UNIVERSITY OF

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Editing of Kinesin Heavy Chain to Elucidate its roles in Neurodegenerative Diseases

A thesis submitted in partial satisfaction of the requirements for the degree of

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In

MOLECULAR, CELL, AND DEVELOPMENTAL BIOLOGY

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Abstract

Neurons are highly specialized and complicated cells. Neurons are composed of a cell body with dendrites that receive signals and a long axon that sends signals. Cellular metabolites, such as mRNAs and proteins, must be transported down the axon of neurons since the majority of them are synthesized solely in the cell body. Axonal transport is carried out by motor proteins, such as dynein and kinesin, that walk along microtubules carrying cargo from the cell body. Motor neuron degenerative diseases have been linked to disruptions in axonal transport from defective motor proteins. To study both the basic mechanism of axonal transport and related neurodegenerative diseases we used gene editing tools to change parts of the *kinesin heavy chain* (Khc) protein that are involved in kinesin regulation and that are known to cause Hereditary Spastic Paraplegia (HSP) in humans. I have already been successful in creating two different mutations in the *Drosophila* kinesin gene involved in kinesin autoregulation. These mutant flies have been tested and were shown to have a disruption in axonal transport. With the completion of ongoing experiments, these mutations will allow for a more in depth understanding of kinesin autoregulation. Investigating the basic functions of kinesin will aid in the development of therapeutic strategies for motor neuron degenerative diseases.

Introduction

Neurodegenerative diseases such as Alzheimer's, Parkinson's and Amyotrophic Lateral Sclerosis are common. Despite the prevalence of these diseases there is still no cure, or even a treatment. A common phenotype in the neurons of patients with neurodegenerative diseases is slowed axonal transport. (Morfini, 2009). Axonal transport is mediated by motor proteins such as kinesin or dynein, which carry cargo in the anterograde (toward the synapse) and retrograde (toward the cell body) directions, respectively. Kinesin has been identified as a large contributor in motor neuron degenerative diseases (Hurd, 2009). For example, patients with Hereditary Spastic Paraplegia have been found to have mutations in their *KIF5A kinesin heavy chain* homologue. By understanding the processes and functionality of kinesin, we may be able to find therapeutic approaches for these diseases.

Motor proteins are vital in a wide array of cellular processes including, mitosis and intracellular transport. Kinesin 1 is essential for the transport of intracellular cargo such as dense core vesicles, or mitochondria in motor neurons. Kinesin 1 is perfectly suited for this transport due to its high processivity. Kinesin 1 walks resolutely in the anterograde direction on microtubules by linking conformational changes to hydrolysis of ATP molecules. It features a heavy chain homodimer with two globular tails and two globular head domains, and two light chains that connect the heavy chains to axonal transport cargoes.

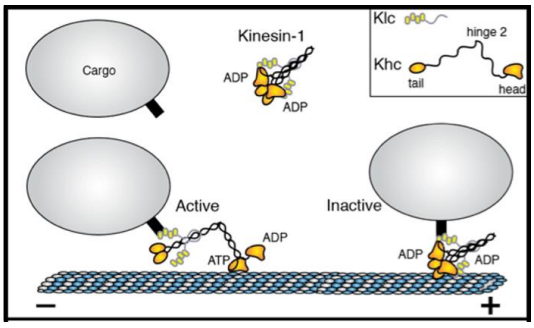

Fig. 1. Kinesin has an auto regulation process where a conserved sequence of amino acids, called the IAK region, in the tail interacts with the motor domain head. This prevents the release of ADP and forward movement on microtubules.

Kinesin undergoes autoregulation *in vitro* by folding on itself at hinge 2 (**Fig. 1**) such that the globular tails interact with the head via a conserved sequence of amino acids in the tail. When this region interacts with the motor domain head, it prevents the release of ADP.

Drosophila melanogaster is a good model organism for studying the mechanisms of kinesin and neurodegenerative diseases. The *Drosophila* kinesin heavy chain gene is a close homolog to the human kinesin heavy chain gene, this leads to similar phenotypes from kinesin mutations within these species. In both human and *Drosophila* kinesin mutants there are common phenotypes in motor neurons such as weak action potential propagations and swellings in axons that are filled with mitochondria, dense core vesicles, and other organic materials. Since *D. melanogaster* larvae are translucent, we can monitor live transport of GFP labeled kinesin cargo, such as mitochondria or dense core vesicles, to observe any disruption in transport caused by the mutations we test. These axonal swellings are called focal accumulations. Focal accumulations are often coupled with a distal paralysis phenotype that causes the larvae's posterior to rise as it attempts to crawl. This action in larva is called tail flipping. Tail flipping can range from a slight upward raise of the posterior to the tail lifting up so that the larva is almost on its head. In the presence of very strong kinesin mutations larvae's body to curve into a C-shape with the posterior end rising towards the anterior end. Mobility is often compromised when the mutations are severe and larva often roll on their sides in an attempt to move or the paralysis is so strong that they can only move their head. Mutations of this severity often result in death during the end of the second instar larvae stage.

Our experiments sought to address two main questions. The first is, what is the role of the loop 12 HSP mutations and kinesin-1 driven axonal transport? Several large studies of patients affected with Hereditary Spastic Paraplegia found mutations in the kinesin heavy chain gene. Many of the patients screened contained missense mutations in the loop 12 region of kinesin 1, which is a key portion of the motor domain head. Our second question is; what is the mechanism involved in the autoregulation of kinesin heavy chain *in vivo*? It is well known how kinesin uses ATP for transport in the cell, but little is known about how kinesin performs its function in cells in order to accomplish transport. Extensive studies of the mechanisms have been done *in vitro*, but have led to conflicting hypotheses (Kaan, 2011;Dietrich, 2008).

Involvement of Motor Proteins in HSP

The causes of motor neuron degenerative diseases are still unknown. These diseases are related to a loss of function of the motor protein kinesin (Hurd and Saxton, 1996). Since this link was discovered, specific mutations in the kinesin heavy chain gene have been identified in families with Hereditary Spastic Paraplegia (Reid et al., 2002). These mutations disrupt the binding of the kinesin heavy chain motor domain to microtubules, and thus the ATP hydrolysis mechanism. Missense mutations in families carrying Hereditary Spastic Paraplegia, such as the missense mutation N256S in the switch II loop of the motor domain, had similar effects when tested in *D. melanogaster* and *C. elegans* (Reid et al., 2002). These mutations are all in the kinesin superfamily protein 5 (Kif5). The majority of the missense mutations are in the motor domains of Kif5A.

Phylogenic analyses have revealed multiple families carrying missense mutations in the Loop 12 region of Kif5. In one family, arginine^{280} in Loop 12 was changed to cysteine (Fichera et al., 2004). Other families carrying Hereditary Spastic Paraplegia also have a missense mutation at arginine^{280}, such as R280H and R280L (Goizet et al., 2009).

Autoinhibition of Kinesin Heavy Chain

The processive transport of dense core vesicles and mitochondria is essential in motor neurons. Unlike other types of cells, simple diffusion does not suffice. The axons of human motor neurons can be up to one meter long, so motor proteins such as kinesin-1, need to travel long distances with high processivity. Kinesin-1 walks in the anterograde direction along microtubules using ATP hydrolysis as an energy source. Kinesin must only walk down the microtubule when cargo is attached. This is regulated by an auto inhibition process (Friedman and Vale, 1999) (**Fig.1)**. The globular tail interacts with the ATPase-domain to prevent the release of ADP (Coy et al., 1999). This is achieved by the interaction of a conserved sequence of amino acids in the globular tail denoted as IAK (^{940}AQIAKPIRS^{948}) with the motor domain heads in order to prevent the release of ADP. Currently, there are two models of how the tail achieves this. The Switch One Model (Dietrich et al., 2008) proposes that the IAK region of each tail interacts with the each of the motor domains (**Fig. 2**). Specifically, the IAK region interacts with

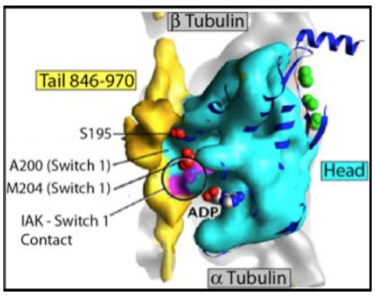

Fig. 2. The Switch I Model (Dietrich, 2008) suggests that the IAK tail region interacts with the Switch I loop in the head domain and blocks the release of ADP.

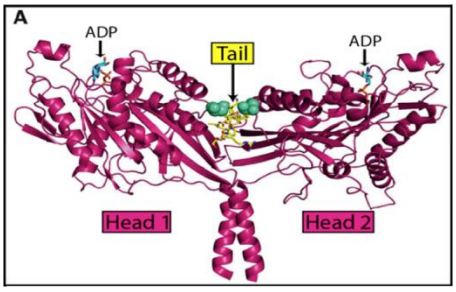

Fig. 3. Head Locking Model (Kaan, 2011) suggesting that one of the globular tails binds the two head domains via the IAK region. This locks them together to prevent a step.

the Switch One loop in the motor domain to block the release of ADP. The Head-Locking Model (Kaan et al., 2011) proposes that only one globular tail interacts with both motor domains (**Fig. 3**). Specifically, the IAK region interacts with both motor domains by hydrogen bonds between amino acids in the head and tail, locking them together. This prevents a step forward, preventing the release of ADP. Since these models were both tested *in vitro* and conflict each other, it is possible that *in vivo* testing might indicate which of these models is correct, if either.

CRISPR/Cas9

The bacteria genus *Streptococcus* developed a very useful enzyme for dealing with bacteriophages, these enzymes are called Cas enzymes. Cas stands for **C**RISPR **as**sociated protein. The most common Cas enzyme that is used is from *Streptococcus pyogenes* and is called Cas9. When viral DNA is introduced into *S. pyogenes*, a 20 nucleotide sequence is copied from the virus and inserted it into the bacterias genome downstream from the Cas9 enzyme. When the Cas9 enzyme is transcribed, so are all of the 20 nucleotide sequences that have been copied from foreign DNA. These 20 nucleotide sequences of RNA are called guide RNA. The Cas9 enzyme takes up the guide RNA and then binds to any matching DNA sequences that contain a protospacer adjacent motif (PAM) site. A PAM site is a sequence of nucleotides adjacent to the 20 nucleotide binding sequence that consists of NGG. Once the enzyme is bound, a double stranded cut is made 5 nucleotides from the PAM site.

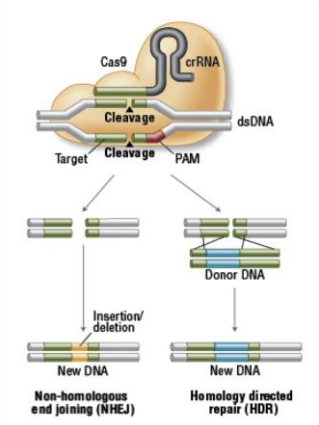

Fig. 4. Cas9 enzyme showing homology directed repair and non-homologous end joining.

With just guide RNA and the Cas9 enzyme, insertions or deletions (indels) in the sequence occur from non-homologous end joining. When mutant donor DNA with

homologous ends is introduced, homology directed repair occurs following a Cas9 cut (**Fig. 4**). The specificity of this enzyme is extremely useful in making precise genome edits. For testing kinesins relevance in Hereditary Spastic Paraplesia, and kinesins autoregulation process, we used the CRISPR/Cas9 method. Guide RNAs were designed for the Loop 12 region of kinesin heavy chain, and for the head and tail region of kinesin heavy chain. Since both experiments required specific missense mutations, a donor DNA sequence was designed as well.

Materials and Methods

CRISPR/Cas9

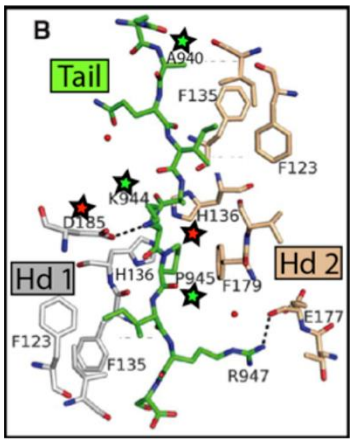

Fig. 5. IAK region showing the amino acid interactions proposed by the Head Locking Model (Kaan, 2011). The dotted lines represent hydrogen bonds between amino acids in the tail and head regions. Starred amino acids will be mutated to test this hypothesis *in vivo*.

The residues involved in the head-locking model are thought to interact via hydrogen bonds. (**Fig. 5**) To test their relevance in the inhibition mechanism, we created missense mutations that change amino acids to oppositely charged amino acids. The starred amino acids in (**Fig. 5)** point to the residues that we plan to mutate. Guide RNA plasmids have been developed for the D185 region and K944 region. Donor DNA has been designed for the following mutations: D185R, D185K, K944E, K944D. The guide RNA and corresponding donor DNA was then injected into flies expressing germline driven Cas9.

In order to test the common mutations of R280 in Hereditary Spastic Paraplegia patients and its relevance to the disease, we will use CRISPR/Cas9 to create these mutations in the kinesin homolog. Three missense mutations are in the process of being developed for R280 and are listed in (**Fig 6.)** The concentraions of donor DNA and guide RNA was based on papers published from other labs. The first three rounds of injections had single stranded donor DNA at 500ng/ul and guide RNA at 100ng/ul (Port, 2014). The last two rounds had a plasmid donor DNA at a concentration of 300ng/ul and guide RNA at 75ng/ul (Ren, 2014). (**Table 1**)

Y276C	L12	USA	pure HSP
R280C	L12	Italy	pure HSP
		France	HSP + MR, PNP
R280L	L12	France	pure HSP
R280H	L12	France	HSP + RP

Fig. 6. Chart of mutations found in patients with Hereditary Spastic Paraplegia. The mutations are missense mutations found in the loop 12 region of kinesin heavy chain. Loop 12 is a key element in microtubule binding.

D. melanogaster Embryo Injections

Initially flies expressing the Cas9 enzyme, either by a germline specific driver (y^1 *M{nos-Cas9.P}ZH-2A w**) or by a ubiquitous driver (y^1*M{Act5C-Cas9.P.RFP-}ZH-2A* w^{1118} *Lig4*^{169}), were injected with guide RNA and donor DNA of varying concentrations that are listed in the results. These injections were done by a company (BestGene Inc.) and in our lab. Later we developed a transgenic line that also expressed the guide RNA for the Loop 12 region (*gRNA-Loop12{v*$^+$*}*). This line was crossed with flies expressing the germline driven Cas9 and then injected in our lab with the donor DNA for the HSP mutations. This was done in parallel with the company injecting guide RNA and donor DNA for HSP mutations into flies expressing germline driven Cas9. The guide RNA and donor DNA were designed for the D185 mutations to test the autoregulation mechanism of kinesin and sent to the company for injections.

The injections were done in embryos within one hour of being laid. They were performed with a micromanipulator into embryos with the chorion intact. Injections were done in the posterior of the embryo to ensure that the developing germline cells would be effected.

Genetic Screenings

The developing flies from the injections only have the mutations in their germline cells. They are therefore crossed with a line of flies (*w*;sna*^{Sco}*/Cyo*) that contain a balancer chromosome for chromosome 2, the chromosome that the kinesin gene is located on.

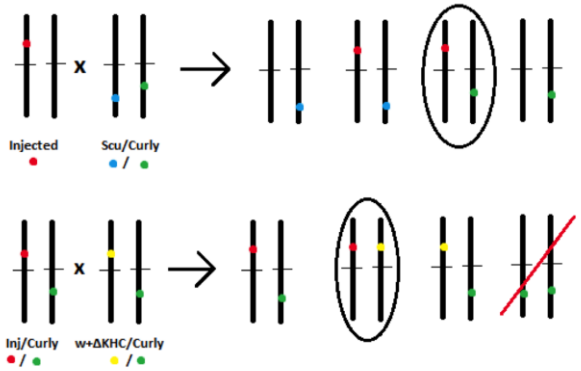

Fig. 7. Possible 2nd chromosomes of injected flies being crossed with flies that have balancers on the second chromosome. The circled genotype of the F1 is then crossed with flies who are null for kinesin heavy chain over a balancer. The circled genotype of the F2 should be lethal if the mutation was made.

The balancer chromosome used, Curly of Oster (CyO), inhibits recombination of chromosomes and has a phenotypic marker that causes curly wings. From this F1 generation, flies expressing the balancer chromosome, CyO, are crossed individually with a line of flies (*ΔKhc-attP(w+)/CyO*) that are heterozygous for a null kinesin mutation and the same CyO balancer chromosome. This development of a F2 generation is necessary because most kinesin mutations are recessive. When the potentially mutated chromosome is over a kinesin null gene, it is essentially hemizygous and is likely to produce a phenotype during larva development. The chromosome with the null kinesin gene also has a red eye color marker (w+) in order to distinguish it from the chromosome of interest. The CyO balancer is lethal over itself, this means that all curly winged flies with orange eyes are heterozygous for our mutation. If no straight winged fly is found, then the mutation is lethal at some point of development before eclosion from the pupa when the mutation is hemizygous. (**Fig. 7**)

Most kinesin mutations result in distal paralysis in the larvae when it is homozygous or hemizygous. We screened for both this distal paralysis in the F2 larvae, and lethality. When these phenotypes were present, we performed a PCR screening. Since mutant phenotypes could also be made from a non-homologous end joining after a successful CRISPR cut, we then take animals expressing the common mutant phenotypes and run a PCR with a mutation specific

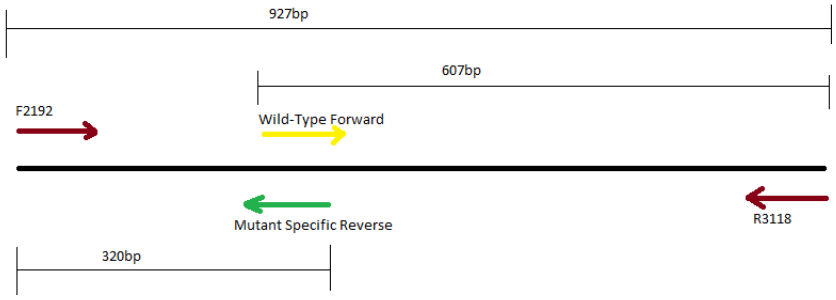

Fig. 8. The figure above is an example of the PCR product sizes with primers for R280H. The outlying forward primer (F2192) and reverse primer (R3118) make a band size of 927bp. The mutant specific primer and F2192 produce a band size of 320bp. The wild type primer and R3118 produce a fragment length of 607bp.

primer, a wildtype specific primer, and two primers that bind outside the region of interest. For R280H, the fragment length of the two outside primers, F2192 and R3118, is 927bp, the length of the mutation specific reverse primer, Loop12-R280H-F, and the F2192 primer is 320bp, and the length of the wildtype specific forward primer, Loop-12-wt-F, and R3118, is 607bp (**Fig. 8**). For D185K and D185R, the fragment length of the two outside primers, F2192 and Loop12-wt-R2, is 630bp, the length of the mutation specific reverse primers, PCR-D185K-Rev and PCR-D185R-Rev, and the F2192 primer is 196bp, and the length of the wildtype specific forward primer, PCR-D185-For, and Loop12-wt-R2, is 467bp. If a band is visible at about 300bp for R280H, or at 200bp for D185R and D185K, when the PCR products are run on a gel, the region of interest is then amplified by PCR using just the two outside primers and sent for sequencing.

Live Image Microscopy

The images were taken on a Nikon Eclipse TE2000-E Inverted Spinning Disk Laser Confocal Fluorescence Microscope. Third instar larvae were placed ventral side down in an imaging chamber, on top of a thin layer of halocarbon oil. A cover slip was placed on top of the larvae, the cover slip had three pieces of layered tape on the edges of one face of the slip. The taped side was placed facing down for spacing. The chamber was then closed which flattened the larvae to the width of the 3 layers of tape. Desflurane was then injected into the chamber for sedation of the larvae. The Desflurane was stored at -20°C, and kept on dry ice during use. The full larva images were taken at 20X air, and the dense core vesicle movies were taken at 60X oil at 2 time points per second. For imaging, flies expressing D185R, D185K, and a wild type control were crossed with *w; Khc^{27}/Cyo, Kr-GFP; P{GawB}D42 Gal4 P{w^{+mC} UAS-ANFGFP}3*

Results

R280 Mutations

Our initial efforts with the CRISPR/Cas9 system involved two sets of guide RNA for the Loop 12 region of kinesin heavy chain (**Table 1**). The donor DNA was single stranded and the Cas9 enzyme was driven by the germline specific driver; vasa. After sequencing some of the F2 from the injected flies, we found that there were single nucleotide polymorphisms (SNPs) where each of the guide RNAs were supposed to bind.

Table 1. CRISPR Trials

Date shipped	Mutation	Strain	Donor	Guide RNA	# of Embryos	# of Larvae	# of Adults	# Fertile	# of indels	# of HDR
3/19/15	R280H	vasa-Cas9	200 bp oligo	gRNA with SNP's	>200	~150	98	11	0	0
6/25/15	R280L	vasa-Cas9	90 bp oligo	Mix of two gRNA	>200	~60	10	5	0	0
6/25/15	R280H	vasa-Cas9	200 bp oligo	Mix of two gRNA	>200	~15				
7/1/15	R280L	nos-Cas9	90 bp oligo	Mix of two gRNA	>200	~130	39	15	0	0
7/1/15	R280H	nos-Cas9	200 bp oligo	Mix of two gRNA	>200	~130	61	32	5	0
9/16/15	R280L	nos-Cas9	plasmid	Reduced concentration	>300	~40	12	6	1	0
9/16/15	R280H	nos-Cas9	plasmid	Reduced concentration	>300	~50	25	8	4	1
2/17/16	D185K	nos-Cas9	plasmid	Reduced concentration	>300	~50	88	60	In progress	2
2/17/16	D185R	nos-Cas9	plasmid	Reduced concentration	>300	~30	76	52	In progress	5

Table 1. The Cas9 enzyme was driven by either nanos, or vasa, both are germline specific. Each round of injections listed on this table is a combination of injections by the company and in lab injections. The first guide RNA (gRNA) was designed from our wild type stock. The flies being injected had single nucleotide polymorphisms (SNPs) in the region the gRNA was supposed to bind. The following gRNA used was corrected for these SNPs. The insertions or deletions (indels) are a results of non-homologous end joining. When our gRNA concentration was lowered we noticed a large drop in viability but an increase in the amount of indels.

New guide RNA was developed incorporating these SNPs. This change lead to a dramatic drop in viability and fertility, still we found no successful cuts from the Cas9 enzyme. We then switched to a different driver; nanos, that is more restricted to the germline and was shown to work for genomic editing of essential genes. (Port, 2014). With this we had our first successful cuts, but the percent was still very low. On the fourth round the guide RNA concentration was reduced from 300ng/µl to 150ng/µl based on optimal concentrations tested by another lab (Ren, 2014). Our percentage of indels increased dramatically. There was one successful homology directed repair but the second guide RNA resulted in an in/del that created a frameshift. This made the mutation useless in testing the mutations relevance in Hereditary Spastic Paraplegia.

In parallel to the round of injections started on 9-16-15, a line of flies was developed with transgenic guide RNA. We then tested the efficiency of cuts by crossing males expressing the guide RNA with females expressing Cas9 driven by either the nanos germline specific driver, or the ubiquitous driver actin with a ligase 4 mutation that prevents non-homologous end joining. When the guide RNA was expressed with the ubiquitous driver there was 100% lethality in the larvae stages. Larvae showed a distal paralysis phenotype similar to Khc null mutants. When the guide RNA was expressed with the germline specific driver, all of the resulting females were sterile. Kinesin plays a role in oogenesis and is required in oocyte streaming. We found that oocytes from females with germline expressed Cas9 and guide RNA completely lack oocyte streaming, again similar to the kinesin null mutant phenotype. Both of these show astounding efficiency of Cas9 cuts and injections of the donor DNA for R280H was started on 1-10-16. On the gel (**Fig. 9**) you can see bands at about 300bp for H6-16, H6-17, H6-19, H2-

11, and H2-12. These five samples were then sent for sequencing. The sequence becomes unreadable after R280 since we were only able to isolate heterozygous flies. This was due to the mutation being lethal in the larval stages when hemizygous and the fact that the heterozygous larva showed distal paralysis phenotypes so it was difficult to tell the difference between heterozygous and hemizygous larvae. With perfect homology directed repair we should see over-lap only in the R280 region, and the sequences would match up again. This is not the case which makes us assume non-homologous end joining occurred, because the overlapping usually is due to a frame shift. The interesting thing about this is, that if you look at the peaks, you can see a red band overlapping with a yellow band after the cysteine in R280 in each of the sequenced flies (**Fig. 10**, **Fig. 11**). This implies that each of the flies are heterozygous for a guanine and adenosine. This was the missense mutation that we were aiming for. It is very unlikely that we would have two separate lines with indels that led to the exact replacement that we were looking for. A second cross is currently being done to screen for hemizygous larvae in order to get a cleaner sequence and determine if a frame shift occurred or if the continued overlap was a sequencing error.

Key of Genotypes for Gel
1: H1-1/CyO
2: H1-14/CyO
3: H1-2/CyO
4: H1-15/CyO
5: H1-7/CyO
6: H1-16/CyO
7: H1-8/CyO
8: H1-17/CyO
9: H1-9/CyO
10: H2-1/CyO
11: H1-10/CyO
12: H2-2/CyO
13: H1-11/CyO
14: H2-6/CyO
15: H1-12/CyO
16: H2-7/CyO
17: H2-8/CyO
18: H2-19/CyO
19: H2-9/CyO
20: H2-20/CyO
21: H2-10/CyO
22: H6-16/CyO
23: H2-11/CyO
24: H6-19/CyO
25: H2-12/CyO
26: H6-17/CyO
27: H2-13/CyO
28: H2-17/CyO
29: H6-17/CyO (larvae)
30: H2-18/CyO
31: H6-19/CyO (larvae)

Fig. 9 L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 L 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 L

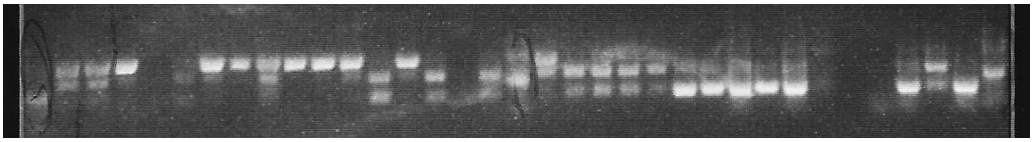

Fig. 9. PCR results of the F2 generation from injections for R280H. The gel was run with products from a PCR that consisted of the wild type specific primer for R280, a mutant specific primer for R280H, and the two out lying primers, F2192 and R3118. The smaller bands at 300bp indicate binding of the mutant specific primer, while bands at 600bp indicate binding of the wild type primer. Bands at 900bp are from the F2192 and R3118 primers.

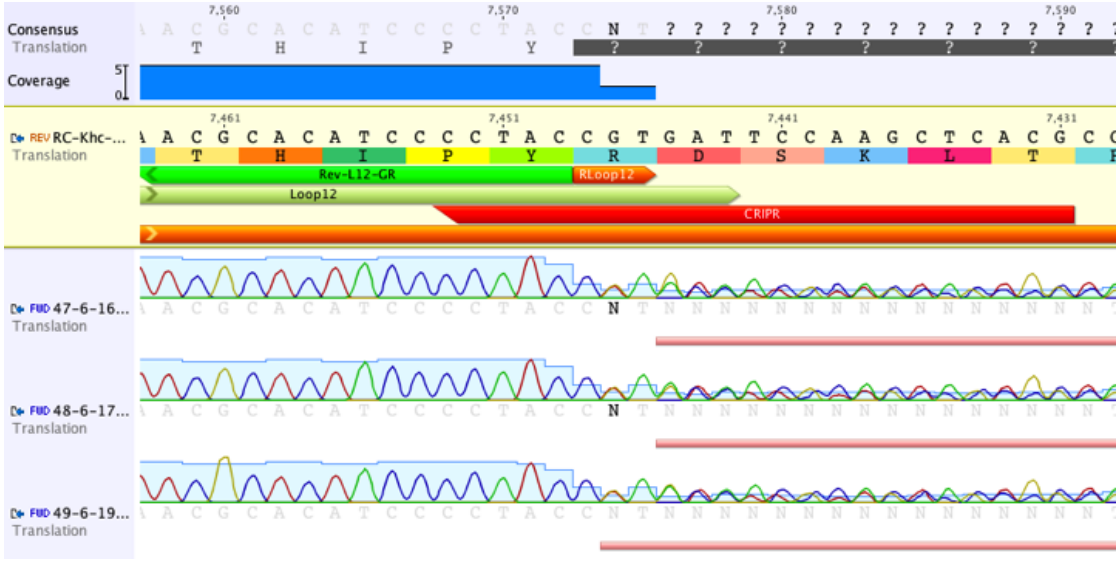

Fig. 10 Sequencing results from the F2 generation of embryos injected for R280H. These are all from the same F1 line, H6. The sequences for heterozygous adult flies; H6-16, H6-17, and H6-19, are aligned against the highlighted wild type sequence. The arginine residue can be seen in the wildtype sequence highlighted with an orange arrow. In the consensus shown at the top you can see that all three of the sequences have the cytosine and thymine of the wildtype **C**G**T**. The donor DNA coded for a single nucleotide polymorphism of CGT to CAT. There are double peaks of a red band (adenosine) and yellow band (guanine) for the nucelotide in between the cytosine and thymine of R280 in all three of the sequences.

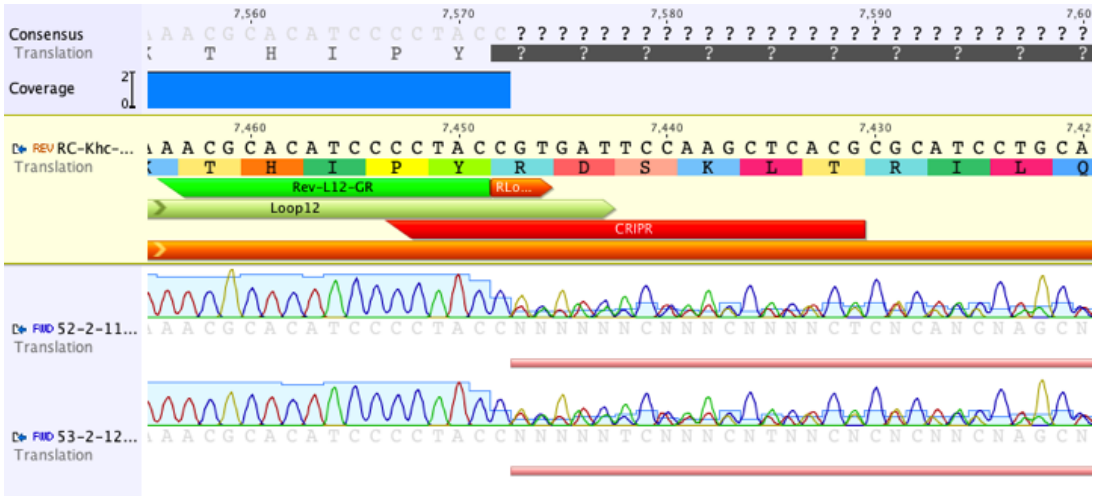

Fig. 11 Sequencing results from the F2 generation of embryos injected for R280H. These are all from the same F1 line, H2. The sequences for heterozygous adult flies; H2-11, and H2-12, are aligned against the highlighted wild type sequence. The arginine residue can be seen in the wildtype sequence highlighted with an orange arrow. In the consensus shown at the top you can see that all three of the sequences have the cystosine of the wildtype **C**GT. The donor DNA coded for a single nucleotide polymorphism of CGT to CAT. There are double peaks of a red band (adenosine) and yellow band (guanine) for the nucleotide after the cytosine of R280 in both of the sequences.

D185 Mutations

On 2-17-16 the company started injecting embryos expressing nanos driven Cas9 with guide RNA and donor DNA for D185R and D185K in order to test the Head-Locking Model of kinesin heavy chains autoregulation mechanism (Kaan, 2011). The screening of the F2 generation is still in progress but so far we have found 2 successful D185K mutations and 5 successful D185R mutations (**Table 1**). On the top gel (**Fig. 12**), which was run with a mutation specific primer for D185R; R71-8, R74-9, and R76-8 had bands visible at 200bp. On the bottom gel (**Fig. 12**), which was run with a mutation specific primer for D185K; K64-3, K64-4, and K65-3 had bands visible at 200bp. For all three of the D185R mutants that were sequenced, you can clearly see the wild type GAT has been successfully been converted to AGG by homology directed repair (**Fig. 13**). For the sequenced D185K mutants, you can also clearly see a mutation of AAG from the wildtype GAT (**Fig. 14**).

The mutant hemizygous larvae for D185K and D185R mutations were both much smaller in size compared to the control and showed very severe distal paralysis (**Fig. 15**). Both of these mutations cause lethality, homozygous or heterozygous animals die at the end of the second instar larvae stage. When observing the transport of dense core vesicles, focal accumulations were present in a much larger number than in the control and axonal transport was significantly reduced (**Fig. 16**). The control is a heterozygous Khc^{27} mutant and thus has a small amount of focal accumulations.

Key of Genotypes for the Gel

1: K64-3/GB2
2: K64-3/Δ
3: K64-4/Δ
4: R71-8/Δ
5: R74-9/Δ
6: R76-8/Δ
7: w*
8: K37-3/Δ
9: K56-4/Δ
10: K65-3/Δ
11: R27-3/Δ
12: R54-5/Δ

GB2- CyO, Kr-GFP
Δ- ΔKhc-attP(w+)

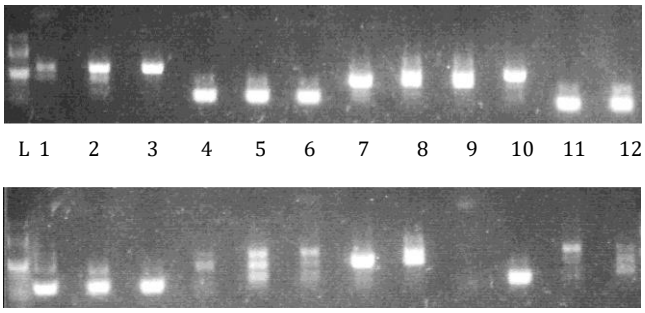

Fig. 12. PCR results of the F2 generation from injections for D185K, and D185R. The top gel was run with products from a PCR that consisted of the wild type specific primer for D185, a mutant specific primer for D185R, and the two out-lying primers, F2192 and Loop12-wt-R2. The bottom gel was run with products from a PCR that consisted of the wild type specific primer for D185, a mutant specific primer for D185K, and the two out lying primers, F2192 and Loop12-wt-R2.
The smaller bands at 200bp indicate binding of the mutant specific primer, while bands at 500bp indicate binding of the wild type primer. Bands at 600bp are from the F2192 and Loop12-wt-R2 primers.

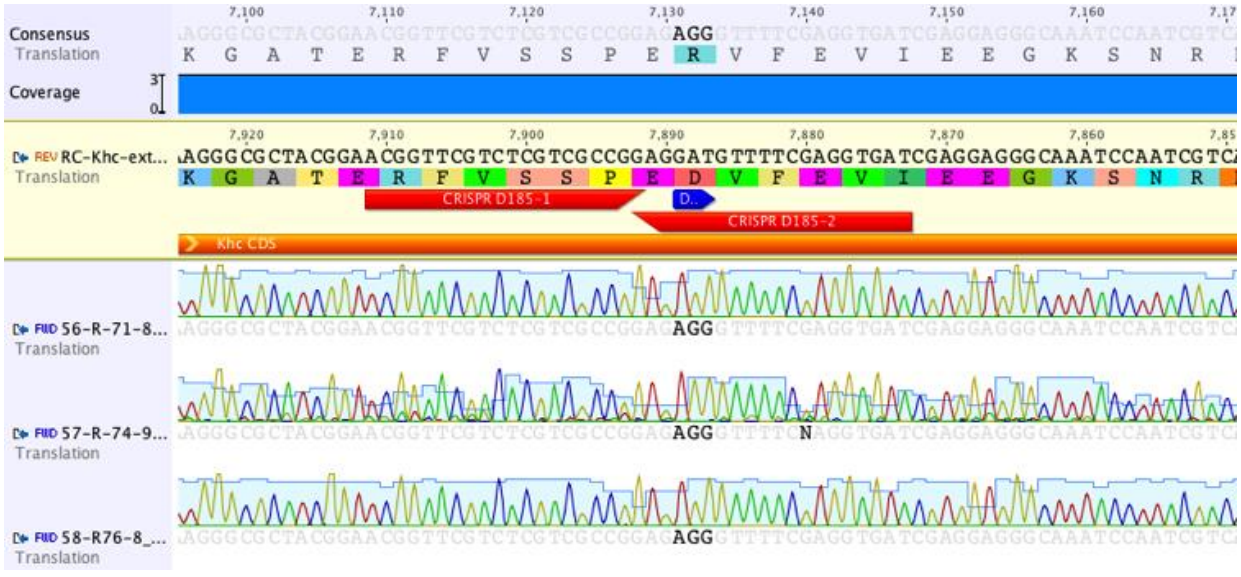

Fig. 13 Sequencing results from the F2 generation of embryos injected for D185R. The sequences for hemizygous larva; R71-8, R76-8, and R74-9, are aligned against the highlighted wild type sequence. The aspartic acid residue can be seen in the wildtype sequence highlighted with a dark blue arrow. The AGG mutation from the donor DNA is written in bold in all three of the sequences. In the consensus shown at the top you can see the translation of the AGG mutation, which codes for arginine.

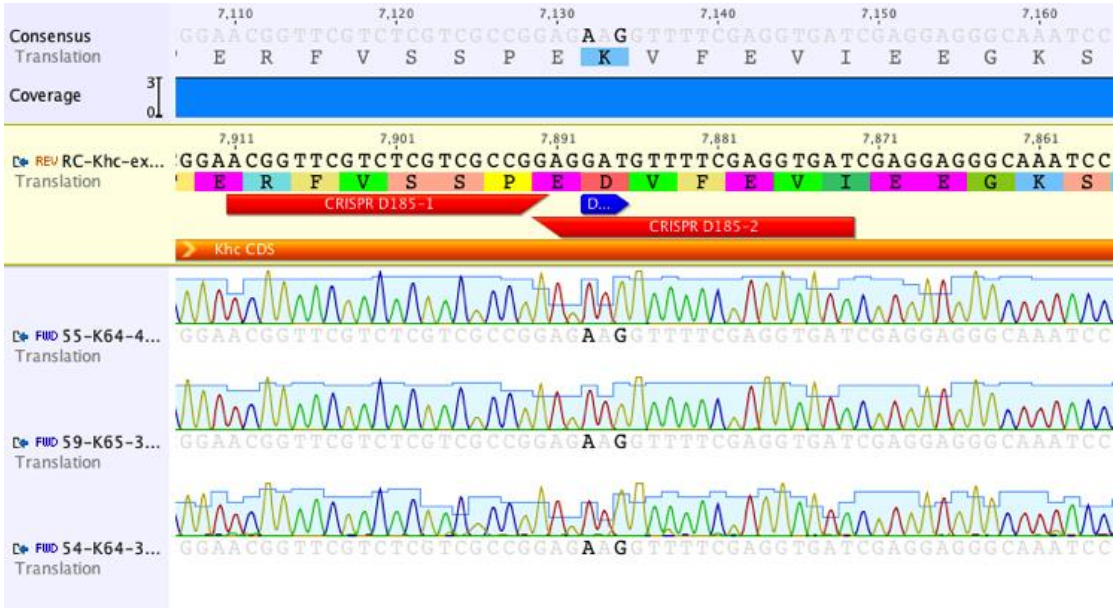

Fig. 14 Sequencing results from the F2 generation of embryos injected for D185K. The sequences for hemizygous larvae; K64-4, K65-3, and K64-3, are aligned against the highlighted wild type sequence. The aspartic acid residue can be seen in the wildtype sequence highlighted with a dark blue arrow. The **AAG** mutation from the donor DNA is written in bold in all three of the sequences. In the consensus shown at the top you can see the translation of the **AAG** mutation, which codes for lysine.

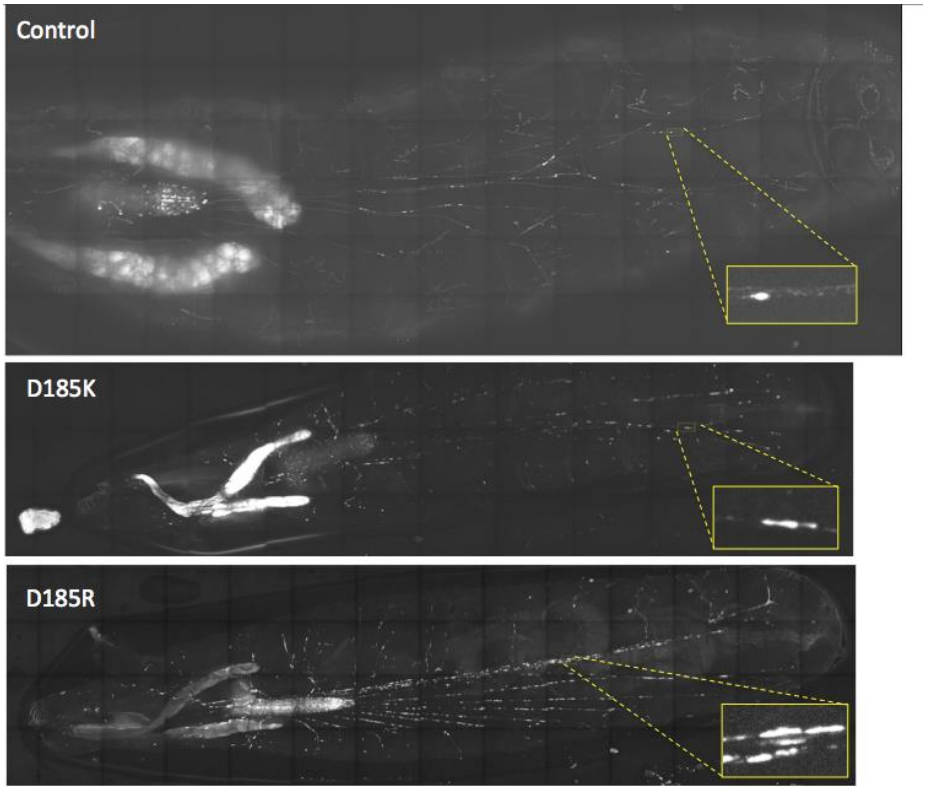

Fig. 15 The top image is a control (Khc^{27}/+) larva, the middle is a D185K mutant, and the bottom image is a D185R mutant with *D42-GAL* driven *UAS-ANF-GFP*. In the yellow square boxes are magnified images of segmental nerves with focal accumulations. These images are a stacked composition of images from different planes in the larva and were taken with a 20X air objective.

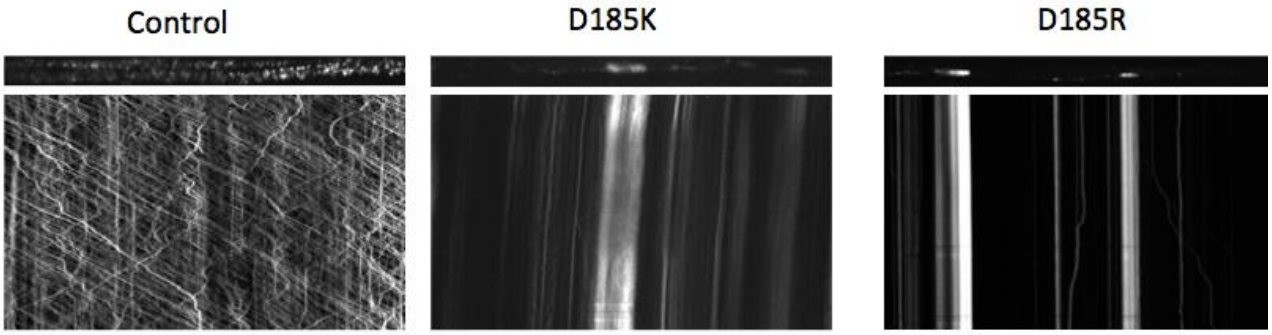

Fig. 16 These are kymographs of dense core vesicle movement in segmental nerves taken with a 60X oil objective. The left image is a control (Khc^{27}/+) larva, the center image is a D185K mutant, and the right image is a D185R mutant with *D42-GAL* driven *UAS-ANF-GFP*. This shows the relation of position versus time of a GFP tagged dense core vesicle. The y-axis is time with zero at the top, and the x- axis represents dense core vesicle movement. Lines with negative slopes indicate anterograde transport and lines with positive slopes indicate retrograde transport. Vertical lines represent non-moving dense core vesicles.

Discussion

The CRISPR/Cas9 system is changing the world of molecular biology. Currently CRISPR is being used to solve a range of health crises, from identifying oncogenes, to developing malaria resistant mosquitos. The possibilities of use are seemingly endless with this system. CRISPR is still a relatively new tool though, and needs a lot more fine-tuning. With our innovation of using transgenic guide RNA, the efficiency of Cas9 cuts has increased dramatically and solved a large issue with the CRISPR system.

There are two main issues with the CRISPR/Cas9 system. The first issue with this system is that after the enzyme has matched up with all of the possible targets, it will begin to make cuts at non-specific sites throughout the genome. This has the potential to destroy vital genes and cause lethality in the organism. Labs at MIT and Harvard have developed an enhanced *S. pyogenes* Cas9 (eSpCas9). The eSpCas9 has three amino acids changed from the wild type Cas9. This modification reduces the chances of off-target cuts to an undetectable amount. However, it looks like in *Drosophila* even the wild type Cas9 is very specific. Our transgenic guide RNA was extremely efficient and due to the high frequency of indels generation lead to *Khc* null-like phenotypes. Changing just a single nucleotide in the fly strain completely changes the outcome: mismatched gRNA was not producing any mutant animals and did not display a mutant phenotype.

The second issue is the efficiency of the enzyme. For the highest efficiency in each model organism, the optimal concentrations of the enzyme and guide RNA differ. With the current optimizations you rarely see a success rate over 50%. For *Drosophila* there are many methods that suggest different concentrations of guide RNA and donor DNA for injections. For the Cas9 enzyme there are three different approaches; inject a

plasmid expressing the Cas9 enzyme, inject the Cas9 enzyme, or to have a transgenic Cas9 gene expressed in the flies. With our innovation of using transgenic guide RNA and transgenic Cas9, we were able to produce 100% efficiency of CRISPR cuts. This will lead to higher success rates for homology directed repair when creating mutations in *Drosophila melanogaster*.

High efficiency of the indel mutations resulting from transgenic gRNA expression suggests that it could be used as a new tool for creating *Drosophila* mutations. In addition to creating stable mutations, this method can be used to turn off gene function in a specific tissue, similar to how the UAS/GAL4 mediated RNAi technique is used now. Based on our results, CRISPR-mediated knockdown can be specific and very efficient. In our experiments we could reproduce *Khc* null phenotypes in the whole animal with ubiquitously expressed Cas9 or only in oocytes with germline-specific Cas9. If new Cas9 transgenes were developed in the future, they could be used to knockdown gene functions in other tissues, like neurons. Potentially the same approach can be used for creating mutations and studying function of any gene.

Many different human diseases are studied using the model organism *Drosophila melanogaster*. With our high efficiency CRISPR method, diseases such as Alzheimer's and Parkinson's may have successful therapeutic strategies in the very near future. Using this CRISPR method we hope to learn more about the causes of neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis and Hereditary Spastic Paraplegia . By using CRISPR to make specific mutations in the motor protein kinesin, we can begin to understand some of the key mechanisms of kinesin that are currently unknown.

One of the most essential mechanisms to understand is the autoregulation of kinesin heavy chain. We will test the Head-Locking Model by creating specific mutations in the head of kinesin heavy chain and performing complementation tests with other *Khc* mutants. Once the tail mutants K944E and K944D are created we will do a complementation test with K944D and D185K. If the Head Locking Hypothesis is correct, we should see a regain of viability and function of kinesin heavy chain if the missense mutations have not destroyed too much of the head and tail structures. This will allow us to test the model *in vivo*. If this model proves to be incorrect we will move forward by creating mutations in amino acids that are proposed to be involved in autoregulation by the Switch I Model. The understanding of this mechanism is integral to understanding the involvement of kinesin in motor neuron degenerative diseases.

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