ABSTRACT

Genetic and Hypoxic Effects on Germline Tumor Development in *Caenorhabditis elegans*.

by

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The process of differentiation of stem cells to committed, progenitor specific cell types is well studied but the reverse process of the dedifferentiation of these committed cells back to the undifferentiated state still remains a major challenge in stem cell biology. In my project, we studied some of the major regulators of the dedifferentiation process taking *C. elegans* germline model system. In *C. elegans*, the germline stem cells at the distal end that initially divide mitotically then enter meiosis and differentiate into gametes as they progress towards the proximal end. In the first part of my project, we proposed that the Ras-ERK MAPK signaling activated by the removal of two negative regulators, PUF-8 RNA-binding protein and LIP-1 dual specificity phosphatase, plays an important role in controlling the dedifferentiation of secondary spermatocytes at the proximal end to a more undifferentiated, multipotent state forming proximal germline tumor. Further, by RNAi screening, the RSKN-1/P90$_{RSK}$, a downstream effector of MPK-1/ERK was identified to be critical for this (already published).

As a continuation of my project, next, the HIF-1 (Hypoxia inducible factor-1) transcription factor that plays a key role in oxygen homeostasis during hypoxic conditions for the survival of the

organism has been studied for its pronounced effect on this dedifferentiation-mediated tumorigenesis process. We found that under hypoxic conditions or under the conditions that mimic hypoxia (by exposing the worms to cobalt chloride, a chemical inducer of hypoxia), the activation of HIF-1 inhibits dedifferentiation-mediated tumorigenesis in *puf-8; lip-1* mutant germline, probably through Ras-ERK MAPK signaling. Besides focusing on the effect of hypoxia on dedifferentiation-mediated tumorigenesis, we also studied the effect of hypoxia on *glp-1(ar202),* also called *glp-1(gf)* gain-of-function mutant - used to study GLP-1/Notch-mediated tumorigenesis where the tumor development occurs via a different pathway where the meiotic entry of germline stem cells are inhibited and instead self-renews and in the process of self-renewal forms the tumor cell-like cells. Interestingly, the activation of HIF-1 by either genetic manipulation or cobalt chloride treatment also inhibited GLP-1/Notch-mediated tumorigenesis. It suggests that there might be a common mechanism underlying the action of HIF-1 in both dedifferentiation-mediated and GLP-1/Notch-mediated germline tumors.

Genetic and Hypoxic Effects on Germline Tumor Development in *Caenorhabditis elegans*.

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by

Udaya Sree Datla

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Dedicated to my family, friends and my mentor.

Dedicated to everyone who are ideal in thought and action.

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LIST OF PUBLICATIONS

- Cha, D.S., **Datla, U.S.**, Hollis, S.E., Kimble, J., and Lee, M.H. (2012) The Ras-ERK MAPK regulatory network controls dedifferentiation in *Caenorhabditis elegans. BBA-Molecular Cell Research* 1823(10):1847-1855.

- Cha, D.S., Hollis, S.E., **Datla, U.S.**, Lee, S., Ryu, J., Jung, H.R., Kim, E., Kim, K., Lee, M., Li, C., and Lee, M.H. (2012) Differential subcellular localization of DNA topoisomerase-1 isoforms and their roles during *Caenorhabditis elegans* development. *Gene Expression Patterns* 12(5-6):189-195.

- **Udaya Sree Datla**, Austin Jon Brokamp, Natasha Carol Scovill, Adam S. Asch, Eunsuk Kim and Myon-Hee Lee. PUF (Pumilio and FBF) as a molecular switch for stem cell control, cell lineage commitment, and dedifferentiation: a lesson from worms. *Journal of Cellular Physiology – Perspective.* (In submission)

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LIST OF SYMBOLS AND ABBREVIATIONS

Puf-8: PUMILIO/FBF domain-containing.

fbf: Fem-3 mRNA Binding Factor.

mex-3: Muscle EXcess.

lip-1: Lateral-signal-Induced Phosphatase.

glp-1: abnormal Germ Line Proliferation.

UNC: UNCoordinated.

mpk-1: Mitogen-activated Protein (MAP) Kinase.

MKP3: MAP Kinase Phosphatase 3.

Ras-ERK: Extracellular signal-regulated Kinase.

RSKN-1: P90RSK, P90Ribosomal S6 Kinase homolog.

mog: Masculinization of Germline.

fog: Feminization of Germline.

cpb-1: CPEB polyA Binding Family.

CPEB: Cytoplasmic Polyadenylation Element Binding proteins.

ife-1: Initiation Factor 4E (eIF4E) family.

eIF4E: Eukaryotic initiation factor.

hif-1: HIF (Hypoxia Inducible Factor) homolog.

vhl-1: Von Hippel-Lindau tumor suppressor homolog.

egl-9: EGg Laying defective.

EGLN: Egg-laying deficiency protein Nine-like protein.

aha-1: Aryl Hydrocarbon receptor Associated protein.

FIH: Factor Inhibiting Hypoxia inducible factor (HIF).

CBP/p300: CREB-1 (CAMP responsive element binding protein 1) Binding Protein.

ARNT: Aryl hydrocarbon Receptor Nuclear Translocator.

ODDD: Oxygen-Dependent Degradation Domain.

HRE: Hypoxia Response Element.

HPH: HIF Prolyl Hydroxylases.

VEGF: Vascular Endothelial Growth Factor.

PHD: Prolyl Hydroxylase Domain enzyme.

PDGF: Platelet-Derived Growth Factor.

EPO: Erythropoietin.

GLUT-1: Glucose Transporter 1.

LDH: Lactate Dehydrogenase.

ENO: Human Enolase.

iNOS: inducible Nitric Oxide Synthase.

NGM: Nematode Growth Medium.

GSCs: Germline Stem Cells.

DTC: Distal Tip Cell.

IPTG: Isopropyl β-D-1-thiogalactopyranoside

DAPI: 4',6-Diamidino-2-Phenylindole, Dihydrochloride.

PFA: Paraformaldehyde.

PTW/PBST: Phosphate Bovine Serum with Tween-20.

BSA: Bovine Serum Albumin.

EDTA: Ethylenediaminetetraaceticacid.

RISC: RNA Induced Silencing Complex.

PVDF: Polyvinylidene difluoride.

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

HRP Antibody system: HorseRadish Peroxidase Antibody system

CHAPTER 1

Overview of *Caenorhabditis elegans* Animal Model System

Introduction

Caenorhabditis elegans is a free-living, non-parasitic, soil nematode which measures approximately 1.3 mm long. It is a cholesterol auxotroph which feeds on organic matter. In lab, it is grown in large numbers on nutrient rich NGM (nematode growth medium) agar plates feeding on *Escherichia coli* bacteria. The average lifespan of the worm is approximately two weeks. *C. elegans* moults through four larval stages before it becomes the adult. It has two sexes: male and hermaphrodite. There are two gonadal arms in an adult worm in which the spermatogenesis begins in the L4 larval stage, which continues throughout lifetime in a male. Whereas in a hermaphrodite, the spermatogenesis ceases and there is a switch to oogenesis in the adult stage and hence can self-fertilize. An adult hermaphrodite worm lays about 300 eggs by self-fertilization and about 1000 eggs by cross-fertilization in a lifetime. A brief review of the lifecycle of the worm – both male and hermaphrodite is shown in ***fig. 2***.

The worm is well suited for phenotypic studies also due to the transparency of the worm. The body of the worm has a feeding tube, the pharynx, which connects to the intestine that runs along the body length which then leads to the rectum and finally ends in an opening, the anus. The anatomy of the worm – both male and hermaphrodite is shown in ***fig. 1***. The generation time of the worm varies with temperature due to its slow growth rate at low temperature and faster growth rate at higher temperature. The generation time is usually 2.5 days at 25ºC, 3.5 days at 20ºC and 5.5 days at 15ºC.

The worm was selected by Sydney Brenner (Nobel Prize winner) for the first time in 1960s. Being the first multicellular organism with completely sequenced genome, the *C. elegans* animal model system is most suited for genetic studies by transgene or transient knockout using RNAi technique. This is directly applied to study the human gene functions due to ~80% homology(Kaletta & Hengartner, 2006). When the worms that are heterozygous for a specific allele gives rise to progeny through self-fertilization, 25% of the progeny will be homozygous for this recessive allele.

C. elegans has five pairs of autosomes and one pair of allosomes. The sex of the worm is determined by the allosome pair which is XX for hermaphrodite and XO for male. Only 0.1% of the total population is males through self-fertilization. However, almost 50% of the progeny are males after cross-fertilization as they do not inherit the X chromosome from their fathers in this case. It is by virtue of this property that it is easy to generate multiple mutations in *C. elegans*. A specific gene function (or the protein encoded by the gene to be more specific) can be studied by silencing the gene (by knockout) and analyzing the relevance in the phenotype of the worm. Another application is to identify the expression pattern of a particular gene by inducing their expression coupled with fluorescent proteins, detected using a fluorescent microscope. The *C. elegans* germline provides a superb model for understanding the molecular controls of stem cells, proliferation, differentiation, and dedifferentiation processes.

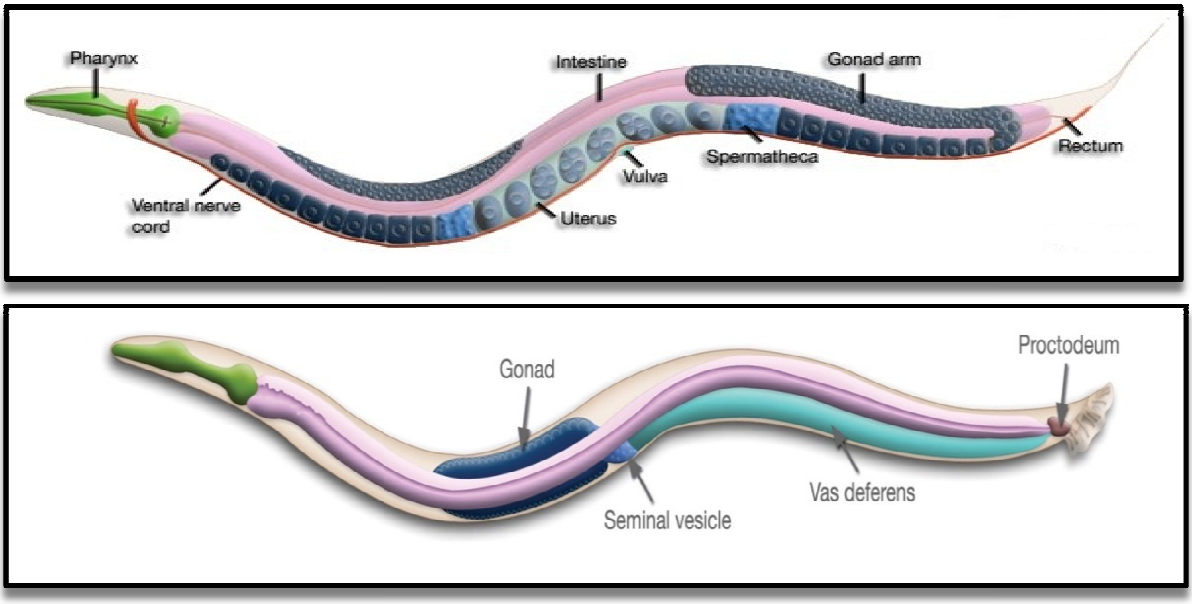


Fig. 1. Anatomy of *Caenorhabditis elegans* – Hermaphrodite (above) and Male (below) (© Wormatlas).

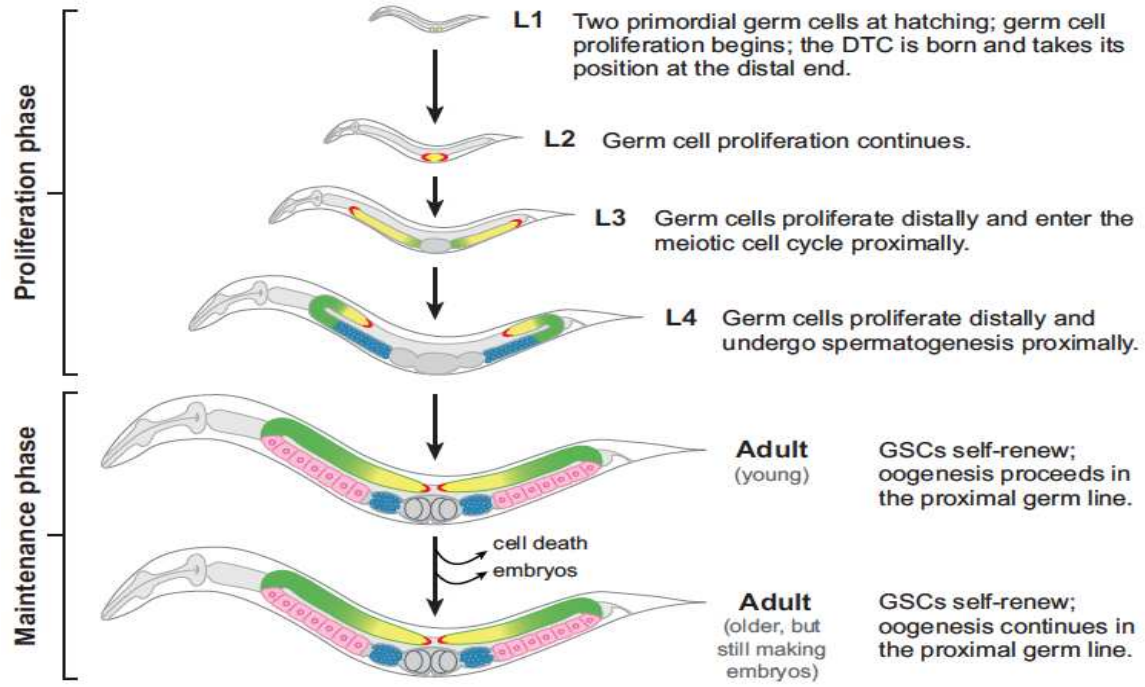


Fig. 2. Review of the life cycle of *Caenorhabditis elegans* – Hermaphrodite and Male(Kimble & Crittenden, 2007).

General Materials and Methods:

Materials Used:

Recipes of Solutions used for Various Experimental Setups:

NGM Agar Plates:

To make 1 liter of NGM Agar solution, about 3 grams of sodium chloride, 2.5 grams of peptone, 17 grams of agar, 1 ml of cholesterol (5 mg/ml in ethanol), 1 ml of 1M calcium chloride (29.4 grams of calcium chloride dihydrate in 200 ml autoclaved distilled water), 1 ml of 1M magnesium sulfate (49.2 grams of magnesium sulfate heptahydrate in 200 ml autoclaved distilled water) and 25 ml of 1M Potassium phosphate buffer (pH 6.0) are added to a large conical flask and the final volume is made up to 1 liter with distilled water and then autoclaved and plated.

OP50 *E. coli* bacteria inoculated in 2XYT buffer is spread on the NGM agar plates to feed *C. elegans.*

[Preparation of 1M Potassium Phosphate Buffer pH 6.0:

In one conical flask, 68 grams of Potassium di-hydrogen phosphate – monobasic phosphate is dissolved in 500 ml of autoclaved distilled water to make 1M Potassium monobasic phosphate solution.

In another conical flask, 87 grams of Di-Potassium hydrogen phosphate is dissolved in 500 ml of autoclaved distilled water to make 1M Potassium dibasic phosphate solution.

About 434 ml of the first solution is mixed with 66 ml of the second solution to make 1M potassium phosphate Buffer and the pH is adjusted to 6.0.]

RNAi Plates:

About 3 grams of sodium chloride, 2.5 grams of peptone, 17 grams of agar, 1 ml of cholesterol (5 mg/ml in ethanol), 1 ml of 1M calcium chloride, 1 ml of 1M magnesium sulfate and 25 ml of 1M potassium phosphate (pH 6.0) are added to a large conical flask and the final volume is made up to 1 liter with distilled water and then autoclaved. After autoclaving, the NGM agar solution is allowed to cool, to which ampicillin (25 mg/ml) and tetracycline (10 mg/ml in 50% ethanol) are added in 1 in 1000 ratio and IPTG in a 1 in 2000 ratio (500 mM) and then plated.

Different kinds of RNAi *E.coli* bacteria inoculated in 2XYT buffer - with ampicillin (25 mg/ml) and tetracycline (10 mg/ml in 50% ethanol) in a 1 in 1000 concentration is spread on the NGM agar plates to feed *C. elegans* to study the RNA interference phenomenon.

[Stock Solutions Preparation:

1M IPTG: 2.38 grams of IPTG powder is dissolved in 10 ml of molecular biology grade water, filter sterilized and stored in 1 ml aliquots at -20ºC.

25 mg/ml Ampicillin: 2.5 grams of ampicillin powder is dissolved in 100 ml of autoclaved distilled water, filter sterilized and stored in 1 ml aliquots at -20ºC.

10 mg/ml Tetracycline: 1 gram of Tetracycline powder is dissolved in 100 ml of 50% ethanol, filter sterilized and stored in 1 ml aliquots at -20ºC.]

2XYT Buffer:

About 10 grams of bacto tryptone, 5 grams of bacto yeast extract and 5 grams of sodium chloride are added to a large conical flask and the final volume is made up to 500 ml with distilled water and then autoclaved and stored at 4ºC after cooling. It serves as a medium good for the growth of *E. coli* bacteria.

M9 Buffer:

About 3 grams of potassium monobasic phosphate, 6 grams of sodium dibasic phosphate and 5 grams of sodium chloride are dissolved in 1 liter distilled water and autoclaved. After autoclaving, 1 ml of 1M magnesium sulfate is added. It is added after autoclaving to avoid precipitation.

Various Methods Followed:

Bacteria Stock:

Bacteria are inoculated to 2XYT buffer and shaking incubated overnight at 37ºC. The next day, 800 µl of this 2XYT buffer concentrated with bacteria is transferred to a stock tube (vial) to which 200 µl of 75% glycerol (should be 20% of the total volume) is added and the vial is then stored at -80ºC.

C. elegans Stock:

A non-contaminated, starved plate with plenty of L1 and L2 stage worms (in dauer stage) of the desired strain is washed with a little more than 500 µl of worm stock solution (S-medium) and is transferred to a stock tube (vial) to which 500 µl of worm freezing solution is added to make 1 ml of the worm stock frozen at -80ºC. A portion of the frozen stock can be test thawed a week later to ensure the viability of worms.

[S-medium: This medium is prepared by adding 10 ml of 1M potassium citrate pH 6.0, 10 ml of trace metals solution, 3 ml of 1M calcium chloride and 3 ml of 1M magnesium sulfate to 1 liter S-basal medium and autoclaved and stored in dark.

S-basal medium: Includes 0.1 M sodium chloride (5.85 grams), 0.05 M potassium phosphate buffer pH 6.0 (1 gram of potassium dibasic phosphate and 6 grams of potassium monobasic phosphate), 1 ml cholesterol (5 mg/ml in ethanol) made up to 1 liter with autoclaved distilled water.

Trace Metals Solution: Includes 5 mM Disodium EDTA (1.86 grams), 2.5 mM ferrous sulfate heptahydrate (0.69 grams), 1mM manganese chloride tetrahydrate (0.2 grams), 1mM zinc sulfate heptahydrate (0.29 grams), copper sulfate pentahydrate (0.025 grams) made to a final volume of 1 liter with autoclaved distilled water.]

DAPI Staining:

The dissected gonads are first washed 1X with PTW/PBST, then fixed in 3% PFA for 10 minutes to 1 hour (time of fixation is based on the germline phenotype – tumorous or non-tumorous) at room temperature, then washed 3X with PTW/PBST and then incubated in cold methanol at -20ºC for 10 min. (Time period of incubation in cold methanol can range over a period of 10 minutes to 1 month). After that, the sample is spun down and washed with PTW. Later, DAPI solution is added to the sample and incubated at room temperature for 10 minutes to 1 hour. Finally, the DAPI stained germlines (stains DNA) are mounted on 2% agarose pads and analyzed for the required phenotype under Nomarski microscope.

[Solutions Used in the Staining Processes:

DAPI working solution: 100 µl of 10X PBS and 20 µl of DAPI in water (stock solution) are added to 880 µl of autoclaved distilled water, vortexed and stored at 4ºC.

1X PTW/PBST: 1 ml of Tween-20 (0.1%) is added to 100 ml of 10X PBS and the final volume is made up to 1 liter with distilled water.

Levamisole/Tetramisole working solution (25 mM): About 60 mg of levamisole hydrochloride powder is dissolved in 10 ml of autoclaved distilled water and stored in 1 ml aliquots at -20ºC.

PTW+Levamisole solution: 1 ml of 25 mM levamisole solution is added to 100 ml of 1X PTW – used for paralyzing/immobilizing the worms to provide a better grip during dissection.

3% PFA: In a 50 ml conical tube, 5 ml of 1M potassium dibasic phosphate solution (pH 7.2), 9.375 ml of 16% PFA and 35.625 ml of autoclaved distilled water are mixed, vortexed and finally stored in 1 ml aliquots at 4ºC.

2% Agar: 2 grams of fine quality agar is dissolved in 100 ml of autoclaved distilled water.

Vecta Shield: Commercially available and is used as mounting medium with DAPI.]

Embryo Isolation (to generate synchronized worm populations):

The plate with a high density of embryos is washed with PTW or M9 solution and the worms with embryos are collected into a micro tube, where they are exposed to the bleaching solution until the adult worm bodies are dissociated (this is further facilitated by frequent vortexing) while the embryos are still intact due to a tough protective coating. The sample is then spun down to remove the supernatant and subsequently washed thrice with M9 solution. In the last washing step, the embryos are synchronized overnight in M9 solution where the L1 stage worms are arrested. They are then plated.

[Bleaching solution protocol: 3.75 ml of 1M sodium chloride and 3 ml bleach (Clorox) are added to 8.25 ml autoclaved distilled water to make the bleaching solution.]

CHAPTER 2

Genetic Analysis of Dedifferentiation-mediated Germline Tumor Development in *Caenorhabditis elegans*

Introduction

PUF proteins are a family of eukaryotic RNA binding proteins that control various physiological processes such as stem cell maintenance/ self-renewal, cell fate specification, differentiation and dedifferentiation. They do so by binding to the PUF binding element (PBE) having the sequence UGUA within the 3' UTR (untranslated region) of the specific mRNA and represses the target mRNA translation or stability(Fan et al., 2013; Spassov & Jurecic, 2003; Whelan, Hollis, Cha, Asch, & Lee, 2012b; Wickens, Bernstein, Kimble, & Parker, 2002). The PUF proteins have post-transcriptional or translational action. PUF proteins are highly conserved ranging from lower organisms to humans and many of their target mRNAs have been shared among several animal model systems(Francischini & Quaggio, 2009; Galgano et al., 2008; Gerber, Herschlag, & Brown, 2004; Gerber, Luschnig, Krasnow, Brown, & Herschlag, 2006; Kershner & Kimble, 2010; Morris, Mukherjee, & Keene, 2008). There are many PUF proteins found in *C. elegans*, *Saccharomyces cerevisiae* and *Arabidopsis thaliana* while there are few of them in vertebrates such as humans and mice – each with two PUF proteins and just one in *Drosophila* - Pumilio and none in Eubacteria and Archaea. Among several PUF proteins in C. elegans, FBF (fem-3 binding factor) was the first one to be identified(B. Zhang et al., 1997).

The structure of a PUF domain first discovered was that of Drosophila Pumilio and human Pum1. It consists of eight repeat motifs with three alpha helices and two pseudo-repeats at the N

and C terminal ends. It is towards the N terminal end that the PUF protein binds to the 3' UTR of the target mRNAs with a binding specificity. A pictorial representation of the structure of *Caenorhabditis elegans* FBF (PUF family) RNA-binding protein bound to its consensus sequence is shown in ***fig. 3.*** (Kaymak, Wee, & Ryder, 2010)(Y. Wang, Opperman, Wickens, & Hall, 2009)**.** In this chapter, PUF-8, the *C. elegans* PUF protein with great similarity to human Pum2, is studied for its role in dedifferentiation in the germline.

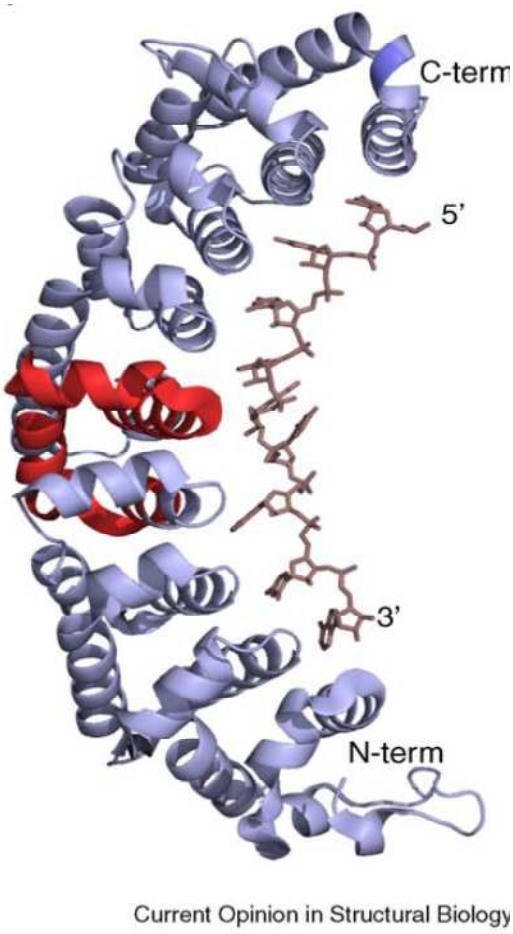


Fig. 3. Structure of *Caenorhabditis elegans* FBF (PUF family) RNA-binding protein bound to the FBE (FBF binding element)(Kaymak et al., 2010; Y. Wang et al., 2009).

Objectives

It has been well established that the stem cells differentiate to form varied cell types through multiple cell lineages. In this process, the undifferentiated stem cells undergo multiple cell divisions to form committed progenitor cells. Nowadays, the topic of interest is how these committed progenitor cells or other intermediate cell types in the differentiation process dedifferentiate back to form cancer stem cell-like cells. This is a challenging topic and not much literature is available to date. Using the *C. elegans* animal model system, we tried to unravel various regulators controlling this dedifferentiation process. Previously, it was shown that PUF-8 RNA-binding protein inhibits the dedifferentiation process due to which some of the *puf-8* knockout single mutants are tumorous at 25^0C where it was hypothesized that it is the primary spermatocytes that dedifferentiate back to form undifferentiated tumor cell-like cells(Subramaniam & Seydoux, 2003). So, we decided to identify other factors in addition to PUF-8 which inhibits this dedifferentiation process and the mechanism involved behind this. To this end, we used RNAi approach and found out that PUF-8 together with LIP-1 (MAPK Phosphatase homologous to the vertebrate dual specificity phosphatase) represses dedifferentiation-mediated germline tumor development. We confirmed this result by generating the *puf-8; lip-1* double mutant. Further, cellular analyses of dedifferentiation will be useful to know in detail about this. To study the mechanism of dedifferentiation regulation, we decided to study the role of MPK-1/ERK MAPK signaling through various downstream targets such as RSKN-1/$P90_{RSK}$ pathway(Arur et al., 2009) to be more specific with our findings. The logic behind this approach is that this regulation may have potential parallel findings in higher animals

where the two putative Pum2 targets - ERK2 MAPK controls cellular dedifferentiation and proliferation(Whelan, Hollis, Cha, Asch, & Lee, 2012a; W. Zhang & Liu, 2002) in differentiated cells such as sertoli cells(X. Zhang et al., 2006), islet cells(Chen et al., 2005) and myoblasts(Hanley, Assouline-Thomas, Makhlin, & Rosenberg, 2011). Thus, these findings in *C. elegans* may have important implications for cell fate reprogramming and cancer therapy in mammals.

Specific Materials and Methods

RNA interference

The phenomenon of RNAi was first discovered by Fire *et al.* 1998 and since then it has become much easier to study the phenotype of the worm by gene inactivation through RNAi approach by knocking out the transcript levels of the gene. It is a post-transcriptional mechanism where the dsRNA is cleaved by the enzyme Dicer into small siRNAs (small interfering RNAs). These siRNAs together with the RISC (RNA induced silencing complex) bind to the mRNAs in the cell by base-pairing. This bound mRNA is then degraded by an enzyme within RISC(Montgomery, 2006). The various methods to trigger RNAi is by injecting the adult worms with dsRNA(Fire et al., 1998), by soaking them in dsRNA solution(Tabara, Grishok, & Mello, 1998), by feeding them with a strain of *E.coli* bacteria engineered to produce large amounts of the specific dsRNA(Timmons & Fire, 1998), or by the transgenesis mechanism(Tavernarakis, Wang, Dorovkov, Ryazanov, & Driscoll, 2000). We used the RNAi of *mpk-1b* and *rskn-1* to study the

role of MPK-1/ERK MAPK signaling in dedifferentiation through RSKN-1/P90$_{RSK}$ pathway and RNAi of *mpk-1, cpb-1, fog-1* and *ife-1* to study the cellular analyses of dedifferentiation.

Results

2.1 PUF-8 and LIP-1 together repress germline tumor development.

Previously Dr. Subrahmaniam *et al.* found that *puf-8(ok302)* single mutant worms, also designated as *puf-8(0)* null mutants that are fertile (produce both sperm and oocytes) at 20^{0}C(Bachorik & Kimble, 2005), when grown at 25^{0}C – some of the worms developed proximal germline tumor(Subramaniam & Seydoux, 2003). But, we found later that by the additional loss of another regulator LIP-1– an ERK/MAPK (Mitogen-activated protein kinase) dual specificity phosphatase homologous to MKP3 (MAP Kinase Phosphatase 3) in mammals, the tumor percentage was highly enhanced. Though we used a different allele *puf-8(q725),* we found similar results where 9% of the *puf-8(q725)* had proximal germline tumor which is far less compared to *puf-8(q725); lip-1(zh15)* worms that were 100% tumorous at 25^{0}C(Cha, Datla, Hollis, Kimble, & Lee, 2012). The results were shown in detail in ***fig. 4***. It is important to note that these *puf-8; lip-1* worms are MOG (Masculinization of gonads: excess sperm and no oocytes) at 20^{0}C(Morgan, Lee, & Kimble, 2009) unlike *puf-8(q725)* single mutants. Independently, *lip-1*(RNAi) treated worms or *lip-1(zh15)* null mutants, also called *lip-1(0)* mutants imitate wild type phenotype – fertile either at 20^{0}C or 25^{0}C(Hajnal & Berset, 2002; M. Lee, Hook, Lamont, Wickens, & Kimble, 2005). So, *puf-8* that has a post-transcriptional

regulation works in conjunction with *lip-1* that acts post-translationally to repress the formation of tumors by dedifferentiation(Cha et al., 2012).

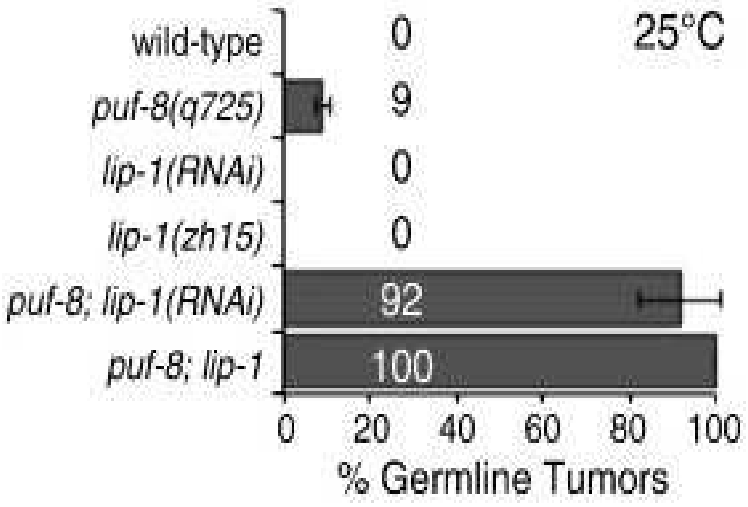


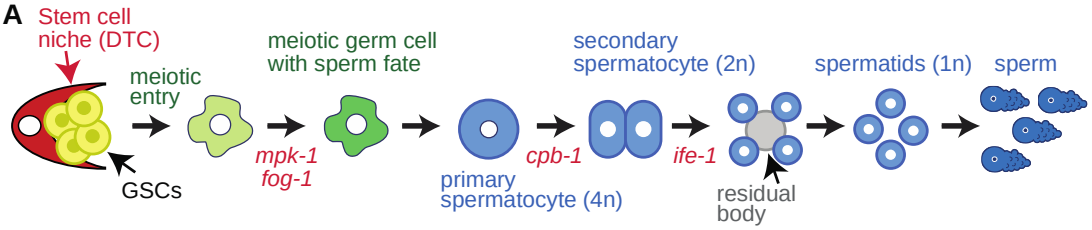
Fig. 4. Graph showing percentage of worms with proximal germline tumor in *puf-8(q725), lip-1(zh15)* single mutants and *puf-8; lip-1* double mutant background with wild type as control at 25^0C – Standard deviation bars were constructed from three independent experiments(Cha et al., 2012).

2.2 Dedifferentiation of secondary spermatocytes to form proximal germline tumor in *puf-8; lip-1* mutant.

Dr. Subrahmaniam *et al.* proposed the theory that it is the primary spermatocytes that dedifferentiate/revert back to form undifferentiated tumor initiating cell-like cells, termed as the proximal germline tumor in *puf-8(0)* mutants(Subramaniam & Seydoux, 2003). To test if the same theory is applicable for germline tumor development in the *puf-8; lip-1* double mutant, we did cellular analyses of dedifferentiation where we blocked the successive key steps of development in spermatogenesis process by doing RNAi to specific genes which regulate this

process(L'Hernault, 2006). A schematic of the spermatogenesis process along with the list of various regulators critical during various stages of spermatogenesis process has been shown in ***fig. 5. a, b***. In the initial stages of spermatogenesis, MPK-1/ERK MAPK signaling controls the progression of meiotic cells through pachytene stage(M. Lee et al., 2007). Progressing forward slowly, the two cytoplasmic polyadenylation element binding (CPEB) proteins, FOG-1 and CPB-1 are necessary for sperm fate specification and differentiation of primary spermatocytes to secondary spermatocytes respectively(Barton & Kimble, 1990; Luitjens, Gallegos, Kraemer, Kimble, & Wickens, 2000). To our surprise, we found that *puf-8; lip-1* mutants in *mpk-1b* RNAi, *fog-1* RNAi or *cpb-1* RNAi had no tumors suggesting that these tumors are formed from the spermatocytes at a stage crossing primary spermatocytes(Cha et al., 2012). So, finally we tested using RNAi of *ife-1*, one of five *C. elegans* mRNA cap binding eIF4E proteins, and found that the *puf-8; lip-1* worms are all tumorous suggesting that it is the 2^0 spermatocytes that dedifferentiated into proximal germline tumors(Cha et al., 2012) since *ife-1* RNAi blocked the progress of germline development by blocking at the 2^0 spermatocyte stage(Henderson et al., 2009). We performed these RNAi experiments by feeding the *puf-8; lip-1* double mutant worms with the specific RNAi starting from L1 stage at 25^0C and then observed the tumorous phenotype by dissecting and DAPI staining 1.5 day adult (1.5 days after L4) worms. The results were shown in detail in ***fig. 5. c.***

A



B

Gene	Homologous	RNAi phenotypes	Ref.
mpk-1	ERK MAPK	arrest germ cells in pachytene	Lee, Min-Ho et. al., 2007.
fog-1	Cytoplasmic polyadenylation element binding protein (CPEB)	switch germ cells from spermatogenesis to oogenesis	Barton and Kimble, 1990.
cpb-1	CPEB	arrest germ cells in 1° spermatocytes	Luitjens et. al., 2000.
ife-1	Cap-binding protein eIF4E	arrest germ cells in 2° spermatocytes	Henderson et. al., 2009

C

Strains	℃	% Germline Tumors	n
puf-8; lip-1; vector(RNAi)	25	100	61
puf-8; lip-1; mpk-1(RNAi)	25	0	56
puf-8; lip-1; fog-1(RNAi)	25	18	17
puf-8; lip-1; cpb-1(RNAi)	25	29	84
puf-8; lip-1; ife-1(RNAi)	25	100	71

Fig. 5. **a.** Schematic of spermatogenesis process in *Caenorhabditis elegans*. **b.** List of various regulators critical during various stages of spermatogenesis process in *Caenorhabditis elegans*. **c.** Germline tumor scoring in *puf-8; lip-1* mutant background exposed to RNAi of the key regulators of spermatogenesis process(Cha et al., 2012).

2.3 MPK-1/ERK MAPK signaling is required for dedifferentiation through RSKN-1/$P90_{RSK}$, a downstream effector of MPK-1/ERK.

Previously it was established that PUF-8 and LIP-1, the two negative regulators of MPK-1/ERK MAPK signaling, work in conjunction to direct/promote the gametogenesis process towards oogenesis and inhibit spermatogenesis(Morgan et al., 2009). This has been determined by observing 100% MOG phenotype in *puf-8; lip-1* knockout mutants at 20ºC(Morgan et al., 2009). So, we wanted to see if similar mechanism is involved in dedifferentiation process at 25^0C. So, we performed RNAi of *mpk-1b* (isoform of *mpk-1* which is expressed in germline(M. Lee et al., 2007)) in *puf-8; lip-1* worms at 25^0C. Interestingly, it was found that the *puf-8; lip-1* tumor phenotype was rescued and all the *puf-8; lip-1* worms were fertile(Cha et al., 2012). To find out the downstream target of MPK-1/ERK MAPK signaling, we selected to perform RNAi of *rskn-1* and found the rescue of tumor phenotype again suggesting that MPK-1/ERK MAPK signaling is required for dedifferentiation and prevents meiotic division through aberrant activation of RSKN-1/$P90_{RSK}$, a downstream effector of MPK-1/ERK(Arur et al., 2009; Cha et al., 2012). The results were shown in detail in **fig. 6. *a, b.*** The reason behind selecting RSKN-1 despite the fact that there are several downstream effectors of MAPK signaling is because $P90_{RSK}$ (P90 Ribosomal S6 Kinase), the RSKN-1 homolog in vertebrates has been identified as a direct ERK substrate which is an effector of ERK–induced transition during meiosis(Maller et al., 2002; Schmitt, Gutierrez, Lénárt, Ellenberg, & Nebreda, 2002).

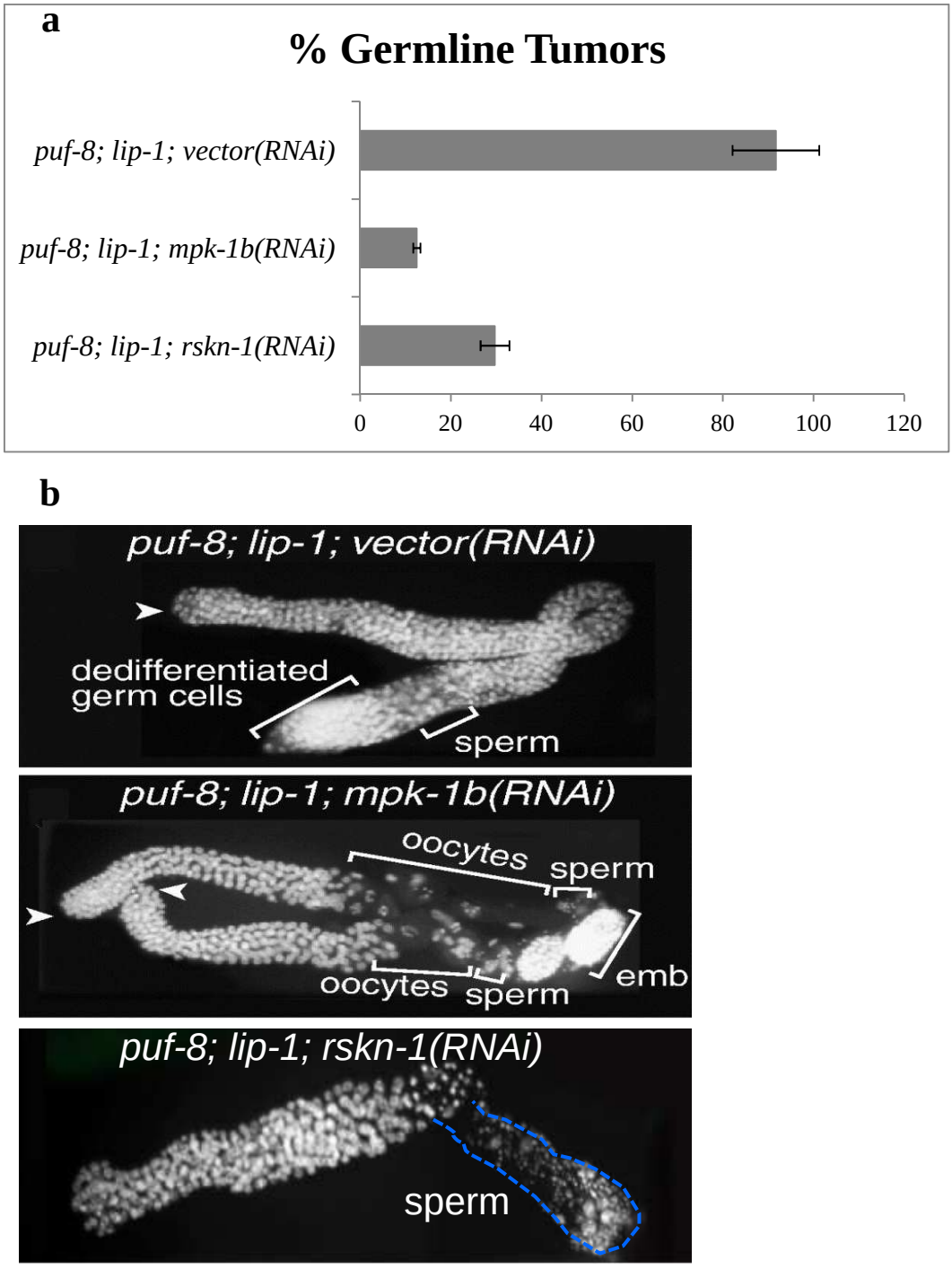


Fig. 6. a. Graph showing percentage of worms with proximal germline tumor in 1 day adult *puf-8; lip-1* mutants that are control (*vector* RNAi) treated, *mpk-1b* RNAi treated and *rskn-1* RNAi treated and raised at 25^0C. Standard deviation bars were constructed from three independent experiments. ***b.*** DAPI stained adult hermaphrodite germlines of *puf-8; lip-1; vector*(RNAi) and *puf-8; lip-1; mpk-1b*(RNAi) and *puf-8; lip-1; rskn-1*(RNAi)(Cha et al., 2012).

Discussion

The tumor cell-like undifferentiated cells formation by dedifferentiation concept which when analyzed in depth can serve the purpose of clarifying more details pertaining to mechanisms of tumor development at an advanced level in higher organisms. The results of this study indicate that *puf-8* exhibits various roles depending on genetic context. It was previously found that in the distal most mitotic region of the *C. elegans* germline where the Germline stem cells self-renew, PUF-8 acts in conjunction with another RNA binding protein MEX-3 (Muscle Excess-3, KH domain containing protein) and function redundantly to maintain the stock of Germline stem cells (GSCs) by promoting their proliferation/self renewal through mitoses(Ariz, Mainpal, & Subramaniam, 2009). Further proximally along the germline, these GSCs undergo meiotic divisions and finally differentiate to form sperm and oocytes. PUF-8 along with LIP-1 are responsible for maintaining this meiotic state by inhibiting dedifferentiation through MPK-1/ERK MAPK signaling(Cha et al., 2012). Thus, PUF-8 has dual opposing roles in the *C. elegans* germline such as the stem cell maintenance by promoting mitosis in the distal mitotic region and maintenance of meiotic state in the meiotic zone where it prevents their exit from meiosis. Similarly, MPK-1/ERK MAPK signaling has various opposite roles. In the mitotic region it promotes differentiation(M. Lee et al., 2005) while it maintains meiosis in pachytene region and promotes oocyte maturation during oogenesis(M. Lee et al., 2007). As such, in *puf-8; lip-1* MOG germlines at 20^0C, the MPK-1/ERK MAPK signaling promotes sperm fate specification(M. Lee et al., 2007) while it promotes dedifferentiation in the same at 25^0C(Cha et al., 2012). This difference can be explained as a result of genetic context.

This regulation is conserved in mammals where ERK2 and p38 MAPKs, two known Pum2 targets promotes differentiation of embryonic stem cells(Burdon, Smith, & Savatier, 2002), but in differentiated cells like Sertoli cells(X. Zhang et al., 2006), myoblasts(Chen et al., 2005) and islet cells(Hanley et al., 2011), activated ERK2 MAPK promotes dedifferentiation and proliferation(Whelan, Hollis, Cha, Asch, & Lee, 2012a; W. Zhang & Liu, 2002). Importantly, combined chemical inhibition of ERK signaling and other signaling pathways has been shown to improve the efficiency of human iPSC (induced pluripotent stem cells) generation from human fibrobrasts(Lin et al., 2009). It speculates that Pum2 may also act as a key regulator for cell fate reprogramming in mammals. Although this idea remains a major challenge, recent discoveries in *C. elegans* may provide an important model for mammalian cell fate reprogramming and may have implications for regenerative medicine and cancer therapy in humans.

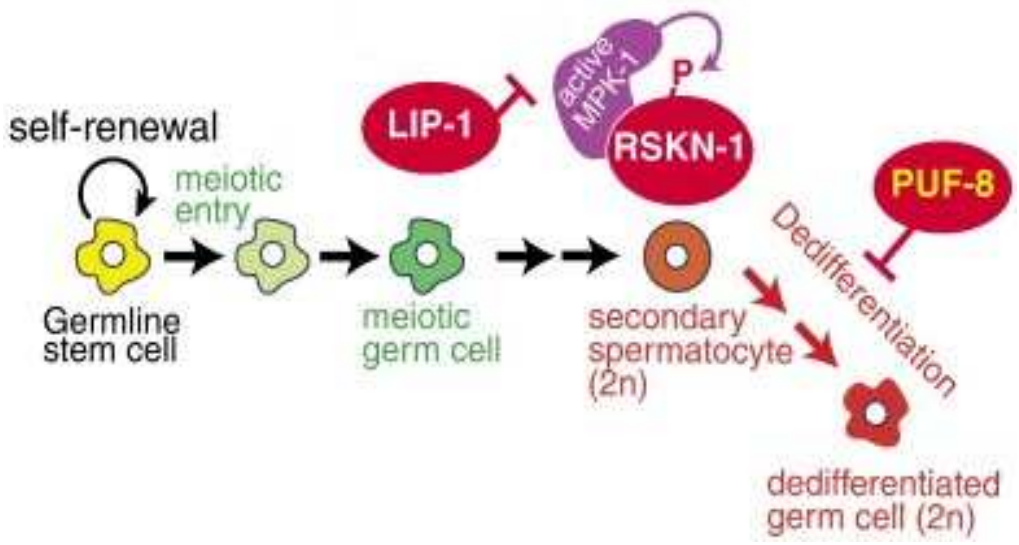


Fig. 7. Overall working model for molecular control of Dedifferentiation-mediated proximal germline tumor development in *Caenorhabditis elegans*(Cha et al., 2012).

CHAPTER 3

Hypoxic Effect on Germline Tumor Development in *Caenorhabditis elegans*.

Introduction

Hypoxia is a condition induced when oxygen levels reach below 2%(Epstein et al., 2001; H. Jiang, Guo, & Powell-Coffman, 2001; Shen & POWELL-COFFMAN, 2003). In hypoxic conditions, the HIF-1 (Hypoxia Inducible factor 1) transcriptional factor that is stabilized is very important to maintain oxygen homeostasis for the survival and normal functioning of cells since oxygen, being the last electron acceptor in aerobic respiration process is required for energy generation(Semenza, 1999). It is a group of heterodimeric transcriptional factors(B. Jiang, Rue, Wang, Roe, & Semenza, 1996; G. L. Wang & Semenza, 1995; G. L. Wang, Jiang, Rue, & Semenza, 1995) controlling the expression of VEGF (vascular endothelial growth factor) which promotes angiogenesis – formation of new blood vessels to enhance oxygen supply to the hypoxic cells, regulates erythropoietin to control formation of red blood cells and also controls the expression of several other genes(Elvidge et al., 2006; Manalo et al., 2005) (detailed in ***fig. 9***). HIF-1 overexpression in tumor cells results in further proliferation of the tumor by enhanced angiogenesis, red blood cell formation and other processes to maintain oxygen homeostasis(Semenza, 2002; Semenza, 2009).

In mammals, there is one of three alpha subunits (HIF-1α, HIF-2α or HIF-3α) and one beta subunit (ARNT or ARNT2). The alpha subunit is regulated by oxygen, and in the absence of oxygen binds to the beta subunit and together, the HIF functions as transcriptional factor. *C. elegans* HIF-1 was first identified by Jiang *et al.* It has two subunits – HIF-1 homologous to HIF-

α subunit and AHA-1 homologous to HIF-1β subunit of mammals(H. Jiang et al., 2001; Powell-Coffman, Bradfield, & Wood, 1998). Not all functions of HIF-1 in higher organisms are conserved in *C. elegans*, yet the mechanism underlying the mode of action of HIF-1 protein, encoded by *hif-1* gene and its role in protection against hypoxia or under stress conditions are conserved.

In normoxia conditions when oxygen is abundant, the PHD (prolyl hydroxylase domain) of *C. elegans* EGL-9 catalyzes a hydroxylation reaction by adding an –OH group to the proline amino acid residue at position 621 in HIF-1 and then VHL-1 that targets the HIF-1 at this position, binds and degrades it. This is how the role of PHD was first discovered in *C. elegans* by studying the mode of action of EGL-9(Epstein et al., 2001). Similarly, in vertebrates, the PHD 1, 2 and 3 (also termed HPH or EGLN) plays the role of EGL-9 in mediating the hydroxylation reaction. First, the two proline residues (Pro-564 and Pro-402) in two oxygen-dependent degradation domains (ODDD) located in the center of HIF-1α and HIF-2α proteins are hydroxylated. They are then degraded by the proteosome pVHL (the Von Hippel-Lindau tumor suppressor protein), a E3 ubiquitin ligase orthologous to VHL-1 in *C. elegans* (***fig. 10***)(Huang, Zhao, Mooney, & Lee, 2002; Ivan et al., 2001; Jaakkola et al., 2001). Whereas in hypoxia, the HIF-1 protein is stabilized and binds to AHA-1 that is homologous to HIF-1β/ARNT of mammals, and then this HIF-1-AHA-1 dimer binds to the DNA sequences that contain hypoxic regulatory elements(H. Jiang et al., 2001). The overall view of HIF-1 (Hypoxia Inducible Factor homolog) regulation during normoxia and hypoxia in *C. elegans* has been shown in ***fig. 8.*** The conservation of all these regulators makes *C. elegans* a good model system for studying hypoxia. It is evident that the *vhl-1(ok161)* deletion mutants have high levels of HIF-1 protein while *hif-1(ia4)* loss-of-

function mutants that appear wild type in normoxia are highly sensitive to hypoxia(Epstein et al., 2001; H. Jiang et al., 2001).

In mammals, it was also found by another group that EGL-9 also acts to regulate HIF-1 activity through a mechanism independent of VHL-1. This was supported by quantitative PCR and protein blot experiments in which the results showed that in *egl-9* mutants, the expression of the target genes of HIF-1 was higher than that in *vhl-1* mutants. The HIF-1 protein expression levels were also higher in *egl-9* mutants compared to *vhl-1* single mutants. Hence, EGL-9 functions to repress transcription of HIF-1 by two independent mechanisms though the mechanism independent of VHL-1 activity still remains unknown(Shao, Zhang, & Powell-Coffman, 2009; Shen, Shao, & Powell-Coffman, 2006).

In mammals, there are other factors that influence stability of HIF-1 as well. In normoxia, the FIH (Factor inhibiting HIF-1) prevents the binding of CBP/p300 transcriptional co-activator to HIF-1 by hydroxylating the asparagine amino acid residue in the transcriptional activation domain towards the C-terminal end of HIF-1(Lando et al., 2002; Lando, Peet, Whelan, Gorman, & Whitelaw, 2002; Mahon, Hirota, & Semenza, 2001). Similarly, the binding of CBP/p300 transcriptional co-activator to HIF-1 is enhanced by phosphorylating the transcriptional activation domain towards the C-terminal end of HIF-1. In hypoxia, these enzymatic regulators of HIF-1, such as the PHD and FIH are inhibited to stabilize HIF-1 and allow it's binding to CBP. Hence, phosphorylation and hydroxylation are the two major post-translational modifications of HIF-1 that regulates stability of HIF-1 based on availability or absence of oxygen(B. Jiang et al., 2001; Treins, Giorgetti-Peraldi, Murdaca, Semenza, & Van Obberghen, 2002; Zhong et al., 2000).

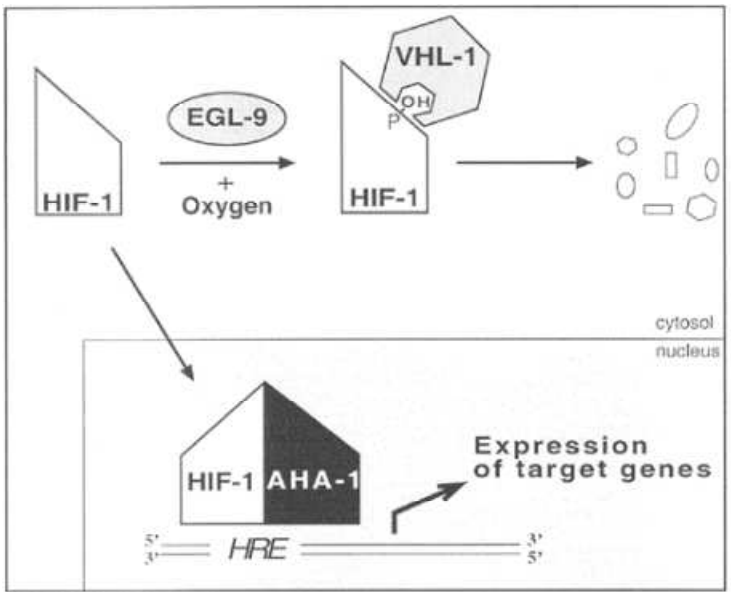


Fig. 8. Overall view of HIF-1 (Hypoxia Inducible Factor homolog) regulation during normoxia and hypoxia in *Caenorhabditis elegans*(Epstein et al., 2001; Shen & POWELL-COFFMAN, 2003).

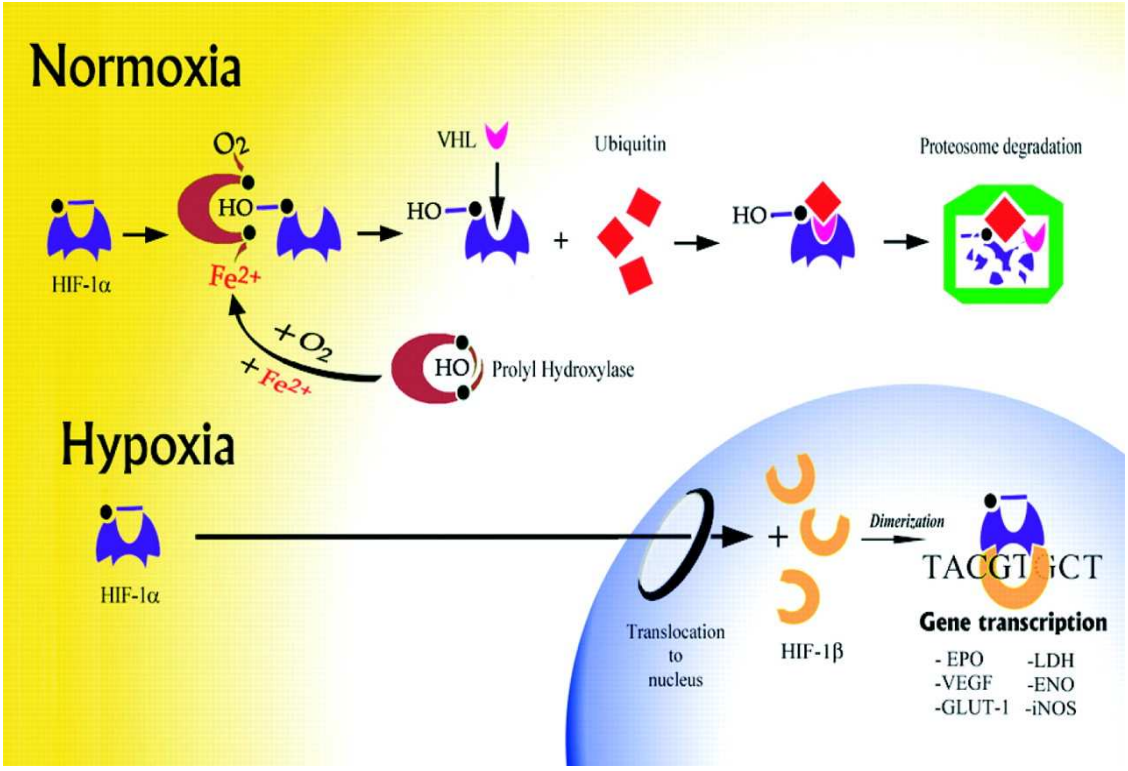


Fig. 9. Schematic of mode of HIF-1 (Hypoxia Inducible Factor homolog) expression of target genes during hypoxia (Drawing by Max. Neal)(LaManna, Chavez, & Pichiule, 2004).

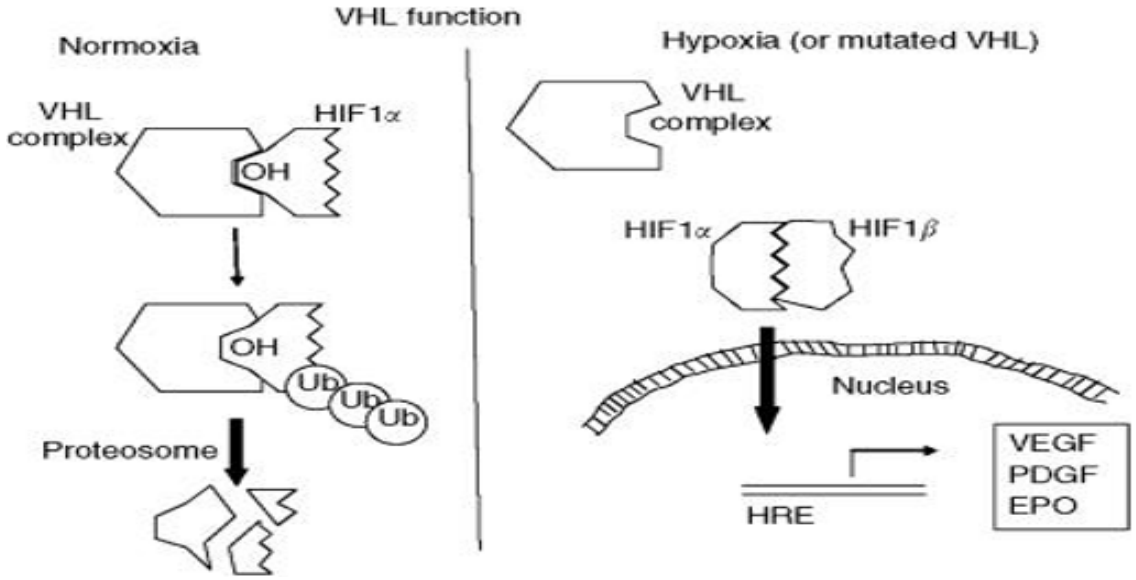


Fig. 10. Diagrammatic representation of mode of VHL-1 (Von Hippel-Lindau tumor suppressor homolog) action during normoxia and hypoxia(Patel, Chaganti, & Motzer, 2006).

Specific Materials and Methods

Cobalt chloride NGM Agar Plates

About 0.059 g of cobalt chloride crystalline solid is dissolved in 10 ml of autoclaved distilled water to make 25mM of cobalt chloride solution. Then, 4 ml of this 25 mM solution is added to 1 liter NGM agar solution (explained in general materials and methods) to make the final concentration to 100µM. This NGM agar solution is then autoclaved and plated. OP50 *E. coli* bacteria inoculated in 2XYT buffer is spread on these cobalt chloride NGM agar plates to feed *C. elegans*

RNAi interference

We used the *vhl-1* RNAi from RNAi feeding library for our experiment with RNA interference and then confirmed it by generating *vhl-1* deletion mutant to avoid the problem of non-specific silencing of the genes by RNAi technique sometimes.

C. elegans strains

All strains were maintained at 20°C as per the standard protocol(Brenner, 1974) except for the transgenic strains which were maintained at 25ºC to prevent the germline silencing of these transgenes(Seydoux & Strome, 1999). The mutants used were: *LGII: puf-8(q725), LGIII: unc-32(e189) glp-1(ar202), LG1V: lip-1(zh15), LGV*: *hif-1(ia4),* and *LGX*: *vhl-1(ok161).* The compound mutants *puf-8(q725); lip-1(zh15), puf-8(q725); lip-1(zh15); hif-1(ia4), puf-8(q725); lip-1(zh15); vhl-1(ok161), puf-8(q725); lip-1(zh15); hif-1(ia4); vhl-1(ok161), unc-32(e189) glp-1(ar202); hif-1(ia4)* and *unc-32(e189) glp-1(ar202); vhl-1(ok161)* that were generated by genetic crosses as described in the results section in this chapter were used to study the phenotype. On the other hand, the transgenic lines HIF-1(WT):myc and HIF-1(P621G):myc(Y. Zhang, Shao, Zhai, Shen, & Powell-Coffman, 2009) were used for immunohistochemistry studies to study the efficacy of hypoxic chamber and cobalt chloride, the chemical inducer of hypoxia.

Single worm PCR

The single worm hypothesized as one of the potential candidates for being the mutant is placed in a PCR tube containing 10 µl of worm lysis buffer with proteinase K at a concentration of 100 µg/ml proteinase K in worm lysis buffer, then frozen at -80ºC for about 30 min. to facilitate proper lysis of the worm. Then, it is heated to 60ºC for about 60 min. to complete cell lysis which is then followed by 15 min. heating to 95ºC in order to inactivate the proteinase K.

About 5µl of this lysate is added to 45 µl of PCR "master mix" in a PCR tube to run the primary PCR (50 µl reaction). The master mix includes 1X PCR buffer, 0.5 µM sense and anti-sense primers, 0.2 mM dNTP mix and 0.001 units Taq DNA polymerase. The samples are rapidly heated to 94°C for 10 min. (pre-denaturation step) and then cycled 30 to 35 times through the following steps:

(i) Denaturation step: 94°C for 30 sec.

(ii) Annealing step: ~5°C less than the least melting temp. of the primers used for 30 sec.

(iii) Polymerization step: 72°C for 45 sec.

The time period for the denaturation, annealing and polymerization steps vary depending on the length of template DNA. Upon completion of the cycle, finally the samples are heated to 72ºC for 10 min. to ensure that the polymerization step is complete.

About 5 µl of the primary PCR product is then used to run the nested PCR (subsequent cycle of amplification to increase the sensitivity and specificity of PCR) and internal PCR (to identify the mutation on both the alleles – for homozygote) reactions independently using a different set of sense and anti-sense primers (***Table 1***) and the respective annealing temperatures (55^0C for hif-1 and 58^0C for vhl-1). About 10- 15 µl of the final PCR product is run on ethidium bromide gel for analysis of the DNA band under UV illumination to identify the sample heterozygous or homozygous for the desired mutation.

[Worm Lysis Buffer: The following ingredients are added to autoclaved distilled water to obtain the final concentrations as mentioned: Potassium chloride at 50 mM, Tris pH 8.3 at 10 mM, magnesium chloride at 2.5 mM, 0.45% NP-40 (IGEPAL)/Triton X-100, 0.45% Tween-20, 0.01% gelatin.

Proteinase K stock solution: 0.02 g of Proteinase K powder is dissolved in 1 ml of TE buffer (10 mM Tris-chloride, pH 7.5 and 1 mM EDTA in autoclaved distilled water) to obtain 20 mg/ml proteinase K stock solution.

50 µl of proteinase K stock solution is added to 10 ml of worm lysis buffer to obtain a solution of 100 µg/ml proteinase K in worm lysis buffer.]

Table 1. Sequence of Oligos (Eurofins MWG operon, salt free and 25 nmol) used for generation of mutants with *hif-1* and *vhl-1* background.

Gene	Sequence (5' → 3')
hif-1(ia4)1S	TGAAGGTGGAGTAGCGCCAG
hif-1(ia4)1AS	GAAAGTGATGCGCATGTCGC
hif-1(ia4)2S	ATGCCGCATGTTCCGATCCC
hif-1(ia4)2AS	GGTATTCAGAGTCCCTGCTC
hif-1(ia4) internal	GACACCACCGGCATCAACAT
vhl-1(ok161)1S	GATCACCAGAAGATCATCAG
vhl-1(ok161)1AS	CATTGCTGAGGTCTCTGGGG
vhl-1(ok161)2S	CTCACGTTGCATCAGGACTG
vhl-1(ok161)2AS	ACTTCAAATTGTAGTTCTCG
vhl-1(ok161) internal	CGGAGCCAGCTCCTCGAATG

Western blot

Mixed stage worms of HIF-1(WT):myc and HIF-1(P621G):myc were exposed to three different conditions: i) Normoxia – by raising them on normal NGM agar plates, ii) 2% Hypoxia – by raising them in hypoxic chamber containing 2% oxygen and 98% nitrogen gas mixture and iii) Hypoxia mimic condition – by raising them on cobalt chloride plates. After exposing the worms to these conditions for about 1.5 – 2 days, then the protein samples were prepared in SDS-PAGE sample buffer. HIF-1 protein has a very short half-life and degrades very quickly upon onset of normoxia and so it's critical to prepare the lysate samples immediately after taking out from hypoxic chamber. The proteins in the samples were then separated on an 8% gel by SDS-PAGE gel electrophoresis and then electrophoretically transferred onto PVDF (Polyvinylidene difluoride) membrane by wet transfer method. The non-specific binding sites on blot were then blocked with 1% skim milk in 1X PBST overnight at 4'c, followed by probing with primary antibody Mouse Anti-c-Myc Monoclonal Antibody (Invitrogen) in 1:5,000 concentration in 1% skim milk in 1X PBS at room temperature for about 2 hours, followed by washing and incubation with HRP-conjugated anti-mouse (Jackson ImmunoResearch) secondary antibody at a concentration of 1:5,000 in 1% skim milk in 1X PBS at room temperature for 1 hour and developed to detect the protein using chemiluminescent substrate, allowing sensitive detection that is documented using X-ray film. Then the blots were stripped in stripping buffer. They were then re-blocked and re-probed with 1:5,000 Mouse monoclonal anti-α-tubulin (Sigma-Aldrich) followed by 1:5,000 HRP-conjugated anti-mouse (Jackson ImmunoResearch) to standardize the total protein concentration in all the samples.

A. Hypoxic Effect on Dedifferentiation-Mediated Germline Tumor Development in *Caenorhabditis elegans*.

Objective

After the findings of genetic effects on dedifferentiation-mediated tumor formation in *C. elegans* germline, we chose to identify other regulators of dedifferentiation process. We mainly focused on hypoxia to check HIF-1 role in tumor development. This idea has been derived from well-established fact that HIF-1 released during hypoxic conditions in tumor promotes further proliferation of the tumor(Semenza, 2009). This aroused the curiosity of whether HIF-1 controls dedifferentiation-mediated tumor development in *C. elegans* germline or not. This novel idea however focuses on the role of HIF-1 in tumor formation via dedifferentiation but does not explain HIF-1 role in proliferation of already formed tumor. The reason for focusing on HIF-1 is to see if HIF-1 also has an opposite role like PUF-8 in mitotic/meiotic switch(Bachorik & Kimble, 2005) and MPK-1/ERK MAPK signaling in it's role in differentiation and dedifferentiation as explained in discussion part in previous chapter(Cha et al., 2012; M. Lee et al., 2005; M. Lee et al., 2007). We wanted to see if HIF-1 promotes or inhibits dedifferentiation process.

Results

3.1 Hypoxic condition suppresses germline tumor development in *puf-8; lip-1* mutant.

For studying the effect of hypoxia on dedifferentiation-mediated germline tumor development, we exposed L4 stage *puf-8; lip-1* worms to normoxia and 2% hypoxic conditions at 25^0C.

Hypoxic condition is maintained by using a hypoxic chamber in which the worms in NGM agar plates are exposed to a mixture of 2% oxygen and 98% nitrogen gases. This mixture of gases is flushed through the hypoxic chamber initially for 5 minutes and then the hypoxic chamber is sealed air-tight by closing the inlet and outlet openings after being filled with this gas mixture. The worms are then dissected after 1.5 days – 1.5 day adult and DAPI stained to observe the phenotype. As per our expectations, we found that the germline tumor percentage in *puf-8; lip-1* worms exposed to hypoxic condition is less than that in normoxia (results not shown).

We faced several drawbacks using the hypoxia chamber like:

a) The worms cannot be checked for starvation once they are placed in the hypoxic chamber as there is a risk of the worms getting exposed to normoxic condition for some time when doing so and this disturbs the continuity of hypoxic condition.

b) It is difficult to identify if the mixture of gases inside the hypoxic chamber can sustain the worms without the necessity of refilling the hypoxic chamber.

c) It is difficult to monitor the stage of the worms due to the risk of the worms getting exposed to normoxic condition for some time when doing so and this disturbs the continuity of hypoxic condition.

3.2 Hypoxia mimic condition using 0.1 mM cobalt chloride suppress germline tumor development in *puf-8; lip-1* mutant.

In order to reduce these experimental setbacks, we proposed to substitute the hypoxic chamber with cobalt chloride, a chemical inducer of hypoxia(G. L. Wang & Semenza, 1993). This idea is implemented by using NGM plates with cobalt chloride (explained in materials and methods

section) to mimic hypoxic conditions. Western blot experiment was performed to confirm if HIF-1 levels are increased in an equal fashion with hypoxic chamber and cobalt chloride. We used HIF-1:myc and HIF-1(P621G):myc transgenic lines with varied expression levels of HIF-1 protein for this experiment. In HIF-1:myc transgenic line resembling wild type, the covalent modification resulted from the hydroxylation of proline amino acid residue at position 621 in HIF-1 by EGL-9 mediates HIF-1 degradation by VHL-1(Epstein et al., 2001). Whereas HIF-1(P621G):myc transgenic line is a gain-of-function mutation where the proline amino acid residue at position 621 is converted to glycine amino acid residue and this prevents *egl-9/vhl-1* mediated degradation of HIF-1 protein and hence this transgenic line exhibit elevated levels of HIF-1 even in normoxic conditions due to the stabilization of the HIF-1 protein(Y. Zhang et al., 2009). A diagram of *hif-1* minigene is shown in ***fig. 11***.

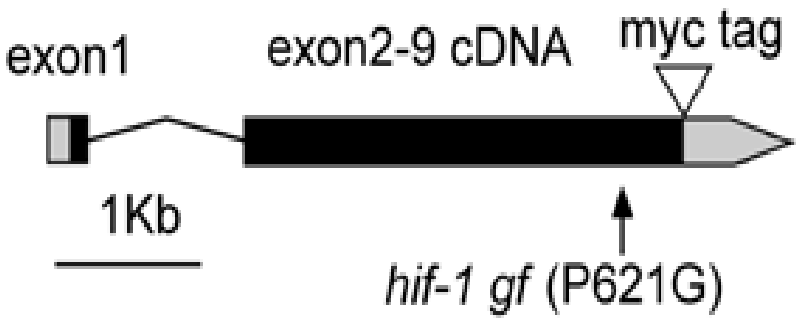


Fig. 11. Pictorial representation of the hif-1 minigene which is a fusion of genomic sequence which includes hif-1 promoter sequence, exon 1, intron 1 and cDNA sequence which includes exons 2-9 for the hif-1 mRNA isoform hif-1a with a c-myc epitope tag inserted(Y. Zhang et al., 2009).

It was observed that in both the transgenic lines, the levels of HIF-1 protein were greatly increased in the 0.1 mM cobalt chloride treated worms and 2% hypoxia exposed worms in an almost equal fashion in comparison with the worms grown in normoxic conditions (***fig. 12***). The

reason behind this indirect approach of using the transgenic lines with myc epitope tag inserted to the *hif-1* gene for the western blot experiment is that it is easier to probe the blot with Mouse Anti-c-Myc Monoclonal Antibody, commercially successful antibody producing good results than Rabbit polyclonal Anti-HIF-1α antibody with which we failed to probe the blot from the protein samples of wild type worms in normoxic, hypoxic and cobalt chloride conditions.

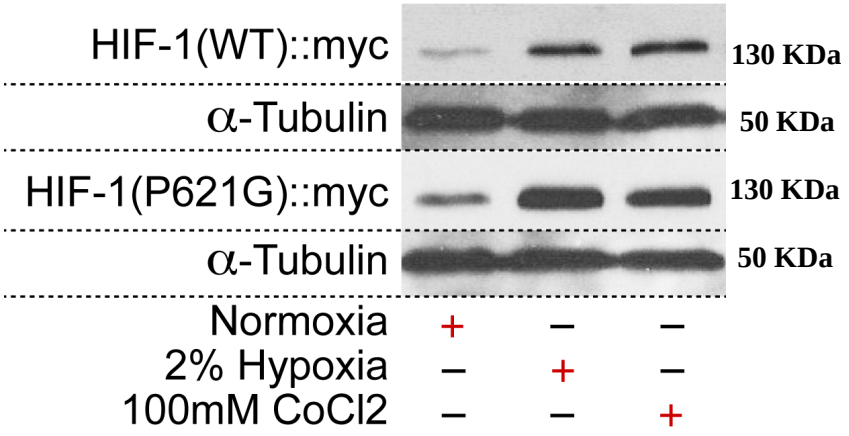


Fig. 12. Western blot for HIF-1 protein estimate in normoxia, 2% hypoxia and hypoxia mimic conditions using 0.1 mM cobalt chloride using the transgenic lines HIF-1(WT):myc and HIF-1(P621G):myc. HIF-1:myc is ~130 kDa and α-tubulin is ~50kDa. In both the transgenic lines – HIF-1(WT):myc and HIF-1(P621G):myc, HIF-1 protein levels are elevated in 2% hypoxic condition using hypoxic chamber and similarly in hypoxia mimic condition.

For producing results, the NGM plate with *puf-8; lip-1* mutant worms was treated with alkaline hypochlorite for embryo isolation, followed by overnight synchronization of the embryos that hatched to L1 larva in M9 buffer. Then the worms were plated and allowed to grow until L4 larval stage at 20^0C when they were divided into two groups – one group exposed to normal conditions and the other group transferred to cobalt chloride plates and upshifted to 25^0C. Later, they were dissected at 1.5 day adult stage and 2 days adult stage and DAPI stained. When scored for tumor percentage, *puf-8; lip-1* worms exposed to cobalt chloride plates had significantly lesser tumor percentage than those in normal NGM agar plates (***fig. 13***). We proposed that HIF-1

(Hypoxia inducible factor – 1) protein has a role in suppressing the germline tumor formation in *C. elegans* after observing consistent results.

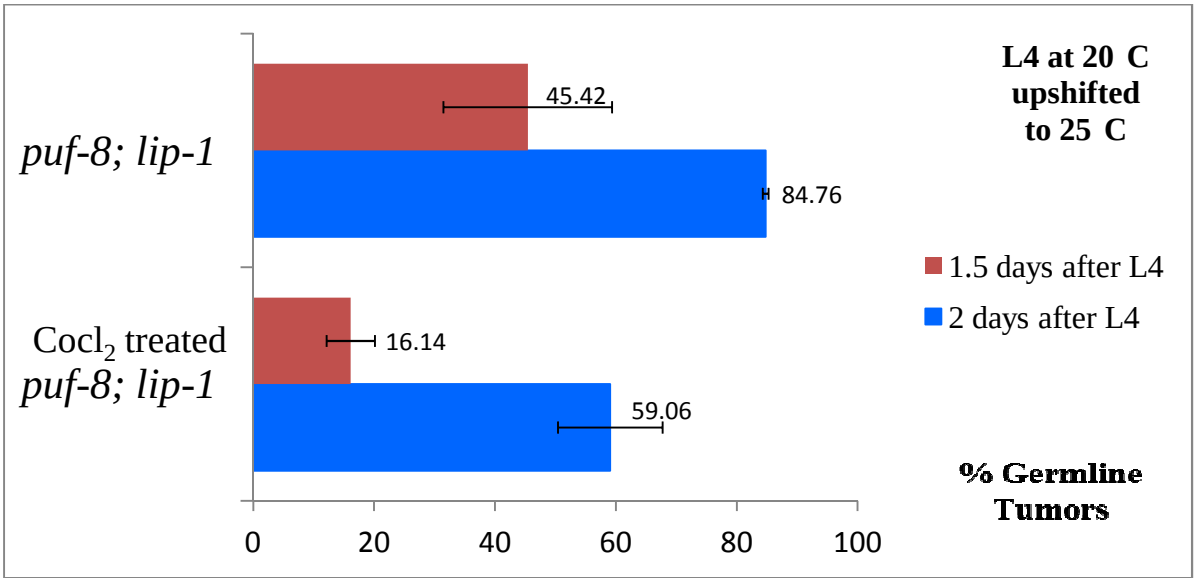


Fig. 13. Graph showing percentage of worms with proximal germline tumor in *puf-8; lip-1* mutants at 1.5 days and 2 days after L4 stage worms upshifted to 25^0C in normoxia and hypoxia mimic condition using cobalt chloride. Standard deviation bars were constructed from three independent experiments.

3.3 Expression/activation of HIF-1 by exposing *puf-8; lip-1* worms to VHL-1 RNAi suppresses germline tumor development.

Hence, we decided to study the hypoxic effect on tumor development through a genetic approach. We used RNAi approach initially to get quick results. We chose to perform RNAi of *vhl-1* in *puf-8; lip-1* and scored tumor percentage and again noticed matching results. In this experiment, the *puf-8; lip-1* worms were raised at 20^0C in *vhl-1* RNAi and then dissected, DAPI stained to score the tumor phenotype at 1D, 2D, 3D and 4D adult stage successively to check the increase in tumor percentage compared to the *puf-8; lip-1* worms exposed to *vector* RNAi (control treatment) at each stage respectively. In the control treated worms, though there was no tumor formation initially in 1 day and 2 days adult worms, majority of them were tumorous on

3rd day and all were tumorous on the 4th day. A similar pattern of increase in tumor percentage was observed in *vhl-1* RNAi treated worms but to a much lesser extent compared to the control (*vector* RNAi) treated worms, implying that the increased levels of HIF-1 protein due to VHL-1 knockout by RNAi has a significant role in tumor suppression (***fig. 14. a, b***). When the same experiment was setup at 25^{0}C, all the *vhl-1* RNAi treated worms were tumorous and this is because the RNAi results are not effective at 25^{0}C i.e. the RNAi effect cannot surpass the temperature effect on tumorigenesis.

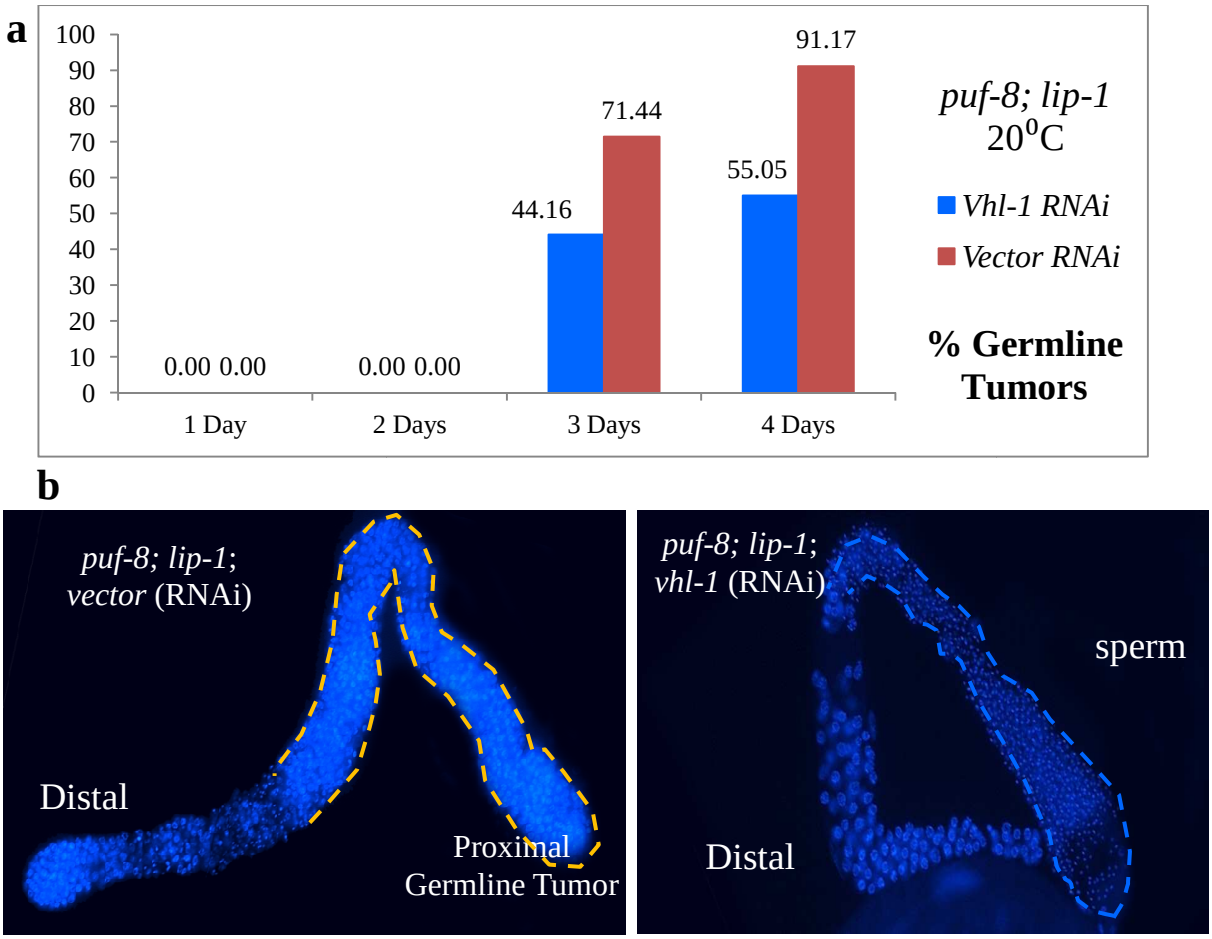


Fig. 14a. Graph showing percentage of worms with proximal germline tumor at 1 day, 2 day, 3 day and 4 day adult *puf-8; lip-1* mutants that are control (*vector* RNAi) and *vhl-1* RNAi treated and raised at 20^{0}C. **b.** DAPI stained adult hermaphrodite germlines of *puf-8; lip-1; vector* (RNAi) and *puf-8; lip-1; vhl-1*(RNAi).

3.4 Expression/activation of HIF-1 by *vhl-1* knockout suppresses germline tumor development in *puf-8; lip-1* mutant.

Since RNAi experiments cannot be completely relied upon; we generated *puf-8(q725); lip-1(zh15); vhl-1(ok161)* knockout mutant as described below (***fig. 15***). The results with the mutant were more pronounced where the germline tumor percentage was greatly decreased in this triple mutant worms in comparison with *puf-8; lip-1* double mutant worms (***fig. 18***).

Genetic cross for the generation of *puf-8(q725); lip-1(zh15); vhl-1(ok161)* mutant.

puf-8; lip-1 green males were crossed with *vhl-1* single mutant hermaphrodites to produce the offspring. Among the offspring, all the non-dumpy, green males represent the genotype *mc6g/+; lip-1/+; vhl-1/+*. They were then allowed to mate with *puf-8; lip-1* non green hermaphrodite worms exposed to *mpk-1b* RNAi. The *puf-8; lip-1* worms which are sterile - Mog (masculinization of the germline: no oocytes and excess of sperm) at 20ºC becomes fertile upon feeding on *mpk-1b* RNAi(M. Lee et al., 2007). So, these fertile *puf-8; lip-1* homozygote worms when crossed with the non-dumpy green male worms that resulted from the first cross generates offspring in which 50% of the worms are homozygous for *lip-1* mutation while 25% are homozygous for *vhl-1* mutation. The non-dumpy, green hermaphrodite worms from progeny of the final cross were singled to identify the required mutant – *puf-8/mc6g; lip-1; vhl*-1 by phenotypic and genotypic analysis. *lip-1* mutation was identified by observing the phenotype of the non-green progeny from each singled worm which should be 100% Mog(Morgan et al., 2009). *vhl-1* mutation was identified by genotyping through polymerase chain reaction (PCR).

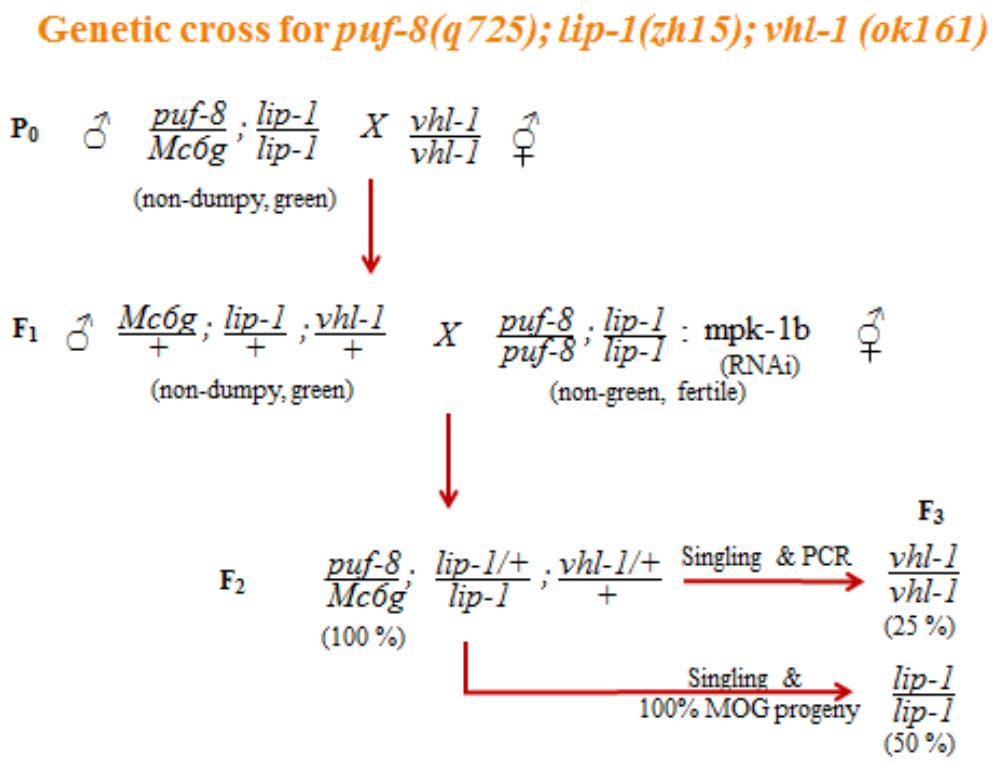


Fig. 15. Schematic of genetic cross for *puf-8(q725); lip-1(zh15); vhl-1(ok161)* mutant.

3.5 Inactivation of HIF-1 by knocking out *hif-1* enhances germline tumor development in *puf-8; lip-1* mutant.

Then we wanted to verify the results in a direct fashion by knocking out *hif-1* expression by generating *puf-8(q725); lip-1(zh15); hif-1(ia4)* triple mutant (***fig. 16***) and when scored for tumor percentage, no change was observed. Similar pattern of tumor percentage was noticed in this triple mutant worms compared to the *puf-8(q725); lip-1(zh15)* worms (***fig. 18***).

Genetic cross for the generation of *puf-8(q725); lip-1(zh15); hif-1(ia4)* mutant.

puf-8; lip-1 green males were crossed with *hif-1* single mutant hermaphrodites to produce offspring. Among the offspring, all the non-dumpy, green males represent the genotype *mc6g/+; lip-1/+; hif-1/+*. They were then allowed to mate with *puf-8; lip-1* non-green hermaphrodite worms exposed to *mpk-1b* RNAi. The *puf-8; lip-1* worms which are sterile - Mog (masculinization of the germline: excess sperm and no oocytes) at 20ºC become fertile upon feeding on *mpk-1b* RNAi(M. Lee et al., 2007). So, these fertile *puf-8; lip-1* homozygote worms when crossed with the non-dumpy green male worms that resulted from the first cross generates offspring in which 50% of the worms are homozygous for *lip-1* mutation while 25% are homozygous for *hif-1* mutation. Hence, the non-dumpy, green hermaphrodite worms from progeny of the final cross were singled to identify the required mutant – *puf-8/mc6g; lip-1; hif*-1 by phenotypic and genotypic analysis. *puf-8; lip-1* mutation was identified by observing the phenotype of the non-green progeny from each singled worm which should be 100% Mog(Morgan et al., 2009). *hif-1* mutation was identified by genotyping through polymerase chain reaction (PCR).

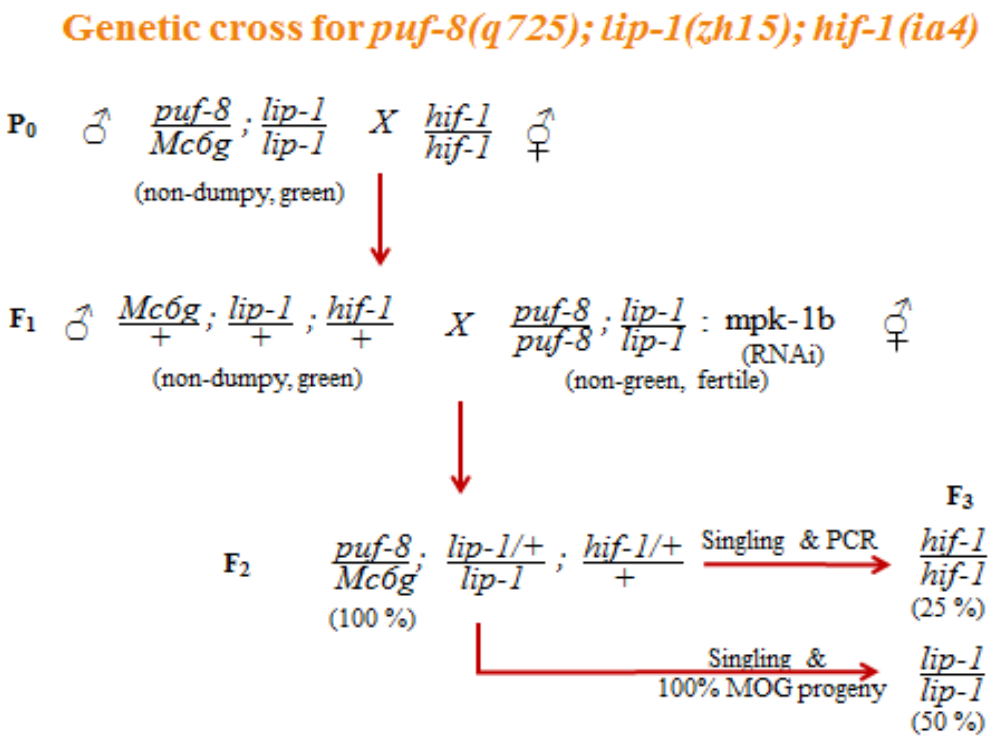


Fig. 16. Schematic of genetic cross for *puf-8(q725); lip-1(zh15); hif-1(ia4)* mutant.

3.6 VHL-1 acts through HIF-1 and not an alternative pathway to suppress germline tumor development in *puf-8; lip-1* mutant.

Next, the question that arises is: If vhl-1 contributes to tumor development through another mechanism independent of HIF-1 and if that is the reason for decreased tumor percentage in the vhl-1 RNAi treated worms or vhl-1 mutant worms. So, to clear this confusion; we generated quadruple mutants with both hif-1 and vhl-1 knocked out (***fig. 17***) and we observed that they are all tumorous indicating that vhl-1 acts through hif-1 (***fig. 18***). Because if there was a mechanism of action of vhl-1 independent of hif-1, then these quadruple mutant worms were supposed to be less tumorous.

Genetic cross for the generation of *puf-8(q725); lip-1(zh15); hif-1(ia4); vhl-1(ok161)* mutant.

puf-8; lip-1; vhl-1 green male worms were crossed with *puf-8; lip-1; hif-1* non-green hermaphrodite worms exposed to *mpk-1b* RNAi. The *puf-8; lip-1; hif-1* worms which are sterile - Mog (masculinization of the germline: excess sperm and no oocytes) at 20ºC become fertile upon feeding on *mpk-1b* RNAi(M. Lee et al., 2007). So, these fertile *puf-8; lip-1; hif-1* homozygote worms when crossed with *puf-8; lip-1; hif-1* green male worms yields the progeny of the genetic constitution *puf-8/mc6g; lip-1; hif-1/+; vhl-1/+*. The non-dumpy, green progeny produced from this cross when singled produces the next generation offspring with a 25% probability for the worms to be *hif-1* homozygous and another 25% probability for the worms to be *vhl-1* homozygous (or) in other words, there is a chance for 1/16 of the offspring to be homozygous for both *hif-1* and *vhl-1* mutations identified by genotyping through polymerase chain reaction (PCR) to obtain the desired quadruple mutant.

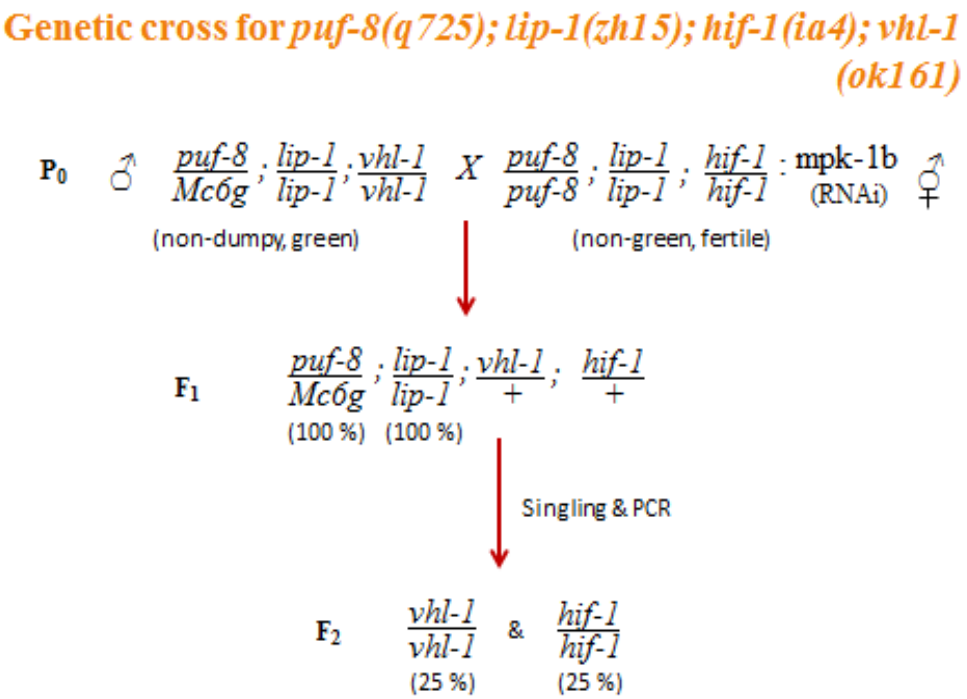


Fig. 17. Schematic of genetic cross for *puf-8(q725); lip-1(zh15); hif-1(ia4); vhl-1(ok161* mutant.

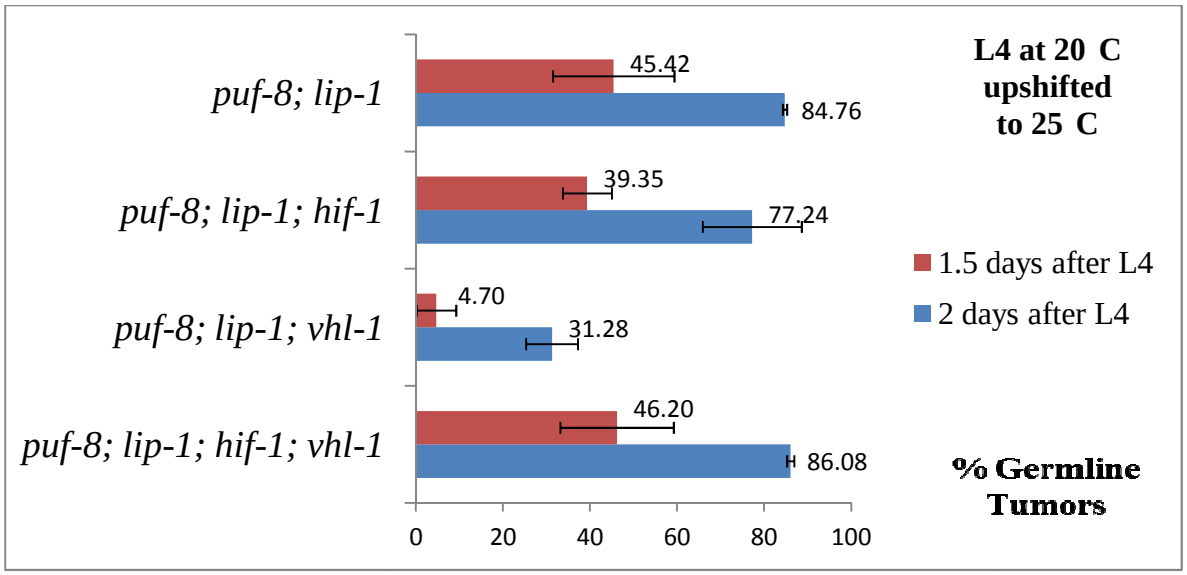


Fig. 18. Graph showing percentage of worms with proximal germline tumor in various *puf-8; lip-1* mutant backgrounds at 1.5 days and 2 days after L4 stage worms upshifted to 25^{0}C. Standard deviation bars were constructed from three independent experiments.

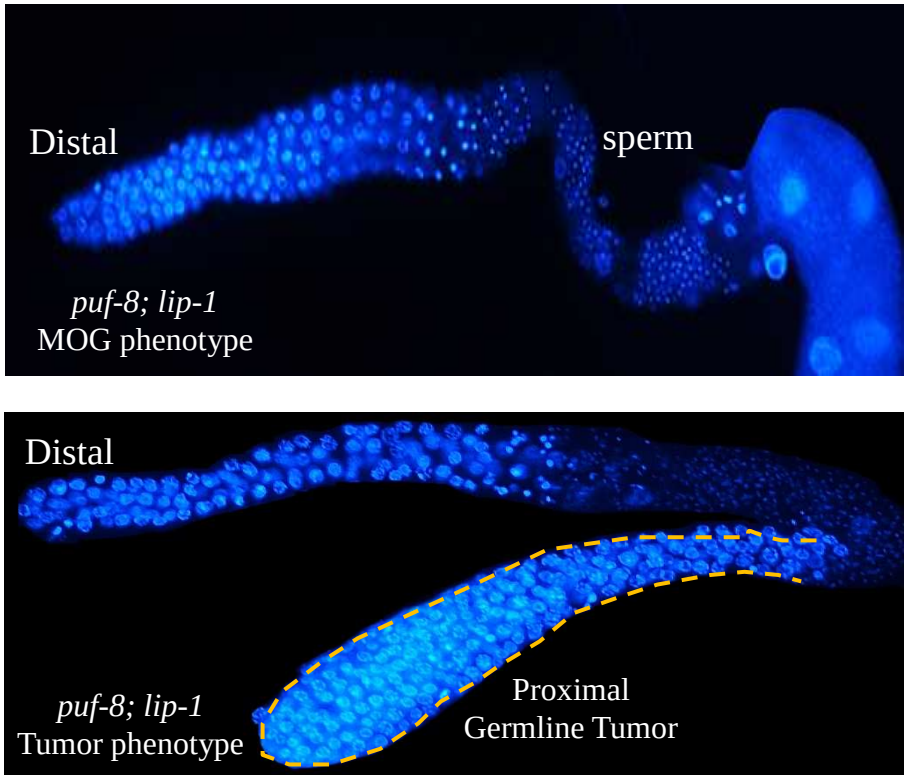


Fig. 19. DAPI stained adult MOG (Masculinization of gonads: excess sperm and no oocytes) and proximal germline tumor phenotypes in *puf-8; lip-1* mutant at 20^{0}C and 25^{0}C respectively.

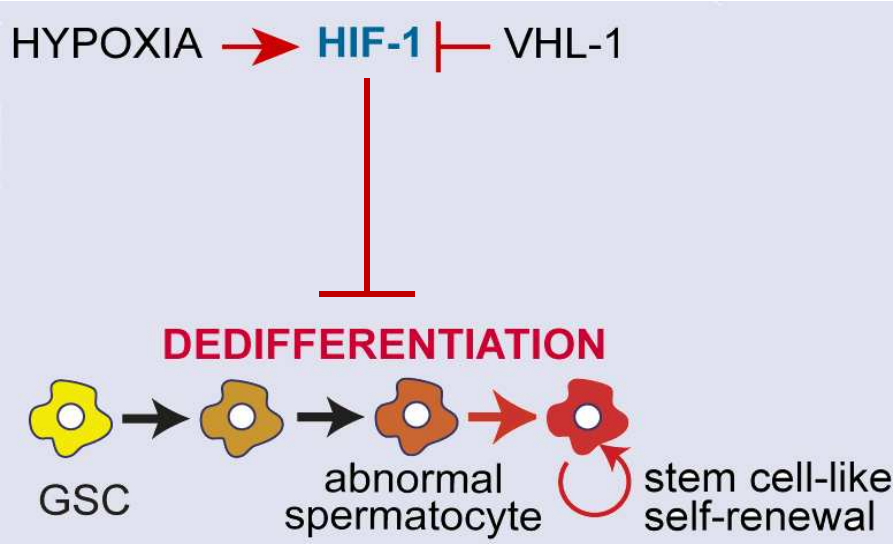


Fig. 20. Overall working model for HIF-1 action on Dedifferentiation-mediated proximal germline tumor development in *Caenorhabditis elegans*.

B. Hypoxic Effect on GLP-1/Notch-Mediated Germline Tumor Development in *Caenorhabditis elegans*.

Objective

Notch signaling plays a key role in the normal development of many tissues and cell types, through diverse effects on proliferation, differentiation, and survival that are highly dependent on signaling strength and cellular context(Allenspach, Maillard, Aster, & Pear, 2002). Aberrant activation of Notch signals potentially contributes to cancer development in several different ways(Allenspach et al., 2002). One example in *C. elegans* germline is that the GLP-1/Notch receptor is highly expressed in the membrane of mitotic germ cells and transduces the GSC niche signal to promote mitotic divisions at the expense of entry into meiosis(Austin & Kimble, 1987; Kimble & Crittenden, 2007; Yochem & Greenwald, 1989). Therefore, hyper-activated GLP-1/Notch (e.g., *glp-1(ar202)* mutant) causes germline tumors characterized by germ cell (or GSC) overproliferation(Berry, Westlund, & Schedl, 1997; Pepper, Killian, & Hubbard, 2003).

So, before generalizing the statement that HIF-1 suppresses germline tumor formation, we decided to test it on germline tumors caused by hyper-activated GLP-1/Notch. This is required to analyze if HIF-1 is specific in action to tumor suppression by acting in conjunction with or parallel to PUF-8 or LIP-1 or both or if HIF-1 acts to repress germline tumor formation through a mechanism independent of PUF-8 and LIP-1. The results are all focused on confirmation of HIF-1 activity on tumor suppression but the mechanism underlying is yet to be unraveled.

Results

3.7 Hypoxia mimic condition using 0.1 mM cobalt chloride suppresses germline tumor development in *unc-32 glp-1(ar202)* mutant.

We followed the same strategy of experiment methodology – step-by-step as in the previous case with *puf-8; lip-1 mutant* i.e. we exposed the *glp-1(ar202)* mutants, henceforth called *glp-1(gf)* gain-of-function worms to normoxic condition and hypoxia mimic condition using cobalt chloride NGM plates and then utilized a genetic approach. While generating the mutants with increased GLP-1/Notch signaling, we used *glp-1(ar202)* worms with a unc (UNCoordinated) mutation in the same chromosome associated with *glp-1* mutation which makes it easy to identify the genotype during the mutant generation process i.e. the worms homozygous for *unc* mutation that have uncoordinated movement indirectly implies homozygosity with respect to *glp-1(ar202)* mutation. The results are discussed in detail.

Embryos of *unc-32 glp-1(ar202)* mutant worms were isolated, plated and allowed to grow until L4 stage at 20^0C and then they were divided into two groups – one group allowed to continue growing on the same NGM agar plates while the other group of worms were transferred onto cobalt chloride NGM agar plates at a concentration of 0.1 mM. They were then upshifted to 25^0C and dissected and DAPI stained 2 days later i.e. 2 day adult stage and scored for tumor percentage. The results were consistent with our previous finding as the worms exposed to cobalt chloride plates (mimic of hypoxia) had less tumor percentage compared to those in normoxia (***fig. 21***). This stresses on the novel finding of suppression of tumor formation by HIF-1 again.

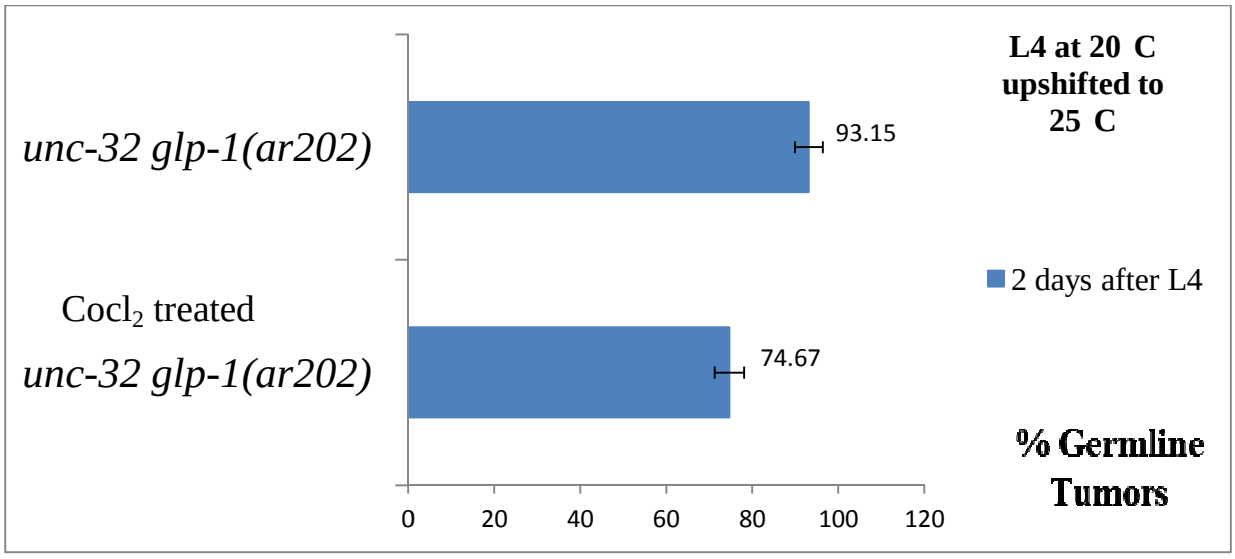


Fig. 21. Graph showing percentage of worms with proximal germline tumor in *unc-32 glp-1(ar202)* mutants at 2 days after L4 stage worms upshifted to 25^0C in normoxia and hypoxia mimic conditions using cobalt chloride. Standard deviation bars were constructed from three independent experiments.

3.8 Expression/activation of HIF-1 by *vhl-1* knockout suppresses germline tumor development in *unc-32 glp-1(ar202)* mutant.

Further, a genetic approach to increase expression of HIF-1 was followed to confirm the preliminary results with hypoxia. We generated *unc-32(e189) glp-1(ar202); vhl-1(ok161)* mutant (***fig. 22***) and noticed that the tumor percentage in this double mutant worms is reduced compared to the *unc-32 glp-1(ar202)* single mutant worms (***fig. 24***).

Genetic cross for the generation of *unc-32(e189) glp-1(ar202); vhl-1(ok161)* mutant

unc-32 glp-1(ar202) worms exposed to *fog-1* RNAi were crossed with *vhl-1* male worms. The *unc-32 glp-1(ar202)* worms which are fertile – has both sperm and oocytes at 20ºC becomes fog (feminization of gonads) upon feeding on *fog-1* RNAi(Barton & Kimble, 1990). So, these feminized *unc-32 glp-1(ar202)* worms which have only oocytes when crossed with *vhl-1* male

worms yields the progeny of the genetic constitution *unc-32 glp-1(ar202)/+; vhl-1/+*. The progeny produced from this cross when singled produces the next generation offspring with a 25% probability for the worms to be *unc-32 glp-1(ar202)* homozygous and another 25% probability for the worms to be *vhl-1* homozygous (or) in other words, there is a chance for 1/16 of the offspring to be homozygous for both *unc-32 glp-1(ar202)* and *vhl-1* mutations in which the *glp-1* mutation associated with *unc* (uncoordinated) is identified by selecting the uncoordinated phenotype worms and *vhl-1* mutation is identified by genotyping through polymerase chain reaction (PCR) to obtain the desired mutant.

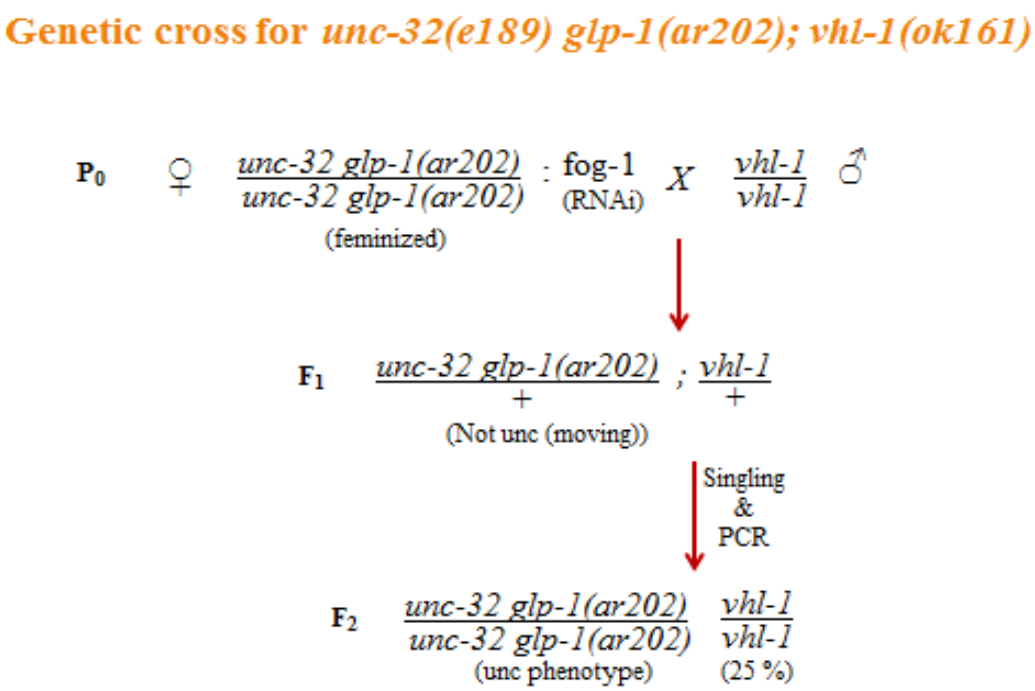


Fig. 22. Schematic of genetic cross for *unc-32(e189) glp-1(ar202); vhl-1(ok161)* mutant.

3.9 Inactivation of HIF-1 by knocking out *hif-1* enhances germline tumor development in *unc-32 glp-1(ar202)* mutant.

Final confirmation was done by generating *unc-32(e189) glp-1(ar202); hif-1(ia4)* mutant (***fig. 23***) to score the tumor percentage which was observed to have collinearity with the *unc-32 glp-1(ar202)* worms i.e. they were all tumorous (***fig. 24***).

Genetic cross for the generation of *unc-32(e189) glp-1(ar202); hif-1(ia4)* mutant.

unc-32 glp-1(ar202) worms exposed to *fog-1* RNAi were crossed with *hif-1* male worms. The *unc-32 glp-1(ar202)* worms which are fertile – has both sperm and oocytes at 20ºC under normal conditions becomes fog (feminization of gonads) upon feeding on *fog-1* RNAi(Barton & Kimble, 1990). So, these feminized *unc-32 glp-1(ar202)* worms which have only oocytes when crossed with *hif-1* male worms yields the progeny of the genetic constitution *unc-32 glp-1(ar202)/+; hif-1/+*. The progeny produced from this cross when singled produces the next generation offspring with a 25% probability for the worms to be *unc-32 glp-1(ar202)* homozygous and another 25% probability for the worms to be *hif-1* homozygous (or) in other words, there is a chance for 1/16 of the offspring to be homozygous for both *unc-32 glp-1(ar202)* and *hif-1* mutations in which the *glp-1(ar202)* mutation associated with *unc* (uncoordinated) is identified by selecting the uncoordinated phenotype worms and *hif-1* mutation is identified by genotyping through polymerase chain reaction (PCR) to obtain the desired mutant.

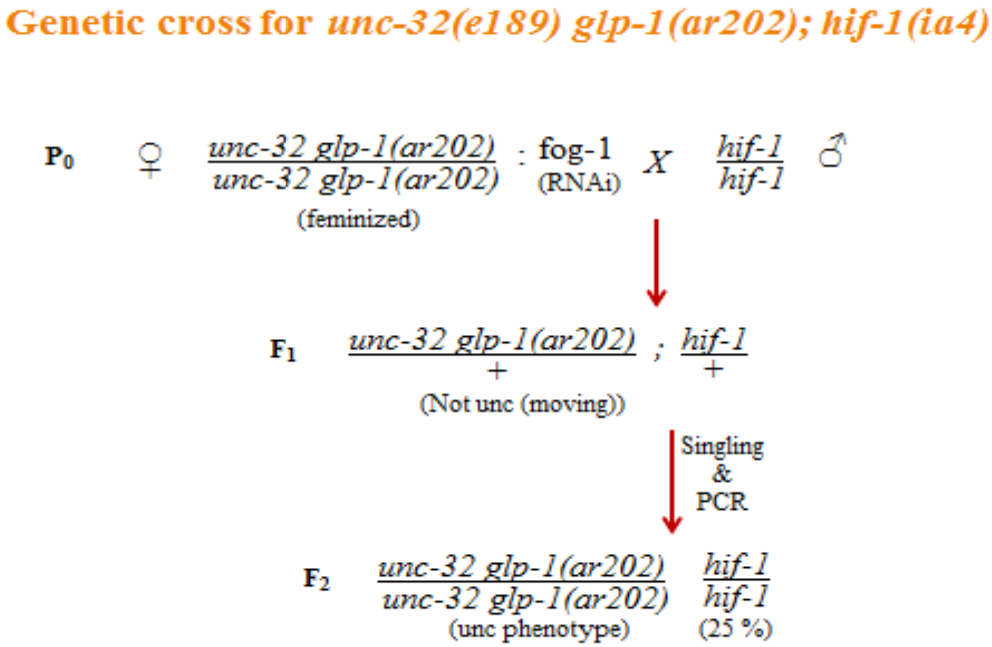


Fig. 23. Schematic of genetic cross for *unc-32(e189) glp-1(ar202); hif-1(ia4)* mutant.

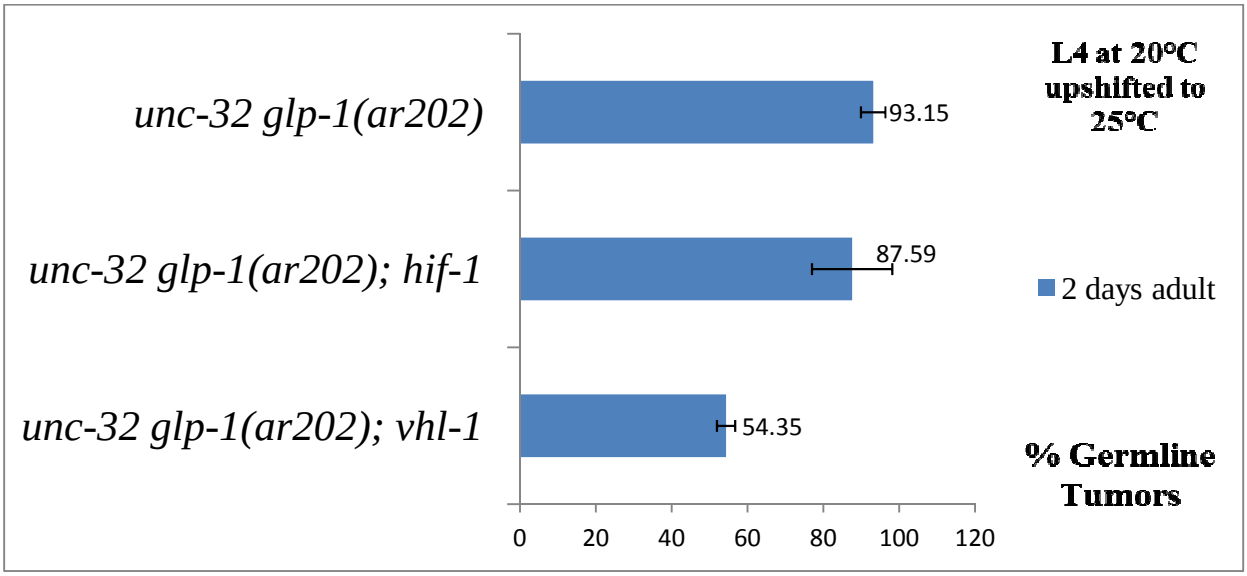


Fig. 24. Graph showing percentage of worms with proximal germline tumor in various *unc-32 glp-1(ar202)* mutant backgrounds at 2 days after L4 stage worms upshifted to 25^0C. Standard deviation bars were constructed from three independent experiments.

NOTE:

It is important to note that in all the above experiments, the worms were grown at 20^0C till L4 larval stage and they were then upshifted to 25^0C and exposed to hypoxic/ cobalt chloride conditions rather than raising them at 25^0C from the initial larval stages in different conditions as these mutant worms exposed to different conditions exhibited varied growth rates at 25^0C and this led to the problem of setting a standard stage for scoring the tumor percentage (explained in detail in discussion section).

The varied tumor percentage in case of treatment with cobalt chloride and genetic mutation is because the genetic effect is more effective when compared to microenvironment effect.

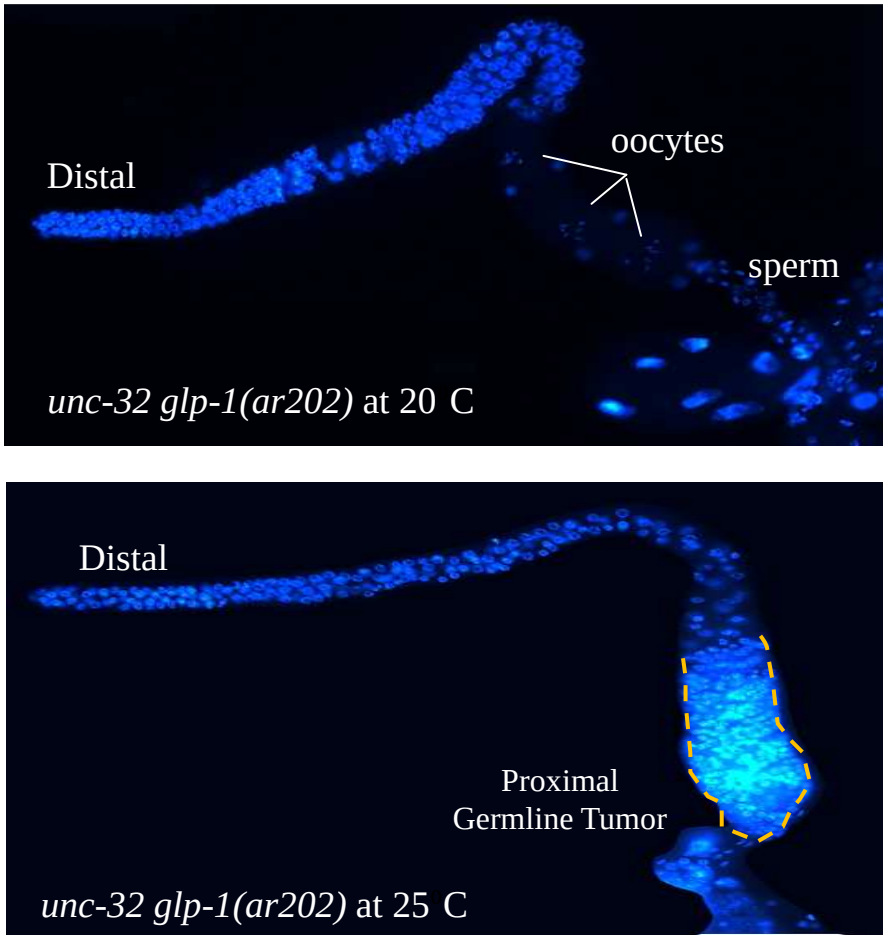


Fig. 25. DAPI stained adult sp/oo (sperm and oocytes) and proximal germline tumor phenotypes in *unc-32 glp-1(ar202)* at 20^0C and 25^0C respectively.

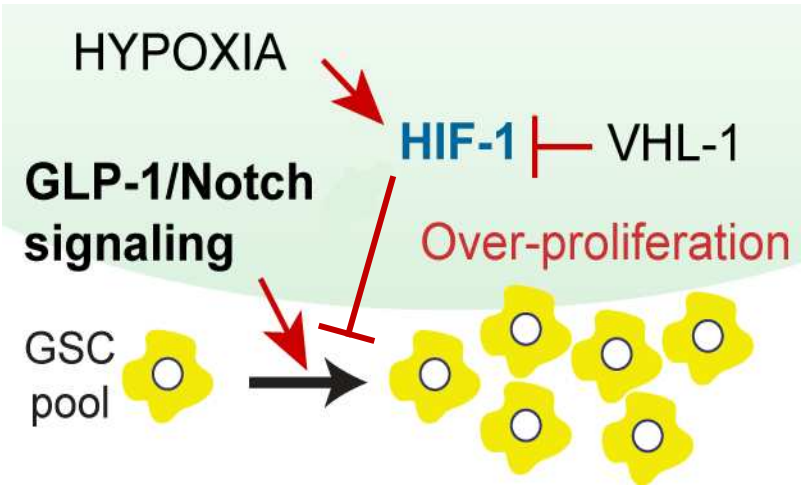


Fig. 26. Overall working model for HIF-1 action on GLP-1/Notch-mediated proximal germline tumor development in *Caenorhabditis elegans*.

Discussion

Irrespective of the mechanism involved behind tumor formation in *C. elegans* germline, the tumor mutants with *vhl-1* mutation or those that are exposed to hypoxic chamber or cobalt chloride (hypoxia mimic condition) exhibited decreased tumor percentage due to increased HIF-1 levels. Another set of results showing decreased tumor percentage in both the *puf-8; lip-1* and *glp-1(ar202)* mutant worms due to alleviated MPK-1 levels when exposed to mpk-1b RNAi indicates the importance of MPK-1 signaling in germline tumor formation irrespective of the mechanism involved behind the tumor formation (dedifferentiation-mediated or GLP-1/Notch-mediated). Connecting the above two results, it could be that HIF-1 may regulate MPK-1/ERK MAPK signaling like PUF-8 and LIP-1, even though they act at different levels. HIF-1 being a

transcriptional factor may act at a transcriptional level to control the MPK-1 activity to repress germline tumor development.

The consistent results supporting the idea that HIF-1 suppresses tumor formation in our results in *C. elegans* contradicts the role of HIF-1 in tumor cells studied in mammals where overexpression of HIF-1 promotes further cancerous tissue growth and its metastasis by supplying oxygen and nutrients to these overproliferating cells(Semenza, 2009). The possible explanation could be that:

1) Though HIF-1 could be a potential tumor suppressor, its function as tumor suppressor is not elicited based on the fact that tumor is developed under normoxic conditions. Once formed, tumor cells face hypoxic conditions in the actively dividing cells leading to shortage in oxygen and nutrient supply and it results in expression of HIF-1 that helps in sustenance of the tumor.
2) Or, germline tumors in *C. elegans*, might or might not be relevant to the somatic tumor model studied in mammals.

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