Genetic Control of Fusion Pore Expansion in the Epidermis of Caenorhabditis elegans

Tamar Gatledge, Aditya Mittal, Clari Valansi, Ken C.Q. Nguyen, David H. Hall, Leonid V. Chernomordik, and Benjamin Podbilewicz

INTRODUCTION

Cell fusion is a ubiquitous and highly controlled process in eukaryotes. Developmental cell fusion is vital for mating and fertilization in yeast and humans, respectively. Cell fusion is required for the formation and maintenance of the muscular-skeletal system in vertebrates, in muscle fibers in Drosophila, and in nearly one third of all cells in Caenorhabditis elegans (Podbilewicz and White, 1994; Heiman and Walter, 2000; Wasserman et al., 2001; Abmayr et al., 2003; Shemer and Podbilewicz, 2003; Chen and Olson, 2005). In C. elegans, diverse cell fusions essential for organ formation and cell fate determination have recently become paradigms for developmental cell fusion (Podbilewicz and White, 1994; Mohler et al., 1998; Nguyen et al., 1999; Sharma-Kishore et al., 1999; Podbilewicz, 2000; Alper and Kenyon, 2002; Mohler et al., 2002; Shemer and Podbilewicz, 2002, 2003; Witz and Rothman, 2002). Cell fusion functions to sculpt organs and to accomplish defined body shapes (Sharma-Kishore et al., 1999; Witz and Rothman, 2002; Shemer and Podbilewicz, 2003). eff-1 (epithelial fusion failure) was identified as a gene encoding type I membrane proteins required for diverse epithelial cell fusion reactions. Mutations in eff-1 result in failure of epithelial cell fusion and developmental defects in organs where cell fusion normally occurs (Mohler et al., 2002; Shemer, 2002; Shemer and Podbilewicz, 2002). The activity of eff-1 is strongly regulated by homeobox containing genes (Shemer and Podbilewicz, 2002, 2003; Cassata et al., 2005). Other transcription and signaling factors have been shown to control the cell fusion process in C. elegans (Nilsson et al., 1998; Ch’ng and Kenyon, 1999; Shemer et al., 2000; Alper and Kenyon, 2001; Chen and Han, 2001; Koh and Rothman, 2001; Alper and Kenyon, 2002; Koh et al., 2002; Zhao et al., 2002; Shemer and Podbilewicz, 2003). The specific roles of different proteins that mediate and control cell fusion and the pathway of this fusion reaction remain unexplored. eff-1 induces cell fusion at distinct developmental times in different organs. Expression of eff-1 using a heat-shock promoter results in cell fusion between different cell types (Shemer et al., 2004; del Campo et al., 2005).

Interestingly, genes homologous to eff-1 have not been identified in Drosophila or vertebrates (Shemer and Podbilewicz, 2003; Podbilewicz and Chernomordik, 2005; Podbilewicz, 2006; Podbilewicz et al., 2006). Genetic screens in Drosophila have identified several genes required for myoblast fusion. Using elegant ultrastructural and developmental studies, it has been determined that the steps affected by these mutants include myoblast differentiation, acquisition of fusion competence,
and recognition and adhesion between myoblasts (Doberstein et al., 1997; Abmayr et al., 2003; Chen et al., 2003). However, genes necessary and sufficient for the actual merger of two plasma membranes into one that has not been reported in other developmental cell fusion reactions outside syncytium-mediated fusion between human trophoblasts (Mi et al., 2000) and eff-1-mediated cell fusion (Shemer et al., 2004; del Campo et al., 2005; Podbilewicz, 2006; Podbilewicz et al., 2006).

Here we hypothesized that since embryonic cell divisions in C. elegans are tightly controlled and invariant (Sulston et al., 1983), we may also find an ordered pattern of cell–cell fusions within the same large syncytium that will be nearly constant between individuals. We apply experimental approaches used for model fusion reactions (Stegmann et al., 1990; Phalen and Kielian, 1991; Frey et al., 1995; Hoekstra et al., 2002; Blumenthal et al., 2003; Chernomordik and Kozlov, 2003; Gibbons et al., 2003; Hu et al., 2003; Jahn et al., 2003; Bonifacino and Glick, 2004; McNerney et al., 2004) to address this hypothesis in living C. elegans. We analyzed cell fusion kinetics in developing embryos and dissected cell membrane fusion into defined stages. Surprisingly, we demonstrate that in the embryonic epidermis of C. elegans a variable cell fuses first, and for each fusogenic cell the anterior and posterior membrane domains fuse independently and asymmetrically. In addition, we found that stable intermediates in late stages of epidermal syncytia formation can be found in larvae and adults of partial loss-of-function eff-1 mutants. Thus, we show that eff-1 is required to initiate, expand and complete syncytia formation in the epidermis.

MATERIALS AND METHODS

Time-Lapse Multifocal Temperature-controlled Confocal Microscopy

Time-lapse movies were recorded using a Nikon Eclipse E-800 with a 60×/1.4 Plan Apo objective using a Bio-Rad MRC1024 confocal microscope with a custom-made copper stage and objective jacket for precise temperature control as described before (Rabin and Podbilewicz, 2000). The resolution of our confocal microscope is ~250 nm. We used and show projections (flattened images) for each time point in all kinetic analyses. Four-dimensional stacks were also analyzed and archives of the complete original data sets are available for further analyses.

The cells responsible for the elongation of the embryo are the hypodermal cells (Sulston et al., 1983; Priess and Hirsh, 1986) and we measured the elongation of the lateral seam cells of the head (Rabin and Podbilewicz, 2000). The whole body elongation rate is 2.5-fold faster than the head elongation rate (Priess and Hirsh, 1986).

The largest syncytium (hyp7) is initiated in the embryo during elongation, and the events occurring from the comma stage to the 1.5–fold stage have been characterized here (Figures 1 and 2). During this time window the wild-type embryo does not move, allowing us to follow the kinetics as described below.

For immunofluorescence we fixed and stained embryos using the methanol/aceton on dry ice protocol (Podbilewicz and White, 1994).

Kinetics of Cell Fusion: The Normal Sweep Method

We developed a semiautomated method to monitor membrane fusion, leading to disappearance of GFP-marked cell junctions in a single C. elegans embryo (Figure 3 and Supplemental Materials and Methods). Using the semiautomated normal sweep method, we could quantify the membrane fusion for each junction as follows:

\[
\text{Fraction of fused junctions} = \frac{\text{Fraction of blank pixels}}{\text{Total pixels along the arc}}
\]

Thus, using this equation for images taken at regular time intervals for each embryo, we obtained the real-time kinetics of cell–cell fusion for multiple junctions in a single embryo. Note that for most of the embryos observed, there is excessive image noise above cell number 4 (as defined in Figure 1A; dorsal cells are numbered in anterior to posterior direction) and for nearly all the embryos, cells number 12 gradually vanish from the dorsal view because of elongation. Therefore, to compare the same multiple fusion events in different embryos, we monitored the kinetics of fusion from junctions between cells 5/6 to cells 10/11 (Figure 3D).

All analyses were done using MATLAB (MathWorks). The m-files are available upon request.

Quantifying the Kinetics of Cell–Cell Fusion

As shown in Figure 3A, first we defined the junctions (blue lines) as circular arcs fitted through three points (red circles defined roughly along the dorsal midline and at the edges of the cells in the dorsal view) that were determined manually for each junction in each individual frame. This is based on the simple mathematical concept that a unique circle passes through every three noncollinear points. Thus, the arc defining each junction was a part of unique circle passing through manually defined points. Because it is impossible to assign fixed geometry to a live biological specimen, especially when monitored in real time, each frame had its own set of arcs characterizing the individual junctions of the embryo. Knowing the total number of pixels along each arc (junction), we measured the apical junction (AJ) discontinuity, representing expanding fusion pores, by looking at appearance of blank pixels (pixel value < threshold; see below), as shown in Figure 3, B and C, by yellow circles along the arcs (blue lines). Because of junctions in live embryos not following strict circular-arcs geometries, for each pixel coordinate along each arc we applied a normal sweep (depending on how good the “blue” arc described a junction by eye): a pixel is scored as blank (i.e., shown in yellow, Figure 3) only if 2–4 pixels above it and below it along the normal from the center of the circle corresponding to that arc and if the pixel value itself is lower than the threshold. Figure 3B shows the normal along a single point on the circle which is always perpendicular to the tangent at that point on the arc.

The threshold was uniformly selected for each junction for all frames as being 1–2 times the average intensity of the whole image, depending on the brightness of the junction (because all the junctions do not have the same GFP intensity). Figure 3C shows the “initiated macrofusion” represented by the yellow circles in absence of the blue line (junction). Supplementary Figure S1 shows four frames from a movie of a developing embryo in which yellow circles are seen to appear as the junctions “dissolve.”

Using our normal sweep method, we found that each fusion process for different junctions in different embryos grown at different temperatures follows sigmoidal kinetics (see examples in Figures 4 and Supplementary Figure S2). To extract the kinetic parameters of the curves we defined the fusion onset, i.e., the lag time \( t_I \), the reach to the \( t_E \) and the time required to reach \( t_T \) saturation. From these, the time required for the termination of the fusion event after the onset was determined as the macrofusion time \( (t_T - t_I) \). This kinetic parameterization for understanding sigmoidal curves was introduced and has been explained in detail (Mittal et al., 2003).

Cell Fusion Analysis (Manual Method)

We measured the length of the discontinuity of the AJ between individual pairs of fusing cells over time and obtained the fusion rate at different temperatures (\( n = 43 \) cell pairs). The simple kinetic analysis described above were done by manually measuring the estimated length of fusion zone cross section from time-lapse movies obtained by confocal microscopy using NIH Image software over time. The linear distances were plotted against time, and the slopes were then used to estimate the rates of disappearance of AJM-1::GFP staining. These rates varied from ~50 nm/min at 11°C to ~1000 nm/min at 25°C. The Arhenius plot obtained by this method gave a slope used to estimate an apparent activation energy of 29.8 kcal/mol (\( \text{e}^{2} \)).

Electron Microscopy

Transmission electron microscopy was performed as described on fourth larval stage and adult \( \text{eff-1(hy21ts50°) } \) mutant animals grown at the semipermissive temperature 20–25°C (Shemer et al., 2004). Two tests were performed to determine whether a candidate microfusion site is indeed a cytoplasmic bridge that may have resulted from an incomplete membrane fusion event. First, the specimen was tilted in the transmission electron microscope (TEM) by \( \pm 20° \) to sharpen up the view of the plasma membrane bordering an apparent cell bridge, further tilting by \( \pm 50° \) showed whether the cell bridge is real. That is to say, if even when the specimen was tilted to extreme angles, we did not find an intact plasma membrane blocking the bridge, this was recorded as a bona fide cytoplasmic bridge. The second test was based on reconstructions in serial sections as previously described (Nguyen et al., 1999). In many instances we found apparently good bridges that failed to meet these criteria.

RESULTS

Experimental Design To Study Syncytogenesi

To identify and study fusion intermediates during embryonic development and to uncover the kinetics of cell fusion in the hypodermis (epidermis) of C. elegans, we imaged transgenic embryos expressing AJM-1::green fluorescent protein and have an extensive collection of raw data including time-lapse movies of individual embryos. These records have been organized to allow a variety of analyses, which are described below.
protein (GFP) on the AJ of these epithelial cells (Knust and Bossinger, 2002). The dynamic behavior of AJs in *C. elegans* is a reliable assay for cell fusion of epithelial cells (Kenyon, 1986; Priess and Hirsh, 1986; Baird *et al.*, 1991; Podbilewicz and White, 1994; Mohler *et al.*, 1998, 2002; Nguyen *et al.*, 1999; Sharma-Kishore *et al.*, 1999; Alper and Kenyon, 2002).

To follow cell fusion by confocal microscopy, we recorded time lapse 4D movies and followed the disappearance of the AJM-1::GFP reporter from the AJ between fusing cells (Hird and White, 1993; Mohler *et al.*, 1998; Rabin and Podbilewicz, 2000; Koppen *et al.*, 2001; Figure 1, A and B, Supplementary Movies S1–S10). Loss of the cell contact zone upon fusion was also followed using immunofluorescence and immunoelectron microscopy with the mAb MH27 that recognizes both endogenous AJM-1 and transgenic AJM-1::GFP proteins (Francis and Waterston, 1991; Koppen *et al.*, 2001). In addition, cytoplasmic content mixing and membrane loss precede AJ disappearance (Mohler *et al.*, 1998, 2002; Shemer *et al.*, 2004), validating AJM-1::GFP loss as an assay for cell–cell fusion in *C. elegans*.

To quantitatively analyze cell fusion during elongation of *C. elegans* embryos, we determined the number of cell pairs that initiated fusion over time. We found a gradual increase in the accumulated number of cells with detectable disruption of AJ continuity. Figure 1C shows the time course for initiation of cell fusion events in a single elongating embryo incubated and imaged at 23°C (see also Figure 2 and Supplementary Movies). Embryonic elongation in *C. elegans* is characterized by defined stages starting with a comma shape (length; time 0 in Figure 2) stage through 1.5-, 2-, 3-, and 4-fold (length) stages of elongation (Sulston *et al.*, 1983; Priess and Hirsh, 1986). Most embryonic epidermal cell fusion events occur from comma to twofold stages (Podbilewicz and White, 1994).

**idf-1 Mutant and Low Temperatures Block Cell Fusion**

*idf-1*(*zu316*), an embryonic lethal mutant, was isolated in a screen for elongation defective embryos (Costa and Priess, personal communication). We found immunostained *idf-1*(*zu316*)-arrested embryos to have fewer dorsal fusions than in wild-type using the MH27 mAb. We genetically mapped...
The temperatures are average 19.9°C. Irregular dorsal fusions along with elongation are observed (Supplementary Movie S8). (E) Cells fused after the embryos began twitching and not before activation of the body wall muscle contraction (n > 100; Supplementary Movie S9). When comparing the dorsal cell fusion defects between different idf-1(−) arrested embryos we found that in the anterior dorsal hypodermis have a higher probability of remaining unfused than cells in the posterior hypodermis (Figures 1E, 2F, and 7A). However, all 21 dorsal epithelial cells that normally form the two major syncytia, hyp6 and hyp7 (Figure 1), are able to express the Idf phenotype (Figure 7A), suggesting that idf-1 activity is involved as part of the dorsal fusion machinery or in its regulation and not as a regional regulator of cell fusion affecting specific cells along the anterior-posterior axis.

Low temperatures have been used to stabilize and identify important steps in viral fusion (Stegmann et al., 1990; Schoch et al., 1992; Chernomordik et al., 1998; Melikyan et al., 2000). To determine the effects of temperature on embryonic epidermal cell fusion in AJM-1::GFP embryos, we incubated comma stage embryos (time = 0; Figure 2) at different temperatures and recorded the changes in dorsal epidermal AJs upon fusion. Neither cell fusion nor embryonic elongation was observed at temperatures below 8.5°C, even after incubation for 24 h (n = 11; Figure 2A). Wild-type embryos incubated between 10 and 25°C reached the twofold stage (halfway through elongation) with most of the dorsal cells fused (n > 100; Figure 2, C and D). We found that cells failed to fuse in wild-type embryos grown at 8.5–10°C; these animals reached the twofold stage of elongation and the muscles twitched demonstrating that the embryos with cells that fail to fuse have some physiological activities (n = 19; Figure 2B).

To compare the “frozen” fusion phenotype obtained at −9°C to the cell fusion defects obtained in eff-1 and idf-1 mutants, we imaged embryonic elongation in eff-1(hy21) mutant embryos expressing AJM-1::GFP. In eff-1(hy21) embryos, dorsal epidermal cells completely failed to fuse at 15 and 25°C (Figures 1D and 2E). Although eff-1(−) embryos elongate and remain dumpy (short and fat) with bulged tail, lumpy body, and other morphological defects maintained during postembryonic development (Mohler et al., 2002; Shemer, 2002), wild-type embryos elongating at −9°C irreversibly arrest at the twofold stage. It seems that elongation is not dependent upon cell fusion, because eff-1 blocks cell fusion but not elongation. It appears that other defects unrelated to epithelial fusion failure may be responsible for the embryonic arrest observed in “frozen” and idf-1(−) embryos (e.g., microtubule depolymerization in the cold).

In summary, we can block cell fusion in wild-type cells at −9°C partially phenocopying eff-1 and idf-1 mutant embryonic cells.

**Kinetics and Temperature Dependence of Cell Fusion In Vivo**

To study intermediates of cell fusion we initiated a kinetic approach in the epidermis of the embryo. For a detailed characterization of cell fusion kinetics, we developed a com-
puter-based method where cell fusion was measured by following the loss of AJ as the appearance of blank pixels in each junction and for each individual frame (see Materials and Methods; Figure 3 and Supplementary Figure S1). Using this semiautomated method, we found that each fusion process for six to nine distinct junctions, in embryos grown at different temperatures, follows sigmoidal kinetics ($n = 74$ cell pairs; Figures 4 and 5A and Supplementary Figure S2).

To extract the kinetic parameters of the curves we defined the delay time or lag ($t_1$) and the time required to reach the sigmoidal saturation ($t_2$). From these, the time required for the termination of the fusion event after the onset was determined and defined as the macrofusion time ($t_2 - t_1$).

This novel semiautomated method was validated by a different manual method to measure macrofusion (see Materials and Methods). Using the semiautomated method, we found that at the fusion-permissive temperatures the macrofusion rate [$k_{Macrofusion} = 1/(t_2 - t_1)$] for each cell pair increased with temperature (Figure 5B). We have analyzed 5–6 pairs of fusing cells in 10 different embryos grown from 13 to 25°C (Figure 4).

To quantify the temperature dependence of developmental cell fusion, we have used conventional Arrhenius plots in which the slope characterizes the apparent activation energy of the rate-limiting step of the process. To obtain an Arrhenius plot, we used the macrofusion rates from Figure 5B. The average macrofusion rates were calculated for each embryo (a total of 6 embryos, 32 junctions). The temperature dependence of the macrofusion rates over the range 13–25°C is linear in a semilog plot with a regression coefficient of $-0.91$; a similar linear slope was independently obtained using a different method to estimate the macrofusion rates over the range of 11–25°C (Figure 5C; see Materials and Methods).

To determine whether our kinetic analyses of cell fusion in vivo have a distinct behavior from other temperature-sensitive processes, we measured the rate of embryonic elongation at different temperatures using the same embryos where we measured the cell fusion rate. The initial rates of embryonic elongation changed from 1.3 nm/min at 8.2°C to 193.5 nm/min at 24°C (Rabin and Podbilewicz, 2000). The temperature dependencies of embryonic elongation have different slopes in different temperature ranges (Figure 5D). This is in contrast to a single apparent rate-limiting step for cell fusion in C. elegans embryonic hypodermis (Figure 5C), implying, along with the cold block of cell fusion (Figure 2B), that cell fusion and embryonic elongation are two distinct processes (see Discussion).

**Kinetic Studies Reveal at Least Three Steps of Cell Fusion**

Is fusion pore expansion (macrofusion) simply a continuation of the same processes that operate during the lag time?
If this were the case, then one would expect the lag time to directly correlate with the macrofusion time. To test this, we plotted macrofusion versus lag times for each junction. For all temperatures studied we found no correlation between lag and macrofusion time, indicating that these are independent kinetic steps (Figure 5F). Moreover, the Arrhenius plot of the lag rates gave a very weak trend (Figure 5E) compared with macrofusion rates (Figure 5C), supporting the interpretation that the lag and macrofusion stages are different mechanistic steps in the process. Because lag and macrofusion are distinct stages, we infer a new stage that occurs during lag time. This microfusion stage cannot be resolved using live confocal microscopy of AJ disappearance. Thus, based on kinetics we have been able to dissect cell fusion into two distinct steps: an early step of microfusion followed by a stage of expanding gap or macrofusion, which we actually measure in our assay.

In various membrane fusion studies, the lag time has proved to be a very significant measurement to investigate when the system is “ready” to fuse (Stegmann et al., 1990; Bron et al., 1993; Danieli et al., 1996; Munoz-Barroso et al., 1998; Parlati et al., 1999). To analyze the lag phase in our system, we pooled the lag time parameters into a cumulative distribution showing a fraction of events that had already occurred by a given time (Supplementary Figure S3). To explain this lag distribution, we investigated different linear kinetic models (see Supplementary Material). We found that the simplest model that fits the data includes two distinct steps. Taken together, this kinetic model and the finding that the Arrhenius plot for lag does not show a linear relationship as would be expected for a single rate-limiting step process (Figure 5E), implying that initiation of cell fusion in C. elegans is at least a two-step process.

In summary, kinetic dissection of cell fusion in C. elegans embryos shows that this is at least a three-step process: Two...
Figure 5. Kinetics of cell fusion events in C. elegans embryos. (A) Solid symbols show kinetics of a single dorsal fusion event measured using the Normal Sweep Method. Solid line shows a theoretical fit to the observed sigmoidal data (see Supplementary Material). To extract the kinetic parameters of the curves we defined lag time for fusion onset \( t_1 \) and time required to reach the sigmoidal saturation \( t_2 \). From these, Macrofusion (MF) time of the fusion event after onset was determined: \( t_2 - t_1 \); Temperature 23.1°C, junction 7/8, Supplementary Movie S6. (B) Temperature dependence of cell fusion for each junction (a color code is defined from black to red with increasing temperature; A–F). From the macrofusion times we obtained the rate constants, given by \( 1/(t_2 - t_1) \), for different junctions (numbers as described in Figure 1A). Junction 8/9 had the fastest macrofusion rate at all temperatures tested. (C) Arrhenius plot for macrofusion rates for junctions 5/6–10/11. Each point represents a single embryo, and error bars are due to multiple junctions. The apparent energy of activation was estimated from the logarithmic form of the Arrhenius equation: \( \ln(k) = \ln(A) - \frac{E*}{RT} \). \( k \) is the rate of the reaction, \( E* \) is the effective activation energy, \( T \) is the absolute temperature (in °K), \( A \) is a pre-exponential factor, and \( R \) is the gas constant (1.9872 calories/°K·mol). \( \ln(k) \) versus \( 1/T \) was used to estimate the apparent energy of activation of the reaction (\( E* \approx 30 \) Kcal/mol) from the slope of the Arrhenius plot. Green triangle, \( idf-1(-) \) at 19.9°C. Blue triangle, \( idf-1(-) \) at 16.8°C. (D) Arrhenius plot for elongation rates of embryos measured at different temperatures (open symbols for 9–13°C; Rabin and Podbilewicz, 2000). At higher temperatures, elongation does not appear to depend on temperature, whereas at lower temperatures, slow elongation has an apparent activation energy of \( \sim 109 \) Kcal/mol based on the slope of the fitted line shown (\( r^2 = 0.8269 \)). (E) Arrhenius plot of the lag does not show a clear linearity. This suggests that lags may involve multiple steps, rather than a single rate-limiting step. Green triangle, \( idf-1(-) \) at 19.9°C. Blue triangle, \( idf-1(-) \) at 16.8°C (with SD \( \approx 0.08 \), not shown). (F) Macrofusion times are plotted versus lag for each junction at each of the temperatures monitored. The lack of correlation indicates that there are at least two distinct mechanistic steps in the reaction: microfusion (\( \mu f \)), which precedes the onset of the first dorsal fusion detectable by our method, and Macrofusion (MF) that is measured by our normal sweep method. Black empty squares, wild-type embryo at 13.6°C. Other colors are for different junctions according to the temperature scale.

steps during the lag stage leading into microfusion and a third step for the actual gap expansion between cells (macrofusion) that results in syncytia formation.

**idf-1 and eff-1 Genetic Interactions during the Epidermal Cell Fusion Process**

To investigate whether \( idf-1 \) and \( eff-1 \) interact genetically, we constructed a strain to study the double mutant \( idf-1; eff-1 \) and compared the embryonic phenotypes of the single mutants with the double mutants at 15 and 20°C (see Materials and Methods). In wild-type embryos most dorsal and ventral fusions take place by the twofold stage of elongation. \( idf-1(-) \) embryos arrest with the characteristic Idf phenotype, namely, irregular dorsal fusions. \( eff-1(-) \) embryos elongate with neither dorsal nor ventral epithelial fusions, and double mutants \( idf-1(-); eff-1(-) \) arrest at the 1.5–3-fold stage of elongation without any cell fusion (Figure 6). At 15 and 20°C the phenotype of the double mutant is a combination of the individual Idf and Eff phenotypes (see Materials and Methods). The lethality associated with \( idf-1(-) \) may not be directly related to cell fusion defects, but rather, might represent an additional function for \( idf-1 \).

To test whether there are cell differentiation changes in \( idf-1(-) \) embryos, we have stained mutant embryos with different tissue-specific monoclonal antibodies (e.g., intestine, pharynx, body wall muscles, lateral epidermis) and we have not found differences with wild-type except for the fusion failure in the dorsal hypodermis. In addition, DIC analyses of \( idf-1(-) \) embryos showed a twisted tail defect different from the Eff-1 tail phenotype (Gattegno, 2003). To test whether \( idf-1 \) and \( eff-1 \) genes function redundantly to promote fusion, we constructed and tested \( hsp::eff-1; idf-1(-); ajm-1::gfp \) animals. We obtained the ectopic fusion phenotype in homozygous \( idf-1(-) \) embryos after heat-shock
Taken together, these results are consistent with *idf-1* and *eff-1* genes acting independently to positively control cell fusion. An alternative explanation is that *idf-1* positively controls *eff-1*.

**Comparative Kinetics of Cell Fusion between idf-1(−) and idf(+ ) Embryos**

To understand the kinetics of cell fusion, it would be useful to have mutations that affect the kinetic parameters. Even the weakest allele of *eff-1* has a complete failure in the initiation of embryonic cell fusion, so we could not use *eff-1* in kinetic analyses. However, in *idf-1(−)* embryos some dorsal epidermal cells are able to fuse several hours after the normal time of fusion during early embryonic elongation (Figure 7A). We imaged nearly 100 embryos and analyzed the kinetics of 10 fusion events from 2 independent embryos that were optimal for quantification using the semiautomated method. We found that these embryos arrested at the twofold stage with characteristic Idf phenotypes (Figure 1E) showing 10 pairs of dorsal cells fusing (Supplementary Movie S9 and Figure 2F). These cell fusions followed characteristic sigmoidal behaviors (see example in Supplementary Figure S2), showing longer lag and macrofusion times than in wild-type embryos imaged at the same temperatures (Figure 7, B and C, and green and blue triangles in Figure 5, C and E). Lag and macrofusion times in *idf(−)* embryos were significantly longer for the fusing pairs of cells than for wild-type (see Supplementary Material).

**Cell Fusion Pioneers Are Variable**

The end result of the cell fusion process is the formation of functional multinucleate cells (syncytia). To define whether there are pioneer junctions (cell pairs) that consistently fuse to form binucleate cells, followed by nonpioneer cells that undergo secondary fusion to binucleate syncytia, we identified 10 junctions that start the formation of binucleate cells...
in different embryos (n = 10). We define pioneer pairs as any two adjacent cells that initiate cell fusion to each other before starting fusion with other cells. We found between one to two pioneers per embryo in the area that we could analyze consisting of dorsal cells 5–11, precursors to hyp7 (see Figure 1A; n = 63 junctions). Only junction 2/3, between cells 2 and 3, was not found to act as a pioneer (n > 1000). Each of the four junctions 6/7–9/10 were pioneers with a similar frequency (0.2–0.3; n = 10). This shows that syncytial precursors of the dorsal hypodermis fuse stochastically (see Figure 4). For each pioneer junction we define anterior and posterior neighbors. The posterior cell fused the fastest (n = 3/10) compared with pioneer and posterior nonpioneer pairs (n = 3/10) or the slowest (n = 4/10) compared with pioneer and posterior nonpioneer pairs (n = 2/10), and in 7 of 10 cases it was the second fusion event in the syncytium. This last observation may explain the apparent anterior to posterior wave of fusion events (Podbielwicz and White, 1994; Mohler et al., 1998; Mohler et al., 2002).

In summary, most junctions in the dorsal hypodermis can initiate a syncytium with a pioneer fusion event. Additional nonpioneer cells fuse to the initial intermediate binucleate syncytium that expands into a giant cell. Our comparison between kinetic parameters of pioneers and nonpioneers indicates that fusion initiation at one side of the cell affects neither the lag time of microfusion nor the macrofusion rate at the other side of the same cell. Autonomous fusion for adjacent junctions argues against the hypothesis that the cell fusion pathway is driven by the lateral tension in the membrane bilayer that might be generated by osmotic effects or by cytoskeleton activity.

**Microfusion: Ultrastructural Intermediate of EFF-1–mediated Epidermal Cell Fusion**

TEM has been previously used to identify structural cell fusion intermediates in yeasts, worms, flies, and mammals (Kalderon and Gilula, 1979; Baron et al., 1986; Doberstein et al., 1997; Gammie et al., 1998; Mohler et al., 1998; Heiman and Walter, 2000). In *C. elegans*, the existence of a distinct stage of microfusion in the fusion pathway is independently supported by kinetic analyses (see above) and the phenotypes in myoepithelial cells observed by TEM in eff-1 conditional mutants that were grown at the semipermissive temperature where microfusion intermediates failed to expand (Shemer et al., 2004). We looked for similar intermediates in the hypodermis of the same fixed and sectioned specimens and found some initiated fusion events that failed to expand (Figures 8 and 9). We conclude that EFF-1 in the hypodermis is required both to initiate cell fusion but also to expand membrane gaps of 20–50 nm to complete macrofusion of around 20,000 nm.

**DISCUSSION**

**Dissection of Cell Membrane Fusion into Three Defined Stages in Developing Embryos**

Here, we have dissected the pathway of cell fusion during embryonic development of *C. elegans*. We developed a new system to simultaneously record, measure, and analyze individually fusing epidermal cells in live embryos. In contrast to studies on simpler fusion systems that investigate maximum pore sizes of a few nanometers (microfusion); here we measure the kinetics of large expanding gaps, of the order of hundreds of nanometers per micron (macrofusion), resulting from single cell–cell fusions critical for animal development. We have found that at these scales, each fusion event follows sigmoidal kinetics in wild-type and idf-1 mutant embryos. On the basis of the sigmoidal behavior, we define lag and macrofusion times as the kinetic parameters for each pair of fusing cells. We found that 9°C incubations block macrofusion but not embryonic elongation, and idf-1 mutations either block early cell fusion steps or slow down macrofusion rates. Moreover, here we found that in the epidermis of eff-1 mutants grown at the semipermissive temperature, there are stable microfusion intermediates similar to ultrastructural microfusions in eff-1–mediated muscle–muscle fusion (Shemer et al., 2004), strengthening the notion that during the lag phase there are two kinetic steps followed by expansion or macrofusion. It is conceivable that additional gene(s) may function in some cell fusion events in epidermal and myoepithelial cells of the pharynx, explaining the stable microfusions in eff-1 mutants (Shemer et al., 2004).

**Genes and Kinetic Behavior Characteristic of Developmental Cell Fusion**

In Figure 10 we propose a model for the cell fusion process in epithelial cells and hypothesize the roles of eff-1 and idf-1 in specific stages based on our findings. Epidermal cell fusion in the embryo is dependent on the activity of EFF-1 (Mohler et al., 2002). Expression of EFF-1 is enough to fuse cells in *C. elegans* and activated EFF-1 primes the system for cell fusion (Shemer et al., 2004; del Campo et al., 2005). We identified three steps in the cell fusion pathway. Two steps in the lag may involve activation of EFF-1 and the initiation of cell fusion or microfusion. Recently we found that expression of EFF-1 on the surface of insect SF9 cells is enough to fuse cells via hemifusion (Podbielwicz et al., 2006). The 9°C cell fusion block we observed in *C. elegans* embryos may be analogous to the 4°C block in influenza virus fusion that freezes the membranes in a hemifusion state (Chernomordik et al., 1998). The discontinuity in the plasma membranes has to expand and this expansion of the microfusion is what we measure in the cell macrofusion assay as the disappearance of the AJ. Although the early stages of membrane fusion are rapid (from fractions of a

**Figure 8.** TEM showing microfusion between hypodermal cells. Micrograph shows microfusion or gap of ~25-nm diameter between two partially fused cells (arrow). Four hypodermal cells (1–4) were pseudocolored to show the borders. For more details see Materials and Methods and Supplementary Figure S4. Bar, 0.1 μm.
second to 1 or 2 min; Kaplan et al., 1991; Plonsky and Zimmerberg, 1996; Mohler et al., 1998; del Campo et al., 2005), the complete disappearance of the membranes and the apical junctions associated to them is a temperature-dependent process that takes several minutes (Figure 4).

Although Arrhenius plot analysis is a convenient way to quantify the temperature-dependence of the process and to compare it with those of other processes, developmental cell fusion is undoubtedly a complex multistep process. The linearity of the Arrhenius plots suggests that the same process is rate limiting over the entire temperature range (Figure 5C). The slope of the plot suggests that this rate-limiting step of macrofusion has an apparent activation energy of 30 kcal/mol. The apparent activation energy of the macrofusion step, 30 kcal/mol, is close to apparent activation energies reported for viral fusion (34–42 kcal/mol), pore formation during exocytosis (23 kcal/mol), Ca²⁺-triggered exocytosis (27 kcal/mol), transferrin endocytosis (30–36 kcal/mol), and fusion of protein-free lipid bilayers (Iacopetta and Morgan, 1983; Clague et al., 1990; Oberhauser et al., 1992; Lee and Lentz, 1998; Earles et al., 2001). idf-1 may affect the lag and the macrofusion stages (Figure 5, C and E). Macrofusion may involve vesiculation of the plasma membrane surrounding the microfusion gap. This is supported by TEM studies of embryonic (Mohler et al., 1998)

Figure 9. Serial section TEMs of eff-1(hy21) animals with microfusions. (A, D, and G) Three serial sections show microfusion (arrows) between two hypodermal precursors in D and G. (B and C) Details of A show intact plasma membrane junctions. (E and F) Higher magnification of D show gap in the cell junctions between cells 1 and 4. (G) Partial fusion between cells 1 and 4. (H and I) Details of G with fusion intermediate. Arrowhead shows abnormal apical junction. a, apical junction; c, cuticle; 1–5, hypodermal cells; m, body wall muscles; e, excretory cell; arrow, microfusion; arrowhead, abnormal apical junction remnant. Bars, 1 μm (A, D, and G); 0.5 μm (B, C, F, H, and I).
cell fusion and intracellular membrane fusion a lot is known about the initial steps leading to fusion pore formation, relatively little is known about fusion pore expansion (Scepek et al., 1998; Dutch and Lamb, 2001; Haller et al., 2001; Gibbons et al., 2004; Jaiswal et al., 2004; Leikina et al., 2004; Nolan et al., 2006).

In summary, independent kinetic, genetic, and ultrastructural studies on cell fusion are consistent with at least three steps in the membrane fusion pathway in C. elegans, with eff-1 acting in early local fusion (microfusion) and late expansion (macrofusion) steps of the pathway.

**Fusion Events Do Not Typically Occur Symmetrically**

We have developed a sensitive and unique paradigm for studying kinetics of cell fusion in living embryos of C. elegans and show that it can be used as a model to analyze how molecules identified in genetic screens for cell fusion defective mutants affect specific steps in the dynamic process of cell fusion. Indeed, in a screen for mutants defective in embryonic morphogenesis (Costa and Priess personal communication; Costa et al., 1998) idf-1(izu316) was identified, and here we show how this mutant gene slows down two distinct kinetic steps of cell fusion in vivo. Unexpectedly, idf-1 mutants affect some cell–cell fusions in a different manner, arbitrarily. Some cell pairs have a complete block, whereas others have a retarded initiation followed by a slow execution. This differential kinetic behavior of cell fusion in the same embryo may reveal intrinsic differences between cells and variability in the trigger of cell fusion in a developing tissue. One explanation for these results is that low density of active EFF-1 on the fusion sites may be sufficient to initiate pore formation but not for their expansion as shown for influenza virus fusion (Kozlov and Chernomordik, 2002; Chernomordik and Kozlov, 2003; Leikina et al., 2004). Alternatively, other genes may also be required to act along with EFF-1 to complete fusion. It is surprising that the arrest phenotype of the idf-1 mutant phenocopies the cold fusion block (9°C). idf-1 has a role in dorsal epithelial fusion and additional essential roles probably unrelated to cell fusion. Future TEM of idf-1–arrested embryos compared with the 9°C block, together with physiological tests to follow lipid and content mixing between fusing cells, should give us a better understanding of the intermediates in cell fusion and the specific roles of EFF-1 and IDF-1 in the process.

**Membrane Domains Fuse Autonomous and Asymmetrically.** Early studies on epithelial cell fusion in C. elegans embryos suggested that there is a variable program in the sequence in which 23 syncytial precursor cells fuse to form the hyp7 syncytium. These studies were based on immunofluorescence of hundreds of fixed specimens at different stages in morphogenesis and reconstruction of the pathways of cell fusion during syncytogenesis (Podbilewicz and White, 1994). More recently, using GFP reporter genes and membrane markers it was possible to follow syncytogenesis of individual embryos in real time (Mohler et al., 1998, 2002; Shemer et al., 2004; del Campo et al., 2005). Taken together these studies showed that the final position, number, and identity of the cells that fuse is invariant during development though the fusion sequence is variable between individuals. In addition, cytoplasmic content mixing followed using GFP reporters is completed in 2–3 min, whereas the complete rearrangement of the plasma membranes and apical junctions takes about 40 min at 23°C (Mohler et al., 1998; Shemer et al., 2004; del Campo et al., 2005).

Here, for all fusion events in wild-type and the idf-1 mutant embryos, we observed sigmoidal kinetics of fusion.
and measured the characteristic parameters: Lag (microfusion) and macrofusion times. Each cell pair fuses with characteristic macrofusion rate in embryos monitored at different temperatures. It appears that the anterior membrane domain of a fusion competent cell fuses or fails to fuse independently of the posterior plasma membrane domain of the same cell. Thus, the anterior or posterior end can fuse faster that the opposite end of the cell, but which end fuses faster is random. Although the lateral membranes are fusion incompetent during embryogenesis in the wild-type, the anterior and posterior membranes develop their fusion competence autonomously and without any apparent symmetry. This control of the fusion competence of specific cells and membrane domains can be overruled by ectopic activity of EFF-1 in fusion-incompetent cells (Shemer et al., 2004). Ectopic expression of eff-1 followed by abnormal tissue-specific cell fusion can also be the result of inactivation of Engrailed/ceh-16-dependent transcriptional repression of eff-1 in lateral seam cells (Cassata et al., 2005), inactivation of vacuolar ATPase in the lateral hypodermis (Kontani et al., 2005), or inactivation of lin-39/Deformed repression of eff-1 in the ventral vulval precursor cells (Shemer and Podbilewicz, 2002).

**Founder Cell Fusion Event Is Variable.** Most dorsal precursor cells of the hyp7 syncytium, except one structural junction, are competent to be pioneers or founder cells. Macrofusion and microfusion rates are not correlated with pioneer and nonpioneer cells having similar probabilities to fuse with the fastest or slowest macrofusion rates. These findings show that there is randomness in the initiation and completion of a genetically programmed sequence of cell fusion events. Localization of EFF-1 in the cell–cell contact zone above a certain threshold may explain these apparently stochastic events (del Campo et al., 2005). The concept of a stochastic epidermal founder cell in *C. elegans* described here is analogous to the founder myoblasts and fusion-competent myoblasts hypothesis in *Drosophila* (Rushton et al., 1995; Abmayr et al., 2003; Chen et al., 2003; Englund et al., 2003). However, our definition of the founder cell is strictly kinetic with respect to the first detectable cell fusion event that occurs after cell fate determination, migration, recognition, adhesion, differentiation, and patterning of the epidermis. In contrast, in the muscles of *Drosophila*, founder cells are pioneers for myogenesis that differ from fusion-competent myoblasts primarily by distinct phenotypes of mutations and differential expression of molecular markers required for recognition, signaling, adhesion, patterning, differentiation, and fusion competence. In contrast to myoblast fusion in *Drosophila*, in the epidermises of *C. elegans*, tightly regulated homotypic expression of EFF-1 initiates and expands cell fusion (Podbilewicz et al., 2003).

In summary, here we have used a new system to study the molecular and cellular mechanisms of cell membrane fusion in developing animals and showed how mutations, temperature blocks, ultrastructural, and kinetic analyses reveal that the first cell fusion event is variable and that membrane fusion events have independent and asymmetric anteroposterior kinetics. Moreover, eff-1 activity is required at early and late stages of the process of epidermal syncytogenesis.

**ACKNOWLEDGMENTS**

We thank M. Costa and J. Priess for kindly providing idf-1(zu316); J. Simkove for lin-1:γf; C. Ramos, E. Leikina, H. Delamoe, E. Zaitseva, M. Glickman, J. Zimmerberg, Y. Rabin, S. Hess, P. Blank, I. Kolotuev, N. Assaf, L. Broday, and M. Suisa for discussions; M. Krause, S. Vogel, M. Kozlov, Y. Graenbaum, K. Melikov, S. Joshua, and G. Shemer for critically reading the manuscript. This work was supported by grants from Israel Science Foundation, The Charles H. Revson Foundation, Binational Science Foundation, Fund for the Promotion of Research at the Technion, and Human Frontier Science Program to B.P. and by the Intramural Research Program of the National Institute of Child Health and Human Development, National Institutes of Health.

**REFERENCES**


Supplementary material

Supplementary Materials and Methods

Strains, plasmids and transgenic animals

N2 Bristol strain, the wild type strain (Brenner, 1974)
SU93 jcIs1 [ajm-1:: GFP, pRF4 rol-6(su1006)] IV (Koppen et al., 2001)
BP8 lon-2(e678)/idf-1(zu316)lon-2(e678) X
BP13 idf-1(zu316) lon-2(e678)/+; tra-2(q276)/+ II (for crosses)
BP26 idf-1(zu316)/+ X, jcIs1 [ajm-1:: GFP, pRF4] IV
BP75 eff-1 hy21 II (Mohler et al., 2002)
BP76 eff-1 hy21 II; jcIs1 [ajm-1:: GFP, pRF4] IV (Mohler et al., 2002)
BP43 idf-1(zu316) dpy-3(e27)/unc-1(e719) X; tra-2(q276)/+ II
BP36 idf-1(zu316) dpy-3(e27)/+ X; eff-1 hy21 II; jcIs1 [ajm-1:: GFP, pRF4] IV
BP28 idf-1(zu316)/ dpy-3(e27)unc-2(e55)X : jcIs1 [ajm-1:: GFP, pRF4] IV
BP45 idf-1(zu316)/ dpy-3(e27)/unc-1(e719) X; tra-2(q276)/+ II; jcIs1 [ajm-1:: GFP, pRF4] IV
JK987 tra-2(q276)/mnC1 dpy-10(e128) unc-52(e444) II (Hodgkin et al., 1994).
BP13 was constructed by crossing BP8 hermaphrodites to tra-2(q276) males.
BP43 was constructed by crossing CB538 unc-1(e538)/unc-1(e538) X to dpy-3(e27) idf-1(zu316)/++ X; tra-2(q276)/tra-2(q276) II (males), that were cloned from the following cross: dpy-3(e27) idf-1(zu316)/dpy-3(e27) hermaphrodites were crossed to tra-2(q276)/tra-2(q276) males.
BP26 was constructed by crossing BP8 hermaphrodites with SU93 roller males, cloning idf-1 heterozygous rollers at the first generation and looked for animals homozygous for the roller phenotype and heterozygous for idf-1.
BP36 was constructed by crossing BP76 hermaphrodites with BP43 tra-2 males. At the first generation idf-1(zu316) dpy-3(e27)/+ + X; eff-1 hy21 / tra-2(q276) II heterozygous were cloned and BP36 animals were cloned according to their tail and progeny.

After counting self-progeny of BP36 animals the following phenotypes were identified:
Dead idf-1;eff-1 homozygous embryos (n=206) out of 758 animals counted (15°C).
Dead idf-1;eff-1 homozygous embryos (n=176) out of 795 animals counted (20°C).

More than 50 dead idf-1;eff-1 homozygous embryos with eff-1 like fusion pattern were analyzed by confocal microscopy. Thus, the phenotype of the double mutant idf-1;eff-1 is embryonic lethality characteristic of Idf phenotype and Eff cell fusion defective phenotype with all epidermal cells unfused.

pME1 hsp16-2::eff-1 was constructed as described (Shemer et al., 2004)
BP204 Ex[hsp16-2::eff-1;rol-6(su1006d)]; jcIs1 [ajm-1:: GFP, pRF4] IV; idf-1(zu316)/+ X.
**Kinetic modeling**

All the embryos were imaged from a well defined developmental stage called the comma stage (Sulston et al., 1983). However, in absence of our knowledge about how different temperatures and mutations may affect the comma stage by itself, we needed to define a rigorous “t=0” for each embryo’s proper kinetic evaluation of the data. Note that in an ideal case scenario, as that in studies on the influenza virus hemagglutinin (HA), t=0 is defined by the time of lowering the pH required for HA mediated fusion. In a much more complex fusion in *C. elegans*, we defined t=0 as the lag time of fusion initiation for the very first observable junction to fuse (i.e. out of the 6 junctions we monitored) for each embryo. By subtracting this time from the lag time of all the subsequent events, we defined “normalized lag” for each of the events that was used to uniformly quantify and compare the kinetics of different junctions in different embryos. Normalized lag is shown only for kinetic modeling in Figure S3.

In Figure S3 the time distribution of all normalized lag times of μf from all wild type embryos is presented as a cumulative distribution i.e., the percentage of the junctions with μf initiated by given time normalized by the total number of all junctions that will eventually fuse is plotted as a function of time. The number of the microfused junctions observed prior to any given time normalized by the total number of the fusion events observed in the experiment is plotted as a function of time. The Y-axis does not start from “zero”, since by definition of normalized lag (see above) one event has already occurred. By 35 min, when μf is initiated in all fusing junctions, the curve reaches 1. Statistical analysis shows that this distribution might be considered as a single normal distribution (see text and Table S2). The fact that the data from independent embryos follows a single normal distribution allows each point on the distribution to be treated as an independent fusion event with the same mechanism as all others. Therefore each point in the distribution might be interpreted as the probability for a given cell pair to initiate fusion at a particular time. For example, 50% (fraction=0.5) of the fusion events were initiated in about 13 min.

Cumulative distribution of normalized lag times were analyzed by an “exhaustive fitting” approach (Bentz, 2000; Mittal and Bentz, 2001; Mittal et al., 2002) using several different mass-action based linear kinetic models (first order single step, n-order single step, n-order 2 step, and a single order 2 step). The “minimalistic” kinetic model that could fit the lag data is:

\[ X \xrightarrow{k_1} Y \xrightarrow{k_2} \mu f \]

μf refers to local fusion, which would be the kinetic step when 2 dorsal cells have initiated fusion. Out of a parameter space of more than 12000 combinations, a little over 4000 best-fits were obtained (Mittal and Bentz, 2001; Mittal et al., 2002). Ranges of the rate constants and parameters from the best fits obtained are \( k_1 = 5.0 \times 10^7 - 6.9 \times 10^8 \text{(min}^{-1}) \), \( k_2 = 1.7 \times 10^8 - 7.0 \times 10^2 \) (min\(^{-1}\)), \( X_0 = 10 - 3.1 \times 10^7 \), \( Y_0 = 1.3 \times 10^4 - 1.0 \times 10^6 \). \( X_0 \) and \( Y_0 \) represent the concentrations (units can be in either molarity or #/µm\(^2\)) of species X and Y in the model. As seen in Figure S3 single step models do not describe the sigmoidal character of our cumulative distribution. Without better understanding of the specific mechanism of
microfusion (µf), the only conclusion we can draw from this analysis is that µf is preceded by at least two distinct kinetic steps.

**Reproducibility of kinetic parameters and statistical analysis**

To test the reproducibility of our kinetic analyses we analyzed cell fusion kinetics in two sets of three wild-type embryos grown at optimal growth conditions. For three embryos grown at average temperatures of 23.3 ± 0.5°C we analyzed a total of n=23 junctions and the average lag obtained was 25.7 ± 10.7 min while the average macrofusion (MF) time was 23.5 ± 12.5 min (±SD). For an independent group of three embryos grown at 20.2 ± 0.5°C (n=19 junctions), we obtained a lag of 22.5 ± 9.3 min and a MF time of 31.8 ± 17.7 min. When we analyzed 10 cell-cell fusion events for idf-1 mutant, MF and microfusion (µf) stages were affected at 20 and 16.8°C (Figures 5 and 7). Unpaired 2-tailed type 3 t-tests show that the pooled lag times for idf-1 vs. wild-type (both at 20 ± 0.5°C) belong to the same underlying distributions with same means (p ~ 0.048 > 0.01). On the other hand, the same t-tests done with MF times for the mutant vs. wild-type give a value of p ~ 0.00008 << 0.01 showing that the MF time data for the mutant belong to a different underlying distribution than the wild-type. The kinetic parameters at 16.8°C show that the idf-1 mutation affects both the µf and MF kinetic stages (compare blue circles with blue triangles in Figures 5C and 5E).

**Supplemental Tables**

**Table S1.** Summary of movies. The movies were generated in wild-type or mutant embryos incubated at the temperatures indicated. The interval between each frame was between 2 to 15 min and the total duration of the time-lapse recording was between 80 to 900 minutes (Rabin and Podbilewicz, 2000). All temperatures are average ± 0.5°C.

**Table S2.** Statistical analysis of kinetic data for the wild type embryos. Data for lag and MF times was pooled for different junctions from different embryos and analyzed as accumulated events resulting in the cumulative distribution function (cdf). An example of this is shown in Figure S3 (see text for details). Whether data from a cdf is due to a similar mechanism (with each point on the cdf signifying the probability of committing to a process via that mechanism) depends on whether the cdf can be explained by a single distribution or multiple distributions (Mittal et al., 2003). To test this in the simplest way, we did Kolmogorov-Smirnov (KS) tests for normality. The KS tests would check whether various cdfs of lag and MF times could be explained by a single normal distribution with the same mean and standard deviation as each respective cdf. We did the KS tests with a confidence interval of 99%. For each distribution, there are standard “Dmax” (KS statistical parameter) values provided for cdfs with different number of data points (Sokal and Rohlf, 1994), which has to be compared to the “Dcrit” value, for a normal (Gaussian) distribution with the same mean and standard deviation, calculated for the cdf at α= 0.01. If Dmax < Dcrit, the cdf can be explained by a single normal distribution and if Dmax ≥ Dcrit, the cdf is a result of multiple normal distributions. From the results shown in the table, cdfs at 16-25°C are
explained by single normal distributions, for both lag and MF times, signifying the same fusion mechanism. This is also supported by the similar phenotypes of embryos at these temperatures (all junctions fuse in these embryos: see Figure 2 and suppl. movies). On the other hand, 13°C affects the kinetics since pooling this data with 16-25°C now is not explained by a single normal distribution. We see that statistical analysis of kinetic results agrees with phenotypic results (e.g. embryo at 13°C shows only partial fusions, i.e. not all dorsal cells fuse by two-fold stage). When the phenotypes of embryos are the same, we find evidence for the same mechanism (single distribution). When phenotypes of embryos are different, we find evidence for multiple mechanisms in either of the two kinetic parameters.

**Supplemental Figure legends**

**Figure S1.** Progression of fusion in the embryo shown in Figure 3. Note that yellow circles appear in different frames in different junctions, i.e. all the junctions are not fusing simultaneously. Scale bars, 10 µm.

**Figure S2.** Kinetics of fusion events shows sigmoidal behavior. In representative examples each panel shows a junction from different embryos (see corresponding movies also). Solid symbols are the fraction of fused junction determined by Eq. 1 given in the Materials and Methods. The solid lines show the fits used to parameterize the sigmoidal curves. Note that time scales are different for different embryos. For measuring the kinetic parameters (see Figure 5A), extrapolation of the sigmoidal data is required to find the time to reach saturation. From the curves shown here, it can be seen that different degrees of extrapolations are required to find the sigmoidal saturation. This reflects the differences in clarity of data obtained, since for some embryos in spite of fusion leading to disappearance of junctions, excessive image noise results in our inability to quantify further using the normal sweep method.

**Figure S3.** Kinetic modeling of lag and macrofusion times. Cumulative distribution of normalized lag times might be interpreted as the probability that a cell pair has already initiated µf by any given time. The pooled lag time data from independent embryos above 16°C follows a single distribution allowing each point on the distribution to be treated as an independent fusion event with the same mechanism as all others. Dashed and solid lines represent the best fitting of this distribution obtained by an “exhaustive fitting” approach for single-step and two-step kinetic models, respectively. While neither of single-step models fits the redistribution, the specific interpretation of the two-step models that fit the lag time data cannot be clarified without further detailed genetic and biochemical work.

**Figure S4.** Detail of original TEM showing a microfusion between hypodermal cells 1 and 4 (h1 and h4). Bar, 100 nm.

**Supplementary Movies**
Movies S1-S10. Representative of a few hundred time-lapse confocal movies obtained at minimum laser intensity as described in the Materials and Methods (Rabin and Podbilewicz, 2000). The technical details of each movie are summarized in Table S1.

Supplementary References


<table>
<thead>
<tr>
<th>Movie File Name</th>
<th>Genotype (wt/Mutant)</th>
<th>Temperature (°C)</th>
<th>Time between Each Frame (min)</th>
<th>Total Movie Duration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Wt</td>
<td>8.2</td>
<td>15</td>
<td>600</td>
</tr>
<tr>
<td>S2</td>
<td>Wt</td>
<td>9.8</td>
<td>15</td>
<td>900</td>
</tr>
<tr>
<td>S3</td>
<td>Wt</td>
<td>13.6</td>
<td>10</td>
<td>300</td>
</tr>
<tr>
<td>S4</td>
<td>Wt</td>
<td>16.8</td>
<td>3</td>
<td>90</td>
</tr>
<tr>
<td>S5</td>
<td>Wt</td>
<td>20.5</td>
<td>2</td>
<td>90</td>
</tr>
<tr>
<td>S6</td>
<td>Wt</td>
<td>23.1</td>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>S7</td>
<td>Wt</td>
<td>23.7</td>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>S8</td>
<td>Wt</td>
<td>25.1</td>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>S9</td>
<td>idf-1(zu316)</td>
<td>19.9</td>
<td>5</td>
<td>250</td>
</tr>
<tr>
<td>S10</td>
<td>eff-1(hy21)</td>
<td>24.9</td>
<td>2</td>
<td>80</td>
</tr>
</tbody>
</table>

Gattegno et al. Table S1
Gattegno et al. Fig. S2
<table>
<thead>
<tr>
<th>Lag cdf</th>
<th>Temperature</th>
<th>16 - 25°C</th>
<th>13 - 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>(D_{\text{max}})</td>
<td></td>
<td>0.12523</td>
<td>0.17980</td>
</tr>
<tr>
<td>(D_{\text{crit}} (\alpha = 0.01))</td>
<td></td>
<td>0.18506</td>
<td>0.17494</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Macrofusion cdf</th>
<th>Temperature</th>
<th>16 - 25°C</th>
<th>13 - 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>(D_{\text{max}})</td>
<td></td>
<td>0.11470</td>
<td>0.23367</td>
</tr>
<tr>
<td>(D_{\text{crit}} (\alpha = 0.01))</td>
<td></td>
<td>0.1715</td>
<td>0.16297</td>
</tr>
</tbody>
</table>
Fraction of Accumulated Events vs. Normalized Lag (min)

Gattegno et al., Fig. S3

$X \rightarrow Y \rightarrow \mu_f$

$X \rightarrow \mu_f$