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AFF-1, a FOS-1-Regulated Fusogen, Mediates Fusion of the Anchor Cell in *C. elegans*

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SUMMARY

Cell fusion is fundamental for reproduction and organ formation. Fusion between most *C. elegans* epithelial cells is mediated by the EFF-1 fusogen. However, fusion between the anchor cell and the utse syncytium that establishes a continuous uterine-vulval tube proceeds normally in *eff-1* mutants. By isolating mutants where the anchor-cell fails to fuse, we identified *aff-1*. AFF-1 ectopic expression results in fusion of cells that normally do not fuse in *C. elegans*. The fusogen activity of AFF-1 was further confirmed by its ability to fuse heterologous cells. AFF-1 and EFF-1 differ in their fusogenic activity and expression patterns but share eight conserved predicted disulfide bonds in their ectodomains, including a putative TGF- β -type-I-Receptor domain. We found that FOS-1, the Fos transcription factor ortholog that controls anchor-cell invasion during nematode development, is a specific activator of *aff-1*-mediated anchor-cell fusion. Thus, FOS-1 links cell invasion and fusion in a developmental cascade.

INTRODUCTION

Most multicellular organisms are comprised of three different germ layers that are organized during development from the outside inward and are separated by basement membranes. The external ectoderm covers and internalizes the mesoderm and endoderm layers. Organs derived from internal germ layers, however, often perform biological functions that rely on connections with the external world, necessitating formation of mixed-layer organs. A prominent example is the birthing of embryos, which first develop within the mesodermal gonad and are then expelled from the body via an ectodermal organ. Despite such a fundamental requirement for establishment of communication and canalization between internal organs and the ectoderm, the molecular mechanisms that govern this process remain poorly understood (Wolpert, 2007).

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In *C. elegans*, uterine-vulval connection is established by the activity of a single cell, the anchor cell (AC), that lies at the interface between the ectodermal vulva and the mesodermal uterus. Upon specification, the AC induces vulva precursor cell (VPC) differentiation by a LIN-3/EGF signal that activates a LET-23/EGF receptor in the VPCs (Sternberg and Horvitz, 1986). This is followed by a second signaling phase where a LAG-2/Delta signal from the AC activates LIN-12/Notch at six surrounding uterine cells, resulting in their differentiation into π cells whose daughters connect to the vulva (Newman et al., 1994). Via these two signaling cascades, the AC synchronizes uterine-vulval development and fixes their relative positions (Figure 1A).

Uterine-vulval connection, continuity, and attachment to the *C. elegans* body are established by two additional activities of the AC. First, the AC induces the localized breakdown of the basement membranes, separating the uterus from the VPCs. The AC then prompts the onset of basolateral processes that invade in between the two central VPCs' descendants (Sherwood and Sternberg, 2003). This invasion is mediated by the activity of the FOS-1 transcription factor in the AC (Sherwood et al., 2005) in a process reminiscent of FOS regulation of cell invasion during normal mammalian development and in tumor metastasis. After completing its signaling and invasion phases, the AC lying between the vulva and uterus lumens must be removed to establish a continuous opening. Its removal is initiated by the fusion of eight π cell daughters, generating the utse syncytium (Figure 1A). The AC subsequently fuses to this syncytium, leaving only a thin layer of cytoplasm in the junction between the lumens (Sharma-Kishore et al., 1999) (Figure 1B). This thin lamina process is broken when the first embryo is laid to establish a direct channel between the two organs.

Cell fusion occurs during the development of multicellular organisms in crucial processes like fertilization and formation of multinucleate cells in muscles, placentas, and bones (Podbilewicz, 2006; Podbilewicz and White, 1994). Despite the detailed characterization of cell-cell fusion events in several organisms, the molecules that directly mediate this process remain uncharacterized (Podbilewicz, 2006; Podbilewicz and White, 1994). The only protein that has been characterized as a fusogen, directly involved in cell-cell fusion, is *C. elegans* EFF-1 (Epithelial Fusion Failure-1) (Mohler et al., 2002). In *eff-1* mutant worms, most of the numerous fusion events characteristic of *C. elegans* development do not occur, while overexpression of *eff-1* results in ectopic fusion of cells that do not normally fuse (del Campo et al., 2005; Shemer et al., 2004). Dynamic expression of *eff-1* in fusing cells links execution of fusion with tight regulation of the process (Podbilewicz, 2006; Shemer and Podbilewicz, 2002). EFF-1 is a type I membrane protein with homologs in other nematodes. EFF-1 fuses heterologous Sf9 insect culture cells, demonstrating that it is a bona fide fusogen (Podbilewicz et al., 2006).

Here we show that in *eff-1* null mutants, several fusion events occur normally. Anchor-cell fusion, the fusion of two vulval rings, and the fusion between the lateral seam cells occur in an EFF-1-independent manner. These processes must therefore be facilitated by the activity of an unidentified fusogen. We have identified a related protein, AFF-1, that is required for AC fusion and other specific fusion events in different organs. Differential and specific expression of these two related fusogens allows a dynamic regulation of most but not all of the 300 stereotyped somatic cell fusion events in *C. elegans*. In an *aff-1* mutant background, AC fusion is blocked while *aff-1* ectopic expression in normally nonfusing cells results in their fusion. This demonstrates both the necessity and sufficiency of *aff-1* for cell fusion in vivo. Moreover, fusion of cultured Sf9 cells upon AFF-1 expression indicates that AFF-1 is a fusogen. The dynamic expression of *aff-1* in the AC and utse is positively regulated by the FOS-1 transcription factor. Thus, FOS-1 governs AC fusion via AFF-1 fusogen, connecting tissue merging, cell invasion, and cell fusion during the formation of a tube.

RESULTS

AC Fusion Failure in *aff-1* Mutants

Previous studies demonstrated that AC fusion occurs normally in *eff-1* hypomorph mutants *np29* and *hy21* (Choi et al., 2006; Shemer et al., 2004). To investigate this further, we examined AC fusion in worms possessing the *eff-1* allele *ok1021*, which is functionally null (A.S. and B.P., unpublished data). We found that in the *eff-1(ok1021)* mutant background, the AC fuses and forms a thin membrane, similar to the wild-type structure (Figures 1C–1F), indicating that AC fusion is mediated by a fusogen distinct from EFF-1.

Failure of AC fusion results in fertilized embryos that cannot exit from the uterus and complete their development inside the body of the mother (Cinar et al., 2003). This was the basis for forward genetic screens in which genes required either directly or indirectly for AC fusion were isolated. One group of genes includes the *cog-2/egl-13* (Cinar et al., 2003; Hanna-Rose and Han, 1999), *lin-11* (Newman et al., 1999) and *lin-29* (Newman et al., 2000) transcription factors, and *smo-1*, encoding a regulator of protein localization (Broday et al., 2004). These mutations disrupt π cell differentiation and localization, including the fusion of their daughters with the AC. *nsf-1*, encoding N-ethylmaleimide-sensitive factor, was isolated as a gene required autonomously for AC fusion (Choi et al., 2006). NSF acts as a regulator of vesicle fusion through binding and disassembly of the SNARE complexes (Sollner et al., 1993). It is unclear whether NSF-1 mediates AC fusion directly or by regulating the traffic and surface expression of a yet-unidentified AC fusogen.

To identify factors that directly mediate AC fusion, we performed a forward genetic screen for an EGg Laying defective (Egl) mutant phenotype. The collection of Egl mutations was refined further for mutations that affect the AC specifically, by following AC fusion directly. We isolated one mutation, *ty4*, in which the AC failed to fuse in 97% (n = 135) of the mutant worms (Figures 1G, 1H, and 2B). The *ty4* mutation was mapped to a genetic interval of 0.24 map unit (about 300 kb) on chromosome II (see Supplemental Experimental Procedures in the Supplemental Data available with this article online). We performed complementation analyses with mutations in this region that correspond to genes that may function in cell fusion. The only mutation failing to complement *ty4* is *tm2214*, a 1 kb deletion in the C44B7.3 predicted gene (Figure 2A). Like *ty4*, *tm2214* mutant worms exhibit a completely penetrant Egl phenotype (Figure 1J; n = 48 and n = 96, respectively), and in 98% of the worms examined, the AC failed to fuse (n = 135; Figure 2B). Sequencing the C44B7.3 gene in the *ty4* mutant revealed a single base substitution of G to A in the putative ATG start codon, resulting in replacement of the initiator methionine with an isoleucine residue. *ty4* therefore presumably represents a null allele. Similarity in the severity of the phenotypes of *ty4*, *tm2214*, and *ty4/tm2214* transheterozygote alleles suggests that the two alleles are functionally null. The AC fusion failure phenotype of *ty4* mutant worms was rescued by introducing an 8 kb genomic fragment harboring the promoter and coding region of the C44B7.3 gene (Figures 2A and 2B). Finally, the AC fusion failure phenotype was detected in worms fed with dsRNA directed against the C44B7.3 transcript (Figure 2B; Table S1). These results demonstrate that the C44B7.3 gene is specifically required for AC fusion, prompting us to rename it *aff-1* (Anchor-cell Fusion Failure-1).

Sequence analysis revealed that *aff-1* encodes a predicted type-I transmembrane protein, with a domain organization similar to that of the EFF-1 fusogen (Figure S3). While the two proteins exhibit only moderate overall primary sequence similarity (26% identity; 46% similarity), we note a striking conservation of cysteine and proline residue positions throughout the two sequences (Figure S3). These observations suggest that AFF-1 and EFF-1 share significant structural similarity and may therefore constitute members of a fusogen family, with distinct functions during development (Figure S4). Figure 2C shows that the ectodomain of AFF-1

contains a domain of ~100 residues with a characteristic pattern of cysteines conserved between EFF-1 and AFF-1 homologs in different nematode species. Moreover, this putative structural region of AFF-1 and EFF-1 ectodomains is a Transforming Growth Factor- β type I Receptor-like (TGF- β RI) structural domain conserved in invertebrates and vertebrates.

AFF-1 Is Required Directly for AC Fusion

Abnormal development or localization of either the AC, the vulval, or the uterine cell types can result indirectly in AC fusion failure. Alternatively, such failure can occur by specifically disrupting the AC fusion process. To explore which of these possibilities is affected by impairment of *aff-1* function, we examined different aspects of vulval, AC, and π /utse cell development and localization prior to the process of AC fusion, in *aff-1* mutant worms. In the two *aff-1* mutant alleles examined, the presence of a bloated shaped AC indicated fusion failure, and the AC did not fuse even during later stages of development (Figure 3H). In 7%–9% (n = 135) of the worms, the unfused AC degenerated (Figure S1) even though AC fate determination, VPC induction, AC invasion, vulva invagination, and eversion occur normally (Figures 3 and 4A–4D). Thus, *aff-1* mutants show specific AC fusion failure.

To determine whether π /utse differentiation was affected in *aff-1* mutants, we followed the expression pattern of *egl-13p::gfp*, which marks the nuclei of utse cells. We found that π /utse differentiation and localization is normal in *aff-1* mutants (data not shown). Additionally, the formation of the utse syncytium, nuclei distal migration, and utse transition into a thin-layer syncytium occurs normally in an *aff-1* background (Figure S1), suggesting that utse syncytiogenesis is facilitated by an *aff-1*-independent mechanism.

Studies of the mechanism of cell fusion in *C. elegans* and during other membrane fusion events revealed that this process is executed via several intermediate steps, including pore formation and pore extension (Mohler et al., 1998; Shemer et al., 2004). In *C. elegans*, cytoplasmic mixing of the fusing cells occurs early on, before the cell shape changes characteristic of syncytia formation (Shemer et al., 2004). To investigate whether cytoplasmic mixing between the AC and utse occurs in an *aff-1* mutant background, we used the AC-specific cytoplasmic marker *cdh-3p::GFP*. In *cdh-3p::GFP* wild-type worms, GFP diffusion from the AC to the utse syncytium is detected (Figure 3C, top arrows), demonstrating the cytoplasmic mixing event. In contrast, in an *aff-1* mutant background, GFP is retained in the AC, indicating that there is no cytoplasmic mixing between the two cell types (Figure 3D, arrowhead). This suggests that AFF-1 is required for early stages of AC fusion, prior to the formation of fusion pores that are large enough to allow diffusion of GFP from the cytoplasm of the AC to the utse cells.

Taken together, these results demonstrate that *aff-1* is required specifically during the process of AC fusion and does not participate in developmental processes occurring before or in parallel to AC fusion. Thus, in the sequence of events leading to fusion, AFF-1 is required prior to pore formation and cytoplasmic content mixing.

AFF-1 Is Required for Specific Vulval and Late Epidermal Seam Cell Fusion

In addition to AC fusion failure, other blocks in specific fusion events were observed in *aff-1* mutant worms. For example, we found specific blocks in fusion of the vulval A and D rings (vulA and vulD) and in the terminal fusion between the entire lateral seam cells, which normally results in the formation of a single epithelial seam syncytium on each side of the animal (Figure 4). As in the case of the AC, these late fusion events occur normally during the late L4-adult transition in an *eff-1* null allele but not in *aff-1* mutant worms (Figures 4D and 4H). The development of these cells before fusion is normal compared with the wild-type (Figure 4), suggesting that *aff-1* acts directly in the fusion process at the late L4 to adult molt. In summary, *aff-1* is required not only for AC fusion but also for other fusion events that are

eff-1 independent. In light of the dual activity of AFF-1 and EFF-1 in different fusion events, a mutation in *aff-1* or *eff-1* alone does not represent complete inhibition of all fusion events. *eff-1 aff-1* double-mutant worms have extremely low viability and can only be maintained due to escapers in contrast to *eff-1* and *aff-1* mutant worms that are viable and semiviable, respectively (Figure 2B and Table S1). utse syncytiogenesis and sperm-egg fusion occur normally in *eff-1* and *aff-1* mutant backgrounds, possibly due to the activity of other fusion factors (Figure S1 and Table S1). This suggests that additional fusogen(s) exist in *C. elegans*.

AFF-1 Is Sufficient to Fuse Cells that Normally Do Not Fuse In Vivo

We have shown that AFF-1 is required for specific fusion events in *C. elegans*. To test whether AFF-1 is also sufficient to fuse cells in *C. elegans*, we cloned the full-length *aff-1* ORF under the regulation of a heat-shock promoter. This construct was expressed in embryos by standard heat-shock protocols, along with the apical junction protein AJM-1::GFP, which marks epithelial cell boundaries (Podbilewicz, 2006). For controls, we examined AJM-1::GFP distribution in non-heat-shocked embryos with the same genotype and in heat-shocked AJM-1::GFP embryos. Ectopic expression of *aff-1* resulted in the disappearance of AJM-1::GFP from the boundaries of hypodermal cells and the redistribution of the marker in large entities that presumably represent the formation of ectopic syncytia (Figure 5B; 26 out of 77 embryos). Normal fusion pattern in the controls demonstrated that the effect is specific to *aff-1* ectopic expression (Figure 5A; n = 100). To exclude the possibility that *aff-1* acts via the activation of *eff-1*, we overexpressed *aff-1* in *eff-1* null mutant embryos and detected the same level of ectopic fusion (Figure 5D; in 40% of the embryos, n = 25); hence AFF-1 fuses cells via an *eff-1*-independent mechanism. In order to examine the dynamic fusogenic activity of *aff-1*, we monitored fusion directly by detecting cytoplasmic mixing between fusing cells. We used a strain in which the *eff-1* promoter drives expression of cytoplasmic GFP in individual cells of the embryonic hypodermis. Dynamic GFP diffusion from the hypodermal cells to the embryonic seam cells, which normally do not express *aff-1*, concomitant with AJM-1::GFP disappearance from the cell junctions shows that AFF-1 induces bona fide cell fusion (Figure 5F, Movie S2). These results demonstrate that AFF-1 is sufficient for the induction of cell fusion in *C. elegans* in an *eff-1*-independent manner.

C. elegans AFF-1 Fuses Heterologous Insect Cells

The necessity of AFF-1 for specific fusion events, combined with its ability to promote ectopic cell fusions, suggests that this protein acts directly in the cell fusion process. To explore this further, we expressed the AFF-1 protein in heterologous cultures of Sf9 insect cells that do not usually undergo cell fusion (Podbilewicz et al., 2006). Western blot analysis detected a single band corresponding to AFF-1 protein at apparent MW of 75 kDa (Figure 5G). Surface biotinylation and immunofluorescence revealed that the protein is distributed both in intracellular compartments and at the surface of Sf9 transfected cells (Figures 5H and 5I). Fusion in culture was estimated by evaluating the relative quantity of multinucleated cells after transfection. Using confocal and fluorescence microscopy, we demonstrated that transfection with *aff-1* generated multinucleate cells containing two to six nuclei (Figures 5J–5M and Figure S6). We detected 20% multinucleation in cells transfected with *aff-1* (Sf9-AFF-1 cells) in comparison to only 3% in cells that were transfected with an empty vector (Figure 5N). To investigate whether AFF-1-induced multinucleation is a result of cell-cell fusion or a failure of cell division, we incubated Sf9-AFF-1 cells in the presence of 5'-fluoro-2'-deoxyuridine (FdUrd) that blocks cell division at the transition between G1 and S phases (Podbilewicz et al., 2006). We found that Sf9-AFF-1 cells treated with FdUrd had a similar proportion of multinucleation as untreated cells (15.8% ± 2.8% without FdUrd and 15% ± 2.4% with FdUrd). Thus, the transfected cells incubated in FdUrd did not show an apparent decrease in the number of multinucleated cells, supporting a mechanism of multinucleation independent from failure in cytokinesis in the presence of karyokinesis.

To compare the fusogenic activity and potency of AFF-1 with that of EFF-1, we measured the percent of multinucleation in plates transfected with EFF-1 or AFF-1 (Podbilewicz et al., 2006). We found that 48 hr posttransfection with 0.5 $\mu\text{g/ml}$ DNA, AFF-1 expression on the surface was ~ 100 times lower than EFF-1 expression, as estimated by western blotting after surface biotinylation (gray bars, Figure 5O). In the same cells, the efficiency of multinucleation was only two times higher for cells transfected with EFF-1 than for cells expressing AFF-1 (white bars, Figure 5O). Low expression of AFF-1 might reflect its cytotoxic effects, observed as loss of cells for higher concentrations of DNA and/or longer posttransfection times (data not shown). To increase the surface expression of AFF-1, we expressed a chimera containing AFF-1 ectodomain with EFF-1 transmembrane and cytoplasmic tail (AFF-1::EFF-1cyto, see Supplemental Data). The AFF-1::EFF-1cyto chimera showed an increase in surface expression together with a significant increase in the level of multinucleated cells. While the level of surface expression was 30 times lower for AFF-1::EFF-1cyto than for EFF-1, we observed a similar level of multinucleation (Figure 5O). Thus, comparable cell fusion efficiency at a much lower surface density suggests that AFF-1 is a more potent fusogen than EFF-1. In conclusion, AFF-1 surface expression in insect cells results in cell fusion and formation of syncytia in vitro, demonstrating that AFF-1 is a bona fide fusogen.

***aff-1* Is Expressed in the AC and utse Cells as They Fuse**

To follow *aff-1* expression, we fused a 4.5 kb fragment of the *aff-1* promoter to GFP and monitored its distribution in transgenic worms. The extent of the *aff-1* promoter was deduced from sequence similarities between *C. elegans aff-1* and its putative ortholog from *C. briggsae*. Specific and continuous expression was detected in the AC from the invasion of the vulval primordium at mid-L3 until its fusion with the utse cells (Figures 6A–6C). As the vulva completes its invagination in the L4, the utse syncytium starts to express *aff-1*, resulting in coexpression of *aff-1* in both cells prior to their fusion (Figure 6D). Since utse *aff-1* expression and AC-utse fusion occur almost simultaneously, it is possible that *aff-1* expression detected in the utse is actually a contribution from the AC cytoplasm after the fusion event. To test this, we examined utse *aff-1* expression in *lin-29(n482)* mutant worms where AC-utse fusion does not occur (Newman et al., 2000). *aff-1* expression in the utse in these mutants indicated that *aff-1* is specifically expressed in the utse cells and is not a consequence of AC to utse cytoplasmic GFP diffusion after fusion (Figure 6F). Moreover, the *lin-29* transcription factor is not required for *aff-1* expression in π /utse cells, as demonstrated by *aff-1* utse expression in *lin-29* mutant worms (Figure 6F). Taken together, the phenotypes in *aff-1* and *lin-29* mutant backgrounds, and *aff-1* expression in the AC followed by expression in the utse, are consistent with a direct function of AFF-1 in the AC-utse fusion process.

Dynamic Expression of *aff-1* in a Selected Group of Cells

To determine the expression of *aff-1* transcripts in *C. elegans*, we expressed a transcriptional *aff-1* promoter::GFP reporter (see Supplemental Data). *aff-1* expression is first detected in the embryonic hyp5 cell and later during larval development in various cell types, including pharyngeal muscles (Pm3 and Pm5), uterine rings (Ut2 and Ut4), head and tail neurons, sheath cells of chemo-sensory neurons, and male tail neurons (Figure S2). *aff-1* is also expressed in vulval vulD (Figures 6C and 6E; arrows) and the seam cells (Figures 6G and 6H) shortly before these cells fuse. In general, the myoepithelial cells of the pharynx and the epithelial cells in the uterus, vulva, and hypodermis that express *aff-1* undergo fusion. In contrast, there is no evidence for cell fusion in neurons that express *aff-1*. The dynamic expression of *aff-1* suggests that *aff-1*-mediated cell fusion is regulated by transcriptional cues. The complementary expression of *aff-1* in cells that fuse independently of *eff-1* suggests that *eff-1* and *aff-1* activities are independent and that their combined autonomous activities account for most somatic cell fusion events in *C. elegans*.

To determine the cellular and subcellular localization of AFF-1 in *C. elegans*, we expressed a translational AFF-1::GFP using a genomic fragment containing the predicted *aff-1* promoter and the ORF, including introns and regulatory sequences (see Supplemental Data). We found that AFF-1::GFP was weakly expressed on the plasma membrane and in intracellular organelles in *hyp5*, seam cells, vulD vulval precursors, and AC before, during, and after cell-cell fusion (Figure S5 and data not shown). Since we found weak expression in different independent transgenic lines, we expressed AFF-1::GFP under a strong AC-specific promoter (Kirouac and Sternberg, 2003). We found the reporter to be strongly expressed in intracellular organelles, and on the surface of the AC from the time of invasion of the vulval primordium to the fusion to the utse (Figures 6I and 6J). Both *aff-1* reporters were expressed in *hyp5*, seam cells, vulD cells, and AC. Taken together, the transcriptional and translational *aff-1* reporter constructs support the model in which AFF-1 is dynamically expressed in a specific group of cells that undergo cell fusion during normal development.

FOS-1 Regulates AFF-1-Mediated AC-utse Fusion

To identify the mechanism controlling *aff-1* expression in the AC, we looked for candidate transcription factors. The early expression of *aff-1* in the AC starting from the preinvasion stage suggests that *aff-1* is regulated by transcription factors that act early during AC development. One such candidate is the FOS-1A transcription factor that is expressed early in the AC to promote basement membrane removal during invasion (Sherwood et al., 2005). To test whether FOS-1 may control *aff-1* expression, we looked for FOS-1-binding sites in *aff-1* promoter using Transfac algorithm (Supplemental Experimental Procedures). We identified a consensus TGA (T)TCA Fos-binding site in position -1866 of *aff-1* promoter that is present in an identical position within the *aff-1* promoter from *C. briggsae*, supporting the hypothesis that FOS-1 controls *aff-1* expression. To further examine this hypothesis, we looked for *aff-1p::GFP* expression in the *fos-1(ar105)* mutant that specifically disrupts *fos-1a* (Sherwood et al., 2005). Strikingly, *aff-1* expression is strongly reduced or undetectable in the AC and other uterine cells in this *fos-1* mutant (Figures 7D and 7E; n = 33). In contrast, *aff-1* expression is retained in VulD and the seam cells (Figure 7E; n = 25), indicating that *fos-1a* positively controls *aff-1* expression in the AC and utse cells. Taken together, these results suggest that FOS-1A controls AC fusion via *aff-1* expression. To test whether *fos-1* is required for AC fusion, we analyzed *fos-1* mutants and found that the AC failed to fuse (Figures 7A–7C, arrowheads; n = 20). Thus, the activity of *fos-1* positively regulates two different aspects of AC development: invasion and fusion.

DISCUSSION

The unique development of the gonadal AC in *C. elegans* serves as a model for numerous fundamental cellular and developmental processes (Kimble, 1981; Seydoux and Greenwald, 1989; Sharma-Kishore et al., 1999; Sherwood et al., 2005; Sternberg and Horvitz, 1986). AC development is terminated by fusion to the utse syncytium (Figure 7F). The discrete morphology of the AC makes it an attractive model for cell fusion studies at single-cell resolution. We propose that AFF-1, together with the related EFF-1 fusogen, represent founding members of a family of developmental fusogens that induce fusion using similar mechanisms. AFF-1 and EFF-1 proteins presumably share a common ectodomain structure that includes a fold similar to TGF- β -type I Receptors. This putative domain represents nearly one-fifth of the EFF-1 and AFF-1 ectodomains. This conserved structure may be utilized by the as-yet-unidentified fusogens in other phyla. In addition, the dual activity of *fos-1* as a regulator of both AC invasion and fusion uncovers a novel molecular connection between these cellular processes. This surprising link may have implications in the understanding of diverse developmental processes such as tube morphogenesis, placentation, angiogenesis, and metastasis (Wolpert, 2007).

Foundation of a Family of Developmental Fusogens

The general structure of EFF-1 and AFF-1 proteins is similar: a signal sequence that is followed by a long extracellular portion, a predicted transmembrane domain, and an intracellular tail (Figure S3). The viral fusogen-like domains in EFF-1 that were previously hypothesized as possible fusion-related domains (Mohler et al., 2002) are only moderately conserved in AFF-1 proteins (Figure S3). In contrast, a striking conservation in the position and number of all 16 cysteines in the extracellular domain of AFF-1 and EFF-1 proteins, along with partial conservation of 11 out of 20 extracellular prolines, may stabilize a similar three-dimensional structure required for fusion facilitation. While there are clear homologs in other nematodes, AFF-1 and EFF-1 exhibit only minor similarity to proteins from other vertebrates and invertebrates, especially to proteins from the TGF- β /activin/BMP type I receptor superfamily (Figure 2C). A TGF- β -RI-like binding domain (Keah and Hearn, 2005) may define an interacting extracellular region conserved between *C. elegans* fusogens EFF-1 and AFF-1 that may be shared with unidentified invertebrate and vertebrate fusogens. Interestingly, TGF- β has been recently found to negatively regulate cell fusion in human endometrial carcinomas (Strick et al., 2007). However, the fact that some critical residues in the TGF- β -RI-like binding domain of FF family members are not conserved (e.g., the Cys-Asn residues; Figure 2C) undercuts the idea that the FF family members will have similar protein-protein interactions as TGF- β -RIs. Perhaps common activities or protein-protein interactions were shared by a common ancestor, but were lost as each family evolved more efficient ways to execute their respective functions.

In summary, EFF-1 and AFF-1 are the founding members of a family of fusogens in *C. elegans* and probably in other nematodes. All the members of the FF family share eight conserved predicted disulfide bonds in their ectodomains, including a putative TGF- β -type-I-Receptor-like domain (Figure 2C). The TGF- β -R1-like domain probably represents a conserved fold that might have implications for the mechanisms of developmental cell fusion.

Why Does *C. elegans* Need More Than One Fusogen?

Based on the conservation of extracellular cysteine and proline residues in the EFF-1 and AFF-1 proteins (Figures S3 and S4), which may stabilize a similar structure, one may hypothesize that fusion between *aff-1*- and *eff-1*-expressing cells may take place. However, several lines of evidence suggest that this heterotypic fusion does not occur and provide an explanation that emerges from biological context for the existence of these two similar fusogens. If heterotypic fusion between EFF-1 and AFF-1 occurred, the *aff-1*-expressing seam cells would fuse with the surrounding *eff-1*-expressing hyp7. However, this is not observed, so AFF-1 activity in the seam cells might allow seam cell fusion without the loss of seam cell identity (Figure 4H). In other cell types examined, such as VulD and embryonic hyp5 cell, there are EFF-1-expressing syncytia adjacent to AFF-1-expressing syncytia, but heterotypic fusions do not occur (Figure 6C and Figure S2). The regulated expression of two fusogens might establish the formation of developmental barriers between adjacent syncytia that may represent a general characteristic of developmental fusion.

The discovery of a new developmental fusogen in *C. elegans* implies that one general-purpose fusogen EFF-1 is not enough to account for all somatic cell fusions. AFF-1 is indeed a specialized fusogen required for particular fusion events. AFF-1-mediated fusions usually involve small membrane domains and limited timing of action, and are tightly controlled by transcriptional and posttranslational mechanisms (e.g., FOS-1 and NSF-1, respectively). Indeed, our experiments in heterologous insect cells suggest that AFF-1 is a more potent but also more toxic fusogen than its relative EFF-1.

FOS-1 Controls Cell Invasion and Cell Fusion

FOS-1A is expressed and required autonomously in the AC for its invasion activity in *C. elegans*. FOS-1A activates a network of target genes that facilitates cell invasion, a process reminiscent of Fos functions in mammalian development. We demonstrated an additional function of FOS-1 transcriptional network-regulating cell fusion after cell invasion (Figure 7F). Hence, the two sequential processes are regulated by the same transcription factor. Cell fusion may be a general safety mechanism to extinguish the invasive cell behavior of cells during normal development. For example, the formation of the syncytial trophoblast in the placenta may extinguish the invasive behavior of the cytotrophoblasts and may also restrict the invasive potential of embryonic cells (Cross et al., 1994). The dual activity of *fos-1*-regulating cell invasion followed by cell fusion uncovers a novel cascade of events that may also be utilized in the normal development of vertebrates and in tumor progression.

EXPERIMENTAL PROCEDURES

aff-1 Gene Characterization and Mutants Isolation

Gene prediction was verified by sequencing three C44B7.3 ESTs (yk474, yk1083, and yk1627). Two full-length ESTs revealed that the ORF starts at an ATG laying 45 bp 5' from the wormbase annotated ATG (Wormbase Release WS167, <http://www.wormbase.org/>). This results in an addition of a signal peptide to the predicted protein.

The *ty4* mutant was isolated in an EMS-based screen described previously (Choi et al., 2006; Cinar et al., 2003). *ty4* was mapped to a genetic interval of about 0.24 map units (about 300 kb) in the center of chromosome II by Tc1 mapping followed by three-factor and deficiency mapping (Supplemental Data). To identify the *ty4* mutation, the entire coding region of the *aff-1* gene (3 kb, containing 12 exons and 11 introns), as well as 5 kb of *aff-1* UTRs and promoter, was amplified using *ty4* mutant as template by single worm PCR and sequenced using relevant primers. The *tm2214* deletion was generated and generously provided by Shohei Mitani, Tokyo Women's Medical University School of Medicine, Japan (<http://www.nbrp.jp/index.jsp>). PCR amplification and sequencing of *tm2214* mutants revealed that a deletion of 999 bp occurs (from base 147 to 1146 in the C44B7.3 sequence where 1 is the A of the ATG) that introduced a stop codon after alanine 47 in the AFF-1 protein. *ty4* and *tm2214* alleles were outcrossed five and ten times, respectively.

For *ty4* rescue experiments, an 8 kb genomic fragment of *aff-1* that includes the promoter and coding region was amplified from wild-type worms. This PCR product was coinjected with pRabGFPrim3' injection marker (Choi et al., 2006) into *ty4* mutants.

Phenotypic Analysis

To measure the percentage of Egl worms, we picked hermaphrodites as L4 larvae onto separate plates. The worms were examined daily for the next 4 days, and all progeny were counted as in Brenner (1974). To analyze AC invasion and fusion, L3 to L4 larvae were examined using Nomarski optics and the AC marker *cdh-3p::GFP* (Pettitt et al., 1996). Cell fusion events in other tissues were examined using the apical junction marker AJM-1::GFP. Nuclear position in the utse was examined using *egl-13p::GFP* (Cinar et al., 2003; Hanna-Rose and Han, 1999).

RNAi experiments were performed as described in Timmons et al. (2001). A 1.5 kb fragment from *aff-1* gene was cloned in L4440 (pPD129.36) cloning vector (kindly provided by A. Fire) and was transformed into the HT1115 bacteria strain. After IPTG induction, the bacterial culture was seeded on NGM agar plates. Worms from the RNAi-sensitive strain *eri-1(mg366)* IV were individually plated, and phenotypes were scored in the F1 progeny.

For heat shock experiments, 20–40 laying hermaphrodites were grown on agar plates at 20°C for 2 hr. After removal of the laying animals, the plates were sealed and subjected to 32°C for 30 min in a water bath and were visualized by transferring post-heat-shock embryos to an egg salt drop on a poly-L-lysine-coated slide or a 3% aga-rose-coated slide. Cell fusion was assayed immediately after heat shock treatments.

Lethality was scored for *ty4* and *tm2214*. For *ty4*: 22% L1 rod-like lethal and 2% embryonic lethal (n = 400). For *tm2214*: 6% L1 rodlike lethal and 0% embryonic lethal (n = 1946).

Identification of the Homology to TGF- β -RI Extracellular Domain

The amino acid sequence of EFF-1A was used to find homologs in *Pristionchus pacificus* using BLAST (http://www.pristionchus.org/cgi-bin/blast_iframe.pl).

A significant alignment was obtained for contig2476 (E Value $1e^{-33}$). We then proceeded to identify the amino acid sequence of the corresponding protein. The program “Augustus” (<http://augustus.gobics.de/>) was used for the gene prediction using the training set for *C. elegans*. We were able to predict a protein 570 aa in length which shows 40% identity to EFF-1A and 22% identity to AFF-1. We used PSI BLAST (Altschul et al., 1997) to verify that this homolog shows significant homology to all known EFF/AFF family (FF Family) members, and indeed, using this sequence, we were able to retrieve eight protein sequences all belonging to the FF family after one iteration. We then proceeded to review the various proteins aligned below the default threshold score of 0.005 (the standard cut-off score of BLAST is an E Value of 10). One protein RE55648p (*Drosophila melanogaster*), a member of the TGF- β -receptor family, with an E Value of 2 was of particular interest because of the high conservation specifically in Cysteine residues and an identity of 24% over a sequence length of 84 aa. To evaluate whether this homology is statistically significant and whether this Cysteine pattern is specific, we aligned this domain, using ClustalW, with other FF family members and created a pattern of the Cysteine and some similar residues:

CXC[EDH]X(0,1)CX(1,3)[TS][ENS]X(1,2)CX(2)[EDS]X[YFH]X(0,5)CX (9,17)CX(12)
CX(8,10)CC

We then utilized this pattern or variations of it to run PHI BLAST (Altschul et al., 1997). We retrieved RE55648p and other members of the TGF- β receptor type I superfamily.

aff-1 eff-1 Double Mutants Have Very Low Viability

Out of 1381 F1 progeny from a balanced strain containing *eff-1(ok1021) aff-1(tm2214)/mnl1 [myo-2::gfp; pes-10::gfp] II*, we obtained only ten non-GFP-homozygous double mutants. From the ten double mutants, we obtained only six F2 animals. The combined progeny (escapers) were all Dpy Unc with Eff tail and comprised two gravid Egl, two L2, and two larvae with posterior paralyzed body. Based on these results, we conclude that cell fusion in *C. elegans* is required for viability.

Cell Culture Assay

Sf9 cells were grown to 50% confluency on 35×10 mm tissue culture plates as recommended by manufacturers. Cells were transfected with cellfectin and with plasmid at 3 μ g/ml (either pIZT-Empty vector, or pIZT-AFF-1), as recommended by Invitrogen, and were analyzed at different times from 18 to 96 hr posttransfection as in Podbilewicz et al. (2006).

To assay syncytium formation and to correlate it with the expression of GFP reporter present in the plasmids used for the transfection, we stained cell nuclei with Hoechst (1 μ g/ml, H3570, Molecular Probes) or DAPI for 10 min at 22°C. Multinucleated cells were assayed between

24 to 48 hr posttransfection, since after extended durations in culture we found high levels of toxicity with pIZT-*aff-1* DNA. We obtained GFP(+) fluorescence (transfected cells), DIC, Hoechst, and phase-contrast images. Low expression of GFP in fusing AFF-1 cells hindered identification of the transfected cells as GFP-expressing cells. Thus, we scored the efficiency of multinucleation for all cells rather than only for cells transfected with AFF-1 protein and, as shown in Podbilewicz et al. (2006) significantly underestimated fusion rates.

The extents of multinucleation defined as the ratio between the number of nuclei in multinucleate cells and the total number of nuclei, are presented as means \pm standard errors of at least seven experimental replicates from the same transfection (Podbilewicz et al., 2006).

Imaging and Software

Fluorescence and Nomarski images were captured using a Hamamatsu ORCA-ER on a Nikon E800 microscope or Zeiss Axiovert 200. Images were merged using Photoshop 8. Confocal microscopy used BioRad MRC1024 or Zeiss LSM 510 META. Sequence alignments were done using ClustalW (<http://www.ebi.ac.uk/clustalw/>) and were edited by the Jalview software (Clamp et al., 2004).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A.S. and J.C. contributed equally to this work. A.S. did the molecular biology work along with phenotypic characterization of all the worm strains. J.C. and A.P.N. isolated and characterized the *ty4* allele and did the *ty4* rescue experiments. E.L. and L.C. designed, performed, and analyzed cell fusion assays in culture. E.L. and C.V. did biochemical experiments. O.A. did the heat shock experiments and bioinformatics. A.S. and B.P. designed and analyzed the experiments and wrote the paper. All authors discussed the results and commented on the manuscript.

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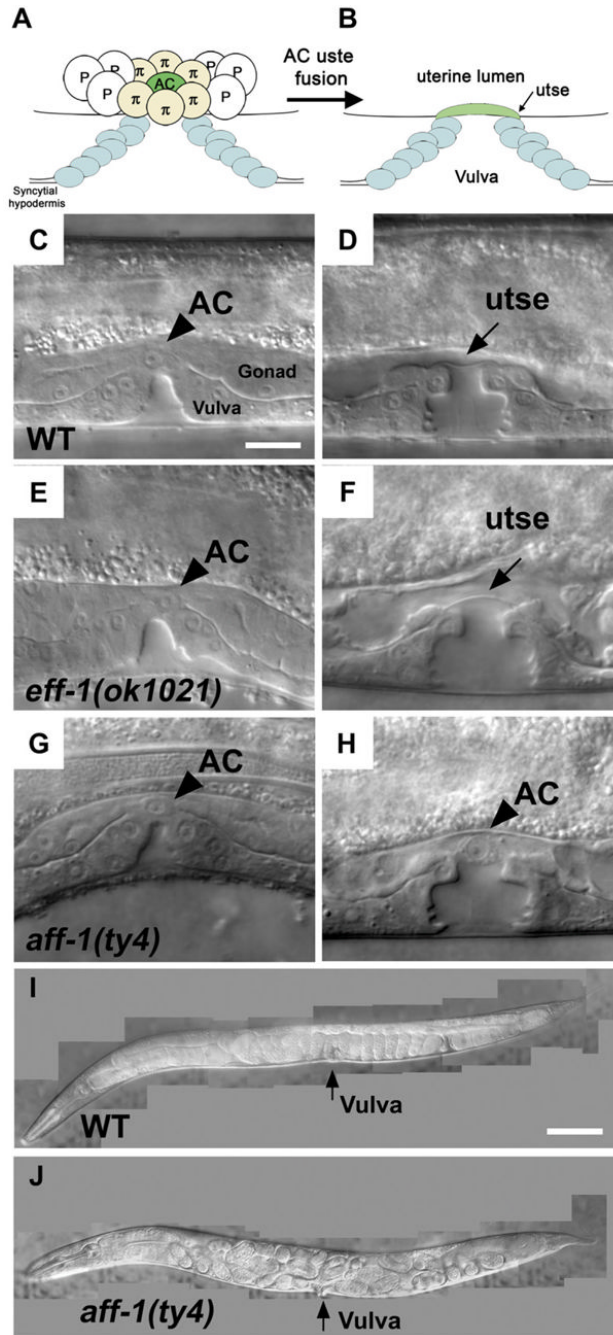


Figure 1. *aff-1* Mutants Exhibit AC Fusion Failure Phenotype

Animals in (C)–(H) are in early- to mid-L4 larval stage, and (I) and (J) are gravid adults. In all the panels hereafter, anterior is to the left; ventral down.

(A) Schematic view of AC (green) and utse cell precursors (π) before cell fusion. The vulval primordial epithelial cells (blue) invaginate connecting the epidermis (hypodermis) with the uterus.

(B) Formation of the utse and cell-cell fusion of AC with π cell daughters connect the uterus through the vulva.

(C, E, and G) In wild-type as in *eff-1* and *aff-1* null alleles the AC is correctly localized on the vulva apex (arrowhead).

(D) In wild-type animal, fusion of the AC to the utse syncytium resulted in the formation of a hymen/utse layer (arrow) between the vulval and uterine lumens.

(F) In *eff-1* mutant, normal AC fusion resulted in hymen formation, indicating that *eff-1* is not required for AC fusion.

(H) In *aff-1* mutant, the AC failed to fuse and was retained at the uterus-vulva junction (arrowhead).

(I) Adult animal exhibiting normal development of embryos that are laid from the uterus at the 50–100 cell stage.

(J) Strong Egl phenotype of *aff-1* mutant hermaphrodite. Embryos complete embryonic development in the uterus, and the larvae hatch inside the mother.

The scale bar represents 5 μm in (A)–(H) and 20 μm in (I) and (J).

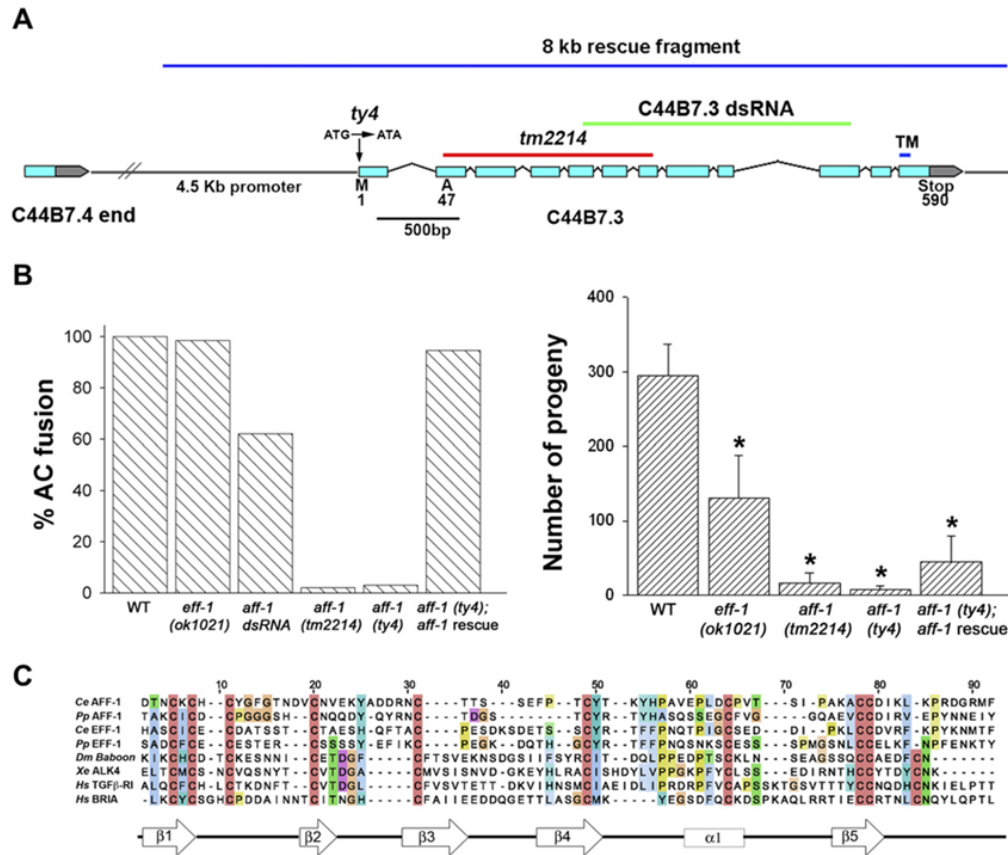


Figure 2. *aff-1* Activity Is Required for AC Fusion

(A) Scheme of *aff-1* gene structure with mutations and construct annotations. The first methionine is substituted to isoleucine in *ty4* mutation while the *tm2214* deletion (red line) introduced a stop codon after alanine 47. The sequence that was used as the template for dsRNA experiments is marked in green and the 8 kb PCR-based rescue fragment in blue.

(B) Phenotypic analysis of *aff-1* alleles. AC fusion failure and low fertility in *ty4* and *tm2214* mutants. *aff-1* dsRNA phenocopy *aff-1* mutant phenotype. *ty4* phenotype is rescued by an 8 kb fragment from *aff-1*. Error bars indicate standard deviation. Asterisks indicate difference from wild-type with statistical significance of $p < 0.001$ according to unpaired two-tailed t test.

(C) Sequence alignment of *C. elegans* fusogens with their putative homologs from *P. pacificus* and selected members of the TGF- β type I receptors. The alignment is limited to the structurally defined part of the Hs BRIA sequence. Secondary structures presented under the alignment refer to the solved crystal structure of BRIA. Nine cysteines (pink) are conserved between all the aligned proteins, suggesting that these proteins share a similar structural fold. An additional cysteine followed by asparagine (green) that are part of TGF- β binding domain are not conserved in EFF-1 and AFF-1 proteins. Abbreviations: *Ce*, *C. elegans*; *Pp*, *P. pacificus*; *Dm*, *D. melanogaster*; *Xe*, *X. laevis*; *Hs*, *H. sapiens*; BRIA, BMP receptor IA extracellular domain; TGF, TGF- β RI. Alignment color code was according to the ClustalX color scheme Jalview software. Accession numbers: *Ce* AFF-1: EF205023; *Pp* AFF-1: contig1480; *Ce* EFF-1: GI:19071563. *Pp* EFF-1: Contig2476 (http://www.pristionchus.org/cgi-bin/seq_retrieval.pl); *Dm* Baboon: gi|33589356; *Xe* ALK4: gi|49903662; TGF *Hs*: gi|4759226; BRIA *Hs*: gi|48425316. See Experimental Procedures.

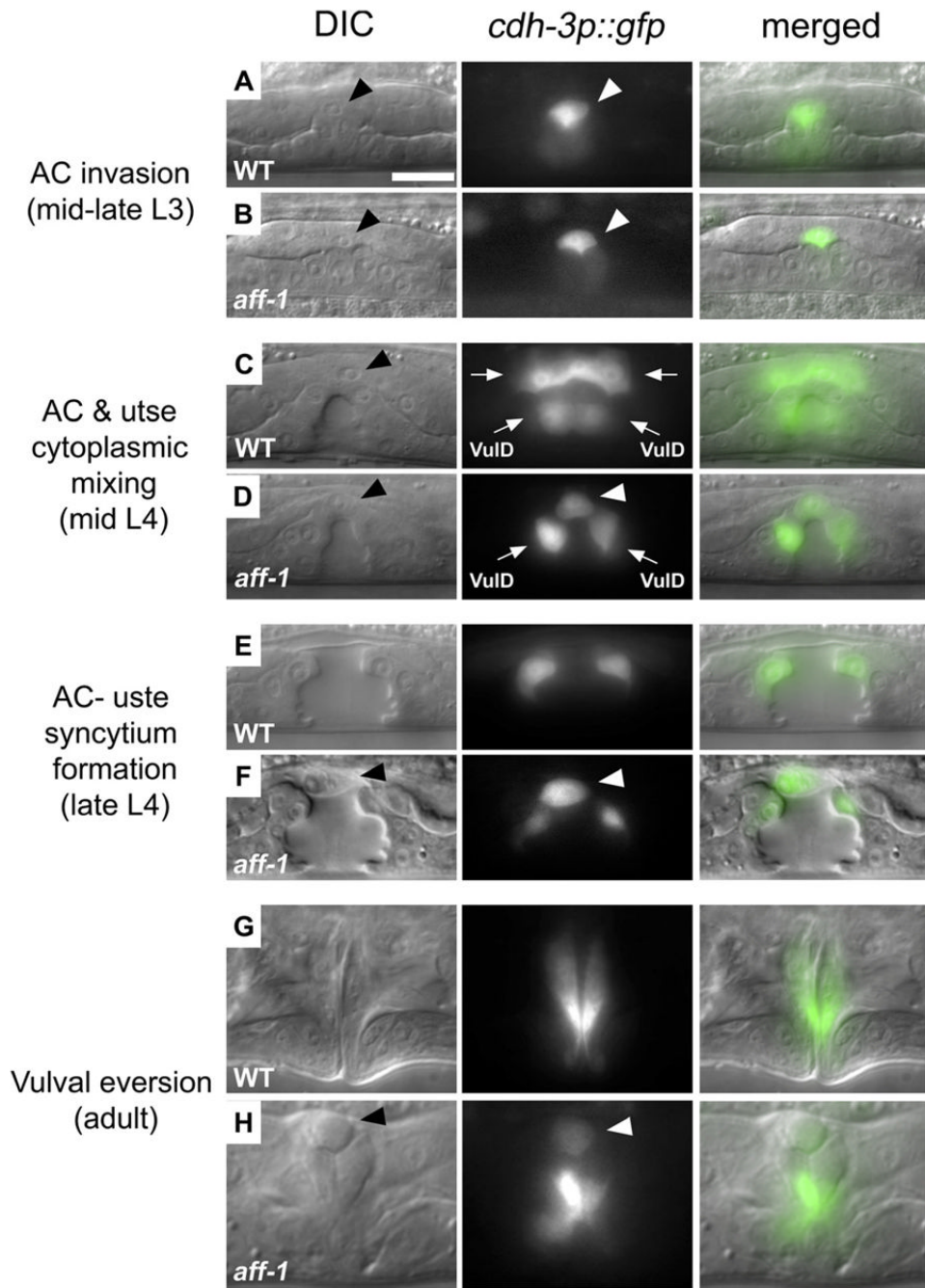


Figure 3. *aff-1* Is Required for AC Fusion prior to Cytoplasmic Mixing

AC, arrowhead; utse syncytium, arrows.

Nomarski (left) fluorescence (center) and overlaid (right) images of vulval-uterine area in critical intermediates of AC development during L3 to adult.

(A) AC invasion in the L3 stage was detected by a cadherin promoter driving GFP expression (*cdh-3p::GFP*; [Sherwood and Sternberg, 2003]).

(B) In *aff-1* mutant, AC invasion is normal.

(C) In wild-type, cytoplasmic mixing between AC and utse cells is detected by diffusion of the AC marker *cdh-3p::GFP* to the utse (arrows); VulD represents vulval ring “D.”

- (D) In *aff-1* mutant, *cdh-3p::GFP* retention in the AC demonstrates that cytoplasmic mixing does not occur (arrowhead).
- (E) At the vulval “Christmas tree” stage, the AC and utse syncytium form a thin layer between vulva and uterus lumens in wild-type.
- (F) In *aff-1* mutant, this layer is not formed and the unfused AC lies at the vulva-uterus junction (arrowhead).
- (G) Normal adult vulva after eversion.
- (H) Unfused AC remains at the apex of the everted *aff-1* vulva (arrowhead). All panels are at the same magnification; the scale bar corresponds to 5 μm .

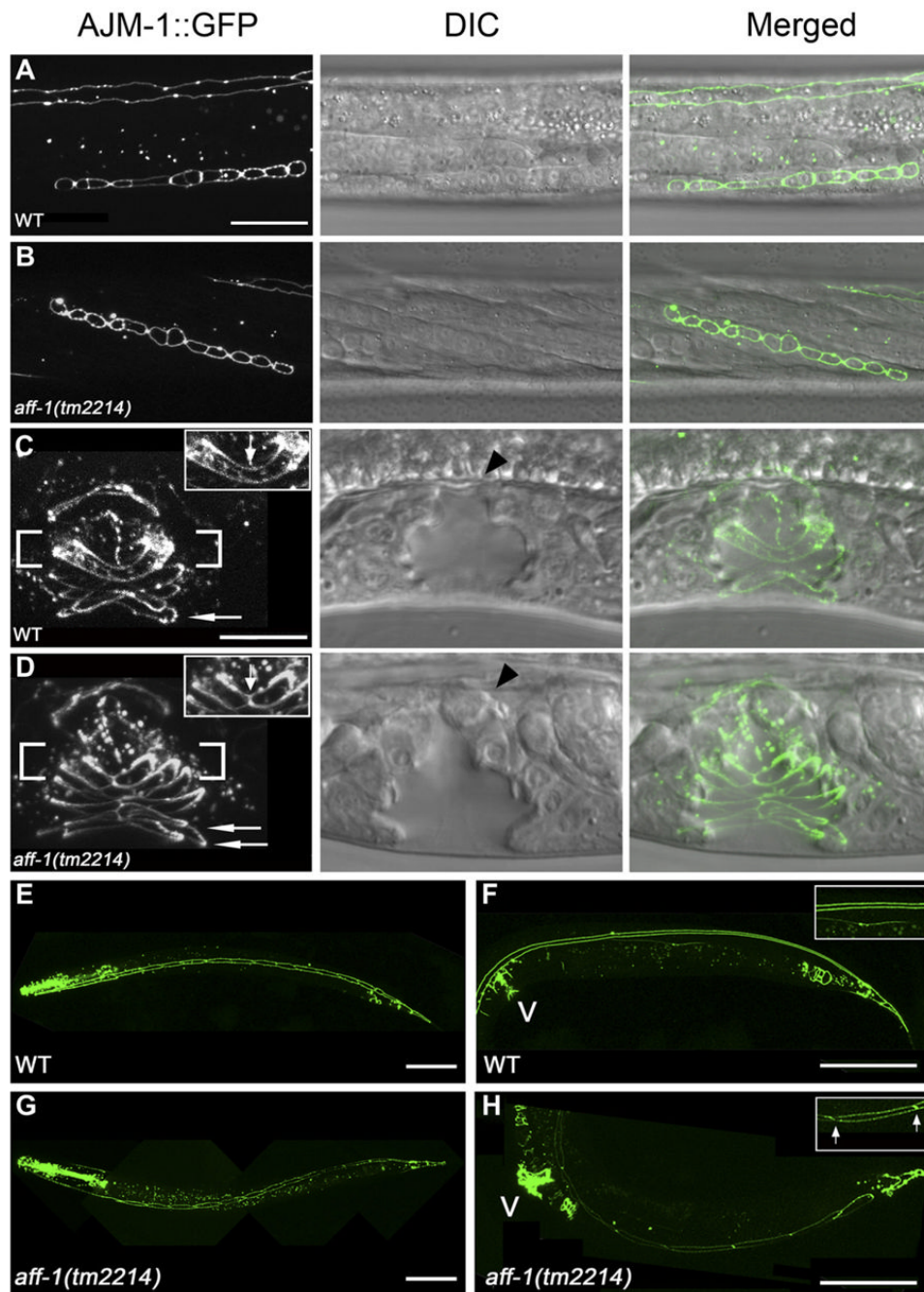


Figure 4. *aff-1* Is Required for Fusion Events in Other Tissues

(A–D) Nomarski (center) and the corresponding fluorescence image in selected stages of vulval development of the apical junction marker AJM-1::GFP that marks epithelia cell borders.

(A) In wild-type worms, 12 primordial vulval cells are located at the ventral side at late L3 stage.

(B) A similar pattern in *aff-1* mutants shows that *aff-1* does not affect VPCs proliferation. In addition, the fusion of 3° fate VPCs to the epidermis is normal.

(C) Fusion of vulval cells results in the formation of vulval rings in wild-type (for example vulD ring, fusion marked with an arrow in inset). vulA represents a single ventral ring (arrow).

(D) In *aff-1* deletion, the two D cells did not fuse; hence the D ring is unfused (arrow in insert). The A cells fail to fuse before ring formation and two vulA rings form instead of one (arrows). (E–H) Selected stages of seam cell development in wild-type (E and F) and *aff-1* mutant worms (G and H) examined by the AJM-1::GFP marker.

(E) In wild-type L3 stage, 16 seam cells are on each side of the body, separated by apical junctions (left view).

(F) During late L4/early adult, seams undergo cell fusion that results in a long syncytium marked by two parallel lines of AJM-1::GFP; see insert and top of (A).

(G) In *aff-1* mutant, early seam development is similar to wild-type.

(H) During late L4, the seam syncytia did not form, so individual cells are detected and remained unfused in adults. Insert shows detail with unfused apical junctions, arrows.

(A, B) and (C, D) are panels with same magnification. The scale bars in (A) and (C) represent 10 μm . (E–H) Scale bar corresponds to 50 μm . V, vulva.

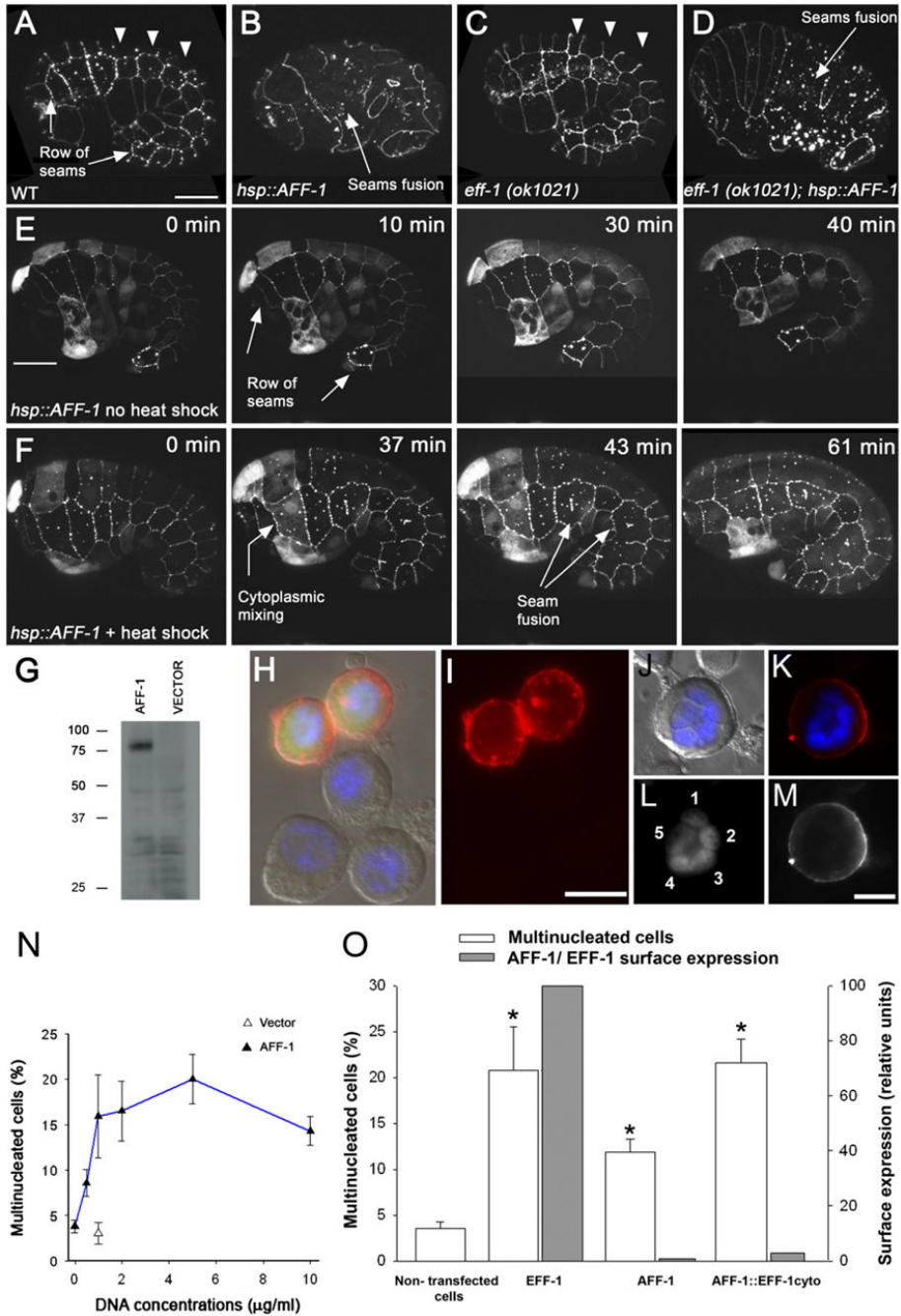


Figure 5. AFF-1 Fuses *C. elegans* Hypodermal Cells and Heterologous Insect Cells
 (A–F) Comma to 1.5-fold stage embryos, anterior to the left and ventral down.
 (A–D) Confocal projections of embryos from different genetic backgrounds all marked with the apical junction marker AJM-1::GFP. Punctuated staining is due to the background of the GFP.
 (A) In wild-type embryos, dorsal hypodermal cells undergo fusion (arrowheads). The seam cells do not fuse during embryogenesis (arrows).
 (B) *hsp::aff-1* embryos after heat shock. The disappearance of apical junction between individual cells suggests that AFF-1 causes fusion of the hypodermal cells.

- (C) *eff-1* mutant embryos where most embryonic fusions do not occur. Arrowheads mark some unfused dorsal cells.
- (D) The lack of *eff-1* does not attenuate AFF-1-induced fusion, indicating that AFF-1 acts in an *eff-1*-independent mechanism.
- (E and F) Individual frames from time-lapse movies (see Supplemental Data) of control (E) and *hsp::eff-1* worms (F) marked by AJM-1::GFP and by *eff-1p::GFP* that is distributed in the cytoplasm of individual hypodermal cells. The time after heat shock appears on top right.
- (E) In non-heat-shocked embryos, dorsal fusion is normal while individual seam cells did not fuse (arrows; see Movie S1).
- (F) In the intermediate step of the heat shock effect (37 min), diffusion of GFP from ventral hypodermal cell to a single seam cell (arrow) concomitant with apical junction removal between these cells indicates that *aff-1* is sufficient to induce cell fusion ectopically. In addition, fusion between seams is observed (arrows). The cytoplasmic GFP diffuses through *hyp6*, *hyp7*, and seam cells (Movie S2).
- (G) AFF-1 protein tagged with V5-6XHis epitopes was expressed in Sf9 cells and detected from the cell lysate as a single specific band of apparent MW of 75 kDa by western blot with anti-V5 antibodies.
- (H) Immunofluorescence with anti-V5 antibodies (red), DAPI staining (blue) on *aff-1*-expressing cells (green). The lower three cells do not express the construct.
- (I) AFF-1 protein (red) is distributed at the cell surface and in intracellular puncta.
- (J–M) AFF-1 expression in a pentanucleate cell.
- (J) Cell nuclei are marked by DAPI staining (blue) merged with DIC.
- (K) AFF-1 protein immunostaining (red) and DAPI (blue).
- (L) Five distinct nuclei (1–5) are detected in the syncytium.
- (M) AFF-1 protein is localized to the plasma membrane.
- (N) Ectopic expression of AFF-1 results in multinucleated Sf9 cells 24 hr after transfection. Percentages of multinucleation with respect to *aff-1* DNA concentration are shown (filled triangles and blue line). The multinucleation of control cultures transfected with empty vector is marked by an empty triangle.
- (O) Cell surface AFF-1 induces multinucleation more potently than EFF-1. Percentages of multinucleated cells (empty columns) and surface expression in relative units (gray columns) of empty vector, EFF-1, AFF-1 and a chimera between AFF-1 extracellular domain and EFF-1 transmembrane and cytoplasmic domain (AFF-1::EFF-1cyto; see Supplemental Data).
- (A–F) Scale bars represent 10 μm ; (H and I) 20 μm ; (J–M) 10 μm .
Error bars represent standard error and stars represent statistical significance of $p < 0.05$.

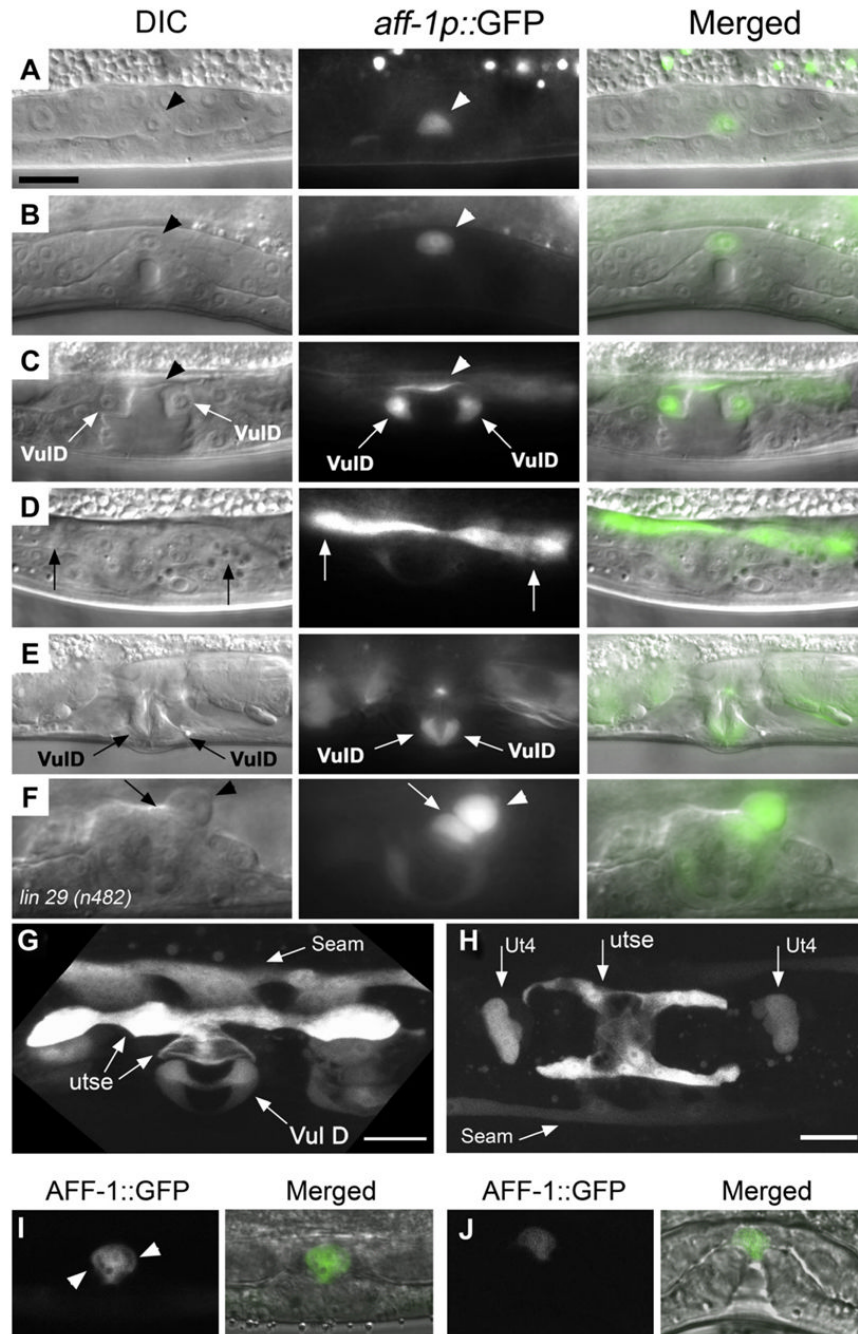


Figure 6. Correlation between *aff-1* Expression and Its Fusogenic Effect

(A–F) Nomarski (left) *aff-1* promoter GFP fusion (*aff-1p::GFP*) fluorescence signal (center) and overlaid (right) images.

(A) At mid-L3, *aff-1* is expressed specifically in the AC (arrowhead).

(B) AC expression is retained while the vulva invaginates during early L4.

(C) Expression of the *aff-1* transcriptional reporter is induced in the vulval D ring cells (arrows) and in the AC-utse syncytium as it is formed in late L4 stage (arrowhead).

(D) A different focal plane from the same worm shown in (C) where the AC-utse expression is highlighted (arrows).

(E) The vulD expression of *aff-1* is retained in adult worms (arrows).

(F) In *lin29(n482)* mutants, AC and utse fusion do not occur (Newman et al., 2000). A single AC (arrowhead) is localized adjacent to a single utse cell (arrow). *aff-1* signal in utse cells that did not fuse with the AC cell demonstrates that *aff-1* is autonomously expressed in the utse cells.

(G) Confocal projection of *aff-1* expression pattern in L4 larva (dorsolateral view). *aff-1* is expressed in VulD ring, the utse, and in the seams. Seam cell expression starts at mid-L4 just before *aff-1*-dependent fusion.

(H) Confocal projection of the H-shaped utse syncytium where *aff-1* is expressed (ventral view). In addition, *aff-1* expression is detected in two rows of lateral seam cells and in the two uterine ut4 toroids (Ut4).

(I) Subcellular localization of AFF-1::GFP protein in the AC during invasion (lateral view of confocal image). The fusion protein was specifically expressed in the AC by the anchor-cell-specific promoter pAC (Kirouac and Sternberg, 2003). AFF-1::GFP protein is localized in intracellular compartments and at the plasma membrane (arrowheads).

(J) Similar subcellular localization of AFF-1::GFP was detected at the time of AC fusion in a confocal image (lateral view).

(A–F) Scale bar 5 μm ; (G) 10 μm ; (H) 20 μm .

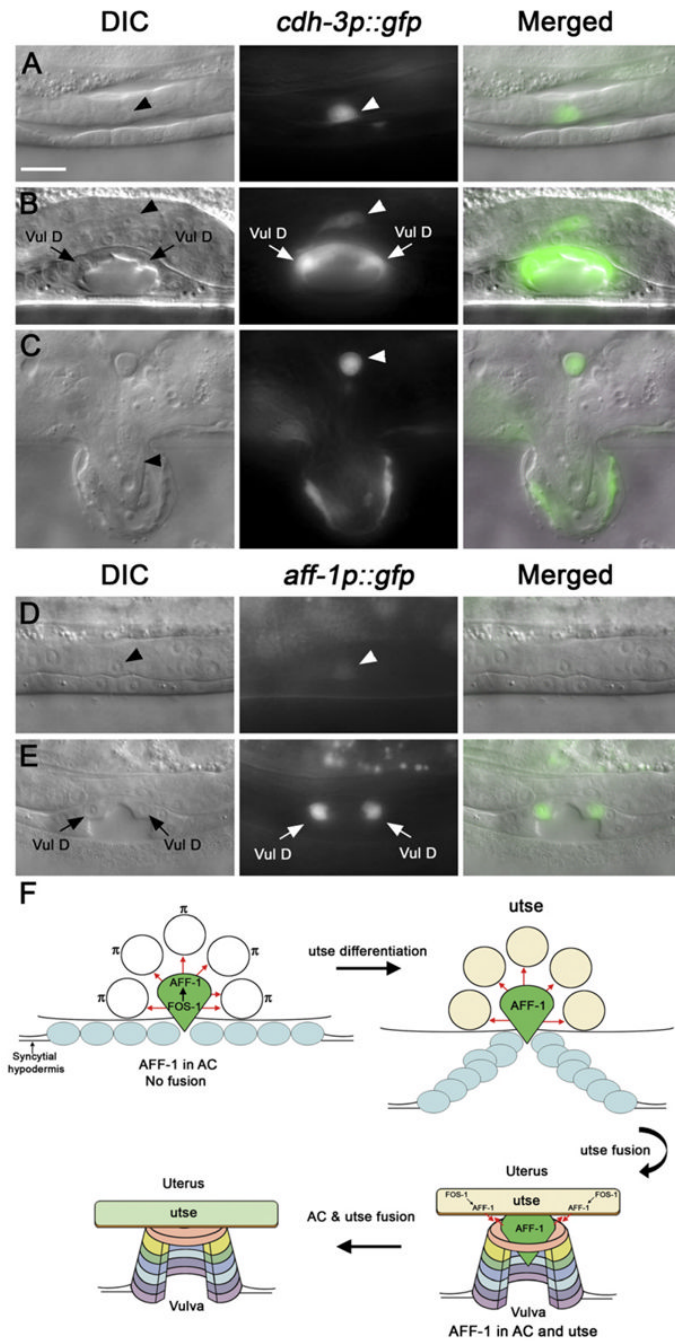


Figure 7. FOS-1 Controls AC Fusion via *aff-1* Expression in utse and AC

(A–C) Nomarski (left), AC marker *cdh-3p::GFP* (center), and overlaid (right) images of *fos-1* (*ar105*) mutant L3 to adult.

(A) AC invasion did not occur in *fos-1* mutants, indicated by the retention of basement membrane between the gonad and the ectoderm (Sherwood et al., 2005) (arrowhead).

(B) In *fos-1* mutant L4 larva, the *cdh-3p::GFP*-labeled AC did not fuse (arrowhead).

(C) This fusion failure persists until adulthood (arrowhead).

(D and E) Images of *fos-1(ar105)* mutants. No-marski (left), *aff-1* promoter GFP (center), and merged (right).

(D) At the stage of AC invasion (L3), AC does not invade in *fos-1* mutant (arrowhead; 100%, n = 36) and expresses *aff-1* very weakly or is undetectable (97%, n = 33) in comparison to wild-type (see Figure 6A).

(E) *aff-1* was not detected in the AC or other uterine cells while still retaining expression in VulD ring and in the seam cells (not shown) compared with wild-type (L4; Figures 6B–6D).

(F) Schematic representation of FOS-1-mediated regulation of *aff-1* RNA (green) and AFF-1 protein (red arrows). FOS-1 controls *aff-1* expression during AC invasion. During π /utse differentiation, AFF-1 is expressed in the AC only. After utse AFF-1-independent fusion, the utse syncytium starts expression of *aff-1* RNA. Once AC and utse syncytium both express AFF-1, AC-utse fusion occurs.

(A)–(E) are with the same magnification. Scale bar represents 5 μ m.