ROLE OF A CONSERVED AMINO ACID MOTIF
IN LOCALIZATION OF HUMAN CLIC5
TO MICROVILLI

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ABSTRACT

Hearing is one of the five senses and is important for survival and enrichment of life in numerous species. It is a complex and intricate biological process that is dependent upon the proper formation and maintenance of many structures. One of these structures is the hair bundle, known as the organelle of hearing. The hair bundle is composed of organized stereocilia, which are actin based projections from the apical surface of hair cells in the cochlea. I focus on the role of the protein Clic5, a member of the Clic family, which localizes to the base of stereocilia and is necessary for hearing in mice. This family is evolutionarily conserved and contains a highly conserved C-P-F-[S/C] motif that is also found in larger families such as the thioredoxin and glutathione S-transferase superfamilies. Because of its conservation and role in redox reactions within the larger families, I have targeted this motif for mutagenesis to determine its role in localization.

I have shown that the conserved CXXS motif in human Clic5 plays a critical role in the localization of Clic5 to microvilli, an actin based structure that can be used as a model for stereocilia. This was done by inducing mutations into the motif and examining the effects on the protein’s localization to microvilli in cultured LLC-PK1 cells. Within the motif, Cys32 is crucial for localization to microvilli, while Ser35 is not necessary for this localization. I have also shown that a S35C mutation, with or without the presence of Cys32, does not have an apparent effect on localization of Clic5 to microvilli.
This thesis is dedicated to my mentor

Dr. Soichi Tanda

Who has helped me every step of the way.

His patience, dedication, and enthusiasm for teaching

has opened up amazing opportunities for me

and provided me with a bright future in medical research.
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INTRODUCTION

I. Importance of Hearing

Hearing is necessary for conventional communication between many animals, and those with hearing loss suffer substantial hindrances in their everyday lives. In numerous species, it affects processes like mating and alerting to the presence of predators, which are crucial to fitness and survival. Although less crucial to our survival, hearing serves significant and life enriching roles in humans. It is important in learning of speech and language, alerts us to danger, allows us to interact and communicate easily, and aids in relaxation.

Hearing loss in humans can occur at any age and has many different causes and forms. Around 250 million people worldwide suffer from hearing impairment, about 50 percent of which has a genetic basis.¹ In the United States, the National Institute on Deafness and Other Communication Disorders (NIDCD) reports that approximately 17 percent of adults experience some form of hearing loss, while other estimates claim that nearly 60 percent of adults age 70+ experience prebycusis (late onset hearing loss).¹ NIDCD also estimates that 26 million Americans are afflicted with hearing loss from exposure to intense noise, such as loud music or working conditions. Regardless of the cause, hearing impairment is prevalent in both children and adults. It can inflict social and economic burdens that affect not only the individuals, but their families, communities, and the countries they live in as well. This impediment of communication may hinder the individual’s progress in school,
work, and life as a whole, and the subsequent cost of special education and the
government’s disability payments places a financial burden on the economy.

Hearing loss is grouped into three categories by the American Speech-
Language-Hearing Association (ASLHA): conductive, sensorineural, and mixed
hearing loss. The groups are organized by what portion of the auditory system is
damaged. Conductive hearing loss encompasses problems within the outer and middle
ear, such as infection, blockage, or malformation, and can usually be fixed with
medication or surgery. Any damage involving the inner ear is classified as
sensorineural hearing loss, which includes hereditary malformations as well as damage
caused by illness, age, head trauma, and loud noise. The inner ear consists of the
cochlea and vestibular systems for hearing and balance, respectively. It is located
within a hollow cavity in the temporal bone of the cranium, making it difficult to
isolate and study in humans. According to the ASLHA, sensorineural hearing loss is
the most common type of permanent hearing loss. This is because it is inherently
difficult to study and treat, therefore medical and surgical cures are not available for
most forms.

II. Hair Cells

A primary cause of sensorineural hearing loss is malfunction within the
cochlea, known as the organ of hearing. The cochlea is a spiral shaped, fluid-filled
cavity with three main compartments: the scala vestibule, scala media, and scala
tympani. The scala media holds the Organ of Corti, which is composed of a variety of
cell types (Figure 1). My research focuses on hair cells, which are the sensory cells of hearing. Humans have between 15,000 and 30,000 of these highly specialized polarized epithelial cells that are named for the hair-like projections, called stereocilia, on their apical surface (Figure 1). Hair cells are responsible for the detection and transduction of sound from mechanical stimulation in the cochlea to electrical impulses in the sensory neurons of the brain. In mammals, hair cells are virtually unable to regenerate, so damage from loud noise, injury, or disease is permanent and irreparable with current medical capabilities.

**Figure 1. Structures of the mammalian ear.** (A) Outer, middle, and inner ear of a human with the spiral shaped cochlea (organ of hearing) labeled. (B) Cross section of the cochlea showing the three fluid filled cavities and the Organ of Corti located within the scala media. (C) Structure of the Organ of Corti showing position of hair cells in relation to basilar and tectorial membranes. (D) Apical view of hair cells showing arrangement of stereocilia and an electron micrograph showing the apical surface of a mouse hair cell. Note the characteristic V shape of the hair bundle (Figure from Brown et al. 2008).

There are two types of hair cells: inner hair cells (IHCs) and outer hair cells (OHCs). Although structurally similar, the two types of hair cells have very different
functions in auditory processing. There is a single row of inner hair cells, which are the primary sensory cells transmitting auditory information to the brain through afferent neurons. The three rows of outer hair cells outnumber inner hair cells, and primarily function to increase hearing sensitivity. Rather than afferently transmitting information to the brain, outer hair cells receive information from the brain via efferent neurons. They have the ability to move in response to the electrical stimulation, which alters movement of the basilar membrane and affects inner hair cells sensing. This leads to amplification and tuning to specific external stimuli.²

III. Stereocilia

Stereocilia project from the apical surface of hair cells into the scala media of the cochlea, where they contact the tectorial membrane and cochlear fluid (Figure 1).¹ On each hair cell there are 3-4 rows of stereocilia of varying heights, organized in a staircase like manner. Altogether, the stereocilia of each hair cell are called a hair bundle, and each hair bundle exhibits a characteristic V or W shape (OHCs) or a slight arch (IHCs). Within the hair bundle, each stereocilia is connected to its neighbors by several linkers, which help maintain the cohesion of the bundle. The hair bundle is plane polarized, meaning its spatial orientation atop the hair cell is tightly controlled. The precise arrangement of the stereocilia within the hair bundle, and of the hair bundle itself, is specifically regulated, evolutionarily conserved, and necessary for proper auditory function.²
The hair bundles are mechanosensory organelles that are a core component of the auditory apparatus. Each stereocilia tapers at its base, which increases the bending in response to fluid movements caused by sound vibrations. When deflection of the hair bundle is in the direction of the tallest row of stereocilia, the movement opens mechanically gated ion channels, allowing an influx of K+ from the cochlear endolymph that depolarizes the cell. The depolarization subsequently causes a receptor potential and release of neurotransmitters from the basal end of the hair cell. The neurotransmitter binds to receptors on postsynaptic neurons which generates neural impulses that the brain can interpret as sound.3

IV. Actin

Stereocilia’s function is highly dependent upon their structure. Actin, a highly conserved protein, is key to proper stereocilia structure. Actin is found in nearly every eukaryote and is important for the survival of these cells.4 It has a wide array of functions in cell structure and movement, and is one of the three major cytoskeletal elements in eukaryotic cells. Its role as a structural protein makes its regulation critical in many cells like hair cells, whose function depends on proper structure. Actin exists as a globular monomer (G-actin) that can spontaneously polymerize into long filaments (F-actin) (Figure 2). Because each polar G-actin monomer orients itself in the same direction, the F-actin filaments are polarized with a plus end and a minus end. In structures like microvilli and stereocilia the filaments undergo a constant treadmilling, with polymerization occuring at the plus end and depolymerization at the
minus end. In order for growth to occur, the rate of monomer addition to the plus end must exceed the rate of removal at the minus end, and for a constant length to be maintained the rate of depolymerization must equal the rate of polymerization. The formation, length, and stability of these filaments is regulated and controlled by over 100 accessory proteins including molecular motors, actin binding proteins, and crosslinking proteins. 4

![Figure 2. Structure of actin.](image)

In stereocilia, each projection is internally supported by a paracrystalline array of actin filaments. 2 At the stereocilia base, only the most central filaments (rootlets) of the densely packed actin protrude into the cell body. The rootlets interact with the cuticular plate, a semi rigid meshwork of actin that lies just below the apical surface of
the hair cell. Of particular interest in regard to stereocilia are the proteins that determine and maintain actin filament length, facilitate its interaction with the plasma membrane, and form the unique tapered base.

**V. Models for Studying Stereocilia**

The location of human hair cells within the skull makes it difficult to explore their development, ultrastructure, and molecular components, so other models and methods have been used to enhance our understanding. Several non-mammalian vertebrates, such as frogs, turtles, chickens, and zebrafish, are used\textsuperscript{2,5}; experimentation with mice may be most common and helpful to understanding hair cells in humans.\textsuperscript{1} At a cellular level, because of their basic structural similarities, microvilli are often used as a model for stereocilia.

Although unique to the inner ear, stereocilia are similar to other actin-rich cellular projections such as microvilli and filopodia (Figure 3).\textsuperscript{6} All three structures exhibit actin treadmilling, which includes polymerization of actin at the tip and depolymerization at the base. However, the stereocilia itself is a stable lasting structure, whereas filopodia can fully disassemble and reform.
During development, stereocilia begin as precursor microvilli. The apical surface of each hair cell is initially covered with precursor microvilli, which through a complex signaling process migrate and arrange to form mature hair bundles (Figure 4). As with all actin-based projections, the microvilli are polarized with the filaments plus ends at the tip and minus ends at the base. This polarization is maintained during their development into stereocilia. The precursor microvilli must go through stages of elongating, thickening of the actin core, tapering of the base, and orienting spatially in order to form a fully functional hair bundle. The elongation and thickening of each actin bundle occurs through polymerization at the plus end of the filaments and addition of filaments around the original actin bundle, respectively. Once the hair
bundle is fully formed, excess microvilli are resorbed into the hair cell so that stereocilia are the only remaining projections on the apical surface.

Figure 4. Formation of stereocilia from precursor microvilli. (A) Schematic diagram showing stereocilia formation in chick hair cells and (B) electron micrographs showing stereocilia formation in hamster hair cells. Note the migration, elongation, and thickening of precursor microvilli to form the mature hair bundle, and the subsequent disappearance of excess microvilli from the apical surface of the hair cell (Frolenkov et al. 2004).

Mice and humans are closely related evolutionarily, with about 99 percent of mouse genes having a human ortholog. Mice and humans share many similarities in the physiology and structure of their auditory systems. The use of mouse models, including spontaneous and experimentally induced mutants, has helped accelerate the discovery and understanding of genes required for hair cell structure and function. Spontaneous mutations found to cause deafness in mice have helped to identify genes critical to human hearing, such as the jerker mouse (an espin mutant). The jerker mouse in turn led to the discovery that espin is a deafness gene in humans. Espin
functions to crosslink actin filaments together, increasing stability of the bundle. When over-expressed, espin causes elongation of actin bundles, and absence of espin, as in the jerker mouse, results in shortening of the stereocilia. \(^1\) Genes identified as critical to human hearing through genetic screening can be experimentally altered in mice for study of their functions. For example, the knockout model for protein tyrosine phosphatase receptor type Q (PTPRQ) showed that absence of functional PTPRQ causes abnormal formation of linkers between stereocilia shafts. From this we learned that PTPRQ is needed for connections between stereocilia and overall bundle organization. \(^1\) Thus far, 43 of the 61+ genes linked to hereditary hearing loss in humans have mouse models that can be used for their study. \(^8; 9\)

Studies using isolation and analysis of proteins within hair cells of the chicken utricle have provided an extensive list of the proteins present in hair cells. Some key proteins identified throughout the studies include radixin, taperin, glutathione S-transferase omega (GST omega), PTPRQ, and Clic5. \(^10; 11\) Of these proteins, PTPRQ, radixin, taperin, and Clic5 localize almost exclusively to the base of stereocilia (Figure 5). \(^10\) Their location suggests these proteins may be interacting and functioning in the formation and maintenance of the tapered base and regulation of actin bundle disassembly at the minus end. In mouse mutants, elimination of any of these proteins causes malformation of hair bundles and deafness. PTPRQ, radixin, and taperin have been identified as deafness genes in humans, but the status of Clic5 as a human deafness gene has yet to be determined.
Figure 5. Proteins localized to the base of stereocilia. (A) Localization of PTPRQ (green) to the base of chicken hair bundles (marked by red actin). (B) Radixin (green) at the base of chicken hair bundles (marked by red actin). (C) Localization of taperin (green) to the base of mouse hair bundles (marked by red actin). (D) Localization of Clic5 (green) to the base of chicken hair bundles (marked by red actin). Adapted from Shin et al. 2007 (A,B, and D) and Rehman et al. 2010 (C).

VI. Other Proteins Critical to Stereocilia Formation and Maintenance

As discussed above, PTPRQ is associated with stereocilia linking, and is implicated in the stability and growth of the hair bundle. Radixin is part of the ezrin/radixin/moesin (ERM) family and is required for the long-term maintenance of hair bundles. The ERM family functions to link actin bundles to the plasma membrane. The C-terminus of ERM proteins binds to actin, while the N-terminus associates with membrane bound proteins. ERM proteins are active only when phosphorylated. There is some functional redundancy within the family, such that a lack of radixin can be compensated for by ezrin during initial development of stereocilia. However, even with ezrin present, radixin is needed for maintenance of
stereocilia past the second week of life in mice. Scanning electron micrograph (SEM) images of radixin-deficient mice show almost complete degeneration of the hair bundles at day 40 (Figure 6).14

![Figure 6. Comparison between hair bundles of wild type and radixin deficient mice.](image)

Figure 6. Comparison between hair bundles of wild type and radixin deficient mice. Scanning electron micrographs of hair bundles in wild type (Rdx +/+ ) and radixin deficient (Rdx +/-) mice at postnatal day 14 (A) and postnatal day 40 (B). (A) Note the early signs of degeneration of the hair bundles in Rdx +/- mice. (B) Note the almost complete degeneration of the hair bundles in older Rdx +/- mice (Adapted from Kitajiri et al. 2004).14

Taperin is a protein found only in vertebrates and is known to bind to and inhibit protein phosphatase 1 (PP1). PP1 dephosphorylates and inactivates members of the ERM family, therefore taperin functions upstream of ERMs.11

Clic5, the focus of this paper, was identified as critical to hearing through mapping of a spontaneous mutation causing deafness in Jitterbug mice. Clic5 and radixin are present in roughly equimolar amounts in chicken hair bundles, and Clic5
has been shown to interact with the C-terminus of radixin.\textsuperscript{15} It is possible Clic5 interacts directly with the actin cytoskeleton as well.\textsuperscript{16} It is crucial that we learn more about each of these proteins to better understand stereocilia and hair cells and implement that knowledge in developing aids and cures for those afflicted with hearing impairments.

\textbf{VII. Clic Family}

The Chloride Intracellular Channel (Clic) Family is characterized by a approximately 240 amino acid residue Clic domain. These proteins belong to the larger glutathione S-transferase (GST) fold superfamily and the thioredoxin-like superfamily.\textsuperscript{16} The first member of the Clic family discovered, bovine Clic5b (originally named p64), was isolated because of its function as an ion channel in membrane vesicles within the bovine kidney cortex. However, subsequent cloning called into question the protein’s role as an ion channel. Its sequence is more similar to that of a soluble globular protein and did not match that of any known ion channels or integral membrane proteins. Further in vitro analysis of the family has shown that Clics exist in a stable globular form, but are capable of functioning as ion channels; however, whether they act as channels in vivo has yet to be definitively determined.

The Clic family is evolutionarily conserved with Clic-like proteins found in some plant species (Arabidopsis) and invertebrates. In most vertebrates, the Clic family is composed of six highly conserved paralogs (Clic1-6) with several splice variants (in Clic2, Clic5, and Clic6) that likely all arose from a single ancestral gene.
Although they are not well understood, the conservative duplication of the genes and their presence in both vertebrates and invertebrates indicates these proteins play an important role. The Clic proteins contain no known signal sequence directing them to a specific cellular location. They have been found in a wide range of locations within cells, including the plasma membrane, intracellular membranes, nucleus, and cytoplasm.

Figure 7. Diagram of human Clic5 amino acid sequence and domains conserved in the thioredoxin and GST superfamilies. Indicates location of the evolutionarily conserved C-P-F-S amino acid motif in the N-terminal region of Clic5 corresponding to the GSH binding site of the thioredoxin family. (Adapted from BLAST search result with Human Clic5)

Along with their putative channel functions, Clics may have an enzymatic function similar to the redox coupling of the GST family. The conserved C-P-F-[S/C] motif located within the N-terminal domain of the Clics resembles the GST family C-P-[aromatic]-[S/C/A/V] active site (Figure 7). Clics have a structural homology to GST omega (which is present in hair cells), where this motif is part of the glutathione (GSH) binding site. The GST family uses GSH in redox reactions, therefore, Clics may also utilize GSH or a GSH-like cofactor. Due to its evolutionary conservation and putative involvement in redox reactions, this motif has been a target in several studies. In Clic1 it was shown that Cys24 (corresponding to Cys32 in Clic5) of this
C-P-F-S motif is capable of forming a mixed disulfide bond with GSH. Mutation of Cys24 to an alanine residue caused electrophysiological changes and eliminated redox sensitivity of channels formed in synthetic lipid bilayers.\textsuperscript{16} In Clic4, the homologous Cys35 residue was shown to be crucial to Clic4 translocation from the cytoplasm to the plasma membrane after lysophosphatidic acid stimulation. My research will focus on Cys32 and Ser35 of the C-P-F-S motif within human Clic5.

\textbf{VIII. Clic5}

Human Clic5 (isoform1) is a 251 amino acid long 32kDa protein encoded on chromosome 6 (Figure 8). It was identified as part of a multi-protein complex in human placental microvilli, and was isolated using affinity chromatography with a GST-ezrin fusion construct. This protein has been found in a wide array of tissues, although it does not appear to be as widely expressed as other members of its family. Tissues and cells found to express Clic5 in humans include heart muscle, skeletal muscle, kidney, and placenta.\textsuperscript{17} In mice, Clic5 has been found in lung, brain, heart, kidney, spleen, bone, adrenal gland, thymus, and inner ear tissue.\textsuperscript{15}

As stated previously, Clic5 was first implicated in hearing through gene mapping of a spontaneous recessive mutation causing deafness in Jitterbug mice. Homozygous recessive mutant mice exhibit hearing loss and vestibular dysfunction due to lack of functional Clic5 protein in the hair cells of the inner ear. These mice also exhibit emphysema-like malformations in their lungs, but a standard pathological analysis showed no other notable abnormalities. During development, stereocilia
Figure 8. Human Clic5 nucleotide and deduced amino acid sequences. Highlighted region indicates the evolutionarily conserved C-P-F-S motif. Sequence available from GenBank under accession number AF216941.

appear to begin forming normally, but subsequently degenerate quickly with malformations detectable as early as day 3 postnatal (Figure 9). Staining with an antibody to radixin showed reduced levels of radixin in Jitterbug mice. This was
consistent with earlier results suggesting an interaction between Clic5 and radixin, linking the plasma membrane to actin bundles at the base of stereocilia.\textsuperscript{15}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{Comparison between hair bundles of wild type and Clic5 mutant mice. Scanning electron micrographs of hair bundles in wild type and Clic5 mutant mice at p17. (A) In outer hair cells of wild type mice the plasma membrane covers each actin bundle individually like a glove, while in mutant mice the plasma membrane covers the entire hair bundle like a mitten. (B) Stereocilia of inner hair cells degenerate in mutant mice, causing apparent gaps in the hair bundle. Scale bars 5 microns. (Mark Berryman, personal communication)}
\end{figure}

\section*{IX. Objectives and Significance}

Study of jitterbug mice showed that loss-of-function mutations of Clic5 in mice lead to deafness.\textsuperscript{15} Given the evolutionary conservation between humans and mice, I expect that Clic5 would be a deafness gene in humans. Thus, a long-term goal in my laboratory is to elucidate molecular and cellular functions of Clic5 in stereocilia formation in the inner ear of the mammals.
My research focused on the CXXS motif within human Clic5. Within the Clic family this motif is highly conserved and takes the form of either CXXS or CXXC. The CXXS motif has been proposed to play a role in function of Clic5. As the localization of Clic5 to the base of stereocilia is likely necessary for its proper function, I investigated the role of the motif in localization of Clic5 to microvilli in cultured cells, a model for stereocilia. Both Cys32 and Ser35 of this motif were targets for mutagenesis because of their conservation, and because of the hypothesized reactivity of Cys32. To better understand the role of this motif, five sets of mutations were made. To determine the role of each residue separately, independent mutations were made to change Cys32 and Ser35 to alanine residues (AXXS and CXXA, respectively). A double mutation was made to alter both Cys32 and Ser35 to alanine residues (AXXA). To address differences in the fourth position of the motif within the Clic family, two mutations were made to alter Ser35 to a cysteine residue, one preserving Cys32 (CXXC) and one also altering the Cys32 residue (AXXC). Finding what effect these mutations have on Clic5 localization will help understand the role of this conserved motif and is a step forward in the path to elucidating the function of Clic5 in stereocilia formation.

Studying how stereocilia are formed and maintained will provide insight toward a comprehensive understanding of the inner ear that can help pave the way for the development of treatment for individuals afflicted with sensorineural hearing loss. This includes a wide range of people such as those born deaf, those who lose hearing with age, and those exposed to loud noises or traumatic head injuries. Therefore,
investigation of Clic5 and how it functions in stereocilia may provide a pathway to
devising a treatment in which we can supplement the loss of one protein by boosting
the amount of another to regrow degenerated stereocilia.
MATERIALS AND METHODS

I. Making mutations to the Clic5 gene

In vitro mutagenesis was done to target the evolutionarily conserved putative redox sensitive motif C-P-F-S near the N-terminus of human Clic5 cDNA. This motif is similar to the thioredoxin/glutaredoxin C-P-[aromatic]-[S/C/A/V] motif, in which the first cysteine residue is redox active. Sets of primers were created to induce 5 different mutations in the wild type CXXS motif (see Table 1).

1. The conserved, polar Cys32 residue was mutated to a proposed non-reactive, non-polar alanine residue (CXXS → AXXS) to determine the role of Cys32 in localization of Clic5 to microvilli.

2. The conserved, polar Ser35 was mutated to an alanine residue (CXXS → CXXA) to determine the role of Ser35 in localization of Clic5 to microvilli.

3. Both Cys32 and Ser35 were mutated to alanine residues (CXXS → AXXA) to determine the overall role of the motif in localization of Clic5 to microvilli.

4. The Ser35 residue was mutated to a cysteine residue (CXXS → CXXC) to determine if converting the motif to more closely resemble other members of the Clic family (CXXC) enhances localization of Clic5 to microvilli.

5. The Cys32 was mutated to an alanine, and the Ser35 to a cysteine (CXXS → AXXC) to determine if absolute location of the cysteine residue within the motif is important to localization of Clic5 to microvilli.
Table 1: Primers used to induce mutations into human Clic5a cDNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AXXS (+ strand)</td>
<td>5'-GGAGAAAGCATCGGCAACGCTCCTTTCTCTCAGCGCTTTCTCATG-3'</td>
</tr>
<tr>
<td>AXXS (- strand)</td>
<td>5'-CATGAAGAGACGTTGCGATGCTTTCTCCGATGCTTTCTCATG-3'</td>
</tr>
<tr>
<td>CXXA (+ strand)</td>
<td>5'-GGAGAAAGCATCGGCAACGTCCCCCTTTCTCTCAGCGCTTTCTCATG-3'</td>
</tr>
<tr>
<td>CXXA (- strand)</td>
<td>5'-CATGAAGAGACGTTGCGATGCTTTCTCCGATGCTTTCTCATG-3'</td>
</tr>
<tr>
<td>AXXA (+ strand)</td>
<td>5'-GGAGAAAGCATCGGCAACGCTCCTTTCTCTCAGCGCTTTCTCATG-3'</td>
</tr>
<tr>
<td>AXXA (- strand)</td>
<td>5'-CATGAAGAGACGTTGCGATGCTTTCTCCGATGCTTTCTCATG-3'</td>
</tr>
<tr>
<td>AXXC (+ strand)</td>
<td>5'-GGAGAAAGCATCGGCAACGCTCCTTTCTCTCAGCGCTTTCTCATG-3'</td>
</tr>
<tr>
<td>AXXC (- strand)</td>
<td>5'-CATGAAGAGACGTTGCGATGCTTTCTCCGATGCTTTCTCATG-3'</td>
</tr>
</tbody>
</table>

Note: **Yellow highlighting** indicates no change in the residue. **Blue highlighting** indicates a mutation made to Cys23. **Green highlighting** indicates a mutation made to Ser 35.

Stratagene Quickchange Mutatgenesis Kit was used to create the five desired mutations in purified Clic5a cDNA in pTriplEx vector (pTriplEx-Clic5). Polymerase Chain Reaction (PCR) was done following kit protocol using 35ng of Clic5a cDNA template and 120ng of each appropriate primer per 50µl reaction. Following initial denaturation at 95°C for 30 seconds (segment 1), segment 2 was set to either 16 cycles (for single residue change mutants AXXS, CXXA, and CXXC) or 18 cycles (for double residue change mutants AXXA and AXXC). Segment 2 consisted of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 68°C for 6 minutes (1min/kb of 6kb plasmid). Following PCR, a DpnI digest was done at 37°C for one hour to eliminate the wild type template DNA.

For transformation 1µl aliquots of DpnI digested DNA were added to 50 µl of XL1 Blue supercompetent cells. Mixtures were incubated on ice for 30 minutes followed by heat pulse at 42°C for 45 seconds and incubated on ice again for 2
minutes. Then 300\textmu l of SOC media was added and mixtures were incubated at 37\degree C for 1 hour with shaking. After incubation 50\textmu l aliquots were plated on Luria-Bertani Medium (LB) growth plates with ampicillin (Amp; 50\textmu g/ml) and incubated overnight at 37\degree C.

Eight isolated colonies of each mutation were selected to grow in 5ml LB with Amp (50\textmu g/ml) at 37\degree C overnight. Plasmid DNA was purified from 1.5ml aliquots of culture using Qiagen Plasmid Miniprep Kit. Purified DNA was eluted with 100\textmu l elution buffer (EB).

Purified plasmids were screened for the presence of Clic5a with a KpnI restriction digest. One KpnI site is present within Clic5a cDNA approximately 930bp from the beginning of cDNA, and one KpnI site is present in the multicloning site (MCS) of the pTriplEx vector. Presence of Clic5a DNA was confirmed by two bands appearing with sizes of approximately 900bp and 5.1kb. Plasmids containing Clic5a cDNA were sequenced at OU genomics facility to confirm the presence of desired mutations and absence of errors in the sequence.

**II. Making Clic5a fusion constructs with GFP reporter gene**

Wild type and mutant Clic5 DNA were placed into green fluorescent protein (GFP) vectors (Table 2) so that Clic5 localization could be visualized in model cells using microscopy. Two different fusion constructs were made for each mutant and wild type Clic5 to ensure that the position of GFP did not affect Clic5 localization. One fusion construct had GFP fused to the C-terminus of Clic5 (Clic5-GFP), and the
other had GFP fused to the N-terminus of Clic5 (GFP-Clic5). Clic5-GFP fusion constructs were favored for experimentation because it placed GFP farthest from the proposed reactive CXXS motif being examined; however certain experiments were done with both constructs to ensure there was no difference in localization based on GFP position.

Table 2. Primers used for amplification and introduction of restriction sites in Clic5 cDNA

<table>
<thead>
<tr>
<th>Fusion Construct</th>
<th>Host vector</th>
<th>Antibiotic resistance</th>
<th>Forward primer to introduce Xho1 restriction site</th>
<th>Reverse primer to introduce BamHI restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clic5-GFP</td>
<td>pEGFP-N3</td>
<td>Kan^-</td>
<td>5’ATGCCTCGAGTCGCCACCA TGGCGACAGACTCGGCGACA GCTAAC-3’</td>
<td>5’ATGCGGATCCGGATCGGC TGAGCGGTTTG-3’</td>
</tr>
<tr>
<td>GFP-Clic5</td>
<td>pEGFP-C3</td>
<td>Kan^-</td>
<td>5’ATGCCTCGAGAAGACTCG CGGACAGCTAAC-3’</td>
<td>5’ATGCGGATCCCAAGGATCGG GCTGAGGCGGTTTG-3’</td>
</tr>
</tbody>
</table>

Once desired mutations were confirmed in pTriplEx-Clic5, Clic5 cDNA was amplified using PCR with Expand High Fidelity Enzyme (Roche). Primers used for amplification contained sequences to introduce restriction sites at both ends of Clic5 DNA (Table 2). An Xho1 site was introduced to the N-terminal end of Clic5, and a BamHI site was introduced to the C-terminal end of Clic5. PCR was done using 1ng of DNA template and a 0.2μM concentration of each appropriate primer per 25μl reaction. Segment 1 was set for one cycle with denaturation at 94°C for 2 minutes, annealing at 55°C for 1 minute, and extension at 72°C for 3 minutes. Segment 2 was set for 29 cycles with denaturation at 94°C for 45 seconds, annealing at 55°C for 1
minute, and extension at 72°C for 1.5 minutes. DNA was purified using QIAgen PCR Purification Kit. Purified DNA was eluted with 100µl EB.

Restriction enzyme digests were done using XhoI and BamHI (New England Biolabs) at 37°C for 1 hour to prepare amplified Clic5 DNA and GFP vectors for fusion. Following digestion, Clic5 and GFP vector DNA was purified using Qiagen PCR Purification Kit. Then ligation reactions were done using T4 DNA ligase (New England Biolabs) at 15°C overnight to fuse Clic5 DNA into GFP vectors. For ligation, 70ng of Clic5 DNA and 150ng of appropriate vector DNA were used per 40µl reaction.

For transformation, 5µl aliquots of ligated DNA were added to 75µl of DH5α competent cells. Mixtures were incubated on ice for 30 minutes followed by heat pulse at 42°C for 45 seconds and incubated on ice again for 2 minutes. Then 300µl of SOC media was added, and mixtures were incubated at 37°C for 1 hour with shaking. After incubation, 50µl aliquots were plated on LB growth plates with kanamycin (Kan; 50µg/ml) and incubated overnight at 37°C.

Eight isolated colonies of each construct were selected to grow in 5ml LB with Kan (50µg/ml) at 37°C overnight. Plasmid DNA was purified from 1.5ml aliquots of culture using Qiagen Plasmid Miniprep Kit. Purified DNA was eluted with 100µl EB.

Purified plasmids were screened for the presence of Clic5a with a KpnI restriction digest (New England Biolabs). One KpnI site is present within Clic5a
cDNA approximately 100bp from the translation initiation site; GFP vectors contained no KpnI sites. Presence of Clic5a DNA was confirmed by one band appearing with a size of approximately 5.5kb. Plasmids containing Clic5a cDNA were sequenced at the Ohio University (OU) genomics facility to confirm the proper ligation into vectors and re-confirm the presence of desired mutations and absence of errors in the sequence.

III. Cell Culture

Cultured LLC-PK1 porcine renal epithelial cells were used as a model because they exhibit numerous microvilli over their surfaces. Microvilli are an appropriate model for the study of stereocilia because both are F-actin rich projections from the cell surface, and stereocilia develop from precursor microvilli. Wild type and mutant Clic5 fusion constructs were transfected into LLC-PK1 cells to determine if the normal and mutated proteins localize to the actin based microvilli.

Cells were grown in 100mm petri dishes at 37°C with 5% carbon dioxide (CO₂) concentration in Minimum Essential Medium Alpha Medium 1X with 2mM L-glutamine and without ribonucleotides and deoxyribonucleotides (Gibco) containing 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals) and the standard amounts of Penicillin/Streptomycin (MEMα Complete). Cell cultures were monitored daily for possible contamination, and split when growth reached approximately 100% confluence. Typically 1:12 splits reach 100% confluence in 3-4 days.

Cell splitting:
When growth reached approximately 100% confluence, cells were split. In a sterile hood, cells were rinsed once with 2ml phosphate buffered saline (PBS), then again with 2ml of 0.05% Trypsin EDTA mix in MEMα (Gibco). Then 2.5ml of 0.05% Trypsin EDTA mix was added and cells were incubated for 7-10 minutes at 37°C with 5% CO₂. Once all cells were detached and free floating in media, 8ml of MEMα complete media was added. For a 1:12 split, 1ml of this mixture was added to a fresh 100mm dish with 11ml MEMα complete media. Cells were then labeled and returned to incubate at 37°C with 5% CO₂.

Seeding for transfections:

Cells were rinsed and trypsinized as described above. A 0.2ml aliquot of trypsinized cells was added to 2.5ml of MEMα complete media in a 35mm dish with three sterile round glass coverslips. Cells were left to incubate at 37°C with 5% CO₂ for 24 hours.

Transfection Protocol:

Cells were transfected 24 hours after seeding. A transfection mixture containing 100µl serum-free MEMα, 750ng of Clic5 fusion construct, and 6µl polyethylenimine (Roche) was prepared and incubated 15 minutes at room temperature. Cells were washed twice with 2ml PBS, and then 1.5ml serum-free MEMα was added to cell dishes. The transfection mixture was then added drop wise to cell dishes. Cells were incubated for 4-5 hours at 37°C with 5%
CO₂. After incubation with transfection mixture, cells were rinsed twice with 2ml PBS, and 2.5ml MEMα complete media was added to cells. Cells are then incubated for 12 hours at 37°C with 5% CO₂.

**Fixation and Permeabilization of Cells:**

Cells were fixed and stained 16 hours after the start of transfection. Cells were fixed for 10 minutes in 4% EM grade formaldehyde (Polysciences) in PBS, rinsed with PBS, and permeabilized for 60 seconds in 0.1% Triton X-100 in PBS. Once permeabilized, cells were rinsed again with PBS and were either be directly mounted (for the microvilli localization study) or stained and then mounted (for the Clic5 and F-actin co-localization study).

**Phalloidin Staining:**

Phalloidin is a marker for F-actin. Once permeabilized and rinsed, cells were stained with a 1:50 mixture of phalloidin-Alexa546 (Invitrogen) in PBS. Approximately 30µl of phalloidin mixture was added to each coverslip and cells were left to stain in a moist chamber in the dark at room temperature for 30 minutes. Once staining was complete, cells were rinsed with PBS and mounted.

**Mounting:**

Cells were mounted on glass slides in 6µl Prolong Gold AntiFade Reagent with DAPI (Invitrogen) and left in the dark at room temperature
overnight. Edges of coverslip were sealed with clear nailpolish and slides were rinsed with ddH₂O to remove residual salts.

IV. Western Blot

For western blot analysis, cells in 100mm culture dishes were transfected with 4.5µg of Clic5 fusion construct. After two days incubation, cells were rinsed two times with PBS and harvested by addition of 300µl of 2x Laemmli sample buffer with 1% 2-mercaptoethanol and subsequent scraping of the dish surface. Cells were transferred to a micro centrifuge tube and placed into a boiling water bath for 5 minutes. Lysis was completed by passage through sequentially smaller gauge needles (20, 23, and 25 gauge) and cellular extract was precipitated by centrifugation at 13,000rpm in a micro centrifuge. Four 20µl aliquots of 1:1 serially diluted cell lysate were loaded onto a 10% SDS-polyacrylamide gel and the gel was run for 1 hour.

Proteins were transferred to a PVDF membrane, 0.45µm pore size (Millipore), by semi-dry transfer at 0.8mAmp of constant current per cm² of gel for 1 hour. The PVDF membrane was prepared for blotting by soaking in methanol for 30 seconds after which it is thoroughly washed with ddH₂O. The PVDF membrane and blotting papers were then soaked in the transfer buffer (48mM Tris, 39mM glycine, 0.1% SDS, pH 9.2), and the gel was soaked in ddH₂O for 15 minutes before the protein transfer. After transfer the membrane was put in a blocking solution containing 5% nonfat dry milk (Carnation) in Tris-Buffered Saline (TBS) (pH 7.45) for 30 to 60 minutes at room temperature. After blocking, the membrane was then incubated overnight at 4°C.
with the primary antibody (anti-Clic5a B-132\textsuperscript{17}, 1:2,000 dilution; mitochondrial ATP Synthase subunit alpha, 1:5,000 dilution, MitoSciences) diluted in 1% nonfat dry milk in Tris-Buffered Saline with 0.01% Tween-20 (TBST). After incubation with the primary antibody, the membrane was rinsed with TBST and then washed three times for 5 minutes each. The membrane was then incubated for one hour at room temperature with secondary Horseradish Peroxidase-Conjugated Protein A (1:5,000 dilution, Sigma) diluted in 1% nonfat dry milk in TBST. After incubation with the secondary, the membrane was rinsed with TBST and then washed three times for 5 minutes each. The bands (or signals) were detected with enhanced chemiluminescence reagent (Amersham Biosciences). Images of bands were taken with BIO-RAD Molecular Imager ChemiDoc XRS+ Imaging System.

V. Microvilli Localization Study

Cells transfected with Clic5 fusion constructs were visualized using Nikon epi-fluorescence microscopy (model Eclipse E600 and Internsilight C-HGFI) at 1,000X magnification. Blue channel was set for excitation from 330-380nm and emission from 435-485nm. Green channel was set for excitation from 460-500nm, and emission from 510-560nm. Red channel was set for excitation from 540-580nm, and emission from 600-660nm. For accuracy, cells were not stained with phalloidin-Alexa546 because the color filter on our microscope allowed bleed through to occur from the red channel to the green channel. Previous comparison between F-actin stained cells and Clic5-GFP fusion protein localization in cells showed enrichment of Clic5-GFP fusion protein in hair-like projections which likely correspond to the microvilli (MV) of the
cells (Figure 10). Whole cells were examined and placed into one of three categories: exhibiting Clic5 fusion protein localization in microvilli-like projections over >50% of the visible cell surface (good MV localization), exhibiting Clic5 fusion protein localization in microvilli-like projections over <50% of the visible cell surface (poor MV localization), or not exhibiting any Clic5 fusion protein localization in microvilli-like projections over the visible cell surface (no MV localization) (Figure 11). Total MV localization was calculated by adding good MV localization and poor MV localization.

**Figure 10. Comparison between F-actin staining and Clic5-GFP fusion protein localization.** Epi-fluorescence images of LLC-PK1 cells (A) Cells stained with phalloidin as a marker for F-actin (red) and DAPI as a marker for DNA (blue). Note the appearance of hair-like projections which represent microvilli. (B) Cells transfected with wild type Clic5-GFP fusion construct (green) and stained with DAPI as a marker for DNA (blue). Note the appearance of hair-like projections (similar to those seen in phalloidin stained cells) which presumably correspond to microvilli. Scale bar 20 µm.
Figure 11. Categories used to classify localization of Clic5 fusion proteins to microvilli-like projections of LLC-PK1 cells. Epi-fluorescence images of LLC-PK1 cells transfected with Clic5-GFP constructs (green) and stained with DAPI as a marker for DNA (blue). (A) Example of good MV localization where Clic5 fusion protein exhibits localization in microvilli-like projections over >50% of the visible cell surface. (B) Example of poor MV localization where Clic5 fusion protein exhibits localization in microvilli-like projections over <50% of the visible cell surface. (C) Example of no MV localization where Clic5 fusion protein does not exhibit localization in any microvilli-like projections over the visible cell surface. Scale bar 20 µm.

VI. Clic5 and F-Actin Co-Localization Study

A selection of representative cells transfected with each Clic5-GFP fusion construct was imaged using a Zeiss confocal microscope (model LSM 510). Green channel excitation was set at 488nm, and emission was from 505-530nm. Red channel excitation was set at 543nm, and emission was at 560nm. Z-stack images (2,048 x 2,048) of approximately 2µm into the apical surface of cells were taken at 0.482µm intervals and compiled as maximum projections for examination. Cells transfected with GFP-Clic5 fusion constructs were also imaged, but not examined in this study. Confocal imaging eliminated the complication caused by bleed through in the original microvilli localization study, so it was possible to stain transfected cells with phalloidin as a marker for F-actin. This was used to confirm that the microvilli-like
projections seen in unstained Clic5-GFP transfected cells did in fact correlate to phalloidin stained microvilli (Figure 12). Confocal images were also used to determine the average percent co-localization between Clic5 and F-actin in microvilli for each fusion construct. From the confocal images, a random selection of cells was used to count the number of microvilli (based on F-actin staining) and the number of microvilli with Clic5 localization (based on observation of Clic5-GFP fusion protein). This was done by magnifying images, increasing brightness and contrast to make projections more clear, separating the red (F-actin) and green (Clic5-GFP) channels, and using ImageJ count function to count each separate projection (Figure 13).

Between 15 and 20 individual cells were examined for each Clic5-GFP fusion construct. Percent co-localization of Clic5-GFP with F-actin in MV was determined by dividing the total number of microvilli with Clic5 localization by the total number of microvilli and multiplying by 100.

Figure 12. Correlation between F-actin staining and Clic5-GFP fusion protein localization in LLC-PK1 cells. Confocal images of LLC-PK1 cells transfected with Clic5-GFP (green) and stained with phalloidin as a marker for F-actin (red). (A) Phalloidin staining of cells showing microvilli. (B) Clic5-GFP fusion protein localization showing appearance of microvilli-like projections. (C) Merge image showing co-localization (orange) of F-actin and Clic5-GFP fusion protein in microvilli of cells. Scale bar 20 μm.
Figure 13. Example of method for determining Clic5-GFP fusion protein and F-actin co-localization in microvilli of LLC-PK1 cells. Confocal images of LLC-PK1 cells transfected with Clic5-GFP fusion construct (green) and stained with phalloidin as a marker for F-actin (red). (A) Original merged confocal image with randomly selected cell boxed in white. Increased brightness, contrast, and magnification of (B) F-actin staining and (C) Clic5-GFP fusion protein localization for selected cell. Note numbers over projections inputted using ImageJ count function. Scale bar 20 µm.

VII. Statistical Analysis

Statistical analyses were performed for each study to compare differences between each fusion construct. In the microvilli localization study, differences in total MV localization and differences in good MV localization were compared among constructs. In the Clic5 and F-actin co-localization study differences in Clic5-GFP co-
localization with F-actin in microvilli were compared among constructs. All comparisons were done using the chi-square test in Microsoft Excel with a significance value set to \( p < 0.05 \).
RESULTS

I. LLC-PK1 cells do not express detectable levels of endogenous Clic5

![Figure 14. Image of actin rich microvilli in LLC-PK1 cells. Epi-fluorescence image of LLC-PK1 cells stained with phalloidin to mark F-actin (red) and DAPI to mark DNA (blue). Note the numerous hair-like projections (microvilli) over the cell surfaces. Scale bar 20 µm.](image)

Western blot analysis with Clic5 antibody shows that the cell line used in transfection experiments (LLC-PK1 porcine renal epithelial cells- Figure 14) does not express detectable levels of endogenous Clic5 or Clic4 (Clic5 antibody is known to cross react with Clic4). No Clic5 or Clic4 is detected in any of the cell extracts; endogenous protein should be similar in size to purified recombinant Clic5 protein (Figure 15, Arrow C at ~30kD) or Clic4 protein (Figure 15, Arrow D at ~28kDa). Note that the theoretical weight of Clic5 is 28kDa, however previous studies have found that it runs at 32kDa on Western blots. Clic5 fusion protein from transfected cell extracts showed the expected size of approximately 54kDa (Figure15, Arrow A). When normalized to the loading control mitochondrial ATP synthase subunit alpha
(Figure 15, Arrow B), all transfected Clic5 fusion constructs are expressed at approximately the same levels in the cells.

Figure 15. Western blot analysis of Clic5 expression in LLC-PK1 cells. Four 1:1 serial dilutions (1-4) were done for each category. Non-transfected cells, mock transfected cells, and pure recombinant Clic5, Clic1, and Clic4 were used as controls; mitochondrial ATP Synthase subunit alpha was used as a loading control. Cells transfected with wild type GFP-Clic5 (WT C3 CXXS), wild type Clic5-GFP (WT N3 CXXS), mutant AXXS Clic5-GFP (N3 AXXS), mutant CXXA Clic5-GFP (N3 CXXA), and mutant AXXA Clic5-GFP (N3 AXXA) were analyzed. Cells were probed with Clic5 antibody. No endogenous Clic5 or Clic4 was detected in cell extracts. Approximately equal amounts of each Clic5 fusion protein were detected in transfected cells. Arrows indicate bands of (A) Clic5 fusion protein at ~54kDa (B) mitochondrial ATP-Synthase subunit alpha at ~50kDa (C) recombinant Clic5 at ~30kDa and (D) recombinant Clic4 at ~28kDa. The mobilities of protein standards are indicated on the left in kilodaltons.

The lack of endogenous Clic5 and Clic4 provides an ideal background for examining effects of transfected wild type and mutant Clic5 constructs as there is no endogenous protein within the cells to interfere with proteins from transfected constructs.

Appearance of Clic5 fusion protein at the expected theoretical molecular weight confirms Clic5-GFP fusion constructs were made and expressed in cells. Clic5 fusion
proteins showed approximately the same levels in each sample. This indicates that there is no difference in cellular expression levels among the constructs and thus differences observed among wild type and mutant Clic5 are unlikely to be derived from inequalities in expression.

II. Wild type Clic5 localizes to microvilli, nucleus, and cytoplasm of LLC-PK1 cells

LLC-PK1 cells were transfected with wild type Clic5-GFP construct to determine the localization of the Clic5-GFP fusion protein. Cells were fixed and stained with phalloidin as a marker for F-actin and DAPI as a marker for DNA. Cells were examined using Nikon epi-fluorescence microscopy (model E600). Wild type Clic5-GFP fusion proteins were seen in the microvilli, nucleus, and cytoplasm of LLC-PK1 cells (Figure 16).

Figure 16. Localization of wild type Clic5-GFP fusion protein in LLC-PK1 cells. Epi-fluorescence images of LLC-PK1 cells transfected with wild type Clic5-GFP fusion construct (green). Cells are fixed and stained with phalloidin to mark F-actin (red) and DAPI to mark DNA (blue). Arrows indicate wild type Clic5-GFP fusion protein localization to (A) microvilli (B) nucleus and (C) cytoplasm of LLC-PK1 cells. Scale bar 20 μm.
Wild type Clic5 enrichment in the microvilli of these cells provides an ideal model for studying Clic5 function in actin-based structures, such stereocilia in hair cells of the inner ear.

III. Position of GFP in fusion constructs does not affect Clic5 subcellular localization

Two GFP fusion constructs were made for each of the five Clic5 mutants and for wild type Clic5. One fusion construct had GFP fused to the C-terminus of Clic5 (Clic5-GFP, N3), and the other had GFP fused to the N-terminus of Clic5 (GFP-Clic5, C3). Western blot analysis of cell extract from transfected LLC-PK1 cells shows that each construct is expressed in approximately equal amounts (Figure 15).

Microvilli localization experiments were done with both fusion configurations to determine if they showed similar distribution in microvilli of LLC-PK1 cells (Table 3). Qualitatively, there appears to be no difference between Clic5-GFP and GFP-Clic5 fusion protein localization using either epi-fluorescence (Figure 17) or confocal imaging (Figure 18). For the microvilli localization study, whole cells were examined and placed into one of three categories: exhibiting Clic5 fusion protein localization in microvilli-like projections over >50% of the visible cell surface (good MV localization), exhibiting Clic5 fusion protein localization in microvilli-like projections over <50% of the visible cell surface (poor MV localization), or not exhibiting any Clic5 fusion protein localization in microvilli-like projections over the visible cell
surface (no MV localization). Total MV localization (good MV localization plus poor MV localization) of Clic5 fusion proteins for each mutant and wild type was compared using a chi-square test (Table 4). This showed no significant difference in localization pattern for wild type CXXS, mutant CXXA, mutant AXXA, and mutant CXXC Clic5 fusion proteins (all have p>0.05). There was a significant difference in localization pattern for both the mutant AXXS and mutant AXXC Clic5 fusion proteins (p <0.001 for both). This is likely due to poor enrichment of these mutant proteins to microvilli. Since there were no notable differences between Clic5-GFP and GFP-Clic5 fusion protein localization, only the Clic5-GFP fusion constructs were used for subsequent studies and for detailed description in further results. Clic5-GFP fusion constructs were preferred because they had GFP located farther from the motif of interest. Additional information about GFP-Clic5 fusion constructs can be found in the appendix.
Figure 17. Comparison of localization to microvilli-like projections for Clic5 fusion proteins. Epi-fluorescence images of LLC-PK1 cells transfected with wild type and five mutant Clic5 fusion constructs (green). GFP is located at either the C-terminus (Clic5-GFP) or N-terminus (GFP-Clic5) of Clic5. Cells are fixed and stained with DAPI to mark DNA (blue). There are no notable qualitative differences between Clic5-GFP and GFP-Clic5 versions of each protein in their localization to microvilli-like projections. Scale bar 20 µm.
Figure 18. Comparison of co-localization with F-actin in microvilli for Clic5 fusion proteins. Confocal images of LLC-PK1 cells transfected with wild type and five mutant Clic5 fusion constructs (green). GFP is located at either the C-terminus (Clic5-GFP) or N-terminus (GFP-Clic5) of Clic5. Cells are stained with phalloidin to mark F-actin (red). There are no notable qualitative differences between Clic5-GFP and GFP-Clic5 versions of each protein in their co-localization (orange) with F-actin in microvilli. Scale bar 20 μm.
IV. Mutations of Cys32 significantly decreases Clic5 localization to microvilli

To determine the functional importance of the evolutionarily conserved CXXS motif in human Clic5, the proposed reactive, polar, hydrophilic Cys32 residue was mutated to a nonpolar, hydrophobic alanine residue (C32A mutation) in three different
constructs (CXXA, AXXA, and AXXC). This resulted in significantly diminished localization to microvilli as compared to wild type Clic5.

![Figure 19. Comparison of localization to microvilli-like projections for wild type and Cys32 mutant Clic5-GFP fusion proteins.](image)

Localization of wild type and Cys32 mutant Clic5-GFP fusion proteins to microvilli-like projections was examined in the microvilli localization study (Table 5, Figure 19), and co-localization between Clic5-GFP and F-actin was examined in the Clic5 and F-actin co-localization study. Statistical comparison between constructs using chi-square tests were done for both studies (Tables 6, 7 and 9). In the microvilli (MV) localization study, wild type CXXS Clic5-GFP was found to exhibit at least some fusion protein localization to microvilli-like projections (total MV localization) in 98.7% of 1886 cells examined, while 76.8% of cells exhibited fusion protein localization to microvilli-like projections over at least 50% of their visible surface (good MV localization). Mutant AXXS Clic5-GFP exhibited the poorest localization to microvilli-like projections of all the constructs, where 66.2% of the 1217 cells
examined exhibited total MV localization, and 24.3% of cells examined exhibited good MV localization. This is a significant decrease in both total MV localization (32.9% decrease) and good MV localization (68.4% decrease) compared to wild type Clic5-GFP (p < 0.001 for both). Mutant AXXA Clic5-GFP exhibited the highest total MV localization among the C32A mutants, where 89.6% of the 1235 cells examined exhibited total MV localization, and 46.3% of cells examined exhibited good MV localization. However, this is still a significant decrease in both total MV localization (9.2% decrease) and good MV localization (39.7% decrease) compared to wild type (p <0.001 for both). Mutant AXXC Clic5-GFP exhibited the highest percentage good MV localization among the C32A mutants, where 82.8% of the 1231 cells examined exhibited total MV localization, while 52.6% of cells examined exhibited good MV localization. This again is a significant decrease in both total MV localization (16.1% decrease) and good MV localization (31.5% decrease) compared to wild type (p <0.001 for both).

Table 5. Localization to Microvilli-Like Projections for Wild Type and Cys32 Mutant Clic5-GFP Fusion Proteins in LLC-PK1 Cells

<table>
<thead>
<tr>
<th>Clic5-GFP Fusion Protein</th>
<th>GOOD MV LOCALIZATION (&gt;50%)</th>
<th>POOR MV LOCALIZATION (&lt;50%)</th>
<th>TOTAL MV LOCALIZATION (Good + Poor)</th>
<th>NO MV LOCALIZATION</th>
<th>TOTAL # OF CELLS COUNTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type CXXS</td>
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<td>1861</td>
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<td>1886</td>
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<tr>
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<td>806</td>
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<td>1217</td>
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<tr>
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<td>572</td>
<td>535</td>
<td>535</td>
<td>128</td>
<td>1235</td>
</tr>
<tr>
<td>Mutant AXXC</td>
<td>648</td>
<td>371</td>
<td>1019</td>
<td>212</td>
<td>1231</td>
</tr>
</tbody>
</table>
Results from transfection of GFP-Clic5 fusion constructs show the same pattern of all C32A mutants having significantly decreased total and good MV localization as compared to wild type, with the only notable difference being that the mutant AXXA GFP-Clic5 exhibited the highest percentage of good MV localization among the C32A mutants. See appendix for results from Clic5-GFP microvilli localization study (Tables A1, A2 and A3, Figure A1).

<table>
<thead>
<tr>
<th>Clic5-GFP Fusion Protein</th>
<th>Mutant AXXS</th>
<th>Mutant AXXA</th>
<th>Mutant AXXC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type CXXS</td>
<td>Yes &lt;0.001</td>
<td>Yes &lt;0.001</td>
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<tr>
<td>Mutant AXXS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant AXXA</td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Clic5-GFP Fusion Protein</th>
<th>Mutant AXXS</th>
<th>Mutant AXXA</th>
<th>Mutant AXXC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type CXXS</td>
<td>Yes &lt;0.001</td>
<td>Yes &lt;0.001</td>
<td>Yes &lt;0.001</td>
</tr>
<tr>
<td>Mutant AXXS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant AXXA</td>
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<td></td>
</tr>
</tbody>
</table>

In summary, C32A mutations significantly reduced Clic5 localization to microvilli-like projections by at least 9.2% (mutant AXXA) in the total MV.
localization category, and by at least 24.2% (mutant AXXC) in the good MV localization category.

In the Clic5-GFP and F-actin co-localization study (Tables 8 and 9, Figure 20), wild type CXXS Clic5-GFP fusion protein exhibited co-localization with F-actin in 81.9% of the 3964 microvilli examined from 18 cells. Cells used for examination were selected randomly from confocal images. Mutant AXXS Clic5-GFP exhibited 25.6% co-localization with F-actin in the 4275 microvilli examined from 19 cells. This is a significant decrease (68.7% decrease) in co-localization compared to wild type (p <0.001). Mutant AXXA Clic5-GFP exhibited the highest co-localization with F-actin among the C32A mutants, with a 47.6% co-localization with F-actin in the 4911 microvilli examined from 15 cells. This is a significant decrease (41.9% decrease) in co-localization compared to wild type (p <0.001). Mutant AXXC Clic5-GFP exhibited the lowest co-localization with F-actin of the C32A mutants, with a 20.5% co-localization with F-actin in the 5369 microvilli examined from 15 cells. This is a significant decrease (75.0% decrease) in co-localization compared to wild type (p <0.001).

GFP-Clic5 constructs were not measured in this study, however qualitative observation of confocal images for GFP-Clic5 fusion constructs appears to show the same pattern. See appendix for confocal images of GFP-Clic5 constructs (Figure A2).
Table 8. Co-Localization with F-Actin in Microvilli for Wild Type and Cys32 Mutant Clic5-GFP Fusion Proteins in LLC-PK1 Cells

<table>
<thead>
<tr>
<th>Clic5-GFP Fusion Protein</th>
<th># MV Exhibiting Co-Localization</th>
<th># MV Not Exhibiting Co-Localization</th>
<th>Total # MV Counted</th>
<th>Total # of Cells Analyzed</th>
<th>% Co-Localization of Clic5-GFP with F-Actin in MV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type CXXS</td>
<td>3246</td>
<td>718</td>
<td>3964</td>
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<td>81.9%</td>
</tr>
<tr>
<td>Mutant AXXS</td>
<td>1093</td>
<td>3182</td>
<td>4275</td>
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<td>25.6%</td>
</tr>
<tr>
<td>Mutant AXXA</td>
<td>2340</td>
<td>2571</td>
<td>4911</td>
<td>15</td>
<td>47.6%</td>
</tr>
<tr>
<td>Mutant AXXC</td>
<td>1386</td>
<td>5369</td>
<td>6755</td>
<td>15</td>
<td>20.5%</td>
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Table 9. Statistical Significance of Differences in Co-Localization with F-Actin for Wild Type and Cys32 Mutant Clic5-GFP Fusion Proteins Using Chi-Square Test

<table>
<thead>
<tr>
<th>Clic5-GFP Fusion Protein</th>
<th>Mutant AXXS</th>
<th>Mutant AXXA</th>
<th>Mutant AXXC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type CXXS</td>
<td>Yes &lt;0.001</td>
<td>Yes &lt;0.001</td>
<td>Yes &lt;0.001</td>
</tr>
<tr>
<td>Mutant AXXS</td>
<td></td>
<td>Yes &lt;0.001</td>
<td>Yes &lt;0.001</td>
</tr>
<tr>
<td>Mutant AXXA</td>
<td></td>
<td></td>
<td>Yes &lt;0.001</td>
</tr>
</tbody>
</table>

The results from both studies show that mutation of the proposed reactive Cys32 residue of the conserved Clic5 CXXS motif is crucial to the localization of Clic5 to microvilli. When Cys32 is mutated to an alanine residue, fusion protein localization to microvilli is significantly diminished for all mutants as compared to the wild type fusion protein. Qualitative analysis shows the localization of the Cys32 mutants appears notably different from that of wild type (Figures 19 and 20).
Although all mutant and wild type proteins are consistently seen in both nucleus and cytoplasm, the C32A mutant fusion constructs are much less apparent in microvilli. When these mutants do exhibit localization to microvilli, it is often only seen in a few small patches over the cell surface, not evenly distributed through all microvilli as is seen in wild type Clic5.
Figure 20. Comparison of co-localization with F-actin in microvilli for wild type and Cys32 mutant Clic5-GFP fusion proteins. Confocal images of LLC-PK1 cells transfected with wild type and Cys32 mutant Clic5-GFP fusion constructs (green). Cells are stained with phalloidin to mark F-actin (red). Note the appearance of co-localization (orange) between Clic5-GFP and F-actin in microvilli of cells transfected with wild type CXXS Clic5-GFP, and the decrease in this co-localization in cells transfected with Cys32 mutant Clic5-GFP. Scale bar 20 μm.
V. Mutation of Ser35 does not cause decreased localization of Clic5 to microvilli

To further study the functional importance of the evolutionarily conserved CXXS motif in human Clic5, the polar, hydrophilic Ser35 was mutated in two different constructs (CXXA and CXXC). The S35A mutation was made to address the role of Ser35 in the motif, and the S35C mutation was made to address the possibility that a cysteine residue could functionally replace the serine. In these constructs there was no significant decrease in localization to microvilli-like projections. There was a significant increase in localization seen for these mutants in the Clic5 and F-actin co-localization study.

![Comparison of localization to microvilli-like projections for wild type and Ser35 mutant Clic5-GFP fusion proteins](image)

Figure 21. Comparison of localization to microvilli-like projections for wild type and Ser35 mutant Clic5-GFP fusion proteins. Epi-fluorescence images of LLC-PK1 cells transfected with wild type and Ser35 mutant Clic5-GFP fusion constructs (green). Cells are stained with DAPI to mark DNA (blue). Note the similarities in appearance of hair-like projections on the surface of cells transfected with wild type CXXS Clic5-GFP and mutant CXXA and CXXC Clic5-GFP. Scale bar 20 µm.

Localization of wild type and Ser35 mutant Clic5-GFP fusion proteins to microvilli-like projections was examined in the microvilli localization study (Table 10,
Figure 21), and co-localization between Clic5-GFP and F-actin was examined in the Clic5 and F-actin co-localization study. Statistical comparisons between constructs using chi-square tests were done for both studies (Tables 11, 12 and 14). In the microvilli (MV) localization study, wild type CXXS Clic5-GFP was found to exhibit at least some fusion protein localization to microvilli-like projections (total MV localization) in 98.7% of 1886 cells examined, while 76.8% of cells exhibited fusion protein localization to microvilli-like projections over at least 50% of their visible surface (good MV localization). For mutant CXXA Clic5-GFP, 98.0% of the 1955 cells examined exhibited total MV localization, and 75.8% of cells examined exhibited good MV localization. This is a 0.7% decrease in total MV localization and a 1.3% decrease in good MV localization compared to wild type, neither of which is a significant decrease (p > 0.05 for both). For mutant CXXC Clic5-GFP, 98.7% of the 1230 cells examined exhibited total MV localization, and 92.4% of cells examined exhibited good MV localization. This is no change in total MV localization and a 20.3% increase in good MV localization compared to wild type. The increase seen in good MV localization of the mutant CXXC is a significant increase (p < 0.001).

Results from transfection of GFP-Clic5 fusion constructs show the same pattern where Ser35 mutants have either a non-significant change in total MV localization, while mutant CXXC GFP-Clic5 showed a significant increase in good MV localization as compared to wild type (p < 0.001). See appendix for results from GFP-Clic5 microvilli localization study (Tables A1, A2, and A3, Figure A1).
In summary, Ser35 mutations do not significantly reduce Clic5 localization to microvilli-like projections where the largest decrease in total MV localization is 1.3% (mutant CXXA), and the largest decrease in good MV localization was 0.7% (mutant CXXA).

<table>
<thead>
<tr>
<th>Table 10. Localization to Microvilli-Like Projections for Wild Type and Ser35 Mutant Clic5-GFP Fusion Proteins in LLC-PK1 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clic5-GFP Fusion Protein</td>
</tr>
<tr>
<td>----------------------------</td>
</tr>
<tr>
<td>Wild Type CXXS</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Mutant CXXA</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Mutant CXXC</td>
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<table>
<thead>
<tr>
<th>Table 11. Statistical Significance of Differences in Total Microvilli Localization for Wild Type and Ser35 Mutant Clic5-GFP Fusion Proteins Using Chi-Square Test</th>
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</thead>
<tbody>
<tr>
<td>Clic5-GFP Fusion Protein</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Wild Type CXXS</td>
</tr>
<tr>
<td>Mutant CXXA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 12 Statistical Significance of Differences in Good Microvilli Localization for Wild Type and Ser35 Mutant Clic5-GFP Fusion Proteins Using Chi-Square Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clic5-GFP Fusion Protein</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Wild Type CXXS</td>
</tr>
<tr>
<td>Mutant CXXA</td>
</tr>
</tbody>
</table>
In the Clic5-GFP and F-actin co-localization study (Tables 13 and 14, Figure 22), wild type CXXS Clic5-GFP fusion protein exhibited co-localization with F-actin in 81.9% of the 3964 microvilli examined from 18 cells. Cells used for examination were randomly selected from confocal images. Mutant CXXA Clic5-GFP fusion protein exhibited the highest percent co-localization with F-actin of all the constructs with 88.8% co-localization with F-actin in the 4031 microvilli examined from 15 cells. This is a significant increase (8.4% increase) in co-localization compared to wild type (p <0.001). Mutant CXXC Clic5-GFP exhibited 84.6% co-localization with F-actin in the 5048 microvilli examined from 15 cells. This is a 3.3% increase in co-localization compared to wild type, which is a non-significant increase (p >0.05). It should be noted that the difference in percent co-localization between mutant CXXA and mutant CXXC is non-significant.

GFP-Clic5 constructs were not examined in this study; however qualitative observation of confocal images for GFP-Clic5 fusion constructs appears to show the same pattern. See appendix for confocal images of GFP-Clic5 constructs (Figure A2).

<table>
<thead>
<tr>
<th>Table 13. Co-Localization with F-Actin in Microcilli for Wild Type and Ser35 Mutant Clic5-GFP Fusion Proteins in LLC-PK1 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clic5- GFP Fusion Protein</td>
</tr>
<tr>
<td>Wild Type CXXS</td>
</tr>
<tr>
<td>Mutant CXXA</td>
</tr>
<tr>
<td>Mutant CXXC</td>
</tr>
</tbody>
</table>
Table 14. Statistical Significance of Differences in Co-Localization with F-Actin for Wild Type and Ser35 Mutant Clic5-GFP Fusion Proteins Using Chi-Square Test

<table>
<thead>
<tr>
<th>Clic5-GFP Fusion Protein</th>
<th>Mutant CXXA</th>
<th>Mutant CXXC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type CXXS</td>
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<td>No</td>
</tr>
<tr>
<td>Mutant CXXA</td>
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<td>&gt;0.05</td>
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</table>

The results indicate that the evolutionarily conserved Ser35 residue of the Clic5 CXXS motif is not critical for localization of Clic5 to microvilli. When Ser35 is mutated to an alanine residue, co-localization with F-actin in microvilli significantly increased compared to wild type. Note that all Clic5-GFP fusion proteins are consistently seen in both nucleus and cytoplasm.
Figure 22. Comparison of co-localization with F-actin in microvilli for wild type and Ser35 mutant Clic5-GFP fusion proteins. Confocal images of LLC-PK1 cells transfected with wild type and Ser53 mutant Clic5-GFP constructs (green). Cells are stained with phalloidin to mark F-actin (red). Note the appearance of co-localization (orange) between Clic5-GFP and F-actin in cells transfected with wild type CXXS, mutant CXXA, and mutant CXXC Clic5-GFP. Scale bar 20 µm.
DISCUSSION

I. Overview

The evolutionarily conserved CXXS motif in human Clic5 plays a role in this protein’s localization to actin-based microvilli, however the motif as a whole is not crucial for microvilli localization. This motif is found within the Clic, thioredoxin, and glutathione S-transferase families, and forms part of a glutathione (GSH) binding site. Interaction between GSH and the thioredoxin and GST families is critical for their enzymatic activity in regulation of cellular oxidation states and the cellular response to stress. The motif’s conservation within the Clic family suggests that the Clic proteins are influenced by the oxidative state of cells and may have a similar enzymatic function involving GSH or a GSH-like binding partner.

Clic5 was found to localize to the base of stereocilia in the hair cells of the inner ear. This localization implies that Clic5’s presence at this specific location is necessary for its essential role in the formation and maintenance of stereocilia, and disruption of its localization may prevent Clic5 from functioning. My project focused on determining if the conserved CXXS motif was critical for the localization of Clic5 to microvilli, an actin based model of stereocilia. This was a step toward elucidating the function of Clic5 and determining the role of the conserved CXXS motif in the Clic family.

Several studies have investigated the role of this motif in other Clics, specifically focusing on the reactive cysteine residue (corresponding to Cys32 in
Clic5).\textsuperscript{18;19} I investigated the role of this motif in Clic5 localization by making mutations to the highly conserved Cys32 and Ser35 residues and examining their effects on the protein’s localization to microvilli of LLC-PK1 cells. I have shown that the Cys32 residue is critical for human Clic5 localization to microvilli while the Ser35 residue is not. I have also shown that S35C mutations, with or without the presence of Cys32, do not have an apparent effect on localization of Clic5 to microvilli.

**II. Cys32 is critical to Clic5 localization to microvilli**

In my experiments, Clic5 fusion proteins with a C32A mutation (AXXS, AXXA, and AXXC) showed significantly decreased localization to microvilli of LLC-PK1 cells. The decrease in localization to microvilli was most significant for the fusion proteins that contained a polar, hydrophilic residue in the 35 position (AXXS and AXXC). The double mutant in which both Cys32 and Ser35 were mutated to alanine resides (AXXA) showed decreased localization to microvilli, however the decrease was not as large as that seen for mutants AXXS and AXXC. Examination of the AXXC mutant showed that absence of Cys32 could not be compensated for by presence of a cysteine in the 35 position. Similar mutations have been made to the reactive cysteine residue of this motif in Clic1, Clic4, and GST omega to determine its structural and functional role in these proteins.\textsuperscript{18;19;20;21}

In GST omega, a member of the GST family, the corresponding Cys32 residue has been shown to form a mixed disulfide bond with GSH.\textsuperscript{22} GST omega is capable of modulating activity of a calcium channel in intracellular membranes. It was found that
a C32A mutation abolishes this modulating activity of GST omega.\textsuperscript{20} This C32A mutation has also been shown to abolish the thioltransferase activity of GST omega.\textsuperscript{21} In Clic1, the Cys24 residue (corresponding to Cys32 in Clic5) is also capable of forming a covalent mixed disulfide bond with GSH under oxidizing conditions.\textsuperscript{20} Cys24 can also form an intramolecular disulfide bond with Cys59, which causes a dramatic structural change allowing non-covalent Clic1 dimers to form under oxidizing conditions. These dimers are capable of forming anion channels \textit{in vitro}. The C24A mutation in Clic1 diminishes the protein’s ability to form these dimers and thus they are unable to form channels \textit{in vitro}.\textsuperscript{18} Clic4 has been found to translocate to the plasma membrane after LPA stimulation, however this translocation is not affected by redox regulation.\textsuperscript{19} It was found that a corresponding mutation (C35A) diminished the ability of Clic4 to translocate to the plasma membrane after LPA stimulation, whereas mutation of two other conserved cysteines farther downstream did not affect Clic4 translocation. As this translocation is not modulated by changes in the oxidative state of cells, and Cys35 was crucial for translocation, it was proposed that Cys35 may serve as an active site for a still unproven enzymatic function affecting translocation.\textsuperscript{19}

In both GST omega and Clic1, the first, conserved cysteine residue in this motif has been found to interact with GSH, indicating this residue is reactive and part of an active site\textsuperscript{18; 20; 22}. To summarize, this cysteine has been shown to be critical to processes like channel modulation (GST omega), enzymatic activity (GST omega), dimerization (Clic1), translocation (Clic4), and localization (Clic5; my study). The inability of C32A mutant Clic5 proteins to localize to microvilli could be caused by a
structural change, a change in their ability to interact with GSH or a GSH-like signaling molecule, or a change in their putative enzymatic function.

III. Ser35 is non-critical to Clic5 localization to microvilli

In my experiments, Clic5 fusion proteins with a Ser35 (CXXA, AXXA, CXXC, AXXC) mutation showed a range of results. Both proteins containing a Ser35 mutation with an unaltered Cys32 (CXXA and CXXC) did not exhibit any significant decrease in localization to microvilli, indicating Ser35 is non-critical to localization. The proteins containing a Ser35 mutation and a Cys32 mutation (AXXA and AXXC) did exhibit significantly decreased localization to microvilli, however this decrease is attributed to the C32A mutation and not to the change in Ser35. Therefore I conclude that Ser35 is not critical to human Clic5 localization to microvilli of LLC-PK1 cells.

In my experiments, the CXXA mutant exhibited significantly improved localization to microvilli compared to the wild type CXXS. In GST omega, which localizes to a variety of subcellular locations including membranes, the motif takes the form CXXA. Since this motif takes the form of CXXS in other GST family members which are primarily cytosolic, the membrane localization of GST omega could be attributed to the alanine residue in the fourth position of this motif. This may explain why the CXXA Clic5 mutant exhibited significantly increased localization to microvilli. It is possible that this motif plays similar roles in GST omega and Clic5. The change from a polar, hydrophilic group to a nonpolar hydrophobic group in the 35 position could allow a conformational change that favors Clic5 interactions with the
plasma membrane or F-actin. This could also explain why the AXXA Clic5 mutant exhibited greater localization to microvilli than did the AXXS and AXXC mutants, both of which retain a polar, hydrophilic group in the 35 position.

IV. S35C mutations do not improve Clic5 localization to microvilli

Within the mammalian Clics, this evolutionarily conserved motif takes the form of either CXXS (Clics 1, 4, 5, and 6) or CXXC (Clics 2 and 3).\(^\text{16}\) The CXXC motif is also found in non-mammalian Clics such as Drosophila.\(^\text{24}\) In the Clic-like protein found in C.elegans, the motif takes the form of DXXC.

It was hypothesized that an S35C mutation may improve localization of Clic5 to microvilli, since other members of the Clic family contain a cysteine (rather than serine) in this position. However, the CXXC mutant did not exhibit any significant change in localization to microvilli compared to CXXS, showing the S35C mutation had no effect on localization. When comparing the AXXC to the AXXS mutant, AXXC showed increased localization in one study and decreased localization in the other. However, qualitatively these mutants appear very similar. This quantitative discrepancy is likely due to the inherent difficulty in evaluating C32A mutants, as their localization to microvilli is very poor. Therefore, I conclude that S35C mutations have no apparent effect on human Clic5 localization to microvilli of LLC-PK1 cells.

In Clic2, where the motif takes the form CXXC, it was found that an intramolecular disulfide bond forms between Cys30 and Cys33 (corresponding to Cys32 and Ser35 in Clic5, respectively) under physiological conditions.\(^\text{25}\) The
crystallized structure of Clic2 did not exhibit binding of GSH to this motif, likely because of the presence of the disulfide bond. Despite lack of evidence for GSH binding, this motif is still thought to be an active site in the protein and may bind to GSH when in a reduced state. It is possible that the S35C mutation and resulting CXXC motif in Clic5 allowed formation of an intramolecular disulfide bond in Clic5 similar to that seen in Clic2. If so, Clic5 may not depend on GSH binding in this motif for localization to microvilli. However, structural analysis needs to be done to show whether or not wild type Clic5 binds GSH in this motif, and if the CXXC mutation in Clic5 forms an intramolecular disulfide bond that prevents GSH binding.

V. The conserved CXXC motif is functionally important in Drosophila

In C. elegans, the Clic-like protein EXC-4 localizes to the luminal membrane and is crucial to the formation of the excretory tubule. Localization to the luminal membrane has been shown to be dependent on the N-terminal quarter of EXC-4, which contains a DXXC motif corresponding to the CXXS motif in Clic5. Mutation of Asp36 (corresponding to Cys32 in Clic5) to an asparagine residue, or mutation of Cys39 (corresponding to Ser35 of Clic5) to an alanine residue did not change localization or disrupt the function of EXC-4 in formation of the excretory tubule. Therefore, the DXXC motif in EXC-4 is not critical to localization or function of the protein. It was also found that expression of human Clic1 in EXC-4 mutant C. elegans did not rescue excretory tubule defect, and human Clic1 did not localize to the luminal membrane. However, when the membrane targeting N-terminal region of EXC-4 was attached to the C-terminal three quarters of human Clic1, the protein was able to
localize to the luminal membrane and rescue the mutant phenotype. Thus, the mechanism of membrane localization of EXC-4 is different from that of mammalian Clic1, but their C-terminal three quarters are functionally interchangable. The high sequence similarity in the N-terminal region between Clic1 and Clic5 predicts that this difference in localization mechanism is likely the same between EXC-4 and Clic5.

In *Drosophila*, the Clic protein has a CXXC motif and is crucial to the organism’s survival, where a Clic null mutation causes 95% lethality at 28°C. Wild type CXXC Clic localizes to the plasma membrane and expression of wild type Clic in the Clic null mutant rescues the lethal phenotype 100%. In the Clic null mutant model, expression of an AXXA mutant Clic rescues the lethal phenotype only up to 50%. This indicates that this motif contributes to at least 50% of *Drosophila* Clic function (Nathan Hansen and Soichi Tanda, personal communication).

Although the DXXC motif seen in the *C.elegans* EXC-4 Clic-like protein was found to be non-critical to its function, the functional important of the CXXC motif in *Drosophila* Clic supports the hypothesis that the conserved cysteine residue in the first position of the motif is important to Clic family protein function in both vertebrates and invertebrates. Thus I propose that this motif plays an important role in both the localization and function of Clic5, and is likely crucial to the intricate process of hearing in mammals. Now that this motif has been identified as critical in Clic5, further research can be done to determine its specific function. This will help better understand Clic5’s role in hearing, and is a step toward elucidating the still undetermined functional roles of other members of the Clic family.
REFERENCES


channel protein CLIC5 is expressed at high levels in hair cell stereocilia and is essential for normal inner ear function. *J Neurosci* **26**, 10188-98.


Table A1. Localization to Microvilli-Like Projections for Wild Type and Mutant GFP-Clic5 Fusion Proteins in LLC-PK1 Cells

<table>
<thead>
<tr>
<th>GFP-Clic5 Fusion Protein</th>
<th>GOOD MV LOCALIZATION (&gt;50%)</th>
<th>POOR MV LOCALIZATION (&lt;50%)</th>
<th>TOTAL MV LOCALIZATION (Good + Poor)</th>
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<th>TOTAL # OF CELLS COUNTED</th>
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<td>78.3%</td>
<td>20.0%</td>
<td>98.3%</td>
<td>1.7%</td>
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</tr>
<tr>
<td>Mutant AXXA</td>
<td>774</td>
<td>669</td>
<td>1443</td>
<td>154</td>
<td>1597</td>
</tr>
<tr>
<td></td>
<td>48.5%</td>
<td>41.9%</td>
<td>90.4%</td>
<td>9.6%</td>
<td></td>
</tr>
<tr>
<td>Mutant CXXC</td>
<td>788</td>
<td>76</td>
<td>864</td>
<td>8</td>
<td>872</td>
</tr>
<tr>
<td></td>
<td>90.4%</td>
<td>8.7%</td>
<td>99.1%</td>
<td>0.9%</td>
<td></td>
</tr>
<tr>
<td>Mutant AXXC</td>
<td>157</td>
<td>695</td>
<td>852</td>
<td>220</td>
<td>1072</td>
</tr>
<tr>
<td></td>
<td>14.6%</td>
<td>64.8%</td>
<td>79.5%</td>
<td>20.5%</td>
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Table A2: Statistical Significance of Differences in Total Microvilli Localization for Wild Type and Mutant GFP-Clic5 Fusion Proteins Using Chi-Square Test

<table>
<thead>
<tr>
<th>Clic5-GFP Fusion Protein</th>
<th>Mutant AXXS</th>
<th>Mutant CXXA</th>
<th>Mutant AXXA</th>
<th>Mutant CXXC</th>
<th>Mutant AXXC</th>
</tr>
</thead>
<tbody>
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<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td></td>
<td>&lt;0.001</td>
<td>&gt;0.05</td>
<td>&lt;0.001</td>
<td>&gt;0.05</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mutant CXXA</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<td>&lt;0.001</td>
<td>&gt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Mutant CXXC</td>
<td>Yes</td>
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<td>Yes</td>
<td>Yes</td>
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<td>Yes</td>
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<tr>
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</table>
Table A3: Statistical Significance of Differences in Good Microvilli Localization for Wild Type and Mutant GFP-Clic5 Fusion Proteins Using Chi Square Test

<table>
<thead>
<tr>
<th>Clic5-GFP Fusion Protein</th>
<th>AXXS Mutant</th>
<th>CXXA Mutant</th>
<th>AXXA Mutant</th>
<th>CXXC Mutant</th>
<th>AXXC Mutant</th>
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</thead>
<tbody>
<tr>
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<td>Yes &lt;0.05</td>
<td>Yes &lt;0.001</td>
<td>Yes &lt;0.001</td>
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<td>Yes &lt;0.001</td>
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<td></td>
<td></td>
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<tr>
<td>CXXA Mutant</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>AXXA Mutant</td>
<td></td>
<td></td>
<td></td>
<td>Yes &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>CXXC Mutant</td>
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<td></td>
<td>Yes &lt;0.001</td>
</tr>
<tr>
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<td>Mutant</td>
<td>Mutant</td>
<td>Mutant</td>
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<tr>
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<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
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</tr>
<tr>
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<td>AXXS</td>
<td>CXXA</td>
<td>AXXA</td>
<td>CXXC</td>
<td>AXXC</td>
</tr>
</tbody>
</table>

**Figure A1. Comparison of localization to microvilli-like projections for wild type and mutant GFP-Clic5 fusion proteins.** Epi-fluorescence images of LLC-PK1 cells transfected with wild type and mutant GFP-Clic5 fusion constructs (green). Cells are stained with DAPI to mark DNA (blue). Note the appearance of hair-like projections on the surface of cells transfected with wild type CXXS, mutant CXXA, and mutant CXXC GFP-Clic5, and the lack of there projections on cells transfected with mutant AXXS, mutant AXXA, and mutant AXXC GFP-Clic5. Scale bar 20 µm.
Figure A2. Comparison of co-localization with F-actin in microvilli for wild type and mutant GFP-Clic5 fusion proteins. Confocal images of LLC-PK1 cells transfected with wild type and mutant GFP-Clic5 fusion constructs (green). Cells are stained with phalloidin to mark F-actin (red). Note the appearance of co-localization (orange) between GFP-Clic5 and F-actin in microvilli of cells transfected with wild type CXXS, mutant CXXA, and mutant CXXC GFP-Clic5, and the decrease in this co-localization in cells transfected with mutant AXXS, mutant AXXA, and mutant AXXC GFP-Clic5. Scale bar 20 μm.