Chapter 2
Heterochronic Control of AFF-1-Mediated Cell-to-Cell Fusion in *C. elegans*

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Abstract In normal development cell fusion is essential for organ formation and sexual reproduction. The nematode *Caenorhabditis elegans* has become an excellent system to study the mechanisms and developmental functions of cell-to-cell fusion. In this review we focus on the heterochronic regulation of cell fusion. Heterochronic genes control the timing of specific developmental events in *C. elegans*. The first microRNAs discovered were found as mutations that affect heterochronic development and cell–cell fusions. In addition numerous heterochronic transcription factors also control specific cell fusion events in *C. elegans*. We describe what is known about the heterochronic regulation of cell fusion of the epidermal seam cells. The fusogen AFF-1 was previously shown to mediate the fusion of the lateral epidermal seam cells. Here we provide evidence supporting the model in which LIN-29, the heterochronic Zinc-finger transcription factor that controls the terminal fusion of the seam cells, stimulates AFF-1 expression in the seam cells before they fuse. Therefore, the heterochronic gene LIN-29 controls AFF-1-mediated cell–cell fusion as part of the terminal differentiation program of the epidermal seam cells.

2.1 Introduction

Throughout development of the nematode *Caenorhabditis elegans* about one third of the somatic cells go through cell to cell fusion (for recent reviews see [1–3]).

Cell fusion events occur during both embryonic and postembryonic development in various organs including the hypodermis, vulva, pharynx and uterus [4–6]. It was shown that the cell fusions in *C. elegans* are mediated by two fusogens EFF-1 and AFF-1 [7, 8]. Mutations that cause ectopic fusion lead to embryonic lethality. In agreement, developmental cell fusion was found to be a tightly regulated process in *C. elegans*. In addition to spatial regulation, the developmental timing of cell fusion events is also critical. Several transcription factors have been found to regulate the precise developmental stage in which fusions occur [1].

2.2 Heterochronic Genes Regulate the Timing of Developmental Events

Heterochrony is a change in the timing of a specific developmental event relative to other developmental events which are not affected [9, 10]. Heterochrony can cause evolutionary variation since a change in timing of a certain developmental event can result in speciation [9] (reviewed in [11, 12]).

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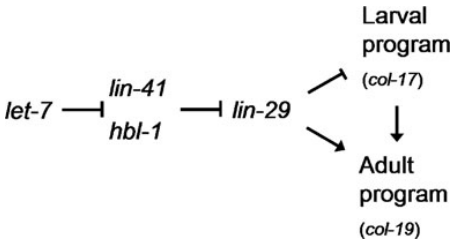

Fig. 2.1 The heterochronic pathway. Simplified model of the heterochronic gene pathway controlling the terminal seam cells differentiation

For example, two nematodes species, *Pristionchus pacificus*, and *C. elegans*, display heterochronic differences between them in several cell lineages such as vulval and gonadal lineages, although the genetic basis for those differences is not known [13].

Heterochronic mutants that control several developmental events were identified in *C. elegans*. Mutations in these heterochronic genes change the time of developmental events while other events in the organism occur in the normal timing of the wild type. Heterochronic mutations can result in two types of phenotypes. In the precocious phenotype the developmental event occurs earlier than in wild type and in the retarded phenotype the event happens in a later stage with respect to the wild type and in addition the event can be reiterated [10] (for recent reviews see [12, 14, 15]).

The heterochronic genes that were identified by genetic approaches control various developmental events among them vulva formation, dauer larva formation, aging and terminal differentiation of the hypodermal seam cells [10, 16–18]. Using epitasis analysis these heterochronic genes were organized into a model of heterochronic pathway (Fig. 2.1).

2.3 The Heterochronic Gene *lin-29* Determines the Final Fate of the Seam Cells

The transcription factor LIN-29 is the most downstream known heterochronic regulator of the seam cells terminal differentiation [18] (Fig. 2.1). By terminal differentiation the seam cells switch from larval seam cells program into adult seam cells program [18]. In *lin-29* mutants the seam cells exhibit retarded phenotype; the seam cells fail to terminally differentiate and the larval program is reiterated [10, 18]. The *lin-29* gene encodes a transcription factor that contains five $(Cys)_2$-$(His)_2$ zinc finger domains [19].

To date the heterochronic pathway is comprised of numerous genes, but here we will describe a brief summary of the pathway. *lin-4* and *let-7,* the first two members discovered in the microRNA (miRNA) family, were identified as members of the heterochronic gene pathway [20, 21]. During the mid-L1 stage *lin-4* is expressed and downregulates the LIN-14 nuclear protein, which specifies the L1 fate [10, 20, 22]. When *lin-4* is mutated, the lineage pattern of the L1 stage is reiterated [23]. In *lin-14* loss of function mutants the L2 pattern occurs precociously in the L1 stage [10]. *lin-4* also represses LIN-28 permitting transition to L3 stage fate [24]. LIN-46 and *let-7* paralogs *mir-84*, *mir-48* and *mir-241* downregulate HBL-1 to control the L2 to L3 transition [25]. In addition DAF-12 while bound to its ligand, also downregulates HBL-1 to control this transition by directly activating *let-7* miRNA family members [26]. Later during development LIN-41 and HBL-1 repress LIN-29 expression thus, specifying late larval fate. Next, *let-7* downregulates LIN-41 and HBL-1 allowing LIN-29 expression that direct the seam cells terminal differentiation at L4 to adult transition [27–29].

In addition to the seam cells terminal differentiation, *lin-29* is also required in other tissues. *lin-29* is necessary in the egg laying system for specification of the utse, regulation of genes expression in the vulval cells at the L4 stage and differentiation of the vulval cells [30]. Furthermore *lin-29* is required in a subset of the lateral seam cells for proper vulva morphogenesis and egg laying [31]. Thus, by

acting in several components of the egg laying system, *lin-29* may coordinate the vulval-uterine-seam cell connection.

Additionally *lin-29* is required for the linker cell death in *C. elegans* male that occur during or just after the L4/adult transition. Since *let-7* also controls the linker cell death, it is likely that the linker cell death is regulated by the heterochronic pathway [32].

2.4 LIN-29 Controls the Terminal Differentiation of the Epidermal Seam Cells

The lateral hypodermal seam cells form two rows of cells one on each side along the body of the worm. The seam cells are hypodermal cells that synthesize and secrete the cuticle [33]. During each of the 3 larval stages (L1–L3), around the time of the molts, the seam cells divide in a stem cell manner producing one daughter cell that retains a seam cell fate and a second cell that either will fuse to the hypodermal hyp7 syncytium or will have other fate [34].

During the larva to adult molt, which is the transition from L4 to adult, the seam cells undergo terminal differentiation. The seam cells stop cell divisions, fuse with each other forming a longitudinal syncytium on each side of the worm (Figs. 2.2 and 2.3a), synthesize adult cuticle which includes secretion of the adult "alae" and stop the molting cycle [18]. The alae are a set of raised cuticular stripes that are positioned along the body of the worm above the seam cells. In addition to this morphological difference, the larval and the adult cuticle are also distinguished in their collagen gene expression [35]. *lin-29* is required for all the events of the terminal differentiation of the seam cells (Fig. 2.1).

Since *lin-29* is a transcription factor it can act by regulating either directly or indirectly the transcription of genes that are required for the terminal differentiation, therefore regulating genes involved in cell cycle exit, cell fusion, switching to the adult cuticle and in the molting cycle. It was found that *lin-29* regulates the transcription of specific collagen genes (*col-17* and *col-19*) at the L4-adult molt [36]. *lin-29* represses *col-17* and activates *col-7* and *col-19* transcription at this stage [36]. It was previously shown that LIN-29 protein binds in vitro to *col-19* and *col-17* promoter sequences [19]. In the case of *col-19* this binding of LIN-29 is to the regulatory sequence which is necessary for in vivo adult-specific activation of the collagen gene *col-19*. These results suggest a direct role of *lin-29* in regulating collagen genes which are required for seam cells terminal differentiation. Additional possible targets of LIN-29 are *nhr-23* and *nhr-25* that encode conserved nuclear hormone receptors which are essential for larval molting. *nhr-23* and *nhr-25* were shown to be downstream effectors of *let-7* and *mir-84*. A possible model is that LIN-29 represses *nhr-23* and *nhr-25* after the forth molt and by that cause exit from the molting cycle [37]. LIN-29 is the best candidate for regulating the seam cells fusion in the L4 to adult switch.

Now two important questions can be asked: how do the seam cells fuse during the terminal differentiation? And – what is the regulation mechanism of the seam cells fusion?

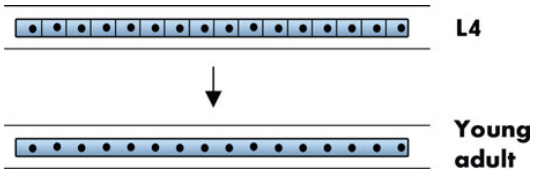

Fig. 2.2 The seam cells terminal fusion. In the L4 stage the lateral hypodermal seam cells form two rows (*left* and *right*) each containing 16 cells. During the transition from L4 to adult, the seam cells fuse with each other forming a longitudinal syncytium on each side of the worm. The seam cells fusion is part of their terminal differentiation process

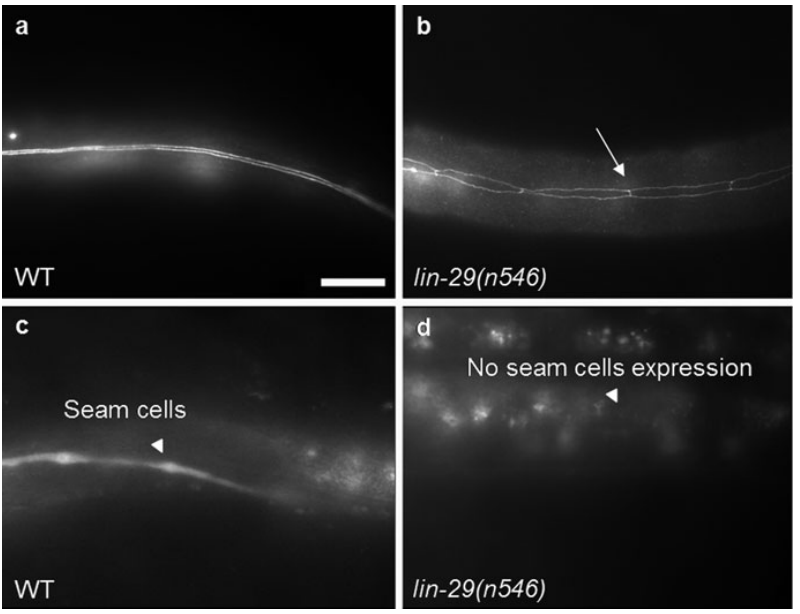

Fig. 2.3 LIN-29 controls AFF-1- mediated fusion of the seam cells. (**a**,**b**) Immunofluorescence of worms stained with MH27 antibody which recognizes an antigen in the adherens junctions of epithelial cells [4, 46, 47]. (**c**,**d**) Transgenic worms expressing *aff-1p::GFP* construct. (**a**) In wild type worms at late L4 stage, the seam cells fused forming a syncytium that is marked by two parallel lines of adherens junctions. (**b**) Young adult *lin-29(n546)* worm in which the seam cells failed to fuse. *Arrow* indicates unfused adherens junction. (**c**) *aff-1promoter::GFP* construct was expressed (*arrowhead*) in wild type worms in the seam cells at late L4 stage. (**d**) Late L4 *lin-29(n546)* mutant in which there was no *aff-1promoter::GFP* expression in the seam cells while *aff-1promoter::GFP* expression was retained in other tissues like the utse (not shown). In (**a**), (**c**) anterior is to *right*, in (**b**), (**d**) to the *left*. *Scale bar* represents 25 μm

2.5 AFF-1 Protein Mediates the Terminal Fusion of the Hypodermal Seam Cells

In order to study cell fusion in *C. elegans* forward genetic screens were performed searching for fusion failure phenotypes. Two genes were identified as encoding proteins that induce cell fusion events in *C. elegans*. One of these proteins, AFF-1 was found to be the protein necessary for the fusion of the seam cells during their terminal differentiation [8].

In addition to the seam cell fusion *aff-1* (**a**nchor cell **f**usion **f**ailure-1) is also required for the fusion of the anchor cell (AC) to the utse syncytium [8]. The AC coordinates the connection between the uterus and the vulva. The AC induces vulval precursor cells to receive vulval fates thereby inducing vulva formation [38, 39]. Next, the AC induces surrounding uterine cells to become π cells that produce the uterine cells (utse, uv1) which connect to the vulva [40, 41]. The final stage in the formation of the connection between the uterus and vulva involves the removal of the AC by cell fusion in order to enable passing of eggs through the egg-laying organ. First, eight π cells progeny fuse to form the utse syncytium, and then the AC fuses to this utse syncytium. The AC-utse syncytium is an H-shaped cell that in its middle region has a thin cytoplasmic process which is located between the vulva and the uterus and serves as the nematode's hymen. While the first egg exits the uterus this hymen is broken generating a connection between the uterine and vulval lumens [41].

Additional fusions events to which *aff-1* is required are the fusion of the vulval rings vulA and vulD that occur in the L4 stage [8]. AFF-1 ectopic expression is sufficient to induce cell fusion in cells that do not normally fuse in *C. elegans*. Moreover, AFF-1 was shown to fuse heterologous tissue culture cells. These observations indicate that AFF-1 serves as a bona fide fusogen [8].

aff-1 is required for only a part of the fusion events in *C. elegans*. The first fusogen identified in *C. elegans* was EFF-1 (**e**pithelial **f**usion **f**ailure 1). EFF-1 is essential for most of epidermal, vulval and pharyngeal cell fusion events [7, 42]. As AFF-1, EFF-1 fuses cells that normally do not fuse in vivo and also causes fusion of heterologous tissue culture cells, therefore, EFF-1 is an actual fusogen [42, 43]. Further research has shown that EFF-1 needs to be expressed in both fusing cells in *C. elegans* and in heterologous cells for cell fusion to occur. Thus, EFF-1 functions in a homotypic fusion mechanism [43].

Recently it was shown that in addition to its role in epithelial cells, EFF-1 also has a role in controlling dendrites structure in *C. elegans* by inducing dendrites retraction and autofusion [44].

eff-1 and *aff-1* genes encode type I transmembrane proteins that share only moderate sequence homology but exhibit significant similarity in their presumptive structure. EFF-1 and AFF-1 proteins show conservation in the number of cysteines and partial conservation of prolines residue number in the extracellular region. In addition the proteins contain a possible TGF-β-type-I-Receptor domain [8]. *aff-1* and *eff-1* represent the first two members of developmental eukaryotic fusogens. Together, *eff-1* and *aff-1* account for most cell fusions in *C. elegans* but not for all. For example, both sperm-egg fusion and π cell daughters fusion forming the utse are carried out in each of *eff-1* mutant and *aff-1* mutant and also in *eff-1 aff-1* double mutant [8]. These observations suggest that there are additional fusogens in *C. elegans*.

What is the regulation mechanism of the seam cells fusion? As mention above, in *lin-29* loss of function mutant worms the seam cells fail to undergo their terminal fusion (Fig. 2.3b). Thus, in order to answer the above question we recently examined the possibility that *lin-29* is regulating *aff-1* in the seam cells. We found that while in wild type worms *aff-1promoter::GFP* is expressed in the seam cells starting from the L4 stage, in *lin-29(n546)* loss of function mutant worms there is partial or no *aff-1p::GFP* expression in the seam cells during this stage (Fig. 2.3c, d) [45]. These results suggest that *lin-29* positively regulates *aff-1* expression in the seam cells during the L4 to young adult transition by transcriptional regulation. Thus, *aff-1* may be an effector of the heterochronic pathway (Friedlander-Shani and Podbilewicz, unpublished results).

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