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Discovering Functional ERK Substrates Regulating *Caenorhabditis elegans* Germline Development

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Abstract

The Rat Sarcoma (RAS) GTPase-mediated extracellular signal-regulated kinase (ERK) pathway regulates multiple biological processes across metazoans. In particular during *Caenorhabditis elegans* oogenesis, ERK signaling has been shown to regulate over seven distinct biological processes in a temporal and sequential manner. To fully elucidate how ERK signaling cascade orchestrates these different biological processes in vivo, identification of the direct functional substrates of the pathway is critical. This chapter describes the methods that were used to identify ERK substrates in a global manner and study their functions in the germline. These approaches can also be generally applied to study ERK-dependent biological processes in other systems.

Keywords

C. elegans oogenesis; ERK substrates; Functional genomics

1 Introduction

The Receptor Tyrosine Kinase (RTK)-RAS-ERK pathway relays extracellular signals through a conserved kinase cascade that results in phosphorylation and activation of the terminal kinase ERK [1]. ERK proteins are members of the conserved proline-directed serine/threonine MAPK (Mitogen Activated Protein Kinase) family, and are directly activated by MAPK Kinase (MEK) via dual phosphorylation on the threonine and tyrosine of the conserved TEY motif [2]. Active ERK (referred to as diphospho-ERK) then phosphorylates downstream substrates to execute multiple cellular and developmental processes [3 – 5]. ERK normally functions to regulate a diverse array of cellular processes, such as cell proliferation, cell death, and cell differentiation [6 – 8]. Inappropriate activation of ERK, due to activating mutations in the Epidermal Growth Factor Receptor (EGFR), RAS, or RAF, contributes significantly to the pathogenesis of many human diseases, including multiple tissue-specific cancers such as colorectal, pancreatic, lung, and thyroid [9 – 11]. While inappropriate ERK activation appears causal to the development of these diseases, the molecules most directly involved in disease etiology are the substrates through which ERK executes its orders. Thus, to obtain a clearer picture of the molecular basis of these diseases, it is imperative to identify ERK substrates and to dissect the genetic and molecular basis of their functions in normal and diseased states.

ERK recognizes its substrates through their possession of specific amino acid signatures, called ERK docking sites [12]. Docking sites enable high-affinity interaction between individual substrates and ERK, enabling efficient substrate phosphorylation on the appropriate phospho-acceptor site(s) (S/TP). To date, all confirmed ERK substrates contain one or more of the three characterized docking sites: the DEF domain, the D domain, the RSK domain, and the putative docking motif in the thyroid hormone receptor [12 – 18]. The functional significance of docking sites has been demonstrated in proteins from *C. elegans* to mammals [19, 20]. And, as detailed below, we leveraged the molecular signature of these docking sites to help identify 30 novel ERK substrates that execute a suite of ERK-dependent biological processes in the *C. elegans* germline [21].

C. elegans is a powerful genetic model system for dissecting the function and regulation of the RAS-ERK signaling pathway [22]. Relative to mammalian systems, which contain multiple genes for RAS and ERK, *C. elegans* contains one RAS gene (*let-60 ras*) and one ERK gene (*mpk-1 erk*), rendering it a genetically more facile system to dissect the function of these genes [23, 24]. The RAS-ERK signaling pathway controls multiple cellular and developmental events in *C. elegans* such as vulval and excretory duct development [25]. In particular, meiotic progression and oocyte development in *C. elegans* is under a tight control of the RAS-ERK signaling pathway and active ERK regulates multiple distinct cellular and developmental processes in the oogenic germline. *C. elegans* germline is essentially a tube that consists of mitotic germline stem cells at its distal end and mature oocytes at its proximal end (Fig. 1) [26]. While total ERK protein is distributed homogeneously throughout the entire germline, the active form of ERK, diphospho-ERK (dpERK), displays a dynamic, stereotyped, and bimodal localization pattern that correlates well with the characterized ERK-dependent processes in the germline (Fig. 1b) [27].

To understand the mechanisms underlying ERK-dependent events, identification of ERK substrates that mediate each of these specific processes becomes crucial. In this chapter we describe the bioinformatic, genetic, and biochemical approaches that we used to identify ERK substrates that regulate oogenesis in *C. elegans*.

2 Materials

2.1 Bioinformatics

1. WormBase.org.
2. SCAN PROSITE: prosite.expasy.org/scanprosite/.
3. Transmembrane domain prediction: www.cbs.dtu.dk/services/TMHMM/ and www.ch.embnet.org/software/TMPRED_form.html.
4. BLAST analysis: www.ncbi.nlm.nih.gov/blast/Blast.cgi.
5. T-Coffee alignment server: <http://tcoffee.crg.cat/apps/tcoffee/do:regular>.
6. Worm in situ hybridization database: nematode.lab.nig.ac.jp/db2/index.php.
7. Microarray database: <http://cmgm.stanford.edu/~kimlab/germline/> [28].
8. PFAM: pfam.xfam.org.

9. Conserved Domain Architecture Retrieval Tool: <http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>.

2.2 RNAi-Based Screen

1. cDNA regions (~500 bp to 1 kb) from candidate genes.
2. pPD128.36 double-T7 promoter vector.
3. Restriction enzymes as needed.
4. T4 DNA ligase.
5. DH10B bacterial competent cells.
6. SOC media.
7. GenElute Plasmid Miniprep Kit.
8. HT115(DE3) competent bacterial cells.
9. LB agar plate with 100 µg/mL ampicillin and 10 µg/mL tetracycline for bacterial selection.
10. LB with 100 µg/mL ampicillin and 10 µg/mL tetracycline.
11. *E. coli* OP50 strain.
12. Normal Growth Medium (NGM) plates for worm growth [29] with 0.2 % lactose and 100 µg/mL ampicillin.
13. *C. elegans* hermaphrodite mutants:
 - *rrf-1(pk1417)* I.
 - *rrf-1(pk1417)* I; *mpk-1(ga111ts)* III.
 - *rrf-1(pk1417)* I; *let-60(ga89ts)* IV.
14. Dissecting microscope.

2.3 Germline Dissection and Immunofluorescence

1. Glass embryo dishes.
2. PBST: PBS with 0.1 % Tween 20.
3. Levamisole: 100 mM stock, store at –20 °C.
4. Syringe needles, 25 G 5/8.
5. Fixation buffer: 3 % paraformaldehyde, 74.6 mM K₂ HPO₄, 29.4 mM KH₂ PO₄, pH 7.2.
6. Glass Conical tube.
7. Clinical bench-top centrifuge.
8. Glass pipets.

9. 100 % Methanol.
10. Glass culture tubes.
11. Goat serum: 30 % solution diluted in PBST.
12. Mouse anti-MAPKYT antibody (Sigma), used for dpMPK-1 analysis.
13. Rabbit anti-SYN-4 antibody (kind gift from Dr. M. Glotzer, University of Chicago).
14. Rabbit anti-PTC-1 antibody (kind gift from Dr. P. Kuwabara, University of Bristol, UK).
15. Donkey anti-mouse Alexa Fluor 594.
16. Goat anti-rabbit Alexa Fluor 488.
17. DAPI: 1 µg/mL solution, store at −20 °C.
18. Vectashield.
19. Microscope slides.
20. Agarose pad: prepared from a 2 % Agarose solution in water.
21. Glass microcapillary pipets.
22. 24 mm × 50 mm cover glass #1.
23. Compound microscope, differential interference contrast (DIC), and fluorescence microscopy.

2.4 in Vitro Kinase Assay

1. pTrcHis Topo Expression Vector.
2. pGEX5-A Expression Vector.
3. LIN-1 protein constructs (kind gift from Dr. Kerry Kornfeld, Washington University, St. Louis).
4. BL21(DE3) bacterial competent cells.
5. Ni-NTA agarose or glutathione sepharose.
6. BCA protein assay kit.
7. Bovine serum albumin.
8. Isopropyl β-D-1-thiogalactopyranoside (dioxane free): prepare 100 mM stock solution in water and use at 1 mM final concentration. Freeze the stock at −20 °C for long-term storage.
9. 10 % SDS-PAGE gel.
10. SimplyBlue SafeStain Coomassie stain.
11. Purified Myelin Basic Protein (Sigma).

12. Purified ERK2 kinase (New England Biolabs).
13. Kinase assay buffer: 50 mM Tris-HCl, 10 mM MgCl₂, 2 mM DTT, 1 mM EGTA, 0.01 % Brij 35, pH 7.5.
14. ATP.
15. [γ -³²P]ATP.
16. Trichloroacetic acid: 10 % solution in water (ice-cold).
17. 2× SDS-sample buffer: 125 mM Tris-HCl, 20 % glycerol, 4 % SDS, 0.005 % bromophenol blue.
18. Whatman P81 cellulose phosphate filter paper.
19. Phosphoric acid: 50 % solution in water (ice-cold).
20. Scintillation counter.
21. X-ray film and accessories.
22. Gel electrophoresis supplies for DNA: horizontal gel electrophoresis tanks, power supply.
23. Agarose for gel electrophoresis.

3 Methods

In the following sections, we describe background, logic, and methods to identify functional targets of MPK-1 ERK during oogenesis (outlined in Fig. 2). The methods include: (1) Bioinformatic approach to identify all potential MPK-1 ERK targets in the *C. elegans* germline, (2) Genetic approach using RNA interference-based enhancer screen to identify targets that genetically/functionally intersect with the RAS-MPK-1 ERK pathway, and (3) Biochemical approach to define the direct substrates of active MPK-1 ERK vs. indirect genetic interactors of the RAS-MPK-1 ERK pathway.

3.1 Bioinformatic Analysis

3.1.1 Identification of Putative Targets with ERK Docking Sites and Phospho-Acceptor Sequences—The MAP kinase phosphorylation sequence on targets is Serine (S) or Threonine (T), followed by a Proline (P, where P is in the +1 position) [30]. However, most S/T P sites on proteins are not phosphorylated and a number of different kinases (e.g., the Cyclin Dependent Kinases and MAP kinases) use the S/T P as the phosphoacceptor site. Therefore the S/T P site is not the primary sequence-specificity determinant on substrates. Peptide library screening does not reveal any strong ERK preferences for residues in the immediate vicinity of the phospho-acceptor, at positions −5, −4, −3, +2, +3, or +4, except for a slight bias towards a hydrophobic amino acid at −2 position [31]. Instead it has been found that distinct recognition motifs, termed “docking sites,” determine specific interactions between ERK and its substrates [13].

Docking sites (DS) are specific sequences that can dramatically increase the efficiency of phosphorylation of a substrate protein at the phospho-acceptor site. Currently there are four

known DS that mediate interactions of ERK with their substrates, the D-domain, ERK2 domain on RSK, the FXFP motif (also known as the DEF domain) and the docking site on thyroid hormone receptor [12 – 16, 32]. The best-characterized ERK docking motif is a short sequence related to the domain originally called the docking or D -domain [12]. The D-domain may appear at some distance from and in any orientation with respect to the phospho-acceptor site in the substrate. The typical D-domain sequence is a cluster of basic residues, usually two or more, followed within a few residues by Leucine-X-Leucine (LXL). However, D-domains can interact with ERK1/2, JNK/SAPK, p38 family members, and perhaps other MAP kinases. Increasing the specificity of interaction with ERK2 can be achieved when the LXL motif is followed within a few residues by hydrophobic amino acids. This modest sequence difference may result in recognition by only one or two of these types of MAP kinases, including ERK and p38 [12, 13]. D-domains (basicX 0–5 LXL and basicX 0–5 LXLXNhydrophobic sequences) are present in numerous ERK substrates including the transcription factors Elk-1, c-Jun and the MEF2 family, and upstream factors such as MEK1/2 and phosphotyrosine phosphatases.

The second motif, LAXRR, and its variants (such as LXXRR) [15] are present in several protein kinases that are ERK substrates. LXXRR is thought to be recognized specifically by ERK1/2 and is found in RSK and paralogs and Mnk2 [14]. LAXRR domain on Rsk binds to ERK1/2 directly and is required for RSK activation by ERK. LAXRR was found to be specific for ERK as p38 and JNK MAP kinase cannot use this DS [14].

The third motif is the FXFP sequence, which appears to interact only with ERK1/2 [16]. This motif is present in ETS transcription factors such as LIN-1, Sap -1, and Elk-1, protein kinases such as A-RAF, and dual-specificity protein phosphatases such as Mkp-1 and DUSP4. This motif has been shown to work independently or in combination with the D-domain to mediate kinase-substrate binding [16].

The fourth motif is the KGFFRR sequence, a basic amino acid-enriched motif first identified in nuclear thyroid hormone receptor TB β 1, and is thought to be specific for the binding of ERK1/2 [18]. Similar docking motifs were found in other members of the nuclear hormone receptor superfamily, including nuclear estrogen, glucocorticoid, and progesterone receptors, all of which are potential MAPK substrates [32].

The importance of DS is further emphasized by the conservation of these sites throughout evolution. The functional significance of such sites has been demonstrated in *C. elegans* LIN-1 protein, the ETS domain transcription factor, and its mammalian homolog, Elk-1. Both have the D-domain and the FXFP characteristic motif and their mechanism of action appears to be identical, although the position of the motifs in the proteins are not identical [19]. Additionally, the *Drosophila* counterparts of the vertebrate MEF2 and RSK proteins contain conserved docking domains based on in vitro biochemical data [17].

To determine the putative ERK substrates that contain the characteristic ERK docking site motifs, computational screens were performed against the *C. elegans* proteome. The docking site motifs used for performing the screen were: (a) basicX₀₋₅ - LXL and basicX₀₋₅ -

LXLNhyrodophobic, (b) FXFP, (c) LXXRR, and (d) KGFFRR, present within 100 amino acid on either N or C terminus of the phospho-acceptor sequence.

1. Log onto SCAN PROSITE: prosite.expasy.org/scanprosite/.
2. Select “Option 2 – submit MOTIFS to scan them against a PROTEIN sequence database.”
3. Under “STEP 1 – Enter a MOTIF or a combination of MOTIFS” box, enter S/T P phospho-acceptor site and one of the four characterized ERK-docking sites: (a) basicX₀₋₅-LXL and basicX₀₋₅-LXLNhyrodophobic, (b) KGFFRR, (c) FXFP, and (d) LXXRR (Fig. 2a).
4. Under STEP2, select “Your protein database” and enter the code “WS249” (or the current version of the worm genome annotation) for the *C. elegans* proteome.
5. Choose the display you prefer in STEP3 and click “START THE SCAN”.
6. Confirm that the ERK-docking site and phospho-acceptor sequences (SP or TP) are within 100 amino acids.
7. This search yields 2058 potential targets.

3.1.2 Identify Potential Targets with Additional Stringent Filters—Given the fairly generic nature of the search, the computational screen often detects multiple proteins. We detected over 2000 proteins that carried at least one of the ERK docking sites. To select for targets that are more likely to be functional ERK substrates in *C. elegans* and to generate a prioritized list for the genetic enhancer screen and the biochemical studies, we applied four additional filters.

First, we excluded proteins with DS on the extracellular side or in the transmembrane region because they likely are inaccessible to active ERK, which usually has cytosolic or nuclear localization. Second, we narrowed down the list to proteins that contain multiple DS, with the idea that at least one of the DS maybe functional in vivo. Third, we focused on targets that are conserved across phylogeny. Finally, since we were interested in identifying functional ERK targets that specifically mediated germline development, we selected for genes that show germline-enriched expression using the available microarray and in situ hybridization databases.

1. Access WormBase.org and download the amino acid sequence of target proteins.
2. Access the TM Pred Server: www.cbs.dtu.dk/services/TMHMM/ and www.ch.embnet.org/software/TMPRED_form.html.
3. Upload the sequence of target protein and the results display the predicted transmembrane helices in that protein.
4. If the DS identified are in the predicted transmembrane region for both searches, remove that protein from the list of potential targets. These searches narrow down the number of potential targets to 1382.
5. Select for proteins with more than one DS, resulting in 623 potential targets.

6. Go to www.ncbi.nlm.nih.gov/blast/Blast.cgi.
7. Select “protein blast”. Put in the sequence of the *C. elegans* protein. Under “Choose Search Set”, select “*homo sapiens* (taxid:9606)” as the organism and run BLAST.
8. Take the sequence of the highest-scoring protein and BLAST it with the *C. elegans* database.
9. If the highest-scoring protein is not the original *C. elegans* protein input, then remove this protein from the list of potential targets.
10. Repeat the reciprocal BLAST analysis (**steps 6 – 9**) with the mouse database.
11. Go to T-coffee web-based alignment tool: <http://tcoffee.crg.cat/apps/tcoffee/do:regular>.
12. Put in the sequences of the *C. elegans* and human proteins and align.
13. If none of the docking sites are conserved between the worm and the human sequences, remove that protein from the list. These searches yield 258 potential targets.
14. Access the Nematode Expression Data Base hosted at: nematode.lab.nig.ac.jp/db2/index.php to access the worm in situ hybridization database.
15. Click on “simple search” and search for the protein of interest.
16. Click on the resulting clusters (CELK numbers) and check for the mRNA expression in adults. Examples of germline-enriched vs. non-germline-enriched expression are shown in Fig. 3.
17. Narrow down the list to targets that show germline-enriched mRNA expression. These searches yielded 161 targets.

3.2 RNAi-Based Genetic Enhancer Screen

Reverse genetics in *C. elegans* has been prevalent since 1998, when it was shown that the introduction of double-stranded RNA into a hermaphrodite worm results in potent and specific inactivation of an endogenous gene with the corresponding sequence [33]. This technique, known as RNA interference (RNAi), enables rapid, targeted gene inactivation and has become an extremely important tool for studying gene function in vivo. RNAi-mediated gene disruption is used in high-throughput screening of computationally identified ERK targets to assess their validity. We employed feeding RNAi as the mode of delivery because it has a number of advantages. First, feeding is far less labor-intensive than microinjection and is convenient for performing RNAi on a large number of worms or testing a large number of different genes. Second, feeding is considerably less expensive than either injection or soaking, which require the in vitro synthesis of dsRNA.

3.2.1 Generating RNAi Clones for Feeding RNAi

1. PCR-amplify 1 kb of cDNA region in the candidate gene with primers containing restriction enzyme sites.
2. Verify the PCR products using gel electrophoresis followed by gel extraction and sequence confirmation.
3. Perform restriction enzyme digestion on both the PCR product and the pPD128.36 vector.
4. Confirm the digested fragments using gel electrophoresis.
5. Follow the NEB T4 DNA ligase protocol to ligate the cut fragments into the linearized plasmid (<https://www.neb.com/protocols/1/01/01/dna-ligation-with-t4-dna-ligase-m0202>).
6. Transform the assembled plasmid into DH10B competent cells, plate the transformed cells onto LB ampicillin plates and incubate overnight at 37 °C.
7. Perform PCR, with primers outside the multiple cloning sites, on the colonies obtained above, followed by gel electrophoresis to confirm the presence of inserts.
8. Replica plate each colony used for colony PCR onto fresh LB ampicillin plate and incubate the replica plate at 37 °C overnight.
9. For each colony that is positive on the PCR, inoculate into 5 mL of LB with ampicillin (100 µg/mL) and incubate overnight at 37 °C.
10. The next morning, centrifuge the cells at $4000 \times g$ for 30 min on a desktop centrifuge and freeze the pellets at -20 °C.
11. Perform plasmid extraction on each.
12. Check the plasmid integrity via gel electrophoresis.
13. Confirm the correct sequence of the insert (see^{Note 1}).

3.2.2 Making RNAi Plates

1. Make fresh NGM plates containing 0.2 % lactose and 100 µg/mL ampicillin (labeled RNAi NGM plates).
2. Transform confirmed RNAi constructs into HT115(DE3) competent bacterial cells, plate on LB ampicillin (100 µg/mL) agar plates and incubate overnight at 37 °C.
3. Inoculate 1–2 colonies into 2–4 mL of LB with ampicillin and grow overnight at 37 °C with shaking (200 rpm).

¹Two existing *C. elegans* RNAi libraries generated by the Ahringer and the Vidal group (Ahringer Library or the Vidal Library) can be used for performing the RNAi analysis. Sequence-verify the integrity of the clone from the library before using it in your experiment.

4. Dilute the overnight culture 1:100 into fresh LB with ampicillin (100 µg/mL) and incubate at 37 °C for 6 h with shaking at 200 rpm. (Six mL of culture is sufficient for seeding approximately 30 lactose RNAi plates.)
5. After 6 h of incubation, seed 250 µL of bacteria onto each RNAi NGM plate.
6. Incubate the seeded plates at room temperature for 3 days, until a dense bacterial growth is evident.

3.2.3 Germline-Specific RNAi

1. Maintain *rrf-1(pk417)*, *rrf-1(pk417); mpk-1(ga111ts)* and *rrf-1(pk417); let-60(ga89ts)* worms at permissive temperature (20 °C) on regular NGM plates seeded with OP50 bacteria (*see* ^{Note 2}).
2. Transfer 3–5 gravid adults onto each RNAi NGM plate. (First transfer to an unseeded NGM plate to eliminate any residual OP50 bacteria.)
3. Incubate at 20 °C and label the plates as Day 0.
4. Transfer worms to new RNAi NGM plates next day and label as Day 1. Repeat on Day 2.
5. Monitor the F1 progenies each day.
6. Pick F1 progeny at mid-L4 stage onto fresh RNAi NGM plates.
7. Let grow for 24 h and score the phenotypes by whole-mount DIC imaging and dissection followed by immunofluorescence staining (*see* ^{Note 3}).

3.2.4 Attenuation of RNAi Feeding—RNAi inactivation of some genes may produce strong phenotypes distinct from *mpk-1* phenotypes in the control *rrf-1* background. To prevent these phenotypes from obscuring the MPK-1-dependent processes, we reduce the time that the worms are exposed to the dsRNA.

1. Transfer control *rrf-1(pk417)* adults to RNAi plates at mid-L4 stage.
2. Examine the animals 48 or 72 h after exposure to dsRNA. If the animals present with no phenotype, move to **step 3**.
3. Using the condition as in **step 2**, retest the target gene into *rrf-1(pk417)*, *rrf-1(pk417);mpk-1(ga111ts)* and *rrf-1(pk417); let-60(ga89ts)* worms.

3.2.5 Microdissection and Immunofluorescence Staining

1. Pick 50 worms directly into a glass embryo dish with 50 µL PBST.
2. Add 1 µL of 100 mM levamisole to the glass dish containing the worms and gently swirl to mix the liquid.

²The standard *E. coli* strain used as worm food is OP50.

³Since the efficiency of RNAi may vary, multiple germlines are needed for careful characterization of the phenotypes under either DIC imaging or dissection followed by immunofluorescence. As a standard practice we dissect over a 100 animals and process their extruded germlines for each experiment.

3. As paralysis sets in, cut the animals between the second and the third pharynx: place the pharynx at the region of the intended cut between two syringe needles and decapitate by moving needles in a scissor like motion (avoid needles with bent tips). For most animals, at least one gonad arm should extrude completely. Finish dissection in 2.5 min.
4. Add 2 mL of fixation buffer directly to the dish and cover the dish.
5. Incubate for 10 min at room temperature
6. After the incubation time, add 3 mL of PBST to the dish and transfer all liquid into a 5 mL glass conical tube using a glass pipet (*see* ^{Note 4}).
7. Centrifuge for 30 s at $2000 \times g$ in a clinical bench top centrifuge.
8. Remove supernatant, fill the tubes with 5 mL of PBST, followed by centrifugation, and remove the supernatant (carefully resuspend the germlines during each wash). Perform this step for a total of three times.
9. After the final wash with PBST, remove as much supernatant as possible.
10. Add 2 mL of ice-cold 100 % methanol directly to the tubes. Mix gently.
11. Incubate the tubes at -20°C for at least 20 min and up to 24 h (*see* ^{Note 5}).
12. Wash three times as described in **step 8**. Leave <0.5 mL of liquid after the last wash.
13. Transfer the germlines into glass culture tubes, 6 mm $\times$ 50 mm, using glass Pasteur pipettes.
14. Let the germlines settle by gravity.
15. Remove as much supernatant as possible.
16. Add 100 μL of 30 % goat serum to the 6 mm $\times$ 50 mm tubes.
17. Incubate at room temperature for 1 h or at 4°C overnight.
18. Dilute primary antibodies in 100 μL of 30 % goat serum (*see* ^{Note 6}).
19. Add the diluted primary antibodies to the tubes and incubate at 4°C overnight. Dilutions used:
 - Mouse anti-MAPKYT antibody, 1:400.
 - Rabbit anti-PTC-1, 1:50.
 - Rabbit anti-SYN-4, 1:400.

⁴Dissected germlines stick to plastic. To prevent losing these, use glass pipets and tubes in all steps.

⁵Incubation of dissected and fixed germlines in methanol (at -20°C) should be for no less than 20 min and no more than 24 h for optimal dpMPK-1 staining. For comparing levels of dpMPK-1 accumulation between RNAi treatments, we recommend the fixation conditions between the genotypes and treatment to be identical.

⁶For immunofluorescence staining, the appropriate dilution of each primary and secondary antibody must be determined on wild-type germlines prior to conduction of the actual experiment.

20. Wash the germlines by first filling the tubes with PBST, letting germlines settle by gravity for 3–4 min (no centrifugation required), and removing the supernatant. Perform the wash for a total of three times.
21. Dilute secondary antibodies (1:400) in 100 μ L of 30 % goat serum.
22. Add the diluted secondary antibodies to the tubes.
23. Incubate the tubes at room temperature for 2 h in dark.
24. Wash three times with PBST as in **step 20**.
25. Add 400 μ L of 0.1 ng/mL DAPI. Incubate in dark at room temperature for 20 min.
26. Wash once with 1 $\times$ PBST and remove as much supernatant as possible.
27. Add one drop of Vectashield to the tubes.
28. Using a drawn capillary pipette or a Pasteur pipette, transfer germlines onto a thin large 2 % agarose pad that covers most of a slide.
29. After drawing off excess liquid from atop the agarose pad with a capillary, an eyelash hair (or finely drawn capillary) can be used to push gonads and intestines away from one another.
30. Cover with a 24 $\times$ 50 mm coverslip, ensuring that the entire agarose pad is covered.
31. Let excess liquid evaporate and germlines flatten by placing the unsealed slides overnight at 4 $^{\circ}$ C in dark.
32. Seal the slide next morning with nail polish along the edges of the coverslip (see Note 7).

3.2.6 Interpretation—For a given cellular process (e.g., germ cell apoptosis) controlled by MPK-1 ERK, it is likely that phosphorylation of multiple targets, rather than a single target, is necessary for the normal biological outcome. Therefore, RNAi based gene inactivation is performed in sensitized genetic backgrounds to permit detection of small or partial changes in a pathway that controls a given cellular process. Downstream targets can be either activated or inactivated by MPK-1 ERK phosphorylation. If activated, then MPK-1 ERK is a positive regulator of the target (Fig. 2b); if inactivated, then MPK-1 ERK is a negative regulator of the target (Fig. 2b). The sensitized backgrounds used in the screen are *mpk-1(ga111ts)* ERK loss-of-function mutation and *let-60(ga89gfts)* RAS gain-of-function mutation, both of which have temperature-sensitive character.

At permissive temperature, *mpk-1(ga111ts)* mutants have wild-type germline phenotypes but reduced level of dpMPK-1 (Fig. 2c). Under this condition, MPK-1 ERK likely phosphorylates substrates at a lower level. Therefore, if RNAi of a candidate gene produces

⁷Do not move or put pressure on the coverslip (covering the fixed and stained germlines on top of the agarose pad) once the slide is made. Slides can be stored at 4 $^{\circ}$ C in dark for up to a week for imaging.

mpk-1 -like loss-of-function phenotype, it normally functions to promote the *mpk-1* -dependent process and is likely activated by MPK-1 ERK phosphorylation.

At permissive temperature, *let-60 (ga89ts)* mutants have wild-type germline phenotypes but elevated dpMPK-1 level and persistent MPK-1 ERK activation at the loop region (Fig. 2c). Under this condition, MPK-1 ERK likely phosphorylates substrates at regions where they are normally dephosphorylated. Therefore, if RNAi of a candidate gene produces *let-60*-like gain-of-function phenotype, it normally functions to inhibit the *mpk-1* -dependent process and is likely inactivated by MPK-1 ERK phosphorylation.

3.3 Biochemical Analysis

The above RNAi experiments demonstrate that target gene products genetically interact with LET-60 RAS-MPK-1 ERK signaling, suggesting that the proteins are MPK-1 ERK phosphorylation substrates. However, it is formally possible that the target protein acts in a parallel pathway or may be further downstream of another direct target. Therefore it is necessary to confirm biochemically that RNAi-validated target proteins are substrates of ERK phosphorylation. ATP phosphotransfer assay is conducted using recombinant active murine ERK2 at manufacturer's recommendations.

The *bona fide* -docking site containing ERK substrate should have a lower kinetic constant (K_m) for phosphorylation than a nonspecific (nondocking site containing) substrate [34] due to better binding. Myelin Basic Protein (MBP) is one such nonspecific substrate (nondocking site substrate), which forms the control in the K_m assay. The K_m is determined to assess the efficiency of phosphorylation of the phospho-acceptor sequence(s). The assay is conducted using filter binding and scintillation counting (counts per minute, CPM) with increasing amounts of the target proteins [34].

3.3.1 Candidate Substrate Protein Production

1. Generate the 6× His or GST recombinant proteins using pTrcHis Topo and pGEX5-A bacterial expression vectors, respectively (*see* ^{Note 8}).
2. Verify all clones by sequencing for orientation and sequence integrity.
3. Express proteins using BL21(DE3) cells, at 20 °C using 1 mM isopropyl β-D -1-thiogalactopyranoside (dioxane free) for 16 h.
4. Proteins are then purified using Ni-NTA agarose or glutathione sepharose [35] (*see* ^{Note 9}).

3.3.2 In Vitro Kinase Analysis Using Activated Murine ERK2

1. Dialyze purified proteins into the Kinase assay buffer for 2–4 h.

⁸In cases where the target proteins are large (>100 kDa) and thus not easily soluble, truncated proteins containing conserved domains including the docking sites and phosphoacceptors can be used for analysis. If the docking domain and phospho-acceptors are on the N-terminal half of the protein, then tag the C-terminal end with 6× His or GST, and *vice versa*.

⁹To estimate the amount of purified protein, conduct bicinchoninic acid (BCA)-based protein assays, as well as run 10 % SDS PAGE with BSA dilution series standards and compare the intensity of bands on a Coomassie stained gel.

2. Set up the kinase reaction. A standard 50- μ L reaction contains 50 ng of purified protein, 100 μ M cold ATP, 1 μ M [γ - 32 P] ATP, 1 $\times$ Kinase buffer and 1 U of active ERK2 enzyme (*see* Note 10). Purified MBP is used as a control. Kinetic analysis is performed using seven concentrations of the test target protein and control MBP ranging from 0.2 to 2 μ M.
3. Incubate the reaction for 15 min at 30 $^{\circ}$ C, at which time 32 P incorporation is linear with respect to time.
4. Calculate the specific activity of 32 P as counts per minute/pmol of ATP stock (usually about 3000 on average, however, this should be determined at the start of each experiment).
5. Terminate the reaction with 2 $\times$ SDS-sample buffer.
6. Separate the samples on a 10 % SDS-PAGE, dry on a gel drier and expose to X-ray film. SDS-PAGE and autoradiography are used to establish that intact fusion protein contains most of the incorporated 32 P.
7. To quantify phosphorylation, stop the reaction with 10 % ice-cold trichloroacetic acid.
8. Collect the precipitated protein by centrifugation at 14,000 rpm or 18,000 $\times g$.
9. Spot the precipitated proteins onto P81 phospho-cellulose paper and wash three times with ice-cold 50 % phosphoric acid.
10. Measure the amount of bound radioactivity with scintillation counter.
11. K_m and V_{max} are calculated from the intercepts of the Lineweaver-Burke Plot and in each case the data closely approximates a straight line. To calculate V_{max} , 1 unit of ERK2 activity is defined as the ability to transfer 200 pmol of phosphate/min to MBP; the activity of ERK2 is calibrated by the manufacturer. V_{max} is determined by assaying the amount of total phosphate incorporation per unit time/enzyme amount (usually calculated to around 0.03 pmol). Total phosphate incorporation is calculated by using the measured CPM, the specific activity of 32 P, and the reaction volume. Relative acceptor ratio is V_{max}/K_m and is normalized by setting value for MBP to 1.0.

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¹⁰Purified ERK2 kinase is purchased from NEB and reactions are carried out as described previously [16, 19, 34].

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Fig. 1. ERK activation pattern in sensitized genetic backgrounds. (a) Schematic of *C. elegans* adult hermaphrodite. (b) Schematic of one adult hermaphroditic germline showing different stages of germ cell development, dp MPK-1 pattern and seven MPK-1-dependent processes throughout germline development. DTC marks distal tip cell. TZ marks transition zone. (c) DAPI staining and activation pattern of dpMPK-1 ERK (red) in wild-type, *mpk-1* loss-of-function and *let-60* gain-of-function alleles at permissive temperature (20 °C). Both mutants have wild-type germline morphology despite decreased or increased/ectopic levels of

dpMPK-1 ERK. *Asterisks* and *white lines* mark the distal and proximal ends of the germline, respectively [36, 37]. Scale bar: 20 μ m

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A. Bioinformatics

To globally identify all potential MPK-1 targets in *C. elegans* germline

1. Proteins in the *C. elegans* proteome containing: **B. RNAi-based genetic screen**

To identify potential targets that genetically/functionally intersect with the RAS/ERK pathway

Background	Candidate gene RNAi	Phenotypes	Interpretation
Wild type		No/Mild	
Low ERK <i>mpk-1</i> loss-of-function		Enhanced	Activated by MPK-1 ERK Promotes ERK-dependent process
High RAS <i>let-60</i> gain-of-function		Enhanced	Inactivated by MPK-1 ERK Inhibits ERK-dependent processes

C. Biochemical approach

To confirm that the substrates are direct targets of ERK

Generate 6X His and GST recombinant proteins

Full-length or truncated

↓

in vitro kinase assay

↓

Calculate Vmax and Km to assay efficiency of phosphorylation

Fig. 2.

Flow chart of experimental procedures. (a) Outline of bioinformatics approach based on defined ERK docking site motifs. (b) Rationale of genetic approach using RNAi-based enhancer screen. (c) Outline of biochemical analysis of the functional interactors [21]

Fig. 3. mRNA expression as assayed by in situ hybridization by the Kohara lab, accessible *via* the Nematode Expression Data Base (NEXTDB). For example, in situ hybridization for *drsh-1*, an RNase III ribonuclease (**a**), reveals germline expression, while that for *mec-7* beta tubulin (**b**) does not. Clone 842a08 and 411c2 were used to detect *drsh-1* and *mec-7* mRNA expression, respectively