Protocol

Induction of Germline Apoptosis in *Caenorhabditis elegans*

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RNA interference (RNAi) is an incredibly powerful tool for rapid and efficient knockdown of gene expression. This technology can be used to induce apoptosis in the germline of *Caenorhabditis elegans*. Genotoxic stressors such as ionizing radiation (IR), ultraviolet light, chemical mutagens (e.g., *N*-ethyl-*N*-nitrosourea [ENU]), and DNA cross-linking reagents can also be used to stimulate apoptosis. These approaches, described here, combined with the powers of in vivo imaging methods, should keep *C. elegans* apoptosis researchers busy for several years, sorting out how various signaling pathways influence life and death decisions in this organism.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Caenorhabditis elegans strain of interest (e.g., *lin-35[n745]*, *rrf-1[pk1417]*, or *rrf-3[pk1426]*)

L1 larval stage worms are typically used for the RNAi procedure (see Step 5), whereas L4 worms are used for IR or ENU treatments (see Steps 6 and 8). The hypochlorite method is a standard technique that is used to synchronize worms to the L1 larval stage, as well as to remove contamination. This procedure, along with many other basic worm handling techniques, can be found in Wormbook (Stiernagle).

ENU (Sigma-Aldrich N3385)

Freshly dilute the ENU stock solution to 5 mM in M9 buffer <R> each time it is used.

HT115 bacteria (see Step 1)
Isopropylthio-β-galactoside (IPTG) (0.1 M)
Luria Bertani (LB) + amp and tet <R>

Prepare both liquid medium and plates containing solid medium.

OP50 bacteria (optional; see Steps 6–9)
Plates containing solid nematode growth medium (NGM) <R>
Plates containing solid RNAi medium <R>
RNAi clones from the *C. elegans* RNAi feeding library (Source BioScience LifeSciences; see Discussion)

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Bacterial RNAi lawns plated on NGM plates remain useful for experiments when stored at 4°C for ~1 mo. However, in our experience, some RNAi bacterial strains do not last this long when stored in the cold. If you plan to use a particular RNAi feeding strain repeatedly, we recommend preparing your own glycerol stock and storing it at −80°C to avoid repeated access of the stock library.

Equipment

Gamma (^{137}Cs source), X, or UV irradiator
Imaging setup (see Protocol: **Visualizing Apoptosis in Embryos and the Germline of *Caenorhabditis elegans*** [Lant and Derry 2014])

For high-resolution imaging and quantification of apoptotic bodies, we recommend a compound microscope that is equipped with differential interference contrast (DIC) optics (63× oil immersion lens).

Incubators at 20°C and 37°C
Microcentrifuge tubes (0.5 mL)
Orbital shaker at 37°C
P200 tips (sterile)
Tubes (15-mL conical)

METHOD

Examining Apoptosis Using RNAi

1. Select a targeted RNAi clone of interest from the *C. elegans* RNAi feeding library. Using a sterile P200 tip, streak from the glycerol stock onto a plate containing LB + amp and tet. Incubate the plates overnight at 37°C.

   Streaking the plate in a cross hatch pattern is optimal for creating single colonies.

   A negative control consisting of an empty RNAi plasmid (or a gene with no strong RNAi phenotype) in the HT115 bacterial strain should be cultured concurrently.

2. On the next day, harvest individual colonies with a sterile P200 tip and transfer to 15-mL conical tubes containing 4 mL of liquid LB + amp and tet. Grow overnight on an orbital shaker at 37°C.

   Liquid RNAi cultures can be stored at 4°C for up to 2 wk for repeated use.

   See Troubleshooting.

3. On the next day, add 20 µL of 0.1 M IPTG to each culture and incubate an additional 4 h on the shaker at 37°C.

   IPTG induces T7 RNA polymerase in the RNAi bacteria.

4. Spread 70 µL of each bacterial culture on plates containing solid RNAi medium, and allow the bacteria to grow overnight at room temperature.

   Ensure that lids are kept on the plates during the overnight growth period to prevent contamination. See Troubleshooting.

5. Add L1-stage worms to the bacterial lawn and allow them to grow until they are at the correct stage to observe apoptosis phenotypes (either young adult for germline apoptosis or after they have begun to lay eggs in the next generation for embryonic apoptosis). Proceed to Step 10.

   Typically worms are placed onto RNAi-expressing bacterial lawns at the L1 stage, but if the targeted gene is essential, plating at the L3 or L4 stage may be necessary to observe a strong loss-of-function phenotype without affecting viability of the organism.

   See Troubleshooting.

Examining Apoptosis with Genotoxic Agents

IR is an efficient genotoxic stressor and causes no obvious morphological changes to somatic tissues. It activates the DNA damage response, thereby stimulating germline apoptosis. As an alternative, alkylating agents such as ENU can be used.

Inducing Apoptosis Using Irradiation

6. One day before irradiation, pick 25 to 40 worms from a plate at the L4 stage. Place the worms on a new NGM plate (seeded with OP50 bacteria if using a mutant strain or the desired RNAi bacterial strain if assessing the apoptosis phenotype of a particular gene after knockdown). Allow the plate to incubate overnight at 20°C.

   See Troubleshooting.

7. On the next day, expose the worms (now at the young adult stage) to a dose of IR (0–120 Gy). Allow them to recover overnight, and proceed to Step 10.

   If there is uncertainty as to what duration of radiation will be most effective to institute a quantifiable effect, the standard starting point should be 60 Gy. If the strain is suspected to have apoptotic resistance, 120 Gy should be applied initially. The irradiated strain should be run alongside equally staged controls (N2) to compare the effects of the treatment.

Inducing Apoptosis Using ENU

8. Pipette 1 µL of 5 mM ENU into the lid of a 0.5-mL microcentrifuge tube anchored on the surface of a small, seeded NGM plate (i.e., in the bacteria). Add 20 worms at the L4 or young adult stage to the ENU solution and incubate in the dark for 4 h at 20°C, with the plate inverted to prevent evaporation.

   Because of the surface tension, using this small volume will prevent the dissolved ENU and worms from falling out of the lid when inverted. Just be careful not to bump the plate too hard when moving.

9. Following incubation, carefully pipette the worms onto a new NGM plate (seeded with OP50 bacteria or target RNAi) and allow them to recover from the ENU treatment. On the next day, proceed to Step 10.

Analyzing Apoptosis

10. Observe and quantify corpses using DIC microscopy with a 63× oil immersion lens.

    Quantification of apoptosis and morphological analysis is described in Protocol: ***Visualizing Apoptosis in Embryos and the Germline of Caenorhabditis elegans*** *(Lant and Derry 2014). It is imperative to perform at least three independent biological replicates when quantifying germline apoptosis, as this tissue shows more variability compared with developmentally programmed cell death in the invariant somatic lineages. For initial characterization of putative apoptosis-defective mutants, we recommend performing a time course analysis (e.g., 6–48 h post-IR) to define the kinetics of corpse formation.*

TROUBLESHOOTING

Problem (Step 2): The bacterial streak is too thick to pick individual colonies.

Solution: This is the result of too many bacteria on the pipette tip or incubating for too long, causing colony overgrowth. Restreak from the stock and incubate for less time (18 h). Make sure the bacterial culture is not grown for longer than 24 h.

Problem (Step 4): Contamination is present on the bacterial plates.

Solution: Make sure the plate lids are closed to prevent foreign particles from contaminating the bacterial plate. If the glycerol stock is contaminated, pick several individual colonies, verify the presence of the plasmid containing the desired RNAi clone by sequencing or restriction mapping, and grow a new liquid culture. It is also possible that the stock solutions used to prepare the plates or liquid RNAi cultures are contaminated. This can be tested by plating solutions on bacterial growth plates to see if contaminating bacteria grow after 24 h at 37°C. Finally, the worms plated may contain contaminating bacteria. Some worm strains have a greater capacity for contamination (because of storage of bacteria in their gut), and as such the bleaching protocol (Stiernagle) can be modified by adding an extra two bleach wash steps followed by three sterile H_2O washes.

Problem (Step 5): RNAi does not provide the expected phenotype, if a phenotype is expected.

Solution: The particular RNAi may not have full penetrance and may require a second generation to show conserved effects. Alternatively, the single colony picked to produce the liquid culture may not have been effective; sometimes, the HT115 strain will mutate plasmids. Pick multiple colonies (making sure to stick to the "lines" of inoculation) and check individually isolated clones for the desired phenotype. However, it may be necessary to reclone the worm gene fragment into a fresh L4440 vector and retransform HT115 bacteria. This has worked well for the *ced-9* RNAi bacteria strain, which we have observed to frequently become ineffective for gene knockdown by the feeding method.

Problem (Step 5): RNAi does not provide a strong phenotype.

Solution: As mentioned above, cultivating the worms on the target RNAi for a further generation may increase the consistency of any phenotypes seen. Alternatively, if the knockdown phenotype is not penetrant we recommend using strains carrying mutations in genes that cause hypersensitivity to RNAi, such as *rrf-3(pk1426)* or *lin-35(n745)* (Simmer et al. 2002; Lehner et al. 2006).

Problem (Step 5): RNAi is too toxic or causes morphological defects that make it difficult to observe apoptosis phenotypes.

Solution: Strains that are resistant to RNAi in the soma, such as the *rrf-1(pk1417)* mutant (Sijen et al. 2001), can be used to selectively ablate genes in the germline. These strains should be used when ablation in the soma causes morphological defects that make assessment of the germline difficult. However, caution should be exercised when using *rrf-1* alleles, as it has been recently reported that these mutants are not resistant to RNAi in all somatic tissues (Kumsta and Hansen 2012).

Problem (Step 6): The L4 stage has been missed, and there is an imperative to irradiate as soon as possible.

Solution: Worms can be irradiated on the same day, provided that they are distinctly at the young adult stage. Hence, the operator must be able to distinguish the stages of adulthood efficiently. Apoptotic nuclei in the germline are difficult to see as the worm ages because the intestine accumulates fat that can occlude the bends of the gonad arms, where apoptosis is detectable.

DISCUSSION

RNAi is not always 100% efficacious, as it causes a reduction in gene function that can be variable in penetrance—compared to the complete loss of function in null mutants or consistent reduction in function of hypomorphic alleles. The main advantages of using RNAi are the incredible ease and speed with which the function of a particular gene can be ascertained. The results from RNAi gene ablation, while not always as penetrant and consistent as those obtained using mutant strains, can be used to systematically screen multiple gene targets concurrently and rapidly. But care must be taken in both culturing RNAi clones and assessing phenotypes. We recommend confirming the identity of RNAi clones by sequencing or restriction mapping and using mutant strains to confirm RNAi phenotypes and epistatic interactions with other genes. Observing a phenotype by RNAi may not be effective or reproducible in the first generation, depending on the nature of the gene or the RNAi clone. This will require troubleshooting, either of the developmental stage when the worms are placed on the RNAi feeding strains or of the number of generations on RNAi necessary to obtain a penetrant phenotype.

The Source BioScience LifeSciences RNAi feeding library provides an extensive array of RNAi clones stored as frozen glycerol stocks in a 384-well plate format. This library represents over 86% of the predicted genes in the *C. elegans* genome, including almost all known apoptosis genes. For control experiments, RNAi ablation of core apoptosis pathway genes (Fig. 1) can be used to ensure that assays

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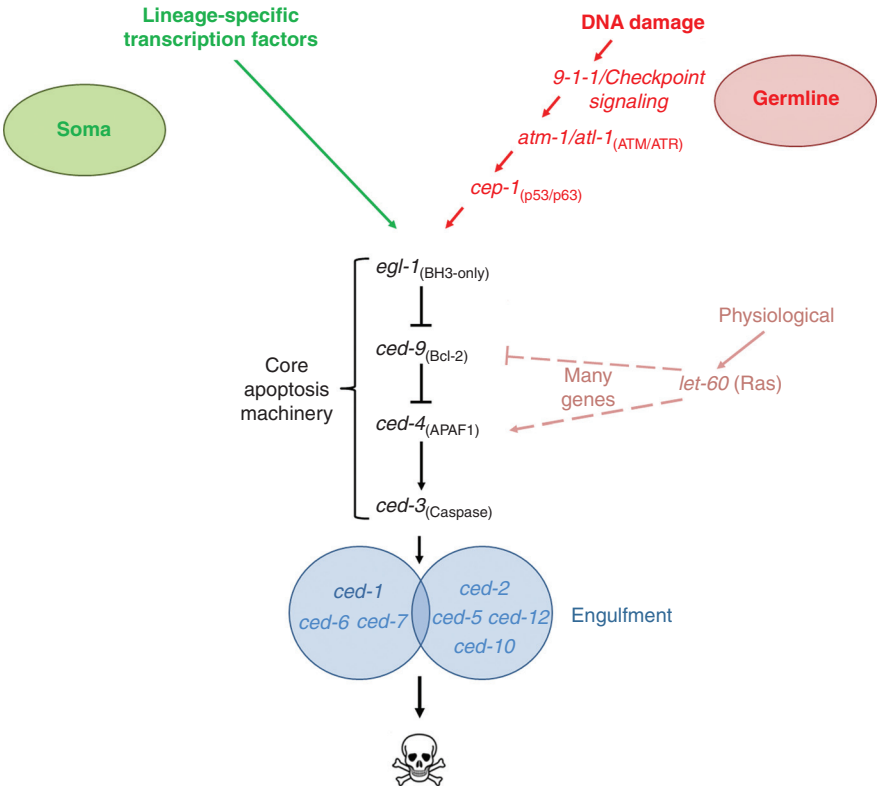

FIGURE 1. Apoptosis signaling in *C. elegans*. Somatic apoptosis uses the core machinery and is regulated by controlling the levels of *egl-1* through lineage-specific transcription factors. Germline apoptosis can be broken into two categories: physiological and stress-induced. Stress-induced apoptosis (indicated as DNA damage) requires the activities of both *cep-1* and *egl-1*, whereas physiological apoptosis is controlled by independent pathways that activate the core apoptotic proteins CED-4 and CED-3 (Gumienny et al. 1999). Following core apoptosis machinery activation and culminating in the activation of the CED-3 executioner caspase, DNA degradation and engulfment machinery is also activated (full components not shown), resulting in the complete death and removal of the cell.

and reagents are working properly. In addition, targeting known apoptosis genes that stimulate or repress apoptosis when knocked down is useful in establishing epistatic order for new apoptosis genes for which mutant strains are available. Whole-genome profiling studies are revealing previously undetected variations in the genomes of various *C. elegans* strains, many of which are predicted to disrupt gene function (Flibotte et al. 2010). Thus, it is also important to confirm that the apoptotic phenotype of a given mutant strain is actually caused by the suspected gene by either rescuing the phenotype with a transgene or validating with different mutant alleles. While rescue of a mutant phenotype by expression of a wild-type transgene is the desirable method to confirm that genotype corresponds to phenotype, it is technically challenging to express genes in the *C. elegans* germline. As an alternative, the RNAi feeding method can be used to confirm that ablation of a suspect gene in wild-type animals can recapitulate the apoptosis phenotype. Whenever possible, apoptosis phenotypes detected using RNAi should be confirmed using strains that contain loss-of-function alleles to the corresponding gene.

Stimulation of apoptosis with genotoxic stress can be achieved through a number of strategies. The source of toxicity should be selected appropriately. We outlined IR treatment here, as it is a common activator of the DNA damage response in the *C. elegans* germline and requires much less optimization than a drug dissolved in buffer, such as ENU. However, RNAi to the *rad-51* gene causes double-strand breaks in DNA that can be used as an alternative to irradiation to activate the *cep-1*-dependent germline apoptosis signaling pathway (Lettre et al. 2004). By stimulating germline apoptosis via irradiation, the effects of a mutation (or gene ablation by RNAi) are exaggerated in proportion with the radiation dose. Of course, using an appropriate control for comparison is imperative, and

provides verifiable and consistent baselines for stress-induced apoptosis (for both qualitative and quantitative metrics).

RECIPES

Luria Bertani (LB) + Amp and Tet

1. To prepare solid medium, mix 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 15 g of agar, and bring to 1 L with H_2O. (To prepare liquid medium, combine all ingredients except agar.)
2. Autoclave for 30 min.
3. Let cool for 1 h in a 55°C water bath.
4. Add 2 mL of 50 mg/mL ampicillin and 2 mL of 5 mg/mL tetracycline.
5. While the agar-containing medium is still warm, pour a thin layer (~0.5 cm) into each Petri dish and let cool to solidify.
6. Store dishes or liquid medium at 4°C for up to 1 mo.

M9 Buffer for Worms

1. Dissolve 3 g of KH_2PO_4, 6 g of Na_2HPO_4, and 5 g of NaCl in 1 L of H_2O.
2. Autoclave for 20 min.
3. Add 1 mL of 1 M $MgSO_4$.
4. Store at room temperature. After 2 wk, check for visible contamination before use.

Nematode Growth Medium (NGM)

1. For solid NGM, mix 3 g of NaCl, 2.5 g of peptone, and 20 g of agar and bring to 1 L with H_2O. (For liquid NGM, prepare without agar.)
2. Autoclave for 1 h.
3. Let cool for 1 h in a 55°C water bath.
4. Add 1 mL of cholesterol (5 mg/mL in ethanol), 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 25 mL of 1 M (pH 6.0) KPO_4, mixing after each addition.
5. Using an automated plate pourer (peristaltic pump), pour the medium into sterile plastic plates to set. During pouring, keep the NGM on a hot plate with stirrer to prevent the medium from solidifying.
6. Store NGM plates at 4°C until use. Seed NGM plates with bacteria the day after pouring. Warm the plates to room temperature before adding the worms. (For liquid NGM, store at room temperature for up to 1 mo. Regularly check for excess cloudiness to ensure bacterial contamination is absent; this is particularly important for liquid RNAi cultures.)

RNAi Medium

1. Prepare 1 L of nematode growth medium (NGM) by following Steps 1–4 of the NGM recipe. <R>
2. Add 2.5 mL of 0.1 M isopropylthio-β-galactoside (IPTG) and 500 µL of 50 mg/mL carbenicillin.
3. Using an automated plate pourer (peristaltic pump), pour the medium into sterile plastic plates to set. During pouring, keep the medium on a hot plate with stirrer to prevent the medium from solidifying.
4. Store RNAi plates for up to 1 mo at 4°C.

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