Investigating the Role of the IP_3 Signalling Pathway in RNA Interference in *C. elegans*

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This thesis is submitted for the degree of *Doctor of Philosophy*
September 2019

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Summary

Alice Elizabeth Mary Rees

Investigating the role of the IP_3 signalling pathway in the regulation of RNA interference in *C. elegans*

Both inositol 1,4,5-trisphosphate (IP_3) mediated signalling and RNA interference are widespread processes with fundamental roles in animal cell function. In the nematode *C. elegans* these two pathways have been shown to intersect such that IP_3 signalling mutants display an altered exogenous RNAi response. IP_3 is a key second messenger in the transduction of intracellular signals. Produced by the hydrolysis of membrane phospholipid PIP_2 by phospholipase C (PLC), IP_3 triggers Ca^{2+}release from internal stores by binding to the IP_3 receptor, a ligand gated calcium channel in the ER, thus triggering downstream calcium signalling pathways. It has been previously shown that loss of function in the IP_3 receptor (ITR-1) or in PLCβ (EGL-8) results in an enhanced response to exogenous dsRNA, with a more robust silencing response than that seen in WT worms. Conversely the potentiation of IP_3 signalling by the loss of function IPP-5, a phosphatase which catalyses the hydrolysis of IP_3, results in resistance to RNAi.

In order to better understand the context of the IP_3 signalling events which are regulating the RNAi response I sought to identify the upstream activator of EGL-8 in this mechanism. EGL-8 is canonically activated by $G\alpha_q$ homologue ELG-30. However, by screening the RNAi responses of Gα signalling mutants using a range of assays, EGL-30 was ruled out as an activator of EGL-8 in the pathway regulating RNAi, since EGL-30 loss of function does not result in an enhanced RNAi response. The $G\alpha_{o/i}$ homologue, GOA-1, was identified as the most likely activator of EGL-8 due to the strongly enhanced RNAi response in *goa-1* loss of function mutants in a number of assays. Other Gα subunits and known regulators of GOA-1 and EGL-30 signalling were also analysed.

The RNAi response is a multistep process in which the exogenous RNAi pathway must compete with the closely related and interlinked endogenous small RNA pathways for resources. With the aim of better understanding how changes in IP_3 signalling might be influencing the RNAi response, small RNA sequencing was performed to look for evidence of change in the endogenous small RNA pathways of *itr-1* mutants, and followed up with qPCR. However, no substantial changes to the endogenous small RNA pathways were found.

Downstream of the production of primary siRNAs from exogenous dsRNA, separate argonautes mediate cytoplasmic and nuclear RNAi responses. I utilised an established assay to test for effects of IP_3 signalling on nuclear RNAi. The results suggest no specific alterations to the nuclear RNAi pathway in these mutants.

Acknowledgements

First and foremost, I would like to thank my supervisor Howard Baylis for his continual support and encouragement throughout this process. As well as teaching me a great deal about *C. elegans* you have always offered the help feedback I needed, often at very short notice. Your support has been invaluable. I would also like to thank Eric Miska and the members of the Miska lab for allowing me join in their lab meetings, and Melanie Tanguy for her guidance in producing the small RNA sequencing libraries. In the Zoology department, I would like to thank James Savage, whose advice on statistics was a huge help when I couldn't decide how best to analyse my data, Riva for broadening my knowledge of fish, ensuring that I have learnt something about animals larger than the worm, and in particular Helen, for keeping me entertained around the lab and through many a tea break and crossword session. Beyond the department, thank you to all the friends who have kept me who have helped and encouraged me, especially Sarah who has been an incredibly kind and supportive friend from the beginning of my time in Cambridge. I would also like to thank my parents, who have supported me through my many years of education.

Thank you to the BBSRC for funding this work.

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Investigating the Role of the IP_3 Signalling Pathway in RNA Interference in *C. elegans*

Chapter 1

Introduction

Introduction

1.1 *C. elegans* as a model organism

C. elegans is a small free-living nematode which feeds on bacteria found in its natural habitat of decaying plant matter throughout the temperate and tropical regions (for a brief review of the natural history of *C. elegans* see (Félix and Braendle, 2010)). Its small size, short generation time, ease of cultivation in the lab, transparency and convenient genetics are just a few of the reasons why it has become established as a popular model organism; used in the study of a range of biological processes since its popularisation as a genetic model by Sydney Brenner in the 1960s (summarised (Brenner, 2002)) .

The lifecycle of *C. elegans* consists of 4 larval stages, L1-L4, and the reproductive adult stage, with the majority of the population consisting of self-fertilising hermaphrodites. Under stress conditions, such as overcrowding and low food availability, or high temperature the L3 stage is replaced by a long lived, stress resistant dauer state characterised by an absence of feeding and morphological changes. In the absence of food L1 worms can also enter diapause (see (Corsi et al., 2015) for a summary of the lifecycle). The timing of the lifecycle varies with temperature and between strains, but for the classical WT strain Bristol N2, at 20°C the time from hatching to egg laying is approximately 3 days in the absence of dauer or L1 diapause (Byerly et al., 1976) (see Figure 1.1).

The anatomy of *C. elegans* is simple, consisting of single layers of cuticle, hypodermis and body wall muscle surrounding a body cavity, intestine and gonad, with a simple pharynx in the head region leading into the intestine and a limited neuronal network (Figure 1.2) The majority of body structure is identical between the male and hermaphrodite, with very few differences detectable before the L4 stage, although cell lineage differences giving rise to the sex specific structures (tail fan, lack of vulva, and lack of oocyte producing structures in male) are present (Sulston and Horvitz, 1977)(Sulston and Horvitz, 1977)(Sulston and Horvitz, 1977) (Sulston and Horvitz, 1977; Sulston et al., 1980). Facultative males are produced as a result of random non-disjunction of the X chromosome during meiosis and occur at a rate of approximately 1:1000. A self-fertilising hermaphrodite can produce about 300 offspring fertilised using stored sperm produced by the ovitestes in the L4 stage before switching to oocyte production, whilst a mated individual can produced up to 1000 (Corsi et al., 2015). As a consequence of the low frequency of males and rarity of outcrossing events, within strain genetic variation is near null. This is especially true in the lab but even in the wild out crossing

between locally adjacent populations appears to be minimal and genetic bottlenecks common (Barrière and Félix, 2005).

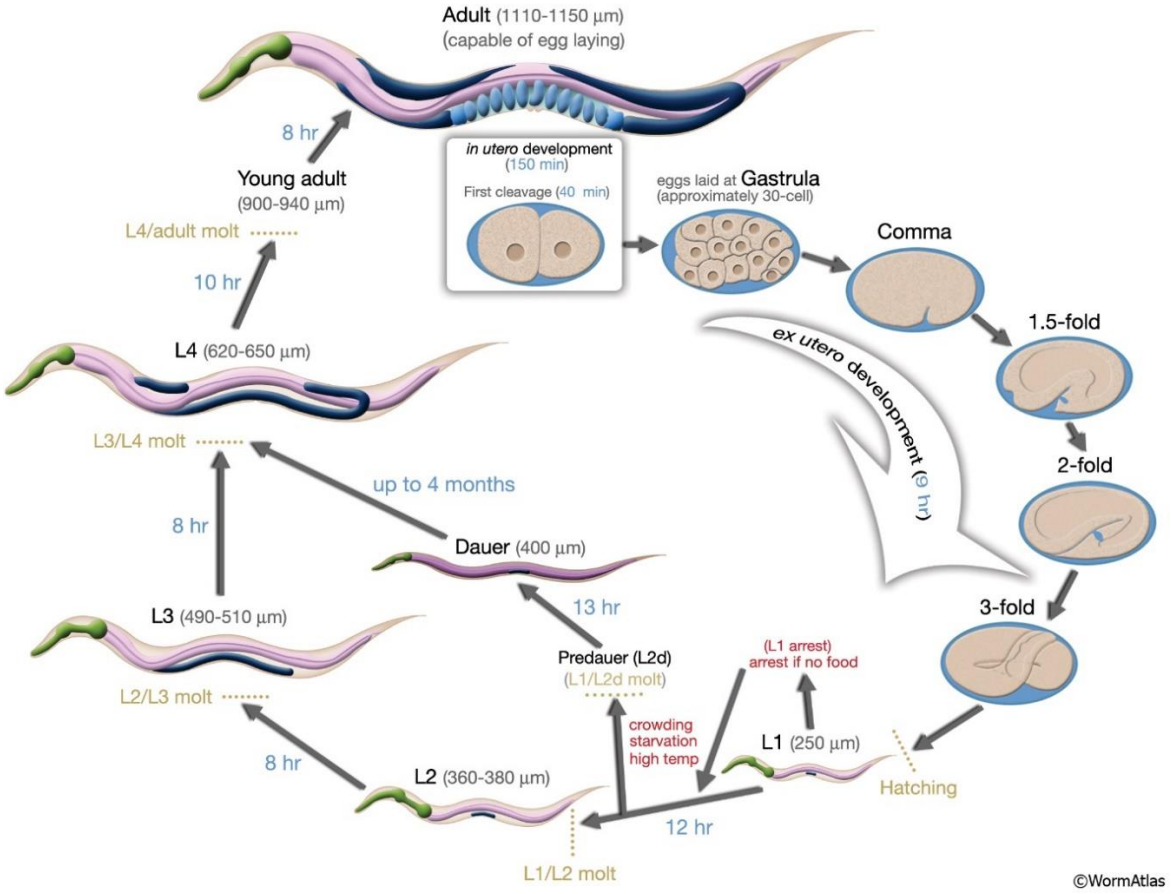


Figure 1.1. Life cycle of *C. elegans* at 22°C under standard conditions. Image reproduced from Introduction to *C. elegans* anatomy (Altun, Z.F. and Hall, D.H. 2009. Introduction. In *WormAtlas*.).

C. elegans development was found to be highly invariant, uniquely so among the model systems, making it a very powerful system to study cell fate. This invariance combined with transparency and limited cell number made it possible for the entire embryonic and postembryonic lineage to be mapped (Sulston and Horvitz, 1977; Sulston et al., 1983). The adult hermaphrodite consists of 949 somatic cells, each of a clearly defined type. Despite its small size, low cell count and simple structure *C. elegans* contains a range of tissue types and displays a surprisingly complex behavioural repertoire. The invariant development and relatively small number of 302 neurones made it possible for the entire neuronal map or connectome to be built using serial electron microscopy (White et al., 1986); a powerful tool to better understand nervous system development and function when coupled to the well-defined behavioural repertoire. The mapping of the patterns of cell division and cell death in *C. elegans* together with a powerful genetic toolkit allowed for the molecular mechanisms of programmed cell death or apoptosis to be investigated and understood (reviewed in(Ellis et al., 1991)).

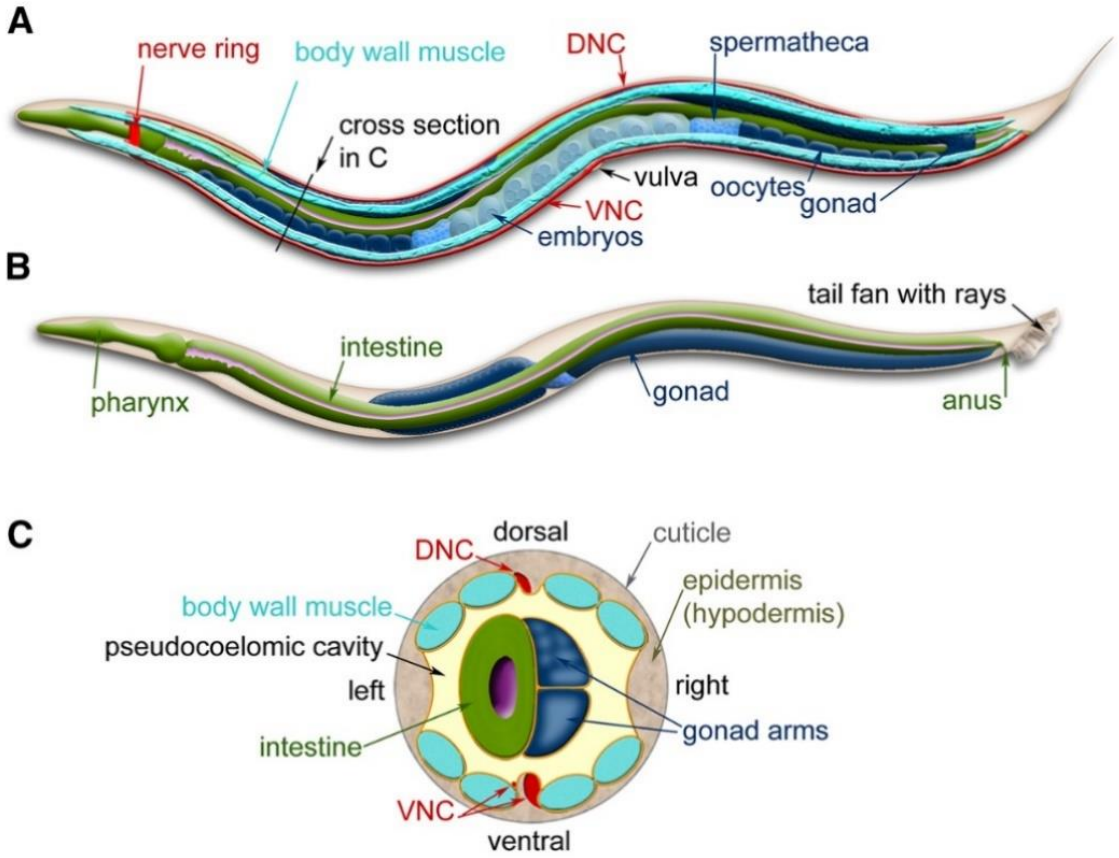


Figure 1.2. Anatomy of C. elegans: **A**- hermaphrodite, **B**- male, **C**- transverse cross section. Image reproduced from (Corsi A.K., Wightman B., and Chalfie M. A Transparent window into biology: A primer on Caenorhabditis elegans (June 18,2015), WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.177.1, http://www.wormbook.org.)

The power of *C. elegans* as a model organism also stems from a well-established genetic map and set of tools to enable genetic manipulation. The diploid genome consists of 5 chromosomes plus the X chromosome (Brenner, 1974). It was also the first animal for which an entire genome sequence was available. The draft genome was completed in 1998 by the *C. elegans* sequencing consortium, paving the way for the Human Genome Project. Despite its apparently simplistic anatomy and physiology and low cell count, the initial genome sequence revealed 19000 protein coding genes; roughly equivalent to the human genome and more than the fly *Drosophila melanogaster* (The *C. elegans* Sequencing Consortium, 1998).

One of the most significant discoveries made in *C. elegans* was that of RNA interference (RNAi)– gene silencing induced by dsRNA (Fire et al., 1998). The potency, specificity and systemic nature of the response in *C. elegans* made it ideally suited to studies probing the mechanisms of RNAi. As the mechanisms of RNAi became clearer so did the fact that the small antisense RNAs, which were identified as the causal agent (Hamilton and Baulcombe, 1999; Zamore et al., 2000), were one of several abundant classes of small silencing RNAs that formed an integral part of the endogenous gene regulatory systems; namely the small-interfering RNAs, microRNAs, and piwi-

related RNAS. The first genes encoding microRNAs had in fact already been identified from *C. elegans* lineage mutants (Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993). The discovery of RNA interference not only unleashed a powerful genetic technique but also precipitated a growing understanding of the mechanisms and roles of the small silencing RNAs. This has been accompanied by the realisation of their extent and importance in endogenous gene regulation and genome defence, not just in *C. elegans* but throughout the eukaryotic kingdom (reviewed in (Ghildiyal and Zamore, 2009; Moazed, 2009; Tabara et al., 1998; Zhuang and Hunter, 2012a)).

1.2 Signal transduction

In order to function efficiently a cell must regulate its behaviour depending on its environmental context. This ability is pivotal to the survival of all forms of life, from unicellular bacteria to multicellular organisms, where each cell has a highly specialised role and must work in concert with surrounding cells and respond appropriately to external environmental cues sensed by distant cells. Effective cell-cell communication and transduction of signals within the cell is therefore crucial, as misinterpretation of a signal by the receiver could lead to aberrant and inappropriate cell activity (for a general discussion see (Marks et al., 2009, chapter 1)). For a cell to respond to an external stimulus a signal must first be detected by the recipient cell. Detection usually occurs at the cell surface membrane- the interface of the inner cell and the outside world- where a great diversity of cell surface receptors have evolved to detect a huge range of highly specific stimuli.

The paradigm of the receptor consists of sensory, transmembrane and catalytic domains, with additional regulatory domains which fine tune the response. Upon stimulation by the agonist a conformational change occurs in the receptor which transmits the signal from the substrate binding or sensory domain, across the cell membrane to intracellular catalytic domain of the receptor which brings about a change inside the cell, often a phosphorylation event. This intracellular change in turn serves as a signal for the activation of further intracellular proteins; triggering a “signalling cascade” which transduces the signal via “second messengers” to effector proteins which bring about the response. The same principles of receptors, transducers, and effectors is also used to transmit signals in response to internal stimuli (such as the metabolic state of the cell), using the same fundamental language of signal transduction: direct contact, conformational changes, phosphorylation events and small molecule second messengers. At each stage along the signalling cascade the receiving party is interpreting and transducing the message. Although the molecular changes seen in members of the signalling cascade are in many ways highly stereotyped the meaning

of the signal is highly context dependent, with meaning attributed to signal by the receiver, not inherent to the messenger (Marks et al., 2009).

As would be expected, detection of a range of unique agonists requires a large repertoire of highly specific receptors, although the majority of this diversity is generated through slight variations of a few very common types of receptor, such as G-proteins coupled receptors or receptor tyrosine kinases; with large gene families and splice variants encoding receptors which have been adapted for each new need. The same theme of re-use and adaptation can be seen in the diversity of molecules and proteins which are involved in signal transduction, with the same, protein types, small molecules, lipids and ions, being utilised again and again in signal transduction across and within the diversity of organisms. In a few cases, such as the nuclear steroid hormone receptors, a single protein acts as both receptor, transducer and final effector. However, the use of second messengers allows for amplification of the initial signal, as well as providing opportunities for integration and divergence of a single signal into a signalling network. Multiple inputs can feed into the same second messenger, allowing for the integration of signalling information via coincidence detection, but also allowing for multiple outputs with parallel, synergistic, or independent signalling roles, as well as multiple stages of regulatory feedback. The same second messenger is even commonly used within the same cell to respond to different stimuli - and yet specificity of outcome is not lost. It follows that the context of the signal, rather than identity of the messenger itself must therefore be crucial to specificity. Identity of the activator(s) and receiver(s), subcellular localisation, timing and spatio-temporal pattering of the signal and cellular context and past exposures all shape the outcome of a signalling cascade. It is also becoming increasingly apparent that cross talk between what were previously considered distinct pathways is commonplace (Hancock, 2010; Marks et al., 2009).

1.2.i Generating diversity and specificity in signalling- calcium signalling

A perfect example of a second messenger being utilised over and over again to achieve a vast variety of different outcomes is Ca^{2+}. Ca^{2+} is a truly ubiquitous second messenger with a key role in the regulation of a vast number of cellular and physiological processes, ranging from immediate effects such as exocytosis and muscle contraction to longer term changes to the transcriptional profile and cell proliferation and development. With such a wide range of potential outcomes how is specificity and discrimination achieved, even when multiple calcium regulated processes co-occur in a single cell? From an extensive toolkit of calcium signalling regulators, buffers, channels, pumps, effectors, transducers and receptors a highly specific combination of proteins and isoforms are expressed and spatially localised in each cell type, influencing the spatio-temporal dynamics of the

production and amplification of the signal as well as the state and availability of the receiver (Berridge et al., 2003).

Ca^{2+} is present in all parts of the cell but at carefully regulated concentrations. The cytoplasmic Ca^{2+} level is kept low, approximately 100nM in most cell types, whilst Ca^{2+} is stored at high concentration in subcellular compartments such as the endoplasmic reticulum and mitochondria. External Ca^{2+} concentration is also higher than cytoplasmic levels, providing two major sources of Ca^{2+} which can be drawn upon for signalling, with the endoplasmic reticulum/sarcoplasmic reticulum being the largest internal reservoir. The relatively low calcium levels compared to potassium, sodium or chloride ions means that most calcium signalling events have little effect on overall membrane potential. The activation of Ca^{2+} ion channels located in the plasma membrane and internal stores allows for cytoplasmic [Ca^{2+}] to be increased (reviewed in (Berridge, 2014)).

Although Ca^{2+} is free to diffuse in the cytoplasm most changes are highly localised and transient, with free calcium quickly bound by local buffers or effectors and then rapidly returned to the resting level by calcium pumps. The temporal pattern and size of the transient signal, playing a key part in signal identity. Protein complexes associated with the channel play a regulatory role in determining the sensitivity of the channel to the agonist or stimulus but also shape the feedback and feedforward loops which regulate the pattern of calcium transient produced. Receptors and effectors too can be closely coupled to the calcium channel though anchoring. Global changes such as calcium waves require the coordinated action of large arrays of channels (reviewed in (Berridge et al., 2003).

1.2.ii The Inositol 1,4,5-trisphosphate signalling pathway

One of the key ligand gated calcium channels is the Inositol 1,4,5-trisphosphate receptor. Inositol 1,4,5-trisphosphate, or IP_3 hereafter, is a small molecule second messenger frequently used in the transduction of a range of cell signalling events throughout the animal kingdom. IP_3 is one of the most frequently utilised members of a family of inositol containing phospholipid derivatives involved in the regulation and transduction of cellular signals (reviewed in (Berridge, 1993; Berridge and Irvine, 1989; Czech, 2000; Hancock, 2010; Payrastre et al., 2001)) (Figure.1.3).

The metabolism and modification of specific phospholipid components of the cell membrane, in particular the phosphoinositides, is crucial to regulating the localisation of key signalling proteins and in the production of small molecule second messengers. The transduction of many extra-cellular signals, including the classical neurotransmitter acetylcholine and the growth

factor EGF, to name but two examples, utilises hydrolysis of phosphoinositides, producing second messengers which trigger a signalling cascade, leading to a range of downstream effects which include calcium mobilisation, protein kinase C activation, arachidonic acid production and guanylate cyclase activation (Berridge, 1984). The inositol containing phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is a minor component of the phospholipid membrane but is highly important in the localisation and activation of key signalling proteins via PH domains, and in the regulation of the actin cytoskeleton and intracellular trafficking (Gilmore and Burridge, 1996; Martin, 2001; Raucher et al., 2000; Toker, 1998).

PIP_2 can be hydrolysed to diacyl glycerol (DAG) and IP_3, catalysed by the phosphodiesterase phospholipase C (PLC). DAG and IP_3 are both able to act as second messengers; with DAG remaining membrane associated and stimulating protein phosphorylation, whilst IP_3 is free to diffuse through the cytoplasm to trigger the mobilisation of intracellular calcium via binding to the IP_3 receptor, a ligand gated calcium channel, localised to the endoplasmic reticulum (ER) . IP_3 production by PLC therefore leads to the release of calcium from the ER to the cytoplasm and the subsequent activation of downstream calcium signalling pathways (Berridge, 1984).

The termination of the IP_3 signal is controlled in part by metabolism of IP_3. IP_3 can be directly converted to inositol 1,3,4,5-tetrakisphosphate (IP_4) by the IP_3 kinase IP3K (*Ife-2* in *C. elegans*) or to inositol 1,4-bisphosphate (IP_2) by inositol polyphosphatase 5-phosphatase (*ipp-5*) (Drayer et al., 1996; Majerus, 1992, 1996) (Figure 1.3). IP3K and IPP-5 can act in different roles to regulate IP_3 levels and the importance of IP3K vs IPP-5 in the negative regulation of IP_3 signalling varies with cellular context (Bui and Sternberg, 2002).

As a frequently utilised second messenger IP_3 has been found to play an essential role in a diverse range of cellular processes; including the initiation of calcium waves accompanying oocyte activation following fertilisation, regulating synaptic plasticity in neurones, platelet activation, cell proliferation, differentiation, metabolism, exocytosis , neuronal response and smooth muscle contraction, in response to a range of neurotransmitters, hormones and growth factors (reviewed in (Berridge, 2009, 2016)).

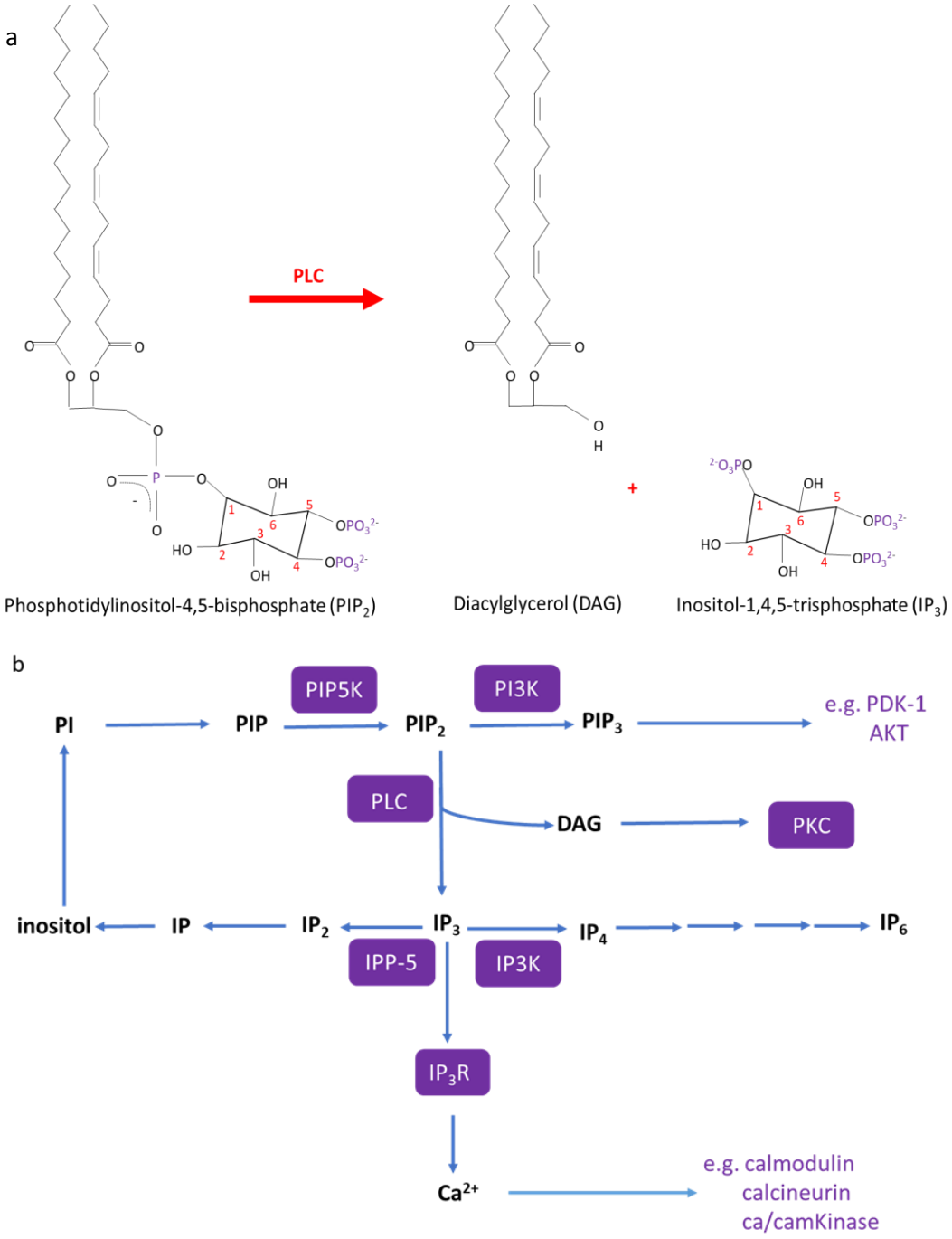


Figure 1.3. Inositol phosphate metabolism. a- The structure of phosphoinositide PIP2 which is broken down to second messengers DAG and IP_3. **b**- Some of the inositol phosphatides, their metabolic relationship, and their downstream effectors.

1.2.iii The IP_3 Receptor

IP_3R was first identified as the protein P400 in mice (Furuichi et al., 1989). A transmembrane glycophosphoprotein, it is most closely related to the ryanodine type Ca2+ receptors (RyR). The IP_3 gated Ca2+ channel follows a popular prototype for cation channels common to both pro and eukaryotes- a functional tetramer, each monomer consisting of 6-span transmembrane channel forming domain and a p-loop which together form a selectivity filter, central cavity and gate. Ligand dependent gating of the C- terminal channel forming domain is regulated by the N-terminal ligand binding domain and modulatory/coupling domains, making for a selective and regulated transmembrane pore (Furuichi et al., 1989, 1994). Specifically each subunit of the IP_3R tetramer can be considered to consist of 5 functional domains as depicted in Figure 1.4 (Uchida et al., 2003), with the latest evidence suggesting that the IP_3 binding sites of all 4 subunits must be filled for effective channel opening (Alzayady et al., 2016). The N-terminal suppressor region is also responsible for the differing IP_3 affinities displayed by the three mammalian IP_3R isoforms (Mikoshiba, 2007).

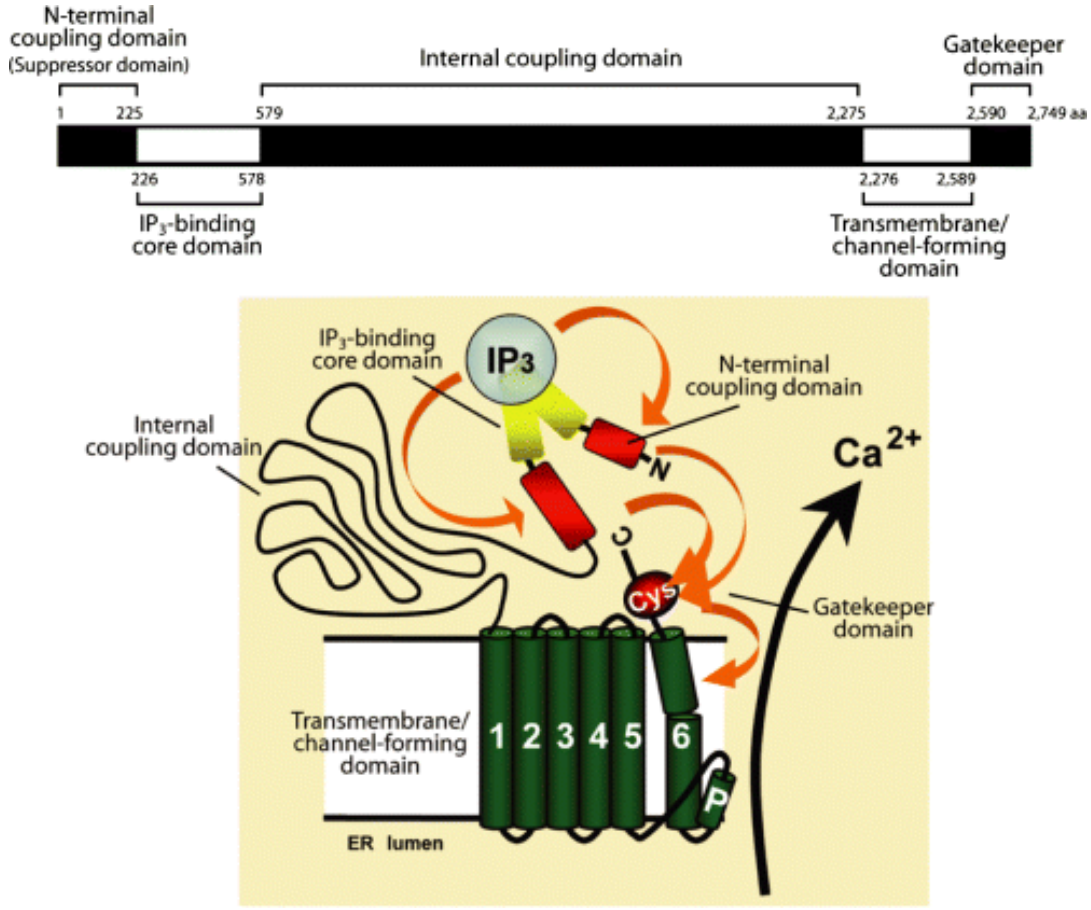


Figure 1.4. The 5 domains model of the mouse IP_3R1 receptor. IP_3 binding triggers a conformational change which is transmitted through the N-terminal coupling and internal coupling domains to the C- terminal gatekeeper domain to allow calcium channel opening. Image reproduced from (Journal of Neurochemistry, Volume: 102, 1426-1446, 2007, DOI: (10.1111/j.1471-4159.2007.04825.x) (Mikoshiba, 2007)).

The IP_3R also displays calcium-induced-calcium-release (CICR) behaviour with calcium acting as an essential co-agonist with IP_3. The bell-shaped response curve of the IP_3R to calcium demonstrates a maximal response around 300nM [Ca^{2+}], after which calcium becomes increasingly inhibitory (Bezprozvanny et al., 1991). However studies using purified IP_3R1 found that high concentrations of Ca^{2+} are not intrinsically inhibitory but require the calcium binding protein Calmodulin to bind bring about this inhibitory effect; with calmodulin binding to IP_3R in a calcium dependent manner at high concentrations of cellular calcium (Michikawa et al., 1999; reviewed in (Taylor and Laude, 2002)). These sequential positive and negative feedback loops are thought to play an essential role in the generation of calcium oscillations, characteristic of signalling via internal store calcium release (Berridge, 1993). For a review of IP_3 receptor structure, function and signalling dynamics see (Mikoshiba, 2007; Prole and Taylor, 2019; Taylor and Tovey, 2010).

As well as being a ligand-gated calcium channel the IP_3R has a central role in the organisation of signalling events as a scaffold for a macro molecular signalling complex, theorised to be important in the tailoring of the outcomes of IP_3 signalling, as is the spatial regulation of calcium release by the subcellular localisation of IP_3R. The regulation of IP_3R localisation through trafficking of ER vesicles and IP_3R mRNA granules along the microtubule cytoskeleton also plays an important role in the regulation of IP_3 signalling (reviewed in (Mikoshiba, 2007; Prole and Taylor, 2016, 2019)).

1.2.iv Phospholipase C (PLC)

A number of different PLC isoforms have been characterised. In mammals these fall into 6 groups: PLC-β, PLC-γ, PLC-ε, PLC-δ, PLC-η and sperm specific PLC-ζ. In *C. elegans* there are 5 PLC genes, plus *pll-1,* a homologue of the catalytically inactive PLC-like protein PLC-L1 (aka PRIP). Of the 5 putative catalytically active PLCs 4 encode clear homologues of the mammalian PLCs: *egl-8 (plc-β), plc-1(plc-ε), plc-3(plc-γ*), *plc-4 (plc-δ), while plc-2* is most similar to *plc-β* (Gower et al., 2005a; Kariya et al., 2004; Lackner et al., 1999; Miller et al., 1999; Shibatohge et al., 1998; Yin et al., 2004) (see Figure 1.5). All PLC isozymes share common structural features, the key characteristic of which are the central catalytic X and Y domains, responsible for the inositol phospholipase cleavage. All but PLCζ also have an N terminal PH domain, which facilitates binding to inositol phospholipids such as PIP_2, followed by an EF hand, calcium binding domain and a more C terminal C2 domain (reviewed in (Katan, 1998; Suh et al., 2008)). Other domains are more type specific and provide specificity of activation (Illenberger et al., 2003; Singer et al., 2002). For instance, each class of PLC is canonically activated by a different class of upstream proteins; PLCγ has been found to be canonically activated by RTKs and contains a number of SH2 and SH3 binding domains. PLCβ has been found to be

activated by heterotrimeric G protein signalling in response to GPCR activation and contains a unique C terminal region shown to be needed for interaction Gα-proteins, and which has GAP activity (reviewed in (Suh et al., 2008)).

1.2.v IP_3 signalling in *C. elegans*

In vertebrates 3 genes encode distinct IP_3R isoforms with different calcium affinities and expression patterns. By contrast in *C. elegans* a single IP_3R gene, *itr-1,* encoding multiple isoforms, has been identified in the *C. elegans* genome (Baylis et al., 1999). Like its mammalian counterparts ITR-1 consists of an N-terminal domain required for IP_3 ligand binding, followed by an internal coupling domain containing MIR and RIH (RyR and IP_3R Homology) domains which are shared between IP_3 receptors and the closely related calcium channels the ryanodine receptors. The C-terminal region is predicted to form an integral ion channel consisting of 6 transmembrane domains and with a pore loop structure between M5 and M6 (Baylis et al., 1999).

The overall expression pattern of *itr-1* is broad with ITR-1 expressed in motor neurones, pharynx, vulva, spermatheca, intestine and rectal epithelial cells and gonad. Numerous alternative isoforms have been identified from *itr-1* cDNA, with variants generated from the use of three alternative start sites and alternative splicing (figure 1.6). 3 alternative promoter regions, upstream of each alternative first exons, direct tissue specific isoform expression. Isoform A is expressed from promoter pA in the vulva, rectal epithelial cells, pharynx terminal bulb, spicule protractor muscles of the proctodeum and in male-specific neuron CP8 or CP9. Expression from promoter pB drives expression in the spermatheca, PDA motor neuron, excretory cells, amphid socket cells, directs expression in the spicule retractor muscles, gubernaculum retractor muscles, posterior oblique muscles, diagonal muscles, and the vas deferens. Expression of isoform D from promoter pC drives expression in the intestine, pharynx isthumus, spermatheca, spermathecal valve and uterine sheath, vas deferens and seminal vesicle (Gower et al., 2001, 2005).

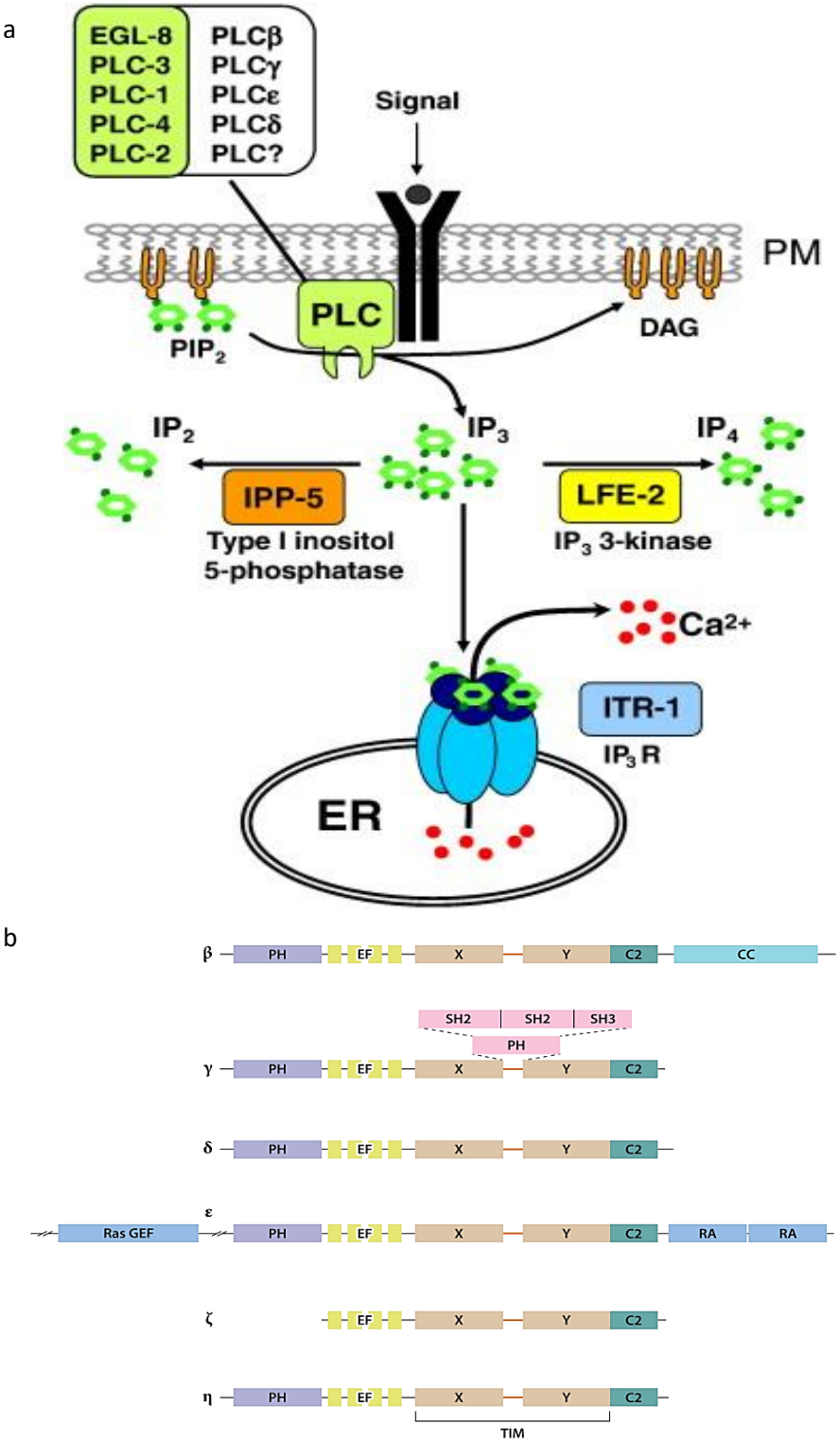


Figure 1.5. PLCs and the IP_3 signalling pathway. a- The 5 *C. elegans* phosoplipase C homologues and their mammalian counterparts catalyse the hydrolysis of PIP_2 to second messengers IP_3 and DAG. IP_3 stimulates release of ca^{2+} from the ER via binding to the IP_3 receptor. IP_3 can be further metabolised by conversion to IP_2 or IP_4. Image reproduced from (Baylis and Vázquez-Manrique, 2012). **b**- The structure of 6 mammalian PLC types, showing key domains. All PLC isoforms contain an EF hand domain and X and Y catalytic domains and C2 domain. All but PLC-ζ also have a PH domain. PLC-β also contains a C-terminal CT domain which allows activation by $G\alpha_q$ and has GAP activity. Reproduced from (Kadamur and Ross, 2013).

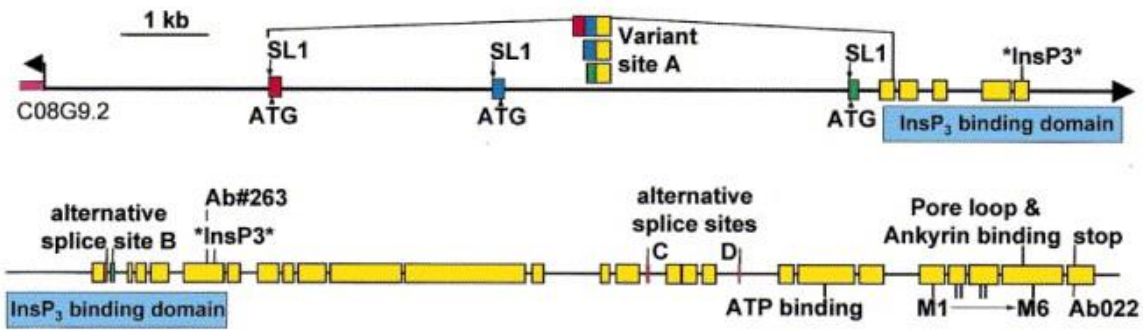


Figure 1.6. **The alternative isoforms of ITR-1** are generated through alternative promoter usage and alternative splicing of the single IP3R gene itr-1. Reproduced from (Baylis et al., 1999).

IP_3 is a key component in the regulation of a number of physiological and cellular processes including, ovulation, contraction of the spermathecal sheath cells, egg laying, movement, pharyngeal pumping, intestinal defecation rhythm, fertility, lifespan and male mating behaviour (for review see (Baylis and Vázquez-Manrique, 2012)). The known IP_3 dependent processes in *C. elegans*, are summarised in table 1.1, together with the upstream PLC and other signalling components.

As is seen in other systems many of the roles of IP_3 and calcium signalling in *C. elegans* display a strong rhythmic component; with the spatio-temporal dynamics of IP_3 induced Ca^{2+} signalling being a key regulator of the frequency and/or pattern of the rhythmic contractions of pharyngeal muscle in pharyngeal pumping, the intestinal muscles in the defecation cycle and the myoepithelial sheath and spermathecal contractions controlling ovulation. In each case disruption of the IP_3 signalling pathway affects the rhythm and/or amplitude of the contractions.

Successful ovulation requires the carefully regulated IP_3/Ca^{2+} signalling driven high frequency contractions of the sheath cell and correct timing and magnitude of the contraction and dilation the spermatheca (Bui and Sternberg, 2002; Clandinin et al., 1998). IP_3 signalling is downstream of two secreted ligands; MSP is produced by sperm in the spermatheca whilst LIN-3 is produced by the proximal maturing oocyte. MSP triggers both IP_3 dependent oocyte maturation and IP_3 dependent basal contractile activity of the sheath cell via PLC-3. LIN-3/LET-23 acts via LIN-1 to trigger IP_3 signalling regulating both sheath cell and spermathecal contractions. The importance of precise IP_3 regulation in this system is highlighted by the complex range of subtly different phenotypes of failure in ovulation seen in the IP_3 signalling mutants (Bui and Sternberg, 2002; Clandinin et al., 1998; Kariya et al., 2004; Yin et al., 2004). Sterility can be rescued by loss of function of IPP-5 but is worsened by LFE-2 mutation, both of which negatively regulate the IP_3 signalling pathways regulating ovulation (Bui and Sternberg, 2002; Clandinin et al., 1998; Kariya et al., 2004a; Yin et al., 2004).

The rate of pharyngeal pumping in *C. elegans* is regulated depending on food availability, increasing from 100 pumps/min to 225 pumps/min when food is present. IP_3 signalling in the

pharynx acts downstream of *egl-30* activated *egl-8* and upstream of serotonin induced contractions of the pharyngeal muscle to regulate this response to food. The IP_3 mediated regulation of the response to food in this instance is dependent on interaction of the IP_3R with myosin II, loss of this interaction leads to a loss of increased pharyngeal pumping in response to food (Walker et al., 2002b, 2002a).

The signals and rhythms which define the defecation cycle have been studied in some detail however some aspects are yet to be fully understood. In WT worms on food a repeated and coordinated pattern of intestinal contractions drive a 50s defecation cycle. The defecation motor program consists of 3 steps: contraction of the posterior body wall muscles (pBoc), contraction of the anterior body wall muscles (aBoc), contraction of the enteric muscles and enteric sphincter relaxation (Exp). Though largely invariant in WT fed worms, lack of food leads to a pause in the cycle while disruption of IP_3 or Ca^{2+} signalling causes mutants to display an altered and dysregulated rhythm. IP_3 dependent calcium release in the intestinal cells is the central timekeeper of this rhythm of which the IP_3R is a central component (Dal Santo et al., 1999). It was suggested that the intestinal rhythm could be regulated by either the rate of activation or rate of shut off of the calcium wave. Further studies have led to a model whereby propagation of the calcium waves from the posterior to the anterior intestinal cells coincides with the initiation of pBoc contraction and is necessary to initiate the further stages of the defecation cycle. IP_3 mediated calcium wave propagation to the anterior of the intestine acts upstream of the DVB and AVL motor neurones to coordinate the pBoc and Exp steps (Teramoto and Iwasaki, 2006; Walker et al., 2002).

A number of roles for IP_3 signalling in development have also been identified. Disruption of IP_3 signalling though expression of an IP_3 sponge results in a high degree of embryonic lethality and PLC-1 dependent IP_3 signalling is also a key signal in the regulation of epidermal morphogenesis (Thomas-Virnig et al., 2004; Vázquez-Manrique et al., 2008; Walker et al., 2002).

IP_3 is also known to act neuronally in the regulation of the avoidance response and male mating behaviours. In the ASH neurones which mediate the avoidance response EGL-8 and EGL-30 both act up stream of ITR-1 to regulate locomotion in response to nose touch and benzaldehayde (Walker et al., 2009). ITR-1 and EGl-8 are also involved in the regulation male mating behaviour, being required for both correct turning behaviour and spicule insertion(Gower et al., 2005).

Many more processes in *C. elegans* are regulated by PLC dependent but not IP_3 dependent pathways, instead acting via DAG and a Protein kinase C triggered phosphorylation cascade. For example EGL-8 is also known to act neuronally, again downstream of its canonical effector EGL-30, in the control of ACh and neuropeptide release from the neurones regulating locomotion, however

DAG is the primary downstream effector in this case (Lackner et al., 1999), although IP_3 signalling also plays a role (Peterkin & Baylis, unpublished). A summary of IP_3 regulated processes in *C. elegans* are given in table 1.1.

Process	Upstream PLC	Other upstream components	Experimental modulation of IP_3 pathway	Notes	References
epidermal migration	*plc-1, egl-8*		*plc-1* overexpression *egl-8* overexpression *itr-1(jc5)* *itr-1(sa73)*		(Thomas-Virnig et al., 2004; Vázquez-Manrique et al., 2008)
gastrulation			IP_3 sponge *itr-1(sa73)*		(Walker et al., 2002)
Other embryonic defects			IP_3 sponge *itr-1(sa73)*		(Thomas-Virnig et al., 2004; Walker et al., 2002)
ovulation (myoepithelial sheath cell signalling)	*plc-3* *plc-1*	*lin-3, let-23, pip5k*	*itr-1 gf (sy327)* *itr-1 (sa73)* *itr-1 RNAi* *ipp-5 (sy605)*	Requires PM Ca^{2+} entry for refill	(Bui and Sternberg, 2002; Chi and Reinke, 2009; Clandinin et al., 1998; Hiatt et al., 2009; Kariya et al., 2004a; Norman et al., 2005; Yin et al., 2004)
Basal contractile activity of sheath cell	*plc-3*	*MSP/vab-1, pip5k*	*IP_3 sponge*		
ovulation (spermathecal cell signalling)	*plc-1*	*Lin-3/ let-23, pip5k*	*Ipp-5(sy605) and rescue* *lfe-2 lof* *itr-1 gof*	Emo phenotype	
oocyte meiotic maturation		*MSP/let-23?*	*itr-1 (sa73)* *itr-1 gof* *itr-1* RNAi		(Corrigan et al., 2005)

defecation	*plc-3*	*vav-1, ced-10, mig-2, rho-1*	*itr-1(sa73)* *itr-1 (jc5)* *itr-1 (n2559)* *ipp-5 lof* *lfe-2 lof* IP_3 sponge	*egl-8* also involved but not via IP3	(Dal Santo et al., 1999; Norman et al., 2005; Teramoto and Iwasaki, 2006; Walker et al., 2002a)
pharyngeal pumping	*egl-8*	*egl-30*	IP_3 sponge *itr-1 (sa73)* *itr-1(tm902)*		(Walker et al., 2002a)
male mating	*egl-8*		*itr-1 gf* *itr-1 (sa73)* *itr-1* RNAi		(Gower et al., 2005)
lifespan/healthspan	*plc-3*	*let-23, lin-3, hpa-1/2*	*itr-1(sa73)* *itr-1 gof*	*daf-16* independent	(Iwasa et al., 2010)
neuronal modulation			*itr-1 (sa73)*		(Hukema et al., 2006; Kindt et al., 2007)
necrosis			*itr-1 (sa73)*	Srp-6 induced cell death	(Luke et al., 2007; Xu et al., 2001)
mechanical avoidance-ASH	*egl-8, plc-3*		*itr-1 RNAi* *egl-8 neurone specific rescue* *plc-3 neurone specific rescue* IP_3 sponge		(Walker et al., 2009)

Table 1.1. The many roles of IP_3 signalling in *C. elegans.*

1.2.vi GPCRs and Heterotrimeric G-Proteins

G-protein coupled receptors are a highly diversified family of 7 span transmembrane receptors which are associated with a membrane bound G-protein heterotrimer through which the signal is transduced. In their inactive state heterotrimeric G proteins consist of a Gβγ dimer bound to a Gα:GDP. In the standard model of heterotrimeric G protein signalling, agonist binding the GPCR causes a conformational change which causes the GPCR to act as a GEF, stimulating GDP to GTP exchange at Gα. GDP for GTP exchange acts as a molecular switch resulting in release of Gα from Gβγ which can each activated downstream effectors. Upon GTP to GDP hydrolysis the Gα re-associates with the Gβγ, terminating the signal. Most Gα subunits have intrinsic GTPase activity which can be accelerated by interaction with GTPase activating proteins (GAPs), such as RGS proteins. The remarkable diversity of the GPCRs is matched by a prolific range of downstream effectors (see Figure 1.7) and a complex network of regulators of G- protein signalling (reviewed in (Marks et al., 2009; Ross and Wilkie, 2000; Siderovski and Willard, 2005; Syrovatkina et al., 2016)).

All 4 classes of mammalian Gα subunits have well established cannonical effectors (summarised (Berridge, 2014; Marks et al., 2009)). $G\alpha_o$ activates adenylyl cyclase, an action which is opposed by $G\alpha_i$. $G\alpha_i$ can also stimulate PI3 kinase γ and PLCβ. $G\alpha_{12/13}$ activates rhoA via rhoGEF. Finally Gαq signals via PLCβ to activate a range of downstream effectors including the IP_3R and DAG kinase or via Trio to stimulate the MAPK pathway (Frooninckx et al., 2012). Known Gβγ effectors are varied and include PLCβ, G-protein coupled receptor kinase (GRK), adenylyl cyclase 2, and ion channels including muscarinic potassium channel GIRK, and calcium channel α1B (Ford et al., 1998). Through these effectors the downstream outcomes of Gβγ activation may be synergistic with those of the Gα subunit or divergent. PLCβ family isozymes can also be activated by both the α and βγ subunits and by Rho family small G proteins (Illenberger et al., 2003).

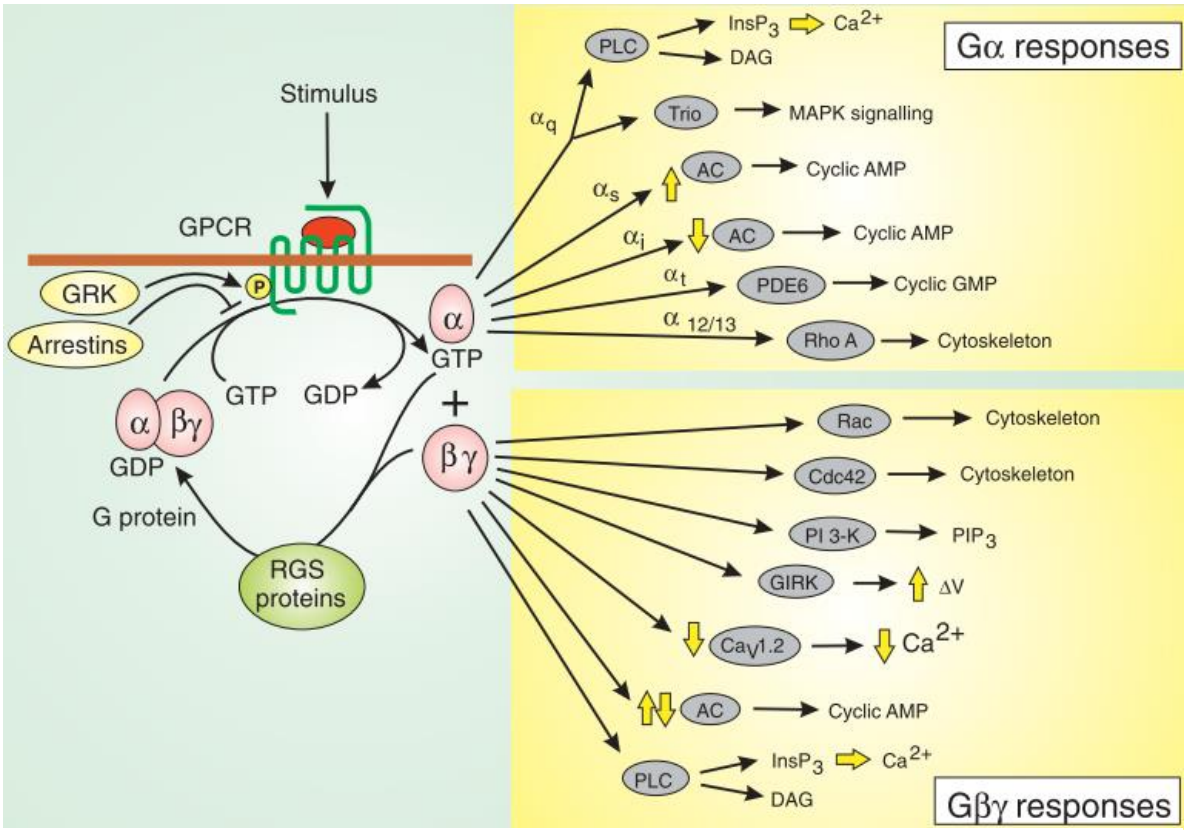


Figure 1.7. The diversity of heterotrimeric G-protein effectors downstream of the GPCR associated G-protein cycle. In the active conformation both $G\alpha^{GTP}$ and Gβγ subunits are known to exert a range of effects. Image reproduced from Berridge, M.J. (2014) Cell Signalling Biology[module 2].

1.2.vii Heterotrimeric G-protein signalling in C. *elegans*

The pairing of different Gα subunit isomers with different Gβ and Gγ subunits is one way of generating specificity and diversity in G-protein signalling as each functional heterotrimer will display slightly different signalling dynamics. The human genome encodes 23 Gα, 5 Gβ and 12 Gγ genes, allowing for a great deal of specificity to be generated through the many possible αβγ combinations. By comparison in the *C. elegans* genome there are only 2 Gβ and 2 Gγ homologues encoded but 21 Gα genes. The Gγ homologue GPC-2 is widely expressed while GPC-1 is only expressed in select neurones. Both GPB-1 and GPB-2 are widely expressed though GPB-2 is not essential for viability. It follows logically that the majority of diversity and specificity must be dependent on the identity of the Gα subunit; with the 21 Gα isomers, representing 1 homologues of each mammalian Gα class- GSA-1 (G_s), GOA-1 (G_i), EGL-30 (G_q) and GPA-12 ($G_{12/13}$)- the remainder show no obvious homology to particular mammalian subunits. This combinational variation, though not insignificant is small compared to the 1300 GPCRs encoded in the *C. elegans* genome, most of which are so far orphan receptors (reviewed in (Bastiani et al., 2006; Koelle, 2016; Wilkie, 2000)).

Of the 21 Gα genes *goa-1, egl-30, gsa-1, gpa-7, gpa-16, gpa-12, gpa-10, gpa-9, gpa-15,* and *gpa-14* are expressed in a range of neuronal and other tissue types whilst *gpa-17* is expressed only in the intestine and *gpa-1*, *gpa-3, gpa-4, gpa-5, gpa-6, gpa-8, gpa-11 and odr-3* are expressed in subsets of neurones, mostly sensory, and seem to be involved principally in chemosensation. GPA-2 and GPA-3 also have roles in the Dauer decision, through their roles in detection of the Dauer pheromone. GPA-5 is also involved in the negative regulation of RAS/MAPK signalling during vulval development (reviewed in (Bastiani et al., 2006)). The roles of each of the mammalian Gα homologues and β/γ effectors in *C. elegans* are many and varied, including many essential developmental processes (reviewed in (Matúš and Prömel, 2018) and numerous physiological and neuronal roles (Koelle, 2016).

In *C. elegans,* as in other systems PLCβ /EGL-8 is canonically activated by heterotrimeric G-protein signalling, specifically by heterotrimers containing the $G\alpha_q$ homologue EGL-30. EGL-30 activated PLCβ signalling has been found to regulate many processes in *C. elegans* including ovulation, movement and viability, intestinal rhythm and the release of neurotransmitter acetylcholine (ACh) in synaptic transmission (Brundage et al., 1996; JH and M, 2000; Lackner et al., 1999). But EGL-30 has also been found to act in a PLCβ independent manner in the regulation of egg laying (Bastiani et al., 2003), acting through the RhoGEF UNC-73(trio) in this and a number of other instances including locomotion and growth (Williams et al., 2007).

Heterotrimeric G protein subunits Gα GOA-1($G\alpha_o$), EGL-30 ($G\alpha_q$), and GPA-12 ($G\alpha_{12}$) and GSA-1 (Gαs) all have roles in the C. *elegans* motor neurones and act pre- and post- synaptically in a variety of behaviours. GOA-1, EGL-30 and GPA-12 all contribute to the regulation of DAG levels in the neurones regulating body wall muscle contractions in locomotion. EGL-30 acts downstream of mAChR to activate EGL-8 leading to DAG production. GOA-1 acts downstream of a serotonin activated GPCR to antagonise EGL-30 signalling. EGL-30 and GPA-12 both activate DAG kinase inhibitor RHO-1, via the RhoGEFs UNC-73/Trio and RHGF-1 respectively, to reduced breakdown of DAG. A similar circuit of G-protein signalling regulates the control of egg laying, with GOA-1 and EGL-30 acting antagonistically upstream of EGL-8. GSA-12 also stimulates neuropeptide release via adenylate cyclase activation leading to a cAMP dependent pathway. GOA-1 and EGL-30 mutants also both show defects in neuronal migration, male mating behaviour and slow pharyngeal pumping. (Perez-Mansilla and Nurrish, 2009).

Outside of the neuronal networks heterotrimeric G- protein signalling is a key regulator of in a diverse range of processes and pathways. EGL-30, GOA-1 and GSA-1, GPA-12, GPA-7 and GPA-16 all act in a variety of muscles. GSA-1 also promotes growth and is required for viability after hatching. It

also has roles in gametogenesis and morphogenesis and excess GSA-1 activity leads to neurotic neuronal cell death. GOA-1 and GPA-16 act redundantly in spindle positioning during embryogenesis, and GOA-1 null mutants display a low frequency of embryonic lethality and partial sterility. EGL-30 is also required for viability after hatching and loss of function mutants frequently arrest during larval development. GOA-1 and EGL-30 also affect vulval development (reviewed in (Bastiani et al., 2006)).

1.2.viii Modulation of G-protein signalling

The activity of heterotrimeric G-protein signalling is initiated by the GEF activity of the agonist bound GPCR with the length of the signal determined by the strength of the GTPase activity of the Gα subunit. This activity is modified by interactions with a range of regulatory proteins. GTPase Activating Proteins (GAPs), including RGS domain containing proteins (Koelle, 1997) act to stimulate the intrinsic GTPase activity of Gα proteins, hastening the conversion of GTP to GDP and hence the termination of the signal. This activity is opposed by guanonucleotide exchange factors (GEFs), which encourage the exchange of GDP for GTP, dissociation of the heterotrimer and initiation of the signalling (reviewed in (Siderovski and Willard, 2005)). Numerous interactions fine tune signal transduction by G-proteins. It is common for effector proteins such as PLCβ to have GAP activity and hence contain an intrinsic negative feedback mechanism for the termination of the signal (Koelle, 1997). In *C. elegans* GEFs such as RIC-8 are in turn regulated by AGS proteins such as AGS-3 (Hofler and Koelle, 2011; Tall et al., 2003) to promote signalling whilst RGS proteins such as RGS-1, RGS-2, EAT-16 and EGL-10 promote its termination. In some cases interaction of the RGS proteins with the Gα subunit is also dependent on the Gβ5 homologue GPB-2 (Van der Linden et al., 2001; Robatzek et al., 2001).

The roles of many G-protein signalling regulators can be clearly seen in the regulation of the locomotive and egg laying networks in *C. elegans* in which GOA-1 and EGL-30 signal antagonistically in a neuronal signalling networks upstream of serotonin. The antagonism between $G\alpha_o$ and $G\alpha_q$ is mediated by a number of The RGS protein EAT-16 was found to supress the lethargic phenotype of constitutively active GOA-1 (Hajdu-Cronin et al., 1999). It was further found that EAT-16 and EGL-10 selectively inhibit action of $G\alpha_q$ and $G\alpha_o$ respectively, with specificity provided by an N-terminal region of each RGS. Inhibition of the opposing Gα subunit by EAT-16 and EGL-10 is necessary for signalling of the opposing subunit. EAT-16 and EGL-10 loss of function mutants display phenotypes similar though less severe to that of GOA-1 and EGL-30 respectively (Dong et al., 2000; Hajdu-Cronin et al., 1999; Patikoglou and Koelle, 2002). The $G\beta_5$ ortholog GPB-2, encoded by *eat-11* interacts with GOA-1, EGL-30, EGL-10 and EAT-16 and is required by the RGS proteins to regulate the antagonism in

this circuit with loss of GPB-2 causing a reduction in EAT-16 and EGL-10 activity (Chase et al., 2001; Robatzek et al., 2001). The membrane targeting subunit RSPB-1 is also necessary for EAT-16 function (Porter and Koelle, 2010). The GEF RIC-8/synembryn activates EGL-30 and is itself regulated by AGS-3 (Hofler and Koelle, 2011; Lackner et al., 1999; Miller et al., 2000; Tall et al., 2003).

The calcium/calmodulin-dependent protein kinase II (CaMKII) and the calcium/ calmodulin-dependent protein phosphatase calcineurin are also involved in the regulation of the EGL-30/GOA-1 signalling network in both locomotion and egg laying. In response to high cellular calcium CaMKII and calcineurin act antagonistically to promote GOA-1 and EGL-30 signalling respectively (Lee et al., 2004; Robatzek and Thomas, 2000).

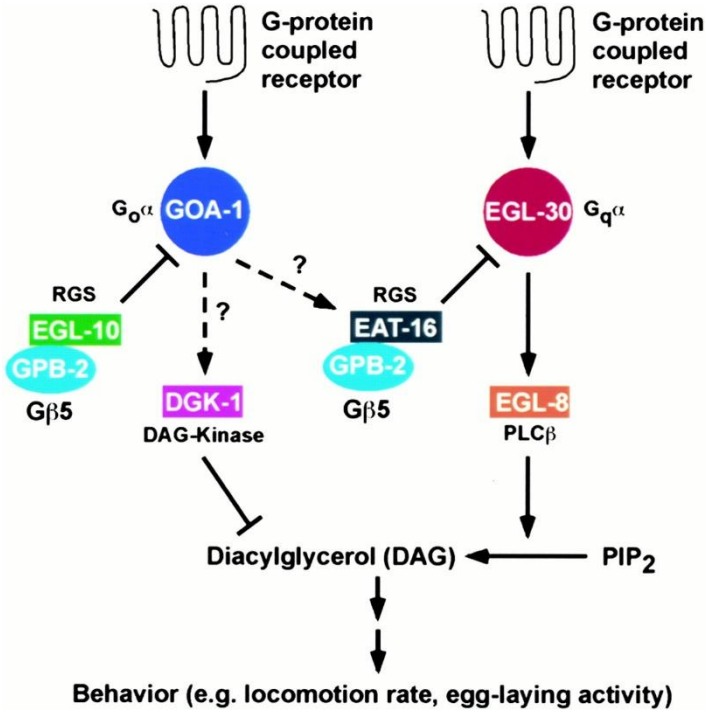


Figure 1.8 GOA-1 and EGL-30 act antagonistically in the neuronal signalling pathways regulating egg-laying and locomotion. Models suggest that the RGS proteins EAT-16 and EGL-10 interact directly with GOA-1 and EGL-30 respectively, facilitated by GPB-2. GOA-1 signalling inhibits the action of the EGL-30 pathway, perhaps by direct activation of EAT-16, or via DAG-kinase. A reciprocal negative regulation of GOA-1 signalling by EGL-30 has also been suggested. Image reproduced from (Van der Linden et al., 2001).

1.3 RNA Interference and small silencing RNAs

One of the key roles of intracellular signalling pathways is the regulation of cell behaviour via the regulation of gene expression. One family of epigenetic regulatory mechanisms, the importance of which has become increasingly apparent over the last two decades, are the small silencing RNAs which act through an RNA induced silencing complex or RISC. This family of RNA interference-based mechanisms are evolutionarily ancient (reviewed in (Carthew and Sontheimer, 2009; Shabalina and Koonin, 2008; Zamore, 2002)), being found throughout the eukaryotic tree, where the processes have diversified from origins as an antiviral defence mechanisms, to play important roles in the regulation of endogenous gene expression, genome surveillance, and protection from foreign genetic elements (reviewed in (Ghildiyal and Zamore, 2009; Moazed, 2009)).

The effects of so called 'antisense RNA' on mediating gene silencing in plants, fungi and animals had been previously reported, and manipulated; however it was only in 1998 that dsRNA was identified as the key trigger in RNA mediated gene silencing or RNA interference (Fire et al., 1998). The observation that long dsRNA could induce gene silencing in *C. elegans* coincided with observations of various forms of virus and transgene induced post transcriptional gene silencing phenomena such as co-suppression, in plants and fungi (Cogoni and Macino, 1997; Ratcliff et al., 1997; Ruiz et al., 1998; Sijen et al., 1996). As understanding of the mechanisms of RNAi grew it was quickly realised that these processes were related and that the same core machinery and mechanisms were also required for maintaining transposon silencing (Ketting and Plasterk, 2000; Ketting et al., 1999; Montgomery and Fire, 1998; Tabara et al., 1999). All were using a conserved mechanism- triggered by dsRNA (Cogoni and Macino, 1999; Matzke et al., 2001; Montgomery and Fire, 1998; Ratcliff et al., 1999; Sharp, 1999; Waterhouse et al., 1998), dependent on the generation of small-interfering RNAs (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000) and utilising shared machinery to silence complementary mRNAs (Catalanotto et al., 2000; Fagard et al., 2000; Ketting and Plasterk, 2000; Ketting et al., 1999; Tabara et al., 1999)

Further still it was found that the same machinery and mechanisms responsible for RNA interference were also involved in the generation and actions of genomically encoded small RNA regulators -the microRNAs (Grishok et al., 2001). *lin-4* and *let-7* had been previously characterised as small temporal RNAs responsible for the regulation of developmental timing in *C. elegans* (Lee et al., 1993; Reinhart et al., 2000). They were subsequently found to be the first of a number of microRNAs to be characterised across the multicellular eukaryotes, further expanding the family of RNAi related mechanisms to include developmental regulators, conserved between species (Pasquinelli et al.,

2000). Concurrently the effects of dsRNA induced gene silencing were being verified in a diverse range of eukaryotic systems including drosophila, trypanosomes, zebra fish and mice and the potential power of RNAi as an experimental technique was realised (reviewed in (Bosher and Labouesse, 2000; Zhuang and Hunter, 2012a)).

The characterisation of the machinery and mechanisms of RNAi not only allowed the full exploitation of a powerful experimental technique, but together with the identification of increasing numbers of endogenous small RNAs, opened the door on the world of small silencing RNAs (reviewed in (Zamore, 2002)). In *C. elegans* the potency of the effect was noted, as was the systemic and environmental nature (Fire et al., 1998; Tabara et al., 1998; Timmons and Fire, 1998). RNAi libraries have been created to allow the targeting of almost every gene in the genome, allowing the phenotypes of many genes to be characterised for the first time (Kamath et al., 2003). Observations of transgenerational effects of RNAi treatment have been reported in several organisms, particularly *C. elegans,* with important implications for transgenerational epigenetic inheritance (reviewed in (Feng and Guang, 2013; Rankin, 2015)).

1.3.i The evolutionarily conserved machinery and mechanisms of RNA interference

The exogenous-siRNA, or RNAi, pathway and the endogenous small silencing RNA pathways have all evolved from common origins, with the overall mechanism and key proteins being well conserved. At the core in all cases short dsRNAs 21-32 nucleotides in length are bound by a protein of the argonaute family. The argonaute cleaves one stand of the short dsRNA to leave an argonaute bound short single stranded guide RNA which forms the central component of the active compound - the RNA induced silencing complex, or RISC. The active RISC is a complex containing a short single stranded (ss)RNA, 21-32nt in length, bound to an argonaute protein together with variable auxiliary factors (reviewed in (Ghildiyal and Zamore, 2009)). Sequence specificity is provided by the guide RNA which targets the RISC to complementary mRNAs via Watson-Crick base paring. A range of classes of small RNAs with diverse targets have been identified (reviewed in (Aalto and Pasquinelli, 2012)). In most (but not all cases) the overall outcome is reduced expression of the target. The precise outcome of RNA targeting by ssRNA/argonaute complex is dependent on Argonaute and auxiliary factor identity and can include mRNA degradation, destabilisation and inhibition of translation in the cytoplasm, or co-transcriptional nuclear effects leading to transcriptional silencing and even the deposition of chromatin marks (reviewed in (Holoch and Moazed, 2015; Meister, 2013; Peters and Meister, 2007)).

The prevailing theory for the original purpose of RNA interference is as a viral defence mechanism. The exogenous siRNA pathway, in which small-interfering RNAs are generated from foreign dsRNAs in the cytoplasm and used to target homologous RNA sequences for silencing, represents an ancient mechanism of genome defence from virus and transposable elements (reviewed in (Obbard et al., 2009; Sarkies and Miska, 2013)). RNAi based mechanisms still play a critical role in viral defence in many invertebrates and plants, which lack a more complex immune response, and are still essential for transgene silencing in vertebrates and invertebrates.

From this ancient origin the endogenous small silencing RNAs have evolved to fulfil diverse roles, centred around genome defence and genetic regulation (reviewed in (Carthew and Sontheimer, 2009; Moazed, 2009; Shabalina and Koonin, 2008). With isolated exceptions, where the RNAi machinery seems to have been lost, organisms from all branches of the eukaryotic tree have been found to be competent of some form of exogenous RNAi and to utilise endogenous RNAi based mechanism of gene regulation and genome defence.

The critical players in all RNAi based pathways are the argonautes. Argonaute proteins are found in all branches of the eukaryotic kingdom and even have prokaryotic homologues (reviewed in(Swarts et al., 2014)). In all RNAi related silencing pathways argonautes are bound to short single stranded RNAs which act as a specificity factor to guide the argonaute to a complementary target transcript. The archetypal design of the eukaryotic argonaute centres around 4 conserved functional domains, the N-terminal, PAZ , MID and PIWI domains. The PAZ and MID domains are involved in guide RNA binding, with the MID domain forming a nucleotide binding pocket. The N-terminal and PIWI domains are critical for target cleavage with PIWI domain containing an RNase H-like active site and DEDX motif needed for the “slicer” activity displayed by many argonautes (reviewed in (Olina et al., 2018; Swarts et al., 2014)). The small RNA/argonaute based regulatory systems are extremely adaptable, as can be seen in their largely independent blooming in both plant and animal lineages and the cross-over in the roles played by different small-RNA classes between species.

Three major types of endogenous small RNA have been characterised in animals, primarily via deep sequencing; microRNAs (miRNAs), piwi-interacting RNAs (piRNAs) and small-interfering RNAs (siRNAs). Of these the endogenous siRNAs bear the closest resemblance to the products and mechanisms of the exogenous RNAi pathway. Endogenous siRNAs are found in plants, animals, fungi and ciliates, and have a diverse set of targets and functions. microRNAs are abundant in both plants and animals where they are key regulators of developmental events (reviewed in (Bartel, 2018; Voinnet, 2009)), while piRNAs are found only in animals and are critical for maintaining germline stability (reviewed in (Weick and Miska, 2014)). More recently extensive deep sequencing has led to

further classes of argonaute associated small silencing RNAs being identified including tRNA derived small RNAs (tsRNAs) which have been found in several organisms, and seem to be involved in transgenerational epigenetic inheritance (Chen et al., 2016; Haussecker et al., 2010; Luo et al., 2018; Sarker et al., 2019) reviewed in (Li et al., 2018)). miRNAs, siRNAs and piRNAs each play varied and sometimes overlapping roles in gene and genome regulation and are distinguished by differences in their biochemical characteristics (length and 3 prime/ 5 prime chemistry) and mechanism of biogenesis. Different classes also typically display difference in argonaute binding partners and degree of target complementarity, which influence their downstream effects (reviewed in (Ghildiyal and Zamore, 2009; Grishok, 2005)).

In both plants and animals small RNA mediated gene silencing can act at either the post transcriptional level (PTGS) or co-transcriptional level to bring about a variety of different outcomes. The effects of RISC targeting include down regulation of gene expression via translational repression and mRNA cleavage or degradation, whilst in the nucleus the RNA- induced initiation of transcriptional silencing (RITS) complex effects can include polymerase destabilisation during transcription and recruitment of further proteins leading to chromatin modification. In some cases small RNA/argonaute complexes can even promote target gene expression via mRNA stabilisation or directing the deposition of activatory chromatin marks. These pleiotropic outcomes are dependent on a diverse set of argonautes together with accessory factors, with argonaute identity and cellular context being the key determinant of the outcome of RISC/RITS mediated regulation of a target gene.

The evolution of the argonaute proteins mirrors the diversifying functions of the small RNA pathways with blooms of argonaute clades arising from duplications and subsequent divergence. The eukaryotic argonautes can be grouped into clades based on homology to give two main families; the members of the AGO clade are found throughout the eukaryotic kingdom, in animals plants and fungi whilst members of the PIWI clade have been found only in the metazoa. Largely independent expansions have taken place in plants and animals. miRNAs bind primarily to AGO clade members in both plants and animals whilst siRNAs in animals bind to members of both clades and the animals specific piRNAs (piwi-interacting RNAs) associate with only the animal specific PIWI clade argonautes for which they are named. In plants the AGO clade has been diversified to fill many functional niches filled by piwi clade argonautes in animals such that there is little meaningful functional difference between the two clades.

1.3.ii Small RNA pathways in *C. elegans*

Within the Nematoda a further argonaute bloom has taken place giving rise to the WAGO clade of argonautes. In *C. elegans* the WAGO (Worm argonaute) clade argonautes are responsible for many of the more derived argonaute functions and are tightly linked to the amplification of the silencing signal via secondary siRNAs to bring about a robust response. The *C. elegans* genome encodes 21 functional Argonaute proteins, some, such as CSR-1, PRG-1, HRDE-1 and RDE-1, NRDE-3 and ERGO-1 have unique functions whilst others such as ALG-1 and ALG-2, ALG-3 and ALG-4, and several members of the WAGO clade have overlapping roles.

Figure 1.9 shows the phylogeny of the C. elegans argonaute lineage and the class of small RNA with which they are most commonly associated. The specifics of each of the small RNA pathways, their associated argonautes and functions shall be discussed in their context in *C. elegans*, much of which is also applicable to other organisms.

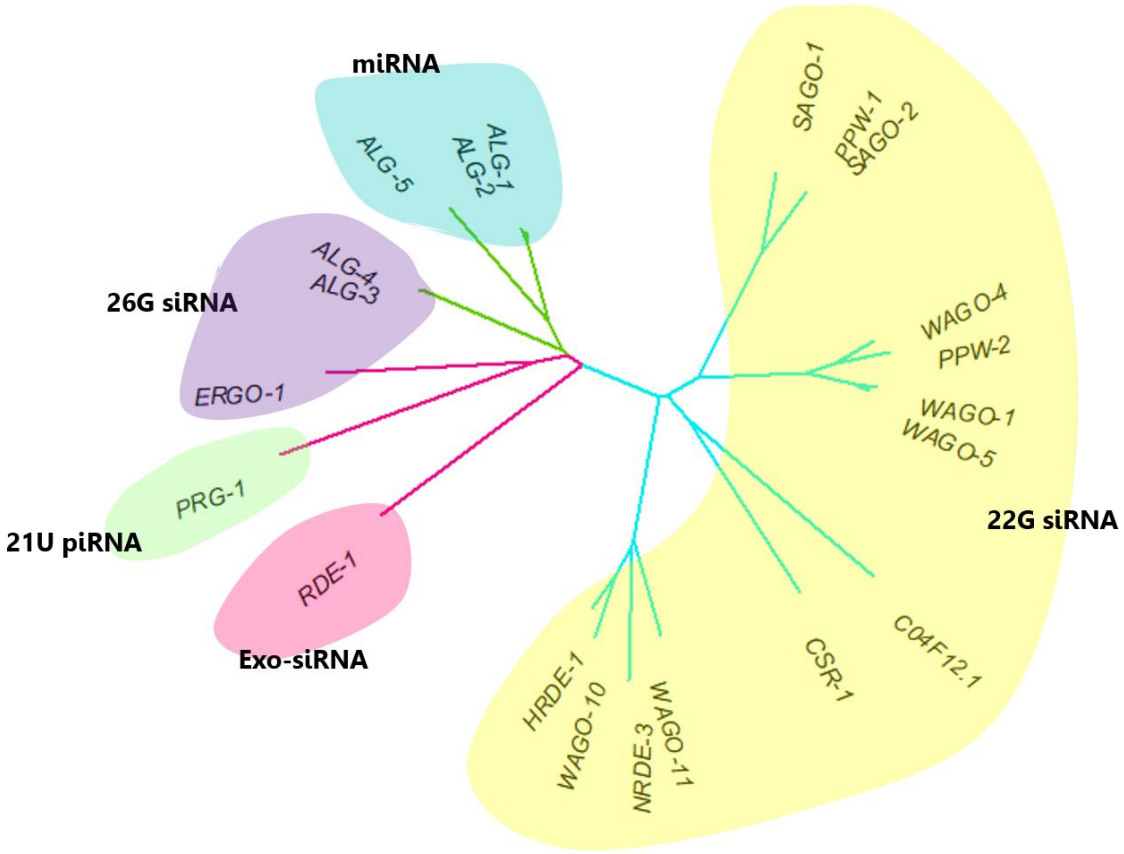


Figure 1.9. An unrooted phylogeny of the argonaute proteins of *C. elegans*. Branch length represents divergence. The 3 major clades of argonaute are represented by different coloured lines: green- the AGO clade common to both plants and animals, pink- the metazoan specific PIWI clade, blue- the nematode specific WAGO clade. The class of small RNA most frequently associated with each argonaute is represented by the navy groupings. In a few cases such as C04F12.1 where there is a lack of experimental evidence this is assumed based on homology to argonaute of better-known function. Phylogeny generated using protein sequences of each argonaute taken from retrieved from WormBase, aligned using Clustal Omega MSA (EMBL-EBI) software and most likely phylogenetic relationship generated using Simple Phylogeny (EMBL-EBI)). Where multiple isoform sequences were available the longest isoform was used.

1.3.iii microRNAs

The first class of the small silencing RNAs to be identified, miRNAs are a highly abundant class of small silencing RNAs in both plants and animals. microRNAs were first identified during the characterisation of heterochronic mutants in *C. elegans*, with the identification of the non-protein coding causal loci *lin-4* and *let-7* (Reinhart et al., 2000; Wightman et al., 1993). *let-7* was found to have highly conserved homologues in many other animal species, in all cases acting late in development (Pasquinelli et al., 2000). Initially known as small temporal RNAs, such small RNA

regulators were renamed microRNAs as further similar RNAs were identified and found to regulate non-temporal genes (Ambros et al., 2003; Lau et al., 2001; Lee and Ambros, 2001). Mature miRNAs are 21-24nt long, derive from highly structured, genomically encoded loci and principally act as developmental regulators through post-transcriptional gene silencing of protein coding genes (reviewed in (Bartel, 2018; Carthew and Sontheimer, 2009; Voinnet, 2009).

miRNAs originate from genomically encoded loci, either from non-protein coding genes or within the introns of coding pre-mRNA. Canonical miRNAs are transcribed by RNA polymerase II, as long primary transcripts complete with 5' cap. These long primary miRNA (pri-miRNA) transcripts contain many repeating and palindromic sequences which fold into secondary structures which always include at least one hairpin stem-loop. The double stranded hairpin structures in long pri-miRNAs are processed into short ds pre-miRNA stem-loops, 60-70nt long, by a 'microprocessor' heterotrimer consisting of RNAase III drosha together with 2 copies of a dsRNA binding domain (dsRDB) protein (known as pasha in flies). Pre-miRNAs are then exported from the nucleus to the cytoplasm where they are processed into mature miRNA 20-24nt in length, with 2 nt 3' overhang on each end by RNAase III Dicer, again assisted by a dsRBD protein. The miRNA duplex is then loaded into an argonaute protein and auxiliaries to form the pre-miRISC, which then becomes the mature miRISC as one strand of the duplex is cleaved to leave only the guide strand.

microRNAs in *C. elegans* are predominantly bound by the AGO class argonautes ALG-1 and ALG-2, with a small number of miRNAs being found to associate with ALG-5 in some studies (Brown et al., 2017). In *C. elegans,* as in other animals, pairing to the target RNA is typically incomplete but with near perfect complementarity within the seed region – a region of 6-8nt at near the 5'end of the miRNA (reviewed in (Bartel, 2018)). In animals pairing most commonly occurs within the 3'UTR of target transcripts. In most cases limited pairing to the target transcript prevents direct cleavage by the miRISC and silencing is instead brought about by translational repression, mRNA deadenylation and mRNA decay. In plants, where high complementarity between miRNA and the target is more common, mRNA cleavage is a common outcome (reviewed in (Ghildiyal and Zamore, 2009; Huntzinger and Izaurralde, 2011)).

Tissue specific differences are also seen in the outcome and mechanism of miRISC targeting. In the *C. elegans* soma ALG-1/2 miRISC targeting typically results in translational repression and mRNA decay whilst in the germline ALG-1/2 miRISC targeting leads to temporary translational repression but mRNA stabilisation. This dramatic difference in behaviour is seen due to differences in auxiliary and associated proteins. In the germline miRNA targeted mRNAs are localised to the p-granules along with protein which inhibit translation whilst also protecting transcripts from

degradation. This allows target mRNAs to accumulate to high levels, and is hypothesised to play a key role in allowing maternal mRNA accumulation in oocytes (Dallaire et al., 2018).

A single pri-miRNA locus can give rise to several mature miRNAs. Such polycistronic miRNAs typically belong to the same 'family', meaning they share closely related mature miRNA sequences, particularly with respect to the seed region, and as such will often target the same set of target genes. As complementarity is only strictly necessary in the short seed region a single miRISC can target a number of genes, often functionally related. So far 253 miRNA precursors producing 437 mature miRNAs have been recorded in *C. elegans,* with 113 precursors meeting stringent confidence criteria (miRbase 22). This is considerably fewer than 1000 (500 high confidence) or more miRNA precursors identified in the human genome. It is predicted that over half of all mRNAs are regulated by miRNA (Bartel, 2018).

There is some uncertainty over whether plant and animal microRNAs have evolved from a common microRNA type ancestral mechanism or if they have been independently derived from adaptation of a more basal mechanism (Millar and Waterhouse, 2005). Despite obvious broad similarities in mechanism, biogenesis and function there are sufficient differences between plant and animal microRNAs that independent evolutions seem more likely. It has even been suggested that the evolution of a miRNA like mechanism could be a prerequisite to the evolution of complex body plans (reviewed in(Carthew and Sontheimer, 2009; Shabalina and Koonin, 2008)).

1.3.iv 21U piRNAs

piRNA were first identified in the mouse where they were found to be critical for maintaining genome stability in the germline, a function which has since been found to apply to flies and worms as well as mammals. piRNAs are so named due to their association with argonautes of the piwi clade, which like piRNAs are found only in metazoans. The critical role of piRNA is to function in the germline to maintain genome stability through transposable element silencing, in particular during spermatogenesis. The rewriting of many silencing chromatin marks during spermatogenesis leaves the genome vulnerable to mobile genetic elements normally silenced. piRNAs appear to play a key role in maintaining and re-establishing this silencing, with loss of the piRNA pathway leading to infertility (reviewed in (Malone and Hannon, 2009; Weick and Miska, 2014).

Mammalian piRNAs are typically 25-30nt long however the *C. elegans* equivalents are 21nt long with a 5' monophophorylated Uracil and undergo 3' 2'-O methylation by HENN-1. The 21U RNAs function exclusively in the germline and bind to the PIWI class argonaute bind PRG-1 to bring

about post transcriptional gene silencing in the cytoplasm. piRNA biogenesis is distinctive and requires neither RdRP nor DCR-1 - primary transcripts are transcribed by RNA polymerase II as individual 26nt capped-small RNAs from specific clusters of non-coding loci on chromosome IV and then processed into 21nt RNAs by cleavage of the 5' nucleotides including the cap. piRNA loci clusters act as a memory of transposable elements in the genome with the piRNAs acting in trans to silence the transposable elements. Accordingly, piRNA loci are depleted for exons but are enriched in the sense orientation at the start and antisense orientation at the end of full length transposon loci of the TC3 class in particular. The sequence of 21nt piRNAs are highly variable, with no evidence of sequence conservation. However loci are identifiable by conserved up-stream motifs - a large AT rich motif, with a short core consensus sequence which is bound by forkhead family transcription factors, followed by the small motif YRNT which acts as the initiator element for pol II transcription and triggers 21U processing (Ruby et al., 2006). A second group of 21U RNA originate from capped-small RNAs which map bi-directionally to transcription start sites of coding genes. These TSS associated 21U RNAs have the small but not the large upstream motif and are a subset of capped-small RNAs from TSS, most of which are not processed into 21U RNAs since they lack the YRNT motif. The significance of these TSS- associated 21U RNAs is unknown (reviewed in (Billi et al., 2014).

The effects of piRNAs are not limited to direct PTGS by 21U RNAs but are amplified by the RdRP dependent production of secondary siRNA from target transcripts. In mammalian systems which lack RdRPs an alternative and distinctive 'ping-pong' mechanism of biogenesis is seen.

1.3.v 26G endogenous siRNAs

In *C. elegans* all primary endo-siRNAs are 26nt in length with a bias for 5' guanine, are 5' monophosphorylated (26G siRNAs) and are produced by RNA dependent RNA transcription from pol II transcribed transcripts. 26G RNAs map predominantly antisense to spliced mRNAs of annotated genes, or to long-intergenic-non-coding RNAs (lincRNAs) and other lncRNAs (reviewed in (Billi et al., 2014)) . Two major classes of 26G RNA have been identified based on differences in the argonaute with which they associate. ERGO-1 class 26G RNAs are abundant in embryos, larva and in the oogenic gonad, whilst ALG-3/4 class 26G RNAs are found in the spermatogenic gonad. The two classes share a core set of biogenesis factors known as the ERI complex.

The production of both classes of 26G RNAs is dependent on the ERI complex consisting of RdRP RRF-3, dicer (DCR-1), dicer-related helicase DRH-3, exonuclease ERI-1, dsRBD protein RDE-4, Tudor domain protein ERI-5, and ERI-3. dsRNAs are produced by RDRP dependent transcription from long transcripts in the cytoplasm, such as mRNAs and lincRNAs, then processed by Dicer to give short dsRNAs with 2nt overhangs at each end. This produces a 26nt siRNA with a monophosphorylated 5'G

terminus. For ERGO-1 class siRNAs the methyltransferase HENN-1 is required to produce a 2'O-trimethylated 3' end whilst ALG-3/4 class siRNAs remain 3' hydroxylated.

The ERI complex dependent mechanism of primary endo-siRNA production seems to be the predominant mechanism In *C. elegans* however other sources of endogenous long dsRNA are also possible including dsRNA produced due to overlapping convergent transcription at transposons, cryptic loci and aberrant transcripts. In *drosophila* some siRNAs are produced from structured loci, an origin more typical of miRNA.

ALG-3/4 siRNAs are produced in the spermatogenic gonad where they are needed for successful spermatogenesis. The paralogues ALG-3 and ALG-4 act largely redundantly but loss of function of both results in severe defects in spermatogenesis, in particular at 25 °C (Conine et al., 2010). ERGO-1 class siRNAs are produced in the oogenic gonad and early embryo but perdure throughout development (reviewed in (Billi et al., 2014)). They map antisense to both coding genes and non-coding regions and likely play a number of roles including the silencing of recently duplicated genes, pseudogenes and transgenes and the maintenance of centromeric regions (reviewed in (Billi et al., 2014; Fischer et al., 2013)).

26G siRNAs display complete or near complete target complementarity and targeting by the 26G/ERGO-1 or 26G ALG-3/4 complexes acts at a post-transcriptional level in the cytoplasm to bring about gene silencing likely via target cleavage. Target mRNAs are then used as substrates for the production of secondary siRNAs which can act at the post-transcriptional or co-transcriptional level due to the actions of a diverse set of argonautes belonging to the WAGO clade (reviewed in (Billi et al., 2014; Fischer et al., 2013)).

1.3.vi The exogenous siRNA pathway

The exogenous siRNA pathway, the primary purpose of which is thought to be as a viral defence mechanism, can be easily manipulated as a way of silencing endogenous genes by the introduction of exogenous dsRNA complementary to the endogenous target.

In *C. elegans* long dsRNAs can be introduced artificially via feeding, soaking, transgene expression or microinjection (reviewed in (Zhuang and Hunter, 2012a)). Once in the cytoplasm the long dsRNA is first bound by the DICER family endonuclease DCR-1 and the dsRNA binding protein RDE-4. Following cleavage by DCR-1 the short dsRNA, which is 21-24nt long with 2nt single stranded overhang at each 3' end, is bound by the argonaute family protein RDE-1 together with auxiliary proteins to form the pre-RISC. Following passenger strand cleavage by argonaute RDE-1 and

methylation of 3' end by HENN-1, the mature RISC made up of a guide RNA, an argonaute and auxiliary factors are formed. The short single stranded RNA targets the RISC to complementary RNA transcripts in the cytoplasm which are bound and cleaved to induce gene silencing. Complementarity between guide and target RNAs in the exogenous pathway is always high, and usually perfect (reviewed in (Ghildiyal and Zamore, 2009)).

RDE-1 is the only Argonaute family member essential for the exo-RNAi pathway (Tabara et al., 1999). It is required for binding primary exo-siRNAs, cleavage of the passenger strand and initiation of the RNAi response but studies have found that it does not directly bring about target cleavage, slicer activity being very low (Steiner et al., 2009). Instead RDE-1 recruits ribonucleause RDE-8 for target cleavage (Tsai et al., 2015).

A number of aspects of the exogenous RNAi response in *C. elegans* contribute to the robust organism wide response, which is less apparent in other, particularly mammalian systems. Firstly, RNAi can be initiated by environmental uptake of dsRNA, either via feeding or soaking. Secondly, the response is systemic - the RNA induced silencing can spread between cells and tissues. This together with the fact that most cell types will readily take up long dsRNA likely contributes to the flexibility and seeming indifference the system displays to the mechanism of exposure to the original trigger. Thirdly, the amplification of the silencing signal through the production of secondary siRNAs coupled to secondary argonautes multiplies the PTGS effects and mediates co-TGS via the nuclear RNAi response (Gu et al., 2012; Pak et al., 2012). This second wave of response is key to the transgenerational heritability of RNAi effects seen in the *C. elegans* germline, with RNAi induced phenotypes being frequently reported up to 4 generations after exposure to exo-RNAi triggers and over 10 generations for piRNA related silencing (Sapetschnig et al., 2015).

1.3.vii Amplification and diversification- 22G secondary siRNAs

The targeting of mRNAs in the cytosol by RDE-1 RISC in the exo-RNAi pathway, PRG-1 RISC in piRNA pathway and ALG-3/4 and EGRO-1 RNA induced silencing complexes in the 26G siRNA pathways in fact represents only the first stage of the effects of each of these pathways. The cleaved target transcript is then used as a template for the production of secondary siRNAs via an RDRP dependent, dicer independent mechanism (Alder et al., 2003; Pak and Fire, 2007; Shi et al., 2013; Sijen et al., 2001, 2007; Tsai et al., 2015b).

These secondary siRNAs are 22nt long, 5' tri-phosphorylated and 3' hydroxylated with 5' guanine bias. 22G siRNAs are produced as short single stranded siRNAs by RdRP dependent

unprimed synthesis from target transcripts. The endoribonuclease RDE-8 is recruited by the RDE-1 RISC to cleave target mRNAs and generate 3' uridylated fragments which are used as templates for 22G production (Tsai et al., 2015) via a mechanisms which requires the RDRPs RRF-1 and/or EGO-1, the dicer-related-helicase DRH-3 and the tudor-domain protein EKL-1 to form an RDRP module (Gu et al., 2009; Pak and Fire, 2007; Sijen et al., 2007). The exact role of EKL-1 in the RDRP module is unknown however it is known to interact with DRH-3. DRH-3 is a key element of both the EGO-1/RRF-1 and RRF-3/ERI complexes and is proposed to act in template secondary structure relaxation, promoting sequential initiation of polymerisation, and 22G dissociation. RRF-1 and EGO-1 are encoded in an operon but show quite distinct expression patterns. Both contribute to the 22G population with the germline expressed EGO-1 alone responsible for CSR-1 class 22G RNA production and RRF-1 responsible for 22G production in the soma (reviewed in (Billi et al., 2014)).

Secondary siRNA production amplifies the silencing signal and are key to generating a robust RNAi response. 22G siRNAs are also the major effectors of the 26G and 21U pathways. Secondary siRNA synthesis also biases the siRNA pool to target expressed genes as only those primary si/piRNAs with an expressed target will be amplified. In addition to amplification of the silencing signal, the secondary siRNA response also allows for a diversification in the downstream effects of RISC targeting via the diverse actions of the WAGO (Worm specific Argonaute) clade argonautes with which they associate (Yigit et al., 2006) and increases the longevity of the silencing signal.

The WAGO clade is a greatly expanded nematode specific argonante clade members of which seem to bind exclusively to 22G siRNAs. Unlike the argonautes which bind to primary siRNA, miRNA or piRNAs which have the conserved residues of the catalytic triad of the endonuclease like PIWI domain necessary for direct target strand cleavage, all but one members of the WAGO clade have lost this 'slicer' ability, hinting at different mechanisms of actions and functional diversity (Aoki et al., 2007; Yigit et al., 2006; reviewed in (Buck and Blaxter, 2013)).

A large number of the WAGO argonautes act semi-redundantly in the cytoplasm to bring about robust PTGS via mechanisms such as translational repression, mRNA destabilisation and indirect target cleavage and degradation via the recruitment of auxiliary proteins to the target bound RISC; enhancing and potentiating the effects of PTGS originated by the primary siRNA/piRNA RISC. Such is the redundancy of these cytoplasmic WAGOs that loss of function of multiple argonautes is needed for the secondary siRNA response to be completely lost, as evidenced by the MAGO mutants (Yigit et al., 2006).

However, a number of other members of the WAGO class act non-redundantly in unique roles. Nuclear targeted argonautes such as NRDE-3, HRDE-1 and CSR-1 are able to target nascent

transcripts and thus orchestrate co-transcriptional effects, including co-TGS and chromatin modifications. The mechanisms by which these nuclear argonautes exert their effects remain poorly understood but they play important roles in a variety of processes including transgenerational gene silencing, nuclear RNAi, germline maintenance and even chromosome segregation (for review see (Cecere and Grishok, 2014; Holoch and Moazed, 2015)).

1.3.viii Nuclear targeted argonautes - Nuclear RNAi, heritability and the csr-1 pathway

The argonautes NRDE-3 and HRDE-1 both contain bipartite Nuclear localisation signals and are imported to the nucleus upon 22G binding (Guang et al., 2008). NRDE-3 acts in the soma while HRDE-1 acts in the germline to mediate transcriptional gene silencing in both exogenous and endogenous RNAi pathways(Buckley et al., 2012). Both engage the same core nuclear silencing machinery- the components of the NRDE (nuclear RNAi defective) to form the RNA induced transcriptional silencing (RITS) complex. Upon nuclear localisation 22G/NRDE-3 and 22G/HRDE-1 bind to nascent complementary transcripts and recruit NRDE-2 which together recruit NRDE-1 to mediate inhibition of transcription elongation. NRDE-1 is also recruited to adjacent chromatin by NRDE-4 to direct further silencing through H3K9 trimethylation at target loci (Burkhart et al., 2011; Burton et al., 2011; Guang et al., 2010; Mao et al., 2015; Zhuang et al., 2013). The nuclear RNAi pathway acts in parallel to the cytoplasmic 22G/WAGO RISCs to ensure robust silencing.

22G siRNAs allow for silencing to perdure after the primary trigger is no longer expressed. NRDE-3 bound 22G siRNAs in the soma ensure that endogenous ERGO-1 targets continue to be effectively silenced throughout larval development, despite the fall in 26G levels after embryogenesis just as somatic exo-RNAi silencing perdures after dsRNA trigger removal. Such perdurance even extends across generations, with silencing chromatin marks induced by RNAi being erased and re-established in the F1 generation via a small RNA dependent mechanism (Burton et al., 2011). The epigenetic inheritance of silencing against somatic targets is NRDE-3 dependent and perdures for a single generation whereas the HRDE-1 dependent inheritance of germline silencing can persists for far longer (Ashe et al., 2012). The mechanisms of the establishment and re-establishment of RNA induced silencing in the F1 generation and beyond remain unclear but interestingly silencing chromatin marks are more robust in the F1 generation than in the parental generation, possibly due to the need for very early exposure to the primary trigger to initiate efficient nuclear silencing (Burton et al., 2011; Shiu and Hunter, 2017). After this in the absence of a primary trigger in the soma silencing chromatin marks along with 22G siRNA are quickly lost in successive generations. It seems that 22G siRNAs in the soma are unable to undergo further rounds of amplification and it is

possible that inherited primary small RNAs are needed to re-establish the secondary siRNA population required for nuclear silencing (Burton et al., 2011).

In the germline nuclear RNAi is essential for effective genome surveillance of foreign elements targeted by the piRNA pathway, acting as an epigenetic memory of non-self which can perdure for many generations even in the absence of primary trigger (Ashe et al., 2012). Loss of function of *hrde-1, nrde-1, nrde-2* or *nrde-4* results in a mrt phenotype equivalent to that seen in mutants of upstream piRNA components such as prg-1, resulting in fertility defects (Buckley et al., 2012). Unlike in the soma, HRDE-1 bound 22G siRNAs in the germline are capable of undergoing further rounds of amplification to form new 22G siRNAs or tertiary siRNAs (Sapetschnig et al., 2015). This seems to be the key to the multigenerational perdurance of silencing since like in the soma new chromatin marks are laid down each generation. The germline expressed cytoplasmic argonaute WAGO-4 also plays a role in the inheritance of silencing, with WAGO-4 bound siRNAs hypothesised to be directly transmitted from parent to progeny (Xu et al., 2018).

Interestingly tertiary siRNAs were shown to map to further from the initial target site than do the secondary siRNAs, demonstrating the *cis* spread of silencing along the target gene. Where silencing has been triggered by piRNAs, such as the silencing of a piRNA sensor transgene, tertiary siRNAs also mediate the *trans* spread of silencing, where by a silenced transgene can trigger the conversion of an expressed transgene to the silenced state even in the absence of the initial piRNA sensor to act as a trigger (Sapetschnig et al., 2015). This pattern of silencing bears a striking resemblance to the phenomenon of paramutation such as that seen in the classic example of epigenetic inheritance the maize b1 locus - a phenomenon now also known to be siRNA dependent.

C. elegans argonaute, chromosome segregation and RNAi deficient (CSR-1) is a unique member of the WAGO clade. CSR-1 is the only *C. elegans* argonaute essential for viability. As the name suggests, loss of CSR-1 leads to defects in chromosome organisation and segregation due to failures in chromosome condensation and kinetochore attachment in both germline and somatic cells. Mutants are sterile, show distinctive defects in p-granule aggregation and loss of exogenous RNAi efficiency, in particular against germline and embryonic targets. CSR-1 seems to act in a number of unconventional roles; it is the only secondary argonaute to retain 'slicer' activity (Aoki et al., 2007) yet it seems that the vast majority of CSR-1 target genes are not silenced. Instead CSR-1 acts co-transcriptionally to promote the transcription target genes (Cecere et al., 2014). CSR-1 also directs histone modification, is required in holocentromere organisation and chromosome segregation (Claycomb et al., 2009; She et al., 2009), plays an important role in the 3' end processing of histone mRNAs (Avgousti et al., 2012), and may also act with pumilio proteins in the cytoplasm to

inhibit translation, as well as having a possible role in the exo-RNAi response (reviewed in (Wedeles et al., 2013a)).

CSR-1 22G siRNAs are preferentially transcribed by EGO-1 and are uridylated by CDE-1, directing them to the CSR-1 pathway (van Wolfswinkel et al., 2009). The gene regulatory effects of CSR-1 are prolific, targeting and promoting the expression of over 4000 genes which are preferentially germline expressed (Cecere et al., 2014; Wedeles et al., 2013a). These include targets of the ALG-3/4 primary siRNA subset and CSR-1 is central to the action of the ALG-3/4 siRNA pathway, with CSR-1 acting in the spermatogenic gonad to upregulate expression of target genes essential for male fertility (Conine et al., 2013) and to provides a memory of male germline gene expression (Conine et al., 2013). CSR-1 is equally important for protecting gene expression in the oogenic germline where it protects germline gene expression (Wedeles et al., 2013b). CSR-1 target transcripts are protected from accidental silencing which could be caused by the spread of tertiary siRNA mediated paramutation downstream of the piRNA pathway, therefore acting in antagonistic concordance with piRNA mediated genome surveillance (Sapetschnig et al., 2015; Seth et al., 2013). Similarly, CSR-1 localisation to chromatin also limits the distribution of centromeric histone identity to bounds suitable for proper kinetochore assembly and chromosome segregation. Antagonism with CDE-1 dependent siRNA degradation helps to maintain this balance (van Wolfswinkel et al., 2009). CSR-1 also regulates oocyte-embryo transcriptional transition during embryonic genome activation (Fassnacht et al., 2018). The diverse actions of CSR-1, protecting gene expression in the male germline via co-transcriptional regulation whilst fine tuning maternal mRNA expression in the embryo via PTGS, with even less conventional roles in histone maturation serves to highlight the complexity of the small RNA mediated control mechanisms.

The full extent of the function of nuclear RNAi in mediating chromatin dynamics is as yet unknown.

1.3.ix The systemic nature of RNA mediated gene silencing and environmental RNAi

In *C. elegans* RNA based silencing signals can be spread between cells, both within the soma and from the soma to the germline, mediating parental RNAi. The molecular basis for the systemic nature of RNAi in *C. elegans* began to be understood with the identification of SID (Systemic RNAi Defective) and RSD (RNA spreading defective) mutants (Tijsterman et al., 2004; Winston et al., 2002). The SID-1, SID-2, SID-3 and SID-5 mutants are defective in different parts of systemic and environmental RNAi (Hunter et al., 2006; Wang and Hunter, 2017). A number of studies suggest that the silencing signal is spread by dsRNA, possibly by a range of different RNA species (Jose et al., 2011), reviewed in (Chitwood and Timmermans, 2010; Sarkies and Miska, 2014).

sid-1 and *sid-2* both encode transmembrane channels. SID-1 is a transmembrane dsRNA import channel needed for import of dsRNA into cells (Shih et al., 2009) . SID-1 is necessary for spread of the silencing signal and systemic RNAi is lost with *sid-1* (Hunter et al., 2006; Winston et al., 2002). Tissues lacking SID-1 transporter expression such as most neurones are refractory to RNAi, but susceptibility can be increased by expression of SID-1 (Calixto et al., 2010). *sid-2* loss of function mutants are defective in RNAi by feeding. SID-2 was found to be a transmembrane transporter for long dsRNA localised to the apical membrane of the intestinal cells, lining the intestinal lumen. SID-2 is required for uptake of dsRNA from the lumen, possibly via endocytosis. Loss of SID-2 function results in an inability for RNAi to be induced via feeding (environmental RNAi) but does not affect the ability of the dsRNA induced silencing to spread from cell to cell (Hunter et al., 2006; Winston et al., 2007).

SID-5 is required for export of the dsRNA silencing signal from intestine, and from other cells and is endosomally localised (Hinas et al., 2012). RSD-3 is a homologue of human Clathrin interactor 1, an ENTH (epsin N-terminal homology) domain containing protein involved in vesicle trafficking. RSD-3 localises to endosomes and the trans-golgi network and is required for the import of silencing RNAs into cells of both the soma and germline, most likely from the pseudocoelom (Imae et al., 2016; Tijsterman et al., 2004). The identification of many endomembrane localised proteins involved in RNAi, including the snare *sec-22* (Zhao et al., 2017) and RISC components has led to the suggestion that rather than taking place freely in the cytoplasm RNAi is a membrane associated process (Rocheleau, 2012).

1.3.x Subcellular localisation/organisation

There is substantial evidence that RNA silencing processes are membrane associated in *C. elegans,* fly, plant and mammalian systems. In *C. elegans* there is a clear role for the endomembrane system in systemic and environmental RNAi as both SID-5, RSD-3 are localised to different compartments of the endomembrane. The Mammalian homologues of SID-1, SIDT2 has also been shown to be localised to the endosome and lysosome (Jialin et al., 2010; Nguyen et al., 2017).

Beyond this many core components of the small silencing RNA machinery are endomembrane associated, including the RISC and dicer. The rough ER is emerging is as the most likely site of translational repression by the miRISC and the siRISC. Mammalian AGO2 was first isolated as a golgi/ER associated protein (Cikaluk et al., 1999) and miRNA/siRNA loaded AGO2, along with dicer and other RISC loading complex (RLC) factors have since been shown to co-sediment with

the rough ER (Stalder et al., 2013). Stalder et al. further show that the siRISC is anchored to the cytosolic side of the rough ER by RLC factors TRBP and PACT. In plants, AGO1 also localises to the ER and the rough ER localised integral membrane protein AMP1 is required for the translational repression of target mRNAs by miRNAs.

Furthermore, the efficiency of silencing by small RNAs is dependent on endosomal trafficking. miRISC components AGO2 and GW182 congregate in GW-bodies which have been shown to correspond the endosome/MVB (Gibbings et al., 2009; Lee et al., 2009). The maturation to multivesicular bodies is necessary for an efficient silencing response; depletion of ESCRT components, required for the sorting of proteins into the MVB, compromises miRNA activity (Gibbings et al., 2009). Whilst MVB to lysosome fusion has a negative effect on the RNAi response. The snare *sec-22* has been found to reduce RNAi efficiency by regulating late endosome/MVB fusion to lysosomes, acting in a SID-5 dependent manner (Zhao et al., 2017). Kim et al. propose that a picture is emerging where in the RNAi mediated processes are closely linked to the endomembrane system; not only in the transfer of the silencing signal in systemic RNAi, but with the rough ER as the site of RISC loading and silencing initiation and the MVBs as sites of RISC recycling (Kim et al., 2014).

The mutator class of genes are required for transposon silencing as well as exogenous RNAi. The six mutator proteins, together with the RDRP RRF-1, and the DEAD box helicases SMUT-1 and RDE-12, co-localise to peri-nuclear localised punctate foci in the *C. elegans* germline known as mutator foci. Mutator foci are proposed to be the key sites of siRNA amplification. The mutator foci are not membrane associated but are instead proposed to exist as phase separated compartments, nucleated by the intrinsically disordered protein MUT-16 (Phillips et al., 2012, 2014; Yang et al., 2014; Zhang et al., 2011).

1.3. xi The efficiency of RNAi - core machinery and competition between pathways

As may have become apparent, the core RNAi machinery of all small RNA pathways in *C. elegans* and other organism consists of the same, or homologues of the same, central protein components. These are RNA endonucleases such as dicer-like-proteins, DEAD/DEAH box helicases, dsRNA binding proteins (typically type II with a helicase domain) and of course argonaute (piwi-argo) family proteins. In *C. elegans* as in other groups RDRPs play key roles in primary and secondary siRNA generation, however insects and mammals have lost all RDRP genes and therefore rely on bidirectional transcription to generate dsRNA duplexes (Lewis et al., 2018). Many such core components were identified in mutants defective in the exo-RNAi response, so called RNAi defective

(RDE) mutants. Since a sole dicer enzyme is responsible for the production a range of different small RNA in *C. elegans* additional factors must direct dicer to generate this specificity.

In *C. elegans,* despite the robust and systemic nature of the RNAi response, it is none the less the case that certain tissues and genes are resistant to RNAi induced knock down, whilst other display almost total knockdown and some a partial knock down. Furthermore the response to RNAi in *C. elegans* displays a stochastic variation or noise; within a population of identical worms exposed to the same stimulus, some will show RNAi induced knockdown whilst other will not (Arbel-Goren et al., 2014). Alterations in the efficiency of RNAi can therefore be seen in changes both in the severity of the RNAi phenotype, caused by the degree of knockdown within that worm and in the percentage of worms in the population displaying a given phenotype.

A range of mutants have been identified which display either an enhanced or reduced efficiency of RNAi (reviewed in Joyce et al., 2006). In an enhanced RNAi (Eri) mutant genes which display little or no knockdown in a WT background can be knocked down efficiently, whilst in mutants with reduced efficiency very little response is seen. Mutations of many core components of the exogenous RNAi pathway leads to complete loss of the RNAi induced phenotype as seen in RNAi defective (Rde) mutants. Mutant phenotypes reveal complex interdependencies and antagonisms between the availability of different pathway components.

As already discussed, loss of function of the Argonaute RDE-1 leads to complete loss of the RNAi response, with primary exo-siRNAs generated in the same quantity but leading to no phenotype. (Parrish and Fire, 2001; Tabara et al., 1999). Whilst loss of multiple WAGO/MAGO class Argonautes downstream leads to a reduced RNAi response, suggesting many WAGO/MAGO class Argonautes each contribute incrementally to RNAi, including SAGO-1, SAGO-2 and PPW-1. MAGO availability appears to be a limiting factor in the efficiency of RNAi which efficiency can be increased by overexpression (Yigit et al., 2006).

In the exo-RNAi pathway the dsRNA binding protein RDE-4 acts as a dimer to bind long dsRNA and recruit DCR-1 (Tabara et al., 1999, 2002). In RDE-4 loss of function mutants primary siRNA are produced at much lower than WT levels in response to injection of dsRNA. The RDE-4 but not the RDE-1 defect can be partially bypassed by addition of siRNAs, suggesting a role for RDE-4 in the recognition of dsRNA leading to siRNA production (Parrish and Fire, 2001).

The DExH/D box RNA helicase DRH-1 (Dicer related helicase) interacts with both RDE-4 and dicer and also plays a role in the RNAi response (Tabara et al., 2002). Deletion of DRH-1 leads to loss of anti-viral immunity in *C. elegans* due to lack of viral dicer targeting of viral RNA (Ashe et al., 2013).

DRH-1 is one of 3 *C. elegans* homologues of the mammalian viral recognition protein RIG-1. DRH-3 is required for an efficient RNAi response and in the endogenous RNAi response in the germline (Gu et al., 2009).

Many RDE proteins have been found to have a role in secondary siRNA production, with some of the same proteins apparently involved in secondary siRNA production in both the exogenous and endogenous siRNA pathways (Zhang et al., 2012). The RDRPs RRF-1 and EGO-1 are required for secondary siRNA production, with EGO-1 playing an essential role in the germline (Aoki et al., 2007; Smardon et al., 2000). Many more factors are also needed for efficient RNAi response such as the ribonuclease RDE-8, which is recruited by RDE-1 to target transcripts and recruits RDRPs (Tsai et al., 2015). The helicases DRH-3 and RDE-12 are also required (Gu et al., 2009; Shirayama et al., 2014; Yang et al., 2014). It is common for genes involved in the production of WAGO class 22G secondary siRNA to show a dosage-sensitive RNAi defective phenotype, as seen in *rde-10, rde-11, rsd-2, rsd-6* and *haf-6* mutants, where injection of high concentration of dsRNA can compensate for the defect (Zhang et al., 2012) .

The secondary siRNA binding argonaute CSR-1 functions mainly in endo-RNAi pathway but also promotes exo-RNAi efficiency in the germline (Gu et al., 2009; Seth et al., 2013; Wedeles et al., 2013a, 2013b; Yigit et al., 2006).

It is thought that RNA interference originally evolved as a viral defence mechanism and competition between viral derived exo-siRNAs and endogenous small RNAs alters endogenous- siRNA and miRNA dependent gene expression in virally infected worms (Sarkies et al., 2013). Furthermore antiviral immunity is lost in strains with a defective RNAi response. (Ashe et al., 2013). This RNAi based antiviral immunity does not require a functional systemic nor transgenerational RNAi response but appears to acts cell autonomously (Ashe et al., 2015).

A range of mutants have also been identified which display an enhanced exo-RNAi response. So-called Enhanced RNA interference (Eri) mutants were first noted for their ability to enhance the efficiency of RNAi screens against refractory or neuronally expressed genes. Many such mutants have been found to be loss of function in endogenous RNAi pathways components, leading to theory of competition for shared resources between the pathways (Sarkies et al., 2013; Zhuang and Hunter, 2012b). In such models loss of function in one pathway frees up shared components for use in the exo-RNAi pathway. The 9 classical *eri* class mutants are all associated with DCR-1 and are thought to be required for endo-siRNA production (Zhuang and Hunter, 2011, 2012a, 2012b).

For example, loss of function mutants of RDRP *rrf-3/ eri-2* display enhanced RNAi induced gene silencing and have been used to enhance RNAi screens (Simmer et al., 2003). RRF-3 is suggested to compete with RDRPs RRF-1 or EGO-1 (Kishore et al., 2006; Massirer et al., 2012; Simmer et al., 2002, 2003). Secondary Argonautes, DICER, and SID-1 are all proposed to be limiting resources as overexpression can increase RNAi efficiency (Zhuang and Hunter, 2012b). Competition is also proposed to differ between tissues, contributing to tissue specific difference in RNAi (Zhuang and Hunter, 2011). All 9 original Eri mutants could be rescued maternally and also enhance RNAi in the germline (Zhuang and Hunter, 2012a). Several including *eri-1, rrf-3, eri-3* and *eri-5* also display a temperature sensitive sterility at 25°C.

Other Eri mutants act by antagonising the RNAi pathway. The RNAse T enzyme ERI-1 degrades siRNAs and is expressed most strongly in the gonad and neurones, likely contributing to neuronal resistance (Jadiya and Nazir, 2014; Kennedy et al., 2004). RNAi mediated gene silencing in the soma is also antagonised by the RNA editing adenosine deaminase ADR-2. Repetitive transgene arrays in the germline are readily silenced whilst the silencing of repetitive arrays in the soma is less common. However, loss of function of ADR-2 or its regulator ADBP-1 generates an RNAi hypersensitive background in which somatic transgenes are silenced via an *rde-1* dependent mechanism (Knight and Bass, 2002; Ohta et al., 2008).

Another group of Eri mutants are transcriptional repressors which act as part of the Rb complex and form a subset of the class B synthetic multivulval gene class (synMuvB). This class of mutants includes Retinoblastoma (Rb) homologue *lin-35,* and *lin-15B.* Loss of function of LIN-35 results in an enhanced exo-RNAi response, including an enhanced germline and neuronal RNAi response, enhanced somatic transgene silencing, reduced function in the endogenous RNAi pathway and temperature sensitive sterility, as seen in other ERI mutants (Joyce et al., 2006; Lehner et al., 2006; Wang et al., 2005). *lin-35* mutants and other SynMuvB mutants which form part of the Rb complex also show misexpression of germline specific P-granules and other germline specific genes. The requirement for RDRP RRF-1 for an efficient RNAi response in the soma is also relaxed in *lin-35* mutants, possibly due to the germline restricted RDRP EGO-1 becoming more widely expressed (Wang et al., 2005; Wu et al., 2012). The enhanced RNAi effects caused by loss of function of the Rb complex are additive to those seen in the classical Eri mutants (Wang et al., 2005). For example the strain KP3948 with genotype *eri-1(mg366) IV; lin-15B(n744)* X is frequently made use of due to its RNAi efficiency being greater than that of eri-1 mutants alone.

LIN-35 is a key tumour suppressor regulating cell cycle transition and has numerous developmental roles. In the developing embryo its expression is regulated by the miR-35-41 set of

miRNAs which have a critical role in early development. The miR-35-41 family of miRNA have also been shown to regulate sensitivity of the exo-RNAi response, further highlighting the interconnectivity between the different small RNA pathways. The loss of *miR-35-41* results in loss of LIN-35 accumulation, enhanced expression of endo-siRNA target genes- suggesting reduced function in the endo-siRNA pathway, and enhanced response to RNAi by feeding; supporting the idea of competition between these pathways influencing exo-RNAi efficiency. This phenotype can be rescued by overexpression of *lin-35* from transgenes or by the maternal rescue to *miR*-35-41 or *lin-35* (Massirer et al., 2012; Mushegian, 2016).

Efficient RNAi in certain tissues, such as the pharynx, and efficient transgenerational RNAi has been shown to require the nuclear RNAi pathway (Burton et al., 2011; Shiu and Hunter, 2017). Initiation of the nuclear RNAi pathway requires early exposure to dsRNA such that the RNAi induced silencing in the pharynx is only seen in the second generation of RNAi by feeding experiments. However *lin-15* and to a lesser extent *eri-1* mutants the sensitivity and/or breadth of the critical window in which the nuclear RNAi pathway may be triggered is increased, allowing some degree of silencing in the first generation (Shiu and Hunter, 2017).

Other mutants with an enhanced RNAi response are involved in the transport of or localisation of the dsRNA or the RNAi machinery. The snare protein SEC-22 was recently found to interact with SID-5. Loss of function of *sec-22* results in a cell autonomous enhanced RNAi response (Zhao et al., 2017). As previously discussed the correct localisation and targeting of both RNAi effectors and targets within the endomembrane system is likely to have an important role in the efficiency of small RNA mediated gene silencing (reviewed in (Kim et al., 2014)). Loss of function of MUT-16 strongly reduces the efficiency of the exogenous RNAi response due to the disruption of the mutator foci and mutator protein complex (Phillips et al., 2012; Zhang et al., 2011).

Interestingly insulin signalling mutants *daf-2* and *age*-1 have also been reported to regulate the RNAi response (Wang and Ruvkun, 2004). The insulin like receptor DAF-2 and the PI3 kinase AGE-1 are known to transduce signals regulating metabolism, development, stress resistance and lifespan but decreased DAF-2/ AGE-1 signalling also enhances the exo-RNAi response. Small RNAs are known to play critical roles in development and the dauer decision.

Environmental conditions are commonly reported to affect the RNAi response and the influence the activity of the small RNA pathways. A number of small RNA regulated processes are temperature dependent. For example at 20°C germline expressed transgenes are rapidly silenced within a few generations of insertion but are not silenced at 25°C (Strome et al., 2001), similarly levels of the siR-1 endogenous siRNA are 10 fold lower at 25°C than at 20°C (Fischer et al., 2013).

Temperature has been shown to affect the RNAi response in mosquitos, leading to increased susceptibility to viral infection (Adelman et al., 2013). Physiological stresses such as growth at 25°C or starvation induced developmental arrest also trigger siRNA dependent changes to gene expression which may be inherited for several generation after the stress is removed (Rechavi et al., 2014; Schott et al., 2014). Starvation has also been reported to affect the exo-RNAi response (Zhuang and Hunter, 2012a) and also leads to heritable changes in the piRNA pathway (Angelo, 2009). Finally Lin-35/Rb expression is also increased in response to starvation (Láscarez-Lagunas et al., 2014).

1.4 The IP_3 signalling pathway regulates RNAi

Work in the Baylis lab found that the mutants of a number of components of the IP_3 signalling pathway displayed an altered RNAi response (Nagy et al., 2015). Loss of function of PLCβ/EGL-8 or the IP_3 receptor ITR-1 results in an enhanced RNAi response to feeding and transgene expression. Whilst loss of IPP-5 leads to an RNAi resistant phenotype. This shows that reduced IP_3 signaling leads to an enhanced exo-RNAi response whilst increased levels of IP_3 as seen in *ipp-5* mutants leads to a reduced response. Furthermore, the enhanced RNAi response of *itr-1* and *egl-8* mutants is additive to that seen in classical Eri mutants, such that *eri-1; itr-1* double mutants are more sensitive to RNAi than *eri-1* or *itr-1* alone, and *eri-1;egl-8;lin-15B* triple mutants are further enhanced beyond *eri-1;lin-15B* double mutants.

Interestingly tissue specific rescue of *itr-1* in the intestine, but not in target cells, was able to rescue the enhanced RNAi phenotype, suggesting that this is the site of action of the IP_3 signalling pathway systemically regulating the RNAi response. This was found to be the case not only in the case of environmental RNAi, where dsRNA triggers must be taken up through the intestine, but also in transgene induced RNAi, with dsRNA produced in the pharynx able to silence GFP in the body wall muscle. This demonstrates that signaling in the intestine acts non-cell autonomously to affect the regulation of RNAi systemically. The intestine plays many roles in *C. elegans,* being not only important in the regulation of defecation but also as a key site of signaling in stress response pathways, host-pathogen interactions and aging. It is also the primary site of the synthesis and export of yolk proteins to the developing oocyte.

These intriguing findings raise a number of questions; foremost, how the IP_3 signaling might be acting to regulate the RNAi response and whether other small RNA pathways are affected and whether the RNAi response is being regulated in response to physiological or environmental conditions via the IP_3 signaling pathway? Although IP_3 is a common second messenger utilized

downstream of a diverse range of cellular receptors the involvement of EGL-8/PLCβ suggests that G-protein signalling is involved.

My aims in this thesis were to better understand the context of the IP_3 signalling events which are regulating the RNAi response and to further investigate which aspects of the endogenous RNAi pathways were altered in IP_3 signalling mutants. Firstly, I sought to identify the upstream activator of EGL-8 in the pathway regulating RNAi by screening Gα signalling mutants for an altered RNAi response, using a range of assays. EGL-8 is canonically activated by $G\alpha_q$ homologue ELG-30. However, EGL-30 was ruled out as an activator of EGL-8 in this mechanism, since EGL-30 loss of function does not result in an enhanced RNAi response. Instead the $G\alpha_{o/l}$ homologue, GOA-1, was identified as the most likely activator of EGL-8 due to the strongly enhanced RNAi response in *goa-1* loss of function mutants. Other Gα subunits and known regulators of GOA-1 and EGL-30 signalling interactions were also analysed and a loss of function mutant of the RGS protein EAT-16 was also found to have an enhanced RNAi response.

Next, with the aim of better understanding how changes in IP_3 signalling might be influencing the RNAi response, I looked to see if the Eri phenotype of *itr-1* mutants could be explained by changes to the endogenous small RNA pathways. Small RNA sequencing was used to look for evidence of such changes, followed up with qPCR. However, no substantial changes to the endogenous small RNA pathways were found.

Finally, following the interesting work of Shui and Hunter suggesting that classical Eri and SynMuvB mutants have an increased sensitivity to nuclear RNAi, I utilised the *myo2p::GFP* transgenic system to test for effects of IP_3 signalling on nuclear RNAi. The results suggest no specific alterations to the nuclear RNAi pathway in these mutants, although *itr-1* mutants do have a mildly increased sensitivity.

Chapter 2

Materials and Methods

Materials and Methods

2.1 *C. elegans* maintenance

All Worms were cultured on NGM (Nematode Growth Media) agar plates spread with OP50 *E. coli* and grown at 20°C unless otherwise stated. Worms were cultured using standard media and standard techniques and were transferred regularly to avoid contamination, starvation or overcrowding as established and outlined in (Brenner, 1974; Lewis and Fleming, 1995). See table 2.1 for all C. *elegans* strains used in this work, with further details of strains given in the strains tables in the relevant chapters.

2.1.i NGM plates

An NGM solution consisting of 1.5g NaCl, 8.5g agar and 1.25g peptone was dissolved in 487.5 ml distilled water, autoclaved and allowed to solidify for storage until needed (produced by the department media facility). This NGM was then microwaved until melted, cooled to 65°C in an incubator, then allowed to stand at room temperature until cool enough to handle. The following were added under aseptic conditions: 500µl 1M $CaCl_2$, 500µl 1M $MgSO_4$, 500µl cholesterol (5mg/ml in ethanol), 12.5ml 1M KPO_4 (pH 6.0). The final NGM solution was poured into petri dishes.

2.1.ii OP50 cultures and seeding

All steps were carried out using aseptic technique. Liquid culture of single OP50 colonies were grown overnight in standard LB broth (Lennox) at 37°C with shaking then stored at 4°C until needed. Liquid OP50 culture was added to each NGM plate, spread and allowed to grow at 30°C overnight until a thin but confluent bacterial lawn was formed (30µl per 50mm plate, 200µl per 100mm plate). Spread plates were stored at 4°C until used.

2.1.iii Embryo preparation

An embryo preparation using hypochlorite solution was used to produce axenised embryos in order to clean contaminated stocks and produce synchronised worm populations. Plates of gravid adults cleared of bacteria were washed into a 15ml falcon tube into a total volume of 3.5ml M9 solution to which was added 0.5ml 5N NaOH and 1ml NaClO (5-10% bleach). Tubes were vortexed intermittently until worms disintegrated to release eggs (~10min) at which point tubes were

centrifuged at 1300g for 30sec, to loosely pellet eggs, then the supernatant removed and replaced with sterile H_2O and mixed. To create synchronised L1 populations centrifugation, supernatant removal and rinsing was repeated the final time adding M9 buffer [3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 litre. Sterilize by autoclaving] to a final volume of 5ml. Axenised eggs in M9 were incubated at 21°C overnight with gentle shaking to hatch into L1s in the absence of food.

2.2 RNAi by feeding

All RNAi experiments were carried out using RNAi by feeding using the system of IPTG inducible dsRNA expressing bacteria of the *E. coli* strain HT115(DE3) carrying modifications of the L4440 vector, as established by Timmons, Court and Fire, 2001. The system works as follows: HT115(DE3) carries a stably inserted and IPTG inducible T7 polymerase gene, the L4440 vector produces dsRNA products transcribed by the T7 polymerase (Figure 2.1) (Timmons et al., 2001). *dpy-13, lin-1, lin-31* and *unc-15* RNAi clones used were from the Ahringer RNAi library (Kamath and Ahringer, 2003), a clone carrying the plasmid pPD128.110 was used for *gfp* RNAi (Timmons et al., 2001), a related clone with an RNAi plasmid carrying the E. *coli chloramphenicol acetyl transferase* (*cat*) gene was used as a control (a gift from A. Fire). All experiments were carried out using NGM worm culture plates with added carbenicillin (50µg/ml) and IPTG (1mM) spread with dsRNA inducing bacteria (RNAi NGM plates).

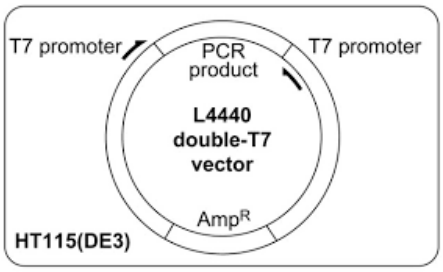


Figure 2.1. The L4440 vector used to express dsRNA. Image reproduced from dnai.org. Copyright 2003, Cold Spring Harbor Laboratory. All rights reserved, noncommercial, educational use only. Material in DNA Interactive may not be used in any form by organizations or commercial concerns, except with express permission.

2.2.i Procedure

RNAi assay plates were established as follows: 6-10 egg laying adults (depending on strain fertility) were placed on an RNAi NGM plate and allowed to lay eggs for approximately 6 hours before being removed. The F1 progeny hatched and matured on the plate and were scored for the relevant phenotype 3 days after setup, at the adult stage.

2.2.ii RNAi bacterial cultures

Cultures were grown on standard LB Agar plates with added carbenicillin (50µg/ml) and tetracycline (12.5µg/ml). Single colonies of bacteria were picked in liquid LB broth (Lennox) with added carbenicillin (50µg/ml) and grown overnight at 37°C with shaking then stored at 4°C until needed. Overnight cultures were seeded (30µl /50mm plate) and spread onto NGM plates with added carbenicillin (50µg/ml) and IPTG (1µM) and grown overnight at 30°C.

2.2.iii RNAi assay scoring - *lin-31, lin-1, dpy-13* and *unc-15*

For *lin-31, lin-1, dpy-13* and *unc-15* assays 3 plates of RNAi treated worms were scored per strain and per repeat. Worms were scored by placing the plate over a transparent grid of 5mmx 5mm squares and tallying according to phenotype of worms visible in each square at that time. Double counting was minimised by working systematically across the grid but could not be ruled out entirely due to the motile nature of the worms. Worms were viewed at 25x magnification using a dissecting microscope. For *lin-1* and *lin-31* RNAi assays worms were counted as WT if no vulval phenotype was visible, MUV if multiple vulvas were visible, or protruding vulva if a prominent vulval bump was visible. For *unc-15* assays worms were scored as WT if no locomotive defect was observed, impaired if locomotion was abnormal, severe if locomotion was not possible but some movement of the head was seen or paralysed if completely immobile. Stationary worms were poked in the tail to encourage locomotion before scoring. For *dpy-13* assays absolute measuring was not possible when scoring by eye alone. Reduction in size was estimated by comparison to an untreated plate of worms of the same strain and worms were scored as severe if estimated to be less than half normal length, moderate if a definite reduction was discernible estimated less than ¾ length, WT if no effect was discernible. The natural variation in length of the strain tested made accurate scoring by eye difficult.

2.2.iv *dpy-13* RNAi - measurement of worm length

dpy-13 RNAi assays were repeated with an improved scoring technique. One plate of *dpy-13* treated worms and one plate of control (*cat*) treated worms were set up per strain per repeat as previously described. Sections of plate containing worms were photographed using a Leica MZ FLIII microscope set to 2.5x magnification paired with a Q imaging micropublisher 5.0 cooled RTV camera and accompanying Q imaging software and saved as a Tiff. Following image capture worm length was

measured using the software ImageJ (ImageJ2,Fiji distribution) (Rueden et al., 2017; Schindelin et al., 2012) by drawing a segmented line along the midline of the worm and measuring line length.

2.2.v GFP RNAi of GABAergic neurones

One plate per strain of worms carrying the *unc-47p::GFP* transgene were treated with *gfp* RNAi or control (*cat*) RNAi. Young adult worms were mounted onto a 10 well multi-test microscope slide (MP biomedicals), in a solution of tricaine/levamisole (0.02% tricaine, 0.001% levamisole, in M9) and viewed with a Zeiss Axioskop 2plus microscope, equipped a Q imaging wLS broad-band LED illumination system, a Zeiss plan NEOFLUAR 20x objective lens, and a Q imaging micropublisher 5.0 cooled RTV camera. In each worm the number of GABAergic neurones with visible GFP fluorescence was counted. Brightly fluorescent cell bodies were scored as 1, weakly fluorescent cell bodies as 0.5. Since the 4 neurones of the head were sometime difficult to distinguish these were excluded form final analysis to give a maximum score of 21.

2.2.vi Measurement of pharyngeal GFP following RNAi

The fluorescence of strains carrying the *myo2p::GFP* transgene (see table 2.1 for strain details) was quantified in the F1 and F2 generation following either *gfp* RNAi or *cat* RNAi treatment for two generations or one generation of *gfp* RNAi treatment followed by second generation *cat* treatment. For the starvation treatment group, prior starvation treatment was carried out by placing mixed populations on plates without food for 10 days. Starved worms were reintroduced to food for one generation before assay set up to allow egg laying to resume prior to RNAi assay set up.

Young adult worms were mounted onto a 10 well multi-test microscope slide (MP biomedicals), in a solution of tricaine/levamisole (0.02% tricaine, 0.001% levamisole, in M9) and viewed with a Zeiss Axioskop 2plus microscope, equipped a Q imaging WLS LED illumination system, a Zeiss plan NEOFLUAR 20x objective lens, and a Q imaging micropublisher 5.0 cooled RTV camera. Worms were imaged with a focus on achieving a clear image of the pharynx whilst also capturing sufficient body to score for the presence of BWM fluorescence. Worms were imaged without brightfield illumination. This helped to maximise the dynamic range of fluorescence which could be captured but meant that in instances where GFP knock down was near total worms could not be seen in images. In such cases a matched image taken under brightfield conditions was also captured. Images were analysed using image J (ImageJ2,Fiji distribution) (Rueden et al., 2017; Schindelin et al., 2012) as follows: the file opened with bioFormats importer, channels were split to create 3 separate images and the channel 1 image was analysed. The rectangular selector and selector rotator tools were used to fit a tight-fitting quadrilateral around the head of the worm from the nose to the base

of the pharyngeal bulb (as seen in Figure 2.2). The measure tool was used to measure fluorescence intensity in this area and 3 background areas of approximately equal size, selected from different points in the image. Where the pharynx was not visible in the dark image the matched light image was used to select the pharyngeal region for measurement in the dark image. The presence of fluorescence in the BWM nuclei was noted as present, absent or partial. For convenience of scale 1 AU was defined as the mean value of HB955 worms on *cat* RNAi across the F1 and F2 generations. Percentage change and normalised values were calculated by dividing the value for each worm by the mean value of *cat* treated worms of that strain in that generation.

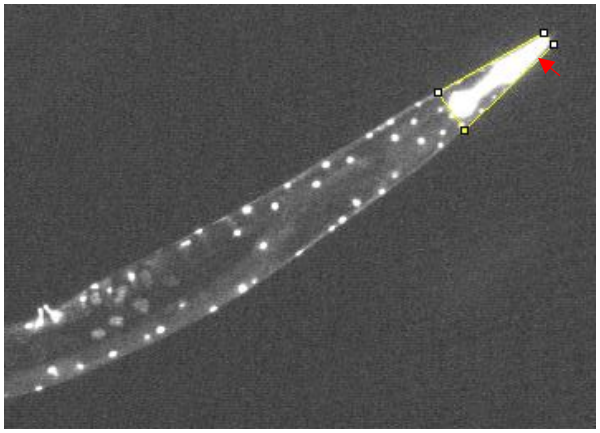


Figure 2.2. The pharyngeal area was selected as shown (yellow outline indicated by red arrow) and the pixel intensity within this area measured.

2.3 Small RNA library preparation

Populations of Bristol N2 and *itr-1(sa73)* worms were synchronised by hypochlorite treatment of fecund adult worms to extract the embryos (as described in section 2.1.iv). Embryos were removed from bleach and allowed to hatch in the absence of food in liquid suspension of M9 buffer at 20°C. L1 stage larvae were then plated onto NGM to grow as a synchronised population of worms. As young adults worms were washed in M9 buffer and centrifuged into a pellet of packed worms. 10ml of TRIzol reagent (Invitrogen) was added per ml of packed worms and the suspension was flash frozen using a dry ice and ethanol bath before being stored at -80°C overnight. Worms were lysed by repeated cycles of freezing in liquid Nitrogen, thawing at 37°C and vortexing for 1 minute. This was repeated 8 times. Chloroform extraction using 0.2ml of chloroform per 1ml of TRIzol reagent was used to extract RNA from the lysed worms into the upper aqueous phase. A second chloroform extraction was carried out with equal volumes chloroform and sample extract and the upper phase again selected. RNA was precipitated using isopropanol and washed in ice cold 75% ethanol. RNA was re-suspended in RNAase free water. Purity of RNA was assessed by measuring the 260/280 nm

absorption reading using NanoDrop spectrophotometer (ND1000, ThermoFisher Scientific). The presence of undegraded RNA in each sample was confirmed by agarose gel electrophoresis and ethidium bromide staining. Samples were stored at -80°C.

For the 5' independent libraries 5µg total RNA was treated with 1µl RNA 5' Polyphosphatase (Epicentre). RNA was then re-extracted by phenol extraction followed by chloroform/isoamyl-alcohol extraction. RNA was then precipitated overnight in ethanol and sodium acetate and re-suspended in RNAase free water. 5' dependent and independent sequence libraries were then prepared as described in the TruSeq Small RNA sample preparation guide: RNA samples were then converted to cDNA sequence libraries and the fraction of the cDNA library corresponding to the small RNA population was selected by band extraction after agarose gel electrophoresis. Quality control was also carried out on the libraries prior to Illumina sequencing. 5' dependent and 5' independent libraries of WT and *itr-1(sa73) C. elegans* strains were prepared. These 4 libraries were then sequenced using high throughput Illumina sequencing, 2 technical replicates per group.

2.4 Quantitative real-time PCR

Total RNA was extracted from synchronised population of N2, *itr-1(sa73), egl-8(e2917), ipp-5(sy605)* L1 worms: worms were synchronised by hypochlorite treatment of fecund adult worms to extract the embryos (as described in section 2.1.iv). Embryos were removed from bleach and allowed to hatch overnight in the absence of food in liquid suspension of M9 buffer at 20°C followed by being centrifuged into a pellet of packed worms. Worms were lysed in 10ml TRIzol per 1ml packed worms by repeated cycles of freezing in liquid Nitrogen, thawing at 37°C and vortexing for 1 minute. This was repeated 8 times. Chloroform extraction using 0.2ml of chloroform per 1ml of TRIzol was used to extract RNA from the lysed worms into the upper aqueous phase. A second chloroform extraction was carried out with equal volumes chloroform and sample extract and the upper phase again selected. RNA was precipitated using isopropanol and washed in ice cold 75% ethanol. cDNA was produced by reverse transcription using the Promega Reverse Transcription System according to manufacturer's protocol and using random primers (Promega, 0.5µg/µl). qPCR reactions were carried out using KAPA SYBR FAST qPCR kit and BioRad iCycler. 30ng total cDNA was used per reaction.

qPCR protocol was as follows: 95.0°C for 05:00 min, 50X (95.0°C for 00:15, 60.0°C for 01:00), 95.0°C for 01:00, 50.0°C for 01:00, 80X(55.0°C for 00:10).

All test and control reactions were carried out in triplicate. Relative abundance of test gene mRNA to the control gene GAPDH mRNA in mutant strains compared to the N2 strain was calculated using the

ΔΔCt method implemented via the R package 'ddCt' (Zhang JD, Biczok R, Ruschhaupt M (2019). *ddCt: The ddCt Algorithm for the Analysis of Quantitative Real-Time PCR (qRT-PCR)*. R package version 1.40.0.). See table 2.2 for qPCR primers used in this work.

2.5 Statistical analysis and data presentation

All Statistical analysis in chapters 3 and 5 was performed in R studio (version 1.2.1335), using R ((version 3.6.1) (R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.))
Most statistical analysis, including the fitting of analysis of variance models, fitting linear models, fitting generalised linear models, Tukey HSD tests and Wilcoxon rank sum tests, was carried out using functions of the R stats package. Additionally, the car package was used for Levene's test (John Fox and Sanford Weisberg (2019). An R Companion to Applied Regression, Third Edition. Thousand Oaks CA: Sage. URL: https://socialsciences.mcmaster.ca/jfox/Books/Companion/), the package lme4 was used to fit generalised mixed linear models (Douglas Bates, Martin Maechler, Ben Bolker, Steve Walker (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, 67(1), 1-48. doi:10.18637/jss.v067.i01.) and the DHARMa package was used to assess the fit of models (Florian Hartig (2019). DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. R package version 0.2.4. https://CRAN.R-project.org/package=DHARMa)

Results figures were produced in R using the ggplot2 package (H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016), the scales package (Hadley Wickham (2018). scales: Scale Functions for Visualization. R package version 1.0.0. https://CRAN.R-project.org/package=scales) and the Viridis colour palette (Simon Garnier (2018). viridis: Default Color Maps from 'matplotlib'. R package version 0.5.1. https://CRAN.R-project.org/package=viridis). The cowplot package was used to assemble multipanel figures (Claus O. Wilke (2019). cowplot: Streamlined Plot Theme and Plot Annotations for 'ggplot2'. R package version 1.0.0. https://CRAN.R-project.org/package=cowplot).

The statistical analysis of the small RNAseq libraries presented in chapter 4 was performed in SeqMonk (Andrews, 2011). An unbiased probe set of continuous 40bp windowed probes covering the entire genome assembly was used to compare libraries. Libraries were corrected for total read count. The 'intensity difference' and 'DeSeq2' (Love, M.I., Huber, W., Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2 Genome Biology 15(12):550 2014) statistical testing tools were used to looks for probes which were differentially expressed

between the JT73 and N2 libraries. Figures not generated using SeqMonk were generated using R after using the package 'ShortRead' to import sequence libraries (M. Morgan, S. Anders, M. Lawrence, P. Aboyoun, H. Pages, and R. Gentleman (2009): "ShortRead: a Bioconductor package for input, quality assessment and exploration of high-throughput sequence data". Bioinformatics 25:2607-2608). GO term enrichment analysis was performed using WormBase (WormBase web site, http://www.wormbase.org, release WS272, date 2019).

Strain	Genotype	Reference	Labelled as
N2	WT (Bristol)	(Brenner, 1974)	WT
YY216	*eri-9(gg106)*	(Pavelec et al., 2009)	*eri-9*
JT73	*itr-1(sa73)*	(Iwasaki et al., 1995; Nagy et al., 2015a)	*itr-1*
CB6614	*egl-8(e2917)*	(Nagy et al., 2015a; Yook and Hodgkin, 2007)	*egl-8*
PS3653	*ipp-5(sy605)*	(Bui and Sternberg, 2002; Nagy et al., 2015a)	*ipp-5*
***Egl-30* mutants screened**			
DA823	*egl-30*(ad805) I.	(Brundage et al., 1996; McMullan et al., 2012)	*egl-30 lof1*
MT1434	*egl-30*(n686) I.	(Brundage et al., 1996), (Trent et al., 1983)	*egl-30 lof2*
DA1084	*egl-30(*ad806) I.	(Brundage et al., 1996)	*egl-30 lof3*
CG21	*egl-30*(tg26) I*; him-5*(e1490) V.	Bastiani et al., 2003)	*egl-30 gof1*
CE1047	*egl-30*(ep271) I.	(Fitzgerald et al., 2006)	*egl-30 gof2*
PS4264	*egl-30*(*sy676md186)* I; *him-5*(e1490) V.	(Bastiani et al., 2003)	*egl-30* lof gof *rescue*
PS4263	*egl-30(md186)* I; *dpy-20(e1282)* IV; syIs105[*egl30::gfp + dpy-20*(+)].	Bastiani et al., 2003)	*egl-30 lof OE rescue*

PS2444	*dpy-20*(e1282) IV; syIs36 [egl-30::egl-30(+)].	(Bastiani et al., 2003; Brundage et al., 1996; Hajdu-Cronin et al., 1999; Robatzek and Thomas, 2000)	*egl-30 OE*
Gα mutants screened			
MT363	*goa-1(n363)*	(Dong et al., 2000; Robatzek and Thomas, 2000; Segalat et al., 1995)	*goa-1 lof1*
DG1856	*goa-1(sa734)*	(Robatzek and Thomas, 2000)	*goa-1 lof2*
MT2426	*goa-1(n1134)*	(Segalat et al., 1995)	*goa-1 lof3*
PS1493	*dpy-20(e1362) IV; syIs9.*	(Hajdu-Cronin et al., 1999; Mendel et al., 1995)	*goa-1 OE*
KG421	*gsa-1(ce81)*	(Schade et al., 2005)	*gsa-1 gof*
NL795	*gpa-7(pk610)*	(Plasterk et al., 1999)	*gpa-7 lof*
NL1147	*gpa-10(pk362)*	(Plasterk et al., 1999)	*gpa-10 lof*
NL594	*gpa-12(pk322)*	(Alam et al., 2016; Plasterk et al., 1999; Yemini et al., 2013; Ziegler et al., 2009a)	*gpa-12 lof*
BW1809	*gpa-16(it143) ; him-5(e1490)*	(Johnston et al., 2008)	*gpa-16 lof*
RB1800	*gpa-17(ok2334)*	(Yemini et al., 2013).	*gpa-17 lof*
Regulators of G-protein signalling mutants screened			
LX147	*rgs-1(nr2017)* III.	(Dong et al., 2000)	*rgs-1 lof*
MT8504	*egl-10(md176*) V.	(Esposito et al., 2010; Koelle and Robert Horvitz, 1996; Porter and Koelle, 2010)	*egl-10 lof1*
JT609	*eat-16(sa609*) I.		*eat-16 lof1*
LX1313	*eat-16(tm761)* I*; egl-10(md176*) V.	(Wani et al., 2012)	*eat-16: egl-10 lof*
RM1702	*ric-8(md303)* IV.	(Miller et al., 2000)	*ric-8 lof1*

RM2209	*ric-8(md1909)* IV.	(Miller et al., 2000)	*ric-8 lof2*
RB1145	*ags-3(ok1169)*	(Hofler and Koelle, 2011)	*ags-3 lof*
JT603	*gpb-2(sa603)* I	(Robatzek et al., 2001)	*gpb-2 lof1*
DA541	*gpb-2(ad541*) I.	(Robatzek et al., 2001)	*gpb-1 lof2*
LX1270	*rsbp-1(vs163*) I.	(Porter and Koelle, 2010)	*rspb-1 lof*
Unc-47::GFP transgenic strains			
EG1285	*lin-15B&lin-15A(n765); oxIs12 (unc-47p::GFP + lin-15(+))*	(McIntire et al., 1997)	*EG1285*
HB900	*itr-1(sa73)IV; him-5(e1462)V; oxIs12X*	(Nagy et al., 2015b)	*Itr-1*
HB450	*goa-1(n363); oxIs12X*	This work	*goa-1 lof1*
HB451	*syIS9; oxIs12X (goa-1 xs)*	This work	*goa-1 OE*
HB452	*egl-30(ad805); oxIs12X*	This work	*egl-30 lof*
Pharyngeal GFP strains			
HB955	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]*	(Nagy et al., 2015a)	*WT 2*
HB953	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]*	(Nagy et al., 2015b)	*WT 1*
HB948	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; egl-8(e2917)V*	(Nagy et al., 2015b)	*egl-8 1*
HB949	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; egl-8(e2917)V*	(Nagy et al., 2015b)	*egl-8 2*
HB951	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; ipp-5(sy605)X*	(Nagy et al., 2015b)	*ipp-5 1*
HB952	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; ipp-5(sy605)X*	(Nagy et al., 2015b)	*ipp-5 2*
HB954	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; itr-1(sa73)IV*	(Nagy et al., 2015b)	*itr-1 1*
HB956	*ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP]; itr-1(sa73)IV*	(Nagy et al., 2015b)	*itr-1 2*

Table 2.1. *C. elegans* strains used in this work. Strains not created in the Baylis lab were supplied by the Caenorhabditis Genetics Centre (University of Minnesota).

Gene	Primer	Sequence
GAPDH	ED2822	TGGAGCCGACTATGTCGTTGAG
	ED2823	GCAGATGGAGCAGAGATGATGAC
T02G6.4	AR2856	TGAATGCCGTAGGAGCAACC
	AR2857	GCATCTCCAAATAGGTGCAG
F55C9.5	AR2858	GGAAACAATACCATTGGAGC
	AR2859	TTGGAAATGGAATCTCCTTC
Y116F11A.1	AR2860	AGCAGCAAAAGCGATCGGAG
	AR2861	GTGCCAGCTCGCTAGGAGTG
C11G6.2	AR2862	AGAGTAGTTTTGATGACTGG
	AR2863	CGATGATAATAAGTCGTGTC
F52G3.4	AR2864	ACAGTCCCGTGCCCATTCTG
	AR2865	TGTCGATTGCACTCCGTGTG
C40A11.10	AR2866	TCGGACAATATCACTGGATC
	AR2867	GGTGTGATGCTATCGCTTAG
Lin-35	AR2868	ACTAATACACCGCCACCATC
	AR2869	TCAAGAGAAACCCGTTGAAC

Table 2.2. qPCR primers used in this work.

Chapter 3

Identification of cell signalling mutants with an altered RNAi response

Identification of cell signalling mutants with an altered RNAi response

3.1 Introduction

IP_3 signalling, both in *C. elegans* and more widely is initiated by the activation of inositol specific phospholipases of the PLC family. As previously discussed (see section 1.2.iv) the *C. elegans* genome encodes 5 putatively active PLC genes, 4 of which are clear homologues of mammalian counterparts, each known to play a role in the regulation of IP_3 signalling in different contexts (Gower et al., 2005; Lackner et al., 1999; Miller et al., 1999; Yin et al., 2004) reviewed in (Baylis and Vázquez-Manrique, 2012)). In each case the PLC is activated by an upstream signal transducer of a particular type, principally heterotrimeric G-proteins, small G-proteins or receptor tyrosine kinases (reviewed in (Suh et al., 2008). Like it's mammalian counterpart PLCβ, EGL-8 is canonically activated by G-protein signalling and has been predicated to be capable of interaction with both the Gαq and Gβγ signalling components (Illenberger et al., 2003; Singer et al., 2002).

Since only plcβ homologue *egl-8* loss-of-function mutants were found to display an enhanced RNAi phenotype comparable to that of an *itr-1* loss-of-function mutant it seems clear that EGL-8 is responsible for the production of IP_3 in the ITR-1 dependent signalling pathway regulating the exogenous RNAi response in *C. elegans* (Nagy et al., 2015). The most obvious candidate for the upstream signalling component responsible for the activation of EGL-8 in this pathway was therefore the well-established canonical activator, the *C. elegans* homologue of mammalian Gαq subunit, EGL-30 (Brundage et al., 1996). However preliminary screens of *egl-30* mutants for an altered RNAi phenotype performed in the lab showed no consistent RNAi phenotype and therefore no clear role for EGL-30 in this pathway, raising the possibility that heterotrimeric G-protein signalling involving a different Gα subunit could be regulating EGL-8 activation in this pathway.

A central goal of my project was therefore to identify the upstream signalling components regulating EGL-8 activation, and therefore IP_3 production, in the signalling pathway modulating the exogenous RNAi response. The approach I took was to screen existing mutant strains of candidate genes for an altered (enhanced or decreased efficacy) RNAi response, using RNAi by feeding to target knock down of genes known to have an incomplete penetrance of the RNAi induced loss of function phenotype in a WT background, but an easily scorable phenotype in an enhanced RNAi background.

A range of such genes have been previously characterised and used in the identification of Eri mutants (Kennedy et al., 2004; Simmer et al., 2002; Wang et al., 2005). It is commonly seen that in such an enhanced RNAi background, where the efficacy of RNA interference is improved, both the frequency of the occurrence of the knockdown phenotype in the population, and the severity of the

phenotype are increased. When screening mutants for a global increase in the efficacy of the exogenous RNAi pathway it is important to characterise the efficacy of RNAi induced knockdown against a range of genes, operating in a variety of different pathways, in order to establish whether it is the RNAi response itself which is altered, or whether a more apparent phenotype is seen due to genetic interaction between the RNAi target gene and the mutant being screened. In the latter case if a mutant is already partially compromised in a pathway regulating a given phenotype then further compromise to that pathway due to even partial RNAi induced knock-down of a second gene is more likely to produce the target phenotype, due to the genetic background being sensitised to that phenotype rather than due to any change in the efficacy of the RNAi. This possibility warrants particular consideration when dealing with mutants of genes known to play diverse and pleiotropic roles in *C. elegans,* such as the Gα proteins and their second messengers.

Using this approach, I screened a range of *egl-30* mutants in order to test the preliminary finding that EGL-30 (Gαq) was not the upstream activator of EGL-8 (PLCβ) in the IP_3 signalling pathway regulating the exogenous RNAi response. I also screened a range of other Gα protein mutants based on the working hypothesis that heterotrimeric G-protein signalling involving a different Gα subunit was the upstream activator of EGL-8, which seemed the most likely scenario given that PLCβ is well established as an effector of GPCR/heterotrimeric G-protein signalling. Finally, I screened a number of mutants of proteins known to be involved in the regulation of heterotrimeric G-protein signalling in order to build a clear picture of the signalling network regulating the RNAi response.

The RNAi target genes utilised were *lin-31*, *lin-1*, *dpy-13* and *unc-15.* All of which are well established as targets for measuring RNAi response; they have been previously used in the characterisation of eri mutants (Kennedy et al., 2004; Simmer et al., 2002) and were used to characterise the altered RNAi response of *itr-1, egl-8* and *ipp-5* mutants (Nagy et al., 2015). *lin-31 and lin-1* encode the heterodimeric components of the transcription factor complex downstream of the MAP kinase signalling pathway involved in vulval induction (Tan et al., 1998). Loss of either *lin-1* or *lin-31* leads to a multivulval phenotype, or in the case of a partial knock down the milder phenotype of a protruding vulva. *Dpy-13* encodes a component of the cuticle collagen, loss of which leads to altered body shape known as dumpy (Dpy) (von Mende et al., 1988). *unc-15* is a paramyosin ortholog loss of which leads to loss of locomotion (Kagawa et al., 1989). Animals display a sluggish locomotion in the mildest case and complete flaccid paralysis in the most severe case, along with a variety of other phenotypes associated with loss of functional myosin such as an Egl phenotype. All strains tested were compared to the wildtype strain Bristol N2, an *eri-9* mutant strain, displaying an enhanced exo-RNAi response, as a positive control and an *ipp-5* lof mutant strain, displaying a greatly

decreased efficiency of exo-RNAi as a comparison for a decreased response. All strains were scored as adults after 1 generation of exposure to dsRNA by feeding.

Neuronal GFP knockdown using GFP RNAi was used to confirm results in cases where an altered RNAi response seemed likely but complex strain phenotypes made phenotype scoring and ruling out compounding genetic interactions difficult. Neuronally expressed genes are generally refractory to RNAi but knockdown is seen in enhanced RNAi backgrounds(Calixto et al., 2010; Kennedy et al., 2004; Lehner et al., 2006). GFP knockdown is a good readout of exogenous RNAi efficacy, with *gfp* expression more likely to be truly independent of signalling pathway activity than endogenous genetic pathways such as those regulating vulval development, cuticle formation, or locomotion.

3.2 Results

3.2.i Investigating the role of EGL-30 (Gαq) in the regulation of the RNAi response

If Gαq *egl-30* were to be acting in its canonical role as the activator of PLCβ *egl-8* in the IP_3 signalling pathway regulating the exo-RNAi response, then it would be expected that loss of function of *egl-30* would phenocopy loss of function of *egl-8* and *itr-1*, resulting in an enhanced RNAi response. To test this hypothesis a range of previously characterised EGL-30 mutant strains were identified and selected for screening. These included loss of function (lof), gain of function (gof), overexpression (OE), and loss of function mutants rescued by either a second masking gain of function mutation or by transgene (lof gof rescue/ OE rescue). Each strain displays varying severities of *egl-30* associated loss of function and gain of function phenotypes, representing a range of pleiotropic effects. Loss of function of *egl-30* is associated with a severe Egl phenotype leading to bloating due to egg retention, lethargy/flaccid paralysis, which becomes more pronounced with age and causes a notably abnormal pattern of locomotion and foraging, reduced rate of pharyngeal pumping, and ovulation defects contributing to reduced fertility and egg laying, and reduced viability. Loss of function of *egl-30* was found to suppress the lethal effects of arecoline seen in *gpb-2* (*eat-11*) lof mutants (Brundage et al., 1996). Gain of function or over expression of *egl-30* causes a set of opposite phenotypes. GOF mutants are hyperactive, spending less time at rest, change direction more frequently and have an exaggerated body bend. They also display a constitutive egg laying phenotype whereby eggs are more frequently and at earlier stages of development than WT strains (<4 cell stage). The strains used are described in Table 3.1.

Strain name	genotype	labelled as	variant/ description	key phenotypes	key references
DA823	*egl-30*(ad805) I	***egl-30 lof 1***	Likely near null. Intronic point mutation predicted to cause defective splicing of intron 2.	Severe flaccid near paralysis, very sluggish with shallow tracks, severely bloated with eggs, reduced pharyngeal pumping, unresponsive to harsh body touch. Reduced behavioural and immune response to bacterial infection. Semi dominant suppressor of *eat-11* (*gpb-2).*	(Brundage et al., 1996; McMullan et al., 2012)
MT1434	*egl-30*(n686) I	***egl-30 lof 2***	lof. Intronic point mutation predicted to cause defective splicing of intron 4.	Sluggish with variable tracks, bloated with eggs, reduced pharyngeal pumping. Semi dominant suppressor of *eat-11* (*gpb-2).*	(Brundage et al., 1996)**,** (Trent et al., 1983)
DA1084	*egl-30(*ad806) I	***egl-30 lof 3***	lof. Point mutation leading to Ser(6) to Phe.	Sluggish, bloated with eggs. Semi dominant suppressor of *eat-11* (*gpb-2).*	(Brundage et al., 1996)
CG21	*egl-30*(tg26) I*; him-5*(e1490) V	***egl-30 gof 1***	g/a substitution. Hypermorph GOF	Hyperactive movement, increased coiling, melatonin resistant, small.	Bastiani et al., 2003)

Table 3.1. *egl-30* mutants used in work described in this chapter.
Descriptions taken from referenced papers or from WormBase.

Strain name	genotype	labelled as	variant/ description	key phenotypes	key references
CE1047	*egl-30*(*ep271*) I	***egl-30 gof 2***	Dominant, gain-of-function g/a substitution leading to M to L substitution.	Hyperactive movement, constitutive egg laying.	(Fitzgerald et al., 2006)
PS4264	*egl-30*(*sy676md186)* I; *him-5*(e1490) V.	***egl-30 lof gof resuce***	Rescuing and gof. *sy676* intragenic revertant in lof mutation md186.	Semi-dominant hyperactive movement with exaggerated body bends and constitutive egg laying.	(Bastiani et al., 2003)
PS4263	*egl-30(md186)* I; *dpy-20(e1282)* IV; syIs105[*egl-30::gfp + dpy-20*(+)]. Integrated transgene	***egl-30 lof OE rescue***	[*egl-30*::GFP + *dpy-20*(+)] including all of the presumptive 5'-transcriptional regulatory sequences, introns, and presumptive 3 regulatory sequences for *egl-30*.	partially rescue egl-30(md186) with respect to pharyngeal pumping, egg laying and movement. Hypersensitive response to neurotransmitters in egg-laying assays.	Bastiani et al., 2003)
PS2444	*dpy-20*(e1282) IV; *syIs36* [*egl-30::egl-30(+)*].	***egl-30 OE***	WT e*gl-30* overexpressed. *syIs36* [(pLB2) *egl-30(+)* + pBS + (pMH86) *dpy-20(+)*].	Hyperactive movement, exaggerated body bends, eggs laid constitutively and early stage, starved appearance.	(Bastiani et al., 2003; Brundage et al., 1996; Hajdu-Cronin et al., 1999; Robatzek and Thomas, 2000)

Table 3.1 (continued). *egl-30* mutants used in work described in this chapter.
Descriptions taken from referenced papers or from WormBase.

The effects of *lin-31* and *lin-1* RNAi were measured by scoring the phenotype of adult worms as a WT (unaffected), multivulval phenotype (MUV) or a single protruding vulval phenotype (protruding). Each worm was scored for the presence or absence of these phenotypes allowing the proportion of worms of each strain population with each phenotype to be calculated. The results of the *lin-31* RNAi assays against the *egl-30* mutants are shown in figure 3.1. All 3 *egl-30 lof* strains consistently show a lower level of response to *lin-31* RNAi treatment than the WT strain, whilst some but not all *egl-30 gof* strains appear to show an enhanced response to *lin-31* RNAi. A higher incidence of MUV and protruding vulval phenotype is seen in the *egl-30 gof 1* and *egl-30 lof OE rescue* strains, with response levels comparable to that of the *eri-9* strains. A statistically significant but less dramatic increase is seen in the strain *egl-30 lof+ gof,* whilst no significant difference in response from the WT is seen in the strains *egl-30 gof 2* and *egl-30 OE.*

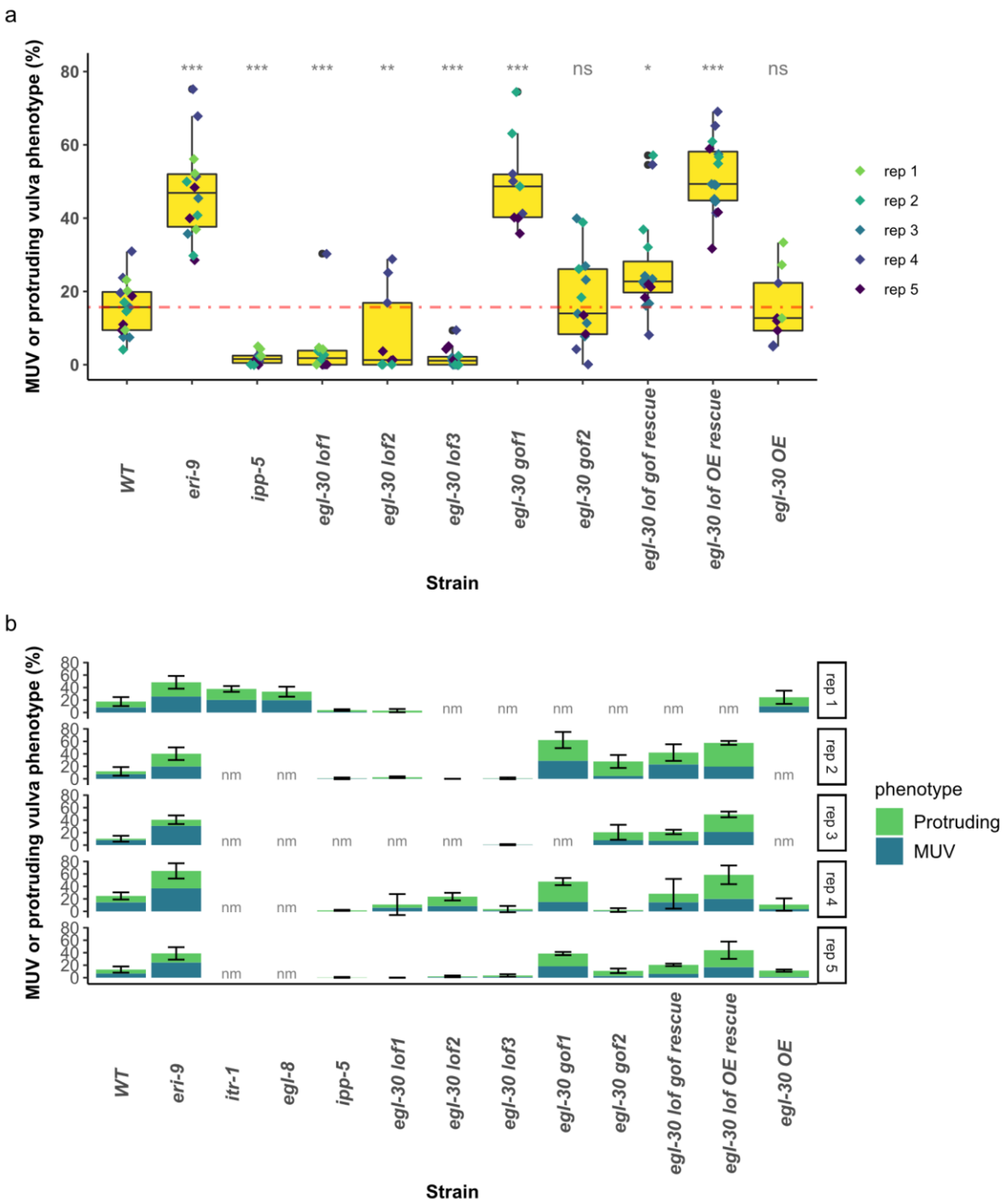


Figure 3.1. Results of *lin-31* RNAi by feeding on *egl-30* mutants. Successful *lin-31* knockdown during development results in a multivulval (MUV) phenotype, or a milder protruding vulva phenotype. **a**- Box plots representing the range of values of the sum MUV or protruding vulval phenotypes seen for each strain. Box edges represent upper and lower quartiles with the central line representing the median. Each coloured dot represents one experimental plate with a median population of 104 worms, range 15-380. Statistical significance was assessed using logistic regression analysis, modelling the proportion of population affected, weighted for plate population, and including observation-level random effect. Statistically significant differences from WT are indicated. **b**- Represents the data shown in **a** but % MUV or protruding vulval is broken down by class. The mean percentage of worms of each strain with a MUV or protruding vulval phenotype is shown, error bars represent standard deviation (SD). Each row represents an independent repeat consisting of 3 replicate plates per strain. "nm" represents strain not tested in this repeat. Data for *egl-8* and *itr-1* strains is shown for comparison.

The response to lin-1 RNAi, seen in figure 3.2, follows a very similar pattern to the response to lin-31 RNAi. The *egl-30* lof strains each exhibit a response comparable to (*egl-30 lof1, egl-30 lof3*) or lower than (*egl-30 lof2*) that seen in the WT strain, whilst the gain of function mutant *egl-30 lof1* (*egl-30(tg26*)) and the *egl-30 lof +OE rescue* strain carrying the, display a significantly enhanced response. These are the same two strains which show the strongest response to *lin-31* RNAi, once again with levels of response comparable to, or higher than, that of the *eri-9* mutant. In the case of both *lin-31* and *lin-1* RNAi treatment an clear increase in proportion of the population with the more severe MUV phenotype as well as an increase in protruding vulval phenotype is seen, as shown in figure 3.1b and 3.2b respectively. The other gain of function and overexpression strains show no significant difference in response to *lin-1* RNAi treatment compared to the WT, as was seen with *lin-31.*

The implication from these results, that reduced *egl-30* signalling may have an inhibitory effect on the RNAi response whilst reduced function may increase efficacy is a surprising result since this is the exact opposite of the response which would be expected if *egl-30* is acting in its canonical role as an activator of IP_3 signalling. These results should be interpreted with the caveat that both *lin-31* and *lin-1* act downstream in the MAPK signalling cascade regulating vulval development. It was to be expected therefore, that a similar pattern of apparent sensitivities to *lin-1* and *lin-31* RNAi would be seen across the *egl-30* mutants, since any potential genetic interactions between the target gene and *egl-30* due to pathway specific interactions or background sensitivity to aberrant vulval development would be expected to be seen in both assays. It was therefore important to assay the RNAi sensitivity of the *egl-30* mutants using a range of independent assays.

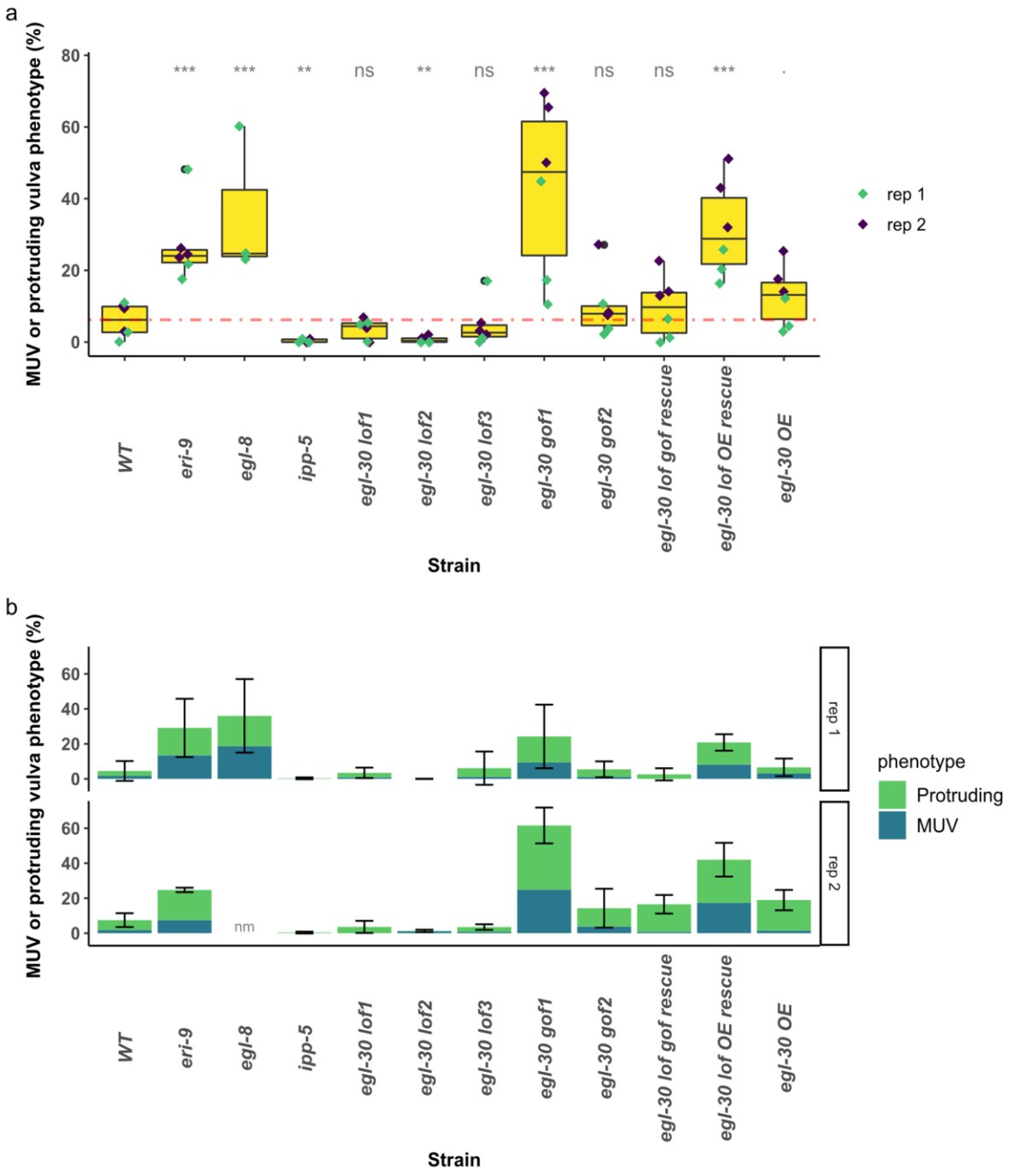


Figure 3.2. Results of *lin-1* RNAi treatment on egl-30 mutants. a- Box plots representing the range of values of the sum MUV or protruding vulval phenotypes seen for each strain. Box edges represent upper and lower quartiles with the central line representing the median. Each coloured dot represents one experimental plate with a median population of 109 worms, range 12-221. Statistical significance was assessed using logistic regression analysis, modelling the proportion of population affected, weighted for plate population, and including observation-level random effect. Statistically significant differences from the WT strain are shown. **b**- Represents the same data shown in **a,** but % MUV or protruding vulval is broken down by class. The mean percentage of worms of each strain with a MUV or protruding vulval phenotype is shown, error bars represent standard deviation (SD). Each row represents an independent repeat consisting of 3 replicate plates per strain. Data for *egl-8* strains is shown for comparison.

The effects of *unc-15* RNAi treatment on the *egl-30* mutants were assayed, (see figure 3.3) and showed a markedly different pattern of sensitivities to the *lin-31* and *lin-1* assays. The *egl-30* lof mutants all showed a high degree of paralysis and impaired locomotion, equivalent to the response seen in the WT strain. This is very different from the reduced response seen to *lin-31* and *lin-1* RNAi. However, this result is less surprising given that loss of *unc-15* and loss of *egl-30* both result in lethargy and flaccid paralysis. The apparent increased sensitivity seen in *egl-30* lof1 mutants therefore is likely not a result of an increased sensitivity to RNAi but to an increased predisposition to the measured phenotype. Conversely the *egl-30 gof2, egl-30 OE,* and *egl-30 lof gof rescue* mutants all show significantly reduced percentage of paralysed and impaired worms compared to the WT. The *egl-30 gof1* overall shows no significant difference to the WT response, however the percentage of paralysed worms is decreased whilst the *egl-30 lof OE rescue shows a greater than WT response*. The reduced paralysis seen in several of the gof strains could conceivably be in part be due to the hyperactive phenotype of these strains compensating for loss of *unc-15* function, making the results of this assay difficult to interpret reliably.

The *dpy-13* RNAi assay provides a more independent assessment of the effect of RNAi sensitivity since the Dpy phenotype does not overlap with any known *egl-30* phenotypes. The results shown in figure 3.4a show that whilst some strains did appear to show a change in the frequency of occurrence of the Dpy phenotype some gof strains, such as *egl-30 gof 1* show an increased frequency of Dpy worms, whilst *egl-30 gof 2* show a decreased response. There is also a significant inconsistency between the behaviour of same strains between repeat experiments (fig 3.4b). I found that the *dpy-13* RNAi phenotype manifested as a full spectrum of alteration in length with a high penetrance of a mild-moderate Dpy phenotype characterised by slightly shorter length but otherwise normal appearance seen in WT populations with a continual spectrum of reduction in length seen through to the severe Dpy phenotype, characterised by worm appearing at least less than half their normal length seen in *eri-9* mutants. The continuous nature of spectrum of this phenotype combined with the natural variations in worm length between strains made reliably scoring the phenotypes by eye into discrete categorisations challenging.

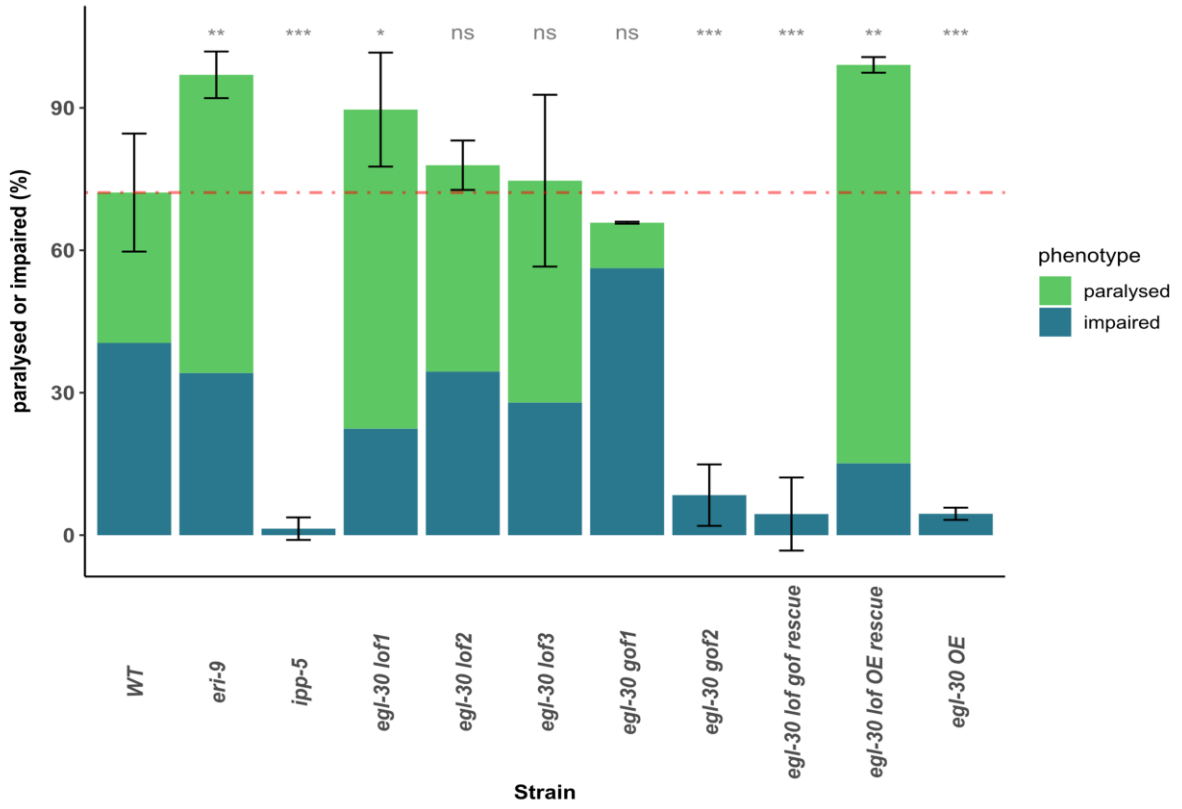


Figure 3.3. Results of *unc-15* RNAi treatment on *egl-30* mutants. Successful *unc-15* knock- down results in a paralysis phenotype, with partial knock-down producing a milder impaired locomotion phenotype. The *mean percentage of worms displaying the paralysed (green bars) or impaired locomotion phenotypes (blue) is shown for each strain, error bars represent standard deviation (SD). Number of worms per plate range 44-287, median 84, with 3 plates per strain.* Statistical significance was assessed using logistic regression analysis, modelling the proportion *of the* population with paralysed or impaired phenotype, weighted for plate population, fitted to a quasibinomial distribution. Statistically significant differences from the WT strain are denoted by "***" ($p<0.001$), "**" ($p<0.01$), "*" ($p<0.05$), "." (not significant $0.05<p<0.1$), "ns" (not significant, $p>0.1$).

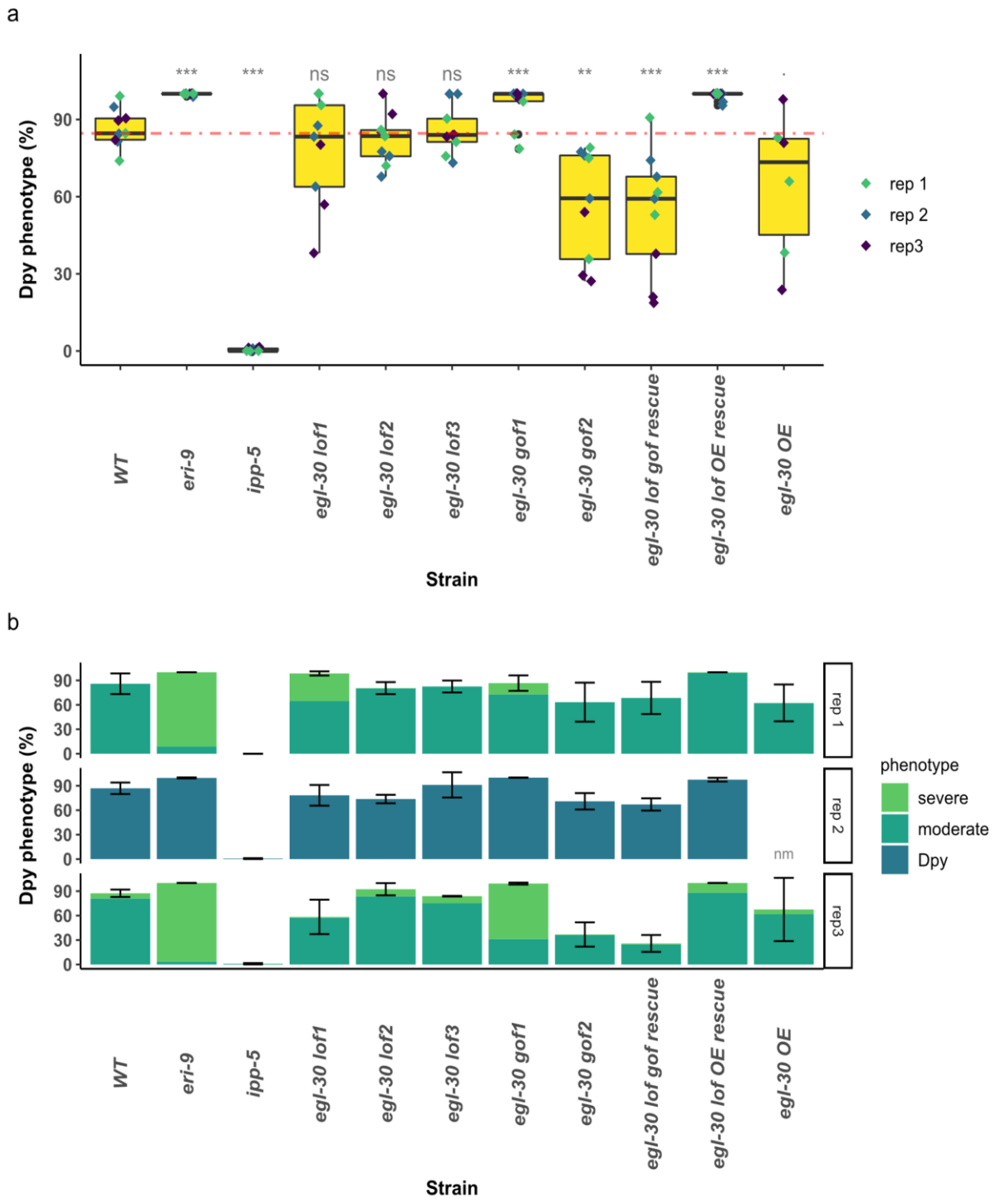


Figure 3.4. Effects of *dpy-13* RNAi by feeding on *egl-30* mutants. a- Box plots representing the range of values of the Dpy phenotypes seen for each strain. Each coloured dot represents one experimental plate with a median population size of 93 worms, range 11-236. Statistical significance was assessed using logistic regression analysis, modelling the proportion *of the* population with paralysed or impaired phenotype, weighted for plate population, fitted to a binomial distribution with strain as a fixed effect and with observational level random effect. Statistically significant differences from the WT strain are as indicated. **b-** Represents the same data shown in **a,** but % Dpy or protruding vulval is broken down by class. The mean percentage of worms of each strain with MUV and protruding vulval phenotypes is shown, error bars represent standard deviation (SD). Each row represents an independent repeat consisting of 3 replicate plates per strain. "nm" represents strain not tested in this repeat.

In order to try to improve the accuracy and the reliability of the *dpy-13* RNAi assay I measured the effect of *dpy-13* RNAi treatment by measuring the length of each worm from an image rather than scoring the phenotype by eye. As can be seen from the variation in the size of the control treatment (CAT) worms in figure 3.5a (blue boxes) there is considerable variation in the natural length of the different strains when grown on the control bacteria, with most strains being significantly shorter than the WT. The mean length of the worms of each strain raised on control bacteria was therefore used to normalise the length measurements for each strain allowing the change in length from expected length for each worm to be calculated (figure 3.5b). As expected, *eri-9* shows a clear reduction in mean length whilst *ipp-5* shows no change. Although several of the strains tested show a significant difference in change in length compared to the WT, in most cases this change in relatively small when compared to the levels change seen *eri-9* mutants (figure 3.5b, 3.6). Figure 3.5a shows the unnormalized data. To better compare the measured and observational counts of the effects of *dpy-13* the change in length was binned into categories and plotted to show the percentage of each strain displaying a reduction in length greater than 20, 40, or 60% (figure 3.6).

The results of these two methods of assaying the effect *dpy-13* RNAi treatments are largely consistent. In figure 3.4 the three *egl-30* loss of function strains show no consistent significant difference from the WT. This is also the case for *egl-30 lo f1* and *egl-30 lof 3* in figure 3.5, where *egl-30 lof 2* shows a very small but significant decrease compared to the WT. The results seen for the *egl-30* gain of function strains are less consistent. *egl-30 gof 1* show a significantly greater reduction in average length upon *dpy-13* than the WT, but *egl-30 gof 2* shows a decreased shows a decreased prevalence of the Dpy phenotype (as seen in figure 3.5 and 3.6) as well as an increased incidence of the more severe Dpy phenotype (fig 3.4a, 3.4b and 3.6). A non-significant and smaller increase in both of these measures is also consistently seen in the *egl-30 lof OE rescue* strain. However, the other *egl-30 gof* or *OE* strains show either a slight decrease in the *dpy-13* RNAi phenotype or no consistent change.

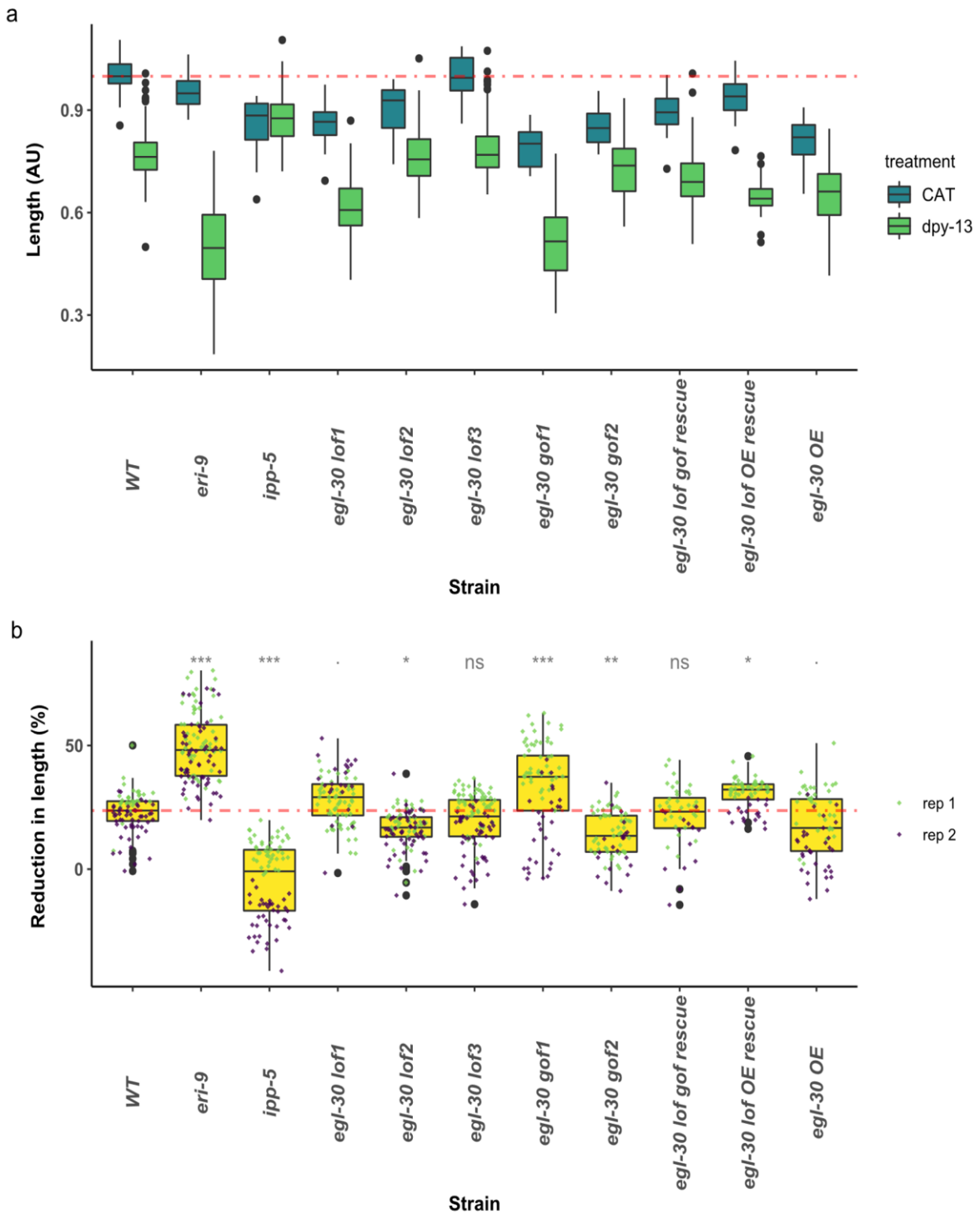


Figure 3.5. Reduction of length caused by *dpy-13* RNAi by feeding treatment on *egl-30* mutants.

a- The length of worms of each strain treated fed either control (*cat*) or *dpy-13* RNAi cultures is shown for comparison. There is considerable variation in the control treated length of worms between strains. **b-** The reduction in length of worms compared to the mean control treated length for that strain in that set. Median n/strain =82, range 48-118. Statistical significance assessed by regression analysis. Linear model accounting for length, RNAi treatment and strain. Overall *dpy-13* treatment results in a significant reduction in length. Statistically significant difference in response to dpy-13 RNAi treatment for the test strains compared to the WT strain are denoted by "***" ($p<0.001$), "**" ($p<0.01$), "*" ($p<0.05$), "." (not significant $0.05<p<0.1$), "ns" (not significant, $p>0.1$).

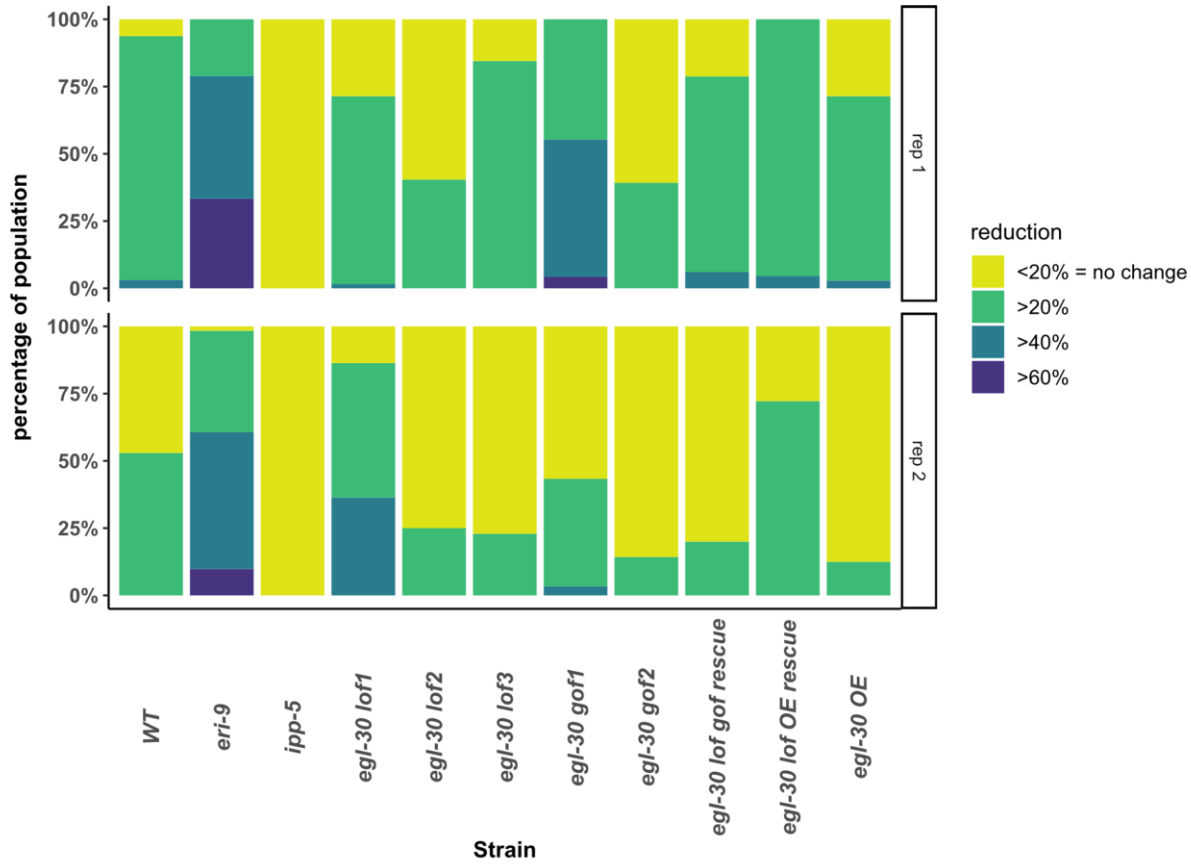


Figure 3. 6. Reduction of length caused by *dpy-13* RNAi by feeding treatment on *egl-30* mutants. The data from figure 3.5b is here represented as the frequency of dpy-13 RNAi treated worms of each strain which show a reduction in length of <20%, >20%, >40%, >60%.

Conclusion – EGL-30 is not the upstream activator of EGL-8 in this pathway

Taken together the results of the *lin-31, lin-1, unc-15* and *dpy-13* RNAi assays against *egl-30* mutants paint an unclear picture (summarised in table 3.2), with inconsistent results seen between different strains of a similar nature in response to the same RNAi target, and with different patterns seen in the response of the same strain to RNAi treatment against different target genes. Comparably inconsistent results were seen in preliminary work (Baylis pers comm.). However, a few strains do show a more consistent pattern. *egl-30 gof 1* shows an increased response to RNAi to all target genes apart from *unc-15* as does the *egl-30 lof OE* rescue strain, which also shows a significant increase in response to *unc-15 RNAi*. *egl-30 gof 1* has the strongest gof phenotype of all the strains tested. By comparison the 3 *egl-30 lof* mutant tested, show a tendency towards a reduced response with the exception of *unc-15* RNAi (which may be an unrepresentative assay for reasons already discussed). Whilst a few gain-of-function and overexpression mutant strains show an enhanced response in to both *lin-31* and *lin-1*, and *dpy-13* RNAi this is not seen for all strains. This could be due to a number of reasons including the range of severities of the mutants, the differing nature of mutations or the tissue of action in the case of OE mutants.

The only firm conclusion that can be drawn is that *egl-30* loss of function mutants certainly do not display a consistent enhanced exo-RNAi response to RNAi by feeding as seen in *itr-1* (IP_3R) and PLCβ *egl-8* loss of function mutants. This makes it unlikely that Gαq EGL-30 is the upstream activator of EGL-8 in the IP_3 signalling pathway regulating the exo-RNAi response. Interestingly however the results do not rule out the possibility that increased EGL-30 function increases the RNAi response. These results leave open the question of the identity of the upstream activator of EGL-8 the most likely candidates being a different Gα protein or a Gβγ dimer.

Strain	RNAi Response			
	lin-31	*lin-1*	*unc-15*	*dpy-13*
eri-9	++	+	++	++
ipp-5 lof	- -	- -	- -	- -
egl-30 lof 1	- -	n.s. (-)	+	n.s. (+)
egl-30 lof 2	-	--	n.s.	-
egl-30 lof 3	- -	n.s. (-)	n.s.	ns
egl-30 gof 1	++	++	n.s. (-)	+
egl-30 gof 2	n.s.	n.s.	- -	-
egl-30 lof gof rescue	+	n.s.	- -	ns
egl-30 lof OE rescue	++	+	+	+
egl-30 OE	n.s.	+ (n.s.)	- -	n.s. (-)

Table 3.2. Summary of the RNAi response of the tested egl-30 mutants.
Semi-quantitative summary of the changes seen in the RNAi response of the strains tested, relative to the WT strain. + significant increase, ++ significant large increase, - significant decrease, -- large significant decrease (minimal response), ns no significant change.

3. 2.ii - Identifying Gα protein mutants with an altered RNAi response

Having ruled out EGL-30 as a direct activator of EGL-8 a range of other mutants of Gα subunits were screened. Candidate genes were selected based on the known expression pattern of the Gα subunit. Of the 21 genes for Gα homologues encoded in *C. elegans* several are known to have a highly restricted expression pattern, being detected in a narrow range of mostly sensory neurones (Plasterk et al., 1999). A neuronal role for the signalling pathway regulating RNA interference is possible however the intestine was previously identified as the most likely site of action of the IP_3 signalling regulating this the RNAi response (Nagy et al., 2015), and no neurones directly innervate the *C. elegans* intestine. Gα subunits with a broad expression pattern or which are known to be expressed in the intestine were therefore prioritised (see table 3.3). As with *egl-30* mutants several of the other Gα mutants also show a broad range of phenotypes. In particular the *goa-1 lof* mutants show an almost identical set of phenotypes as the *egl-30 gof* mutants, including hyperactive movement, constitutive egg laying and exaggerated body bend, whilst the *goa-1* OE mutant phenocopies *egl-30 lof*. The strains tested and their key phenotypes are further summarised in Table 3.3.

Strain name	genotype	labelled as	variant/ description	key phenotypes	key references
MT363	*goa-1(n363)*	***goa-1 lof 1***	Null. Large deletion of entire coding region.	Hyperactive movement, constitutive egg laying, early stage eggs, increased amplitude of tracks.	(Dong et al., 2000; Robatzek and Thomas, 2000; Segalat et al., 1995)
DG1856	*goa-1(sa734)*	***goa-1 lof 2***	Likely null. Substitution in aa52 causing early stop.	Hyperactive movement, constitutive egg laying of early stage eggs, increased amplitude of tracks.	(Robatzek and Thomas, 2000)
MT2426	*goa-1(n1134)*	***goa-1 lof 3***	Likely partial LOF G to A substitution in the initiation codon of *goa-1.*	Hyperactive, constitutive egg laying, early stage eggs. Less severe phenotype than n363 or sa734.	(Segalat et al., 1995)
PS1493	*dpy-20(e1362) IV; syIs9.*	***goa-1 OE***	Integrated transgene carrying several copies of constitutively active *goa-1.* under control of goa-1 promoter.	Lethargic/flacid paralysis, retain eggs.	(Hajdu-Cronin et al., 1999; Mendel et al., 1995)
KG421	*gsa-1(ce81)*	***gsa-1 gof***	gof hypermorph Point mutation c/t substitution.	Hyperactive locomotion, aldicarb hypersensitive, levamisole hypersensitive, oocyte meiotic maturation variant.	(Schade et al., 2005)
NL795	*gpa-7(pk610)*	***gpa-7 lof***	lof. 961 bp deletion.	Body posture and locomotion variations, melatonin resistant, egg laying defective.	(Plasterk et al., 1999)

Table 3.3. Gα mutant strains used in work described in this chapter.
(not including the *egl-30* mutants, see table 3.1). Descriptions taken from referenced papers or from WormBase.

Strain name	genotype	labelled as	variant/ description	key phenotypes	key references

NL1147	*gpa-10(pk362)*	***gpa-10 lof***	lof. 2895 bp deletion and unknown 445bp insertion	minor Body posture and locomotion variant- increased forward locomotion.	(Plasterk et al., 1999)
NL594	*gpa-12(pk322)*	***gpa-12 lof***	Presumed lof. 1935 bp deletion (Tc1 transposon excision)	Minor body posture and locomotion variations. Axon regeneration defective, defective antifungal innate immunity.	(Alam et al., 2016; Plasterk et al., 1999; Yemini et al., 2013; Ziegler et al., 2009)
BW1809	*gpa-16(it143); him-5(e1490)*	***gpa-16 lof***	Presumed lof. g/a substitution exonic	Spindle orientation defective in early embryo.	(Johnston et al., 2008)
RB1800	*gpa-17 (ok2334)*	***gpa-17 lof***	Presumed lof. 1693bp deletion.	Minor body posture and locomotion variants. carbon dioxide avoidance variant.	(Yemini et al., 2013)**.**

Table 3.3 (continued). Gα mutant strains used in work described in this chapter
(not including the *egl-30* mutants, see table 3.1). Descriptions taken from referenced papers or from WormBase.

When challenged with *lin-31* RNAi treatment multiple Gα mutant strains show a significantly higher incidence of MUV or protruding vulva phenotype than the WT reference strain N2 (figure 3.7a). The two *goa-1 lof* mutants, the *gsa-1 gof* mutant and *gpa-16 lof* mutants all show a significant increase in incidence of MUV/ protruding vulva phenotype, comparable to the *eri-9* positive control. Comparison of the incidence of MUV phenotype compared to protruding vulval phenotype as shown in figure 3.7b reveals that in the case of *gpa-16* the increased effects seen are largely due to an increase in the incidence of the milder protruding vulval phenotype. Whilst in the case of *goa-1 lof1, goa-1 lof 2* and *gsa-1 gof* an increase is seen in the incidence of both the MUV and protruding vulval phenotypes, with a significant increase in MUV phenotype compare to the WT. However both *goa-1 lof* and *gsa-1 gof* mutants also display a higher than WT incidence of vulval bump or protruding vulva when not treated with *lin-31* RNAi (known phenotype, data not shown) which could suggest an increased susceptibility to the MUV phenotype and makes the *lin-31* assay a less reliable indicator of any change in the underlying RNAi phenotype in these strains. *gpa-12* shows a significantly lower incidence of the phenotype whilst *gpa-7, gpa-10* and *gpa-17* show no significant difference in response.

By comparison, in response to *unc-15* RNAi treatment (figure 3.8) two out of three *goa-1* strains (*goa-1 lof2* and *goa-1 lof3)* show an increased response with an increased incidence of both complete paralysis and significantly impaired locomotion caused by partial paralysis. *gpa-12* mutants again show a dramatically decreased response. Whilst *goa-1 lof1, gpa-7 and gpa-17* shows no significant difference compared to the WT, *gpa-10* shows a non-significant but noticeable increase compared to the WT.

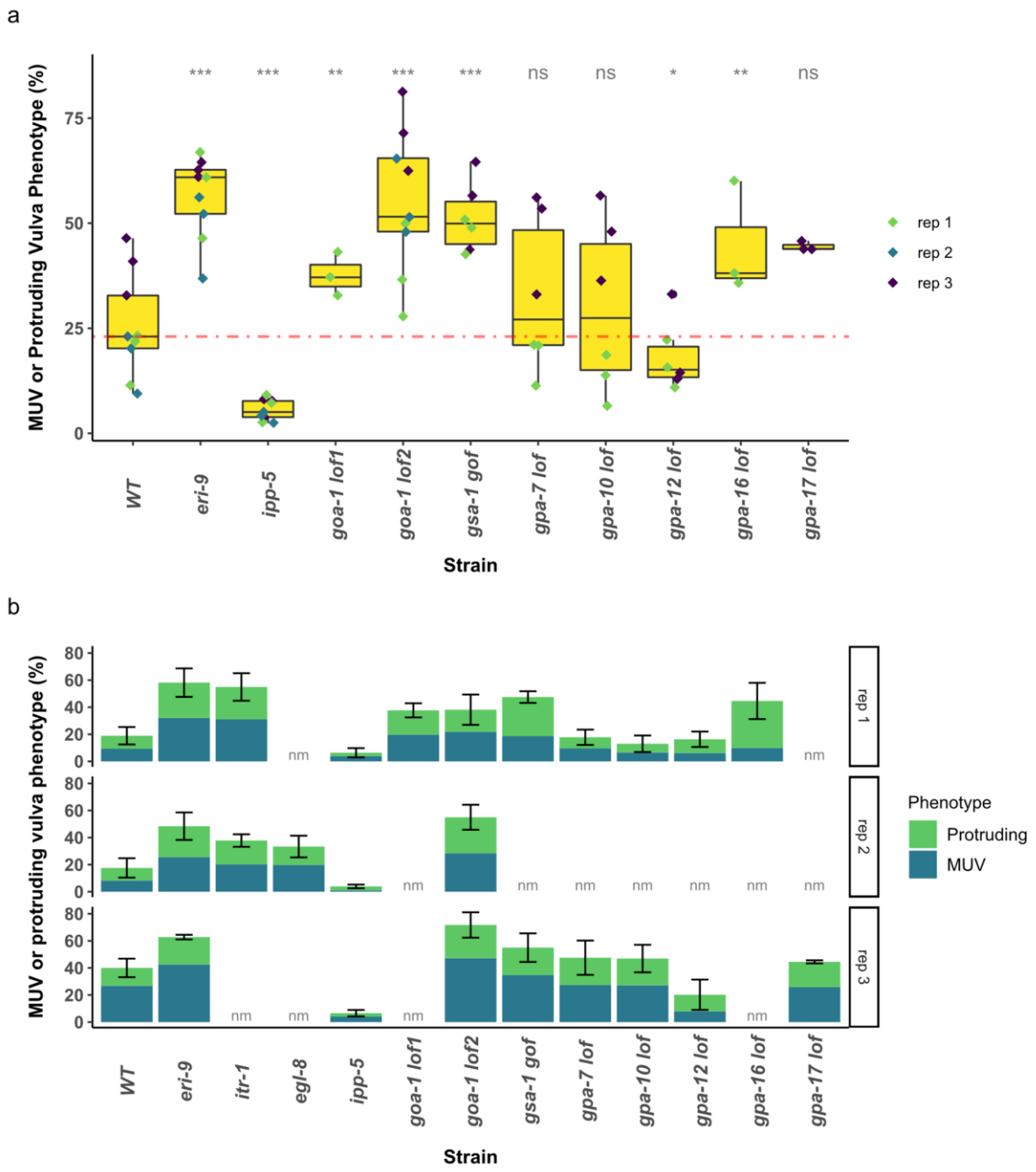


Figure 3.7. Results of *lin-31* RNAi by feeding on Gα mutants. Successful *lin-31* knockdown during development results in a multivulval (MUV) phenotype, or a milder protruding vulva phenotype. **a**- Box plots representing the range of values of the sum MUV or protruding vulval phenotypes seen for each strain. Box edges represent upper and lower quartiles with the central line representing the median. Each coloured dot represents one experimental plate with a median population of 99 worms, range 14-293. Statistical significance was assessed using logistic regression analysis, modelling the proportion of population affected, weighted for plate population, and including observation-level random effect. Statistically significant differences from the WT strain are shown, denoted by "***" ($p<0.001$), "**" ($p<0.01$), "*" ($p<0.05$), "." (not significant $0.05<p<0.1$), "ns" (not significant, $p>0.1$). **b**- Represents the same data shown in **a** but % MUV or protruding vulval is broken down by class. Additionally, results of *itr-1* and *egl-8* strains are shown for comparison. The mean percentage of worms of each strain with a MUV or protruding vulval phenotype is shown. Error bars represent standard deviation (SD) Each row represent an independent repeat consisting of 3 replicate plates per strain. "nm" represents strain not tested in this repeat.

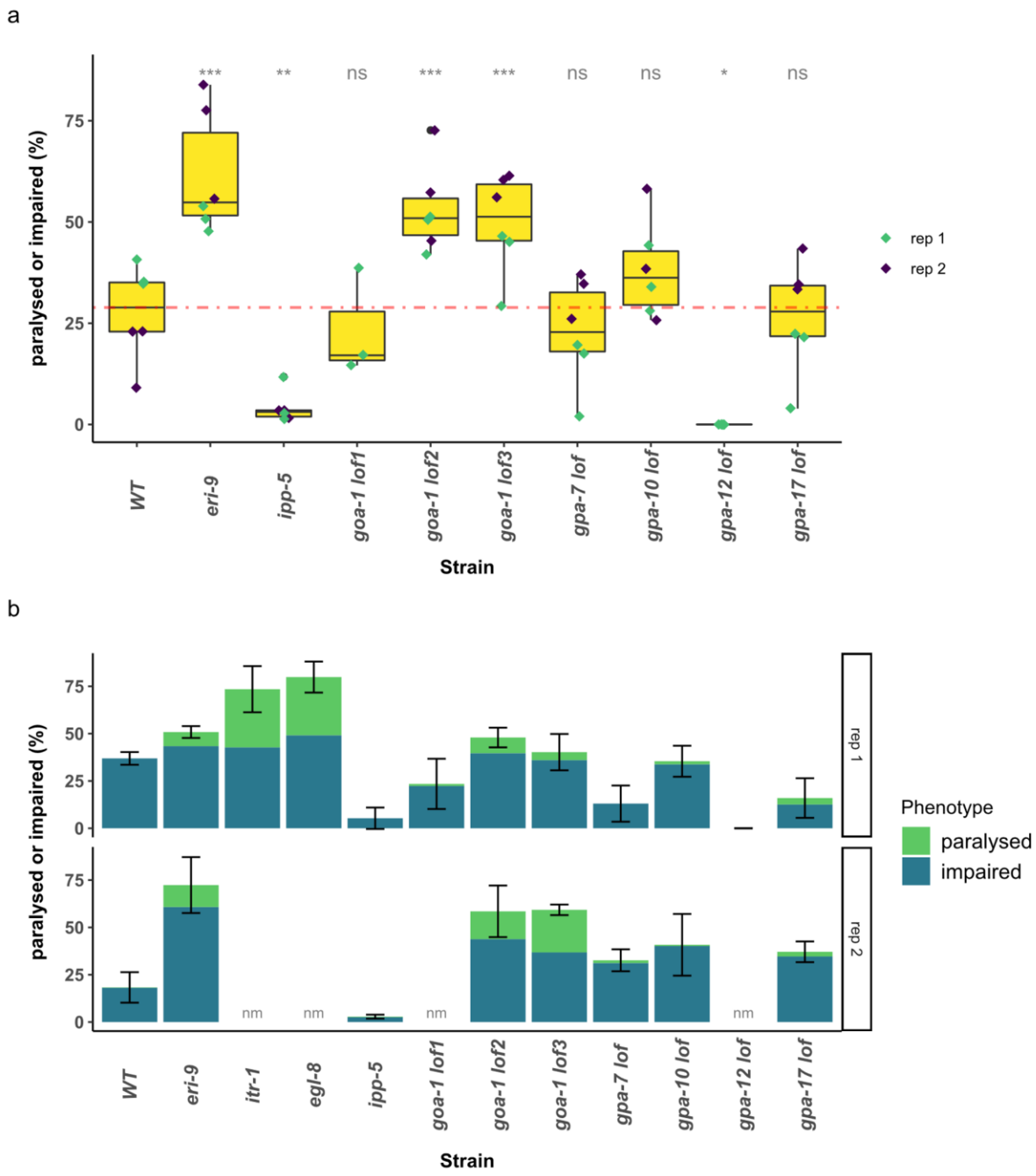


Figure 3.8. Results of *unc-15* RNAi by feeding treatment on Gα mutants. Successful *unc-15* knock- down results in a paralysis phenotype, with partial knock-down producing a milder impaired locomotion phenotype. **a-** box plots represent the range of values for the % of worms of each strain displaying either a paralysed or severely impaired phenotype. Each coloured dot represents one plate with a median population of 71 worms, with 3 plates per strain. Statistical significance was assessed using regression analysis, modelling the proportion *of the* population with paralysed or impaired phenotype, weighted for plate population, fitted to a gaussian distribution. Statistically significant differences from the WT strain are denoted by "***" ($p<0.001$), "**" ($p<0.01$), "*" ($p<0.05$), "**.**" (not significant $0.05<p<0.1$), "ns" (not significant, $p>0.1$). **b -** The mean percentage of worms displaying the paralysed (green bars) or impaired locomotion phenotypes (blue) is shown for each strain, error bars represent standard deviation (SD). Results of *itr-1* and *egl-8* strains are shown for comparison. "nm" represents strain not tested in this repeat.

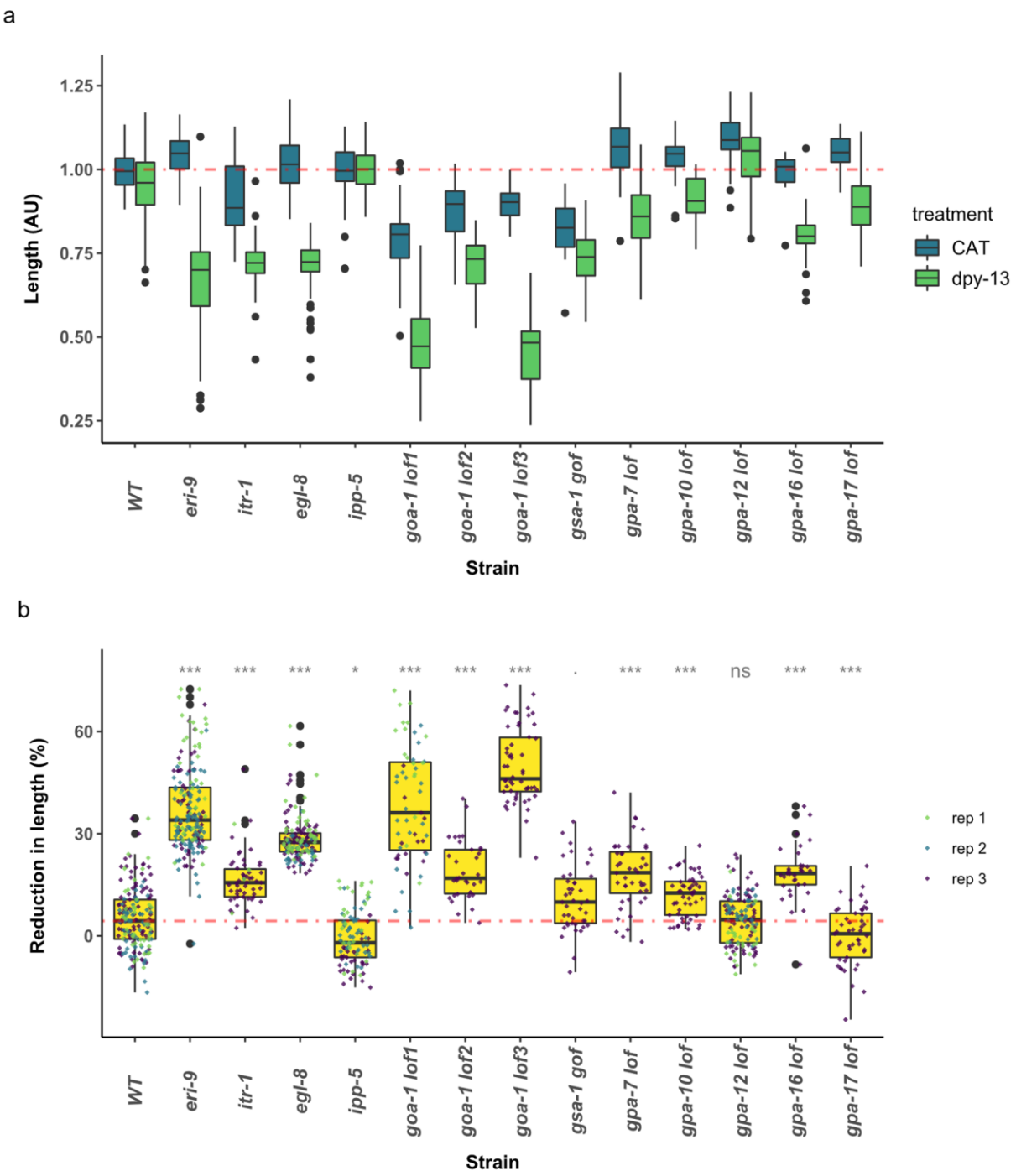


Figure 3.9. Effect of *dpy-13* RNAi treatment on G-alpha mutant strains. a- The length of worms of each strain treated fed either control (cat) or *dpy-13* RNAi cultures is shown for comparison. There is considerable variation in the control treated length of worms between strains. **b-** *The reduction in length of worms compared to the mean control treated length for that strain in that set. Median n/strain =54, range 33-186. Statistical significance assessed by regression analysis, linear model accounting for length, RNAi treatment and strain. Overall dpy-13 treatment results in a significant reduction in length.* Statistically significant difference in response to dpy-13 RNAi treatment for the test strains compared to the WT strain are denoted by "***" (p<0.001), "**" (p<0.01), "*" (p<0.05), "**.**" (not significant 0.05<p<0.1), "ns" (not significant, p>0.1).

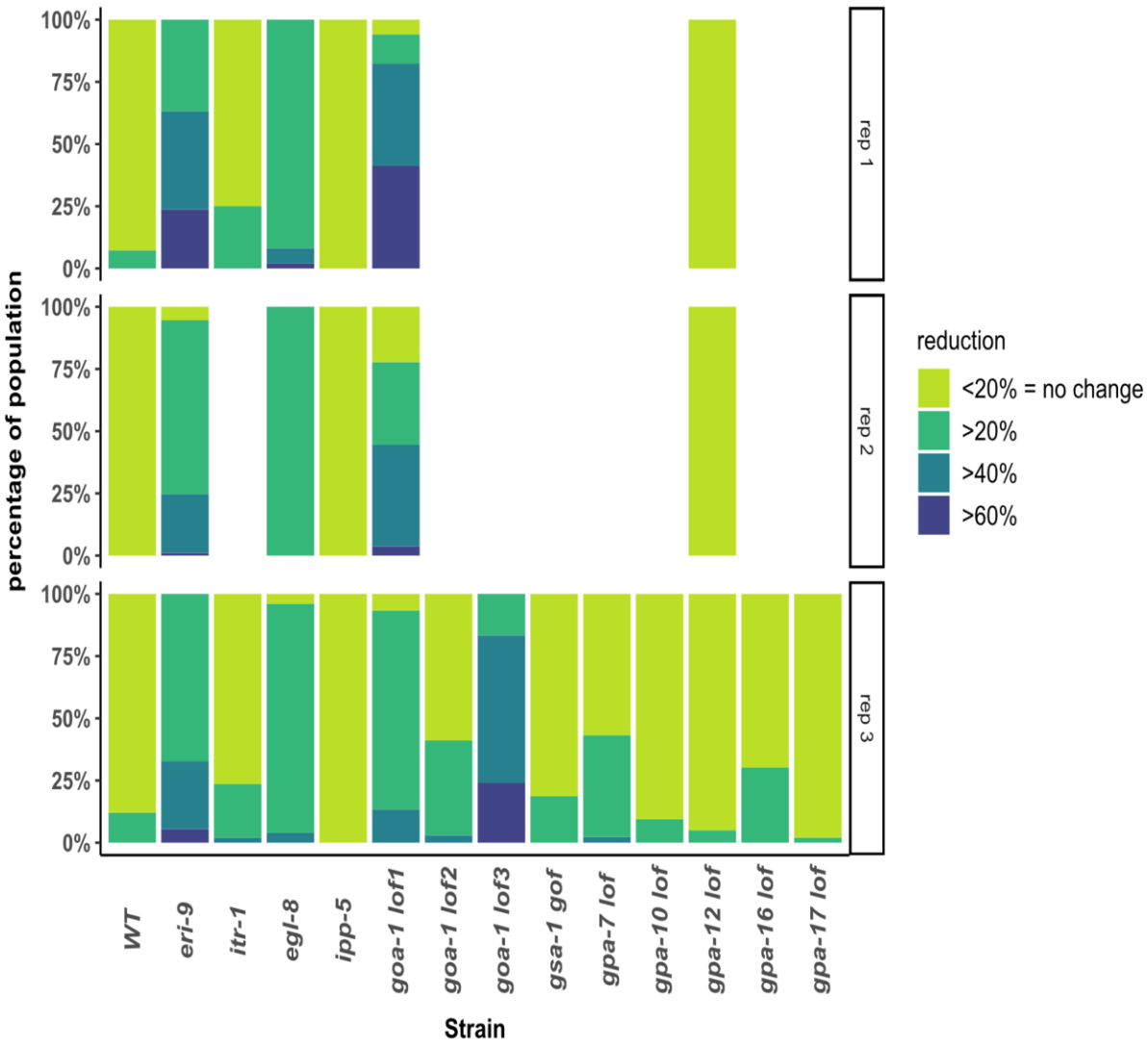


Figure 3.10. Reduction of length caused by dpy-13 RNAi by feeding treatment against egl-30 mutants. The data from figure 3.9 is shown again, here represented as the frequency of dpy-13 RNAi treated worms of each strain which show a reduction in length of <20%, >20%, >40% or >60%. Only eri-9, goa-1 lof1 and the goa-1 lof3 p*opulations contain worms with* the most severe reductions in length, > 60 %.

The results of the *dpy-13* RNAi treatment assayed by measuring length show that, as was seen in the *egl-30* mutants, there is significant variation in the length of the control treated worms (figure 3.9a, blue bars). Even more variation is seen in the length of the *dpy-13* RNAi treated worms. In order to easily compare the changes in length seen for each strain the measured length of each worm (length AU) was normalised according to mean length of the worms of each strain raised on control bacteria. This allowed the relative change in length from expected length for each worm to be calculated as seen in figure 3.9b. The majority of strains also show significant differences in response to *dpy-13* RNAi treatment compared to the wildtype (figure 3.9a, green bars and 3.9b).

However, in most cases these differences are small, with only 2 strains of the *goa-1 lof 1* and *goa-1 lof 3* showing mutants showing an average reduction comparable to that of the *eri-9* mutant. These strains are also the only ones to show incidences of reduction in length greater than 60% as seen in figure 3.10. The *gpa-12 lof* mutant which was significantly reduced RNAi response compared to WT upon *unc-15* and *lin-31* RNAi treatment does not show a significant decrease in length upon *dpy-13* RNAi treatment in comparison to the WT. Very little change was also seen in the WT in this set of *dpy-13* assays, making, a reduction in efficiency of RNAi difficult to detect, although a significant difference was still seen between *ipp-5* and the WT.

Conclusion – *goa-1 lof* strains frequently show a strongly enhanced RNAi response

The results of the RNAi assays for the different Gα strains tested are summarised in table 3.4. The majority of Gα strains tested show no consistent pattern of an altered RNAi response compared to the WT strain across the three different RNAi treatments. *gpa-7 and gpa-10* show no significant difference to the WT in the frequency or severity of their response phenotypes to *lin-31 or unc-15* RNAi treatment, with a slight lightly enhanced response to dpy-13 RNAi treatment. The *gpa-12 lof* strain shows a significant decrease in response to both *lin-31* and *unc-15* RNAi treatment but the response to dpy-13 RNAi treatment is very closely aligned to that seen in the WT strain. *gsa-1 and gpa-16* both show an enhanced response to *lin-31* RNAi treatment, with *gpa-16* also showing an enhanced response to *dpy-13* RNAi treatment. Unfortunately, the response of *gpa-16* to *unc-15* RNAi was not tested due to the temporary loss of this line. *goa-1 lof2* shows a significantly and dramatic increased response to all the RNAi treatments tested, whilst *goa-1 lof3* shows a similarly clear enhancement for the two RNAi treatments against which it was tested. *goa-1 lof1* shows a clear enhancement in its RNAi response to lin-*31* or *dpy-13* RNAi treatment, but this enhancement was not detected in response to *unc-15* RNAi treatment. Nevertheless, the results of the 3 *goa-1* lof strains taken together point to a strongly enhanced RNAi response.

Strain	RNAi response		
	lin-31	*unc-15*	*dpy-13*
eri-9	++	++	++
ipp-5	- -	- -	-
goa-1 lof1	+	Ns (-)	++
goa-1 lof2	++	++	+
goa-1 lof3	nm	++	++
gsa-1 gof	++	nm	ns (+)
gpa-7 lof	ns	ns (-)	+
gpa-10 lof	ns	ns (+)	+
gpa-12 lof	-	- -	ns
gpa-16 lof	+	nm	+
gpa-17 lof	ns	ns	-

Table 3.4. Summary of the RNAi response of the tested G-alpha mutants. Semi-quantitative summary of the changes seen in the RNAi response of the strains tested, relative to the WT strain. + significant increase, ++ significant large increase, - significant decrease, -- large significant decrease (minimal response), ns no significant change.

3.2.iii Confirmation of results using the *unc-47p::GFP* transgenic system

The complex phenotypes and potential pleiotropic interactions of the *egl-30* and *goa-1* mutants with the RNAi targets make reliably interpreting the results of the RNAi assays based on developmental and behavioural phenotypes difficult. I therefore wished to confirm that *goa-1* lof mutants were indeed enhanced in their RNAi response using a method not based around the knock down of an endogenous gene. The *unc-47p::GFP* transgenic system developed by the Ruvkun lab, where GFP is expressed in only the GABAergic neurones has been previously used in the characterisation of Eri mutants such as *eri-1* (Kennedy et al., 2004). It is therefore a well characterised system for assessing the efficacy of enhanced RNAi response by quantifying the loss of GFP fluorescence from the neurones upon GFP RNAi treatment. *unc-47* is expressed in all the GABAergic neurons of adult and larval stages. The 26 GABAergic neurons of the adult hermaphrodite comprise of the RIS interneuron and ALV, DVB, 4 RME, 6DD and 3VD motor neurons which can each be easily visualised (White et al., 1986). The neurons of *C. elegans* have been found to be refractory to RNAi in a WT background but knockdown is possible when the exo-RNAi response is enhanced (Kennedy et al., 2004). Both *itr-1 and egl-8* mutants show enhanced knockdown of *unc-47p::GFP*(Nagy et al., 2015). The *unc-47p::GFP* transgene was first crossed into a *goa-1* lof, *goa-1* OE

and *egl-30* lof background. The *itr-1; unc-47p::GFP* strain had been previously constructed. The strain EG1285 (*lin-15B&lin-15A(n765); oxIs12 (unc-47p::GFP + lin-15(+))),* which does not show an enhanced RNAi response was used as the reference to which levels of *unc-47p::GFP* knockdown were compared. RNAi by feeding using a GFP dsRNA construct was used to induce GFP RNAi. The effects were quantified by scoring the 22 D-type, DVB, ALV and RIS neuron cell bodies for the presence of absence of visible fluorescence. As a control the number of fluorescent neurones were also counted in control RNAi treated worms. All strains showed similar and consistently high numbers of fluorescent neurones (data not shown).

Figure 3.11a shows the response of GFP RNAi on the GFP fluorescence in the GABAergic neurones in EG1285, *itr-1(sa73)* lof, *goa-1* lof, *egl-30 lof* and *goa-1 OE* backgrounds. In EG1285 loss of fluorescence from any neurons is rare, with the number of GFP positive neurones counted ranging from 22 to 16, with an average of 20 fluorescent neurones. The median number of GFP positive neurones following GFP RNAi treatment in the *itr-1* and *goa-1* lof backgrounds is dramatically reduced, to 14 and 12 respectively. Notably the individual variation is extremely high, reflecting the stochastic nature of the RNAi process in each neuron. The median number of GFP positive neurones in the *egl-30 lof* and *goa-1 OE* backgrounds is 20, showing no difference from that seen in EG1285 although the distribution of the observed values is narrower, being comparable to that seen in untreated worms.

This assay clearly supports the findings that *goa-1* loss of function results in an enhanced RNAi phenotype, and that neither *egl-30* loss of function nor *goa-1* OE cause an enhanced RNAi phenotype. However, it is not possible to conclude whether *egl-30* loss of function nor *goa-1 OE* results in a reduced sensitivity to RNAi due to the unidirectional nature of this assay.

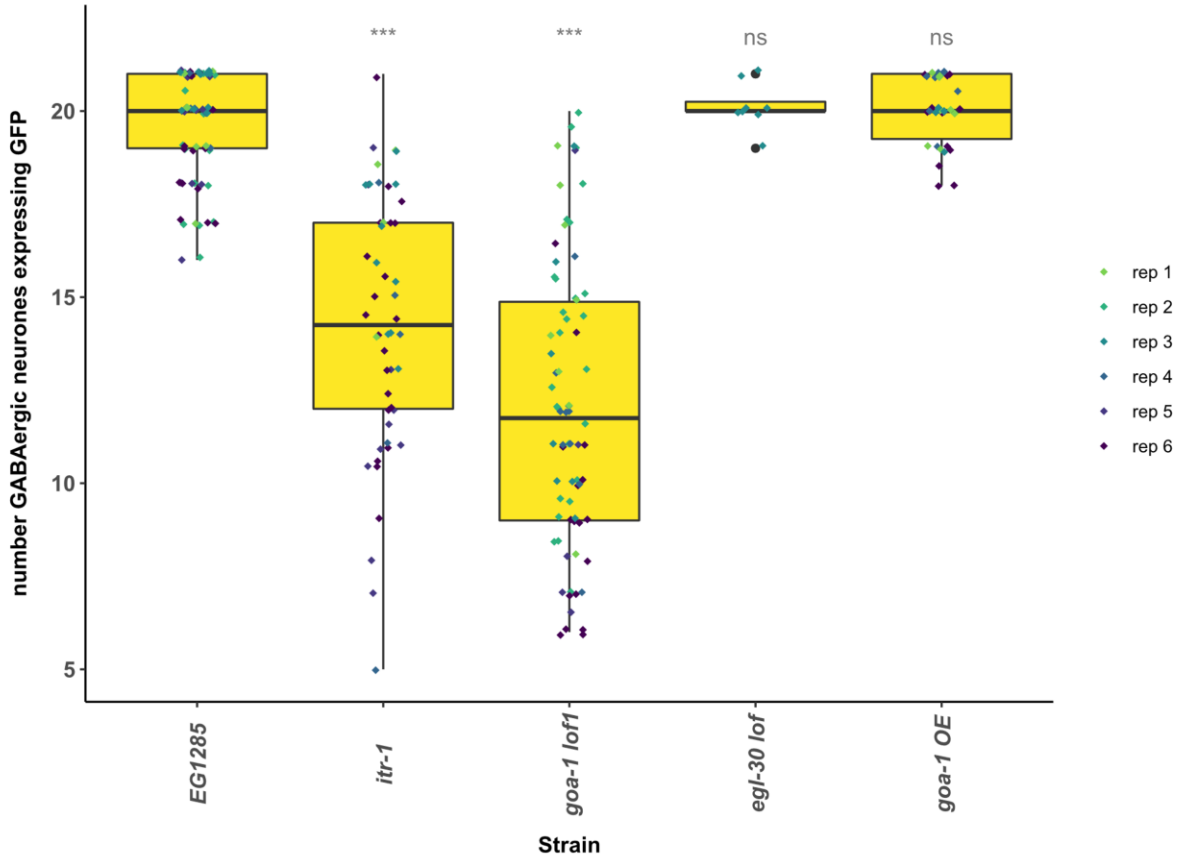


Figure 3.11. Knockdown of GFP flourescence in GABAergic neurones due to GFP RNAi by feeding.
A significant loss of flouresecnce is seen in *itr-1* lof ($p<0.001$) and *goa-1 lof (*$p<0.001$) backgrounds but not in the *egl-30* lof and *goa-1* OE backgrounds, compared to EG1285. The 22 D-type, DVB, ALV, RIS neurones were scored for their presence or absence of GFP. Stastistical significance tested by ANOVA followed by Tukey HSD test.

3.2.iv Investigating a role for regulators of the *GOA-1* / EGL-30 antagonism – Regulators of G-protein signalling

The finding that *goa-1* loss of function leads to an increased efficiency in the exo-RNAi response suggests that *goa-1*, or the active Gβγ dimer from a $G\alpha_o$ containing heterotrimer (see chapter 1 -1.2.vii) is likely acting to activate *egl-8* in the pathway regulating the RNAi response. Signalling by heterotrimeric G-proteins is carefully regulated by a range of proteins acting as activators or inhibitors of G-protein signalling- in particular of the Gα subunit. To better understand how GOA-1 may be regulating EGL-8 activity I investigated whether any known regulators of GOA-1 heterotrimeric signalling had an altered RNAi response. Since GOA-1 and EGL-30 are known to signal antagonistically in a number of pathways in *C. elegans* known regulators of the $G\alpha_o/G\alpha_q$ network were focussed on (as discussed in chapter 1 -1.2.viii).

One of the key ways in which G-protein signalling is regulated is through influencing the GTP/GDP state of the Gα subunit. Conformational change in the subcellular domains of the GPCR upon agonist binding triggers GTP/GDP exchange leading to dissociation of the active $G\alpha_{GTP}$ and Gβγ subunits. Gα subunits have inherent GTPase activity and so will in time return to GDP state, leading to reformation of the inactive heterotrimer. In *vivo* interactions with accessory proteins serve to further regulate the timing and specificity of G-protein activation and interactions with effector proteins in a number of ways, including influencing the GDP/GTP state of the Gα subunit through GAP (GTPase activating protein) and GEF (Guanonucleotide exchange factor) activity (reviewed in (Lanier, 2004)). GEFs promote formation of the active $G\alpha_{GTP}$ state, complementing or substituting for the role of the GPCR and potentiating the time the G-proteins spend in the active conformation. Conversely RGS (regulator of G-protein signalling) proteins act as GAPs, stimulating the GTPase activity of Gα, accelerating the return to the inactive state and the termination of signalling (Watson et al., 1996).

In *C. elegans* the antagonism between GOA-1 and EGL-30 signalling, which through the regulation of neurotransmitter secretion regulates, locomotive and egg laying behaviours is mediated by the GGL-domain containing R7 RGS proteins EAT-16, EGL-10 and RGS-1. EAT-16 is an inhibitor of EGL-30 whilst EGL-10 is an inhibitor of GOA-1. An interaction with GPB-2, the orthologue of mammalian $G\beta_5$, is required for both EAT-16 and EGL-10 function (Chase et al., 2001; Robatzek et al., 2001). The R7 anchoring protein RSBP-1, a homologue of mammalian R7BP, is required to target EAT-16 to the plasma membrane (Porter and Koelle, 2010). Other known regulators of this network include the GEFs RIC-8 and AGS-3 which together with GOA-1 also regulate spindle formation during embryogenesis (Hofler and Koelle, 2011; Miller and Rand, 2000; Miller et al., 2000), promote

GTP/GDP exchange and dissociation of the heterotrimer respectively. Like EGL-10, RGS-1 is also a negative regulator of *GOA-1*. The strains tested are described in table 3.5. This group of strains was tested using the *lin-31* and *dpy-13* RNAi assays.

Strain	genotype	labelled as	variant/ description	key phenotypes	key references
LX147	*rgs-1(nr2017)* III.	***rgs-1 lof***	Likely null. 638bp deletion with 35bp insertion.	Weak egg laying defects.	(Dong et al., 2000)
MT8504	*egl-10(md176*) V.	**egl-10 *lof***	Likely null. Deleted or severely rearranged.	Retains eggs, slow, sluggish locomotion and foraging behaviour with shallow tracks, Long. Defective avoidance response.	(Esposito et al., 2010; Koelle and Robert Horvitz, 1996; Porter and Koelle, 2010)
JT609	*eat-16(sa609*) I.	**eat-16 *lof***	lof c/t substitution.	Hyperactive, lays early stage eggs, adicarb hypersensitive, levamisole hypersensitive. Eat	
LX1313	*eat-16(tm761)* I*; egl-10(md176*) V.	***eat-16: egl-10 lof***	Likely double null. *tm761* is a 1225bp deletion/ 16bp insertion.		(Wani et al., 2012)
RM1702	*ric-8(md303)* IV.	***ric-8 lof 1***	Strong lof. C/A substitution that converts alanine 275 to glutamic acid.	Retains eggs, sluggish locomotion, near paralysis in older worms, reduced body flexion/straight posture, reduced pharyngeal pumping and growth rate. Some embryonic lethality and early defects.	(Miller et al., 2000)

Table 3.5. Regulators of G-protein signalling mutant strains used in work described in this chapter. Descriptions taken from referenced papers or from WormBase.

Strain	genotype	labelled as	variant/ description	key phenotypes	key references
RM2209	*ric-8(md1909)* IV.	***ric-8 lof2***	lof. 1.6 kb Tc1 transposon insertion following amino acid.	Poor growth and fertility at 25°C. Milder forms of the *md303* phenotypes.	(Miller et al., 2000)
RB1145	*ags-3(ok1169)*	***ags-3 lof***	Null. Estimated 1200bp deletion.	Partial defects in food search behaviours after food deprivation.	(Hofler and Koelle, 2011)
JT603	*gpb-2(sa603)* I	***gpb-2 lof1***	Likely null. g/a substitution causing premature stop codon after second WD40.	Variable locomotion ranging from lethargic to hyperactive, intermittent exaggerated body bends. Egg laid slightly late stage, Eat.	(Robatzek et al., 2001)
DA541	*gpb-2(ad541)* I.	***gpb-1 lof2***	lof. Substitution causing W368X.	Starvation hypersensitive, arecoline hypersensitive, intermittent exaggerated body bends, eggs laid slightly late stage pharyngeal pumping variant.	(Robatzek et al., 2001)
LX1270	*rsbp-1(vs163)* I.	***rsbp-1 lof***	Null. 169 bp deletion removing exon 2 and causing frameshift.	Eggs laid early, hyperactive movement, slow development, Eat. Phenocopies *eat-16 null.*	(Porter and Koelle, 2010)

Table 3.5 (continued). Regulators of G-protein signalling mutant strains used in work described in this chapter. Descriptions taken from referenced papers or from WormBase.

Figure 3.12 shows the results of the *lin-31* RNAi assays. The *eri-9* and *ipp-5* lof strains behave as expected consistently showing a dramatic increase and decrease respectively, compared to the WT, in the percentage of multivulval or protruding vulval phenotypes observed. *eat-16 lof* also shows consistently high levels of MUV and protruding vulval phenotypes at levels comparable to those seen in the *eri-9* mutant strain and is significantly enhanced compared to the WT in respect to incidence of these phenotypes. A significant change in the opposite direction is seen in *egl-10 lof,* which shows lower than WT levels of the response to *lin-31* RNAi, though not to the extreme of zero incidence seen in the *ipp-5* mutant. The striking difference in effect of *lin-31* RNAi on *eat-16 lof* and *egl-10 lof* would seem to reflect the opposing roles of these two RGS proteins. The *eat-16;egl-10 lof* double mutant shows a significantly enhanced response to *lin-31*, similar to that seen in *eat-16 lof.* As might be expected given the role known roles of both GPB-2 and RSBP-1 in assisting EAT-16 function, *gpb-1 lof1, gpb-1 lof2, rsbp-1 lof* all show a significant enhancement of incidence of MUV and protruding vulva phenotype, similar to that seen in *eat-16 lof.* No significant difference from the WT is seen in *rgs-1 lof.* Among the GEFs tested, neither of the *ric-8 lof* mutants show a significant difference from the WT whilst *ags-3* shows a subtle but significant increase.

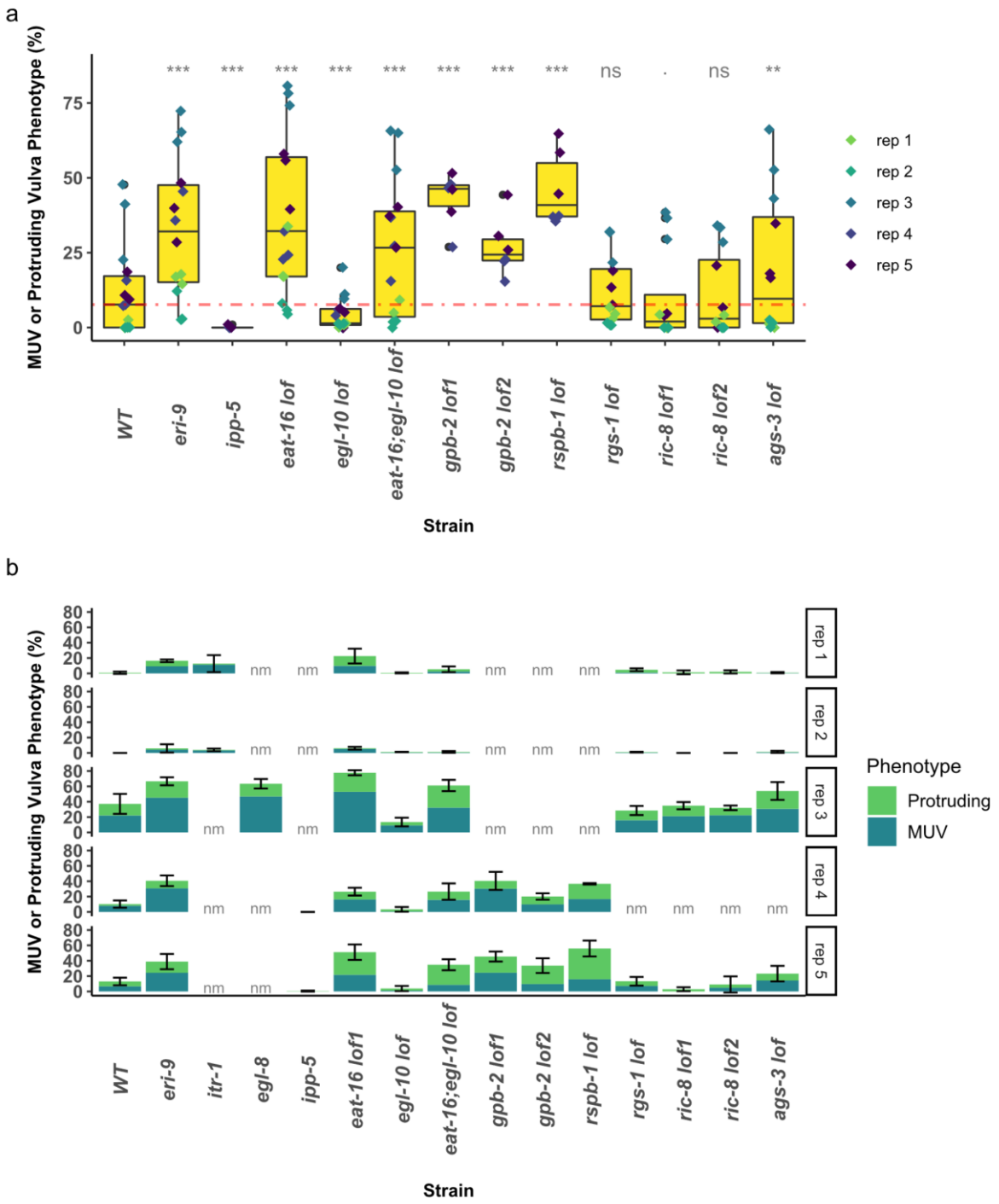


Figure 3.12. Results of *lin-31* RNAi by feeding against mutants of G-protein regulators. a- Box plots representing the range of values of the sum MUV or protruding vulval phenotypes seen for each strain. Box edges represent upper and lower quartiles with the central line representing the median Each coloured dot represents one experimental plate with a median population of 67 worms, range 15-297. Statistical significance was assessed using logistic regression analysis, modelling the proportion of population affected, weighted for plate population, and including observation-level random effect and rep as random effects. Statistically significant differences from the WT strain are shown. **b**- Represents the same data shown in **a**, but % MUV or protruding vulval is broken down by class. The mean percentage of worms of each strain with a MUV or protruding vulval phenotype is shown, error bars represent standard deviation (SD) Each row represent an independent repeat consisting of 3 replicate plates per strain. "nm" represents strain not tested in this repeat. Results of *egl-8 lof* and *itr-1 lof* strains are shown for comparison.

The results of *dpy-13* RNAi treatment assayed by scoring phenotype presence by eye (figure 3. 13 and by measuring (figure 3.14, 3.15) are largely concordant. Once again figure 3.14a shows significant variation between strains in the average length of worms grown on control bacteria. This variation likely had a confounding effect on the scoring of the milder *dpy-13* phenotypes by eye and requires the normalisation of the measured lengths by the within strain average in order to patterns in the change in length upon *dpy-13* RNAi treatment. A significant increase in % reduction in length compared to the WT is seen across *eat-16 lof, egl-10 lof, rgs-1 lof, ags-3 lof and ric-8 lof 2* mutants, with both *ric-8 lof1* and *eat-16;egl-10 lof* showing a non-significant increase in average reduction in length (see figure 3.14b). Both *gpb-2 lof* mutants show no significant change from the WT reduction in length, whilst *rspb-1 lof* is the only strain to show a significant decrease in reduction in length, compared to the WT strain. A closer look at the distribution of the change in length of *dpy-13* RNAi treated worms, seen in figure 3.15 shows that for most strains the vast majority of worms show no more variation length than that seen in control treated worms (<20% = no change). Even for strains which show a significant increase in reduction in length than that seen in the WT, differences are slight with an increase in the percentage of the population showing a moderate reduction in length of between 20-40%. Only *eat-16 lof1* and *eri-9* populations consistently contain worms with the more dramatic Dpy phenotypes – reductions in length greater than 40 or even 60%.

These results of the *dpy-13* measured assays are largely compatible with those seen in figure 3.13, where *dpy-13* RNAi assays have been scored by eye. The most notable exceptions to this are *ags-3 lof* and *rsbp-1 lof, with ags-3 s*howing a non-significant slight decrease in incidence of Dpy phenotype whilst *rsbp-1* shows a significant increase in incidence of Dpy phenotype. These effects are the opposite to those seen when length is accurately measured. In the case of the *rsbp-1 lof* this conflict could in part me explained by the mis-scoring of worms of this strain as mildly Dpy due to the significantly shorter length of the control treated worms compared to the wildtype. The variation in the measured length of *dpy-13* treated worms of this strain is also highly variable.

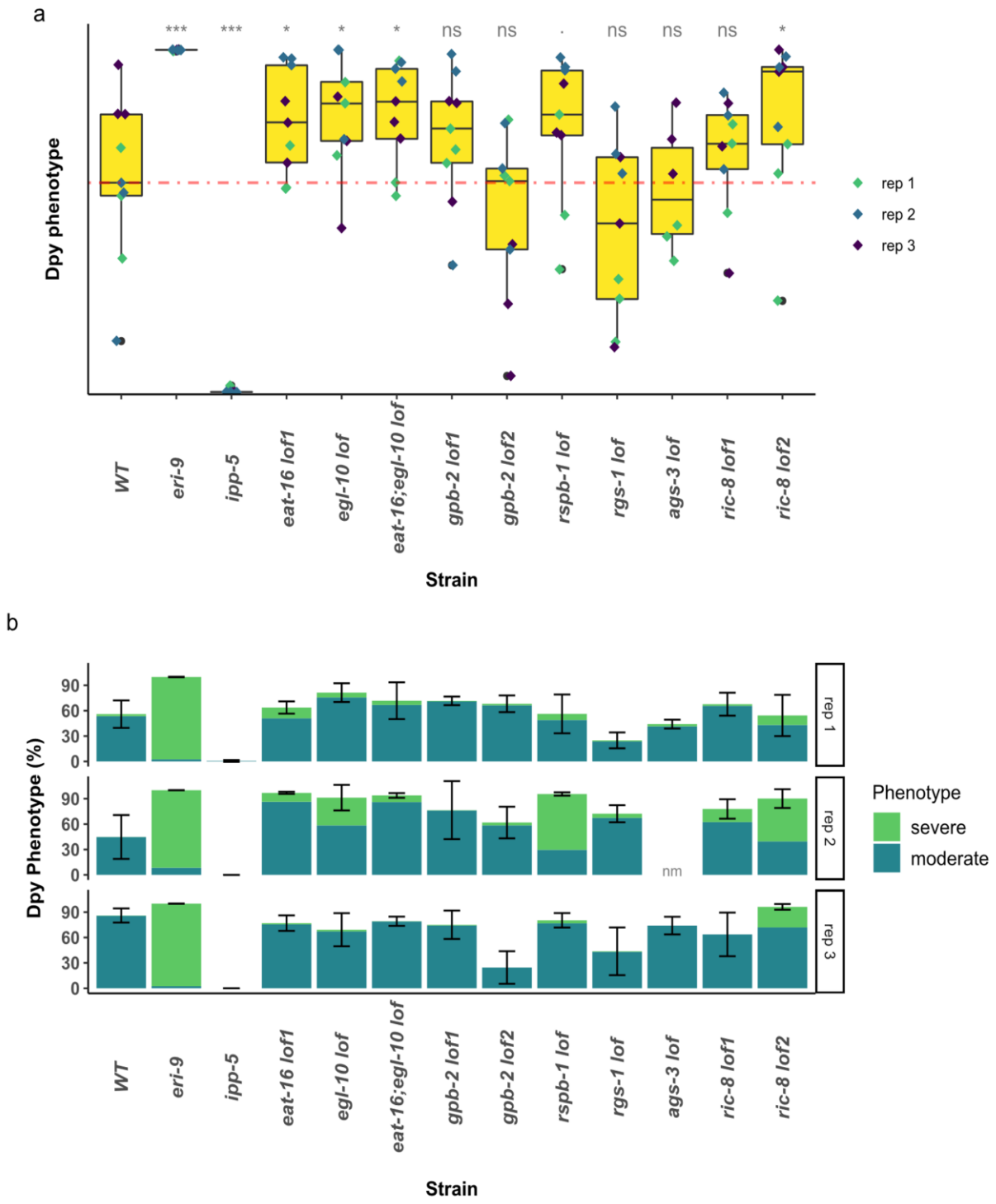


Figure 3.13. Effects of *dpy-13* RNAi by feeding on mutants of regulators of G-protein signalling mutants. Successful *dpy-13* knockdown results in a distinctive Dpy phenotype. **a**- Box plots representing the range of values of the Dpy phenotypes seen for each strain. Each coloured dot represents one experimental plate with a median population size of 112 worms, range 40-288. Statistical significance was assessed using logistic regression analysis, modelling the proportion of the population with paralysed or impaired phenotype, weighted for plate population, fitted to a glm of a binomial distribution with strain as a fixed effect and with rep and observational level as random effects. Statistically significant differences from the WT strain are shown. **b-** % dpy is broken down by class. The mean percentage of worms of each strain with MUV and protruding vulval phenotypes is shown, error bars represent standard deviation (SD). Each row represents an independent repeat consisting of 3 replicate plates per strain. "nm" represents strain not tested in this repeat.

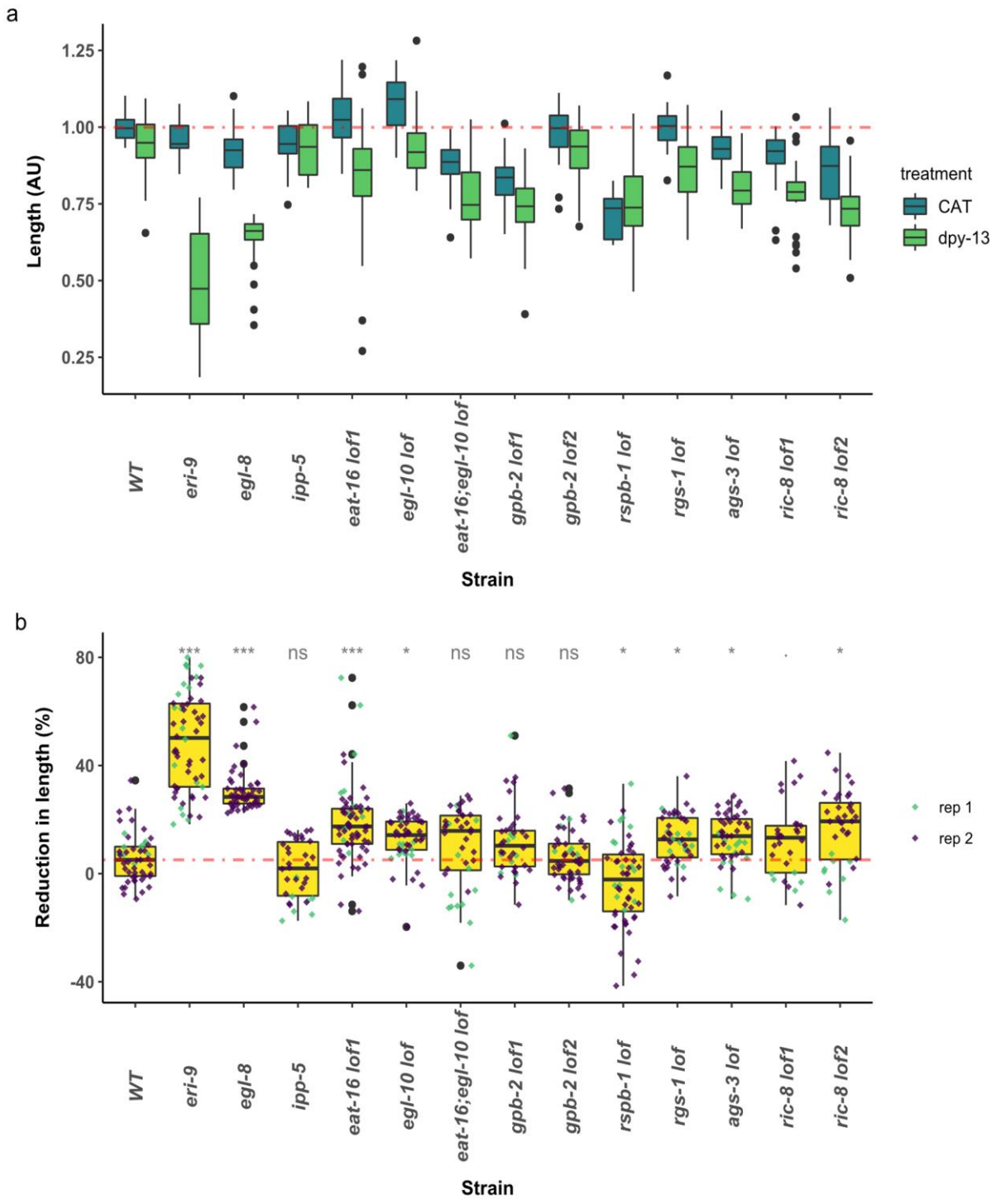


Figure 3.14. **Effect of *dpy-13* RNAi treatment on Regulators of Gα signalling mutant strains.** ***a***- The length of worm*s* of each strain treated fed either control *(cat)* or dpy-13 RNAi cultures is shown for comparison. There is considerable variation in the control treated length of worms between strain*s.* **b-** The reduction in length of worms compared to the mean control treated length for that strain in that set. Median n/strain =46, range 26-58. Statistical significance assessed by regression analysis, linear model accounting for length, RNAi treatment and strain. Overall dpy-13 treatment results in a significant reduction in length. Statistically significant difference in response to *dpy-13* RNAi treatment for the test strains compared to the WT strain are shown. Results of *egl-8 lof* strain are shown for comparison.

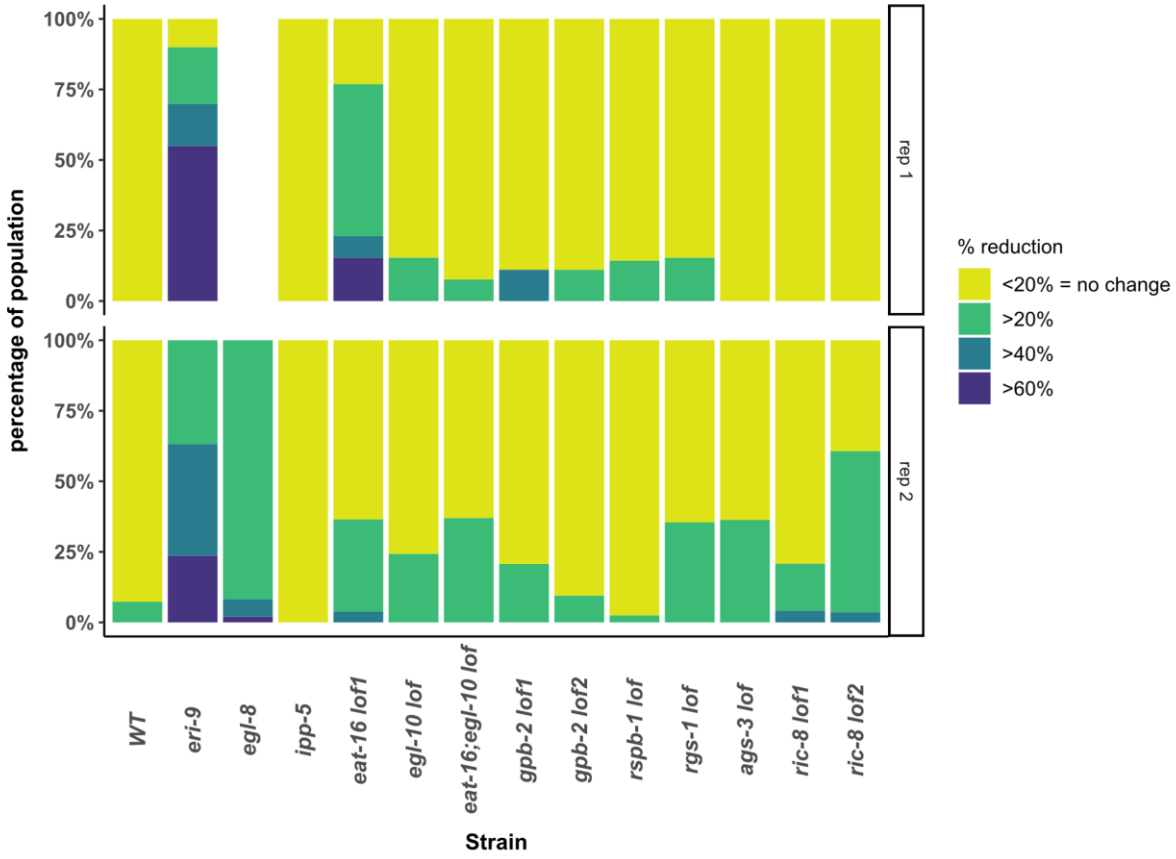


Figure 3.15. Reduction of length caused by *dpy-13* RNAi by feeding treatment against mutants of G-protein regulators. The data from figure 14 is shown again, this time showing this time the frequency of *dpy-13* RNAi treated worms of each strain which show a reduction in length <20%, >20%, >40%, 60%.

Conclusion – *eat-16 lof results in an enhanced RNAi response.*

The results of the RNAi assays testing the response of the known regulatory mutants of the GOA-1 and EGL-30 antagonism to RNAi treatment are summarised in table 3.6. The *eat-16 lof* strain alone shows a consistent and clear enhancement in the RNAi response to both *lin-31* and *dpy-13* RNAi treatments. *ags-3 lof* also shows a less dramatic but significantly increased response to both *lin-31* RNAi and *dpy-13* RNAi (detected when length measured), while *ric-8 lof1* consistently shows no significant change from the wildtype in response to both treatments. A consistent trend is also seen in the *eat-16;egl10 lof* double mutant. However, all the strains tested show almost no consistency between response to *lin-31* RNAi treatment and response to *dpy-13* RNAi treatment, relative to the WT strain.

Strain	**RNAi Response**		
	lin-31	*dpy-13*	*dpy-13* (measured)
eri-9	++	++	++
ipp-5	- -	- -	-
eat-16 lof	++	+	++
egl-10 lof	-	+	+
eat-16; egl-10 lof	++	ns (+)	ns (+)
gpb-2 lof1	++	ns (+)	ns
gpb-2 lof2	++	ns	ns
rspb-1 lof	++	ns (+)	-
rgs-1 lof	ns	ns (-)	+
ric-8 lof1	ns	ns (+)	ns (+)
ric-8 lof2	ns	+	+
ags-3 lof	+	ns (-)	+

Table 3.6. Summary of the RNAi response of the tested regulators of G-protein signalling mutants. Semi-quantitative summary of the changes seen in the RNAi response of the strains tested, relative to the WT strain. + significant increase, ++ significant large increase, - significant decrease, -- large significant decrease (minimal response), ns no significant change.

3.3 Conclusions – *goα-1* lof and *eat-16* lof but not *egl-30* lof results in an enhanced RNAi response

Drawing together the results of the Gα proteins screened for an altered RNAi response the *goα-1* lof mutants standout for both the strength and penetrance of their response to *lin-31, unc-15* and *dpy-13* RNAi treatment, with responses consistently comparable to the responses seen in the *eri-9* mutant strain, and in *itr-1(sa73)* and *egl-8(e2917)* lof mutants. That this apparent increase in response to RNAi treatment is also seen in *GFP* RNAi knockdown in the *unc-47p::GFP* system, a phenotype independent assay, confirms that *goa-1* loss of function does result in a general increase in the efficiency of the gene silencing response to exogenous dsRNA, similar to that seen in the *itr-1* and *egl-8* lof mutants.

Although several other Gα mutants show an increased response to RNAi treatment against one or more targets, including the *gsa*-1 gof, *gpa-10* lof, *gpa-7* lof, *gpa-16* lof and *gpa-17 lof,* none show a consistently increased response comparable to that seen in the *goa-1* mutants. *gpa-12* stands out for consistently showing a markedly low response to all RNAi treatments tested, although when length is measured there is no significant change in reduction in length caused by *dpy-13* RNAi compared to the WT. The lack of response to *unc-15* RNAi treatment is particularly striking, although it should be noted that this was only tested on one occasion (figure 3.8). Whether this reflects a decrease in the efficiency of the exo-RNAi response as is seen in the *ipp-5* mutants is uncertain, since the changes seen in response to *lin-31* and *dpy-13* RNAi treatment are less dramatic. GPA-12 is known to act, with RACK-1, upstream of EGL-8 and PLC-3 in a conserved signalling pathway regulating an innate immune response to injury and infection (Ziegler et al., 2009).

Amongst the *egl-30* mutant strains the general trend seen in response to *lin-31* and *lin-1* RNAi treatment, of a decreased response amongst loss of function mutant and an increased response amongst some of the gain of function and OE mutants is poorly reproduced in other assays. Amongst the lof mutants none show a significant reduction in response to *dpy-13* RNAi treatment, and only *egl-30 lof 2* shows a significant decrease in response to *dpy-13* RNAi treatment. Of the *egl-30* gof and OE strains only *egl-30 lof OE* rescue shows a consistently enhanced response to all 4 treatment conditions compared to the WT strain. e*gl-30 gof 1* also shows a trend for enhanced response, being significantly increased in response to all treatments but *unc-15* to which there is no significant difference to the WT. However there seems to be no discernible consistent trend for any of the other *egl-30* mutants. The *unc-47p*::GFP assay confirmed that *egl-30* lof does not enhanced the RNAi response but any reduction in response was unable to be confirmed due to limitations of the assay.

The potential confounding influence of the pleiotropic phenotypes of the *egl-30* and *goa-1* mutants, as well as several of the other Gα mutants on the scoring and interpretation of the RNAi assays should be considered. It is possible that one or more of the Gα mutants could have an unidentified role in the regulation of vulval development, whilst several of the mutants tested are known to display movement variants, and to have varying degrees of altered body length/shape. The most obvious of these are the near paralysis seen in severe *egl-3*0 mutants or *goa-1* hypermorphic mutants which make it very difficult to reliably score or draw meaningful conclusions from the *unc-15* RNAi assay. It should also be noted that both the *egl-30 OE* and *goa-1 OE* transgenes contain *dpy-20* and are carried in a *dpy-20 lof* background, and as such could be potentially sensitised to the induction of the Dpy phenotype by *dpy-13* RNAi. For all of these reasons the testing of the suspected enhanced RNAi response using a system independent of confounding phenotypes, such as GFP knockdown in the *unc-47p::GFP* system is the crucial.

On top of the differing patterns of response seen to the different RNAi targets for many strains it is worth noting the considerable variation seen across replicates of the same assay including in some cases contradictory results seen for some strains in different sets. Sources of variation include different RNAi cultures, different IPTG batches and experimental error as well as natural variation resulting in part from the stochastic nature of the biological processes involved.

What can be concluded is from these results and the result of the *unc-47p::GFP* GFP RNAi assays in an *egl-30* lof background is that *egl-30* lof mutants do not show an enhanced RNAi response as is seen in *egl-8* and *itr-1* lof mutants. In fact, the balance of evidence points towards a potential RNAi resistant phenotype in *egl-30* lof mutants and a possible enhanced RNAi phenotype in *egl-30* gof mutants. Therefore, counter to the canonical, *egl-30* cannot be the upstream activator of *egl-8* in the IP_3 signalling pathway regulating the RNAi response. Instead *goa-1* has emerged as the most likely candidate for the upstream activator of *egl-8*, with *goa-1 lof* mutants showing an even greater enhanced RNAi response than that seen in *itr-1(sa73)* mutants at 20°C.

GOA-1 and EGL-30 are known to signal antagonistically to regulate several independent processes in *C. elegans* including the modulation of locomotion and egg laying, L4 to adult lethargus, the ASH mediated avoidance response. Cross talk in this antagonistic system occurs at a number of levels, downstream of EGL-30 activated EGL-8 signalling via the GOA-1 induced inhibition of DAG via DAG kinase, but also upstream at the level of GOA-1 vs EGL-30 activation, mediated by the RGS proteins EAT-16, EGL-10, RGS-1 and RGS-2 (not tested), and by the GEFs RIC-8 and AGS-3, with GPB-2 required by both EAT-16 and EGL-10, and RSBP-1 required by only EAT-16. Several of these regulators therefore phenocopy *goa-1* and *egl-30* mutants to varying degrees.

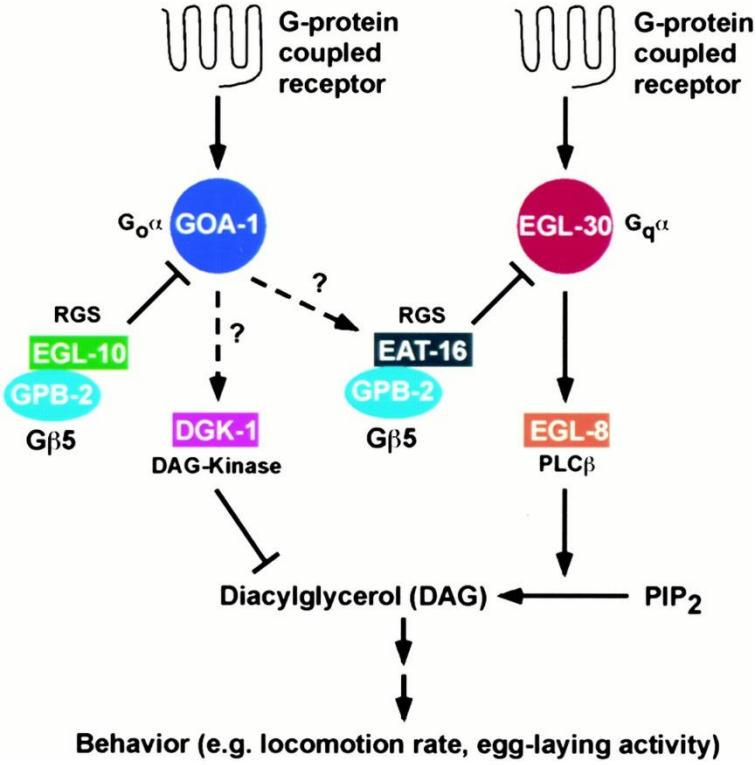


Figure 3.16. The antagonism between the goa-1 and egl-30 signalling pathways is hypothesised to be mediated by GPB-2, EAT-16 and EGL-10. Reproduced from (Van der Linden et al., 2001).

Given the variation seen in many of the *egl-30* strains in particular perhaps it is unsurprising that the responses of the regulators of G-protein signalling, selected for their known roles in the regulation of GOA-1 and/or EGL-30 signalling, to the *lin-31* and *dpy-13* RNAi assays are similarly mixed. *egl-10 lof,* which generally phenocopies *egl-30* lof due to EGL-10s role in the inhibition of GOA-1, shows a decreased response to *lin-31* RNAi but a slight increase in response to *dpy-13* RNAi. *eat-16 lof , which* as negative regulator of *egl-30*, phenocopies *goa-1 lof* (Hajdu-Cronin et al., 1999), shows an opposite phenotype to *egl-10* with respect to *lin-31* RNAi treatment but the *eat-16, egl-10* and *eat-16;egl-10* double mutant all show very similar responses to dpy-13 RNAi treatment. Loss of function mutants of *gpb-2* and *rsbp-1,* binding partners of *egl-10 ,* and *egl-10* and *eat-16*, respectively, show a clear increase in response to *lin-31* RNAi treatment and no change, or a decrease in the case of *rsbp-1,* in response to *dpy-13* RNAi treatment. Whilst *rgs-1* lof shows no change in response compared to the WT for *lin-31 treatment,* a small increase in response to *dpy-13* RNAi, a pattern also seen in both *ric-8* mutants. Although the opposing function of *egl-10* and *eat-16* are not seen in the *dpy-13* RNAi assays the strongly enhanced response of the *eat-16 lof* mutant in response to both *lin-31* RNAi and *dpy-13* RNAi treatments is strikingly similar to that seen in *goa*-1 lof mutants and suggests a possible general enhanced RNAi response.

The *unc-47p::GFP* system could be used in In order to confirm whether the results seen for *eat-16 lof* so far reflect an enhanced RNAi phenotype in accordance with that seen in *goa-1 lof.* The involvement of eat-16 would strengthen the suggestion that *egl-30* lof mutants show a decrease efficacy of RNAi. The *unc-47p::GFP* transgenic system was unable to confirm this due to the unidirectional nature of this assay. A better system to confirm the results of possible decreased RNAi mutants such as *egl-30 lof*, *goa-1 OE/ gof*, and potentially *gpa-12 lof,* whilst still being free of confounding phenotypic variants would be a non-neuronal GFP transgenic system, in which *GFP* RNAi produces a strong but not total reduction in fluorescence in a WT background.

There are a number of possible way in which *goa-1* could be acting to regulate the *egl-8/*IP_3 signalling pathway in the regulation of the RNAi response. One possibility is that *goa-1* is acting directly upstream of *egl-8.* Another is that *goa-1* activity may influence *egl-8* activity in a more distal manner, perhaps acting at a different of the signalling pathway in a different cell or even a different tissue. It is also possible that *goa-1* is regulating the RNAi response via an independent pathway to *egl-8,* such that multiple signalling pathway converge on the regulation of the RNAi response. Ultimately epistasis analysis of *goa-1; egl-8* or *goa-1; ipp-5* double mutants would be needed to confirm whether *goa-1* is acting upstream or in parallel to the IP_3 signalling pathway to regulate the RNAi response. Tissue specific transgenes could be used to attempt to rescue *goa-1* in the intestine to see whether this is able to rescue the systemic RNAi phenotype, as it does in the case of *itr*-1 mutants.

Chapter 4

Do *itr-1* mutants have an altered small RNA profile?

Do *itr-1* mutants have an altered small RNA profile?

4.1 Introduction

Small RNA sequencing has been used extensively in *C. elegans* and other organisms to gain better understanding of the nature, origins, diversity and function of small endogenous RNAs (e.g. (Gent et al., 2010; Ruby et al., 2006; Sarkies et al., 2015; Shi et al., 2013)). Small RNA sequence profiles are indicative of cell type, cell state and, at the organismal or population level, developmental state or population profile. Small RNA sequence profiling can also reveal deficiencies in one or more branches of the endogenous small RNA pathways. For example, loss of function mutants in components unique to the 26G siRNA pathway such as *ergo-1, rrf-3, eri-1, eri-9* fail to accumulate 26G small RNAs, a change which is clearly evident by small RNA sequencing (Gent et al., 2010).

Whilst some RNAi pathway components are unique to a given pathway others are shared between pathways and act as a limiting resource to generate competition between pathways (as discussed in section 1.3.xi). There is evidence for such competition for shared resources between the exo-RNAi and endo-siRNA pathways and between both the exo-RNAi, endo-siRNA and miRNA pathways (Zhuang and Hunter, 2012). For example the classical Eri mutants such as *eri-1, rrf-3* and *eri-9* whilst enhanced for exogenous RNAi are defective in endogenous siRNA induced silencing (Lee et al., 2006). One possible explanation for the altered exogenous RNAi efficiency of IP_3 pathway mutants is that it is underpinned by changes in the endogenous small RNA pathways. Changes such as a decrease in endogenous siRNAs would increase the availability of shared components for the exogenous pathway as is seen in the Eri mutants. If there is a change in one or more branches of the endogenous RNAi pathways this change may be detectable via high throughput sequencing of small RNA libraries. The endogenous classes of small silencing RNAs in C. elegans and their key characteristics are summarised in table 4.1. For a full discussion of the different endogenous small RNA types please see section 1.3.ii-1.3.vii.

itr-1(sa73) is a temperature sensitive loss of function mutation in the IP_3 receptor which acts as near WT at 16°C and near null at 25°C. Even at the less restrictive temperature of 20°C *itr-1(sa73)* displays a strong Eri phenotype comparable to that of classical Eri mutants. In order to look for differences in the endogenous small RNA population that might provide clues as to the mechanism through which the enhanced RNAi phenotype of *itr-1* mutants is achieved, I prepared small RNA libraries of WT and *itr-1(sa73)* mutant worms. Following small RNA sequence analysis, a qPCR based

experiment was designed to follow up on potential changes identified by small RNA sequencing using a broader range of IP_3 pathway mutants, *itr-1, egl-8* and *ipp-5*.

Small RNA type	**Length and 5' end chemistry**	**Biogenesis**	**Associated argonautes**	**Known functions**
miRNA	21-24 nt, most 22 nt 5'mono P	Primary transcripts from pri-miRNA encoding loci	ALG-1, ALG-2, ALG-5	Development
26G siRNA	26 nt 5' mono p	RDRP from pol II transcripts followed by Dicer cleavage.	ERGO-1, ALG-3, ALG-4	Oogenic gonad and throughout development Spermatogenesis
22G siRNA	22 nt 5' tri P	RDRP from 26G siRNA, piRNA and exo-siRNA target transcripts	WAGO clade	Various
21U piRNA	21 5'mono P	piRNA clusters	PRG-1	Silence transposons in the germline
sdRNAs (sno RNA derived)	Various 5' mono P	Cleavage of Small nucleolar RNAs by Dicer		miRNA like
tsRNA (tRNA derived)	28–36 nt (tiRNAs) 14–30 nt (tRF) 5' mono P	Cleavage of tRNAs by Dicer		Various including ribosome biogenesis, intergenerational inheritance (Li et al., 2018)

Table 4.1. The endogenous small RNAs of C. elegans.

4.2 Results of small RNA sequencing

As small RNA expression is dependent on the stage of the worms it was important that the WT and *itr-1(sa73)* populations were comparable. Since the growth defects of *itr-1(sa73)* worms are such that comparable populations could not be achieved on mixed-stage plates libraries were prepared from synchronised populations of young adult worms grown at 20°C. The adapter ligation reaction used to prepare the libraries for sequencing requires molecules to have 5' mono-phosphate and 3' hydroxyl groups, which are naturally present on the subset of small RNAs which are processed by Dicer. Two different library types were prepared from the WT and *itr-1* RNA extracts. 5' independent libraries were phosphatase treated to convert 5' tri-phosphate group to mono-phosphate prior to adaptor ligation and therefore include all small RNAs, whilst the untreated 5 prime dependent library captures only the subset of small RNAs with a 5' mono-phosphate group (and does not include the 22G siRNAs). I made both 5' dependent and 5' independent libraries of WT *itr-1(sa73) C. elegans* strains. These 4 libraries were then sequenced using high throughput Illumina sequencing, with 2 technical replicates of each library.

N2 5' dependent, JT73 5' dependent and JT73 5' independent libraries all showed good overall read count. In each case over 95% of reads mapped to the genome. The N2 5' independent library unfortunately had far lower total read count (circa 10 fold) and only 50% of reads successfully mapped to the genome. The dramatically lower total read count and mapping efficiency of this library is indicative of the lower quality and it is therefore difficult to draw reliable direct comparisons between the N2 and JT73 5' dependent libraries. In spite of this, when distributions are corrected for total read count the quantile-quantile distribution of all 4 libraries is very similar, as is the profile of read length and other properties.

4.2.i N2 and *itr-1* small RNA populations showed no obvious differences

Figure 4.1 shows the distribution of total reads in each library by length and 5' nucleotide. Both the distribution of reads by length and the 5' end composition of those reads is well matched between N2 and *itr-1(sa73)* populations in the 5' end dependent libraries (Figure 4.1 a, b), showing no obvious differences. Clear differences are evident between the 5' end dependent and 5' end independent libraries (a and b, c and d). The most obvious difference being an increased representation of reads with a 5' G. Many of these are likely to the secondary siRNAs, typically 22G reads which are not represented in the 5' dependent library due to their 5' end chemistry. If the

dramatic difference in representation of the 36nt peak is ignored, then the distribution of reads in the N2 and JT73 5' independent libraries is largely similar.

A relatively small number of reads with high copy number account for the majority of total reads in both the 5' dependent and 5' independent libraries. These highly expressed reads belong mostly to the miRNA class. *Figure 5.2* shows the distribution of unique reads by length and 5' nucleotide. Here once again the distributions of reads in the N2 and JT73 libraries are extremely similar in both the 5' dependent and 5' independent libraries with the only obvious difference being the different representation of the 36nt reads between the 5' independent libraries. This difference seems likely to be an artefact created by the lower read count of the N2 5' dependent library.

Differing methods of biogenesis mean that whilst miRNAs are expressed in high copy number 26G siRNAs are largely expressed as unique reads or in low copy number, with amplification following 22G production. Comparing the histograms of total and unique reads the peak of 26G reads siRNAs is far more evident in the 5' dependent library unique read distribution. The presence of additional 22G reads in the 5' independent libraries is even more evident in the distribution of unique reads than in figure 4.1.

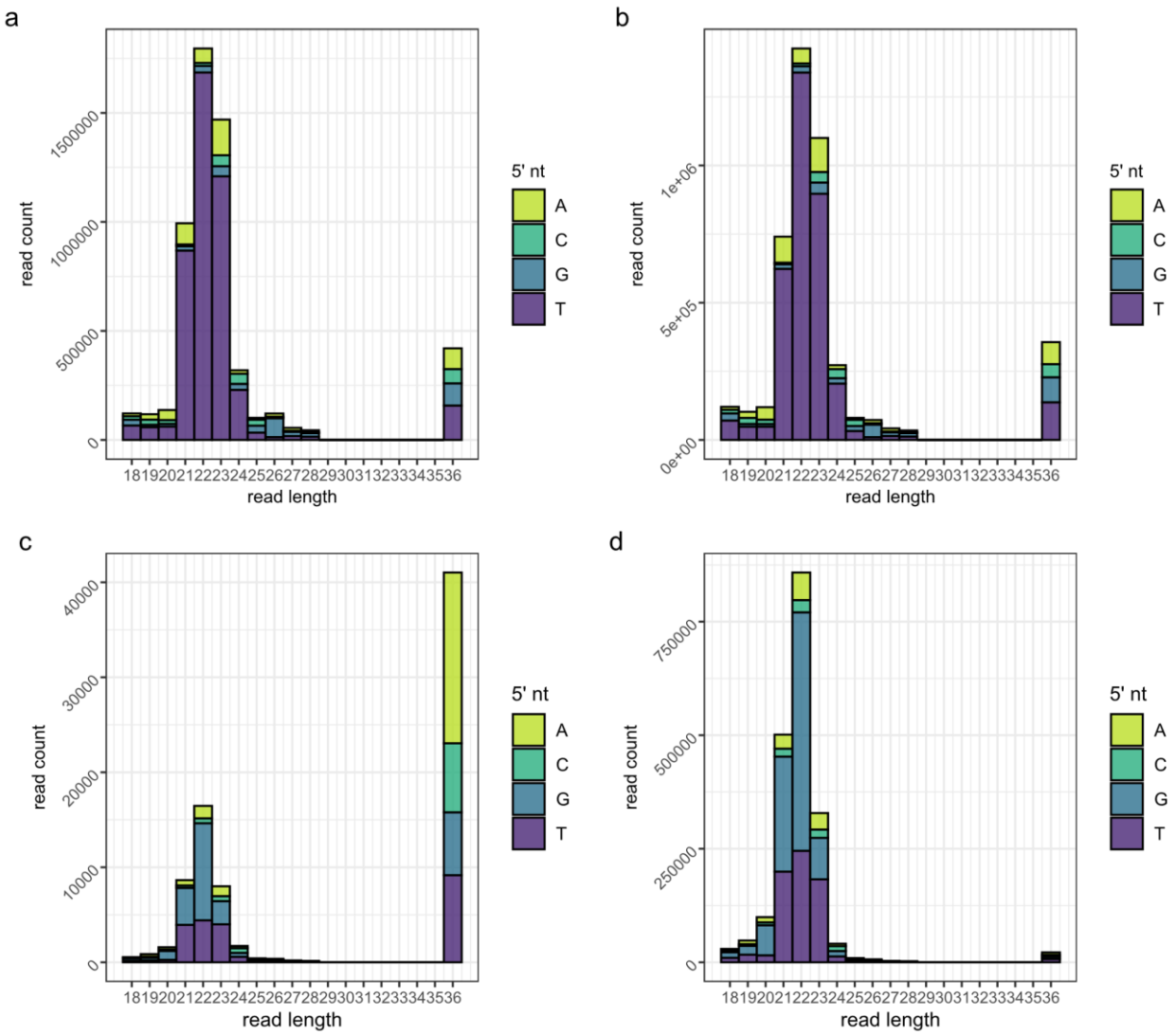


Figure 4.1. Histograms showing total read counts by length and 5' nucleotide. a) N2 5' dependent, b) JT73 5' dependent, c) N2 5' independent, d) JT73 5' independent. The increased proportion of 22G reads in the 5' independent libraries is apparent in both the N2 and JT73 libraries. There are no apparent differences in the distribution of reads between the 5' dependent libraries. Despite large differences in read count the composition of reads in 18-26nt range of N2 and JT73 5' independent libraries is very similar.

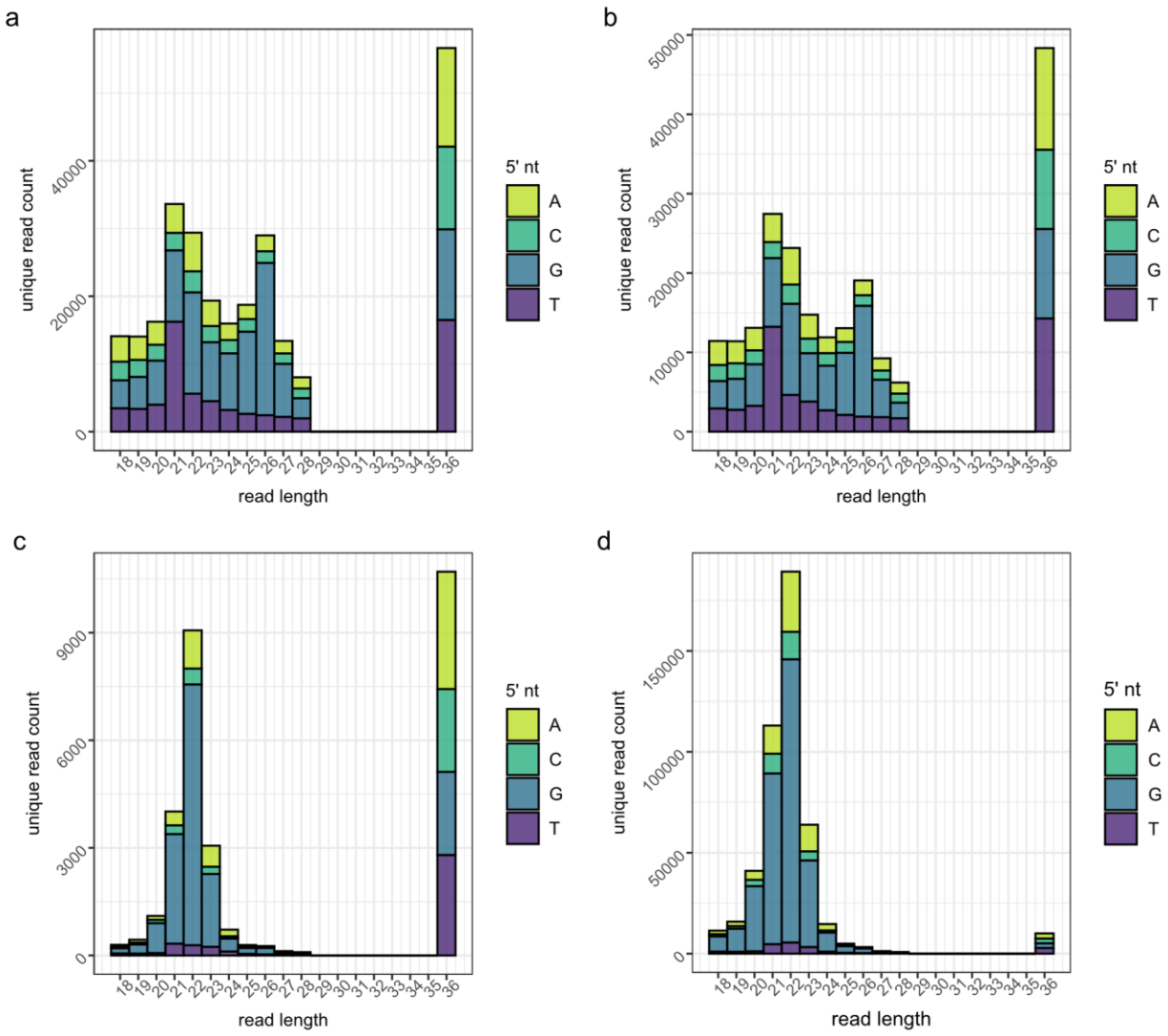


Figure 4.2. Histograms showing unique read counts by length and 5' nucleotide. a) N2 5' dependent, b) JT73 5' dependent, c) N2 5' independent, d) JT73 5' independent. In both a and a large contribution of 21U, and 26G RNAs can be seen. There is no apparent difference in the distribution of reads between the N2 and JT73 5' dependent libraries. In both b and c the increased proportion of 22G reads in the 5' independent libraries compared to the 5' dependent libraries a and b is clear. A dramatic difference in 36nt reads is seen in the N2 vs JT73 library, but despite large differences in read count the composition of reads in 18-26nt range of N2 and JT73 5' independent libraries is very similar.

Although no obvious changes to the overall small RNA profile were apparent it was possible that there were specific changes to a subset of small RNAs. The program SeqMonk (Andrews, 2011) - a tool to visualise and analyse high throughput mapped sequence data was used to carry out more in-depth analysis. Specifically, in order to look in more detail at where in the genome reads were mapping to and whether there were any changes in the relative depth at each point between the N2 and *itr-1* libraries.

Reads mapping to continuous 40bp windowed probes covering the entire genome assembly were compared. *Figure 5.3a* shows a scatter plot of the number of reads (corrected for total read count) which map to each genomic location coloured by the genomic feature to which they map. This allows the composition of the libraries to be inferred and is consistent with the profiles seen in the read length distributions. The majority of highly expressed reads map to genes encoding miRNA. A large number of reads also map to piRNAs of both high and low expression. A mixture of sense, and majoritively, antisense oriented reads map to mRNA and pseudogenes representing siRNA targets. Smaller numbers of reads map to tRNAs, small nucleolar (sno) RNAs and rRNAs. In the 5' independent library (*figure 5.3 b)* a greater proportion of reads map to the mRNAs and pseudogenes, as would be expected due to the inclusion of 22G secondary siRNAs in the library.

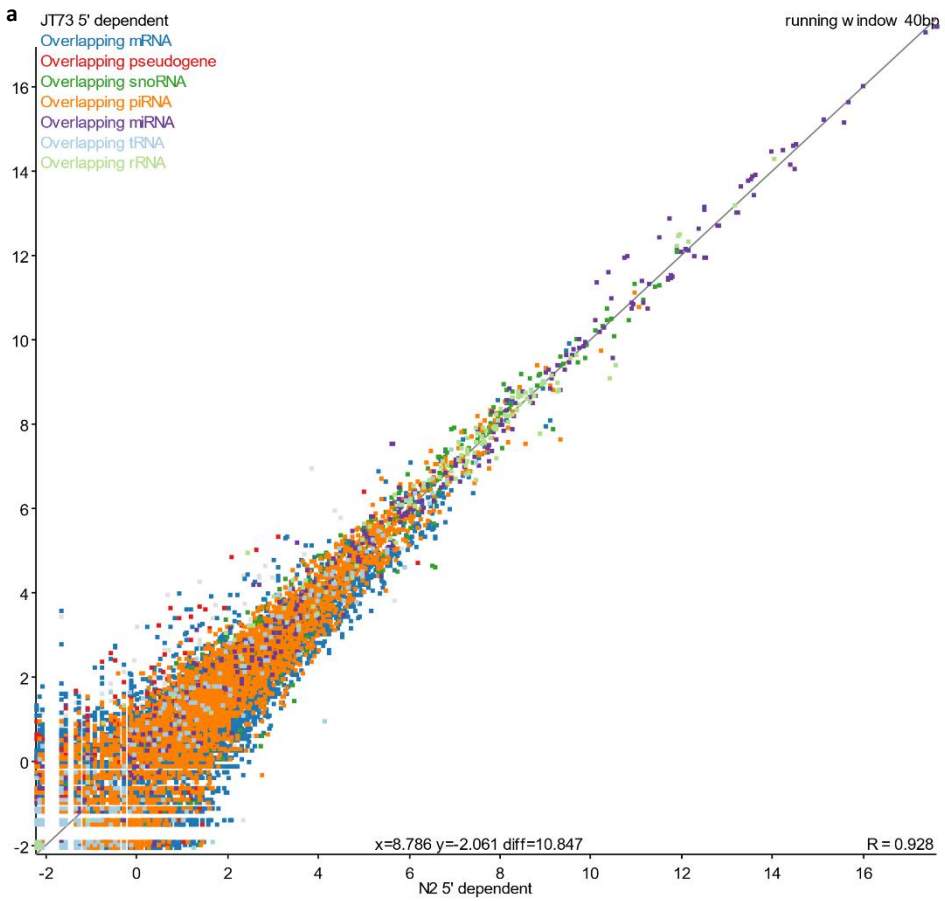


Figure 4.3. Scatter plots of read count coloured by the genomic feature to which they map.

a- 5' dependent libraries.

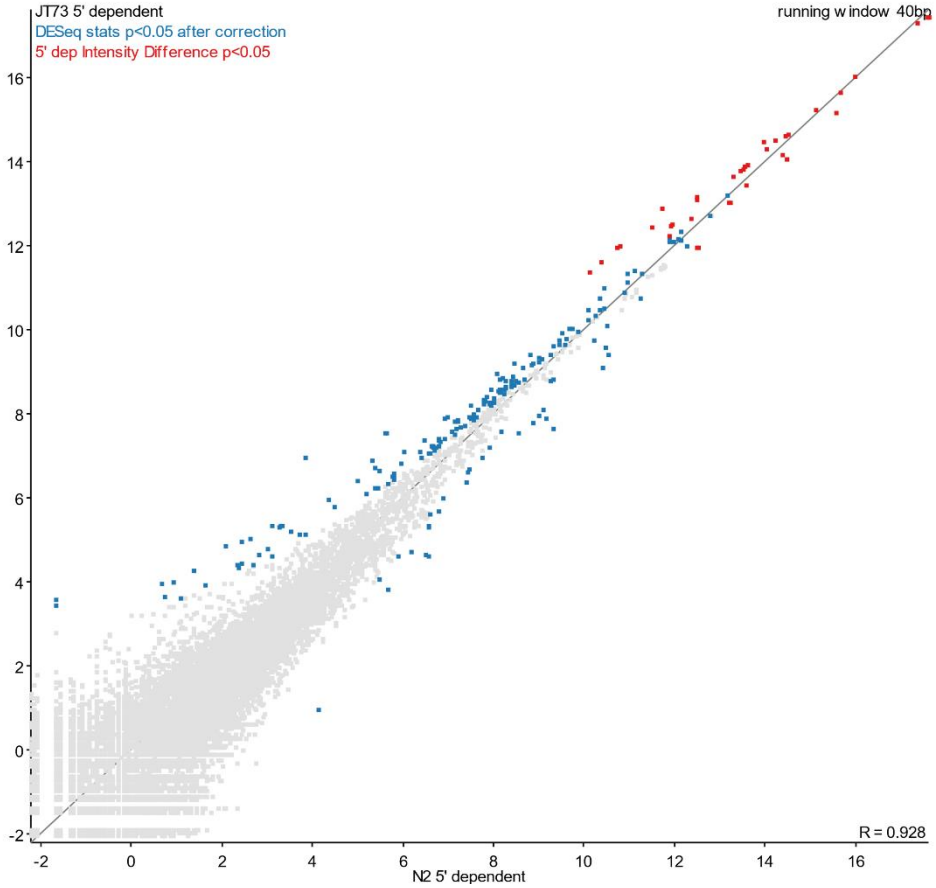
b- 5' independent libraries. In both a and b JT73 library is mapped to the Y axis and the N2 library to the X axis. Axes scales are logarithmic.

4.2.ii *itr-1* small RNA populations are enriched for subclasses of miRNA and siRNAs

Although the scatter plot of normalised read counts for each probe position show no points of dramatic difference there are groups of points which are could be differentially expressed. Using SeqMonk I tested for any statistically significant differences in read count between the N2 and JT73 5' dependent libraries, at each probe location, using the intensity difference and DeSeq2 tools. Intensity difference testing identified a small subset of points which appear to be differentially expressed between the two groups. An alternative statistical analysis (DESeq2) identified a larger set of significant points as shown in *figure 5.4.*

Notably Included in these statistically significant probe sets are a subset of microRNAs which form the *mir-35-41* family. All 7 of the miRNAs in this cluster have a slightly higher expression in the JT73 5' dependent library than in the N2. This set is of interest since the *mir-35-41* family miRNAs have been shown to regulate RNAi sensitivity via the actions of LIN-35/Rb, a synMUV B gene and core component of a chromatin complex. *mir-35-41* family miRNAs positively regulate the accumulation of retinoblastoma homologue LIN-35/Rb and both *mir-35-41* and *lin-35* loss of function results in an enhanced RNAi response, upregulation of endo-siRNA target genes (Grishok et al., 2008; Lehner et al., 2006; Massirer et al., 2012; Wang et al., 2005; Wu et al., 2012). However the *mir-35* family are also important developmental regulators and are strongly expressed in oocytes and the early embryo. It was therefore possible that differences in mir-35 family expression could be accounted for by changes in embryo number or stage between the two samples. However, the far lower fertility of *itr-1(sa73)* mutants made this unlikely and levels of other miRNAs known to be expressed in the early embryo were no higher in the JT73 library than the N2 library (data not shown).

The set of probes identified as significantly different by the DESeq2 analysis included reads which map to know ERGO-1 class siRNA targets. One of the two major branches of primary siRNAs, ERGO-1 class siRNAs are produced in the oogenic gonad and early embryo but persist throughout development. Highlighting other probes mapping to known ERGO-1 class siRNA targets (as identified in (Vasale et al., 2010)) shows that a large cluster of reads slightly more highly expressed in the JT73 library 5' dependent library as seen in *figure 5.4b.*



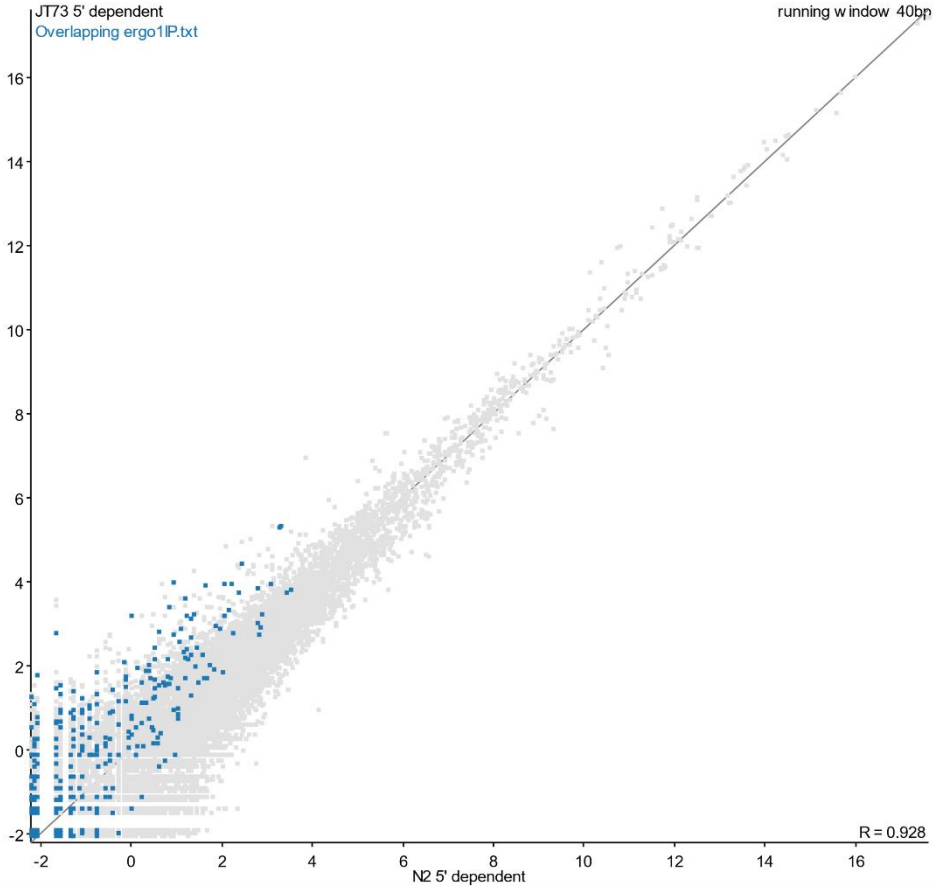


Figure 4.4. Scatter plots of read intensity at each genomic location for the of N2 and JT73 5' dependent libraries.

a- Probes with significantly different reads intensity are highlighted. Blue - probes identified by only DESeq2 analysis, red -probes identified in the intensity difference analysis.

b- probes mapping to ERGO-1 class siRNA targets are highlighted. All axes scales are logarithmic.

Since a mild enhancement of small RNAs mapping to *ergo-1* targets and a more definite enhancement of miR-35-41 family miRNAs was seen in the JT73 library compared to the WT library I decided to use quantitative real-time PCR to investigate whether these changes translated to changes in mRNA levels of *lin-35* and a small selection of previously identified ERGO-1 target genes. *lin-35* was an obvious target for further investigation due to its role as the regulator of RNAi downstream of *mir-35-41* whilst ERGO-1 target genes were selected based on a combination of differential target siRNA expression in the small RNA sequencing data and target siRNAs which were found to be strongly enriched in an ERGO-1 Immuno-Precipitation experiment performed by (Vasale et al., 2010) and were therefore high-confidence ERGO-1 class siRNA targets.

4.2.iii IP_3 signalling mutants show no change in *lin-35* or ERGO-1 target gene mRNA levels

qPCR primers were designed against *lin-35* and four ERGO-1 target genes: F55C9.5, Y116F11A.1, C11G6.2 and C40A11.10. To avoid any potential bias in enrichment created by different embryonic stages or numbers in adult worms and to maximise synchronicity qPCR was performed using RNA harvested from L1 worms. qPCR was performed on N2, *itr-1(sa73)*, egl-8*(e2917)*, and ipp-5(sy605) mutants, using the housekeeping gene GAPDH as a control (see table 4.2 for strain details). The ΔΔCt method was used to calculate relative abundance of mRNA levels in the mutant compared to the WT samples. The results of the qPCR analysis are shown in *figure 5.5.* No significant change in relative abundance (expression fold change, tested by t-test) was detected in any of the target genes.

Strain	Genotype	Variant/description	Reference	Labelled as
N2	WT (Bristol)		(Brenner, 1974)	WT
JT73	*itr-1(sa73)*	Near null at 25°C, partial lof at 20°C. Substitution leading to TS lof.	(Iwasaki et al., 1995; Nagy et al., 2015)	*itr-1*
CB6614	*egl-8(e2917)*	Likely Null. MOS1 transposon insertion.	(Nagy et al., 2015; Yook and Hodgkin, 2007)	*egl-8*
PS3653	*ipp-5(sy605)*	Likely Null. 951 bp deletion.	(Bui and Sternberg, 2002; Nagy et al., 2015)	*ipp-5*

Table 4.2. Strains used in this chapter.

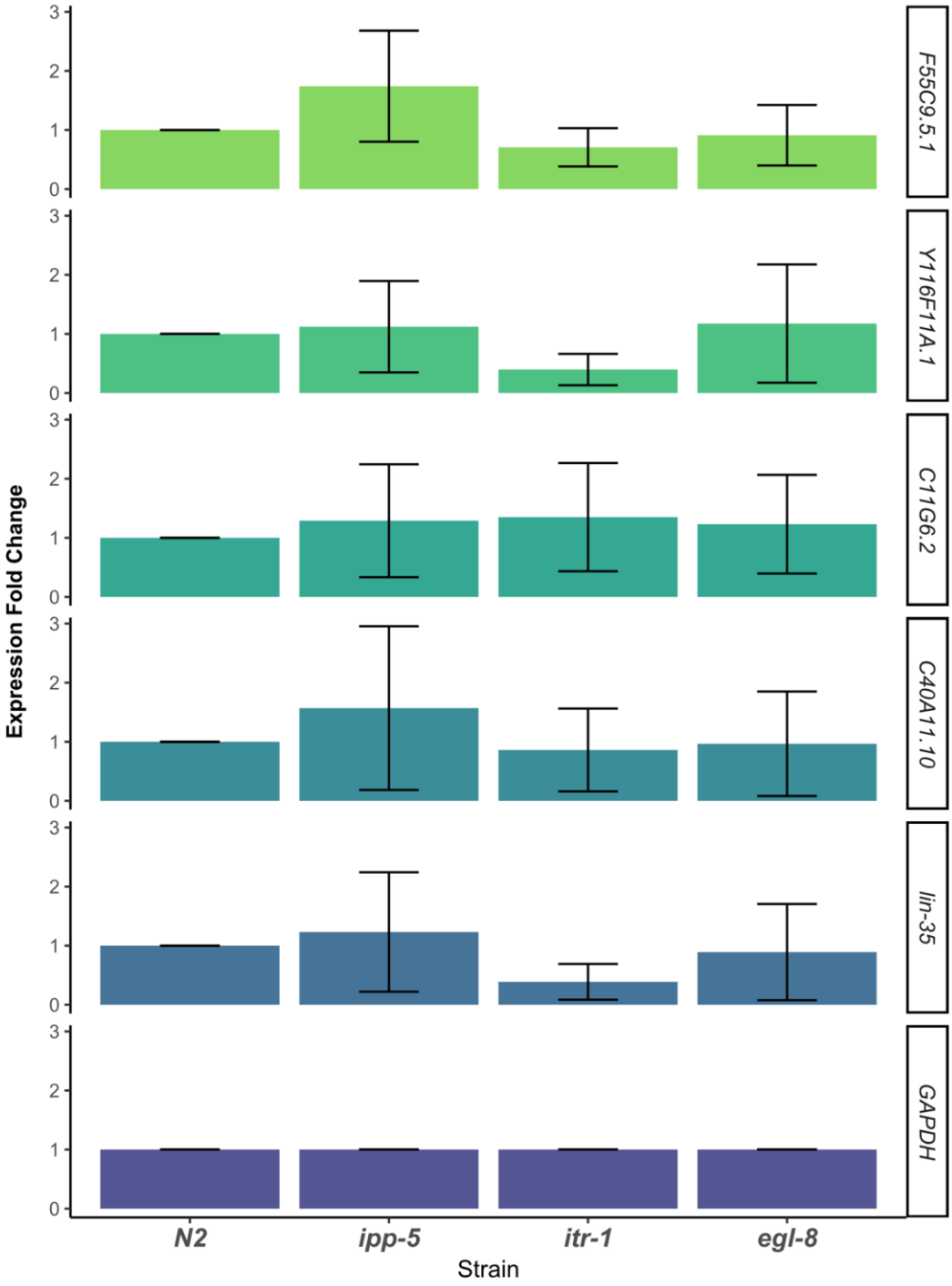


Figure 4.5. The Expression fold change of target gene transcripts as measured by qPCR and calculated by the ΔΔCt method. Ct values were normalised relative to the housekeeping control gene GAPDH and the WT strain N2 before expression fold change was calculated. No change significant difference in fold change was detected for any of the 5 target genes.

Since the results of the qPCR did not support the changes detected in the small RNA-seq analysis I looked for alternative explanations for the differences seen. As previously mentioned differences in oocyte or embryo numbers or stage would offer one explanation for the changes however other miRNAs expressed in the early embryo showed no differential expression and the fertility defects of *itr-1* analysis seemed to make this explanation unlikely since you would not expect higher number of developing embryos in *itr-1* animals. To look for alternative explanations for the differences detected in the small RNA libraries, I used WormBase to perform gene set enrichment analysis for the genes to which probes with significantly higher expression in the *itr-1* 5 prime dependent library mapped. Phenotypes for which this set of genes were enriched included endomitotic oocytes (Emo) and decreased oocyte number. These phenotypes are consistent with those of *itr-1* mutants and so are not unexpected however they could be sufficient to explain the enrichment of *mir35-41* since upon further investigation, as well as in developing embryos and oocytes, high expression of *mir-35-41* genes are also associated with an Emo phenotype, as seen in *itr-1* mutants.

Other GO terms for which the genes to which probes with significantly higher expression in the *itr-1* 5 prime dependent library mapped were enhanced for were biological adhesion, peptide biosynthesis, actin based processes, and regulation of cellular amide metabolic processes. With the set of genes including cadherin *cdh-1, ints-8* and *hsp-1*, all of which have links to membrane transport/ localisation.

Since the results of the qPCR make it unlikely that the enhanced RNAi phenotype of *itr-1* mutants can be explained by changes to either the ERGO-1 siRNA pathway or changes to *lin-35* levels I looked for genes showing large differences in small RNA read mappings which were not part of the mir-35-41 or ERGO-1 pathways. Levels of reads mapping to the long intergenic region *linc-12* were significantly higher in the JT73 5 prime dep library as were reads mapping to Y116A8B.1 and T05E12.8. All three genes are associated with the *daf-2* the insulin like receptor pathway. Mutation of this pathway also show to enhanced RNAi response (Wang and Ruvkun, 2004). However, such changes to the reads mapping to these genes could simply be consistent with the altered germline composition of *itr-1* mutants stemming from the severe fertility defects.

4.3 Conclusions - changes to the endogenous small RNA pathways of IP_3 signalling mutants remain elusive

Although analysis of Small RNA-seq data of WT and *itr-1* adult worm populations suggested an enhancement of a subset of small RNAs in JT73 worms, including the miRNAs mir-35-41 and ERGO-1 class siRNAs a follow up study using Q-PCR to look at transcript levels of *lin-35* and *ergo-1* target genes showed no changes between WT, *itr-1, egl-8, and ipp-5* populations of L1 worms. Since the Q-PCR was performed at a different developmental stage to the RNA-seq it is possible that robust changes would be seen in adult worms or at a different stage. The L1 stage was chosen to maximise synchronicity between strains with variable developmental timing and therefore maximise comparability between the samples without any confounding factors such as differences in germline composition or developmental stage. However, this approach comes with the limitation that although all the target genes are expressed in the L1 stage several are more strongly expressed in other stages. Another consideration is that although *mir-35-41* family mutants are decreased in LIN-35/Rb protein levels no change in *lin-35* transcript levels was detected by Massirer et al. who propose that *lin-35* regulation by *mir-35-41* occurs at a post-transcriptional level, possibly via intermediates. Given this it is possible that even if mir-35-41 levels are altered in IP_3 signalling mutants you would not see a change in *lin-35* transcript levels.

However, a major caveat to the small RNA sequencing data is that although technical replicates were sequenced both WT and *itr-1* libraries come from a single biological replicate of each and as such the differences seen may not be a reliable representation of the differences between the two strains. Although statistically significant, the changes seen are relatively slight and repeat sequencing with more biological replicates would be needed to ensure that the changes detected are robust and representative. Ideally synchronised mixed populations would be used to create the libraries in order to capture the small RNA state at all developmental stages. However, the fairly severe growth defects of the *itr-1(sa73)* worms, which are slower to develop and more variable, meant that comparable populations could not be achieved on mixed-stage plates. Instead I used synchronised populations of young adult worms for sequencing, however even achieving equivalent populations at the young adult stage was challenging due to the variability in growth and development of *itr-1(sa73)* mutant, such that the time period during which all worms complete their final molt is far broader than for WT worms. This makes achieving perfectly synchronised populations beyond the L1 challenging and was the reason why L1 worms were chosen for the qPCR experiment, in order to maximise perfect synchronicity and reduce stage dependent variability.

In summary no gross changes to the small RNA profile of *itr-1* mutants were detected by small RNA sequencing suggesting no drastic changes in levels of any of the endogenous small RNA pathways and as such no impairments to the miRNA, 26G siRNA or piRNA pathways were detected. The lower quality of the N2 5' independent library makes it more difficult to reliably compare changes to the 22G siRNA population of the *itr-1* and WT worms however no change was suggested. Although a subset of reads were identified as enhanced in the *itr-1* 5' dependent library subsequent qPCR failed to confirm the changes suggested to mir-35-41 and ERGO-1 subsets and it is possible the enhanced genes can be explained by the Emo and other germline phenotypes of the *itr-1* mutants. Overall, no satisfactory explanation for the enhanced RNAi phenotype of *itr-1* mutants could be found by small RNA sequencing analysis.

Chapter 5

Do IP_3 signalling mutants display an altered nuclear RNAi response?

Do IP_3 signalling mutants display an altered nuclear RNAi response?

5.1 Introduction - The nuclear RNAi pathway

In addition to the mechanisms of small RNA directed post-transcriptional gene silencing which take place in the cytoplasm, both the exogenous and endogenous RNAi pathways can also direct transcriptional gene silencing (TGS) and other effects within the nucleus. miRNAs, siRNAs and piRNAs have all been found capable of triggering TGS, either directly or indirectly in a variety of systems. Having multiple levels at which the expression of target genes can be regulated allows for fine-tuned control in endogenous gene regulation and for a more robust silencing response in the exogenous RNAi pathway. In *C. elegans* the major effectors of piRNA, endo-siRNA and exogenous-siRNA types are the 22G secondary siRNAs which bind to WAGO clade argonautes to form the central components of a number of downstream pathways – the WAGO, CSR-1 and nuclear RNAi pathways. The WAGO and nuclear RNAi pathways act in parallel to mediate silencing in the cytoplasm and nucleus respectively whilst the CSR-1 pathway protects targets from aberrant silencing.

Evidence of a link between RNA induced silencing and changes to transcriptional activity was first seen in plants and yeast with very similar effects now established in *C. elegans* as well as *Drosophila* and mammalian cells. In *Arabidopsis* dsRNA was shown to lead to changes in target gene transcription and promoter methylation (Mette *et al.*, 1999, 2000; Mette, Matzke and Matzke, 2001). In the yeast *S. pombe* loss of key RNAi pathway components was found to lead to de-repression of silenced genes, with alterations in histone methylation and surrounding chromatin state, and non-coding RNAs produced from the cen region in *S. pombe* have been found to be important for the maintenance of centromeric heterochromatin and the silencing of transgenes, and maintenance of the histone methylation in these regions in an RNAi dependent manner (Reinhart and Bartel, 2002; Volpe *et al.*, 2002).

In *C. elegans* the first evidence for RNAi silencing of nuclear transcripts came from observations of the co-silencing of genes expressed in an operon, and co-transcribed into a single pre-mRNA, such as *lin-1/lin-26*. RNAi directed against either mature mRNA induces the same lethal phenotype as a *lin-26* null mutation, which cannot be explained by loss of *lin-1* alone (Bosher *et al.*, 1999). A similar result is seen with the silencing of the *lin-15* bicistron by RNAi against *lin-15a* or *lin-15b.* Observations that co-suppression acted via an RNAi related mechanism further supported a nuclear role for RNAi (Ketting and Plasterk, 2000). Evidence of RNAi induced TGS in the soma came from observations of the unexpected silencing of the *elt-2::GFP* reporter by RNAi by feeding

constructs not targeting *GFP* and from observations of that co-suppression. The *elt-2::GFP* reporter transgene is expressed in the intestinal cells of *C. elegans.* Silencing was found to be inducible by feeding on bacteria expressing dsRNA complementary to only the repetitive transgene backbone. Silencing was found to be occurring at the transcriptional level with decreased levels of pre-mRNA and changes to the chromatin state including decreased histone H4 acetylation, and to be dependent on *dcr-1* and a number of other genes implicated in RNAi and chromatin remodelling (Grishok, Sinskey and Sharp, 2005).

RNAi induced TGS is dependent upon the production of secondary 22G siRNAs (as discussed in section 1.3.vii) The production of new siRNAs from the target mRNA enables a phenomenon known as transitive RNAi whereby silencing can spread beyond the initial target site, along the mRNA. Further work using the *elt-2::GFP* transgenic system found that RNAi by feeding targeting GFP (GFP dsRNA treatment) silenced not only the *elt-2::GFP* transgene but also the endogenous *elt-2* gene due to the transitive spread of the silencing signal to sequences expressed in *cis* to the original target sequence; this led to the silencing of complementary sequences expressed in *trans*. Such treatment results in the deposition of histone marks and second generation silencing is dependent on the efficiency of nuclear silencing (Burton, Burkhart and Kennedy, 2011; Zhuang, Banse and Hunter, 2013; Mao *et al.*, 2015).

Forward genetic screens for mutants deficient in nuclear RNA silencing lead to the isolation of NRDE (Nuclear RNAi Deficient) mutants, each a critical component in the nuclear arm of the exogenous RNAi response (Guang *et al.*, 2008, 2010). Characterisation of such mutants has shed light on the mechanisms of the nuclear RNAi pathway in *C. elegans* and has revealed that two parallel pathways act in the soma and germline, with many shared NRDE components but with a different central argonaute. In the soma the Argonaute NRDE-3 is the central component of the nuclear siRISC. Whilst in the germline HRDE-1 acts in a similar and extended role to also permit transgenerational inheritance of silencing (as discussed in section 1.3.viii)

Primary exo-siRNA, endo-siRNAs, piRNAs all trigger secondary siRNA production upon binding to target mRNAs in the cytoplasm. These 22G siRNAs are produced through un-primed synthesis by RdRPs *rrf-1* and *ego-1* from the target mRNA template. 22G siRNAs in the cytoplasm are bound by a range of WAGO clade argonautes to amplify the initial silencing response, including the nuclear argonautes NRDE-3 in the soma and HRDE-1 in the germline. NRDE-3 contains a bipartite nuclear localisation signal (NLS) but is cytoplasmically localised in the absence of a 22G small RNAs. The presence and binding of siRNAs allows both siRNA /NRDE-3 to be transported into the nucleus (Guang *et al.*, 2008). In the nucleus siRNA/NRDE-3 binds to complementary sequences of nascent

pre-mRNA transcripts and recruit further effector proteins including NRDE-2, NRDE-4 and NRDE-1 to form the RNA induced transcriptional silencing complex or RITS. NRDE-2 associates with siRNA/NRDE-3 complex and is required for co-transcriptional silencing of the nascent pre-mRNA via mechanisms which include inhibition of RNA pol II during transcript elongation (Guang *et al.*, 2010).

In addition to polymerase inhibition nuclear RNAi activity includes changes to chromatin at the target locus. The pre-mRNA associated complex of siRNA/NRDE-3 and NRDE-2 further recruits NRDE-1 and NRDE-4, all of which are required for chromatin association of NRDE-1 and the associated H3K9 trimethylation and H3K27 trimethylation, markers of gene silencing (Burkhart *et al.*, 2011; Mao *et al.*, 2015). Chromatin methylation requires recruitment of additional factors including the methyltransferases SET-25, SET-32 and MET-2 which all contribute to H3K9me3. Whilst histone methylation was shown not to be essential for efficient nuclear silencing (Kalinava *et al.*, 2017), factors mediating histone methylation and other chromatin modifications do play an important role in the transgenerational maintenance/re-establishment of silencing (Ashe *et al.*, 2012; Luteijn *et al.*, 2012; Shirayama *et al.*, 2012; Mao *et al.*, 2015; Woodhouse *et al.*, 2018).

The methylation marks directed by nuclear RNAi are especially evident in the F1 progeny of worms exposed to the primary trigger (Mao *et al.*, 2015). Interestingly, marks seem to be redeposited in a secondary siRNA dependent process each generation. Primary siRNAs may also be passed down. In the soma silencing is only effectively maintained for a single generation however additional mechanisms are in play in the germline (Sapetschnig *et al.*, 2015; Xu *et al.*, 2018).

In the germline HRDE-1 acts in place of NRDE-3 as the argonaute at the heart of the nuclear RNAi pathway. There are a number of differences in the behaviour of this HRDE-1/germline pathway compared to its somatic counterpart; most notably the ability for stable transgenerational silencing of germline genes. Whilst somatic nuclear silencing induces and strengthens silencing in the generation following dsRNA exposure, silencing is quickly lost in subsequent generations. The nature of the inheritance of RNA induced silencing in both the germline and soma remains only partially understood. A combination of cytoplasmic and nuclear WAGO clade argonautes are required for efficient transgenerational inheritance (Buckley *et al.*, 2012; Xu *et al.*, 2018). It may be the primary siRNAs which are passed onto the F1 to establish the silencing of somatic genes and re-establish the secondary siRNA population. In the soma secondary siRNA do not appear to be transmitted/ capable of further amplification in future generations. By contrast the production of tertiary siRNA in the germline allows the re-establishment of the siRNA pool in subsequent generations and hence the maintenance of silencing (Sapetschnig, Sarkies, Nicolas J. Lehrbach, *et al.*, 2015).

The endogenous functions of the nuclear RNAi/argonaute pathways are varied (as discussed in section 1.3.viii). Loss of function of any of the core NRDE components leads to a mortal germline (mrt) phenotype with the gradual loss of fertility over several generations. Many of these phenotypes closely align with those seen in piRNA mutants and are likely caused by loss of transposon silencing in the germline. Loss of NRDE-3 which functions only in the soma results in a milder phenotype than other NRDE mutants. There are likely many somatic endogenous roles of nuclear RNAi. In *C. elegans* NRDE-3 bound siRNAs target pre-rRNAs to regulate rRNA homeostasis (Zhou *et al.*, 2017) and in human cells nuclear localised argonautes have been shown to direct alternative splicing (Alló *et al.*, 2009; Ameyar-Zazoua *et al.*, 2012). miRNAs have also been found to play important roles in the regulation of gene expression through TGS in the nucleus (reviewed (Huang and Li, 2012)).

5.1.i The nuclear RNAi pathway in response to exogenous RNAi

The majority of tissues in *C. elegans,* with the notable exception of the neurones, are competent for silencing by exo-RNAi, however a few including the pharynx and vulval muscle have been reported to be resistant. Shiu and Hunter recently showed that whilst GFP expression in the pharynx is resistant to silencing in the first generation of exposure to the dsRNA trigger, GFP is efficiently silenced in the second generation of exposure and that this silencing requires the nuclear RNAi pathway (Shiu and Hunter, 2017). Their finding show that a critical window exists early in the lifecycle of *C. elegans* during which the worm is competent for nuclear RNAi, and pharyngeal GFP can be silenced, with the pathway becoming increasing incompetent from hatching onwards. They also found that in Eri mutants of the SynMuvB class, such as *lin-35* to extend the competent window.

My aim was to use the *myo2p::GFP* transgenic system to probe whether background mutations in my genes of interest affected the efficiency of RNAi induced gene silencing in the pharynx, as a proxy for the nuclear RNAi response. I confirmed findings that pharyngeal GFP is only substantially reduced in the second generation of RNAi by feeding and also investigated whether prior starvation of worms, a strong environmental signal, had any effect on the efficiency of RNAi in the pharynx.

5.2 Results

I used strains carrying the *myo-2p::GFP* transgene *mIS12,* and *myo-3p::GFP* transgene *ccIs4251,* which express GFP in the pharyngeal muscle, and in all muscle with nuclear and mitochondrial localisation, respectively. A strain carrying both of these transgenes had been previously crossed to *ipp-5(sy605), itr-1(sa73), egl-8(e2917)* strains to generate new strains carrying the transgenes in *ipp-5(sy605), itr-1(sa73), egl-8(e2917)* and WT genetic backgrounds. Details of the strains used, their genotype and the cross from which they derived are given in table 5.1.

RNAi was induced by feeding on bacteria expressing dsRNA complementary to *gfp* or to the bacterial gene *cat* as a control. Adult worms were placed on plates, allowed to lay eggs for approximately 6 hours then removed. The progeny of these worms are labelled as F1, and their progeny as F2. Worms were fed either control RNAi bacteria for two generations, *gfp* RNAi bacteria for two generations, or *gfp* RNAi in the F1 followed by control RNAi in the F2. Live worms were imaged, and the level of pharyngeal fluorescence quantified using ImageJ. Whilst fluorescence intensity of the pharynx was precisely quantified, expression of the pan-muscular *myo-3p::GFP* was simply scored as present, absent or partial (if knockdown was evident but incomplete). A representative image showing GFP expression in a control treated worm is shown in figure 5.1.

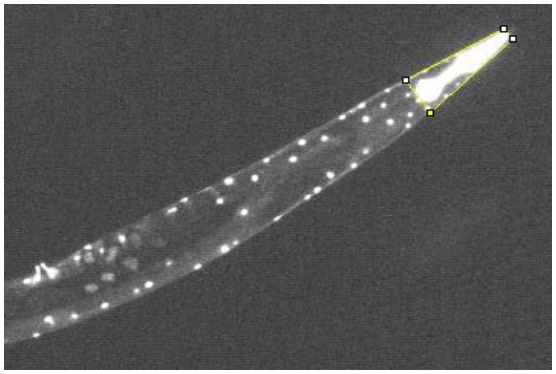


Figure 5.1. A representative image of a WT control treated worm. GFP expression is seen clearly in the pharyngeal muscles and localised to the nuclei of the body wall and other muscles The area in which pharyngeal fluorescence was measured in shown by the yellow outline.

Strain name	genotype	Labelled as	Variant description	From same cross as
HB955	ccIs4251 I; mIs12 II	***WT 2***	ccIs4251 I [myo-3p::mitoGFP]; mIs12 II [myo-2p::GFP] ccIs4251	HB954, HB956
HB953	ccIs4251 I; mIs12 II	***WT 1***		HB951, HB952
HB948	ccIs4251 I; mIs12 II; *egl-8(e2917)*V	***egl-8 1***	Likely null. MOS1 transposon insertion.	HB949
HB949	ccIs4251 I; mIs12 II; *egl-8(e2917)*V	***egl-8 2***		HB948
HB951	ccIs4251 I; mIs12 II; *ipp-5(sy605)*X	***ipp-5 1***	Likely null. 951 bp deletion.	HB953, HB952
HB952	ccIs4251 I; mIs12 II; *ipp-5(sy605)*X	***ipp-5 2***		HB953, HB951
HB954	ccIs4251 I; mIs12 II; *itr-1(sa73)*IV	***itr-1 1***	Near null at 25°C, partial lof at 20°C. Substitution leading to TS lof.	HB956, HB955
HB956	ccIs4251 I; mIs12 II; *itr-1(sa73)*IV	***itr-1 2***		HB954, HB956

Table 5.1. The *C. elegans* strains used in this chapter. See (Nagy *et al.*, 2015) for details of these strains.

5.2.i Levels of transgene expression vary between strains.

In order to establish a baseline level of GFP expression from the transgene in the pharynx the level of fluorescence of the pharynx was quantified in worms following control RNAi treatment. There is a high degree of variability in the expression of the GFP transgene in the pharynx, both within and between strains, as shown in figure 5.1. The level of pharyngeal fluorescence is significantly lower in the WT 2 strain, than in the mutant strains and WT 1 strain. There are also significant but less dramatic differences in fluorescence between the WT 1 strain and the two *itr-1* strains as seen in figure 5.2). The reasons for these differences are unclear. Shui and Hunter found that the transgene was expressed more strongly amongst mutants defective in transgene silencing, however, the differences in behaviour between the two WT strains suggests that a significant amount of variation in fluorescence, and presumably transgene expression, may be due to random variation in silencing behaviour, or other differences in the genetic background rather than being attributable to the gene of interest. The very high degree of variability in fluorescence intensity measured between worms of the same strain supports the theory of an inherently stochastic mechanism of somatic transgene silencing. The fluorescence intensity of the *myo-3::GFP* transgene, which is expressed outside of the pharynx, was not quantified precisely however expression was scored as present, absent or partial. *myo-3::GFP* transgene was visibly expressed in all control treated worms.

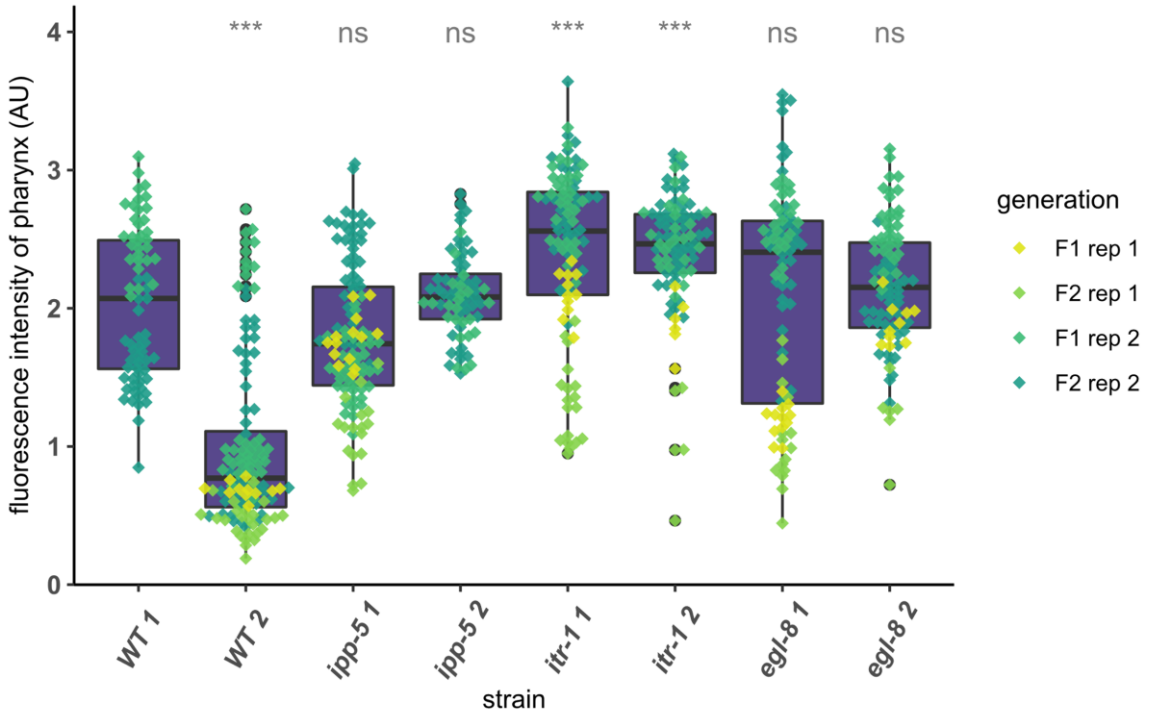


Figure 5.2. Difference in fluorescence intensity of control treated worms. There is a high degree of variability both within and between strains. Between strain differences were tested statistically using anova followed by Tukey HSD test. All strains show a significantly higher level of fluoresence than the strain *WT 2*, and several also show significant differences from strain *WT 1*, as indicated in the plot. The box plots represent the range of the recorded data points. The central line represents the median with the box edges representing the upper and lower quartiles. Coloured dots represent individual worms with colour indicating the data set and generation in which they were recorded. Strains *WT 1* and *ipp-5* 2 were included in only the rep 2 data set.

5.2.ii Effects of RNAi treatment in the first generation

As would be expected given the reports of the pharynx being resistant to RNAi, gfp RNAi treatment had only a limited effect on the fluorescence intensity of the pharynx in the F1, despite clear loss of fluorescence in the body wall muscles. As shown in figure 5.3.a, following a single generation of either *gfp* RNAi or control treatment most strains showed a small reduction in fluorescence intensity of in the pharynx, however not all strains behaved the same. Significance testing using an analysis of variance model followed by post-hoc testing shows a significant global effect of *gfp* compared to control treatment. Looking at the effect on each strain no significant change is seen in either of WT or *egl-8* strains, while both of the *itr-1* strains show significantly lower levels of pharyngeal fluorescence following *gfp* treatment than control treatment. Neither *ipp-5* strains shows any decrease in fluorescence following gfp treatment. In the strain *ipp-5 1* significantly

higher levels of fluorescence were recorded after gfp treatment than after control treatments whilst in *ipp-5 2* there is no significant change. With the exception of the *ipp-5* strains, all other strains show a trend for a small reduction in pharyngeal fluorescence intensity following treatment with *gfp* RNAi. The effects of *gfp* RNAi on GFP fluorescence outside of the pharynx were in line with the expected behaviour of *itr-1, egl-8* and *ipp-5* mutants (see figure 5.3.b). Individuals from both WT strains show a range of *gfp* knockdown, from complete loss of visible fluorescence in the bwm, to the loss of fluorescence in only some bwm nuclei. All individuals from the *itr-1* and *egl-8* strains showed a complete loss of GFP fluorescence in the bwm, whilst the majority of worms belonging to the *ipp-5* strains showed no loss of fluorescence.

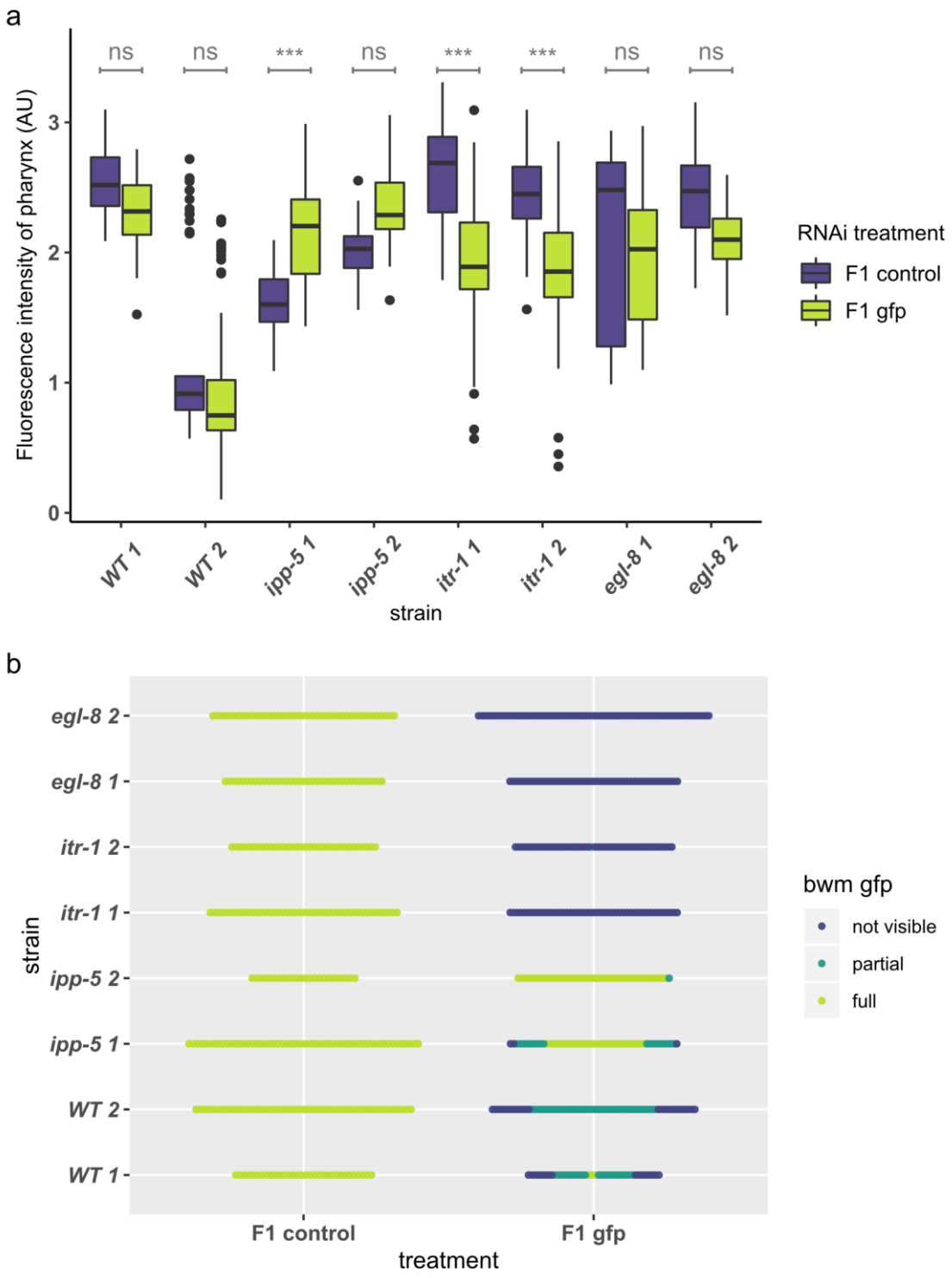


Figure 5.3. **a - Effect of *gfp* RNAi treatment on the fluorescence intensity of the pharynx in the F1 generation.** In the F1 generation *gfp* RNAi treatment has a small yet significant overall effect. Significant difference between the control and *gfp* treated groups was found in some strains, as indicated. Statistical significance of treatment, strain and interaction thereof was tested using an anova followed by Tukey test. Box plots representing the range of the recorded data points are shown. The cetral line represents the median with the box edges representing the upper and lower quartiles. Outliers to the expected data range are shown as points. **b- Effect of *gfp* RNAi treatment on the fluorescence of the bwm in the F1 generation.** GFP expression in the body wall muscle of each worm was scored as not visible, partial (visible in some nuclei) or full (no visible reduction). Lines are constructed of dots coloured according to bwm GFP state, each dot representing a single worm.

5.2.iii Effects of RNAi treatment in the second generation

Although *gfp* RNAi treatment had little effect on pharyngeal fluorescence in the first generation, due to the resistance of the pharynx to RNAi, exposure in the first generation would be expected to trigger knockdown of GFP expression in the second generation, due to the production of secondary siRNAs engaging the nuclear RNAi response (Shiu and Hunter, 2017). Consistent with the findings of Shiu and Hunter that RNAi in the pharynx requires the nuclear RNAi pathway, which can only be triggered during a critical period early in development, the overall effect of gfp RNAi treatment in the F1 generation was a dramatic reduction in pharyngeal fluorescence in the F2 generation ($p<<0.001$). As can be seen in figure 4.3.a *gfp* RNAi treatment in both the F1 and F2 generation, or in the F1 generation alone resulted in a clear and significant reduction in the level of pharyngeal fluorescence intensity across all strains tested, with the notable exception of *ipp-5 2.* In all strains a large variability in fluorescence levels is seen following both control or *gfp* treatment.

Across the WT 1, WT 2, *itr-1* 1, *itr-1* 2, e*gl-8* 1, *egl-8* 2 and *ipp-5* 1 strains the same general trend in response to *gfp* RNAi treatment is seen, whilst the strain *ipp-5* 2 shows no significant reduction in response to either one or two generations of exposure to *gfp* dsRNA. Since the baseline level of fluorescence expression differs between strains, the level of pharyngeal fluorescence as a percentage of the mean fluorescence of control treated worms of that strain was calculated and is shown in figure 5.4.b. This allows any differences in response between strains to be better compared. Whilst the difference in behaviour of the *ipp-5* 2 strain is most obvious there is also a statistically significant differences in behaviour between the WT 1 and several other strains, though these are small compared to the overall effects of treatment. There is no significant difference between the response of the *ipp-5 1* strain and either WT strain. In response to the 'F1 gfp, F2 gfp' RNAi treatment both *itr-1 1, itr-1 2* and *egl-8 2* have a significantly greater mean percentage reduction fluorescence. This difference is also seen in response to 'F1 gfp, F2 control' treatment in both *itr-1* and both *egl-8* strains.

A high variability in response is seen across all strains, with some individuals from all strains showing no detectable reduction in pharyngeal GFP in response to either *gfp* treatment condition, whilst the majority show substantial GFP knockdown. In the strain *ipp-5 1* the median response to both 'f1 gfp, f2 gfp' and 'f1 gfp, f2 control' treatments is slightly less than that seen in the WT strains and is greatly less in *ipp-5 2* where no response is seen. Conversely the median response is slightly greater in all *itr-1* and *egl-8* strains.

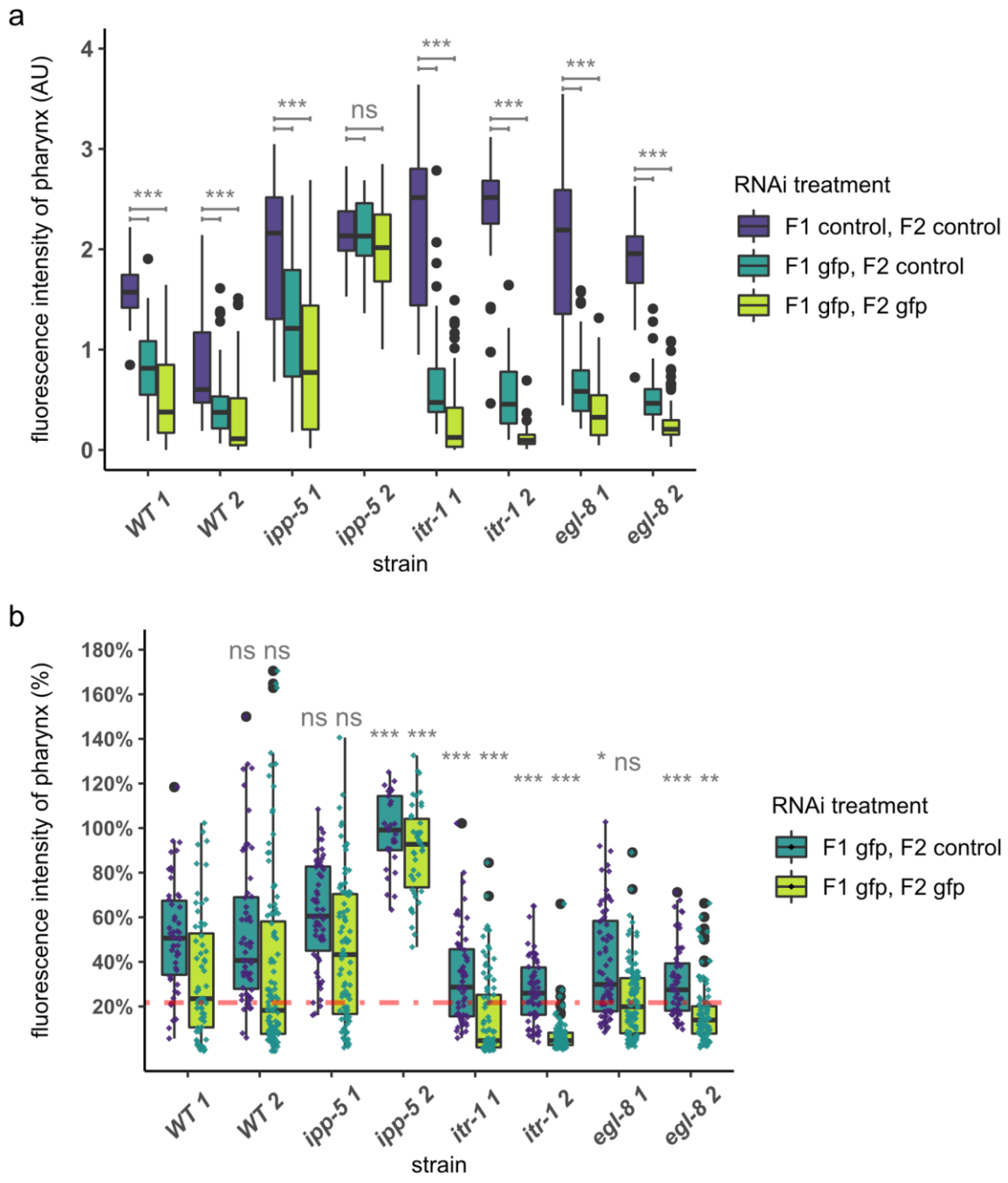


Figure 5.4. Effect of gfp RNAi treatment on the fluorescence intensity of the pharynx in the F2 generation. **a**-Across all strains except ipp-5 2, the expected response of a significant difference in fluorescence intensity of the pharynx of gfp vs control treated worms is seen in the second. Exposure to gfp dsRNA in only the first generation leads to a clear decrease in GFP expression ($p<<0.001$), whilst exposure in the first and second generations leads to and even great decrease ($p<<0.001$). Significance of response, both globally and for each strain (as shown) was tested statistically using anova followed by Tukey test. **b**- The fluorescence intensity of the pharynx following one or two generation of RNAi treatment is shown as a percentage of the mean value for control treated worms of that strain. The red line indicates the median level of fluorescence of 'F1 gfp, F2 gfp' treated worms of both WT strains combined. Significance values indicate difference from the equivalent treatment group of the WT 1 strain, tested using Wilcoxon rank sum test.

In order to test whether the nuclear RNAi response is entirely dependent on inherited silencing signals or whether re-exposure strengthens the response worms exposed to GFP RNAi treatment in the F1 were treated with either *gfp* or control RNAi bacteria in the F2 generation. Exposure to *gfp* RNAi treatment in the only the first generation ('F1 gfp*,* F2 control') still results in a clear reduction in pharyngeal fluorescence in the F2 generation, clearly demonstrating the hereditary component of the nuclear RNAi pathway, even when targeting a somatically expressed gene. However interestingly the level of apparent GFP knock down resulting from only F1 exposure ('F1 gfp, F2 gfp') is significantly less than that seen in worms exposed to GFP dsRNA for both generations ($p<<0.001$). These differences can be seen clearly in the density chart in figure 5.5.a which shows that the three different treatment conditions result in distinguishable populations with separate peaks but overlapping density distributions.

Shui and Hunter reported that early exposure to dsRNA expressed from a heat inducible promoter can trigger nuclear RNAi in that same generation. Differences in the fluorescence intensity seen between worms exposed for GFP RNAi treatment for both generations and those exposed in only the f1 generation could suggest an additive effect of distinct inherited and early exposure components contributing to silencing, or could reflect differences in levels of initial trigger, simply resulting from length of exposure.

Unlike the fluorescence in the pharynx, fluorescence in the body wall muscles was substantially recovered in the F2 generation of 'F1 *gfp,* F2 *cat'* treated worms (see figure 5.5b, compared to 5.3b) with most worms showing partial of full fluorescence. As in the F1 generation the proportion of worms showing no visible fluorescence, partial fluorescence and full bwm fluorescence, following either 'F1 gfp, F2 *cat*', or 'F1 gfp, F2 gfp' treatment, varies by strain. As would be expected the *itr-1* and *egl-8* RNAi enhanced strains show the strongest response, whilst the strain *ipp-5 2* shows almost no response. The *ipp-5 1* strain shows a strength of response intermediate between the WT strains and i*pp-5 2*. These results show the same pattern of response as was seen in the pharynx.

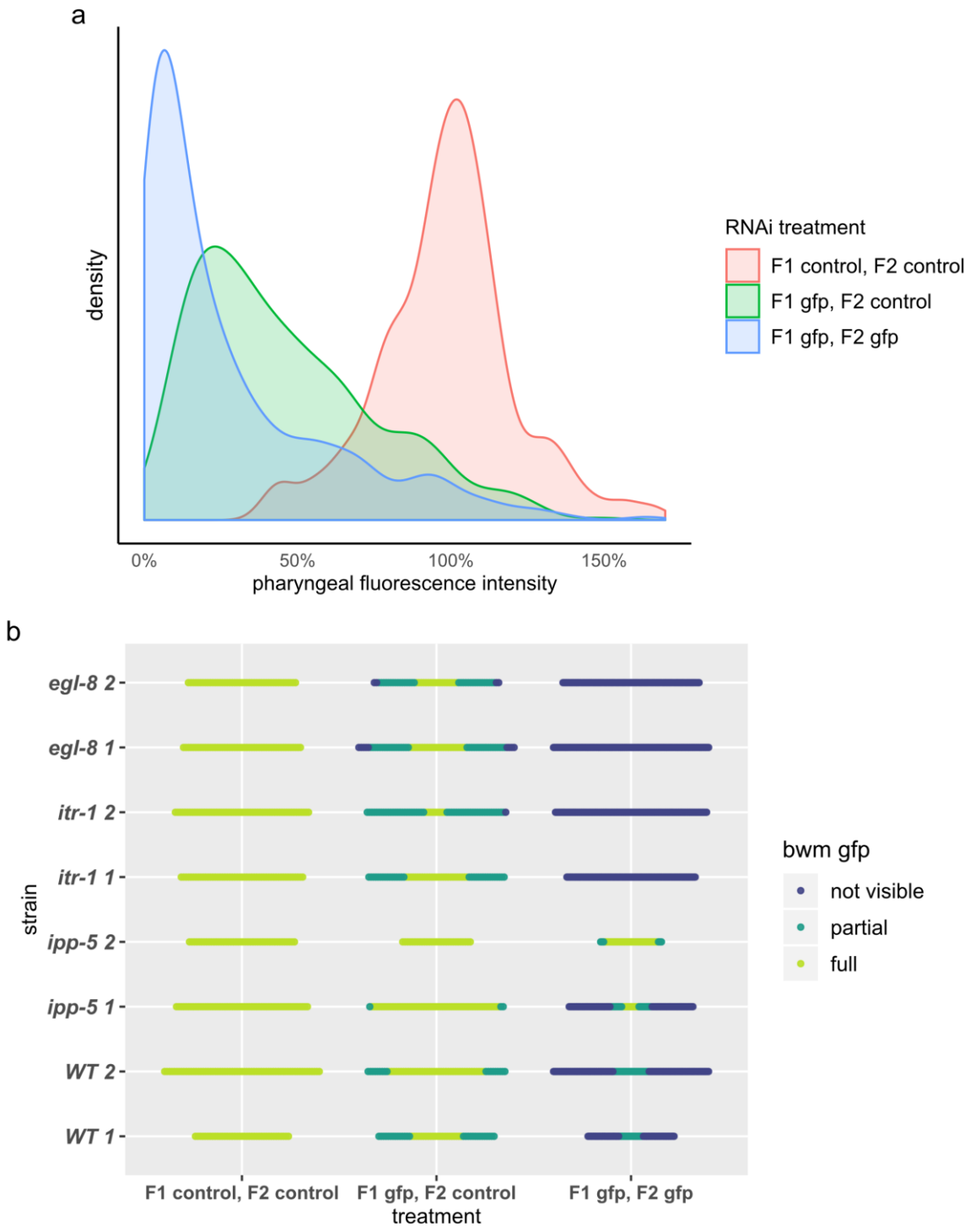


Figure 5.5. **a- Re-exposure to primary dsRNA stimulus aids silencing in the second generation.** The combined density distribution for normalised pharyngeal fluorescence intensity in the f2 generation of all strains tested, coloured by treatment, reveals distinct peaks for 'F1 control F2 control', 'F1 gfp, F2 control', 'F1 gfp, F2 gfp' treated populations. Exposure to gfp primary dsRNA in the first and second generation is the treatment most likely to result in maximal silencing. The wide distribution of the intensity values recorded for control treated worms show the high degree of variability in the level of transgene expression. **b- GFP expression in the body wall muscle in the F2**. Worms were scored as not visible, partial (visible in some nuclei) or full (no visible reduction). Lines are constructed of dots coloured according to bwm gfp phenotype, each dot representing a single worm. bwm fluorescence was substantially recovered in the F2 generation of F1 gfp, F2 control treated worms (compared to 5.3.b).

5.2.iv Does starvation affect the nuclear RNAi response?

Starvation is an environmental cue and a physiological stress which has been shown to trigger the production and transgenerational inheritance of siRNAs in *C. elegans* (Rechavi *et al.*, 2014). In order to test whether the prior starvation of worm populations has any inherited effect on the efficiency of the nuclear RNAi response populations of worms belonging to the WT, *ipp-5, itr-1 1, itr-1 2* and *egl-8 2* strains were first starved for 10 days then transferred to OP50 plates for 2 days to allow an egg laying adult state to be reached before being transferred to *cat* or *gfp RNAi* plates and allowed to lay eggs.

Prior starvation did not have a significant effect on the mean levels of fluorescence intensity measured in the pharynx in either generation of most strain. As can be seen in figure 4.6.a a few strains did show weakly significant difference ($p<0.05$) in the mean level of pharyngeal fluorescence in either the F1 or F2 generation, however, there seems to be no pattern to these changes. Although there is no significant difference in the mean of the starved and unstarved treatment groups of the *cat* treated *itr-1 2* worms there is significant heteroskedasity, tested using Levene's test. This heteroskedacity is also seen in the *gfp* treatment group of *itr-1 2,* which also shown a significant difference in mean and in the *cat* treatment group of the *ipp-5* strain. All other strains however show no significant difference in either mean or heteroskedacity. No differences were seen in the gfp fluorescence outside of the pharynx.

In the f2 generation no significant differences in heteroskedacity or mean are seen between the starved and unstarved treatment groups for among the *cat* RNAi treated worms, however some small but significant difference in mean and detected in the *gfp* RNAi groups. Prior starvation seems to increase the pharyngeal fluorescence intensity of *gfp* treated *itr-1 1* strain worms, whilst it is decreased in *egl-8 2* strain worms. No other strains show any significant difference.

Overall there seems to be no effect of starvation on the nuclear RNAi response however the increased variation in pharyngeal GFP intensity following starvation which is seen in the f1 generation but not the f2 may indicate a more general effect of starvation on transgene expression.

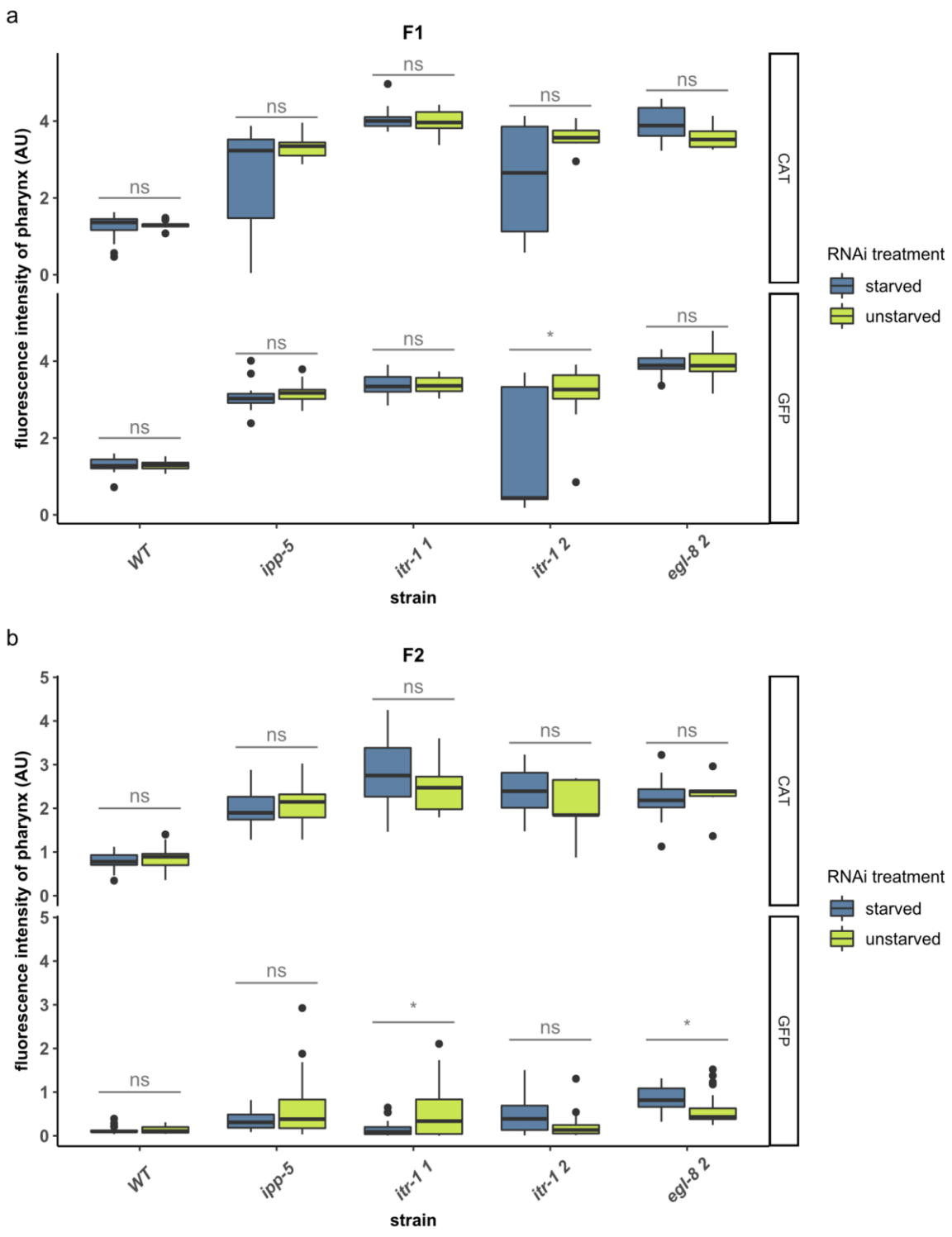


Figure 5.6. The effect of starvation on pharyngeal fluorescence intensity. Starvation increases the variation in the levels of pharyngeal intensity measured in the f1 but not the f2 generation as measured by the Levene's test, in particular in the strain *itr-1 2*. Statistical significance of difference in mean was tested using the pairwise Wilcoxon signed-rank tests and is indicated as ns=not significant, *=p<0.05.

5.3 Conclusions

The IP_3 signalling mutants tested do appear to show some changes in the strength of nuclear RNAi induced silencing as *itr-1 1, itr-1 2,* and *egl-8 2* mutants all show a significantly greater mean percentage change in pharyngeal fluorescence intensity in the F2 generation, following either *gfp* RNAi treatment than the WT strains. One of the two *ipp-5* mutant strain tested, *ipp-5 2* was shown to be almost entirely resistant RNAi induced GFP knockdown in the pharynx whilst the *ipp-5 1* strain showed a response more similar to the WT strains.

The compromised nuclear RNAi response seen in the *ipp-5 2* strain is comparable to that seen in both *nrde-3* and *rde-1* mutants (Shiu and Hunter, 2017); but as in the case of *rde-1* mutants this change is likely the result of up-stream changes in the exogenous RNAi response altering the availability of material for engagement by the nuclear RNAi pathway. Why a comparable response is not seen in the strain *ipp-5* 1 is unclear. It is worth noting that the *ipp-5* 1 strain was also less resistant than *ipp-5 2* to RNAi in the body wall muscle in both the F1 and F2 generation, although both strains were still less sensitive than the WT strains. The reasons for the differences in behaviour of the two *ipp-5* strains will need to be resolved in order to confirm that *ipp-5* loss of function decreases sensitivity to nuclear RNAi. The first step in resolving these discrepancies would be to sequence both strains to confirm that both as homozygous for the same *ipp-5* lof mutation, as expected.

RNAi enhanced mutants belonging to the Eri and SynMuvB classes show clear evidence of silencing in the pharynx in the F1 generation, proposed to be due to an extension of the critical window for initiating the nuclear RNAi response (Shiu and Hunter, 2017). Such a change is not seen in the IP_3 signalling mutants tested. No change is seen in the *egl-8* strains and although some *itr-1 1* and *itr-1 2* worms show evidence of slight pharyngeal silencing, the mean level of pharyngeal fluorescence of *gfp* treated groups being significantly lower than control groups, this is very different from the dramatic silencing seen across WT, *itr-1* and *egl-8* strains in the F2 generation.

Additional controls with a known specific effect on the nuclear RNAi pathway would allow for better evaluation of the differences in pharyngeal silencing seen in IP_3 signalling mutants in the first and second generation. A nuclear RNAi defective mutant such as *nrde-3* should be used as a negative control whilst *lin-35* should be used as a positive control for enhanced nuclear RNAi. Without these controls it is difficult to judge whether the changes seen to pharyngeal GFP silencing are due to any specific effect on the nuclear RNAi pathway.

The subtle enhancement of pharyngeal GFP silencing seen in the *itr-1* and *egl-8* mutants in the F2, and the lack of silencing in *ipp-5 2,* seem more likely to reflect changes in the RNAi response upstream of the engagement of the NRDE-3 pathway. Changes which increase the response to the primary siRNA pathway are likely to result in an increase in secondary siRNA production and therefore increase the availability of 22G siRNAs for engagement by the NRDE-3 pathway. Such changes would be expected to strengthen the nuclear RNAi response even without changes to the critical window for nuclear RNAi. Overall, this data suggests that the IP_3 signalling pathway has no specific effect on nuclear RNAi, but that the general changes in RNAi sensitivity seen in IP_3 signalling mutants do extend to the nuclear RNAi response.

Chapter 6

Discussion

How does IP_3 signalling regulate the RNAi response?

Discussion- How does IP_3 signalling regulate the RNAi response?

Reduced IP_3 signalling as seen in *itr-1* (IP_3 receptor) or *egl-8* (PLCβ) loss-of-function mutants leads to an enhanced response to RNAi. This enhancement is also proportional to the degree of ITR-1 function. In the temperature sensitive mutant *itr-1(sa73)* no change to the RNAi response is seen at 16°C, a strong enhancement is seen at 20°C, and the strongest response is seen at 25°C, equivalent to that seen in other *itr-1* null mutants. Conversely increasing IP_3 signalling by overexpressing *itr-1* or by loss of function of the Inositol phosphatase *ipp-5*, strongly reduces the efficacy of the RNAi response. It was previously shown that rescuing IP_3 signalling in the intestine by expression of WT *itr-1* from the intestinal specific promoter *vha-6p* is sufficient to restore the WT RNAi response in other tissues. Rescuing IP_3 signalling in the intestine resulted in WT RNAi response to both endogenous genes introduced by feeding and WT silencing of GFP transgenes in neurones, body wall muscle (BWM) and the pharynx, even when dsRNA is produced from transgene expression in the pharynx, rather than being introduced by feeding. Rescuing IP_3 signalling in neurones did not affect the RNAi response (Nagy et al., 2015). In the work presented in this thesis I have screened a number of cell signalling mutants, potential regulators of the IP_3 signalling pathway, for an altered response to RNAi by feeding, analysed the small RNA profile of *itr-1* mutant worms and assayed the nuclear RNAi response of *itr-1, egl-8* and *ipp-5* mutants, with the aim of better understanding how alterations in IP_3 signalling causes changes the RNAi response.

6.1 Conclusions and future work

6.1.i G-protein signalling mutants with an altered RNAi response

As the well-established upstream activator of EGL-8 (PLCβ), EGL-30 (Gαq) loss-of-function was expected to lead to an enhanced RNAi response, comparable to that seen in *egl-8* or *itr-1* lof mutants. However this is not the case. Of the three *egl-30* lof mutants screened, which range in severity from a likely null mutation to partial loss-of-function, none displayed a consistent enhanced response to RNAi by feeding targeting *lin-1, lin-31* or *dpy-13*. The responses of the five different *egl-30* gof and overexpression mutants tested were more variable and ranged from a largely WT response, to a consistently enhanced RNAi response seen in the strain *egl30 gof1, and egl30 lof OE rescue*. The only RNAi target gene where this pattern of results was not seen was in response to *unc-15* silencing. However, the flaccid paralysis and hypermobility seen in *egl-30* lof and gof mutants respectively, makes this an unsuitable assay for testing the RNAi response in these mutants. The lack

of an enhanced RNAi response in *egl-30* lof mutants was confirmed assaying neuronal GFP knockdown in the *unc-47::gfp* transgenic system. Overall, these results demonstrate that *elg-30* loss-of-function does not cause an enhanced RNAi response and may cause a mild reduction in RNAi response, whilst *egl-30* gain-of-function or overexpression may lead to an enhanced RNAi response.

It is clear that EGL-30 is not the upstream activator of EGL-8 in the pathway regulating RNAi sensitivity. Alternative Gα subunits, which could be acting as the activator of *egl-8*, either directly or through Gβγ, were therefore screened for an altered RNAi response. I found that loss of function of the $G\alpha_0$ homologue *goa-1* results in an enhanced RNAi phenotype analogous to that seen in egl-8 mutants. This enhanced RNAi phenotype was robust, seen in several different *goa-1* lof in response to *lin-31*, *unc-15* and *dpy-13* RNAi and was confirmed using the *unc-47p::GFP* transgenic system. Other Gα mutants produced more mixed results. Several showed an altered response in one or more RNAi assay, which in some cases could be due to specific genetic interactions with the target. G*pa-12* showed a reduced response to *lin-31* and *unc-15* RNAi, but this was not replicated in *dpy-13* assays. *gpa-16* showed a moderate enhancement in response to both *lin-31* and *dpy-13* assays.

Working from the hypothesis that *goa-1* and *egl-30* may be signalling antagonistically in the pathway regulating RNAi sensitivity, as is known to be the case in the regulation of egg-laying and locomotion, I tested proteins known to regulate this antagonism for an altered RNAi response. These included the RGS family GAPS EAT-16, EGL-10 and RGS-1, the GEFS RIC-8 and AGS-3, GPB-2, the Gβ orthologue and homologue of mammalian $G\beta_5$, which interacts with both EAT-16 and EGL-10 to mediate their repression of EGL-30 and GOA-1 respectively, and RSPB-1, required for the localisation of EAT-16 to the cell plasma membrane. Loss-of-function mutants of *eat-16, gpb-2* and *rspb-2* all showed a strongly enhanced response to *lin-31* RNAi, whilst *egl-10* showed a reduced response, results which strongly support the hypothesis of *goa-1* and *egl-30* signalling antagonistically. However, this pattern of results was not replicated in *dpy-13* RNAi assays, where both *eat-16* and *egl-10* showed an enhanced response. This enhancement was strong in the case of *eat-16* and milder in *egl-10* and no significant change in response was seen in the *eat-16;egl-10* double mutant nor in either *gpb-2* mutant. The mixed results of these mutants mean that need further investigation is needed to clarify a potential role for *eat-16* in the regulation of RNAi sensitivity.

The pleiotropic effects of many Gα proteins and their regulators make accurately assaying alterations in the RNAi silencing of many endogenous genes difficult. The locomotive phenotypes of *goa-1*, *egl-30*, *egl-10* and *eat-16* among others complicate the scoring of Unc assays and differences in sizes make accurately scoring Dpy phenotypes by eye difficult. Even when assays can be scored accurately it is difficult to rule out a more specific regulatory role for the gene in question in the

developmental or neuronal signalling pathway being assayed, which is why it is important to test the RNAi response of mutants using a variety of target genes.

However, the scoring accuracy of the assays used could be improved in some ways. Measuring the length of worms in the *dpy-13* RNAi assay from images of worms, is time consuming but improves the accuracy of this assay. It may be possible to automate the measurement process using image analysis software. MUV phenotypes could be assayed more accurately by testing alleles which seem to alter the RNAi response in a strain carrying a fluorescently expressed vulva marker. This would allow multivulval phenotypes to be scored definitively without relying on the protruding vulva phenotype. Since Gα signalling is likely to have a role in vulval development it is also important to confirm that there is no increased incidence of MUV phenotypes in the untreated or control treated worms.

The use of the *unc47p::GFP* transgenic system is a powerful tool for confirming the enhanced RNAi phenotype of mutants however an improved assay is needed for measuring reduced RNAi phenotypes due to the very low knockdown neuronal GFP seen in WT backgrounds. Assaying GFP knockdown in a non-neuronal tissue such as the BWM, with the *myo3p::GFP* transgene *ccIs3251* would be a good option. When this transgene was used as a control for knockdown in the neuronal RNAi assay intermediate partial to complete knockdown was seen in a WT background, complete knockdown was seen in an enhanced RNAi background such as *itr-1* and no knockdown or partial knockdown was seen in the reduced RNAi background of *ipp-5* mutants. Assaying silencing of this transgene in the BWM by GFP RNAi therefore allows for bi-directional modulation in the RNAi response to be accurately detected. This system could be used to confirm the potentially reduced RNAi sensitivity of *egl-30* lof mutants and *goa-1* OE mutants, and the potentially enhanced RNAi sensitivity of *egl-30* gof mutants. The *gpa-12* lof mutant should also be tested using this system using this system to confirm or rule out a reduced RNAi response. *gpa-16* lof and *eat-16* lof should be tested for enhanced RNAi response using the *unc-47p::GFP* system. It is important to control for any underlying differences in transgene expression by scoring GFP knock down in worms fed on control expressing dsRNA against a control target gene (such as the *cat (Escherichia coli chloramphenicol actetyltransferase*) gene).

6.1.ii - How Could GOA-1 be regulating EGL-8 activity?

Although *goa-1*, *egl-8* and *itr-1* all show an enhanced response to RNAi further work is needed to confirm that they are acting in the same pathway. Epistasis analysis of *goa-1;egl-8* and *goa-1;ipp-5* will shed light on whether *goa-1* is acting in the same pathway or in parallel to IP_3 signalling to regulate RNAi. If *goa-1* is acting in the same pathway as *egl-8* it is likely to be acting as the direct upstream activator, although a more distal role is also possible. *goa-1* could even be acting in the same pathway as *egl-8* but in another tissue, downstream of an extracellular signal produced in response to IP_3 signalling in the intestine. The *unc-47p::GFP* system should be used to confirm whether the RNAi response of *goa-1:egl-8* double mutants is greater than that seen in either single mutant. An additive effect would suggest they are acting in independent pathways to regulate RNAi. *goa-1;eri-1* and *egl-8;eri-1* double mutants should be used as controls, as the *egl-8;eri*-1 double mutants is already known to have an additive effect the RNAi response. Analysis of the RNAi phenotype of *goa-1;ipp*-5 as compared to *egl-8;ipp-5* double mutants will provide additional information. If *goa-1* is in fact acting downstream of IP_3 signalling, then the enhanced RNAi phenotype of *goa-1* lof would be expected to be epistatic to the reduced RNAi phenotype of *ipp-5* lof. Due to the manner in which IPP-*5* acts to influence abundance of IP_3, as opposed to being part of the direct signalling pathway if *goa-1* is acting upstream of *ipp-5, ipp-5* lof is unlikely to completely rescue the *goa-1* enhanced RNAi phenotype. It will therefore be important to compare the RNAi phenotype of *goa-1;ipp-5* and *egl-8;ipp-5* double mutants. If the results of the analysis of double mutant strains suggest that *goa-1* is acting upstream of *egl-8* in the IP_3 signalling pathway regulating RNAi sensitivity, then tissue specific rescue experiments, expressing goa-1 from the *vha-6p* intestinal promoter, could be used to confirm the likelihood of *goa-1* acting in the same cell as *egl-8* and *itr-1* in the regulation of this response.

If GOA-1 is acting directly upstream of EGL-8 how might it be positively regulating EGL-8? PLCβ is canonically activated by EGL-30 via an interaction mediated by the PLCβ specific CTD. However the regulation of PLCβ enzymes is a complex process, controlled by two autoinhibitory domains, interactions with the plasma membrane, interactions with scaffold proteins, negative regulators, and activation by Gβγ subunits and Rho family small G-proteins in addition to $G\alpha_q$ (reviewed (Lyon and Tesmer, 2013)). In particular the Gβγ subunit of mammalian $G\alpha_i$ containing heterotrimers is has been shown to regulate Ca^{2+} signalling via PLCβ1 in synergy to $G\alpha_q$ (Rebres et al., 2011) . Since *goa-1* is the *C. elegans* homologue of mammalian $G\alpha_{i/o}$ activation by the Gβγ subunit of a GOA-1 heterotrimeric G-protein is a possibility. It is also possible that GOA-1 could directly activate EGL-8 or recruit a further factor(s) to do so. It is becoming increasingly clear that in many cases cell signalling pathways are not linear but exist as part of complex and overlapping networks, mediated in

part by interactions with scaffolding proteins to form signalling complexes. How these signalling complexes affect regulation of PLCβ and other signalling enzymes is not yet understood.

Though results are mixed, the data suggests EGL-30 may be acting in opposition to GOA-1 and PLCβ EGL-30 in this pathway. One explanation for this behaviour could be that EGL-30 completes for binding of PLCβ, with GOA-1 or Gβγ. There are numerous instances of GOA-1 and EGL-30 signalling antagonistically in *C. elegans* however the nature of the interactions mediating this antagonism are not well understood(Collins et al., 2016; Esposito et al., 2010; JH and M, 2000; Miller et al., 1999, 2000; Tanis et al., 2008). Competition for binding of GPB-2 between the EGL-30 inhibitor EAT-16 and the GOA-1 inhibitor EGL-10, regulating the activation state of GOA-1 vs EGL-30 has shown to be a regulator of this antagonism in some behaviours (Chase et al., 2001; Van der Linden et al., 2001; Robatzek et al., 2001; Zwaal et al., 1996). Confirmation of if and how *egl-30* and *eat-16* and *egl-10 may* affect RNAi response may help to better understand how *goa-1* may be interacting with *egl-8*.

Whether by GOA-1, the Gβγ subunit, or another means, EGL-8 is being activated in response to an unknown upstream stimulus, most likely acting via a GPCR. The *C.* elegans genome encodes approximately 1300 putative GPCRs. Most of these are orphan receptors with no known ligand and in most cases unknown roles. This is partly because studies of loss of function mutations in *C. elegans* GPCRs suggest that mutation in a single GPCR rarely produced a noticeable phenotype, however some phenotypes have been noted in multi-mutants. Approximately 1200 of these are predicted to be chemosensory receptors, and several more are expressed in only a narrow subset of neurones. Based on the available data of known expression patterns and G-protein coupling, the list of candidate GPCRs for an initial screen can be narrowed down substantially. Mutants for some of these candidate GPCRs are available and for some and multi mutants are available. The available candidate mutants should be screened for an altered RNAi response. Failing any promising results utilising existing mutants, knockout could be generated for candidate GPCRs for which mutants are unavailable. Should this fail to yield results a broader reverse or forward genetic screen for mutants with an enhanced RNAi response could be carried out in an attempt to identify the receptor upstream of the IP_3 signalling pathway regulating sensitivity to RNAi. A reverse genetic screen of strains engineered to carry multiple GPCR would likely be the best approach and is increasingly feasible given development of genetic engineering techniques such as CRISPR based approaches which have already been used successfully in *C. elegans*.

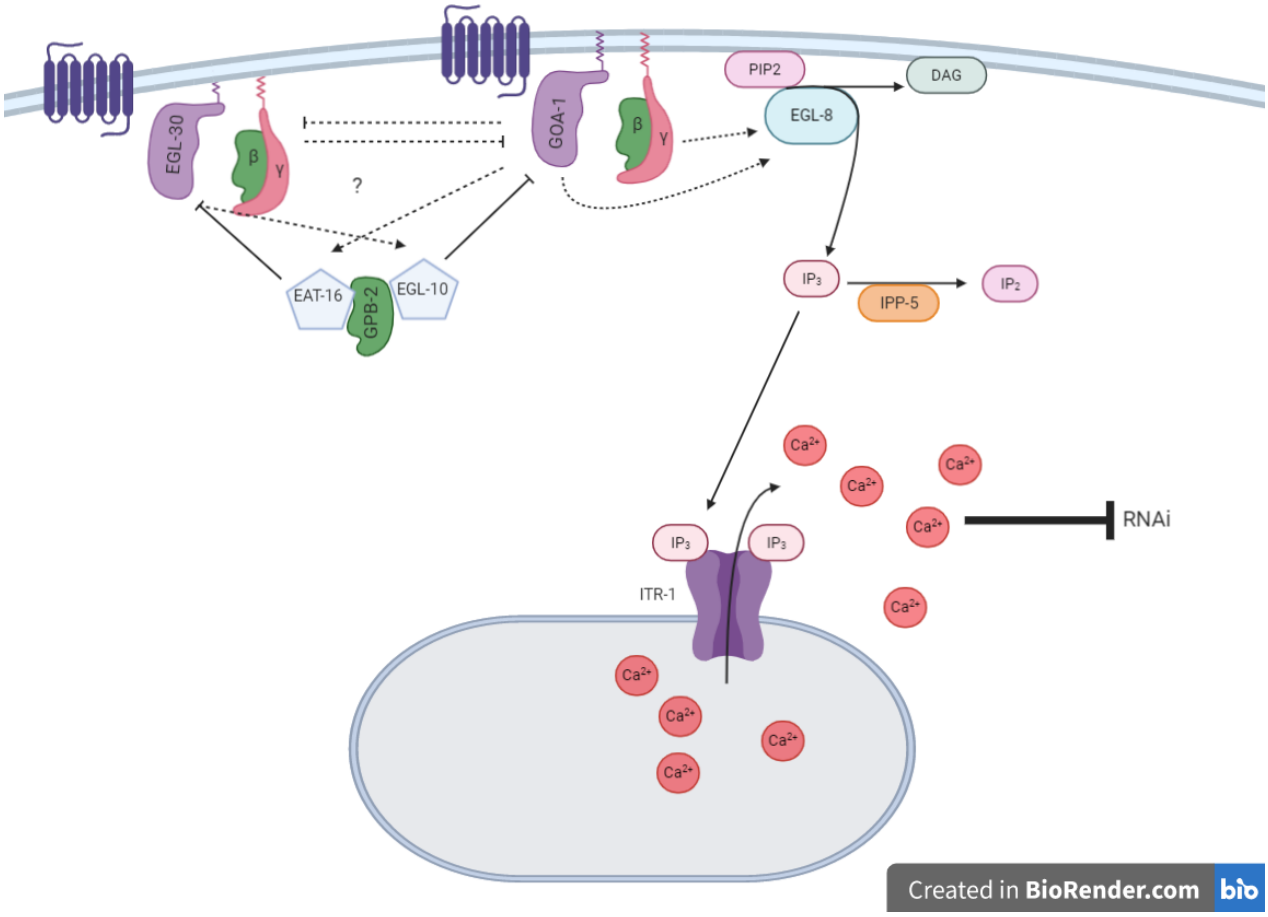


Figure 6.1. Propposed model for the regulation of EGL-8 by GOA-1 in an IP_3 signalling pathway regulating the RNAi response. If *goa-1* is confirmed to be acting as the upstream activator of *egl-8* this could be via a direct interaction or via Gβγ. A possible antagonism between *goa-1* and the canonical activator of *egl-8, egl-30* could be mediated by the GEFs *eat-16* and *egl-10*.

6.1.iii The small RNA profile of *itr-1* worms

Comparison of the small RNA populations of *itr-1* and WT worms, in the absence of stresses or exo-RNAi stimulation showed no gross changes. Changes to the endogenous small RNA population would be expected if one of the endogenous RNAi pathways was compromised in *itr-1* mutants. Although some more specific enhancements were found these were not dramatic and qPCR based follow up experiments no differences between WT and IP_3 signalling mutants.

The major caveat of this work is that the growth and fertility defects of *itr-1(sa73)* are severe enough, even at the intermediate temperature of 20°C, that it was not possible to generate matched mixed populations of WT and *itr-1* worms, which would have captured changes at every developmental stage. Instead synchronised populations of young adult worms were used. miRNA expression is a tightly controlled developmental regulator and as such profiles are tissue stage

specific. It is therefore possible that changes to specific miRNA populations may have been missed by sequencing only young adult worms.

Given the lack of changes to endo-siRNAs profiles of *itr-1* mutants it is very unlikely that the enhanced RNAi response seen in *itr-1 and egl-8* can be accounted for by decreased IP_3 signalling leading to a down regulation in the endo-siRNA pathway, increasing availability of resources to be redirected to the exo-siRNA pathway. However, it does remain possible, if unlikely, that sufficient resources are available for WT endo-siRNA function in the absence of an RNAi stimulus, and that reduced IP_3 signalling favours the redistribution of resources to the exogenous RNAi pathway only when it is active. The failure of the WT 5' independent library means that differences in the 22G secondary siRNA population (which is not captured in the 5' dependent libraries) could not be accurately compared. The up regulation of secondary siRNA production or the up regulation of core components of the exo-RNAi pathway in response to reduced IP_3 signalling therefore both remain possibilities. These possibilities could be tested by the sequencing and comparison of 5' dependent and 5' independent small RNA libraries from WT and IP_3 signalling mutant worms treated with RNAi against an endogenous target gene. The choice of target gene must be made carefully so as to limit knock on affects which could affect small RNA profiles, developmental targets such as *lin-31* for example would be unsuitable since this leads to a change in the developmental identity of a number of cells, whilst a widely expressed neuronal target such as *unc-15* could be suitable choice. The use of a non-endogenous target such as a GFP expressing transgene could be analytically difficult as these sequences would not map to the C. elegans genome. The *egl-8(e2917)* may be a better choice than *itr-1(sa73)* for sequencing experiments since it displays milder growth and fertility defects, but a similarly altered RNAi response. It may therefore be possible to generate well matched mixed populations of WT and *egl-8* worms which would allow changes across all developmental stages to be captured.

6.1.iv The nuclear RNAi response

Testing the nuclear RNAi response, utilising fact that RNAi silencing in the pharynx is dependent on nuclear RNAi, showed a modest alteration in nuclear RNAi mediated silencing in IP_3 signalling mutants. Shi and Hunter found that in WT worms GFP silencing in the pharynx is only seen in the second generation of exposure to dsRNA due to the narrow developmental window, early in development, during which dsRNA exposure is needed in order to engage the nuclear RNAi response (Shiu and Hunter, 2017). I found a small but significant decrease to pharyngeal fluorescence in the F1 generation of *itr-1* mutants; however, this decrease was not seen in the *egl-8* strains. In the F2 generation, enhanced silencing was detected in *itr-1* and *egl-8* mutants, and a resistance to silencing

was clear in one of the two *ipp-5* mutant strains (possible reasons for and work needed to resolve this discrepancy is discussed in chapter 5). Specific changes to the developmental window during which dsRNA exposure is needed in order to initiate the nuclear RNAi pathway, as is seen in *lin-35* mutants were not tested for, but it is possible that a similar change is present in IP_3 mutants. Alternatively it is possible that enhanced sensitivity at an earlier stage of the RNAi pathway, increasing the availability of siRNAs to be engaged by the nuclear RNAi machinery is sufficient to account for the changes seen, without any specific alterations to nuclear RNAi machinery. If there were significant changes to the window of opportunity for initiating nuclear RNAi in *itr-1* and *egl-8* mutants I would expect to see a more first-generation silencing, as was reported for lin-35 mutants (Shiu and Hunter, 2017).

To better understand how IP_3 signalling is affecting the nuclear RNAi response these experiments should first be repeated with the inclusion of a *lin-35* mutant strain so that F1 silencing can be directly compared between *lin-35* and *itr-1* mutants. *nrde-3* (nuclear argonaute) mutants, defective in nuclear RNAi should also be included as a control.

The finding that IP_3 mutants display an altered nuclear RNAi response is further evidence of a clear role for IP_3 signalling in the modulation of the exogenous RNAi response. However, the specifics of the interaction between IP_3 signalling and the RNAi response remain elusive. The lack of major changes to the endogenous small RNA profiles of *itr-1* mutants suggest that the endogenous RNAi pathways are not compromised in IP_3 signalling mutants. This is consistent with the hypothesis that an IP_3 signalling pathway is specifically regulating the exogenous RNAi response. To better understand the possible ways in which IP_3 signalling could regulate the RNAi response, known mechanisms of regulation should be considered.

6.2 How could IP_3 signalling influence the RNAi response?

6.2.i Influences on the efficacy of the RNAi response in *C. elegans*

To better understand how IP_3 signalling, most likely in the intestine, might be affecting the RNAi response throughout the organism we must consider all the potential points of regulation of the exogenous RNAi response, both systemically and intracellularly. The different ways in which other characterised RNAi mutants exert their effects must also be considered. Three factors principally determine the efficacy of the RNAi response in *C. elegans*: 1- Exposure and availability of dsRNA to initiate silencing. Transport mutants affect the availability of dsRNA to the silencing cells. 2- sensitivity of the silencing cell to the dsRNA. The recipient cell must be competent for RNAi response and must have sufficient resource of RNAi machinery. 3- the target gene. Some genes are less receptive to silencing but why is unclear. In many cases increasing either availability of dsRNA or sensitivity can overcome this, as is demonstrated to by the use of genes which are not effectively silenced in the WT background to screen for enhanced RNAi mutants. Most RNAi mutants fit into one of two categories – either they act on or in the core exo-RNAi pathway or endogenous RNAi pathways, or they act in the intercellular and/or intracellular transport of silencing RNAs. Figure 6.2 outlines the key stages of the exo-RNAi response in *C. elegans.* Any stage in the transport, amplification and silencing process is a potential point of regulation as efficiency at each stage contributes to a robust silencing response.

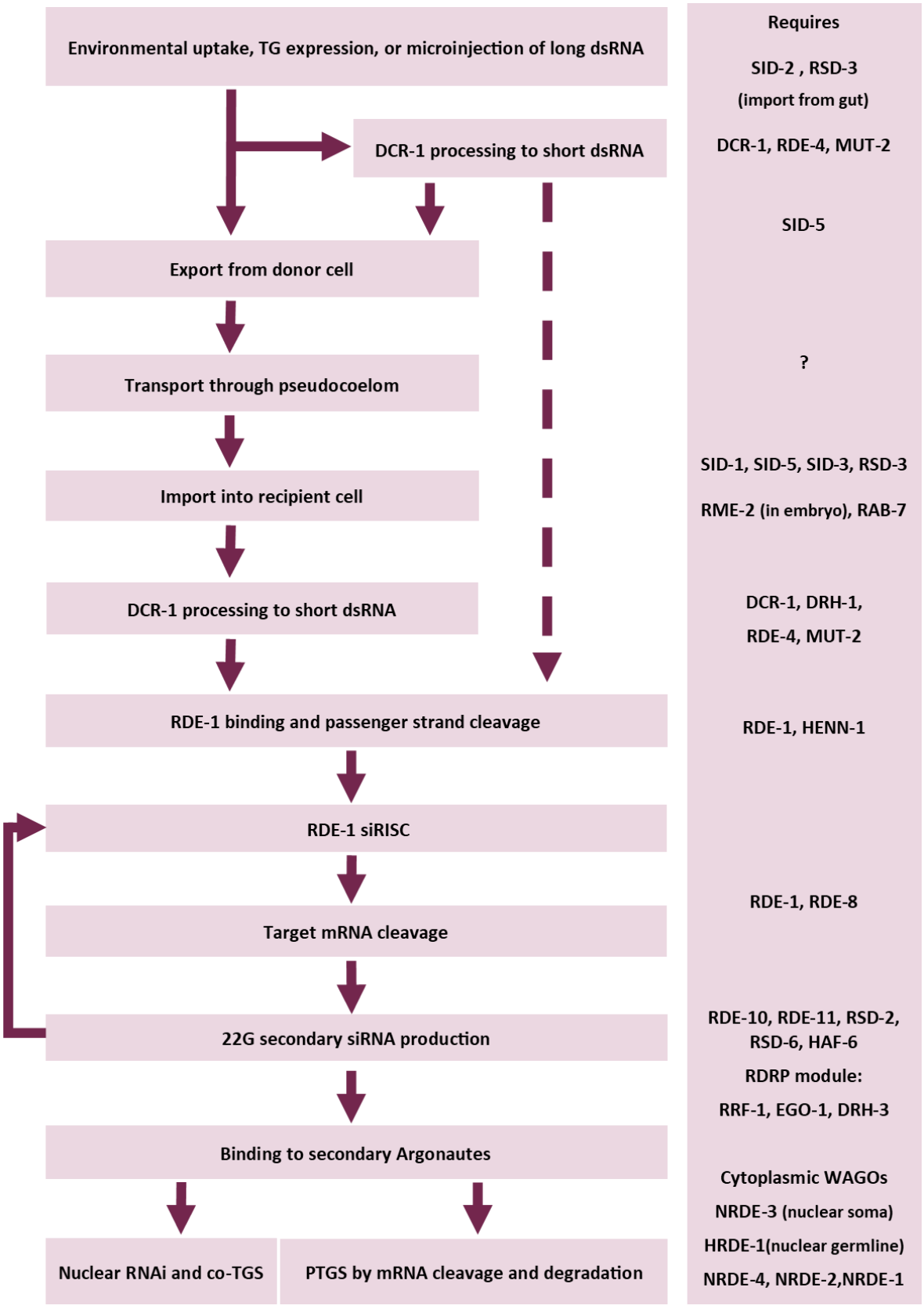


Figure 6.2. The exogenous RNAi pathway in *C. elegans.*
Each stage in the RNAi pathway is a potential point of regulation. On the left some of the proteins known to contribute to each stage of the RNAi process. Though not all are essential, all contribute to an efficient and effective silencing response.

6.2.ii Availability of dsRNA trigger

It is clear from the strong systemic response to RNAi in *C. elegans* that silencing RNAs are spread between cells and tissues. Although much has been learnt from the identification of SID and RSD mutants many aspects of the mechanisms involved in intercellular spread remain poorly understood. What is known about the proteins required for an effective systemic response suggests there may be several mechanisms of import, export and transport involved.

dsRNA dosage at the receiving cells depends on both the initial exposure/uptake and the efficiency of delivery to the effector cell. Although in many cases a relatively small amount of initial dsRNA trigger is need to trigger the RNAi response due to the efficient amplification mechanism, initial dosage is none the less an important factor in the strength of response, with a higher dose of dsRNA leading to a stronger response via all methods of induction.

Could the altered RNAi response of the IP_3 signalling mutants be explained by alterations in exposure to dsRNA trigger? In the case of RNAi by feeding increased environmental uptake of dsRNAs, either by increased consumption of bacteria, the amount of time bacteria spend in the gut, or increased expression of SID-2 could lead to a higher dosage of dsRNA being delivered to the intestinal cells and therefore a stronger RNAi response. *itr-1 mutants* are constipated due to reduced IP_3 signalling leading to a lengthened defection cycle (Dal Santo et al., 1999; Espelt et al., 2005; Teramoto and Iwasaki, 2006) and are also slower to develop such that the time period of exposure to dsRNA when measuring KD of a developmental gene is slightly lengthened. However these possibilities were investigated by Nagy et al. and mutant animals with similar defecation and growth phenotypes to *itr-1* were not found to have an increased RNAi response (Nagy et al., 2015). Consistent with this the growth and defecation phenotypes seen in *egl-8* mutants are milder than those seen in *egl-8* mutants but the enhancement to the RNAi response is comparable. The canonical *egl-30, egl-8*, *itr-1* signalling pathway regulates pharyngeal pumping, with loss of function of any of these genes leading to reduced feeding. Differences in feeding therefore cannot explain the enhanced response to RNAi by feeding seen in *itr-1*, and *egl-8* lof mutants. Therefore whilst it is important to consider the effect which defection and growth phenotypes may have, when combined with the reduced feeding seen in *itr-1* mutants, and more mildly in *egl-8* mutants the dosage of dsRNA to which *egl-8* and *itr-1* mutants are likely to be exposed to remains lower than would be expected for e WT animal. Furthermore changes in feeding, defecation or environmental uptake of dsRNA from the gut are clearly insufficient to explain the enhanced RNAi phenotype of *egl-8* and *itr-1* mutants since this is also seen in response to other methods of induction, such as transgene expression.

SID-2 is a dsRNA receptor found in membranes facing the intestinal lumen. It is only required for the initiation of RNAi by feeding where is actively transports long dsRNAs from the lumen to the intestinal cells. (Hunter et al., 2006; McEwan et al., 2012; Winston et al., 2007). SID-2 dependent uptake is dependent on the PH of the lumen and is likely by endocytosis. Increased expression of SID-2 would increase uptake and therefore increase the response to RNAi by feeding. However, a change in SID-2 expression would not explain the increased response of IP_3 and *egl-8* mutants, and decreased response of *ipp-5* mutants to dsRNA introduced by injection or transgene expression. This phenomenon cannot then be explained as a simple dosage effect.

Efficient systemic response by any method of induction requires export from the donor cell, stable transport through the pseudocoelom and import to the recipient cell. Changes to any one of these stages could alter the dosage of RNA trigger received. The most likely conduit for the spread of RNA species around *C. elegans* is the pseudocoelom or PC, a fluid filled intercellular space. The relatively low abundance of initial trigger needed and dependence on amplification and secondary siRNA production for a strong response might logically lead to the suggestion that siRNA species might be transported. However, experimental evidence points instead to dsRNAs being the main mobile species in *C. elegans*, with amplification occurring as a downstream event within the recipient cell where silencing is occurring.

By studying the silencing response in different RNAi mutants with mosaic rescue, Jose, Garcia and Hunter (Jose et al., 2011) found that long dsRNA and at least one other species of dsRNA is transported intercellularly. This second species is an intermediate in the production of the mature siRNA, having already been processed by DCR-1 (dicer), the long dsRNA binding protein RDE-4 and the nucleotidyl transferase MUT-2/RDE-3 before export, bypassing the requirement for these proteins in the receiving cell (Jose et al., 2011). RDE-4 is required for recruitment of dicer whilst, MUT-2 modifies the RNA. The RDE-4 and MUT-2/RDE-3 mutants display an interesting third tissue rescue phenotype, whereby rescuing expression in tissues containing neither the donor not receiving cell can rescue the defects in the RNAi pathway. Presumably this is mediated by the export of dsRNA from the donor cell, import and processing into short dsRNA in the tertiary cell, re-export and import into the recipient cell to initiate silencing. The ways in which RNAs are recruited to become mobile RNAs and if they are specifically modified for this purpose are unknown.

Efficient delivery of sufficient dsRNA trigger to each cell is therefore an important step in initiating robust silencing. Mutants which cause changes to export from the donor cells or uptake into the recipient cell alter the efficacy of the systemic RNAi response. One key protein for an efficient systemic RNAi response in *C. elegans*, regardless of the initiation method, is SID-1. SID-1 is

an integral membrane channel, strongly selective for dsRNA. The selectivity of SID-1 for dsRNA supports the finding that transport of single stranded small RNA species, such as the active siRISC bound siRNA or secondary siRNAs, is unlikely to be contribute to the systemic RNAi response in *C. elegans.* SID-1 is not required for export of dsRNA from donor cells into the pseudocoelom but is required for entry into the recipient cells, an in particular entry into the cytosol (either from vesicles or external). Evidence of a length bias in SID-1 transport is mixed, with some studies finding some selectivity for longer dsRNAs whilst other studies suggest no selectivity, and even a faster rate of shorter dsRNA transport in vitro (Jose et al., 2009; Li et al., 2015; Shih et al., 2009; Winston et al., 2002). Loss of function of SID-1 leads to a complete loss of systemic RNAi. SID-1 is expressed in the PM of most cells in *C. elegans* with the notable exception of neurones. This lack of SID-1 expression is a limiting factor in the RNAi response to neuronal genes, since overexpression of SID-1 in neuronal cells can overcome the natural tendency of neurones to be refractory to RNAi (Calixto et al., 2010). IP_3 signalling mutants also display enhanced response to neuronal RNAi, however this phenotype is also seen in the classical Eri mutants. Regardless of the low or absent SID-1 expression in neurones some dsRNA must be able to enter cells and initiate a response in mutants where cells are more sensitive. Whist SID-1 expression is important for an effective systemic RNAi response SID-1 transport from the PC directly into the cytosol is not the only dsRNA import pathway. Changes to SID-1 expression are therefore one way in which sensitivity to RNAi can be altered. Interestingly enhancing SID-1 expression in neurones was found to cause a reduced RNAi phenotype more generally. This is thought to be due to the increase uptake RNA into cells overexpressing SID acting as a sink for dsRNA, reducing the availability of dsRNA to be taken up by other cells (Calixto et al., 2010).

Studies of the requirements of SID-1 in parental RNAi demonstrate multiple functional roles. In the embryo, the yolk protein receptor RME-2 functions in dsRNA import, with RAB-7, SID-5 and SID-1 needed for trafficking and release into the cytosol from the endosome (Wang and Hunter, 2017).

Trafficking and localisation within the endomembrane system is clearly an important part of the RNAi response. RSD-3 is an epsinR homologue which localises to the trans-golgi network and is thought to be required for endocytosis of dsRNAs via clathrin coated vesicles (Imae et al., 2016). SID-5 seems to be expressed in all tissues where it localises to the late endosome. SID-5 is required for export of dsRNAs from donor cells and it is also hypothesised to mediate the release of RNA from endosomes into the cytosol (Hinas et al., 2012). Loss of SID-5 leads to loss of the systemic RNAi response whilst loss of the endomembrane protein SEC-22 leads to an enhanced RNAi response (Zhao et al., 2017). SID-3 is an effector protein Kinase, homologue of mammalian ACK1. Like ACK1 in mammals, SID-3 regulates actin dynamics downstream of CDC42 (Lažetić et al., 2018).

How dsRNAs are recruited or modified to become mobile is poorly understood, as is the way in which they are transported between cells. Changes to the efficiency of transport of the mobile dsRNA responsible for the spread of silencing is therefore a possible way in which the systemic RNAi response could be modulated. dsRNAs appear to be stable in the extracellular environment of the pseudocoelom but how this is achieved is unknown. Regulation of the stability of mobile dsRNAs is another possible point of control which could be influenced by IP_3 signalling.

6.2.iii Changes to the RNAi sensitivity at the level of the effector cell

Many of the proteins identified from mutant screens for enhanced RNAi- the classical Eri mutants - are now thought to form part of the ERI complex, the core set of biogenesis factors required for 26G siRNA production. Whilst some ERI complex components are required for both endogenous and exogenous small RNA production such as DCR-1, DRH-3 and RDE-4, other components including the RdRP RRF-3, exonuclease ERI-1 and Tudor domain proteins ERI-5 and ERI-3 are only required in endo- siRNA production (Pavelec et al., 2009). Loss of function of these components compromises the endogenous siRNA pathway but leads to an enhanced sensitivity to exogenous dsRNA. The competition model postulates that the lack of, for example RRF-3, makes components such as DRH-3, DCR-1, and RDE-4 more available to process exo-dsRNA. This suggests a finely balanced antagonism between the endogenous and exogenous RNAi pathways where influencing the availability of siRNA biogenesis factors could regulate efficiency of both pathway (Zhuang and Hunter, 2012). 22G siRNAs produced downstream of both the endogenous and exogenous RNAi pathways are also likely to compete for binding to secondary argonautes. Competition also differs between tissues. Of the *eri-1, rrf-3, eri-3, eri-5, eri-6/7, eri-8, eri-9* and *eri-11* mutants, not all show an enhanced RNAi phenotype in all tissues and the degree of enhancement differs by both strain and tissue. Interestingly all 8 of these mutants show strong somatic maternal rescue of the Eri phenotype in response to *dpy-13, unc-73* and *hmr-1* RNAi, but not of the spermatogenesis defects shared by *eri-1, rrf-3* and *eri-3* mutants (Zhuang and Hunter, 2011). The ability for Eri defects to be maternally rescued demonstrates that either the maternal contribution to the embryo includes either the Eri components themselves or the 26G siRNAs in sufficient quantities to compete with the exo-siRNA pathway and restore the balance of competition to WT levels.

Beyond its role in the ERI complex the RNA exonuclease Eri-1 is thought to further antagonise the exogenous RNAi pathway by degrading exo-siRNAs and miRNAs. Eri-1 is widely expressed but is particularly strongly expressed in the gonad and in neurones, where RNA degradation is thought to

contribute to the low sensitivity of these cells to RNAi (Kennedy et al., 2004). Reduction in Eri-1 is another way in which RNAi sensitivity can be increased (Jadiya and Nazir, 2014).

Following primary siRNA biogenesis, the amplification of silencing through the production of secondary siRNAs is a further point of potential regulation. Mutants including RDE-10, RDE-11, RSD-6, RSD-4 and HAF-6 are compromised in this stage and exhibit a far weaker RNAi response. RSD-6 and RSD-4 were originally thought to be defective in the spread of silencing due to their ability to respond to RNAi by injection but not to feeding. However, a better understanding of the stages in the RNAi pathway has allowed this to be refined. Mutants defective in the amplification of silencing are can be described as dosage sensitive since the much higher dosage of dsRNA provided by injection rather than feeding allows these silencing defects to be overcome (Han et al., 2008; Zhang et al., 2012).

A further set of enhanced RNAi mutants belong to the synMUVB class of mutants and form part of the Rb complex (Ceron et al., 2007; Lehner et al., 2006). These include *lin-35/Rb* and *lin-15b*. The Rb complex is a transcriptional repressor but the precise mechanisms by which *lin-35/Rb* regulates RNAi is still poorly understood. In part it may be due to the misregualtion of normally germline restricted genes for RNAi such as the RdRP *ego-1* (Wu et al., 2012). EGO-1 functions in place of RRF-1 in the RdRP module for secondary siRNA production in the germline, possibly allowing for increased production of secondary siRNAs in the soma of Lin-35 mutants. Consistent with this the Nuclear RNAi response which is dependent of secondary siRNA production is enhanced in LIN-35 mutants. The time sensitive window during which dsRNA exposure is needed to trigger nuclear RNAi is also relaxed in LIN-35 mutants (Shiu and Hunter, 2017). Similarly to the classical Eri mutants, maternal rescue of *lin-35/Rb* reduces RNAi hypersensitivity (Massirer et al., 2012).

In the analysis of the *itr-1* small RNA seq library I identified the *miR-35-41* miRNA family as mildly enhanced compared to N2 worms. *miR-35-41* regulates *lin-35* expression and deletion of *mir-35-41* results in an enhanced RNAi phenotype (Massirer et al., 2012). This therefore suggested a possible link between IP_3 signalling and the regulation of the exo-RNAi response via the *miR-35-41/lin-35* pathway. However, qPCR failed to detect and changes in *lin-35* transcript levels and whilst there are a number of reasons why this could be the case (as discussed in chapter 4) the endomitotic phenotype of *itr-1* mutants is a more likely source of the changes in miR-35-41 expression seen. IP_3 signalling cannot be acting via the same route as either Rb complex mutants (*lin-35, lin-15b*) or *eri-1* to regulate the RNAi response, since the very strongly enhanced RNAi response of the *eri-1(mg366); lin-15b(n744)* double mutant is further enhanced in *the eri-1(mg366); lin-15b(n744); egl-8(e2917)* triple mutant. Therefore, IP_3 signalling must be regulating RNAi sensitivity by another mechanism.

As already discussed the lack of any significant changes to endo-siRNAs profile detected seen in the small RNA seq experiment make it unlikely that IP_3 signalling down regulates the endo siRNA pathway, increasing availability of resources to be redirected to the exo-siRNA pathway. The up regulation of secondary siRNA production or the up regulation of core components of the exo-RNAi pathway in response to reduced IP_3 signalling both remain possibilities. As discussed, this could be tested by the sequencing and comparison of 5' dependent and 5' independent small RNA libraries from WT and IP_3 signalling mutant worms treated with RNAi against an endogenous target gene.

Correct trafficking on dsRNAs within the endomembrane system is likely to be important for both the export of mobile dsRNA and the import of dsRNAs for siRNA production. Correct sub-cellular localisation of siRNAs and components of the siRICS is also is also likely to be important for an efficient silencing response, although much about the subcellular localisation of siRNA processing remains poorly understood. In mammalian cells the Rough ER has been shown by Stalder et al. to be the major site of siRISC loading and silencing. They also showed that whilst subcellular organisation is important for the efficiency of RNAi response it is not essential for silencing- disruption of loading of the siRISC at rER by depletion of components of the RISC-loading complex makes the slows silencing, as does nocodazole treatment. Whilst perturbation of ante-retrograde transport with brefeldin A leads to accumulation of products in ER and increased RNAi efficiency (Stalder et al., 2013). The ER is also the largest store of intracellular calcium, and therefore the location of many calcium channels including the IP_3 receptor. What is more, the dynamic structure of the ER is a key aspect of cell function, changing with cell state and in response to intracellular signals, including in response to calcium signalling (reviewed (Schwarz and Blower, 2016)).

The insulin-like signalling pathway regulates response to stress and longevity in *C.* elegans via the regulation of transcription factor DAF-16. Loss of function of the insulin-like receptor DAF-2 or the downstream PI3-kinase AGE-1 results in an enhanced RNAi response which is not seen in the absence of DAF-16 function (Wang and Ruvkun, 2004). How the many DAF-16 regulated changes in gene expression lead to changes in RNAi response is unknown, however since DAF-16 regulated processes include the dauer response which is dependent on the endogenous siRNA pathway, this regulation may not be specific to the exo-RNAi response (Bharadwaj and Hall, 2017).

6.2.iv Role of the intestine in regulating RNAi

Despite its small cell number, the *C. elegans* intestine is not merely a primitive digestive system but a complex factory of many overlapping roles. These include acting as a protein factory for the production and export of yolk proteins for the oocytes, as a signalling hub for the regulation of various physiological processes, and as the interface between the worm and the surrounding world. All three of the *Caenorhabditis* virus so far discovered target intestinal cells (Félix and Wang, 2019).

Rescuing IP_3 signalling in the intestine by expression of WT *itr-1* from the well characterised intestinal specific promoter *vha-6p* is sufficient to rescue the RNAi phenotype systemically, restoring the WT RNAi response in other tissues. This is seen not only in response to RNAi by feeding but also in response to dsRNAs produced intracellularly. Complete rescue of the RNAi response was demonstrated for GFP silencing in neurones, in response to RNAi by feeding and in both the BWM and the pharynx, in a transgenic system whereby dsRNA is produced from a transgene in the pharynx, and partially rescue was seen in response to *lin-1* RNAi by feeding.

Therefore, the best evidence for the site of action of the IP_3 signalling pathway regulating RNAi sensitivity is that it is acting non-cell autonomously in the intestine. Since the effects of *itr-1, egl-8* and *ipp-5* mutants are seen not only in response to RNAi by feeding, these effects cannot be explained by the regulation of the import of dsRNA from the intestine. However, several other possibilities remain open. I propose four hypotheses for how IP_3 signalling in the intestine could be exerting a systemic effect on RNAi.

6.2.v Hypotheses for the role of IP_3 signalling in the regulation of RNAi

1- Oocyte provisioning

Given the role of the intestine in the production of yolk proteins for oocyte provisioning, the role demonstrated for the yolk protein receptor RME-2 in the entry of dsRNAs into the embryo, and the maternal rescue phenotype seen in several Eri mutants, the possibility that IP_3 signalling could be regulating the RNAi response via oocyte provision should be considered. Changes to maternal factors -the proteins or RNAs produced in the intestine and imported into the embryo- could lead to changes in the expression of components of the exo-RNAi pathway in the next generation, altering the sensitivity of the cells to respond to dsRNA. In this case *itr-1*, *egl-8* and *ipp-5* mutants would be acting as maternal effect mutants. This could be confirmed by maternal rescue experiments, involving crossing WT and IP_3 signalling mutants. The temperature sensitive mutant *itr-1(sa73)* could

also be utilised be used If signalling occurs in the parent then embryos laid at 16 but raised at 20 will show a WT response.

2- Intestinal sink

Enhancing *sid*-1 expression in neurones was found to cause a reduced RNAi phenotype more generally. This is thought to be due to the increase uptake RNA into cells overexpressing SID acting as a sink for dsRNA, reducing the availability of dsRNA to be taken up by other cells (Calixto et al., 2010). Could IP_3 signalling in the intestine lead to changes in the expression of *sid-1* which reduce or increase the availability of mobile dsRNAs for uptake by other cells? If this were the case changes to RNAi sensitivity in the intestine would show the opposite pattern to other tissues. This hypothesis is easily tested by assaying the response of i*tr-1, egl-8, ipp-5,* and *goa-1* mutants in response to RNAi of intestinal gene targets such as *act-5, gtl-1,* and *ife-2*.

3- Changes to the stability or transport of mobile RNAs

Very little is known about factors which affect the stability of mobile dsRNA in the extracellular environment of the pseudocoelom, however the stability of these mobile RNAs is crucial for an effective systemic RNAi response. It is conceivable that IP_3 signalling in the intestine could regulate the secretion of RNA binding proteins, or RNases which influence the stability of mobile RNAs and therefore the systemic RNAi response. If such stability factors are factors are required for the efficient transport of mobile RNAs through the extracellular space then I argue that the unique position of the intestine as the gateway of the environmental RNAi response would make this the most likely tissue to produce such factors.

In this hypothesis the enhanced RNAi phenotype of *itr-1, egl-8* and *goa-1* mutants is entirely mediated via changes to the systemic transport of mobile RNAs. Counter to this idea is the observation that changes to GFP silencing in the pharynx of *itr-1* and *ipp-5* mutants is also seen when silencing is induced by dsRNAs expressed in that same tissue. In this scenario a substantial proportion of the dsRNA produced would have to be exported to the pseudocoelom and reimported into cells of the same tissue, in order for changes to mobile RNA transport to influence silencing. However, there are reasons to think that this may be the case. The ability of silencing to be rescued in *rde-4* and *mut-2* mutants by expression in a third tissue demonstrates that there must be sufficient long dsRNA exported from the donor cells, imported into the third tissue for processing by dicer, re-exported as short dsRNAs and imported into the receiving cell in sufficient quantities to compensate for a lack of primary exo-siRNA production in the donor and recipient cells. This implies a very active flow mobile RNAs between cells, and there is no a priori reason to assume that export of dsRNAs

should be any less prevalent when dsRNA is produced in the same cell as the target gene as in other scenarios.

The hypothesis that IP_3 signalling is regulating changes to the stability or systemic transport of mobile RNAs could be tested in *sid-1* mutant background. If silencing of GFP in the pharynx in response to dsRNAs expressed in the same tissue is still enhanced in *itr-1* mutants in a *sid-1* null background, when no systemic spread of mobile RNAs can occur then IP_3 signalling is not regulating the RNAi response via changes to the systemic spread of mobile RNAs.

4- Humoral signal altering RNAi sensitivity in distal cells

Arguably the most likely scenario for how IP_3 signalling in the intestine could alter the RNAi sensitivity of cells in other tissues is via the production of a humoral signal. In response to this signal the RNAi sensitivity of receiving cells could be altered in a number of ways; changes to degradation or intracellular trafficking of exo-siRNAs, or dsRNA intermediates, changes to availability of core component of the exo-RNAi pathway involved in primary or secondary siRNA production. For example the enhanced RNAi phenotype of Eri mutants such as RRF-3 suggests that components of the RdRP module responsible for secondary siRNA are a limited resource, increased expression of the RdRP module would therefore be expected to enhance production of secondary exo-siRNAs without compromising the endogenous siRNA pathway. Such a model is consistent with the changes seen in the nuclear RNAi response of IP_3 signalling mutants. In this model it is possible that g*oa-1* is acting at the level of the receiving cell to regulate the response to the humoral signal.

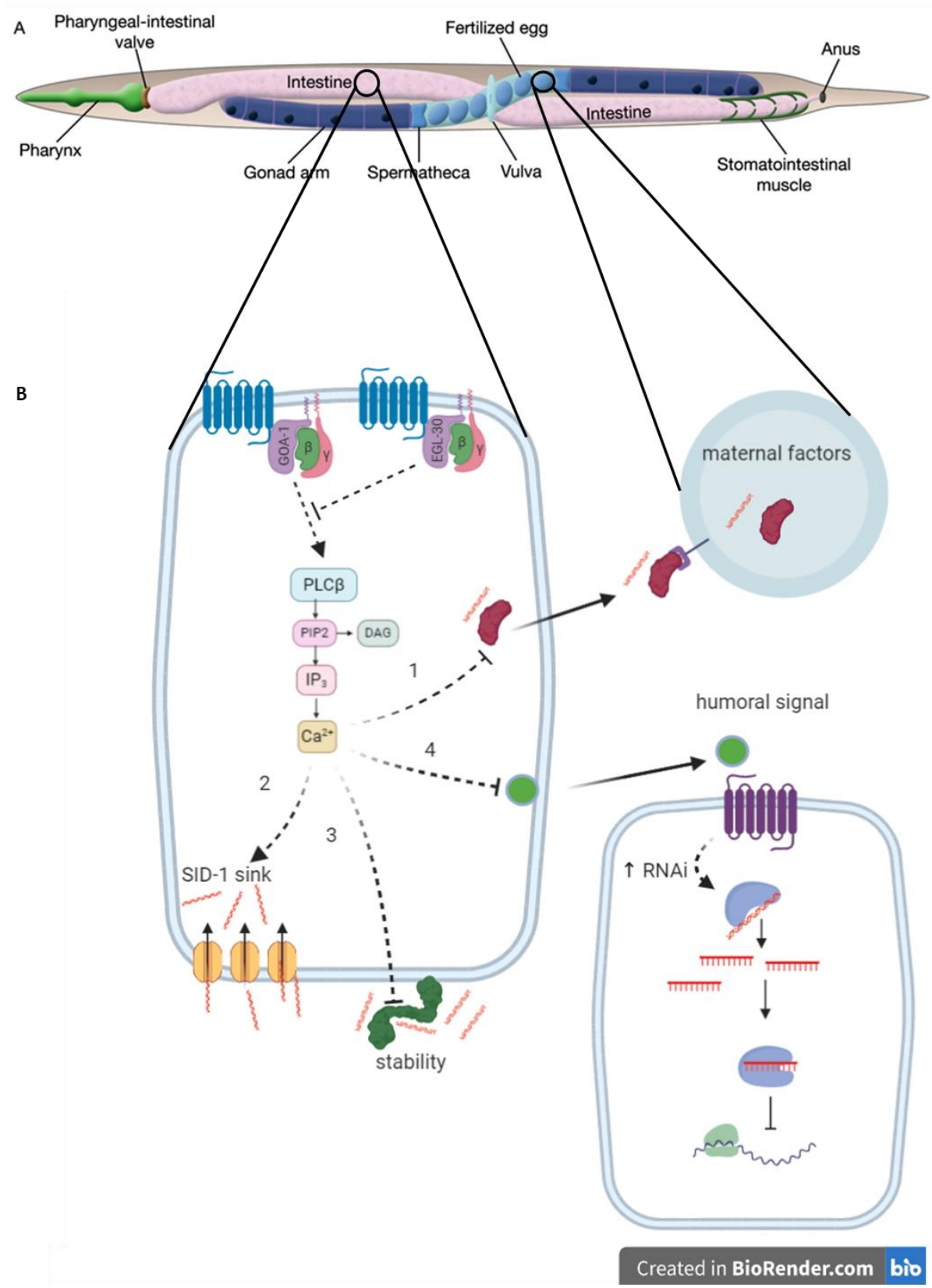


Figure 6.3. Possible models for the regulation of RNAi sensitivity by IP_3 signalling in *C. elegans*.
A- The anatomy of the alimentary canal and germline. Image Modified from Worm Atlas (wormatlas.org). **B-** Signalling in the intestine influences RNAi sensitivity in the other tissues. This could be achieved by changes in efficiency of transport of mobile dsRNAs (2,3), or changes to the sensitivity of the receiving cell either in response to a humoral signal or changes in maternal factors (1,4).

6.3 Beyond *C. elegans* - Cell signalling regulating RNAi pathways

A growing body of evidence form studies in mammalian and other in neuronal systems suggests that the rapid regulation of miRNA levels is a key mechanism in neuronal plasticity. In clear examples of cell signalling pathways altering gene expression via miRNA pathways, several different studies have found that neuronal stimulation via diverse extracellular signals such as Serotonin, NmDA, mGluR and BDNF can lead to rapid degradation of specific miRNAs, reversing transcriptional silencing and altering gene expression (reviewed (Fu et al., 2016)). Fu, Shah and Baraban suggest that the miRNA system in itself could be thought of as a second messenger system, responding to extracellular stimuli to regulate gene expression. This intriguing idea towards a more generalised and complex view of the cellular signalling pathways, reflecting the reactive and dynamic role small RNA pathway seem to play in regulating gene expression.

Brain derived neurotrophic-factor (BDNF) is a widely expressed secreted protein important for healthy brain function, including differentiation and neuronal plasticity. BDNF stimulation of neurones in the hippocampus leads to BDNF dependent aborization via specific changes in gene expression to allow dendrite arborization which are regulated by rapid changes in miRNA activity. BDNF, signalling via a RTK/ MAPK signalling pathway regulates miRNA activity in two ways. Increased expression of miRNA maturation factors, dicer and TRBP, both part of the risc loading complex acts non-specifically to increase global miRNA production, thereby repressing the expression of a broad set of genes. This is accompanied by the simultaneous activation of the lin-28 pathway, leading to the specific degradation of let-7 family miRNAs and therefore increase translation of let-7 target transcripts. Combination of global changes via altering dicer expression and specific changes. (Huang et al., 2012).

The process of long-term potentiation (LTP) in neurones, a form of synaptic plasticity, is known to be highly dependent on IP_3 and Ca^{2+} signalling events. The NMDA receptor is a ligand gated calcium channel and a major mediator of excitatory neurotransmission, whilst the mGluR receptor family are glutamate stimulated GPCRs, which can regulate calcium signalling via both Gαq activation of PLCβ and via interactions with NMDAR via a protein scaffold (reviewed (Willard and Koochekpour, 2013)) . Studies of LTP in the rat hippocampus found that activation of the NMDAR bidirectionally regulates changes to miRNAs associated with the Ago2-RISC. NMDA signalling promotes decay of some mature miRNAs, whilst other miRNAs show increased expression and increased association with Ago2. Group 1 mGluR signalling was also shown to alter miRNA expression during LTP. Expression of the primary miRNAs corresponding to the mature miRNAs which are downregulated in response to NMDAR signalling are upregulated by group 1 mGluR signalling (Pai et al., 2014; Wibrand

et al., 2010). The direct mechanisms of this regulation are unknown, although NMDAR signalling has also been shown to alter P-body localisation and composition. Interestingly blockade of NMDAR signalling is known to increase expression of the IP_3 receptor in hippocampal neurones (Cai et al., 2004), whilst group 1 mGluR receptors activate the Gαq/PLCβ/IP_3 signalling cascade. A role for IP_3 signalling in the regulation of miRNA expression in mammalian neurones is therefore likely.

One of the mechanisms through which signalling pathways have been shown to regulate small RNA levels in neuronal cells is via the translin/TRAX complex, or C3PO. C3PO is an octameric RNase involved in small RNA processing. Both the function and the constituent components, translin and closely related protein TRAX, of C3PO appear to be well conserved with homologues identified in yeast, flies and humans. In human cell lines and *D. melanogaster* translin/TRAX increases the rate of siRNA passenger strand degradation, promoting the formation of the active RISC in the exo-RNAi pathway (Liu et al., 2009). In human cell lines translin/TRAX can also degrade pre-miRNAs by targeting mismatched stem bulges for cleavage (Asada et al., 2014), whilst in *D. melanogaster* C3PO also functions in passenger strand cleavage in the endo-siRNA pathway (Liu et al., 2019).

Very interestingly a direct interaction between PLCβ and C3PO has been shown by to be involved in the regulation of both the exo-siRNA and miRNA pathways in mammalian neuronal cell lines (Aisiku et al., 2010; Philip et al., 2012, 2013). In mammalian systems there are 4 PLCβ genes, with further alternative isoforms. Whilst Gαq localisation appears to be limited to the PM, together with the majority of PLCβ, a significant proportion of PLCβ is also localised to the cytosol and the nucleus. In an attempt to identify potential alternative activators or biding partners of cytosolic PLCβ Aisiku, Runnels and Scarlata carried out a yeast two hybrid screen using the C terminal helical domain of PLCβ1 as bait. This screen identified C3PO component TRAX as a high affinity binding partner of PLCβ1, an association verified in vivo, where cytosolic PLCB colocalises with C3PO (Aisiku et al., 2010). Furthermore, TRAX and competes with Gαq for binding of PLCβ. Overexpression of TRAX leads to a reduction Gαq/ PLCβ signalling as demonstrated by a reduction in both PIP2 hydrolysis and Ca^{2+} signalling following stimulation. Conversely overexpression of Gαq leads to a reduction in cytosolic PLCβ expression. The result of PLCβ interaction with C3PO is to reduce the rate of hydrolysis of the passenger strand of exo-siRNAs, leading to a reduction in siRNA activity. This reduction in catalytic activity appears to be specific to rapidly hydrolysed siRNA, with changes in Gαq/PLCβ signalling only affecting the RNAi of some genes (Philip et al., 2012, 2016; Sahu et al., 2014). PLCβ regulation of trax also has endogenous roles, regulating the expression of some miRNAs and in the regulation of cell cycle progression. In PC12 cells PLCβ/TRAX was found to associated with different CDKs at different points of cell cycle to regulate progression (Scarlata et al., 2016, 2018). A direct role for PLCβ1 and PKCα in the control of erythropoiesis via the regulation of miR210 levels has

also been demonstrated. MTH induced erythroid differentiation is inhibited by enhanced PLCβ signalling or decreased PKCα, leading to a loss of miR-210 expression via an unknown mechanism (Bavelloni et al., 2014).

Whilst this connection between G-protein signalling, PLCβ and RNAi is intriguing, neither components of the C3PO heteromer, TRAX and translin appear to be present in *C. elegans*. Despite a seemingly well conserved role for the translin/TRAX (C3PO) complexes from human to drosophila no role for translin/TRAX complexes in RNAi has been reported in *C. elegans* and neither translin nor TRAX homologues have been identified in the *C. elegans* genome. Consistent with this, I carried out blast searches which failed to find a likely homologue despite translin/TRAX homologues being present in many non-mammalian lineages, including other nematodes. Further, in this system the binding of PLCβ to TRAX in the cytosol, rather than to G-proteins at cell membrane, would lead to a reduction in both IP_3 production and the RNAi response, the opposite of the pattern seen in *C. elegans.*

Despite the apparent lack of direct translatability of this mechanism of regulation to *C. elegans*, this does highlight the potential role of non-canonical interactions in the regulation of cell signalling or RNAi. In particular, the competition for binding of PLCβ between Gαq and TRAX seen here leads to PLCβ and its canonical activator Gαq acting in opposition in the regulation of RNAi. In the case of PLCβ/TRAX signalling is not occurring via IP_3 production, however this none the less demonstrates that the interactions between Gαq and PLCβ are not limited to the roles of activator and effector but can and are utilised in more complex systems of regulation. Could a similar competitive binding model account for the difference in the roles of *egl-8* and *egl-30* in RNAi regulation in C. elegans? In such a model *goa-1* or the GBγ subunit from a *goa-1* heterotrimeric G-protein could directly compete with *egl-30* for the binding and activation of *egl-8*.

In plants, a number of cellular regulators of RNAi have been identified including the conserved protein AtRLI2, and calmodulin-like protein rgs-CaM (regulator of gene silencing–calmodulin-like protein). AtRLI2 and its mammalian homologue ABCE1 (ATP-binding cassette sub-family E member 1) have multiple conserved functions in RNA processing and also both act as suppressors of RNAi. Furthermore, overexpression of human ABCE1 from the *myo-3* promoter in *C. elegans* inhibits GFP RNAi in the BWM (Kärblane et al., 2015). Several virally encoded suppressors of PTGS have been identified in plants. A search for plant proteins which interact with one such suppressor identified the Calmodulin related proteins rgs-CaM, and rgs-CaM itself was found to act acts as a suppressor of RNAi (Anandalakshmi et al., 2000). As a calmodulin-like proteins rgs-CaM

contains multiple EF-hand calcium binding domains, demonstrating a direct link between calcium signalling and control of RNAi in plants.

6.2.4 Why regulate the RNAi response?

Whether by the production of a humoral signal to regulate the RNAi response throughout the organism, or by an alternative mechanism, such a change would be expected to be carefully regulated and could be acting in response to physiological or environmental factors. However, speculation of what such an upstream stimulus is likely to be is hampered by the poor understanding of the endogenous roles of the exogenous RNAi response. The RNAi response is an important antiviral defence, not only in *C. elegans* but in plants and other animals. In *C. elegans* mammalian RIG-1 homologue DRH-1 is essential for initiation of the antiviral response but is dispensable for RNAi (Ashe et al., 2013). This implies that there may be endogenous roles of exo-RNAi beyond an antiviral response. Environmental RNAi is clearly not an accidental side-effect of the systemic transport of endogenous dsRNAs, since it relies on the expression and localisation of the specialised dsRNA transported SID-2 in cell membranes facing the intestinal lumen. Understanding the reasons for environmental RNAi may shed light on why and when regulation of the RNAi response is required.

There has been speculation about the potential for silencing RNA species themselves to act as messengers at the intracellular, intercellular, and even transgenerational level. The systemic nature of the RNAi response allows dsRNAs to transmit information about the state of one cell to, other cells in the organism, where it influences the expression of genes in that cell in response – essentially acting as a humoral signal. miRNAs have been shown to act as intercellular signals in plants and the detection miRNAs in extracellular fluids in mammals, has led to the suggestion that they may also do so in animals. As with other signals there must be careful regulation not only at the level of the producing cell but at the level of the receiving cell, which must be competent to interpret that signal and respond correctly.

In conclusion the negative regulation of an IP_3 signalling pathway in *C. elegans* results in a systemic enhancement of RNAi response, whilst increased IP_3 signalling results in resistance to RNAi. This response appears to be specific to the exogenous RNAi pathway since no changes were detected in the endogenous small RNA profiles of *itr-1* mutants. Counter to the canonical model of PLCβ *egl-8* activation by $G\alpha_q$ homologue *egl-30*, the $G\alpha_{i/o}$ homologue *goa-1* is proposed to act as the upstream activator of PLCβ *egl-8,* with possible negative regulation by *egl-30*. This signalling pathway most likely acts in the intestine to regulate RNAi sensitivity systemically, either through changes tot eh

efficiency of transport of mobile RNAs or through changes to sensitivity of distal cells to these mobile RNAs, either in response to a humoral signal in the adult worm or though changes in gene expression in the adult worm resulting from maternal factors.

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