Mol. Cells *28*, 455-461, November 30, 2009
DOI/10.1007/s10059-009-0145-5

Molecules and Cells

Identification and Characterization of a Putative Basic Helix-Loop-Helix (bHLH) Transcription Factor Interacting with Calcineurin in *C. elegans*

Soo-Ung Lee[1,6], Hyun-Ok Song[2,6], Wonhae Lee[2,5], Gunasekaran Singaravelu[2,3], Jae-Ran Yu[1,*], and Woo-Yoon Park[4,*]

Calcineurin is a Ca^{2+}/Calmodulin activated Ser/Thr phosphatase that is well conserved from yeast to human. It is composed of catalytic subunit A (CnA) and regulatory subunit B (CnB). *C. elegans* homolog of CnA and CnB has been annotated to *tax-6* and *cnb-1*, respectively and *in vivo* function of both genes has been intensively studied. In *C. elegans*, calcineurin play roles in various signaling pathways such as fertility, movement, body size regulation and serotonin-mediated egg laying. In order to understand additional signaling pathway(s) in which calcineurin functions, we screened for binding proteins of TAX-6 and found a novel binding protein, HLH-11. The HLH-11, a member of basic helix-loop-helix (bHLH) proteins, is a putative counterpart of human AP4 transcription factor. Previously bHLH transcription factors have been implicated to regulate many developmental processes such as cell proliferation and differentiation, sex determination and myogenesis. However, the *in vivo* function of *hlh-11* is largely unknown. Here, we show that *hlh-11* is expressed in pharynx, intestine, nerve cords, anal depressor and vuvla muscles where calcineurin is also expressed. Mutant analyses reveal that *hlh-11* may have role(s) in regulating body size and reproduction. More interestingly, genetic epistasis suggests that *hlh-11* may function to regulate serotonin-mediated egg laying at the downstream of *tax-6*.

INTRODUCTION

Calcineurin is a serine/threonine protein phosphatase which functions in diverse Ca^{2+}-mediated signaling pathways such as T-cell activation (Im and Rao, 2004), hippocampal long-term deppression (Mulkey et al., 1994) and muscle development (Schulz and Yutzey, 2004). It is highly conserved from yeast to human and is composed of two subunits: catalytic subunit, calcineurin A (CnA) and regulatory subunit, calcineurin B (CnB) (Crabtree, 1999; Klee et al., 1979; 1998; Stewart et al., 1982). The CnA contains a catalytic phosphatase domain and regulatory domain which is composed of a CnB binding domain, a calmodulin (CaM)-binding domain and an auto-inhibitory domain (AI) (Hashimoto et al., 1990; Hubbard and Klee, 1989). In the absence of Ca^{2+}/CaM, the AI domain occupies the active site thereby inhibits calcineurin enzyme activity. However, the binding of Ca^{2+}/CaM causes conformational change of calcineurin to displace AI domain from active site thereby activate calcineurin enzyme activity.

In *C. elegans*, CnA and CnB are encoded by single gene, *tax-6* and *cnb-1*, respectively. Previous studies have shown that calcineurin plays roles in reproduction, body size regulation, movement, egg laying in response to serotonin, and chemo/thermotaxis in *C. elegans* (Bandyopadhyay et al., 2002; Kuhara et al., 2002; Lee et al., 2004). In order to identify additional signaling pathways in which calcineurin functions, we screened for proteins interacting with TAX-6 by the yeast two-hybrid system. One of these interacting proteins was identified as F58A4.7. (Kim et al., 2008) The *hlh-11* mapped to the F58A4.7 and it has been annotated as *C. elegans* homolog of AP4, which is one of the basic helix-loop-helix (bHLH) transcription factor family members.

The bHLH transcription factors are specified by highly conserved structural motifs including a basic region for DNA binding and HLH domain to form dimer (HLH) (Ledent and Vervoort, 2001; Massari and Murre, 2000; Murre et al., 1994). In various metazoan including fly, nematode and vertebrates, the bHLH transcription factors have been implicated in many developmental processes such as cell proliferation and differentiation, sex determination, neurogenesis and myogenesis (Ciarapica et al., 2003; Hallam et al., 2000; Jan and Jan, 1993; Massari and Murre, 2000).

The *C. elegans* HLH-11 is approximately 71% identical in bHLH domain and 21% identical to human AP4 in overall amino acid sequences. Until now, 39 different bHLH genes have been predicted from the *C. elegans* genome (Ledent et al., 2002). Some HLH proteins such as HLH-1 (Chen et al., 1992;

[1]Department of Environmental and Tropical Medicine, Konkuk University School of Medicine, Seoul 143-701, Korea, [2]Department of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea, [3]Waksman Institute, Rutgers University, Piscataway, NJ 08854, USA, [4]Department of Radiation Oncology, College of Medicine, Chungbuk National University, Cheongju, 361-763, Korea, [5]Present address: Division of Gene Analysis, National Institute of Scientific Investigation, Seoul 158-707, Korea, [6]These authors contributed equally to this work.
*Correspondence: maria205@kku.ac.kr (JY); wynpark@chungbuk.ac.kr (WP)

Received July 30, 2009; accepted August 31, 2009; published online October 21, 2009

Keywords: basic helix-loop-helix, calcineurin, *C. elegans*, egg laying, HLH-11

1994; Fukushige and Krause, 2005; Harfe et al., 1998a; Krause et al., 1994; Zhang et al., 1999), HLH-2/HLH-3 (Karp and Greenwald, 2003; 2004; Krause et al., 1997; Portman and Emmons, 2000; Thellmann et al., 2003) and HLH-8 (Corsi et al., 2000; 2002; Gort et al., 2008; Harfe et al., 1998b; Solari et al., 1999; Wang et al., 2006; Zhao et al., 2007) had been extensively characterized for their specific functions in *C. elegans*. However, to the best of our knowledge, it is still largely unknown about the *in vivo* functions of *hlh-11* gene in *C. elegans*. Here we report the molecular characteristics of HLH-11, the expression patterns of the *hlh-11* and the characterization of *hlh-11* deletion mutant which has been isolated in this present work. We further propose the novel function of *hlh-11* as the protein interacting with calcineurin in a set of calcineurin-mediated signaling pathways.

MATERIALS AND METHODS

Genetics and maintenance of *C. elegans* strains

The following *C. elegans* strains were obtained from CGC (Caenorhabditis Genetics Center) at the University of Mi-nnesota (USA): Bristol type N2, PR675 *tax-6(p675)* IV and CB4088 *him-5(e1490)* V. KJ306 *tax-6(jh107)* IV was previously isolated (Lee et al., 2004). KJ663 *hlh-11(jh139)* III was isolated by reverse genetic method (Park et al., 2000) and out-crossed more than six times. The worms were grown on standard NGM (Nematode Growth Medium) seeded with *E. coli* OP50. Worms were handled according to the established methods (Brenner, 1974).

Yeast two-hybrid analysis

Yeast two-hybrid screening was performed using full length of TAX-6 cloned into pAS2-1 as bait and *C. elegans* cDNA library cloned into pACT as prey vector. The screening was done as per the manufacturer's protocol (Clontech, USA) (Singaravelu et al., 2007).

Isolation of *hlh-11* deletion mutant from TMP/UV mutagenized library

TMP (Trimethylpsoralen)/UV method was used to generate *C. elegans* deletion mutants. Screening of mutants from the mutagenized library was carried out by a nested PCR-based method and subsequent sib selections. Primers were designed based on the predicted sequences spanning the full genomic DNA of *hlh-11*(F58A4.7); outer upstream primer (5′-TCG GCA TTT GAT TTG ACG GCT TCT-3′) and outer downstream primer (5′-ACG AGC GAT GTC TGT GAG GTT GGT-3′), inner upstream primer (5′-TTC GTT GCG TTC TGT CTT GCC TAC-3′) and inner downstream primer (5′-GGT TGG TGG AGG AGA TGT TGG AAT-3′). Deletion downstream primer (5′-TAT TGT TTG GAG ATG TGC TAT-3′) was paired with inner upstream primer. Homozygous line was out-crossed at least six times to wild-type animals to establish the strain *hlh-11(jh139)* and was used in subsequent analysis. Deletion region for the *hlh-11* hermaphrodites was determined by nested PCR followed by sequencing of the PCR products.

Construction of GFP reporter plasmid and microinjection

The cosmid F58A4 was obtained from Alan Coulson (The Sanger Center, UK). To obtain a full-length of *hlh-11*, the genomic DNA fragment containing 5,353 bp of upstream sequence and the full length of *hlh-11* gene product was amplified by nested PCR using the following primer sets based on F58A4.7; outer upstream primer (5′-TCG AGA TAA CTT TGG AAA GTG CAG TG-3′), outer downstream primer (5′-GAT ACT GTG CCT GGT GAG AGA GAG-3′) and inner upstream primer (5′-GGC GAT TAT TCA AGC AGG TCT AGA-3′), inner downstream primer (5′- ACG GAC CCG GGC GAG CGA TGT CTG-3′). To construct transcriptional GFP fusion of *hlh-11* gene, another inner downstream primer (5′-CCC GGG CTT CTG CGC TAT CCG AAC GAA C-3′) was paired with same inner upstream primer. The amplified product was subcloned into pGEM-T easy vector (Promega, USA) and sequenced. Transcriptional and translational *hlh-11* gene was fused to a promoterless green fluorescence protein (GFP) vector, pPD95.75 (kindly provided by A. Fire) using the *Xba*I and *Sma*I sites. Microinjection of fusion plasmids and transformation marker pRF4 (dominant *rol-6*) was performed as described (Mello and Fire, 1995). The plasmid construct was injected at 150 ng/μl concentration mixed with 450 ng/μl pRF4 plasmid following standard methods (Mello and Fire, 1995). After the stable transgenic line was obtained, worms were treated with 1% (w/v) levamisole to immobilize and were visualized by fluorescence confocal microscopy (Nikon, Japan). Male expression was observed in transgenic *him-5(e1490)* male worms.

Reverse transcriptase PCR

Total RNA from N2 and *hlh-11(jh139)* mutants were extracted according to manufacture-provided instructions using TRI-Reagent (MRC, USA). Then the extracted RNA from each worm pool was reverse-transcribed by a Sensiscript RT kit (Qiagen, USA) with an oligo(dT) primer. The completed reverse transcription reaction mix was then used as a DNA template for general PCR method with primers designed to amplify the deletion region of *hlh-11*; upstream primer (5′-ATT TGA TCT AAC TGA AGA GGA C-3′) and downstream primer (5′-TGT TGG AAT GAA ATG ACT TC-3′). The RT-PCR products were confirmed by DNA sequencing.

Phenotypic analysis

The two-day old adult animals were examined for measuring body length. The worms were immobilized on 2% agarose pads with 10 mM levamisole and then length was measured under a Normaski microscope, Carl Zeiss (Axio Imager. A1). The brood sizes were examined as described (Bandyopadhyay et al., 2002; Lee et al., 2004). Briefly, the brood size was determined by counting the number of F_1 progenies after placing and daily transferring individual P_0 worms on seeded plates and allowed to self-fertilize at 20°C. The one-day old adult worms were examined for serotonin mediated egg laying behavior. Serotonin mediated egg laying phenotypes were examined as described (Singaravelu et al., 2007; Trent et al., 1983). Briefly, animals were immersed into a control M9 buffer (3 × g KH_2PO_4, 6 × g Na_2HPO_4, 5 × g NaCl, 1 ml of 1 M $MgSO_4$ per liter) or a 25 mM serotonin (5-HT) solution (Sigma) for 90 min, after which the number of eggs laid by each worm was counted.

RESULTS AND DISCUSSION

The regulatory subunit of *C. elegans* calcineurin, TAX-6, physically interacts with the HLH-11

The yeast two hybrid screening using TAX-6 as bait has isolated a novel gene, the *hlh-11*. It was recovered from the *C. elegans* cDNA library and confirmed by sequencing. To further test the interacting regions between TAX-6 and HLH-11, we used the following three constructs of TAX-6 as the bait: the full length of TAX-6, the construct lacking the auto-inhibitory region and finally the construct having only the catalytic domain. By testing the TAX-6 deletion constructs through yeast two hybrid tests, we found that indeed TAX-6 physically interacts with HLH-11 and the auto inhibitory domain is not required for this interaction. This was evidenced by the growth on synthetic drop

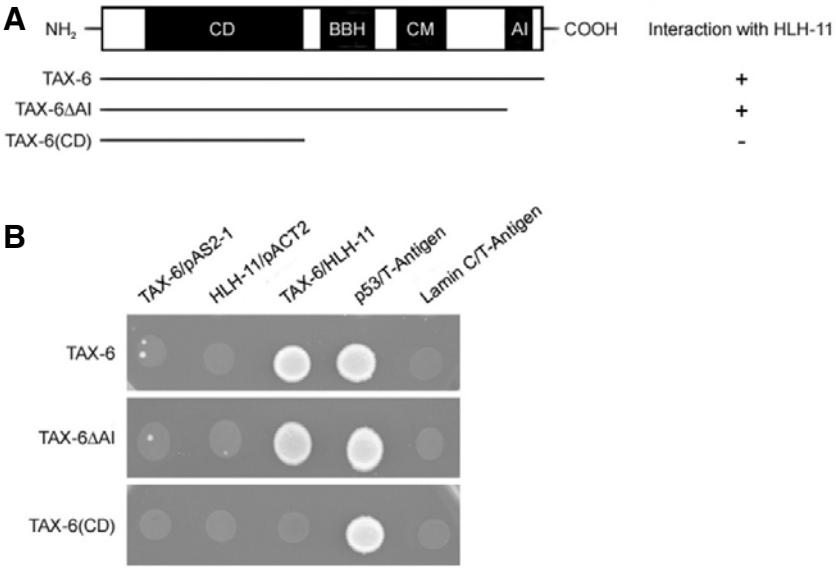

Fig. 1. HLH-11 binds to TAX-6. (A) Schematic diagram of TAX-6 showing individual domains. Domains are: CD, catalytic domain; BBH, calcineurin B binding helix; CM, calmodulin binding motif; AI, auto inhibitory domain. The binding ability or disability of TAX-6 to HLH-11 are indicated as (+) or (-). (B) Yeast two hybrid analysis of TAX-6 and HLH-11. The bait and prey vectors are indicated as 'bait/prey' above each lane. The identity of TAX-6 bait vectors is shown in left of each lane.

out media with the bait lacking auto inhibitory domain (Figs. 1A and 1B). The p53/T-antigen interaction was used as a positive control and Lamin C/T-antigen interaction was used as a negative control. As shown by the yeast two-hybrid analysis, HLH-11 binds to TAX-6 through the regulatory region containing calcineurin B binding helix and calmodulin binding domain.

C. elegans HLH-11 is a homolog of human AP4

The *hlh-11* gene is mapped to the cosmid F58A4.7 and is located between *hcp-3* and *tbg-1* gene on chromosome III (Fig. 2A). The predicted *hlh-11* gene is composed of four alternatively spliced isoforms: *hlh-11a* (F58A4.7a), *hlh-11b* (F58A4.7b), *hlh-11c* (F58A.7c) and *hlh-11d* (F58A4.7d). The three isoforms (*hlh-11a, hlh-11b, hlh-11c*) have 6 exons encoding 431 amino acids for isoform b, 429 amino acids for isoform a (with deletion of two amino acids at position 110 and 111 with respect to isoform b) and 428 amino acids for isoform c (with deletion of three amino acids at position 110, 111 and 391 with respect to isoform b). The forth isoform d is the shortest with 3 exons encoding only 136 amino acids (Figs. 2A and 2B). The alignment analysis revealed that CeHLH-11 is 20.9% identical to human AP4 in the overall amino acid seqeunces. However, CeHLH-11 showed 70.6% identity in bHLH (basic Helix-Loop-Helix) domain compared to that of human AP4 (Fig. 2C). This suggests that CeHLH-11 has a well conserved bHLH domain of human AP4, which might function as a putative transcription factor.

The *hlh-11* gene is expressed in pharynx, vuvla muscles, intestine and nerve cords

To investigate the expression pattern of the *hlh-11* gene *in vivo*, we constructed a transcriptional and translational GFP fusion of the *hlh-11* gene. The transcriptional and translation GFP fusion of the *hlh-11* showed similar expression patterns. The *hlh-11* gene is expressed in pharynx (Fig. 3A), intestine (Fig. 3A) and nerve cords (Fig. 3B). It is also expressed in H-shaped excretory cell (Fig. 3C), vulva muscles (Fig. 3D), anal depressor (Fig. 3E), male spicules and especially in hyp7 cells of hypodermis in males (Fig. 3F). Previously we and others have shown that TAX-6 is also expressed in pharynx, intestine, nerve cords, anal depressor and vulva muscle (Bandyopadhyay et al., 2002; Kuhara et al., 2002; Lee et al., 2005). Therefore these results suggest that along with the physical interaction between TAX-6 and HLH-11, the *hlh-11* probably functions with calcineurin in those specific tissues.

hlh-11(jh139) deletion mutant is a loss of function mutant

In order to examine the *in vivo* function of *hlh-11* gene, we screened the deletion mutant of *hlh-11* from TMP/UV-mutagenized worm library by using nested PCR method (Fig. 4A) and successfully isolated an *hlh-11* deletion mutant (*jh139*) (see "Materials and Methods"). The *hlh-11(jh139)* mutant bears a 1,847 bp deletion which spans from the beginning of the 3rd intron to the last intron (Figs. 4A and 4B). Since this deletion can be easily detected by nested PCR at a single animal level, heterozygous and homozygous mutant animals were identified (Fig. 4D). In order to locate the deleted region precisely, we have cloned and sequenced RT-PCR products from the *hlh-11(jh139)* mutant mRNAs (Fig. 4C). Sequencing data revealed that the forth and the fifth exons were deleted resulting in the truncated transcript (Figs. 4B and 4C). These results suggest that *hlh-11(jh139)* mutant is likely to be a loss of function mutant.

HLH-11 may negatively regulate serotonin mediated egg laying at the downstream of TAX-6

Since the HLH-11 was isolated as a novel binding partner of calcineurin and the expression pattern of the *hlh-11* gene was similar to that of calcineurin, we looked for the calcineurin-related phenotypes of *hlh-11(jh139)* mutant in specific biological pathways such as body length, brood size and serotonin mediated egg laying behavior which have been known to be regulated by calcineurin in *C. elegans* (Bandyopadhyay et al., 2002; Lee et al., 2004). In addition, we made double mutants between *hlh-11(jh139)* and calcineurin A gain of function mutant, *tax-6(jh107)* or calcineurin A loss of function mutant, *tax-6(p675)* to examine their genetic interactions in those particular pathways.

The body length of *hlh-11(jh139)* mutant animals was significantly smaller (801 $\pm$ 17, n = 18) than that of wild type animals (947 $\pm$ 12, n = 18) (Fig. 5A) suggesting its possible role in regulating body size as calcienurin does. Interestingly, the body length of *hlh-11(jh139);tax-6(p675)* double mutants (593 $\pm$ 10, n = 11) was similar to that of *tax-6(p675)* mutants (517 $\pm$ 08, n = 18) rather than *hlh-11(jh139)* mutants (Fig. 5A). Furthermore, *hlh-11(jh139);tax-6(jh107)* double mutants also showed similar

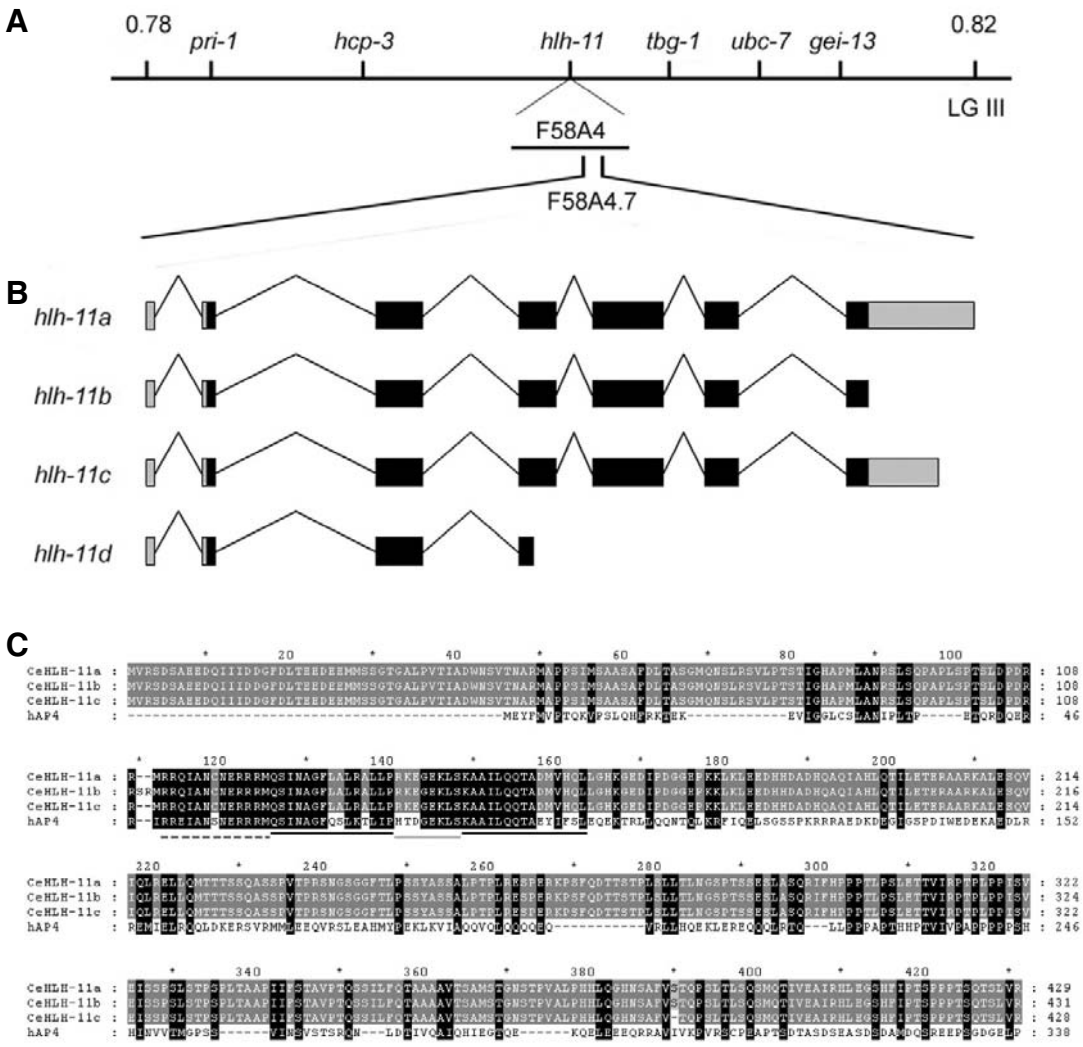

Fig. 2. Genomic organization of *hlh-11* and multiple alignment of HLH-11 amino acid sequences. (A) *hlh-11* gene is located in between *hcp-3* and *tbg-1* gene on F58A4.7 cosmid in chromosome III. (B) *hlh-11* gene is composed of six exons and has four isoforms. (C) The predicted amino acid sequence of CeHLH-11 isoforms (HLH-1a, Accession No. CAA80167; HLH-11b, Accession No. CAA80170; HLH-11c, Accession No. CAD90178) are aligned with AP4 of *Homo sapiens* (Accession No. Q01664). Shaded amino acids indicate identical amino acid residues and conserved repeats. Dashed and grey line indicates basic and loop domain, respectively. Each solid line indicates first and second helix domain.

body length (1025 ± 20, n = 16) to that of *tax-6(jh107)* mutants (1004 ± 11, n = 14) (Fig. 5A). These results suggest that calcineurin is epistatic to *hlh-11* in the regulating pathway for body size.

Next we analyzed the brood size of *hlh-11(jh139)* mutants and found that it was significantly reduced (227.6 ± 5.4, n = 43) than that of the wild type animals (298.9 ± 4.9, n = 32) indicating that like calcineurin, the *hlh-11* may be also important to regulate fertility in *C. elegans*. To test whether they function together in regulating brood size, we investigated the brood size of double mutants. *hlh-11(jh139);tax-6(jh107)* double mutants exhibited similar brood size (274.2 ± 5.2, n = 35) to that of *tax-6(jh107)* single mutants (279.9 ± 4.9, n = 35) whereas *hlh-11(jh139);tax-6(p675)* double mutants showed significantly restored brood size (153.1 ± 3.8, n = 28) compared to that of *tax-6(p675)* mutants (109.1 ± 4.6, n = 28). However, the brood size of *hlh-11(jh139);tax-6(p675)* double mutants was also significantly reduced compared to that of *hlh-11(jh139)* mutants. Therefore, these results suggest that the *hlh-11* and calcineurin may function in more than one pathway to regulate fertility in *C. elegans*.

Finally, we examined the serotonin mediated egg laying phenotype of the *hlh-11(jh139)* mutant animals. Exogenous treatment of serotonin is known to stimulate egg laying in wild type worms. We found that *hlh-11(jh139)* mutants showed similar sensitivity to serotonin as wild type animals. As previously reported (Bandyopadhyay et al., 2002; Lee et al., 2004), *tax-6(jh107)* mutants were hypersensitive and *tax-6(p675)* mutants were resistant to the treatment of serotonin. Interestingly, *hlh-11(jh139);tax-6(p675)* double mutants showed normal response to serotonin as *hlh-11(jh139)* mutants did. Furthermore, *hlh-11(jh139);tax-6(jh107)* double mutants showed significantly reduced hypersensitivity to serotonin compared to *tax-6(jh107)* mutants. Although *hlh-11(jh139);tax-6(jh107)* double mutants displayed Egl (egg laying defective) phenotype so that they lay many eggs even in M9 control buffer, that Egl phenotype did

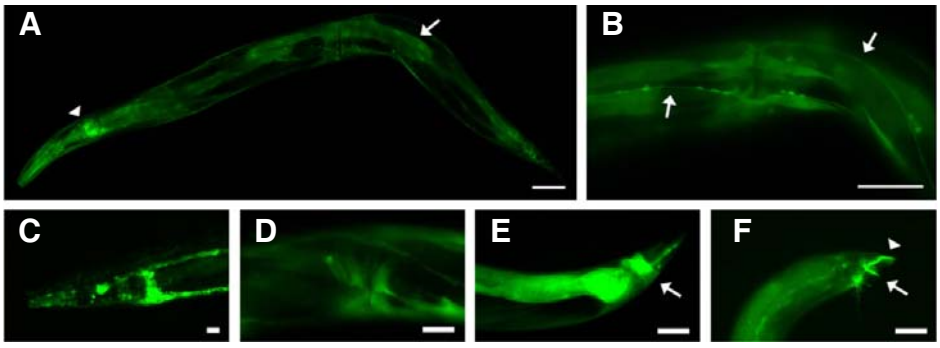

Fig. 3. Expression pattern of *hlh-11* gene. (A-B) Translation GFP fusion of *hlh-11*. (C-F) Transcriptional GFP fusion of *hlh-11*. (A) *hlh-11* gene is expressed broadly including pharynx (arrowhead) and intestine (arrow). *hlh-11* gene is expressed in nerve cords (B; arrows), H-shaped excretory cell (C), vulva muscle (D) and anal depressor (E; arrow) of hermaphrodites. (F) *hlh-11* gene is also expressed in spicule (F; arrow) and hypodermis (especially hyp7; arrowhead) of male tail. Scale bars, 50 μm (A, B) and 25 μm (C-F).

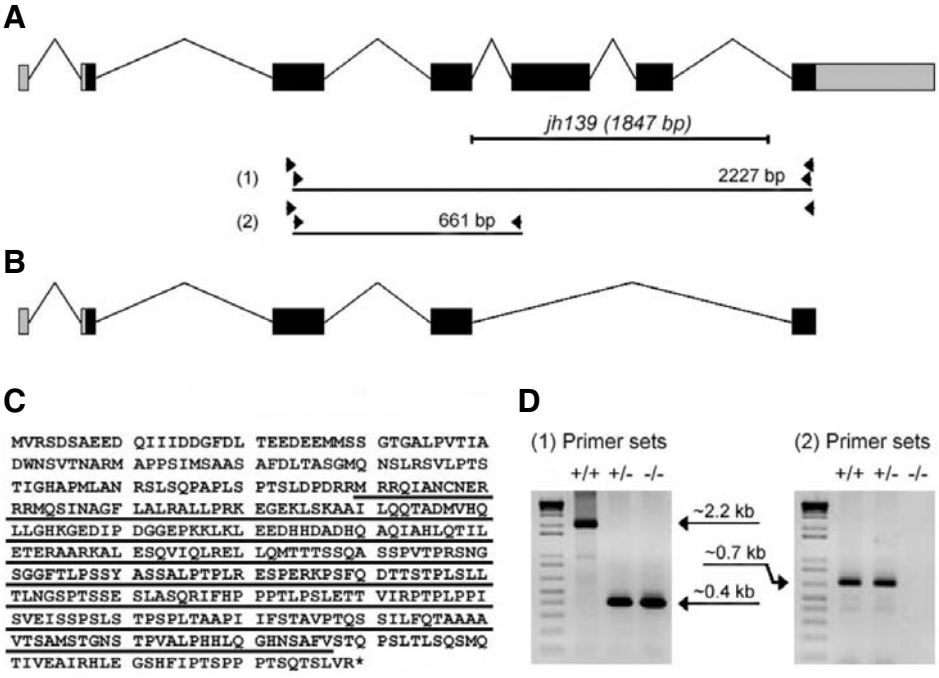

Fig. 4. Isolation of *hlh-11(jh139)* mutant. (A) Deleted region of *hlh-11* mutant are indicated with vertical line. Primer sets used to identify deletion mutant are shown as (1) and (2). (B) Truncated transcript of *hlh-11(jh139)* mutant. The fourth and fifth exons are deleted in *hlh-11(jh139)* mutant. (C) Amino acid sequence of HLH-11. Deleted amino acid sequences of *hlh-11(jh139)* mutant are underlined. (D) Nested PCR results of wild type (+/+), heterozygous (+/-) and homozygous (-/-) *hlh-11(jh139)* mutant.

not enhance the response to serotonin. Normalized results indicate that indeed, the serotonin sensitivity of *tax-6(jh107)* mutants was partially suppressed under *hlh-11(jh139)* mutant background (data not shown). Along with the same expression of both *tax-6* and *hlh-11* in vuvla muslces, these results suggest that *hlh-11* may negatively regulate serotonin-mediated egg laying at the downstream of calcienruin.

HLH-11 may regulate TAX-6 as a transcription factor and/or a functional regulator

In this study, we examined three different phenotypes to test the genetic interaction between *hlh-11* and *tax-6*. Since the molecular characteristic of HLH-11 is a putative transcription factor and calcineurin is a cytosolic phosphatase, we simply expected that *hlh-11* may be epistatic to *tax-6*. However, our results did not show the consistent epistasis between two genes. For example, *hlh-11* is epistatic to *tax-6* in the regulation of serotonin-mediated egg laying whereas *tax-6* is likely to be epistatic to *hlh-11* in body size regulation. Therefore, we assume that there might be more complex genetic interaction between *hlh-11* and *tax-6*. In order to explain this complexity we first questioned, based on the molecular characteristic of HLH-11, whether *hlh-11* can regulate *tax-6* at the transcriptional level. We analyzed promoter region of *tax-6* (softwares, TFBIND and TFSEARCH) and found a potential AP4 binding sequence (called as E-box, CANNTG) at -149 upstream from the ATG (data not shown). This may suggest a possibility that *tax-6* could be activated by the *hlh-11* transcription factor in certain signaling pathway such as body size regulation which we have shown here.

HLH-11 was found to bind to the regulatory domain of calcineurin implicating that it may function as a regulator rather than a substrate of calcineurin. Furthermore, we found that like RCAN-1, a well known regulator of calcineurin (Davies et al., 2007), HLH-11 has a calcineurin binding motif (PxIxxT) at amino acid position 339 (a and c isoform) and 341 (b isoform). Therefore, we also considered the possibility that HLH-11 may not be a transcription factor but act as a regulatory protein. Although HLH-11 has a well conserved bHLH domain which is required for DNA binding, the subcellular localization of HLH-11 has not yet been confirmed *in vivo* and/or *in vitro*. Thus, the possibility that HLH-11 may act as a signaling protein remains to be elucidated. Until now we did not see any inhibitory and/or activating function of HLH-11 to TAX-6. However, we have shown that

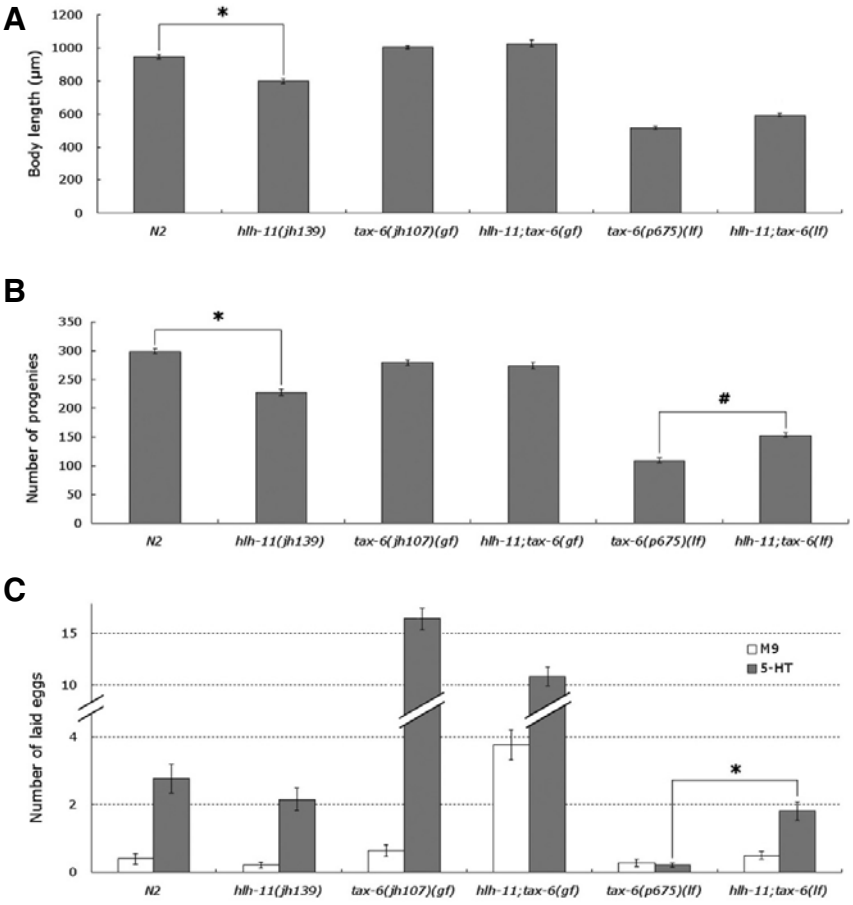

Fig. 5. Characterization of *hlh-11(jh139)* mutant. (A) Body length of mutants. *, $p < 0.0001$. (B) Brood size of mutants. *, $p < 0.0001$. #, $p < 0.0001$. (C) Serotonin-mediated egg laying behavior of mutants. *, $p < 0.0001$.

both *hlh-11* and *tax-6* are expressed in many common tissues in *C. elegans*. In addition, many possible signaling pathways which calcineurin may function in those specific tissues have not yet been fully investigated. Therefore, further analyses could reveal new signaling pathway(s) that HLH-11 function with TAX-6 as a regulator.

ACKNOWLEDGMENTS

We thank CGC for strains, Dr. Yuji Kohara for providing cDNA, Dr. Alan Coulson for cosmid clone and Dr. Andrew Fire for GFP expression vector. Authors are grateful to Dr. Meenakshi Dwivedi for critical reading of the manuscript. This work was supported by the program of Basic Atomic Energy Research Institute, which is a part of the Nuclear R&D Programs funded by the Ministry of Education, Science and Technology of Korea in 2009.

REFERENCES

Bandyopadhyay, J., Lee, J., Lee, J.I., Yu, J.R., Jee, C., Cho, J.H., Jung, S., Lee, M.H., Zannoni, S., Singson, A., et al. (2002). Calcineurin, a calcium/calmodulin-dependent protein phosphatase, is involved in movement, fertility, egg laying, and growth in *Caenorhabditis elegans*. Mol. Biol. Cell *13*, 3281-3293.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. Genetics *77*, 71-94.

Chen, L., Krause, M., Draper, B., Weintraub, H., and Fire, A. (1992). Body-wall muscle formation in *Caenorhabditis elegans* embryos that lack the MyoD homolog *hlh-1*. Science *256*, 240-243.

Chen, L., Krause, M., Sepanski, M., and Fire, A. (1994). The *Caenorhabditis elegans* MYOD homologue HLH-1 is essential for proper muscle function and complete morphogenesis. Development *120*, 1631-1641.

Ciarapica, R., Rosati, J., Cesareni, G., and Nasi, S. (2003). Molecular recognition in helix-loop-helix and helix-loop-helix-leucine zipper domains. Design of repertoires and selection of high affinity ligands for natural proteins. J. Biol. Chem. *278*, 12182-12190.

Corsi, A.K., Kostas, S.A., Fire, A., and Krause, M. (2000). *Caenorhabditis elegans* twist plays an essential role in non-striated muscle development. Development *127*, 2041-2051.

Corsi, A.K., Brodigan, T.M., Jorgensen, E.M., and Krause, M. (2002). Characterization of a dominant negative *C. elegans* Twist mutant protein with implications for human Saethre-Chotzen syndrome. Development *129*, 2761-2772.

Crabtree, G.R. (1999). Generic signals and specific outcomes: signaling through Ca^{2+}, calcineurin, and NF-AT. Cell *96*, 611-614.

Davies, K.J., Ermak, G., Rothermel, B.A., Pritchard, M., Heitman, J., Ahnn, J., Henrique-Silva, F., Crawford, D., Canaider, S., Strippoli, P., et al. (2007). Renaming the DSCR1/Adapt78 gene family as RCAN: regulators of calcineurin. FASEB J. *21*, 3023-3028.

Fukushige, T., and Krause, M. (2005). The myogenic potency of HLH-1 reveals wide-spread developmental plasticity in early *C. elegans* embryos. Development *132,* 1795-1805.

Gort, E.H., van Haaften, G., Verlaan, I., Groot, A.J., Plasterk, R.H., Shvarts, A., Suijkerbuijk, K.P., van Laar, T., van der Wall, E., Raman, V., et al. (2008). The TWIST1 oncogene is a direct target of hypoxia-inducible factor-2alpha. Oncogene *27*, 1501-1510.

Hallam, S., Singer, E., Waring, D., and Jin, Y. (2000). The *C. elegans* NeuroD homolog *cnd-1* functions in multiple aspects of motor neuron fate specification. Development *127*, 4239-4252.

Harfe, B.D., Branda, C.S., Krause, M., Stern, M.J., and Fire, A. (1998a). MyoD and the specification of muscle and non-muscle fates during postembryonic development of the *C. elegans* mesoderm. Development *125,* 2479-2488.

Harfe, B.D., Vaz Gomes, A., Kenyon, C., Liu, J., Krause, M., and Fire, A. (1998b). Analysis of a *Caenorhabditis elegans* Twist homolog identifies conserved and divergent aspects of mesodermal patterning. Genes Dev. *12,* 2623-2635.

Hashimoto, Y., Perrino, B.A., and Soderling, T.R. (1990). Identification of an autoinhibitory domain in calcineurin. J. Biol. Chem. *265*, 1924-1927.

Hubbard, M.J., and Klee, C.B. (1989). Functional domain structure of calcineurin A: mapping by limited proteolysis. Biochemistry *28*, 1868-1874.

Im, S.H., and Rao, A. (2004). Activation and deactivation of gene expression by Ca^{2+}/calcineurin-NFAT-mediated signaling. Mol. Cells *18,* 1-9.

Jan, Y.N., and Jan, L.Y. (1993). HLH proteins, fly neurogenesis, and vertebrate myogenesis. Cell *75*, 827-830.

Karp, X., and Greenwald, I. (2003). Post-transcriptional regulation of the E/Daughterless ortholog HLH-2, negative feedback, and birth order bias during the AC/VU decision in *C. elegans*. Genes Dev. *17,* 3100-3111.

Karp, X., and Greenwald, I. (2004). Multiple roles for the E/Daughterless ortholog HLH-2 during *C. elegans* gonadogenesis. Dev. Biol. *272,* 460-469.

Kim, Y.H., Song, H.O., Ko, K.M., Singaravelu, G., Jee, C., Kang, J., and Ahnn, J. (2008). A novel calcineurin-interacting protein, CNP-3, modulates calcineurin deficient phenotypes in *Caenorhabditis elegans*. Mol. Cells *25*, 566-571.

Klee, C.B., Crouch, T.H., and Krinks, M.H. (1979). Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. Proc. Natl. Acad. Sci. USA *76,* 6270-6273.

Klee, C.B., Ren, H., and Wang, X. (1998). Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. J. Biol. Chem. *273,* 13367-13370.

Krause, M., Harrison, S.W., Xu, S.Q., Chen, L., and Fire, A. (1994). Elements regulating cell- and stage-specific expression of the *C. elegans* MyoD family homolog hlh-1. Dev. Biol. *166,* 133-148.

Krause, M., Park, M., Zhang, J.M., Yuan, J., Harfe, B., Xu, S.Q., Greenwald, I., Cole, M., Paterson, B., and Fire, A. (1997). A *C. elegans* E/Daughterless bHLH protein marks neuronal but not striated muscle development. Development *124,* 2179-2189.

Kuhara, A., Inada, H., Katsura, I., and Mori, I. (2002). Negative regulation and gain control of sensory neurons by the *C. elegans* calcineurin TAX-6. Neuron *33,* 751-763.

Ledent, V., and Vervoort, M. (2001). The basic helix-loop-helix protein family: comparative genomics and phylogenetic analysis. Genome Res. *11,* 754-770.

Ledent, V., Paquet, O., and Vervoort, M. (2002). Phylogenetic analysis of the human basic helix-loop-helix proteins. Genome Biol. *3,* RESEARCH0030.

Lee, J., Jee, C., Song, H.O., Bandyopadhyay, J., Lee, J.I., Yu, J.R., Park, B.J., and Ahnn, J. (2004). Opposing functions of calcineurin and CaMKII regulate G-protein signaling in egg-laying behavior of *C. elegans*. J. Mol. Biol. *344,* 585-595.

Lee, J., Song, H.O., Jee, C., Vanoaica, L., and Ahnn, J. (2005). Calcineurin regulates enteric muscle contraction through EXP-1, excitatory GABA-gated channel, in *C. elegans*. J. Mol. Biol. *352,* 313-318.

Massari, M.E., and Murre, C. (2000). Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. Mol. Cell. Biol. *20,* 429-440.

Mello, C., and Fire, A. (1995). DNA transformation. Methods Cell Biol. *48,* 451-482.

Mulkey, R.M., Endo, S., Shenolikar, S., and Malenka, R.C. (1994). Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. Nature *369,* 486-488.

Murre, C., Bain, G., van Dijk, M.A., Engel, I., Furnari, B.A., Massari, M.E., Matthews, J.R., Quong, M.W., Rivera, R.R., and Stuiver, M.H. (1994). Structure and function of helix-loop-helix proteins. Biochim. Biophys. Acta *1218,* 129-135.

Park, B.J., Lee, J., Lee, J., Kim, S., Choi, K., Park, C.S., and Ahnn, J. (2000). Isolation of deletion mutants by reverse genetics in *Caenorhabditis elegans*. Korean J. Biol. Sci. *5*, 5.

Portman, D.S., and Emmons, S.W. (2000). The basic helix-loop-helix transcription factors LIN-32 and HLH-2 function together in multiple steps of a *C. elegans* neuronal sublineage. Development *127,* 5415-5426.

Schulz, R.A., and Yutzey, K.E. (2004). Calcineurin signaling and NFAT activation in cardiovascular and skeletal muscle development. Dev. Biol. *266,* 1-16.

Singaravelu, G., Song, H.O., Ji, Y.J., Jee, C., Park, B.J., and Ahnn, J. (2007). Calcineurin interacts with KIN-29, a Ser/Thr kinase, in *Caenorhabditis elegans*. Biochem. Biophys. Res. Commun. *352,* 29-35.

Solari, F., Bateman, A., and Ahringer, J. (1999). The *Caenorhabditis elegans* genes *egl-27* and *egr-1* are similar to MTA1, a member of a chromatin regulatory complex, and are redundantly required for embryonic patterning. Development *126,* 2483-2494.

Stewart, A.A., Ingebritsen, T.S., Manalan, A., Klee, C.B., and Cohen, P. (1982). Discovery of a Ca^{2+}- and calmodulin-dependent protein phosphatase: probable identity with calcineurin (CaM-BP80). FEBS Lett. *137,* 80-84.

Thellmann, M., Hatzold, J., and Conradt, B. (2003). The Snail-like CES-1 protein of *C. elegans* can block the expression of the BH3-only cell-death activator gene *egl-1* by antagonizing the function of bHLH proteins. Development *130,* 4057-4071.

Trent, C., Tsuing, N., and Horvitz, H.R. (1983). Egg-laying defective mutants of the nematode *Caenorhabditis elegans*. Genetics *104,* 619-647.

Wang, D., Claus, C.L., Vaccarelli, G., Braunstein, M., Schmitt, T.M., Zuniga-Pflucker, J.C., Rothenberg, E.V., and Anderson, M.K. (2006). The basic helix-loop-helix transcription factor HEBAlt is expressed in pro-T cells and enhances the generation of T cell precursors. J. Immunol. *177,* 109-119.

Zhang, J.M., Chen, L., Krause, M., Fire, A., and Paterson, B.M. (1999). Evolutionary conservation of MyoD function and differential utilization of E proteins. Dev. Biol. *208,* 465-472.

Zhao, J., Wang, P., and Corsi, A.K. (2007). The *C. elegans* Twist target gene, *arg-1*, is regulated by distinct E box promoter elements. Mech. Dev. *124,* 377-389.