IP₃ 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 (IP₃) signalling alters RNAi sensitivity in Caenorhabditis elegans. Reducing IP₃ signalling enhances sensitivity to RNAi in a broad range of genes and tissues. Conversely up-regulating IP₃ signalling decreases sensitivity. Tissue-specific rescue experiments suggest IP₃ functions in the intestine. We also exploit IP₃ 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 C. elegans; calcium signalling; enhanced RNAi; inositol 1,4,5-trisphosphate; RNA interference

Subject Categories RNA Biology; Physiology

DOI 10.15252/embr.201439585 | Received 15 September 2014 | Revised 4 December 2014 | Accepted 15 December 2014 | Published online 21 January 2015


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 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 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 [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 (IP₃) is an important second messenger in animals. IP₃ 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). IP₃ production leads to Ca²⁺ release from the endoplasmic reticulum (ER) through a ligand-gated ion channel receptor, the IP₃R, which regulates a wide range of processes in animals including C. elegans [23]. Here, we show that reducing or increasing IP₃ signalling enhances or suppresses the sensitivity of 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 ([IP₃]Rs) 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 hypervensitive 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)* (Table 1). Assays were carried out at 20°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 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 *rf3*, *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 *rf3*) 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 defecation (*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 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 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 *mys-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 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 IP$_3$R 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. IP$_3$ 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>egf-8 (e2917)</th>
<th>rrf-3 (pk1426)</th>
<th>eri-1(mg366); lin-15b(n744)</th>
<th>lin-158(n744) + unc-119;p:sid-1</th>
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<tbody>
<tr>
<td>lin-1</td>
<td>Muv</td>
<td>−</td>
<td>++</td>
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<tr>
<td>lin-31</td>
<td>Muv</td>
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<td>Dpy</td>
<td>−</td>
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<td>Ste</td>
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<tr>
<td>unc-15</td>
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<td>+++</td>
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<td>+++</td>
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<tr>
<td>unc-55</td>
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<td>+</td>
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<tr>
<td>unc-119</td>
<td>Unc</td>
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<td>+</td>
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<td>+</td>
<td>++</td>
<td>+++ probe</td>
</tr>
<tr>
<td>unc-14</td>
<td>Unc</td>
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<td>+</td>
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<td>nd</td>
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</tr>
<tr>
<td>unc-58</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.
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, iir-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.

Figure 2. IP₃ signalling modulates sensitivity to internally induced RNAi.
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 function might first 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::dsRNA/GFP) [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 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 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). Furthermore, vha-6p::itr-1 partially rescued sensitivity to lin-1 RNAi by feeding (Fig 3B). 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 2B and C). Expression of itr-1 in the intestine restores normal sensitivity in both tissues (Fig 3C).

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 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.

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**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 8 worms; error bars denote SEM. Number of worms left to right: 7, 5, 6, 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* ≥ 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

**Acknowledgements**

We thank A. Fire, K. Ford, S. Mitani and H. Peterkin for the provision of plasmids and strains. Some strains were provided by the CGC, which is
funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Other strains were provided by the Mitani Lab through the National Bio-Resource Project of the MEXT, Japan. We are grateful to J. Ahringer, B. Olofsson and members of the Baylis group for helpful discussions. AIN was funded by Trinity Hall College, Cambridge and the Cambridge European Trust. The work of MDS and RPV-M was partially funded by a Miguel Servet Grant (CP11/00090) from the Health Research Institute Carlos III, which is partially supported by the European Regional Development Fund. RPV-M is a Marie Curie fellow (CIG322034). RG was funded by the MRC (G0601106).

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.

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