Studying the Behavioral Role of the KCNN2 Gene in Zebrafish Embryos

Gina Dragonette

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Studying the behavioral role of the KCNN2 gene in zebrafish embryos

Gina Dragonette

A thesis presented in partial fulfillment of the requirements of the Undergraduate Honors Program at the University of New Haven.

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ABSTRACT

Introduction Mutations in the KCNN2 gene have been linked to phenotypes of movement disorders and autism. The goal of this experiment was to use the CRISPR-Cas9 system to create a deletion within the KCNN2 gene and to observe the effect on the expression of movement in zebrafish embryos. Methods Linearized Cas9 DNA and guide DNA underwent in vitro transcription (IVT) reactions to produce RNA to be injected into zebrafish embryos at the one cell stage. KCNN guide RNAs were produced for the KCNN2 and KCNN3 genes (to prevent KCNN3 protein function from compensating for loss of KCNN2’s protein function), and the SLC45A2 gene, which was used as a positive control to indicate success of reagent preparation and Cas9 function. Genotypic and phenotypic analyses were performed 2 days post-fertilization (dpf).

Results No notable difference was seen with genotypic analysis, but KCNN-injected embryos moved less in unprovoked and provoked phenotypic movement tests than wild type (WT) embryos. Discussion Microdeletions may have been the cause of the observed phenotypic difference between KCNN-injected and WT embryos, however, statistical analysis showed that the observed differences in quantity of movement were not statistically significant. Conclusion The data collected in this experiment is not sufficient to conclude whether or not microdeletions in the KCNN2 gene were produced or may have contributed to a difference in the expression of movement in zebrafish embryos. Future research should be conducted targeting different guide sequences and using more sophisticated genotypic and phenotypic analyses.

Keywords: CRISPR-Cas9, KCNN2, zebrafish
INTRODUCTION

This experiment examined the effect of the disruption of the KCNN2 gene in zebrafish embryos. Each of the following sections of the introduction provides further detail on (1) zebrafish as a model organism, (2) the CRISPR-Cas9 system, and (3) the KCNN2 gene.

Zebrasfish as a Model Organism

Danio rerio, zebrafish, can be used in research as a model organism. Zebrafish and humans share 70% of their genes, and 84% of human genes associated with disease have analogous genes in zebrafish, enabling researchers to use zebrafish to study human diseases.\(^1\)\(^2\) Zebrafish have many of the same body systems and carry out many of the same functions as humans, and the pathways and genes associated with development and disease in these body systems are highly conserved between zebrafish and humans, making zebrafish ideal for studying human disease.\(^2\) In addition, zebrafish are relatively easy to maintain, develop quickly, are transparent in early development, reach reproductive age quickly, and produce abundant offspring in a short amount of time.\(^4\) Because zebrafish reproduce via external fertilization, their embryos can easily be modified at an early stage, allowing for whole-organism genetic modification.\(^2\) The full sequence of the zebrafish genome is known, so any mutations produced in their genes that lead to the development of disease can be identified.\(^1\) Zebrafish have been used to study genetics, cancers, and heart, muscle, brain, blood, cognitive, and neurological diseases.\(^1\)\(^3\)\(^4\)\(^5\)
**CRISPR-Cas9**

Clustered regularly interspaced short palindromic repeats (CRISPR) is an immune response used in prokaryotes to prevent viral infections.\(^6\) CRISPR systems use any one of several different nucleases to cut nucleic acids in the cell; one such nuclease is Cas9.\(^7\) The Cas9 system is part of the type II CRISPR-Cas systems, in which the Cas protein uses the host’s cellular enzymes and molecules to process the CRISPR RNA.\(^8\) Researchers can use CRISPR-Cas systems to alter a genome’s DNA sequence by targeting and cutting at specific DNA sequences to either change their sequence, knock it out of the genome, or add a sequence to the genome.

The CRISPR-Cas9 system works by using guide RNAs to guide the Cas9 nuclease to a specific location in the genome, where the Cas9 will make a cut in the DNA sequence.\(^7\) The DNA sequence will be modified by the cut because the DNA repair mechanisms will try to fix the damaged DNA, but the edits made to fix the sequence will likely not be the same as the initial DNA sequence. This leads to the DNA sequence becoming altered from that of the WT sequence. The DNA then does not produce the same RNA sequence as a WT DNA sequence would, so the protein that is produced from the altered DNA sequence is different from the protein produced in a WT organism, meaning that its function will be modified.

**The KCNN2 Gene**

The KCNN2 gene encodes for proteins called small-conductance calcium-activated potassium type 2 channels (SK2) that are highly expressed in the human brain and have been associated with neurotransmitter release, synaptic plasticity,
and memory formation.\textsuperscript{9} There are four isoforms (functionally similar proteins with similar amino acid sequences) of the SK2 protein coded for by the KCNN2 gene that are produced via different splicing patterns of the mRNA: the standard SK2 isoform, a long isoform, and two short isoforms.\textsuperscript{9}

The KCNN2 gene has been linked to autism in multiple genome-wide association studies, which look for genetic markers that are present in people with a particular phenotype and absent in people not expressing the phenotype.\textsuperscript{10,11} This indicates that mutations in the KCNN2 gene may have a role in the development of autism in humans, which frequently expresses an atypical movement phenotype, such as lack of coordination and balance.\textsuperscript{12} In addition, several mutations in the KCNN2 gene (including nonsense, splice site, frameshift deletion, in-frame deletion, and missense mutations) have been associated with phenotypes of the movement disorder dystonia and muscle spasms and tremors.\textsuperscript{13,14}

In zebrafish, the model organism used in this experiment, the KCNN2 gene has three paralogs, or homologous genes: KCNN3, KCNN1, and KCNN4.\textsuperscript{15} These paralogs are derived from the same genetic origin, but produce different proteins. In humans, the KCNN3 paralog has the most similar expression pattern to KCNN2, so it was also targeted in this experiment to avoid KCNN3 expression compensating for any phenotypic effect caused by deletions in the KCNN2 gene.\textsuperscript{16,17}

It was hypothesized that the disruption of the KCNN2 gene using the CRISPR-Cas9 system would produce a phenotypic difference in the injected embryos' expression of movement.
METHODS

This experiment had six unique parts: (1) guide selection and PCR primer generation, (2) Cas9 IVT, (3) guide IVT, (4) microinjection, (5) genotypic analysis, and (6) phenotypic analysis.

**Guide Selection and PCR Primer Generation**

Guide DNAs were selected using CRISPRScan. Two guides each were chosen for KCNN2 and KCNN3 from the largest exon within each gene. Guides with the highest CRISPRScan score and lowest CFO off-target score were chosen, with the exception of those that were within 20 nucleotides of either end of the exon. The guides were then modified with the addition of the following sequences: 5'-TAATACGACTCACTATAGG(N18)GTTTTAGAGCTAGAA-3'. SLC45A2 guide DNAs from previous research were used.

Forward and reverse PCR primers were designed for both the KCNN2 and KCNN3 genes. Primers were designed to be located between the end of the exon and the nearest guide, and to be 22 nucleotides in length with approximately equal proportions of A/T and C/G, with G and C on the ends, ensuring that no primer would base pair with any other primer. Guides and PCR primers were resuspended to 100 mM in nuclease free water and stored at -20°C.

The primers were tested via a 25 ul PCR reaction (2 ul template DNA, 12.5 ul 2x clear GoTaq master mix, 9.5 ul nuclease free water, 0.5 ul forward primer, 0.5 ul reverse primer) with DNA from a WT zebrafish fin to ensure that they would amplify the desired region of the genome. The PCR parameters were (1) 95°C, 2 minutes; (2) 95°C, 30
seconds; (3) 58°C, 30 seconds; (4) 72°C, 2 minutes; (5) Go to step 2, 34x; (6) 72°C, 10 minutes; (7) 12°C, unlimited time. *These PCR parameters were used for all PCR reactions in this procedure.

**Cas9 IVT**

An overnight culture of *E. coli* containing the pT3TS-nCas9n plasmid was prepared. Cas9 DNA was extracted following the QIAGEN QIAprep Spin Miniprep Kit instructions. DNA was eluted in nuclease free water and quantified via nanodrop. A 40 uL linearization reaction was performed by incubating 7 - 10 ug of plasmid DNA in 4 ul 10x CutSmart buffer, 1 ul Xbal, and the remaining volume of nuclease free water at 37°C overnight. The reaction was then spiked by adding an additional 17 ul nuclease free water, 2 ul 10x CutSmart buffer, and 1 ul Xbal and incubating for 30 minutes at 37°C. The DNA was purified following the QIAGEN QIAquick PCR Purification Kit instructions. DNA was eluted in nuclease free water and quantified via nanodrop. Linearization was confirmed by running the product on a 1% agarose gel.

The IVT reaction was performed following the Ambion mMessage mMachine T3 kit instructions for a 20 ul reaction, using up to 1 ug of DNA, 10 ul 2x NTP/cap mix, 2 ul 10x transcription buffer, 2 ul 10x T3 enzyme mix, and the remainder nuclease free water. The reaction was incubated at 37°C for 3 hours. To the reaction, 1 ul Turbo DNase was added, then incubated at 37°C for 15 minutes. The reaction was cleaned using ammonium acetate/ethanol precipitation by adding 10 ul ammonium acetate, then 60 ul 100% ethanol to the reaction and incubating at -80°C for at least 20 minutes. The solution was centrifuged at max speed at 4°C for 15 minutes and the supernatant was discarded. The
pellet was dislodged with the addition of 1 ml 70% ethanol, and the solution was centrifuged at max speed at 4°C for 5 minutes. The supernatant was removed, then dried for 2 minutes at room temperature. The pellet was resuspended in 50 ul nuclease free water, quantified via nanodrop, and run on a 1% agarose gel to ensure degradation of the product was not present. mRNA was aliquoted in 2 ul volumes and stored at -80°C.

**Guide IVT**

Two PCR reactions (one for the KCNN guides [both KCNN2 and KCNN3], and one for the SLC45A2 guides) were run using 25 ul 2x clear GoTaq master mix, 5 ul universal oligos (5’-AAAAAGCAACCGACTCGGTGCCACTITTTCAAGTTGATAACGGACTAGCCTTATTTAACTTTGCTATTTCTAGCTCTAAAAAC-3’), 15 ul nuclease free water, and 5 ul oligo master mix. The oligo master mix contained equal volumes and concentrations of each primer. One oligo master mix was prepared for KCNN guides, and a second was prepared for SLC45A2 guides. The PCR product was purified using the RNase free QIAGEN QIAquick PCR purification kit. DNA was eluted in nuclease free water and quantified via nanodrop. The PCR product was run on a 2% agarose gel to ensure that the guides were amplified.

A 20 ul IVT reaction was set up at room temperature using up to 1 ug PCR product, 2 ul 10x transcription reaction buffer, 2 ul 100 mM DTT, 1.8 ul each of 100 mM ATP, CTP, GTP, and UTP, 0.5 ul RiboGuard RNase Inhibitor, 2 ul T7 RNA polymerase, and the remainder of nuclease free water. The reaction was incubated at 37°C for 12 - 16 hours, then digested with 1 ul Turbo DNase at 37°C for 15 minutes. To the reaction, 80 ul nuclease free water was added, then 10 ul 5 M ammonium acetate and 300 ul 100%
ethanol were added. The reaction was incubated for at least 1 hour at -80°C. The solution was centrifuged at maximum speed at 4°C for 15 minutes, then the supernatant was discarded, and the pellet was washed two times with 1 ml 70% ethanol, completely removing the supernatant each time. The pellet was air dried for up to 10 minutes, then resuspended in 30 ul nuclease free water. The mRNA was run on a 2% agarose gel to ensure degradation of the product was not present. mRNA was aliquoted in 3 ul volumes and stored at -80°C.

**Microinjection**

Zebrafish crosses were set up one night prior to injection, and a single male and female were housed separately in one tank. In the morning, when the lights went on, the separation panel was removed and the fish were allowed to spawn. Embryos were then collected for injection.

Two injection mixes were prepared, one with the KCNN guide mix, and a second with the SLC45A2 guide mix. The injection mixes were prepared to have 200 ng/ul of Cas9 mRNA and 40 ng/ul of guide RNA. Microinjection was used to inject 1 nl of injection mix into each embryo. The injected embryos were moved to a petri dish of blue water, and uninjected embryos were added to a separate petri dish of blue water. The embryos were incubated at 28°C and debris and dead embryos were removed frequently during the first 2 dpf.
Genotypic Analysis

At 48 hours post fertilization, 12 embryos (2 WT and 10 KCNN-injected embryos) representative of their population were selected for genotyping. Each embryo was added to its own aliquot of 50 ul 100 mM NaOH, then enough nuclease free water to make a total volume of 100 ul was added. The embryos were heated in solution at 95°C for 10 minutes. After incubation, the solutions were placed on ice and 10 ul 1M Tris-HCL, pH 8.0 was mixed into each solution.

Each DNA sample was run in two 25 ul PCR reactions with 2 ul template, 12.5 ul 2x clear GoTaq master mix, and 9.5 ul nuclease free water. To one reaction for each sample, 0.5 ul each of KCNN2 forward and reverse primers were added, and to the second reaction for each sample, 0.5 ul each of KCNN3 forward and reverse primers were added. The PCR products were then run on a 2% agarose gel.

Phenotypic Analysis

After 2 dpf, phenotypic observation of movement was performed on 12 KCNN-injected embryos and 12 WT embryos that were representative of their population. Each embryo was placed in a petri dish of blue water and allowed to sit undisturbed for 2 minutes. The number of times each embryo moved in the 2 minute resting period was noted. The embryos were then observed for an additional 10 minutes, and the number of unprovoked motions (movement in a circle or straight line, or significant movement of the body without a change in location) was recorded, as well as the total unique instances of motion. After 10 minutes of observation, the side of the petri dish was tapped, and the number of embryos that reacted to the provocation was
recorded. Then, a pipette tip was used to tap the dish next to each embryo, and whether or not each embryo reacted to the provocation was recorded.

RESULTS

This section details the data collected and the findings of the experiment.

Guide Selection and PCR Primer Generation

Schematics of the KCNN2 gene (Figure 1A) and the KCNN3 gene (Figure 1B) show each gene’s relative intron and exon locations, with DNA guides and PCR primers indicated in their relative location on each gene’s largest exon. Two guide DNAs were chosen for both the KCNN2 and KCNN3 genes. Each guide DNA was 23 base pairs in length. For the KCNN2 gene, the two guide DNAs that were selected were (1) GGTACA ATCGCAGAAACATGGGG(−) located at bases 10:17638886-17638909, with a CRISPRScan score of 50, and a CFD off-target score of 5.95; and (2) GATGCTGCCTG ATGAAGCATCGG(−) located at bases 10:17638948-17638971, with a CRISPRScan score of 60, and a CFD off-target score of 3.40. For the KCNN3 gene, the two guide DNAs that were selected were (1) GGCGGTTACCCCGGTGCCGGGG(+) located at bases 16:23663859-23663882, with a CRISPRScan score of 70, and a CFD off-target score of 1.14; and (2) AGGTAGAGTCTAAGAAACATGGG(−) located at bases 16:23663968-23663991, with a CRISPRScan score of 51, and a CFD off-target score of 5.04. The SLC45A2 guide DNAs that were used are (1) TAATACGACTCACTATAGGA GCCTCCGAGGCGCTCTAGTTTAGAGCTAGAAA(+) and (2) TAATACGACTCACC TATAGGGGAAGGTTGATTATGCACGTTTAGAGCTAGAA(+).
The PCR primers that were designed were each 22 base pairs in length. The primers that were designed for the KCNN2 gene were CAGTTATTCAATGGTGACAC G(+) and GGTCTTCATAACAAATCGTGTG(-), and the primers that were designed for the KCNN3 gene were GAATTGGCCATGACCATGGAGAG(+) and CCGTGGAAGAGCT TGCTATGTAG(-).

**IVT Reactions**

The pT3TS-nCas9 plasmid (Figure 2A), which is 7332 base pairs, was used to generate linearized Cas9 DNA. The plasmid was linearized with XbaI, then run on a 1% agarose gel, and a band was visualized between 6000 and 8000 base pairs (Figure 2B). The Cas9 DNA concentration was 92.1 ng/ul, as determined by nanodrop. SLC45A2 and KCNN guide DNAs were amplified via PCR reactions, then run on 2% agarose gels, producing bands around 100 base pairs (Figures 3A and 3C). IVT reactions were performed to transcribe Cas9 and guide DNAs into mRNA. Cas9 and guide mRNAs were run on a 2% agarose gel and each mRNA product produced a single band on the gel (Figures 2C, 3B, and 3D).

**Microinjection**

SLC45A2-injected embryos had little to no dark pigmentation characteristic of zebrafish, while all KCNN-injected embryos and WT embryos had dark eyes and dark markings across their bodies (Figure 4). Embryos injected with either SLC45A2 or KCNN guides, as well as WT embryos appeared to be the same size at the same developmental phase for all observed stages of development up to 3 dpf.
Genotypic Analysis

PCR reactions were used to genotype 2 WT and 10 KCNN-injected embryos. Each of the WT and KCNN-injected embryo’s DNA produced a band on the gel around 300 base pairs when run with KCNN2 PCR primers (Figure 5A), and around 200 base pairs when run with KCNN3 PCR primers (Figure 5B), while no band was produced for the negative no template control when run with either set of PCR primers.

Phenotypic Analysis

When phenotypic analysis was performed on the WT embryos, 6 of the 12 embryos moved a total of 23 times with 11 unique movements during the 12 minutes of unprovoked observation (Figure 6). A single unique embryo responded to each of the two provocation tests. When the KCNN-injected embryos were observed, 4 of the 12 embryos moved a total of 6 times with 6 unique movements during the 12 minutes of unprovoked observation. A single unique embryo responded to the dish being tapped, and no embryos responded to a pipette tip tapping the dish next to them.

DISCUSSION

This section analyzes and interprets the results of this experiment and discusses their implications.

Guide Selection and PCR Primer Generation

The KCNN2 guides that were selected were both located on the largest exon of the KCNN2 gene (Figure 1A). The first was located 197 base pairs away from the 5’ end
of the exon, and the second was located 137 base pairs away from the 3’ end of the exon. The guides were located 39 base pairs away from each other, with a maximum 85 base pair deletion expected between the far ends of the guides. The KCNN3 guides that were selected were both located on the largest exon of the KCNN3 gene (Figure 1B). The first was located 89 base pairs away from the 5’ end of the exon, and the second was located 76 base pairs away from the 3’ end of the exon. The guides were located 86 base pairs away from each other, with a maximum 132 base pair deletion expected between the far ends of the guides.

The forward and reverse primers for each of the genes were tested using WT zebrafish DNA. A 333 nucleotide amplification was expected for WT DNA when the KCNN2 forward and reverse primers were used; the PCR product produced a band around 300 base pairs when run on an agarose gel. A 228 nucleotide amplification was expected for WT DNA when the KCNN3 forward and reverse primers were used; the PCR product produced a band around 200 base pairs when run on an agarose gel. These results indicated that the PCR primers that were generated for both the KCNN2 and KCNN3 genes successfully amplified their target regions. Therefore, when used on DNA exposed to CRISPR-Cas9, a minimum 246 nucleotide amplification was expected when the KCNN2 primers were used, and a minimum 96 nucleotide amplification was expected when the KCNN3 primers were used.

**IVT Reactions**

The pT3TS-nCas9 plasmid (Figure 2A) was amplified in *E. coli* grown in LB broth with ampicillin so that only *E. coli* containing the plasmid would grow, as the
plasmid, in addition to having the the Cas9 gene, had an AmpR gene that allowed it to grow in the presence of ampicillin. When the plasmid was isolated and linearized with Xbal, a band equivalent in size to the size of the plasmid (7332 base pairs) was expected. When the DNA was run on an agarose gel (Figure 2B), a band was seen at about 7000 base pairs, indicating successful linearization of the plasmid. If the plasmid had not been successfully linearized, the plasmid DNA would have migrated in a distinctly different manner than the linearized DNA and would have run further on the gel, appearing to be smaller than 7332 base pairs in length.

The successfully linearized DNA was then used in an IVT reaction to produce Cas9 mRNA. When the Cas9 mRNA was run on an agarose gel, a single band was produced without significant smearing present on the gel (Figure 2C). This indicates that the mRNA was successfully prepared without degradation, as degradation of the mRNA would have produced smearing on the gel. Because RNA runs differently than DNA on an agarose gel, the size of the mRNA could not be determined using this method, but the absence of contamination, as indicated by the single band produced, indicates that only Cas9 mRNA was present in the mRNA product.

After amplification, SLC45A2 and KCNN guide DNAs produced bands at about 100 base pairs (Figures 3A and 3C). This was the expected size of the amplicons for the guide DNAs, indicating that the PCR amplification was successful. The SLC45A2 and KCNN guide DNAs were then subjected to IVT reactions to produce guide RNA. When the mRNAs produced from these reactions were run on agarose gels, each produced a single band (Figures 3B and 3D). No significant smearing was noted on these gels,
indicating that the mRNA was not degraded, and the single band indicated that contamination was not present.

**Microinjection**

The zebrafish embryos injected with SLC45A2 guides were expected to lack significant dark markings and to express an albino phenotype. When the embryos injected with SLC45A2 guide mRNA were viewed at 2 dpf, they lacked significant pigmentation that were present on both the KCNN injected and WT embryos (Figure 4). The completeness of the albino phenotype expressed in these embryos indicated that the Cas9 mRNA was successfully translated into the Cas9 protein in the zebrafish embryos.

At 2 dpf, all three embryo groups (SLC45A2-injected, KCNN-injected, and WT) appeared to be the same size and shape. No deformities were observed in either the SLC45A2- or KCNN-injected embryos as compared to the WT embryos, indicating that the microinjection process and presence of the Cas9 protein had no significant impact on the development of the embryos.

**Genotypic Analysis**

Each of the WT and KCNN-injected embryo DNAs that were genotyped with KCNN2 PCR primers produced bands at about 300 base pairs, which was the expected amplicon size for WT DNA, but not for KCNN-injected DNA (Figure 5A). When the same DNAs were genotyped with KCNN3 primers, all produced bands around 200 base pairs, which was the expected amplicon size for WT DNA, but not KCNN-injected DNA (Figure 5B). The consistency of the band sizes produced by WT and KCNN-injected
DNA suggest that no significant deletions were created by the CRISPR-Cas9 system in the KCNN-injected embryos. However, microdeletions (base pair and small nucleotide sequence deletions) are difficult to identify when DNA is run on agarose gels. Therefore, the absence of a significant difference in the size of bands produced by WT and KCNN-injected embryos does not prove that no deletions were produced by injection with CRISPR-Cas9 and KCNN guide mRNA, and that small deletions may have been present that contributed to the observed difference in the movement phenotype. Larger deletions may not have been observed due to the guides not leading the Cas9 nuclease to the DNA sequences as expected, efficient DNA repair mechanisms in the embryos, or unsuccessful guide selection. Faulty Cas9 and guide mRNA preparation was likely not a cause for the absence of significant deletions due to the success of the expression of the albino phenotype in SLC45A2-injected embryos.

Phenotypic Analysis

A phenotypic difference was expected between the KCNN-injected and WT embryos; however, the similarity of appearance between the two groups of embryos indicated that any phenotypic difference in appearance between the two groups of embryos was not apparent after 2 dpf.

When movement of the KCNN-injected and WT embryos was assessed 2 dpf, KCNN-injected embryos expressed less movement for each test than WT embryos (Figure 6). This indicates that although no difference was observed in the physical appearance of the two groups of embryos, a phenotypic difference may be present in the expression of movement between the two groups. A t-test was performed on each data set.
between the two groups using 22 degrees of freedom. None of the individual tests resulted in a t-score greater than 2.074, the required t-score to prove statistical significance with a confidence interval of 95%, indicating that the observed phenotypic difference in the movement of the zebrafish embryos was not statistically significant. This suggests that the observed phenotypic difference may have been a result of chance, rather than any microdeletions that may have been created in the gene.

CONCLUSION

The results of this experiment indicate that microdeletions in the KCNN2 gene may have been produced by the CRISPR-Cas9 system in the zebrafish embryos, resulting in the KCNN-injected embryos expressing less movement than WT embryos. However, the data from the phenotypic behavioral observations were found not to be statistically significant. Thus, it cannot be certain whether or not the CRISPR-Cas9 system made microdeletions in the KCNN2 gene, or whether or not those deletions are responsible for behavioral differences between KCNN-injected and WT embryos. Further research into this topic should assess different CRISPR guides and perform more thorough genotypic and phenotypic analyses, such as using hairpin PCR primers and software that can calculate different components of movement, such as speed and distance traveled.
Figure 1 DNA guide and PCR forward and reverse primer generation for KCNN2 and KCNN3 genes. A KCNN2 gene with two guides selected from the largest exon, and PCR primers designed on either end of the largest exon. B KCNN3 gene with two guides selected from the largest exon, and PCR primers designed on either end of the largest exon.
**IVT Reactions**

**A**

**Figure 2** The plasmid from which Cas9 DNA was linearized and mRNA was transcribed. A The pT3TS-nCas9n plasmid is 7332 base pairs. B Cas9 plasmid was linearized by Xbal and run on a 1% agarose gel. Cas9 DNA concentration was 92.1 ng/ul, as determined by nanodrop. C Purified Cas9 mRNA produced by IVT was run on a 2% agarose gel. Cas9 mRNA concentration was 1992.5 ng/ul, as determined by nanodrop.
Figure 3  KCNN and SLC45A2 amplified guide DNA and transcribed guide mRNA was visualized by agarose gel electrophoresis.  
A  Amplified KCNN guide DNA was run on a 2% agarose gel.  
B  Purified KCNN guide mRNA produced by IVT was run on a 2% agarose gel.  
C  Amplified SLC45A2 guide DNA was run on a 2% agarose gel.  
D  Purified SLC45A2 guide mRNA produced by IVT was run on a 2% agarose gel.
Microinjection

Figure 4 All three groups of embryos developed at the same rate. The WT embryos exhibited typical developmental features. The SLC45A2-injected embryos expressed an albino phenotype. The KCNN-injected embryos expressed no substantially different phenotype than the WT embryos.
**Genotypic Analysis**

**A**

KCNN2 primers

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Figure 5 DNA was extracted from 2 WT embryos and 10 KCNN-injected embryos and amplified with PCR reactions using either KCNN2 or KCNN3 primers. **A** The PCR products of the reactions using KCNN2 primers were run on a 2% agarose gel. **B** The PCR products of the reactions using KCNN3 primers were run on a 2% agarose gel.
Figure 6 Fewer KCNN-injected embryos expressed movement in total and in each test, provoked and unprovoked, than WT embryos.
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