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| Citation       | Cohn, Jesse et al., "Activity-Dependent Regulation of the Proapoptotic BH3-Only Gene egl-1 in a Living Neuron Pair in Caenorhabditis elegans." G3: Genes, Genomes, Genetics 9, 11 (November 2019): 3703-3714 © 2019 The Author(s) |
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| As Published   | https://dx.doi.org/10.1534/g3.119.400654                                                                                                                                                                                                                     |
| Publisher      | Genetics Society of America                                                                                                                                                                                                                                  |
| Version        | Final published version                                                                                                                                                                                                                                    |
| Citable link   | https://hdl.handle.net/1721.1/125707                                                                                                                                                             |
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Activity-Dependent Regulation of the Proapoptotic BH3-Only Gene egl-1 in a Living Neuron Pair in Caenorhabditis elegans

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ABSTRACT  The BH3-only family of proteins is key for initiating apoptosis in a variety of contexts, and may also contribute to non-apoptotic cellular processes. Historically, the nematode Caenorhabditis elegans has provided a powerful system for studying and identifying conserved regulators of BH3-only proteins. In C. elegans, the BH3-only protein egl-1 is expressed during development to cell-autonomously trigger most developmental cell deaths. Here we provide evidence that egl-1 is also transcribed after development in the sensory neuron pair URX without inducing apoptosis. We used genetic screening and epistasis analysis to determine that its transcription is regulated in URX by neuronal activity and/or in parallel by orthologs of Protein Kinase G and the Salt-Inducible Kinase family. Because several BH3-only family proteins are also expressed in the adult nervous system of mammals, we suggest that studying egl-1 expression in URX may shed light on mechanisms that regulate conserved family members in higher organisms.

KEYWORDS  BH3-only egl-1 C. elegans URX apoptosis

Members of the BH3-only family of proteins are essential regulators of cell death in nearly all metazoans. Each member of this family shares the “BCL-2 homology” domain BH3, which allows them to bind the anti-apoptotic members of the BCL-2 family and block their activity, thereby initiating apoptosis (Lettre and Hengartner 2006; Giam et al. 2008). In mammals, there are at least eight BH3-only proteins, with some estimates ranging higher (Aouacheria et al. 2005; Hoppo et al. 2012). These proteins are regulated in a number of ways, such as transcriptionally (Oda et al. 2000; Nakano and Vousden 2001; Sturm et al. 2006; Borensztajn et al. 2007), post-transcriptionally (Ventura et al. 2008; Sherrard et al. 2017), and post-translationally (Verma et al. 2001; Lowman et al. 2010). This complex regulation allows for cells to properly induce cell death during development and in response to a wide variety of contexts and circumstances. Additionally, mounting evidence suggests that some BH3-only proteins may contribute to non-apoptotic cellular processes as well, such as glucose metabolism (Danial et al. 2003; Danial et al. 2008; Giménez-Cassina et al. 2014), autophagy (Maiuri et al. 2007; Sinha et al. 2008; Lindqvist et al. 2014), and lipid transport (Espoti et al. 2001). Despite their important role in initiating programmed cell death and likely other cellular processes, our understanding of the factors that regulate BH3-only proteins is incomplete.

The nematode C. elegans has historically played an important role in understanding the mechanisms of programmed cell death and the role of BH3-only proteins (Lettre and Hengartner 2006). Genetic screens in the 1980s and 1990s using C. elegans uncovered a core pathway through which programmed cell death is initiated and executed (Ellis and Horvitz 1986; Yuan and Horvitz 1990; Hengartner et al. 1992; Yuan and Horvitz 1992; Conradt and Horvitz 1998), the main features of which are well conserved all the way through mammals (Czabotar et al. 2006).
et al. 2014). The primary initiator of this cell death pathway was found to be a BH3-only protein called egl-1 (egg-laying defective-1). A gain-of-function mutation in the regulatory region of egl-1 causes the inappropriate death of neurons in the egg-laying circuit, while subsequent loss-of-function mutations in egl-1 prevented this cell death (Conradt and Horvitz 1998; Conradt and Horvitz 1999). egl-1 was found to be necessary for most developmental cell deaths in the worm, and sufficient to induce apoptosis cell-autonomously when expressed ectopically in vivo (Conradt and Horvitz 1998; Chang et al. 2006; Lomonosova and Chinnadurai 2008).

So far, evidence exists for egl-1 regulation at the transcriptional and the post-transcriptional level (Nehme and Conradt 2008; Nehme and Horvitz 1991; Metzstein et al. 2006; Wu et al. 2007; Sitwala et al. 2008). The URX neurons are necessary for worms to orient properly to different oxygen levels in their environment (Gray et al. 2013). With the use of a fluorocent transcriptional reporter for egl-1, we first transformed the above PCR product into N2 WT by microinjection at a concentration of 2 ng/μl along with a Punc-122::GFP co-injection marker to generate strain JPS600 carrying the extra-chromosomal array with Punc-122::GFP fused product were: AGGCCTGATCATAGTTTCTGCCATTTG.

Because the study of egl-1 regulation has historically yielded insights into factors that control apoptosis in higher animals, we propose that this novel adult transcription of egl-1 in URX may further our understanding of roles and regulatory mechanisms of BH3-only proteins in apototic and potentially non-apototic contexts.

MATERIALS AND METHODS

Strains Used

The following strains were used: N2 Bristol as wild type; JPS600 vxEx600[Pgl-1:mCherry Punc-122::GFP]; JPS601 vxEs601[Pgl-1:mCherry Punc-122::GFP]; JPS602 vxEx602[Pgl-1:mCherry Punc-122::GFP Pgcy-32::GFP]; JPS620 ced-6(n1813) III; vxEx602[Pgl-1:mCherry Punc-122::GFP Pgcy-32::GFP]; MT22516 him-8(e1489) V; mls343[Pegl-1::4xNLS::GFP]; MT8735 egl-1(n1084n3082) V; RB1305 egl-1(ok1418) V; RA629 dbEx614[Pgcy-37::YC2.60;unc-54UTR Punc-122::RFP]; JPS1191 egl-1(n1084n3082) V; dbEx614[Pgcy-37::YC2.60;unc-54UTR Punc-122::RFP]; JPS879 vxEx879[ Pgcy-32::GFP Punc-122::GFP]; JPS1106 egl-1(n1084n3082) V; vxEx1106[Pgcy-32::GFP Pfat-7::GFP]; JPS1031 vxEx1031[Pgcy-32::RAB-3::GFP Pgcy-32::mCherry]; JPS1070 egl-1(n1084n3082) V; vxEx1031[Pgcy-32::RAB-3::GFP Pgcy-32::mCherry]; JPS806 cng-1(v3x3) V; vx6601[Pgl-1::mCherry Punc-122::GFP]; JPS803 cng-1(v3x3) V; vx6601[Pgl-1::mCherry Punc-122::GFP]; JPS793 cng-1(v3x3)Pgcy-32::CNG-1(+); Peft-2::GFP; JPS909 cng-1(v3x3)db42 X; vx6600[Pgl-1::mCherry Punc-122::GFP]; JPS812 egl-1(n582IV); vx6600[Pgl-1::mCherry Punc-122::GFP]; JPS839 egl-1(n2368IV); vx6600[Pgl-1::mCherry Punc-122::GFP]; JPS937 egl-4(n477) V; vx6601[Pgl-1::mCherry Punc-122::GFP]; JPS1124 kin-29(oy38) X; vx6601[Pgl-1::mCherry Punc-122::GFP]; JPS1040 egl-4(n477) V; kin-29(oy38) X; vx6601[Pgl-1::mCherry Punc-122::GFP]; JPS893 egl-4(4xv19) X; cng-1(v3x3) V; vx6601[Pgl-1::mCherry Punc-122::GFP]; JPS1190 egl-4(n477) V; cng-1(j1h111) V; vx6601[Pgl-1::mCherry Punc-122::GFP]; JPS1127 cng-1(j1h111) V; kin-29(oy38) X; vx6601[Pgl-1::mCherry Punc-122::GFP]; JPS1126 cng-1(j1h111) V; kin-29(oy38) X; vx6601[Pgl-1::mCherry Punc-122::GFP]

Molecular Biology and Transgenic Strain Construction

The egl-1 reporter construct was generated by long PCR fusion of three fragments: 1042-bp upstream of the egl-1 start codon amplified from N2 DNA lysate, worm-optimized mCherry amplified from plasmid pCFJ90, and 5744-bp of the 3’ downstream region of egl-1 genomic DNA including the 3’UTR of egl-1 amplified from N2 lysate (Shevchuk et al. 2004). Primers used to generate the final fused product were: AGGCCTGATCATAGTTTCTGCCATTTG and ATCCCTAAGATTTTCTCAAGATCAGATGTTCATC.

To generate the strain JPS601 with the integrated egl-1 reporter transgene vxEs601, we first transformed the above PCR product into N2 WT by microinjection at a concentration of 2 ng/μl along with a Punc-122::GFP co-injection marker to generate strain JPS600 carrying the extra-chromosomal array vxEx600. This array was then integrated by UV irradiation using a UV Stratalinker 2400 (Stratagene), and the resulting integrated strain was then outcrossed to N2 six times before any further use (Mariol et al. 2013).

URX neuron labeling constructs were generated by using an 876-bp fragment of the gcy-32 promoter established from earlier studies (Yu et al. 1997). The promoter fragment was amplified from N2 gDNA and then PCR fused with either worm-optimized mCherry amplified from pCFJ90 or worm-optimized GFP amplified from pPD95.75. The fused products were then subcloned into the pCR-Blunt vector using a Zero Blunt PCR Cloning Kit from ThermoFisher Scientific. These constructs were used in injection mixes at a concentration of 20 ng/μl.

The URX-targeted synaptic marker RAB-3::GFP was generated by digesting plasmid NM1028 with PstI and NcoI and inserting the gcy-32 promoter with Gibson Assembly. This construct was injected at a concentration of 25 ng/μl. NM1028 was a kind gift of Michael Nonet (Washington University in St. Louis).

The construct to cell-specifically rescue cng-1 in URX was generated by amplifying the full coding region of cng-1 and the gcy-32 promoter each from N2 gDNA. These were then assembled by Gibson Assembly with a pPD95.75 backbone that had been digested with XbaI and EcoRI. This was then injected at a concentration of 20 ng/μl.

Mutations were followed in crosses by PCR genotyping and/or by phenotype where applicable.
Mutagenesis and Mutant Identification

To screen for mutations that affect expression of *vxIs60J* in URX, we mutagenized the otherwise wild-type strain JP5601 with 0.5-mM N-ethyl-N-nitrosourea (ENU) in M9 buffer for 4 hr and then examined F2 progeny using a fluorescence dissection microscope. We looked for animals with a loss or diminishment of *mCherry* expression in URX. We followed the strategy outlined in Zuryn, et al. (2010), to identify candidate causal mutations. We identified allele *vx*3 as an A-to-C mutation 651 bp from the start codon of *cng-1* predicted to convert the aspartic acid at position 162 to an alanine.

For our reversion screen, *cng-1(vx3)* mutant animals were backcrossed to strain JP5601 six times and then subjected to ENU treatment as above. F2 progeny were screened on a fluorescence dissection microscope to look for recovery of fluorescence in URX. All revertant mutants displayed a slight egg-laying defect and a longer body than wild type – common characteristics of the *egl-4* mutant. Revertant mutants failed to complement the *egl-4(nv477)* allele when tested for *vxIs60J* expression in URX. One member of this complementation group, *vx19*, showed a T-to-G early stop-codon mutation at position 2389 of the *egl-4 F55A8.2a.1* transcript. This mutation truncates the final 18 amino acids from the protein.

General Microscopy

Worms were mounted on 2% agarose pads and anesthetized with 30-mM sodium azide in NGM. Epifluorescent images were taken on an Olympus IX51 inverted microscope equipped with an X-Cite FIRE LED Illuminator (Excellitas Technologies Corp.) using an Olympus UPlanFL N 40X/0.75 NA objective and QCapture Pro 6.0 software. Confocal images were taken with a Zeiss LSM 710 microscope equipped with a Plan-Apochromat 40x/1.4 Oil objective and Zen Software.

*egl-1* Reporter Fluorescence Quantification and Oxygen Experiments

For fluorescence quantification of the *egl-1* reporter in URX, animals were synchronized by timed egg laying and then imaged three days later. *kin-29* mutants develop significantly slower than wild-type worms or *egl-4* mutants, however because reporter expression level is a function of the amount of time an animal is exposed to oxygen as opposed to a particular age, we compared day 1 wild-type and *egl-1* adults to L2-L3 *kin-29* animals that had been synchronized by egg-laying.

Fluorescence intensity was quantified with FIJI software as previously described (Mccoy et al. 2014). Briefly, the cell body was outlined and then the background was subtracted to give a final fluorescence intensity for each cell. These values were then normalized to wild-type animals grown in parallel at 21% oxygen for comparison purposes. Pictures shown in figures are to show presence/absence of reporter expression; non-saturated photos were used for quantification.

For experiments in which the oxygen environment was different than 21%, worms were grown or maintained in a Modular Incubator Chamber (Billups-Rothenberg) attached to oxygen tanks containing 100% medical grade O₂ (Praxair), or either 1% or 10% O₂ balanced with nitrogen (Airgas).

Single Molecule FISH

smFISH experiments were performed as described elsewhere (Raj et al. 2008). Fixed mixed stage animals were incubated overnight at 30°C with previously designed *egl-1* probes (Johnsen and Horvitz 2016). Animals were subsequently mounted for imaging after two washes lasting 30 min each. Image acquisition was performed on a Nikon TE-2000 inverted microscope with a 100x objective (Nikon, NA 1.4). A Pixis 1024 camera (Princeton Instruments) controlled by MetaMorph software (Molecular Devices) was used to detect smFISH signal and acquire images with an exposure time of 2 sec. Images were processed and prepared for publication using ImageJ software (NIH).

UX Calcium Imaging

To image young adults, we picked L4 animals expressing the YC2.60 Ca²⁺ sensor 24 hr before imaging. On the day of the assay, 5 – 10 day 1 adult animals were glued to agarose pads (2% in M9 buffer, 1 mM CaCl₂), using Dermabond tissue adhesive, with their body immersed in OP50 washed off from a seeded plate using M9 buffer. The animals were quickly covered with a PDMS microfluidic chamber and 7% O₂ was pumped into the chamber for 2 min before we began imaging, to allow animals to adjust to the new conditions. Neural activity was recorded for 9 min with switches in O₂ concentration every 2 min.

Imaging was performed on an AZ100 microscope (Nikon) bearing a TwinCam adaptor (Cairn Research, UK), two ORCAFlash4.0 V2 digital cameras (Hamamatsu, Japan), and an AZ Plan Fluor 2X lens with 2x zoom. Recordings were at 2Hz. Excitation light from a Lambda LS xenon arc lamp (Sutter) was passed through a 438/24 nm filter and an FF458DiO2 dichroic (Semrock). Emitted light was passed to a DC/T510LPXRXTUf2 dichroic filter in the TwinCam adaptor cube and then filtered using a 483/32 nm filter (CFP), or 542/27 nm filter (YFP) before collection on the cameras. Recordings were analyzed using Neuron Analyzer, a custom-written Matlab program available at https://github.com/neuronanalyzer/neuronanalyzer.

Lifespan Assays on Pseudomonas aeruginosa

Assays were performed as previously described (Styer et al. 2008). *P. aeruginosa* was grown overnight at 37°C and then seeded onto NGM-agar plates modified to contain 0.35% peptone. After seeding, plates were placed at 37°C overnight and moved in the morning to 25°C to equilibrate temperature. At least 10 gravid adults were placed on each plate with 4-5 replicate plates per strain per assay. Animals were assessed every 4-8 hr for survival. Those that failed to respond to touch were counted as dead, and animals that crawled up the sides of the plate and desiccated were censored. Animals were maintained at 25°C and moved to fresh plates every other day over the course of the assay.

Dendrite Scoring Assays

URX dendrites in day 4 adults carrying a *Pgcy-32::GFP* transgene were visualized and scored for tip morphology. Dendritic endings with a secondary branch that extended at least 5 microns from the primary branch were scored as "complex", otherwise they were scored as "simple". About 17% of URX dendrites in the *egl-1* mutant failed to extend all the way to the tip of the nose, possibly because of extra undead cells interfering with dendritic attachments to glia. These animals were not included in the determination of complex/simple ratios.

Data Availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article and figures. Supplemental material available at FigShare: https://doi.org/10.25387/g3.9816401.

RESULTS

egl-1 is transcribed in URX neurons post-developationally

To investigate the expression pattern of *egl-1*, we generated a transcriptional reporter construct consisting of 1.1 kb of the upstream promoter region, as well as 5.5 kb of the region downstream of the gene.
The coding region of egl-1 was replaced with a C. elegans-optimized version of mCherry (Figure 1A). We integrated this construct to create the transgenic reporter vxls601 (Mariol et al. 2013). To ensure that vxls601 faithfully reported true egl-1 expression, we visualized apoptotic cells in a ced-6(n1813) mutant background in which dead cell corpses persist due to a defect in engulfment (Figure 1A') (Liu and Hengartner 1998). We found that apoptotic cell corpses reliably showed high mCherry expression, indicating that vxls601 correctly reflects egl-1 transcription in at least the developmental cell deaths we examined. For simplicity and clarity, we refer to vxls601 as the egl-1 reporter from hereon.

To our surprise, we also found that the egl-1 reporter was expressed in adult animals in a very small subset of neurons, and in one pair especially strongly in all individuals (Figure 1A''). This expression persisted throughout the entire life of the worm. We used cell-specific reporters and morphological characterization to identify the neurons most brightly expressing the reporter as the bilaterally symmetric neuron pair URX (Yu et al. 1997). By examining eggs, we found that egl-1 reporter expression was detectable in URX by at least the threefold stage, albeit at extremely faint levels compared to later expression (Supplemental Figure 1). In addition to URX, we also found that AQG, PQR, and one of the AWCon/off neuron pair expressed the reporter less often (see Figure 1B, top, for an example). Of these neurons, the expression in the URX neuron pair was the most consistent and robust, so we chose to characterize the adult expression of egl-1 by focusing our attention specifically on URX.

Reporter genes are occasionally expressed in cells that do not normally express the endogenous gene because the reporter lacks regulatory regions that repress the endogenous gene (Tursun et al. 2009). To test this possibility for our egl-1 reporter, we used single molecule fluorescent in situ hybridization (smFISH) to confirm whether endogenous egl-1 mRNA transcripts could be found in URX (Johnsen and Horvitz 2016). To test the co-localization of the egl-1 fluorescent reporter and endogenous egl-1 mRNA, we used animals that carry the egl-1 reporter transgene nIs343, which expresses nuclear-localized GFP using 6.5 kb of the upstream promoter region of egl-1 (Hirose and Horvitz 2013). These animals showed the same expression pattern as our egl-1 reporter described above (vxls601), including expression of GFP in a small number of neurons persisting past development, and especially strongly in URX. We found that the GFP expression always (8/8 worms) co-localized with the smFISH signal from probes targeted to the egl-1 transcript (Figure 1C). Notably, this expression continued past the L2 larval stage, which is when the last somatic cell deaths in the hermaphrodite occur (Solston and Horvitz 1977).

Past studies have identified genomic regulatory regions around egl-1 that control expression in certain apoptotic cells, so we performed promoter truncation experiments to determine whether egl-1 expression in URX is dependent on regions previously identified as important for egl-1 transcription in other cells (Boulin et al. 2006). We identified a 20-bp region ~85 bp upstream from the egl-1 transcriptional start site that was necessary for reporter expression in URX (Figure S1). This region has not been identified as necessary for any cell deaths examined in the worm thus far, and it is near but distinct from a previously identified binding site of the transcription factor SPTF-3 (Hirose and Horvitz 2013). This suggests that egl-1 transcription is regulated in URX by different regulatory regions than have been previously described in developmental cell deaths, though this area may still be necessary for egl-1 expression during development in cells other than URX.
overexpressing egl-1 in those cells. Wild type (n = 50) vs. egl-1(ok1418) (n = 45) vs. URX(-) (n = 49), P = 0.99 as determined by the Mantel-Cox test. C. Dendritic morphology assay of day 4 adults. Complex dendrites have at least one secondary branch that extends at least 5 μm from the primary dendritic stalk, simple dendrites do not. Wild-type (n = 159) vs. egl-1(n1084n3082) (n = 173), P < 0.01 as determined by Fisher’s exact test. D. Representative photos of URX synapses (green) in wild type and egl-1(n1084n3082) day 1 adults. Both strains carry a [Pgcy-32::mCherry Pgcy-32::RAB-3::GFP] reporter transgene. Arrows point to the URX axon (red) in the nerve ring. All scale bars are 5 μm.

We tried several approaches to determine whether the EGL-1 protein was also produced in URX; however, due to technical difficulties we were unable to make this assessment (see Discussion for more details). Nevertheless, we conclude that endogenous egl-1 is transcribed post-developmentally in the URX neuron pair without initiating apoptosis.

**No obvious role for egl-1 in URX neurons**

The URX neurons do not die under normal conditions. This leaves open the possibility that egl-1 may play a non-apoptotic role in URX. To address this hypothesis, we examined the egl-1 loss-of-function mutant with regard to a variety of URX- and egl-1-related phenotypes. We examined either the loss-of-function egl-1(n1084n3082) allele, which has an insertion that causes a frameshift and loss of the BH3 domain of the protein, or the presumed null egl-1(ok1418) allele which deletes a portion of the egl-1 promoter and coding region.

URX is the main class of oxygen-sensing neurons in *C. elegans*. Increases in environmental oxygen lead to increases in calcium influx into the neurons, which can be measured by expressing the ratiometric calcium sensor YC2.60 in URX (Nagai et al. 2004). We tested whether egl-1 plays a role in oxygen sensation by comparing oxygen responses in wild-type and egl-1(n1084n3082) mutant day 1 adults (Figure 2A and 2A'). We exposed worms to several oxygen concentrations from 7% to 21% oxygen, but found no significant difference in the URX oxygen response of egl-1(n1084n3082) mutant worms at any oxygen level we tested. We conclude from these results that egl-1 is not involved in oxygen sensation in URX.

URX has also been suggested to have a role in the *C. elegans* innate immune response. Transgenic worms lacking the URX, AQR, and PQR neurons were shown to survive longer than wild-type animals on the pathological bacterium *Pseudomonas aeruginosa* (Styer et al. 2008). We found that the egl-1(ok1418) mutant has similar survival rates as wild type on *P. aeruginosa* (Figure 2B), though we note that we were unable to replicate the previous finding that worms lacking URX, AQR, and PQR survive longer on *P. aeruginosa*, despite several attempts. Thus, we cannot definitively conclude whether egl-1 is necessary for URX to contribute to the innate immune response in *C. elegans*; however, we think it is unlikely that egl-1 plays this role. We also examined URX morphology to look for any signs of neurodegeneration after incubation for 24 hr on *P. aeruginosa*, but found no obvious defects in any worms (40/40).

URX has been described as having a characteristic variable, branched dendritic ending at the tip of the nose ("complex" endings) (Ward et al. 1975; White et al. 1986; Doroquez et al. 2014). In our parallel submitted work, we show that URX dendritic tips fail to branch in certain genetic and environmental conditions ("simple" endings). We examined the egl-1(n1084n3082) mutant for dendritic tip morphology and found that it had "simple", unbranched tips more often than wild-type animals (22% simple vs. 6.3% simple, P < 0.01 Fisher’s exact test) (Figure 2C). However, the effect size was much smaller relative to what we found in other genetic background and environmental conditions. Moreover, the effect could be due to a number of other factors such as supernumerary cells in the head region of the egl-1 mutant interfering with proper tip elaboration in certain individuals, so we did not characterize this phenotype further in the egl-1 mutant.

Finally, egl-1 has previously been suggested to have a role in synapse pruning during the development of the RME neurons (Meng et al. 2015). Using a GFP-tagged version of the synaptic marker RAB-3 expressed specifically in the URX neurons (Bounoutas et al. 2009), we visually compared URX synapses in the nerve ring in wild type and the egl-1(n1084n3082) mutant, but found no gross defects in synapse localization or abundance in the egl-1 mutant (Figure 2D). This suggests that egl-1 does not play an obvious role in synapse pruning in URX under basal circumstances.
**C. elegans** gated calcium channel in long-term oxygen sensation eventually gates the only L-type voltage-heterodimerize and produce cGMP in response to binding molecular of this mutation by cell-specific rescue. We found that near complete lack of gcy-35(ok769) URX to respond to changes in environmental oxygen. We found that diminished or lost in URX. We recovered several mutants, including a missense mutation in gcy-35 (Cho et al. 2005; Wojtyniak et al. 2013). The vx3 allele of gcy-35 is an A-to-C transversion in the third exon, changing an aspartic acid to an alanine. We confirmed the causal nature of this mutation by cell-specific rescue in URX (Figure 3A), and by phenocopy with the canonical jh111 deletion allele of cng-1 (data not shown).

CNG-1 is a cyclic nucleotide-gated ion channel that is an essential component of the oxygen sensation pathway in URX (Busch et al. 2012; Couto et al. 2013). Thus, we hypothesized that perhaps egl-1 expression was driven by oxygen sensation and neuronal activity. We tested this hypothesis by quantifying reporter expression in other mutants defective in oxygen sensation, as well as in wild-type worms maintained in varying levels of environmental oxygen.

First, we quantified egl-1 reporter expression in mutants lacking gcy-35 or gcy-36. These genes encode soluble guanylyl-cyclases that heterodimerize and produce cGMP in response to binding molecular oxygen (Cheung et al. 2004). Both gcy-35 and gcy-36 are necessary for URX to respond to changes in environmental oxygen. We found that both gcy-35(ok769) and gcy-36(db42) loss-of-function mutants had a near complete lack of egl-1 reporter expression in URX (Figure 3B).

Prolonged calcium entry into URX through CNG-1 in response to long-term oxygen sensation eventually gates the only L-type voltage-gated calcium channel in C. elegans, EGL-19 (Busch et al. 2012). We tested a role for EGL-19 in egl-1 expression in URX by quantifying egl-1 reporter expression in both a loss-of-function allele of egl-19, as well as a gain-of-function allele which has an increased open probability (Lainé et al. 2014). We found that egl-1 reporter expression in URX was significantly decreased in the egl-19(n582) loss-of-function allele, and significantly increased in the egl-19(ad2368g) gain-of-function allele. These results signify that EGL-19 activation downstream of prolonged oxygen sensation is necessary for egl-1 transcription in URX (Figure 3C).

We next directly assessed whether environmental oxygen sensation drives egl-1 expression in URX by cultivating wild-type animals with the egl-1 reporter in different oxygen environments and quantifying reporter expression on day 1 of adulthood. Wild isolates of C. elegans have a preferency for 5–12% O2 conditions, which is thought to reflect the environments most favorable to their survival (Gray et al. 2004). These strains will avoid both hypoxia (<1% O2) and oxygen levels over 21% O2, including the 21% O2 conditions normally found at the lab benchtop. The lab strain N2 has a dampened capability to avoid 21% oxygen due to a gain-of-function mutation in the gene npr-1 (De Bono and Bargmann 1998). This difference in behavior is not explained by differences in the URX neuron itself however, as both N2 and the npr-1 loss-of-function mutant (which mimics the npr-1 allele found in natural isolates) display similar URX calcium dynamics in response to oxygen (Jang et al. 2017). Instead, the difference in behavior is thought to originate from functional differences downstream of URX. For these reasons, we grew N2 animals in 1%, 10%, or 21% oxygen environments. We also grew worms in 100% oxygen to test whether the egl-1 reporter would be expressed higher than at 21% conditions, and may possibly lead to cell death of URX. This oxygen condition is likely much higher than worms experience in their natural environment.
We found that egl-1 reporter expression levels reflected the cultivation level of oxygen up to 21% oxygen, with day 1 animals grown in 1% oxygen having only eight percent the level of reporter expression as those grown in 21% oxygen (Figure 3D). Interestingly, worms grown at 100% oxygen actually had slightly lower expression of the reporter than those grown at 21%, and the morphology of URX was normal in these worms.

We considered the possibility that growth from egg to adulthood in these different environments had caused developmental differences in the URX neuron that led to the differences we observed in reporter expression. Therefore, we carried out a complementary set of experiments where populations were grown at one oxygen concentration until day 1 of adulthood and then shifted to another oxygen level until day 2. Worms grown at 10% or 21% oxygen, and then maintained at those same levels until day 2 had an increase of reporter expression of about thirty percent over that period, while populations grown at 10% oxygen then moved to 21% oxygen had a mean increase of over 150% from day 1 to day 2 (Figure 3E). Taken together, these experiments strongly suggest that environmental oxygen sensation by URX is necessary for expression of egl-1 in URX.

EGL-4 and KIN-29 regulate egl-1 expression in URX partially in parallel

We undertook two approaches to identify other genetic pathways that may regulate egl-1 expression in URX: a reversion screen where we screened for mutants that recovered egl-1 reporter expression in a cng-1(vx3) mutant background, and a candidate screen focused on other genes known to regulate activity-dependent genes.

Our reversion screen recovered several alleles of egl-4, the worm homolog of Protein Kinase G, as determined by complementation tests and phenocopy (Daniels et al. 2000; Stansberry et al. 2001; L’etoile et al. 2002). We sequenced the egl-4 genomic area in one of these recovered mutants, vx15, and found a T-to-G transversion that introduces a stop codon eliminating the final 18 amino acids of the protein. This region is part of the “AGC-kinase C-terminus” domain, which is shared among related kinases and is highly conserved through humans (Parker and Parkinson 2001). Both egl-4(n477);cng-1(jh111) double and egl-4(n477) loss-of-function single mutants expressed the egl-1 reporter higher than wild type, though expression was decreased in the double mutant compared to the egl-4 single mutant (Figure 4A, 4D). This indicates that EGL-4 likely acts partially in parallel with CNG-1 and functions to repress expression of egl-1. This is consistent with past studies that have outlined how EGL-4 translocates into the nucleus to affect gene expression in response to long-term neuronal activity (O’Halloran et al. 2012). We confirmed expression of egl-1 in URX in the egl-4(n477) mutant by smFISH (Figure 4B), further affirming the fidelity of the egl-1 reporter to endogenous egl-1 transcription.

Separately, we also examined egl-1 reporter expression in another kinase pathway mutant that has been previously implicated in regulating activity-dependent gene expression. KIN-29 is the worm homolog of the Salt-Inducible Kinase (SIK) family of proteins, and like EGL-4, has been shown to translocate to the nucleus to affect gene transcription in certain contexts (Katoh et al. 2002; Van Der Linden et al. 2007; Van Der Linden et al. 2008). We found that the kin-29(oy38) loss-of-function mutant had increased egl-1 reporter expression in URX compared to wild-type, though less than the egl-4 mutant (Figure 4A).

EGL-4 and KIN-29 have previously been shown to regulate expression of the str-1 chemoreceptor in the AWB sensory neuron by phosphorylating the MEF-2 transcription factor, antagonizing its activity (Van Der Linden et al. 2007). If MEF-2 were also involved in expression of egl-1 in URX, we would expect it to have the opposite phenotype of EGL-4, which would be decreased expression of the egl-1 reporter at 21% oxygen. However, the mef-2(gv1) loss-of-function mutant had the same level of egl-1 reporter expression as wild type (Figure 4C). This suggests that EGL-4 likely does not act through MEF-2 in regulating egl-1 expression as it does str-1.

Several pieces of evidence suggest that egl-4 and kin-29 act at least partially in parallel to regulate egl-1 transcription in URX. First, we found that the egl-4;kin-29 double mutant had higher egl-1 reporter expression than either single mutant (Figure 4A). Reporter intensity at 21% oxygen normalized to wild type was 10.7 ± 2.6 (st. dev.) for the egl-4;kin-29 double mutant, compared to 5.0 ± 1.0 for egl-4 and 2.7 ± 0.6 for kin-29 single mutants. Also, we found that the cng-1;kin-29 double mutant had very low expression of the egl-1 reporter, in contrast to the egl-4;cng-1 double mutant which had higher expression of the reporter than wild type (Figure 4D). These results strongly suggest that egl-4 and kin-29 act through partially separate pathways to repress expression of egl-1 in URX.

Above, we found that the egl-1 reporter was expressed higher than wild type in the egl-4 mutant even when the URX oxygen transcription pathway was severely compromised with the cng-1 mutation. This made us wonder whether environmental oxygen could still regulate egl-1 transcription in URX in mutants without the egl-4 and kin-29 repressor pathways. We hypothesized that the egl-1 reporter would be expressed highly even at low oxygen in these mutants. However we found that egl-4 and kin-29 single mutants as well as the egl-4;kin-29 double mutant still showed decreased reporter expression in low oxygen conditions (Figure 4E). This provides evidence for at least a third, oxygen-dependent mechanism to regulate egl-1 expression in URX. As an independent test for this hypothesis, we asked if the egl-1 reporter was still expressed in egl-4;kin-29 after mutation of the oxygen sensor GCY-35. We found that the triple mutant gcy-35egl-4;kin-29 showed vastly decreased reporter expression compared to the egl-4;kin-29 double mutant (Figure 4F). Interestingly, the triple mutant still had reporter expression at about wild-type levels, and higher than the gcy-35 single mutant. These results are most consistent with the possibility that GCY-35 is upstream of at least one repressive pathway other than the egl-4 and kin-29 pathways, and that this unknown pathway can regulate egl-1 expression in URX in an oxygen-dependent manner (Figure 4G).

DISCUSSION

egl-1 is transcribed in the URX neurons post-developmentally without killing them

Here we described the unexpected transcription of the proapoptotic BH3-only gene egl-1 in the URX neuron pair in post-developmental C. elegans. We also note that while this paper was under review, Taylor, et al. published the first single-cell resolution transcriptome of L4-larval stage C. elegans (Taylor et al. 2019). Out of all of the cells analyzed, this data set reports the highest expression of egl-1 in URX, and to a lesser extent in AW/Coff, AQR, PQR and AIA neurons. This independent approach strikingly confirms the expression pattern that we found for egl-1 using transcriptional reporters and smFISH.

This expression of egl-1 in URX is unusual because it stands in contrast to the typical context of egl-1 expression, which mostly occurs during development to trigger apoptosis. Previous work has suggested that both pro- and anti-apoptotic genes are present in developing cells (Shaham and Horvitz 1996). They function antagonistically, keeping a cell alive unless the apoptotic cascade is initiated by egl-1 expression. However, egl-1 transcription in URX, which continues past when...
somatic cell deaths in the hermaphrodite worm cease, does not lead to cell death in any of the circumstances we examined.

In mammals, several BH3-only proteins are expressed in the adult animal in various tissues, including the nervous system (O’Reilly et al. 2000; Nääpänkangas et al. 2003; Coulitas et al. 2004; Sturm et al. 2006; Lein et al. 2007; Hawrylycz et al. 2012). It is difficult to assess the implication of this expression because many mammalian BH3-only proteins are regulated post-transcriptionally; however, this area of implication of this expression because many mammalian BH3-only proteins is largely unexamined and could prove to be of significance. With at least eight family members, studying BH3-only proteins in mammals can be difficult due to redundancy in their functions (Giam et al. 2008). In contrast, *C. elegans* has only two BH3-only proteins, *egl-1* and *ced-13* (Schumacher et al. 2005). *egl-1* is necessary for nearly all developmental somatic cell deaths while *CED-13* seems to function in germline cell death and does not appear to overlap with the activity of *egl-1* (Schumacher et al. 2005; King et al. 2018). These factors make studying the roles of BH3-only proteins more straightforward in *C. elegans*.

Intriguingly, transgenic expression of *egl-1* has been previously used to genetically ablate the URX neurons. The transgene array *qaIs2241* drives *egl-1* expression from the *gcy-36* promoter, and the death of URX in these animals was evident by both visual inspection and by URX-specific behavioral deficits (Chang et al. 2006; Syer et al. 2008; Carrillo et al. 2013; Zhao et al. 2018). Albeit artificial, this shows that URX is capable of undergoing apoptosis in an *egl-1*-dependent manner.

We propose that the most likely explanation for why this *egl-1* transgene array kills URX while the endogenous expression of *egl-1* we describe in this study does not is related to the expression level of *egl-1* in URX. The *gcy-36* gene is highly expressed in URX and *qaIs2241* contains multiple copies of *Pgcy-36::egl-1* in an array, so transgenic *egl-1* is likely expressed much higher than what occurs endogenously.

A recent study showed a threshold effect for *egl-1* expression, such that some cells during development transiently express very low levels of *egl-1* and do not die, but their daughter cells that do die highly upregulate *egl-1* to initiate cell death (Sherrard et al. 2017). This phenomenon could explain why URX survives development even though we can detect faint expression of our reporter in URX in eggs, when other cell deaths are occurring in the embryo.

### egl-1 transcription in URX is regulated by neuronal activity, EGL-4, and/or KIN-29

We found that sensory transduction, the worm PKG homolog EGL-4, and the worm SIK homolog *KIN-29* all regulate *egl-1* expression in URX in a complex manner. We did not directly test whether EGL-4 and KIN-29 act cell-autonomously in URX to regulate *egl-1*, which leaves open the possibility that they function in other cells to do so. Previous work has shown that both EGL-4 and KIN-29 can act in sensory neurons to cell-autonomously regulate chemoreceptor expression (Lanjuin and Sengupta 2002; Van Der Linden et al. 2008). Furthermore, the cellular localization of EGL-4 has previously been studied directly in AW sensory neurons, where it was shown to translocate to the nucleus when intracellular cGMP decreased with prolonged exposure to an AWC-sensed odorant (O’Halloran et al. 2012). Interestingly, URX has a constitutively high level of cGMP in wild-type animals at 21% oxygen and even higher cGMP production in a *egl-1* mutant.
The circumstantial role in programmed cell death. Though the transcription of family members regulate BH3-only proteins in other systems may be investigated. Our results suggest that exploring whether PKG and SIK 2018); however, their relation to BH3-only proteins has largely not been mRNAs we see in URX is translated to a protein. We attempted to activity-dependent gene expression in C. elegans. This suggests that studying egl-1 expression in URX can offer a complementary system to identify factors that regulate activity-dependent gene expression in C. elegans. Both Protein Kinase G and the Salt-Inducible Kinase family have been shown to be involved in cell death regulation, in both repressive and activating roles in other model systems (Fiscus 2002; Deguchi et al. 2004; Cheng et al. 2009; Fallahian et al. 2011; Du et al. 2016; Tarumoto et al. 2018); however, their relation to BH3-only proteins has largely not been investigated. Our results suggest that exploring whether PKG and SIK family members regulate BH3-only proteins in other systems may be a worthwhile area of study. Though the transcription of egl-1 in URX that we describe does not lead to apoptosis, it is feasible that these/such conserved regulators in other animals may have evolved a general or circumstantial role in programmed cell death.

The egl-1 mutant is not impaired for multiple URX-related phenotypes

One important question that remains unanswered is whether or not the egl-1 mRNA we see in URX is translated to a protein. We attempted to answer this question using a variety of approaches, such as immunohistochemistry using commercially available antibodies, tagging the endogenous protein with CRISPR, and transgenically expressing a tagged version of the protein. Unfortunately these attempts were unsuccessful, so we were unable to definitively assess whether the egl-1 protein product is present in URX. Other labs have also reported being unable to tag the egl-1 protein (B. Conrad, personal communication).

Despite the lack of a clear answer about the presence of egl-1 protein in URX, we nevertheless compared the egl-1 mutant and wild-type worms with regard to the known URX-related functions of oxygen sensation and immune response to pathogenic bacteria. For both cases, we found no differences. We also examined URX at the cellular level in the egl-1 mutant by looking at dendrite and synapse morphology. General synapse morphology looked normal in the egl-1 mutant, though there was a slight defect in dendritic tip morphology compared to wild type. However, this defect could have several origins that are unrelated to an action of egl-1 in URX, so we did not follow this subtle phenotype further.

In the absence of a clear abnormal phenotype of the egl-1 mutant with regard to URX function, we can only speculate about why egl-1 is transcribed in this living pair of neurons. One possibility is that whereas egl-1 normally triggers cell death during development, its expression in URX represents an atypically regulated form of apoptosis for which the egl-1 transcript is maintained in the cell so that in response to some additional trigger the translated protein product then induces apoptosis. There is at least one other example for which egl-1 expression does not cause immediate cell death (Johnsen and Horvitz 2016); the cell Bal/rapaav in the male tail expresses egl-1 but persists in a poised state unless a neighboring cell begins to engulf it, at which point cell death occurs – a process termed "assisted suicide." The URX neuron pair might similarly exist in a poised state in the wild-type worm. In this model, one or more unknown signals would act as the "trigger" that causes translation of egl-1 and possibly cell death. Because wild isolates of C. elegans typically prefer 5–12% O2 conditions and URX is tonically active, one trigger might be prolonged exposure to higher concentrations of oxygen. However, URX neurons show no evidence of death or dysfunction when cultivated at 21% or 100% oxygen. Also, we found that exposure to 100% oxygen did not lead to higher egl-1 reporter expression than at 21% oxygen. Thus, high oxygen levels do not appear to serve as an apoptotic trigger for URX, at least alone.

One potential problem with this apoptotic-trigger hypothesis, however, is that the canonical downstream binding partner of egl-1, CED-9, and the downstream effector CED-4 are both thought to no longer be expressed in somatic tissue past early development (Chen et al. 2000), so egl-1 protein expressed in URX in late larval and adult C. elegans might not induce apoptosis.

An alternate possibility is that egl-1 expression in URX represents a moonlighting function for egl-1, similar to the non-apoptotic roles that are beginning to be uncovered for mammalian BH3-only proteins (Espositi et al. 2001; Danial et al. 2003; Mairui et al. 2007; Danial et al. 2008; Sinha et al. 2008; Giménez-Cassina et al. 2014; Lindqvist et al. 2014). The apoptotic-trigger and moonlighting possibilities are not mutually exclusive.

Activity-dependent egl-1 transcription in post-developmental C. elegans might provide a new system for the study of mechanisms that regulate activity-dependent gene expression and might also possibly reveal a non-apoptotic role for a BH3-only protein. Our future work will focus on identifying the mechanisms and transcription factors by which neuronal activity controls egl-1 transcription in URX, and on what role egl-1 might play in this neuron.

ACKNOWLEDGMENTS

We would like to thank Lina Gomez and Luisa Scott for helpful discussions, Michael Nonet for reagents, and Susan Rozen for expert assistance. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Additional funds were provided by NIH-NIA grants RF1AG057355 and R01AG041135. V.K.D. was a Howard Hughes Medical Institute International Student Research fellow. H.R.H. is an Investigator of the Howard Hughes Medical Institute.

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