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A phenotype-based RNAi screening for Ras-ERK/MAPK signaling-associated stem cell regulators in *C. elegans*

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Summary

Stem cells have the ability to self-renew and to generate differentiated cell types. A regulatory network that controls this balance is critical for stem cell homeostasis and normal animal development. Particularly, Ras-ERK/MAPK signaling pathway is critical for stem cell self-renewal and differentiation in mammals, including humans. Aberrant regulation of Ras-ERK/MAPK signaling pathway results in either stem cell or overproliferation. Therefore, the identification of Ras-ERK/MAPK signaling pathway-associated regulators is critical to understand the mechanism of stem cell (possibly cancer stem cell) control. In this report, using the nematode *C. elegans* mutants, we developed a methodology for a phenotype-based RNAi screening that identifies stem cell regulator genes associated with Ras-ERK/MAPK signaling within the context of a whole organism. Importantly, this phenotype-based RNAi screening can be applied for other stem cell-associated signaling pathways such as Wnt/ β -catenin and Notch using the *C. elegans*.

Keywords

Stem cell; Ras-ERK/MAPK signaling; high-throughput RNAi screening; *Caenorhabditis elegans*

1. Introduction

Stem cells are defined by their ability to make more stem cells (a property known as “self-renewal”), retain the potential to generate specific cell types (termed “pluripotency”), and give rise to specific cell type (termed “differentiation”) [1] (Fig. 1A). Stem cell self-renewal, pluripotency, and differentiation are tightly controlled by intrinsic regulatory networks that respond to various extrinsic signaling stimuli, including Notch, LIF/STAT3, Wnt/ β -catenin, FGF/ERK, TGF/SMAD and PKC signaling [2] (Fig. 1A). Abnormalities in these pathways may cause the formation of cancer stem cells and tumors [3]. Therefore, understanding the signaling pathways and identifying important factors help to understand stem cell self-renewal, pluripotency, and differentiation for tissue engineering as well as application for regenerative medicine and cancer therapy.

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Ras-ERK/MAPK signaling pathway is crucial for stem cell regulation [4–6]. For example, Ras-ERK/MAPK signaling pathway plays an important role in driving the extracellular matrix (ECM)-induced osteogenic differentiation of human mesenchymal stem cells (hMSCs) [7]. In addition, suppression of Ras-ERK/MAPK signaling pathway by chemical inhibitors (e.g., U0126, PD98059, PD184352, or PD0325901) promotes self-renewal and pluripotency maintenance of mouse embryonic stem cells (mESCs) [6,4,8,9] and improves fibroblast reprogramming [10] (Fig. 1A). In contrast to mESCs, high basal ERK/MAPK activity is required for maintaining human ESCs (hESCs) in an undifferentiated state [5] (Fig. 1A). Moreover, inhibition of ERK/MAPK activity by chemical inhibitors rapidly caused the loss of hESC self-renewal capacity [5]. Therefore, Ras-ERK/MAPK signaling pathway controls both self-renewal and differentiation of mammalian stem cells, depending on the cellular context (Fig. 1A).

Ras-ERK/MAPK signaling pathway is also highly conserved in the nematode *Caenorhabditis elegans* (*C. elegans*) (Fig. 1B). In *C. elegans*, two different receptor tyrosine kinases (RTKs; LET-23/EGFR and EGL-1/FGFR) stimulate LET-60/Ras and an MAPK cascade that consists of LIN-45/RAF, MEK-2/MEK and MPK-1/ERK [11,12] (Fig. 1B). This Ras-ERK/MAPK signaling pathway controls multiple cellular processes, including induction of vulval, uterine, spicule, P12 and excretory duct cell fates, control of sex myoblast migration and axon guidance, as well as regulation of germline stem cells and their differentiation [11,13,12,14]. Particularly, Ras-ERK/MAPK signaling pathway controls the self-renewal and differentiation of GSCs, depending on the genetic context: MPK-1/ERK activation promotes the proliferation of GSCs in *puf-8*; *gap-3* mutant background [15], but it also promotes the differentiation of GSCs in wild-type [14] and *puf-8*; *lip-1* mutant background [16]. Notably, aberrant MPK-1/ERK activation in *puf-8*; *lip-1* mutants promotes sperm fate [16], resulting in masculinization of germline (Mog) sterility at permissive temperature (20°C) (Fig. 2C and 2D). Genetic mutation (e.g., *mpk-1*) or chemical inhibition (e.g., U0126) of Ras-ERK/MAPK signaling pathway is able to reprogram germ cells from sperm fate to oogenic fate, and restores fertility to *puf-8*; *lip-1* mutants, like wild-type [16] (Fig. 2C and 2D). Interestingly, *puf-8*; *lip-1* mutants also develop germline tumors via the dedifferentiation of spermatocytes at restrictive temperature (25°C) [13]. This dedifferentiation mechanism has been implicated in cancer stem cell formation and tumorigenesis [17,18]. Therefore, *puf-8*; *lip-1* animals may facilitate the identification of novel candidate genes that may be involved in Ras-ERK/MAPK signaling-mediated stem cell regulation and tumorigenesis. To date, several conserved regulators that affect Ras-ERK/MAPK signaling pathway have been identified in diverse model system [19–22]. However, current approaches are mostly focused on a target specific screening or forward/reverse genetic analyses, and are labor and time intensive. In addition, the use of live organisms for genetic screening is technically challenging.

In this report, we developed a phenotype-based RNAi screening method to identify stem cell regulators that are associated with Ras-ERK/MAPK signaling pathway using *C. elegans* as a model system. This feeding RNAi approach provides several advantages over RNAi method using double strand (ds) RNA microinjection. Those include: 1) rapid and convenient knock-down of large numbers of genes, 2) convenient screening of wells using a fluorescence microscope, and 3) ease of multiple knock-downs. Moreover, Ras-ERK/MAPK signaling

pathway and its positive and negative regulators are closely associated with stem cell control and tumorigenesis. Therefore, identification and characterization of Ras-ERK/MAPK signaling-associated regulators provide a promise for stem cell control, tumorigenesis, and regenerative medicine.

2. Materials

2.1. *C. elegans* mutants

For an RNAi-based screening, several Ras/MAPK signaling-associated mutants will be used (Table 1). *C. elegans* strains of interest can be obtained from Caenorhabditis Genetics Center (CGC): <http://cbs.umn.edu/cgc/home>.

2.2. Nematode Growth Media (NGM)

For 1 L of NGM agar media, mix 3 g NaCl, 2.5 g Bacto Peptone, 17 g agar, 1 mL 1 M CaCl₂, 1 mL 1M MgSO₄, 25 mL potassium phosphate (pH 6.0), and dd H₂O to a final volume of 971 mL, and autoclave. After autoclaving and cooling to ~55°C, add 1 mL of 5 mg/mL Cholesterol in 95% Ethanol (EtOH). Pour NGM media to 6 mm Petridishes manually or using a pouring system.

2.3. Luria Bertani (LB) for OP50 *E. coli* bacteria culture

For 1 L of LB media, mix 10g Bacto Tryptone, 5 g Bacto Yeast, 10 g NaCl, and dd H₂O to a final volume of 1 L, and autoclave.

2.4. M9 buffer

For 1 L of M9 buffer, mix 5 g NaCl, 6 g Na₂HPO₄, 3 g KH₂PO₄, and dd H₂O to a final volume of 999 mL, and autoclave. After autoclaving, add 1 mL of autoclaved 1 M MgSO₄.

2.5. Bleaching solution

For 1 L of bleaching solution, mix 245.9 mL of 1N Sodium hydroxide, 196.7 mL of Sodium hypochloride (household bleach) and 557.4 mL of autoclaved ddH₂O in a sterilized bottle.

2.6. RNAi plates

After making normal NGM media and autoclaving, add 1 ml of ampicillin (100 mg/mL) and 1 ml of 0.5 M IPTG when autoclaved NGM media are cooled down to 55°C and pour 96-well deep-well plates or 35 mm Petridishes manually or using a pouring system.

2.7. RNAi libraries (see Table 2)

2.8. Equipment

Autoclave machine

Automated worm sorter, COPASTM BIOSORT (Union Biometric)

ArrayScan V^{TI} HCS Reader (Cellomics, ThermoFisher)

Centrifuge machine

Fluorescence microscopy
 Moist sealed plastic containers
 Optical microscope
 PCR machine
 Robotic liquid handling system
 SpotDetector BioApplication
 Temperature-controlled incubator for worm maintenance
 Temperature-controlled shaking incubator for bacteria cultivation
 ThinSeal (Sigma-Aldrich)

3. Methods

3.1. *C. elegans* culture

Worms are cultured in the laboratory on 100 mm NGM plates seeded with OP50 *E. coli* bacteria at permissive temperature (20°C), as described [23].

1. Inoculate OP50 *E. coli* bacteria colony in 100 mL LB media.
2. Culture at 37°C with shaking for overnight.
3. Apply approximately 0.5 ml of OP50 *E. coli* bacteria liquid culture to 60 mm NGM plates using pipet (see Note 1).
4. Allow the OP50 *E. coli* bacteria lawn to grow overnight at room temperature (see Note 2).
5. Transfer worms to NGM plates with OP50 *E. coli* bacteria and culture at 20°C.

3.2. Generation of *puf-8*; *lip-1*; [*Pmyo-2::mCherry*] transgenic mutants

Wild-type adult hermaphrodites produce both sperm and oocytes. They are therefore self-fertile (Fig. 2A and 2D). Since *puf-8(q725)* single homozygote mutants are self-fertile like wild-type [24] (Table 1; Fig. 2B and 2D), these animals can be used directly in RNAi screening to identify candidate genes that may inhibit Ras-ERK/MAPK signaling pathway (Fig. 2B). For example, RNAi of *lip-1* in *puf-8* mutants produces only sperm (Mog phenotype) and they are sterile (Fig. 2B and 2D). *lip-1* encodes a dual-specificity phosphatase (a mitogen-activated protein kinase phosphatase (MKP) homolog), which inhibits an ERK phosphorylation [25]. Consistent with RNAi result, 100 % of *puf-8(q725)*; *lip-1(zh15)* double mutants exhibit Mog phenotype and are all sterile (Table 1; Fig. 2C and 2D). The inhibition of Ras-ERK/MAPK signaling pathway by either genetic mutation (*mpk-1b* RNAi) or chemical inhibition (U0126) reprogram sperm cell fate into an oogenic cell fate, and *puf-8*; *lip-1* mutants regain fertility [16] (Fig. 2C and 2D). Therefore, *puf-8*

¹For general strain maintenance, medium size plates (60 mm diameter) are useful. For a high-throughput RNAi screening, 96-well or 24-well microtiter plates (Nunc MicroWell 96) are desirable. To confirm the RNAi results, small plates (35 mm diameter) are useful.

²Seeded plates can be stored at the cold room until needed.

single or *puf-8; lip-1* double mutants may facilitate the identification of novel candidate genes that either inhibit or activate Ras-ERK/MAPK signaling pathway.

To enable efficient worm sorting and image acquisition of progeny on plates, an integrated copy of the *Pmyo-2::mCherry* transgene (expressing red-fluorescent protein, mCherry in the pharynx) could be integrated into the *puf-8; lip-1* mutant background (Fig. 3A and 3B) [26]. Homozygous *puf-8; lip-1* mutants are sterile (Fig. 3D) and must be maintained as heterozygotes using a balancer chromosome *mIn[mIs14(Pmyo-2::GFP) dpy-10(e128)]*, (henceforth called “*mc6g*”) (Fig. 3C). Homozygous *mc6g* mutants have an abnormal, dumpy (*dpy*) body and express GFP in the pharynx. Therefore, *puf-8; lip-1* homozygote mutants have non-GFP and non-dumpy (Fig. 3D). The incorporation of the *Pmyo-2::mCherry* marker will help simplify assay development for the detection of *puf-8; lip-1* progeny that were difficult to identify using bright field optics (Fig. 3E and Fig. 4). Although this protocol mostly describes a methodology to identify Ras-ERK/MAPK signaling-associated activators using *puf-8; lip-1* double mutants, *puf-8* single mutants can also be used to identify Ras-ERK/MAPK signaling-associated inhibitors (Fig. 2B). To generate *puf-8(q725)/mc6g; lip-1; [Pmyo-2::mCherry]* transgenic mutant,

1. Pick approximately ten adult *puf-8/mc6g; lip-1* heterozygote hermaphrodites and transfer them to a new NGM agar plate with OP50 *E. coli* bacteria (henceforth called NGM/OP50).
2. Pick and transfer five *Pmyo-2::mCherry* male transgenic worms to the NGM/OP50 plate with ten adult *puf-8/mc6g; lip-1* hermaphrodites.
3. Incubate them for 3–5 days in 20°C.
4. F1 singling: Pick approximately four F1 adult males (L3-L4 stages), expressing *Pmyo-2::mCherry* but not *Pmyo-2::GFP* (a balancer chromosome) from the plate and transfer them to new NGM/OP50 plates. Genotype of F1 males will be *puf-8/+; lip-1/+ ::[Pmyo-2::mCherry]*.
5. Pick and transfer approximately ten adult *mc6g; lip-1* hermaphrodites (GFP(+) in the pharynx) with dumpy phenotype to the NGM/OP50 plate with *puf-8/+; lip-1/+ ::[Pmyo-2::mCherry]* males.
6. Incubate at 20°C until F2 progeny are L3-L4 stage (typically, this takes about 2–3 days). (see Note 3)
7. F2 singling: Pick approximately twenty F2 hermaphrodites worms (L3-L4 stages) expressing both mCherry and GFP from the plates and transfer them to new NGM/OP50 plates (one worm per plate).
8. Incubate F2 hermaphrodites for 2 days at 20°C.
9. Genotype by single worm PCR and sequencing.
10. Maintain *puf-8/mc6g; lip-1/lip-1; [Pmyo-2::mCherry]* worms at 20°C.

³Approximately 25% of F2 cross progeny expressing both mCherry and GFP will be *puf-8/mc6g; lip-1/lip-1; [Pmyo-2::mCherry]* worms.

3.3. RNAi bacterial preparation

RNAi libraries (Table 2) can be purchased from BioScience. For a high-throughput feeding RNAi screening,

1. Prepare 96-well deep-well plates containing 400 μ l of LB media and ampicillin (100 μ g/mL final) using a robotic liquid handling system (a sterile pinning device), but can also be performed manually if necessary.
2. Redistribute the RNAi clones into 96-well deep-well plates containing LB/ampicillin using a robotic liquid handling system, but can also be performed manually if necessary.
3. Seal each plate with ThinSeal (Sigma-Aldrich, St. Louis, MO, USA) film and incubate at 37°C with shaking for overnight.
4. Add IPTG (a final concentration of 4 mM) to each well.
5. Reseal each plate and incubate for 1 h with shaking to induce production of dsRNAs.
6. Centrifuge the plates for 5 min at 3,500 RPM to pellet the bacteria.
7. Carefully remove the supernatant and resuspend the bacterial pellet with 400 μ l of LB/100 μ g/mL ampicillin/4mM IPTG.

3.4. Preparation of L4 staged *puf-8*; *lip-1*; [*Pmyo-2::mCherry*] for a high-throughput feeding RNAi screening

A. Embryo isolation—

1. Culture *puf-8/mc6g*; *lip-1*; [*Pmyo-2::mCherry*] transgenic mutants on ten 100 mm NGM/OP50 plates at 20°C until there are many healthy, non-starved adult worms on the plates.
2. Collect mixed-staged worms into a 15 ml conical tube using an M9 buffer (see Materials) and spin down using a swing bucket centrifuge.
3. After removing M9 buffer carefully, add 5 ml of bleaching solution (see Materials) to the tube and mix the tube by inverting gently for about 5–6 minutes or until intact adult worms are dissolved.
4. Once most of the bodies have dissolved, centrifuge them at 1,000 RPM for 1 minute, remove bleaching solution, and immediately add 10 ml of M9 buffer to the tube and mix well.
5. To remove the remaining bleaching solution, wash three times with M9 buffer.
6. Finally, add 5–10 ml of M9 buffer to the tube and allow the eggs hatch overnight with gentle rocking in a 20°C incubator.

B. Worm sorting and RNAi—Most *puf-8* single mutants are self-fertile [27,24] (Fig. 2B), but *puf-8*; *lip-1* homozygous mutants are sterile [16] (Fig. 2C and 3D). Thus, *puf-8*; *lip-1* homozygous mutants are maintained as heterozygotes using a balancer chromosome,

“*mc6g (mIn[mIs14(Pmyo-2::GFP) dpy-10(e128)])*”. Homozygotes for experimentation are selected by picking GFP(-), non-dpy animals using a fluorescence microscope. However, for large-scale animal handling, it would be advantageous to automatically identify homozygous animals using a fluorescent marker (Fig. 3). To this end, *puf-8/mc6g; lip-1/lip-1; [P_{Pmyo-2}::mCherry]* worms expressing both a red-fluorescent protein, mCherry, in the pharynx will be used (Fig. 3). The incorporation of the red-head marker, allows effective isolation of homozygous *puf-8; lip-1* animals [GFP(-) and mCherry(+)] (Fig. 3D) using an automated worm sorter, COPAS™ BIOSORT (Union Biometric, Holliston, MA, USA). Furthermore, the mCherry head marker also simplified detection of *puf-8; lip-1* progeny that was difficult to identify using bright field optics (Fig. 4).

1. The hatched L1 larvae (from Step 3.4-A) are plated on fifteen 100 mm NGM/OP50 plates and incubated at 20°C for 2 days (the majority of the animals will be at the L4 larval stage). (see Note 4)
2. Sort approximately twelve L4 transgenic *puf-8; lip-1; [P_{Pmyo-2}::mCherry]* worms into each well of a 96-well (Nunc MicroWell 96) with 40 ul of the induced RNAi culture (see Step 3.3) using COPAS™ BIOSORT, which enables worm sorting into a more homogeneous population based on animal size and fluorescence intensity [26]. However, it can also be performed manually if necessary (see Note 5)
3. Place the plates into moist sealed plastic containers to avoid evaporation and incubate in a 25°C shaking incubator for 2–3 days.

3.5. Image acquisition and identification of initial hits

1. Anesthetize worms with 4 mM levamisole or 50 mM sodium azide prior to image capture.
2. Acquire images with the ArrayScan V^{TI} HCS Reader (Cellomics, ThermoFisher, Pittsburgh, PA, USA) fitted with a 2.5× objective and a 0.63× coupler. The images are captured utilizing TRITC channel assay [26]. It can also be performed manually using a dissecting fluorescence microscope if necessary.

3.6. RNAi hit validation

RNAi clones yielding absolute z-score of 0.5 are verified by repeating the assay three times. These values are then compared with the transgenic strain fed on positive (e.g., *mpk-1b RNAi*) and negative (e.g., *vector RNAi*) controls for statistical significance through

⁴Theoretically, approximately 50% of the total worm population will have *puf-8/mc6g; lip-1; [P_{Pmyo-2}::mCherry]* genotype, approximately 25% of them will have *mc6g; lip-1; [P_{Pmyo-2}::mCherry]* genotype, and approximately 25% of them will have *puf-8; lip-1; [P_{Pmyo-2}::mCherry]* genotype.)

⁵To sort homozygous *puf-8; lip-1; [P_{Pmyo-2}::mCherry]* mutants manually, approximately several hundreds young adult *puf-8; lip-1; [P_{Pmyo-2}::mCherry]* sterile homozygotes are cultured in 100 ml K-media with 50 uM U0126 (an MEK1/2 chemical inhibitor) for 5–6 days (every one or two days, add U0126 chemicals). The chemical inhibition of MPK-1/ERK will fully rescue the sterility of *puf-8; lip-1* homozygotes as we reported in [16]. Once *puf-8; lip-1* fertile homozygotes are approximately more than 10,000, embryos are isolated by worm bleaching and allowed to hatch at 20°C for 12 hours. The resulting L1-staged worms will be then put on the 48-well microtiter RNAi plates (20 worms/each RNAi well). The *puf-8; lip-1* progeny arising from chemically reprogrammed worms are Mog sterile as adults because they make only sperm in the absence of U0126 at 20°C [16,35]. This method will prepare enough *puf-8; lip-1* homozygotes for a large-scale RNAi screening. Sterility will be confirmed by *vector (RNAi)*.

unpaired Student's two-tailed *t*-test ($P < 0.05$). RNAi clones that do not show statistically significant data in two of three experiments are excluded from further analysis. The gene ontology of the validated hits is analyzed as described in O'Reilly et al. [28].

1. Culture bacteria with a hit RNAi clone in LB/ampicillin media at 37°C with shaking for overnight.
2. Spin down at 4,400 RPM for 10 minutes.
3. Carefully remove the supernatant and resuspend the RNAi bacterial pellet with 400 µl of LB/ampicillin media.
4. Seed resuspended bacteria on RNAi plates (approximately 100 µl per plate) and induce the production of dsRNAs at room temperature for 1–2 days.
5. Place L4 staged *puf-8; lip-1; [Pmyo-2::mCherry]* worms (non-GFP) on RNAi plates and incubate them at 20°C for 2–3 days.
6. Analyze the effect of RNAi on Ras-ERK/MAPK suppression by measuring the number of viable progeny in wells.
7. Determine their germline phenotypes by staining dissected gonads with gametes markers (anti-RME-2 for oocytes and anti-SP56 for sperm) and DAPI.

3.7. Genetic analysis of RNAi hits

To test whether RNAi hits are associated with Ras-ERK/MAPK signaling pathway in *C. elegans* germline, several mutant strains can be employed: for example, *mpk-1(ga111)* temperature-sensitive, loss-of-function mutant [29] and *let-60(ga89)* temperature-sensitive, gain-of-function mutant [30] (Table 1). At 20°C, most *mpk-1(ga111)* and *let-60(ga89)* mutants are typically fertile, but at 25°C, aberrant Ras-ERK/MAPK signaling makes them sterile: *mpk-1(ga111)* mutants exhibit pachytene germ cell arrest at 25°C [29] and the *let-60(ga89)* mutants exhibit a multivulva and germline sterile phenotype at 25°C [30]. If the RNAi hit enhances *mpk-1(ga111)* sterility even at 20°C, it will suggest that the target gene normally promotes Ras-ERK/MAPK signaling in *C. elegans* germline. This pachytene arrest phenotype can be determined by staining dissected gonads with membrane marker (e.g., anti-SYN-4 [31]), gamete markers (anti-RME-2 for oocytes [32] and anti-SP56 for sperm [33]) or DAPI. This result can also be confirmed by the RNAi hit in *let-60(ga89)* at 25°C: RNAi hit may suppress the multivulva phenotype and sterility of *let-60(ga89)* at 25°C.

4. Future directions and possible applications

This phenotype-based feeding RNAi screening will be suitable for the identification of new candidate genes associated with the Ras-ERK/MAPK signaling pathway that have undetected in conventional screening and/or due to multiorgan complexity. Once candidate genes are identified, future directions may include 1) analysis of their expression patterns using a single molecule fluorescent *in situ* RNA hybridization (smFISH) and/or immunofluorescence (Yoon et al., unpublished results), 2) biochemical analysis to test whether the candidate genes are putative MPK-1/ERK substrates [20], and 3) functional analysis of the candidate genes in mammalian stem cells.

Phenotype-based feeding RNAi screening method can be applied for other signaling pathways such as Wnt and Notch using *C. elegans*. Like Ras-ERK/MAPK signaling pathway, the aberrant regulation of these signaling pathways results in sterility [34]. Therefore, phenotype (sterility to fertility)-based RNAi screening may provide a read-out for other signaling pathways through the detection of viable progeny in a high-throughput format. In addition, this phenotype-based screen method can be easily modified for identification of small-molecules that influence Ras-ERK/MAPK, Notch, or Wnt/ β -catenin signaling pathways [34]. Therefore, this approach will provide valuable insight into genetic factors that are associated with conserved signaling pathways and accelerating the discovery of new therapeutics for cancer and other diseases caused by aberrant regulation of Ras-ERK/MAPK, Notch, or Wnt/ β -catenin signaling pathways that are well conserved in *C. elegans*.

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Figure 1. Stem cell regulation and Ras-ERK/MAPK signaling

(A) A simple regulatory network that controls stem cell self-renewal, pluripotency, and differentiation. Stem cells have the capacity to self-renew and give rise to specific cell types (differentiation). This network is tightly regulated by diverse extrinsic signaling pathways (e.g., Ras-ERK/MAPK) and intrinsic factors (e.g., transcriptional regulators). In particular, Ras-ERK/MAPK signaling pathway can either promote or inhibit stem cell differentiation, depending cellular context. (B) Conserved Ras-ERK/MAPK signaling pathway in mammals and *C. elegans*. In *C. elegans* germline, Ras-ERK/MAPK signaling pathway is critical for meiotic germ cell progression, oocyte maturation, and sperm fate specification.

Figure 2. A phenotype-based RNAi screening that identifies Ras-ERK/MAPK regulators
 (A) Schematic of adult *C. elegans* hermaphrodite. Cells at the distal end (*) of the germline, including germline stem cells (GSCs), divide mitotically (yellow). As cell move proximally, they enter meiosis (green) and differentiate into either sperm (blue) or oocytes (pink). (B–C) Strategy for the phenotype-based identification of genes that either inhibit (B) or activate (C) Ras-ERK/MAPK signaling using *C. elegans* mutants.

Figure 3. *puf-8/mc6g; lip-1/lip-1; [Pmyo-2::mCherry]* transgenic mutants

(A) Schematic of *C. elegans* head. (B) Reporter transgenes. *puf-8* mutants are maintained using a chromosome II balancer, mc6g, which is marked with *Pmyo-2::GFP* and *dpy-10(e128)*. *puf-8* heterozygotes and *lip-1* homozygotes (*puf-8/mc6g; lip-1/lip-1; [Pmyo-2::mCherry]*) have normal body shape and express both GFP and mCherry (red) in the pharynx and they are fertile (C). *puf-8* homozygotes and *lip-1* homozygotes (*puf-8/puf-8; lip-1/lip-1; [Pmyo-2::mCherry]*) have normal body shape and express only mCherry in the pharynx and they are sterile (D). Inhibition of MPK-1/ERK activity by either *mpk-1b*(RNAi) or U0126 treatment rescues *puf-8; lip-1* sterility (E) and their progeny have *Pmyo-2::mCherry* (Red) fluorescence in the pharynx, which enables effective measuring of the number of viable progeny in wells. Homozygotes for the mc6g balancer also express both GFP and mCherry in the pharynx, but they have an abnormal, dumpy (dpy) body (not shown).

Figure 4. An image-based approach for detecting progeny

Well images are acquired using the Arrayscan V^{Ti} and analyzed for the presence of progeny using the SpotDetector BioApplication, but can also be performed manually using fluorescence dissecting stereo-microscopy. *mpk-1b*(RNAi) rescues *puf-8; lip-1* Mog sterility and their progeny that express mCherry are detected by SpotDetector BioApplication (Artificial picture).

Table 1

A list of representative Ras-ERK/MAPK signaling-associated mutants

Genotype	Strain	Ref.
<i>puf-8(q725)</i>	JK3231	[24]
<i>lip-1(zh15)</i>	AH102	[25]
<i>puf-8(q725)/mIn1 [dpy-10(e128) mIs14]; lip-1</i>	JK3961	[16]
<i>puf-8(q725)/mIn1 [dpy-10(e128) mIs14]; lip-1; [Pmyo-2::mCherry]</i>	MHL44	[26]
<i>mpk-1(ga111) unc-79(e1068)</i>	SD939	[29]
<i>let-60(ga89)</i>	SD551	[30]
<i>puf-8(q725)/mIn1 [dpy-10(e128) mIs14]; lip-1; mpk-1(ga111) unc-79(e1068)</i>	JK3997	[13,16]

Table 2*C. elegans* RNAi libraries

<i>C. elegans</i> feeding RNAi collection	Source	Approximate clone number
Ahringer lab RNAi library	Source BioScience	23,269 (including 3,507 supplementary sets)
ORFeome RNAi library	Dharmacon	11,000