IP$_3$ signalling regulates exogenous RNAi in \textit{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 \textit{Caenorhabditis 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 conserved cell signalling pathways can modify RNAi responses, implying that RNAi responses may be influenced by an animal’s physiology or environment.

Keywords \textit{C. elegans}; calcium signalling; enhanced RNAi; inositol 1,4,5-trisphosphate; RNA interference

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 \textit{Caenorhabditis 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 \textit{C. elegans} and other organisms to exhibit environmental RNAi [5]. For example, feeding \textit{C. elegans} on bacteria expressing dsRNA initiates a widespread RNAi response [8,9]. Studies in \textit{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 \textit{C. elegans} and other animals remains unclear [14].

Whilst \textit{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 \textit{rrf-3} [15] and \textit{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 [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 1A). IP$_3$ production leads to Ca$^{2+}$ release from the endoplasmic reticulum (ER) through a ligand-gated ion channel receptor, the IP$_3$R, which regulates a wide range of processes in animals including \textit{C. elegans} [23]. Here, we show that reducing or increasing IP$_3$ signalling enhances or suppresses the sensitivity of \textit{C. elegans} to RNAi in a broad range of genes and...
tissues. Tissue-specific rescue suggests that IP₃ 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

IP₃ receptor mutants have enhanced RNAi sensitivity

In C. elegans, IP₃ receptors (IP₃Rs) are encoded by a single gene itr-1 (Fig 1A) [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 1). RNAi was performed by feeding in an itr-1 temperature-sensitive allele itr-1(sa73), a further reduction in mRNA levels in itr-1(sa73) exhibits a partial reduction-of-function phenotype but is reasonably healthy. Other widely used RNAi-sensitive strains were also tested (Table 1). We found that itr-1(sa73) worms generally showed stronger and more penetrant RNAi phenotypes than wild-type animals (Table 1, Fig 1B). 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 1C), that is 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 defeca-
tion (kqt-3) [28] and growth (dbl-1) (Supplementary Fig S1) showed that these do not result in increased sensitivity to lin-1 RNAi (Fig 1B).

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 IP₃, 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 1B and 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 1D). 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 1E). Secondly, we used a direct read-out of gene expression by performing RNAI of GFP in animals carrying GFP markers. In one system an unc-47p::GFP transgenic reporter expressed in GABergic 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 1F and J). itr-1(sa73); unc-47p::GFP animals showed a similar reduction in response to GFP RNAI (Fig 1F and J). In a second system (Fig 2A), we measured RNAI of GFP expressed in the body wall muscles using a myo-3p::GFP construct [30,31]. Again itr-1 mutants show an increased effect (Fig 1I).

PLC-β/egl-8 mutants show increased RNAI responses

IP₃ is produced from phosphatidylinositol 4,5-bisphosphate (PIP₂) by a family of phospholipase C enzymes which are activated by cell surface receptors (Fig 1A). To investigate whether ITR-1 modifies RNAI through a canonical IP₃-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 1G). 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 1G) 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 showed increased sensitivity to a wide range of genes (Table 1). egl-8(e2917) animals also showed significant increases in sensitivity in both the unc-47p::GFP reporter (Fig 1F) and body wall muscle GFP systems (Fig 1I). 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 1G and 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 knock-down of 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 1G). 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 IP₃ signalling causes RNAI resistance

To ascertain whether IP₃ signalling is capable of modulating the RNAI response in both directions, we tested whether increasing IP₃,
Figure 1.
signalling could reduce RNAi sensitivity. Initially, we increased expression of the IP₃R 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 1H). Wild-type worms carrying extra itr-1 genes show reduced expression of the unc-15 phenotype. To confirm this result,

Table 1. IP₃ signalling mutants are hypersensitive to RNAi for a variety of genes.

<table>
<thead>
<tr>
<th>RNAi</th>
<th>Phenotype</th>
<th>N2</th>
<th>itr-1 (sa73)</th>
<th>egl-8 (e2917)</th>
<th>rrf-3 (pk1426)</th>
<th>eri-1(mg366); lin-15b(n744)</th>
<th>lin-15b(n744) + unc-119p::sid-1</th>
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<tr>
<td>lin-1</td>
<td>Muv</td>
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<td>lin-31</td>
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<td>Ste</td>
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<tr>
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<tr>
<td>unc-119</td>
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<td>++</td>
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</tr>
<tr>
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<tr>
<td>unc-58</td>
<td>Unc</td>
<td>—</td>
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− = 0–4.9%, + = 5–24.9%, ++ = 25–49.9%, +++ = 50–74.9%, ++++ = 75–100%

RNAi was induced by feeding. Adult Caenorhabditis elegans were placed on a lawn of Escherichia 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, embryonic 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.
**Figure 2.** IP₃ signalling modulates sensitivity to internally induced RNAi.

A RNAi was induced and measured 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 IP₃, itr-1 worms show increased knock-down 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, and the middle line indicates the median. Outliers were not removed. Total number of worms left to right: 38, 43, 41, 39. Data were collected from three independent plates of worms.

C Inositol 5-phosphatase, ipp-5, mutant animals show decreased knock-down 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 in (B). Total number of worms left to right: 40, 62, 41, 62. Data were collected from three independent plates of worms.

Data information: Significance was assessed using Mann–Whitney U-tests and shown as ***P < 0.001. In each case, means were also compared using Student’s t-tests and shown to have similar levels of significance.
we used ipp-5 mutants. IP₃ is metabolised to IP₂ and IP₄ by the enzymes inositol polyphosphate 5-phosphatase encoded by ipp-5 and IP₃ 3-kinase encoded by lfe-2, respectively (Fig 1A). ipp-5 loss-of-function mutant animals are therefore assumed to have increased IP₃ levels (see [26] for discussion). ipp-5 mutants show a substantially reduced response to unc-15 RNAi (Fig 1H). lfe-2 mutants which may also have increased IP₃ 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 1I). Thus, increased IP₃ signalling leads to decreased RNAi sensitivity, and decreased IP₃ signalling causes increased sensitivity demonstrating that IP₃ signalling is able to modulate RNAi sensitivity in worms. We note that we and others have used RNAi in IP₃ signalling mutants to dissect IP₃-mediated signalling pathways [26]. The results of such studies should now be reviewed in the light of these results.

**IP₃ 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 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 2A) [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 2B). Similarly, ipp-5 mutants show decreased sensitivity in both the pharynx and bwm (Fig 2C). Thus, itr-1 mutants show increased sensitivity to internally produced RNAi.

**itr-1 acts in the intestine to modify RNAi responses**

We sought to further clarify the mechanism of IP₃ 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 neurons and the intestine (Ford, Peterkin and Baylis, unpublished data). 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 neurons (unc-47p::itr-1) or in the nervous system in general (unc-119p::itr-1) failed to restore normal RNAi sensitivity (Fig 3A). 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 3A).

**Using IP₃ 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 double mutant 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 4A and 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 animals carrying this allele 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 4C). 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 IP₃ 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, neurons are relatively inaccessible to nucleic acids hampering attempts to modify neurodegenerative diseases. The experiments presented in this work show that the IP₃/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.

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 neurons. However, expression in the GFP-expressing neurons using either the GABAergic neuron promoter unc-47p or a pan-neuronal promoter unc-119p failed to rescue sensitivity. Average of n worms; error bars denote SEM. Number of worms left to right: 7, 5, 6, 7, 33, 18, 39, 42, 41. Data were 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 SEM. 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, and line shows median. Outliers were not removed. Total number of worms left to right: 38, 43, 19, 39, 22. Data were collected from three independent plates of worms.

Data information: Significance was assessed using an unpaired, two-tailed Student’s t-test (A, B) or a Mann–Whitney U-test (C). The results of significance tests are presented as follows: ns, not significant \( P \geq 0.05 \); \(* P = 0.01–0.05\); \(** P = 0.001–0.01\); \(*** P < 0.001\). For data in (C), means were also compared using Student’s t-tests and shown to have similar levels of significance.
Materials and 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 Supplementary Table S1. Worms were cultured using standard techniques [39]. All experiments were performed at 20°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 h 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. 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.

**Supplementary information** for this article is available online: http://embor.embopress.org

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

Conflict of interest
The authors declare that they have no conflict of interest.

References


