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EFF-1 Is Sufficient to Initiate and Execute Tissue-Specific Cell Fusion in *C. elegans*

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Summary

Despite the identification of essential processes in which cell fusion plays spectacular roles such as in fertilization and development of muscle, bone, and placenta [1–5], there are no identified proteins that directly mediate developmental cell fusion reactions. *C. elegans* has recently become among the best-characterized models to use for studying developmental cell fusion [5–15]. The *eff-1* (epithelial *f*usion *f*ailure) gene encodes novel type I membrane proteins required for epithelial cell fusion. Analysis of *eff-1* mutants showed that cell fusion normally restricts routes for cell migration and establishes body and organ shape and size [5, 8, 9, 11]. Here, we explored cell fusion by using time-lapse confocal and electron microscopy of different organs. We found that ectopic expression of *eff-1* is sufficient to fuse epithelial cells that do not normally fuse. This ectopic fusion results in cytoplasmic content mixing and disappearance of apical junctions, starting less than 50 min after the start of *eff-1* transcription. We found that *eff-1* is necessary to initiate and expand multiple microfusion events between pharyngeal muscle cells. Surprisingly, *eff-1* is not required to fuse the gonadal anchor cell to uterine cells. Thus, *eff-1* is sufficient and essential for most but not all cell fusion events during *C. elegans* development.

Results and Discussion

The activity of *eff-1* is tightly regulated in time and space to prevent cell fusion of certain epithelial cells, demonstrating that negative regulation of *eff-1* is as important as positive regulation for normal development ([9]; Cassata et al., unpublished data). Other transcription and signaling factors have been shown to regulate specific cell fusion events in *C. elegans* [5, 10, 16–18] and *Drosophila* [4, 19, 20]. However, little is known regarding the underlying molecular details.

Most of what is known about membrane fusion came from studying relatively simple fusion reactions mediated by enveloped viruses such as HIV, Influenza, and Semliki Forest viruses and intracellular SNAREs [21–27]. Only the SNARE complex and viral fusogens have been shown to be both necessary and sufficient for membrane fusion.

eff-1 Is Sufficient to Initiate Epithelial Cell Fusion

We have shown earlier that *eff-1* is necessary for certain cell fusion events in *C. elegans* [8, 14]. To test whether *eff-1* is also sufficient, we expressed the *eff-1* ORF under control of a heat shock promoter (*hsp2-16*; a kind gift from A. Fire). To express *hsp::EFF-1*, the transgenic lines were subjected to heat shock cycles and stained for immunofluorescence with the antibody MH27 (see Experimental Procedures). Untreated transgenic worms, heat-shocked wild-type, and *eff-1(−)* animals served as negative controls. In worms subjected to heat shock, seam cells (lateral epidermis) ectopically fused to the surrounding epidermis (n = 26; Figure 1C), resulting in discontinuities in the anterior-posterior rows of lateral cells. These abnormal *eff-1*-dependent cell fusions were induced after heat shock in all larval stages except in late L4 larvae (n = 30).

To test whether nonepidermal cells can become fusogenic after ectopic expression of EFF-1, we analyzed vulval precursor cells (VPCs) in the ventral side of L3 larval-stage animals. We found that VPCs in *hsp::EFF-1* animals fused ectopically to the epidermis, resulting in aberrant vulvae (n = 11/32, Figure 1G). Thus, *eff-1* ectopic expression promotes ectopic fusion in normally nonfusogenic cells (Figures 1C and 1G). To determine whether ectopic EFF-1 is sufficient to fuse cells that have never expressed wild-type EFF-1, we repeated the same experiments but in an *eff-1(−)* background. We found that after heat shock in *eff-1* mutants, ectopic expression of *eff-1* is able to induce cell fusion in two distinct epithelial ectoblastic cells: the epidermal seam cells and the VPCs (n = 22, Figures 1D and 1H).

Is *eff-1*-dependent ectopic fusion restricted to postembryonic development? To test this, we analyzed live *eff-1(−);hsp::EFF-1;ajm-1::gfp* embryos subjected to heat shock treatments. We found that such embryos showed ectopic fusions in dorsal, lateral (seam), and ventral epithelia (n = 25; Figure 1J) including fusions between normally distinct epidermal compartments. These ectopic fusions resulted in abnormal embryonic morphogenesis followed by embryonic lethality. Although heat shock treatment by itself can lead to embryonic lethality, control worms did not show the ectopic fusion phenotype (n = 43; Figure S5 in the Supplemental Data available with this article online).

In order to ascertain that *eff-1* induces true cell fusion (and not just deterioration of the apical junctions), we

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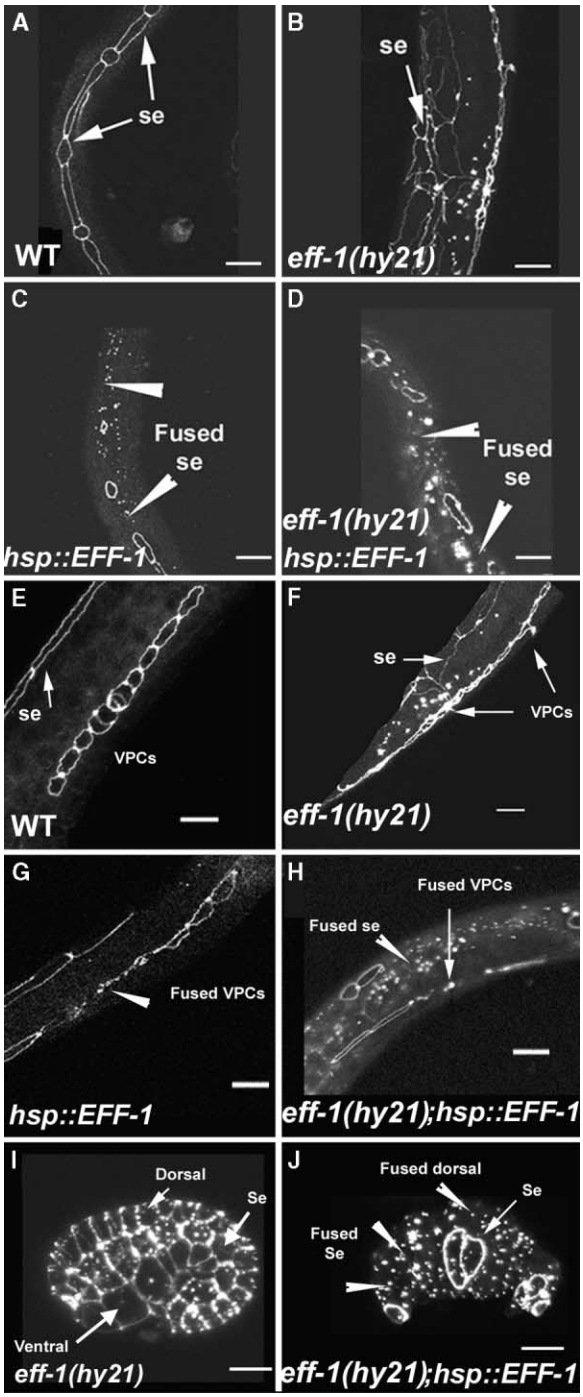

Figure 1. *eff-1* Is Sufficient to Promote Epithelial Cell Fusion

In wild-type worms, the epithelial seam cells divide during each larval molt, yielding epidermal daughter cells, which fuse to the hypodermis, and ectoblastic daughter cells, which stay unfused, forming single, lengthwise rows along the left and right sides. During the L4 stage, the cells inside the rows fuse between themselves and form two syncytia that will form specific cuticle structures [1]. In *eff-1(−)* mutants all the seam cells fail to fuse [8]. Transgenic worms, carrying a *hsp::EFF-1* construct either in a wild-type or an *eff-1(−)* background were subjected to heat shock treatment. In control heat-shocked animals and in untreated transgenic worms, the fusion pattern of epithelia was normal. Specifically, the unfused postembryonic seam cells were arranged in two lateral rows in wild-type ([A] only left side is seen in this focal plane), or as a network of unfused cells in *eff-1(−)* mutants (B). In worms, after heat shock, seam cells ectopically fused to the epidermis. This was true for the wild-type background (C) and for the *eff-1(−)* background (D). In wild-type worms, three specific VPCs divide three times (E), yielding a 22-cell vulval primordium that undergoes organogenesis to form a stack of seven vulval rings, some of them syncytial [13]. (F) Epithelial cells fail to fuse in *eff-1(hy21)* mutants grown at the restrictive temperature. The unfused VPCs connect through processes with dorsal and lateral seam epidermal cells that also fail to fuse. After heat shock treatment, worms carrying the *hsp::EFF-1* construct showed ectopic fusion of the VPCs to the hypodermis. This was true for the wild-type background (G) and for the *eff-1(−)* background (H). Thus, when ectopically expressed, *eff-1* is sufficient to promote epithelial cell fusion in normally unfused cells throughout vulval and seam cell development. (I) *eff-1(hy21)* mutant embryos at the comma stage expressing *ajm-1::gfp* and showing three rows of nonfusing epithelial cells (dorsal, lateral, and ventral). (J) Embryos carrying an *hsp::EFF-1* construct after heat shock treatment. Excluding several cells in the head and the tail and two seam cells, all the epithelial cells have fused to the hypodermis. Punctuated staining is due to background of the GFP. (A–D) Anterior is up, and dorsal is left. (E–J) Dorsal is up, and anterior is left. "Se" indicates seam cells, arrows indicate unfused cells, and arrowheads indicate ectopically fused cells. The scale bars represent 10 μm.

tested cytoplasmic content mixing in embryos expressing *hsp::eff-1; eff-1p::GFP; ajm-1::GFP; eff-1(hy21)* (Figure 2). We found that cytosolic GFP (*eff-1p::GFP)* did spread between ectopically fused epithelial cells, accompanied by disappearance of AJM-1::GFP from the apical junctions (AJs), showing these are real fusion events. To determine the minimal time required to get ectopic fusion from the initiation of heat shock to the start of cell fusion, we tried different times of the heat shock period and imaged cell fusion and cytoplasmic content mixing in live embryos using time-lapse microscopy. We detected such events already 50 min after the start of the heat shock treatment (which by itself must take place for at least 35 min). EFF-1-dependent cell fusion events occurred before morphogenesis between tens of cells that are not normally fusogenic in wild-type embryos.

In summary, *eff-1* is not only necessary but it is also sufficient to promote cell fusion in vivo. Being sufficient does not necessarily mean *eff-1* acts alone. Our results mean that if other components exist in the fusion machinery, they are already present in nonfusing cells and require direct *eff-1* activity to initiate fusion events. Alternatively, *eff-1* could serve as a signaling molecule, activating a cascade, which results in the transcription of fusion genes and recruitment of these gene products to promote cell fusion. However, a short time window between *eff-1* expression and fusion events (≤50 min), together with evidence showing that different mutations prevent downregulation of cell fusion and *eff-1* expression throughout development ([5, 9]; Cassata et al., unpublished data), suggest that most cells are fusion competent and only need *eff-1* expression to fuse during embryonic and larval development.

Tissue-Specific Activities of *eff-1* Initiate and Expand Cell Membrane Fusion

To identify the stage in epidermal cell fusion in which *eff-1* is active and to characterize tissue-specific *eff-1-*

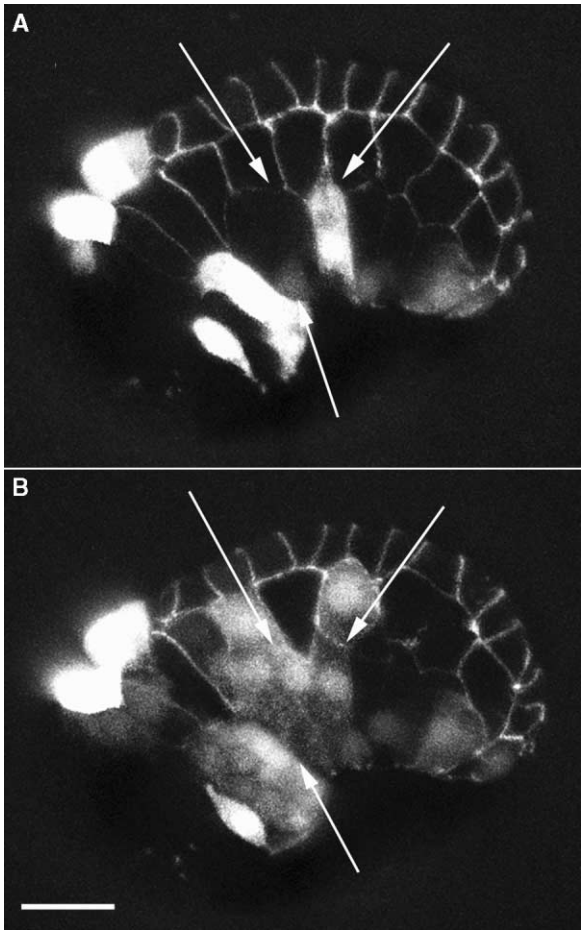

Figure 2. *eff-1*-Mediated Ectopic Fusion Leads to Mixing of Cytoplasmic Contents

eff-1(hy21); hsp::EFF-1 embryo expressing *ajm-1::gfp* and *eff-1p::gfp*. Following heat shock, ectopic fusion between normally nonfusing cells (A) (embryonic dorsal with lateral cells) allows mixing of cytoplasmic contents (B). *eff-1p::gfp* transcriptional construct served as a cytoplasmic marker. This marker is not expressed normally in the embryonic lateral seam cells of wild-type, *eff-1(−)*, and control animals ([8] and data not shown). The cytoplasmic mixing is coupled to disappearance of apical junctions (arrows). Dorsal is up and anterior is left. The scale bar represents 10 μm.

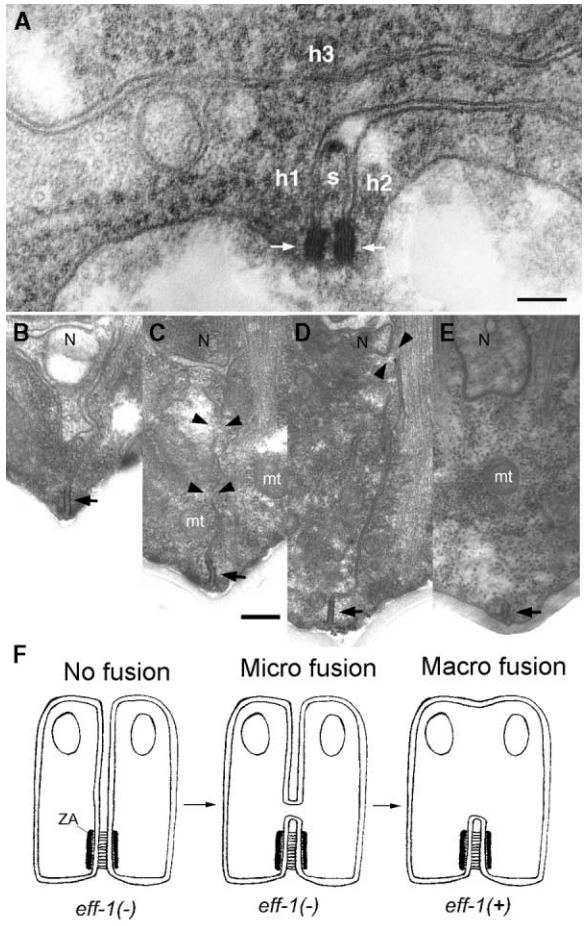

Figure 3. TEM of Unfused Epidermal and Muscle Cells

(A) Transverse thin section of mutant *eff-1(hy21)* L4 grown at the restrictive temperature shows normal ultrastructure of the hypodermal membranes, where the separation between apposing plasma membranes that failed to fuse is generally between 9 to 13 nm as in wild-type. The ultrastructure of the AJs (arrows) and membranes appears normal. Here, a seam cell process (s) narrows and three neighboring hypodermal cells (h1, h2, h3) remain unfused along the lateral bodywall. Lateral cuticle lies along bottom edge of panel.

(B–F) TEM of pharyngeal muscle pairs shows variable fusion failure in a mutant *eff-1(hy21)* adult grown at the restrictive temperature or successful fusion in wild-type cells.

(B) Two cells have failed to fuse, leaving a persistent cell border running from the neuron process (N) to the apical junction (AJ indicated by arrow).

(C–D) Above the AJ, two pairs of cells *eff-1(hy21)* mutant cells have formed microfusions (arrowheads), which are so small that no mitochondria (mt) could pass.

(E) Two wild-type cells have fully fused below the neuron process (N), leaving behind a complete AJ on the plasma membrane of the fused cell pair.

(F) Model showing a pathway for pharyngeal muscle homotypic fusion based on TEM intermediates. Zonula adherens (ZA) is a component of the AJ [34] (B–E). The scale bars represent 100 nm.

mediated cell fusion in *C. elegans*, we studied the ultrastructure of unfused epithelial cell membranes in *eff-1* mutants. We examined transverse sections of L4 and young adult animals by transmission electron microscopy (TEM) and found extra epithelial cells separated by intact plasma membrane borders and linked by normal AJs (Figure 3A, arrows). "Unfused" lateral epidermal seam cells produce defective cuticular structures (alae; Figure S1), and ectopic cell junctions persist between the progeny of the seam cells and other epithelial cells even in the adult. The morphology of the unfused epithelial *eff-1* mutant cells by light and electron microscopy was similar to wild-type cells except that the plasma membranes and AJs remained intact and unfused throughout development. Some cells showed local increases in autophagy, perhaps due to limited opportunities to deliver secretory products to the cuticle as the epithelium stratifies (Figure S2). Moreover, the distance between unfused membranes (9–13 nm) was undistinguishable from wild-type (Figure 3A). Based on these observations, we conclude that *eff-1* mediates epithelial cell fusion before or at the initiation stage.

To determine whether *eff-1* is essential for the initia-

tion of cell fusion in nonepithelial tissues, we examined ultrastructure of pharyngeal muscles in *eff-1(−)* animals. In wild-type animals pharyngeal muscles are myoepithelial cells that do not lose the AJ between them during and after cell fusion (D.H.H., Z. Altun, *see AlimFIG12 & 13 at wormatlas.org/handbook/alimentary/alimentary1*.). Figures 3B, S3, and S4 show that the AJs remained in between the pairs of pharyngeal muscles and the membranes remained unfused in 17/30 pairs of cells. Therefore, *eff-1* is also required to initiate muscle-muscle fusion in the pharynx. However, in 6/21 cases cell fusion was initiated but its expansion was blocked revealing a distinct morphological intermediate (Figures 3C and 3D). We refer to this step as microfusion. By serial TEM, we observed multiple sites of fusion initiation, some sites stopping as microfusions while others progress further. In one *eff-1(hy21)* animal grown at the semi-permissive temperature of 22°C, we found 7/9 cell pairs fusing, and no examples of microfusions as seen in the wild-type (Figure 3E). Thus, in pharyngeal myoepithelial cells *eff-1* is required both for cell fusion initiation and expansion from microfusion to a step that we refer as macrofusion (Figure 3F).

To determine whether *eff-1* is required for cell fusion in other tissues in addition to epithelia and muscles, we explored whether the gonadal anchor cell (AC) fuses in *eff-1(−)* animals. The AC normally induces and invades the vulva primordium and eventually fuses in the late L4 stage to eight uterine cells (utse) to form a hymen that separates the uterus from the vulva [13, 28, 29]. Anna Newman found that the AC undergoes normal cell fusion in *eff-1(hy21)* (A.P. Newman, personal communication), and we confirmed this surprising result in two stronger alleles of *eff-1* including *eff-1(np29)*, which is predicted to be null (n = 10), showing that *eff-1* is not required for the heterotypic fusion of the AC with the utse cells. In addition, we have shown that the AC normally fused in *rrf-3* animals hypersensitive to RNA interference and that had an otherwise strong Eff-1 phenotype in the hypodermis and vulva after feeding on bacteria that produce *eff-1*(dsRNA). A complete deletion of *eff-1* will be necessary to bring the ultimate proof of the *eff-1*-independence of the AC fusion.

Why and How Do Cells Fuse?

Surprisingly, we do not have satisfying answers for these basic questions, although synctia formation is essential in humans for muscle formation, bone homeostasis, nourishment of the embryo, and fertilization, among others. Here, we answered two questions regarding developmental cell fusion in a multicellular organism: Is *eff-1* sufficient for cell fusion in vivo, and is *eff-1* required for cell fusion events in different cell types?

Future in vivo and in vitro studies on how EFF-1 proteins initiate tissue-specific cell fusion and drive it through a stable microfusion intermediate that expands into a complete macrofusion may provide a precise picture of how membrane fusion works in multiple tissues in *C. elegans*. Finally, EFF-1-like proteins and fusion pathways explored here in *C. elegans* may be shared by unexplored cell fusion reactions in mammalian trophoblasts, myoblasts, osteoclasts, and stem cells.

Experimental Procedures

Time-Lapse Multifocal Temperature-Controlled Confocal Microscopy

Time-lapse movies were recorded using a Nikon Eclipse E-800 with a 60X/1.40 Plan Apo objective using a BioRad MRC1024 confocal microscope [30]. When using GFP, we estimate the resolution of our confocal microscope to be ~250 nm.

Heat Shock Experiments

Mixed or synchronized populations of larvae were heat shocked for 1 hr at 31°C, followed by 2 hr recovery at 20°C, another 1 hr heat shock cycle at 31°C, and a final 2 hr recovery at 20°C. Embryonic fusion was assayed after similar heat shock treatments or after one cycle of 1 hr or shorter periods at 32°C. The worms were then subjected to live fluorescence analysis or were fixed using the Finney-Ruvkun protocol [13, 31], mounted on slides and subjected to fluorescence analysis using the MRC-1024 (Bio-Rad) laser confocal scanning microscope. In cases where *ajm-1::gfp* was absent, worms were stained with the mouse MH27 monoclonal antibody as described earlier [1, 18, 32]. All heat shock experiments were repeated at least four times, yielding reproducible results.

To fix and stain embryos, we used the methanol/acetone on dry ice protocol [1]. Embryos were collected from plates into an egg salt drop on a poly-L-lysine coated slide, with a mouth pipette, a platinum pick, or by scrubbing and washing plates.

Electron Microscopy

All TEM procedures were performed in multiwell glass slides as described [33]. In brief, *eff-1(hy21)* worms grown at 25°C were washed in M9 buffer, which was replaced with the aldehyde fixative (0.2 M HEPES with 2.5% glutaraldehyde, 2% formaldehyde) for 1–2 hr at room temperature. The animals were cut open with a razor blade, to increase penetration of fixatives. After buffer washes, samples were fixed in osmium tetroxide fixative (0.1 M HEPES with 1% osmium tetroxide, 0.5% $KFe(CN)_6$) for 1–2 hr at room temperature and washed in buffer. After en bloc staining in uranyl acetate for 1 hr, animals were grouped in 2% agarose blocks and then infiltrated with Scipoxy resin (Energy Beam Sciences), placed into molds, and heated for 3 days in a 60°C oven. Some *eff-1* samples were also fixed for TEM by high-pressure freezing intact animals in a Bal-Tec HPM 010 device, followed by freeze substitution into 0.8% osmium + 2% glutaraldehyde in acetone/methanol (80/20), and infiltration into LX112 resin. Transverse thin sections were collected on the formvar-coated grids, and poststained with 5% uranyl acetate and lead citrate. The specimens were examined in a Philips CM10 electron microscope at the Center for *C. elegans* Anatomy in Albert Einstein College of Medicine (Bronx, NY) or in a Philips CM120 cryo-electron microscope in the Krumholz Advanced Microscopy Center at the Chemical Engineering Department (Technion, Haifa).

For scanning electron microscopy (SEM), animals were prepared without any cutting via the same fixation steps, then washed in dH_2O after osmication, critical-point dried, and sputter coated with gold before examination in a JEOL 6400 SEM.

Supplemental Data

Supplemental Data including Supplemental Experimental Procedures and five additional figures are available at http://www.current-biology.com/cgi/content/full/14/17/1587/DC1/.

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