IP_3 signalling regulates exogenous RNAi in *Caenorhabditis elegans*

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Abstract

RNA interference (RNAi) is a widespread and widely exploited phenomenon. Here we show that changing inositol 1,4,5-trisphosphate (IP_3) signalling alters RNAi sensitivity in *C. elegans*. Reducing IP_3 signalling enhances sensitivity to RNAi in a broad range of genes and tissues. Conversely up-regulating IP_3 signalling decreases sensitivity. Tissue specific rescue experiments suggest IP_3 functions in the intestine. We also exploit IP_3 signalling mutants to further enhance the sensitivity of RNAi hypersensitive strains. These results demonstrate that a conserved cell signalling pathways can modify RNAi responses, implying that RNAi responses may be influenced by an animal's physiology or environment.

Keywords: *C. elegans*/ Enhanced RNAi/ Inositol 1,4,5-trisphosphate/ RNA interference/ calcium signalling.

Introduction

RNA interference (RNAi) is a widespread and widely exploited phenomenon which has potential as a strategy for both the treatment of disease and pest control. RNAi results in down-regulation of a specific gene in response to the production of small interfering RNAs (siRNAs). RNAi is one of a family of processes mediated by small non-coding RNAs [1, 2]. In *C. elegans*, and in a number of other organisms, RNAi is systemic so that the introduction of dsRNA into one tissue triggers gene silencing in other tissues [3-7]. Furthermore, systemic RNAi enables *C. elegans* and other organisms to exhibit environmental RNAi [5]. For example, feeding *C. elegans* on bacteria expressing dsRNA initiates a widespread RNAi response [8, 9]. Studies in *C. elegans* and other organisms have provided mechanistic insights into RNAi [4, 10-13], although, the role of exogenous RNAi in the normal life of *C. elegans* and other animals remains unclear [14].

Whilst *C. elegans* mounts a robust and widespread RNAi response for many genes it is clear that its sensitivity to RNAi can be modified. Enhancers of RNAi such as *rrf-3* [15] and *eri-1* [16] are believed to act cell autonomously and are thought to enhance exogenous RNAi by releasing components that are normally shared between the exogenous- and endogenous-RNAi pathways [17]. A group of retinoblastoma (Rb) pathway genes also give rise to enhanced sensitivity when mutated [18, 19]. These genes and others (e.g. [20]) appear to influence RNAi sensitivity through core sncRNA pathways, gene regulatory mechanisms or RNA transport. Whether the response can also be altered by the broader physiological state of an animal remains unclear. Some hints that this may be the case come from observations that environmental or other factors may

influence RNAi [21]. For example, temperature can affect the RNAi response to particular genes in certain backgrounds [21, 22] and it is a common observation that temperature can affect the results of RNAi experiments.

Inositol 1,4,5-trisphosphate (IP_3) is an important second messenger in animals. IP_3 is generated by the action of phospholipase C (PLC) in response to a diverse range of extracellular stimuli, including neurotransmitters and hormones acting on G-protein or tyrosine kinase coupled receptors (Fig **1**A). IP_3 production leads to Ca^{2+} release from the endoplasmic reticulum (ER) through a ligand-gated ion channel receptor, the IP_3R, which regulates a wide range of processes in animals including *C. elegans* [23]. Here we show that reducing or increasing IP_3 signalling enhances or suppresses the sensitivity of *C. elegans* to RNAi in a broad range of genes and tissues. Tissue specific rescue suggests that IP_3 signalling acts non-cell autonomously and that it acts in the intestine. Our results imply that an animal's exogenous RNAi response may be influenced by its physiology or environment.

Results and Discussion

IP_3 receptor mutants have enhanced RNAi sensitivity

In *C. elegans* IP_3 receptors (IP_3Rs) are encoded by a single gene *itr-1* (Fig **1**A) [24-26]. Whilst investigating *itr-1* we observed that an unexpectedly large number of genes showed apparent interactions with *itr-1* in RNAi experiments. This led us to hypothesise that *itr-1* reduction-of-function mutants have enhanced RNAi responses. To test this we selected a group of RNAi targets, which had been reported to be refractory to RNAi in wild-type (WT) worms but sensitive to RNAi in hypersensitive strains [18, 27]. We selected genes that act in a range of processes and tissues, including genes that cause embryonic lethality (*apr-1*, *qua-1*, and *hmr-1)*, sterility (*arf-3* and *mys-1*), defects in vulval development (*lin-1* and *lin-31*), a dumpy phenotype (*dpy-13*) or neuronal Unc phenotypes (Table I). RNAi was performed by feeding in an *itr-1* temperature sensitive allele *itr-1(sa73)* (Table I). Assays were carried out at 20^{o}C at which temperature *itr-1(sa73)* exhibits a partial reduction-of-function phenotype but is reasonably healthy. Other widely used RNAi sensitive strains were also tested (Table I). We found that *itr-1(sa73)* worms generally showed stronger and more penetrant RNAi phenotypes than wild-type animals (Table I, Fig **1**B). In comparison with the RNAi sensitive strain *rrf-3*, *itr-1(sa73)* often showed similar sensitivity (e.g. *qua-1* RNAi caused 23.3 ± 12% lethality in *itr-1* and 22.9 ± 2.5% in *rrf-3*) but often showed less enhancement than *eri-1; lin-15b* worms (e.g. *lin-31* RNAi caused 22.8 ± 0.6 multivulval worms in *itr-1* and 56.1 ± 6.1% in *eri-1; lin-15b*). Thus *itr-1(sa73)* animals show a broad enhancement of the RNAi effect in a range of target tissues including the nervous system.

In our standard assay adult worms are placed on RNAi plates and allowed to lay eggs, which subsequently develop on the same plate. We see the same effect within a single generation (Fig **1**C) i.e. the effect is independent of any generational effects. *itr-1(sa73)* animals are slow growing and constipated [25]. These phenotypes might increase RNAi by increasing exposure to dsRNA. Analysis of RNAi sensitivity in animals carrying mutations which cause *itr-1*-like phenotypes in defecation (*kqt-3*) [28] and growth (*dbl-1*) (Fig **E1**) showed that these do not result in increased sensitivity to *lin-1* RNAi (Fig **1**B).

To test whether the RNAi sensitivity of *itr-1* mutants correlates with the degree of reduction in *itr-1* function we tested other alleles of *itr-1*. We used *lin-1* as a standard test RNAi target. *lin-1* is involved in vulval development and depletion causes a multivulval (Muv) phenotype [29]. *itr-1* is not known to be involved in vulval development so that any effect of IP_3 signalling on the *lin-1* Muv phenotype should be independent of vulval development. *itr-1(sa73)* worms show a significantly stronger RNAi response to *lin-1* RNAi than wild-type animals (Fig **1**B**,**D). Two putative null or near null alleles, *itr-1(tm902)* and *itr-1(n2559)* showed significantly stronger RNAi sensitivity than *itr-1(sa73)*. Expression of a genomic *itr-1(+)* transgene in *itr-1(sa73)* restores normal RNAi sensitivity (Fig **1**D). Thus *itr-1* mutants show increased sensitivity to RNAi, which is proportional to the degree of *itr-1* function.

Increases in RNAi phenotypes in *itr-1* mutants result from reduced target gene expression

To confirm that the increased sensitivity of *itr-1* mutants did indeed result from reduction in gene expression we used two approaches. First, we used qRT-PCR to demonstrate that RNAi of *dpy-13* causes a further reduction in mRNA levels in *itr-1* and,

as a positive control, eri-*1;lin-15b* mutants compared to that seen in wild-type animals (Fig **1**E). Secondly we used a direct readout of gene expression by performing RNAi of GFP in animals carrying GFP markers. An *unc-47p::GFP* transgenic reporter expressed exclusively in GABAergic neurons [16] was used to test knock-down in the nervous system. Wild-type animals expressing this construct show very little reduction in GFP fluorescence after feeding of GFP dsRNA whereas in *eri-1(mg366)* animals the number of fluorescent neurons is significantly reduced after GFP RNAi [16] (Fig **1**F, J**)**. *itr-1(sa73); unc-47p::GFP* animals showed a similar reduction in response to GFP RNAi **(**Fig **1**F, J**)**. In a second system (Fig **2**A) we measured the ability of GFP RNAi to deplete GFP expressed in the body wall muscles using a *myo-3p::GFP* construct [30, 31] again *itr-1* mutants show an increased effect (Fig **1**I).

PLC-β / EGL-8 mutants show increased RNAi responses

IP_3 is produced from phosphatidylinositol 4,5-bisphosphate (PIP_2) by a family of phospholipase C enzymes which are activated by cell surface receptors (Fig **1**A). To investigate whether ITR-1 modifies RNAi through a canonical IP_3-mediated pathway we tested whether phospholipase C (PLC) also interacts with the RNAi pathway. We tested mutants of each of the five *C. elegans* PLC genes [32] for increased sensitivity to *lin-1* dsRNA **(**Fig **1**G**)**. Only loss of PLC-β, *egl-8* resulted in a significant increase in multivulval animals. Three different alleles of *egl-8* showed increased RNAi sensitivity (Fig **1**G) with the strongest phenotype in the putative null allele *egl-8(e2917*), and lesser effects in two partial loss-of-function mutants [33]. *egl-8(e2917)* animals show increased sensitivity to a wide range of genes (Table I). *egl-8(e2917)* animals also showed significant increases in sensitivity in both the *unc-47p::GFP* reporter (Fig **1**F) and body wall muscle GFP systems (Fig 1**I**). Thus, *egl-8* also modulates RNAi sensitivity. The

level of reduction in the null allele *egl-8(e2917)* is not as severe as that in *itr-1* null alleles (compare Fig **1**G and **1**D), thus other PLCs may be compensating for the loss of EGL-8. RNAi tests of the remaining PLCs using *lin-1* in an *egl-8* background revealed that only PLC-γ, *plc-3* enhanced the RNAi response further. We therefore tested *plc-3(tm753); egl-8(n488)* worms and found that they also have increased RNAi sensitivity over either single mutant (Fig **1**G). Thus *plc-3* is able to compensate for the loss of *egl-8* in RNAi sensitivity. PLC-β is usually activated by heterotrimeric G-proteins acting downstream of GPCRs [34]. Thus signalling through a GPCR may be important to the alterations in RNAi sensitivity.

Increased IP_3 signalling causes RNAi resistance.

To ascertain whether IP_3 signalling is capable of modulating the RNAi response in both directions we tested whether increasing IP_3 signalling could reduce RNAi sensitivity. Initially we increased expression of the IP_3R by introducing transgenes carrying the whole *itr-1* gene into wild-type worms. Such transgenes tend to be toxic when introduced at high level and thus overexpression is likely to be modest. To test for reduced RNAi we used RNAi of *unc-15* by feeding which produces an intermediate phenotype in wild-type worms (Fig **1**H). Wild-type worms carrying extra *itr-1* genes show reduced expression of the *unc-15* phenotype. To confirm this result we used *ipp-5* mutants. IP_3 is metabolised to IP_2 and IP_4 by the enzymes inositol polyphosphate 5-phosphatase, encoded by *ipp-5* and IP_3 3-kinase encoded by *lfe-2* respectively (Fig **1**A). *ipp-5* loss-of-function mutant animals are therefore assumed to have increased IP_3 levels (see [26] for discussion). *ipp-5* mutants show a substantially reduced response to *unc-15*

RNAi (Fig **1**H). *lfe-2* mutants which may also have increased IP_3 levels in some tissues, did not change sensitivity to *unc-15* RNAi. To test whether reduced sensitivity in *ipp-5* mutants was due to changes in gene expression we used the body wall muscle GFP reporter and showed reduced knock-down in *ipp-5(sy605)* animals (Fig **1**I). Thus, increased IP_3 signalling leads to decreased RNAi sensitivity and decreased IP_3 signalling causes increased sensitivity demonstrating that IP_3 signalling is able to modulate RNAi sensitivity in worms. We note that we and others have used RNAi in IP_3 signalling mutants to dissect IP_3-mediated signalling pathways [26]. The results of such studies should now be reviewed in the light of these results.

IP_3 signalling modifies RNAi induced by internally produced dsRNA

Widespread RNAi induced, as above, by the feeding of bacteria carrying dsRNA consists of a number of steps in which *itr-1* might function. First dsRNA is absorbed from the environment through the intestine. Next the RNAi signal is transported between cells through a process requiring the production, export and import of the RNAi signal. Finally cell autonomous processes leading to mRNA destruction are required.

To narrow down the step at which *itr-1* functions we asked whether sensitivity to RNAi induced by dsRNA introduced by other methods was also altered. We used a system designed to assay RNAi spreading (Fig **2**A) [31] in which GFP is expressed in the pharynx (*myo-2p::GFP*) and body wall muscle (bwm) (*myo-3p::GFP*). RNAi is then induced by expressing sense and antisense RNA for GFP in the pharynx (*myo-2p::dsRNAGFP*) [30]. Thus, knock down of GFP in the pharynx is, presumably, primarily, cell autonomous whilst knock down in the body wall muscle requires RNAi spreading. We observed increased knock down in *itr-1* mutants in both the pharynx and

bwm (Fig **2**B). Similarly *ipp-5* mutants show decreased sensitivity in both the pharynx and bwm (Fig **2**C). Thus *itr-1* mutants show increased sensitivity to internally induced RNAi.

itr-1 acts in the intestine to modify RNAi responses

We sought to further clarify the mechanism of IP_3 action by investigating the site of action at a tissue level. We used tissue specific promoters to express an *itr-1* cDNA that had previously been shown to rescue other phenotypes in both neurones and the intestine (Ford, Peterkin and Baylis unpublished). Using the *unc-47p::GFP* system and RNAi by feeding we tested for the ability of *itr-1* to restore normal sensitivity in the target cells. Expression of *itr-1* in *unc-47* expressing neurones (*unc-47p::itr-1*) or in the nervous system in general (*unc-119p::itr-1*) failed to restore normal RNAi sensitivity (Fig **3**A). Whilst, this suggests that *itr-1* does not act in the target cells we cannot exclude other possibilities such as insufficient expression, although the ability of the *unc-119p::itr-1* construct to rescue other neuronal phenotypes makes this less unlikely. In contrast expression from a well-characterised intestine specific promoter, *vha-6p* [35] was able to restore normal sensitivity (Fig **3**A). Furthermore *vha-6p::itr-1* partially rescued sensitivity to *lin-1* RNAi by feeding (Fig **3**B). In both of these experiments we induced RNAi by feeding and thus *itr-1* might function in the gut to improve dsRNA uptake. We therefore tested whether expression in the intestine could rescue sensitivity induced in response to an internal dsRNA trigger. We used the GFP pharynx and bwm system in an *itr-1* background. As shown above *itr-1* mutants show increased sensitivity in both the pharynx (producing tissue) and bwm (non-producing tissue) (Fig **2**B,C). Expression of *itr-1* in the intestine restores normal sensitivity in both tissues (Fig **3**C).

Thus, IP_3 signalling in the intestine appears to play a role in the modulation of RNAi sensitivity. Since intestinal *itr-1* function influences RNAi induced by either external or internal dsRNA it seems unlikely that this is exclusively due to modified uptake from the gut although we cannot exclude such a role. For example, even in the internal dsRNA system it is possible that dsRNA is released into the environment and subsequently taken up by other worms. In the internal dsRNA system RNAi responses in both producing and target cells are increased in *itr-1* mutants. This suggests that IP_3 signalling is not altering spreading, although, again we cannot exclude an indirect route to RNAi in the producing cells. Overall, therefore we consider the most likely explanation for our data is that IP_3 signalling in the intestine is involved in the production or release of some humoral signal, which then modifies RNAi responses in cells elsewhere in the body.

Using IP_3 signalling mutants to produce further increases in RNAi sensitivity.

RNAi sensitive strains have been important tools in the analysis of gene function. In some cases sensitivity can be increased in an additive fashion by combining mutations, notably in the example of *eri-1* and *lin-15B* and similar strains [18, 19, 36]. We therefore tested whether *itr-1* is able to further enhance hypersensitivity in *eri-1* mutants. *itr-1(sa73)*; *eri-1(mg366)* double mutants show an increased sensitivity to both *lin-1* and *GFP* dsRNA (Fig **4**A, B). We therefore attempted to make a strain with further increased sensitivity over currently available sensitised strains. *eri-1(mg366); lin-15b(n744)* strains are commonly used in RNAi screens. Since *itr-1* mutants have pleiotropic phenotypes we used *egl-8(e2917)* as these animals are relatively healthy. We produced two individual isolates of a strain with the genotype *eri-1(mg366); lin-15b(n744); egl-8(e2917)*, HB946 and HB947. Tests of these strains using *lin-1* and *lin-31* show that they have higher sensitivity than the *eri-1(mg366); lin-15b(n744)* strain (Fig **4**C). Use of this strain may be

advantageous in RNAi screening experiments, although it carries mutations in three pathways and would need to be used with care. These results also suggest that *itr-1* functions through a different mechanism than either *eri-1* or *lin-15B.*

Conclusions

Our results provide the first clear example of a signal transduction pathway acting in the regulation of RNAi. This discovery raises the possibility that an animal's response to exogenous dsRNA may be modified by changes in the animal's environment or internal physiology as reflected in intercellular signals which require intracellular IP_3 signalling. The discoveries that *itr-1* mutants strongly enhance the RNAi response and that this sensitivity can be further improved by combining *itr-1* with other RNAi sensitive mutations may prove useful in the application of RNAi to the treatment of disease and the control of pests, including other nematodes. For example, RNAi based strategies to treat human disease are currently being explored but at therapeutic doses tissue accessibility varies. For example, RNAi can efficiently reduce the function of a liver-derived enzyme involved in cholesterol synthesis in hepatocytes [37, 38]. In contrast neurones are relatively inaccessible to nucleic acids hampering attempts to modify neurodegenerative diseases. The experiments presented in this work shows that the IP_3/calcium signalling pathway enables RNAi in refractory tissues. This pathway is conserved from invertebrates to humans, and therefore our results may have relevance to developing methods of RNAi intervention in difficult to reach tissues in humans and other animals.

Methods

Detailed methods are given in the supplementary methods.

C. elegans culture and strains

Full details of strains used in this work are given in Table E1. Worms were cultured using standard techniques [39]. All experiments were performed at 20^{o}C.

RNAi induced by exogenous dsRNA

RNAi by feeding [40] was carried out using bacterial RNAi feeding strains from the Ahringer library [41]. Adult animals were placed on plates seeded with bacteria expressing dsRNA and allowed to lay eggs for between 2 and 6 hours before removal. The resulting progeny were scored for the relevant phenotypes.

RNAi induced by endogenous dsRNA, fluorescent microscopy and image analysis

We used a system similar to that developed by Hunter and colleagues in which GFP reporters are present in the pharynx and body wall muscle of the animals [31]. L4 animals were imaged. Image collection was optimised independently for the pharynx and body wall muscle respectively. In the case of the *ipp-5* experiments worms were synchronised and starved to increase the RNAi effect [31] and thus the range of detection for resistance.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

AIN, RPV-M, RG, HAB designed experiments, AEH, AIN, CC, HAB, MB, MDS, MH, ML, RG RPV-M, performed experiments. AIN, HAB and RPV-M wrote the manuscript. HAB instigated and oversaw project.

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Figure legends

Figure 1 - IP_3 signalling regulates RNAi sensitivity

(A) Diagram of the IP_3 signalling pathway showing key components and *C. elegans* gene names.

(B) Mutants in the IP_3 receptor gene, *itr-1,* are hypersensitive to RNAi induced by feeding. Reduction of function of *lin-1* and *lin-31* give a multivulval phenotype, *dpy-13* gives short worms. For other genes see Table I. Average of 5 repeats unless marked below. Error bars denote S.E.M. Total number of worms left to right: 363, 424, 835, 389, 312, 291*, 465* - 352, 190, 235, 296, 185 – 293*, 776, 323, 191, 560. * 4 repeats.

(C) RNAi hypersensitivity in *itr-1* mutants is observed within a single generation. L1 animals were exposed to GFP (RNAi) and the number of GFP positive GABAergic neurones (*unc-47p::GFP*) counted in young adults. The *cat* (*E. coli* chloramphenicol actetyltransferase) gene was used as a negative control. Average of n worms, error bars denote S.E.M. Number of worms left to right: 9,10,9 – 30,30,30. Data was collected from three independent plates of worms.

(D) The increase in RNAi sensitivity for *lin-1* correlates with the severity of the *itr-1* mutant allele. Rescue of *itr-1* by an extrachromsomal transgene carrying a genomic copy of the *itr-1* gene (*itr-1(+)*) restores normal sensitivity. Average of 3 repeats. Error bars denote S.E.M. Total number of worms left to right: 346, 251, 133, 155, 122.

(E) qPCR of *dpy-13* mRNA shows that increased RNAi sensitivity in *itr-1* animals is caused by increased target mRNA knock-down. Average of three experiments, each sample was replicated in triplicate in each experiment.

(F) Knock-down of GFP expression in the GFP positive GABAergic neurones (*unc-47p::GFP*) system is enhanced by *itr-1* and *egl-8* mutants. Average of n worms, error

bars denote S.E.M. Number of worms left to right: 8,7,6,10,19,20,22,43. Data was collected from four independent plates of worms.

(G) *egl-8,* phospholipase Cβ is the primary PLC involved in the RNAi hypersensitivity pathway. Loss of function mutants in all *C. elegans* phospholipase C (PLC) genes were tested for RNAi hypersensitivity. *egl-8* mutants show increased sensitivity. *plc-3*, phospholipase Cγ mutants show no increase in sensitivity alone but are able to further enhance sensitivity in *egl-8* mutants. Average of 3 repeats unless marked below. Error bars denote S.E.M. Total number of worms left to right: 259, 127, 231, 71, 225, 351, 273, 335, 46*. * 5 repeats.

(H) Increased IP_3 signalling causes RNAi resistance. Worms were exposed to *unc-15* RNAi by feeding. Worms were scored as normal/mildly uncoordinated or severely uncoordinated/ paralysed. Statistical significance is against wild-type unless otherwise indicated. Average of 5 repeats unless marked below. Error bars denote S.E.M. Total number of worms left to right: 1016*, 609, 144, 174, 436, 394, 466, 177, 332. *10 repeats.

(I) Changes in RNAi sensitivity in IP_3 signalling mutants are associated with alterations in gene knockdown. Worms expressing GFP in their body wall muscles (bwm) using a *myo-3p::GFP* transgene were exposed to GFP RNAi by feeding. The level of GFP fluorescence was measured and normalised to control worms. *ipp-5* worms show reduced knockdown compared to wild-type worms whilst *itr-1* and *egl-8* worms show increased knockdown. Average of 20 worms taken from two independent plates of worms. Error bars denote S.E.M. Experiment was performed on two occasions on two individual lines of each genotype. Data for one set of genotypes is shown. Statistical significance is against to wild-type.

(J) Representative images of the *unc-47p::GFP* GABAergic worms showing untreated animals and the effect of GFP RNAi in a wild-type and *itr-1* background. Scale bar =10µm.

Where shown, significance was assessed using an unpaired, two-tail Student's t-test. The results are presented as: ns, not significant $p \geq 0.05$, * significant p=0.01 to 0.05, **very significant p=0.001 to 0.01, *** extremely significant $p < 0.001$.

Figure 2 - IP_3 signalling modulates sensitivity to internally induced RNAi

(A) RNAi was induced and measure internally using a system developed to test the spreading of RNAi [31]. Integrated transgenes express GFP in the pharynx (*myo-2p::GFP*) and the body wall muscle (*myo-3p::GFP*). dsRNA is produced in the pharynx by an array carrying both sense and antisense fragments of the GFP gene driven by the *myo-2* promoter. The degree of knock-down in the two tissues is measured using digital imaging.

(B) IP_3R, *itr-1*, worms show increased knockdown in both (i) pharynx and (ii) body wall muscle. Well fed L4 animals were imaged and analysed. Knock-down was calculated relative to control animals in which the dsRNA producing array was absent. Data are shown as box and whisker plots. Whiskers represent min to max, box represents 25-75 percentiles, the middle line indicates the median. Outliers were not removed. Total number of worms left to right: 38,43,41,39. Data was collected from three independent plates of worms.

(C) Inositol 5-phosphatase, *ipp-5*, mutant animals show decreased knockdown in both (i) pharynx and (ii) body wall muscle. Animals were synchronised and starved L4 animals were imaged and analysed. Knock-down was calculated relative to control animals in which the dsRNA producing array was absent. Data are shown as for panel B. Total number of worms left to right: 40,62,41,62. Data was collected from three independent plates of worms.

Significance was assessed using Mann-Whitney U tests and shown as *** extremely significant $p<0.001$. In each case means were also compared using Students t-tests and shown to have similar levels of significance.

Figure 3 - Expression of the itr-1 cDNA in the intestine restores normal RNAi sensitivity in itr-1 mutants

(A) Expression of a rescuing *itr-1* cDNA in the intestine (*vha-6p::itr-1*) restores normal sensitivity to GFP RNAi of GFP tagged neurones. However, expression in the GFP expressing neurones using either the GABAergic neurone promoter *unc-47p* or a pan-neuronal promoter *unc-119p* failed to rescue sensitivity. Average of n worms, error bars denote S.E.M. Number of worms left to right: 7,5,6,6,7 – 33,18,39,42,41. Data was collected from two independent plates of worms.

(B) Expression of a rescuing *itr-1* cDNA in the intestine (*vha-6p::itr-1*) partially restores normal sensitivity to *lin-1* RNAi. Average of 5 repeats. Error bars denote S.E.M. Total number of worms left to right: 202, 482, 91.

(C) Expression of a rescuing *itr-1* cDNA in the intestine (*vha-6p::itr-1*) restores normal sensitivity to internally induced RNAi in both the producing cells (pharynx) **(i)** and target body wall muscle cells **(ii)**. Data are shown as box and whisker plots. Whiskers represent min to max, box represents 25-75 percentiles, line shows median. Outliers were not removed. Total number of worms left to right: 38,43,19 – 39,39,22. Data was collected from three independent plates of worms.

Significance was assessed using an unpaired, two-tail Student's t-test, panels A and B or a Mann-Whitney U test, panel C. The results of significance tests are presented as: ns, not significant $p \geq 0.05$, * significant $p=0.01$ to 0.05, **very significant $p=0.001$ to 0.01, *** extremely significant $p<0.001$. For data in panel C means were also compared using Students t-tests and shown to have similar levels of significance.

Figure 4 - IP_3 signalling mutations act additively with other RNAi enhancing alleles to increase RNAi sensitivity.

(**A**) *itr-1* and *eri-1* act together to further enhance RNAi sensitivity as measured by *lin-1*RNAi. Average of 5 repeats. Error bars denote S.E.M. Total number of worms left to right: 299, 68, 211.

(**B**) *itr-1* and *eri-1* act together to further enhance RNAi sensitivity as measured by GFP RNAi of GFP tagged neurones. Average of n worms, error bars represent S.E.M. Number

of worms left to right: 12,20,19,22. Data was collected from four independent plates of worms.

.

(C) Combining an *egl-8* (PLC) mutation with the *eri-1; lin-15b* double mutation results in a strain with further enhanced RNAi to *lin-1 and lin-31*. Average of 5 repeats. Error bars denote S.E.M. Total number of worms left to right: 153, 303, 269, 401, 329 – 426, 657, 458, 448, 500.

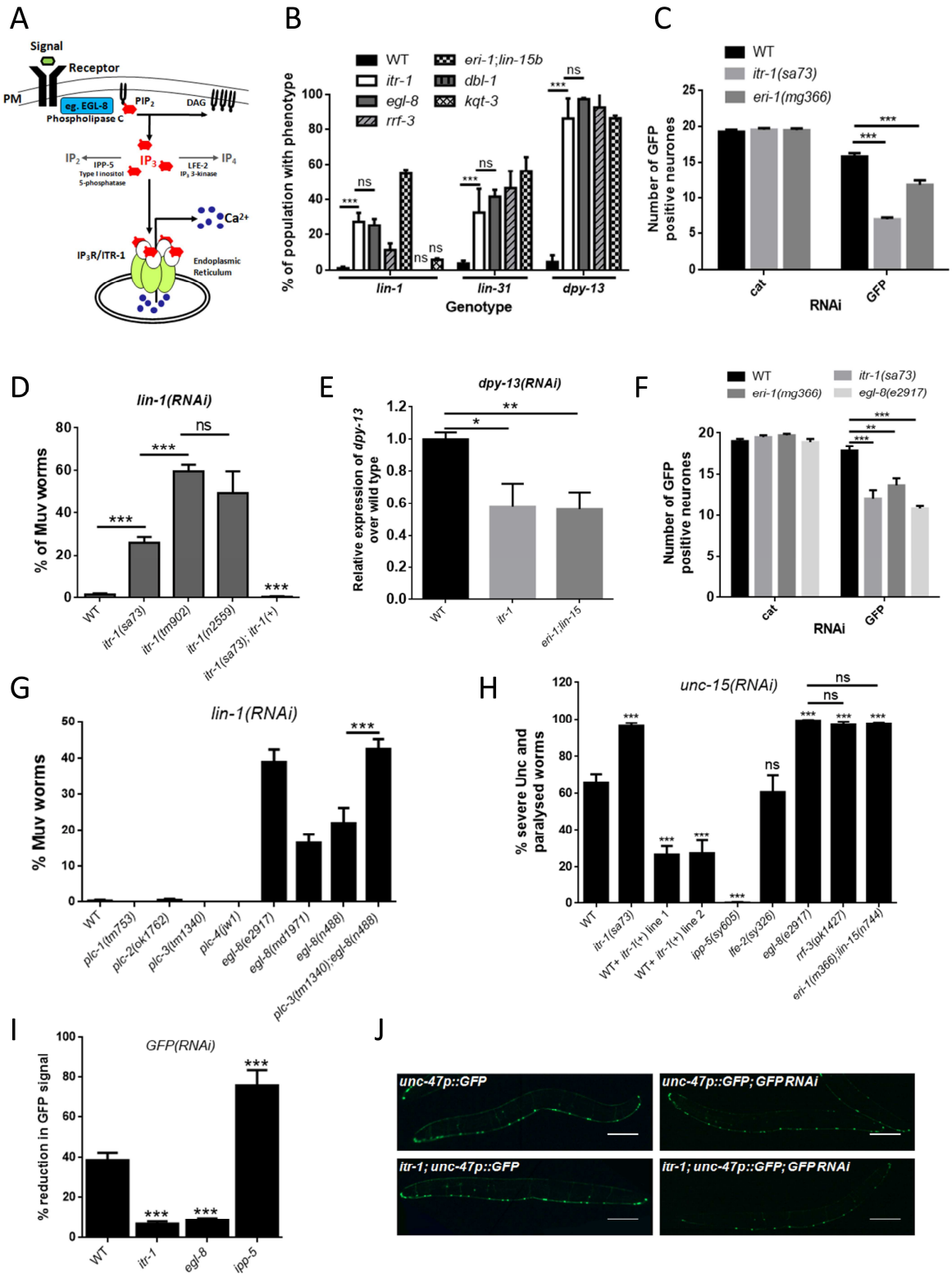
Where shown, significance was assessed using an unpaired, two-tail Student's t-test. The results of all significance tests are presented as: ns, not significant $p \geq 0.05$, * significant $p = 0.01$ to 0.05, **very significant $p = 0.001$ to 0.01, *** extremely significant $p < 0.001$.

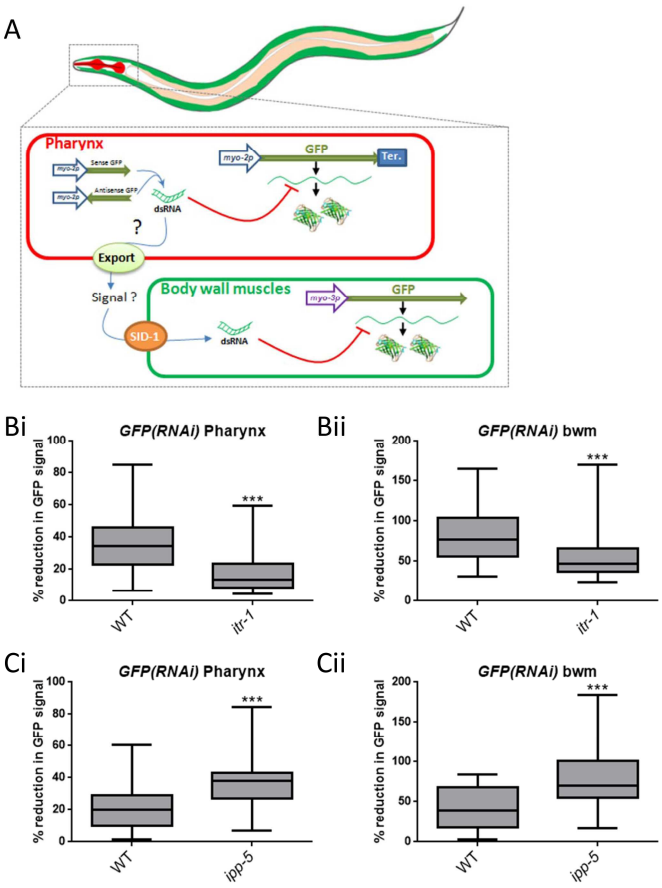
Table I. IP_3 signalling mutants are hypersensitive to RNAi for a variety of genes.

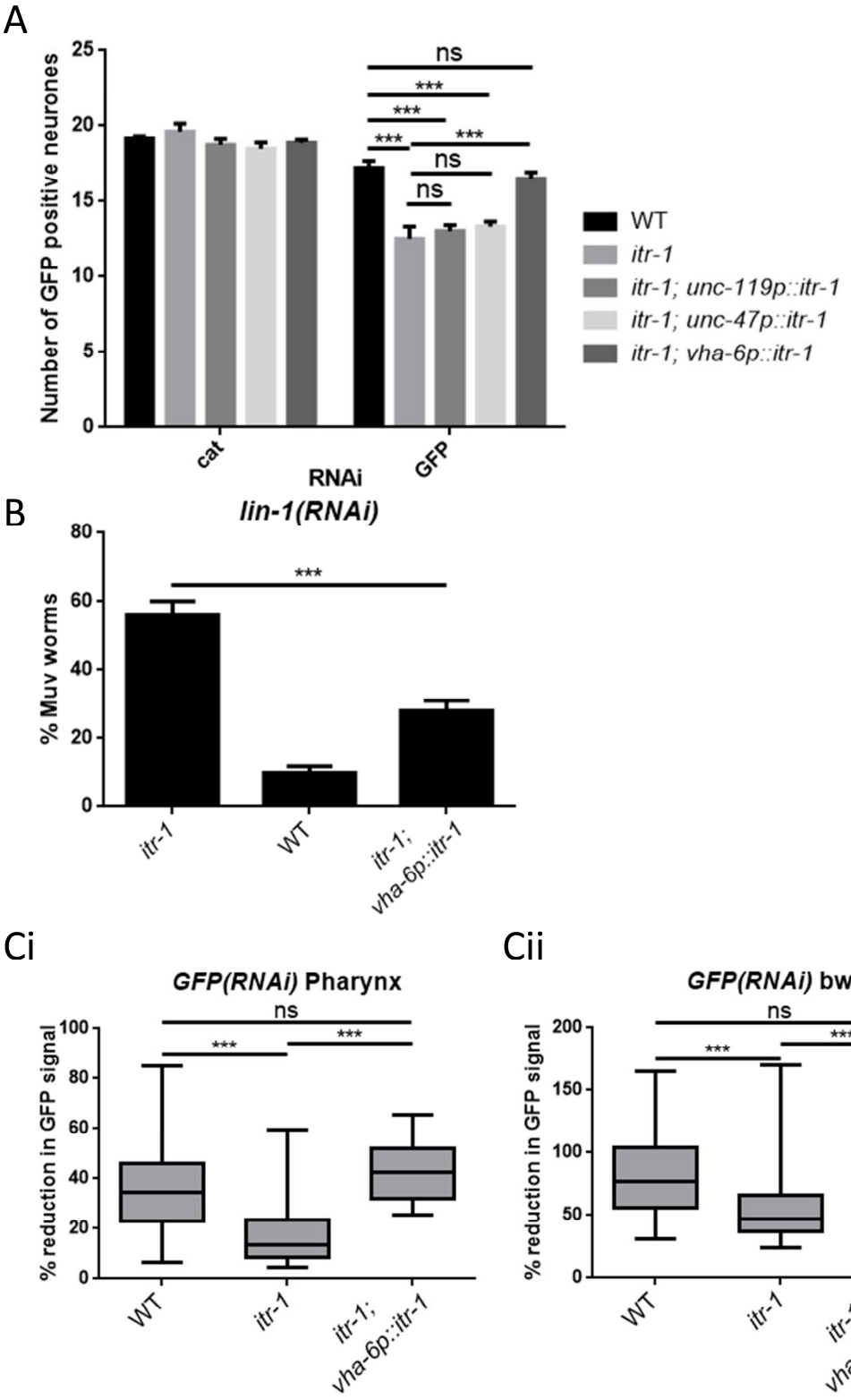
RNAi	Phenotype	N2	*itr-1 (sa73)*	*egl-8 (e2917)*	*rrf-3 (pk1426)*	*eri-1(mg366); lin-15b(n744)*	*lin-15B(n744) + unc-119p::sid-1*
lin-1	Muv	-	++	++	+	+++	nd
lin-31	Muv	-	++	++	++	+++	nd
dpy-13	Dpy	-	++++	++++	++++	++++	nd
arf-3	Ste	+	++++	++++	++++	++++	nd
mys-1	Ste	-	++	++	+	++	nd
apr-1	Emb	-	+	+	+	++	nd
qua-1	Emb	-	+ *	+	+	++	nd
hmr-1	Emb	-	+	++	++	+++	nd
unc-15	Unc + Par	+++	++++	++++	++++	++++	++++
unc-55	Unc	-	+++	+++	nd	+++	++++
unc-119	Unc	-	++	++	nd	+++	++++
unc-14	Unc	-	++	+	nd	+++	++++
unc-58	Unc	-	+++	+++	nd	++++	++++

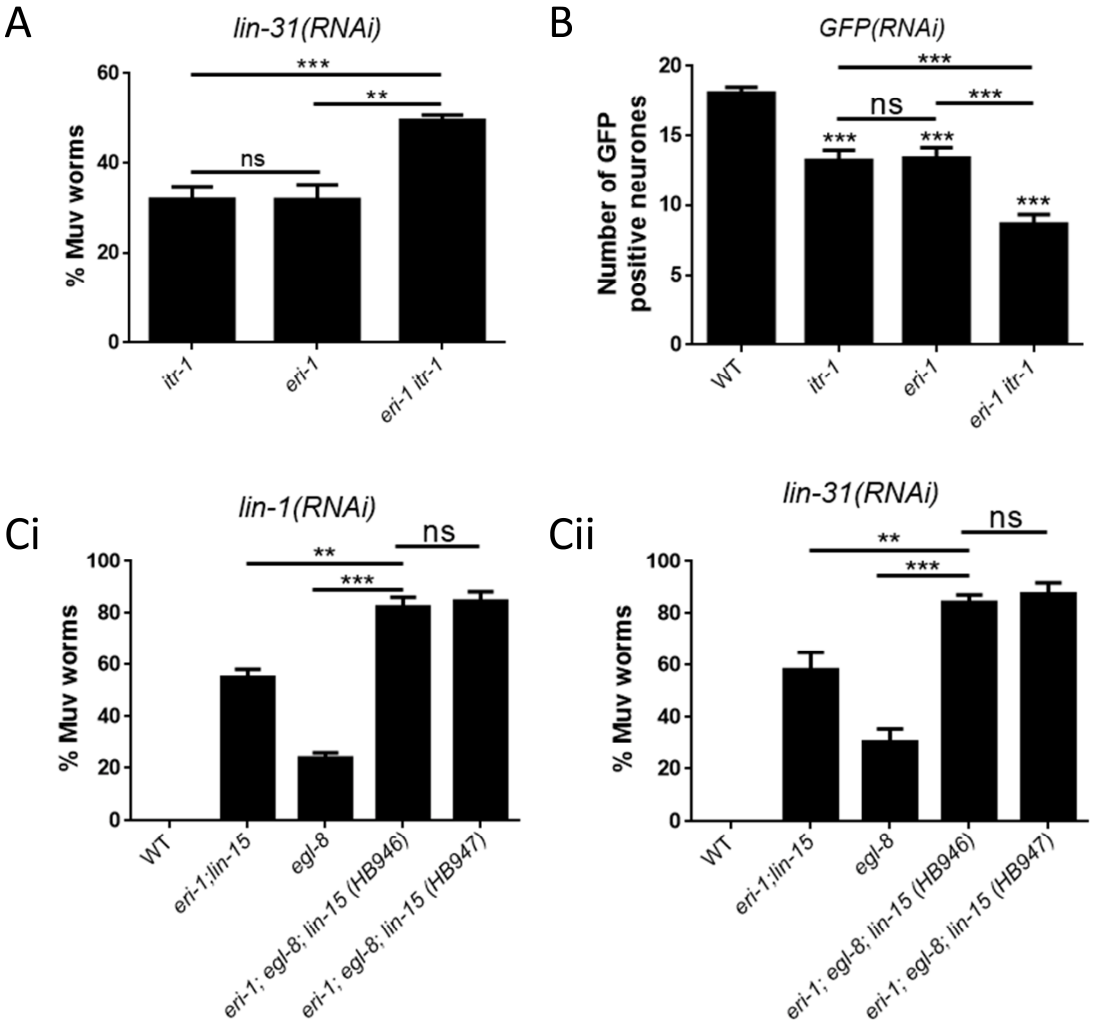
- = 0-4.9%, + = 5-24.9%, ++ = 25-49.9%, +++ = 50-74.9%, ++++ = 75-100%

RNAi was induced by feeding. Adult *C. elegans* were placed on a lawn of *E. coli* expressing dsRNA for the target gene. For each gene 5 repeats were performed. Each repeat had between 30 and 100 worms. The percentage of offspring showing the RNAi phenotype is indicated. Phenotypes: Muv, multivulval; Dpy, dumpy; Ste, sterile; Emb, embroynic lethality; Unc, uncoordinated; Par, paralysed. * RNAi of *qua-1* in *itr-1(sa73)* worms resulted in an additional Ste phenotype. It is unknown whether this is an RNAi sensitivity effect or a genetic interaction.


A
Signal
Receptor
PM
eg. EGL-8
Phospholipase C
PIP2
DAG
IP2
IPP-5
Type I inositol 5-phosphatase
IP3
LFE-2
IP3 3-kinase
IP4
Ca2+
IP3R/ITR-1
Endoplasmic Reticulum
B
WT
itr-1
egl-8
rrf-3
eri-1;lin-15b
dbl-1
kqt-3
% of population with phenotype
lin-1
lin-31
dpy-13
Genotype
C
WT
itr-1(sa73)
eri-1(mg366)
Number of GFP positive neurones
cat
GFP
RNAi
D
lin-1(RNAi)
% of Muv worms
WT
itr-1(sa73)
itr-1(tm902)
itr-1(n2559)
itr-1(sa73); itr-1(+)
E
dpy-13(RNAi)
Relative expression of dpy-13 over wild type
WT
itr-1
eri-1;lin-15
F
WT
eri-1(mg366)
itr-1(sa73)
egl-8(e2917)
Number of GFP positive neurones
cat
GFP
RNAi
G
lin-1(RNAi)
% Muv worms
WT
plc-1(tm753)
plc-2(ok1762)
plc-3(tm1340)
plc-4(jw1)
egl-8(e2917)
egl-8(md1971)
egl-8(n488)
plc-3(tm1340);egl-8(n488)
H
unc-15(RNAi)
% severe Unc and paralysed worms
WT
itr-1(sa73)
WT+ itr-1(+) line 1
WT+ itr-1(+) line 2
ipp-5(sy605)
lfe-2(sy326)
egl-8(e2917)
rrf-3(ok1427)
eri-1(m366);lin-15(n744)
I
GFP(RNAi)
% reduction in GFP signal
WT
itr-1
egl-8
ipp-5
J
unc-47p::GFP
unc-47p::GFP; GFP RNAi
itr-1; unc-47p::GFP
itr-1; unc-47p::GFP; GFP RNAi


A
Pharynx
Sense GFP
Antisense GFP
dsRNA
?
myo-2p
GFP
Ter.
Export
Signal ?
Body wall muscles
SID-1
dsRNA
myo-3p
GFP
Bi
GFP(RNAi) Pharynx
% reduction in GFP signal
***
WT
itr-1
Bii
GFP(RNAi) bwm
% reduction in GFP signal
***
WT
itr-1
Ci
GFP(RNAi) Pharynx
% reduction in GFP signal
***
WT
ipp-5
Cii
GFP(RNAi) bwm
% reduction in GFP signal
***
WT
ipp-5


A
Number of GFP positive neurones
25
20
15
10
5
0
ns
***
***
***
***
ns
ns
WT
itr-1
itr-1; unc-119p::itr-1
itr-1; unc-47p::itr-1
itr-1; vha-6p::itr-1
cat
GFP
RNAi
B
lin-1(RNAi)
% Muv worms
80
60
40
20
0
***
itr-1
WT
itr-1;
vha-6p::itr-1
Ci
GFP(RNAi) Pharynx
% reduction in GFP signal
100
80
60
40
20
0
ns
***
***
WT
itr-1
itr-1;
vha-6p::itr-1
Cii
GFP(RNAi) bwm
% reduction in GFP signal
200
150
100
50
0
ns
***
***
WT
itr-1
itr-1;
vha-6p::itr-1


A
lin-31(RNAi)
% Muv worms
60
40
20
0
***
**
ns
itr-1
eri-1
eri-1 itr-1
B
GFP(RNAi)
Number of GFP positive neurones
20
15
10
5
0
***
ns
***
***
***
***
WT
itr-1
eri-1
eri-1 itr-1
Ci
lin-1(RNAi)
% Muv worms
100
80
60
40
20
0
**
***
ns
WT
eri-1;lin-15
egl-8
eri-1; egl-8; lin-15 (HB946)
eri-1; egl-8; lin-15 (HB947)
Cii
lin-31(RNAi)
% Muv worms
100
80
60
40
20
0
**
***
ns
WT
eri-1;lin-15
egl-8
eri-1; egl-8; lin-15 (HB946)
eri-1; egl-8; lin-15 (HB947)

Expanded View

IP_3 signalling regulates exogenous RNAi in *Caenorhabditis elegans*

Anikó I. Nagy, Rafael P. Vázquez-Manrique, Marie Lopez, Christo Christov, María Dolores Sequedo, Mareike Herzog, Anna E Herlihy, Maxime Bodak, Roxani Gatsi and Howard A. Baylis

CONTENTS

SUPPLEMENTARY FIGURE

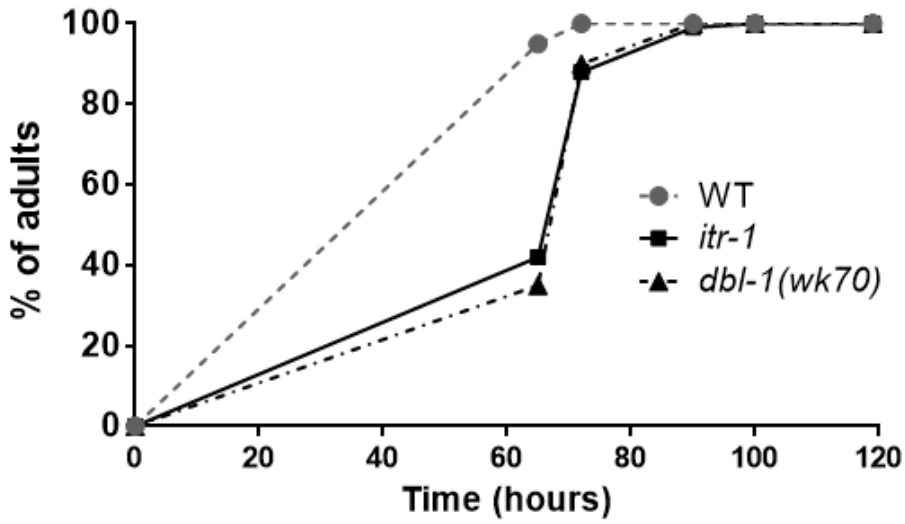


Figure E1. *dbl-1(wk70)* animals have a similar growth rate to *itr-1(sa73)* at 20°C. We confirmed that both *itr-1* and *dbl-1* have slower growth rates. A synchronized population of each genotype was incubated at 20°C and the percentage of adult animals was scored at the indicated time points. n=1.

SUPPLEMENTARY METHODS

Nematode culture and strains

Full details of strains used in this work are given in Table E1. Worms were cultured using standard techniques and media [1]. Crosses were performed using standard techniques. Genotypes were confirmed by PCR and/or DNA sequencing. All experiments were performed at 20^{o}C.

RNAi induced by exogenous dsRNA

RNAi by feeding [2] was carried out using bacterial RNAi feeding strains from the Ahringer library [3]. The identity of clones was confirmed by DNA sequencing. The chloramphenicol acetyl transferase (*cat*) gene from *E. coli* was used as a control [2]. RNAi of GFP used the plasmid pPD128.110 [2]. Adult animals were placed on plates seeded with bacteria expressing dsRNA and allowed to lay eggs for between 2 and 6 hours before removal. The resulting F1 progeny were scored for the relevant phenotypes. In the case of strains which were maintained as heterozygotes (e.g. some *itr-1* and *plc-3 genotypes*), heterozygous adults were allowed to lay eggs but only homozygous offspring were scored.

To test whether the enhanced RNAi in *itr-1(sa73)* animals is also seen within a single generation worms were synchronised by bleaching fecund adults to produce embryos which were then allowed to hatch in the absence of food. Starved L1 larvae were placed on dsRNA GFP producing bacteria and the adults assayed.

qRT- PCR

qRT-PCR of *dpy-13* was used to investigate changes in mRNA levels. Large amounts of each strain were grown and then synchronised by bleaching. The resulting L1 larvae were plated straight onto feeding plates with *dpy-13* RNAi bacteria or with the *cat* control. We collected and washed L2 larvae just before they began the second larval molt. Total RNA was isolated using the NucleoSpin RNA II kit, from Macherey-Nagel (Düren, Germany). 1 µg of RNA was reverse-transcribed using the GeneAmp Gold RNA PCR Reagent kit, from Applied Biosystems (Foster City, Ca, United States). To measure *dpy-13* cDNA levels we used TaqMan® primers and probe, kit Ce02462719_g1, Life Technologies Corporation, (Carlsbad, Ca, USA) and a Real-Time PCR Platform Applied Biosystems ViiATM. We used *pmp-3* as a "housekeeping gene" [4, 5] primers and probe set Ce02485188_m1.

Rescue and tissue specific expression of *itr-1*

Rescuing arrays of *itr-1* (*itr-1(+)*) (eg HB800 and HB905) contained pJL52, a BAC carrying a 31.2kb *Stu*I genomic fragment which contains the entire *itr-1* genomic DNA, including the coding sequences, 3´ regions and all known promoter regions. A 2.35 kb promoter region of *unc-119* (terminal sequences: ggatccctcttggggaaaacgggcg and ctctgccttcatatatgctgttgtagctg) [6], a 2.8 kb promoter region of *vha-6* (terminal sequences: cgcgttcaccactcgaccaccgaac and tttttatgggttttggtaggttttag) [7], a 1.19 kb promoter region of *unc-47* (terminal sequences: gatcccggaacagtcg and ctgtaatgaaataaatgt) were amplified from genomic DNA and cloned upstream of the *itr-1* cDNA using restriction enzyme splicing or Gateway technology.

Transgenic animals were generated by injecting DNA into the germ line using standard methods [8]. The strain HB836 carries an extrachomosomal array derived from *vha-6p::itr-1* injected at 1 ng/µl and a marker pHAB200 [*mec-7p::GFP*] injected at 120 ng/µl.

This array has been shown to rescue the expulsion and growth defects of *itr-1(sa73)* mutants confirming that it restores *itr-1* function in the intestinal cells (K. Ford and R. Gatsi pers. comm.). HB905 carries *itr-1(+)* injected at 5 ng/µl and pRF4 [*rol-6* semi-dominant marker] injected at 120 ng/µl. HB906 carries *unc-47p::itr-1,* injected at 5 ng/µl and pRF4 injected at 120 ng/µl. HB907 carries *unc-119p::itr-1,* injected at 5 ng/µl and pHAB200 [*mec-7p::GFP*] injected at 120 ng/µl. *The unc-119p::itr-1* construct has been shown to rescue levamisole resistance of *itr-1(sa73)* mutants confirming that it restores *itr-1* function in neuronal cells (H Peterkin pers. Comm.). The *unc-47* promoter was separately fused to GFP to confirm that it produces the same expression pattern as the *oxls12 unc-47p::GFP* reporter.

RNAi induced by endogenous dsRNA

We used a system similar to that developed by Hunter and colleagues in which GFP reporters are present in the pharynx and body wall muscle of the animals [9]. To provide body wall muscle fluorescence we used the same chromosomal insertion, *ccIs4251 I,* but to avoid difficulties in some crosses we used a very similar insertion on chromosome II, *mIs12,* to that used by Winston and colleagues (*mIS11*) [9] to provide pharyngeal GFP. Strains were produced from SD1347 and CB5584. All strains were checked for homozygosity and by PCR and/or sequencing for mutant alleles.

To produce dsRNA from the pharynx we introduced equal quantities of sense and antisense fragments of the *GFP* gene driven from the *myo-2* promoter using the approach of Jose *et al,* [10]. We used the same primers and amplified the *myo-2* promoter from gDNA and GFP from the plasmid pPD118.33 (a gift of A. Fire). To create dsRNA producing arrays each fusion PCR product was microinjected at 2.5 ng/µl together with a coelomocyte specific red fluorescent construct to produce *Ex[myo-2p::GFP for; myo-*

2p::GFP rev; unc112p::mCherry] arrays. To perform tissue specific expression of *itr-1*, *vha6p::itr-1* constructs produced as described above were injected with the above components at 5 ng/µl. Two or more individual lines of each genotype were isolated.

Fluorescent microscopy and image analysis

GFP expression was analysed using a Zeiss Axioskop 2 Plus upright microscope equipped with a Q-Imaging Micropublisher 5.0 RTV CCD camera. Images were collected using Q-imaging software with identical camera settings for each experiment. Two sets of camera setting were used in pharynx/body wall muscle experiments to optimise collection from the pharynx and body wall muscle respectively. Image analysis was performed using Image J. In the case of experiments using the pharynx/ body wall muscle GFP system fluorescence was normalised to the appropriate parental strain without the *Ex[myo-2p::GFP for; myo-2p::GFP rev; unc-112p::mCherry]* array. In the case of the *ipp-5* experiments worms were synchronised and starved to increase the RNAi effect [9] and thus the range of detection for resistance.

Table EI, Strains used in this work

Strain[a]	**Genotype**	**Reference**
Bristol N2	wild type	[11]
CB138	*unc-24(e138)IV*	[11]
CB540	*unc-68(e540)V*	[11]
CB6614	*egl-8(e2917)V*	[12]
CB5584	*mIS12 II [myo-2::GFP; pes-10::GFP; gut::GFP]*	Edgley et al pers comm. and CGC.
CZ252	*itr-1(n2559)/lin-45(n2018); unc-24(e138)IV*	[13]
EG1285	*lin-15B(n765) oxIs12 [unc-47p::GFP + lin-15(+)]X*	[14]
HB349	*plc-4(jw1)IV*	[15]

HB372	*plc-1(tm753)X*	[16]
HB390	*itr-1 (tm902); unc-24 (e138)IV*	Vazquez-Manrique unpublished
HB652	*plc-3(tm1340)II/mIn1[dpy-10(e128)mIs14]II*	[16]
HB679	*plc-2(ok1761)V*	[16]
HB715	*plc-3(tm1340)II/mIn1[dpy-10(e128)mIs14]II; egl-8(n488)V*	[17]
HB800	*itr-1(sa73)IV; jwEx800[itr-1(+) pJL52]*	K. Ford unpublished
HB836	*itr-1(sa73)IV; jwEx648[vha-6p::itr-1(+)]*	K. Ford unpublished
HB900	*itr-1(sa73)IV; him-5(e1462)V; oxls12X*	This work
HB901	*eri-1(mg366)IV; him-5(e1462)V; oxls12X*	This work
HB902	*eri-1(mg366) itr-1(sa73) IV ; him-5(e1462)V; oxls12X*	This work
HB903	*egl-8(e2917)V; oxls12X*	This work
HB904	*itr-1(sa73); egl-8(e2917)V; oxls12X*	This work
HB905	*itr-1(sa73)IV; him-5(e1467)*V*;oxls12X; jwEx608[itr-1(+)pJL52]*	This work
HB906	*itr-1(sa73)IV; him-5(e1467)V;oxls12X; jwEx906[unc-47p::itr-1(+)]*	This work
HB907	*itr-1(sa73)IV; him-5(e1462)V;oxls12*X *; jwEx628[unc-119p::itr-1(+)]*	This work
HB940	*jwEx800[itr-1(+)pJL52]*	This work
HB943	*jwEx800[itr-1(+)pJL52]*	This work
HB945	*itr-1(sa73)IV; oxls12X; vha6p::itr-1; jwEx648[vha-6p::itr-1(+)]*	This work
HB946	*eri-1(mg366)IV; lin-15b(n744) egl-8(e2917)X*	This work
HB947	*eri-1(mg366)IV; lin-15b(n744) egl-8(e2917)X*	This work
HB948 [b]	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; egl-8(e2917)V*	This work
HB951 [b]	*Ipp-5 ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; ipp-5(sy605)X*	This work
HB954 [b]	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; itr-1(sa73)IV*	This work
HB955 [b]	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]*	This work
HB958 [b]	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; itr-1(sa73)IV; Ex[myo-2p::GFP for; myo-2p::GFP rev]*	This work
HB961 [b]	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; Ex[myo-2p::GFP for; myo-2p::GFP rev]*	This work
HB968 [b]	*Ipp-5 ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; ipp-5(sy605)X; Ex[myo-2p::GFP for; myo-2p::GFP rev]*	This work
HB971 [b]	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; itr-1(sa73)IV; Ex[myo-2p::GFP for; myo-2p::GFP rev; myo-2p-*	This work

	::itr-1(cdna)	
HB973 [b]	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; itr-1(sa73)IV; Ex[myo-2p::GFP for; myo-2p::GFP rev; myo-3p-::itr-1(cdna)*	This work
HB974 [b]	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; itr-1(sa73)IV; Ex[myo-2p::GFP for; myo-2p::GFP rev; vha-6p::itr-1(cdna)*	This work
HK330	*unc-68(hk30)V*	[18]
JT73	*itr-1(sa73)IV*	[19]
KP3948	*eri-1(mg366)IV; lin-15B(n744)X*	[20]
LT121	*dbl-1(wk70)V*	[21]
MT1083	*egl-8(n488)V*	[22]
NL2099	*rrf-3(pk1427)II*	[23]
PS3653	*ipp-5(sy605)X*	[24]
PS2286	*unc-38(x20) lfe-2(sy326)I*	[25]
RM2221	*egl-8(md1971)IV*	[26]
SD1347	*ccIs4251 I [myo-3p::GFP-LacZ(NLS)) + pSAK4 (myo-3p::mitochondrial GFP) + dpy-20(+)]*	[27]
TM542	*kqt-3(tm0542)II*	S. Mitani unpublished
TU3335	*lin-15B(n744) X; uIs57 [unc-119p::YFP + unc-119p::sid-1 + mec-6p::mec-6]*	[28]

Notes:

[a.] N2, CB138, CB540, CB6614, CB5584, CZ252, EG1285, HK30, JT73, LT121, KP3948, MT1083, NL2099. PS2286, PS3653, RM2221, SD1347 were supplied by the *Caenorhabditis* Genetics Centre (University of Minnesota). TM542 was a gift of Prof. S. Mitani at the NBRP (Tokyo Women's Medical University School of Medicine).

[b.] Only relevant insert details are given here.

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