JCB Journal of Cell Biology

SPOTLIGHT

RTN3 and RTN4: Architects of SARS-CoV-2 replication organelles

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SARS-CoV-2 depends on host proteins for successful replication. In this issue, Williams et al. (2023. *J. Cell Biol.* https://doi.org/10.1083/jcb.202203060) report that the ER membrane-modulating proteins RTN3 and RTN4 are required for the formation of SARS-CoV-2 replication organelles via direct interaction with viral proteins NSP3 and NSP4.

Replication of the coronaviral genome during the virus life cycle results in the production and release of new infectious virus particles (1). SARS-CoV-2 binds to its cellular receptor angiotensin-converting enzyme 2 (ACE2), and assisted by cell surface proteases, the virus and cell membranes fuse to enable the viral genome to enter the host cell cytoplasm. Here, the viral genome is directly translated to produce the viral proteins that make up the replication and transcription complex (RTC), namely the non-structural proteins (NSPs) 1–16 (2). By a not yet well understood process, the NSPs 2–16 engage host cell ER membranes to form the coronavirus replication organelles, e.g., perinuclear double membrane vesicles (DMVs), where viral RNA replication and transcription takes place. It is known that NSPs 3, 4, and 6 are involved in the formation of DMVs (3), but exactly how the virus can hijack the host ER membranes, and which host cell proteins may play a role, remains elusive.

Therefore, two most intriguing questions in the coronavirus field are how these dynamic replication organelles (ROs) are formed during viral infection and whether these structures are indeed the specific location of RNA replication. Here, Williams et al. (4) contribute some missing pieces to these open questions and pave the way for a clearer understanding of DMV formation by analyzing reticulons (RTNs), host proteins that are involved in ER membrane reshaping.

RTN3 and RTN4 play a role in ER membrane reshaping and have been previously shown to be in close proximity to the coronaviral RTC (5–7). Are these proteins part of the missing pieces that facilitate virus-induced remodelling of ER membranes to create the coronavirus replication organelles? Starting with this hypothesis, Williams et al. (4) first showed that siRNA-mediated knockdown (KD) of RTN3 and RTN4 reduced replication of an early SARS-CoV-2 isolate (USA-WA1/2020), as well as replication of Alpha, Beta, and Delta variants of concern (4). They conclude that SARS-CoV-2 depends on RTN3 and RTN4 for infection and further questioned whether these proteins are involved in viral genome replication. Double-stranded RNAs are intermediate products that are formed during coronaviral genome replication and transcription and are most likely located within DMVs. This dsRNA formation was decreased in RTN KD cells at late stages of SARS-CoV-2 replication and correlated with overall reduced viral genomic RNA levels. Interestingly, RTNs share a conserved structural domain: reticulon homology domain (RHD), which is involved in ER membrane curvature (8). By analyzing different truncated versions of RTN3 and RTN4, Williams et al. (4) show that the RHD domain is sufficient for supporting SARS-CoV-2 genome replication. As RTN3 and RTN4 are ER membrane-shaping proteins, they might play a role in shaping and forming coronaviral DMVs. Stunningly, transmission electron microscopy of infected RTN3 KD and RTN4 KD cells showed an almost complete lack of DMVs at any analyzed time point up to 48 h after infection, while the general ER morphology remained intact. This is particularly surprising because at earlier time points, until 12 h after infection, no differences were found in the amount of NSPs in infected RTN KD compared with infected untreated cells. Although not directly shown, this observation strongly suggests that a considerable level of coronaviral genome replication and translation can occur in the absence of DMVs. Finally, RTN3 and RTN4 were shown to directly bind to NSP3 and NSP4, the two major viral proteins involved in DMV formation. These results clearly demonstrate the importance of RTN3 and RTN4 for SARS-CoV-2 infection and relate their specific function to viral genome replication and DMV formation (Fig.1).

Williams et al. (4) identified the ER membrane-modulating proteins RTN3 and RTN4 to facilitate SARS-CoV-2 genome replication via triggering DMV formation, potentially through membrane rearrangements and direct interaction with NSP3 and NSP4. The RHD domain was shown to be the main structural part involved in SARS-CoV-2 replication. Strikingly, FAM134B, another ER

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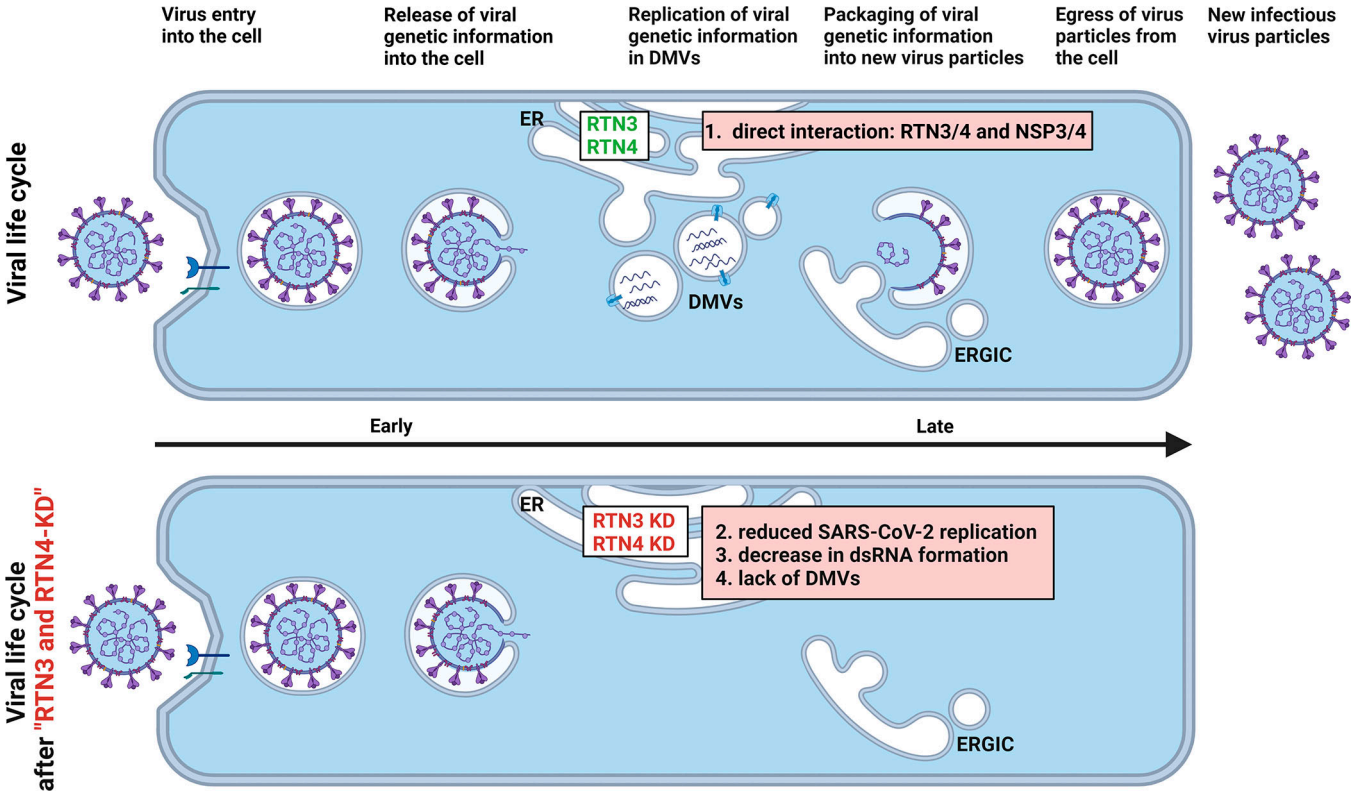

Figure 1. **RTN3 and RTN4 are key players of the SARS-CoV-2 life cycle.** Illustration of the SARS-CoV-2 life cycle in the presence (upper panel) and absence (knockdown; lower panel) of RTN3 and RTN4. While the early viral life cycle is not impacted by RTN3 and RTN4, both proteins play a role in the later phase, during the formation of replication organelles (DMVs), via direct interaction with NSPs 3 and 4. In the absence of RTN3 and RTN4, DMV formation is impaired and results in reduced SARS-CoV-2 replication. This figure was created with Biorender.com.

protein harboring a RHD domain, was not involved in virus infection, indicating a very specific function of RTN3 and RTN4. It is merely astonishing that a KD of either RTN3 or RTN4 results in such a significant change of infectivity, even though each KD phenotype could be rescued by overexpression of the other. The most intriguing fact is that early replication (up to 12 hpi) does not seem to be affected by depletion of RTN3 or RTN4. While DMV formation is absent in these conditions, comparable levels of NSPs were found in KD and WT cell lines, indicating similar levels of translation. Williams et al. (4) hypothesize that potentially other membranous structures or ROs, such as convoluted membranes, small spherule invaginations, zippered ER compartments, or double membrane spherules, serve as additional coronavirus replication compartments. These structures are a complex interconnected vesiculotubular network that can be visualized by cryoelectron microscopy upon coronavirus infection (3). DMVs were shown to harbour "fibrillar material," potentially RNA, but the same has not (yet) been found in the other RO structures. However, it was shown previously that the number and size of DMVs does not correlate with the amount of RNA during infection (9), suggesting other sites of RNA replication might exist. To confirm the authors' hypothesis, it would be interesting to analyze whether other types of ROs are present at early time points after infection in the RTN3 and RTN4 deficient cells, and whether, e.g., these potential replication sites correlate with detection of dsRNA. These processes are dynamic and short lived and have been difficult to visualize using different EM techniques. A visual absence of these structures would thus not necessarily mean that they don't exist.

Reticulons have been previously shown to impact replication of flaviviruses, a family of viruses that also has membrane-bound replication with very similar replication organelles. Further reticulons were observed in proximity to perinuclear DMVs during SARS-CoV-2 infection, as well as to the murine hepatitis virus RTC. With their study, Williams et al. (4) put the spotlight on the reticulons RTN3 and RTN4 for an active role during SARS-CoV-2 infection and raise new questions on the site of viral RNA replication during the early phase of coronavirus infection.

Acknowledgments

This work was supported by the Swiss National Science Foundation (grants #310030B_201278 and #51NF40-205601).

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