

UNCOVERING THE CRITICAL AMINO ACIDS IN THE BETA SUBUNIT OF THE
EPITHELIAL SODIUM CHANNEL (ENaC)

By

Adaobi Okonkwo, B.S.

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Committee Members:

Rachell Booth, Chair

Kevin Lewis

Wendi David

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ABSTRACT

A high intake of sodium into the body can lead to high blood pressure that could result to stroke, kidney and heart disease. Sodium balance in the body is partially regulated in the distal tubules of the nephron in the kidney via a membrane channel called the epithelial sodium channel (ENaC). The structure, regulation and assembly of ENaC's subunits is not yet fully understood. In this study, error prone polymerase chain reaction (EP-PCR) was used to introduce random mutations in the extracellular loop of the beta subunit of ENaC in order to identify residues that may be critical to proper function. A yeast screen, dilution survival pronging assay, was used to visualize the level of growth inhibition of yeast cells expressing α -ENaC (i.e. ENaC function) with wild type or various β -ENaC mutants. Also, a western blot was used to confirm mutated ENaC expression in yeast cells. The potential β -ENaC were identified in these preliminary studies and should be further characterized in additional dilution pronging assays and expression studies.

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Sodium ions are important electrolytes that helps in regulating the volume of fluid in the body. Regulation of sodium ions in cells is critical because an imbalance of sodium ions poses serious health problems in mammalian systems. An increase in blood sodium ion levels corresponds to an increase in the volume of water in the blood resulting in increased blood volume (i.e. pressure) pumped through the blood vessels hence leading to high blood pressure (1 - 2). Abnormal increases in sodium ion levels can ultimately result in heart and kidney failure (3).

Reabsorption of sodium ions is achieved in the functional unit of the kidney (4). The kidney is made of millions of functional units called nephrons that extend through the medulla and cortex of the kidney (Fig 1). The nephron consists of aggregates of capillaries called glomerulus that is surrounded by the Bowman's capsule. Unfiltered blood enters the nephron and is filtered out through the glomerulus. The filtrate which contains water, sodium ions, urea and other small molecules and ions leaves the glomerulus and is transported to the proximal tubule where the majority of the sodium and water is reabsorbed. The remaining filtrate travels along the loop of Henle, where about 25-30% of sodium ions are also reabsorbed. The remaining 2-5% of sodium ions are reabsorbed in the distal convoluted tubule. The sodium ions reabsorbed in the distal tubule is needed for fine tuning homeostasis in the body (5 - 7). After the final reabsorption occurs, the remaining filtrate is excreted from the kidney as urine (Fig 1) (6).

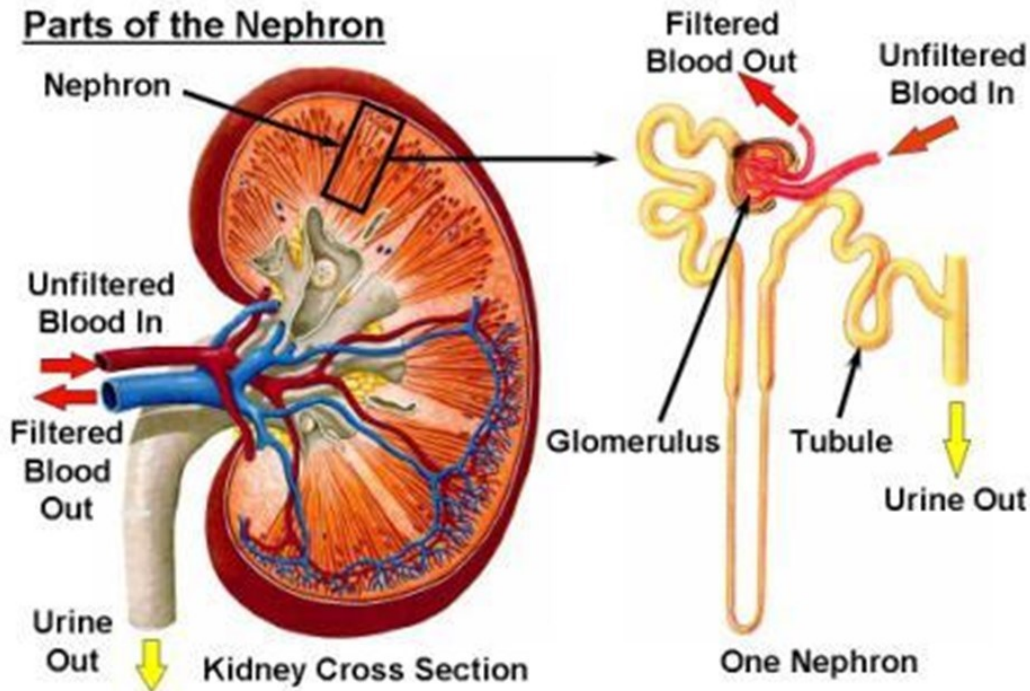


Figure 1: Schematic diagram of the kidney and its functional unit. Blood enters the nephron and is filtered out through the glomerulus. The filtrate which contains salts, water and other small molecules travels through the proximal tubule where about 60 -70% of sodium and water is reabsorbed. The remaining filtrate then travels down the loop of Henle and then the distal convoluted tubule where the sodium needed for homeostasis is reabsorbed. The remaining filtrates that was not absorbed is excreted as urine (6)

Membrane ion channels, present in the distal tubule, are responsible for reabsorbing of sodium ions needed for maintaining homeostasis. Membrane ion channels are proteins embedded within the lipid bilayers of cells that regulate the passage of inorganic ions such as Na^+ and K^+ across the lipid bilayers of the cells. The function of a membrane ion channel is dependent on the position of the cell's subunits, its stoichiometry and its location in the cell (8). The membrane-bound amiloride-sensitive Na^+ ion channel present in the distal tubules is called the epithelial sodium channel (ENaC) (5, 9 - 10). ENaC is found in epithelial cells that line organs separating fluids in the kidneys, lungs, sweat glands, gastro-intestinal tract, colon and salivary ducts (4, 9 -10,

12). The transport of sodium ions via ENaC in the distal tubule of the kidney is rate limiting step in reabsorption of sodium (4, 7, 10 - 12).

ENaC is located on the apical membrane (the side where urine passes through) of the epithelial cells that line the interior walls of the nephron (Fig 2). Sodium ions passively enter the cell through the pore of ENaC (2). As sodium ions flows into the cell, water is also reabsorbed through the aquaporin channels that are also located on the apical membrane. Sodium ions flow through the cell and enter the bloodstream on the basolateral side through a Na^+ / K^+ ATPase channel that regulates the concentration of sodium ions in the cell (13). The ATPase pumps two potassium ions into the cell and three sodium ions into the bloodstream using active transport.

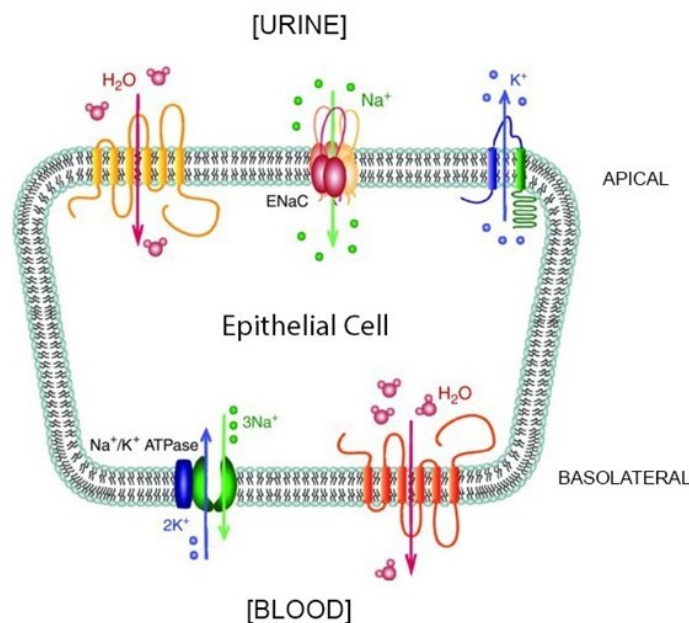


Figure 2: Schematic representation of the sodium ion reabsorption system in epithelial cells. Sodium from the excreted filtrate is reabsorbed through ENaC into the epithelial cells. Water is reabsorbed via the aquaporin channels. Sodium ion is pumped into blood through the Na^+/K^+ ATPase channel (34).

ENaC is a sodium selective amiloride sensitive channel that consists of three homologous subunits, α , β and γ (Fig 3) (15). The three subunits share a 30 – 40% sequence homology (2, 5, 16 – 17). In order to achieve a maximal activity, the subunits of ENaC assemble together to form a heterotrimeric structure in a 1 α :1 β :1 γ ratio (7, 18). Studies have shown that the activity of ENaC is dependent on the presence of the α subunit whereas the β and γ subunits are important accessory subunits for the regulation of the channel. The α - subunit by itself can form a homotrimeric channel although the β and γ subunits by themselves cannot form a functional channel (16, 19). The presence of a fourth subunit called the δ subunit has been shown to exist, though its role is still uncertain (2). The δ subunit is highly expressed in the testis, ovaries, pancreas and brain (68).

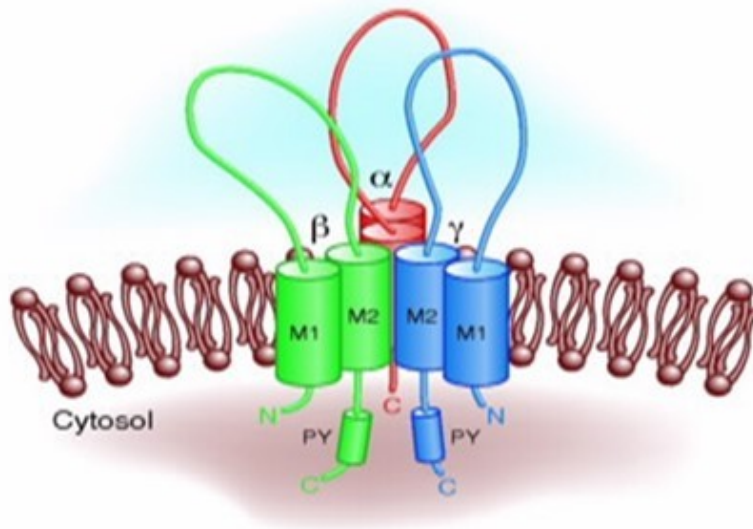


Figure 3: Structural features of the epithelial sodium channel. ENaC consists of three homologous subunits, α , β , and γ , that can exist as a heterotrimeric protein in a 1:1:1 ratio. Each subunit has a short cytoplasmic N and C terminus, two transmembrane domains (M1 and M2) and a large extracellular loop. The “PY” motif in the C-termini of the β and γ - subunits plays an important role in regulation (13).

ENaC belongs to the ENaC/degenerin gene (ENaC/DEG) superfamily of ion channels. Members of this superfamily transport sodium and are expressed in epithelial cells within the nervous system (i.e., sensory neurons) and muscles (21). Members of this family share structural similarities including a short intracellular N-terminus and C-terminus, a large cysteine rich extracellular loop, and two transmembrane domains called M1 and M2 (Fig 4A) (2, 9, 15). The cysteine-rich domains (CRDs) found within the extracellular loop that connect the two transmembrane domains are thought to form disulfide bonds that help stabilize the tertiary structure of the loop (Fig 4B) (22).

Members of this superfamily include the acid sensing ion channel (ASIC), which plays a role in sensory neurons and is activated by protons (i.e., acidic environment), the degenerin found in *C. elegans* that transports sodium in response to touch and other mechanosensory behaviors, and the ripped pocket/picked pocket channel present in the testis and ovaries of *Drosophilla*, which functions through transduction of mechanical stimuli from heat (22).

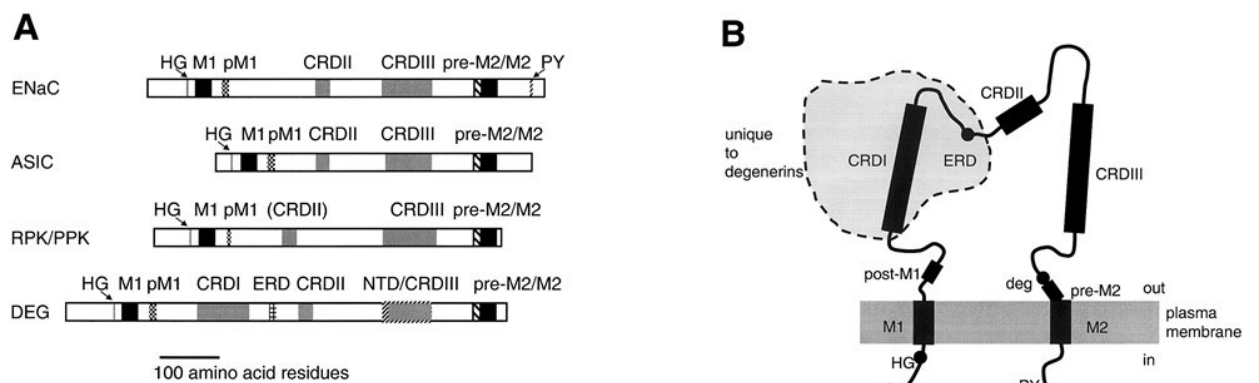


Figure 4: Conserved domains of ENaC/DEG superfamily. A: linear illustration of the homologous regions of the ENaC/DEG superfamily. B: The topology of an individual ENaC/DEG subunit. CRD: Cysteine-rich domain. ERD: Extracellular regulatory domain (22).

ENaC is highly selective for Na^+ and Li^+ ions over K^+ and is blocked by amiloride, a diuretic that leads to the wasting of sodium ions (17). Studies have shown that a decrease in amiloride affinity for the pore of ENaC increases the transport of sodium ions hence resulting in a competition between the amiloride and sodium ions that are transported (25).

The stoichiometry of the ENaC subunits is not yet proven. Previous studies have shown that ENaC channels are comprised of either three, four (26, 27, 28), eight or nine subunits (29, 30). The idea of eight or nine subunits is as a result of the subunits of ENaC coming together to form a structure consisting of two tetramers or three trimers, respectively (31). Although the crystal structure of ENaC has not been solved, a structural model has been developed (FIG 5) (32). One proposed model of ENaC and previous studies suggests that the stoichiometry is 1:1:1 (18, 32) based on the crystal structure of ASIC that belongs to the same superfamily as ENaC.

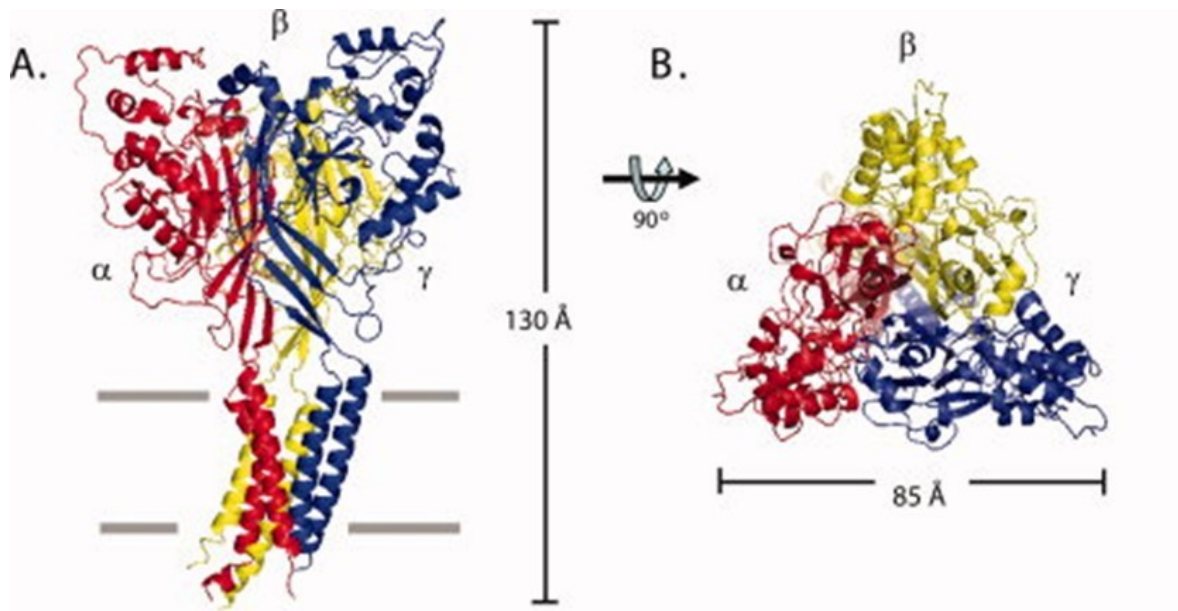


Figure 5: The proposed structure of ENaC. The ribbon models of the proposed structure of ENaC showing the side view (A) and the top view (B) (32).

Misregulation of ENaC channel can lead to disorders in the function of ENaC. Two monogenetic diseases are known to be associated with mutations of ENaC, Pseudohypoaldosterism type 1 (PHA-1) and Liddle's syndrome. PHA-1 is a low blood pressure disease that is caused by a frameshift or deletion mutation in the α , β , or γ subunits of ENaC (34). This mutation causes a decrease in the function of the sodium channel resulting in a decreased reabsorption of sodium ions although there is an increased level of aldosterone in the blood (34, 35). PHA-1 is characterized by dehydration, metabolic acidosis and increase in potassium level in the blood. PHA-1 can also occur as a result of mutation in the mineralocorticoid receptor (9,35).

Liddle's syndrome on the other hand is a high blood pressure disease that is caused by either a frameshift or missense mutation or by introduction of a stop codon in the C- terminus of the β or γ subunit (16). This mutation affects the PY motif that obviates the degradation of ENaC by ubiquitin ligase and NEDD4-2, hence causing ENaC to be hyperactive (36). When ENaC is hyperactive, there is an immoderate reabsorption of sodium that results in hypertension (34). Liddle's syndrome is characterized by hypokalemia, metabolic alkalosis, hypertension, and low levels of aldosterone.

ENaC is constitutively active, so a ligand or external factors are not required to open the channel. For this reason, ENaC is tightly regulated in order to maintain the concentration of sodium ions within the cell. Hormones and regulatory proteins play a role in the regulation of ENaC (25). The mineralocorticoid hormone aldosterone is the most physiologically important activator of ENaC (25, 37). The mineralocorticoid

receptor (MR) in the cytosol is activated once aldosterone is bound to it and then transported to the nucleus. Upon entering the nucleus, the activated MR either inhibits or activates the transcription of specific genes and increases the transcription of α ENaC (25, 38). The number of ENaC channels at the cell surface increase with an increase in aldosterone levels. Aldosterone does not only increase the total abundance of α ENaC but also causes the proteins that regulate ENaC trafficking to be transcribed (25). Aldosterone also increases the mRNA level of an aldosterone induced protein called serum-glucocorticoid induced-kinase I (SGK I). The mechanism of ENaC activation directly by SGK1 is not yet known (11, 25), but it is thought aldosterone activates SGK1, which can in turn either activate ENaC directly or inactivate NEDD4-2 via phosphorylation (Fig 6) (1, 40, 41). The phosphorylation of NEDD4-2 produces a binding site for 14-3-3 proteins and obstructs an interaction between ENaC and NEDD4-2 hence resulting in accumulation of ENaC at the cell surface and an increase in sodium reabsorption (41).

NEDD4-2 is an E3 ubiquitin ligase that is expressed in organs such as the brain, colon, kidney and lungs (25, 42) and it plays an important role in the regulation of ENaC. NEDD4-2 is comprised of about 3 – 4 WW domains, a C-2 domain, and a Hect domain (Fig 6) (43, 44, 45). The WW domain aids in recognition of PY motifs in other proteins while the C-2 is thought to play a role in trafficking and localization (46). The WW domain consists of two conserved tryptophan residues that are spaced apart by 20 – 22 amino acids (2, 46). The C-terminus of β - and γ -ENaC has a PY motif that interacts with the WW domains. The binding of NEDD4-2 to ENaC enables lysine residues in the N-termini of the α and γ ENaC subunits to be ubiquitinated by the Hect domain on NEDD4-

2 (43, 46). The ubiquitination of the α -subunit is probably dependent on that of γ subunit (2, 25). Although β -ENaC has a PY motif, a previous study showed that when the lysine residues in the N-terminal of β -ENaC were mutated, the lysine residues were not ubiquitinated and did not result in a decrease in the activity of ENaC channel (48). This observation led to the suggestion that the regulation of ENaC by ubiquitination is indirectly targeting the intracellular portion of ENaC. Studies have shown that an increase in the activity of ENaC associated with Liddle's syndrome is as a result of NEDD4-2 not being able to bind to the PY motif hence resulting in an increased reabsorption of sodium (10, 14, 48).

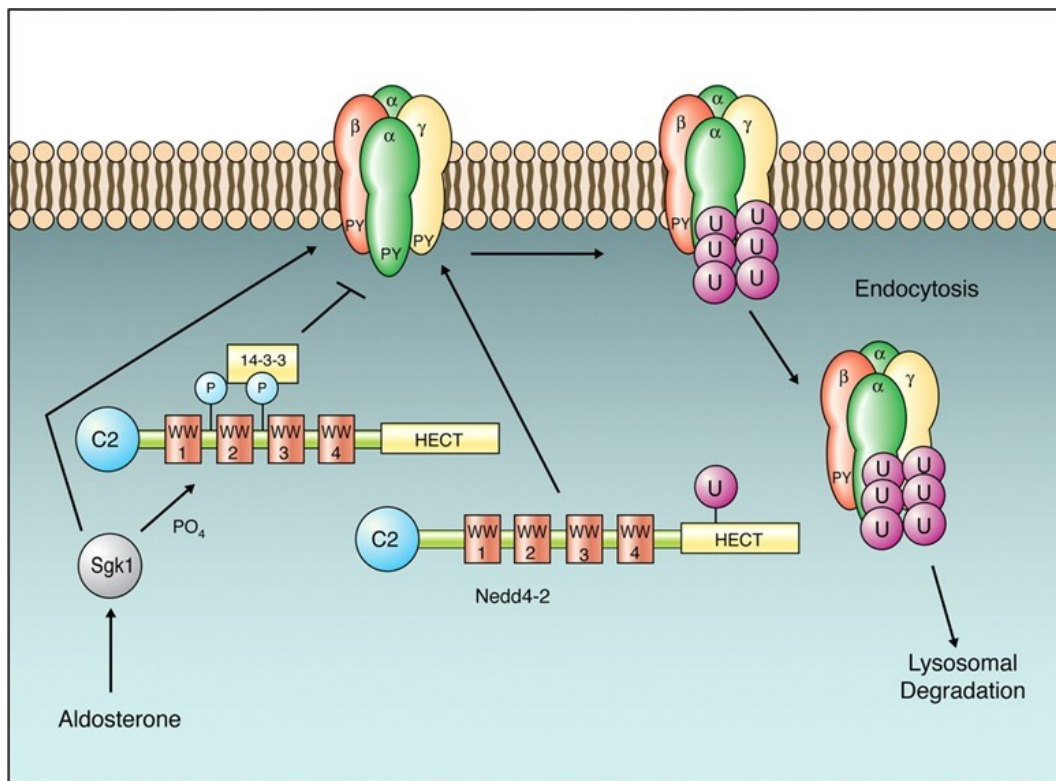


Figure 6: Regulation of ENaC. SGK1 is activated by aldosterone. Activated SGK1 can either phosphorylate ENaC directly or NEDD4-2. WW domains of NEDD4-2 interacts with the PY motifs in the ENaC subunits, resulting in the ubiquitination of the N-terminus of α - and γ -ENaC. Ubiquitination reduces the activity of ENaC and causes ENaC to be targeted for degradation (49).

Once ENaC is ubiquitinated, it is trafficked into the endosomal compartments where it is recycled or targeted for degradation in the lysosomes (47). Three deubiquitinating enzymes are known to affect ENaC activity; UCH-L3, USP2-45 and USP-10. USP2-45 and USP-10 increase the activity of ENaC (14, 47). During exocytosis, endocytosis, and secretion in eukaryotic cells, Rab proteins which are GTPases, play a role in membrane trafficking (69). Rab proteins are known to play an important role in the recycling of ENaC channels to the apical membrane (10). ENaC subunits are recycled from the endosomes to the apical surface after deubiquitination (10, 47).

Understanding the arrangement and structural assembly of ENaC and its subunits will give a better insight to understanding its regulation and function. Random mutagenesis combined with a functional screening method is an unbiased approach to study the function of a biological macromolecule or structure (50). Random mutations can be generated using error-prone PCR (EP-PCR), a modification of the standard polymerase chain reaction (51). EP-PCR was designed in such a way to reduce the fidelity of Taq DNA polymerase without considerably reducing the rate of amplification (Fig 7) (50, 52). The polymerase used in EP-PCR does not have 3'-5' exonuclease activity and so is unable to proofread mismatched bases thus increasing the mutation rate (50). Mutation rate can also be increased by adding uneven concentrations of nucleotides, increasing the elongation time, adding Mn^{2+} , or increasing the concentration of Mg^{2+} (50, 51, 52).

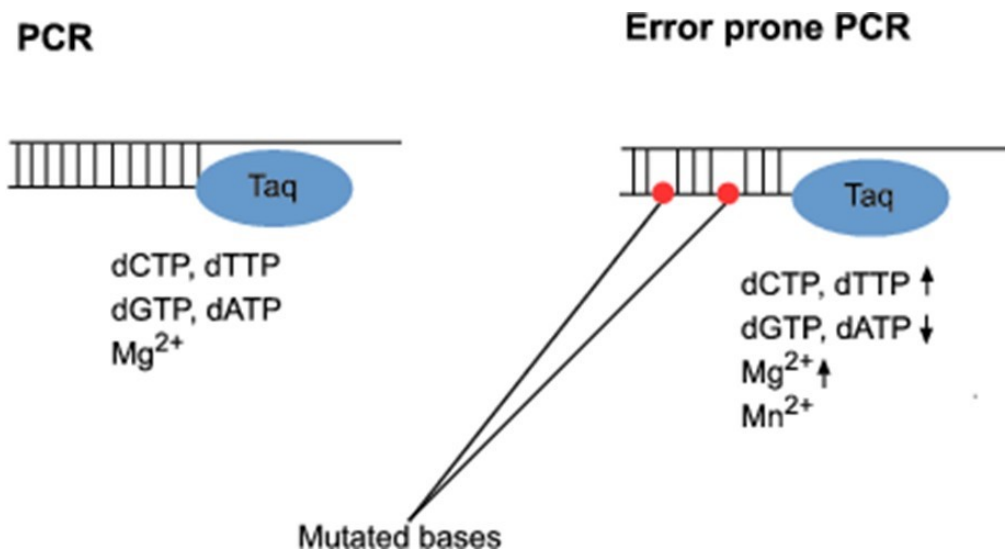


Figure 7: Comparison between standard PCR and error- prone PCR. In EP-PCR, unequal concentration of dNTPs is added, concentration of Mg^{2+} is increased and Mn^{2+} is also added to increase the error rate of Taq DNA polymerase (53).

A novel yeast screen that allows the activity (i.e. sodium ion transport) of ENaC mutants to be screened was developed by Raquel Ybanez (54). This screen demonstrates the induced salt-sensitive phenotype in yeast cells that express ENaC. The extent of growth inhibition when yeast cells that express ENaC are grown on a salt media is visually observed through the use of an assay called a survival pronging dilution assay (Fig 8). This screen is a tool used to uncover for residues critical for the function of ENaC to be identified (54).

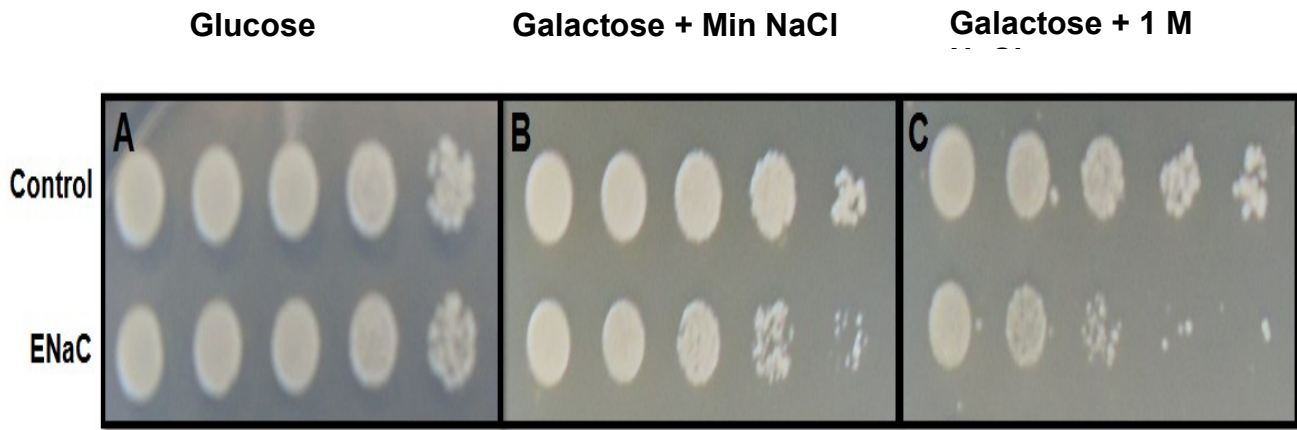


Figure 8: Pronging assay showing salt sensitivity. S1InsE4A yeast cell was transformed with pYES2NTvector (Control) and pYES2NT-alpha ENaC (ENaC). The cells were grown on; (A) - glucose, (B) - galactose and (C) - galactose + 1M NaCl media (54).

Most studies on ENaC have focused on identifying and understanding the roles of the amino acid residues found within the intracellular region. About 70% of the amino acid residues of ENaC are found within the extracellular region and this region is important for the functioning of ENaC channel, though little is known about the role of extracellular loop (14, 24, 55). The goal of this project was to identify the critical residues within the extracellular loop of the beta subunit of ENaC. This study utilized EP-PCR to generate random mutations. Mutants generated were screened for gain or loss of function using the pronging dilution assay and then sequenced to determine the location and type of mutation.

CHAPTER II

MATERIALS AND METHODS

Preparation of Competent ER2925 *E. Coli* Cells

Non-methylated ER2925 cells were grown shaking overnight at 37 °C in 5 mL of Luria-Bertani (LB) broth with no antibiotics added. The overnight culture was diluted 1:50 in LB broth and then incubated again 2-3 hours to obtain an OD₅₅₀ of 0.3-0.5. The cells were pelleted by centrifugation at approximately 1902 x g for 5 minutes. The cell pellet was then resuspended in 1/20th volume of cold LB/10 % PEG/5 % w/v DMSO solution and placed on ice for about 5 minutes. The cells were aliquoted into micro centrifuge tubes and then stored at -80 °C.

Transformation of pESC-Leu/ β -ENaC plasmid DNA into *E. coli*

Competent ER2925 cells were thawed on ice for 10 minutes and then transformed with 613 ng of pESC-Leu/ β -ENaC plasmid DNA. The mixture was incubated on ice for 15 minutes and then in room temperature water for 10 minutes. Next, 0.9 mL of LB broth was added to the reaction mixture and shaken for 50 minutes at 37 °C. Then 20-250 μ L were plated on LB plates containing 100 μ g/mL of ampicillin and incubated overnight at 37 °C.

Isolation of Plasmid DNA from *E. coli* Cells

A single colony was picked from a LB plate with transformed cells and incubated with shaking overnight at 37 °C in 5 mL of LB broth with addition of 100 μ g/mL ampicillin. The plasmid DNA was isolated from ER2925 *E. coli* cells using the Qiagen

QIAprep spin Miniprep Kit (Valencia, CA) according to the manufacturer's protocol. DNA was eluted with 50 μ L sterile water. The plasmid was quantitated with the Nanodrop 2000 spectrophotometer from Thermo Fisher Scientific (Wilmington, DE).

Double Digestion of pESC-Leu/ β -ENaC

Double digestion reactions for pESC-Leu/ β -ENaC were set up using 6.5 μ g of pESC-Leu/ β -ENaC DNA, 1X Cutsmart buffer (50 mM potassium Acetate, 20 mM Tris-Acetate, 10 mM Magnesium Acetate, 100 μ g/ml BSA, pH 7.9 at 25 $^{\circ}$ C) from New England BioLabs (Ipswich, MA), 20 units of ZRA1 (New England BioLabs), and 10 units of SexA1 (New England BioLabs) in a total reaction volume of 50 μ L. The reaction mixtures were incubated overnight at 37 $^{\circ}$ C and stopped with 1X endorstop (10 mM EDTA, 5% v/v glycerol, 0.1% w/v SDS, 0.01% w/v bromophenol blue, pH 8.0). Digested plasmids were analyzed using horizontal gel electrophoresis.

Horizontal Gel Electrophoresis

DNA samples were loaded into a 0.8% (w/v) Tris-acetate-EDTA (TAE) agarose gel and separated in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) (Thermo Scientific) using the JSB-30 Mini Gel-O Submarine System from IBI Scientific (Peosta, IA). The gel was allowed to run for 50 minutes at 110 volts. The gel was stained with ethidium bromide (0.25 mg EtBr/ml) for 20 minutes, rinsed in ddH₂O for 10 minutes and then imaged using the Molecular Imager[®] ChemiDoc[™] XRS-Imaging System from Bio-Rad (Hercules, CA).

Gel Extraction

DNA fragments were excised from the gel and cleaned using the QIAEX II Extraction (Qiagen) per manufacturer's protocol. DNA was eluted with 40 μ L sterile water and quantitated using Nanodrop 2000 spectrophotometer to verify purity and yield of the DNA fragments.

Error-Prone PCR (EP-PCR)

PCR products were generated by using 100 ng of template DNA, 1X Standard Taq Reaction Buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.3 at 25 °C) (New England Biolabs), 200 μ M dNTPs (New England Biolabs), 0.2 μ M forward and reverse primers (listed in table 1) from IDT Integrated DNA Technologies, Inc (Coralville, IA), and 5 units of Taq DNA polymerase (New England Biolabs) in a total reaction volume of 50 μ L. The reaction was subjected to the following conditions: 95 °C for 30 seconds, 35 cycles of 95 °C for 30 seconds, 60 °C for 60 seconds, 68 °C for 90 seconds and 1 cycle of 68 °C for 10 minutes. The PCR reaction mixture was analyzed using horizontal gel electrophoresis and gel extracted. The PCR product was further cleaned using an ethanol precipitation protocol. One-tenth volume of 3 M sodium acetate was added to the PCR product along with 2.5 volumes of absolute ethanol. The reaction mixture was placed in the -20°C for at least 10 minutes and then centrifuged 21000 x g for 15 minutes. The supernatant was discarded and the pellet was allowed to air dry for approximately 20 minutes. The pellet was resuspended with sterile water in 1/5th of the original volume. The product was quantitated using a Nanodrop 2000 spectrophotometer.

Table 1: Primers Used for EP-PCR Reaction

Primer Name	Primer Sequence
UP BmENaC Random	5' – CCAGGACCCTGAACTTTACC-3'
DN BmENaC Random	5' – GCCTATTGCTATCTAAACCTGC-3'

Transformation of Digested Plasmid and EP-PCR product into Yeast

The digested pESC-Leu/ β -ENaC plasmid and beta mutant PCR product was co-transformed into BY4742 yeast cells using the lithium acetate transformation protocol. The yeast cells were incubated overnight in YPDA broth (1% w/v yeast extract, 2% w/v peptone, 0.02% w/v adenine and 2% w/v glucose) shaking at 30 °C. One milliliter of the overnight culture was centrifuged at 21000 x g for 30 seconds and supernatants were discarded. To the cell pellet, the following reagents were added: 33% PEG 3350 (Mallinckrodt Baker Inc., Phillipsburg, NJ), 0.1 M lithium acetate (Sigma-Aldrich, St Louis, MO), 0.28 mg/ml sonicated salmon sperm DNA (Agilent Technologies, Santa Clara, CA), 2.8 mg/ml β -mercaptoethanol (MP Biomedicals, LLC, Salon, OH), 288 ng of digested pESC-Leu/ β -ENaC plasmid, 288 ng of EP-PCR beta and ddH₂O to a total reaction volume of 360 μ L. The reaction mixture was vortexed for 1 minute and incubated for 20 minutes at 42°C. The reaction mixture was spun down at 3756 x g for 2 minutes, supernatant was discarded and pellets resuspended in 200 μ L ddH₂O. Next, the cells were plated on selective media (2% w/v glucose, 0.67% w/v yeast nitrogen base, 0.01% w/v of tryptophan, 0.005% w/v histidine, 0.14% w/v yeast synthetic drop out media, and 2.5% w/v bacto agar) and incubated for 4 days at 30 °C.

Dilution Pronging Survival Assays

Ten different colonies were picked from the transformed yeast plates and grown overnight in a selective media containing 2% w/v glu-leu-ura with shaking at 30 °C. Patch plates containing streaks of the different colonies from the transformed yeast plates was prepared on selective media and grown at 30 °C for 2 days. A toothpick was used to scrape yeast cells, about the size of a peppercorn, from the patch plates, they were resuspended in 0.5 mL of ddH₂O, and then diluted 40-fold. The cells were sonicated for 8 seconds at 20% amplitude using VIBRA cell probe sonicator (Newtown, CT) and then counted using a hemocytometer under a microscope. Based on the average cell count, yeast cells were added to a 96-well plate to obtain a concentration of 2×10^7 cells per 220 μ L and then diluted 5-fold across 7 wells. Next, cells were pronged onto selective leu-ura plates containing either 2% w/v glucose or 2% w/v galactose plus 0.5 M salt concentration to identify differences in growth. The plates were incubated for 2-6 days at 30 °C.

Expression of ENaC in Yeast

The different mutants of interest were patched onto a selective media plates containing 2 % w/v glu-leu-ura and incubated for 4 days at 30 °C. Cells were scraped from the patch plate and grown in 15 ml glu-leu-ura media overnight at 30 °C with shaking. The OD₆₀₀ was measured the next day and the amount of overnight culture required to get an OD₆₀₀ of 0.4 in 50 ml induction media was used. The aliquot of overnight culture needed was centrifuged at 1500 x g for 5 mins at 4 °C. The cell pellet

was resuspended in 50 ml selective media containing 2% w/v galactose and incubated for 8 hours with shaking at 30 °C. At the end of the 8 hours, the OD₆₀₀ for the samples was determined. The cells were again centrifuged 1500 x g for 5 mins at 4 °C. The cell pellet was resuspended in 500 µl of sterile water. The cells were centrifuged at 21130 x g for 30 secs. The supernatant was discarded and the pellets were stored at -80 °C until ready for use.

Post Alkaline Protein Extraction

The cell pellets were resuspended in 600 µl ddH₂O, 600 µl 0.2 M NaOH and incubated for 5 mins at room temperature. The reaction mixture was then pelleted at 3000 x g for 5 mins and the pellet was resuspended in 600 µl SDS sample buffer (2 % SDS, 0.006 mM Tris-HCl pH 6.8, 5 % (v/v) glycerol, 4 % (v/v) beta-mercaptoethanol), incubated at 95 °C for 3 mins, pelleted by centrifugation at 18000 x g for 10 mins and the supernatant was collected.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Ten microliters of the post-alkaline supernatant was mixed with 1X NuPage LDS sample buffer (Invitrogen Carlsbad, CA) and 5 % (v/v) beta-mercaptoethanol. The sample was heated at 95 °C for 5 mins and then loaded onto a Mini-PROTEAN[®] TGX Stain-Free[™] 4 – 15 % Precast Gels (Bio-Rad). The gel was ran using a Mini-PROTEAN[®] Tetra Vertical Electrophoresis Cell (Bio-Rad) and then allowed to run at 100 volts for 70 mins in 1X running buffer (25 mM Tris-HCl, 192 mM glycine and 0.1 % (w/v) SDS, pH 8.3). The gel was transferred to a 0.45 µm nitrocellulose membrane using

the Trans-Blot® Turbo™ RTA Transfer Kit (Bio-Rad) via the Trans-Blot® Turbo™ Transfer System (Bio-Rad) for 20 mins at 1.3 Amps and 20 volts.

Western Blot

The nitrocellulose membrane was incubated in dry milk (5 % (w/v) blocking solution, 1X Tris Buffered Saline (TBS) (50 mM Tris-HCl pH 7.4, 150 mM NaCl), 0.1 % (v/v) Tween-20 (TBST)) for 30 mins at room temperature with agitation. A 1:1000 dilution of anti-beta ENaC (StressMarq Biosciences, Victoria BC, Canada) in blocking solution was added to the blot and incubated overnight at 4°C with agitation. The next day, the membrane was washed three times for a total of 15 mins with agitation. A 1:20000 dilution of affinity purified antibody peroxidase labeled goat anti-rabbit (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to 20 ml blocking solution and incubated with blot for an hour with agitation at room temperature. The membrane was washed three times in 20 ml for a total of 15 mins and then equilibrated for five mins in 20 ml 1X TBS. Equal volumes of each of the Western Lightning Plus-ECL Enhanced Sensitivity Chemiluminescence Substrate (PerkinElmer, Waltham, MA) reagents was mixed and dispensed on the membrane for one min in a dark room. The membrane was imaged using the ChemiDoc™ XRS - Imaging System (Bio-Rad).

Isolation of Mutant Plasmid from Yeast and Transformation into *E. coli* Cells

A patch plate was prepared by growing yeast cells containing the beta mutants on a SC-leu-ura plate containing 2% w/v glucose and incubated at 30 °C for 3 days. An overnight culture of the cells was grown on SC-leu-ura media containing 2% w/v glucose at 30 °C. The cells were centrifuged at 8000 x g for 3 minutes and the cell pellet was

resuspended in 100 mM sodium phosphate buffer, pH 7.5 with 1500 units / μL of zymolase which helps to break down the yeast cell wall. The resuspended pellet was incubated for 2 hours at 37 °C. The plasmid DNA was then isolated from yeast using the Qiagen QIAprep spin Miniprep Kit according to the manufacturer's protocol. The plasmid DNA was eluted with water and the DNA was quantitated. The plasmid was transformed into Top 10 *E. coli* cells and then isolated from *E. coli* also as described above. The isolated plasmid was then quantitated, verified in a horizontal gel and sequenced.

Digestion of Mutant Plasmid

Two and half micrograms of the isolated plasmids were digested with 20 units of XhoI and 10 units of NheI. The digestion reactions were incubated at 37 °C for 4 hours. The reaction was inactivated by adding 1X endorstop sample buffer to the reactions. The digestion products were then analyzed on a 0.8 % w/v TAE Agarose gel.

Sequencing of Mutant Plasmid

To sequence the beta-ENaC gene, 80 ng/ μL of isolated pESC-Leu/ β -ENaC mutants and 5 μM of sequencing primers (Table 2) were sent to Quintara Biosciences (Albany, CA). The sequence was analyzed by aligning the sequence data with pESC-Leu/ β -ENaC and beta-ENaC genes using the MUSCLE program on the European Bioinformatics Institute's website (<http://www.ebi.ac.uk/Tools/msa/muscle/>).

Table 2: Primers Used for Sequencing

Primer Name	Primer Sequence
Beta internal	5'-ATGGA ACTGTGTGTACCTTGC-3'
Mutant Sequencing	5'-ATGAGCTAATGGAGGCAGTCCTGG-3'
Internal Sequencing	5'-AATTCAGTCCCAGGGTTGGCAGG-3'

CHAPTER III

RESULTS AND DISCUSSION

In order to understand the structure-function relationship of ENaC, preliminary random mutagenesis studies were done to identify the amino acids within the extracellular loop of beta-ENaC that are potentially critical to the overall structure/function of the protein.

pESC-Leu Vector

The beta-ENaC gene used for this research was subcloned into pESC-Leu by Esther Lee (56). The pESC vector has two galactose promoters, *GAL1* and *GAL10* that are in opposite orientation (Fig 9). This vector allows one or two different genes to be simultaneously expressed in yeast. The beta-ENaC gene follows the c-MYC epitope under the *GAL1* promoter while the gamma-ENaC gene follows the FLAG epitope under the *GAL10* promoter. These engineered epitopes are positioned within the two multiple cloning sites (MCS) that are located downstream of each of promoter.

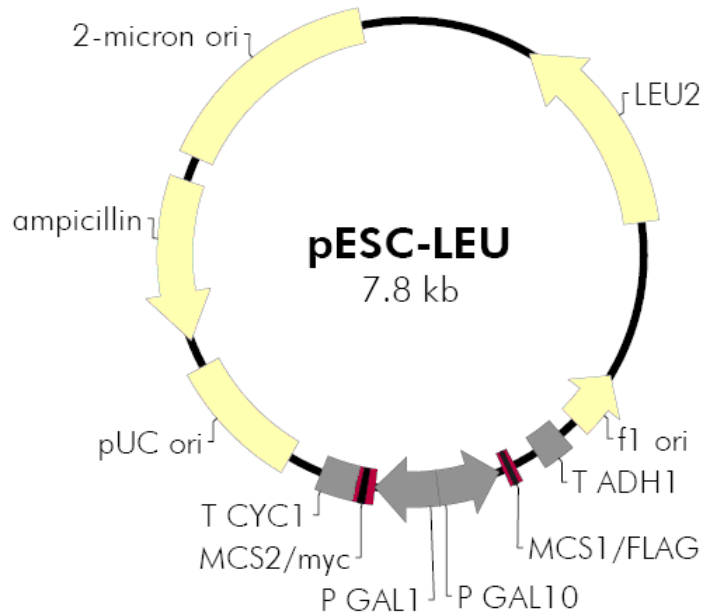


Figure 9: Vector map of pESC-Leu (57).

Error-Prone PCR (EP-PCR) of β -ENaC Gene

Random mutations in the β -ENaC gene were generated using EP-PCR. EP-PCR is a modification of the standard PCR that requires a Taq polymerase, a polymerase without the ability to proofread, an increased concentration of MgCl_2 and/or MnCl_2 . In a previous study done by Ty Whitesnant (58), EP-PCR was optimized under several conditions by adding increased concentrations of Mg^{2+} alone or Mg^{2+} combined with Mn^{2+} to determine mutation frequency. The optimal conditions for EP-PCR reactions leading to 2 – 3 mutations in 700 bp was using 1.5 mM Mg^{2+} alone and these conditions were used in the current study.



Figure 10: Cartoon map showing the positions and directions of random beta primers within the beta-ENaC gene. The Beta- ENaC gene has about 2704 bp. The UP BmENaC random is found at positions 518-537 while the DN BmENaC primer is found at positions 1372-1393.

The beta mutant PCR products were analyzed using gel electrophoresis and the DNA fragment migration corresponded to an 804 bp fragment (Fig 11 lane 4-6) based on the 1 kb DNA ladder (Fig 11, lane 1). The expected the fragment size was 875 bp. The PCR products were excised from the gel, cleaned up using QIAEX 11 gel extraction kit (QIAGEN) and then quantitated. The yield of the PCR product was 0.9 µg and its purity based on the 260/280 was 2.1.

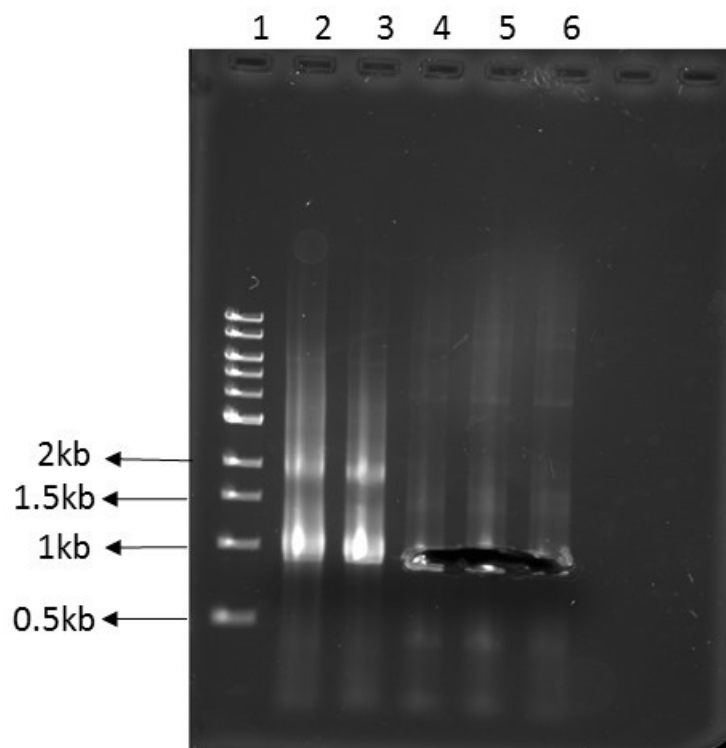


Figure 11: Beta-ENaC EP- PCR products. The PCR products were loaded onto a 0.8 % TAE Agarose gel. Lane 1, 1 kb DNA ladder (NEB). Lane 2-3, PCR products of pCMV/alpha-ENaC. Lane 4-6, PCR products of pCMV/beta-ENaC. The gel was stained 0.25 mg EtBr/ml. The primers used for this reaction were the UP BmENaC random primers and the DN BmENaC random primers.

In an effort to get a higher DNA yield for future experiments, the beta mutant PCR product was directly cleaned using the QiaQuick gel extraction kit (QIAGEN) followed by an ethanol precipitation protocol. This gave a much higher yield of 13.5 μ g and a 260/280 of 1.8.

Digestion of pESC-Leu/ β -ENaC

The pESC-Leu/ β -ENaC was digested with SexA1 and Zra1 which are both unique restriction enzymes that cut the β -ENaC gene at positions 730 and 1054, respectively. SexA1 is sensitive to dam methylation, so the plasmid was transformed and grown in a

non-methylated strain of *E. coli* ER2925 from NEB. Then 6.5 µg of pESC-Leu/β-ENaC was digested with 20 units of Zra1 and 10 units of SexA1 overnight at 37 °C. The digested product was analyzed in a 0.8% w/v 1X TAE agarose gel. The pESC-Leu/β-ENaC migrated at ~ 10 kb, which is around the expected value which was 9.8 kb (Fig 12). The digested plasmids were excised from the gel and cleaned up using the Qiagen QIAEX II gel extraction kit and then quantitated. The yield of the digested plasmid was 2.1 µg and a 260/280 of 1.7 was obtained.

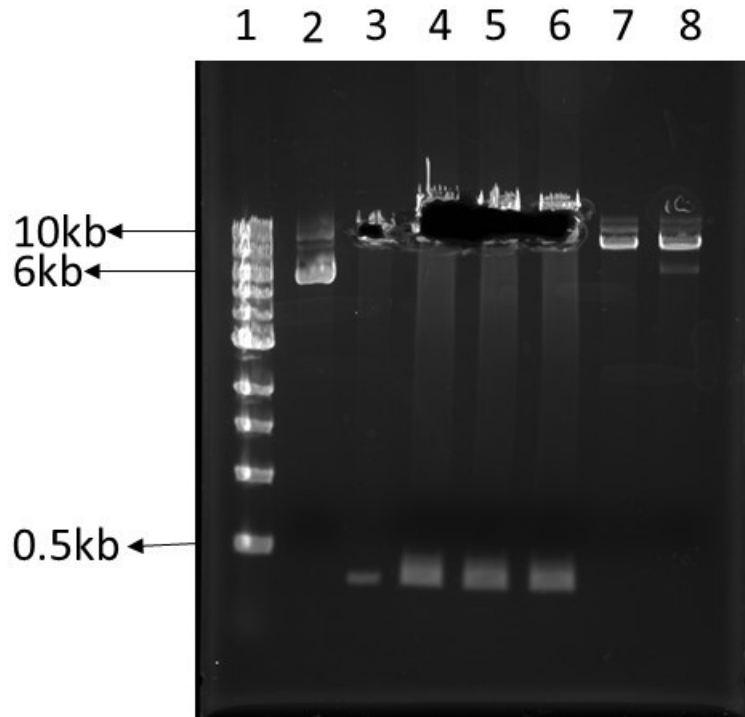


Figure 12: Gel extraction of digested pESC-Leu/β-ENaC. The digested products were ran on a 0.8% w/v TAE agarose gel. Lane 1- 1kb DNA ladder, lane 2- control uncut pESC-Leu/β-ENaC, lanes 3-6 -pESC-Leu/β-ENaC digested with SexA1 and Zra1, lane 7- pESC-Leu/β-ENaC digested with Zra1, lane 8- pESC-Leu/β-ENaC digested with SexA1. The gel was stained with 0.25 mg EtBr/ml.

The purified digested pESC-Leu/β-ENaC and the β-ENaC EP-PCR product were co-transformed into BY4742 yeast cells using a highly efficient lithium acetate transformation protocol. Ten individual colonies were selected for further

characterization via dilution pronging survival assays. These yeast colonies which each possess a different mutant clone were then grown overnight in 2% w/v glu-leu-ura media at 30°C and then re-transformed with pYES2/NTA/ α -ENaC using the same transformation protocol. Also, yeast cells containing pESC-Leu/ β -ENaC were transformed with pYES2/NTA/ α -ENaC, which was used as the wild-type control for the pronging assays.

Dilution Pronging Survival Assays

Pronging assays were performed to determine the function of mutated pESC-Leu/ β -ENaC by comparing growth of yeast cells expressing α -ENaC and mutated β -ENaC to the wild type yeast cells expressing α -ENaC and β -ENaC. Cells were plated onto SC-leu-ura plates that contains 2% glucose (no expression, control) or 2% galactose (turns on the expression of ENaC) (Fig 13 and 14).

The cells expressing $\alpha\beta_{\text{mutants}}$ that were grown in the presence of glucose and 0.5 M salt showed similar growth as compared to the $\alpha\beta$ wild type control (Fig 13A and B) indicating that ENaC was not expressed. Hence, irrespective of the mutant genes being present, the cells grew normally. However, in the presence of galactose only (i.e. expression of ENaC), cells expressing beta mutants 2 and 3 exhibited similar growth inhibition like the wild type (Fig 13C rows 1, 3 and 4) while beta mutants 1, 4 and 5 grew slightly better than the control (Fig 13C rows 2, 4, 5 and 6).

In the presence of galactose and salt, salt sensitivity of cells expressing ENaC was increased (Fig 13D). Beta mutants 1, 4 and 5 displayed a slight better growth as compared to the wild type. (Fig 13D rows 2, 5 and 6).

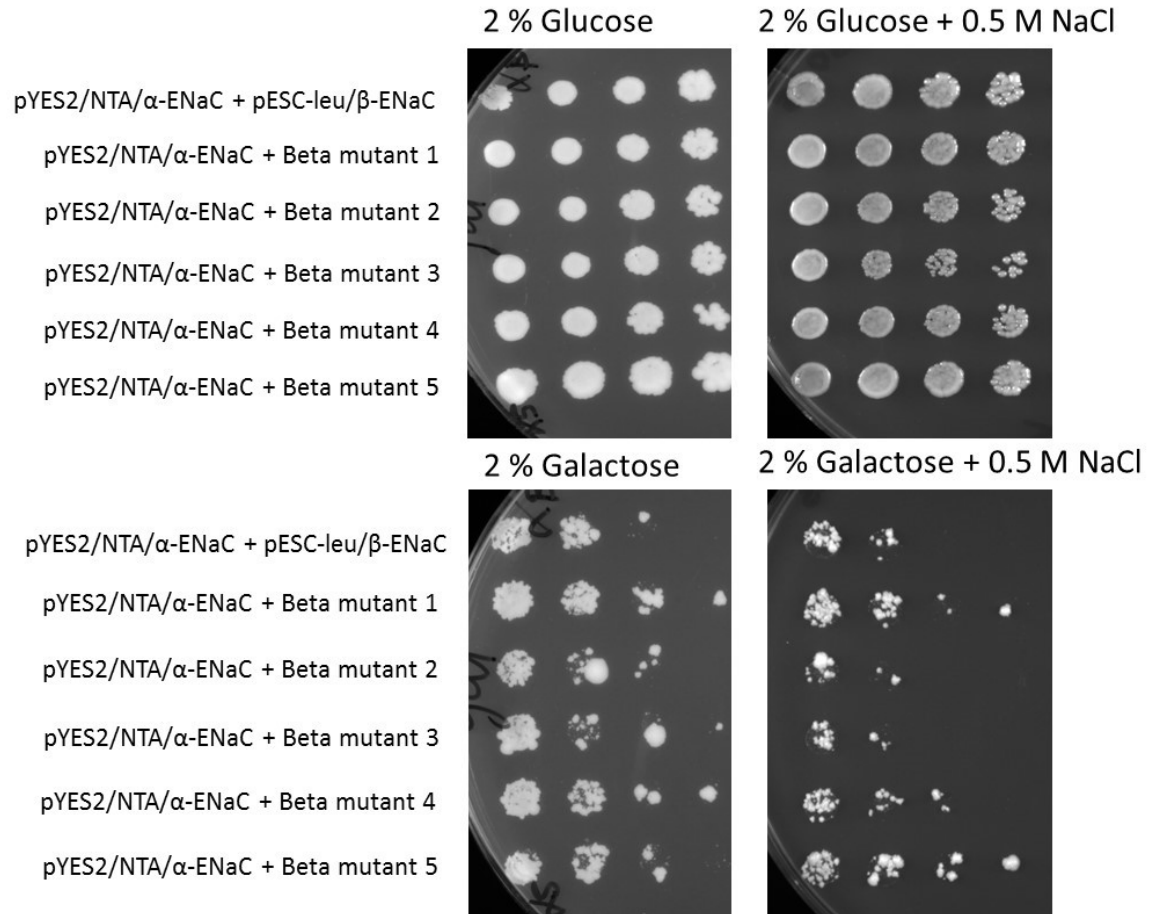


Figure 13: Dilution pronging survival assay of pESC-Leu/β mutants 1-5. PESC-Leu/β-mutants was transformed into BY4742 cells and then retransformed with pYES2/NTA/αENaC. Cells were plated on selective plates to observe differences in growth as compared to the control. **A** - Cells grown on SC-Leucine-Uracil with 2% glucose. **B** - Cells grown on SC- Leucine-Uracil with 2% glucose plus 0.5 M NaCl **C** - Cells grown on SC- Leucine-Uracil with 2% galactose. **D** - Cells grown on SC Leucine--Uracil with 2% galactose plus additional 0.5 M NaCl. The plates were imaged using the Molecular Imager® ChemiDoc™ XRS-Imaging System from Bio-Rad (Hercules, CA).

Cells expressing beta mutants 6-10 exhibited similar growth (no expression) as compared to the wild type controls when grown in the presence of glucose and 0.5 M salt (Fig 14A and B). In the presence of galactose and 0.5 M salt, beta mutants 6-8 and

mutant 10 exhibited similar growth inhibition as compared to the wild type controls (Fig 14D, rows 2, 3, 4, and 6) Beta mutant 9 displayed better growth than the control and the other mutants (Fig 14D, row 5). This indicates that the mutant is causing a decrease in ENaC function unlike the control which is overexpressing ENaC. The same results were seen in the presence of galactose only.

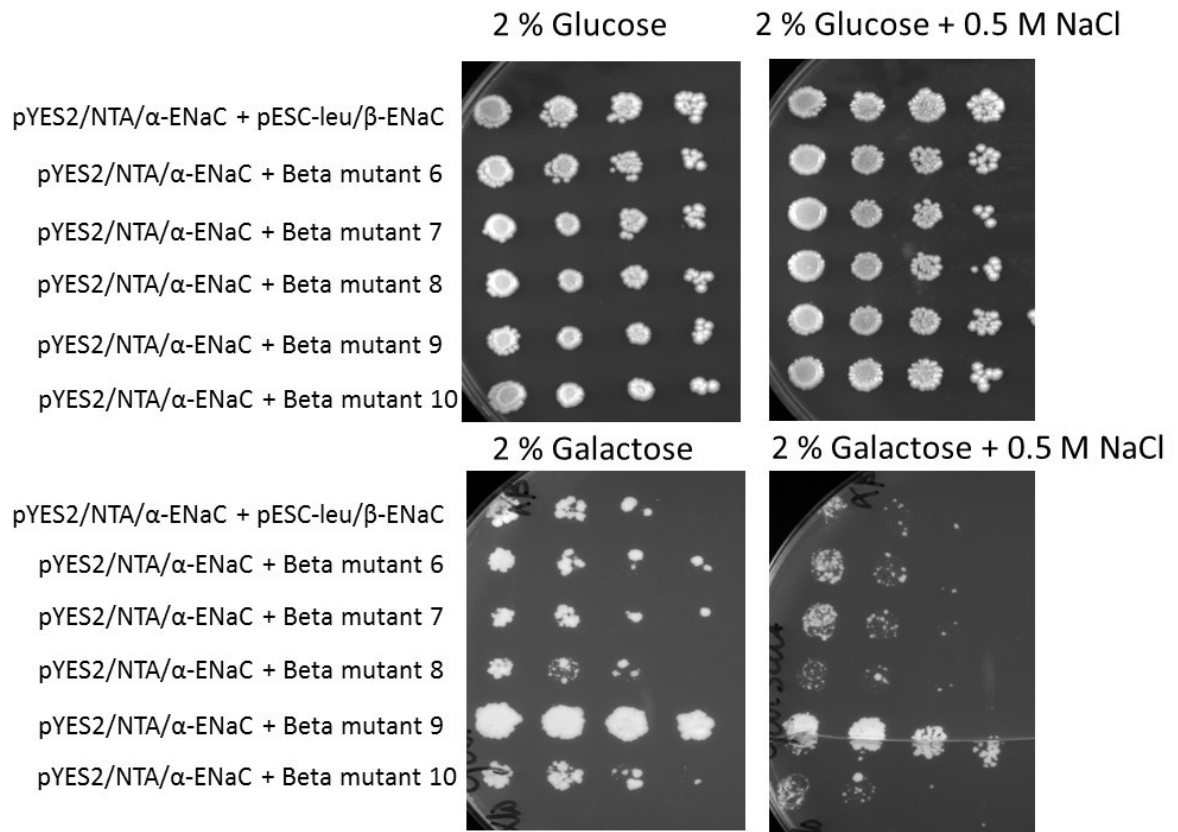


Figure 14: Dilution pronging survival assay of pESC-Leu/β-mutants 6-10. PESC-Leu/β-mutants was transformed into BY4742 cells and then retransformed with pYES2/NTA/αENaC. Cells were plated on selective plates to observe differences in growth as compared to the control. **A** - Cells grown on SC- Leucine-Uracil with 2% glucose. **B** - Cells grown on SC- Leucine-Uracil with 2% glucose plus 0.5 M NaCl **C** - Cells grown on SC- Leucine-Uracil with 2% galactose. **D** - Cells grown on SC Leucine--Uracil with 2% galactose plus additional 0.5 M NaCl.

Also, a repeat of the pronging assays was performed to confirm similar trends in an independent experiment. Cells expressing the wild type pYES2/NTA/α-ENaC +

pESC-Leu/ $\beta\gamma$ -ENaC that has the three subunits of ENaC present were used as the controls, but are not comparable. Cells were plated onto SC-leu-ura plates that contained 2% glucose (control) or 2% galactose. The growth trends of cells expressing mutant β -ENaCs were similar to results seen when the mutated beta mutants were compared to each other in fig 13 and 14 (Fig 15 and 16).

The cells expressing $\alpha\beta_{\text{mutants}}$ that were grown in the presence of glucose and 0.5 M salt showed similar growth to each other and the $\alpha\beta\gamma$ wild type control (Fig 15A and B) indicating that ENaC was not expressed. In the presence of galactose and 0.5 M salt, cells with beta mutants 1, 4 and 5 grew slightly better cells with mutants 2 and 3 (Fig 15D rows 2, 4, 5 and 6) as seen in the prior experiment (Fig 13 and 14).

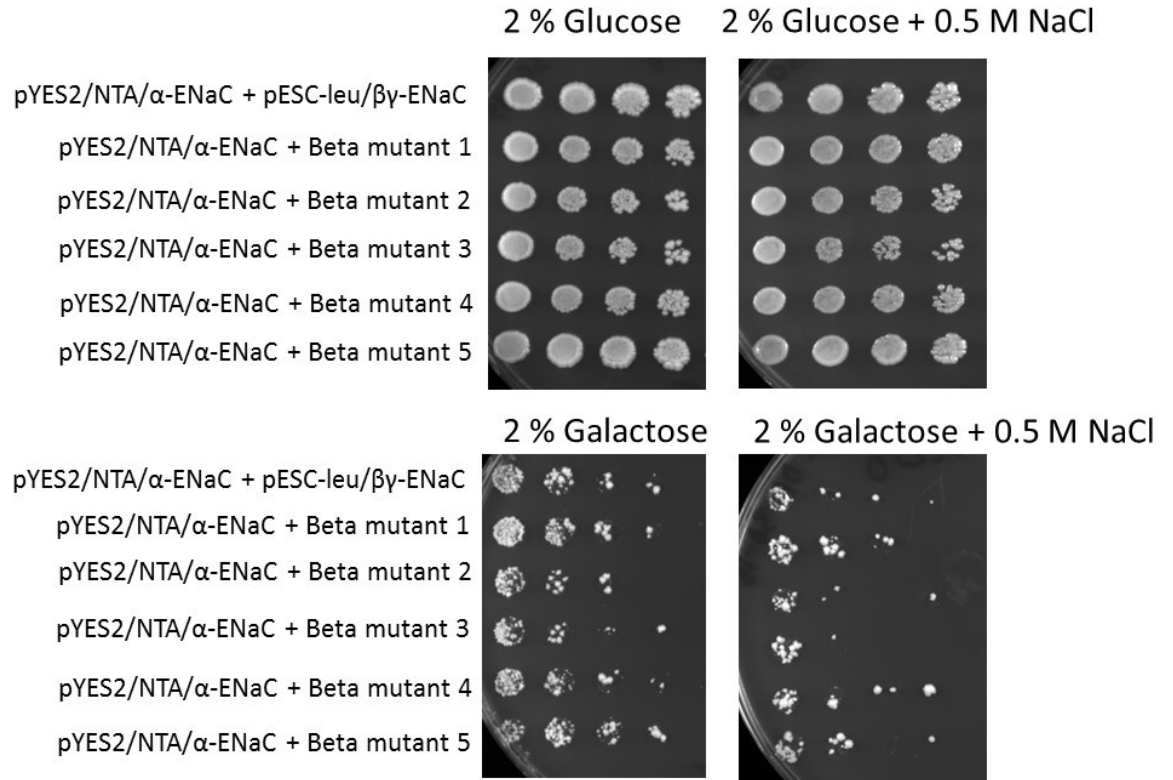


Figure 15: Dilution pronging survival assay of pESC-Leu/β mutants 1-5. PESC-Leu/β-mutants was transformed into BY4742 cells and then retransformed with pYES2/NTA/αENaC. Cells were plated on selective plates to observe differences in growth as compared to the control. *A* - Cells grown on SC-Leucine-Uracil with 2% glucose. *B* - Cells grown on SC- Leucine-Uracil with 2% glucose plus 0.5 M NaCl. *C* - Cells grown on SC- Leucine-Uracil with 2% galactose. *D* - Cells grown on SC Leucine--Uracil with 2% galactose plus additional 0.5 M NaCl. The plates were imaged using the Molecular Imager® ChemiDoc™ XRS-Imaging System from Bio-Rad (Hercules, CA).

Beta mutants 6-8 and mutant 10 also caused similar growth inhibition as compared to each other (Fig 16D, rows 2, 3, 4, and 6) in the presence of galactose only and galactose with salt while beta mutant 9 displayed better growth than the the other mutants (Fig 16D, row 5).

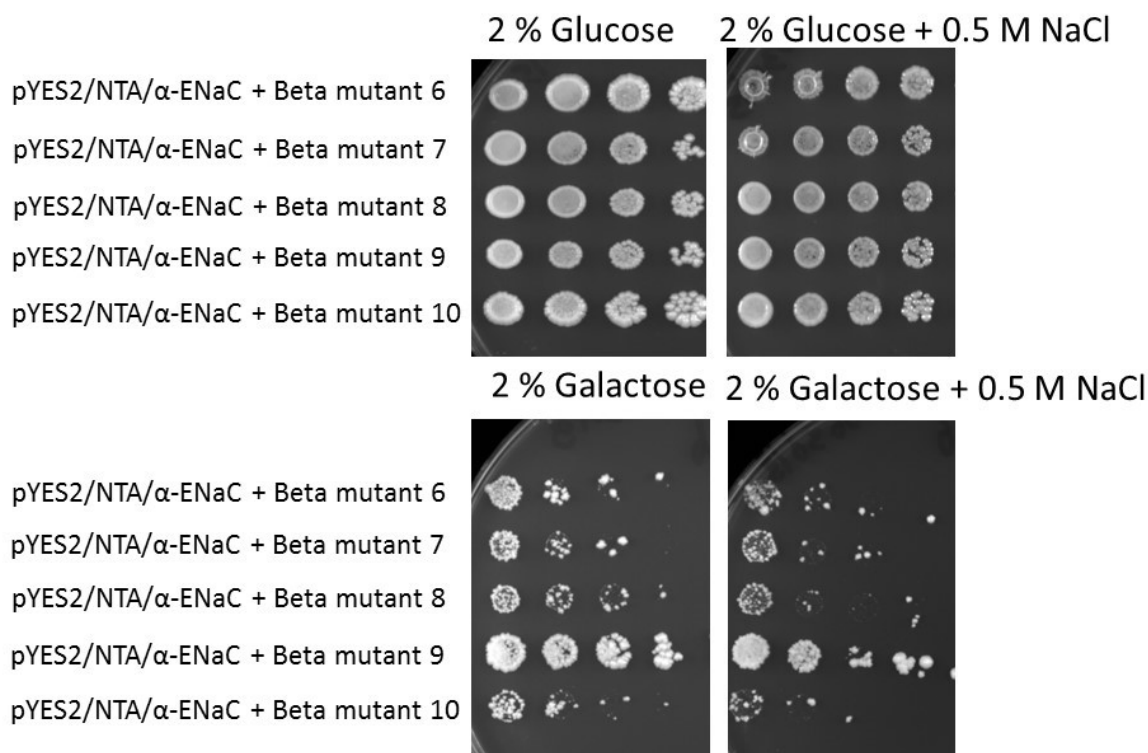


Figure 16: Dilution pronging survival assay of pESC-Leu/β mutants 6-10. PESC-Leu/β-mutants was transformed into BY4742 cells and then retransformed pYES2/NTA/αENaC. Cells were plated on selective plates to observe differences in growth as compared to the control. **A** - Cells grown on SC-Leucine-Uracil with 2% glucose. **B** - Cells grown on SC- Leucine-Uracil with 2% glucose plus 0.5 M NaCl **C** - Cells grown on SC- Leucine-Uracil with 2% galactose. **D** - Cells grown on SC Leucine--Uracil with 2% galactose plus additional 0.5 M NaCl. The plates were imaged using the Molecular Imager® ChemiDoc™ XRS-Imaging System from Bio-Rad (Hercules, CA).

The wild type pYES2/NTA/α-ENaC + pESC-Leu/βγ-ENaC displayed a very dramatic growth inhibition because it has the three subunits present which is expected. The characterization displayed by the control is due to the fact that ENaC co-assembles as a heterotrimeric protein to achieve full activity (59).

Expression of pESC-Leu/β-ENaC in Yeast

Yeast cells containing beta mutants of interest were patched onto a glu-leu-ura plates containing 2% w/v glucose for 3 – 4 days. Colonies were scraped and grown

overnight at 30 °C in 2% glu-leu-ura media with shaking. The OD₆₀₀ reading was determined and was used to calculate the amount of overnight culture needed to obtain an OD₆₀₀ of 0.4. The cells were spun down and the media was changed to 2% gal-leu-ura media. The cells were then allowed to grown for 8 hrs at 30 °C with shaking. The proteins were extracted from the yeast cells using post alkaline extraction method as described. The protein was loaded onto a 4 – 15 % reducing SDS PAGE gel and then transferred to a nitrocellulose membrane.

To confirm the expression of pESC-Leu/ β -ENaC mutants in yeast, a western blot was performed. The membrane was probed with anti-beta ENaC primary antibody and anti-rabbit HRP secondary antibody. Beta-ENaC mutants 1, 4 and 5 exhibited two forms of beta-ENaC proteins; a non-glycosylated form at 70 kDa and a glycosylated form at 100 kDa (Fig 17, lanes 2 – 4, 60) while only non-specific binding was seen for mutant 9 (Fig 17, lane 5). No control was expressed in yeast. The theoretical molecular weight of beta-ENaC is 72.2 kDa, but due to varying glycosylation, the proteins consistently migrate higher in SDS-PAGE. The molecular weights of the two bands of beta mutant 1 were calculated to be 71.45 kDa and 98.79 kDa (Fig 17, lane 2) while the molecular weights for beta mutant 4 were 74.07 kDa and 95.29 kDa (Fig 17, lane 3). For beta mutant 5, the molecular weights were calculated to be 74.07 kDa and 102.4 kDa (Fig 17, lane 4), respectively. For beta mutant 9 where no ENaC expression is seen, it is possible that the protein is not being expressed or being degraded immediately. In either case, the pronging assay results are consistent in that there was no growth inhibition for beta mutant 9. The results obtained from the western blot for the other mutants corresponds to the results from the dilution pronging survival assay in that there was a slight growth.

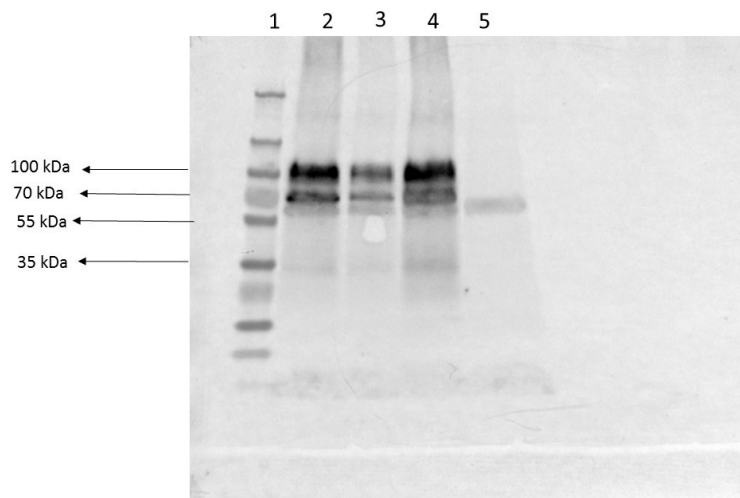


Figure 17: Beta-ENaC mutant expression. The protein samples were loaded onto a 4-15 % reducing SDS PAGE. The gel was transferred to a nitrocellulose membrane and probed with an anti-beta ENaC (primary antibody) and an anti-rabbit horseradish peroxidase (secondary antibody). Lane 1, PageRuler plus Prestained Protein Ladder. Lane 2, pYES2/NTA/ α -ENaC + beta mutant 1. Lane 3, pYES2/NTA/ α -ENaC + beta mutant 4. Lane 4, pYES2/NTA/ α -ENaC + beta mutant 5. Lane 5, pYES2/NTA/ α -ENaC + beta mutant 9.

Isolation of Mutant plasmid from *S. cerevisiae*

Beta mutants 1, 4, 5 and 9 were chosen as mutants of interest because they showed either slight or no growth inhibition as compared to the wild-type control. Cells of mutants of interest were scraped from a patch plate containing glu-leu-ura and grown in selective glu-leu-ura media overnight at 30°C with shaking. The cell walls of yeast are difficult to disrupt and so an enzyme, zymolase, was used. The plasmid DNA was then isolated from yeast cells using the Qiagen Qiaprep spin miniprep kit. The yield of the isolated plasmids, beta mutants 1, 4, 5 and 9, obtained were 16.0 μ g, 15.2 μ g, 10.0 μ g and 16.6 μ g, respectively, while the purity obtained were 2.0, 2.0, 2.1 and 2.0, respectively (Table 3).

Table 3: Yield and Purity of Mutant Plasmid DNA Isolated from Yeast

Mutant	Yield (µg)	Purity (260/280)
Beta mutant 1	16.0	2.0
Beta Mutant 4	15.2	2.0
Beta Mutant 5	10.0	2.1
Beta Mutant 9	16.6	2.0

As expected, chromosomal DNA that appeared as smears was seen (Fig 18) in each sample. In eukaryotic cells, separation of plasmid and chromosomal DNA is very difficult which is why for further characterization, the resulting DNA was transformed into *E.coli* cells to isolate only the plasmid DNAs from the yeast cells.

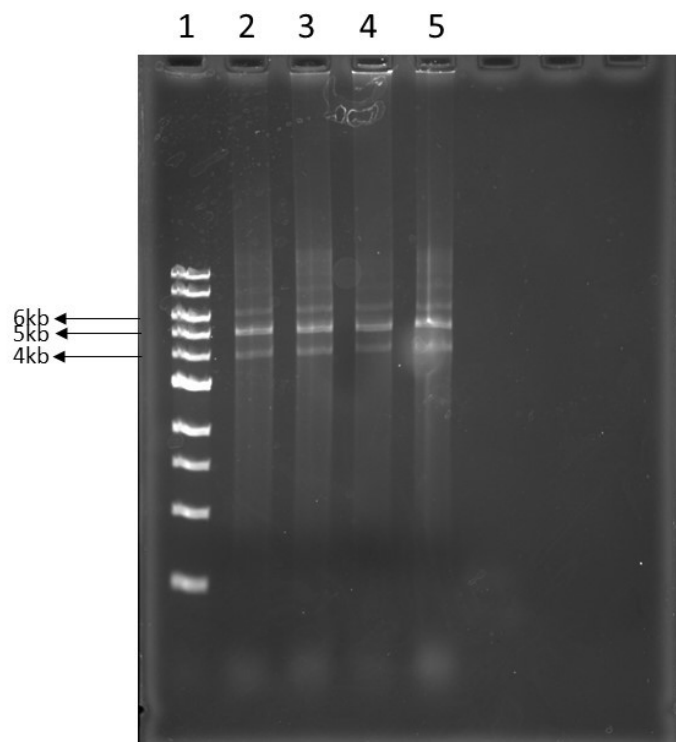


Figure 18: Plasmid DNA of beta mutants 1, 4, 5 and 9 from yeast. The isolated DNA plasmids were ran on a 0.8 % TAE Agarose gel. Lane 1, 1 kb DNA ladder. Lane 2, pYES2/NTA/ α -ENaC + beta mutant 1. Lane 3, pYES2/NTA/ α -ENaC + beta mutant 4. Lane 4, pYES2/NTA/ α -ENaC + beta mutant 5. Lane 5, pYES2/NTA/ α -ENaC + beta mutant 9. The gel was stained with 0.25 mg EtBr/ml.

Digestion of Mutant Plasmid

The plasmids containing mutant beta-ENaCs were transformed into Top 10 *E. coli* cells and 500 μ l – 750 μ l of the reaction mixture was spread on LB plates supplemented with 100 μ g/ml ampicillin and grown overnight at 37 °C. DNA plasmid was isolated from a single colony of *E. coli* using the Qiagen QIAprep spin Miniprep Kit and the plasmid concentrations was quantified. The yield of beta mutant 1, 4, 5 and 9 plasmid DNAs were 26.3 μ g, 27.6 μ g, 33.5 μ g and 28.1 μ g, respectively, while the purity of the plasmids DNA obtained were 1.9, 1.8, 1.8 and 1.9, respectively (Table 4).

Table 4: Yield and Purity of Mutant Plasmid DNA Isolated from Top 10 *E. coli* cells

Mutant	Yield (µg)	Purity (260/280)
Beta Mutant 1	26.3	1.9
Beta Mutant 4	27.6	1.8
Beta Mutant 5	33.5	1.8
Beta Mutant 9	28.1	1.9

Two and one-half micrograms of each plasmid DNA was digested with Xho1 and Nhe1 at 37 °C for 4 hrs. The digestion reaction was inactivated with 1X endorstop sample buffer. The digestion products were analyzed on a 0.8 % w/v TAE horizontal gel electrophoresis. Theoretically, Xho1 and Nhe1 should cut pESC-Leu/ β -ENaC to produce two fragments at 2 kb and 7 kb. As expected, two fragments at ~2 kb and 7 kb were observed for beta mutant 5 (Fig 19, lane 6). The control has the γ -subunit present which is about 2 kb and this explains why the fragment at 9.8 kb for the control is larger than the 7 kb fragment that is expected. Beta mutants 1, 4 and 9 had three fragments at ~ 2.5 kb, 2.8 kb, and 3.6 kb (Fig 19, lane 4, 5 and 7). The three fragments seen for mutants 1, 4 and 9 suggests that a plasmid other than β -ENaC was isolated from yeast.

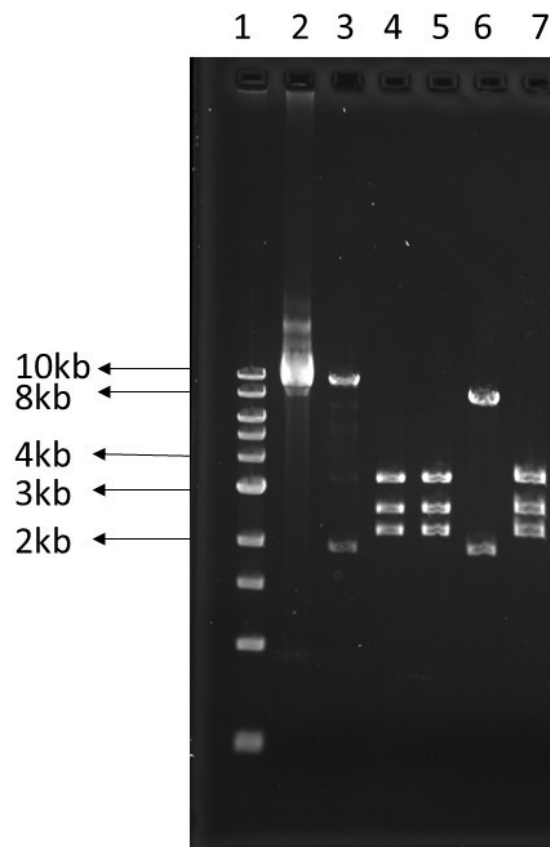


Figure 19: Gel electrophoresis of digested mutants 1, 4, 5 and 9 plasmids with XhoI and NheI. The digested products were ran on a 0.8 % TAE Agarose gel. Lane 1, 1 kb DNA ladder. Lane 2, uncut pESC-Leu/ β / γ -ENaC. Lane 3, Digested pESC-Leu/ β / γ -ENaC. Lane 4, pYES2/NTA/ α -ENaC + beta mutant 1. Lane 5, pYES2/NTA/ α -ENaC + beta mutant 4. Lane 6, pYES2/NTA/ α -ENaC + beta mutant 5. Lane 7, pYES2/NTA/ α -ENaC + beta mutant 9. The gel was stained with 0.25 mg EtBr/ml.

In order to understand why the mutants produced three fragments instead of two, the mutant plasmid DNAs were sent to Quintara Biosciences to be sequenced. The sequence data were translated using Swiss Institute of Bioinformatics tool (59) and compared against a protein sequence database. The result showed that the three fragments produced were from the pYESNTA/ α -ENaC that was co-transformed with the pESC-Leu β -ENaC mutant. pYESNTA/ α -ENaC sequence was theoretically digested with XhoI and NheI using the NEBcutter V2.0 and the expected fragments were at 2194, 2563 and 3363

bp and these fragments corresponds to those seen on the gel (Fig 19, lane 4, 5 and 7).

These results show that the plasmid DNA isolated from yeast and transformed into *E. coli* cells, depending on what colony was grown in LB broth, isolated, and digested, could either be β -ENaC or α -ENaC.

In order to obtain only beta mutants that will be used for further characterization, multiple colonies from the *E.coli* transformation plates (after plasmid isolation from yeast) were grown separately in LB broth overnight at 37 °C, plasmid DNA from each was isolated and quantitated. One thousand nanograms of each plasmid DNA was digested with Xho1 and Nhe1 for 4 hrs at 37 °C. The digestion reactions were inactivated with 1X endorstop buffer and analyzed on a 0.8 % w/v TAE agarose gel. Out of the seven different colonies of beta mutant 1 analyzed, only four were consistent with beta-ENaC sequences (Fig 20, lanes 2, 3, 5 and 7) while the other three were consistent with alpha-ENaC sequences (Fig 20, lanes 4, 6 and 8).

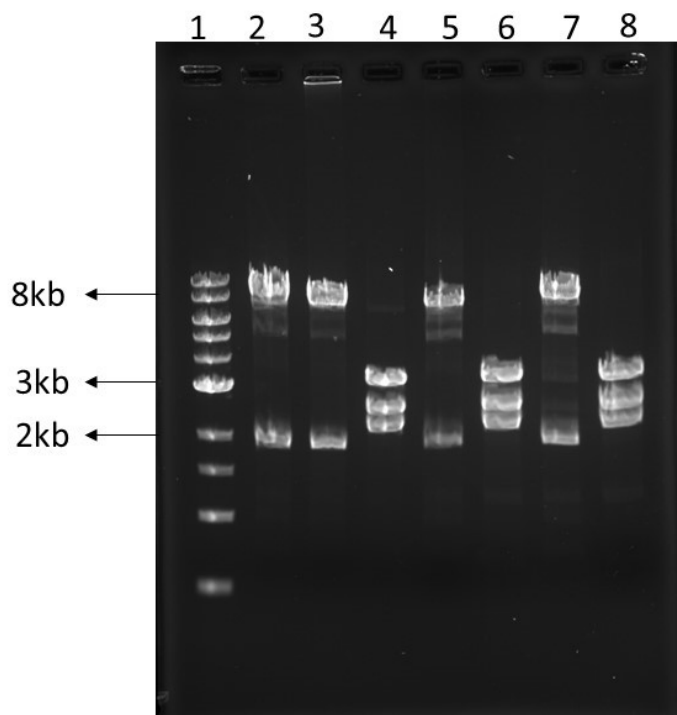


Figure 20: Beta-ENaC mutant 1 clones digested with XhoI and NheI. The plasmid DNAs of beta mutant 1 was ran on a 0.8 % TAE Agarose gel. Lane 1, 1 kb DNA ladder. Lane 2 - 8, multiple colonies of pYES2/NTA/ α -ENaC + beta mutant 1. The gel was stained with 0.25 mg EtBr/ ml.

For beta-ENaC mutant 4, only one of the colonies was found to be beta ENaC (Fig 21, Lane 9). Nine were found to be alpha ENaC (Fig 21, Lanes 2,4,5,6,7,8,10, 11 and 12) while one was seen to be a mixture of α and β -ENaC (Fig 21 lane 3). When two colonies that are in close enough proximity are picked and isolated, there would be a mixture of plasmids after isolation. This explains why in lane 3, we have a mixture of α and β -ENaC.

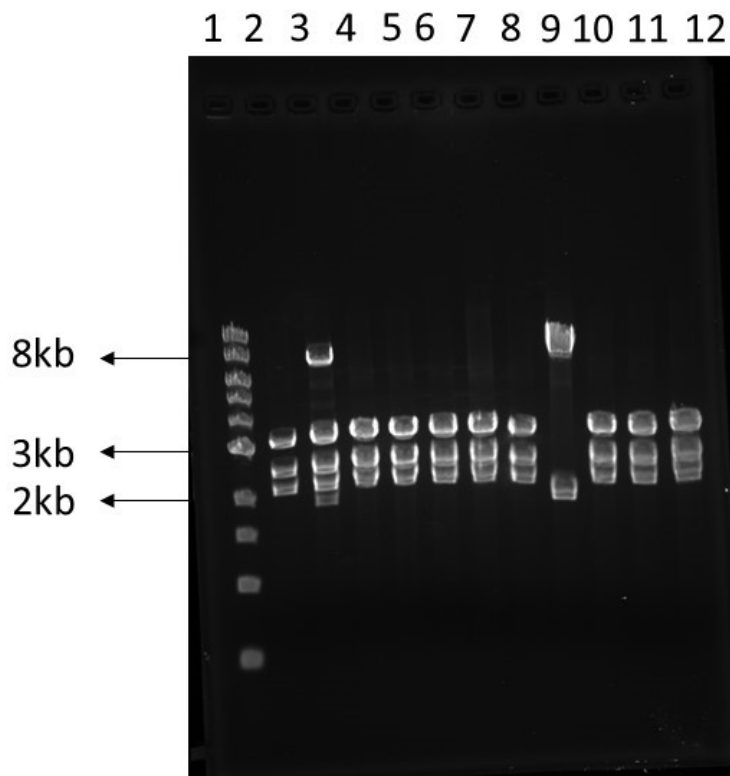


Figure 21: Beta-ENaC mutant 4 plasmid clones digested with Xho1 and Nhe1. The plasmid DNAs of beta mutant 4 were ran on a 0.8 % TAE Agarose gel. Lane 1, 1 kb DNA ladder. Lane 2 -12, multiple colonies of pYES2/NTA/ α -ENaC + beta mutant 4. The gel was stained with 0.25 mg EtBr/ml.

Only two colonies of beta ENaC mutant 5 were found to be beta ENaC (Fig 22, lanes 4 and 5), the remaining five were alpha ENaC (Fig 22, lanes 2, 3, 6, 7 and 8).

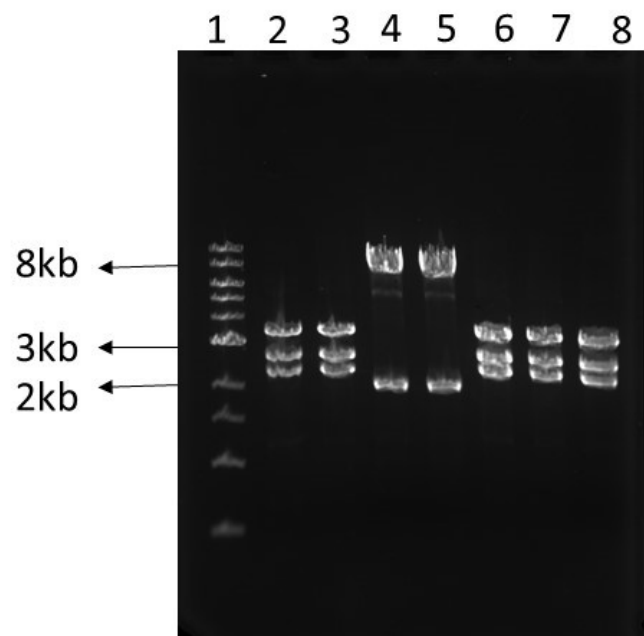


Figure 22: Beta-ENaC mutant 5 plasmid clones digested with Xho1 and Nhe1. The plasmid DNAs of beta mutant 5 were ran on a 0.8 % TAE Agarose gel. Lane 1, 1 kb DNA ladder. Lane 2 - 8, multiple colonies of pYES2/NTA/ α -ENaC + beta mutant 5. The gel was stained with 0.25 mg EtBr/ml.

For beta-ENaC mutant 9, only four were observed to be beta-ENaC (Fig 23, lanes 2, 4, 6 and 7) while the other five were alpha-ENaC (Fig 23, lanes 3, 5, 8, 9 and 10).

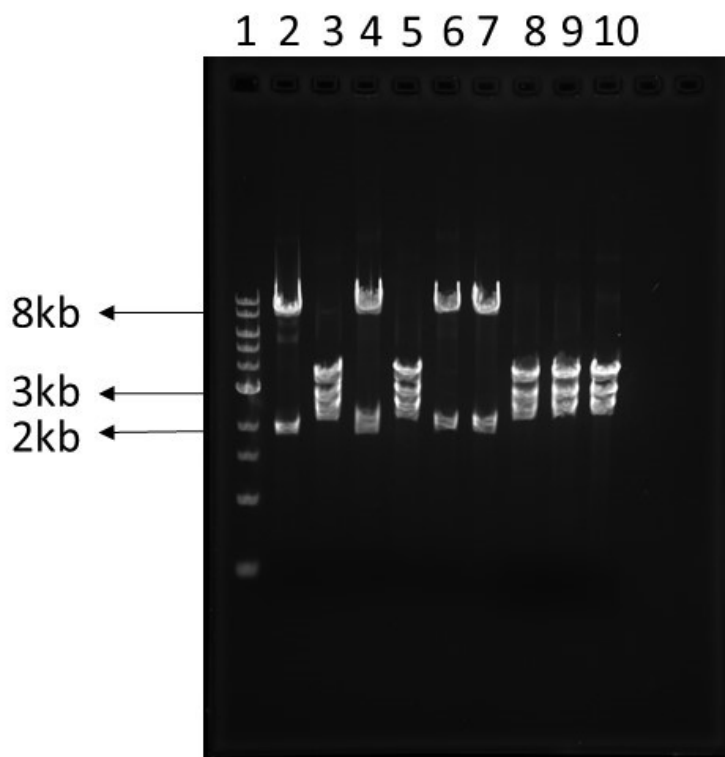


Figure 23: Beta-ENaC mutant 9 plasmid clones digested with Xho1 and Nhe1. The plasmid DNAs of beta mutant 9 were ran on a 0.8 % TAE Agarose gel. Lane 1, 1 kb DNA ladder. Lane 2 - 8, multiple colonies of pYES2/NTA/ α -ENaC + beta mutant 9. The gel was stained with 0.25 mg EtBr/ml.

Sequencing and Alignment of Beta Mutant ENaCs

Out of the eleven different clones identified as mutant beta-ENaC from the digestion of beta mutants 1, 4, 5 and 9, one of each of the mutants was selected for DNA sequencing. The mutants were sequenced using three different primers; beta internal primer, mutant sequencing primer, and beta internal sequencing primer. The different primers were used to ensure that the region that contains both the forward and reverse random primers were targeted (Fig 24).

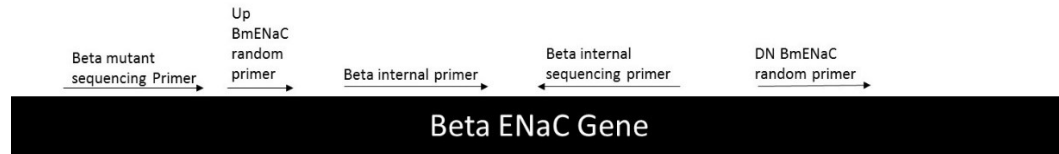


Figure 24: Cartoon map showing the locations and directions of primers. Sequencing primers; Beta mutant sequencing primer, Beta internal primer and Beta internal sequencing primer. PCR primers; Up BmENaC random primer and DN BmENaC primer.

The sequencing data were first translated to the protein sequence in all reading frames and then blasted against a protein database using BLASTx (61) to ensure that all the mutants selected were actually beta-ENaC. Once identified that they were all beta-ENaC, the DNA sequencing data were then compared to wild type *Mus Musculus* beta ENaC mRNA using BLAST 2 sequence software (62, 63) to identify point mutations within the mutant beta DNA. Also, the translated sequence using ExPASy translate tool (64) was aligned to the wild type *Mus Musculus* beta amino acid sequence using BLASTp (63) to identify amino acid changes within the protein. The first and last 100 DNA bp of the sequence were not considered when identifying the points of mutation because of potential errors that may have occurred during sequencing.

Alignment of Point Mutations for All Mutants

As mentioned earlier, only four of the beta ENaC mutants identified as beta plasmids were sequenced. The nucleotide sequence data were aligned using Multiple Sequence Comparison by Log Expectation (65) (Fig 25, 27, 29). The nucleotide sequence data were then translated to amino acids using EXPASY (64) and then aligned using Clustal Omega software (66) (Fig 26, 28, 30).

Beta-ENaC	GGAAGTGTGTGTACCTTGCGGAACCTTACCAGTGCCACCCAGGCCGTAAGTGTAGTGGTAC
Beta-mutant-1	-----TCTCAGTGCA--CCAGGCCGT-ACTGAGTGGTAC
Beta-mutant-4	-----ATTAGTGCCACCAGGCCGT-ACTGAGTGGTAC
Beta-mutant-5	-----GTTCGTGCCCCCAGGCCGT-ACTGAGTGGTAC
Beta-mutant-9	-----AGAAGGGCACAGGCCGT-ACTGAGTGGTAC
	* *****
Beta-ENaC	ATCCTGCAGGCCACCAACATCTTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGC
Beta-mutant-1	ATCCTGCAGGCCACCAACATCTTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGC
Beta-mutant-4	ATCCTGCAGGCCACCAACATCTTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGC
Beta-mutant-5	ATCCTGCAGGCCACCAACATCTTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGC
Beta-mutant-9	ATCCTGCAGGCCACCAACATCTTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGC

Beta-ENaC	TATGCCCTGATCGCATAATCCTAGCTTGCTGTGTTGGAACAGAGCCCTGCAGTCATCGG
Beta-mutant-1	TATGCCCTGATCGCATAATCCTAGCTTGCTGTGTTGGAACAGAGCCCTGCAGTCATCGG
Beta-mutant-4	TATGCCCTGATCGCATAATCCTAGCTTGCTGTGTTGGAACAGAGCCCTGCAGTCATCGG
Beta-mutant-5	TATGCCCTGATCGCATAATCCTAGCTTGCTGTGTTGGAACAGAGCCCTGCAGTCATCGG
Beta-mutant-9	TATGCCCTGATCGCATAATCCTAGCTTGCTGTGTTGGAACAGAGCCCTGCAGTCATCGG

Beta-ENaC	AACTTCACGCCTATCTTCTACCCTGATTATGGCAACTGTACATCTTCAACTGGGGCATG
Beta-mutant-1	AACTTCACGCCTATCTTCTACCCTGATTATGGCAACTGTACATCTTCAACTGGGGCATG
Beta-mutant-4	AACTTCACGCCTATCTTCTACCCTGATTATGGCAACTGTACATCTTCAACTGGGGCATG
Beta-mutant-5	AACTTCACGCCTATCTTCTACCCTGATTATGGCAACTGTACATCTTCAACTGGGGCATG
Beta-mutant-9	AACTTCACGCCTATCTTCTACCCTGATTATGGCAACTGTACATCTTCAACTGGGGCATG

Beta-ENaC	ACAGAGGAGACACTTCCTTCTGCCAACCTGGGACTGAATTTGGTCTCAAGTTGATCCTG
Beta-mutant-1	ACAGAGGAGACACTTCCTTCTGCCAACCTGGGACTGAATTTGGTCTCAAGTTGATCCTG
Beta-mutant-4	ACAGAGGAGACACTTCCTTCTGCCAACCTGGGACTGAATTTGGTCTCAAGTTGATCCTG
Beta-mutant-5	ACAGAGGAGACACTTCCTTCTGCCAACCTGGGACTGAATTTGGTCTCAAGTTGATCCTG
Beta-mutant-9	ACAGAGGAGACACTTCCTTCTGCCAACCTGGGACTGAATTTGGTCTCAAGTTGATCCTG

Beta-ENaC	GACATCGGTCAGGAGGACTATGTCCCCTTCCTTGCGTCCACAGCAGGGGCTAGGCTGATG
Beta-mutant-1	GACATCGGTCAGGAGGACTATGTCCCCTTCCTTGCGTCCACAGCAGGGGCTAGGCTGATG
Beta-mutant-4	GACATCGGTCAGGAGGACTATGTCCCCTTCCTTGCGTCCACAGCAGGGGCTAGGCTGATG
Beta-mutant-5	GACATCGGTCAGGAGGACTATGTCCCCTTCCTTGCGTCCACAGCAGGGGCTAGGCTGATG
Beta-mutant-9	GACATCGGTCAGGAGGACTATGTCCCCTTCCTTGCGTCCACAGCAGGGGCTAGGCTGATG

Beta-ENaC	CTTCATGAGCAGAGGACGTACCCCTTCATTAGAGAGGAGGGCATCTATGCCATGGCAGGA
Beta-mutant-1	CTTCATGAGCAGAGGACGTACCCCTTCATTAGAGAGGAGGGCATCTATGCCATGGCAGGA
Beta-mutant-4	CTTCATGAGCAGAGGACGTACCCCTTCATTAGAGAGGAGGGCATCTATGCCATGGCAGGA
Beta-mutant-5	CTTCATGAGCAGAGGACGTACCCCTTCATTAGAGAGGAGGGCATCTATGCCATGGCAGGA
Beta-mutant-9	CTTCATGAGCAGAGGACGTACCCCTTCATTAGAGAGGAGGGCATCTATGCCATGGCAGGA

Beta-ENaC	ACTGAGACGTCTATTGGGGTGCTGGTGGACAAGCTGCAACGCAAGGGGGAGCCCTACAGT
Beta-mutant-1	ACTGAGACGTCTATTGGGGTGCTGGTGGACAAGCTGCAACGCAAGGGGGAGCCCTACAGT
Beta-mutant-4	ACTGAGACGTCTATTGGGGTGCTGGTGGACAAGCTGCAACGCAAGGGGGAGCCCTACAGT
Beta-mutant-5	ACTGAGACGTCTATTGGGGTGCTGGTGGACAAGCTGCAACGCAAGGGGGAGCCCTACAGT
Beta-mutant-9	ACTGAGACGTCTATTGGGGTGCTGGTGGACAAGCTGCAACGCAAGGGGGAGCCCTACAGT

Beta-ENaC	CCCTGCACCATGAACGGCTCCGATGTTGCCATAAAGAACCTCTACAGTGTCTACAACACC
Beta-mutant-1	CCCTGCACCATGAACGGCTCCGATGTTGCCATAAAGAACCTCTACAGTGTCTACAACACC
Beta-mutant-4	CCCTGCACCATGAACGGCTCCGATGTTGCCATAAAGAACCTCTACAGTGTCTACAACACC
Beta-mutant-5	CCCTGCACCATGAACGGCTCCGATGTTGCCATAAAGAACCTCTACAGTGTCTACAACACC
Beta-mutant-9	CCCTGCACCATGAACGGCTCCGATGTTGCCATAAAGAACCTCTACAGTGTCTACAACACC

Beta-ENaC	ACCTATTCCATCCAGGCCGTGCTTCATTCTGTTTCCAAGACCACATGATCCGTAAGTGC
Beta-mutant-1	ACCTATTCCATCCAGGCCGTGCTTCATTCTGTTTCCAAGACCACATGATCCGTAAGTGC
Beta-mutant-4	ACCTATTCCATCCAGGCCGTGCTTCATTCTGTTTCCAAGACCACATGATCCGTAAGTGC
Beta-mutant-5	ACCTATTCCATCCAGGCCGTGCTTCATTCTGTTTCCAAGACCACATGATCCGTAAGTGC
Beta-mutant-9	ACCTATTCCATCCAGGCCGTGCTTCATTCTGTTTCCAAGACCACATGATCCGTAAGTGC

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Beta-ENaC      AGTTGTGGCCACTACTTATACCCACTGCCTGAAGGAGAGAAATACTGCAACAACAGGGAC
Beta-mutant-1  AGTTGTGGCCACTACTTATACCCACTGCCTGAAGGAGAGAAATACTGCAACAACAGGGAC
Beta-mutant-4  AGTTGTGGCCACTACTTATACCCACTGCCTGAAGGAGAGAAATACTGCAACAACAGGGAC
Beta-mutant-5  AGTTGTGGCCACTACTTATACCCACTGCCTGAAGGAGAGAAATACTGCAACAACAGGGAC
Beta-mutant-9  AGTTGTGGCCACTACTTATACCCACTGCCTGAAGGAGAGAAATACTGCAACAACAGGGAC
                *****

Beta-ENaC      TTCCAGACTGGGCCTATTGCTATCTAAACCTGCAGATGAGTGTGACCCAGAGAGAGACC
Beta-mutant-1  TTCCAGACTGGGCCTATTGCTATCTAAACCTGCAGATGAGTGTGACCCAGAGAGAGACC
Beta-mutant-4  TTCCAGACTGGGCCTATTGCTATCTAAACCTGCAGATGAGTGTGACCCAGAGAGAGACC
Beta-mutant-5  TTCCAGACTGGGCCTATTGCTATCTAAACCTGCAGATGAGTGTGACCCAGAGAGAGACC
Beta-mutant-9  TTCCAGACTGGGCCTATTGCTATCTAAACCTGCAGATGAGTGTGACCCAGAGAGAGACC
                *****

Beta-ENaC      TGCCTCAGCATGTGC-AAGGAGTCCTGCAATGACACCCAGTATAAGATGACCATCTCCAT
Beta-mutant-1  TGCCTCAGCATGTGC-AAGGAGTCCTGCAATGACACCCAGTATAAGATGACCATCTCCAT
Beta-mutant-4  TGCCTCAGCATGTGC-AAGGAGTCCTGCAATGACACCCAGTATAAGATGACCATCTCCAT
Beta-mutant-5  TGCCTCAGCATGTGC-AAGGAGTCCTGCAATGACACCCAGTATAAGATGACCATCTCCAT
Beta-mutant-9  TGCCTCAGCATGTGC-AAGGAGTCCTGCAATGACACCCAGTATAAGATGACCATCTCCAT
                *****

Beta-ENaC      -GGCTGACTGGCCATCTGAGGCCCTCTGAGGATTGGATCTTACATGTCTCTGTCTCAGGAGC
Beta-mutant-1  -GGCTGACTGGCCATCTGAGGCCCTCTGAGGATTGGATCTTACATGTCTCTGTCTCA-GAGC
Beta-mutant-4  -GGCTGACTGGCCATCTGAGGCCCTCTGAGGATTGGATCTTACATGTCTCTGTCTCAGAGCC
Beta-mutant-5  -GGCTGACTGGCCATCTGAGGCCCTCTGAGGATTGGATCTTACATGTCTCTGTCTCA-GAGC
Beta-mutant-9  -GGCTGACTGGCCATCTGAGGCCCTCTGAGGATTGGATCTTACATGTCTCTGTCTCAGGAGC
                *****

Beta-ENaC      GGGACCAGAGCTCGAATATCACCCCTGAGCAGG-AAGGGTATTGTCAAGCTCAATATCTAC
Beta-mutant-1  GGGACCAGAGCTCGAATATCACCCCTGAGCAGG-AAGGGTATTGTCAAGCTCAATATCTAC
Beta-mutant-4  GGGACCAGAGCTCGAATATCACCCCTGAGCAGG-AAGGGTATTGTCAAGCTCAATATCTAC
Beta-mutant-5  GGGACCAGAGCTCGAATATCACCCCTGAGCAGG-AAGGGTATTGTCAAGCTCAATATCTAC
Beta-mutant-9  GGGACCAGAGCTCGAATATCACCCCTGAGCAGG-AAGGGTATTGTCAAGCTCAATATCTAC
                *****

Beta-ENaC      TTCCAAGAGTTCAACTACCGTACCATTGAGGAATCTCCAGCCAACAAT-ATTGTGT-GGC
Beta-mutant-1  TTCCAAGAGTTCAACTACCGTACCATTGAGGATCTCCAGCCAACAAT-ATTGTGT-GGC
Beta-mutant-4  TTCCAAGAGTTCAACTACCGTACCATTGAGGAATCTCCAGCCAACAAT-ATTGTGT-GGC
Beta-mutant-5  TTCCAAGAGTTCAACTACCGTACCATTGA-GAATCTCCAACAACAAT-ATTGTGTGGGC
Beta-mutant-9  TTCCAAGAGTTCAACTACCGTACCATTGAGGAATCTCCAGCCAACAAT-ATTGTGT-GGC
                *****

Beta-ENaC      TGCTCTCTAACC-TGGGGGGCCAGTTTGGCTTCT-GGATGGGGGGCTCGG-TGCTGTGCC
Beta-mutant-1  TGCTCTCTAACC-TGGGGGGCCAGTTTGGCTTCTGGATGGGGGGCTCGG-TGCTGTGCC
Beta-mutant-4  TGCTCTCTAACC-TGGGGGGCCAGTTTGGCTTCTCT-GGATGGGGGGCTCGG-TGCTGTGCC
Beta-mutant-5  TGCTCTCTAACC-TGGGGGGCCAGTTTGGCTTCTGGATGGGGGGCTCGG-TGCTGTGCC
Beta-mutant-9  TGCTCTCTAACC-TGGGGGGCCAGTTTGGCTTCT-GGATGGGGGGCTCGG-TGCTGTGCC
                *****

Beta-ENaC      TCATTGAGTTTGGGGAGATCATTTATCGACTTCATTTGGATCACCATCATCAAGCTGGTGG
Beta-mutant-1  TCATTGAGTTTGGGGAGA-----
Beta-mutant-4  TCATTGAGTTTGGGGAGA-----
Beta-mutant-5  TCATTGAATTTTGGG-----
Beta-mutant-9  TCATTGAGTTTGGGGAGAT-----
                *****

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Figure 25: DNA alignment of beta mutant plasmids when sequenced with beta internal primer.

Beta ENaC mutants were aligned against wild type *Mus musculus* beta ENaC mRNA (Accession number: NM_011325.2). The highlighted nucleotides show the different points of mutation. Green- Beta mutant 1, Turquoise- Beta mutant 4, Yellow- Beta mutant 5, Pink- Beta mutant 9. The asterisk (*) beneath the alignment shows conserved DNA bases. The dash (-) is a gap.

```

Beta-ENaC      DRIILACLFGTGPCSHRNFTPIFYPDYGNCYIFNWGMTTEETLPSANPGTEFGLKLILDIG
Beta-mutant-1  DRIILACLFGTGPCSHRNFTPIFYPDYGNCYIFNWGMTTEETLPSANPGTEFGLKLILDIG
Beta-mutant-4  DRIILACLFGTGPCSHRNFTPIFYPDYGNCYIFNWGMTTEETLPSANPGTEFGLKLILDIG
Beta-mutant-5  DRIILACLFGTGPCSHRNFTPIFYPDYGNCYIFNWGMTTEETLPSANPGTEFGLKLILDIG
Beta-mutant-9  -----MTEETLPSANPGTEFGLKLILDIG
                *****

Beta-ENaC      QEDYVPFLASTAGARLMLHEQRTYPFIREEGIYAMAGTETSIGVLVDKLQRKGEPYSPCT
Beta-mutant-1  QEDYVPFLASTAGARLMLHEQRTYPFIREEGIYAMAGTETSIGVLVDKLQRKGEPYSPCT
Beta-mutant-4  QEDYVPFLASTAGARLMLHEQRTYPFIREEGIYAMAGTETSIGVLVDKLQRKGEPYSPCT
Beta-mutant-5  QEDYVPFLASTAGARLMLHEQRTYPFIREEGIYAMAGTETSIGVLVDKLQRKGEPYSPCT
Beta-mutant-9  QEDYVPFLASTAGARLMLHEQRTYPFIREEGIYAMAGTETSIGVLVDKLQRKGEPYSPCT
                *****

Beta-ENaC      MNGSDVAIKNLYSVYNTTYSIQACLHSCFQDHMIRNCSCGHYLYPLPEGEKYCNNRDFPD
Beta-mutant-1  MNGSDVAIKNLYSVYNTTYSIQACLHSCFQDHMIRNCSCGHYLYPLPEGEKYCNNRDFPD
Beta-mutant-4  MNGSDVAIKNLYSVYNTTYSIQACLHSCFQDHMIRNCSCGHYLYPLPEGEKYCNNRDFPD
Beta-mutant-5  MNGSDVAIKNLYSVYNTTYSIQACLHSCFQDHMIRNCSCGHYLYPLPEGEKYCNNRDFPD
Beta-mutant-9  MNGSDVAIKNLYSVYNTTYSIQACLHSCFQDHMIRNCSCGHYLYPLPEGEKYCNNRDFPD
                *****

Beta-ENaC      WAYCYLNLQMSVTQRETCLSMCKESCNDTQYKMTISMADWPSEASEDWILHVLSQERDQS
Beta-mutant-1  WAYCYLNLQMSVTQRETCLSMCKESCNDTQYKMTISMADWPSEASEDWILHVLSQSGTRA
Beta-mutant-4  WAYCYLNLQMSVTQRETCLSMCKESCNDTQYKMTISMADWPSEASEDWILHVLSQSGDQS
Beta-mutant-5  WAYCYLNLQMSVTQRETCLSMCKGVLQ-----
Beta-mutant-9  WAYCYLNLQMSVTQRETCLSMCKESCNDTQYKMTISMADWPSEASEDWILHVLSQERDQS
                *****      :

Beta-ENaC      SNITLSRKGIVKLNIFYQEFTTVPLRNLQPTILCGCSLTWGLASGLSGWARGCCASLSLGR
Beta-mutant-1  RISP-----
Beta-mutant-4  SNITLSRKGVCQAQYLLPRV-QLPYH-----
Beta-mutant-5  -----
Beta-mutant-9  SNITLSREGIVKLNIFYQEFNYRTIEESPAN----NIVWLLSNLGG-----

```

Figure 26: Protein alignment of beta mutant plasmids when sequenced with beta internal primer.

Beta ENaC mutants were aligned against wild type *Mus musculus* beta ENaC amino acid (Accession number: NP_035455.1). The asterisk (*) beneath the alignment shows conserved amino acid residues. The Colon (:) displays conserved amino acid residues with strong chemical properties. The dash (-) is a gap.

The DNA sequencing data for beta mutant 1 when sequenced with beta mutant sequencing primer were aligned separately against the wild type *Mus Musculus* beta ENaC mRNA because the data obtained were short as compared to the other mutants sequenced with the same primer.

```

Beta-ENaC      AAGATTCTGGCTCCAGAGGCCAGCCACAGCAACACCACCAGGACCCTGAACTTTACCATC
Beta-mutant-1  -----CAGCCACAGC-ACACCACCAGGACCCTGAACTTTACCATC
                *****

Beta-ENaC      TGGAACCACACACCCTGGTCCTTATTGATGAGCGGAACCCTGACCACCCGGTGGTTCTC
Beta-mutant-1  TGGAACCACACACCCTGGTCCTTATTGATGAGCGGAACCCTGACCACCCGGTGGTTCTC
                *****

Beta-ENaC      AATTGTTTGGGGACAGCCACAACAGCAGCAACCCGGCTCCAGGAAGCACCTGCAATGCC
Beta-mutant-1  AATTGTTTGGGGACAGCCACAACAGCAGCAACCCGGCTCCAGGAAGCACCTGCAATGCC
                *****

Beta-ENaC      CAGGGATGCAAAGTGGCCATGAGGCTGTGCAGTGCCAATGGAAGTGTGTGTACCTTGCGG
Beta-mutant-1  CAGGGATGCAAAGTGGCCATGAGGCTGTGCAGTGCCAATGGAAGTGTGTGTACCTTGCGG
                *****

```


Beta-ENaC	AACTTCACCAGTGCCACCCAGGCCGTAAGTGGTACATCCTGCAGGCCACCAACATC
Beta-mutant-1	AACTTCACCAGTGCCACCCAGGCCGTAAGTGGTACATCCTGAGGCCACCAACATC *****
Beta-ENaC	TTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGCTATGCCCTGATCGCATAATC
Beta-mutant-1	TTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGCTATGCCCTGATCGCATAATC *****
Beta-ENaC	CTAGCTTGCCTGTTTGAACAGAGCCCTGCAGTCATCGGAACCTCAGCCTATCTTCTAC
Beta-mutant-1	CTAGCTTGCCTGTTTGAACAGAGCCCTGCAGTCATCGGAACCTCAGCCTATCTTCTAC **** *
Beta-ENaC	CCTGATTATGGCAACTGCTACATCTTCAACTGGGGCATGACAGAGGAGACACTTCCTTCT
Beta-mutant-1	CCTGATTATGGCAACTGCTACATCTTCAACTGGGGCATGACAGAGGAGACACTTCCTTCT *****
Beta-ENaC	GCCAACCCCTGGGACTGAATTTGGTCTCAAGTTGATCCTGGACATCGGTGAGGAGACTAT
Beta-mutant-1	GCCAACCCCTGGGACTGAATTTGGTCTCAAGTTGATCCTGGACATCGGTGAGGAGACTAT *****
Beta-ENaC	GTCCCTTCCTTGCCTCCACAGCAGGGGCTAGGCTGATGCTTCATGAGCAGAGGACGTAC
Beta-mutant-1	GTCCCTTCCTTGCCTCCACAGCAGGGGCTAGGCTGATGCTTCATGAGCAGAGGACGTAC *****
Beta-ENaC	CCCTTCATTAGAGAGGAGGGCATCTATGCCATGGCAGGAAGTGAACGCTCTATTGGGGTG
Beta-mutant-1	CCCTTCATTAGAGAGGAGGGCATCTATGCCATGGCAGGAAGTGAACGCTCTATTGGGGTG *****
Beta-ENaC	CTGGTGGACAAGC-TGCAACGCAAGGGGGAG-CCCTACAGTCCCTGCACCATGAACGGCT
Beta-mutant-1	CTGGTGGACAAGC-TGCAACGCAAGGGGGAG-CCCTACAGTCCCTGCACCATGAACGGCT **
Beta-ENaC	CCGATGTTGCCATAAAGAACCCTCTACAGTGTCTACAACACCACCTATTCCATCCAGGCCCT
Beta-mutant-1	CCGATGTTGCCATAAAGAACCCTCTACAGTGTCTACAACACCACCTATTCCATCCAGGCCCT *****
Beta-ENaC	GTCTTCATTCTGTGTTCCAAGACCACATGATCCGTAAGTGCAGTTGTGGCCACTACTTAT
Beta-mutant-1	GTCTTCATTCTGTGTTCCAAGACCACATGATCCGTAAGTGCAGTTGTGGCCACTACTTAT ***
Beta-ENaC	AAGATTCTGGCTCCAGAGGCCAGCCACAGCAACACCACCAGGACCCCTGAACCTTTACCATC
Beta-mutant-4	-----GTAGTCAGCCACAGCAGCACCACCAGGACCCCTGAACCTTTACCATC
Beta-mutant-5	-----GTAG-CAGTCACAGCA-CACCACCAGGACCCCTGAACCTTTACCATC
Beta-mutant-9	-----GTAG-CAGCCACAGCA-CACCACCAGGACCCCTGAACCTTTACCATC * * *
Beta-ENaC	TGGAACCACACACCCTGGTCCTTATTGATGAGCGGAACCCCTGACCACCCGGTGGTTCTC
Beta-mutant-4	TGGAACCACACACCCTGGTCCTTATTGATGAGCGGAACCCCTGACCACCCGGTGGTTCTC
Beta-mutant-5	TGGAACCACACACCCTGGTCCTTATTGATGAGCGGAACCCCTGACCACCCGGTGGTTCTC
Beta-mutant-9	TGGAACCACACACCCTGGTCCTTATTGATGAGCGGAACCCCTGACCACCCGGTGGTTCTC *****
Beta-ENaC	AATTGTTTGGGGACAGCCACAACAGCAGCAACCCGGCTCCAGGAAGCACCTGCAATGCC
Beta-mutant-4	AATTGTTTGGGGACAGCCACAACAGCAGCAACCCGGCTCCAGGAAGCACCTGCAATGCC
Beta-mutant-5	AATTGTTTGGGGACAGCCACAACAGCAGCAACCCGGCTCCAGGAAGCACCTGCAATGCC
Beta-mutant-9	AATTGTTTGGGGACAGCCACAACAGCAGCAACCCGGCTCCAGGAAGCACCTGCAATGCC *****
Beta-ENaC	CAGGGATGCAAAGTGGCCATGAGGCTGTGCAGTGCCAATGGAACGTGTGTACCTTGCGG
Beta-mutant-4	CAGGGATGCAAAGTGGCCATGAGGCTGTGCAGTGCCAATGGAACGTGTGTACCTTGCGG
Beta-mutant-5	CAGGGATGCAAAGTGGCCATGAGGCTGTGCAGTGCCAATGGAACGTGTGTACCTTGCGG
Beta-mutant-9	CAGGGATGCAAAGTGGCCATGAGGCTGTGCAGTGCCAATGGAACGTGTGTACCTTGCGG *****
Beta-ENaC	AACTTCACCAGTGCCACCCAGGCCGTAAGTGGTACATCCTGCAGGCCACCAACATC
Beta-mutant-4	AACTTCACCAGTGCCACCCAGGCCGTAAGTGGTACATCCTGCAGGCCACCAACATC
Beta-mutant-5	AACTTCACCAGTGCCACCCAGGCCGTAAGTGGTACATCCTGCAGGCCACCAACATC
Beta-mutant-9	AACTTCACCAGTGCCACCCAGGCCGTAAGTGGTACATCCTGCAGGCCACCAACATC *****

Beta-ENaC	TTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGCTATGCCCTGATCGCATAATC
Beta-mutant-4	TTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGCTATGCCCTGATCGCATAATC
Beta-mutant-5	TTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGCTATGCCCTGATCGCATAATC
Beta-mutant-9	TTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGCTATGCCCTGATCGCATAATC

Beta-ENaC	CTAGCTTGCCCTGTTTGGAAACAGAGCCCTGCAGTCATCGGAACCTCACGCCTATCTTCTAC
Beta-mutant-4	CTAGCTTGCCCTGTTTGGAAACAGAGCCCTGCAGTCATCGGAACCTCACGCCTATCTTCTAC
Beta-mutant-5	CTAGCTTGCCCTGTTTGGAAACAGAGCCCTGCAGTCATCGGAACCTCACGCCTATCTTCTAC
Beta-mutant-9	CTAGCTTGCCCTGTTTGGAAACAGAGCCCTGCAGTCATCGGAACCTCACGCCTATCTTCTAC

Beta-ENaC	CCTGATTATGGCAACTGCTACATCTTCAACTGGGGCATGACAGAGGAGACACTTCCTTCT
Beta-mutant-4	CCTGATTATGGCAACTGCTACATCTTCAACTGGGGCATGACAGAGGAGACACTTCCTTCT
Beta-mutant-5	CCTGATTATGGCAACTGCTACATCTTCAACTGGGGCATGACAGAGGAGACACTTCCTTCT
Beta-mutant-9	CCTGATTATGGCAACTGCTACATCTTCAACTGGGGCATGACAGAGGAGACACTTCCTTCT

Beta-ENaC	GCCAACCCTGGGACTGAATTTGGTCTCAAGTTGATCCTGGACATCGGTGAGGAGGACTAT
Beta-mutant-4	GCCAACCCTGGGACTGAATTTGGTCTCAAGTTGATCCTGGACATCGGTGAGGAGGACTAT
Beta-mutant-5	GCCAACCCTGGGACTGAATTTGGTCTCAAGTTGATCCTGGACATCGGTGAGGAGGACTAT
Beta-mutant-9	GCCAACCCTGGGACTGAATTTGGTCTCAAGTTGATCCTGGACATCGGTGAGGAGGACTAT

Beta-ENaC	GTCCCTTCCTTGCGTCCACAGCAGGGGCTAGGCTGATGCTTCATGAGCAGAGGACGTAC
Beta-mutant-4	GTCCCTTCCTTGCGTCCACAGCAGGGGCTAGGCTGATGCTTCATGAGCAGAGGACGTAC
Beta-mutant-5	GTCCCTTCCTTGCGTCCACAGCAGGGGCTAGGCTGATGCTTCATGAGCAGAGGACGTAC
Beta-mutant-9	GTCCCTTCCTTGCGTCCACAGCAGGGGCTAGGCTGATGCTTCATGAGCAGAGGACGTAC

Beta-ENaC	CCCTTCATTAGAGAGGAGGGCATCTATGCCATGGCAGGAAGTGAACGCTCTATTGGGGTG
Beta-mutant-4	CCCTTCATTAGAGAGGAGGGCATCTATGCCATGGCAGGAAGTGAACGCTCTATTGGGGTG
Beta-mutant-5	CCCTTCATTAGAGAGGAGGGCATCTATGCCATGGCAGGAAGTGAACGCTCTATTGGGGTG
Beta-mutant-9	CCCTTCATTAGAGAGGAGGGCATCTATGCCATGGCAGGAAGTGAACGCTCTATTGGGGTG

Beta-ENaC	CTGGTGGACAAGCTGCAACGCAAGGGGGAGCCCTACAGTCCCTGCACCATGAACGGCTCC
Beta-mutant-4	CTGGTGGACAAGCTGCAACGCAAGGGGGAGCCCTACAGTCCCTGCACCATGAACGGCTCC
Beta-mutant-5	CTGGTGGACAAGCTGCAACGCAAGGGGGAGCCCTACAGTCCCTGCACCATGAACGGCTCC
Beta-mutant-9	CTGGTGGACAAGCTGCAACGCAAGGGGGAGCCCTACAGTCCCTGCACCATGAACGGCTCC

Beta-ENaC	GATGTTGCCATAAAGAACCCTCTACAGTGTCTACAACACCACCTATTCCATCCAGGCCTGT
Beta-mutant-4	GATGTTGCCATAAAGAACCCTCTACAGTGTCTACAACACCACCTATTCCATCCAGGCCTGT
Beta-mutant-5	GATGTTGCCATAAAGAACCCTCTACAGTGTCTACAACACCACCTATTCCATCCAGGCCTGT
Beta-mutant-9	GATGTTGCCATAAAGAACCCTCTACAGTGTCTACAACACCACCTATTCCATCCAGGCCTGT

Beta-ENaC	CTTCATTCTGTTTCCAAGACCACATGATCCGTAAGTGCAGTTGTGGCCACTACTTATAC
Beta-mutant-4	CTTCATTCTGTTTCCAAGACCACATGATCCGTAAGTGCAGTTGTGGCCACTACTTATAC
Beta-mutant-5	CTTCATTCTGTTTCCAAGACCACATGATCCGTAAGTGCAGTTGTGGCCACTACTTATAC
Beta-mutant-	CTTCATTCTGTTTCCAAGACCACATGATCCGTAAGTGCAGTTGTGGCCACTACTTATAC

Beta-ENaC	CCACTGCCTG-AAGGAGAGAAATACTGCAACAACAGGGACTTCCCAGA-CTGGGCCTATT
Beta-mutant-4	CCACTGCCTG-AAGGAGAGAAATACTGCAACAACAGGGACTTCCCAGA-CTGGGCCTATT
Beta-mutant-5	CCACTGCCTG-AAGGAGAGAAATACTGCAACAACAGGGACTTCCCAGA-CTGGGCCTATT
Beta-mutant-9	CCACTGCCTG-AAAGAGAGAAATACTGCAACAACAGGGACTTCCCAGA-CTGGGCCTATT

Beta-ENaC	GCTATCT-AAACCTGCAGATGAGTGTGACCC-AGAGAGAGACCTGCCTC--AGCATGTGC
Beta-mutant-4	GCTATCT-AAACCTGCAGATGAGTGTGACCC-AGAGAGAGACCTGCCTC--AGCATGTGC
Beta-mutant-5	GCTATCT-AAACCTGCAGATGAGTGTGACCC-AAAGAGAGAGACCTGCCTC--AGCATGTGC
Beta-mutant-9	GCTATCT-AAACCTGCAGATGAGTGTGACCC-AGAGAGAGACCTGCCTC--AGCATGTGC

```

Beta-ENaC      AAGGAGT-CCTGCAATG-AC-ACCCAGTATAAGATGACCATCTCCATGGCTGACTGGCCA
Beta-mutant-4  AAGGAGTCCCTGCAATGAACAACCCATATAAAGATGACCATCTCCAT-----
Beta-mutant-5  AAGGAGTTCTTGCAATG-ACAACCCA-----
Beta-mutant-9  AAGGAGT-CCTGCAATG-AC-ACCCAGTATAAGATGACCATCTCCATGGCTG-----
***** * ***** ** *****

```

Figure 27: DNA alignment of beta mutant plasmids when sequenced with beta mutant sequencing primer. Beta ENaC mutants were aligned against wild type *Mus musculus* beta ENaC mRNA (Accession number: NM_011325.2). The highlighted nucleotides show the different points of mutation. Green- Beta mutant 1, Turquoise- Beta mutant 4, Yellow- Beta mutant 5, Pink- Beta mutant 9. The asterisk (*) beneath the alignment shows conserved DNA bases. The dash (-) is a gap.

```

Beta-ENaC      AVLEKILAPEASHSNTTRTLNFTIWNHTPLVLIDERNPDHPVVLNLFGDHNSNPAPGS
Beta-Mutant-1  -----QPQHTTRTLNFTIWNHTPLVLIDERNPDHPVVLNLFGDHNSNPAPGS
               . . .*****

Beta-ENaC      TCNAQGCKVAMRLCSANGTVCTLRNFTSATQAVTEWYILQATNIFSQVLPQDLVGMGYAP
Beta-Mutant-1  TCNAQGCKVAMRLCSANGTVCTLRNFTSATQAVTEWYILKATNIFSQVLPQDLVGMGYAP
               *****:*****

Beta-ENaC      DRIILACLFGTPEPCSHRNFTPIFYPDYGNCYIFNWGMTEETLPSANPGTEFGLKLILDIG
Beta-Mutant-1  DRIILDYLFGTDPCHRNFTPIF-----
               *****:*****

Beta-ENaC      TCNAQGCKVAMRLCSANGTVCTLRNFTSATQAVTEWYILQATNIFSQVLPQDLVGMGYAP
Beta-mutant-4  -----MRLCSANGTVCTLRNFTSATQAVTEWYILQATNIFSQVLPQDLVGMGYAP
Beta-mutant-5  -----MRLCSANGTVCTLRNFTSATQAVTEWYILQATNIFSQVLPQDLVGMGYAP
Beta-mutant-9  -----

Beta-ENaC      DRIILACLFGTPEPCSHRNFTPIFYPDYGNCYIFNWGMTEETLPSANPGTEFGLKLILDIG
Beta-mutant-4  DRIILACLFGTPEPCSHRNFTPIFYPDYGNCYIFNWGMTEETLPSANPGTEFGLKLILDIG
Beta-mutant-5  DRIILACLFGTPEPCSHRNFTPIFYPDYGNCYIFNWGMTEETLPSANPGTEFGLKLILDIG
Beta-mutant-9  -----MTEETLPSANPGTEFGLKLILDIG
               *****

Beta-ENaC      QEDYVPFLASTAGARLMLHEQRTYPFIREEGIYAMAGTETSIGVLVDKLQRKGEPYSPCT
Beta-mutant-4  QEDYVPFLASTAGARLMLHEQRTYPFIREEGIYAMAGTETSIGVLVDKLQRKGEPYSPCT
Beta-mutant-5  QEDYVPFLASTAGARLMLHEQRTYPFIREEGIYAMAGTETSIGVLVDKLQRKGEPYSPCT
Beta-mutant-9  QEDYVPFLASTAGARLMLHEQRTYPFIREEGIYAMAGTETSIGVLVDKLQRKGEPYSPCT
               *****

Beta-ENaC      MNGSDVAIKNLYSVYNTTYSIQACLHSCFQDHMIRNCSCGHYLYPLPEGEKYCNRDFPD
Beta-mutant-4  MNGSDVAIKNLYSVYNTTYSIQACLHSCFQDHMIRNCSCGHYLYPLPEGEKYCNRDFPE
Beta-mutant-5  MNGSDVAIKNLYSVYNTTYSIQACLHSCFQDHMIRNCSCGHYLYPLPERREILQQQGLPR
Beta-mutant-9  MNGSDVAIKNLYSVYNTTYSIQACLHSCFQDHMIRNCSCGHYLYPLPERREKYCNRDFPR
               *****.::::*

Beta-ENaC      WAYCYLNLQMSVTQRETCLSMCKESCNDTQYKMTISMADWPSEASEDWILHVLSQERDQS
Beta-mutant-4  LGLLLSKT-----CR-----
Beta-mutant-5  LGPIAI-----
Beta-mutant-9  LGLLLSKP-----CR-----

```

Figure 28: Protein alignment of beta mutant plasmids when sequenced with beta mutant sequencing primer. Beta ENaC mutants were aligned against wild type *Mus musculus* beta ENaC amino acid (Accession number: NP_035455.1). The asterisk (*) beneath the alignment shows conserved amino acid residues. The Colon (:) displays conserved amino acid residues with strong chemical properties. The dash (-) is a gap.

Beta-ENaC	AAGTACCTCCTGAAGTGCCTGCACCGGCTGCAGAAGGGCCCAGGCTACACCTACAAGGAG
Beta-mutant-1	-----CTACAAGGAG
Beta-mutant-4	AAGTACCT-CTGAAGTG-CTGCACCGGCTGCAGAAGGGCACAGGCTACACCTACAAGGAG
Beta-mutant-5	-----TGCACGGGTTACACTTTCATGGAAG
Beta-mutant-9	AAGTACCTCCTGAAGTGCCTGCACCGGCTGCAGAAGGGCCCAGGCTACACCTACAAGGAG
	* * *
Beta-ENaC	CTGCTAGTGTGGTACTGCAATAACACCAACACCCACGGCCCCAAACGCATCATCTGTGAG
Beta-mutant-1	CTGCTAGTGTGGTACTGCAATAACACCAACACCCACGGCCCCAAACGCATCATCTGTGAG
Beta-mutant-4	CTGCTAGTGTGGTACTGCAATAACACCAACACCCACGGCCCCAAACGCATCATCTGTGAG
Beta-mutant-5	CTGCTAGTGTGGTAAAGCAAT-ATACCAACACGCTCGGCTTCAATGCATCATCTGTGAG
Beta-mutant-9	CTGCTAGTGTGGTACTGCAATAACACCAACACCCACGGCCCCAAACGCATCATCTGTGAG
	***** * * * * *
Beta-ENaC	GGGCCCCAAGAAGAAGGCCATGTGGTTCCTGCTTACGCTGCTCTTCGCCTGCCTGGTGTGC
Beta-mutant-1	GGGCCCCAAGAAGAAGGCCATGTGGTTCCTGCTTACGCTGCTCTTCGCCTGCCTGGTGTGC
Beta-mutant-4	GGGCCCCAAGAAGAAGGCCATGTGGTTCCTGCTTACGCTGCTCTTCGCCTGCCTGGTGTGC
Beta-mutant-5	GGGCCTAAGAAGAAGGCCAAGTTTTCCTGATTACGCTGCTCTTCGCCTGCCTGGTGTGT
Beta-mutant-9	GGGCCCCAAGAAGAAGGCCATGTGGTTCCTGCTTACGCTGCTCTTCGCCTGCCTGGTGTGC
	***** * * * * *
Beta-ENaC	TGGCAGTGGGGAGTCTTTCATCCAGACCTACCTGAGCTGGGAGGTCAGCGTCTCGCTCTCC
Beta-mutant-1	TGGCAGTGGGGAGTCTTTCATCCAGACCTACCTGAGCTGGGAGGTCAGCGTCTCGCTCTCC
Beta-mutant-4	TGGCAGTGGGGAGTCTTTCATCCAGACCTACCTGAGCTGGGAGGTCAGCGTCTCGCTCTCC
Beta-mutant-5	CGGCAGTGGGGAGTCTTCTTCCAGACTTACCTGAGCTGGGAGGTCAGTGTCTCGCTCTCC
Beta-mutant-9	TGGCAGTGGGGAGTCTTTCATCCAGACCTACCTGAGCTGGGAGGTCAGCGTCTCGCTCTCC
	***** * * * * *
Beta-ENaC	ATGGGCTTCAAGACGATGAACTTCCCGGCGGTACCGTCTGCAATTCCAGCCCCCTTCCAG
Beta-mutant-1	ATGGGCTTCAAGACGATGAACTTCCCGGCGGTACCGTCTGCAATTCCAGCCCCCTTCCAG
Beta-mutant-4	ATGGGCTTCAAGACGATGAACTTCCCGGCGGTACCGTCTGCAATTCCAGCCCCCTTCCAG
Beta-mutant-5	ATGGGCTTCAAGACGATGAACTTCCCGGCGGTACCGTCTGCAATTCCAGCCCCCTTCCAG
Beta-mutant-9	ATGGGCTTCAAGACGATGAACTTCCCGGCGGTACCGTCTGCAATTCCAGCCCCCTTCCAG

Beta-ENaC	TACTCCAAGGTCAAGCACTTGCTCAAGGACTTGGATGAGCTAATGGAGGCAGTCTGGAA
Beta-mutant-1	TACTCCAAGGTCAAGCACTTGCTCAAGGACTTGGATGAGCTAATGGAGGCAGTCTGGAA
Beta-mutant-4	TACTCCAAGGTCAAGCACTTGCTCAAGGACTTGGATGAGCTAATGGAGGCAGTCTGGAA
Beta-mutant-5	TACTCCAAGGTCAAGCACTTGCTCAAGGACTTGGATGAGCTAATGGAGGCAGTCTGGAA
Beta-mutant-9	TACTCCAAGGTCAAGCACTTGCTCAAGGACTTGGATGAGCTAATGGAGGCAGTCTGGAA

Beta-ENaC	AAGATTCTGGCTCCAGAGGCCAGCCACAGCAACACCACCAGGACCCTGAACTTTACCATC
Beta-mutant-1	AAGATTCTGGCTCCAGAGGCCAGCCACAGCAACACCACCAGGACCCTGAACTTTACCATC
Beta-mutant-4	AAGATTCTGGCTCCAGAGGCCAGCCACAGCAACACCACCAGGACCCTGAACTTTACCATC
Beta-mutant-5	AAGATTCTGGCTCCAGAGGCCAGCCACAGCAACACCACCAGGACCCTGAACTTTACCATC
Beta-mutant-9	AAGATTCTGGCTCCAGAGGCCAGCCACAGCAACACCACCAGGACCCTGAACTTTACCATC

Beta-ENaC	TGGAACCACACACCCTGGTCCTTATTGATGAGCGGAACCCTGACCACCCGGTGGTTCTC
Beta-mutant-1	TGGAACCACACACCCTGGTCCTTATTGATGAGCGGAACCCTGACCACCCGGTGGTTCTC
Beta-mutant-4	TGGAACCACACACCCTGGTCCTTATTGATGAGCGGAACCCTGACCACCCGGTGGTTCTC
Beta-mutant-5	TGGAACCACACACCCTGGTCCTTATTGATGAGCGGAACCCTGACCACCCGGTGGTTCTC
Beta-mutant-9	TGGAACCACACACCCTGGTCCTTATTGATGAGCGGAACCCTGACCACCCGGTGGTTCTC

Beta-ENaC	AATTGTGTTGGGGACAGCCACAACAGCAGCAACCCGGCTCCAGGAAGCACCTGCAATGCC
Beta-mutant-1	AATTGTGTTGGGGACAGCCACAACAGCAGCAACCCGGCTCCAGGAAGCACCTGCAATGCC
Beta-mutant-4	AATTGTGTTGGGGACAGCCACAACAGCAGCAACCCGGCTCCAGGAAGCACCTGCAATGCC
Beta-mutant-5	AATTGTGTTGGGGACAGCCACAACAGCAGCAACCCGGCTCCAGGAAGCACCTGCAATGCC
Beta-mutant-9	AATTGTGTTGGGGACAGTCAACAACAGCAGCAACCCGGCTCCAGGAAGCACCTGCAATGCC

Beta-ENaC	CAGGGATGCAAAGTGGCCATGAGGCTGTGCAGTGCCAATGGAAGTGTGTGTACCTTGCGG
Beta-mutant-1	CAGGGATGCAAAGTGGCCATGAGGCTGTGCAGTGCCAATGGAAGTGTGTGTACCTTGCGG
Beta-mutant-4	CAGGGATGCAAAGTGGCCATGAGGCTGTGCAGTGCCAATGGAAGTGTGTGTACCTTGCGG
Beta-mutant-5	CAGGGATGCAAAGTGGCCATGAGGCTGTGCAGTGCCAATGGAAGTGTGTGTACCTTGCGG
Beta-mutant-9	CAGGGATGCAAAGTGGCCATGAGGCTGTGCAGTGCCAATGGAAGTGTGTGTACCTTGCGG

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Beta-ENaC      AACTTCACCAGTGCCACCCAGGCCGTAAGTGAAGTGGTACATCCTGCAGGCCACCAACATC
Beta-mutant-1  AACTTCACCAGTGCCACCCAGGCCGTAAGTGAAGTGGTACATCCTGCAGGCCACCAACATC
Beta-mutant-4  AACTTCACCAGTGCCACCCAGGCCGTAAGTGAAGTGGTACATCCTGCAGGCCACCAACATC
Beta-mutant-5  AACTTCACCAGTGCCACCCAGGCCGTAAGTGAAGTGGTACATCCTGCAGGCCACCAACATC
Beta-mutant-9  AACTTCACCAGTGCCACCCAGGCCGTAAGTGAAGTGGTACATCCTGCAGGCCACCAACATC
                *****

Beta-ENaC      TTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGCTATGCCCCTGATCGCATAATC
Beta-mutant-1  TTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGCTATGCCCCTGATCGCATAATC
Beta-mutant-4  TTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGCTATGCCCCTGATCGCATAATC
Beta-mutant-5  TTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGCTATGCCCCTGATCGCATAATC
Beta-mutant-9  TTCTCACAAGTGCTTCCTCAGGACCTGGTGGGGATGGGCTATGCCCCTGATCGCATAATC
                *****

Beta-ENaC      CTAGCTTGCTGTGTTGGAACAGAGCCCTGCAGTCATCGGAACCTCACGCCTATCTTCTAC
Beta-mutant-1  CTAGCTTGCTGTGTTGGAACAGAGCCCTGCAGTCATCGGAACCTCACGCCTATCTTCTAC
Beta-mutant-4  CTAGCTTGCTGTGTTGGAACAGAGCCCTGCAGTCATCGGAACCTCACGCCTATCTTCTAC
Beta-mutant-5  CTAGCTTGCTGTGTTGGAACAGAGCCCTGCAGTCATCGGAACCTCACGCCTATCTTCTAC
Beta-mutant-9  CTAGCTTGCTGTGTTGGAACAGAGCCCTGCAGTCATCGGAACCTCACGCCTATCTTCTAC
                *****

Beta-ENaC      CCTGATTATGGCAACTGCTACATCTTCAACTGGGGCATGACAGAGGAGACACTTCCTTTCT
Beta-mutant-1  CCTGATTATGGCAACTGCTACATC-TCAACTGGG-----
Beta-mutant-4  CCTGATTATGGCAACTGCTACATC-TCAACTGGG-----
Beta-mutant-5  CCTGATTATGGCAACTGCTACATC-TCAACTGGG-----
Beta-mutant-9  CCTGATTAT-GCAACTGCTACATC-TCAACTGGG-----
                *****

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Figure 29: DNA alignment of beta mutant plasmids when sequenced with beta internal sequencing primer. Beta ENaC mutants were aligned against wild type *Mus musculus* beta ENaC mRNA (Accession number: NM_011325.2). The highlighted nucleotides show the different points of mutation. Green- Beta mutant 1, Turquoise- Beta mutant 4, Yellow- Beta mutant 5, Pink- Beta mutant 9. The asterisk (*) beneath the alignment shows conserved DNA bases. The dash (-) is a gap.

```

Beta-ENaC      -----MPVKKYLLKCLHRLQKPGYTYKELLVWYCINNNTNTHGPKRIICEGPK
Beta-mutant-1  -----
Beta-mutant-4  -----
Beta-mutant-5  -----MEASVVKQYTNLTGLTLCIICEGPK
Beta-mutant-9  MEQKLISEEDLELMPVKKYLLKCLHRLQKPGYTYKELLVWYCINNNTNTHGPKRIICEGPK

Beta-ENaC      KKAMWFLLTLLFACLVCWQWGVFIQTYLSWEVSVLSMGFKTMNFPVAVTVCNSSPFQYSK
Beta-mutant-1  ---MWFLLTLLFACLVCWQWGVFIQTYLSWEVSVLSMGFKTMNFPVAVTVCNSSPFQYSK
Beta-mutant-4  ---MWFLLTLLFACLVCWQWGVFIQTYLSWEVSVLSMGFKTMNFPVAVTVCNSSPFQYSK
Beta-mutant-5  KKAKFLITLLFACLVCRQWGVFQTYLSWEVSVLSMGFKTMNFPVAVTVCNSSPFQYSK
Beta-mutant-9  KKAMWFLLTLLFACLVCWQWGVFIQTYLSWEVSVLSMGFKTMNFPVAVTVCNSSPFQYSK
                :*:*****

Beta-ENaC      VKHLLKDLDELMEAVLEKILAPEASHSNTTRTLNTIWNHTPLVLIDERNPDHPVVLNLF
Beta-mutant-1  VHHLLKDLDELMEAVLEKILAPEASHSNTTRTLNTIWNHTPLVLIDERNPDHPVVLNLF
Beta-mutant-4  VRHLLKDLDELMEAVLEKILAPEASHSNTTRTLNTIWNHTPLVLIDERNPDHPVVLNLF
Beta-mutant-5  VRHLLKDLDELMEAVLEKILAPEASHSNTTRTLNTIWNHTPLVLIDERNPDHPVVLNLF
Beta-mutant-9  VHHLLKDLDELMEAVLEKILAPEASHSNTTRTLNTIWNHTPLVLIDERNPDHPVVLNLF
                *:*****

Beta-ENaC      GDSHNSSNPAPGSTCNAQGCKVAMRLCSANGTVCTLRNFTSATQAVTEWYILQATNIFSQ
Beta-mutant-1  GDSHNSSNPAPGSTCNAQGCKVAMRLCSANGTVCTLRNFTSATQAVTEWYILQATNIFSQ
Beta-mutant-4  GDSHNSSNPAPGSTCNAQGCKVAMRLCSANGTVCTLRNFTSATQAVTEWYILQATNIFSQ
Beta-mutant-5  GDSHNSSNPAPGSTCNAQGCKVAMRLCSANGTVCTLRNFTSATQAVTEWYILQATNIFSQ
Beta-mutant-9  GDSHNSSNPAPGSTCNAQGCKVAMRLCSANGTVCTLRNFTSATQAVTEWYILQATNIFSQ
                *****

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Beta-ENaC      VLPQDLVGMGYAPDRIILACLFGEPCSHRNFTPIFYPDYGNCYIFNWMTEETLPSANP
Beta-mutant-1  VLPQDLVGMGYAPDRIILACLFGEPCSHRNFTPIFYPDYGNCYISTGHTERN-----
Beta-mutant-4  VLPQDLVGMGYAPDRIILACLFGEPCSHRNFTPIFYPDYGNCYISTGHTERI-----
Beta-mutant-5  VLPQDLVGMGYAPDRIILACLFGEPCSHRNFTPIFYPDYGNCYISTGHRDETP-----
Beta-mutant-9  VLPQDLVGMGYAPDRIILACLFGEPCSHRNFTPIFYPDYATATSQLGIQRER-----
                *****...

```

Figure 30: Protein alignment of beta mutant plasmids when sequenced with beta internal sequencing primer. Beta ENaC mutants were aligned against wild type *Mus musculus* beta amino acid (Accession number: NP_035455.1). The asterisk (*) beneath the alignment shows conserved amino acid residues. The Colon (:) displays conserved amino acid residues with strong chemical properties. The period (.) displays conserved amino acid residues with weak chemical properties. The dash (-) is a gap.

Table 5: Summary of Nucleotide Changes, Amino acid Transition and Types of Mutations

Beta Mutant	Nucleotide Change	Protein Change	Type of Mutation	Growth Inhibition as Compared to the Control
1	A434G, C766A, G801T, C845A, G848A, G864C, G888C, C900A, G971C, T984G, T986G, A988G, A989G, G993C, G1000A, G1035A, A1041G, G1052T, G1077A, C1080A, C1086A, T1088A, G1091A, G1096A, A1111G, T1112A, G1113A, G1125A, G1128A, G1136A, G1143A, C1156T, G1160A, G1164T	K109R, Q220K, Q231H, A246D, C247Y, E252D, Y264stop	Missense mutations, Silent mutation, Nonsense mutation and a truncated Protein	Slight Inhibition
4	A434G, G1534A, A1535G, G1536C, C1537G,	K109R, D420E, E476S, R477G	Missense mutations, Silent mutations	Slight Inhibition
5	C271A, C300T, T301C, A319T, C327T, C348T, A434G,	L55I, W65R, I71F, K109R, D448Stop	Missense mutations, Silent mutations, 1 Nonsense mutation and a truncated Protein	Slight Inhibition
9	A434G, C618T, G910(-), G1333A, C1362T	K109R, G268A, G409R, K488E	Missense mutations, Silent mutation	No Inhibition

Beta mutants 1 and 5 both contained a nonsense mutation that resulted in the generation of a premature stop codon hence causing the protein to be shortened though this doesn't correlate with the western blot. These mutants also contained silent and

missense mutations. Studies have shown that a truncation in α , β and γ -subunits of ENaC have been associated with Liddle's syndrome and PHA1 (16, 34) though these truncations are not found in the extracellular loop.

Beta mutants 4 and 9 contained both silent and missense mutations. This finding corresponds to the results from the western blot and yeast pronging assay where beta mutants 4 and 9 exhibited a slight (Fig 13D, row 5 and fig 17) and a no growth inhibition (Fig 14D, row 5 and fig 17), respectively. In all the mutants, a K109R mutation was seen. Thus, this does not seem to be a mutation but a variant form of beta-ENaC from the reported sequence (PubMed).

Beta mutant 4 had a mutation at position 420 where an aspartate was replaced with a glutamate (D420E). Aspartate (D) and glutamate (E) share chemical similarities and so the D420E mutation is less likely to affect the function of ENaC. Beta mutant 4 also possessed an E476S and a R477G mutation found in the knuckle domain of ENaC. Glutamate (E) and arginine (R) are negatively and positively charged amino acids, respectively, that participate in electrostatic interaction as well as hydrogen bond interactions. Serine (S) participates only in a hydrogen bond interaction while glycine does not participate in either electrostatic interaction or hydrogen bond formation. These mutation changes have a greater potential of altering the structure/function of ENaC. Further studies will need to be done to ascertain if these mutations are critical to ENaC function.

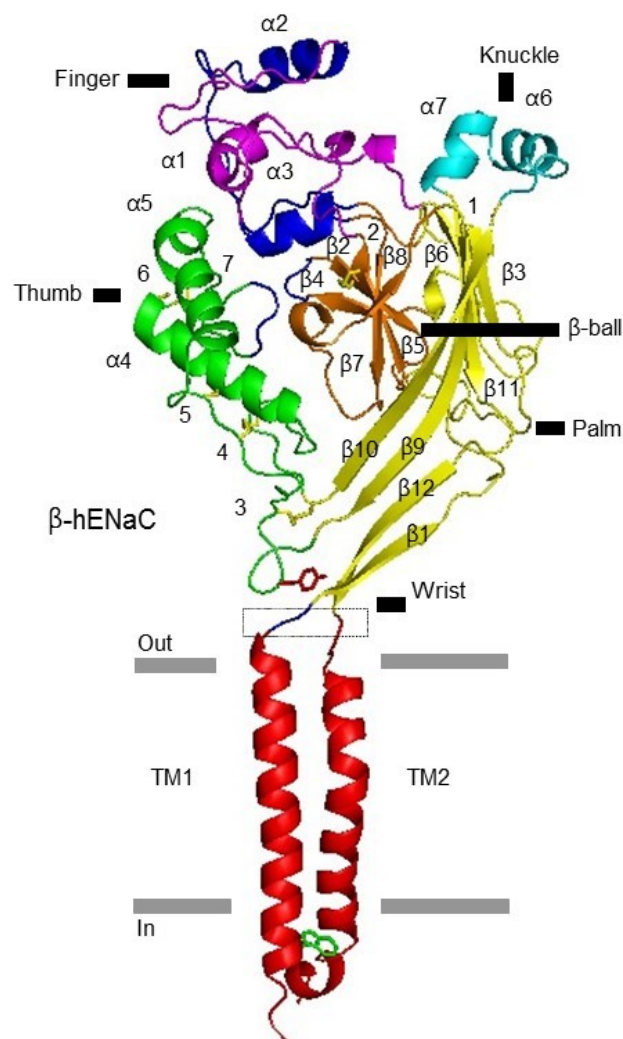


Figure 31: Predicted model structure of beta-human ENaC. This model is proposed based on the crystal structure of the chicken acid sensing ion channel (cASIC1) A monomer (using 2QTS coordinates). The transmembrane domains (TM1 and TM2) and linker regions are colored in red. The palm, knuckle and thumb domain are colored in yellow, cyan and green, respectively. The β -ball region is colored in orange while the finger domain is colored in magenta and blue (32).

Beta mutant 9 possesses three mutations G268A, G409R and K488E. The glycine (G) of G268A mutation is a conserved residue found in the $\beta 5$ strand of the β -ball of ENaC (Fig 31). Glycine and alanine (A) are non-polar amino acids that share chemical properties. They both participate in hydrophobic interactions. G268A mutation is less likely to affect the function of ENaC but might affect the structure since glycine is usually found in beta turns due to their flexibility and not alanine. Since the exact

structure of ENaC is not yet known, the importance of this amino acid needs to be further examined. G409R mutation is found in a region between the $\alpha 4$ and $\alpha 5$ helices of the thumb domain. Arginine is a polar/charged amino while glycine is not, so this mutation is more likely to affect the structure/function of the protein. The K488E mutation of beta mutant 9 is found in the $\alpha 7$ of the knuckle domain. Lysine (K) is a positively charged amino while glutamate (E) is a negatively charged, so this mutation is also likely to affect the structure/function of the protein. Lysine attracts negatively charged amino acids, so substituting with glutamate is going to result in a repulsion. The results obtained from the pronging assay which shows no growth inhibition for beta mutant 9 and the western blot correlates. These findings suggest that the mutations of beta mutant 9 were critical for proper functioning of the protein and should be the focus of future studies.

CHAPTER IV

CONCLUSIONS

Studies on ENaC have been done mostly on the α -subunit because it is required for ENaC to be a functional channel. About 70% of the amino acid residues that make up ENaC are found in the extracellular region and little is known about this region. This preliminary study, focused on the extracellular region of the β -subunit.

In this study, random beta mutants were generated using EP-PCR that utilizes a Taq polymerase that does not proofread mismatched DNA base pairs. pESC-Leu/ β -ENaC and β -ENaC EP-PCR products were co-transformed into BY4742 yeast cells and then retransformed with pYES2/NTA/ α -ENaC. A yeast screen and a western blot were used to ascertain function and expression of ENaC, respectively. Mutants of interest, beta mutants 1, 4, 5 and 9, were sequenced and the sequencing data were compared to the wild type pESC-Leu/ β -ENaC to identify changes in the amino acid sequence.

Beta mutants 1, 4, and 5 displayed a slight growth inhibition as compared to wild type pESC-Leu/ β -ENaC. When sequenced and compared to the amino acid sequence of the wild type, beta mutants 1 and 5 were found to have a nonsense mutation that introduced a premature stop codon that caused the protein to be truncated though this result did not correspond to the western blot. Beta mutant 9 showed no growth inhibition when compared to the wild type, when sequenced and compared to the amino acid sequence of the wild type, both a silent and missense mutations were present. These findings are also consistent with the result obtained from the western blot which showed that the protein was not detected.

The K109R mutation found in all the mutants is likely a variant of the beta-ENaC and a mutation created by the EP-PCR. The mutations of beta mutant 4; E476S and R477G, may alter the function and structure of the protein. Mutation G268A of beta mutant 9 is not likely to significantly alter the structure/function of ENaC. This is because both glycine and alanine share chemical properties. G409R and K488E are more likely to have an effect on the structure/function of ENaC.

For future studies, the mutated pESC-Leu/ β -ENaC + pYES2/NTA/ α -ENaC can be retransformed with the gamma subunit of ENaC to determine the effects of these mutations when the three subunits are present. These different mutants can further be characterized using a patch-clamp technique to determine their effects in mammalian cells as well as determine how these various mutants contribute to the proper structure/function of ENaC. Also, a cell surface biotinylation and a confocal microscope can be used to track the localization of ENaC. These suggestions can give more insights as to why the results obtained from the western blot did not correspond to the mutations found within the proteins of beta mutants 1 and 5.

The difference in growth among beta mutants 1-10 seen from the pronging assays results were very subtle. So a future study needs to be done to clarify these differences, possibly by adjusting the conditions of the plates in which the yeast cells used for the pronging assays are grown. This also needs to be done to demonstrate that this subtle differences are reproducible.

Also, the mutant plasmids of beta mutants 1, 4, 5 and 9 needs to be re-transformed back into BY4742 yeast cells to see if the transformants still give the same initial

phenotype. Because the initial phenotypes are such small subtle effects, it is quite possible that they will not be seen again. False positive results are very common in these kinds of mutant screens. The results of this study are preliminary and may have identified several interesting beta mutants, but they haven't yet been confirmed and will require future work to establish that the phenotypes are reproducible.

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