**Specific Aims**

Aminoacyl tRNA Synthetases attach amino acids to their cognate tRNA molecules in an early step during protein synthesis that essentially establishes the genetic code (Ibba and Soll, 2000). In addition to this critical role, most tRNA synthetases carry out additional functions important to a cell’s physiology, such as regulation of angiogenesis, inflammation, amino acid metabolism, and translation (Guo and Schimmel, 2013; Mukhopadhyay, et al., 2009; Xu, et al., 2012). Recently, there have been a number of human genetic disorders associated with mutations in tRNA synthetase genes, a high proportion of which target nervous tissue (Antonellis, et al., 2008). The fact that aminoacyl tRNA synthetases can do more than aminoacylate tRNA and their associations with human disease indicates that we do not yet fully understand the functions of these enzymes *in vivo*. Specifically, several mutations in Histidyl-tRNA Synthetase, the enzyme responsible for attaching the amino acid histidine to its cognate tRNA, have been implicated in neurological disorders (Puffenberger, et al., 2012; Vester, et al., 2013). One of these disorders is a combined deafness-blindness disorder called Usher Syndrome Type 3B (USH3B). The symptoms of USH3B (progressive loss of hearing and vision) imply that HARS is particularly important for the function of the eye and the ear, but we do not yet know in what capacity. On-going work has begun to elucidate the effects of the USH3B mutation at the molecular level, but animal models are needed to help us understand effects of the mutation at the organismal level (Puffenberger, et al., 2012). We plan to generate a zebrafish line with the analogous mutation using the targeted gene editing system CRISPR/Cas9. Using zebrafish as our model will allow us to visualize and characterize phenotypes caused by the mutation *in vivo*, thanks to their optical clarity and external development. We will also use this model in conjunction with a proteomic approach, through which we will identify HARS binding partners that could reveal novel functions of the protein related to vison and hearing. **We hypothesize that HARS provides an important function unique to the visual and auditory systems, either via a novel mechanism or through tissue specific regulation of processes related to protein synthesis.**

**Aim 1: Establish zebrafish line that expresses HARS carrying the mutation analogous to the human USH3B mutation**

a. Using CRISPR/Cas9 and the Tol2 Transposase system we will create a zebrafish line that expresses the USH3B mutation. We will generate mutants on a previously established transgenic line that express fluorescent proteins in retinal neurons and hair cells, which will allow us to directly observe the effect of the mutation on the morphology of these tissues.

b. Evidence suggests that the USH3B mutation disrupts HARS thermos-stability and this could contribute to the disease. We plan to heat shock larvae to assess whether this exacerbates the mutant phenotypes.

**Aim 2: Evaluate effect of USH3B mutation on the Unfolded Protein Response (UPR) and Endoplasmic Reticulum (ER) Stress**

a. Disruptions to protein synthesis often induce the ER stress response and UPR. To determine whether the USH3B mutation impacts protein synthesis we will use qPCR to assess whether the USH3B mutation affects the expression of ER stress genes. We will use immunohistochemistry and in situ hybridization to determine whether there are spatial differences in the expression of ER stress/UPR markers in the zebrafish.

b. We will induce ER stress/UPR using a pharmacological approach to test whether ER stress is sufficient to induce an USH3B phenotype. If it is, these results would suggest that ER stress is the main molecular cause of USH3B.

**Aim 3: Identify HARS binding partners and the significance of these interactions**

a. We will perform a HARS pull-down assay with lysates from whole zebrafish larvae to isolate proteins HARS interactors and use mass spectrometry to identify these proteins. To validate identified binding partners, we will use western blotting and immunohistochemistry to assess co-localization.

b. We will use biochemical and genetic approaches to test the functional relevance of these interactions.

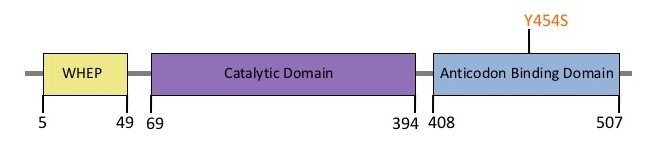
**Background**

***Aminoacyl tRNA Synthetases***

During the process of mRNA translation, tRNA molecules deliver amino acids to the ribosome to be added to the growing polypeptide chain. They accomplish this task via complementary base pairing between the anticodon of the tRNA and codon of the mRNA template, which codes for a specific amino acid. tRNAs act as the adapters that translate the mRNA codon into the corresponding amino acid. In order to ensure accurate translation, amino acids must be attached to tRNA molecules with specific anticodons. This is achieved by enzymes called aminoacyl tRNA synthetases. Each of the 20 common amino acids has a corresponding synthetase, which catalyzes the highly specific reaction that ligates an amino acid to a cognate tRNA molecule. All aminoacyl tRNA synthetases attach their amino acid to their tRNA molecules by essentially the same two-step mechanism. First, they activate their amino acid by using ATP to form an aminoacyl adenylate, then they transfer the amino acid to the acceptor stem of a cognate tRNA molecule (Ibba and Soll, 2000). These enzymes are therefore essential for maintaining the genetic code and proper protein synthesis.

As if serving this critical function was not enough, these enzymes have adopted a number of extra roles during their evolution (Brown, et al., 2010; Francklyn, 1997; Francklyn, et al., 2002; Guo and Schimmel, 2013). Many of these functions take advantage of properties of the enzymes’ canonical activity, such as amino acid and RNA binding, which occur in the synthetases’ core domains (Brown, et al., 2010; Carter, 1993; Francklyn, 1997; Francklyn, et al., 2002; Guo and Schimmel, 2013). Other non-canonical functions are dependent on various domains that synthetases in higher organisms have acquired (Brown, et al., 2010; Franckly, 1997; Francklyn, et al., 2002; Guo and Schimmel, 2013). These extra roles tie synthetases to a wide range of cellular processes from vascular patterning to inflammation (Brown, et al., 2010). Along with the unearthing of novel synthetase functions, a growing number of human disorders have been linked to mutations in synthetase genes (Antonellis and Green, 2008). Given their importance, it is not surprising that defects to synthetases result in disease, but it is surprising that many of these mutations are associated with rather tissue-specific disorders; specifically, there seems to be a bias towawrds neurological disorders (Antonellis and Green, 2008). The elucidation of new functions and the connections to disease remind us that there is still much to learn about the diverse roles these proteins play.

Of particular interest to us is Histidyl tRNA Synthetase (HARS), which attaches the amino acid histidine to its associated tRNA molecules. HARS functions as a homodimer and, in animals, each HARS monomer is composed of three domains (Guo, et al., 2010; Ibba and Soll, 2000). At the N-terminus there is a WHEP domain, which is a domain found on several synthetases but is not essential for tRNA aminoacylation (Raben, et al., 1994). This domain is followed by the catalytic domain, which contains the amino acid and ATP binding pocket, and then an anticodon binding domain at the C-terminus (Figure 1) (Raben, et al., 1994).



N

C

**Figure 1:** Linear representation of the HARS domain structure and the location of the USH3B mutation.

HARS has been found to exhibit pro-inflammatory activity, but there is currently little known about non-canonical functions of HARS (Brown, et al., 2010; Howard, et al., 2010). Interestingly, mutations in *HARS* have recently been associated with two different neurological disorders, a deafness-blindness disorder and a peripheral neuropathy (Puffenberger, et al., 2012; Vester, et al., 2013). The deafness-blindness disorder has been classified as Usher Syndrome Type IIIB (USH3B) and is associated with a point mutation that causes a Tyr to Ser substitution at position 454 (Y454S), a residue that provides an indirect interaction between the anticodon binding domain of one monomer and the catalytic domain of the other (Figure 1) (Pufffenberger, et al. 2012). This association raises the question of why a mutation in an enzyme required for protein synthesis throughout the body would cause a tissue specific disorder such as Usher Syndrome.

***Usher Syndrome***

Usher Syndromes are a group of genetic disorders characterized by combined deafness and blindness that follow an autosomal recessive pattern of inheiritance. Usher Syndromes are clinically and genetically diverse and are divided into three general groups based on characteristics of the symptoms. Type I is the most severe and is defined by profound deafness from birth and pre-pubertal vision loss (Ahmed, et al., 2003; Petit, 2001; Reiners, et al., 2006; Williams, 2008). Type II is more moderate and is distinguished from Type I by later onset of moderate to severe hearing loss as well as later onset of vision loss (Ahmed, et al., 2003; Petit, 2001; Pieke-Dahl, et al., 2000; Reiners, et al., 2006; Reisser, et al., 2002; Williams, 2008). Finally, Type III is the mildest form and is characterized by variable, progressive hearing and vision loss (Ahmed, et al. 2003, Pakarinen, et al., 1995; Petit, 2001; Reiners, et al., 2006, Williams, 2008). Studies in both humans and animal models of Usher Syndrome have shown that the hearing loss component of the disorder is due to disorganization and degeneration of sensory hair bundles in the cochlea (Bonnet and Amraoui, 2012; McGee, et al., 2006; Wagenaar, et al., 2000). The vision loss is due to retinitis pigmentosa, which is caused by the degeneration of retinal photoreceptors and presents as the development of night blindness followed by progressive tunneling of the visual field (Bonnet and Amraoui, 2012; Van Soest, et al., 1999).

Each of the three Usher Syndrome types is heterogeneous and all together there are currently 13 genes associated with the disorder (Bonnet and Amraoui, 2012; Mathur and Yang, 2014). The products of Usher genes perform a wide variety of functions (Bonnet and Amraoui, 2012; Mathur and Yang, 2014). They include an unconventional myosin, a G-protein coupled receptor, scaffold proteins, cell adhesion proteins, and other uncharacterized proteins (Hasson, et al., 1995; Mathur and Yang, 2014; Weil, et al., 1995; Weston, et al., 2004). Interestingly, studies have shown that many of these proteins actually form complexes within photoreceptors and hair cells and that disrupting the function of one of the components can disrupt the entire complex (Adato, et al, 2005; Blanco-Sanchez, et al., 2014; Boeda, et al., 2002; Grati, et al., 2012; Maerker, et al., 2008). It will be interesting to see if newer Usher proteins fit into these complexes as well.

HARS was linked to USH3B in 2012, making it a relatively recent addition to the list of Usher proteins (Puffenberger, et al., 2012). USH3B patients appear to lose hearing early in childhood, though the progression is not well characterized as there is no data on hearing function at birth (Puffenberger, et al., 2012). Vision defects are evident during childhood, but the rate of degeneration among these patients is highly variable (Puffenberger, et al., 2012). We should note that the visual defects do not perfectly match retinitis pigmentosa symptoms and that there are also reports of USH3B patients having febrile seizures and hallucinations. These reports make this disorder an unusual form of Usher Syndrome and there is some doubt as to whether these patients are true Usher pateints or if they suffer from another disorder (Mathur and Yang, 2014; Puffenberger, et al., 2012) Genetic mapping revealed that these USH3B patients all had a point mutation in the gene for *HARS* that caused the Y454S substitution described above (Puffenberger, et al., 2012). Initial biochemical characterization of the mutant enzyme indicates that it maintains essentially normal function. The protein has the same localization pattern as the wildtype in mIMCD3 cells, it dimerizes as expected, and kinetically it is almost indistinguishable from the wildtype protein (Puffenberger, et al., 2012; Robey-Bond, personal communication). One defect that has been found comes from recent thermal shift experiments, which indicate that the USH3B HARS mutant has reduced thermo-stability (Abbot J, unpublished). However, it remains to be seen whether this defect has any functional impact on the eye or ear.

***Visual and Auditory Systems***

How the Usher proteins contribute to visual and auditory function is still being elucidated and regardless of whether HARS remains on the list of Usher proteins, the symptoms seen in the individuals with the HARS Y454S mutation indicate that this protein also carries out an important function in these two sensory systems. Visual function is derived from the neurons in the retina, the structure at the back of the eye that detects light. Photoreceptor cells in the outermost layer of the retina are highly specialized neurons that contain photopigments in the membrane of their outer segments. These pigments undergo a conformational change when they absorb light, which induces a signaling cascade that ultimately affects the synaptic transmission of the photoreceptor, thereby transforming the light stimuli into an electrical one. A collection of interneurons (horizontal, bipolar, and amacrine cells) in the inner nuclear layer of the retina help process the stimuli, which is finally transmitted to the brain by retinal ganglion cells. Most Usher Syndrome patients exhibit retinitis pigmentosa (night blindness followed by progressive reduction of peripheral vision), which indicates degeneration of the photoreceptor cells (Bonnet and Amraoui, 2012). Curiously, recent visual testing on children with USH3B who had not reported visual symptoms yet suggests that they have defects in the inner nuclear layer (which contains the interneurons) rather than in the photoreceptors (Robey-Bond, personal communication). It may be that this is just a correlational finding and that photoreceptor defects will present later on, but further investigation is necessary to determine what cell types are affected by the USH3B mutation.

Auditory function comes from the ear, where sound waves physically move tiny bones in the middle ear, which in turn produced pressure changes in the cochlea, a helical, fluid-filled structure within the inner ear. These pressure waves displace the basilar membrane, an elastic membrane running down the middle of the cochlea. On the basilar membrane is the Organ of Corti, which is comprised of mechanosensory hair cells and support cells. The hair cells are responsible for translating the pressure waves (sound waves) into electrical stimuli. Like the photoreceptors in the retina, sensory hair cells are also specialized neurons. They have a graded collection of microvilli structures, called stereocilia, on their apical surface. When the basilar membrane moves, it moves the hair cell stereocilia across another membrane that sits just above them. The sterocilia are connected to one another such that when one moves it pulls on the tip of the next. At the tip are mechanosensitive ion channels, which open in response to the pulling motion and activate the hair cell. Unlike the photoreceptors, which transmit electrical stimuli to a number of interneurons before it goes to retinal ganglion cells and the brain, cochlear hair cells transmit electrical stimuli directly to spiral ganglion cells that project back to the brain. In most Usher Syndromes, it has been fairly well established that the affected auditory cells are the sensory hair cells (Mathur and Yang, 2014). It appears that many of the Usher proteins are involved with establishing the structure and organization of stereocilia and mechanoelectrical transduction activity of the hair cells (Grillet, et al., 2009; McGee, et al., 2006; Tian, et al., 2009). Affected hair cells are not able to function properly and ultimately degenerate (McGee, et al., 2006). The etiology of USH3B hearing loss is also likely due to degeneration of the hair cells, but we do not yet know why they degenerate.

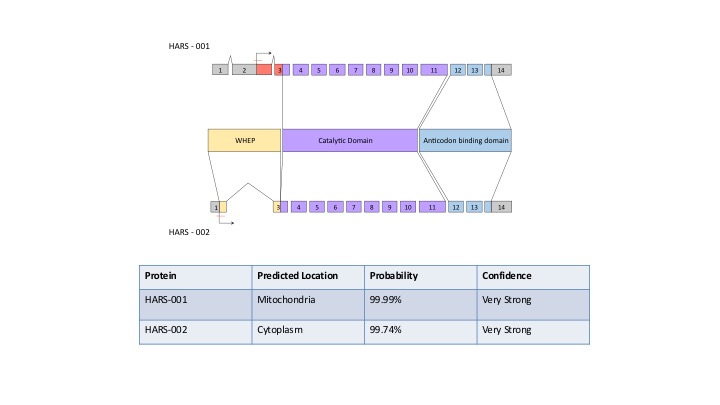
There are a few similarities between the sensory cells of each system that could explain why Usher mutations cause defects in these two distinct sensory systems. Both hair cells and photoreceptors harbor their sensory receptors in specialized structures that rely on both actin and microtubule based projections. In the photoreceptor, the outer segment, which harbors the photopigments, is connected to the inner segment by the connecting cilium. Also protruding up from the inner segment are microvilli-like processes that wrap around the base of the outer segment. The hair bundle on the apices of a hair cell is composed of both the microvilli-like stereocilia as well as a transient, microtubule-based kinocilium, which is important for the development of the hair bundle (Denman-Johnson and Forge, 1999; Lelli, et al., 2016). Many Usher proteins have been localized to the eye and ear analogs of these structures (Maerker, et al., 2006; Grillet, et al., 2009; Sahly, et al., 2012). Animal models have shown that these proteins are important for the development of these structures in the ear, however, less is known about their importance in photoreceptor development (Bonnet and Amraoui, 2012). Another similarity, and another structure where Usher proteins have been found, is at an atypical synaptic structure called a synaptic ribbon. Ribbon synapses allow for tonic release of neurotransmitter. This function is important for both hair cells and photoreceptors because they both exhibit continuous release of neurotransmitter that varies based on graded changes in the electrical state of the cell. The structure and function of both of hair cells and photoreceptors also means that they have high metabolic demands and protein turn-over. Mutations in several Usher proteins have been found to cause ER stress-induced apoptosis in zebrafish hair cells, suggesting that the inability to keep up with the demands of these cells could contribute to the degeneration of these sensory cells (Blanco-Sanchez, et al., 2014). This association seems particularly relevant for the USH3B mutation since HARS is integral to protein synthesis and disrupting HARS function is predicted to disrupt this process.

**Significance**

Vision and hearing are possibly the two most valued senses we possess as they are central to human communication. The systems that allow us to see and hear the world around us are sensitive to genetic and environmental insults, but there are few available therapies for vision and hearing loss. A better understanding of the molecular mechanisms that enable visual and auditory function will inevitably aid in the development of new therapies. Genetic disorder, while devastating, offer us a hint at what molecules are important for the function of these sensory systems. The finding that a globally important protein such as HARS is associated with a disorder characterized by defects to both vision and hearing is intriguing. Elucidating the role of HARS in the eye and ear will provide valuable insight into how visual and auditory sensory cells work, and also possible functions of tRNA synthetases in neural tissues. We will be using the model organism *Danio rerio* (zebrafish) to investigate HARS function, which allows us to ask questions about HARS at both the molecular and organismal level. This breadth will provide a more complete picture of the roles HARS plays in sensory systems.

**Preliminary Studies**

One of the benefits of using zebrafish as a model organism is their homology to humans. Aminoacyl tRNA synthetases are, in general, highly conserved between species, so we predicted that human and zebrafish *HARS* would be similar. What we found was that, from a genomic perspective, humans and zebrafish actually have a significant difference in regards to *HARS*. The human genome contains two *HARS* genes (*HARS and HARS2*) arranged in a head-to-head conformation (O’Hanlon, et al., 1995; 2002). The main difference between these two genes is that *HARS* codes for a cytoplasmic protein, while *HARS2* codes for a mitochondrial protein (O’Hanlon, et al., 2002). In contrast, the zebrafish genome contains only one *hars* gene, which appears to undergo alternative splicing to generate both a cytoplasmic and mitochondrial HARS protein (Figure 2). Despite this difference, when we align the amino acid sequences for the human and zebrafish cytoplasmic HARS proteins, they share 77% amino acid identity. This high a degree of similarity throughout protein suggests that both species proteins have retained similar functions despite the difference in genomic organization.



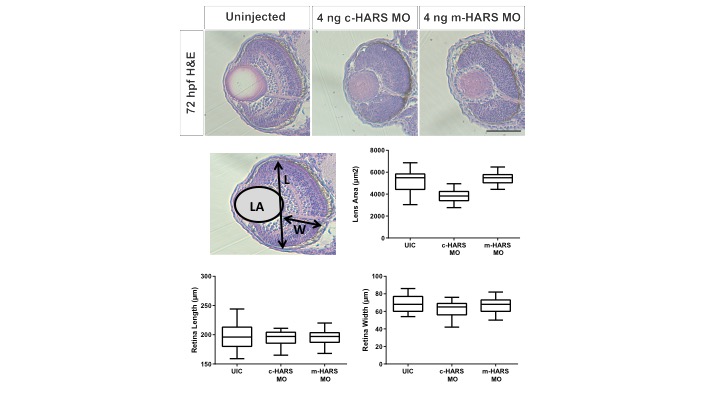
A

**Figure 2:** (A)The exon structure of the two transcripts generated from zebrafish *hars* in relation to the protein domains show that alternative splicing gives rise to proteins which differ only at their N-termini. (B) Subcellular localization predictors, predict with high confidence that HARS-001 and HARS-002 localize to the mitochondria and cytoplasmrespectively.

B

We have also begun functionally characterizing HARS in the zebrafish by using antisense morpholino oligonucleotides that block translation of the cytoplasmic *hars* transcript. We have found that when we inject embryos with a low concentration of cytoplasmic HARS morpholino the larvae have retinal lamination defects and fewer neuromasts (external sensory structures that are analogous to auditory hair cells) at 72 hours post fertilization (hpf), but that the overall morphology of the fish appears to be comparable to uninjected siblings (Figure 2). We have also used CRISPR/Cas9 mutagenesis to knock-out *hars* and seen a similar phenotype in the injected larvae (Figure 2). Additionally, *in situ* hybridization for *hars* shows strong expression in the developing eye and ear in both mouse and zebrafish (Diez-Roux, et al., 2011; Thisse and Thisse, 2004). These observations support the ideas that a) HARS has particularly important role in the eye and ear and b) that this role is conserved in the zebrafish.

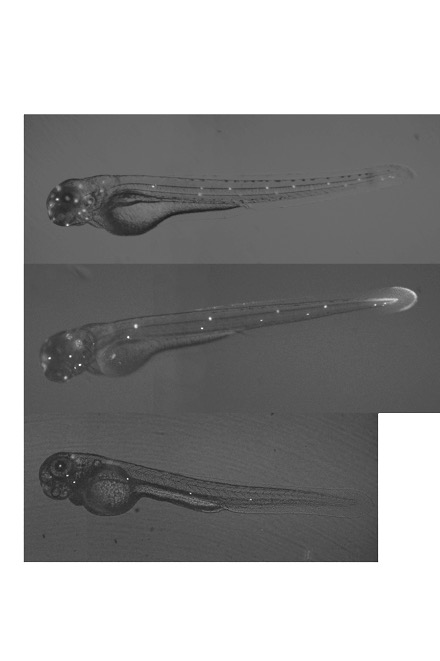
**Approach**



**Figure 3:** Using a fluorescent dye that selectively stains neuromasts and a general histological procedure we have found that the knock-down of *hars* results in (A) fewer neuromasts (arrows) down the posterior lateral line and (B) retinal lamination defects in 72 hpf larvae.

A

B



72 hpf 4-Di-2-ASP

Uninjected

4 ng c-HARS MO

HARS Mutant

(Mosaic)

**Aim 1: Generate zebrafish line that expresses the HARS mutation analogous to the USH3B mutation**

**Rationale**: As evidenced by our preliminary studies, HARS is highly conserved between humans and zebrafish and likely performs similar functions in the eye and ear of both organisms. Based on these observations we predict that introducing a mutation analogous to the USH3B mutation will result in zebrafish with visual and auditory defects similar to those seen in humans. Clinical and biochemical evidence suggests that this mutation may be thermosensitive, a hypothesis that we can test by comparing how wildtype and “USH3B” zebrafish respond to heat-shock. The zebrafish will allow us to look more closely at which cell types are affected by the mutation and in what capacity. A deeper understanding of the phenotype will provide clues as to what molecular mechanisms may be causing these phenotypes. Ultimately, our findings will provide insight into the etiology of the human USH3B condition.

**Methods 1a:** We will use the targeted gene editing system CRISPR/Cas9 in combination with single-stranded DNA oligonucleotides (ssODNs) to generate an “USH3B” zebrafish line. The idea behind this technique is that we can use CRISPR/Cas9 to induce a double-strand break (DSB) at a specific position in *hars*, which the cell will repair in one of two ways: nonhomologous end-joining (NHEJ) or homology-dependent repair (HDR). We can also supply the cell with an ssODN that is homologous to the region surrounding the DSB except that it contains the base-pair change we wish to induce. The cell will use this ssODN to undergo HDR and then mismatch repair to replace the wildtype sequence with our desired sequence. This technique has been used in cell culture to induce specific base pair substitutions and should be possible in zebrafish embryos (Bialk, et al., 2015). With the help of the Kmiec Lab, who developed this system, we have designed ssODNs and CRISPR guides that will allow us to induce the base pair change that will create the “USH3B” mutation. However, because this will cause the mutation to affect both the cytoplasmic and mitochondrial versions of zebrafish HARS, we will then use Gateway cloning and transposase-mediated transgenesis to insert the *hars* coding sequence with for the wildtype mitochondrial HARS. We will create this line on the *Tg(pou4f3:GFP)* zebrafish line, which expresses GFP in sensory hair cells and retinal ganglion cells. This background will allow us to directly observe the effect of our manipulations on the morphology and organization of the hair cells, which we expect to be disturbed, given the human phenotypes. It is currently unclear how the retina is affected in USH3B patients, but clinical tests indicate inner nuclear layer defects. To determine how the retina is affected in zebrafish, we will perform immunohistochemistry with retinal cell-type specific antibodies to assess the patterning of the retina and morphology of affected cells. It could be that mispatterning or “mis-wiring” of the retina during development results in a faulty circuit later in life that causes the visual impairment seen in USH3B patients.

**Alternative Approach:** In the event that we are unable to generate the specific mutant/transgenic line that we are aiming for, we can utilize the cytosolic-*hars* morpholino used in our preliminary studies to knock-down endogenous *hars* and co-inject with mRNA for wildtype or mutant *hars*. This approach will effectively result in “USH3B” zebrafish that we can then use to analyze phenotypes.

**Methods 1b:** There is strong evidence that the USH3B mutation reduces HARS’ thermostability, but that providing excess amounts of one of its substrates can stabilize the protein. To test this *in vivo* we will expose USH3B and wildtype zebrafish larvae to increasing temperatures and perform the same techniques used in aim **1a** to assess the phenotypic effect on the retina and hair cells. We expect that larvae expressing mutant *hars* will present defects at a lower temperature than wildtype larvae. Additionally, we can treat larvae with excess histidine and assess whether this alleviates the heat-shock induced phenotype. If this outcome is what we observe it would imply that the instability of mutant HARS contributes to USH3B symptoms.

**Aim 2: Determine whether the USH3B mutation induces the Unfolded Protein Response (UPR) and endoplasmic reticulum (ER) stress in the zebrafish and whether the eye and ear are more sensitive to this stress than other tissues**

**Rationale:** The USH3B mutation appears to render the HARS protein less thermo-stable and it is predicted that this instability leads to unfolding of the protein under temperature stress conditions. Unfolded HARS would be non-functional and most likely targeted for degredation. The result would be less functional HARS available to aminoacylate tRNAHis in the cell. A reduction in aminoacylated tRNAHis is predicted to disrupt protein synthesis, resulting in an accumulation of erroneous proteins, which in turn would induce ER stress and UPR. These responses initially induce mechanisms that will boost the cell’s ability to cope with misfolded proteins, however they can eventually lead to apoptosis (Lin, et al., 2007). Unaminoacylated tRNAcan also induce the amino acid starvation response (AAR), which converges with the UPR/ER stress upon inhibition of the translation initiation factor eIF2α and increase in the translation of the transcription factor ATF4 (Lin, et al., 2007; Sundred, et al., 2009). If the eye and ear have a heightened AAR/UPR/ER stress response, then this could explain the hearing and vision loss seen in USH3B patients. In contrast, if these tissues have a lesser response, the defects could be a result from excessive misfolding of proteins important to the function of the eye and the ear.

**Methods 2a:** These cellular stress responses promote an increase in the expression of a stereotypic set of genes that we can measure by qPCR to assess whether the response has been induced and compare between treatments. A member of the Francklyn lab recently used qPCR to measure the ATF4 target genes *asns*, *gpt2*, and *eif4ebp1* in zebrafish treated with inhibitors of another tRNA synthetase, indicating that this technique is a feasible method to measure these stress responses in our system (Mirando, et al., 2015). We can perform whole mount *in situ* hybridization for the same set of genes in order to visualize the expression levels and to assess expression localization. We predict that USH3B larvae will have an increased UPR/ER stress response overall, but that there may be differential expression across tissues that could provide insight into the USH3B phenotype.

**Methods 2b:** We will determine to what extent the ER stress response contributes to the USH3B phenotype by pharmacologically induce ER stress using thapsigargin (Tg), which reduces calcium-dependent chaperone activity in the ER by inhibiting a calcium channel in the ER membrane (Vacaru, et al., 2014). If the phenotype is mainly due to an accumulation of misfolded proteins and ER stress, then we expect that wildtype larvae treated with Tg will phenocopy USH3B mutants. Given this hypothesis, we also predict that Tg treatment will exacerbate the mutant larvae phenotype as it would be adding to the already increased load of misfolded proteins. Other groups have shown that the cytotoxic effects of unresolved UPR can be alleviated by decreasing protein synthesis rates (Parzych, et al., 2015). With this in mind, if the USH3B phenotype is a result of unchecked UPR, then we predict that inhibiting protein synthesis in USH3B mutant zebrafish will mitigate the phenotypes. We can test this prediction by treating fish with the translation inhibitor cycloheximide. Along with observing the morphological effects of these treatments, we will also use qPCR and *in situ* hybridization as in **2a** to measure and observe the transcriptional response to our manipulations.

**Alternative Approach:** Pharmacologically inducing ER stress globally will inevitably cause other defects in zebrafish larvae. We would expect the eye and ear to exhibit defects at a lower dose than other tissues if ER

stress is the cause of USH3B phenotypes, but it may be that these inhibitors are not sensitive enough to induce subtle phenotypes. In this case, we can turn to photo-morpholinos to genetically and locally manipulate components of UPR and protein synthesis in the eye and in hair cells of the ear. While this method would be technically challenging, it would allow us to assess how ER stress affects these tissues specifically.

**Aim 3: Identify HARS binding partners unique to tissues affected in USH3B**

**Rationale:** It has become clear that tRNA synthetases serve essential functions beyond tRNA aminoacylation. Therefore, it is plausible that the USH3B mutation could be disrupting a non-canonical function of HARS that is unique to retinal neurons and hair cells. Several other Usher Syndrome proteins have been found to complex with each other and these complexes appear to be important for maintaining auditory and visual function because mutations which disrupt the ability for these complexes to form appear to underlie different types of Usher Syndrome (see **Background** for citations). To date HARS has not been found to interact with any of the other Usher proteins, but it’s association with Usher Syndrome is relatively recent and so would likely have been overlooked in previous studies. If HARS is found to interact with other Usher proteins and these interactions are disrupted by the USH3B mutation, then this finding would indicate a possible mechanism that causes visual and auditory system defects and also a novel function of this protein. Our plan is to use a proteomics approach to identify HARS binding partners in zebrafish, which allows us to both look specifically for Usher protein interactions as well as screen for other binding partners of HARS.

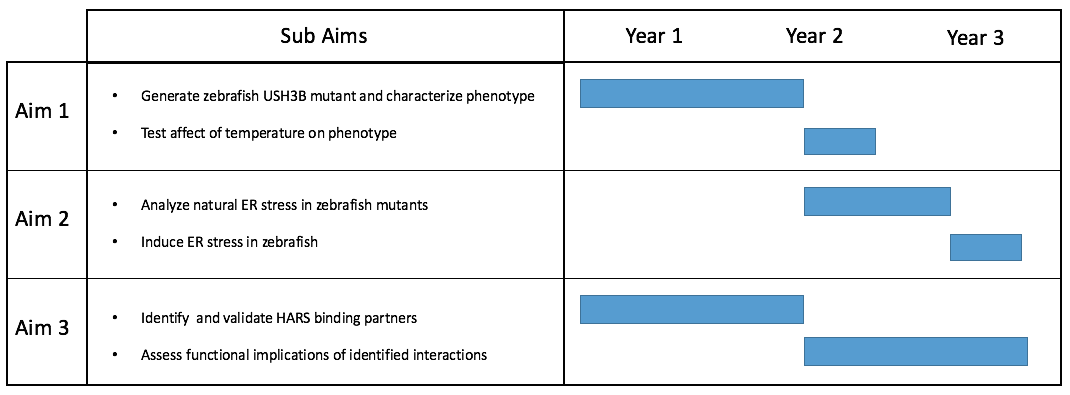
**Methods 3a:**  We plan to capture HARS binding partners using a pulldown assay with affinity purified GST-tagged zebrafish HARS as bait and zebrafish lysate as the source of prey proteins. As negative controls we will run the assay with GST alone and with lysate alone so that we can subtract out proteins that do not specifically interact with HARS in our downstream analyses. As a positive control we can use western blotting to confirm binding of a known HARS interactor, such as elongation factor 1β (Lee, et al., 2002). Proteins collected in our pulldown assay will be identified by mass spectrometry using equipment available from VGN and protocols used extensively by a collaborator’s lab (Cheerathodi and Ballif, 2011). We expect to identify components of translation machinery, but also novel binding partners which may include known Usher proteins or proteins that could indicate a role for HARS in sensory receptor function or neuronal function in general. To validate binding interactions, we will express epitope-tagged HARS in cell culture and in zebrafish larvae using standard protocols and perform both co-immunoprecipitation followed by western blotting as well as immunohistochemistry and co-localization analysis.

**Alternative Approaches:** If we are not able to purify sufficient amounts of GST-tagged HARS protein, there are alternative tags that we can use. For example, the Francklyn lab has use Halo Tag technology to perform pulldowns with human HARS. The Francklyn lab has also been validating new HARS antibodies and testing their affinity for zebrafish HARS. If we find that any of these perform well, we could perform co-immunoprecipitation to isolate endogenous HARS and binding partners directly from zebrafish lysate instead of performing a pulldown.

**Methods 3b:** After validating HARS binding partners, we can test the functional relevance of these interactions and how they may apply to USH3B in a couple ways. First, we will perform a pulldown with the USH3B mutant instead of wildtype and use western blotting to assess whether the mutation alters these interactions. We can also use morpholinos to knock-down expression of the binding partner. We would expect that if this interaction is important for HARS’ function in the eye and ear then reduced expression in the associated proteins would cause phenotypes similar to the USH3B mutation.

**Alternative Approaches:** We can also perform similar experiments in cell culture, using a cell line derived from inner ear progenitors, which our collaborators have used in the past. We can over express epitope-tagged wildtype or mutant HARS and perform co-immunoprecipitation from these cells. This could prove to be a more efficient method than using zebrafish, though it raises the issue of having overexpressed the protein and being in culture rather than in an organism. However, these cells are ear specific, and so theoretically would express a greater proportion of the relevant proteins, making it more likely for us to observe relevant interactions.

**Timeline**

****

**Works Cited**

Adato A, Michel V, Kikkawa Y, Reiners J, Alagramam KN, Weil D, Yonekawa H, Wolfrum U, El-Amraoui A, Petit C (2006) Interactions in the network of Usher syndrome type 1 proteins. Human Molecular Genetics 14:347–356.

Ahmed, Riazuddin, Riazuddin, Wilcox (2003) The molecular genetics of Usher syndrome. Clin Genet 63:431–444.

Antonellis A, Green E (2008) The Role of Aminoacyl-tRNA Synthetases in Genetic Diseases. Annu Rev Genom Hum G 9:87–107.

Bialk P, Rivera-Torres N, Strouse B, Kmiec EB (n.d.) Regulation of Gene Editing Activity Directed by Single-Stranded Oligonucleotides and CRISPR/Cas9 Systems. PloS one 10:e0129308.

Blanco-Sánchez B, Clément A, Fierro J, Washbourne P, Westerfield M (2014) Complexes of Usher proteins preassemble at the endoplasmic reticulum and are required for trafficking and ER homeostasis. Disease Models & Mechanisms 7:547–559.

Boeda B, El-Amraoui A, Bahloul A, Goodyear R, Daviet L, Blanchard S, Perfettini I, Fath KR, Shorte S, Reiners J, Houdusse A, Legrain P, Wolfrum U, Richardson G, Petit C (2002) Myosin VIIa, harmonin and cadherin 23, three Usher I gene products that cooperate to shape the sensory hair cell bundle. Embo j 21:6689–6699.

Brown M, Reader J, Tzima E (2010) Mammalian aminoacyl-tRNA synthetases: Cell signaling functions of the protein translation machinery. Vasc Pharmacol 52:21–26.

Bonnet C, El-Amraoui A (2012) Usher syndrome (sensorineural deafness and retinitis pigmentosa): pathogenesis, molecular diagnosis and therapeutic approaches. Curr Opin Neurol 25:42.

Carter CW (1993) Cognition, mechanism, and evolutionary relationships in aminoacyl-tRNA synthetases. Annu Rev Biochem 62:715–748.

Cheerathodi M, Ballif B (2011) Identification of CrkL-SH3 binding proteins from embryonic murine brain: implications for Reelin signaling during brain development. J Proteome Res 10:4453–4462.

Denman-Johnson K, Forge A (1999) Establishment of hair bundle polarity and orientation in the developing vestibular system of the mouse. J Neurocytol 28:821–835.

Diez-Roux G et al. (2011) A high-resolution anatomical atlas of the transcriptome in the mouse embryo. Plos Biol 9:e1000582.

Francklyn C, Musier-Forsyth K, Martinis SA (1997) Aminoacyl-tRNA synthetases in biology and disease: new evidence for structural and functional diversity in an ancient family of enzymes. RNA 3:954–960.

Francklyn C, Perona J, Puetz J, Hou Y-M (2002) Aminoacyl-tRNA synthetases: versatile players in the changing theater of translation. Rna New York N Y 8:1363–1372.

Grati M, Shin J-B, Weston M, Green J, Bhat M, Gillespie P, Kachar B (2012) Localization of PDZD7 to the stereocilia ankle-link associates this scaffolding protein with the Usher syndrome protein network. The Journal of Neuroscience 32: 14288-14293.

Grillet N, Xiong W, Reynolds A, Kazmierczak P, Sato T, Lillo C, Dumont RA, Hintermann E, Sczaniecka A, Schwander M (2009) Harmonin mutations cause mechanotransduction defects in cochlear hair cells. Neuron 62:375–387.

Guo M, Yang X-L, Schimmel P (2010) New functions of aminoacyl-tRNA synthetases beyond translation. Nature reviews Molecular cell biology.

Guo M, Schimmel P (2013) Essential nontranslational functions of tRNA synthetases. Nature chemical biology 9:145–153.

Hasson T, Heintzelman MB, Santos-Sacchi J, Corey DP, Mooseker MS (1995) Expression in cochlea and retina of myosin VIIa, the gene product defective in Usher syndrome type 1B. Proceedings of the National Academy of Sciences 92:9815–9819.

Howard OM, Dong H, Yang D, Raben N, Nagaraju K, Rosen A, Casciola-Rosen L, Härtlein M, Kron M, Yang D, Yiadom K, Dwivedi S, Plotz P, Oppenheim J (2002) Histidyl–tRNA Synthetase and Asparaginyl–tRNA Synthetase, Autoantigens in Myositis, Activate Chemokine Receptors on T Lymphocytes and Immature Dendritic Cells. J Exp Medicine 196:781–791.

Ibba M, Soll D (2000) Aminoacyl-tRNA synthesis. Annu Rev Biochem 69:617–650.

Lelli A, Michel V, Monvel J, Cortese M, Bosch-Grau M, Aghaie A, Perfettini I, Dupont T, Avan P, El-Amraoui A, Petit C (2016) Class III myosins shape the auditory hair bundles by limiting microvilli and stereocilia growth. J Cell Biology 212:231–244.

Lin JH, Li H, Yasumura D, Cohen HR, Zhang C (2007) IRE1 signaling affects cell fate during the unfolded protein response. Science 318: 944-949.

Maerker T, van Wijk E, Overlack N, Kersten F, McGee J, Goldmann T, Sehn E, Roepman R, Walsh E, Kremer H, Wolfrum U (2008) A novel Usher protein network at the periciliary reloading point between molecular transport machineries in vertebrate photoreceptor cells. Human Molecular Genetics 17:71–86.

Mathur P, Yang J (2014) Usher Syndrome: Hearing loss, retinal degeneration, and associated abnormalities. Biochimica et Biophysica Acta.

McGee J, Goodyear R, McMillan R, Stauffer E, Holt J, Locke K, Birch D, Legan K, White P, Walsh E, Richardson G (2006) The Very Large G-Protein-Coupled Receptor VLGR1: A Component of the Ankle Link Complex Required for the Normal Development of Auditory Hair Bundles. The Journal of Neuroscience 26:6543–6553.

Mirando A, Fang P, Williams T, Baldor L, Howe A, Ebert A, Wilkinson B, Lounsbury K, Guo M, Francklyn C (2015) Aminoacyl-tRNA synthetase dependent angiogenesis revealed by a bioengineered macrolide inhibitor. Scientific Reports 5:13160.

Mukhopadhyay R, Jia J, Arif A, Ray PS, Fox PL (2009) The GAIT system: a gatekeeper of inflammatory gene expression. Trends Biochem Sci 34:324–331.

O’Hanlon TP, Raben N, Miller FW (1901) A Novel Gene Oriented in a Head-to-Head Configuration with the Human Histidyl-tRNA Synthetase (HRS) Gene Encodes an mRNA That Predicts a Polypeptide Homologous to HRS. Biochemical and Biophysical Research Communications 210:556–566.

O’Hanlon T, Miller F (2002) Genomic organization, transcriptional mapping, and evolutionary implications of the human bi-directional histidyl-tRNA synthetase locus (HARS/HARSL). Biochemical and biophysical research communications 294.

Pakarinen, Karjalainen, Simola, Laippala, Kaitalo (1995) Usher’s syndrome type 3 in Finland. Laryngoscope 105:613–617.

Parzych, Chinn, Chen, Loaiza, Porsch, Valbuena, Kleijnen, Karadimitris, Gentleman, Keun, Auner (2015) Inadequate fine-tuning of protein synthesis and failure of amino acid homeostasis following inhibition of the ATPase VCP/p97. Cell Death Dis 6:e2031.

Pieke-Dahl, Möller, Kelley, Astuto, Cremers, Gorin, Kimberling (2000) Genetic heterogeneity of Usher syndrome type II: localisation to chromosome 5q. J Med Genet 37:256–262.

Petit C (2001) Usher syndrome: from genetics to pathogenesis. Annu Rev Genomics Hum Genet 2:271–297.

Puffenberger E, Jinks F, Sougnez C, Cibulskis K, Willert R, Achilly N, Cassidy R, Fiorentini C, Heiken K, Lawrence J, Mahoney M, Miller C, Nair D, Politi K, Worcester K, Setton R, DiPiazza R, Sherman E, Eastman J, Francklyn C, Robey-Bond S, Rider N, Gabriel S, Morton D, Strauss K (2012) Genetic Mapping and Exome Sequencing Identify Variants Associated with Five Novel Diseases. PLoS ONE 7:e28936.

Raben N, Nichols R, Dohlman J, McPhie P, Sridhar V, Hyde C, Leff R, Plotz P (1994) A motif in human histidyl-tRNA synthetase which is shared among several aminoacyl-tRNA synthetases is a coiled-coil that is essential for enzymatic activity and contains the major autoantigenic epitope. Journal of Biological Chemistry 269:24277–24283.

Reiners J, Nagel-Wolfrum K, Jürgens K, Märker T, Wolfrum U (2006) Molecular basis of human Usher syndrome: deciphering the meshes of the Usher protein network provides insights into the pathomechanisms of the Usher disease. Experimental eye research 83:97–119.

Reisser, Kimberling, Otterstedde (2002) Hearing Loss in Usher Syndrome Type II is Nonprogressive. Ann Otology Rhinology Laryngology 111:1108–1111.

Sahly I, Dufour E, Schietroma C, Michel V, Bahloul A, Perfettini I, Pepermans E, Estivalet A, Carette D, Aghaie A, Ebermann I, Lelli A, Iribarne M, Hardelin JP, Weil D, Sahel JA, El-Amraoui A, Petit C (2012) Localization of Usher 1 proteins to the photoreceptor calyceal processes, which are absent from mice. J Cell Biol 199:381–399.

Sundrud MS, Koralov SB, Feuerer M, Calado DP, Kozhaya AE, Rhule-Smith A, Lefebvre RE, Unutmaz D, Mazitschek R, Waldner H, Whitman M, Keller T, Rao A (2009) Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response. Science 324:1334–1338.

Thisse, B., Thisse, C. (2004) Fast Release Clones: A High Throughput Expression Analysis. ZFIN Direct Data Submission

Tian G, Zhou Y, Hajkova D, Miyagi M, Dinculescu A, Hauswirth WW, Palczewski K, Geng R, Alagramam KN, Isosomppi J, Sankila E-M, Flannery JG, Imanishi Y (2009) Clarin-1, Encoded by the Usher Syndrome III Causative Gene, Forms a Membranous Microdomain: POSSIBLE ROLE OF CLARIN-1 IN ORGANIZING THE ACTIN CYTOSKELETON. Journal of Biological Chemistry 284:18980–18993.

Vacaru A, Narzo A, Howarth D, Tsedensodnom O, Imrie D, Cinaroglu A, Amin S, Hao K, Sadler K (2014) Molecularly defined unfolded protein response subclasses have distinct correlations with fatty liver disease in zebrafish. Dis Model Mech 7:823–835.

Van Soest S, Westerveld A, Jong P, Bleeker-Wagemakers E, Bergen A (1999) Retinitis Pigmentosa Defined From a Molecular Point of View. Surv Ophthalmol 43:321–334.

Vester A, Velez‐Ruiz G, McLaughlin H, Program N, Lupski J, Talbot K, Vance J, Züchner S, Roda R, Fischbeck K, Biesecker L, Nicholson G, Beg A, Antonellis A (2013) A Loss‐of‐Function Variant in the Human Histidyl‐tRNA Synthetase (HARS) Gene is Neurotoxic In Vivo. Hum Mutat 34:191–199.

Wagenaar M, Schuknecht H, Nadol J, Rens M, Pieke-Dahl S, Kimberling W, Cremers C (2000) Histopathologic Features of the Temporal Bone in Usher Syndrome Type I. Archives Otolaryngology Head Neck Surg 126:1018–1023.

Weil D, Blanchard S, Kaplan J, Guilford P, Gibson F, Walsh J, Mburu P, Varela A, Levilliers J, Weston MD (1995) Defective myosin VIIA gene responsible for Usher syndrome type 1B. Nature 374:60–61.

Weston MD, Luijendijk M, Humphrey KD (2004) Mutations in the VLGR1 gene implicate G-protein signaling in the pathogenesis of Usher syndrome type II. The American Journal of Human Genetics 74:357-366

Williams DS (2008) Usher syndrome: Animal models, retinal function of Usher proteins, and prospects for gene therapy. Vision Research 48:433–441.

Xu X, Shi Y, Zhang H-M, Swindell EC, Marshall AG, Guo M, Kishi S, Yang X-L (2012) Unique domain appended to vertebrate tRNA synthetase is essential for vascular development. Nature communications 3:681.