

# The CONJUDOR pipeline for multiplexed knockdown of gene pairs identifies RBBP-5 as a germ cell reprogramming barrier in *C. elegans*

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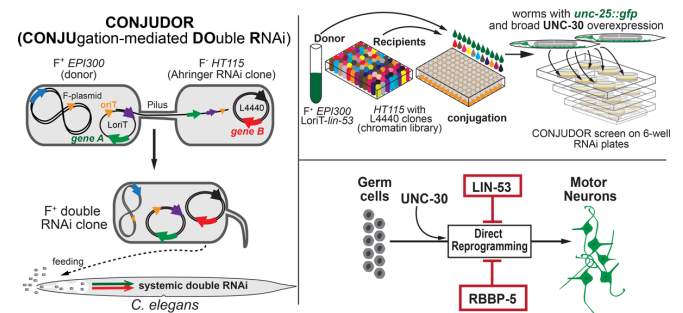
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## ABSTRACT

Multiple gene activities control complex biological processes such as cell fate specification during development and cellular reprogramming. Investigating the manifold gene functions in biological systems requires also simultaneous depletion of two or more gene activities. RNA interference-mediated knockdown (RNAi) is commonly used in *Caenorhabditis elegans* to assess essential genes, which otherwise lead to lethality or developmental arrest upon full knockout. RNAi application is straightforward by feeding worms with RNAi plasmid-containing bacteria. However, the general approach of mixing bacterial RNAi clones to deplete two genes simultaneously often yields poor results. To address this issue, we developed a bacterial conjugation-mediated double RNAi technique ‘CONJUDOR’. It allows combining RNAi bacteria for robust double RNAi with high-throughput. To demonstrate the power of CONJUDOR for large scale double RNAi screens we conjugated RNAi against the histone chaperone gene *lin-53* with more than 700 other chromatin factor genes. Thereby, we identified the Set1/MLL methyltransferase complex member RBBP-5 as a novel germ cell reprogramming barrier. Our findings demonstrate that CONJUDOR increases efficiency and versatility of RNAi screens to examine interconnected biological processes in *C. elegans* with high-throughput.

## GRAPHICAL ABSTRACT

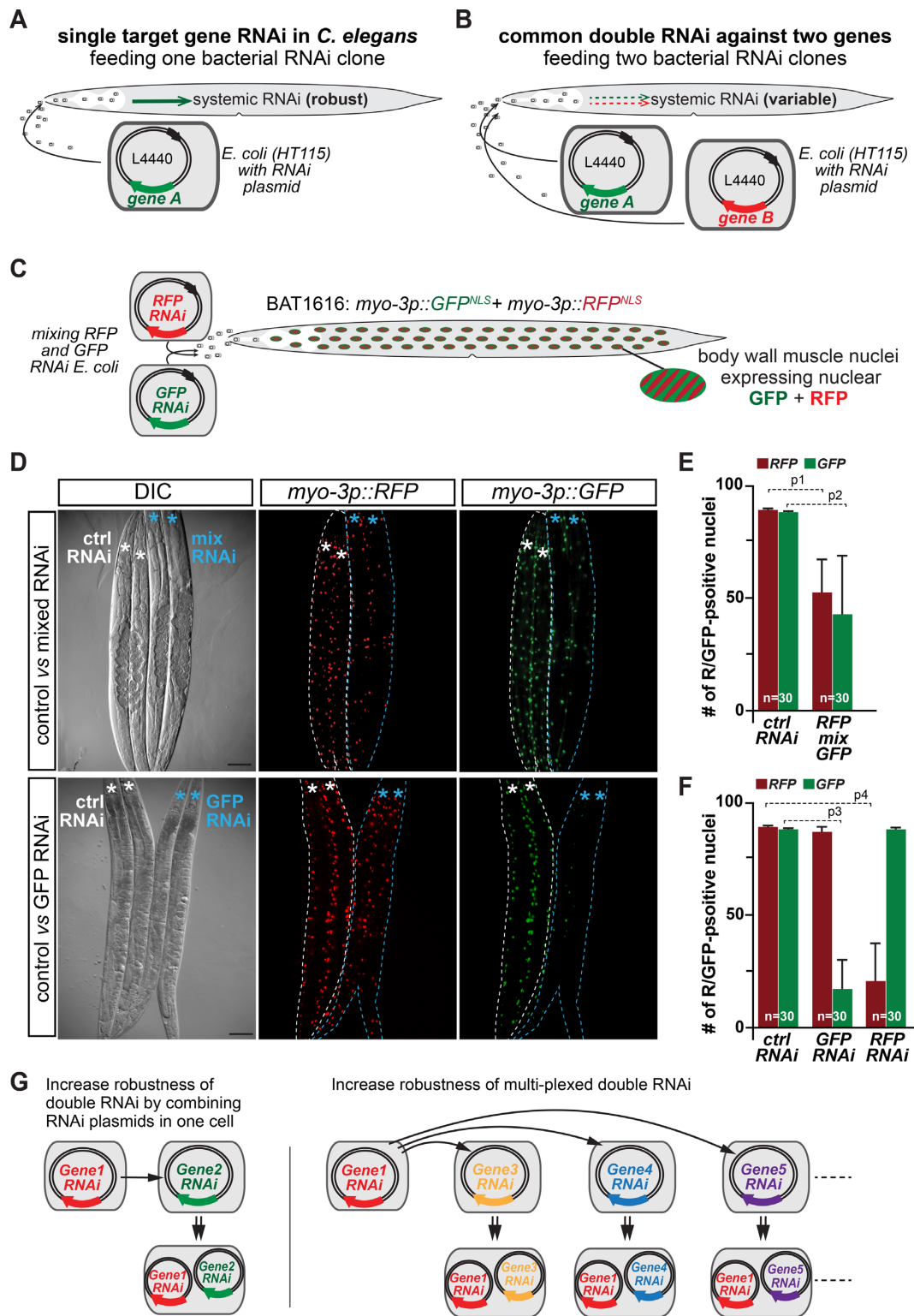


## INTRODUCTION

Most biological processes such as development, cell fate specification, aging, and behavior are controlled by the activity of multiple genes. One approach to investigate the implication of genes in regulating such complex processes is their inactivation. Assessment of phenotypes caused by gene inactivations allows inference of possible roles of the investigated genes in respective processes (1).

Reverse genetics by RNAi is an essential tool to at least partially inactivate genes in the nematode *Caenorhabditis elegans*, which has been used as a powerful model organism to reveal highly conserved molecular mechanisms and gene regulatory pathways (2,3). To perform RNAi in *C. elegans*, animals can be soaked or injected with dsRNA (4) as well as fed with individual *Escherichia coli* bacterial strains producing dsRNA against only one specific gene (5,6) (Figure 1A). Based on the procedure RNAi can cause partial knockdown allowing the investigation of genes, which would cause early developmental arrest, sterility, or even lethality when fully depleted. This RNAi feature is an important benefit compared to genetic screens based on mutagenesis or gene editing causing deletions. Mutagenizing chemical compounds or CRISPR/Cas9 can lead to a full gene knockout, and hence, reduce the possibility to study essential genes during

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**Figure 1.** Double RNAi in *C. elegans* by feeding mixed RNAi bacteria. (A) RNAi in *C. elegans* is straightforward by feeding dsRNA-producing *E. coli* (*HT115* strain). dsRNA against the target gene is produced from the L4440 RNAi plasmid. (B) Double RNAi to knockdown two genes simultaneously by mixing two RNAi bacterial clones. (C) Illustration of transgenic *BAT1616* worms expressing RFP and GFP in nuclei of muscles. Using the *myo-3* promoter 95 body wall muscle nuclei in hermaphrodites are labelled. (D) Representative pictures of DIC and fluorescent signals of *BAT1616* fed with RNAi bacteria against RFP and GFP either mixed or individually. Asterisks label pharynx of simultaneously imaged animals. Scale bars are 20  $\mu$ m. (E and F) Quantification of muscle nuclei number with depleted GFP or RFP signals. See methods and Supplementary Table S4 for details. Control: Rluc RNAi. Single RNAi was mixed 1:1 with Rluc. Statistics: Multiple t-test with two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. p1, p2, p3, p4 =  $1 \times 10^{-15}$ . Total analyzed animals per experiment (triplicate)  $n = 30$ . Error bars represent SEM. For detailed scoring numbers see Supplementary Table S4. (G) Illustration of proposition to increase robustness of double RNAi by combining two RNAi plasmids in bacterial cells.

biological processes post-embryonically or in adult animals (1).

Simultaneous RNAi-mediated knockdown of two genes in *C. elegans* is generally applied by mixing two bacterial strains that contain specific dsRNA-producing plasmids targeting an individual gene (7) (Figure 1B). However, this approach is not reliable and often yields inefficient knockdown of both genes (8,9). This inefficiency can be overcome by generating a single plasmid producing both dsRNAs against the targeted genes (8). While ‘stitching’ target genes together on one RNAi plasmid mediates robust double RNAi, it is not feasible for large scale screens, as it would require high-throughput plasmid cloning.

In order to reconcile double RNAi robustness with high-throughput screening, we developed a CONJUGATION-mediated DOuble RNAi technique, which we term ‘CONJUDOR’. CONJUDOR generates double RNAi bacteria clones in high-throughput and significantly reduces the amount of time and reagents compared to plasmid cloning. At the same time CONJUDOR provides simultaneous knockdown of a large set of two-gene combinations in a robust manner.

To examine the efficiency of CONJUDOR for large-scale screening, we investigated the knockdown of ~700 chromatin factors in combination with RNAi against the histone chaperone LIN-53 in *C. elegans*. LIN-53, in conjunction with the chromatin silencer PRC2, was previously shown to prevent transcription factor-induced (TF) conversion of germ cells into neuron-like cells (10–12). RNAi against *lin-53* alone allows efficient germ cell conversion to glutamatergic neurons (termed ASE) only upon overexpression of the TF CHE-1 using a heat-shock-inducible transgene (10,13,14). In contrast, only limited conversion to GABAergic motor neurons by the Pitx-type homeodomain TF UNC-30 could be observed in LIN-53-depleted animals. We hypothesized that depletion of additional chromatin regulators together with *lin-53* may increase germ cell reprogramming to GABAergic neurons. Indeed, our CONJUDOR screen revealed that co-depletion of the Set1/MLL methyltransferase complex member RBBP-5 together with LIN-53 significantly increased germ cell reprogramming to GABAergic neurons.

Chromatin factors have been identified in *C. elegans* and mammals as cellular reprogramming barriers (15,16) and possible interplay of epigenetic mechanisms remains to be an important research aspect of safeguarding cell fates. CONJUDOR provides the multiplexed interrogation of combinatorial gene knockdowns for investigating such aspects, but can be also applied in the context of other biological phenomena. Genes may act in distinct or similar pathways with identical or converging physiological functions. Hence, it is important to study their relationship in order to better understand underlying mechanisms of biological processes such as development and cell fate specification, which may also be relevant for addressing open questions in biomedical research.

## MATERIALS AND METHODS

### Nematode cultures

Animals were maintained according to standard procedures (17). Heat-shock sensitive strains were kept at 15°C.

### *Caenorhabditis elegans* (*C. elegans*) worm strains

N2: wild isolate, Bristol variant.

BAT28: *otIs305* [*hsp-16.2p::che-1::3xHA*, *rol-6*(*su1006*)] *ntIs1* [*gcy-5p::gfp*, *lin-15*(+)] *V* BAT256: *otIs355* [*rab-3::NLS::TagRFP*] *IV*

BAT684: *juIs8* [*unc-25::GFP*]; *barEx147* [*hsp-16.2/4::unc-30*, *rol-6*(*su1006*)]

BAT1616: *ccls4251* [*myo-3p::NLS::gfp*] *I*; *barIs112* [*myo-3p::NLS::tagRFP*, *HygR*] *X*;

### Synchronized worm population

Synchronized worms were obtained by bleaching hermaphrodites with eggs or by L1 arrest. Gravid hermaphrodites were treated with household bleach (5% sodium hypochlorite) mixed with 1M NaOH and water (3:2:5) Following worm lysis, eggs were washed three times with M9 buffer. For harvesting L1 worms, plates with freshly hatched L1 larvae were collected by washing off with M9 buffer + gelatin. Arrested L1 larvae and bleached eggs were either applied directly onto RNAi or regular NGM plates.

### *Escherichia coli* (*E. coli*) bacterial strains

*OP50*: uracil auxotroph

*HT115*: F-, *mcrA*, *mcrB*, *IN*(*rrnD-rrnE*)1, *rnc14::Tn10*(DE3 lysogen: *lacUV5* promoter -T7 polymerase) (IPTG-inducible T7 polymerase) (RNAse III minus).

*EPI300*: F- *mcrA*  $\Delta$ (*mrr-hsdRMS-mcrBC*)  $\Phi$ 80*dlacZ* $\Delta$ *M15*  $\Delta$ *lacX74* *recA1* *endA1* *araD139*

*SW105*: *SW103*  $\Delta$ *galK*

### Generation of pRK24-KanR and *LoriT*

pRK24-KanR (dBT847 Tursun lab name) was constructed by recombineering to replace Ampicillin resistance (*AmpR*) of *pRK24* (18) with Kanamycin resistance (*KanR*) because *L4440* used in the standard ‘Ahringer’ *C. elegans* RNAi library (5) already carries *AmpR*. Recombineering was performed as previously described (19). Primer to PRC amplify the *KanR* cassette for recombineering were:

FWD: GAA GTT TTA AAT CAA TCT AAA GTA TAT  
ATG AGT AA ACT TGG TCT GAC AGt tat tag aaa aat  
tea tcc agc aga cg;

REV: TGT ATT TAG AAA AAT AAA CAA ATA GG  
GGT TCC GCG CAC ATT TCC CCGAAA AGc gcg gaa  
ccc cta ttt gt tta ttt ttc.

Generating the ‘donor’ RNAi plasmid *LoriT*, required addition of the *oriT* which allows transfer by conjugation. *AmpR* of *L4440* was replaced with Chloramphenicol (*CamR*) resistance to allow selection for presence of *LoriT* together with the resident *AmpR*-containing *L4440* RNAi plasmid after conjugation. Primers used to PCR amplify *oriT* and *CamR* for GIBSON cloning were: *oritFWD*: cca cgc gtt cca tgg GGC GCT CGG TCT TGC CTT;

*oritREV*: cca cgc gtc acg tgg AGC GCT TTT CCG CTG  
CAT AAC.

Further information can be found in Supplemental Figure S2B, C and in Supplementary Table S2. *prK24-KanR* and *LoriT* will be made available through Addgene upon publication of this manuscript.



### Generation of donor bacteria: F<sup>+</sup> *EPI300* with *LoriT*

The recombination deficient *E. coli* strain *EPI300* is transformed with *pRK24-KanR* to generate a stable F<sup>+</sup> strain, which can conjugate with other bacteria. F<sup>+</sup> *EPI300* (containing *pRK24-KanR*) was made electrocompetent for transformation and aliquots were frozen as previously described (19). Alternatively, F<sup>+</sup> *EPI300* can be kept as a standard glycerol stocks and made electrocompetent when needed (see below). Gene sequence of the target gene is inserted into the multiple cloning site of the plasmid *LoriT* (L4440 plasmid derivative containing *oriT* and *CamR* instead of *AmpR*). The RNAi plasmid *LoriT-target-gene* is electroporated into F<sup>+</sup> *E. coli EPI300* to generate the donor RNAi bacteria. In brief, bacteria are grown until reaching an OD<sub>600</sub> between 0.4 and 0.8, put on ice for 15 min. The cells are pelleted at 4°C for 15 min and washed with ice-cold ddH<sub>2</sub>O. The cells are pelleted once more at 4°C and the supernatant is removed except ~0.5 ml. Into an aliquot of 100 μl of F<sup>+</sup> *EPI300*, 50 ng of donor RNAi plasmid (*LoriT-target-gene*) DNA is being added, transferred to electroporation cuvette (0.2 cm electrode gap) and incubated for 2 min on ice. The sample is then electroporated by pulsing with 2.5 kV using a standard electroporator. After electroporation 900 μl of LB medium are added and the bacteria are incubated at 37°C for 1 h under shaking. The cells are plated on selective LB-KanR/CamR plates and incubated overnight at 37°C. Single colonies are picked and grown to prepare glycerol stocks of F<sup>+</sup> *EPI300* bacteria that are competent for conjugation and can transfer *LoriT-target-gene*.

### Generation of double RNAi bacteria clones by conjugation

On day one 96-well plates filled with 100 μl LB agar are prepared and dried overnight. Donor F<sup>+</sup> *EPI300* (with e.g. *LoriT-lin-53* as used in this study) and recipient F<sup>-</sup> *HT115* bacteria of the Ahringer RNAi library clones were grown overnight at 37°C to saturation. Depending on the number of conjugations, the F<sup>-</sup> *HT115* bacteria with Ahringer RNAi clones should be grown in a 96-well format (deep-well). Donor F<sup>+</sup> bacteria are pelleted at 4°C and 80% of supernatant is removed resulting in an approximately 5× up-concentration. 5 μl of donor and recipient bacteria are pipetted in equal amounts of on LB agar containing 96-well plates, covered with a lid or aluminum foil and incubated for 1 h at 37°C. After incubation, 100 μl of LB-Amp/Tet/Cam medium is added and the plates are incubated for another 6 h under mild shaking. Afterwards, ~5 μl from each well are transferred into a new 96-deep-well plate containing LB-Amp/Cam. The second selection step does not contain tetracycline (Tet) as this affects the worms negatively. The 96-deep-well plates are incubated overnight at 37°C under shaking and the conjugated RNAi bacteria are ready to be seeded onto NGM RNAi plates. For further information of RNAi clones used in the study see Supplementary Table S1.

### Generation of double RNAi bacteria clones by stitching

In order to fuse the sequences of two RNAi target genes (*oma-1 + oma-2* and *lin-53 + rbbp-5*) into the L4440 plasmid as described previously (8) we used Gibson cloning.

The NEBuilder® Assembly Tool (<http://nebuilder.neb.com>) was used to design specific primers (see Supplementary Table S3) to amplify DNA fragments with overhangs. The L4440 vector DNA was digested with restriction enzymes and PCR-amplified fragments were combined with vector and 2× Gibson Mastermix (NEB). The Gibson ligation was used for transformation of *HT115 E. coli*.

### Determining and confirming successful conjugation (for proof of concept in this study only)

Conjugated RNAi plasmid-containing bacteria clones were confirmed by colony PCR upon growth on Amp/Cam. Donor RNAi plasmid containing bacteria (e.g. *LoriT-lin-53* as used in this study) in the F-plasmid *pRK24-KanR*-containing *EPI300* bacteria were grown overnight to saturation, mixed at a 1:5 ratio on LB agar plates, incubated for 1 h at 37°C, washed off and plated on LB-Kan/Amp/Cam plates to select for conjugated bacteria clones containing the donor RNAi plasmid and the F-plasmid *pRK24-KanR*.

### RNA interference experiments

The reprogramming experiments were carried out by feeding the animals with bacteria containing conjugated or standard RNAi clones. Generally, we performed F1 RNAi by exposing L4 stage larval animals to RNAi plates. For germ cell reprogramming experiments, plates were kept at 15°C and the following F1 generation was heat shocked at L3/L4 stage for 30 min at 37°C. Afterwards animals were kept at 25°C overnight and scored 24 h post heat-shock for ectopic induction of *gcy-5::GFP* or *unc-25::GFP*. For double RNAi by mixing, bacteria were grown as saturated cultures. The OD<sub>600</sub> was measured to ensure that bacteria were mixed at an equal ratio and seeded on standard RNAi plates (20). For experiments to compare single RNAi versus mixed RNAi, the single RNAi bacteria were mixed in equal ratio 1:1 with the control RNAi bacteria *Rluc* (in *HT115* bacteria). For further information on media recipes used in the study see Supplementary Table S3. In all RNAi experiments, RNAi against *Renilla luciferase* (*Rluc*) was used as control to avoid unspecific effects by empty L4440 (21).

### RNAi screening

The RNAi screening was performed as described previously for the whole-genome and chromatin-library RNAi screening (20,22). In brief, conjugation of *LoriT-lin-53* (*EPI300* bacteria) with the chromatin library (all in *HT115* bacteria) was performed as described above. The conjugated clones were used to generate six-well RNAi NGM agar plates supplemented with 1mM IPTG and 50 μg/ml Carbenicilin. Plates were seeded with conjugated RNAi bacteria and *Renilla luciferase* (*Rluc*) RNAi was used as control. The CONJUDOR RNAi screen for germ cell reprogramming barriers was performed as an F1 screen using a standard RNAi feeding protocol (23). Screening was performed in duplicates using synchronized L1 animals which were grown at 15°C on normal food. After reaching the L4 larval stage animals were transferred to RNAi plates and grown on RNAi



at 15°C until the F1 progeny reached the L4 stage. Subsequently, animals were heat-shocked at 37°C for 30 min and afterwards incubated at 25°C until the next day as described before (13,22). Plates were screened for presence of ectopic GFP in the germline using a fluorescence stereo microscope such as the M205 FCA (Leica) as described in previous studies to screen for this phenotype (10–12,20,22).

### Scoring of RNAi phenotypes and muscle nuclei for RFP / GFP

Quantification of nuclei expressing GFP and RFP in the strain BAT1616 was performed by epifluorescence microscopy using a ZEISS Axio Imager.M2 (Zeiss) equipped with the Sensicam CCD camera by PCO Imaging. Z-stack image acquisitions were performed using the MicroManager plugin in ImageJ and acquired pictures were processed using Fiji (<http://fiji.sc>) (24,25). Nuclei were quantified with the help of the multi-selection plugin tool in Fiji to mark counted nuclei.

Quantification of other RNAi phenotypes relating to the germline teratoma and reprogramming were performed using the fluorescence stereo microscopes M205 FCA (Leica). Other phenotypes relating to the 'oma' and lethality phenotypes were scored using standard stereo microscopes.

### General microscopy

For imaging, worms were mounted on freshly made 2% agarose pads using 10 mM tetramizole hydrochloride in M9 buffer to anesthetize animals. Microscopy analyses were performed using the ZEISS Axio Imager.M2 (Zeiss) equipped with the Sensicam CCD camera by PCO Imaging. For image acquisition MicroManager plugin in ImageJ was used (24,25). Acquired picture were processed using ImageJ.

### Knockdown analysis by quantitative RT-PCR

RNAi was performed as described above with Rluc as control, which was also used to mix 1:1 with single RNAi experiments. RNA was isolated as described previously using Trizol (Thermo Scientific) (12). RNA samples were subsequently treated with DNase I (Thermo Scientific), including RiboLock RNase inhibitor (Thermo Scientific), according to the manufacturer's instructions. cDNA was prepared with GoScript reverse transcription kit (Promega), using Oligo(dT)18 (Thermo Scientific) as primer and including RiboLock RNase inhibitor according to the manufacturer's instructions. Finally, qPCR was carried out using Maxima Green/ROX 2× Master Mix (Thermo Scientific) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). As reference genes *cdc-42* and *pmp-3* were used (26) and analysis was done using the  $2^{-\Delta\Delta CT}$  method by Schmittgen & Livak (27) with relative expression compared to animals treated with control RNAi (Rluc). 4 biological repeats each with triplicate measurements were performed. Statistical analysis was performed using Student's *t*-test with two-tailed distribution with homoscedastic variance. Primer sequences are provided in Supplementary Table S2.

### Statistical analysis

RNAi phenotype experiments were performed with repeats as outlined in the source data in Supplemental Table S4. Numbers of animals (n) used for each group in repeat experiment are indicated in the figures and Supplementary Table S4. Rounded *P*-values can be found in the figure legends of the respective data illustration. Statistical methodology is indicated in Supplementary Table S4, where either multiple *t*-test with two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with  $Q = 1\%$  (each row was analyzed individually, without assuming a consistent SD) using Prism7 (GraphPad) has been performed, or Student's *t*-Test with two-tailed distribution with homoscedastic variance using Excel (Microsoft).

## RESULTS

### Double RNAi by mixing bacterial strains is inefficient

In order to assess the degree of double RNAi robustness we generated the strain BAT1616, which expresses red fluorescent protein (RFP) as well as green fluorescent protein (GFP) in muscles using the *myo-3* promoter (Figure 1C). For easy assessment of fluorescence signal intensities both fluorescent proteins are localized to the nuclei of all 95 body wall muscles (Figure 1C-D). To simultaneously deplete RFP and GFP signals in the muscle nuclei we applied a 1:1 mix of RNAi bacterial clones, based the 'Ahringer' RNAi library *HT115 E. coli* strains, each containing RNAi plasmids against *RFP* and *GFP* (Figure 1C-F) (Supplementary Figure S1). Around 50% of muscle nuclei lost RFP and GFP signals in F1 RNAi animals (Figure 1C-F). In contrast, feeding *HT115 E. coli* with GFP or RFP RNAi-plasmids individually reduced GFP and RFP signals more efficiently in approximately 75% of muscle nuclei, respectively (Figure 1F). This outcome confirmed that mixing RNAi bacteria attenuates knockdown efficiency of individual genes as previously reported (8).

To solve the issue of decreased RNAi efficiency upon mixing different RNAi bacteria, we assumed that combining two different RNAi plasmids in the same cell may increase robustness and efficiency of double RNAi (Figure 1G).

### Bacterial conjugation to combine RNAi plasmids

Generating hundreds or thousands of new plasmids as described previously (8) to combine a target gene of interest with a set of other targets is not feasible for large-scale double RNAi screens.

In order to develop a pipeline that allows combining RNAi plasmids with high-throughput, which provides robust double RNAi knockdowns, we sought for a method that consumes low amount of time and reagents. One such approach is bacterial conjugation, which allows the transfer of plasmids with an *origin of transfer (oriT)* among bacterial cells. Competence for bacterial conjugation requires presence of the fertility factor, also termed F-plasmid, which contains several genes of the *tra* locus for the formation of a pilus appendage. Bacteria with the F-plasmid are denoted as F<sup>+</sup> (donor) and connect via the pilus to F<sup>-</sup> bacteria (recipient) to transfer plasmids or other genetic material containing an *oriT* to the recipient (Supplementary Figure S2).

To adopt bacterial conjugation for combining RNAi plasmids, we made the F-plasmid *pRK24* (18) and the RNAi plasmid *L4440* to be compatible with each other. We first replaced the Ampicillin resistance (AmpR) of *pRK24* with Kanamycin resistance (KanR) because *L4440* used in the standard ‘Ahringer’ *C. elegans* RNAi library (5) already carries AmpR. To exchange AmpR with KanR we used recombineering, as previously described, due to the extensive size of *pRK24* (Figure 2A) (19).

The generation of a selectable ‘donor’ RNAi plasmid, which can be transferred by conjugation, needed the addition of the *oriT* and replacement of AmpR with Chloramphenicol (CamR) resistance. This allows selection for presence of the transferred RNAi plasmid together with the resident AmpR-containing *L4440* RNAi plasmid after conjugation. We termed the newly generated donor plasmid ‘*LoriT*’, which is basically *L4440* carrying *oriT* and CamR instead AmpR (Figure 2B). Additionally, we figured that maintaining a large episome such as the F-plasmid requires stable conditions in bacteria - optimally preventing recombination events. Therefore, we used the *E. coli* strain *EPI300*, which is deficient of recombinases and has proven to maintain large fosmids in a stable manner (Figure 2C) (19). After cloning the target gene into *LoriT*, it is transformed to F<sup>+</sup> *EPI300* bacteria (contain *pRK24-Kan*). This creates the donor strain that is ready to be conjugated with the receiving ‘Ahringer’ RNAi bacteria (in *HT115 E. coli* strain) for combining the target gene of interest with any other target gene for double RNAi (Figure 2C).

Next, we tested which conjugation procedure yields the most efficient transfer of the target gene-containing *LoriT* (Figure 2D, Supplementary Figure S3A–I). By analyzing a number of variations including antibiotics at different steps and performing conjugation in liquid versus on solid media we determined the most efficient procedure (Supplementary Figure S3A–I). Combining a 5:1 ratio of donor:recipient bacterial culture on solid LB agar for 1h with subsequent selection (Cam/Amp in liquid for 1h) yielded 100% conjugation efficiency (Figure 2D, Supplementary Figure S3A–I).

Overall, our adaption of the bacterial conjugation system to combine RNAi plasmids in bacteria is robust and straightforward to generate a large set of bacterial cells simultaneously producing dsRNA against two target genes. We termed our new technique CONJUDOR, which stands for CONJUgation-mediated DOuble RNAi.

### Robust knockdown of GFP and RFP in muscles by CONJUDOR

To demonstrate that CONJUDOR provides efficient knockdown of two genes simultaneously we co-depleted GFP and RFP expressed in muscle nuclei as described before. Additionally, we generated a *LoriT-GFP* RNAi plasmid and transformed into the F<sup>+</sup> *EPI300 E. coli* strain (containing *pRK24-KanR*) (Figure 3A). Subsequent conjugation with standard *HT115 E. coli* containing *L4440-RFP* RNAi plasmid generated bacterial cells producing dsRNA against both GFP and RFP (*GFP\_CON\_RFP*) (Figure 3A and B). To assess and compare knockdown efficiencies, the conjugated bacteria and mixed GFP+RFP RNAi bacteria

were used for double RNAi in BAT1616 worms (Figure 3B–C). A similar result for mixed RNAi bacteria was observed as described before, where around 50% of nuclei showed simultaneous GFP/RFP signal depletion (Figures 1D and E; 3C and D). In contrast, the conjugated bacteria simultaneously depleted GFP and RFP signals in more than 90% of the cells.

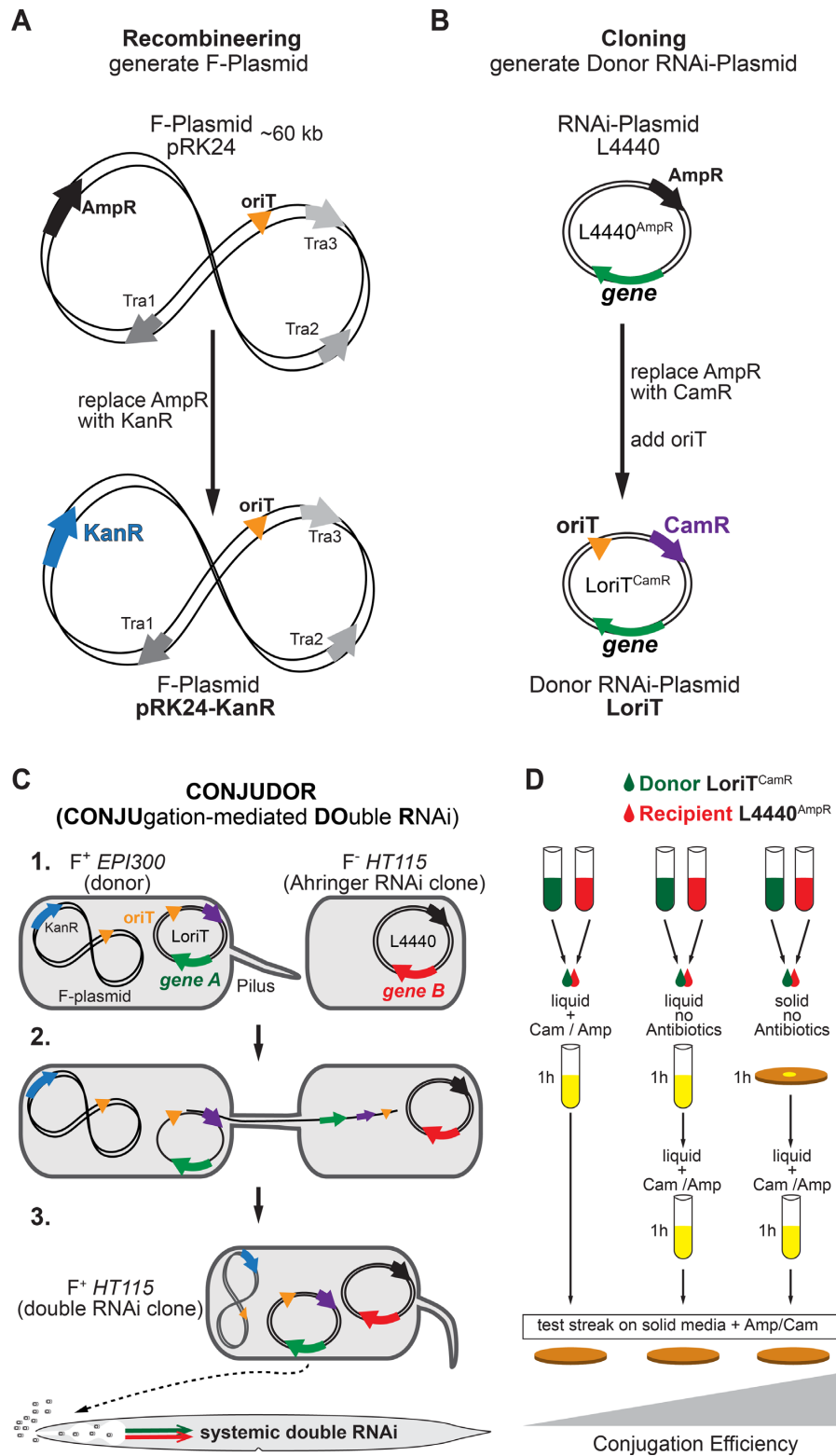
The outcome of testing BAT1616 worms for double RNAi against GFP and RFP indicates a very robust double RNAi knockdown by combining RNAi plasmids in one cell via conjugation using CONJUDOR.

### CONJUDOR is a robust double RNAi approach also for other phenotypes

To assess the efficiency of CONJUDOR in targeting endogenous genes, we decided to target *oma-1* and *oma-2*, which are redundantly required for oocyte maturation (28). Double mutants lacking *oma-1* and *oma-2* were described to be fully sterile due to immature oocytes, which accumulate in the gonads (28). In contrast, animals missing only *oma-1* or *oma-2* are fertile (28) (Figure 4A). Wild-type worms were subjected to F1 double RNAi targeting *oma-1* and *oma-2* either by mixing the individual *oma-1* and *oma-2* RNAi bacteria, or by using conjugated *oma-1\_CON\_oma-2* RNAi bacteria (Figure 4B–D).

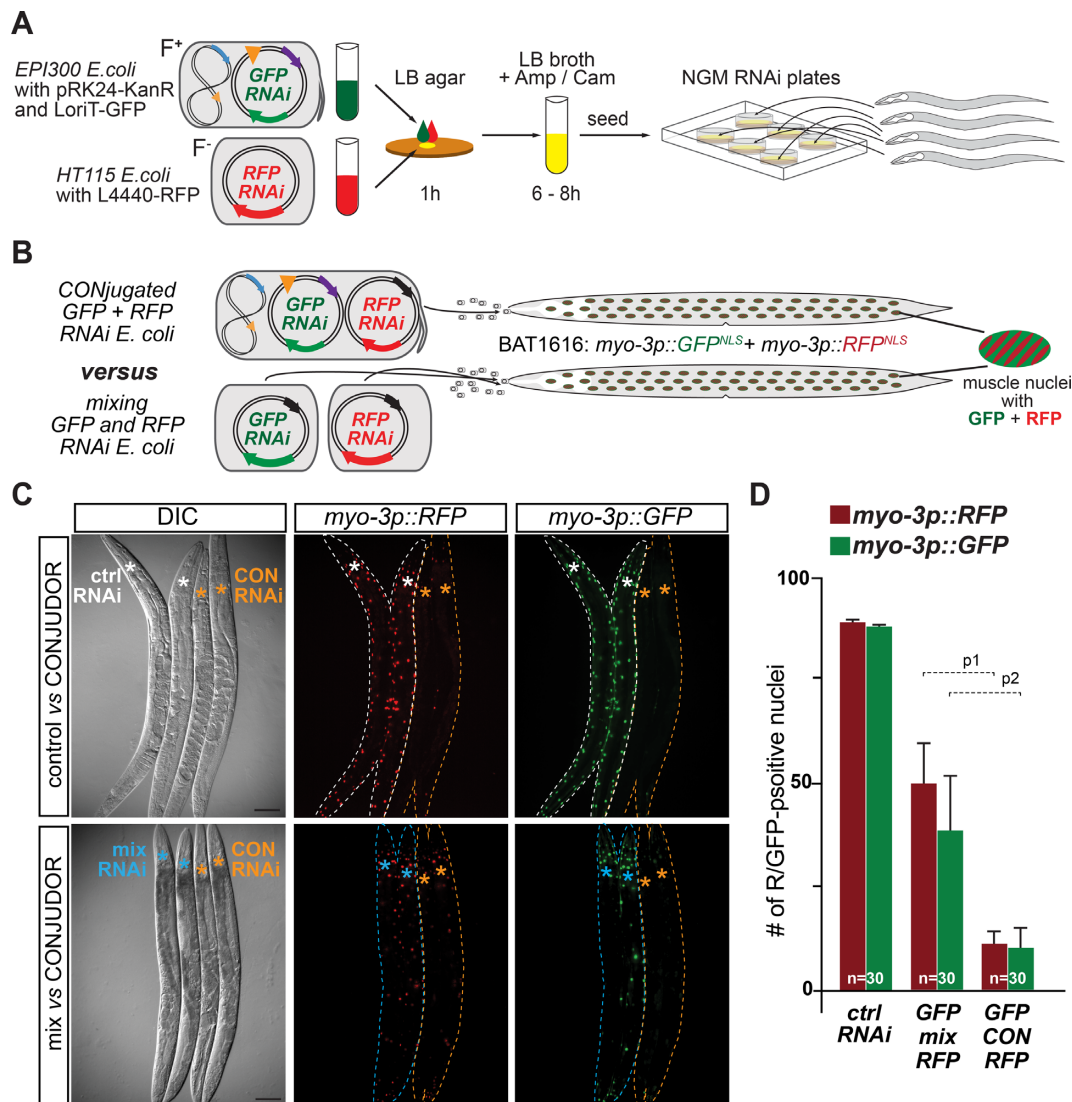
In this context, we also tested whether different double RNAi clones generated by conjugation are equally effective. While mixed RNAi caused an arrest of oocyte maturation in around 25% of animals, three different conjugation-derived double RNAi bacteria clones against *oma-1\_CON\_oma-2* caused around 60% sterility in a reproducible manner (Figure 4C and D). Notably, animals showed the characteristic ‘*oma*’ phenotype, which leads to accumulation of immature oocytes in the gonad, indicating that sterility was indeed caused due to depletion of *oma-1* and *oma-2* (Figure 4D). Additionally, we compared CONJUDOR-mediated co-depletion of *oma-1* and *oma-2* with the ‘stitching’ approach. Stitching had been reported previously to be more efficient than mixing RNAi bacteria against *oma-1* and *oma-2* (8). Stitching requires the combination of the two target gene sequences on the *L4440* plasmid to generate dsRNAs targeting both genes simultaneously (8). Feeding of *HT115* bacteria containing the stitched *L4440-oma-1::oma-2* plasmid revealed that CONJUDOR-caused sterility by *oma-1\_CON\_oma-2* is comparable to the efficiency of stitching, which is marginally higher (Figure 4C).

Furthermore, we assessed teratoma formation of germ cells upon depletion of the translational regulators GLD-1 and MEX-3. Mutants carrying both mutations *gld-1(q485)* and *mex-3(or20)* alleles have been shown to develop teratomas in their germline that can be visualized based on expression of pan-neuronal reporters (29). We used worms, which express RFP in neuronal nuclei under the control of the pan-neuronal *rab-3* gene promoter (Figure 4E and F). Using CONJUDOR to target *gld-1* and *mex-3* simultaneously caused significantly more animals (35%) with germline teratomas than mixing RNAi bacteria against both genes (5%) (Figure 4F and G). Additionally, we also targeted the 26S-Proteasome subunit genes *rpn-10* and *rpn-12*, which cause synthetic lethality when co-depleted (30).



**Figure 2.** Creating a double RNAi system by bacterial conjugation. (A) The F-plasmid (fertility factor) encodes for components of the conjugation machinery to transfer *oriT*-containing genetic material. Recombineering was used to replace Ampicillin resistance (AmpR) with Kanamycin resistance (KanR) to allow combination with RNAi plasmids. (B) *LoriT* plasmid: we equipped the *L4440* RNAi plasmid (used for clones of the Ahringer RNAi library) with *oriT* and exchanged AmpR with Chloramphenicol resistance (CamR). (C) *pRK24-KanR*-containing *EPI300* *E. coli* are F<sup>+</sup> and can conjugate with *HT115* RNAi bacteria clones of the Ahringer RNAi library, which are F<sup>-</sup>. Conjugated bacteria are selected based on CamR/AmpR. (D) Different conjugation procedures were evaluated to find the most efficient transfer of *LoriT* to recipient RNAi bacteria. For detailed description and results see Supplemental Figure S3.





**Figure 3.** CONJUDOR knockdown of nuclear GFP and RFP in muscles. (A) Schematic illustration of CONJUDOR to generate *GFP* and *RFP* double RNAi bacteria. (B) Double RNAi against *GFP* and *RFP* in muscle nuclei of BAT1616 by CONJUDOR versus mixing individual RNAi bacteria. (C) Representative pictures of DIC and fluorescent signals of BAT1616 fed with RNAi bacteria against *RFP* and *GFP* either mixed or conjugated. Asterisks label pharynx of simultaneously imaged animals. Scale bars are 20  $\mu$ m. (D) Quantification of muscle nuclei number with depleted *GFP* or *RFP* signals. See methods and Supplementary Table S4 for details. Control: *Rluc* RNAi. Statistics: Multiple *t*-test with two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli,  $p_1, p_2 = 1 \times 10^{-15}$ . Total analyzed animals (triplicate)  $n = 30$ . For detailed scoring numbers see Supplementary Table S4. CONJUDOR is depleting *GFP* and *RFP* more efficiently than mixing RNAi bacteria. Error bars represent SEM.

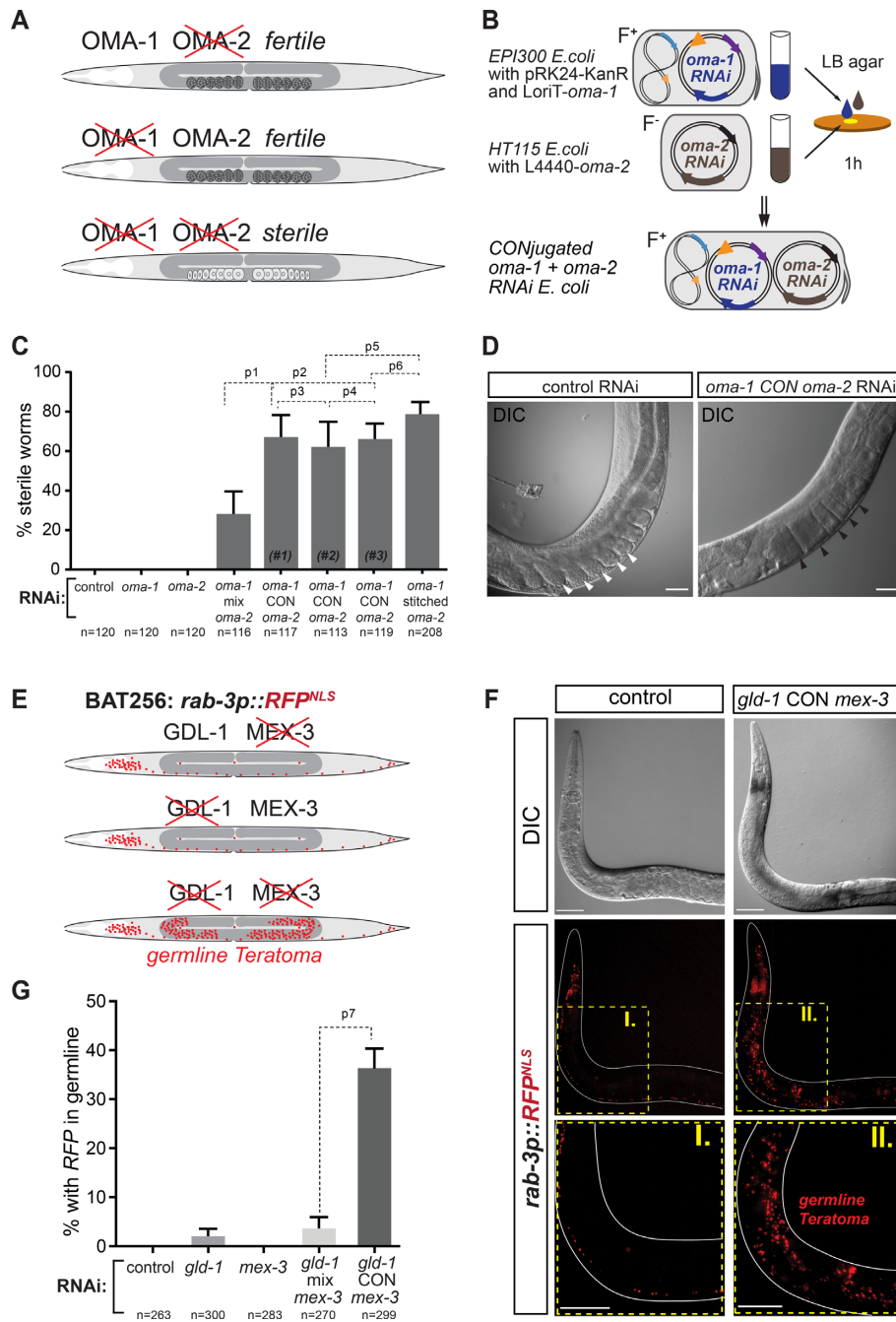
While CONJUDOR-mediated simultaneous knockdown of *rpn-10* and *rpn-12* reduced survival by ~50%, only 25% of the animals fed with mixed *rpn-10* and *rpn-12* RNAi bacteria died (Supplementary Figure S4).

Overall, our results provide evidence that CONJUDOR is a highly robust technique for simultaneous knockdown of endogenous genes by double RNAi.

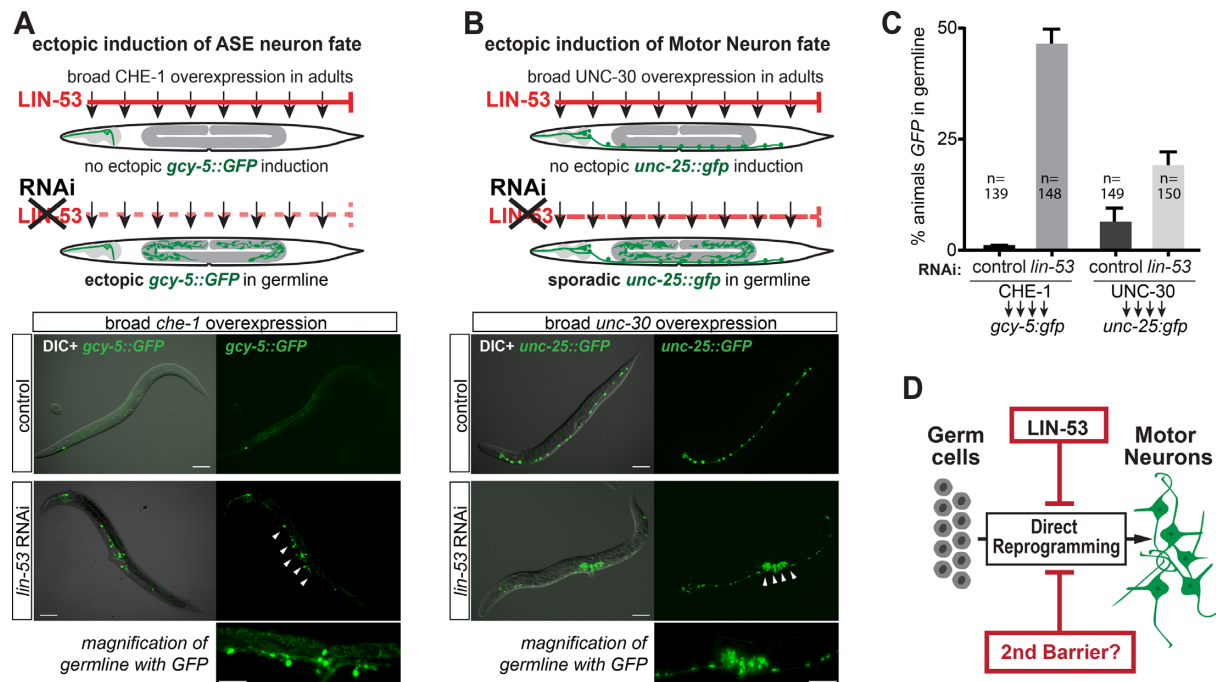
### Epigenetic barriers of germ cell reprogramming to neuron-like cells

It was previously discovered that the epigenetic factor LIN-53, which can directly bind to histones, acts as a reprogramming barrier in the germline. RNAi depletion of *lin-53* along with transgenic over-expression of specific transcrip-

tion factors (TF) allows germ cell reprogramming to defined types of neuronal cells (10,11). Overexpression of the Zn-finger TF CHE-1 using a heatshock activatable promoter induces conversion to glutamatergic ASE neuron-like cells labelled by expression of the ASE neuron-specific reporter *gcy-5::GFP* (Figure 5A) (10). The Pitx-type homeodomain TF UNC-30 is required for the specification of GABAergic motor neurons. Consequently, heat-shock induced UNC-30 overexpression in *lin-53* RNAi animals induces the GABA fate marker *unc-25::GFP* in germ cells (10) (Figure 5B). However, the induction of *unc-25::GFP* by UNC-30 is less efficient than *gcy-5::GFP* induction by CHE-1 (Figure 5C). We speculated that this discrepancy may be due to additional epigenetic barriers that limit ectopic induction of the GABAergic motor neuron fate (Figure 5D).



**Figure 4.** CONJUDOR knockdown of two endogenous genes. (A) Double depletion of *oma-1* and *oma-2* causes sterility due to immature oocytes. (B) Schematic illustration of CONJUDOR to generate *oma-1*\_CON\_*oma-2* double RNAi bacteria. (C) Three independent *oma-1*\_CON\_*oma-2* bacteria clones were tested and compared to mixing RNAi bacteria against *oma-1* and *oma-2*. Quantification of sterile animals displays higher efficiency of CONJUDOR for all three tested *oma-1*\_CON\_*oma-2* clones compared to mixed RNAi bacteria. Control: Rluc RNAi. Statistics: unpaired *t*-test; \*\**P* < 0.001; *n* = 140, *ns* = not significant. Error bars represent SEM. (D) Representative DIC pictures of gonad region of control animals and *oma-1*\_CON\_*oma-2* double RNAi treated animals. White arrow heads depict embryos, black arrow heads indicate accumulation of immature oocytes. Scale bars are 10  $\mu$ m. (E) Double depletion of *gld-1* and *mex-3* leads to teratoma formation in the germline. (F) Representative fluorescence signal pictures of animals expressing the pan-neuronal reporter *rab-3p::RFP<sup>NLS</sup>*. Double RNAi using CONJUDOR against *gld-1* and *mex-3* leads to teratoma formation visualized by the expression of neuronal RFP signals in the germline. Scale bars are 10  $\mu$ m. (G) Quantification of teratoma formation confirms significantly increased induction of teratoma formation upon feeding with *gld-1*\_CON\_*mex-3* bacteria compared to mixed RNAi bacteria against *gld-1* and *mex-3*. Control: Rluc RNAi. Statistics: *t*-test with two-tailed distribution. p1 = 0.008; p2 = 0.015; p3 = 0.422; p4 = 0.509; p5 = 0.031; p6 = 0.032; p7 = 0.0006. Total analyzed animals (triplicate) in C is *n* = 1033, in G is *n* = 1415. Error bars represent SEM. For detailed scoring numbers see Supplementary Table S4.



**Figure 5.** Epigenetic barriers of germ cell to neuron reprogramming in *C. elegans*. (A) Schematic illustration of transgenic animals expressing the glutamatergic ASE neuron fate marker *gcy-5::GFP* and allowing heat-shock-inducible broad CHE-1 overexpression. DIC / GFP pictures of animals with germ cells reprogrammed to ASE neurons upon depletion of the histone chaperone LIN-53 and broad overexpression of CHE-1. White arrow heads indicate germline with reprogrammed cells (this area is magnified below). (B) Schematic illustration of transgenic animals expressing the GABAergic motor neuron fate marker *unc-25::GFP* and allowing heat-shock-inducible broad UNC-30 overexpression. DIC/GFP signal pictures of animals with germ cells reprogrammed to GABAergic neurons upon depletion of LIN-53 and broad overexpression of UNC-30. White arrow heads indicate germline with reprogrammed cells (area is shown in magnification below). (C) Quantification of germ cell to neuron reprogramming by CHE-1 and UNC-30 upon *lin-53* RNAi. Induction of the GABA fate marker by UNC-30 is less efficient. Total analyzed animals (triplicate)  $n = 587$ . See Supplementary Table S4 for details. Control: Rluc RNAi. Error bars are SEM. (D) A second barrier may decrease germ cell to GABAergic motor neuron conversion.

The task to screen for a putative ‘2nd barrier’ by co-depleting *lin-53* with other chromatin regulators provided an attractive test case to perform CONJUDOR for a large set of double RNAi knockdowns.

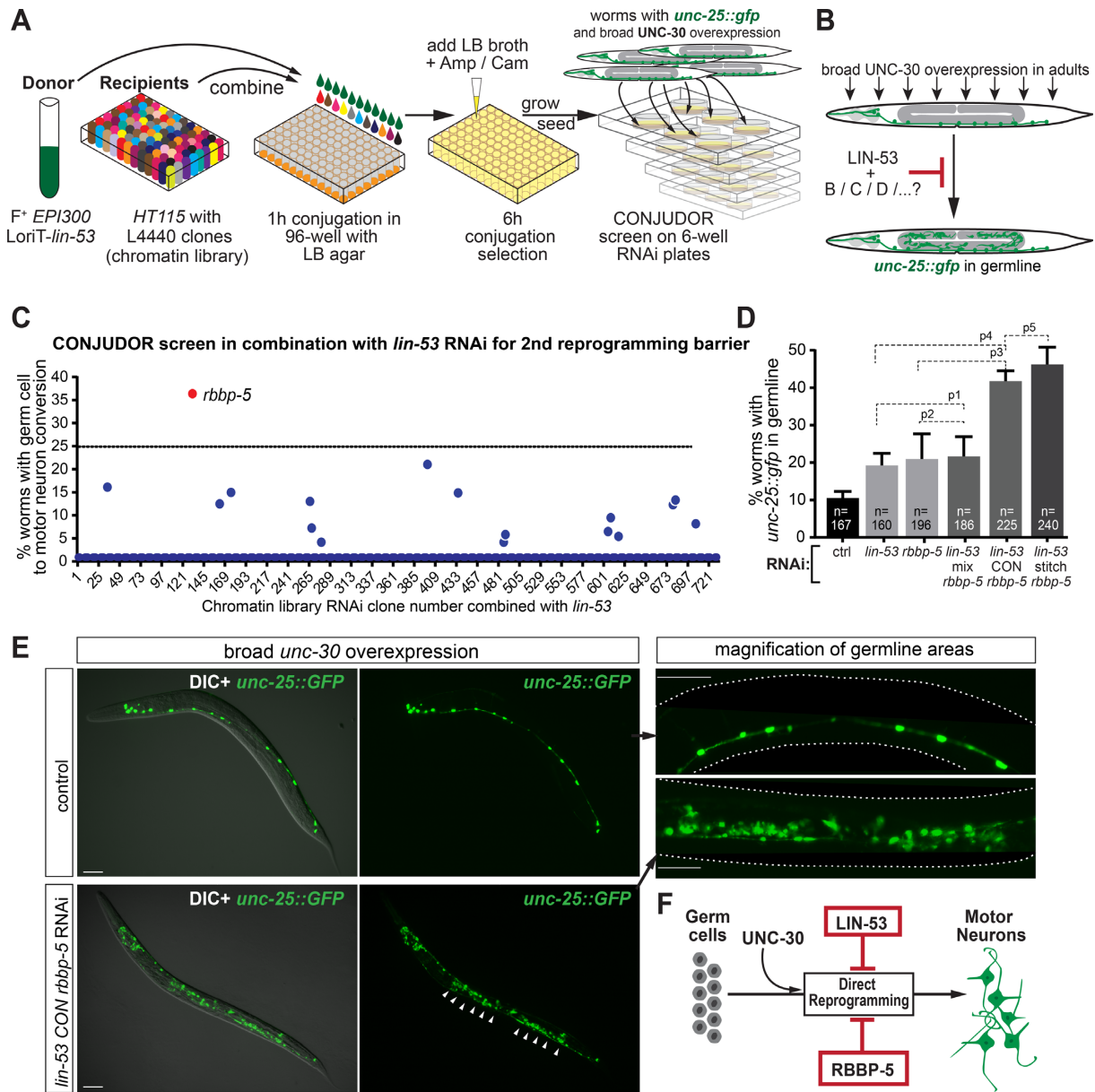
### CONJUDOR identifies RBBP-5 as a novel reprogramming barrier

To identify additional epigenetic regulators, which may be involved in limiting the conversion of germ cells to GABAergic neurons, we decided to conjugate the *LoriT-lin-53* plasmid with ~700 other RNAi clones that target chromatin-related genes based on our previously described chromatin RNAi library (20) (Figure 6A).

Worms were subjected to CONJUDOR in an F1 RNAi screen and heat-shocked as young adults to induce broad expression of the GABA motor neuron fate-inducing TF UNC-30 (Figure 6A and B). One day later we scored for animals showing ectopic *unc-25::GFP* expression in their germline (Figure 6C). Among the more than 700 tested *lin-53* co-depletions, we found that worms fed with bacteria derived from conjugating *LoriT-lin-53* with *L4440-rbbp-5* (*lin-53\_CON\_rbbp-5*) showed a marked increase of the *unc-25::GFP* induction rate in the germline (Figure 6C). The *rbbp-5* gene encodes for the Set1/MLL methyltransferase complex member RBBP-5 and is the ortholog of human RBBP5 (RB binding protein 5) (31). Next, we compared

*lin-53\_CON\_rbbp-5* to single RNAi against *rbbp-5*, *lin-53* or *L4440-lin-53* mixed with *L4440-rbbp-5* RNAi bacteria (Figure 6D). We noticed that knockdown of *rbbp-5* alone provides a similar number of animals with germlines positive for *unc-25::GFP* as RNAi against *lin-53* (Figure 6D). However, CONJUDOR-mediated knockdown of *lin-53* and *rbbp-5* simultaneously (*lin-53\_CON\_rbbp-5*) almost doubled the efficiencies of single RNAi knockdowns (Figure 6D). Knockdown efficiencies of *rbbp-5* and *lin-53* were also assessed based on mRNA levels using quantitative PCR (qPCR) (Supplementary Figure S5). This approach confirmed that simultaneous depletion of both genes is more efficient by CONJUDOR compared to mixing RNAi bacteria (Supplementary Figure S5). Notably, double RNAi by mixing bacteria yields a similar number of animals with germlines positive for *unc-25::GFP* as single RNAi against *lin-53* or *rbbp-5* (Figure 6D). A lack of additive or synergistic enhancement could be interpreted as a cooperative function of LIN-53 and RBBP-5 in the same pathway or complex to counteract germ cell conversion. However, CONJUDOR against *lin-53* and *rbbp-5* indicates an additive effect compared to single RNAi against *lin-53* and *rbbp-5*, rather suggesting functions in separate processes. Such additive effect is also obtained when using an RNAi clone with stitched *lin-53* and *rbbp-5* in the *L4440* plasmid (Figure 6D). Together with previous comparisons of double RNAi by mixing versus CONJUDOR our results provide evidence that CONJUDOR is suitable for double RNAi to assess ge-





**Figure 6.** CONJUDOR identifies RBBP-5 as a novel barrier of germ cell to neuron reprogramming. (A) Illustration of CONJUDOR to generate double RNAi bacteria targeting *lin-53* together with ~700 other chromatin regulators. F<sup>+</sup> *EPI300* bacteria containing *LoriT-lin-53* conjugation with *HT1115* RNAi bacteria clones from the previously published chromatin RNAi library (16). (B) Transgenic animals expressing the GABAergic motor neuron fate marker *unc-25::GFP* and allowing heat-shock-inducible broad *UNC-30* overexpression were fed with conjugated bacteria to assess germ cell to neuron conversion. (C) CONJUDOR screening for animals with GABAergic fate marker *unc-25::GFP* ectopic expression in the germline. Feeding of *LoriT-lin-53* conjugated with *rbbp-5* RNAi bacteria resulted in a marked increase of *unc-25::GFP* expression induction in the germline. The stippled line indicates the chosen cut-off for enhancement. See material and methods and Supplementary Table S4 for details. (D) Direct comparison of *lin-53*.CON.*rbbp-5* to single RNAi against *rbbp-5*, *lin-53*, and *L4440-lin-53* mixed with *L4440-rbbp-5* *HT1115* RNAi bacteria. CONJUDOR is allowing GABAergic fate reporter expression in the germline more efficiently than mixing RNAi bacteria. Control: Rluc RNAi. Statistics: Multiple t-test with two-tailed distribution. p1 = 0.612; p2 = 0.864; p3 = 0.001; p4 = 0.0004; p5 = 0.202. Total analyzed animals (triplicate) in (D) n = 949. Error bars represent SEM. For details on scoring and screening see Supplementary Table S4. (E) Representative DIC / GFP signal pictures of animals with germ cells expressing the GABAergic neuron fate reporter *unc-25::GFP* upon simultaneous depletion of LIN-53 and RBBP-5 by CONJUDOR and broad overexpression of *UNC-30*. White arrow heads indicate germline with reprogrammed cells. White stippled line in magnification of germline areas outline bodywalls. (F) Model of preventing germ cell conversion to GABAergic motor neurons by RBBP-5 and LIN-53.

netic interactions, which may be more accurate compared to mixing RNAi bacteria clones.

Overall, we show that our newly developed CONJUDOR technique is highly efficient to conduct high-throughput double RNAi screens. By using CONJUDOR we identified the Set1/MLL methyltransferase complex member RBBP-5 as a previously undiscovered germ cell reprogramming barrier demonstrating versatility and robustness of this novel double RNAi technique.

## DISCUSSION

Depletion of gene activities by RNAi-mediated knockdown is important for investigating gene functions. In particular, genes that cause embryonic lethality or developmental arrest when fully eliminated by knockout approaches (e.g. by mutagenesis or CRISPR/Cas9-mediated excision) can often not be interrogated for their implication in post-developmental processes. Yet, RNAi-mediated co-depletion of such essential genes to investigate complex biological processes in *C. elegans* is challenging. Previous approaches, such as feeding mixed RNAi bacteria, suffer from lack of robustness, as we demonstrate also in this study, or are not practical for high-throughput screens. For instance, ‘stitching’ together two target genes by cloning into the *L4440* plasmid to produce dsRNA against both targets in the same bacterial cell has been shown to mediate robust double RNAi (8). While this approach is certainly a reliable method for double RNAi, generating, e.g., 700 new plasmids each containing two target genes with subsequent bacterial transformations limits practicability and flexibility for high-throughput double RNAi screens.

We developed a new method of conducting combinatorial RNAi in *C. elegans* based on bacterial conjugation, which we termed CONJUDOR. By creating a two-component system consisting of conjugation-competent F<sup>+</sup> *E. coli* (based on *EPI300*), which contain the F-plasmid *pRK24-KanR*, and a modified RNAi donor plasmid *LoriT*, we are able to co-deplete genes simultaneously in a robust manner. We demonstrate CONJUDOR for model targets (*GFP* and *RFP* in muscle nuclei) as well for several endogenous genes. Sterility due to defective oocyte maturation is caused when both *oma-1* and *oma-2* genes are co-depleted (28). This ‘*oma*’ phenotype is caused with significantly higher efficiency by CONJUDOR when compared to mixing the two *oma-1* and *oma-2* *HT115* RNAi bacteria. While the stitched version of *oma-1::oma-2* appeared to be slightly more efficient in inducing sterility, we could not obtain a 100% sterility effect for both double RNAi approaches as shown previously for the double mutants *oma-1(te33)*, *oma-2(te51)* (8,28). A possible explanation for this outcome is that RNAi causes a knockdown effect, which allows varying degrees of remaining transcripts as compared to full knockouts.

Furthermore, feeding conjugated bacteria versus mixed bacteria consistently induced double RNAi with higher efficiency against all tested gene combinations such as *mex-3* and *gld-1* (germline teratomas) as well as *rpn-10* and *rpn-12* (lethality).

Based on the robustness of CONJUDOR we were able to identify the Set1/MLL methyltransferase complex mem-

ber RBBP-5 (31,32) as a novel germ cell reprogramming barrier. The rationale for using CONJUDOR to screen for chromatin factors that counteract germ cell reprogramming was based on our observation that overexpression of UNC-30 in animals with RNAi against the previously identified germ cell reprogramming barrier LIN-53 (10–12) yielded only a limited number of animals with GABAergic motor neuron fate in germ cells. Feeding worms with conjugated bacteria containing *lin-53* RNAi (*LoriT-lin-53*) and *rbbp-5* RNAi (*L4440-rbbp-5*) led to increased ectopic induction of the GABAergic neuron fate in germ cells.

Notably, comparison of *lin-53.CON\_rbbp-5* to single or mixed RNAi against *rbbp-5*, *lin-53* or *L4440-lin-53* mixed with *L4440-rbbp-5* RNAi bacteria revealed almost a doubling of the number of animals with germlines positive for the GABA neuron fate reporter *unc-25::GFP*. In contrast, mixed double RNAi against *lin-53* and *rbbp-5* showed similar numbers as single RNAi against *lin-53* or *rbbp-5*. Such lack of enhancement during genetic interaction testing is usually being interpreted as LIN-53 and RBBP-5 functioning in the same pathway or complex. Generally, it should be mentioned here that RNAi is not ideal to examine genetic interactions. To assess synergistic, synthetic, or additive effects upon loss of two genes, principally the use of null-mutants allows more consistent conclusions. Yet, knockdown by CONJUDOR of *lin-53* and *rbbp-5* revealed an additive effect compared to mixed double RNAi. This result suggests functions of the chromatin-regulating factors LIN-53 and RBBP-5 in separate regulatory pathways. This notion is supported by previous studies showing that LIN-53 cooperates with the PRC2 chromatin silencer to safeguard the germ cell fate and counteract conversion to neurons (11,12). Depletion of LIN-53 or PRC2 subunits resulted in a global loss of chromatin silencing in the germline as revealed by abolished H3K27 methylation (11). In contrast, it was demonstrated that RNAi against *rbbp-5*, which is part of the chromatin-regulating complex SET1/MLL/COMPASS (31,33), reduces H3K4 methylation in the germline. Thus, the enhancement observed upon simultaneous RNAi knockdown of *lin-53* together with *rbbp-5* is likely due to distinct effects on germline chromatin. The finding that LIN-53 and RBBP-5 may act in parallel pathways due to the observed additive effect suggests that CONJUDOR provides a reliable technique for double RNAi to assess genetic interactions. Mutants of these essential genes can otherwise not be tested in the context of germline safeguarding in adult animals due to lethality or early developmental arrest (33,34). Generally, double RNAi by CONJUDOR may provide more accurate genetic interaction testing of essential genes as compared to mixing RNAi bacteria clones. Alternatively, stitching the gene pairs such as *lin-53* and *rbbp-5*, or *oma-1* and *oma-2* in the *L4440* RNAi plasmid provides another highly robust and efficient double RNAi. This approach could be considered for assessments where high-throughput combinations of gene pairs are not required. Also, where possible, the use of a null-mutant in combination with RNAi against the second gene may provide more reliable genetic interaction assessments.

The exact mechanism of how RBBP-5 safeguards the germline to prevent conversion to GABAergic motor

neuron-like cells remains to be determined and will be subject of future research. It is possible that RBBP-5 limits reprogramming of germ cells to GABAergic neurons because the Pitx-type homeodomain TF UNC-30 activates gene expression differently than the Zn-finger TF CHE-1. Further investigation will shed light on possible molecular mechanisms of germ cell safeguarding by RBBP-5. Here, we used the identification of RBBP-5 to highlight the power and versatility of CONJUDOR and provide evidence for its efficiency. As most biological processes are co-regulated by the orchestrated activity of several genes, CONJUDOR opens new perspectives for all research fields that make use of the genetic model *C. elegans* to address open question *in vivo*. Moreover, robust triple RNAi could be performed by ‘stitching’ two target genes into *LoriT* with subsequent conjugation to other RNAi clones thereby further increasing the multiplexing of knockdowns.

Overall, further developments such as CONJUDOR, possibly in combination with applications such as inducible degrons introduced by gene editing (35), are likely to increase the complexity of RNAi-based screens for investigating biological processes in an unprecedented manner.

## DATA AVAILABILITY

Scoring of RNAi phenotypes are provided in Supplementary Table S4, which also contains information of repeat experiments and number of tested animals (n).

*C. elegans* strains generated in this study will be made available through CGC (<https://cgc.umn.edu>). The plasmids *pRK24-KanR* (163692) and *LoriT* (163691) are available via Addgene ([www.addgene.org](http://www.addgene.org)).

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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*Conflict of interest statement.* None declared.

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