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Lysosomal proteins GLO-1 and GLO-4 are involved in RNA interference in *C. elegans*

Benjamin Holmgren

Degree project in biology, Master of science (2 years), 2012

Examensarbete i biologi 45 hp till masterexamen, 2012

Biology Education Centre and Department of Medical Biochemistry and Microbiology, Uppsala University

Supervisor: Andrea Hinas

Summary

RNA interference is a sequence-specific gene silencing mechanism, acting in areas ranging from developmental control to virus defense. In recent studies, a high proportion of lysosomes to multivesicular bodies has been shown to decrease RNA interference in *Drosophila melanogaster* and mammalian cells, and a depletion of lysosomes enhances RNA interference. In order to determine whether this is true also in the nematode *Caenorhabditis elegans*, I have studied the role of the lysosome biogenesis proteins GLO-1 and GLO-4, in *C. elegans* RNA interference. I measured the efficiency of RNA interference-mediated silencing against two different target genes (*pos-1* and *dpy-13*) in GLO-1 and GLO-4 deficient animals, expecting their RNA interference to be stronger than normal because of their lysosome deficiency. I found that while animals deficient in GLO-1 had an enhanced RNA interference against *dpy-13*, they did not exhibit the same RNA interference enhancement with *pos-1* as target gene. Conversely, animals with a GLO-4 deficiency had an enhanced RNA interference against *pos-1*, but not *dpy-13*. While the enhanced RNA interference shows that GLO-1 and GLO-4 have a role in RNA interference, the fact that they respond differently to different target genes suggests that they play a more complex role than first thought.

Introduction

RNA interference (RNAi) is a sequence-specific gene silencing mechanism, with an RNA guide strand acting as sequence determinant (Ghildiyal and Zamore, 2009). RNAi silences gene expression either by degrading messenger RNA (mRNA) or by effecting translational inhibition. RNAi exists in slightly different shapes in most eukaryotes, acting in areas ranging from developmental control to virus defense. RNAi is mediated by small RNAs, which can be subdivided into different classes based on structure and origin. MicroRNAs (miRNAs) are endogenous 20-24 nucleotides long RNAs that are completely or partially complementary to the 3' Untranslated Region of the target mRNA. Silencing is then achieved by degrading the mRNA or by preventing translation. In contrast to the endogenous miRNAs, short interfering RNAs (siRNAs) are cleaved from long double-stranded RNA (dsRNA) and need to be completely complementary to the target mRNA.

Recent studies have implicated late endosomes and multivesicular bodies (MVB) in RNAi. The blocking of MVB maturation to lysosomes causes an increased RNAi efficiency, whereas blocking MVB formation decreases RNAi efficiency in *Drosophila melanogaster* and mammalian cells (Lee et al., 2009, Gibbings et al., 2009). It is therefore feasible to suggest a general rule that RNAi efficiency is affected by the relative amounts of MVBs and lysosomes, where a high proportion of lysosomes cause more of the RNA species to be degraded, rather than incorporated in a downstream RNAi pathway.

Systemic spread of RNAi between cells and tissues has been shown in plants and certain animals such as the nematode *Caenorhabditis elegans* (Jose and Hunter, 2007), but recently there have been observations of systemic spread also in mammals. miRNA has been found in exosomes secreted by human glioblastoma cells (Valadi et al., 2007), and a recent study shows that RNAi can spread via microvesicles from tumor-associated macrophages to breast cancer cells in humans (Yang et al., 2011). Exosomes from self-derived dendritic cells have also been tested as a therapeutic delivery mechanism for siRNA in mice (Alvarez-Erviti et al., 2011). Another recent study describes how miRNA from rice has been found in human blood, and this miRNA was even found to negatively regulate the expression of an endogenous liver protein (Zhang et al., 2011).

C. elegans is a commonly used model organism when studying RNAi and RNAi transport. While being a small and relatively simple organism, *C. elegans* is differentiated into several different tissues, which is important in order to investigate systemic RNAi. It is also trivial to induce RNAi in *C. elegans*, either by feeding them bacteria expressing dsRNA, soaking them in dsRNA or through microinjection of dsRNA (Zhuang and Hunter, 2011). RNAi feeding against genes with an easily scored phenotype in combination with forward genetics has enabled the identification of *C. elegans* strains that are either less sensitive to RNAi or that have an enhanced RNAi phenotype (Kennedy et al., 2004).

In this project, I have studied the role in RNAi of the Rab GTPase GLO-1, and GLO-1's guanine nucleotide exchange factor GLO-4, in *C. elegans*. The protein GLO-1 is required for the biogenesis of gut granules (lysosome-related gut organelles) in *C. elegans* (Hermann et al., 2005). If the efficiency of RNAi is dependent on the proportion of lysosomes to MVBs, animals deficient in gut granules, such as animals with a nonfunctional mutant *glo-1* gene, should have an enhanced RNAi phenotype. Previous results (Y. Zhao, unpublished results) indicated that *glo-1* animals do have an enhanced RNAi phenotype, but the strain used in that

experiment had not been outcrossed after mutagenesis, and only one gene was targeted with dsRNA.

I have compared the RNAi efficiency of *glo-1* animals to that of wild-type animals, using dsRNA targeting two genes, *dpy-13* and *pos-1*. Both of these produce an easily scored phenotype when silenced and are expressed in different tissues; *pos-1* in germ cells and *dpy-13* in epidermal cells. I have also performed the same tests on *glo-4* animals and *glo-1* animals carrying a gut-specific *glo-1* rescue construct. GLO-4 is a putative guanine nucleotide exchange factor to GLO-1, and *glo-4* mutant animals have similar gut granule phenotypes as *glo-1* mutant animals (Hermann et al., 2005). My results show that the outcrossed *glo-1* mutant strain has an enhanced RNAi phenotype against one target gene, in concordance with the preliminary result by Yani Zhao. However, the same strain is not more sensitive to RNAi against a germ line expressed gene. Moreover and in contrast to expected results, *glo-4* mutant animals are more sensitive to RNAi against the germ line cell expressed gene, but show no higher sensitivity to RNAi against the epidermal target. In addition, the rescue construct, which restores gut granule levels in *glo-1* mutant animals, fails to restore wild-type RNAi sensitivity. In conclusion, while these results do not support the original hypothesis, *glo-1* and *glo-4* still appear to have some function in RNAi. More work is required to elucidate exactly what their function is.

Results

Outcrossing *glo-1* to reduce effect of secondary mutations

A previous result with *dpy-13* dsRNA feeding indicated that *glo-1* mutants have an enhanced RNAi phenotype (Y. Zhao, personal communication). However, the *glo-1* mutant strain in use was created by treating wild-type animals with the mutagen Ethyl methanesulfonate (EMS), and had not yet been outcrossed with a wild-type strain. Mutant strains that are generated through random mutagenesis carry many mutations (Shaham, 2007). It can therefore be difficult to be certain that a phenotype in such a strain derives from the studied gene and is not the effect of mutations in one or many other genes. In order to reduce the risk that the observed enhanced *dpy-13* RNAi phenotype observed in the *glo-1* mutant strain is caused by a secondary mutation, the *glo-1* mutant strain was outcrossed twice to the reference wild-type strain N2. The *glo-1* gene is located on the X chromosome. *C. elegans* has an XO sex-determination system, where hermaphrodites carry two X chromosomes and males are hemizygous for X. One consequence of this is that the X chromosome is not subject to homologous recombination in males, which complicates crosses with genes on the X chromosome. Therefore, all crosses required one generation of hermaphrodite worms. The crossing scheme is shown in Fig. 1. Worms homozygous for the *glo-1* mutation are deficient in uptake of LysoTracker Red, which selectively stains acidic lysosomes such as gut granules in wild-type worms (Fig. 2A, (Hermann et al., 2005)). Therefore, I used LysoTracker Red to distinguish homozygous *glo-1* mutants from heterozygous or wild-type animals throughout the outcrossing. Two separate strains were isolated from this cross. Both strains were confirmed by sequencing to be homozygous for the *glo-1* mutation.

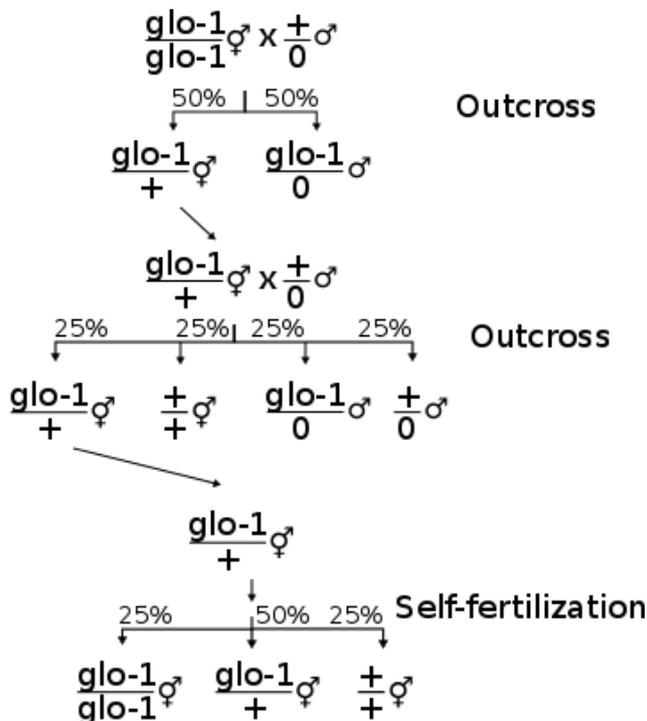


Figure 1. Crossing scheme used to outcross the *glo-1* strain zu391 with the wild-type strain N2. *glo-1* is located on the X chromosome, of which *C. elegans* males are hemizygous. Therefore, *glo-1* is only properly outcrossed in hermaphrodites, where chromosomal crossover of the X chromosome can occur.

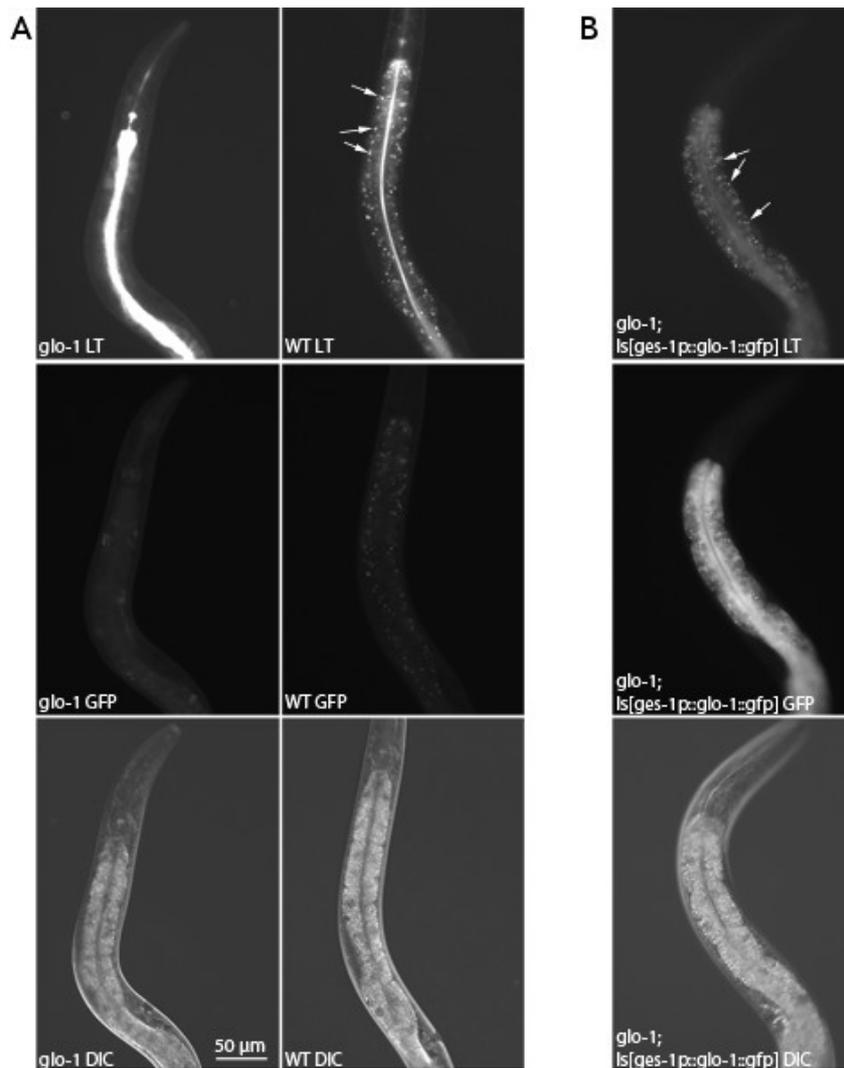


Figure 2. LysoTracker Red selectively stains gut granules. Microscope images were used to genotype progeny from crosses. A: *glo-1* mutants in comparison with wild-type animals. Top row: LysoTracker Red visualizes gut granules (arrows), which are missing in *glo-1* mutants. The lumen is brightly stained by residual LysoTracker Red. Middle row: the GFP filter shows that gut granules are slightly autofluorescent. Bottom row: Differential Interference Contrast images. B: *glo-1::gfp* construct rescues gut granule formation in *glo-1* animals. The distribution of *glo-1::gfp* can be seen with the GFP filter (middle row).

glo-1* mutant animals are more sensitive to RNAi against *dpy-13*, but not against *pos-1

To see whether the outcrossed *glo-1* mutant strain still showed the enhanced RNAi phenotype, *glo-1* animals were grown on plates with a lawn of bacteria expressing *dpy-13* dsRNA. *dpy-13* is an epidermally expressed gene, the silencing of which yields short and stout (“dumpy”) animals (von Mende et al., 1988). After three days, plates were scored for animals at the late larval L4 stage exhibiting the dumpy phenotype. A significantly higher number of *glo-1* animals developed dumpiness compared to a wild-type strain, indicating that the outcrossed *glo-1* animals were more susceptible to RNAi against the epidermally expressed *dpy-13* gene (Fig. 3A).

In order to determine whether the enhanced RNAi phenotype of *glo-1* shown against an epidermally expressed gene could also be seen against genes in other tissues, *glo-1* animals

were grown on plates with a lawn of bacteria expressing *pos-1* dsRNA. *pos-1* is a gene expressed in the germ line cells and *pos-1* silencing increases embryonal and larval lethality in the offspring (Tabara et al., 1999). After three days, the total amount of animals that had survived until the L4 stage or later was scored. Surprisingly, the enhanced RNAi phenotype observed when targeting *dpy-13* could not be seen when targeting *pos-1*. No significant difference in brood size could be seen between *glo-1* animals and wild-type, meaning that RNAi sensitivity against *pos-1* was unaffected (Fig. 3B). *glo-1* animals are therefore more sensitive to RNAi against some genes, such as *dpy-13*, while having wild-type sensitivity for RNAi against other genes, such as *pos-1*.

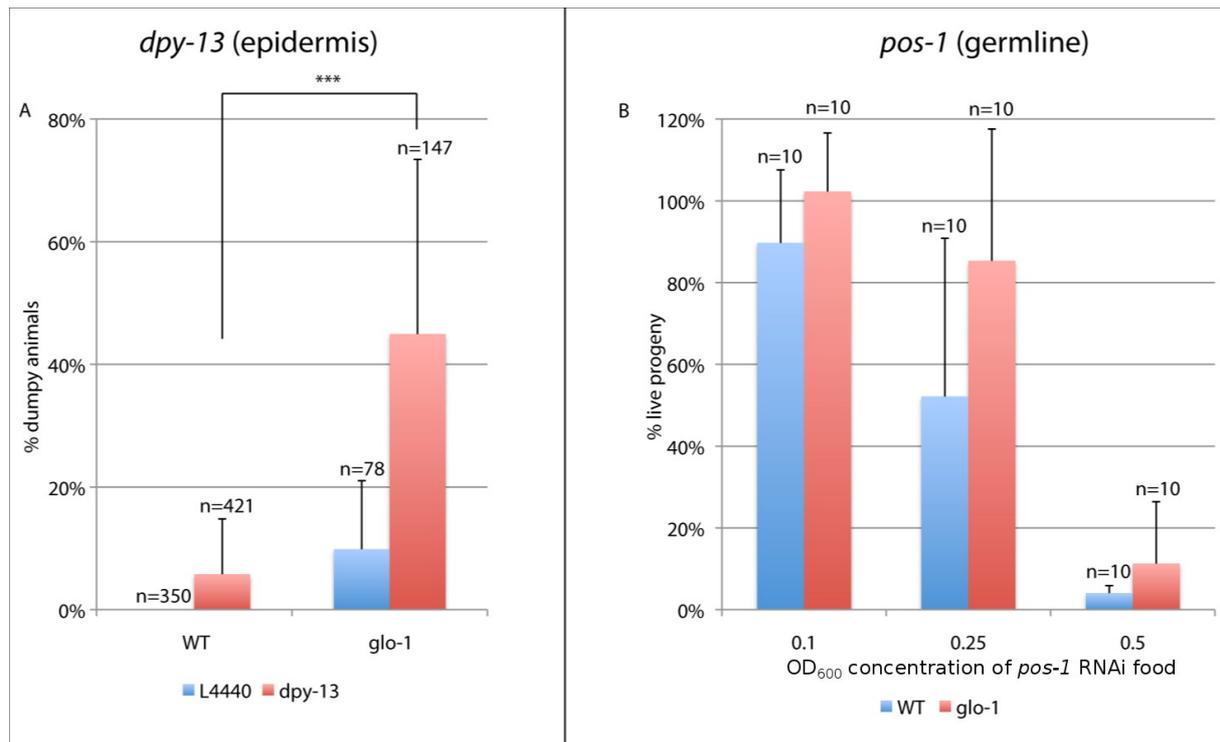


Figure 3. *glo-1* mutant animals are more sensitive to RNAi against *dpy-13*, but not against *pos-1*. RNAi feeding was performed on *glo-1* animals, using both *dpy-13* and *pos-1* dsRNA. A: Percentage of animals scored as having the "dumpy" phenotype following feeding with *dpy-13* dsRNA. *glo-1* mutants are significantly more affected by *dpy-13* RNAi ($p=0.000942582$) than wild-type (WT) animals are. B: Brood size of *glo-1* animals fed *pos-1* dsRNA, in percentage of brood size when fed vector control food. Three different dilutions of RNAi food were used. In contrast with *dpy-13* dsRNA feeding, *pos-1* dsRNA did not elicit a stronger RNAi phenotype in *glo-1* mutants than it did in wild-type animals. p =probability that there is no actual difference between populations, determined using two-tailed Student's t -test. A p -value less than 0.05 is considered significant. n =number of animals scored. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Error bars are standard deviation.

glo-4* mutants are not more susceptible to *dpy-13* RNAi feeding, but more sensitive to *pos-1

GLO-4 is a likely Guanidine Exchange Factor to the Rab-like GTPase GLO-1, and *glo-4* mutants are phenotypically similar to *glo-1* mutants in regards to gut granule loss (Hermann et al., 2005). I was therefore interested to test whether *glo-4* mutants, as *glo-1* mutants, show an enhanced RNAi phenotype when fed *dpy-13* dsRNA. However, in contrast to *glo-1* animals, *glo-4* animals were not more sensitive to *dpy-13* RNAi than wild-type animals (Fig. 4A). I decided to test whether *glo-4* would also have a wild-type RNAi sensitivity to the germ line expressed *pos-1* gene. Interestingly, there was a significantly smaller brood size from *glo-4*

animals when compared to wild-type animals, meaning that the effect of the RNAi against *pos-1* was stronger in *glo-4* animals (Fig. 4B). Some worm strains in use have been shown to carry a certain deletion mutation in the *mut-16* promoter, which renders them insensitive to RNA interference in somatic cells (Gabel et al., 2009). Such a background mutation could mask an enhanced RNAi phenotype and thus explain the discrepancy between the *dpy-13* and *pos-1* RNAi. Therefore, to determine whether the *glo-4* mutant strain carries the *mut-16* promoter deletion, whole-worm Polymerase Chain Reaction (PCR) was performed with primers flanking the potential deletion site. The *glo-4* strain in use produced PCR fragments of the same size as wild-type worms, indicating that the *glo-4* strain in use does not carry the *mut-16* deletion (Fig. 5). The *glo-1* strain was also found to have the wild-type *mut-16* size. While *glo-1* is sensitive to *dpy-13* RNAi and resistant to *pos-1* RNAi, *glo-4* paradoxically acts the opposite way; being more sensitive to *pos-1*, but not *dpy-13*. This is surprising, as they otherwise have identical phenotypes in other areas such as gut granule loss and embryonal lethality.

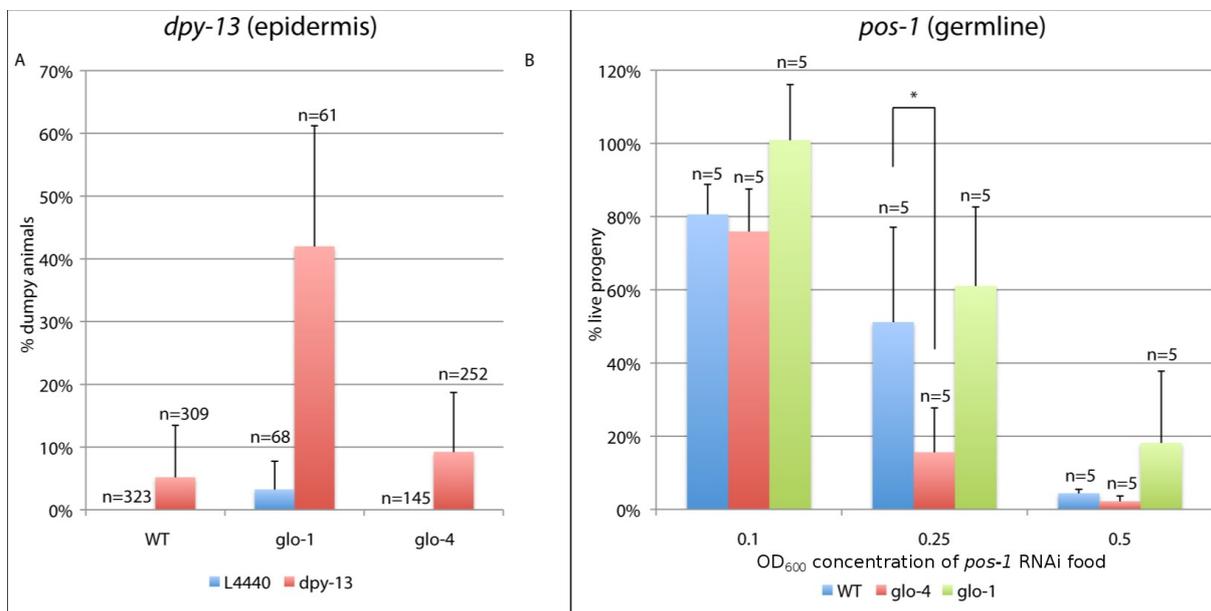


Figure 4. *glo-4* mutants are not more susceptible to *dpy-13* RNAi feeding, but more sensitive to *pos-1*. RNAi feeding was performed on *glo-4* mutant animal, using both *dpy-13* and *pos-1* dsRNA. A: Percentage of worms scored as "dumpy" following *dpy-13* dsRNA feeding. *glo-4* animals are no more sensitive to *dpy-13* dsRNA than wild-type (WT) animals. B: Efficiency of feed-mediated *pos-1* RNAi, at three different dilutions of *pos-1* bacteria, measured in brood size normalized to negative control brood size. *glo-1* mutants are significantly more affected than wild-type animals at 0.25 *pos-1* dilution ($p=0.033920135$). p =probability that there is no actual difference between populations, determined using two-tailed Student's t-test. A p -value less than 0.05 is considered significant. n =number of animals scored. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Error bars are standard deviation.

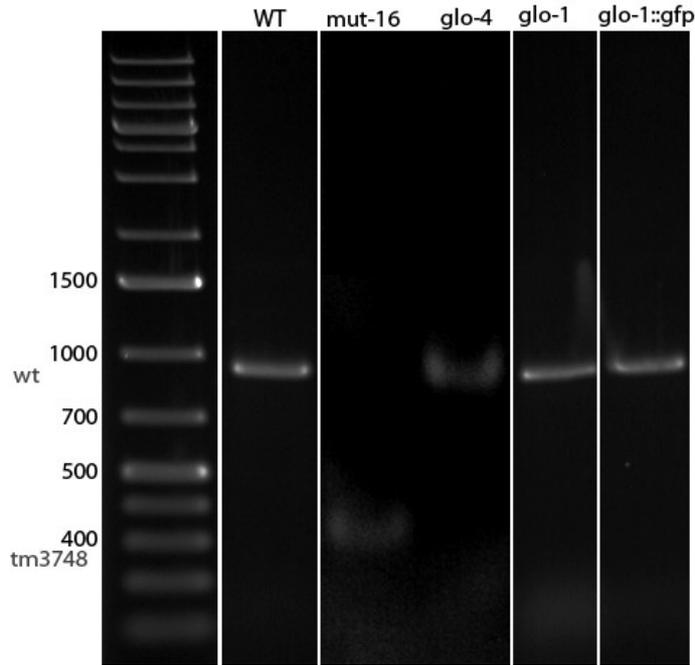


Figure 5. All strains used in the study have wild-type copies of the *mut-16* promoter. Whole-worm PCR was performed with *mut-16* primers, in order to determine whether any of them carried a common secondary deletion.. *glo-1* (zu391), *glo-1::gfp* (hjIs9), *glo-4* (ok623) and wild type (N2) strains used all carried the wild-type *mut-16* gene.

An intestine-expressed *glo-1::gfp* fusion rescues the gut granule loss phenotype of the *glo-1* mutant

While *glo-1* expression occurs predominantly in the gut, *glo-1* is also expressed in other tissues, such as neurons (Grill et al., 2007). In order to determine whether the enhanced RNAi phenotype of *glo-1* is a result of decreased lysosomal activity in the gut, I attempted to restore *glo-1* levels specifically in the gut by crossing the *glo-1* mutant strain to a strain carrying a translational *glo-1::gfp* construct, driven by the gut-specific promoter *ges-1p* (Zhang et al., 2010). This genetic construct codes for a GLO-1 protein fused with a Green Fluorescent Protein (GFP) marker protein. This fusion protein is visible using fluorescent microscopy, and is thought to function as the native GLO-1 protein. Genetic crosses require different methods depending on whether the two markers, in this case *glo-1* and *glo-1::gfp*, are located on the same chromosome. In order to test whether the *glo-1::gfp* transgene is, like *glo-1*, located on the X chromosome, a genetic cross was performed between wild-type hermaphrodites and males derived from a *glo-1::gfp*/wild-type cross (Fig. 6). If *glo-1::gfp* were on the X chromosome, none of the male progeny from this cross would express *glo-1::gfp*. Otherwise, half of the male progeny would express *glo-1::gfp*. The progeny was examined by fluorescent microscopy, and roughly half of the male progeny were indeed GFP(+), meaning that the *glo-1::gfp* is not on the X chromosome.

After having established that *glo-1* and *glo-1::gfp* are located on different chromosomes, a strain carrying both markers was constructed according to the scheme in Fig. 5. To verify that the *ges-1p::glo-1::gfp* rescued the gut granule loss phenotype of the *glo-1* strain, the new *glo-1; ges-1p::glo-1::gfp* strain was stained with LysoTracker Red, which selectively stains acidic

lysosomes. The *glo-1; ges-1p::glo-1::gfp* strain did indeed show rescue of the gut granule loss phenotype of *glo-1*, indicating that the *glo-1::gfp* fusion protein is functional (Fig. 2B).

***glo-1::gfp* does not rescue the enhanced RNAi phenotype of *glo-1* against *dpy-13*, but confers sensitivity to *pos-1* RNAi**

I had thus seen that the *glo-1::gfp* construct can rescue the gut granule loss phenotype of *glo-1* animals. In order to determine whether the *glo-1::gfp* construct with a gut-specific promoter could also rescue the enhanced RNAi phenotype of *glo-1*, the *glo-1; ges-1p::glo-1::gfp* animals were subjected to *dpy-13* RNAi as described above. Surprisingly, the *glo-1::gfp* construct, when driven by a gut-specific promoter, could not restore RNAi sensitivity to wild-type levels. In fact, the *glo-1::gfp* construct instead unexpectedly appeared to increase RNAi sensitivity, rather than decreasing it (Fig. 7A). The RNAi efficiency of *glo-1; ges-1p::glo-1::gfp* against the germ line-expressed *pos-1* was also examined as previously with *glo-1*. Whereas *glo-1* animals had no increased sensitivity to *pos-1* RNAi, *glo-1; ges-1p::glo-1::gfp* animals had significantly smaller brood sizes than *glo-1* animals without the rescue construct (Fig. 7B). Seeing how *glo-1; ges-1p::glo-1::gfp* were more sensitive to RNAi than both wild-type animals and *glo-1* mutants, I tested the efficiency of RNAi against *dpy-13* of the strain carrying the *ges-1p::glo-1::gfp* construct, but with a functional *glo-1* gene. There was no increase in the number of dumpy animals when fed *dpy-13* dsRNA expressing bacteria when compared to wild-type worms (Fig. 7A). In fact, no dumpy animal could be seen at all among the *ges-1p::glo-1::gfp* animals. The fact that this strain is not more sensitive to RNAi suggests the possibility of a secondary mutation, masking the phenotype. One such candidate mutation is the previously mentioned *mut-16* deletion, however, whole-worm PCR with primers flanking the common *mut-16* deletion gave a PCR fragment of wild-type size (Fig. 5).

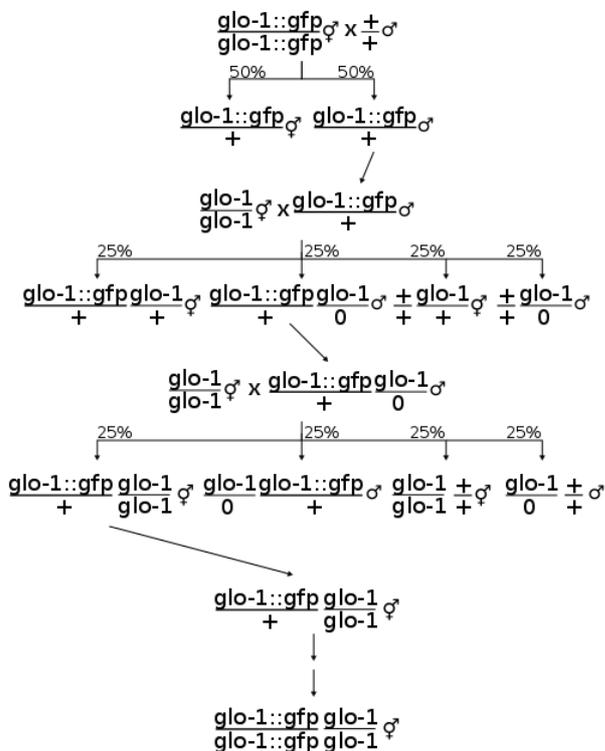


Figure 6. Crossing scheme used to cross *glo-1* into *glo-1::gfp* strain. This was used to produce a *glo-1::gfp* strain lacking a functional wild-type *glo-1* gene.

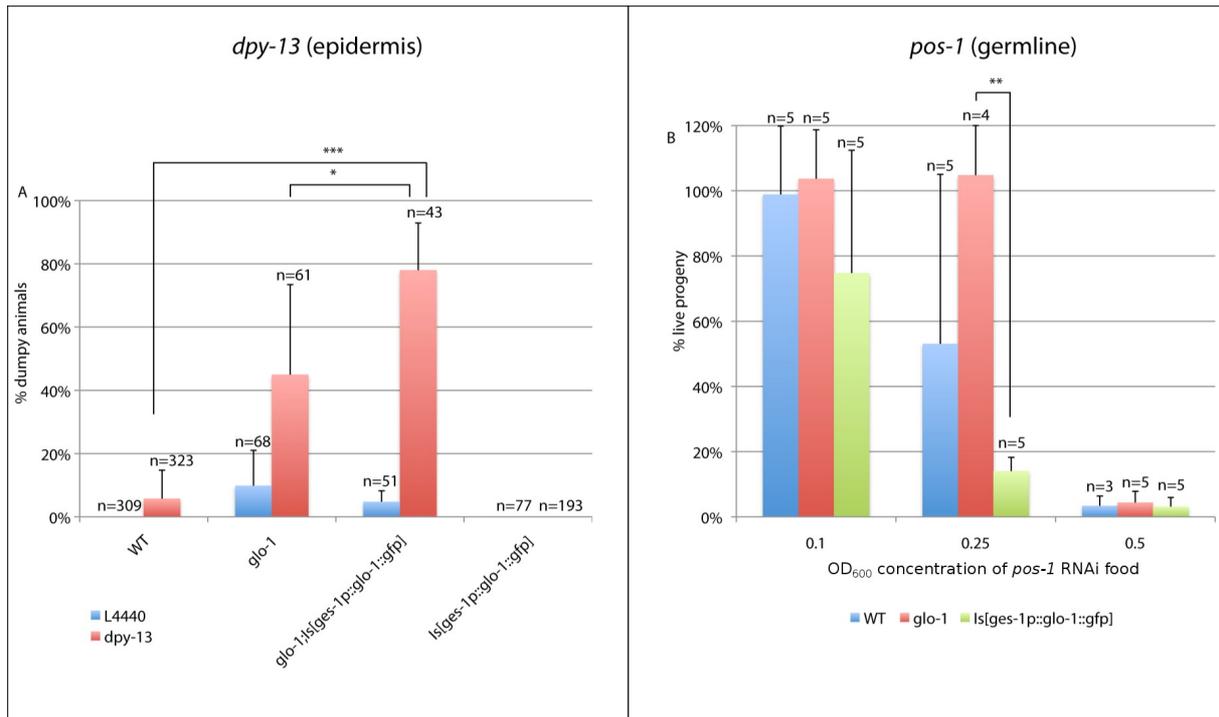


Figure 7. *glo-1::gfp* does not rescue the enhanced RNAi phenotype of *glo-1* against *dpy-13*, but confers sensitivity to *pos-1* RNAi. RNAi feeding was performed on *glo-1::gfp* transgenic animals, using *dpy.13* and *pos-1* dsRNA. A: Proportion of dumpy animals following *dpy-13* dsRNA feeding. *glo-1* animals are significantly more sensitive to *dpy-13* RNAi than wild-type (WT) after being crossed with a strain carrying a gut-expressed *glo-1::gfp* rescue construct ($p=0,004300803$ between *glo-1* and *glo-1;Is[glo-1::gfp]*, $p=1,59589*10^{-8}$ between wild-type and *glo-1;Is[glo-1::gfp]*). B: *pos-1* dsRNA feeding with different dilutions of *pos-1* dsRNA. RNAi efficiency is measured in total brood size normalized to brood size when fed negative control bacteria. As with *dpy-13* feeding, *glo-1;Is[glo-1::gfp]* is significantly more sensitive to *pos-1* dsRNA than *glo-1* mutant animals ($p=0,001157005$). p =probability that there is no actual difference between populations, determined using two-tailed Student's t-test. A p -value less than 0.05 is considered significant. n =number of animals scored. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Error bars are standard deviation.

Discussion

In this project, I have tested whether the efficiency of RNAi is enhanced in animals with defective gut granule biogenesis. The working hypothesis was that with fewer lysosomes, more dsRNA would be incorporated into the RNAi pathway rather than being degraded, giving an enhanced RNAi phenotype. I tested the efficiency of RNAi by feeding dsRNA to *glo-1* mutant animals, which are deficient in gut granule biogenesis. Two different endogenous genes were used as RNAi targets: *pos-1* and *dpy-13*. The *glo-1* mutant strain used in this study does indeed show an enhanced RNAi phenotype, but this effect is only apparent when fed dsRNA against the epidermally expressed *dpy-13* gene, while the efficiency of RNAi against the germ line gene *pos-1* remains at wild-type levels. The cause of this discrepancy has yet to be illuminated, but a few possible explanations come to mind. It is possible that the *glo-1* mutation actually confers enhanced RNAi against both targets, but this effect is masked by confounding factors against certain targets. Perhaps the strain of *glo-1* used carries a secondary mutation that persisted through the outcrossings, making the RNAi silencing against *dpy-13* more penetrant. The confounding effect of secondary mutations can be eliminated by, rather than using a *glo-1* strain generated through random mutagenesis, using wild-type animals in which *glo-1* has been silenced through RNAi, or by using a different *glo-1* mutant strain.

In addition, I have also compared the RNAi efficiency of *glo-1* mutants with that of *glo-4* mutants. GLO-1 is a small GTPase of the Rab family and GLO-4 is a Guanine nucleotide Exchange Factor thought to act in concert with *glo-1*. In my tests, *glo-4* mutant animals exhibit a different enhanced RNAi phenotype than *glo-1* mutant animals. Rather than being more sensitive to *dpy-13* dsRNA and no more sensitive to *pos-1* dsRNA than wild type, *glo-4* mutants are sensitive to dsRNA against *pos-1*, but not *dpy-13*. As with *glo-1*, it is impossible to rule out effects from background mutations from these results. A *mut-16* promoter deletion is one example of a mutation that confers resistance to RNAi only in somatic cells, leaving germ cells with a wild type RNAi efficiency. Many commonly used strains of *C. elegans* have been found to carry a deletion mutation in *mut-16*, rendering it inactive. The *glo-4* strain in use in this study was tested negative for this particular deletion, but it is still possible that some other background mutation would confer a similar phenotype. This possible explanation can be tested by repeating the experiments either on another *glo-4* mutant strain or a wild-type strain where *glo-4* has been silenced by RNAi.

Whereas the gut-expressed *glo-1::gfp* transgene does indeed rescue the gut granule loss phenotype of *glo-1* mutant animals, it merely exacerbates the enhanced RNAi phenotype against *dpy-13*, and introduced an enhanced RNAi phenotype against *pos-1*. Paradoxically, when I tested the RNAi sensitivity of the original *glo-1(+);glo-1::gfp* strain which was crossed with the *glo-1* mutant strain to produce a *glo-1;glo-1::gfp* strain, it did not show an enhanced response to dsRNA against *dpy-13*. While a commonly found deletion in the *mut-16* promoter was ruled out as a possible explanation for this, it is still possible that the strain carries a background mutation with the same phenotype as a *mut-16* deletion. If it does carry such a mutation, it would hide any effect the *glo-1::gfp* insert has in animals with a functional endogenous *glo-1* gene. In that case, the strain would either need to be outcrossed before RNAi studies on the *glo-1::gfp* strain can be properly performed, or the transgene could be injected into a wild-type strain. One can speculate that since *glo-1::gfp*, driven by a gut-specific promoter, makes *glo-1* animals more sensitive to RNAi, *glo-1* could have different effects on RNAi efficiency when expressed or silenced in different tissues, such that an

animal with GLO-1 only in the gut is more sensitive than an animal completely lacking GLO-1 or one that has a wild-type expression. Conversely, one would then expect that an animal with *glo-1* silenced specifically in the gut would be less sensitive than a wild-type animal.

This project has yielded some interesting and unexpected results, most of which lack an explanation. In all, they do not support the thesis that the efficiency of RNAi transport is proportional to the lysosomal activity in the gut. While animals deficient in *glo-1*, therefore lacking gut granules, did acquire an enhanced RNAi efficiency, a transgenic *glo-1::gfp* construct with a gut-specific promoter did not rescue this phenotype even though it rescued the gut granule loss phenotype. It seems clear however that *glo-1* and *glo-4* do have a function in RNAi. Whether they have a role in general RNAi or the transport of dsRNA remains to be elucidated. So far, only two genes have been used as RNAi targets, both with different results. Future studies should include a larger and more diverse set of genes as RNAi targets.

Materials and Methods

C. elegans strains

N2 was used as the reference wild-type strain (Brenner, 1974). Mutant alleles used were *eri-1(mg366)*, *glo-1(zu391)*, *glo-4(ok623)* and *hjls9[pges-1::glo-1::gfp]* (Zhang et al., 2010). Strain CB3388 was used as *mut-16* positive control.

Bacterial strains

For regular maintenance, the uracil deficient *Escherichia coli* strain OP50 is used as food for the animals, as it grows slowly and forms a thin "lawn" on agar plates. For RNAi feeding, the following bacterial HT115 clones were used: *pos-1(V-6A23)*, *dpy-13(IV-2K17)*. Both strains originate from the Ahringer RNAi library (Kamath and Ahringer, 2003). In addition, a HT115 strain with an empty L4440 vector was used as negative control. Strains from the Ahringer RNAi library carry cDNA of the target gene in an L4440 plasmid vector, flanked on both sides by T7 promoter sites. The vector also carries an ampicillin marker. The HT115 *E. coli* strain is deficient in RNase III, which would otherwise digest the dsRNA. HT115 carries a viral T7 RNA polymerase that can be selectively induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG).

Primers

All primers were ordered from Life Technologies. Primer sequences (5' to 3') were *mut-16* forward: CCCGCCGATACAGAACTAA; *mut-16* reverse: AATATTCGATCGGCAAGCAG; *glo-1* forward: CAAGATTCACGTTGCCTTCA; *glo-1* reverse: TTCAATGTGCATTGCTGAGTT.

Microscopy

A Nikon Eclipse 90i microscope was used for all microscopy. Microscope slides were prepared by dropping 2% agarose onto them and placing another microscope slide on top, creating an agar pad. Before placing worms on the agar pad, 10 μ l of 2 mM levamisole (Sigma-Aldrich) in M9 (86 mM NaCl (Fluka), 40 mM Na₂HPO₄ (Merck) 20 mM KH₂PO₄ (Sigma)) was applied to the agar pads to paralyze the worms. 20-30 L3-Adult worms were added to each slide and a cover slip was applied. Slides were examined with microscope within an hour.

Whole-worm PCR

Five adult animals from each strain to be tested were picked to a PCR tube and treated with 2.5 μ l 100 μ g/ml proteinase K (Sigma-Aldrich) in 10 mM Tris-HCl (pH 8.5). The PCR tubes were then frozen to -80°C for more than ten minutes, at which point they were thawed and incubated at 65°C for 60 minutes, then 95°C for 15 minutes. To each PCR tube, 23 μ l PCR master mix was added (1x DreamTaq Green buffer (Fermentas), 1 μ M dNTP mix (Fermentas), 1 μ mol/ml forward primer, 1 μ mol/ml reverse primer, and 0.75 U DreamTaq (Fermentas)). For *mut-16* PCR, the following PCR program was run on an Applied Biosystems GeneAmp PCR System 9700: initial denaturation at 95°C for 30 seconds; 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72 °C for 1 minute, and a final elongation at 72°C for 5 minutes. The same program was used for the *glo-1* PCR except that the annealing temperature was 53°C and the machine used was an Eppendorf Mastercycler gradient PCR.

Gel extraction of DNA and sequencing

PCR products were run on 1.8% agarose/TBE (89 mM Tris-borate (Sigma), 89 mM boric acid (Sigma), 20 mM EDTA (Sigma)) gels. The bands were excised and the DNA was purified using GeneJet™ Gel Extraction Kit (Fermentas) according to manufacturer's instructions. The purified DNA was sent to Macrogen for sequencing.

Generation of *C. elegans* strains by genetic crosses

N2 males to be used in genetic crosses were generated as follows. Eight N2 worms in the L4 larval stage were moved to each of three medium NG (1.7% (w/v) Bacteriological agar (Oxoid), 0.25% (w/v) Bacto-peptone (BD), 50mM NaCl (Fluka), 0.5% (w/v) Cholesterol (Sigma-Aldrich), 1 mM CaCl₂ (Merck), 1 mM MgSO₄ (Aldrich) 25 mM Potassium Phosphate (Sigma) buffer pH 6.0) plates seeded with OP50. The worms were then heat shocked at 30°C for 5 hours, and then placed at 25°C. After two days, plates were inspected to identify male progeny.

A crossing scheme for outcrossing *glo-1* is outlined in Figure 1. Crosses were performed on 55 mm NG (1.7% (w/v) Bacteriological agar (Oxoid), 0.25% (w/v) Bacto-peptone (BD), 50mM NaCl (Fluka), 0.5% (w/v) Cholesterol (Sigma-Aldrich), 1 mM CaCl₂ (Merck), 1 mM MgSO₄ (Aldrich) 25 mM Potassium Phosphate (Sigma) buffer pH 6.0) plates seeded with OP50 culture in the center. For the first cross, three plates were prepared with one *glo-1* hermaphrodite and four N2 males each. For the second cross, six plates were prepared with one hermaphrodite and four males. For the remaining crosses, eight plates were used with the same number of hermaphrodites and males as the second cross. To isolate heterozygous *glo-1* animals after the final cross, L4 hermaphrodite offspring from the cross were singled to plates and kept overnight to lay embryos, to be used for future crosses. After this, they were singled to 1 μM LysoTracker (Invitrogen) plates and kept overnight to lay embryos for genotyping. Two days later, the offspring on the LysoTracker plates were subjected to microscopy. Homozygous *glo-1* animals were identified as having fewer gut granules than wild-type animals. Assuming Mendelian inheritance, 25% of the offspring from *glo-1* heterozygous animals would be homozygous for *glo-1*. L4 animals on the non-LysoTracker plate from animals that had been shown to be heterozygous for *glo-1* were then singled to new plates, allowed to lay embryos overnight and then singled to new LysoTracker plates. After two days, the animals on the new LysoTracker plates were subjected to microscopy and the proportion of animals with gut granule loss were scored. Animals with 100% *glo-1/glo-1* offspring ($n > 10/\text{plate}$) were assumed to be homozygous for *glo-1*, and the offspring on the non-LysoTracker plate from those animals were saved as outcrossed *glo-1* animals.

Crossing scheme for creating the *glo-1;glo-1::gfp* strain is outlined in Figure 6. All crosses were performed in triplicate, with one hermaphrodite and four male animals on each small plate. For selecting GFP(+) or GFP(-) animals, low-magnification fluorescence microscopy was used.

Bacteria-mediated RNAi

HT115 *E. coli* clone selectively expressing *dpy-13* dsRNA and a HT115 clone carrying the empty L4440 vector for negative control were restreaked from -80°C freezer onto LB+Ampicillin (0.5% (w/v) yeast extract (Oxoid), 1% (w/v) Tryptone (Oxoid), 170 mM NaCl (Fluka), 10 mM Tris (Sigma) pH 8.0, 50 μg/ml ampicillin) plates, and grown at 37°C overnight. From these, one colony was picked using sterile technique and incubated no more

than 16 hours in 2 ml LB with 50 µg/ml ampicillin (as above). These cultures were used to seed 55 mm NG/Ampicillin/IPTG (1.7% (w/v) Bacteriological agar (Oxoid), 0.25% (w/v) Bacto-peptone (BD), 50 mM NaCl (Fluka), 0.5% (w/v) Cholesterol (Sigma-Aldrich), 1 mM CaCl₂ (Merck), 1 mM MgSO₄ (Aldrich) 25 mM Potassium Phosphate (Sigma) buffer pH 6.0, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich) and 50 µg/ml ampicillin) plates. All *dpy-13* experiments were performed in triplicates, and with an equal number of L4440 vector controls. Five gravid adults were singled to each RNAi plate. To ensure that the worms were synchronized in age, they had been isolated prior to becoming adults. After 3 hours, more than 20 embryos had usually been laid on each plate, and the gravid adults were removed. The plates were placed in 20 °C incubator and offspring were scored for dumpiness three days later.

For *pos-1* RNAi, cultures with *pos-1* dsRNA and negative control cultures were prepared as with *dpy-13* RNAi. The cultures' Optical Densities (OD) at 600 nm were measured, and they were diluted with LB to OD₆₀₀=1. The *pos-1* culture was subsequently diluted 0.5, 0.25 and 0.10 with the negative control culture. The diluted cultures and the L4440 negative control culture were then used to seed plates. Plates with the three dilutions of *pos-1* and negative controls were used for subsequent experiments. All *pos-1* experiments were performed in sets of five and with equally many negative control plates. One L4 hermaphrodite was singled to an RNAi plate which was placed in 20 °C over night. The now gravid adult was then removed. After three days, the total number of offspring L4 or older was scored.

Acknowledgments

I am grateful for all the help, support and guidance my supervisor Andrea Hinas has provided me with. I would also like to thank Yani Zhao for her insight, knowledge, friendship and OP50. Also thanks to coordinator Lars Liljas and Nicusor Timneau and opponents Wael Kamel and Beer Chakra Sen for their helpful suggestions. Furthermore, I am thankful for everyone in the D7:3 corridor for all the help they have provided, in particular Gordon Virgo, Nizar Enweji and Sahil Aery.

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