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# Tetraspanins: Architects of Viral Entry and Exit Platforms

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**ABSTRACT** Host factors render cells susceptible to viral infection. One family of susceptibility factors, the tetraspanin proteins, facilitate enveloped virus entry by promoting virus-cell membrane fusion. They also facilitate viral egress from infected cells. In this Gem, we discuss recent insights into how tetraspanins assemble viral entry and exit platforms on cell membranes, and we speculate that tetraspanins contribute to nonviral membrane fusions by similar mechanisms.

**KEYWORDS** coronavirus, membrane fusion, tetraspanin, virus entry

For enveloped viruses, host factors determining susceptibility to infection include several “activators” which operate at particular subcellular sites to stimulate virus-cell membrane fusion. Depending on the infecting virus, activators may be cellular receptors, cations, or proteases which engage viral fusion proteins and trigger their refolding into forms that catalyze membrane coalescence. For many viruses, multiple activators are utilized in rapid succession. In these cases, activators must be in close proximity to each other, and to the incoming viral fusion proteins, to allow for rapid and efficient viral entry. Recent research identified the role of cellular proteins, including tetraspanins, in coalescing these activators. In this Gem, we discuss how tetraspanins facilitate efficient viral infection by organizing activators on host cell membranes.

## TETRASPANINS

The tetraspanins are membrane proteins; they have four (*tetra*) transmembrane spans linked extracellularly by one large and one small extracellular loop (termed LEL and SEL, respectively) (Fig. 1, inset). They are ubiquitously present in eukaryotes (1). Their conserved structures and interacting partners suggest that they perform central roles in controlling membrane architecture. For example, they induce positive (outward) membrane curvatures (2), potentially through their cone-shaped transmembrane domains (3). Tetraspanins further influence membrane architecture by interacting more directly with lipids and with other integral membrane proteins. The tetraspanin CD81 binds cholesterol, which incorporates into a pocket between the tetraspanin transmembrane domains and alters LEL conformations (3). Several other tetraspanins associate with palmitic acid, which covalently links to cytoplasmic domains (4, 5). In conjunction with cholesterol and palmitate, tetraspanins interact with several so-called “partner” proteins. Distinct LEL- and cytoplasmic tail-dependent interactions with integrins (6, 7), adhesion molecules (8), and other tetraspanins and their associated partners (9) give rise to web-like tetraspanin-enriched microdomains (TEMs) on cellular membranes (10, 11). It has been proposed that these TEMs might be platforms for virus entry (12, 13), particularly for viruses that directly use tetraspanins as receptors (14). Yet there have been several reports that both enveloped and nonenveloped viruses preferentially enter cells through TEMs, presumably without directly binding a tetraspanin molecule (15–18). These intriguing reports stimulated our interest in the mechanisms by which viruses use TEMs for cell entry.

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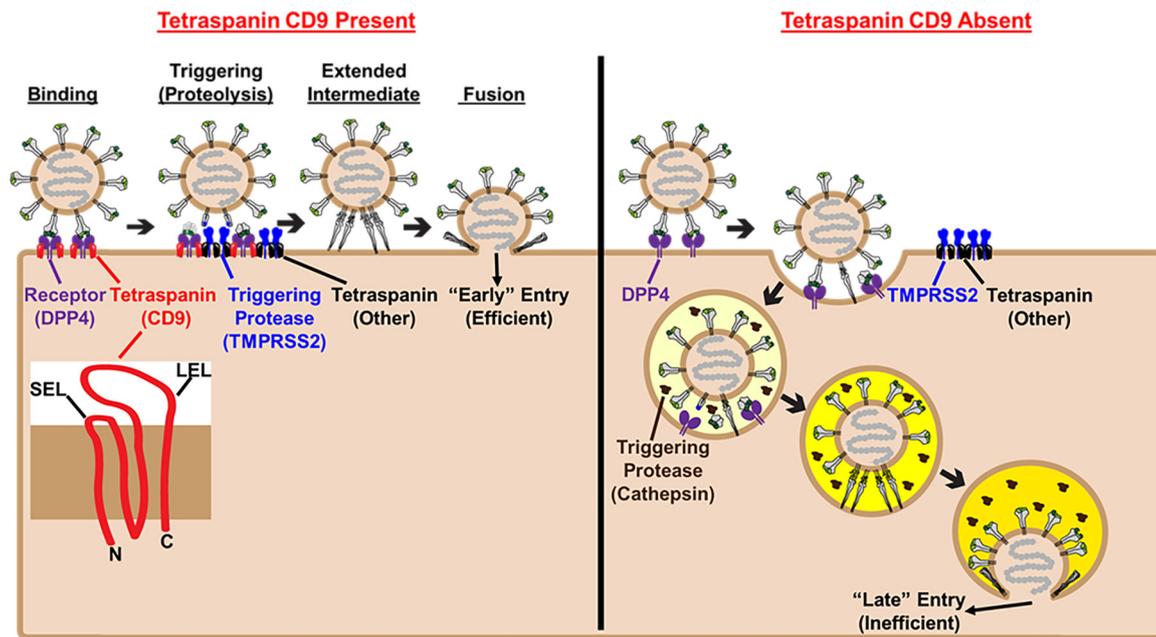
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**FIG 1** Tetraspanins construct virus entry platforms and dictate CoV entry routes. (Inset) Tetraspanins are composed of four transmembrane spans connected by one large extracellular loop (LEL) and one small extracellular loop (SEL), with short N- and C-terminal tails protruding into the cytosol. (Left) To drive viral entry into host cells, MERS-CoV S (MERS-S) proteins (gray) bind dipeptidyl peptidase 4 (DPP4) receptors (purple) via their receptor binding domains (green). Receptor engagement exposes substrates (blue stars) susceptible to cleavage by cellular proteases, including type II transmembrane protease serine subtype 2 (TMPRSS2) (blue). The tetraspanin CD9 (red) links DPP4 to TMPRSS2 on the plasma membrane. DPP4-TMPRSS2 linkages facilitate the rapid proteolytic triggering of MERS-S proteins after DPP4 binding. Proteolytically triggered MERS-S relocates its receptor binding domains and unfolds into an extended intermediate structure that embeds hydrophobic fusion peptides into target cell membranes. Refolding of intermediates then pulls virus and cell membranes together to catalyze membrane fusion and “early” entry at or near the cell surface. (Right) In the absence of CD9, DPP4 and TMPRSS2 are not linked, and MERS-S proteins are not efficiently triggered on the cell surface. MERS-CoVs are instead endocytosed and thus encounter endosomal cathepsins (brown). At low pH (yellow), cathepsins cleave MERS-S proteins, triggering inefficient, “late” entry in the endosomal network.

## CORONAVIRUS ENTRY

Our inroads into mechanisms by which tetraspanins facilitate virus entry came from investigations of coronaviruses (CoVs). These are enveloped viruses that cause respiratory and enteric infections in humans and animals (19); life-threatening severe acute respiratory syndrome CoV (SARS-CoV) and Middle East respiratory syndrome CoV (MERS-CoV) are notable members. CoV infections are driven by spike (S) fusion glycoproteins. Extending from virions, these S proteins bind target cell receptors and then encounter cellular activators. The activators are proteases which cleave S proteins in ways that liberate domains catalyzing virus-cell fusion (20). Frequently, and in MERS-CoV infections, this proteolytic activation process takes place in stages. Furin and related proprotein convertases cleave S proteins during virus egress from producing cells (a “priming” step) (21) and then, after secreted viruses attach to target cells, serine or cysteine proteases cleave S proteins again (a “triggering” step) (22–25). Here, at the second triggering step, is where the tetraspanins come into play (Fig. 1, left panel).

## TETRASPININS PROMOTE CoV ENTRY BY LINKING CoV RECEPTORS AND PROTEASES

Since receptor-bound CoV S proteins are susceptible to triggering cleavages (26, 27), we surmised that receptors and S-cleaving proteases must be in close proximity to trigger membrane fusion. Tetraspanins appeared to be reasonable candidates for bringing receptors and proteases together. Indeed, there were good suggestions that the CoV receptors and proteases are coalesced within TEMs (28–31). Therefore, we hypothesized that tetraspanins condense CoV entry factors into localized positions whereby effective spatiotemporal activation of viral fusion takes place.

We first explored this hypothesis by using biochemical approaches. We isolated

TEMs from cells containing CoV receptors and activating proteases and determined whether they localized to TEMs. Indeed, two CoV-activating proteases and the receptors of MERS-, SARS-, 229E-, and murine hepatitis virus (MHV)-CoVs were all found in TEMs (32). TEMs, while containing only ~20% of all plasma membrane proteins, contained 50% to 90% of the CoV receptors and proteases, indicating that CoV activators are targeted to TEMs. Notably, isolated TEMs activated CoV S proteins *in vitro*, allowing these viruses to enter cells in a manner independent of proteases on target cells. TEM activating potential also correlated with protease abundance (32). These findings indicated that TEMs act as platforms for fusion-activating CoV S protein proteolysis.

We next used genetic manipulation to consider whether individual tetraspanins are required for fusion activation and virus entry. Given the specificity of tetraspanin-partner protein interactions (33, 34), it was likely that individual tetraspanins are responsible for ferrying particular CoV receptors into TEMs. In support of this hypothesis, the MERS-CoV receptor dipeptidyl peptidase 4 (DPP4) and the 229E-CoV receptor APN localized to TEMs only in the presence of a particular tetraspanin, CD9 (35). Furthermore, cells lacking CD9 were resistant to MERS and 229E pseudovirus entry, making it clear that these viruses needed CD9, and probably TEM-localized receptors, for their entry. In contrast, deletion of a related CD81 tetraspanin did not confer resistance to virus entry. Other CoVs, however, apparently use distinct tetraspanins, because omission of CD9 did not relocalize SARS-CoV and MHV receptors, nor did it affect susceptibility to SARS and MHV pseudoviruses. With these findings, tetraspanins were further elucidated as partners for ferrying of specific CoV receptors into TEMs.

The idea that tetraspanins bring CoV receptors to TEM-associated proteases was further supported by the discovery that a well-known CoV-activating protease, type II transmembrane protease serine subtype 2 (TMPRSS2), was adjacent to the MERS-CoV receptor DPP4 as measured by proximity ligation assays (35). Yet in cells lacking CD9, this colocalization was not observed, and even though CD9 knockout cells retained cell surface DPP4 and TMPRSS2, they were resistant to virus entry. Only when excess TMPRSS2 was supplied through transfection was virus susceptibility restored. Therefore, these findings demonstrated that CD9 was required to coalesce DPP4 and TMPRSS2, with this juxtaposition of entry factors being necessary for robust MERS-CoV membrane fusion and infection (Fig. 1, left panel). It is conceivable that all CoVs utilize closely juxtaposed entry factors. This may explain, in part, why most known CoV receptors are transmembrane peptidases, even though their peptidase activities are not used to activate CoV fusion proteins (36, 37). The subcellular localization of transmembrane peptidases appears to be tightly controlled (38), and the CoV receptors we surveyed all localized to TEMs (32). It is possible that CoVs have selected peptidases so that virus entry is localized to protease-rich TEMs.

### TETRASPANINS DICTATE CoV ENTRY ROUTES

The proteases that cleave CoV S proteins and activate virus cell entry are present at several subcellular locations. On CoV-susceptible cells, there may be serine-class TMPRs at or near plasma membranes (39, 40), and there may also be cysteine-class cathepsin proteases within endosomes (22, 39). Therefore, depending on the abundance of these proteases, a CoV can be activated for entry at different locations along the endocytic pathway, either early (TMPR mediated) or late (cathepsin mediated) after virus endocytosis (Fig. 1, right panel). Notably, in the presence of CD9, we found that MERS pseudovirus entry was early and was suppressed by serine protease inhibitors, while in the absence of CD9, entry was late and was unaffected by serine protease inhibitors but rather was blocked by cathepsin inhibitors and endosome-neutralizing agents (35). These findings fit well with the identification of CD9 as an agent of DPP4 and TMPR colocalization: by linking the receptors and TMPR proteases at or near the plasma membrane, the CD9 tetraspanin facilitated early CoV entry into target cells. Note that the early entry route is far more likely to result in productive infection, as demonstrated by Shirato and colleagues in separate studies of HCoV-229E, -OC43, and -HKU1 (41, 42).

Late entry, at least for some CoVs, appears to be a lower-efficiency, last-chance infection route before virus destruction in lysosomes.

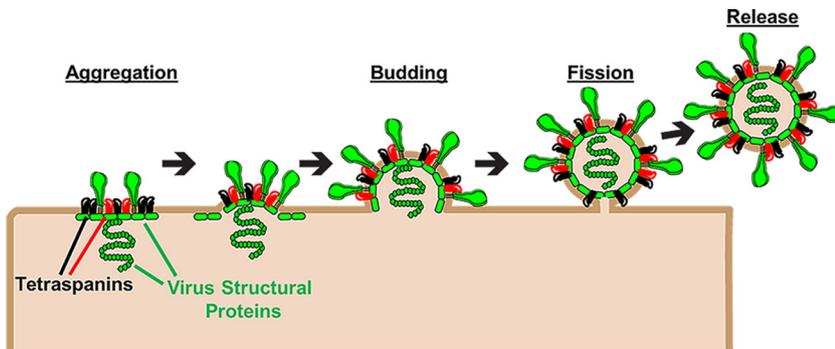
### TETRASPANINS CONTRIBUTE TO CoV INFECTION AND PATHOGENESIS *IN VIVO*

The *in vitro* finding that CD9 facilitated the highly infectious early entry route suggested that CD9 and other tetraspanins might also be *in vivo* CoV susceptibility factors. We expected that this would be the case, as strong evidence from protease inhibitor studies indicated that early-acting TMPRs might be central for respiratory CoV infections (43). Indeed, we did find key roles for CD9 and TMPRSS2 in MERS-CoV infections. Using adenoviral vectors expressing short hairpin RNAs (shRNAs), we transiently depleted either CD9 or TMPRSS2 in mouse lungs and then determined their susceptibility to MERS-CoV infection. Depleting CD9 and TMPRSS2 reduced MERS-CoV lung titers by ~90% and ~80%, respectively (35). Clearly, the vast majority of MERS-CoVs enter lung epithelial cells through the early entry route, and late entry pathways cannot compensate for early entry blockade. Given that humans express 33 tetraspanins and 19 serine TMPRs, it is remarkable that MERS-CoV is so highly dependent on a single tetraspanin and a single protease to infect the lung. Further investigation is needed to determine whether other CoVs utilize particular tetraspanins and proteases to enter cells *in vivo*.

The importance of early entry for MERS-CoV pathogenesis was further highlighted by the mutations that occur upon adaptation of this virus to the mouse lung environment. MERS-CoVs can infect mice when the human DPP4 (hDPP4) receptor is present, but these infections usually do not cause overt respiratory disease. Serial passage of MERS-CoVs in hDPP4 mouse lungs, however, generated mouse-adapted (MA) MERS-CoVs that caused severe lung disease characteristic of acute respiratory distress in humans (44). Notably, MA viruses accumulated S protein mutations that facilitated rapid, CD9-dependent early entry (35). In contrast, the avirulent cell culture-adapted (CCA) MERS-CoVs took 3 times longer to enter cells, and their infection was not supported by CD9. It is noteworthy that Vero cells, in which CCA MERS-CoVs were adapted, have low TMPRSS2 expression levels (39), as do several other cell lines routinely used to investigate CoV entry mechanisms *in vitro* (27, 39). TMPR<sup>+</sup> cell culture models may better reflect the *in vivo* environment of CoV entry, since CoVs utilize TMPR-mediated “early” entry routes in the lung (35, 43).

### TETRASPANINS AS GENERAL PROMOTERS OF VIRUS ENTRY

Accumulating evidence indicates that tetraspanins promote the entry of multiple viruses, including influenza A virus (IAV) (15, 32), human cytomegalovirus (HCMV) (45), human papillomavirus (HPV) (17, 18), hepatitis C virus (HCV) (14, 46, 47), Lujo virus (48), and several alphaviruses (49, 50). In some of these cases, the tetraspanins may be proviral because they coalesce the multiple host factors required to catalyze membrane fusion and infection. For example, IAV hemagglutinin (HA) proteins require proteolytic cleavage to undergo fusion-catalyzing structural transitions, and these proteolytic cleavages occur in TEMs (32). Depleting the tetraspanin CD81 from target cells restricts ~90% of IAV entry (15), suggesting that this tetraspanin may aid in localizing IAV-activating proteases with sialate receptors. Tetraspanins can also condense virus-associated membrane proteins into complexes prior to endocytosis. The tetraspanin CD151 provides an example of this, as it drives HPV endocytosis by coalescing receptor-bound viruses and integrin coreceptors targeted for endocytosis. HCV and the tetraspanin CD81 set another illustration. CD81 acts as the primary HCV receptor, binding directly to the HCV E2 glycoprotein (14), yet CD81 also coalesces the HCV coreceptors claudin-1, syndecan-1, and scavenger receptor B1 into a complex required for subsequent HCV endocytosis (46, 47, 51, 52). We speculate that many viruses have evolved to utilize entry factors that reside in close juxtaposition and that tetraspanins act to aggregate these entry factors into TEMs.



**FIG 2** Tetraspanins construct virus exit platforms. Tetraspanins (black and red) associate with viral structural proteins (green), leading to their aggregation into TEMs. Tetraspanins also induce positive membrane curvatures that may promote nascent virus budding. Finally, tetraspanins facilitate membrane fission, allowing for virus release.

### TETRASPANINS ARE TARGETED BY THE INNATE IMMUNE RESPONSE

Cells have evolved to target tetraspanins to restrict viral infection. Among the antiviral type I interferon-stimulated gene products are the interferon-induced transmembrane proteins (IFITMs), which are small alpha-helical proteins composed of ~130 residues that inhibit several virus entry processes at the level of virus-cell membrane fusion (53, 54). Intriguingly, IFITMs intercalate into TEMs, disrupting tetraspanin interactions with their partner proteins (55). One can speculate that as these intruding IFITMs accumulate during innate immune responses, they might separate or disorganize virus entry factors on target cell membranes. Notably, it is known that IFITM1 alters HCV coreceptor interactions, in correlation with restricted HCV-cell membrane fusion (56). It is possible that TEM disruption is a common mechanism by which the IFITMs restrict viral entry and infection.

### TETRASPANINS IN VIRAL EGRESS

In addition to promoting virus entry into target cells, tetraspanins also facilitate viral assembly and egress (Fig. 2). Many enveloped viruses, including IAV (15), human immunodeficiency virus (HIV) (16, 57), and herpes simplex virus 1 (HSV-1) (58), bud out of TEMs. Viruses may utilize these TEMs to facilitate vesicle budding and release. One possible mechanism by which this takes place is through tetraspanin-mediated membrane remodeling. The four tetraspanin transmembrane domains form two pairs of antiparallel helices, which adopt a cone-shaped structure opening toward the outer leaflet of the lipid bilayer (3). This architecture may facilitate membrane curvatures that may promote enveloped virus vesicle formation and membrane fission (2). Investigations into IAV egress support this speculative role for tetraspanins in virus budding. IAV HA localizes to TEMs containing CD81, and this tetraspanin is required for successful virion release (15). In the absence of CD81, virions bud from the plasma membrane but do not undergo fission to be released from the cell (15). They instead become elongated, with no fission at a membrane neck. These findings suggest that tetraspanins facilitate the membrane curvatures required for membrane fission and virus release.

Another way in which tetraspanins facilitate viral egress is by aggregating viral structural proteins. For example, the HSV-1 capsid protein VP26 interacts with the tetraspanin TSPAN7, leading to its accumulation on the nuclear membrane (58). This interaction is required for viral budding and egress from the nucleus. In the absence of TSPAN7 or upon mutation of VP26, HSV-1 capsids are sequestered in the nucleus, unable to exit (58). In addition, filamentous influenza virus strains accumulate CD81 at the ends of budding virions (15), along with the viral M2 protein, which mediates scission of IAV membranes from host cells (59). It is conceivable that CD81 facilitates M2 accumulation at the site of virus-cell membrane scission (15). These investigations highlight the ability of tetraspanins to facilitate multiple stages of the viral life cycle.

During viral biogenesis, tetraspanins incorporate into nascent virions (15, 57, 60, 61), suggesting that they may function in the context of the extracellular virion, perhaps at the virion cell entry stage. Intriguingly, virus-incorporated tetraspanins suppress rather than promote membrane fusion and virus entry (62, 63). In the context of HIV, overexpressing the tetraspanins CD9, CD63, and CD81 in virus producer cells inhibits HIV envelope-mediated membrane fusion (62–65), while depleting tetraspanins by use of small interfering RNAs (siRNAs) promotes membrane fusion (63, 65). We speculate that the high abundance of tetraspanins in HIV membranes may lead to these inhibitory effects. HIV Gag actively recruits tetraspanins to viral assembly sites, leading to the aggregation of large TEMs in HIV membranes (16). Notably, overexpressed tetraspanins inhibit HIV membrane fusion only in the presence of Gag (63). It is possible that Gag concentrates tetraspanins and cholesterol to rigidify HIV membranes, rendering them incompatible with membrane merger. Antibody-mediated tetraspanin cross-linking aggregates TEMs and suppresses CoV entry (32), supporting this notion. Further investigation is needed to determine whether the relative abundance of tetraspanins may dictate their pro- or antifusion activities.

### TETRASPANINS IN NONVIRAL MEMBRANE FUSIONS

Tetraspanins and assembled TEMs contribute to a wide variety of biological processes, including those involved in cell metabolism, signal transduction, and intercellular adhesion. One of their additional critical roles is in controlling nonviral cellular membrane fusions. Cell-cell fusions generate many biologically important structures, including fertilized zygotes (66), skeletal muscles (67), placentae (68), and multinucleated macrophages (69). Notably, tetraspanins are known regulators of many cell-cell fusion reactions (70–73). Their role is most striking during fertilization, which absolutely requires the tetraspanin CD9 (70). CD9 knockout mice are infertile, and eggs lacking CD9 are unable to fuse with sperm (70). CD9-laden extracellular vesicles, however, can compensate for loss of CD9 by bridging sperm and egg cells and subsequently fusing them together, in a process known as “fusion from without” (71). CD9 overexpression stimulates monocyte cell-cell fusions (72), which lead to the formation of multinucleated osteoclasts that resorb bone. Similarly, antibodies targeting CD9 suppress the fusion of myoblasts into the large syncytia required to form skeletal muscle (73). While the mechanisms behind tetraspanin-facilitated cell-cell fusions are not fully elucidated, we speculate that tetraspanins generally regulate membrane fusion events through their ability to bend membranes and cluster the factors required for membrane fusion catalysis. Surprisingly, many eukaryotic cell-cell fusion catalysts remain unidentified. Proteomic analysis of TEMs from fusogenic cells, along with molecular genetic approaches, may identify these elusive endogenous cellular fusogens.

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