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.

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, vulD, and vulE 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...
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 1st 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::YPFP 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 2nd vulval descendents. From this screen, we identified nhr-67 as a gene necessary for negative regulation of ceh-2 expression in the 1st 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 2nd lineage-specific genes in the 1st-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 1st 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.
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-67*...
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 Apes-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 vulB), 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 vulB and vulD expression (and occasional vulE 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::Apes-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::Apes-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::Apes-10 constructs. Besides the previously reported expression in head neurons, we observed expression in the
Construct (D) contains 6-kb sequence upstream of the predicted first ATG of egl-17. Construct (C) is an nhr-67 transcriptional reporter driven by 1 kb of the native promoter region (red rectangle) and contains regulatory sequences of the fourth exon (sequences downstream of the orange vertical bar) and the native 3’ noncoding region (yellow rectangle). Construct (B) spans from the fourth intron (gene sequence downstream of the orange vertical bar) and the native 3’ noncoding region (yellow hatched rectangle) attached to minimal Δpes-10::GFP. Construct (A) consists of 1 kb upstream promoter sequence (red rectangle), the entire coding region (blue rectangles) and 2 kb of the 3’ noncoding region (black lines). 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 2 kb of the 3’ noncoding region (yellow hatched rectangle) attached to minimal Δpes-10::GFP.

Noncoding Region

- **egl-17**

  - **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° descendants 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,
VulF is eliminated. (H) In lin-11(n389) (G) In both vulE and vulF cells.

 EGL-38(n578); nhr-67 (F) Derepression phenotype in both 1 (D) absent in vulF cells. In contrast, (E) In cog-1(sy275) (C) In nhr-67 in the vulE and vulF cells. (B) In egl-38(n578); nhr-67 RNAi doubles show a markedly stronger derepression in all the vulF cells and vulF descendants.

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 in their background. (A) In wild-type animals. (B) egl-17 expression is invariably off in both vulE and vulF 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.

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 analyzing 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        | +         | +           | +           |                          |
| +             | +         | +           | +           |                          |
| RNAi          | +         | +           | +           |                          |
| sy275         | +         | +           | +           |                          |
| n578          | +         | +           | +           |                          |
| n389          | +         | +           | +           |                          |
| +             | +         | +           | +           |                          |
| +             | n578      | +           | +           |                          |
| +             | +         | n389        | +           |                          |
| RNAi          | sy275     | +           | +           |                          |
| n578          | +         | +           | +           |                          |
| RNAi          | sy275     | +           | +           |                          |
| n578          | +         | +           | +           |                          |
| RNAi          | sy275     | n578        | +           |                          |
| RNAi          | sy275     | n389        | +           |                          |

All animals carry the syh54 transgene in their background. Boldface indicates that p-values are significant.

lin-11(n389) is a strong loss-of-function allele. Both nhr-67(n389) and egl-38(n578) are missense mutations in the homeodomain.

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        | +         | +           | +           |                          |
| +             | +         | +           | +           |                          |
| RNAi          | +         | +           | +           |                          |
| sy275         | +         | +           | +           |                          |
| n578          | +         | +           | +           |                          |
| n389          | +         | +           | +           |                          |
| +             | sy275     | +           | +           |                          |
| n578          | +         | +           | +           |                          |
| RNAi          | sy275     | +           | +           |                          |
| n389          | +         | +           | +           |                          |
| RNAi          | sy275     | n578        | +           |                          |
| RNAi          | sy275     | n389        | +           |                          |

All animals carry the syh54 transgene in their background. Boldface indicates that p-values are significant.

This column refers to the % of animals that displayed ectopic ceh-2 expression in any of the four vulF cells.
Table 3. Regulatory Interactions between Known Components of the Vulval Patterning Network during L4

| Genotype                        | GFP Reporter | vulA | % on (n) | p-Value | Intensity | vulB1 | % on (n) | p-Value | Intensity | vulB2 | % on (n) | p-Value | Intensity |
|---------------------------------|--------------|------|----------|---------|-----------|-------|----------|---------|-----------|-------|----------|---------|-----------|
| +                               | lin-1::GFP   | 7    | 42       | NA      | ++        | 98    | 42       | NA      | ++        | 98    | 42       | NA      | ++       |
| nhr-67 RNAi                     | lin-1::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-1(n389)                     | cog-1::GFP   | 0    | 30       | 1.000   | –         | 0     | 30       | 1.000   | –         | 0     | 30       | 1.000   | –        |
| nhr-67 RNAi                     | cog-1::GFP   | 0    | 28       | 1.000   | –         | 0     | 28       | 1.000   | –         | 0     | 28       | 1.000   | –        |
| cog-1(sy275)                    | 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)                    | 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-1(n389)                     | nhr-67::GFP  | 29   | 24       | 0.017   | –/–b      | 0     | 24       | 0.040   | –         | 0     | 24       | 1.000   | –        |
| cog-1(sy275)                    | nhr-67::GFP  | 88   | 52       | 1.000   | ++        | 13    | 52       | 0.503   | ++        | 0     | 52       | 1.000   | –        |
| egl-38(n578)                    | nhr-67::GFP  | 92   | 39       | 0.890   | ++        | 46    | 39       | 0.031   | ++        | 0     | 39       | 1.000   | –        |
| nhr-67 RNAi                     | nhr-67::GFP  | 91   | 89       | 0.830   | ++        | 21    | 89       | 0.861   | ++        | 0     | 89       | 1.000   | –        |
| lin-1(n389); nhr-67 RNAi        | nhr-67::GFP  | 0    | 58       | 0.000   | –         | 0     | 58       | 0.000   | –         | 0     | 58       | 1.000   | –        |
| cog-1(sy275); nhr-67 RNAi       | 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.

*Transgenes used were lin-11::GFP(syIs80), cog-1::GFP(syIs63), syIs64, and nhr-67::GFP (syEx716 and syEx749).

**Overall cog-1 and nhr-67 expression levels in the vulval cells are significantly decreased compared to their respective wild-type controls.

vulC expression scored prior to L4 lethargus.

p-Values are calculated using Fisher’s exact test.

*Compared to the respective wild-type GFP reporter controls.

*lin-11(n389)RNAi doubly perturbed animals compared to nhr-67 RNAi–treated animals.

*egl-1::nhr-67(RNAi) doubly perturbed animals compared to single cog-1 mutant or nhr-67 RNAi background.

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Figure 6. cog-1 Levels in the 1° Vulval 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).

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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
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<sup>_vul</sup> lineage) are nonautonomous. For example, the AC generates EGF and Wnt signals and is required to differentiate vulE and vulF nonautonomous. For example, the AC generates EGF and possible that the effects (particularly on the 1<sup>_vul</sup> lineage) are nonautonomous. For example, the AC generates EGF and possible that the effects (particularly on the 1<sup>_vul</sup> lineage) are nonautonomous.

**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<sup>_vul</sup> vulval lineages (Figure 1), but it also promotes gene expression of the EGF protein LIN-3, which is necessary for vul 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 regulatory gene that operates on at least four of the seven.

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 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* inhibits 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).

Table 3. Extended.

|     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| vulC |     |     |     |     |     |     |     |     |     |
| % on | *P*- | Intensity | % on | *P*- | Intensity | % on | *P*- | Intensity | % on | *P*- | Intensity |
|      | Value |          |      | Value |          |      | Value |          |      | Value |          |
|      |       |          |      |       |          |      |       |          |      |       |          |
| 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 | ++/– | 0 (30) | 0.000 | – | 73 (30) | 0.337 | +/+ | 50 (30) | 0.000 | +/– |
| 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) | 0.20 | ++ | 12 (43) | 1.000 | +/+ | 9 (43) | 0.117 | + |
| 0 (95) | NA | – | 0 (95) | NA | – | 0 (95) | NA | – | 0 (95) | NA | – |
| 21 (24) | 0.001 | +/– | 0 (24) | 1.000 | – | 0 (24) | 1.000 | – | 0 (24) | 1.000 | – |
| 10 (52) | 0.007 | ++ | 40 (52) | 0.000 | ++ | 23 (52) | 0.000 | ++ | 4 (52) | 0.130 | + |
| 8 (39) | 0.027 | ++ | 41 (39) | 0.000 | ++ | 0 (39) | 1.000 | – | 0 (39) | 1.000 | – |
| 11 (89) | 0.002 | ++ | 11 (89) | 0.002 | ++ | 10 (89) | 0.003 | ++ | 11 (89) | 0.002 | ++ |
| 22 (58) | 0.172 | + | 0 (58) | 0.014 | – | 0 (58) | 0.027 | – | 0 (58) | 0.014 | – |
| 54 (24) | 0.003 | ++ | 63 (24) | 0.396 | ++ | 46 (24) | 0.213 | ++ | 42 (24) | 0.011 | ++ |
In contrast to their different autoregulatory properties, we find that certain genetic interactions are indeed conserved between the \emph{D. melanogaster} \textit{tailless} and \emph{C. elegans} \textit{nhr-67}; namely \textit{tailless} restricts the expression domain of \textit{ems} in the head segments [44], which is comparable to \textit{nhr-67} repressing the worm \textit{ems} ortholog \textit{ceh-2} in the inappropriate vulval cells. Additional \textit{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 invertebrate 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 \textit{trans-regulation} experiments reveal that both \textit{egl-17} and \textit{ceh-2} contain cis-regulatory elements that are directly or indirectly dependent on \textit{cog-1} (Nkx6.1/6.2), \textit{egl-38} (Pax2/5/8), \textit{nhr-67} (\textit{tll}), and \textit{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\textsuperscript{st} vulval lineage involves both a short-range AC-dependent signal using the Ras pathway as well as \textit{lin-17} (Wnt) signaling [10]. In the context of \textit{egl-17} gene expression, \textit{cog-1} single mutants exhibit increased levels in the 2\textsuperscript{nd} vulF cells. In contrast, \textit{nhr-67} RNAi appears to exclusively affect \textit{egl-17} expression in the vulE cells (arrows).

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

\textit{nhr-67} activity is included along with the other vulval patterning genes: \textit{lin-11}, \textit{cog-1}, and \textit{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 \textit{egl-38} regulating \textit{egl-17} and \textit{ceh-2} gene expression in vulE is revealed by analysis of \textit{egl-38}(n578) animals treated with \textit{nhr-67} RNAi and \textit{cog-1}(sy275); \textit{egl-38}(n578) double mutants.

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**Figure 7. \textit{nhr-67} Is Differentially Regulated in the 1\textsuperscript{st} and 2\textsuperscript{nd} 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) \textit{nhr-67::GFP} transgene in their background. (A) Wild-type animals treated with control fos-1 RNAi show no alteration of \textit{nhr-67} vulval expression compared to non-RNAi–treated animals. fos-1 RNAi animals exhibit abnormal vulval morphology, yet show wild-type \textit{nhr-67} expression in vulA cells (arrows). (B) \textit{lin-11}(n389) mutants partially eliminate the vulA-specific expression of \textit{nhr-67} (arrow). (C) In a \textit{lin-11}(n389) background, premature vulC expression (arrows) is observed sometimes during L4 stage. (D) \textit{cog-1}(sy275) mutants misexpress \textit{nhr-67} in the 2\textsuperscript{nd} vulD cells (arrows). (E) In a \textit{cog-1}(sy275) background, \textit{nhr-67} levels are highly elevated in the 1\textsuperscript{st} vulE lineages (arrows). (F) \textit{egl-38}(n578) mutants show ectopic \textit{nhr-67} expression in vulD cells (arrows). (G) \textit{nhr-67} RNAi feeding results in the robust increase in its own expression levels in vulF cells (arrow).

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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 temporarily 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].

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.

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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 1º vulval cell fate execution. This might account for the elaborate autoregulatory and trans-regulatory interactions specifically seen in 1º vulval descendants, as opposed to their 2º-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 1º vulF cell type may entail low levels of LIN-11 and NHR-67 along with low doses of LIN-11 and NHR-67. In contrast, the 1º cell type may entail low levels of LIN-11 and NHR-67 along with 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 and COG-1, and we are unable to precisely define what LIN-11 levels as opposed to the reverse situation in vulB1 and 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 deciphered by rigorously elucidating the genomic networks that operate within the seven distinct 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 (htp://www.nomarski. com). Photographs were taken with a monochrome Hamamatsu digital camera (htp://www.hamamatsu.com) and Improvment Openlab 4.0.4 software (htp://www.improvement.com). The fluorescent images were overlaid with their respective DIC images using Adobe photoshop 7.0.1 (htp://www.adobe.com). The vulval expression patterns for all strains except syIs49 were visualized during the late L4 stage. In the case of syEx76, the vulval expression was also examined during L4 lethargus and adult stage. In syIs49 animals, vulA-specific 5'-3'-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 [cgh-2::GFP], syIs55 [cgh-2::YFP], syIs51 [cdh-3::GFP], syIs49 [zmp-1::GFP], syIs57 [zmp-1::YFP], syIs59 [egl-17::GFP] [9], syIs78 [am-1::GFP] [26], syIs107 [lin-3::GFP] [58], syIs4 [egl-17::GFP] [16], guEx64 [pre-2::GFP] (gift from Chamberlin lab), kuts36 [gen-26::GFP] [18], syIs63 and syIs64 [cog-1::GFP] [27], syIs80 [lin-11::GFP] [59], syIs76 [8-kb nhr-67::ZPG-10::GFP], syIs749 [8-kb nhr-67::ZPG-10::GFP], syIs744 [nhr-67 intron-4 Aop-10::GFP], syIs825 [6 kb upstream nhr-67::GFP + 1 kb nhr-67::ZPG-10::GFP], syIs865 [nhr-67::GFP::nhr-67 intr-3::end], and syIs756 [unc-53::GFP]. Alleles used in this study: LGI, lin-11(n389); LGII, cog-1(y275), eff-1(y21); LGIII, unc-119(64d); LGIV, unc-31(e169), egl-38(n578), dpy-1(e1166sd), dpy-20(e1282); LGV, hml-e(1490). 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 nPn11 construct [61] into unc-119(+) [62] and pBBSK+ (Strategene, ht://www.strategene.com) as coinjection markers. A reverse genetics screen was conducted against 508 transcription factors (Table S1) from the Ahringer library (Medical Research Council Genservice) 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 hatched by bleeding 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::Aop-10::GFP reporter gene constructs: The pPD77–78 vector, which includes the Aop-10 basal promoter driving GFP and the unc-54 3′ UTR (gift from Fire lab), was used as a template to generate 2-kb Aop-10::GFP products. The primers used for amplification are 5′-GCTTTGATCCGTCAGCCGCTTGC-3′ and 5′-AAGGGCCCGTAGCCGACACTAGGAG-3′. All nhr-67 gene fragments were amplified from the C08F8 cosmid and were stitched together with the Aop-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 Aop-10::GFP. The primers used to amplify this template are 5′-TGCTTGGATACTGATCCGCCCTC-3′ (forward) and 5′-CAAGGCGTTCAGCATGCAAGCTTAAA-GAACTACCTGATTTTTG-3′ (reverse). Construct (2) spans from the fourth intron to the 3′ noncoding region fused to minimal Aop-10::GFP. This product was generated using the forward primer 5′-GCTTTGATCCGTCAGCCGCTTGC-3′ and the reverse primer as construct (1). Construct (3) is an nhr-67::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 Aop-10::GFP. The following primers were used to amplify this product: 5′-GAAGCCCGAGAATGGGGGATC-3′ and 5′-CAAGGCGTTCAGCATGCAAGCTTAAA-GAACTACCTGATTTTTG-3′ (reverse). Reporter constructs were injected into unc-119(+/+) [62] and pBBSK+ (Strategene) as coinjection markers. lin-11(n389); syIs76 and syIs49 were injected to construct into unc-119(n389); unc-119(+/+) [62], and hml-e(1490) background using unc-119(+e) 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 TIFF).

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.
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: pax-2 (NM_001026999; NM_001027000; NM_001026999; WBGene00006957); naa-57 (WBGene00006788; NM_001027000; NM_001026999); and zmp-1 (WBGene00006897; NM_171138).

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