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REVIEW

The hallmarks of cell-cell fusion

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ABSTRACT

Cell-cell fusion is essential for fertilization and organ development. Dedicated proteins known as fusogens are responsible for mediating membrane fusion. However, until recently, these proteins either remained unidentified or were poorly understood at the mechanistic level. Here, we review how fusogens surmount multiple energy barriers to mediate cell-cell fusion. We describe how early preparatory steps bring membranes to a distance of ~10 nm, while fusogens act in the final approach between membranes. The mechanical force exerted by cell fusogens and the accompanying lipidic rearrangements constitute the hallmarks of cell-cell fusion. Finally, we discuss the relationship between viral and eukaryotic fusogens, highlight a classification scheme regrouping a superfamily of fusogens called Fusexins, and propose new questions and avenues of enquiry.

KEY WORDS: Fertilization, Fusogen, Gamete fusion, Mating, Organogenesis, Cell-cell fusion, Syncytin, Fusexins, EFF-1, HAP2, GCS1, AFF-1, Myomaker, Myomixer, Myomerger, Minion, Placenta, Muscle, Virus-cell fusion, Tick-borne encephalitis, Zika, Influenza, Dengue viruses, SNAREs, Hemifusion, Pore formation

Introduction

The vast majority of cells are capable of cell division. However, only a select group of cell types undergo the opposite process – fusion between cells. Membrane fusion involves the physical merging of two membranes into a single bilayer, allowing the exchange of luminal contents. Cell fusion is a fundamental process for development and sexual reproduction and probably even in the origin of the first eukaryotic cell (Radzvilavicius, 2016). Despite these important functions, the molecular mechanisms that underlie cell-cell fusion are only just beginning to be uncovered. In this Review, we focus on fusogens – specialized proteins that function to directly fuse membranes. We begin by reviewing established mechanisms of membrane fusion mediated by well-studied viral and intracellular fusogens, arguing that there are multiple energy barriers that need to be surmounted to complete fusion. We distinguish cellular processes that are needed to prepare cells for fusion but which are not directly involved in the physical merging of the membranes, and propose that there are at least three hallmarks of cell-cell fusion which are characterized by the action of fusogens in the final ~10 nm of plasma membrane separation (Fig. 1). Finally, we discuss findings on the identification of new cell fusogens that are both necessary and sufficient to fuse cells during development, and can act either bilaterally (i.e. are required on both fusing membranes) or unilaterally (i.e. are required on just one of the fusing membranes).

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Influenza HA2 and SNAREs: bona fide fusogens

Much of what we know about membrane fusion has come from studies of enveloped viruses fusing with their targets and of the fusion of intracellular membranes. In these contexts, fusion is mediated by proteins called fusogens, the majority of which contain transmembrane domains (TMDs) (Martens and McMahon, 2008). Among viral fusogens, the Hemagglutinin HA2 subunit from the influenza virus is arguably the best understood, while the conserved SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) family of fusogens is well studied for its role in driving intracellular fusion events (Stein et al., 2009; Weber et al., 1998) (Fig. 2A,B). These fusogens assemble into either a unilateral homotypic complex (the HA2 trimer that forms a six-helical bundle on the viral envelope) or a bilateral heterotypic complex (the so-called four-helical bundle SNARE complex). Both fusogens are targeted to defined sites of fusion, with HA2 residing on the viral membrane, while SNAREs are enriched at specific membrane compartments. Viral fusogens and SNAREs are necessary for membrane fusion but, more importantly, both are sufficient for fusion, meaning that when incorporated into membranes that otherwise would not merge, the proteins induce fusion (Nussbaum et al., 1987; Weber et al., 1998). These two operational criteria (necessity and sufficiency) lie at the very core of widely adhered benchmarks that a bona fide fusogen must fulfill (Oren-Suissa and Podbilewicz, 2007; Rizo, 2006).

In pioneering crystallographic work, the Hemagglutinin HA2 subunit was found to assemble into trimers consisting of a coiled-coil of α-helices (Wilson et al., 1981). The first demonstration that HA2 trimers are sufficient to mediate fusion was performed by cloning of the gene followed by its ectopic expression in simian cells, resulting in the formation of multinucleated syncytia (White et al., 1982). Following on from this, efforts to understand how HA2 fuses membranes have focused on structural comparisons of the metastable prefusion and the low-energy postfusion conformational states (Harrison, 2008; Podbilewicz, 2014; White et al., 2008), leading to the development of the spring-loaded model (Bullough et al., 1994; Carr and Kim, 1993). This model proposes that a low pH-induced conformational change triggers the exposure of previously hidden hydrophobic residues that are together known as a fusion peptide. The peptide extends into the target membrane and this is followed by a hairpin-like fold-back of HA2 trimers that pulls the membranes together. These studies have been limited, however, by the use of truncated fusogens without their TMD, making the question of how these conformational transitions are mechanically coupled to membrane fusion difficult to assess. A combination of theoretical, functional and genetic analyses has therefore been necessary to bridge the gap between the structural rearrangement of fusogens and membrane fusion intermediates. Insightful information was revealed when HA2 was anchored to the external leaflet of fibroblast plasma membranes by replacing its TMD with glycosylphosphatidylinositol (Kemble et al., 1994; Melikyan et al., 1995). When added to red blood cells, no cytoplasmic exchange between cells was detected but proximal leaflets of the bilayers became merged, a state known as

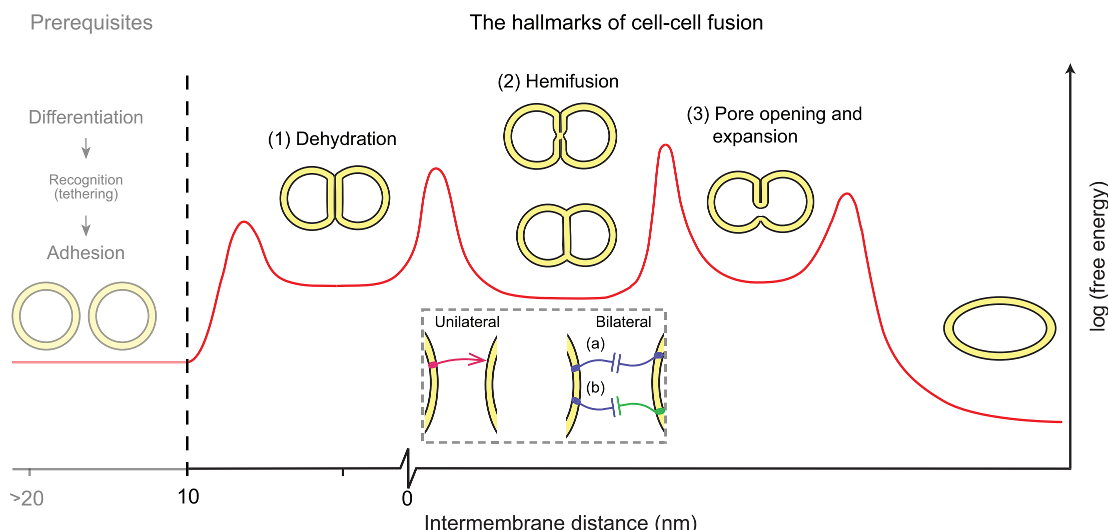

Fig. 1. The hallmarks of cell-cell fusion. The pathway to cell-cell fusion begins with determination of the cell fusion fate (differentiation), recognition between the cells that are destined to fuse, and tight adhesion between the neighboring cells. At the end of these prefusion events, the two plasma membranes are positioned at a distance not closer than ∼10 nm. Biological fusogens overcome at least four energetic barriers (red peaks in the schematic energy plot) during the cell fusion pathway. The promotion of the three lipidic intermediates mediated by cell fusogens constitute the hallmarks of cell-cell fusion: (1) dehydration of contacting plasma membranes, bringing the phospholipid heads to distances of close to 0 nm; (2) merger of the outer monolayers or hemifusion via a stalk and/or diaphragm intermediates; (3) opening and expansion of fusion pore(s) from nanometer diameter to multiple microns. The insert shows two topological types of fusogens, unilateral and bilateral, which can be distinguished between (a) homotypic and (b) heterotypic fusogen complexes (colors depict different generic membrane proteins). All known biological fusogens or fusogen complexes are both essential and sufficient to overcome multiple energy barriers for complete fusion.

hemifusion, which had been theoretically formulated a decade earlier (Chernomordik et al., 1986; Kozlov et al., 1989; Kozlov and Markin, 1983) (Fig. 2C). This hemifusion state can also be arrested by mutating amino acids in either the TMD region or the fusion peptide (Kemble et al., 1994; Melikyan et al., 2000; Qiao et al., 1999), demonstrating that hemifusion represents an on-pathway lipidic intermediate and that HA2 trimers require complete membrane insertion in order to overcome this energy barrier.

The idea that SNAREs are necessary and sufficient for membrane fusion was demonstrated by biochemical reconstitution using liposomes (Weber et al., 1998). Fusion is observed when a specific combination of SNAREs is present on both fusing membranes, with individual SNAREs contributing one or two helices that assemble into a coiled-coil. SNAREs assemble bilaterally in a zipper-like fashion in the N- to C-terminal direction, and are able to interact in *trans* when opposing membranes are ∼8 nm apart (Li et al., 2007). Similarly to HA2, perturbation of the SNARE complex can result in fusion arrest at defined intermediates (Lu et al., 2005; Reese et al., 2005). In a reconstituted SNARE-mediated fusion system, a single amino acid deletion results in arrest before the onset of hemifusion, with membranes remaining ∼1 nm apart (Hernandez et al., 2012). It is likely that the only molecules separating the bilayers in this context are those comprising a thin hydration shell that covers the lipid head groups (Leikin et al., 1987). Hydrostatic forces strongly repel two approaching membranes to an extent that to reduce the membrane distance to ∼0.5 nm, an exponentially increasing force spanning four orders of magnitude is needed (LeNeveu et al., 1976; Shrestha and Banquy, 2016). At distances of ∼1 nm, early signs of hemifusion stalk formation begin to emerge, strongly indicating that dehydration of the polar head groups precedes the initiation of hemifusion (Donaldson et al., 2011). Thus, dehydration is another high-energy intermediate state that fusogens must overcome (Fig. 1).

Once fusogens initiate hemifusion, they need to exert additional force to open a pore between the membranes to allow soluble content exchange and complete fusion. Theoretical and experimental studies have proposed that, depending on the lipid composition, the energy requirements for pore opening are at least as great as those needed for hemifusion and the preceding dehydration step (Chernomordik and Kozlov, 2003, 2005; Lu et al., 2005; Reese et al., 2005; Reese and Mayer, 2005). One demonstration of this is the report of long-lived hemifusion intermediates resulting from low surface densities of fusogens, which can be opened into a pore at higher densities (Chernomordik et al., 1998; Leikina and Chernomordik, 2000). However, experimental data showing how conformational changes of fusogens are mechanically coupled to pore opening and expansion are still lacking. High-resolution ultrastructural analysis has uncovered snapshots of different stages of the pore opening process in a hybrid-reconstituted system with HA2 (Calder and Rosenthal, 2016; Chlanda et al., 2016) (Fig. 2D). Nevertheless, what is pending for any fusogen is the identification of perturbations that can arrest a nascent or expanding pore in a similar way to that achieved for the preceding steps.

The hallmarks of cell-cell fusion

The basic thermodynamic and biophysical requirements encountered during the membrane fusion of enveloped viruses and intracellular vesicles are expected to be the same as those occurring during fusion between cells. Fundamentally, any cellular machinery with the task of fusing plasma membranes will have to carry out at least three energetically costly tasks, which both HA2 and SNAREs have evolved to do: (1) dehydrate polar head groups, (2) promote a hemifusion stalk, and (3) open and expand pores between fusing cells. We have schematically portrayed these hallmarks as intermediate stages in an energy diagram (Fig. 1). For cell-cell fusion (and likely all other types of fusion), the presence of a single energy barrier is probably too simplistic to represent the complex merging of the membranes. Rather, as we have discussed above, a large body of research on SNAREs and viral fusogens suggests that fusogens must overcome these energy

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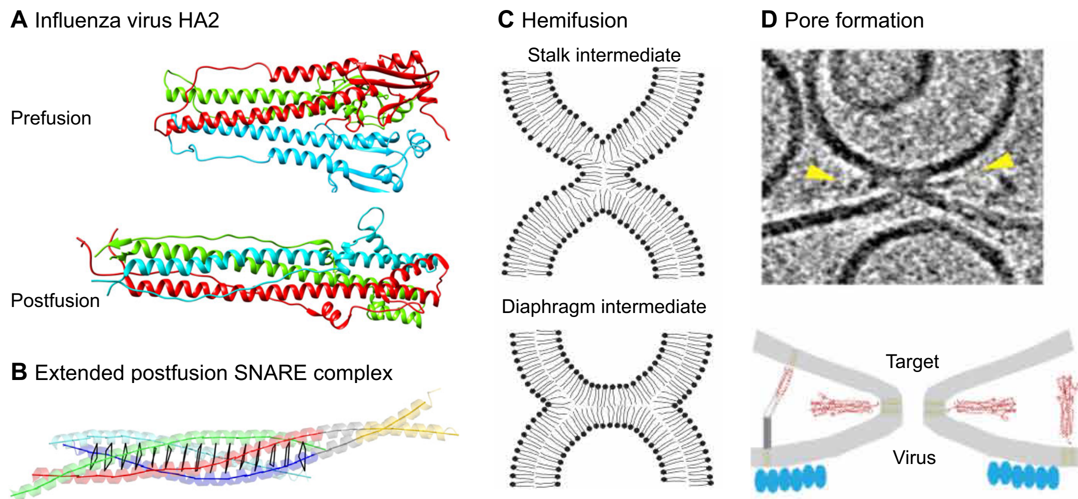

Fig. 2. Well-characterized fusogens: influenza Hemagglutinin and the SNARE complex. (A) Crystal structures of the ectodomain of influenza virus Hemagglutinin HA2 in the prefusion (neutral pH) and postfusion (low pH) conformations. The three HA2 monomers are in red, green and cyan. In the prefusion state of HA2, the C-terminal ends that link to the viral membrane are on the right. In the postfusion state, the C-terminal ends and the fusion peptides are together on the left. (B) SNARE complex of four alpha-helices from rat syntaxin-1A (red), SNAP-25 (cyan and green) and synaptobrevin-2 (violet), with the C-terminal linkers (gray) and transmembrane regions (yellow) on the right; the helical bundle is stabilized by interactions between side chains. PBD accession numbers for the HA2 trimers are 2HMG [prefusion (Weis et al., 1990)] and 1QU1 [postfusion (Chen et al., 1999)]; for SNARE complex is 3IPD (Stein et al., 2009). (C) Two different structures of the hemifusion lipidic intermediate in which only the contacting (proximal) monolayers have fused without the opening of a pore. (Top) An emerging point-wise hemifusion intermediate is called a stalk. (Bottom) Radial expansion of a stalk gives rise to a hemifusion diaphragm. A fusion pore can originate from either a stalk or a diaphragm. (D) (Top) Section from an electron microscopy tomogram showing an emerging pore between a HA2-containing enveloped virus and a liposome with electron-dense bars protruding from the contact points (arrowheads). (Bottom) Proposed protein intermediate associated with membrane merging and formation of a fusion pore. Shown are an HA2 extended trimer (left) and foldback structure ('trimer of hairpins'; see A, postfusion) inserted around the fusion pore or in the virus membrane. The virus matrix (blue ovals) is disrupted at the fusion pore. Reproduced with permission from Calder and Rosenthal (2016).

barriers. Importantly, as we shall argue below, the thermodynamic origin of these hallmarks is a function of intermembrane distance. We contend that at distances greater than ∼10 nm, repulsive forces that prevent cell membranes from being brought together can be readily overcome by well-known cellular machineries, notably adhesion complexes and cytoskeletal structures. Below this distance, however, the energetic barriers increase exponentially, requiring a different class of proteins: cell fusogens. It is the action of these fusogens, which begins at ∼10 nm of membrane separation, that constitute the three hallmarks of cell-cell fusion.

Early preparatory steps for cell-cell fusion

Although we do not consider the molecular steps preceding tight adhesion and polar head group dehydration to be distinctive hallmarks of cell-cell fusion, it is essential to distinguish the early preparatory steps of cell-cell fusion from those processes involved in the mechanical exertion of force and which contribute to, and are sufficient for, membrane fusion (in contrast to only being necessary). Historically, this distinction has not always been clear-cut, as exemplified by the names given to the first genes associated with defects in fusion, such as the FUS genes (e.g. in yeasts, worms and green algae), which were later found not to take part in the mechanics of membrane fusion (see e.g. Kontani et al., 2005; Misamore et al., 2003; Trueheart and Fink, 1989). Several preparatory steps have already been identified as basic prerequisites for cell-cell fusion in a wide range of biological scenarios (Aguilar et al., 2013; Zito et al., 2016). Here we regroup them into three broad preparatory stages: differentiation, recognition and adhesion. We argue that the main aim of these processes is to localize and target cell fusogens to sites of fusion within a critical distance of 10 nm. Below, we provide a walkthrough of how select cell fusion systems adopt different strategies to prepare cells for fusion with one ultimate goal: to merge two cells into one.

Bringing membranes close enough: insights from *Chlamydomonas*

The green algae *Chlamydomonas reinhardtii* is a single-celled organism that can reproduce via the fusion of two gametes of opposite mating types, termed *mt*+ and *mt*−. For cell-cell fusion to occur during fertilization, precursor gamete cells must first divide and differentiate to enter a cellular program that initiates the pathway towards fusion. In *C. reinhardtii*, the *mt*+ and *mt*− haploid cells begin differentiation following depletion of environmental nitrogen. In addition to triggering the expression of genes that help the cell to cope with nitrogen starvation, differentiation results in the expression of genes required for mating (Saito and Matsuda, 1991). This includes expression of the *mt*+ agglutinin SAG1 and the *mt*− agglutinin SAD1 (Ferris et al., 2005; Lin and Goodenough, 2007). During this process, a subpopulation of SAG1 and SAD1 is transported to the flagella, allowing gametes to recognize and physically attach to their mating partner (Wang et al., 2006). Another change during differentiation is the expression of the membrane proteins FUS1 (*mt*+) and HAP2 (*mt*−), which localize to polarized patches of the cell body and which both give rise to mating defects when deleted (Buchanan et al., 1989; Ferris et al., 1996; Misamore et al., 2003) (Fig. 3A). Agglutinin-mediated contact between *mt*+ and *mt*− gametes in *C. reinhardtii* then triggers a new activation signal, which is transduced along the flagellum (Pan and Snell, 2002; Wang et al., 2006). SAG1 and SAD1 are further relocated to the flagella from the cell body plasma membrane (Hunnicutt et al., 1990). An increase in cAMP concentration triggers two crucial prerequisites for fusion: (1) dissolution of the cell wall and (2) assembly of actin-filled mating structures on both

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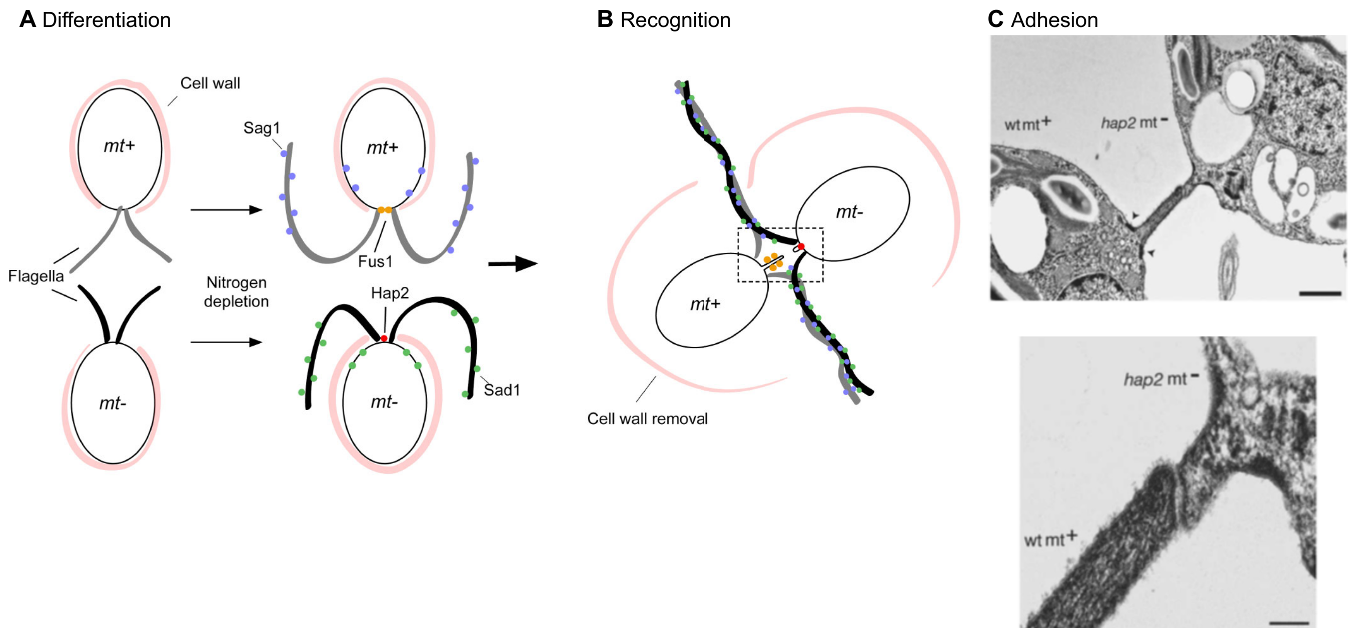

Fig. 3. Early preparatory steps before gamete fusion during *Chlamydomonas reinhardtii* mating. During mating in *C. reinhardtii*, three early steps that precede membrane fusion can be identified that help to bring membranes to within 10 nm of each other. (A) Gametes from two mating types (*mt*+ and *mt*−) enter a differentiation program following nitrogen depletion, which initiates or enhances the expression of genes required for sexual reproduction. Differentiation of precursor gametes induces or enhances the expression of proteins (e.g. Sag1, Fus1, Hap2, Sad1) that are required at different stages of the fusion pathway. Differentiation also contributes to the targeting of these proteins to predefined sites of fusion. (B) Partner recognition then involves contact-dependent signaling that triggers a complex biochemical cascade, which leads to the formation of mating projections on both mating types. (C) Adhesion between the two mating projections then occurs; this can be arrested by deletion of HAP2 from the *mt*− gamete, resulting in an arrest in mating with projections found 10 nm apart. (Top) The tip of the fertilization tubule on a wild-type *mt*+ gamete is tightly associated with the apex of the mating structure of a *hap2 mt*− gamete. Arrowheads indicate the doublet zone bar. (Bottom) A higher magnification view shows that the membranes of the two mating structures are separated by ~10 nm. Scale bars: 200 nm, top; 50 nm, bottom. Reproduced with permission from Liu et al. (2008).

mt+ and *mt*− cells (Fig. 3B). The *mt*− mating structure consists of a cup-shaped membrane projection extending ~0.5 µm, while the longer (~3 µm) actin filament-filled *mt*+ mating structure, known as a fertilization tubule, extends towards the mating partner forming a cellular junction (Detmers et al., 1983; Goodenough et al., 1982). In wild-type matings, appropriately timed addition of actin polymerization inhibitors after initiation of tubule assembly prolongs the junction lifetime, revealing the presence of electron-dense material (referred to as ‘fringes’) that holds the membranes ~10 nm apart (Detmers et al., 1983; Goodenough et al., 1982). In *fus1* knockouts, the fringes are absent, the fertilization tubule retracts and fusion is impaired, suggesting that FUS1 is a structural constituent of the junction with an adhesive function (Goodenough et al., 1982; Misamore et al., 2003). The fusion-relevant interaction is uncovered in *hap2* knockouts, where mating is arrested and stable fringes are observed but with membranes adhered to one another at a distance of ~10 nm (Fig. 3C). Thus, HAP2 takes part in the fusion process and operates after formation of the fertilization tubule at a step downstream of FUS1 after junction assembly (Liu et al., 2008).

What can we learn from the example of *C. reinhardtii*? The most important lesson here is that adhesion (and all preceding steps) is both functionally and biochemically dissectible from membrane fusion. Functionally, we can recognize three early steps that precede membrane fusion: differentiation, recognition and adhesion. These steps culminate in the assembly of a junction between opposing mating structures at the plasma membrane. Biochemically, neither FUS1-mediated tethering nor actin-generated propulsion of the fertilization tubule contributes to the mechanics of fusion, whereas HAP2 is essential for fusion at the step where the junction is established. Thus, the function of the mating junction is to bring the membranes within a critical distance (~10 nm) to allow the fusogen (s) to initiate membrane fusion. As we shall examine later in more detail, this fusogen is the protein HAP2.

Immunoglobulin superfamily receptors mediate myoblast-myoblast adhesion during *Drosophila* myogenesis but fusogens remain unidentified

We now turn to a developmental process involving the fusion of somatic cells: myoblast fusion (Abmayr and Pavlath, 2012; Kim et al., 2015a; Onel et al., 2014). In *Drosophila*, genetic studies have determined an asymmetric interaction between fusing cells during the formation of diverse embryonic and adult muscles (Schejter, 2016). A central precept is that precursor myoblasts differentiate into founder cells (FCs) and fusion-competent myoblasts (FCMs) through cross-talk between the Ras and Notch signaling pathways (Carmena et al., 2002; Ciglar et al., 2014). After the first fusion event between an FC and an FCM, the resulting binucleate cell continues to fuse with neighboring FCMs, giving rise to a multinucleate myotube (Fig. 4). Differentiation results in the expression and targeting of an adhesion machinery concomitant with major cytoskeletal rearrangements, imparting FCs/myotubes and FCMs with the capacity to migrate and recognize each other. The main adhesion membrane protein of FCs and myotubes is Kirre (Dumbfounded) (Ruiz-Gómez et al., 2000), a member of the immunoglobulin superfamily (IgSF). Through *trans* association with the FCM-specific IgSF protein Sticks and stones (Sns) (Bour et al., 2000), recognition and adhesion (see Box 1) between myotubes and FCMs is established.

No equivalent to *C. reinhardtii* HAP2 has been identified in *Drosophila* as displaying a fusion failure phenotype after the

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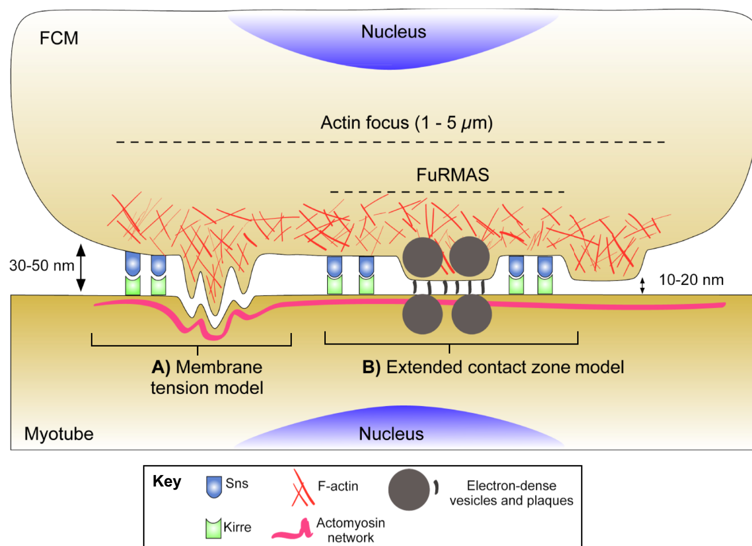

Fig. 4. Models for the prefusion stages of *Drosophila* myoblast-myotube adhesion. While the overall framework is shared among different myogenic settings, the structural details shown here correspond primarily to the *Drosophila* system, and may differ substantially in other tissues or systems. Various programs of myogenic differentiation (mediated by the Notch and other developmental signaling pathways) underlie the expression of key participants in the fusion process. A major initial event is recognition between a fusion-competent myoblast (FCM) and a multinucleated myotube, which is mediated by distinct members of the immunoglobulin superfamily (IgSF, e.g. Sns and Kirre). There are two contending models for how membranes are then organized for fusion, both of which portray actin structures as pivotal to the focusing of the fusion machinery. (A) The membrane tension model involves invasive protrusions that penetrate into the myotube and are counterbalanced by an actomyosin network to increase membrane tension. The increased tension might be important for the actual merging of the membranes. (B) The extended contact zone model portrays adhesion between the membranes held together by actin-mediated forces in conjunction with IgSF complexes forming structures known as FuRMAS. Electron-dense vesicles and plaques, possibly containing the essential proteins mediating fusion, may also be present. Fusion pore opening may initiate at a single or at multiple locations at either extended adhesive zones and/or at the tips of the invasive protrusions.

membrane adhesion step. This is despite some similarities in the steps preceding membrane fusion. For example, electron micrographs have revealed the presence of electron-dense material (reminiscent of fringes) at focused contact sites (Doberstein et al., 1997), which are held together by supramolecular assemblies of IgSF proteins connected to actin nucleation factors known as FuRMAS (Kesper et al., 2007). The presence of actin-filled finger-like protrusions emanating from embryonic FCMs into myotubes (reminiscent of fertilization tubules) has also been reported (Haralalka et al., 2011; Jin et al., 2011; Sens et al., 2010). These protrusions, extending 1-3 µm, are assembled from F-actin foci on the FCMs and are spatially correlated with proteins involved in actin polymerization (Gildor et al., 2009; Haralalka et al., 2014; Kim et al., 2007; Massarwa et al., 2007; Onel et al., 2011).

Based on a combination of ultrastructural and genetic evidence, two prevalent models have emerged describing where and how fusion takes place in *Drosophila* myoblasts. The 'membrane tension' model (Fig. 4A) emphasizes the penetrating nature of the protrusions and an increase in membrane tension generated by a myotube actomyosin network on the opposing cell. Membrane tension in this model contributes substantially to overcoming the fusion energy barriers at the tip of the protrusions (Kim et al., 2015b). An 'extended contact zone' model (Fig. 4B) emphasizes the presence of extended (flattened) adhesion zones between the myotube and myoblasts and contends that actin and adhesive structures organize and predefine sites for membrane fusion mediated by currently unknown factors (Dhanyasi et al., 2015; Kesper et al., 2007). An insightful and rather counter-intuitive finding reported in adult indirect flight muscles is the RNAi-mediated perturbation of the adhesive IgSF machinery, which arrests the myotube/myoblast intermembrane distance at ~40-50 nm (Dhanyasi et al., 2015). The interference of branched actin polymerization, on the other hand, results in a distance of ~10-20 nm, indicating that branched actin structures bring the membranes even closer together than the adhesion machinery, up to a distance of ~10 nm (Schejter, 2016). In unperturbed flies, electron-dense contact sites are observed over these extended zones where pores are thought to open.

Both models discussed above have weaknesses. For instance, the intermediate stages of the fusion pore opening process at either electron-dense contact sites or at the tips of the finger-like protrusions have not been reported. The proposed fusogenic character of the finger-like protrusions is inconsistent with the observation that the intermembrane spacing in the protrusions is constant throughout their entire length. If actin-driven membrane tension contributes directly to fusion, we would expect a gradual

Box 1. Adhesion machineries connect fusion-fated cells and confer specificity of recognition

An important clue into understanding what confers specificity between fusion-fated cells is provided by the expression profile of adhesive proteins. We have already seen that the agglutinins SAG1 and SAD1 are differentially expressed in haploid cells in *C. reinhardtii*, while *Drosophila* myoblasts contain specific IgSF proteins. In vertebrate myogenesis, the zebrafish IgSF proteins Jamb (Jam2) and Jamc (Jam3) are required for adhesion of precursor muscle cells (Powell and Wright, 2011). Thus, it appears that recognition specificity is controlled by the formation of heterotypic complexes bridging two distinct cells. In mammalian sperm-egg fertilization, the adhesive complex formed between IZUMO1 in the sperm and JUNO (IZUMO1R) in the egg is essential for fertilization (Bianchi et al., 2014; Inoue et al., 2005), while sequence variations at the IZUMO-JUNO complex interface have been proposed to control recognition specificity between different mammalian species (Aydin et al., 2016). The structural biology of such adhesion and recognition machineries reveals how fusion-fated cells maintain close separation (Aydin et al., 2016; Kato et al., 2016; Nishimura et al., 2016; Ohto et al., 2016; Raj et al., 2017), a prerequisite before initiation of membrane fusion. However, there is weak support for the idea that adhesive complexes themselves are mechanically involved in membrane fusion. First, the expression and formation of IgSF and other adhesive complexes occur in tissues that normally do not fuse and where they serve distinct physiological functions, such as the establishment of tight junctions (Powell and Wright, 2011). Second, adhesive functionality per se is not dependent on a TMD, as shown for Kirre and Sns for which cell recognition is still achievable by replacing the TMDs with a GPI anchor (Galletta et al., 2004). This is in contrast to fusogens, where TMDs are important for their activity. Lastly, the lack of clearance of adhesion proteins at fusion sites can sterically impede closer membrane contact and even impair cell fusion (Dottermusch-Heidel et al., 2012).

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reduction between opposing membranes as the protrusions are extended deeper into the myotubes, a scenario that is not supported by the available ultrastructural data. In line with the idea that protrusions are insufficient to drive fusion, a culture-based assay consisting of a non-fusing cell line derived from *Drosophila* embryos revealed that actin-propelled protrusions are observed between adhered cells but without fusion (Shilagardi et al., 2013). However, if the cells are transfected with a known fusogen – the epithelial cell fusogen EFF-1 identified in *C. elegans* (discussed below) – efficient cell fusion is then observed (Shilagardi et al., 2013). Thus, we conclude that bona fide fusogens remain to be identified in *Drosophila* myoblasts.

Cell fusogens in development and fertilization: engines for membrane fusion

At the turn of this century, at least four families of fusogens that drive developmental cell-cell fusion had been reported: (1) fusion family proteins in *C. elegans* [e.g. EFF-1 and AFF-1 (Mohler et al., 2002; Sapir et al., 2007)] and other organisms [e.g. nematodes, cephalochordates, arthropods, ctenophores and a protist (Avinoam et al., 2011)], which mediate somatic cell fusions during organ formation, tubulogenesis and neuronal repair (Avinoam and Podbilewicz, 2011; Kravtsov et al., 2017; Oren-Suissa et al., 2017; Smurova and Podbilewicz, 2016a); (2) HAP2 (GCS1) in plants, invertebrates, algae and protists, which mediates gamete fusion (Fedry et al., 2017; Johnson et al., 2004; Mori et al., 2006; Pinello et al., 2017; Valansi et al., 2017); (3) Syncytins in mammals, which mediate cytotrophoblast (CTB) fusion in placentation (Blond et al., 2000; Mi et al., 2000); and (4) Myomixer/Minion/Myomerger and Myomaker, which mediate myoblast fusion in vertebrates (Bi et al., 2017; Gamage et al., 2017; Millay et al., 2013; Quinn et al., 2017; Zhang et al., 2017). Interestingly, gamete fusogens in fungi, nematodes and vertebrates have not been identified. In addition, fusogens required for osteoclasteogenesis, eye lens formation and neuronal fusion in vertebrates, myoblast fusion in invertebrates, wound healing, inflammation, cancer, and stem cell fusion have not been described. Thus, most cell fusogens await to be discovered. Below, we provide an overview of these four families of fusogens and discuss how they drive cell-cell fusion during development.

EFF-1 and AFF-1 fusogens in epithelial and myoepithelial cells: fusion via a bilateral mechanism

In *C. elegans*, cell-cell fusion plays an important role in sculpting tissues and organs during development; indeed, one third of the somatic cells generated during development undergo programmed cell-cell fusion events during embryogenesis and postembryonic development (Podbilewicz, 2000; Podbilewicz and White, 1994; Shinn-Thomas and Mohler, 2011). Using forward genetic screens, epithelial fusion failure 1 (EFF-1) (Mohler et al., 2002) and its paralog anchor cell fusion failure 1 (AFF-1) (Sapir et al., 2007) were identified as genes that are necessary for cell-cell fusion. EFF-1 mediates fusion in 23 epidermal cells in the embryo (Fig. 5A), and during larval development it acts to fuse 116 additional cells to form the hyp7 syncytium that envelops most of the animal, as well as fusion of other epithelial and myoepithelial cells in the pharynx, epidermis, glands, vulva, uterus and other organs (Podbilewicz and White, 1994; Sharma-Kishore et al., 1999; Yochem et al., 1998). AFF-1 in turn mediates fusion of embryonic epidermal cells, some

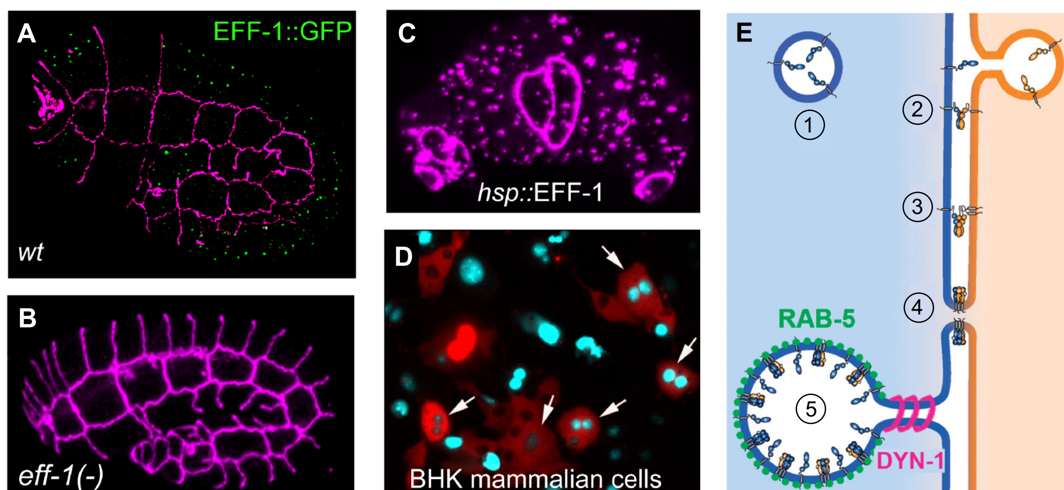

Fig. 5. EFF-1 is necessary and sufficient for cell-cell fusion in *C. elegans*. (A) Localization of EFF-1::GFP (green) in early endosomes following dorsal hypodermal fusion (top) in an elongating *C. elegans* wild-type embryo. Apical junctions between epidermal cells are marked with DLG-1::dsRed (magenta). The image was obtained by structural illumination microscopy (see Smurova and Podbilewicz, 2016b). (B) The epithelial fusion failure phenotype in an *eff-1* null mutant demonstrates that EFF-1 is required for cell-cell fusion. Anti-AJM-1 antibody labels apical junctions (magenta). Immunofluorescent image obtained by K. Smurova (see Smurova and Podbilewicz, 2016b). (C) EFF-1 overexpression is sufficient to fuse cells that normally do not fuse in *eff-1(−)* mutant embryos. Most epithelial junctions have disappeared following heat shock induction of EFF-1 expression, resulting in hyperfusion. Reproduced with permission from Shemer et al. (2004). (D) AFF-1-expressing cells (red cytoplasm) and EFF-1-expressing baby hamster kidney (BHK) cells (cyan nuclei) can fuse, demonstrating that both EFF-1 and AFF-1 are bilaterally sufficient to fuse heterologous mammalian cells. Arrows point to cells with content mixing (red cytoplasm and cyan nuclei). Reproduced with permission from Avinoam et al. (2011). (E) Model for EFF-1 localization and fusion mechanism. The cytoplasm and plasma membrane of two fusing cells are represented in orange and blue. (1) EFF-1 monomers (blue and orange complexes) are expressed by the two fusing cells and are targeted to the plasma membranes via vesicular transport. (2) Fusion is initiated by the assembly of two monomers of EFF-1 in *trans* into a dimer followed by (3) the incorporation of a third EFF-1 monomer (to form a trimer). (4) A conformational change of the EFF-1 trimer induces fusion of opposing membranes and mixing of the cytoplasms by opening of a fusion pore, which may require the cooperative action of several trimers. The bilateral design allows precise control of EFF-1 *trans* interaction, preventing excessive fusion. (5) Monomeric and oligomeric EFF-1 in postfusion states are actively removed from the cell surface via receptor-mediated endocytosis in a dynamin- and RAB-5-dependent mechanism, resulting in EFF-1 accumulation in early endosomes. RAB-5 is in green, dynamin (DYN-1) in magenta. Reproduced with permission from Smurova and Podbilewicz (2016a).

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myoblasts of the pharynx, and glial cells in the dauer stage, among other cells/organs (Abdus-Saboor et al., 2011; Chiorazzi et al., 2013; Procko et al., 2011; Sapir et al., 2007). EFF-1 and AFF-1 also mediate self-fusion during neurite regeneration, dendritic pruning and tubulogenesis in the excretory, reproductive and intestinal systems (Ghosh-Roy and Chisholm, 2010; Neumann et al., 2015; Oren-Suissa et al., 2017, 2010; Rasmussen et al., 2008; Stone et al., 2009). Interestingly, self-fusions have been reported during blood vessel pruning during vertebrate vascular differentiation, although the fusogens implicated in this context remain unknown (Lenard et al., 2015).

Misregulation of the EFF-1/AFF-1-based cell fusion machineries by interference of EGF/RAS/MAPK, Notch and Wnt signaling can result in either excessive fusion or fusion failure, causing, for instance, vulvaless or multi-vulva phenotypes, respectively (Dalpe et al., 2005; Escobar-Restrepo and Hajnal, 2014; Koh et al., 2004; Kolotuev and Podbilewicz, 2004, 2008; Myers and Greenwald, 2005; Schmid and Hajnal, 2015; Weinstein and Podbilewicz, 2016). In larval epidermal lateral seam cells, actin fibers crosslinked by Spectroplakin (VAB-10A) interact with EFF-1 to increase its apical distribution and assist in reducing the intermembrane distance (Yang et al., 2017). In *eff-1* mutants, tight adhesion of cells programmed to fuse is unaffected (Fig. 5B), with the intermembrane distance spaced at ∼10 nm (Podbilewicz et al., 2006; Shemer et al., 2004). Failure in cell-cell fusion in *eff-1* and *aff-1* mutants results in multiple phenotypes that affect the sculpting of organs and cell fates, and eventually causes defective ectopic migration and defects in intracellular trafficking in the cells that fail to fuse (Cassata et al., 2005; Mohler et al., 2002; Sapir et al., 2007; Shemer and Podbilewicz, 2002; Smurova and Podbilewicz, 2016b).

EFF-1 and AFF-1 comprise the first eukaryotic fusion family (FF) to be identified (Sapir et al., 2007; White, 2007). FF proteins were the first cell fusogens for which the sufficiency criterion was corroborated stringently based on three lines of evidence: (1) both EFF-1 and AFF-1, when expressed in cells that normally do not express them, can fuse the cells in *C. elegans* as long as the cells are in close contact (Avinoam et al., 2011; del Campo et al., 2005; Sapir et al., 2007; Shemer et al., 2004) (Fig. 5C); (2) ectopic expression of EFF-1 fuses both mammalian (Fig. 5D) and insect cultured cells (Avinoam et al., 2011; Podbilewicz et al., 2006; Sapir et al., 2007); (3) pseudotyped vesicular stomatitis virus (VSV) mediates viral envelope fusion with the host cell when the viral fusogen glycoprotein G is replaced with EFF-1 or AFF-1, although FF proteins must be present on both the viral and host membranes (Avinoam et al., 2011). Genetic mosaics in *C. elegans* confirm the bilateral requirement of EFF-1 for fusion *in vivo* (Podbilewicz et al., 2006), while it has also been shown that EFF-1 can interact promiscuously in *trans* with AFF-1 to mediate cell-cell and virus-cell fusion (Avinoam et al., 2011). Providing support for a common biological fusion mechanism, it was further demonstrated that EFF-1 initiates the formation of fusion pores via a hemifusion intermediate (Podbilewicz et al., 2006). Small lipidic pores open within seconds, while a higher concentration of EFF-1 on both plasma membranes is needed to continue expanding micron-sized pores within minutes (del Campo et al., 2005; Gattegno et al., 2007; Podbilewicz et al., 2006; Smurova and Podbilewicz, 2016b). Taken together, these findings reveal that FF proteins are bilaterally sufficient for cell-cell fusion and interact both homotypically and heterotypically to mediate fusion via common biological lipid intermediates (Fig. 5E).

More detailed insights into the molecular nature of FF protein *trans* interactions have been obtained through biochemical and structural analyses. The atomic structure of the ectodomain of EFF-1 (Pérez-Vargas et al., 2014) reveals a striking homology to class II viral fusion proteins, such as those found in Zika, Semliki forest, rubella and dengue viruses. Despite exhibiting very low sequence homology to viral fusogens, EFF-1 monomers assemble into trimers resembling the postfusion hairpin trimer conformation typical of class II viral glycoproteins (Fig. 5E). Thus, the structural similarity between EFF-1 and class II viral fusogens includes highly conserved secondary, tertiary and quaternary structural features (Fig. 6). Soluble monomers of the EFF-1 extracellular domain were found to inhibit cell-cell fusion, suggesting that trimer assembly of membrane-embedded proteins is essential for the initiation of fusion (Pérez-Vargas et al., 2014).

Of importance for understanding how EFF-1 complex assembly is coupled to membrane fusion is defining the timing of the assembly process. For instance, using cryogenic transmission electron microscopy, upright monomers can be observed on fragments of plasma membrane (Zeev-Ben-Mordehai et al., 2014), suggesting that assembly of EFF-1 monomers in *cis* is prevented, by unknown mechanisms. The addition of soluble domain III (Ig domain) of EFF-1 inhibits fusion, probably by blocking a conformational rearrangement from a prefusion monomer to a postfusion trimer of hairpins formed in *trans* (Pérez-Vargas et al., 2014), supporting a model whereby the assembly of EFF-1 complexes zippers the membranes in a SNARE-like manner (Fig. 5E). Consistent with this view, EFF-1 does not contain bulky hydrophobic amino acids comprising a fusion loop that inserts into the host membrane. Nevertheless, alternative

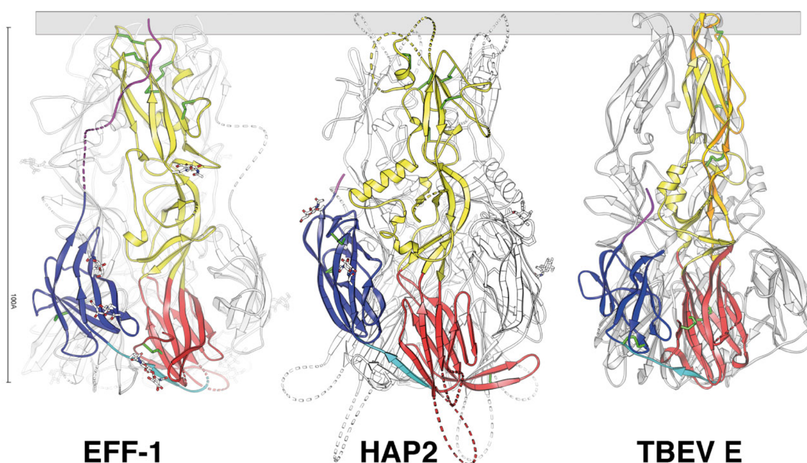

Fig. 6. The somatic fusogen EFF-1 from *C. elegans*, the sexual gamete fusogen HAP2 from *C. reinhardtii* and the tick-borne encephalitis virus E glycoprotein (TBEV E) share a similar structural fold. All three proteins are trimeric; shown here are their ectodomains with one monomer colored according to the class II viral convention: domain I (red, DI), domain II (yellow, DII) and domain III (blue, DIII). The amphipathic loops of HAP2 and TBEV E that are depicted on top of the proteins are predicted to interact with the membrane (gray bar). By contrast, for EFF-1 the loops are negatively charged and therefore are not predicted to interact with the membrane; at the end of the EFF-1 stem (magenta) is the C-terminus that is close to the membrane where the TMD is predicted to be. Disordered regions (dashed tubes), disulfide bonds (green sticks) and linkers (cyan) connect DIII and DI. Reproduced with permission from Fedry et al. (2017).

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models are conceivable and future structure-function studies should be helpful in understanding how the assembly of FF proteins is coupled to the mechanics of membrane fusion.

HAP2 in gametes: cell fusion with unilateral and bilateral requirements

Hapless 2/Germ cell-specific 1 (HAP2/GCS1), henceforth HAP2, is a conserved gamete type I transmembrane protein for which a wealth of genetic evidence suggests an essential role in gamete fusion in flowering plants and protists, including the thale cress *Arabidopsis thaliana*, the malaria parasite *Plasmodium*, the slime mold *Dictyostelium* and the aforementioned algae *Chlamydomonas* (Hirai et al., 2008; Johnson et al., 2004; Liu et al., 2008; Mori et al., 2006; Okamoto et al., 2016; von Besser et al., 2006). Most genetic and cell biological experiments in plants, *Chlamydomonas* and *Plasmodium* point towards a requirement for HAP2 on only one gamete, i.e. a unilateral requirement in male gametes (Hirai et al., 2008; Liu et al., 2008; Orias, 2014; von Besser et al., 2006; Wong et al., 2010). However, HAP2 has also been detected in ovules in *Arabidopsis* and maize (Borges et al., 2008; Moiseeva et al., 2017). Conflicting views have also been reported for *Chlamydomonas*: most studies suggest that HAP2 is present only in the *mt−* gamete, but low expression levels have been detected in the *mt+* gamete (Mori et al., 2006). In the ciliated single-celled protozoan *Tetrahymena*, both fusing gametes require surface expression of HAP2 to result in efficient fusion (Cole et al., 2014).

In *Arabidopsis*, structure-function studies suggest that the N-terminal ectodomain of sperm HAP2 interacts with proteins on the surface of the egg (Mori et al., 2010; Wong et al., 2010). To test sufficiency and understand the mechanistic basis of HAP2 from *Arabidopsis*, it was expressed in mammalian cells. Upon HAP2 overexpression, multinucleation and cytoplasmic content mixing are observed but only when the protein is present on opposing cells (Valansi et al., 2017). *Arabidopsis* HAP2 has also been expressed on the surface of pseudotyped vesicular stomatitis virus (VSV), and the resulting pseudotyped virus VSVΔG-HAP2 is able to fuse to BHK hamster cells, but only when HAP2 is simultaneously expressed on the surface of the target cells (Valansi et al., 2017). Thus, all the sufficiency-based assays suggest that *Arabidopsis* HAP2 mimics an EFF-1-like bilateral mechanism (Fig. 7).

In recent years, structural studies have provided some insight into how HAP2 proteins might function during cell-cell fusion. For instance, the recently determined structure of the *Chlamydomonas* HAP2 ectodomain uncovered the presence of trimers of hairpins highly homologous to EFF-1 and class II viral fusogens (Fig. 6); these were also predicted using bioinformatic modeling (Fedry et al., 2017; Pinello et al., 2017; Valansi et al., 2017). It was proposed that *Chlamydomonas* HAP2 contains two amphipathic loops that closely mimic fusion loops found at the end of domain 2 of class II viral fusogens (Fedry et al., 2017). In viruses, these loops contain bulky hydrophobic residues that have evolved to insert into the target membrane, a crucial step during the pre- to postfusion conformational transition (Harrison, 2008; White et al., 2008). If the bulky hydrophobic residues are mutated, the ability of enveloped viruses to infect and fuse is abrogated (Costin et al., 2013; Delos et al., 2000; Gregory et al., 2011; Hannah et al., 2007; Steinhauer et al., 1995). Although difficult to firmly conclude, owing to an unresolved region of the protein structure, the two amphipathic loops in *Chlamydomonas* HAP2 are depicted as being exposed and inserted into the membrane (Fedry et al., 2017). The ectodomain of *Chlamydomonas* HAP2 is monomeric and binds liposomes only when assembled into trimers (Fedry et al., 2017). Moreover, when two bulky residues in the amphipathic loops of *Chlamydomonas* HAP2 are mutated (F192A-W193A), binding is abrogated, supporting a viral-like insertion mechanism for HAP2 (Fedry et al., 2017). However, a crucial difference with the viral counterpart is that the double F192A-W193A mutant is still able to mediate gamete fusion at ∼65% of the efficiency of the wild type, whereas for viral fusogens such as those found in flaviviruses and alphaviruses, equivalent mutations severely impair fusion (Kielian et al., 1996; Stiasny et al., 1996). The membrane-penetrating activity conferred by the bulky residues, although stimulatory, cannot therefore comprise the core mechanism of action by which HAP2 fuses membranes.

As mentioned above, HAP2 is present on both of the fusing cells in *Tetrahymena*. However, the deletion of HAP2 from one cell is still able to support fusion, although not as efficiently, implicating a unilateral mechanism of fusion (Pinello et al., 2017). The presence of viral-like fusion loops in *Tetrahymena* HAP2 has been proposed, the mutations of which give rise to fusion defects. However, a mutation that affects the folding of the same region in the bilateral fusogen EFF-1 also gives rise to a fusion defect (Pérez-Vargas et al., 2014), and thus a unilateral viral-like mechanism cannot be concluded based only on the impairment of fusion as a result of disrupting the putative fusion loops. Interestingly, peptides obtained from the predicted fusion loop of *Tetrahymena* HAP2 can induce membrane merging to the same extent as peptides derived from viral fusion loops (Pinello et al., 2017), suggesting a fusion-promoting activity of the loops. Nonetheless, we must caution that other peptides exhibiting fusogenic activities with model membranes

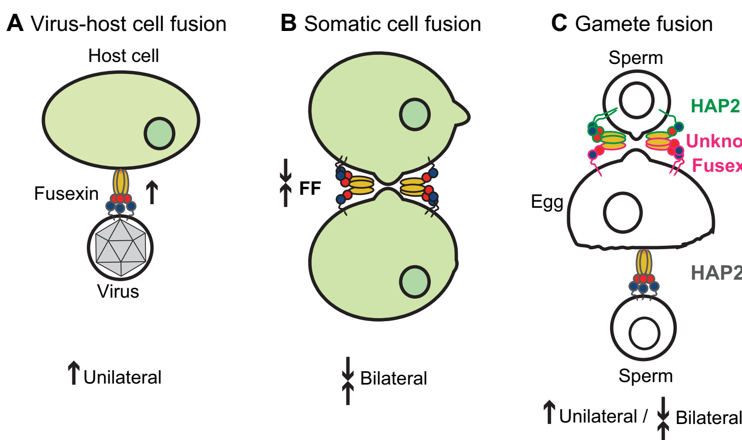

Fig. 7. Fusexins share a postfusion structure but achieve it via different mechanisms. (A) Virus-host cell fusion is unilateral and the fusogen is present only in the viral envelope. (B) Somatic cell-cell fusion is mediated by FF proteins (EFF-1 and AFF-1) that exhibit bilateral activity; *Arabidopsis* HAP2 introduced into mammalian cells also exhibits bilateral fusogenic activity. (C) Two models for HAP2-mediated gamete fusion. (Bottom) Genetic analyses in several sexually reproducing organisms indicate that HAP2 is present or required in one gamete only (unilateral model). (Top) It is conceivable that unidentified Fusexins (or other unidentified proteins) are present in the opposite gamete (bilateral model), as suggested by sufficiency tests in mammalian cells. Adapted with permission from Valansi et al. (2017).

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were later found not to be fusogenic *in vivo* (Blobel et al., 1992; Cho et al., 2000; Muga et al., 1994). Thus, a more stringent unilateral sufficiency test, such as one involving pseudotyped viruses as described above, would allow a more conclusive assessment of a viral-like fusion mechanism of *Tetrahymena* HAP2.

The requirements for specific residues in the postulated fusion loop among different HAP2 proteins might reflect evolutionarily distinct strategies to fuse cells (Fig. 7). The most striking example of this is the role of a conserved arginine between the predicted loops; the mutation of this residue (R185A) blocks gamete fusion in *Chlamydomonas* (Fedry et al., 2017), whereas the equivalent mutation (R164A) in *Tetrahymena* shows no defect (Pinello et al., 2017). A detailed analysis of this region in *Arabidopsis* HAP2 is pending, but the ability of heterologously expressed HAP2 to mediate fusion with cells expressing EFF-1 shows that if a membrane-penetrating function is present, it appears to be overridden by the ability of HAP2 to interact in *trans* with a bilateral fusogen (Valansi et al., 2017). Likewise, the bilateral requirement of HAP2 in heterologous cells does not preclude a unilateral mechanism in the natural physiological context either, as it is not possible to discard the potential involvement of other proteins (such as unknown receptors) that might be important for the positioning or assembly of HAP2 across the opposing membrane (Fig. 7C). A curious observation is that in bilateral crosses of *hap2* knockout *Tetrahymena* cells, a severe adhesion defect is observed, whereas when HAP2 is present on at least one cell the adhesion efficiency is unaffected (Pinello et al., 2017). This might be indicative of the presence of an unidentified protein interacting in *trans* with HAP2, as has been proposed for *Arabidopsis* HAP2 (Fig. 7C). Thus, further analyses using more stringent unilateral and bilateral sufficiency tests may be necessary to determine whether there is a bi-modal mechanism of fusion among HAP2 proteins or if the fusion mechanism has diverged to fit specific physiological contexts, as previously suggested (Pinello et al., 2017).

Syncytins: placental unilateral fusogens

The placenta is a transient organ that is essential for fetal development (Cross et al., 1994). In humans, cell-cell fusion is required for CTB fusion and formation of the syncytiotrophoblast (STB) – the outer epithelial layer of the placental villi (Potgens et al., 2004). A number of years ago, the analysis of a set of human endogenous retroviral elements expressed in placental tissues led to the identification of a gene, named *Syncytin-1* (*ERVW-1*), that was predicted to encode a viral envelope protein capable of acting as a fusogen (Blond et al., 1999; Mi et al., 2000). Transfection of several cell lines with Syncytin-1 gave rise to multinucleated syncytia when these cells were mixed with cells expressing the receptor Ala/Ser/Cys/Thr transporter type 1 and 2 (ASCT1 and ASCT2; also known as SLC1A4 and SLC1A5) (Blond et al., 2000), suggesting a conserved mechanism of action with viral fusogens. Remarkably, the receptor requirement can be bypassed in certain cell lines and also when Syncytin-1-expressing cells are incubated with protein-free liposomes, conclusively showing that Syncytin-1 is unilaterally sufficient for membrane fusion (Mi et al., 2000). Soon after this discovery, a second human Syncytin (Syncytin-2; or ERVFRD-1) was identified and was also shown to be sufficient for the fusion of cells expressing the MFSD2A receptor (Blaise et al., 2003; Esnault et al., 2008). In mice, two independent Syncytins (A and B) are required for normal placenta formation and are sufficient to fuse cells (Dupressoir et al., 2005, 2011; Peng et al., 2007). Intriguingly, Syncytins have recently been implicated in cell fusion during formation of multinucleated osteoclasts (Møller et al., 2017; Søe et al., 2011), in the pathogenesis of cancer (Bolze et al., 2016; Larsson et al., 2007a,b) and even in enhanced myoblast fusion in male mice (Redelsperger et al., 2016).

A number of factors that regulate Syncytins have been identified. For instance, Syncytins are known to be glycosylated, and the degree and positioning of glycosylation regulates fusogenic activity and interactions with cognate receptors (Cui et al., 2016). The transcription factor GCM1 is the major regulator of Syncytins during STB formation and, in turn, is regulated by the cAMP/PKA signaling pathway (Liang et al., 2010; Yu et al., 2002). Growth factors, cytokines, nuclear hormone receptors, the actin cytoskeleton and adhesion molecules – all of which are required for preparatory stages of CTB fusion – are also likely to mediate their effects via Syncytins (Dalton et al., 2007; Ruebner et al., 2012; Shibukawa et al., 2010). Mechanistically, however, less is known about Syncytins from a structure-function standpoint. The structural profiling of Syncytins is close to that of the HIV-1 envelope glycoprotein (gp) 160, the precursor of gp41, which contains a fusion peptide, a TMD and folding elements that make up the structural core of viral class I fusogens (Gong et al., 2005). Although there is a certain degree of assumption that Syncytins operate like class I fusogens, further systematic biochemical experiments would be helpful to distinguish any adaptations of a Syncytin-based fusion machinery to the specific context of STB formation.

Myomaker and its associated micropeptide: the machinery of myoblast fusion in mice

In contrast to *Drosophila*, myoblast fusion in vertebrates is homotypic, and there is no evidence for 'founder' (FC-like) and 'feeder' (FCM-like) subpopulations. Myomaker – a multispan transmembrane protein expressed on the plasma membrane of myoblasts – was revealed to be required for bilateral myoblast fusion in mouse and zebrafish (Goh and Millay, 2017; Millay et al., 2016, 2013, 2014; Mitani et al., 2017; Zhang and Roy, 2017). Three groups have recently reported that a micropeptide (Myomerger/Myomixer/Minion; the three groups gave this small protein of ∼100 residues different names) is required in conjunction with Myomaker to fuse myoblasts (Bi et al., 2017; Quinn et al., 2017; Zhang et al., 2017). Both Myomaker and the micropeptide can mediate myoblast-fibroblast and fibroblast-fibroblast fusion. This is in contrast to Myomaker alone, which can only mediate myoblast-fibroblast fusion when fibroblasts are transfected with Myomaker (Millay et al., 2013, 2014). There is also evidence that Myomaker and the micropeptide physically interact to control the formation of multinucleate skeletal muscles (Bi et al., 2017), although conclusive biochemical evidence demonstrating that an interaction between Myomaker and the micropeptide is directly coupled to the merging process awaits to be demonstrated.

Myomaker and the micropeptide can induce fusion of non-myogenic cells that normally do not fuse, demonstrating sufficiency for fusion (Bi et al., 2017; Quinn et al., 2017; Zhang et al., 2017). Myomaker was shown to localize to the Golgi apparatus and is transported to the plasma membrane where it acts at or before the hemifusion stage between C2C12 myoblasts (Gamage et al., 2017). Another protein implicated in myoblast fusion is fibroblast growth factor receptor-like 1 (FGFRL1), which can fuse CHO cells, although in knockout mice a defect is only observed in slow muscle fibers; premature death prevents evaluating a role for FGFRL1 at later stages of myogenic development (Steinberg et al., 2010a,b; Zhuang et al., 2015). Surprisingly, the TMD and specific

hydrophobic amino acids in an Ig domain (Ig3) are essential for its fusogenic activity, although no receptor partners have been identified (Zhuang and Trueb, 2017). Additional experiments are thus needed to determine how the Myomaker-micropeptide system mediates fusion and whether it acts in conjunction with FGFRL1 or if they display non-overlapping functions in different myogenic tissues. Importantly, it will be interesting to determine if similar fusogens are also involved in *Drosophila* myoblast fusion.

The origins and evolution of cell fusogens

The structural and functional similarities between EFF-1, HAP2 and viral class II fusion proteins suggest a common ancestry to this diverse group of fusogens. This has led to a reclassification scheme comprising a superfamily of proteins called Fusexins: fusion proteins essential for sexual reproduction and exoplasmic merger of plasma membranes (Valansi et al., 2017) (Fig. 8). Despite the evident conservation of their structural folds, the Fusexins appear to have diverged mechanistically to adapt to specific biological scenarios. Viruses, for instance, use a 'self-sufficient' unilateral strategy by inserting fusion loops into cellular target membranes (Harrison, 2008; Kielian and Rey, 2006; Podbilewicz, 2014; Stegmann et al., 1989; White et al., 2008) (Fig. 7A). Genetic evidence is consistent with a viral-like unilateral mechanism for *Chlamydomonas*, *Arabidopsis*, *Plasmodium* and *Tetrahymena* HAP2-mediated gamete fusion, a fusion strategy that is reminiscent of one gamete 'taking the lead' in fertilization (Fedry et al., 2017; Hirai et al., 2008; Liu et al., 2008; Mori et al., 2006; Pinello et al., 2017; von Besser et al., 2006). By contrast, *C. elegans* FF proteins and *Arabidopsis* HAP2 appear to engage in a bilateral mechanism, as concluded from sufficiency-based fusion assays, a strategy that enables tighter spatial and temporal control of cell fusion (Avinoam et al., 2011; Podbilewicz et al., 2006; Valansi et al., 2017). Lastly, *Tetrahymena* HAP2 may adopt a bi-modal mechanism in which EFF-1-like *trans* interactions pull the membranes together, with the insertion of fusion loops facilitating membrane merging (Pinello et al., 2017). Future structure-function analyses, including more comprehensive fusion sufficiency tests for all Fusexins, are needed to determine the degree of mechanistic promiscuity of the Fusexins.

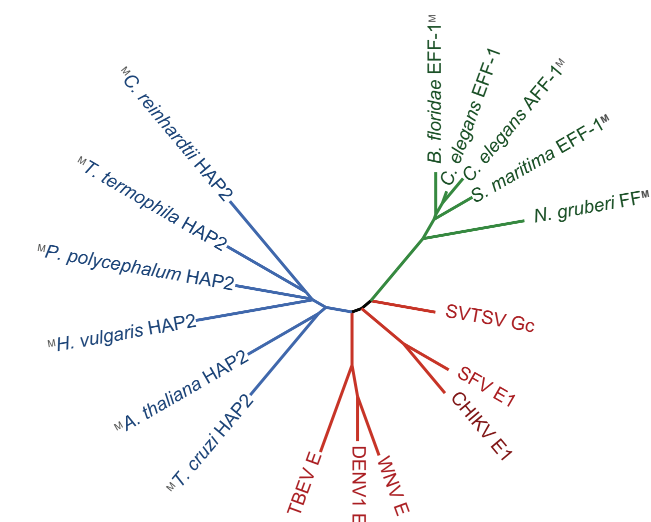

Fig. 8. Structural relationships between viral, sexual and somatic Fusexins. Unrooted tree inferred using a distance matrix. Superscript M indicates a modeled structure; HAP2, blue; EFF-1/AFF-1/FF, green; class II viral fusogens, red. Reproduced with permission from Valansi et al. (2017).

Fusexins are present in very diverse viral groups (Modis, 2014) and in most eukaryotic lineages (Speijer et al., 2015). Current phylogenetic studies are unable to track whether the original Fusexin was forged within a cellular or a viral genome. The 'cell hypothesis' proposes that viral Fusexins are derived from different lineages via the capture of cellular genes encoding eukaryotic fusogens (Frame et al., 2001; Pinello et al., 2017). Sexual Fusexins (HAP2) have been identified in the basal lineages of almost all eukaryotic kingdoms except for fungi (Steele and Dana, 2009; Wong and Johnson, 2010). Thus, it is conceivable that sexual Fusexins originated from an ancient gene that is at the base of the last common eukaryote but which was later lost in fungi. This ancestral Fusexin was also likely to be crucial for the evolution of eukaryotes and to the origin of sexual reproduction (Pinello et al., 2017).

An alternative 'virus hypothesis' proposes that sexual Fusexins are of viral origin and appeared before modern eukaryotic sexual reproduction (Koonin and Dolja, 2013; Koonin et al., 2015; Nasir and Caetano-Anolles, 2015). The presence of endogenous viral signatures in eukaryotic genomes is extensive and consistent with such a scenario (Frame et al., 2001). The Syncytins are a unique example of virus-to-cell gene transfer, representing genes that were captured and lost independently several times in different placental mammals (Esnault et al., 2013). A current evolutionary model proposes that ∼150 million years ago, a founding retroviral envelope gene enabled the transition from egg-laying to placental animals. During the radiation of different mammalian lineages, this ancestral Syncytin gene was replaced on multiple occasions by adoption of newer retroviral envelope genes, perhaps by genes encoding progressively more efficient Syncytins (Cornelis et al., 2015). In this way, mammals acquired class I fusion glycoproteins related to HIV envelope genes (Nakaya and Miyazawa, 2015; Renard et al., 2005). Likewise, it is conceivable that an ancient virus might have transferred a Fusexin gene to sexually reproducing eukaryotes and replaced a less efficient fusion machinery that became extinct (Doms, 2017).

AFF-1- and EFF-1-like proteins were probably the last Fusexins to appear, since proto-viruses and gamete fusion preceded multicellularity. Modern somatic Fusexins (FF proteins) are presently restricted to multicellular invertebrates (nematodes, arthropods, ctenophores and the chordate *Branchiostoma*), with the exception of *Naegleria* (a flagellated amoeba) (Avinoam et al., 2011). Given that HAP2 was not recognized as a family member until recently, it is possible that additional somatic Fusexins are present in other organisms but are difficult to identify due to low sequence similarity. Alternatively, the distribution pattern indicates that Fusexins were either introduced later in evolution into certain multicellular organisms by horizontal gene transfer or that they were lost from other lineages of multicellular eukaryotes (Podbilewicz, 2014). Indeed, the absence of identified sexual fusogens in fungi and vertebrates leaves the window wide open for the presence of new fusogen families with entirely different design principles. This appears to be the case with the Myomaker-micropeptide machinery, while FAST fusogens from non-enveloped viruses that mediate cell-cell fusion are a reminder that multiple evolutionarily unrelated families of fusogens may have arisen (Podbilewicz, 2014; Shmulevitz and Duncan, 2000).

Conclusions

The field of membrane fusion was inspired for decades by work on viral and then later intracellular fusion machineries. The

classification of these proteins as fusogens was gradual and a result of careful genetic, structural and biochemical analyses. In this Review, we have applied the same level of stringency in assessing bona fide cell fusogens, concluding that a hallmark of cell-cell fusion is the presence of a fusogen consisting of either a single protein or a multiprotein complex, engendered with the energetically costly tasks of dehydrating the polar head groups, promoting hemifusion stalks and opening and expanding pores (Fig. 1). Despite obvious physiological differences between the different types of cell fusion systems, we do not regard as coincidence the fact that disruption of many fusion machineries consistently impedes plasma membranes from getting closer together than 10 nm. This commonality serves to highlight that membrane fusion entails biophysical forces that are conserved across a wide spectrum of biological fusion reactions.

With the sufficiency tests completed for Syncytins, Fusexins and now Myomaker-micropeptide, these are exciting times for research in the cell-cell fusion field. Several mechanistic questions need to be resolved, but the key outstanding questions are no longer about ‘if’ but rather ‘how’ these proteins mediate membrane fusion. In addition, the evolutionary connection of Syncytins and Fusexins with viral fusogens could provide clues to finding new candidates, in particular the identification of cell fusogens in vertebrates and in fungi, where a few candidates have been found but for which sufficiency tests are still lacking. Indeed, it is likely that surprising and exciting findings in the coming years will lead to the discovery of these missing fusogens, as well as enlighten us as to how cell fusogens work at the molecular level.

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Competing interests
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