**C. ELEGANS MAINTENANCE**

C elegans strains were maintained at 20C on nematode growth media (NGM) seeded with OP50 bacteria (Stiernagle2006). Strains are listed in table x

**RNA INTERFERENCE**

Experiments:

* Par-2 rundowns
* PH rundowns
* PAR-2 and PAR-6 rundowns looking at PH ring fragment

RNAi was performed using the feeding method described in Kamath2003. Bacterial feeding clones were grown in LB liquid culture with ampicillin (50ug/mL) for 16 hours at 37C in a shaking incubator. dsRNA expression was then induced with IPTG (5mM), and 150mL bacteria were struck onto 60mm NGM agar plates, which were then incubated at room temperature for 24 hours. To obtain complete gene depletions, L4 worms were added to plates and incubated at 20C for 24 hours before imaging. To obtain graded depletions, adult/L4 worms were left on plates for 0-24 hours.

**CRISPR**

**PAR-2 point mutants**

PAR-2 point mutations (AxA, R163A, R183-5A, C56S, K351A, K94A, L50R and L109R) were carried out by CRISPR. In general, this process involved designing CRISPR guides to target the site of the mutation, designing a repair template to add the point mutation, injecting, screening progeny by PCR and sequencing.

Guide RNAs were identified using the CRISPOR tool (Concordet 2018). Two guides close to the target site were selected for each mutation, and ordered from IDT as Alt-R crRNA. Point mutations were incorporated into 200 bp repair templates homologous to the target region, which were ordered as ultramer oligomers from IDT. To aid screening, silent restriction sites were also added to each repair template close to the target site. CRISPR target sites were also silently mutated to prevent repaired sequences from being re-targeted, either through silent mutation to the PAM sites, or a series of silent mutations to the guide target sites.

crRNA guides were annealed with tracrRNA (IDT) by combining 0.5 uL tracrRNA (4 mg/ml) with 2.75 uL guide and 2.75 uL duplex buffer (IDT), and incubating at 95C for 5 minutes. 20 ul of injection mix was prepared containing annealed crRNA, the repair template and Cas9 enzyme (recipe in table x). Injection mixes also contained constructs for dpy-10 co-CRISPR using the strategy described in Arribere2014, which is designed to produce a dominant roller phenotype following successful CRISPR. Injection mixes were incubated at 37C for 10 minutes and centrifuged at 14,000 rpm for 10 minutes. The gonads of 10-20 young adult worms were injected, and worms were left at 20C.

Approximately 50 F1 roller worms were singled from each set of injections, left for 24 hours to lay eggs, and screened by single worm PCR using appropriate primers and restriction digest using the appropriate restriction enzyme. Candidate heterozygous lines could be identified based on the cutting pattern of the PCR product. F2 worms were then screened in a similar way to test for homozygosity, and homozygous worms sequenced to confirm incorporation of the intended DNA sequence. Up to two independent lines were saved for each mutant.

I designed constructs and performed CRISPR for four point mutations (K351A, K94A, L50R and L109R), and performed CRISPR for one other with constructs designed by Nisha Hirani (C56S). Other mutants were designed and performed by Nisha Hirani (AxA, R163A, R183-5A). A summary of the reagents used for each mutant is shown in table x. Guide RNA sequences are shown in table x. Repair template sequences are shown in table x. PCR screening primers are shown in table x.

**Mos site insertions**

Mitochondrial GBP

I codon optimised amino acids 1 to 55 from the C. elegans tomm-20 gene, corrected for piRNA sites using the online pirScan tool (Wu2018) and ordered as a gBlock from IDT. I PCR amplified the sequence, and cloned it into plasmid pNG0019, previously used to construct PH::GBP::mKate2 (Rodriguez2017), replacing the PH plasma membrane localisation signal with TOMM-20(1-55). I inserted the resulting plasmid (pTB1) at the ttTi5605 mos1 site locus of HT1593 unc-119(ed3) worms by CRISPR, using the strategy described by Dickinson2013.

Aiming to create an untagged version, I initially repeated the above procedure, cloning the TOMM20 1-55 sequence into the pNG0020 vector (PH::GBP), again removing PH and replacing with TOMM20 1-55, yielding the plamid pTB2 (TOMM20::GBP). Unfortunately, this construct failed to express in vivo, likely due to germline silencing. I hypothesised that the mKate sequence might prevent germline silencing in the full construct, possibly due the inclusion of introns. Therefore, as an alternative strategy to create an untagged line, I aimed to mutate the chromophore region of mKate to remove fluorescent signal (Pletnev2008). To do so, I designed guide RNAs to target the chromophore region of mKate (TB34, TB35), and designed a repair template (TB33) which included a Y65A mutation, changing the chromophore from MYG to MAG. Following the strategy described in section x, I successfully carried out the point mutation, and this was successful in eliminating fluorescence whilst maintaining expression.

Membrane tethered PAR-2 RING fragment

Firstly, I amplified GFP from pDD282 by PCR. Secondly, I codon optimised sequences for PH and PAR-2 (1-177), corrected for piRNAs, ordered PH::PAR-2(1-177) as a gBlock and amplified by PCR. Next, using Gibson assembly, I cloned both products into pRI21, a vector designed for inserting genes at the ttTi5605 mos1 locus under control of a mex-5 promoter and a nmy-2 3’ UTR. I inserted the resulting plasmid (pTB5) into the C. elegans genome by CRISPR.

To express a C56S form of the construct, I performed site directed mutagenesis on pTB5, generating pTB6, which was inserted by CRISPR in the same way.

**glh-1 site insertions**

Germline silencing common limits, or entirely prevents expression of transgenes in early embryos. Recently, Goudeau have developed an expression approach that takes advantage of glh-1, which encodes for a germline helicase protein which is highly expressed in the germline. The method involves inserting coding sequence for a gene of interest at the 3’ end of the endogenous glh-1 gene (immediately prior to the stop codon), preceded by a T2A ribosome skipping sequence. High expression of GLH-1 yields high expression of the protein of interest, and T2A promotes ribosome skipping to separates the protein from GLH-1. This leaves a 17 amino acid scar at the C-terminus of GLH-1, but only a single proline at the N-terminus of the second protein.

To insert sequences at this site, I use the strategy described by Dokshin. This is similar to the approach used in section x, but uses PCR to generate a double-stranded repair template with single-strand overhangs, which allows longer sequences to be inserted. For each insert, this procedure involves generating a PCR product containing the insert sequence (short product), and a separate PCR product containing the insert flanked by additional 100 bp homology arms (long product). The two PCR products are then PCR purified (Qiagen, QIAquick PCR purification kit), mixed in equimolar amounts, denatured at 95C, and annealed by gradually cooled to room temperature. This creates a mixture double stranded molecules, approximately half of which will be short/long molecules with single stranded overhangs. Using the entire reaction mix as a repair template, the rest of the procedure is similar to section x.

To test the potential utility of this method, and to serve as a base for future line generation, I began by inserting an mNG sequence at the glh-1 site. I targeted the 3’ end of glh-1 with guides TB0105 and TB0106. To generate the short repair template product, I designed primers (TB0107 and TB0108) to amplify the mNG sequence from plasmid pDD268 (Dan Dickinson). To promote ribosomal skipping, I included a T2A sequence in the forward primer upstream of mNG. To aid future insertions, I also included INPP4A, an optimised guide RNA sequence (ref), downstream of the mNG sequence in the reverse primer. The long product was generated by amplifying the short product with primers TB0109 and TB0110 adding 100bp homology arms to the forward and reverse primers. The overall insert sequence is shown in figure x.

Following the protocol described above, I successfully inserted T2A::mNG::INPP4A at the glh-1 site. Imaging embryos revealed high expression of mNG, but also revealed puncta in the posterior of embryos representing a small amount of glh-1::mNG fusion protein (fig x). Whilst highly efficient in vitro, 2A peptides have been shown to result in incomplete ribosome skipping in a range of in vivo systems, which can lead to a small amount of unseparated fusion protein (Kim2011). In mouse and HeLa cell lines, the efficiency of ribosome skipping has been shown to increase by including an additional P2A sequence (an alternative ribosome skipping sequence) in tandem with T2A (known as tandem P2A-T2A, or tPT2A), which entirely removes fusion protein expression without affecting overall expression levels (Liu2017, Pan2017).

To test this approach, I inserted a P2A sequence upstream of T2A by CRISPR, using an approach similar to section x, targeting the site with guides TB0122 and TB0123, and using ultramer oligo x as a repair template (made by IDT). After inserting this additional sequence, visible expression of glh-1::mNG fusion protein is lost (fig xB), without any change in overall expression levels (fig xC).

To express N-terminal fusion proteins, the INPP4A site can then be targeted with guide TB0113, which targets the INPP4A site, and sequences inserted via a repair template with 5’ homology to mNG and 3’ homology to the glh-1 3’ UTR (including the stop codon). Long sequences should be inserted using the Dokshin method described above, although shorter sequences may be inserted using an ultramer oligomer repair template.

To express mNG::PAR-2(1-177), I amplified the PAR-2(1-177) sequence from plasmid pTB5 using two sets of primers to create short (TB0115 and TB0144) and long (TB0117 and TB0145) products for the repair template. The sequence was then inserted using the Dokshin protocol described above.

**LIVE IMAGING**

Two methods were used to mount embryos for microscopy. In some experiments, embryos were dissected from adult worms in 5 uL M9 buffer on a coverslip and mounted on 2% agarose pads (Zipperlen2001). In other experiments, embryos were dissected in 8 uL egg buffer, and mounted between a slide and coverslip with 20 um polystyrene beads.

Midplane confocal images were captured using 60x or 100x objective lenses on a Nikon TiE system equipped with an ﻿X-Light V1 spinning disk system (CrestOptics) with 50 um slits, Obis 488/561 nm fiber-coupled diode lasers (Coherent) and an Evolve delta camera (Photometrics). The system was controlled using Metamorph (Molecular Devices) and configured by Cairn Research.

**BROOD SIZE ASSAYS**

Young adult worms were left on individual plates for 5 hours to lay eggs. The worms were then removed from the plates, and the plates were left at 20C. After two days, the number of L4s and unhatched eggs on the plates were counted, and embryonic lethality was calculated as the fraction of total progeny that failed to hatch (i.e. unhatched eggs / (L4s + unhatched eggs)). On the third day, the number of gravid and sterile adults were counted, and adult sterility was calculated as the fraction of sterile adults amongst the total population of adult worms (i.e. sterile / (gravid + sterile)).