Biochemical and Genetic Characterization of *Caenorhabditis elegans* Homeodomain protein DVE-1

Role of epigenetics in *C. elegans* Development and Aging

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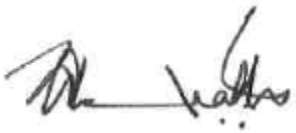
CERTIFICATE

This is to certify that this dissertation entitled **"Biochemical and Genetic Characterization of *Caenorhabditis elegans* Homeodomain protein DVE-1: Role of epigenetics in *C. elegans* Development and Aging"** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by Miss. Kavya Leo Vakkayil at Indian Institute of Science Education and Research(IISER Pune) under the supervision of Dr. G P Manjunath, Scientist-C, Biology department, Indian Institute of Science education and Research (IISER PUNE) during the academic year 2014-2015.



Signature of the Supervisor
Dr G P MANJUNATH

Date:25-3-15

DECLARATION

I hereby declare that the matter embodied in the report entitled **"Biochemical and Genetic Characterization of *Caenorhabditis elegans* Homeodomain protein DVE-1: Role of epigenetics in *C. elegans* Development and Aging"** are the results of the investigations carried out by me at the Department of Biology, Indian Institute of Science education and Research (IISER PUNE) under the supervision of Dr. G P Manjunath, Scientist-C, Indian Institute of Science education and Research (IISER PUNE) and the same has not been submitted elsewhere for any other degree.

Signature of the Student Date:25-3-2015

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ABSTRACT

Insulin/Insulin like growth factor signaling (IIS) has been implicated in several models of organismal aging. The mechanistic details of this pathway as well as its role in regulating lifespan have been established conclusively over the last two decades. Recent studies have indicated that chromatin-organizing molecules play an important role in regulating life span via IIS pathway. We have identified a role for *C. elegans* Homeodomain protein Defective Proventriculus (DVE-1) in regulating transcription in response to IGF signaling. Experiments reported in this document focuses on DVE-1, and its potential role in regulating the lifespan via IGF signaling pathway in *C. elegans*.

Our results indicate that DVE-1 is expressed in all larval (L1, L2, L3, L4) and adult stages in *C. elegans*. The protein is expressed more in the adult stage when compared to the larval stages. The bioinformatics analysis on transcription factors that bind to 2000 bases of the 5' UTR of *dve-1* showed hits for those transcription factors, which are important players in insulin signaling indicating DVE-1 levels may be regulated by *DAF-2* signaling. An interaction network was also constructed using co-expression, physical interaction and common biological functions as criteria. The interactome revealed several factors known to participate in *DAF-2* signaling, further confirming the participation of DVE-1 in calorie restriction mediated regulation of lifespan. Abrogation of DVE-1 activity by using a dominant negative allele resulted in increase in the lifespan of *C. elegans* by 40% when compared to the control animals, confirming DVE-1 as a suppressor of *DAF-2* signaling.

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1. INTRODUCTION

Calorie restriction as a regime to regulate organismal life span has been known to be effective across a wide range of model organism including humans. Insulin and Insulin like growth factor (IGF) mediated signaling is one of the primary sensors of metabolic flux in several model organisms. It is often invoked during regulation of organismal life span by calorie restriction.

Extensive research over the past two decades indicates that calorie restriction mediated increase in life span is caused by the down regulation of the IGFR mediated signaling. The down regulation of the IGFR pathway prevents phosphorylation of the FOXO family of transcription factors by both Akt/PI3K and JNK pathways. While there does not seem to be a universal mechanism for the phosphorylation mediated localization of these transcription factors, it is observed in most cases that phosphorylation leads to the retention of these factors in the cytosol due to their association with protein chaperones. The loss of the phosphate groups on the Foxo's leads to the disruption of their association with protein chaperones followed by their translocation into the nucleus(Gami & Wolkow, 2006).

In *C. elegans, the* IGF pathway is mediated by a receptor called DAF-2. Ligand binding to DAF-2 induces a cascade of phosphorylation events that lead to retention of a FOXO family transcription factor DAF-16 in the cytosol. DAF-16 binds to a common motif in the promoter element of *several* genes, called the DAF-16 binding element (DBE) TTGTTTAC. Down regulation of IGF pathway either due to calorie restriction or ablation of DAF-2 either by siRNA or by gene deletions results in the down regulation of protein kinase activity resident downstream of DAF-2(Gami & Wolkow, 2006) The resultant translocation of DAF-16 into the nuclease results in enhanced life span. The insulin/IGF1 pathway exerts its effect on life span by up regulating genes that involve cell stress response, anti-microbial genes, fat and steroid hormone synthesis and by down regulating genes whose activity causes shortening of life span. Although insulin signaling happens to be one of the primary cues for regulating life span, the neuronal network and the germ cells play a role. In *C. elegans* ablation of sensory neurons results in the long lived phenotype that is obtained by CR or knockdown of

daf2(Wiesner, 2013). A similar effect is obtained by the ablation of germ cells(Berman & Kenyon, 2006). Organisms that show enhanced life span are also observed to be more resistant to tumor formation and reactive oxidative species (ROS) as compared to their wild type counterparts. Taken together these observations indicate an integrated and evolutionarily conserved pathway for regulating life span. In summary, it is now generally accepted in the area of ageing that primary control of ageing resides in the brain, which receives its cues from nutritional status of the organisms. Based on the nutritional status the reproductive functions of the organism are regulated. Once the organism has fulfilled its reproductive functions, increased susceptibility to DNA damaging agents and tumor formation, induces aging and death(Wiesner, 2013).

1.1 IGFR Signaling in *C. elegans* and higher order chromatin architecture:

The list of genes that are effected by and effect life span extension, in both *C. elegans* and mice is rather extensive and now includes sirtuins, *skn-1*, Pha4/Foxa AMPK, RHEB, daf16/Foxa1 and *hsf-1*. The coordinated expression of several genes that regulate aging, suggests that in addition to transcription factors, this phenomenon must also involve the participation of proteins that regulate higher order chromatin architecture. Of the several proteins that have been studied as part of the mechanism that regulates aging, a few show very strong correlation with aging and associated reduction in disease burden. A microarray survey reveals that along with 40 other transcription factors CBP and its co factors are induced by nutritional deprivation in mouse hypothalamus. Consistent with the fact that nutritional deprivation is often correlated with increased life span, hypothalamic expression of CBP and SATB1 display a strong and positive correlation of 84% and 81% respectively with lifespan variance in five strains of mice. The corresponding study involving worm analogs of these proteins in *C. elegans* using RNA interference corroborates these results. Expression of both CBP and SATB1 can be used a marker for age, is reduced with aging and diabetes and can be induced by CR and ablation of *daf2* activity. In addition, down regulation of CBP protein and CBP interacting factors including worm homolog of SATB1, DVE-1, by RNA interference results in suppression of extended lifespan resulting from ablation of DAF-2 activity. Ablation of histone deacytylase by RNAi or inhibition of its activity by

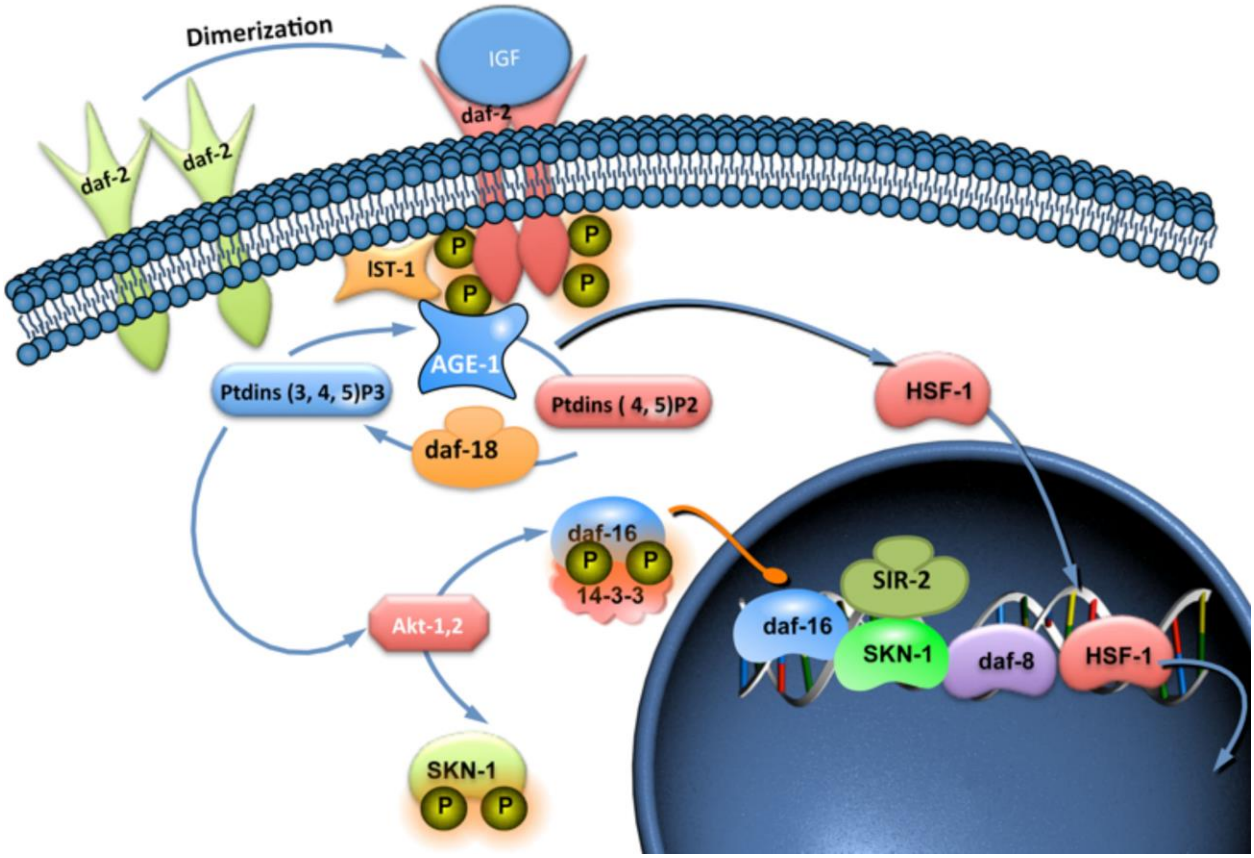


Figure 1: IGFR signaling pathway in C. elegans. Binding of Insulin like growth factor (IGF) to its receptor IGFR (DAF-2) results in DAF-2 self-phosphorylation and dimerization and to the recruitment of the phosphatidylinositol 3-kinase AGE-1 and the insulin receptor substrate 1 orthologous IST-1. AGE-1 results in the generation of phosphatidylinositol-3, 4, 5-triphosphate (Ptdins (3, 4, 5) P_3) that activates AKT family. The phosphatase DAF-18 antagonizes AGE-1. In the presence of IGF, the combined action of AGE-1 and DAF-18 results in phosphorylation of Forkhead family transcription factor DAF-16 that stabilizes its interaction with protein chaperone 14-3-3, resulting in its retention in the cytoplasm. Inhibition of IGF binding to IGFR prevents phosphorylation of DAF-16 and causes its dissociation from 14-3-3, thus allowing DAF-16 to translocate into the nucleus. DAF-16 binds to a conserved sequence called the DAF-16 binding element (DBE) also called the Insulin responsive sequence (IRS) in higher eukaryotes and up regulate genes responsible for enhanced lifespan.

(HDAC) inhibitors that inhibit histone acetylation levels are able to enhance life span and delay age related pathology. Further studies have shown that sir 2.1 functions upstream of daf-16 and enhances lifespan in *C. elegans*(Tissenbaum & Guarente, 2001) *daf-2* mutation also causes increased expression of CBP-1 (creb binding protein) in *C. elegans*, a histone acetyl transferase that enhances lifespan by chromatin modifications. Inhibition of four genes namely *cbp-1, daf-16, hsf-1* and *dve-1* reduced the viability of adult worms. DVE-1 plays an important role in attenuating the lifespan extension in *daf-2* mutants ((Zhang et al., 2009) indicating that DVE-1 regulates lifespan via a crosstalk with insulin signaling pathway.

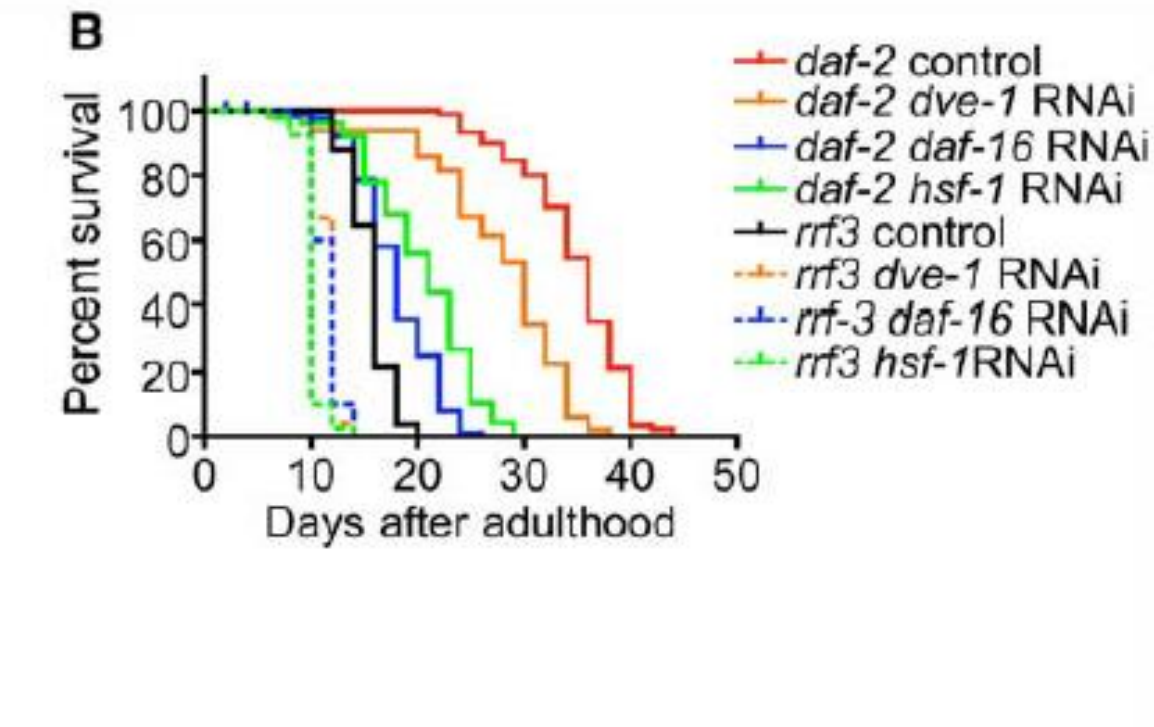


Figure 2: Inhibition of CBP-interacting factors attenuates lifespan extension by bacterial dietary restriction. (A) *daf-16*, *hsf-1*, and *dve-1* RNAi attenuate lifespan extension by bDR. **(B)** *daf-16* and *hsf-1* but not *dve-1* RNAi blocks lifespan extension by the *daf-2* mutation (Image from Zhang et al., PLoS Biology (2009))

1.3 Biological functions of DVE and DVE-1:

dve-1 via alternative splicing encodes three proteins with a COMPASS (CMP) domain and two homeodomains.(Nakagawa et al., 2011) DVE-1 interacts with two or more components of the EGF/RAS signaling pathway, and is required for such signaling in vulval development. The CMP domain is found in the N-terminus of SATB-class CUT Homeodomain proteins namely the human SATB1 and in *Drosophila* Defective Proventriculus (DVE).It is proposed that DVE-1 may be orthologous to these proteins. Both DVE and DVE-1 encode two highly divergent homeodomains that are homologous between species, which might be a consequence of intragenic duplication. Mutant phenotype analysis has shown that DVE-1 is required for larval development, vulval development and reproduction. Loss of Defective ventriculus-1 activity leads to defects during both worm and fly development.

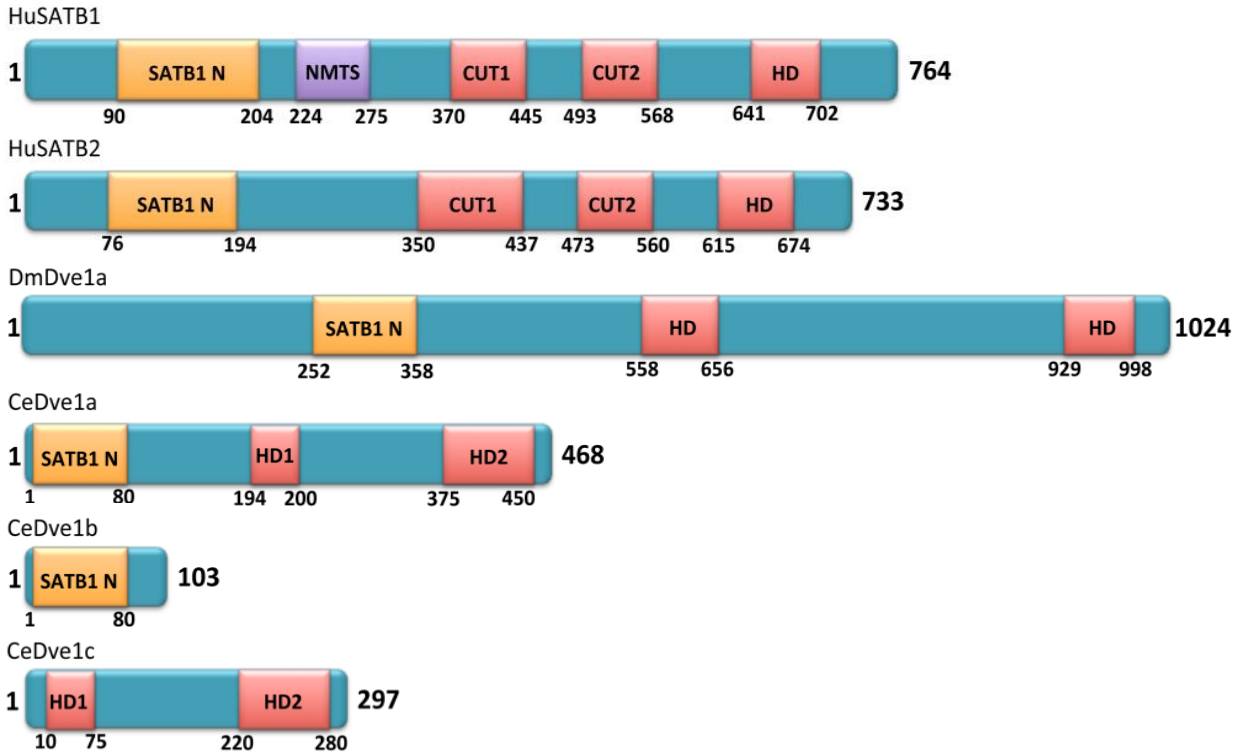


Figure 3: Domain organization in SATB family proteins. Both mouse and human SATB proteins contain both CUT and homeodomains, the CUT domain is absent in the fly DVE and worm DVE-1. The most conserved region is present in the N-terminal, which has been found to be essential for protein-protein interaction.

A large number of studies have been done to reveal the roles of DVE in *D. melanogaster*. DVE is expressed in ventral ectoderm and midgut. The expression is dependent on differential EGFR signaling(Shirai et al., 2003).The expression of DVE in proventriculus and gut is activated by Wingless whereas Dpp activates DVE and helps in development of middle mid-gut (Nakagoshi, Shirai, Nabeshima, & Matsuzaki, 2002). DVE also suppresses joint formation whereas Notch inhibits DVE expression leading to joint formation in drosophila leg(Shirai, Yorimitsu, & Kiritooshi, 2007).The protein also plays a role in specifying ocellar region in *Drosophila* head (Yorimitsu, Kiritooshi, & Nakagoshi, 2011).DVE acts as a node in regulating rhodopsin expression (Jr et al., 2011).*Drosophila* genome project have identified two transcripts namely type A (4.9kb) and type B (3.5kb) from the *dve* gene locus. The middle midgut expresses DVE-A and proventriculus expresses DVE-B during embryogenesis. The protein plays an important role in male accessory gland development which caused enhanced fecundity in Drosophila (Minami, Wakabayashi, Sugimori, Taniguchi, & Kokuryo, 2012).Recent studies have shown that the ortholog of DVE in *C. elegans* namely DVE-1 plays an important role in mitochondrial unfolded protein response. Upon mitochondrial stress, a complex is formed between DVE-1 and UBL-5 which binds to the promoter region of chaperones namely *hsp60* and *hsp6* (Broadley & Hartl, n.d.)It has also been found that DVE-1 regulates the expression of *mab-9* T box gene.

1.4 Relevance and aims of the present study:

Among the chromatin factors that play crucial role in *C. elegans,* only a few including PHA4 and SWI/SNF have been elucidated to play a role in IGF signaling (Cui, Han, & Hughes, n.d.) Even though this study proposes DVE-1 as a chromatin organizer that regulates lifespan via IGF signaling the mechanistic details of DVE-1 function remain largely unknown. Identifying small molecules involved in these pathways and their characterization have a larger significance as these pathways are involved in even diseases like cancer and diabetes. Biochemical and genetic characterization of proteins that crosstalk with IGF signaling and identifying functional homologs in mammals will have implications in developing drugs and thereby to achieve pharmacological

manipulation of lifespan (Gami & Wolkow, 2006). The primary experimental objectives of this study are

1. Identification of the Spatio-temporal expression pattern of DVE-1.
2. Codon optimization, cloning and purification of DVE-1.
3. Identification of physical, functional and genetic interactions of DVE-1

2. MATERIALS AND METHODS

2.1 Materials

Oligonucleotides were obtained from SIGMA and Integrated DNA technologies. Restriction endonucleases, DNA polymerases and ligase were obtained from New England Biolabs (NEB). Fine chemicals were obtained from SIGMA, Invitrogen and Thermo Scientific.

2.2 *C. elegans* strains and culture conditions

All strains were obtained from Caenorhabditis Genetics Center (CGC) at University of Minnesota. The worms were maintained on *E. coli* strain OP50 (*ura*$^-$) on Nematode growth medium plates (NGM). The worms were maintained at specified temperatures (15°C-25°C) depending on the genotype of the worm strain. Further details of the strains can be found in appendix A.

2.2.2: Preparation of NGM plates:

	Components	**Amount to be added for 1 liter of NGM medium**
1	Distilled Water	975ml
2	NaCl	3g
3	Agar	17g
4	Peptone/Tryptone	2.5g
5	IM $CaCl_2$	1ml
6	5mg/ml cholesterol in ethanol	1ml
7	IM $MgSO_4$	1ml
8	1M KPO_4 buffer	25ml
9	Nyastatin	1ml

The components 1-4 were added in an Erlenmeyer flask. The media was autoclaved and cooled it by keeping it in 55°C water bath for 15 minutes. Components 5-9 were

added subsequently and the mix poured (30 mL in each plate) into 100mm Petri plates. The plates were kept at room temperature for overnight drying or at 37°C for 8 hours.

2.2.3 Seeding NGM plates:

The NGM plates were seeded using *Escherichia coli* strain OP50. 200µL of bacterial suspension was added using a pipette and spread using a glass rod. Necessary care was given not to spread the lawn to the edges. Bacteria were killed by exposure to UV light for 2 minutes using a UV Crosslinker. The plates were kept at room temperature for overnight drying or at 37°C for 8 hours.

2.3 Assay for lifespan

Lifespan was assayed on solid medium according to published protocol. (Sutphin and Kaeberlein, 2009). Briefly worms were seeded on *Escherichia coli* seeded on nematode growth medium in the presence of FUDR. Live worms were counted on every alternate day. Survival curves were constructed using Kaplan meier analysis.

2.3 Cloning and purification of DVE-1

ORF for CeDVE-1 was amplified using cDNA prepared from RNA isolated using mixed stage worm cultures. Codon usage analysis was performed using JCAT (http://www.jcat.de/), an online tool for codon usage analysis. The complete coding sequence was assembled using overlapping oligonucleotides. The Overlap Extension PCR (as explained in 2.3.1) was performed using KOD Hot Start DNA polymerase (Cellbiosis).The ORF was fused to Sumo Tag using OEP and cloned into bacterial expression vector pThioHisA (Novagen). Expression of fusion protein was verified in E. coli expression strain DH5-α. DH5-α cells were transformed with plasmid pMGCE1 harboring codon optimized CeDVE-1 fused to Sumo. Heterologous protein expression was induced by addition of 1mM IPTG to the bacterial culture. Cells were harvested after 6 hours of growth, suspended in lysis buffer (Refer 2.3.2) and sonicated by a probe

sonicator (Refer 2.3.3). Soluble protein mixture was resolved on a 10% SDS PAGE (Refer 2.3.4).

2.3.1 Overlap Extension PCR:

Primers were designed to bridge the necessary parts. Two primers that are compliment of one another are used. The end primers used have restriction sites. The necessary fragments are amplified via extension PCR that were purified using a DNA purification column. The overlap PCR will help to anneal the purified fragments. Purification PCR is carried out after adding end primers. The correct size fragments were gel extracted and cloned into desired vector.

2.3.2 Purification of DVE-1:

Codon optimized *C elegans* DVE-1 fused to S. cerevisiae SUMO expressed as His Tag fusion using pET28a. The protein was found to be present in pellet when induced with 1mM IPTG at 37C. Protein Induction was carried out at 18°C using auto-induction media. Approximately half of total protein was found to be present in soluble fraction.

Protein purification was performed as follows; Bacterial pellet was resuspended in lysis buffer (50mM TrisHCl pH8, 150 mM NaCl, 1X Protease Inhibitor mix) (4 mL per gram of wet cell paste). Cells were lysed by sonication and cell lysate was clarified of debris by centrifugation at 37000 rpm at 4°C for 45 minutes in Ti45 Rotor. Sumo-DVE-1 was precipitated from the solution using saturated ammonium sulphate to a final saturation of 30%. The ammonium sulphate pellet was dissolved in 50 mL of Ni-NTA loading buffer, (20mM NaPO4, 100mM NaCl, 5% Glycerol, 1mM 2 mercapto-ethanol). The protein sample was dialysed against the loading buffer for 12 hours with two changes. The dialysate was loaded on a Ni-NTA column (Tricorn XK 10/100). Protein was eluted with a linear gradient of 20-200mM Imidazole. Fractions were checked on a 7.5% SDS-PAGE and fractions containing DVE-1 were pooled and loaded on Resource Q. The bound protein was eluted using a linear gradient of 100 to 750 mM NaCl. Sumo DVE-1 eluted as a single peak. 5 mL of the Resource Q eluate was loaded on a Sephadex-75

column and eluted with a flow rate of 1 mL per minute. The fractions were checked on a 7.5% SDS-PAGE. A single band corresponding to a protein with a molecular weight of approximately 60 kDa (Sumo-DVE-1) was visible.The protein was found to be prone to cleavage into two fragments of approximately equal molecular weight.

2.4 Standard Worm protocols:

2.4.1 Synchronization of worm cultures

The eggs were collected using the standard hypochlorite treatment of gravid worms. The adult worms were flushed using 4ml of M9buffer and were collected in 1.5ml micro centrifuge tubes. The hypochlorite solution was prepared (0.6ml sterile water, 0.5ml 4% bleach and 0.4ml of 5N NaOH) and 500microliter was added to each of the tubes. Each tube was agitated on a vortex mixer for 10minutes with 2 min interval and centrifuged at 3200 rpm for 30 seconds. The supernatant was discarded leaving 100microliter to which 1ml sterile water was added. This was again centrifuged at 3200rpm for 30seconds.The pellet was suspended in M9 buffer and the eggs were spotted on fresh NGM plates.

2.4.1a Composition of M9 buffer:

Distilled water	999ml
KH_2PO_4	3g
Na_2HPO_4	6g
NaCl	5g
1M $MgSO_4$	1ml

The buffer was autoclaved before use.

2.4.2 Glycerol stocks preparation

The glycerol stocks were prepared using soft agar freezing solution protocol. (Stiernagle, 2006). The soft agar freezing solution was melted and cooled at 50degree Celsius for 30 minutes.2-3 plates of freshly starved L1-L2 animals were washed using 0.6ml (per vial of stock) of S buffer and kept in ice for 15minutes in a cryovial..0.6ml of soft agar freezing solution was added and mixed. The cryotubes were placed in a small

Styrofoam box with slots for holding micro tubes after filling ¾ of the box with isopropanol. The stocks were maintained at -80°C.

2.4.2a Composition of Soft Agar Freezing solution:

Water	Make up to 100ml
NaCl	0.58g
KH_2PO_4	0.68g
Glycerol	30g
1M NaOH	0.56ml
Agar	0.4g

The solution was autoclaved before use.

2.4.2b Composition of S buffer

K_2HPO_4	129ml of 0.05M stock
KH_2PO_4	871ml of 0.05M stock
NaCl	5.85g

The solution was autoclaved before use

2.5 Microscopy

The *C. elegans* strain namely SJ-4197 was used for the expression analysis. The worms were synchronized and then collected at different stages. The worms were washed using 1X PBS, centrifuged at 1500rpm (30 seconds,3 times).The worms were fixed using 4% PFA solution(45minutes,RT),washed using 1X PBS after fixing and mounted using Vectashield™ mounting solution. The prepared slides were preserved at 4°C.The Axiovision™ software was used for image analysis and digital microscopy. The images were captured using an Apo tome or confocal microscope.

2.6 RNA isolation and cDNA synthesis

Populations of N2 (Bristol) (wt) strain of *C. elegans were* synchronized using sodium hydroxide and sodium hypochlorite treatment. Worm populations were harvested at various larval (L1, L2, L3 and L4) and adult stages. Total RNA was isolated using commercial RNA isolation kits (Refer: 2.7.1) RNA quality/concentration was determined using Nanodrop™. RNA was reverse transcribed using commercial cDNA synthesis kit (Refer 2.7.2).

2.6.1 RNA Isolation:

Worms from 2-3 plates were washed using M9 medium and centrifuged at 3000rpm for 5minutes.The supernatant was removed and the step was repeated for 2times to get rid of bacteria.1ml of Trizol™ reagent was added to each 1ml and vortexed. The tubes were kept at 37°C for 2-3 hours till the worm tissue was completely dissolved. The tubes where spun at 12000g for 10 minutes at 4°C. The supernatant was transferred to a new tube and 200µl of chloroform was added. The mixture was agitated for 15 seconds and incubated at room temperature for 5 minutes. The tubes were centrifuged at 12,000g for 15 minutes and the top aqueous layer was transferred to a new micro centrifuge tube.500µL of isopropanol was added and mixed by inverting. This was again centrifuged at 12,000g for 15 minutes at 4°C. The RNA pellet was washed using 75% ethanol and centrifuged at 7500g for 5 minutes at 4°C. The pellet was dried at 37°C for not more than 15 minutes and then dissolved in 50µL of DEPC treated water at 55°C. The samples were frozen and stored at -80°C.

All plastic ware were autoclaved. The M9 medium and 75% ethanol was made in DEPC treated water.

2.6.2 cDNA Synthesis:

The RNA isolated and preserved at -80°C was kept at 55°C for 10 minutes. The concentration was estimated and 1µg of RNA was used for cDNA synthesis.

RNA (1µ gm)	xµl
Poly dT	2µl
10Mm dNTP	2µl
Nuclease free Water	12-(4+x)µl

The reaction was set for 70°C for 5minutes. After 5 minutes the following components were added:

1X Protoscript™ buffer	4µL of 5X stock
0.1mM DTT	2µL of 1M DTT stock
Protoscript™ Reverse transcriptase	1µL

cDNA synthesis was performed at 42°C for 60 minutes, followed by heat inactivation of enzymes at 95°C for 5 minutes.

2.7 *In silico* Analysis

2.7.1. Identification of Transcription Factor Binding sites (TFBs) upstream of dve-1: 2kb of genomic DNA upstream of DVE-1 TSS was obtained using UCSC genome browser. The nucleotide sequence was scanned for presence of TFBs using the nematode database of Transfac. The top ten hits were rated based on the conservation of consensus recognition sequence for nematode transcription factors.

2.7.2. Analysis of ChIP seq data for identification of Transcriptional targets of DVE-1: We analyzed the data generated by the modENCODE project to deduce sequence preference of DVE-1. These studies were done at the late embryo (8789 sequences) and L4 larval stage (7394 sequences) in a nematode strain expressing DVE-1 with a GFP tag and employed antibodies generated against GFP. We subjected the sequences from the peaks identified by the modENCODE project to MEME-ChIP, DREME, and CentriMo to identify motifs that are enriched. We rejected peaks less than 10 bases long as they are likely to be spurious and therefore biologically irrelevant.

The peaks generated by analysis of ChIP seq data were aligned to *C. elegans* genome. Coding sequences present downstream of DVE-1 peaks were identified. We were able to identify 2611 coding sequences that showed occupancy of DVE-1 and could be potentially regulated by DVE-1. These genes were clustered using common biological processes by employing Bingo and visualized using Cytoscape.

3. RESULTS

3.1 Expression pattern of DVE-1:

The spatial expression of DVE-1 was captured during different life stages of *C. elegans*. The Axiovision™ software was used for image analysis and digital microscopy. The images were captured using an Apo tome.

The expression of DVE-1 was predominantly observed in the anterior and posterior ganglion as well as along the dorsal and ventral nerve cords in all the larval and adult stages. The expression analysis was performed using Image-J software. GFP fluorescence and differential interference contrast images were acquired independently and merged using the software provided by the manufacturer. The 3D object counter plugin was used to detect the integrated density of fluorescence after adjusting the threshold.

From the preliminary observations, the amount of expression is more in the adult when compared to the larval stages in all the three regions namely anterior, posterior and middle region.

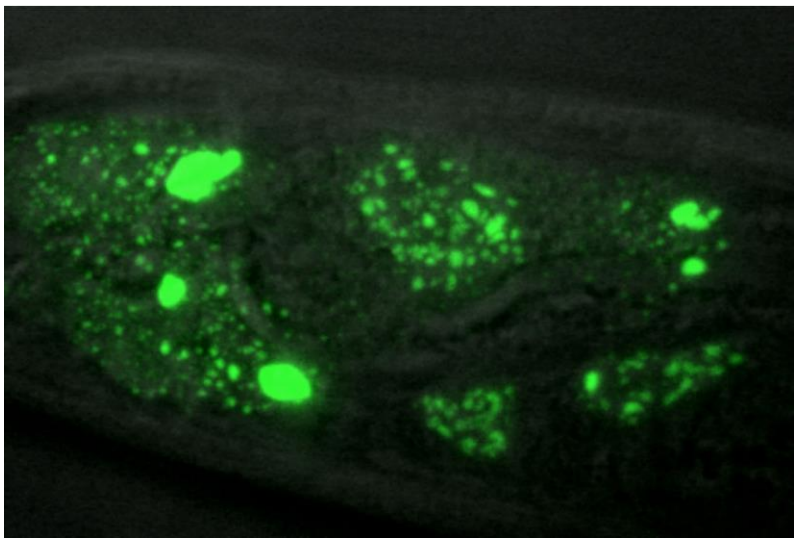
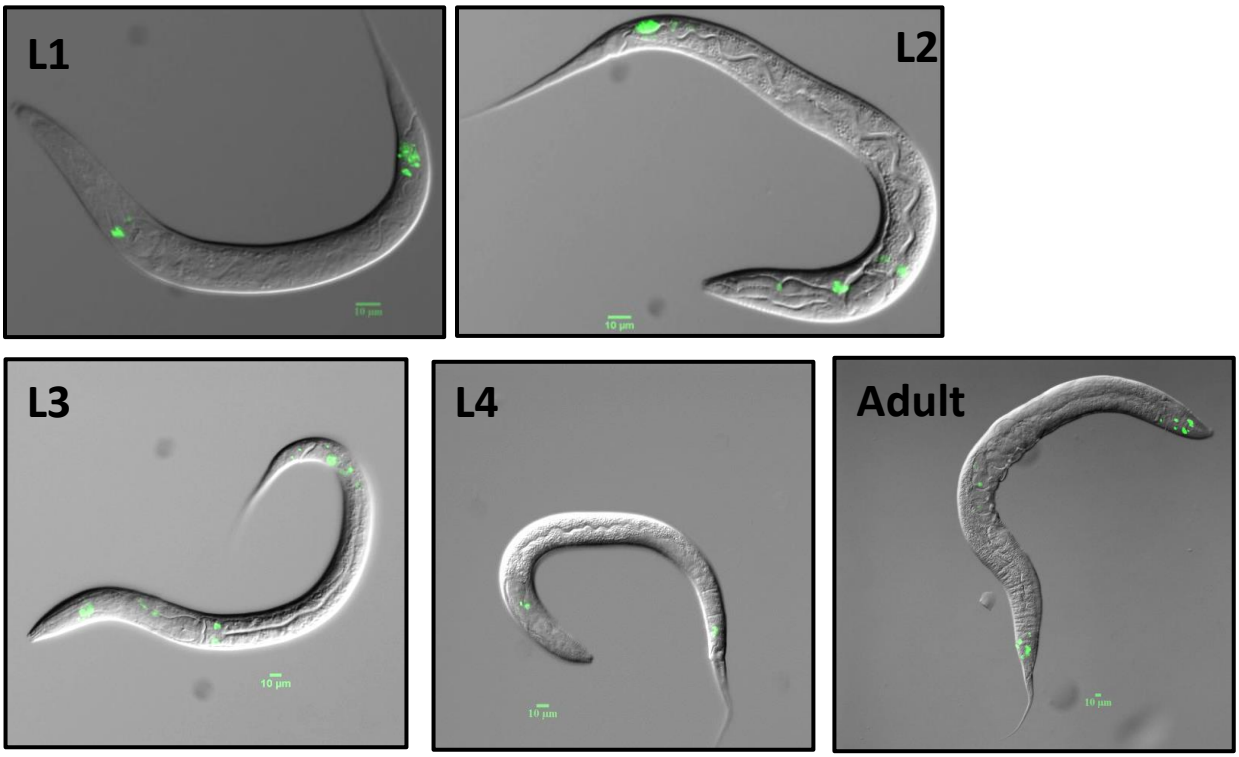


Figure 4: DVE-1 expression in the head ganglion of adult worm. The image was acquired using a Ziess Confocal microscope at a magnification of 100X. The DVE-1expression can be seen in specific subsets of cells around the head (anterior) ganglion.



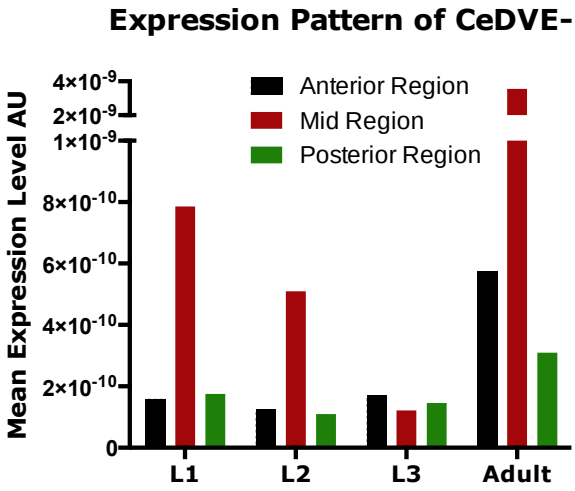
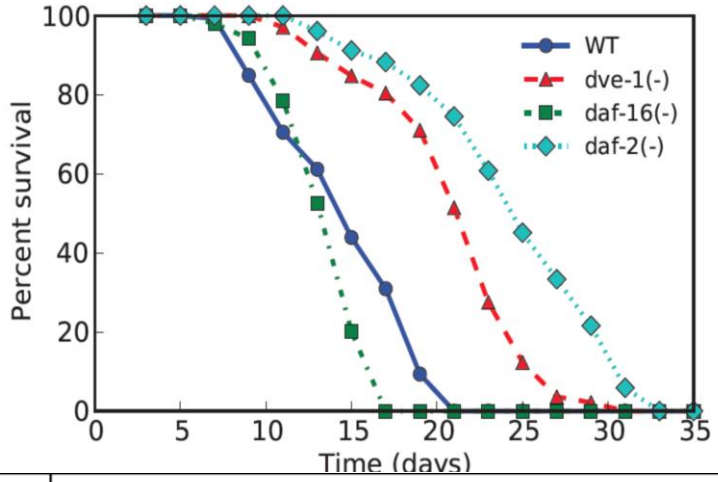


Figure 5: Expression pattern of DVE-1. Mixed population of SJ 4197 strain of *C. elegans* were synchronized and developmental stages **(A)** L1 (40X), **(B)** L2 (40X), **(C)** L3 (20X), **(D)** L4 (20X) and **(E)** Adult (10X) were imaged using apotome microscopy. The Z-stack images were captured for the head (anterior), tail (posterior) and middle

region of the worms in different life stages. **(F)** The integrated density of fluorescence was calculated and the amount of expression estimated for each stages.

3.2 Lifespan Assay (Performed by Ms Vina Tikiyani at IISER-Mohali):

Lifespan was assayed on solid media using the wild type N2 bristol strain as well as genetic loss of function mutants in *daf-16* and *daf-2*. We observed that the presence of an allele of *dve-1* with a 508 base deletion such that only the N terminal region is expressed acts as a dominant negative allele. The mean lifespan for the wild type N2 Bristol was 15 days, whereas the *daf16(-)* showed a lower lifespan of 13.87 days in agreement with published reports. *daf-2*(-) showed an increase in lifespan again in agreement with published reports. tm4803 strain that harbored a deletion in the *dve-1* loci showed an increase in lifespan with a mean lifespan of 21.42 days, indicating that DVE-1 may act as a suppressor of increased lifespan and inhibition of its activity either by a loss of function or a dominant negative allele results in alleviation of this repressive activity resulting in enhanced lifespan.



Name	Restricted Mean		
	Days	Standard Error	95% CI
wt	15.00	0.34	14.34 ~ 15.66
dve-1 (-)	21.42	0.38	20.68 ~ 22.16
daf-16 (-)	13.87	0.19	13.49 ~ 14.25
daf-2 (-)	25.51	0.48	24.57 ~ 26.45

Figure 6: Inhibition of DVE-1 function results in enhanced life span: Life span was analyzed in tm 4803 strain of *C. elegans* which harbors a truncation in the genomic copy of *DVE-1* resulting in a DVE-1 devoid of the DNA binding domains. This allele acts as a dominant negative suppressor of wild type *DVE-1* resulting in

loss of DVE-1 functions. WT N2 Bristol, *daf-16 (-)* and *daf-2* (-) strains of *C. elegans* were used as experimental controls. Lifespan analysis was performed using Online Application for Survival Analysis (OASIS) (Yang *et al.,* PLoS One (6)8) by employing Kaplan Meier test.

3.3 Expression and Purification of CeDVE-1:

Coding sequence for CeDVE-1 optimized for expression in *E. coli* was ligated into bacterial expression plasmid pET28a with an N-terminal Sumo tag. The protein showed robust expression. However the protein underwent rapid auto-proteolysis to yield two fragments of approximately equal molecular weight.

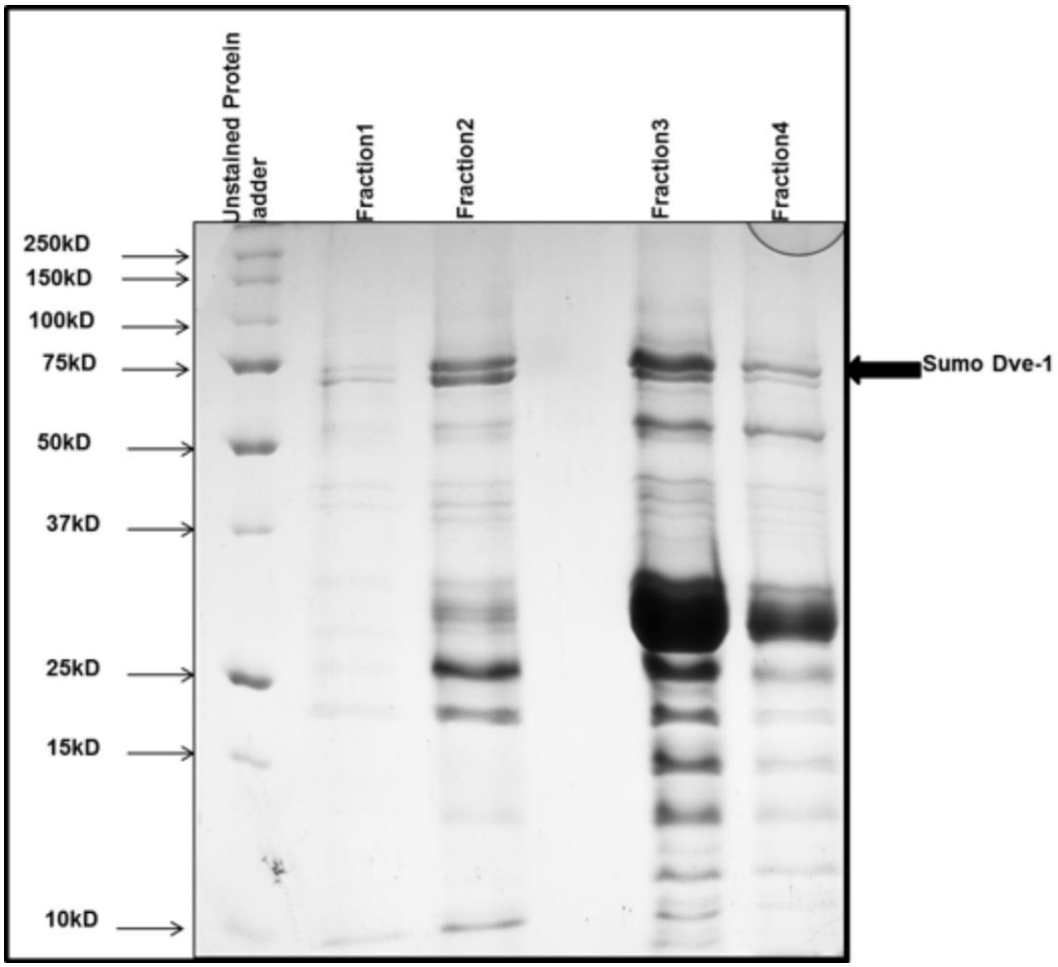


Figure 7: Expression and purification of Codon optimized Sumo: CeDVE-1. DVE-1 expressed as fusion with His-tagged yeast Sumo protein was expressed in E. coli BL21 (DE3). The protein interacted with Ni-NTA agarose and could be purified by employing affinity purification. The protein was however liable to auto-proteolytic cleavage resulting in two fragments of approximately equal molecular weight.

3.4 Identification of Transcriptional Regulators of DVE-1

The top ten transcription factors based on the conservation of their binding sites in the genomic sequence upstream of DVE-1 start site are listed in Table 1. The most conserved binding sites were that corresponding to DAF-12 and CHE-1, whereas the sites for EOR-1 and TRA-1 were most represented in the 2kb region upstream of DVE-1 TSS. In addition binding sites were also found for PHA-4 and SKN-1, transcription factors that are involved in regulating transcription in response to IGF signaling in *C. elegans.*

Transcription Factor	Binding Sequence	Identification	Number of Sites
daf-12		ChIP-Seq	1
che-1		compiled matrix imported from public database	1
eor-1		ChIP-Seq	9
pha-4		ChIP-Seq	1
Tra-1		SELEX (CASTing, SAAB, TDA, Target detection assay)	21
blmp-1		ChIP-Seq	4
efl-1		ChIP-Seq	1
lin-14		SELEX (CASTing, SAAB, TDA, Target detection assay)	1
hlh-2:hlh-19		universal protein binding microarrays (PBM)	1
skn-1		ChIP-Seq	1

Table 1: Identification of Transcription Factors that can Potentially act as Regulators of DVE-1 Expression. 2 kb of genomic sequence upstream of DVE-1 TSS was analyzed for the presence of known Transcription Factor Binding sites (TFBs) using the Transfac database of known nematode transcription factors. The top 10 hits were selected for validation by PCR amplification and generation of RNAi constructs for bacterial fed RNAi knockdown and subsequence analysis of the knockdown on DVE-1 expression.

3.5 Analysis of DVE-1 ChIP-seq Datasets and Identification of transcriptional targets of DVE-1

The results of our analysis show that DVE-1 prefers DNA sequences with a GAGA repeat and in some cases binds to palindromic sequences indicating that DVE-1 might function as a dimer or a tetramer. This is in agreement with the presence of the SATB like domain in its N-terminal (Figure 3) that is known to function as a tetramer. The closest matches to the motifs identified are those for EOR-1, GEI-1, and DAF-12. Interestingly, these proteins were also present in the list of genes identified as potential interacting partners of DVE-1 indicating that DVE-1 may bind to DNA as part of a multiprotein complex.

A major caveat of our findings, however, is that these experiments were performed using DVE-1 tagged to GFP. GFP being a large molecule is very likely to interfere with DVE-1's ability to interact with both DNA as well as other proteins. Gene ontology analysis of the proteins that were found to be potentially regulated by DVE-1 revealed that 7.1 % of the total number of genes was involved in regulation of adult lifespan (Appendix). In addition to regulation of adult lifespan DVE-1 appears to be important for *C. elegans* development (43.0 %), post embryonic development (19.8 %) and development of reproductive organs (9.0 %). Interestingly a small percent of these genes (1.4 %) were also involved in chromatin organization, a function ascribed to SATB family of proteins that we postulate to be a functional homolog of DVE-1 in higher eukaryotes.

Motif	Logo	E Value
BAGGMTTA		1.7e-236
AKACGCAG		2.1e-160
TYTCTCB		2.1e-092
TGTKTR		1.2e-071
ARCCTAAG		3.8e-061

Table 2: DNA Motifs Enriched in DVE-1 ChIP seq Datasets.16183 sequences were analyzed for the presence of DNA motifs that may correspond to the recognition motif for DVE-1. These included sequences from late embryo (8789) and L4 stages (7394) of worm development. Enriched motifs contained GAGA repeats and several displayed palindromic repeats indicating that DVE-1 may bind sequences as a dimer or tetramer. In addition to novel motifs, we also found recognition motifs for DAF-12, EOR-1 and GEI-1 indicating these proteins may be present in a complex with DVE-1.

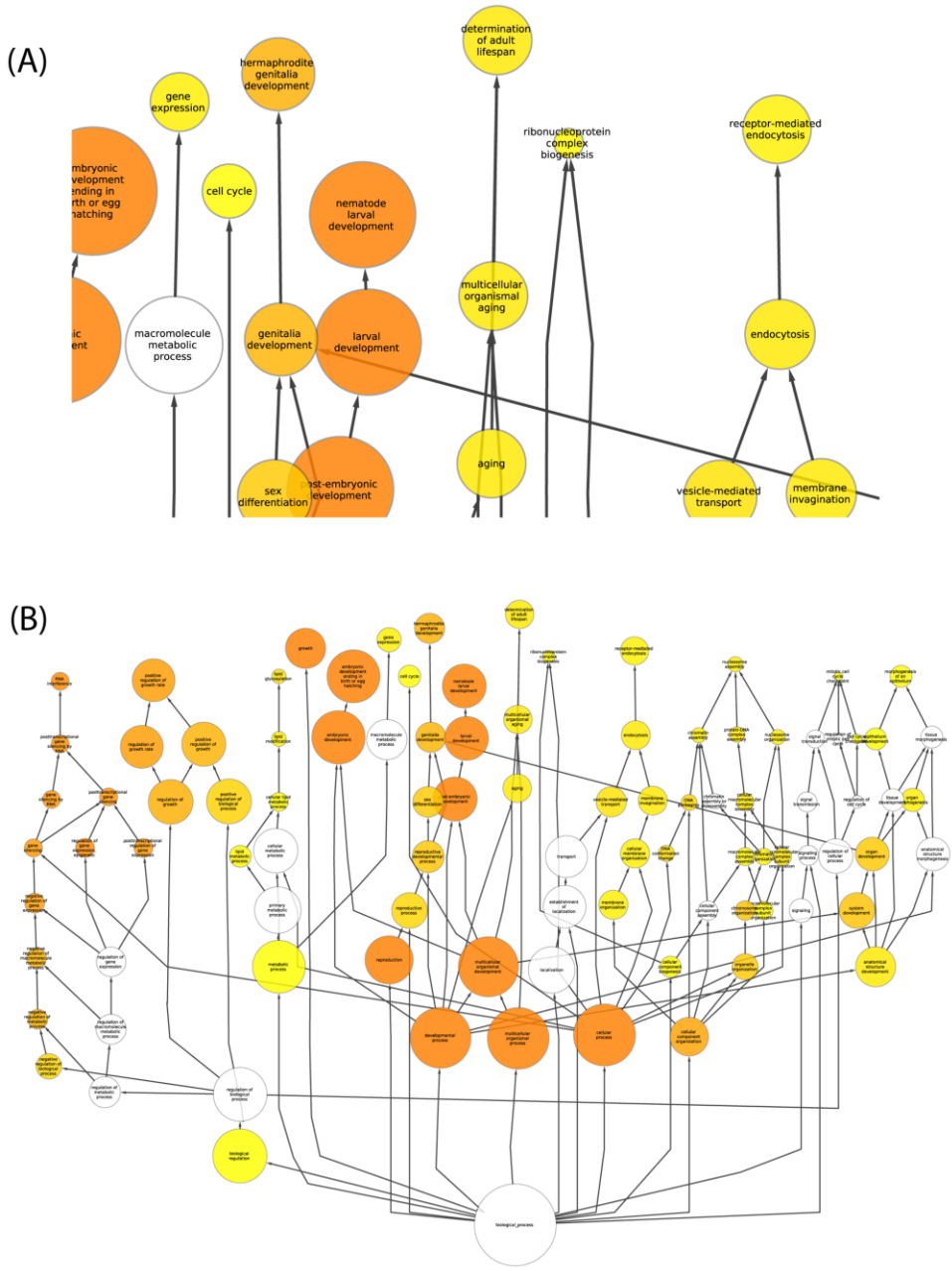


Figure 8: Gene Ontology analysis of DVE-1 Targets ChIP sequencing data was analyzed from the modEncode dataset to deduce potential targets of DVE-1. The peaks were aligned to the *C. elegans* genome and open reading frames within one kb of the peak were identified. We identified 2611 potential transcriptional targets of DVE-1. These were grouped on the basis of common biological functions and used to generate hierarchical gene ontology network **(B).** 7.1 % of the total numbers of genes identified were involved in organismal aging **(A).**

4. DISCUSSION

This study presents novel insights on the expression pattern of DVE-1 in *C. elegans*.DVE-1:: GFP expression was much predominantly in the head(anterior) and the tail(posterior) nerve ganglion followed by dorsal and ventral nerve cords. This pattern of expression was found to be consistent in all stages of worm development. However, preliminary experiments were performed at a relatively low magnification of 20X and cannot rule out expression of DVE-1 in other organs. Within the head (anterior) nerve ganglion, the expression of DVE-1 can be seen in a subset of neurons. The pattern of expression in the dorsal, ventral nerve cords, head (anterior) and tail(posterior) nerve ganglions were obtained by taking z stack images. The protein expression was higher in adults when compared to the larval stages. Further experiments will involve injection of neuron specific fluorescent markers followed by co-localization of Dve-1 with such markers. A major caveat of our findings, however, is that these experiments were performed using DVE-1 tagged to GFP. GFP being a large molecule is very likely to interfere with DVE-1's ability to interact with both DNA as well as other proteins.

This protein seems to play an important role in development as well as maintenance of nervous system as the pattern observed signifies the expression throughout the nerve cord and in the ganglions. Previous studies have shown that neurons are key players in mediating increased lifespan in *C. elegans*. Dietary restriction and insulin signaling have been well studied in *C. elegans.* It has been shown that two sensory neurons help to sense redox stress." Interestingly, in the same line the Dillin group had shown before that genetic interference with the respiratory chain specifically in neurons activates a beneficial, mitochondrial unfolded protein response in distant tissues, postulating a diffusible, however still unidentified, ''mitokine" that target a mitochondrial pro-longevity signal"(Wiesner, 2013).DVE-1 has been shown to have a well-established role in mitochondrial unfolded protein response (UFR). The neuronal expression pattern as well as its role in UFR gives a strong evidence to hypothesize DVE-1 to be the unidentified player.

2000 bases of the 5' UTR of *dve-1* was analyzed for the presence of worm transcription factors in the Transfac database. Studies have shown that DAF-12 acts upstream of DAF-16 and mediates IGF signaling.CHE-1 plays an important role in chemosensory neuron development as well as functioning.LIN14 binds directly to DNA and regulates transcription from genes that respond to insulin. The factors were involved in IGF signaling and hence it suggests that further understanding of these factors may help to understand if DVE-1 expression is regulated in an IGF dependent manner. The analysis of all top 10 hits will help to get a better understanding on players that regulate DVE-1 expression in *C. elegans*.

DVE-1 ORF was successfully codon optimized for expression in *E. coli*. A protein corresponding to an approximate molecular weight of 60kDa, corresponding to Sumo-DVE-1 fusion protein was observed in the induced lane. Further standardization of purification protocol is being attempted as this protein is unstable.

The gene ontology analysis has shown that DVE-1 interacts with genes involved in regulating adult lifespan (7%).But a large number of genes that interact with DVE-1 were involved in regulating the development of reproductive system. Recent studies have shown that reproductive system plays an important role in regulating the lifespan. Even though the mechanism by which different tissues interact with each other to regulate lifespan is unknown, it has been shown that in *C. elegans* the germ cell removal extends lifespan(Berman & Kenyon, 2006).Bioinformatic prediction of DVE-1 regulating large number of genes involved in reproductive organ development suggests that DVE-1 might be regulating lifespan via fine tuning the genes involved in development of reproductive system either by forming multiprotein complexes or via crosstalking with IGF signaling in the germ cells. Immunoprecipitation studies using DVE-1will help in further understanding the partner proteins to which DVE-1 may bind.

Due to the paucity of information about DVE-1, the exact mechanism by which this protein participates in IGF signaling is unclear at this point of time. As a first step, we tried to study the role of DVE-1 in *C. elegans* biology as well as characterize its expression pattern and biochemical properties. We propose a hypothesis on how

SATB1/DVE-1 may participate in the insulin signaling pathway. In the presence of IGF the transcription factors associated with insulin signaling remains in the cytoplasm.DVE-1 along with other proteins repress transcription from IRS loci at this point. In the absence of IGF binding to its receptor DAF2, DAF16 (the FOXO homolog in *C. elegans*) dissociate from chaperons which sequester them in the cytoplasm. This causes nuclear localization of DAF-16, which displaces DVE-1 and signals the transcription of genes that results in enhanced lifespan.

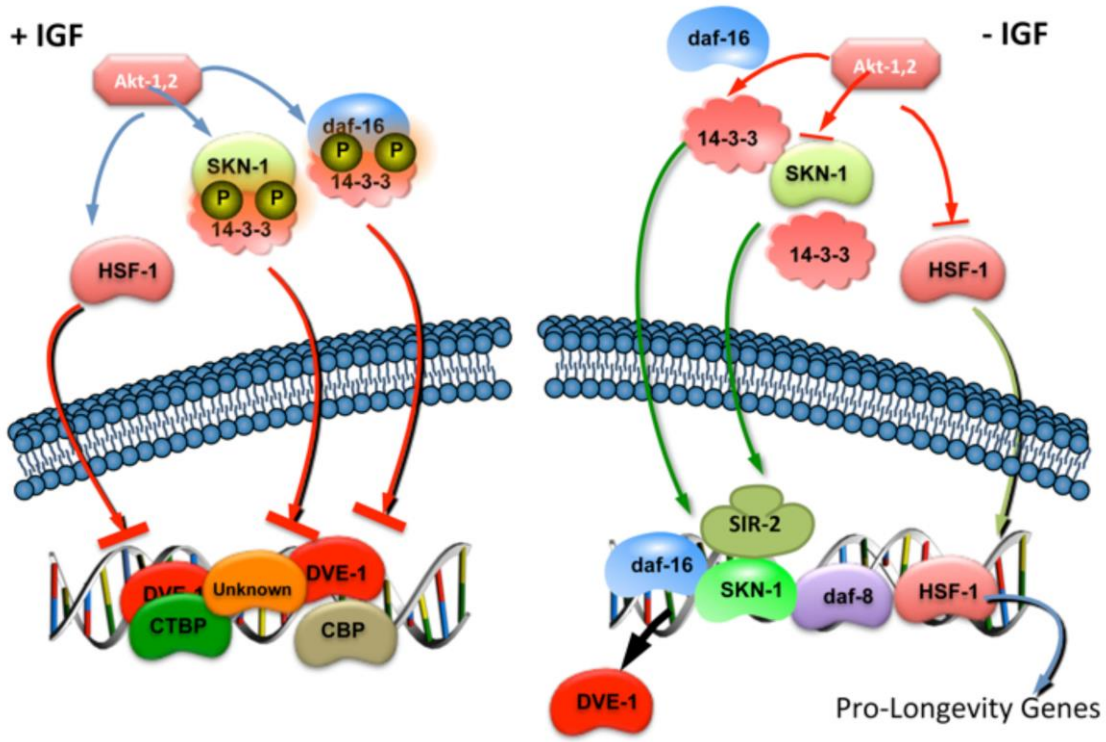


Figure 9: Working Hypothesis for the Role of SATB/DVE-1 in IGF signaling: In the presence of IGF, transcription factors associated with IGF signaling are retained in the cytoplasm. At the same time the IRS loci are occupied by DVE-1 and associated repressor proteins preventing any transcription from such loci. In the absence of IGF binding to IGFR (DAF-2), transcription factors such as DAF-16 dissociate from their chaperons such as 14-3-3 and enter the nucleus, displacing DVE-1 and associated repressor complex resulting in the expression of pro-longevity genes.

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APPENDIX A

1. Amplification of Interacting partners of DVE-1

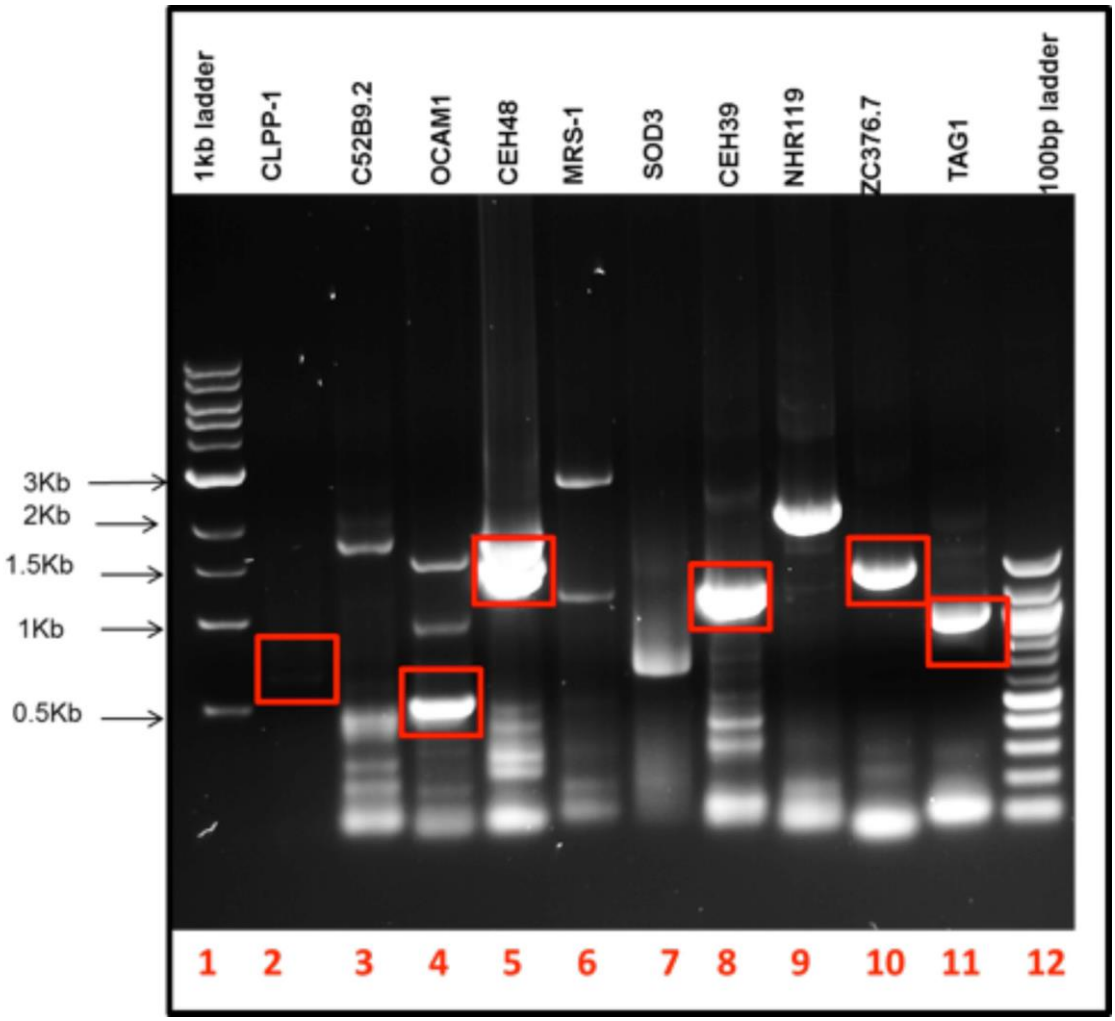

Figure:1 Amplification of Interacting partners of DVE-1

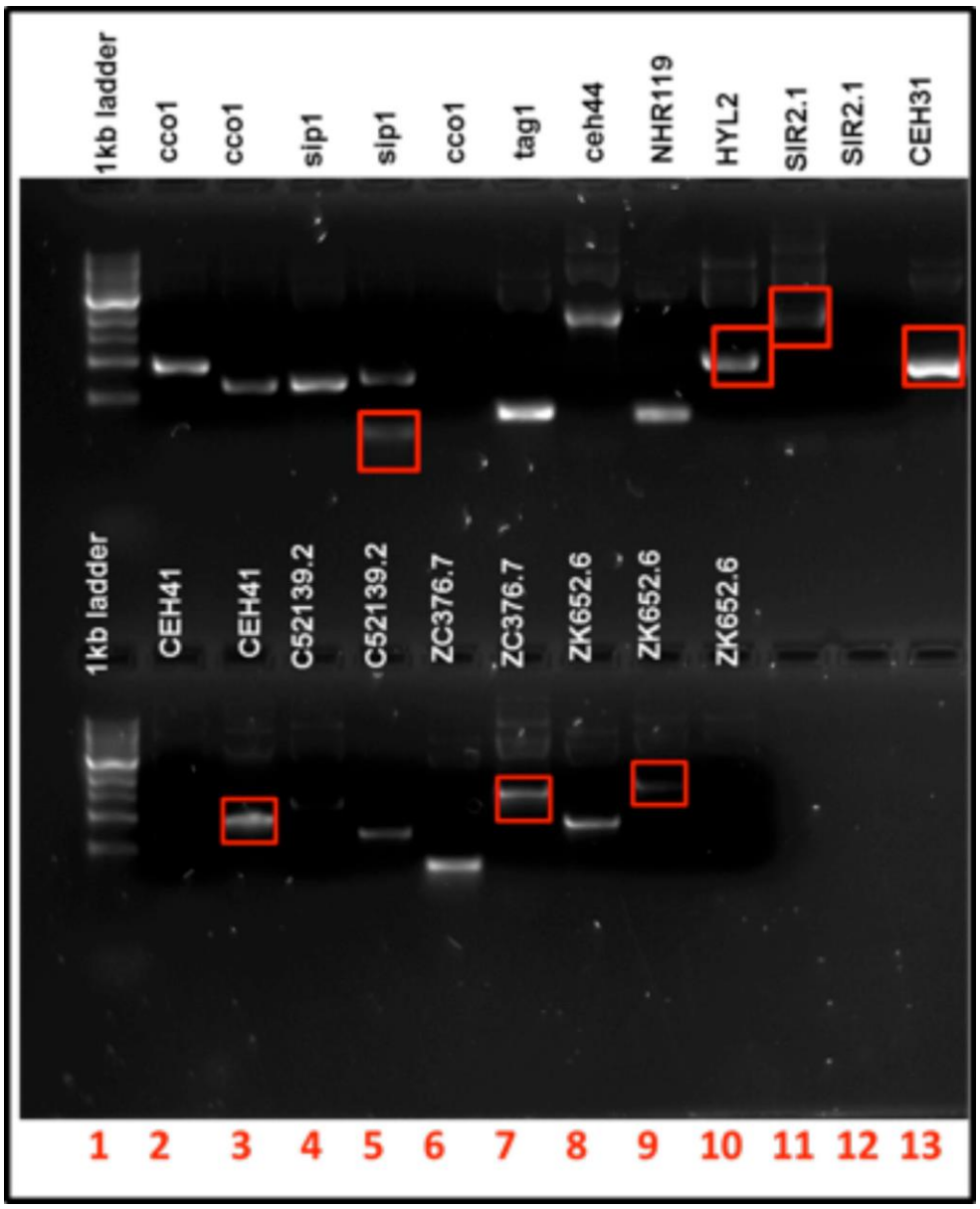

Figure: 2 A PCR was performed using colonies from transformed plates for the interacting partners of DVE1. The PCR amplified product was loaded on a gel to confirm the amplification.

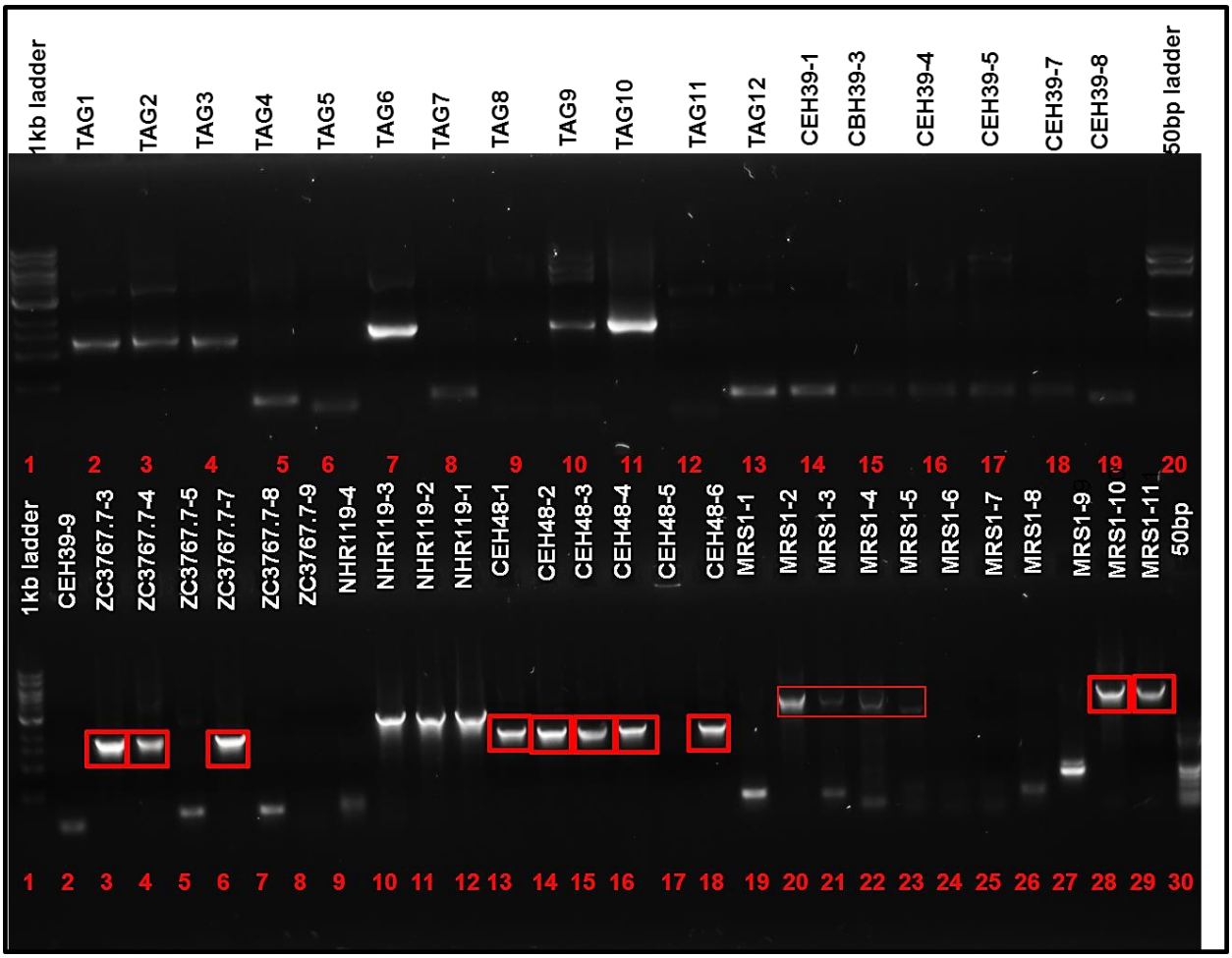

Figure: 3 Plasmid DNA was isolated using Miniprep protocol. PCR was performed using the isolated DNA as the template for the interacting partners of DVE1. The PCR amplified product was loaded on a gel to confirm the amplification.

1. **Amplification of the Transcription factors obtained as hits in the Transfac analysis**

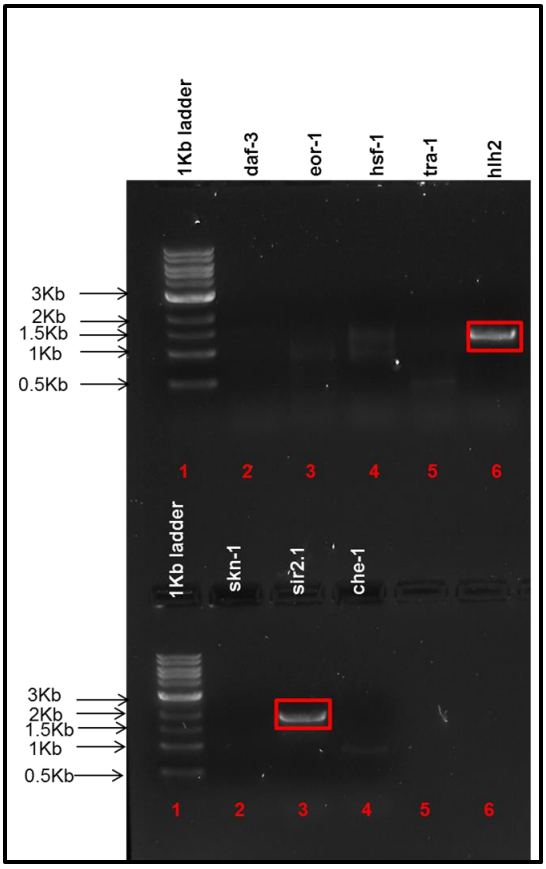

Figure 4: The cDNA isolated from mixed stage worms were used as a template to amplify the transcription factors using gene specific primers. The PCR product was loaded on a 0.8% agarose gel to confirm the amplification.

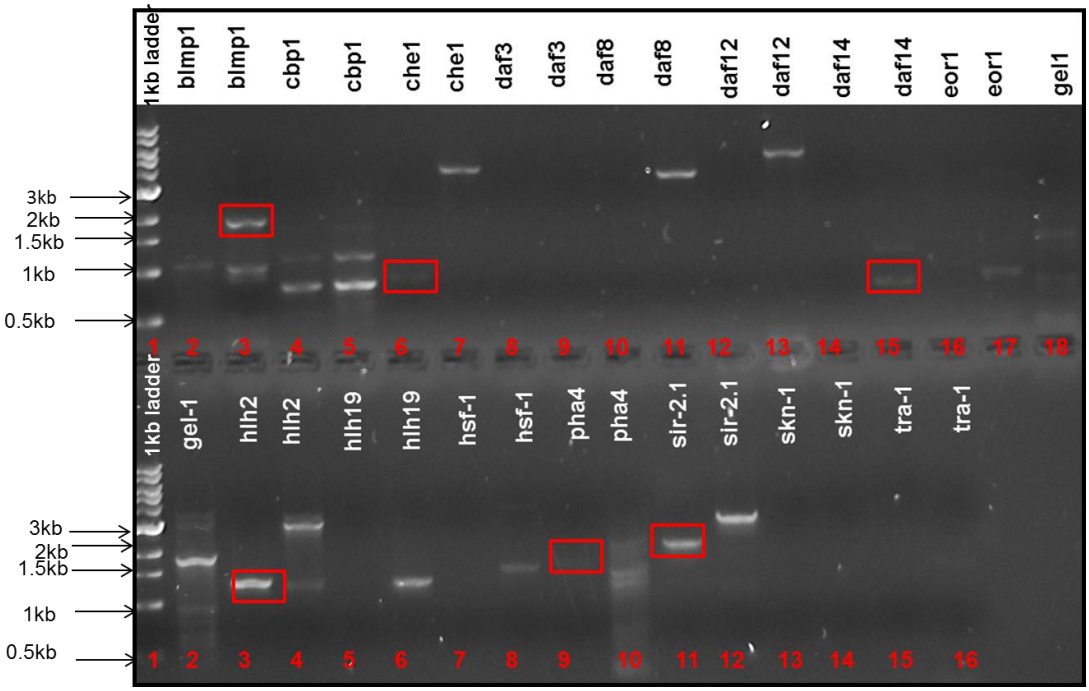

Figure 5: The cDNA and genomic DNA isolated from mixed stage worms were used as a template to amplify the transcription factors using gene specific primers. The PCR product was loaded on a 0.8% agarose gel to confirm the amplification.

1. Lane 1 representing each gene contains PCR product obtained using cDNA as the template.
2. Lane 2 representing each gene contains PCR product obtained using genomic DNA as the template.

APPENDIX B

Potential transcriptional targets of DVE-1 that are involved in regulation of adult lifespan in *Caenorhabditis elegans*

nol1	**The protein product have S-adenosylmethionine dependent methyl transferase activity** • **RNA binding and processing**
d1069.3	**This gene encodes a protein that localizes to the membrane. Invivo function is unknown.**
ges-1	**Encodes a gut specific carboxylesterase** • **lineage specific differentiation.**
tag-307	**Involved in determination of adult lifespan**
sid2	**SID-2 is an intestinal luminal transmembrane protein** • **environmental RNAi in *C. elegans*.**
unc-112	**unc112 encodes components of cell adhesion matrix structures.**
rpn-11	**Encodes a predicted non-ATPase subunit of the 19S regulatory complex of the proteasome** • **adult viability, osmoregulation, embryonic viability, and movement.**
sas-5	**sas-5 encodes a coiled-coil protein daughter centriole formation.**
ins-7	**Encodes protein that regulates aging and crosstalk with IIS.**
lys-7	**lys-7 encodes an enzyme homologous to an antimicrobial lysozyme.**
sir2.1	**sir2.1 acts in the same genetic pathway as daf-2 and daf-16 and regulates lifespan.**
tpi-1	**This gene encodes an enzyme involved in glycolysis and gluconeogenesis.**
let-363	**let-363 encodes proteins with C-terminal similarity to phosphatidylinositol kinase (PIK). Normal development and physiology of the intestine.**
elt-2	**elt-2 encodes a GATA-type**

	transcription factor required for initiating and maintaining terminal differentiation of the intestine.
pqn-41	**The protein product of this gene is predicted to contain a glutamine/asparagine (Q/N)-rich ('prion') domain.**
y39h10a.6	**The gene has been proposed to participate in protein amino acid phosphorylation.**
sbds-1	**This gene protein that function in RNA metabolism.**
cyc-2.1	**The protein product of this gene is predicted to function in the electron transport chain and is essential for normal brood sizes and growth rates.**
lbp-7	**lbp-7 encodes a predicted intracellular fatty acid binding protein. The role in *C. elegans* development and/or behavior is not yet known.**
ifc-2	**ifc-2 encodes intermediate filament protein dispensable for viability but required for normal movement, growth rate, body size, body shape, and cuticle strength.**
asm3	**The protein product act as a positive regulator of the DAF-2/IIS pathway in *C. elegans***
rps15	**Functionally, the gene encodes a protein that has been proposed to participate in a protein biosynthesis.**
ctl-1	**ctl-1 encodes CTL-1 which exhibits catalase activity in vitro, and functions as an antioxidant enzyme.**
wts-1	**The gene has been proposed to participate in protein amino acid phosphorylation.**
f08b4.7	**-**
pat4	**The pat-4 gene encodes a serine/threonine kinase required for required for formation of integrin-mediated muscle cell attachments during embryogenesis.**
acdh-1	**acdh-1 encodes a short-chain acyl-CoA dehydrogenase and plays a role in energy production.**

vps16	**vps-16 activity is required for biogenesis of the lysosome-related gut granules and for embryonic development**
drr1	**The gene is associated to extension in adult lifespan, reduced fecundity and Dietary Restriction Response.**
drr2	**This gene product is involved in nucleic acid binding activity.**
lin-40	**lin-40 encodes a protein which is expected to have DNA binding activity.**
eif-3b	**Encodes protein involved in protein binding and translation initiation factor activity.**
ril-2	**Encodes protein involved in determination of lifespan, protein metabolic processes and development.**
y53g8al.2	**Encodes a Putative uncharacterized protein**
rps22	**Encodes protein involved in translation.**
rps23	**Encodes protein involved in translation.**
rpl6	**Encodes protein involved in translation**
plk-1	**Encodes serine/threonine protein kinases that perform important functions in cell cycle.**
y53g8al.3	**Encodes protein involved in oxidative stress response.**
cyp-34a9	**-**
dod-3	**Encodes proteins that regulate lifespan and act downstream Of DAF-16.**
rpn-8	**Encodes a protein coding proteosomal regulatory subunit.**
c32h11.9	**-**
hsp-70	**Encodes heat shock proteins**
vit-2	**Encodes protein that has a role in lipid transport**
pbs-5	**Encodes protein involved in ubiquitin dependent protein catabolism**
y43f4b.7	**Encodes protein involved in regulating lifespan.**
pbs4	**Encodes protein involved in ubiquitin dependent protein catabolism,threonine-endopeptidase activity**

rpn9	**rpn-9 is predicted to encode a non-ATPase subunit of the 19S regulatory complex of the proteasome that that affects body morphology, embryonic viability, locomotion, larval viability, fertility, and growth;**
ubc-12	**The ubc-12 gene encodes a ubiquitin-conjugating enzyme whose substrate is NED-8, and which is required for both embryogenesis and terminal hypodermal differentiation;**
ctl-2	**Encodes choline transporter protein**
ada-2	**Encodes subunits of nuclear histone acetyl transferase.**
y77e11a.7	**-**
y43f8b.12	**-**
mag-1	**Encodes DNA-3-methyladenine glycosylase that helps to prevent alkylation of DNA**
him-6	**Encodes DNA damage response proteins.**
f56a4.3	**-**
dod-6	**Encodes protein involved in regulating lifespan.**
cpr-1	**cpr-1 encodes a cysteine protease of the cathepsin B-like cysteine protease family and plays a role in proteolysis and peptidolysis**
tufm-1	**This gene encodes a protein which participates in protein translation in mitochondria**
mcm-2	**The protein encoded by this gene is one of the highly conserved mini-chromosome maintenance proteins**
hsp-6	**Encodes proteins involved in heat shock response.**
sqv-6	**sqv-6 encodes a xylosyltransferase, active in cell culture, that is required for cytokinesis**
npl-4.2	**-**
daf15	**daf-15 is required for non-dauer**

	development,
cgh-1	**cgh-1 encodes a putative DEAD-box RNA helicase,**
cct-6	**Encodes a protein involved in ATP binding activity, protein binding activity**
atp-3	**atp-3 encodes a mitochondrial ATP synthase and is part of a regulatory system that controls and establishes rates of respiration, behavior and aging during development that persists during adulthood.**
vps-32.2	**-**
cul-4	**Encodes a protein whose activity is essential for negative regulation of DNA-replication licensing.**
k12h4.5	**Encodes Probable signal peptidase complex subunit 3**
cul-3	**Encodes a component of a ubiquitin E3 ligase that is essential for mitotic division**
zk1127.5	**Encodes a Probable RNA 3'-terminal phosphate cyclase-like protein;**
gst-5	**Encodes glutathione S transferase and plays a role in lifespan extension.**
c42d8.9	**-**
t20h4.5	**This gene encodes a 23 kDa subunit of mitochondrial complex I that is required for oxidative phosphorylation and for resistance to volatile anesthetics**
c36e8.1	**Encodes an uncharacterized protein.**
ntl-2	**Encodes a protein involved in regulation of transcription.**
rpl-30	**Encodes ribosomal proteins**
rps-5	**Encodes a disease resistance protein**
npp-3	**Encode pyro phosphatase/phosphodiesterase**
f19b6.1	**-**
rpl-34	**Encodes a ribosomal protein 34**
f28b3.5	**Encodes a protein involved in regulating adult lifespan**
hrp1	**Encodes RNA-binding protein, which is involved in the polyadenylation.**
sdc-2	**The protein encoded by this gene is a**

	transmembrane (type I) heparin sulfate proteoglycan and is a member of the syndecan proteoglycan family
dod-21	**Encodes proteins Involved in regulating adult lifespan.**
aat-8	**Encodes protein involved in organismal aging.**
f59c6.5	**-**
dod-24	**Life span extension**
dod-23	**Adult lifespan**
spp-1	**The protein encoded by this gene is involved in the attachment of osteoclasts**
cyp33-e2	**Encodes protein that helps in eicosanoid formation**
c29f9.1	**Adult lifespan**
c29f9.2	**Adult lifespan**
h28o16.1	**Adult lifespan**
y53g8ar.7	**Adult lifespan and transmembrane transport**
y53g8ar.8	**Adult lifespan**
pes-8	**PES-8 may be required for normal morphology of the openings of the spermathecal valve, the vulva, and the rectum,**
clec-186	**Encodes a C-type lectin family member**
c09b7.2	**Encodes a transposase.**
skr-1	**Encodes a protein ivolved in cell proliferation.**
y71h2ar.2	**Encodes a protein involved in cysteine type peptidase activity.**
igg-3	**-**
hsp16.2	**Encodes a heat shock protein**
dod-19	**Encodes a protein involved in insulin signaling**
c06a5.1	**-**
egl-4	**Functions in chemo sensation**
f37c4.4	**-**
gst-10	**gst-10 encodes a predicted glutathione S-transferase**
nuc-1	**Encodes proteins involved in peroxisome proliferation.**
cdr-6	**Encodes Cadmium Responsive, encoding cadmium-inducible lysosomal**

	protein CDR-3 family member.
smc-3	**Encodes proteins involved in structural maintenance of chromosomes.**
thn-1	**Encodes proteins having direct anti-microbial activity**
lem-2	**Encodes a LEM domain containing protein**
mtl-2	**Encodes metallothionin.**