Genetic Control of Fusion Pore Expansion in the Epidermis of *Caenorhabditis elegans*[□] [▽]

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Developmental cell fusion is found in germlines, muscles, bones, placenta, and stem cells. In *Caenorhabditis elegans* 300 somatic cells fuse during development. Although there is extensive information on the early intermediates of viral-induced and intracellular membrane fusion, little is known about late stages in membrane fusion. To dissect the pathway of cell fusion in *C. elegans* embryos, we use genetic and kinetic analyses using live-confocal and electron microscopy. We simultaneously monitor the rates of multiple cell fusions in developing embryos and find kinetically distinct stages of initiation and completion of membrane fusion in the epidermis. The stages of cell fusion are differentially blocked or retarded in *eff-1* and *idf-1* mutants. We generate kinetic cell fusion maps for embryos grown at different temperatures. Different sides of the same cell differ in their fusogenicity: the left and right membrane domains are fusion-incompetent, whereas the anterior and posterior membrane domains fuse with autonomous kinetics in embryos. All but one cell pair can initiate the formation of the largest syncytium. The first cell fusion does not trigger a wave of orderly fusions in either direction. Ultrastructural studies show that epidermal syncytiogenesis require *eff-1* activities to initiate and expand membrane merger.

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; Wassarman *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; Witze and Rothman, 2002). Cell fusion functions to sculpt organs and to accomplish defined body shapes (Sharma-Kishore *et al.*, 1999; Witze 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, acquirement of fusion competence,

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Abbreviations used: AJ, apical junction; *eff-1*, epithelial fusion failure-1; *idf-1*, irregular dorsal fusion-1; TEM, transmission electron microscopy.

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 have not been reported in other developmental cell fusion reactions outside syncytin-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; Bonifacio and Glick, 2004; McInerney *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×/1.40 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/acetone 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 semi-automated normal sweep method, we could quantify the membrane fusion for each junction as follows:

Fraction of fused junctions = Fraction of blank pixels

$$= \frac{\text{Number of blank pixels}}{\text{Total pixels along the arc}}$$

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 beyond 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 monitoring it 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-arc 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 arc, 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_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 as the macrofusion time ($t_2 - t_1$). 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 analyses 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 a straight line was fitted. 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 Arrhenius plot obtained by this method gave a slope used to estimate an apparent activation energy of 29.8 kcal/mol ($r^2 = 0.9474$).

Electron Microscopy

Transmission electron microscopy was performed as described on fourth larval stage and adult *eff-1(hy21ts)* mutant animals grown at the semipermissive temperature 20–23°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 $\pm 10^\circ$ to sharpen up the view of the plasma membrane bordering an apparent cell bridge, further tilting by $\pm 50^\circ$ 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 Syncytiogenesis

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

Figure 1. Fusion between dorsal epithelial cells and elongation in *C. elegans* embryos. (A) Cylindrical projection of comma stage embryo before elongation and cell fusion. The dorsal epithelial cells are numbered so apical junctions (AJs) are readily identified. For example, junction 7/8 refers to AJ between cells 7 and 8. Red and yellow: dorsal cells (a–d) will fuse to form hyp6 syncytium (orange). Blue: dorsal cells (1–17) that will form hyp7 syncytium (light blue). Black circles, nuclei. (A, B, D, and E) Anterior is to the top of page. (B) A wild-type embryo elongates to the twofold stage and hypodermal cells fuse. (C) Kinetics of multiple cell fusion events and embryonic elongation. The number of accumulated fusion events in the dorsal hypodermis (●) and the increase in body length (□) for the same wild-type embryo are shown. Developing embryo expressing AJM-1::GFP was recorded at 23.1°C. A fusion event is defined as appearance of detectable AJ discontinuity. (D) Wild-type embryo (~9°C) or *eff-1*(–) (15–25°C) with epidermal fusion failure. (E) *idf-1(zu316)* embryo (20°C) arrested at twofold stage of elongation with irregular dorsal fusions (Idf). Only some junctions fuse (light blue). Note that head and tail are not shown in cylindrical projections.

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 (~50-μm length; time = 0 in Figure 2) stage through 1.5-, 2-, 3-, and 4-fold (~200-μm 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

Figure 2. In vivo cell fusion monitored by staining of AJs using AJM-1::GFP. Wild-type (A–D); *eff-1(hy21)* (E); and *idf-1(zu316)* mutant (F). The numbers on left corners of each image show time in minutes after the comma stage ($t = 0$). (A) At 8.2°C both dorsal fusions and elongation of the embryo are blocked (see Supplementary Movie S1). (B) At 9.8°C dorsal fusions are blocked but elongation is observed (Supplementary Movie S2). (C) At 13.6°C some dorsal fusions are observed along with embryo elongation (Supplementary Movie S3). (D) At 25.1°C normal dorsal fusions are observed along with embryonic elongation. This is the phenotype that results in optimal embryonic development (Supplementary Movie S8). (E) *eff-1(-)* at 24.9°C. No dorsal fusion with normal embryonic elongation is observed (Supplementary Movie S10). (F) *idf-1(-)* at 19.9°C. Irregular dorsal fusions along with elongation are observed (Supplementary Movie S9). The temperatures are average $\pm 0.5^\circ\text{C}$. Eggs are $\sim 50\ \mu\text{m}$ long. (A–C and E) Lateral views; dorsal is left. (D and F) Dorsolateral views; anterior is up. Arrows, unfused junctions; brackets, fused regions.

idf-1(zu316) to the left arm of chromosome X and found that *idf-1* acts recessively. Mutant embryos arrest between the 1.5–3-fold stage of elongation with fewer cell fusions in the dorsal hypodermal cells and posterior defects (Figure 1E; $n > 1000$). To test whether *idf-1(zu316)* is a hypomorph or a knockout, we constructed an *idf-1* over deficiency (deletion) strain and found that the arrested *idf-1(zu316)/deficiency* embryos arrested earlier than the homozygous *idf-1(zu316)* embryos (data not shown). Thus, *idf-1(zu316)* is a hypomorph. The molecular identity of *idf-1* has not been determined. Irregular cell fusion events occurring in *idf-1* mutants were retarded compared with the timing of expected fusion events in wild type. *idf-1(-)* 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 cells 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 $\sim 9^\circ\text{C}$ 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 $\sim 9^\circ\text{C}$ 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 $\sim 9^\circ\text{C}$ 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-

Figure 3. Semiautomated normal sweep method to follow cell fusion in vivo. (A) Each junction is defined by an arc shown by the blue line, passing through three manually determined points (in red) in the dorsal view for each frame. The points need to be defined manually because the embryos twitch, move, and elongate, leading to deviations in locations of the same junction in different frames. (B) We look at 2–4 pixels above and below each point on the arc, along the normal drawn from the center of the circle to that particular point on the arc, to quantify presence or absence of fusion as described in experimental procedures section. (C) The progression of fusion is shown by drawing yellow circles that correspond to each point on the arc where the pixels are scored as blank (see Supplementary Material). (D) Junctions 5/6–10/11 in the dorsal view of an embryo. Note that one of the junctions above 5/6 is already partially fused (see arrow) in this embryo. (E) Disappearance of junctions 5/6–10/11 due to fusion. (F) Modeling the embryo junctions of interest as arcs. (G) Quantifying progression of fusion. Note the excessive image noise toward the anterior part of the embryo (above junctions 5/6). This image noise was observed in most of the embryos we imaged. Therefore, for consistent and proper comparison between different embryos we compared the fusion between junctions 5/6–10/11. Bars, 10 μm .

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 [$k_{\text{Macrofusion}} = 1/(t_2 - t_1)$] 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?

Figure 4. Kinetic map of dorsal cell fusions in developing *C. elegans* embryos. The curves shown are sigmoidal fits to the kinetic data for the fusing junctions in different wild-type embryos grown and imaged at different temperatures (see Supplementary Movies and Supplementary Table S1). Supplementary Movies: S4, 16.8°C; S5, 20.5°C; S6, 23.1°C; S7, 23.7°C; and S8, 25.1°C. This figure highlights that using the semiautomated method we simultaneously characterize single cell–cell fusion events for multiple junctions of live embryos. Each panel represents a single embryo and the curves represent the “kinetic map” of hypodermal cell fusions.

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: $\ln(k) = \ln A - E^*/(RT)$. 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 ($E^* = \sim 30$ 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 ~ 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 = 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 (μf), 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

Figure 6. Genetic interaction between *idf-1* and *eff-1* mutants. Confocal projections of representative wild-type, single, and double mutant embryos grown at 15°C. (A) Wild-type embryo, most epidermal fusions have occurred in the hypodermis. (B) *eff-1(hy21)* embryo with all junctions unfused. (C) *idf-1*-arrested embryo with some unfused dorsal cells. (D) Double mutant arrested with a round shape; the animal was moving and all hypodermis is unfused. Dorsal partial projections of the embryos are shown. Anterior is to the left. Arrowheads, unfused junctions. (A–C) MH27 immunofluorescence. (D) AJM-1::GFP background. Egg length, ~50 μ m.

Figure 7. Characterization of the cell fusion defective phenotypes in *idf-1(zu316)* mutant. (A) Frequencies of dorsal hypodermal unfused junctions in *idf-1(-)* homozygous embryos ($n = 64$; ■) compared with wild type embryos ($n = 15$; □) at similar elongation stages at 15°C: 1.88 ± 0.14 - and 1.89 ± 0.22 -fold elongation for wild type and *idf-1(zu316)*, respectively. Cells a–d are part of hyp6; cells 1–17 are part of hyp7 (Figure 1A). There is high variability in the dorsal unfused pattern, although the occurrence of unfused junctions is higher at the anterior part of hyp7; these results may imply that *idf-1* is crucially required for proper fusion in these cells. (B and C) Kinetics of cell fusion in *idf-1* embryos is defective as seen for both the lag and macrofusion times. Note that our single event kinetic measurements were done on an *idf-1* embryo for which 5/6 did not fuse (■). These embryos were imaged at $20 \pm 0.5^\circ\text{C}$. Wild-type embryos were triplicates (□).

($n = 14/84$). 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-1(+)* 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-1(-)* 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

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 μ m.

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 neighboring nonpioneer junctions that fuse later. Pioneers can have the fastest ($n = 3/10$) or the slowest ($n = 5/10$) macrofusion rates compared with their nonpioneer anterior and posterior neighbors. The anterior cell that fuses to a pioneer syncytium was the fastest ($n = 3/10$) or slowest ($n = 3/10$) compared with pioneer and posterior nonpioneer cells. The posterior cell fused the fastest ($n = 4/10$) or slowest ($n = 2/10$), 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 (Podbilewicz 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 yeast, 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 (Podbilewicz *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 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, E, F, H, and I).

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 macro-

fusion 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), Ca^{2+} -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 10. Working model for dorsal epithelial cell-cell fusion in developing *C. elegans* embryos. Kinetic modeling has shown a three-step kinetic process. The initial state has two cells adhering to each other. There is a transition from the initial state to a state where EFF-1 is “activated.” The activated protein(s) mediate microfusion. The kinetically defined lag time comprises two steps culminating in microfusion. Microfusion (μf) is transformed into Macrofusion (MF). Because no macrofusion is observed for the embryos at 9°C, this temperature may affect either, or both, of the first two steps. The *idf-1* mutants show variable fusion events in embryos, apparently with both lag and macrofusion steps affected. Statistical analysis suggests that *idf-1* mutants are affected in the lag and macrofusion steps (see Supplementary Material).

and postembryonic (Nguyen *et al.*, 1999) epidermal cells that have revealed the appearance of vesicles in the gap between fusing cells, suggesting that vesiculation after initial microfusion may be a critical step in syncytia formation and morphogenesis (Podbilewicz, 2000, 2006; Podbilewicz and Chernomordik, 2005).

Actin contraction may be the rate-limiting step for the actin-mediated elongation of *C. elegans* embryos (Priess and Hirsh, 1986; Costa *et al.*, 1998; McKeown *et al.*, 1998). Morphogenesis is strongly temperature dependent only between 8 and 15°C with an apparent activation energy of 110 kcal/mol (Figure 5D), similar to the apparent activation energy of F-actin movement on rabbit skeletal myosin (Anson, 1992). At temperatures in the range of 15–25°C, the rate of elongation is weakly temperature-dependent with an estimated apparent activation energy of 1.5 kcal/mol and an average elongation rate of 160 ± 23 nm/min. In contrast to elongation, the Arrhenius plots for the macrofusion step were linear at 13–25°C using the semiautomated method and at 11–25°C using the manual method, indicating that within this temperature range cell fusion in *C. elegans* has the same rate-limiting step.

Using stringent criteria including careful tiltings and reconstructions in serial sections (*Materials and Methods*), we found that microfusion events in *eff-1* mutants do occur between epidermal cells that fail to complete syncytia formation. In multiple cases we were able to distinguish places where the membrane just twists into a new plane of section (detectable when tilting the TEM grid) from true microfusion sites where fields of cytoplasmic ribosomes span the gap across the cell border. Thus, although the unfused cells bear irregular and convoluted common borders not found in wild-type animals, we can confirm multiple microfusions that reveal another function for EFF-1 in the expansion from microfusion to complete fusion. There appear to be multiple chances for such microfusions to occur between the same hypodermal cells as their common borders are very extensive. Structurally similar microfusions have been identified in cells infected with human cytomegalovirus and measles virus (Firsching *et al.*, 1999; Gerna *et al.*, 2000; Ehrenguber *et al.*, 2002). It is believed that viral induced microfusions may be involved in viral spreading. In addition, stable microfusion-like structures have been described between fibroblasts and cardiomyocytes (Driesen *et al.*, 2005). Although in virus-

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(zu316)* 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 epidermal 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 Autonomously and Asymmetrically. Early studies on epidermal 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 syncytiogenesis (Podbilewicz and White, 1994). More recently, using GFP reporter genes and membrane markers it was possible to follow syncytiogenesis 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 than 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 epidermis of *C. elegans*, tightly regulated homotypic expression of EFF-1 initiates and expands cell fusion (Podbilewicz *et al.*, 2006).

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 syncytiogenesis.

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REFERENCES

- Abmayr, S. M., Balagopalan, L., Galletta, B. J., and Hong, S. J. (2003). Cell and molecular biology of myoblast fusion. *Int. Rev. Cytol.* 225, 33–89.
- Alper, S., and Kenyon, C. (2001). REF-1, a protein with two bHLH domains, alters the pattern of cell fusion in *C. elegans* by regulating Hox protein activity. *Development* 128, 1793–1804.
- Alper, S., and Kenyon, C. (2002). The zinc finger protein REF-2 functions with the Hox genes to inhibit cell fusion in the ventral epidermis of *C. elegans*. *Development* 129, 3335–3348.
- Anson, M. (1992). Temperature dependence and Arrhenius activation energy of F-actin velocity generated in vitro by skeletal myosin. *J. Mol. Biol.* 224, 1029–1038.
- Baird, S. E., Fitch, D. A., Kassem, I. A. A., and Emmons, S. W. (1991). Pattern formation in the nematode epidermis: determination of the arrangement of peripheral sense organs in the *C. elegans* male tail. *Development* 113, 515–526.
- Baron, R., Neff, L., Van, P. T., Nefussi, J., and Vignery, A. (1986). Kinetic and cytochemical identification of osteoclast precursors and their differentiation into multinucleated osteoclasts. *Am. J. Pathol.* 122, 363–378.
- Blumenthal, R., Clague, M. J., Durell, S. R., and Epand, R. M. (2003). Membrane fusion. *Chem. Rev.* 103, 53–69.
- Bonifacio, J. S., and Glick, B. S. (2004). The mechanisms of vesicle budding and fusion. *Cell* 116, 153–166.
- Bron, R., Wahlberg, J. M., Garoff, H., and Wilschut, J. (1993). Membrane fusion of Semliki Forest virus in a model system: correlation between fusion kinetics and structural changes in the envelope glycoprotein. *EMBO J.* 12, 693–701.
- Cassata, G., Shemer, G., Morandi, P., Donhauser, R., Podbilewicz, B., and Baumeister, R. (2005). *ceh-16/engrailed* patterns the embryonic epidermis of *Caenorhabditis elegans*. *Development* 132, 739–749.
- Ch'ng, Q., and Kenyon, C. (1999). *egl-27* generates anteroposterior patterns of cell fusion in *C. elegans* by regulating *Hox* gene expression and *Hox* protein function. *Development* 126, 3303–3312.
- Chen, E. H., and Olson, E. N. (2005). Unveiling the mechanisms of cell-cell fusion. *Science* 308, 369–373.
- Chen, E. H., Pryce, B. A., Tzeng, J. A., Gonzalez, G. A., and Olson, E. N. (2003). Control of myoblast fusion by a guanine nucleotide exchange factor, *loner*, and its effector ARF6. *Cell* 114, 751–762.
- Chen, Z., and Han, M. (2001). *C. elegans* Rb, NuRD, and Ras regulate *lin-39*-mediated cell fusion during vulval fate specification. *Curr. Biol.* 11, 1874–1879.
- Chernomordik, L. V., Frolov, V. A., Leikina, E., Bronk, P., and Zimmerberg, J. (1998). The pathway of membrane fusion catalyzed by influenza hemagglutinin: restriction of lipids, hemifusion, and lipidic fusion pore formation. *J. Cell Biol.* 140, 1369–1382.
- Chernomordik, L. V., and Kozlov, M. M. (2003). Protein-lipid interplay in fusion and fission of biological membranes. *Annu. Rev. Biochem.* 72, 175–207.
- Clague, M. J., Schoch, C., Zech, L., and Blumenthal, R. (1990). Gating kinetics of pH-activated membrane fusion of vesicular stomatitis virus with cells: stopped-flow measurements by dequenching of octadecylrhodamine fluorescence. *Biochemistry* 29, 1303–1308.
- Costa, M., Raich, W., Agbunag, C., Leung, B., Hardin, J., and Priess, J. R. (1998). A putative catenin-cadherin system mediates morphogenesis of the *Caenorhabditis elegans* embryo. *J. Cell Biol.* 141, 297–308.
- Danieli, T., Pelletier, S. L., Henis, Y. I., and White, J. M. (1996). Membrane fusion mediated by the influenza virus hemagglutinin requires the concerted action of at least three hemagglutinin trimers. *J. Cell Biol.* 133, 559–569.
- del Campo, J. J., Opoku-Serebuoh, E., Isaacson, A. B., Scranton, V. L., Tucker, M., Han, M., and Mohler, W. A. (2005). Fusogenic activity of EFF-1 is regulated via dynamic localization in fusing somatic cells of *C. elegans*. *Curr. Biol.* 15, 413–423.
- Doberstein, S. K., Fetter, R. D., Mehta, A. Y., and Goodman, C. S. (1997). Genetic analysis of myoblast fusion: *blown fuse* is required for progression beyond the prefusion complex. *J. Cell Biol.* 136, 1249–1261.
- Driesen, R. B., Dispersyn, G. D., Verheyen, F. K., van den Eijnde, S. M., Hofstra, L., Thone, F., Dijkstra, P., Debie, W., Borgers, M., and Ramaekers, F. C. (2005). Partial cell fusion: a newly recognized type of communication

- between dedifferentiating cardiomyocytes and fibroblasts. *Cardiovasc. Res.* 68, 37–46.
- Dutch, R. E., and Lamb, R. A. (2001). Deletion of the cytoplasmic tail of the fusion protein of the paramyxovirus simian virus 5 affects fusion pore enlargement. *J. Virol.* 75, 5363–5369.
- Earles, C. A., Bai, J., Wang, P., and Chapman, E. R. (2001). The tandem C2 domains of synaptotagmin contain redundant Ca²⁺ binding sites that cooperate to engage t-SNAREs and trigger exocytosis. *J. Cell Biol.* 154, 1117–1123.
- Ehrensgruber, M. U., Ehler, E., Billeter, M. A., and Naim, H. Y. (2002). Measles virus spreads in rat hippocampal neurons by cell-to-cell contact and in a polarized fashion. *J. Virol.* 76, 5720–5728.
- Englund, C., Loren, C. E., Grabbe, C., Varshney, G. K., Deleuil, F., Hallberg, B., and Palmer, R. H. (2003). Jeb signals through the Alk receptor tyrosine kinase to drive visceral muscle fusion. *Nature* 425, 512–516.
- Firsching, R., Buchholz, C. J., Schneider, U., Cattaneo, R., ter Meulen, V., and Schneider-Schaulies, J. (1999). Measles virus spread by cell-cell contacts: uncoupling of contact-mediated receptor (CD46) downregulation from virus uptake. *J. Virol.* 73, 5265–5273.
- Francis, G. R., and Waterston, R. H. (1991). Muscle cell attachment in *Caenorhabditis elegans*. *J. Cell Biol.* 114, 465–479.
- Frey, S., Marsh, M., Gunther, S., Pelchen-Matthews, A., Stephens, P., Ortlepp, S., and Stegmann, T. (1995). Temperature dependence of cell-cell fusion induced by the envelope glycoprotein of human immunodeficiency virus type 1. *J. Virol.* 69, 1462–1472.
- Gammie, A. E., Brizzio, V., and Rose, M. D. (1998). Distinct morphological phenotypes of cell fusion mutants. *Mol. Biol. Cell* 9, 1395–1410.
- Gattegno, T. (2003). Isolation and characterization of cell fusion mutants in *C. elegans*. Ph.D. thesis, Haifa: Technion-Israel Institute of Technology.
- Gerna, G., Percivalle, E., Baldanti, F., Sozzani, S., Lanzarini, P., Genini, E., Lillieri, D., and Revello, M. G. (2000). Human cytomegalovirus replicates abortively in polymorphonuclear leukocytes after transfer from infected endothelial cells via transient microfusion events. *J. Virol.* 74, 5629–5638.
- Gibbons, D., Erk, I., Reilly, B., Navaza, J., Kielian, M., Rey, F., and Lepault, J. (2003). Visualization of the target-membrane-inserted fusion protein of Semliki Forest Virus by combined electron microscopy and crystallography. *Cell* 114, 573–583.
- Gibbons, D. L., Vaney, M. C., Roussel, A., Vigouroux, A., Reilly, B., Lepault, J., Kielian, M., and Rey, F. A. (2004). Conformational change and protein-protein interactions of the fusion protein of Semliki Forest virus. *Nature* 427, 320–325.
- Haller, T., Dietl, P., Pfaller, K., Frick, M., Mair, N., Paulmichl, M., Hess, M. W., Furst, J., and Maly, K. (2001). Fusion pore expansion is a slow, discontinuous, and Ca²⁺-dependent process regulating secretion from alveolar type II cells. *J. Cell Biol.* 155, 279–289.
- Heiman, M. G., and Walter, P. (2000). Prm1p, a pheromone-regulated multi-spanning membrane protein, facilitates plasma membrane fusion during yeast mating. *J. Cell Biol.* 151, 719–730.
- Hird, S. N., and White, J. G. (1993). Cortical and cytoplasmic flow polarity in early embryonic cells of *Caenorhabditis elegans*. *J. Cell Biol.* 121, 1343–1355.
- Hoekstra, D., Martin, I., Kahya, N., Ruysschaert, J. M., and Pecheur, E. (2002). On the mechanism of protein-induced membrane fusion: from model to biological membranes. *Cell. Mol. Biol. Lett.* 7, 231–232.
- Hu, C., Ahmed, M., Melia, T. J., Sollner, T. H., Mayer, T., and Rothman, J. E. (2003). Fusion of cells by flipped SNAREs. *Science* 300, 1745–1749.
- Iacopetta, B. J., and Morgan, E. H. (1983). The kinetics of transferrin endocytosis and iron uptake from transferrin in rabbit reticulocytes. *J. Biol. Chem.* 258, 9108–9115.
- Jahn, R., Lang, T., and Sudhof, T. C. (2003). Membrane fusion. *Cell* 112, 519–533.
- Jaiswal, J. K., Chakrabarti, S., Andrews, N. W., and Simon, S. M. (2004). Synaptotagmin VII restricts fusion pore expansion during lysosomal exocytosis. *PLoS Biol.* 2, E233.
- Kalderon, N., and Gilula, N. B. (1979). Membrane events involved in myoblast fusion. *J. Cell Biol.* 81, 411–425.
- Kaplan, D., Zimmerberg, J., Puri, A., Sarkar, D. P., and Blumenthal, R. (1991). Single cell fusion events induced by influenza hemagglutinin: studies with rapid-flow, quantitative fluorescence microscopy. *Exp. Cell Res.* 195, 137–144.
- Kenyon, C. (1986). A gene involved in the development of the posterior body region of *C. elegans*. *Cell* 46, 477–487.
- Knust, E., and Bossinger, O. (2002). Composition and formation of intercellular junctions in epithelial cells. *Science* 298, 1955–1959.
- Koh, K., Peyrot, S. M., Wood, C. G., Wagmaster, J. A., Maduro, M. F., Eisenmann, D. M., and Rothman, J. H. (2002). Cell fates and fusion in the *C. elegans* vulval primordium are regulated by the EGL-18 and the ELT-6 GATA factors—apparent direct targets of the LIN-39 Hox protein. *Development* 129, 5171–5180.
- Koh, K., and Rothman, J. H. (2001). ELT-5 and ELT-6 are required continuously to regulate epidermal seam cell differentiation and cell fusion in *C. elegans*. *Development* 128, 2867–2880.
- Kontani, K., Moskowitz, I. P., and Rothman, J. H. (2005). Repression of cell-cell fusion by components of the *C. elegans* vacuolar ATPase complex. *Dev. Cell* 8, 787–794.
- Koppen, M., Simske, J. S., Sims, P. A., Firestein, B. L., Hall, D. H., Radice, A. D., Rongo, C., and Hardin, J. D. (2001). Cooperative regulation of AJM-1 controls junctional integrity in *Caenorhabditis elegans* epithelia. *Nat. Cell Biol.* 3, 983–991.
- Kozlov, M. M., and Chernomordik, L. V. (2002). The protein coat in membrane fusion: lessons from fission. *Traffic* 3, 256–267.
- Lee, J., and Lentz, B. R. (1998). Secretory and viral fusion may share mechanistic events with fusion between curved lipid bilayers. *Proc. Natl. Acad. Sci. USA* 95, 9274–9279.
- Leikina, E., Mittal, A., Cho, M. S., Melikov, K., Kozlov, M. M., and Chernomordik, L. V. (2004). Influenza hemagglutinins outside of the contact zone are necessary for fusion pore expansion. *J. Biol. Chem.* 279, 26526–26532.
- McInerney, G. M., Smit, J. M., Liljestrom, P., and Wilschut, J. (2004). Semliki Forest virus produced in the absence of the 6K protein has an altered spike structure as revealed by decreased membrane fusion capacity. *Virology* 325, 200–206.
- McKeown, C., Praitis, V., and Austin, J. (1998). sma-1 encodes a betaH-spectrin homolog required for *Caenorhabditis elegans* morphogenesis. *Development* 125, 2087–2098.
- Melikyan, G. B., Markosyan, R. M., Hemmati, H., Delmedico, M. K., Lambert, D. M., and Cohen, F. S. (2000). Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. *J. Cell Biol.* 151, 413–423.
- Mi, S. et al. (2000). Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature* 403, 785–789.
- Mittal, A., Leikina, E., Chernomordik, L. V., and Bentz, J. (2003). Kinetically differentiating influenza hemagglutinin fusion and hemifusion machines. *Biophys. J.* 85, 1713–1724.
- Mohler, W. A., Shemer, G., del Campo, J., Valansi, C., Opoku-Serebuoh, E., Scranton, V., Assaf, N., White, J. G., and Podbilewicz, B. (2002). The type I membrane protein EFF-1 is essential for developmental cell fusion in *C. elegans*. *Dev. Cell* 2, 355–362.
- Mohler, W. A., Simske, J. S., Williams-Masson, E. M., Hardin, J. D., and White, J. G. (1998). Dynamics and ultrastructure of developmental cell fusions in the *Caenorhabditis elegans* hypodermis. *Curr. Biol.* 8, 1087–1090.
- Munoz-Barroso, I., Durell, S., Sakaguchi, K., Appella, E., and Blumenthal, R. (1998). Dilation of the human immunodeficiency virus-1 envelope glycoprotein fusion pore revealed by the inhibitory action of a synthetic peptide from gp41. *J. Cell Biol.* 140, 315–323.
- Nguyen, C. Q., Hall, D. H., Yang, Y., and Fitch, D. H. A. (1999). Morphogenesis of the *Caenorhabditis elegans* male tail tip. *Dev. Biol.* 207, 86–106.
- Nilsson, L., Li, X., Tiensuu, T., Auty, R., Greenwald, I., and Tuck, S. (1998). *Caenorhabditis elegans* lin-25: cellular focus, protein expression and requirement for *sur-2* during induction of vulval fates. *Development* 125, 4809–4819.
- Nolan, S., Cowan, A. E., Koppel, D. E., Jin, H., and Grote, E. (2006). FUS1 regulates the opening and expansion of fusion pores between mating yeast. *Mol. Biol. Cell* 17, 2439–2450.
- Oberhauser, A. F., Monck, J. R., and Fernandez, J. M. (1992). Events leading to the opening and closing of the exocytotic fusion pore have markedly different temperature dependencies. *Biophys. J.* 61, 800–809.
- Parlati, F., Weber, T., McNew, J. A., Westermann, B., Sollner, T. H., and Rothman, J. E. (1999). Rapid and efficient fusion of phospholipid vesicles by the alpha-helical core of a SNARE complex in the absence of an N-terminal regulatory domain. *Proc. Natl. Acad. Sci. USA* 96, 12565–12570.
- Phalen, T., and Kielian, M. (1991). Cholesterol is required for infection by semliki forest virus. *J. Cell Biol.* 112, 615–623.
- Plonsky, I., and Zimmerberg, J. (1996). The initial fusion pore induced by baculovirus GP64 is large and forms quickly. *J. Cell Biol.* 135, 1831–1839.
- Podbilewicz, B. (2000). Membrane fusion as a morphogenetic force in nematode development. *Nematology* 2, 99–111.

- Podbilewicz, B. (2006). Cell fusion. In: WormBook, ed. The *C. elegans* Research Community: WormBook, doi/10.1895/wormbook.1.52.1, <http://www.wormbook.org>.
- Podbilewicz, B., and Chernomordik, L. V. (2005). Cell fusion in development and disease. In: Protein-Lipids Interactions, ed. L. K. Tamm, New York: Wiley-VCH, 221–244.
- 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.
- Podbilewicz, B., and White, J. G. (1994). Cell fusions in the developing epithelia of *C. elegans*. *Dev. Biol.* 161, 408–424.
- Priess, J. R., and Hirsh, D. I. (1986). *Caenorhabditis elegans* morphogenesis: The role of cytoskeleton in elongation of the embryo. *Dev. Biol.* 117, 156–173.
- Rabin, Y., and Podbilewicz, B. (2000). Temperature-controlled microscopy for imaging living cells: apparatus, thermal analysis, and temperature dependency of embryonic elongation in *C. elegans*. *J. Microsc.* 199(Pt 3), 214–223.
- Rushton, E., Drysdale, R., Abmayr, S. M., Michelson, A. M., and Bate, M. (1995). Mutations in a novel gene, myoblast city, provide evidence in support of the founder cell hypothesis for *Drosophila* muscle development. *Development* 121, 1979–1988.
- Scepek, S., Coorssen, J. R., and Lindau, M. (1998). Fusion pore expansion in horse eosinophils is modulated by Ca²⁺ and protein kinase C via distinct mechanisms. *EMBO J.* 17, 4340–4345.
- Schoch, C., Blumenthal, R., and Clague, M. J. (1992). A long-lived state for influenza virus-erythrocyte complexes committed to fusion at neutral pH. *FEBS Lett.* 311, 221–225.
- Sharma-Kishore, R., White, J. G., Southgate, E., and Podbilewicz, B. (1999). Formation of the vulva in *C. elegans*: a paradigm for organogenesis. *Development* 126, 691–699.
- Shemer, G. (2002). Cell fusion and organogenesis in *Caenorhabditis elegans*. Ph.D. thesis, Haifa: Technion-Israel Institute for Technology.
- Shemer, G., Kishore, R., and Podbilewicz, B. (2000). Ring formation drives invagination of the vulva in *C. elegans*: Ras, cell fusion and cell migration determine structural fates. *Dev. Biol.* 221, 233–248.
- Shemer, G., and Podbilewicz, B. (2002). LIN-39/Hox triggers cell division and represses EFF-1/Fusogen-dependent vulval cell fusion. *Genes Dev.* 16, 3136–3141.
- Shemer, G., and Podbilewicz, B. (2003). The story of cell fusion: big lessons from little worms. *BioEssays* 25, 672–682.
- Shemer, G., Suissa, M., Kolotuev, I., Nguyen, K.C.Q., Hall, D. H., and Podbilewicz, B. (2004). EFF-1 is sufficient to initiate and execute tissue-specific cell fusion in *C. elegans*. *Curr. Biol.* 14, 1587–1591.
- Stegmann, T., White, J. M., and Helenius, A. (1990). Intermediates in Influenza induced membrane fusion. *EMBO J.* 9, 4231–4241.
- Sulston, J. E., Schierenberg, E., White, J. G., and Thomson, J. N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* 100, 64–119.
- Wassarman, P. M., Jovine, L., and Litscher, E. S. (2001). A profile of fertilization in mammals. *Nat. Cell Biol.* 3, E59–E64.
- Witze, E., and Rothman, J. H. (2002). Cell Fusion: an Efficient sculptor. *Curr. Biol.* 12, R467–R469.
- Zhao, X., Yang, Y., Fitch, D. A., and Herman, M. E. (2002). TLP-1 is an asymmetric cell fate determinant that responds to Wnt signals and controls male tail tip morphogenesis. *Development* 129, 1497–1508.