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Han Ting Chou
*Georgia State University*, hchou2@gsu.edu

Raymarie Gomez Vazquez
*Georgia State University*, rgomezvazquez1@gsu.edu

Kun Wang
*New York University School of Medicine*, kwang4@student.gsu.edu

Richard Campbell
*Georgia State University*, rcampbell15@student.gsu.edu

Gaolin Zheng Milledge
*North Carolina Central University*

*See next page for additional authors*

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Authors
Han Ting Chou, Raymarie Gomez Vazquez, Kun Wang, Richard Campbell, Gaolin Zheng Milledge, Walter W. Walthall, and Casonya Matese Johnson

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HES-mediated Repression of Pten in *C. elegans*

Han Ting Chou§, Raymarie Gomez Vazquez’, Kun Wang#, Richard Campbell’, Gaolin Zheng Milledge†, Walter W. Walthall’, Casonya M. Johnson§

†Department of Biology and §Center for Obesity Reversal, College of Arts and Sciences, Georgia State University, Atlanta, GA 30303, USA

#Department of Environmental Health Science, Division of Biostatistics, New York University School of Medicine, New York, NY 10016, USA

†Department of Mathematics and Computer Science, North Carolina Central University, Durham, NC 27707, USA
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Corresponding Author:
Casonya M. Johnson
Georgia State University
Department of Biology
161 Jesse Hill Jr. Dr., SE
Atlanta, GA 30303
Phone: 404-413-5426
Electronic mail: cjohnson113@gsu.edu
ABSTRACT The Hairy/Enhancer-of-Split (HES) group of transcription factors controls embryonic development, often by acting downstream of the Notch signaling pathway; however, little is known about postembryonic roles of these proteins. In Caenorhabditis elegans, the six proteins that make up the REF-1 family are considered to be HES orthologs that act in both Notch dependent and Notch-independent pathways to regulate embryonic events. To further our understanding of how the REF-1 family works to coordinate post-embryonic cellular events, we performed a functional characterization of the REF-1 family member, HLH-25. We show that, after embryogenesis, hlh-25 expression persists throughout every developmental stage, including dauer, into adulthood. Like animals that carry loss-of-function alleles in genes required for normal cell cycle progression, the phenotypes of hlh-25 animals include reduced brood size, unfertilized oocytes, and abnormal gonad morphology. Using gene expression microarray, we show that the HLH-25 transcriptional network correlates with the phenotypes of hlh-25 animals, and that the C. elegans Pten ortholog, daf-18, is one major hub in the network. Finally, we show that HLH-25 regulates C. elegans lifespan and dauer recovery, which correlates with a role in the transcriptional repression of daf-18 activity. Collectively, these data provide the first genetic evidence that HLH-25 may be a functional ortholog of mammalian HES1, which represses PTEN activity in mice and human cells.

INTRODUCTION
In Caenorhabditis elegans, HLH-25 is one of six members of the REF-1 family of basic helix-loop-helix (bHLH) transcription factors (ALPER and KENYON 2001). This family is characterized by the presence of two bHLH domains, and are considered functional orthologs of the Hairy Enhancer of Split (HES) family, partly because of their roles as HES-like transducers of Notch signaling in early embryos (NEVES and PRIESS 2005). REF-1, the first family member to be identified via genetic screens, acts downstream of both Notch and GATA signaling to direct endoderm specification (HERMANN et al. 2000; NEVES et al. 2007) and to direct neuronal lineage decisions (LANJUN et al. 2006), including the V-ray lineage in C. elegans males (ROSS et al. 2005). In addition to acting downstream of embryonic Notch signaling events, the family member HLH-29 is targeted by LIN-12/Notch during vulva muscle formation in L4 stage animals (LI et al. 2013). HLH-29 also acts post-embryonically to transcriptionally regulate a diverse set of genes, including those required for chemorepulsive behaviors (McMILLER et al. 2007), IP3-mediated movement of unfertilized oocytes and fertilized eggs through the spermatheca (WHITE et al. 2012), iron homeostasis, and oxidative stress response (QUACH et al. 2013).

Biological roles have not yet been identified for HLH-25. Notch-dependent, embryonic expression of the gene hlh-25 occurs in descendants of the AB blastomeres, cells that eventually give rise to the pharynx, the nervous system, and the hypodermis (NEVES and PRIESS 2005; SCHNABEL and PRIESS 1997). Notch-independent, embryonic expression of hlh-25 is mediated by the GATA factor MED-1 and occurs in descendants of the MS blastomere, which also give rise to the pharynx and muscle and to the somatic gonad (BROITMAN-MADURO et al. 2005; SCHNABEL and PRIESS 1997). Yeast two-hybrid analysis and protein binding microarrays suggest that the REF-1 family proteins fail to heterodimerize with other bHLH proteins and instead bind E-box-like sequences as homodimers (GROVE et al. 2009; POWELL et al. 2004). Of all the REF-1 family proteins, HLH-25 has the highest degree of DNA-binding promiscuity and tightly binds to five E-box-like sequences. As a comparison, REF-1 binds tightly and HLH-26
binds weakly to the same E-box-like sequence, while HLH-29 only binds weakly to two sequences (GROVE et al. 2009). Gene ontology of the genes containing HLH-25 binding sites predicts that HLH-25 plays critical roles in cell division and development, including embryonic development, larval development, growth, and specification of cell fate (GROVE et al. 2009).

HLH-25’s putative role in regulating cell division and embryonic and post-embryonic development supports earlier studies suggesting that the C. elegans REF-1 family proteins are orthologs of the mammalian HES protein family (NEVES et al. 2007). Like the other REF-1 family proteins, the sequence similarity between HLH-25 and the HES protein family lies within the bHLH domain.

HLH-25 is more similar to HES1 than to the other mouse HES proteins, but is most similar to the mouse Atonal homolog protein Math1 when amino acid residues flanking the bHLH domain are included in the analysis. Both Atonal1 and Hes1 play well established roles in directing neuronal cell fate decisions (AKAZAWA et al. 1995; BEN-ARIE et al. 2000; OUJI et al. 2013; TOMITA et al. 2000; WANG et al. 2001), as do the respective, previously characterized C. elegans orthologs LIN-32 and LIN-22 (MILLER and PORTMAN 2011; WRISCHNIK and KENYON 1997). Other studies suggest that the human and mouse HES1 proteins are oncogenic (AXELSON 2004; GAO et al. 2014; SANG et al. 2010), underscoring the roles that these proteins play in regulating cell division and cell proliferation.

In this study, we describe a functional characterization of HLH-25 using reverse genetics and molecular approaches. We show that hlh-25 is expressed post-embryonically in body wall muscles and in neurons, and that the phenotypes of hlh-25 animals include reduced brood size, unfertilized oocytes, and abnormal gonad morphology, all of which are consistent with defective cell cycle or cell division events during embryogenesis. We

show that the predicted HLH-25 transcriptional network, based on gene expression microarray, includes the Pten ortholog DAF-18 and other genes that mediate cell division, embryonic development, and larval development. Finally, we show that the ability of HLH-25 to facilitate dauer recovery and lifespan correlates with our mRNA studies suggesting that HLH-25 transcriptionally represses daf-18/Pten expression.

RESULTS

Phenotypes of hlh-25(ok1710) animals and Phlh-25::GFP activity suggest roles for HLH-25 in reproduction. Previous studies indicate that hlh-25 is expressed in 8-cell stage embryos in response to Notch signalling, and in later stage embryos in response to GATA signalling (BROITMAN-MADURO et al. 2005; NEVES and PRIESS 2005). Using an hlh-25::GFP transcriptional reporter cmjEx31 (Figure S1), we detected post-embryonic expression of hlh-25 at all larval and adult stages, including dauer stage, in unidentified neurons of the head and tail, the anal depressor muscle, anterior and posterior intestinal cells, and the head and body wall muscles of transgenic animals (Figure 1). We detected expression in the PDE sensory neuron pairs and rare, transient expression in migrating distal tip cells of L3/L4 stage animals. We also detected stage-specific hlh-25 expression in multiple neurons of the ventral nerve cord in starvation-induced dauer stage animals. We were able to use cell body location and commissure morphology to identify three of the motor neurons as VD1, DA3, and DA7. Expression in the body wall and head muscles and in the PDE neuron pair was not evident in dauer larvae.

The hlh-25(ok1710) allele is a null allele that was generated by EMS mutagenesis. It is a 1550 bp deletion that spans from 292 bp upstream of the initiator codon to 348 bp downstream of the terminator codon (Figure
S1). We outcrossed these animals 10 times, confirmed homozygosity for the ok1710 allele by PCR, and then examined them for mutant phenotypes. *hlh-25(ok1710)* animals produced fewer live progeny at 20°C than wild-type animals (Figure 2A, P-value = 0.0027) while laying more unfertilized oocytes (P-value = 0.00014) throughout the egg-laying period.

Adult hermaphrodites produce more oocytes than sperm, and will continue to lay unfertilized eggs for a short period after depleting their sperm supply. Under conditions where food is plentiful, and when cultured at 20°C, wild-type hermaphrodites lay eggs over the first five days of adulthood (WARD and CARREL 1979; WHITE et al. 2012). As previously described, we found that wild-type animals lay an increasing number of unfertilized eggs as the sperm supply diminishes. In our assays, wild-type animals continued to lay a small number of unfertilized eggs for one additional day after the active egg-laying period ended (egg-laying day 6), to produce an average total of 49 unfertilized oocytes (Figure 2B, Table S1). *hlh-25(ok1710)* animals continued to produce unfertilized oocytes for four days after the active egg-laying period ended (egg-laying day 9), and produced an average total of 232 unfertilized oocytes. This phenotype has been recently named uno-o (RIESEN et al. 2014), and is also seen in animals that carry null alleles of the REF-1 family protein HLH-29 (WHITE et al. 2012). We were able to partially rescue the uno-o phenotype in *hlh-25(ok1710)* animals by expressing *hlh-25* from an extrachromosomal array, cmjEx32, so that transgenic animals produced an average of 38% fewer unfertilized oocytes than *hlh-25(ok1710)* animals (Figure 2A).

We also examined the gonad arms of adult hermaphrodites. In wild-type animals, oocytes production occurs as progressive movement through two U-shaped gonad arms, starting with the production of germline nuclei at the distal end of each gonad arm, and ending with the ovulation of the oocyte into the sperm-filled spermatheca at the ends proximal to the uterus (HUBBARD and GREENSTEIN 2000). In the distal, syncytial region of each arm, the germline nuclei go through mitotic and meiotic divisions while being surrounded by a commonly shared cytoplasmic core. Nuclei start to compartmentalize into oocytes as they move progressively closer to a bend in the gonad arm, and are fully compartmentalized, or budded, as they leave the bend and proceed towards the spermatheca (GREEN et al. 2011). We found that organization of the gonad arm and germline progression were abnormal in 63% of *hlh-25(ok1710)* animals. As shown in Figure 3 and in Figures S2 and S3, gonad architecture was variably abnormal and included constrictions in the distal and proximal gonad arms, increased apoptosis, increased oocyte number, delayed oocyte compartmentalization, abnormal chromatin condensation, and arrested embryos in the uterus.

Other phenotypes of *hlh-25(ok1710)* animals included decreased movement as monitored by thrashing and locomotion assays (Figure S4), leading us to classify *hlh-25* mutants as locomotion variants. This phenotype correlates with the expression of cmjEx31 in the body wall muscles and in head neurons, but we could not accurately assay for rescue of this phenotype by transgenic expression of *hlh-25* because transgenic animals also carried the rol-6 marker, which causes a dominant locomotor defect. We did not pursue the locomotion phenotype further in this study.

**DAF-18 is one major hub in the *hlh-25* transcriptional network.** We used gene expression microarray to compare the expression profiles of late L4/young-adult stage *hlh-25(ok1710)* animals to profiles of age-matched, wild-type animals. We found
that the expression of 630 genes, 506 of which were up-regulated and 124 of which were down-regulated, was altered by at least 2.0 fold (Table S2), and confirmed by RT-qPCR that the expression of 21 genes was significantly affected in L4-stage hlh-25(ok1710) animals (Table 1). As shown in Table 1, the direction of the change in expression was the same for 18 of the 21 genes. It is possible that the opposite, but significant change in the three remaining genes was due to differences in the developmental age of animals at the times of RNA extraction. Interestingly, 152 of these genes, indicated by asterisks in Tables 1 and S2, contain predicted HLH-25 binding sites within their putative promoters (GROVE et al. 2009). Hypergeometric analysis, indicated that this overlap of 152 genes was significantly lower than expected when compared to an overlap between the list of genes identified by protein binding microarray to a randomly chosen gene set of the same size (P-value < 2.5 x 10^{-05}).

We clustered related target genes based on enriched gene ontology (GO) terms. A total of 328 genes were organized into 10 annotation clusters that all fit within the broad category of ‘Development’ (top eight clusters are shown in Figure 4A). Using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING 9.0) to identify predicted and known interactions between proteins encoded by the target genes (SZKLARCZYK et al. 2011), we found that the hlh-25 regulatory network has a high degree of overlap and interconnectivity. For example, 38 genes clustered under the GO term ‘SEXUAL REPRODUCTION.’ Of those, 22 genes (58%) formed a single, highly interconnected, interaction network (Figure 4B) and were also associated with at least two other clusters. This trend of high connectivity among genes within a cluster and significant overlap among genes between clusters was evident for the entire network. We also identified six genes that we considered to be major hubs (Table 2). These genes were associated with at least four of the GO clusters and formed predicted interactions with at least 35 other targets in the network. Importantly, the hub genes underscore the developmental influence of the hlh-25 regulatory network, encoding helicases, cyclins, and RNA binding proteins with established roles in embryogenesis (Table 2). The C. elegans homolog of the mammalian Pten tumor suppressor, DAF-18 (MASSE et al. 2005), is one of the major hubs, forming connections with 62 proteins distributed across six annotation clusters (Figure 4C, bold genes in Table S2).

HLH-25 functions upstream of DAF-18 to regulate dauer recovery. It is not clear whether the REF-1 proteins and the HES proteins arose from the same parent gene; however, conservation within the helix-loop-helix domains and the ability to transduce Notch signals suggest that REF-1 proteins and HES proteins are members of the same functional family. Importantly, the identification of daf-18 as a major hub in the HLH-25 transcriptional network, together with the identification of predicted HLH-25 binding sites in the daf-18 promoter (GROVE et al. 2009), underscores the functional homology between the HLH-25 and vertebrate HES1 protein. In the mouse thymus, HES1 acts downstream of Notch signalling to repress Pten activity during T-cell differentiation (WONG et al. 2012), and the resistance of T-cell acute lymphoblastic leukemia (T-ALL) cells to Notch inhibition depends on functional HES1 binding sites in the Pten promoter (PALOMERO et al. 2007). Therefore, we further characterized the genetic interaction between the genes encoding the HES homolog HLH-25 and the PTEN homolog DAF-18.

Using RT-qPCR we found that the levels of daf-18 mRNA increase greater than 2-fold in embryos (data not shown) and in L4 stage animals (Table 1) compared to age-
matched controls. The levels of daf-18 mRNA also increased in wild-type animals that were subjected to hlh-25 RNAi (Figure S5), suggesting that the increased daf-18 activity is due to loss of HLH-25 in hlh-25(ok1710) animals. We failed to detect differences in daf-18 transcriptional activity in wild-type and hlh-25 animals expressing Pdaf-18::GFP via a previously described, integrated transgene. These animals were generated in a daf-2 loss-of-function background, which increases the transcriptional activity of the daf-18 promoter (SOLARI et al. 2005). It is possible that loss of daf-2 masks the effects of HLH-25. Unfortunately, we have not yet been successful in generating stably transgenic lines expressing Pdaf-18::GFP in animals that produce functional DAF-2.

In C. elegans, longevity and entry into dauer stage are controlled through the insulin signalling pathway, and depend on the insulin receptor DAF-2. Decreased insulin signalling, via loss-of-function mutations in daf-2, increases C. elegans lifespan and causes a dauer-constitutive phenotype. These phenotypes are suppressed in daf-18 animals: daf-18 animals have reduced lifespan (SOLARI et al. 2005) and animals that carry strong loss-of-function alleles are unable to undergo dauer development (GIL et al. 1999; MIHAYLOVA et al. 1999; ROUAULT et al. 1999). The reduced lifespan of daf-18 animals can be rescued, and slightly extended beyond the wild-type lifespan, by the overexpression of daf-18 from the extra-chromosomal array quls18 (BRISBIN et al. 2009; SOLARI et al. 2005). We reasoned that since hlh-25(ok1710) animals have increased daf-18 activity, they should have an extended lifespan. As expected, the lifespan of hlh-25(ok1710) animals (median LS = 19 days) is significantly longer than the lifespan of wild-animals (median LS = 16 days, P-value <0.0001) grown at 20°C (Figure 5A). This result is comparable to the extended lifespan previously reported for C. elegans quls18 females (BRISBIN et al. 2009).

Normally when wild-type, starvation-induced dauer larvae are moved to favorable growth conditions, they take approximately 60 minutes to commit to exiting the dauer stage at 25°C, and they molt into the L4 stage at approximately ten hours (GOLDEN and RIDDLE 1982; GOLDEN and RIDDLE 1984a; GOLDEN and RIDDLE 1984b; GOLDEN and RIDDLE 1984c). As animals begin to exit dauer stage and enter into the L4 molt, fat accumulation and pharyngeal pumping begin after approximately 60 minutes and three hours, respectively, of making the dauer recovery/exit decision (PROUDFOOT et al. 1993). The onset of vulva formation is detectable within twelve hours of the exit decision, and recovering dauers enter late L4/early adult stage within 24 hours of the decision to exit dauer stage.

Because daf-18(ok480) do not form dauers under starvation conditions, we used heat stress to induce dauer formation via an IIS-independent pathway in wild-type, hlh-25(ok1710), daf-18(ok480), and quls18 animals, and compared their dauer recovery phenotypes. We found that at least 80% of wild-type animals (n = 1199) reached early L4 stage within 12 hours after the switch to 20°C. As expected, dauer recovery was similar to wild-type in daf-18(ok480) animals (n = 485) but was longer in quls18 animals (n = 955). At 12 hours, approximately 65% of the daf-18(ok480) animals, but only 10% of the quls18 animals, reached early L4. Interestingly, the dauer recovery timing of the hlh-25(ok1710) animals (n = 738) was very similar to the quls18 animals: only 21% of hlh-25(ok1710) animals reached L4 stage within 12 hours, and only 45% reached L4 stage within 24 hours. This delayed dauer recovery phenotype in hlh-25(ok1710) animals was partially rescued by transgenic expression of hlh-25 (cmjEx32): 70% of transgenic animals (n = 300) reached L4 within 24 hours. For a more quantitative
approach, we monitored the onset of pharyngeal pumping during dauer recovery. Dauer recovery in daf-18(ok480) animals was not significantly different from recovery in wild-type animals; however, it took 1.5 times longer for the onset of pharyngeal pumping to occur in 50% of hlh-25(ok1710) animals (P-value < 0.0001) than in wild-type animals (Figure 5B). This delay was rescued by transgenic expression of hlh-25 (Figure 5C). These results, together with our gene expression data, suggest that HLH-25 acts upstream of daf-18 to negatively regulate lifespan and dauer recovery.

DISCUSSION

This study is a functional characterization of HLH-25, a member of the C. elegans REF-1 family of transcriptional regulators. Previous studies have shown that the gene hlh-25 is transcriptionally activated through Notch-signaling in cells that eventually give rise to the pharynx and nervous system, and through the Notch-independent GATA family of transcription factors in tissues that give rise to the pharynx, muscle, and somatic gonad. Here we show that expression of hlh-25 persists throughout the C. elegans life cycle. Although we identified a number of HLH-25 targets that are germline expressed and whose mRNAs are stored in oocytes prior to fertilization, we were unable to recover transgenic lines that report germline expression of hlh-25. Future studies may further define the role of HLH-25 in embryogenesis, which includes determining whether the maternal contribution of HLH-25 is required for proper embryogenesis.

Our results show that the phenotypes of hlh-25 animals include reduced brood size, unfertilized oocytes in the uterus, abnormal gonad morphology/architecture, extended lifespan, and delayed dauer recovery. In previous studies, functional annotation of candidate target genes suggested overlapping roles in cell signaling events for all of the REF-1 family proteins, and HES-like roles for HLH-25 and HLH-29 in regulating embryonic and larval development, growth, cell fate specification, and reproductive behaviors (GROVE et al. 2009). Interestingly, this functional overlap is suggested, but not clearly evident, in the phenotypes of REF-1 family mutants that have been characterized to date. Both ref-1 and hlh-29 animals, for example, show varying degrees of embryonic lethality that would ultimately lead to reduced brood size (ALPER and KENYON 2001; McMILLER et al. 2007; WHITE et al. 2012); however the reduced brood size in hlh-25 animals is not due to lethality during embryogenesis, but to abnormalities that occur prior to fertilization. Likewise, mutations in ref-1, hlh-29, and hlh-25 cause morphological defects that affect sexual reproduction. Loss of ref-1 affects cell fusion events during vulva morphogenesis (ALPER and KENYON 2001) and ray lineage decisions during morphogenesis of the tail (ROSS et al. 2005). Loss of either hlh-29 or hlh-25 results in unfertilized oocytes, abnormal gonad morphologies, and the exploded through vulva phenotype; however, the underlying mechanisms of these phenotypes appears to differ in hlh-25 and hlh-29 animals. The unfertilized oocyte phenotype in hlh-29 animals, for example, is caused in part by altered expression of genes required for inositol 1, 4, 5-triphosphate (IP₃) signaling, which affects the ability of oocytes and eggs to move through the spermatheca (WHITE et al. 2012). Based on our observations of independent ovulation events, however, spermatheca function in hlh-25 animals appears normal, and genes required for IP₃ signaling are not affected by loss of HLH-25. These data suggest that the REF-1 family proteins do not act within a single transcriptional cascade to regulate specific events, and that each family member plays unique, stage-specific roles in C. elegans development.
Our gene expression microarray analysis suggests that there are six major hubs in the HLH-25 transcriptional network, including the *C. elegans* PTEN homolog, *daf-18*. We demonstrated by RT-qPCR that *daf-18* expression is normally repressed by HLH-25. This result is the first direct correlation between a HES protein target gene in mammals and a REF-1 family target gene in *C. elegans*. Hes1 directs cell division, in part, by repressing *Pten* expression in mouse (Wong et al. 2012), in human large cell neuroendocrine carcinoma (Nasgashio et al. 2011), and in regenerating Clara cells of wild-type lungs (Xing et al. 2012). In *C. elegans*, DAF-18 is required during embryogenesis to maintain G2 arrest in germline precursor cells (Fukuyama et al. 2006) via inhibition of Tor complex 1 signaling (Fukuyama et al. 2012). We did not examine embryonic or L1-stage phenotypes of *hlh-25* animals; however, we show that, like animals that overexpress *daf-18* (Brisbin et al. 2009; Liu and Chin-Sang 2015), *hlh-25* animals have an extended lifespan and take longer to exit dauer stage. Further examination of both the embryonic and post-embryonic regulation of *daf-18* by HLH-25 will further our understanding of the mechanisms and biological significance of HES-dependent activation and repression of PTEN.

*C. elegans* enter the dauer stage in response to adverse growth conditions, maintain this state while conditions remain undesirable, and then exit, or recover from dauer stage when environmental conditions are more favorable (Hu 2007; Ouellet et al. 2008). The decision and ability to enter into dauer stage requires the integration of dietary and environmental signals through multiple molecular pathways, including insulin/IGF, TGF-β, and guanylyl cyclase, and the molecular details of dauer entry in response to starvation, heat stress, and overcrowding have been well established (reviewed in FieLENbach and AntEBi 2008). Less is known about the molecular requirements for dauer maintenance and dauer recovery. Studies suggest that GLP-1 dependent Notch activation in chemosensory neurons is required for dauer maintenance (Ouellet et al. 2008), and that metabotropic acetylcholine signaling is required for the induction of an insulin-like signal during dauer recovery (Tissenbaum et al. 2000). Previous studies have also shown that dauer recovery requires LIN-12 dependent Notch activation, though the downstream target of this signaling event has not been identified (Ouellet et al. 2008). Future studies will determine whether HLH-25 acts downstream of Notch to modulate dauer recovery by repressing *daf-18* activity.

Finally, gene ontology and protein interaction analysis of the HLH-25 network suggests that HLH-25 affects genes required for three highly connected processes: cell division, cell cycle regulation, and reproduction, including gamete formation. The connectivity between the genes in these processes suggests that the HLH-25 regulatory network is amendable to change without the corresponding disruption in overall functionality. This feature is illustrated by the hub gene *daf-18*, and the hub genes *cgh-1, cyb-2.1, cyb-3, mcm-3*, and *put-3*, which encode proteins required for early embryonic cytokinesis, meiosis II, G2/mitosis transition, licensing of DNA replication, and spindle positioning, respectively (Audhya et al. 2005; Boag et al. 2005; Hubstenberger et al. 2012; Kaitna et al. 2002; Sonnevile et al. 2012; Van der Voet et al. 2009). Though mutations in any of the hub genes, or in any of the nodes connected to them, result in severe phenotypes, including sterility, mutant animals are viable and have functional cell cycles. Future work will include further defining and validating the HLH-25 transcriptional network and correlating this network with the post-embryonic functions of HLH-25.

Taken together, our results demonstrate that the *C. elegans* HES
homolog, HLH-25, functions post-embryonically to transcriptionally regulate genes required for cell division, cell cycle regulation, and sexual reproduction, including the gene encoding the C. elegans Pten homolog, DAF-18.

MATERIALS AND METHODS

C. elegans Growth and Culture Conditions

The following strains were used: N2 Bristol wild-type (BRENNER 1974); VC1220, hlh-25(ok1710) II; RB712, daf-18(ek480) IV; IC748, quIs18; CMJ3001, cmjEX31(Phlh-25::GFP::unc-54 'UTR in pPD95.67 + pRF4); CMJ3002, hlh-25(ok1710) II; cmjEx32[(Phlh-25::hlh-25) + (Pmyo2::mCherry::unc-54 3'UTR)]; and CMJ3003, hlh-25(ok1710) II; daf-18(ek480). The extrachromosomal array cmjEx31 includes 1540 nucleotides upstream of the predicted initiation codon, plus the first 30 nucleotides of the coding region for hlh-25, fused in-frame to sequences coding GFP. The extrachromosomal array cmjEx32 includes the genomic DNA sequences spanning from 1540 nucleotides upstream of the initiator codon to 573 nucleotides downstream of the predicted stop codon for hlh-25. VC1220 animals were outcrossed 10 times to N2 animals, and final homozygosity was confirmed by PCR. Animals were maintained at 20°C on NGM agar plates seeded with Escherichia coli strain OP50 and were synchronized by alkaline hypochlorite treatment as previously described (LEWIS and FLEMING 1995). Bacteria mediated RNAi was performed as previously described (KAMATH et al. 2003; QUACH et al. 2013) using HT115 producing dsRNA for either the control gene unc-55 or for hlh-25/hlh-27. The genes hlh-25 and hlh-27 are predicted to produce identical mRNAs and it is impossible to selectively silence hlh-25.

Microscopy

For imaging, animals were anesthetized either with 0.2% levamisole (GOVINDAN et al. 2009) or with 10 mM NaN₃, mounted on 2% agarose pads containing 10 mM NaN₃, and imaged using either a 40X or a 60X water-immersion objective with the Nikon Eclipse 90i microscope equipped with a Nikon CoolSnap CCD camera. Z projections (sums of intensity) were obtained using NIS-Elements AR, version 4.0. For expression profiling, animals were cultured for at least 2 generations at 16°C and then were synchronized and allowed to grow to the appropriate stage (L1 to L4). Forty animals per larval stage were examined for expression. For dauer stage expression, 10 starvation-induced dauer were imaged.

Egg Laying Assays

L4-stage animals were selected to individual NGM plates and allowed to molt to adulthood at 20°C. At the start of the egg laying period, each animal was moved daily to a new plate, already seeded with OP50, until 3 days after eggs or unfertilized oocytes were no longer detected. Eggs and oocytes were counted on the day that the hermaphrodite was removed. The plate was then incubated at 20°C for 24 hours, and the numbers of live progeny and unhatched eggs were counted. Eggs that did not hatch within the 24 hour period were scored as dead, while the number of live progeny was checked again in the next 24 hours and then removed. For rescue assays, the ratio of unfertilized oocytes to fertilized eggs was calculated daily.

Lifespan Assays

Lifespan assays were performed as previously reported (QUACH et al. 2013). Lifespan survival comparisons were done individually for each of three biological replicates using the log rank test in GraphPad Prism 6 (GraphPad Software, Inc) and were then repeated with the data from all biological replicates combined into one experiment. Combined data are presented here.
Dauer Recovery Assays
To induce dauer formation, embryos were collected by hypochlorite treatment, washed extensively in water, and then approximately 200 embryos were placed onto an NGM plate containing a sparse lawn of OP50 (~10 µL of saturated OP50, 24 hours before use). Embryos were allowed to hatch, and the dauer larva to develop, undisturbed at 27°C for at least 96 hours. Cultures were visually examined to ensure that at least 90% of the larvae were arrested as dauer. Failure to include OP50 on the plate resulted in animals that arrested as starved L1 stage larvae. For dauer recovery, 25 dauers per strain were individually moved to a plate with a thick lawn of OP50 and incubated at 20°C. Dauers were examined every three hours over a twenty-four hour period for fat accumulation, pharyngeal pumping, crescent formation, and then at 8 hour intervals for another 24 hours for oocyte/embryo formation, and onset of egg-laying. The rates of onset of pharyngeal pumping were plotted as survival from dauer, and were compared by log-rank test using GraphPad Prism 6. The timing of dauer recovery varied with the day of the assay, with the onset of pumping occurring in 50% of wild-type animals as early as 3 hours or as late as 8.5 hours. In all assays the trend was the same, and the data shown are representative of one experiment.

Total RNA Isolation, cDNA Synthesis, Reverse Transcription, and Quantitative PCR
Total RNA extraction and cDNA synthesis were carried out as previously described (Quach et al. 2013) except total RNA was extracted from synchronized L4 stage animals. After cDNA synthesis, qPCR assays were performed with Taqman Gene Expression Assays (Invitrogen) for detection of amplicon, using relative quantitation with normalization against the endogenous control gene pmp-3 (Hooge wijs et al. 2008). Values shown represent the averages of at least three different experiments. The ID numbers for the Taqman Assays used in this study are available upon request.

Gene Expression Microarray
Gene expression microarray (GEO Accession #GSE65417), including probe preparation, hybridization, fluidics run and chip scan, was performed by Georgia State University DNA/Protein Core Facility. Global gene expression in synchronized populations of VC1220 animals was compared to expression in N2 (wild-type) animals using GeneChip C. elegans Genome Array (Affymetrix). Data collection was carried out using GCOS 1.4 software (Affymetrix). Data analysis was performed using GeneSpring GX 11 Software (Agilent, Palo Alto, CA), and probe intensity values were normalized using Robust Multichip Average (RMA)-algorithm. The quality controls on samples and on probe sets were performed stepwise to detect the outlying samples and the poor probe sets. The Principal Components Analysis (PCA) score plot and hybridization controls plot were applied for sample detection. Those probe sets passing minimal detection cutoffs and quality control measurements subsequently were used for statistical analysis. The student’s t-test was performed to find the candidates for differential expression, and genes with significant signal level between different conditions (p<0.05) were collected. In addition, fold change analysis were performed on the genes with significant expression, and all genes showing greater than two-fold change were considered putative targets.

Functional Analysis by Gene Ontology
Gene Ontology (GO) analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID), version 6.7, to cluster related target genes (Huang da et al. 2009a; Huang da et al.
Additional GO terms and functional information for putative targets were assigned based on Wormbase annotations (HARRIS et al. 2010). Interactions and connectivity between target gene products were identified using the Search Tool for the Retrieval of Interacting Genes/Proteins (SZKLARCZYK et al. 2011) and redrawn manually.

**Mobility Assays**
For locomotion assays, individual L4 stage animals were placed by platinum wire onto an unseeded NGM plate, allowed to recover for 5 minutes, and then examined after the start of forward locomotion for 30-seconds. For thrashing assays, individual animals were placed into 50 µL of M9 medium. Each lateral movement of the head was counted as one thrash, and movement was monitored for one 30 second interval per animal. Each biological replica included at least 25 animals per strain, and each assay was repeated for three biological replicas.

**ACKNOWLEDGEMENTS**
We gratefully acknowledge our undergraduate research assistants, Emma Blasingame and Thai Tran for help with assays for brood size and embryonic viability, and the technical assistance of the Georgia State University microarray facility coordinator, Ms. Sonja Young. *Caenorhabditis elegans* strains used in this study were provided by the Caenorhabditis Genetics Center (funded by the NIH National Center for Research Resources, USA), Dr. I.D. Chin-Sang (Queens University), and Dr. F. Solari (Claude Bernard University). Portions of this work served to partially fulfill the requirements for the Masters of Science degree for R. Gomez Vazquez and K. Wang. The National Science Foundation grants MCB0919413 and MCB1139184 to C. Johnson supported this research and HT Chou, and provided stipend support for K. Wang and R. Gomez Vazquez, respectively. R. Campbell is a GSU Brains and Behavior Fellow.

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Table 1. Validation of randomly selected HLH-25 targets by RT-qPCR

<table>
<thead>
<tr>
<th>WormBase ID</th>
<th>Sequence ID</th>
<th>Gene Name</th>
<th>log$_2$ FC (microarray)</th>
<th>log$_2$ FC (RT-qPCR)</th>
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<tr>
<td>WBGene00000230</td>
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<td>T12D8.5</td>
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*target previously predicted by protein binding microarray (GROVE et al. 2009)
Table 2. Hub Genes in the HLH-25 Network

<table>
<thead>
<tr>
<th>Wormbase ID</th>
<th>Sequence ID</th>
<th>Gene Name</th>
<th>Functional Summary^</th>
<th>GO Cluster Association*</th>
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<tr>
<td>WBGene000000479</td>
<td>C07H6.5.2</td>
<td>cgh-1</td>
<td>ATP-dependent helicase; prevents apoptosis of developing embryos</td>
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<td>WBGene00000866</td>
<td>Y43E12A.1</td>
<td>cyb-2.1</td>
<td>Cyclin B family; required for oocyte maturation</td>
<td>X X X X X</td>
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<td>WBGene00000868</td>
<td>T06E6.2a.2</td>
<td>cyb-3</td>
<td>G2/mitotic-specific, Cyclin B3</td>
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</tr>
<tr>
<td>WBGene00000913</td>
<td>T07A9.6</td>
<td>daf-18</td>
<td>Phosphatase; negative regulator of insulin-like signaling</td>
<td>X X X</td>
</tr>
<tr>
<td>WBGene00003155</td>
<td>C25D7.6.2</td>
<td>mcm-3</td>
<td>DNA replication licensing factor</td>
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<tr>
<td>WBGene00006239</td>
<td>Y45F10A.2.1</td>
<td>put-3</td>
<td>RNA bindig protein; required for cell cycle timing, spindle positioning, pronuclei formation</td>
<td>X X X</td>
</tr>
</tbody>
</table>

^Functional summaries are based on Wormbase annotations (www.wormbase.org)

*ED, embryonic development ending in birth; S, sexual reproduction; P, nucleoside, proton transport; M, meiosis; G, regulation of growth; C, cell cycle
Figure 1. Postembryonic expression of Phlh-25::GFP. A-D) Nomarski images (left) accompanied by GFP fluorescent images (right). A) Expression of cmjEx31 in multiple head neurons (arrowheads). This pattern was detected in animals at all stages, with a few additional neurons at dauer stage. B) L3 expression in distal tip cell (arrow, red dashed circle), unidentified head neuron (arrowhead) and head muscles (asterisk). C) L3 stage expression in PDE (arrow). Arrowheads indicate PDE processes. D) Dauer stage expression in ventral nerve cord neurons. In all images anterior is to the right, dorsal is up. Scale bar represents 50 µm.
Figure 2. Brood size and unfertilized oocytes hlh-25(ok1710) animals. A) hlh-25(ok1710) animals have fewer live progeny and lay more unfertilized eggs than wild-type animals. Expression of the extrachromosomal array cmjEx32, which carries hlh-25 genomic sequences, rescues this phenotype in hlh-25(ok1710) animals. Graph shows mean of total numbers of live progeny (dark bars) and unfertilized eggs (light bars) produced during the entire lifespans of wild-type (n = 7), hlh-25(ok1710) (n = 12), and hlh-25(ok1710; cmjEx32) animals (n = 9). * P-value <0.05, **P-value <0.005. B) Average numbers of live progeny (solid lines) and unfertilized eggs (dashed lines) produced per day of egg laying by the same hlh-25(ok1710) animals (triangles) and wild-type animals (circles) as
Figure 3. Gonad and oocyte morphologies of *hlh-25(ok1710)* animals. Nomarski (A, C) and merged fluorescent (B,D) images of representative gonad architecture and oocyte morphology in wild-type animals (A,B) as analyzed in a strain co-expressing a fluorescent chromosome marker (mCherry-Histone H2b) and a marker targeting GFP to the plasma membrane (PI4,5P2) (GREEN et al. 2011), (B). Representative gonad architecture and oocyte morphology in *hlh-25(ok1710)* animals (C,D). Phenotypes include increased oocyte number, distal gonad arm constriction (arrow), increased apoptosis (^), and arrested embryos in uterus (#). Insert in C) shows magnified view of distal gonad arm constriction. Inserts in B) and D) show embryonic nuclei. In all images, ventral is up, anterior is to the right, scale bar = 50 μm.
Figure 4. Functional annotation of HLH-25 targets. A) Distribution of HLH-25 targets into top eight GO clusters. B) Predicted and known interactions between genes in the ‘SEXUAL REPRODUCTION’ cluster. The hub genes cgh-1, cyb-2.1, cyb-2.2, cyb-3, and puf-3 are outlined in red. The hub gene daf-18 is not a part of the ‘SEXUAL REPRODUCTION’ cluster. C) Representative genes from the 62 HLH-25 targets that form predicted or known interactions with daf-18. All genes, except daf-18, clustered under “EMBRYONIC DEVELOPMENT ENDING IN BIRTH (EDB)” Most genes also clustered under at least one other top GO category, including “NUCLEOSIDE/NUCLEOTIDE TRANSPORT” (green ovals).
Figure 5. HLH-25 regulates lifespan and dauer recovery. A) 
*hlh-25(ok1710)* animals (n = 282) have longer than lifespans than wild-type animals (n = 264, P-value <0.0001). B) Representative experiment showing the percentage of animals with pharyngeal pumping during dauer recovery at 20°C. P-value <0.0001 and P-value = 0.938 when *hlh-25(ok1710)* animals and *daf-18(ok480)* animals, respectively, were compared to wild-type. C) Representative experiment showing the percentage of animals with pharyngeal pumping during dauer recovery at 20°C. P-value <0.0001 and P-value = 0.2771 when *hlh-25(ok1710)* and *hlh-25(ok1710;cmjEx32)* animals, respectively, were compared to wild-type.