5-6-2011

Characterization of Expression of the KCNE Gene Family in Zebrafish, Danio rerio

Lauren Vernlund
Roger Williams University, lvernlund703@r.wu.edu

Follow this and additional works at: http://docs.rwu.edu/honors_theses
Part of the Biochemistry, Biophysics, and Structural Biology Commons, and the Biology Commons

Recommended Citation
http://docs.rwu.edu/honors_theses/15

This Thesis is brought to you for free and open access by the RWU Theses at DOCS@RWU. It has been accepted for inclusion in Honors Theses by an authorized administrator of DOCS@RWU. For more information, please contact mwu@rwu.edu.
Characterization of Expression of the KCNE Gene Family in Zebrafish, *Danio rerio*

Lauren Vernlund

Bachelor of Science in Biology and Chemistry
Department of Biology, Marine Biology, and Environmental Science

Feinstein College of Arts and Sciences
Roger Williams University
May 2011
The thesis of Lauren Vernlund was reviewed and approved by the following:

_________________________________ Date ______________________

Dr. Kerri S. Warren
Associate Professor of Biology
Thesis Advisor

_________________________________ Date ______________________

Dr. Thomas Sorger
Professor of Biology

_________________________________ Date ______________________

Dr. Lauren Rossi
Assistant Professor of Chemistry
Acknowledgements

I would like to thank Dr. Kerri S. Warren for her guidance, knowledge, and unsurpassed dedication to this project and its success. Thank you to Dr. Tom Sorger and Dr. Lauren Rossi for a critical reading of the manuscript, and to Caitlin Gosselin, Erica Ryke, and Ana Malone Oliver for their laboratory assistance. Funding was provided by the Roger Williams University Honors program.
# Table of Contents

Abstract .......................................................................................................................... v

Background ..................................................................................................................... 1-5

Research Methods and Procedures .............................................................................. 5-11

Results ............................................................................................................................. 11-23

Figure 1. Agarose gel electrophoresis separation of amplified KCNE4 from wildtype zebrafish DNA .................. 13

Figure 2. KCNE1 and KCNE3 PCR product gel electrophoresis confirmation for two separate colonies of *E. coli* transfected with the zebrafish Chromosome 23 BAC ...................................................... 14

Figure 3. Agarose gel electrophoresis analysis of the KCNE3 FA/RB PCR products amplified from seven colonies of *E. coli* transfected with the zebrafish Chromosome 23 BAC ...................................................... 15

Figure 4. BLAST alignment of sequenced KCNE4 from wildtype zebrafish with KCNE4 sequence from the Zebrafish Genomic Library (NCBI) .................................................................................................................. 16

Figure 5. Phylogenetic tree alignment of KCNQ1, KCNQ2, KCNQ3, KCNQ4 and KCNQ5 genes in human, mouse, xenopus, chicken, carp and zebrafish ........................................................................................... 17

Figure 6. Phylogenetic tree alignment of the HCN1, HCN2, HCN3 and HCN4 gene members in human, mouse, rainbow trout and zebrafish .......................................................................................... 18

Figure 7. Phylogenetic tree alignment of the KCNE1, KCNE2, KCNE3, KCNE4 and KCNE5 gene members in human, mouse, xenopus, chicken, salmon, carp and zebrafish .................................................................................. 19

Figure 8. KCNE1, KCNE3 and Cytochrome Oxidase expression via agarose gel electrophoresis separation of rtPCR products from heart cDNA .................................................................................. 20

Figure 9. KCNE1, KCNE3 and cytochrome oxidase expression via agarose gel electrophoresis separation of rtPCR products from liver cDNA .................................................................................. 21

Figure 10. The Syntenic Analysis of the *Slow mo* region on Chromosome 23 in Zebrafish, *Danio rerio* revealed that taz, ATP64P1, GDI1, and FAM50A are located on chromosome 23 in zebrafish and the X chromosomes of humans and mice in non-contig portions .................................................................................. 23

Discussion & Conclusion .............................................................................................. 23-32

Literature Cited ................................................................................................................. 33-34

Appendix .......................................................................................................................... 35-38

Glossary of Molecular Genetics Terms ........................................................................ 39
Abstract

The KCNE gene family codes for five transmembrane accessory proteins, “minK related peptides” or Mirps, involved in the modification of voltage-gated potassium (Kv) channels, K⁺ selective pores vital in the regulation of membrane potential and repolarization in all organisms. In mammals, all five KCNE gene members are conserved and active in the heart. In the zebrafish Danio rerio, there are no apparent orthologs for KCNE2 or KCNE5, yet they contain Kv channels with homologous structure, function, and Mirp regulatory behavior to other organisms. Sequence analysis of wildtype zebrafish KCNE1, 3 and 4, and rtPCR on RNA from zebrafish tissues to assess adult expression led to the identification of the Mirps in zebrafish and a depiction of their expression patterns. Specifically, zebrafish were phylogenetically identified as homologs to KCNE1 and KCNE4 from other species and KCNE1 and KCNE3 cDNA showed expression in wildtype adult zebrafish heart tissue, implicating that MinK, Mirp2, and Mirp3 play active roles in the regulation of voltage-gated potassium channels in zebrafish, Danio rerio.
Background

Potassium channels have important physiological roles in all living organisms. Specifically, this channel family utilizes a diverse and highly conserved class of membrane proteins, which include more than 75 pore-forming subunits (Lundquist et al. 2006). Voltage-gated potassium (Kv) channels such as the potassium voltage-gated channel, KQT-like subfamily ( KCNQ) channels and Hyperpolarization-activated cyclic nucleotide gated (HCN) channels encompass a large portion of this family and work to govern resting membrane potential as well as to repolarize excitable membranes (McCrossan and Abbott, 2004). Kv channels are comprised of a tetramer of α subunits consisting of 6 transmembrane domains encoded by a large number of genes (Abbott et al. 2007). Furthermore, the modification of Kv channels involves, in part, small single transmembrane accessory proteins called “minK related peptides” or Mirps, translated from members of the potassium voltage-gated subfamily E (KCNE) gene family. This family consists of five peptide members: MinK (KCNE1), Mirp1 (KCNE2), Mirp2 (KCNE3), Mirp3 (KCNE4), and Mirp4 (KCNE5). All five gene members are conserved in mammals, with at least some annotated members present in fish, flies, amphibians and birds.

In humans, the expression of all five KCNE genes in varying combinations has been observed in excitable tissues as reviewed by Meir 2005. Specifically, KCNE1 expression has been localized to the heart, lungs, kidneys, testis, ovaries, small intestine, and peripheral blood leukocytes. KCNE2 is highly expressed in brain, heart, skeletal muscle, pancreas, placenta, kidney, colon, and thymus tissue. Additionally, KCNE2 is less significantly expressed in liver, ovaries, testis, prostate, small intestine and leukocytes and is nearly undetectable in the lungs and spleen. KCNE3 is widely expressed in skeletal muscle, brain, and heart, with the highest levels
in kidney and moderate levels in the small intestine. KCNE4 is predominately expressed in embryo and adult uterus, with low expression found in the kidneys, small intestine, lungs and heart. KCNE5 is highly expressed in the heart, skeletal muscle, brain, spinal cord, and placenta. The KCNE family members have been functionally linked to specific Kv channels, which is supported by the overlap in KCNE gene expression observed by Meir in 2005 in human excitable tissues and implies that each member plays specific inhibitory or enhancement roles in voltage-gated potassium channels (Appendix 1).

Furthermore, a study on the expression of multiple KCNE genes participating in the $I_{Ks}$ current in the human heart found that relative expression varied between regions in the heart and co-expression of KCNE1, KCNE3, KCNE4, and KCNE5 resulted in varying functional effects ranging from constitutive activation to suppression (Lundquist et al. 2005). Specifically, KCNE3 resulted in rapidly activating the $I_{Ks}$ current, KCNE4 or KCNE5 suppressed the $I_{Ks}$ current, and co-transfection of KCNE3, KCNE4, or KCNE5 with KCNE1 resulted in rapid activation or significant inhibition of the $I_{Ks}$ current in comparison to the expression of KCNE1 alone (Lundquist et al. 2005). The results of Lundquist et al.’s study imply that each Mirp peptide plays an individual role in the modification of other family members and, together, the set of peptides achieve the overall regulation of specific potassium currents in humans.

While humans contain all five KCNE gene family members, many other organisms have been observed to possess only a few of the KCNEs. Particularly, mouse Mus musculus, have been reported to heavily express the voltage-gated potassium channel KCNQ1 in the stomach, small intestine, and colon and KCNE3 has been shown to be expressed in the colon and to a lesser extent in the small intestine. In a study, it was determined that KCNQ1 and KCNE3 co-localize in the colon and small intestine (Dedek and Waldegger, 2001). In the same study, it was
found that co-expression of KCNE2 and KCNQ1 yield potassium currents that were open at resting voltages (Dedek and Waldegger, 2001).

Similarly, *Xenopus laevis* conserve KCNE1, KCNE3, and KCNE5 and in a study on the Kv2.1 voltage-gated potassium channel, it was shown that MinK (from KCNE1) and Mirp2 (from KCNE3) work additively to slow the activation of the channel in oocytes (Gordon et al. 2006). The constitutive activation observed in the Kv2.1 channel indicates that MinK, and Mirp2 interact directly in the channel. Moreover, an extensive study in *Xenopus* oocytes demonstrated that KCNE1 and KCNE3 affect channel KCNQ5 (Kv7.5) currents. Specifically, KCNE1 slows activation and suppresses inward rectification and KCNE3 inhibits Kv7.5 currents, lending further implication that the KCNE family members in *Xenopus* have functional interactions (Roura-Ferrer et al. 2009). The study of the KCNE gene members in *Mus musculus* and *Xenopus laevis* confirm that KCNE genes play an active role in the modification of voltage-gated potassium channels in various species and, furthermore, the KCNE gene products show interactive and constitutive modification.

**Pre-Thesis Research**

In collaboration with Caitlin Gosselin, it was determined that fish, flies, amphibians, and birds have been found to conserve at least a few members of the KCNE gene family but not all (Appendix 2). Specifically, in zebrafish *Danio rerio*, genomic evidence only supports the presence of KCNE1 (MinK) on Chromosome 9, KCNE3 (Mirp2) on Chromosome 23, and KCNE4 (Mirp3) on Chromosome 18. KCNE orthologs in zebrafish have not yet been published although homologs to KCNE1 (Chromosome 9) and KCNE4 (Chromosome 18) are readily apparent from sequence alignments to human KCNE1 and KCNE4 (Gosselin and Warren,
unpublished). Chained tBLASTn searches (NCBI, 2009) of the genome reveal KCNE3-like sequence on Chromosome 23. (Gosselin, Warren, & Vernlund, unpublished). To date, the entire zebrafish genome has been sequenced, the latest versions possess dense gene coverage and it appears that there are no orthologs for KCNE2 or KCNE5 (Warren, Gosselin, & Vernlund unpublished).

The absence of KCNE2 and KCNE5 in the zebrafish genome could be considered to represent the absence of a specific voltage-gated potassium (Kv) channel. However, it has been observed in electrophysiology studies that zebrafish contain Kv channels with homologous function to other organisms (Appendix 3) (Warren and Baker, unpublished observation). Additionally, the cardiac pacemaker currents in zebrafish behave as if they are regulated by Mirps (Baker et al, 1997). Moreover, the Slow mo mutant zebrafish displays a severe reduction in the fast kinetic component of the $I_h$ current while the slow component remains intact (Baker et al. 1997). The $I_h$ current is modified by Mirps and is responsible for the slow depolarization of heart tissue which is due to nonselective Na$^+$/K$^+$ conductance activated upon hyperpolarization. The selective attenuation of one component of the $I_h$ current in the Slow mo mutant is indicative of a missing voltage-gated potassium channel modifier, making the members of the KCNE gene family ideal candidates for the genetic mutation responsible for the bradycardia observed in Slow mo zebrafish (Baker et al. 1997). Additionally, the Slow mo mutation maps to a region of Chromosome 23 corresponding to KCNE3-like sequence (Gosselin, Warren & Vernlund, unpublished) and is available in the Bacterial Artificial Chromosome (BAC) clone CH211-117K14 (Warren, unpublished).

If the conservation of each of the five KCNE gene family members in mammals plays such a vital role in the regulation of voltage-gated potassium channels, then how do the only
existing peptides in zebrafish compensate for the missing functional peptides of Mirp1 and Mirp4? Or does the diminished number of such genes in zebrafish comparative to mammals represent an adaptive loss of genes relative to a common vertebrate ancestor? In the theory of Natural Selection, it is expected that more complex organisms diverging from common ancestors will conserve genetic mutations that lend to an increase in fitness for that organism. In comparison to zebrafish, it would be expected that higher evolved species should contain not only more genes, but more specialization within gene families. Identification of the Mirps in zebrafish and a characterization of their expression patterns, will allow for a comparison between the functional roles of known Kv channel accessory proteins and insight into the regulation of voltage-gated potassium channels in zebrafish, a model organism for cardiac drug interactions in humans.

**Research Methods and Procedures**

*In silico Target Gene Primer Designs*

Primer sets for KCNE4 were designed and checked for the appropriately sized Polymerase Chain Reaction (PCR) product using the National Center for Biotechnology Information Primer Designing tool (Appendix 4) (NCBI, 2009). Additionally, primer sets designed for a previous study for KCNE1 and KCNE3 using Integrated DNA Technologies Primerquest (IDT, 2011) against zebrafish cDNA sequence were included in this study (Appendix 4). The primers were obtained from Invitrogen, re-suspended to 100µM concentrations, diluted to 10µM working concentrations, and stored at -20°C.
PCR Amplification of KCNE Gene Sequences in Zebrafish

Genomic DNA, extracted from adult tail fin clips from three frozen, previously euthanized *wildtype* adult fish under a separate IACUC-approved protocol, was subjected to overnight heat digestion at 50°C in extraction buffer with proteinase K, followed by centrifugation and dilution of the supernatant. The genomic DNA from two of the three zebrafish was used in PCR to amplify KCNE1 and KCNE4. Additionally, *E. coli* transfected with the BAC DNA from the Chromosome 23 BAC Clone (CH211-117K14) obtained from BACPAC Resources were plated and grown on LB agar plates prepared with chloramphenicol. Several colonies were selected for colony-based PCR. The PCR products for all three target genes were produced by combining 19µL of master mix, 5µL of DNA, 0.5µL of 10µM forward primer and 0.5µL of 10µM reverse primer. The master mix solution was sufficient for 15 reactions and was prepared by combining 30 µL 10X Buffer, 11.4 µL 50mM MgCl₂, 3 µL 10mM dNTPs, 1.5 µL 5units/µL Taq Polymerase, and 182.1 µL deionized water. After combination, the solution contained 1X Buffer, 1.9mM MgCl₂, 0.1mM dNTPs, 0.025units/µL Taq Polymerase, 100nM forward primer and 100nM reverse primer. The PCR with a 60°C annealing temperature was run on 32 amplification cycles beginning with 10 minutes at 95°C for DNA denaturing and following with cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes, and finished with extension at 72°C for 7 minutes. Agarose gel electrophoresis was used for confirmation for the amplification of KCNE1, KCNE3, and KCNE4, as well as, confirmation that the BAC we received contained DNA from Chromosome 23. Specifically, each sample well in the 2% agarose, Ethidium bromide containing gel was filled with 10µL of a PCR sample. The Ethidium Bromide dye fluoresces under Ultraviolet (UV) radiation, allowing the DNA bands to be visualized.
TOPO TA Cloning of PCR Products

To confirm the presence of KCNE1, KCNE3, and KCNE4 and to build tools to observe the expression of each gene in zebrafish, the PCR products from 3 *wildtype* DNA templates were cloned into the TOPO transcription vector using the Invitrogen pCR®II-TOPO TA cloning kit and transformed into chemically competent *E. coli*. This cloning kit was selected for the inserted Sp6 and T7 promoter regions on the plasmid vector. These promoters have the potential to be used to produce antisense (mRNA) and sense (negative control sequence) probes for *in situ* hybridization. All reagents used were included in the kit and no further modification of these reagents took place. Preparation involved incubating a solution of 1.5µL of the previously produced PCR product, 0.5µL salt solution and 0.5µL of plasmid vector for each target gene at room temperature for 10 minutes to allow for insertion of the PCR product into the plasmid vector. After the 10 minute incubation period, the 0.5mL microcentrifuge tubes were placed on ice while 20mL of *E. coli* cells were added to each vial. The solution was incubated on ice for 5 minutes and then heat shocked in a 42°C water bath for 30 seconds. The tubes were placed back on ice and 250µL of room temperature SOC medium was added to each tube. The tubes were agitated in an orbital shaker for 1 hour at 37°C. After the 1 hour incubation, the cells were plated on sGal/LB agar plates containing Ampicillin by pipetting 20µL and 100µL onto two separate plates for each vial. The cells were incubated overnight at 37°C. Confirmation of the presence of the KCNE1, KCNE3, and KCNE4 PCR product transformation into the chemically competent *E. coli* was indicated by the presence of white colonies.
**Plasmid Isolation**

Plasmid DNA for KCNE1, KCNE3 and KCNE4 were isolated for sequencing using the Purelink Quick plasmid preparation kit (Invitrogen). Colonies of the chemically transfected *E. coli* for each target gene were selected, placed in culture tubes containing 4mL of LB media spiked with Ampicillin, and incubated overnight at 37°C in an orbital shaker. Isolation of the plasmid DNA involved transferring the bacterial cultures to 2.0mL tubes and centrifuging for four minutes. The supernatant was poured off from each tube, leaving cell pellets in the tubes. The QIA Spin Miniprep Kit protocol was used for purification of high-copy plasmid DNA. The cell pellets were re-suspended in 250µL of P1 buffer and transferred to microcentrifuge tubes. To these solutions, 250µL of P2 buffer was added and each tube was gently inverted six times until the solution became viscous and clear. This step was followed by the addition of 350µL of N3 buffer and each tube was immediately inverted six times to avoid localized precipitation. The solutions were centrifuged for 10 minutes at 13,000rpm in a table-top microcentrifuge. Each of the supernatants was applied to a separate QIAprep Spin Column by pipetting. The columns were centrifuged for 60 seconds and the flow-through was discarded. The QIAprep Spin Columns were washed with 0.5mL of PB buffer. The columns were centrifuged for an additional 60 seconds and the flow-through was discarded. The columns were rewashed with 0.75mL of PE buffer and centrifuged for 60 seconds. The flow-through was discarded and the columns were centrifuged for an additional minute to remove residual wash buffer. The QIAprep columns were placed in clean 1.5mL microcentrifuge tubes and the DNA was eluted by adding 50µL of EB buffer to each column.
Sequencing and Comparison of KCNE members to Zebrafish Genomic Library

The purified plasmids were confirmed to be of ample concentration using the Nano Drop 1000 DNA-50 program and diluted to the appropriate sequencing concentration of 4.0 ng/µL with nanopure water. The plasmids were submitted to the Genome Sequencing Center at the University of Rhode Island. Once the sequences were obtained, they were compared to the Zebrafish genomic library using the NCBI Basic Local Alignment Search Tool (BLAST) alignment for confirmation of the presence of coding sequence, and compared to KCNE members from other species to examine relationships between and among family members (NCBI, 2009).

In silico Phylogenetic Analysis of the KCNE, HCN, and KCNQ Gene Family Members in Various Organisms

Annotated amino acid sequences for the five KCNE family members, the four HCN family members, and the five KCNQ family members present in zebrafish Danio rerio, human Homo sapiens, mouse Mus musculus, chicken Gallus gallus, Xenopus laevis, carp Carassius carassius, salmon Salmo salar, rainbow trout Oncorhynchus mykiss were obtained from NCBI (NCBI, 2009). In some instances, only nucleotide sequences were annotated and required translation to amino acid sequence using EMBOSS Transeq from the European Bioinformatics Institute (EBI, 2011). The amino acid sequences for each of the gene family members present in each of the previously mentioned organism were confirmed to encode the protein using BLASTp (NCBI, 2009). The KCNE1, KCNE3, and KCNE4 zebrafish sequences obtained from the Genome Sequencing Center were translated to amino acid sequence using EMBOSS Transeq (EBI, 2011) and confirmed using BLASTp (NCBI, 2009). The sequences from KCNE, HCN,
and KCNQ for each organism were aligned using the MUSCLE program and Phylogeny.FR in separate phylogenetic trees (Phylogeny.FR, 2011).

RT-PCR Analysis of Expression of the KCNE Gene Members in Wildtype and Slow mo Mutant Zebrafish

RNA isolated using the RNeasy Protect Mini Kit from Qiagen from adult wildtype zebrafish heart and liver tissue for a separate, IACUC-approved Warren lab project was used for cDNA synthesis and examination of KCNE1, KCNE3, and KCNE4 expression. RNA purification and rtPCR utilized the Qiagen QuantiTect® Reverse Transcription Kit. All reagents were provided in the kit and no further modification was implemented. Template RNA was thawed on ice along with room temperature (20°C) thawing of the gDNA Wipeout Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water. The genomic DNA elimination reaction was prepared on ice by combining 2µL of 7X gDNA Wipeout Buffer, 1µg template RNA, and 12µL of RNase-free water. The solution was incubated for 2 minutes at 42°C and placed immediately on ice. The reverse-transcription master mix was prepared on ice by combining 1µL of Quantiscript Reverse Transcriptase, 4µL of 5X Quantiscript RT Buffer and 1 µL of RT primer mix. The template RNA was added to each tube containing reverse-transcription master mix and was incubated for 30 minutes at 42°C. The tubes were then incubated at 95°C to inactivate Quantiscript Reverse Transcriptase.

A 2µL aliquot of each finished reverse-transcription reaction was added to the rtPCR mix which contained primers for KCNE1, KCNE3, KCNE4 and cytochrome oxidase used as a control. Cytochrome oxidase was chosen as a control because it is highly abundant in all cells that contain mitochondria, and is therefore, expected to be present in both zebrafish heart and
liver. The PCR with a 60°C annealing temperature was run for 32 amplification cycles beginning with 10 minutes at 95°C for DNA denaturing and following with cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes, and finished with extension at 72°C for 7 minutes. Agarose (2%) gel electrophoresis, spiked with Ethidium bromide, was used to qualitatively view the PCR products as an indicator of cDNA levels, mRNA levels and therefore KCNE gene expression in the zebrafish.

**Syntenic Analysis of Zebrafish Chromosome 23 BAC locus and Various Mammalian Genomes**

Synteny was used to determine the co-localization of genes contained within the Slow mo region of Chromosome 23 (CH211-117K14) and the chromosomes of human *Homo sapiens* and mouse *Mus musculus*. The Map Viewer from the NBCI website (NCBI, 2009) was used to compare selected regions of the chromosome to the corresponding regions of various mammalian species. Additionally, the homology between the genes contained in the Slow mo region of Chromosome 23 and the corresponding genes of various mammalian species were determined using the HomoloGene application on the NCBI website (NCBI, 2009).

**Results**

**KCNE1, KCNE3, and KCNE4 Primer Confirmation and Amplification**

The Forward1/Reverse1 (F1/R1) and Forward2/Reverse2 (F2/R2) primers designed for KCNE4 (Appendix 4) were confirmed to amplify sequence from genomic DNA collected from three separate wildtype zebrafish in PCR by 2% agarose containing Ethidium bromide gel
electrophoresis (Figure 1). The zebrafish BAC was received and confirmed to contain the sequence from Chromosome 23. Primers from KCNE1 (negative control) and KCNE3 (expected to amplify) were used in a colony-based PCR analysis and products run through gel electrophoresis to check for amplification. PCR on DNA from two BAC colonies (1 and 2) showed amplification of KCNE3 and no amplification of KCNE1 as detailed in Figure 2. As aforementioned, KCNE1 is mapped to Chromosome 9 and KCNE3 is mapped to Chromosome 23. These results confirm that the BAC contains the piece of Chromosome 23 previously identified as containing the Slow mo gene mutation. In order to determine the reliability of the primer used to amplify KCNE3, and the uniformity of the BAC colonies, colony PCR utilizing the KCNE3 Forward A/Reverse B primer (Appendix 4) was performed and products examined via gel electrophoresis. Six colonies were tested and all showed amplification of KCNE3. These results are displayed in Figure 3.
Figure 1. Agarose gel electrophoresis separation of amplified KCNE4 from frozen fin clip DNA from two previously euthanized, adult *wildtype* zebrafish identified as wt Y and wt Z. The KCNE4 Forward1/Reverse1 and KCNE4 Forward2/Reverse2 primers designed for this study showed clear band separation of DNA dyed with Ethidium Bromide and visualized using UV fluorescence. These results confirm the efficacy of the F1/R1 and F2/R2 primers in amplifying the KCNE4 gene from two sets of zebrafish DNA.
Figure 2. KCNE1 and KCNE3 PCR product gel electrophoresis confirmation for two separate colonies of *E. coli* transfected with the zebrafish Chromosome 23 BAC using the KCNE3 ForwardA/ReverseB and KCNE3 ForwardB/ReverseA primers designed in a previous study. All KCNE3 PCR products separated into distinct bands implying that KCNE3 was amplified from the BAC DNA containing Chromosome 23 from both colony 1 and 2.
Figure 3. Agarose gel electrophoresis analysis of the KCNE3 ForwardA/ReverseB PCR products amplified from seven colonies of *E. coli* transfected with the zebrafish Chromosome 23 BAC. All colonies were amplified for KCNE3 from the Chromosome 23 BAC DNA, indicating that the BAC contained sequence from the zebrafish Chromosome 23 and that the KCNE3 ForwardA/ReverseB primers amplify sequence from multiple sources.

**Comparison of KCNE Gene members to the Zebrafish Genomic Library**

Comparison of the sequenced KCNE1, KCNE3, and KCNE4 to the zebrafish Genomic Library resulted in confirmation that KCNE1 maps to Chromosome 9, KCNE3 maps to Chromosome 23 and KCNE4 maps to Chromosome 18 (NCBI, 2009). The sequences received for zebrafish KCNE4 showed 99% identity with annotated KCNE4 gene sequence from the zebrafish genome (Figure 4). The KCNE3 sequences were linked to the portion of DNA contained on the Chromosome 23 BAC leading to further confirmation that the primers designed for KCNE3 amplify sequence from Chromosome 23. Currently, the KCNE3-like coding
sequence is annotated in the Zebrafish Genomic Library but was not identified as open reading frame in the open reading frame databases.

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Zebrafish KCNE4</td>
<td>Genomic Library</td>
<td>1</td>
<td>GCCAAACGCAACATCCGCTCCGGAGTGAAGAGCTCACGCTTGGTGTTCACCAGCAG</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequenced</td>
<td>Zebrafish KCNE4</td>
<td>454</td>
<td>GCCAAACGCAACATCCGCTCCGGAGTGAAGAGCTCACGCTTGGTGTTCACCAGCAG</td>
<td>395</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zebrafish KCNE4</td>
<td>Genomic Library</td>
<td>61</td>
<td>AGCTCCACATCGCAAGCCGCCCGCCCTCTCAACATGAAAAGGACCGCGCTGGAGGC</td>
<td>119</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequenced</td>
<td>Zebrafish KCNE4</td>
<td>394</td>
<td>AGCTCCACATCGCAAGCCGCCCGCCCTCTCAACATGAAAAGGACCGCGCTGGAGG</td>
<td>335</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zebrafish KCNE4</td>
<td>Genomic Library</td>
<td>120</td>
<td>AGCCCGGAACACGCGAAACTGCTTGGTGGCGCCCGTGGGCGACTCGGC</td>
<td>179</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequenced</td>
<td>Zebrafish KCNE4</td>
<td>334</td>
<td>AGCCCGGAACACGCGAAACTGCTTGGTGGCGCCCGTGGGCGACTCGGC</td>
<td>275</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zebrafish KCNE4</td>
<td>Genomic Library</td>
<td>180</td>
<td>TGCTCTCCCTCTGCAGAGCCCTGAGCTGACCGGCTTTTGAGGCCTTTTCTCGCGGT</td>
<td>239</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequenced</td>
<td>Zebrafish KCNE4</td>
<td>274</td>
<td>TGCTCTCCCTCTGCAGAGCCCTGAGCTGACCGGCTTTTGAGGCCTTTTCTCGCGGT</td>
<td>216</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zebrafish KCNE4</td>
<td>Genomic Library</td>
<td>240</td>
<td>GGTCCGCTCATACGACATGCTCCGATGACGGAAGACTCCGTAAGGACACCCAGAT</td>
<td>299</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequenced</td>
<td>Zebrafish KCNE4</td>
<td>215</td>
<td>GGTCCGCTCATACGACATGCTCCGATGACGGAAGACTCCGTAAGGACACCCAGAT</td>
<td>156</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zebrafish KCNE4</td>
<td>Genomic Library</td>
<td>300</td>
<td>GAGCGATGTAAGTCCGCTGATTCTCGCCTGCGGATAAGGGAATTCGCTGCTG</td>
<td>359</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequenced</td>
<td>Zebrafish KCNE4</td>
<td>155</td>
<td>GAGCGATGTAAGTCCGCTGATTCTCGCCTGCGGATAAGGGAATTCGCTGCTG</td>
<td>96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zebrafish KCNE4</td>
<td>Genomic Library</td>
<td>360</td>
<td>AGCCGATGAAACCGCGCTG</td>
<td>379</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sequenced</td>
<td>Zebrafish KCNE4</td>
<td>95</td>
<td>AGCCGATGAAACCGCGCTG</td>
<td>76</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.** BLAST alignment of sequenced KCNE4 from wildtype zebrafish with KCNE4 sequence from the Zebrafish Genomic Library (NCBI, 2009). Alignment confirmed that the sequenced KCNE4 matched with 99% identity to the annotated KCNE4 sequence.

**Phylogenetic Analysis of the Voltage-gated Potassium Channel KCNQ Gene Members**

MUSCLE alignment of annotated protein sequence for the five KCNQ channel gene members in human *Homo sapien*, mouse *Mus musculus*, African clawed frog *Xenopus laevis*, chicken *Gallus gallus*, carp *Carassius carassius* and zebrafish *Danio rerio* revealed various combinations of the five family members in each organism and distinct taxonomic units for each of the five family members (Figure 5). Specifically, human and mouse were found to contain all five KCNQ family members with high relatedness. Xenopus was found to only contain KCNQ1 and chicken was found to only contain KCNQ4. Zebrafish was determined to contain KCNQ1
with high relation to Carp KCNQ1. Search of the zebrafish genome revealed KCNQ2-like and KCNQ5-like protein sequence, which showed moderate phylogenetic relation to the KCNQ2 proteins in human and mouse.

**Figure 5.** Phylogenetic tree alignment of KCNQ1, KCNQ2, KCNQ3, KCNQ4 and KCNQ5 genes in human, mouse, Xenopus, chicken, carp and zebrafish with specific nodes for each KCNQ family member. It was determined that humans and mice contain KCNQ1, 2, 3, 4, and 5 with high relation, Xenopus contain KCNQ1, chicken contain KCNQ4, and carp contain KCNQ1. Zebrafish were found to contain KCNQ1 with highly related KCNQ2-like and KCNQ5-like proteins to the KCNQ2 in human and mouse.

**Phylogenetic Analysis of the “Pacemaker Channel” HCN Gene Members**

MUSCLE alignment of annotated protein sequence (Phylogeny.FR, 2011) for the four HCN channel gene members in human *Homo sapiens*, mouse *Mus musculus*, rainbow trout *Oncorhynchus mykiss* and zebrafish *Danio rerio* revealed various combinations of the four members in each organism (Figure 6). Mouse and human were found to conserve all four HCN channel proteins with relatively high relatedness. The zebrafish was found to conserve HCN2
and HCN4-like protein. The HCN4-like sequence was distantly linked to the HCN4 sequence in human and mouse. Rainbow trout was found to only conserve HCN1 and HCN2 with relatively high relation to the zebrafish HCN2. Due to the presence of HCN1 in rainbow trout, it can be inferred that zebrafish may contain an un-annotated HCN1 member. It should be noted that no annotated HCN gene members were found for carp, Xenopus, or chicken.

**Figure 6.** Phylogenetic tree alignment of the HCN1, HCN2, HCN3 and HCN4 gene members in human, mouse, rainbow trout and zebrafish. Human and mouse were found to contain HCN1, 2, 3, and 4 with high relation, zebrafish and rainbow trout contain HCN2 with high relation and the zebrafish showed HCN4-like protein as well.

**Phylogenetic Analysis of the KCNE Gene Members**

CLUSTALW alignment of annotated protein sequences (EBI, 2011) for the five KCNE gene members in human *Homo sapiens*, mouse *Mus musculus*, African clawed frog *Xenopus laevis*, chicken *Gallus gallus*, salmon *Salmo salar*, carp *Carassius carassius* and zebrafish *Danio rerio* revealed distinct conservation of the KCNE gene members (Figure 7). Humans and mice
were confirmed to contain all five members with close relation. Xenopus was determined to conserve KCNE1, KCNE3 and KCNE5. Chicken was found to conserve KCNE1, KCNE2, KCNE3, and KCNE4. The tBLASTn of the zebrafish sequences for KCNE1 and KCNE4 were determined to code for protein (NCBI, 2009). Genomic searches reveal annotation for only KCNE4 in zebrafish. Carp was confirmed to contain KCNE1 with high relation to the zebrafish KCNE1. Salmon was found to contain KCNE4 with relation to the zebrafish KCNE4. Specifically, zebrafish nucleotide sequences for KCNE1, KCNE3, and KCNE4 were translated to amino acid using EMBOSS Transeq (EBI, 2011) for phylogenetic analysis resulting in the identification of KCNE1 and KCNE4 only in alignment.

Figure 7. Phylogenetic tree alignment of the KCNE1, KCNE2, KCNE3, KCNE4, and KCNE5 gene members in human, mouse, xenopus, chicken, salmon, carp and zebrafish. Zebrafish nucleotide sequences for KCNE1, KCNE3, and KCNE4 were translated to amino acid using EMBOSS Transeq resulting in the identification of only KCNE1 and KCNE4.
RT-PCR Analysis of Expression of the KCNE Gene Members in *Wildtype* Zebrafish

Analysis of mRNA produced from reverse transcription-polychain reaction revealed full gene expression of the cytochrome oxidase (control) in the heart and liver (Appendix 4). Additionally, KCNE1 and KCNE3 showed amplification in the heart, confirming that the contig portions of Chromosome 23 that link to the KCNE3 gene are expressed (Figure 8). Conversely, KCNE1 and KCNE3 were not found to be expressed in the liver (Figure 9). It should be noted that the KCNE4 primers did not show amplification of the wildtype tail fin clip DNA or the cDNA and was therefore not included in this analysis.

![Image of gel electrophoresis](image)

**Figure 8.** KCNE1, KCNE3 and cytochrome oxidase expression via 2% agarose gel electrophoresis separation of rtPCR products from heart cDNA dyed with Ethidium bromide and visualized with UV fluorescence. KCNE1 and KCNE3 revealed expression in the heart (RT+).
cDNA in addition to the positive controls of cytochrome oxidase and wildtype zebrafish DNA. All primers run with cDNA in the absence of reverse transcriptase were negative as expected.

**Figure 9.** KCNE1, KCNE3 and cytochrome oxidase expression via 2% agarose gel electrophoresis separation of rtPCR products from liver cDNA. KCNE1 and KCNE3 revealed no expression in the liver tissue. Cytochrome oxidase and wildtype tail fin clip DNA showed bands at the appropriate basepair lengths confirming the efficacy of the KCNE3 primers as well as the production of cDNA from harvested mRNA.

**Syntenic Analysis of Zebrafish Chromosome 23 BAC locus and Various Mammalian Species**

The determination of physical co-localization of genetic loci surrounding the BAC region of Chromosome 23 in zebrafish *Danio rerio* revealed a gain in syteny in comparison to human *Homo sapiens* and mouse *Mus Musculus*. Specifically, the CR354596.12 region of Chromosome
23 in zebrafish is bordered by or contains RAF1a, synaptin II, SLC6A2, troponinC slow, GDI1, ATP6AP1, tafazzin, DNase1-like1, and neurotrophin7. The homology of these genes and the genetic loci of humans and mice revealed clear evolutionary genomic rearrangements. Specifically, taz, ATP64P1, GDI1, and FAM50A are located on Chromosome 23 in zebrafish and the X Chromosomes of humans and mice, however, it should be noted that these are not completely continuous portions of the chromosome (Figure 10). Additionally, RAFR1a, SynaptinII, CHCHD6, SLC41A3 and K1F15 are located on human Chromosome 3 and mouse Chromosome 6 which shows a clear break and inversion of ancestral DNA (Figure 10). These portions of microsynteny show an inherited link between humans and mice. More interestingly, the mapped position of KCNE3 on Chromosome 23 in zebrafish appears to lie directly beside ATP4P1. This is an area that is found between ATP4P1 and GDI1 on the X Chromosome of both humans and mice, however, it borders an apparent break in synteny on Chromosome 23 in zebrafish, implying that KCNE3 in zebrafish is an insertion. This break in synteny is evident due to the fact that additional genes found in this region of the X Chromosome in humans and mice are located a great distance away from one another on Chromosome 23 in zebrafish.
Figure 10. The Syntenic Analysis of the *Slow mo* region on Chromosome 23 in Zebrafish, *Danio rerio* revealed that ATP64P1, GDI1, FAM50A and DNase1-like1 are located on Chromosome 23 in zebrafish and the X chromosomes of humans and mice in non-contig portions. Additionally, RAF1a, SynaptinII, CHCHD6, SLC41A3, and K1F15, found on chromosome 23 of zebrafish, are located on human chromosome 3 and mouse chromosome 6 indicating a break and inversion of ancestral DNA. The mapped position of KCNE3 on Chromosome 23 in zebrafish appears to border a break in synteny and this apparent gene insertion is supported by the presence of co-localization of its surrounding genes on the X chromosomes of humans and mice.

**Discussion & Conclusion**

In the characterization of the KCNE gene family in Zebrafish, *Danio rerio* sequence amplification, phylogeny, rtPCR and synteny were used to confirm the presence of KCNE1, KCNE3, and KCNE4 in the Zebrafish genome. Specifically, KCNE1, a previously un-annotated gene in the zebrafish genome, was phylogenetically linked with high relation to carp KCNE1 and was demonstrated to be expressed in the heart. Additionally, KCNE1 sequence amplified in a previous study was confirmed to have 99% identity with the zebrafish genome.

Similarly, KCNE4 sequence was confirmed to have 99% identity with annotated sequence in the genome and was phylogenetically linked with high relation to salmon KCNE4.
The KCNE4 primers utilized in the rtPCR amplification of cDNA from *wildtype* heart and liver tissue did not successfully amplify the expected 400 base pair long product. This was due to the presence of large cDNA in the preparation utilizing a cDNA synthesis kit optimized for production of cDNA less than 200bp in length.

Furthermore, KCNE3 sequence was found on the BAC-containing portion of Chromosome 23 and KCNE3 mRNA was indirectly shown to be present in *wildtype* heart tissue. In the phylogenetic analysis of KCNE3, it was determined that the sequence amplified from genomic DNA, when translated, has homology to KCNE3-like sequence. From these results, the KCNE3 gene amplified from the primers designed for this study will be considered a KCNE3-like family member.

The phylogenetic analysis of the KCNQ and HCN voltage gated potassium channels showed distinctly grouped family members among various species, implicating that the protein sequences for these channels are highly conserved. In zebrafish, phylogeny revealed that zebrafish contain HCN2, HCN4-like, KCNQ1, KCNQ2-like, and KCNQ5-like, confirming that zebrafish have pacemaker channels and voltage-gated potassium channels with similar structure and function to other organisms.

The implications of this study are such that zebrafish contain three members of the five members in the KCNE gene family, a trend that has been validated in other species. Yet, this once again raises the question, if zebrafish have all of the pacemaker currents that are regulated by the five KCNE gene family members in mammals, then why do not all five members appear in their genome? It has become apparent that in order to fully understand the KCNE gene family in zebrafish, the evolutionary development of the zebrafish genome must be analyzed.
In conventional evolution, it is expected through the theory of Natural Selection that higher organisms diverging from common ancestors will conserve genetic mutations resulting in an increase in fitness for that organism. This genetic diversification may be the product of point mutations, chromosomal recombination, or gene duplication. More specifically, these incidences imply that Natural Selection can lead to both pruning of genes as well as diversification, which accounts for gene loss and specialization. In comparison to zebrafish, it would be expected that humans, a higher evolved species, should contain not only more genes, but more specialization within gene families. These well-documented trends may account for the presence of five KCNE gene members in mammals and only three in zebrafish. However, there have been several genomic events in the evolution of the zebrafish genome which have accounted for diversion from the expected evolutionary path. Specifically, it has been hypothesized that there has been three rounds of Whole Genome Duplication in evolution, and the third round is considered to be the point of origin of the teleost fish, the class to which zebrafish belong (Jaillon et al. 2009). Consideration of the possible outcomes of Whole Genome Duplication may lead to more insight into the development and functional roles of the KCNE gene family members in zebrafish.

**Whole Genome Duplication**

Whole Genome Duplication involves the immediate amplification of chromosomes, doubling each gene of an individual. The result of Whole Genome Duplication is the transformation of a diploid species to tetraploidy, a gene dosage situation that has many outcomes over a long period of time. Specifically, duplicate genes may undergo neofunctionalization, subfunctionalization, or nonfunctionalization (Jaillon et al. 2009).
Neofunctionalization refers to the event where several gene copies are retained causing mutations to accumulate in a sibling copy resulting in a new function, which is eventually preferentially selected. Conversely, subfunctionalization involves distribution of distinct functions from preduplicated genes between two genes (Jaillon et al. 2009). Both neofunctionalization and subfunctionalization tend towards genetic specialization.

While specialization is one possible outcome of Whole Genome Duplication, the most common result is gene loss through nonfunctionalization. This model involves the retained copies of genes becoming pseudogenes, which may eventually be lost. Over time, species who have undergone Whole Genome Duplication eventually return to diploidy from tetraploidy status while retaining hybridization between sub-populations having different losses of duplicates, leading to genetic combinations that result in speciation (Jallion et al. 2009). In characterizing the KCNE gene family in Zebrafish, the neofunctionalization, subfunctionalization, and nonfunctionalization consequences of the Whole Genome Duplication of teleost fish must be taken into consideration.

**Gene Loss**

In evolution, lineage-specific gene loss plays a major role in the differences in gene repertoires between genomes. The susceptibility of a gene to be lost during evolution is a direct reflection of the importance of the gene to the viability of the organism. If there are at least two genes with homologous function, one of the two redundant genes may take over the functional role of the counterpart, ultimately ending in the loss of the less utilized gene. Gene loss in genomes that have undergone Whole Genome Duplication like that of the zebrafish, is often manifested through reciprocal gene loss. Specifically, reciprocal gene loss occurs when two
paralogs are retained until speciation, after which each species loses a different copy (Sémon & Wolfe, 2007). In some instances, the paralogs lost in speciation may be responsible for missing orthology between two species. In the case of zebrafish, some genes appear to lack human orthologs and are considered to be novel genes; however, their origin is most likely the result of the third Whole Genome Duplication (Postlethwait, 2007). From this it can be inferred that the KCNE3-like gene did not phylogenetically map to other KCNE genes as a result, in part, of reciprocal gene loss.

In a study examining reciprocal gene loss between tetradon from the tetraodontidae family, and zebrafish from the teleost family after Whole Genome Duplication, Sémon and Wolfe estimated that approximately 1700 ancestral loci have undergone reciprocal gene loss (2007). The results of this study imply that reciprocal gene loss at duplicated loci is probably a contributing factor to all speciation events occurring between the teleost whole genome duplication and sometime after tetraodon and zebrafish diverged (Sémon & Wolfe, 2007). Moreover, Sémon and Wolfe discuss the fact that the human lineage has undergone interchromosomal rearrangements which tend to obscure the syntenic relationship with zebrafish, who are more susceptible to inversions than interchromosomal rearrangements (2007). The implications of this study are such that the blurred lines in synteny between the KCNE3 region of the zebrafish Chromosome 23 and that of humans and mice may be the result of reciprocal gene loss and interchromosomal rearrangements in the mammalian lineage.

**Gene Duplication and Specialization**

In evolution, gene duplication often results in the creation of a copy of a gene that is free from selective pressures which would traditionally have a deleterious effect on mutations. This
gene plasticity is the foundation for the specialization of specific genes within a family of genes in organisms that have undergone duplication. The effects of specialization from gene duplication are most apparent in the evolution of the family of *Hox* genes, which are responsible for anterior-posterior specification and segment identity in the development of metazoans (Jaillon et al. 2009). Specifically, *Hox* genes represent one copy in invertebrates, four copies in mammals, and more than four copies in teleost fishes (Jaillon et al. 2009). The evolution of the family of *Hox* genes implies that vertebrate ancestors probably had single copies of genes that are now found in multiple copies in vertebrates and gene maps suggest that this occurred by polyploidization (Postlethwait et al. 1998).

Furthermore, in a study on the investigation of troponin C1 as the possible gene candidate for the zebrafish mutant *Silent Partner*, Sogah et al. determined that the duplication of troponin C1 in zebrafish resulted in subfunctionalization, or tissue specific partitioning of troponin C expression and function, which they termed Troponin C1a and Troponin C1b (2010). Specifically, TNNC1a is seen throughout the heart and TNNC1b is expressed mainly in skeletal but not cardiac muscle (Sogah et al. 2010). In contrast, humans contain a single troponin C1 (TNNC1) gene, which is expressed in both cardiac and skeletal muscle (Sogah et al. 2010). More interestingly, the two copies of troponin C1 in zebrafish are located on Chromosome 23 in the same region as the KCNE3-like gene, suggesting that this is a region of an ancestral duplication event.

**In vitro Functional Promiscuity of the KCNE gene Family Members**

Specialization within gene families as a result of Whole Genome Duplication has often led to the retention of mutations within genes coding for proteins. Slight variation within protein
structure may be the result of added functionality or tissue specific modification. The possibility that Mirps may have the genetic plasticity to alter function as a result of speciation is imperative to this study. In a study on the evolution of the KCNE gene family, Cai et al. report that mammalian KCNE1 proteins can assemble with putative Xenopus KCNQ homologues and, reciprocally, Xenopus KCNE proteins affect the gating of several heterologously expressed human pore-forming subunits (2006). Additionally, human KCNE proteins have been shown to individually assemble with KCNQ1 in heterologous expression systems in vitro and in the case of KCNE1 and KCNE2 also in native tissues showing a versatility of function within the family (Cai et al. 2006). From the in vitro promiscuity of Mirps, it can be inferred that the interactions of Mirps with voltage-gated potassium channel subunits result from tertiary protein structure and may not be completely sequence specific. Moreover, these results imply that Mirps have the ability to affect several potassium channel properties simultaneously, control channel modulation by signaling molecules, as well as, to cause congenital or acquired channelopathies (Cai et al. 2006).

In zebrafish, it can be assumed that MinK, Mirp2, and Mirp3 will display the same form of functional promiscuity of the KCNE gene members found in other species. Considering the fact that zebrafish conserve sequence coding for voltage-gated potassium channels and have $I_h$ currents with similar structure and function to that of other species, it can be assumed that the three Mirps present in their genome are functional and compensate for the missing Mirp1 and Mirp4 peptides. Further investigation of MinK, Mirp2, and Mirp3 through in situ hybridization must be conducted to confirm and characterize the functional roles of the KCNE gene family members found in the zebrafish genome.
KCNE3: a Candidate Gene for the *Slow mo* Mutation causing Cardiac Arrhythmia in Zebrafish

While it is apparent from the results of this study that zebrafish do possess and most likely utilize KCNE1 and KCNE4 in the regulation of voltage-gated potassium channels, the presence and role of KCNE3 in the zebrafish genome is more uncertain. It could be argued that the “KCNE3” gene is a novel gene and may serve alternative functions in cardiac tissue in zebrafish. However, zebrafish are missing the genes encoding Mirp1 and Mirp4 and have an \( I_h \) current that looks like a pacemaker channel modified by Mirp1, as well as, a mutant whose pacemaker channels behave as if they are missing Mirp1. In a study comparing the effect of Mirp1 loss on the \( I_h \) current in mice, Yu et al. showed that Mirp1 is responsible for severe reduction in the fast component of the \( I_h \) current (2001). Similarly, an electrophysiological recording of the \( I_h \) current in the *Slow mo* mutant zebrafish shows a severe reduction in the fast component of the \( I_h \) current (Appendix 5). More interestingly, the *Slow mo* mutation has been physically mapped to the same region of Chromosome 23 in which the zebrafish KCNE3 gene is located and KCNE3 cDNA from heart tissue showed amplification. Additionally, in mice and humans, the \( I_h \) current is produced from the HCN2 voltage gated potassium channel which is only modified by Mirp1 in humans (Appendix 1). HCN2 and HCN4-like channel sequences have been annotated in the zebrafish genome, yet KCNE2 is not present in their genome. It is apparent that Mirp1 plays a vital role in the regulation of voltage gated potassium channels in other species. It is possible that the evolutionary events that have resulted in the speciation of zebrafish have selectively pressured KCNE3 in zebrafish to take on, in part, the functional roles of KCNE2 observed in other species.
If KCNE3 could be potentially responsible for modifying vital cardiac currents in zebrafish, then why is it not more prominent in the transcription map of the region of genomic sources? There are several characteristics of KCNE3 that can account for its inaccessibility. Specifically, KCNE3 is a small gene comprised of less than 200bp and as a result leads to the transcription of a very small RNA transcript. Additionally, KCNE3 is mapped to a region of high repetition on Chromosome 23 and is located at an intersection of synteny breaks where a section found on the human X chromosome section split and is juxtaposed to a Chromosome 3 fragment and mouse X chromosome sequences reside next to Chromosome 6. Furthermore, there is a known duplication in this region of Chromosome 23 as outlined in the discovery of the subfunctionalization of troponin C1 in zebrafish by Sogah et al. (2010). From these characteristics, it is apparent that in order to support the presence of a functional KCNE3 in the zebrafish genome, RNA extracted from a Slow mo embryo heart must be analyzed for expression of KCNE3. Further investigation would also include in situ hybridization of Slow mo embryos to characterize the overall expression of the KCNE gene members present in the mutant.

Overall, the results of this study offer strong evidence that zebrafish Danio rerio not only contain three members of the KCNE gene family within their genome, but utilize them in a variety of functional roles. Understanding the expression and interactions of KCNE1, KCNE3 and KCNE4 in zebrafish will lead to insight into the fine-tuned regulation of the repolarization of excitable membranes within the species. As a model organism for humans, it is imperative that the functional role of the vital zebrafish voltage-gated potassium modifiers, like Mirps, be taken into consideration in clinical trials. Moreover, confirmation that KCNE3 is responsible for the Slow mo mutation has the potential to be utilized in the development of a cardiac-specific blood
pressure medication, an ingenuity that would mitigate the adverse corporal effects of current treatment options.
Literature Cited


European Bioinformatics Institute (2011) European Molecular Biology Laboratory, http://www.ebi.ac.uk


Appendix 1 (Modified from Meir, 2005)

KCNE Subunits and their Interactions in Human K\(_v\) Channels

<table>
<thead>
<tr>
<th>Gene</th>
<th>Peptide</th>
<th>Associated Channel</th>
<th>Functional Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNE1</td>
<td>MinK</td>
<td>Kv3.1</td>
<td>Inhibitory-slower activation, stronger inactivation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv3.2</td>
<td>Inhibitory-slower activation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv4.3</td>
<td>Increased current</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv7.1</td>
<td>Slower activation, increased conductance</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv11.1</td>
<td>Increased current</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv3.1</td>
<td>Inhibitory- slower activation, stronger inactivation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv3.2</td>
<td>Inhibitory-slower activation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv4.2</td>
<td>Inhibitory but enhances at v rest due to slow inactivation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv4.3</td>
<td>Increased current</td>
</tr>
<tr>
<td>KCNE2</td>
<td>Mirp1</td>
<td>Kv7.1</td>
<td>Losing voltage dependence, increased current at v rest</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv11.1</td>
<td>Inhibitory</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HCN1</td>
<td>Increased current</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HCN2</td>
<td>Increased current</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HCN4</td>
<td>Increased current</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv2.1</td>
<td>Inhibitory</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv3.1</td>
<td>Inhibitory</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv3.2</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>KCNE3</td>
<td>Mirp2</td>
<td>Kv3.4</td>
<td>Enhancing current at rest</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv7.1</td>
<td>Losing voltage dependence, increased current at v rest</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv7.4</td>
<td>Inhibitory</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv11.1</td>
<td>Inhibitory</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv1.1</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>KCNE4</td>
<td>Mirp3</td>
<td>Kv1.3</td>
<td>Inhibitory</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kv7.1</td>
<td>Slower activation</td>
</tr>
<tr>
<td>KCNE5</td>
<td>Mirp4</td>
<td>Kv7.1</td>
<td>Inhibitory</td>
</tr>
</tbody>
</table>

Appendix 2
Currently known KCNE Gene Members in the Genomes of Various Organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>KCNE Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human <em>Homo sapiens</em></td>
<td>KCNE1, KCNE2, KCNE3, KCNE4, KCNE5</td>
</tr>
<tr>
<td>African Clawed Frogs <em>Xenopus laevis</em></td>
<td>KCNE1, KCNE3, KCNE5</td>
</tr>
<tr>
<td>Chicken <em>Gallus gallus domesticus</em></td>
<td>KCNE1, KCNE2, KCNE3, KCNE4</td>
</tr>
<tr>
<td>Zebrfish <em>Danio rerio</em></td>
<td>KCNE1, KCNE3, KCNE4</td>
</tr>
</tbody>
</table>
Appendix 3 (Warren, Wilcox & L’Italien, unpublished 2008)

Human KCNE proteins mapped to *Danio rerio* regions by chained tBLASTn at the USCS Genome Browser site (genome.ucsc.edu)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Peptide</th>
<th>Map position</th>
<th>Cardiac role</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNE1</td>
<td>MinK</td>
<td>Chr 9</td>
<td>$I_{ks}$, $I_{kr}$</td>
</tr>
<tr>
<td>KCNE2</td>
<td>MiRP1</td>
<td>-----</td>
<td>$I_{h}$, $I_{kr}$</td>
</tr>
<tr>
<td>KCNE3</td>
<td>MiRP2</td>
<td>Chr 23</td>
<td>$I_{ks}$, $I_{kr}$</td>
</tr>
<tr>
<td>KCNE4</td>
<td>MiRP3</td>
<td>Chr 18</td>
<td>-----</td>
</tr>
<tr>
<td>KCNE5</td>
<td>MiRP4</td>
<td>-----</td>
<td>-----</td>
</tr>
</tbody>
</table>

Appendix 4

Designed KCNE1 Primer Sets

**Primer Pair 1**

Forward primer: ATGCTGGAGCAGAACTCCAC

Reverse primer: GCGGATGTAGCTGAACATGA

**Primer Pair 2**

Forward primer: ACAGATGTGCATTCCCTGCT

Reverse primer: CACCTTCTTTGAGCGGATGT

Designed KCNE3 Primer Sets

**Primer Pair 1**

Forward primer: GCGATGTTTCAGACCAACTT

Reverse primer: AACCCAACGAAAAAGCAATG

**Primer Pair 2**

Forward primer: GCACTCAATGCGATGTTCAG

Reverse primer: GCACTCAATGCGATGTTCAG
Designed KCNE4 Primer Sets

Primer Pair 6  
Product length = 381 bp

Forward primer 1  CCACCGCGCTTTTCATCGCCT  20  
Template 76 .................. 95

Reverse primer 1  TGGCGAAACGCACATCCGCT  20  
Template 456 .................. 437

Primer Pair 7  
Product length = 299 bp

Forward primer 1  CCCCTTATCCGGCGGAAGCG  20  
Template 110 .................. 129

Reverse primer 1  GCTCCACCGAGCAGAGTGCG  20  
Template 408 .................. 389

Purchased Cytochrome Oxidase Primer Set

Primer Pair 1

Forward primer  TTGGGGCCCCCGATATGGCA

Reverse primer  CCCCTCCTGCGGGGTCAAG
Appendix 5 (Modified from Yu et al. 2001)

**Figure 11.** Electrophysiological recording of wildtype zebrafish $I_h$ current and *Slow mo* mutant zebrafish $I_h$ current in comparison with the $I_h$ current observed in the HCN2 channel of mouse *Mus musculus* with and without the modification of Mirp1. The observed $I_h$ current in the *Slow mo* is notably similar to that of the current produced by the mouse HCN2 channel in the absence of Mirp1.
Glossary of Molecular Genetics Terms

**BLAST**
NCBI program that allows analyst to search a nucleotide database using a nucleotide query (NCBI, 2009).

**BLASTp**
NCBI program that allows analyst to search a protein database using a protein query (NCBI, 2009).

**CLUSTALW**
EBI general purpose, multiple sequence alignment program for DNA or proteins (EBI, 2011).

**EMBOSS Transeq**
EBI program that translates nucleic acid sequences to the corresponding peptide sequence. It can translate in any of the 3 forward or three reverse sense frames, or in all three forward or reverse frames, or in all six frames (EBI, 2011).

**Gel Electrophoresis**
A process used for the separation of biological macromolecules in which the gel acts as sieving medium (Encyclopedia Britannica, 2011).

**Homolog**
A gene related to a second gene by descent from a common ancestral DNA sequence. The term, homolog, may apply to the relationship between genes separated by the event of speciation or to the relationship between genes separated by the event of genetic duplication (Lewis, 2011).

**Homologene**
A NCBI gene homology tool that compares nucleotide sequences between pairs of organisms in order to identify putative orthologs. Curated orthologs are incorporated from a variety of sources via the Gene database (NCBI, 2009).

**MUSCLE**
Phylogeny.FR multiple alignment tool that has the capability of aligning nucleic sequences between 200 and 6000bp, as well as, protein sequences between 200 and 2000bp (Phylogeny.FR, 2011).

**Ortholog**
Orthologs are genes in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function in the course of evolution. Identification of orthologs is critical for reliable prediction of gene function in newly sequenced genomes (Lewis, 2011).

**Paralog**
Paralogs are genes related by duplication within a genome. Orthologs retain the same function in the course of evolution, whereas paralogs evolve new functions, even if these are related to the original one (Lewis, 2011).

**Synteny**
Genomic sequencing and mapping have enabled comparison of the general structures of genomes of many different species. Study of synteny can show how the genome is cut and pasted in the course of evolution (Encyclopedia Britannica, 2011).