An expression screen for RhoGEF genes involved in *C. elegans* gonadogenesis

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**Abstract**

The gonad in *Caenorhabditis elegans* is an important model system for understanding complex morphogenetic processes including cellular movement, cell fusion, cell invasion and cell polarity during development. One class of signaling proteins known to be critical for the cellular events underlying morphogenesis is the Rho family GTPases, particularly RhoA, Rac and Cdc42. In *C. elegans* orthologues of these genes have been shown to be important for gonad development. In our current study we have extended those findings by examining the patterns of 5' cis-regulatory element (5'CRE) activity associated with nineteen putative guanine nucleotide exchange factors (GEFs) encoded by the *C. elegans* genome predicted to activate Rho family GTPases. Here we identify 13 RhoGEF genes that are expressed during gonadogenesis and characterize the cells in which their 5'CREs are active. These data provide the basis for designing experiments to examine Rho GTPase activation during morphogenetic processes central to normal gonad development.

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1. Results and discussion

The gonad in *Caenorhabditis elegans* is an excellent model for elucidating the genetic and cell-biological mechanisms underlying organogenesis (Hubbard and Greenstein, 2000). The somatic tissues of the adult hermaphrodite gonad are generated through stereotyped division, rearrangement and patterning of the progeny of two somatic gonadal cells known as Z1 and Z4 (Kimble and Hirsh, 1979). These cells encircle the primordial germ cells near the end of embryogenesis to form the gonad primordium (Fig. 1A and E) (Kimble and Hirsh, 1979). After hatching, development in *C. elegans* proceeds through four distinct larval growth stages (L1–L4) separated by cuticular molts. During the L1 stage, Z1 and Z4 begin a process of oriented cell division that generates the cells whose progeny give rise to the tissues of the gonad (Fig. 1A and E) (Kimble and Hirsh, 1979). Cell division continues during the L2 stage, but this developmental window is also characterized by early morphogenetic changes within the gonad. Of particular importance are the movements of cells that give rise to the uterine and spermathecal epithelia as well as the gonad sheath cells. During the late L2 stage, these 10 cells come to completely occupy the central region of the gonad, forming the somatic gonad primordium in a process coincident with exclusion of the germ cells into the gonad arms (Fig. 1B and F) (Kimble and Hirsh, 1979). Also at this time, elongation of the gonad arms along the anterior–posterior axis occurs and is guided by two specialized cells known as distal tip cells (DTCs). The DTCs are located at the extreme anterior and posterior ends of the gonad (one on each gonad arm) and migrate along the body wall during larval development (Fig. 1B–D and G). During this process gonad sheath cells flatten and migrate along the extending gonad, ultimately encircling the adult gonad arms (Fig. 1C, D, G and H) (Hedgecock et al., 1987).

Successful passage of fertilized eggs out of the adult hermaphrodite requires connection of the uterus to the vulva, which forms as a separate epithelial tube in the hypodermis directly ventral to

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the uterine precursors. A specialized uterine cell, the anchor cell (AC), is central to the development of both tissues (Newman et al., 1995; Sherwood and Sternberg, 2003; Sundaram, 2004). During the L2–L3 molt, the AC induces vulval tissue by secreting the EGF-like ligand LIN-3 (Sundaram, 2004). Later, during the mid-L3 stage, the AC plays a second critical role in establishing the initial connection between the uterus and vulva by invading through the basement membranes separating the gonad and hypodermis (Sherwood et al., 2005; Sherwood and Sternberg, 2003; Ziel et al., 2009). Following basement membrane removal and attachment to the central vulval cells, the AC fuses with surrounding uterine cells, which, during the late L3 and L4 stages, begin to form the

Fig. 1. (A–D) Schematic representation of larval gonad development in *C. elegans*. (A) After hatching, during the L1 stage, the somatic gonad precursor cells Z1 and Z4 surround the primordial germ cells and are located on the anterior and posterior sides of the early gonad. (B) During the L2 stage these cells give rise to somatic lineages of the gonad including the distal tip cells (DTCs) and the cells of the somatic gonad primordium. (C) During the L3 stage the cells of the somatic gonad primordium divide to form the dorsal and ventral uterine cells (DU/VU cells) as well as the gonad sheath and spermathecal cells. (D) During the L4 stage, the uterine and spermathecal epithelium form mature lumens. The DTCs and the gonad sheath cells complete their migrations. (E–H) DIC micrographs overlaid with LAM-1::GFP fluorescence labeling the basement membrane which surrounds and outlines the gonad. RhoGEF 5 CRE activity was scored in: (E) Z1 and Z4 at the L1 stage; (F) the somatic gonad primordium at the L2 stage; (G) the AC, VU/DU cells, the spermathecal and sheath lineage and the DTCs during the L3 stage and (F) the uterine and spermathecal epithelium at the L4 stage. (I–L) Selected examples of RhoGEF 5 CRE activity during gonadogenesis: (I) 5 CREs from C28C12.10 were active in Z1 and Z4; (J) C02F12.4 was detected in the somatic gonad primordium; (K) T19E10.1/ect-2, K07D4.7b, F55C7.7a/unc-73 and C11D9.1 5 CRE activity detected in the DU/VU cells, AC, DTC and spermathecal and sheath lineages respectively; (L) K11E4.4/pix-1 5 CREs were active broadly within the uterine epithelium, whereas elements from C11D9.1 were active specifically within the spermatheca. Scale bar in (E) represents 20 μm.

Abbreviations: epithelium, Ep; distal tip cell, DTC; anchor cell, AC; dorsal uterine cell, DU; ventral uterine cell, VU in this and all subsequent figures.
mature epithelium characteristic of adult worms (Fig. 1D and H) (Newman and Sternberg, 1996; Sharma-Kishore et al., 1999). This event occurs at the same time as maturation of the spermathecal epithelia and their connection to the uterus (Fig. 1D and H) (Hubbard and Greenstein, 2000).

The Rho family of small GTPases (Rho GTPases) are known to be critical for key steps in hermaphroditic gonadogenesis (Blelloch et al., 1999; Welchman et al., 2007; Ziel et al., 2009). The C. elegans genome contains clear orthologs of vertebrate racs (mig-2, ced-10 and rac-2), rhoA (rho-1) and cdc42 (cdc-42) (Lundquist, 2006). Both mig-2 and ced-10 function during DTC migration and AC invasion, while cdc-42 has been shown to regulate early gonad organization (Blelloch et al., 1999; Welchman et al., 2007; Ziel et al., 2009). However, the early embryonic lethality associated with complete loss-of-function mutations in ced-10 and cdc-42 or with rho-1 RNAi treatment, has complicated functional analysis of these genes during gonadogenesis.

An alternative approach to further understand Rho GTPase signaling during development is to examine the regulatory proteins that control their activity. Rho family members are molecular switches that interact with their effector proteins in the GTP-bound state. Activation of Rho GTPases is controlled by guanine nucleotide exchange factors (GEFs), while inactivation is regulated by GTPase activating proteins (GAPs) (Narumiya, 1996). Of particular interest are the GEF proteins, as these molecules are thought to couple Rho GTPase activity to specific signaling events, such as those controlling developmental timing or directional migration (Schiller, 2006). Thus, mutations in individual GEF genes may be used to uncover functions for Rho GTPases in a specific subset of developmental or cellular events. Because most genomes contain a much larger complement of RhoGEFs than Rho family GTPases, designing experiments to determine the individual or combinatorial contribution of specific RhoGEFs to Rho GTPase regulation is difficult without prior documentation of expression patterns (Schmidt and Hall, 2002). To this end, we have characterized 5’ cis-regulatory element (5’CRE) activity from 19 putative RhoGEF genes during gonadogenesis in C. elegans. Our results provide evidence for a wide range of tissue and temporal specificity amongst these 5’CREs. We anticipate that the profiles described here will aid in designing experiments to determine the genetic requirements for Rho GTPase regulation by GEF proteins during organogenesis in vivo.

1.1. Identification of likely RhoGEF proteins in C. elegans

To identify likely RhoGEF-encoding genes in C. elegans we searched the SMART database for C. elegans proteins predicted to contain a dbl-homology (DH) domain, a characteristic feature of GEF proteins for Rho-type GTPases (Letunic et al., 2009). From this search we identified sixteen C. elegans genes likely to encode typical RhoGEF proteins (Fig. 2). As expected, most of the predicted proteins encoded by these genes also contains a pleckstrin-homology (PH) domain in close proximity to the DH domain, which aids in the nucleotide exchange reaction (Schmidt and Hall, 2002). In addition, a second family of proteins related to vertebrate DOCK180 and C. elegans CED-5 has been shown to function as GEFs for some Rho family members (Cote and Vuori, 2007). For this reason, we examined 5’CRE activity from the three genes in this family (C02F4.1/ced-5, F22G12.5 and F46H5.4) that are encoded by the C. elegans genome (Cote and Vuori, 2007). While some of these genes (C02F4.1/ced-5, C33D9.1/exc-5, T19E10.1/ect-2, K11E4.4/pix-1, F32F2.1/ugl-1, F55C7.7/unc-73, C09D1.1/unc-89 and C35B8.2/vav-1) have been identified by genetic screens or other loss-of-function approaches, eleven have not been previously characterized (Benian et al., 1996; Hikita et al., 2005; Lucanic and Cheng, 2008; Morita et al., 2005; Steven et al., 1998, 2005; Yoo and Greenwald, 2005).

1.2. Identification of RhoGEF 5’CRE activity during gonadogenesis

To determine which RhoGEF proteins might be important during gonadogenesis, we sought to examine their spatial and temporal expression during larval development. For this purpose, we took advantage of the fact that 5’CRE activity can be efficiently assayed in C. elegans by linking putative regulatory elements to the coding sequences for the green fluorescent protein (GFP) in transgenic worms (Hunt-Newbury et al., 2007). In this way we examined 5’CREs from each of the nineteen RhoGEF genes identified due to the presence of a DH domain or similarity to CED-5 and DOCK180. In total we examined 24 transgenic lines representing 5’CREs from all 19 genes, encompassing 28 of the 34 total RhoGEF 5’CREs based upon current transcript predictions in Wormbase (WS159). A schematic view of each RhoGEF gene and detailed information regarding each associated reporter construct is available in Supplemental Fig. 1 and Supplemental Table 1.

In our preliminary screen (see Section 2) we identified 5’CREs from 13 of these 19 RhoGEFs that were active during gonadogenesis (Fig. 2). Importantly, all 5’CREs examined drove GFP expression in at least some cells during larval development with the exception of those from Y95B8A.12 (data not shown). The lack of expression across multiple transgenic lines indicated that this gene may not be expressed in larvae or that our construct lacked the complete cis-regulatory architecture necessary to drive expression. For those RhoGEF 5’CREs that were active during gonadogenesis, we conducted a more detailed analysis of their expression patterns by focusing on critical developmental landmarks during larval gonad formation (examples of 5’CRE activity in each cell type examined are shown in Fig. 1 I–L and summarized in Fig. 2; all raw scoring data is provided in Supplemental Table 2).

1.3. RhoGEF 5’CRE activity during early gonadogenesis

To examine RhoGEF 5’CRE activity during early events in the organization of the somatic gonad, we scored GFP expression in Z1 and Z4 and in the somatic primordium during the L1 and L2 larval stages, respectively (Fig. 1A, B, E, F and I). RhoGEFs expressed during these stages could contribute to early cell movements required for general somatic gonad organization and exclusion of the germ-cells into the gonad arms (Hubbard and Greenstein, 2000; Welchman et al., 2007). 5’CREs from four genes (C14A11.3, C28C12.10, K11E4.4/pix-1 and T19E10.1/ect-2) were active in Z1 and Z4 (Figs. 1I and 2). Interestingly, only the construct corresponding to the short transcripts of C14A11.3 (C14A11.3a,c) was active during gonadogenesis, indicating the potential for isoform-specific activity during gonad development (Fig. 2). During the L2 stage we detected continued GFP expression directed by 5’CREs from C14A11.3a,c, C28C12.10 and K11E4.4/pix-1 within the somatic gonad primordium. In addition, regulatory regions corresponding to C02F12.4, C11D9.1, C33D9.1/exc-5 and K07D4.7a became active at this time (Figs. 1J and 2). Notably, the 5’CRE from T19E10.1/ect-2 was silent within these cells, but was reactivated within their progeny during the L3 stage, highlighting the potential...
for dynamic regulation of RhoGEF genes during gonadogenesis (Fig. 2).

1.4. RhoGEF 5 CRE activity during DTC migration

We also identified RhoGEF 5 CREs that were active within the DTCs, where Rho GTPase function has been clearly demonstrated by loss-of-function studies (Blelloch et al., 1999). Six RhoGEF 5 CREs (C14A11.3a,c, C28C12.10, F55C7.7a,b/unc-73, K11E4.4/pix-1, K07D4.7b and C02F4.1/ced-5) drove GFP expression within the DTCs during the L3 stage, when mig-2 and ced-10 regulate DTC turning and directional migration (Figs. 1K and 2) (Reddien and Horvitz, 2000; Zipkin et al., 1997). Importantly, these data were consistent with previous reports of DTC phenotypes caused by mutations in C02F4.1/ced-5 and double-stranded RNA targeting F55C7.7/unc-73, which show defects DTC migration and guidance, and mutations in K11E4.4/pix-1, which manifest DTC morphology defects as well as guidance defects. (Cram et al., 2006; Lucaníc and Cheng, 2008; Wu and Horvitz, 1998).

1.5. RhoGEF 5 CRE activity during AC invasion

We have shown previously that mig-2 and ced-10 function to promote basement membrane removal by the AC (Ziel et al., 2009). cdc-42 also plays an uncharacterized role in this process (J.W.Z., unpublished observations). How these proteins might be activated in response to the pathways that promote the timing and targeting of invasion is not known. Analysis of RhoGEF 5 CRE activity at the time of AC invasion in the mid-to-late L3 stage identified eight RhoGEF 5 CREs from seven genes capable of driving AC expression (Figs. 1K, 2 and 3A–H). In most cases (C02F12.4, C14A11.3a,c, C28C12.10, F55C7.7a,b/unc-73, K07D4.7b and C02F4.1/ced-5) GFP expression was also detected amongst the dorsal and ventral uterine cells and thus might reflect enhancer elements broadly active within the central somatic cells of the
gonad (Figs. 2 and 3A–G). In contrast, a 5′CRE construct from K07D4.7b, highly similar to Ephexin and a human gene ARHGEF16, showed a high degree of specificity within the gonad. This 5′CRE, corresponding to the smaller transcript of K07D4.7, was only expressed within the AC and the DTCs (Figs. 2 and 3H).

1.6. RhoGEF 5′CRE activity during Uterine, Spermathecal and Gonad Sheath development

The gonad sheath as well as the uterine and spermathecal epithelia all derive from the somatic gonad primordium (Kimble and Hirsh, 1979). During gonad arm elongation, sheath cells, which derive from the same precursor cells as some spermathecal cells, ultimately form a contractile apparatus around the gonad arms (Kimble and Hirsh, 1979). Also during the late L3 stage and continuing into the L4 stage, cells of the dorsal and ventral uterus form a mature, lumenalized epithelial tube from a previously uniform cluster of cells (Newman et al., 1996). Importantly, Rho GTPase signaling is critical for establishing the apical-basolateral polarity characteristic of cells within mature epithelia (Van Aelst and Simmons, 2002). During the late L3 stage we detected 5′CRE activity from a large number of RhoGEF genes within this tissue. In total eight 5′CREs ( C02F12.4, C14A11.3a,c, C28C12.10, F55C7.7a,b/unc-73, K11E4.4/pix-1, T19E10.1/ect-2 and K07D4.7a) were active in the VU/DU cells prior to epithelialization during the L3 stage (Figs. 1K and 2). Some of these elements were also active in the spermathecal and sheath lineages (C14A11.3a,c, C33D9.1/exc-5, F55C7.7ab/unc-73, K07D4.7a, K11E4.4/pix-1 and T19E10.1/ect-2) at the L3 stage (Fig. 2). 5′CREs from C11D9.1 and F22G12.5 were active in the spermathecal and sheath lineages but not within the DU/VU cells (Figs. 1K and 2).

5′CREs from C02F12.4, C14A11.3a,c, C28C12.10, F55C7.7a,b/unc-73, K07D4.7a, K11E4.4/pix-1 and T19E10.1/ect-2 were active in the VU/DU cells prior to epithelialization during the L3 stage (Figs. 1K and 2). Some of these elements were also active in the spermathecal and sheath lineages (C14A11.3a,c, C33D9.1/exc-5, K11E4.4/pix-1 and T19E10.1/ect-2) at the L3 stage (Fig. 2). 5′CREs from C11D9.1 and F22G12.5 were active in the spermathecal and sheath lineages but not within the DU/VU cells (Figs. 1K and 2).

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(from C11D9.1, C35B8.2/vav-1, F55C7.7e/unc-73 and F13E6.6) were active only in the spermathecal epithelia during the L4 stage (Figs. 1L and 2).

1.7. Conclusion

During gonadogenesis, Rho family GTPases are known to regulate early gonad organization, DTC migration and AC invasion (Welchman et al., 2007; Ziel et al., 2009; Zipkin et al., 1997). Based on experimental results from other systems, they are also predicted to regulate AC fusion to its neighboring uterine cells as well as lumenalization of the uterine and spermathecal epithelia (Cote and Vuori, 2007; Van Aelst and Symons, 2002). Thus, the Rho GTPases are expected to have multiple functions during gonad organogenesis. How these functions would be coordinated and segregated during development, however, remains unclear. In our current study we provide the first comprehensive examination of RhoGEF 5’CRE activity during a complex developmental process in C. elegans. Our data show that a large portion of the C. elegans RhoGEF complement is expressed during gonad formation. 5’CRE activity associated with these genes is largely overlapping, both temporally and spatially, and all cell types examined express multiple RhoGEFs. Together these results support the idea that distinct RhoGEFs may be used to activate Rho GTPases for specific functions during development. The fact that multiple RhoGEF genes are expressed in most gonadal cell types also raises the possibility of extensive genetic redundancy. Consistent with this notion, only five of these 19 RhoGEF genes (C09D1.1/unc-89, C33D9.1/exc-5, F55C7.7e/unc-73, T19E10.1(ect-2) and C02F4.1(ect-5)) have been identified by traditional forward genetic screens. Given this possibility, the 5’CRE activity profiles described here, as well as the transgenic reporter lines developed during this study will be useful in designing genetic experiments to examine the combinatorial functions of RhoGEF proteins during gonadogenesis and other developmental processes in C. elegans.

2. Materials and methods

2.1. Generation of C. elegans RhoGEF 5’CRE-reporter transgenes

Some strains (indicated by the prefix sls or sEx) were provided by the Caenorhabditis Genetics Center and were made available by the Genome B.C. C. elegans Gene Expression Consortium (Hunt-Newbury et al., 2007). 5’CRE-reporter transgenes were generated using a previously reported PCR fusion strategy (Hobert, 2002). Briefly, putative regulatory sequences upstream of each transcript were amplified from N2 genomic DNA using a special primer nested within the 5’CRE amplicon and a reverse primer nested within the GFP amplicon. The resulting PCR products were typically either the intergenic region or approximately 2.5 kb of sequence upstream of each transcript.

Extrachromosomal arrays carrying each 5’CRE-reporter transgene were generated by germ-line transformation of unc-119(ed4) mutants. A standard injection mix containing 1–10 ng/μl 5’CRE-reporter DNA, 75 ng/μl pBSKK-, and 50 ng/μl unc-119-rescuing sequence (pDP#MM016B) was used. Transformants were distinguished by their non-Unc phenotype and transgenic lines were established from single non-Unc F2 progeny. These strains were propagated using standard techniques (Brenner, 1974). A complete list of primers, 5’CRE sizes, and resulting extrachromosomal arrays is presented in Table S1. Schematic representations of each 5’CRE-reporter construct in the context of the endogenous locus are presented in Supplemental Fig. 1.

2.2. Phylogenetic analysis of predicted RhoGEF proteins

An amino acid alignment of the ~200–amino acid (AA) DH domains from the C. elegans DH-domain containing RhoGEF proteins was generated using MUSCLE v3.6 (Edgar, 2004). Bayesian phylogenetic analyses were conducted using MrBayes 3.1.2 via a web portal at the CBSU Web Computing Resources Microsoft High-Performance Computing Institute (http://cbsuapps.tc.cor nell.edu/index.aspx) (Ronquist and Huelsenbeck, 2003). The Bayesian analysis was conducted using a mixed amino acid model with gamma which selected the WAG model of amino acid evolution with a 100% posterior probability with 2,000,000 generations sampled every 100 generations with four chains over four independent runs. A summary tree was produced from the final 18,000 trees representing 1,800,000 stationary generations per run, and 72,000 trees representing 7,200,000 stationary generations for the consensus tree. In addition, neighbor joining (NJ) (using mean AA distances) was performed using PAUP- 4.0b10 (Swofford, 2000). ProtTest selected the LG+H model of amino acid evolution, which was used for a maximum likelihood (ML) bootstrap analysis using RaXML v7.0.4 (Stamatakis, 2006; Abascal et al., 2005; Le and Gascuel, 2008) performed on the Duke Shared Cluster Resource. The LG model was provided at http://www.kramer.in.tum.de/exelixis/software.html. Domain architecture of the C. elegans RhoGEF proteins was determined using the SMART database or the NCBI Conserved Domain Database (Marchler-Bauer et al., 2009).

2.3. Scoring of RhoGEF 5’CRE activity during gonadogenesis

Living worms were examined and imaged using a Zeiss AxioImager A1 microscope with a 100 plan-apochromat objective and a Zeiss AxioCam MRm CCD camera, controlled by Zeiss Axiovision software (Zeiss Microimaging), or using a Yokogawa spinning disk confocal mounted on a Zeiss AxioImager A1 microscope controlled by the iVision software package (Biovision Technologies). Images were processed and overlaid using Photoshop 8.0 (Adobe Systems).

RhoGEF 5’CRE-reporter transgenes were first screened for general gonadal expression by examining at least thirty transgenic worms across the four larval stages. 5’CRE-reporter transgenes that were active during any stage and in any gonadal cell-type were subjected to a more detailed screening process at the discrete developmental stages described in Fig. 1. Because extrachromosomal arrays are unstable and can be lost during mitosis, at least 10 animals were examined at each specific stage. Raw scoring data for each 5’CRE-reporter construct is provided in Supplemental Table 2.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gep.2009.06.005.

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