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# Fusogenic Reoviruses and Their Fusion-Associated Small Transmembrane (FAST) Proteins

Roy Duncan

Department of Microbiology & Immunology, Department of Biochemistry & Molecular Biology, and Department of Pediatrics, Dalhousie University, Halifax, Nova Scotia, Canada, B3H 4R2; email: roy.duncan@dal.ca

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## Keywords

viral membrane fusion proteins, cell-cell fusion, syncytium formation, viroporins, fusogenic reoviruses

## Abstract

With no limiting membrane surrounding virions, nonenveloped viruses have no need for membrane fusion to gain access to intracellular replication compartments. Consequently, nonenveloped viruses do not encode membrane fusion proteins. The only exception to this dogma is the fusogenic reoviruses that encode fusion-associated small transmembrane (FAST) proteins that induce syncytium formation. FAST proteins are the smallest viral membrane fusion proteins and, unlike their enveloped virus counterparts, are nonstructural proteins that evolved specifically to induce cell-to-cell, not virus-cell, membrane fusion. This distinct evolutionary imperative is reflected in structural and functional features that distinguish this singular family of viral fusogens from all other protein fusogens. These rudimentary fusogens comprise specific combinations of different membrane effector motifs assembled into small, modular membrane fusogens. FAST proteins offer a minimalist model to better understand the ubiquitous process of protein-mediated membrane fusion and to reveal novel mechanisms of nonenveloped virus dissemination that contribute to virulence.

## 1. INTRODUCTION

**FAST:** fusion-associated small transmembrane

**FP:** fusion peptide

**TM:** transmembrane

**MPER:** membrane-proximal external region

The family *Reoviridae* comprises 15 genera divided into two subfamilies, the *Spinareovirinae* and *Sedoreovirinae* (1). Common features of these viruses are a nonenveloped capsid surrounding a double-stranded RNA genome of 9–12 individual segments present at equimolar ratios. Members in two of these genera, *Aquareoviruses* and *Orthoreoviruses*, are the only known examples of nonenveloped viruses that induce syncytium formation. Syncytiogenesis is attributable to an unusual family of viral membrane fusion proteins, the fusion-associated small transmembrane (FAST) proteins (2). Unlike enveloped virus fusogens (i.e., membrane fusion proteins) that evolved to mediate virus-cell fusion during the entry process, FAST proteins are nonstructural, accessory proteins that induce cell-cell fusion (3). These rudimentary viral fusogens bear no structural similarity or evolutionary relationship to enveloped virus fusogens, resembling instead a large collection of small, unrelated viral membrane remodeling proteins collectively referred to as viroporins (4). Phylogenetic analyses suggest the extant FAST proteins arose as the only fusogenic viroporins by two or more separate gain-of-fusion events, assembling specific combinations of distinct membrane-interactive motifs into minimalist membrane fusion machines. This review focuses on evolutionary aspects of the FAST proteins and the fusogenic reoviruses that encode them, on our current understanding of how FAST proteins promote virus dissemination and virulence, and on how the varied FAST protein membrane-interactive motifs function in a coordinated manner to mediate cell-cell fusion and syncytium formation.

## 2. MEMBRANE FUSION AND ENVELOPED VIRUS FUSOGENS

Numerous physiological processes such as the formation and maintenance of muscle, bone, and placenta require cell-cell membrane fusion (5). To fuse membranes, polar lipid headgroups must be dehydrated and membrane bilayers must be distorted into thermodynamically unfavorable structures. In the prevailing fusion-through-hemifusion model, negative curvature in the outer bilayer leaflet (i.e., the leaflet curves outward from the plane of the membrane) creates a highly curved dimple (6). The strong positive curvature at the apex of the dimple forces apart lipid headgroups, exposing the underlying acyl chains to water. These hydrophobic defects and membrane stresses promote merging of the outer leaflets of the two bilayers to create an unstable hemifusion intermediate. Induction of positive curvature in the inner leaflets leads to resolution of the hemifusion intermediate and formation of a stable pore, the major energy barrier in the fusion reaction (7, 8).

Physiological membrane fusion requires protein fusogens to alter membrane curvature and induce membrane stresses during the fusion reaction (9). Enveloped viruses use complex, multimeric glycoproteins to mediate virus-cell fusion. The different classes of enveloped virus fusogens differ considerably in their tertiary and quaternary structures but are remarkably similar in their presumed mechanism of action (10). Receptor binding and/or low pH in the endocytic pathway trigger dramatic, irreversible conformational rearrangements, converting the prefusion structure into an extended intermediate with a fusion peptide (FP) motif at its tip. These FPs contain hydrophobic residues that insert into the target membrane. FPs are structurally dynamic in different environments, and they share a high degree of amino acid similarity within members of the same fusogen class (11–13). This extended intermediate tethers the two membranes together, anchored by the transmembrane (TM) domain in the virus envelope and to the cell membrane via the FP. Fold-back of the extended intermediate into a post-fusion trimeric hairpin draws the two membranes together and positions the FP and TM domain in close proximity. How membrane merger occurs is unknown, but the membrane-proximal external region (MPER), TM domain, and FP motif are all implicated in this process. Evidence suggests hydrophobic (frequently aromatic) amino acid side chains in both the FP and MPER insert into the outer leaflet of the two

bilayers, inducing curvature stresses and altering lipid headgroup hydration (14, 15). Juxtaposition of these three motifs in the trimeric hairpin structure and their structural plasticity allows interactions between the FP and the TM domain or MPER (15, 16), promoting the lipid rearrangements needed for pore formation.

## 3. AQUAREOVIRUSES AND ORTHOREOVIRUSES

The 15 genera in the family *Reoviridae* are highly diverged, sharing only vestigial sequence similarity between homologous proteins. The genera *Aquareovirus* and *Orthoreovirus* are notable exceptions, sharing highly similar capsid structures and sequence similarity in their nine homologous proteins (17–19). The seven recognized species of aquareoviruses (AqRVs) contain 11 genome segments and infect saltwater and freshwater fish. Orthoreoviruses (ORVs) contain 10 genome segments and infect a wide range of vertebrate hosts (**Figure 1*b***), including humans (MRV, *Mammalian orthoreovirus*), reptiles (RRV, *Reptilian orthoreovirus*), baboons (BRV, *Baboon orthoreovirus*), bats (NBV, *Nelson Bay orthoreovirus*), domesticated land- and waterfowl (ARV, *Avian orthoreovirus*), and a newly recognized species (PRV, *Piscine orthoreovirus*) that infects fish (20). It is unclear whether a recent species isolated from bat flies (MaRV, *Mahlapitisi orthoreovirus*) extends the host range of ORVs to invertebrates (21) or if MaRV was obtained passively following a blood meal from an infected vertebrate host. Three additional tentative ORV species broaden the host range of this genus (**Figure 1*b***). A tortoise isolate (22) extends the reptilian ORV host range from Lepidosauria (snakes and lizards) to Testudines (turtles and tortoises), and three wild bird isolates that group together as a species extend the avian ORV hosts from Galloanserae (water- and landfowl) to Neoaves (23–25). The third tentative species (BrRV, *Broome orthoreovirus*) was isolated from fruit bats (26), as was NBV (27), but these viruses share <25% amino acid identity in their outer capsid clamp proteins and each possesses unique 5′-terminal signature sequences. Such terminal sequences are conserved in all 10 genome segments within, but not between, ORV species and constitute one of the polythetic criteria used to define a new ORV species (28, 29).

**AqRV:** aquareovirus

**ORV:** orthoreovirus

**MRV:** mammalian orthoreovirus

**RRV:** reptilian orthoreovirus

**BRV:** baboon orthoreovirus

**NBV:** Nelson Bay orthoreovirus

**ARV:** avian orthoreovirus

**PRV:** piscine orthoreovirus

**MaRV:** Mahlapitsi orthoreovirus

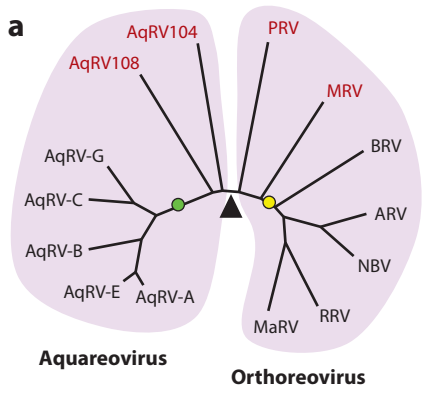
**BrRV:** Broome orthoreovirus

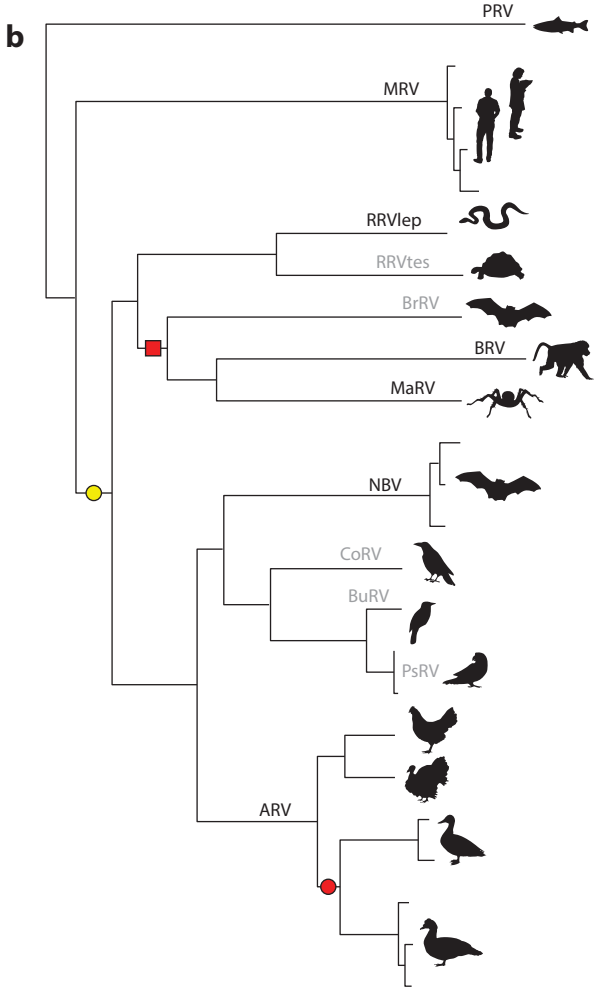
## 4. THE FUSOGENIC REOVIRUSES

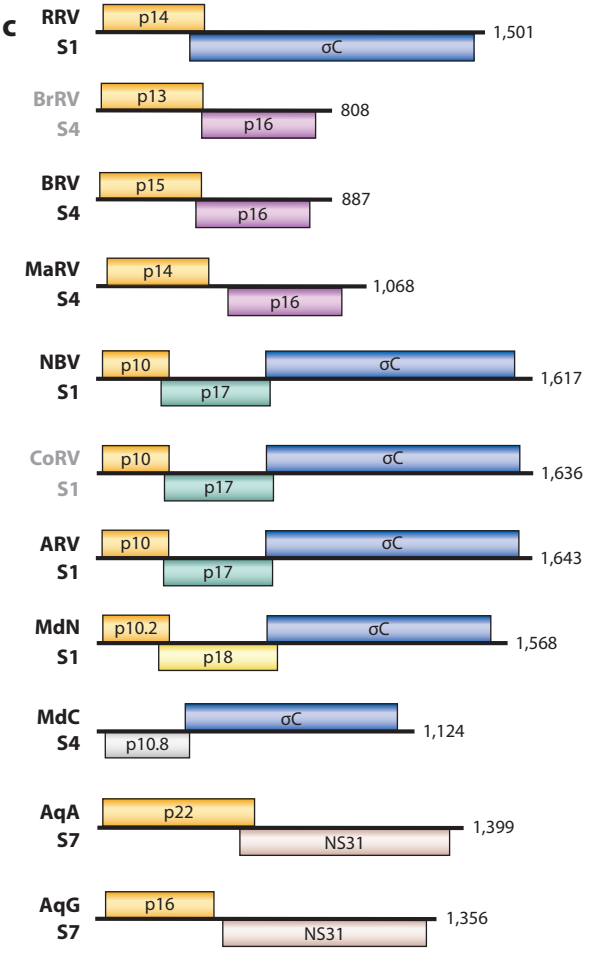
The majority, but not all, of the AqRV and ORV species induce syncytium formation (**Figure 1*a***). The prototypical MRVs and PRV are both nonfusogenic (30), as are two recent isolates of AqRVs (AqRV104 and AqRV108) from grass carp (19, 31). The remaining species in both genera constitute the known fusogenic reoviruses. Phylograms constructed using concatenated sequences of homologous proteins, including those from other *Reoviridae* genera as outgroup viruses, revealed a well-defined branch point between the AqRVs and ORVs (19). The two species of AqRV and ORV closest to this branchpoint are nonfusogenic, while the more distally branching species are all syncytiogenic (**Figure 1*a***), suggesting two separate gain-of-fusion events led to evolution of the extant fusogenic AqRVs and ORVs from an ancestral nonfusogenic virus. Similar phylogenetic analysis also reveals an apparent loss-of-fusion event. The ARV species is more diverse than other species, with isolates from water- and landfowl segregating to distinct clades, as do their host species (**Figure 1*b***). While all landfowl isolates are syncytiogenic, reported waterfowl isolates lack cell-cell fusion capacity (32). The most parsimonious explanation is a single loss-of-fusion event as these two viruses and their respective hosts diverged from each other.

### 4.1. Pathogenicity and Biological Features of the Fusogenic Reoviruses

For the most part, fusogenic and nonfusogenic reoviruses have similar replication strategies and share the same constellation of genes, excluding the gene that confers syncytiogenic capability to




(*Caption appears on following page*)

**Figure 1** (*Figure appears on preceding page*)

Phylogenetic relationships in the fusogenic AqRVs and ORVs. (*a*) Phylogenetic relationships between the seven recognized ORV species and five sequenced AqRV species plus two tentative new AqRV species (AqRV104 and AqRV108). The phylogram was constructed using amino acid sequences of the homologous inner capsid clamp proteins and number of differences method. Species in red are nonfusogenic, while those in black are fusogenic. The black arrowhead indicates the branchpoint leading to outgroup viruses from other genera in the family, all of which are nonfusogenic. Colored circles indicate presumed gain-of-fusion events leading to the fusogenic species. (*b*) Phylogram of the recognized and tentative (*gray lettering*) ORV species based on the outer capsid clamp protein, constructed using the number of differences method. Recognized hosts for each virus species are indicated with images. The yellow circle corresponds to the gain-of-fusion event depicted in panel *a*. The red circle is the predicted loss-of-fusion event leading to waterfowl isolates of ARV. The red box is the presumed recombination event leading to loss of σC and gain of p16 proteins diagrammed in panel *c*. (*c*) Polycistronic genome segments of the indicated fusogenic ORVs and AqRVs. Abbreviated virus names and their respective genome segments (S1, S4, or S7) are on the left. Names in gray are tentative species. The solid line represents the double-stranded RNA, and numbers indicate the nucleotide length of the genome segment. Rectangles depict the ORFs named according to their translation products. The orange ORFs encode the FAST proteins. Other ORFs are color-coded to indicate homologous proteins. RRV isolates from Testudines (RRVtes) and Lepidosauria (RRVlep) share the same depicted gene arrangement. The BuRV and PsRV Neoaves isolates share the depicted gene arrangement for CoRV. AqRV-E and AqRV-B share the arrangement of ORFs shown for AqRV-A (AqA), while AqRV-G (AqG) has the same arrangement as AqRV-C. The ORF arrangements of novel and classical MdRVs (MdN and MdC, respectively) are also depicted. Abbreviations: AqRV, aquareovirus; ARV, avian orthoreovirus; BrRV, Broome orthoreovirus; BRV, baboon orthoreovirus; BuRV, bulbul orthoreovirus; CoRV, corvid orthoreovirus; FAST, fusion-associated small transmembrane; MaRV, Mahlapitsi orthoreovirus; MdRV, Muscovy duck orthoreovirus; MRV, mammalian orthoreovirus; NBV, Nelson Bay orthoreovirus; ORF, open reading frame; ORV, orthoreovirus; PRV, piscine orthoreovirus; PsRV, psittacine orthoreovirus; RRV, reptilian orthoreovirus.



the fusogenic reoviruses. Furthermore, interventions that inhibit cell-cell fusion induced by ARV and RRV have no significant effect on the virus replication cycle or infectious progeny virus production (3). Thus, syncytium formation is an accessory, not an essential, biological property of fusogenic reoviruses, a conclusion supported by the presumed gain and then loss of MdRV fusion ability (**Figure 1*b***). The fact that 10 of the 14 ORV and AqRV species acquired and maintained this biological property, however, suggests that cell-cell fusion confers some competitive advantage to the virus. While cell-cell fusion is not required for virus-induced cytopathology and virus egress, both of these processes are markedly enhanced by syncytium formation (33, 34). Syncytia maintain essential gene expression and metabolic functions, meaning they provide an expanded localized environment for virus replication. When syncytia become very large, they rupture, leading to a burst of progeny virus release for systemic dissemination of the infection (34). By promoting both localized and systemic virus spread, cell-cell fusion provides the fusogenic reoviruses with novel means to enhance nonenveloped virus dissemination within an infected host. Increased spread of the infection due to cell-cell fusion is a likely contributor to the virulence of the fusogenic reoviruses, all of which are associated with pathologies following natural infections. Several NBVs have been isolated from humans with acute respiratory infections (35–41), and serosurveys indicate zoonotic transmissions are frequent and may be a cause for concern (42). BRV causes meningoencephalomyelitis in nonhuman primates (43–45), RRV causes pneumonia and neurological dysfunctions in snakes (46, 47), and ARV induces a range of pathologies in commercial poultry flocks including enteritis, tenosynovitis, and myocarditis (48). AqRV infections are generally asymptomatic but have been associated with hepatic necrosis (49). In support of cell-cell fusion as a virulence determinant of the fusogenic reoviruses, a recombinant vesicular stomatitis virus expressing the RRV p14 FAST protein is more neurovirulent following intranasal inoculation of mice (50). Recent development of a reverse genetics system for fusogenic reoviruses and a mouse model for pathogenic NBV isolates should allow more direct assessment of the role of cell-cell fusion in virulence (51, 52).

## 4.2. Evolutionary Relationships of the Fusogenic Reoviruses

**ORF:** open reading frame

**MdRV:** Muscovy duck orthoreovirus

The viral genome segments responsible for syncytium formation are all polycistronic, with a remarkable diversity in the composition and arrangement of open reading frames (ORFs) (**Figure 1*c***). The five sequenced species of AqRVs segregate into two clades, both of which have bicistronic S7 genome segments whose 3′-proximal ORF encodes homologous NS31 nonstructural proteins of no defined function. These two clades differ in their 5′-proximal ORFs that encode distinct, but related, p22 or p16 FAST proteins (53–55). There are also two clades of fusogenic ORVs. The first comprises RRV [both Lepidosauria (RRVlep) and Testudine (RRVtes) isolates], BRV, MaRV, and the new tentative bat species, BrRV, all of which have a bicistronic gene arrangement (**Figure 1*c***). Note that in this clade, BRV is monophyletic in six of the nine homologous proteins, as shown in the outer capsid clamp protein phylogram (**Figure 1*b***), but paraphyletic in the core clamp protein phylogram (**Figure 1*a***), presumably a reflection of genome segment reassortment during evolution. All members of this clade encode a related FAST protein in their first ORF, but the downstream ORF encodes either the σC cell attachment fiber protein in RRV (28, 56) or an unrelated p16 protein in the other three species (57) (**Figure 1*c***). A single recombination event would explain concurrent loss of the fiber protein and gain of p16 in this monophyletic group.

ARV and NBV isolates constitute the second clade of fusogenic ORVs. This clade has a tricistronic gene arrangement with the 5′- and 3′-proximal ORFs encoding homologous FAST proteins and fiber proteins, respectively, and the central ORF encoding homologous p17 nonstructural proteins (58, 59) (**Figure 1*c***). This tricistronic, partially overlapping gene arrangement poses interesting issues regarding how ribosomes bypass two functional upstream ORFs to access the σC translation start site (60, 61). The Muscovy duck ORVs (MdRVs) are an anomaly in this clade as they are nonfusogenic. As with other members of this clade, the so-called novel MdRVs have a similar tricistronic S1 genome segment with 5′- and 3′-proximal ORFs encoding a FAST protein homolog and fiber protein, respectively (32), although the FAST protein is not functional. The central ORF encodes a p18 protein of unknown function that bears no resemblance to the p17 proteins encoded by other clade members. In contrast, the so-called classical MdRVs, the first to be sequenced (62), have a bicistronic S4 genome segment encoding the fiber protein and a p10.8 protein lacking any of the hallmark features of a FAST protein (63). The diversity in the number of ORFs on their polycistronic RNAs and the proteins they encode implies that recombination events, in addition to genome segment reassortment (i.e., exchange of entire genome segments), substantially contributed to fusogenic ORV evolution.

# 5. REOVIRUS FUSION-ASSOCIATED SMALL TRANSMEMBRANE PROTEINS

The fusogenic reoviruses encode six different FAST proteins composed of three small functional domains—an ectodomain, TM domain, and endodomain—all of which function as fusion modules (26, 28, 53, 54, 64–66). Despite limited sequence similarity and a diversity of membrane remodeling motifs, comparable domains between different FAST proteins are functionally interchangeable, although not all combinations are tolerated (55, 67–70), suggesting that a functional FAST protein requires specific combinations of membrane remodeling motifs. The ability of FAST proteins to promote cell-cell virus transmission and/or induce cytopathic effects has been exploited to enhance oncolytic virotherapy (50, 71, 72) and to establish a long sought-after reverse genetics system for rotaviruses (73). FAST proteins are promiscuous membrane fusogens that fuse almost all types of cells, a beneficial feature used to develop a lipid nanoparticle intracellular delivery

platform (74–76). These small, modular membrane fusion machines also provide a toolbox for membrane protein bioengineering and synthetic biology (77). For example, FAST proteins contain specific peptide motifs that govern partitioning into lipid rafts, homotypic multimerization, trafficking from the Golgi complex to the plasma membrane, and induction or sensing of membrane curvature (see below). These motifs could be used in research or clinical applications to alter the subcellular localization, stability, or multimeric status of cellular membrane proteins or to activate or inhibit signaling of membrane receptors. The following sections highlight some interesting evolutionary, structural, and functional features of FAST proteins, concluding with a model of how motifs in these three fusion modules function in a coordinated manner from both sides of the membrane to mediate cell-cell fusion.

## 5.1. Hallmark Features of Fusion-Associated Small Transmembrane Proteins

FAST proteins are all small (~100–200 residues), nonstructural viral proteins expressed inside virus-infected cells and trafficked through the endoplasmic reticulum (ER)-Golgi secretory pathway to the plasma membrane (78). A single TM domain serves as a reverse signal-anchor sequence to direct a bitopic $N_{out}/C_{in}$ type I topology in the membrane. This topology localizes a very small N-terminal ectodomain (~20–40 residues) external to the plasma membrane and positions considerably longer (~40–140 residues) C-terminal endodomains in the cytoplasm (**Figure 2**). As shown for ARV p10, RRV p14, and BRV p15, the ectodomains are small, amphiphilic peptides that share

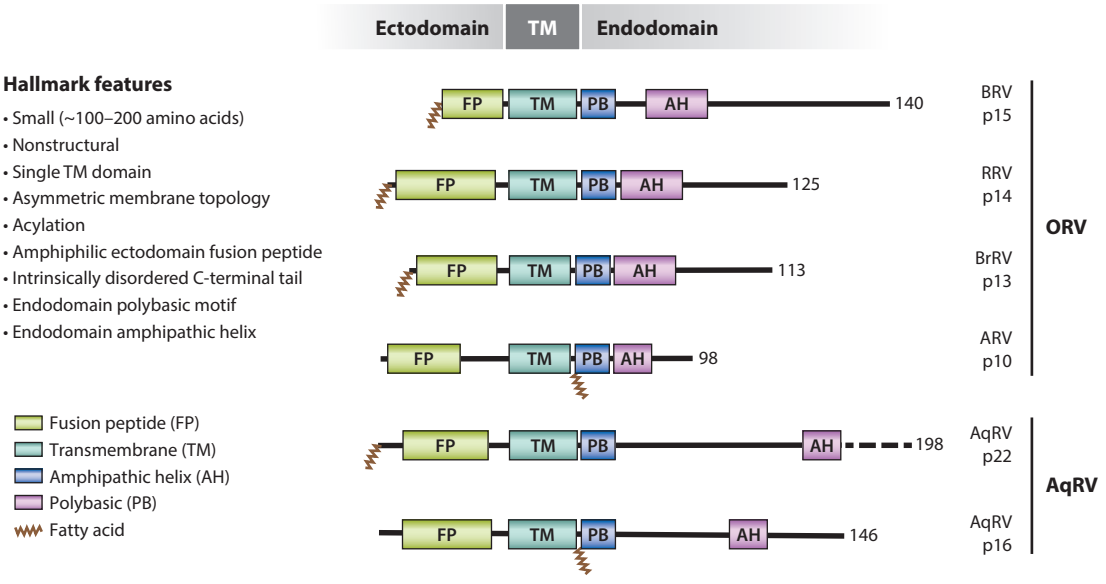



**Figure 2**

Hallmark features of FAST proteins. The hallmark features of FAST proteins are listed on the left, and the location and arrangement of key structural and functional motifs in the six distinct FAST proteins encoded by the ORVs and AqRVs are shown on the right, drawn to approximate scale and aligned at their TM domains. Numbers indicate the number of residues in each protein. The dashed line in AqRV p22 indicates an extended C-terminal tail. Boundaries of the motifs are approximate. Functional FP motifs and acylation sites have so far been confirmed for only BRV p14, RRV p14, and ARV p10. The approximate boundaries of the ectodomain, TM domain, and endodomain are diagrammed at the top. Abbreviations: AH, amphipathic helix; AqRV, aquareovirus; ARV, avian orthoreovirus; BrRv, Broome orthoreovirus; BRV, baboon orthoreovirus; FAST, fusion-associated small transmembrane; FP, fusion peptide; ORV, orthoreovirus; PB, polybasic; RRV, reptilian orthoreovirus; TM, transmembrane.

**PB:** polybasic

**AH:** amphipathic helix

**CM:** conserved motif

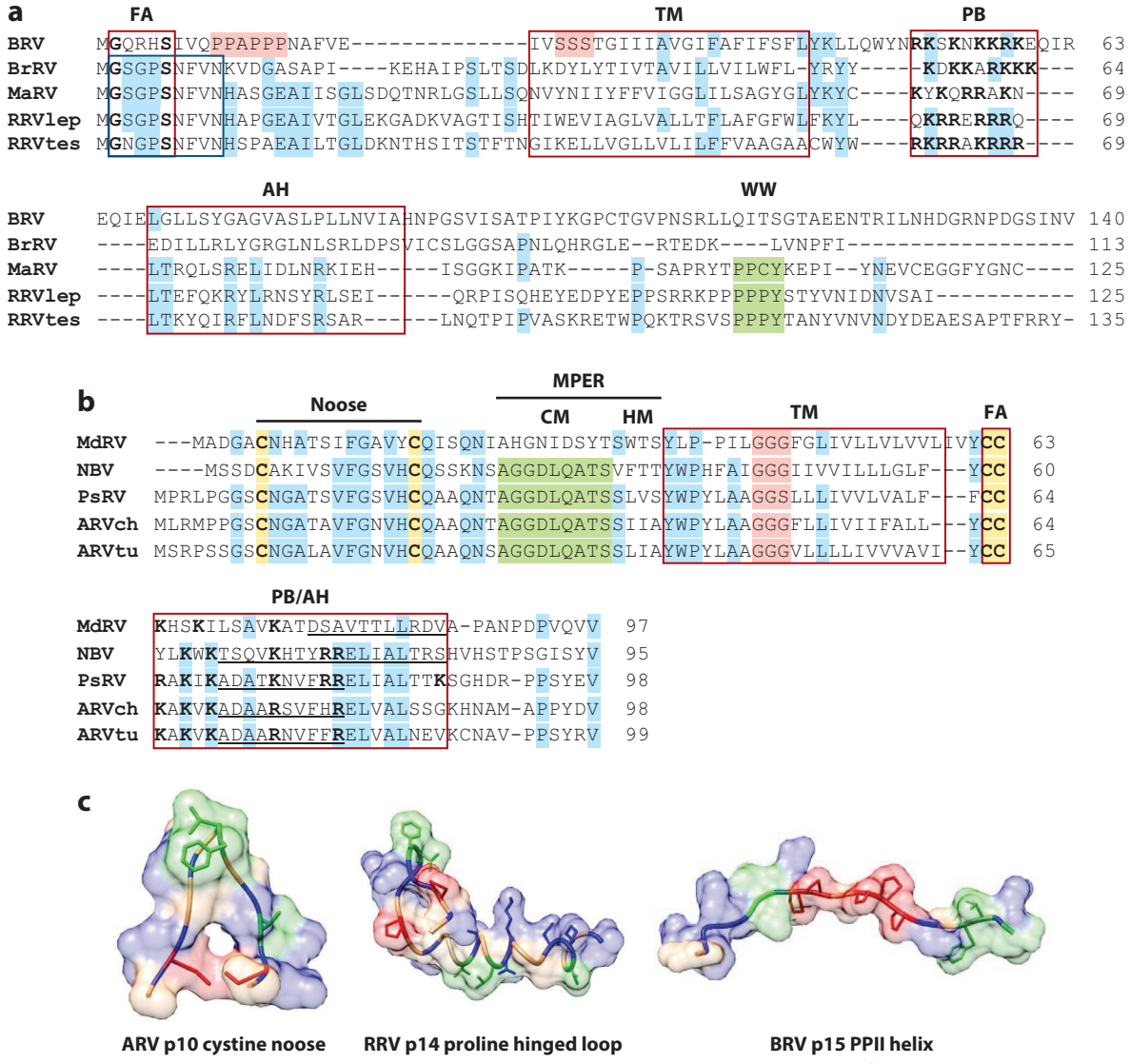
functional attributes with FPs (79–82). In the majority of cases, myristoylation of the ectodomain at a penultimate N-terminal glycine is required for fusion activity (28, 66, 69). There are three hallmark features of a FAST protein endodomain. First, the cytoplasmic tails are predicted to be intrinsically disordered, a common attribute of proteins with multiple interaction partners (83, 84). Second, all FAST protein endodomains contain a juxtamembrane polybasic (PB) motif (**Figure 2**), which in RRV p14 functions as a novel *trans*-Golgi export signal (78). A minimum of three basic residues in the p14 PB motif function in a sequence-independent, membrane-proximal manner to direct p14 interactions with Rab11 and sorting of p14 into AP1-coated vesicles for anterograde transport to the plasma membrane (78, 85, 86). Third, FAST protein endodomains contain at least one region that can be modeled as an amphipathic α-helix (AH), frequently referred to as a hydrophobic patch, that functions to promote pore formation (87). The p10 FAST proteins of the ARV/NBV clade vary slightly in two of the above hallmark features (**Figure 2**). The p10 proteins have equivalent-sized endo- and ectodomains of ∼40 residues instead of an asymmetric membrane topology, and fusion activity requires palmitoylation of a juxtamembrane di-cysteine motif in the endodomain instead of N-terminal myristoylation of the ectodomain (88). The AqRV p16 FAST proteins also replace an N-terminal myristoylation consensus sequence with a juxtamembrane di-cysteine motif that may be palmitoylated (53, 89).

## 5.2. Evolution of Fusion-Associated Small Transmembrane Proteins and Viroporins

As with the viruses that encode them, the p16 and p22 AqRV FAST proteins represent two distinct clades (**Figure 1*a*,*c***). A similar arrangement and repertoire of structural motifs coupled with conserved, albeit limited, sequence identity in their ectodomains implies the AqRV proteins arose from a common ancestor (55). The situation is not so clear with ORV FAST proteins that also form two clades, neither of which share discernible sequence similarity to the AqRV FAST proteins. One clade comprises the p13, p14, and p15 FAST proteins of BrRV, RRV and MaRV, and BRV, respectively. The p13 and p14 FAST proteins share vestigial sequence conservation (**Figure 3*a***) and contain a conserved N-terminal decapeptide containing the myristoylation site (26), suggesting they arose by divergent evolution from a common progenitor. BRV p15 lacks both sequence conservation with p13 and p14 and the conserved decapeptide (**Figure 3*a***), but retains a functional myristoylation motif (55). It also has a truncated ectodomain of <20 residues (versus ∼40 residues for all other FAST proteins) that contains a unique polyproline type II (PPII) helix (66, 82). The homologous p10 FAST proteins of the ARV/NBV clade share no sequence similarity to the p13/p14/p15 FAST protein clade (**Figure 3*b***), their ectodomains contain a unique disulfide bond-stabilized loop and membrane-proximal conserved motif (CM), and p10 replaces N-terminal myristoylation with palmitoylation of an endodomain di-cysteine motif (64, 90, 91). It is unclear whether p10, and possibly p15, evolved by additional independent gain-of-fusion events.

The last common ancestor of FAST proteins was presumably a small, nonfusogenic, membrane-associated protein. Almost all viruses encode such proteins. Examples include influenza virus M2, human immunodeficiency virus vpu, hepatitis C virus p7, rotavirus nsp4, and picornavirus 2B. Collectively, these proteins are referred to as viroporins since a common feature is their ability to form oligomeric pores and/or alter the capacity of membranes to control ion flow (4, 92). Viroporins share no sequence similarity and vary widely in their atomic structures and membrane topologies, suggesting they arose by convergent evolution. Viroporins use similar motifs to those present in FAST proteins (e.g., TM domains, AHs) to alter membrane curvature and remodel intracellular membranes. A well-studied example is the influenza virus M2 viroporin. This 97-residue protein has a single TM domain connecting a 24-residue N-terminal ectodomain to a

54-residue endodomain that has a predicted disordered C-terminal tail and membrane-proximal AH, all features of FAST proteins. The M2 AH clusters at the boundary of membrane microdomains, so-called lipid rafts (93), and localizes to the neck of virions budding from the plasma membrane (94). Membrane insertion of the AH induces membrane strain and curvature to promote fission of the bud neck and virus release (95). It is plausible that FAST proteins evolved to become the only fusogenic viroporins through the assembly of specific combinations of protein trafficking and membrane remodeling motifs required to generate the curvature stresses in plasma membranes needed to drive their fusion. A similar situation may apply to the small, bitopic cellular protein myomerger required for myoblast fusion into multinucleated myotubes, which shares several features with FAST proteins and viroporins (96, 97).




(*Caption appears on following page*)

**Figure 3** (*Figure appears on preceding page*)

FAST protein structural and functional motifs. Panels *a* and *b* depict sequence alignments of the two FAST protein clades. (*a*) One clade comprises the BRV p15, BrRV p13, MaRV, RRVlep, and RRVtes p14 FAST proteins. (*b*) The second clade comprises the homologous p10 FAST proteins encoded by some bat orthoreoviruses (NBV) and all avian orthoreoviruses (MdRV, PsRV, and chicken and turkey isolates of ARV, ARVch and ARVtu, respectively). Alignments were generated using Clustal omega and manually refined based on demonstrated or presumed structural and functional motifs characteristic of FAST proteins. Residues conserved in three or more clade members (*a*) or in four or five of the avian and bat p10 sequences (*b*) are shaded blue. Motifs that are hallmark features of FAST proteins are labeled and boxed in red. In panel *a*, the N-terminal decapeptide conserved in the p13 and p14 FAST proteins is boxed in blue; this sequence contains the conserved myristoylation consensus sequence (GXXXS/T, *boxed in red*) that is also present in BRV p15. The unique p15 ectodomain polyproline helix FP and TM domain tri-serine motifs are shaded purple. A WW domain ligand motif (PPXY) is conserved in the p14 FAST proteins and is shaded green—interaction partners for this motif have not been identified. Panel *b* depicts unique p10 motifs. The four conserved cysteine residues are shaded yellow; the two in the ectodomain form an intramolecular disulfide bond generating a cystine noose FP while the juxtamembrane di-cysteine motif in the endodomain is the site of essential palmitoylation. The MPER contains a nine-residue CM present in all p10 isolates except those from nonfusogenic MdRV isolates; this motif drives p10 multimerization in membrane microdomains, with the adjacent tetrapeptide dictating HM. All p10 isolates contain an essential tri-glycine motif in their TM domain (*purple*), and in some isolates their PB motif partially overlaps with the predicted AH motif (*underlined*); the precise boundaries of these conjoined motifs are approximate. Panel *c* shows volume projections of NMR conformers of the indicated FAST protein FPs in aqueous environments, visualized using UCSF Chimera. Residues are color-coded green (hydrophobic), blue (hydrophilic), and orange (amphiphilic). Side chains of the important cystine (p10) or proline (p14 and p15) residues (*red*) and the phenylalanine and valine residues at the apex of the p10 and p14 loop regions (and other hydrophobic residues) are shown embedded within the volume projections. Abbreviations: AH, amphipathic helix; ARV, avian orthoreovirus; BrRV, Broome orthoreovirus; BRV, baboon orthoreovirus; CM, conserved motif; FA, fatty acid; FAST, fusion-associated small transmembrane; FP, fusion peptide; HM, homomultimerization motif; MaRV, Mahlapitsi orthoreovirus; MdRV, Muscovy duck orthoreovirus; MPER, membrane-proximal external region; NBV, Nelson Bay orthoreovirus; NMR, nuclear magnetic resonance; PB, polybasic motif (basic residues in boldface); PsRV, psittacine orthoreovirus; RRVlep, reptilian orthoreovirus Lepidosauria; RRVtes, reptilian orthoreovirus Testudines; TM, transmembrane domain.



### 5.3. Fusion-Associated Small Transmembrane Proteins Are Promiscuous Bona Fide Fusogens

FAST proteins fulfill the requirements of bona fide fusogens (98), as shown by the ability of purified RRV p14 reconstituted into liposome membranes to induce liposome-cell fusion (76). RRV p14 can also induce liposome-liposome fusion when divalent cations are used to promote aggregation of anionic liposomes. Atomic force microscopy reveals that the p14 ectodomain extends <1.5 nm from liposome membranes, explaining the need for liposome aggregation to promote close membrane apposition (99). These promiscuous fusogens can fuse almost all cell types at neutral pH, an exception being the AqRV-G p16 protein that requires slightly acidic pH to induce syncytium formation (89). While enveloped virus fusion complexes mediate both prefusion membrane attachment and membrane fusion (100), FAST proteins lack receptor-binding activity (101), consistent with their promiscuous fusion activity. In the absence of a specific trigger such as receptor binding and/or low pH, syncytiogenesis is regulated in part by the rate of FAST protein accumulation in the plasma membrane, which is influenced by translation from suboptimal start codons and by the ER-associated degradation (ERAD) pathway (3, 53, 54, 58, 102).

### 5.4. Fusion-Associated Small Transmembrane Proteins Form Multimeric Membrane Fusion Platforms

FAST proteins form multimeric complexes and associate with membrane microdomains, but the relationship between these two processes varies somewhat between different FAST proteins. RRV p14 partitions into liposome membrane microdomains with biophysical properties (i.e., increased thickness) resembling cholesterol- and sphingolipid-enriched lipid rafts (93), and in transfected cells p14 segregates into plasma membrane microdomains (99). Co-immunoprecipitation using

chimeric p14/p10 FAST proteins indicates the p14 ectodomain directs formation of homomultimers. These multimers are stable following detergent treatments that disrupt lipid rafts but are not formed in the presence of brefeldin A, which disrupts ER-Golgi trafficking, consistent with lipid raft assembly and p14 multimerization in the Golgi complex (70, 103).

Cholesterol depletion/repletion studies, fluorescence resonance energy transfer (FRET) analysis, and immunofluorescence microscopy indicate the ARV and NBV p10 proteins assemble homomultimeric fusion platforms (estimates suggest octameric assemblies) in separate plasma membrane microdomains (91). Unlike p14, cholesterol-dependent p10 homomultimerization and membrane microdomain association are codependent, meaning p10 multimeric complexes are stable only in lipid rafts. Formation of multimeric fusion platforms is dictated by the 13-residue MPER of p10 (**Figure 3*b***). The MPER contains the 9-residue CM present in all ARV and NBV p10 proteins. Absence of this motif, which presumably provides a binding interface suitable for p10 multimerization, explains why the MdRV p10.2 FAST protein is nonfunctional (32). The four diverged juxtamembrane MPER residues function as a homomultimerization motif (HM) and govern formation of homotypic ARV or NBV p10 fusion platforms; exchange of just these four residues allows heteromultimerization between ARV and NBV p10 (91). The FAST protein fusion platforms could merely serve to assemble multimeric fusion complexes, or their specific features may contribute to the fusion reaction. For example, the phase boundaries at the edge of lipid rafts are regions of membrane distortion due to the differing heights of the raft lipids and adjacent membrane lipids (104). As previously mentioned, the influenza virus M2 viroporin preferentially promotes membrane budding and fission at these phase boundaries (105), and enveloped virus FPs may preferentially target these same regions to promote membrane fusion (106). Further studies are needed to establish whether similar raft interactions are a feature of the FAST proteins.

The prevailing view of protein-membrane microdomains is that membrane proteins and lipids coalesce in a sterically favored lipid environment best adapted to match the length and contours of their TM domains (93, 107). Protein multimerization and protein-lipid or lipid-lipid interactions promote further protein segregation due to hydrophobic mismatch between membrane thickness and the length of the TM domain. Consequently, segregation of the ARV and NBV p10 proteins to distinct microdomains may reflect differences in the distribution of polar and apolar residues in their juxtamembrane HM resulting in recruitment of lipids with different acyl chain lengths. FAST protein TM domains may also play a role in raft localization. The RRV p14 TM domain can be functionally replaced with other FAST protein TM domains but not by heterologous TM domains from other viral fusogens, implying there are family-specific features of FAST protein TM domains (67). The MPER-adjacent ends of FAST protein TM domains contain residues essential for fusion activity, including a conserved tri-glycine motif in p10 and hydrophobic beta-branched residues and a tri-serine motif in p15 (67, 68, 88). Whether these motifs function in conjunction with the MPER to assemble FAST protein fusion platforms or play a more direct role in the fusion process, as shown for several enveloped virus fusogens (15, 16, 108, 109), has not been established.

## 5.5. The Prefusion Stage of Fusion-Associated Small Transmembrane Protein Syncytiogenesis Requires Surrogate Cell-Cell Adhesins

FAST proteins are both necessary and sufficient to mediate liposome fusion, but cell-cell fusion requires surrogate adhesins and active actin remodeling to mediate the prefusion stages of cell-cell fusion. Unlike the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor proteins that form homotypic bilateral interactions to drive intracellular vesicle fusion (110), FAST proteins are unilateral fusogens and do not bind to specific receptors (81, 101). The short distance

AX1: annexin A1

that FAST protein ectodomains extend from the membrane is insufficient to span the intercellular gap between adjacent cells. They overcome this restriction by localizing to adherens junctions, a ubiquitous cell-cell junctional complex stabilized by bilateral, calcium-dependent interactions between cadherins (111). These extended regions of cell-cell contact bring membranes to within ~15–30 nm and trigger actin remodeling (112, 113). Blocking cadherin engagement or actin dynamics impedes FAST protein-mediated syncytium formation (101), suggesting that actin-driven membrane dynamics at adherens junctions brings membranes into close enough proximity to trigger cell-cell fusion by FAST proteins. The unidentified fusogens mediating macrophage fusion to form osteoclasts and giant cells also rely on E-cadherins (114, 115). Other surrogate adhesins that promote cell-cell attachment in the absence of cadherin engagement also support FAST protein syncytiogenesis (101), indicating FAST proteins function as opportunistic fusogens that convert existing cell-cell junctional complexes into fusion synapses.

## 5.6. Pore Expansion Leading to Syncytium Formation Is Calcium and Annexin Dependent

Completion of the actual membrane fusion reaction generates a stable fusion pore with a diameter of only a few nanometers. Syncytium formation requires postfusion expansion of these micropores into micrometer-sized macropores. With intercellular distances at adherens junctions of ~20 nm nascent fusion pores would have a highly stressed rim with a radius of curvature of ~10 nm. A yeast two-hybrid screen identified annexin A1 (AX1) as a genetic interaction partner of the intrinsically disordered RRV p14 endodomain. Calcium-dependent interaction of AX1 with membranes alters curvature and promotes membrane aggregation. This membrane sculpting affects numerous cellular processes including plasma membrane repair, exo- and endocytosis, and actin dynamics (116–118). FRET analysis revealed calcium-dependent interactions between AX1 and p14, and knockdown of AX1 or chelation of intracellular calcium inhibited syncytiogenesis but not intercellular transfer of small aqueous fluors (119). Similar results were obtained for pore formation and syncytiogenesis induced by the measles virus F and H proteins. Thus, AX1 involvement in pore expansion and syncytium formation may represent a generalized cellular response to nascent cell-cell fusion pores. Studies with enveloped virus fusogens and during cell-cell fusion of macrophages and myoblasts also reveal a role for cellular proteins that alter membrane curvature during postfusion pore expansion (120–122).

Exactly how such membrane-aggregating, curvature-inducing agents promote pore expansion has not been determined. The source of the increased intracellular calcium needed for pore expansion is also unclear. One possibility is calcium influx through the plasma membrane via FAST protein porin activity or by leaky membrane fusion. Another is calcium release from intracellular storage compartments, as shown for the nsp4 and 2B viroporins of rotaviruses and picornaviruses, respectively, that use their membrane-proximal AHs to form calcium-conducting ion channels (123, 124). Aside from influencing pore expansion, viroporin disruption of calcium homeostasis induces apoptosis, inflammasome activation, autophagy, and actin disruption (125, 126). Similar effects resulting from FAST protein alterations to calcium homeostasis could be major contributors to FAST protein-induced cytopathic effects or to the ability of FAST proteins to enhance oncolytic virotherapy (72).

## 5.7. Fusion-Associated Small Transmembrane Protein Ectodomain Fusion Peptides

The rudimentary FAST protein ectodomains are all small, amphiphilic peptides. These domains in p10, p14, and p15 are sensitive to substitution and in liposome fusion assays induce robust lipid

mixing, two characteristics of enveloped virus FPs. They do so, however, using remarkably diverse structures. While structurally distinct, these unusual viral FPs all appear to be dynamic structures, and all three contain a pair of apolar aromatic and beta-branched amino acids (e.g., Phe and Val).

The ARV/NBV p10 FP comprises the N-terminal ~25 residues, connected by a 2-residue linker to the MPER multimerization motif. Contained within this FP are two conserved cysteine residues that form an intramolecular disulfide bond, creating an 11-residue amphiphilic loop with conserved Val/Ile-Phe residues at the apex of the loop (90) (**Figure 3*c***). The ectodomain targets p10 for rapid degradation by the ERAD pathway, which responds to exposed hydrophobic residues as a sign of protein misfolding (102, 127). Substitutions that decrease the hydrophobicity of this loop increase p10 stability but decrease cell-cell fusion. Biophysical analysis indicates that disulfide bond formation exposes hydrophobic residues to water and is required for peptide partitioning into liposome membranes and for peptide-induced liposome fusion (80, 81). These results imply that the p10 FP forms a geometrically constrained cystine noose that forces solvent exposure of hydrophobic residues for membrane insertion (128). The sensitivity of p10-mediated cell-cell fusion to membrane-impermeable inhibitors of thioredoxins suggests this noose structure may need to be disrupted at some stage of the fusion reaction (90).

The p14 and p15 FAST proteins use two very different types of FPs, both of which require N-terminal myristoylation and function in a sequence-specific manner to destabilize lipid bilayers. These are the only known viral FPs that require myristoylation for function. Nuclear magnetic resonance (NMR) analyses of a p14 ectodomain peptide, which displays robust activity in liposome fusion assays, indicate this amphiphilic peptide assumes different structures in aqueous and membrane mimetic environments. Under aqueous conditions, the N terminus of this peptide forms a 7-residue proline-hinged loop with conserved Phe-Val residues near the tip of the loop flanked by a disordered 25-residue MPER (**Figure 3*c***). When analyzed in a membrane mimetic environment, the proline loop region becomes disordered and the MPER assumes an AH-kink-helix structure (79, 129). Conversely, the BRV p15 ~20-residue ectodomain contains a PPAPPP motif that exists as a left-handed PPII helix in both aqueous and membrane mimetic environments (82) (**Figure 3*c***). Proline residues are tolerant of substitution, but multiple substitutions that disrupt the helix structure ablate p15-induced syncytiogenesis. The PPII helix is flanked by short, disordered amphiphilic N and C termini, with the C-terminal MPER region containing a Phe-Val sequence. Despite the dramatic differences in their structures, the p15 ectodomain can be functionally replaced by that of p14, but the converse substitution is nonfusogenic (55), evidence that specific combinations of different ecto-, endo-, and TM domain motifs are needed to generate a functional FAST protein.

## 5.8. Multifunctional Fusion-Associated Small Transmembrane Protein Endodomains

With asymmetric membrane topologies biased toward their C-terminal cytoplasmic domains, it is unsurprising that FAST protein endodomains play a prominent role in the fusion process. This contrasts markedly with the majority of enveloped virus fusogens whose topology is ectodomain biased and whose cytoplasmic tails are generally either dispensable for, or inhibit, their fusion activity (130–134). As already discussed, the RRV p14 endodomain interacts with AX1 to promote pore expansion, and the juxtamembrane PB motif mediates Golgi export. The PB motif could also play a more direct role in the fusion reaction through electrostatic interactions with anionic phospholipids in the cytoplasmic leaflet of membrane bilayers, as occurs during intracellular membrane fusion events (135). Three additional features of these endodomains are noteworthy. Progressive C-terminal truncation of the p10 and p14 FAST proteins results in a progressive

loss of pore formation (84), indicating that this intrinsically disordered region not only promotes pore expansion by recruiting AX1 but also plays an additional undefined role in pore formation. FAST protein endodomains also function in *trans* to promote pore expansion. A small percentage of the p14 FAST protein is proteolytically cleaved near the endodomain/TM domain boundary to generate a soluble endodomain fragment, in both p14-transfected and virus-infected cells (136). This endodomain fragment is nonfusogenic on its own, but when coexpressed with diverse functional fusogens (including other FAST proteins and influenza HA and during developmental fusion of muscle myoblast into myotubes) functions downstream of pore formation to enhance pore expansion. FAST protein endodomains clearly provide a useful tool to define generic cellular processes involved in pore expansion and syncytiogenesis.

**FLiPS:** fusion-inducing lipid packing sensor

FAST proteins endodomains also contain a membrane-proximal AH that functions as a lipid packing sensor to promote pore formation. Lipid packing sensors are AHs that partition into hydrophobic defects present in highly curved membranes, such as those present in small vesicles associated with intracellular trafficking (137, 138). The high degree of membrane curvature at the rim of a nascent fusion pore generates a stressed membrane structure with hydrophobic defects and presents a major energy barrier to completing the fusion reaction. Studies using liposomes with different radii of curvature indicate that the p15 AH preferentially partitions into the membranes of smaller liposomes that have high positive curvature (87). NMR and circular dichroism spectroscopy supported by mutational studies indicate that this motif is highly dynamic, with conformers ranging from a linear, AH-kink-helix structure to an amphipathic helical hairpin. Mutations that disrupt this structure in the BRV p15 FAST protein ablate cell-cell fusion, and this motif can be functionally replaced by the predicted linear AH in the p14 FAST protein endodomain and by several cellular lipid packing sensors (87). Anchored near the membrane by the FAST protein TM domain, the endodomain AHs are perfectly positioned to sense and respond to the increasing positive curvature generated in the cytoplasmic leaflet by nascent pore formation. By functioning as a fusion-inducing lipid packing sensor (FLiPS), these AHs lower the energy barrier to stable pore formation.

## 6. PATHWAY OF FUSION-ASSOCIATED SMALL TRANSMEMBRANE PROTEIN-MEDIATED MEMBRANE FUSION

A model of how FAST proteins mediate cell-cell membrane fusion and syncytium formation has emerged from the structural and functional studies described above (**Figure 4**). This model accounts for the limited constraints on evolution of the FAST proteins and the diversity of functional motifs they have assembled for a specific purpose—to make cytoplasmic connections between cells that promote cell-cell virus transmission. The model shares similarities with the current perception of how enveloped virus fusogens mediate this process, with some notable exceptions. The prefusion step in cell-cell fusion requires adhesion molecules to tether the donor and target membranes together and then reduce interbilayer distances to <2 nm, the critical repulsive range of hydrated phospholipid headgroups (10, 139). Receptor binding and conformational changes in enveloped virus fusion complexes mediate these events. In contrast, FAST proteins function specifically as cell-cell, not virus-cell, fusogens, allowing them to evolve as minimalist membrane fusion machines that assemble multimeric fusion platforms at adherens junctions where they can exploit cadherins and actin remodeling to mediate this prefusion stage. Assembly of these multimeric platforms is reversible (**Figure 4**), as are the fusion complexes involved in yeast peroxisome fusion (140). Similar to the influenza virus M2 viroporin (94), FAST proteins may partition to the boundary of lipid rafts, an area prone to membrane deformation. While ectodomain features govern FAST protein oligomerization, specific features of a FAST protein TM domain and/or

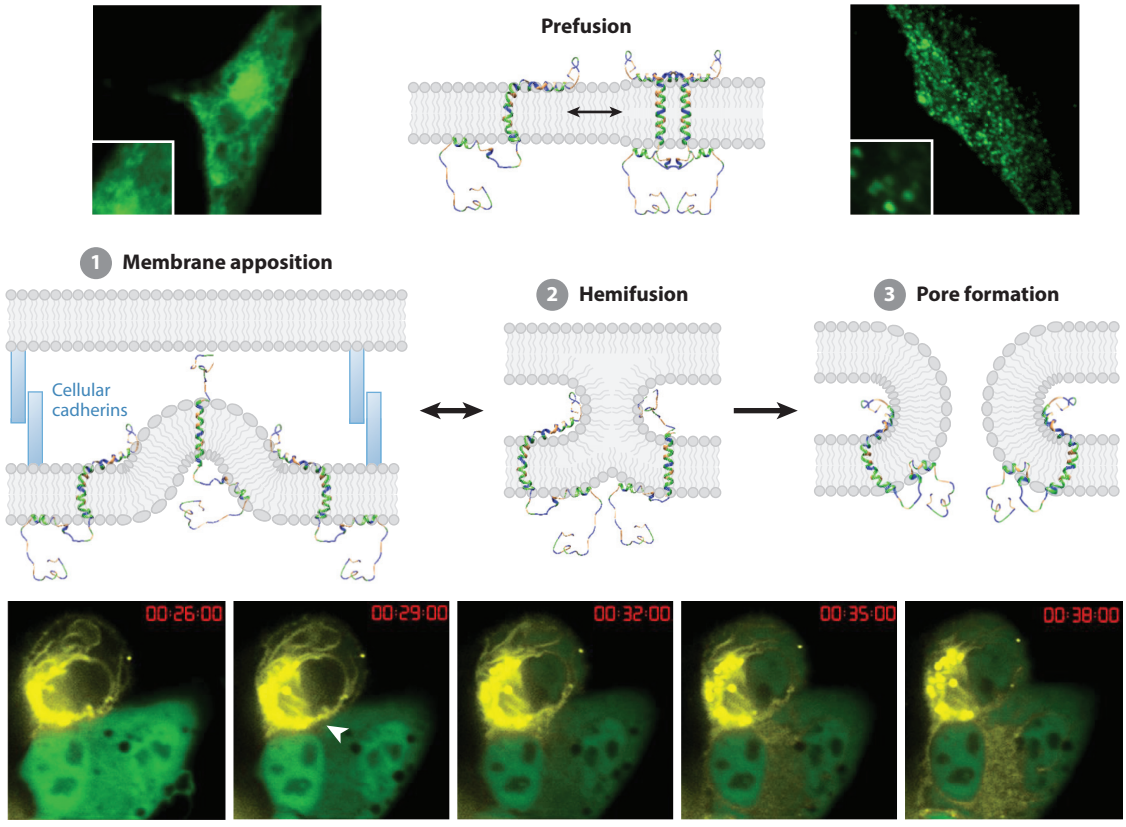



**Figure 4**

FAST protein-mediated cell-cell fusion. FAST proteins in the plasma membrane are depicted as composites of NMR conformers of the RRV p14 ectodomain and TM domain, and the BRV p15 cytoplasmic amphipathic helix, joined together by flexible linkers. The C-terminal cytoplasmic tails are predicted to be intrinsically disordered and have been modeled as such. In the prefusion stage (*top*), FAST proteins cluster together in cholesterol-dependent membrane microdomains (i.e., lipid rafts) that are thicker than the adjacent normal lipid bilayers and assemble multimeric fusion platforms. Immunofluorescent images at the top show surface staining of the p14 FAST protein (*green*) indicating punctate staining of multimeric fusion platforms (*top right*) that are disrupted by extraction of membrane cholesterol using methyl-b-cyclodextrin (*top left*). Insets are magnifications. The membrane fusion reaction (*middle* panels) initiates with close membrane apposition mediated by cellular cadherins (*shaded blue rectangles*). FAST proteins nonspecifically localize to these adherens junctions where the extended zone of contact between cells promotes close membrane apposition. Random or actin-mediated membrane protrusions are presumed to decrease the intermembrane distance and serve as the trigger for the fusion reaction. Motifs present in the ectodomain (FPs and MPER) and TM domain are envisioned to cooperate in altering lipid hydration and lipid packing, thereby inducing curvature stresses in the membrane, drawn as a protruding dimple. The ectodomains are structurally dynamic, as shown here by two different conformers of the p14 ectodomain, which may permit these ectodomains to reversibly interact with the donor membrane and with a closely apposed target membrane. Following the fusion-through-hemifusion paradigm, these membrane stresses would induce initial lipid mixing between the contacting leaflets of the two bilayers, generating a transient hemifusion intermediate shown as a stalk-like structure. This unstable intermediate must either revert to two planar bilayers or progress to pore formation. By partitioning into hydrophobic defects that emerge in the highly curved rim of nascent fusion pores, the FAST protein endodomain amphipathic helix functions as a FLiPS to lower the energy barrier to pore formation. Fluorescent images in the lower panels show the time course (in minutes) of heterotypic fusion between quail cells cotransfected with plasmids expressing EGFP and p14 FAST protein and Vero cells stained with the fluorescent lipid dye DiI (*yellow*). The white arrow in the second image indicates a site of fusion pore formation resulting in transfer of the soluble and lipid fluors between cells. Abbreviations: BRV, baboon orthoreovirus; EGFP, enhanced green fluorescent protein; FAST, fusion-associated small transmembrane; FLiPS, fusion-inducing lipid packing sensor; FP, fusion peptide; MPER, membrane-proximal external region; NMR, nuclear magnetic resonance; RRV, reptilian orthoreovirus; TM, transmembrane.

MPER are also likely to influence the spatiotemporal arrangement of these fusion platforms. In the absence of a closely apposed target membrane, FAST protein ectodomains are closely associated with the membrane in which they reside. These ectodomains are structurally malleable and contain FPs that expose hydrophobic residues for membrane insertion. At the prefusion stage, these hydrophobic residues are presumably sequestered from water by interaction with the donor membrane or within the multimeric complexes present in microdomain fusion platforms.

In the extended zone of cell-cell contact at adherens junctions, random or actin-driven membrane protrusions that decrease the intermembrane distance likely serve to trigger the fusion reaction. Most FAST protein ectodomains contain an N-terminal myristate whose short 14-carbon acyl chain has a very low dissociation constant for membranes (141), suggesting the ectodomain FPs may reversibly partition into the donor membrane and into a closely apposed target membrane facilitated by their inherent structural plasticity (**Figure 4**). Thus, FAST protein FPs and possibly the MPER and/or adjacent TM domain features (e.g., essential hydrophobic β-branched residues, tri-serine and tri-glycine motifs) provide a means to alter the hydration status, lamellar arrangement, and curvature of both donor and target membranes to generate a favorable environment for lipid mixing. FAST proteins are insensitive to agents such as lysophosphatidylcholine that promote positive curvature and inhibit stalk formation in other systems (142), suggesting some difference in the nature of a presumed hemifusion intermediate or in how this intermediate transitions to pore formation.

Regardless of how it occurs, lipid mixing would generate a transient, thermodynamically unfavorable bilayer arrangement that needs to be rapidly resolved, either by reversion to two planar bilayers or by progression to pore formation. The high degree of positive curvature in the inside rim of a nascent fusion pore presents a major barrier to pore formation. How enveloped virus fusogens overcome this barrier is unclear, but current models suggest interactions between the FP, TM domain, and MPER may all be involved. For FAST proteins, the endodomain AH is a key player in pore formation. All FAST proteins have a predicted or demonstrated AH in their endodomains, and, as shown for RRV p14 and BRV p15, this AH serves as a FLiPS and partitions into highly curved membranes (**Figure 4**). In so doing, the FLiPS motif masks hydrophobic defects as they appear in nascent fusion pores and stabilizes membrane curvature, making the forward reaction to pore formation a more favorable means of resolving the hemifusion state. This novel feature of FAST proteins may partly explain the difficulty in detecting a hemifusion intermediate, with lipid mixing being tightly coupled to stable pore formation.

## 7. CONCLUDING REMARKS

Enveloped virus fusion proteins need to mediate virus-cell fusion using the fixed quantity of fusion proteins present in the virion envelope, and they need to mediate both the prefusion and the actual membrane merger phases of the fusion process. These constraints led to a mechanism that relies on rapid and triggered unfolding and refolding of complex, multimeric ectodomain structures. In contrast, FAST proteins evolved specifically to mediate cell-cell membrane fusion. This distinct evolutionary imperative means FAST proteins can use protein expression and trafficking to accumulate multimeric fusion platforms in specific regions of the plasma membrane, and they can use surrogate adhesion proteins and membrane fluctuations to establish a favorable fusion environment. They are also not constrained by the space available inside a virus particle, allowing them to evolve extended cytoplasmic tails that play a prominent role in the fusion reaction. As virus-encoded cellular fusion proteins, the FAST proteins have therefore assembled a diverse repertoire of membrane-modifying motifs into minimalist fusion machines that attack the membrane from all sides to drive cell-cell membrane fusion.

## DISCLOSURE STATEMENT

The author is a cofounder of Entos Pharmaceuticals, which holds intellectual property related to FAST proteins.

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