DISSECTION OF GENE REGULATORY NETWORKS UNDERLYING PATTERNING AND MORPHOGENESIS IN THE *C. ELEGANS* VULVA

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Proverbs 6:20-22

"My son, keep your father's commands and do not forsake your mother's teaching. When you walk, they will guide you; when you sleep, they will watch over you; when you awake, they will speak to you."

Abstract

During development, in the course of which the single-celled egg generates a whole organism, cells become different from each other and form patterns of types of cells. It is these spatially defined cell-fate patterns that underlie the generation of complex organs. The mechanisms that establish these precise spatial patterning events depend on the implementation of diverse 'gene regulatory networks' (a consequence of functional interconnections between regulatory genes (transcription factors) and their target genes). Dissection of gene regulatory networks that control patterning of gene expression and differentiation would thus help us understand how cells generate a spatially defined pattern of cell fates during organ formation. Resources such as diverse spatial and temporal cell-fate markers, reverse genetics (RNAi), trans-genesis, and the ease of manipulation at the single-cell level make *C. elegans* a tractable system for studying the execution of cell-type-specific gene expression programs that occur during organogenesis. Consider the *C. elegans* vulva, a postembryonically derived organ that invariantly consists of seven distinct vulval cell types (vulA, vulB1, vulB2, vulC, vulD, vulE and vulF), each with its own unique gene expression profile. These features make the *C. elegans* vulva a particularly attractive model for dissecting the postembryonic gene regulatory networks involved in patterning and organ morphogenesis**.**

This thesis focuses on elucidating the regulatory networks that control gene expression in the seven vulval cell types of *C. elegans* during organogenesis. The transcription factors *lin-11*(LIM), *cog-1*(Nkx6.1/6.2), and *egl-38*(Pax2/5/8) have been previously implicated as key regulators of gene expression in the vulva. Identification of additional regulatory factors is warranted, so as to rigorously dissect the mechanisms that

specify the spatial fate patterns of terminally differentiated cell types. To this end, I systematically disrupted the gene activity of 508 transcription factors via RNAi and assayed the expression of *ceh-2,* a readout for vulB fate during the L4 stage. From this screen, I identified the *tailless* ortholog *nhr-67* as a novel regulator of vulval gene expression. *nhr-67* acts in combination with *cog-1, egl-38,* and *lin-11* to execute accurate patterning of gene expression of their downstream targets. The pair-wise interactions between these regulatory genes are complex and vary among the seven cell types. One of the ways in which *nhr-67* maintains cell identity is through restriction of inappropriate cell fusion events in specific vulval cells (namely vulE and vulF). The cell fusion defects observed in an *nhr-67* RNAi background can be partially attributed to deregulation of fusogens. *cog-1* and *lin-11* (but not *egl-38*) mutants also show heterotypic fusion defects to different degrees. I also discovered a striking regulatory circuit that affects a subset of the vulval lineages: *cog-1* and *nhr-67* inhibit both one another and themselves. We argue that the 1° vulval cells (vulE and vulF) utilize this novel regulatory motif to rapidly switch fates in response to transient inputs. We also speculate that the built-in flexibility of this circuit acts as a failsafe mechanism (in the event of cell damage) in the vulE and vulF cells. We postulate that the differential levels and combinatorial patterns of *lin-11, cog-1, egl-38,* and *nhr-67* expression are a part of a regulatory code for the mature vulval cell types.

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CHAPTER I:

Role of Gene Regulatory Networks in Development

Introduction

Genomic DNA contains the blueprints and necessary information to generate an organism of a given species. During the course of development, an organism successively progresses through multiple regulatory states. These regulatory states are determined by the presence and activities of sequence-specific transcription factors and cell-signaling pathways operating at specific times and places. Cis-regulatory sequences (that are encoded in the genome) interpret these dynamic regulatory states by interacting with transcription factors to produce complex spatial and temporal gene expression patterns (Levine and Davidson, 2005). These individual elements are hardwired with the correct response to every possible situation the organism will encounter during development. Execution of complex developmental processes such as differentiation and morphogenesis depend on how cis-regulatory information is processed both at the individual gene level and at the gene network (systems) level. Dissecting these hard-wired genomic regulatory codes would provide useful insights as to how and why precise spatial fate patterns are established during organogenesis and what accounts for morphological differences observed among related species.

In general, cis-regulatory elements are organized into promoters, enhancers, and repressors. Promoters serve as loading sites for the basal transcriptional machinery (RNA polymerases) and are located immediately upstream of the transcription start site. Enhancers tend to reside at various distances from the promoter and are found in introns, upstream sequences, and 3’ non-coding regions of the genes they regulate. Enhancer elements interact with the basal transcription apparatus at the promoter to drive tissue-

and stage-specific gene expression. Repressor elements typically inhibit transcriptional activity by blocking the interactions between the enhancer elements and the basal transcriptional apparatus. Repression is generally used as a strategy to delineate spatial boundaries. Cis-regulatory elements or modules are typically several hundred base pairs long and contain multiple binding sites for transcription factors (Davidson, 2006; Small et al., 1992). These cis-regulatory DNAs act as information processing devices in that they integrate diverse inputs (in the form of regulatory factors), process the information (e.g., using logic functions such as “and”, “or”, “not”) and output the appropriate expression pattern of their target genes (Davidson, 2006). Interaction of several such modules can result in expression patterns of greater complexity. Gene regulatory networks consist of a multitude of these information processing devices that act in concert to specify an information cascade that drives development forward.

Gene regulatory networks are logic maps that elaborate all the functional connections between the regulatory genes and the associated regulatory modules of their target genes. Each cis-regulatory module functions as a unique node in the network and several such nodes function together as subcircuits that generate accurate cell fate patterns, launch differentiation programs and coordinate morphogenesis (Davidson, 2006). These regulatory subcircuits provide a causal answer as to why, when, and where genes are expressed in a developing organism. Although each of the individual circuits contributes to the system, the overall logic of the network can be better appreciated from a global perspective. The complete network architecture reveals properties (e.g., feedback loops, differentiation batteries) that can never be appreciated at the level of any individual gene.

Investigating development from the perspective of gene regulatory networks illuminates how static information contained within the genomic DNA translates into the dynamic regulatory states of a developing organism. Dissection of these networks reveals the underlying logic that is used to produce complex morphologies (Levine and Davidson, 2005). Gene regulatory networks also uncover differentiation pathways that can be fine tuned to generate diverse cell types, which is an area of intense study in the field of stem-cell biology (Matthias and Rolink, 2005; Shaywitz and Melton, 2005). Lastly, studying evolutionary changes in gene regulatory networks would provide a foundation for understanding morphological diversity (Carroll, 1990; Hinman et al., 2003; Levine and Davidson, 2005).

The recent availability of diverse animal genomes has demonstrated that bilaterians ranging from nematodes to mammals use the same assortment of transcription factors and signaling molecules to transmit spatial and temporal information over the course of development (Erwin and Davidson, 2002). Developmental complexity is not accomplished with increases in genome or proteome sizes. Rather, complexity is achieved by taking a finite repertoire of transcription factors and utilizing them in multiple unrelated processes during development. Thus within and among diverse species, every regulatory gene responds to diverse regulatory inputs that control its expression in different spatial domains at various developmental stages. Cross-species comparisons of gene regulatory networks can identify common strategies (Arkin et al., 1998; Koide et al., 2005; Levine and Davidson, 2005). For example, sea urchins and frogs utilize signaling pathways to establish precise spatial gene expression patterns. As more gene networks become available for a broad range of developmental processes in

diverse species, the common themes that might emerge from these analyses would enhance our understanding of organogenesis (especially in experimentally intractable systems like humans).

Differentiation Batteries and "Lock-down" Mechanisms

Fate specification of different cell populations and territories underlies the generation of complex body structures. These specialized populations differentiate from other populations by terminally expressing diverse combinations of effector genes. These precise sets of effector genes define unique attributes of each cell type. At a systems level, differentiation can be viewed as the final output of all gene regulatory networks that control development. A differentiation event in development can be fully understood by mapping out all the regulatory interactions in the network architecture (from the onset of specification to the organized expression of the effector gene set).

A differentiation battery refers to a set of functionally related effector genes that are expressed in a given cell type (Davidson, 2006). These genes are coordinately expressed because their cis-regulatory elements respond to similar transcription factor inputs. All genomes are hypothesized to employ various "lock-down" mechanisms to ensure the maintenance and enhancement of these differentiated regulatory states over time. Lock-down mechanisms are also used to restrict any inappropriate transcriptional activity in terminally differentiated structures. Furthermore, lock-down mechanisms also transmit these differentiated regulatory states to future cell generations, allowing efficient proliferation of terminally differentiated tissue structures during organogenesis. Thus disruption of lock-down mechanisms results in fatal developmental errors and has devastating consequences on the viability of the organism.

Understanding the diverse strategies gene networks use to establish and lock-down regulatory states would advance our knowledge of tissue differentiation and organogenesis (Stathopoulos and Levine, 2005).

1. General Circuits and Strategies in Developmental Regulatory Networks

No individual gene is capable of communicating sufficient spatial information to generate complex patterns of cell fates, a prerequisite for organogenesis. Rather, a single tissue or organ is the outcome of the expression and activities of a multitude of genes. Network logic directs the formation of complex morphologies through consecutive rounds of pattern formation. In this way, information from the previous round of specification gets locked in and integrated with additional information from concurrent specification events. Depicting gene regulatory networks as circuit diagrams is especially informative as it documents all the regulatory inputs and outputs from each constituent gene and provides a comprehensive view of how diverse cell types acquire their identity (Longabaugh et al., 2005). Here I discuss some general strategies that organisms use to establish spatially defined patterns of developmental gene expression.

1-1. Gradients

All developmental control systems function as gene regulatory networks in that each regulatory gene has both multiple inputs and outputs. Gradients are one of the mechanisms embryos use to produce transient and dynamic expression of downstream genes in different spatial domains, as they progress through successive regulatory states. The usage of gradients also demonstrates how cis-regulatory elements can be precisely

modulated to respond to dynamic conditions (e.g., differential morphogen levels) (Jiang and Levine, 1993; Stathopoulos and Levine, 2002).

Dorsal network

Dorsal-ventral patterning in *Drosophila* is mediated by the graded distribution of a maternal transcription factor, Dorsal (an ortholog of NF-κB), which ultimately results in the differentiation of specialized tissues, including the mesoderm, the neurogenic ectoderm and the dorsal ectoderm (Stathopoulos and Levine, 2002). Roughly half of the 50 Dorsal target genes encode transcription factors, while the other half encode components of the FGF, EGF, and Dpp (TGFβ) signaling pathways (Stathopoulos and Levine, 2004). The enhancer regions of these downstream genes consist of target sites that have a range of sensitivities to Dorsal (and other inputs), thereby restricting their expression in specific spatial domains in the embryo. For example, Twist enhancers contain several low-affinity Dorsal binding sites and are only activated by high Dorsal levels present in the presumptive mesoderm (Jiang et al., 1991). In contrast, Rhomboid enhancers contain higher-affinity Dorsal and Twist binding sites and are activated by intermediate levels of the Dorsal gradient in ventral neurogenic ectoderm. The Snail repressor directly competes with Twist for the same binding sites and keeps Rhomboid expression off in the mesoderm (Ip et al., 1992). The different threshold readouts of the Dorsal gradient are partly dependent on the quality of individual Dorsal binding sites (Papatsenko and Levine, 2005).

The above scenario illustrates how a concentration gradient allows a single gene to convey different positional signals to a whole array of targets. The Dorsal gradient also

demonstrates that cis-regulatory elements can be sensitive not only to the abundance of an activator, but to dynamic cellular conditions as well.

1-2. Boundary Repression

Boundary repression is one of several architectural motifs that come to light while dissecting gene regulatory networks. Morphogen gradients alone cannot account for the strict expression patterns of genes that specify cell fates. In general, transcriptional repressors are used to generate and maintain boundaries between cells with different fate patterns. The mechanisms that enforce boundary repression can be divergent among the different species.

D/V patterning in *Drosophila*

For example in *Drosophila,* transcriptional repression has an extensive role in the initial patterning of the syncytial embryo. Since cell boundaries are not yet established, intercellular signaling plays a limited role in the early spatial patterning events. In this developmental system, boundaries of expression patterns are regulated by multiple tiers of transcriptional repression. An excellent example would be the regulatory interactions among the five repressors Snail, Vnd, Brk, Ind, and Shn during dorsal-ventral patterning (Cowden and Levine, 2003; Kosman et al., 1991; Pyrowolakis et al., 2004; Zhang et al., 2001). The direct cross inhibition between these repressors results in the formation of four distinct tissue territories: mesoderm, ventral neurogenic ectoderm, dorsal neurogenic ectoderm, and dorsal ectoderm. Consequently, multipotent nuclei are rapidly transformed into specific cell identities.

Neural crest specification in vertebrates

Unlike the *Drosophila* embryo, intercellular signaling plays a crucial role in defining boundaries in organisms like vertebrates. An interesting illustration of this sort of boundary repression is found in neural crest formation in vertebrates (Fig. 1) (Meulemans and Bronner-Fraser, 2004). The mesoderm and non-neural ectoderm secrete inductive signals such as BMP to specify the neural crest fate (Bonstein et al., 1998; Liem et al., 1995; Marchant et al., 1998). Differential levels of BMP signaling are required to induce neural plate and neural crest gene expression. Inhibition of BMP signaling induces expression of *Sox2* in the neural plate, which is required to promote expression of neural differentiation genes such as *N-CAM* and *N-tubulin* (Mizuseki et al., 1998). *Sox2* also indirectly represses expression of the neural crest specifier *Slug* in the neural plate (Wakamatsu et al., 2004). In the presumptive neural crest (neural plate border), intermediate levels of BMP induces high levels of *Slug* and other neural crest specifiers such as *Snail, Sox9,* etc. (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Nguyen et al., 1998). In turn, these neural crest specifiers perform two important regulatory functions: (a) repression of *Sox2* function (LaBonne and Bronner-Fraser, 2000; Spokony et al., 2002) and (b) regulation of effector genes that control different aspects of the neural crest (Aoki et al., 2003; Britsch et al., 2001; Fukata and Kaibuchi, 2001; Lang and Epstein, 2003; Ng et al., 1997). Neural crest and neural plate fates are thus demarcated using a combination of intercellular signaling and cross-regulation.

1-3. Stable Feedback Loops

A key property of all gene regulatory networks is that they strictly enforce progression throughout development. Stable feedback loops are a mechanism that is typically used to drive development forward (Fig. 2A). In general, signal transduction activates expression of regulatory gene A. Gene A promotes expression of gene B (a target), which in turn activates a cascade of downstream target genes. However, gene B positively feedbacks to gene A, thereby "locking down" the differentiation state established by the original signal. Once the feedback loop is activated, the downstream genes are no longer dependent on the initial transient inputs. A benefit of this developmental strategy is that transient signals are converted into stable circuits. Additionally, the regulatory proteins that function in early development are then free to be reutilized in later specification and differentiation events without conflict. Most gene regulatory networks require the maintenance of stable feedback loops or the differentiation state is disrupted.

Skeletal muscle differentiation in vertebrates

Regulation of skeletal muscle specification and differentiation in vertebrates is dependent on a regulatory network that consists of two families of transcription factors: bHLH muscle regulatory factors (MRFs: MyoD, myogenin, MRF4) and the myocyte enhancer factor 2 (MEF2) group of MADS-box regulators. Members of this network interact with each other genetically and physically, and together they cooperate to positively regulate transcription of downstream muscle-specific differentiation genes (adapted from Yun and Wold, 1996) (Fig. 2B). MyoD acts as a muscle specification gene which activates expression of the differentiation gene myogenin. Myogenin subsequently

activates MEF2 and MRF4, which are required for the execution of terminal muscle differentiation programs. Additionally, MRF4 and myogenin positively regulate the activity of MyoD. In this fashion, skeletal muscle cells are engaged in a self-sustaining positive loop that drives the expression of muscle differentiation genes and thus locks down the newly established differentiation state. The outcome of such positive feedback loops is that the embryo acquires control of its new regulatory state and is relieved from its dependence on transiently expressed inputs.

1-4. Autoregulation

Another variation of stable feedback loops is autoregulation, which is defined as the regulation of a gene by its own gene product. Negative autoregulation occurs when a transcription factor binds its own promoter to reduce production of its own mRNA. As a result, the higher the concentration of a given transcription factor, the lower its production rate. By contrast, a regulatory factor positively autoregulates when it activates its own transcription. The initial dynamics are slow, but as the transcription factor levels rise, its production rate increases due to the positive autoregulation loop.

The steady state levels of a protein are typically important for its optimal function. Negative autoregulation speeds up the response time of gene circuits (Rosenfeld et al., 2002). For example, a strong promoter can provide rapid production of its own mRNA and use autorepression to reach the desired steady-state levels. This can be compared to a simple regulated gene with a weaker promoter that is set to reach steady-state levels at a slower rate. Negative autoregulation also reduces variations of steady-

state expression levels due to fluctuations in the production rate (Becskei and Serrano, 2000).

A positive autoregulatory circuit has the opposite effect, in that it slows down response times. Slow dynamics can be particularly useful in time-consuming developmental processes, such as the cell cycle. Such slow processes can benefit from extended delays in the production of proteins responsible for different stages of the process. Positive autoregulation (which exceeds the rate of protein turnover) also confers the advantage of achieving bistability: positive autoregulation maintains robust expression levels of the gene, even after the initial activator inputs are gone (Becskei et al., 2001). Many transcription networks utilize this type of circuit to make irreversible decisions in development, such as execution of cell fate.

2. Emerging Gene Networks in *C. elegans*

Microarray analysis, trans-genesis, systematic gene disruption (via RNAi), and invariant cell lineage are features that make *C. elegans* a tractable model system for elucidating gene regulatory networks that govern fate specification and differentiation of complex organs (Lee et al., 2004). The availability of genome sequences for several divergent nematodes, *C. briggsae* (Gupta and Sternberg, 2003) and *C. remanei* permits the rapid identification of conserved non-coding enhancer elements. Here I describe the advances made in several emerging gene regulatory networks (GRNs) in *C. elegans*:

2-1. Embryonic GRNs:

***pal-1*: Specification of C-lineage**

Embryonic gene regulatory networks are typically set into motion by the graded distribution of maternally deposited RNA/protein. Microarray and RNAi experiments in *C. elegans* embryos have been used to describe a provisional network for the specification and differentiation of the C-blastomere lineage (Baugh et al., 2005a) (Fig. 3). The C-lineage blastomeres develop into muscle and some epidermal cell types. PAL-1, a homeodomain transcription factor, is required for the specification of the C-lineage blastomere (Hunter and Kenyon, 1996). Maternal *pal-1* RNAs are evenly distributed throughout the unfertilized oocyte and eventually become restricted to the C-blastomere due to transcriptional and translational regulation. Maternal PAL-1 activates zygotic transcription of many target genes in the C-blastomere (including zygotic *pal-1* via positive autoregulation). Regulatory genes such as *tbx-8*, *tbx-9*, and *elt-1* may be direct targets of PAL-1. ELT-1, a GATA transcription factor is necessary for the differentiation of specific epidermal cell types. PAL-1 also promotes expression of *hnd-1*(Hand-like bHLH), which subsequently leads to the activation of *unc-120* (MADS) and *hlh-1*(MyoD) in developing muscles. Similar to their vertebrate orthologs, HND-1, UNC-120, and HLH-1 represent a regulatory module that is dedicated to muscle differentiation (Baugh et al., 2005b). Dissection of the cis-regulatory elements of PAL-1 targets would further elaborate this provisional network.

***pha4*: Foregut differentiation**

The pharynx is a feeding organ in *C. elegans* that consists of gut epithelial cells, muscles and neurons, and is an excellent system for unraveling genomic networks that

control organogenesis. All these cell types are similar in that they all express the organ identity gene *pha-4,* an ortholog of FoxA (Mango et al., 1994). Microarray assays identified around 350 pharynx-enriched genes with diverse spatial and temporal expression patterns (Gaudet and Mango, 2002). Many of these target genes carry copies of the PHA-4 consensus binding sequence TRTTTRY in their cis-regulatory regions. The affinities of the individual PHA-4 binding sites influence the temporal specification patterns during pharyngeal differentiation. Higher- and lower-affinity PHA-4 sites are correlated with earlier and later pharyngeal expression, respectively. Furthermore, altering the affinity of these binding sites causes changes in the temporal expression pattern of target genes.

Binding affinity is not the sole determinant of *pha-4* dependent gene expression. The enhancers of some early activated *pha-4* targets contain other shared motifs that are necessary for driving expression during early development (Gaudet et al., 2004). Additionally, some of the late *pha-4* targets contain a repressor element that blocks precocious expression at the early stages of pharyngeal differentiation.

Approximately, 10% of the PHA-4 targets encode putative transcription factors. The genetic interactions between these regulatory genes and their individual roles in the diverse pharyngeal cell types are still unknown. Further characterization of these regulatory factors would reveal how they coordinate with PHA-4 and each other to activate expression downstream effector genes and mediate organogenesis.

2-2. ASE Specification: A Postembryonic GRN

Unlike embryonic GRNs, postembryonic networks are not dependent on maternal inputs. The two main gustatory neurons of *C. elegans*, ASE left (ASEL) and ASE right (ASER), are symmetric in many different regards, like cell position, axo-dendritic morphology, and synaptic connectivity. However both neurons express a distinct assortment of putative chemoreceptors at the adult stage, which is necessary when navigating through complex sensory environments (Pierce-Shimomura et al., 2001; Yu et al., 1997). These asymmetric molecular profiles correlate with the functional asymmetry of the two neurons. Forward genetic screens have been successfully used to define the gene regulatory network that diversifies ASEL and ASER (Chang et al., 2004; Chang et al., 2003; Johnston and Hobert, 2003; Johnston et al., 2005). The ASEL/R regulatory network utilizes feedback loops to progress from an equipotent, hybrid precursor state to irreversible terminally differentiated cellular states (Fig. 4). The stable nature of these feedback mechanisms allows the system to respond to transient inputs and adopt one of two discrete end states (bistable system). Here, ASER is biased to have higher levels of *cog-1* which turns off ASEL-like features by repression of the activator *die-1*. Additionally, the positive autoregulation of *cog-1* decisively locks down the ASER cell fate choice. In ASEL, spatially precise gene expression requires the function of *die-1* and the absence of *cog-1* repressor activity. In addition, the homeobox gene *lim-6* (a *die-1* target) reinforces the ASEL regulatory state via inhibition of *cog-1* activity. As mentioned earlier, these stable feedback loops ensure that the system is no longer dependent on transient inputs. The identity of the inputs that trigger the differential activity of *cog-1* vs. *die-1* in the ASE neurons is unknown. Cis-regulatory analyses of

these network components would unveil the nature of their upstream regulatory inputs (possibly other spatially restricted transcription factors or signaling pathways).

3. The Vulva: A Postembryonic Paradigm for Organogenesis

Recent studies in *C. elegans* have attempted to build regulatory networks using high-throughput global analyses such as microarray data, yeast one hybrid interactions, ChIP, and bioinformatics (Ao et al., 2004; Lee et al., 2003; McElwee et al., 2003; Murphy et al., 2003; Wook Oh et al., 2006). These are useful and effective in terms of identifying relevant targets affected by upstream regulatory factors. However, these analyses have limitations in that they do not always reflect normal physiology. Secondly, the data acquired are typically at a lower resolution and can provide an oversimplified framework not necessarily relevant to diverse cell types and varying developmental states. Stage- and tissue-specific regulatory programs (such as stable feedback loops, boundary repression, and “lock down” subroutines) would not be detected through these high-throughput approaches. An alternative approach to constructing genomic networks is through the investigation of multiple pairwise interactions (Johnston et al., 2005; Rivera-Pomar and Jackle, 1996; Vallstedt et al., 2001). This more-traditional approach can yield data of higher resolution, reflecting precise spatial and temporal gene expression in an intact organism. Thus the resulting network would be more refined and would unveil the subtle differences that exist in the diverse cell types at specific stages. Resources such as diverse spatial and temporal vulval cell-fate markers, reverse genetics (RNAi), and the ease of manipulation at the single-cell level provide powerful tools for studying the execution of cell-type-specific gene expression programs (Inoue et al.,

2005). These features make the *C. elegans* vulva useful for dissecting the gene regulatory networks involved in patterning and organ morphogenesis. Comparisons of the vulval network with those reported in genome-wide studies in other model systems would highlight both differences and similarities in tissue differentiation and organogenesis (Stathopoulos and Levine, 2005).

The *C. elegans* vulva is postembryonically derived from six multipotent vulval precursor cells (VPCs) P3.p–P8.p. The three central VPCs, P5.p–P7.p are induced to adopt vulval fates, whereas the remaining precursors fuse with the hypodermal syncytium hyp7. The 1° (primary) and 2° (secondary) vulval fates are generated via EGF and Notch signaling respectively (Sternberg, 2005). The vulva is an intact organ that invariantly consists of seven distinct cell types, each with its own pattern of gene expression and morphogenetic migrations (Inoue et al., 2002; Sharma-Kishore et al., 1999; Sulston and Horvitz, 1977). The P6.p 1° lineages generate the vulE and vulF cells, while the P5.p and P7.p 2° lineages generate the vulA, vulB1, vulB2, vulC, and vulD cells. The diverse cell types appear to have different physiological roles that affect the morphology and function of the hermaphrodite reproductive organ. For example, EGF signaling in the vulF cells induces the formation uv1 uterine cells (Chang et al., 1999). The 1° vulval cells initialize the uterine-vulval connection by triggering and directing anchor cell (AC) invasion (Sherwood and Sternberg, 2003). The vulE and vulF lineages are also required for the proper migration of the sex myoblasts (Burdine et al., 1998) and establishing contacts with the HSN and VC neurons (egg-laying motor neurons) (Colavita and Tessier-Lavigne, 2003; Shen et al., 2004). Vulval muscles that are associated with vulC and vulD regulate egg-laying behavior. vulA, the outermost vulval cell-type, forms the attachment

with the cuticle. A complete picture that depicts the functional relevance of all the vulval cell types is still lacking. Differential gene expression is thought to be the driving force behind the specialization of these epithelial cells. The finite number of cell types in this complex organ provides us with an elegant model for dissecting cell-specific gene regulatory networks. Teasing apart the vulval network would provide us with valuable insights into postembryonic organogenesis.

The spatially defined pattern of cell fates in the vulva is an outcome of transcription factor networks that operate in the individual cell types (Inoue et al., 2005). While the signaling network that initiates vulval development and sets the gross pattern of cell differentiation is well understood, the gene regulatory network that specifies the final seven cell fates is not understood (Sternberg, 2005). Both Ras and Wnt pathways are required for the precise spatial patterning of the 1° vulE and vulF cells (Wang and Sternberg, 2000), and both Wnt/Ryk and Wnt/Frizzled signaling pathways are necessary for patterning the P7.p 2° vulA-vulD cells (Ferguson et al., 1987; Inoue et al., 2004; Sawa et al., 1996). Several transcription factors that regulate spatio-temporal gene expression in the vulva have been already described (Cui and Han, 2003; Inoue et al., 2005; Tiensuu et al., 2005). *lin-29* encodes a C2H2 Zn-finger transcription factor and affects gene expression in vulC, vulD, and vulE cells (Newman et al., 2000). *lin-11*, a LIM homeobox transcription factor, drives gene expression in all seven vulval cell types (Freyd et al., 1990; Gupta et al., 2003). The Nkx6.1/Nkx6.2 homeodomain gene, *cog-1,* regulates gene expression in vulB, vulC, vulD, vulE, and vulF cells (Inoue et al., 2005; Palmer et al., 2002). By contrast, the PAX2/5/8 protein EGL-38, appears to be the only known example of a cell-type specific regulatory factor; it promotes expression of certain

target genes and inhibits inappropriate expression of other targets exclusively in vulF cells (Chamberlin et al., 1997; Chang et al., 1999; Inoue et al., 2005). Our knowledge about the regulatory interactions between these transcription factors and how they go about generating the complex expression patterns of their targets is limited. Furthermore, an appreciation for the differences in the network architecture for each of the cell types requires the identification of novel transcriptional regulators.

Thesis Prelude

Comparison of gene networks among a broad spectrum of species is useful in deciphering the regulatory logic that ultimately gives rise to complex functional morphologies. However, a majority of the most intensively studied gene regulatory networks are embryonic networks. To date, the *C. elegans* vulva is the first excellent model for elucidating postembryonic gene regulatory networks that control organogenesis. Using a high-resolution reverse genetics approach, I successfully identified the *tailless* ortholog *nhr-67* as a new node in the vulval patterning network. In addition to isolating and characterizing a novel component of the vulval regulatory network, my work illustrates the recurrence of certain network motifs/strategies such as autoregulation, stable feedback loops, boundary repression, functional redundancy, and combinatorial control of effector gene expression during vulval morphogenesis. I also discovered a previously unreported regulatory circuit in a specific subset of vulval cells: cross inhibition in conjunction with dual negative autoregulation. The provisional vulval network described in this thesis provides a very solid framework for future work. Pushing our proposed regulatory network forward would entail identifying other potential factors,

integrating all the interactions between the components, and assaying the output expression of multiple target genes.

References:

Ao, W., Gaudet, J., Kent, W. J., Muttumu, S., and Mango, S. E. (2004). Environmentally induced foregut remodeling by PHA-4/FoxA and DAF-12/NHR. Science *305*, 1743–1746.

Aoki, Y., Saint-Germain, N., Gyda, M., Magner-Fink, E., Lee, Y. H., Credidio, C., and Saint-Jeannet, J. P. (2003). Sox10 regulates the development of neural crest-derived melanocytes in *Xenopus*. Dev Biol *259*, 19–33.

Arkin, A., Ross, J., and McAdams, H. H. (1998). Stochastic kinetic analysis of developmental pathway bifurcation in phage lambda-infected *Escherichia coli* cells. Genetics *149*, 1633–1648.

Baugh, L. R., Hill, A. A., Claggett, J. M., Hill-Harfe, K., Wen, J. C., Slonim, D. K., Brown, E. L., and Hunter, C. P. (2005a). The homeodomain protein PAL-1 specifies a lineage-specific regulatory network in the *C. elegans* embryo. Development *132*, 1843–1854.

Baugh, L. R., Wen, J. C., Hill, A. A., Slonim, D. K., Brown, E. L., and Hunter, C. P. (2005b). Synthetic lethal analysis of *Caenorhabditis elegans* posterior embryonic patterning genes identifies conserved genetic interactions. Genome Biol *6*, R45.

Becskei, A., Seraphin, B., and Serrano, L. (2001). Positive feedback in eukaryotic gene networks: cell differentiation by graded to binary response conversion. Embo J *20*, 2528–2535.

Becskei, A., and Serrano, L. (2000). Engineering stability in gene networks by autoregulation. Nature *405*, 590–593.

Bonstein, L., Elias, S., and Frank, D. (1998). Paraxial-fated mesoderm is required for neural crest induction in *Xenopus* embryos. Dev Biol *193*, 156–168.

Britsch, S., Goerich, D. E., Riethmacher, D., Peirano, R. I., Rossner, M., Nave, K. A., Birchmeier, C., and Wegner, M. (2001). The transcription factor Sox10 is a key regulator of peripheral glial development. Genes Dev *15*, 66–78.

Burdine, R. D., Branda, C. S., and Stern, M. J. (1998). EGL-17(FGF) expression coordinates the attraction of the migrating sex myoblasts with vulval induction in *C. elegans*. Development *125*, 1083–1093.

Carroll, S. B. (1990). Zebra patterns in fly embryos: activation of stripes or repression of interstripes? Cell *60*, 9–16.

Chamberlin, H. M., Palmer, R. E., Newman, A. P., Sternberg, P. W., Baillie, D. L., and Thomas, J. H. (1997). The PAX gene *egl-38* mediates developmental patterning in *Caenorhabditis elegans*. Development *124*, 3919–3928.

Chang, C., Newman, A. P., and Sternberg, P. W. (1999). Reciprocal EGF signaling back to the uterus from the induced *C. elegans* vulva coordinates morphogenesis of epithelia. Curr Biol *9*, 237–246.

Chang, S., Johnston, R. J., Jr., Frokjaer-Jensen, C., Lockery, S., and Hobert, O. (2004). MicroRNAs act sequentially and asymmetrically to control chemosensory laterality in the nematode. Nature *430*, 785–789.

Chang, S., Johnston, R. J., Jr., and Hobert, O. (2003). A transcriptional regulatory cascade that controls left/right asymmetry in chemosensory neurons of *C. elegans*. Genes Dev *17*, 2123–2137.

Colavita, A., and Tessier-Lavigne, M. (2003). A Neurexin-related protein, BAM-2, terminates axonal branches in *C. elegans*. Science *302*, 293–296.

Cowden, J., and Levine, M. (2003). Ventral dominance governs sequential patterns of gene expression across the dorsal-ventral axis of the neuroectoderm in the *Drosophila* embryo. Dev Biol *262*, 335–349.

Cui, M., and Han, M. (2003). Cis regulatory requirements for vulval cell-specific expression of the *Caenorhabditis elegans* fibroblast growth factor gene *egl-17*. Dev Biol *257*, 104–116.

Davidson, E. H. (2006). Genomic Regulatory Systems: Development and Evolution, 2nd edn (San Diego, Academic Press).

Erwin, D. H., and Davidson, E. H. (2002). The last common bilaterian ancestor. Development *129*, 3021–3032.

Ferguson, E. L., Sternberg, P. W., and Horvitz, H. R. (1987). A genetic pathway for the specification of the vulval cell lineages of *Caenorhabditis elegans*. Nature *326*, 259–267.

Freyd, G., Kim, S. K., and Horvitz, H. R. (1990). Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene *lin-11*. Nature *344*, 876–879.

Fukata, M., and Kaibuchi, K. (2001). Rho-family GTPases in cadherin-mediated cell-cell adhesion. Nat Rev Mol Cell Biol *2*, 887–897.

Gaudet, J., and Mango, S. E. (2002). Regulation of organogenesis by the *Caenorhabditis elegans* FoxA protein PHA-4. Science *295*, 821–825.

Gaudet, J., Muttumu, S., Horner, M., and Mango, S. E. (2004). Whole-genome analysis of temporal gene expression during foregut development. PLoS Biol *2*, e352.

Gupta, B. P., and Sternberg, P. W. (2003). The draft genome sequence of the nematode *Caenorhabditis briggsae*, a companion to *C. elegans*. Genome Biol *4*, 238.

Gupta, B. P., Wang, M., and Sternberg, P. W. (2003). The *C. elegans* LIM homeobox gene *lin-11* specifies multiple cell fates during vulval development. Development *130*, 2589–2601.

Hinman, V. F., Nguyen, A. T., Cameron, R. A., and Davidson, E. H. (2003). Developmental gene regulatory network architecture across 500 million years of echinoderm evolution. Proc Natl Acad Sci U S A *100*, 13356–13361.

Hunter, C. P., and Kenyon, C. (1996). Spatial and temporal controls target *pal-1* blastomere-specification activity to a single blastomere lineage in *C. elegans* embryos. Cell *87*, 217–226.

Inoue, T., Oz, H. S., Wiland, D., Gharib, S., Deshpande, R., Hill, R. J., Katz, W. S., and Sternberg, P. W. (2004). *C. elegans* LIN-18 is a Ryk ortholog and functions in parallel to LIN-17/Frizzled in Wnt signaling. Cell *118*, 795–806.

Inoue, T., Sherwood, D. R., Aspock, G., Butler, J. A., Gupta, B. P., Kirouac, M., Wang, M., Lee, P. Y., Kramer, J. M., Hope, I., et al. (2002). Gene expression markers for *Caenorhabditis elegans* vulval cells. Mech Dev *119 Suppl 1*, S203–209.

Inoue, T., Wang, M., Ririe, T. O., Fernandes, J. S., and Sternberg, P. W. (2005). Transcriptional network underlying *Caenorhabditis elegans* vulval development. Proc Natl Acad Sci U S A *102*, 4972–4977.

Ip, Y. T., Park, R. E., Kosman, D., Bier, E., and Levine, M. (1992). The *dorsal* gradient morphogen regulates stripes of *rhomboid* expression in the presumptive neuroectoderm of the *Drosophila* embryo. Genes Dev *6*, 1728–1739.

Jiang, J., Kosman, D., Ip, Y. T., and Levine, M. (1991). The *dorsal* morphogen gradient regulates the mesoderm determinant *twist* in early *Drosophila* embryos. Genes Dev *5*, 1881–1891.

Jiang, J., and Levine, M. (1993). Binding affinities and cooperative interactions with bHLH activators delimit threshold responses to the *dorsal* gradient morphogen. Cell *72*, 741–752.

Johnston, R. J., and Hobert, O. (2003). A microRNA controlling left/right neuronal asymmetry in *Caenorhabditis elegans*. Nature *426*, 845–849.

Johnston, R. J., Jr., Chang, S., Etchberger, J. F., Ortiz, C. O., and Hobert, O. (2005). MicroRNAs acting in a double-negative feedback loop to control a neuronal cell fate decision. Proc Natl Acad Sci U S A *102*, 12449–12454.

Koide, T., Hayata, T., and Cho, K. W. (2005). *Xenopus* as a model system to study transcriptional regulatory networks. Proc Natl Acad Sci U S A *102*, 4943–4948.

Kosman, D., Ip, Y. T., Levine, M., and Arora, K. (1991). Establishment of the mesoderm-neuroectoderm boundary in the *Drosophila* embryo. Science *254*, 118–122.

LaBonne, C., and Bronner-Fraser, M. (1998). Neural crest induction in *Xenopus*: evidence for a two-signal model. Development *125*, 2403–2414.

LaBonne, C., and Bronner-Fraser, M. (2000). Snail-related transcriptional repressors are required in *Xenopus* for both the induction of the neural crest and its subsequent migration. Dev Biol *221*, 195–205.

Lang, D., and Epstein, J. A. (2003). Sox10 and Pax3 physically interact to mediate activation of a conserved c-RET enhancer. Hum Mol Genet *12*, 937–945.

Lee, J., Nam, S., Hwang, S. B., Hong, M., Kwon, J. Y., Joeng, K. S., Im, S. H., Shim, J., and Park, M. C. (2004). Functional genomic approaches using the nematode *Caenorhabditis elegans* as a model system. J Biochem Mol Biol *37*, 107–113.

Lee, S. S., Kennedy, S., Tolonen, A. C., and Ruvkun, G. (2003). DAF-16 target genes that control *C. elegans* life-span and metabolism. Science *300*, 644–647.

Levine, M., and Davidson, E. H. (2005). Gene regulatory networks for development. Proc Natl Acad Sci U S A *102*, 4936–4942.

Liem, K. F., Jr., Tremml, G., Roelink, H., and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell *82*, 969–979.

Longabaugh, W. J., Davidson, E. H., and Bolouri, H. (2005). Computational representation of developmental genetic regulatory networks. Dev Biol *283*, 1–16.

Mango, S. E., Lambie, E. J., and Kimble, J. (1994). The *pha-4* gene is required to generate the pharyngeal primordium of Caenorhabditis elegans. Development *120*, 3019–3031.

Marchant, L., Linker, C., Ruiz, P., Guerrero, N., and Mayor, R. (1998). The inductive properties of mesoderm suggest that the neural crest cells are specified by a BMP gradient. Dev Biol *198*, 319–329.

Matthias, P., and Rolink, A. G. (2005). Transcriptional networks in developing and mature B cells. Nat Rev Immunol *5*, 497–508.

McElwee, J., Bubb, K., and Thomas, J. H. (2003). Transcriptional outputs of the *Caenorhabditis elegans* forkhead protein DAF-16. Aging Cell *2*, 111–121.

Meulemans, D., and Bronner-Fraser, M. (2004). Gene-regulatory interactions in neural crest evolution and development. Dev Cell *7*, 291–299.

Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S., and Sasai, Y. (1998). *Xenopus* Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. Development *125*, 579–587.

Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., Li, H., and Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. Nature *424*, 277–283.

Newman, A. P., Inoue, T., Wang, M., and Sternberg, P. W. (2000). The *Caenorhabditis elegans* heterochronic gene *lin-29* coordinates the vulval-uterine-epidermal connections. Curr Biol *10*, 1479–1488.

Ng, L. J., Wheatley, S., Muscat, G. E., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D. M., Tam, P. P., Cheah, K. S., and Koopman, P. (1997). SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. Dev Biol *183*, 108–121.

Nguyen, V. H., Schmid, B., Trout, J., Connors, S. A., Ekker, M., and Mullins, M. C. (1998). Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a *bmp2b/swirl* pathway of genes. Dev Biol *199*, 93–110.

Palmer, R. E., Inoue, T., Sherwood, D. R., Jiang, L. I., and Sternberg, P. W. (2002). *Caenorhabditis elegans cog-1* locus encodes GTX/Nkx6.1 homeodomain proteins and regulates multiple aspects of reproductive system development. Dev Biol *252*, 202–213.

Papatsenko, D., and Levine, M. (2005). Quantitative analysis of binding motifs mediating diverse spatial readouts of the Dorsal gradient in the *Drosophila* embryo. Proc Natl Acad Sci U S A *102*, 4966–4971.

Pierce-Shimomura, J. T., Faumont, S., Gaston, M. R., Pearson, B. J., and Lockery, S. R. (2001). The homeobox gene *lim-6* is required for distinct chemosensory representations in *C. elegans*. Nature *410*, 694–698.

Pyrowolakis, G., Hartmann, B., Muller, B., Basler, K., and Affolter, M. (2004). A simple molecular complex mediates widespread BMP-induced repression during *Drosophila* development. Dev Cell *7*, 229–240.

Rivera-Pomar, R., and Jackle, H. (1996). From gradients to stripes in *Drosophila* embryogenesis: filling in the gaps. Trends Genet *12*, 478–483.

Rosenfeld, N., Elowitz, M. B., and Alon, U. (2002). Negative autoregulation speeds the response times of transcription networks. J Mol Biol *323*, 785–793.

Sawa, H., Lobel, L., and Horvitz, H. R. (1996). The *Caenorhabditis elegans* gene *lin-17*, which is required for certain asymmetric cell divisions, encodes a putative seven-transmembrane protein similar to the *Drosophila frizzled* protein. Genes Dev *10*, 2189–2197.

Sharma-Kishore, R., White, J. G., Southgate, E., and Podbilewicz, B. (1999). Formation of the vulva in *Caenorhabditis elegans*: a paradigm for organogenesis. Development *126*, 691–699.

Shaywitz, D. A., and Melton, D. A. (2005). The molecular biography of the cell. Cell *120*, 729–731.

Shen, K., Fetter, R. D., and Bargmann, C. I. (2004). Synaptic specificity is generated by the synaptic guidepost protein SYG-2 and its receptor, SYG-1. Cell *116*, 869–881.

Sherwood, D. R., and Sternberg, P. W. (2003). Anchor cell invasion into the vulval epithelium in *C. elegans*. Dev Cell *5*, 21–31.

Small, S., Blair, A., and Levine, M. (1992). Regulation of *even-skipped* stripe 2 in the *Drosophila* embryo. Embo J *11*, 4047–4057.

Spokony, R. F., Aoki, Y., Saint-Germain, N., Magner-Fink, E., and Saint-Jeannet, J. P. (2002). The transcription factor Sox9 is required for cranial neural crest development in *Xenopus*. Development *129*, 421–432.

Stathopoulos, A., and Levine, M. (2002). Dorsal gradient networks in the *Drosophila* embryo. Dev Biol *246*, 57–67.

Stathopoulos, A., and Levine, M. (2004). Whole-genome analysis of *Drosophila* gastrulation. Curr Opin Genet Dev *14*, 477–484.

Stathopoulos, A., and Levine, M. (2005). Genomic regulatory networks and animal development. Dev Cell *9*, 449–462.

Sternberg, P. W. (2005). Vulval development, *Wormbook*, ed., (The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.6.1, www.wormbook.org).

Sulston, J. E., and Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. Dev Biol *56*, 110–156.

Tiensuu, T., Larsen, M. K., Vernersson, E., and Tuck, S. (2005). *lin-1* has both positive and negative functions in specifying multiple cell fates induced by Ras/MAP kinase signaling in *C. elegans*. Dev Biol *286*, 338–351.

Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T. M., and Ericson, J. (2001). Different levels of repressor activity assign redundant and specific roles to *Nkx6* genes in motor neuron and interneuron specification. Neuron *31*, 743–755.

Wakamatsu, Y., Endo, Y., Osumi, N., and Weston, J. A. (2004). Multiple roles of Sox2, an HMG-box transcription factor in avian neural crest development. Dev Dyn *229*, 74–86.

Wang, M., and Sternberg, P. W. (2000). Patterning of the *C. elegans* 1^{o} vulval lineage by RAS and Wnt pathways. Development *127*, 5047–5058.

Wook Oh, S., Mukhopadhyay, A., Dixit, B. L., Raha, T., Green, M. R., and Tissenbaum, H. A. (2006). Identification of direct DAF-16 targets controlling longevity, metabolism and diapause by chromatin immunoprecipitation. Nat Genet *38*, 251–257.

Yu, S., Avery, L., Baude, E., and Garbers, D. L. (1997). Guanylyl cyclase expression in specific sensory neurons: a new family of chemosensory receptors. Proc Natl Acad Sci U S A *94*, 3384–3387.

Yun, K., and Wold, B. (1996). Skeletal muscle determination and differentiation: story of a core regulatory network and its context. Curr Opin Cell Biol *8*, 877–889.

Zhang, H., Levine, M., and Ashe, H. L. (2001). Brinker is a sequence-specific transcriptional repressor in the *Drosophila* embryo. Genes Dev *15*, 261–266.

Figure Legends:

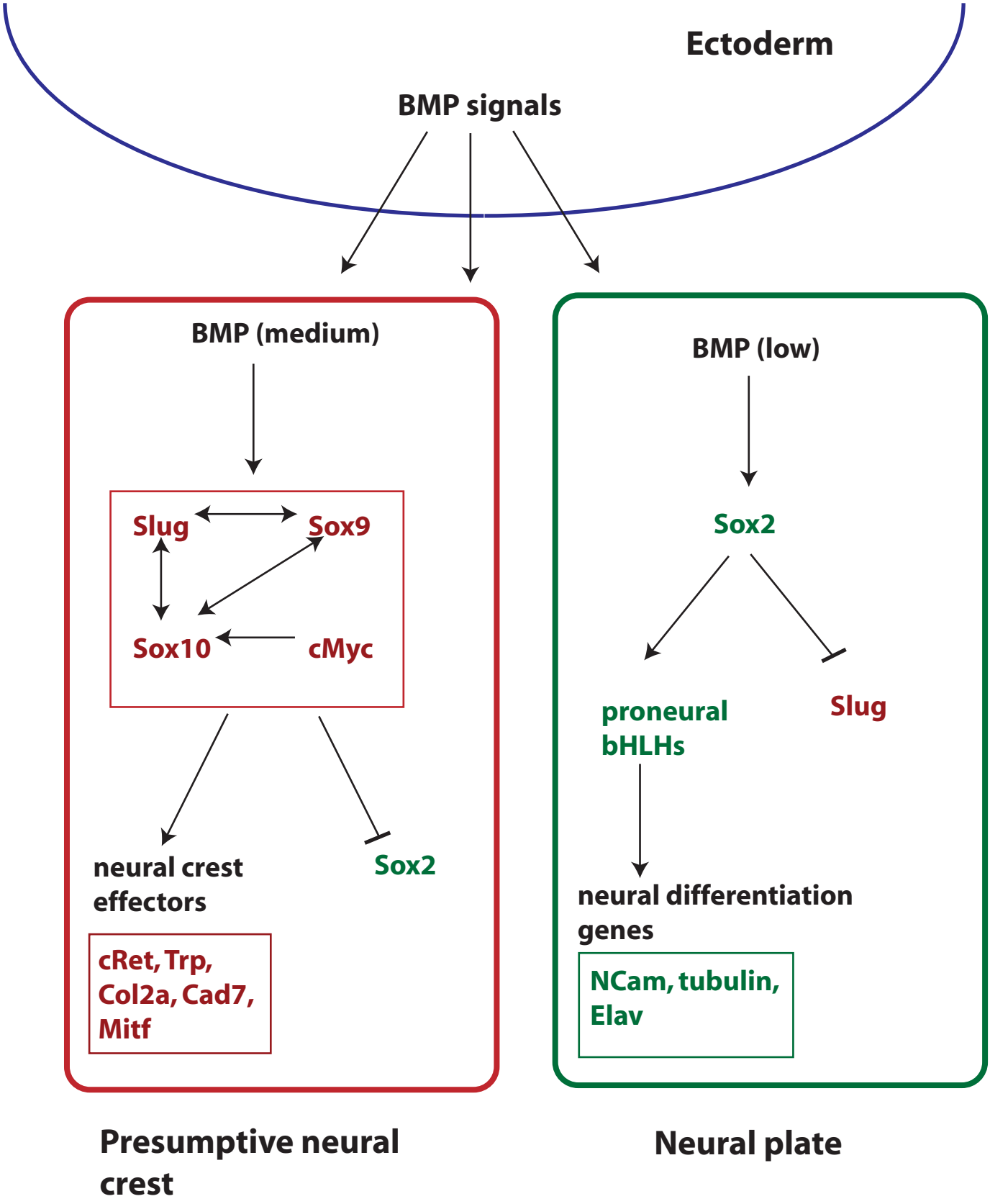
Fig. 1: Comparison of the gene regulatory interactions that occur during boundary repression at the neural plate border (presumptive neural crest) vs. the neural plate in vertebrates. Vertebrates utilize intercellular signaling (e.g., BMP) to define boundaries during neural crest specification. Arrows represent positive regulatory interactions and block arrows represent inhibitory inputs. Regulatory genes that promote neural crest specification are in red, whereas genes that regulate patterning and differentiation neural plate are in green.

Fig. 2: Stable feedback loops. (A) An initial input activates expression of regulatory gene A, which in turn promotes the expression of gene B. Besides acting on downstream target genes, gene B positively feedbacks to gene A, thereby “locking down” the regulatory state established by the original signal. When the initial input subsides, these genes will remain activated. (B) Stable positive feedback loops in skeletal muscle specification and differentiation in vertebrates. Arrows represent positive regulatory

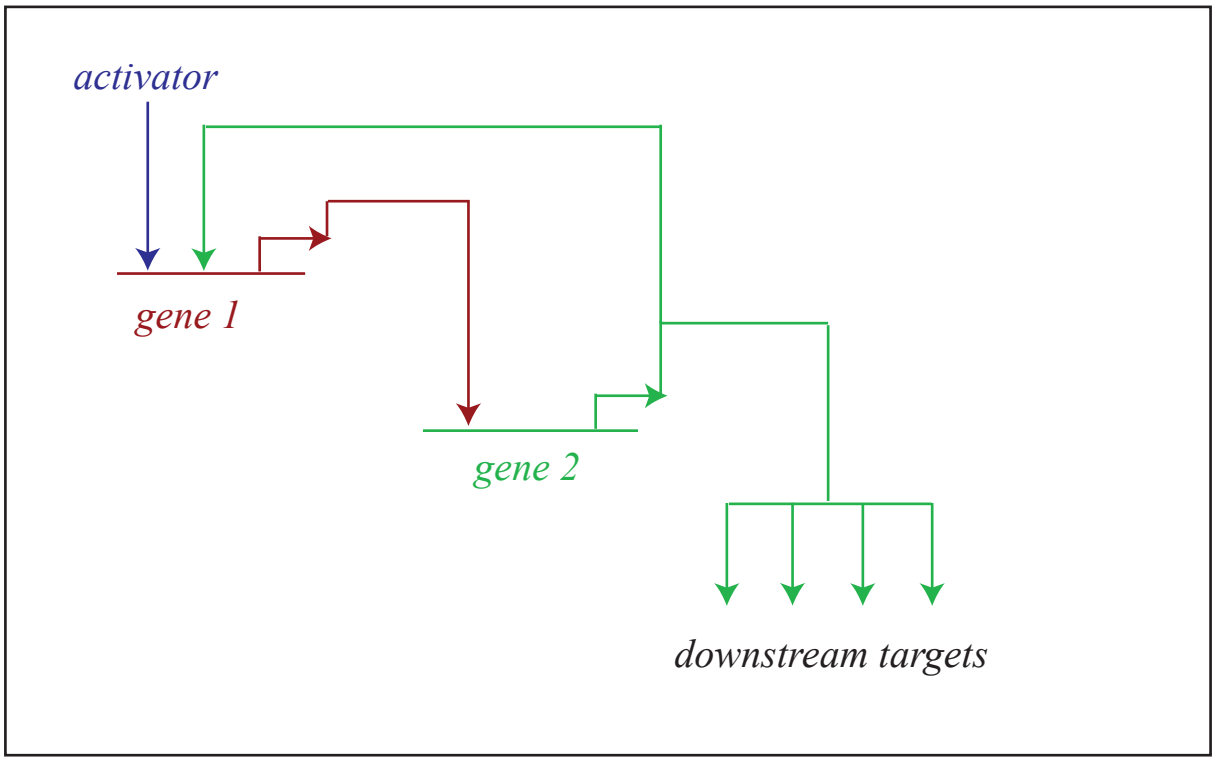
interactions. The displayed interactions in this simplified network only include cross regulatory loops. (Adapted from Yun K. and Wold B., 1996.)

Fig. 3: Proposed regulatory network specified by *pal-1* during C-lineage specification. (This figure is taken from Baugh et. al., 2005.) The predicted regulatory interactions are based on microarray data, RNAi experiments and bioinformatics. The temporal phases are indicated on the left. Lines with arrows represent cell-autonomous interactions and lines with dots indicate regulation by intercellular signaling pathways.

Fig. 4: A summary of the feedback mechanisms in the ASE bistable system. Arrows represent positive regulatory interactions and block arrows represent inhibitory interactions. The gray font indicates inactive components in the network and the black font indicates active components in the network. (Adapted from Johnston et. al., 2005.)


Ectoderm
BMP signals
BMP (medium)
Slug
Sox9
Sox10
cMyc
neural crest effectors
Sox2
cRet, Trp, Col2a, Cad7, Mitf
BMP (low)
Sox2
proneural bHLHs
Slug
neural differentiation genes
NCam, tubulin, Elav
Presumptive neural crest
Neural plate

A.


activator
gene 1
gene 2
downstream targets

B.

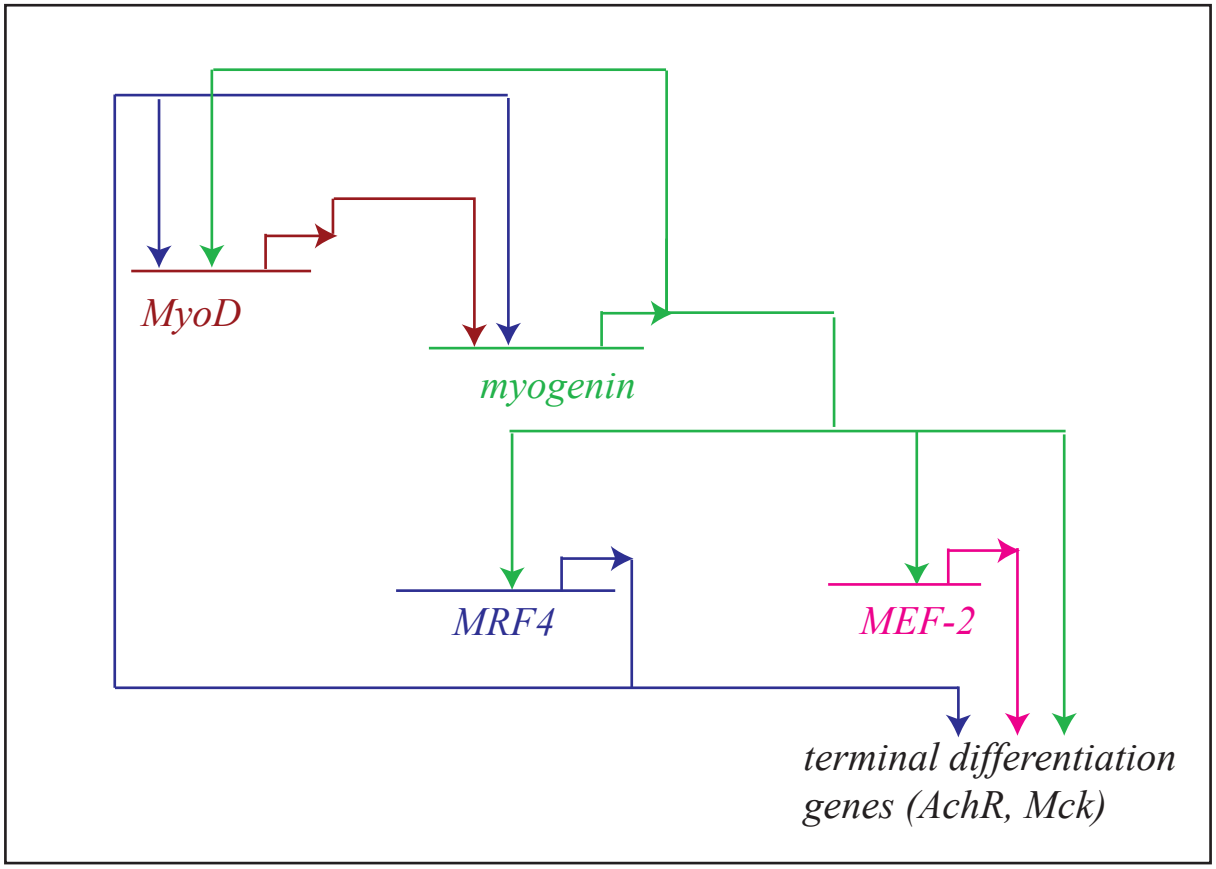
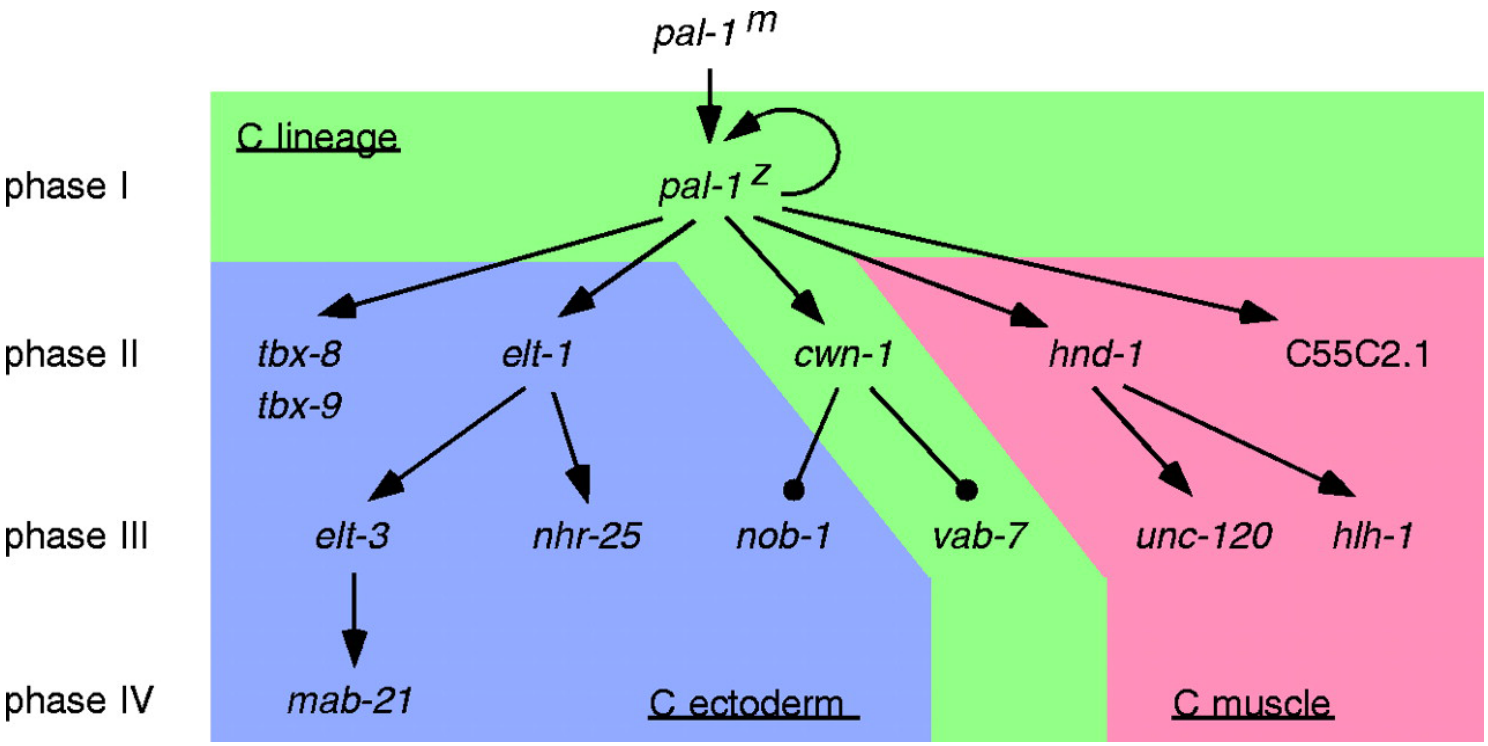
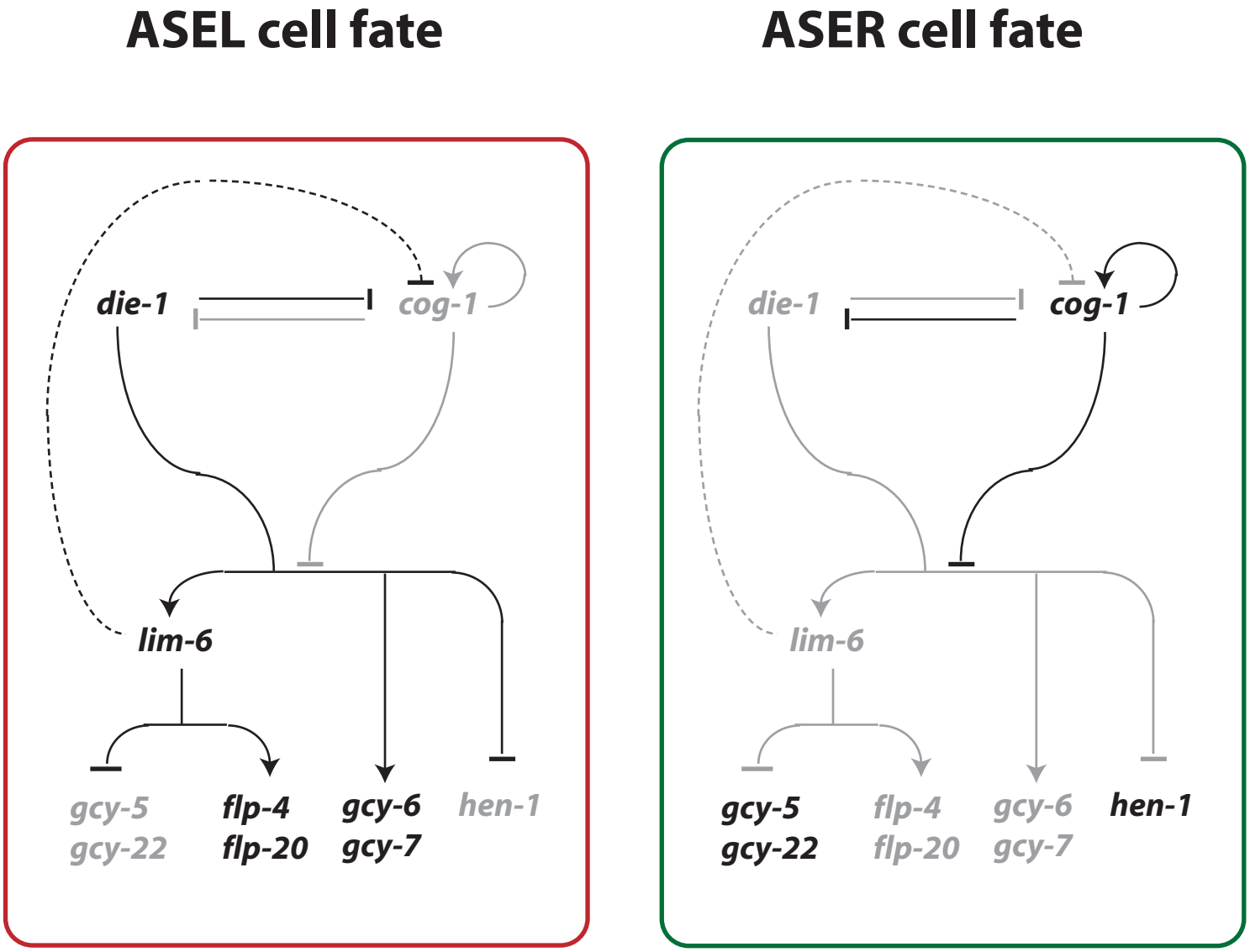

MyoD
myogenin
MRF4
MEF-2
terminal differentiation
genes (AchR, Mck)

Fig. 3



Adapted from Baugh et. al, 2005


ASEL cell fate
die-1
cog-1
lim-6
gcy-5
gcy-22
flp-4
flp-20
gcy-6
gcy-7
hen-1
ASER cell fate
die-1
cog-1
lim-6
gcy-5
gcy-22
flp-4
flp-20
gcy-6
gcy-7
hen-1

Appendix:

Transcriptional Network Underlying *Caenorhabditis elegans* Vulval Development

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Gene Regulatory Networks Special Feature: Transcriptional network underlying Caenorhabditis elegans vulval development

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Transcriptional network underlying *Caenorhabditis elegans* vulval development

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The vulval development of *Caenorhabditis elegans* provides an opportunity to investigate genetic networks that control gene expression during organogenesis. During the fourth larval stage (L4), seven vulval cell types are produced, each of which executes a distinct gene expression program. We analyze how the expression of cell-type-specific genes is regulated. Ras and Wnt signaling pathways play major roles in generating the spatial pattern of cell types and regulate gene expression through a network of transcription factors. One transcription factor (*lin-29*) primarily controls the temporal expression pattern. Other transcription factors (*lin-11*, *cog-1*, and *egl-38*) act in combination to control cell-type-specific gene expression. The complexity of the network arises in part because of the dynamic nature of gene expression, in part because of the presence of seven cell types, and also because there are multiple regulatory paths for gene expression within each cell type.

organogenesis | signaling pathways | transcription

Developmental events are driven by spatially and temporally regulated gene expression. Understanding how complex patterns of expression are produced is therefore a critical part of deciphering mechanisms of development. In general, intercellular signaling mechanisms interact with a network of transcription factors to generate cell-type-specific patterns of gene expression. The late stage of *Caenorhabditis elegans* vulval development offers a useful model in which to study this process. During this period of vulval development, seven distinct cell types are produced that express unique combinations of genes. Over the last several years, a number of genes were discovered that are expressed in cell-type and stage-specific patterns in the vulva, and several transcription factors were found to regulate these genes. In this paper, we synthesize and extend our current knowledge of this genetic network.

The *C. elegans* vulva connects the uterine lumen to the outside, allowing for passage of sperm and fertilized eggs (1). Vulval cells are generated postembryonically from precursor cells P3.p P4.p, P5.p, P6.p, P7.p, and P8.p [also called vulval precursor cells (VPC)]. During the mid-third larval (L3) stage, EGF and Notch signaling induces the middle three VPCs (P5.p, P6.p, and P7.p) to adopt vulval fates, whereas P3.p, P4.p, and P8.p fuse with the hypodermal syncytium, hyp7 (2–6).

During the late-L3 to the early-L4 stage, P5.p, P6.p, and P7.p undergo two or three rounds of cell division to produce 22 nuclei (7) (Fig. 1*A*). These nuclei are in cells of seven types (vulA, vulB1, vulB2, vulC, vulD, vulE, and vulF), as evidenced by subsequent morphogenetic movements and by the pattern of gene expression (8, 9) (Fig. 1*B*). The seven cell types that are present in the adult vulva represent specializations within the general epithelial cell class. These cells exhibit cell-type general features; for example, each expresses *ajm-1*, a component of the apical junction that connects neighboring cells in epithelial tissues (8). However, in addition, each cell type exhibits functional specializations: vulF cells, which form the innermost section of the vulva, connect directly with cells of the uterus. vulE cells form structural attachments to lateral hypodermal (seam) cells. vulC and vulD cells attach to vulval muscles that open the vulva for the passage of eggs. vulA cells form attachment to the hyp7 syncytium. It is expected that gene expression differences underlie these specializations.

Here, we are concerned with the execution of cell-type-specific gene expression programs during the late L3 and L4 stages, mostly after the terminal division of vulval cells. During this period, each cell type exhibits a cell-type-specific pattern of gene expression, and several transcription factors are known that regulate the expression of these cell-type-specific genes. We bring together our current knowledge of this system to produce the framework in which to investigate the gene regulatory network controlling vulval organogenesis.

Materials and Methods

Determination of Gene Expression Patterns. Essentially all gene expression analyses described in this paper (including data from other papers) were carried out by using *gfp* reporter transgenes. For all results, it is possible that reporter expression does not accurately reflect the expression pattern of the endogenous gene. For simplicity, we refer to the reporter by the corresponding gene name.

The expression pattern of C55C3.5 was determined by using *gfp* reporter clone pUL#G221N (I. Hope, personal communication). This plasmid was injected into *unc-119*(*ed4*) animals by using the plasmid pDP#MM016B [*unc-119*(+)] as a coinjection marker (10). Of genes listed in Fig. 1*B* and in the main text, we have not examined the expression pattern of *syg-2*, *bam-2*, and *sqv-4*. Because GFP is likely to be stable for many hours, the time at which expression is turned off is not reliably indicated by decreased GFP expression. For most genes we analyzed, GFP fluorescence persists into the adult stage.

Genotypes. For Tables 1–3, *gfp* reporter transgenes used were *ayIs4[egl-17::gfp]*, *syIs50[cdh-3::gfp]*, *syIs49[zmp-1::gfp]*, and *syIs54[ceh-2::gfp]* (9). The *egl-26::gfp* transgenic line analyzed was *kuIs36* (11). Mutations used are; *cog-1*(*sy275*), *cog-1*(*sy607*), *lin-29*(*sy292*), *lin-11*(*n389*), and *egl-38*(*n578*). Of two *cog-1* transcripts, the longer *cog-1A* transcript contains a corepressor-binding domain, whereas the shorter *cog-1B* transcript does not (12). *sy275* is a missense mutation predicted to affect both transcripts. *sy607* is a deletion that eliminates the *cog-1A* transcript. The two alleles exhibit complementary defects in vulval development (13). Although both alleles are recessive, it is not known whether the loss of *cog-1* function causes observed phenotypes. *lin-29*(*sy292*) and *lin-11*(*n389*) are strong loss-of-function alleles, and *egl-38*(*n578*) is a reduction-of-function allele. Strains were constructed by using standard methods.

Results and Discussion

Vulval Cell-Type-Specific Gene Expression. A number of genes are expressed in specific subsets of vulval cells (Fig. 1*B*). Previously

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Abbreviations: VPC, vulval precursor cell; L*n* stage, larval *n* stage.

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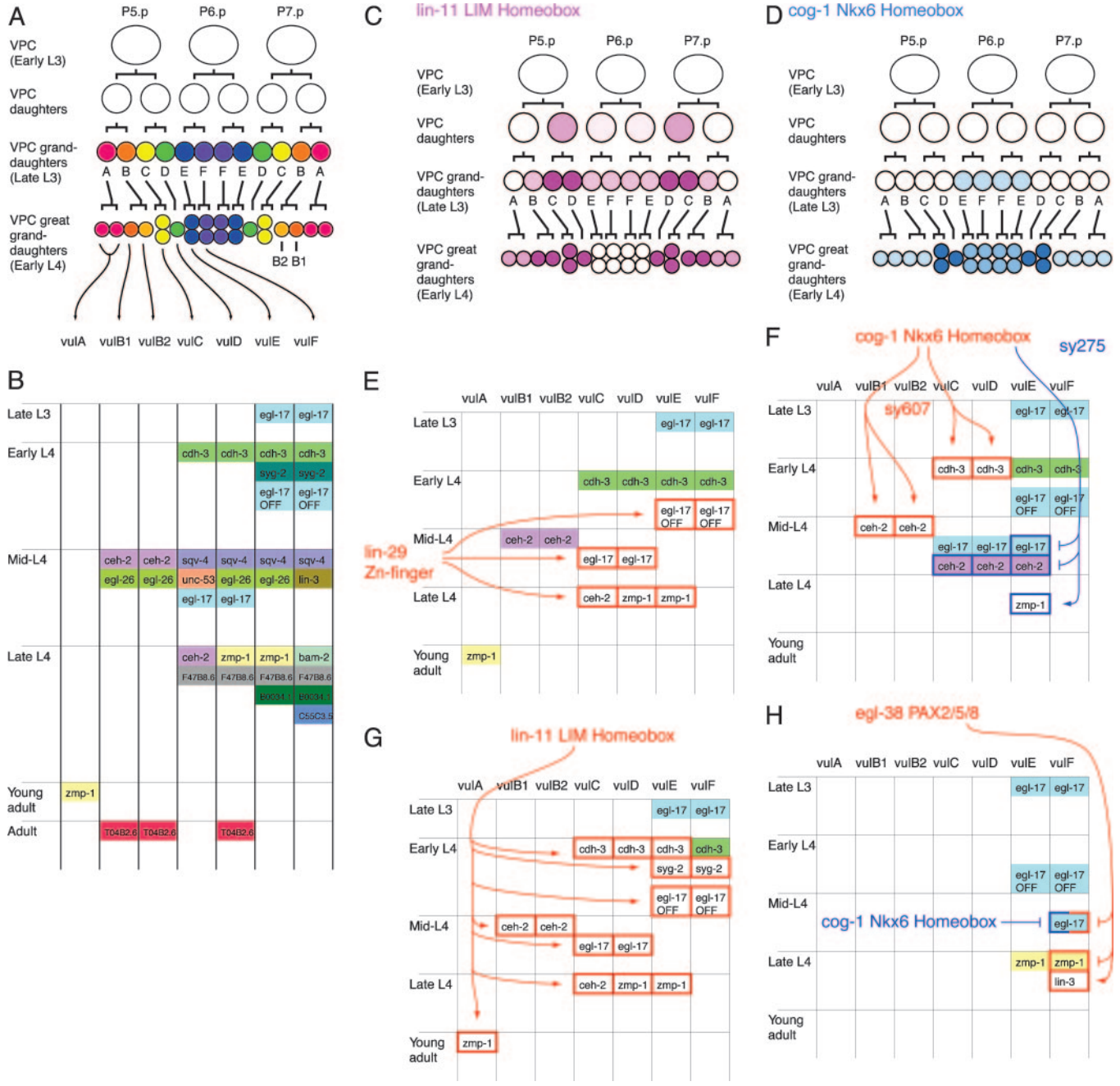

Fig. 1. The pattern of gene expression during late stages of vulval development. (*A*) An overview of vulval development. Lineal origins of 22 vulval nuclei are indicated. ''ABCDEFFEDCBA'' refer to vulval cell types vulA, vulB1, vulB2, vulC, vulD, vulE, and vulF. vulB is the only case in which a single VPC granddaughter gives rise to two cell types (8). Vulval cell nuclei at each stage are positioned as indicated (left, anterior; right, posterior). (*B*) A summary of cell-type specificity and timing of expression in the wild type (*Materials and Methods*) (9, 11, 13–21, 24–26). Boxes indicate stages at which gene expression is activated. The vertical order of events within each time block is arbitrary. For *egl-17*, vulE/vulF expression begins in P6.p (early L3) and persists in their descendants (vulE and vulF) until turned off in the early L4 stage. This inactivation, which is regulated by *lin-29* and *lin-11*, is indicated by the box marked ''*egl-17* OFF.'' *ceh-2* is expressed at a higher level in vulB1 compared with vulB2. (*C*) Expression pattern of *lin-11*. The diagrammed pattern is based on the *lin-11*::*gfp* transgene *syIs80* (26). (*D*) Expression pattern of *cog-1*. The pattern is based on the *cog-1*::*gfp* transgene *syIs63* (13). (*E*) The altered pattern of gene expression in *lin-29* mutants (9, 23) (Tables 1 and 2). White boxes with the red outlines indicate loss of expression and loss of *egl-17* down-regulation in the *lin-29* mutant. *lin-29* appears to regulate events that occur during the mid-L4 to the late L4 stage. (*F*) The altered pattern of gene expression in *cog-1* mutants (Table 3 and Fig. 2). Arrows are drawn with the assumption that both *sy607* and *sy275* phenotypes are caused by different reduction of function of the *cog-1* gene. Filled boxes with red or blue outline indicate ectopic expression. (*G*) Altered pattern of gene expression in *lin-11* mutants (26). (*H*) Altered pattern of gene expression in the *egl-38* mutant (16) (Tables 1 and 2). *egl-17* expression in vulF is observed in the *cog-1*(*sy275*); *egl-38* double mutant, suggesting a redundant repression mechanism.

described genes of this type include *cdh-3* (14), *egl-17* (15), *lin-3* (16), *zmp-1* (9, 17), *ceh-2* (9), T04B2.6 (9), F47B8.6 (9), B0034.1 (9), *unc-53* (18), *egl-26* (11), *sqv-4* (19), *bam-2* (20), and *syg-2* (21). *egl-26* was previously reported to express in vulE and vulB2 cells (11). We found that a nuclear-localized *egl-26*::*gfp* transcriptional fusion expressed in vulB1, vulB2, vulD, and vulE cells (*Materials and Methods*). The expression was somewhat variable and was observed starting from the mid-L4 stage and continuing

Table 1. Expression of *zmp-1* in vulE and vulF cells

Genotype	vulE	vulF	No. of animals
Wild type	+	−	80
lin-11	−	−	55*
lin-29	−	−	50†
cog-1 (sy275)	−	−	52
egl-38	+	+	48
lin-11; egl-38	−	−	52
lin-29; egl-38	−	−	56
cog-1; egl-38	−	−	56

*Ref. 26.
†Ref. 9.

into the adult stage. The C55C3.5 gene encoding a novel protein was previously found to express in vulval cells (I. Hope, personal communication). We found that *C55C3.5*::*gfp* was expressed in vulF cells, starting from the late-L4 and continuing into the adult stage.

Several conclusions can be drawn from Fig. 1*B*. First, all seven cell types exhibit distinct programs of gene expression, despite the fact that these cells are related by cell lineage and function. [vulB1 and vulB2 differ in the level of *ceh-2* expression but otherwise have similar expression profiles (9)]. Distinct expression profiles likely underlie distinct functions of vulval cell types. For example, *lin-3*, which encodes an EGF-related signaling protein, is expressed in vulF cells in the mid-L4 stage (16). This signal is required for a vulva-to-uterus signaling that induces a specific fate, uv1, in uterine cells adjacent to vulF.

The pattern of marker expression also reveals a strict temporal regulation of gene expression (Fig. 1*B*). For example, *cdh-3* is expressed in early L4, F47B8.6 is expressed in late L4, and T04B2.6 is expressed ≈1 day after the L4-to-adult molt (9). For *egl-17*, *ceh-2*, *zmp-1*, and *sqv-4*, the timing of gene expression is different for different vulval cells (9, 15, 19). For example, *egl-17* is expressed in vulE and vulF cells in the L3 stage and in vulC and vulD in the L4 stage.

Trans-Regulation of Vulva Gene Expression. The analysis of the regulatory network controlling the pattern of gene expression in the vulva has focused primarily on the effect of transcription factor mutations on gene expression reporter transgenes. In most cases, a direct transcriptional regulation of the target has not been demonstrated. Key results are summarized in Fig. 1 *E–H*. So far, important regulators are *lin-29* (encoding Zn-finger transcription factor; Fig. 1*E*) (9, 22, 23), *cog-1* (Nkx6 homeodomain; Fig. 1 *D* and *F*) (13), *lin-11* (LIM homeodomain; Fig. 1 *C* and *G*) (24–26), and *egl-38* (PAX 2/5/8; Fig. 1*H*) (16, 27).

Table 2. Expression of *egl-17* in vulE and vulF cells (L4)

Genotype	vulE	vulF	No. of animals
Wild type	−	−	59
cog-1 (sy275)	+++	−	46
egl-38	−	−	38
cog-1; egl-38	+++	+++	37
lin-11	—*	—*	45†
lin-29	—*	—*	43‡
lin-11; lin-29	—*	—*	38
lin-11; cog-1	—*	—*	43
lin-29; cog-1	—*	—*	40
lin-11; cog-1; egl-38	—*	—*	35
lin-29; cog-1; egl-38	—*	—*	36

*These cells express *egl-17*::*gfp* at a low level. We interpret these as the persistence of L3 expression.
†Ref. 26.
‡Ref. 23.

Table 3. Expression of *egl-17, ceh-2,* and *cdh-3* in *cog-1* mutants

Reporter	Mutations	vulA	vulB1 and vulB2	vulC	vulD	vulE	vulF
egl-17	+	0	0	100	100	0	0
egl-17	*cog-1 (sy275)*	0	0	100	92	92	0
egl-17	*cog-1 (sy607)*	0	0	93	100	0	0
ceh-2	+	0	100	0	0	0	0
ceh-2	*cog-1 (sy275)*	20	90	80	80	88	0
ceh-2	*cog-1 (sy607)*	0	0	0	0	0	0
cdh-3	+	0	0	100	100	100	100
cdh-3	*cog-1 (sy275)*	0	0	100	100	100	100
cdh-3	*cog-1 (sy607)*	0	0	14	14	71	94

Percentages of cells in mid-L4 animals that expressed *egl-17*::*gfp*, *ceh-2*::*gfp* and *cdh-3*::*gfp*. See Table 4, which is published as supporting information on the PNAS web site, for number of cells scored.

A Temporal Regulator of Gene Expression. *lin-29* is required for the expression of *egl-17* in vulC and vulD (23), *ceh-2* in vulC (9), and *zmp-1* in vulD and vulE (Fig. 1*E*, Tables 1 and 2, and Fig. 5, which is published as supporting information on the PNAS web site) (9). By contrast, *lin-29* in not required for the expression of *cdh-3* in vulC, vulD, vulE, vulF (9), *ceh-2* in vulB (9), *egl-17* in vulE and vulF (23), and *zmp-1* in vulA (9). Moreover, the expression of *egl-17* in vulE and vulF is observed during the L4 stage (23), suggesting that the mechanism that turns off *egl-17* expression in these cells is compromised (Fig. 5). These *lin-29* phenotypes are not easily explained by cell fate changes between vulval cell types but suggest a temporal regulatory defect: *lin-29* mutations cause loss of events associated with the mid-to-late L4 time points. This interpretation of these data is particularly attractive, because *lin-29* mutations are known to cause heterochronic defects in other tissues, specifically in the L4-to-adult transition in the lateral hypodermis (22, 28, 29). *lin-29* is expressed in all vulval cells, starting in the mid-L3 stage and continuing through the L4 stage (30).

Cell-Type-Specific Regulators of Gene Expression. We analyzed the effect of two *cog-1* (Nkx6.1/6.2 homeodomain) mutations on the expression of vulval-cell-specific gene expression reporters (Fig. 1*F*, Table 3, and *Materials and Methods*). *cog-1*(*sy275*) is a missense mutation in the homeodomain, and *cog-1*(*sy607*) is a small deletion that eliminates one of two *cog-1* transcripts (13). We found that in the mid-L4 stage, *cog-1*(*sy275*) caused ectopic expression of *egl-17* in vulE cells (Fig. 2) and ectopic expression of *ceh-2* in vulC, vulD, and vulE cells and loss of *zmp-1* expression in vulE cells. In contrast, *cog-1*(*sy607*) caused loss of *cdh-3* expression in vulC, vulD, and vulE cells and loss of *ceh-2* expression in vulB. These results indicate that *egl-17*, *cdh-3*, *ceh-2*, and *zmp-1* are regulated by the *cog-1* gene. Although some *cog-1* expression is observed in all vulval cells, *gfp* reporters suggest that *cog-1* is most strongly expressed in vulC and vulD and weakly in vulE and vulF, implying a cell-type-specific function (13) (Fig. 1*D*).

A somewhat similar situation is presented with *lin-11* (LIM-homeodomain) (Fig. 1 *C* and *G*). During the L4 stage, *lin-11* is expressed strongly in vulB, vulC, and vulD and weakly in other vulval cells, suggesting that *lin-11* is involved in the specification of these cell types (24, 26). However, unexpectedly, *lin-11* is cell-autonomously required for expression of most vulval genes tested, including in cells where the *lin-11* level is low (26).

egl-38 is a PAX2/5/8 transcription factor required for expres-

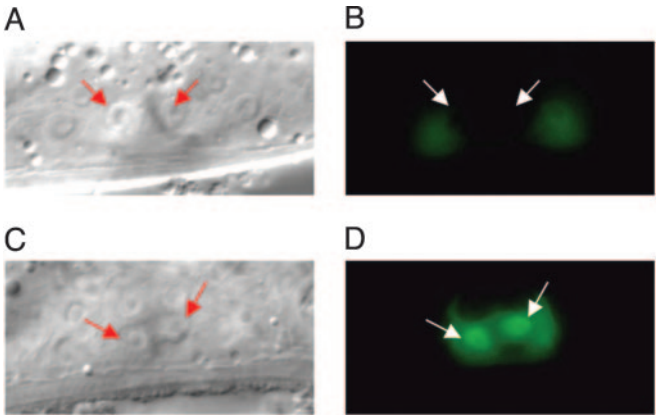

Fig. 2. Regulation of *egl-17* by *cog-1*. (*A* and *B*) Nomarski and epifluorescence images of wild-type mid-L4 animal carrying the *egl-17*::*gfp* transgene. Arrows point to vulE nuclei. vulE cells are not fluorescent. (*C* and *D*) *cog-1*(*sy275*) animals at the same stage carrying the *egl-17*::*gfp* transgene. vulE cells are fluorescent.

sion of the *lin-3* gene in vulF cells (16, 27). We found that *egl-38* represses expression of *zmp-1* in vulF cells, indicated by ectopic *zmp-1* expression in *egl-38* mutants (Fig. 1*G* and Table 2). In addition, in an *egl-38*; *cog-1* double mutant, *egl-17* is expressed in both vulE and vulF cells. Thus, *egl-38* is also capable of repressing *egl-17* expression in vulF cells, although in the wild type, this function is redundant with the *cog-1*-dependent mechanism that restricts *egl-17* expression to vulC and vulD. *egl-38* is currently the best example of cell-type-specific factors, promoting expression of some genes (*lin-3*) and repressing expression of others (*zmp-1*, *egl-17*) in a single cell type, vulF.

Regulators of the Transcription Factor Network. The transcription factor network that regulates gene expression in individual cell types must be regulated by the cell-fate-patterning mechanism that specifies each cell to a specific fate and does so in a spatially precise pattern. In the vulva, the cell types occur in a specific ABCD-EFFE-DCBA pattern (Fig. 1*A*). Although the full mechanism that establishes this pattern is not known, Wnt signals, mediated by *lin-17* (Frizzled-type Wnt receptor) and *lin-18* (Ryk-type Wnt receptor), control the anterior/posterior order of cell types among P7.p descendants (31, 32) (Fig. 3). Analysis of *cog-1* (31) and *lin-11* (25) expression in *lin-17* and *lin-18* mutants indicates that Wnt signaling establishes the correct spatial pattern of transcription factor expression. As described above (Tables 1 and 3 and Fig. 1 *F* and *G*) (26), *cog-1* and *lin-11*, in turn, control the expression pattern of *egl-17* and *cdh-3*. Patterns of *egl-17* and *cdh-3* expression observed in *lin-17* and *lin-18* mutants are consistent with high levels of *cog-1* and *lin-11* turning on the expression of these genes (31, 32). Another set of cell-fate-patterning mechanisms controlling gene expression was revealed by the analysis of vulE vs. vulF fate specification using the *zmp-1* reporter. A dominant-negative Ras or the ablation of the anchor cell disrupts the pattern of *zmp-1* expression in presumptive vulE and vulF cells, indicating that a Ras-mediated signal, probably from the anchor cell, establishes the spatial pattern of cell fates (17).

These results confirm that cell–cell communication is important in patterning cell fates, and that signaling pathways operate through the transcription factor network to control the pattern of gene expression. Expression patterns of various genes (Fig. 1 *B–D*) suggest that transcription factors are expressed in all vulval cells at different levels, whereas genes regulated by them have relatively simple on/off patterns of expression. This difference suggests that the spatial pattern becomes progressively more refined as the information is passed through the regulatory network. This progressive refinement of pattern is likely a consequence of integration of information from multiple regulatory mechanisms, such as intercellular communication and feedback regulation. Many of these disparate data inputs are likely processed at the level of cis-regulatory modules. Thus, the

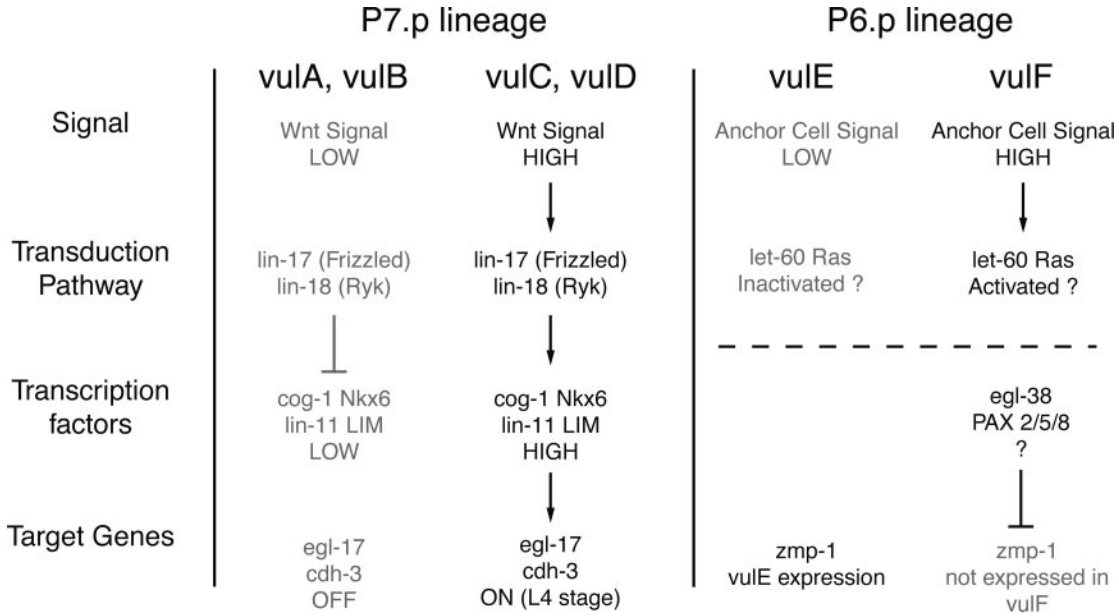

Fig. 3. Link between cell fate patterning mechanisms and gene expression. In general, inductive signals regulate transcription factor networks to regulate gene expression. In the P7.p (but not P5.p) lineage, Wnt signals transduced by *lin-17* and *lin-18* control the pattern of *cog-1* and *lin-11* expression (25, 31). *cog-1* and *lin-11* in turn regulate *egl-17* and *cdh-3* expression (Table 3) (26). It has not been determined whether *cog-1* and *lin-11* regulate each other. In the P6.p lineage, an anchor cell signal and a *let-60* Ras signal transduction pathway are required to establish the correct pattern of *zmp-1* expression pattern (17). *zmp-1* expression is also repressed in vulF by *egl-38* PAX2/5/8 (Table 1). It is not known whether the patterning mechanism acts through *egl-38*. The expression pattern of *egl-38* is also not known.

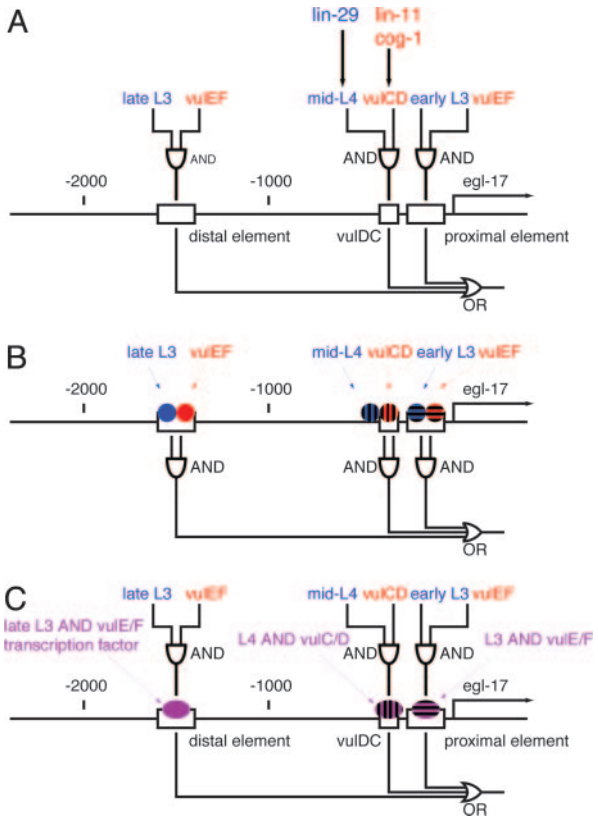

Fig. 4. cis-regulatory elements of *egl-17*. (*A*) A map of the *egl-17* 5′ regulatory region. Boxes indicate enhancer elements defined by Cui and Han (34) and Kirouac and Sternberg (33). ''AND'' and ''OR'' logic gate symbols indicate sites and logic of information integration. Temporal (blue) and spatial (red) information is integrated as indicated by the logic circuit diagram to produce the complete *egl-17* expression pattern. In one model (*B*), spatially and temporally regulated transcription factors each bind directly to the *egl-17* cis-regulatory region. The integration of information takes place on enhancer elements. In the alternative model (*C*), spatial and temporal cues are integrated at the transcription factor level. These transcription factors (purple) with both spatially and temporally restricted activity regulate each enhancer element.

spatial pattern of transcription factor effects becomes more restricted than the spatial pattern of transcription factor expression. This hypothesis is consistent with the observation that cells affected by *lin-11* and *cog-1* mutations do not correspond directly to cells that express high levels of *lin-11* and *cog-1*.

cis-Regulation of Vulva Gene Expression. cis-regulatory elements (e.g., enhancers) have been analyzed in detail for several genes expressed in the vulva, most notably *egl-17*, *cdh-3*, and *zmp-1*, using transgenic assays (33, 34). A comparative genomics analysis of the regulatory region of orthologs from *C. elegans* and *Caenorhabditis briggsae* has also proved useful.

Here, we focus on the analysis of the *egl-17* gene. As shown in Fig. 1*B*, this gene is expressed in vulE and vulF cells during the L3 stage and in vulC and vulD cells during the L4 stage. Dissection of the 5′ regulatory region revealed that there are three separable enhancer elements, two driving expression in vulE and vulF and one driving expression in vulC and vulD (33, 34) (Fig. 4*A*). Notably, each of these elements drives expression at different times. The distal vulE/vulF element drives expression in the mid-L3 to early-L4 and the proximal vulE/vulF element drives expression in the early to mid-L3 stage (34). The vulDC element drives expression in the mid-L4 stage. Thus, the expression of *egl-17* is produced by the composite activity of three discrete enhancers, each of which drives both spatially and temporally restricted pattern of expression. We propose two models for how the information that operates on these enhancers is integrated. In one model (Fig. 4*B*), temporal (blue) and spatial (red) regulators both bind directly to the *egl-17* promoter, and information integration is achieved directly on the cis-regulatory element. Alternatively, transcription factors that bind to each of these promoters may already combine temporal and spatial information (Fig. 4*C*). Our results indicate that the vulDC element regulating mid-L4 expression is likely regulated by *lin-29*, *lin-11*, *cog-1*, and *egl-38*. Additional experiments are necessary to determine the molecular mechanism of information integration.

Conclusion

The late vulval development of *C. elegans* offers an excellent system in which to investigate cell fate determination and regulation of cell-type-specific gene expression. In particular, this system combines single-cell resolution with a high degree of temporal resolution in an easily manipulated model organism. In many respects, vulval development is reminiscent of other systems in that transcription factors are expressed in overlapping domains, and the identity of each domain is established combinatorially by the presence or absence of specific subsets of these transcription factors. One interesting example with possible parallels to the vulva is the fate-specification mechanism in the vertebrate ventral neural tube (35). In this system, Nkx6.1 and Nkx6.2 homeodomain proteins (homologs of *cog-1*) interact with transcription factors Dbx1 and Dbx2 in a mutually repressive network, and different activities of repressor proteins help establish the spatial pattern of cell fates (36, 37). It is possible that *C. elegans cog-1* functions in a similar manner in the vulva.

Analysis of vulval development also highlights several features that are not necessarily evident in other systems. First, analysis of vulval development has revealed a highly complex pattern of temporal regulation, which is undoubtedly a feature of most organogenetic processes (for example, see refs. 38 and 39). The involvement of *lin-29*, a known regulator of stage-specific development in *C. elegans*, suggests that the global mechanism of temporal regulation feeds into the development of this particular organ. Additional mechanisms probably exist that control expression at other time points. Whether these other time points are regulated by a global mechanism or in an organ-autonomous manner is not yet clear.

One concept that has been invoked in analyses of cell or organ fate specification is that of ground state and selector genes. For example, in *Drosophila* appendage development, it has been proposed that a default "ground state" exists and is modified by "selector" genes to produce an antenna or a leg (40). The concept can be applied to the level of individual cell types as well (for example, ref. 41). From this point of view, the cell-type-specific transcription factors *cog-1*, *lin-11*, and *egl-38* can be thought of as selector genes for subsets of vulval cell types. What is the ground state of vulval cells in the absence of selector genes? A cell in such a state presumably will not express the cell-type-specific genes described in Fig. 1 but will retain the epithelial identity common to all vulval cells. It is unclear whether such a state has been observed in any of the mutants. Vulval cells in *lin-11* mutants lack most cell-type-specific expression but retain the ability to undergo some morphogenetic movements characteristic of vulval cells and thus may most closely resemble the ground state.

In other systems, analyses of coregulated genes have successfully identified "gene batteries" (42), sets of genes with common cis-regulatory elements that are coexpressed (for example, ref. 43). However, our understanding of vulval development is still limited, relative to the number of cell types

and the number of distinct stages that require different gene expression patterns. Consequently, within the relatively small number of functionally unrelated genes analyzed so far, genes are more likely to be regulated by distinct mechanisms. Thus, although gene batteries with multiple genes probably exist in this system, their analysis requires knowledge of more genes and a detailed understanding of which transcription factors regulate their expression.

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1. Wood, W. B. (1988) *The Nematode Caenorhabditis elegans* (Cold Spring Harbor Lab. Press, Plainview, NY).
2. Kimble, J. (1981) *Dev. Biol.* **87,** 286–300.
3. Sternberg, P. W. & Horvitz, H. R. (1986) *Cell* **44,** 761–772.
4. Sternberg, P. W. (1988) *Nature* **335,** 551–554.
5. Yochem, J., Weston, K. & Greenwald, I. (1988) *Nature* **335,** 547–550.
6. Hill, R. & Sternberg, P. (1992) *Nature* **358,** 470–476.
7. Sulston, J. & Horvitz, H. R. (1977) *Dev. Biol.* **56,** 110–156.
8. Sharma-Kishore, R., White, J., Southgate, E. & Podbilewicz, B. (1999) *Development (Cambridge, U.K.)* **126,** 691–699.
9. Inoue, T., Sherwood, D., Aspöck, G., Butler, J., Gupta, B. P., Kirouac, M., Wang, M., Lee, P.-Y., Kramer, J. M., Bürglin, T., *et al*. (2002) *Mech. Dev.* **119S,** S203–S209.
10. Maduro, M. & Pilgrim, D. (1995) *Genetics* **141,** 977–988.
11. Hanna-Rose, W. & Han, M. (2002) *Dev. Biol.* **241,** 247–258.
12. Chang, S., Johnston, R. J., Jr. & Hobert, O. (2003) *Genes Dev.* **17,** 2123–2137.
13. Palmer, R. E., Inoue, T., Sherwood, D. R., Jiang, L. I. & Sternberg, P. W. (2002) *Dev. Biol.* **252,** 202–213.
14. Pettitt, J., Wood, W. & Plasterk, R. (1996) *Development (Cambridge, U.K.)* **122,** 4149–4157.
15. Burdine, R. D., Branda, C. S. & Stern, M. J. (1998) *Development (Cambridge, U.K.)* **125,** 1083–1093.
16. Chang, C., Newman, A. P. & Sternberg, P. W. (1999) *Curr. Biol.* **9,** 237–246.
17. Wang, M. & Sternberg, P. W. (2000) *Development (Cambridge, U.K.)* **127,** 5047–5058.
18. Stringham, E., Pujol, N., Vandekerckhove, J. & Bogaert, T. (2002) *Development (Cambridge, U.K.)* **129,** 3367.
19. Hwang, H. Y. & Horvitz, H. R. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 14224–14229.
20. Colavita, A. & Tessier-Lavigne, M. (2003) *Science* **302,** 293–296.
21. Shen, K., Fetter, R. D. & Bargmann, C. I. (2004) *Cell* **116,** 869–881.
22. Rougvie, A. E. & Ambros, V. (1995) *Development (Cambridge, U.K.)* **121,** 2491–2500.
23. Newman, A. P., Inoue, T., Wang, M. & Sternberg, P. W. (2000) *Curr. Biol.* **10,** 1479–1488.
24. Freyd, G., Kim, S. & Horvitz, H. R. (1990) *Nature* **344,** 876–879.
25. Gupta, B. P. & Sternberg, P. W. (2002) *Dev. Biol.* **247,** 102–115.
26. Gupta, B. P., Wang, M. & Sternberg, P. W. (2003) *Development (Cambridge, U.K.)* **130,** 2589–2601.
27. Chamberlin, H. M., Palmer, R. E., Newman, A. P., Sternberg, P. W., Baillie, D. L. & Thomas, J. H. (1997) *Development (Cambridge, U.K.)* **124,** 3919–3928.
28. Ambros, V. & Horvitz, H. R. (1984) *Science* **226,** 409–416.
29. Ambros, V. (1989) *Cell* **57,** 49–57.
30. Bettinger, J. C., Euling, S. & Rougvie, A. E. (1997) *Development (Cambridge, U.K.)* **124,** 4333–4342.
31. Inoue, T., Oz, H. S., Wiland, D., Gharib, S., Deshpande, R., Hill, R. J., Katz, W. S. & Sternberg, P. W. (2004) *Cell* **118,** 795–806.
32. Deshpande, R., Inoue, T., Priess, J. R. & Hill, R. J. (2005) *Dev. Biol.* **278,** 118–129.
33. Kirouac, M. & Sternberg, P. W. (2003) *Dev. Biol.* **257,** 85–103.
34. Cui, M. & Han, M. (2003) *Dev. Biol.* **257,** 104–116.
35. Briscoe, J., Pierani, A., Jessell, T. M. & Ericson, J. (2000) *Cell* **101,** 435–445.
36. Muhr, J., Andersson, E., Persson, M., Jessell, T. M. & Ericson, J. (2001) *Cell* **104,** 861–873.
37. Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T. M. & Ericson, J. (2001) *Neuron* **31,** 743–755.
38. Aurelio, O., Boulin, T. & Hobert, O. (2003) *Development (Cambridge, U.K.)* **130,** 599–610.
39. Bateman, J. M. & McNeill, H. (2004) *Cell* **119,** 87–96.
40. Casares, F. & Mann, R. S. (2001) *Science* **293,** 1477–1480.
41. Lints, R., Jia, L., Kim, K., Li, C. & Emmons, S. W. (2004) *Dev. Biol.* **269,** 137–151.
42. Davidson, E. H. (2001) *Genomic Regulatory Systems: Development and Evolution* (Academic, San Diego).
43. Wenick, A. S. & Hobert, O. (2004) *Dev. Cell* **6,** 757–770.

CHAPTER II:

The *tailless* Ortholog *nhr-67* Regulates Patterning of Gene Expression and Morphogenesis in the *C. elegans* Vulva

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The *tailless* Ortholog *nhr-67* Regulates Patterning of Gene Expression and Morphogenesis in the *C. elegans* Vulva

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Regulation of spatio-temporal gene expression in diverse cell and tissue types is a critical aspect of development. Progression through *Caenorhabditis elegans* vulval development leads to the generation of seven distinct vulval cell types (vulA, vulB1, vulB2, vulC, vulD, vulE, and vulF), each with its own unique gene expression profile. The mechanisms that establish the precise spatial patterning of these mature cell types are largely unknown. Dissection of the gene regulatory networks involved in vulval patterning and differentiation would help us understand how cells generate a spatially defined pattern of cell fates during organogenesis. We disrupted the activity of 508 transcription factors via RNAi and assayed the expression of *ceh-2,* a marker for vulB fate during the L4 stage. From this screen, we identified the *tailless* ortholog *nhr-67* as a novel regulator of gene expression in multiple vulval cell types. We find that one way in which *nhr-67* maintains cell identity is by restricting inappropriate cell fusion events in specific vulval cells, namely vulE and vulF. *nhr-67* exhibits a dynamic expression pattern in the vulval cells and interacts with three other transcriptional regulators *cog-1* (Nkx6.1/6.2), *lin-11* (LIM), and *egl-38* (Pax2/5/8) to generate the composite expression patterns of their downstream targets. We provide evidence that *egl-38* regulates gene expression in vulB1, vulC, vulD, vulE, as well as vulF cells. We demonstrate that the pairwise interactions between these regulatory genes are complex and vary among the seven cell types. We also discovered a striking regulatory circuit that affects a subset of the vulval lineages: *cog-1* and *nhr-67* inhibit both one another and themselves. We postulate that the differential levels and combinatorial patterns of *lin-11, cog-1,* and *nhr-67* expression are a part of a regulatory code for the mature vulval cell types.

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Introduction

Complex gene regulatory networks operating in diverse cell types and tissues are crucial for development. Diverse intercellular signals and transcription factor networks control gene expression within individual cell types, acting on *cis*-regulatory modules of target genes [1]. Understanding such regulation first requires documenting all the regulatory inputs and outputs from each gene [2]. This information allows circuit diagrams to be constructed that provide a global perspective on how diverse cell types acquire their identity. Gene regulatory networks have been well studied in a wide range of biological model systems such as endomesoderm specification in the sea urchin embryo [3], dorso-ventral patterning in the *Drosophila* embryo [4], and mesoderm specification in *Xenopus* [5]. The common themes that might emerge from these studies would advance our understanding of organogenesis in vertebrates.

The *Caenorhabditis elegans* vulva is postembryonically derived from six vulval precursor cells P3.p–P8.p. The central three vulval precursor cells P5.p–P7.p are induced to adopt 1° (primary) and 2° (secondary) vulval fates via epidermal growth factor (EGF) and Notch signaling, whereas the remaining precursors fuse with the hypodermal syncytium hyp7 [6]. The vulva is composed of seven distinct cell types, each with its own set of expressed genes and morphogenetic migrations [7–9]. The P6.p 1° lineages generate the vulE and vulF cells, while the P5.p and P7.p 2° lineages generate the vulA, vulB1, vulB2, vulC, and vulD cells. The signals that induce 1° versus 2° fates in the primordial vulval precursor cells are known. However, the processes that govern patterning and differentiation of the mature vulval cell types are largely unknown [6]. Both Ras and Wnt pathways are required for the precise spatial patterning of the 1° vulE and vulF cells [10], and both Wnt/Ryk and Wnt/Frizzled signaling pathways are necessary for patterning the P7.p 2° vulA–vulD cells [11–13].

Genes expressed in the mature vulval cell types include some with known functions and many others without known physiological roles. *lin-3* (EGF) is expressed in vulF and is required to signal from vulF to uterine uv1 cells [14,15]. *egl-17* encodes a fibroblast growth factor (FGF)-like protein that is

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Abbreviations: AC, anchor cell; EGF, epidermal growth factor; Egl, egg laying; FGF, fibroblast growth factor; GFP, green fluorescent protein; Pvl, protruding vulva; RNAi, RNA interference

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Author Summary

During development, in which the single-celled egg generates a whole organism, cells become different from each other and form patterns of types of cells. It is these spatially defined fate patterns that underlie the formation of complex organs. Regulatory molecules called transcription factors influence the fate patterns that cells adopt. Understanding the role of these transcription factors and their interactions with other genes could tell us how cells establish a certain pattern of cell fates. This study focuses on studying how the seven cell types of the *Caenorhabditis elegans* vulva arise. This organ is one of the most intensively studied, and while the signaling network that initiates vulval development and sets the gross pattern of cell differentiation is well understood, the network of transcription factors that specifies the final cell fates is not understood. Here, we identify *nhr-67,* a new transcription factor that regulates patterning of cell fates in this organ. Transcription factors do not necessarily act alone, and we explore how NHR-67 works with three other regulatory factors (each with human homologs) to specify the different properties of the vulval cells. We also demonstrate that the interconnections of these transcription factors differ between these seven diverse cell types, which may partially account for how these cells acquire a certain pattern of cell fates.

required for migration of the sex myoblasts to their precise final positions [16,17]. *egl-17* is initially expressed in the 1° vulval lineages and is shut off during the L4 stage. Expression in vulC and vulD is observed during early L4 and persists throughout adulthood. The vulval expression correlates with the sites of muscle attachment. *egl-26* encodes a novel protein that contains an H box/NC domain and is expressed in vulB1, vulB2, vulD, and vulE cells [18,19]. *zmp-1* encodes a zinc metalloprotease and is expressed in vulD and vulE during the L4 stage and in vulA in adults [9]. *ceh-2* encodes a homeodomain protein that is related to *Drosophila empty spiracles* and is expressed in vulB1 and vulB2 cells during the L4 stage and in vulC upon entry into L4 lethargus [9,20]. *pax-2* is a recent gene duplication of the PAX2/5/8 protein EGL-38 [21] and is expressed exclusively in the vulD cells. *zmp-1, ceh-2, egl-26,* and *pax-2* have no known function in the vulva.

Transcription factor networks in individual vulval cell types somehow generate a spatially precise pattern of cell fates [19]. Several transcription factors that regulate gene expression in the diverse vulval cell types have already been described [19,22–24]. *lin-11,* a LIM homeobox transcription factor, regulates gene expression in all seven vulval cell types [25,26]. The Nkx6.1/Nkx6.2 homeodomain gene, *cog-1,* regulates gene expression in vulB, vulC, vulD, vulE, and vulF cells [19,27]. In contrast, *egl-38* encodes a PAX2/5/8 protein that appears to be the only known example of a vulval cell type–specific regulatory factor; it promotes expression of certain target genes and restricts expression of other targets exclusively in vulF cells [14,19,28]. Additional regulatory factors need to be identified to elucidate the precise spatial patterning of the mature vulval cell types.

Here, we identify *nhr-67* as a component of the gene regulatory networks underlying vulval patterning and differentiation. *nhr-67* is required for the accurate patterning of gene expression and regulation of cell fusion in several vulval cell types and is dynamically expressed in the vulva. *nhr-67* interacts genetically with *cog-1, egl-38,* and *lin-11* to produce the complex expression patterns of their downstream targets. We demonstrate that the pairwise interactions between these four regulatory genes vary among the diverse vulval cell types. These results indicate that *nhr-67, cog-1, lin-11,* and *egl-38* form a part of a genetic network that generates different patterns of gene expression in each of the seven cell types.

Results

nhr-67 Regulates Gene Expression in Multiple Vulval Cell Types

An RNA interference (RNAi) screen of 508 known and putative transcription factors encoded in the *C. elegans* genome (see Table S1) was conducted in a *ceh-2::YFP* reporter background. At the time we performed the screen, this was the best available set. *ceh-2* encodes a homeodomain protein orthologous to *Drosophila* Empty Spiracles (EMS) and vertebrate EMX1 and EMX2 and serves as a readout for vulB fate during the L4 stage [20]. Modifiers of *ceh-2* expression are good candidates for genes involved in patterning and/or differentiation of 2° vulval descendents. From this screen, we identified *nhr-67* as a gene necessary for negative regulation of *ceh-2* expression in the 1° vulE and vulF cells (Figure 1A–1B). Reciprocal BLAST searches indicate that *nhr-67* encodes an ortholog of the *tailless* hormone receptor, which consists of an N-terminal transactivation domain, a centrally positioned DNA-binding domain, and a C-terminal ligand-binding domain. The only other positive was the GATA-type transcription factor *egl-18,* which was previously shown to be involved in vulval development [29–31]. Other genes that should have been positive in the screen *(lin-11* and *cog-1)* were not isolated from the RNAi screen, thus indicating a high false-negative rate. Analysis of the *nhr-67* deletion allele *ok631* revealed severe defects in early larval development (L1 lethality and/or arrest). In order to bypass this early larval arrest phenotype, we resorted to feeding young L1 larvae with *nhr-67* RNAi and assayed for defects in vulval gene expression. *nhr-67* was also found to be required for negative regulation of two additional L4-specific markers: *egl-26* (wild-type expression in vulB, vulD, and vulE cells) (Figure 1C–1D) and *egl-17* (wild-type expression in vulC and vulD cells) in the vulF cells. Thus, *nhr-67* activity is necessary for the negative regulation of expression of several 2° lineage-specific genes in the 1°-derived vulval cells during the L4 stage. Consistent with previous reports, *nhr-67* RNAi results in a highly penetrant protruding vulva (Pvl) and egg-laying (Egl) defective phenotype [32] (Figure S1). However, other transcription factors exhibiting a Pvl RNAi phenotype, such as *fos-1, egl-43,* and *unc-62,* have normal vulval gene expression (unpublished data).

In addition to its negative regulatory role, we also found that *nhr-67* is necessary for promoting expression of specific genes. For example, *nhr-67* is necessary for *zmp-1* expression in vulA during the adult stage (Figure 1E–1F). *nhr-67* is also required for vulD-specific expression of *pax-2* and *egl-17* during the L4 stage (Figure 1G–1J). These examples show that *nhr-67* positively regulates gene expression in the secondary vulA and vulD cells. *nhr-67* is also required for positively regulating gene expression in the 1° vulval cells, namely vulF-specific expression of *lin-3,* an EGF-like protein (Figure 1K and 1L). Therefore, *nhr-67* regulates gene expression in at least four of the seven vulval cell types.

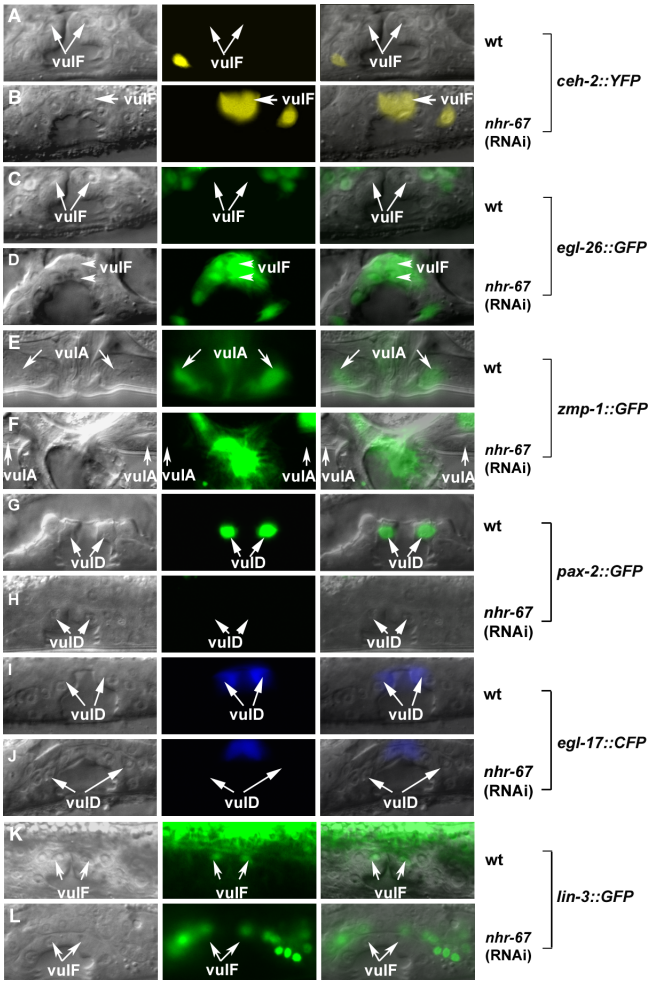

Figure 1. *nhr-67* Is Required for Proper Gene Expression in Multiple Vulval Cell Types

Lateral images of the developing vulva during the L4 (A–D and G–L) and the adult stage (E and F). (A–L) Nomarski (left), fluorescence (center), and overlaid (right). Expression of vulval cell fate markers in wild-type (A, C, E, G, I, and K) and *nhr-67* RNAi–treated animals (B, D, F, H, J, and L). In *nhr-67* RNAi–treated animals, the vulval morphology is abnormal compared to wild-type; namely, the migration of vulF cells is defective. (A) In wild-type animals, *syIs55 [ceh-2::YFP]* expression is off in the 1° vulF cells (arrows).
(B) *syIs55* animals treated with *nhr-67* RNAi show ectopic *ceh-2* expression in the 1° vulF cells (arrow).
(C) *kuIs36 [egl-26::GFP]* expression is completely absent in the vulF cells (arrows).
(D) *nhr-67* RNAi results in misexpression of *egl-26* in the vulF lineages (arrowheads).
(E) Wild-type *zmp-1::GFP (syIs49)* expression is observed in the vulA cells (arrows).
(F) In contrast, *nhr-67* RNAi abolishes the vulA-specific expression (arrows) of *zmp-1*.
(G) *guEx64 [pax-2::GFP]* is expressed exclusively in vulD cells (arrows) in wild-type animals.
(H) *pax-2* expression in vulD is abolished in an *nhr-67* (RNAi) background (arrows).
(I) Wild-type *egl-17::GFP (syIs59)* expression is observed in the vulD cells (arrows).
(J) *nhr-67* RNAi results in the loss of *egl-17* expression in the vulD cells (arrows).
(K) In wild-type animals, *lin-3::GFP (syIs107)* is expressed solely in vulF cells (arrows).
(L) *lin-3* expression in vulF cells is eliminated when treated with *nhr-67* RNAi (arrows).
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In the L3 stage, the early 1° and 2° vulval cell fates can be distinguished by the patterns of cell division of their descendents. The 1° fated cell typically gives rise to four granddaughters that divide transversely (left-right axes); whereas a subset of the granddaughters derived from a 2° cell divide longitudinally (anterior-posterior axes). To determine if *nhr-67*-dependent alterations in gene expression are a consequence of fate transformations in the early 1° and 2° vulval lineages, we monitored the pattern of the vulval cell divisions in an *nhr-67* RNAi background. In the absence of *nhr-67,* the vulval cell lineages appear wild-type in terms of both cell number and orientation of cell division (unpublished data). Thus, the perturbations in gene expression caused by reduced *nhr-67* function are not the result of gross abnormalities in the early vulval cell lineages.

nhr-67 Prevents Inappropriate Fusion Events between the 1° Vulval Cells

During the L4 stage, the seven vulval cell types invaginate cooperatively to assume a characteristic morphology. The similar cell types subsequently fuse, generating toroid rings that line the vulval cavity [8]. We wanted to ascertain if the observed cell fate transformations in *nhr-67*(RNAi) animals were possibly due to improper fusion events between the wrong cell types. Cell fusion defects can be assayed using *ajm-1::GFP* (an adherens junction marker) to visualize the cell number and architecture of the vulval toroids. When observing the mid-sagittal plane of wild-type animals, *ajm-1::GFP* appears as dots between cells. The eight dots on either side correspond to the seven distinct vulval cell types (Figure 2A). Most *nhr-67* RNAi–treated animals do not exhibit dramatic defects in cell fusion (Figure 2B). The 2° vulval lineage–derived cells (vulA, vulB1, vulB2, vulC, and vulD) consistently generate mature toroids. However, inappropriate fusion often occurs (65%, $n = 17$) between the presumptive vulE and vulF cells (indicated by the missing dots at the top of the vulval invagination) (Figure 2C). Since *nhr-67* regulates gene expression in vulval cells other than vulE and vulF, improper cell fusion events cannot fully account for all its altered gene expression patterns.

We then wanted to determine if the altered gene expression occurring in the 1° vulval cells was dependent on these improper fusion events. We attempted to address this question using two approaches: (a) by analyzing the effect of *nhr-67* RNAi on the expression of *egl-17* and *ceh-2* transgenes in an *eff-1(hy21)* background, and (b) by monitoring the vulval expression levels of *eff-1* in animals with reduced *nhr-67* activity. *eff-1* is a type I membrane protein necessary for cell fusion [33]. Disruption of *nhr-67* function in an *eff-1*-deficient background is still sufficient to cause upregulation of both *egl-17* (Figure 2D and 2E) and *ceh-2* (Figure 2F and 2G) in the 1° vulval cells. Thus, the *nhr-67*-dependent alterations in gene expression are not dependent on *eff-1*-mediated cell fusion. We also observed that *eff-1* levels (strong expression in vulA and vulC cells, weak expression in vulF cells) are highly elevated in vulD and vulF cells when *nhr-67* gene activity is compromised (Figure 2H and 2I). However, we also note that *eff-1* is not sufficient to rescue the vulE-vulF fusion defects observed in *nhr-67* (RNAi) background (unpublished data). One possibility is that *eff-1(hy27)* is a temperature-sensitive allele that fails to completely eliminate cell fusion. Another possibility is that in addition to *eff-1, nhr-*

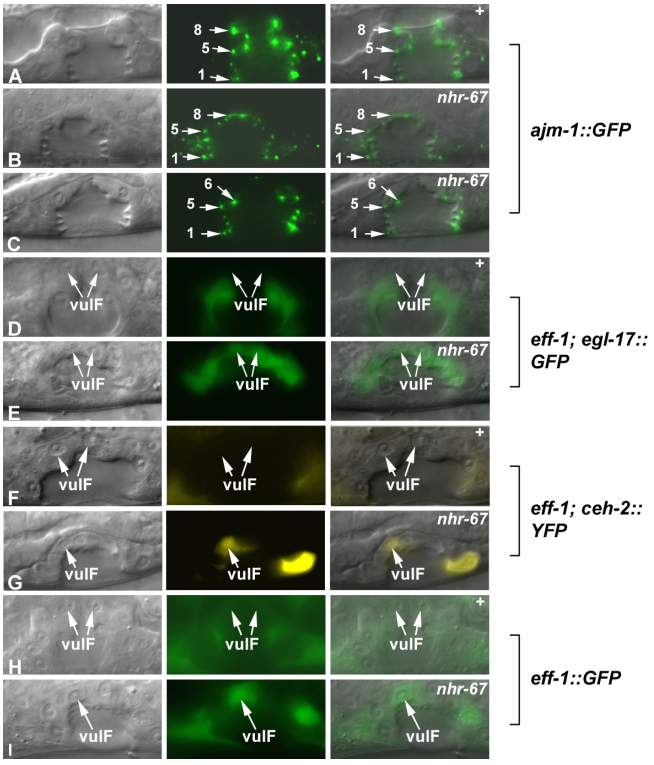

Figure 2. *nhr-67* Prevents Inappropriate Fusion Events between the 1° Vulval Cells

(A–I) Nomarski (left), fluorescence (center), and overlaid (right). The adherens junction marker *ajm-1::GFP* is used to visualize the cell number and architecture of the vulval toroids in wild-type (A) and *nhr-67* RNAi–treated animals (B and C). When observing a mid-sagittal optical section of L4 hermaphrodites, *ajm-1::GFP* appears as dots between the vulval cells. Loss of adherens junction expression signifies a reduction in the cell number due to a cell fusion defect. (A) In wild-type animals, the eight dots on either side correspond to the seven distinct vulval cell types (arrows). The overall vulval morphology of *nhr-67* RNAi–treated animals appears abnormal compared to wild-type.

(B) In some cases, the number of adherens junctions is normal in *nhr-67* RNAi–treated animals (arrows).

(C) Reduction of *nhr-67* sometimes results in the loss of dots at the top of the vulval invagination (which indicates an inappropriate fusion event between the vulE and vulF cells) (arrows). However, the altered gene expression observed in an *nhr-67* RNAi background does not appear to be dependent on cell fusion defects.

(D) In the absence of *eff-1*-mediated fusion, *ayIs4 [egl-17::GFP]* expression is completely absent in the vulF cells (arrows).

(E) In contrast, depletion of *nhr-67* activity in an *eff-1* mutant background is sufficient to cause derepression of *egl-17* in the 1° vulF cells (arrows).

(F) In *eff-1* mutants, *ceh-2* expression is absent in vulF cells (arrows).

(G) Reduction of *nhr-67* activity in an *eff-1* mutant background results in ectopic *ceh-2* expression in vulF cells (arrow).

(H) In wild-type animals, *eff-1::GFP* is not expressed in vulF cells (arrows).

(I) *eff-1* levels are elevated in vulF cells when *nhr-67* activity is compromised (arrow).

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67 negatively regulates other target genes that mediate cell fusion.

nhr-67 Is Dynamically Expressed in Multiple Vulval Cells

Previous work reported that an *nhr-67* construct containing 6 kb of the promoter region directs expression in several head neurons [34]. We generated several additional transcriptional reporter constructs that tested the entire *nhr-67* coding region, introns and the 3′ noncoding region for enhancer activity using the *Δpes-10* basal promoter [35]. An 8-kb fragment that consisted of 1-kb 5′ sequence, the entire coding region and introns, and 2 kb of the 3′ noncoding region yielded expression in the vulva, the hyp7 epidermal syncytium, late stage embryos, and the male tail (Figures 3 and 4A). This *nhr-67* construct exhibits a dynamic expression pattern in the vulval cells. During the late L4 stage, *nhr-67* is first observed in vulA cells (Figure 3A) (and occasionally in vulB1), and this expression is maintained throughout adulthood. Expression in vulC is only seen upon entry into L4 lethargus and persists in adults (Figure 3B). Strong vulB1 and vulB2 expression (and occasional vulD expression) is observed only in young adults (Figure 3C). A 4.5-kb reporter construct that spans from the fourth intron to the 3′ noncoding region is sufficient to drive expression in the same tissues as seen with the 8-kb fragment (Figure 4B). No expression is seen in the vulC, vulD, vulE, and vulF cells during the L4 stage unless *nhr-67* or *cog-1* activity is eliminated (see below). Thus, the *cis*-elements driving the vulval expression of *nhr-67* appear to be located in the region spanning the fourth intron to the 3′ noncoding region. We then wanted to confirm if these regulatory elements were capable of interacting with the endogenous promoter of *nhr-67* in order to promote its transcription in the vulva. To test this, we generated an *nhr-67* transcriptional reporter driven by 1 kb of its native promoter and containing regulatory sequences downstream of the fourth exon in their normal context. The *nhr-67* transcriptional construct containing the endogenous promoter recapitulated the vulval and embryonic expression pattern observed with the *nhr-67::Δpes-10* constructs (Figure 4C). We also examined whether the upstream regulatory sequences of *nhr-67* interact with the downstream regulatory elements to influence its vulval expression. This test was accomplished by coinjecting a transcriptional green fluorescent protein (GFP) construct that contains a 6-kb upstream sequence of *nhr-67* (Figure 4D) with the 8-kb *nhr-67::Δpes-10* construct (Figure 4A) described above. We find that in the presence of the 6-kb promoter region, the vulval expression is identical to that of the 8-kb *nhr-67::Δpes-10* constructs. Besides the previously reported expression in head neurons, we observed expression in the

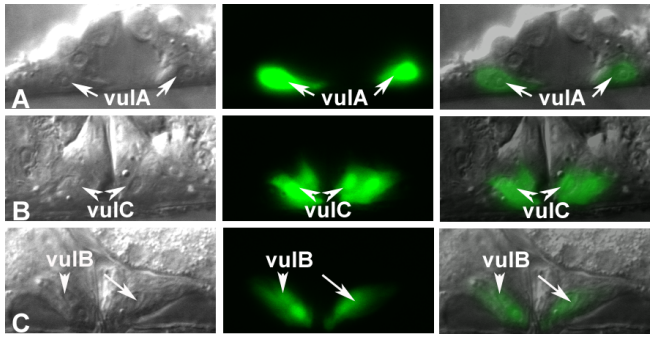

Figure 3. *nhr-67* Is Dynamically Expressed in Multiple Vulval Cell Types

(A–C) Nomarski (left), fluorescence (center), and overlaid (right). All animals displayed carry the *syEx716* transgene in their background. (A) *nhr-67* is robustly expressed in the vulA cells (arrows) during the L4 stage.

(B) vulC expression (arrowheads) is visible upon entry into L4 lethargus.

(C) High levels of *nhr-67* expression in vulB1 and vulB2 cells (arrowhead and arrow) are detectable in young adults.

doi:10.1371/journal.pgen.0030069.g003

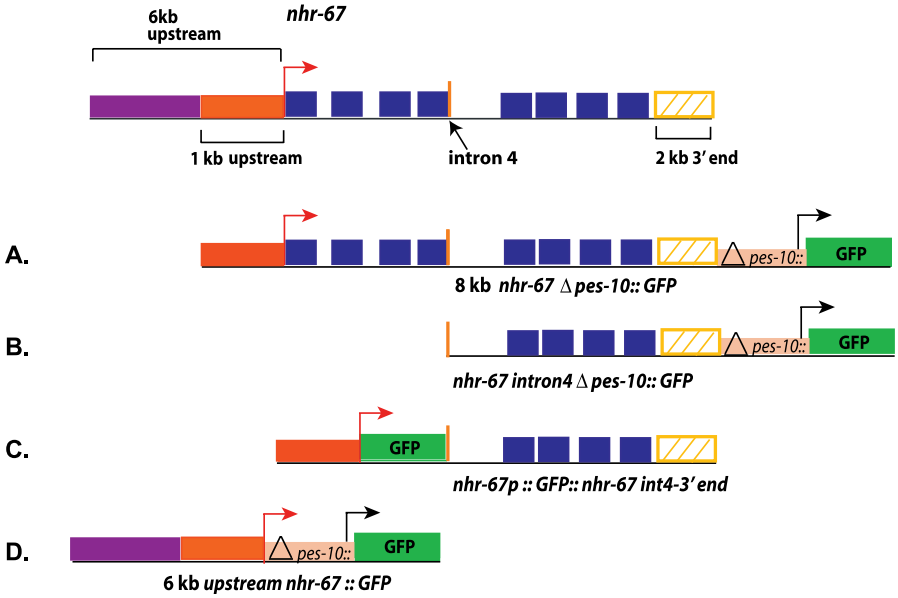

Figure 4. The Regulatory Element(s) Driving the Vulval Expression of *nhr-67* Is Present in the Region That Spans from the Fourth Intron to the 3′ Noncoding Region

Several transcriptional reporter constructs containing the *nhr-67* coding exons (blue rectangles), introns (black lines), and the 3′ noncoding region (yellow hatched rectangle) were generated. The red arrow indicates the presumptive promoter of *nhr-67* and the black arrow is proximal to the minimal *Δpes-10 promoter*. The yellow rectangle includes 2 kb of the 3′ noncoding region. The orange vertical bar indicates the junction between the fourth exon and fourth intron. Construct (A) consists of 1 kb upstream promoter sequence (red rectangle), the entire coding region (blue rectangles) and introns (black lines), and 2 kb of the 3′ noncoding region (yellow hatched rectangle) attached to minimal *Δpes-10::GFP*.
Construct (B) spans from the fourth intron (gene sequence downstream of the orange vertical bar) to the 3′ noncoding region (yellow rectangle) fused to minimal *Δpes-10::GFP*.
Construct (C) is an *nhr-67::GFP* transcriptional reporter driven by 1 kb of the native promoter region (red rectangle) and contains regulatory sequences 3′ of the fourth exon (sequences downstream of the orange vertical bar) and the native 3′ noncoding region (yellow rectangle).
Construct (D) contains 6-kb sequence upstream of the predicted first ATG of *nhr-67* (purple and red rectangles) appended to minimal *Δpes-10::GFP*.
doi:10.1371/journal.pgen.0030069.g004

anchor cell (AC) (during mid–late L3 stage) in hermaphrodites and the linker cell in males (Figure S2A and S2B).

Regulation of *egl-17* and *ceh-2* Expression in the 1° Vulval Cells

We attempted to understand the *trans*-regulation of vulval expression in the diverse cell types by analyzing the regulation of two target genes in detail: *egl-17* and *ceh-2*. To dissect the *trans*-regulation of these target genes, we constructed various double and triple mutant/RNAi combinations and assayed for alterations in gene expression in the 1° vulval cells.

During the L4 stage, the *egl-17* transcriptional reporter is expressed solely in vulC and vulD, being absent in both vulE and vulF (Figure 5 and Table 1). *nhr-67* RNAi in an otherwise wild-type background results in an increase of *egl-17* expression in the vulF cells (Figure 5 and Table 1). In those *nhr-67* RNAi animals, only one of the four vulF cells exhibits this ectopic *egl-17* expression during the L4 stage. *egl-17* expression is consistently absent in the vulF cells of *cog-1* and *egl-38* hypomorphic alleles (Figure 5 and Table 1). In comparison, *cog-1* animals treated with *nhr-67* RNAi are qualitatively enhanced (i.e., several vulF cells misexpress *egl-17*), whereas *egl-38* animals treated with *nhr-67* RNAi displayed a qualitatively and quantitatively higher *egl-17* expression in the vulF cells (Figure 5 and Table 1). *cog-1* is necessary for negatively regulating *egl-17* expression in the vulE cells and acts redundantly with *egl-38* to negatively regulate *egl-17* in the vulF cells [19] (Figure 5 and Table 1). We also observed frequent *egl-17* upregulation in the vulE cells of *egl-38; nhr-67* (RNAi) doubly perturbed hermaphrodites (Table 1), which is invariably absent in either singly perturbed background. Our study provides the first example of *egl-38* modulating gene expression in the vulE cell type. Hence, *egl-38*, *nhr-67*, and *cog-1* act together to negatively regulate *egl-17* expression in the 1° vulval lineages during the L4 stage. Loss of *lin-11* function leads to complete abolition of *egl-17* gene expression in all vulval cells [26] (Figure 5 and Table 1). Lastly, the ectopic *egl-17* expression visualized in the 1° descendents of *cog-1*-, *egl-38*-, and *nhr-67*-depleted backgrounds is dependent on *lin-11* activity (Figure 5 and Table 1). Loss of *nhr-67* in combination with *lin-11* yields rare *egl-17* expression in apparently random vulval cell types (~4% of animals).

In wild-type L4 hermaphrodites, *ceh-2::YFP* expression is only observed in the vulB cells and is invariably absent in both vulE and vulF cells. *nhr-67* RNAi results in a moderate frequency of ectopic *ceh-2* expression in the vulE and vulF cells (Table 2). Eliminating *lin-11* function leads to complete loss of ectopic *ceh-2* expression in the 1° vulval lineages of *nhr-67* RNAi animals (Table 2). *ceh-2* expression is consistently absent in the 1° vulF cells of *cog-1* and *egl-38* single mutants (Table 2). *cog-1* mutants exhibit a moderate increase of *ceh-2* expression in the vulE cells [19] (Table 2). We also found that 90% of *cog-1; egl-38* doubles show increased *ceh-2* expression in vulE cells compared to *cog-1* (32%) or *egl-38* (0%) single mutants (Table 2). Thus, analysis of these double mutants provides us with a second example of *egl-38* regulating gene expression in the vulE cells. As with the *egl-17* reporter,

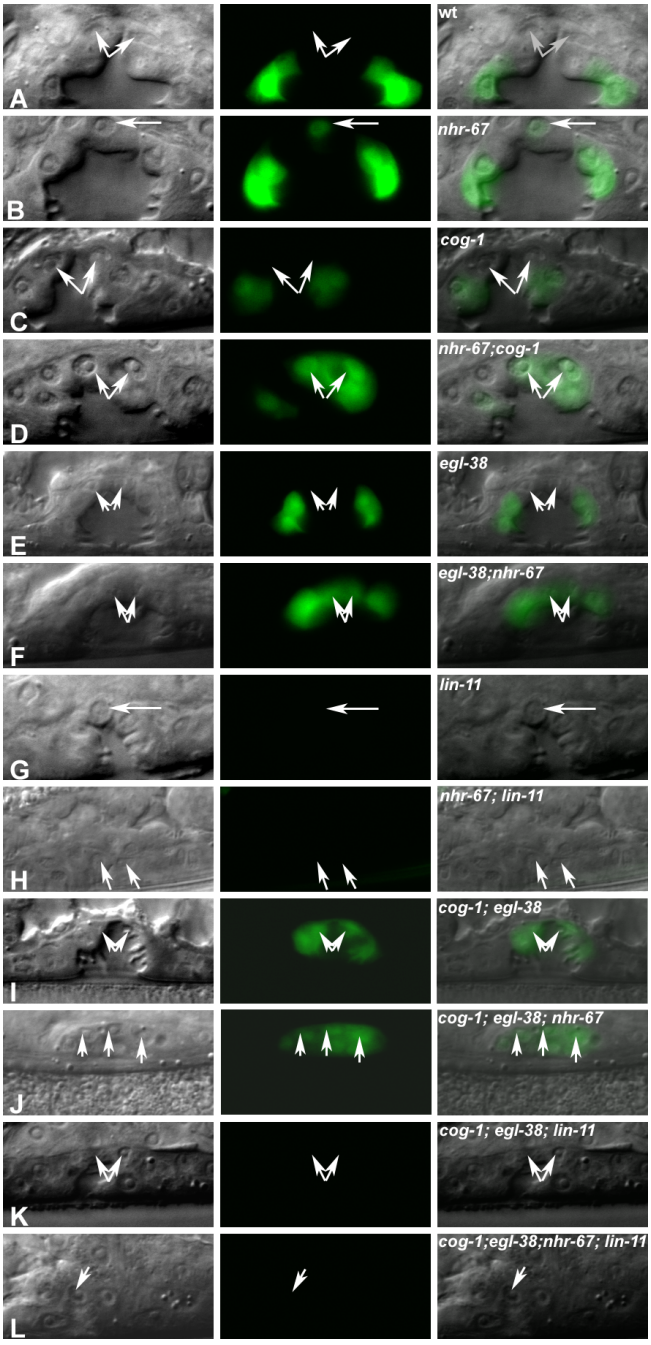

Figure 5. Regulation of *egl-17* Expression in the 1° Vulval Cells

(A–L) Nomarski (left), fluorescence (center), and overlaid (right). Display animals from Table 1. Arrows indicate the position of the visible vulF cells during the mid L4 stage. All animals displayed carry the *ayIs4* transgene in their background. (A) In wild-type animals *egl-17* expression is absent in the vulE and vulF cells.

(B) *nhr-67* RNAi typically results in ectopic *egl-17* expression in one out of four vulF cells. In contrast, *egl-17* remains off in the vulE cells.

(C) In *cog-1(sy275)* mutants, *egl-17* is misexpressed in vulE cells and is absent in vulF cells.

(D) *cog-1(sy275); nhr-67* RNAi doubles show a markedly stronger derepression phenotype in both 1° vulE and vulF lineages.

(E) In *egl-38(n578)* mutants, *egl-17* expression is invariably off in both vulE and vulF cells.

(F) *egl-38(n578); nhr-67* RNAi doubles show robust expression of *egl-17* in both vulE and vulF cells.

(G) In *lin-11(n389)* animals, *egl-17* expression is absent.

(H) In *lin-11(n389); nhr-67* RNAi doubles, the ectopic *egl-17* expression in vulF is eliminated.

(I) *cog-1(sy275); egl-38(n578)* mutants misexpress *egl-17* in vulE and vulF cells.

(J) The *cog-1 (sy275); egl-38(n578); nhr-67* RNAi triple shows complete *egl-17* derepression in all the vulE and vulF descendants.

(K) In *cog-1(sy275); egl-38(n578); lin-11(n389)* mutants, *egl-17* is completely absent.

(L) In *cog-1(sy275); egl-38(n578); lin-11(n389); nhr-67* RNAi quadruples, *egl-17* expression in the vulva is abolished.

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simultaneous depletion of *cog-1* and *egl-38* activities results in a high frequency of *ceh-2* misexpression in the vulF cells (Table 2). Both *cog-1* and *egl-38* are thus required for negative regulation of *ceh-2* expression in the vulF cells.

Regulatory Interactions between Known Components of the Vulval Patterning Network during L4

cog-1, *lin-11*, and *nhr-67*, all of which regulate different aspects of vulval gene expression, exhibit dynamic spatial and temporal expression patterns in the developing vulva [26,27]. *egl-38* expression has been observed in the vulF cells [15]. As mentioned previously, *nhr-67* expression is primarily restricted to vulA (and occasionally vulB1) cells during L4 stage. Yet numerous perturbations in gene expression are observed in *nhr-67* RNAi–treated animals, suggesting that *nhr-67* is indeed functional during the L4 stage in other mature vulval cell types besides vulA (Figure 1). A similar observation can be made about *cog-1*. Wild-type animals occasionally exhibit weak *cog-1* expression in vulE cells but none in vulF cells (Table 3). However, *cog-1* synergistically interacts with *egl-38* and *nhr-67* to regulate *egl-17* expression in the vulF cells (Figure 5 and Table 1). One attractive hypothesis is that levels of both these transcription factors are maintained under strict spatio-temporal control. We thus set out to investigate the interactions among these regulatory factors by assaying for alterations in the reporter gene expression in various mutant backgrounds.

During the L4 stage, *lin-11* is consistently expressed in the 2° vulB, vulC, and vulD lineages, and occasionally in the vulA and vulF cells. Neither *cog-1* nor *egl-38* mutations alter *lin-11* vulval expression [36]. Similarly, reduction of *nhr-67* gene activity also does not impact *lin-11* expression in the vulva (Table 3).

The *cog-1* translational reporter is strongly expressed in vulC and vulD, weakly expressed in vulE, and undetectable in vulF cells during L4 (Figure 6 and Table 3). We found that *cog-1* levels are increased in the 1° vulF cells of *nhr-67* RNAi–treated hermaphrodites as well as in *lin-11* and *egl-38* mutants (Figure 6 and Table 3). *nhr-67* RNAi–treated animals also showed elevated *cog-1* expression in the vulE cells (Table 3). In *lin-11* mutants, *cog-1* levels in vulD are completely abolished as opposed to the vulC-specific expression, which is only partially affected (~57% of animals) (Table 3). Overall *cog-1* expression levels in *lin-11* loss-of-function mutants are noticeably reduced when compared to the wild-type reporter background. The frequency of vulD-specific *cog-1* expression is significantly increased in *egl-38* mutants (Table 3). *cog-1* negatively autoregulates in vulA, vulB1, and vulB2 cells (Table 3).

nhr-67::GFP expression is consistently observed in vulA during the L4 stage (Table 3). *lin-11* mutants only partially eliminate the vulA-specific expression of *nhr-67* (Figure 7 and

Table 1. Regulation of *egl-17* Expression in the 1° Vulval Lineages

Genotype				Phenotype		*p*-Value vulE	*p*-Value vulF
nhr-67	***cog-1***	***egl-38***	***lin-11***	**% vulE on *(n)*[a]**	**% vulF on *(n)*[b]**		
+	+	+	+	0 (18)	0 (18)	NA	NA
RNAi[c]	+	+	+	0 (28)	14 (28)	1.000[e]	**0.018[e]**
+	*sy275*	+	+	83 (12)	0 (12)	**0.001[e]**	1.000[e]
+	+	*n578*	+	0 (25)	0 (25)	1.000[e]	1.000[e]
+	+	+	*n389*	0 (30)	0 (30)	1.000[e]	1.000[e]
RNAi[d]	*sy275*	+	+	75 (32)	9 (32)	1.000[f]	0.701[f]
RNAi	+	*n578*	+	54 (28)	68 (28)	**0.000[g]**	**0.011[g]**
RNAi	+	+	*n389*	0 (76)	0 (76)	1.000[h]	**.001[h]**
+	*sy275*	*n578*	+	58 (55)	56 (55)	0.472[i]	**0.009[i]**
RNAi	*sy275*	*n578*	+	74 (39)	79 (39)	1.000[j]	0.849[j]
+	*sy275*	*n578*	*n389*	0 (44)	0 (44)	**0.000[k]**	**0.000[k]**
RNAi	*sy275*	*n578*	*n389*	0 (44)	0 (44)	**0.000[l]**	**0.000[l]**

All animals carry the *ayIs4* transgene in their background. Boldface indicates that *p*-values are significant.
lin-11(n389) is a strong loss-of-function allele. Both *cog-1* and *egl-38* are reduction of function alleles:
cog-1(sy275) is a missense mutation in the homeodomain and *egl-38(n578)* is a missense mutation in the paired domain.
[a]This column refers to the % of animals that displayed ectopic *egl-17* expression in any of the four vulE cells.
[b]This column refers to the % of animals that displayed ectopic *egl-17* expression in any of the four vulF cells.
[c]In *nhr-67* RNAi–treated animals, only one out of four vulF cells exhibit the derepression phenotype.
[d]In *cog-1(sy275); nhr-67* (RNAi) doubles the derepression phenotype is qualitatively enhanced, i.e., several vulF cells misexpress *egl-17*.
p-Values were evaluated using Fisher's exact test:
[e]Single mutant/RNAi background compared to wild-type controls.
[f]*nhr-67* (RNAi)–treated *cog-1* mutants compared to *cog-1* single mutants and *nhr-67* RNAi background.
[g]*nhr-67* (RNAi)–treated *egl-38* mutants compared to *nhr-67* RNAi–treated animals and *egl-38* single mutants.
[h]*nhr-67* (RNAi)–treated *lin-11* mutants compared to *nhr-67* RNAi background.
[i]*cog-1; egl-38* double mutants compared to *cog-1* and *egl-38* single mutants.
[j]*cog-1; egl-38; nhr-67* (RNAi) triply perturbed background compared to *nhr-67*(RNAi)–treated *egl-38* animals.
[k]*lin-11; cog-1; egl-38* triple mutants compared to *cog-1; egl-38* double mutants.
[l]*nhr-67*(RNAi)–treated *cog-1; egl-38; lin-11* mutants compared to *cog-1; egl-38; nhr-67* (RNAi) triply perturbed background.
doi:10.1371/journal.pgen.0030069.t001

Table 3). *nhr-67* expression in vulA is completely abolished only in the absence of both *lin-11* and its positive autoregulatory activity (Table 3). Overall, *nhr-67* expression levels in *lin-11* loss-of-function mutants are noticeably reduced when compared to a *lin-11*(+) background. *lin-11* activity is also required for directing the ectopic *nhr-67* expression in the 1° lineages when the autoregulatory loop is compromised (Table 3, see below). Also, loss of *lin-11* sometimes caused premature vulC expression of *nhr-67* during L4 stage, which can be interpreted either as a cell type or a temporal regulatory defect (Figure 7 and Table 3). Reduction of *cog-1* function results in increased expression of *nhr-67* in vulC and

Table 2. Regulation of *ceh-2* Expression in the 1° Vulval Lineages

Genotype				Phenotype		*p*-Value vulE	*p*-Value vulF
nhr-67	***cog-1***	***egl-38***	***lin-11***	**% vulE on *(n)*[a]**	**% vulF on *(n)*[b]**		
+	+	+	+	0 (40)	0 (40)	NA	NA
RNAi	+	+	+	15 (40)	28 (40)	**0.028[c]**	**0.002[c]**
+	*sy275*	+	+	32 (41)	0 (41)	**0.001[c]**	1.000 [c]
+	+	*n578*	+	0 (41)	0 (41)	1.000 [c]	1.000 [c]
+	+	+	*n389*	0 (40)	0 (40)	1.000 [c]	1.000 [c]
RNAi	*sy275*	+	+	44 (48)	19 (48)	0.543[d]	0.467[d]
RNAi	+	*n578*	+	34 (38)	29 (38)	0.135[e]	1.000[e]
RNAi	+	+	*n389*	0 (50)	0 (50)	**0.010[f]**	**0.001[f]**
+	*sy275*	*n578*	+	90 (21)	86 (21)	**0.027[g]**	**0.000[g]**

All animals carry the *syIs54* transgene in their background. Boldface indicates that *p*-values are significant.
[a]This column refers to the % of animals that displayed ectopic *ceh-2* expression in any of the four vulE cells.
[b]This column refers to the % of animals that displayed ectopic *ceh-2* expression in any of the four vulF cells.
p-Values were evaluated using Fisher's exact test:
[c]Single mutant/RNAi background compared to wild-type controls.
[d]*nhr-67* RNAi–treated *cog-1* mutants compared to *cog-1* single mutant and *nhr-67* RNAi background.
[e]*nhr-67* RNAi–treated *egl-38* mutants compared to *egl-38* single mutant and *nhr-67* RNAi background.
[f]*nhr-67* RNAi–treated *lin-11* mutants compared to *nhr-67* RNAi–treated animals.
[g]*cog-1;egl-38* double mutants compared to *cog-1* and *egl-38* single mutants.
doi:10.1371/journal.pgen.0030069.t002

Table 3. Regulatory Interactions between Known Components of the Vulval Patterning Network during L4

Genotype	GFP Reporter[a]	vulA			vulB1			vulB2		
		% on *(n)*	*p*-Value	Intensity	% on *(n)*	*p*-Value	Intensity	% on *(n)*	*p*-Value	Intensity
+	*lin-11::GFP*	7 (42)	NA	++	98 (42)	NA	++	98 (42)	NA	++
nhr-67 RNAi[d]	*lin-11::GFP*	10 (30)	0.694	++	90 (30)	0.864	++	97 (30)	1.000	++
+	*cog-1::GFP*	0 (43)	NA	−	0 (43)	NA	−	0 (43)	NA	−
lin-11(n389)[d]	*cog-1::GFP*	0 (30)	1.000	−	0 (30)	1.000	−	0 (30)	1.000	−
nhr-67 RNAi[d]	*cog-1::GFP*	0 (28)	1.000	−	0 (28)	1.000	−	0 (28)	1.000	−
cog-1(sy275)[d]	*cog-1::GFP*	16 (31)	**0.017**	+	42 (31)	**0.000**	+	32 (31)	**0.000**	+
+	*cog-1::GFP*	0 (45)	NA	−	0 (45)	NA	−	0 (45)	NA	−
egl-38(n578)[d]	*cog-1::GFP*	0 (43)	1.000	−	0 (43)	1.000	−	0 (43)	1.000	−
+	*nhr-67::GFP*	86 (95)	NA	++	20 (95)	NA	++	0 (95)	NA	−
lin-11(n389)[d]	*nhr-67::GFP*	29 (24)	**0.017**	+/−[b]	0 (24)	**0.040**	−	0 (24)	1.000	−
cog-1(sy275)[d]	*nhr-67::GFP*	88 (52)	1.000	++	13 (52)	0.503	++	0 (52)	1.000	−
egl-38(n578)[d]	*nhr-67::GFP*	92 (39)	0.890	++	46 (39)	**0.031**	++	0 (39)	1.000	−
nhr-67 RNAi[d]	*nhr-67::GFP*	91 (89)	0.830	++	21 (89)	0.861	++	0 (89)	1.000	−
lin-11(n389); nhr-67 RNAi[e]	*nhr-67::GFP*	0 (58)	**0.000**	−	0 (58)	**0.000**	−	0 (58)	1.000	−
cog-1(sy275); nhr-67 RNAi[f]	*nhr-67::GFP*	88 (24)	1.000	++	17 (24)	0.784	++	0 (24)	1.000	−

Intensity key: ++ = very bright GFP expression, + = low GFP expression, +/− = weak GFP expression, − = no detectable GFP expression.
In the *p*-value columns, boldface indicates that *p*-values are significant.
In the intensity columns, any alterations in signal intensity are indicated by boldface.
[a]Transgenes used were *lin-11::GFP(syIs80), cog-1 ::GFP(syIs63* and *syIs64),* and *nhr-67::GFP (syEx716* and *syEx749).*
[b]Overall *cog-1* and *nhr-67* expression levels in the vulval cells are significantly decreased compared to their respective wild-type controls.
[c]vulC expression scored prior to L4 lethargus.
p-Values are calculated using Fisher's exact test:
[d]Compared to the respective wild-type GFP reporter controls.
[e]*lin-11;nhr-67*(RNAi) doubly perturbed animals compared to *nhr-67* RNAi–treated animals,
[f]*cog-1;nhr-67*(RNAi) doubly perturbed animals compared to single *cog-1* mutant or *nhr-67* RNAi background.
doi:10.1371/journal.pgen.0030069.t003

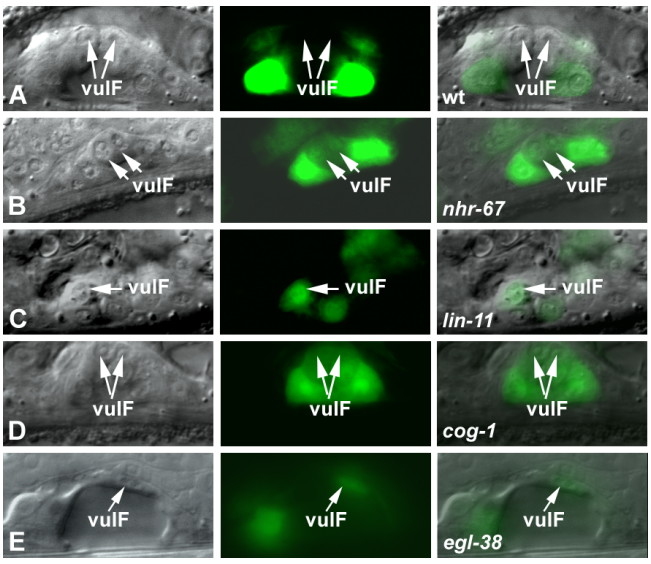

Figure 6. *cog-1* Levels in the 1° VulF Cells Are Antagonized by Multiple Genes

(A–E) Nomarski (left), fluorescence (center), and overlaid (right). Display animals from Table 3. All animals displayed carry either the *syIs63* (A–D) or *syIs64* (E) transgene in their background. (A) In wild-type animals, *cog-1* expression is absent in the vulF cells (arrows).
(B) *nhr-67* RNAi results in the derepression of *cog-1* levels in the vulF cells (arrows).
(C) *lin-11(n389)* mutants show ectopic *cog-1* expression in vulF (arrow).
(D) *cog-1(sy275)* mutants lose the ability to negatively autoregulate their expression levels in both vulE and vulF (arrows).
(E) *cog-1* is ectopically expressed in vulF in an *egl-38* mutant background (arrow).
doi:10.1371/journal.pgen.0030069.g006

vulD during the L4 stage and vulE and vulF during L4 lethargus (Figure 7 and Table 3). Depletion of both *cog-1* and *nhr-67* activities leads to a more robust increase in *nhr-67* levels in the vulF cells (Table 3). *egl-38* mutants sometimes showed ectopic *nhr-67* expression in vulC and vulD cells during the L4 stage (Figure 7 and Table 3) and significantly increased its frequency of expression in vulB1 cells (Table 3).

Negative Autoregulation of *nhr-67* and *cog-1* in 1° Vulval Lineages

In addition to the cross-inhibitory interactions between *cog-1* and *nhr-67* in both the 1° vulE and vulF cells, we also discovered that they both negatively autoregulate in the same cell types. Inhibition of *nhr-67* by RNAi feeding results in the robust increase of *nhr-67::GFP* expression levels in both vulE and vulF cells (Figure 7 and Table 3). Elevation of *nhr-67* transcriptional levels is also visible in the vulC and vulD lineages of *nhr-67* (RNAi) animals during L4 stage. Upregulation of *nhr-67* expression in vulC, vulD, vulE, and vulF cells is also visible with the 4.5-kb *nhr-67* transcriptional reporter construct (Figure 4B) in an *nhr-67*(RNAi) background (unpublished data). We used *fos-1* RNAi feeding as a control to exclude the possibility that the observed negative autoregulation was a nonspecific effect of inducing RNAi. *fos-1* RNAi–treated animals exhibited a strong Pvl phenotype (at least in part due to its AC invasion phenotype) [37] and did not alter *nhr-67* levels in the 1° lineages (Figure 7 and Table 3). Similarly, ectopic expression of *cog-1::GFP* in all 1° vulval descendents is consistently observed when *cog-1* activity is compromised (Figure 6 and Table 3). Thus, *nhr-67* and *cog-1* appear to be activated in all the mature vulval cell types but

Table 3. Extended.

vulC			vulD			vulE			vulF		
% on *(n)*	*p*-Value	Intensity	% on *(n)*	*p*-Value	Intensity	% on *(n)*	*p*-Value	Intensity	% on *(n)*	*p*-Value	Intensity
95 (42)	NA	++	100 (42)	NA	++	7(42)	NA	+/−	14 (42)	NA	+/−
87 (30)	0.863	++	93 (30)	0.866	++	0(30)	0.270	−	7 (30)	0.466	+/−
100 (43)	NA	++	63 (43)	NA	++	49(43)	NA	+/−	0 (43)	NA	−
57 (30)	0.147	+[b]	0 (30)	**0.000**	−	73 (30)	0.337	+/−	50 (30)	**0.000**	+[b]
96 (28)	1.000	++	86 (28)	0.460	++	43 (28)	0.831	++	29 (28)	**0.001**	++
90 (31)	0.866	++	100 (31)	0.22	++	97 (31)	0.071	++	71 (31)	**0.000**	+
100 (45)	NA	++	44 (45)	NA	++	11 (45)	NA	+/−	0 (45)	NA	−
100 (43)	1.000	++	100 (43)	**0.020**	++	12 (43)	1.000	+/−	9 (43)	0.117	+
0 (95)[c]	NA	−	0 (95)	NA	−	0 (95)	NA	−	0 (95)	NA	−
21 (24)[c]	**0.001**	+[b]	0 (24)	1.000	−	0(24)	1.000	−	0 (24)	1.000	−
10 (52)[c]	**0.007**	++	40 (52)	**0.000**	++	23(52)	**0.000**	++	4 (52)	0.130	+
8 (39)[c]	**0.027**	++	41 (39)	**0.000**	++	0(39)	1.000	−	0 (39)	1.000	−
11 (89)[c]	**0.002**	++	11 (89)	**0.002**	++	10 (89)	**0.003**	++	11 (89)	**0.002**	++
22 (58)[c]	0.172	+	0 (58)	**0.014**	−	0(58)	**0.027**	−	0 (58)	**0.014**	−
54 (24)[c]	**0.003**	++	63 (24)	0.396	++	46 (24)	0.213	++	42 (24)	**0.011**	++

are then restricted by both autoregulatory and *trans*-regulatory mechanisms.

Discussion

nhr-67: A Novel Regulator of Vulval Patterning in *C. elegans*

nhr-67 encodes a *C. elegans* ortholog of *tailless,* a crucial regulator of blastoderm patterning in the terminal pathway of *Drosophila* embryogenesis as well as neuronal development. We find that *nhr-67* activity is required for the regulation of gene expression in several mature vulval cell types and is dynamically expressed in the vulva. For technical reasons, we have been unable to determine whether *nhr-67* acts in the vulval cells for these functions. However, the expression of *nhr-67* in the vulva and the complexity of the interactions are most consistent with a primarily autonomous action of *nhr-67*. However, given the expression of *nhr-67* in the AC, it is possible that the effects (particularly on the 1° lineage) are nonautonomous. For example, the AC generates EGF and Wnt signals and is required to differentiate vulE and vulF cells, presumably via these signals [10]. Loss of vulF-specific *lin-3* expression in an *nhr-67* RNAi background is certainly consistent with this model. The AC also promotes 1° over 2° fate [38]. The ectopic expression of 2° lineage-specific genes *ceh-2* and *egl-17* in the 1° vulval cells is also consistent with this model. However, lineage analysis of *nhr-67* (RNAi) hermaphrodites argues that these alterations are not full 1° to 2° cell fate transformations in the early vulval lineages. In addition, the observed effects on *pax-2* and *zmp-1* expression are inconsistent with this model. It remains a formal possibility that some of *nhr-67* effects in the vulva are due to a role in the AC.

Our data are consistent with the function of *Drosophila tailless,* which facilitates proper gap gene expression at the posterior end of the blastoderm embryo via its dual activator/repressor activity [39–41]. Specifically, *tailless* blocks segmentation and maintains the identity of the terminal boundaries via repression of *Kruppel* and *knirps* activity and promotes *hunchback* expression, which is necessary for the establishment of terminal-specific structures [42,43]. *tailless* is also necessary for regulating gene expression during the generation of head segments as well as anterior brain development [44]. We also find that *nhr-67* prohibits improper fusion events between related cell lineages, at least partly due to strict spatial regulation of the fusogen *eff-1* in certain vulval cell types.

As discussed below, *nhr-67* interacts genetically with three other transcriptional regulators, *cog-1, egl-38,* and *lin-11,* to produce complex patterns of gene expression, probably through *trans*-regulation of cell type–specific enhancers (Figure 8).

Comparison of the Vulval Network with Other Genomic Networks

We have uncovered a novel set of genetic interactions between *nhr-67,* several transcription factors, and many target genes that contribute to the identity of distinct vulval cell types. For example, *nhr-67* appears to be particularly important in the execution of vulF fate and maintaining its cellular identity via regulation of gene expression and fusion events between distinct cell types. Not only does *nhr-67* inhibit inappropriate gene expression that is associated with the 2° vulval lineages (Figure 1), but it also promotes gene expression of the EGF protein LIN-3, which is necessary for uv1 fate specification and facilitates proper vulval-uterine connection during development [14]. The functional data obtained from numerous RNAi experiments demonstrates that *nhr-67* (like its *Drosophila* ortholog) is a versatile regulatory gene that operates on at least four of the seven vulval cell types (vulA, vulD, vulE, and vulF). However, we have not tested whether any of these approximately ten interactions are direct.

An interesting feature of the network is our suggestion that both *nhr-67* and *cog-1* might negatively autoregulate in the same vulE and vulF cells. *Drosophila melanogaster tailless* does not regulate itself [45], suggesting that *nhr-67* autoregulation is a developmental phenomenon unique to nematodes *(C. elegans).* This apparent divergence in *tailless* regulation between phyla suggests that a more precise fine-tuning of *tailless* levels is required for the execution of accurate

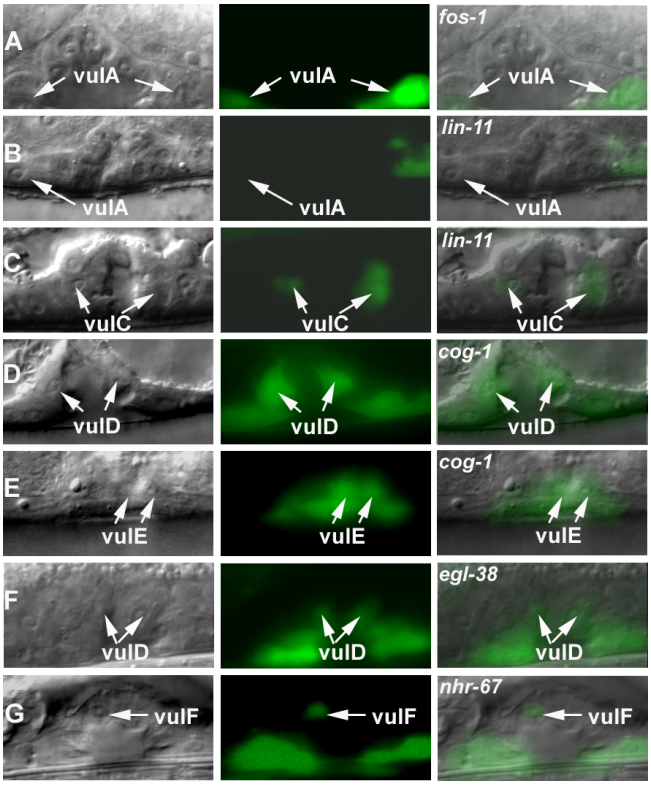

Figure 7. *nhr-67* Is Differentially Regulated in the 1° and 2° Vulval Lineages

(A–G) Nomarski (left), fluorescence (center), and overlaid (right). Display animals from Table 3. All animals displayed carry either *syEx716* (A, D, E, and F) or *syEx749* (B and C) *[nhr-67::GFP]* transgene in their background.
(A) Wild-type animals treated with control *fos-1* RNAi show no alteration of *nhr-67* vulval expression compared to non-RNAi–treated animals. *fos-1* RNAi animals exhibit abnormal vulval morphology, yet show wild-type *nhr-67* expression in vulA cells (arrows).
(B) *lin-11(n389)* mutants partially eliminate the vulA-specific expression of *nhr-67* (arrow).
(C) In a *lin-11(n389)* background, premature vulC expression (arrows) is observed sometimes during L4 stage.
(D) *cog-1(sy275)* mutants misexpress *nhr-67* in the 2° vulD cells (arrows).
(E) In a *cog-1(sy275)* background, *nhr-67* levels are highly elevated in the 1° vulE lineages (arrows).
(F) *egl-38(n578)* mutants show ectopic *nhr-67* expression in vulD cells (arrows).
(G) *nhr-67* RNAi feeding results in the robust increase in its own expression levels in vulF cells (arrow).
doi:10.1371/journal.pgen.0030069.g007

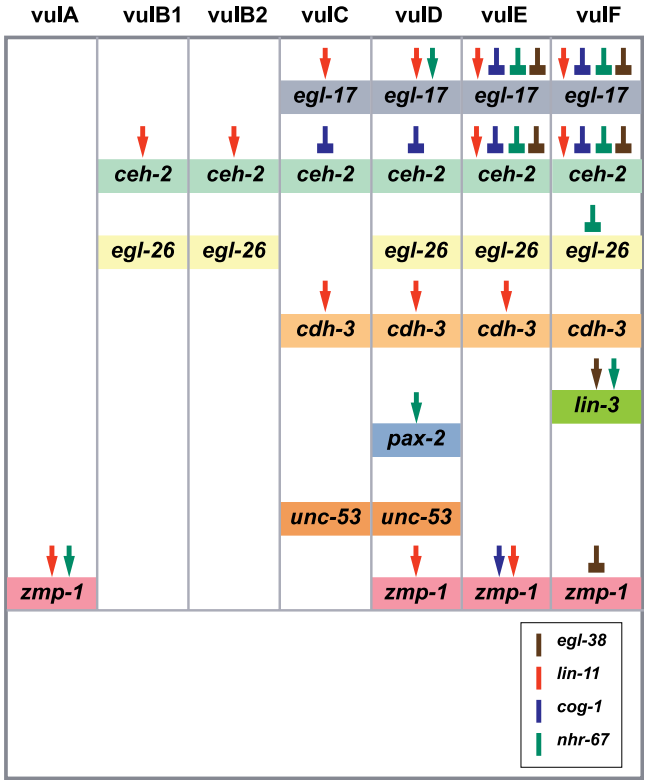

Figure 8. A Summary of the Gene Regulatory Network That Functions during Vulval Patterning and Differentiation in *C. elegans*

nhr-67 activity is included along with the other vulval patterning genes: *lin-11, cog-1, and egl-38.* Colored arrows represent positive inputs and colored block arrows represent repressor inputs for target gene expression in the distinct vulval cell types. The role of *egl-38* regulating *egl-17* and *ceh-2* gene expression in vulE is revealed by analysis of *egl-38(n578)* animals treated with *nhr-67* RNAi and *cog-1(sy275); egl-38(n578)* double mutants.
doi:10.1371/journal.pgen.0030069.g008

patterning in the *C. elegans* vulva. In contrast to their different autoregulatory properties, we find that certain genetic interactions are indeed conserved between the *D. melanogaster tailless* and *C. elegans nhr-67;* namely *tailless* restricts the expression domain of *ems* in the head segments [44], which is comparable to *nhr-67* repressing the worm *ems* ortholog *ceh-2* in the inappropriate vulval cells. Additional *tailless* targets from other organisms [40,46,47] may also have an impact on vulval patterning. Predictions can also be made in the reciprocal direction and used to elucidate vertebrate development. For example, FGF signaling is required for both vertebrate and inverterbrate heart development [48,49]. The LIM domain protein ISL1 promotes differentiation in a subset of cardiac progenitor cells and transcriptionally activates several FGF genes in mice [50]. Our *trans*-regulation experiments reveal that both *egl-17* and *ceh-2* contain *cis*-regulatory elements that are directly or indirectly dependent on *cog-1* (Nkx6.1/6.2), *egl-38* (Pax2/5/8), *nhr-67 (tll),* and *lin-11* (LIM) activity. These data may provide further insights into the elaborate regulation of classic developmental genes such as FGF and EMS, both of which have multiple roles in metazoan development.

Patterning in vulE versus vulF Lineages

Previous work demonstrated that patterning of the E and F descendents of the 1° vulval lineage involves both a short-range AC-dependent signal using the Ras pathway as well as *lin-17* (Wnt) signaling [10]. In the context of *egl-17* gene expression, *cog-1* single mutants exhibit increased levels in the vulE cells only. In contrast, *nhr-67* RNAi appears to exclusively affect *egl-17* expression in the vulF cells. The negative regulatory activities of *cog-1* in vulF and *nhr-67* in vulE only become apparent in an *egl-38* mutant background (which shows no phenotype on its own). This difference suggests that *cog-1*-mediated negative regulation plays a greater role in vulE cells whereas *nhr-67*-mediated negative regulation functions primarily in vulF cells. One hypothesis is that vulF cells are biased by proximity to the AC to have higher levels of *nhr-67* compared to *cog-1* (Figure 9). The genetic regulatory interactions within the vulval network

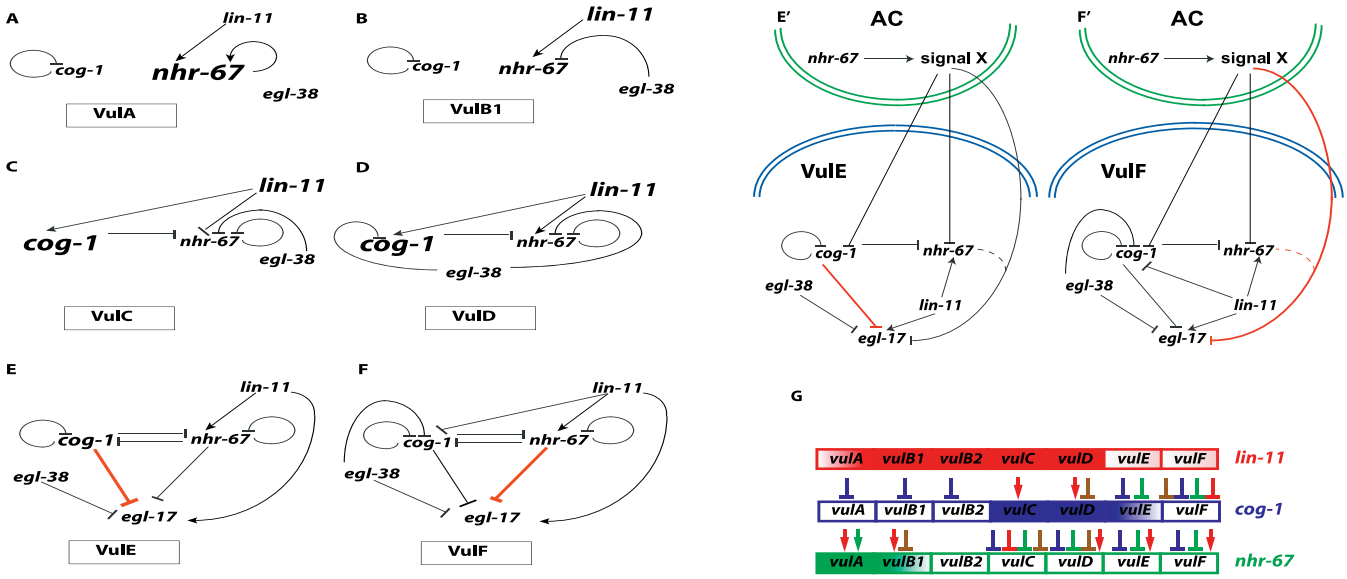

Figure 9. A Summary of the Identified Genetic Regulatory Interactions That Affect Some of the Distinct Vulval Cell Types

In (A–F and E′ and F′), black arrows represent positive inputs and black block arrows represent repressor inputs for target gene expression that are functional within a given cell type: vulA (A), vulB (B), vulC (C), vulD (D), vulE (E and E′), and vulF (F and F′).

In (A–D), the larger font size depicts high levels of expression of the represented patterning gene within the specified cell type.

(E and F) This model assumes that the interactions mediated by *nhr-67* occur within the vulE and vulF cells. Red block arrows (E and F) indicate that a specific regulatory factor (*nhr-67* in vulF and *cog-1* in vulE) acts as a major repressor within the specified cell type. (E′ and F′) This alternate model presumes that *nhr-67* acts in the AC to differentiate between vulE and vulF cells. Signal X could be Ras, Wnt, or some other signaling pathway. Red block arrows (E′ and F′) indicate that the activity associated with a specific regulatory factor (*nhr-67* in vulF and *cog-1* in vulE) plays a major role in patterning gene expression with in the specified cell type.

(G) A color-coded summary of the distinct expression patterns of *lin-11* (red), *cog-1* (blue), and *nhr-67* (green). Each box represents one of the seven vulval cell types. The colored boxes with the graded pattern represent rare weak expression of the transcriptional reporter, whereas boxes with solid colors represent robust expression. Colored arrows and block arrows are used to document all the identified regulatory interactions that occur during L4 patterning in the diverse vulval cell types: *lin-11* (red), *cog-1*(blue), *egl-38* (brown), and *nhr-67* (green). This model illustrates multiple patterning differences between the seven mature vulval cell types.

doi:10.1371/journal.pgen.0030069.g009

demonstrate that *cog-1* levels are negatively regulated in vulF cells via four inputs: *lin-11, egl-38, nhr-67, and cog-1*. In comparison, *nhr-67* expression in vulF cells is modulated by two antagonistic inputs *(cog-1* and *nhr-67)* and one positive input *(lin-11),* thus possibly resulting in its higher levels. These observations are consistent with a model where *nhr-67* acts as the major negative regulator in vulF cells.

nhr-67 and *cog-1* cross-inhibit each other's transcriptional activities, specifically in the vulE and vulF cells, implying that a mutually antagonistic feedback loop exists that exclusively affects the cells of the 1° vulval lineages. Both *cog-1* and its mammalian ortholog *Nkx6.1* have been previously implicated in bistable loops that reinforce one of two possible stable end states [51,52]. The cross-inhibitory interactions between *nhr-67* and *cog-1* might be relevant in the specification of vulE versus vulF cell fates. The nature of the bistable loop between *cog-1* and *nhr-67,* however, is unknown. In particular, the bistable loop may be a consequence of either direct transcriptional regulation (as implied in Figure 9) or indirect regulation through an unknown intermediate regulatory factor.

However, the above observation does not rule out the possibility that additional regulatory factors might also contribute to proper patterning of 1° lineages. These other inputs could presumably operate via several potential mechanisms such as modulating the balance between *cog-1* versus *nhr-67* levels, being exclusively active in one 1° cell type, and interacting at distinct *cis*-regulatory elements of the downstream targets.

Patterning Differences between 1° and 2° Vulval Lineages

Given the complexity of the observed vulval regulatory interactions, we propose that the network operating on each vulval cell type is unique (Figure 9). A single regulatory factor may have differential functions in terms of executing accurate spatio-temporal gene expression in diverse cells. For instance, *lin-11* may upregulate *cog-1* levels in the 2° vulC and vulD cells while antagonizing them in the 1° vulF cells. A similar argument can be made about the *lin-11*-dependent regulation of *nhr-67*. *lin-11* may temporally regulate *nhr-67* by inhibiting its vulC-specific expression during the L4 stage. In contrast, *lin-11* is clearly critical for the positive regulation of *nhr-67* expression in both vulE and vulF cells.

Both *cog-1* and *nhr-67* are present at high levels in a subset of the 2° vulval cells, yet are barely detectable in the 1° vulval cells. Nevertheless, the disruption of either factor yields obvious defects in 1° vulval cell–specific gene expression. A cross inhibition circuit, such as we propose for *cog-1* and *nhr-67,* can be bistable, with stable states that tolerate inherent fluxes in gene expression (i.e., it would not randomly oscillate between states) [53–55]. Negative autoregulatory circuits have been shown to reduce cell–cell fluctuations in the steady-state level of transcription factors [56] and can speed up the response times of transcription networks without incurring the cost of constant protein production and turnover [57].

These two distinct circuits might enable cells to reach a developmental state with built-in flexibility, allowing rapid switching of their fate upon transient inputs (as opposed to sustained inductive inputs that are metabolically costly). In this model, dynamic levels of *cog-1* and/or *nhr-67* expression could correlate with particular aspects of 1° vulval cell fate execution. This might account for the elaborate autoregulatory and *trans*-regulatory interactions specifically seen in 1° vulval descendents, as opposed to their 2°-derived counterparts. We postulate that although all the vulval cells appear to use the same regulatory factors, their differential effects on the diverse cell types is what results in accurate gene expression.

Regulatory Code for the Seven Vulval Cell Types?

During the L4 stage, the gradient of *nhr-67* expression is opposite to that of either *cog-1* or *lin-11*. This difference in gene expression domain raises the question of whether the levels of these factors are critical for vulval development. For example, high levels of *lin-11* result in misexpression of *egl-17* in vulA and abnormal vulval invagination [26]. Different concentrations and combinatorial expression patterns of *lin-11, cog-1,* and *nhr-67* might thus encode mature vulval cell types (Figure 9). For example, differentiation to the 1° vulF cell type may entail low levels of LIN-11 and NHR-67 along with lower levels of COG-1. In contrast, the 1° vulE cells require medium levels of COG-1 along with low doses of LIN-11 and NHR-67. vulA and vulB are similar to each other with respect to maintaining low COG-1 levels. However, vulA cells are characterized by their high NHR-67 levels and medium LIN-11 levels as opposed to the reverse situation in vulB1 and vulB2 cells (medium-low NHR-67, high LIN-11). Lastly, both vulC and vulD have indistinguishably high levels of LIN-11 and COG-1, and we are unable to precisely define what distinguishes these two cell types from each other. One hypothesis is the differential regulation of NHR-67 and COG-1 in both cell types: COG-1 levels are impacted by *egl-38* in vulD (but not vulC), whereas NHR-67 levels are negatively regulated by *lin-11* in vulC (but not vulD). An obvious limitation of this proposed regulatory code is that it does not take into account other transcription factors that may potentially mediate vulval patterning.

The intricacies of vulval organogenesis can be deconstructed by rigorously elucidating the genomic networks that operate within the seven mature vulval cell types. Deciphering this regulatory code will provide valuable information on network connections and might provide insights into other examples of organogenesis.

Materials and Methods

Microscopy. Transgenic worms were anesthetized using 3 mM levamisole and observed using Nomarski optics (http://www.nomarski.com). Photographs were taken with a monochrome Hamamatsu digital camera (http://www.hamamatsu.com) and Improvision Openlab 4.0.4 software (http://www.improvision.com). The fluorescent images were overlaid with their respective DIC images using Adobe photoshop 7.0.1 (http://www.adobe.com). The vulval expression patterns for all strains except *syIs49* were visualized during the late L4 stage. In the case of *syEx716,* the vulval expression was also examined during L4 lethargus and adult stage. In *syIs49* animals, vulA-specific *zmp-1::GFP* expression was scored in adults only.

Genetics and RNAi. *C. elegans* strains were cultured at 20 °C using standard protocols (Brenner, 1974). Transgenes used in this study are as follows: *syIs54 [ceh-2::GFP], syIs55 [ceh-2::YFP], syIs51 [cdh-3::CFP], syIs49 [zmp-1::GFP], syIs77 [zmp-1::YFP], syIs59 [egl-17::CFP]* [9], *syIs78 [ajm-1::GFP]* [26], *syIs107 [lin-3::GFP]* [58], *ayIs4 [egl-17::GFP]* [16], *guEx64 [pax-2::GFP]* (gift from Chamberlin lab), *kuIs36 [egl-26::GFP]* [18], *syIs63* and *syIs64 [cog-1::GFP]* [27], *syIs80 [lin-11::GFP]* [59], *syEx716* [8-kb *nhr-67Δpes-10::GFP*], *syEx749* [8-kb *nhr-67Δpes-10::GFP*], *syEx744 [nhr-67* intron4 *Δpes-10::GFP], syEx925* [6 kb upstream *nhr-67::GFP* + 8 kb *nhr-67Δpes-10::GFP*], *syEx865* [*nhr-67p::GFP::nhr-67* int4–3′end], and *syEx756 [unc-53::GFP].* Alleles used in this study: LGI, *lin-11(n389);* LGII, *cog-1(sy275), eff-1(hy21);* LGIII, *unc-119(ed4);* LGIV, *unc-31(e169), egl-38(n578), dpy-4(e1166sd), dpy-20(e1282);* LGV, *him-5(e1490).* A complete list of strains is included in Table S2.

Transgenic lines were generated using standard microinjection protocol that produces high-copy number extrachromosomal arrays [60]. *syEx756* was generated by injecting the pNP10 construct [61] into *unc-119(ed4); him-5* background using *unc-119*(+) [62] and pBSK+ (Stratagene, http://www.stratagene.com) as coinjection markers.

A reverse genetics screen was conducted against 508 transcription factors (Table S1) from the Ahringer library (Medical Research Council Geneservice) to assay for alterations in vulval expression patterns for the *ceh-2::YFP* transgene. RNAi feeding protocol is similar to that previously described [32]. Embryos were harvested by bleaching gravid adults and were placed on a lawn of *Escherichia coli* strain expressing double-stranded RNA at 20 °C. Animals were scored after 36 h (during the L4 stage) using Nomarski microscopy. We resorted to *nhr-67* RNAi feeding for the rest of this study since the *nhr-67* deletion allele *(ok631)* results in L1 lethality and/or arrest (International *C. elegans* Knockout Consortium). All subsequent *nhr-67* RNAi feeding experiments were done as described above. *nhr-67* RNAi feeding experiments that entailed the restriction of cell fusion (via a temperature-sensitive allele of *eff-1*) were conducted at 25 °C.

Generation of *nhr-67* reporter transgenes. *nhr-67::Δpes-10::GFP* reporter gene constructs: The pPD97–78 vector, which includes the *Δpes-10* basal promoter driving GFP and the *unc-54* 3′ UTR (gift from Fire lab), was used as a template to generate 2-kb *Δpes-10::GFP* products. The primers used for amplification are 5′-GCTTGCATGCCTGCAGGCCTTG-3′ and 5′-AAGGGCCCG-TACGGCCGACTAGTAGG-3′. All *nhr-67* gene fragments were amplified from the C08F8 cosmid and were stitched together with the *Δpes-10::GFP* fragment via PCR fusion [63] and were designated as "pdd-1 constructs." Construct (1) consists of 1-kb promoter sequence, the entire coding region, and introns and 2 kb of the 3′ noncoding region attached to minimal *Δpes-10::GFP.* The primers used to amplify this template are 5′-CTGCTCAAAACTTTTGCTCC-3′ (forward) and 5′-CAAGGCCTGCAGGCATGCAAGCTTAAA-GAACTACTGTAGTTTTTG-3′ (reverse). Construct (2) spans from the fourth intron to the 3′ noncoding region fused to minimal *Δpes-10::GFP.* This product was generated using the forward primer 5′-GTTCGATCATGGATCCTCTCC-3′ and the same reverse primer as construct (1). Construct (3) is an *nhr-67p::GFP* reporter that contains 1 kb of the native promoter stitched in-frame with a 700-bp coding fragment of GFP (amplified from the pPD95–69 vector, a gift from Fire lab). The resulting 1.7-kb gene product was subsequently fused to 4.5 kb of *nhr-67* regulatory sequences (that span from the fourth intron to the 3′ noncoding region) via PCR. Construct (4) contains 6-kb sequence upstream of the predicted first ATG of *nhr-67,* appended to minimal *Δpes-10::GFP.* The following primers were used to amplify this product: 5′-GAACCCGGCGACGTTACGGGGCTTC-3′ and 5′-CAAGGCCTGCAGGCATGCAAGCCATCTGTGAAACCGCAGTCAT-CAT-3′.

Reporter constructs were injected into *unc-119(ed4); him-5* worms using *unc-119*(+) [62] and pBSK+ (Stratagene) as coinjection markers. *lin-11(n389); syEx749* doubles were constructed by injecting the 8-kb *nhr-67::Δpes-10::GFP* construct into *lin-11(n389); unc-119(ed4); him-5* background using *unc-119*(+) as a rescue marker.

Supporting Information

Figure S1. *nhr-67* RNAi Results in a Highly Penetrant Pvl and Egl Phenotype

A mid-sagittal optical view of an adult *nhr-67* RNAi–treated hermaphrodite.

Found at doi:10.1371/journal.pgen.0030069.sg001 (5.3 MB TIF).

Figure S2. The Upstream Regulatory Sequence Drives *nhr-67* Expression in the Gonad

(A and B) Nomarski (left), fluorescence (center), and overlaid (right). (A) *nhr-67* is expressed in the AC in hermaphrodites and (B) in the linker cell in males.

Found at doi:10.1371/journal.pgen.0030069.sg002 (3.8 MB TIF).

Table S1. List of Screened Transcription Factor RNAi Clones

Found at doi:10.1371/journal.pgen.0030069.st001 (51 KB XLS).

Table S2. Strain List

Found at doi:10.1371/journal.pgen.0030069.st002 (22 KB XLS).

Accession Numbers

The WormBase Gene IDs (www.wormbase.org) as well as the Refseq accession numbers (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide) for the genes described in this study are *ajm-1:*WBGene00000100 (NM__077135; NM__077137; NM__077136; NM__171966); *cdh-3:*WBGene00000395 (NM__066286); *ceh-2:*WBGene00000429 (NM__059345); *cog-1:*WBGene00000584 (NM__182115); *eff-1:*WBGene00001159 (NM__001026819); *egl-17:*WBGene00001185(NM__075706); *egl-26:*WBGene00001193 (NM__061251); *egl-38:*WBGene00001204 (NM__069435); *lin-3:*WBGene00002992 (NM__171418;NM__171919;NM__171918); *lin-11:*WBGene00003000 (NM__060295); *nhr-67:* WBGene00003657 (NM__069693); *pax-2:*WBGene00003938 (NM__068112); *unc-53:* WBGene00006788 (NM__001027000;NM__001026999); and *zmp-1:*WBGene00006987 (NM__171138).

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Author contributions. JSF and PWS conceived and designed the experiments, analyzed the data, and wrote the paper. JSF performed the experiments.

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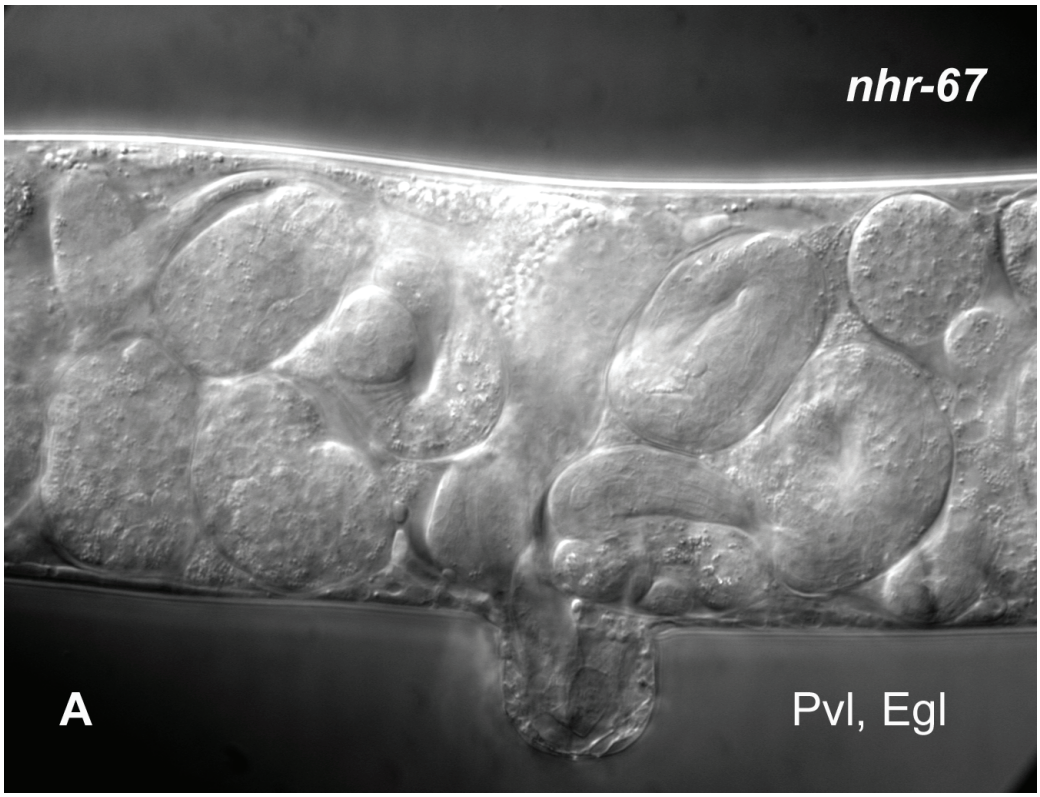
Competing interests. The authors have declared that no competing interests exist.

References

1. Levine M, Davidson EH (2005) Gene regulatory networks for development. Proc Natl Acad Sci U S A 102: 4936–4942.
2. Longabaugh WJ, Davidson EH, Bolouri H (2005) Computational representation of developmental genetic regulatory networks. Dev Biol 283: 1–16.
3. Oliveri P, Davidson EH (2004) Gene regulatory network controlling embryonic specification in the sea urchin. Curr Opin Genet Dev 14: 351–360.
4. Stathopoulos A, Levine M (2004) Whole-genome analysis of *Drosophila* gastrulation. Curr Opin Genet Dev 14: 477–484.
5. Koide T, Hayata T, Cho KW (2005) *Xenopus* as a model system to study transcriptional regulatory networks. Proc Natl Acad Sci U S A 102: 4943–4948.
6. Sternberg PW (2005) Vulval development. The *C. elegans* Research Community, WormBook. doi/10.1895/wormbook.1.6.1, http://www.wormbook.org.
7. Sulston JE, Horvitz HR (1977) Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. Dev Biol 56: 110–156.
8. Sharma-Kishore R, White JG, Southgate E, Podbilewicz B (1999) Formation of the vulva in *Caenorhabditis elegans:* A paradigm for organogenesis. Development 126: 691–699.
9. Inoue T, Sherwood DR, Aspock G, Butler JA, Gupta BP, et al. (2002) Gene expression markers for *Caenorhabditis elegans* vulval cells. Mech Dev 119 (Suppl 1): S203–S209.
10. Wang M, Sternberg PW (2000) Patterning of the *C. elegans* 1° vulval lineage by Ras and Wnt pathways. Development 127: 5047–5058.
11. Inoue T, Oz HS, Wiland D, Gharib S, Deshpande R, et al. (2004) *C. elegans* LIN-18 is a Ryk ortholog and functions in parallel to LIN-17/Frizzled in Wnt signaling. Cell 118: 795–806.
12. Ferguson EL, Sternberg PW, Horvitz HR (1987) A genetic pathway for the specification of the vulval cell lineages of *Caenorhabditis elegans*. Nature 326: 259–267.
13. Sawa H, Lobel L, Horvitz HR (1996) The *Caenorhabditis elegans* gene *lin-17*, which is required for certain asymmetric cell divisions, encodes a putative seven-transmembrane protein similar to the *Drosophila* Frizzled protein. Genes Dev 10: 2189–2197.
14. Chang C, Newman AP, Sternberg PW (1999) Reciprocal EGF signaling back to the uterus from the induced *C. elegans* vulva coordinates morphogenesis of epithelia. Curr Biol 9: 237–246.
15. Rajakumar V, Chamberlin HM (2006) The Pax2/5/8 gene *egl-38* coordinates organogenesis of the *C. elegans* egg-laying system. Dev Biol 301: 240–253.
16. Burdine RD, Branda CS, Stern MJ (1998) EGL-17(FGF) expression coordinates the attraction of the migrating sex myoblasts with vulval induction in *C. elegans*. Development 125: 1083–1093.
17. Burdine RD, Chen EB, Kwok SF, Stern MJ (1997) *egl-17* encodes an invertebrate fibroblast growth factor family member required specifically for sex myoblast migration in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 94: 2433–2437.
18. Hanna-Rose W, Han M (2002) The *Caenorhabditis elegans* EGL-26 protein mediates vulval cell morphogenesis. Dev Biol 241: 247–258.
19. Inoue T, Wang M, Ririe TO, Fernandes JS, Sternberg PW (2005) Transcriptional network underlying *Caenorhabditis elegans* vulval development. Proc Natl Acad Sci U S A 102: 4972–4977.
20. Aspöck G, Ruvkun G, Burglin TR (2003) The *Caenorhabditis elegans ems* class homeobox gene *ceh-2* is required for M3 pharynx motoneuron function. Development 130: 3369–3378.
21. Wang X, Greenberg JF, Chamberlin HM (2004) Evolution of regulatory elements producing a conserved gene expression pattern in *Caenorhabditis*. Evol Dev 6: 237–245.
22. Tiensuu T, Larsen MK, Vernersson E, Tuck S (2005) *lin-1* has both positive and negative functions in specifying multiple cell fates induced by Ras/MAP kinase signaling in *C. elegans*. Dev Biol 286: 338–351.
23. Newman AP, Inoue T, Wang M, Sternberg PW (2000) The *Caenorhabditis elegans* heterochronic gene *lin-29* coordinates the vulval-uterine-epidermal connections. Curr Biol 10: 1479–1488.
24. Cui M, Han M (2003) *Cis*-regulatory requirements for vulval cell-specific expression of the *Caenorhabditis elegans* fibroblast growth factor gene *egl-17*. Dev Biol 257: 104–116.
25. Freyd G, Kim SK, Horvitz HR (1990) Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene *lin-11*. Nature 344: 876–879.
26. Gupta BP, Wang M, Sternberg PW (2003) The *C. elegans* LIM homeobox gene *lin-11* specifies multiple cell fates during vulval development. Development 130: 2589–2601.
27. Palmer RE, Inoue T, Sherwood DR, Jiang LI, Sternberg PW (2002) *Caenorhabditis elegans cog-1* locus encodes GTX/Nkx6.1 homeodomain proteins and regulates multiple aspects of reproductive system development. Dev Biol 252: 202–213.
28. Chamberlin HM, Palmer RE, Newman AP, Sternberg PW, Baillie DL, et al. (1997) The PAX gene *egl-38* mediates developmental patterning in *Caenorhabditis elegans*. Development 124: 3919–3928.
29. Trent C, Tsuing N, Horvitz HR (1983) Egg-laying defective mutants of the nematode *Caenorhabditis elegans*. Genetics 104: 619–647.
30. Eisenmann DM, Kim SK (2000) Protruding vulva mutants identify novel loci and Wnt signaling factors that function during *Caenorhabditis elegans* vulva development. Genetics 156: 1097–1116.
31. Koh K, Peyrot SM, Wood CG, Wagmaister JA, Maduro MF, et al. (2002) Cell fates and fusion in the *C. elegans* vulval primordium are regulated by the EGL-18 and ELT-6 GATA factors—apparent direct targets of the LIN-39 Hox protein. Development 129: 5171–5180.
32. Simmer F, Moorman C, van der Linden AM, Kuijk E, van den Berghe PV, et al. (2003) Genome-wide RNAi of *C. elegans* using the hypersensitive *rrf-3* strain reveals novel gene functions. PLoS Biol 1: e12. doi:10.1371/journal.pbio.0000012
33. Mohler WA, Shemer G, del Campo JJ, Valansi C, Opoku-Serebuoh E, et al. (2002) The type I membrane protein EFF-1 is essential for developmental cell fusion. Dev Cell 2: 355–362.
34. Gissendanner CR, Crossgrove K, Kraus KA, Maina CV, Sluder AE (2004) Expression and function of conserved nuclear receptor genes in *Caenorhabditis elegans*. Dev Biol 266: 399–416.
35. Fire A, Ahnn J, Kelly WG, Harfe B, Kostas SA, et al. (1998) GFP applications in *C. elegans*. In: Kain MCS, editor. GFP strategies and applications. New York: John Wiley and Sons. pp. 153–168.
36. Wang M (2000) Pattern formation during *Caenorhabditis elegans* vulval development [PhD thesis]. California Institute of Technology.
37. Sherwood DR, Butler JA, Kramer JM, Sternberg PW (2005) FOS-1 promotes basement-membrane removal during anchor-cell invasion in *C. elegans*. Cell 121: 951–962.
38. Sternberg PW, Horvitz HR (1986) Pattern formation during vulval development in *C. elegans*. Cell 44: 761–772.
39. Weigel D, Jurgens G, Klingler M, Jackle H (1990) Two gap genes mediate maternal terminal pattern information in *Drosophila*. Science 248: 495–498.
40. Steingrimsson E, Pignoni F, Liaw GJ, Lengyel JA (1991) Dual role of the *Drosophila* pattern gene *tailless* in embryonic termini. Science 254: 418–421.

41. Pignoni F, Baldarelli RM, Steingrimsson E, Diaz RJ, Patapoutian A, et al. (1990) The *Drosophila* gene *tailless* is expressed at the embryonic termini and is a member of the steroid receptor superfamily. Cell 62: 151–163.
42. Casanova J (1990) Pattern formation under the control of the terminal system in the *Drosophila* embryo. Development 110: 621–628.
43. Lehmann R, Nusslein-Volhard C (1987) *hunchback*, a gene required for segmentation of an anterior and posterior region of the *Drosophila* embryo. Dev Biol 119: 402–417.
44. Hartmann B, Reichert H, Walldorf U (2001) Interaction of gap genes in the *Drosophila* head: *tailless* regulates expression of *empty spiracles* in early embryonic patterning and brain development. Mech Dev 109: 161–172.
45. Rudolph KM, Liaw GJ, Daniel A, Green P, Courey AJ, et al. (1997) Complex regulatory region mediating *tailless* expression in early embryonic patterning and brain development. Development 124: 4297–4308.
46. Hoch M, Gerwin N, Taubert H, Jackle H (1992) Competition for overlapping sites in the regulatory region of the *Drosophila* gene *Kruppel*. Science 256: 94–97.
47. Goriely A, Stella M, Coffinier C, Kessler D, Mailhos C, et al. (1996) A functional homolog of *goosecoid* in *Drosophila*. Development 122: 1641–1650.
48. Yutzey KE, Kirby ML (2002) Wherefore heart thou? Embryonic origins of cardiogenic mesoderm. Dev Dyn 223: 307–320.
49. Kirby ML (2002) Molecular embryogenesis of the heart. Pediatr Dev Pathol 5: 516–543.
50. Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, et al. (2003) Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell 5: 877–889.
51. Johnston RJ Jr, Chang S, Etchberger JF, Ortiz CO, Hobert O (2005) MicroRNAs acting in a double-negative feedback loop to control a neuronal cell fate decision. Proc Natl Acad Sci U S A 102: 12449–12454.
52. Vallstedt A, Muhr J, Pattyn A, Pierani A, Mendelsohn M, et al. (2001) Different levels of repressor activity assign redundant and specific roles to *Nkx6* genes in motor neuron and interneuron specification. Neuron 31: 743–755.
53. Ptashne M (2004) A genetic switch: Phage lambda revisited. Cold Spring Harbor (New York): Cold Spring Harbor Laboratory Press. 154 p.
54. Kobayashi H, Kaern M, Araki M, Chung K, Gardner TS, et al. (2004) Programmable cells: Interfacing natural and engineered gene networks. Proc Natl Acad Sci U S A 101: 8414–8419.
55. Gardner TS, Cantor CR, Collins JJ (2000) Construction of a genetic toggle switch in *Escherichia coli*. Nature 403: 339–342.
56. Becskei A, Serrano L (2000) Engineering stability in gene networks by autoregulation. Nature 405: 590–593.
57. Rosenfeld N, Elowitz MB, Alon U (2002) Negative autoregulation speeds the response times of transcription networks. J Mol Biol 323: 785–793.
58. Hwang BJ, Sternberg PW (2004) A cell-specific enhancer that specifies *lin-3* expression in the *C. elegans* anchor cell for vulval development. Development 131: 143–151.
59. Gupta BP, Sternberg PW (2002) Tissue-specific regulation of the LIM homeobox gene *lin-11* during development of the *Caenorhabditis elegans* egg-laying system. Dev Biol 247: 102–115.
60. Mello CC, Kramer JM, Stinchcomb D, Ambros V (1991) Efficient gene transfer in *C. elegans:* Extrachromosomal maintenance and integration of transforming sequences. Embo J 10: 3959–3970.
61. Stringham E, Pujol N, Vandekerckhove J, Bogaert T (2002) *unc-53* controls longitudinal migration in *C. elegans*. Development 129: 3367–3379.
62. Maduro M, Pilgrim D (1995) Identification and cloning of *unc-119,* a gene expressed in the *Caenorhabditis elegans* nervous system. Genetics 141: 977–988.
63. Hobert O (2002) PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic *C. elegans*. Biotechniques 32: 728–730.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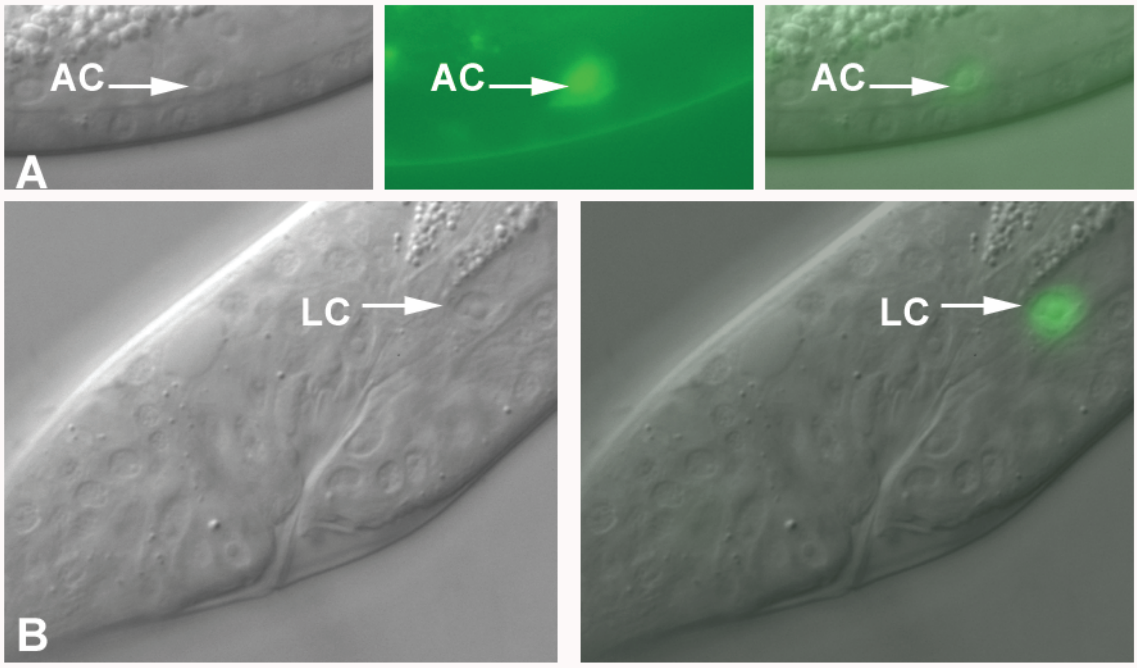
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CHAPTER III:

Gene Regulatory Networks Controlling Vulval Patterning and Morphogenesis

Summary

Regulation of gene expression (pattern formation) is responsible for the spatial organization of specialized structures (e.g., organs) in the overall body plan. Spatially defined gene expression patterns are the product of transcription factor networks operating within specific boundaries. It is thus necessary to evaluate the different roles of these transcription factors so as to deconstruct the patterning events that lead up to organogenesis. In this chapter, we extend our knowledge of the gene regulatory network that controls vulval patterning and morphogenesis. Several key transcription factors (*lin-11*, *nhr-67,* and *cog-1*) maintain cell identity by regulating target gene expression and restricting inappropriate fusion between the distinct vulval cell types. We uncover additional regulatory interactions with *egl-38*, further illustrating recurrent network themes such as boundary repression, lockdown mechanisms and, cell-type specific differentiation programs. Here I highlight the similarities and differences of the vulval network strategies (compared to other well-characterized networks) and speculate on the causality behind the network architecture in a specific subset of the vulval cells (vulE and vulF). We argue that the collaboration of these general network motifs with the novel regulatory motif (cross inhibition in conjunction with dual negative autoregulation, described in Chapter II) is what rapidly differentiates the 1° vulE and vulF lineages from each other.

Introduction

Complex morphologies are the product of spatially defined fate patterns in diverse cell- and tissue-types. Establishment of these precise fate patterns is dependent on the accurate execution of spatio-temporal gene expression. Understanding how complex gene

expression patterns are generated is crucial in deciphering events that lead up to organogenesis. Typically, intercellular signals and transcription factor networks act on cis-regulatory modules to regulate target gene expression within individual cell types (Levine and Davidson, 2005). Gene regulatory networks orchestrate diverse processes such as fate specification, patterning of gene expression, differentiation, and morphogenesis. These networks also provide direct visualization of all the elaborate interconnections between regulatory genes, signaling components, and their downstream effectors. Dorsal-ventral patterning and segmentation in *Drosophila*, endomesoderm specification in *Strongylocentrotus purpuratus*, and mesoderm specification in *Xenopus* represent a few of the contemporary regulatory networks that have been well characterized (Koide et al., 2005; Ochoa-Espinosa et al., 2005; Oliveri and Davidson, 2004; Stathopoulos and Levine, 2004). However, the vast majority of these intensively studied examples feature embryonic networks. Comparison of gene regulatory networks in diverse species at distinct developmental stages is necessary so as to broaden our general views on organ morphogenesis.

The postembryonic *C. elegans* vulva is an elegant model to dissect the genomic networks that regulate gene expression and organogenesis. High-resolution experimental approaches are plausible in the nematode because of features like reverse genetics, trans-genesis and invariant cell lineage (Lee et al., 2004). The *C. elegans* vulva is the organ that serves as a channel for transport of fertilized eggs and sperm and connects the uterine lumen to the external environment. The vulval cells arise from a group of precursor cells (P3.p–P8.p) generally referred to as vulval precursor cells (VPCs). During the mid- third larval stage, the central three VPCs P5.p–P7.p are induced to adopt vulval fates via EGF

and Notch signaling, whereas the remaining three VPCs adopt non-vulval fates (Sternberg, 2005). During the L4 (fourth larval) stage, seven terminally differentiated vulval cell types arise, each with its own unique gene expression profile and morphogenetic migration patterns (Inoue et al., 2002; Sharma-Kishore et al., 1999). The P6.p descendents constitute the 1° vulE and vulF cells, whereas the P5.p and P7.p descendents constitute the 2° vulA, vulB1, vulB2, vulC, and vulD cells. While the signaling network that initiates vulval development and sets the gross pattern of cell differentiation is well characterized, the network of transcription factors that specifies the final seven cell fates is not understood (Sternberg, 2005).

Each vulval cell type can be described as a specialized epithelial cell with its own set of properties. For example, the vulA cells which form the base of the reproductive organ, attach to the hypodermal syncytium. In contrast, the vulF cells which comprise the innermost portion of the vulva directly connect to the uterus. The differential gene expression programs in these discrete cell types might explain their underlying specialized functions. In this chapter, we broaden our knowledge of the vulval regulatory network that differentiates the mature cell types and directs organogenesis. The intricate connections between the different transcription factors and their targets demonstrate that the network architecture for each of the vulval cell types is distinct.

Materials and Methods

Microscopy

Transgenic worms were anesthetized using 3 mM levamisole and observed using Nomarski optics (http://www.nomarski.com). Photographs were taken with a

monochrome Hamamatsu digital camera (http://www.hamamatsu.com) and Improvision Openlab 4.0.4 software (http://www.improvision.com). The fluorescent images were overlaid with their respective DIC images using Adobe Photoshop 7.0.1 (http://www.adobe.com).

Genetics and RNAi

C. elegans strains were cultured at 20°C using standard protocols (Brenner, 1974). Transgenes used in this study are as follows: *syIs54 [ceh-2::GFP]*, *syIs51 [cdh-3::CFP]*, *syIs101[dhs-31/T04B2.6::yfp]*, *syIs61[F47B8.6::gfp]* (Inoue et al., 2002), *syIs78 [ajm-1::GFP]* (Gupta et al., 2003), *syIs107 [lin-3::GFP]* (Hwang and Sternberg, 2004), *guEx64 [pax-2::GFP]* (gift from Chamberlin lab), *guEx877 [egl-38::GFP]* (gift from Chamberlin lab), *kuIs36 [egl-26::GFP]* (Hanna-Rose and Han, 2002), *syEx724 [bam-2::GFP]*, *syEx756 [unc-53::GFP]*. Alleles used in this study: LGI, *lin-11(n389);* LGII, *cog-1(sy275), lin-29(n333), lin-29 (sy292);* LGIII, *unc-119(ed4);* LGIV, *egl-38(n578);* LGV, *him-5(e1490).*

Transgenic lines were generated using standard microinjection protocol that produces high-copy-number extrachromosomal arrays (Mello et al., 1991). *syEx756, syEx724,* and *syEx[TRB202]* were generated by reinjecting the constructs pNP10 (Stringham et al., 2002) and pAC13 (Colavita and Tessier-Lavigne, 2003) respectively into *unc-119(ed4); him-5* background using *unc-119*(+) (Maduro and Pilgrim, 1995) and pBSK+ (Stratagene, http://www.stratagene.com) as coinjection markers.

We resorted to *nhr-67* RNAi feeding for this study since the *nhr-67* deletion allele *(ok631)* results in L1 lethality and/or arrest (International *C. elegans* Knockout

Consortium). The *nhr-67* RNAi feeding protocol is similar to that previously described (Simmer et al., 2003). Briefly, embryos were harvested by bleaching gravid adults and were placed on a lawn of *Escherichia coli* strain expressing double-stranded RNA at 20°C. Animals were scored using Nomarski microscopy after 36 h (during the L4 stage).

Results

lin-11, lin-29, cog-1, egl-38, and *nhr-67* form a transcriptional network that regulate vulval patterning of gene expression

There are currently around 32 reporter genes that are expressed during different temporal windows in the diverse subsets of the vulval cells (Table 1). A quarter of these transgenic reporters encode sequence-specific transcription factors and the rest encode an assortment of catalytic proteases, structural proteins, signaling molecules and guidance cues. Although the physiological relevance for some of these vulval genes is not entirely understood, it is presumed that they all contribute in some way to the specialization of the discrete vulval cell types. *lin-11* (Freyd et al., 1990; Gupta et al., 2003), *lin-29* (Newman et al., 2000), *cog-1* (Inoue et al., 2005; Palmer et al., 2002), *egl-38* (Chamberlin et al., 1997; Chang et al., 1999; Inoue et al., 2005) were previously shown to regulate expression of a small subset of these vulval genes. *nhr-67*, the *tailless* hormone receptor was initially identified in a reverse genetics screen as a regulator of *ceh-2* expression (described in Chapter II). In collaboration with Ted Ririe, we sought to expand the vulval network by genetically perturbing the above four transcription factors in various transgenic reporter backgrounds. Our collective findings are all summarized in Fig. 1. The current trend of the network depicts *lin-11* as the major positive regulator of vulval

gene expression whereas *cog-1*, *nhr-67* and *egl-38* function as boundary repressors, particularly in the 1° vulE and vulF lineages.

Components of the vulval network regulate cell fusion

During the L4 stage, the specialized vulval cell types invaginate to assume a characteristic morphology. The similar cell-types fuse together and generate seven sequential toroid rings that line the vulval lumen. One of the general features all the vulval cell-types share is the expression of *ajm-1*, a component of the apical junction that connects to adjacent cells in epithelial tissues. We wanted to ascertain whether the components of the transcription factor network also play a role in maintaining the general attributes of the vulval epithelial cells in addition to cell-type specific gene expression. The cell number and architecture of the vulval toroids can be assessed using the *ajm-1::GFP* reporter, which localizes to adherens junctions (Fig. 2). We previously found that *nhr-67* RNAi-treated animals sometimes display cell-fusion defects that are restricted to the vulE and vulF cell types (Chapter II). *egl-38* mutants lack any fusion defects, although the vulF cells are mispositioned (Rajakumar and Chamberlin, 2006). *cog-1(sy275)* mutants were frequently missing 1–2 toroid rings as opposed to wild-type hermaphrodites (Fig. 2). Unlike *nhr-67*, cell fusion is defective in a broader range of cell types (vulA, vulB, vulC, vulD, and vulE). *lin-11(n389)* mutants consistently displayed the most dramatic fusion defects in that the majority of the toroid rings were missing (as many as 5 toroid rings) (Fig. 2) (Gupta et al., 2003). Fusion defects in *lin-29* mutants have not been assessed. We thus demonstrate that several components of the vulval

transcription factor network maintain the general identity of the vulval cells by regulating cell fusion, albeit to different degrees.

Regulatory interactions with *egl-38*

As described above and in Chapter II, a network of mutually interacting transcription factors regulates patterning of gene expression in the diverse vulval cell types. *lin-11, cog-1, egl-38,* and *nhr-67* are immersed in a complex network of mutual and autoregulatory interactions (Chapter II). This elaborate network establishes cell identity and ensures the execution of cell-type-specific gene expression programs. The recent availability of an *egl-38::GFP* transcriptional reporter allows us to investigate the effects of the different transcription factors on its vulval expression (Rajakumar and Chamberlin, 2006). During the L4 stage, *egl-38* expression has been detected exclusively in the vulF cells (Fig. 3). Disruption of *nhr-67* gene activity via RNAi results in the abolition of vulF-specific *egl-38* expression (Fig. 3). *cog-1(sy275)* mutants did not alter *egl-38* vulval expression. *egl-38* levels in the vulva are downregulated when *egl-38* function is compromised, indicating that it positively autoregulates in vulF cells (Fig. 3). The effects of *lin-11* on *egl-38* transcriptional activity have not been evaluated.

Discussion

Network strategies employed during vulval patterning and morphogenesis

The vulval network encompasses a broad spectrum of network strategies such as boundary repression, stable feedback loops, combinatorial control, and negative autoregulation. For example, the expression boundary of *cog-1* is reinforced by an

assembly of mutual and autoregulatory interactions, thereby preventing any inappropriate transcriptional activity in the terminally differentiated vulA, vulB, vulE, and vulF cell types. An illustration of a stable feedback loop in the vulval network would be the “and” logic circuit observed in the vulA cells. *lin-11* activity is necessary to drive vulA-specific expression of *nhr-67*, which is subsequently required to turn on *zmp-1* (an effector gene). In addition to the *lin-11* input, *nhr-67* positively autoregulates in vulA, thus creating a stable feedback loop that maintains its differentiated regulatory state over time. Combinatorial control circuits are also a prominent feature of the vulval patterning network. For instance, both *cog-1* and *egl-38* single mutants lack vulF specific defects in *egl-17* expression. Their regulatory roles in the vulF cells become unmasked only when both of their activities are compromised. The advantage of having such built-in redundancy is that it ensures the accurate execution of gene expression programs. Lastly, negative autoregulation appears to be a prevalent network motif in several vulval cell types, especially vulE and vulF cells. This regulatory motif speeds up the response time of gene circuits and is particularly useful when making rapid decisions about cell fates (Rosenfeld et al., 2002).

Thus the vulval network appears to utilize many of the differentiation strategies employed by other model systems. We also note that the combination and architecture of the various network strategies is divergent within the seven cell types. We postulate that these differences in the network architecture will partly account for how and why the diverse vulval cell types execute their final fates.

Plausible links between cell fusion and cell fate transformation?

Three of the four tested transcriptional regulators (*lin-11, nhr-67,* and *cog-1*) have demonstrable function when it comes to restricting inappropriate heterotypic cell fusion events. One plausible explanation for cell fate transformation is the occurrence of improper fusion events between the wrong cell types. In the case of reduced *nhr-67* function, the cell fusion defects are specific to the 1° vulval lineages and cannot account for the altered gene expression patterns observed in the 2° vulval lineages (vulA and vulD). The role of *nhr-67* in cell fusion is partially attributable to deregulation of the fusogen *eff-1* (Chapter II). Loss of *eff-1* however is not sufficient to rescue fusion defects in *nhr-67* RNAi animals, which in turn suggests that *nhr-67* negatively regulates additional targets that mediate fusion (data not shown). In contrast, *egl-38* impacts gene expression in vulB, vulC, vulD, vulE and vulF (Chapter II). The alteration of these vulval expression patterns in *egl-38* mutants is not the consequence of fusion defects since the cell number and overall architecture of the toroid rings is wild-type (Rajakumar and Chamberlin, 2006). At present, we lack any conclusive evidence that shows a correlation between cell fusion defects and perturbed gene expression patterns in *cog-1* and *lin-11* mutants. One possibility is that *cog-1* and *lin-11* impede cell fusion events so as to forestall any cell fate transformations. Alternatively, the two regulatory factors might function in two discrete processes during vulval organogenesis: regulation of cell fusion and patterning of gene expression.

eff-1 encodes a type I membrane protein that is essential for developmental cell fusion. Homotypic cell fusion events between the seven vulval cell types are completely blocked in an *eff-1* mutant background (Mohler et al., 2002), and overexpression of *eff-1*

via heat shock is sufficient to fuse vulval cells that do not normally fuse (Shemer et al., 2004). Strict spatial control of such a fusogen is thus essential to maintain the structural integrity of the vulva. We have shown that *eff-1* is one of the two physiologically relevant targets of *nhr-67* during vulval morphogenesis (Chapter II). Monitoring *eff-1* levels in *cog-1* and *lin-11* mutants would address whether these transcription factors also regulate the activity of the fusogen by spatially restricting its expression.

Flexibility between vulE and vulF fates

Our group previously demonstrated that patterning between the E and F lineages requires both a short-range anchor cell (AC) dependent signal, as well as Wnt signaling (Wang, 2000). However, the downstream transcription factor network that specifies the properties of the 1° vulval cells was unknown. The regulatory functions of *nhr-67*, *cog-l*, and *egl-38* in the 1° vulval cells are similar in that they inhibit the expression of several L4-specific genes (*ceh-2* and *egl-17*), that are hallmarks of 2° fate (Chapter 2). However, our understanding as to how these regulatory genes differentiate between the two possible 1° vulval cell fates was limited. As described in Chapter II, *cog-1* and *nhr-67* inhibit both one another and themselves exclusively in the 1° vulE and vulF cells. We speculated that these two distinct circuits might enable cells to reach a regulatory state with built-in flexibility, allowing rapid switching of their fates upon transient inputs. Our recent interaction data with *egl-38* provides us with some insight into the possible relevance of this striking biological circuit (Fig. 4).

We previously hypothesized that the vulF cells are biased by proximity to the AC to have higher levels of *nhr-67* compared to *cog-1*. Conversely, we argued that elevated

levels of *cog-1* compared to *nhr-67* correlate with vulE fate. We have now shown that *nhr-67* activates *egl-38* expression in the vulF cells, which is subsequently necessary for uv1 fate specification (Chang et al., 1999). Establishment of a proper vulval-uterine connection is a key property that is associated with execution of vulF fate. *egl-38* is also required for the repression of certain 'vulE characteristics' in the vulF cells, namely inhibition of *zmp-1* expression. Furthermore, *egl-38* positively autoregulates in vulF cells, thereby locking down its differentiation state and eliminating any dependence on initial transient inputs. These data are thus consistent with our previous model where elevated *nhr-67* levels promote the execution of vulF fate in the 1° vulval cells. In light of these new findings, it is even more conceivable that the mutual and auto-inhibitory circuits of *nhr-67* and *cog-1* promote rapid fate switching in response to altered intra/intercellular inputs. The observation of E lineages compensating for uv1 fate specification (a vulF specific property) when F lineages are disrupted, is consistent with this model (Chang et al., 1999). A rapid fate switching mechanism in the 1° vulval cells would ensure the establishment of a proper vulval-uterine connection especially in the event where the vulF cells are damaged. This feature of built-in flexibility serves as a failsafe mechanism to guard against environmental damage or internal errors that occur during organogenesis.

References

Chamberlin, H. M., Palmer, R. E., Newman, A. P., Sternberg, P. W., Baillie, D. L., and Thomas, J. H. (1997). The PAX gene *egl-38* mediates developmental patterning in *Caenorhabditis elegans*. Development *124*, 3919–3928.

Chang, C., Newman, A. P., and Sternberg, P. W. (1999). Reciprocal EGF signaling back to the uterus from the induced *C. elegans* vulva coordinates morphogenesis of epithelia. Curr Biol *9*, 237–246.

Colavita, A., and Tessier-Lavigne, M. (2003). A Neurexin-related protein, BAM-2, terminates axonal branches in *C. elegans*. Science *302*, 293–296.

Freyd, G., Kim, S. K., and Horvitz, H. R. (1990). Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene *lin-11*. Nature *344*, 876–879.

Gupta, B. P., Wang, M., and Sternberg, P. W. (2003). The *C. elegans* LIM homeobox gene *lin-11* specifies multiple cell fates during vulval development. Development *130*, 2589–2601.

Hanna-Rose, W., and Han, M. (2002). The *Caenorhabditis elegans* EGL-26 protein mediates vulval cell morphogenesis. Dev Biol *241*, 247–258.

Hwang, B. J., and Sternberg, P. W. (2004). A cell-specific enhancer that specifies *lin-3* expression in the *C. elegans* anchor cell for vulval development. Development *131*, 143–151.

Inoue, T., Sherwood, D. R., Aspock, G., Butler, J. A., Gupta, B. P., Kirouac, M., Wang, M., Lee, P. Y., Kramer, J. M., Hope, I., et al. (2002). Gene expression markers for *Caenorhabditis elegans* vulval cells. Mech Dev *119 Suppl 1*, S203–209.

Inoue, T., Wang, M., Ririe, T. O., Fernandes, J. S., and Sternberg, P. W. (2005). Transcriptional network underlying *Caenorhabditis elegans* vulval development. Proc Natl Acad Sci U S A *102*, 4972–4977.

Koide, T., Hayata, T., and Cho, K. W. (2005). *Xenopus* as a model system to study transcriptional regulatory networks. Proc Natl Acad Sci U S A *102*, 4943–4948.

Lee, J., Nam, S., Hwang, S. B., Hong, M., Kwon, J. Y., Joeng, K. S., Im, S. H., Shim, J., and Park, M. C. (2004). Functional genomic approaches using the nematode *Caenorhabditis elegans* as a model system. J Biochem Mol Biol *37*, 107–113.

Levine, M., and Davidson, E. H. (2005). Gene regulatory networks for development. Proc Natl Acad Sci U S A *102*, 4936–4942.

Maduro, M., and Pilgrim, D. (1995). Identification and cloning of *unc-119*, a gene expressed in the *Caenorhabditis elegans* nervous system. Genetics *141*, 977–988.

Mello, C. C., Kramer, J. M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. Embo J *10*, 3959–3970.

Mohler, W. A., Shemer, G., del Campo, J. J., Valansi, C., Opoku-Serebuoh, E., Scranton, V., Assaf, N., White, J. G., and Podbilewicz, B. (2002). The type I membrane protein EFF-1 is essential for developmental cell fusion. Dev Cell *2*, 355–362.

Newman, A. P., Inoue, T., Wang, M., and Sternberg, P. W. (2000). The *Caenorhabditis elegans* heterochronic gene *lin-29* coordinates the vulval-uterine-epidermal connections. Curr Biol *10*, 1479–1488.

Ochoa-Espinosa, A., Yucel, G., Kaplan, L., Pare, A., Pura, N., Oberstein, A., Papatsenko, D., and Small, S. (2005). The role of binding site cluster strength in Bicoid-dependent patterning in *Drosophila*. Proc Natl Acad Sci U S A *102*, 4960–4965.

Oliveri, P., and Davidson, E. H. (2004). Gene regulatory network controlling embryonic specification in the sea urchin. Curr Opin Genet Dev *14*, 351–360.

Palmer, R. E., Inoue, T., Sherwood, D. R., Jiang, L. I., and Sternberg, P. W. (2002). *Caenorhabditis elegans cog-1* locus encodes GTX/Nkx6.1 homeodomain proteins and regulates multiple aspects of reproductive system development. Dev Biol *252*, 202–213.

Rajakumar, V., and Chamberlin, H. M. (2006). The Pax2/5/8 gene *egl-38* coordinates organogenesis of the *C. elegans* egg-laying system. Dev Biol *301*, 240–253.

Rosenfeld, N., Elowitz, M. B., and Alon, U. (2002). Negative autoregulation speeds the response times of transcription networks. J Mol Biol *323*, 785–793.

Sharma-Kishore, R., White, J. G., Southgate, E., and Podbilewicz, B. (1999). Formation of the vulva in *Caenorhabditis elegans*: a paradigm for organogenesis. Development *126*, 691–699.

Shemer, G., Suissa, M., Kolotuev, I., Nguyen, K. C., Hall, D. H., and Podbilewicz, B. (2004). EFF-1 is sufficient to initiate and execute tissue-specific cell fusion in C. elegans. Curr Biol *14*, 1587–1591.

Simmer, F., Moorman, C., van der Linden, A. M., Kuijk, E., van den Berghe, P. V., Kamath, R. S., Fraser, A. G., Ahringer, J., and Plasterk, R. H. (2003). Genome-

wide RNAi of *C. elegans* using the hypersensitive *rrf-3* strain reveals novel gene functions. PLoS Biol *1*, E12.

Stathopoulos, A., and Levine, M. (2004). Whole-genome analysis of *Drosophila* gastrulation. Curr Opin Genet Dev *14*, 477–484.

Sternberg, P. W. (2005). Vulval development, *Wormbook*, ed., (The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.6.1, www.wormbook.org).

Stringham, E., Pujol, N., Vandekerckhove, J., and Bogaert, T. (2002). *unc-53* controls longitudinal migration in *C. elegans*. Development *129*, 3367–3379.

Wang, M. (2000). Pattern Formation during *Caenorhabditis elegans* Vulval Development. PhD Thesis, California Institute of Technology.

Figure Legends:

Fig. 1: Summary of network that functions during vulval patterning and differentiation in *C. elegans.* Diagram includes the activities of *cog-1, nhr-67, egl-38, lin-11,* and *lin-29*. Colored arrows represent positive inputs and colored block arrows represent repressor inputs for target gene expression in the distinct vulval cell types.

Fig. 2: Components of the vulval patterning network regulate cell fusion. (A–C) Nomarski (left), fluorescence (center), and overlaid (right). The adherens junction marker *ajm-1::GFP* is used to visualize the cell number and architecture of the vulval toroids in wild-type (A), *cog-1(sy275)* (B), and *lin-11(n389)* (C) mutants. When observing a mid-sagittal optical section of L4 hermaphrodites, *ajm-1::GFP* appears as dots between the vulval cells. Loss of adherens junction expression signifies a reduction in the cell number due to a cell fusion defect. (A) In wild-type animals, the eight dots on either side correspond to the seven distinct vulval cell types (arrows). The overall vulval morphology of *cog-1 and lin-11* mutants appears abnormal compared to wild-type. (B)

Disruption of *cog-1* activity often results in the loss of a single toroid ring. The arrow indicates an inappropriate fusion event between the vulC and vulD cells. (C) *lin-11* mutants exhibit the most dramatic fusion defects as they lack most of the vulval toroid rings (arrows).

Fig. 3: *egl-38* expression in the vulF cells is dependent on several regulatory inputs. (A–C) Nomarski (left), fluorescence (center), and overlaid (right). All animals displayed carry the *guEx877 [egl-38::GFP]* transgene in their background. (A) In wild-type animals, *egl-38* expression is detected exclusively in the vulF cells (arrows). (B) *nhr-67* RNAi results in the abolition of *egl-38* expression in the vulF cells (arrows). (C) *egl-38 (n578)* mutants lose the ability to positively autoregulate their expression levels in vulF (arrows).

Fig. 4: Differentiation of vulE vs. vulF. Arrows represent positive inputs and block arrows represent repressor inputs for target gene expression. Gray font indicates no detectable expression within the cell type whereas black font indicates detectable expression levels. Red circle indicates that the activity associated with a specific regulatory factor (*nhr-67* in vulF and *cog-1* in vulE) plays a major role in patterning gene expression within the specified cell type. (A and B) This model assumes that the interactions mediated by *nhr-67* occur autonomously in the vulE and vulF cells. (C and D) This alternate model presumes that *nhr-67* acts in the AC to differentiate between vulE and vulF cells. Signal X could be Ras, Wnt, or some other signaling pathway.

Table 1:

Gene	Expression (L4, adult)
bam-2	B,D,E, F
C55C3.5	F
unc-53	C, D
lin-3	F
egl-38	F
nhr-67	A, B, C
lin-39	A
lin-29	all vulval cells
nas-37	B
cog-1	C, D, E
col-7	A
dhs-31	B1, B2, D
daf-6	E, F
pax-2	D
B0034.1	E, F
egl-26	B, D, E
zmp-1	A, D, E
ceh-2	B1, B2, C
cdh-3	C, D, E, F
col-48	B1, B2, C, D
F48B9.5	C, D
grd-5	B1, B2
grl-1	B2
sqv-4	C, D, E, F
syg-2	E, F
grl-4	A, B1, B2, D
grl-10	A, B1
grl-12	C
grl-15	A, B1, B2, C, D
egl-17	C, D (E, F in late L3)
F47B8.6	C, D, E, F
lin-11	all vulval cells

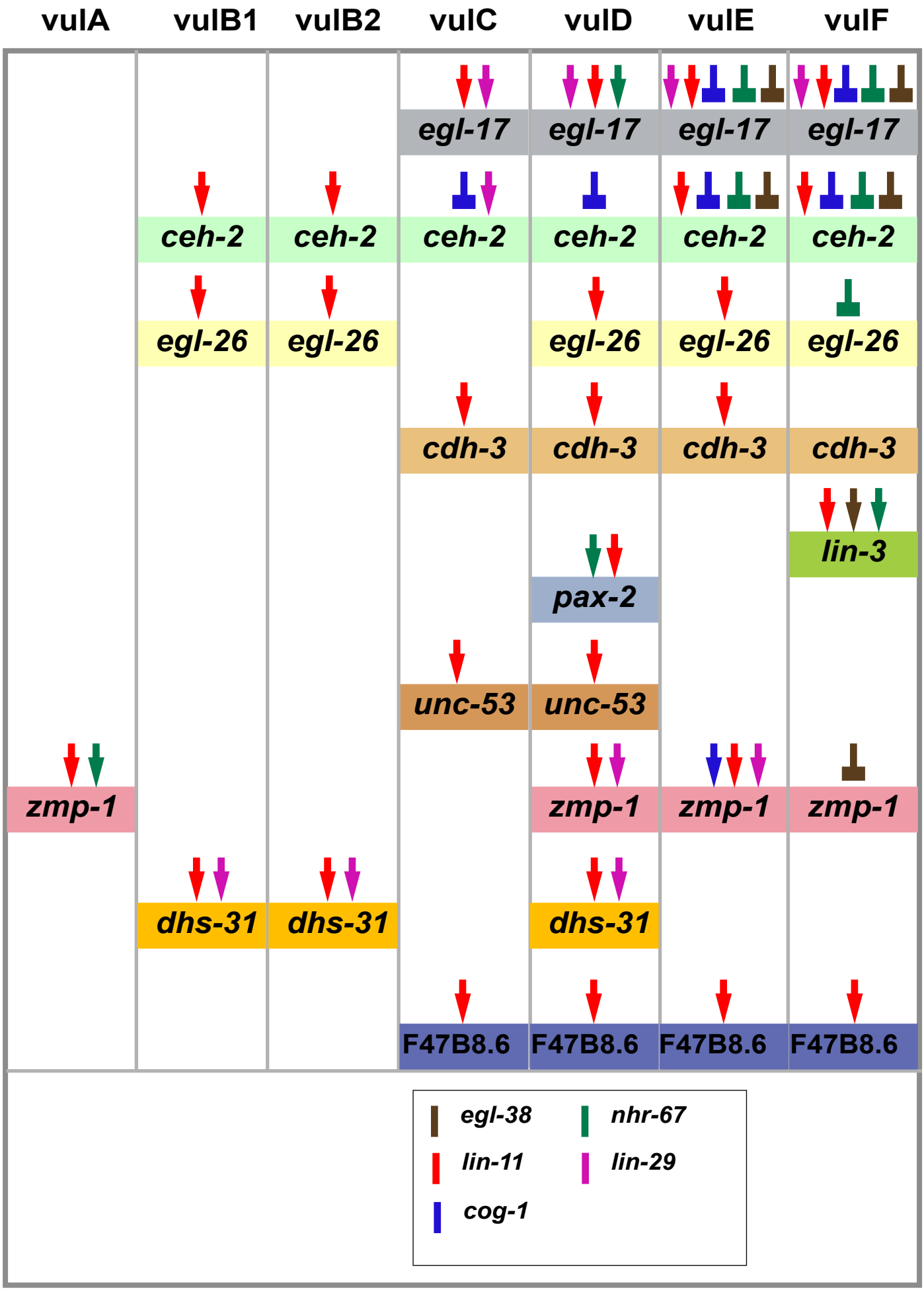
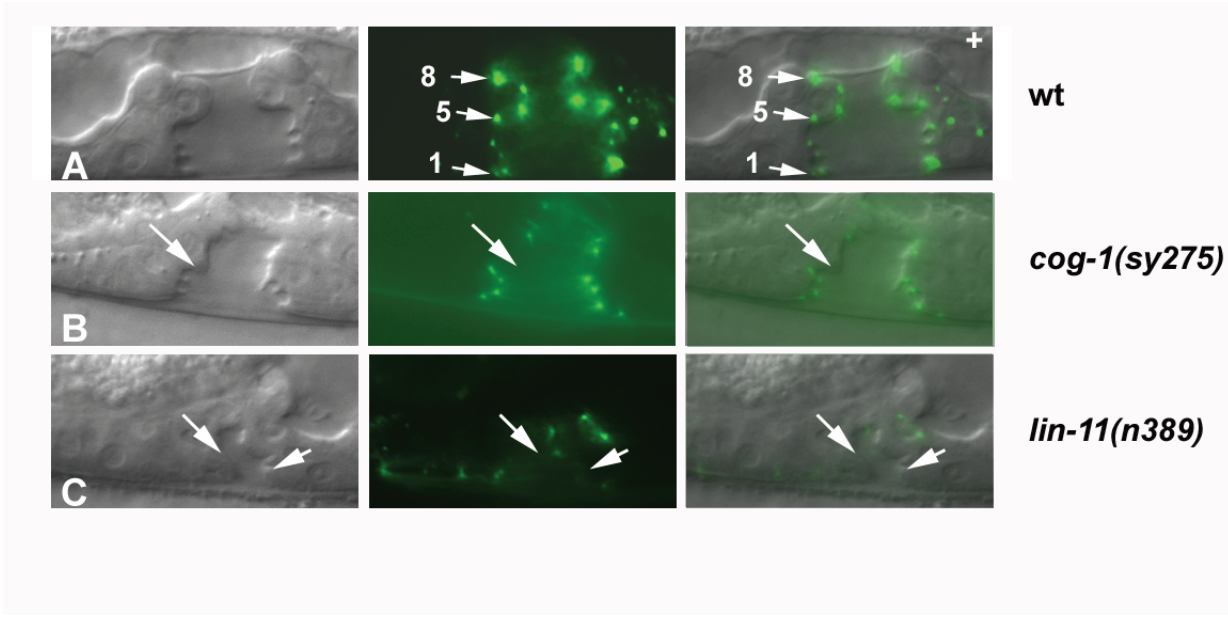
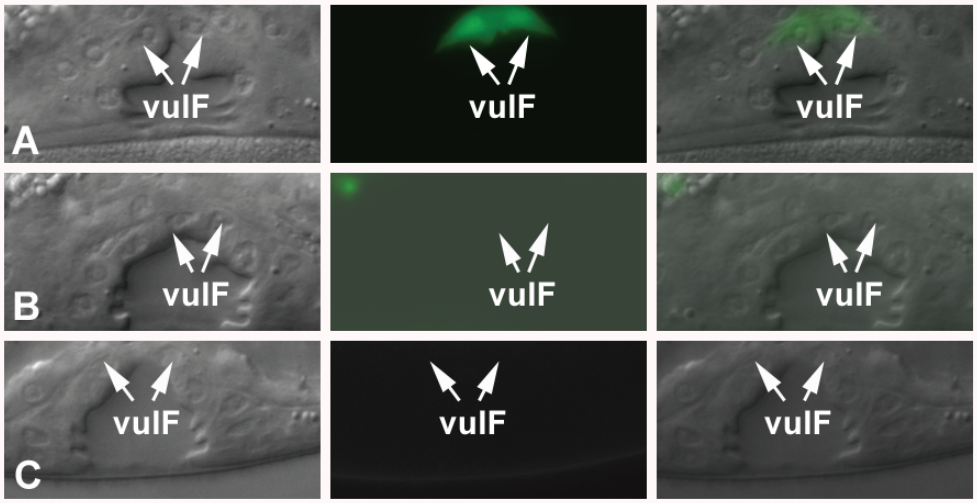
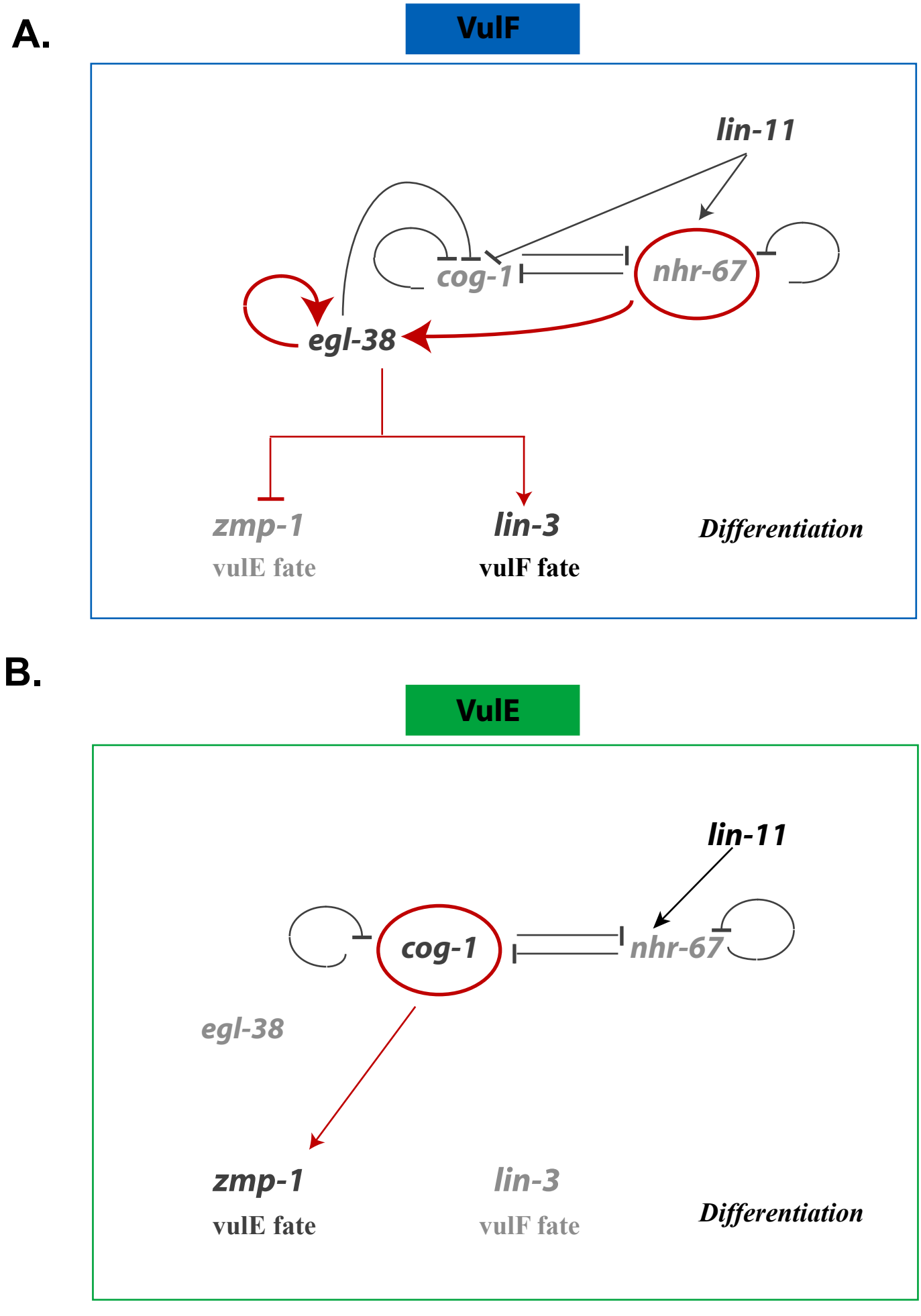
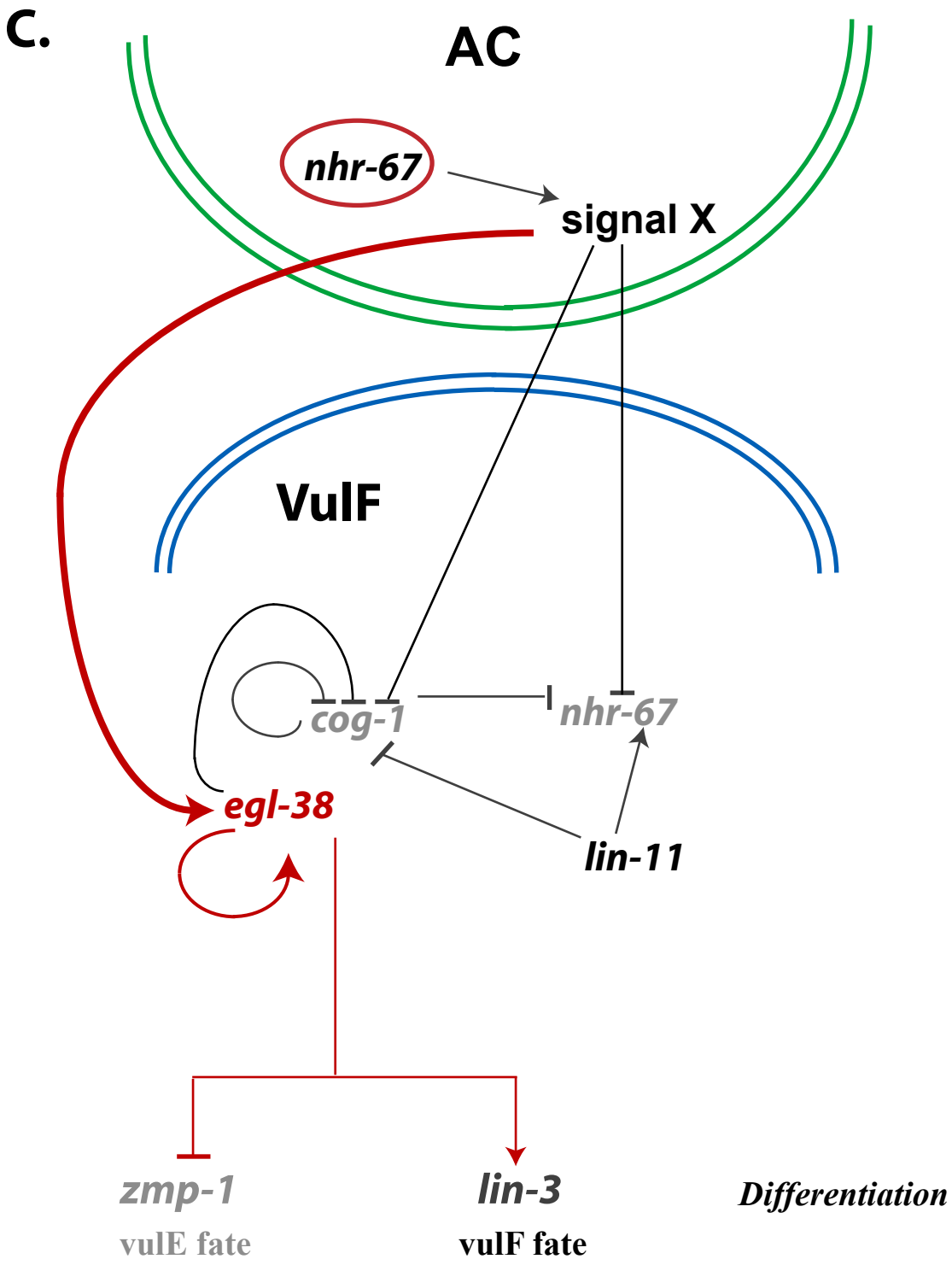

vulA
vulB1
vulB2
vulC
vulD
vulE
vulF
egl-17
ceh-2
egl-26
cdh-3
lin-3
pax-2
unc-53
zmp-1
dhs-31
F47B8.6
egl-38
nhr-67
lin-11
lin-29
cog-1

Fig. 2

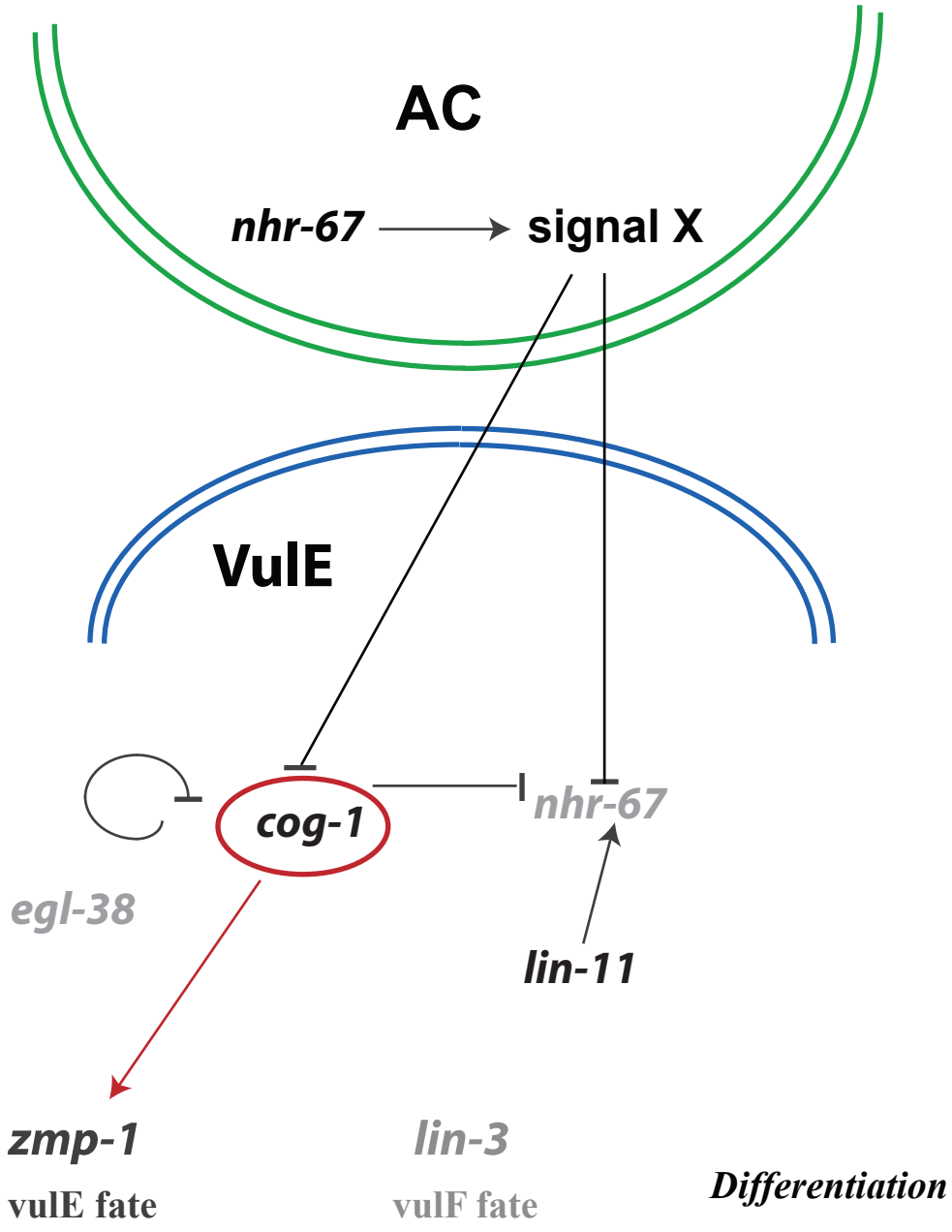



vulF
A
vulF
vulF
wt
vulF
B
vulF
vulF
nhr-67
vulF
C
vulF
vulF
egl-38


A.
VulF
lin-11
cog-1
nhr-67
egl-38
zmp-1
vulE fate
lin-3
vulF fate
Differentiation
B.
VulE
lin-11
cog-1
nhr-67
egl-38
zmp-1
vulE fate
lin-3
vulF fate
Differentiation


C.
AC
nhr-67
signal X
VulF
cog-1
nhr-67
egl-38
lin-11
zmp-1
vulE fate
lin-3
vulF fate
Differentiation

D.


AC
nhr-67
signal X
VulE
cog-1
nhr-67
egl-38
lin-11
zmp-1
vulE fate
lin-3
vulF fate
Differentiation

Appendix:

Cis-regulatory analysis of *ceh-2*

Preliminary cis-regulatory analysis of *ceh-2* demonstrates how different tissue-specific regulatory elements integrate diverse trans-acting inputs and generate a specific output, a crucial determinant of pattern formation.

Generation of *ceh-2* reporter transgenes

ceh-2::Δpes-10::GFP reporter gene constructs: The pPD97-78 vector, which includes the *Δpes-10* basal promoter driving GFP and the *unc-54* 3′ UTR (gift from Fire lab), was used as a template to generate 2-kb *Δpes-10::GFP* products. The primers used for amplification are 5′ GCTTGCATGCCTGCAGGCCTTG 3′ and 5′ AAGGGCCCGTACGGCCGACTAGTAGG 3′. All *ceh-2* gene fragments were amplified from the C27A12 cosmid and were stitched together with the *Δpes-10::GFP* fragment via PCR fusion (Hobert, 2002). The *syIs54* reporter contains ~ 10 kb genomic sequence that lies immediately upstream of the *ceh-2* translational start site (Inoue et al., 2005). The 5.3 kb promoter sequence that is proximal to the translational start site was amplified with the following primers: (forward) 5’ GACAAAACTGGCATGAGCC 3’ and (reverse) 5’CAAGGCCTGCAGGCATGCAAGCATGTTGCCATACAGATCCGC 3’.

The remaining 4.7 kb distal upstream fragment was generated using the following primers 5’ CTAAAAATGCTCGGGATGCTC 3’ (forward) and 5’ CAAGGCCTGCAGGCATGCAAGCCATGCCAGTTTTGTCCACAAG 3’ (reverse). The pTRB202 plasmid was a gift from the Burglin lab (Aspöck et al., 2003). Reporter

constructs were injected into *unc-119(ed4); him-5* worms using *unc-119*(+) (Maduro and Pilgrim, 1995) and pBSK+ (Stratagene) as coinjection markers.

Results:

Cis-regulatory analysis of L4 specific *ceh-2* expression:

Genotype (n)	Transgene	vulA	vulB	vulC	vulD	vulE	vulF
+ (41)	*syExTRB202*	-	+	-	-	-	-
nhr-67 (RNAi) (44)	*syExTRB202*	-	+	-	-	-	+
+ (40)	*syIs54*	-	++	-	-	-	-
nhr-67(RNAi) (40)	*syIs54*	-	++	-	-	++	++
cog-1 (sy275) (41)	*syIs54*	-	++	++	++	++	-
lin-11(n389)(40)	*syIs54*	-	-	-	-	-	-
+ (24)	*syEx 5.3k ceh-2*	-	++	++	++	-	-
nhr-67 (RNAi) (22)	*syEx 5.3k ceh-2*	-	++	++	++	++	++
+ (21)	*syEx 4.7k ceh-2*	-	-	-	-	-	-

Key:

++ : Robust expression

+: Faint expression

- : No expression

The TRB202 *ceh-2* translational fusion, which includes 5 kb of the upstream region up to the third intron, drives vulB expression at very low frequencies. However disruption of *nhr-67* activity in this transgenic background occasionally results in the derepression of

ceh-2 levels in vulF. The transcriptional reporter *syIs54* contains 10 kb sequence upstream of the translational start site. This particular reporter (as opposed to TRB202) displays frequent vulB expression during L4 stage. As described in Chapter II, this transgene contains elements that are responsive to *lin-11, cog-1,* and *nhr-67* function. We then split the 10 kb upstream sequence into two fragments: *syEx 5.3k* and *syEx 4.7k*. The *syEx 5.3k* construct contains the 5.3 kb sequence proximal to the translational start site. *syEx4.7kb* contains the 4.7 kb distal upstream sequence that immediately flanks the *syEx5.3k* sequence. *syEx4.7kb* transgene did not display any vulval expression. *syEx5.3k* displayed robust vulval expression in vulB and additional expression in vulC and vulD. Reduction of *nhr-67* activity in the *syEx5.3k* transgenic background resulted in ectopic expression of vulE and vulF. These data indicate that the 5.3 kb immediately upstream of the *ceh-2* translational site contains the *nhr-67* responsive elements, and that the third intron of *ceh-2* contains vulval repressor elements that are separable from those in the upstream promoter region. Further dissection of the *ceh-2* upstream regulatory sequences and isolating promoter fragments that respond to the activities of *lin-11, nhr-67*, *cog-1,* and *egl-38* is the next step to further characterizing the mechanism by which these transcription factors regulate *ceh-2* expression.

Vulval genes that were not perturbed by loss of *lin-11*, *lin-29*, *nhr-67*, and *cog-1*

nhr-67 RNAi failed to affect the expression of the following vulval genes: *cdh-3* (wild-type expression in vulC, vulD, vulE, and vulF), *unc-53* (wild-type expression in vulC and vulD), *bam-2* (variable expression in vulB, vulD, vulE, and vulF) and *dhs-31* (vulB and vulD). *cog-1(sy275)* mutants did not impact the expression of *pax-2* (wild-type expression in vulD). *lin-29* did not impact expression of F47B8.6, *egl-26, lin-3,* and *unc-*

53. Thus *nhr-67*, *cog-1,* and *lin-29* do not appear to be master regulators of spatial gene expression in any particular subset of vulval cells.

<u>*nhr-67* translational GFP reporter</u>

A 3'GFP tagged *nhr-67* translational reporter was constructed via PCR stitching. Briefly, the pPD95-69 vector (gift from the Fire lab) was used as a template to amplify an 870 bp GFP fragment. The primers used for this amplification were 5'GAGAGTGTTAATGTTGAAGAGGTTATGAGTAAAGGAGAAGAAC 3' (forward) and 5' GAATTTACTATCTAAACCTCTTATTTGTATAGTTCATCCATGCCATG 3' (reverse). All *nhr-67* gene fragments were amplified from the C08F8 cosmid. A 2 kb PCR fragment that includes the 3' non-coding region of *nhr-67* was generated with the following primers: 5' GAGGTTTAGATAGTAAATTC 3' (forward) and 5' CAAGGCCTGCAGGCATGCAAGCTTAAAGAACTACTGTAGTTTTTG 3' (reverse). The 870 bp GFP fragment was fused to the 2 kb 3' non coding region of *nhr-67* via PCR. The resulting 2.9 kb gene product was subsequently stitched in frame with a 6 kb *nhr-67* gene fragment that contained 1 kb of the endogenous promoter and all the exons and introns. The forward and reverse primers used to generate the 6 kb *nhr-67* product were 5'CTGCTCAAAACTTTTGCTCC 3' and 5' AACCTCTTCAACATTAACACTCTC 3', respectively. The *nhr-67* translational reporter was injected into *unc-119(ed4); him-5(e1490)* at several concentrations along with *unc-119(+)* [40 ng/μl] and *pBSK+* [7.5 ng/ μl] as co-injection markers. We were unable to generate stable transgenic lines due to the toxicity of this reporter construct. However, about a 1/3 of the F1 adult transformants

(that were rescued for the *unc-119* phenotype) displayed vulval expression that was identical to the *nhr-67* transcriptional reporters.

Overexpression studies with *nhr-67*

We find that misexpression of *nhr-67* cDNA in the vulva using a *lin-11* promoter had no/little alteration on the expression of two tested vulval markers *lin-3::GFP* (n = 43) and *zmp-1::GFP* (n = 40). Only 2/43 animals showed ectopic *lin-3* expression in vulD.

***nhr-67* site of action studies**

We attempted to determine the site of action for *nhr-67* by resorting to multiple approaches:

A. Mosaic analysis

Homozygotes of the *nhr-67(ok631)* deletion allele (obtained from the CGC) exhibit an early larval lethality/arrest phenotype. The *ok631* allele was rebalanced with the double recessive marker LGIV, *unc-30(e191) dpy4(e1166)*. (Rebalancing was necessary since the original GFP linked nT1 dominant balancer predominantly gave rise to aneuploid progeny, rendering the strain unsuitable for routine germline rescue). The 33 kb C08F8 cosmid sequence includes the upstream sequence, the open reading frame and the 3' non-coding region of *nhr-67* along with many other surrounding genes. The C08F8 cosmid was injected into the *nhr-67(ok631)/unc-30(e191) dpy-4(e1166)* heterozygotes at 10 ng/µl using *sur-5::GFP* (pTG96 at 80 ng/ µl), *myo-2::GFP* (10 ng/µl), and pBSK+ (7.5 ng/ µl) as coinjection markers. *sur-5::GFP* is an ubiquitously expressed nuclear marker and *myo-2::GFP* is a cytoplasmically expressed pharyngeal marker. The strategy was to isolate transformants that were homozygous for the *nhr-67(ok631)* allele but were

rescued by the C08F8 cosmid. Germline transformants were selected by picking *myo-2::GFP* (+), *sur-5::GFP* (+) non Dpy-Unc hermaphrodites, and were cultured individually on NGM plates. However, analysis of our transgenic stable lines revealed that the C08F8 cosmid failed to rescue the larval lethality that is associated with *ok631* allele. The failure to rescue might be due to a background mutation in the deletion allele or rearrangements within the cosmid.

B. Tissue specific RNAi in the vulva

We attempted to address the site of action of *nhr-67* by resorting to tissue-specific RNAi. The general strategy was to produce constructs containing an *nhr-67* inverted repeat with a 500 bp intervening sequence, driven by different vulval promoters. Non-proofreading Taq polymerase (Roche) was used during PCR amplification of the *nhr-67* insert so as to retain the 3' A overhangs for subsequent TOPO TA cloning. An *nhr-67* cDNA construct (OpenBiosystems) was used as a template for amplifying a 700 bp gene product with a flanking AvrII site at the 3' end. A 500 bp GST fragment (the spacer sequence) with flanking 5' and 3' NheI restriction sites was PCR amplified from the pGEX2T plasmid. The *nhr-67* and the GST products were mixed together in a 2:1 ratio, digested with AvrII and NheI, and ligated in the presence of dATP. The resulting 1.9 kb hairpin fragment was then cloned into the TOPO vector pCR2.1 (Invitrogen) via standard TOPO TA cloning. The *nhr-67* hairpin in the TOPO vector was subsequently cloned into the pJFBR69 vector (pBR322 backbone) via SpeI and XhoI restriction digests to generate and pJFBR67pin, respectively. The *daf-6* promoter (vulE and vulF specific) from pCK1 (gift from Felix lab) was subcloned into the pJFBR67pin vector using SphI and NcoI restriction sites to generate *daf-6p::67pin* construct. The *lin-11p::67pin* construct was

generated by subcloning the *lin-11* promoter (pan-vulval) from pPGF11-13 into the pJFBR67pin vector via SphI and XhoI restriction digests.

The *daf-6p::67pin* construct was injected into *unc-119(ed4);him-5(e1490)* along with *unc-119*(+) [40 ng/µl] and pBSK(+) [7.5 ng/µl] as coinjection markers. Stable lines were crossed into hermaphrodites carrying 8 kb *nhr-67Δpes-10::GFP* transgenes and were assayed for changes in *nhr-67* expression in the 1° lineages. Only 2/27 hermaphrodites displayed weak expression in vulE (not as reliable as the results seen with *nhr-67* RNAi feeding). The *lin-11p::67pin* construct was injected into *unc-119(ed4);him-5(e1490)* along with *unc-119*(+) [40 ng/µl], *nhr-67 intron4Δ pes-10::GFP* [33ng/µl], and pBSK(+) [7.5 ng/µl] as coinjection markers. Stable transgenic lines were assessed for the effect of vulval specific RNAi on the expression of *nhr-67 intron4Δ pes-10::GFP*. *nhr-67* expression in the *lin-11::67pin* strains was identical to their respective wild-type controls. Unlike *nhr-67* RNAi feeding, the vulval cell type specific *nhr-67* hairpin experiments proved to be ineffective, possibly due to inefficacy of the tested promoters. Thus these data cannot be conclusively used to rule out site of action in the vulva.

C. Gonad ablations in *lin-15(n309)* hermaphrodites

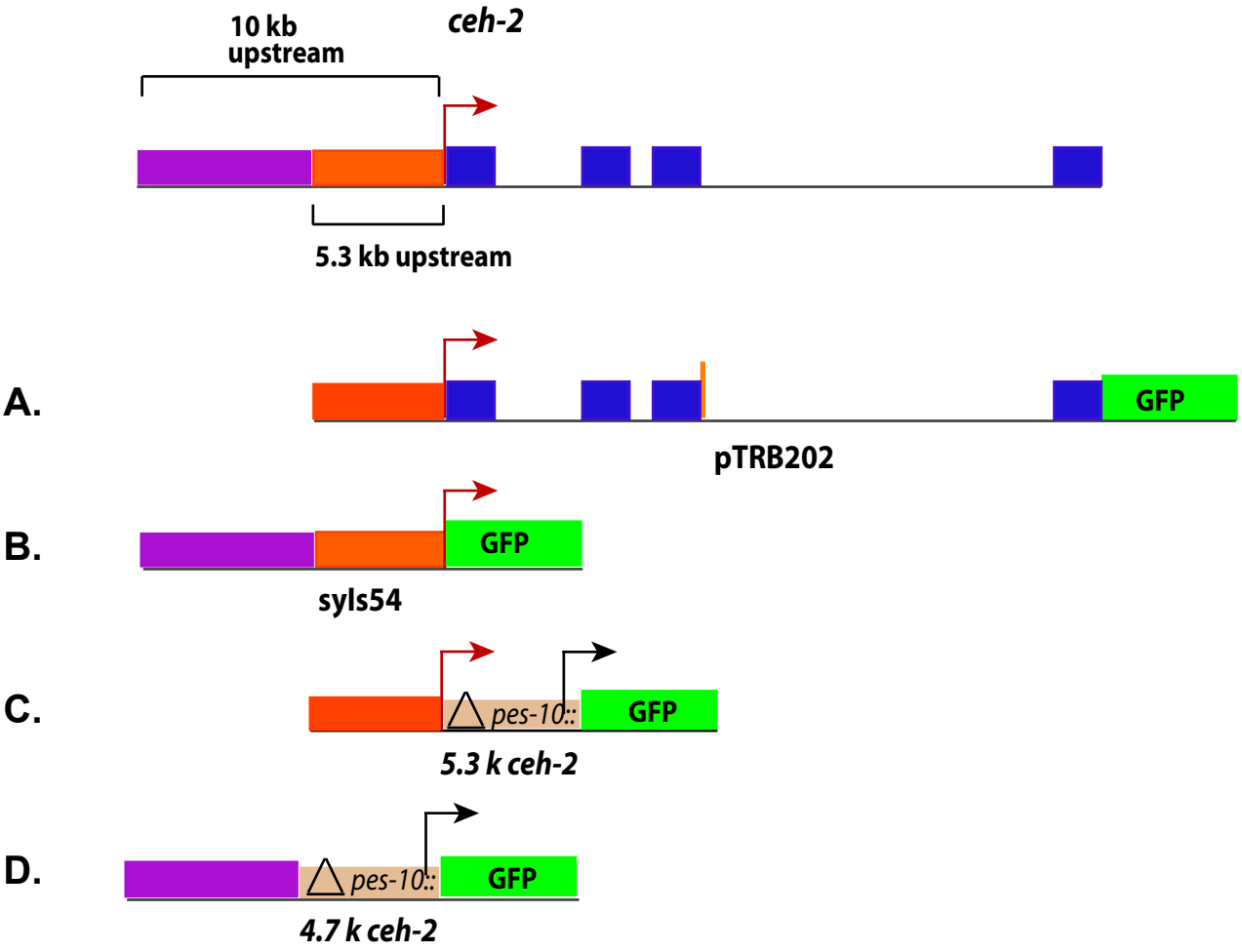
Lastly, we tried to exclude the gonad as a possible site for action for *nhr-67*. We resorted to ablating the gonad (Z1, Z2, Z3, and Z4) in L1 larvae in an *nhr-67(RNAi); lin-15(n309)* background, and assayed for *ceh-2* expression levels in the 1° vulval lineages. Gonad-independent vulval induction occurs in *lin-15* mutants due to ectopic *lin-3* levels generated in the hypodermal syncytium (Cui et al., 2006). Gonad ablations in *lin-15* mutants did not affect *ceh-2* expression in the vulval cells and looked identical to the

unablated *lin-15* controls. Unlike our *nhr-67* RNAi controls (wild-type animals treated with *nhr-67* RNAi), the *lin-15; nhr-67* RNAi-treated unablated controls fail to cause any derepression of *ceh-2* levels in the vulE and vulF cells. *nhr-67* RNAi feeding is ineffective in a *lin-15* mutant background and thus cannot be used to exclude the gonad as the site of action. We note that *ceh-2* levels in the vulvae are completely abolished when the gonad is ablated in *nhr-67(RNAi); lin-15(n309)* hermaphrodites.

Due to the various technical reasons listed above, we have been unable to conclusively determine the site of action for *nhr-67*.

Figure legend: Cis-regulatory analysis of *ceh-2*

Several *ceh-2* reporter constructs containing the upstream regulatory sequences were generated (purple and red rectangles). The *ceh-2* coding exons are represented by blue rectangles and introns are represented by black lines. The red arrow indicates the presumptive promoter of *ceh-2* and the black arrow is proximal to the minimal *Δpes-10* promoter. Construct (A) is a translational reporter that consists of 5.3 kb upstream promoter sequence (red rectangle), the entire coding region (blue rectangles), and introns (black lines) attached in frame with *GFP*. Construct (B) contains 10 kb sequence that is upstream of the predicted translational start site (purple and red rectangles) of *ceh-2* fused to *GFP*. Construct (C) contains 5.3 kb sequence upstream of the predicted first ATG of *nhr-67* (red rectangle) appended to minimal *Δpes-10::GFP*. Construct (D) contains the 4.7 kb distal upstream sequence that immediately flanks the 5.3 kb sequence in construct (C) (purple rectangle) fused to minimal *Δpes-10::GFP*.


10 kb
upstream
ceh-2
5.3 kb upstream
A.
GFP
pTRB202
B.
GFP
syIs54
C.
pes-10::
GFP
5.3 k ceh-2
D.
pes-10::
GFP
4.7 k ceh-2

CHAPTER IV:

Summary

As an organism traverses through multiple developmental states, cells differentiate from each other, generating diverse cell and tissue types. Such spatially defined fate patterns form the basis of specialized structures (e.g., organs) and are the product of gene networks operating in a variety of cell types. Gene regulatory networks are logic maps that illustrate all the functional interconnections between the regulatory genes and the cis-regulatory modules of their target genes. Dissection of these regulatory networks would help us comprehend how cells generate precise cell-fate patterns during organogenesis. Although gene regulatory networks have been described in a fair number of biological systems, the majority of these represent embryonic networks. My thesis work is the first example of a postembryonic gene regulatory network that controls organogenesis.

The seven *C. elegans* vulval cell types are an excellent system to elucidate the genomic networks that regulate patterning of gene expression and morphogenesis. While the signaling networks that establish the early fate patterns have been well characterized, little is known about the gene regulatory network that specifies the properties of terminally differentiated seven cell fates. In Chapter I we review the preliminary regulatory network that drives gene expression programs in the specific vulval cell types. We describe several transcription factors that are known to regulate spatio-temporal gene expression in the mature vulval cell types. Information on the regulatory interactions between these transcription factors and their combined activities to regulate downstream targets was limited prior to this thesis work. Chapter II reports on the isolation of *nhr-67*, a novel component of the vulval regulatory network. *nhr-67* was recovered during a high-resolution reverse-genetics screen and was found to regulate patterning of gene

expression and morphogenesis in the vulva. The pairwise interactions between *nhr-67* and the known components of the vulval network are complex and vary among the seven cell types. Integration of this new regulatory factor into the network helps visualize the differences in the network architecture for each of the cell types. In Chapter III, we investigate the different roles of these transcription factors in an attempt in deconstruct the patterning events that lead up to organogenesis. We find that several transcription factors (*lin-11*, *nhr-67,* and *cog-1*) maintain cell identity by regulating gene expression and restricting inappropriate fusion between the distinct vulval cell types. This postembryonic network shows a recurrence of certain network motifs/strategies that are also observed in embryonic networks. These network motifs include autoregulation, stable feedback loops, boundary repression, functional redundancy, and combinatorial control of effector gene expression. I also describe a striking regulatory circuit that has not been previously reported in the regulatory networks of other biological systems. Both *nhr-67* and *cog-1* engage in cross inhibition as well as negative autoregulation in the 1° vulval cells. We argue that the network architecture in the vulE and vulF cells confers several advantages, such as built-in flexibility, as well as rapid fate switching in response to ephemeral inputs. The provisional circuit diagrams (generated by this thesis work) provide a very solid foundation for future refinements to the vulval network. We postulate that the differential levels and combinatorial patterns of *lin-11, cog-1, egl-38,* and *nhr-67* expression are a part of a regulatory code for the terminally differentiated vulval cell types. Pushing our proposed regulatory network forward necessitates the identification of other potential factors, dissection of their regulatory mechanisms, and cis-regulatory analysis of various targets and regulatory genes.

Future Directions

Role of the AC on the expression of regulatory components of the vulval network

One of the most interesting problems is to further tease apart the role of the anchor cell (AC) in the patterning of the 1° vulval cell types. The patterning of E and F descendents of the 1^{o} vulval lineage involves both a short-range anchor cell-dependent signal using the RAS pathway, as well as *lin-17* (Wnt) signaling (Wang and Sternberg, 2000). An attractive hypothesis is that vulF cells are biased by proximity to the AC to have higher levels of *nhr-67* compared to *cog-1*. The most informative approach would be to conduct AC ablations and evaluate the expression levels of *nhr-67, cog-1,* and *egl-38*, all of which specify the properties of the vulE and vulF. Alterations in the expression pattern of any these regulatory factors would argue that the AC impacts the patterning of the vulE and vulF lineages by regulating the activities of some of the transcription factors that function in these vulval cells. Alternatively, the absence of an effect suggests that patterning mechanism of the AC may be distinct from that regulated by the current vulval transcription factor network. However, given the expression of *nhr-67* in the AC, it is possible that its effects (particularly on the 1^{o} lineage) are a non-autonomous upstream event. Namely, *nhr-67* activates EGF, Wnt, or some other signal in the AC, which in turn differentiates between vulE and vulF cells (Chapters II and III).

Dissecting the role of the AC can be supplemented by identifying the pivotal signal(s) that differentiates between the two 1° vulval cell types. The two likely candidates for this process would be signaling via RAS and Wnt pathways (Wang and Sternberg, 2000). It would be useful to evaluate the vulval expression levels of both the regulatory genes and their targets in genetically perturbed RAS and Wnt backgrounds.

These experiments may reveal additional insights into the later signaling events that impact the patterning of differentiated 1° and 2° vulval cell types. For example, both Wnt/Ryk and Wnt/Frizzled signaling pathways are necessary for patterning of target gene expression (*ceh-2* and *cdh-3*) in the P7.p 2^{o} vulA-vulD cells (Inoue et al., 2004). The expression pattern of the regulatory factor *lin-11* is also perturbed in the P7.p 2° lineages in a *lin-17* (Frizzled) mutant background (Gupta and Sternberg, 2002). Disruption of both Wnt pathways in a *lin-17; lin-18* double-mutant background also alters the expression of the differentiation gene *zmp-1* in the 1° vulval cells (Wang and Sternberg, 2000). An interesting hypothesis would be that Wnt patterns vulE and vulF cells by modulating the activities of different components of the transcription factor network. Deciphering the nature of these signals and linking them to our network of transcription factors would provide us with a more comprehensive view of the regulatory network architecture for each of the terminally differentiated vulval cell types.

Future dissection of the vulval patterning network

Our provisional network provides us with opportunity to deconstruct the intricacies of vulval patterning and organogenesis. Deciphering the vulval regulatory code will provide valuable information on network connections and might also provide insights into other examples of genomic networks. For example, a lot of interesting parallels may be drawn by comparing the vulval patterning network to the ASE specification network in *C. elegans*. In the ASE bistable system, the Nkx6.1/6.2 homeodomain gene *cog-1* specifies the ASER fate over the ASEL fate. The cross-inhibitory interactions between *nhr-67* and *cog-1* in the 1° vulval cells might be relevant in the specification of vulE versus vulF cell fates. A key distinction is that *cog-1*

positively autoregulates in the ASER neuron, whereas it displays autoinhibitory activity in vulA, vulB, vulE, and vulF. It would be interesting to evaluate the role of *nhr-67* in ASE specification since it is also expressed in several head neurons (Gissendanner et al., 2004). Likewise, the transcription factors that affect the ASE fate decision (*die-1* and *lim-6*) may also play a role in vulval patterning.

An investigation of the role of *pax-2* in the vulval patterning network may be informative in terms of evolutionary comparison of organogenesis between nematodes. Unlike *C. elegans*, the closely related species *C. briggsae* has a single PAX2/5/8 ortholog. *pax-2* (a recent gene duplication of *egl-38*) is expressed in the 2° vulD cells (Wang et al., 2004), whereas *egl-38* expression is detected only in the 1° vulF cells. In terms of network regulatory interactions, both genes are positively regulated by *nhr-67* and are unaffected by *cog-1* activity (Chapters II and III). It would be interesting to test if *pax-2* (like its relative) engages in positive autoregulation and impacts gene expression in a particular subset of the vulval cells (e.g., vulD). Another open question is whether the two PAX proteins overlap with each other in terms of certain functions. For example, both *pax-2* and *egl-38* have been implicated in the regulation of apoptosis in somatic and germline cells (Park et al., 2006). Elucidating the role of *pax-2* in vulval gene expression would be interesting not only in terms of identifying a novel component of the vulval network, but also in illustrating the evolutionary diversification of gene regulatory networks controlling the generation of the same organ.

Another interesting direction to pursue is the investigation of the actual regulatory mechanism of these vulval transcription factors. We demonstrate that *nhr-67, cog-1, lin-11,* and *egl-38* form a part of a genetic network that generates different patterns of gene

expression in each of the seven vulval cell types. The directness between the regulatory genes and their numerous targets needs to be assessed. Firstly, the consensus binding sites of these regulatory factors need to be determined through methods like SELEX-SAGE. The vulval elements of the different targets can be dissected via cis-regulatory analysis and be computationally screened for these consensus sites and tested by site-directed mutagenesis, gel shift assays (EMSAs), as well as ChIP (chromatin IP). Certain genetic interactions in the vulval network which have been demonstrated to be direct in other biological systems would be prioritized. For example, *tailless* directly binds the head segment enhancer element of *ems* and restricts its expression domain in *Drosophila* (Hartmann et al., 2001). This is analogous to *nhr-67* repressing the worm *ems* ortholog *ceh-2* in the inappropriate vulval cells. Continual dissection of the vulval element of *ceh-2* (Chapter III Appendix) would facilitate these mechanistic studies.

nhr-67 and *cog-1* both maintain a critical status in the vulval network as their activities confer specific properties in the diverse cell types. Yet not much is known about the inputs that initially establish their spatial domains. Cis-regulatory analyses of both these regulatory genes would unveil the conserved vulval elements that can subsequently be assessed for conserved motifs. The upstream trans-acting factors can then be identified via yeast-one hybrid studies (Deplancke et al., 2004). The relevance of these physical interactions can be confirmed using biochemical and genetic approaches.

Characterization of these novel regulatory factors would fill in the gaps of the provisional network and thus extend our knowledge on vulval patterning and organogenesis. Comparison of the vulval network to gene regulatory networks in other

biological systems may be useful in dissecting the regulatory logic used to generate complex morphological diversities in the animal kingdom.

References

Deplancke, B., Dupuy, D., Vidal, M., and Walhout, A. J. (2004). A gateway-compatible yeast one-hybrid system. Genome Res *14*, 2093–2101.

Gissendanner, C. R., Crossgrove, K., Kraus, K. A., Maina, C. V., and Sluder, A. E. (2004). Expression and function of conserved nuclear receptor genes in *Caenorhabditis elegans*. Dev Biol *266*, 399–416.

Gupta, B. P., and Sternberg, P. W. (2002). Tissue-specific regulation of the LIM homeobox gene *lin-11* during development of the *Caenorhabditis elegans* egg-laying system. Dev Biol *247*, 102–115.

Hartmann, B., Reichert, H., and Walldorf, U. (2001). Interaction of gap genes in the *Drosophila* head: *tailless* regulates expression of *empty spiracles* in early embryonic patterning and brain development. Mech Dev *109*, 161–172.

Inoue, T., Oz, H. S., Wiland, D., Gharib, S., Deshpande, R., Hill, R. J., Katz, W. S., and Sternberg, P. W. (2004). *C. elegans* LIN-18 is a Ryk ortholog and functions in parallel to LIN-17/Frizzled in Wnt signaling. Cell *118*, 795–806.

Park, D., Jia, H., Rajakumar, V., and Chamberlin, H. M. (2006). Pax2/5/8 proteins promote cell survival in *C. elegans*. Development *133*, 4193–4202.

Wang, M., and Sternberg, P. W. (2000). Patterning of the *C. elegans* 1^{o} vulval lineage by RAS and Wnt pathways. Development *127*, 5047–5058.

Wang, X., Greenberg, J. F., and Chamberlin, H. M. (2004). Evolution of regulatory elements producing a conserved gene expression pattern in *Caenorhabditis*. Evol Dev *6*, 237–245.