A recombineering pipeline for functional genomics applied to *Caenorhabditis elegans*

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We present a new concept in DNA engineering based on a pipeline of serial recombineering steps in liquid culture. This approach is fast, straightforward and facilitates simultaneous processing of multiple samples in parallel. We validated the approach by generating green fluorescent protein (GFP)-tagged transgenes from *Caenorhabditis briggsae* genomic clones in a multistep pipeline that takes only 4 d. The transgenes were engineered with minimal disturbance to the natural genomic context so that the correct level and pattern of expression will be secured after transgenesis. An example transgene for the *C. briggsae* ortholog of *lin-59* was used for ballistic transformation in *Caenorhabditis elegans*. We show that the cross-species transgene is correctly expressed and rescues RNA interference (RNAi)-mediated knockdown of the endogenous *C. elegans* gene. The strategy that we describe adapts the power of recombineering in *Escherichia coli* for fluent DNA engineering to a format that can be directly scaled up for genomic projects.

Genome sequencing and annotation projects define complete lists of protein and RNA components for living systems. They also present the challenge to generate functional information for thousands of previously uncharacterized genes. Protein tagging with fluorescent or affinity tags provides a generic way to describe protein expression and localization patterns, and protein-protein interactions. In yeast, where gene tagging by homologous recombination is straightforward, this approach has been applied on a genome scale[1–4].

In most higher eukaryotes, homologous recombination is too inefficient for large-scale applications. Protein tagging has been typically achieved with cDNA transgenes. cDNA transgenes, however, do not recapitulate endogenous regulation by alternative splicing or *cis* regulatory sequences, and often contain viral or nonspecific promoters, which deliver inappropriately high expression and do not respond to signals. Thus, they do not recapitulate correctly the endogenous regulation, pattern or level of expression.

Constructs based on large genomic clones such as bacterial artificial chromosomes (BACs) can be better transgenes. They can contain all of the regulatory sequences and thus are likely to result in highly accurate expression patterns at physiological levels. Indeed, the use of BAC transgenes in the mouse has been routinely successful for recapitulating endogenous expression levels, patterns and regulation[5].

The development of DNA engineering methods based on homologous recombination in *E. coli*[6–9], termed recombineering[10,11], has simplified the manipulation of large genomic clones and facilitated their application as transgenes.

Recently, mouse BAC transgenes have been used in human tissue-culture cells in combination with RNAi to exploit cross-species sequence differences, facilitating selective knockdown of the endogenous gene[12]. Thus, the 'third allele' presented by the transgene can become the primary expressed allele[13].

Extending this approach to model systems that permit RNAi, such as *C. elegans*, provides a simple platform for protein tagging and functional studies. Owing to its small size and invariant cell lineage, *C. elegans* is an excellent model for protein localization studies using reporter genes. Algorithms have been developed that facilitate automatic four-dimensional lineage tracing of fluorescent signals[14]. Protein purification methods based on generic tandem affinity tags have also been adapted to *C. elegans*[15].

Here we describe an efficient recombineering pipeline for generating tagged transgenes by engineering of *C. briggsae* genomic BAC clones and show that integrative transformation of an example transgene correctly recapitulates the function of its endogenous counterpart. This method can be easily scaled up for parallel processing of multiple genes.

RESULTS

BAC clone mapping

We generated an interactive clone map of the *C. briggsae* BAC library[16], which can be visualized in the Wormbase genome browser (**Supplementary Fig. 1** online). We mapped 2,769 clones, which collectively contain 225 Mbp and represent approximately twofold coverage of the predicted genome size[16]. To confirm that the mapping was correct, we analyzed 30 clones by restriction digest and found that all of them had the expected pattern (data not shown).

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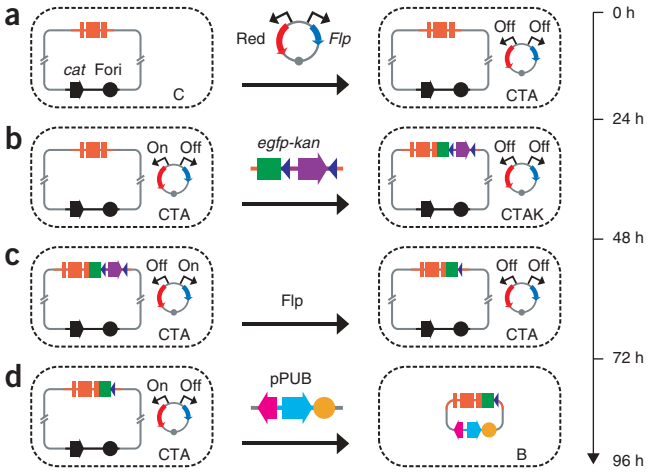

Figure 1 | Recombineering pipeline. A suitable BAC clone for the gene of interest (orange) is chosen and all recombineering steps are done in the original BAC host cells (broken line). (**a**) The host is transformed with the dual expression plasmid pRedFlp. Fori, BAC replication origin; *cat*, chloramphenicol resistance gene. (**b**) Expression of the Red operon (red) is induced with rhamnose and the cells are then electroporated with the *egfp-kan* cassette. (**c**) Expression of Flp recombinase is induced with anhydrotetracycline. Flp binds to the *FRT* sites (blue triangles) and excises the *kan* gene. (**d**) Expression of the Red operon is again induced with rhamnose; the cells are then electroporated with the pPUB subcloning vector, followed by selection for blasticidin, temperature shift to 37 °C and omission of all other antibiotics. The antibiotics included at each stage are indicated: C, chloramphenicol; T, trimethoprim; A, ampicillin; K, kanamycin; B, blasticidin.

Recombineering pipeline for generating tagged transgenes

We generated tagged transgenes from BAC clones by a combination of homologous recombination and Flp-*FRT*–mediated site-specific recombination in a 'recombineering pipeline', which takes just 4 d (**Fig. 1**). We carried out all steps in liquid culture using nonclonal selection. To facilitate a multistep protocol, we created a pRedFlp expression plasmid (**Fig. 2a**), which contains a modified Red operon[17] under the rhamnose-inducible promoter[18], and the debilitated Flp recombinase F70L[19] under a tetracycline-inducible promoter[20]. This configuration permits selective expression from either promoter by using different ligands, as confirmed by western blot and functional analysis (**Supplementary Fig. 2** online). Transformation of the pRedFlp plasmid into the original BAC host strain provided the recombination potential for all cloning steps (**Fig. 1a**). The plasmid has the temperature-sensitive pSC101 origin, which facilitates its removal from the cells in the last step by temperature shift in the absence of selection[21].

We performed the tagging step on single-copy BAC clones by rhamnose induction of Red expression, followed by electroporation of a carboxy-terminal tagging cassette (**Fig. 1b**) consisting of the enhanced GFP gene (*egfp*) and an *FRT*-flanked kanamycin resistance gene (*kan*). The cassette was amplified by polymerase chain reaction (PCR) with primers containing 50-nucleotide homology arms for insertion in front of the stop codon. The PCR template plasmid (**Fig. 2b**) has an R6K origin of replication[22], which cannot replicate in the BAC host strain DH10B, thereby eliminating the main source of background in recombineering caused by carryover of the PCR template[17]. We then removed the *kan* gene by tetracycline induction of Flp-*FRT*–mediated site-specific recombination (**Fig. 1c**).

The final subcloning step (**Fig. 1d**) used gap repair mediated by the Red proteins[8]. We again induced Red expression with rhamnose before electroporating a subcloning vector based on the p15A moderate-copy replication origin, which contains the *unc-119* transformation marker for *C. elegans* plus the blasticidin resistance gene (*bsd*) for selection in *E. coli*, and a unique site for the meganuclease I-*Sce*I (**Fig. 2c**). Because the *unc-119* genomic fragment that is widely used as a rescue marker for transgenesis is 5.7 kbp (ref. 23), we used the *C. briggsae* ortholog *unc-119*, which encodes an almost identical protein but has much smaller introns, to reduce the size of the subcloning vector. The genomic region that we used has been shown to rescue completely the *unc-119* loss-of-function phenotype in *C. elegans*[24]. The final size of the subcloning cassette was 3.2 kbp, which is small enough to permit addition of the subcloning homology arms by oligonucleotides and PCR amplification. We selected these homology arms to subclone the genomic region from the closest upstream gene stop or start codon to the closest downstream gene stop or start codon, unless the tagged gene was part of an operon, in which case the whole operon was subcloned in the same way.

We designed the liquid culture recombineering pipeline to permit simultaneous processing of several genes. To evaluate the efficiency of the protocol, we processed 12 genes in parallel in two independent experiments, using 1-ml cultures in Eppendorf tubes (**Fig. 3**). Nine of the genes were successfully tagged and

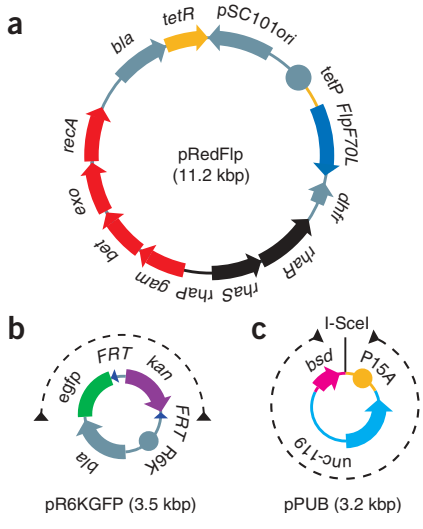

Figure 2 | Recombineering toolkit. The plasmids used are drawn to scale. Origins are indicated by circles and genes are shown as blocks. (**a**) pRedFlp, the dual vector for Red and Flp expression. Expression of the modified red operon[17] (*gam*, *bet*, *exo*, *recA*) is regulated by the rhamnose-inducible promoter (rhaP) and the *rhaS* and *rhaR* genes. Expression of Flp F70L recombinase is controlled by the tetracycline-inducible promoter (tetP) and the tet repressor (*tetR*). The plasmid contains ampicillin resistance (*bla*) and trimethoprim resistance (*dhfr*) genes and the pSC101 replication origin. (**b**) pR6KGFP, template plasmid for PCR-based generation of the *egfp-kan* tagging cassette containing the R6K origin of replication. (**c**) pPUB, subcloning plasmid containing the p15A origin of replication, the *unc-119* transformation marker and the blasticidin resistance gene (*bsd*). Broken lines represent the extent of PCR products obtained from the template plasmids.

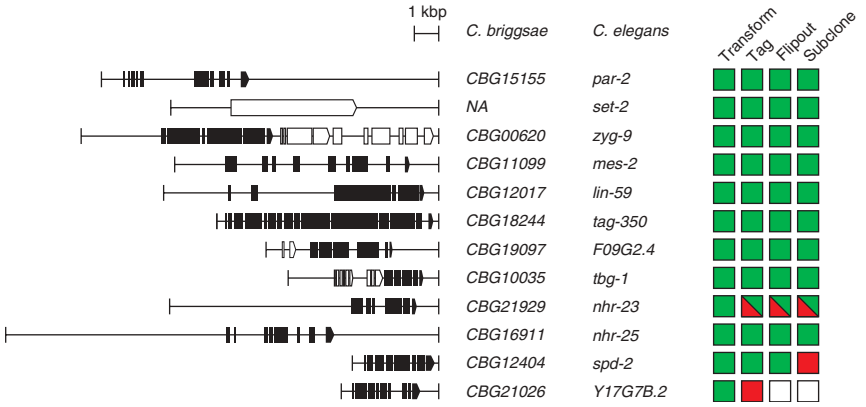

Figure 3 | Parallel processing in liquid culture. Left, exon structure and subcloned regions for the 12 genes tested in the recombineering pipeline. The target gene exons are shown as filled boxes. Exons of additional genes involved in the same operon are shown as open boxes except for *set-2*, for which the whole gene is shown as a single open box because the exact exon structure is not known. Middle, gene names of the tagged *C. briggsae* proteins and of their *C. elegans* orthologs. Right, outcomes of the steps in **Figure 1** in two independent experiments. Green indicates growth in selection as expected; red indicates no growth; half red/half green indicates that the step was successful in only one of the two experiments.

subcloned in both experiments. Tagging of one of the genes failed in one of the experiments, but was successful in the other (*CBG21929*). Two genes failed in both experiments, either at the tagging step (*CBG21026*) or at the subcloning step (*CBG12404*), because the original BAC clone was mutated or incorrectly assigned (data not shown). Failures at the recombineering steps were easy to observe because the cultures were empty. The final cultures were streaked onto plates, and two colonies for each product were examined for correct recombination by restriction digest. All clones were correct (**Supplementary Fig. 3** online and data not shown). Additionally we analyzed 24 single clones for one of the genes: all clones had lost the dual pRedFlp plasmid and some had also lost the BAC (**Supplementary Fig. 4** online).

Generation of transgenic worm lines

To validate the strategy, we generated transgenic worms with the EGFP-tagged *C. briggsae* ortholog of *lin-59*. Stably integrated worm lines were generated by ballistic transformation[23]. Transformation efficiency was close to that achieved with the commonly used *unc-119* vector pAZ132 (ref. 23). We tested whether I-SceI–mediated linearization of the construct before bombardment could increase the number of integrated and/or GFP-positive lines, but no significant improvement was observed (**Supplementary Table 1** online).

All GFP-positive lines showed the same expression pattern (**Fig. 4**). A weak nuclear signal was observed first at mid-blastula stage and then in many cells throughout development and into adulthood, but expression was stronger in neurons, hypodermal cells and some unidentified cells in the head and the tail. The expression pattern was similar to that of a *lin-59* promoter::*gfp* reporter transgenic line[25].

Transgene rescue of RNAi-induced phenotype

To test whether the transgene was functional, we used RNAi by feeding to knock down expression of the endogenous gene. Although the two genes share good overall homology in DNA sequence, no single stretch of 20 identical nucleotides could be found. Therefore, double-stranded RNA targeted to the *C. elegans lin-59* gene was expected to have no effect on the *C. briggsae* transgene (**Fig. 5a**).

As previously shown[26], feeding wild-type *C. elegans* with *lin-59* dsRNA resulted in no obvious phenotype; however, various phenotypes were observed in the RNAi-hypersensitive strain *rrf-3*$^{-/-}$,

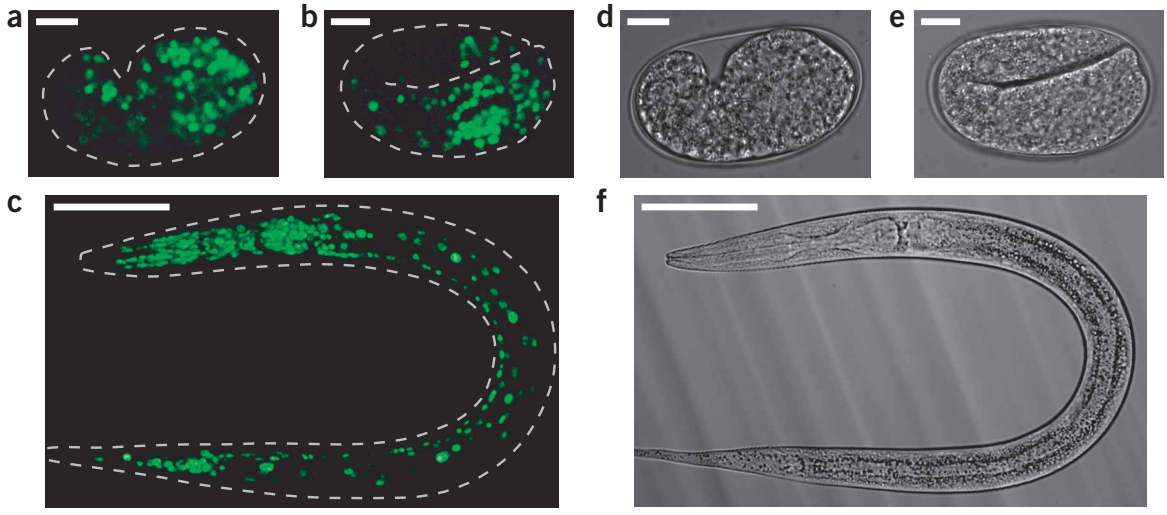

Figure 4 | Expression pattern of the c*blin-59*::*egfp* transgene. (**a**–**f**) GFP fluorescence (**a**–**c**) and the corresponding bright-field image (**d**–**f**) at different stages of development. Onset of morphogenesis (**a**,**d**); elongation (**b**,**e**); larva (**c**,**f**). Image acquisition settings were adjusted to compensate for the different expression levels throughout development and the GFP signals in **a**–**c** are not directly comparable. Scale bars, 10 μm (**a**,**b**,**d**,**e**); 50 μm (**c**,**f**).

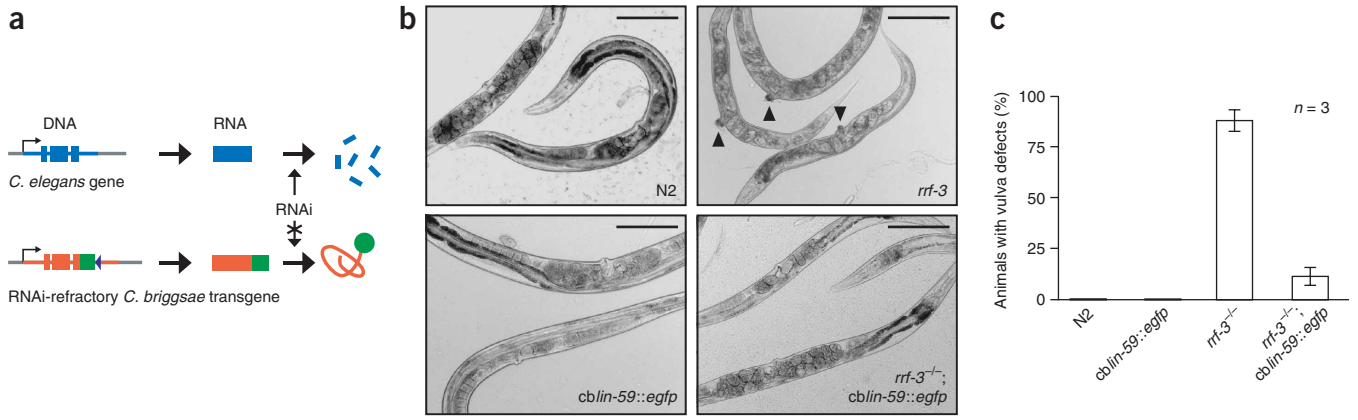

Figure 5 | Rescue of an RNAi-induced phenotype by a cross-species third allele. (**a**) Third allele strategy. Expression of the host alleles is knocked down by RNAi. Because sequence differences in the cross-species ortholog preclude RNAi knockdown, the transgenic third allele can replace the endogenous protein. (**b**) Knockdown of *lin-59* produces defects in vulva formation (arrowheads) in the RNAi hypersensitive *rrf-3*$^{-/-}$ strain, but not in wild-type N2, cb*lin-59*::*egfp* or *rrf-3*$^{-/-}$;cb*lin-59*::*egfp*. (**c**) Penetrance of the defective vulva phenotype in three independent experiments. Error bars show the highest and lowest values. Scale bars, 200 μm.

including defects in vulva development (**Fig. 5b**). We crossed the transgenic line to *rrf-3*$^{-/-}$ and compared the effect of *lin-59* RNAi in the presence and the absence of the transgene. In three independent experiments, the number of worms with vulval defects after *lin-59* RNAi was reduced from almost 90% for *rrf-3*$^{-/-}$ to just above 10% in the *rrf-3*$^{-/-}$;*lin-59*::*egfp* strain (**Fig. 5b**,**c**). Expression levels of GFP in the *rrf-3*$^{-/-}$;*lin-59*::*egfp* were not obviously affected (data not shown); thus, the *C. briggsae* transgene was not affected by RNAi against *C. elegans lin-59*.

DISCUSSION

Multistep DNA engineering by either conventional or recombineering approaches has so far involved clonal selection on plates and screening of individual clones after each step. Previously we, and others, have reported the very high fidelity of Red/ET recombineering[6–11]; that is, the frequency of illegitimate recombination is much less than the intended, homologous event mediated by Red or RecET proteins. Here we have shown that this high fidelity facilitates a way to engineer DNA by using sequential steps in liquid culture without cloning or checking until the final product. Liquid culture cloning is faster and much easier to scale up for parallel processing of many genes.

The recombineering pipeline that we have developed is directed towards making GFP-tagged transgenes for ballistic transformation in *C. elegans* from an indexed *C. briggsae* BAC library. Applications other than protein tagging are clearly possible; for example, transgenes carrying site-directed mutations or deletions aimed at mapping of *cis* regulatory elements could be generated. Different applications will probably require alterations to the pipeline strategy and different expression plasmids. On the basis of our experience here, we confidently predict that other schemes can be developed and that DNA engineering is about to enter a new phase.

A few points underlying the design of this pipeline contributed to the high recombination efficiency necessary for nonclonal selection. First, by introducing an expression plasmid, all steps were done in the original BAC host strain. This approach avoids the more difficult isolation and retransformation of the BAC into a special host such as DY380 (ref. 9) and the concomitant need to recheck the integrity of the BAC.

Second, the tagging was performed on single-copy BAC clones. This approach avoids the complication encountered with recombineering multicopy targets, owing to the host cell containing copies of both the original and the modified plasmid.

Third, the tag was inserted as a cassette with an excisable selection gene, which was then removed by Flp recombination. We used Flp-*FRT* and not Cre-*loxP* because the BAC vector backbone contains *lox* sites. In *E. coli*, Flp excision can be almost 100% efficient even without selection[19]. To regulate Flp recombination strictly, however, we had to use a debilitated Flp variant[19] because the basal expression of wild-type Flp before tetracycline induction was sufficient to drive excision against kanamycin selection at the tagging step (data not shown).

Last, the most common problem encountered with gap repair subcloning is self closure of the linear subcloning vector, mediated by homologous recombination between short internal repeats[8]. To avoid this source of background, we minimized the distance between the terminal subcloning homology arms and either the origin or the selection gene. This construction ensures that any intramolecular recombination event will produce a dysfunctional plasmid lacking a functional origin or antibiotic resistance gene and so cannot be propagated under selection.

By using a cross-species *C. briggsae* transgene, we could selectively knock down the endogenous *C. elegans* gene by RNAi. Thus, the transgene became the primary expressed copy of the gene. Because the transgene should include all of the regulatory elements required for appropriate expression, this third-allele strategy presents a convenient methodological alternative to homologous recombination of the endogenous gene.

Although the possibility of using RNAi to knock down the endogenous gene is an advantage of cross-species transgenes, the same strategy can be applied to transgenes based on *C. elegans* genomic DNA in a loss-of-function mutant background. A *C. elegans* fosmid library has been recently constructed (http://www.geneservice.co.uk/products/clones/Celegans_Fos.jsp): like BACs, these clones

are based on the F-plasmid single-copy origin of replication. These fosmids contain an arabinose-inducible circuit for copy number amplification. We therefore placed the Red operon under rhamnose induction because our previous use[6,17] of arabinose for Red operon induction would be incompatible with the fosmid library. So far, the rhamnose system performs as well as, if not better than, the arabinose system for regulated expression of the Red operon and consequent control over the recombinogenic window.

Our method compares favorably with existing approaches for protein tagging in *C. elegans*. The only method applicable for high-throughput generation of protein fusions, based on Gateway cloning[27], suffers from the limitations inherent in a cDNA approach. A yeast-based recombineering approach has been used to engineer *C. elegans* yeast artificial chromosome clones[28]; however, cloning in yeast is slow and inefficient as compared with recombineering in bacteria. In addition, yeasts are constitutively recombinogenic. Thus, cloned DNA sequences, especially large ones, are continuously prone to rearrangement and need to be carefully monitored. By contrast, our approach combines the advantages of authentic regulation with an application of recombineering that is simple, fast and can be easily scaled up for automated large-scale tagging. Generation of a tagged transgene for each of the 19,735 protein-coding genes in *C. elegans* is now feasible and can be achieved at a relatively low cost.

METHODS

Bioinformatics. We used automated BLAST to align each BAC end sequence to the CB25 *C. briggsae* genome assembly. To improve the quality of the map, we excluded hits with a BLAST *e* value other than 0, and hits that were shorter than 300 bp or that mapped to multiple positions. We further filtered out BAC pairs that were less than 10 and more than 300 kbp apart. The BAC clone map is available as **Supplementary Data** online.

Strains and reagents. All worm strains were from the Caenorhabditis Genetics Center and were maintained as described[29]. *C. briggsae* BAC clones were from the BACPAC Resource Center. The plasmid for production of *lin-59* dsRNAi was from Geneservice. The sequences of the primers used for tagging and subcloning are available in **Supplementary Methods** online.

We used Red/ET recombination to generate the dual pRedFlp using fragments of the following vectors: pASK3 (IBA), pSC101gbaA[17], pOG44 (Stratagene) and pSCrhaB2 (ref. 18). We generated the *egfp-FRT-kan-FRT* targeting cassette by fusion PCR using the *egfp* sequence from pEGFP (Clontech) and the kanamycin resistance gene from pJP5603 (ref. 22). We used Red/ET recombination to recombine the cassette together with the R6K origin from pJP5603 (ref. 22) and the ampicillin resistance gene from pBAD24 (Invitrogen). We constructed the subcloning vector pPUB by ligation of the *C. briggsae unc-119* ortholog (*CBG18291*), which was amplified by PCR from genomic DNA, the p15A origin of replication from pACYC184 (NEB) and the blasticidin resistance gene from pcDNA (Invitrogen).

We used previously characterized antibodies[17,19] for western blot analysis of Redβ and Flp expression.

Suppression of RNAi induced phenotype by *C. briggsae* transgene. We used a published dsRNA feeding protocol[30] to feed wild-type N2, *cblin-59*::*egfp*, *rrf-3*$^{-/-}$ or *rrf-3*$^{-/-}$;*cblin-59*::*egfp* worms with bacteria carrying either the construct expressing *C. elegans lin-59* dsRNA or empty vector. In three independent experiments, we scored 500 second-generation worms of each strain.

Additonal methods. A detailed protocol for liquid culture recombineering, a modified version of the previous method[23] for generating integrated worm lines by ballistic transformation, and detailed instructions on how to use the BAC clone map are available in **Supplementary Methods**.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

M.S. performed the experiments in **Figures 1**,**3**–**5**; M.S., S.S. and A.P. generated the reagents used in **Figure 2**; A.R. generated the BAC clone map; S.E. performed the bombardment experiments; Y.Z. provided reagents and essential expertise in Red/ET recombineering; M.S, A.A.H. and A.F.S. conceived the project and prepared the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

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