University of Alberta

Characterization of a newly identified kidney Anion Exchanger 1 mutant, C479W

by

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Abstract:

Anion Exchanger 1, AE1, is a membrane glycoprotein that functions as a dimer in the red blood cells (RBC) as well the kidney. It functions to exchange Cl^- for HCO_3^- in an electroneutral manner, with the RBC AE1 having an additional function in maintaining its biconcave shape. Mutations in AE1 can cause Hereditary Spherocytosis (HS) and distal Renal Tubular Acidosis (dRTA). A mutation, C479W, has been discovered in Edmonton that caused the rare incidence of HS and dRTA in young patient who is heterozygous for C479W and G701D, a recessive dRTA mutation. Expression in MDCK cells has demonstrated that C479W AE1 is retained intracellularly, is misfolded, but can dimerize. C479W AE1's trafficking to the plasma membrane is not rescued by interaction with WT protein, or the small molecules glycerol and DMSO, or by reduced temperature. C479W AE1 also has an increased interaction with the ER chaperone protein, Calnexin.

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Abbreviations:

α-IC: alpha-intercalated cells

AE1: Anion Exchanger 1

AP: Alkaline Phosphatase

CA II: carbonic anhydrase II

CTD: C-terminal domain

Co-IP: co-immunoprecipitation

dRTA: distal Renal Tubular Acidosis

Endo H: Endoglycosidase H

ER: Endoplasmic reticulum

ERAD: ER-associated degradation

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GPA: glycophorin A

HEK: Human Embryonic Kidney

HPLC: High performance liquid chromatography

HS: Hereditary Spherocytosis

IF: Immunofluorescence

ILK: Integrin-Linked Kinase

ISH: *in situ* hybridization

MDCK: Madin Darby Canine kidney

MEF: Mouse Embryonic Fibroblast

NDI: Nephrogenic Diabetes Insipidus

NTD: N-terminal domain

PCR: Polymerase Chain Reaction

PNGase F: Peptide N-Glycosidase F

RNA: Ribonucleic Acid

RBC: Red blood cell

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

I – Introduction

i - Anion Exchanger 1 background in red blood cells

Anion exchanger 1, AE1, is a membrane glycoprotein comprised of 911 amino acids containing a single glycosylation site at Asparagine (Asn) 642 [1]. It functions in the electroneutral exchange of chloride for bicarbonate in the membrane of red blood cells (RBC) as well as the alpha-intercalated cells of the kidney [2]. The erythrocyte isoform of AE1 was cloned and its base pair sequence was elucidated in 1989 [3]. The focus of this study is on the kidney isoform of AE1 (kAE1), however there is a wealth of studies on the erythrocyte AE1 (eAE1) that can be used in the understanding of kAE1; therefore the preliminary background will begin with eAE1.

a. Function of eAE1:

eAE1 catalyzes the electroneutral exchange of one Cl^- ion for one HCO_3^- ion in the membrane of RBCs (figure 1) [2]. The transport of Cl^- into RBC occurs at a rate of 10^{11} molecules per second [4]. This requires a transporter concentration to be 10% of membrane protein, which limits the number of potential membrane proteins responsible for the transport. Ho and Guidotti suspected band 3 [4]. Using RBC from donor blood, they were able to study phosphate transport, similar to transport of Cl^-, into the cell by measuring the quantity of remaining [^{32}P]orthophosphate in the surrounding media. In addition, the anion transport could be inhibited by a sulfanilate anion that competes for binding with phosphate on the extracellular

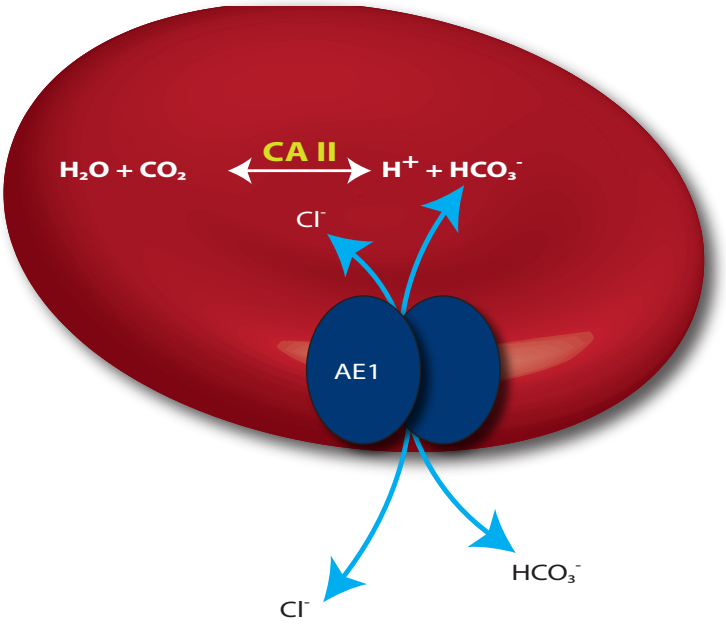

Figure 1. *The function of eAE1 in RBCs:* Carbonic anhydrase (CAII) converts water and carbon dioxide into a proton and bicarbonate. AE1 then exchanges that bicarbonate for extracellular chloride.

surface of the RBCs, presumably at the phosphate binding site [4]. The source of anion transport and inhibition was localized to a 100 kDa band, otherwise known as eAE1, by separation on a Sepharose 4B column. It is now known that eAE1 functions in the membrane of RBC to exchange Cl^- for HCO_3^- in a 1:1 electroneutral exchange.

eAE1 has an additional function in RBC; it interacts with the cytoskeleton that is essential for the RBCs biconcave shape. (see *Structural domains)*

b. Oligomeric State of eAE1 protein:

First, AE1 exists in a predominantly dimeric state, with the erythrocyte isoform having an additional tetrameric state that plays an integral role in RBC shape [5],[6]. Using a non-ionic detergent, nonaethyleneglycol dodecyl ether ($C_{12}E_9$) to purify eAE1 from bovine blood and a dissociating reagent, dimethylmaleic anhydride (DMMA), to separate possible oligomers, it was determined through an SDS-PAGE gel that AE1 is found in the plasma membrane as both a dimer and a tetramer [7]. Size exclusion high performance liquid chromatography (HPLC) was used to elucidate the oligomeric state of human eAE1. Blood samples were received from donors and eAE1 was isolated from red blood cell ghosts to be applied to size exclusion HPLC [8]. The fractions obtained were subjected to 215 nm wavelengths of UV light to measure absorbance from which oligomer associations can be revealed. The major peak on the chromatogram corresponded to eAE1 as a dimer. This was confirmed through identification of the mass of the polypeptide. Larger

oligomeric forms were also found, but to a lesser extent than the dimer. Through crystal formation, it was resolved that residues 314-344 are responsible for dimerization of the cytoplasmic domain [6]. The identification of a physiological tetramer was not confirmed [6].

c. Trafficking pathway of membrane-bound glycoprotein's and the importance of glycosylation:

Each monomer of AE1 carries a single N-glycosylation at Asn642 [1]. Concurrent with translation of AE1 in the Endoplasmic Reticulum (ER), an addition of a sugar made up of 3 glucose, 9 mannose and 2 *N*-acetylglucosamine sugar residues is added to the polypeptide [9]. The three glucose residues are used as a marker in folding of the protein, and once correct tertiary structure is achieved, the protein can escape the ER (figure 2). Further trafficking of the protein to the Golgi will allow for conversion of the high mannose sugar to a complex oligosaccharide by the Golgi resident proteins, mannosidases [5].

The purpose of the glycosylation site in terms of function or trafficking of eAE1 was investigated by Groves and Tanner [10]. They generated mutants of eAE1 that disrupted the consensus sequence, Asn-X-Ser/Thr (X is any amino acid except Proline) at position 642. *Xenopus* oocytes were injected with cRNA for WT and the glycosylated mutant. Quantifying eAE1 that was susceptible to Chymostrypsin cleavage demonstrated that these two forms of the protein traffic to the cell surface. No difference was observed in the percentage of protein found at the plasma

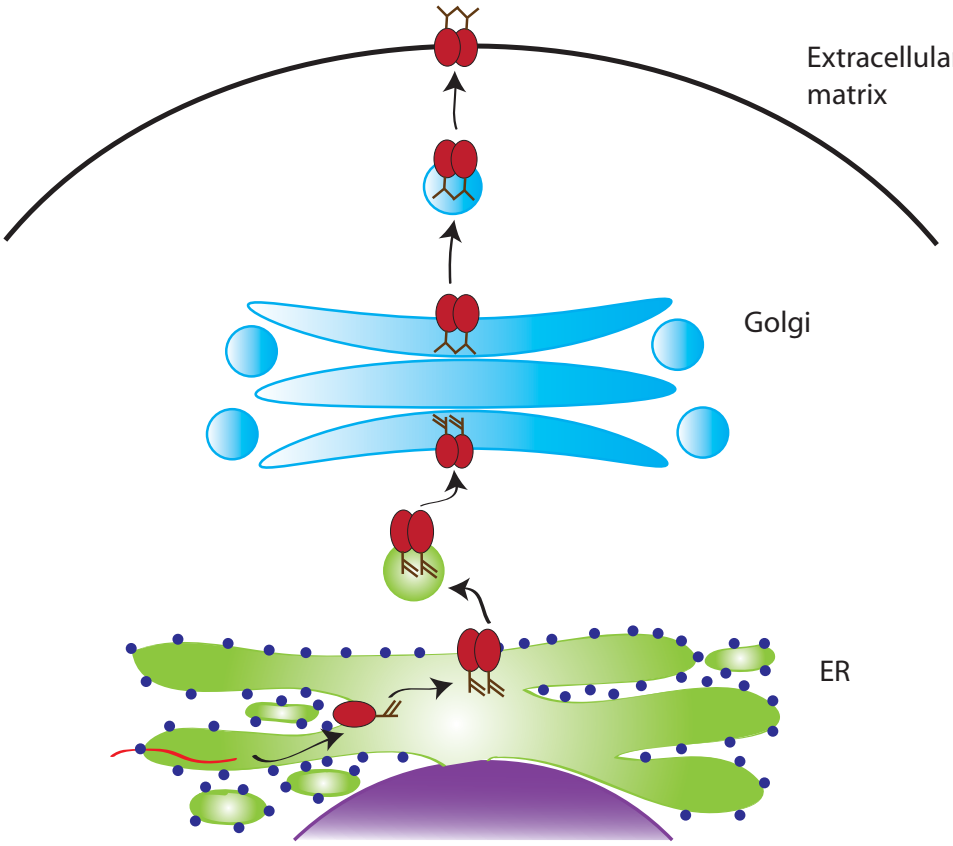

Figure 2. *The pathway of glycosylation of membrane-bound proteins*: Proteins destined for the plasma membrane are translated in the endoplasmic reticulum where glycoproteins receive high mannose residues. The newly synthesized glycoprotein is then sent to the Golgi apparatus where the high mannose oligosaccharides are converted to complex oligosaccharides via mannosidases and the protein is packaged for transport to the plasma membrane. The vesicle then fuses to the plasma membrane ebedding the protein.

membrane. Importantly, Glycophorin A (GPA), a chaperone-like protein (*see below*), was also added to the oocytes from which they noted that GPA enhanced the trafficking of both proteins equally. To test the effects of glycosylation on anion transport, oocytes were injected again with cRNA for mutant or WT eAE1 as well as GPA. Their transport activity was measured in the presence and absence of a stilbene inhibitor known to prevent anion exchange. Although both the WT and the mutant eAE1, in the absence of inhibitor, were functional, the mutant lacking glycosylation had transport rates approximately 30-40 % lower than WT. They also proved to be more sensitive to inhibition. Based on the above results, glycosylation of eAE1 is not required for the trafficking of the protein, nor is it imperative for the function of the protein.

To aid in the trafficking of eAE1, RBCs have an integral membrane protein, GPA, which acts as a chaperone-like protein and assists in the trafficking and of eAE1 to the plasma membrane [11]. GPA presence does not affect total eAE1 at the plasma membrane, however it increased the rate of accumulation of eAE1 and its activity [11].

d. Structural domains:

Within a monomer, AE1 consists of two structural domains, a 52 kDa C-terminal domain (CTD) consisting of amino acids 360-911 and a 43 kDa N-terminal domain (NTD), which consists of the first 359 amino acids [12]. The CTD is comprised of 12-14 transmembrane segments and is responsible for the ion exchange of Cl^- for HCO_3^-

which increases RBC capacity for CO_2 [11, 13]. The membrane-spanning domain may also be involved in the removal of senescent RBC by the spleen [6]. In addition, the short 33 amino acid C-terminal tail of the CTD has a pertinent role in the binding of carbonic anhydrase II (CAII) [14]. The NTD serves as a binding site for glycolytic enzymes such as glyceraldehyde-3-phosphate (GAPDH), phosphofructokinase, aldolase, hemoglobin, and others, as well as acting as a binding site for cytoskeletal proteins (figure 3) [15]. The interaction between this domain and the cytoskeleton is crucial for the additional function of AE1 in RBC, maintenance of the biconcave shape [6]. The N terminal region of eAE1 binds to protein 4.1 when in a dimeric state and additionally binds ankyrin and protein 4.2 when it is in a tetrameric state [11, 15]. Protein 4.2 functions to increase anion transport activity of eAE1 [16]. Ankyrin in turn binds directly to the cytoskeletal protein, spectrin therefore acting as the link between eAE1 and the cytoskeleton [15]. It is the interaction between eAE1, ankyrin, and spectrin that offers the RBC its essential qualities of stability and flexibility [15]. Interestingly, a single tetramer of eAE1 is not large enough to accommodate all peripheral protein binding [16]. The abundance of eAE1 in the plasma membrane, comprising 25 % of total membrane protein [6] in RBCs, allows for the interactions with the cytoskeleton [16].

e. Structure of eAE1:

Experimentally, it is more difficult to elucidate membrane protein structure relative to cytosolic protein's structure due to the hydrophobicity of membrane spanning regions of membrane proteins. For eAE1, a crystal structure has been developed for

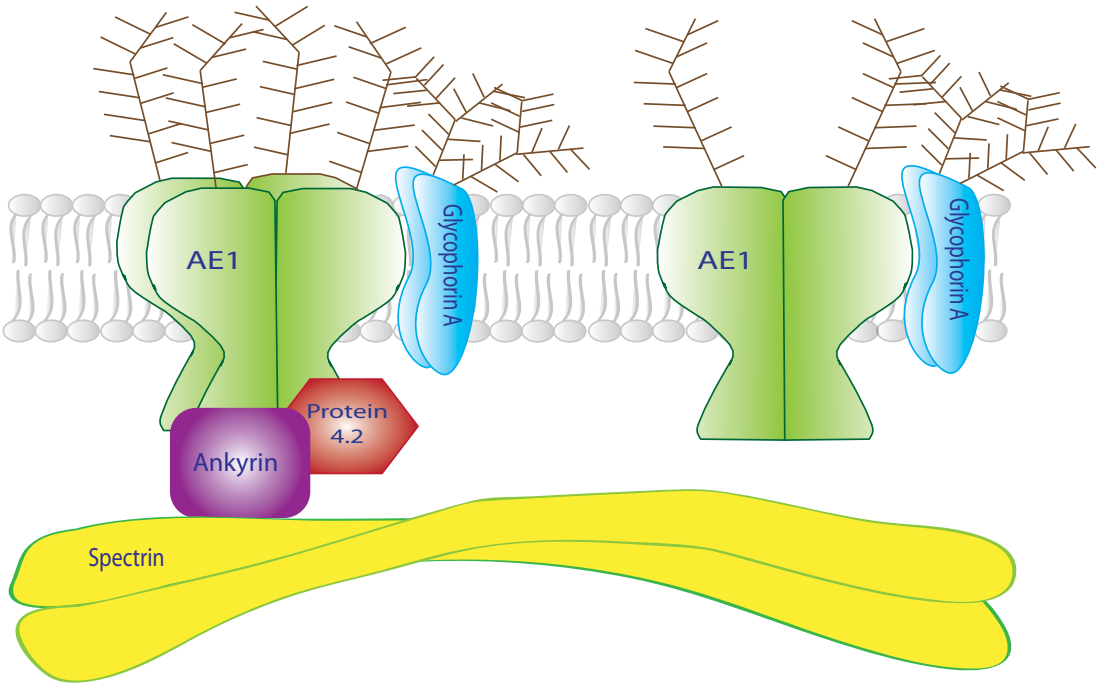

Figure 3. *The interaction of eAE1 with glycophorin A and the cytoskeleton*: AE1 functions in the membrane of RBC where it interacts with glycophorin A, a chaperone-like protein, as well as protein 4.2 and ankyrin. Ankyrin links eAE1 to spectrin, a major protein of the cytoskeleton.

both structural domains, CTD and NTD [6]. The NTD, being cytoplasmic is resolved to 2.6 Å and includes 11 β-strands and 10 helical segments (figure 4a) [6]. Residues 202-211 were not resolved owing to the NTD's flexibility in movement that facilitates ligand binding [6]. As for the crystal structure of the membrane spanning region, a 3-dimensional model has been proposed based on the reconstitution of 2-dimensional crystals with a resolution of 20 Å (figure 4b) [17]. The authors were able to confirm the dimeric nature of AE1; as well they could infer that eAE1 had a membrane-spanning region of 110 Å in length with a cytosolic protrusion on each end, proposed to be the N and C termini of the protein [17]. The limitation of this structure is the resolution of 20 Å. This is insufficient to determine secondary structure of the protein, which is ultimately the goal of crystal formation.

f. Inhibitor binding:

Stilbene disulfonates are inhibitors of various anion transporters including AE1 [18]. The most widely studied inhibitor is DIDS (4,4'-diisothiocyanato-2,2'-stilbenedisulfonate) and its derivative H_2DIDS (4,4'-diisothiocyanatodihydrostilbene-2,2'-disulfonate), which contain an isothiocyano R-group that reacts with lysine residues. These inhibitors bind tightly and rapidly to form a 1:1 complex with each AE1 subunit. Functional studies have shown that DIDS and H_2DIDS binding to AE1 exhibit a linear relationship with anion exchange [19]. DIDS and H_2DIDS bind to AE1 with the same affinity. Stilbene disulfonate inhibitor binds to Lys-851 and Lys-359 in AE1. One of the Lyseine residues, Lys-851, is involved in the control of anion exchange. Evidence is now mounting that

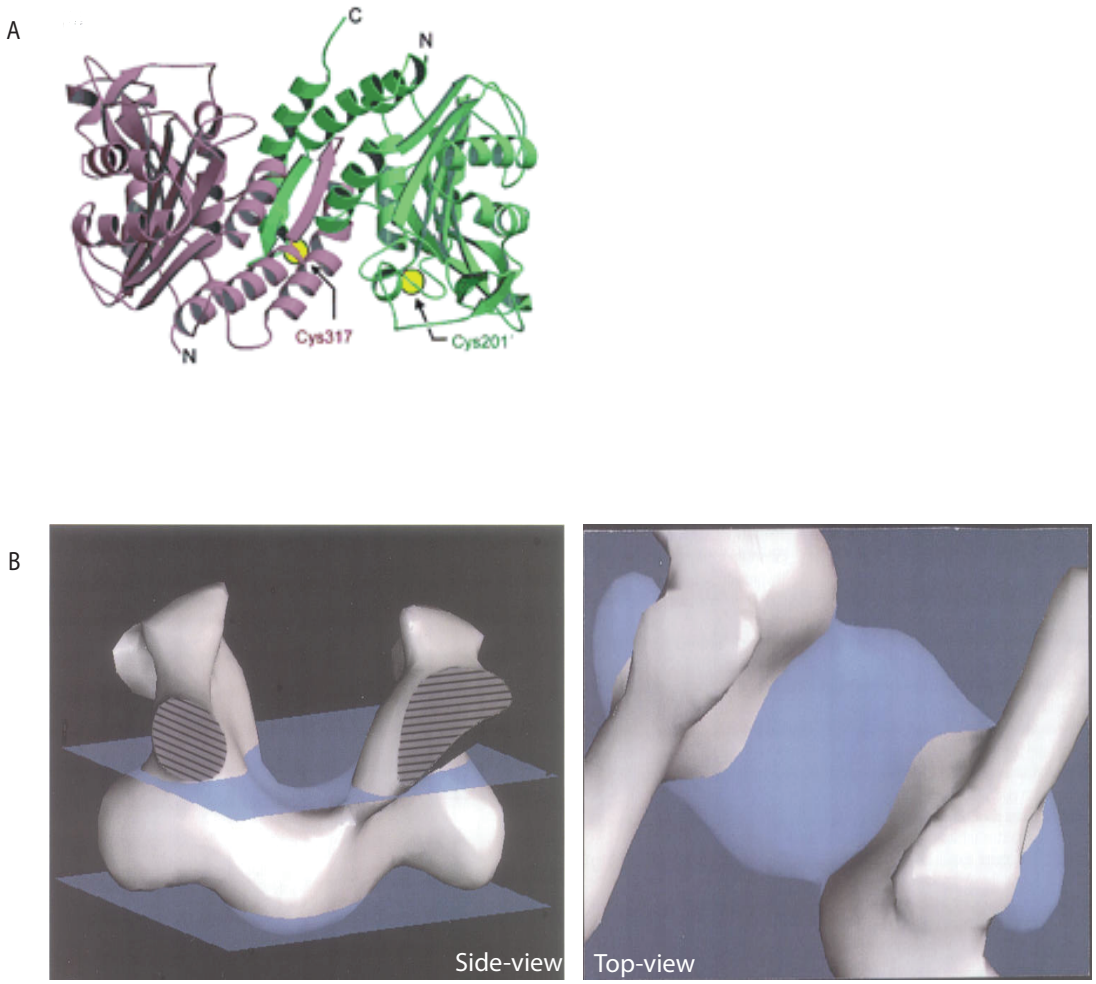

Figure 4. *3D imaging showing tertiary structure of eAE1and a ribbon diagram of the cytoplasmic domain of eAE1 dimer*: A) Ribbon diagram of the cytoplasmic domain of eAE1 dimer showing interactions between interlocking dimerization arms. B) A 3D map of the dimeric membrane protein eAE1. Side-view shows both the N and C-termini facing the cytosolic side of the cell with a bulky region embedded within the membrane (represented by two blue planes). Striped regions represent areas that form protein-protein interactions. Top-view showing the N and C termini that protrude into the cytoplasm. Dimensions of the protein are 60 x 110̊ A with a thickness of 80 Å.
[6, 28]

shows that these inhibitors do not bind directly to the active site of AE1, but bind allosterically, inhibiting the binding of anions [18].

ii - Kidney Anion Exchanger 1 background:

a. Identification of AE1 in the kidney:

With the increasing knowledge of eAE1 and the understanding of this protein's importance in the RBCs, came an interest in whether this protein is expressed in other tissues. Using Immunoblotting analysis, Cox *et al*. tested different tissues of adult chickens including skeletal muscle, spinal cord, cerebellum, lens, retina, and kidney [20]. Using an antibody against eAE1 the results revealed no presence of an eAE1 homologue in any of the above tested tissues except the kidney. This newly discovered protein runs on an SDS-PAGE gel at approximately 115 kDa, whereas the avian eAE1 runs at approximately 105 kDa. Further experimentation showed that the kidney form (kAE1) of the avian eAE1 differed in the size of its transcript and polypeptide, and that this new isoform is also expressed in a specific region within the kidney [20].

A couple of months after Cox *et al.* published, another paper was made public. This time, using immunostaining it was discovered that eAE1 has an analogue in mammalian (rat) kidney cells that is localized to the basolateral membrane of the intercalated cells of the collecting duct [21]. In addition, this kidney isoform co-

localized in kidney cross-sections with ankyrin and spectrin, two proteins known to interact with eAE1.

It was not until a couple of years later that kidney tissues from patients were tested for the presence of kAE1. Digested human tissue samples were purified to obtain protein contents for further treatment with antibodies known to bind different eAE1 fragments [22]. Immunoblotting revealed that a portion of the NTD of kAE1 is missing, this information corroborates previous evidence that kAE1 migrates faster on an SDS-PAGE gel due to truncation of the kidney isoform. Immunofluoresence of kidney sections obtained from a patient revealed that kAE1 localized to the cortical collecting duct of the alpha intercalated cells on the basolateral membrane [22].

b. The function of the intercalated cells in the collecting duct of the kidney:

The collecting duct within the kidney is a tightly regulated epithelium that plays a major role in excretion of acids, and is responsible for the acidification of urine [23]. Within the collecting duct are intercalated cells, alpha and beta; the beta cells are responsible for base secretion, while the alpha cells are responsible for acid secretion [23]. These polarized, intercalated cells may be able to convert between alpha and beta type intercalated cells based on external environmental cues [24]. Within the alpha-intercalated cells (α-IC) are multiple proteins that work in concert to acidify the urine as well as buffer the blood (figure 5). Located in the apical membrane is a proton pump, a vacuolar type H^+-ATPase, that is responsible for pumping H^+ into the urine in an ATP dependent manner. The inner medullary-

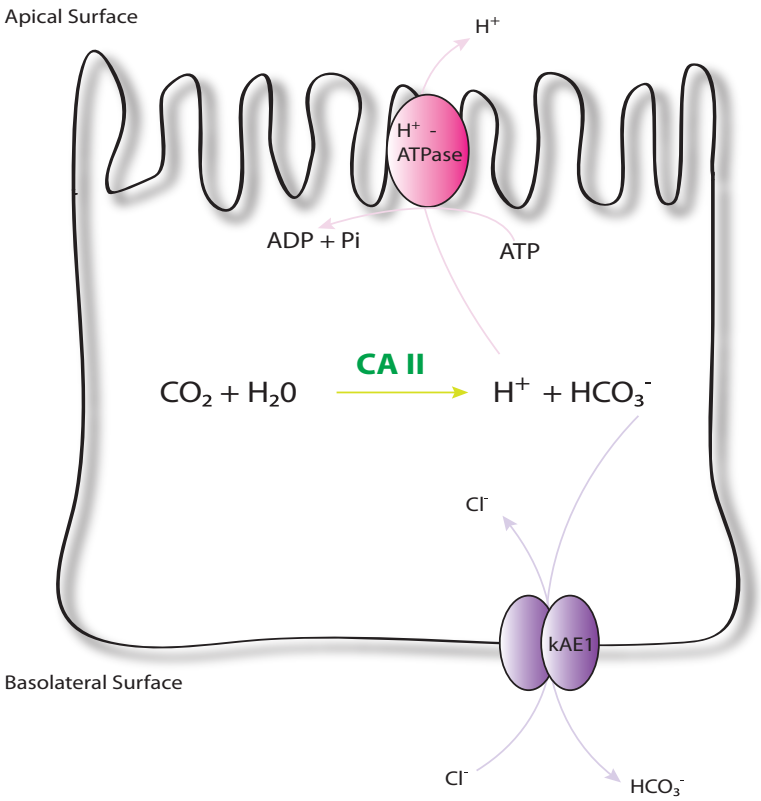

Figure 5. *Function of kAE1 in α-intercalated cells:* kAE1 functions in the basolateral surface of alpha-intercalated cells of the collecting duct of the kidney. Carbonic anhydrase II (CAII) is a cytosolic enzyme that converts CO_2 and H_2O into a H^+ and HCO_3^-. THe H^+-ATPase located in the apical membrane of the polarized cell then pumps the proton into the urine with the hydrolysis of ATP. CAII's second product, HCO_3^- is tranported into the blood via kAE1, which exchanges one extracellular Cl^- for one HCO_3^- molecule.

collecting duct is responsible for 80 % of acid secretion into the urine [23]. Carbonic anhydrase II (CAII) catalyzes the reaction between water and CO_2 producing H^+ as well as a HCO_3^-. The HCO_3^- is then exported from the cell via a basolateral Cl^-/HCO_3^- exchanger [23]. It is important to note that approximately 80% of HCO_3^- is reabsorbed in the proximal tubule of the kidney, about 10 % is reabsorbed in the thick ascending limb, leaving the remaining 10 % to be reabsorbed in the collecting duct so that the urine is free of HCO_3^- [25]. The collecting duct is important for the fine-tuning of bicarbonate reabsorption as shown by the onset of disease when this process is interrupted (see *Disease*).

c. The function of kAE1 in the collecting duct:

To determine whether kAE1 is responsible for the Cl^-/HCO_3^- in the α-IC of the collecting duct, enzyme linked *in situ* hybridization (ISH) and immunofluoresence (IF) were performed on both the medullary and cortical regions of the collecting duct, both of which contain α-IC [26]. Using sections from rat kidney, RNA probes specific for kidney and erythrocyte isoforms of AE1 linked to alkaline phosphatase (AP) were added and incubated. The endogenous mRNA hybridized to the RNA probes was then visualized using an anti-AP antibody linked to a fluorescent molecule. Sections were taken from control rats and compared with sections taken from rats that were either acidotic or alkalotic. From this experiment it was determined that medullary IC express twice the quantity of kAE1 mRNA than cortical IC.

Huber *et al.* also performed IF using antibodies against both rat isoforms of AE1 as well as an antibody against human AE1 (detects eAE1 and kAE1) [26]. These antibodies were added to rat sections that had been treated the same as the previous ISH experiment (Control, acidotic, and alkalotic). It was determined that the α-IC in the cortex had decreased AE1 staining under alkalotic conditions compared to both the control and acidotic sections. For the medullary α-IC, the authors noted no difference in staining under either of the conditions. It was concluded based on the above evidence that α-IC of the collecting duct, both medullary and cortical regions show the capacity to respond to changes in acid-base status [26].

d. Interactions of kAE1 with the cytoskeleton:

It has been proposed that an interaction between kAE1 and ankyrin could contribute to the polarization of the α-IC of the kidney in addition to localizing membrane proteins to certain regions of the plasma membrane [21]. This theory is based on the relationship between eAE1 and ankyrin, where ankyrin links eAE1 to a cytoskeleton member, spectrin [15]. This relationship is integral to the maintenance of the RBC 's biconcave shape. Tissue sections of rat medulla were first subjected to antibodies specific for the eAE1 binding site of ankyrin. The sections were then stripped and re-stained with a rat anti-AE1 antibody. The images showed co-localization to the basolateral membrane of the medulla α-IC. The authors concluded that this interaction can limit the lateral mobility of the membrane proteins and may contribute to their localization within the membrane [21].

To strengthen the authors' arguments for an interaction between ankyrin and kAE1, a study looking at the binding of these two proteins was required. This is precisely what Ding *et al.* did using HEK293 (human embryonic kidney) cells [27]. This cell line does not express AE1 or ankyrin as determined by western blot. HEK 293 cells were transfected with eAE1, kAE1 or AE1m (containing only the last 18 residues of the NTD and the complete membrane domain) as well as a fragment of ankyrin (ANK1) containing 12 of the 24 tandem repeats known to bind AE1. They were able to determine quantitatively that the full-length eAE1 binds the ankyrin fragment, however kAE1 did not; neither did the control, AE1m. From this it was deduced that kAE1 does not bind ANK1 and that the initial 79 amino acids of eAE1 are essential for this interaction [27]. Another study using human kAE1 looked at the interaction of this truncated form with the glycolytic enzyme, aldolase, and protein 4.1 [28]. Based on competitive binding of these proteins for AE1 versus kAE1, it was concluded that the initial 65 amino acids of AE1 are required for these interactions.

e. Interaction of kAE1 with ILK and kanadaptin:

To investigate proteins that could interact with kAE1, a yeast two-hybrid screen was performed using the NTD of kAE1 [29]. Through this method, it was discovered that kAE1 interacts with Integrin-Linked Kinase (ILK), a protein that phosphorylates Serine and Threonine and is a known actin-binding protein. This interaction was confirmed using co-IP in HEK cells. To identify the region of ILK responsible for this interaction, the first 192 amino acids of ILK were deleted and co-transfected with kAE1 into HEK cells. The C-terminal domain (CTD) was sufficient to co-IP kAE1.

The role of ILK in kAE1 trafficking and localization was ascertained using cell surface biotinylation on HEK cells expressing either kAE1 alone or with ILK (this causes an over-expression of ILK as HEK cells express it endogenously). ILK increased the amount of kAE1 at the plasma membrane from 32 % to 50 % of total kAE1, but does not act on kAE1 function. Using pulse-chase it was learned that after 1 hour, ILK and kAE1 co-IPed, showing early association. This leads to the deduction that ILK may affect the trafficking of kAE1. In addition to its possible trafficking capabilities, ILK is associated with the actin cytoskeleton; therefore it is possible that ILK acts as an intermediate between the cytoskeleton and kAE1. Indeed, in HEK cells kAE1 associates with the actin cytoskeleton via ILK, paxillin, and actopaxin. Sections from normal human kidney were taken and immunofluorescence was performed to visualize a co-localization *in vivo* of ILK and kAE1 [29].

A yeast-2-hybrid assay was also used to identify an interaction between the mouse kAE1 and a new protein kanadaptin (kidney anion exchanger adaptor protein) [30]. Using immunocytochemistry on rabbit kidney cortex slices, it was determined that kanadaptin interacted with kAE1 early when it was localized to cytoplasmic vesicles. The colocalization between kanadaptin and kAE1 was gone when kAE1 localized to the basolateral membrane of the intercalated cells of the rabbit, bringing about the theory that kanadaptin assists kAE1 in its targeting to the plasma membrane. A few years later, researchers studied the interaction and effects of human kanadaptin on human kAE1 in HEK cells. Immunofluorescence showed that kanadaptin is largely

localized to the nucleus, but still had minimal colocalization with kAE1 in cytoplasmic compartments [31]. Pulse-chase experiments were used to determine that the presence of kanadaptin in HEK cells did not improve the rate of trafficking of kAE1, as well, co-IP experiments were performed and neither protein immunoprecipitated the other, therefore it remains controversial as to whether kanadaptin is a mediator of kAE1 trafficking.

Based on the above evidence, kAE1 is localized to the α intercalated cells of the collecting duct of the kidney where it is responsible for Cl^-/HCO_3^- exchange and can respond to changes in the acid-base environment. In addition, kAE1 does not interact with ankyrin, aldolase, or protein 4.1, therefore not supporting the theory that the interaction between kAE1 and ankyrin contributes to the polarization and localization of membrane proteins. However, ILK is a strong candidate for this role. Furthermore, kAE1 may not interact with kanadaptin.

f. Oligomeric State of kAE1:

The erythrocyte AE1 exists mainly as a dimer with the potential to form tetramers. For the kAE1 isomer it is expected that the main functional state would be a dimer as well. In 2002, Quilty *et al.* constructed eAE1 with a poly-Histidine (His-tag) tail at its C-terminus and transfected HEK cells along with kAE1 (without a tag) [32]. eAE1 was eluted and the samples were run on a western blot probing for kAE1. It was confirmed that kAE1 could form a dimer with eAE1 showing that kAE1 can dimerize, which is consistent with what was seen in erythrocytes.

g. Structure of kAE1:

Because eAE1 and kAE1 are isoforms, their primary structure is the same except for the N-terminal deletion of 65 amino acids [28]. These two proteins have additional similarities in their secondary structure. Using differential absorption of polarized light to determine secondary structure of the two forms, it was concluded that they both have similar α-helical and β-sheet representation [28]. Furthermore, eAE1 undergoes a conformational change when pH changes from 6.5 – 10.5 as determined by Stoke's radius (55-66 Å). When kAE1 was subjected to the same conditions the Stoke's radius was measured, and it was observed that it too undergoes a conformational change similar to that of eAE1 (56-66 Å) [28]. Using sedimentation analysis it was confirmed that the NTD kAE1 could still dimerize [12], same as was seen in the crystal structure of eAE1 [6].

However, there are points of dissimilarity between the structures of eAE1 and kAE1. Firstly, because kAE1 lacks the initial 65 amino acids at its N-terminus, it is also lacking a β-strand (amino acids 55-65) [12]. Within the crystal structure, this β-strand is present in the core of the protein and is now known to function in the stability of the protein based on deletion experiments and thermal stability [12]. Another distinction in kAE1's tertiary structure is the proximity of two Cysteine (Cys) residues, 201 and 317. Under oxidizing conditions, eAE1 can form an inter-subunit disulfide bond between these Cys residues; kAE1 does not form this bond, possibly making the tertiary structure of kAE1 less stable [28].

h. Trafficking of kAE1 to the plasma membrane:

Wild type kAE1 in mammalian kidney cells and polarized Madin Darby Canine Kidney (MDCK) cells is targeted to the basolateral membrane [21, 33]. When the last 11 residues from the C-terminus of kAE1 are deleted, this mutant R901X, is trafficked to the apical membrane of MDCK type 1 cells [34]. This C-terminal region contains within it, a Tyrosine sorting motif and a putative class II PDZ interacting domain. The Tyrosine sorting motif, YxxΦ (where x is any amino acid and Φ is a bulky hydrophobic amino acid) is a recognition sequence for μ subunit of an adaptor protein (AP). These AP's are responsible for endocytosis, which occurs in vesicle trafficking and internalization of nutrients and signals, as well as trafficking to the plasma membrane [35]. PDZ domains are structural domains that are found in signaling proteins that function to anchor transmembrane proteins to the cytoskeleton and to hold together signaling molecules. They are more variable in their primary sequence than the Tyrosine sorting motif, but often terminate with a hydrophobic residue. This residue inserts into a pocket of its interacting partner; the pocket of the partner protein is responsible for the specificity of the interaction [36].

A double mutant was generated that contained amino acid substitutions of key residues to alanine within the putative Tyrosine sorting motif (Y904A and V907A) [34]. These constructs were expressed in polarized MDCK cells and using immunofluoresence it was noted that kAE1 trafficked to the apical membrane. Additionally, constructs containing one of the above mutations were tested in

polarized MDCK cells. Using immunofluoresence, it was observed that V907A kAE1 protein was trafficked to the basolateral membrane and Y904A was intracellularly retained. Toye *et al.* concluded based on the above observations that Y904 is essential for basolateral targeting of kAE1 in polarized MDCK cells [34]. The PDZ-binding domain was determined to be non-essential for basolateral localization, however it was noted to increase efficiency of trafficking of kAE1 by decreasing amount of intracellular protein.

A second putative Tyrosine sorting motif exists within kAE1, located in the NTD at amino acid 359, YKGL [34]. It was established that Y359 is essential for basolateral targeting because polarized MDCK cells expressing kAE1 with the Y359A substitution are targeted to the apical membrane as visualized through immunofluorescence microscopy [37]. When the NTD is deleted (amino acids 65-360), kAE1 localizes to the apical membrane in polarized MDCK cells [34].

It was first confirmed that these two residues, Y359 and Y904, could be phosphorylated when MDCK cells expressing kAE1 are treated with pervanadate, a phosphatase inhibitor. Immunoprecipitation experiment exhibited a trend; the longer the incubation period of MDCK cells with pervanadate, the more-phosphorylated kAE1 was present as determined by western blot. Interestingly, Williamson *et al.* could see through immunofluorescence that the phosphorylation of kAE1 caused the internalization of this protein. In addition, pervanadate treatment had no effect on the localization of the apically targeted mutants (Y359A,

Y904A/V907A, and R901X). This observation is consistent with another observation made by Williamson *et al.* that treatment of MDCK cells expressing kAE1 with $NaHCO_3^-$ to create a basic environment induces phosphorylation of Y359 and to a lesser extent, Y904 [37].

There are essential regions within the N and C terminal domains that are required for its basolateral targeting; the exact mechanism has yet to be elucidated. There exists, however, two putative Tyrosine sorting motifs that can be phosphorylated, and they appear to play a role in the localization of kAE1.

In summary, the erythrocyte AE1 has an isoform expressed exclusively in the α-intercalated cells of the collecting duct of the kidney, which is truncated by the first 65 amino acids. It functions at the basolateral surface as a dimer where it exchanges Cl^- for HCO_3^- in an electroneutral manner. It may interact with the cytoskeleton through its interaction with ILK, much like the interaction between eAE1 and ankyrin; its trafficking however, is independent of kanadaptin in human fibroblast cells. Both the N and C terminal domains have a key role in the trafficking of kAE1 to the basolateral membrane as shown in polarized MDCK cells.

iii – Diseases caused by mutations in AE1

a. Hereditary Spherocytosis:

Hereditary spherocytosis (HS) is an inherited hemolytic anemia that causes RBCs to take on a spheroidal shape and to become osmotically fragile [5]. The first known cause of HS was due to a deficiency in spectrin, a major protein of the cytoskeleton, or ankyrin, a linker of spectrin to the membrane [38, 39]. More recently it has been discovered that HS can be caused by a deficiency in eAE1 [38]. HS caused by eAE1 deficiencies accounts for 20 % of all HS cases [15]. The disease is characterized by hyperhemolysis and can be treated by blood transfusions or in more severe cases, splenectomy [15]. Blood samples taken from patients and run on an SDS-PAGE gel showed that the amount of eAE1 protein was reduced 20-30% compared to a normal individual. In addition, eAE1 reduction is associated with a proportional reduction in protein 4.2. The expression of seven missense mutations in HEK cells was used to determine surface content of eAE1 [5]. Immunofluorescence and cell surface biotinylation revealed that these mutants are defective in their trafficking in that they were localized to a peri-nuclear region and had no cell surface representation. Further testing of the trafficking of the mutants using glycosylation status and Endo-H sensitivity revealed that they did not reach the medial Golgi. HS can be caused by a deficiency in eAE1 due to its intracellular retention causing a decrease in eAE1 and cytoskeletal interactions.

b. Southeast Asian Ovalocytosis:

Southeast asian ovalocytosis (SAO) is a condition of the red blood cells that affects individuals of Southeast Asian descent and is marked by rigid blood cell morphology [40]. In addition, SAO, which is caused by a deletion of amino acids 400-408 at the

interface between the NTD and the first transmembrane segment, affords its carriers a resistance to the invasion of malarial parasites. Individuals carrying this deletion do not exhibit any disease phenotypes. When SAO is co-expressed with WT in MDCK cells, WT protein can assist SAO trafficking to the basolateral membrane, conversely when expressed alone, SAO is retained within the ER [41]. A homozygous patient for this mutation has not been found; it is thought to be lethal.

c. distal Renal Tubular acidosis:

dRTA is an inherited defect of renal function, however drugs or pathogens are also causes of this disease [42], characterized by alkaline urine, systemic metabolic acidosis, and nephrocalcinosis that can cause renal failure. The duration of illness is life-long and treatment with citrates and alkalizing salts produces an improvement in symptoms. There is no cure. One cause of dRTA was not discovered until many decades after the characterization of the disease. Blood samples were collected from family members of four distinct families with inherited dRTA and PCR (Polymerase Chain Reaction) were used to look for mutations in eAE1 [43]. All affected individuals were heterozygous, with the families carrying different mutations: R589H, R589C, and S613F. Using the blood samples of the dRTA patients as well as blood from non-dRTA patients, the activity of eAE1 was determined based on the influx of [^{35}S]-Sulfate. Three of the families all carry a mutation in R589, and functional analysis determined reduced sulfate transport for these RBC; approximately 76 % of the control. The S613F individual had increased transport of sulfate, 262 % of the control transport. All affected individuals had similar numbers

of RBC membrane eAE1 compared to control. Constructs of the above mutations were made for the RBC isoform and the kidney isoform and expressed in *Xenopus* oocytes to study Cl^- flux. Functional results were similar between isoforms, with R589H producing 40 % of WT activity and the other two mutants, R589C and S613F, displaying control values for Cl^- flux. This was the first association made between AE1 and dRTA.

In order to understand the effects of these dominant mutations within the cell, R589H was expressed in HEK cells. Interestingly, eAE1 R589H trafficked to the surface of the cell as observed by immunofluorescence and cell surface biotinylation however, the kAE1 R589H colocalized with Calnexin and had no mutant protein was present at the cell surface, as shown in the cell surface biotinylation [44]. The dominant effects of this mutant were confirmed when kAE1 WT and R589H were expressed together in HEK cells and no visible kAE1 was detected at the plasma membrane because of heterodimerization. Results were similar when the mutants R589H and S613F were expressed in polarized and non-polarized MDCK cells; both dominant mutants were retained intracellularly and colocalized with Calnexin in immunofluorescence studies [33]. In addition, when R589H was expressed in MDCK cells with WT, R589H retained the WT within the ER in polarized and non-polarized cells. There have been other dominant dRTA mutants that include: R901X, A858D, G609R, and A888L/A889X all of which display the similar dominant characteristics seen in R589H and S613F (Table 1).

Table 1. *Recessive and dominant mutations causing dRTA:* All known mutations that cause dRTA, both recessively and dominantly are listed. Information about their localization within polarized and non-polarized MDCK cells as well as their function within X*enopus* oocytes is displayed.

Dominant	**MDCK location** nonpolarized/polarized	**Function in *Xenopus***	**References**
R589H, R589C, R589S	ER/intracellular	Normal	[33, 42]
S613F	ER/intracellular	Slightly reduced	[33, 42]
R901X	ER/apical	Normal	[77, 34]
A858D	plasma membrane/basolateral	Low	[78, 79]
A888L/A889X	unknown	unknown	[64]
G609R	unknown/apical and basolateral	Normal	[81]
Recessive	**MDCK location** nonpolarized/polarized	**Function**	**References**
G701D	Golgi/Golgi	None	[60, 33]
S773P	ER/basolateral	None (HEK)	[31, 33]
V488M	ER/ER	unknown	[64]
R602H	plasma membrane/basolateral	unknown	[64, 80]
ΔV850	Intracellular/ Intracellular	Low	[78, 64, 79]
C479W	ER/ER	None	[61]
E522K	plasma membrane/basolateral	unknown	[66]
SAO (Δ400-408)	Intracellular/ Intracellular	None	[80, 79]
S667F	ER, plasma membrane/ ER	Near zero	[65]

The recessive mutations of AE1 that cause dRTA have different trafficking patterns (Table 1). When G701D and S773P were expressed in HEK cells, cell surface biotinylation reveals that the mutant protein cannot traffic to the plasma membrane [31]. This observation was confirmed with immunofluoresence showing that S773P AE1 colocalized with Calnexin, and G701D was retained intracellularly in a peri-nuclear region. Similar results were obtained when the two mutants were expressed in non-polarized MDCK cells [33]. Importantly, in polarized MDCK cells, S773P AE1 traffics to the basolateral membrane where it colocalizes with E-cadherin. G701D AE1 is retained intracellularly. Because kAE1 exists as a dimer, it is important to note the effects of a recessive mutant on the WT protein. When G701D kAE1 co-expressed with WT in polarized MDCK cells, WT rescues the trafficking of this mutant. S773P AE1 is already expressed at the basolateral membrane in polarized MDCK cells, and when co-expressed with WT, the results are the same. There are still many other recessive mutations of AE1 that cause dRTA (Table 1) that display varying degrees of retention within the cell. However, all of them (V488M, R602H, ΔV850, C479W, E522K, and S667P) when expressed as a homodimer or as a compound heterodimer can cause dRTA. When AE1-SAO is co-expressed with the recessive dRTA mutant, G701D, they can form heterodimers and are also retained intracellularly [45]. This is explained in patients with the SAO/G701D genotype who suffer from dRTA.

Further investigations have shown more mutations within AE1 that cause dRTA both recessively and dominantly (Table 1) with their position within the protein

playing a key role in the effects of the mutation (figure 6). It is important to note, that dRTA can be caused by mutations in the H^+-ATPase or CAII as their relationship is described previously.

iv – Case study report

The case of a young woman of Scandinavian-Caucasian descent, who was diagnosed with HS and dRTA (figure 7), was brought to the Cordat lab based on the unusual presence of these two diseases in a single individual. The lab received blood from the patient in addition to her father and mother and two siblings. Samples were sent to Dr. Yenchitsomanus' lab in Thailand where it was sequenced. We discovered that the patient carried a previously unknown mutation, C479W, and a known recessive mutation, G701D. To our knowledge, this is the first reported individual of non-Asian descent to carry the G701D mutation.

The patient was first diagnosed with severe HS shortly after birth. She was initially treated with regular blood transfusions, however they proved insufficient and at the age of three she underwent a splenectomy, improving her condition. The patient was also diagnosed at the age of 2 ½ with dRTA based on symptoms of nephrocalcinosis and failure to thrive. Her condition improved with oral bicarbonate supplementation. Her father, who carries the newly identified

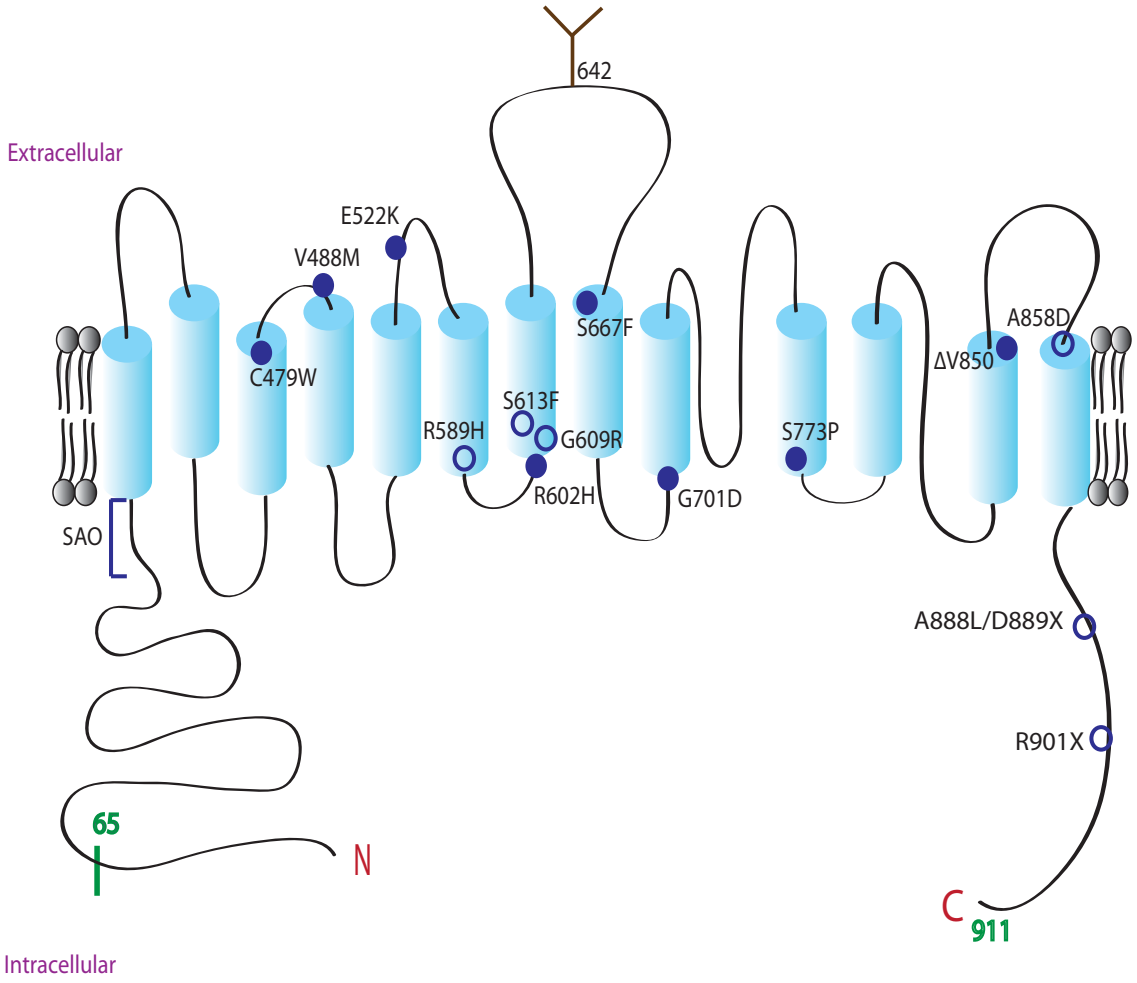

Figure 6. *Topology model of AE1 showing dominant and recessive mutations:* AE1 is a 911 amino acid membrane protein comprised of 13 transmembrane sections with an intracellular N and C termini. The fourth extracellular loop is glycosylated at Asn642. The kidney isoform is 65 amino acids shorter at the N terminus. ● denotes recessive distal renal tubuar acidosis (dRTA) mutations, ○ denotes dominant dRTA mutations.

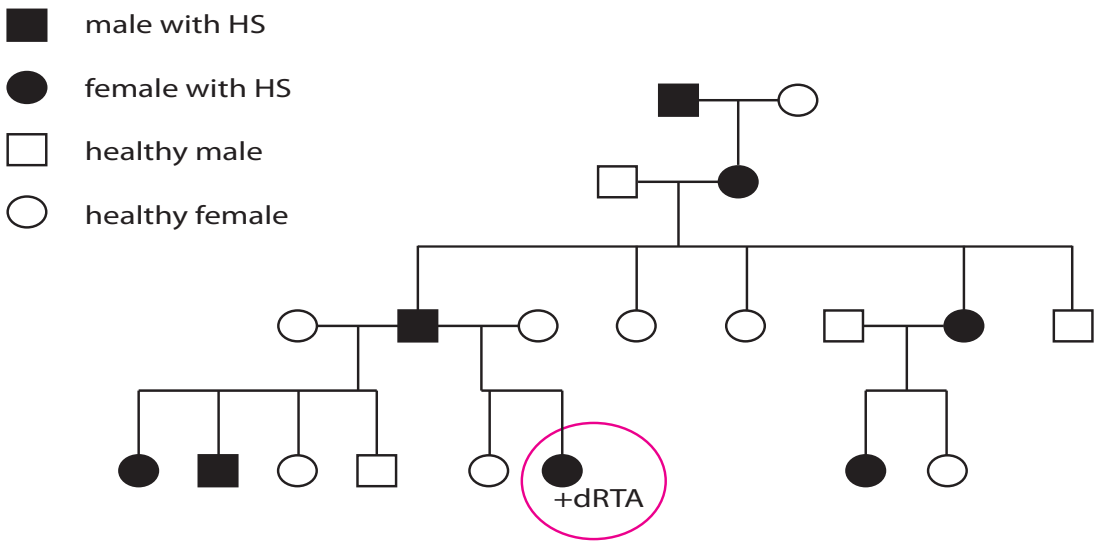

Figure 7. *Family pedigree*: Patient is afflicted with HS as well as dRTA, indicated by the circle.

mutation, C479W (Table 2), was diagnosed with a milder HS condition with no dRTA. Her mother is healthy as are her siblings.

v - Small molecule rescue

In an effort to improve trafficking of kAE1 mutant proteins, we looked to other exemplar membrane proteins, and used their methods and treatments as inspiration in the design of our own experiments.

a. Cystic Fibrosis Transmembrane Conductance Regulator background:

CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) is a cAMP regulated Cl^- channel that utilizes one ATP molecule per Cl^- transported [46]. CFTR is mainly expressed in the airways, pancreas, and sweat glands with the most severe phenotypes presenting in the lungs [47]. Abnormalities in the CFTR protein predispose patients' airways to chronic, recurrent infections with associated tissue inflammation ultimately leading to pulmonary failure. Immunofluorescence indicated that WT CFTR is at the apical membrane in polarized airway epithelium [48]. The most common mutation in CF cases, ΔF508, is carried by 90 % of patients and when tested for surface expression by immunofluorescence ΔF508 CFTR showed no surface staining. Further experimentation determined that this common mutation is retained intracellularly within a perinuclear region [48]. Using *Xenopus* oocytes injected with either WT or ΔF508 RNA, Drumm *et al*. were able to activate the Cl^- channel conductance using Forskolin, an activator of adenylate cyclase, and

Table 2. *Genotype and health conditions of the patient and her family:* The patient has genotype C479W/G701D. She inherited the mutation, C479W from her father who also suffers from HS. She inherited the recessive mutation, G701D mutation from her mother who is healthy. Her siblings are also healthy.

Family Member	Condition	Genotype
Mother	Healthy	G701D/WT
Sister	Healthy	WT/unknown
Brother	Healthy	WT/unknown
Father	HS	C479W/WT
Patient	HS/dRTA	C479W/G701D

observe Cl^- uptake by the oocytes therefore if located at the cell surface, the ΔF508 CFTR mutant is functional [49]. However, ΔF508 function differed greatly from that of WT exhibiting a slower rate of increase of activity and a lower level of steady-state flow. However, this mutant could establish a Cl^- current compared to the negative control and, therefore is a candidate for pharmacological therapy given its breadth in CF patients.

b. Methods of rescue in the trafficking of CFTR:

The processing of CFTR includes glycosylation at two different sites on the protein [50]. Glycosylation status can indicate processing of the protein and is often used as a marker of trafficking; newly synthesized proteins run further on an SDS-PAGE gel than a mature, fully glycosylated protein that has trafficked beyond the ER. Decreasing growth temperature has proved to be an effective method of restoring membrane protein trafficking either through conveying kinetic or thermodynamic stability to the protein, or changing the activities of endogenous chaperone proteins [51]. 3T3 fibroblasts expressing either WT or ΔF508 were incubated at variable temperatures (37°C, 30°C, 26°C, and 23°C) for 48 hours, then lysed, precipitated and run on an SDS-PAGE gel [52]. Looking at glycosylation as a marker for protein trafficking, it was observed that growing cells expressing the mutant ΔF508 increased the quantity of fully glycosylated protein that has trafficked beyond the ER. The quantity of WT protein remained unchanged.

Another mode of rescue is the use of small molecules that have the potential for pharmacological therapies. IB3 cells (bronchial epithelium of CF patient) that were treated with 1M glycerol for 24 hours followed by an IP to precipitate CFTR, showed an increase in mature, glycosylated protein versus newly synthesized CFTR [50]. The only drawback of this treatment is the high concentration of glycerol used to elicit a trafficking rescue cannot be considered as a potential therapy, however it is the first step towards identification of specific therapies

c. Vasopressin-2 Receptor background:

Arginine vasopressin (AVP) is a hormone that regulates urine volume by increasing reabsorption of water in the collecting duct of the nephron through stimulation of the Vasopressin-2 receptor (V2R) [53]. V2R is located on the basolateral surface of the collecting tubule cells and functions to bring about the insertion of Aquaporin-2 (AQP-2) membrane protein into the apical membrane of the collecting tubule allowing for the reabsorption of water [53, 54]. The majority of V2R missense mutations result in misfolded protein that remains trapped in the ER causing X-linked nephrogenic diabetes insipidus (X-NDI).

d. Methods of rescue in the trafficking of V2R:

Nine missense mutations of V2R that are retained within the ER were studied for rescue by 4 % glycerol, 1 % DMSO, and 27°C [55]. The method of monitoring the maturation of protein by its glycosylation status was employed to determine improved trafficking of the mutants. The mutants were expressed in the MDCK type

I cell line and subjected to each of the above treatments for 16 hours before the protein was collected and a western blot was performed. Of the nine mutants studied, only the V206D mutation of V2R was susceptible to trafficking improvement by the treatments, glycerol and DMSO. Decreasing the temperature increased the amount of newly synthesized protein, however that result did not translate into an improvement in the maturation of V2R. Another lab studying V2R trafficking rescue, expressed mutants, A98P and L274P, in transfected COS-7 cells and subsequently treated them with 2 % DMSO, 1M glycerol, or reduced temperature (26°C) for six hours [56]. The ability of the chemical chaperones to rescue the trafficking of the V2R mutants was observed by immunofluorescence and compared to WT surface expression. Prior to treatment with DMSO, glycerol and reduced temperature, A98P and L274P were not expressed at the plasma membrane, whereas the WT was. Following treatment with DMSO and decreased temperature there was a marked improvement in the trafficking of the mutants indicated by the increased surface staining resembling the WT staining. Glycerol was ineffective as a rescue treatment on both mutants.

e. Aquaporin-2 background and trafficking rescue:

Arginine vasopressin acts on V2R, which causes the movement of AQP-2 membrane protein water channel to move from endosomal vesicles to the apical membrane of the collecting duct cells in the nephron where water can subsequently be reabsorbed [57]. Malfunctions in AQP-2 cause autosomal nephrogenic diabetes insipidus (A-NDI), a rare form of NDI. Point mutations associated with A-NDI have

been identified and noted that when expressed in *Xenopus* oocytes trafficking was impaired; the mutants were retained within the ER. Based on immunoblot analysis of density gradient fractionated vesicles from WT or T126M AQP-2 transfected CHO cells (Chinese Hamster Ovary), 1 M glycerol changed the localization from the ER fractions to the endosome and plasma membrane fractions. These results were confirmed with immunofluorescence and functional analysis was performed. Measuring cell swelling in WT and T126M AQP-2 expressing CHO cells following a decrease in osmolarity of surrounding solution showed that T126M alone is non-functional demonstrating cell volumes resembling the negative control. However, when these cells were treated with 1 M glycerol, its apparent water transport rate increased to a value not significantly different than that of WT. In addition to glycerol, 2 % DMSO and decreased temperatures were tested for their affects on the trafficking of WT and T126M. DMSO was an effective treatment shown by decreasing ER staining of the mutant protein, T126M. Temperature, however was ineffective as a rescue treatment, in fact, growing MDCK cells expressing either WT or T126M at 26 or 30°C caused the accumulation of both proteins in the ER, whereas at 37°C, WT has the ability to traffic to the plasma membrane [57].

II - Materials and Methods

i - Cloning for kAE1

Plasmids were a gift from the Reithmeier Lab [58]. Retroviral plasmids pFBNeo-kAE1-HA557 or pFBNeo-kAE1-myc557 were constructed by shuttling the cDNA encoding the entire human kidney anion exchanger carrying an external HA or myc tag, respectively. Tags were inserted into the XhoI site of the retroviral expression vector pFBneo at site 557 (Stratagene, La Jolla, CA, USA). The pFBneo-kAE1HA557 or pFBneo-kAE1myc557 dominant and recessive mutants were constructed using Stratagene QuickChange site-directed mutagenesis kit and confirmed by automated sequencing (ACGT Corporation, Toronto, Canada).

ii - Cell Culture

In preparation of stable cells lines expressing kAE1 protein, HEK 293 cells grown to 30-50 % confluency in 75 cm^2 flasks were transfected with 4 μg each of pVPack – GP, pVPack-VSVG, and pFBNeo-kAE1-HA557 (stratagene) using the transfection reagent, FuGene 6 (Roche Diagnostics Corporation, Indianapolis, IN, USA). After 24-48 hours, the supernatant containing the virus was collected and filtered through a 0.45 μm filter removing cell debris, but not the virus. The virus plus 8 μg/ml polybrene were then added to MDCK cells grown in 25 cm^2 flasks at 30-50 % confluency and left for infection for 24 hours followed by the addition of selection

media containing 1 mg/mL of geneticin (G418, Sigma Aldrich). Cells stably expressed kAE1 protein for 2-3 weeks. MDCK cells are grown in DMEM F12 supplemented with Penicillin/Streptomycin (1 mg/ml) + 10 % FBS. HEK cells are grown in DMEM + Penicillin/Streptomycin (1 mg/ml) + 10 % FBS.

iii - Cell lysate

Cells were grown on a 10 cm dishes until confluent. Cells were collected in PBS (140 mL NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and spun down at 1000 rpm for 5 minutes. Lysis buffer was added (PBS, 1 % Triton X-100, 1/100 Proteinase Inhibitor (Sigma Aldrich, St. Louis, MO, USA)) and samples were put on ice for 15 minutes then centrifuged at 14000 rpm for 15 minutes at 4°C. Supernatant was collected and BCA assay was performed to determine protein concentration. 125 μL was collected and added to equivalent volume of Laemmli sample buffer. Samples of equivalent protein concentrations were run on 8 % SDS-PAGE gel, transferred to a nitrocellulose membrane and protein was detected using mouse anti-HA antibody (Covance, Emeryville, CA, USA).

iv - Cell surface biotinylation

MDCK cells expressing either kAE1 WT-HA or one of the kAE1 mutants, C479W-HA or G701D-HA, were incubated with 0.5 mg/mL of EZ-link NHS-SS-Biotin reagent (MJS Biolynx, Brockville, ON, Canada) for 15 minutes on ice in Borate buffer (10 mM

boric acid, 154 mM NaCl, 7.2 mM KCl, 1.8 mM $CaCl_2$, pH 9). Following, the reaction was quenched with 1X PBS with 1% Bovine Serum Albumin. Cells were lysed using RIPA buffer (1 % deoxycholic acid (w/v), 1 % Triton X-100 (w/v), 0.1 % SDS (w/v), 0.15 M NaCl, 1 mM EDTA, 10 mM Tris-Cl, pH 7.5) with 1/100 Proteinase Inhibitor and centrifuged. A sample of 50 μL was saved in 50 μL Laemmli sample buffer for Western blot. Remaining cell lysate was incubated with UltraLink Immobilized monomeric avidin (ThermoScientific, Rockford, IL, USA) for 1 hour at 4°C. Samples were then centrifuged and a second sample was saved for Western blotting. Beads were washed with RIPA three times and protein was eluted using Laemmli sample buffer. Samples were run on an 8 % SDS-PAGE gel, transferred to a nitrocellulose membrane and protein was detected with mouse anti-HA antibody (Covance, Emeryville, CA, USA) followed by a goat anti-mouse antibody coupled to horseradish peroxidase (HRP).

v - Co-immunoprecipitation

MDCK cells co-expressing kAE1 WT-HA/WT-myc, kAE1 WT-myc/C479W-HA, or kAE1 G701D-myc/C479W-HA were scraped into microcentrifuge tubes using 1X PBS and centrifuged at 1000 rpm for 5 minutes. Cells were lysed using 1X PBS, containing 1 % Triton X-100 and 1/100 Proteinase Inhibitor and incubated on ice for 15 minutes. Cells were then centrifuged again at 14000 rpm for 15 minutes. Supernatant was collected and a 50 μL sample was saved for Western blotting. Remaining cell lysate was incubated with rabbit anti-myc antibody (Santa Cruz

Biotechnologies, Santa Cruz, CA, USA) for 1 or 2 hours at 4°C. Protein G-Sepharose beads were added to the lysate and incubated at 4°C for 1 hour. Beads were centrifuged and a 50 μL sample was saved for Western blotting. The beads were washed three times using 1X PBS + 0.1 % Triton X-100. Protein was eluted using Laemmli sample buffer. Samples were electrophoresed on an 8 % SDS-PAGE gel, transferred to a nitrocellulose membrane, followed by detection using mouse anti-HA antibody (Covance, Emeryville, CA, USA) followed by a goat anti-mouse antibody coupled to HRP.

vi – Immunocytochemistry

MDCK cells expressing kAE1 WT-HA and/or one of the kAE1 mutants (C479W-HA, G701D-HA, R589H-HA) were grown on either glass coverslips or semi-permeable Transwell polycarbonate filters. Cells were fixed with 4 % Paraformaldehyde and quenched with 100 mM glycine in PBS (pH 2.95). They were then either permeabilized or kept intact followed by blocking with 1X PBS + 1 % BSA. Cells were then incubated with mouse anti-HA antibody (Covance, Emeryville, CA, USA), rat anti-HA antibody (Roche, Basel, Switzerland), rabbit anti-calnexin antibody (generous gift from David Williams, University of Toronto), rabbit anti-giantin antibody (Covance, Emeryville, CA, USA), or rat anti-E-Cadherin antibody (Sigma Aldrich, St; Louis, MO, USA) for 30 minutes. Following washing with 1X PBS, the cells were incubated with secondary antibody; anti-mouse Cy3 antibody (Jackson Immunoresearch Laboratories Inc. West Grove, PA, USA), anti-rat Cy5 antibody

(Jackson Immunoresearch Laboratories Inc. West Grove, PA, USA), anti-mouse-Alexa 488 antibody (Molecular Probes, Carlsbad, CA, USA), anti-rabbit Alexa-488 antibody (Molecular Probes, Carlsbad, CA, USA), or anti-rat Alexa-488 (Molecular Probes, Carlsbad, CA, USA). Samples were washed again and 4'-6-Diamidino-2-phenylindole (DAPI) (Invitrogen) was added for 5 minutes. Glass coverslips were mounted onto slides using mounting medium (DAKO North America Inc., Carpinteria, CA, USA). Samples were visualized using an Olympus 1X81 microscope equipped with a Nipkow spinning-disk optimized by Quorum Technologies using 60X objective lens (Guelph, ON, Canada).

vii – Immunoprecipitations

MDCK cells expressing either kAE1 WT-HA or one of the kAE1 mutants (C479W-HA G701D-HA, R589H-HA) were collected and lysed in PBS, containing 1 % Triton X-100 and 1/100 proteinase Inhibitor. Protein concentration was determined using a BCA assay followed by extraction of a 50 μl sample to be saved for western blotting. The remaining lysate was incubated with rat anti-HA antibody for 1-2 hours. Our protein of interest was precipitated using Protein G-Sepharose and eluted with Laemmli sample buffer. Western blots were performed in tandem, transferred to a nitrocellulose membrane and blottied with either mouse anti-HA antibody or rabbit anti-Calnexin antibody.

viii - SITS binding assay

MDCK cells expressing either kAE1 WT-HA or a kAE1 mutant (C479W-HA or G701D-HA) were collected and lysed using PBS, containing 1 % Triton X-100 and 1/100 Proteinase Inhibitor. Cell lysates were incubated on ice for 30 minutes, and then centrifuged and the supernatant was collected. 100 μl was preincubated with 100 mM H_2DIDS for one hour prior to incubation with SITS affi-beads [5]. Protein was eluted with Laemmli buffer and Western blot was run and transferred to a nitrocellulose membrane. Protein was detected using mouse anti-HA antibody followed by a goat anti-mouse antibody coupled to HRP.

ix - BCA assay

To determine protein concentration, two samples from each cell lysate sample were taken at different dilutions and added to 200 μl BCA solution (Thermo Scientific). Samples were incubated at 37°C for 30 minutes followed by the reading of their absorbance from which protein concentration was calculated, based on a BSA standard curve.

x - Chemical treatments

For concentration dependent experiments, MDCK cells grown in DMEM F12 supplemented with Penicillin/Streptomycin (1 mg/ml) and 10 % FBS were treated with 1 %, 2 %, 3 %, 4 % or 5 % glycerol in DMEM F12 with Penicillin/Streptomycin (1 mg/ml) and 10 % FBS for 16 hours. DMSO was added at concentrations of 0.5 %,

1 %, 1.5 %, and 2 % for 16 h to the DMEM F12 supplemented with Penicillin/Streptomycin (1 mg/mL) and 10% FBS. Cells incubated at 30°C were originally kept at 37°C until they were moved to 30°C for 48 h, 24 h, 16 h, or 4 h. For the Calnexin co-IP experiments MDCK cells were incubated with 1 % DMSO, 3 % glycerol, and 30°C for 16 h. For immunofluorescence cells were incubated with 1 % DMSO, 1 % glycerol, and 30°C for 16 h.

III - Results Part 1-Characterization of kAE1 mutants C479W and G701D

All of the experiments described were carried out in MDCK (Madin Darby Canine Kidney) cells. This model cell line was chosen because they are mammalian renal cells that can polarize forming a tight epithelial layer functioning similar to the collecting duct. They also do not express endogenous AE1. Furthermore, our protein constructs contain either an HA epitope or a myc epitope at position 557, which does not interfere with the proteins trafficking to the plasma membrane or its function. The results detailed in Chapter II are published in Chu *et al*, 2009.

i - Cell surface expression of kAE1 WT and mutants G701D and C479W

a. Cell surface biotinylation determining kAE1 presence at the plasma membrane:

In order for kAE1 to exert its function in the kidney, there has to be protein present at the plasma membrane. To determine if the two mutants of kAE1, G701D and C479W, can traffic to the plasma membrane in non-polarized MDCK cells in the same manner as the kAE1 WT, we first performed a cell surface biotinylation experiment to measure surface expression of the protein. Cells were grown to approximately 90 % confluency; biotinylating reagent was then added to intact MDCK cells expressing either kAE1 WT-HA or one of the kAE1 mutants, G701D-HA or C479W-HA before permeabilizing and precipitating biotin bound protein with streptavidin beads. Three samples were taken throughout the experiment: T, represents the total amount of cell kAE1 protein present; B, represents the amount

of kAE1 that was present at the plasma membrane that bound to the biotin reagent and that was precipitated; and S, represents the difference between total protein and the amount of protein precipitated. On the Western blot (figure 8), for kAE1 WT, we see two bands, a higher molecular weight band, which is kAE1 with a complex oligosaccharide glycosylation and a lower molecular weight band, which represents newly synthesized kAE1 that contains a high mannose oligosaccharide. The complex oligosaccharide corresponds to protein that has trafficked beyond the ER while high mannose oligosaccharide containing protein corresponds to protein within the ER.

For kAE1 WT, we see that there is surface expression as evidenced by the presence of the complex oligosaccharide band in the B fraction (figure 8). For the two mutants, however we do not see the presence of the complex oligosaccharide band in the B fraction (figure 8) suggesting that both kAE1 C479W and kAE1 G701D are not trafficked to the plasma membrane, but retained intracellularly.

b. Colocalization of WT or mutant kAE1 with ER marker, Calnexin:

To visualize where in the cell these two mutants reside, we performed immunofluorescence looking at the ER, the candidate organelle where mis-folded secretory or membrane-bound proteins are retained and subsequently degraded by ER-associated degradation (ERAD) [9]. MDCK cells expressing either WT or mutant kAE1 were grown to approximately 70 % confluency before they were fixed and permeabilized. Antibodies were used against the ER marker, Calnexin, and against

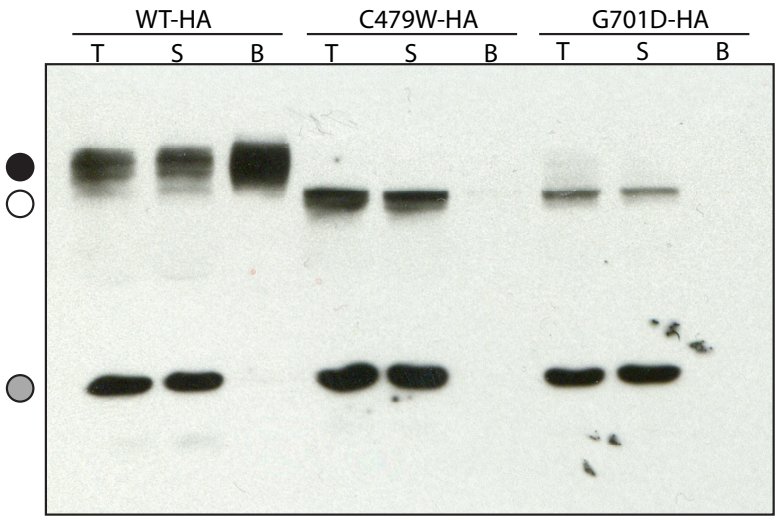

Figure 8. *Cell Surface Biotinylation of kAE1* : HA-tagged kAE1 expression in MDCK cells determining cell surface expression of WT, G701D, and C479W by cell surface biotinylation experiment. Biotin reagant was added to intact cells, they were then permeabiilized and biotin bound protein was precipitated using streptavidin beads followed by elution with 2x sample buffer. The total cell lysate (T) represents all AE1 present in the cell; The supernatant (S) represents all AE1 that was not precipitated by the streptavidin beads; and all AE1 precipitated is represented in the B fraction, which represents surface AE1 protein. The WTHA protein has 2 bands, the complex oligosaccharide form of the protein represents AE1 that has travelled beyond the ER (denoted by ●). The newly synthesized form of AE1 contains a high mannose oligosaccharide and represents AE1 that has not left the ER (denoted by ○). ◍ Denotes GAPDH, a loading control and an indicator of whether biotin reagent has leaked into the cells.

the HA tagged WT or mutant kAE1. We visualized the cells using an Olympus 1X81 microscope equipped with a Nipkow spinning disk and a 60X objective lens. Colocalization of kAE1 with the ER marker, Calnexin, is indicated by a yellow color.

kAE1 WT does not co-localize with Calnexin (figure 9a), as expected confirming cell surface biotinylation results of kAE1 WT trafficking to the plasma membrane. The newly identified mutation, kAE1 C479W, does co-localize with Calnexin (figure 9b) noted by the presence of yellow in the image, and kAE1 G701D does not co-localize with Calnexin (figure 9c). From the results of cell surface biotinylation and immunofluorescence, we can conclude that kAE1 C479W does not traffic to the plasma membrane because it is retained within the ER and kAE1 G701D although is not present at the plasma membrane at least during its maturation is transported further along on the membrane protein trafficking pathway beyond the ER as shown by its glycosylation bands in figure 8.

c. Colocalization of WT or mutant kAE1 with Golgi marker, giantin:

After a protein leaves the ER, it is transported to the Golgi apparatus where its high mannose sugar is converted to a complex oligosaccharide before it is transported to the plasma membrane to exert its function [9]. We looked next for co-localization between kAE1 WT-HA, kAE1 C479W-HA, and kAE1 G701D-HA with the Golgi marker, giantin following the same process as performed for colocalization with calnexin. kAE1 WT, again does not co-localize based on the absence of yellow staining (figure 10a), but has surface staining consistent with previous results.

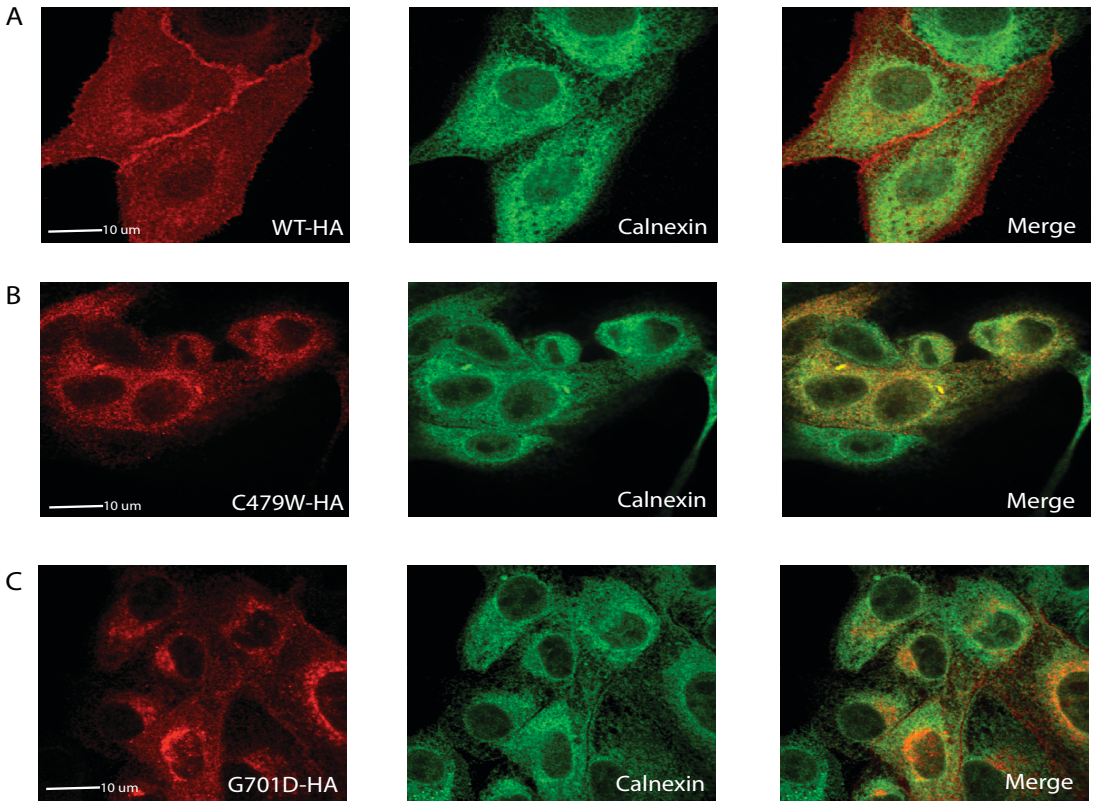

Figure 9. *Colocalization of kAE1 WT or mutants with the ER marker, Calnexin:* MDCK cells expressing WT-HA or mutant forms of kAE1 (C479W-HA or G701D-HA) were detected with rat anti-HA anitbody colocalized with the ER marker, Calnexin, which is detected with rabbit anti-calnexin. A) WT-HA when merged with Calnexin does not show colocalization. B) C479W-HA when merged with Calnexin does show colocalization. C) G701D-HA when merged with Calnexin does not show colocalization. Cy3 conjugated anti-rat and Alexa fluor-488 conjugated anti-rabbit secondary antibodies were used. Yellow in the merged image denotes colocalization between kAE1 and Calnexin. Detected by an Olympus 1X81 microscope with a 60X objective lens.

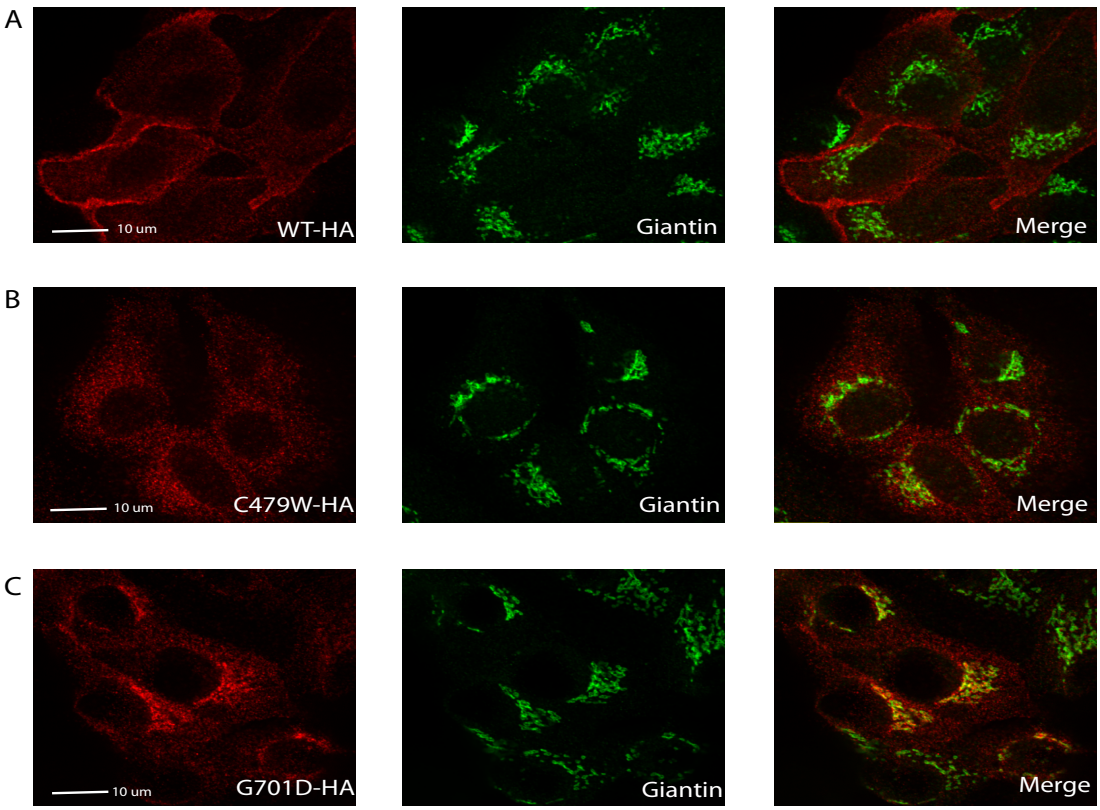

Figure 10. *Colocalization of kAE1 WT or mutants with the Golgi marker, giantin:* MDCK cells expressing WT-HA or mutant forms of kAE1 (C479W-HA or G701D-HA) were detected with rat anti-HA anitbody colocalized with the Golgi marker, giantin, which is detected with rabbit anti-giantin. A) WT-HA when merged with giantin does not show colocalization. B) C479W-HA when merged with giantin does not show colocalization. C) G701D-HA when merged with giantin does show colocalization. Cy3 conjugated anti-rat and Alexa fluor-488 conjugated anti-rabbit secondary antibodies were used. Yellow in the merged image denotes colocalization between kAE1 and giantin. Detected with an Olympus 1X81 microscope with a 60X objective lens.

kAE1 C479W does not co-localize with giantin (figure 10b) as expected because it does not appear to escape the ER. Lastly, kAE1 G701D does co-localize with the Golgi marker, giantin based on the yellow staining in the merged image (figure 10c). This is consistent with our results as there is no detectable protein at the plasma membrane, but it is not retained within the ER.

d. Localization of WT and mutant kAE1 in polarized MDCK cells:

Because the α-ICs of the kidney are polarized, it is important to note the trafficking of the mutants in polarized cells [21]. MDCK cells were grown on semi-permeable transwell plates until polarized, the cells were then fixed and permeabilized and antibodies were added to detect both kAE1-HA protein and E-Cadherin, a basolateral membrane marker protein.

Figure 11 shows that in polarized MDCK cells expressing kAE1 WT protein, as expected, there is co-localization between E-Cadherin and kAE1 WT evidenced by the yellow color in the image. For both of the kAE1 mutants, C479W and G701D, we do not see an overlapping of staining (figure 11), therefore we can conclude that neither of the above mutants traffic to the plasma membrane in polarized cells, substantiating the results we saw in non-polarized MDCK cells.

ii - Conformation of kAE1:

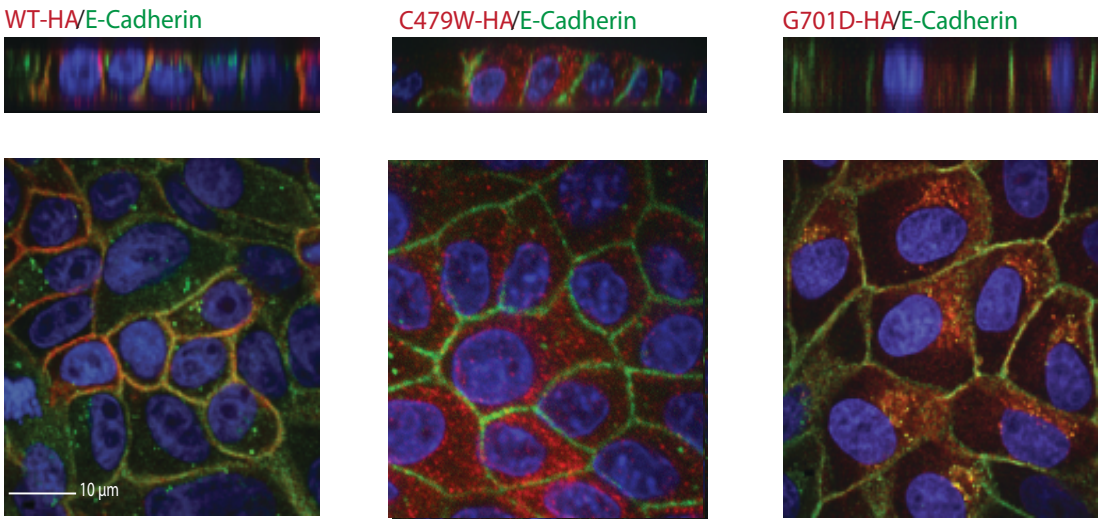

Figure 11. *Polarized MDCK cells expressing kAE1 WT or mutants colocalized with E-Cadherin, a basolateral membrane protein*: Cells were grown in semi-permeable transwell plates expressing either WT or mutant (C479W or G701D) protein. Cells were permeabilized then kAE1 was labeled with mouse anti-HA primary antibody followed by anti-mouse Cy3 (red). E-Cadherin protein was labeled with rat anti-E-Cadherin, followed by anti-rat Alexa 488 (green). Yellow denotes colocalization. Cells were visualized using Olympus 1X81 microscope. Upper image is side view of cells and lower image is top view of cells.

a. Binding ability of kAE1 with stilbene inhibitors as an indicator of proper tertiary structure:

kAE1 binds stilbene inhibitors and its ability to bind these inhibitors acts as a strong indicator of proper protein conformation because these inhibitors bind near the active site of kAE1 [59]. In this experiment we used H_2DIDS and SITS that are stilbene inhibitors known to inhibit kAE1 protein. For each of our MDCK cell lines expressing kAE1 WT, kAE1 C479W, or kAE1 G701D we separated them into two lysate samples, one pre-incubated with H_2DIDS before the addition of SITS beads and the other just had SITS beads added, without pre-incubation with H_2DIDS. AE1 binds both H_2DIDS and SITS beads with high affinity [59]. If the protein is properly folded, we expect that those pre-incubated samples will not bind SITS beads because they are already bound to soluble H_2DIDS. This is what we see for the WT kAE1 (figure 12). In our fraction incubated only with SITS beads (B) we see approximately the same quantity of protein as we see in the total cell lysate fraction (T). We also do see a very small quantity of protein in the sample pre-incubated with H_2DIDS (S). Looking next at the C479W mutant (figure 12), we see that the affinity of the SITS beads for the mutant protein is decreased as compared to the kAE1 WT when collating the B fraction with the T fraction. Experiments in *Xenopus* oocytes have shown that the kAE1 C479W is not functional at the plasma membrane [59]. For the kAE1 G701D mutant, we observe a decreased binding affinity to SITS beads compared to kAE1 WT (figure 12). We conclude then that the conformation of the kAE1 mutants, C479W and G701D is abnormal, which is substantiated by their decreased affinity for the stilbene inhibitor, SITS.

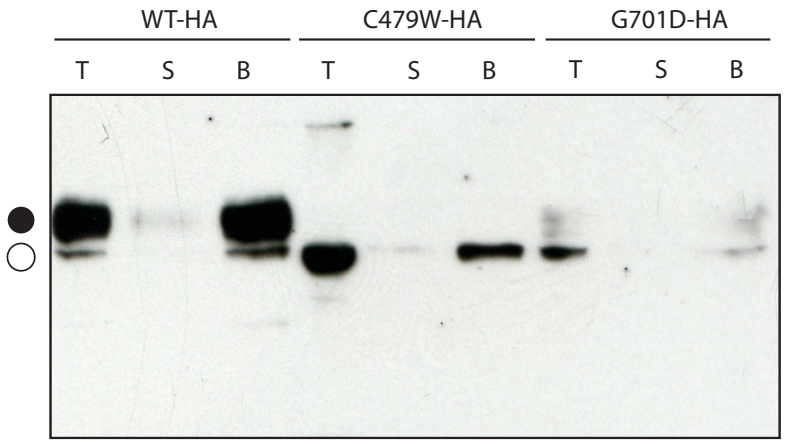

Figure 12. *SITS binding assay demonstrating mis-folding of kAE1 mutants*: MDCK cells expressing either WT-HA, C479W-HA, or G701D-HA were lysed. T fraction is total cell lysate, S fraction corresponds to cell lysate that was pre-incubated with H_2DIDS then incubated with SITS beads. B fraction corresponds to remaining cell lysate that was only incubated with SITS beads. Western blot was performed immunoblotting with mouse anti-HA detecting kAE1. ● corresponds to kAE1 with complex oligosaccharide, ○ corresponds to kAE1 with high mannose sugar.

iii - Co-expression of kAE1 WT with the kAE1 mutants C479W and G701D

a. Co-immunoprecipitation of kAE1 WT with each kAE1 mutant to look for dimerization:

To better understand how these proteins traffic in the kidney of the patient and her family, we mimicked the genotype of the father (WT/C479W), who suffers from HS and the genotype of the patient (G701D/C479W), who was diagnosed with both HS and dRTA. The first step was to ascertain whether the two mutants, which appear to have an irregular conformation, could form a dimer with either each other or if kAE1 C479W can form a dimer with kAE1 WT. To this aim, we introduced HA and myc tags into WT and mutant protein at position 557. Following the addition of rabbit anti-myc antibody to the cell lysate, we precipitated all protein that is bound to the primary antibody using Protein G beads. We then ran a Western blot using mouse anti-HA antibody to detect kAE1. We expect when we precipitate a myc-tagged protein and subsequently blot for an HA-tagged protein those mutants that can form dimers will appear as a band on the blot. If the mutant is unable to form a dimer, we will not see a band in the B fraction. Our control, kAE1 WT-myc/kAE1 WT-HA, shows that when we precipitate the kAE1 WT-myc (figure 13a, fraction B), we co-precipitate the kAE1 WT-HA, therefore we can conclude, as we would expect, that WT kAE1 could form a homodimer. Looking at kAE1 WT-myc/kAE1 C479W-HA, we can see that when we precipitate the kAE1 WT-myc, we also precipitate the kAE1 C479W-HA (figure 13a, fraction B). Similar results are

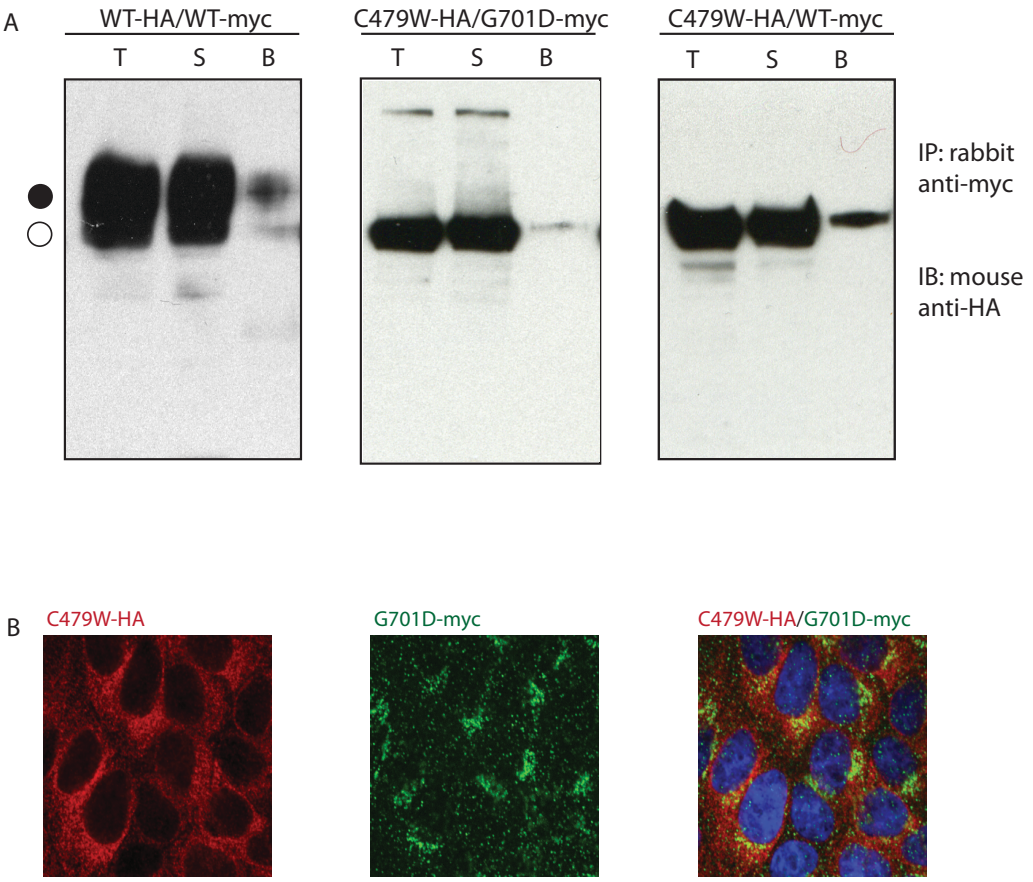

Figure 13. *Co-Immunoprecipitation of WT kAE1 with either WT or mutant kAE1, localization in MDCK cells of C479W and G701D*: A) MDCK cells expressing either WT-HA/WT-myc, C479W-HA/G701D-myc, or C479W-HA/WT-myc were precipitated using a rabbit anti-myc antibody followed by a Western Blot using mouse anti-HA antibody. T represents total cell kAE1 protein, B represents immunoprecipitated kAE1, and S represents the difference between total kAE1 and precipitated kAE1. ● corresponds to kAE1 with complex oligosaccharide, ○ corresponds to newly synthesized kAE1 with high mannose oligosaccharide. B) Non-polarized MDCK cells expressing G701D-myc and C479W-HA were stained with mouse anti-myc and rat anti-HA antibodies, followed by anti-mouse Alexa 488 (green) and anti-rat Cy3 (red) antibodies. Cells were visualized with an Olympus 1X81 microscope using a 60X objective. DAPI (blue) represents the nucleus.

seen for the kAE1 G701D-myc/kAE1 C479W-HA where the two proteins are precipitated together (figure 13a, fraction B).

In conclusion, in the case of the father, because kAE1 WT can form a heterodimer with kAE1 C479W, there is the potential for at least partial rescue of the mutant to the plasma membrane. In the case of the patient, kAE1 G701D can form a heterodimer with kAE1 C479W, however, because both are retained intracellularly it is expected that they would remain intracellular (figure 13b). This information will be important later when looking at methods of rescuing trafficking of the mutants to the plasma membrane.

b. Co-expression of WT kAE1 with WT or mutant kAE1 looking at surface versus total protein expression:

To visualize the interaction of our dually expressed proteins in MDCK cells, we used immunofluorescence and stained for surface versus total protein expression of our mutants. MDCK cells co-expressing either kAE1 WT-myc/kAE1 WT-HA, kAE1 WT-myc/kAE1 C479W-HA, or kAE1 G701D-myc/kAE1 WT-HA were grown to approximately 70 % confluency before they were fixed. Antibody was added to intact cells to detect mutant protein at the plasma membrane, as well as WT kAE1 in the control. This was followed by permeabilization of cells and detection of total kAE1, mutant and WT, within the cell using two different colours.

As a control, we stained surface and total protein of kAE1 WT-myc/kAE1 WT-HA (figure 14a) cells. As expected they are both expressed at the cell surface and in the merged image, they co-localize. We also tested the effects of kAE1 WT expression on the trafficking of each of the kAE1 mutants of interest, C479W and G701D. kAE1 C479W surface staining is absent (figure 14c). Therefore, we can conclude that there is no surface expression of kAE1 C479W and kAE1 WT does not rescue the trafficking of this mutant. What is interesting about this finding is that kAE1 C479W, although it is dominant for HS in the RBC, it is recessive for dRTA in the collecting duct of the kidney as the father, whom is a compound heterozygote with C479W/WT does not exhibit any symptoms of dRTA. Despite the evidence that shows that these two forms of kAE1 are interact, there must still be sufficient kAE1 protein at the basolateral membrane to stave off dRTA. Our other mutant of interest, kAE1 G701D, shows a more typical recessive pattern where the WT kAE1 can rescue the mutant trafficking to the plasma membrane as evidenced by the surface expression of the protein in MDCK cells when co-expressed with kAE1 WT (figure 14b).

What we learned about the new mutant, kAE1 C479W is that it is retained intracellularly within the ER and is not trafficked to the plasma membrane when co-expressed with kAE1 WT (figure 14 and 15) although these two forms of kAE1 do have the ability to form a dimer. However, because kAE1 C479W is recessive for dRTA in the α-IC, there must be sufficient kAE1 WT protein present at the plasma

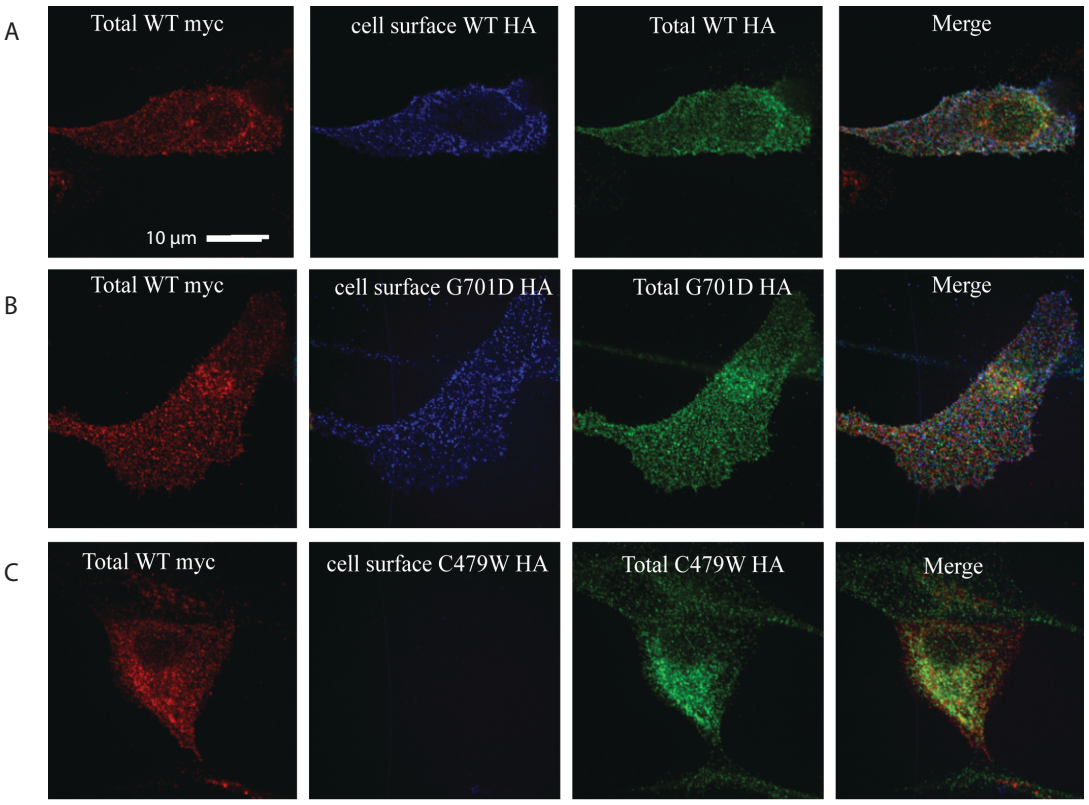

Figure 14. *Non-polarized MDCK cells co-expressing WT and WT or mutant kAE1 protein looking at surface versus total protein expression*: Detected surface protein using either rat anti-HA or mouse anti-myc. Cells were permeabilized and detected myc tagged protein with mouse anti-myc and HA-tagged protein using rat anti-HA. A) WT-myc surface expression versus total WT-HA expression, expression of WT-myc at the cell surface. B) G701D-myc surface expression versus total WT-HA expression, total G701D-myc shows surface expression of the mutant. C) C479W-HA surface expression versus total WT-myc expression, no surface expression of the mutant. Alexa fluor-405 and Alexa fluo-488 conjugated anti-rat and Cy3 conjugated to anti-mouse secondary antibodies were used. Detected using an Olympus 1X81 microscope with a 60X objective lens.

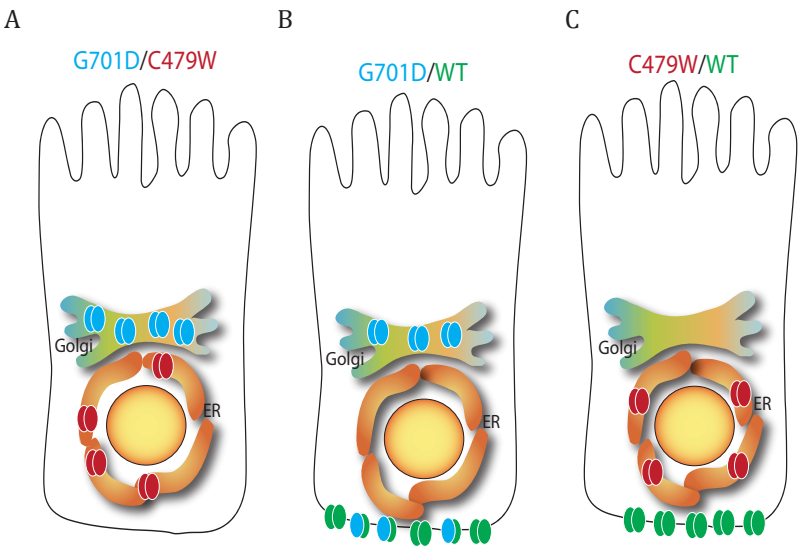

Figure 15. *Summary of localization of kAE1 dimers:* A) C479W homodimers (red) localize to the ER, G701D homodimers (blue) localize to the Golgi. B) G701D homodimers (blue) localize to the Golgi and G701D/WT heterodimers as well as WT heterodimers (green) traffic to the basolateral membrane. C) C479W homodimers (red) localize to the ER and WT homodimers (green) localize to the basolateral membrane. Although C479W can form heterodimers with G701D and WT, they do not escape the ER and are believed to be degraded quickly.

membrane to prevent the disease phenotype. As for kAE1 G701D, this mutant when expressed alone in MDCK cells is also retained intracellularly, however in the Golgi,

not the ER. It also has the ability to form dimers with kAE1 WT and kAE1 C479W and when expressed in MDCK cells with kAE1 WT, its trafficking can be rescued to the plasma membrane (figure 14 and 15). However, because both kAE1 G701D and kAE1 C479W are retained intracellularly, the patient has no kAE1 at the plasma membrane in MDCK cells and therefore suffers from dRTA (figure 13b and 15).

IV - Results Part 2-Trafficking rescue of mutants and interaction with ER chaperone, Calnexin

i - Trafficking rescue of the kAE1 mutants C479W, G701D, and R589H when treated with DMSO, Glycerol, and decreased temperature.

In an effort to find treatment for patients afflicted with HS and/or dRTA, we wanted to study the effects that small molecules have on the trafficking of these mutant AE1 proteins to the plasma membrane. Our inspiration came from other well-studied proteins CFTR, AQP-2, and V2R and the results that have been seen with the treatment of different small molecules and reduced temperature. Our results were quantified using image J software.

a. Effects of varying glycerol concentrations on the trafficking of WT or mutant kAE1:

We chose to test the previously characterized kAE1 mutants, C479W and G701D, for the effects of small molecules and reduced temperature; in addition we also included the previously characterized dominant dRTA mutation kAE1 R589H, whose kidney isoform is retained within the ER, but erythrocyte isoform traffics to the plasma membrane where it maintains its ability to exchange ions as shown in *Xenopus* oocytes [43, 44].

Beginning with MDCK cells expressing one of WT, C479W, G701D, or R589H forms of kAE1, we then grew them to confluency followed by cell lysis. We measured

protein concentration using a BCA assay and ran equivalent quantities of protein on an SDS-PAGE gel followed by a Western blot. Results depicting rescue of the trafficking of the mutants show an increase in the quantity of complex oligosaccharide kAE1 protein, which signifies escape of the protein from the ER to the Golgi where its high mannose oligosaccharide is converted to a complex oligosaccharide. For the glycerol condition, we used 1 %, 2 %, 3 %, and 4 % concentrations based on previous findings that 1 M of glycerol (equivalent to approximately 1 %) up to 4 % glycerol was effective in restoring trafficking of mutant V2R and AQP-2 protein [55, 57]. It was found that for the kAE1 WT, there was no noticeable increase in complex versus high mannose oligosaccharide bands of the protein (figure 16). What we found for the mutants varies depending on the mutant. The recessive mutant, kAE1 G701D, which is retained intracellularly in the Golgi, had increased complex band versus high mannose oligosaccharide under 1 % and 2 % conditions (figure 16). 3 % and 4 % glycerol was ineffective in its ability to rescue the trafficking likely because at these percentages, glycerol can be cytotoxic causing the separation of the cells from the flask surface. The other recessive mutant, kAE1 C479W, which is retained in the ER, was found to have no noticeable complex band under any concentrations of glycerol (figure 16). The third mutant, kAE1 R589H, is dominant and is also retained in the ER and under control conditions, exhibits no complex oligosaccharide band, however, under select glycerol concentrations, 2 % and 3 %, kAE1 R589H exhibits the presence of complex band (figure 16).

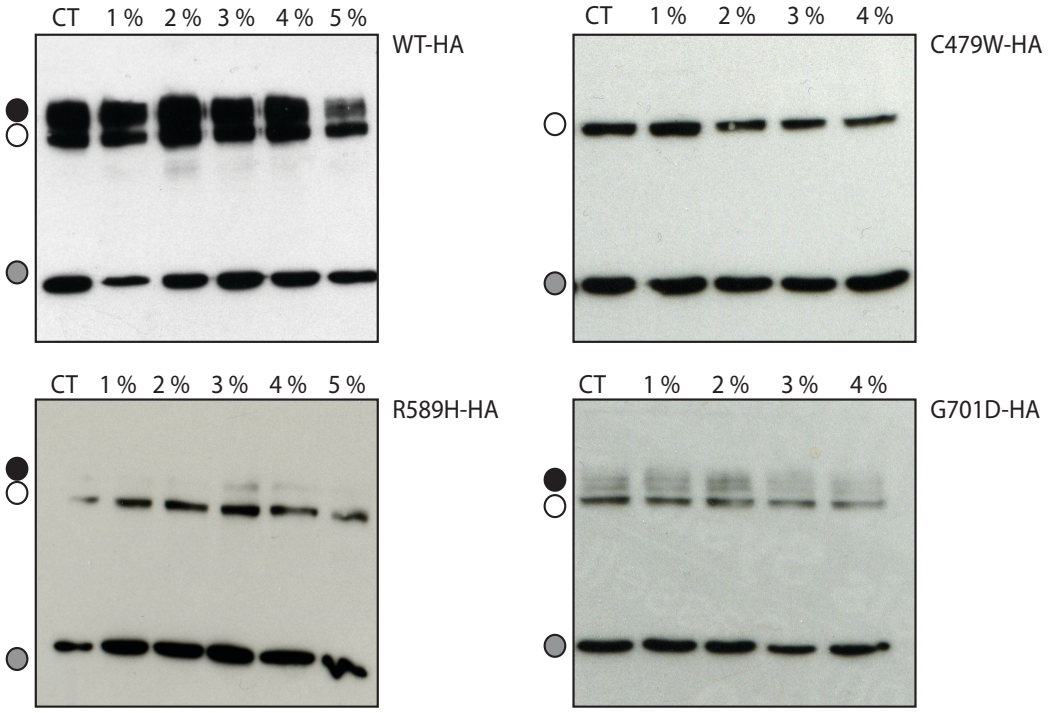

Figure 16. *kAE1 expressing cells treated for 16 hours with varying concentrations of glycerol*: MDCK cells expressing WT or mutant (C479W, G701D, R589H) forms of kAE1 treated for 16 hours 1 %, 2 %, 3 %, 4 %, or 5 % glycerol. Cells were lysed and western blot was performed. probed with mouse anti-HA antibody. ● represents complex oligosaccharide form of kAE1. ○ represents high mannose form of kAE1. ◍ represents GAPDH, a loading control. CT=control.

b. Effects of varying DMSO concentrations on the trafficking of mutant kAE1:

DMSO has also been shown to rescue trafficking of mutant proteins [55-57]. So, we next tested the effects of this small molecule on the dRTA mutants. We chose to test 0.5 %, 1 %, 1.5 %, and 2 % based on the literature that has shown that DMSO is an effective chemical chaperone at 1 % and 2 % concentrations [55, 56]. For kAE1 WT there was no change in quantity of complex kAE1 between control and the addition of all concentrations of DMSO (figure 17). For kAE1 G701D, we found that the amount of complex carbohydrate band does increase with the addition of all concentrations (figure 17). Again for kAE1 C479W (figure 17) we found no detectable complex kAE1 and for R589H, we found that under 1 % and 1.5 % DMSO treatments, there is a conversion of high mannose kAE1 to complex kAE1 (figure 17).

c. Effects of 30℃ temperature on the trafficking of the kAE1 mutants:

Our last testing condition was 30°C temperature for different time points, 48 h, 24 h, 16 h and 4 h. Previous work demonstrated that reduced temperature is effective in restoring trafficking of mutant membrane proteins when incubated between 6 hours and 21 hours. We found that the kAE1 WT, again, is unchanged (figure 18). kAE1 G701D shows an increase in the amount of complex oligosaccharide kAE1 band (figure 18). kAE1 C479W is, again, unchanged under all time points at 30°C (figure 18). Lastly, kAE1 R589H, like kAE1 C479W, shows no complex band at any of the time points (figure 18).

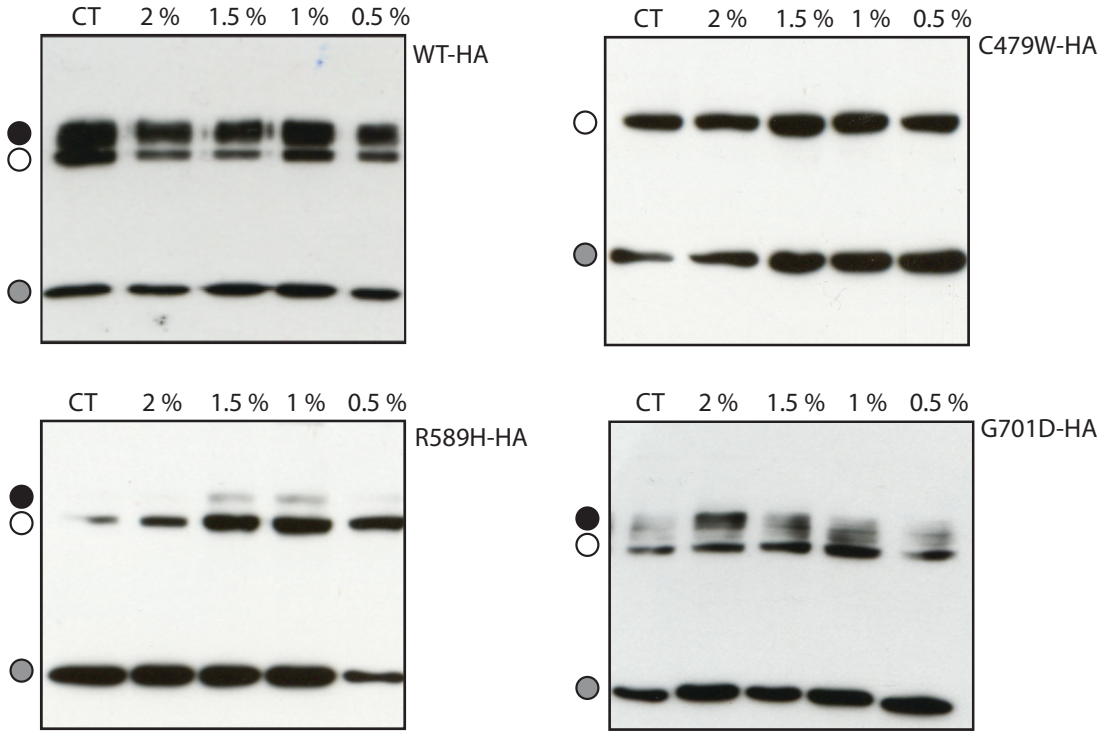

Figure 17. *kAE1 expressing cells treated for 16 hours with varying concentrations of DMSO*: MDCK cells expressing WT or mutant (C479W, G701D, R589H) forms of kAE1 treated for 16 hours with 2 %, 1.5 %, 1 %, or 0.5 % DMSO. Cells were lysed and western blot was performed. Probed with mouse anti-HA antibody. ● represents complex oligosaccharide form of kAE1. ○ represents high mannose form of kAE1. ◍ represents GAPDH, a loading control. CT = control.

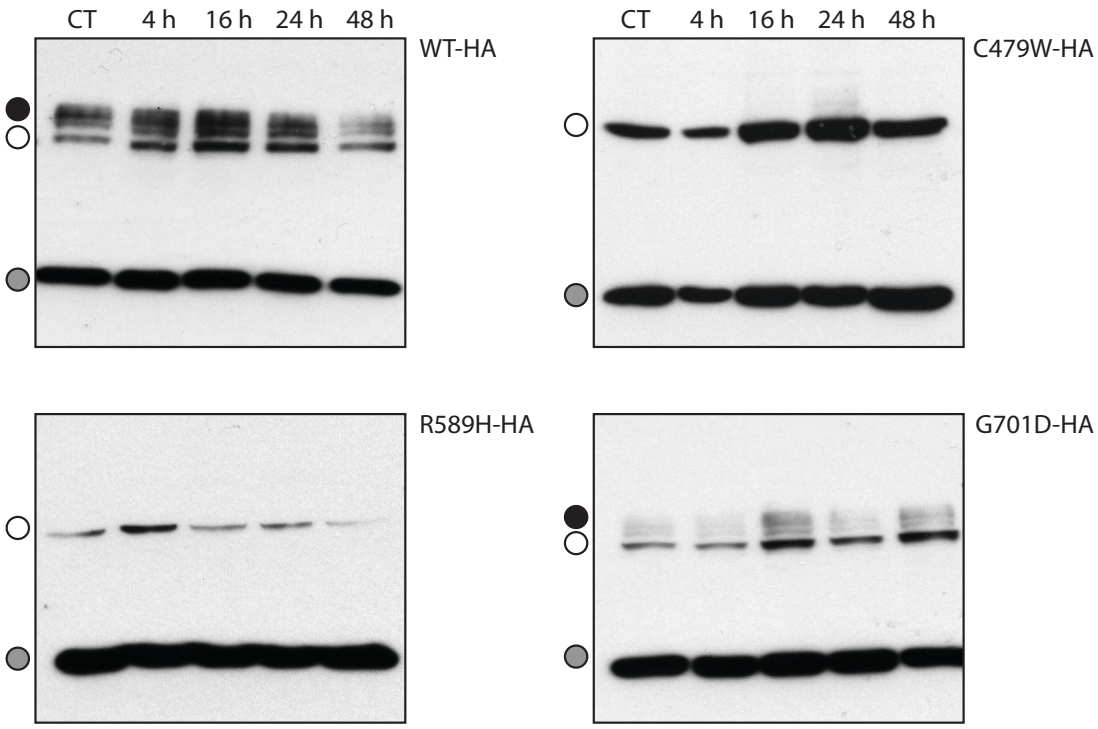

Figure 18. *kAE1 expressing cells incubated at 30˚C for varying times:* MDCK cells expressing WT or mutant (C479W, G701D, R589H) forms of kAE1 treated for 4, 16, 24, 48 hours at 30˚C. Cells were lysed and western blot was performed. Immunoblotted with mouse anti-HA antibody. ● represents complex oligosaccharide form of kAE1. ○ represents high mannose form of kAE1. ◍ represents GAPDH, a loading control. CT = control.

d. Histogram of trends of rescue for kAE1 WT and each of the kAE1 mutants:

When we combine the data from three independent experiments we can visualize the resulting trend of rescue of each of our treatments on each of the mutants. Figure 19 depicts the trends of rescue for kAE1 WT and each of the kAE1 mutants by quantifying the intensity of the complex oligosaccharide band versus total protein expression bands and converting the ratio into a percentage. The kAE1 WT protein in Western blotting showed no change in its trafficking under any treatment compared to control conditions. This is exemplified in the histogram (figure 19), which depicts approximately equivalent percentages of complex versus total protein expression. kAE1 C479W shows a similar trend as the kAE1 WT, showing no rescue by any treatments.

Based on the histogram, we can conclude for kAE1 G701D that DMSO is effective at increasing its trafficking. The same is true of all incubation periods at reduced temperature. However, for glycerol, 1 % and 2 % show improvement in trafficking, but 3 % and 4 % have the same percentage of complex versus total protein expression as the control. This lack of rescue could be attributed to glycerol's cytotoxic effects at high concentrations. kAE1 R589H proved to be a contender for the reception of small molecule rescue based on the observation that certain concentrations of DMSO and glycerol could increase its trafficking. Studying the trend depicted in the histogram, DMSO at concentrations between 0.5 % and 1.5 %, have the ability to increase the percentage of complex versus total protein expression. Same result was seen for glycerol at 2 % and 3 %. Reduced

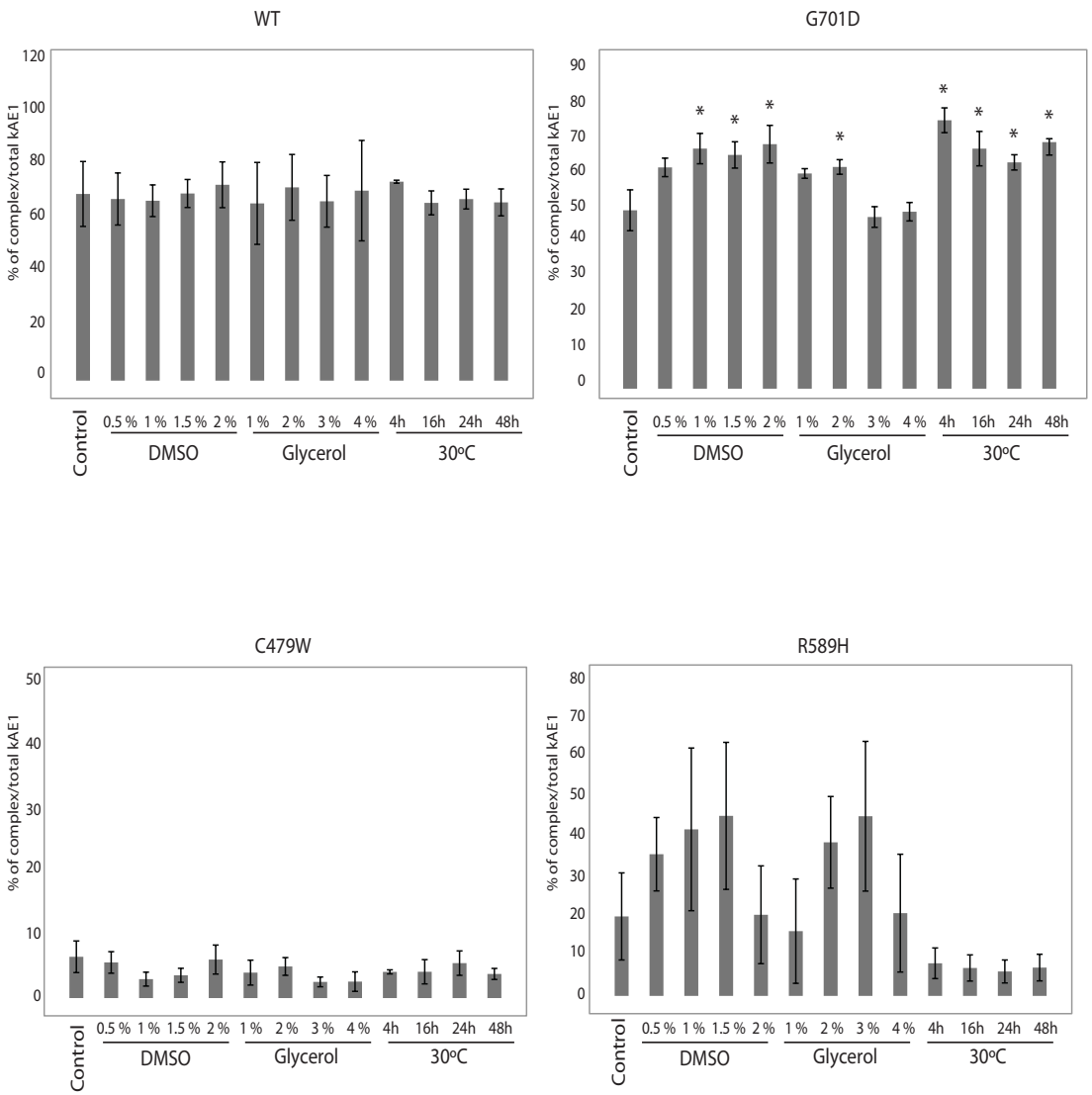

Figure 19. *Histograms showing rescuing trend of Glycerol, DMSO, and reduced temperature on mutants*: Amount of complex kAE1/total kAE1in MDCK cells under control conditions and varying concentrations of DMSO and glycerol, and varying incubation times at 30°C. Error bars, SD (n=3). * = statistically significant difference ($p \leq 0.05$).

temperature was an ineffective method of rescue for kAE1 R589H. It should be noted that for kAE1 R589H, the error bars are larger for this kAE1 mutant than the other kAE1 mutants. This is due to kAE1 R589H's low expression level in MDCK cells, which results in longer error bars.

e. Surface versus total protein expression of kAE1 WT and each kAE1 mutant following treatment of DMSO, glycerol, and reduced temperature:

The transition from a high mannose glycosylation to a complex oligosaccharide glycosylation occurs in the Golgi; therefore we cannot say for certain that an increase in complex band of kAE1 is associated with trafficking of the protein to the plasma membrane. To elucidate whether there is full trafficking rescue, we used immunofluorescence to visualize where our protein was being targeted under control conditions as well as under the influence of 1 % DMSO, 1 % glycerol and 16 hour incubation at 30°C. The conditions were chosen based on their ability to rescue the trafficking of kAE1 R589H, however due to 3 % glycerol's cytotoxic effects on MDCK cells, we used 1 % glycerol instead of 3 % glycerol. We began with the addition of rat anti-HA antibody to intact non-polarized MDCK cells to detect surface kAE1 WT or kAE1 mutant protein. This was followed by the addition of the secondary antibody, anti-rat Alexa 488 (green) (figure 20). The cells were then permeabilized and rat anti-HA antibody was added again, now to detect all kAE1 protein, both extracellularly and intracellularly. This was followed by the addition of the secondary antibody, anti-rat Cy3 (red). Under these conditions, improvement

in green staining depicts an increase in protein trafficking to the plasma membrane and red staining indicates expressed kAE1 protein.

Using an Olympus 1X81 microscope with a 60X objective, we were able to visualize our protein's location within the cell. For the kAE1 WT, as expected it was at the plasma membrane with some intracellular staining of newly synthesized protein (figure 20a). We observed no difference in surface staining of kAE1 WT protein under any of the rescue treatments (figure 20b-d). One of the promising mutants for these treatments, kAE1 G701D, showed very little surface staining under control conditions (figure 21a), however under treatment with 1 % DMSO, 3 % glycerol, and 30°C temperature, there is a distinct increase in surface staining of kAE1 G701D (figure 21b-d). Another promising protein for rescue is kAE1 R589H, which shows under control conditions, no surface staining of protein (figure 22a), however when treated with 1% DMSO, there is some rescue of the ER retained protein to the plasma membrane (figure 22b). The results for reduced temperature and 1% glycerol are similar to that of the control conditions, no noticeable kAE1 R589H at the plasma membrane (figure 22c and d). Lastly, kAE1 C479W, which showed no complex band on our western blot under control or treatment conditions, showed the same results with immunostaining. The control kAE1 C479W protein was retained intracellularly (figure 23a) as well the same results were seen for the three treatments (figure 23b-d).

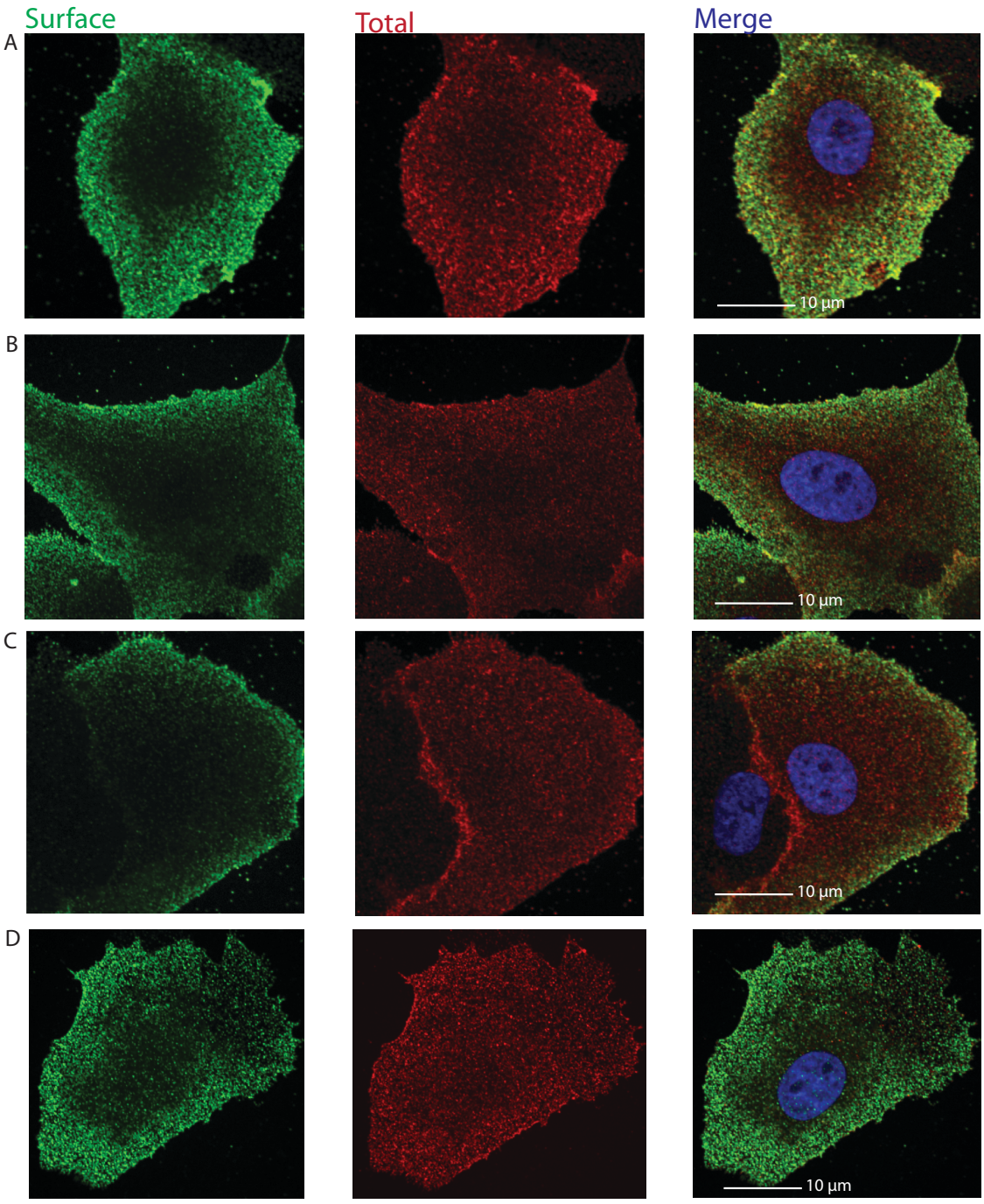

Figure 20. *Non-polarized MDCK cells expressing WT kAE1 showing surface versus total protein expression when treated with DMSO, glycerol or 30°C temperature*: non-polarized MDCK cells expressing WT-HA. Surface kAE1 protein was labeled with rat anti-HA followed by anti-rat Alexa 488 (green). Cells were permeabilized and again labeled with rat anti-HA followed by anti-rat Cy3 (red) and DAPI, which labels DNA (blue). A) Control cells, no treatment. B) Cells were treated with 1% DMSO for 16 h. C) Cells were grown at 30°C for 16 h. D) Cells were treated with 1% Glycerol for 16 h. Cells were visualized with an Olympus 1X81 microscope with 60X objective lens.

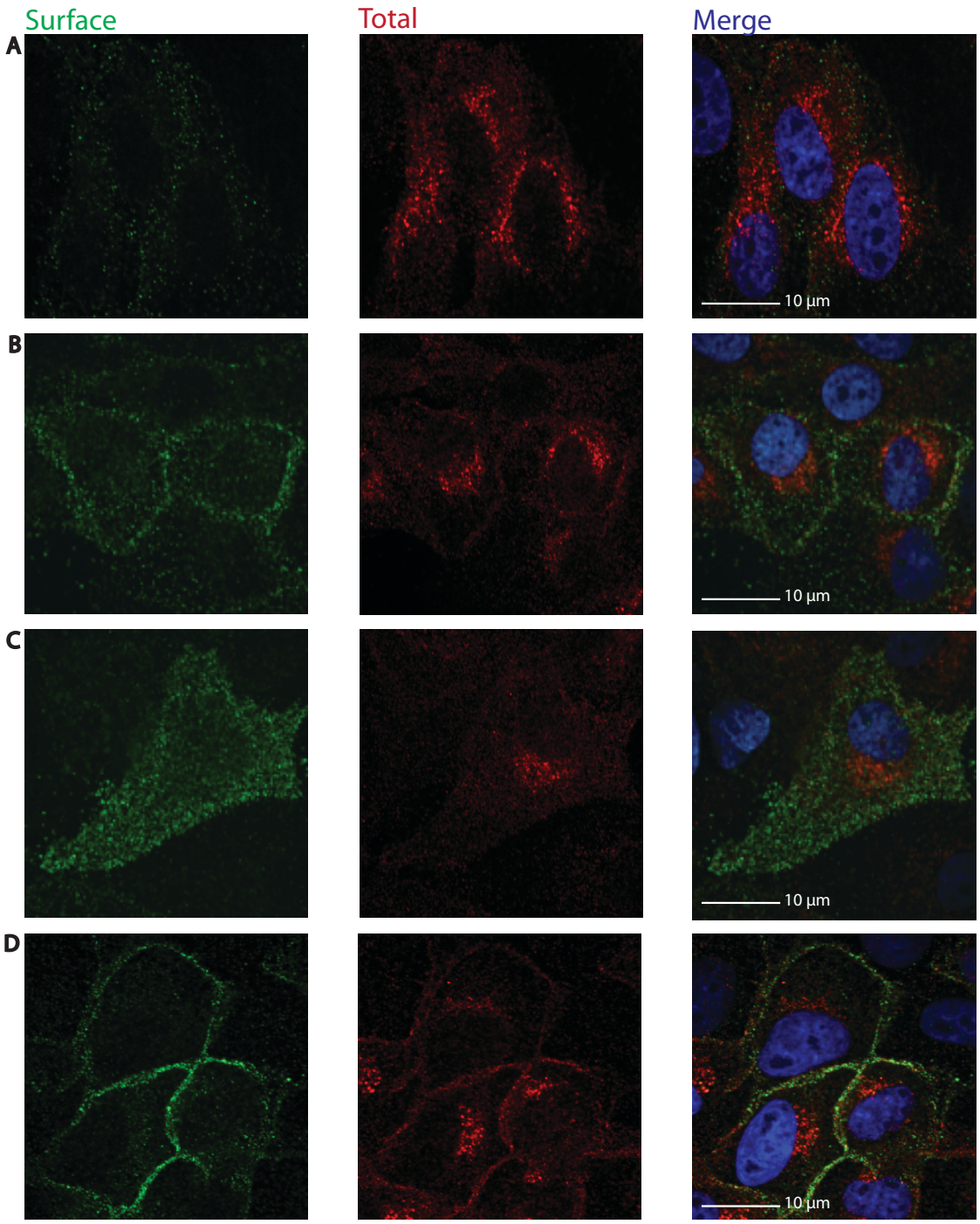

Figure 21. *Non-polarized MDCK cells expressing G701D kAE1 showing surface versus total protein expression when treated with DMSO, glycerol or 30°C temperature*: non-polarized MDCK cells expressing G701D-HA. Surface kAE1 protein was labeled with rat anti-HA followed by anti-rat Alexa 488 (green). Cells were permeabilized and again labeled with rat anti-HA followed by anti-rat Cy3 (red) and DAPI, which labels DNA (blue). A) Control cells, no treatment. B) Cells were treated with 1% DMSO for 16 h. C) Cells were grown at 30°C for 16 h. D) Cells were treated with 1% Glycerol for 16 h. Cells were visualized with an Olympus 1X81 microscope with a 60X objective lens.

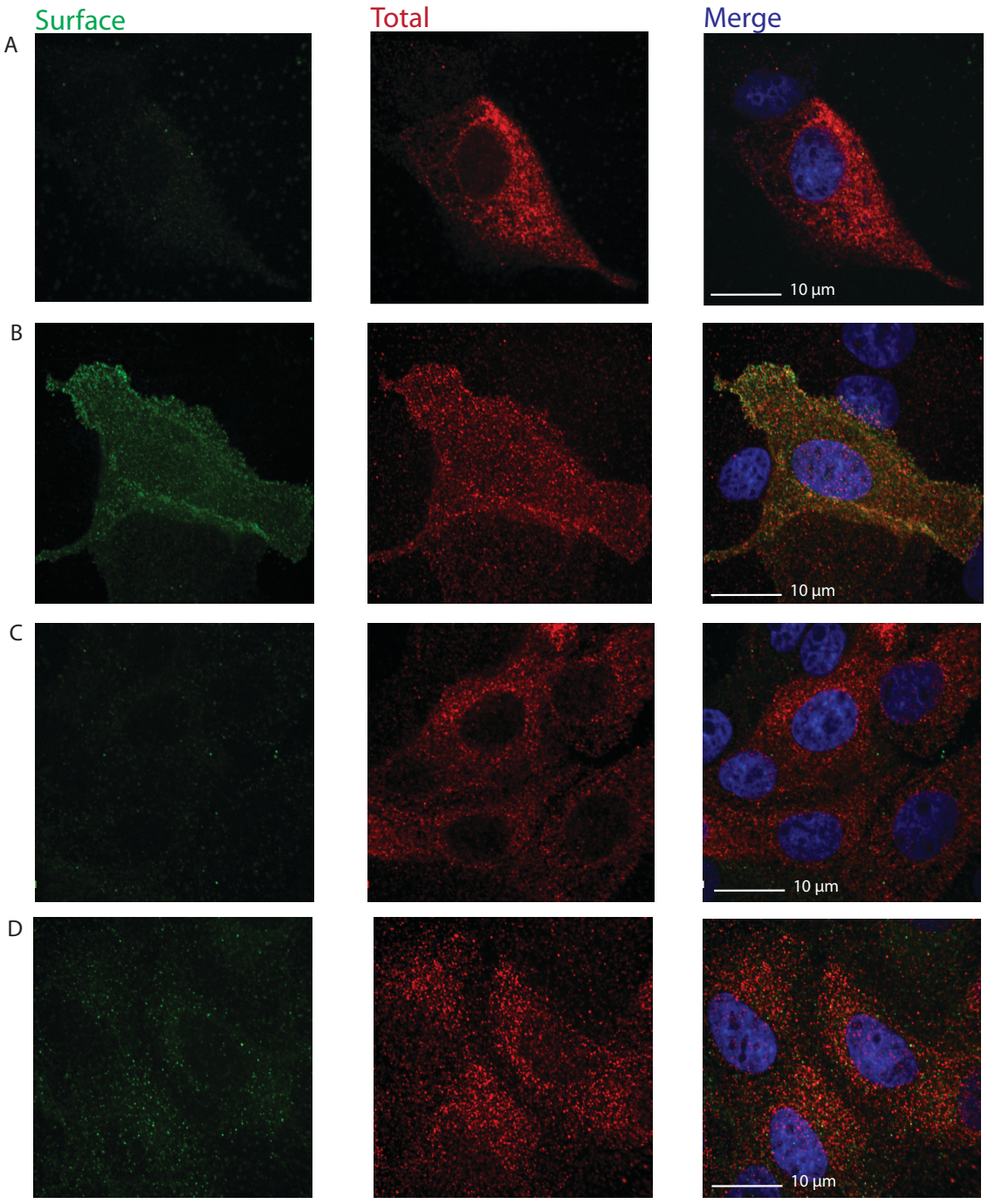

Figure 22. *Non-polarized MDCK cells expressing R589H kAE1 showing surface versus total protein expression when treated with DMSO, glycerol or 30°C temperature*: non-polarized MDCK cells expressing R589H-HA. Surface kAE1 protein was labeled with rat anti-HA followed by anti-rat Alexa 488 (green). Cells were permeabilized and again labeled with rat anti-HA followed by anti-rat Cy3 (red) and DAPI, which labels DNA (blue). A) Control cells, no treatment. B) Cells were treated with 1% DMSO for 16 h. C) Cells were grown at 30°C for 16 h. D) Cells were treated with 1% Glycerol for 16 h. Cells were visualized with an Olympus 1X81 microscope with a 60X objective.

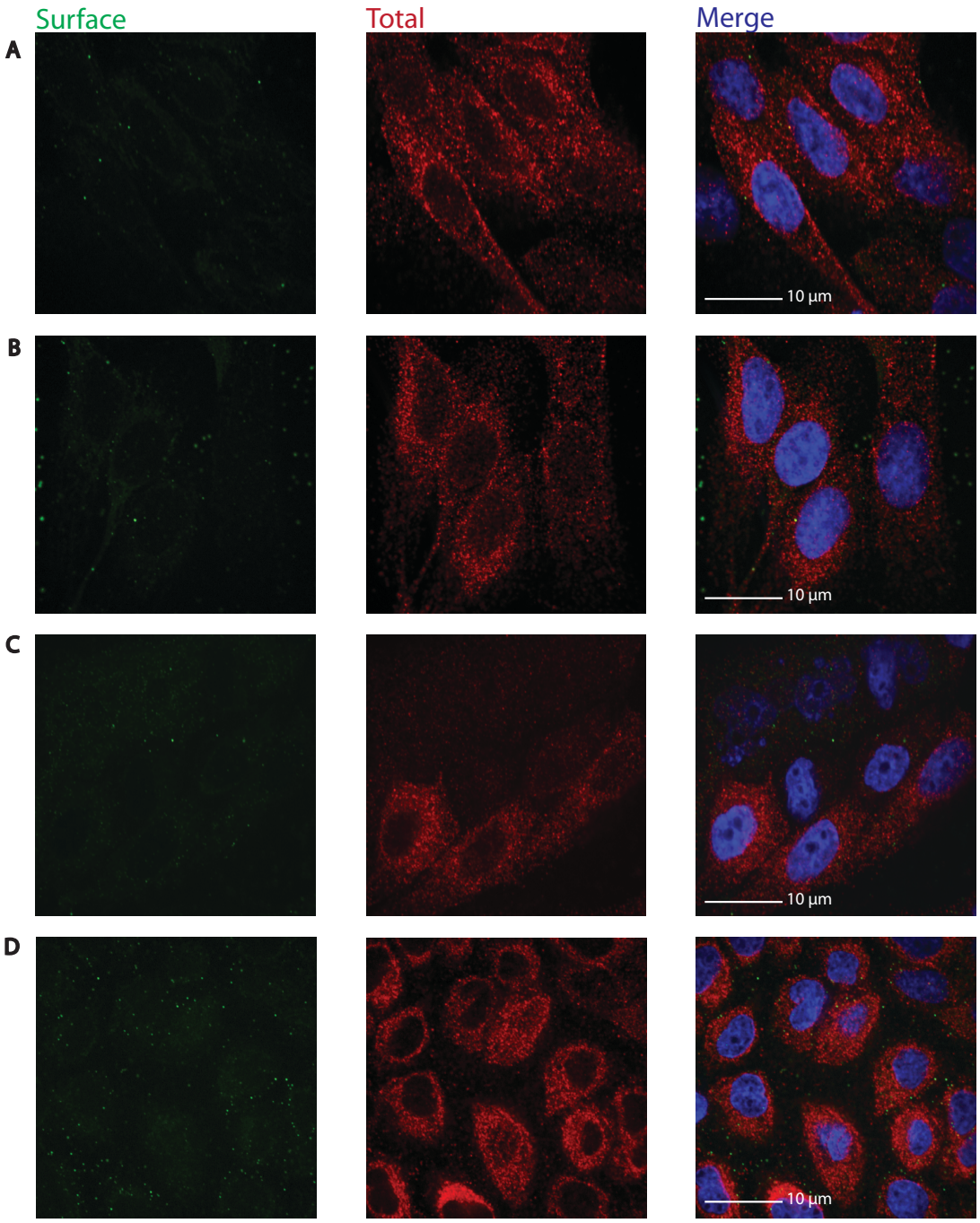

Figure 23. *Non-polarized MDCK cells expressing C479W kAE1 showing surface versus total protein expression when treated with DMSO, glycerol or 30°C temperature*: non-polarized MDCK cells expressing C479W-HA. Surface kAE1 protein was labeled with rat anti-HA followed by anti-rat Alexa 488 (green). Cells were permeabilized and again labeled with rat anti-HA followed by anti-rat Cy3 (red) and DAPI, which labels DNA (blue). A) Control cells, no treatment. B) Cells were treated with 1% DMSO for 16 h. C) Cells were grown at 30°C for 16 h. D) Cells were treated with 1% Glycerol for 16 h. Cells were visualized with an Olympus 1X81 microscope with a 60X objective.

So far we have seen that the kAE1 WT protein expression is unchanged under the treatment of Glycerol, DMSO, and lower temperature. Two of the kAE1 mutants, G701D and R589H, show promise in terms of increasing the trafficking of the protein to the plasma membrane. kAE1 G701D exhibits an increase in complex band under all treatments, glycerol, DMSO, and lower temperature; this was confirmed to be a rescue of trafficking to the plasma membrane by immunofluorescence, not just an increase in complex oligosaccharide protein. kAE1 R589H revealed to only be rescued by DMSO and glycerol and only under certain concentrations of these molecules. Temperature appears to have no effect. As for kAE1 C479W, so far no treatment has been shown to enable departure from the ER.

ii - Interaction of kAE1 with calnexin under treatment of 1 % DMSO, 3 % glycerol, and 30°C temperature for 16 hours.

Calnexin is an important ER-resident protein that functions, with calreticulin, to ensure the proper folding of glycoproteins [9]. A newly translated glycoprotein in the ER will enter the calnexin/calreticulin (CNX/CRT) cycle where the protein will undergo a series of glucose additions and removals by other proteins they recruit (figure 24); this cycle will continue until the protein is folded properly upon which it will be trafficked to the Golgi [9]. If the protein is unable to fold properly, it will be degraded in the cytosol [9].

a. Interaction of CNX with eAE1:

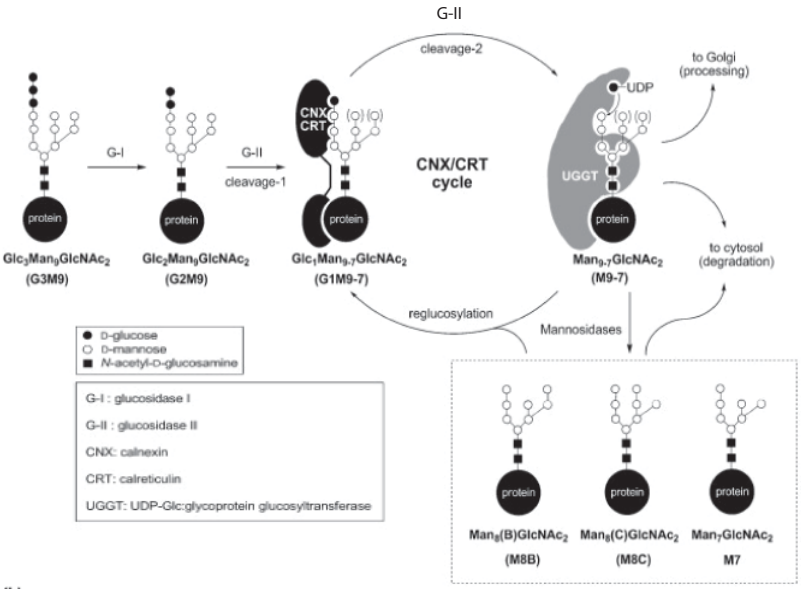

Figure 24. *Calnexin/Calreticulin cycle demonstrating the process of quality control in the ER for glycoproteins*: Membrane-bound glycoproteins have their folding monitored by the CNX/CRT cycle based on glucosylation. Properly folded proteins are further trafficked to the Golgi, those proteins that are mis-folded are either re-glucosylated and re-enter the CNX/CRT cycle, or they are targeted for degradation. [9]

The relationship between CNX and eAE1 has been recently reported using K562 cells (Erythroleukemia cells line). It detailed an interaction between CNX and eAE1 through co-immunoprecipitation, an interaction that was maintained with the two mutants that the authors studied, SAO and R760Q [60]. In cells lacking CNX there was no change in the trafficking of the kAE1 WT protein to the plasma membrane, but in MEF (Mouse Embryonic Fibroblast) cells deficient in CNX, the expression level of eAE1 was 75 % of MEF cells expressing CNX. Another interesting discovery was that the mutants did not have an enhanced interaction with CNX compared to kAE1 WT protein in K562 cells. This evidence indicates that eAE1 is a potential substrate for CNX.

We hypothesize that in MDCK cells, small molecule treatment may change the time of the interaction of kAE1 with CNX, therefore providing misfolded proteins a better chance of escaping the ER. To test this, we performed an immunoprecipitation of MDCK cells expressing one of kAE1 WT, kAE1 C479W, kAE1 G701D, or kAE1 R589H and treated for 16 hours with either 1 % DMSO, 3 % glycerol, or 30°C temperature, or they received no treatment at all. Cells were grown to confluency, then collected and lysed. Protein concentration was determined using a BCA assay. To precipitate kAE1 rat anti-HA antibody was added to bind HA-tagged kAE1. This was followed by incubation of the cell lysates with protein G beads. kAE1 was eluted using Laemmli sample buffer. Two Western blots were performed loading equal amounts of protein per lane. We then detected kAE1 and CNX on separate blots, using mouse anti-HA for kAE1 detection and rabbit anti-CNX for CNX detection.

Under control conditions, for MDCK cells expressing kAE1 WT protein, there appears to be no interaction with CNX (figure 25). This could be due to the interaction between kAE1 WT protein and CNX being too transient and therefore difficult to co-IP. For kAE1 C479W under all treatments, an interaction with CNX was observed as evidenced by the band in the B fraction of the Western blot (figure 25). For the control, we do not see any apparent CNX. kAE1 G701D interacts with CNX more strongly than kAE1 WT, however this interaction is not enhanced under any of the treatments, as shown in the B fraction (figure 25). For kAE1 R589H, we can see that in the B fraction of our Western blot there is an interaction between kAE1 R589H and CNX under treatment conditions (figure 25). Based on our hypothesis, we expect to see an interaction of kAE1 WT protein with CNX and to increase this interaction under small molecule treatment conditions. The same trend is expected for our mutants whose trafficking is enhanced by our chosen treatments.

b. Histogram depicting trend of interaction of kAE1 WT or kAE1 mutant protein with CNX:

When we combine data from 3 independent experiments we can see the resulting trend in the interaction between CNX and kAE1 protein. In parallel with Western blot detecting CNX, we ran the same samples on a separate gel and performed a western blot detecting kAE1 protein. From the two blots, we quantified the intensity of the co-immunoprecipitated CNX band as well as the precipitated kAE1 band (not shown) and represented this information in a ratio of CNX/kAE1 for each

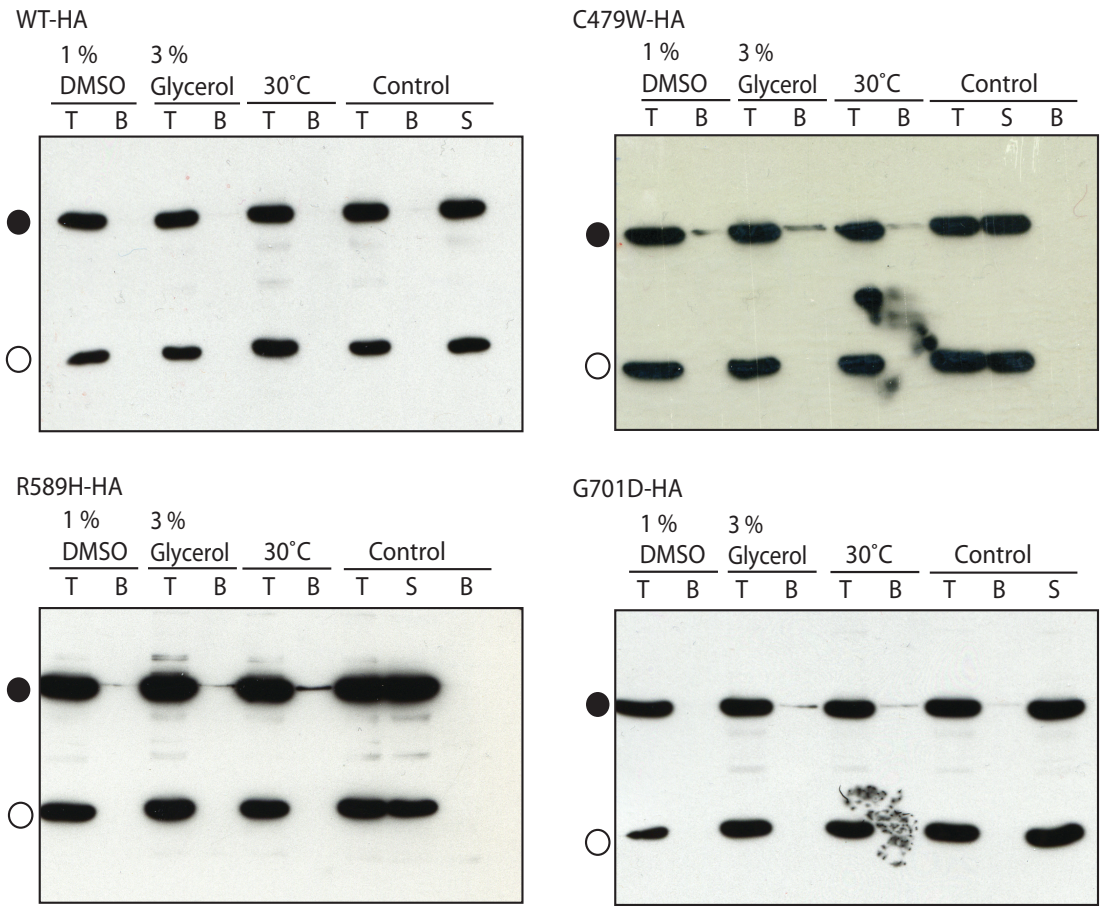

Figure 25. *Western blots showing calnexin co-precipitated by kAE1*: MDCK cells expressing WT or mutant AE1 were collected and precipitated with rat anti-HA antibody. Western blot was performed and immunoblotted with rabbit anti-calnexin (MW = 90 kDa) and mouse anti-GAPDH. T represents total expressed Calnexin, B represents Calnexin that was precipitated by kAE1, and S represents the difference between the T fraction and the B fraction.● represents calnexin, ○ represents GAPDH, a loading control.

condition and each mutant. For the kAE1 WT protein, its interaction with CNX is consistent throughout control and treatment scenarios (figure 26). kAE1 G701D exhibits an interesting trend with its interaction with CNX decreased under treatment conditions, however its interaction with CNX is still greater than that of kAE1 WT (figure 26). The remaining two mutants showed variation in their interaction with CNX. kAE1 C479W, a mutant that has thus far shown no trafficking beyond the ER, depicted an increased interaction with CNX compared to control conditions (figure 26). Conversely, kAE1 R589H showed an enhanced interaction with CNX under reduced temperatures and to a lesser extent treatment with DMSO (figure 26). For those kAE1 mutants that are rescued by small molecule treatment, their interaction with CNX is decreased. Conversely, those kAE1 mutants that are not rescued by small molecules show an increase in their interaction with CNX. In order to further the understanding of this relationship between kAE1 and CNX, additional experimentation is required.

What we know is that CNX does interact with kAE1, both mutant forms as well as WT forms. Whether this interaction is pertinent to the trafficking rescue of kAE1 is still not known. One important trend to note is that of CNX and kAE1 G701D. The treatments showed an increase in the trafficking of kAE1 G701D to the plasma membrane as shown in immunofluorescence. This in conjunction with the reduced interaction with CNX could be, at least in part, the reason for the increased trafficking of kAE1 G701D to the cell surface and therefore deserves more attention.

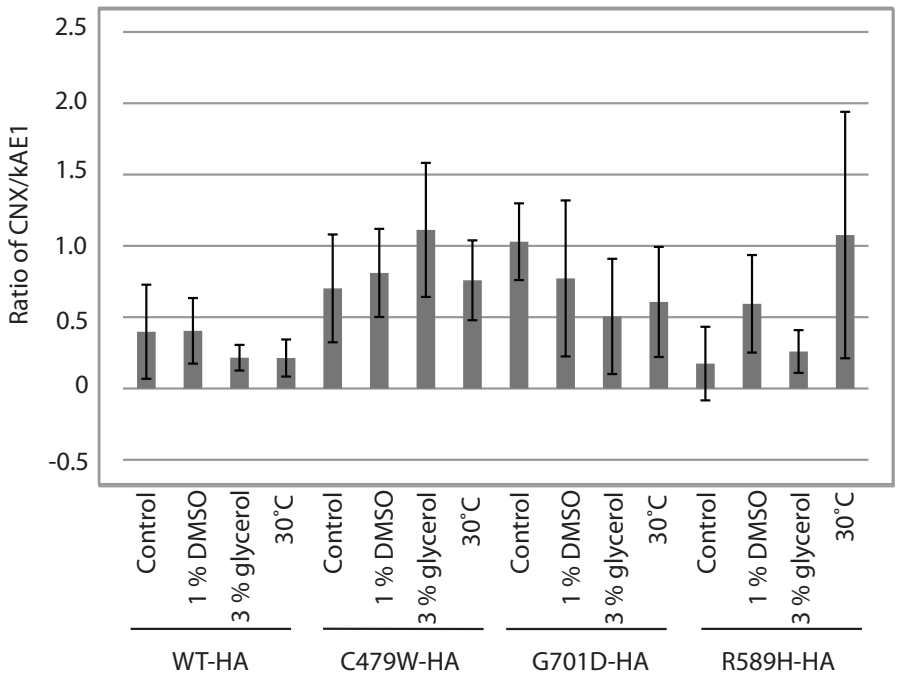

Figure 26. *Histogram showing the trend of interaction of WT kAE1 and each mutant with Calnexin:* MDCK cells expressing either WT or mutant kAE1 were treated with 1 % DMSO, 3 % glycerol, or 30˚C for 16 hours. Quantity of Calnexin is expressed as a ratio of quantity of kAE1. Error bars = SD (n=3).

V – Discussion

i - Characterization of the kAE1 mutants C479W and G701D

a. Classification and functionality of the kAE1 mutants, C479W and G701D:

In an effort to understand the cause of the disease state of the patient, we looked at the localization of the mutants in MDCK cells. There are three major classes of mutants to which they could be classified under: Class I mutants that result in prematurely truncated proteins, Class II mutants have impaired trafficking, and Class III mutants are full length proteins that have little to no function [61]. Utilizing cell surface biotinylation and immunofluorescence we were able to localize the newly identified kAE1 C479W mutant to the ER and discover that it is not expressed at the surface of MDCK cells classifying kAE1 C479W as a Class II mutant. A previously studied mutant, kAE1 G701D was localized largely to the Golgi and also displayed no surface expression exhibited by immunofluorescence, thereby classifying it as a Class II mutant as well.

Through SITS inhibitor binding we were able to confirm that these two mutant proteins are misfolded (figure 12) as evidenced by the inhibitor having reduced affinity for the mutants [59]. Functional experiments using *Xenopus* oocytes demonstrated that the mutant kAE1 G701D when co-expressed with GPA was able to traffic to the plasma membrane and once there, it was functional [62]. Similarly, the newly identified kAE1 mutant, C479W, was expressed in *Xenopus* oocytes in the

presence and absence of GPA; it was found that this novel mutant could traffic to the plasma membrane without the chaperone, GPA, however it remained functionally inactive at the plasma membrane [63].

b. C479's position within the membrane-spanning region of AE1:

Cysteine mutagenesis studies on eAE1 revealed that when all five Cysteine residues are mutated to serine, including C479, the protein remains functionally normal and can still traffic to the plasma membrane of HEK cells [64]. C479 is located in the putative transmembrane segment 3 and it has been suggested that it is a pore lining helix [60]; the substitution to serine conserves its hydrogen bonding abilities and explains the preservation of function of Cysteine-less eAE1's. However, when C479 is mutated to Tryptophan, a non-polar amino acid unable to hydrogen bond, the function of the protein is lost, corroborating the supposition that C479 could be a pore-lining residue.

c. kAE1 C479W and its role in HS and dRTA:

The interesting characteristic of the newly identified kAE1 mutant C479W is that it can cause both HS as well as dRTA, however it is dominant for HS and recessive for dRTA. This is exhibited by the phenotype of the father whom is heterozygous for kAE1 C479W with kAE1 WT, but only has HS and no kidney problems. A unique aspect of the patient is that HS and dRTA are normally mutually exclusive diseases; this is only the fourth case that characterizes a mutation that has the potential to cause both diseases in a single individual. The first mutation was Band 3 Coimbra,

or kAE1 V488M, which in its heterozygous state causes only HS and in its homozygous state causes HS and dRTA [65]. This patient requires regular blood transfusions as well as an oral bicarbonate supplement to thrive [65]. The relief of metabolic acidosis normalized the calcium in the urine and subsequently prevented further nephrocalcinosis. Expression of this mutant in polarized and non-polarized MDCK cells revealed that V488M is retained within the ER when expressed alone [66]. Additionally, interaction with kAE1 WT was limited as evidenced by co-IP and minimal co-localization of kAE1 WT with kAE1 V488M in immunofluorescence imaging. SITS affinity binding demonstrated that kAE1 V488M is not grossly misfolded, which in conjunction with the small amount of kAE1 WT/kAE1 V488M heterodimers formed appears sufficient to stave off dRTA. More eAE1 is probably required in RBC membranes due to its integral role in maintaining the biconcave shape explaining kAE1 V488M's dominant inheritance for HS. We see a similar pattern with kAE1 C479W. When in the heterozygous state with kAE1 WT, there appears to be sufficient kAE1 trafficking to the plasma membrane, but not adequate for eAE1's function in the RBCs.

kAE1 C479W also has the ability to form dimers with kAE1 WT and kAE1 G701D, as shown in co-IP experiments (figure 13a), however in immunofluorescence studies we do not see any colocalization between kAE1 WT and kAE1 C479W at the plasma membrane (figure 14). kAE1 C479W has a dominant effect on the kAE1 WT protein, although the father (C479W/WT) does not exhibit any dRTA phenotypes, therefore classifying kAE1 C479W as a recessive mutation for dRTA. We suspect that because

the only available protein at the plasma membrane is eAE1 WT homodimers, this quantity of protein is insufficient for eAE1's interaction with the cytoskeleton preventing the maintenance of the RBC shape. However, the approximately 25% of protein available in the father's kidney cells is enough to prevent the onset of dRTA.

The second mutation found to cause HS and an incomplete form of dRTA (displaying symptoms of dRTA only when the body is challenged with high acidity) in the homozygous state is Band 3 Courcouronnes, or kAE1 S667F, [67]. This patient received 11 blood transfusions before a splenectomy was performed at the age of 9 months. Acidosis was noticed, but partially receded after a few months and oral bicarbonate supplements were discontinued. The patient tested negative at 10 and 22 months for nephrocalcinosis. To understand this mutation, a functional assay was performed expressing kAE1 S667F in *Xenopus* oocytes, establishing no measurable activity. However, when expressed in this same cell line, but in conjunction with GPA, activity was noted, admittedly approximately 55% of normal kAE1 WT function. Interestingly, this change is not due to an increased presence of kAE1 S667F at the plasma membrane, but is due to GPA increasing the activity of the mutant. The same was seen when kAE1 S667F was expressed with WT in *Xenopus* oocytes, an increase in activity without an increase in surface expression. In MDCK cells, the majority of kAE1 S667F is retained within the ER of both polarized and non-polarized cells and to a lesser extent the Golgi and plasma membrane.

Most recently, a new mutation has been discovered, kAE1 E522K, that when in the heterozygous state with kAE1 G701D causes both dRTA and HS [68]. This patient requires daily bicarbonate supplements in addition to regular blood transfusions, but exhibits no nephrocalcinosis. When kAE1 E522K is expressed in MDCK cells alone, it traffics to the plasma membrane in non-polarized and polarized cells, albeit to a lesser extent, approximately half of that seen in kAE1 WT [68]. The mutant kAE1 protein was also able to traffic to the plasma membrane when expressed with kAE1 WT. When the genotype of the patient was mimicked in MDCK cells (E522K/G701D), no kAE1 was expressed at the cell surface. Pulse-chase experiments revealed that the half-life of kAE1 G701D is approximately five hours and that of kAE1 WT or kAE1 E522K is 11 hours [68]. When kAE1 G701D forms heterodimers with kAE1 E522K, kAE1 G701D decreases its stability, making the protein susceptible to degradation [68].

d. kAE1 G701D is retained intracellularly possibly due to a Golgi retention signal:

Our findings for kAE1 G701D agree with the findings of other researchers. kAE1 G701D is retained intracellularly when expressed in MDCK cells; its trafficking can be rescued by kAE1 WT protein explaining why the mother, whom is WT/G701D, exhibits no symptoms of either HS or dRTA. Through co-IP experiments, we discovered that kAE1 C479W and kAE1 G701D can form heterodimers (figure 13a), however immunofluorescence illustrated that each mutant still remained intracellular (figure 13b). In an effort to understand why kAE1 G701D is impaired in its trafficking, a series of substitution experiments have previously been

performed replacing the non-polar Glycine residue with one of four amino acids: Glutamic acid, another negatively charged amino acid, Arginine, a positively charged amino acid, or Alanine or Lysine, both of which are non-polar amino acids [33]. According to the current topology models of kAE1, G701D is located at the cytosolic end of transmembrane 9 [69]. The charged substitutions, G701E and G701R, were retained within the Golgi, same as G701D [33]. However, when Glycine is mutated to a non-polar amino acid, the protein was able to traffic to the surface of non-polar MDCK cells and the basolateral membrane of polarized MDCK cells. Work on the bovine β-1,4-Galactosyltransferase, a Golgi resident protein, revealed a possible correlation between transmembrane length and cell localization [70]. Proteins targeted to the plasma membrane have a longer hydrophobic transmembrane domain compared to proteins destined for the Golgi, which tend to have a shorter hydrophobic span. Mutating G701 to a charged amino acid decreased its hydrophobic span possibly creating a Golgi retention signal. This is supported by the observation that G701 being mutated to a different non-polar amino acid residue does not impair its trafficking to the plasma membrane in MDCK cells [33].

ii - Small molecule rescue of the kAE1 mutants C479W, G701D, and R589H

The second part of this project incorporated another mutant, kAE1 R589H. This mutant is a dominant mutation causing dRTA due to the kidney isoform of AE1 with the R589H mutation being retained within the ER of HEK cells [44]. Interestingly, eAE1 R589H can traffic to the plasma membrane in HEK cells and it retains its anion

transport function once there. Due to its dominant nature, the kAE1 R589H mutant cannot be rescued by co-expression with kAE1 WT protein, but was functional when present at the plasma membrane of *Xenopus* oocytes [43]. Although it does have minimal trafficking to the apical membrane of polarized MDCK cells, the majority of protein was retained within the ER [33]. Due to the potential that the kAE1 R589H mutant has for possible rescue, we chose to examine the effects of small molecules on this mutation in addition to the recessive kAE1 mutations we were currently studying, C479W and G701D.

a. Glycerol as a candidate for small molecule rescue:

Glycerol, a polyol that is suspected of working as a rescuing molecule by stabilizing proteins against denaturation [50], proved to be an effective treatment for the kAE1 G701D and kAE1 R589H mutants by increasing the quantity of complex oligosaccharide band of kAE1 (figure 16). kAE1 C479W remained within the ER; glycerol had no effect on the trafficking of this mutant (figure 16). Previously, glycerol proved to be a strong rescuer of protein trafficking for Cystic Fibrosis Transmembrane Conductance Regulator, Vasopressin 2 Receptor, and Aquaporin-2 [39, 40, 50]. For CFTR, high concentrations of glycerol were required in order to induce a positive change in the trafficking of the ΔF508 CFTR mutant in IB3 cells. The consequence of using high concentrations is it limits the application for use in humans. In addition, the 1 M concentration was toxic to the cells causing the majority of the cells to detach before the 24 hour incubation period had expired [50]. Indeed, with our own mutants we occasionally experienced this cytotoxic

effect of glycerol on our MDCK cells at concentrations of 3 % and 4 % (equal to 0.32M and 0.43M, respectively).

Certain mutations of the V2R membrane protein when expressed in MDCK cells, benefited from treatment with 4 % glycerol when incubated for 16 hours [55]. Of the nine mutants that were studied, only the V2R-V206D mutation showed a significant increase in the maturation of the protein, evidenced by the presence of the mature band of protein on an immunoblot. More recent research on V2R and small molecule rescue observed that 1 M glycerol was ineffective in rescuing three different mutations of interest when expressed in COS-7 cells [56].

A third protein with the potential of benefiting from small molecule rescue is AQP-2. This membrane protein resides in the collecting duct of the nephron where, under the influence of vasopressin, AQP-2 travels from vesicles to the apical membrane [57]. When ER retained mutants of AQP-2 are expressed in CHO cells and subsequently treated with 1 M glycerol, a redistribution of protein from the ER to endosomes and the plasma membrane is observed. We learned in studying the effects of glycerol on different proteins expressed in different cell lines containing different mutations, that glycerol appears to have a variable effect that is dependent upon the mutation studied and possibly dependent upon the cell line used.

b. DMSO as a candidate for small molecule rescue:

Robben *et al.* used the model cell line, MDCK, to study nine different mutations of V2R. They found that the effect of 4 % glycerol is mutation specific, rescuing the trafficking of only V2R-V206D, however they also looked at the effects of six other chemical chaperones including DMSO [55]. Their findings revealed that of the nine mutants, only V2R-V206D was susceptible to being rescued which included 1 % DMSO and Thapsigargin, an inhibitor of the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA), in addition to glycerol. This pattern of mutation specific rescue is evident in our research with kAE1. We found that the kAE1 mutation C479W is not prone to having its trafficking rescued by any of the tested methods (figure 19), including DMSO, whereas both R589H and G701D kAE1 mutations have both demonstrated a propensity to trafficking rescue by the chemical chaperones tested (figure 19). These findings strengthen the supposition that chemical chaperones act in a mutation specific manner.

c. Temperature as a candidate for trafficking rescue of mutant proteins:

Reduction in medium temperature is thought to promote ER-associated protein folding by conveying kinetic or thermodynamic stability, allowing for mutated proteins to fold in a manner resembling the wildtype protein form [51]. We found that when we subjected kAE1 WT and the kAE1 mutants to growth conditions at a reduced temperature, 30°C, the kAE1 G701D mutant had an increase in its complex oligosaccharide band (figure 18) leading to trafficking of the protein to the plasma membrane as evidenced by immunofluorescence (figure 21). This follows the trend seen for both V2R mutants (A98P, L274P, and R113W) and ΔF508 CFTR in that

decreasing temperature appears to be a potent rescuer of mutant protein trafficking [51, 56].

Our evidence suggests that the ability of a mutants trafficking to be affected by small molecules, such as DMSO and glycerol, or reduced temperature is largely mutation specific and appears to be independent of dominant versus recessive mutations. kAE1 C479W, a recessive mutation with dominant characteristics based on the lack of rescue of trafficking by kAE1 WT, was unable to be rescued by any of the chosen treatments. kAE1 G701D and kAE1 R589H however showed that despite one mutation being recessive and the other dominant, both were susceptible to treatment, thus improving the ratio of surface protein to total protein. To further understand the effects of rescuing treatment, it would be beneficial to look at the effects of these treatments on the mutations studied in a different cell line. This could tell us if the ability to rescue trafficking of mutants is not only mutation specific, but perhaps also cell line specific. It would also be helpful to quantify transport activity of the mutants under control and treatment conditions. In the kidney, these treatments could be beneficial in a patient if there is increased activity at the plasma membrane.

d. Calnexin as a possible target for treatments by changing its chaperone activities:

Calnexin is an ER resident chaperone protein that plays an integral role in Calnexin/Calreticulin (CNX/CRT) cycle (figure 24). Because of CNX's role in assisting the folding of membrane proteins within the ER, it has been postulated

that the knock out of CNX would allow mutant proteins to escape the ER to the plasma membrane where they can exert their function [71]. A study utilizing CNX with a deletion of the amino acids 185-520 showed an increase in the amount of ΔF508 CFTR at the plasma membrane of CHO (Chinese Hamster Ovary) cells without interrupting their ability to interact [71]. It was also demonstrated that the mature form of ΔF508 CFTR that could traffic to the plasma membrane was functional, however not to the same extent as WT-CFTR. What is crucial here is the ability of the mutant CFTR to escape the ER in order to traffic to the plasma membrane. CNX associates with newly synthesized WT and mutant CFTR, but maintains this association for the lifetime of the mutant whereas WT is able to escape its association with CNX allowing it to move to the Golgi for further processing [72].

There is some debate in the field however, that perhaps CNX is only involved in the efficient folding of WT protein and that ERAD of mutant proteins is CNX-independent by occurring before the CNX/CRT cycle [73]. Using RNAi to down regulate CNX in CHO cells, the maturation of both WT and ΔF508CFTR was not effected, however the processing of the WT CFTR to its mature form was hindered from 30 % to 20 %. In a second model, again using CHO cells however in this case ones with a knockout (KO) of CNX, the ΔF508 CFTR quantity and maturation were unchanged compared to normal CHO cells [74]. In addition, the amount and maturity of WT-CFTR decreased from CHO cells to CNX-KO CHO cells. These results

were supported in functional assays that confirmed no change in ΔF508 CFTR in CNX-KO CHO cells and decreased WT-CFTR activity in the same cell line.

The classical view of CNX is that it functions as an ER chaperone. Evidence has shown that although CNX interacts with partially folded proteins in a transient manner based on its glucosylation status, it is its interactions with other ER-resident chaperones that confers CNX's ability to retain misfolded proteins and to act at targeting those misfolded proteins for degradation [75]. CNX may act as a chaperone itself by possessing a polypeptide-binding site that allows it to bind to soluble proteins, lacking any glycosylation. It is its role as a chaperone that has inspired us to test its effects on the trafficking of kAE1 mutant proteins.

In our work with kAE1, we looked at the interaction between CNX and either WT or mutant kAE1 in MDCK cells through the use of co-immunoprecipitation in control or treated (1 % DMSO, 3 % glycerol, 30°C for 16 h) cells. We chose CNX as a candidate chaperone from the ER based on the fact that CNX is the only chaperone that was demonstrated to physically interact with AE1 protein [76]. Based on our results we were able to see a trend in the interaction of kAE1 with CNX (figure 26) in the steady state. kAE1 WT appeared to have a transient or weak interaction with CNX based on the inability to co-IP CNX with kAE1 WT under control conditions as well as treatment with our trafficking rescuers.

The kAE1 mutants displayed a different trend; kAE1 C479W and kAE1 G701D increased their interaction with CNX under control conditions, while kAE1 R589H displayed similar control conditions to the kAE1 WT. The interaction of kAE1 G701D decreased with treatment, that also allowed plasma membrane trafficking of the mutant; kAE1 C479W and kAE1 R589H had an increased interaction with CNX under certain treatments, specifically those that do not promote trafficking rescue to the plasma membrane. The results with kAE1 G701D support the theory that decreasing CNXs involvement in the folding of proteins could increase the trafficking by allowing the protein to escape the ER [71].

For the other two kAE1 mutants, however, we see an increase in their interaction with CNX under control and treatment conditions compared to kAE1 WT. This agrees with our original hypothesis that increasing CNX's chaperone activities could increase the trafficking of our mutants by allowing them to fold properly. In order to make any conclusions about the interaction between CNX and kAE1 further experimentation is required. It would be beneficial to look at the time-dependence of this interaction using pulse-chase. Although we see some kAE1 mutants increasing their interaction with CNX, is this interaction shorter or longer than that seen in kAE1 WT or under control conditions? It would also be useful to look at the effects of trafficking of kAE1 mutants in MDCK cells when CNX is decreased through the use of siRNA. These experiments combined will give a broader picture as to the relationship between kAE1 and CNX.

VI– Conclusions

We were able to characterize the newly identified kAE1 C479W, which we discovered, like other previously described mutants, is retained within the ER. Mimicking the genotype of the patient (C479W/G701D) who suffers from HS and dRTA and the genotype of the father (WT/C479W) who suffers from HS, we were able to look at the interaction of kAE1 C479W with kAE1 G701D and kAE1 WT to observe how the behavior of kAE1 C479W can be changed. Expression with kAE1 G701D had no impact on the trafficking kAE1 C479W protein, maintaining a membrane with little or no kAE1 present. kAE1 WT, although able to form a heterodimer with kAE1 C479W, did not improve its trafficking. From this we can conclude that for the patient, in both RBCs and kidney cells, she does not have any functional AE1 at the plasma membrane leading to the onset of HS and dRTA. As for the father, the homodimers of kAE1 WT that traffic to the plasma membrane appear to be sufficient for function in the kidney, however insufficient for its function in the RBC.

We also studied the ability of chemical chaperones to improve the trafficking of these mutants for the purpose of developing future pharmacological therapies for the dRTA patients. kAE1 G701D proved to be a strong candidate for future rescue therapy demonstrated by its ability to be rescued by glycerol, DMSO and low temperatures. kAE1 C479W was quite the opposite, proving that the methods chosen are insufficient to rescue its trafficking, leaving the opportunity for more

options to be tested. Although in *Xenopus* oocytes it was noted that kAE1 C479W was non-functional [63], there is still hope that if the trafficking can be improved, perhaps the mutant could interact with the cytoskeleton in RBC thereby reversing HS. We also looked at a dominant kAE1 mutant, R589H, which is retained within the ER and is known to cause dRTA, but not HS. The fact that glycerol and DMSO were able to improve its trafficking in MDCK cells also makes it a contender for future pharmacological therapy. As well, our results support the theory that chemical rescue is mutation specific.

In addition to small molecules, we hypothesized that a possible reason for retention within the ER is the interaction between the mutant and the chaperone protein, Calnexin. We noticed a trend that was mutation specific. kAE1 G701D decreased its interaction with CNX under treatment conditions consistent with its escape from the ER and subsequent trafficking to the plasma membrane. kAE1 C479W and kAE1 R589H under conditions that did not improve trafficking to the plasma membrane increased their interaction with CNX. It should be noted that the interaction of the mutants with CNX was greater than that seen with kAE1 WT. This could indicate a possible pathway of our mutants such that in future study, this interaction could be key to the trafficking of kAE1.

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