University of Nevada—Reno

Drosophila RET Mutants as a Model for Hirschsprung's Disease

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by

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Abstract

Mutant RET protein can fail to activate neural migration pathways causing the newborn colon to lack neurons for peristalsis. Considered as Hirschsprung's disease, no treatment exists to induce proper migration thus requiring resection of the non-innervated colon. *Drosophila melanogaster*'s RET is a close homolog to human RET. Fly RET mutants were characterized as possible models for Hirschsprung's disease treatment testing. The mutants had five distinct characteristics relative to wild type: foraging behavior and decreased neuronal vesiculation, movement of gut food, larval growth, and survival time. The mutants were used to create a behavioral assay for treatment testing. Mutant *Msn* and *GAP1* (RET phenotype enhancers) were tested as possible rescues but failed to better the RET mutant phenotype. The mutants' problems in moving food, very similar to Hirschsprung's disease patients that cannot move food past the colon, implies promise that the fly *RET* mutant can act as a model with five dimensions for treatment testing.

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Introduction

Rearranged during Transfection (RET) is a receptor tyrosine kinase. Like many receptor tyrosine kinases that use extracellular signal to activate intracellular pathways, RET does the same but with a high neurodevelopmental focus (Lemmon & Schlessinger, 2010). It is involved in neuronal survival, proliferation, and migration (Ibanez, 2013). The mechanism of signal transduction involves a family of co-receptors (GFRs) that bind to a family of extracellular ligands (GFLs) (Knowles et al., 2006). This complex then binds to RET to activate it and, in turn, activate intracellular pathways like neuronal migration (Ibanez, 2013; Mason, 2000). When RET is mutated and loses its ability to initiate the pathways in the developing fetus, Hirschsprung's disease (HSCR) sometimes develops (Bordeaux et al., 2000; Carlomagno et al., 1996). In humans, this causes migrating neural precursors to fail to reach and survive in the colon of the developing fetus. Surgical resection of the colon is necessary for portions that lacks a certain level of function. No treatment to artificially migrate the neurons or innervate the colon exists. However, *Drosophila melanogaster*'s RET (dRET) shows high homology to human RET due to similar protein domains (hRET) (Abrescia, Sjöstrand, Kjaer, & Ibáñez, 2005).

The dRET pathway involves the same general RET, GFL, and GFR mechanism. The difference is that *Drosophila*'s GFL is still unknown and the dRET extracellular domain is different enough to not be able to bind mammalian GFLs (Abrescia, Sjostrand, Kjaer, & Ibanez, 2005). However, hRET and dRET

seem to initiate many of the same pathways due to the similar dRET intracellular domain. These underactive pathways in mutated hRET are responsible for HSCR. Therefore, the fruit fly might be an adequate model for understanding more about the pathways and possible treatment of HSCR with the benefit of fast *Drosopihila* generation times. Some genes, when mutated to produce 60% less respective protein, enhance an eye cancer caused by mutant (overactive) *dRET* in the fruit fly (Read et al., 2005; Santoro et al., 1995). These mutant enhancer proteins of the genes are enhancers when having no effect, and are therefore presumed suppressors of RET pathways when not mutated and fully active. This paves the way for these suppressor genes to be suitable drug targets; i.e., if the suppressor genes can be blocked from expressing, the downstream pathways that RET activates can be overexpressed to make up for a loss-of-function mutated *RET*. This would then allow neurons to migrate to and survive properly in the colon. Our lab has created fruit fly *dRET* mutants via CRISPER-Cas9. One set (LMx) of three *dRET* mutants with an additional off-target mutation (LM1, LM2, LM3). The off-target mutation was repaired and another set (LMx.x) of three *dRET* mutants was made (LM1.1a, LM2.1m, LM3.2d). The first step is to characterize any phenotype(s) of the 6 *dRET* mutants as a model for HSCR. The second is to prepare an efficient assay that can reliably show changes in phenotype(s) when treatments are put in. Finally, wo of the mutant enhancer genes, *msn** and *GAP1**, will be used in a rescue test. Overall, the goal is to be able to use *Drosophila RET* mutants as a model for HSCR treatment testing and to understand the complex interactions for artificial neuronal migration.

Results

The LMx mutants were tested for rate of survival in the early neurodevelopmental stage as the first step in characterizing any defects. The homozygous variants were used due to their lethality. Early neurodevelopmental stage was decided as from 1st instar stage to 3rd instar stage which was 24 hours to 72 hours after embryo laying, respectively. Therefore, 1st instar homozygous LMx larvae were tested on an agar plate with red-dyed food and counted for percent alive at 24 hours later (48-hour designation) and 24 hours after that (72-hour designation)—considered as the behavioral assay. Wild type (WT) was included as a negative control for comparing results to normal survival rates. RET Deficiency (Df) was included because its *RET* gene is completely deleted among some surrounding DNA and can thus act as the positive control for what a *dRET* phenotype should probably be similar to.
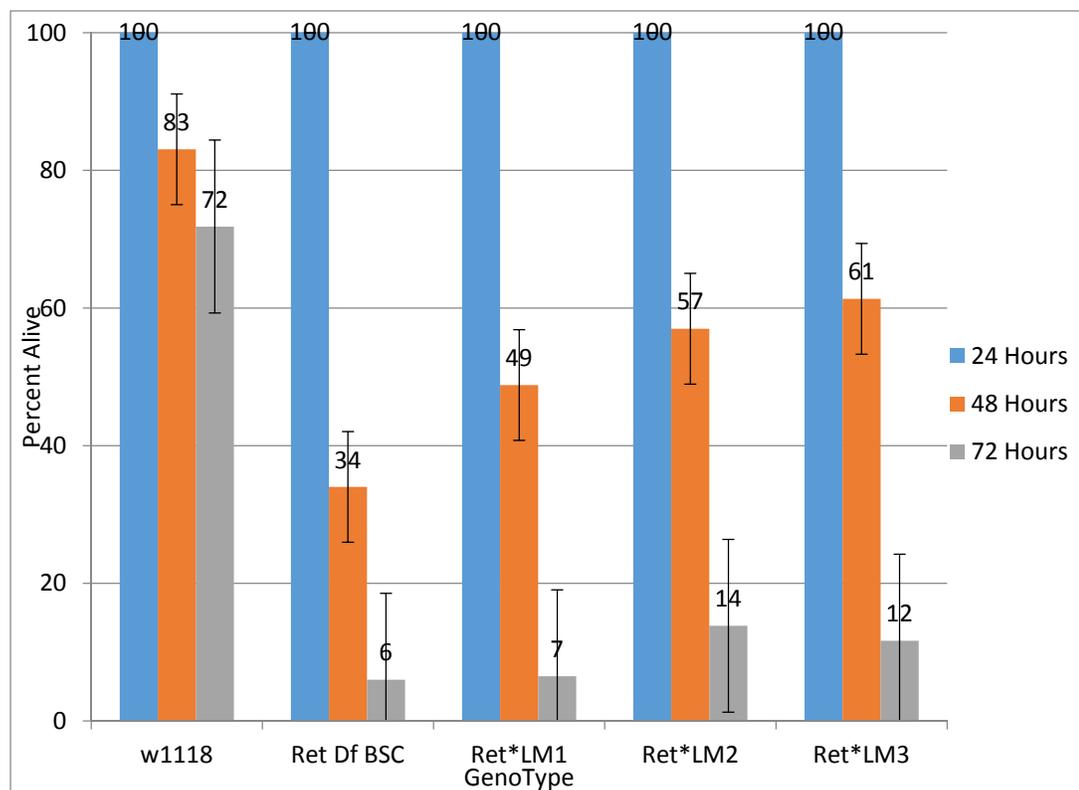
Figure 1. Percent alive of homozygous LMx and Df larvae with food at 24, 48, and 72 hours. The behavioral assay was used to measure the percentage of homozygous larval mutants alive at 24, 48, and 72 hours after embryo laying. Overall, Df and LMx larvae had significantly less survival at each time point relative to WT. LMx and Df did not survive into normal 3rd instar stage compared to WT. We tested 180 WT, 150 Df, 178 LM1, 157 LM2, and 160 LM3 larvae.

The behavioral assay found that LMx and Df had higher mortality rates than WT (Fig. 1). At the 48-hour point, LMx had 22-34% less larvae alive and Df had 49% less larvae alive relative to WT. At 72 hours, LMx had 58-65% and Df had 66% less larvae alive than WT. A 95% confidence interval test on this data shows that it should be accurate by about 10% higher or lower. It was then presumed highly likely that the RET protein in fruit flies is crucial for overall survival relative to normal levels of survival shown by the WT rates and thus the

first step towards the HSCR model. The similarity in Df and LMx survival rates helped support that all three LMx mutants were indeed *dRET* mutants since Df was already outright missing *RET* from its genome. However, we further questioned the mutation in LMx as a valid *dRET* mutant.
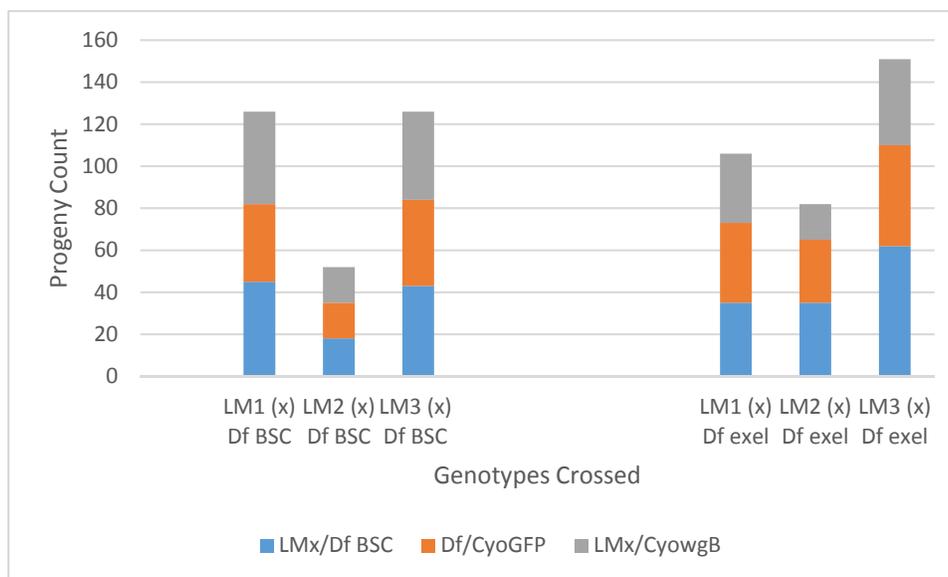
Figure 2. Progeny counts of adult-stage flies when LMx was crossed with Df BSC or exel. Two RET Df lines (BSC and exel balanced with Cyowgβ) were crossed with all three LMx lines (balanced with CyoGFP) to test the lethality of LMx/Df BSC or exel (functional homozygotes). Neither LMx/Df BSC nor exel showed lethality at adult stage since they made up about one-third of the total progeny. CyoGFP/CyowgB was lethal due to the Cyo balancer being lethal when homozygous.

We tested if progeny with an LMx allele and Df allele (LMx/Df) would act as functional homozygotes. Full lethality was expected like in the homozygous variants of each mutant with itself (e.g., LM1/LM1) at the time point of adult stage. To our massive surprise, LMx/Df made it to adulthood (Fig. 2). Not only that, they represented about one-third of the three possible progeny genotypes as if nothing was wrong. This indicates that two copies of (presumed) mutant

RET shown to already be lethal on their own, just like the RET Df homozygotes, were now not lethal when put together. These results prompted suspicion of an off-site mutation that is not all too uncommon in CRISPR DNA editing (Cho et al., 2014). DNA sequencing of the entire genome of all three LMx lines was not a viable option so the genome of the LMx lines were repaired for all but the *RET* mutation via homologous recombination. Three new, extra mutants were generated from each original LMx line.
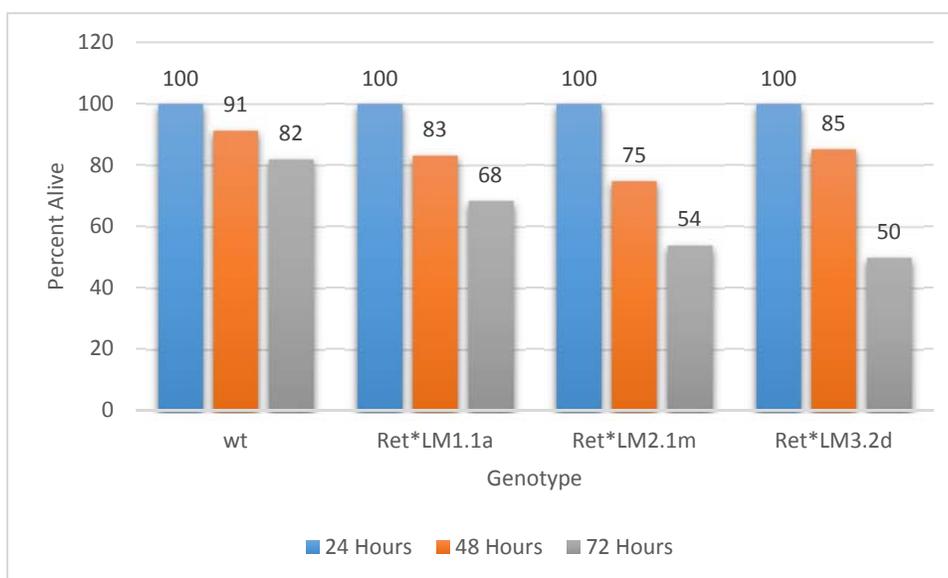

Figure 3. Percent alive of homozygous LMx.x larvae with food at 24, 48, and 72 hours. Each LMx.x line was its LMx counterpart but without any off-site mutations. Each were retested for survival to see if there was any change in survival. LMx.x generally showed lower survival rates than WT. LMx.x had higher survival than LMx likely due to an off-site mutation in the LMx lines. Variations between each LMx.x mutant are likely due to random chance. Some mutant larvae survived into the adult stage in every assay and thus the mutation is not fully homozygous lethal anymore. W1118 (wt) was measured again as a control and showed slightly higher or statistically similar results as in the previous assay (Fig. 1). N=150 (wt), 250 (1.1a), 100 (2.1m), and 150 (3.2d) larvae.

The behavioral assay was rerun with the three new LMx.x lines as a controlled experiment testing if there was an off-cite mutation in LMx. It was

highly suspected as such because adult homozygous flies were present in the population of each LMx.x stock. Indeed, the LMx.x mutant larvae had noticeably higher survival rates than LMx but not much less than WT (Fig. 3). LMx.x survived only 8-16% less than WT at 48 hours and 24-32% less at 72 hours. Relative to LMx, LMx.x survived 8-26% better at 48 hours and 26-41% better at 72 hours. There is still no doubt that the mutated *RET* in LMx.x is hindering overall survival; it is just not as severe.

At this point, since *dRET* is already a close homolog to *hRET*, survival rates of LMx and LMx.x could be used as a baseline control for treatment testing that significantly increase the mutants' survivability. However, we noticed behavioral phenotypes very similar to people with HSCR and so continued to investigate for more characteristics and answers to what the off-site mutation is really causing.

Foraging and Feeding Defects
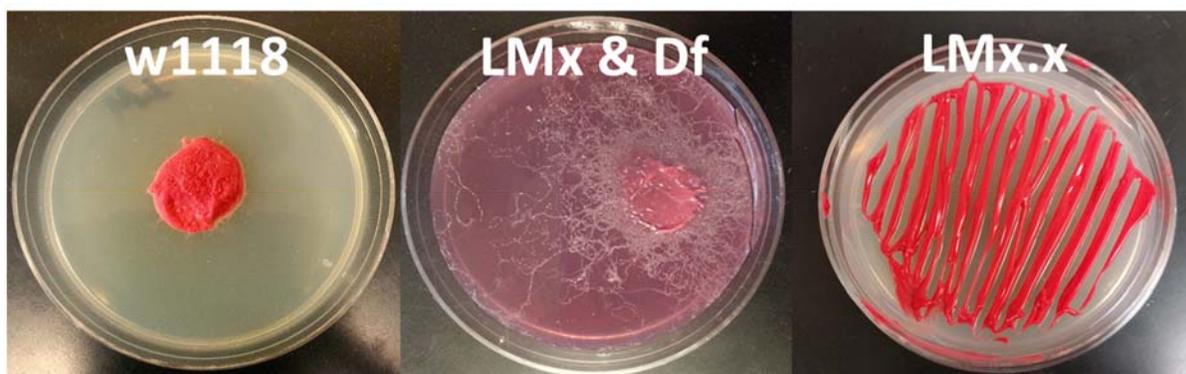
Figure 4. Foraging behavior of LMx and Df at 48 hours. Whiter tracks on agar plates represent the movement of larvae at 48 hours. WT appeared stationary due to having no tracks in the agar (left). LMx and Df showed larval tracks indicating a foraging behavior (mid). Foraging in LMx.x could not be measured due to the food in their behavioral assay plating scheme taking up the whole plate (right).

At first, we noticed tracks on the LMx behavioral assay that turned out to likely be a foraging behavior (Fig. 4). The LMx and Df agar plates contained the tracks or streaks on the agar indicating paths the larvae took when moving with track density around the food. Another observation was that the WT actually ate all their food by 48 hours and required an almost completely-covered plate of food added for the last 24 hours until 72 hours or it resulted in rampant digging into the agar gel. The LMx and Df plates had a just-noticeable amount of less food after each day likely due to very little eating and did not require any added food and showed no digging into the agar. The foraging, lowered food consumption, and lack of digging when no extra food was added by LMx relative to WT implied that the LMx had a feeding defect. LMx.x ate just about as much food as WT, which also explained LMx.x higher survival rates than their LMx counterpart (Fig. 1, 3). However, LMx.x lines were created months after LMx was tested; by the start of the LMx.x behavioral assay, a new plating scheme was being used for faster larvae counting that inadvertently cannot test for foraging well. We also did not realize this at the time due to being entranced by the closest HSCR phenotype in the mutants yet. A closer look at the mutants revealed the speculated feeding defect

The behavioral assay presented us with the complicated spectrum of feeding and growth defects in all 6 mutants (Fig. 5). One observation was that the LMx.x larval plates had some larvae growing as well as WT (Fig. 5A left arrow) and some permanently stalled at 1st instar size as the most severe of the spectrum (Fig. 5A right arrow). Within this LMx.x spectrum though lay more variation. The smaller ones (close to or at 1st instar size) would be barely alive at
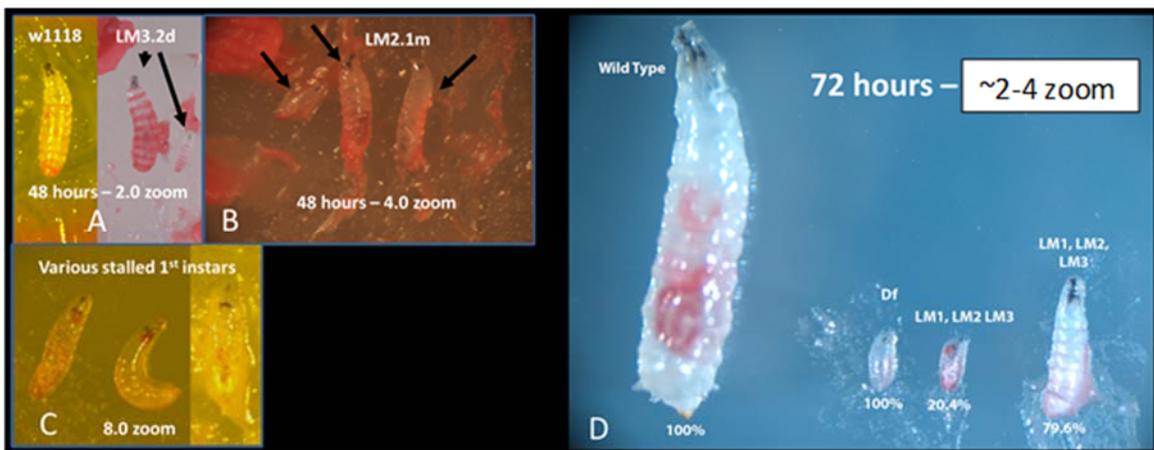
Figure 5. The spectrum of the feeding and growth defect in larval mutants and rescue assay. The pictures were captured at specific zoom levels for better comparison during observation. (A) LM3.2d growth was similar to WT (left arrow) or stalled at 1st instar (right arrow). (B) LM2.1m had some larvae that could not eat at all (left), some passed red food into the gut (mid), and some could not pass much food but still grew past 1st instar (right). (C) General dRET* spectrum of multiple genotypes showing stalled 1st instars and an impassability of food in the mouth (right two) or some passage but no growth (right). (D) LMx differences in growth, incidence of common sizes, and feeding phenotypes seen by 72 hours. (E) GAP1* failed to rescue; there was no apparent change in size difference between stalled 1st instar and large larvae, relative to LMx size differences shown in Fig. 5D.

48 hours and mostly dead by 72 hours; some could fill their gut with food yet still not grow much (Fig. 5B mid arrow) while some did not have any significant amount of food in their gut but still grew just a little past the stalled 1st

instar size (Fig. 5B right arrow). The stalled 1st instar larvae showed a very clear

phenotype of not being able to move any red food past their pharynx or

esophagus like a human HSCR colon (Fig. 5C left, mid), or some could get

specks into the gut but not actually grow from it (Fig. 5C right).

We observed that the LMx lines were a more severe version of the LMx.x

feeding phenotype but not different in phenotype. The spectrum of growth in LMx

did not keep up with WT's growth and instead reached its max size of a late 1st to

early 2nd instar (Fig. 5D right). Rarely did the LMx larvae have visible red food in

their gut and instead were almost always empty yet still led to decent growth (Fig.

5D right). Stalled 1st instars from LMx with and without food stuck in the mouth

were present though only the "with-food" was shown (Fig. 5D 2nd from right).

Also, the feeding and growth defects of both sets of mutants did not differ within

a set; i.e., LM1, LM2, and LM3 had no visible difference from each other. These

observations painted a clear picture that two larvae with the same mutant *dRET*

2nd chromosome could differ in the severity but not of the type of defect: feeding

and growth. This started the idea that the off-site mutation was oddly specific to

the LMx.x feeding and growth defect and thus *dRET* because it only made those

two defects worse while not resulting in a single other (observable) difference.

On top of that, there was no difference between the three LMx lines; this implied

that the CRISPR assay that accidentally generated them when targeting *RET*

made the same off-site mutations. Chances of this having been random were

nearly impossible, and thus made us extremely curious of LMx's off-site

mutation's relevance to mutant *RET* phenotypes. Given that the stalled 1st instars

were always the same size rather than a hard-to-define larval size or gut food content, and were the pinnacle of the *dRET* mutant phenotype for its similarity to HSCR, we tested them for incidence as another baseline variable.

The 8-20% incidence of stalled 1st instars was very similar between the LMx and LMx.x lines (Fig. 6). There was no significant difference between the size of the stalled 1st instars between any of the mutants or Df measured (Fig. 5). LM2.1m showed the first distinction between other *dRET* mutants though; almost all of its stalled 1st instars would be dead at 48 hours while the rest of the LMx.x and LMx mutants' stalled 1st instars survived at least into 72 hours regularly before starving to death. The Df line stalled at 100% incidence and had absolutely no ability to eat any food—including not even getting it in the mouth at least (Fig. 5C left, mid). This test provided the largest difference in phenotype between Df and the mutants, and the first mutant, LM2, with a consistent difference in a phenotype. Using the only available quick treatment, we attempted to rescue the mutants with the two enhancer mutant genes.
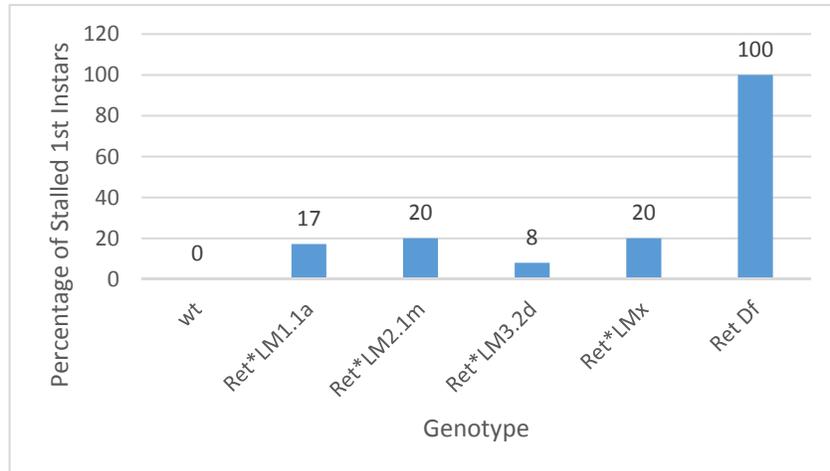
Figure 6. Percentage of stalled 1st instars of LMx, LMx.x, and Df. As a definitive marker of the most severe *dRET* feeding defect, the incidence of stalled 1st instars was measured. WT did not have any as expected. LMx and LMx.x had generally the same incidence of stalled 1st instars. Df had a 100% incidence. LM2.1m's stalled 1st instars were seen usually dead by 48 hours while all other mutants' stalled 1st instars usually survived into 72 hours.

Rescue attempt with *msn** and *GAP1**
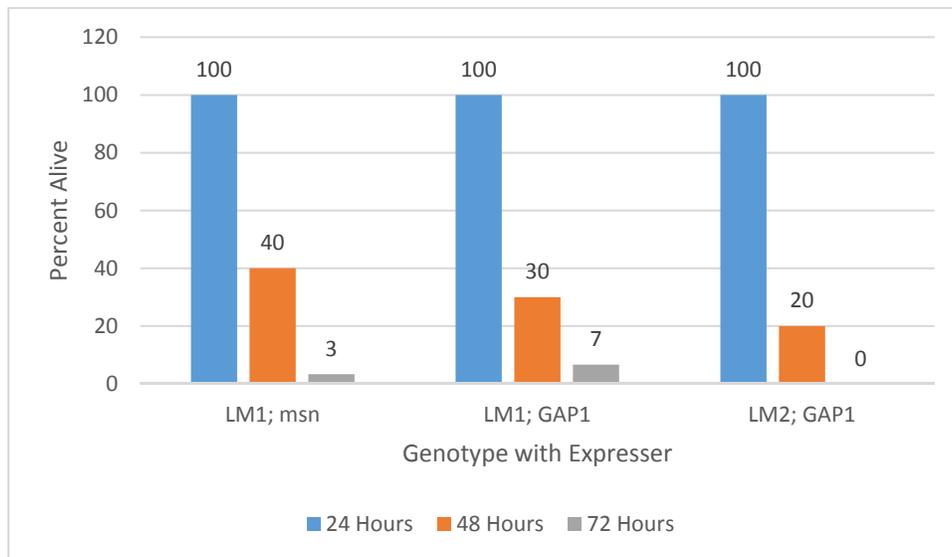
Figure 7. Survival of LMx with GAP1* or msn* on 3rd chromosome. A behavioral assay was used to measure survival rates of LMx with a copy of either enhancer on its 3rd chromosome as a potential rescue method. Unfortunately *GAP1** or *msn** failed to increase baseline survival rates of LMx (Fig. 1) and caused possible worsening or no change in survival rate. Actual genotypes were Ret*LMx; gene-or-wt/wt where "gene" was GAP1* or msn*. None survived into adults and thus there were no full rescues either. N=30 for each of the three genotypes tested.

The rescue attempt ultimately failed. Homozygous LMx mutants were given one copy of either enhancer onto their 3rd chromosome. This allowed us to keep both copies of the mutated *dRET* in LMx since *dRET* is on their 2nd chromosome. LM1; msn had a 7% decrease in survival at 48 hours and 4% decrease at 72 hours (Fig. 7). LM1; GAP1 had a 19% drop in survival at 48 hours and a 0% change at 72 hours. LM2; GAP2 had a 37% drop in survival at 48 hours and a 14% drop in survival at 72 hours. There was an obvious trend of worse survival rates but likely not out of the realm of random chance. None of the larvae survived into adult hood or a larger growth stage such as 3rd instar (Fig. 8). The difference in size between the stalled 1st instars and largest larvae present at 72 hours in LMx was characterized in Fig. 5D. Therefore, when we saw no significant increase in the difference between the largest larvae and the stalled 1st instars on the rescue plates, we determined there was no partial rescue either. As a final test, the stalled 1st instar incidence was measured (Fig. 9). The incidence ranged from 7% lower to 13% higher of observed stalled 1st instars. Since there was no significant trend of rescue, both *msn** and *GAP1** were deemed as failing to rescue the survival, feeding, or growth defect of LMx mutants.
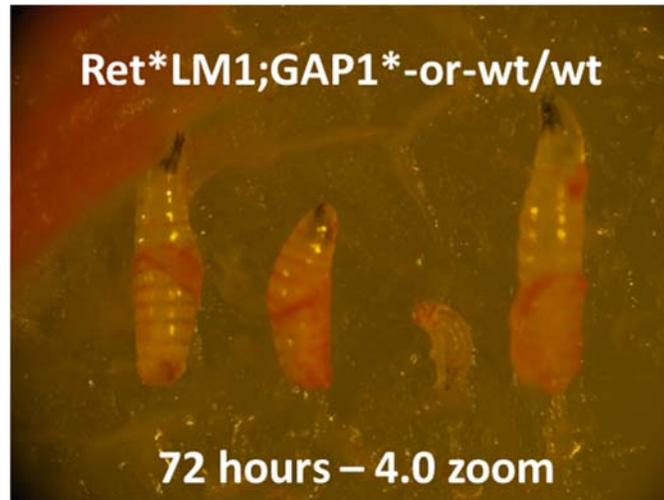
Figure 8. Size comparison of LM1; GAP1* larvae from enhancer test. A picture was taken of the general range of sizes from all the behavioral assays of the enhancer test at 72 hours. The larvae are dead in the picture at 72 hours as most were. Stalled 1st instar larvae were still observed (2nd from right). Size differences between the stalled 1st instar and the largest larvae (right) are not significantly different from size differences of the same two types in LMx (Fig. 5D 2nd from right and right). Therefore, there was no partial or full rescue observable.
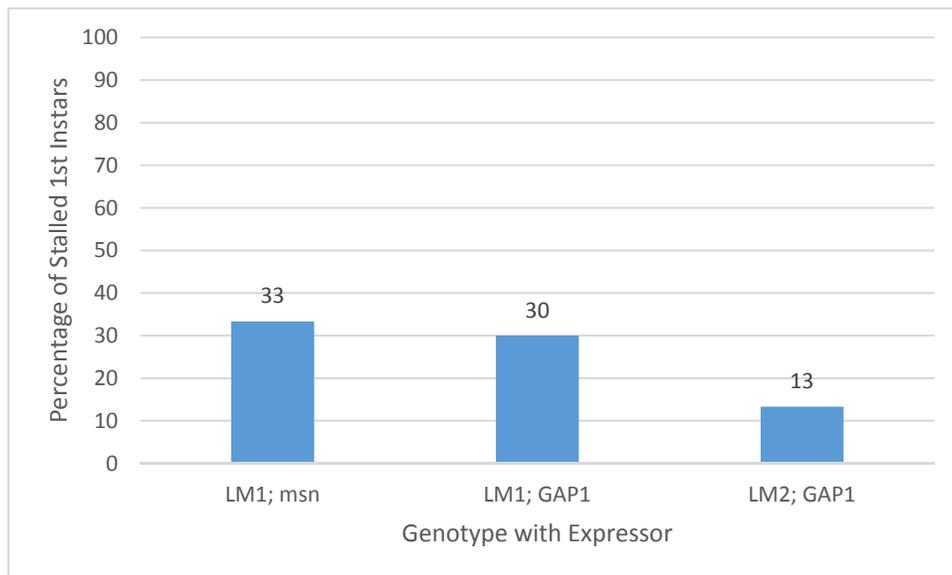
Figure 9. Incidence of stalled 1st instars in LMx with an enhancer gene. One of the enhancer genes were put on the 3rd chromosome of the LMx mutants as possible rescues and measured for incidence of stalled 1st instars as the expected variable change. The measurement showed no significant decrease in stalled 1st instars incidence (Fig. 6) and thus was branded as failing to rescue LMx. N=30 larvae for each of the three genotypes tested.

Neuronal Stains

The previous characterization was of overall, large-scale phenotypes like

feeding and growth that require the work of far more than just RET protein for full

function. Therefore, we wanted to see if the *dRET* mutants had neurons that

failed to migrate or survive in some way to bridge the cellular aspect of *dRET* to
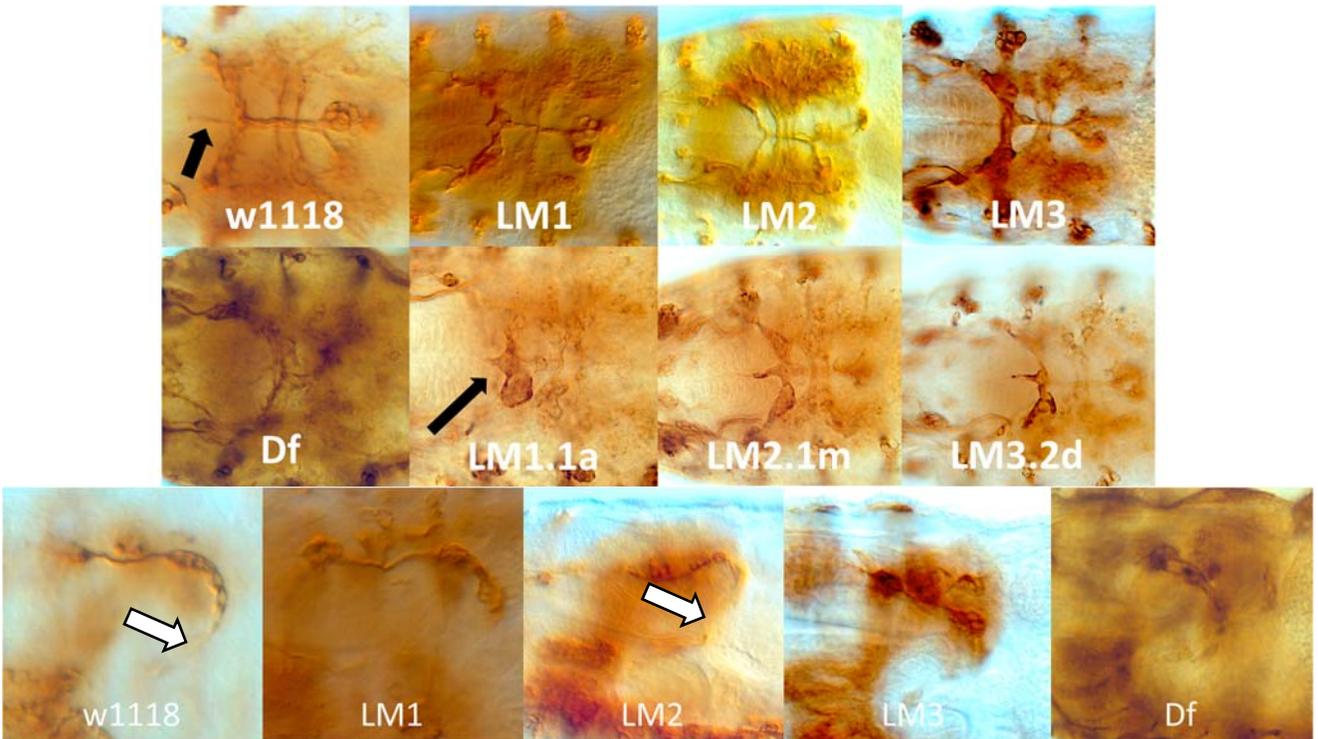
Figure 10. 22c10 stains of WT, Df, LMx, and LMx.x stage 17 embryonic stromatogastric nervous system (SNS). The neurons of WT, Df, LMx, LMx.x were stained to identify if the mutants had dysfunctional neural migration. The mutants and Df had a shortened or highly-disfigured frontal nerve/ganglia (black arrow) relative to WT. The mutants and Df also show no staining in the end of the esophagus (white arrow) unlike WT. Stains and images acquired by Logan Myers.

hRET and HSCR. The late-stage embryos of mutants, Df, and WT were stained

with an antibody for the stomatogastric nervous system (SNS), considered the

equivalent of the human enteric nervous system. The stains showed that WT had

a frontal nerve growing out at a certain length (Fig. 10 w1118) and LMx, LMx.x,

and Df had disfigured frontal nerves (Fig. 10 LM1.1a black arrow). This is

important because the frontal nerve functions in contraction of the pharynx, and the stalled 1st instars usually could not push the food past their pharynx or esophagus (Fig. 5C) (Spiess, Schoofs, & Heinzel, 2008). Other stains also showed that WT had neurons migrate down the esophagus (the neurons curving downwards, bottom row, w1118) and generally made it to the section the white arrow is pointing to. LMx neurons did not migrate that far as it seems. However, Logan Myers finds pictures where none of these neuronal migration problems are seen. That might explain why the *dRET* phenotype we had observed was always a spectrum of various aspects.

Discussion

The problems of Df and LM mutants

There are many aspects of the data that are extremely confusing and inconclusive, but do not necessarily ruin the possibility of a fly HSCR model. One of the main reasons for using Df throughout the experiment was its expected role as a negative control; i.e. a tool to know what phenotype(s) a *dRET* mutant would probably have. The problem though was that the Df flies had more than just their *RET* gene cut out. Therefore, it was not a perfect negative control, and instead had to be used as a general guideline but not definitive of what was a *dRET* mutant phenotype and what was not. Ironically, we never can have a perfect negative control because the perfect *dRET* mutant is what we tried to make. Therefore, this heavily complicates the data by questioning why the LMx and LMx.x (LM mutants) failed to perfectly match the Df phenotypes. For example,

when survival rates showed that LMx.x did worse than WT but far better than Df (Fig. 1, 3). Was LMx.x failing as a *dRET* mutant or a HSCR model, or is Df too deficient in unrelated genes?

One of the biggest mysteries is that Df had a 100% incidence of stalled 1st instars and absolutely none had any food in their mouth or gut. This is essentially the perfect behavioral defect of a HSCR model because Df's complete loss of *RET* implies that they lack proper innervation in their gut for peristalsis similar to humans and colons. However, it could be that the outside deletions of Df resulted in that by making something else dysfunctional that effects growth and feeding like the ability to open the mouth, pheromone detection, appetite level, and so on. For comparison, human HSCR patients have more variation than Df due to only having to cut some of their colon out as the severity varies person to person (McKeown, Stamp, Hao, & Young, 2013). Referring to the methods, LM1 and LM3's nonsense mutation early in *RET* sequence might not have been completely degraded after finishing translation and is instead floating around having made some effect. LM2 is only missing a cysteine for proper folding that should get degraded, yet LM2.2m's stalled 1st instar seem to die over 24 hours sooner than all other mutants. This is why we could not perfectly conclude that LMx and LMx.x can be used as HSCR models because of the massive range in phenotype severity and its difference from the almost-negative control (Df). Logan Myers will eventually figure out where these proteins are localizing if not being degraded after transcription and what they are doing though via cell culture.

Phenotypic similarities with HSCR

However, there is no doubt about the similarity of a fly with mutant *RET* and a human with mutant *RET*. HSCR patients cannot move waste material past their colon. Similarly, the *dRET* mutants had about a 20% incidence of larvae stalled at 1st instar presumably because they could not move food through their gut as Fig. 5C shows. However, almost all of LMx had no food in their gut but still grew to at least early 2nd instar. This could be evidence that ability to eat does not correlate with growth but seems like a violation of the conservation of mass. The explanation is most likely that the larval gut still retained full absorption rates of WT. Therefore, when the larvae that can eat and push just a little food but not enough to clump like in Fig. 5C then their gut might absorb it so fast that it seems like they cannot eat at all. That is why this feeding defect should be seen as a sign of approval as an HSCR model but not one that can be quantified as well. If a treatment mechanism caused the incidence of stalled 1st instar to drop to 0% throughout multiple trials though it would likely have grounds for not being random chance.

At first, when the LMx off-cite mutation was realized, it showed to be dropping survival rates of the truer *dRET* mutants (LMx.x) and thus useless in the pursuit of an HSCR model (Fig. 3). However, the incidence of stalled 1st instars is not different between LMx and LMx.x (Fig. 6). If incidence of stalled 1st instars is considered a core phenotype of the fly HSCR model, then one could say the off-

site mutation is unrelated to *RET* and therefore HSCR. However, if it was truly an

unrelated mutation, then we probably should have seen a different phenotype in

them, or solely lower survival rates. That was not the case. LMx larval

observations had foraging and almost no larvae with food in the gut (Fig. 4-5);

i.e., more severe versions of LMx.x feeding defects where only some of the

larvae had no food in the gut. We did not find any vastly different phenotypes in

LMx unrelated to LMx.x nor only lower survival. It seems reasonable to conclude

that CRISPR made an off-target cut to some gene that is related to feeding

and/or gut peristalsis. And given that it seems to have made the same off-cite

mutation in LM1, LM2, and LM3 despite being made during different events

means that there is some specificity. It is a lot of speculation but it would be good

to use some clever genetics and molecular biology to find that off-cite mutation

and find out its relation to *RET*, which is exactly what the lab plans to do.

　　Addressing the failure of the rescue assay is critical to defending *dRET* as

a mechanism of HSCR treatment testing. It was expected to enhance RET's

downstream signaling somehow and function as an enhancer of the phenotype

for rescue, but it did not work. One problem was that only one copy (one allele) of

each enhancer gene was able to be used. Combine that with a 100% lethal,

homozygous LMx with an off-site mutation that may or may not be affected by

the enhancer gene and it is very likely that one copy simply could not rescue

such a severe phenotype in these scenarios. There is also the fact that the

enhancer is really the lack of the corresponding gene (*msn* or *GAP1*). If *msn* or

GAP1 provide a necessary function for something else that affects the very

general concept of feeding and survival, then it might simply make the flies worse regardless of being an enhancer of RET phenotypes. That would explain the general trend of the worsening of LMx during the rescue assays (Fig. 7-9).

In the end, the similarity to Df, the similarity to HSCR patients' gut phenotype, and the neuronal stains showing likely failure of neuronal migration or degeneration in two key places related to gut food movement paint a pretty promising result that the *Drosophila RET* mutants can be used as an HSCR model. For the emphasis on treatment testing, if larvae survive into 96 hours, stop foraging, have significantly less stalled 1st instar, grow into 3rd instar stage, or if embryonic neuronal stains showing migration and innervation similar to wild type then it would probably be a compound or mechanism that is a step in the right direction for treating human dysfunctional RET.

Methods

Behavioral & mortality assay. The homozygous larvae, at approximately 24 hours old (1st instar), were placed in a white or grape agar petri dish containing red-dyed yeast. The red-dyed yeast was made with 1g yeast paste and .003g carmine. The red yeast was placed in the middle of the petri dish in a 1cm^2 square with 20-50 larvae placed around the edges of said food square. Food square changed to strips of food throughout plate for hungrier larvae like WT and LMx.x (Fig. 4 right). Data is then gathered at 48 hours of larval age (1 day after plating), and 72 hours (2 days after plating) on percent alive, incidence of stalled 1st instar, pictures of larvae for growth and feeding defects, and foraging behavior.

Rescue assay. Larvae were made and picked with the genotype of Ret*LMx-statGFP/Ret*LMx; gene-or-WT/WT with gene being msn* or GAP1* and used in the behavioral assay with no difference.

Progeny count. Virgin female adult flies of one genotype were mated with adult male flies of the other genotype in a tube of fly food and the offspring counted by genotype (determined by phenotypic markers such as curly wings for Cyo and eye color for GFP and Df exel).

22c10 SNS stains. Embryos of interest were stained with 22c10 antibodies, put in 70% glycerol, bathed in RapidClear for a few minutes or a day, and pictures were taken afterwards with a microscope.

CRISPR *dRET* Mutants and Df. LM1 had a nonsense mutation in *dRET* exon 3. LM2 had a missing cysteine residue in exon 5 for causing possible miss-folding. LM3 had a nonsense mutation in exon 5. LMx.x have these same mutations in *dRET*. Df exel and BSC obtained from Bloomington Stock Company.

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