10
Cell Fusion in Development and Disease

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10.1
Introduction

Cell fusion is a key stage of many fundamental developmental processes. In humans cell fusion is essential in fertilization, placentation, myogenesis and osteogenesis. Here we discuss different developmental fusions with special emphasis on muscle and epithelial fusion in *Drosophila* and in *Caenorhabditis elegans*. We then describe the emerging understanding of membrane fusion in simpler fusion reactions, highlighting cell fusion mediated by viral glycoproteins. While developmental cell fusion is an example of fusion in normal cell physiology, viral fusion represents an example of a fusion-based pathological process. In the last section we discuss lessons from the developmental cell fusion field to viral membrane fusion and vice versa. First, we analyze approaches to separate expression of a candidate developmental cell fusion protein from its activation (if any) at the time of fusion. Second, we review approaches to block developmental fusion at different stages and to uncouple local fusion events from subsequent stages of fusion pore expansion. Third, we discuss the importance of dividing the role of cell adhesion mechanisms from the machinery that mediates actual fusion.

10.2
Developmental Cell Fusion for Health

While most cells in a multicellular organism will not fuse during normal development, cell fusion plays a central function as part of the differentiation of specific cells at determined times during development. Currently, for most cell fusion events it is not known what makes a particular cell membrane fusogenic, while most cell membranes will never fuse. This is in contrast to the highly fusogenic intracellular membranes that are constantly fusing during exocytic and endocytic transport events.

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From fertilization to organogenesis, cell fusion has important developmental functions. Little is known about the molecular and cellular mechanisms that prevent most cells from fusing, and how membrane fusion is triggered, executed and completed in certain cells. Healthy individuals require normal sperm–egg fusion to procreate and defective fertilization may identify the proteins required during sperm–egg fusion. In addition, lack of homotypic fusion between myoblasts, pre-osteoclasts or trophoblasts will certainly result in lethality or major diseases. However, human diseases where any cell fusion is clearly defective have not been characterized, probably due to early embryonic lethality or severe placentation defects. Candidate diseases where cell fusion may be compromised include infertility, muscle diseases with small or immature muscle fibers, cancers with giant multinucleated cells, preclampsia and placental implantation defects.

While many cell adhesion molecules have been implicated in binding between gametes of opposite sexes from yeast to vertebrates [1–7], there is little evidence for these molecules being responsible for post-binding membrane merger activities. Members of the ADAM family of type I membrane glycoproteins with metalloprotease and disintegrin domains have been considered as candidate fusion proteins (fusogens or fusases) for sperm–egg and myoblast–myoblast fusion in vertebrates [8–11]. However, homologs in invertebrates do not appear to participate in cell fusion [12–18] and recent studies in vertebrates have not demonstrated a direct role for ADAM proteins in cell membrane merger [19–23]. Recently, tetraspanin surface receptors have been implicated in sperm–egg interactions [24–26]; however, studies in phagocytes suggest that CD9 and CD81 tetraspanins may prevent and not promote cell fusion [27]. Other adhesion membrane glycoproteins, including CD47 and CD44, have been implicated in cell–cell interactions required for mononuclear pre-osteoclasts binding and cell fusion in bones [28–31]. Here, we will further discuss and compare the process of cell fusion in the formation of muscles and epithelial organs in vertebrates and invertebrates.

10.2.1 Muscles

10.2.1.1 **Vertebrates**

During embryonic development stem cells differentiate into muscle cells that will form muscle fibers. These muscle fibers cannot proliferate, so the number of fibers is determined embryonically in vertebrates. In the first stage of muscle development, embryonic muscle cells fuse to form primary fibers. In the second stage of fetal development, several secondary fibers grow on each primary fiber [32]. Muscle fibers are formed by the fusion of myoblasts and each muscle fiber is a syncytial cell containing thousands of nuclei. In humans, each skeletal muscle contains many long and tubular muscle fibers. Each muscle fiber ranges in size from only about 1 mm to a few centimeters in length and their diameters vary from ten to a few hundred micrometers. While muscle fibers cannot

divide, in times of muscle growth and increased protein synthesis, additional nuclei are provided by cell fusion of muscle satellite cells formed during embryogenesis. Thus, post-embryonic cell fusion appears to be a strategy to increase the size of differentiated muscle fibers that cannot proliferate and this process is believed to occur during hypertrophy (e.g. exercise), in degenerative muscular diseases and in injuries [32].

Many studies in vertebrate muscle cells in tissue culture have identified numerous adhesion molecules, proteases, kinases, phospholipases, channels, growth factors and signaling molecules implicated in myoblast fusion [9, 33–43]. It remains to be determined whether any of these *in vitro* studies are applicable to *in vivo* model systems [44–46].

10.2.1.2 *Drosophila*

In the fruit fly, muscle formation can be studied *in vivo* and many mutations that affect myoblast fusion have been identified [44, 46–49]. The muscle fibers in *Drosophila* are smaller syncytial cells that contain fewer nuclei than in vertebrates, and its development takes hours compared to days and weeks in vertebrates [46]. Based on elegant molecular genetic studies combined with ultrastructural analyses of wild-type and mutants in *Drosophila* (Fig. 10.1), researchers have proposed a path-

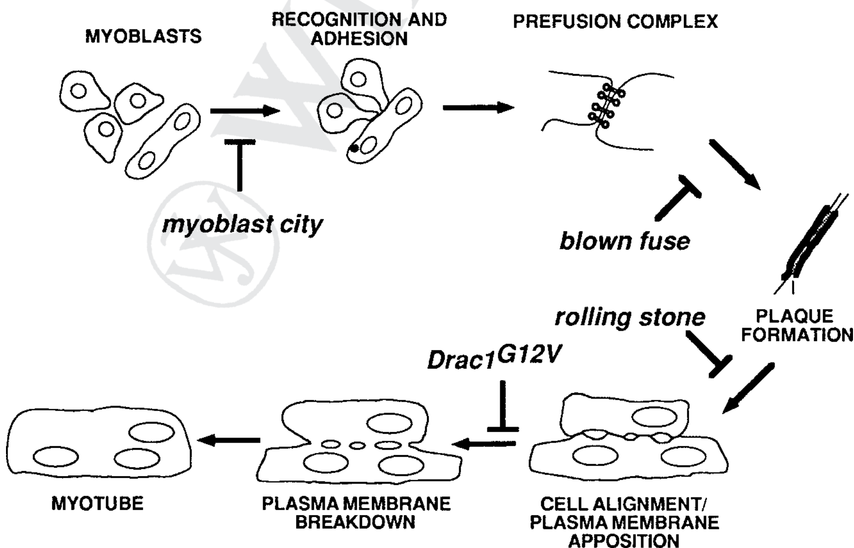

Fig. 10.1 Model of intermediate steps in myoblast fusion in *Drosophila*. Proposed schematic of the steps of myoblast fusion at the ultrastructural level, indicating the action point of each mutant. (Reprinted from [50] © 1997, with permission from Rockefeller University Press).

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way that includes recognition, adhesion and transduction of putative fusion signals from the cell surface to the cytoskeleton [46, 50, 51].

Myoblast Fate Determination, Attraction, Recognition and Adhesion Two populations of myoblasts have been characterized in *Drosophila*: muscle founder/pioneer cells and fusion-competent cells [44–46, 52]. Determination of the fate of the fusion-competent cells and the founder cells depends on the activities of transcription factors and cell–cell interactions [44–46, 52]. Founder cells expressing specific identity or selector genes attract fusion-competent cells using membrane receptors containing immunoglobulin superfamily (IgSF) domains [53, 54]. In turn, fusion-competent cells expressing different IgSF receptors [55–57] migrate extending filopodia, recognize and adhere to the founder cell forming the contact zone (CZ) [50]. It has been shown that cell–cell recognition and adhesion is mediated by specific IgSF receptors [47, 57]. Interestingly, formation of multinucleated osteoclasts in vertebrates, involves transmembrane glycoproteins that also belong to the IgSF [29].

Vesicle Pairs in a Pre-fusion Complex The next step in myogenesis is the formation of a characteristic pre-fusion complex containing pairs of vesicles of around 40 nm diameter containing electron dense "coats" (Fig. 10.2 A–C). These pairs of coated vesicles appear symmetrically aligned along the juxtaposed cell membranes [50].

Myoblast city, a cytoplasmic protein with Src homology (SH3), Crk and Docker domains and homologous to the vertebrate Dock180 and the *C. elegans* protein CED-5 required for phagocytosis of cell corpses, appears to have a function in this stage [44, 58]. Other activities that are required in the early pre-fusion stages involve the intracellular protein Antisocial/Rolling pebbles expressed in the founder cell and containing a lipolytic domain, ATP- and GTP-binding sites, ankyrin repeats, and coiled-coil domains [59, 60].

Formation of an Electron-dense Plaque or Junctional Complex The next morphological intermediate appears to be the formation of a 10-nm thick and around 500-nm long electron-dense plaque extending along the cytoplasmic face of the apposed plasma membranes in the CZ with diffuse electron-dense material also present in the extracellular space (Figs. 10.1 and 10.2 D) [50]. It is not known what the molecular components of this electron-dense plaque are, but it is tempting to speculate that the IgSF membrane receptors, other adhesion molecules and cytoskeletal components may be associated to these structures.

This step requires the activity of Blown fuse, a cytoplasmic protein with a plexin homology (PH) domain [50]. The involvement of the intracellular proteins Blown fuse and Antisocial in the pre-fusion complex formation as well as other cytoskeletal (D-Titin and Paramyosin) and signal transduction components such as Loner, a founder cell-specific ADP-ribosylation factor GDP-exchange protein (ARF-GEP) and three different GTPases (Drac1, Drac2 and dARF6) suggests that there are signal transduction pathways linked to myoblast fusion [51, 61]. In particular, it ap-

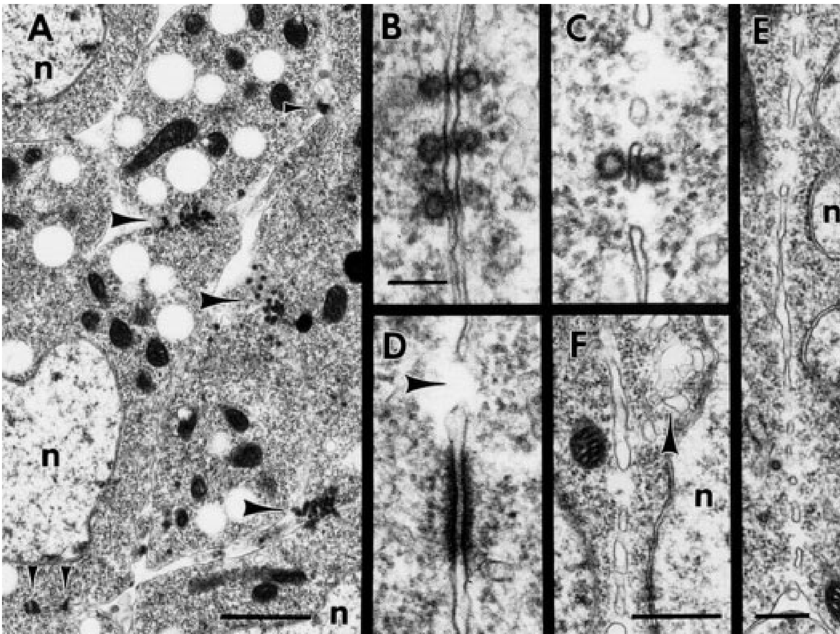

Fig. 10.2 Ultrastructure of intermediate steps in myoblast fusion in *Drosophila*. Electron micrographs of wild-type myoblast fusion in early stage 13 embryos. All the stages of the fusion process occur simultaneously in various parts of the developing musculature. (A) Myoblasts in early stage of fusion. Note pre-fusion complexes at points of cell–cell contact (arrowheads); n = myoblast nuclei. (B) Three sets of paired vesicles. Note electron-dense material in the extracellular space between pairs of vesicles. (C) Paired vesicles oriented across a vesiculating pair of plasma membranes. (D) An electron-dense plaque near a region of actively fusing membrane; note fusion pore (arrow). (E) Fusion pores in a vesiculating plasma membrane. (F) Later-stage vesiculating plasma membrane. The membrane sacs have increased in width and a group of irregular clear vesicles is present (arrowhead). Bars: (A) 1 μm; (B–D) 100 nm; (E) 250 nm; (F) 500 nm. (Reprinted from [50] © 1997, with permission from Rockefeller University Press).

pears that Antisocial and Loner are recruited independently to the sites of fusion by one of the IgSF receptors [51]. The presence of a lipolytic enzyme signature sequence in Antisocial suggests that a cytoplasmic lipase could modify the inner leaflet, catalyzing fusion from the opposite side of the plasma membranes. In summary, the proposed signaling pathway would be initiated in the IgSF membrane receptors through adaptors and intracellular signals that would be transduced to the cytoskeleton [46]. Cytoskeleton modifications may be required for plaque formation and/or for the next steps in the process.

Cell Alignment, Plasma Membrane Apposition and Pore Formation After or during the formation of the plaques, the cells align longitudinally closely apposing their cell membranes in the CZ. This is followed by the formation of multiple small pores or microfusions that appear to have a diameter between 20 and 50 nm by transmission electron microscopy (TEM) (Fig. 10.2 C–D). From three-dimensional reconstructions of serial sections it is not clear whether the pores are circular in cross-section. It is conceivable that the pores are connected or that each pore originated from an independent membrane fusion event [50].

In *rolling stone/rost* mutant embryos, there is accumulation of extensive electron-dense plaques and the plasma membranes between aligned myoblasts appear closer than in wild-type with little fusion detected [50]. It is proposed that removal of membrane glycoproteins from the CZ is required before close apposition of the membranes. This could be accomplished by proteolysis or by movement of the proteins outside the CZ. In *rost* mutants the removal of the proteins in the CZ may be eventually completed, explaining the closer than normal distance between the membranes after disappearance of the plaques [50]. *rost* molecular identity is not clear [49, 62, 63] and, based on the phenotypes observed by TEM, *rost* may be involved in removal of the electron dense plaques or in membrane merger [50].

In *drac1* dominant-negative mutant embryos [61], the plaques appear to form, elongate and align normally (Fig. 10.1). However, the juxtaposed cell membranes in the CZ have abnormal morphology with few or no pores and it was estimated that fusion failed in 90% of the myoblasts. It was proposed that Drac1 is required for a late step in plasma membrane pore formation [50].

Vesiculation: Microfusion Expansion to Macrofusion The next step is vesiculation (also called plasma membrane breakdown; Fig. 10.2 C, E and F). The plasma membranes form vesicles along their shared lengths, and the plasma membrane remnants are probably recycled resulting in vesicles and the formation of a myotube [50]. Candidate mutations affecting this stage have not been described.

Later in development, additional mononucleated fusion-competent cells extend filopodia towards multinucleated muscle precursors initiating a second wave of cell fusion that results in growth of syncytial myotubes [46, 52].

10.2.2
C. elegans

C. elegans, a free-living nematode worm, is a robust genetic system to analyze developmental cell fusion because it has a high number of invariant epithelial and myoepithelial cell fusions [64]. Three hundred somatic cell fusion events occur during different stages of development in distinct tissues from the mouth to the tail [64–73]. While in *Drosophila* cell fusion has been described in muscles and in humans cell fusions have been confirmed in only three organs, i.e. skeletal muscles, placenta and bones, in *C. elegans* one-third of all somatic cells

reproducibly fuse in muscles, gland and epithelia of the pharynx, epidermis (hypodermis), uterus, vulva, excretory gland, and male tail. Interestingly, the body wall muscle cells responsible for locomotion are not syncytial and only a few pharyngeal muscles fuse in *C. elegans*. Both epithelial and muscle developmental cell fusion in *C. elegans*, as in other organisms, has been divided in the following steps: (1) proliferation, cell fate determination and differentiation; (2) cell migration; (3) cell–cell recognition and adhesion; (4) membrane fusion; and (5) mixing of cytoplasmic contents and rearrangement of the cytoskeleton. For the purpose of this chapter we will concentrate on the last two steps.

10.2.2.1 **Epithelial Cell Fusion Assay in *C. elegans***

Most developmental fusions in *C. elegans* are between cells that first differentiate into components of polarized epithelial tissues. There are apical junctions (AJ) marking the apical borders between epithelial cells [74]. After cell–cell fusion the AJ between any two cells disassembles, indicating the fusion of the juxtaposing membranes. TEM of cells before and after fusion has demonstrated that disappearance of AJ correlates with membrane fusion. The dynamic behavior of AJs during cell fusion has been well documented [64, 69, 70, 75–81].

10.2.2.2 **Control of Cell Fusion**

As in *Drosophila* and vertebrates, a number of transcription factors and signaling pathways are known that control epithelial and muscle differentiation and distinct cell fusion events in *C. elegans* [82–101]. Signaling pathways active upstream of cell membrane fusion will not be discussed. The identified cell-specific regulators of cell fusion affect a few restricted events resulting in hyperfusion when there is loss of function of the regulator. However, until recently no proteins involved in the actual membrane merger event had been identified. The prediction is that knocking down any gene required for cell fusion will result in hypofusion or complete fusion failure.

10.2.2.3 **Developmental Genetics of Cell Fusion in *C. elegans***

Screens using mutagenized transgenic *C. elegans* strains expressing AJM-1::GFP were designed to identify mutants in which epithelial cells are properly differentiated and patterned but fail to fuse. Two such mutants, *eff-1(oj55)* and *eff-1(hy21)*, were isolated in independent screens and failed to complement each other. *Eff-1(oj55)* was isolated by Bill Mohler and John White in a screen for embryonic epidermal fusion defects, while *eff-1(hy21)* was isolated by Gidi Shemer in a distinct conditional screen for vulva morphogenesis and cell fusion defects. Both mutations result in subviable worms that have deformed bodies and behavioral defects associated with cell fusion failure [102]. Additional alleles of *eff-1* have been recently isolated in different screens (Gattegno, Assaf and Podbilewicz, unpublished results).

10.2.2.4 *eff-1* Mutant Epidermal Cells do not Initiate Cell Membrane Fusion

eff-1 mutant embryos have a specific block in all epidermal cell fusion events. In both wild-type and *eff-1* mutant embryos the epidermal cells are born, and undergo normal migration and patterning events forming an epidermal monolayer. However, all of the 43 cells that would normally fuse to form eight syncytia containing between two to 23 nuclei remain distinct in *eff-1*, failing to fuse as the embryo elongates.

Mixing of cytoplasm between Green Fluorescent Protein (GFP)-labeled and unlabeled cells in live embryos can be followed in wild-type cells as cell fusion progresses. In contrast, in *eff-1* mutant embryos cytoplasmic GFP remains contained within single epidermal cells, demonstrating that cell fusion was blocked after recognition, AJ formation and tight adhesion in the CZ, but before membrane merger and cytoplasmic content mixing [102].

To determine whether the effects of *eff-1(hy21)* on larval epidermal cells were specific to the process of cell fusion, the behavior of cells and their AJs in larvae at different stages of development was studied. These epithelial cells were generated at the right stages of development, exhibited their appropriate cell lineage division patterns and interacted normally within the epithelium, making the correct cell contacts and migrations. However, the cells failed to fuse and abnormal ectopic extensions connect all regions of the epidermis. Thus, failures in cell fusion and syncytium formation result in the presence of "unfused" cells in the epidermis of *eff-1(hy21)* worms. Unfused seam cells produce defective cuticular structures, and ectopic cell junctions persist between the progeny of the seam cells and ventral cells even in the adult. In summary, the morphology and behavior of the unfused epithelial cells by light microscopy appears normal, except that the plasma membranes, CZs and cell junctional complexes remain intact and unfused cells send processes that abnormally link epidermal cells [88, 102, 103].

TEM of mutant *eff-1(hy21)* L4 grown at the restrictive temperature shows normal ultrastructure of the hypodermal membranes (Fig. 10.3 A). The extra seam cells that failed to fuse form multilayered stratified epithelia that are not seen in wild-type animals containing the characteristic epithelial monolayer. Higher magnification of the unfused epithelial cells shows that the distance between two plasma membranes in the CZs that failed to fuse is, as in wild-type, between 9 and 13 nm. The ultrastructure of the AJ and membranes appear normal except for the appearance of autophagocytic organelles that may be a way in which cells try to dispose excess membranes [103].

10.2.2.5 *eff-1*-mediated Cell Fusion is Essential for Healthy Organogenesis

Widespread, but precise, cell fusion failure in epidermal, tail and vulval cells is consistent with the interpretation that a block in cell fusion may prevent normal morphogenesis in these organs. The general anatomy and behavior are also compromised since *eff-1* animals are deformed, dumpy and uncoordinated, suggesting that *eff-1* activity is also required for normal growth, organ morphology and movement [73, 88, 102, 103].

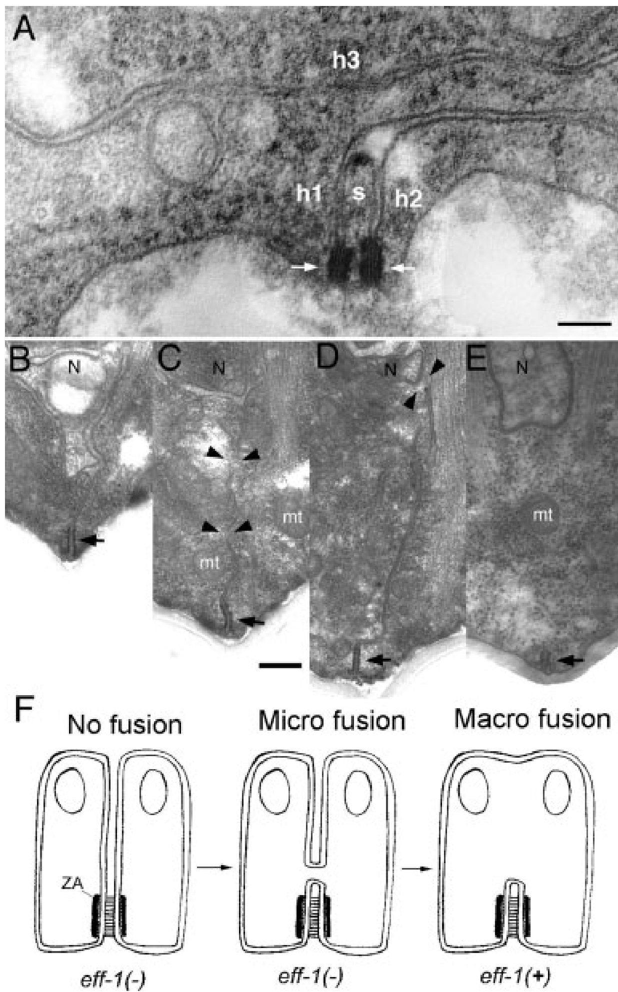

Fig. 10.3 TEM of unfused epidermal and muscle cells.
(A) Transverse thin section of mutant *eff-1(hy21)* L4 grown at the restrictive temperature shows normal ultrastructure of the hypodermal membranes, where the separation between apposing plasma membranes that failed to fuse is generally between 9 and 13 nm as in wild-type. The ultrastructure of the AJs (arrows) and membranes appears normal. Here a seam cell process (s) narrows and three neighboring hypodermal cells (h1, h2, h3) remain unfused along the lateral body wall. The lateral cuticle lies along the bottom edge of the panel.
(B–F) TEM of pharyngeal muscle pairs shows variable fusion failure in a mutant *eff-1(hy21)* adult grown at the restrictive temperature or successful fusion in wild-type cells. (B) Two cells have failed to fuse, leaving a persistent cell border running from the neuron process (N) to the AJ (arrows). (C and D) Two pairs of cells have formed microfusions (arrowheads) above the AJ, which are so small that no mitochondria (mt) could pass. (E) Two wild-type cells have fully fused below the neuron process (N), leaving behind a complete AJ on the plasma membrane of the fused cell pair.
(F) Model showing a pathway for pharyngeal muscle homotypic fusion based on TEM intermediates. ZA, zonula adherens is a component of the AJ [74] (B–E). Scale bars: 100 nm. (Reprinted from [103] © 2004, with permission from Elsevier).

10.2.2.6 *eff-1* Encodes Novel Type I Membrane and Secreted Proteins

eff-1 was cloned to study its molecular activity in epithelial cell fusion using transformation rescue, RNA interference and sequencing of mutant alleles compared to wild-type [102]. *eff-1* encodes four alternatively spliced isoforms – two predicted type-I membrane proteins and two secreted proteins (WormBase 2003; http://www.wormbase.org). Homologs of *eff-1* were identified in the closely related free-living nematode *C. briggsae* and in a variety of animal and plant parasitic nematodes [88]. The EFF-1 predicted membrane proteins in *C. elegans* and *C. briggsae* share a single transmembrane domain and a large N-terminal ectodomain. This ectodomain contains cysteines that may form disulfide bonds, potential glycosylation sites and a 22-amino-acid internal putative fusion peptide [104]. A predicted coiled-coil domain characteristic of viral class I fusogens [105] was not found in neither of the predicted EFF-1 isoforms. Sequencing the coding regions from homozygous *eff-1* mutant worms revealed mutations of the putative proteins: from Pro183 to Leu in *eff-1(hy21)* and from Ser441 to Leu in *eff-1(oj55)* [102].

EFF-1 isoforms in *C. elegans* and in *C. briggsae* contain a predicted consensus phospholipase A_2 (PLA_2) aspartic acid active site. Although they lack other domains required for catalytic activity [106], the presence of the PLA_2 consensus raises two functional possibilities: (1) that EFF-1 may associate with another protein to reconstitute PLA_2 activity and (2) that this domain could act by interacting with phospholipids within membranes.

10.2.2.7 *eff-1* is Highly Expressed in Epidermal Cells Ready to Fuse

Expression of *eff-1p::gfp* was visualized in wild-type embryos and worms by confocal microscopy, and three- and four-dimensional reconstruction [73, 88, 102, 103, 107]. *eff-1p::gfp* expression was silent throughout the first third of embryogenesis, first appearing about 4 h after fertilization in a subset of epidermal precursor cells. Over the next 3 h, these and additional fluorescent cells were observed to migrate over the ventral and dorsal surfaces of the embryo, and most of the GFP-positive cells fused to form the hyp6 and hyp7 syncytia. Mononucleated epithelial cells remained non-fluorescent until shortly before initiation of cell fusion. Expression was seen in epithelial and non-epithelial organs known to fuse [102].

10.2.2.8 *eff-1* is Sufficient for Cell Membrane Fusion *in vivo*

It was shown that *eff-1* is required for cell fusion in *C. elegans*. To test whether *eff-1* is also sufficient to promote cell fusion, *eff-1* was ectopically expressed in wild-type and *eff-1* mutant animals using a heat-shock promoter. Less than 1 h after heat shock, epithelial seam cells ectopically fused to the epidermis, resulting in discontinuities in the anterior–posterior rows of lateral cells and confirmed by ectopic cytoplasmic content mixing between cells that do not normally fuse. This ectopic fusion was also apparent in the ventral epithelia as vul-

val precursor cells fused ectopically to the epidermis, resulting in aberrant vulvae. Thus, *eff-1* ectopic expression promotes ectopic fusion in normally non-fusogenic cells [103].

In summary, *eff-1* is not only necessary, but it is also sufficient to promote epithelial fusion *in vivo*. These experiments, together with evidence showing that different mutations in the Hox gene *lin-39/deformed/hoxD4* and other homeobox containing genes prevent downregulation of *eff-1* expression and suppression of specific epithelial cells fusions during restricted stages in development [73, 88, 107], strongly suggest that many epithelial cells are fusion competent and *eff-1* expression is sufficient to fuse cells [103].

10.2.2.9 **Tissue-specific Fusogenic Activity of *eff-1* in Pharyngeal Muscles**

In *C. elegans*, strong *eff-1* mutations block cytoplasmic content mixing, initiation of pore formation and plasma membrane breakdown in epithelial and myoepithelial cells (Fig. 10.3 A and B) [103]. Surprisingly, multiple stable 20- to 50-nm microfusions have been characterized in *eff-1* conditional mutants at the semi-restrictive temperature in pharyngeal muscles (myoepithelial cells; Fig. 10.3 C and D), but not in epidermal cells [103]. Thus, *eff-1* is required to initiate and expand cell–cell fusion in the muscles (Fig. 10.3 F). The mechanism of fusion pore expansion from a microfusion, that is not large enough to allow the free passage of mitochondria, to a completed macrofusion in myotubes or syncytial hypodermal cells is not known (see Fig. 10.5). It is interesting that the dense plaque (AJ) disappears during syncytia formation in epithelial cells of *C. elegans* as also occurs with the dense plaques in *Drosophila* myoblasts. However, in wild-type pharyngeal muscles of *C. elegans*, the AJ plaques remain even in the adult, marking the positions where the plasma membranes used to be before syncytia formation (Fig. 10.3 E and F).

Finally, there is tissue specificity in the fusogenic activity of this gene. While *eff-1* is essential and sufficient to initiate and complete cell fusion in epidermal and vulval cells, *eff-1* is not essential for the fusion of the gonadal anchor cell to uterine cells [103].

10.2.3
Comparison between Cell Fusion in a Worm, a Fly and Vertebrates

The major steps of developmental cell fusion are conserved in worms, flies and humans, and include differentiation, recognition, adhesion, alignment, membrane fusion and rearrangement of the cytoskeleton.

In human skeletal muscles each syncytial fiber contains thousands of nuclei, compared to around 30 nuclei in an insect muscle cell and exactly two, four, or 139 nuclei in each specific epithelial or myoepithelial syncytia. Something unique about cell fusion in worms is that the identity, number, position and behavior of the cells that fuse are invariant.

Pairing of vesicles in the CZ before cell membrane fusion is distinctive for *Drosophila* myoblasts [50]. While in *C. elegans* coated vesicles along the cytoplasmic domains of the plasma membranes have not been observed [70, 102, 103], in the muscle cell line L6 [108] and in primary cultures of quail myoblasts [109] vesicles with electron-dense materials have been observed and associated with myoblast fusion. It is not known what is the molecular and subcellular nature of the paired vesicles in the pre-fusion complex. It has been hypothesized that the symmetry of the paired vesicles may be due to the mechanics of homotypic cell fusion [50].

The electron-dense plaques formed in *Drosophila* prior to myoblast cell fusion are similar to plaques previously described in vertebrate myoblasts [110]. Interestingly, in *C. elegans* pharyngeal muscles, there is an AJ containing AJM-1 and other proteins characteristic of the zonula adherens forming an electron-dense plaque that is present before, during and after cell fusion [67, 103]. In contrast, in the epithelial cell fusions in *C. elegans*, the AJ disappears as the membrane fusion site expands from microfusion (25–50 nm) detected in electron micrographs to expanding macrofusion (250–20 000 nm) detected by confocal microscopy (Fig. 10.3) [70, 79, 81, 102, 103].

It is believed that specific membrane proteins are required to maintain cells in close proximity in the CZ before fusogenic proteins can act. In *blown fuse* mutants electron-dense paired vesicles lining the cell border accumulate in *Drosophila* myoblasts. In *rolling stone* mutants, electron-dense plaques between unfused myoblasts accumulate to abnormally high levels. In *myoblast city* all membrane-associated pre-fusion complexes are absent [50]. These symmetrical junctional structures are postulated to be part of the pre-fusion complex in *Drosophila* myoblasts, perhaps delivering or holding fusogenic molecules in place on both membranes prior to fusion [50]. Similarly, gap junctions have been reported to be a necessary precursor to a fusion event in some vertebrate myoblasts and specific channel blockers can inhibit cell fusions [42, 111, 112]. These dense plaques are similar to adherens junctions and may be required at some sites of cell fusion, apparently to localize signaling or fusogenic molecules [50]. It has been proposed that adherens junctions provide such a function in the tail tip cells [70]. Unlike *Drosophila* myoblast fusions, no obvious pre-fusion complexes or vesicles participate in initiating cell fusions in the embryo or on the tail tip of *C. elegans*. Thus, in both the tail tip and the embryonic cell fusions, the fusing membranes appear to be maintained closely together by an adherens plaque or AJ, with fusion pore formation occurring at or very near the AJ [70, 79]. However, during the embryonic fusions but not in the tail tip, the AJs do not immediately disappear, but retreat away from the apical side toward more basal portions of the cell border, leading a front of fusions between adjacent cells beginning apically and progressing basally. In addition, vesiculation in the *C. elegans* embryonic hypodermal syncytia result in irregular 20- to 50-nm vesicles or tubules along the area formerly covered by the apposed plasma membranes similar to the vesicles observed in *Drosophila* myoblasts [50, 79]. However, in the tail tip, larger vesicles appear in the fusing cells, but not only where the apposed fusing membranes were localized [70].

Microfusions, also called fusion pores, ranging from 25 to 50 nm have been observed in *Drosophila* [50] and vertebrate myoblasts [40]. In *Drosophila*, a dominant-negative mutation in *drac1* appears to block pore formation. Interestingly, in *C. elegans* pharyngeal muscles, a complete block in *eff-1* activity results in a total failure in the initiation of cell fusion with no apparent discontinuities in the apposed plasma membranes [103]. However, partial loss of function in *eff-1* blocks cell fusion in a microfusion stage showing multiple 25- to 50-nm pores, very similar to the ones observed in myoblasts from *Drosophila* and vertebrates (Figs. 10.1–10.3). It is conceivable that the same protein, EFF-1, may be essential to initiate membrane merger and/or microfusion formation and also to expand these microfusions to a macrofusion in a syncytium.

After the cell membranes fuse there is a change in syncytial cell shapes within muscle fibers, epidermal syncytia, osteoclasts and syncytial trophoblasts [71–73], and in some cases there are simultaneous nuclear migrations within the newly formed syncytia [70]. These changes in syncytia shape must be accompanied by active changes in the cytoskeleton in these cells. The changes in cell shape may result from a migratory behavior, similar to other cell migrations involving a blunt leading edge or filopodia with the cell nucleus pressed close to the front and with thinner processes trailing behind the cells [70, 113]. In addition, the cell shape changes upon fusion may involve redistribution of large amounts of membranes from the site of fusion to a different domain of the newly formed syncytial cell [71, 72].

10.3 Cell Fusion in Diseases

Infertility, cancer, preeclampsia, muscle diseases, and parasitic, bacterial and viral infections are some of the diseases that may involve cell–cell fusion defects as part of their pathogenesis [104, 105, 114–117]. Here, we will discuss how enveloped viruses cause cell fusion.

10.3.1 Cell Fusion Mediated by Enveloped Viruses

Arguably, fusion mediated by viral glycoproteins remains the best-characterized example of a fusion reaction. Recent years have brought new experimental approaches to dissecting pathways of membrane and protein rearrangements in fusion. New findings indicate that fusion proteins drive the entire reaction and that completion of the fusion reaction is a harder job than the initiation of fusion.

Membrane fusion is an important stage of enveloped virus infection (see Chapter 12). While some viruses (e.g. influenza) first enter the cell by endocytosis and then fuse their envelope with the endosomal membrane upon acidification of the endosome, other viruses (e.g. HIV, measles and mumps) fuse direct-

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ly with the plasma membrane. During replication of the viruses such as measles and mumps, expression of the fusion protein at the cell membrane can result in the fusion of neighboring cells, and the formation of multinucleated cells or syncytia that might be important for viral cell–cell spread and pathogenicity. Since several of the fusogenic envelope glycoproteins [e.g. influenza hemagglutinin (HA), HIV gp120/41 protein and E1 of Semliki Forrest virus] are among the best-characterized membrane proteins, exploration of viral fusion reactions is important not only for developing new antivirals but also as a model for ubiquitous biological fusion.

10.3.1.1 Dissection of Viral Membrane Fusion

Viral fusion is usually studied *in vitro* where the virus fuses with liposomes or target cells. In an alternative and widely used approach, viral fusion proteins are expressed in cells. Fusion of virus protein-expressing cells with target cells or with lipid bilayers is assayed as redistribution of membrane and aqueous content probes using fluorescence microscopy, spectrofluorimetry and electrophysiology. In many studies on the dissection of the viral fusion pathway, cell–cell fusion was slowed down or blocked at different stages by lowering temperature, modifying fusogenic proteins, decreasing their numbers, blocking their conformational changes by specific peptides and/or altering lipid composition to that unsuitable for fusion [118–128]. These studies suggest that viral fusion proceeds through a hemifusion intermediate (fusion of contacting membrane monolayers without merger of the inner monolayers), similar to those identified for fusion of protein-free lipid bilayers (for review, see [123]). HA-mediated hemifusion requires only a fraction of all HAs needed for complete fusion. Additional activated HAs stabilize the hemifusion connection and then expand it to allow lipid redistribution through it. HAs in still greater numbers advance the reaction beyond hemifusion to an opening and then irreversible expansion of a fusion pore connecting two aqueous volumes initially separated by the membranes. In brief, local merger of membranes into hemifusion and then fusion pore connections appear to be much less energy intensive than the subsequent stage of fusion pore expansion, still driven by the fusion proteins. The emerging pathway of viral fusion and the notion of the fusion pore expansion as the most demanding job for fusion proteins are further substantiated by studies on cell fusion mediated by HIV env proteins [129, 130] and on fusion mediated by retroviral envelope glycoproteins [128, 131, 132].

10.3.1.2 Initiation and Expansion of Membrane Fusion

Neither the mechanism(s) by which viral envelope glycoproteins form early fusion intermediates nor the mechanism(s) by which the proteins drive fusion pore expansion are known. However, while the former have been the subject of several hypotheses, the latter remain almost unexplored. Recent work on the mechanisms of protein-driven pore expansion was based on contrasting fusion with another

kind of membrane remodeling, i.e. fission of one membrane into two. Analysis of the best-characterized biological processes that involve membrane budding-off and fission emphasizes the critical role of protein coats. Formation of vesicles out of a plasma membrane and *trans*-Golgi membranes, trafficking of membrane vehicles between the endoplasmic reticulum, *cis*-Golgi and the Golgi cisternae, and budding of and release of enveloped viruses at the latest stages of viral morphogenesis all involve self-assembly of the coat proteins at the membrane surface. The protein coat spontaneously acquires a strongly curved shape, bends the membrane into a bud with a constricted neck and, finally, mediates lipid bilayer fission either on its own or with involvement of additional proteins. Since local fusion/expansion of the fusion pore and budding/fission rearrangements involve similar topological stages organized in the opposite order, a recent study suggests that an interconnected coat formed by membrane-bound activated fusion proteins generates the driving force for fusion [133].

10.3.1.3 **Protein–Protein and Protein–Lipid Interactions in Membrane Fusion**

The protein coat model of membrane fusion [133] assumes that activated fusion proteins form a dense interconnected protein coat (Fig. 10.4). This coat surrounds the developing fusion site and has an intrinsic shape, which is strongly curved in the direction opposite to that of the coat driving membrane budding and fission. The bending of the protein coat deforms the underlying lipid bi-

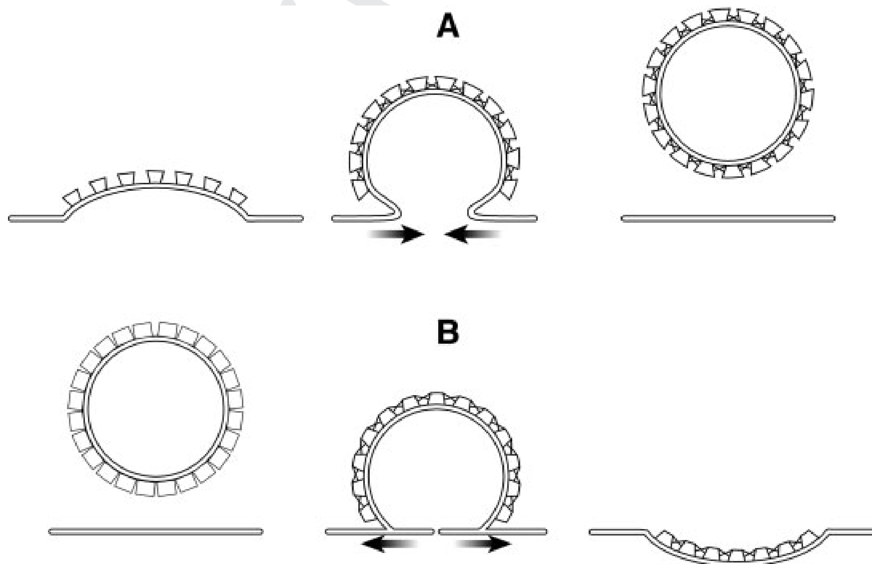

Fig. 10.4 Coat mechanism for membrane remodeling. (A) Budding – fission. (B) Fusion – expansion of the fusion pore. (Reprinted from [123] with permission from Annual Reviews).

layer, and produces tension that drives fusion and expands the fusion pore. In contrast to the models that describe only the earliest fusion stages that might yield local fusion intermediates, the protein coat model accounts for the force driving the fusion pore expansion until it reaches the dimension of the coat itself. For a virus, whose surface is completely covered by the coat, this means a complete insertion of the viral membrane into the target one.

10.3.1.4 The Role of Fusion Proteins Outside the Fusion Site

One of the most unexpected predictions of the fusion coat hypothesis is the possible functional role suggested for fusion proteins located outside of the fusion site. Based on the literature, it appears that proteins driving membrane merger in fusion and fission do it in radically different ways. Fission can and likely is mediated by proteins, which are not located between merging membranes [134, 135]. In contrast, fusion has been generally believed to result from the local action of only those fusion proteins, which are located in the CZ between the membranes and interact directly with the target membrane [136–140]. However, until recently the role of the fusion proteins outside of the CZ had not been tested.

The role of the "outsider" fusion proteins was studied for HA-mediated fusion in a recent study [141]. While both outsiders and insiders undergo similar restructuring at the pH of fusion [142], the inter-membrane contact in the CZ involves only a fragment of the viral or HA expressing cell surface, so that only some of the HAs, referred to as "insiders", are situated inside the CZ, while the rest – the "outsiders" – cannot directly interact with the target membrane. To differentiate between the fusogenic actions of the insider and the outsider HAs, different agents that inhibit or promote HA fusogeneity were conjugated to polystyrene beads too large to enter very tight and extended CZs between HA cells and bound red blood cells. Bead-conjugated antibodies that bind to HA, and proteases that cleave HA, specifically target HA outsiders, but leave HA insiders intact. The results in this experimental system indicate that changes in the fusogenic properties of HA outsiders have significant effects on fusion with the latest fusion stage of a fusion pore expansion being most sensitive [141].

10.3.1.5 HA Insiders Initiate Hemifusion and HA Outsiders Expand Fusion Pores

The mechanisms by which the proteins that at the time of the activation are located outside of the CZ directly influence the most energy-intensive stages of fusion leading to opening and expansion of a fusion pore remain to be understood. Since early fusion intermediates are not affected by the HA outsider-targeting agents, fusion might involve two radically different activities of the same fusion protein HA. First, local action of HA insiders generates local hemifusion. In contrast, the subsequent transition from hemifusion to the expanding fusion pore might be driven by HAs located outside of the initial fusion site. The achieved fusion phenotype might depend on the total number of activated HAs including both insiders and outsiders. At the highest HA densities, only the most advanced

fusion stage, an expansion of the fusion pore, depends on HA outsiders and is inhibited by the bead-conjugated agents. For lower density of activated HAs, outsiders are required even for lipid mixing. While the discovered involvement of HA outsiders in fusion can be readily explained by the fusion coat mechanism, it is also possible that HA-mediated fusion develops along the circumference rather than in the central region of the CZ, allowing HA outsiders located in the immediate proximity to the CZ circumference to significantly affect fusion. The functional role of fusion proteins located outside of the CZ strengthens a tempting hypothesis that the oppositely directed processes of membrane fusion and fission work according to a common principle: the proteins drive membrane remodeling from outside of the zone of the actual membrane rearrangement.

Clearly, opening of an initial fusion pore of around 0.5 nm is only the beginning of the fusion reaction [143]. Even after reaching 15 nm in diameter, fusion pores can still close [144]. The transition from an initial flickering pore to an irreversibly expanding fusion pore depends on having sufficient number of activated fusion proteins (see above) and on the structure of the fusion proteins. Mutations and deletions in different regions of viral envelope glycoproteins, i.e. fusion peptide [145], transmembrane domain [127, 146] and cytoplasmic region [125, 147, 148], block fusion pore dilation. In addition, fusion pore expansion versus pore resealing depends on the lipid composition of the membranes. For instance, the incorporation of cholesterol into the target lipid bilayer greatly decreases the number of flickers and the first pore formed usually irreversibly expands [149].

10.3.1.6 Models for Final Expansion of Fusion Pores

Even when fusion connections become irreversible and the fusion pore reaches the largest size resolvable by electrophysiological assays, and by following redistribution of usual aqueous probes (e.g. 6-nm diameter, 70-kDa dextran [150]) with fluorescence microscopy, further enlargement is required for successful completion of the fusion reaction. The characteristic size of a rod-shaped influenza virus ribonucleoprotein that is delivered into cytosol of the infected cell by endosome/envelope fusion is around 10–20 nm in diameter and 20–80 nm in length [151]. Syncytia formation involves the enlargement of a cytoplasmic bridge between cells to the cell-size scale of many microns.

The mechanisms that underlie these late fusion stages and their dependence on the proteins that initiated fusion remain almost unexplored. In general, the final enlargement of an aqueous connection between fusing membranes can proceed by four radically different scenarios (Fig. 10.5). First, it can be a radial expansion of a single fusion pore formed in the central part of the CZ. Second, the enlargement of the cytoplasmic bridge might involve excising of a CZ disk by a pore(s) that form(s) at and grow(s) along the CZ edge. This unexpected pathway was discovered for vacuole fusion, where proteins that regulate docking and fusion accumulate at the edge of the CZ [152]. The third scenario suggests that after earlier fusion stages, two membranes in the CZ are connected by multiple expanding fusion pores. Further development of these pores is topolog-

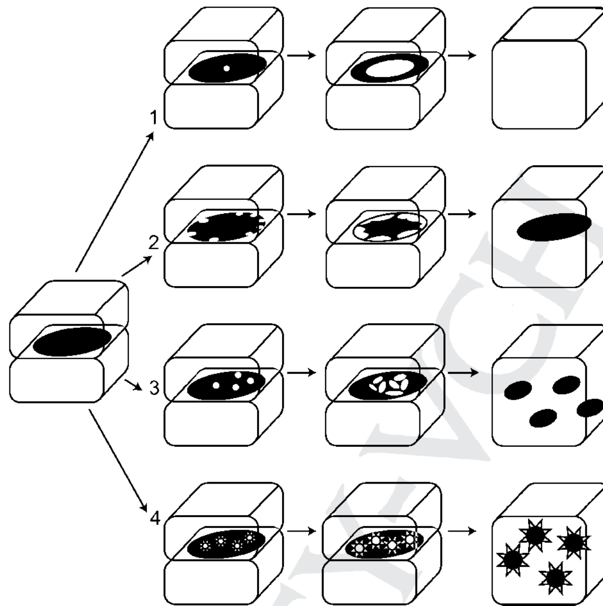

Fig. 10.5 Models of the final enlargement of fusion pores. We show here four different pathways by which two contacting cells at the left (the tight CZ is shown in black) can completely join their volumes in a final syncytium at the right. (1) Opening of a single pore within a CZ is followed by its radial expansion over the entire CZ. (2) Initial pores are formed only along the edge of the CZ. Enlargement of the pores proceeds along the edge and finally excises a CZ membrane disk released into the volume of the fused cell as a membrane vesicle. (3) Expansion of multiple fusion pores formed within CZ results in its breakdown into vesicles. (4) Vesiculation of the CZ is controlled and catalyzed by cell protein machinery shown as triangular shapes surrounding pores and then vesicles.

ically impossible and subsequent widening of the cytoplasmic bridge involves spontaneous vesiculation of the CZ [153]. While the fourth scenario also suggests vesiculation of the CZ, in this case the vesiculation is controlled and catalyzed by the cell machinery that might include dynamin, actin and other proteins involved in intracellular processes of membrane fission. Note that resealing of wounds in the plasma membrane, a process analogous to membrane vesiculation, but oppositely directed, is driven by the intracellular fusion machinery [154]. The CZ disassembly by cell-controlled vesiculation might be triggered by opening of a single or multiple fusion pores.

The specific mechanisms of the late fusion stages, including the nature of the driving force, and the involvement of the cytoskeleton and membrane trafficking machinery of the cell, in the generation and control of this force remain to be understood. For syncytia formation, late fusion stages most likely involve dynamic changes in cytoskeletal structures. As shown for cell fusion induced by an electric field [155, 156], a few minutes after fusion initiation bundles of microtubules be-

gin to extend into the cytoplasmic bridges between the cells, establishing connection between cytoskeletal networks of two cells. Actin bundles condensed at cell edges might widen the bridges by lamellipodial extension. In contrast to electrofusion initiation that can be achieved at 4 °C, the cytoskeleton-dependent stages of syncytia formation downstream of local fusion proceed only upon raising the temperature to 37 °C [157]. This interesting finding along with a recent discovery of long-living intercellular connections between cells of 50–200 nm diameter [158] indicate that both stabilization and widening of these connections might be controlled by the cellular machinery. Thus, late stages of cell fusion likely involve proteins different from the fusion machinery that generated the fusion pores.

10.4 Dissection of Developmental Fusion Based on Viral Fusion Analogies

In this section we will discuss different strategies already tested in viral systems that can be applied to study more complex developmental cell fusion systems.

10.4.1 Activation of a Developmental Fusogen

EFF-1 is a unique candidate for a developmental cell fusogen or fusase. This is because (1) *eff-1* mutants block cell fusion at a stage that is after recognition and adhesion, (2) cell fusion fails before early membrane fusion events, prior to microfusion (pore formation) as detected by TEM and prior to cytoplasmic content mixing as detected by fluorescence microscopy, (3) *eff-1* is expressed in cells prior to cell fusion, and (4) ectopic expression of EFF-1 proteins in cells that do not normally fuse induces ectopic fusion, thus *eff-1* is both necessary and sufficient for cell fusion in *C. elegans*. To test whether EFF-1 needs to be activated at the time of fusion it will be necessary to identify the trigger. The trigger could be a certain threshold concentration of protons or calcium. Alternatively, binding to an unknown EFF-1 receptor could trigger activation of the candidate fusogen. In addition, it is conceivable that a certain concentration of EFF-1 in the membrane is necessary to initiate the fusion reaction.

10.4.2 Dissection of Developmental Cell Fusion

To separate the process of developmental cell fusion into additional stages it would be useful to describe the kinetics of the process and change conditions that will block or slow down the rates of initiation of cell fusion, microfusion (pore formation) and macrofusion (pore expansion). This can be accomplished using different temperatures, additional mutations, changing the concentrations of EFF-1, changing the lipid composition of the membranes and other treatments that have been extensively used for viral fusion reactions.

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10.4.3
Direct Cell Fusion Promotion or Indirect Relaxation of Fusion Blocks

It is conceivable that cells fuse when a fusogen is expressed and activated as occurs in viral-induced membrane fusion. Alternatively, the fusogen could be lifting mechanisms that prevent cell fusion by maintaining the plasma membranes separated. Interestingly, in *Drosophila* several adhesion molecules from the IgSF have been found to participate in the recognition and adhesion between myoblasts. It is conceivable that if the adhesion is too strong and stable, it will prevent cells from fusing. Different mutations and concentrations of adhesion receptors and fusion inducers may play a role in maintaining the cells separate or triggering cell–cell fusion. Finally, the apparent morphological similarity between the myoblast–myoblast electron-dense plaques in vertebrates and *Drosophila* compared to the electron-dense AJs in *C. elegans* myoepithelial pharyngeal muscles and epithelial cells suggests a simple mechanistic similarity with the coat hypothesis of membrane fusion and fission. Microfusions of 20–50 nm diameter appear near or at the electron-dense plaques (Fig. 12.2 C and D), from this region the pore(s) expand, and it is tempting to hypothesize that the coat of electron-dense material may have an active role in the expansion of the fusion pore during developmental and viral induced cell fusions.

10.5
Concluding Remarks

Undoubtedly the mechanisms of most important developmental fusion reactions remain obscure at present. However, recent advances in the characterization of specific developmental fusion reactions along with methodology and mechanistic motifs emerging from the work on simpler examples of cell fusion suggest that this research field is ready for rapid progress. Cross-fertilization between developmental systems that have identified numerous pre-fusion molecules involved in recognition, binding, signal transduction and cytoskeletal reorganization (e.g. *Drosophila*) could help other systems where the pre-fusion genes have not been identified (e.g. *C. elegans*), but where a strong candidate for a fusion protein has been identified. Finally, all of the developmental systems will benefit from using the expertise generated over several decades on the studies of viral fusogens. Clearly, in all complex and simpler systems the late stages of cell membrane fusion deserve more attention because initiating membrane fusion reactions is not sufficient to complete the formation of a syncytial cell.

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