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Cell fusion during development

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Most readers of this review originated from a sperm–egg fusion event. Cell fusion is a process that is crucial at many intersections later during development. However, we do not know which molecules (fusogens) fuse the membranes of gametes to form zygotes, myoblasts to form myotubes in muscles, macrophages to form osteoclasts in bones, or cytotrophoblasts to form syncytiotrophoblasts in placentas. There are five gold standards that can be applied for the identification of genuine fusogens. Based on these criteria, a numerical score can be used to assess the likelihood of protein fusogenicity. We compare distinct families of candidate developmental, viral and intracellular fusogens and analyze current models of membrane fusion.

Introduction

Whether a plant, protist or primate, sexual reproduction begins with the simple process of membrane fusion, forming the first cellular unit of the organism by mixing two cells with different genetic and cytoplasmic contents [1] (Figure 1). During early embryonic development, cell fusion is involved in muscle formation [2], and continuous development depends upon correct fusion events. For example, in mammals, macrophage fusion forms the bone-resorbing osteoclasts [1], and trophoblast cells {see Glossary} fuse to generate the placental syncytiotrophoblast layer that serves as a barrier between maternal and fetal environments [3]. In nematodes, fused epithelial cells form barriers and constrain cell migration, by forming the skin, vulva, uterus, hymen and glands of the worm (Figures 2) [4]. In some sponges, cells (blastomeres) fuse early in development, and somatic cell fusions have been detected in fungi, leeches, insects and many other organisms (Figure 3) [5–8].

Most of our knowledge on the mechanism of cell fusion comes from intracellular vesicle fusion and viral–host cell fusion [9]. Cell fusion processes generally involve bringing two lipid bilayers into close proximity, followed by the formation of a fusion pore and cytoplasmic mixing, to form a syncytium (Figure 4). The molecules that take part in the fusion reaction can be divided into three groups according to their involvement in the fusion stages. The first group is composed of molecules that function before fusion, such as adhesion molecules. The second group includes the mediators of fusion that directly rearrange the lipid bilayers and lead to the formation of fusion pores. These proteins are defined as fusogens and are the focus here. The third group consists of molecules that lead to extension of the fusion pores, and to complete disassembly of membranes.

Developmental cell fusion is still poorly understood, and although many proteins required for various cell–cell fusion events have been identified over the past decades, only a few have been thoroughly characterized and defined as fusogens. Defining whether a protein is a true fusogen has proven to be a difficult task. A primary obstacle is how to distinguish between proteins which function in fusion rather than adhesion. In sperm–egg fusion, for example, impairment of either fusion or adhesion will lead to a reduction in fertility and, as a consequence, to a small brood size. Recently, the generation of knockout mice has proved useful in identifying proteins directly involved in sperm–egg fusion, and refuted several proteins previously identified as fusogens [10].

Here, we suggest the use of five defined criteria for the identification of genuine fusogens (Box 1). Although some proteins which meet these criteria have been identified for viral and intracellular membrane fusion, for cell–cell fusion only a few exist that meet most criteria (Table 1). We discuss current candidate fusogens for various cell fusion events in different organisms, and evaluate how well the best candidates meet the five criteria for *bona fide* fusogens.

Gamete fusion during mating and fertilization

Sexual reproduction usually occurs through a sequence of fast occurring events that include gamete attraction

Glossary

Acrosome reaction: The binding of the sperm to the zona pellucida triggers the fusion of the acrosomal vesicle found at the sperm head with the sperm cell membrane. The fusion results in the release of the acrosomal proteolytic enzymes that lyze the zona pellucida, enabling the sperm to reach the egg membrane.
ADAM proteins: a family of membrane proteins with a disintegrin and metalloprotease domain. The ADAMs are involved in diverse processes such as development and cell–cell interactions.
Cytotrophoblast: the inner layer of trophoblast cells.
FF family: a novel family of nematode-specific type-1 membrane proteins, necessary and sufficient for cell fusion. These proteins contain a conserved signature of cysteines at their ectodomain.
HERV: the human endogenous retroviral protein family.
HERV-W: a new family of HERVs, that is not replication competent.
IgSF proteins: the immunoglobulin superfamily is a large group of proteins that share structural similarities, all possessing the immunoglobulin domain. The family includes cell surface and soluble proteins involved in adhesion, recognition and binding of cells.
SPE-9: defective spermatogenesis 9 protein.
FER-1: spermatogenesis or fertilization defective 1 protein.
Syncytiotrophoblast: multinucleated layer formed by the fusion of trophoblast cells.
Trophoblast cells: epithelial cells that form the outer layer surrounding the blastocyst and attach the embryo to the uterus wall. The trophoblasts provide nourishment from the mother and develop into a large part of the placenta.
Utse: an 'H'-shaped syncytium (hymen) that connects the uterus to the vagina and attaches to the lateral epidermal seam cells in *C. elegans*.
Zona pellucida: a thick glycoprotein outer layer surrounding the mammalian oocyte. The sperm binds to the zona pellucida, and this binding triggers the acrosomal reaction, which enables the sperm to penetrate.

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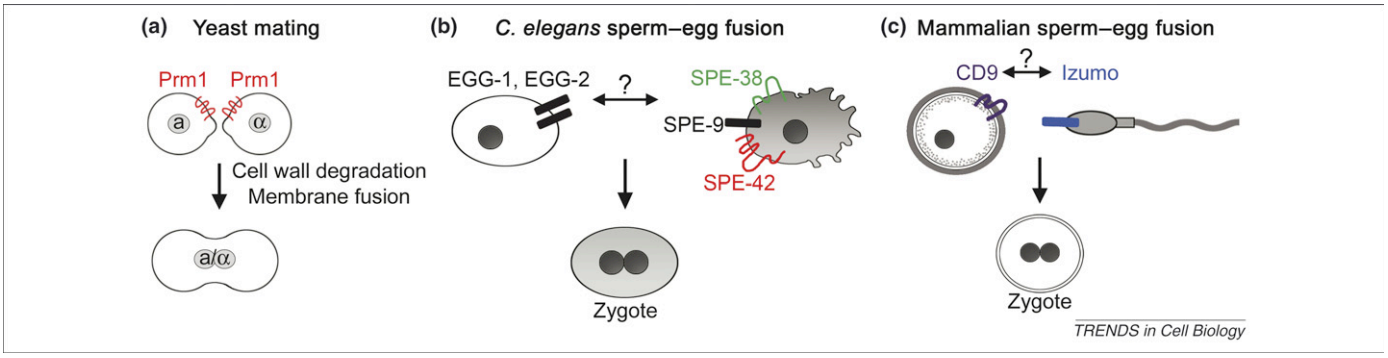

Figure 1. Gamete fusion in various organisms. **(a)** Prm1 (red) is localized to the site of cell fusion. In prm1 mutants, the cell wall degrades but fusion does not occur [1,12,13]. **(b)** Sperm–egg fusion in *C. elegans* requires the expression of EGG-1 and EGG-2 in the egg, and SPE-9, SPE-38 and SPE-42 in the sperm [18,21,22,24]. It is unknown whether sperm proteins interact with oocyte EGG-1 and EGG-2, and the ligands for SPE-9, SPE-38 and SPE-42 have not yet been identified. **(c)** In mice, CD9 is expressed in the microvillar region of the egg [39]. Izumo becomes detectable after the acrosomal reaction on the sperm surface [35]. It is not clear whether Izumo and CD9 interact and whether this interaction is required for the fusion process.

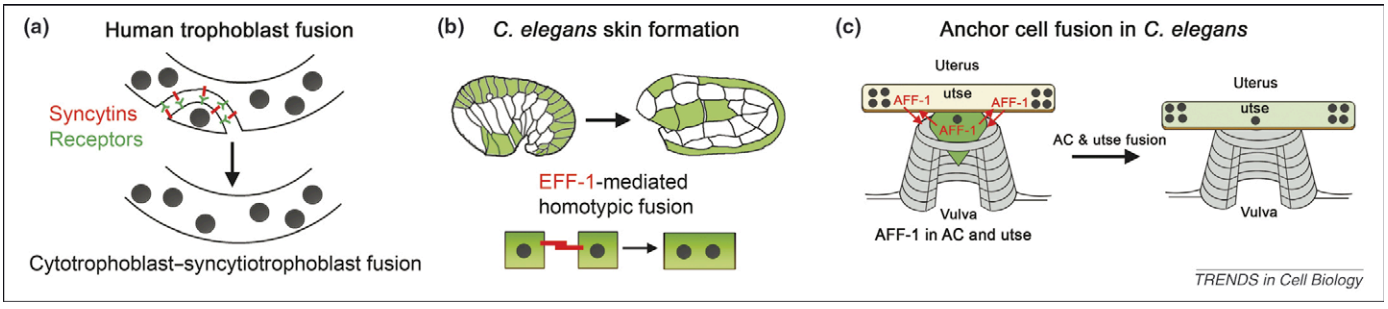

Figure 2. Epithelial cell fusion in humans and worms. **(a)** In the formation of the human placenta, secondary trophoblast fusion involves fusion of cytotrophoblasts with the syncytiotrophoblast layer. Syncytins are the best fusogenic candidates identified for these cell fusion events, and several receptors have been identified [3,46]. **(b)** EFF-1 is necessary and sufficient for homotypic epithelial cell fusion in *C. elegans* [15,56]. In the embryo, most of the dorsal cells and some of the ventral cells (green) fuse to form the hypodermal syncytia [4,87]. Modified, with permission, from Ref. [29]. **(c)** In *C. elegans*, fusion between the AC and the utse occurs only after they both express AFF-1. Other epithelial fusions are also mediated by AFF-1 in *C. elegans* [16]. Modified, with permission, from Ref. [16].

and migration, recognition, attachment and adhesion, followed by fusion. Gamete fusion is a unique heterotypic fusion process that merges two genetically different cells into one. Studying gamete fusion requires detailed analysis of membrane dynamics, using light and electron microscopy. However, owing to the dynamic nature of these events, it is still difficult to differentiate between gamete fusion failure and defects in the processes that precede fusion.

Yeast mating and membrane fusion

Mating in the yeast *Saccharomyces cerevisiae* involves secretion of pheromones by α- and a-mating type haploid cells. Pheromone detection activates a signaling cascade that leads to the activation of gene transcription, cell cycle arrest and cell polarization. The mating partners adhere, the cell wall is degraded and fusion occurs [11]. A reverse genetic screen designed to identify uncharacterized pheromone-regulated membrane proteins [12] uncovered a candidate fusogen, Prm1, a multispanning transmembrane protein, which localizes to the site of membrane fusion after pheromone induction (Figure 1a). In *prm1* mutants, the cell wall is degraded but the frequency of fusion between the apposing membranes is decreased. However, Prm1 is not essential for fusion because a mutation in both mating partners results only in ~50% fusion failure,

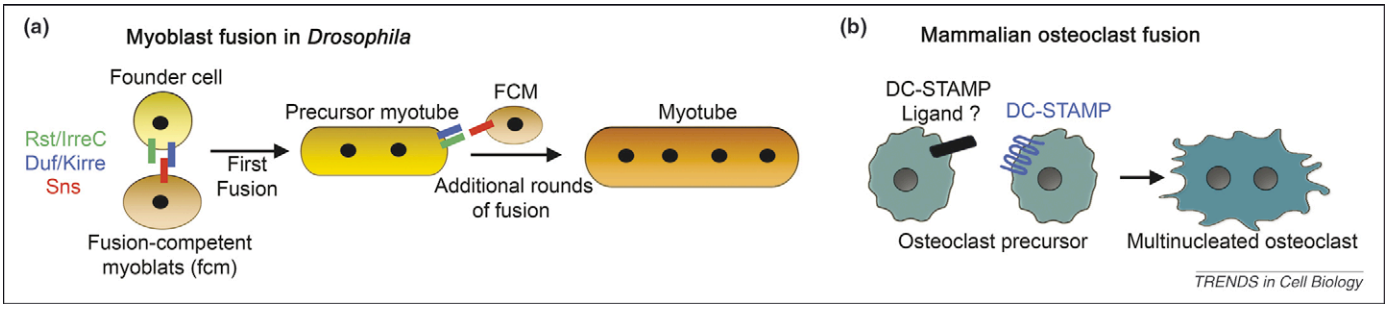

Figure 3. Mesodermal cell fusions in flies and mammals. **(a)** Myoblast fusion in *Drosophila* begins with recognition and attachment of the founder cell and fusion-competent cells. Sns is expressed on the surface of the FCM, and Duf/kirre and Rst/IrreC are expressed on the surface of the founder cell. The first round of fusion leads to the formation of a precursor myotube, and additional rounds of fusion between FCMs and nascent myotubes form a mature myotube [1]. **(b)** Mononuclear osteoclasts fuse to form bone-resorbing, multinucleate osteoclasts in mammals. DC-STAMP is required for the fusion process, and fusion can occur only if DC-STAMP is expressed in one of the fusing cells, suggesting interaction through an unknown ligand [23].

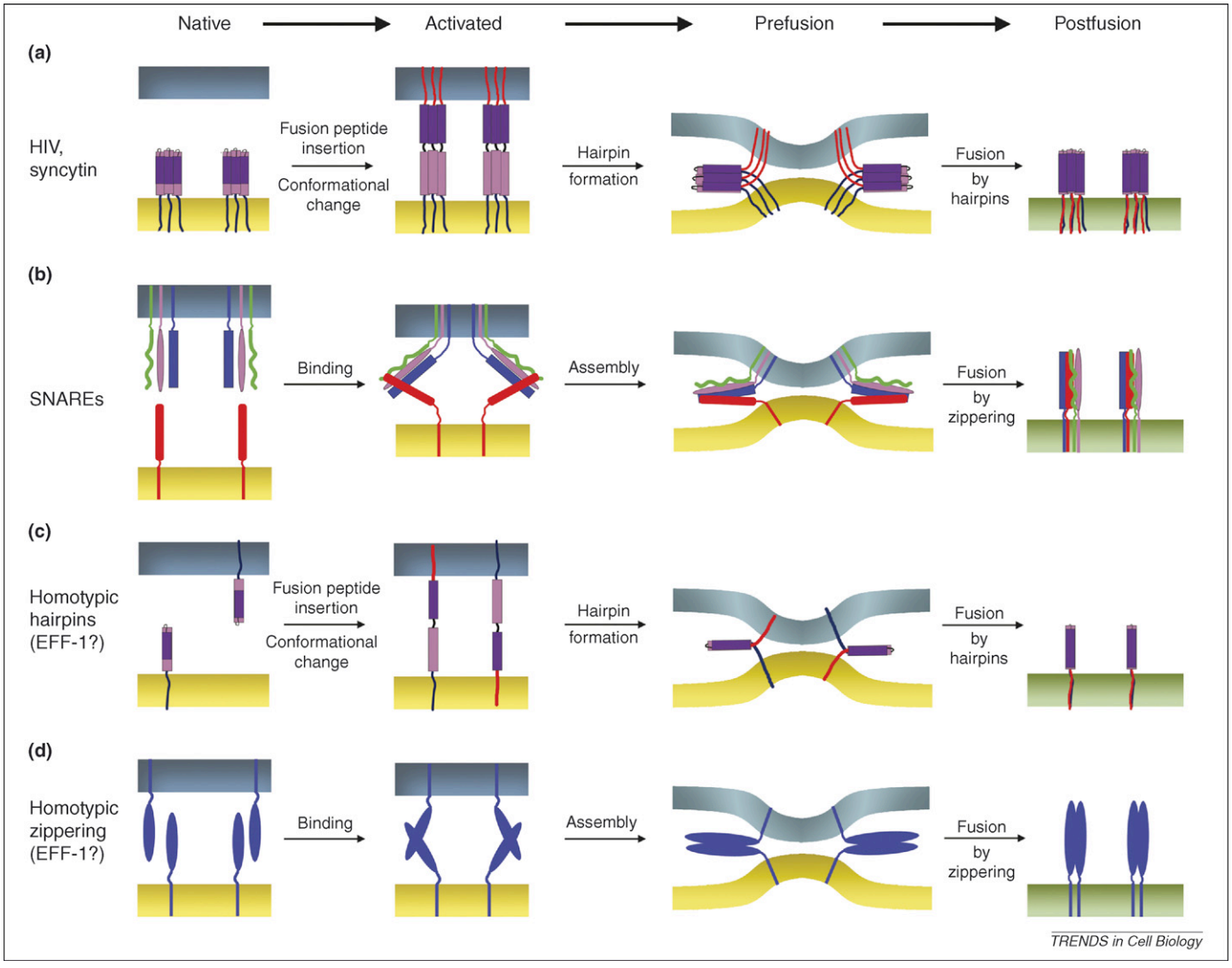

Figure 4. Simplified comparison between proposed models for viral, intracellular and developmental membrane fusion mechanisms. **(a)** The Env proteins of retroviruses (e.g. HIV) and probably Syncytin exist in the native conformation before being triggered by binding to a receptor (not shown) that induces a dramatic conformational change. This conformational change involves the formation of coiled coils and insertion of amphipathic fusion peptides (red) into the target membranes (only two fusogenic trimeric proteins are shown). Formation of hairpins and six-helix bundles induce tightening of the membranes followed by membrane merger [88–90]. **(b)** Native heterotrimeric tSNAREs (blue, green and violet) bind to the vSNAREs (red). Activation by coiled-coil formation assembles the complexes in a prefusion conformation that involves bending and tightening of the membranes. Zippering of the complexes is believed to induce hemifusion (not shown), followed by complete membrane merger (postfusion) [84]. **(c)** Homotypic model for EFF-1-mediated fusion based on heterotypic hairpins shown in (a). EFF-1 proteins (only two monomers are shown) undergo conformational changes and insertion of putative fusion peptides or loops (red) into the apposing membrane. Hypothetical refolding and hairpin formation results in membrane tightening followed by hemifusion (not shown) and complete merger of the membranes. **(d)** Model for homotypic binding between FF proteins based on the zippering mechanism shown in (b). Homotypic interactions activate EFF-1 proteins in both cell membranes. Assembly of complexes bends the membranes (only two *trans*-homodimers are shown). Zippering of the complexes induces fusion through hemifusion (not shown). In addition to the four models shown here, a hybrid mechanism involving homotypic zippering of hairpins (c+d) or other novel mechanisms are conceivable for FF and other fusogens [58].

and when missing from one mating cell, the fusion levels decrease only slightly [12].

An additional screen for mutants that enhance the *prm1*-dependent fusion-failure phenotype identified Kex-2 [13]. *kex2* mutations enhance the *prm1* phenotype but do not completely block fusion. Its molecular function as a Golgi protease suggests the existence of a proteolitically activated protein involved in the cell fusion machinery. For the above reasons, it is not clear if Prm1 is the yeast fusogen, or whether Kex-2 is responsible for the processing of an unidentified fusogen. Recently, the activated pheromone receptors were shown to have a novel and surprising late role in yeast mating, apparently at the stage of cell wall digestion and membrane juxtaposition before membrane fusion [14].

Fertilization in Caenorhabditis elegans

In recent years, the nematode *C. elegans* has proven to be a useful model for studying the process of fertilization. Sperm–egg fusion in *C. elegans* is independent of known fusogens that function in somatic fusion [i.e epithelial fusion failure 1 (EFF-1) and anchor cell fusion failure 1 (AFF-1) [15,16]; see later]. *C. elegans* sperm lacks a flagellum and an acrosome, and uses a single pseudopod for amoeboid motility. Nonetheless, it carries out all essential reproduction steps common to most spermatozoa: migration to the site of fertilization, recognition, adhesion and gamete fusion. Moreover, because *C. elegans* hermaphrodites are self-fertile, isolation of mutants affected only in spermatogenesis is relatively simple because such mutant hermaphrodites

Box 1. The gold standards of a *bona fide* membrane fusogen

Several criteria must be met to define a protein properly as a *bona fide* fusogen [58]. First, the protein must be essential for membrane fusion. Second, the fusogen should be expressed at the site and time of fusion. Third, the molecule should be sufficient to fuse cells that normally do not fuse *in situ*. Fourth, expression of the protein in heterologous cells must lead to fusion of these cells, scored by the formation of multinucleate cells. Fifth, biochemical reconstitution of the candidate fusogen(s) into an isolated *in vitro* system such as liposomes must fuse them. Exceptions might exist – for example, for proteins that are fusogens but have not been shown to be essential for fusion – because there are several redundant proteins. Additional examples include the formation of protein complexes required for fusion, or the requirement of fusion cofactors. These proteins cannot be evaluated under the same criteria, and their identification as fusogens will be more complicated.

Each candidate fusogen scores two points for fulfilling each criterion. Proteins that fuse cells in a heterologous system will score two points for this criterion only if it has been shown that multinucleation was a result of fusion and not failed cytokinesis (Table 1).

produce viable offspring when mated with a wild-type male [17].

Genetic screens have identified several genes required for fertilization. The best characterized *C. elegans* candidate fusogen so far is SPE-9 (see Glossary), a sperm-specific single transmembrane protein containing ten epidermal growth factor (EGF)-like repeats, with similarity to ligands of the Notch receptor [18] (Figure 1b). *spe-9* mutant hermaphrodites lay unfertilized oocytes, whereas males are completely sterile. *spe-9* mutant sperm show normal morphology and motility, and are able to compete with hermaphrodite sperm. After sperm activation, SPE-9 localizes to the pseudopod [18,19]. Although *spe-9* is required specifically for fertilization, it is unclear whether it is required for the membrane fusion step or for earlier stages such as recognition and tight adhesion, as might be interpreted from function of other EGF-motif-containing proteins that are known to mediate adhesive or ligand–receptor interactions. However, no receptor for SPE-9 on the surface of oocytes has yet been identified [20].

Additional sperm proteins required for fertilization are SPE-38 and SPE-42 (Figure 1b). Similarly to *spe-9* mutants, *spe-38* and *spe-42* mutant sperm are fully motile and able to migrate to the site of fertilization but are incapable of fertilizing oocytes [21,22]. SPE-38 and SPE-42 proteins share structural features with proteins in other phyla that have been linked to membrane fusion. SPE-38 is predicted to encode a novel four-pass integral membrane protein with some structural similarities to the yeast Prm1 [12] and [21]. However, SPE-38 is not a member of the tetraspanin family, which has been implicated in the process of mammalian sperm–egg fusion (see later). SPE-42, a predicted seven-pass transmembrane protein, has a DC-STAMP-like domain. The DC-STAMP protein is required for osteoclast and macrophage fusion (see later) [23]. Although their precise function in fertilization, or whether they interact with one another, is unknown, it is possible that these proteins function during the fusion process and that their function has been conserved in evolution.

The first-discovered egg components required for fertilization were EGG-1 and EGG-2 type II transmembrane, low-density lipoprotein (LDL) receptor repeat-containing proteins [24]. Hermaphrodites that lack these mutually redundant proteins are completely sterile. A model in which EGG-1 and EGG-2 function as receptors for unknown sperm ligands can be deduced from their specific egg surface expression and the molecular nature of LDL receptor-related molecules. So far, no interaction has been established between any egg and sperm components in *C. elegans*, so it will be interesting to see whether any interaction exists between SPE-38, SPE-42 or SPE-9 and EGG-1 or EGG-2, which seem to be the best candidate proteins to be directly involved in *C. elegans* sperm–egg fusion.

Fertilization in Drosophila

Although *Drosophila melanogaster* has served as a powerful model organism for over a century, little is known about the mechanisms underlying sperm–egg fusion. More than 30 years ago, Perotti's [25] classic ultrastructural studies of fertilization in *Drosophila* revealed that the whole sperm enters the egg, and showed that the sperm membrane around the tail is intact. However, because the ultrastructure of the sperm membrane at the fusion stage could not be visualized, this does not serve as sufficient proof for lack of fusion between sperm and oocyte membranes (M-E. Perotti, personal communication) [25]. Intriguingly, several recent papers have claimed that the sperm enters the egg without membrane fusion, by puncturing a hole in the oocyte membrane, followed by sperm membrane breakdown [26,27]. Plasma membrane breakdown and vesiculation probably occur after membrane fusion events required to remove and recycle the sperm plasma membrane, as occurs in other cell fusion processes in mammals, flies and worms (see later) [28,29]. Plasma membrane breakdown in *Drosophila* requires the proteins Sneaky and Misfire [26,27]. Sneaky shares domain similarity with DC-STAMP and SPE-42 (Table 1), and one of the homologs of Misfire is *C. elegans* FER-1, a protein required for the fusion of a membranous organelle during maturation of spermatids to motile spermatozoa [30]. Taken together, there is no proof for the absence of sperm–egg fusion in *Drosophila*. The homology between the proteins required for membrane breakdown in *Drosophila* and proteins implicated in fusion in other organisms implies conserved protein function. The proteins that mediate sperm–egg fusion in *Drosophila* remain to be identified.

Sperm–egg fusion in mammals

Fertilization in mammals includes migration and activation of sperm in the female tract, penetration through cell layers surrounding the egg, recognition, binding and induction of the acrosomal reaction, which enables penetration through the zona pellucida, and, finally, sperm–egg fusion [31]. Although much attention has been given over the years to several fusion candidate proteins such as the ADAM proteins on the sperm (fertilins) and the egg integrins, knockout mouse experiments showed that these proteins are not essential for cell fusion [10,32,33]. Recently, the immunoglobulin superfamily (IgSF)

Table 1. Examples of candidate fusogens[a]

Candidate fusogen	Family	Organism	Expression	Fusogenic score	Essential for fusion	Expressed at the time and place of fusion	Sufficient *in situ*	Fuse cells (heterologous)	*In vitro* liposome fusion	Homotypic/ heterotypic	Refs
Env	Class I enveloped Viruses	HIV	CD4+ cells	10	+	+	+	+	+	Heterotypic	[83,91]
HA		Influenza	Lung, epithelia	10	+	+	+	+	+	Heterotypic	[9,83]
vSNAREs, tSNAREs	SNAREs	Eukaryotes	Intracellular	10	+	+	+	+	+	Heterotypic	[9,84]
Syncytin-1	Syncytins (retroviruses)	*Homo sapiens*	Cytotrophoblasts and syncytiotrophoblast	7	±	+	ND	+	+	Heterotypic (?)	[44,45,50]
EFF-1	FF	*C. elegans*	Epithelia, vulva, pharynx	8	+	+	+	+	ND	Homotypic	[15,56–58]
AFF-1			Epithelia, AC–utse, vulva	8	+	+	+	+	ND	ND	[16]
Examples of plasma membrane proteins required for fusion											
Duf/Kirre	IgSF	*D. melanogaster*	Founder cell	2	–	+	–	–	ND	Homo/Hetero[b]	[66,69,92]
Sns			Fusion-competent myoblasts (FCM)	4	+	+	–	–	ND	Heterotypic	[68,69]
Rst/IrreC			Founder cell and FCM	2	–	+	–	–	ND	Heterotypic	[67]
IZUMO		*M. musculus*	Sperm or testis	4	+	+	ND	ND	ND	Heterotypic	[35]
CD9	Tetraspanin	*M. musculus*	Multiple tissues, oocyte microvilli	4	+	+	ND	ND	ND	Heterotypic (?)	[36,37]
SPE-38	4 TM	*C. elegans*	Sperm	4	+	+	ND	ND	ND	Heterotypic	[21]
Prm1	5 TM	*S. cerevisiae*	Mating cells	3	±	+	ND	ND	ND	ND	[12]
SPE-42	Multispan DC-STAMP-like	*C. elegans*	Sperm	2	+	ND	ND	ND	ND	Heterotypic	[22]
DC-STAMP		*M. musculus*	Dendritic cells, macrophages, osteoclasts	3	+	±	ND	ND	ND	Heterotypic	[23,93]
SPE-9	EGF repeats	*C. elegans*	Sperm	4	+	+	ND	ND	ND	Heterotypic	[18,94]
EGG-1; EGG-2	LDL repeats	*C. elegans*	Oocyte	4	+	+	ND	ND	ND	Heterotypic	[24]

[a]The fusogenic score (0–10) was calculated using the following scoring system: + (requirement fulfilled) = 2 points, – (requirement not fulfilled) = 0 points, ND (not determined) = 0 points and ± (requirement partially fulfilled) = 1 point.
[b]Abbreviations: homo/hetero, homotypic/heterotypic molecular recognition; TM, transmembrane; utse, uterine seam cells;?, contradictory results.

membrane glycoprotein Izumo was identified using a mouse monoclonal antibody that specifically inhibits sperm–egg fusion [34] (Figure 1c). Izumo is sperm specific, and is detectable on the sperm surface only after sperm have undergone the acrosomal reaction [35]. Whereas *Izumo*-deficient female mice show normal fertility, *Izumo*-deficient male mice are completely sterile, despite normal mating behavior and normal sperm migration and motility. Accumulation of sperm in the perivitelline space suggests that Izumo is required for fusion but not for adhesion or zona penetration. *Izumo*(−) sperm injected directly into wild-type eggs produced fertilized eggs and normally developed embryos, ruling out an Izumo-induced defect in later development. The human Izumo homolog is also detectable specifically in human sperm. When adding polyclonal antibodies against the human Izumo to a xeno-species fusion system containing zona-free hamster eggs and human sperm, no fusion occurs [35]. Thus, it seems that Izumo represents the best sperm–egg candidate fusogen identified so far, being the first mammalian sperm protein essential for fusion (Table 1). However, the question of whether Izumo is the sperm–egg fusogen requires further investigation.

On the egg plasma membrane, CD9 is a tetraspanin protein family member that has been shown to be essential for fertilization (Figure 1c). Three different laboratories have generated CD9 knockout mice, which show severely reduced fertility (up to 98%) [36–38]. CD9 contains four transmembrane domains and two extracellular loops of unequal size. Many possibilities exist for the involvement of CD9 in sperm–egg fusion. CD9 localizes to the microvillar region of the oocyte [39], the area to which the sperm binds and later fuses with. In CD9 knockout mice, microvilli morphology is impaired and their curvature altered [39]. In addition, it was shown that CD9 associates with the IgSF proteins EWI-2 and EWI-F, which bind to actin filaments in microvilli [31]. This association links CD9 to the actin cytoskeleton network, suggesting a possible function in remodeling of surface curvature, to enable membrane fusion. Although known to associate with IgSF molecules, no connection between CD9 and Izumo has yet been shown (Figure 1c). Recent work has shown that membrane fragments containing CD9 from the oocyte plasma membrane are transferred to the fertilizing sperm found in the perivitelline space [40]. This intriguing phenomenon might be important for reorganizing membrane domains on the sperm necessary for the fusion process.

Another link between the tetraspanin CD9 and the fusion machinery comes from *in vitro* assays in which knocking down expression of CD9 and an additional tetraspanin, CD81, resulted in enhanced syncytia formation and viral entry induced by HIV-1 envelope proteins [41], and, in another case, knockdown enhanced fusion of mononuclear phagocytes [42]. Thus, CD9 might be involved in various aspects of the fusion process, with pro- or anti-fusogenic activities in different experimental systems.

Early development

Epithelial fusion

1. Trophoblast fusion in placenta formation Trophoblast fusion in the mammalian placenta can be divided into two modes. In the first, neighboring trophoblasts of the blastocyst undergo cell–cell fusion to form the syncytiotrophoblast (Figure 2a). The second occurs when cytotrophoblasts fuse with the syncytiotrophoblast for expansion and maintenance of the syncytiotrophoblast, which serves as a barrier between maternal and fetal blood vessels [3]. The best characterized candidate fusogen for trophoblast fusion in humans is Syncytin-1, encoded by the HERV-W retroviral element, and belonging to the class I viral fusogens, as HIV [43] (Table 1). Syncytin-1 transfection into various cell lines results in the formation of multinucleate cells [44,45], demonstrating the fusogenic properties of Syncytin-1. Conflicting results have been obtained regarding the cellular localization of Syncytin-1 but the majority of expression seems to be in the syncytiotrophoblast and the cytotrophoblasts [46].

Several receptors for Syncytin-1 have been identified, including the D mammalian retrovirus receptor RDR [44] and ASCT1 [47–49] (Figure 2a). Rat sarcoma cells, which do not fuse when expressing Syncytin-1, showed elevated fusion activity when cotransfected with the RDR receptor, suggesting a heterotypic mechanism of fusion for Syncytin-1 [44]. Although Syncyin-1 inhibition in primary cultures by antisense oligonucleotides [50] and by anti-Syncytin antiserum in human trophoblastic cell line leads to a decrease in cell fusion, it is unclear whether Syncytin-1 is essential for trophoblast fusion *in situ*.

Another, less characterized retroviral gene expressed in cytotrophoblastic cells is Syncytin-2, encoded by the retroviral *env* gene HERV-FRD [51,52]. Transient transfection of cell lines with Syncytin-2 also leads to ectopic fusion [51], suggesting a fusogenic role for Syncytin-2. Syncytin-1 and -2 are found only in highly evolved primates [51], leading to the question of how placenta–trophoblast fusion occurs in other species, such as rodents. Recently, two murine viral envelope genes have been discovered, encoding Syncytin-A and -B [53]. Although evolutionarily distinct from the genes encoding Syncytin-1 and -2, both proteins display fusogenic activity following expression in transfected cells. No receptor has yet been identified for Syncytin-A and -B [53]. As occurs in the case of Syncytin-1, inhibition of Syncytin-A by antisense oligonucleotides and anti-Syncytin-A antiserum in culture leads to a decrease in cell fusion [54]. Structural and functional studies of Syncytin-A show that the heptad repeats region can form a stable α-helical complex, typical of class I viral fusion proteins [55].

It seems that at least twice during evolution, retroviral genes have adapted independently to perform apparently similar functions in trophoblast fusion. The mysterious existence of two independent Syncytins, in two different species, poses an intriguing question: is there a specific requirement for two proteins, each complementing the fusogenic activity of the other, or is the dual existence a fail-safe mechanism for such an important process? In summary, Syncytins in rodents and primates are related to retroviral glycoproteins from the class I viral fusogens and are sufficient to fuse heterologous cells in culture. To determine whether mouse Syncytins are also essential for trophoblast fusion, detailed analyses of knockout mice of both Syncytins is required (Table 1).

2. C. elegans skin formation In *C. elegans*, epithelial cells start forming the syncytial hypodermis 340 min after the first cleavage [4]. The hypodermis is the outer layer of the body, which establishes the body shape, secretes the cuticle and stores nutrients ([4] and the Wormatlas website, http://www.wormatlas.org). Many cells in the hypodermis form large syncytia; for example, 23 hypodermal cells fuse during embryonic development and 116 additional cells fuse to this giant syncytium during larval development (Figure 2b). Genetic screens for fusion failure resulted in the identification of EFF-1, a type I membrane protein essential for epithelial cell fusion [15]. In *eff-1* mutants, most of the fusion events do not occur, affecting the elongation and motility of larvae and adults. Further research demonstrated that *eff-1* is also sufficient to induce embryonic and postembryonic cell fusions in *C. elegans* [56,57]. Moreover, expression in heterologous tissue culture cells results in ectopic fusion and the formation of multinucleate syncytia [58]. Unlike viral fusion and Syncytin-mediated fusion, which promote fusion through a heterotypic mechanism, it was demonstrated that *eff-1* is required for fusion in both fusing cells, both *in vivo* and in tissue culture [58]. In addition, green fluorescent protein-tagged EFF-1 has been shown to accumulate at the contact zone between fusing cells, before fusion [57]. Meeting four of the five gold standards for proteins involved in membrane fusion, EFF-1 is most likely to be a genuine fusogen (Table 1; Box 1).

Late development

Anchor cell fusion in C. elegans

In addition to the hypodermis, fusion was observed in the formation of the vulva, excretory gland, male tail, anchor cell (AC), uterus and pharynx [4,59–61]. In the postembryonic stages, *eff-1* is required for fusion of additional epithelial cells, such as the hypodermis and the seam cells, most of the vulval cells and cells of the tail [15]. However, *eff-1* is not required for fusion of the AC to the uterine seam cell (utse) or hymen [16,56]. In *C. elegans*, the AC is responsible for establishing the physical connection between the uterus and the vulva. Serving as an 'organizing center', the AC induces cell fate change, and differentiation of the vulva precursor cells and uterine cells [59,62]. Uterine–vulval continuity is further established by fusion of eight uterine π–cell daughters, generating the utse syncytium, and fusion of the AC to this syncytium leads to the formation of a thin hymen layer between the vulva and the uterus [59,60] (Figure 2c). The first laid egg ruptures this hymen, establishing a direct vulval–uterine connection. A genetic screen for egg-laying defects identified AFF-1, a type I transmembrane protein [16]. AFF-1 is essential for AC fusion, for fusion of the vulval rings vulA and vulD, and for fusion of the hypodermal seam cells into a continuous row, forming two extended seam cell syncytia on the lateral sides of the animal. Similarly to EFF-1, AFF-1 expression in transfected Sf9 cells is sufficient to induce multinucleate cell formation (Table 1). AFF-1 and EFF-1 are homologs with moderate sequence conservation but they share a remarkable putative structural conservation, in that all 16 cysteines and 11 out of 22 proline residues are conserved in their ectodomains. Together, EFF-1 and AFF-1 represent the founding members of a family of developmental fusogens, mediating cell–cell fusion [16]. As for the Syncytins, it is not clear why two fusogens are required to perform similar fusion events. It will be interesting to see whether the constitution of the FF family will lead to the discovery of additional family members that fuse cells in different organisms.

Continuous development, tissue remodeling and repair

Myoblast fusion

During myogenesis in *Drosophila*, mononucleate myoblasts fuse to form multinucleate muscle fibers (Figure 3a). This process involves 'founder cell' myoblasts, and 'fusion competent myoblasts' (FCM). The founder cell functions as the organizer of muscle formation, attracting the FCMs, which migrate toward it and, after recognition, fuse with the founder cell. Embryonic myoblast fusion starts with the founder cell fusing with one to two FCMs, to generate a muscle precursor. In the subsequent steps, FCMs fuse with the precursors, to generate multinucleate myotubes [63]. Extensive reviews of *Drosophila* myoblast fusion are provided by Chen *et al.* [1] and Horsley and Pavlath [2].

Using electron microscopy, Doberstein *et al.* [28] identified an intermediate step in myoblast fusion of paired vesicles that align on apposing membranes before fusion. It has been proposed that the myoblast fusogen resides within those vesicles, and localizes to the plasma membrane before membrane fusion [28,64]. Recently, Estrada *et al.* [65] identified Singles Bar, a candidate protein required for fusion of the paired vesicles to the plasma membrane.

Several IgSF proteins have been found to be required for myoblast fusion in *Drosophila*: Dumbfounded (Duf), also called Kin of Irregular chiasm C (Kirre) and Roughest (Rst), also called Irregular chiasm C (IrreC), in the founder cell, and Sticks and stones (Sns) in the FCM [66–68]. Although single mutations of Duf and Rst show that they are not essential for myoblast fusion, when both proteins are mutated, severe myoblast fusion defects are observed [67], suggesting redundant function in myoblast formation. Sns was shown to be essential for cell fusion [68], and to interact with Duf *in vitro* [69]. However, expression of Sns, Duf and Rst in S2 culture cells mediates adhesion and aggregation but not fusion, suggesting that Duf, Rst and Sns function by mediating recognition, attraction or tight binding rather than cell fusion [69]. To date, no candidate proteins have been found to be both necessary and sufficient for myoblast fusion (Table 1).

Recently, more light has been shed on the mechanism of myoblast fusion in *Drosophila*, linking myoblast fusion and cytoskeleton remodeling [64,70], through D-WIP/Sltr and WASp–ARP2/3 association and F-actin polymerization. D-WIP/Sltr is recruited to the sites of myoblast fusion and regulates the actin polymerization machinery.

Similarly to *Drosophila*, mammalian muscle development is also a multistage process, in which mononucleate myoblasts fuse with one another to form nascent myotubes. Multinucleate myotubes undergo additional rounds of fusion, leading to an increase in size and maturation [2]. As for the sperm–egg fusion, many proteins have

been implicated in myoblast fusion, including members of the ADAM family, integrins and various adhesion molecules. None of these proteins, however, have been shown to be essential for myoblast fusion, and knockout of most of them in mice revealed no apparent musculature defects [71–75]. Recently, Kirrel, a homolog of *Drosophila* Duf/Kirre and Rst/IrreC, has been shown to be required for myoblast fusion in Zebrafish [76]. In morpholino-injected embryos directed against Kirrel, a large number of unfused mononucleate monocytes were detected. These findings reveal an evolutionary conservation in the regulation of muscle fusion. Nonetheless, the myoblast fusogen remains to be identified.

Macrophage fusion: multinucleation of osteoclasts

Macrophages fuse and differentiate to form multinucleate osteoclasts (bone-resorbing cells) in bones or giant cells in different tissues (Figure 3b). Both types of syncytia have resorption abilities, such as resorption of the mineralized matrix of the bone or of invading pathogens, and thus have an important role in bone remodeling and immune defense [1]. Several proteins have been identified that have a role in macrophage fusion. Macrophage fusion receptor (MFR) and its ligand CD47 are both IgSF cell-surface proteins. Whereas MFR expression rises before fusion, the levels of CD47 remain constant [1,77]. An extracellular peptide form of CD47 binds to macrophages, associates with MFR and prevents multinucleation [78], and the extracellular domain of MFR prevents fusion of macrophages *in vitro* [77]. However, no further evidence exists to support a direct involvement in the fusion machinery. CD44 is a hyaluronan receptor adhesion protein involved in osteoclast fusion. It is also expressed strongly at the onset of fusion, and antibodies against CD44 were shown to inhibit osteoclast formation [79], although knockout mice do not support a direct involvement in the fusion process [80]. Recently, a seven-transmembrane protein, DC-STAMP, was found to be required for cell fusion of osteoclasts and macrophage giant cells. DC-STAMP knockout mice do not form multinucleate osteoclasts, and show increased bone mass (less bone-resorbing activity) and a mild osteopetrosis phenotype [23]. DC-STAMP was shown to be necessary in only one of the fusing cells for fusion to occur. However, DC-STAMP localization to the plasma membrane during fusion, and the identification of a ligand, remain to be explored (Figure 3b; Table 1). Despite the present lack of definitive evidence, such as fusion of heterologous cells, DC-STAMP is emerging as a promising candidate for the macrophage and osteoclast fusogen.

Concluding remarks and future directions

Cell fusion failure in humans might be associated with diseases such as myopathies, osteopetrosis, infertility and preeclampsia. However, the involvement of mutated candidate fusogens in the pathogenesis of these diseases is speculative. The potential therapeutic applications of cell fusion in tissue repair using stem cells, and in the treatment of some cancers, are controversial [81,82] and require fusogen identification and mechanistic characterization before we can understand and modulate these physiological and pathological cell fusion events.

Currently, there are two major models by which genuine fusion proteins (fusogens) function. The first model is based on studies of viral fusogens belonging to three structural classes. Fusogens of enveloped viruses form 'hairpins', which 'fold back' through diverse conformational changes to bend and eventually fuse the membranes. Figure 4a shows a simplified model for class I and retroviral fusogens. Fusogen activation involves a conformational change in which coiled coils cause the fusogens to become erect, resulting in the insertion of the amphipathic fusion peptides or loops into the target membrane. Folding of the trimeric fusogens results in the formation of six-helix bundles, which bend the membranes toward each other, leading to hemifusion (fusion of the outer leaflets) followed by a complete merger of the membranes. Viral fusogens from classes II and III form hairpin structures using different structural intermediates [83]. Figure 4b shows the second model for the merger of intracellular membranes by the formation of heterotrimeric tSNAREs. This assembly induces 'zippering' – that is, approach of the membranes followed by hemifusion, and complete merger of the bilayers without the insertion of amphipathic peptides [84]. Based on these models (Figure 4a,b), we suggest three alternative hypothetical FF homotypic fusion mechanisms. The first model involves fusion peptide insertion and hairpin formation (Figure 4c). The second hypothesis comprises zippering of FF proteins with or without amphipathic fusion peptides (Figure 4d). A third model (not shown) involves a combination of the previous two (hairpins–zippers). Finally, it is conceivable that FF-mediated fusion follows a completely different mechanism. Dynamic structural analyses of FF proteins during fusion, combined with the identification of functional motifs in these fusogens, will help to define the mechanism of FF-mediated cell fusion. This cell fusion process might serve as a paradigm for the yet unidentified developmental fusogens. Because this mechanistic characterization is complex, it should be implemented only on strong fusogen candidates, fulfilling five gold standards (Box 1):

(i) Is it essential for fusion *in vivo*?
(ii) Is it expressed at the time and place of fusion?
(iii) Is the protein sufficient to fuse cells that normally do not fuse *in situ*?
(iv) Can the protein fuse heterologous cells in tissue culture?
(v) Can the protein fuse liposomes *in vitro*?

Because developmental cell–cell fusogens remain, with the exception of Syncytins and FFs, hypothetical and undiscovered, it is conceivable that the molecular mediators of cell membrane fusion are not only hidden, but might also be completely different from the known viral and intracellular membrane-fusion proteins. Modifiers of membrane lipid composition and proteolipid channels have been proposed as alternative mediators of membrane fusion [85,86]. However, we believe that the evidence for viral, intracellular and developmental fusogens support the hypothesis of a unified concept in which viral-like or SNARE-like proteins fuse biological membranes through hairpins, zippers, hairpins–zippers or novel fusogenic mechanisms.

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