.0 (0.79, 0.80, and 0.86 respectively). Therefore,
we concluded that while using the design of 5 images per culture produced a sufficiently reliable data set, the most precise data set for ASL height measurement was obtained when acquiring 20 images/culture. Additionally, since HBECs can vary in cell layer thickness, even within 1 culture, it sometimes happens that 1-2 images per culture are out of the field of view and/or unreadable. However, even with this error rate, this still leaves enough ASL images to perform a thorough analysis, and in the interest of throughput, we have found that it is better to discard unusable images, rather than to try to reimage them.

2.4.4. Application analysis

Using our method, we were able to rapidly generate a time-course to show that SPLUNC1’s ability to inhibit ENaC lasted for 8 h and a dose-response curve to show that the maximum efficacy of SPLUNC1 for inhibiting ENaC was at 25 μM and with an IC$_{50}$ of 6.53 μM (Figure 2.7A, B). Moreover, our method is suitable to study the physiological function of ion channels such as ENaC. We were also able to demonstrate that our program can detect differences in ASL height between normal and CF HBECs. As previously described (Matsui et al., 1998a), basal ASL height was significantly different in normal and CF HBECs (Figure 2.7C). We next tested whether our method could detect changes in ASL height following the activation of Cl$^{-}$ secretion with mucosal ATP. Both normal and CF HBECs elicited a significant response to ATP (Figure 2.7C). However, the magnitude of the response was significantly greater in normal and CF HBECs (Figure 2.7C). ATP is rapidly metabolized to adenosine by ecto-enzymes in the ASL (Lazarowski and Boucher, 2009). As such, ATP (and its breakdown product adenosine) are predicted to activate CaCC and CFTR respectively in normal airway epithelia. In contrast, ATP can activate CaCC in CF HBECs, and while adenosine is likely still formed, adenosine’s effector (CFTR) is absent from CF HBECs.
2.4.5. **Concluding remarks**

For this paper, we automatically acquired ASL images from one 12 mm diameter culture at a time, which was placed in a chamber on the inverted confocal microscope prior to imaging. While our method can be theoretically applied to cultures placed in 12 well dishes or in other multi-well systems, the cultures would need to be sufficiently close to the bottom of the chamber to stay within the working distance of the objective lens (our 63 x glycerol objective lens has a working distance of 300 µm). When cultures are placed in commercially available 12-well plates, the distance between the objective lens and the ASL exceeds this range and the ASL cannot be imaged. However, should new 12, 24 or 96-well plates become available which were redesigned to place the cultures closer to the lens, then our technique could be modified appropriately, which would be predicted to significantly increase the throughput of the imaging.

In conclusion, our automatic ASL measurement method combines the usage of an automatic stage on a XZ-scanning confocal microscope for ASL image acquisition and our novel code for automatic image analysis of ASL heights. We have shown here that our method generates ASL height measurements with consistency, accuracy, speed without the need for extensive computing power. Our method will increase the throughput of ASL measurements, which can be applied for a fast screening of therapeutic drugs and for the study of airway physiology. All the codes and program will soon be released online at [http://pogary.yu.ac.kr](http://pogary.yu.ac.kr).
<table>
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<th>64 subsections</th>
<th>128 subsections</th>
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<td>56.1 ms</td>
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Table 2.1. The average of elapsed times to measure ASL height of an image in the test image set according to the subsection configuration.
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<tr>
<th>Image Name</th>
<th>Average of Inliers (pixel/µm)</th>
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<th>Subregion2 (pixel/µm)</th>
<th>Subregion3 (pixel/µm)</th>
<th>~</th>
<th>Subregion30 (pixel/µm)</th>
<th>Subregion31 (pixel/µm)</th>
<th>Subregion32 (pixel/µm)</th>
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<td>62/16.95</td>
<td>62/16.95</td>
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<td>78/21.33</td>
<td>81/22.15</td>
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<td>85/23.24</td>
<td>87/23.79</td>
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</tr>
<tr>
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<td>89/24.34</td>
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Table 2.2. The result of height measurements with 32 subsections and the representative ASL heights of the test image set obtained using our program.
Figure 2.1. Initial processing of confocal micrographs using Otsu’s threshold. (A) Representative fluorescent ASL images. The red-colored band represents the ASL region. ASL height is measured in pixels and converted into a real length according to the ratio of pixel-to-length. In this case, 512 pixels = 145 µm. Images can have an ASL region of varying height from a narrow band (top right) to a wide band (bottom right). (B) Binarized image taken from A (upper left). (C) Binarized image taken from B after morphological operations with several subsections of the ASL region labeled. (D-G) Histogram profiles of the subsections of ASL regions shown in C.
Figure 2.2. Analysis of histogram profiles using a 7-Step filter. (A) Histogram profile of subsection 2 (Taken from Figure 2.1C). (B) Histogram showing the result of a 5-point running average. (C) Bipolar profile after thresholding of Figure 2.1C. (D-E) After applying 7-point step function, values are shown either as 1 or -1. D shows the rising edge and E shows the falling edge. (F) Graph showing the result of filtering with the 7-point rising edge step function. (G) Graph showing the result of filtering with the 7-point falling edge step function.
Figure 2.3. ASL height measurements according to different subsection configurations. (A-E) Examples of the detected subsection boundaries (green), and average boundary positions (white) in a fluorescent image using A:8, B:16, C:32, D:64, E:128 subsections. (F) An example of a 32 subsection configuration. (G) The average and maximum of relative differences in the measured heights of two neighboring subsection configurations for all the test images.
Figure 2.4. The distribution of inlier and outlier subsections in a 2-dimensional plane. Difference of height is the absolute value of the difference between the height of subsection and the initial average of heights. Relative difference of height is the absolute value of the difference between the height of a subsection and the initial average of heights divided by the initial average of heights. The ground truth inliers (blue circles) and outliers (red asterisks) are sub-optimally separated by union of the two thresholded regions bounded by the red lines. Therefore, any subsections located within the dotted square are considered outliers. In the process of defining inliers and outliers, inaccurate selection occurred as shown here at point \(~(0.12, 40;\text{ see arrow})\). However, the accuracy of the mean ASL height was not altered as our algorithm is dependent on the distribution of all collected data points.
Figure 2.5. Examples of the detected boundaries of different ASL regions obtained from the test image set. (A) Broad ASL region, (B) Moderate ASL region, (C) Narrow ASL region from normal HBECs. (D) Very narrow ASL region from CF HBECs. The thickness of the ASL band did not affect the detection of accurate boundaries (green lines), average boundaries (white line), and outlier subsections (blue).
Figure 2.6. A comparison of sample size versus mean ASL height. (A) Mark-and-Find designs for acquiring different amounts of fluorescent images per culture (left). Each dot represents a location where an image is acquired. Histograms of measured ASL heights from all of the acquired images corresponding to each Mark-and-Find design using \( n = 5 - 40 \) images per culture (right). (B) Graph showing mean ASL heights versus the number of data points used, based on the different Mark-and-Find designs shown in A. Each data point represents a mean ASL height from each culture. The same cultures were reimaged using each of the 5 different Mark and Find algorithms.
Figure 2.7. Validation of Our Approach: Inhibition or activation of ENaC and stimulation of CFTR/Cacc on ASL height. (A) ASL height in the presence of varying concentrations of recombinant SPLUNC1 (all n = 24). Compared to 0.1 μM: *** P < 0.001, difference to 100 μM. † P < 0.05, difference to 10 μM and 25 μM. (B) Dose response for ASL height following 4 h recombinant SPLUNC1 exposure on HBECs (all n = 28). (C) Representative western blot of αENaC (upper) with or without dexamethasone treatment. GAPDH (lower) was blotted as a loading control. (E) ASL height in the presence and absence of 300 μM of ATP on normal and CF HBECs (n = 30 for normal and n = 20 for CF). **** P < 0.0001 and * P < 0.05.
Chapter 3: SPLUNC1 Dissociates and Endocytoses ENaC

3.1. Introduction

Cystic fibrosis (CF) is the most common fatal genetic disease in Caucasians and affects epithelia of multiple organs, including the pancreas, lungs, sweat glands, gastrointestinal tract, liver, and reproductive tract. The CF gene product, the cystic fibrosis transmembrane conductance regulator (CFTR), is an ATP-binding cassette (ABC) transporter that acts as an anion channel. The lack of a functional CFTR not only diminishes anion secretion but also causes excessive Na$^+$ absorption through ENaCs (Knowles and Boucher, 2002). Consequently, ASL is dehydrated, contributing to mucus stasis and an increased chance of bacterial infection (Boucher, 2007). The airways of patients with CF are mildly acidic due to the lack of bicarbonate transport through the CFTR (Coakley et al., 2003; Poulsen et al., 1994). CFTR directly inhibits ENaC and therefore, lacking or reduced function of CFTR also contributes to ENaC dysregulation and ASL dehydration (Garland et al., 2013; Gentzsch et al., 2010). Acidic ASL, together with ASL dehydration, impairs the innate defense system of lungs in patients with CF and leads to chronic colonization with opportunistic bacteria (Delhaes et al., 2012; Govan and Deretic, 1996).

ENaCs are expressed in the apical membrane of epithelia in the colon, sweat glands, salivary duct, kidney, and lung, where they function as the rate-limiting factor for Na$^+$ absorption across epithelia (Butterworth, 2010; Eaton et al., 2010; Rossier and Stutts, 2009). An ENaC is a
heterotrimeric sodium channel comprised of α, β, and γ subunits (Butterworth, 2010; Rossier and Stutts, 2009). Each subunit has two transmembrane domains, a large extracellular loop, and intracellular N- and C-termini (Butterworth, 2010; Rossier and Stutts, 2009). Alone, αENaCs can conduct Na⁺, but at a reduced level compared to αβγENaCs, while βENaCs alone is nonfunctional (Canessa et al., 1994; Firsov et al., 1996). To optimally conduct Na⁺, the expression of α, β and γ subunits is required (Firsov, 1998; Kellenberger and Schild, 2002). At the plasma membrane of airway epithelia, ENaCs are finely regulated by changing the channel open probability (Po) and/or the number of plasma membrane ENaCs (N). While Po is mostly determined by proteolytic cleavages at αγENaC extracellular loops, N is mostly determined by ubiquitination-dependent endocytosis (Butterworth, 2010; Eaton et al., 2010; Palmer et al., 2012; Staub et al., 2000). Ubiquitination of ENaCs is mediated by the ubiquitin ligase Nedd4-2, known to interact with the PY motif located at each subunit’s C-terminus (Snyder et al., 2004b). A failure to appropriately ubiquitinate an ENaC due to mutations in the β and/or γENaC leads to Liddle’s syndrome: overactive ENaCs in the kidney. This leads to Na⁺-dependent hypertension (Knight et al., 2006; Lu et al., 2007; Staub et al., 2000). Furthermore, it has also been demonstrated that Nedd4-2 knockout mice exhibit salt-induced hypertension and elevated ENaC activity, leading to lung dehydration (Boase et al., 2011; Kimura et al., 2011; Shi et al., 2008).

Short palate, lung and nasal epithelium clone 1 is a secreted protein that is highly expressed in large airways (Gakhar et al., 2010). While SPLUNC1 was originally thought to play a role in the innate defense of airways by demonstrating antimicrobial activity against Gram-negative bacteria (Di, 2011; Gakhar et al., 2010; McGillivary and Bakaletz, 2010), it has been demonstrated that SPLUNC1 can also modulate salt/water balance and airway hydration by inhibiting ENaCs in airways (Garcia-Caballero et al., 2009; Garland et al., 2013; Hobbs et al., 2013). Previous studies
have demonstrated that recombinant SPLUNC1 (rSPLUNC1) inhibits ENaCs through its ENaC inhibitory domain, a region of 18 amino acids from G22 to A39 that we named S18, by binding extracellularly to βENaCs and thereby limiting transepithelial Na⁺ and water movement across airway epithelia (Garland et al., 2013; Hobbs et al., 2013).

In collaboration with the Redinbo laboratory in the Department of Chemistry, we previously solved the crystal structure of SPLUNC1 and determined that its charged surface enables it to interact with ENaCs in a pH-dependent fashion to maintain airway hydration, a mechanism that contributes to expelling inhaled pathogens (Garland et al., 2013; Tarran and Redinbo, 2014). The S18 region is unavailable to bind to and regulates ENaCs in the acidic environment of CF lungs, one of the causes for CF ASL volume depletion (Garland et al., 2013). While SPLUNC1’s ability to regulate ENaCs has been described, the mode of inhibition remains obscure. The current study investigates how SPLUNC1 negatively regulates ENaC. We determined that SPLUNC1 serves as an allosteric regulator of ENaCs that selectively internalizes and degrades α and γENaCs via the lysosome while leaving SPLUNC1 and βENaCs together at the plasma membrane.

3.2. Method

3.2.1. Cell Culture

As previously described, HEK293T cells were cultured in MEM-α media with 10% FBS, 100 units/mL penicillin and streptomycin at 37°C with 5% CO₂ (Hobbs et al., 2013). For surface biotinylation and immunoprecipitation experiments, HEK293T cells were seeded on Corning® tissue-culture treated 60 mm x 15 mm dishes. For microscopy experiments, HEK293T cells were seeded on #1.5 glass cover slips (0.13–0.16 mm thick) placed in plastic six or twelve-well plates. HEK293T cells were transfected when 70% confluent, 16–18 h before the experiment using
Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer’s protocol as previously described (Garland et al., 2013).

Normal human bronchial epithelial cells (HBECs) were obtained from main stem bronchi following protocols approved by UNC’s Committee on the Protection of the Rights of Human Subjects (Garcia-Caballero et al., 2009) and cultured as previously described (Fulcher et al., 2005). The HBECs were seeded on 12-mm T-clear inserts (Corning-Costar) in a modified bronchial epithelial growth medium at 37°C/5% CO₂. When cells reached 100% confluency, HBECs were maintained at an air-liquid interface (ALI) and all experiments were performed within ~4 weeks after seeding.

3.2.2. Constructs

For double tagged subunits, human α-, β-, and γENaCs were each tagged with HA on the N-terminus and V5 on the C-terminus (Hobbs et al., 2013). For single tagged subunits, human α- and γENaCs were tagged with V5 on C-termini and βENaCs were tagged with 6X His on the C-terminus. To test the effect of the C-terminus on ubiquitination, αENaCs were truncated after Proline-595 and a V5 epitope were added to the C-terminus. For fluorescently tagged subunits, α- and βENaCs were labelled with GFP in the C-termini and γENaCs were labelled with mCherry on its C-terminus. Human Anoctamin-1 (Ano1) was tagged with mCherry on its C-terminus. The dominant negative form of Nedd4-2 had amino acids 2–59 deleted along with Ser-221, Thr-246, and Ser-327 phosphorylation sites mutated to Ala (Snyder et al., 2004a). Ubiquitin was tagged with HA on its N-terminus.
3.2.3. Expression and Purification of Human SPLUNC1

Recombinant human SPLUNC1 (rSPLUNC1) and rSPLUNC1 without its ENaC inhibitory domain (\(^{\Delta 44}\)rSPLUNC1) were expressed in *E. coli* and purified as previously described (Garland et al., 2013). SPLUNC1 was labelled with DyLight 594 NHS Ester following the manufacturer’s protocol (Thermo Fisher Scientific).

3.2.4. Surface Biotinylation

HEK293T cells were transfected with either (1) human HA-αENaC-V5, βENaC, and γENaC, (2) human αENaC, HA-βENaC-V5, and γENaC, (3) human αENaC, βENaC, and HA-γENaC-V5, (4) human HA-αENaC-V5, βENaC, γENaC, and Nedd4-2 Dominant Negative, or (5) human -αENaC-P595X-V5, βENaC, and γENaC. HEK293T cells were treated with SPLUNC1 (10 μM) in MEM-α media and HBECs were apically treated with SPLUNC1 in Ringer’s solution at 37°C/5% CO\(_2\) at timed intervals. Surface biotinylation was performed as described previously (Garland et al., 2013). Briefly, cells were washed with ice-cold phosphate buffered saline (PBS) with 1 mM MgCl\(_2\) and 1 mM CaCl (PBS+++) and incubated with NHS-Biotin (0.5 mg/mL) (Thermo Fisher Scientific) in a borate buffer for 20 min on ice. For experiments using HBECs, NHS-Biotin (1 mg/mL) was incubated apically while 10% FBS in PBS++ was added basolaterally for 30 min on ice. Biotinylation was quenched with 10% FBS in PBS++ for 20 min on ice. Cells were lysed with biotinylation lysis buffer (100–200 μL) (10 mM TRIS-HCl at pH 7.4, 0.4% sodium deoxycholate, 1% NP-40, 50 mM EGTA with protease inhibitor cocktail (Roche)). Lysed cells were centrifuged for 10 min at 5000 x g and supernatants were subject to a bicinchoninic acid (BCA) protein assay and an equal amount of proteins were incubated with Neutravidin agarose (75-100 μL) overnight at 4°C under rotation (Thermo Fisher Scientific). From each sample, 20 μg of protein was saved separately to blot whole cell lysate ENaCs. Proteins were eluted from the
beads using 2x laemmli sample buffer (Biorad) by boiling at 95°C for 10 min. Neutravidin beads were separated from the eluted proteins by centrifugation at 14,000 x g for 3 min at room temperature. Supernatants were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Anti-V5 (Thermo Fisher Scientific), anti-HIS (Cell Signaling), anti-ubiquitin (Enzo), and anti-GAPDH (cell signaling) antibodies were used for immunoblotting. Endogenous human αENaCs were probed using an antibody that recognizes the complete cytosolic N-terminus of human αENaC (UNC1 19.2.1) (Gentzsch et al., 2010).

3.2.5. Immunoprecipitation

HEK293T cells were transfected with either (1) human HA-αENaC-V5, βENaC, and γENaC, (2) human αENaC, HA-βENaC-V5, and γENaC, (3) human αENaC, βENaC, and HA-γENaC-V5, (4) human HA-αENaC-V5, βENaC, γENaC, and Nedd4-2 Dominant Negative, (5) human αENaC-P595X-V5, βENaC, and γENaC, (6) human αENaC-V5, βENaC, γENaC, and HA-ubiquitin, (7) human αENaC, βENaC-HIS, γENaC, and HA-ubiquitin, (8) human αENaC, βENaC, γENaC-V5, and HA-ubiquitin, or (9) human αENaC-P595X-V5, βENaC, γENaC, HA-ubiquitin. Cells were treated with SPLUNC1 (10 μM) in MEM-α media with 10% FBS for 1 h at 37°C/5% CO2. Cells were washed with ice-cold PBS, lysed in 200 μL 2% SDS in Tris-buffered saline (TBS) with 1 mM DTT. Lysed samples were incubated at 95°C for 10 min. Samples were cooled, sonicated for 20 sec, and then subject to BCA assays for the protein concentration measurement. From each sample, 20 μg of protein was saved separately to blot whole cell lysate ENaCs. To dilute SDS, 750 μL of TBS with 1% Triton-X-100 and 1 mM DTT was added at equal amounts to the samples. The samples were pre-cleared using A/G Agarose (Santa Cruz) for 30 min at 4°C and immunoprecipitated with either 1:200 dilution anti-V5 (Thermo Fisher Scientific) or 1:100 dilution anti-HIS (Cell Signaling) antibody overnight at 4°C under rotation. The beads were washed once
with TBS with 0.5 M LiCl and 1 mM DTT and twice with TBS with 1% Triton X-100 and 1 mM DTT. Proteins were eluted in 2x laemmli sample buffer by boiling at 95°C for 5 min and separated from the beads by centrifugation at 13000 x g for 10 min. Supernatants were subjected to SDS-PAGE. Endogenous ubiquitin was detected using anti-Ubiquitin antibody (Enzo) and overexpressing HA-ubiquitin was detected using anti-HA antibody (Covence).

### 3.2.6. Binding Assay

HEK293T cells were seeded on glass cover slips and transfected with human αENaC, βENaC-GFP, and γENaC. Cells were incubated with rSPLUNC1 (10 μM) labelled with DyLight 594. Cells were washed three times with PBS and fixed with 4% paraformaldehyde (PFA) for 5 min at room temperature.

### 3.2.7. Immunostaining

#### 3.2.7.1 The Effects of MG-115 and chloroquine:

HEK293T cells were seeded on glass cover slips and transfected with human αENaC-V5, βENaC-GFP, and γENaC. Cells were pretreated with the proteasome inhibitor MG-115 (30 μM) and the lysosome inhibitor chloroquine (200 nM) for 30 min in MEM-α media with 10% FBS at 37°C/5% CO₂. Following pre-treatment with inhibitors, cells were treated with SPLUNC1 (10 μM) for 3 h then fixed with 4% PFA for 5 min at room temperature. Cells were permeabilized using permeabilizing solution (PBS with 5% normal goat serum, 1% bovine serum albumin, and 0.1% Triton X-100) for 1 h at room temperature under gentle agitation. Anti-V5 mouse antibody (Thermo Fisher Scientific) was incubated overnight at 4°C in permeabilizing solution at 1:500 dilution. Cells were washed with PBS and treated with anti-mouse secondary antibody conjugated
with DyLight 649 (Jackson ImmunoResearch Laboratories) for 1 h at room temperature in permeabilizing solution at 1:200 dilution.

3.2.7.2 Colocalization:

HEK293T cells were transfected with either (1) human αENaC-V5, βENaC, and γENaC-GFP to examine α- and γENaC colocalization or (2) human αENaC-GFP, βENaC, and γENaC to examine αENaC and LAMP1 colocalization. After treating cells with SPLUNC1 (10 μM) for 1.5 h in MEM-α media with 10% FBS, cells were fixed with 4% PFA, blocked for 1 h at room temperature under gentle agitation using permeabilizing solution, and incubated overnight at 4°C with either anti-V5 mouse antibody (Thermo Fisher Scientific) at 1:500 dilution or anti-lysosomal membrane protein 1 (LAMP1) rabbit antibody (Cell Signaling) at 1:200 dilution in permeabilizing solution. Anti-mouse or anti-rabbit secondary antibody conjugated with DyLight 649 (Jackson ImmunoResearch Laboratories) was treated at 1:200 dilution in permeabilizing solution for 1 h at room temperature. Cells were counterstained with DAPI (1 μg/mL) for 5 min at room temperature.

3.2.8. Acquiring and Analyzing Confocal Images

Cover slips were mounted on slides using FluorSave™ (Milipore) or on a ring chamber with PBS on top. Images were acquired with a Leica SP5 or SP8 confocal microscope using a 63x/1.3 NA glycerol immersion lens or 63x/1.4 NA oil immersion lens, respectively. Images were analyzed using Image-J software (NIH) or the Leica Application Suite (Leica).

3.2.9. Acceptor Photobleaching Förster Resonance Energy Transfer

HEK293T cells were transfected with the following combinations of constructs: (1) Human αENaC-GFP, βENaC, and γENaC-mCherry, (2) human αENaC, βENaC-GFP, and γENaC-mCherry, (3) human αENaC, βENaC, and γ-mCherry and GFP alone, or (4) human αENaC,
βENaC-GFP, γENaC, and mCherry alone. Following treatment with SPLUNC1 (10 μM) in MEM-α media with 10% FBS for 5, 15, 30, or 60 min at 37°C/5% CO₂, cells were fixed with 4% PFA for 5 min at room temperature. Acceptor Photobleaching Förster Resonance Energy Transfer (FRET) was performed using a Leica SP5 confocal microscope with a 63x/1.3 NA glycerol immersion objective, as previously described (Staruschenko et al., 2004). Briefly, the donor (GFP) was excited at 488 nm and emission collected from 495–549 nm. The acceptor (mCherry) was excited at 561 nm and emission collected from 530–645 nm. Images of the donor and acceptor before and after photobleaching were obtained. The percentage FRET efficiency was calculated as \[
\frac{\text{Donor}^{\text{postbleach}} - \text{Donor}^{\text{prebleach}}}{\text{Donor}^{\text{postbleach}}} \cdot 100.
\]

3.2.10. Statistical Analysis

All experiments were repeated on 3–6 separate occasions. Experiments conducted using HBECs were repeated with 4–5 different donors with triplicates per donor. All immunostaining and FRET experiments were done in triplicates where each replicate counts as a cover slip. For each replicate, 10–20 cells were selected for analysis. All image analyses were performed using Image J. Densitometry analyses on surface biotinylation normalized to GAPDH. Immunoprecipitation densitometry analyses were normalized to the respective immunoblot of the pull-downed protein except for in the time course experiment.

Outliers were identified and excluded using the robust nonlinear regression outlier test (Motulsky et al., 2006). The D’Agostino-Pearson omnibus normality test identified the data set distribution. When comparing two groups, we used a non-paired, two tail student t-test for normally distributed data and the Kolmogorov-Smirnov test for non-normally distributed data. For multiple group comparisons, we used ANOVA on data with a normal distribution and the Kruskal-
Wallis test on data with a non-normal distribution. Either * or + was used to notate \( P \) values: */+ \( P < 0.05 \), **/+ \( P < 0.01 \), ***/+///+ \( P < 0.001 \), ****/+////+ \( P < 0.0001 \). All data are shown as mean ± SEM.

3.3. Results

3.3.1. Characteristics of ENaC subunit internalization by SPLUNC1

3.3.1.1. ENaC internalization and degradation by SPLUNC1 on HEK293T cells

The ENaC inhibitory domain of SPLUNC1, known as S18, reduces ENaC currents even after treatment with MTSET, a sulfhydryl reagent that locks ENaC in an open position when βENaCs have S518C point mutation (Hobbs et al., 2013). This suggests that SPLUNC1 inhibits ENaC by reducing the number of plasma membrane ENaCs. To investigate whether SPLUNC1 internalizes endogenous plasma membrane ENaCs, we performed surface biotinylation after treating HBECs with vehicle or recombinant SPLUNC1 (rSPLUNC1) or rSPLUNC1 mutants lacking S18 (Δ44rSPLUNC1) (Garland et al., 2013; Hobbs et al., 2013). We decided to run the two proteins on a native gel to detect each protein at a non-denatured state. The wild type rSPLUNC1 was found to have a molecular weight of ~25 kDa while Δ44rSPLUNC1 was found with a lower molecular weight of ~20 kDa (Figure 3. 1). As previous studies have demonstrated that a minimum of 2 h incubation with SPLUNC1 hydrates ASL, we performed surface biotinylation after 3 h of incubation with rSPLUNC1 (Garcia-Caballero et al., 2009; Garland et al., 2013; Hobbs et al., 2013). We have previously shown that the IC\(_{50}\) and IC\(_{100}\) of SPLUNC1 is 6.53 µM and 11.94 µM, respectively (Chapter 2; Choi et al., 2015). Therefore, we decided to treat cells with 10 µM of SPLUNC1, a value higher than IC\(_{50}\) but not exceeding IC\(_{100}\).
To look at the effects of SPLUNC1 on all three subunits, we transfected HEK293T cells with $\alpha\beta\gamma$ENaC with only one subunit tagged with HA/V5 per transfection. We used the V5 tag to probe for each subunit at the plasma membrane and in the whole cell lysate after 3 h exposure to rSPLUNC1. We used GAPDH to ensure that the membrane fraction was clean and subsequently, all densitometry values were normalized to GAPDH as loading controls. We used non-transfected cells to show the specificity of the primary antibody.

A 3 h incubation with rSPLUNC1 but not $\Delta^{44}$rSPLUNC1 significantly decreased the number of plasma membrane and the whole cell lysate $\alpha$ENaCs (Figure 3. 2A). Both cleaved (molecular weight $\sim$75 kDa) and uncleaved (molecular weight $\sim$100 kDa) versions of $\alpha$ENaC were detected at the plasma membrane while only the uncleaved version was detected in the lysate (Figure 3. 2A). Ultimately, rSPLUNC1 was able to reduce both cleaved and uncleaved versions of plasma membrane $\alpha$ENaCs (Figure 3. 2A). Since the levels of both cleaved and uncleaved versions of plasma membrane $\alpha$ENaCs were reduced, when obtaining densitometry values, values of both cleaved and uncleaved versions were combined to generate a single value. The densitometry analysis indicated that rSPLUNC1 reduces plasma membrane and lysate $\alpha$ENaCs by $\sim$2-fold. On the other hand, the number of plasma membrane and lysate $\beta$ENaCs remained unchanged (Figure 3. 2A,C). For $\beta$ENaCs, only the full length version was detected as the $\beta$ENaC does not undergo proteolytic cleavages due to heavy glycosylation (Figure 3. 2A) (Rossier and Stutts, 2009). Additionally, plasma membrane and lysate $\gamma$ENaC levels were reduced with rSPLUNC1 but not $\Delta^{44}$rSPLUNC1 treatment (Figure 3. 2A,D). For $\gamma$ENaCs, only the uncleaved version was detected at the plasma membrane and lysate (Figure 3. 2A). Densitometry analysis indicated that SPLUNC1 reduces the plasma membrane and lysate $\gamma$ENaCs by $\sim$3-fold and $\sim$2-fold respectively (Figure 3. 2A,D). The combined data suggest that S18 is responsible for the internalization of $\alpha$- and $\gamma$ENaCs.
We investigated whether αENaCs go into the insoluble fraction, which may lead the decreased levels detected in the lysate. To do so, we further lysed the pellet with 5% SDS followed by sonication and probed for αENaCs. We did not detect αENaCs in the pellet, suggesting that αENaCs are not present in the insoluble portion of cells (Figure 3.3). This data suggests that decreased levels of lysate α- and γENaCs are a result of the degradation of α- and γENaCs.

We further investigated the timing of rSPLUNC1-induced internalization of αENaCs by performing a surface biotinylation over 3 h. We observed a linear decrease in both the plasma membrane and whole cell lysate αENaCs during this period (Figure 3.4). Interestingly, in this case, we were only able to detect the cleaved version of αENaCs in the membrane. This may be because these experiments were performed with HEK293T cells that has been passaged for a longer period of time. Moreover, the cleavage pattern is heavily dependent on the condition of the antibody. Therefore, rather than making detailed interpretations of the cleavage pattern, we simply note the trend of the internalization with this data. The first significant decrease was observed after 2 h of incubation with rSPLUNC1 for plasma membrane αENaCs and 3 h of incubation was necessary to see the first significant decrease in whole cell lysate αENaCs (Figure 3.4).

As another control, we investigated whether SPLUNC1 affects the number of plasma membrane and whole cell lysate Anoctamine-1 (Ano1), a multimeric ion channel expressed in the apical membrane of airway epithelia. We transfected HEK293T cells with Ano1 tagged with mCherry and incubated with rSPLUNC1 for 3 h. We detected both monomer (molecular weight ~150 kDa) and dimer (molecular weight ~250 kDa) forms in agreement with previous studies (Fallah et al., 2011; Perez-Cornejo et al., 2012). Our data revealed that rSPLUNC1 did not change the levels of plasma membrane and whole cell lysate Ano1 (Figure 3.5).
In order to investigate if rSPLUNC1 functions similarly on endogenous ENaCs, we used HBECs that endogenously express αβγENaCs. Again, we treated HBECs with either rSPLUNC1 or Δ44rSPLUNC1 for 3 h. We observed that both plasma membrane and whole cell lysate αENaCs significantly decreased by ~5 fold and ~2 fold, respectively, after 3 h of incubation with rSPLUNC1 (Figure 3.6A,B), suggesting that rSPLUNC1 internalizes and degrades endogenous αENaCs. In HBECs, only the cleaved version of αENaCs were detected for membrane fraction samples (Figure 3.6A). Although both uncleaved and cleaved versions of the αENaC subunit were detected in lysate samples, the uncleaved band appeared faintly, suggesting that the majority of lysate αENaCs exist in a cleaved state (Figure 3.6A). As rSPLUNC1 effectively reduced both cleaved and uncleaved αENaCs, cleavage activity on αENaCs likely does not interfere with SPLUNC1’s ability to internalize and degrade the αENaC (Figure 3.6A). The control protein, Δ44rSPLUNC1, did not affect either membrane or lysate αENaCs (Figure 3.6A), suggesting that SPLUNC1’s ENaC inhibitory domain is important for the internalization of endogenous αENaCs by rSPLUNC1. Conversely, we saw no changes in the number of either the plasma membrane or whole cell lysate βENaCs after rSPLUNC1 treatment (Figure 3.6C). Again, due to heavy glycosylation, βENaCs were detected at their full length (Figure 3.6C). Unfortunately, we were unable to blot γENaCs as a reliable antibody could not be obtained. We probed intracellular GAPDH to ensure that the plasma membrane fraction was clean and the densitometry value was normalized to GAPDH as a loading control. When obtaining densitometry values, the values of cleaved and uncleaved bands were combined. Densitometry indicated that rSPLUNC1 treatment significantly reduces the number of both membrane and lysate endogenous αENaCs but not βENaCs (Figure 3.6B,D).
3.3.2. ENaC internalization by SPLUNC1 requires βENaC

Previous studies by Hobbs et al. demonstrated that rSPLUNC1 only binds to the βENaC (Hobbs et al., 2013). To investigate whether this interaction between rSPLUNC1 and the βENaC plays a role in the process of αγENaC internalization, we transfected HEK293T cells with and without βENaCs and performed surface biotinylation to observe the plasma membrane the whole cell lysate αENaCs after 3 h incubation with rSPLUNC1. In agreement with previous studies (Weisz and Johnson, 2003), overall αENaC production was decreased in the absence of βENaCs and only non-cleaved αENaCs were detected at a molecular weight of ~100kDa (Figure 3. 7A). Moreover, in the absence of βENaCs, rSPLUNC1 failed to decrease plasma membrane and whole cell lysate αENaCs, suggesting that the binding of rSPLUNC1 to βENaCs is crucial for the overall ENaC internalization/degradation process (Figure 3. 7A-C).

3.3.3. Binding of SPLUNC1 to βENaC

Our data, together with previous data by Hobb et al., thus demonstrate that SPLUNC1 binds to the extracellular domain of the βENaC to internalize and degrade α- and γENaCs while leaving βENaCs at the plasma membrane (Figure 3. 2,4,6) (Hobbs et al., 2013). Hobbs et al. previously demonstrated that SPLUNC1 binds to βENaCs but not to α and γENaCs. As our data so far showed that βENaCs remain at the plasma membrane, we then investigated whether SPLUNC1 also remains at the plasma membrane. Accordingly, we performed a binding assay with HEK293T cells transfected with α-, β-GFP-, and γENaCs and incubated with rSPLUNC1 labelled with DyLight 594 over 2 h. Non-transfected cells were used to show the specificity of SPLUNC1 binding to ENaC. Our data revealed that (i) rSPLUNC1 binds to βENaCs within 30 min of incubation and (ii) that the level of binding remained constant between 30 min and 60 min of incubation (Figure 3. 8). However, 120 min of rSPLUNC1 incubation further increased the level
of binding by ~2 fold (Figure 3. 8). Qualitatively, rSPLUNC1 coated the plasma membrane of transfected HEK293T cells and remained at the surface throughout the experiment (Figure 3. 8). Furthermore, rSPLUNC1 did not bind to non-transfected HEK293T cells, indicating that there is no specific binding to non-ENaC expressing HEK293T cells (Figure 3. 8).

3.3.4. The fate of internalized αγENaC following SPLUNC1 exposure

3.3.4.1 The effect of MG-115 or chloroquine on α- and βENaC

Our data has thus far suggested that SPLUNC1 engages βENaCs and causes α- and γENaCs to be internalized and degraded (Figure 3. 2,4,6). Since there are two major pathways for protein degradation—proteasomal and lysosomal—we sought to determine the degradation pathway of α- and γENaC subunits after being internalized by rSPLUNC1 treatment. To differentiate between the two pathways, we pre-treated HEK293T cells expressing αENaC-V5, βENaC-GFP, and γENaCs with either MG-115 or chloroquine to inhibit proteasome or the lysosome pathways, respectively. The cells were then exposed to rSPLUNC1 or vehicle for 3 h, fixed with PFA, and αENaCs were immunostained using a V5 epitope. After acquiring images, we measured the relative fluorescence intensity of plasma membrane and intracellular α- and βENaCs for quantitative analysis. In agreement with our surface biotinylation data (Figure 3. 2), without any inhibitor pre-treatments, rSPLUNC1 significantly decreased the fluorescence intensity of the plasma membrane and intracellular αENaCs (Figure 3. 9A,B). Furthermore, similar to our surface biotinylation data (Figure 3. 2), without any inhibitor pre-treatments, βENaCs did not undergo internalization or degradation by rSPLUNC1 (Figure 3. 9A,C).

Compared to vehicle alone, MG-115 pre-treatment followed by vehicle increased the fluorescent intensity of α- and βENaCs at the plasma membrane, suggesting that proteasome
degradation plays a role in the normal biosynthesis and trafficking of α- and βENaCs (Figure 3. 9A-C). Pre-treatment with MG-115 did not affect rSPLUNC1’s ability to decrease the fluorescent intensity of plasma membrane αENaCs, suggesting that MG-115 pre-treatment does not inhibit the internalization process of αENaCs by rSPLUNC1 (Figure 3. 9A,B). Intracellularly, MG-115 pre-treatment interfered with SPLUNC1’s ability to degrade αENaCs (Figure 3. 9A,B). Pre-treatment with MG-115 did not affect intracellular βENaC levels (Figure 3. 9A,C).

Chloroquine pre-treatment followed by vehicle and vehicle alone without any inhibitor pre-treatment resulted in a similar fluorescent intensity of plasma membrane αENaCs, suggesting that chloroquine has a minimal effect on the trafficking pathways of αENaCs (Figure 3. 9A,B). Also, similar to rSPLUNC1 exposure alone, chloroquine pre-treatment with rSPLUNC1 exposure decreased the fluorescent intensity of plasma membrane αENaCs, suggesting that chloroquine does not inhibit rSPLUNC1’s ability to internalize αENaCs (Figure 3. 9A,B). However, chloroquine pre-treatment inhibited rSPLUNC1’s ability to decrease the fluorescent intensity of intracellular αENaCs, suggesting that the αENaC undergoes lysosomal degradation after being internalized by SPLUNC1 (Figure 3. 9A,B). Interestingly, there was a significant increase in plasma membrane βENaC fluorescent intensity with chloroquine pre-treatment compared to no inhibitor pre-treatment, suggesting that lysosomes play a role in the disposal of βENaCs (Figure 3. 9A,C). Conversely, intracellular βENaCs showed no changes following the pre-treatment with chloroquine (Figure 3. 9A,C).

3.3.4.2 Colocalization of αENaC and LAMP1

The previous set of MG-115/chloroquine data suggest that SPLUNC1-exposed αENaCs are subjected to lysosomal degradation. To validate this finding, we performed immunostaining of
LAMP1, a well-characterized lysosome marker, in the absence or presence of rSPLUNC1 in HEK293T cells expressing αENaC-GFP, βENaC, and γENaC. We chose the earlier time point of 1.5 h rather than 3 h since αENaCs signals are lost due to degradation after 3 h exposure to rSPLUNC1. To show the specificity of the secondary antibody, we immunostained cells only with a secondary antibody. After acquiring the images, we used Pearson’s correlation coefficient to quantify colocalization (Dunn et al., 2011). Our data demonstrated that rSPLUNC1 treatment significantly increased colocalization between αENaCs and LAMP1 (Figure 3. 10). Furthermore, qualitatively, we observed that rSPLUNC1 treatment increased the puncta formation. Puncta were marked with both αENaC and LAMP1 (Figure 3. 10).

### 3.3.4.3 Colocalization of α- and γENaC

Since we demonstrated that the αENaC was degraded by rSPLUNC1 via the lysosome (Figure 3. 9-10), we next tested whether γENaCs were degraded similar to αENaC after rSPLUNC1 exposure. We investigated the localization of γENaCs in relation to αENaCs by immunostaining after rSPLUNC1 treatment for 1.5 h on HEK293T cells expressing αENaC-GFP, βENaC, and γENaC-V5. Similar to the colocalization of αENaC and LAMP1 (Figure 3. 10B), we performed quantitative colocalization analysis using Pearson’s correlation coefficient. Our data revealed that the colocalization percentage between α- and γENaCs remained the same in the presence or absence of rSPLUNC1, suggesting that these two subunits remain in close proximity even after SPLUNC1-induced internalization (Figure 3. 11B). Qualitatively, similar to αENaC and LAMP1 colocalization (Figure 3. 10A), we observed that there was an increased formation of puncta marked by both α- and γENaCs (Figure 3. 11A). Furthermore, using the previous data set (shown in Figure 3. 9), we performed colocalization analysis between α- and βENaCs. The data revealed that the colocalization percentage of α- and βENaCs decreased after treatment with
rSPLUNC1, suggesting that these two subunits are dissociated after SPLUNC1 exposure and have different fates (Figure 3.11).

3.3.5. **Endocytosis of αENaC via ubiquitination**

### 3.3.5.1 ENaC subunit ubiquitination

Our data had thus far suggested that α- and γENaCs are subject to lysosomal degradation. We next sought to determine how SPLUNC1 initiated the internalization and degradation of α- and γENaCs. Although ENaCs can utilize a ubiquitin independent endocytosis pathway, such as through caveolae, ENaCs are most commonly endocytosed via a ubiquitin-dependent pathway (Eaton et al., 2010). Therefore, to investigate whether SPLUNC1 ubiquitinated ENaCs, we overexpressed αβγENaCs with one subunit tagged with V5 per transfection and the other two untagged and ubiquitin tagged with HA. As surface biotinylation and immunostaining data revealed that 1.5–2 h was sufficient for α- and γENaCs to be internalized, we chose an earlier time point, namely 1 h, to look at the ubiquitination levels of ENaC subunits. Accordingly, we treated transfected cells with rSPLUNC1 for 1 h and immunoprecipitated each subunit using its V5 tag. We probed for ubiquitin using HA epitope and used non-transfected (NT) cells to look at the specificity of the antibody detection.

In agreement with previous studies, we observed a smear above the molecular weight ~100 kDa ladder mark on the western blots of each subunit when treated with vehicle (Wiemuth et al., 2007; Zhou et al., 2007). Our surface biotinylation data revealed that the degradation of lysate α- and γENaCs takes place after 3 h of incubation with SPLUNC1 (Figure 3.4). Thus, when looking at the lysate of α- and γENaC after 1 h incubation with SPLUNC1, we did not observe any reduction (Figure 3.12A). Interestingly, with SPLUNC1 treatment, we observed a significant, ~2
fold increase in the ubiquitination of αENaCs (Figure 3. 12). By contrast, βENaC ubiquitination was significantly decreased following rSPLUNC1 treatment (Figure 3. 12). Interestingly, rSPLUNC1 treatment did not result in any difference in γENaC ubiquitination levels (Figure 3. 12). We also repeated this experiment but probed for the endogenous ubiquitination level of each ENaC subunit rather than for overexpressed ubiquitin. As expected, the level of endogenous ubiquitin detected after immunoprecipitation was weak (Figure 3. 13A). However, similar to the overexpression data, rSPLUNC1 significantly increased endogenous ubiquitination of αENaCs while having no effect on γENaC ubiquitination (Figure 3. 13A,B). On the other hand, unlike the overexpressed ubiquitin data, rSPLUNC1 did not change the ubiquitination level of βENaC (Figure 3. 13A,B). We speculate that this is due to the overall weak signal of endogenous ubiquitination, as detected by western blot.

3.3.5.2 The effect of Nedd4-2 inhibition on ubiquitination and internalization

Nedd4-2 binds to PY-motifs located at the C-termini of α, β, and γENaCs and ubiquitinates the N-terminal lysines of these subunits. Although there are evidence for trans-acting Nedd4-2 that binds to one subunit but ubiquitinates another, other studies have shown that Nedd4-2’s binding to one subunit impacts the ubiquitination of its binding subunit (Wiemuth et al., 2007; Lu et al., 2007; Zhou et al., 2007). Here, we decided to truncate αENaCs’ PY-motifs to see if this affects αENaC ubiquitination. We truncated αENaCs at P595 to remove the intracellular C-terminus region containing the PY-motif which has the molecular weight of ~7 kDa. In agreement with a previous study demonstrating that the absence of the PY motif increases Po by increasing proteolytic cleavages of the extracellular loop of αENaCs (Abriel et al., 1999), when αENaCs’ PY-motifs were truncated, only the cleaved version of αENaCs were detected by western blot in the lysate at ~70 kDa (Figure 3. 12-13). This maneuver prevented rSPLUNC1 from ubiquitinating
αENaCs (Figure 3. 12-13). Here, we used an ubiquitin antibody that can detect both mono- and poly-ubiquitination. We selected this antibody to observe the overall levels of ubiquitination. Therefore, the smearing on the western blots of wild type and truncated αENaCs detecting for ubiquitin did not exhibit different patterns but rather exhibited different levels of detection. To validate this experiment, we co-transfected αβγENaCs with a Nedd4-2 negative dominant construct, which contained all WW domains but lacked the HECT domain, responsible for catalyzing ENaC ubiquitination (Snyder et al., 2004b). When co-transfecting the Nedd4-2 dominant negative construct with αβγENaCs, we were only able to look at the endogenous ubiquitin levels due to the overall low transfection efficiency when attempting to transfect five different constructs, i.e. human α-, β-, γENaC, HA-ubiquitin, and Nedd4-2 dominant negative constructs. Although we observed weaker ubiquitination signals when looking at endogenous ubiquitin levels, similar to the overexpressed ubiquitin data, we observed that inhibiting the Nedd4-2 function abolished rSPLUNC1’s ability to ubiquitinate αENaCs (Figure 3. 13C,D).

To confirm that αENaC ubiquitination leads to its internalization, we performed surface biotinylation on HEK293T cells transfected with either truncated α-, β-, and γENaC or wild type α-, β-, γENaC and Nedd4-2 dominant negative constructs. The truncated αENaC was found only as a cleaved version at the plasma membrane while wild type αENaCs were found both as cleaved and uncleaved versions at the plasma membrane (Figure 3. 14A). Here, we observed that cleaved αENaCs were detected in the lysate (Figure 3. 14A). However, the uncleaved αENaC band was ~2.5 fold stronger in detection, suggesting that the majority of lysate αENaCs existed uncleaved (Figure 3. 14A). Interestingly, when the Nedd4-2 dominant negative construct was co-expressed with αβγENaCs, the cleaved version of αENaCs were the majority at the plasma membrane (Figure 3. 14). This is in agreement with previous studies demonstrating that the inhibition of Nedd4-2
leads to an increased activation of αENaCs (Abriel et al., 1999). In both cases, rSPLUNC1 was unable to internalize and degrade αENaCs, suggesting that the interaction between Nedd4-2 and αENaCs’ PY-motifs is important for rSPLUNC1-induced αENaC internalization and degradation (Figure 3. 14A-C).

3.3.5.3 The time course of αENaC ubiquitination by rSPLUNC1

To investigate the timing of αENaC ubiquitination following rSPLUNC1 exposure, we conducted a time course study of immunoprecipitation over 3 h. We observed an increasing trend for the first 60 min. After 60 min of rSPLUNC1 exposure, we observed the first significant change in αENaC ubiquitination, also the time-point with the maximum ubiquitination (Figure 3. 15). After 60 min incubation with rSPLUNC1, ubiquitination began to decrease continuously for the next 120 min (Figure 3. 15). In agreement with our previous data (Figure 3. 4), after 120 min of rSPLUNC1 incubation, whole cell lysate αENaCs began to decrease and therefore the number of pulled down αENaCs and the αENaC ubiquitination level decreased accordingly (Figure 3. 15). GAPDH was blotted to show that an equal amount was loaded.

3.3.6. SPLUNC1 is an Allosteric Modulator of ENaC

3.3.6.1 FRET efficiency between different ENaC subunits

Our data had thus far indicated that SPLUNC1 dissociates αβγENaCs. We next sought to determine how SPLUNC1 induced this change. Because SPLUNC1 binds extracellularly to βENaC, yet the majority of known ENaC trafficking mechanisms are intracellular, we hypothesized that SPLUNC1 is an allosteric regulator of ENaC. To test this hypothesis, we measured FRET efficiency between ENaC subunits labelled C-terminally with either GFP or mCherry. As controls, we measured FRET efficiency between one ENaC subunit labelled with
either GFP or mCherry and free mCherry or GFP. As we observed that αENaC ubiquitination takes place as soon as 1 h after incubation with rSPLUNC1 (Figure 3.15), we looked at FRET efficiencies of different ENaC subunit combinations at the plasma membrane after 1 h incubation with SPLUNC1.

Positive FRET values can result from the crowding effect, a phenomenon observed when the acceptor fluorophore is close in distance to the donor fluorophore by random chance due to a high level of protein expression. We investigated if the ENaC subunits FRET pair and control FRET pair resulted in positive FRET values due to the crowding effect. To do so, we plotted the acceptor fluorescent intensity against the FRET efficiency (Figure 3.16A). We observed that there was a positive linear relationship between the control FRET pair, suggesting the measurable change was due to the crowding effect (Figure 3.16A). However, when we plotted the acceptor fluorescent intensity against the FRET efficiency between the αENaC-GFP and γENaC-mCherry FRET pair, we did not observe a positive relationship (Figure 3.16B). This data indicates that the change in ENaC FRET efficiency was not due to the crowding effect and instead was a strategic interaction (Figure 3.16B).

We observed a significant decrease in FRET efficiencies between (i) the αENaC-GFP and γENaC-mCherry FRET pair and (ii) the βENaC-GFP and γENaC-mCherry FRET pair at the plasma membrane after 1 h incubation with rSPLUNC1 (Figure 3.17B). Similarly, intracellular FRET efficiencies between (i) the αENaC-GFP and γENaC-mCherry FRET pair and (ii) the βENaC-GFP and γENaC-mCherry FRET pair significantly decreased after 1 h incubation with rSPLUNC1 (Figure 3.17A,C). At the intracellular level, all control FRET pairs displayed a low level of FRET efficiency with vehicle treatment. Incubation with rSPLUNC1 did not yield any further changes, suggesting that SPLUNC1 exerts its effect within the ENaC subunits (Figure 3.
17A,C). At the plasma membrane, the βENaC-GFP and mCherry control FRET pair only showed a low level of FRET efficiency with vehicle treatment and incubation with rSPLUNC1 did not yield any significant changes in FRET efficiency (Figure 3. 17A,B). While the γENaC-mCherry and GFP control FRET pair also showed a low level of FRET efficiency with vehicle treatment at the plasma membrane, we detected a decrease in the FRET efficiency with rSPLUNC1 incubation (Figure 3. 17A,B). Based on our surface biotinylation data, βENaCs remain at the plasma membrane while γENaCs are internalized. Therefore, we speculate that the change in FRET efficiency between the γENaC-mCherry and GFP pair is due to the movement γENaCs take in preparation for internalization.

3.3.6.2 The time course of FRET efficiency

Since a greater change in the FRET efficiency was observed between the βENaC-GFP and γENaC-mCherry FRET pair than the αENaC-GFP and γENaC-mCherry FRET pair, the former was chosen to evaluate the time course of FRET efficiency at the plasma membrane to delineate the time line of SPLUNC1’s effect on the conformation of ENaCs (Figure 3. 18). Our data indicated that the FRET efficiency decreased linearly over 60 min with rSPLUNC1 incubation, with the first significant change occurring at 30 min after rSPLUNC1 incubation (Figure 3. 18).

3.4. Discussion

3.4.1. SPLUNC1’s order of action on ENaC

We used the FRET technique to determine whether conformational change was induced by rSPLUNC1, surface biotinylation to observe the change in plasma membrane ENaC levels, and immunoprecipitation to investigate ENaCs’ ubiquitination by rSPLUNC1. In particular, we focused on the time course of these experiments to fully understand the sequence of events (Figure 3. 4, 15, 18). Taking these data sets together, we conclude that rSPLUNC1’s binding to
βENaCs leads to the conformational change of ENaCs, promoting the ubiquitination of αENaCs by increasing their interaction within the PY-motif and the ubiquitin ligase Nedd4-2. This ubiquitination results in the internalization and degradation of α- and γ-ENaCs while leaving βENaC at the plasma membrane (Figure 3. 2, 6, 12-15).

3.4.2. Non-coordinated subunit regulation

Here, we have demonstrated that SPLUNC1 dissociates both native ENaCs in HBECs and overexpressed ENaCs in HEK293T cells. Other researchers have indicated that ENaC subunits may be regulated in a non-coordinated manner (Lebowitz et al., 2003; Peters et al., 2014; Stockand et al., 2000; Weisz and Johnson, 2003). However, our finding is novel in that we directly demonstrated that SPLUNC1 induces α- and γENaC internalization and degradation by lysosomes while leaving SPLUNC1 and βENaCs at the plasma membrane. Furthermore, it is of interest that we demonstrated that ENaCs’ conformational change leads to the physical dissociation of the heterotrimeric channel. Based on our data, in addition to previously published data revealing that SPLUNC1 binds to βENaC (Hobbs et al., 2013), we hypothesize that SPLUNC1 binding to βENaC generates a new and relatively stable complex (i.e. SPLUNC1-βENaC) at the expense of αγENaCs, which are degraded. While αβγENaC is typically thought to be the functional unit of the ENaC, it has previously been proposed that ENaCs may also exist in trimers of trimers (Staruschenko et al., 2004). Thus, depending on SPLUNC1 concentration in ASL, there may also be an additional mixed population of SPLUNC1-βENaCs and/or internalized αγENaCs, although whether they still associate as hetero-multimers (i.e. trimers of trimers) remains to be determined.

3.4.3. Ubiquitination and protein control

Ubiquitination of ENaCs has long been recognized as important for regulating ENaC surface densities. This is illustrated by the gain of function mutations in Liddle’s disease, wherein
deletion/disruption of the C-terminal PY-motif in β- and/or γENaCs prevents Nedd4-2-dependent ubiquitination of ENaCs, leading to more functional ENaCs at the plasma membrane (Eaton et al., 2010; Knight et al., 2006). It has been previously proposed that unassembled subunits went to the proteasome while assembled αβγENaCs went to the lysosome (Eaton et al., 2010; Rotin and Staub, 2011; Staub, 1997). In agreement, we noted that both lysosomal and proteasomal inhibitors increased basal ENaC surface densities (Figure 3.9). However, only lysosomal inhibition prevented SPLUNC1-induced degradation of αγENaCs. Butterworth et al. demonstrated that the inhibition of deubiquitinating enzymes increased ubiquitination and decreased the surface expression of αβγENaCs (Butterworth et al., 2007). Interestingly, this maneuver only decreased surface expression and did not alter the intracellular pool, unlike our observations that SPLUNC1-exposed αγENaCs were trafficked to lysosomes. While all ENaC subunits can be ubiquitinated at the plasma membrane (Staub et al., 2000; Wiemuth et al., 2007), the observation that SPLUNC1 only triggered the ubiquitination of αENaCs is novel. Furthermore, it is a newly acknowledged phenomenon that αENaC ubiquitination results in the endocytosis and degradation of not only the α- but also γENaC. We chose to truncate the C-terminus of αENaCs to mimic the premature stop mutation introduced to truncate the C-terminus of β- and γENaC, found in Liddle’s syndrome patients. However, point mutations on putative ubiquitin-receiving lysine residues on αENaCs will further characterize the exact interaction of Nedd4-2 with αENaCs induced by SPLUNC1. Additionally, investigating the effects of the removal of PY-motifs on β- and/or γENaCs on SPLUNC1’s ability to ubiquitinate αENaCs will provide insights to the overall ENaC conformation in relation to ubiquitination and may explain SPLUNC1’s inability to negatively regulate ENaCs in the kidneys of Liddle’s syndrome patients when SPLUNC1 is found in the kidney.
3.4.4. SPLUNC1-induced ENaC’s conformational change

Our data indicated that SPLUNC1’s extracellular binding to βENaCs triggered ENaCs’ conformational change, as indicated by changes in the plasma membrane FRET efficiency between α-γ and β-γ ENaC subunits (Figure 3.17A,B). Traditionally, this data has only indicated that either the distance or orientation between GFP and mCherry was altered. However, since these FRET probes were linked to the C-termini of different ENaC constructs, it is likely that the C-termini were also altered together with the probes. Therefore, we were able to use FRET efficiency as a measure of ENaCs’ conformational change. Importantly, this data demonstrated that SPLUNC1 is an allosteric modulator of ENaC since the binding of SPLUNC1 to βENaC was extracellular while changes in FRET efficiency occurred intracellularly.

As observed in Figure 3.13-14, the truncation of αENaCs’ PY-motifs did not completely abolish the ubiquitination of αENaCs at the basal level. This may be due to the interaction between Nedd4-2 and PY-motifs on β- and/or γENaCs. However, the fact that the truncation of αENaCs’ PY-motifs hindered SPLUNC1 from further ubiquitinating αENaCs suggests that the conformational change of ENaCs by SPLUNC1 disables αENaCs from receiving ubiquitin from Nedd4-2 binding β- and/or γ-ENaCs. Thus, we hypothesized that the conformational change by rSPLUNC1 allows αENaC’s intracellular terminus to become more accessible to Nedd4-2-dependent ubiquitination. It is common that lysine residues on the target protein receive ubiquitin from the ubiquitin ligase (Hochstrasser, 1996). Therefore, investigating the effects of point-mutations in αENaCs’ intracellular lysine residues on ubiquitination will help in determining how αENaCs receive ubiquitin and how SPLUNC1-induced conformational change plays a role in the ubiquitination process.
3.4.5. Concluding remarks

To date, ENaC binding compounds that directly inhibit ENaCs are limited to amiloride and its analogues (Althaus, 2013). These compounds are channel pore blockers that inhibit ENaC function by decreasing Po. These characteristics have led to the development of amiloride and its analogues as therapeutic agents for CF patients whose ASL is severely dehydrated due to hyperactive ENaCs. However, attempts have been thus far unsuccessful due to the rapid clearance of amiloride from the airway (Noone et al., 1997). Unlike these channel blockers, our data suggest that SPLUNC1 inhibits ENaC through the regulation of ENaC trafficking and protein degradation. Although βENaCs are left at the plasma membrane by SPLUNC1, they cannot function alone as an ion channel. Thus, inhibition of ENaC function by SPLUNC1 is likely long-lasting, suggesting SPLUNC1 as a potential therapeutic agent for CF patients. However, it has been revealed that SPLUNC1 is a pH sensitive protein and that it cannot inhibit ENaCs in an environment where the pH is lower than 6 (Garland et al., 2013; Hobbs et al., 2013). Since the pH of CF airways is also acidic, with a pH of 6.5 or lower, it seems more promising to utilize SPLUNC1’s ENaC inhibitory domain peptide, demonstrated to be pH-insensitive, for CF therapy (Garland et al., 2013; Hobbs et al., 2013).
Figure 3. 1. Native gel electrophoretic mobility shifts of SPLUNC1 and Δ44SPLUNC1. Wild type SPLUNC1 runs at ~25 kDa while Δ44SPLUNC1 runs at a lower molecular weight of ~20 kDa.
Figure 3.2. rSPLUNC1 internalizes α and γENaC, leaving βENaC at the plasma membrane in HEK293T cells. (A) Representative immunoblots of HEK293T cells transfected with ENaC subunits differentially tagged and probed with a V5 antibody: Plasma membrane fraction (above the dotted line) and whole cell lysate (below the dotted line) after 3 h incubation with vehicle, △44rSPLUNC or rSPLUNC1. GAPDH was blotted as a loading control and to ensure that the membrane fraction was free of intracellular proteins. (B-D) Densitometry of plasma membrane and whole cell lysate of (B) α-, (C) β-, and (D) γENaC corresponding to panel A (n = 4). Densitometry values of the cleaved and un-cleaved bands were combined. * P < 0.5 and ** P < 0.01. All data are shown as mean ± SEM.
Figure 3. 3. αENaC is not found in the insoluble fraction independent of SPLUNC1 treatment for 3 h. HEK293T cells were transfected with α-V5, β-, and γENaC and treated with or without SPLUNC1 for 3 h. The cells were lysed using surface biotinylation lysis buffer and centrifuged to collect supernatant. The insoluble pellet was further lysed with 5% SDS followed by sonication. Western blot of V5 probing for αENaC and GAPDH of both samples.
**Figure 3.4.** rSPLUNC1 internalizes αENaC in a time-dependent manner in HEK293T cells.  
(A) Representative immunoblot of plasma membrane (above the dotted line) and whole cell lysate (below the dotted line) αENaC-V5 after 1-3 h exposure to rSPLUNC1. GAPDH was blotted as a loading control and to ensure that the membrane fraction was free of intracellular proteins. (B) Densitometry of plasma membrane (red) and whole cell lysate (blue) αENaC with rSPLUNC1 corresponding to panel A (n = 3). *P < 0.5 and +++P < 0.001. * compared surface membrane to 0 h. + compared whole cell lysate to 0 h. All data are shown as mean ± SEM.
Figure 3.5. rSPLUNC1 does not internalize Ano1 in HEK293T cells. (A) Immunoblot of Ano1 tagged with mCherry from plasma membrane and whole cell lysate (upper) after 3 h incubation with vehicle or rSPLUNC1. GAPDH was blotted as a loading control and to ensure that the membrane fraction was free of intracellular proteins. (B) Densitometry of plasma membrane and whole cell lysate Ano1 corresponding to panel A (n = 3). Densitometry values of the monomer and dimer bands were combined. All data are shown as mean ± SEM.
Figure 3. 6. rSPLUNC1 reduces endogenous αENaC but not βENaC in HBECs. (A) Immunoblot of endogenous αENaC from plasma membrane and whole cell lysate after treatment with vehicle, Δ^44 rSPLUNC1, or rSPLUNC1. Whole cell lysate GAPDH was blotted as a loading control and to ensure the membrane fraction was free of intracellular proteins. (B) Densitometry of αENaC corresponding to panel A (n = 6). * P < 0.5 and ** P < 0.01. (C) Immunoblot of endogenous βENaC from plasma membrane and whole cell lysate after treatment with vehicle, Δ^44 rSPLUNC1, or rSPLUNC1. GAPDH was blotted as a loading control and to ensure that the membrane fraction was free of intracellular proteins. (D) Densitometry of βENaC corresponding to panel C (n = 6). All data are shown as mean ± SEM.
Figure 3. rSPLUNC1 internalizes αENaC through the interaction with βENaC in HEK293T cells. (A) Representative immunoblot of plasma membrane αENaC-V5 (above the dotted line), whole cell lysate αENaC-V5 (below the dotted line) after 3 h treatment with vehicle or rSPLUNC1 in the presence or absence of βENaC. GAPDH was blotted as a loading control and to ensure that the membrane fraction was free of intracellular proteins. (B-C) Densitometry of (B) plasma membrane and (C) whole cell lysate αENaC-V5 in different transfection and treatment conditions corresponding to panel A (n = 3). * P < 0.5. Densitometry values of the cleaved and un-cleaved bands were combined. All data are shown as mean ± SEM.
Figure 3. 8. rSPLUNC1 remains at the plasma membrane on HEK293T cells. (A) Representative fluorescent images of βENaC-GFP (green), rSPLUNC1-DyLight594 (red), DAPI (blue), and merged channels in the presence or absence of rSPLUNC1-DyLight594. (B) Quantification of average fluorescence intensity of rSPLUNC1-DyLight594 corresponding to panel A (n = 3). * P < 0.5 and ** P < 0.01. All data are shown as mean ± SEM.
Figure 3. 9. Inhibition of lysosomes but not proteasomes prevents αENaC degradation in HEK293T cells. (A) Representative fluorescent images of αENaC-V5 (red), βENaC-GFP (green), and merged channels in the presence or absence of rSPLUNC1 obtained by confocal microscopy. HEK293T cells were pre-treated with MG-115 and chloroquine for 30 min prior to a 3 h incubation with rSPLUNC1. (B-C) Quantification of average fluorescence intensity of plasma membrane and intracellular (B) αENaC and (C) βENaC corresponding to panel A (n = 4). * P < 0.5, ** P < 0.01, *** P < 0.001 and **** P < 0.0001. All data are shown as mean ± SEM.
Figure 3. 10. αENaC colocalizes with LAMP1 in HEK293T cells. (A) Representative confocal fluorescent images of αENaC-GFP (green), LAMP1 labeled using anti-rabbit DyLight649 (red), DAPI (blue) and merged channels after 1.5 h incubation with vehicle or rSPLUNC1 (left). As a control, we imaged HEK293T cells incubated with secondary antibodies only (right). (B) % colocalization between αENaC and LAMP1 with or without rSPLUNC1 corresponding to panel A (n = 3). ** P < 0.01. All data are shown as mean ± SEM.
Figure 3.11. α- and γENaC colocalize in HEK293T cells. (A) Representative fluorescent images of αENaC-GFP (green), γENaC-V5 labeled using anti-mouse DyLight649 (red), DAPI (blue) and merged channels after 1.5 h incubation with vehicle or rSPLUNC1. (B) Colocalization between α- and βENaC (left) and α- and γENaC (right) with or without rSPLUNC1 corresponding to Figure 3.11 panel A and Figure 3.9 panel A respectively (n = 3). **** P < 0.0001. All data are shown as mean ± SEM.
Figure 3. 12. Detection of increased overexpressed ubiquitin on αENaC by rSPLUNC1 in HEK293T cells. Red squares on immunoblot panels indicate the region used for densitometry analysis. (A) Representative immunoblot of overexpressed HA-ubiquitin (upper) after immunoprecipitating ENaC subunits using V5 or HIS and whole cell lysate of V5 or HIS tagged ENaC subunits (lower) after ~1 h incubation with rSPLUNC1. (B) Densitometry of overexpressed HA-ubiquitin (n = 3). * P < 0.5. All data are shown as mean ± SEM.
Figure 3. 13. Detection of increased endogenous ubiquitin on αENaC by rSPLUNC1 in HEK293T cells. Red squares on immunoblot panels indicate the region used for densitometry analysis. (A) Representative immunoblot of endogenous ubiquitin (upper) after immunoprecipitating each ENaC subunit using V5 tag and whole cell lysate immunoblot of V5 tagged ENaC subunit (lower) in the presence or absence of rSPLUNC1. (B) Densitometry of endogenous ubiquitin corresponding to panel A (n = 4). *** P < 0.001. (C) Representative immunoblot of endogenous ubiquitin on truncated αENaC or wild type αENaC co-expressed with a Nedd4-2 dominant negative construct (upper) after immunoprecipitating αENaC-V5 and whole cell lysate αENaC-V5 (lower) in the presence or absence of rSPLUNC1. (D) Densitometry of endogenous ubiquitin corresponding to panel C (n = 3). ** P < 0.01. All data are shown as mean ± SEM.
Figure 3. 14. Deletion of αENaC’s C-terminus or co-expression of a Nedd4-2 dominant negative construct abolishes the internalization of αγENaC by rSPLUNC1 in HEK293T cells.

(A) Immunoblot of plasma membrane (upper) or whole cell lysate (lower) αENaC-V5 or truncated αENaC-V5 after 3 h incubation with vehicle, ∆44 rSPLUNC1 or rSPLUNC1. GAPDH was blotted as a loading control and to ensure the membrane fraction was free of intracellular proteins. (B) Densitometry of plasma membrane full length or truncated αENaC for each treatment corresponding to panel A (n = 3). ** P < 0.01. Densitometry values of the cleaved and un-cleaved bands were combined. (C) Densitometry of whole cell lysate αENaC or truncated αENaC for each treatment corresponding to panel A (n = 3). * P < 0.5. Densitometry values of the cleaved and un-cleaved bands were combined. All data are shown as mean ± SEM.
Figure 3. 15. rSPLUNC1 ubiquitinates αENaC after ~1 h of incubation in HEK293T cells. (A) Representative immunoblot of HA-ubiquitin (upper) and V5 (lower) after immunoprecipitating each ENaC subunit using its V5 tag post-incubation with rSPLUNC1 for 0, 30, 60, 120 and 180 min. Separated by the dotted line, whole cell lysate immunoblot of V5 tagged ENaC subunit (upper) and GAPDH (lower) after the treatment with rSPLUNC1 for 0, 30, 60, 120 and 180 min. GAPDH was blotted as a loading control. (B) Densitometry of ubiquitin over the course of 180 min of rSPLUNC1 incubation (n = 3). ** P < 0.01. * compared to time 0 min. All data are shown as mean ± SEM.
Figure 3. 16. The change in the FRET efficiency between the γENaC-mCherry and free GFP FRET pair is due to the crowding effect while that of the αENaC-GFP and γENaC-mCherry FRET pair is not. The FRET efficiency between the donor (GFP) and the acceptor (mCherry) was plotted in relation to the brightness of the acceptor fluorophore (mCherry). Positive but not zero correlation suggests the crowding effect since the FRET efficiency increases as the brightness of the acceptor increases. The function of the fitted regression line is shown together with the coefficient of determination ($R^2$). The relationship between (A) γ-mCherry and free GFP and (B) α-GFP and γ-mCherry are shown above.
Figure 3. rSPLUNC1 is an allosteric modulator of ENaC in HEK293T cells. (A) Representative fluorescent images of donor (GFP) and acceptor (mCherry) before and after acceptor photobleaching between (1) αENaC-GFP and γENaC-mCherry, (2) βENaC-GFP and γENaC-mCherry, (3) αENaC-GFP and mCherry alone, and (4) GFP alone and γENaC-mCherry. (B-C) FRET efficiency of different ENaC combinations from the (B) plasma membrane or (C) intracellular after a 60 min incubation with vehicle or rSPLUNC1 (n = 3-4). * P < 0.5 and *** P < 0.001. All data are shown as mean ± SEM.
Figure 3. SPLUNC1 induces ENaC conformational change in a time-dependent manner in HEK293T cells. Plasma membrane FRET efficiency between βENaC-GFP and γENaC-mCherry in the presence of rSPLUNC1 over the course of 60 min ($n = 3$). ** $P < 0.01$ and *** $P < 0.001$. * compared to time 0 min. All data are shown as mean ± SEM.
**Figure 3. 19. SPLUNC1’s Order of Action.** The right axis of the graph (red) plots the relative densitometry of HA-ubiquitin from Figure 3. 15 (hollow triangle), and the plasma membrane αENaC-V5 from Figure 3. 4 (solid square). The left axis of the graph (black) plots % FRET efficiency from Figure 3. 18 (solid circle). All data are shown as mean ± SEM.
Chapter 4: Overall Conclusions and Future Directions

In this dissertation, we sought to investigate the inhibitory effects of SPLUNC1 on ENaCs. The major findings demonstrate that the binding of SPLUNC1 to the β subunits of ENaCs causes the internalization/degradation of α- and γENaCs. Unlike amiloride, which is rapidly cleared from the airways and can therefore only inhibit ENaC for a short period of time (Noone et al., 1997), SPLUNC1’s ability to induce α- and γENaC degradation suggests SPLUNC1 is a promising therapeutic agent for CF. However, unfortunately, SPLUNC1 is a pH-sensitive protein that loses its ability to inhibit ENaC in acidic CF airways (Garland et al., 2013). Since it is already known that SPLUNC1’s ENaC inhibitory domain is pH-insensitive (Garland et al., 2013), investigating whether this domain employs the same mechanism to inhibit ENaC will likely help in the development of a novel therapeutic approach to CF to enhance airway hydration and thus improve mucociliary clearance.

The physical dissociation of heterotrimeric αβγENaCs is a key finding of this dissertation. This observation is novel as we are the first to directly demonstrate the physical dissociation of αβγENaCs. While it has been traditionally believed that ENaCs are a stable complex, our observation is aligned with biological observations of other multi-subunit proteins that are stable yet able to dissociate. For example, G proteins exist as a stable heterotrimer comprised of α, β, and γ subunits, but have been shown to dissociate (Chisari et al., 2009; Digby et al., 2006). The dissociated Gβγ functions separately from the Ga subunit in a variety of different cellular processes, including but not limited to cell division and endosomal signaling (Khan et al., 2013). Additionally,
voltage gated Ca\textsuperscript{++} channels expressed in neurons are known to be a stable multimer comprised of \(\alpha_1, \alpha_2, \beta, \delta\) and \(\gamma\) subunits (Dolphin, 2013). However, interestingly, the \(\alpha_2\) and \(\delta\) subunits can form a separate complex and function as a receptor for drugs such as pregabalin and modulate synaptogenesis (Dolphin, 2013; Stahl et al., 2013). Similarly, we suggest that \(\beta\)ENaCs function separately as a receptor for SPLUNC1. Furthermore, since subunits of a complex with additional roles are a relatively common phenomenon in biology, we speculate that the \(\beta\)ENaC, when bound to its ligand SPLUNC1, may exert additional functions.

Previous studies have demonstrated that SPLUNC1 has an antimicrobial effect against \textit{B. cenocepacia} (Ahmad et al., 2016; Walton et al., 2016). While the rate of CF patients infected with \textit{B. cenocepacia} is lower than other bacteria such as \textit{P. aeruginosa}, \textit{B. cenocepacia} infection results in a high mortality rate due to its ability to invade host cells and cause septic shock (Burns et al., 1996; Govan and Deretic, 1996; Holden et al., 2009). Our preliminary data suggest that SPLUNC1, when present together with ENaCs, reduces the detection of \textit{B. cenocepacia} inside cells (Figure 4. 1-2). Qualitatively, we observed that \textit{B. cenocepacia} signals were reduced when SPLUNC1 was pre-treated on HEK293T cells transfected with ENaCs compared to no SPLUNC1 pre-treatment (Figure 4. 1-2). Additionally, SPLUNC1 pre-treatment on non-ENaC expressing HEK293T cells was unable to reduce \textit{B. cenocepacia} signals (Figure 4. 1-2). However, we do not have appropriate controls to be conclusive, i.e. cells not infected with \textit{B. cenocepacia} to observe the level of damage that the bacteria has on infected cells. Furthermore, a kinetic progression of \textit{B. cenocepacia} invasion of cells and observations of the effect of SPLUNC1 will help understand whether SPLUNC1 plays a protective role in \textit{B. cenocepacia} invasion. Based on previous studies that have shown SPLUNC1 to be a bacteriostatic agent, and the binding of SPLUNC1 to the LPS of \textit{P. aeruginosa}, one could expect that SPLUNC1 binds to \textit{B. cenocepacia} and thereby reduced the
cellular invasion of the bacteria (Ahmad et al., 2016; Di, 2011; Ning et al., 2014; Walton et al., 2016).

Further investigation is necessary to confirm this hypothesis and elucidate if the SPLUNC1-βENaC complex plays a role in the protection of the cellular invasion of *B. cenocepacia*. One excellent area to investigate is the ratio of cell-invaded *B. cenocepacia* to extracellular *B. cenocepacia*. Determining this ratio will help delineate whether the SPLUNC1-βENaC complex reduces the opportunity for *B. cenocepacia* to bind to the plasma membrane of host cells and thus reduce the rate of bacterial invasion, or if SPLUNC1 reduces the number of extracellular *B. cenocepacia* and thus the rate of bacterial invasion. Furthermore, investigating the effect of the SPLUNC1-βENaC complex on *B. cenocepacia* at multiple time points with varying concentrations of SPLUNC1 treatment and/or varying quantities of *B. cenocepacia* infected cells will help understand the efficacy of SPLUNC1’s antimicrobial function against *B. cenocepacia*.

The data in this thesis, and the work of other researchers, have led to the delineation of how SPLUNC1 is able to inhibit ENaCs in the lung (Garcia-Caballero et al., 2009; Garland et al., 2013; Hobbs et al., 2013). However, the physiological impacts of the ability of SPLUNC1 to inhibit ENaCs in organs other than the lung are less understood. For example, ENaCs expressed in the neuronal membrane modulate the presynaptic membrane potential and consequently regulates the release of neurotransmitters (Younger et al., 2013). Interestingly, the inhibition of ENaCs expressed in neurons relieves the symptoms of multiple sclerosis, suggesting that ENaCs may contribute to the pathogenesis of multiple sclerosis (Boiko et al., 2013). Furthermore, ENaCs are expressed in the vascular endothelium where it has been suggested they help in regulating arterial blood pressure. Moreover, overexpression of ENaCs in the vascular endothelium leads to arterial hypertension (Chen et al., 2004; Kusche-Vihrog et al., 2008). Therefore, investigating the effect
of SPLUNC1 on ENaC expressing neurons or endothelial cells will provide insight into the development of therapy for multiple sclerosis and arterial hypertension.

In this thesis, we used a cell culture model to delineate detailed mechanisms. The major advantage of using a cell culture model is the ease of manipulating and controlling conditions, producing high levels of consistency and reproducibility. However, immortalized cell lines, such as HEK293T cells, have acquired mutations that allow these cells to proliferate indefinitely and therefore may behave differently than in vivo cells. Primary cells that were freshly isolated from human lungs were used as an alternative method since these cells are more similar to in vivo cells. Nonetheless, cell culturing environments are very different compared to human tissues and therefore freshly isolated cells eventually lose the characteristics of their parent tissue. Therefore, studies on SPLUNC1 and ENaC in animal models are required to understand in vivo effects in the lung and other organs. For example, investigating the effects of SPLUNC1 in βENaC overexpressed mice, which develops CF-like lung, and/or the consequences of SPLUNC1 knockdown mice in body fluid regulation, will help explain how ENaCs and SPLUNC1 take part in the pathogenesis of the disease and provide insight into new therapeutic approaches.
Figure 4. 1. SPLUNC1 reduces overall *B. cenocepacia* GFP signals in HEK293T cells when ENaC is co-expressed in HEK293T cells. Representative xy-scan confocal images of either non-transfected (control) or αβγENaC transfected HEK293T cells incubated with rSPLUNC1 tagged with DyLight 594 (red) after infecting cells with GFP expressing *B. cenocepacia* strain J2315 (green) for 2 h.
Figure 4. 2. SPLUNC1 reduces *B. cenocepacia* GFP signals inside of HEK293T cells when ENaC is co-expressed in HEK293T cells. Representative confocal images of HEK293T cells that were treated the same as Figure 4.1 but obtained using a zy-scan. Red indicates rSPLUNC1 tagged with DyLight 594 and green indicates GFP expressing *B. cenocepacia* strain J2315 after 2 h of infection.
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