

ABSTRACT

Title of Document: IDENTIFICATION AND CHARACTERIZATION OF A HEME RESPONSIVE ELEMENT IN THE *hrg-1* PROMOTER

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Despite its biological significance, little is known about how animals sense and respond to heme to maintain homeostasis. *C. elegans* is a heme auxotroph, which makes it an excellent model to identify and dissect heme homeostasis pathways. Using *C. elegans* we have identified HRG-1, a vesicular heme transporter that is transcriptionally upregulated when environmental heme is low. The current study seeks to address how *hrg-1* is regulated by heme. Here, we show that a putative 23 base pair (bp) heme-responsive element (HRE) and GATA-binding motifs are necessary for heme-dependent regulation of *hrg-1*. The HRE comprises both enhancer and repressor elements and works in conjunction with ELT-2 to regulate *hrg-1* expression. We propose that the HRE could be used as a molecular tool in *C. elegans* to tightly regulate internal gene expression by modulating environmental heme. Our ultimate goal is to identify the *trans*-acting factor to eventually create a whole animal sensor for monitoring organismal heme homeostasis.

IDENTIFICATION AND CHARACTERIZATION OF A HEME RESPONSIVE
ELEMENT IN THE *hrg-1* PROMOTER

By

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Dedication

To my parents, thank you for all your support

Acknowledgments

Thank you to my advisor Iqbal, who has helped me a great deal in developing my scientific thinking since joining the lab.

To the other members of my committee, Dr. Krause and Dr. Song, who have given me valuable advice regarding my Master's work.

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Table of Contents

Dedication.....	ii
Acknowledgements.....	iii
Table of contents.....	iv
List of Tables.....	v
List of Figures.....	vi
Chapter 1. Literature review.....	1
Introduction.....	1
Heme biosynthesis.....	3
Differential regulation of the heme synthetic pathway in erythroid and non-erythroid cells.....	8
Heme as a transcriptional regulator.....	17
Post-transcriptional regulation.....	21
Heme regulates translation in erythroid cells.....	23
Post-translational regulation by heme.....	24
Heme transport.....	25
Chapter 2. Methods.....	31
Nematode growth conditions.....	31
Preparation of hemin-chloride solution.....	31
Bleaching.....	31
RNA-mediated interference.....	32
Bombardment.....	32
Injection.....	33
Cloning.....	34
Heme response.....	36
Chapter 3. Results.....	37
Locating the heme responsive <i>cis</i> element in the <i>hrg-1</i> promoter.....	37
The conserved 23-bp element is essential for expression of <i>hrg-1</i>	40
Heme response of the <i>hrg-1</i> promoter is controlled by enhancers and repressors.....	41
Chapter 4. Discussion.....	78
Appendices	
Appendix A: <i>C. elegans</i> strains.....	84
Appendix B: Reporter constructs.....	85
References.....	86

List of Tables

Table 1. Summary of GFP expression in transgenic worms expressing deletion constructs.....	76
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List of Figures

Chapter 1

Figure 1. Eukaryotic heme biosynthesis pathway.....	4
Figure 2. Mammals have two ALAS synthases.....	10
Figure 3. The splice variants of the second, third, and fourth enzymes in the heme biosynthesis pathway.....	13

Chapter 3

Figure 1. Alignment of the <i>hrg-1</i> promoter in three nematode species.....	43
Figure 2. ELT is required for the upregulation of GFP in low heme.....	48
Figure 3. A 254 bp region within the <i>hrg-1</i> promoter is sufficient to elicit heme responsiveness.....	50
Figure 4. 159 bp of the of the <i>hrg-1</i> promoter is sufficient for heme response.....	52
Figure 5. The heme responsive element of the <i>hrg-1</i> promoter lies upstream of -98b.....	54
Figure 6. The position of the heme responsive element is not essential for heme responsiveness.....	56
Figure 7. Schematic representing the heme responsiveness of various reporters.....	58
Figure 8. A single GATA site is not sufficient for expression of the <i>hrg-1</i> promoter.....	60
Figure 9. The 23-bp element has two inverted repeats.....	62
Figure 10. Mutation of the 23-bp element abolishes expression of <i>hrg-1</i>	64
Figure 11. The 5 terminal 3' bp of the 23-bp conserved element are not required for repression.....	66
Figure 12. The 159 bp heme-responsive fragment of the <i>hrg-1</i> promoter utilizes enhancement and repression in reponse to heme.....	69
Figure 13. The <i>hrg-1</i> promoter does not confer a heme-response to the <i>vha-6</i> promoter when placed upstream.....	72

Chapter 4

Figure 1. Proposed model of <i>hrg-1</i> regulation by heme.....	82
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Chapter 1: Literature Review

Introduction

Iron is an essential nutrient for almost all living organisms. Physiologically, the most important characteristic of iron is that it can exist in two oxidation states, Fe^{3+} and Fe^{2+} . This allows it to bind and release oxygen, which makes iron absolutely essential for oxidative metabolism. Despite its necessity, however, iron deficiency remains a worldwide problem. According to the World Health Organization, about thirty percent of the world's population are anemic, mainly due to iron deficiency (1). A large contributing factor to this widespread iron deficiency is that an estimated four billion people live on a primarily plant based diet (2). Although iron is abundant in plants, plants also contain organic compounds, such as phytic acid, that reduce the bioavailability of iron. Consequently, between only one to fifteen percent of iron consumed from a plant diet can be absorbed by the body (3). This is in contrast to a diet containing sufficient amounts of animal protein, particularly red meat, which is rich in heme-iron. Fifteen to forty percent of heme-iron that is ingested is absorbed through the intestine (4).

In humans, sixty-five to seventy-five percent of iron in the body is incorporated into heme (5). Heme is a tetrapyrrole that serves as a cofactor for numerous proteins, including the globins and cytochromes. Heme-containing proteins exhibit a wide array of functions including oxidative metabolism, gene regulation, cell differentiation, and gas sensing (6-9). Within the cell, heme functions as both a catalyst for reduction-oxidation reactions and an electron carrier (10).

The heme synthesis pathway has been studied in detail. There are eight genes in the synthesis pathway, all of which have been cloned, and the enzymes they encode for have been crystallized (10). Several diseases, such as X-linked sideroblastic anemia and the porphyrias, are associated with mutations in the heme synthesis pathway (11,12). Despite the progress made in deciphering the synthesis pathway, the intercellular and intracellular transport of heme remains poorly understood. The transport of heme is likely to be highly regulated due to the hydrophobic and cytotoxic nature of heme. As a hydrophobic molecule, heme cannot simply diffuse throughout the aqueous environment of the cytoplasm. Additionally, each heme ring contains an iron molecule that can generate free radicals within the cell via peroxidase reactions. Therefore, it is likely that, as with iron, heme is sequestered and protected for safe and efficient delivery to different cellular compartments for incorporation into hemoproteins.

Studying cellular heme transport and homeostasis has been difficult due to the challenges in uncoupling heme synthesis from trafficking. The difficulty occurs because heme synthesis is highly regulated, and several mechanisms exist to ensure that cells maintain proper physiological heme levels. Therefore, depleting cells of endogenous heme causes many pleiotropic effects, which prevents the identification of specific trafficking molecules. However, we have previously shown that *C. elegans* is a heme auxotroph, making it an excellent genetic model for studying heme trafficking (13). As heme trafficking molecules are identified, the genomic sequences that code for these proteins can be analyzed to determine how they are regulated. One mode of regulation would be at the transcriptional level. Thus, uncovering functional, non-coding DNA

sequences, such as enhancer and repressor elements, of heme transport genes will give a better understanding of how organisms maintain heme homeostasis.

Studies that identify molecules involved in heme trafficking and homeostasis have important nutritional and biomedical ramifications. Discovering human orthologs involved in heme homeostasis could facilitate the creation of iron sources that are more bioavailable to humans. Additionally, nematode specific-genes may be potential drug targets for helminthic infections. It is not only soil-dwelling nematodes that require exogenous heme, but several species of parasitic nematodes require heme from the diet (13). Parasitic nematodes have become increasingly resistant to drugs, yet development of new anthelmintic drugs has remained slow for the past 25 years (14). Targeting worms' need for exogenous heme may represent a novel therapeutic way to combat helminthic infections.

Heme biosynthesis

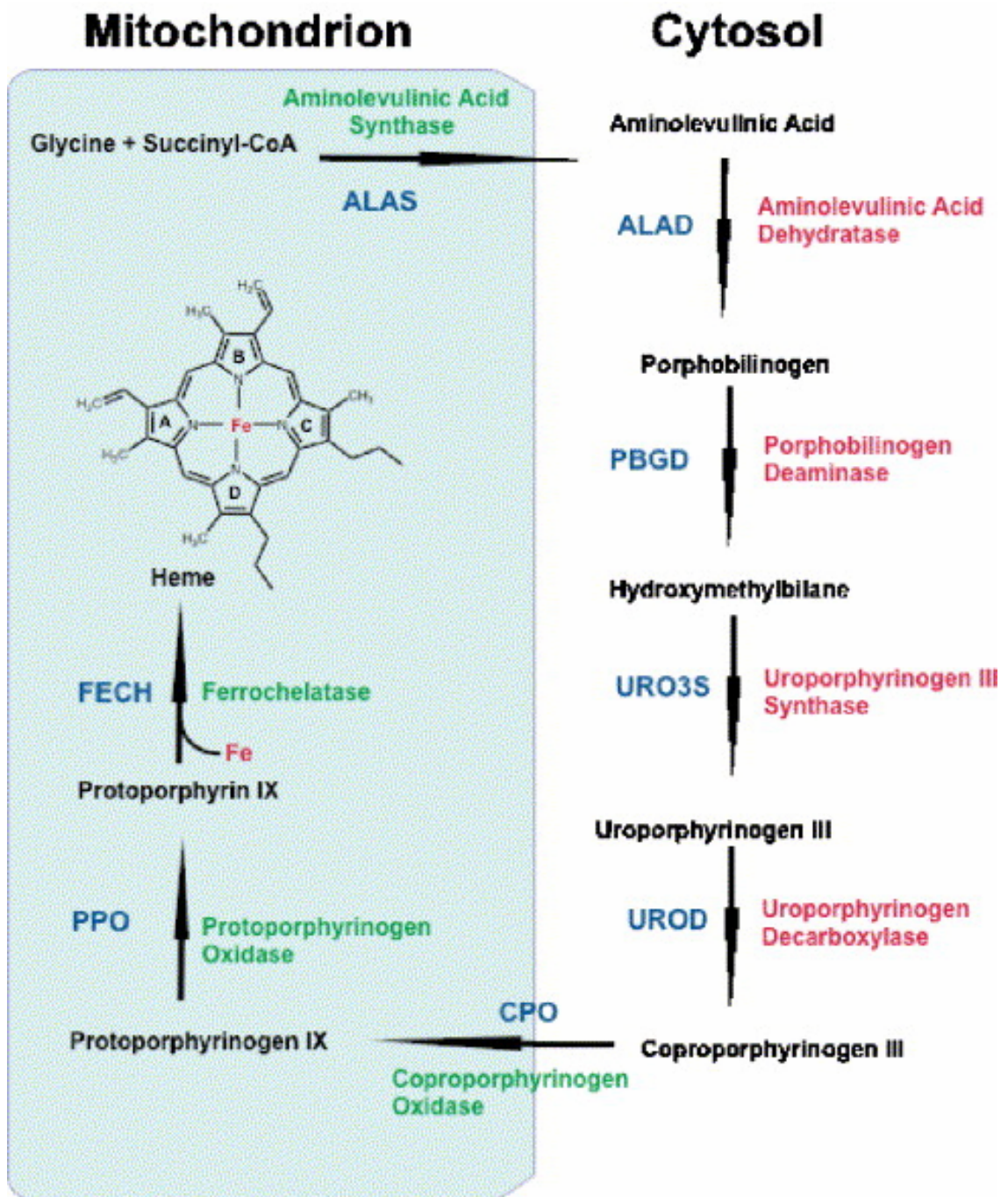
In most eukaryotes and prokaryotes, heme is synthesized via a conserved eight-step pathway. The pathway is outlined in Figure 1. In eukaryotes, the first reaction and the last three reactions take place in the mitochondria, while the remainder of the reactions occurs in the cytosol. All eight genes in the heme synthesis pathway have been cloned, and the associated enzymes have been crystallized (10). The first step in heme biosynthesis is the reaction between glycine and succinyl-coenzyme A (succinyl-CoA), which results in the formation of 5-aminolevulinic acid (ALA). This reaction is catalyzed by aminolevulinic acid synthase (ALAS). ALAS utilizes the active form of vitamin B₆, pyridoxal 5-phosphate (PLP), as a cofactor. PLP binds to a lysine of ALAS, leading to

Figure 1: Eukaryotic heme biosynthesis pathway.

The heme biosynthetic pathway occurs in eight conserved steps. The first and last three steps take place in the mitochondria. The middle four steps take place in the cytosol.

Mitochondrial enzymes are depicted in green and cytosolic enzymes in red.

Abbreviations used in the text are capitalized (adapted from ref. 9).



the formation of a PLP-glycine Schiff base complex. The complex then interacts with succinyl-CoA, catalyzing the formation of ALA. Following synthesis, ALA is exported from the mitochondria by an unknown mechanism.

Once in the cytosol, aminolevulinic acid dehydratase (ALAD) catalyzes the condensation of two ALA molecules to form the pyrrole porphobilinogen (PBG). Zinc is required for ALAD function, consequently heme production is dependent on the availability of zinc (15). ALAD is composed of four homodimers, with one active site per dimer (16). Each active site binds two ALA molecules. One ALA molecule will contribute the acetate and amino-methyl group of PBG, while the other molecule will be responsible for the propionate side chain and the pyrrole nitrogen (10). This reaction completes the formation of a pyrrole.

The next two steps in the synthesis pathway are responsible for combining four porphobilinogen molecules to form a tetrapyrrole. The first of the two reactions is catalyzed by porphobilinogen deaminase (PBGD), which catalyzes the formation of a polymer of four molecules of PBG. The product of this reaction is hydroxymethylbilane (HMB). PBGD utilizes a dipyrrole cofactor derived from PBG molecules. The enzyme binds an HMB molecule, then subsequently adds two additional pyrroles to form a hexapyrrole. The four pyrroles distal from the active site of PBGD are cleaved, forming HMB. The two proximal pyrroles are retained for the active site (17). HMB released from PBGD is unstable and must be quickly converted to uroporphyrinogen III (UROgenIII) by the enzyme uroporphyrinogen III synthase (UROS). UROS flips the “D” ring of HMB and catalyzes ring closure to form a tetrapyrrole macrocycle.

UROgenIII can enter different synthesis pathways. In mammals, UROgenIII can enter the heme biosynthetic pathway or the corrin synthesis pathway. In photosynthetic organisms UROgenIII can also enter the chlorophyll synthesis pathway (10)

The next two reactions in the heme synthesis pathway modify the side chains of the tetrapyrrole. In the first reaction, UROgenIII is converted to coproporphyrinogen III (CPgenIII) by the enzyme uroporphyrinogen decarboxylase (UROD), which removes the carboxylic acid groups of the tetrapyrrole's four acetic acid side chains. The next side chain modification is catalyzed by coproporphyrinogen oxidase (CPOX), which is located on the intramembrane space side of the outer mitochondrial membrane. In order for CPOX to have access to CPgenIII, the substrate must be imported into the mitochondria. It has been reported that the ATP-binding cassette transporter ABCB6 is the CPgenIII mitochondrial transporter (18). However, this finding has been controversial due to the substrate used for binding and uptake assays. The study used planar, oxidized coproporphyrin III instead of the physiological CPgenIII, which is reduced and non-planar (19). CPOX catalyzes the oxidative decarboxylation of the propionate groups of pyrroles A and B, which results in the formation of protoporphyrinogen IX (PPgenIX). CPOX has been reported as the rate limiting enzyme in heme synthesis during erythroid differentiation (20). The second to last step of the pathway is the oxidation of PPgenIX by protoporphyrinogen oxidase (PPOX) to form protoporphyrin IX (PPIX). PPOX is located on the outer surface of the inner mitochondrial membrane and functions as a homodimer (21).

The final step of heme biosynthesis is the addition of iron into PPIX to form heme. The reaction is catalyzed by ferrochelatase (FECH). Ferrochelatase is located on

matrix side of the mitochondrial inner membrane. Due to the spatial arrangement of the final three enzymes and the chemical reactivity of intermediates, it has been suggested that CPOX, PPOX and FECH may form a complex to facilitate the terminal steps of heme synthesis (21,22)

Differential regulation of the heme synthetic pathway in erythroid and non-erythroid cells

By necessity, the heme synthesis pathway is a highly regulated process. Heme production must be coordinated with different environmental conditions, especially iron availability. Accumulation of porphyrins, iron, or heme itself is toxic to the cell. As a result, synthesis must be tightly controlled. Additionally, cells of different tissues have different heme requirements, and the pathway must be regulated accordingly.

Non-erythroid cells, especially hepatic cells, need to adapt to the changing metabolic state of the organism and, therefore, need to adjust the amount of heme that is synthesized. Heme produced in non-erythroid tissue is primarily for use within each cell for incorporation into proteins such as the cytochromes.

Erythroid precursors need to produce a greater amount of heme than other tissues for insertion into hemoglobin. In contrast to other cell types, heme produced in erythroid tissue will be used to deliver oxygen to all tissues of the body. Therefore, erythroid precursors produce heme as long as iron is available. Upregulation of heme synthesis in erythrocytes at the transcriptional level is mainly achieved via erythroid-specific transcription factors.

Differential transcriptional regulation is found in the first enzyme of the heme synthesis pathway, ALAS. As shown in Figure 2, mammals have two forms of ALAS which are differentially expressed depending on cell type. ALAS1 is expressed ubiquitously, and is the rate-limiting enzyme of heme biosynthesis in non-erythroid tissue (10). ALAS1 has been shown to be downregulated by heme at the transcriptional and post transcriptional levels (23,24). The mechanism that governs downregulation has not been determined. Heme also post-translationally regulates ALAS1 by binding to a heme-regulatory motif (HRM) (25). Heme binding to the HRM blocks mitochondrial import, resulting in post-translational feedback inhibition.

In addition to heme, *ALAS1* is responsive to glucose (26). The transcription factor PGC-1 α is upregulated when glucose levels are low, which in turn upregulates *ALAS1*. Interestingly, *ALAS1* is also upregulated by the NPAS-2/BMAL1 complex, which is part of the core clock mechanism that controls circadian rhythm (27). Rev-erba is a nuclear hormone receptor that negatively regulates the NPAS-2/BMAL1 complex in the presence of high heme, ultimately leading to a repression of gluconeogenic genes (28). This may be the system that coordinates glucose and heme homeostasis with the core clock mechanism, as both processes are at least partly under circadian control.

ALAS2 is an erythroid-specific gene and, therefore, has a different regulation pattern than *ALAS1*. At the transcriptional level, upregulation is achieved during erythropoiesis through the binding of erythroid-specific factors to their target sequences (23). Erythroid-specific transcription factors, such as GATA-1 and NF-E1, are synthesized specifically in erythroid tissue during erythropoiesis. Genes such as *ALAS2*

Figure 2: Mammals have two ALAS synthases.

ALAS1 is a ubiquitous ALAS synthase, the gene of which is located on chromosome 3.

ALAS2 is an erythroid-specific ALAS synthase whose gene is located on the X chromosome. Chr. = chromosome location. ATG = translational start site. IRE = Iron response element. H = Housekeeping transcriptional start site. E = Erythroid transcriptional start site. Blue background = housekeeping specific exons. Red background = Erythroid-specific background (adapted from ref 9).



that have promoters containing binding sites for these transcription factors will be upregulated in differentiating erythroid tissue.

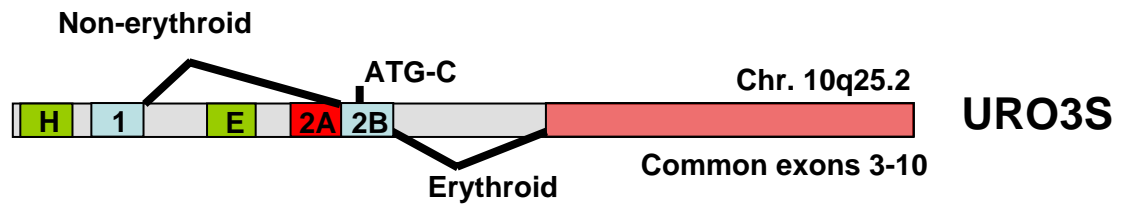
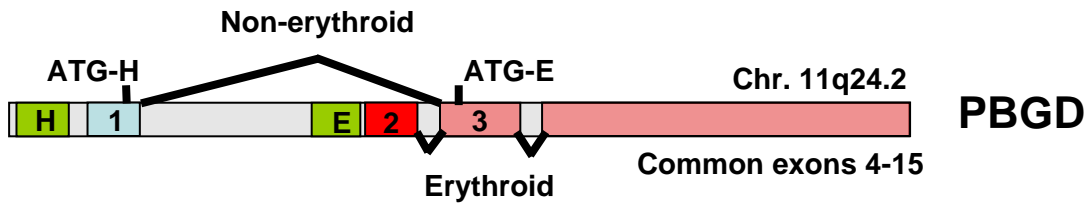
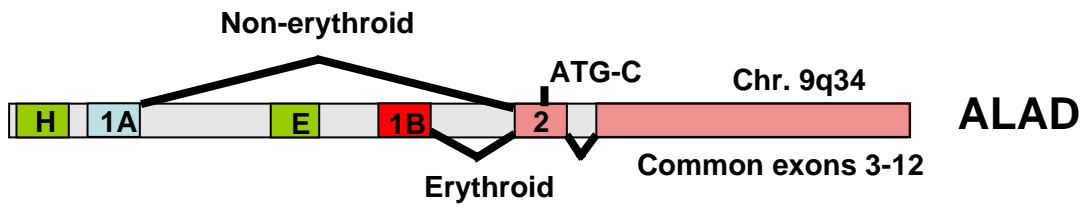
ALAS2 is regulated at the post-transcriptional level by the IRP/IRE system (29). The *ALAS2* transcript contains an iron response element (IRE) in the 5' untranslated region (UTR) near the translational start site, which binds an iron response protein (IRP) when iron is depleted. The presence of an IRP near the translational start site of the *ALAS2* mRNA sterically hinders the initiation complex from forming, thereby resulting in *ALAS2* downregulation in low iron conditions. When iron levels are sufficient, IRP can no longer bind mRNA, which results in the translation of *ALAS2*. Thus, erythroid levels of *ALAS2* are directly linked to iron availability. This is important physiologically. Post-transcriptional regulation of *ALAS2* allows cells to quickly adjust the heme synthesis pathway according to iron levels.

ALAD encodes the second enzyme in the heme synthesis pathway, and consists of eleven coding exons and two non-coding exons (1A and 1B). Differential regulation is achieved by utilizing two different promoters. In non-erythroid tissue, the gene is regulated by a housekeeping promoter located upstream from exon 1A, the transcript of which does not contain exon 1B. The erythroid-specific promoter is located between exon 1 and 2, and contains GATA-1 binding sites. Although the transcripts differ to allow for upregulation specifically in erythropoietic tissue, the resulting proteins are identical because the translational start site for both transcripts is in exon 2 (30).

The third and fourth genes in the pathway, *PBGD* and *UROS*, are transcriptionally regulated in a manner similar to *ALAD* by a housekeeping promoter in all cell types and an erythroid-specific promoter in erythroid tissue, as shown in Figure 3. In *PBGD*, the

Figure 3: The splice variants of the second, third, and fourth enzymes in the heme biosynthetic pathway.

ALAD, *PBGD*, and *UROS* achieve erythroid-specific expression by utilizing erythroid-specific promoters that result in splice variants. Chr. = chromosome location. ATG-C = Common transcriptional start site. ATG-H = Housekeeping transcriptional start site. ATG-E = Erythroid transcriptional start site. Blue background = housekeeping specific exons. Red background = Erythroid-specific exons. Pink background = common exons (adapted from ref. 9).



housekeeping promoter is located upstream of exon 1, while the erythroid-specific promoter is in the intron between exon 1 and exon 2 (31). There are two *cis* acting sequences in the erythroid-specific promoter that are responsible for upregulating transcription in erythroid tissue during erythropoiesis. The first element is located 70 bp upstream of the transcriptional start site and binds the erythroid transcription factor NF-E1. The second element is located 170 bp upstream of the transcriptional start site and binds the erythroid-specific transcription factors NF-E2 and the ubiquitous transcription factor AP1 (31). Unlike *ALAD*, alternative splicing leads to proteins that differ in the N-terminus (32).

In *UROS*, the housekeeping promoter is located upstream of exon 1 and has binding sites for the ubiquitous transcription factors NF1, SP1, AP1, OCT1, and NFR2. The resulting mRNA contains exon 1A or exon 1A fused to exon 2B. The erythroid-specific promoter, located in intron 1, contains nine GATA-1 binding sites. These sites are sufficient to drive erythroid-specific expression. mRNA transcribed from the erythroid-specific promoter contains exon 2A. Both the ubiquitous and erythroid-specific transcripts have exon 2B, which contains the translational start site. Consequently, like *ALAD*, the two transcripts code for identical proteins (33).

The next gene in the pathway, uroporphyrinogen III synthase (*UROS*), does not have an erythroid-specific promoter. However, transcription is increased in erythroid tissues. The mechanism of regulation remains unclear (34).

The sixth gene in the heme synthesis pathway, coproporphyrinogen oxidase (*CPOX*), has only one promoter but achieves differential expression through binding of erythroid-specific transcription factors to enhancer elements. The promoter of *CPOX* has

a SP1-like element, a GATA-1 site, and a novel regulatory element, CPRE, which work together to regulate transcription (35). Differential expression in erythroid MEL cells and non-erythroid NIH3T3 cells suggests differential regulation between erythroid tissue and other cells types, with the GATA-1 site only important in erythroid cells.

Interestingly, the CPRE element is also found in the human β -globin promoter, another gene that is upregulated in erythroid tissue (35). The transcription factor that binds the CPRE element has been shown to be Klp1, which acts as an activator when bound to the CPRE (36).

The second to last gene in the heme synthesis pathway, protoporphyrinogen oxidase (*PPOX*), has also been shown to be differentially expressed in erythroid and non-erythroid cells. *PPOX* is upregulated in erythroid cells when compared to other tissue due to two GATA sites contained within exon 1 of the *PPOX* (37).

The final gene in the pathway, ferrochelatase (*FECH*), achieves differential expression in a manner similar to the two previous enzymes in the pathway. There is a single promoter which contains binding sites for SP1, NF-E2, and GATA-1. These elements have shown different results depending upon the system used. In K562 cells, a erythroleukemia cell line, the SP1 binding site was sufficient to drive erythroid expression of *FECH* (38). It has also been shown that a point mutation in one of the SP1 binding sites disrupts proper transcription of the *FECH* mRNA and leads to erythropoietic protoporphyria (39). In mouse embryonic stem cells, it was shown that the NF-E2 binding site is necessary for both basal expression in pluripotent cells and erythroid-specific upregulation. The same study also demonstrated that GATA SITE 1 binding element acts as a repressor in pluripotent cells, but an enhancer during

erythropoietic development.(40). FECH expression is also upregulated by iron sufficiency and hypoxia. The iron dependent regulation of FECH is due to an iron-sulfur cluster in its C-terminus (41). It is induced by hypoxia due to two hypoxia inducible factor (HIF) binding sites in its promoter (42). It is not surprising that the synthesis of heme is coordinated with iron and oxygen levels because all three molecules are tightly linked during aerobic respiration.

Heme as a transcriptional regulator

Heme has been shown to be a key regulator of several processes at the transcriptional level. Intracellular heme levels are important for the transcriptional control of processes such as oxidative metabolism and circadian rhythm. Heme may also promote erythroid differentiation by promoting the expression of p18 and p21 which are negative regulators of the cell cycle . DNA microarray data from K562 cells has shown that the expression of several hundred genes was altered due to heme. When K562 cells were treated with heme, some genes were transcriptionally upregulated, while others were downregulated, indicating a dual regulatory role for heme (43). Additionally, 370 genes in *C. elegans* were shown to be transcriptionally regulated by heme. These heme responsive genes (*hrgs*) could be grouped into eight categories depending on their transcriptional profile under varying heme concentrations (44). These studies suggest that heme plays a role in the transcriptional regulation of many genes. Further research is needed to uncover the mechanisms that govern heme-dependent transcription, which responses are primary, secondary, and tertiary, and the functions of the genes that are regulated by heme.

The first evidence that heme regulates transcription came from the iso-1-cytochrome *c* gene (*CYCI*) of the electron transport chain in yeast. Cyc1p is the heme binding protein of the cytochrome *b-c1* complex. It was shown that *CYCI* was transcriptionally upregulated by heme through the heme activation protein 2 (Hap2p) by binding to an upstream activation site. Transcription of *CYCI* was reduced 200-fold in cells grown in heme deficient conditions (6). Although the mechanism of heme-response has not been deciphered for Hap2p, the proposed mechanism for the heme regulation by the Hap2p homologue, Hap1p, is that heme binding causes a conformational change which exposes the DNA binding domain. This structural alteration allows Hap1p to bind DNA and act as an enhancer of its target genes (45).

Transcriptional control of the electron transport chain in response to intracellular heme levels is essential for the cell to maintain proper metabolism. Heme is required for aerobic respiration. In low heme conditions, it is a poor use of energy to produce proteins that cannot be utilized. Subsequently it was shown that *COX4*, *COX5*, *COR1*, and *COX6*, which like *CYCI* encode components of the electron transport chain, are transcriptionally regulated by heme (46-48). *COX4*, *COX5*, and *COR1* do not encode hemoproteins, showing that yeast regulation by heme is a general mechanism for components of the electron transport chain, not just hemoproteins.

In mammals, respiration is also transcriptionally controlled by the amount of available heme through the control of hemoglobin production. Adult hemoglobin is produced in erythroid tissue and consists of a heme molecule bound to a tetramer of two α -globin chains and two β -globin chains. In low heme conditions, it is necessary to downregulate globin chain production because the aggregation of globin chains is toxic to

the cell (49). The transcriptional regulation of globin chain production by heme is mediated through Bach1, the first mammalian transcription factor shown to bind heme. The mechanism by which Bach1 regulates globin chain production has been demonstrated (50-52). The β -globin gene locus consists of five genes: ϵ -globin, $G\gamma$ and $A\gamma$ -globin, β -globin, and the δ -globin gene which are differentially regulated during development. Transcription of all genes in the locus is controlled by a locus control region (LCR), which transcriptionally regulates gene expression from a distance of ~60 kb (53). Within the LCR there are MARE sequences (MAf Recognition Element) which bind the Maf class proteins. The Maf class proteins have been shown to be repressors or activators depending on which partner they heterodimerize to. One such partner is p45, the large subunit of the erythroid-specific transcription factor NF-E2. The recognition sequence for this heterodimer is TGCTGA(G/C)TCA(T/C), which is found in the β -globin LCR and the α -globin promoter (51,54). When the Maf / p45 complex binds to its target sequence, it will act as an activator. Members of the Maf family can also interact with Bach1 (50). The Maf / Bach1 heterodimer will bind to the same recognition sequence as Maf / p45, but acts as a repressor. Bach1 can only bind DNA and repress transcription when it is not bound to heme. Therefore Bach1 mediated repression only occurs in low heme conditions. In addition to dissociating from DNA, Bach1 is exported from the nucleus upon heme binding (55). When heme levels are sufficient, heme will bind to Bach1, causing it to dissociate from the MARE elements. The Maf /p45 heterodimer can then bind to the MARE elements and activate transcription. It has been shown that Bach1 has four cysteine proline (CP) HRMs which are responsible for heme

binding. Deletion of these motifs inhibited Bach1 dissociation from MAREs upon the addition of excess heme.

Heme Oxygenase 1 (HO-1), which degrades heme into iron, CO, and biliverdin, is another gene repressed by Bach1 (56). Accumulation of heme in the cell is toxic due to free radical production. When heme levels are high, Bach1 dependent repression of HO is relieved by a similar mechanism to that of globin chain regulation by Bach1.

Ferritin, the protein which is responsible for sequestering free iron in the cell and preventing it from causing oxidative damage, is transcriptionally controlled by heme through Bach1. Because heme contains an iron molecule, cells can only produce heme when sufficient iron is available. Consequently, when cellular heme levels are enough to degrade Bach1, there is most likely also excess iron in the cell. Therefore, ferritin is upregulated under high heme conditions to sequester excess iron (57).

Recently work has been done to demonstrate that heme affects the transcriptional regulation of circadian clock-controlled genes through the nuclear hormone receptor REV-ERB α . In these studies gluconeogenic genes were downregulated when heme is bound to REV-ERB α , probably through the transcription factor BMAL1 (28). BMAL1 is a key component of the circadian rhythm feedback loop, and has been shown to be a target of REV-ERB α . In mammals, heme binding to REV-ERB α allows it to recruit its binding partner, NCoR-HDAC3. This complex will repress transcription when bound to its target sequence.

Heme production and oxidative metabolism have been previously shown to be under circadian control (58-61). It has also been demonstrated that mice deficient in *Bmal1* have abnormal metabolic patterns (62). Despite this evidence linking metabolism

to circadian rhythm, how these processes are integrated is not understood. REV-ERB α may coordinate the circadian rhythm with heme production and glucose production, thereby linking circadian rhythm and oxidative metabolism (28).

Heme has also been shown to repress transcription of tartrate-resistant phosphatase (TRAP) (63). TRAP is an iron-containing protein that may function in iron homeostasis (64). Heme has been shown to repress *TRAP* in peripheral mononuclear cells through the heme-responsive element binding proteins (HREBP), which are composed of subunits of the Ku antigen (KuAg) and Ref-1 protein. HREBP interacts with a 27 bp heme responsive element (tandem repeats of GAGGC) that represses transcription in the presence of heme. Although the mechanism which governs the heme response is unknown, it has been suggested that heme is responsible for bringing all the subunits of HREBP together (65).

Interestingly, TRAP and uteroferrin are transcribed from the same gene but have different post-translation modifications. These modifications lead to the secretion of uteroferrin, while TRAP localizes to the lysosomes (66). If uteroferrin, like TRAP, is downregulated by heme, heme concentration may play a role in the regulation of maternal iron transfer.

Post -transcriptional regulation

Iron and heme homeostasis are intricately linked. In humans, most dietary iron comes from heme. Sixty-five to seventy-five of this iron will be incorporated back into heme. Most of the remaining iron will be stored in the liver as ferritin (5). Furthermore, iron recycling occurs through heme degradation by heme oxygenase 1 (HO-1). These

two molecules are interdependent on each other and need to be co-regulated. One way of achieving reciprocal regulation is through the well characterized-post-transcriptional IRP / IRE system. Initially characterized as an iron regulatory mechanism, it is now clear that heme also plays a crucial role in this system. Mammals have two iron response proteins, IRP1 and IRP2, which are post-translationally regulated by cellular iron levels. In low iron conditions, both IRPs bind to the Iron Response Element (IRE) located in the mRNAs of proteins involved in iron metabolism and homeostasis. When bound to the IRE, IRP1 and IRP2 will upregulate or downregulate an mRNA, depending on where in the transcript the IRE is located. If an mRNA has an IRE in its 5' UTR, binding of an IRP prevents the mRNA from being translated. Consequently, these gene products are downregulated in low iron. If a gene product has an IRE in its 3' UTR, it prevents the mRNA from being degraded. Gene products with a 3'UTR IRE are therefore upregulated when iron is low (67).

It has been demonstrated that IRP2 is the stronger regulator of iron homeostasis, and its expression is dependent on heme (68,69). There is no evidence that IRP1 is also regulated by heme. High iron conditions lead to the degradation of IRP2 by the proteasome. Heme binding to an iron dependent domain (IDD) in IRP2 is required for this degradation (70). Interestingly, the IDD shows similar sequence identity to a typical HRM. Heme binding to the IDD causes the oxidation of IRP2, which allows it to be recognized by the E3 ligase HOIL-1 and degraded by the proteasome (71). When iron levels are sufficient, heme will be synthesized and consequently bind to IRP2, leading to its degradation by the proteasome. The degradation of IRP2 will cause a shift in iron metabolism.

This IRP / IRE system is further integrated with heme production through ALAS2. ALAS2 has been shown to have an IRE in its 5' flanking sequence that is sufficient to confer an iron dependent response in erythroid cells (72,73). Thus, in iron deplete conditions heme synthesis is down-regulated at least in part by the inhibited translation of ALAS2.

Heme has also been shown to be involved in microRNA processing. MicroRNAs are short RNAs that play a role in gene regulation at the transcriptional and post-transcriptional levels. This process is at least in part regulated by heme. The RNA binding protein DiGeorge critical region-8 (DGCR8) is only active as a dimer. To dimerize, it requires heme. Once DGCR8 dimerizes, it can bind to primary microRNAs, resulting in cleavage by DROSHA to produce mature microRNAs. These microRNAs will then regulate their target genes (74). Two microRNAs, miR144 and miR451, have been shown to be involved in erythropoiesis in zebrafish (75). An upstream regulatory element which binds GATA-1 is required for the upregulation of a single pri-miRNA (precursor-RNA) that encodes both miR144 and miR451. Zebrafish depleted of miR451 showed impaired maturation of erythroid precursors, indicating that post-transcriptional regulation by microRNAs is essential for erythropoiesis.

Heme regulates translation in erythroid cells

In erythropoiesis, late-stage erythroblasts lose their nuclei to become reticulocytes. As they mature into erythrocytes over the next few days, they synthesize hemoglobin and other erythroid-specific proteins. Hemoglobin production must be coordinated with heme availability because aggregation of globin chains is toxic to the

cell (49). As has been previously discussed, globin chain production is regulated by heme at the transcriptional level by Bach1 binding to the promoters of the α -globin and β -globin genes. However, mechanisms must exist to regulate globin production after the cell has lost its nucleus and transcriptional regulation is no longer possible. In reticulocytes, this regulation is achieved at the translational level by heme regulated eIF-2 (HRI) kinase (76). In low heme conditions, when synthesis of globin chains needs to be downregulated, HRI is activated. This allows it to phosphorylate serine 51 of the α -subunit of the initiation factor (eIF-2). eIF-2 is normally responsible for recruiting the initiator tRNA to the pre-initiation complex, but phosphorylation prevents eIF-2 from being utilized for translation, resulting in global reduction in mRNA translation.

Although the mechanism HRI uses for heme sensing remains to be elucidated, it has recently been demonstrated that His 119, His 120, and Cys 409 are the ligands for heme binding. The proline residue adjacent to the Cys 409 is also important for heme Fe^{3+} complex binding (77).

Post-translational regulation by heme

In order for proper heme homeostasis to occur, the cell must have a way of sensing intracellular heme concentration. This is accomplished by a group of proteins that have HRMs. Not surprisingly, since many cellular processes are regulated by heme, proteins with HRMs serve a wide a variety of functions. The HRM is a short stretch of amino acids that is degenerate in most positions, but has an absolutely conserved Cys-Pro core and usually a hydrophobic amino acid at the fourth position (78). The first HRM to be discovered was in the yeast transcription factor Hap1p and contains the sequence

Lys/Arg-Cys-Pro-Val/Ile-Asp-His (79,80). This motif is repeated six times in the N-terminus of Hap1p. Importantly, HRMs do not bind heme as tightly as the histidine coordination ligands of the cytochromes and globins. In order to act as sensors, sensor proteins need to be able to release heme, and, consequently, not bind heme as tightly as hemoproteins which utilize heme as a cofactor.

Several proteins that have been discussed in previous sections utilize the HRM to sense heme. The transcriptional repressor Bach1 is exported from the nucleus when heme is bound to the six HRMs present in Bach1 (55,81). IRP2, which regulates iron-homeostatic genes post-transcriptionally, is degraded upon heme binding to its HRM (68). Heme binding to the HRM of HRI kinase, the protein that coordinates hemoglobin production to heme availability in reticulocytes, causes inactivation of the kinase (76). ALAS1, the first enzyme in the heme biosynthetic pathway, is no longer targeted to the mitochondria when heme is bound to its HRM (25).

Heme Transport

Despite attempts to study heme transport in a wide variety of organisms, success has been limited to prokaryotes. Most organisms are heme prototrophs and can synthesize heme endogenously. Heme synthesis and degradation are highly regulated, which makes it difficult to distinguish these intracellular processes from heme transport (19). Although a limited number of proteins have been shown to be important for heme transport in eukaryotes, there has been greater success in identifying and characterizing heme transport in bacteria.

Several bacteria species have developed heme transport systems as a way of acquiring iron from their surroundings. Most of the heme transport systems have been characterized in pathogenic species because most of the bioavailable iron these species encounter within the host organism is in the form of heme. Since heme is toxic and consequently unlikely to be found free within the host environment, pathogenic bacteria must be able to acquire it from hemoproteins such as hemoglobin, hemopexin, haptoglobin, and albumin. Gram positive bacteria which lack an outer membrane have receptors anchored on the inner membrane that recognize heme. The heme is transported across the membrane by ABC permeases (82). Heme transport has been more extensively studied in gram negative bacteria, which utilize specific outer membrane receptors to acquire heme (83). These receptors can acquire heme directly from hemoproteins or from hemophores. Hemophores are extracellular heme-binding proteins produced by the bacteria. Once bound to its receptor, the energized conformation of TonB in the TonB-ExbB-ExbD provides the energy necessary to move the heme or the hemophore into the cell (84). The outer membrane receptors have a TonB box, which is the domain of the receptor that interacts directly with TonB. Once heme has been transported across the inner membrane, it is most likely degraded by a bacterial heme-oxygenase, which releases iron and protoporphyrin IX (85).

One hemophore system that has been characterized is the heme acquisition system (HAS). The HAS pathway is utilized by several bacteria species, including the pathogens *Serratia marcescens* and *Pseudomonas aeruginosa* (86,87). Several of the genes in this system are encoded by the *has* operon. The operon is under transcriptional control of the Fur repressor protein in conjunction with the ECF sigma and anti-sigma factors (88,89).

The Has system has several components that are utilized for heme uptake. HasA is a secreted hemophore that binds to heme outside the cell. HasA is proposed to remove the heme from its carrier, as opposed to forming a stable complex with hemoproteins (90). HasA is secreted from the cell by an ABC transporter complex. The complex consists of three proteins, HasD, HasE, and HasF (87). HasD and HasE comprise the inner membrane portion of the secretory apparatus. HasF, which is not part of the *has* operon, codes for the outer membrane portion of secretory complex (91). HasR is the specific receptor for the HasA/heme complex. HasB, a TonB homolog, provides the energy for movement of heme into the cell (92). The process by which heme is transported from the periplasmic space across the inner membrane has not been well described.

A heme uptake system has also been described in the gram negative pathogen *Shigella dysenteriae*. It was demonstrated that ShuA, an outer membrane receptor, utilizes hemoglobin as a substrate. Once hemoglobin is bound, ShuA utilizes His86 and His420 for heme extraction from hemoglobin and subsequent transport across the outer membrane and into the periplasm (93). Once in the periplasm, heme is sequestered by the periplasmic binding protein ShuT. Heme is then transferred from ShuT to ShuUV, a heme-specific ABC transporter. ShuUV utilizes His252 and His262 to transport heme across the inner membrane to ShuS, the cytoplasmic heme binding protein (94). To release iron from the heme molecule, the bacteria most likely employs a heme oxygenase.

Not all bacteria that require heme utilize it solely as an iron source. Heme can also serve as a protoporphyrin source for several species of bacteria that are lacking enzymes needed for tetrapyrrole synthesis (95). The opportunistic pathogen *Haemophilus influenzae* is a heme auxotroph and, therefore, requires exogenous heme for

aerobic metabolism (95). A hemophore system has been described in *H. Influenzae* that binds hemopexin. To obtain heme from host hemopexin, it utilizes a gene cluster called *HxuABC*. The *HxuA* gene codes for a 100 kDa secreted hemophore that binds hemopexin. HxuB is a membrane protein that forms a pore and allows HxuA to be secreted from the cell. HxuC is the receptor for the HxuA:hemopexin complex (96-98). Further research is needed to determine the mechanisms responsible for heme transport across the inner membrane and incorporated into hemoproteins

Despite these extensive studies of heme uptake and transport in bacteria, proteins with similar functions in eukaryotes have only recently been identified. A heme uptake system has been described in the opportunistic pathogenic fungus, *Candida albicans*. Like its bacterial counterparts, *C. albicans* utilizes heme as an iron source because this is the most abundant form of bioavailable iron within its human host. The two proteins involved, Rtb5p and Rbt51p, are glycoproteins attached to the plasma membrane by a glycosphosphatidylinositol (GPI) anchor. Rbt5p, which appears to be the dominant protein in heme utilization, is induced when iron is limiting (99). Although the mechanism of heme uptake by these proteins is not characterized, several mechanisms have been proposed. Rtb5p/Rbt51p could tether heme to the cell surface, facilitating the diffusion of heme across the plasma membrane or allowing uptake by an unidentified receptor. The Rtb5p/Rbt51p/heme complex could also be internalized and heme released into the cytoplasm (99,100). More studies are needed to determine the mechanism of heme uptake in *C. albicans* after heme is bound to Rtb5p/Rbt51p.

In addition to pathogenic bacteria and fungi, metazoans may also utilize heme as an iron source. Studies have indicated the existence of an intestinal heme receptor in the

duodenum of pigs and humans, although the molecule has remained elusive (101,102). Recently, it was reported that solute carrier 46A1 (SLC46A1) is the mammalian intestinal heme transporter, which the authors named heme carrier protein 1 (HCP1) (103). However, SLC46A1 was actually found to transport folic acid with high affinity (104). SLC46A1 has a much greater affinity for folate than heme, and children with a hereditary loss of function in SLC46A1 needed supplemental folate but did not have any defects in iron metabolism. Therefore, the intestinal heme transporter still remains to be identified.

In eukaryotes, how heme, synthesized in the mitochondria, is exported and subsequently trafficked to other intracellular organelles and possibly to other cells is poorly understood.

Recently, two molecules have been implicated in exporting heme from the cell. The feline leukemia virus receptor subgroup C (FLVCR) was shown to mediate the efflux of the fluorescent heme analog, zinc mesoporphyrin (ZnMP), from K562 cells and rat renal epithelial cells (105). FLVCR null mice have deformities similar to humans with Black-Diamond anemia and die in midgestation (106). Additionally, these mice exhibit proerythroblast maturation arrest. This data suggest that heme efflux is essential for erythropoiesis. ABCG2, which is also called the breast cancer resistance gene (*bcrp1*), is an ABC protein that confers drug resistance to tumor cells. ABCG2/BCRP1 can interact with heme and other porphyrins. Furthermore, *Abcg2/Bcrp1* knockout mice accumulate porphyrins. Based on these observations, it has been hypothesized that ABCG2/BCRP1, like FLVCR, functions by exporting porphyrins out of the cell (107).

The conserved transmembrane-protein HRG-1 has recently been shown to be a vesicular heme transporter (44). *C. elegans* deficient in HRG-1 had increased

accumulation of ZnMP in the intestine, indicating an aberration in heme trafficking. Strikingly, when HRG-1 is knocked-down in zebrafish they showed defects in yolk tube formation, hydrocephalus, and erythropoiesis. It is hypothesized that HRG-1 mediates cellular heme availability by regulating the movement of heme from an uncharacterized endosomal compartment.

In *C. elegans*, HRG-1 has been shown to be transcriptionally upregulated >50-fold by heme (44). Heme concentrations of ≥ 10 μM results in little or no *hrg-1* expression. These results are corroborated by expression of *hrg-1::gfp* transcriptional reporter in worms. These results indicate that *hrg-1* is regulated primarily at the transcriptional level either by activation under low heme, repression under high heme, or a combination of both. The present study was conducted to identify the molecular mechanism of *hrg-1* regulation. Insights gained from these studies could provide a better understanding for how organisms sense and respond to changes in cellular heme levels.

Chapter 2: Methods

Nematode growth conditions

All experiments except for RNA interference assays were performed with *Caenorhabditis elegans* grown axenically in mCeHR-2 liquid medium supplemented with 20 μ M heme at 20°C (108).

Preparation of hemin-chloride solution

130 mg hemin chloride (Frontier CAS# 16009-13-8) was added to 18 ml of 0.3 M NH_4OH . Concentrated HCl was added until the solution had a pH of 7.5 – 8.0. The solution was brought to 20 ml with 0.3M NH_4OH , pH 8.0, to give a final concentration of 10 mM hemin chloride.

Bleaching

Nematode suspensions were centrifuged at 800 x g for 5 min at 4°C. After aspiration of the supernatant, 10 ml of 0.1 M NaCl was added to the pellet. The nematodes were resuspended and allowed to settle on ice for 5 min. After incubation, the supernatant was aspirated and 6 ml of 0.1 M NaCl was added to the pellet. In a separate tube, 1 ml of 5 M NaOH was mixed with 2 ml 5 % Chlorox bleach solution. The NaOH / bleach solution was mixed with water (3 ml / 6 ml) and added to the nematode population. The suspension was intermittently vortexed for 5 min until the cuticles of gravid worms dissolved and the eggs were released, then immediately centrifuged at 800 x g for 45 s at 4°C. The supernatant was aspirated, and 10 ml sterile water was added to the egg pellet and vortexed for 5 s. The suspension was centrifuged at 800 x g for 45 s at 4°C. After

aspiration of the supernatant, the egg pellet was washed again with 10 ml sterile water and centrifuged at 800 x *g* for 45 s at 4°C. The supernatant was aspirated, and the pellet was resuspended in 10 ml M9 salt solution (86 mM NaCl, 42 mM Na₂HPO₄, 22 mM KH₂PO₄, 1 mM MgSO₄ 7H₂O). The eggs were hatched overnight in the M9 solution at 20°C on a platform shaker set to 70 rpm.

RNA-mediated interference

E. coli HT115(*DE3*) expressing *elt-1*, *elt-2*, *elt-6* double-stranded RNA from the Ahringer library were grown in LB broth supplemented with 4 or 25 μM heme for 5.5 h (109). Two wells per construct of a 12 well nematode growth medium (NGM) agar plate were spotted with the bacteria and allowed to induce for 24 h. Forty L3 larvae expressing 3 kb of the *hrg-1* promoter fused to GFP and the *unc-54* UTR (strain IQ6011) grown in mCeHR-2 media supplemented with 10 μM heme were placed in each well. Worms were analyzed 48 h after RNAi feeding with a Leica MZ16FA stereo-microscope. Images were acquired by an inverted Leica DMIRE2 fluorescence microscope using a plan 20x objective (NA = 0.4).

Bombardment

Worms with an *unc-119* (*ed3*) mutation, which causes an impaired mobility phenotype, were grown in two flasks, each containing 90 ml mCeHR-2 media and 20 μM heme, until the population contained ~30,000 gravid worms / ml. The worms were then transferred to 50 ml conical tubes and allowed to settle. The supernatant was aspirated to remove larvae, which take longer to settle than gravid worms. *unc-119* worms were not bleached

due to the limited amount of progeny they produce. About 6,000,000 gravids were spread evenly onto a 10 cm² NGM agar plate and incubated on ice for 30 min. During the incubation, plasmid DNA (prepared from *E. coli* cultures using Macherey-Nagel midi-prep kits) was prepared for bombardment. Reporter plasmids (10 µg) were mixed with 5 µg *unc-119* rescue plasmid. Mutant worms that are successfully transformed with the wild-type *unc-119* gene show normal movement (110). The DNA mixture was coated onto 2 µm gold beads (Ferro Corporation, Germany). The DNA coated gold beads and *unc-119* worms were loaded into the Bio-Rad PDS-1000 particle delivery system. Pressure was built inside the system to 14000 psi, at which point the coated gold beads were fired into the worms. Upon completion of the bombardment, 10 ml of M9 solution was added to the bombardment plate. The M9 / worm mixture was spread evenly onto 20 NGM agar 10 cm² plates with a lawn of *E. coli* strain JM109. After a two-week incubation at 25°C, worms with wild-type movement, which indicated successful transformation, were placed onto individual NGM plates. Only worms exhibiting both wild-type movement and GFP expression were propagated for experiments. Each bombardment produced about 5-6 lines carrying extrachromosomal arrays. An integrated line was obtained in ~ 25% of the bombardments.

Injection

N2 wild-type worms were bleached and incubated at 20°C in M9 buffer overnight to synchronize the population. The following day, ~1000 L1 larvae/ml were placed in mCeHR-2 medium supplemented with 20 µM heme for four days, at which time the population had developed into young gravid worms. Roughly 100 worms were aliquoted

from the mCeHR-2 media and placed on an NGM plate. Worms to be injected were removed from the NGM plate and placed on a 2 % agarose pad coated with a thin layer of immersion oil. Plasmid DNA for injection was prepared with the Qiagen midi-prep kit. The reporter construct was co-injected with a dominant marker, *rol-6*, into wild-type N2 worms (111). Transgenic lines carrying the *rol-6* marker can be easily identified by the rolling phenotype. The final concentration of each plasmid was 50 ng / μ l. The DNA mixture was loaded into a microinjection needle and manipulated with a Narisihge hanging joystick micromanipulator. The DNA ($\sim 10^{-9}$ nl) was injected into the gonads of young gravid worms (P_0) with a Femtojet (Eppendorf) set at 20.5 psi. After injection, worms were allowed to recover by placing them on NGM agar plates spotted with *E. coli* strain OP50. Three days after injection, F_1 progeny with the rolling phenotype were transferred to individual OP50 plates. After three days, plates were checked for F_2 progeny with the rolling phenotype. F_2 rollers are an indication that the transgenes were segregating. These worms were maintained as extrachromosomal lines. Each F_2 roller was further propagated as a separate transgenic line for experiments.

Cloning

Constructs *hrg-1::gfp*, *hrg-1 Δ (-254 - -160)::gfp*, *hrg-1 Δ (-254 - -99)::gfp*, *hrg-1 Δ (-91 - -16)::gfp*, *hrg-1 Δ (-254 - -160, -91 - -16)::gfp*, *hrg-1m(-159 - -137)::gfp* and *vha-6:gfp* were created using the GATEWAY cloning system. The promoter of interest, GFP, and the *unc-54* 3' untranslated region (UTR) were cloned by recombination into the entry vectors pDONR P4-P1R, pDONR 221, and pDONR P2R-P3, respectively, using the GATEWAY BP Clonase kit. The three entry clones were then recombined into a single

destination vector, pDEST R4-R3, using the GATEWAY LR Clonase II plus enzyme kit. The mutation of the 23-bp element in construct *hrg-1m(-159 – -137)::gfp* was generated by mutating T→G, A→C, G→G, and C→A. Constructs *hrg-1Δ(-254 – -160)::vha-6::gfp*, *vha-6::hrg-1Δ(-254 – -160)::gfp*, and *hrg-1Δ(-254 – -160)::egl-18::gfp* were created by restriction enzyme cloning. Fusion PCR was used to create PCR products with 159 bp of *hrg-1* promoter placed 5' or 3' of the *vha-6* promoter. These products were cloned into the Fire vector pPD95.67 (purchased from Addgene). Construct *hrg-1Δ(-254 – -160)::egl-18::gfp* was created by cloning the 159 bp fragment of the *hrg-1* promoter into the pKKMCS vector (a kind gift from Dr. David Eisenmann). Constructs *hrg-1m(-159 – -155)::gfp*, *hrg-1m(-154 – -150)::gfp*, *hrg-1m(-149 – -145)::gfp*, *hrg-1m(-144 – -140)::gfp*, and *hrg-1m(-141 – -137)::gfp* were synthesized by site-directed mutagenesis PCR (112,113). Partially overlapping primers were designed to bind to the *hrg-1* promoter but included each 5-bp mutation (114). These primer pairs were used in a PCR reaction with *hrg-1::gfp* as template. The partially overlapping primers were extended in the PCR reaction, creating linear plasmids with the desired mutations.

Heme response

To assay the heme response of transgenic *C. elegans* transformed with the reporter constructs, worms were bleached to make them axenic and then placed in M9 buffer overnight for synchronization. The following day, synchronized L1 larvae were placed in mCeHR-2 media with either 4 or 100 μM heme. After 72 hours, worms were removed from media and placed on unseeded NGM agar plates. Worms carrying the transgene of interest were separated from non-transgenic worms and analyzed for GFP expression by

fluorescence microscopy with a Leica MZ16FA stereo-microscope. For acquiring images of each line, worms were picked from NGM plates onto 2 % agarose pads spotted with 10 μ l of 10 mM levamisole. Differential interference contrast (DIC) and fluorescence images were acquired with an inverted Leica DMIRE2 fluorescence microscope using a plan 20x (NA = 0.4) or plan 40x (NA=0.55) objectives.

Chapter 3: Results

Locating the heme responsive *cis* element in the *hrg-1* promoter

The conservation of DNA in non-coding regions can indicate regulatory function. Such regions can be identified by comparative genomics, a tool that is especially powerful in *Caenorhabditis* because the genomes of several species have been sequenced (115). To determine the potential heme responsive *cis* elements in the *hrg-1* promoter, we conducted a DNA sequence alignment using 3 kb of the 5' upstream sequence from the ATG translational start site of *C. elegans hrg-1* with its homologs in *Caenorhabditis briggsae* and *Caenorhabditis remanei*. The alignment results showed strong conservation in the first 200 bp with respect to the ATG start site of the *hrg-1* promoter (Figure 1). Within this region were five putative binding *cis* elements for ELT-2, which binds the consensus sequence TGATAA (GATA site). ELT-2 is a GATA family transcription factor that is responsible for the intestinal-specific expression of genes in *C. elegans* (116,117). We called these GATA sites GATA SITE 1 to GATA SITE 5, with GATA SITE 1 being the element closest to the ATG translational start site. GATA SITE 3, GATA SITE 4, and GATA 5 were conserved in *C. briggsae and remanei* and flanked a 23-bp conserved element. GATA SITE 1 and GATA SITE 2 were absent in the *C. briggsae* and *C. remanei* promoters.

C. elegans has seven GATA family transcription factors which are expressed in specific tissues. To determine if ELT-2, which is intestinal specific, is required for *hrg-1* expression under low heme conditions, a transgenic *C. elegans* strain (IQ6011), harboring an integrated transgene with 3 kb of the *hrg-1* promoter fused to *gfp* and the *unc-54* 3' UTR, was fed bacteria synthesizing double stranded RNA for *elt-1*, *elt-2*, *elt-6*, or vector

only. ELT-1 and ELT-6 are GATA-family transcription factors which are expressed in the hypodermis and seam cells, respectively (118,119). IQ6011 fed with either vector, *elt-1*, or *elt-6* showed strong GFP expression in the intestine at 4 μ M heme. However, IQ6011 fed with *elt-2* dsRNA showed attenuated expression of GFP at 4 μ M heme (Figure 2). These results suggest that ELT-2 is required for *hrg-1* expression at low heme.

In addition to the GATA sites, there was a contiguous stretch of 23 nucleotides that was also conserved in the *hrg-1* promoter sequence from *C. elegans*, *C. briggsae*, and *C. remanei*. To investigate whether this conserved region containing the GATA sites and the 23-bp element was sufficient for heme responsiveness, the first 254 bp of the *hrg-1* promoter upstream of ATG start site was fused to *gfp* and the *unc-54* 3' UTR. This construct was used to generate transgenic worms by microinjections or microparticle bombardment using biolistics. The transgenic lines generated by both transformation methods were placed in either 4 or 100 μ M heme for 72 h to evaluate GFP expression. Worms were then collected for analysis by fluorescence microscopy. GFP expression was only seen in worms in 4 μ M heme. This result indicates that 254 bp of the *hrg-1* promoter was sufficient for heme responsiveness (Figure 3). These 254 nucleotides will henceforth be referred to as the *hrg-1* promoter (*hrg-1::gfp*). All the subsequent constructs were named according to the regions deleted from *hrg-1::gfp* (Table 1).

We next sought to delineate the minimal heme-responsive region within the *hrg-1* promoter. If the five putative GATA sites were important for intestinal expression but not heme-responsiveness, then the *hrg-1* promoter should still show a heme response even if the two GATA sites upstream of the 23-bp element were removed. A deletion

was made in the *hrg-1::gfp* construct to remove 95 nucleotides between -254 bp and -160 bp. This construct was named *hrg-1Δ(-254 – -160)::gfp*. This deletion removed GATA SITE 4 and GATA SITE 5, which are located directly upstream of the 23-bp element. Transgenic worms transformed with *hrg-1Δ(-254 – -160)::gfp* expressed GFP in 4 μM heme, but not in 100 μM heme (Figure 4). This indicated that the 159 bp of the *hrg-1* promoter upstream of the ATG start codon was sufficient to confer heme responsiveness.

We then sought to determine if the remainder of the conserved region, which contained the 23-bp element and GATA SITE 3, was important for heme response. A deletion was made in the *hrg-1* promoter which removed the 23-bp conserved sequence and GATA SITE 3. We called this construct *hrg-1Δ(-254 – -99)::gfp*. Transgenic worms expressing *hrg-1Δ(-254 – -99)::gfp* expressed GFP constitutively at low levels in 4 μM and 100 μM heme (Figure 5). The absence of heme responsiveness and low GFP expression indicated that the region located between -159 bp and -98 bp of the *hrg-1* promoter may be necessary for both heme regulation and enhanced *hrg-1* expression.

We investigated whether the relative spatial position of the 23-bp element with respect to the translational start site was important. A stretch of non-conserved bases, -92bp to -16bp, which included GATA SITE 1 and GATA SITE 2, between the 23-bp conserved element and the ATG start codon was deleted. This construct was named *hrg-1Δ(-91 – -16)::gfp*. Transgenic worms expressing this GFP construct displayed heme responsiveness but it was severely attenuated, suggesting that the 23-bp element may have spatial constraints or that GATA SITE 1 and GATA SITE 2 are essential for robust expression (Figure 6). Taken together, these results indicate that the heme-responsive element was within a 67 bp region in the *hrg-1* promoter (Figure 7).

To further confirm our results, deletions from -254 bp to -169 bp and -92 bp to -15 bp were made in the *hrg-1* promoter. This deletion removed the GATA SITE 1, GATA SITE 2, GATA SITE 4, and GATA SITE 5 sites, but retained GATA SITE 3. Transgenic worms expressing *hrg-1* $\Delta(-254 - -169, -92 - -15)::gfp$ had no detectable GFP in the presence or absence of heme. These results suggest that the 23-bp putative heme responsive element and GATA SITE 3 are insufficient by themselves for GFP expression and that other regions within the deleted portion of the promoter, including the GATA elements, are also required for *hrg-1* expression and regulation by heme (Figure 8).

The conserved 23-bp element is essential for the expression of *hrg-1*

Our deletion analysis suggests that the 23-bp region, between -159 bp and -137 bp, may be responsible for heme dependent *hrg-1* regulation. This element comprises two inverted repeats (Figure 9). To determine whether the 23-bp element was necessary for heme-responsiveness, all 23 nucleotides were changed from a purine to a pyrimidine using PCR-based site directed mutagenesis. This construct was named *hrg-1m(-159 - -137)::gfp*. Stable worm lines, transformed with the mutated element, expressed GFP in the anterior and posterior intestine which did not appear to be heme responsive (Figure 10). This result indicated that the 23-bp region was necessary for *hrg-1* expression. We next wanted to ascertain if a specific portion of the 23-bp conserved region was important for heme-responsiveness. We mutated the 23-bp element five consecutive nucleotides at a time. The only construct that showed a different expression pattern than *hrg-1m(-159 - -137)::gfp* was *hrg-1m(-141 - -137)::gfp*, in which five nucleotides at the 3' end of the

23-bp element were mutated (Figure 11). Importantly, GFP expression was repressed in the presence of heme in these transgenic worms. Moreover, these worms expressed GFP at a much lower level when exposed to 4 μ M heme compared to the *hrg-1::gfp* construct. These results suggest that the five mutated nucleotides at the 3' end of the 23-bp element were important for normal levels of GFP expression, even though heme-mediated repression remained intact.

Heme response of the *hrg-1* promoter is controlled by enhancers and repressors

The *elt-2* RNAi results suggested that ELT-2, possibly by working through GATA sites, is important for *hrg-1* expression in the intestine (Figure 2). The loss of GFP expression at 4 μ M in worms expressing the mutated 23-bp element indicated that this region may contain enhancer elements (Figure 11). However, mutation of the five nucleotides at the 3' end of the 23-bp element leads to low levels of GFP expression suggesting that the 23-bp element may also contain repressor element(s). To confirm these results the *hrg-1* $\Delta(-254 - -160)$ deletion construct was fused upstream of the *egl-18* minimal promoter to generate *hrg-1* $\Delta(-254 - -160)::egl-18::gfp$. The *egl-18* minimal promoter drives GFP expression in the intestine and several other tissues but is not heme responsive (Figure 12) (119). In 4 μ M heme, worms transformed with *hrg-1* $\Delta(-254 - -160)::egl-18::gfp$ showed greater GFP expression than worms transformed with *egl-18::gfp*. Conversely, GFP expression was attenuated at 100 μ M heme compared to worms expressing *egl-18::gfp* (Figure 12). Based on these observations, we conclude that the heme-responsiveness of the *hrg-1* promoter is mediated by both enhancer and repressor elements.

The *C. elegans* gene *vha-6* encodes for a subunit of the vacuolar ATPase that is expressed specifically in the worm intestine (120). The *vha-6::gfp* reporter is strongly expressed in worms grown in both low and high heme (Figure 13). Importantly, even though the *vha-6* promoter contains multiple GATA sites and is regulated by ELT-2, this feature by itself is insufficient to confer heme responsiveness. To further investigate the repressor activity of the *hrg-1* promoter, *hrg-1Δ(-254 – -160)::gfp* was either placed upstream or downstream of the *vha-6* promoter to generate constructs *vha-6::hrg-1Δ(-254 – -160)::gfp* and *hrg-1Δ(-254 – -160)::vha-6::gfp*, respectively. We found that *hrg-1Δ(-254 – -160)* was not heme responsive when it was situated upstream from the *vha-6* promoter. However, heme-responsiveness was restored when *hrg-1Δ(-254 – -160)* was positioned downstream from the *vha-6* promoter (Figure 13). We infer that the proximity of the *hrg-1* promoter to the ATG start site may control GFP expression. Altogether, our results indicate that a 23-bp conserved element and the GATA sites work synergistically and are essential for the heme-dependent regulation of *hrg-1* (Table 2).

Figure 1: Alignment of the *hrg-1* promoter in three nematode species

Three kilobases of the 5' flanking sequence of *hrg-1* from *C. elegans*, *C. remanei*, and *C. briggsae* were aligned using ClustalW and Boxshade 3.2.1. A region of several conserved elements was found in a 200-bp region directly upstream of the translational ATG start site. Conserved bases are indicated by a black box. A highly conserved 23-bp region is boxed. GATA sites are highlighted with forward or reverse arrows indicating top or bottom strand orientation, respectively. A conserved spliced leader 1 sequence is underlined.

C. briggsae -3030 TGACAAC TGTAGAATATTGGTGA TAAATATGATGAGTAAATTA TCAGTTGACATTTTCTT
C. remanei -2992 -----TAAATAGTTTT-CAATTAGGTCAACGCTGGA--TTTTCTA
C. elegans -3077 -----GACGGTTCC-GGATACCTATAACGTAAAAACCTAAAAAT

C. briggsae -2970 GCTATTTTTCATAGAAATCAAGATAATTGAGCTC---AGATTGACGGTGTGGGTGGGC
C. remanei -2969 GCTGGAGTT-GTTGAGCGAAAGCGGATAGAGTTTGATGAAATGAGAGATTATGAGAAGAT
C. elegans -3004 GGTTCGAAITTTGTAAATGAAACTTCTATAAAATTTTTTGAAAGATTCTGTGGTCGTTCAAA

C. briggsae -2914 TTATGTCGAGGGAAATACGGTATCAGTGACGTCAAAAACCTAGTCCAGGTTTTAGTTCT
C. remanei -2910 TTAAGAAGAAATGCTATTTTTTCACGAGCAGGAATTGGAACCTACTCCGCGAGATCCCTACTT
C. elegans -2944 ATTTCCAAAACCTAACTTTTTTACTAACCTT-----TTTTTTGGAATTATCATCCCTT

C. briggsae -2854 TCACGGTCTCTCTTACAGTAGCTCCACGTTTTCTCAAGTACCGCAGGTGCCGTAAAGAC
C. remanei -2850 ACTCCACCATCTTTTCACTTTTCAATTTAAAAACTAACTGTTATA--CTCTGCAAGGGT
C. elegans -2862 ATTGAAAACCTTTGTAAATTTATCTTACGTTAT-ATCAAATAATTA-AGGTACTTTGAAAAAC

C. briggsae -2794 TCTGACAG---AACGCCACTTGGACCGTCCACTTTGAGCTTATGAATCTCACTTCTTTTT
C. remanei -2792 TCCTGCAGTCCCAAGTGTAGCTAGGCGGTCTACCATATA--TCTCCGTACTGGGTTCAGCA
C. elegans -2804 AGTTGCCCTTAAATGCT--TTAAATGAATTACTTAAATCAACTGAAT--AACGTAAGAA

C. briggsae -2738 GAAAGATGTCAAAAAAT--TGTCGATTAAGAAAAATGTTTATCATGATATCAGCAAGATTT
C. remanei -2734 AAATATCCGGTGTCTGT--TGCTGGTTGATTCTCCGGTCTTAGCACCTTGACTTTGATAC
C. elegans -2748 AAACGCCCAAAATATGCAATAAAGAAATAAAGTACTTTTCAAAAAAAAAGTCAATTT

C. briggsae -2680 T--ATTAGTTAACCTATTTTGAATATCTTTACGGAAATGGGATACATGGGCTCAGTTTTT
C. remanei -2676 C--TTTGTGTGATTTCTCTT--TTGCTCCCGTGTGAACTTCTGTAGATTCG-TTATCAGA
C. elegans -2688 CCAACCGCTCCGACACAGAAAAATGGTTAAAATTTGAAAAAGGTTTGCTAAAAGTTTTCCG

C. briggsae -2622 GACGATTCGGGTA GCGTTC TAG--TAGAGTGTTTACTGT-AGTTGAATTTGCTATGATATA
C. remanei -2621 AGCAGTGCCTGTCATCTGTAACATTTGAGTACAACCTTT-TTACCACAAAATCTATTTG
C. elegans -2628 AAGATTTATTACAAGATCGCTTATTTGAGCACATTTTAGTCCGTTACTTGTCTCTTAA

C. briggsae -2566 CTGTAA-TACCAGCCAGTACCACATCCCTAG-TCAAAAATGTTTTCTAATCCCTCCCGT
C. remanei -2562 GTTTAACTACCTTCTGAACGGCAGGTGTTGAAATCCAAAAGTTTAGCTGACCGTTTTCTG
C. elegans -2568 ACTTGA--ACAATTCAAGT--CAATTTTTGGAACTCTGCAAG---AGTTAACTAATTCGAA

C. briggsae -2506 TCTACCCTCCAAAAATCCCTCTGAAACAGAATAAACTAACTTTGTAATCATCAAAG
C. remanei -2502 GGAGCGCTCTTTTTGGTTC---AAATCTTTGCTTCTATTTCTTCACT---TCGATT
C. elegans -2513 GTCGAATCGGCATTTATAAAAAGAAACGCGGAAATGCAAACTGTGAGAAAGGAGGAACA

C. briggsae -2446 TGTCTCGGTTCACGATTGACCATCTCCGTCAGATAATATCCATATTCCTGTCCTGCG-A
C. remanei -2449 TGTTTCGGTCCCT-----TCTC-----GACATCTTTCTATCTTTCTTCT-CG-G
C. elegans -2453 AGCAACGAACTCGAGGT--AGCGGGGAGGCGAGAGGGTTTCAGATTTTTGATCTGCTGTG

C. briggsae -2387 AAAAATTTGGCGCTGTCACTGGCATGTTTTCCGGACGGACCCTTTCACTTCTTTTGT
C. remanei -2404 TAACTTCCACAGTCC--ACAAACATGGCATTTGAATGAAGAG----ACCTGGACTTGT
C. elegans -2395 AAAGCTTTACAGTTGG-AAAAAGGAACCTTTGTTTCTAAGCCA--AGTGGAGAGTACA

C. briggsae -2327 TGGAGTCTTCTCGGATTTGGTATCTTCTCCTTTTTTCATCGGAATAAGTTTCGCCCTCCA
C. remanei -2351 ---GATCCATACCCAGTGTCCGATTTGACTGCCATATGTGCGGAAAACAACTTTCTCTC
C. elegans -2339 ---GTACCACCAACAATGTAAGAGTGTAACTTGTTTTTGGTTCAAAACATAA-ACATACA

C. briggsae -2267 TTTTGAACATATCCACAAAGTTTTCGGAGAACCCTTTTTTCTTCAAGTTCAGCAATTCGGG
C. remanei -2294 TTTTCATAGTCTTTTCAGACGAGCTTTCAG---CTTGCGCGGAGCTGCATGTTGATCAG-
C. elegans -2280 TAACGAAAGCTATGGTACGAATAATTAAAAAATCATTGGCCAGCCAGACTGTTATCAA-

C. briggsae -2207 CTTCTCTATTTTCGTTTCTTCTCACTGTCATTTTGTGCGAGTGCCTGGTGGAAAT
C. remanei -2239 -CTCCCCAAGTTTCAAATCAGTGAAGATTGAATAATCGCTGGTACGAC--GAACAGCTC
C. elegans -2221 -AACTGTGATGATAACAAGCGGTATTAAGAAGAACGACATAATCAAC-----CTTGTTC

C. briggsae -2147 TCGAAAAATAGTCGGACGTGGGCTCA--CTGATGAATACCCGCTGTGAGAGTTTGTGCG
C. remanei -2182 CAGAACGGTTTCGTGCTCAGAGGGATCA--CTGCAGAAGATCTCATATCGACATTTCTCCA
C. elegans -2166 CCGACCATCTCTCTTCTATAACCTTAATTTGTCAAGAACTCTCACTCAGTTTTTGTCA

C. briggsae -2089 GCAGCGAGCCTCTGCCTGAGCCGAACGCAAGGTTCTGTTG-CTATCATAGCCAAGT-TCC
C. remanei -2124 TTCAAACCTTCTTCCAACATACGGAGTCTCTTTCTTTTAACTGGCATTGAGATTCTTC
C. elegans -2106 ATTCTAAATCC-----AAACAAAAGTTGTTTTTAAAAAACAATAAATCTCGATTCTTC

C. briggsae -2031 AAT-TGATTCGAAGACGAGTA--AGAACTATTTCTCCGACATTAECTCTTTTGTGATC
C. remanei -2064 GGC-GCATTTGATGATTGTCTCC-ACTGTTATCTTTGAGAATATATGACGACGTAACATCT
C. elegans -2051 AGCAGTAACTCTTAAACCATAGAAAATAATTTCACTAGAAAATAAATGCCGATAGATTTT

C. briggsae -1973 CGTTTGGAGTAGAAAAACAACCTTTGACGCGTTTGTGACAGAATGCGAGATCCTTGA
C. remanei -2006 GAGATAGTGTTCGGTCCACTTCTTCTCTCATTTGTTGGATTCTCTGCAGAAAGTAAT
C. elegans -1991 CAAACAAAGTACATGAAAAGAGCGGCCACTCGGTTCCGAAACCATTAGC--GGGCCCGA-

C. briggsae -1913 CGATTCTTTCCGATGGGGCGAAAACCTTCTTAGGAGACATTGTCTTCTCTGGTCTGCTCT
C. remanei -1946 CCCGACAGATCCATCTGTAGTTTACACCTA--TAACAATATTTGGAAACGATAACATACA
C. elegans -1933 --CTGTAAAACAAATGCACTGACAAAAGCTGAAAGGCCGATAAATCACGTGATTTCTA

C. briggsae -1853 TCTGGTAACGTTGGCAGTGGTTTCTTATCCATTATGGCTGATACCTGGAAGATGAAGAGT
C. remanei -1888 TATGAAGATACTTAAACGGGAAGAAATAAGGAAAAGTACGGA-AGAAAACAGAAGAGAAAA
C. elegans -1875 GGCATTCTAGACCATCGCAATGATTTCAAGGAGCCATGAAACATTGATACCAAAGAAAAA

C. briggsae -1793 GAGGATAGGCAAGATTAGAATTCGCGG--CTGTGAAACAGGGAGGCGAGAGGA--GAG
C. remanei -1829 GAAAGAGAAAGAGAGCTCGCCCGTTCAGTCTATGTCGGAACCGACCGAAAAACCGAA
C. elegans -1815 ACAGAGAGAAAGAGAATGAAAGGCCA----TGTGAACGA-CGAGCGCAAAACA--GAA

C. briggsae -1738 CCGTTACTATTGGCAAAACGGTTATACCAGAAAACCTG-ACTGAACGCGCAACCGGCATCGG
C. remanei -1769 CGAATGCCAACGGCCAAAGGGGGGGTCAATAAAGCA-AAACAGTGCCAGAAAAGAGAGG
C. elegans -1763 TGGTCCCGCGC-CGAATCAATGAATTATGAGTGAGTGGACGACATCTCTTGCCTGGCAA

C. briggsae -1679 CCTTGGGGCGGAGCTATAGACA-----GCCGAAC--TAGAGAGTATAGCTGTGTAGA
C. remanei -1710 GCGCCACCTGCATAGATTGTCTTTTCTCTCAAGTGTGCAAAAATGAGGTATTGATTTGA
C. elegans -1704 AACTGTGCGCGAGATGAGTTACTTTT-CTCACGT-CTCCTTTCATAATCTCCCTCTCT

C. briggsae -1626 GGGTATGTGTGGATCTTCGTTTCGCTATGGAATAATATCAATTGTGATG---AGTTAGA
C. remanei -1650 TGG-ATGCAATAGAGGTATGTTTT--TTTGAAGAATGGCAACAGTGTGTTTCAATCAA
C. elegans -1646 C---TCTCTCGCGCTGTTCTTCTCTCTCTCTCTATTTCTACAATGTC-----TCCAT

C. briggsae -1570 GGCTATTGGAGAGACAACTTAAAAGTAGGAGAAGTACGAAATGTTGCTTTATAAACCCTTA-
C. remanei -1593 AGTGTTTTGCCAAAAACCGTCGAG--AGAGGAAACCGTTTATGGTCCGATAAAAACATCG
C. elegans -1598 TATATTACATTAGGTCACTCGTTGTTGCAATTGACCGCTGTAAATC--TATATTCTCTCT

C. briggsae -1511 -AAATTTAGTAAGAAAGCAAGCTAAACCAAAAAGCCAAAATGAGAGATTTTCATTGGTCA
C. remanei -1535 TAAACTCTGCAAAATCCAGAACATCCATCGTGAACCGCTAATCATTGAT---AAATGATTA
C. elegans -1540 GAATATCGACA-----GAAGGTGAGCAATGTTTTTGTGATGTTAGATTGCAATAAGTTG

C. briggsae -1452 AATTCAAAATAATAAACTAGGCGTTGCGTTGGTCATTGCGACTGAGGGACACATCTTAA
C. remanei -1478 ATATCAGGCGGAAAGCATCTAAAGAATCAAAAACAATTTGGTTCAAACCAAGGATCCGAA
C. elegans -1486 AAGATAATATCAGAG-ATCGATCATCTAAATAGTAGCTTTTTTTAAAGAGAAAAC--A

C. briggsae -1392 CAATGACAAAGTGTAAACAT---TTTGTGGTCCAAACATACACAATGAACA-GTCTAAACG
C. remanei -1418 CAATGGAAAAGTGTAAATATGTATTTTGTGGGTCAAACAGCCCAAAATA-GTAATAGAA
C. elegans -1429 TAGTTTTCAGTTTAAA-----TTAAAAATTTACATTTTCGAAGCATATGTTGGTCAA

C. briggsae -1336 TCTTAACCAAAAGTTCAACGTTATTGACCAACCCGGTGTTCATCATATTTGTGATTAATAA
C. remanei -1359 AGTTA-CCAAAAGTTCGA-AATAATAACGACCCGGTGTTCATTAAAAGTATGATGATAG
C. elegans -1376 TGCCG-TAAAAACGTGAAAAAT----GCAACGTCTATTTTGGT'TTTACAAACCTGATTT

C. briggsae -1276 AAAAGGAGTAAG---AAAGTGATA--ACTCTCTGGCCATCAGAAATCCTTGGTCAATTGT
C. remanei -1301 ACGAGAGATGGGTGAAAAGTGATAFAACCTTCTA-TCTTCATA-----TCGCCAATCCT
C. elegans -1321 GAAATAATGCGTCATGAATCATAACAATTTCTA----TGAAA--TCTTTCCAAAGT

C. briggsae -1221 ATCTTTCGAGAAATATTATCGAGATATCAGTCAACCTTCCCTCCCTTTTGT----
C. remanei -1247 TGCCCTCGCCG---TATTGTGGGAAATCAGTCAACTTTTTCCTTTTGTGGCCA
C. elegans -1267 AGGCGGAGGCTTGAAA CAAGGCAAATAA--ATCACATGCAATTTTCCAAATTTTGGTTT

C. briggsae -1167 AAGATAAACAGTTTACTGTCCGAAAAACCTGAAGTTCACCCCTCGTATTTCTGAATCAAA
C. remanei -1190 AAAGAAAACAGTTTATGTTAAAAATGATAT-----TTTGTTCCTTAC-TTCTCTATCAAA
C. elegans -1207 AATTTTAGTTGCTCAGTACTGTAAATTTA-----GTGTTAACCATGTTGGCATTTA--

C. briggsae -1107 CGATCATAAAGATCCATGATTTTCTCTTTCTGTGGAAAGATCCCAAATAATGGTTGC
C. remanei -1137 CCATCATAACTATCCATGATCTT-TTTCTCAACCAGCATCCCAA-----T
C. elegans -1156 ---CTAAAAATGTTGAAAGAACAT----ACTGAACCAG-ACGTCTAGGCATGTGGTACAT

C. briggsae -1047 TTTTCGGCAAAAATACAAAACAAAAAAGAAATGCACATCCGGTTCCTCGATAATGCGAACA
C. remanei -1087 TTTTCACAGAAAAGACAAAATACATGGCGCATCCGGCTGGCTTCCAAAACGAAAAGATG
C. elegans -1103 AGTTTACAATCATGAGATGCGTAGCGTAAGCCTGCTAAAAATCAAAAGAGAGCTGGCA

C. briggsae -987 AAGTCAAAAATCGGCAGCAAAAATCATAAACCATCCAAAAATTGAACTGTTGGGGAAG
C. remanei -1027 CATGGGAACCTGTGCACCGACAAAGAGATTGAAATTTGGGAGAAATCGTTCACGTGAAT
C. elegans -1043 AAC---ACACTTTGAAACAAAGCTGTGAAACACTAACAGAAAGTCCCAACTATTTTTGT

C. briggsae -927 CACGTGGAATAACAGGAGGAATATGAAACCCTGTATTATTGCCTCGGTTTCTTCTCTT
C. remanei -967 CTTATCCATCACTCGACAATCCGAAAAATAC--CTTTGGTACTGTAGATCTAACATCATG
C. elegans -986 AATCATCATTCCTCCGCAATGTGTGGAAATTTTCTGCCGACCAGACGCTAGACATG

C. briggsae -867 TTTACATGTACCAAAAAAGCCAGGAGGGGTTTCGATGAATGAACAGAGATTAATTTG
C. remanei -909 TCTCTCCAAATCGGAGAGGAAAAAGATAGATTTTGGGAAGTGTAAAGAG-----TGTG
C. elegans -926 TAGCATATGGTCTTG---GAACATGAAAAGGTACAGCCAGCTTCTGTGAG---CTCAA

C. briggsae -807 TGCGTCATAAAAATCGTTTTTTTTCTGGAATGATTACAGTATCTAGATTTCCGAAAAGA
C. remanei -854 TACTTCATAGATATGTATGCTCATTGTAAGTACAAAAAT-----CTTGGTGGCGA
C. elegans -873 AAGTACAAAGAT-TGGATAAAGTTTGT'TTCTTACCTCAAT-----GTTGGGCCAATG

C. briggsae -747 GACAACGGGTAACAGCTATCTTATTCCGAACAGGAGACGCTCTCTCTACCT-TCTTTCTC
C. remanei -801 CACCTCTTCCCAGACA-CATGAACACATGAACACA-ACTCTGTGTAT-CGTTTCTC
C. elegans -820 TTCGAACCAATCTGAAAAGAAATAC-CGGATGACTTCAAATTTTAAAAATGTGCCCGAG

C. briggsae -688 TAGCACCTCT--TCGTTTCTA-GTCTA--TTTTTAGGCATGGA-GGTCAACATCT-G
C. remanei -744 TGGCACTCTCCAAGTGTCTCTTTACTCTT--CTGCAAACGATAAAGGTACCTGCTAG
C. elegans -761 TATCGAAGTATATAAGAATCTTAACTTTCGGTACATAAAATAAATAAATAATTTTGG

C. briggsae -635 CGGACCGTCTGTGTGTGTAGTTGATCGTCTTCTCTCATAGAACGGACCACT--TTAT
C. remanei -686 TGGCCCTCTCGCATCCATCTCTC--TTTTCCATCATCGGAAAGAGGGAGTACCCCTTAT
C. elegans -701 AAAATTTAGTTAAAGGGTTTGATAAATTGCCGAATTTCCAAAAAACAGTTCTTTTCGA

C. briggsae -577 CGATTTTCTGTATTTCTGTCTGCTAAAAACGCCT--TC---TGG---CACTCGCAG
C. remanei -628 TGATTTTTTTCACATTTTCAT-ATCCAAGAAAAGCCT--TTGGCCTCC---TGATTTCTGA
C. elegans -641 GATGTTTGTSCAATCTCTTATGTCTGACCCCTATATAGTTGGTTTGAGCATGATTTGGAA

C. briggsae -524 AACAA--TT-TGTAAGCTGGTT-TTCAGGTGAGTCTTCAACA-TGTTATGAGTAGAAAATA
C. remanei -574 GGTAAGATT-TGGGATTTTCTCATTTTATGAGTTTAAAGGAGCTTTTGTCTCCATTAA
C. elegans -581 TGCAAAGTTGTAGAAAACCAAATTTGATGAGCTCTCAAAA--TTATGAGTGGCAAAA

C. briggsae -467 TTTTTGAGTGGTTTCCCATGTA-AAACTTATAGTAAAATACAAAACATC---TTATTGG
C. remanei -515 ---TTGGGTTTCTCGGCTCTT-ACGTTTAGATAATTCTATAAACGTTGTATTTTTTA
C. elegans -523 CGAAACAATTTCCCAATTTTCAAAATTTTACTGCGGTATATGATTGCTCAATCATA

C. briggsae -412 AAAA-----TCCCA--TCGATCGTTGTCCGAA--GATTAAATAGTCACACCTT
C. remanei -458 AAAGCGTTGTTCTTCCAG--TCGATCGTTCTTATAAATGTTTATAGTTCCCTGGTTGA
C. elegans -463 TATGA-----TATCCAAAGTTTGTATTTCATGTTGCAACTTGAAAATCTTTTTTTGTT

C. briggsae -365 CAGCT-----TGAGATCTTTGACATTTTTCACAAAATCATTGTGCATTACATT-
C. remanei -400 CAACTATGATAAAATAAACCCCTTGCAATTTTTCATAAACAGTTTACTTCTGTACTTG
C. elegans -409 TCACCAT-----CTACACTGGTTTCAAAAATACATACAGTGTACCACCCTCAGTTTTTC

C. briggsae -315 -----ATTATCGGA-----AAAAC TTC-----TTCTCCACTAGG
C. remanei -340 CGCACAAAAGTTTCATAATTGAAC TACAAAAGAGACACACATACATTTCTTTATCATG
C. elegans -355 AAATTCAAAAGTACAGAGATCGGAC-----AAAAC TCTCCACATTTCTTAATAAT

C. briggsae -265 CC--AACAC--TTTCCATTTTCTTGA AATTGACAC-----TCCACCGTCCGGCT----
C. remanei -280 TC--ATCGTCATTTCAACTTCTCACGCAGGCAACACAAAATTTCTGAATCGACATTTAT
C. elegans -304 TCACA CAATTGTTTCAACCTTTCTGTCAAGAGCACAG--TTCTGTGAAACGAAAG-AT

C. briggsae -218 -GCCCTTAAAC-----AATG-----G-AGAGGAGGGCCGTAAAAACAATAGGGGA
C. remanei -222 GACTCCTCCCC-----TGTGTGCCCTA-AAACAAAGGCGGTGAAAACAAT---GGGA
C. elegans -247 GACGCCTCCTCTGGTATGAGTGTGTGATATGAACAAAGCCCGTGAAAACAATGAGAAA

C. briggsae -174 AGCTGATAAGTTTGATAGTGAGAAG---AGCCATATGGTGCAACAATGATT TTGTGATGA
C. remanei -174 AAGTGATAAGTT-GATAGTGGAACTGAGCCATATGGTGCAACAATGATT TTGCAATGA
C. elegans -187 GTCCGATAAGTT-GATAGTGGAACTCGAGCCATATGGTGCAACAATGATT TTGCAATGA
G5 G4 G3

C. briggsae -117 TAACA ACTTGAACGCATATAAAGTGGTCTGAGGAAGCGGCTTCTAGTCAGAAA---TTTC
C. remanei -115 TAACAGTCTGAAAACATATAAATA TGGGAC-AGAAGTGTAGTTTGGGTCTA---TCTC
C. elegans -127 TAACAGACC GAAAACATATAAATA TGGGGGAGTATGTGCTTTGGGTTCTAAGGTTCTT
G3

C. briggsae -61 G---TACTCT-CTATCCCTCCCTTAACAGACATATTTTCAAATAT--TCTCATTTT-
C. remanei -59 G---TACCG---CATTCGGTACTTTTGTGCTTACTTTCTAATTTCA-TGTATTTTTT
C. elegans -67 AATTATTCATATCACAATGCACCTTTTCTGACCGTTTCTTTTCTAAATTTGTTTTTTT
G2 G1

C. briggsae -6 -CAGTGCATG
C. remanei -7 CCAGTGCATG
C. elegans -7 TCAGTGTATG

Figure 2: ELT-2 is required for the upregulation of GFP in low heme.

Transgenic *C. elegans* strain IQ6011 was generated using 3 kb of the *hrg-1* 5' flanking sequence and fused to *gfp* and the *unc-54* 3' UTR. IQ6011 worms were initially cultured axenically in mCeHR-2 liquid medium supplemented with 10 μ M heme. Synchronized L1 larvae were then exposed to HT115 (DE3) bacteria grown in the presence of 4 μ M heme and induced to produce double stranded RNA against either *elt-1*, *elt-2*, or *elt-6*. DIC (left panel) and GFP (right panel) images are 20x. For fluorescence, images were captured using gain = 18, exposure time = 0.03 s. n = 80 P0 worms per treatment. These results are representative of two separate experiments.

RNAi

4 μ M heme

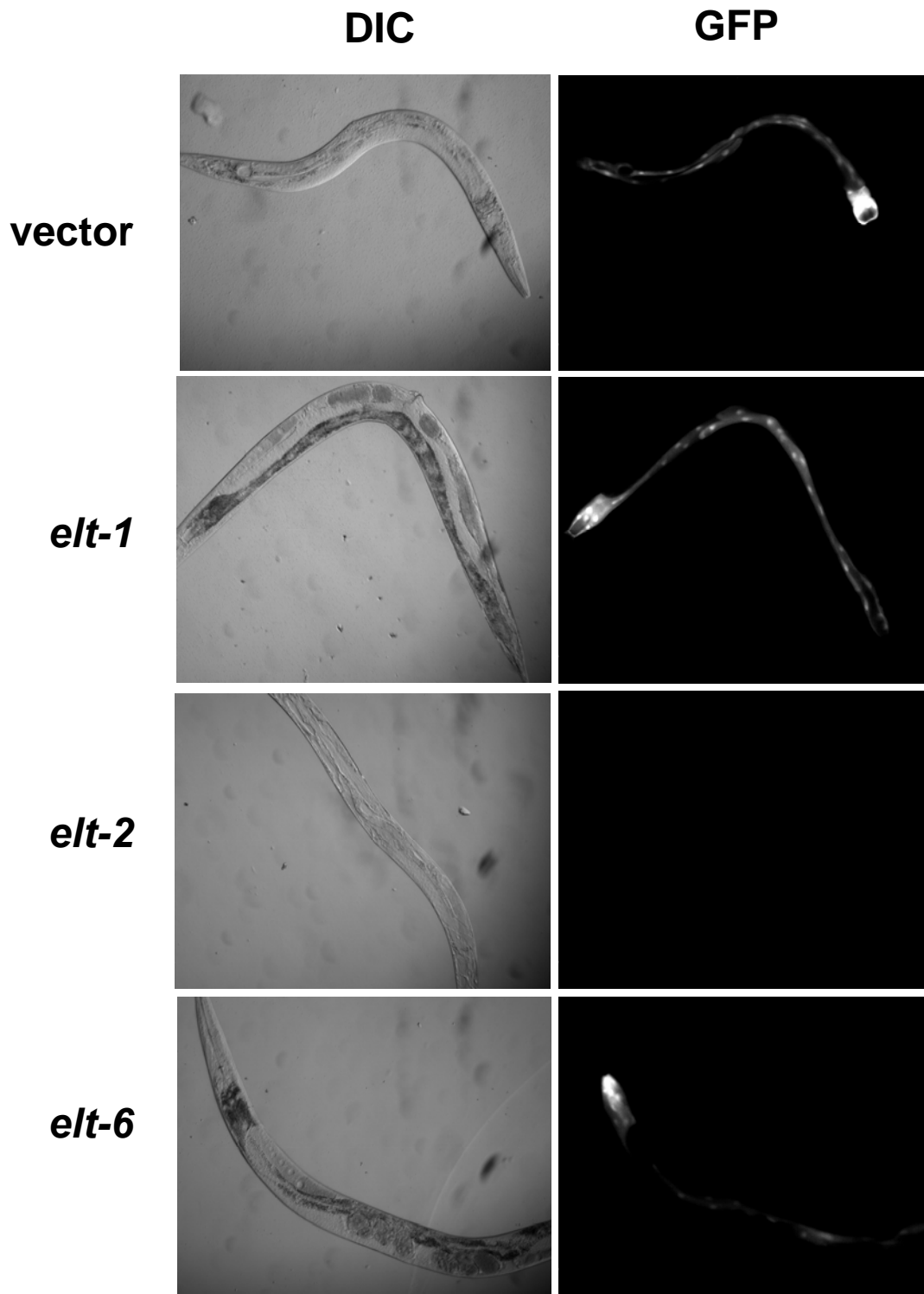


Figure 3: A 254 bp region within the *hrg-1* promoter is sufficient to elicit heme-responsiveness.

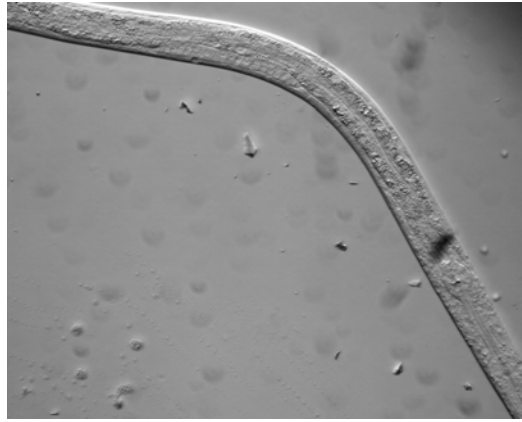
hrg-1 5' flanking sequence (254 bp) was fused to GFP and *the unc-54* 3' UTR.

Transgenic *C. elegans* strains carrying this *hrg-1::gfp* construct were made axenic and simultaneously synchronized. L1 larvae were placed in either 4 μ M or 100 μ M heme for 72 h. GFP expression in the worms was analyzed by microscopy. DIC (left panel) and GFP (right panel) images are 40x. For fluorescence, images were captured using gain = 18, exposure time = 0.09 s. n = 20 worms per treatment. The results are representative of two experiments from three injected and one bombarded line of transgenic *C. elegans*. G = GATA site.

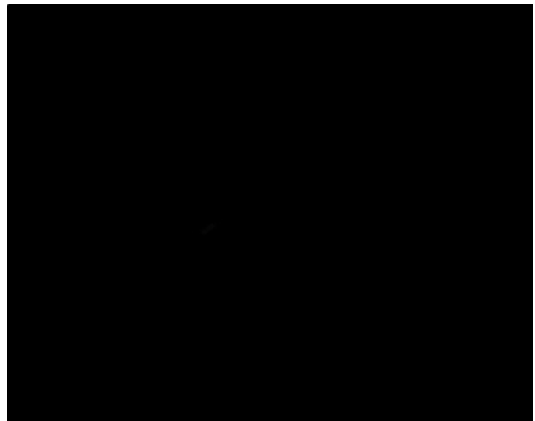
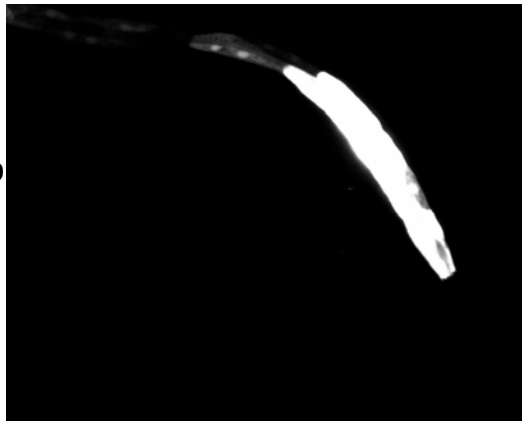
4 μ M heme

100 μ M heme

DIC



GFP



hrg-1::gfp

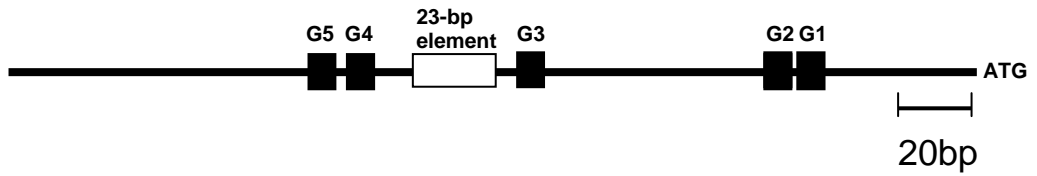
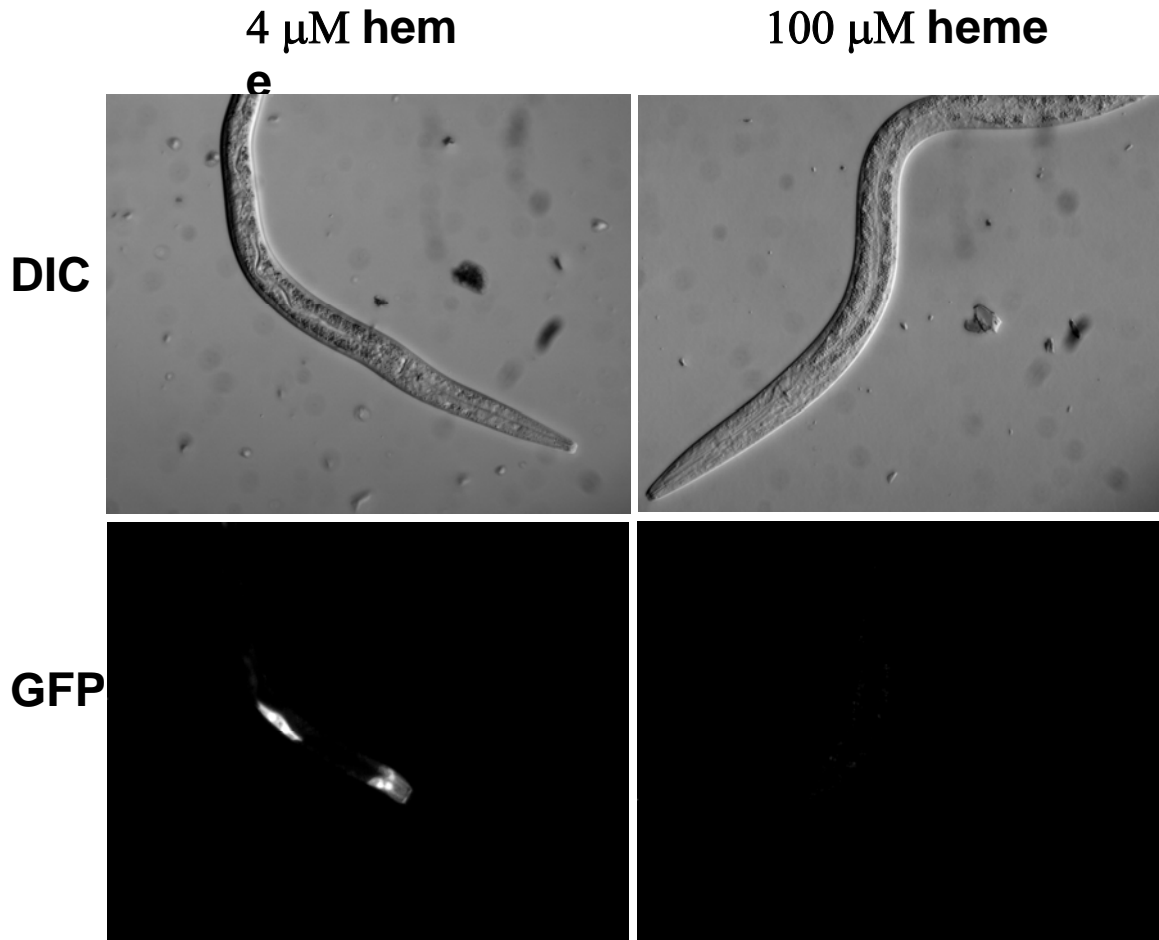


Figure 4: 159 bp of the *hrg-1* promoter is sufficient for heme-response.

A deletion was made in *hrg-1::gfp* from -254 bp to -160 bp, leaving the 159 bp directly 5' of the translational ATG. Transgenic *C. elegans* strains carrying this *hrg-1Δ(-254 – -160)::gfp* construct were made axenic and simultaneously synchronized. L1 larvae were placed in either 4 μM or 100 μM heme for 72 h. GFP expression in the worms was analyzed by microscopy. DIC (left panel) and GFP (right panel) images are 40x. For fluorescence, images were captured using gain = 18, exposure time = 0.09 s. n = 20 worms per treatment. The results are representative of two experiments from two bombarded lines of transgenic *C. elegans*. G = GATA site.



***hrg-1* $\Delta(-254 - -160)::gfp$**

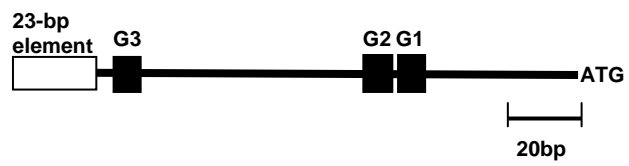


Figure 5: The heme responsive element of the *hrg-1* promoter lies upstream of – 98bp.

A deletion was made in *hrg-1::gfp* from -254 bp to -99 bp, leaving the 98 bp directly 5' of the translational ATG. Transgenic *C. elegans* strains carrying this *hrg-1Δ(-254 – -99)::gfp* construct were made axenic and simultaneously synchronized. L1 larvae were placed in either 4 μM or 100 μM heme for 72 h. GFP expression in the worms was analyzed by microscopy. DIC (left panel) and GFP (right panel) images are 40x. For fluorescence, images were captured using gain = 18, exposure time = 0.09 s. n = 20 worms per treatment. The results are representative of two separate experiments from an injected line of *C. elegans*. G = GATA

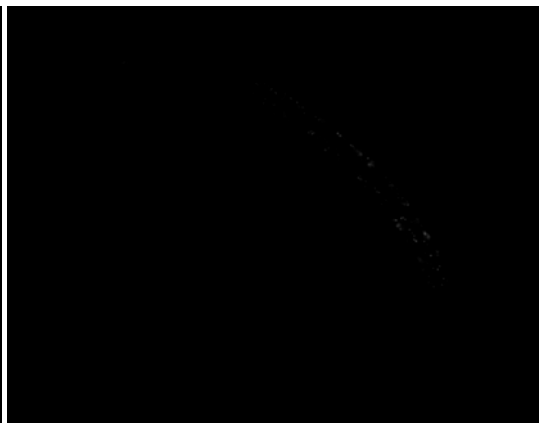
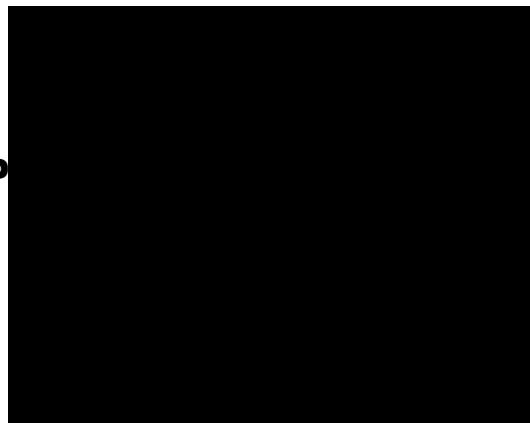
4 μ M heme

100 μ M heme

DIC



GFP



hrg-1 $\Delta(-254 - -99)::gfp$

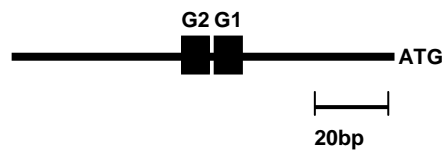


Figure 6: The position of the heme responsive element is not essential for heme responsiveness.

A deletion was made in *hrg-1::gfp* from -91 bp to -16 bp. Transgenic *C. elegans* strains carrying this *hrg-1Δ(-91 – -16)::gfp* construct were made axenic and simultaneously synchronized. L1 larvae were placed in either 4 μM or 100 μM heme for 72 h. GFP expression in the worms was analyzed by microscopy. DIC (left panel) and GFP (right panel) images are 40x. For fluorescence, images were captured using gain = 18, exposure time = 0.3 s. n = 20 worms per treatment. The results are representative of two separate experiments from an integrated line of *C. elegans*. G = GATA. Because GFP expression in this transgenic strain was weak, a higher exposure time was used to acquire the fluorescence images. Even though worms at 100 μM heme do not express any detectable GFP, the autofluorescence from the intestinal gut granules is visible.

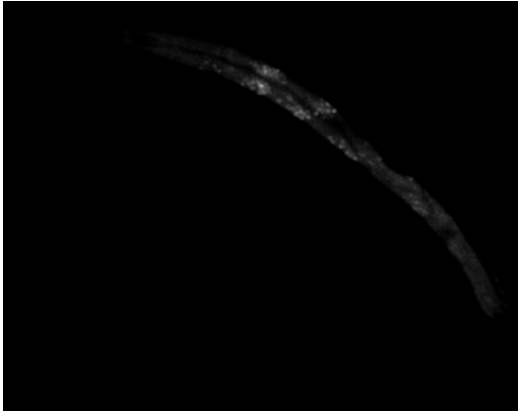
4 μ M heme

100 μ M heme

DIC



GFP



hrg-1 $\Delta(-91 - -16)::gfp$

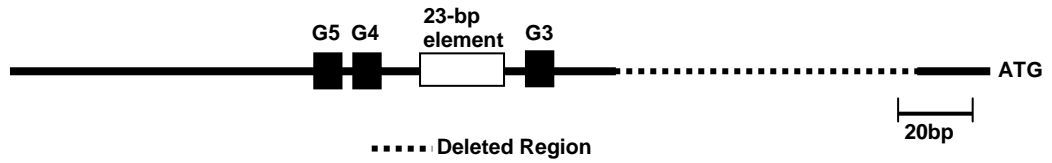
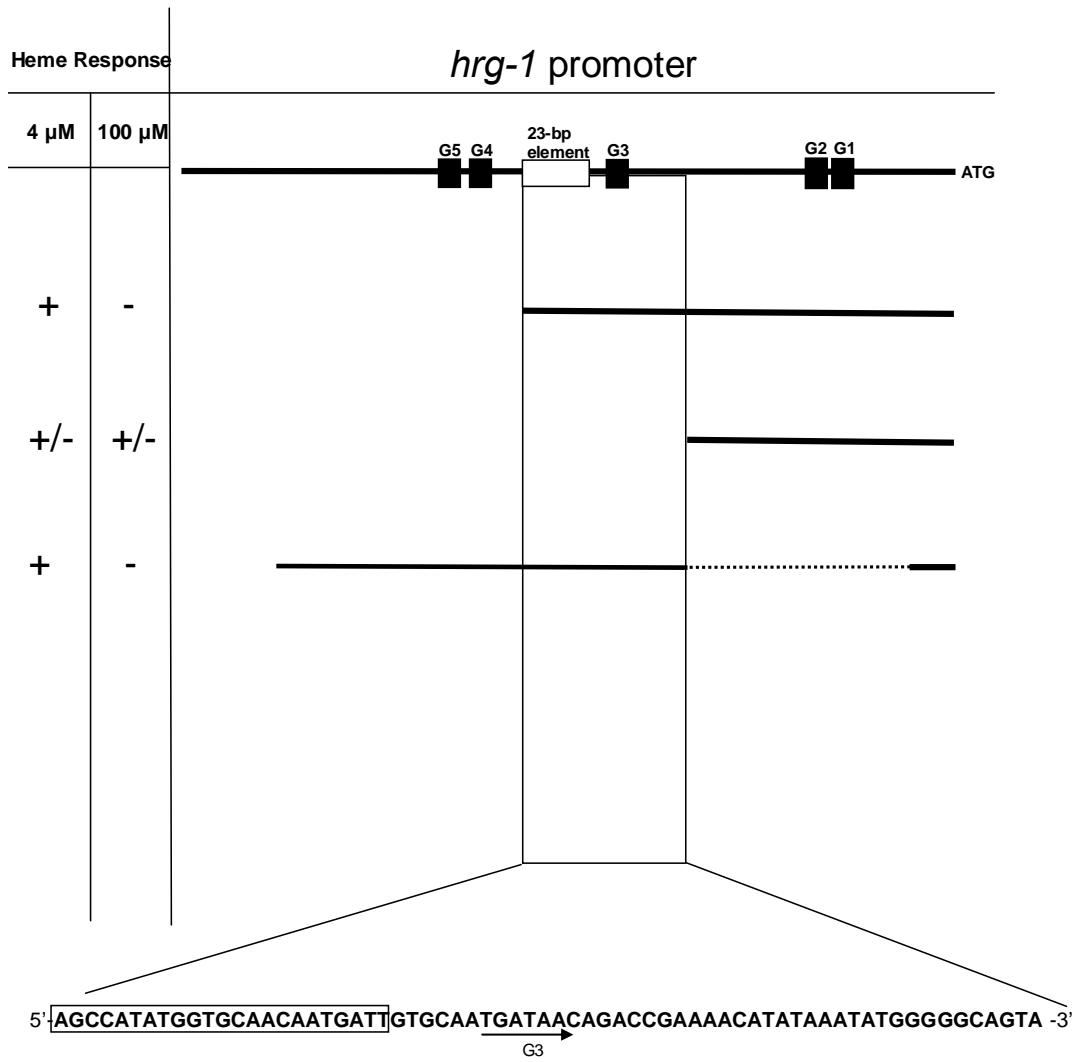


Figure 7: Schematic representing the heme responsiveness of various reporters

From the previous constructs analyzed, we inferred that the HRE lies within a region of 67 bp. The blue box represents the inferred region containing the HRE. The 23-bp conserved element is boxed. GATA SITE 3 is highlighted with a forward arrow.



□ 23-bp conserved element → GATA-3

⋯ Deleted region

Figure 8: A single GATA site is not sufficient for expression of the *hrg-1* promoter.

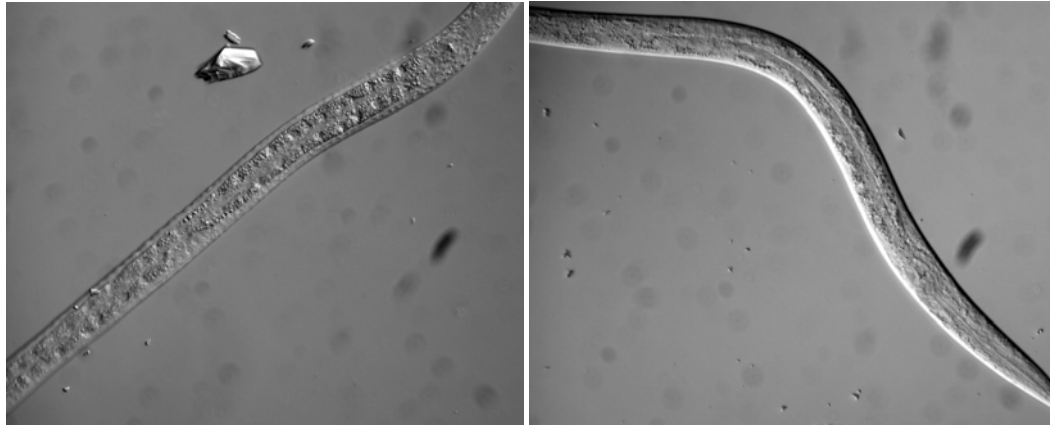
A deletion was made in *hrg-1::gfp* from -254 bp to -160 bp and -92 to -16 bp.

Transgenic *C. elegans* strains carrying this *hrg-1Δ(-254 – -159, -92 – -16)::gfp* construct were made axenic and simultaneously synchronized. L1 larvae were placed in either 4 μM or 100 μM heme for 72 h. GFP expression in the worms was analyzed by microscopy. DIC (left panel) and GFP (right panel) images are 40x. For fluorescence, images were captured using gain = 18, exposure time = 0.3 s. n = 20 worms per treatment. The results are representative of two separate experiments from two bombarded lines of transgenic *C. elegans*. G = GATA. Even though worms at 4 μM and 100 μM heme do not express any detectable GFP, the autofluorescence from the intestinal gut granules is visible.

4 μ M heme

100 μ M heme

DIC



GFP



hrg-1 $\Delta(-254 - -160, -92 - -16)::gfp$

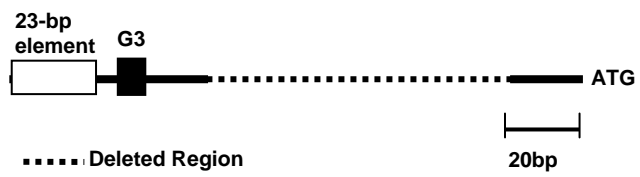


Figure 9: The 23-bp element of the *hrg-1* promoter has two inverted repeats

Within the 23-bp element of the *hrg-1* promoter are two inverted repeats, which can function as binding sites for transcription factors. The inverted repeats are shown by bold italics and opposing arrows.

→
AG**CCATATGG**TGCAACAATGATT
TC**GGTATACC**ACGTTGTTACTAA
←

→
AGCCATATGGTGCAACA**AATGATT**
TCGGTATACCACGTTG**TACTAA**
←

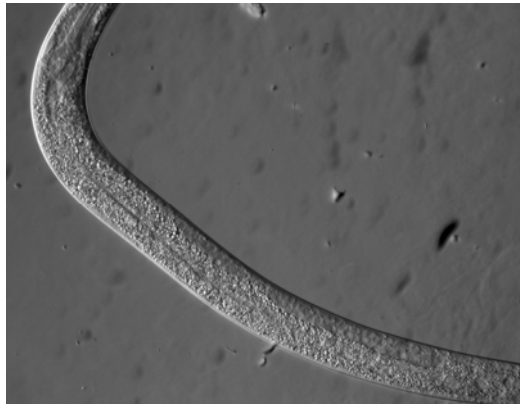
Figure 10: Mutation of the 23-bp conserved element abolishes expression of *hrg-1*.

The 23-bp conserved element was mutated in *hrg-1::gfp*. Transgenic *C. elegans* strains carrying this *hrg-1m(-159 – -137)::gfp* construct were made axenic and simultaneously synchronized. L1 larvae were placed in either 4 μM or 100 μM heme for 72 h. GFP expression in the worms was analyzed by microscopy. DIC (left panel) and GFP (right panel) images are 40x. For fluorescence, images were captured using gain = 18, exposure time = 0.09 s. n = 20 worms per treatment. The results are representative of two separate experiments from two injected lines and two bombarded lines of transgenic *C. elegans*. G = GATA.

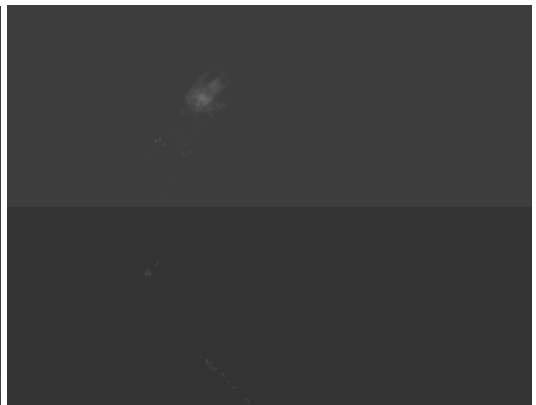
4 μ M heme

100 μ M heme

DIC



GFP



hrg-1m(-159 – -137)::gfp

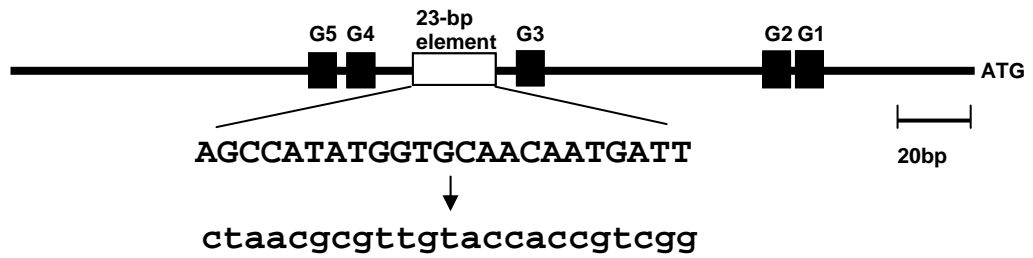


Figure 11: The 5 terminal 3' bp of the 23-bp conserved element are not required for repression

The 23-bp conserved element was mutated five consecutive nucleotides at a time. Transgenic *C. elegans* strains carrying these constructs were made axenic and simultaneously synchronized. L1 larvae were placed in either 4 μ M or 100 μ M heme for 72 h. GFP expression in the worms was analyzed by microscopy. Only images for mutation of the 3' terminal nucleotides are (11B) shown because all other constructs displayed an identical heme-response as the 23-bp mutation (11A). DIC (left panel) and GFP (right panel) images are 40x. For fluorescence, images were captured using gain = 18, exposure time = 0.09 s. n = 20 worms per treatment. The results are representative of two separate experiments from an injected line of *C.elegans*. G = GATA. mt = mutation. Mutated sequence is represented by lowercase italics.

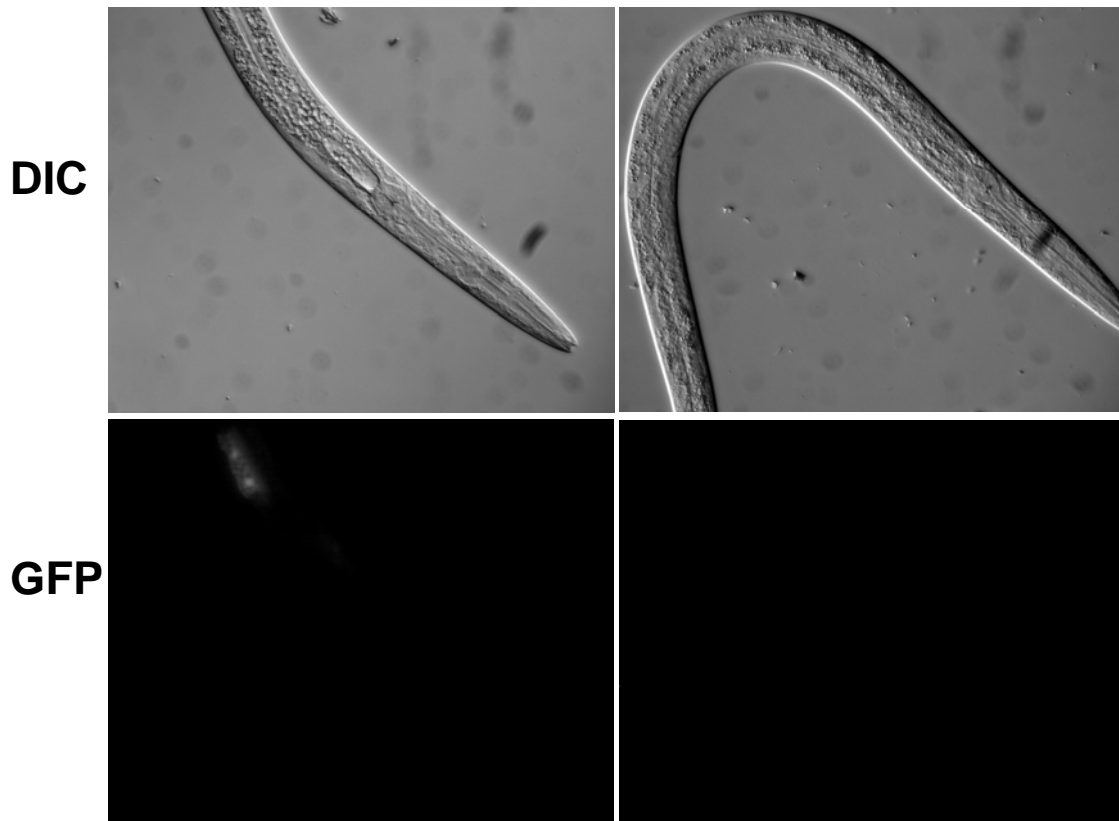
11A

	23-bp conserved element	Heme Response	
		4 μ M	100 μ M
Wild-type	AGCCATATGGTGCAACAATGATT		
mt1	ctaac	+/-	+/-
mt2	gcggt	+/-	+/-
mt3	gtacc	+/-	+/-
mt4	accgt	+/-	+/-
mt5	gtcgg	+/-	-

11B

4 μ M heme

100 μ M heme



hrg-1m(-141 – -137)::gfp

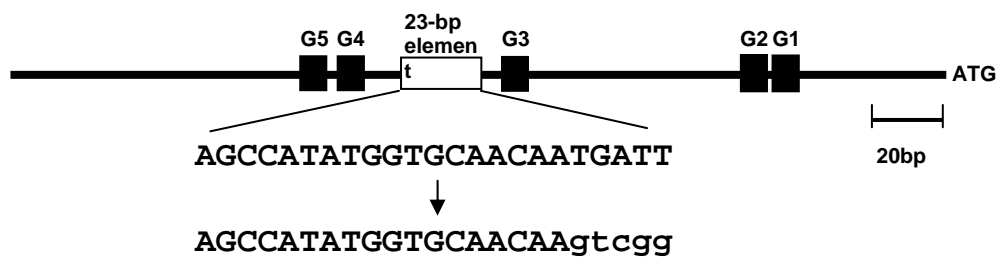


Figure 12: The 159 bp heme-responsive fragment of the *hrg-1* promoter utilizes both enhancement and repression in response to heme.

159 bp heme-responsive fragment of the *hrg-1* promoter was placed upstream of the *egl-18* minimal promoter. Transgenic *C. elegans* strains carrying *egl-18::gfp* (12A) or *hrg-1Δ (-254 - -160)::egl-18::gfp* (12B) were made axenic and simultaneously synchronized. L1 larvae were placed in either 4 μM or 100 μM heme for 72 h. GFP expression in the worms was analyzed by microscopy. DIC (left panel) and GFP (right panel) images are 40x. For fluorescence, images were captured using gain = 18, exposure time = 0.09 s. n = 20 worms per treatment. The results are representative of two separate experiments from one injected line and one bombarded line of transgenic *C. elegans*. G = GATA.

12A

4 μ M heme

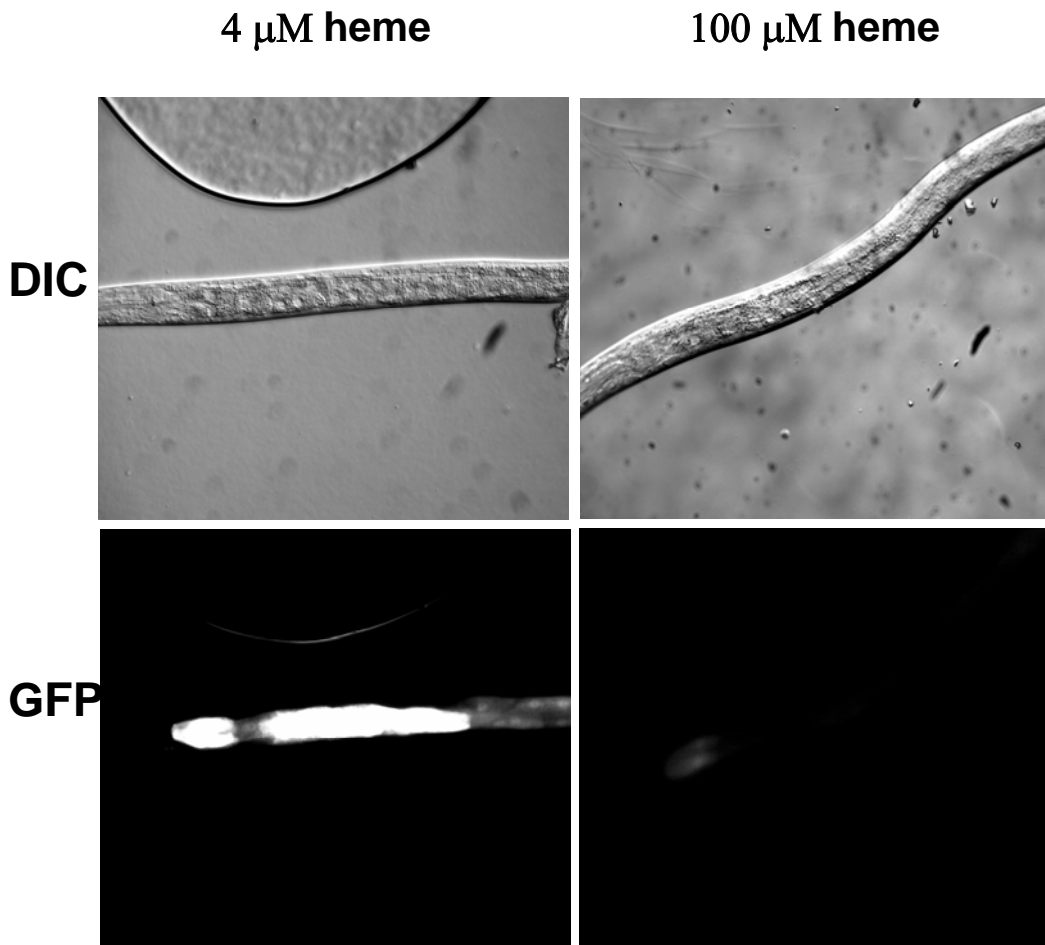
100 μ M heme



egl-18::gfp



12B



hrg-1 Δ (-254 – -160):*egl-18*::*gfp*



Figure 13: The *hrg-1* promoter does not confer a heme response to the *vha-6* promoter when placed upstream.

The 159 bp heme-responsive fragment of *the hrg-1* was placed 5' (13B) and 3' (13C) of the *vha-6* promoter. Transgenic *C. elegans* strains carrying either one of these two constructs or the *vha-6::gfp* (13A) were made axenic and simultaneously synchronized. L1 larvae were placed in either 4 μ M or 100 μ M heme for 72 h. GFP expression in the worms was analyzed by microscopy. *vha-6::gfp* DIC (left panel) and GFP (right panel) images are 20x. For fluorescence, images were captured using gain = 18, exposure time = 0.09 s. n = 20 worms per treatment. The results are representative of two separate experiments from two bombarded lines of transgenic *C. elegans*. *hrg-1* $\Delta(-254 - -160)::vha-6::gfp$ and *vha-6::hrg-1* $\Delta(-254 - -160)::gfp$ DIC (left panel) and GFP (right panel) images are 40x. For fluorescence, images were captured using gain = 18, exposure time = 0.09 s. n = 20 worms per treatment. The results are representative of two separate experiments from two injected lines of transgenic *C. elegans*. G = GATA.

13A

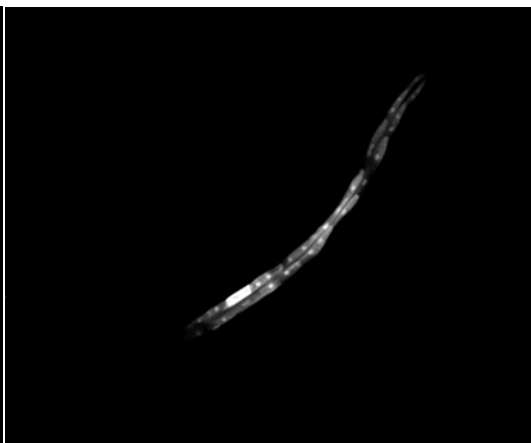
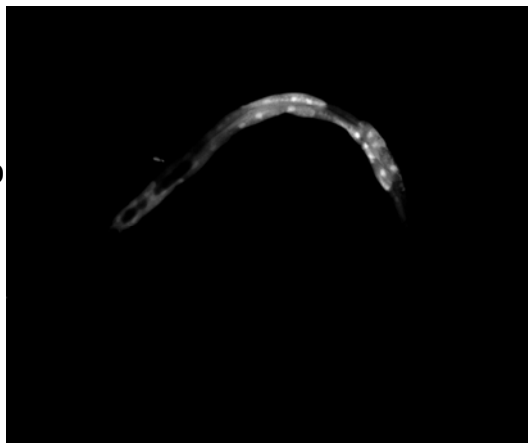
4 μ M heme

100 μ M heme

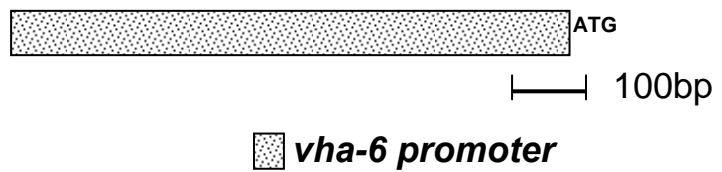
DIC



GFP



vha-6::gfp



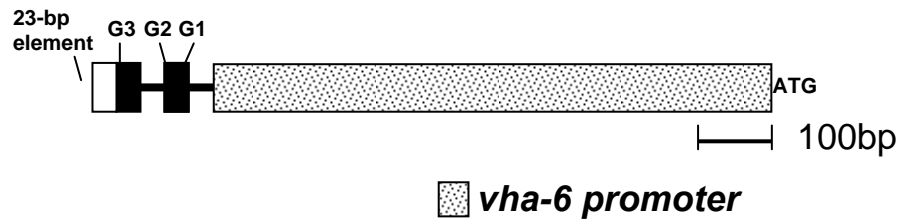
13B

4 μ M heme

100 μ M heme



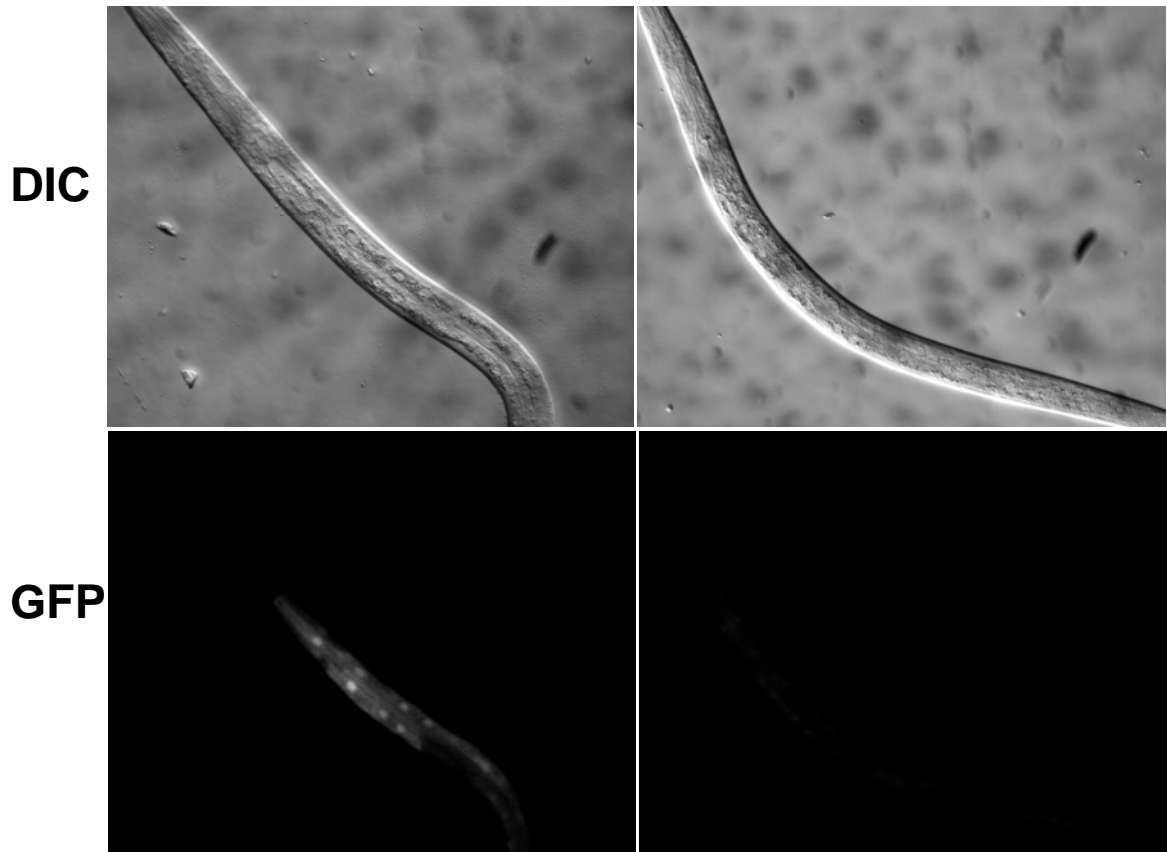
hrg-1 $\Delta(-254 - -160)::vha-6::gfp$



13C

4 μ M heme

100 μ M heme



vha-6::hrg-1 Δ (-254 – -160) ::gfp

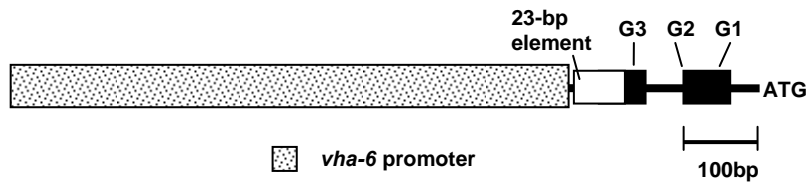


Table 1: Summary of GFP expression in transgenic worms expressing deletion constructs.

The intensity of heme-dependent-GFP expression was quantified by visual inspection.

++++ = 100% GFP expression. +++ = 75% GFP expression. ++ = 50% GFP expression.

+ = 25% GFP expression. +/- = <10% GFP expression. - = no GFP expression. White

box = 23-bp element. Black boxes = GATA sites.

Construct	GFP expression	
	4 μ M heme	100 μ M heme
	++++	-
	+++	-
	+/-	+/-
	+	-
	-	-
 AGCCATATGGTGCAACAATGATT ↓ ctaacgcgttgtaccaccgtcgg	+/-	+/-
 AGCCATATGGTGCAACAATGATT ↓ AGCCATATGGTGCAACAAGtcgg	+	-
	++++	-
	++++	++++
	+	+++

Chapter 4: Discussion

Heme is a tetrapyrrole that serves as a co-factor for many proteins. Although the heme synthesis pathway has been studied in detail, little is known about how organisms maintain heme homeostasis, partly because only a handful of regulatory molecules have been identified in metazoans. We have established *C. elegans* as a genetically tractable animal model to identify the regulatory molecules and dissect the cellular pathways that modulate heme homeostasis. Using microarrays, we identified ~370 genes that are transcriptionally responsive to environmental heme (44). *hrg-1*, which encodes for a heme transporter, is greater than 40 fold upregulated under heme limited conditions but is undetectable when environmental heme is ≥ 10 μ M heme. These results suggest that *hrg-1* transcription is either enhanced under low heme, repressed under high heme, or a combination of both. In this study, we define the minimal promoter of *hrg-1* and identify an evolutionarily conserved heme responsive element (HRE) in the *hrg-1* promoter.

The regulatory elements of the *hrg-1* promoter are conserved in three *Caenorhabditis* species (Results Figure 1). These three species reside within the same habitat, micro-organism rich soil, and consequently are subject to the limited amounts of bioavailable heme. As heme auxotrophs, the physiological utilization of this heme is critical for the worms' survival. Therefore, it is not surprising that the regulatory pathways for heme utilization are conserved. Although parasitic nematodes reside in a different environment than soil-dwelling species, they are also heme auxotrophs. Thus, it is feasible that these helminths may also share similar heme regulatory mechanisms with free-living nematodes. If *hrg* homologs in parasitic helminths are regulated in a similar manner to *hrgs* in *C. elegans*, our studies in *C. elegans* could be used to establish a

paradigm for how other parasitic nematodes forage and ingest dietary heme for growth and reproduction, which will eventually aid in the development of anthelmintics to combat worm infections.

We have shown that *hrg-1* is regulated by ELT-2, the transcription factor proposed to control all intestinal specific regulation in *C. elegans*. Although there are five putative ELT-2 binding elements (GATA sites) in the *hrg-1* promoter, they may not all be functionally equivalent in *hrg-1* regulation. Other genes, such as *pho-1* and *ges-1*, also have multiple GATA sites but it was shown that a specific GATA site is important for the regulation of each gene (121). For both genes, the critical GATA site has the sequence ACTGATAA. In the *hrg-1* promoter, the closest sequence to this is GATA SITE 3 (AATGATAA). When the region containing GATA SITE 3 is moved from its endogenous position, expression in the intestine is significantly reduced. However, whether this is due to the spatial rearrangement of GATA SITE 3 or the deletion of GATA SITE 1 and GATA SITE 2 needs to be further examined. It has been shown that the ELT-2 homolog of the parasitic species *Haemonchus contortus* is functionally similar to *C. elegans* ELT-2, providing evidence of similar regulatory mechanisms utilized by both *C. elegans* and parasitic nematodes (122).

In addition to GATA sites, a 23-bp conserved element is essential for the expression of *hrg-1*. Data suggest that the spatial position of the element is not critical for heme response, but may be important for the enhanced gene expression. Using BLAST searches we have not been able to find this 23-bp element in other *hrgs* that share a similar heme-dependent regulation as *hrg-1*. It seems unlikely that *hrg-1* would have a unique system of regulation when there are other *hrgs* are also regulated by heme in a

similar manner. We speculate that the binding-site for the *trans*-acting factor which binds to the 23-bp element may be degenerate.

Preliminary data has suggested that the 23-bp element cannot function independent of GATA sites in larvae and adult worms. However, *hrg-1* is expressed as early as the 128 stage embryo in all cell types, possibly due to an embryo specific transcript, suggesting that during early development *hrg-1* expression is independent of ELT-2 (O'Connell, Hamza unpublished data). This is in contrast to other intestinal genes such as *pho-1*, which is gut specific during late embryogenesis (121). The difference in the spatial regulation of *hrg-1* may be based upon the source of heme. Adult worms ingest exogenous heme through the intestine. Heme must pass through these intestinal cells before it can be allocated to other cells, so it is logical that transport molecules would be highly regulated in the intestine. Developing embryos, however, acquire heme and other nutrients from their mothers. All embryonic cells may have direct access to exogenous heme. It is, therefore, advantageous to have transport molecules such as HRG-1 expressed in all cells. Because *hrg-1* is expressed initially in all cells during embryogenesis, it may be possible to mimic this expression pattern in adult worms. This may facilitate the development of GFP-based heme-sensors in extra-intestinal cells to elucidate the intercellular heme transport pathways. It is also equally likely that the putative *trans*-acting enhancer and repressor are expressed only in the intestine from the L1 larvae stage to adult. In this case, these factors may work synergistically with ELT-2 to specify intestinal expression of *hrg-1*. Identifying these factors will be a future priority to aid in generating a whole animal heme sensor.

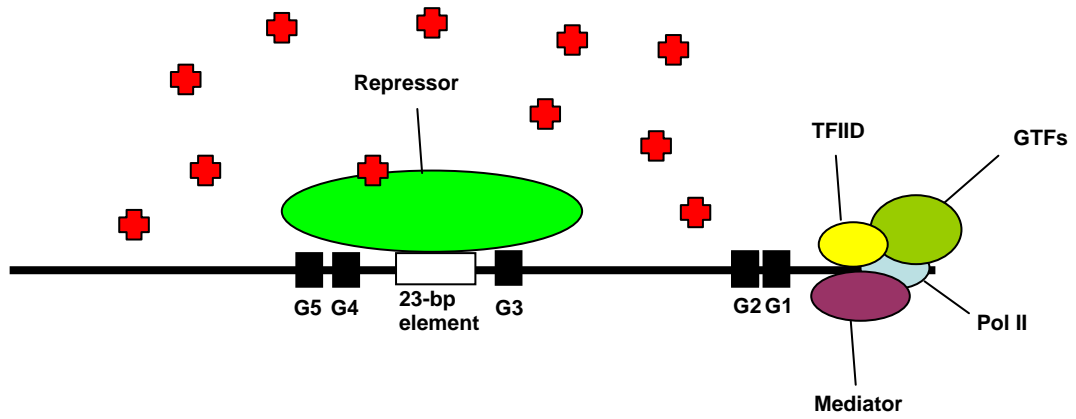
Bases on our results, we propose a model in which the regulation of *hrg-1* regulation may be comparable to genes that are regulated by the heme-sensitive repressor, Bach1 (Figure 1). In the presence of high heme, Bach1 is displaced from DNA, which allows the Maf / p45 complex to upregulate its target genes. We speculate that a repressor could occupy the 23-bp element of the *hrg-1* promoter in the presence of high heme levels. As heme concentration decreases, the repressor is displaced by an enhancer which recruits ELT-2. When both ELT-2 and the enhancers are bound to their respective elements, *hrg-1* is transcriptionally upregulated. Another possibility is that ELT-2 is always bound to the GATA sites, but the transcription factor that binds the 23-bp element controls the rate of transcription under different heme concentrations. A system in which GATA elements work in concert with other factors may be a general form of regulation in *C. elegans*. ELT-2 controls all intestinal genes. The specific control of these genes under different environmental conditions could be achieved through the orchestrated recruitment and interaction with distinct enhancers, repressors, or modifiers. Further studies on *hrg-1* and other ELT-2 regulated genes will help elucidate the mechanisms of regulation.

An RNAi screen is currently ongoing in our group to identify the transcription factor(s) that bind to the 23-bp element. These studies will help us understand how animals sense and respond to changes in heme levels by altering the expression of transporters and other molecules that work together to regulate heme homeostasis. An in-depth knowledge of intestinal heme homeostasis may one day lead to the development of bioavailable sources of iron that are more efficiently absorbed by humans.

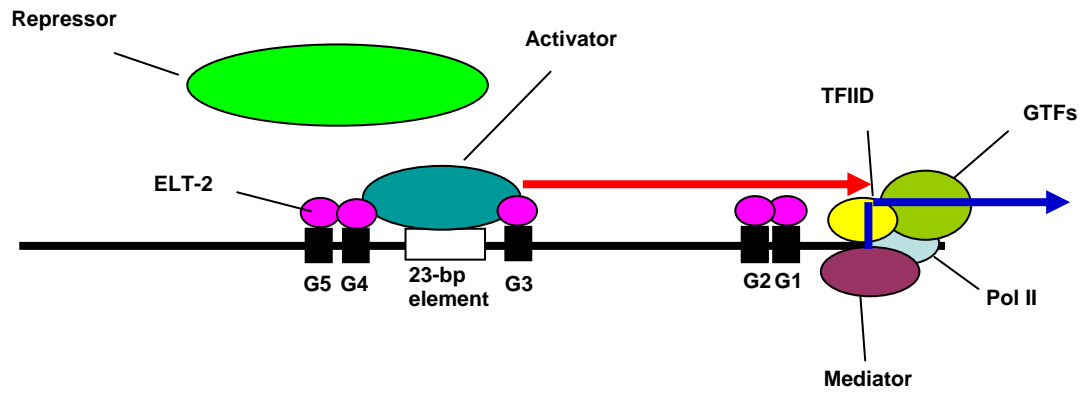
Figure 1: Proposed model of *hrg-1* regulation by heme

A repressor binds the 23-bp element in high heme which out competes the binding of an enhancer to this same element. In low heme, the repressor can no longer bind DNA. An enhancer binds the 23-bp element and subsequently recruits ELT-2 to enhance transcription of *hrg-1*. Red arrow = enhancement. Blue arrow = transcription. Red crosses = heme. TFIID = transcription factor II D. GTFs = general transcription factors. Pol II = RNA polymerase II. Mediator = a transcriptional coactivator.

100 μ M heme



4 μ M heme



Appendix A: *C. elegans* strains.

Strain name	Genotype
IQ6011-1	<i>ihEx01(hrg-1::gfp, rol-6+)</i>
IQ6011-2	<i>unc-119(ed3) III; ihEx02(hrg-1::gfp, unc-119+)</i>
IQ6011-3	<i>unc-119(ed3) III; ihEx03(hrg-1Δ(-254 – -160)::gfp, unc-119+)</i>
IQ6011-4	<i>ihEx04(hrg-1 Δ(-254 – -99)::gfp, rol-6+)</i>
IQ6011-5	<i>unc-119(ed3) III; ihIs05(hrg-1 Δ(-91 – -16)::gfp, unc-119+)</i>
IQ6011-6	<i>unc-119(ed3) III; ihEx06(hrg-1Δ(-254 – -160, -92 – -16)::gfp,unc-119+)</i>
IQ6011-7	<i>ihEx07(hrg-1m(-159 – -137)::gfp, rol-6+)</i>
IQ6011-8	<i>unc-119(ed3) III; ihEx08(hrg-1m(-159 – -137)::gfp,unc-119+)</i>
IQ6011-9	<i>ihEx09(hrg-1m(-159 – -155)::gfp, rol-6+)</i>
IQ6011-10	<i>ihEx10(hrg-1m(-154 – -150)::gfp, rol-6+)</i>
IQ6011-11	<i>ihEx11(hrg-1m(-149 – -145)::gfp, rol-6+)</i>
IQ6011-12	<i>ihEx12(hrg-1m(-144 – -140)::gfp, rol-6+)</i>
IQ6011-13	<i>ihEx13(hrg-1m(-141 – -137)::gfp, rol-6+)</i>
IQ7081	<i>ihEx81(egl-18::gfp, rol-6+)</i>
IQ7082	<i>unc-119(ed3) III; ihEx82(egl-18::gfp,unc-119+)</i>
IQ7083	<i>ihEx83(hrg-1Δ (-254 – -160)::egl-18::gfp, rol-6+)</i>
IQ7084	<i>unc-119(ed3) III; ihEx84(hrg-1Δ (-254 – -160)::egl-18::gfp,unc-119+)</i>
IQ7061	<i>unc-119(ed3) III; ihEx61(vha-6::gfp,unc-119+)</i>
IQ7062	<i>ihEx62(hrg-1Δ(-254 – -160)::vha-6::gfp, rol-6+)</i>
IQ7063	<i>ihEx63(vha-6::hrg-1Δ (-254 – -160)::gfp, rol-6+)</i>

Appendix B: Reporter constructs.

Construct	Description	Transfection method
<i>hrg-1::gfp</i>	Contains -254 bp – 0 bp of the <i>hrg-1</i> 5' flanking sequence fused to <i>gfp</i>	Injection, Bombardment
<i>hrg-1Δ(-254 – -160)::gfp</i>	Contains -159 bp – 0 bp of the <i>hrg-1</i> promoter	Bombardment
<i>hrg-1Δ(-254 – -99)::gfp</i>	-98 bp – 0 bp of the <i>hrg-1</i> 5' promoter	Injection
<i>hrg-1Δ(-91 – -16)::gfp</i>	Contains -254 bp – -92 bp, -15 bp – -0 bp of the promoter	Bombardment
<i>hrg-1Δ(-254 – -160, -91 – -16)::gfp</i>	Contains -159 bp – -92 bp, -15 bp – -0 bp of the <i>hrg-1</i> promoter	Bombardment
<i>hrg-1m(-159 – -137)::gfp</i>	The 23-bp element of the <i>hrg-1</i> promoter was mutated	Bombardment, Injection
<i>hrg-1m(-159 – -155)::gfp</i>	bp 1-5 of the 23-bp element were mutated	Injection
<i>hrg-1m(-154 – -150)::gfp</i>	bp 6-10 of the 23-bp element were mutated	Injection
<i>hrg-1m(-149 – -145)::gfp</i>	bp 11-15 of the 23-bp element were mutated	Injection
<i>hrg-1m(-144 – -140)::gfp</i>	bp 16-20 of the 23-bp element were mutated	Injection
<i>hrg-1m(-141 – -137)::gfp</i>	bp 19-23 of the 23-bp element were mutated	Injection
<i>egl-18::gfp</i>	The <i>egl-18</i> minimal promoter fused to <i>gfp</i>	Bombardment, Injection
<i>hrg-1Δ(-254 – -160)::egl-18::gfp</i>	The 159-bp heme responsive fragment of the <i>hrg-1</i> promoter upstream of the <i>egl-18</i> promoter	Bombardment, Injection
<i>vha-6::gfp</i>	The <i>vha-6</i> promoter fused to <i>gfp</i>	bombardment
<i>hrg-1Δ(-254 – -160)::vha-6::gfp</i>	The 159-bp heme responsive fragment of the <i>hrg-1</i> promoter upstream of the <i>vha-6</i> promoter	Injection
<i>vha-6::hrg-1Δ(-254 – -160)::gfp</i>	The 159-bp heme responsive fragment of the <i>hrg-1</i> promoter downstream of the <i>vha-6</i> promoter	Injection

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