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An Evolutionary Comparative Study of Congenital Stationary Night Blindness-Associated TRPM1 Genetic Variants of Uncertain Significance in Horses and Humans Utilizing Caenorhabditis elegans

Gabrielle Davis
gdavis4@stu.jsu.edu

Jacksonville State University
Graduate Studies
700 Pelham Rd N., 320 Bibb Graves Hall, Jacksonville, AL 36265
Phone: (256) 782-5348
http://www.jsu.edu/graduate/index.html

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THESIS APPROVAL

Candidate: Gabrielle Elise Davis

Major: Biology

Thesis Title: An Evolutionary Comparative Study of Congenital Stationary Night Blindness-Associated *TRPM1* Genetic Variants of Uncertain Significance in Horses and Humans Utilizing *Caenorhabditis elegans*

Approval:

_____

Dr. Ashley N. Turner — Date
Assistant Professor of Biology, Major Professor

_____

Dr. Michael E. Burns — Date
Associate Professor of Biology

_____

Dr. Chris A. Murdock — Date
Professor of Biology

_____

Dr. Channing R. Ford — Date
Dean, Graduate Studies

AN EVOLUTIONARY COMPARATIVE STUDY OF CONGENITAL STATIONARY NIGHT BLINDNESS-ASSOCIATED *TRPM1* GENETIC VARIANTS OF UNCERTAIN SIGNIFICANCE IN HORSES AND HUMANS UTILIZING *CAENORHABDITIS ELEGANS*

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GABRIELLE ELISE DAVIS

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Gabrielle Elise Davis August 4, 2023

ABSTRACT

Congenital stationary night blindness (CSNB) is a heterogeneous collection of genetic diseases affecting the eyes and vision in horses and humans. Current research has implicated several genetic mutations impacting different genes involved in phototransduction and signal transmission, including *TRPM1*. In horses, genetic mutations in *TRPM1* also result in a leopard spotting pattern or leopard complex. The goal of this study is to examine the potential impact of CSNB associated *TRPM1* missense variants of uncertain significance (VUS). Previous research in *Caenorhabditis elegans* have revealed an orthologous *TRPM1* gene known as *gon-2* that allows for comparative studies. The evolutionary relationship of *TRPM1* and other orthologous genes were examined along with the evolutionary conservation of *TRPM1* missense VUS. Three *TRPM1* VUS were identified in conserved loci across human, horse, *C. elegans*, and other species. A gene mutational analysis, predictive missense variant analysis, and protein modeling predict *TRPM1* the c.2572A>G (p.Ile875Val) to be likely pathogenic or damaging. These findings support further *in vivo* assessment of the VUS. To assess this, a CRISPR-Cas9-engineered *C. elegans* model containing the *TRPM1* missense VUS in the nematode loci of *gon-2* was proposed. Two sets of DNA primers were designed and tested to amplify the VUS region in *gon-2* using polymerase chain reaction (PCR) and gel electrophoresis. CRISPR RNA guides were also designed to target *gon-2* and will be used in future microinjection experiments. A PCR assay was optimized to be utilized for downstream screening and genotyping to identify the *gon-2* VUS *C. elegans* strain. This mutant strain will allow for further *in vivo* investigation of the missense VUS in the *C. elegans* model.

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This is dedicated to a wonderful woman who was full of life, and someone that I cannot remember without having a smile on her face. She told me to never give up on my dreams. Thank you, Nannie.

Gabrielle Davis

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I. INTRODUCTION

The impact of most genetic variants identified within a particular species is still largely unknown. Learning more about genetic variants and their potential impact on gene structure and function is important for understanding the cause, pathology, and potential treatment of a particular disease. Congenital stationary night blindness (CSNB) is a heterogeneous collection of genetic diseases affecting the eyes and vision in humans and horses (Zeitz et al., 2015). Current research has implicated several genetic mutations impacting different genes involved in phototransduction and signal transmission (Lamb, 2020). This includes autosomal recessive mutations in the *TRPM1* gene, which is an ion channel expressed on the dendrites of bipolar cells involved in signal transmission from rods (Nakamura et al., 2010). Bipolar cells are the central neurons of the retina that have light-elicited photoreceptors and are also found in olfactory epithelium and ganglia of the vestibular-cochlear nerve in both humans and horses (Nelson & Connaugton, n.d.).

In horses, genetic mutations in *TRPM1* also result in a leopard-spotted coat and body pattern or leopard complex (Figure 1 A-C) through TRP proteins that are thought to have a role in controlling calcium concentration (Bellone et al., 2008). The TRP channel family itself is involved with multiple intracellular expressions and responses within multiple species. The channels within the TRP family can permit the entry of Ca^{2+} into hyperpolarized cells, resulting in intracellular responses found within the phosphatidylinositol and protein kinase C pathways (Bellone et al., 2008). Decreased expression of these proteins could potentially cause a decrease in melanocyte function, along with bipolar cell signaling within different areas of the body causing the leopard

spotted skin condition (Bellone et al., 2008). The *TRPM1* gene also activates a cation channel known as transient receptor potential cation channel subfamily M member 1 that is expressed in ON Bipolar Cells, transporting positively charged atoms into cells (Bellone et al., 2010). Since horses are a prey animal, they rely on their senses to assess their environment (*Vision in the Equine*, n.d.). Their eyesight is a large factor of this assessment, considering that their eyes are eight times the size of humans, and are needed to detect motion and distance of their environmental surroundings (*Vision in the Equine*, n.d.). These TRP channels are so important that they are found to be in all animal groups, but with different functions including hearing, vision, taste, temperature-sensitivity, and osmoregulation. The decreased expression and function of *TRPM1* within specific locations, such as the eye and skin, could alter bipolar cell signaling and the function of melanocytes.

The goal of this project was to examine CSNB associated variants of uncertain significance (VUS) identified in horses, *Equus caballus*. However, there were only a few CSNB associated genetic variants identified in horses available and published in the literature. One *TRPM1* missense variant associated with CSNB in horses was identified in the literature, and it was discovered to not be in an evolutionary conserved locus between horses and nematodes (Bellone et al., 2010). There has largely been less gene and genome sequencing in horses compared to humans, with even less sequencing associated with a horse disease in a clinical setting. The horse genome was first sequence in 2006 and the Horse Genome Project released the full map in 2009, EquCab1.0 (Wade et al., 2009). Fast-forward fifteen years, and the most current horse genome version is

EquCab3.0, the third one (Beeson et al., 2020). In comparison, the human genome was first released in 2001 and completed in 2003 by the Human Genome Project (Lander et al., 2001). The human reference genome has been continually updated and improved over the past two decades with the most current human genome version being GRCh38.p13, the thirty-eighth one (Schneider et al., 2017). With personalized medicine in humans, DNA sequencing has allowed clinicians to better understand a patient's condition to impact prevention, diagnosis, and treatment based on genetic and genomic medicine. This has led to a large, public dataset of human genetic variants associated with conditions (Landrum et al., 2018). A gap exists in publicly available and clinically relevant genetic variants associated with conditions that are identified in horses. Due to this obstacle, CSNB associated *TRPM1* VUS identified in humans were utilized to determine if a particular missense mutation might impact *TRPM1* function and lead to CSNB in horses.

The *TRPM1* gene is also found within humans and is also related to CSNB. Within humans, symptoms such as lack of rod function, nystagmus, and amblyopia can all first be seen during childhood (Nakamura et al., 2010). More research has also shown that this disease can have different hereditary patterns such as X-linked recessive and autosomal recessive, but an essential aspect into understanding this gene is the pathway that is regulates using ON bipolar cells (Nakamura et al., 2010). Visual separation using ON and OFF bipolar cell pathways is a key part of the function of *TRPM1* that is found in both horses and humans. Within humans, the release of glutamate from photoreceptors regulates the responses of ON bipolar cells using a glutamate receptor and a G-protein. New research within mice has indicated that *TRPM1* is associated with the dendritic tips

of ON bipolar cells (Nakamura et al., 2010). Mice were then manipulated to be null *TRPM1* mutant mice, which showed lack of photo response and therefore the TRPM1 channel being negatively regulated by the activated G-protein (Nakamura et al., 2010). Animal models, such as mice and other non-human animals, have served in research to help us understand and improve human biology and health (Phillips & Roth, 2019).

One major model organism in biology has been the *Caenorhabditis elegans* (Figure 1D) (Ankeny, 2001). This species is one of the most abundant and diverse on the planet, while having an impact on ecosystems, economies, and human health (Murfin et al., 2012). The global importance of these organisms, along with their major experimental tractability have made them one of the main scientific research models for different experiments using mutualistic, symbiotic, and pathogenic relationships (Murfin et al., 2012). *C. elegans* were the first metazoan with a sequenced genome, allowing it to also become one of the major model organisms for genetic and biomedical research (Kiontke & Fitch, 2013). A combination of simplicity, having a sequenced genome, and the ideal environment for rapid and effective experiments for gene studies led to this organism becoming very popular amongst the science community (Aboobaker & Blaxter, 2000). Studies of *C. elegans* have revealed an orthologous *TRPM1* gene known as *gon-2* that regulates a transient receptor potential channel required for the initiation and continuation of postembryonic cell divisions of gonad cells (Kemp et al., 2009). This allows further comparative studies of *TRPM1* and *gon-2* between horses, humans, and nematodes.

If a *TRPM1* missense VUS has been associated with CSNB in humans, then the same genetic variant might result in CSNB and/or spotted coated patterns in horses. If that *TRPM1* VUS is conserved across horses to humans to nematodes, then the genetic variant will impact *gon-2* gene and protein structure and function. This mutation placed in the context of the *gon-2 C. elegans* ortholog could result in a mutant phenotype compared to the wildtype nematode animal. Identification of conserved regions of the *TRPM1* gene and missense mutations within these regions can identify and determine VUS in humans to explore further *in silico* and *in vivo*.

II. MATERIALS AND METHODS

Phylogenetic Analysis of *TRPM1* Orthologous Genes

A systematic study was carried out to determine the relationship of *TRPM1* and related genes across multiple species. The programs Benchling (Benchling [Biology Software]. (2022)) and MEGA version 4 (Tamura, Dudley, Nei, and Kumar 2007)) were used for sequence files and the estimation and construction of phylogenetic trees. Coding DNA (cDNA) gene sequence files for 23 species were obtained from Ensembl through importing into Benchling. The following gene IDs were using to import each species cDNA file: human (ENSG00000134160), gorilla (ENSGGOG00000016762), chimpanzee (ENSPTRG00000006859), macaque (ENSMMUG00000009430), cow (ENSBTAG00000001639), pig (ENSSSCG00000004843), horse (ENSECAG00000007539), dog (ENSCAFG00845020749), cat (ENSFCAG00000003436), rabbit (ENSOCUG00000004314), rat (ENSRNOG00000015829), mouse (ENSMUSG00000030523), opossum (ENSMODG00000011776), platypus (ENSOANG00000010363), tuatara (ENSSPUG00000019094), Chinese softshell turtle (ENSPSIG00000015045), chicken (ENSGALG00010024748), zebra finch (ENSTGUG00000005474), collared flycatcher (ENSFALG00000012539), tropical clawed frog (ENSXETG00000016479), zebrafish (ENSDARG00000103476), fruit fly (FBgn0265194), and nematode (WBGene00001651). Each cDNA gene sequence file was exported as a FASTA file and imported into MEGA (Kumar et al., 2018). With MEGA, files were assembled and aligned using ClustalW (Larkin et al., 2007). A Maximum Likelihood method with

bootstrap analysis was created using the Jukes-Cantor Method, with a total of 23 gene sequence files. (Thomson, 2013) (Ripplinger et al., 2010). The bootstrap was inferred from 500 replicates, and the final data set concluded with 8,810 positions.

Identification of Disease Variants within *TRPM1* in Conserved Location

CSNB associated missense VUS previously identified in humans were obtained from ClinVar (Landrum et al., 2018). Evolutionary conservation of each VUS locus across horse, human, and nematode was examined through multiple sequence alignment (MSA), shown in Table 2. The three cDNA gene files of horse, human, and nematode were imported into Benchling using the species gene IDs reported in the previous section. A MSA was constructed through a consensus alignment utilizing the MAFFT alignment program (Katoh & Standley, 2013). Conservation at the DNA and amino acid levels were assessed.

PolyPhen-2 Analysis

PolyPhen-2 was used to predict the possible impact of an amino acid change on the structure and function of the human *TRPM1* protein. The *TRPM1* protein identifier (Q7Z4N2) and amino acid substitution position (875) was input, along with the specific amino acid substitution (isoleucine to valine), into the PolyPhen-2 online server (I. Adzhubei et al., 2013). The TRPM1 protein sequence had 1,603 amino acids.

Protein Modeling

Protein three-dimensional structure predictions of wildtype human TRPM1 (1,603 amino acids) and mutant VUS proteins were generated using SWISS-MODEL (Benkert et al., 2011; Bertoni et al., 2017; Bienert et al., 2017; Guex et al., 2009; Mariani et al.,

2013; Studer et al., 2014, 2020, 2021; Waterhouse et al., 2018). For the wildtype protein modeling, the TRPM1 protein identifier (Q7Z4N2) was input into the SWISS-MODEL online server. For the mutant protein modeling, the complete target amino acid FASTA sequence was input with the amino acid substitution into the SWISS-MODEL online server. Template-based threading and modeling were performed for both wildtype and mutant protein modeling using the top structure templates identified by the Local Meta-Threading-Server (LOMETS), TRPM3 ion channel (PDBID: 8ddx). Wildtype and mutant protein models were exported as pdb files from SWISS-MODEL. Pdb files were taken into PyMOL (Version 2.5.1, Schrödinger) to visualize, compare, and analyze the two protein models.

Once the template was retrieved, a model was built and transferred into the 3D imaging database, PYMOL (*PyMOL | Pymol.Org*, n.d.). PYMOL was used to determine root mean square deviation (RMSD) values, and to create 3D images of both wildtype and variant protein models to structural analysis. With this protein models, shown in Figure 4, structural changes within the protein can determine if changes to the protein structure are damaging, which in turn can cause functionality problems for the protein as well.

Polymerase Chain Reactions

DNA primers were designed using the design tool within Benchling to amplify the VUS locus in the *gon-2* gene. Two primer sets were designed, selected, and tested through polymerase chain reaction (PCR) experiments. Genomic DNA from N2 *C. elegans* were used as the template in 12.5 µL PCR reactions with 2x Taq Master Mix

(Cat. # M0270S, New England Biology) and DNA primers at a 10 µM starting concentration. Genomic DNA was obtained by placing 5-10 adults in a PCR tube with 30 µL of lysis solution containing Proteinase K (Cat. # V302B, Promega) and incubated at 65°Cfor 2 hours, followed by 95°C for 10 minutes. A small volume (1 µL) of this solution was used directly as a template for PCR. An initial PCR was carried out with annealing temperature of 55 °C for 30 cycles. Subsequent PCRs were conducted to optimize the annealing temperature for each primer set across a temperature gradient. The PCR reactions were resolved and analyzed on 6% polyacrylamide gels through electrophoresis. Gels were stained with GelRed (Cat. # 41003, Biotium) and observed and imaged with a UV gel documentation system.

CRISPR Guide Design

CRISPR targets were identified surrounding the VUS locus in the *gon-2* gene using the design tool within Benchling. Two guides were designed and selected to target the VUS region.

III. RESULTS

A systematic study was carried out to determine the relationship of horse *TRPM1* and related genes across different species. Maximum Likelihood and bootstrap analysis were conducted to examine evolutionary relatedness of *TRPM1* and its orthologs. Phylogenetic analyses revealed *TRPM1* orthologs of human and horse share a more recent ancestral connection compared to nematode (Figure 2). Nematode share a more recent ancestral connection with fruit fly compared to horse and human (Figure 2).

Identification of CSNB associated VUS within conserved regions of the *TRPM1* gene in human, horse, and nematode were identified through MSA. A total of 31 missense variants with clinical relevance ranging from benign to uncertain to pathogenic were initially examined. Three VUS were identified to be in a conserved locus across all 3 species: E1324K, H1195R, and I875V. Following further analysis, the two variants H1195R and I875V appeared mostly likely to be pathogenic variants reported through ClinVar. Table 1 displays the level of conservation at the DNA and amino acid levels for the VUS I875V locus across *TRPM1* orthologs in human, horse, and nematode. The specific genetic location within each species' ortholog was determined (Table 2).

To examine the potential impact of the amino acid substitution I875V on the structure and function of the human *TRPM1* protein, predictive missense variant analysis was carried out through Polyphen-2. The HumDiv model predicted I875V to be possibly damaging to the protein with a score of 0.775 (Figure 3).

To further assess the potential structural impact of I875V on the human *TRPM1* protein, we modeled 3D structures of a wildtype *TRPM1* protein and the mutant VUS I875V protein (Figure 4A). When we overlaid the two protein structures of the wildtype and mutant proteins, structural differences were observed and the calculated RMSD is 0.038. The missense VUS results in the amino acid substitution of isoleucine to valine (Figure 4B).

With *in silico* studies supporting further assessment of the impact of *TRPM1* VUS I875V, experiments were carried out to design and generate a mutant *C. elegans* strain harboring I875V in the *gon-2* locus to allow for *in vivo* investigation.

Two sets of DNA primers were designed to amplify the *TRPM1* VUS region within *gon-2* in the nematode (Table 3). Each primer set was created with the forward and reverse primers flanking the VUS locus in the middle of the PCR amplicon. The expected amplicon sizes from the primer sets are 373 base pairs from primer set 1 and 973 base pairs from primer set 2 (Table 3).

Initial PCRs were carried out to assess the amplification and specificity of each primer set. PCR bands were present for both primer sets amplifying nematode genomic DNA (Figure 5). Primer pair 1 yielded a single, prominent DNA band approximately ~450 base pairs in size, whereas primer pair 2 yielded a faint DNA band approximately ~1,500 base pairs with numerous faint lower bands (Figure 5). To optimize the amplification conditions for each primer set, gradient PCR was then performed to identify the optimal annealing temperature to reduce nonspecific amplification. Figure 6

displays the gradient PCR results of primer set 1 and the optimal annealing temperature was identified at 58.1 ℃. Identifying an optimal annealing temperature for primer set 2 required a much wider temperature range to be tested. Following three rounds of gradient PCR, 67.4 ℃ was identified as the optimum (Figure 7).

RNA guides were designed to target the *TRPM1* VUS region within *gon-2* in the *C. elegans* model through CRISPR-Cas9 editing. Two guides were selected that target the region within 30 base pairs of the VUS location in *gon-2* (Figure 8). Each RNA guide is complementary to the binding site with the protospacer adjacent motif (PAM) that Cas9 binds to cut the specified DNA target (Table 4, Figure 8).

IV. CONCLUSIONS

From this study, phylogenetic analyses revealed *TRPM1* orthologs of horse and human to share a distant ancestral connection with nematode and fruit fly. The Maximum likelihood tree represents a hypothesis on the evolutionary history of *TRPM1* according to the underlying model would have most likely given rise to the *TRPM1* orthologs sequence data. For this experiment, the selected model was Jukes-Cantor as it is a common model for the evolution of DNA sequences (Thomson, 2013). It also assumes that the probability is the same for the transition of nucleotides. In comparison with the other trees that were created, it is the one that follows the evolutionary flow of species the most, and it is the most detail oriented based on the locality of nodes and sister taxa (Thomson, 2013). It appears the *TRPM1* gene for horse and other species share a common ancestor and this tree depicts the evolutionary relationships among the species. For most of the evolutionary relationships, the nodes had good support by bootstrap, however a few had weak support. For example, two species' orthologs and their relationships that had low support were opossum and platypus. Additional species' orthologs could be added to this analysis to improve and refine relationships.

Comparing nematode to human, it is predicted that nematode and human genomes contain the same number of genes even though their genome sizes vary (Sommer & Streit, 2011). It has been reported the size of an average exon in nematode is comparable to a human exon, whereas the introns and intergenic regions tend to be considerably smaller in nematode (Sommer & Streit, 2011). Intron size and the number of introns found within genomes when compared to the exons give evidence that the introns help

with gene regulation and expression (Carmel et al., 2007). Introns and their length are also involved in the efficiency of natural selection and can help determine if genes are more conserved across species (Carmel et al., 2007). *TRPM1* and its orthologous genes appear to be fairly conserved within the coding exons based on observations. Conservation of a gene is a crucial aspect to examine to better understand its structure and functionality. This information helps decipher the similarities and differences of the gene as it pertains to different species. A previous study reported on the high level of sequence and structural conservation in TRP channels, including TRMP1, across multiple species (Cabezas-Bratesco et al., 2022).

Upon evolutionary conservation analysis of the *TRMP1* VUS c. 2624A>G (p.Ile875Val), it was observed the amino acid isoleucine was present at each locus across multiple orthologous genes, including horse, human, and nematode. Among the three species, the only DNA sequence difference observed within the codon occurs in the third position for *gon-2* (ATC instead of ATA), or the "wobble position." The wobble position is the third spot within a codon that leads to degeneracy and redundancy of codons (26.1, 2018). When this position is different, the first and second positions that help code for the same amino acid help conserve the encoded amino acid and protein structure. Therefore, a change in the first or second position could result in the encoded protein having structural and possible functional changes (26.1, 2018). Within *gon-2*, the codon ATC is still in-frame to encode for isoleucine.

This *TRPM1* VUS is a missense variant where a single DNA base pair change results in an amino acid shift. As with changing the first or second position of a codon, a

missense variant can result in a similar outcome. Specifically, a missense mutation is located at a positional change within the codon resulting in a new amino acid, and therefore can cause a structural change within the protein encoded (Iqbal et al., 2020). Missense mutations within highly conserved genes may influence gene regulation and expression, or can potentially cause structural and functional damage to the encoded protein (Iqbal et al., 2020). The *TRPM1* VUS occurs in a highly conserved gene region across multiple species. Important to note, the VUS results in an amino acid shift of isoleucine to valine, both of which are nonpolar, hydrophobic amino acids.

Discovering evolutionary conservation of the *TRPM1* VUS locus, predictive missense variant analysis was carried out to examine the potential impact of this amino acid substitution on the human *TRPM1* protein. A public server and tool exist for predicting the outcome of missense variants on human proteins, PolyPhen-2, that was utilized to further examine the *TRPM1* missense VUS I875V (I. A. Adzhubei et al., 2010). These results predicted a change from isoleucine to valine within the human *TRPM1* protein to be possibly damaging. There are two scores returned in the analysis report, HumDiv and HumVar, which had different predictions for the VUS based on the two datasets used to train and build these prediction models. HumDiv is better suited for the assessment of variants for Mendelian diseases, whereas HumVar is better suited for evaluating rare alleles and variants identified in genome-wide association studies (I. A. Adzhubei et al., 2010). With CSNB associated with *TRPM1* mutations being an autosomal recessive condition in horses and humans, the HumDiv score provides a better evaluation for the missense VUS on impacting the human protein (Sandmeyer et al.,

2012) (Bellone et al., 2008) (Li et al., 2009) (Audo et al., 2009) (van Genderen et al., 2009). Therefore, the VUS is predicted to be possibly damaging to the human protein.

With the *TRPM1* VUS being predicted to be damaging to the encoded human protein, homology generated models of the native human *TRPM1* protein and the VUS mutant protein were constructed and compared to identify potential structural differences between the two proteins. Comparing the protein models, the calculated RMSD value was greater than zero indicating a structural change where atoms shifted in 3D space between the constructed protein models. Mentioned previously, the VUS results in an amino acid shift of isoleucine to valine, both of which are nonpolar, hydrophobic amino acids. So, the two amino acids are like one another in chemical properties. Importantly, isoleucine and valine are two of the three branched-chain amino acids that are important to the formation of proteins (Keating & Cronan, 1996). With their structure and function being important to the overall formation of proteins, the differences between these two non-polar amino acids within the *TRPM1* protein could have more of an impact than expected (Keating & Cronan, 1996). This could especially be the case for this amino acid substitution in the protein as this locus is in a highly ordered alpha helix structure that is a component of one of the transmembrane domains of the human *TRPM1* protein (Agosto et al., 2018). Information gathered from UniProt shows the examined isoleucine for the *TRPM1* VUS I875V is the sixth residue of twenty-one amino acids total that comprise the first transmembrane domain of the protein (I. A. Adzhubei et al., 2010). The observed structural differences between the wildtype and VUS mutant *TRPM1* protein models could impact the overall function of the protein.

The *in silico* observations and findings provided evidence to support further *in vivo* assessment of the *TRMP1* VUS c. 2624A>G (p.Ile875Val). With the VUS in a conserved locus from horse to human to nematode, the *C. elegans* model was selected for further testing in the *TRPM1* ortholog, *gon-2*. This system is commonly used as a model for a phenotypic mutation to be examined and observed (Markaki & Tavernarakis, 2020). Therefore, if a mutation is seen within the *C. elegans* model after DNA manipulation, evidence could suggest the same for other species with the same orthologous gene and amino acid change (Markaki & Tavernarakis, 2020).

To do this, a CRISPR-Cas9-engineered *C. elegans* model was designed containing the *TRPM1* missense VUS in the nematode loci of *gon-2*. The design for this type of mutant strain has been utilized to generate point mutations in the model and previously described (Paix et al., 2017) (Ghanta et al., 2021). Two CRISPR RNA guides were designed and identified around the VUS loci within *gon-2*. A DNA repair template has also been designed and will be used for microinjection of nematodes along with the Cas9 protein and CRISPR RNA guide to generate the potential mutant VUS strain.

A PCR-based screening assay was also designed and optimized for downstream mutant nematode screening (Ghanta et al., 2021). PCR produces copies of DNA through exponential amplification of a specific region being examined in research (Green & Sambrook, 2018). An initial PCR was carried out with the annealing temperature of 55℃ for both primer sets amplifying the *gon-2* region surrounding the VUS location. Each primer set was later optimized through gradient PCR. Thermal gradient PCR is used to optimize annealing temperatures to increase the specificity of a primer set (Chang & Lee,

2005). The setup for this type of PCR experiment is similar to the previous PCR conducted at a controlled temperature; however, a range of temperatures are used on the same primers within the template DNA to find the correct banding pattern needed (Chauhan, 2019). If the primers bind to the desired region (or segment) of DNA within the gradient PCR, then the specific temperatures and the range used are looked at to determine which temperature creates a better amplification with less banding patterns of other amplicons (Chauhan, 2019). An optimum annealing temperature was acquired for primer set 1 using the range of 55 – 65℃, whereas primer set 2 required two rounds of gradient PCR with a higher range of 65 – 75℃ to identify the best temperature. This optimization step is crucial for use of these primers in the downstream PCR-based screening assay. A single, specific *gon-2* PCR amplicon from wildtype N2 *C. elegans* DNA resolved on a polyacrylamide gel will be the starting control for the comparison and detection of the edited, mutant VUS strain and CRISPR-Cas9 activity in nematodes (Ghanta et al., 2021). Both primer sets will be used for initial mutant screening. One final step that could be carried out to confirm the exact genetic locus being amplified is DNA sequencing of the PCR amplicons from each primer set. The DNA sequence obtained from this could be aligned and compared to the *gon-2* gene file for analysis and confirmation.

Future experiments will include analyzing the phenotypic differences present in the mutant VUS nematode strain compared to the wildtype N2 strain. If the VUS does impact function of *gon-2*, then it is expected to impact gonadal and vulva development. This study has provided *in silico* examination and will provide *in vivo* assessment of this

CSNB-associated VUS shedding light on its clinical significance for horses and humans as well as the conservation of gene and protein structure and function of these orthologs across species.

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APPENDIX A: TABLES

Table 1

Evolutionary conservation of TRPM1 VUS locus across 3 species

Human Disease Variant	**TAC** Tyr	**ACA** Thr	**GTA** Val	**TCA** Ser	**TAC** Tyr
Human (*H. sapiens*)	**TAC** Tyr	**ACA** Thr	**ATA** Ile	**TCA** Ser	**TAC** Tyr
Horse (*E. callabus*)	**TAC** Tyr	**ACA** Thr	**ATA** Ile	**TCG** Ser	**TAC** Tyr
Nematode (*C. elegans*)	**TGG** Trp	**ACT** Thr	**ATC** Ile	**TCA** Ser	**TCC** Phe

Table 2

Genetic location of VUS within TRPM1 orthologs across 3 species

Species	**Gene Ortholog**	**Variant Location**
Humans (*H. sapiens*)	*TRPM1*	c. 2624A>G (p.Ile875Val)
Horses (*E. callabus*)	*TRPM1*	c. 2629A>G (p.Ile877Val)
Nematode (*C. elegans*)	*gon-2*	c. 3943A>G (p.Ile1315Val)

Table 3

DNA primer design for targeting the TRPM1 VUS locus within gon-2

	Primer Set 1	**Primer Set 2**
Forward Primer	5'-TCACTTCGACGGATCGGCCAAA-3’	5'-TCACAGCAGACGACGAAAGGCA-3'
Reverse Primer	5'-AGAACGGTTTCGCGTCTGACAT-3'	5'-AGAACGGTTTCGCGTCTGACAT-3'
Expected PCR Product Size	373 base pairs	973 base pairs

Table 4

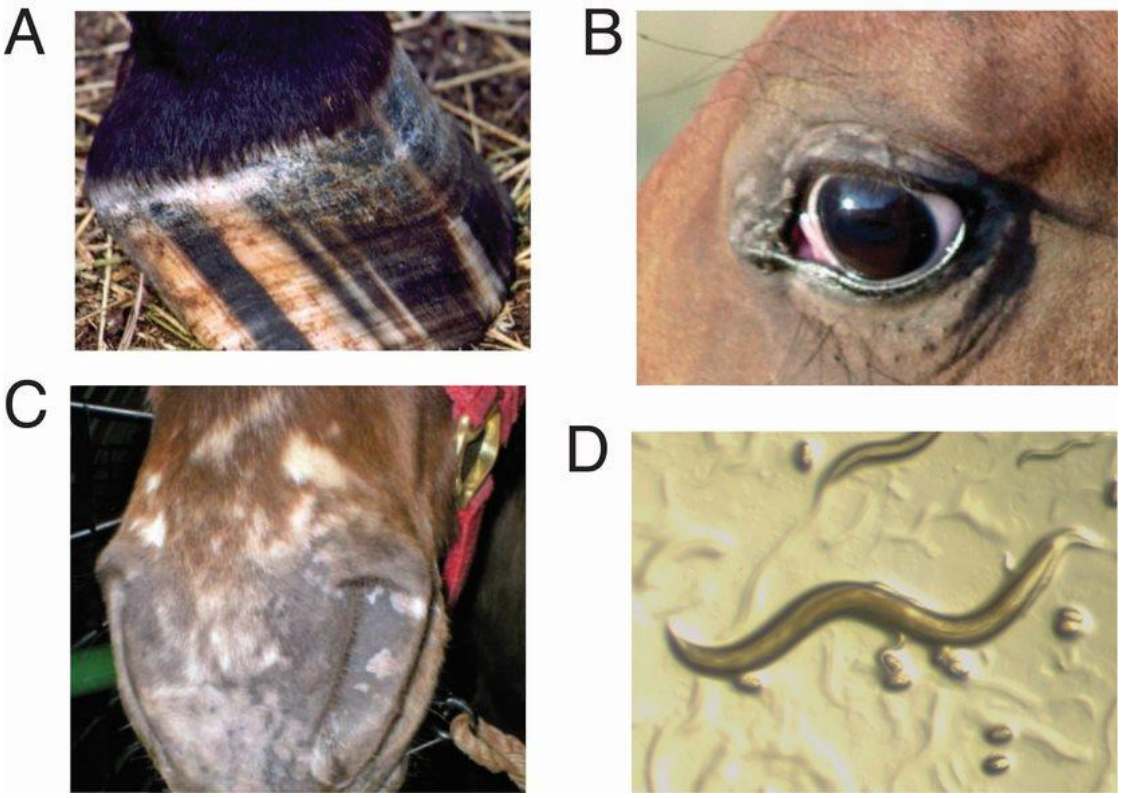
CRISPR RNA guide design for gon-2 targeting

Cut Position (DNA base pair)	**DNA Strand**	**CRISPR RNA Guide Sequence**	**PAM**
17755	bottom	CATGACCAAAATGTTGAAAT	TGG
17767	top	CAATTTCAACATTTTGGTCA	TGG

APPENDIX B: FIGURES

Figure 1

Images of leopard complex associated characteristics in horses and the C. elegans model

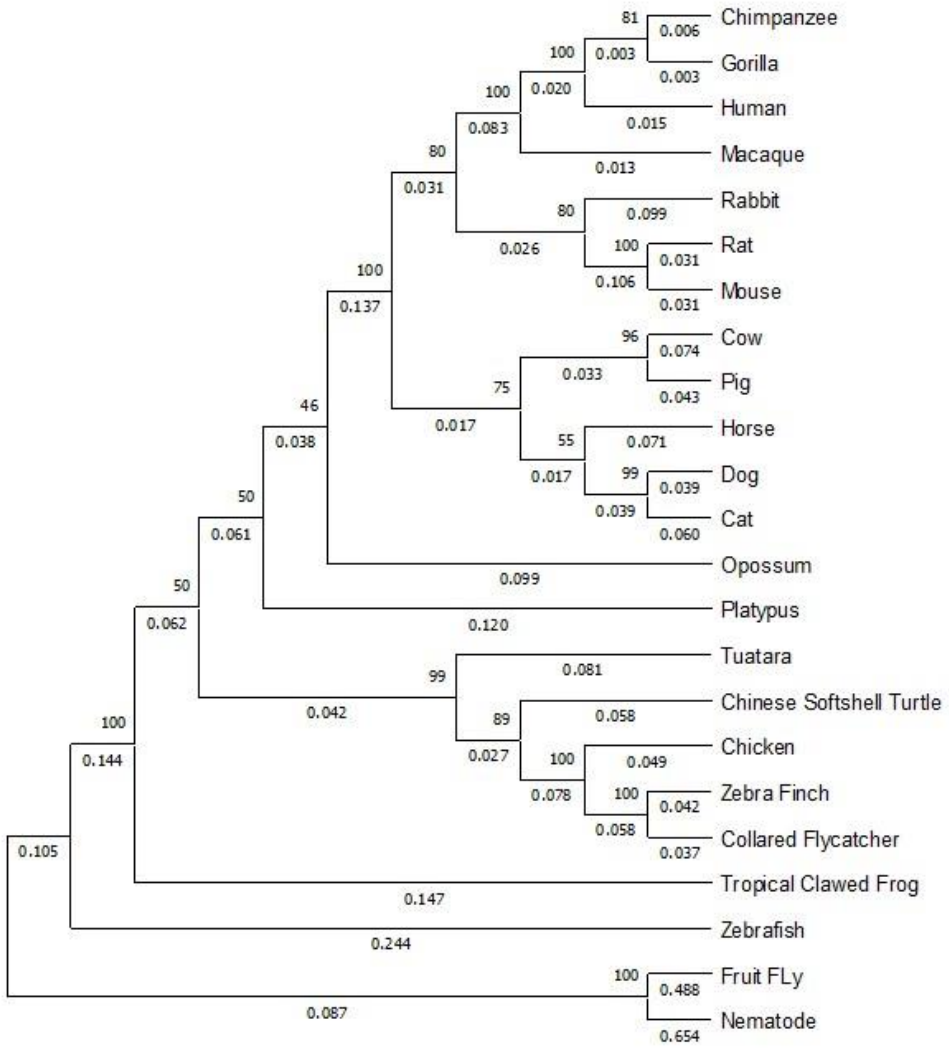


The spotting pattern can appear as striped hooves (A), white sclera in the eye (B), and mottled skin (C). Mottled skin can occur around different body areas, including the eyes and muzzle (B, C). This study proposes to examine a horse condition through the lens of the nematode *C. elegans* model. Adult N2 hermaphrodite animal imaged at 50x with a dissecting microscope and a Motic camera (D). Horse photos reproduced from Bellone et al., Fine-mapping and mutational analysis of TRPM1: A candidate gene for leopard complex (LP) spotting and congenital stationary night blindness in horses, *Briefings in*

Functional Genomics, 2010, Volume 9, Issue 3, Pages 193-207, by permission of Oxford University Press.

Figure 2

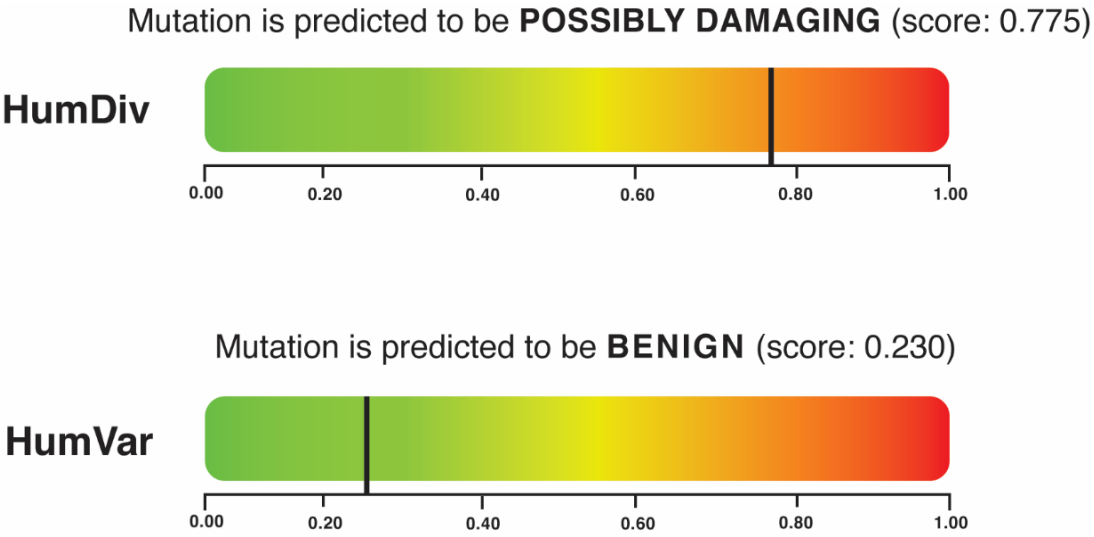
Phylogenetic analysis of TRPM1 orthologs across multiple species



Phylogenetic tree estimated by the Maximum Likelihood method in MEGA based on *TRPM1* orthologs coding sequence data. The bootstrap consensus tree was inferred from 500 replicates using the Jukes-Cantor model. This analysis included 23 gene sequences, with a total of 8810 positions within the final data set. Numbers above branches indicate bootstrap support.

Figure 3

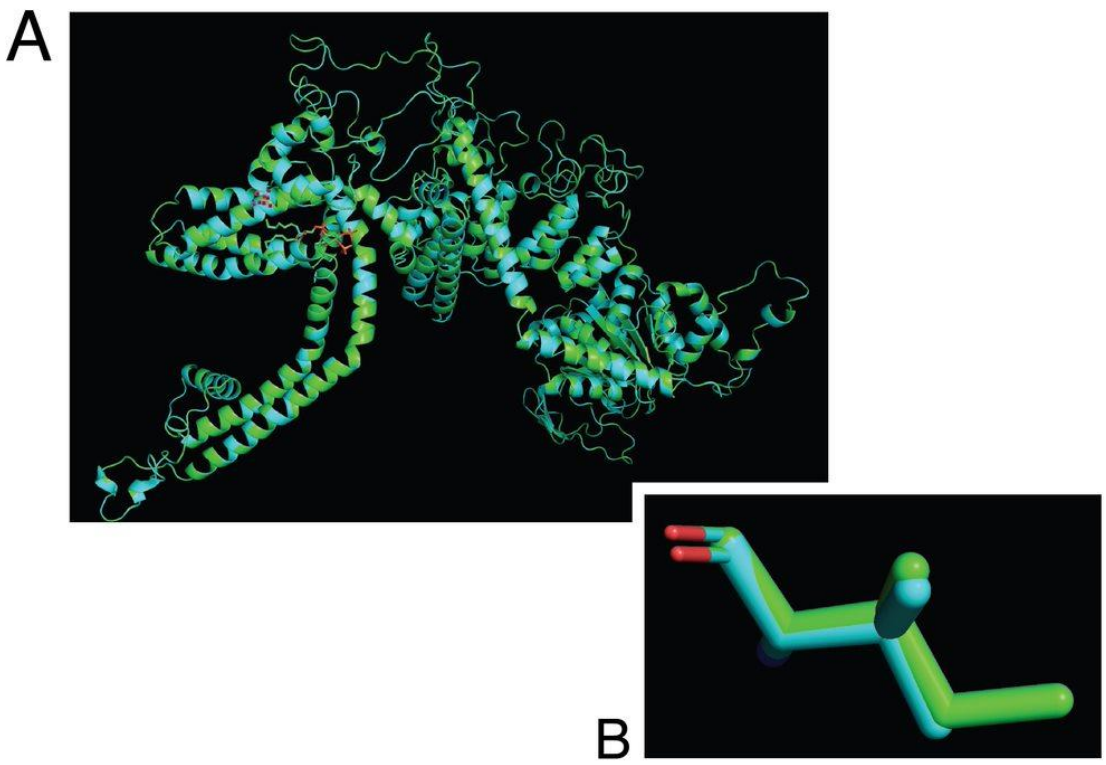
Results of the PolyPhen-2 analysis predicting the pathogenicity of the TRMP1 VUS p.Ile875Val substitution on the human TRMP1 protein



HumDiv and HumVar scores are reported from the analysis.

Figure 4

Predicted 3D structure of the human wildtype TRPM1 protein and the mutant VUS protein



The protein structures were generated with the *TRPM1* amino acid sequence for wildtype and the identified mutant *TRPM1* VUS (A). The two protein models were compared and aligned (lime green = wildtype protein, teal blue = mutant protein). The *TRPM1* VUS region is highlighted by the cluster of pink dots on the alpha helix for both proteins toward the top left. The *TRPM1* VUS leads to an amino acid shift from isoleucine to valine (B).

Figure 5

Initial PCR results of the two designed DNA primer sets amplifying the TRPM1 VUS locus in gon-2 through gel electrophoresis

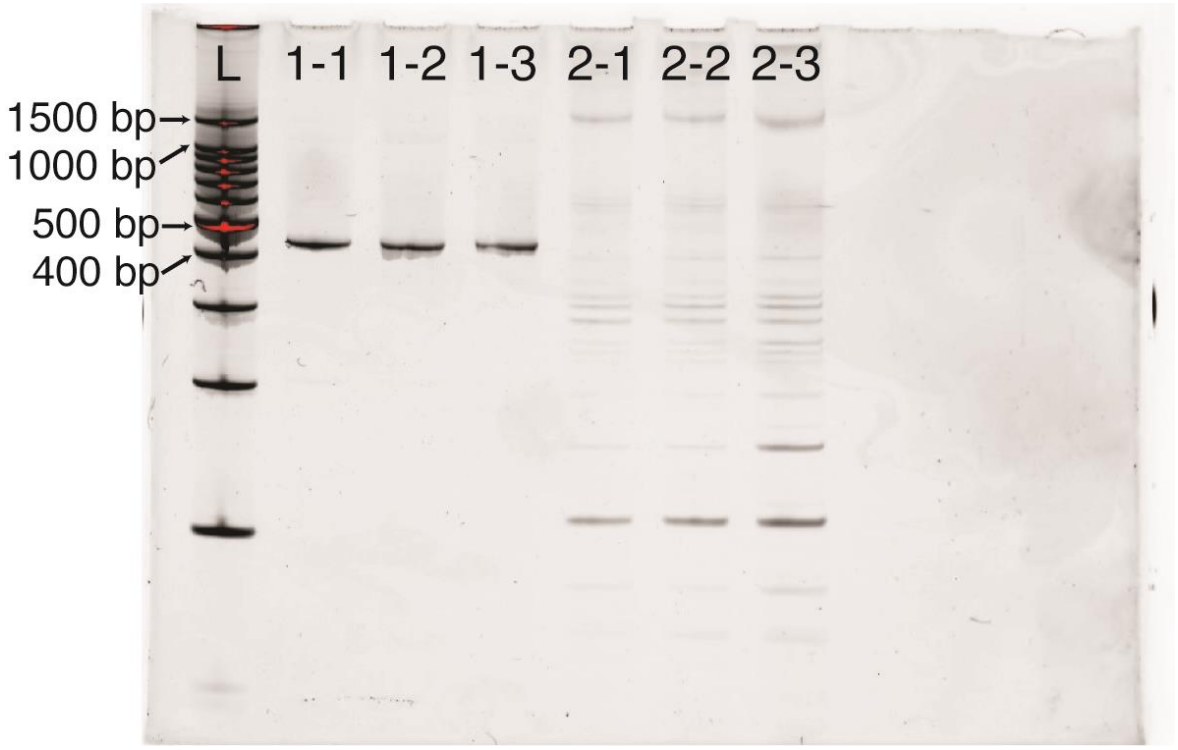


Image of GelRed stained polyacrylamide gel showing initial PCR amplification for each primer set using a 55 ºC annealing temperature. Wildtype N2 *C. elegans* DNA template was used across three PCR reactions for each primer set. L = 100 bp ladder, 1-1/1-2/1-3 = primer set 1 PCR products, 2-1/2-2/2-3 = primer set 2 PCR products.

Figure 6

PCR results and annealing temperature optimization of primer set 1 amplification of the TRPM1 VUS locus in gon-2 through gel electrophoresis

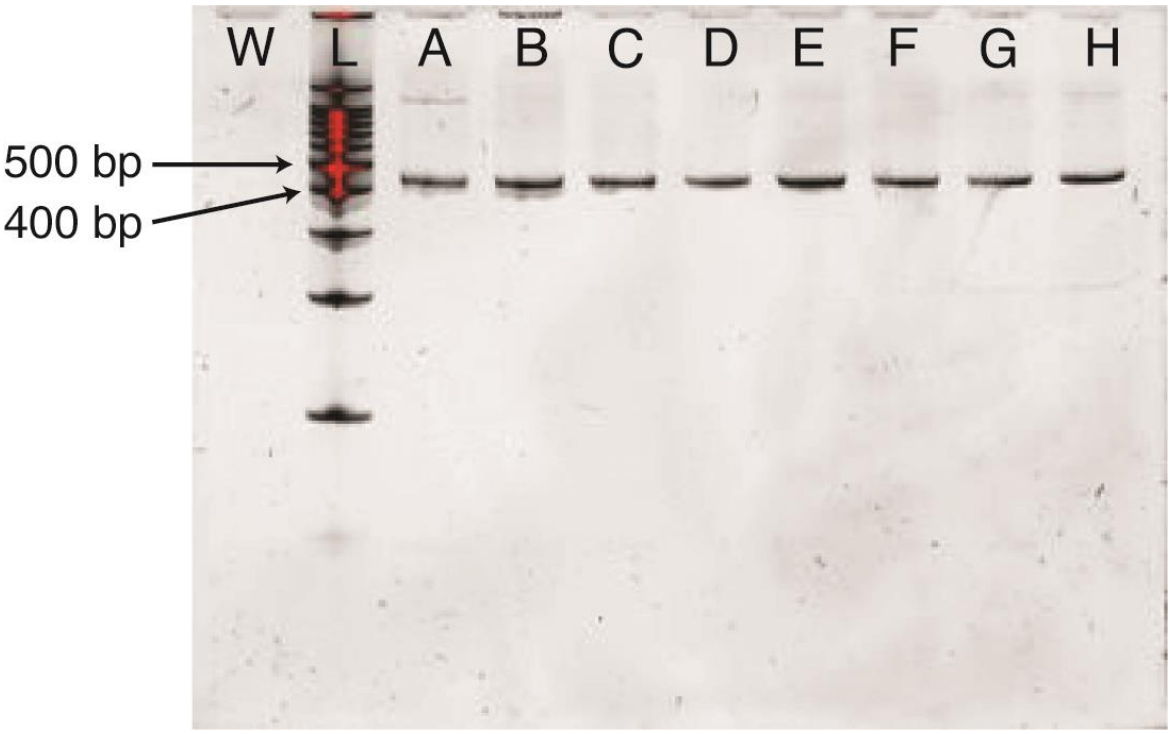
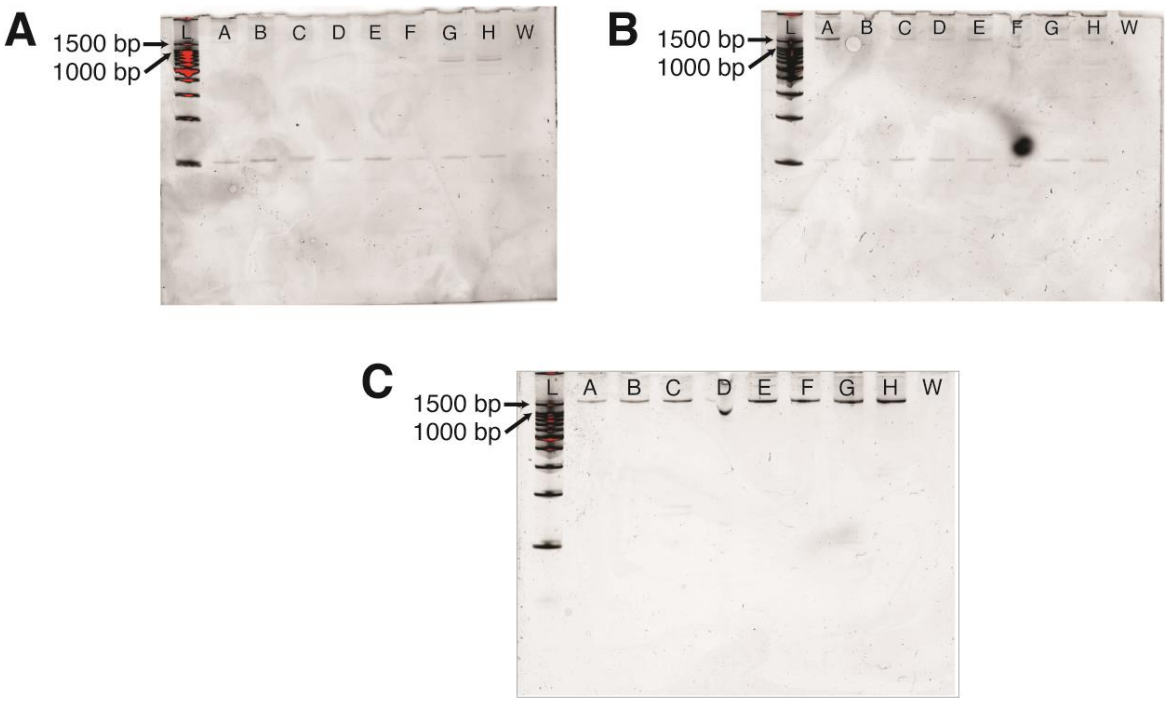


Image of GelRed stained polyacrylamide gel showing PCR amplification for primer set 1 using a thermal gradient for the annealing temperature. Wildtype N2 *C. elegans* DNA template was used for eight PCR reactions across a thermal gradient. Annealing temperatures tested in each lane for primer set 1 include: A = 60 ºC, B = 59.3 ºC, C = 58.1 ºC, D = 56.3 ºC, E = 54 ºC, F = 52.3 ºC, G = 50.9 ºC, and H = 50 ºC. L = 100 bp ladder, W = water (no template control).

Figure 7

PCR results and annealing temperature optimization of primer set 2 amplifications of the TRPM1 VUS locus in gon-2 through gel electrophoresis

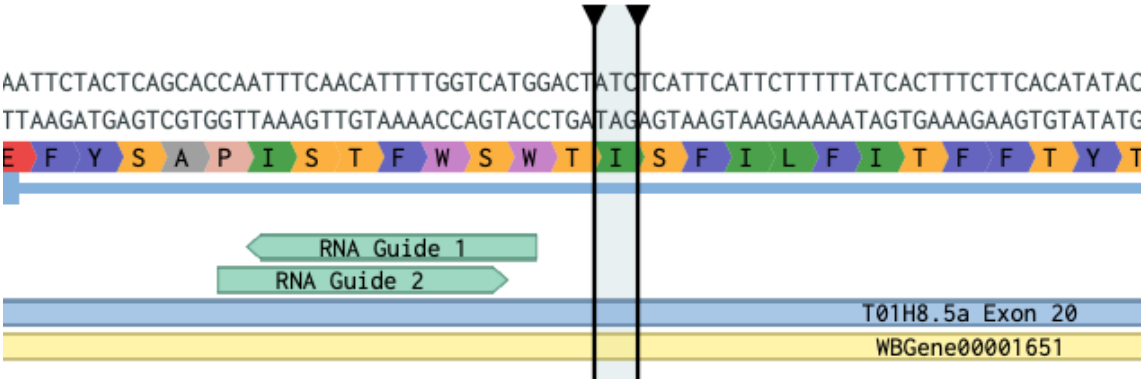


Images of GelRed stained polyacrylamide gels showing PCR amplification for primer set 2 using a series of thermal gradients for the annealing temperature (A-C). Wildtype N2 *C. elegans* DNA template was used for eight PCR reactions across three thermal gradients. (A) Annealing temperatures tested in each lane for primer set 2 across 50 – 60 ºC gradient include: A = 60 ºC, B = 59.3 ºC, C = 58.1 ºC, D = 56.3 ºC, E = 54 ºC, F = 52.3 ºC, G = 50.9 ºC, and H = 50 ºC. (B) Annealing temperatures tested in each lane for primer set 2 across 55 – 65 ºC include: A = 65 ºC, B = 64.3 ºC, C = 63.1 ºC, D = 61.3 ºC, E = 59 ºC, F = 57.3 ºC, G = 56 ºC, and H = 55 ºC. (C) Annealing temperatures tested in

each lane for primer set 2 across 65 – 75 ºC include: A = 75 ºC, B = 74.4 ºC, C = 73.1 ºC, D = 71.3 ºC, E = 69 ºC, F = 67.4 ºC, G = 66 ºC, and H = 65 ºC. L = 100 bp ladder, W = water (no template control).

Figure 8

CRISPR targeting in the gon-2 gene



Schematic showing the *gon-2* gene, the *TRPM1* VUS location (highlighted), and the CRISPR targeting regions flanking that region (green arrows). Image obtained from design and analysis within Benchling.