# INTERMEMBRANE OLIGOMERIZATION OF SARS-COV-2 M-PROTEIN: POSSIBLE ROLE IN VIRAL BUDDING

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Despite the extensive research spurred by the catastrophic effects of COVID-19 pandemic, precise molecular mechanisms of some stages in SARS-CoV-2 life cycle remain elusive. One of such stages is the detachment of viral particles during budding. Using confocal fluorescence microscopy, we observed formation of specific structures by endoplasmic reticulum in human cells expressing SARS-CoV-2 M-protein, implicating oligomerization of M-protein across parallel membranes. In our opinion, such intermembrane oligomerization may provide a driving force for pinching off the viral particles during SARS-CoV-2 budding.

**Keywords:** SARS-CoV-2 membrane protein, protein-protein interactions, fluorescence microscopy, endoplasmic reticulum, OSER structures, viral budding

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# МЕЖМЕМБРАННАЯ ОЛИГОМЕРИЗАЦИЯ М-БЕЛКА КОРОНАВИРУСА SARS-COV-2: ВОЗМОЖНАЯ РОЛЬ В ПОЧКОВАНИИ ВИРУСА

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Несмотря на интенсивные исследования, стимулированные катастрофическими последствиями пандемии COVID-19, точные молекулярные механизмы некоторых стадий жизненного цикла коронавируса SARS-CoV-2, в частности, отрыва вирусных частиц при почковании, остаются неизвестными. При экспрессии М-белка SARS-CoV-2 в клетках человека мы наблюдали образование специфических структур эндоплазматического ретикулума, появление которых свидетельствует о способности М-белка к олигомеризации в составе параллельных мембран. На наш взгляд, такая межмембранная олигомеризация М-белка может быть движущей силой для сближения и слияния мембран при отшнуровывании вирусных частиц SARS-CoV-2.

**Ключевые слова:** мембранный белок SARS-CoV-2, белок-белковые взаимодействия, флуоресцентная микроскопия, эндоплазматический ретикулум, почкование вирусных частиц

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The ongoing SARS-CoV-2 pandemic constantly reminds us that our current means of dealing with life-threatening zoonotic viral infections are critically limited. The combined administration of drugs targeting diverse molecular entities appears a promising strategy of preventing 'quick' resistance. In this regard, the development of screening platforms and the search for inhibitors with novel mechanisms of action are highly relevant.

A mature SARS-CoV-2 virion carries single-stranded RNA genome (coding strand) and four structural proteins designated N, M, S and E [1, 2]. The soluble nucleocapsid protein (N) ensures binding and compactization of the viral RNA. The rest structural proteins are integral membrane polypeptides. The homotrimeric spike protein (S) ensures specific binding of the particle with ACE2 receptor at the cell surface and subsequent internalization of the complex by fusion of viral and host cell membranes. S-protein is large (1272 amino acid residues, a.a.r.) and has a prominent extracellular N-terminal portion followed by transmembrane helix and small cytoplasmic C-terminal domain. M-protein, the highest-copied, is thought to coordinate the viral particle assembly through interactions with N-protein (and accordingly with the viral genome) and other structural proteins. M-protein (222 a.a.r.) consists of short extracellular N-terminal portion, three transmembrane segments and cytoplasmic C-terminal domain; its structure has not been determined experimentally but relies on available models for homologous proteins. E-protein of SARS-CoV-2 is small (75 a.a.r.), low-copied (few molecules per virion) and forms homopentamers. Its N-terminus exposed to extracellular space is followed by a single transmembrane segment and small cytoplasmic C-terminal domain. E-protein has been implicated in the viral particle assembly and viroporin ion channel functionalities.

Despite the most diligent research efforts of the last two years, some steps in the SARS-CoV-2 life cycle are still poorly understood. For instance, exact molecular mechanisms of the viral budding remain elusive. The release of membrane-enveloped viral particles involves two principal steps: formation of spherical protrusions at the membrane and subsequent detachment of the enveloped particles from the host membrane [3]. Many viral proteins reveal a capacity of deforming the membrane to initiate budding, but only few of them can mediate membrane fission for the final detachment. Most enveloped viruses use the host cell 'endosomal sorting complex required for transport' (ESCRT) apparatus to promote the membrane scission [4]. However, in certain taxa including coronaviruses this stage involves viral proteins only [5, 6].

A recent computational modeling study has demonstrated that E-protein can both sense and induce the membrane curvature, as its cytoplasmic C-terminus 'prefers' convex

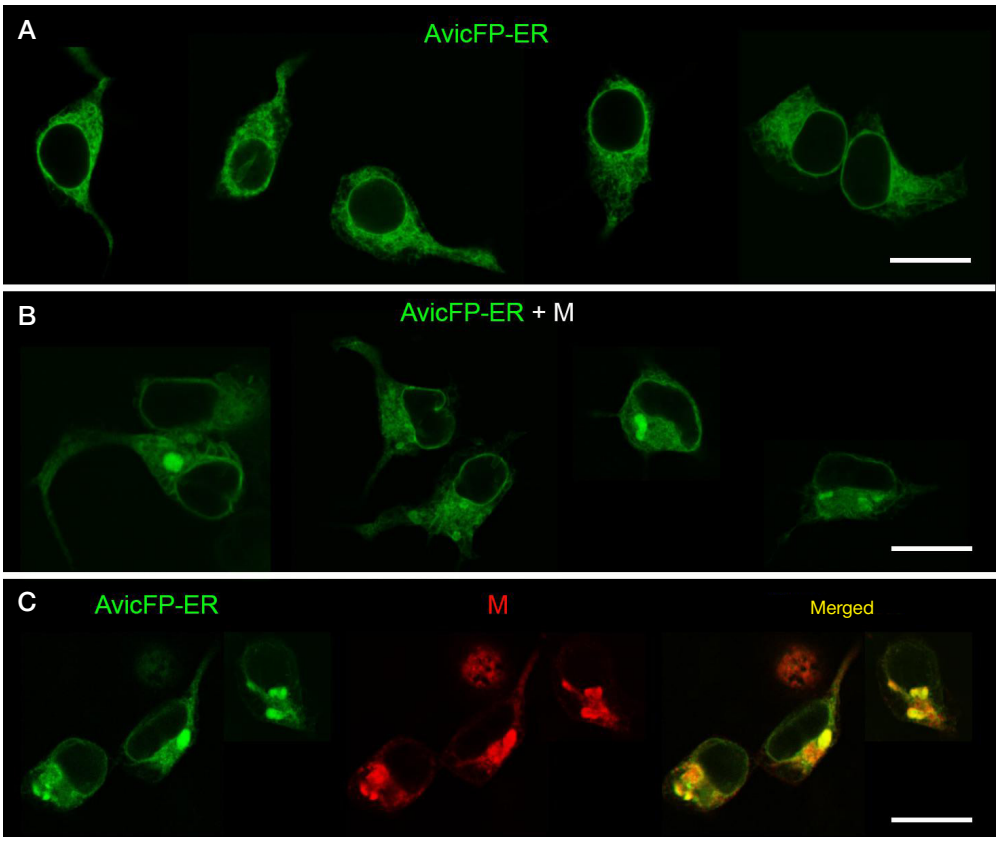


**Fig. 1.** Confocal fluorescence microscopy of HEK293T cells transfected with expression constructs. Scale bars, 20 μm. **A**. Transfection with plasmid encoding fluorescent protein AvicFP-ER localized to ER (*green* channel). **B**. Cotransfection with plasmids encoding AvicFP-ER (*green* channel) and M-protein. **C**. Cotransfection with plasmids encoding AvicFP-ER (*green* channel) and M-protein with subsequent immunostaining for M-protein (*red* channel; a merged channel image is shown on the *right*). The images are collages of four (**A**), four (**B**) and two (**C**) fields of view, showing representative results of each experiment.

regions of the membrane [7]. Apart from a circular convex region (neck) which connects the budding virion with the host cell membrane, the inside of the budding particles is concave. The curvature-induced segregation effect may explain the fewness of E-protein molecules in the virion; importantly, it also suggests active participation of E-protein in the neck formation and scission [8].

In this study, we used fluorescence microscopy of human cells expressing SARS-CoV-2 M-protein. Compared with the full-fledged viral infections of cell cultures, transfection models have certain advantages. Firstly, a particular target protein can be studied as a separate entity uninfluenced by other viral components, which affords a reduction in research complexity and a higher degree of accuracy. Secondly, the absence of infectious agent eliminates the safety concerns and allows making research in a conventional laboratory. Thirdly, such single-component experimental systems can be readily transformed into safe and straightforwardly scalable screening platforms for corresponding inhibitors. On the other hand, the reductionist models based on individual viral genes have serious limitations and all findings obtained with such models require verification in more sophisticated settings.

HEK293T cells were transfected with expression vector encoding SARS-CoV-2 M-protein (pGBW-m4134547, AddGene plasmid #152583) and green fluorescent protein mAvicFP1 with endoplasmic reticulum (ER) localization signal (mAvicFP1-ER) to enable ER visualization [9]. The confocal microscopy observations were carried out with Leica DMIRE2 TCS SP2 inverted microscope (Leica; Germany) in green (excitation 488 nm, detection 500–535 nm) and red (excitation 543 nm, detection 560–660 nm) channels. Preliminary control transfections with mAvicFP1-ER produced the expected patterns of cytoplasmic fluorescence shaped as a delicate network morphologically corresponding to ER (Fig. 1A). However, in cotransfections with the M-protein-encoding construct, the mAvicFP1-ER signal looked different. The network was preserved, but the majority of cells presented with bright rounded structures 1–5 μm in size (Fig. 1B). Immunostaining with anti-M-protein antibody (polyclonal rabbit IgG, ABIN6952906; antibodies-online Inc., USA) topped with goat secondary antibodies conjugated to Alexa Fluor 594 red-fluorescent dye (A-11012; Invitrogen, USA) revealed colocalization of M-protein and mAvicFP1-ER (Fig. 1C). Apparently, M-protein was associated with ER membranes and somehow promoted their agglomeration.

Similar structures were previously described in cells expressing certain proteins anchored in ER membranes and prone to oligomerization. The effect was explained by interactions of protein subunits across ER membranes resulting in agglomeration of the membranes into multishaped multilayered arrays — the so-called 'organised smooth endoplasmic reticulum' (OSER) structures [9, 10] (Fig. 2A).

SARS-CoV-2 M-protein is known to be capable of dimerization within a membrane [11]. Our findings indicate that M-protein is also prone to oligomerization across two membranes. The interaction is likely to involve C-terminal domains protruding into cytosol.

Effective scission of a membranous vesicle requires membrane convergence to a 1–5 nm distance [12]. As C-terminal domain in M-protein extends by 3 nm from the membrane surface [11], intermembrane interactions between C-terminal domains can bring the opposite membranes as close as 3–6 nm. Therefore, intermembrane oligomerization of M-protein may provide a driving force for the membrane convergence, which enables scission of SARS-CoV-2 particles during budding (Fig. 2B).

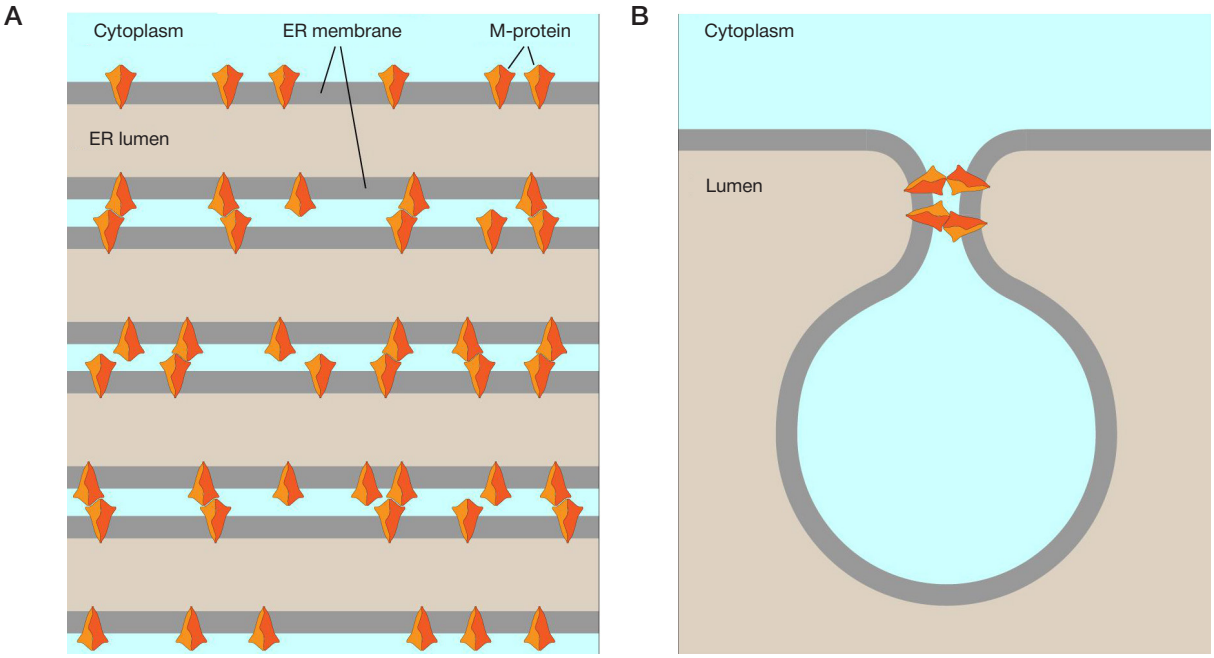


**Fig. 2.** A scheme of SARS-CoV-2 M-protein intermembrane oligomerization. **A**. Stacking of ER membranes into OSER structure through interactions of M-protein dimers in adjacent membranes. **B**. Hypothetical participation of the M-protein intermembrane oligomers in the neck formation during budding (M-protein molecules at other locations are omitted for clearance).

This hypothesis can be tested by mutagenesis of M-protein at the putative intermembrane interaction interfaces (the external amino acid positions of C-terminal domain unengaged in dimerization). The developed test for OSER structures may provide a simple means to assess the influence of particular amino acid substitutions on the intermembrane oligomerization capacity. In the case of confirmed functional significance, visualization of OSER structures in M-protein-expressing cells can be used as a safe functional assay in high-throughput screenings of the candidate inhibitors.

## CONCLUSION

Here we describe the formation of OSER structures in human cells expressing SARS-CoV-2 M-protein. The confocal microscopy observations indicate effective oligomerization of M-protein subunits across adjacent ER membranes. We believe that such interactions may promote membrane convergence and ultimately facilitate the detachment of viral particles during budding. If true, the developed test for OSER structures can be used in high-throughput screenings of M-protein oligomerization inhibitors.

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