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Phenotyping of the *fld-1* mutant previously isolated in a *paqr-2* suppressor screen

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Abstract

The Adiponectin receptors AdipoR1 and AdipoR2 in mammals are studied primarily because of their suspected anti-diabetic functions. The *C. elegans* AdipoR2 homolog *paqr-2* has previously been described and shows three distinct phenotypes: a withered tail-tip, intolerance to temperatures under 15 °C and intolerance to glucose. The *paqr-2* mutant shows an increase of saturated fatty acids (SFA) and this increase could be linked to both the cold intolerance and glucose intolerance. When grown on culture plates containing glucose the amount of SFA in the membrane phospholipids of the *paqr-2* mutant increases even more, as though this mutant cannot regulate the levels of SFAs. Recently, several mutations in the *fld-1* gene were discovered to suppress *paqr-2* phenotypes, including the glucose sensitivity. In other words, the *fld-1* mutations rescue the *paqr-2* mutant and act as suppressors of the glucose intolerance in that mutant. The aim of the present project was to try to discover phenotypes in the *fld-1* single mutant using a variety of assays. Unexpectedly, no obvious differences between wild type worms and the *fld-1* mutant worms were found. Specifically, *fld-1* mutants had normal life span, brood size, defecation rates, pharyngeal pumping rates, locomotion rate, temperature tolerance and tolerance to a mild detergent. The function of *fld-1* remains therefore unclear.

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Introduction

The *paqr-2* gene

The *paqr-2* gene was first studied because of its similarity to the mammalian homologs *ADIPOR1* and *ADIPOR2*. Currently our interest in this gene relates to a possible connection between its role in membrane fluidity regulation and its role in type II diabetes [1, 2]. The PAQR-2 protein has seven transmembrane domains but is unrelated to G protein coupled receptor. The PAQR-2 protein acts together with another protein called IGLR-2 on the plasma membrane, as shown in previous studies [3]. The mutant is glucose intolerant: glucose causes growth arrest of *paqr-2* worms at concentrations as low as 20 mM [3]. *paqr-2* mutant worms contain a abnormally high levels of saturated fatty acids (SFAs) among their phospholipids and are only sensitive to D-glucose; L-glucose has no effect.

The *fld-1* gene

The *fld-1* mutation in *C. elegans* is a suppressor of *paqr-2* mutant phenotypes. The Pilon lab has found seven independent alleles of this gene in a *paqr-2* suppressor screen (unpublished). The *fld-1* mutation is the most potent suppressor of glucose intolerance in the *paqr-2* mutant, but also suppresses the withered tail tip and cold intolerance. However, the precise function of *fld-1* is unknown. The aim of this project was therefore to identify phenotypes for the *fld-1* mutant.

One aspect of the *paqr-2;fld-1* double mutant that guided some of our experiments is that it has a higher ratio of unsaturated fatty acids (UFAs) in membranes that lead to their improved fluidity. This suggests that the *fld-1* mutations may make the worm more sensitive to heat or to diets rich in unsaturated fatty acids. Some of the experiments will therefore specifically investigate this general hypothesis in the hope that they may lead us closer to the function of the gene and the mechanism by which it suppresses the glucose intolerance of the *paqr-2* mutant. The mechanism of glucose toxicity in diabetes is not well understood, but we hope that elucidating how *fld-1* protects *paqr-2* mutants from glucose toxicity will provide useful new insights into this phenomenon [4].

The *fld-1* gene encodes a protein that shows similarities to the TLCD1 and TLCD2 proteins that are members of a protein family characterized by the presence of a TLC (TRAM, LAG1, CLN8) domain in their sequence (**Fig. 1.**) [5]. Some proteins with this domain have important roles in lipid synthesis [6]. Another, TLCD1, also known as calfacilitin in mouse and human, is not itself an ion channel but enhances Ca^{2+} influx in $Ca_V1.2$ channels, The protein may be needed for regulating Ca^{2+} signalling [6]. The TRAM1 is located in the ER and linked to the export of misfolded polypetides that are targeted for degradation [7]. When TRAM1 is mutated there is an upregulation of UPR (unfolded protein response) in the ER. TRAM1 is normally upregulated during ER stress and if the cells lack the TRAM1 protein the ER shows defects in the removal of misfolded polypeptides, leading to an upregulation of the UPR [7]. The insulin producing β-cells have also shown to be dependent on this mechanism for coping with their high secretory demand. The secreting β-cells have shown to be affected by UPR-dependent cell death [8]. The CLN8 protein is also located in the ER and

regulates the activity of ceramide synthases. Mutations in CLN8 are associated with neurological diseases such as progressive epilepsy with mental retardation (EPMR) (aka Northern epilepsy syndrome) [9-12]. Ceramide synthases also contain a TLC domain and are responsible sphinogolipid biosynthesis. Furthermore, ceramides are important signalling molecule and consist of a sphingoid base attached to a fatty acid via an amide bond [5]. Note that the FLD-1 protein that is the subject of the present study is mostly closely related to TLCD1 (aka calfacilitin) and TLCD2 in terms of sequence homology.

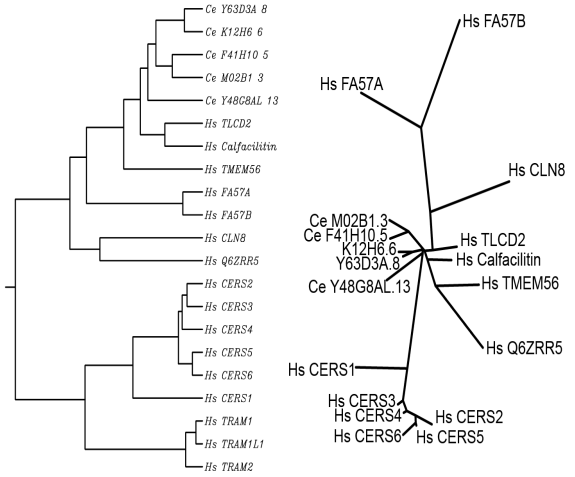

Fig. 1. Phylogenetic tree borrowed from Marc Pilon. The figure shows a rooted and un-rooted phylogenetic tree of the 16 human and 5 TLC domain proteins.

The relationship between membrane fluidity and type II diabetes

There is a possible link between cell membrane fluidity and diabetes. It is crucial for cells that their membrane properties are maintained at near-optimum so that interactions and processes that are necessary can be preformed. Many cellular processes that can be linked to type II diabetes, such as glucose transport and insulin secretion by pancreatic beta cells, depend crucially on optimal membrane properties [13]. The lipids therefore have an important role in membrane function, protein signalling and protection of the cell [8]. For example, if cell membrane is too rigid due to a higher amount of SFAs, then both the affinity of the receptor for insulin and their activity upon insulin binding decrease [14]. More generally, membrane homeostasis is linked to diet (dietary fats become incorporated into cellular membranes) and thus could contribute to the risk of developing type II diabetes [13, 15].

Caenorhabditis elegans as a model organism

C. elegans is used as a model in all experiments presented here. *C. elegans* is a great model for studying different genes that can be linked to their mammalian homologs [16]. It is a 1 mm long non-parasitic nematode that exists in two sexes: self-reproducing hermaphrodites or males. In the laboratory, *C. elegans* typically lives on a diet of *Escherichia coli* and its life cycle is 3.5 days when cultivated at 20 °C. In other words a newly laid egg requires 3.5 days to grow into an adult that starts laying its own eggs. The organism has a fixed numbers of cells (959 in hermaphrodites) and is especially useful for genetic experiments and observations because it is so easy to propagate in vast numbers [17]. The life cycle can be divided into the larval stages L1-L4 followed by the fertile adult stage with an average life span of about 2 weeks [18].

Materials and Methods

Worms

The worms used were the wild type animals variety Bristol, strain N2, as well as mutant strains carrying the *paqr-2(tm3410), fld-1(et48), fld-1(et49) and* the double mutants *paqr-2(tm3410);fld-1(et48)* and *paqr-2(tm3410);fld-1(et49)*. The *paqr-2(tm3410)* mutant will henceforth be simply referred to as *paqr-2*. Synchronized worms were always used, and produced either by bleaching pregnant mothers and allowing their progeny to hatch overnight before spotting L1s on test plates [16], by allowing adult worms to lay eggs for 1,5-2 hours and then removing them, or by picking L4s. All of the assays were done trice or more. Worms were maintained on NGM plates as described unless other stated [16].

Locomotion assay

One-day old adults were used. 9 cm NGM-plates were marked with genotype and a starting point and the worms were allowed to move freely for an hour, while being monitored and their movements traced on the plate with a felt pen. After one hour the worms were removed and the plates scanned. ImageJ was used to measure the lengths of the traces [19].

Brood size assay

Individual L4s were put on 10 separate plates for all six studied genotypes then kept on the plates for 24 hours, by which time they began laying eggs. The worms were moved daily until they stopped laying eggs, and the progeny of each worm were counted 3 days after removal of the mother, as per a published protocol [20]. The brood size assay was also performed in a different way: the progeny was counted and removed as soon as they were visible on the plate; this allowed the adult worm to live and lay eggs undisturbed by being picked and transferred daily (which poses a risk of injury).

Life span assay

Five L4s of the *fld-1(et48)* strain and five worms of the N2 strain were put on 20 NGM plates for a total 100 worms of each genotype. The worms were moved to new plates every second day until the end of the fertile period, after which time they were moved only once a week. The worms were scored daily for viability: a worm was declared dead if it didn't move when gently poked with a worm pick on the nose and tail [20].

Pharyngeal pumping rate

L4s were picked to new plates the day prior to the assay, which was performed on young adults. Ten worms for each of the six genotypes were scored by counting the number of pharyngeal pumping events during 20 seconds periods [21].

Defecation rate

Synchronized one day adults were used for the defecation rate assay by picking L4s onto new plates the day prior to the assay. Five worms of each of the six genotypes were used. The time between defecation was measured five times for each worm and a mean calculated for each worm [21].

Detergent in NGM

Different concentrations of the supplement was diluted in the agar before pouring the plates, as follows: 0%, 0,01%, 0,05%, 0,1% and 0,2% Nonidet P-40 (NP-40) [22]. L1s obtained by bleaching gravid mothers were spotted on the plates and allowed to grow for 3 days until they were one-day old adults. The worms were placed on 2

% agarose pads in a drop of 100 mM levamisole and covered with a coverslip as described [23]. The Zeiss Axiophot microscope was used and the images taken with the Axiovision program. 20 worms of each genotype were then measured in ImageJ.

Temperature tolerance

The worms were synchronized by letting adult worms lay eggs for 1,5-2 hours and then removed. After three days, the one-day adults were put in 37°C for 1, 2, 4, 6 and 8 hours. 20 individuals were scored per plate and observed for motion; unmoving worms scored as dead [24].

Results

The *fld-1* mutant did not show any significant difference from the control worms in any of the experiments performed during this project.

The *fld-1* mutant has a normal tolerance to Nonidet P-40

As expected the *paqr-2* mutant shows an increase in health when cultivated in the presence of small amounts of the mild detergent Nonidet P-40 (NP-40) [22]. The *fld-1* mutant does not show any different reaction to the detergent then the wild type worm, at least as determined by the length of the worms 72 hours of cultivation of L1s (**Fig.2A**).

The *fld-1* mutant has a normal tolerance to heat shock

The six genotypes were put in 37 C° for different amounts of time then scored for survival. The worms survived as long as 8 hours, and there was no genotype that differed from the wild type in this assay (**Fig. 2B**).

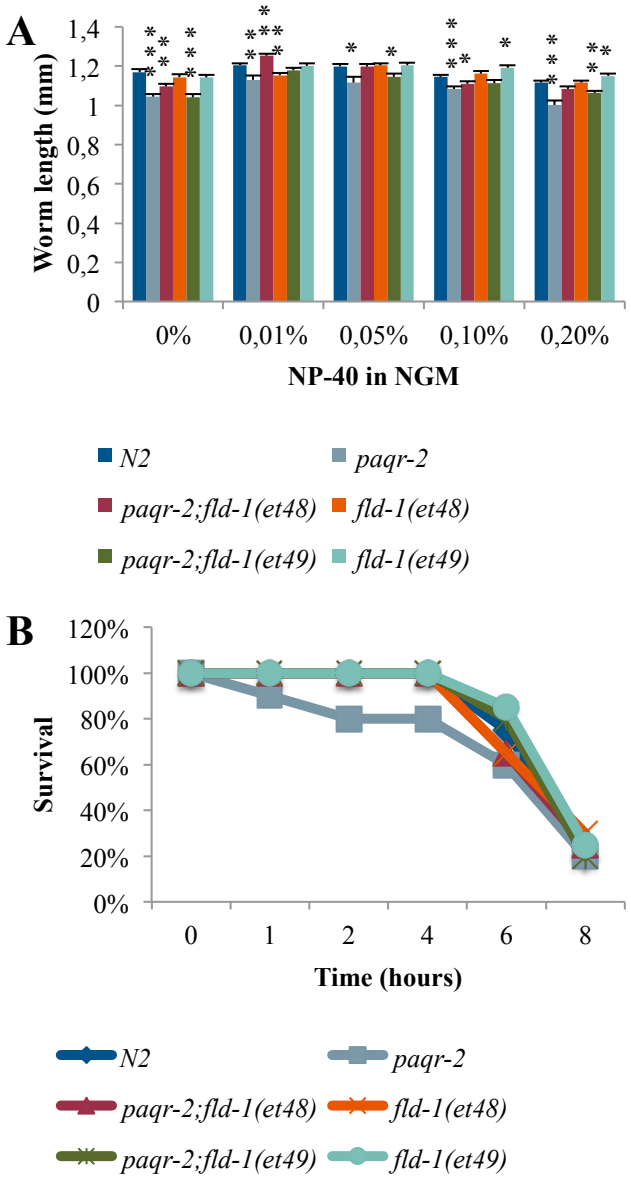

Fig.2. (A) Detergent may show a little increase in health for the different genotypes. There is a little but not obvious difference in worm length in the wild type and mutants. The different experiment shows small differences between the *fld-1* mutants and wild type but they're not consistent. **(B) Temperature tolerance assay.** The six genotypes N2, *paqr-2, paqr-2;fld-1(et48), paqr-2;fld-1(et49), fld-1(et48)* and *fld-1(et49)* are placed in 37 C° during an amount of time.

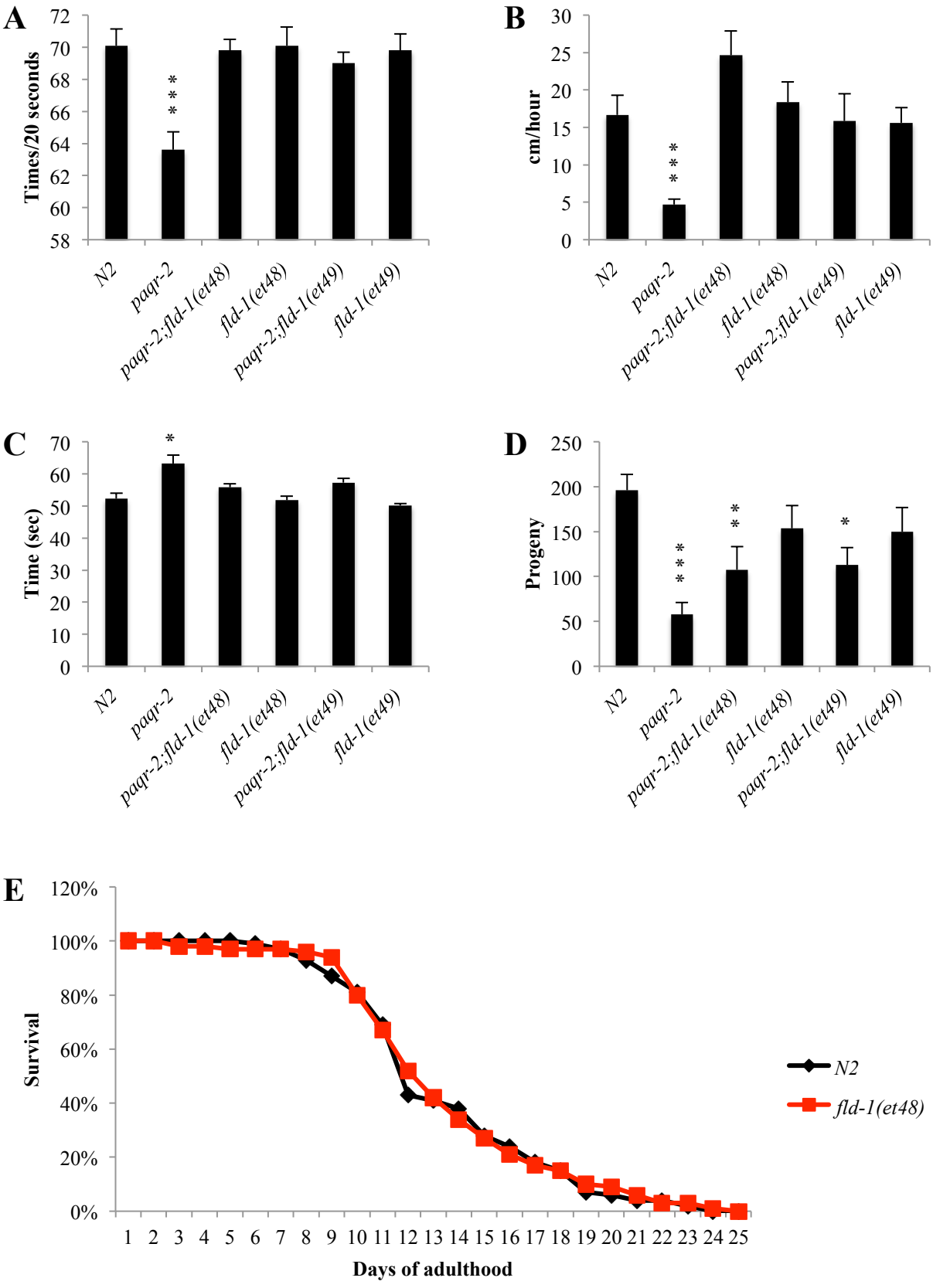

Fig. 3. The *fld-1* mutant shows no significant difference from the wild type. The mutants expect *paqr-2*; show no difference in pharyngeal pumping rate **(A)**, no difference in the locomotion assay **(B)**, defecation rate **(C)**, brood size **(D)** or life span **(E)**. The results show a particularly healthy genotype.

The *fld-1* mutants has a normal pharyngeal pumping rate

The *fld-1* mutants showed no significant difference from the control genotypes in this assay, neither did the double mutants. Only the *paqr-2* mutant showed a decrease in pumping rate (**Fig. 3A**).

The *fld-1* mutant has normal locomotion

The worms were allowed to move freely for an hour, tracing the tracks with a felt pen. The plates were scanned and the results were put in a graph. None of the genotypes showed a significant difference from the wild type expect the *paqr-2* mutant, which moved poorly as expected (**Fig 3B**).

The *fld*-1 mutant has a normal defecation rate

The *fld-1* mutant did not show any difference in defecation rate. Only *paqr-2* showed an increase in time between the defecations (**Fig. 3C**).

The *fld-1* mutants has a normal brood size

In this assay the N2 have given less progeny than it should compared to other studies, this could have to do with the health of the worms. The *fld-1* single mutants did not show a significant difference in the amount of progeny compare to the control (**Fig. 3D**).

The *fld-1* mutant has a normal life span

The life span assay was performed on *fld-1(et48)* mutants and N2 as a control. There was no significant difference between the two genotypes (**Fig. 3E**).

Discussion

The only known phenotype of the *fld-1* mutant is that it acts as a *paqr-2* suppressor. The goal with this project was to find out which other phenotypes it may have. Some of the hypotheses that we had initially were that mutations in the *fld-1* gene may promote membrane fluidity or impair uptake of dietary lipids. The first hypothesis predicts that the *fld-1* mutant could have difficulties surviving at high temperatures or in the presence of detergent, since both treatments could increase membrane fluidity in this mutant presumed to already have high fluidity. This was however not the case.

The mutant did not show any reduced locomotion which indicates that there is nothing wrong with the musculature or motor neurons [25]. Defecation rate should be once every 45 seconds, however, the worms that were looked at in this assay had a longer period (>50s); these results could mean that the worms that were used (even wild-type N2) were not completely healthy or their environment not optimal [26]. However, the mutants did not show any differences in the defecation rate compared to the wild type strain. This could mean that there is no major defect in the defecation motor program (DMP) that consist of three parts of muscle**,** posterior muscle body contraction, anterior muscle body contraction and the expulsion muscle contraction, which also indicate that there is nothing wrong with the motor neurons regulating this process [26]. The pharyngeal pumping rate assay also showed reason to believe that there is nothing wrong with the motor system or the rhythmic muscle contractions of the worm. Brood size was no different from the N2 strain; the mutant worms had no problem with the egg laying nor the production of eggs. This could be linked to the motor system and the reproduction system, another result that shows a normal motor system

and a healthy worm [27]. Furthermore, the *fld-1(et48)* mutant lives as long as the control, which also provides evidence that this is a particularly healthy mutant. A decrease in life span would have indicated sickness not visible to the naked eye, but in this case there is no difference between the two genotypes, which means that the gene is not essential.

As mentioned earlier, the temperature tolerance assay was important because of the hypothesis about the increased fluidity in the membrane compared to wild type. The mutant worm should in that case not tolerate the heat as good as the N2 or the *paqr-2* mutant because of its smaller amount of saturated fatty acids (SFA), the regulation between the SFA and the unsaturated fatty acids is crucial for adaption in rise or reduction of temperature [22]. Somewhat disappointingly, the *fld-1* mutants did not show a lack of tolerance for the higher temperature, but instead tolerated the heat just as well as the other genotypes, including wild type. This could give us a clue about the membrane fluidity or the regulation of fatty acids in membrane fluidity. This mutant likely can regulate its membrane fluidity and maintain it at near optimal levels even under high, fluidizing temperatures. It therefore does not seem likely that the membrane gets more fluid when the *fld-1* mutant worms are exposed to environments that would normally increase the fluidity. This might mean that the mutation of this gene does not actually make the membrane more fluid by having a higher ratio of unsaturated fatty acids but instead regulate its amount of saturated fatty acids to make sure it does not get too rigid. This could explain why it is capable of surviving in 37 Cº as long as the wild type and also why it is not affected by fluidizing amounts of NP-40.

Others have shown that the *fld-1* gene affects the membrane lipid composition in a way that makes the membrane more fluid in the *paqr-2* mutant, and the unsaturated fatty acids ratio becomes higher in comparison to the saturated fatty acids or at least equal (unpublished). The cell signalling pathways that are involved in the lipid distribution of SFA and UFAs should therefore be relatively functional or even improved in the double mutant compared to the *paqr-2* single mutant.

The relationship between the *paqr-2* gene and the *fld-1* gene seems somewhat similar to that between two "selfish genes" recently described in Science [28]. A selfish gene is a gene that lives for its own benefit [28]. If it were a selfish element it would explain why it shows no other phenotype than supressing the *paqr-2* phenotypes. A gene that lacks no significant function does not exist for a long time, the fact that the *fld-1* gene exists even though it doesn't show any obvious function or purpose could indicate that it is a selfish gene. The *fld-1* gene could be toxic to the worm by moderating the membrane fluidity and making it more rigid and making the worm sick. The *paqr-2* gene along with the *iglr*-2 gene could then work as an antidote and counteract the effect of the *fld-1* gene. Perhaps the activity of the FLD-1 protein is toxic for the worms unless the PAQR-2 and IGLR-2 complex, acting as an "antidote" is also functional. The *paqr-2* gene should then in itself not be an essential gene but its purpose would be as an antidote to the toxic selfish gene *fld-1,* maybe by interacting and inhibiting a FLD-1 regulated pathway. If this is the

case as referred to the article written by Ben-David et al. the double mutant *paqr-2;fld-1* should show no phenotypes compared to the N2 worm. This is not strictly the case since the double mutant, though healthier then the *paqr-2* single mutant, still shows a decrease in health in several assays. Perhaps a related protein that is not as toxic as the FLD-1 protein but works along side it can be mutated and create a triple mutant that will supress more of the phenotypes and be as healthy as the N2 [28]. In any case, the *fld-1* gene exists in the worm, and when it is mutated it shows no other phenotype than supressing the phenotypes of the *paqr-2* mutant. It is a healthy worm that appears to be just as good as the wild type.

In conclusions, I did not find any new phenotypes for the *fld-1* mutants during this project, nor did I find out the function of this mysterious gene. One of the hypotheses was that the *fld-1* mutant would not tolerate higher temperatures as good as the wild type, but the assays this not confirm this expectation. There might be an interest for future experiment to investigate the "selfish gene" hypothesis more closely. There could also be a reason to do another life span with glucose plates instead of standard NGM plates to see if there is a difference in life span between the wild type and the mutant. An assay for tolerance to oleic acid could also be interesting because of the presumed higher membrane fluidity in the *fld-1* mutant.

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