The essential functions of bHLH-PAS proteins in *Caenorhabditis elegans*

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ABSTRACT

The basic-helix-loop-helix PAS (bHLH-PAS) proteins are a family of transcription factors that mediate diverse processes, including cellular adaptations to environmental signals and developmental cell fate decisions. The *C. elegans* genome encodes 5 bHLH-PAS proteins. They are AHR-1, HIF-1, AHA-1, C15C8.2/CKY-1 and T01D3.2. The AHA-1 protein is the *C.elegans* ortholog of the mammalian aryl hydrocarbon receptor nuclear translocator (ARNT). AHA-1 can dimerize with multiple bHLH-PAS members such as AHR-1 and HIF-1, and the complexes have important functions. Although *ahr-1*, *hif-1* double loss-of-function mutants are viable, animals homozygous for null mutations in *aha-1* arrest development as young larvae. This data indicates that AHA-1 has other functions in addition to its functions in *ahr-1* and *hif-1* signaling. In this dissertation, I continue the analysis of AHA-1. I confirmed that *aha-1* loss-of-function animals arrest at first or second larval stage with small body size and cuticle defects. The *aha-1*-defective worms do no have obvious feeding defects prior to arrestment. AHA-1 can form DNA-binding complexes when expressed *in vitro*. AHA-1 is also expressed in germ-line cells in addition to most somatic cells. C15C8.2/CKY-1 is another bHLH-PAS protein in *C.elegans*. It has been shown that CKY-1 is mainly expressed in the worm's feeding organ-pharynx and the chimeric gene *cky-1:aha-1* can rescue *aha-1* loss-of-function phenotype. By RNAi experiment, I show that RNAi-treated worms showed obvious delays in development and some of them had cuticle defects. Expression of AHA-1 from two other promoters, *myo-2* and T01D3.2 didn't rescue the larval arrest phenotype of *aha-1-* defective worms. We predict that CKY-1 has essential functions in *C.elegans* development.

CHAPTER 1

General Introduction

During development, the individual cells of any living organism need to sense environmental and developmental signals and make appropriate responses, such as initiating cellular division, differentiation, proliferation, or apoptosis. There are many ways to perform such tasks. Among them, changing the activity of transcription factors and, therefore, altering gene expression is one essential and effective method. The bHLH-PAS proteins are a family of transcription factors that contains a basic-helix-loop-helix motif and a PAS domain. These proteins control a series of important processes such as toxin metabolism, response to hypoxia and circadian rhythms. In this dissertation, I continue the analysis of the bHLH-PAS proteins in a powerful genetic system, the nematode *Caenorhabditis elegans*. I characterize the essential functions of AHA-1, a bHLH-PAS protein. I also assess the requirement for C15C8.2/CKY-1, another bHLH-PAS protein and an AHA-1 dimerization partner, during *C.elegans* development.

LITERATURE REVIEW

The model organism *Caenorhabditis elegans*

C.elegans is a small, rapidly growing soil nematode found commonly in many parts of the world. It is almost invisible to the naked eye, and is commonly viewed under a microscope. The food source for *C.elegans* is OP50, a bacteria, so it can be conveniently maintained in the laboratory. Adults are transparent and about 1mm in length. The worms can be propagated on agar plates with a bacteria lawn. *C.elegans* has two sexes,

hermaphrodite and male. Males arise spontaneously in hermaphrodite populations with a very low frequency (~1/500). Hermaphrodites can produce both oocytes and sperm and self fertilize, while males only produce sperm and need to mate with hermaphrodites for proliferation. The life cycle for *C.elegans* is about 3 days under optimal conditions. After embryogenesis, newly hatched larvae grow quickly through a series of four molts to become adult animals. This short life cycle makes *C.elegans* a good model for genetic analysis (Wood et al. 1988).

C.elegans is a simple organism. The adult hermaphrodite has only 959 somatic cells, and the adult male has only 1031 somatic cells. The complete anatomy of *C.elegans* is known with the help of electron microscope (White et al. 1976). The location and characteristics of all somatic cells in the adult hermaphrodite and male, and the complete cell lineage have been well studied and are all clear by now (Sulston & Horvitz 1977; Sulston et al. 1983).

C.elegans is a good model for both forward and reverse genetic analysis. Chemical mutagens such as ethylmethanesulfonate (EMS) induce mutations at a high frequency. In some strains, transposons are mobile. Transposon insertion is also an effective method to create mutations in *C.elegans*. Once the mutant is isolated, it can be frozen and stored in liquid nitrogen for years and still keep its appropriate phenotype after thawing and recovery (Epstein et al. 1995).

Sequencing of the *C.elegans* genome was essentially complete by 1998. At that time, *C.elegans* was the only multicellular organism for which the sequence was known. It was completed in Nov 2002 (WormBase News and Notes). The 97-megabase sequence predicts over 19,000 genes (The *C.elegans* Sequence consortium, 1998). Over 17300 genes have been

shown to encode mRNAs, as determined by open-reading-frame sequence tags (Reboul et al. 2001). There are several internet resource sites. Wormbase is one powerful resource for *C.elegans* genomic information (Stein et al. 2001). Knowing the genomic sequence of *C.elegans* is only the first step. The next challenge is to determine exactly what all of these genes do in terms of the development and physiological functioning of the organism. Large-scale double-stranded RNA interference (RNAi) and microarray experiments are two important ways to assign functions to specific genes.

RNAi is the process by which double-stranded RNA (dsRNA) induces the homology-dependent degradation of cognate mRNA. This phenomenon was first discovered in *C.elegans* by Fire and his colleagues in 1998 (Fire et al. 1998). At that time, people already knew that both sense RNA and antisense RNA could suppress specific gene expression in worms (Guo et al. 1995). It was surprising to find that dsRNA was at least tenfold more potent as a silencing trigger than were sense or antisense alone. From then on, double-stranded RNA interference became a valuable and powerful tool in the analysis of gene function in *C.elegans*. There are 3 ways to introduce dsRNA into worms: injection, soaking and feeding. Introduction by injection is injecting dsRNA into the gonad of hermaphrodites (Fire et al. 1998). Soaking requires soaking worms in buffer containing dsRNA (Tabara et al. 1998). Through RNAi-by-soaking, 2500 genes were analyzed and 27% of them showed detectable phenotypes (Maeda et al. 2001). Worms can also be fed with bacteria expressing dsRNA (Timmons et al. 1998). Large-scale functional genomic analysis by feeding RNAi was performed. A RNAi library of 16757 bacteria clones was constructed, representing ~86% o f the 19427 predicted genes in *C.elegans*. Mutant phenotypes were observed for 1722 genes. (Kamath et al. 2003).

To determine the efficiency of the screen, Kamath and his colleagues assessed their ability to identify correctly the known loss-of-function phenotypes for previously studied loci. They detected RNAi phenotypes for 63.5% of 323 detectable loci. 92% of the detected RNAi phenotypes were similar to the known mutant phenotypes. They found that RNAi is more effective for analyzing genes that can cause embryonic or larval lethality and sterility than genes involved in post-embryonic development. 77.9% of the known genes with lethal phenotype were detected by RNAi. Less than half (42.2%) of the genes that have post-embryonic functions have RNAi phenotype (Kamath et al. 2003). Also, genes involved in neuronal functions are more resistant to RNAi than other cell types (Tavernarakis et al. 2000, Timmons et al., 2001). Thus, the worm strain that has RNAi phenotypes for genes with post-embryonic functions or neuronal functions was needed. Through studying the mechanism of RNAi, researchers found that RNA directed RNA polymerase plays an essential role in this process (Sijen et al. 2001). Since only a few trigger dsRNA molecules are sufficient to inactivate a continuously transcribed target mRNA for long periods of time, there must be some amplification processes involved. It turns out that dsRNA is cleaved into small fragments of 21-23 nucleotides (siRNA) (Zamore et al. 2000), and can serve as primers and initiate the RNA-directed RNA polymerase chain reaction to amplify the interference (Lipardi et al. 2001). Loss of the putative RNA-directed RNA polymerase encoded by the *rrf-3* gene makes *C.elegans* hypersensitive to RNAi. This hypersensitive strain is especially effective for genes that have post-embryonic phenotypes and the genes that are involved in neuronal function. Of the 80 dsRNAs analyzed, there were 26 that induced phenotypes in a wild type genetic background. There were additional 23 in *rrf-3* mutant background. For

post-embryonic phenotypes, there were 4 detected in wild type, while 18 were detected in mutant worms (Simmer et al. 2002).

basic helix-loop-helix-PAS (bHLH-PAS) proteins

The family of transcription factors that contain bHLH and PAS motifs controls a variety of developmental and physiological events. The term PAS is an acronym derived from the three first identified proteins in this family: PER (the product of the *Drosophila Period* gene), ARNT (mammalian Aryl hydrocarbon receptor nuclear translocator) and SIM (the product of *Drosophila single-minded* gene). The PAS domain is a multi-functional interaction domain found in a broad range of organisms, from bacteria to human. The family of PAS-containing proteins is proposed to mediate many important processes (Crews et al., 1999; Gu et al., 2000).

The known bHLH-PAS proteins perform their functions by forming heterodimeric DNA-binding complexes. One partner of the heterodimer is constantly expressed while the other partner's expression is precisely regulated by developmental or environmental signals (Crews 1998). The PAS domain is used for protein dimerization and ligand binding (Huang et al. 1993; Reisz-Porszasz et al. 1994). The PAS domain is about 260-310 amino acids and contains two well-conserved repeats: PASA and PASB. Each repeat is about 50 amino acid in length (Nambu et al. 1991; Crews et al. 1988). The helix-loop-helix domain is located near the N-terminus and promotes dimerization. The basic domain is the DNA binding domain. Each basic domain in a dimer binds its own half site in a sequence-specific manner (Murre et al. 1994).

The bHLH-PAS proteins are a family of transcription factors that can alter gene expression to adapt to environmental and developmental changes. The most well understood pathways are the aryl hydrocarbon receptor pathway and the hypoxia response pathway.

Aryl hydrocarbon receptor pathway

The mammalian aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor. It mediates many of the carcinogenic and teratogenic effects of certain environmental pollutants. In the absence of ligand, AHR is found in the cytoplasm complexed to 90 kDa heat shock protein (Hsp90) and AHR interaction factor. Association with these proteins is a prerequisite state for AHR's activation (Perdew GH et al. 1988; Carver LA et al. 1994; Denis M et al. 1998; Carver LA et al. 1997,1998). The endogenous ligands for AHR aren't known. However, an AHR- activating compound was successfully identified from porcine lung recently (Song J et al. 2002). Certain harmful chemicals, such as 2,3,7,8-tetrachlorodibenzo-p-dixon (TCDD) and benzopyrene, are AHR-activating ligands (Birnbaum et al. 1994; Bock et al. 1994). Upon binding ligand, AHR translocates to the nucleus, dissociates from Hsp90, and forms a heterodimer with the aryl hydrocarbon receptor translocator (ARNT) (Hankinson et al. 1995; Swanson et al. 1993). The complex then binds DNA elements called xenobiotic response elements (XRE) of target genes and changes genes transcription (Mclane KE et al. 1994; Shen ES et al. 1992). The consensus XRE sequence is TNGCGTG. AHR binds to the TNGC half site, and ARNT binds to the GCG half site (Swanson et al. 1995; Bacsi et al. 1995). The target genes of AHR:ARNT complex include genes encoding xenobiotic metabolizing enzymes (XME). The up-regulation of such enzymes can increase metabolism

of certain insulting chemicals and decrease their biological half-life (Fujisawa-Sehara et al. 1987; Lusska et al. 1993).

Besides its function in toxin metabolism, AHR also has an important role in development. *ahr*-defective mice strains have been developed by three independent laboratories. Although there are some phenotypic differences, these mice commonly show defects in liver growth and development (Fernandez-Salguero et al. 1995; Schmidt et al. 1996). Researchers found that AHR plays a role in the resolution of fetal vascular structures during development. The smaller hepatocyte size is the result of massive portosystemic shunting in null animals (Lahvis et al. 2000). As expected, *ahr* null mice exhibit decreased constitutive expression of XMEs in response to exposure to dioxins (Mimura et al. 1997).

C.elegans AHR-1 and AHA-1 are orthologs of mammalian AHR and ARNT. These two proteins are encoded by the *ahr-1* and *aha-1* genes respectively, and they share biochemical properties with their mammalian cognates. AHR-1 binds Hsp90 *in vitro,* and AHR-1 and AHA-1 can interact to bind XRE sequences specifically (Powell-Coffman et al. 1998). In *C.elegans*, AHR-1 is expressed in a subset of neurons. Animals lacking *ahr-1* functions show specific defects in neuronal differentiation. (Qin and Powell-Coffman submitted, 2003)

The AHR homolog in *Drosophila* is the gene product of *spineless-aristapedia (ss)*. The ARNT homolog in *Drosophila* is the gene product of *tango (tgo)*. Ss and Tgo can form heterodimers *in vitro,* and the complexes cause transcriptional activation of reporters containing mammalian AHR:ARNT binding sites (Emmons et al. 1999). This indicates that Ss:Tgo heterodimers are very similar to AHR:ARNT heterodimers in DNA-binding specificity and transcriptional activation ability. Null mutants of *ss* are viable and show some

defective phenotypes: The distal region of the leg is deleted, and sensory bristles are reduced in size. The most striking mutant phenotype is a transformation of distal antenna into distal leg. These effects suggest that Ss plays a role in specifying distal antennal identity (Duncan et al. 1998).

The function of the bHLH-PAS protein in hypoxia response

Oxygen homeostasis is essential for many living organisms, from bacteria to mammals. Oxygen deprivation endangers the survival of animals, because it is required for the oxidative phosphorylation, which generates ATP. Many diseases that are common causes of mortality, such as heart disease, cancer and cerebrovascular disease, are related to oxygen homeostasis (Semenza et al. 2000). Hypoxia inducible factor 1 (HIF-1) mediates cellular and systemic homeostatic responses to reduced oxygen availability. HIF-1 contains two subunits: HIF-1 alpha and HIF-1 beta. Both of them are bHLH-PAS proteins (Wang et al. 1995). HIF-1 beta is another name for the aryl hydrocarbon receptor nucleus translocator (ARNT). HIF-1 beta is constantly expressed in most somatic cells under hypoxia or normoxia, while the level of HIF-1 alpha is precisely regulated by the cellular oxygen level (Wang et al. 1995). Under normoxic conditions, the Von Hippel-Lindau tumor suppressor protein (pVHL) binds directly to the alpha subunit and targets its ubiquitination and proteosomal degradation (Huang et al. 1998). Thus the steady-state levels of HIF-1 alpha are low, and relatively little transcription complex is formed (Salceda et al. 1997). Under hypoxic conditions, the ubiquitin-proteasome pathway is inhibited, and HIF-1 alpha translocates into the nucleus and forms heterodimers with ARNT (Sutter et al. 2000). The complex then binds to the core DNA sequence A/(G)CGTG, this sequence is known as hypoxia response element (HRE).

Thus, target genes expression is altered (Semenza et al. 1992; Ho et al. 1995). The products of some target genes, such as EPO, VEGF, promote oxygen delivery. Some other target genes have functions in switching metabolism to low oxygen consumption.

The oxygen sensor that responds to HIF-1 activation has been sought for a long time. One breakthrough was the identification of an oxygen-dependent degradation (ODD) domain within HIF-1 alpha that controls its degradation by the ubiquitin-proteasome pathway. The ODD domain is located in the central region of HIF-1 but does not overlap with the PAS domain (Huang et al. 1998). Comparison of the available ODD sequences of human and mouse HIF-alpha proteins revealed a domain of 15 amino acids with strong sequence conservation between all the members. Selective alanine substitutions of 8 amino acids (561-568) stabilized the protein in normoxic conditions (Srinivas et al. 1999). An alanine scan of this region showed that Leu562 and Pro564 were essential for specific binding to pVHL. The interaction between human pVHL and the ODD domain of the HIF-1 alpha subunit requires hydroxylation of the proline residue (P564) by a prolyl-4-hydroxylase (Jaakkola et al. 2001). Since the process of hydroxylation requires oxygen as a co-substrate and iron as a co-factor, the HIF-1-prolyl-hydroxylase (PH) is proposed to function as a cellular oxygen sensor (Ivan et al. 2001).

In *C.elegans*, a similar complex to mammalian HIF-1 mediates the response to hypoxia. This complex consists of HIF-1 and AHA-1, which are the *C.elegans* homologs of HIF-1 alpha and HIF-1 beta respectively. This suggests that the mechanism of hypoxia signaling be likely conserved among metazoans (Jiang et al. 2001). The enzyme and the putative oxygen sensor that hydroxylates a proline within the ODD domain were first identified in *C.elegans* (Epstein et al. 2001). It is encoded by *egl-9* gene, and *egl-9* mutants

show striking up-regulation of HIF-1 levels in *C.elegans*. This enzyme is well conserved between species. Using *egl-9* sequence to search human genomic databases, three mammalian homologs designated "prolyl hydroxylase containing" (PH) 1, 2 and 3 were identified (Epstein et al. 2001; Bruick et al. 2001).

Besides HIF-1 stability, the activation of HIF-1 may also be important to hypoxic response. There are two transcription activity domains in HIF-1 alpha. One overlaps with the ODD domain, and the second is near the c-terminus (Pugh et al. 1997). Phosphorylation of HIF alpha may regulate its transcriptional activity (Wang et al. 1995; Salceda et al. 1997). Recruitment of general transcriptional co-activators including CBP/p300, SRC-1 or TIF2 had been shown to potentiate HIF-1 alpha transcriptional activity (Ema et al. 1999; Carrero et al. 2000; Gu et al. 2001).

HIF-1 plays an important role in embryonic vascularization. Early mammalian embryonic development proceeds under reduced oxygen levels. HIF-1 alpha null mutant mice arrest and die at day 11 of gestation with neural tube defects, cardiovascular malformations, and marked cell death within the cephalic mesenchyme (Iyer et al. 1998; Ryan et al. 1998). HIF-1 alpha is also required for efficient tumor formation. Angiogenesis is a crucial step in tumor growth and progression. HIF-1 regulates angiogenesis by controlling the expression of vascular endothelial cell growth factor (VEGF). Thus, the study of HIF-1 and hypoxia-response pathway has important meaning for anticancer study.

ARNT is an important bHLH-PAS protein

The aryl hydrocarbon nuclear translocator protein (ARNT) is a founding member of the bHLH-PAS family. ARNT is a well-conserved protein between species.

The *Drosophila* ARNT homolog is called Tango. Its bHLH and PAS domains are 92%

and 53% identical to its mammalian homologs, respectively (Sonnenfeld et al. 1997).

The *C.elegans* ARNT homolog is called AHA-1. Its bHLH and PAS domains are 84%

and 41% identical to its mammalian homologs, respectively (Powell-Coffman et al.

1998). ARNT and its homologs are common dimerization partners for multiple

bHLH-PAS proteins. The dimers have specific cellular or developmental functions.

ARNT family proteins can form heterodimers with the aryl hydrocarbon receptor

(AHR), HIF-1alpha, and mammalian single-minded homolog Sim1. The

corresponding complexes mediate toxin metabolism, hypoxia response and neuronal

development respectively (Hankinson et al. 1995;Wang et al. 1995; Probst et al.

1997). Co- immunoprecipitation and HPLC molecular mass assays showed that

ARNT also forms homodimers *in vitro*(Sogawa et al.1995). No *in vivo* function for

ARNT homodimer has been reported.

Drosophila Tgo is able to interact with the AHR homolog Spineless-aristapedia

(Ss), Single-minded (Sim), and Trachealess (Trh). The corresponding complexes

regulate distal antennal identity, CNS midline development, and tracheal development

respectively (Emmons et al.1999; Margaret et al. 1997; Sonnenfeld et al. 1997). Tgo can

also interact with the *Drosophila* HIF-1alpha homolog Similar (Sima) *in vitro* (Lavista-

Llanos et al. 2002).

In *C.elegans*, AHA-1 had been shown to interact with AHR-1 and HIF-1. The

corresponding complexes mediate neuronal development and hypoxia response (Qin and

Powell-Coffman submitted; Jiang et al. 2001). In *hif-1* loss-of-function animals, AHA-1

is not efficiently nuclear localized in intestinal cells (Jiang et al. 2001). Thus, in this cell

type, AHA-1 nuclear localization is dependent on its dimerization partner HIF-1.

What's already known about *C.elegans* AHA-1

To understand the function of AHA-1 in *C.elegans* development, *aha-1* deletion

mutations were generated. *aha-1(ia01)* homozygous mutants arrest their development as

young larvae and the penetrance is 100%. Most of the worms arrest at first or second larval

stage. The remainder arrest before adulthood. The arrested worms have feeding defects. The

longer the time of arrest, the severer the phenotype. 35% (n=94) of the terminally arrested

larvae had a "stuffed" pharynx 3-4 days after hatching. In these animals, bacteria food was

visibly lodged in the pharynx (Jiang 2002).

To test if *aha-1* mutant phenotype can be rescued by *aha-1* genomic sequence, a

DNA construct containing the *aha-1* genomic sequence was injected into the gonad of *aha-1(ia01)* heterozygous worms. The *aha-1 (ia01)* homozygous loss-of-function progeny

carrying the extrachromosomal array of *aha-1* genomic sequence can develop to adults and

give rise to progeny. However, this strain grows slower than wild-type worms (Jiang 2002).

A former graduate student in the lab, Huaqi Jiang, proposed that AHA-1 might

execute its essential developmental function with another bHLH-PAS protein,

C15C8.2/CKY-1. He generated *cky-1:gfp* transgenic worms, this reporter is expressed in a

subset of pharyngeal cells, including muscle cells, g2 gland cell, hypodermal cell, marginal

cells and pharyngeal-intestinal valve cells. In order to test whether AHA-1 can form

heterodimers with CKY-1 *in vitro,* he performed the electrophoretic mobility shift assays.

When CKY-1 and AHA-1 were incubated together, they bound labeled probe that contained

the sequence 5'-TGCGTG, thereby decreased the mobility of the probe. Formation of the

DNA-binding complex requires both AHA-1 and CKY-1 because neither CKY-1 nor AHA

alone can bind to the probe to alter its mobility. Also, this DNA binding is sequence specific.

To test whether expression of AHA-1 only in the pharynx could rescue the *aha-1* loss-

of-function phenotype, a chimeric gene was generated. The *cky-1* promoter was fused to the

aha-1 coding region, in order to direct the expression of AHA-1 in the pharynx. This

construct could rescue the larval arrest *aha-1(ia01)* phenotype (Jiang 2002).

DISSERTATION ORGANIZATION

The *C.elegans* genome encodes 5 bHLH-PAS proteins, which are AHR-1, HIF-1,

AHA-1, CKY-1 and T01D3.2. In my research, I focus on the analysis of AHA-1 and CKY-1.

Chapter I is the literature review. First, I introduce the genetic model organism-

C.elegans, including the general description, the genome, and the important approach used

for genomic functional assay---RNAi. Then I describe the bHLH-PAS transcription factors,

including their structure and function, the two well-studied pathways: aryl hydrocarbon

receptor (AHR) pathway and hypoxia pathway. Finally I describe the importance of the

ARNT protein and the experiments of this project done by a former graduate student in the

lab.

In chapter II, I describe the ARNT and its *Drosophila* and *C.elegans* homolog in

detail. I list the results of my experiments, which include the phenotype of *aha-1* mutant, the

ink feeding assay of the rescued *aha-1* mutants, the gel mobility shift assays of AHA-1 and

AHA-1 immunolocalization data. Finally I discuss the results.

In chapter III, I give an introduction of another bHLH-PAS protein-CKY-1. I explain that the nematode pharynx, *Drosophila* dorsal vessel and vertebrate heart are evolutionary conserved. Then I describe the RNAi data of *cky-1*, the rescue data of two chimeric genes and the antibody staining data of *cky-1:aha-1* rescued worms. Finally I discuss the results.

Chapter IV is the general conclusion. I summarize the results of my experiments.
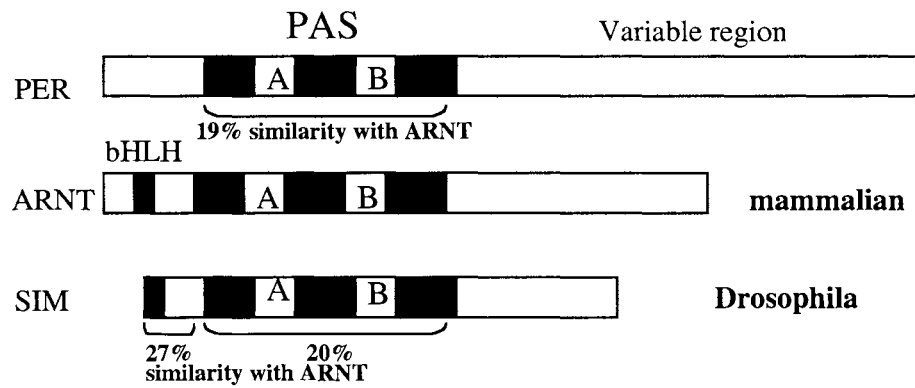
Figure 1. Structure of the three founding members of bHLH-PAS proteins. Shown are PER, Drosophila Period protein; ARNT, mammalian aryl hydrocarbon receptor nuclear translocator; SIM, Drosophila single-minded protein. The bHLH and the PAS domain are indicated in the figure. The percentage of identities of PER and SIM in the bHLH and PAS domain, as compared with ARNT, are indicated.
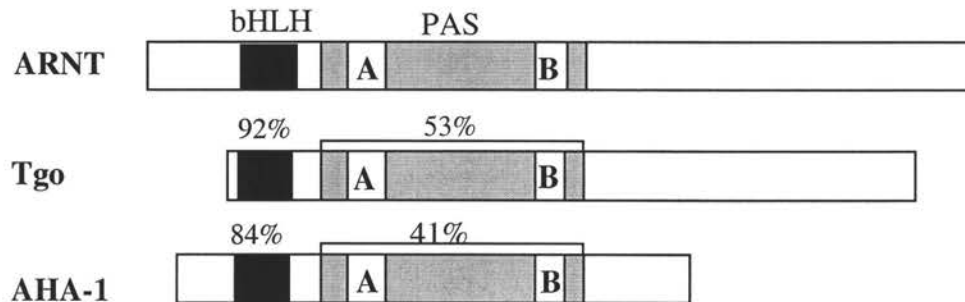
Figure 2. Structure of ARNT bHLH-PAS protein subfamily. Shown are ARNT, mammalian aryl hydrocarbon receptor nuclear translocator (ARNT); Tgo, Drosophila Tango protein; AHA-1, *C. elegans* AHA-1 protein. The conserved basic-helix-loop-helix (bHLH) domains are represented by black boxes. The Per-Arnt-Sim (PAS) domains are indicated by gray boxes, which contain two imperfect repeats called PAS core domains, A and B. The percentage of identities of Tgo and AHA-1 in the bHLH and PAS domain, as compared with ARNT, are indicated.

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CHAPTER 2

AHA-1 plays an essential role in *C.elegans* development.

ABSTRACT

To understand the essential functions of *aha-1*, I further characterized the *aha-1*-defective phenotype and the AHA-1 expression pattern. I confirmed that *aha-1* loss-of-function animals arrest at first or second larval stage with small body size and cuticle defects. In a prior study, terminally arrested *aha-1*-defective worms were shown to have feeding defects. Here, I demonstrate that the arrest phenotype occurs prior to these defects. When AHA-1 is expressed *in vitro*, it can bind DNA containing two ARNT half sites. It was shown before that AHA-1 is expressed in most, if not all, somatic cells. Here, I show that it is also expressed in the germline cells.

INTRODUCTION

Sensing and adapting to environmental stress and developmental signals is essential for the survival of any living organism. In many cases, this process involves changing gene expression. bHLH-PAS proteins are a family of transcription factors that mediate diverse processes including cellular division, differentiation, proliferation, and apoptosis (Jin et al. 2001; Kolluri et al 1999; Brusselmans et al 2001). The aryl hydrocarbon receptor nuclear translocator (ARNT) is a founding member of the bHLH-PAS family. ARNT can dimerize with multiple bHLH-PAS proteins. The mammalian AHR:ARNT complex is the most studied dimer of this family. This complex controls metabolism of some toxins. AHR is a ligand-activated receptor. Some man-made contaminants such as TCDD can activate AHR.

The activated AHR then translocates to the nucleus and dimerizes with ARNT. The heterodimer binds specifically to the xenobiotic response elements (XRE) of target genes such as CYP1A1, and regulates gene expression (Hankinson et al. 1995). ARNT can also form heterodimers with hypoxia inducible factor 1(HIF-1), and the corresponding complexes mediate response to hypoxia (Wang et al. 1995). HIF-1 is also a bHLH-PAS protein, and the HIF-1:ARNT complex is required for embryonic vascularization and tumor formation (Ryan et al. 1998). Homozygous Arnt-/- knockout mice are not viable. Usually these mice die in utero between 9.5 and 10.5 days of gestation. Many defects were detected. The lethality is due to defective placental vascularization. This is related to ARNT's known role in hypoxic induction of angiogenesis (Maltepe et al. 1997).

The *Drosophila* ortholog of ARNT is called Tango (Tgo). The *Drosophila single-minded* (*sim*) and *tracheless* (*trh*) genes both encode bHLH-PAS proteins. These two proteins control the development of the central nervous system (CNS) midline cell lineage and tracheal tubules respectively (Nambu et al. 1991; Wilk et al. 1996). Yeast two-hybrid and co-immunoprecipitation experiments showed that Tgo can interact with Sim and Trh individually *in vitro*. Also, *tgo* loss-of-function mutations showed CNS midline and tracheal defects (Sonnenfeld et al. 1997). These data support a model in which Sim and Trh may function as heterodimers with Tgo. The *Drosophila* AHR homolog is encoded by *spineless-aristapedia (ss)*, which plays a role in specifying distal antennal identity (Duncan et al. 1998). *tgo*-mutant somatic clones showed antennal, leg, and bristle defects that were almost identical to those caused by loss-of-function mutations in *ss*. Yeast two-hybrid assays indicated that Ss and Tgo proteins interact directly. These data support a model in which Ss functions as heterodimers with Tgo (Emmons et al. 1999).

ARNT2 and ARNT3 are also bHLH-PAS proteins. Murine ARNT2 is expressed in the brain and kidney (Hirose et al. 1996). It is similar but distinct from ARNT. ARNT2 polypeptide carries a characteristic basic helix-loop-helix (bHLH)/PAS motif in its N-terminal region with close similarity (81% identity) to that of mouse ARNT and has an overall sequence identity of 57% with ARNT. ARNT2 can interact with AHR and mouse Sim as efficiently as ARNT *in vitro*. The complex can recognize and bind a specific DNA sequence (Hirose et al. 1996). ARNT2 can also form functional HIF complexes in neurons, and the complexes play an integral role in hypoxia responses in CNS (Maltepe et al. 2000). Homozygous *Arnt2* knockout mice survive up to birth with thymic defects. ARNT2 controls the development of the secretory neurons at the later or final stages of differentiation (Wines et al. 1998). ARNT3 is also called brain muscle ARNT-like protein 1 (BMAL) or MOP3. It is mainly expressed in the brain and muscle, and it forms heterodimers with Clock, a bHLH-PAS protein, to regulate circadian rhythms (Ikeda et al., 1997; Takahata et al. 2000).

The *C.elegans* ortholog of ARNT is called AHA-1. The *C.elegans* genome encodes 5 bHLH-PAS proteins. They are AHR-1, HIF-1, AHA-1, C15C8.2/CKY-1 and T01D3.2 (Jiang et, al. 2001). It has been shown that AHA-1 can interact with *C.elegans* AHR-1 *in vitro* and form complexes that bind specifically to the xenobiotic response elements (Powell-Coffman et al. 1998). Also, AHA-1 can bind HIF-1 *in vitro*. HIF-1 is the *C.elegans* ortholog of mammalian HIF-1 alpha. The corresponding complex is proposed to mediate responses to hypoxia (Jiang et al., 2001). A former graduate student in the lab, Huaqi Jiang, demonstrated that animals carrying loss-of-function mutations in both *ahr-1* and *hif-1* are viable, but the *aha-1* null mutants are larvae lethal (Jiang 2002). This suggests that in addition to its functions in *ahr-1* and *hif-1* signaling pathways, *aha-1* may have other functions. Here, I

further characterize the *aha-1* loss-of-function mutant phenotype. *aha-1*-defective animals have small body sizes, pale intestines and cuticle defects. In a previous study, terminally arrested *aha-1* null mutants were shown to have feeding defects (Jiang 2002). Here, I show that null mutant worms feed normally before arrest. Also, I demonstrate that AHA-1 can form DNA binding complexes *in vitro*, and the DNA recognition sequence has two ARNT half sites. Antibody staining data shows that AHA-1 is expressed in germline cells in addition to most somatic cells.

MATERIALS AND METHODS

Worm strains

Worm strains used in this experiment are described below: wild type, Bristol N2; ZG7:*aha-1(ia01)/lin-11(n566)* I.

In addition, I created ZG109, which is *aha-1(ia01)/aha-1(ia01)* animals carrying the rescuing extrachromosomal array of *pHJ28* (*aha-1* genomic sequence), PD4251 (*myo-3:gfp*) and pRF4 (Mello et al. 1991).

All the strains were grown under standard conditions (Wood et al. 1988) and fed with bacteria OP50.

Ink feeding assay

The ink-feeding assay was modified from the methods originally described by Avery and Horvitz in 1990. The worm strain used for this experiment is ZG 109, which is the rescued *aha-1/aha-1* homozygous loss-of-function mutants carrying extrachromosomal array of pHJ28 (*aha-1* genomic sequence), PD4251 (*myo-3:gfp*, expressing *gfp* only in the body

wall muscle) and pRF4. Synchronized worms were grown on 2X peptone plates with bacteria OP50. When most of the worms were adults and there were enough eggs on the plates, adults and larvae were washed away from the plates with M9 buffer (Wood et al. 1998). Plates were washed two additional times to make sure that only eggs were left on the plates. The plates were incubated at 20°C to allow the eggs to hatch. Eighteen hrs later, plates were washed with M9 buffer, and newly hatched larvae were collected into a 15ml centrifuge tube. After two washes, larvae were spun down. All but 500 μl of supernatant was removed and worms were transferred to a 1.5-ml microcentrifuge tube. 500μl S basal buffer (Wood et al. 1988) and 20μl 50X concentrated bacteria were added, the tube was rotated at 20°C for 15 minutes. India ink (1:100) was added. After 15 minutes of rotation, worms were quickly paralyzed by adding sodium azide to final concentration at 0.05%. The number of worms that had ink particles in their gut was counted, using a Nikon E800 microscope.

AHA-1 antibody staining

The AHA-1-specific monoclonal antibody 10H8 (Jiang et al., 2001) was used for *in situ* staining. Immunostaining protocol was modified from the method described by Finney in 1991. The concentration of the witches brew was 0.5X instead of 1X. The fixed worms were incubated in 1 ml 1% βME/Tris-Triton overnight at 37°C rotating. The concentration of the primary antibody 10H8 was 1:100. The concentration of the secondary antibody (rhodamine donkey anti mouse, bought from Jackson Lab) was 1:250. The image was photographed using a Spot RT camera (Roche Dignosis) on a Nikon E800 microscope.

Gel mobility shift assay

AHA-1 was expressed from the expression construct pJ343 in rabbit reticulocyte

lysates (Promega TNT system). The gel mobility shift assays were performed as described

(Powell-Coffman et al.1998). The AHA-1 wild type probe was: 5'-

TCGACAAAGGT**CACGTG**ATTGTGG, annealed to 5'-

TCGACCACAAT**CACGTG**ACCTTTG. The AHA mut1 competitor was 5'-

TCGACAAAGGT**CA**TA**TG**ATTGTGG, annealed to 5'-

TCGACCACAAT**CA**TA**TG**ACCTTTG. Another competitor, AHA-1mut2, was 5'-

TCGACAAAGGT**CAC**A**TG**ATTGTGG, annealed to 5'-

TCGACCACAAT**CA**T**GTG**ACCTTTG. The wild type and mutant probes were

radioactively labeled by using DNA polymerase I Klenow and [_-^{32}P] dCTP. For supershift

assays, 1_l of rabbit anti-AHA-1 polyclonal antibody (Jiang 2002) or 10H8 was added to the

reaction mix. 4E10 is a negative control monoclonal antibody, which recognizes AHR-1.

aha-1 mutant phenotype observation.

The worm strain used for phenotypic analysis was ZG7 *aha-1 (ia01)/lin11 (n566)*.

ZG7 worms were raised on 2x peptone plates (Wood et al. 1988). When there were enough

eggs on the plates, worms were washed away from the plates with M9 buffer. The plates

were washed 2 additional times to make sure only eggs were left on the plates. The plates

were incubated at 20°C for 1 hour. Then the plates were washed with M9 to collect the newly

hatched larvae. The larvae were transferred to a new plate with OP50 and incubated at 20°C.

after 18 hrs, the arrested worms were observed under Nikon E800 microscope.

RESULTS

aha-1 mutant phenotype assay

A former student in the lab, Huaqi Jiang, generated two deletion alleles of *aha-1*, *aha-1(ia01)* and *aha-1(ia02)*. Both of them are predicted to be strong loss-of-function alleles (Jiang 2002). In order to better understand the AHA-1 function, I analyzed the *aha-1 (ia01)* loss-of-function mutant phenotype. Homozygous *aha-1(ia01)* animals arrest as young larvae. The strain was kept as *aha-1(ia01)/lin11(n566)* heterozygotes (Jiang 2002). One hour after hatching, 11.4% (n=272) of the progeny of *aha-1(ia01)/lin-11(n566)* worms had cuticle defects. It was noted that there were creases in their cuticles. The body sizes of the worms that had cuticle defects were smaller than the normal worms. When newly hatched N2 were used for analysis as a control, no worm with cuticle defects was observed (n=133). Eighteen hrs after hatching, some *aha-1(ia01)/lin-11(n566)* progeny were clearly smaller than the others. 74.1% (n=58) of the small worms had cuticle defects (Figure 1B). After arrest, the *aha-1*-defective worms had pale intestine and small body size as compared to their wild type siblings (Figure 1A). No obvious defects in the pharynx were detected at this stage.

Ink feeding assay

Since the arrested worms usually have pale intestines, which are also seen in starving worms, it is reasonable to predict that these worms may suffer from feeding defects. One main reason that causes feeding defects is abnormalities in either the structure or the function of the pharynx. The pharynx is a neuromuscular organ in the anterior of the worm, and it is used for ingesting and concentrating food by its pumping movement. In order to analyze the pharynx of *aha-1(ia01)* worms, a former graduate student in the lab, Huaqi Jiang, observed

the structure of the *aha-1(ia01)* mutant pharynx. There was no obvious abnormality detected in the arrested worms. He also performed an ink feeding assay to determine whether *aha-1(ia01)* worms have defects in the pharyngeal functions. After feeding worms with foods of bacteria mixed with ink, over 97% of wild type first and second stage larvae had ink particles in their intestines. In contrast, 24 hrs after hatching, less than 50% of the *aha-1(ia01)* worms contained ink particles in their intestines. In these assays, *aha-1(ia01)* homozygotes were distinguished from their phenotypically wild type siblings using morphological criteria. The *aha-1(ia01)* worms had already arrested development at the time of assays. 35% (n=94) of the terminally arrested larvae had ink particles clogged in the gut lumen 3-4 days after hatching (Jiang 2002).

The data above shows that arrested *aha-1* mutants have feeding defects. However, it is not clear whether arrest is due to starvation. To address this, it was important to assay feeding prior to arrest. This required that *aha-1*-defective animals could be distinguished from their wild-type siblings. In order to solve this problem, I generated a strain in which *aha-1(ia01)* worms were rescued with an array containing *aha-1* genomic sequence and marked with the PD4251, a *myo-3:gfp* marker, which is expressed in body wall muscles. pHJ28, which contains *aha-1* genomic sequence, PD4251 and pRF4 were co-injected into the gonad of *aha-1(ia01)/lin11* worms. Progeny that were homozygous *aha-1(ia01)/aha-1(ia01)* carrying the rescuing extrachromosomal array were recovered. In this strain, I could distinguish the *aha-1*-defective worms that don't express GFP from the rescued worms before they arrest development. The timing used in ink feeding assays was 7-11 hours after hatching. At this time point, the worms haven't arrested development. After feeding worms with bacteria mixed with ink for 15 minutes, 91.1% (n=158) of the worms without the

extrachromosomal array contained ink particles in their intestine. 98.8% (n=239) of the worms that carry the extrachromosomal array had ink particles in their intestine. Using two-sample t-test to compare the two numbers, there is significant difference between them (P<0.05). But, since the difference is relatively small, it can not account for the 100% larval arrest phenotype.

Antibody staining

Previously in Dr. Powell-Coffman lab, it was detected that AHA-1 is broadly and constantly expressed in most, if not all, somatic cells, including hypodermal cells, intestinal cells, pharynx cells and neurons. In some cell types, the efficient nuclear localization of AHA-1 depends on its dimerization partner HIF-1 (Jiang et al. 2001). I further characterized that besides in somatic cells (Figure 2), AHA-1 is also expressed in germline cells. The newly hatched worms have a gonadal primordium consisting of 4 cells: two precursors of the somatic gonad, Z1 and Z4, and two precursors of the germ-line tissue, Z2 and Z3. The hermaphrodite adult reproductive system consists of two tubular ovotestes, one anterior and one posterior. The ovotestes are joined centrally by two spermathecae on both sides and a uterus in the center. The uterus opens mid-ventrally to the exterior via the vulva (Figure 3A). The somatic distal tip cells are located at the distal end of each arm. The germ–line nuclei of the distal arm include mitotic nuclei most distally and meiotic nuclei more proximally (Figure 3B). The images in figure 3 C&D show the anterior arm of the gonad of *hif-1* mutant worms. AHA-1 is co-localized with DAPI in both mitotically and meiotically dividing cells. The co-localization with DAPI occurs at all stages of germline development.

AHA-1 can form homodimers *in vitro*

To test whether AHA-1 can form a DNA binding complex *in vitro*, I expressed AHA-1 in rabbit reticulocyte lysates and performed electrophoretic mobility shift assays (EMSA). ARNT binds the DNA half site 5' GTG (Basci et al. 1995). AHA-1 can bind this same sequence (Powell-Coffman et al. 1998). Thus, a homodimer was predicted to bind 5'-CACGTG. I assayed AHA-1 binding to a probe containing this sequence. The experiment was repeated for more than 30 times. A representative result is shown in figure 4. The complex could bind to radioactively labeled DNA, causing reduced mobility in a non-denaturing gel (lane 3). The complex was progressively competed away by the addition of 10X, 50X and 200X unlabeled wild type probe (lane 4,5,6). To assess the specificity of this complex, two mutant competitors were assayed: mut1 has mutations in both half sites and mut2 has a mutation only in one half site. The two mutant oligos did not compete for binding to AHA-1 (lane 7, 8). Since mutation in one half site abolishes binding, this data supports the model that AHA-1 binds this sequence as a dimer. Adding AHA-1 specific monoclonal antibody to the reaction caused the complex to run slower (line 9). Adding AHA-1 specific polyclonal antibody generated several supershift bands (line 10). There is no supershift when control antibody 4E10, which binds specifically to AHR, was added into the reaction (line 11). The positions of probe and supershift (SS) are shown by arrow.

DISCUSSION

AHA-1 has essential function

Prior analysis of the *aha-1*-defective phenotype indicated that terminally arrested *aha-1* loss-of-function mutant larvae had feeding defects (Jiang 2002). There are two possibilities to explain this finding. One is that *aha-1* has an essential function in the pharynx. When this gene is knocked out, worms can not feed normally and eventually arrest due to starvation. The other possibility is that *aha-1* has other essential functions in *C.elegans* development, and developmental arrest was not caused by feeding defects. After arrest, the worm is too sick to eat. In order to further test these two models, I generated a strain in which the larval arrest phenotype is rescued by *aha-1* genomic sequence. The rescued worms also carry a GFP marker. With the help of the GFP marker, I could distinguish the mutant worms from the wild type worms before the developmental arrest. After feeding worms with bacteria mixed with ink for 15 minutes, 91.1% (n=158) of the worms without the extrachromosomal array contained ink particles in their intestine. 98.8% (n=239) of the rescued worms had ink particles in their intestine. Although by two sample t-test, the two numbers are different (p<0.05), but the differences can not fully account for the 100% larval arrest phenotype. I conclude that the mutant worms do not have obvious feeding defects before arrest.

Cuticle defects in *aha-1* defective-larvae

The *aha-1*–defective worms arrest at L1 or L2 stage. I observed the newly hatched worms and arrested L1 larvae. One hour after hatching, some worms have cuticle defects. It is reasonable to predict these worms are *aha-1(ia01)* homozygotes because of two reasons:

1). The wild type control worms do not show the cuticle defects phenotype. 2). Eighteen hrs

after hatching, the *aha-1(ia01)* animals have arrested, are smaller than their siblings, and

have similar cuticle defects. The cuticle is an exoskeleton that is synthesized by the

underlying epithelial tissue, called the hypodermis. The cuticle consists predominantly of

small collagen-like proteins that are extensively crosslinked. The *C.elegans* genome encodes

over 150 collagen genes. The cuticular collagen genes are subject to strict spatial and

temporal modes of regulation. During the life cycle, a cuticle is synthesized five times. Once

in the embryo before hatching, and then at the end of each of the four larval stages before

molting (Johnstone IL, 1994). Since the newly hatched *aha-1(ia01)* mutants have cuticle

defects, it is possible that AHA-1 may regulate some essential collagen genes. Loss-of-

function mutations in *aha-1* cause the misexpression of some collagen genes. Thus, the

worms show cuticle defects.

AHA-1 can binds DNA as homodimers

AHA-1 can form homodimers *in vitro*. In 1995, Sogawa and his colleagues showed by

co-immunoprecipitation and HPLC molecular mass assay that ARNT could form

homodimers. This complex can bind the E box core sequence 5' CACGTG in an adenovirus

major late promoter. Also, they showed that ARNT homodimers have

transcription–enhancing activity *in vitro* (Sogawa et al. 1995). In 2001, Huffman and his

colleagues synthesized a 56-amino acid peptide that contained the bHLH domain of ARNT.

In the absence of DNA, the ARNT-bHLH peptide was shown to form homodimers in lower

ionic strength buffers, as evidenced by dynamic light scattering analysis. The complex can

bind E-box DNA with high specificity and affinity. At this time, no *in vivo* targets of ARNT homodimers have been identified.

AHA-1 is the dimerization partner of multiple bHLH-PAS proteins

In *C.elegans*, there are five bHLH-PAS proteins. They are AHR-1, HIF-1, AHA-1, CKY-1 and T01D3.2. AHA-1 was shown to be the dimerization partner of AHR-1, HIF-1 and CKY-1 *in vitro* (figure 5). AHA-1 can interact with AHR-1 *in vitro* and the complexes mediate neuronal development. AHA-1 can form DNA-binding heterodimers with HIF-1 *in vitro*. The complexes have functions in responses to hypoxia. AHA-1 can also interact with CKY-1 *in vitro* and the complexes were predicted to have pharyngeal functions. In addition to dimerize with other protein, AHA-1 can form DNA-binding homodimers. The *in vivo* functions of the homodimers are unknown yet. The *in vitro* expression of T01D3.2 is not successful, so whether AHA-1 can form DNA-binding complexes with T01D3.2 or whether the complexes have any *in vivo* functions are not clear yet.
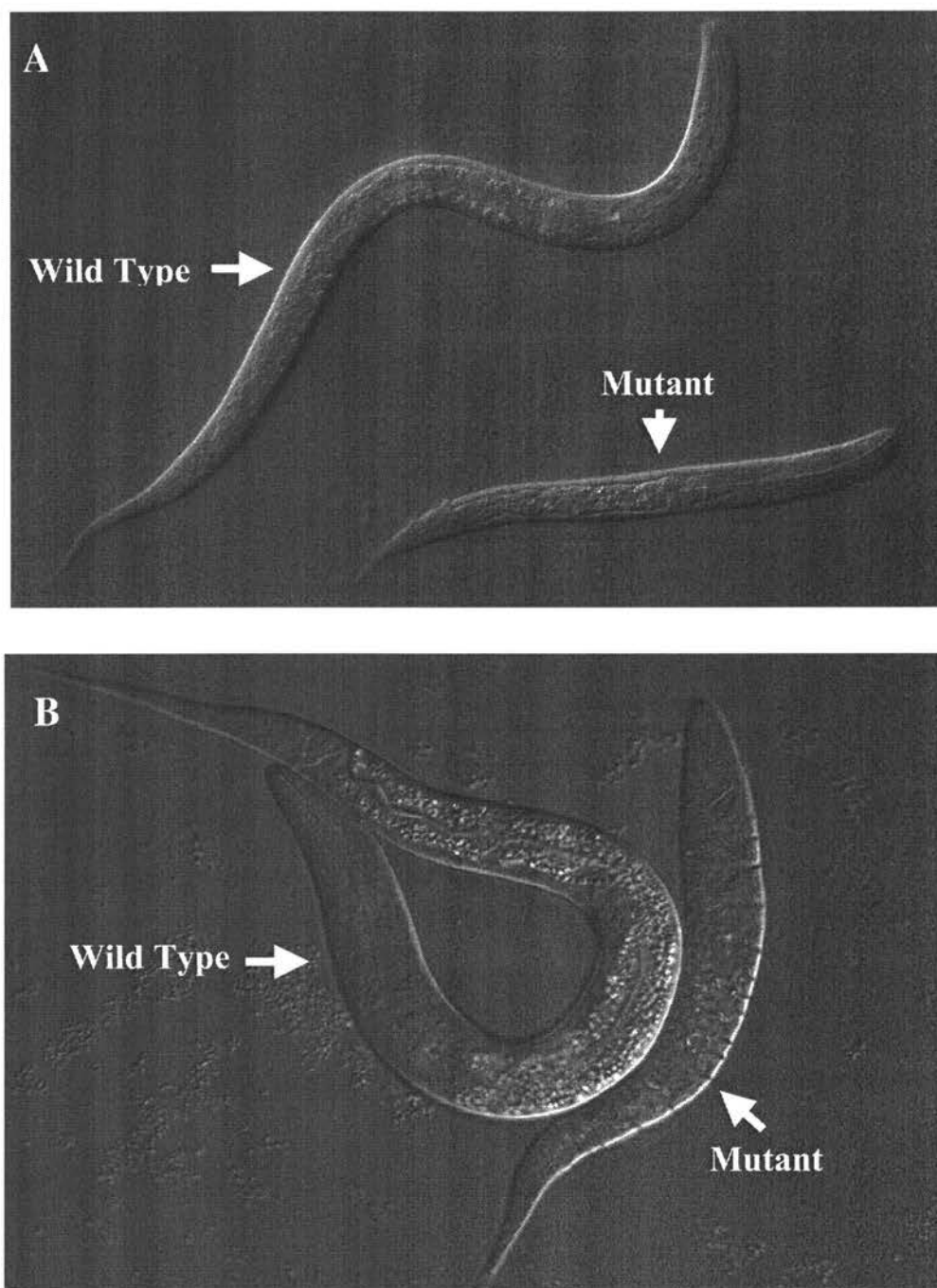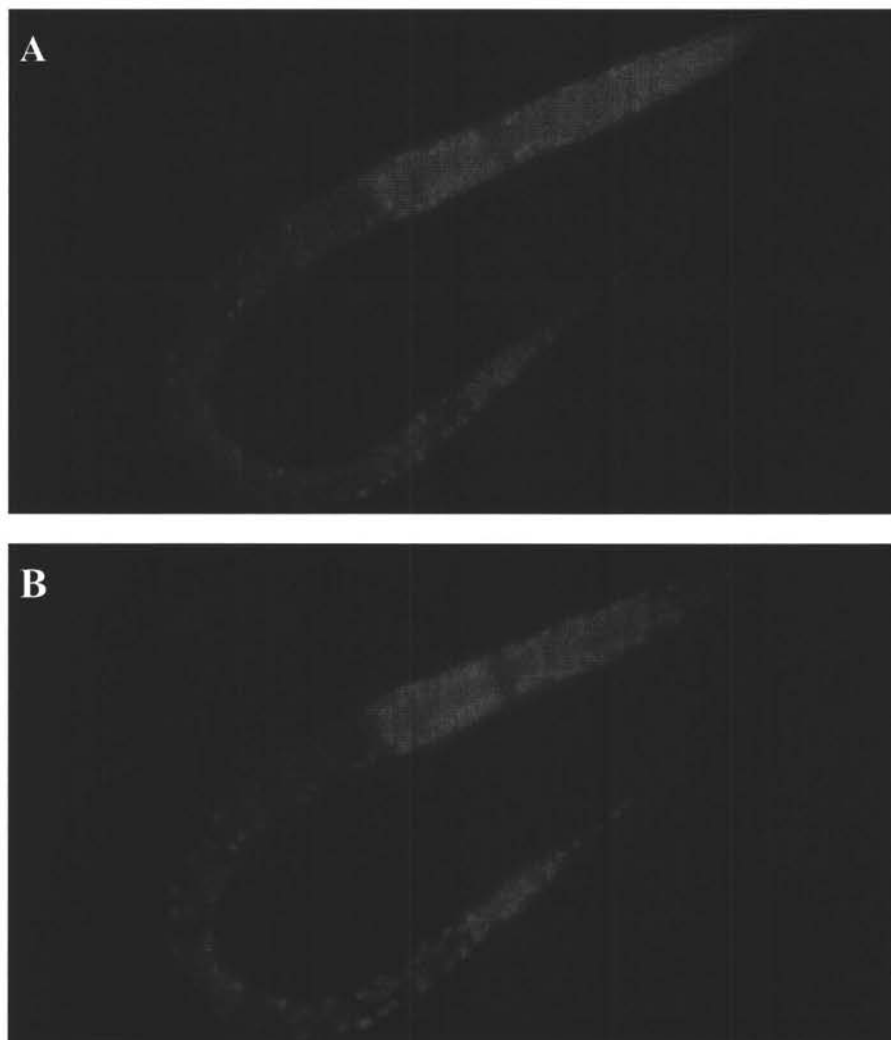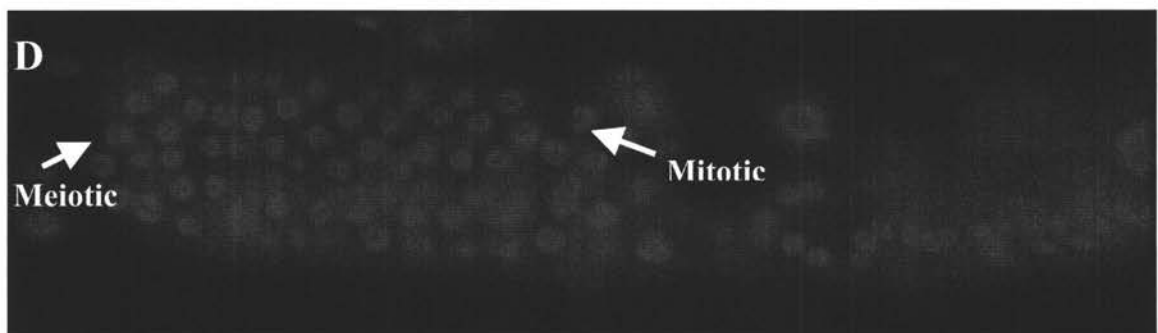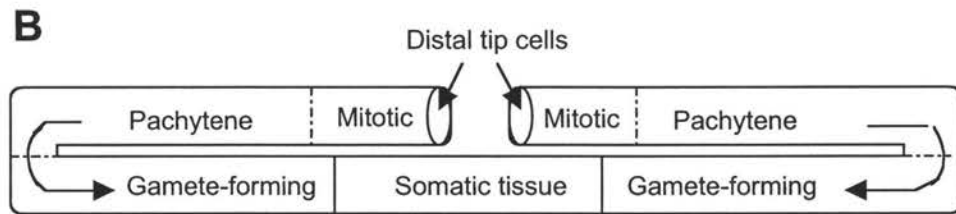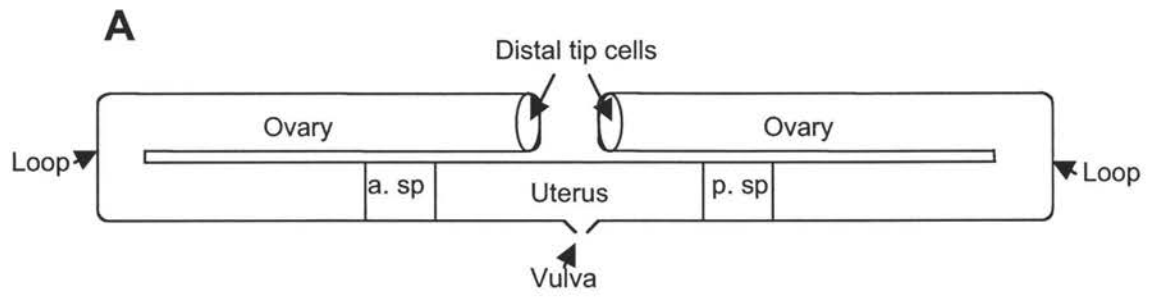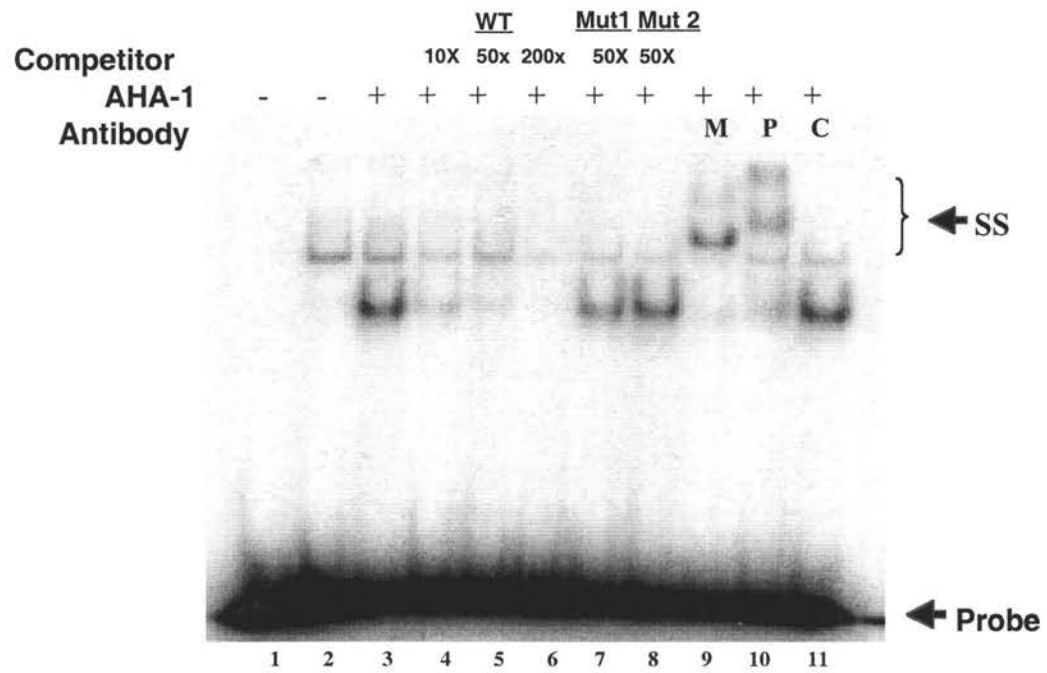
Figure 1

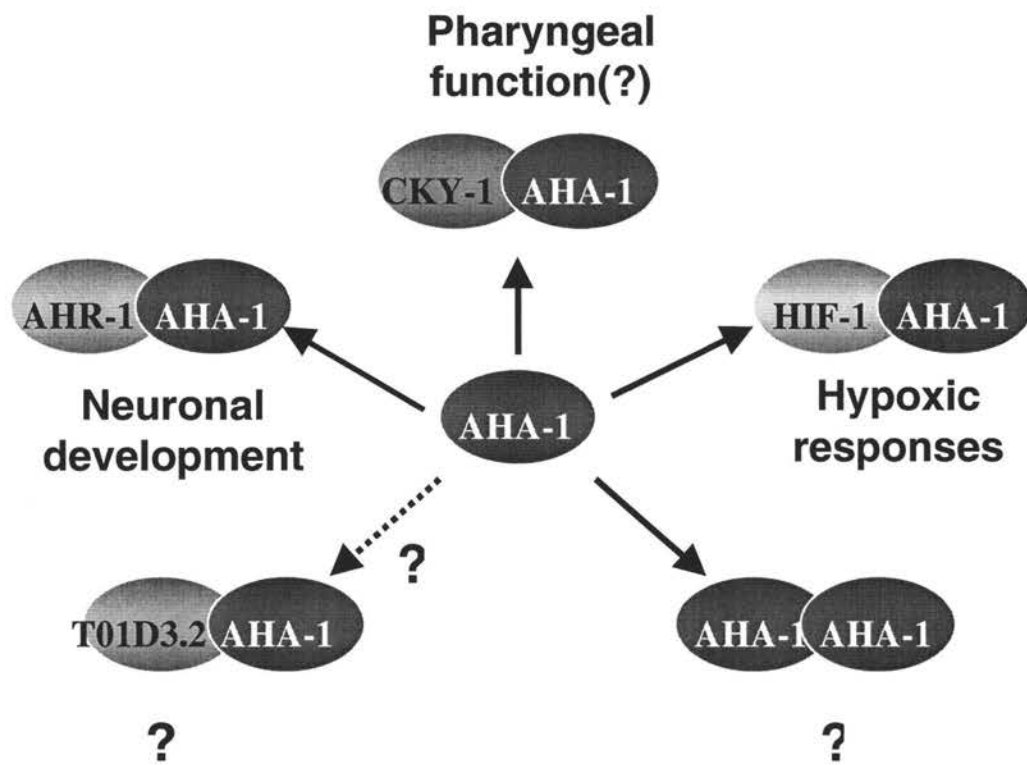Figure 2

Figure 3

Figure 4

Figure 5

FIGURE LEGENDS

Figure 1. *aha-1* loss-of-function phenotypes.

    A. 18 hrs after hatching, *aha-1*-defective animal is smaller than its wild type sibling.

    B. 74.1% (n=58) of *aha-1*-defective worms have cuticle defects at L1 stage.

Figure 2. Immunolocalization of AHA-1 in wild type worm using the monoclonal antibody 10H8.

    A. AHA-1 is present in the nuclei of most somatic cells. A second stage larva is shown here. The anterior is at top right.

    B. DAPI staining of A.

Figure 3. Immunolocalization of AHA-1 in the germline of *hif-1* loss-of-function worm using the monoclonal antibody 10H8.

    A. The structure of the gonad of adult hermaphrodite. The ovary, anterior spermathecae (a. sp), posterior spermathecae (p. sp), uterus and vulva are indicated in the diagram. The anterior is to the left. The dorsal is up. The four diagrams are in the same direction.

    B. Spatial organization of adult gonad. The distal tip cells, mitotic region, meiotic region (pachytene), gamete-forming region are indicated in the diagram. Regions are demarcated by dashed lines.

C. AHA-1 is present in the nuclei of germ-line cells. The anterior arm of an adult

hermaphrodite gonad is shown here. The mitotic region and meiotic region are

indicated in the images.

D. DAPI staining of C.

Figure 4. AHA-1 can form homodimer *in vitro*.

AHA-1 was expressed in rabbit reticulocyte lysates (Promega TNT system) and incubated

with labeled AHA-1 wild type probe (lane 3). Wild type and mutant unlabeled competitor

were added in some reaction as showed in picture. To create supershift, 1ul of AHA-1

specific monoclonal antibody 10H8 (M) or polyclonal antibody (P) were added, 4E10 (C),

which is a monoclonal antibody recognizes AHR, was added as a control. This reaction

mixes were analyzed by nondenaturing polyacrylamide gels. Arrows indicate supershift (SS)

and probe (Probe) respectively.

Figure 5. AHA-1 is the dimerization partner of multiple bHLH-PAS proteins.

AHA-1 can dimerize with AHR-1, HIF, or CKY-1 *in vitro*. The function of each complex is

indicated in the figure. The question marks indicates the facts that whether AHA-1 can

interact with T01D3.2 and whether the corresponding complex has *in vivo* functions, whether

AHA-1 homodimer has *in vivo* functions, are unknown.

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CHAPTER 3

Characterization of C15C8.2/CKY-1, a pharyngeally expressed bHLH-PAS Protein in *C.elegans*

ABSTRACT

The *C.elegans* genome encodes five proteins that contain basic-helix-loop-helix and PAS motifs. They are AHR-1, HIF-1, AHA-1, CKY-1 and T01D3.2. The AHA-1 bHLH-PAS protein has been shown to interact with three other family members, AHR-1, HIF-1 and C15C8.2/CKY-1. Although *ahr-1* and *hif-1* loss-of-function double mutants are viable, animals lacking *aha-1* functions arrest during larval development. A former student in the lab, Huaqi Jiang, showed that CKY-1 is mainly expressed in the pharynx of *C.elegans*. CKY-1 can form DNA-binding complexes with AHA-1 *in vitro,* and a *cky-1:aha-1* chimeric gene can rescue *aha-1* loss-of-function phenotype. Here, I extend those studies. I show that treatment of worms with *cky-1* RNAi results in larval arrest and cuticle defects. Expression of AHA-1 from two other promoters, *myo-2* or *T01D3.2,* didn't rescue the larval arrest phenotype of *aha-1(ia01)* null mutants. The AHA-1 antibody staining of the *cky-1:aha-1* rescued worms confirmed the CKY-1 expression pattern.

INTRODUCTION

C15C8.2/CKY-1 is a bHLH-PAS protein. Analysis of a GFP reporter construct indicated that CKY-1:GFP was expressed in all pharyngeal muscle cells, g2 gland cells, epithelial cells, marginal cells and pharyngeal-intestinal valve cells. CKY-1:GFP expression was not detectable in any of the pharyngeal neurons or in the g1 gland cells. 5-10 cells

immediately outside of the pharynx also expressed CKY-1:GFP (Jiang 2002). The pharynx is

the worm's feeding organ. It contracts rhythmically and pumps bacterial food into the

intestine (Avery et al 1993). The *C.elegans* pharynx, *Drosophila* dorsal vessel and vertebrate

heart are proposed to arise from a common ancestral structure, despite great differences in

morphology. The vertebrate heart is a pulsatile organ that can pump blood throughout the

body. The circulatory system of *Drosophila* is open. The hemolymph is pumped throughout

the body by the dorsal vessel, which is also a pulsatile organ. There is more evidence to

support this model at the molecular level. *ceh-22* is a *C.elegans* gene that is expressed in the

muscle cells of the pharynx. CEH-22 activates gene expression in pharyngeal muscle cells

and is required for normal pharyngeal development (Okkema et al. 1993, 1997). *tinman* is a

Drosophila gene that is expressed in visceral mesoderm and the heart. It has been shown that

the function of *tinman* is required for visceral muscle and heart development (Bodmer R. et

al 1994). In vertebrates, there are six *tinman* homologs. *nkx2.5* is the most highly conserved

among diverse vertebrate species and has been shown to be essential for myogenic and

morphogenetic differentiation of the mammalian heart (Evans et al. 1999, Harvey 1996). The

three genes mentioned above, *ceh22, tinman* and *nkx2.5,* all belong to the same family: NK-2

homeobox genes. Also, the fact that zebra fish *nkx2.5* can efficiently rescue a *ceh-22* mutant

when expressed in pharyngeal muscle indicates that *nkx2.5* and *ceh-22* provide a single

conserved molecular function. All the data above suggests that an evolutionarily conserved

mechanism underlies heart development in vertebrate and insects and pharyngeal

development in nematodes (Haun et al. 1998).

MATERIALS AND METHODS

Worm Strains

Worm strains used in this experiment are described below: wild type, Bristol N2;

ZG7 *aha-1(ia01)/lin-11(n566)* I; ZG62 *aha-1(ia01)/ aha-1(ia01)* pHJ32 Ex10 (Jiang 2002);

ZG 113 *aha-1(ia01)/ lin11(n566)* pHJ36 Ex1; ZG114 *aha-1(ia01)/ lin11(n566)* pHJ36 Ex22;

ZG115 *aha-1(ia01)/ lin11(n566)* pHJ36 Ex28; ZG116 *aha-1(ia01)/ lin11(n566)* pHJ38 Ex2;

ZG117 *aha-1(ia01)/ lin11(n566)* pHJ38 Ex3; RNAi hypersensitive strain, *NL2099, rrf-*

3(pk1426) II

All the strains were grown under standard conditions (Wood et al. 1988) and fed with

bacteria OP50. The DNA constructs for rescue assay were co-injected with pRF4 (Mello et

al. 1991).

Prepare bacteria express dsRNA of cky-1

cky-1 cDNA is about 2.2kb in length. A 1.3kb SacI/EcoRI fragment near the 5' end

was cloned into the SacI/EcoRI site of pPSK79 (a plasmid that has T7 promoters flanking the

polylinker and can express double strand RNA in the presence of IPTG) to create the plasmid

pSW01. Then pSW01 was transformed into the bacteria strain BL21DE3.

Northern blot

To determine whether the RNAi treatment reduced the level of endogenous *cky-1*

RNA, Northern blots were performed. The Northern blot was probed with a 3' end fragment

of the *cky-1* cDNA (0.8kb EcoRI/ XbaI fragment) that did not overlap with the sequence in

pSW01. Bacteria that expressed ds *cky-1* from pSW01 were incubated at 37°C for 6-8 hrs,

and then the bacteria were seeded on 2x peptone plates. The plates were placed at room temperate overnight. Then N2 synchronized young larvae were transferred to the plates and incubated at 20°C. When most of the worms were adults and had enough eggs, the adults were treated with alkaline hypochlorite solution and eggs were collected (Sulston and Hodgkin, 1988). RNA was extracted from the embryos using TRIZOL based method (Burdine et al 1996). RNA blot was performed (Hirose et al 1996).

Prepare of ds RNA of C15C8.2, T01D3.2 and *gfp*

The complementary DNAs for C15C8.2 and T01D3.2 were isolated from an embryonic cDNA expression library (Jiang 2002). Each cDNA was amplified by PCR using two primers T3: 5'-CGC AAT TAA CCC TCA CAT AAG GG-3' and T7: 5'-GTA ATA CGA CTC ACT ATA GGG CG-3'. Then the PCR products were used as templates for *in vitro* transcription of mRNA (Promega riboprobe system kit). RNase-free DNase was added to the systems at the end of reaction to remove DNA templates. Then each reaction was extracted with phenol/chloroform and chloroform. Each strand of RNA was precipitated by isopropanol separately. The RNAs were passed through a centrifuge tube with a 0.22um cellulose acetate filter, and the complementary RNA strands were annealed *in vitro*. The final concentration of the dsRNA was between 0.6~0.7ug/ul. The cDNA of *gfp* was a gift from the Andy Norris lab. *gfp* dsRNA was made by the same protocol as above, the final concentration was between 0.6~0.7ug/ul.

Microinjection

Double-stranded RNA was injected into the gonad of young adults (Esptein et al. 1995). After injection, the worms were allowed to lay eggs for 6-8 hours before progeny were scored. Each worm was placed on a separate plate and incubated at 20°C. Every 6-8 hrs, the injected worms were transferred to a new plate and the eggs it laid were scored. Each worm was transferred twice. About 40 hrs after the first transfer, the L4 worms or beyond on each plate were scored and then transferred to new plates. The plates were continuously screened until there was no L4 worm appeared.

Antibody staining of the *cky-1:aha-1* rescued worms

The AHA-1-specific monoclonal antibody 10H8 (Jiang et al., 2001) was used for *in situ* staining. Immunostaining was performed essentially as described by Finney in 1990. The worm strain used for staining was ZG62: *cky-1:aha-1* rescued *aha-1* mutants. The concentration of the witches brew was 0.5X instead of 1X. The fixed worms were incubated in 1 ml 1% βME/tris-triton overnight at 37°C rotating. The concentration of the primary antibody 10H8 was 1/100, the concentration of the secondary antibody (rhodamine donkey anti mouse, bought from Jackson Lab) was 1/250. The image was photographed using a Spot RT camera (Roche Dignosis) on a Nikon E800 microscope.

Constructs used in *aha-1(ia01)* rescue assays

Two constructs were used in *aha-1(ia01)* rescue assays.

(i)   pHJ36 was designed to express AHA-1 only in the muscle cells of the

pharynx. *myo-2* is a gene expressed only in *C.elegans* pharyngeal muscle

(Okkame et al.1993). A 4.1kb ApaI/KpnI fragment (containing *myo-2*

promoter region) from pPD122.11 (Jiang 2002) was fused to a 2.7kb

ApaI/KpnI fragment (containing part of the *aha-1* genomic sequence) from

pHJ32 (Jiang 2002) to create pHJ35. Then a 6.6kb BalI/KpnI fragment from

pHJ35 was fused to a 2.3kb SacI/KpnI fragment (containing the other part of

aha-1 genomic sequence) of pHJ32 to create pHJ36.

(ii)   pHJ38 was designed to express AHA-1 only in two AVH interneurons in the

head (Jiang 2002). The T01D3.2 promoter fragment was amplified using

primers: PASA10R: CAT GGA TCC ATA AAT ATA TCC TGA GCC ATT

TCT CAA GTG GTT ATA AGT C and PASA9F: GTG GAG TTT GGA

TCC TTC GAA TCG. A 10.2kb BamHI fragment (containing the *aha-1*

genomic sequence) from pHJ32 was fused to a BamHI fragment of PCR

product to generate the plasmid pHJ37. Then a 0.8kb SalI fragment

(containing the T01D3.2 promoter region) of pR14 (Jiang 2002) was fused to

a 9.5 kb SalI fragment of pHJ37 (containing the *aha-1* genomic sequence) to

generate pHJ38.

Rescue assays

The worms used for injection were ZG7 *aha-1(ia01)/lin11(n566)*. PRF4 (Mello et al,

1991) was co-injected with the rescue constructs as a marker. Worms that carried stable

extrachromosomal arrays were screened. Several lines were generated for each injection. For

each line, healthy rollers were each placed on a separate plate and allowed to lay as many

eggs as it could. The eggs on each plate were scored. When the eggs hatched and the worms

grew to L4, each L4 worm was placed on a separate plate. Several days later, the progeny on each plate was observed. If all the progeny on the plate were rollers and there were no bagging worms (the phenotype of *lin-11* homozygous worm), then it was a rescued line.

RESULTS

CKY-1 is required for developmental progression

To test whether *cky-1* has a function in *C.elegans* development, dsRNA mediated interference was performed. When wild type worms were used for RNAi treatment, feeding, injection and soaking were all tried but there was no detectable difference between the experiment group and the control group: GFP RNAi. The progeny of the treated animals were viable, and there were no obvious defects. For feeding RNAi, in order to test its efficiency, northern blots were performed using the RNA extracted from the embryos of experiment and control groups. The results showed that there was no detectable difference of the *cky-1* RNA levels between the two groups. This indicates the RNAi method doesn't work for this gene in wild type background. Also *aha-1(ia01)/lin11(n566)* worms were used for RNAi treatment based on the model that CKY-1 may form heterodimers with AHA-1 to perform its function. The rationale was that when its dimerization partner's dosage decreased, a slight decrease of CKY-1 might result in a detectable defect. But the results were same as using wild type worms.

Recently, animals deficient in *rrf-3* functions were shown to be more sensitive to RNAi than wild type worms (Simmer et al. 2002). When the RNAi hypersensitive worm strain was available, RNAi was performed by injection. The progeny of the *dscky-1* injected worms showed obvious delays in development. The data is summarized in table 1. Three

days after egg laying, 94.5 % of hatched larvae (n=636) had developed to fourth larval stage

(L4) or adults in the *dsgfp* treated group (control). However, only 24.6% of hatched larvae

(n=862) of the *dscky-1* treated group had grown to L4 or adults by this time point. The

developmentally delayed worms were not as healthy as wild type. These worms moved

slowly, and had pale bodies. Some worms eventually developed to adults but were fertile.

The pharynxes of the arrested worms did not have obvious defects. 64.8% of the slow

growing worms had cuticle defects when observed under the microscope (n=54) (Figure 1).

The observation time was 48 hrs after egg laying and the worms were about at the L2 stage at

this time point. This phenotype was not detected in any newly hatched *cky-1* RNAi worms

(n=109). T01D3.2 is another bHLH-PAS protein encoded by *C.elegans* genome. To test

whether this gene has essential functions, RNAi experiments were performed. The growth

rate of T01D3.2 treated worms was similar to that of the *gfp* control group, but the embryonic

lethality was higher. The results of the *dscky-1*, dsT*01D3.2, dsgfp* RNAi and uninjected

worms are summarized in table 1.

Table 1. *dscky-1* RNAi treated worms delay development.

dsRNA	Embryonic lethality, %	Hatched	L4 or adults at 3 days. %	L4 or adults at 5 days. %
dscky-1	484 36.0% (n=1346)	862	24.6% (n=862)	44.0% (n=862)
dsgfp	362 36.3% (n=998)	636	94.5% (n=636)	95.6% (n=636)
dsT01D3. 2	500 43.6% (n=1148)	648	92.9% (n=648)	93.5% (n=648)
uninjected	360 27.5% (n=1311)	951	94% (n=951)	95.1% (n=951)

Note: 3 days is defined as 72 hrs after egg laying. 5 days is defined as 120 hrs after egg laying.

Antibody staining

In order to confirm the expression pattern of CKY-1, the 10H8 monoclonal antibody, which recognizes AHA-1 (Jiang et al. 2001), was used to stain the *cky-1:aha-1* rescued worms. Compared to the wild type staining, in which AHA-1 is expressed in most somatic cells, AHA-1 is only expressed in the pharynx of the *cky-1:aha-1* rescued worms (Figure 2).

Rescue assays

Previous studies demonstrated that *aha-1* genomic sequence or *cky-1:aha-1* can rescue the *aha-1(ia01)* larval arrest phenotype (Jiang 2002). A former graduate student, Huaqi Jiang, generated two other *aha-1* expression constructs. One was used to test whether expressing *aha-1* only in pharyngeal muscle cells can rescue the larval arrest phenotype. The construct was *myo-2: aha-1,* in which the promoter of the *myo-2* gene was fused to the *aha-1* coding sequence. This construct should express AHA-1 protein only in the pharyngeal muscle cells. I did the rescue assay using 3 different lines (table 2). The worms that have same strain name in the table were different worms from same line. There was no rescued worm detected. The rescue efficiency of the *cky-1:aha-1* construct in about 10%. Based on the number of worms screened, if pHJ36 can rescue the *aha-1(ia01)* loss-of –function phenotype and the rescue ratio is 10%, there should be about 12 *aha-1(ia01)/ aha-1(ia01)* worms carrying arrays been rescued. The other construct was *T01D3.2: aha-1*, in which the T01D3.2 promoter was fused to the *aha-1* coding sequence. This construct was predicted to express AHA-1 only in two interneurons in the head. I didn't detect any rescued worm. The results are summarized in Table 2.

Table 2. m*yo-2:aha-1* and *T01D3.2:aha-1* rescue assay.

Construct name	promoter	Expression pattern	Strain name	Transmission frequency	# of rescued/ # of assayed adults
PHJ36	*myo-2*	Pharynx muscle cells	ZG113	52.1%	0/23
			ZG114	65.0%	0/146
			ZG114	37.4%	0/198
			ZG115	49.5%	0/214
			ZG115	52.0%	0/182
PHJ38	*T01D3.2*	Two interneurons in head	ZG115	64.0%	0/32
			ZG115	73.1%	0/52
			ZG116	94.9%	0/39

Note: # of assayed adults indicates all the worms that could develop to adulthood and give rise to progeny.

DISCUSSION

cky-1 has essential function in *C.elegans* development

The RNAi data indicates that *cky-1* has essential functions in *C.elegans* development. In the RNAi experiment, by the end of the third day, there were 94.5% (n=636) of the *gfp* RNAi treated worms grew to L4 stage or beyond. While in the *cky-1* RNAi treated group, the number was only 24.6% (n=862). *cky-1* RNAi treated worms showed obvious delays in development. CKY-1 and AHA-1 can form DNA-binding heterodimers *in vitro* (Jiang 2002). This suggests that CKY-1 might also interact with AHA-1 *in vivo,* and that the complex has functions in *C.elegans* development. The *cky-1:aha-1* rescued worms can develop to the adult stage and give rise to progeny, but the worms grow slower than wild type. This can be explained by two reasons:

i). Expression of AHA-1 only in the pharynx is not enough for normal growth. AHA-1 is also needed in other tissues or pathways during development.

ii). Extrachromosomal arrays are not as stable as chromosome. Different cells may get different copies of arrays. Mosaic expression in the pharyngeal cells may cause insufficient dosage of AHA-1 in some cell types and affect normal functions.

Cuticle defects

The slow-growing *cky-1* RNAi worms were not as healthy as the worms in the control group. Generally they had pale intestine and moved slowly. 74% of such slow-growing worms had cuticle defects. The newly hatched RNAi treated worms did not have this phenotype. The cuticle defects were not like those of the *aha-1(ia01)* worms. The typical phenotype of these defects was that there is a small piece of cuticle still attached to the worms' mouth. It looks like molting defects. In *C.elegans*, there are four molts prior to adulthood. Each larval stage is characterized by stage-specific cuticle formation, including the expression of distinct sets of collagen genes. The heterochronic genes control the timing of the developmental events including molting (Ambros V, 2000). These molting defects in *cky-1* RNAi worms maybe related to the heterochronic pathway. It is possible that CKY-1 interacts with some proteins in the pathway or *cky-1* controls some target genes that are involved in the molting event. So *cky-1*-defective worms have molting defects.

Rescue assay

A DNA construct containing the *aha-1* genome sequence can rescue the *aha-1(ia01)* larval lethality phenotype. But the brood size and growth rate is smaller and slower than the wild type. This maybe because the expression pattern of AHA-1 is mosaic, so in some cells, the AHA-1dosage is insufficient and therefore the corresponding complexes are not enough

for normal functions. Also, in normal growing worms, such as wild type worms or *hif-1*

mutants, the AHA-1 is normally expressed in the germline cells (Chapter 2).

Extrachromosomal arrays are not stable or expressed well in the germline (Miller et al.

1996). This may affect the cell growth and proliferation. The *cky-1: aha-1* constructs can also

rescue the mutant phenotype. This indicates *aha-1* has essential functions in the pharynx, so

only recovering its function in pharynx is enough for the viability of the mutant worms.

T01D3.2 can not rescue the loss-of-function mutant phenotype. T01D3.2 is only expressed in

two interneurons, it is unlikely that only expressing AHA-1 in two cells can rescue the arrest

phenotype. The pHJ36 construct is expressed in the muscle cells of the pharynx. Based on

the data analyzed, it can not rescue the *aha-1 (ia01)* loss-of-function phenotype. The

difference between *cky-1* and *myo-2* is that *myo-2* is only expressed in pharyngeal muscle

cells while *cky-1* is expressed in g2 gland cell, epithelial cells, marginal cells and pharyngeal-

intestinal valve cells in addition to muscle cells. It has been shown that the *kel-1* gene, which

is expressed exclusively in the g1 pharynx gland cell, and is proposed to secrete material

aiding digestion, is essential for larval development. The *kel-1* mutant has a phenotype

similar to *aha*-1 mutant. The pharynx structure is normal but the feeding is inefficient

(Ohmachi et al. 2001). It is possible that g2 gland cell, epithelial cells, marginal cells and

pharyngeal-intestinal valve cells also have functions in *C.elegans* development. Thus,

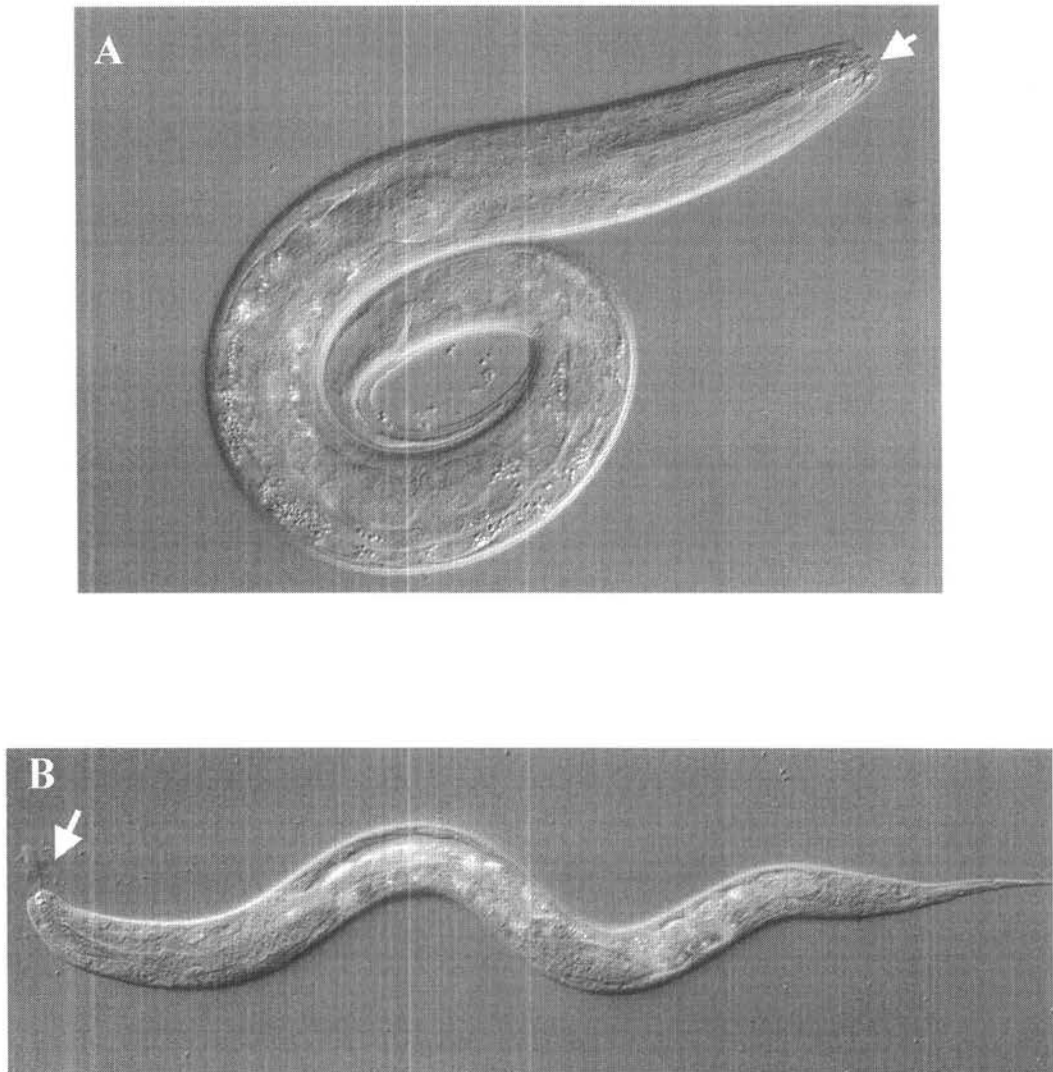without the expression of AHA-1 in these cells, the loss-of-function worms can not be
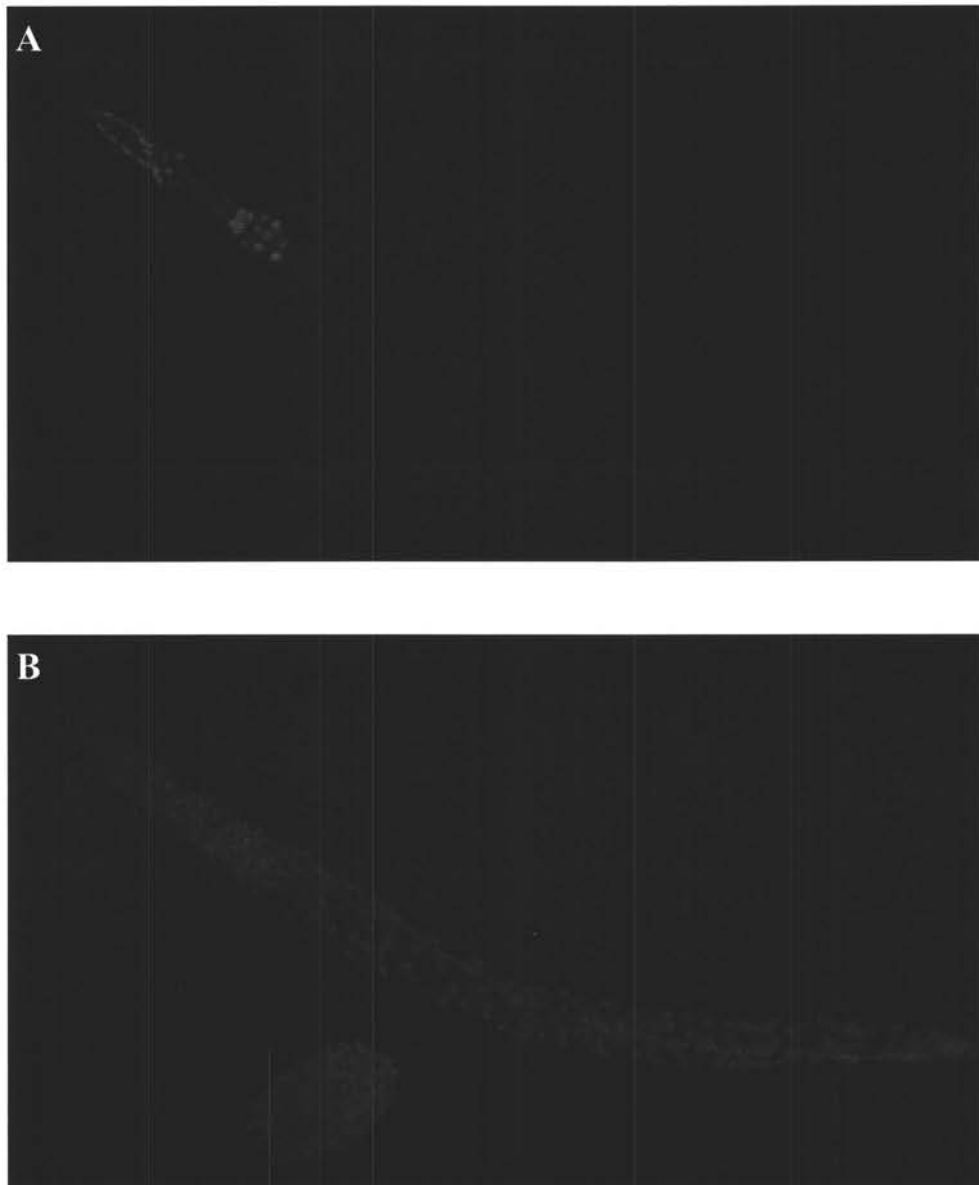
rescued.

Figure 1

Figure 2

FIGURE LEGENDS

Figure 1. Phenotypes of the progeny of *cky-1* RNAi-treated worm

    A. The worm is about 44 hrs after egg-laying. It has cuticle defect as indicated by an

    arrow in the picture.

    B. The worm has a cuticle attached to its mouth. This phenotype is like molting

    defects.

Figure 2. Immunolocalization of AHA-1 in *cky-1:aha-1* rescued worm using the monoclonal

antibody 10H8.

    A. AHA-1 is present only in the nuclei of a subset pharyngeal cells. A second stage

    larva is shown here. The anterior is at the left top.

    B. DAPI staining of A.

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CHAPTER 4

General Conclusion

SUMMARY

The *C.elegans* genome encodes five basic-helix-loop-helix PAS proteins. They are

AHR-1, HIF-1, AHA-1, CKY-1 and T01D3.2. In this dissertation, I focus on the study of two

of them: AHA-1 and CKY-1. The major findings for AHA-1 include: 1) *aha-1 (ia01)*

defective animals have small body size and cuticle defects. 2) The larval arrest of *aha-1*

(ia01) loss-of-function mutants is not due to feeding defects. 3) AHA-1 is expressed in the

germline cells in addition to most somatic cells. 4) AHA-1 can form DNA binding

complexes *in vitro*. The findings for CKY-1 include: 1) *cky-1* is required for *C.elegans*

development. 2) *cky-1* RNAi treated worms have cuticle defects. 3) Expressing AHA-1 in

pharyngeal muscle cells or two interneurons in the head can not rescue the larval lethality

phenotype.

aha-1 is essential in *C.elegans* development

aha-1(ia01) loss-of-function mutants are larval lethal. Most of the worms arrest their

development at L1 or L2 stage (Jiang 2002). I characterized the phenotypes of the arrested

mutant worms. These worms are obviously smaller than their wild type siblings. Their

intestine are pale and there are creases in their cuticle. A previous study showed that the

arrested *aha-1* loss-of-function mutants have feeding defects. There are bacteria lodged in

their pharynx (Jiang 2002). Using an experimental strategy that allows me to distinguish the

aha-1 defective mutants from the mutants rescued by *aha-1* genomic sequence, I determined

that worms do not have obvious feeding defects before arrest of development. Previous study

showed that AHA-1 is expressed in most of the somatic cells include pharyngeal cells,

intestine cells, hypodermal cells and neurons (Jiang, 2002). I detected that AHA-1 is also

nuclear enriched in germline cells in both wild type worms and *hif-1* defective worms. It has

been shown that AHA-1 can form DNA-binding complex with AHR-1, HIF-1 and CKY-1 *in*

vitro (Powell-Coffman et al. 1998; Jiang et al. 2001; Qin and Powell-Coffman submitted,

2003). I performed an electrophoretic mobility shift assay and demonstrated that AHA-1 can

form DNA-binding homodimers *in vitro*. It is not clear whether AHA-1 homodimers have

any *in vivo* functions.

cky-1 has functions in *C.elegans* pharynx

cky-1 double-stranded RNA mediated interference was performed. At first, the worms

used in this experiments were wild type and *aha-1(ia01)/lin11(n566)*. Different methods,

including feeding, soaking and injection, were used to introduce double-stranded RNA into

worms. Also, different stages of worms, including embryos, L1 larvae, L2 larvae, L3 larvae,

L4 larvae and adults, were used for RNAi treatment. Each treatment was repeated for at least

twice. There were no obvious differences between the experiment group and the control

group (*dsgfp* RNAi-treated). The negative results indicated that *cky-1* is a gene that is

resistant to RNAi treatment. When RNAi hypersensitive strain (*rrf-3*) was available, RNAi

experiments were performed by injection. The progeny of the *dscky-1* RNAi-treated worms

showed obvious delays in development compared to the control group (*dsgfp* RNAi treated).

cky-1 RNAi arrested worms had cuticle defects. These data indicated that *cky-1* has an

essential role in *C.elegans* development. CKY-1 can form DNA-binding complexes with

AHA-1 *in vitro*, and the chimeric gene, in which the promoter of *cky-1* directs the expression

of AHA-1, can efficiently rescue the *aha-1(ia01)* mutant phenotype. The rescued worms can

develop to adults and have progeny (Jiang 2002). The data above indicated that CKY-1

might interact with AHA-1 *in vivo* and the complexes regulate some target genes in the

pharynx. The AHA-1 antibody staining of *cky-1* rescued worms showed that *cky-1* is

expressed in pharyngeal cells. Two other constructs, pHJ36 and pHJ38, were generated for

rescue assays. PHJ36 contained a chimeric gene *myo-2:aha-1,* in which a *myo-2* promoter

directs the expression of AHA-1 protein. AHA-1 should be expressed only in the pharyngeal

muscle cells. The other construct pHJ38 contained a chimeric gene *T01D3.2: aha-1*, in which

the T01D3.2 promoter directs the AHA-1 expression. AHA-1 should be expressed only in

two interneurons in the head. Both of these two constructs could not rescue the *aha-1* larval

lethal phenotype.

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