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The *Caenorhabditis elegans* Nuclear Receptor Gene *nhr-25* Regulates Epidermal Cell Development

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The development of the epidermis of *Caenorhabditis elegans* involves cell fusion, migration, and differentiation events. To understand the mechanisms underlying these processes, we characterized the roles of NHR-25, a member of the nuclear receptor family of transcription factors. The NHR-25 homologs Ftz-F1 in *Drosophila* and SF-1 in mammals are involved in various biological processes, including regulation of patterning during development, reproduction, metabolism, metamorphosis, and homeostasis. Impairment of *nhr-25* activity leads to severe phenotypes in embryos and many postembryonic tissues. Further analysis has indicated that *nhr-25* activity is required for the proper development, including cell-cell fusion, of several epidermal cell types, such as the epidermal syncytial, seam, and Pn.p cells. Our results also suggest that *nhr-25* is likely to regulate cell-cell junctions and/or fusion. In a subset of Pn.p cells, called vulval precursor cells, *nhr-25* acts collaboratively with the *lin-39* Hox gene in regulating vulval cell differentiation. Additionally, our data suggest that *nhr-25* may also function with another Hox gene, *nob-1*, during embryogenesis. Overall, our results indicate that *nhr-25* plays an integral role in regulating cellular processes of epidermal cells.

Members of the nuclear receptor (NR) superfamily of transcription factors (also known as nuclear hormone receptors [NHRs]) share some highly conserved peptide motifs, including DNA-binding, ligand-binding, and transactivation domains (for a review, see reference 44). However, these proteins carry out diverse physiological functions (for a review, see references 8, 44, and 60). As more NRs have been identified, the features of the ligands for these receptors have been shown to be more chemically diverse, and not exclusively endocrine (for a review, see references 8 and 15). For many of the identified NRs, no apparent ligands have been found, and these proteins are therefore referred to as orphan nuclear receptors (for a review, see references 8 and 43).

Members of one subfamily of the orphan receptors bind to nonrepetitive DNA sequences as monomers and are constitutively localized to the nucleus; these members include *Drosophila* αFtz-F1 (fushi tarazu factor 1) and mammalian SF-1 (steroidogenic factor 1) (42). Previous studies of *Drosophila* have shown that αFtz-F1 functions collaboratively with a homeodomain protein, fushi tarazu (Ftz), to promote cell fate specification in fly embryos (25, 70). Mutations in *ftz* or α*ftz-f1* cause a common pair rule phenotype, which is associated with defects in alternate segments of the embryos. The alternatively spliced β isoform of Ftz-F1 has been found to regulate ecdysone-induced gene expression and therefore is involved in the metamorphosis of flies (4). Mammalian homologues of *ftz-f1* include the SF-1 and LRH-1 (liver receptor homologue 1) proteins. SF-1 functions in regulating the transcription of some steroidogenic enzymes and plays an essential role in the development of the adrenal gland and gonad (28, 30, 37). LRH-1 has been shown to play a key role in regulating bile acid synthesis and cholesterol homeostasis (22, 39). In addition to two highly conserved zinc finger motifs found in all NRs (43), the DNA-binding domains (DBDs) of αFtz-F1 and SF-1 contain a basic amino acid-rich region called the Ftz-F1 box (63). The only *Caenorhabditis elegans* homologue of the Ftz-F1/SF-1 subfamily is encoded by the *nhr-25* locus. NHR-25 contains a putative DBD that is ~75% identical to those of *Drosophila* Ftz-F1 and human SF-1. NHR-25 also shares a less conserved ligand-binding domain and a transcriptional activation domain with the other members of this subfamily (31).

Previous studies of *C. elegans* have shown that eliminating *nhr-25* gene activity with double-stranded RNA (dsRNA) interference (RNAi) or a deletion mutation results in gross defects in the animals, including embryonic lethality, defects in molting between larval stages, sterility, and vulval abnormality, indicating that this *C. elegans* NR homologue plays multiple roles throughout *C. elegans* development (1, 21). However, the cellular function of *nhr-25* in these developmental events remains largely unknown. Here, our study focuses on the requirement for *nhr-25* for the normal functions of the epidermal cells. In *C. elegans*, most epidermal (also known as hypodermal) cells fuse to form multinucleate syncytia, which play essential roles in protecting the animal's body by secreting collagens that make up the cuticle surrounding the body (36); in regulating cell fate specification of the neighboring cells, including the seam cells and the Pn.p cells (see below) (23, 46); and in taking up dietary sterols (69). A major epidermal syncytium, hyp7, is formed by the fusion of >100 cells, and it covers the majority of the animal's body (55). Our study revealed that *nhr-25* mutants display defects in the ventral epidermal cells in embryos and in the epidermal syncytial cells, the seam cells, and the Pn.p cells in larvae. The seam cells and

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Pn.p cells are specialized epidermal cells. Cell fusion defects were often observed in the epidermal cells in *nhr-25* mutants, which might reflect the involvement of *nhr-25* in cell-cell junction or fusion.

MATERIALS AND METHODS

***C. elegans* strains.** *C. elegans* strains were maintained according to the standard protocol (3). All genetic analyses were performed at 20°C, unless otherwise noted. The *ku217* allele was isolated in a screen for temperature-sensitive mutants defective in vulval morphogenesis following ethyl methanesulfonate mutagenesis (26).

The alleles used in this study were as follows (52): LGIII *unc-119*(*ed3*), *nob-1*(*ct230*), *lin-39*(*n709*), and *lin-39*(*n1760*) (11); LGX *dpy-6*(*e14*) and *unc-9*(*e101*). An integrated *ajm-1*::*gfp* {pJS191 [*ajm-1*::*gfp*] plus pDP#MM016B [*unc-119*(+)]} was used to visualize adherens junctions (40, 50; our unpublished results). An integrated seam cell marker-green fluorescent protein (SCM::GFP) strain, JR 672, was used to score the presence of seam cell nuclei. The SCM construct in this strain contains GFP with a nuclear localization signal driven by an undefined fragment of *C. elegans* genomic DNA (59; J. Rothman, personal communication). This reporter specifically marks the seam cells from the twofold stage through adulthood.

Molecular cloning of the gene defined by *ku217*. Standard three-point mapping techniques were used to locate the mutation in the *ku217* allele. From *dpy-6 unc-9*/*ku217* heterozygotes, 2 out of 38 Unc non-Dpy recombinant progeny segregated in the Egl (egg-laying-defective) phenotype of *ku217*, and 33 out of 35 Dpy non-Unc progeny segregated in the Egl phenotype of *ku217*. We thus mapped *ku217* to a genetic location of 10.9 map units on chromosome X.

In DNA-mediated germ line transformation experiments (45), cosmids that contained genomic sequences in the corresponding regions were microinjected into *ku217* hermaphrodites, together with a *sur-5*::*gfp* reporter, pTG96 (24). The concentrations used were 15 ng/μl for each cosmid and 40 ng/μl for the reporter construct, and at least three independent lines were examined for all experiments. A single cosmid, F11C1, rescued the egg-laying defects associated with *ku217* mutants (7% Egl; $n = 44$). A deletion subclone of F11C1 generated by XbaI digestion that removed all but one predicted open reading frame, F11C1.6, retained the ability to rescue the *ku217* mutant egg-laying defects.

Two cDNA clones that represent the coding sequence of F11C1.6, yk175f2 and yk342d8, were obtained from Y. Kohara (National Institute of Genetics, Mishima, Japan). Both of them contained a stop codon and a poly(A) tail at their 3′ ends but lacked an ATG at their 5′ ends. We PCR amplified the upstream cDNA sequence from a *C. elegans* mixed-staged cDNA library (a gift from R. Barstead) using an SL1 (a spliced leader sequence in *C. elegans*) primer and a unique sequence found in both of the cDNA clones (5′-CAGTGCGTTTGAA GAAGCC-3′). A single PCR product that corresponds to the coding sequence of *nhr-25* was isolated, which contained the predicted start codon and an in-frame stop codon immediately in front of the ATG. This clone was ligated with the cDNA clones from Y. Kohara to generate a full-length cDNA for F11C1.6. The resulting cDNA sequence, together with 3 kb of upstream sequence, rescued the sterility, egg-laying defects, larval lethality, and embryonic lethality for >80% of *ku217* transgenic animals from three independent lines. To determine the molecular lesion in *ku217* animals, mutant genomic DNA corresponding to all of the exons and intron-exon boundaries was amplified. In addition, ~3 kb of sequence upstream of the start codon and 1 kb of sequence downstream of the stop codon were examined. Only one mutation was found in two independent PCR and sequencing experiments, and the antisense strand of DNA was further sequenced to confirm the result.

EMSA. Electrophoresis mobility shift assays (EMSAs) were carried out as previously described (2) with oligonucleotides purchased from Operon (Alameda, Calif.). The wild-type DNA sequence contained the in vivo target nucleotides of *Drosophila* Ftz-F1 (5′-TTGCAGCACCGTCTCAAGGTCGCCGAGTA GGAG-3′). A single-nucleotide switch (G to T; in italics below) was introduced into the wild-type DNA sequence to generate the mutant DNA sequence (5′-T TGCAGCACCGTCTCAAG*T*TCGCCGAGTAGGAG-3′). Oligonucleotides were end labeled with [α-^{32}P]dATP (Perkin-Elmer Life Sciences Inc., Boston, Mass.) by fill-in reactions using Klenow DNA polymerase (Gibco BRL, Carlsbad, Calif.). *nhr-25* full-length cDNA sequence (either wild type or mutant) was inserted into a pGSTag2 vector (a gift from D. Ron and H. Dressler) for expression of a glutathione *S*-transferase (GST)::NHR-25 fusion protein. This fusion protein was expressed in the *Escherichia coli* BL21 strain and was purified according to the protocol of Ausubel et al. with some modifications. Briefly, glutathione-Sepharose resin (Amersham Biosciences, Piscataway, N.J.) was loaded into a Poly-Prep chromatography column from Bio-Rad (Hercules, Calif.). Bacterial lysate containing GST::NHR-25 fusion protein was added to the column and was washed on the column. The purified protein was eluted off the column by adding 50 mM reduced glutathione. The eluate was run on a sodium dodecyl sulfate–10% polyacrylamide gel and was visualized by staining it with Coomassie blue. The protein concentration in the eluate was determined by comparing it with bovine serum albumin proteins at various concentrations in Coomassie blue staining. The purified GST::NHR-25 protein was then incubated with 0.05 pmol of labeled oligonucleotides in 20 μl of incubation buffer (2) for 30 min at 20°C, and 10 μl of the reaction mixture was loaded onto a 0.5% polyacrylamide gel containing 0.5 mM $MgCl_2$. The gel was then dried and exposed to a phosphorimager screen (Amersham Biosciences).

GFP reporters. Full-length cDNA sequence of *nhr-25*, together with 3 kb of upstream regulatory sequence, was inserted into the pPD95.77 vector (a gift from A. Fire), which contains the GFP coding sequence (7) and the 3′ untranslated region of *unc-54* (16) at the 3′ end. A second GFP reporter construct was generated by inserting the *gfp* coding sequence, which was excised from the plasmid pPD103.87 (a gift from A. Fire), in front of the stop codon of *nhr-25*. This construct also contained the native 3′ untranslated region of *nhr-25*. The two constructs were injected into *unc-119*(*ed3*) animals at 30 ng/μl, together with an *unc-119*(+) plasmid, pDP#MM016B (40), at 40 ng/μl.

RNAi. PCR primers that each contained a T7 promoter sequence and a unique *nhr-25* cDNA sequence were used to amplify the first 0.9 kb of the *nhr-25* coding sequence. dsRNA was generated from the 0.9-kb template using a large-scale T7 transcription kit (Novagen, Madison, Wis.). RNAi by dsRNA injection was carried out as described previously (17). The injected animals were transferred to individual fresh plates after 16 h, and their progeny were scored for the mutant phenotype.

For RNAi feeding, the first 1.4 kb of *nhr-25* coding sequence was inserted into the vector pPD129.36 (a gift from A. Fire). The resulting plasmids were transformed into an *E. coli* strain, HT115, and the bacteria were streaked on agar plates containing 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 50 μg of ampicillin/ml to induce the production of dsRNA (32, 61). Worms were raised on these plates for at least two generations before the phenotype was scored to ensure the effectiveness of RNAi.

For tissue-specific RNAi, the first 0.9 kb of *nhr-25* coding sequence was fused in the sense and antisense orientations to the *col-10* promoter (a gift from V. Ambros). The *col-10* promoter contains 1.2 kb of *col-10* genomic DNA upstream of its ATG. Both constructs were injected at 50 ng/μl, and a *sur-5*::*gfp* transcriptional fusion construct was used as a coinjection marker (24).

MH27 antibody staining. Large-scale embryo preparations were fixed and stained according to the method of Miller and Shakes (47). The monoclonal antibody MH27, which recognizes adherens junctions, was a generous gift of R. Waterston and was used at a dilution of 1:250 (19).

GST pull down. Both wild-type and mutant NHR-25 and GST proteins were expressed and purified on glutathione-Sepharose resin as described above. Full-length LIN-39, NOB-1, and luciferase were in vitro translated using a TNT-coupled reticulocyte lysate system from Promega (Madison, Wis.) and labeled with [^{35}S]methionine (Promega). Thirty microliters of a 1:1 slurry of Sepharose resin was incubated with 5 μl of the labeled protein in the incubation buffer (250 mM NaCl, 50 mM Tris · HCl [pH 7.5], 0.5 mM sucrose, 1 mM dithiothreitol, 5% glycerol) for 2 h at 4°C. Subsequently, the reaction mixture was washed five times in the incubation buffer at 4°C, sodium dodecyl sulfate sample buffer was added, and the Sepharose resin was spun down. The supernatant was run on a 10% polyacrylamide gel to separate proteins retained on the resin. The resulting gel was dried and exposed to film.

RESULTS

***ku217* mutants were associated with embryonic and larval lethality, sterility, egg-laying defects, and male tail abnormalities.** We initiated our study of the *nhr-25* gene by isolating the *ku217* allele in a screen for temperature-sensitive mutants defective in vulval development (9, 26). At 20°C, 74% of adult *ku217* homozygous hermaphrodites were egg-laying defective, and some of them were sterile (Table 1). Lethality at either embryonic or larval stages was also observed (Table 1 and Fig. 1B and E). The severity of the above-mentioned phenotypes increased at higher temperatures (Table 1), suggesting that *ku217* is a temperature-sensitive allele. *nhr-25*(*ku217*) mutant

TABLE 1. Phenotypes of *nhr-25* mutants

Genotype	Temp (°C)	*n*	% Phenotype[a]:			
			Emb let	Lar let	Egl	Sterility
N2	20	196	<1	0	0	0
	15	215	13	2	30	5
nhr-25(*ku217*)	20	228	14	2	74	9
	25	118	29	6	39	26
nhr-25(*jm2389*/+)	20	176	22	2	0	0
nhr-25(RNAi injection)	20	372	5	6	ND	ND
nhr-25(*ku217* RNAi injection)	20	67	100	0	ND	ND
nhr-25(*jm2389*/+ RNAi injection)	20	53	100	0	ND	ND
nhr-25(*ku217* RNAi feeding)	20	149	32	31	13	24
nhr-25(*jm2389*/+ RNAi feeding)	20	96	33	30	16	21
nhr-25 (*ku217*) *unc-22*(RNAi)	20	177	10	3	ND	ND

[a] Emb let, embryonic lethality; Lar let, larval lethality; Egl, egg-laying defective; ND, no data.

males were sterile at all temperatures; several structures located at the tail that are required for copulation, including spicules, sensory rays, and the hook, were aberrant (data not shown). Arrested *ku217* larvae were observed with an apparent molting defect; the mutants were unable to shed the old cuticles even when the new ones had fully formed (Fig. 1E). *ku217* animals that survived further to adulthood were sometimes sterile. In these sterile animals, gonadal cells appeared to undergo excess proliferation. The resulting tumorous gonad often failed to elongate and migrate correctly to form functional tissue (reference 21 and data not shown). Vulval development in 9% ($n = 22$) of *ku217* animals was completely absent, possibly due to the lack of differentiation of the anchor cell in the gonad, which is required for signaling the vulval precursor cells to adopt the vulval fate (23). However, all *ku217* animals that were examined appeared to have a normal anchor cell. In the majority of *ku217* animals where vulval differentiation did initiate, cell division sometimes prematurely stopped. Vulval precursor cells underwent fewer rounds of cell division and therefore generated fewer progeny than in wild-type animals (see below). Furthermore, vulval cells in *ku217* hermaphrodites sometimes did not migrate to the right positions so that the invagination formed by these cells was abnormally wide (Fig. 1G).

***ku217* is a reduction-of-function mutation in the *nhr-25* nuclear receptor locus.** A single open reading frame that includes the *nhr-25* gene, F11C1.6, fully rescued the mutant phenotypes of *ku217* (see Materials and Methods). This locus is predicted by the genome-sequencing consortium to encode a 572-amino-acid-long peptide (21). A full-length cDNA clone that correlates with the predicted coding sequence of *nhr-25* was isolated, and it retained the ability to rescue the *ku217* mutant phenotypes (Fig. 2A) (see Materials and Methods). The *nhr-25* gene product is homologous to *Drosophila* Ftz-F1 and mammalian SF-1 in a putative DBD at the N terminus and a transcriptional activation domain at the C terminus. The DBD in these proteins comprises two C2C2-type zinc fingers and a basic amino acid-rich domain called the Ftz-F1 box (Fig. 2B) (63).

Previously, it was shown that deletion of the *nhr-25* locus results in completely penetrant embryonic lethality (1), suggesting that this locus is essential for early embryonic development. To investigate the genetic nature of the *ku217* allele,

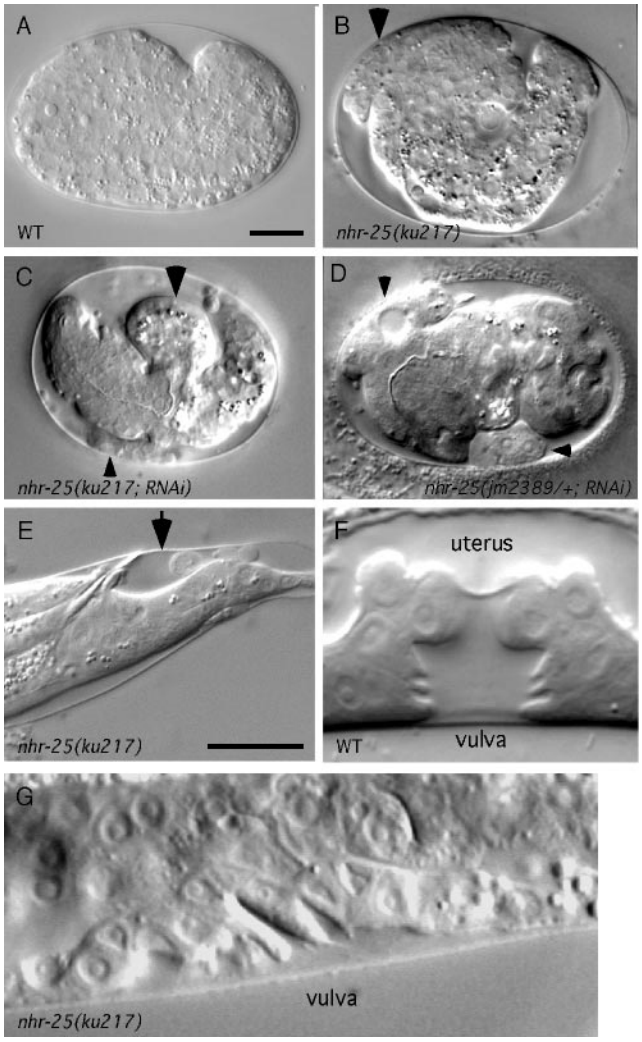


FIG. 1. Phenotypes of *nhr-25* mutant animals. (A to D) Wild-type, *nhr-25*(*ku217*), *nhr-25*(*ku217* RNAi), and *nhr-25*(*jm2389*/+ RNAi) embryos, respectively, during elongation. The arrowheads point to the positions where the animals ruptured. (E) Molting defect of an *nhr-25*(*ku217*) larva during the L2 stage. The arrow indicates the old cuticle that remained surrounding the tail of the animal. (F and G) Vulva morphology of wild-type and *nhr-25*(*ku217*) animals, respectively, during L4. Anterior is to the left. Scale bars, 10 μm.

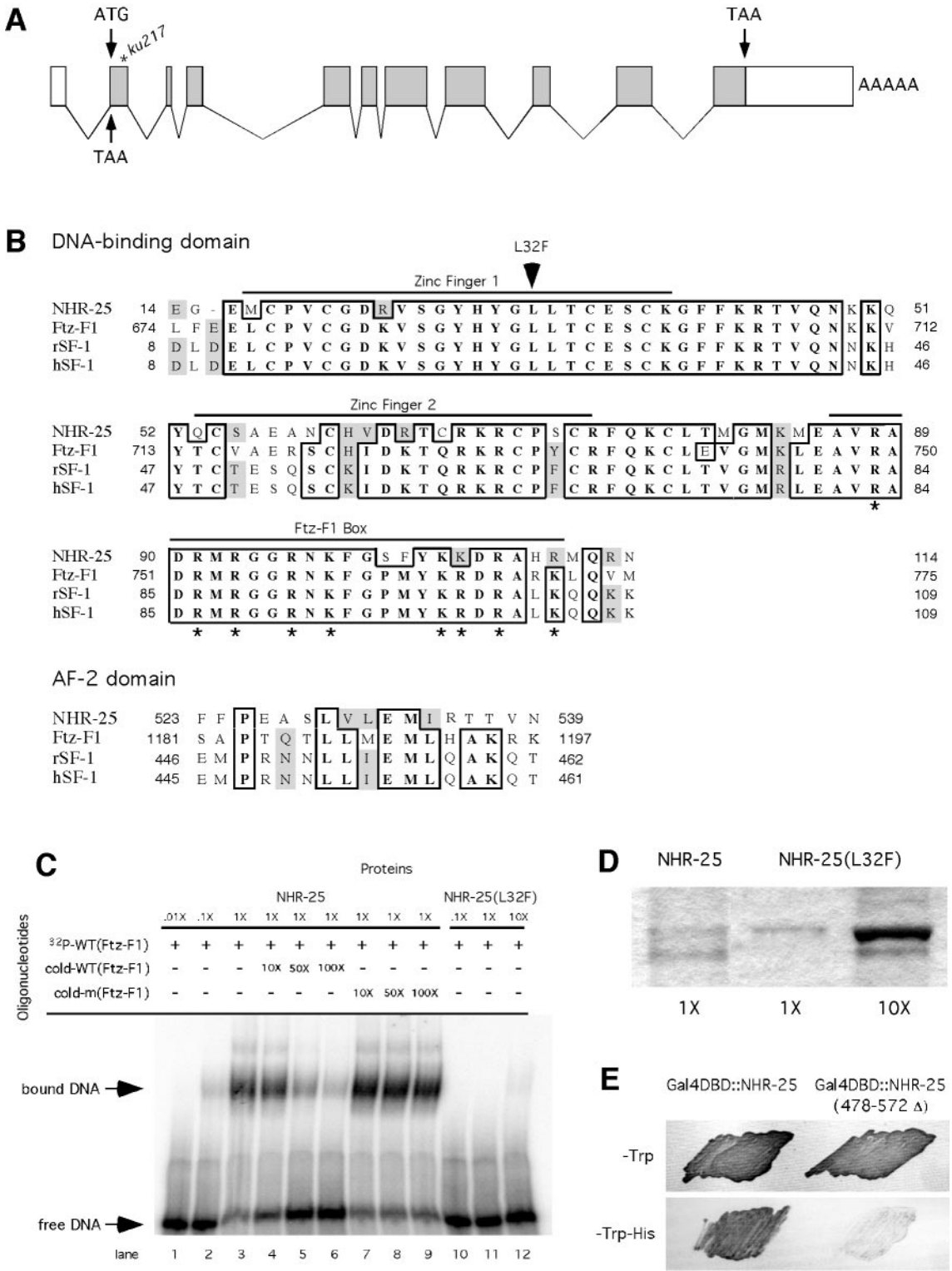


FIG. 2. Gene structure of the *nhr-25* locus and biochemical properties of the NHR-25 protein. (A) *nhr-25* cDNA contains an in-frame stop codon in front of ATG and a poly(A) tail at the 3′ end, suggesting that the cDNA is full length. The *ku217* mutation was a C-to-T transition in the first exon and caused a Leu-to-Phe change in the protein sequence. (B) Alignment of peptide sequences of the DBD and transcriptional activation domain (AF-2) from *C. elegans* NHR-25, *Drosophila* Ftz-F1, and rat and human SF-1 (also see reference 21). Identical residues are boxed, and similar residues are shaded. The DBD consists of two C2C2-type zinc fingers and a basic amino acid-rich domain (Ftz-F1 box). *ku217* was associated with a missense mutation (L32F) in the first zinc finger. The AF-2 domain contains an invariant glutamic acid residue and is flanked by several other conserved amino acids. (C) DNA-binding activities of wild-type NHR-25 and mutant NHR-25 [NHR-25(L32F)] generated by the *ku217* mutation. In an electrophoresis motility shift assay, wild-type NHR-25 specifically bound to a DNA sequence containing the highly conserved core recognition sequence (PyCAAGGPyCPu) of Ftz-F1. NHR-25(L32F) bound poorly to this DNA sequence. A 1× amount of GST::NHR-25

we constructed transheterozygous animals bearing the *ku217* mutation and the *jm2389* deletion allele (1). These transheterozygous animals arrested during embryogenesis, and only a few could survive to late larval stages, indicating that *ku217* is a partial loss-of-function mutation in the *nhr-25* locus. Animals subjected to RNAi against *nhr-25* by injection (see Materials and Methods) in a wild-type background displayed less severe embryonic and larval phenotypes (Table 1 and data not shown). However, in combination with molecular lesions in *nhr-25*, either *ku217* or *jm2389*/+ *nhr-25*(RNAi) resulted in increased lethality (Fig. 1C and D and Table 1), similar to what has been observed in *nhr-25*(*jm2389*) deletion mutants. RNAi of an unrelated gene, *unc-22*, had little effect on lethality (Table 1). Taken together, impairment of *nhr-25* function, either by RNAi or by *ku217* and *jm2389* lesions, had a common effect during embryonic and larval development, although with different severities.

The *ku217* mutation disrupted the DNA-binding ability of NHR-25. A single-nucleotide switch (C to T) was found in the first exon of the *nhr-25* coding sequence in *ku217* mutants, and it was predicted to change a highly conserved leucine residue to phenylalanine in the putative DBD of NHR-25 (Fig. 2A and B). We investigated the DNA-binding abilities of the resulting mutant protein and wild-type NHR-25 using an EMSA (Fig. 2C). As the downstream targets of *nhr-25* in *C. elegans* have not been identified, in this assay we made use of a 33-bp oligonucleotide sequence taken from the upstream promoter of *ftz*, which has been shown to be an in vivo target of *Drosophila* Ftz-F1 (62). In vitro DNA-binding assays have suggested that Ftz-F1 and SF-1 recognize and bind to a consensus 9-bp DNA sequence (PyCAAGGPyCPu) as a monomer (62, 67). Wild-type NHR-25 was able to bind to the oligonucleotide containing the 9-bp sequence, generating a slowly migrating species in the gel (Fig. 2C, lanes 1 to 3). The binding between a ^{32}P-labeled oligonucleotide and NHR-25 could be competed away by adding an excess amount of cold oligonucleotide (Fig. 2C, lanes 4 to 6). A mutant sequence bearing a single-nucleotide change (G to T) in the consensus sequence (see Materials and Methods) was unable to affect the binding between the wild-type DNA sequence and NHR-25 even when it was 100-fold in excess (Fig. 2C, lanes 7 to 9). On the other hand, the mutant NHR-25(L32F) protein interacted with the target DNA sequence at an extremely low affinity (Fig. 2C, lanes 10 to 12, and D). Therefore, the missense mutation (L32F) in the *ku217* mutant protein, located in the highly conserved zinc finger motif, greatly compromised the DNA-binding ability of NHR-25. These data suggest that, like its homologues in flies and in mammals, NHR-25 binds to DNA in a sequence-specific manner and that disruption of the DNA-binding activity leads to the loss-of-function phenotypes observed in *ku217* animals. However, the dramatic >100-fold loss of affinity for DNA by NHR-25(L32F) is somewhat surprising given the partial loss-of-function phenotype in *ku217* animals. This discrepancy could simply be a result of the differences between in vitro and in vivo experiments or a consequence of the use of the *Drosophila* Ftz-F1 binding site in our assay, which might differ somewhat from a true high-affinity NHR-25 site in vivo. Alternatively, NHR-25 could have a DNA-independent function in vivo that is not disrupted in *ku217* mutants.

NHR-25 is a nuclear protein that is likely to function as a transcriptional activator. *C. elegans* NHR-25 also contains a putative transcriptional activation motif, called the AF-2 domain (31). When fused to the GAL4 DBD, full-length NHR-25 was able to activate the *HIS3* reporter gene expression driven by a GAL4 gene promoter (Fig. 2E). Deletion of a peptide sequence (after amino acid 477) of NHR-25 that contains the AF-2 domain greatly compromised the reporter gene transcription (Fig. 2E). Therefore, NHR-25 is likely to function as a transcriptional activator.

In support of the idea of NHR-25 being a transcription factor, an *nhr-25*::*gfp* reporter was found to be expressed in the nuclei of many epidermal cells, including the epidermal syncytial cells, the seam cells, and all the Pn.p cells and their progeny. Many cells in the head and tail also showed *nhr-25*::*gfp* expression (reference 21 and data not shown). *nhr-25* expression was first observed during embryogenesis around the 100-cell stage (data not shown) and continued to be expressed throughout development in the epidermal and seam cells. In the Pn.p-derived vulval cells, when vulval morphogenesis (including cell division, fusion, and migration) was almost complete during the L4-to-adult transition, *nhr-25*::*gfp* expression significantly decreased, whereas in other Pn.p cells that fused with hyp7, *nhr-25*::*gfp* expression remained largely unchanged (data not shown).

***nhr-25* mutants display defects in a subset of embryonic-cell fusion events.** To find out why impairment of *nhr-25* function led to embryonic lethality, we first examined cell division and differentiation during early embryogenesis. Using a four-dimensional Nomarski DIC microscope to record early development of embryos, we found no apparent defect in cell division and differentiation (data not shown). However, later during embryo morphogenesis, the mutant animals often ruptured at the onset of body elongation along the A-P axis (Fig. 1B to D). Such a phenotype often reflects a deficit or malfunction of the epidermal cells, since the external epithelium in the embryos is crucial in sustaining the pressure generated during elongation and keeping the proper shape of the body (for a review, see references 46 and 54). Before embryo elongation initiates, the worm body is covered by six longitudinal rows of epidermal cells, two on the dorsal side, and two on each of the lateral sides. The ventral-lateral epidermal cells elongate and migrate circumferentially to meet at the ventral midline and form adherens junctions at their adjoining plasma membrane. This developmental process is referred to as ventral enclosure (66).

or GST::NHR-25(L32F) is equal to ~50 ng of protein. (D) Coomassie blue staining of the NHR-25 proteins used in panel C. Note that NHR-25(L32F) was used in 10-fold excess compared to wild-type NHR-25 protein for the binding assay in panel C. (E) Transactivation activities of NHR-25 and NHR-25(478-572Δ). NHR-25(478-572Δ), which lacks the AF-2 domain, only weakly activated transcription of the *HIS3* reporter gene, and therefore, the yeast strain (Y190) carrying this truncated form of NHR-25 grew poorly in media lacking histidine (−His). Yeast strains expressing NHR-25 or NHR-25(478-572Δ) grew equally well in media supplemented with histidine. The growth media lacked tryptophan in order to select strains expressing the NHR-25 proteins.

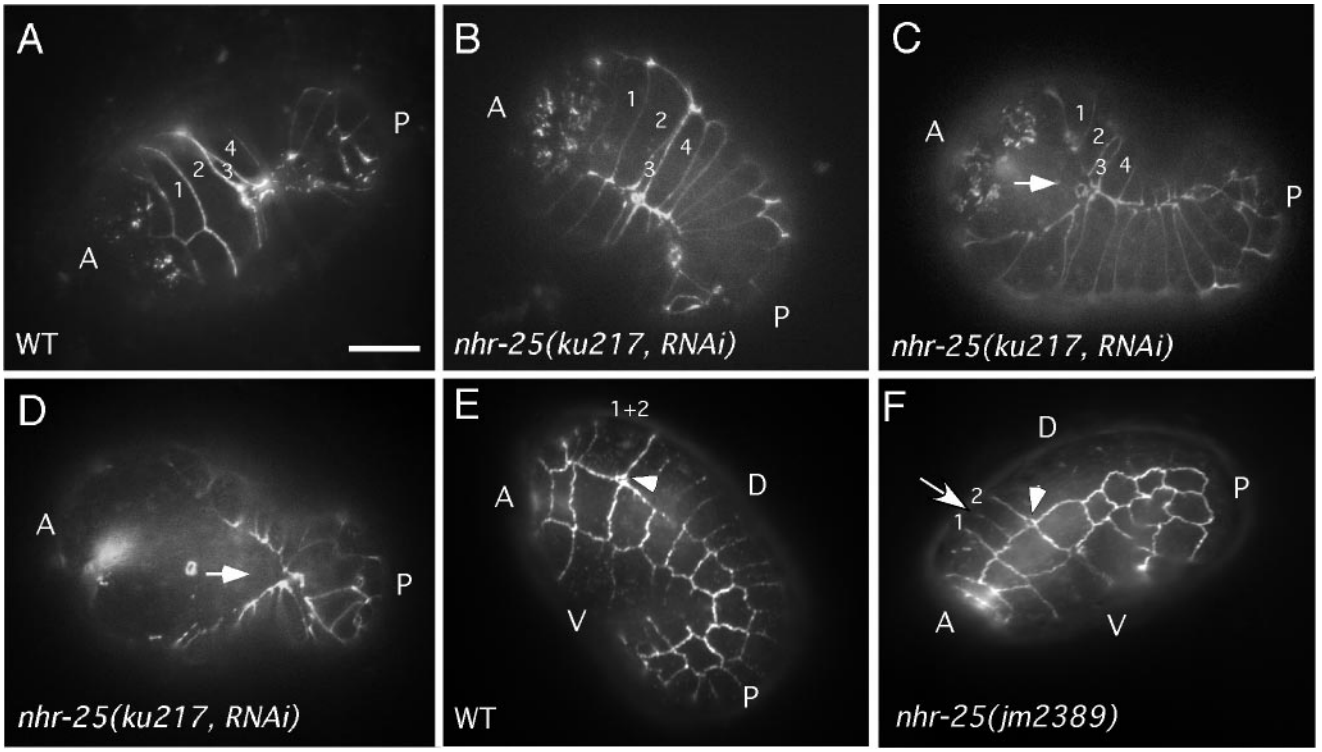
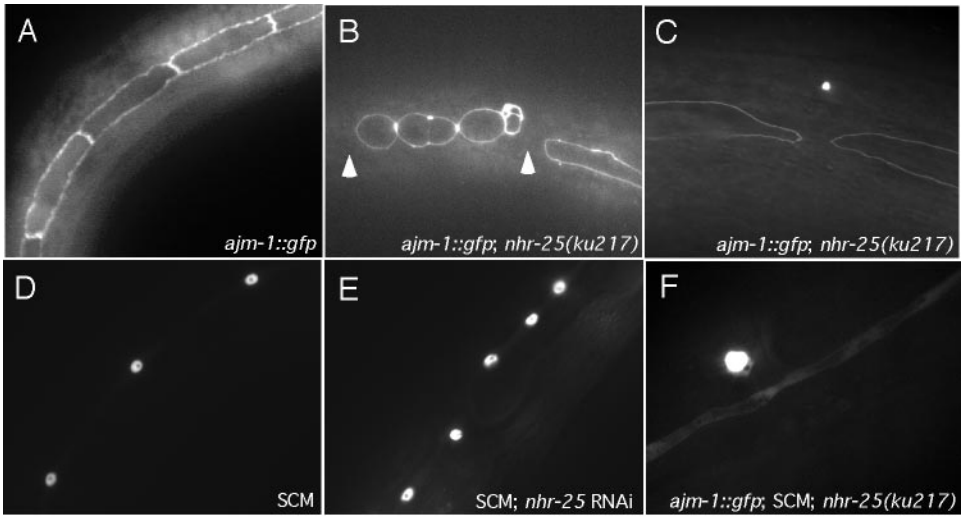


FIG. 3. Fusion defects of *nhr-25* mutant embryos. A and P, anterior and posterior, respectively. (A to D) Ventral side. (A) In wild-type (WT) embryos, ventral enclosure initiates at the anterior end and proceeds to the posterior end. Note that the pair 2 epidermal cells had already fused with one another when the posterior cells started to make contact. The pair 4 cells eventually fused. The other ventral epidermal cells remained unfused. (B) In an *nhr-25*(*ku217* RNAi) animal, pair 2 and 4 cells did not fuse to each other, even after posterior cells had already formed adherens junctions at the adjoining membranes. (C) At a later time point, when the embryo shown in panel B attempted to elongate, the junction between anterior ventral epidermal cells ripped open, as indicated by the arrow. (D) Finally, the posterior epidermal cells could no longer maintain the adherens junctions between them, and the ventral side of the animal was no longer covered by any epidermal cells. (E and F) Fusion defect in the embryonic dorsal epidermis. The arrowheads indicate the positions of deirid sensillae. Dorsal epidermal cells 1 and 2 are located just anterior of the deirid sensillae. D and V, dorsal and ventral, respectively. (E) Lateral view of 1.5-fold MH27-stained wild-type embryo that has lost adherens junctions between dorsal epidermal cells 1 and 2 prior to any of the dorsal epidermal cells that are posterior to the deirid sensillae. (F) Lateral view of 1.5-fold MH27-stained embryo from an *nhr-25*(*jm2389*/+) parent that still retains the adherens junction between dorsal epidermal cells 1 and 2 (arrow) despite the fusion of most of the posterior dorsal epidermal cells. Scale bar, 10 μm.

In wild-type animals, ventral enclosure occurs first in the anterior end and proceeds to the posterior. Two pairs of the most anterior epidermal cells fuse (Fig. 3A, pairs 2 and 4) when the posterior cells start making contact with each other. The normal junction and/or fusion of the anterior epidermal cells allows them to withstand the pressure when cells squeeze into a long tubular worm shape and may be necessary for the enclosure in the posterior end (66).

To investigate cell differentiation, formation of adherens junction, and fusion of the epidermal cells in *nhr-25* mutant embryos, we made use of an *ajm-1*::*gfp* reporter, which is present in the apical junctions between epithelial cells (50). We found that the right number of cells expressed AJM-1 at their peripheries at the proper stage (data not shown), suggesting that the defect seen in mutants was not due to epidermal cell proliferation. In addition, the ventral-lateral epidermal cells in *nhr-25* mutant embryos were properly localized and were able to meet at the ventral midline and form adherens junctions as in wild-type animals (Fig. 3B to D), arguing against any possible defect in cell migration. However, the anterior ventral epidermal cells that normally fuse to each other, i.e., pairs 2 and 4, failed to fuse in some *nhr-25* mutants. By the time posterior epidermal cells enclosed, the anterior cells detached from each other (Fig. 3B to D), and the embryos died shortly thereafter with a morphology identical to that of the embryos shown in Fig. 1B to D. The presence of a morphology associated with a failure in ventral enclosure was observed in 19% ($n = 43$) of the embryos from *nhr-25*(*ku217*) animals injected with *nhr-25* dsRNA and 7% ($n = 43$) of the embryos from an *nhr-25*(*jm2389*/+) animal. A further 16% ($n = 43$) of the *nhr-25*(*ku217* RNAi) embryos appeared to have ruptured during or shortly after the onset of embryonic elongation at a location distinct from the ventral midline.

Since some *nhr-25* mutant embryos rupture on the dorsal side, we used the MH27 antibody that recognizes adherens junctions to investigate contacts and fusion events in the dorsal epidermal cells. In wild-type animals, the dorsal epidermal cells 1 and 2, lying just anterior to the deirid sensillae, usually fuse to each other shortly after the fusion of the pair 2 ventral epidermal cells involved in ventral enclosure (51). Further fusions of the dorsal epidermal cells occur in an anterior-to-posterior manner. Thirteen percent ($n = 45$) of wild-type embryos at the 1.5-fold stage had MH27 staining which indicated the presence of a cell boundary between the two cells (Fig. 3E).



G

Genotype	Avg. # of seam cell nuclei per lateral side	High	Low	(n)
SCM	15.9	16	15	23
ajm-1::gfp; SCM; *nhr-25(ku217)*	17.4	20	15	35
SCM; *nhr-25* RNAi	20.1	32	16	36

FIG. 4. *ajm-1*::*gfp* and SCM expression in seam cells. (A) *ajm-1*::*gfp* expression in seam cells in a wild-type animal. (B) *ajm1*::*gfp* expression in seam cells of *nhr-25*(*ku217*) animals at L1. Note that the seam cells in the mutant had a round shape and were not always connected to other seam cells. The arrowheads indicate gaps between seam cells. (C) Adult *nhr-25*(*ku217*) animal that still had a gap in a lateral syncytial seam cell. (D) Evenly spaced SCM in a wild-type adult animal. (E) Presence of extra adult seam cell nuclei as indicated by the SCM following injection of *nhr-25* dsRNA. (F) SCM was occasionally observed outside of the *ajm-1*::*gfp*-delineated boundary of adult syncytial seam cells in *nhr-25*(*ku217*) mutants. (G) On average, more SCM-positive nuclei were observed in adult syncytial seam cells of *nhr-25* mutants than in wild-type animals.

In contrast, 56% ($n = 41$) of 1.5-fold embryos from an *nhr-25*(*jm2389*/+) parent had a failure or delay in fusion between cells 1 and 2 (Fig. 3F). These two dorsal epidermal cells mirror the pair 2 cells of the ventral epidermis with respect to their position along the anterior-posterior axis and the time at which fusion occurs. It is possible that *nhr-25* is required specifically for the differentiation and/or fusion of these two groups of cells. Another possibility is that these two groups of cells are the most sensitive to fusion defects in general and are therefore most easily assayed as being defective in an *nhr-25* mutant background.

***nhr-25*(*ku217*) mutants had abnormal seam cell morphology and a greater number of adult seam cell nuclei.** The two rows of dorsal epidermal cells interdigitate and fuse to generate the dorsal epidermal syncytium during embryogenesis (58). The ventral epidermal cells either fuse during embryogenesis (as described above) or, except for six pairs (referred to as the P cells [see below]), interdigitate and fuse to form the ventral epidermal syncytium during the L1 larval stage. Epidermal cells that lie on the lateral side of the body (10 on each lateral side) do not fuse and are referred to as the seam cells. The seam cells remain as stem cells during larval stages and divide asymmetrically to generate one stem cell and one daughter cell that differentiates into other cell types. Most of the differentiated daughter cells fuse to the surrounding epidermal syncytium; some, in males, give rise to the male-specific ray sensory neurons in the tail (14, 58). The seam cells connect to both dorsal and ventral syncytia and are therefore important for regulating the body shape of the worms. Additionally, the seam cells of L1 larvae and dauer worms are responsible for producing a specialized cuticle structure called the alae on the lateral surface of the animal. During the L4-to-adult transition, the seam cells eventually fuse together, and this syncytial cell is responsible for producing the adult alae (57). In *nhr-25*(*ku217*) *ajm-1*::*gfp* animals, the lateral epidermal cells appeared normal during embryogenesis. However, as the worm hatched and increased in size, particularly in length, the seam cells failed to elongate along the A-P axis (Fig. 4B). These seam cells had a round instead of a rectangular and elongated shape (Fig. 4A and B). As the worm body further elongated when the animal went through the four larval stages, junctions between neighboring seam cells were no longer maintained, so that gaps between seam cells were often observed even after seam cell fusion (Fig. 4C). Gaps in adult syncytial seam cells were seen in 76% ($n = 38$) of *nhr-25*(*ku217*) animals examined. Injection of *nhr-25* dsRNA caused a seam cell phenotype similar to that seen in *nhr-25*(*ku217*) mutant animals. In the adult *nhr-25*(*ku217*) or *nhr-25* dsRNA-injected animals, the lateral surface of the body was also characterized by patches of alae instead of a continuous one as in wild-type animals. The seam cell morphology and alar defects suggest that seam cell differ-

TABLE 2. Pn.p cell fusion in *nhr-25* mutants

Genotype	% Unfused Pn.ps[a]											
	1	2	3	4	5	6	7	8	9	10	11	*n*
Wild type	0	0	38	100	100	100	100	100	0	0	0	56
nhr-25(*ku217* RNAi feeding)	0	4	75	89	93	93	96	71	11	4	25	28
nhr-25(*ku217*)	0	0	70	100	100	100	100	97	3	0	3	31
lin-39(*n709*)	0	0	53	100	100	100	95	100	0	0	0	38
lin-39(*n709*) *nhr-25*(*ku217*)	0	0	50	91	91	91	82	65	0	3	6	34

[a] 1 to 11, P1.p to P11.p; *n*, number of animals scored.

entiation and/or execution of seam cell function is compromised in *nhr-25* mutants.

We took advantage of a number of GFP markers for seam cell differentiation to better understand the nature of the seam cell defects in *nhr-25* mutant animals. Using SCM (see Materials and Methods) (59), we determined that seam cells were able to differentiate properly in *nhr-25*(*ku217*) and *nhr-25* RNAi animals (Fig. 4E), and the number of seam cells present at the time of hatching in *nhr-25*(*ku217*) animals was not noticeably different from that in wild-type animals (data not shown). However, when *nhr-25*(*ku217*) or *nhr-25* dsRNA-injected young adults were scored for the number of SCM-expressing nuclei in their syncytial seam cells, it was observed that they had a higher number of SCM nuclei than did wild-type animals (Fig. 4E and G). Furthermore, SCM was occasionally expressed outside of the syncytial seam cell, as delineated by *ajm-1*::*gfp* (Fig. 4F). These defects could indicate a failure of the seam cell daughters to properly differentiate and fuse with the epidermal syncytia; hence, they abnormally maintained the seam cell fate. In support of the idea that seam cell differentiation did occur in *nhr-25* compromised animals, the expression patterns of the seam cell markers *elt-5*::*gfp*, *nhr-73*::*gfp*, *nhr-75*::*gfp*, and *nhr-77*::*gfp* were not reproducibly altered when subjected to *nhr-25* RNAi (references 34 and 48 and data not shown). It is unlikely that the GATA transcription factor ELT-5/EGL-18 regulates *nhr-25* transcription, since injection of *elt-5* dsRNA did not disrupt the expression of *nhr-25*::*gfp* in the embryo or in the larval stages of RNAi escapers (data not shown).

Pn.p and vulval cell differentiation was aberrant in *nhr-25*(*ku217*) mutants. Six pairs of the ventral epidermal cells do not fuse during embryogenesis; they interdigitate and line up on the ventral midline of the animal at the end of the L1 stage (58). These cells are referred to as the P1 through P12 cells, anterior to posterior. Their posterior daughters (Pn.p cells) can adopt either the epidermal cell fate or the vulval cell fate (for a review, see reference 23). In wild-type animals, P(1, 2, 9 to 11).p cells fuse to hyp7 during L1 and lose their potential to adopt the vulval fate. The other six Pn.ps, P3.p through P8.p (also called vulval precursor cells [VPCs]), remain unfused with the surrounding hyp7 until L2-L3 and are competent to adopt the vulval fate. Using *ajm-1*::*gfp* to visualize the fusion of Pn.ps, we found that in *nhr-25* mutants, some Pn.ps that normally fuse to hyp7 during L1 did not fuse as late as L2 (Table 2). Conversely, P4.p through P8.p, which should always remain unfused until late L2, sometimes fused to hyp7 during L1 or early L2 and therefore could no longer become vulval cells. In wild-type animals, three of the six VPCs, P(3, 4, 8).p, adopt the epidermal cell fate during the L3 stage; they divide only once and fuse to hyp7. The remaining three VPCs, P(5 to 7).p, are induced by an epidermal growth factor signal to become vulval cells and undergo a total of three rounds of cell division and a series of subsequent morphogenetic events (23, 53). In the *ku217* mutants, fewer VPCs (an average of 2.7 cells; $n = 39$) were induced to become vulval cells at 20°C, consistent with the fact that some of the VPCs fused earlier and therefore lost their competence to acquire the vulval fate. Further lineage analysis revealed that some vulval cells prematurely stopped dividing in the *ku217* mutants, particularly those that normally divide transversely, i.e., vulC, -E, and -F cells (Table 3). Using *ajm-1*::*gfp*, we investigated whether the defect in vulval cell division is accompanied by abnormal cell fusion. As shown in Fig. 5C, the boundary between vulE, vulF, and hyp7 was missing, indicative of abnormal fusion between these vulval cells and hyp7.

In wild-type animals, as the vulval cells finish dividing, they fuse with each other in an invariant pattern to form nine multinucleate syncytial cells. During L4, sister cells from the third division (except for vulB1 and vulB2) on either the anterior or posterior side of the vulva first fuse with each other (Fig. 5B). These cells then send out cytoplasmic processes to the other side of the vulva and fuse with their counterparts. Although the division pattern of the vulA, vulB1, and vulB2 cells did not seem to be much affected by the *nhr-25*(*ku217*) mutation, they sometimes abnormally fused after division and formed a large syncytial cell (Fig. 5C). As mentioned above, migration of the vulval cells was also disrupted by the *nhr-25*(*ku217*) mutation so that the vulval cells on either the anterior or posterior side of the vulva often did not make contact and therefore did not fuse with each other (Fig. 5C).

Major adherens junction components appeared to be normal in *nhr-25* mutants. To test the possibility that the abnormalities in various epidermal cells are a result of disruption of adherens junctions that bring two neighboring cells together, we examined the expression level and localization of adherens junction components in *nhr-25* mutant animals. At least two layers of physical contacts are present at the adherens junctions in *C. elegans*, one consisting of HMR-1, HMP-1, and HMP-2 (12) and the other consisting of AJM-1 (50). The complex formed by HMR-1 (E-cadherin), HMP-1 (α-catenin), and HMP-2 (β-catenin) has been found to connect the intracellular actin network to the extracellular matrix (12). A number of membrane-associated proteins have also been implicated in the proper assembly of the adherens junctions. For example, DLG-1 and LET-413 are required for the proper organization of the actin cytoskeleton, and they cooperatively

TABLE 3. *nhr-25* and *lin-39* mutations disrupt vulval cell induction and division

Genotype	Vulval cell identity[a]												*n*
	P5.p				P6.p				P7.p				
	A	B	C	D	E	F	F	E	D	C	B	A	
Wild-type	L	L	T	N	T	T	T	T	N	T	L	L	10
nhr-25(*ku217*)	L	L	**N**	N	T	**N**	**N**	T	N	**N**	L	L	3
	L	L	**N**	N	T	**N**	**N**	**N**	N	**N**	L	L	3
	L	L	**N**	N	**N**	**N**	**N**	**N**	N	**N**	L	L	2
	L	L	**N**	N	**N**	**N**	**N**	T	N	**N**	L	L	1
	L	L	**N**	N	T	**N**	**N**	**N**	N	T	L	L	1
	L	L	**N**	N	T	**O**	**N**	T	N	**N**	L	L	1
	S			**S**	**N**	**N**	**N**	T	N	**N**	L	L	1
	S			**S**		**S**		**S**		**S**		**S**	1
lin-39(*n709*)	L	L	T	N	T	T	T	T	N	T	L	L	13
	L	L	T	N	T	T	T	T		**S**		**S**	4
	L	L	**N**	N	T	T	T	T	N	T	L	L	1
	N	**N**	**N**	N	T	T	T	T	N	T	L	L	1
	N	**N**	**N**	N	T	T	T	T		**S**		**S**	1
	S			**S**	T	T	T	T	N	T	L	L	1
	S			**S**	T	T	T	T		**S**		**S**	1
lin-39(*n709*); *nhr-25*(*ku217*)	**S**			**S**	**N**	**N**	**N**	**N**			**S**		3
	S			**S**	**N**	**N**	**N**	**N**		**S**		**S**	2
		S			**N**	**N**	**N**	**N**	N	**N**	**N**	**N**	2
	N	**N**	**N**	N	**N**	**N**	**N**	**N**		**S**		**S**	2
	N	**N**	**N**	N	**N**	**N**	**N**	**N**			**S**		2
	L	L	**N**	N	**N**	**N**	**N**	**N**			**S**		1
	S		**N**	N	**N**	**N**	**N**	**N**		**S**		**S**	1
	S		**N**	N	**N**	**N**	**N**	**N**		**S**		**S**	1
	S			**S**		**S**		**S**	N	**N**		**S**	1
	S			**S**		**S**	**N**	**N**		**S**	**N**	**N**	1
		S			**N**	**N**	**N**	**N**			**S**		1
		S				**S**	**N**	**N**		**S**		**S**	1
lin-11(*n566*)[b]	L	L	**L**	**L**	T	T	T	T	**L**	**L**	L	L	1
	L	L	**L**	**L**	T	T	T	T	N	T	L	L	1
	L	L	**L**	N	T	T	T	T	N	**L**	L	L	2
nhr-25(*ku217*) *lin-11*(*n566*)	L	L	**L**	**L**	**N**	**N**	**N**	**N**	**L**	**L**	L	L	1
	L	L	**L**	**L**	T	**N**	**N**	**N**	**L**	**L**	L	L	1
	L	L	**L**	N	**N**	T	**N**	T	N	**L**	L	L	1
	L	L	**L**	N	T	T	**N**	T	**L**	**L**	L	L	1
	L	L	**L**	N	**N**	**N**	**N**	**N**	N	**L**	L	L	1
	L	L	**L**	N	T	**N**	**N**	**N**	N	**L**	L	L	1
	L	L	**L**	N	T	**N**	**N**	T	N	**L**	L	L	1
	L	L	**L**	N	T	**N**	**N**	**N**		**S**		**S**	1
	S			**S**		**S**		**S**		**S**		**S**	1

[a] L, T, N, and O represent the division axes of the vulval cells during the third round of cell division. L, longitudinal division; T, transverse division; N, no division; O, oblique division axis; S, no vulval induction. Abnormal axes are highlighted in boldface. The number of letters in each cell indicates the number of progeny of each Pn.p cell.

[b] See Ferguson et al. (15a) for lineage data for *lin-11*(*382*) and *lin-11*(*n389*).

control AJM-1 localization in the apical junction (18, 35, 38). Mutations in the above-mentioned genes often result in the rupture of the animal body during embryonic elongation, a phenotype similar to that observed in the *nhr-25* mutant animals. Using antibodies against HMR-1, HMP-1, and HMP-2, we found that the expression levels, as well as the localizations, of all three proteins in *ku217* animals injected with *nhr-25* dsRNA, which caused fully penetrant embryonic lethality, were indistinguishable from those in wild-type animals (data not shown). Even in arrested embryos, adherens junctions, as marked by the antibodies of these three proteins, were still clearly visible. As mentioned above, the *ajm-1* expression pattern was not affected in the embryonic epidermal cells, as well as the seam and vulval cells, of larvae (Fig. 3, 4, 5, and 6). Taken together, epidermal cells in *nhr-25* mutants appeared to have properly differentiated and were able to establish the initial physical contact with each other. However, these contacts were often disturbed when the cells underwent extensive morphological changes.

***nhr-25* likely functions cell autonomously in epidermal cells.** Although *nhr-25* is expressed throughout the epidermis, we sought to further investigate where *nhr-25* function is required during development. The gene *sid-1* was shown to be essential for systemic, but not cell-autonomous, RNAi in *C. elegans* (68). We used a mutant *sid-1*(*qt2*) strain to perform tissue-specific RNAi by expressing the sense and antisense strands of partial

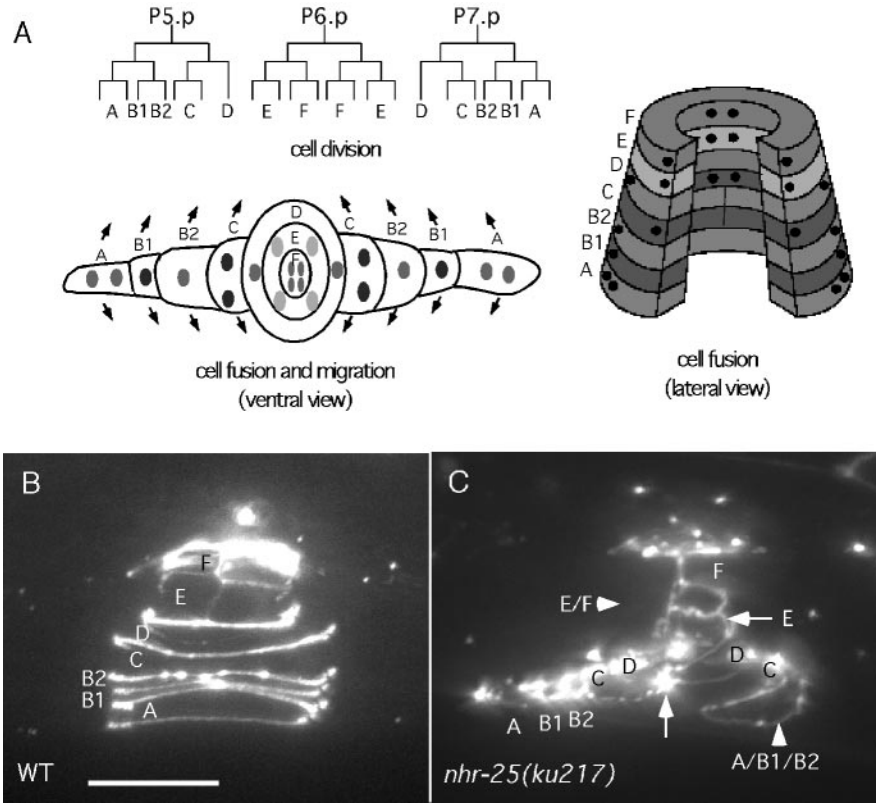


FIG. 5. Vulval cell division, fusion, and migration. (A) Twenty-two vulval cells are generated from three rounds of cell division. Except for vulB1 and vulB2, vulval cells that adopt the same cell fate, e.g., the two vulA cells, on either the anterior or posterior side of the vulva first fuse. These cells then send out cytoplasmic processes to the other side and fuse to the corresponding cells. The arrows indicate the directions of outgrowth of the cytoplasmic processes. A total of nine syncytial cells are generated, and they stack into seven toroidal rings (A through F). (B) *ajm-1*::*gfp* expression in a wild-type (WT) vulva (lateral view). The letters between rings of *ajm-1*::*gfp*-expressing membranes represent the identities of the syncytia. (C) *ajm-1*::*gfp* expression in an *nhr-25*(*ku217*) mutant (dorsal-lateral view). The arrowheads indicate where abnormal cell fusion occurred, and the arrows point to the junctions between cells that failed to fuse. Note that some vulC, -E, and -F cells are no longer enclosed by *ajm-1*::*gfp*-expressing membranes, suggesting that these cells have already fused to the surrounding hyp7. The posterior vulA, -B1, and -B2 abnormally fused together. In contrast, vulD cells and the posterior vulE cells failed to fuse. Anterior is to the left, and ventral is down. Scale bars, 10 μm.

nhr-25 coding sequence under the control of the *col-10* promoter (see Materials and Methods). *col-10* codes for a collagen protein produced by the epidermal cells during embryonic and larval stages (V. Ambros, personal communication). Larval-stage expression of a GFP reporter under the control of the *col-10* promoter appeared to be restricted to hyp7, seam cells, and Pn.ps (data not shown). We found that, in the background of *sid-1*(*qt2*), animals expressing the *pcol-10*::*nhr-25* sense and antisense constructs sometimes had a shorter body length and were more severely defective in the posterior end (Fig. 6C). Ten percent (n = 191) of progeny segregating from an animal carrying *pcol-10*::*nhr-25*(sense/antisense) constructs displayed a larval molting defect at various stages (Fig. 6A and B), suggesting that inhibition of *nhr-25* activity in the epidermal cells interfered with the normal function of these cells. Additionally, 22% (n = 143) of animals positive for *pcol-10*::*nhr-25*(sense/antisense) constructs were Egl. Although these animals were not specifically examined for fusion defects using an adherens junction marker, a number of them had failures in Pn.p cell division and vulval morphogenesis similar to the fusion-defective *nhr-25* RNAi or *nhr-25*(*ku217*) mutants (Fig. 6D). Control animals carrying empty *pcol-10* vector did not display molting defects (0%; n = 301) or the Egl phenotype (<1%; n = 301). Overall, these results suggest that *nhr-25* function is likely to be required cell autonomously in the epidermal cells and that the vulval defects seen in *nhr-25* mutants are probably not the result of defective anchor cell signaling. In further support of this hypothesis, a recent study has shown that the anchor cell expression pattern of a *lin-3*::*gfp* reporter was unchanged in an *nhr-25* RNAi background, where vulval differentiation was greatly compromised (29).

***nhr-25* and *lin-39* act collaboratively in regulating VPC fusion.** As discussed above, in the *nhr-25* mutant, vulval precursor cells sometimes abnormally fused to hyp7; as a result, these cells could no longer acquire the vulval fate and failed to go through all three rounds of cell division. Such a phenotype was also observed in *lin-39* Hox mutants (11, 65). We went on to test possible genetic and physical interactions between *nhr-25* and *lin-39*. We first constructed and examined the phenotypes of double mutants for the two genes. When *nhr-25* RNAi was carried out in the background of a *lin-39*(*n1760*) null mutant, which has not been found to be associated with any embryonic defects, only mild embryonic lethality was observed, as in *nhr-25* dsRNA-treated animals alone, suggesting that these

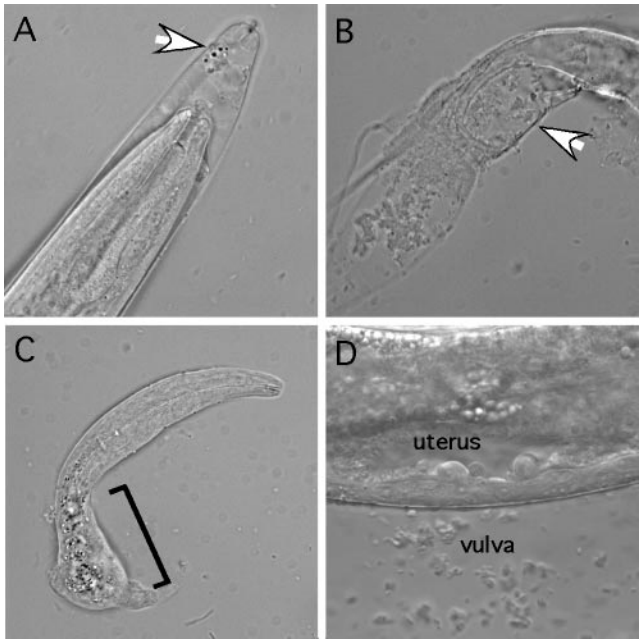


FIG. 6. Disruption of *nhr-25* function through tissue-specific RNAi treatment. (A to D) Phenotypes caused by expression of partial *nhr-25* sense and antisense sequence under the control of the *col-10* promoter in a *sid-1*(*qt2*) background. (A and B) Arrows indicate old cuticle still attached to the anterior (A) and the posterior (B) of the worm. (C) L1 larvae had normal anterior structures but had a shortened posterior half, as indicated by the bracket. (D) Adult hermaphrodite with no recognizable vulval differentiation, very similar to *nhr-25*(*ku217*) mutant animals. Anterior is to the right.

two genes are unlikely to have synergistic or redundant functions during embryogenesis. However, in *lin-39*(*n709*); *nhr-25*(*ku217*) double mutants, both alleles being partial loss-of-function mutations, a higher percentage of VPCs failed to adopt the vulval fate (Table 3). P5.p and P7.p sometimes fused to hyp7 before they underwent any division, and P6.p never divided more than twice (Table 3). We further characterized the fusion defects of P1.p to P11.p in a *lin-39*(*n709*); *nhr-25*(*ku217*) background by using the *ajm-1*::*gfp* marker. The VPC fusion defects seen in this double-mutant background were more severe than in any of the single weak loss-of-function mutants alone but no more severe than a strong loss-of-function in *nhr-25* (i.e., RNA: in the *ku217* background) (Table 2). These data suggest that *lin-39* and *nhr-25* may function collaboratively in preventing P(3 to 8).p cell fusion with the hyp7 cell and subsequently maintaining their competence for the vulval fate.

Secondly, a GST pull-down experiment was carried out to examine the direct physical interaction between NHR-25 and LIN-39. As shown in Fig. 7A, ^{35}S-labeled LIN-39, but not luciferase, was retained on the glutathione-Sepharose resin that was conjugated with wild-type NHR-25, suggesting that NHR-25 specifically interacts with LIN-39 (Fig. 7A). The L32F mutation in the *ku217* allele, which greatly compromised the DNA-binding activity of the NHR-25 protein, did not significantly affect the binding of LIN-39 (Fig. 7A). These data, in conjunction with the genetic evidence, suggest that NHR-25 and LIN-39 likely function together in a complex to regulate the postembryonic development of the VPCs.

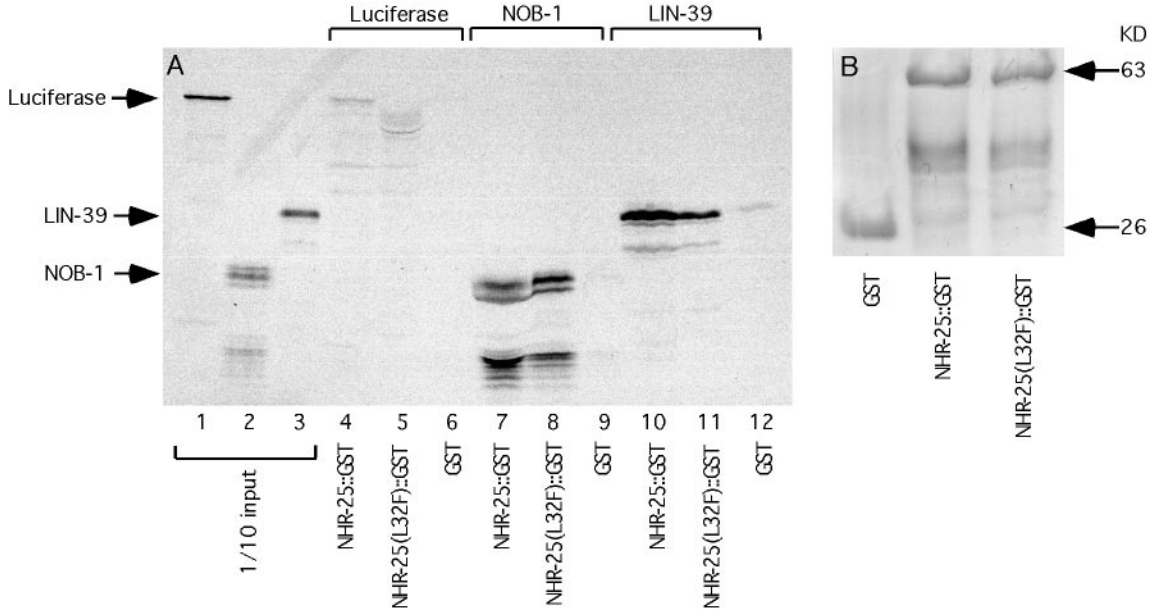


FIG. 7. NHR-25 physically interacts with LIN-39 and NOB-1. (A) Binding between LIN-39 and NHR-25. Lanes 1, 2, and 3, 1/10 total input of the in vitro-translated luciferase, NOB-1, and LIN-39, respectively; lanes 4, 5, and 6, amounts of luciferase protein retained on the Sepharose resin conjugated with NHR-25, NHR-25(L32F), and GST, respectively. None of the three proteins interacted with luciferase. Lanes 7, 8, and 9, amounts of NOB-1 retained on the Sepharose resin conjugated with NHR-25, NHR-25(L32F), and GST, respectively; lanes 10, 11, and 12, amounts of LIN-39 retained on the Sepharose resin conjugated with NHR-25, NHR-25(L32F), and GST, respectively. Only NHR-25 and NHR-25(L32F) (and not GST alone) were able to interact with NOB-1 and LIN-39. Note that the NHR-25(L32F) mutation resides in the DBD of the protein. (B) Similar amounts of GST, NHR-25::GST, and NHR-25(L32F)::GST were used for incubation with in vitro-translated proteins.

TABLE 4. Genetic interaction between *nhr-25* and *nob-1* during embryogenesis

Genotype	% Phenotype[a]		*n*
	Emb let	Nob	
nhr-25 RNAi	11	<1	562
nob-1(*ct230*)	12	6	663
nob-1(*ct230*) *nhr-25* RNAi	56	10	714
nob-1(*ct230*) *unc-22* RNAi	14	5	145

[a] Emb let, embryonic lethality; Nob, no back end.

***nhr-25* and *nob-1* act together to promote embryogenesis.** Because *nhr-25* mutant phenotypes were seen in a number of cell types throughout development, we sought to determine if NHR-25 was functioning with Hox proteins other than LIN-39. We chose to investigate the Hox genes *ceh-13*, *nob-1*, and *mab-5* due to their phenotypes and/or expression patterns. The Hox protein CEH-13 is an attractive candidate for functioning with NHR-25 in the embryo and seam cells due to the *ceh-13* loss-of-function phenotypes, which include rupture of the embryonic epidermis, fusion defects, and seam cell abnormalities (6). MAB-5, another Hox protein, is also known to regulate differentiation of the seam cells (33). However, we failed to observe genetic interactions between *nhr-25* and the Hox genes *ceh-13* and *mab-5* (data not shown). The posterior-group Hox gene *nob-1* was previously shown to have an important role in embryonic morphogenesis (64). *nob-1*(*ct230*), a weak loss-of-function allele, results in animals that are embryonic lethal and larval lethal (with missing posterior regions) and viable animals with variably misshapen tails. Because *nhr-25* mutants sometimes have shortened posterior ends (data not shown and Fig. 6C), we looked for genetic interactions between *nhr-25* RNAi and *nob-1*(*ct230*). We observed enhanced embryonic lethality in a *nob-1*(*ct230*); *nhr-25* RNAi background that was considerably greater than the sum of the lethalities seen in the single mutants (Table 4). Dead embryos from this double-mutant background appeared to be similar to those resulting from a strong loss of function in *nhr-25*. A control RNAi experiment directed against *unc-22* did not show increased lethality in *nob-1*(*ct230*) animals (Table 4). Thus, these results suggest the possibility that *nhr-25* and *nob-1* function together to promote embryogenesis; conversely, *nhr-25* is not likely to function with *ceh-13* or *mab-5*.

To further support the genetic results discussed above, we performed GST pull-down experiments. ^{35}S-labeled NOB-1, but not luciferase, was retained on the glutathione-Sepharose resin that was conjugated with wild-type NHR-25 (Fig. 7A). Again, the L32F mutation in the *ku217* allele did not affect the binding of NOB-1 (Fig. 7A). Thus, NHR-25 may function in a complex with NOB-1 to promote some aspect of embryonic development. As expected, CEH-13 and MAB-5 displayed no physical interaction in the GST pull-down assay (data not shown).

DISCUSSION

Cell fusion defects were observed in the ventral and dorsal embryonic epidermal cells, Pn.p cells, and vulval cells of *nhr-25* mutants. It is possible that the presence of extra SCM-positive nuclei in the adult syncytial seam cell is caused by a failure of the seam cell daughters to properly fuse to hyp7 during larval development, thus maintaining the seam cell fate. Mutations in other genes that disrupt cell fusion, such as *eff-1*, lead to the presence of extra seam cells (49). Alternatively, some seam cell daughters may not be able to properly adopt the cell fate in the first place and consequently are unable to fuse to hyp7. The mutant phenotypes in the male tail may also result from cell fusion defects, since cell fusion plays an important role in the morphogenesis of the male tail (14). However, outside of these defects, fusion in general does not appear to be affected, and no gross changes in *eff-1*::*gfp* expression were observed in *nhr-25* mutant embryos (data not shown). Additionally, *nhr-25* appears to both promote and inhibit Pn.p and vulval cell fusion. Taken together, these results suggest that there is a failure in the regulation of fusion and differentiation and not a specific defect in the fusion machinery. The altered morphology of seam cells, improper migration of Pn.ps, and alar and molting defects in *nhr-25* mutants all support the idea that *nhr-25* functions in additional aspects of normal epidermal cell function that remain to be understood. However, *nhr-25* function is not likely to dramatically control cell fate determination, given the presence of most epidermal cell junction components and seam cell markers in mutant *nhr-25* animals.

It has been suggested that syncytial cells formed by cell-cell fusion are in general in a nonproliferative state (for a review, see reference 55). Therefore, the fusion between a syncytial cell and its neighboring cells may play an important role in controlling cell differentiation of the neighboring cells. During L3, the epidermal growth factor/Ras/MAPK pathway, together with the activities of genes in several other regulatory pathways, including *lin-39*, *lin-12*/*Notch*, and components of the Wnt pathway, actively promote the vulval fate in P5.p to P7.p and repress the alternative epidermal fate by preventing cell fusion between Pn.ps and hyp7 (10, 13, 23, 27, 41). Reduction of *nhr-25* function in the vulva often led to a failure in cell division during the last round of cell division; the undivided cells appeared to have fused with neighboring hyp7 syncytium. Therefore, *nhr-25* is likely to repress cell fusion in order to maintain the proliferating capability of the vulval cells. We noticed that in the mutant vulC cells that stopped dividing, a vulva-specific gene, *egl-17*, was no longer expressed (data not shown). Since vulD cells, the nondividing sister cells of vulC, did express *egl-17* in both mutant and wild-type animals, it suggests that the mutant vulC cells did not transform into a cell fate similar to their sisters' vulval cells. Instead, these mutant vulC cells might have adopted an epidermal cell fate and therefore fused to the hyp7 syncytial cell. A similar choice between fusion and division is also critical for seam cell differentiation: the *elt-5* and *-6* genes are known to be essential for repressing seam-hypodermis fusion and for promoting proper seam cell differentiation (34). However, it remains a question whether the fusion defect seen in the *nhr-25* mutant vulval cells is a consequence of failure in cell division. Previous work by G. Shemer and colleagues has suggested that fusion is not a default state after failure in cell division in the Pn.ps (54). Therefore, we favor the model that the fusion defect is causal in the division defects seen in *nhr-25* mutants.

lin-39 activity in P3.p to P8.p, during the L1 and L2 larval stages, prevents these six cells from fusing to hyp7 and maintains their ability to divide and differentiate into vulval cells. Without *lin-39* function, such as in *lin-39* mutants or in P(1, 2, 9 to 11).p,

the Pn.p cells fuse to hyp7 and can no longer divide (11, 65). *nhr-25* mutants reveal a similar function for *nhr-25* in repressing fusion in the VPCs. Ftz-F1 and its mammalian homologues are known to act as cofactors for other transcriptional regulators (22, 25, 70). For example, Ftz-F1 interacts physically and acts collaboratively with Ftz, a homeodomain-containing protein, to regulate gene expression in every other segment of fly embryos (25, 70). Our results suggest that NHR-25 may function as a cofactor for a Hox protein, LIN-39, in the vulval lineage, based on the physical interaction between the two proteins and the genetic synergy of fusion defects seen with partial loss-of-function mutations in the two genes. However, unlike *lin-39*, *nhr-25* appears to simultaneously promote fusion of the non-vulval-lineage Pn.p cells, P(1, 2, 9 to 11).p. Thus, an attractive model for *nhr-25* regulation of Pn.p cell fusion consists of NHR-25 functioning with LIN-39 to repress fusion of P3.p to P8.p while acting with another transcription factor in the non-VPC Pn.p cells (or in other cell types, such as the seam cells) to promote their fusion to hyp7. The identification of downstream target genes for NHR-25 and LIN-39 is likely to be crucial in further investigating how they cooperate in controlling vulval cell fusion and differentiation. It has been shown that *lin-39* acts to repress Pn.p cell fusion by repressing the expression of EFF-1, a membrane protein directly involved in cell fusion (49, 56). However, we did not observe significant changes in *eff-1*::*gfp* expression in an *nhr-25* mutant background (due to the embryonic lethality, we were unable to use the null mutation to assay the effect of completely eliminating *nhr-25* activity on *eff-1* expression), although this does not exclude the possibility that *nhr-25* acts on other targets that are involved in cell fusion. A recent study of a novel member of the angiotensin-converting enzyme family, *acn-1*, has suggested that NHR-25 positively regulates *acn-1* expression (5). This study also demonstrated that loss of *acn-1* function results in a set of molting, vulval, and seam cell defects similar to those seen in *nhr-25* mutants. Therefore, the *acn-1* gene might define at least one potentially important transcriptional target of NHR-25.

Given the fact that LIN-39 activity is restricted to postembryonic stages in the Pn.p cells, it is possible that NHR-25 functions together with other factors, likely other Hox proteins, during embryogenesis and in different tissues. Indeed, we observed a physical and genetic interaction with the Hox gene *nob-1*. A thorough analysis of *nob-1* expression and function has yet to be completed; therefore, it is difficult to speculate how or in what cells NHR-25 and NOB-1 might function together to promote embryogenesis. NHR-25 complex formation with various Hox or homeodomain-containing proteins might be an important and conserved mechanism for the regulation of NHR-25 function throughout development and in various cell types. However, experiments to look for *ceh-13* or *mab-5* genetic interactions with *nhr-25* mutants, as well as in vitro binding assays to look for the physical interactions of CEH-13 and MAB-5 with NHR-25, were both negative (data not shown).

It remains unknown why the vulC, -E, and -F cells, which normally switch their division axes from the longitudinal to the transverse orientation, were more affected than the vulA and -B cells that continue to divide longitudinally in *nhr-25* mutants (Table 3 and Fig. 6). The difference may imply that *nhr-25* activity is essential in cells that undergo extensive cytoskeleton reorganization during division. Such an activity of *nhr-25* is no longer required once vulC transforms to become vulA/B, such as in the *lin-11* mutant background (20), since the transformed vulC regained its ability to divide along the longitudinal axis even when *nhr-25* function was impaired (i.e., vulC divided longitudinally in both *lin-11* and *lin-11 nhr-25* animals [Table 3]). *nhr-25* activity might also be required for the extensive cytoskeleton reorganization during and after migration and elongation of the ventral epidermal cells, for the elongation of seam cells, and in the migration of induced Pn.p cells.

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