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Supplemental Data

AFF-1, a FOS-1-regulated Fusogen

Mediates Fusion of the Anchor Cell in *C. elegans*

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Supplemental Experimental Procedures

Worm handling and strains

All *C. elegans* strains were cultured and visualized at 20°C as in (Brenner, 1974). Strains used in this study were:
LGII: *eff-1(ok1021)* (Podbilewicz et al., 2006)
aff-1(tm2214) II, eff-1(ok1021)/mIn1[mIs14 dpy-10(e128)] II
aff-1(ty4) II; tyEx14[aff-1 PCR.P + pRabGFPrim3']
aff-1(tm2214) II; jcIs1[ajm-1::GFP + pRF4(rol-6(su1006))] IV
aff-1(tm2214) II; kuIs29[unc-119(+) + egl-13p::GFP(pWH17)] V
aff-1(tm2214) II; syIs50 [cdh-3p::GFP] X
LGIV*: eri-1(mg366) IV*
LGV: kuIs29[*egl-13p::*GFP] (Hanna-Rose and Han, 1999), *fos-1(ar105)*(Sherwood et al., 2005)
fos-1(ar105) V; hyEx167[aff-1p::GFP + pRF4(rol-6(su1006))]
LGX: syIs50[*cdh-3p::*GFP] (Sherwood and Sternberg, 2003)
syIs50[cdh-3p::GFP] X; eri-1(mg366) IV

Mapping the *ty4* mutation

To identify the gene mutated in *ty4* background, we performed Tc1 mapping analysis. We assigned the *ty4* mutation to chromosome II and positioned it between the *stP101*(-4.5) and *stP* (1.8) genetic markers. Next, we executed a three-factor mapping and deficiency mapping to further refine the region to which *ty4* maps. The data suggest that *ty4* is positioned between *dpy-2* (0.05) and *vab-13* (0.29), a genetic interval of 0.24 map unit (about 300 kb) to the right of the center of chromosome II. We performed complementation analyses on several candidate genes within or near the mapping region that were known to be involved in cell fusion or whose absence was known to result in an Egl defect. The mutations used were as follows: *eff-1(hy21), egl-27(n170), pvl-5(de4),* and *snt-1(ad596, md290, n2665).* All of the above mutations complemented the *ty4* mutation, suggesting that *ty4* was not an allele of the corresponding genes. The *ty4* mutation did not complement *tm2214*, a deletion in *C44B7.3* gene that is localized to this region. *ty4/ tm2214* transheterozygous worms exhibited strong Egl phenotype similar to the phenotype of the individual alleles as homozygotes (n=20). This suggests that *ty4* and *tm2214* are alleles of the same gene- *C44B7.3*. The base

substitution in *C44B7.3* gene that resulted in *ty4* mutation was identified by sequencing (See Experimental Procedures).

***aff-1* molecular analysis and constructs**

In all constructs, the DNA was amplified using DNA Expand Taq (Roche) and was sequenced before use. Injection, handling, and characterization of transgenenic strains were done according to standard procedures.
The borders of *aff-1* promoter were predicted by promoter comparison between *aff-1* and its putative *C. briggsae* homologue *CBG11169* using Blast two sequences algorithm in NCBI (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Nine short conserved regions were identified in a 4.5Kb region upstream to *aff-1* start codon suggesting that the promoter lays in this interval. To identify candidate transcription factor binding sites we used the online software tool TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess).

aff-1p::gfp was constructed by PCR amplification of this 4.5Kb fragment from C44B7 cosmid and fusion with GFP following (Hobert, 2002). 10ng/μl of *aff-1p::gfp* was co-injected with 26ng/μl genomic DNA and with 80ng/μl of the transformation marker pRF4 [rol-6(su1006)] (hyEx167).

To drive *aff-1* expression ectopically, full length *aff-1* was amplified from C44B7 cosmid and sub cloned into pPD49.78 plasmid (kindly provided by A. Fire) downstream of the heat-shock promoter. 10ng/μ l from this construct was co-injected with 10ng/μ l of the apical junction marker AJM-1::GFP and with 80ng/ pRF4 [rol-6(su1006)] (hyEx173). Expression of the reporter *aff-1p*::GFP was also observed in embryonic hyp5, sheath cells, head interneuron 5, chemosensory neurons from the head and tail, pharyngeal cells, and L4/adult posterior male tail.

To drive AFF-1::GFP expression specifically in the AC we cloned upstream to *aff-1* genomic sequence a 1.5 kb cis-regulatory region of the *cdh-3* gene (pAC) which drives expression specifically in the AC from L2 to L4 larval stages (Kirouac and Sternberg, 2003). Next we added GFP in frame after the 3' end of the *aff-1* gene by fusion PCR to create the pAC::AFF-1::GFP construct (Hobert, 2002).

To construct *aff-1* promoter::AFF-1::GFP we amplified *aff-1* promoter and coding region from the C44B7 cosmid by PCR. GFP was fused in frame to the 3' end of the last exon of *aff-1* by PCR, to create *aff-1* promoter::AFF-1::GFP.

For Sf9 experiments *aff-1* cDNA was constructed in two steps. In the first step two base substitutions in the EST clone yk747 (G in 1217 instead of A) and (G in 1705 instead of A) were corrected by a standard site directed mutagenesis method. The 3' of this corrected EST was then ligated with 5' of yk1083 to give the correct *aff-1* ORF that was sub cloned into pIZT plasmid to form pIZT::AFF-1-V5-6Xhis (Podbilewicz et al., 2006).

To construct AFF-1::EFF-1cyto, the extracellular portion of AFF-1 ending in isoleucine 540 (see Figure S3) was fused by PCR to EFF-1 transmembrane and intracellular domains starting from serine 550 and cloned into the pIZT vector.

Supplemental References

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. Genetics *77*, 71-94.

Cinar, H. N., Richards, K. L., Oommen, K. S., and Newman, A. P. (2003). The EGL-13 SOX Domain Transcription Factor Affects the Uterine {pi} Cell Lineages in Caenorhabditis elegans. Genetics *165*, 1623-1628.

Clamp, M., Cuff, J., Searle, S. M., and Barton, G. J. (2004). The Jalview Java alignment editor. Bioinformatics *20*, 426-427.

Hanna-Rose, W., and Han, M. (1999). COG-2, a Sox domain protein necessary for establishing a functional vulval-uterine connection in *Caenorhabditis elegans*. Development *126*, 169-179.

Hobert, O. (2002). PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic C. elegans. Biotechniques *32*, 728-730.

Kirouac, M., and Sternberg, P. W. (2003). cis-Regulatory control of three cell fate-specific genes in vulval organogenesis of Caenorhabditis elegans and C. briggsae. Dev Biol *257*, 85-103.

Podbilewicz, B., Leikina, E., Sapir, A., Valansi, C., Suissa, M., Shemer, G., and Chernomordik, L. V. (2006). The *C. elegans* developmental fusogen EFF-1 mediates homotypic fusion in heterologous cells and in vivo. Dev Cell *11*, 471-481.

Sherwood, D. R., Butler, J. A., Kramer, J. M., and Sternberg, P. W. (2005). FOS-1 promotes basement-membrane removal during anchor-cell invasion in C. elegans. Cell *121*, 951-962.

Sherwood, D. R., and Sternberg, P. W. (2003). Anchor cell invasion into the vulval epithelium in C. elegans. Dev Cell *5*, 21-31.

Table S1 Characterization of *aff-1* and *eff-1* loss-of-function phenotypes

Strain/treatment	% AC Fusion Failure (Aff) (n)	% Egl (n)	% Sterility (n)	Number of Progeny ± SD (n)
Wild-type (N2)	0 (135)	0 (59)	0 (59)	294 ± 43 (59)
eff-1 (ok1021)	1.5* (135)	ND	3.8 (52)	131 ± 57 (52)
Empty vector, *eri-1*	0 (66)	4 (117)	ND	ND
aff-1(RNAi), *eri-1*	38 (77)	79 (130)	ND	ND
aff-1 (tm2214)	98** (135)	100 (48)	3.4 (117)	16 ± 14 (117)
aff-1 (ty4)	97*** (135)	100 (91)	0 (46)	7 ± 5 (46)
aff-1 (ty4); aff-1 rescue	5.4 (135)	6 (52)	3 (52)	45 ± 35 (52)

* In these two *ok1021* worms where the uterus and vulva structure was highly malformed, the AC failed to fuse.
** Of the 132 non-fused anchor cells 124 exhibited bloated like shape while the other eight had the shape of a degenerated cell.
*** 132 non-fused anchor cells, 120 exhibited bloated like shape and 12 had degenerated cell shape.

Table S1 **Characterization of *aff-1* and *eff-1* loss-of-function phenotypes**

All strains were grown at 20°C and visualized by Nomarski microscopy. In all wild type worms examined the AC fuses with the uste syncytium resulting in formation of a hymen that permits the laying of ~300 progeny. Normal AC fusion was observed in *eff-1 (ok1021)* mutant worms where a moderate reduction in progeny number is resulting from different abnormalities. Attenuating *aff-1* activity by RNAi or mutations resulted in AC fusion failure concomitant with strong EGg Laying defective (Egl) phenotype and gross reduction in the number of progeny.

Supplemental Figures

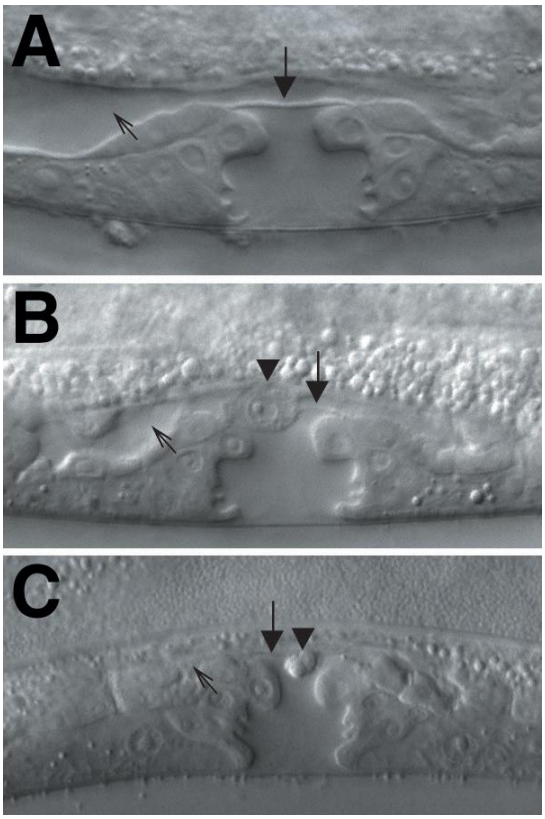

Figure S1 Formation of utse syncytium in *aff-1* mutant worms

(A) Wild-type worm at the L4 stage. Thin laminar process of the utse (large arrow) separates the uterine and vulval lumens. The AC has fused with the utse and is not visible as a distinct cell. Uterus lumen is marked by small arrow.

(B) *aff-1(ty4)* mutant at the L4 stage. The arrowhead indicates unfused AC. The thin laminar process characteristic of utse syncytium formation is evident (large arrow) suggesting that utse syncytium formation is *aff-1*-independent. Uterus lumen is marked by small arrow.

(C) In 12% of *aff-1* mutant worms the AC has a shape of a degenerative cell (arrowhead) while utse syncytium formation occurs (large arrow). It is not clear what mechanism mediates AC degeneration. Uterus is marked by small arrow.

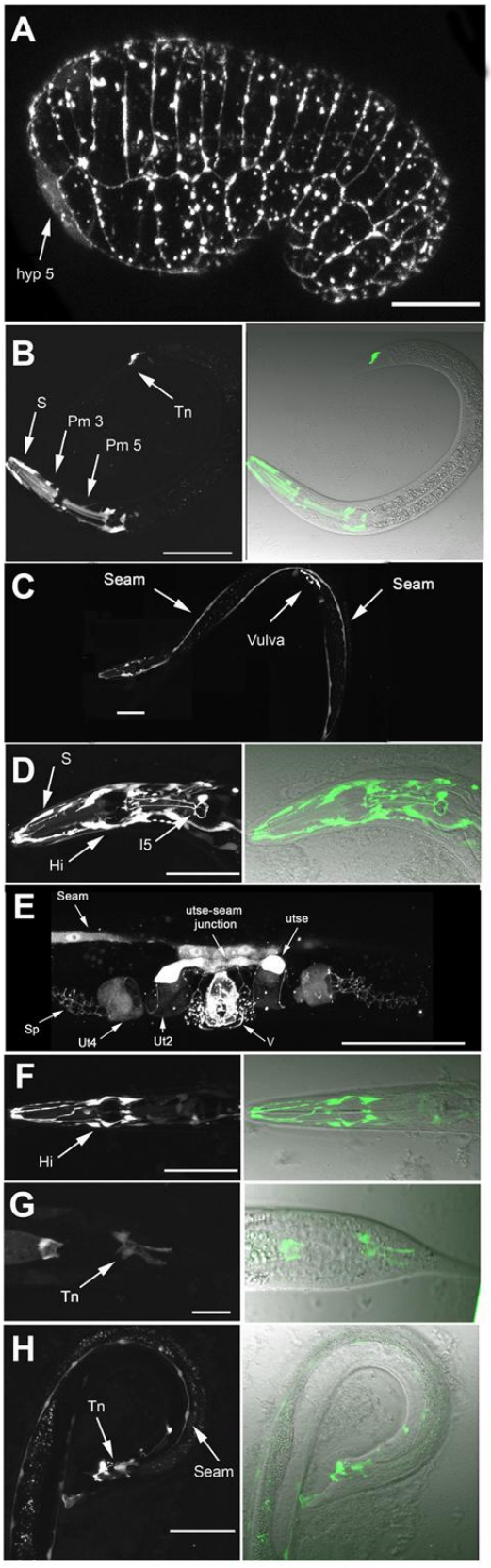

Figure S2 Expression patterns of *aff-1* at different developmental stages

(A) In comma stage embryo *aff-1* is expressed specifically in the two hyp5 cells that undergo fusion to form the hyp5 syncytium. *aff-1* expression is marked by cytoplasmic GFP in hyp5 (arrow). Embryonic cell junctions are marked by AJM-1::GFP.

(B) In L1 hermaphrodite larva *aff-1* is expressed in pharyngeal muscle 3, and 5 (Pm3, Pm5), in sheath cells of chemosensory neurons (S), and in tail neurons (Tn). *aff-1* reporter signal decays in Pm3 and Pm5 until complete disappearance at L4, while the expression in the sheath cells and in head inter neurons is retained until adulthood.

(C) In young adult hermaphrodite *aff-1* is expressed in the two seam cell syncytia that run on each side of the animal body along with expression in the utse syncytium and in vulD vulval ring.

(D) Pharyngeal *aff-1* expression in young adult. *aff*-1 is expressed in the sheath cells of chemosensory neurons (S) and in head inter neurons (I5 and Hi).

(E) *aff-1* is expressed in the uterus ut2 (Ut2) and ut4 toroids (Ut4), in the vulva VulD (V), utse, and seam cells.

(F-H) Expression patterns of *aff-1* in the male.

(F) Head interneurons (Hi) and chemosensory neurons expression of *aff-1* in adult pharynx.

(G) *aff-1* is expressed in neurons in the L4 male tail.

(H) In adult male *aff-1* is expressed in the seam cells and in tail neurons (Tn). Autofluorescence was observed in the spicule and male fan (Af).

Scale bars represent 10μ m in A, E, G and 50μ m in B, C, D, F, H.

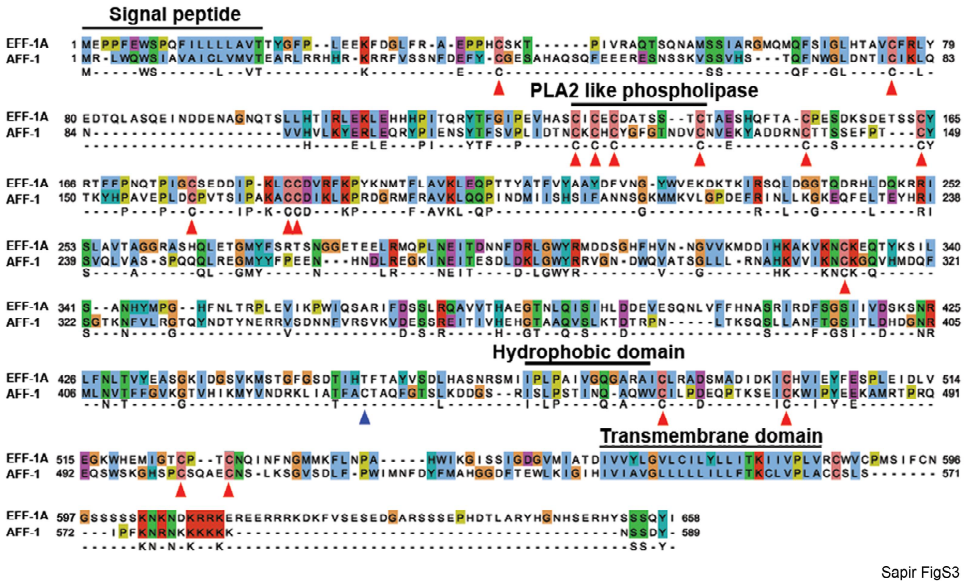

Figure S3 EFF-1 and AFF-1 sequence comparison

EFF-1 and AFF-1 proteins exhibit 26% identity and 46% similarity. EFF-1 putative domains are marked by an annotated line above the sequence. The signal peptide and transmembrane domains are conserved while the PLA-2 active site and the hydrophobic domain are only moderately conserved. Sixteen conserved cysteines in the extracellular domains are marked with red arrowheads while a single non-conserved cysteine in *aff-1* is marked with a blue arrow. In addition to cysteines conservation, partial conservation in 11 out of 22 extracellular prolines was detected. Alignment color code was according to the Clustal X color scheme in Jalview software (Clamp et al., 2004). Abbreviations: *Ce*, *C. elegans*. Accession numbers: Ce EFF-1: C26D10.5 (WP:CE03028); Ce AFF-1: EF205023.

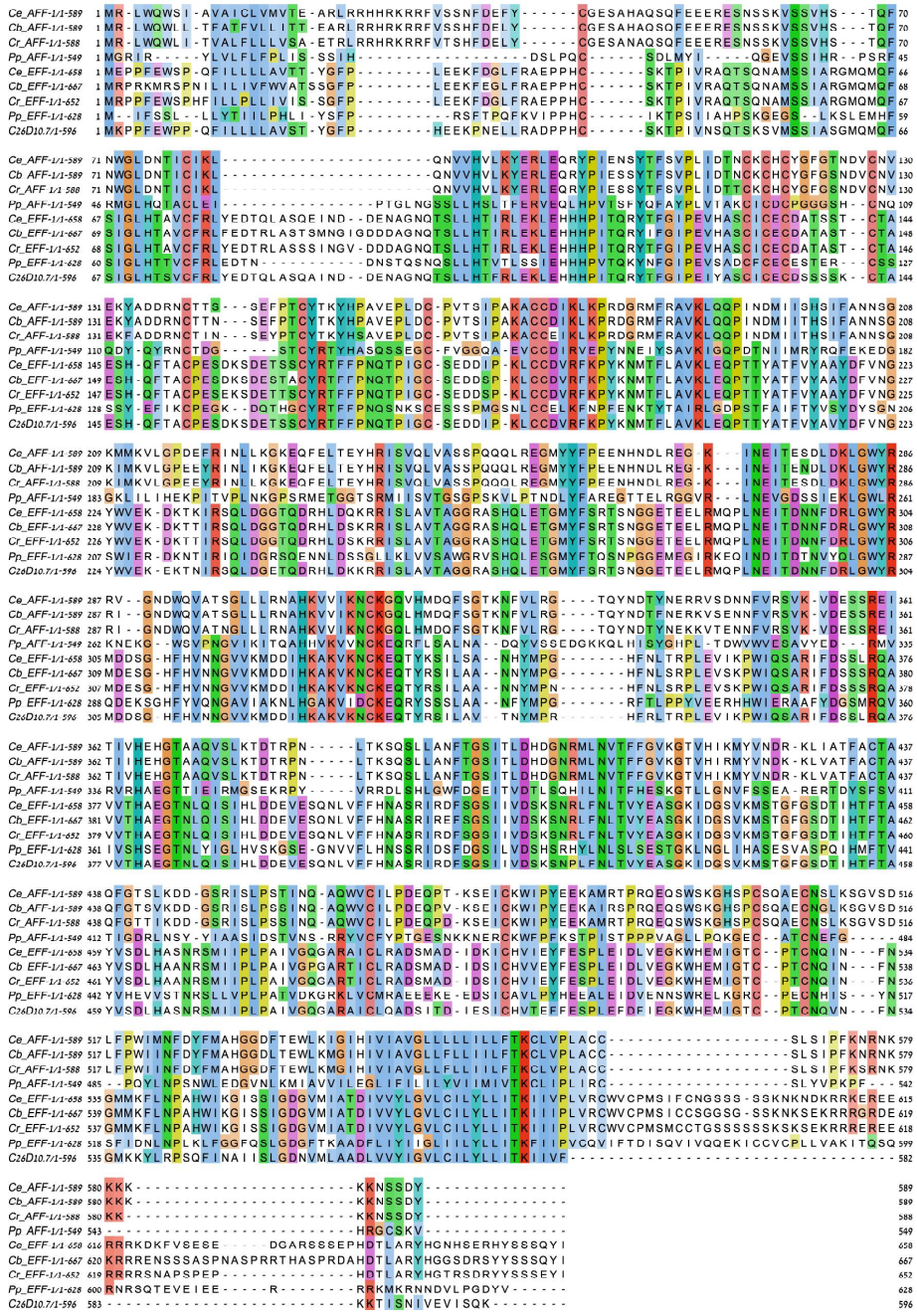

Figure S4 Protein sequence alignment of the FF family in nematodes

Alignment color code was according to the Clustal X color scheme with 10% conservation color increment in Jalview software (Clamp et al., 2004). Abbreviations: *Ce*, *C. elegans*; *Pp*, *P. pacificus*; *Cr*, *C. remanei*; *Cb. C. briggsae*. Accession numbers: *Ce* AFF-1: EF205023; *Cb* AFF-1: CBG11169 (BP:CBP17138); *Cr* AFF-1: Supercontig3 (cr01.sctg3.wum.206.1); *Pp* AFF-1: contig1480; *Ce* EFF-1: C26D10.5 (WP:CE03028); *Cb* EFF-1: BP:CBP05786 (CBG00700) ; *Cr* EFF-1: Supercontig2 (cr01.sctg2.wum.648.1); *Pp* EFF-1: Contig2476.

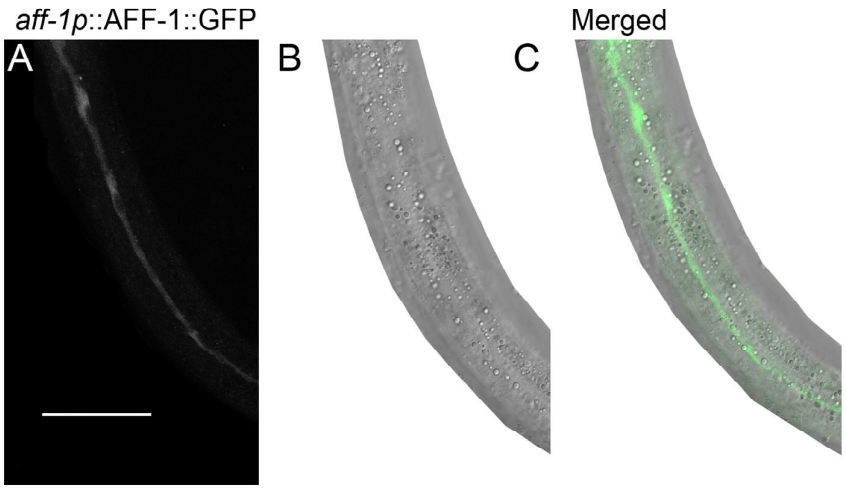

Figure S5 AFF-1 protein localization in L4 seam cells

(A) Dorsal view of L4 larva expressing AFF-1::GFP under the regulation of AFF-1 endogenous promoter. AFF-1 expression in the seam cells correlates with the time of seam cells fusion (L4).

(B) Nomarski image of the same larva.

(C) Merged view demonstrating the distribution of AFF-1 protein in the seam cells (side view).
Scale bar 50 ⌈m.

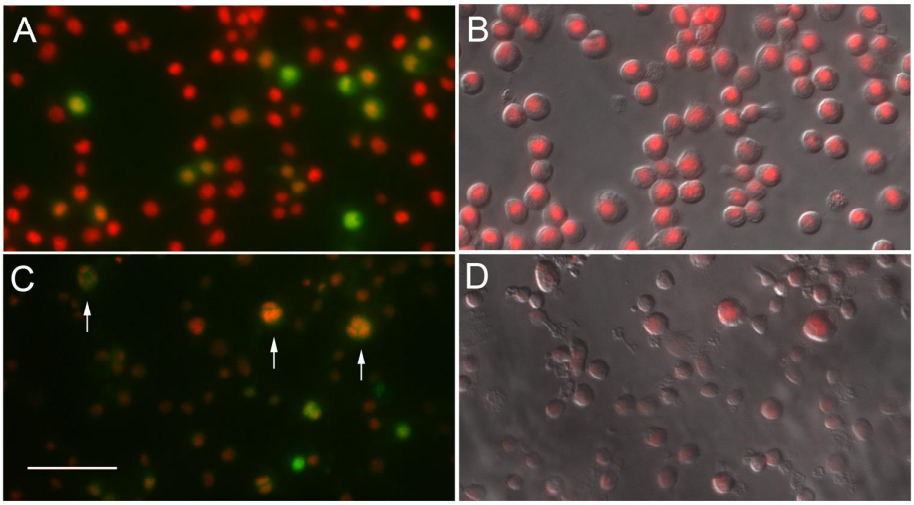

Figure S6 Expression of AFF-1 in Sf9 cells results in multinucleation

(A) Insect Sf9 cells transfected with pIZT/v5-His empty vector shows GFP expression (green) and nuclei (red; Hoechst).

(B) Merged image between nuclei and DIC images of the same field indicates that all cells (transfected and non-transfected) are mononucleated.

(C) Cells transfected with pIZT-AFF-1 (green) became multinucleated (arrows).

(D) Multinucleate AFF-1 expressing cells where 2-4 nuclei are detected per single cell.

All panels are with the same magnifications, scale bar 50 μ m.

Supplemental Movie Legends

Supplemental movie 1 shown in Figure 5E

In non heat shocked *hsp::aff-1* embryo there is no ectopic fusion. Time-animated volume projections are shown from a three dimensional confocal recording of a control live embryo 0 to 40 minutes covering comma to 1.5 fold stage transition. All GFPs are shown as white signal. Cytoplasmic GFP is *eff-1p::GFP* that labels individual cells cytoplasm. AJM-1::GFP at cell-cell junctions mark the cell borders.

Supplemental movie 2 shown in Figure 5F

In heat shocked *hsp::aff-1* embryo there is cytoplasmic mixing and ectopic fusion of most embryonic cells. Time-animated volume projections are shown from a three dimensional confocal recording of a live embryo 0 to 61 minutes covering comma to 1.5 fold stage transition.

Movies may be navigated by clicking and dragging (time-animation) by using corresponding arrows on the keyboard. Requires QuickTime version 4 or later (http://www.apple.com/quicktime/).