

## Video Article

# Application of RNAi and Heat-shock-induced Transcription Factor Expression to Reprogram Germ Cells to Neurons in *C. elegans*

Ena Kolundzic<sup>1</sup>, Stefanie Seelk<sup>1</sup>, Baris Tursun<sup>1</sup><sup>1</sup>Berlin Institute for Medical Systems Biology, Max Delbrück Center for Molecular Medicine in the Helmholtz AssociationCorrespondence to: Baris Tursun at [baris.tursun@mdc-berlin.de](mailto:baris.tursun@mdc-berlin.de)URL: <https://www.jove.com/video/56889>DOI: [doi:10.3791/56889](https://doi.org/10.3791/56889)Keywords: Developmental Biology, Issue 131, Germ Cell, Transcription Factor-Induced Reprogramming, *Caenorhabditis elegans*, Epigenetic Reprogramming Barrier, RNA Interference, Heat-Shock Induction, Neuronal Reprogramming

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## Abstract

Studying the cell biological processes during converting the identities of specific cell types provides important insights into mechanism that maintain and protect cellular identities. The conversion of germ cells into specific neurons in the nematode *Caenorhabditis elegans* (*C. elegans*) is a powerful tool for performing genetic screens in order to dissect regulatory pathways that safeguard established cell identities. Reprogramming of germ cells to a specific type of neurons termed ASE requires transgenic animals that allow broad over-expression of the Zn-finger transcription factor (TF) CHE-1. Endogenous CHE-1 is expressed exclusively in two head neurons and is required to specify the glutamatergic ASE neurons fate, which can easily be visualized by the *gcy-5prom::gfp* reporter. A trans gene containing the heat-shock promoter-driven *che-1* gene expression construct allows broad mis-expression of CHE-1 in the entire animal upon heat-shock treatment. The combination of RNAi against the chromatin-regulating factor LIN-53 and heat-shock-induced *che-1* over-expression leads to reprogramming of germ cell into ASE neuron-like cells. We describe here the specific RNAi procedure and appropriate conditions for heat-shock treatment of transgenic animals in order to successfully induce germ cell to neuron conversion.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/56889/>

## Introduction

Cell fate reprogramming by ectopic TF expression such as the myogenic TF MyoD, which, when overexpressed, converts fibroblasts directly into muscle-like cells<sup>1</sup>, has been a research focus for decades. However, differentiated cells are often refractory to this process, due to mechanisms that safeguard their cellular identity. Germ cells show a similar degree of protection and there are underlying mechanisms that often act as a barrier to prevent germ cell conversion into other cell types upon over-expression of a fate-inducing TF. Typically, epigenetic regulation such as histone modifications and chromatin structure imposes an impediment for the conversion of cell fates<sup>2,3</sup>. We have previously applied reverse genetics using RNAi knock-downs in *C. elegans* and identified factors that safeguard cellular identities and thereby counteract reprogramming of germ cells into neurons<sup>4</sup>. Specifically, the polycomb repressive complex 2 (PRC2) and the highly conserved histone chaperone LIN-53 (CAF-1/RBBP4/7 in humans) prevent the direct conversion of germ cells into specific somatic cell types such as glutamatergic taste neurons in *C. elegans*<sup>4,5</sup>. To identify these germ cell reprogramming barrier factors, we used transgenic animals carrying the heat-shock inducible Zn-finger TF CHE-1. CHE-1 is normally expressed in two head neurons of *C. elegans*, where it specifies the fate of glutamatergic taste neurons termed ASE<sup>6</sup>. The ASE neuron fate can be visualized by expression of the ASE-specific fluorescent reporter *gcy-5prom::gfp*. GCY-5 is a chemoreceptor only expressed in ASE right neuron<sup>7</sup>. Upon *lin-53* depletion by RNAi and induction of broad ectopic expression of CHE-1 by heat-shock, germ cells can be converted into neurons *in vivo*<sup>4,5</sup>. Importantly, timing of RNAi treatment is crucial as only adult worms (P0 generation) exposed to *lin-53* RNAi give rise to F1 progeny animals with a germline that is permissive for reprogramming into neurons<sup>4</sup>.

The above-described germ cell to neuron conversion procedure in *C. elegans* can be used to study a variety of biological questions such as the implication of signaling pathways in cell fate reprogramming. For instance, we used the CHE-1-induced germ cell reprogramming into ASE neurons upon RNAi against *lin-53* in order to study the implication of the Notch signaling pathway in the process of germ cell reprogramming<sup>8</sup>. By performing double RNAi to co-deplete *lin-53* and the histone demethylase-encoding gene *utx-1* we showed that the Notch signaling pathway counteracts PRC2-mediated gene silencing by activating expression of UTX-1 in the germline<sup>8</sup>. This finding demonstrates that the germ cell conversion to neurons described in this protocol could be used to study a complex biological process such as cellular reprogramming in an intact organism and in the context of different developmental and environmental conditions. Furthermore, it provides the perspective to challenge germ cells by over-expressing different fate-inducing TFs to study their role in restriction of cellular plasticity<sup>9</sup>, or to assess their potential for inducing reprogramming of germ cells to other cell types *in vivo*.

While mammalian tissue cultures also allow studying different aspects of cell fate reprogramming, cells grown in culture have limited capacity to mimic signaling pathway conditions compared to an intact organism. Therefore, *C. elegans* is a versatile model for examination of the roles of

various biological processes such as Notch signaling during reprogramming *in vivo*. Additionally, it is a powerful model for genetic screens that is relatively easy to maintain and genetically modify for low costs.

## Protocol

All methods described here with regards to animal care have been approved by the LaGeSo Berlin, Germany

### 1. Solution Preparation

1. **NGM-Agar plates (1 L)**
  1. Add 3 g of NaCl, 2.5 g of peptone media (e.g., Bacto-Peptone), and 20 g of agar. After autoclaving, add 1 mL of cholesterol (5 mg/mL in 95% EtOH stock), 1 mL of 1 M MgSO<sub>4</sub>, 1 mL of 1 M CaCl<sub>2</sub>, 25 mL of 1 M K<sub>2</sub>PO<sub>4</sub>, and 1 mL of amphotericin B (2.5 mg/mL stock).
2. **NGM-Agar RNAi plates (1 L)**
  1. Add 3 g of NaCl, 2.5 g of peptone media, and 20 g of agar. After autoclaving add, 1 mL of cholesterol (5 mg/mL in 95% EtOH stock), 1 mL of 1 M MgSO<sub>4</sub>, 1 mL of 1 M CaCl<sub>2</sub>, 25 mL of 1 M K<sub>2</sub>PO<sub>4</sub>, 1 mL of amphotericin B (2.5 mg/mL stock), 1 mL of 1 M IPTG, and 1 mL carbenicillin (50 mg/mL).
3. **LB-Agar (1 L)**
  1. Add 10 g of peptone media, 5 g of yeast extract, 5 g of NaCl, 20 g of agar, 10 mL of 1 M Tris pH 8.0. Add H<sub>2</sub>O to 1 L. After autoclaving, add 1 mL of 50 mg/mL carbenicillin and 2.5 mL of 5 mg/mL tetracycline.
4. **LB Medium (1 L)**
  1. Add 10 g of peptone media, 5 g of yeast extract, 5 g of NaCl, and 10 mL of 1 M Tris pH 8.0. Add H<sub>2</sub>O to 1 L. After autoclaving, add 1 mL of 50 mg/mL carbenicillin.
5. **M9-buffer (1 L)**
  1. Add 6.0 g of Na<sub>2</sub>HPO<sub>4</sub>, 3 g of KH<sub>2</sub>PO<sub>4</sub>, 5 g of NaCl, and 50 mg of gelatin. Before usage, add 1 mL of 1 M MgSO<sub>4</sub>.
6. **Bleaching solution (10 mL)**
  1. Add 1 mL of NaClO and 2 mL of 5 N NaOH. Add H<sub>2</sub>O to 10 mL.

### 2. Preparation of RNAi Plates

Note: *C. elegans* is typically cultured in the laboratory on 6 cm Petri plates containing 7.5 mL of Nematode Growth Medium Agar (NGM-Agar)<sup>10,11</sup>. This protocol is optimized for worms kept at 15 °C. To prepare plates with NGM, use standard sterile techniques to prevent fungal and bacterial contamination. The following is the protocol to prepare NGM plates supplemented with reagents for RNAi.

1. **Prepare 6-cm NGM-Agar RNAi plates containing 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 50 μg/mL carbenicillin. Let them dry for 24 – 48 h at room temperature in the dark<sup>12</sup>.**
  1. Keep RNAi plates at 4 °C in the dark. Do not use if older than 14 days.
2. **Select the RNAi bacteria (*Escherichia coli* HT115) clone containing the L4440 plasmid with the *lin-53* gene DNA sequence from the frozen glycerol stock from the available RNAi library<sup>13</sup> on LB-agar plates containing 50 μg/mL carbenicillin and 12.5 μg/mL tetracycline by using the three-phase streaking pattern. Grow the bacteria at 37 °C overnight<sup>12</sup>. This clone allows IPTG-dependent production of *lin-53* dsRNA in the bacteria.**
  1. Additionally, grow RNAi bacteria that contain the L4440 plasmid without any gene sequence as the empty vector control<sup>14</sup>.
3. The next day, pick a single colony from *lin-53* or empty vector LB-agar plates using a 200 μL pipette tip and inoculate each of them into a separate culture tube containing 2 mL of liquid LB medium<sup>14</sup> supplemented with 50 μg/mL carbenicillin. Grow cultures overnight at 37 °C until they reach an optical density (OD) at 600 nm of 0.6 – 0.8. Measure the OD using a spectrophotometer<sup>15</sup>.  
CAUTION: The liquid LB media should not contain tetracycline in contrast to the LB-agar plate used for streaking the bacteria from the glycerol stock, since inclusion of tetracycline during feeding leads to a decreased RNAi efficiency<sup>12</sup>
4. **Add 500 μL of each bacterial culture (*lin-53* or empty vector) to 6 cm NGM-Agar RNAi plates using a multipipette. Incubate plates with a closed lid overnight at room temperature in the dark to dry. During this time, the IPTG in the NGM plates will induce production of dsRNA in the bacteria.**
  1. Use at least 3 plates per bacterial culture per experiment. This will provide 3 technical replicates for each experiment.
5. Store dried NGM-agar RNAi plates with bacteria at 4 °C in the dark for up to two weeks.

### 3. Preparation of *C. elegans* Strain BAT28

1. **Maintain the worm strain BAT28<sup>8</sup> containing the transgenes *otIs305* [*hsp-16.2prom::che-1, rol-6(su1006)*] and *ntl1* [*gcy-5prom::gfp*] on OP50 bacteria using standard NGM-Agar plates at 15 °C.**  
Note: For detailed protocol on Nematode culture, see<sup>10</sup>. The *rol-6(su1006)* is a dominant allele and commonly used phenotypic injection marker<sup>16</sup> that causes the typical rolling movement. It is used in the *otIs305* transgene to track *hsp-16.2prom::che-1*.

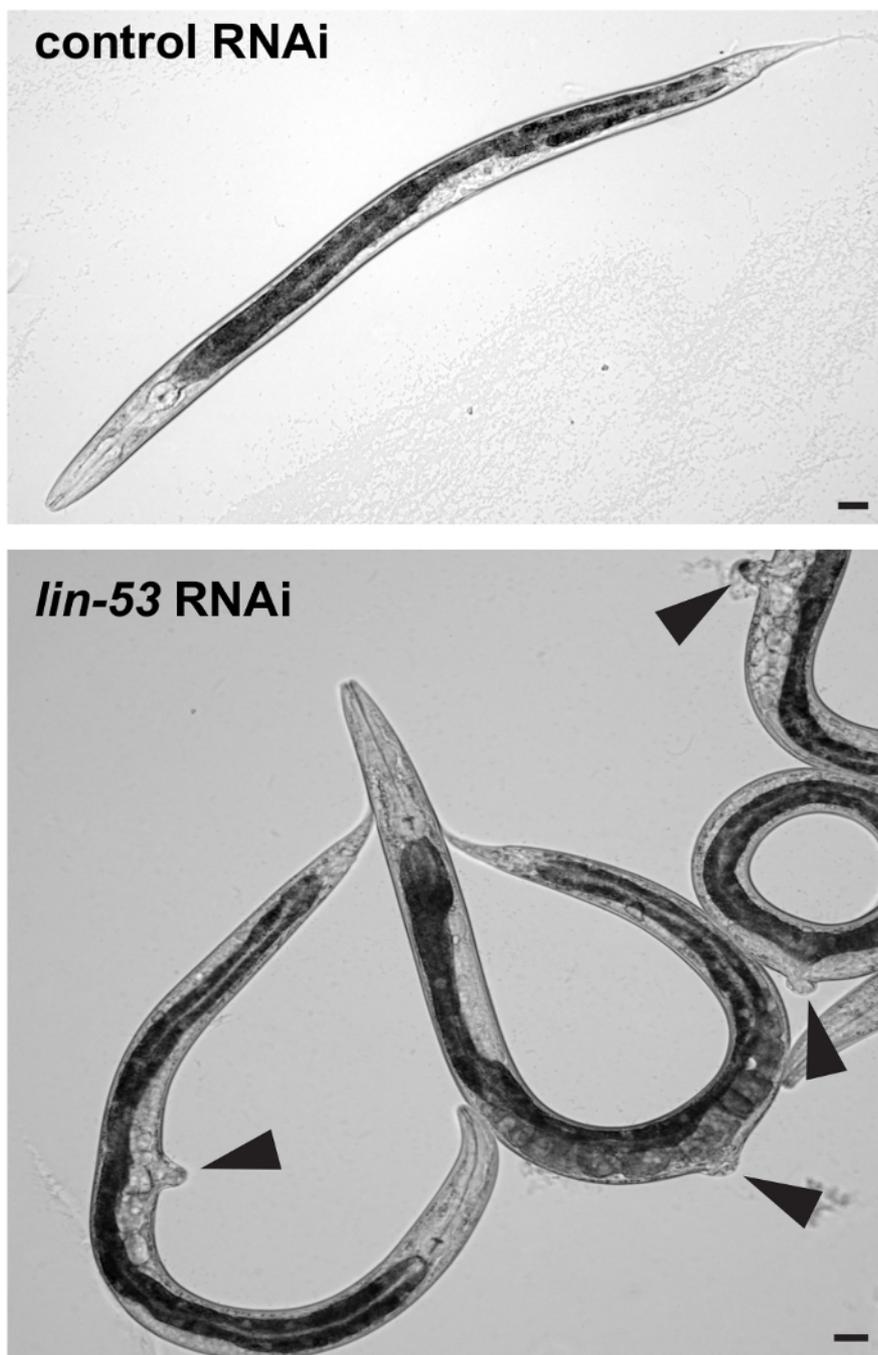
1. Keep the BAT28 strain at 15 °C at all times in order to prevent precocious activation of the *hsp-16.2prom::che-1* transgene. Reduce exposure to temperatures higher than 15 °C while handling the strain as much as possible.
2. **To age-synchronize worms, use the bleaching technique<sup>17</sup>.**
  1. Wash off 6 cm NGM-Agar plates containing adults and eggs of BAT28 using 900 µL M9 buffer. Pellet worms by centrifuging at 900 x g for 1 min. Remove the supernatant.
  2. Add 0.5 – 1 mL of bleaching solution and shake the tube for approximately 1 min until adult worms start to burst open. Monitor using a standard stereomicroscope.
  3. Pellet worms by centrifuging at 900 x g for 1 min. Remove the supernatant.
  4. Wash the worm pellet 3 times by adding 800 µL of M9 buffer and centrifuging at 900 x g.
  5. Place the cleaned eggs on fresh NGM-plates seeded with OP50 bacteria. Grow a bleached population on OP50 bacteria at 15 °C until they reach L4 stage (approximately 4 days). L4 larvae can be recognized by a white patch approximately halfway along the ventral side of the worm<sup>18</sup> using a standard stereomicroscope.  
Note: To achieve the germ cell to neuron conversion phenotype upon depletion of *lin-53*, it needs to be depleted in the parental generation (P0). The scoring for the conversion phenotype is undertaken in the following generation (filial generation F1). To achieve the deplete *lin-53* already in the P0, L4 animals are subjected to RNAi.
3. **Manually transfer 50 L4 stage worms per replicate using a platinum wire to an NGM plate that does not contain any bacteria and let worms move away from any transferred OP50 bacteria. Let worms move on the plate for around 5 minutes. Use bacteria from the NGM-Agar RNAi plates previously seeded with *lin-53* RNAi bacteria (or empty vector control) to transfer to the respective RNAi plates.**
  1. Avoid transferring OP50 bacteria to the actual RNAi plates. Work fast to minimize exposure time of the strain to temperatures higher than 15 °C.
4. **Incubate worms on NGM-Agar RNAi plates at 15 °C for approximately 7 days until F1 progeny of the worms reaches L3 – L4 stage. They can be clearly separated from the P0 animals since they are bigger and thicker than the F1 progeny.**
  1. Visually check under a standard stereomicroscope whether F1 progeny worms show the protruding vulva (*pvul*) phenotype as shown in **Figure 1**. RNAi against *lin-53* has pleiotropic effects and causes the *pvul* phenotype which can be used to assess whether RNAi against *lin-53* has been successful.  
Note: RNAi against *lin-53* can also cause lethality of F1 embryos. This effect increases if plates are exposed to higher degrees than 15 °C before animals reached the L3 – L4 stage. Dead embryos can be recognized by arrested development and lack of hatching.
  2. Incubate plates in the dark since IPTG is light sensitive.

## 4. Induction of Germ Cell Reprogramming

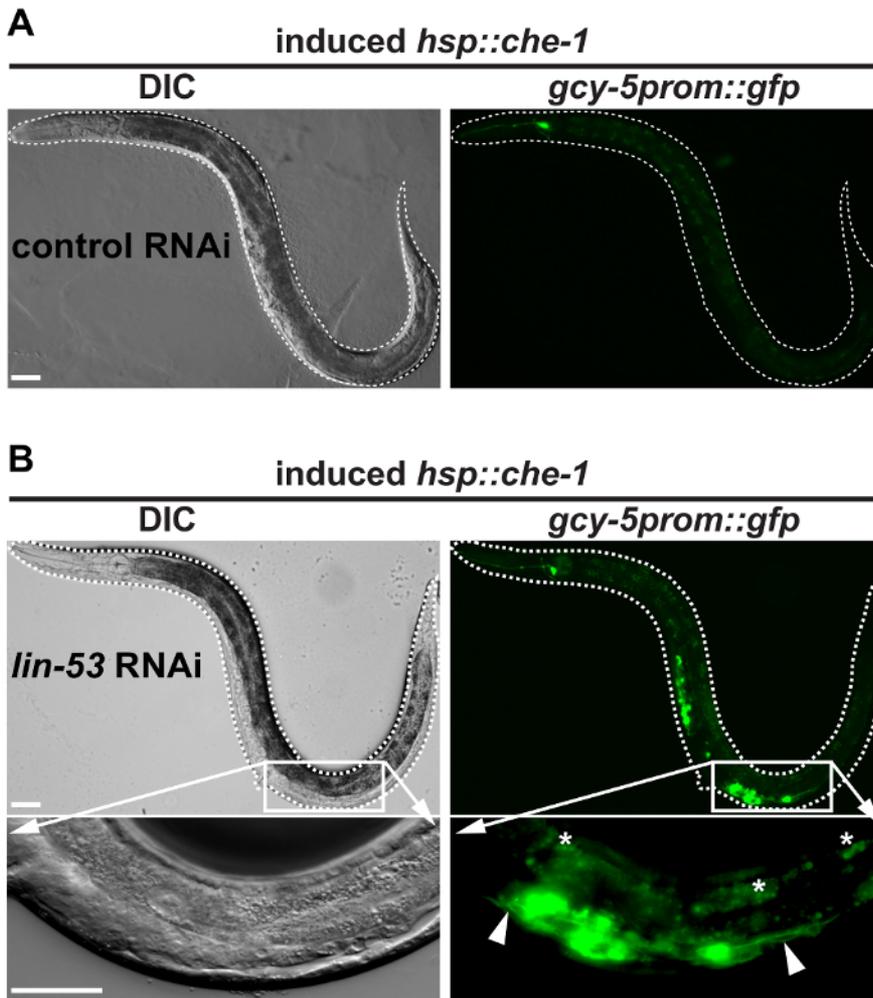
1. Incubate examined RNAi plates containing *lin-53* and empty vector RNAi-treated F1 progeny for 30 min at 37 °C in the dark to activate CHE-1. Use a vented incubator since it allows for a more efficient heat-shock.
2. Allow heat-shocked animals to recover for 30 min at room temperature in the dark and then incubate plates at 25 °C overnight in the dark.
3. Using a standard stereomicroscope coupled to a fluorescence light source, examine animals under the GFP filter for *gcy-5prom::gfp*-derived signals in the mid-body area of the worms as shown in **Figure 2**. Set microscope for GFP signals: excitation maximum at 488 nm with emission maximum at 509 nm.
4. **Evaluate the degree of germ cell to neuron conversion by mounting animals with GFP signals in the mid-body area on agar pad-containing microscopy slides<sup>19</sup>.**
  1. Count the number of animals showing the germ cell to neuron conversion vs. the dark animals to assess the phenotype penetrance. Typically, around 30% of the animals show discrete GFP signals in the germline upon *lin-53* depletion, whereas not more than 5% of the animals show diffuse GFP signals in the germline upon empty vector RNAi.

## Representative Results

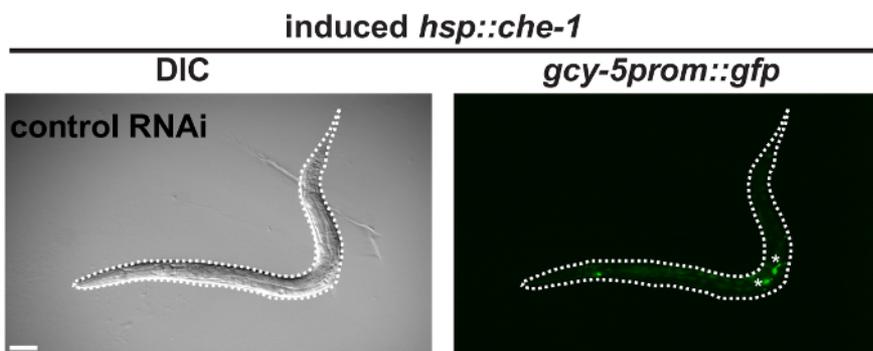
As shown in **Figure 1**, F1 animals that exhibit the *pvul* phenotype illustrate successful application of *lin-53* RNAi. These animals are prone to allow conversion of their germ cells into ASE neuron-like cells upon heat-shock induction of *che-1* over-expression as illustrated in **Figure 2**. In contrast to worms that grew on the control empty vector RNAi *lin-53* RNAi treatment will yield animals that display ectopic GFP-signals in the mid-body area after heat-shock and 25 °C incubation overnight as described earlier<sup>4,8</sup>. Closer examination under an epifluorescence microscope of these worms reveals that the cells showing GFP signals also display neuron-like projections (**Figure 2B**, white arrows) that are typical for neuronal cells. If RNAi against *lin-53* was not successful or heat-shock conditions were inappropriate GFP signals will resemble to those in control RNAi treated animals (**Figure 2**). Importantly, avoid inducing over-expression of *che-1* by heat induction before animals reach mid L3 stage. Animals that were too young at the time of *che-1* over-expression induction can display *ectopic gcy-5prom::gfp* expression in other regions of the body such as the developing vulva as shown in **Figure 3**<sup>13</sup>.



**Figure 1: Pvl phenotype caused by RNAi against *lin-53*.** (Top) DIC picture of L4/young adult stage F1 progeny worms derived from control or *lin-53* RNAi treated mothers. Scale bars = 20  $\mu$ m. (Bottom) Animals treated with *lin-53* RNAi display the protruding vulva (*pvl*) phenotype (black arrow-heads). The *pvl* phenotype confirms that RNAi against *lin-53* was successful. Scale bars = 20  $\mu$ m. [Please click here to view a larger version of this figure.](#)



**Figure 2: Worms with successfully reprogrammed germ cell into neurons.** (A) BAT28 strain animals grown on the empty vector do not show GFP signals in the germline after heat-shock induction of *che-1* overexpression. Dashed white line outlines worm. Scale bars = 20  $\mu$ m. (B) In contrast to BAT28 strain animals grown on the empty vector control RNAi animals grown on *lin-53* RNAi display *gcy-5prom::gfp* signals in the mid-body area. Pictures of the mid-body section with GFP signals taken at 63X magnification reveal GFP-positive cells showing protrusions (white arrow-heads). This morphological feature indicates that germ cells underwent conversion into ASE neuron-like cells. White asterisks mark intestine-derived auto-fluorescence. All pictures were acquired using an epifluorescence microscope with 20X magnification and 63X magnification. Dashed white line outlines worms. Scale bars = 20  $\mu$ m. [Please click here to view a larger version of this figure.](#)



**Figure 3: Early stage worm with ectopic GFP.** BAT28 strain animals grown on the empty vector RNAi bacteria that were heat-shock induced for *che-1* overexpression at early larval stages such as L2 show GFP signals in the mid-body area (white asterisks). These signals are not due to germ cell reprogramming. Dashed white line outlines worm. Scale bars = 20  $\mu$ m. [Please click here to view a larger version of this figure.](#)

## Discussion

While the described protocol using the transgenic *C. elegans* strain BAT28 and RNAi against *lin-53* is straightforward, a number of steps are critical in order to ensure the expected result of germ cell to neuron reprogramming. It is important that the strain BAT28 is kept at 15 °C at all times prior to the experiments. During handling and maintenance of the strain, the time at temperatures above 15 °C needs to be minimized as much as possible. Proper heat-shock conditions are important since insufficient induction of *che-1* overexpression will not result in germ cell to ASE neuron conversion. This can be detected by measuring the phenotype penetrance as described in the protocol. Extensive heat-shock can lead to lethality of the animals. For instance, plates that have been placed near the inner walls or on the bottom ground of a static air incubator often experience extensive heat treatment. Therefore, it is recommended to use a vented heat incubator. Additionally, timing of the heat-shock induction is critical since over-expression of *che-1* during larval stages earlier than L3 can lead to ectopic *gcy-5prom::gfp* induction in different body regions such as the vulva (Figure 3)<sup>9</sup>. Furthermore, extensive heat-shock can be detected by the phenotype penetrance in empty vector RNAi animals which should not extend 5% and increased auto-fluorescence of other tissues, namely the intestine.

Sporadically, RNAi bacteria can lose their activity to efficiently produce dsRNA of the target gene. Unfortunately, this can not be detected prior to the RNAi experiment. In such cases a fresh streak from the glycerol should be grown as described in Section 2 of the protocol. If there is still no RNAi-caused pleiotropic phenotype visible such as the *pvul* phenotype caused by successful RNAi against *lin-53*, then a plasmid DNA isolation from the respective bacteria should be performed and fresh HT115 bacteria need to be transformed with the L4400 plasmid containing the *lin-53* sequence.

Importantly, the BAT28 strain has a the roller phenotype caused by the injection marker pRF4, which was used during transgenesis of the animals with the *hsp-16.2prom::che-1* DNA construct. Occasionally, animals lose the rolling phenotype, which can indicate silencing of the *hsp-16.2prom::che-1* transgene. To properly maintain the BAT28 strain, pick 10 roller animals to a fresh plate and propagate selecting for rolling animals.

The application of the protocol is limited to the F1 RNAi procedure, which means that animals whose parents (P0 generation) have not been exposed to *lin-53* RNAi will not show germ cell to neuron conversion due to maternal rescue<sup>4</sup>. An alternative procedure to convert germ cells to neurons has been described by Ciosk and colleagues<sup>15</sup> using RNAi knock-down of *gld-1* and *mex-3* without over-expression of a transcription factor. However, the obtained neurons do not belong to a specific type of neurons and germ cells also convert to muscle-like cells upon RNAi-mediated depletion of *gld-1* and *mex-3*<sup>15</sup>. Previously, it has been demonstrated the RNAi against *lin-53* allowed germ cell conversion into GABAergic neuron-like cells upon over-expression of the Pitx-type homeodomain transcription factor UNC-30<sup>16</sup> instead of CHE-1<sup>4</sup>. For future directions, different fate-inducing transcription factors can be tested whether *lin-53* depleted germ cells can be converted to other cell types than specific neurons. In this context, overexpression of the myogenic bHLH transcription factor HLH-1, homolog of the mammalian MyoD<sup>17</sup>, induces conversion of germ cell to muscles upon *lin-53* RNAi<sup>5</sup>. The established reprogramming of germ cells to a specific somatic cell type upon *lin-53* RNAi and over-expression of an appropriate fate-inducing transcription factor can be used for a number of different genetic screens. Such suppressor or enhancer screens can help dissecting regulatory pathways that play a role during cell fate conversion.

## Disclosures

The authors have declared that no competing interests exist.

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