Since the initial publication of our work, additional data have accumulated suggesting that deleting the cytoplasmic tail of SARS-CoV-2 Spike improves the titers of Spike-pseudotyped viruses1–3. Given these additional data, we decided to test the effects of deleting the cytomplasmic tail from Spike in our pseudotyped lentivirus system. Other groups have tested deleting the last 18, 19, or 21 amino acids of Spike in VSV-2,4,5, MLV-3, or HIV-based6,7 pseudotyped virus systems. We chose to test the 18 and 21 amino acid truncations, hereafter referred to as Δ18 and Δ21.

We found that the cytoplasmic tail truncations increased Spike-pseudotyped lentiviral titers by ~10-fold compared to the full-length Spike without affecting neutralization sensitivity (**Fig. A1**). We also found that the Δ21 truncation resulted in slightly higher titers than the Δ18 Spike variant. We carried out these experiments according to the protocols in the original manuscript with a couple modifications noted in the figure legend.

It is hypothesized that truncating Spike’s cytoplasmic tail increases titers by increasing Spike-incorporation into budding virions through removing the ER retrieval signal (KxHxx) at the 3’ end of Spike8. In our initial work, we mutated this ER retrieval signal to AxAxx in our Spike-ALAYT construct. This targeted mutation did not increase Spike-pseudotyped lentivirus titers compared to full-length Spike. However, as we present here, truncating the last 18 or 21 amino acids of the Spike cytoplasmic tail does increase Spike-pseudotyped lentivirus titers, indicating that other portions of Spike’s cytoplasmic tail likely play a role in Spike trafficking.

**Figure A1.** Titers of Spike-pseudotyped lentiviral particles in 293T-ACE2 cells (A, B) and neutralization of these viruses with serum from an individual previously infected with SARS-CoV-2 (C). (A) Titers of pseudotyped lentivirus with the ZsGreen backbone pseudotyped with full-length Spike, Spike with either of the two truncations, VSV G, or no viral entry protein. Titers were determined as in **Fig. 3** except cells were counted at 60 h post-infection. The “n.d.” indicates that the titer was not detectable. Data shown are from a single representative example. (B) Titers of the Luciferase-IRES-ZsGreen backbone virus pseudotyped with the specified viral entry proteins. Titers were determined by measuring relative luciferase units (RLUs) per mL and then normalizing to the titers of full-length Spike pseudotyped lentivirus. RLUs were determined at 52 h post-infection. The RLUs per mL for the Spike-pseudotyped viruses are the average of seven wells of a 1:3 dilution of virus in a total volume of 150 μL. For the VSV G-pseudotyped virus, RLUs per mL were averaged from six three-fold dilutions starting at a 1:48 dilution in a total volume of 150 μL. The luciferase assay was carried out as described in the original protocol, *except* the assay was carried out in a black-walled, clear-bottom plate (Greiner, 655090) coated with poly-L-lysine and luciferase readings were measured in these plates without transferring to an opaque-bottom plate. These plates have slightly higher background than opaque plates, resulting in a very low, but detectable titer for viral particles produced without adding a viral entry protein. (C) Neutralization assay with serum collected from an individual previously infected with SARS-CoV-2, 43 days post-symptom onset (p.s.o.). The neutralization assay was carried out as described in the original manuscript except for using black-walled, clear-bottom plates as mentioned in B. Here, we started with a 1:20 serum dilution and did 6, 3-fold dilutions to make the neutralization curve. The full-length neutralization curve shows data averaged from duplicate measurements. The Δ18 and Δ21 neutralization curves display data from a single replicate. The IC50s for the full-length, Δ18, and Δ21 viruses are 1:345, 1:345, and 1:370, respectively. These values all fall within the range of IC50 values we have measured previously for this same serum sample with virus pseudotyped with full-length Spike (1:320-1:375). This serum sample was collected at the University of Washington under IRB…. We thank Dr. David Koelle, Dr. Anna Wald, and Dr. Jim Boonyakornkit for sharing this sample with us.

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