CHAPTER 9

Eukaryotic Cell–Cell Fusion Families

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I. OVERVIEW

The capacity of cells to unite in a regulated fashion, through fusion of their plasma membranes, is essential for reproduction and development in eukaryotes. Yet, the majority of proteins that mediate this process (i.e. fusogens) remain unidentified or uncharacterized. The Fusion Family (FF) and the Syncytins are the first families of cell-cell fusogens. The FF fusion proteins are required in both fusing cell membranes and their structure reveals the formation of flower-like super-complexes. The Syncytins were recently acquired from retroviruses and are similar to the Env glycoprotein of HIV. While there are apparent similarities between these families, their mechanisms are different. These similar and disparate principles may be relevant to other cell fusion events. Thus, FF proteins and Syncytins serve as paradigms for the mechanisms of eukaryotic cell fusion and provide insight to the ongoing quest for the missing fusogens.

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II. INTRODUCTION

Omnis cellula e cellula (all cells are derived from cells) conventionally reflects the significance of cell division to cell biology. However, this key concept is rarely considered in the context of multinucleated or hybrid cells, that rise through fusion of two or more cells into one cell. Cell fusion is critical for many developmental processes such as, fertilization, muscle development, bone maintenance, and immune function in humans (Brodbeck & Anderson, 2009; Helming & Gordon, 2009; Pavlath, 2010; Rochlin, Yu, Roy, & Baylies, 2010; Schejter & Baylies, 2010; Sutovsky, 2009; Vignery, 2000, 2005, 2008). It has also been implicated in neuronal function, stem-cell reprogramming, and carcinogenesis (Ambrosi & Rasmussen, 2005; Lu & Kang, 2009; Oren-Suissa, Hall, Treinin, Shemer, & Podbilewicz, 2010; Rodic, Rutenberg, & Terada, 2004). Accordingly, cell–cell fusion is necessary for embryonic and postembryonic development. While it is evident that cell-to-cell fusion occurs in most eukaryotes, the molecular mechanism is not well-understood. A prerequisite of cell fusion is the capacity to mediate specific and nontoxic membrane bilayer fusion. This process is thought to depend on the function of specialized proteins that act on the bilayers directly (e.g., fusogens). The role of fusogenic proteins is predominantly supported by experimental evidence from a plethora of viral membrane glycoproteins that mediate virus–target–cell fusion or cell-to-cell fusion following infection (Backovic & Jardetzky, 2009; Barry, Key, Haddad, & Duncan, 2010; Clancy, Barry, Ciechonska, & Duncan, 2010; Harrison, 2005, 2008; Haywood, 2010; Kielian, 2006; Martens & McMahon, 2008; Sapir, Avinoam, Podbilewicz, & Chernomordik, 2008; Weissenhorn, Hinz, & Gaudin, 2007; White, Delos, Brecher, & Schornberg, 2008). Additional support for the roles of proteins in membrane fusion is found in protein-mediated membrane fusion that occurs between intracellular membranes (Hughson & Reinisch, 2010; Jena, 2009; Scott & Youle, 2010; Westermann, 2010; Wickner & Schekman, 2008). In consequence, the search for the missing cell–cell fusogens in eukaryotes relies on insights from these models in conjunction with a classical forward genetics approach (Bate, 1990; Baylies, Bate, & Ruiz Gomez, 1998; Chen, Grote, Mohler, & Vignery, 2007; Chen & Olson, 2005; Onel & Renkawitz-Pohl, 2009; Oren-Suissa & Podbilewicz, 2007, 2010; Rochlin et al., 2010; Sapir et al., 2008; Schejter & Baylies, 2010). Moreover, many resident *e*ndogenous *r*etro-*v*iruses (ERVs) can be identified in the genomes of eukaryotes. Human ERVs (HERV) make up 8% of the human genome, of which, 18 HERV elements have been shown to encode intact viral envelope glycoproteins (ENVs) that can potentially mediate cell–cell fusion upon expression (Bannert & Kurth, 2004; Benit, Dessen, & Heidmann, 2001; Blaise, de Parseval, Benit, & Heidmann, 2003; Blaise, de Parseval, & Heidmann, 2005; de Parseval & Heidmann, 2005; de Parseval, Lazar, Casella, Benit, & Heidmann,

2003; Villesen, Aagaard, Wiuf, & Pedersen, 2004). This line of evidence lead to the identification of Syncytin as a functional fusogenic glycoprotein derived from a provirus (Blond et al., 1999; Mi et al., 2000). Syncytin is but one example of a retroviral fusogen that has been selectively preserved while the *gag* and *pol* genes accumulated inactivating mutations. Nevertheless, the biological significance of ERV-derived ENVs in the evolutionary acquisition of cell fusion is unclear. With the exception of these virus-derived fusogens, the only other identified family of fusogens is the ''fusion family'' (FF) from *Caenorhabditis elegans* (Avinoam et al., 2011; Sapir et al., 2007, 2008). Thus, the large majority of fusogens remain unidentified or uncharacterized. Here, we review the chronological steps leading to the identification and characterization of these founding families of eukaryotic cell–cell fusogens.

III. CHOOSING A MODEL SYSTEM: *C. ELEGANS* AS AN ORGANISM TO STUDY CELL–CELL FUSION

Since its introduction in 1963 by Sydney Brenner, *C. elegans* proved to be an outstanding model system to study biological processes. Distinctively, when the detailed cell lineage was completed by John Sulston and collaborators, in the 1980s, it became apparent that *C. elegans* is particularly compelling for the study of cell–cell fusion. For the first time, the morphology and development of each of the 959 somatic nuclei could be studied from cell birth to terminal cell fate through the transparent worm and in the context of the whole animal. Between 1970 and 1988 essentially all syncytial tissues were revealed using serial-section electron microscopy (Sulston & Horvitz, 1977; Sulston, Schierenberg, White, & Thomson, 1983; White, Southgate, Thomson, & Brenner, 1986). By the year 1999, 44 syncytial cells, containing 300 different somatic nuclei that account for approximately 30% of the soma, were reported in the *C. elegans* hermaphrodite alone (Podbilewicz, 2000, 2006; Shemer & Podbilewicz, 2003).

A complete and detailed account of all syncytial tissues was essential for further exploration. In 1994 a spatial and temporal account of embryonic and postembryonic cell-to-cell fusion of epithelial cells was generated (Podbilewicz & White, 1994). Since it is not possible to reliably resolve membrane fusion using Nomarski optics, immunofluorescence of an antigen, that is associated with the apical junctions, was used (MH27; Hall, 1996; Labouesse, 1997). Developing a method to follow the membranes and adding live-temporal-data proved critical to facilitate a forward-genetics approach to seek out the missing fusogens. In 1995, the vital fluorescent membrane probe FM 4–64 was introduced, providing the means for live imaging of cell fusion *in vivo* (Mohler, Simske, Williams-Masson, Hardin, & White, 1998; Vida & Emr, 1995). In

parallel, a transgenic worm strain, expressing a reporter construct combining the AJM-1 apical junction protein (MH27 antigen) with GFP, was constructed (AJM-1::GFP). These reporters were coupled to transmission electron microscopy (TEM) to generate an in-depth analysis of fusion in the epithelia of *C. elegans*. It was observed that fusion begins at a single location on the apical cell membrane and spreads both laterally and basally, reminiscent of viral fusion pores (Chernomordik & Kozlov, 2003, 2005; Chernomordik, Zimmerberg, & Kozlov, 2006; Earp, Delos, Park, & White, 2005; Mohler et al., 1998). Although this apical fusion pore could form at any position along the length of the membrane the correlation with the apical junction marker revealed that the site of initiation was either at or directly apical to the cell junction. In contrast, other studies in *C. elegans* embryos and in cells in tissue culture, support the model in which several fusion pores may form simultaneously and then coalesce as they expand (Cassata et al., 2005; Chen et al., 2008; Gattegno et al., 2007). In addition, since an accumulation of vesicles was observed at the site of fusion it was suggested that plasma membrane vesiculation may play a role in the process of pore expansion (Mohler et al., 1998). In 1999, this study was complemented by a detailed description of male-tail and vulva formation that engage several fusion events, necessary for the formation of functional copulation apparatuses. Serial TEM, immunofluorescence (IF), and AJM-1::GFP transgenic worms were used to characterize the sequence of cell–cell fusion events. In the male tail spatial correlation between sites of fusion and apical junction also suggested that fusion initiates at or near the apical junction, however, plasma membrane vesiculation was not observed (Nguyen, Hall, Yang, & Fitch, 1999). Interestingly, this study found sex-specific differences in the timing and sequence of fusion events during tail formation suggesting a sex-specific mode of regulation. Vulva morphogenesis in hermaphrodites also involves a sequence of cell fusions (Kimble, 1981; Sharma-Kishore, White, Southgate, & Podbilewicz, 1999; Sulston & Horvitz, 1977; Sulston & White, 1980). Initially, 5 of the 12 ventral P(n).p cells fuse with the surrounding syncytium (hyp7). Subsequently, three out of six vulva precursor cells (VPCs; P(3,4,8).p) divide and contribute six additional nuclei by fusion to hyp7. Finally, the 22 cells derived from the remaining 3 VPCs (P(5–7).p) migrate and form seven ring-shaped cells. These stacked concentric rings sculpt a tube that connects the uterus with the external world (Kiontke et al., 2007; Kolotuev & Podbilewicz, 2008; Sharma-Kishore et al., 1999). Strikingly, one of the signals that induces the VPCs to adopt the vulval cell fate originates from a cell that is also destined to fuse (anchor cell, AC) (see Section III. C; Newman & Sternberg, 1996; Sharma-Kishore et al., 1999). If the VPCs are not exposed to AC-derived EGF signaling they adopt a different cell fate and fuse to the hypodermis (Sternberg, 2005). A complex pattern of cell fusions, utilized to sculpt a functional tube by forming rings and eliminating cells via fusion, was also observed

in other organs in *C. elegans* such as the pharynx (Albertson & Thomson, 1976; Rasmussen, English, Tenlen, & Priess, 2008; Sulston & Horvitz, 1977; Sulston et al., 1983), the male tail (Nguyen et al., 1999), and uterus (Newman & Sternberg, 1996; Newman, White, & Sternberg, 1996; Sapir et al., 2007). Thus, the evolution and function of cell fusion and organ formation are tightly coupled in *C. elegans* and related nematodes (Felix et al., 2000; Kiontke et al., 2007; Kolotuev & Podbilewicz, 2004, 2008; Louvet-Vallee, Kolotuev, Podbilewicz, & Felix, 2003).

The critical role of cell fusion in the structure and function of organs was also observed in humans where some myoblasts, macrophages, and trophoblasts undergo programmed cell fusion fates during the organogenesis or maintenance of the muscles, bones, and placenta. Therefore, cell-to-cell fusion emerges as a process that complements cell recognition, migration, invasion, apoptosis, and attachment in the morphogenesis of tissues. In order to identify the mediators of cell fusion an efficient screen was required to discriminate between loss-of-fusion phenotypes and wild-type, and to recover mutant progeny specifically affected in somatic cell fusion.

A. *Genetic Screens for Cell Fusion Defects in* C. elegans

Forward genetic screening for fusion defective mutants requires several crucial tools. First, it is necessary to effectively visualize dynamic changes in cell boundaries (Podbilewicz & White, 1994). This can be achieved by using reliable fluorescent membrane markers (Mohler et al., 2002; Shemer & Podbilewicz, 2000). Second, it is important to discriminate between genes encoding proteins, that specifically induce cell fusion and proteins that affect migration, signaling, and attachment or phenotypes associated with variation in cell fate (Mohler et al., 2002; Shemer, Kishore, & Podbilewicz, 2000). In this respect, *C. elegans* is a practical model because cells can be followed from birth to their prefusion state and ultrastructural studies can be performed to determine whether a candidate mutation affected the acquisition of fusion competence or the distinct stages of membrane fusion (Podbilewicz & White, 1994). Additionally, in order to discriminate between the initiation and completion of membrane fusion and to avoid misinterpreting artifacts associated with the dynamic displacement of membrane markers it is necessary to evaluate the level of mixing between fusing cytoplasms (Mohler et al., 1998, 2002). Conventionally, cytoplasmic mixing is evaluated by following the diffusion of fluorescent markers from one cell to the other or by measuring the reaction output generated by the encounter of molecules (e.g., enzyme and substrate) expressed differentially in the two fusing cells (Avinoam et al.,

2011; Hu et al., 2003; Mohler et al., 2002; Podbilewicz et al., 2006; Shemer & Podbilewicz, 2000; Shemer et al., 2004).

B. EFF-1

The possibility to follow cell migration, attachment, and fusion *in vivo* facilitated the endeavor to differentiate between gene functions associated with differentiation and pattering that precede fusion and membrane fusion *per se*. Subsequently, two *e*pithelial *f*usion-*f*ailure (*eff*) mutants were isolated in independent screens and upon failure to complement each other were attributed to the same gene and annotated as *eff-1(oj55)* and *eff-1(hy21)* (Mohler et al., 2002). The publication of the essentially complete *C. elegans* genome in 1998 and a detailed physical map of the genome obtained by Alan Coulson facilitated the mapping and identification of *eff-1* by transformation rescue (Coulson, Sulston, Brenner, & Karn, 1986; The-*C.-elegans*-Sequencing-Consortium, 1998). By 2002, *eff-1* and its promoter were cloned and transgenic animals, expressing an *eff-1* promoter fused to GFP (*eff-1*p::GFP), were generated. The observed green fluorescent cells confirmed that *eff-1* RNA was expressed in most of the known fusing cells and assisted the identification of cells that were unknown to fuse at the time. Most puzzling was the putative expression of *eff-1* in neurons, which was not predicted by previous works (Mohler et al., 2002). In 2004, an elegant assay to test whether *eff-1* is sufficient to fuse cells that normally do not fuse *in vivo* was established. To ubiquitously express *eff-1*, it was placed under the control of an endogenous heat shock inducible promoter (Fire, Harrison, & Dixon, 1990; Jones, Russnak, Kay, & Candido, 1986). This inducible construct was used to generate transgenic animals also carrying the membrane marker AJM-1::GFP, the cytoplasmic marker *eff-1*p::GFP, and the *eff-1* mutant allele *hy21*. Induction by heat shock treatment resulted in fusion of most embryonic cells. Ectopic fusion was also detected in larvae and in adult animals (Shemer et al., 2004). These results taken together suggested that most cells *in vivo* are fusion-competent and that EFF-1 expression in these cells is sufficient to fuse them. In this same study, thin TEM sectioning of wild type and *eff-1* mutant animals revealed that *eff-1* was required both for initiating and expansion of fusion, from an initial pore to complete membrane merger. EFF-1-induced cell fusion *in vivo* was further demonstrated by an independent study (del Campo et al., 2005); del Campo et al. showed that *eff-1* ectopic expression is sufficient to fuse the cells of the early embryo (at the ∼100 cell stage) suggesting that all embryonic cells are fusion competent. This study made two additional contributions by showing both the behavior of an EFF-1::GFP translational fusion construct and the

effect of several mutations on the behavior of EFF-1. However, the dynamic subcellular localization of EFF-1::GFP does not match observations made by IF staining using anti EFF-1 monoclonal antibodies (Valansi, Smurova, Gonzalez, & Podbilewicz, unpublished results). Furthermore, the EFF-1:: GFP construct harbors two point mutations at highly conserved sites, T176A and N529D, and does not rescue *eff-1(hy21)* animals (OA, unpublished results). In addition, the structure–function relationship of putative motifs in *eff-1* (del Campo et al., 2005) remained elusive as mutations mostly resulted in EFF-1 protein misfolding and retention in the endoplasmic reticulum.

The accumulation of evidence suggesting that EFF-1 is a *bona fide* fusogen necessitated the utilization of research tools designed for the characterization of viral fusogens. Consequently, in 2006, *eff-1* was expressed in heterologous insect cells that do not fuse under normal growth conditions (Podbilewicz et al., 2006). *eff-1* expression at the cell membrane was sufficient to fuse these cells. Furthermore, fusion between cells was shown to depend on expression of *eff-1* in both fusing partners. This was shown by using a combination of cytoplasmic markers in Sf9 insect cells and complemented with classical genetic mosaic analysis *in vivo* (in *C. elegans* embryos, larvae, and adults), which yielded a similar conclusion. Researchers also reported that EFF-1 forms complexes on the membrane and that the extracellular domain stimulates fusion when added to cells expressing the full length transmembrane isoforms (Figure 2) (Podbilewicz et al., 2006). These results provided insights into the mechanism of EFF-1-mediated fusion. Most striking is the evidence showing that *eff-1*-mediated fusion proceeds through a hemifusion intermediate. This was deduced from experiments showing that membrane lipid mixing between fusing partners occurred prior to cytoplasmic mixing. Thus, it seems that while *eff-1* displays a novel homotypic mechanism it converges with viral and intracellular fusogens at the molecular level through the conserved hemifusion intermediate (Podbilewicz et al., 2006). More studies are required to determine the structure–function relationship of EFF-1 and to understand its activity at the molecular level. Furthermore, *eff-1*'s role in the nervous system and the function of its alternative-splicing variants (isoforms) require further examination.

The characterization of cell fusion in a variety of *eff-1* null-alleles (*np29* and *ok1021*) suggested that some cells still fuse in the absence of *eff-1* (del Campo et al., 2005; Mason, Rabinowitz, & Portman, 2008; Mohler et al., 2002; Oren-Suissa et al., 2010; Podbilewicz et al., 2006; Sapir et al., 2007; Shemer et al., 2004). These findings coupled to the annotation of EFF-1's sequence provided the basis for sequence similarity searches to other predicted or known proteins in *C. elegans* that may function as fusogens.

C. AFF-1

Two primary observations supported the hypothesis that other fusogens are encoded in the *C. elegans* genome. First, some of the cells that were known to fuse did not express the *eff-1p*::GFP construct, suggesting that EFF-1 was not acting in these cells. Second, these cells reproducibly fused in worms carrying strong loss of function *eff-1* mutations, suggesting that a different fusogen was acting in these cells. Between 2004 and 2006, attention focused on the finding that EFF-1 was not required for sperm–egg fusion and for AC fusion to the gonadal–utse cell that forms the worm hymen (Choi, 2006; del Campo et al., 2005; Podbilewicz et al., 2006; Shemer et al., 2004). At the time, two putative open reading frames with homology to *eff-1* could be identified by sequence comparisons.

The predicted gene *C26D10.7* shares 90% identity and 95% similarity with *eff-1*. It is located directly 12,596 bp upstream and next to *eff-1*. These results suggest that *C26D10.7* represents a gene duplication event that occurred relatively recently in the evolutionary history of *C. elegans*. It had been speculated that *C26D10.7* is a pseudogene (Mohler et al., 2002; Sapir et al., 2007). However, this is unlikely because variations in the DNA sequence occurred mostly in introns suggesting that C26D10.7 is responsive to selection forces.

The predicted gene C44B7.3, also located on chromosome II, showed only moderate homology to EFF-1 (26% identity and 46% similarity) (Mohler et al., 2002; Sapir et al., 2007). In 2003, Choi and Newman performed forward genetic screens for mutants in which the vulva and uterus failed to connect properly. The screens yielded two mutations that demonstrated complete AC fusion failure and defective connection between the gonad and the vulva. The first mutation identified was in the *N*-ethylmaleimide-*S*ensitive-*F*actor (*nsf-1*; Choi, Richards, Cinar, & Newman, 2006) and the second mutation was mapped to C44B7.3 (Sapir et al., 2007). This mutant specifically failed to complement a deletion in C44B7.3 and had the same phenotype as did RNAi directed against C44B7.3 (Sapir et al., 2007). Therefore, the gene was named *aff-1* (*A*C-*f*usion-*f*ailure). Subsequently, it was determined that AFF-1 is also required for fusion of two out of the seven rings that form the vulva, some hypodermal cells in the embryo and the lateral seam cells at the L4 stage. Following *aff-1*p::GFP transcriptional fusion revealed an intricate interplay between *aff-1* and *eff-1* expression which, sometimes occurs simultaneously in adjacent cells, possibly to prevent undesirable fusion (Sapir et al., 2007). Perhaps the most striking examples of this are the adjacent toroidal (ring-shaped) cells of the vulva. In addition, other rings in the pharynx and connecting to the intestine show a similar behavior. pm8 and vp1 are pharyngeal ring-shaped cells that express

AFF-1 and EFF-1, respectively (Rasmussen et al., 2008). Notch signaling in pm8 induces AFF-1 expression while repressing EFF-1. Thus, each cell expresses a different fusogen and fuses to itself to form a ring. Since AFF-1 and EFF-1 are homotypic fusogens, we propose that this is due to posttranslational regulation at the level of protein transport. AFF-1 and EFF-1 may contain transport/sorting signals that result in a different membrane localization (Avinoam et al., 2011; Podbilewicz et al., 2006; Sapir et al., 2007).

EFF-1 and AFF-1 revealed a novel form of cell fusion, namely, auto-fusion (e.g., when a single cell fuses with itself) (Oren-Suissa & Podbilewicz, 2010). Thus, membrane fusogens are not just utilized to sculpt whole tissues, by removing or merging cells, but intricate cell shapes such as toroids can be formed by auto-fusion (Nguyen et al., 1999; Oren-Suissa & Podbilewicz, 2010; Podbilewicz & White, 1994; Rasmussen et al., 2008; Sharma-Kishore et al., 1999). This may implicate membrane fusion in a variety of biological processes not yet completely understood, such as neuronal and immune function, angiogenesis of small capillaries, and long-range cell communication, possibly challenging the common view in these fields.

AFF-1 and EFF-1 display fundamental differences in their activity. For example, ectopic expression of AFF-1 seemed to result in a stronger fusion reaction, both *in vivo* and in heterologous cells (Sapir et al., 2007). This phenomenon was explored in tissue culture by correlating AFF-1 and EFF-1 levels of membrane expression, with their fusogenic activity. It was observed that low levels of AFF-1 on the cell surface are sufficient to mediate robust fusion compared to EFF-1. Consequently, artificially elevating the level of AFF-1 expression on the cell surface resulted in stronger fusion (Sapir et al., 2007).

However different, AFF-1 and EFF-1 also display similarities. AFF-1 expression is also required in both fusing partners (homotypic). This hypothesis is predominantly supported by the observation that *aff-1p::GFP* expression is always observed in at least two partners prior to fusion (e.g., *hyp5*, VulA, VulD, and AC-utse). In addition, we recently showed that AFF-1 can mediate homotypic cell fusion of baby hamster kidney cells (BHK; Avinoam et al., 2011). In summary, AFF-1 and EFF-1 emerge as a novel family of cellular fusogens operating via homotypic interactions between cells to mediate a fusion reaction through a canonical hemifusion intermediate (Podbilewicz et al., 2006; Sapir et al., 2007).

The lack of robust sequence homology between these paralogs and the conserved pattern of cysteins along the extracellular domain, suggest that their function is tightly associated with structure rather than sequence. A similar lack in sequence homology is common among viral fusogens, even those belonging to the same structural class (Roussel et al., 2006; Sapir et al., 2008; White et al., 2008). Therefore, identifying distant FF proteins in other organisms cannot rely on sequence homology alone.

D. Beyond the Nematode Phylum

The *C. elegans* functional paralogs EFF-1 and AFF-1 are the founding members of a cell–cell fusion protein family (Sapir et al., 2007). By 2011, 35 nematode species with putative FF orthologs were identified, suggesting that the FF family is conserved in the phylum nematoda. Remarkably, beyond this phylum putative orthologs could only be identified in two arthropods, a ctenophore, a chordate, and a protist (Fig. 1A) (Avinoam et al., 2011; Sapir et al., 2008). This unique dispersal can be explained by five alternative hypotheses. First, lack of sufficient sequence conservation, poor genome annotation, or coverage. Second, FFs may have been replaced by other fusogens through the course of evolution. Third, DNA or RNA from nematode parasites may have contaminated the genome/EST sequence libraries. Fourth, FF may have independent origins by convergent evolution to similar protein structures that can fuse cell membranes. Fifth, horizontal gene transfer (HGT) may have occurred. It is noteworthy that, the Syncytin family of fusion proteins (see Section IV), was apparently acquired in this manner from viruses and the possibility that FFs share structural similarities with viral fusogens cannot be ruled out (Avinoam et al., 2011; Kielian, 2006) and should be pursued experimentally.

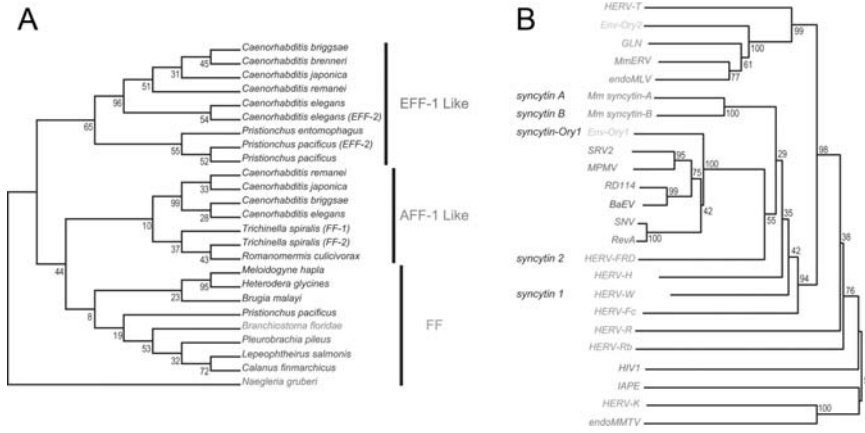

FIGURE 1 Phylogenetic relationships of FFs and Syncytins (A) Phylogenic relationships of FFs (Avinoam et al., 2011). From top to bottom - twenty nematodes, a chordate, a ctenophore, two arthropods, and a protist. [Black - Nematodes, Purple - Chordate, Green - ctenophore, Brown - arthropods, Blue - protest] (B) Phylogenic relationships of ERVs and viruses (Modified from (Heidmann et al., 2009)) are shown. BaEV - Baboon; HERV-T, GLN, HERV-FRD, HERV-H, HERV-W, HERV-Fc, HERV-R, HERV-Rb, HERV-K - Human; MmERV, endoMLV, Mm syncytin-A, Mm syncytin-B, IAPE, endoMMTV - Mouse; Env-Ory2, Env-Ory1 - Rabbit; SRV2, MPMV, RD114, SNV, RevA, HIV1 - Virus [Dark green - Baboon, Light green - Human, Orange - Mouse, Blue - Rabbit, Red - Virus]. (See Color Insert.)

FF proteins share several structural characteristics and a common organization of putative domains (Fig. 2). In general, FFs are type I transmembrane (TM) proteins with an extracellular portion approximately 520–540 aa long that contains four or five regions of elevated sequence homology (R1–R5; Fig. 2). R1 and R5 (CeAFF-1 Arg449–Leu538) are weakly conserved; however, they harbor an even number of conserved cysteines (Cys) that are probably important

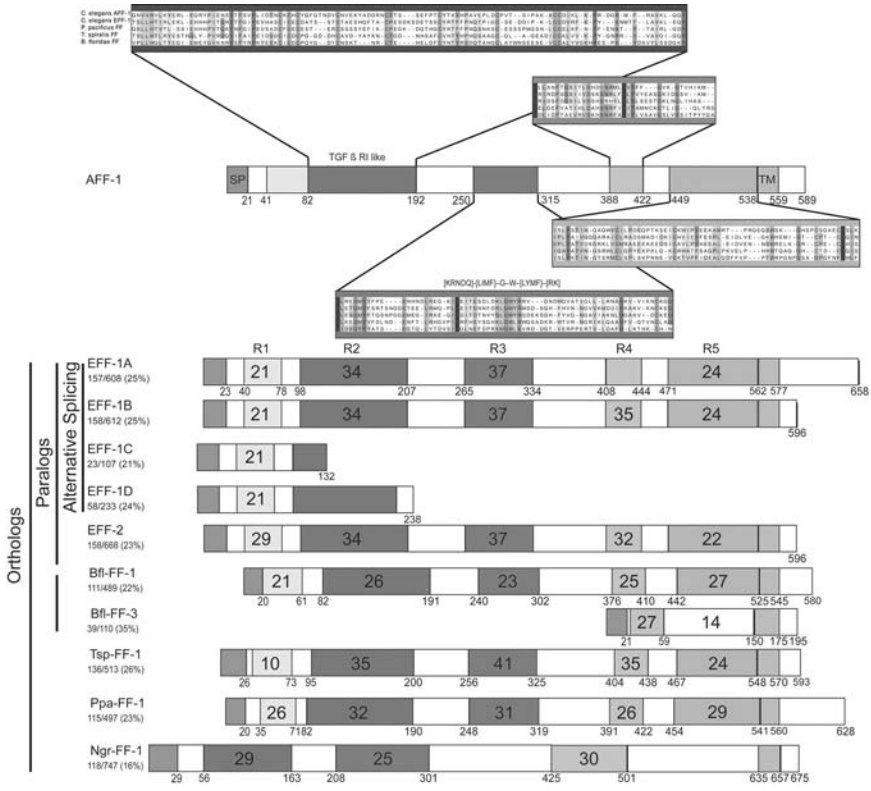

FIGURE 2 Distribution of conserved sequence motifs in FF paralogs and orthologs. Top - schematic representation of the multiple sequence alignment of FF proteins. Alignments of regions R2-R5 of five representative sequences are shown. SP - signal peptide, TM - transmembrane domain. Global sequence identity to AFF-1 is indicated under the name of the protein. Local sequence identity to AFF-1 is indicated within each domain (%). Sequence limits are indicated under the illustration unless identical to previously shown. EFF-2, Bfl- FF-1 and FF-3, Tsp-FF-1, Ppa-FF-1 and Ngr-FF-1 are according to (Avinoam et al., 2011). Multiple sequence alignment of conserved sequence motifs is shown. [Alignment color code was according to the Clustal X color scheme with 40% conservation color increment in Jalview software (Waterhouse et al., 2009). Green - Signal peptide (SP), Pink - R1, Brown - R2, Yellow- R3, Cyan - R4, Purple - R5, Ocher - Transmembrane domain (TM). (Modified from Avinoam et al., 2011).] (See Color Insert.)

for structure and function. R2 (CeAFF-1 Gln82–Pro192) is a Cys-rich domain, R3 (CeAFF-1 Gln250–Gln315) contains a conserved palindromic [KRDENQ]–[LMFI]–G–W–[LYMF]–[RK] pattern, and R4 (CeAFF-1 Ser388–Met422) is moderately hydrophobic (Fig. 2). In addition, *eff-1* encodes four alternative splicing variants (EFF-1 A to D) two of which lack the transmembrane domain (EFF-1 C and D). Both EFF-1C and EFF-1D lack R3–R5, conversely, while EFF-1D contains R1 and R2, EFF-1C only contains R1 and a part of R2. In addition, the *Branchiostoma floridae* genome encodes a truncated form containing a membrane anchored R4 (Fig. 2). It was shown that the two transmembrane isoforms of EFF-1 (EFF-1A and B) can fuse heterologous insect cells in culture, while the EFF-1C secreted isoform has no fusogenic activity (Podbilewicz et al., 2006). The role of these alternative isoforms in *C. elegans* is still unknown and the possibility remains that some are not involved in cell fusion.

To complete the picture, FFs from the human parasitic nematode *Trichinella spiralis* (*Tsp-ff-1*) and the chordate *B. floridae* (*Bfl-ff-1*) have been shown to induce fusion of BHK cells (Avinoam et al., 2011). TSP-FF-1 and BFL-FF-1 share only 26% and 22% sequence identity with AFF-1, respectively. *C. elegans* and *T. spiralis* are among some of the most distant species within nematoda (Mitreva & Jasmer, 2006). These results taken together suggest that FFs are functional orthologs that mediate cell fusion in different organisms. Furthermore, unidentified FFs may turn out to be the missing eukaryotic fusogens (Avinoam et al., 2011).

E. Are FFs Sufficient to Fuse Isolated Membranes?

By biochemical standards to define a protein as a fusogen it must be able to fuse isolated membranes *in vitro*. The experimental design should assure that the protein acts directly on the membranes themselves without additional cellular cofactors (Oren-Suissa & Podbilewicz, 2007). One approach is to reconstitute the candidate fusogen into liposomes and measure membrane fusion. An alternative approach, which has been extensively used in this context, is to express a candidate fusogen on the surface of a heterologous viral envelope. This process is generally referred to as pseudotyping (Fukushi et al., 2005; Matsuura et al., 2001; Schnell, Buonocore, Kretzschmar, Johnson, & Rose, 1996; Takada et al., 1997; Zavada, 1972). If a candidate fusogen is efficiently incorporated into the viral envelope and mediates fusion of the virus to target cells then the interpretation would be that it functions independently and directly on the membranes themselves. A cellular fusogen has never been shown to complement a virus probably because it is difficult to imagine that it would incorporate into a viral envelope. Furthermore, it is unknown whether viral–cell and cell–cell fusion share similar mechanisms.

Vesicular stomatitis virus (VSV) has been widely used to produce pseudotyped viruses presenting a variety of exogenous viral glycoproteins. This is likely because VSV can incorporate other membrane proteins into its envelope (Lodish & Porter, 1980; Schnell et al., 1996; Takada et al., 1997; Tamura et al., 2005). Recently, VSV pseudotyped viruses presenting AFF-1 and EFF-1 were generated. Both FF proteins were efficiently incorporated into the VSV envelope and mediated homotypic virus–cell fusion (Avinoam et al., 2011). AFF-1 pseudotyped viruses also infected EFF-1 expressing cells. This heterotypic interaction between AFF-1 and EFF-1 was independently demonstrated by showing that BHK cells expressing AFF-1 can fuse with cells expressing EFF-1 (Avinoam et al., 2011).

Virions also appear to be an attractive platform to study the ultrastructure of surface proteins. AFF-1 pseudoviruses were examined by negative stain transmission electron microscopy (N-TEM), cryo-TEM, and cryo electron tomography (Avinoam et al., 2011). These analyses revealed that AFF-1 forms distinct supercomplexes, seen as bulky spikes that resemble flowers or rosettes (Fig. 3A). These complexes are reminiscent of assemblies previously observed on viruses and are probably important for the fusogenic activity of these proteins (Gibbons et al., 2003).

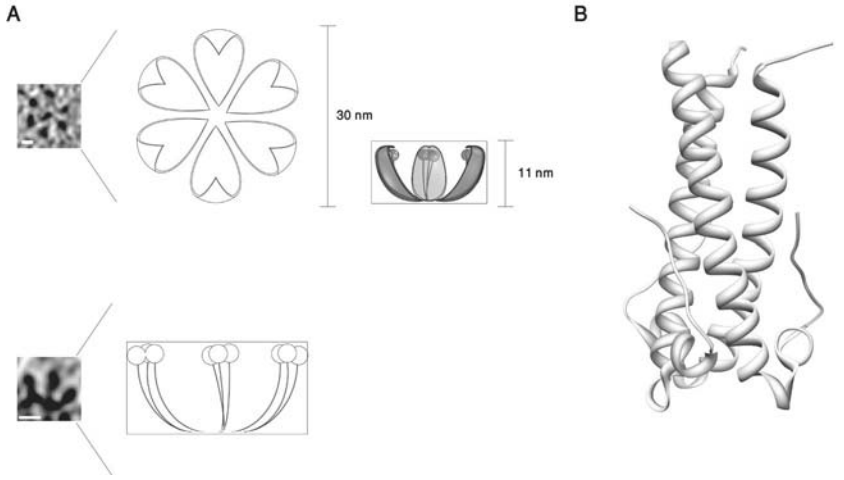

FIGURE 3 Structures of AFF-1 and Syncytin-2. (A) Model of AFF-1 based on the cryo electron tomography. Putative AFF-1 trimers form rosettes that resemble hexameric flowers. Top (left) and side view (right and bottom) (modified from Avinoam et al., 2011). (B) Ectodomain of the transmembrane subunit (residues 391–443) of Syncytin-2 based on chimera rendering of PDB 1Y4 M (Pettersen et al., 2004; Renard et al., 2005). Molecular graphics image was produced using UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco.

To summarize, AFF-1 and EFF-1 can fuse viral envelopes to cells. Fusion is dependent on the presence of either AFF-1 or EFF-1 in both fusing membranes suggesting that FF-mediated fusion depends on homotypic interactions between fusogens. These results further suggest that FF proteins share some similarities with viral fusogens, at least at the ultrastructural level. However, it remains to be seen whether these families are linked by convergent or divergent evolution.

F. *What Other Functions can Fusogens Perform? The Role of* eff-1 *in the Nervous System of* C. elegans

The possible implications of the surprising observation made in 2002 that *eff-1* RNA may be expressed in neurons, was not immediately realized. It is possible to speculate that this was due to the difficulty in conceiving the potential role that a cell–cell fusogen might be playing in the nervous system. The question of fusion of neuronal processes has received little attention so far and the potential functions of fusogens in sculpting membranes independently of fusion was not predicted. In addition, *aff-1* also showed neuronal expression with no noticeable overlap with *eff-1*. In 2010, a study describing the function of EFF-1 in four specialized mechanosensory neurons in *C. elegans* called the PVDs and FLPs was published (Oren-Suissa et al., 2010). The PVDs are an attractive model system to explore neuronal phenotypes. The PVDs are mechanoreceptors comprised of repetitive menorah-like structures containing stereotypic 30–80 nm (diameter) dendritic extensions or branches. The branches of the menorahs show right angle turns in wild-type animals. In *eff-1* mutant-animals these ordered structures are disrupted by excessive branching and variable turn angles. EFF-1 is expressed in the PVDs and its ectopic expression in *eff-1(hy21)* mutants partially rescues the disordered and excessive branching phenotypes. Strikingly, an increased dose leads to the reverse phenotype of hypobranching (reduction in the number of branches). Therefore, EFF-1 functions in a cell autonomous manner in the PVD neurons to restrict branching. Serial section TEM in wild-type *C. elegans* demonstrated that terminal dendrites at the ends of menorahs autofuse forming grids. The mechanosensory function of these ''autofused dendrites'' has not been determined. This is the first demonstration that neuronal branches fuse in wild-type animals (Oren-Suissa et al., 2010). By following the behavior of these extensions using three-dimensional live imaging, it was also observed that the dendritic branches undergo fission, retraction, and fusion. These processes depended on EFF-1 although its primary function in the context of the PVDs seemed to be retraction and not fusion (Oren-Suissa et al., 2010). Thus, FF proteins may yet reveal novel roles that fusogens fulfill; to sculpt cell membrane in parallel to fusion. These findings may also call for the reexamination of conventional doctrines concerning neuronal interconnectivity. The

reticular theory conceived by Camillo Golgi over a hundred years ago, which suggested that neuronal processes fuse to form an elaborate syncytium may have been neglected prematurely. It is still quite possible that neurite–neurite fusion or its membrane intermediates play a role in neuronal function.

IV. SYNCYTINS: AN EXPANDING METAZOAN FUSION FAMILY

In mammals, several tissues and cell types are known to rely on cell–cell fusion for their function. Research has mostly focused on cell fusion that occurs between gametes during fertilization, myoblasts during muscle fiber formation, macrophages during bone maintenance, and trophoblasts during placenta development (Blobel, Myles, Primakoff, & White, 1990; Brodbeck & Anderson, 2009; Helming & Gordon, 2009; Huppertz, Bartz, & Kokozidou, 2006; Huppertz & Borges, 2008; Oren-Suissa & Podbilewicz, 2007; Pavlath, 2010; Primakoff & Myles, 2007; Rochlin et al., 2010; Schejter & Baylies, 2010; Shemer & Podbilewicz, 2000; Sutovsky, 2009; Vignery, 2000, 2005, 2008; Yanagimachi, 1988). The placenta is a vascular organ that develops in gestating females of most mammals, except marsupials and monotremes (Mess & Ferner, 2010). It connects the developing fetus to the uterine wall, thus, allowing nutrient uptake and waste elimination through the mother's circulation. Regardless of the compound difficulties that arise during the cytological analysis of an adult organ *in vivo*, it is the placenta (syncytiotrophoblast fusion) that emerges as an accessible organ for which the fusogens are well characterized (Huppertz et al., 2006; Huppertz & Borges, 2008; Knerr et al., 2004; Orendi, Gauster, Moser, Meiri, & Huppertz, 2010; Potgens, Drewlo, Kokozidou, & Kaufmann, 2004; Stoye, 2009).

The discovery of the syncytiotrophoblast is somewhat unique because it stands as one of the earliest accounts of multinucleated cells, dating back to the early decades of the nineteenth century. The placenta, as well as several other histological accounts, provoked the original question of whether these cells originate from successive mitoses without concomitant cytoplasmic divisions or from fusion of preexisting mononucleated cells. The emergence of electron microscopy techniques in the late 1930s and early 1940s, and their subsequent application in the study of biological material confirmed the syncytial nature of the syncytiotrophoblast. However, it was unclear whether fusion was involved until the 1970s (Boyd & Hughes, 1954; Carr, 1967; Cavicchia, 1971; Rhodin & Terzakis, 1962).

The molecular characterization of cell fusion did not advance significantly until the late 1990s when replication-competent *H*uman *E*ndogenous *R*etro-**V**iruses (HERV) were implicated in cell fusion. In 1998, Blond et al., found that a full length *env* gene, captured in the human genome, HERV-W, showed specific placental expression (Blond et al., 1999). This expression pattern was confirmed in parallel by an additional independent study (Mi et al., 2000). The

possibility, that an ancient viral sequence, incorporated into the germ cell genomes of our ancestors, and adapted from its original function, was perceived immediately (Blond et al., 1999, 2000; Mi et al., 2000). Moreover, the fact that HERV-W encoded a putatively active envelope protein (ENV), that normally mediates viral fusion to target cells, suggested some role in the formation of the syncytiotrophoblast. Thus, HERV-W became the leading candidate fusogen of the syncytiotrophoblast and was hence called *Syncytin* (Mi et al., 2000). Further studies showed that *Syncytin* was sufficient to induce cell-to-cell fusion in several cell lines suggesting that it is capable of fusing plasma membranes of individual cells to form syncytia (Blond et al., 2000; Mi et al., 2000). *Syncytin* also mediates fusion of pseudotyped immunodeficiency viruses to target cells (Dewannieux, Blaise, & Heidmann, 2005). These results taken together suggest that *Syncytin* retained a structure and function that allows it to be efficiently incorporated into viral envelopes and mediate virus binding and fusion. In addition, antisense knockdown of *Syncytin* in primary cytotrophoblast cells reduces fusion (Frendo et al., 2003). This result demonstrates that *Syncytin* is necessary, at least in part, for fusion of cytotrophoblast cells.

Syncytins and class I viral fusogens share similar structures and undergo the same characteristic proteolytic processing; hence, it is considered as a *bona fide* class I viral fusogen directly descendant from viruses (Figs 1B and 3B) (Chang, Chen, Chang, Huang, & Chen, 2004; Cheynet et al., 2005; Gong et al., 2005; Renard et al., 2005).

In 2003, *Syncytin*-2 was discovered and *Syncytin*, now *Syncytin*-1, became the founding member of a family of virus-captured Hominoid fusogens (HERV-FRD; Blaise et al., 2003). *Syncytin*-1 and *Syncytin*-2 penetrated the primate genome recently, >25 and >40 million years (MY) ago, respectively; after the divergence of hominoids and old world monkeys (Goodman et al., 1998; Kim, Takenaka, & Crow, 1999; Voisset et al., 1999). Surprisingly, syncytial trophoblasts had also been observed in Muridae and Leporidae and *Syncytin* related genes such as *Syncytin* A, B (mouse), and *Syncytin*-OryI (Rabbit) have been identified (Fig. 1B) (Dupressoir et al., 2005; Heidmann et al., 2009). *Syncytin* A and B appear to be conserved in all Muridae and it is estimated that they entered the genome 20 MY ago. They are expressed at the time and place of cytotrophoblasts fusion and their expression is sufficient to fuse cells in culture (Dupressoir et al., 2005). *Syncytin* A antisense-knockdown and knockout mice display severe morphological defects in the maternal–fetal interface (Dupressoir et al., 2009; Gong et al., 2007). Taking into account its fusogenic function, these results strongly suggest that it is essential for syncytiotrophoblast morphogenesis in mouse. The discovery of two murine and one leporid ERVs, homologous but not orthologous, to the human *Syncytin* genes confirms that the acquisition of fusogens from viruses occurred repeatedly through the course of evolution. It is particularly noteworthy that despite the mechanistic differences between FFs

TABLE I
Comparison between Eukaryotic Cell–Cell Fusion Families

	Syncytins	FFs
Type I membrane glycoprotein	+	+
Secondary structure	Mainly alpha helices	Mainly beta sheets
Viral-class similarity	I	II, III, or novel
Mode of action	Unilateral (receptor mediated)	Homotypic
Hemifusion intermediate	+	+
Sequence similarity	HIV ENV	None
Loss of function phenotype (essential)	Maternal–fetal interface structural defects	Fusion failure in *C. elegans*
Gain of function phenotype (sufficient to fuse heterologous cells)	+	+
Can substitute a fusogen in a pseudotyped virus and mediates virus–cell fusion	+	+

and viral fusogens (homotypic/bilateral vs. unilateral mechanisms), it seems that the two major fusogenic families, FF proteins and *Syncytins* are viral-like or viral derived (Table I). This may open up new research avenues for future evolutionary and mechanistic studies. Do FFs also have viral origins or have they evolved separately and converged to similar structures? Are the missing fusogens in other systems, viral-like, or did novel mechanisms evolve? Furthermore, *Syncytins* represent 2 out of 16 coding *env* genes currently known in the human genome and *Syncytin*-mediated fusion has not been ruled out in other tissues (Soe et al., 2010). Although, we cannot exclude the possibility that the latter were inactivated or adapted to perform other functions, it seems likely that at least some of them are fusogens.

V. REGULATION OF CELL–CELL FUSION IN NEMATODES AND MAMMALS

Although the regulation of Syncytins and FF proteins is beyond the scope of this review, it is noteworthy that many major conserved molecular pathways have been implicated in the transcriptional regulation of FF proteins in *C. elegans*. Among these are the sex determination, Wnt signaling, Notch signaling, Ras/MAPK signaling, heterochronic pathway, chromatin remodeling pathways, and homeotic genes (Alper & Kenyon, 2002; Cassata et al., 2005;

Hedgecock & White, 1985; Koh et al., 2002; Koh & Rothman, 2001; Margalit et al., 2007; Podbilewicz et al., 2006; Rasmussen et al., 2008; Sapir et al., 2007; Shemer & Podbilewicz, 2002). Reviewed in Alper and Podbilewicz (2008), Friedlander-Shani and Podbilewicz (2011), and Shemer and Podbilewicz (2003). In addition, the c-FOS oncogenic paralog FOS-1 regulates the expression of AFF-1 in the invading AC (Sapir et al., 2007).

In parallel to transcriptional regulation several lines of evidence point to dynamic regulation at the protein level. *fus-1*, a gene encoding a vacuolar–ATPase complex subunit, represses EFF-1 (Kontani, Moskowitz, & Rothman, 2005; Kontani & Rothman, 2005). EFF-1 is localized predominantly in intracellular compartments associated with the apical membrane (Valansi, Smurova, Gonzalez, & Podbilewicz, unpublished results) suggesting tight regulation of EFF-1 dynamics or activity. In addition, del Campo et al. (2005) showed evidence that EFF-1 retention in the plasma membrane may depend on trans interactions with EFF-1 on opposing cell membranes. AFF-1 may also be subject to posttranslational regulation. *nsf-1*, the gene encoding a NEM-sensitive factor homolog in worms, is required for AFF-1-mediated fusion of the AC (Choi et al., 2006). However, the implications of these forms of regulation await further illumination. Nevertheless, it is tempting to speculate that cell–cell fusion in other organisms is regulated by orthologous pathways and that these findings will aid in the identification of the mammalian fusogens.

The regulation of Syncytins in the placenta may share some pathways with the transcriptional control of FF expression and activity. For example, the placental transcription factor GCM1 regulates Syncytin 2 and its receptor MFSD2A in human placenta (Liang et al., 2010). In addition, there is evidence for a role of chromatin remodeling and maternal imprinting in the regulation of syncytin expression (Chang, Chuang, Yu, Yao, & Chen, 2005; Smallwood et al., 2003).

VI. CONCLUDING REMARKS

Here we chronologically reviewed the major research strategies applied through the fruitful discovery of eukaryotic cell–cell fusogens in the past two decades. The major difference between these approaches is that while one relies on a reverse genetics approach to study the function of proviral genes in mammals (Syncytins) the other relies on a forward genetics approach to study fusion failure phenotypes in *C. elegans* (FFs). Nevertheless these fusogens are similar in being the minimal machineries necessary and sufficient for cell-to-cell membrane fusion. It is likely that other fusogens in eukaryotes will follow similar paradigms and that they only remain elusive because they are necessary for viability or are redundant. In addition, their function may rely on multiprotein mechanisms, or unknown cofactors, therefore, it is not possible to

determine if they are sufficient for membrane fusion. However, we speculate that cell–cell fusion in other organisms and tissues will depend on similar protein architectures that arose through convergent evolution or linear decent from viruses.

Future work will determine how FFs and Syncytins act to fuse membranes in diverse epithelial cells (e.g., epidermis in nematodes and placenta in mammals) and how the expression and activities of these diverse fusogens are tightly regulated. Lessons learned from studies of syncytins and FFs may help to identify missing fusogens that mediate fusion of myoblasts, macrophages, stem cells, and gametes across the tree of life.

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