Preliminary data on feasibility:

Our team has the ***expertise*** to handle all data processing on our own as shown below and in our biosketches. Arresting S maturation intermediates in distinct ER-Golgi-plasma membrane compartments **(Fig. A)** has been made possible by an innovative construct design that retains the native trafficking signals in the cytosolic tail by inserting an affinity purification tag in an internal site rather than the tail C-terminus **(Fig. B)**. Using this approach, we have generated an S construct that is retained in ER and cis-Golgi and displays immature, high-mannose glycans (S_{Imm}), a construct that is rapidly exported to the plasma membrane without repeated recycling in the glycan modifying compartments and displays mature, complex-type glycans (S_{Mat}), the S_{WT} construct that recycles in these compartments prior to export to plasma membrane, and an S ecto-domain (S_{ecto}) as a control to compare with massive amounts of published data on S_{ecto}. The S_{WT} construct differs from S_{Imm} at one residue (His1271 in S_{WT} substituted by Lys1271 in S_{Imm}) whereas it differs from S_{Mat} at two residues (Lys1269/His1271 in S_{WT} substituted to Ala1269/Ala1271 in S_{Mat}). As shown in our initial GUP1 application (**NCCAT-GUP1-SH220622**), glycan analysis by mass spectrometry ***validates*** the processing of these S maturation constructs. ***Single particle cryoEM data*** have been collected on S_{ecto} and S_{Mat} at NCCAT and on S_{Imm} at PNCC, on grids ***prepared in-house***. The S_{ecto} reconstruction is at a resolution of 2.5Å **(Fig. C, D)**. The structure of this internally tagged S_{ecto} construct is consistent with previously published C-terminally tagged S_{ecto} structures, which ***validates*** our internal tagging approach in maintaining the tertiary and quaternary organization of the spike. This dataset displays a ***predominantly one-up state of the RBDs*** upon separation of S_{ecto} conformational states by heterogeneous refinement, 3D classification, and 3D variability analysis. In contrast, the S_{Imm} dataset from PNCC, reconstructed to a resolution of 3.2Å, displays a ***wide conformational diversity*** in the RBDs **(Fig. E, F)**. As S_{Imm} has ***~1.7 fold more*** immature glycans than S_{ecto}, this suggests that ***glycan maturation changes*** during inter-organelle trafficking play a critical role in ***modulating spike RBD conformations.*** This glycan maturation difference is ***larger*** between full-length S_{Imm}, S_{WT}, and S_{Mat}, especially at regulatory glycan sites such as at Asn343. An on-the-fly S_{Mat} reconstruction by the NCCAT staff during the cryosparc-live session shows an architecture of characteristic of the spike protein **(Fig. G)**. These S_{Mat} data are currently in early stages of processing in our lab. ***Hence, the S_{WT} data collected under this RAP proposal will complete the entire panel of spike glycan maturation intermediates.***

Status of ready-to-go grids for S_{WT}: The S_{WT} protein has been purified under the conditions established for S_{Imm} and S_{Mat} **(Fig. H)**, which are the two spike constructs we have characterized both biochemically and by single particle cryoEM. Freezing of grids for S_{WT} have also been prepared under the same conditions as the grids for S_{Imm}, S_{Mat}, and S_{ecto}. Our data show that all the full-length spike constructs demonstrate similar ice thickness and particle distribution on the cryoEM grids whereas S_{ecto} has a higher particle density. ***The S_{WT} grids are in storage and ready to be shipped within a 24-hour notice.***

Preliminary Data

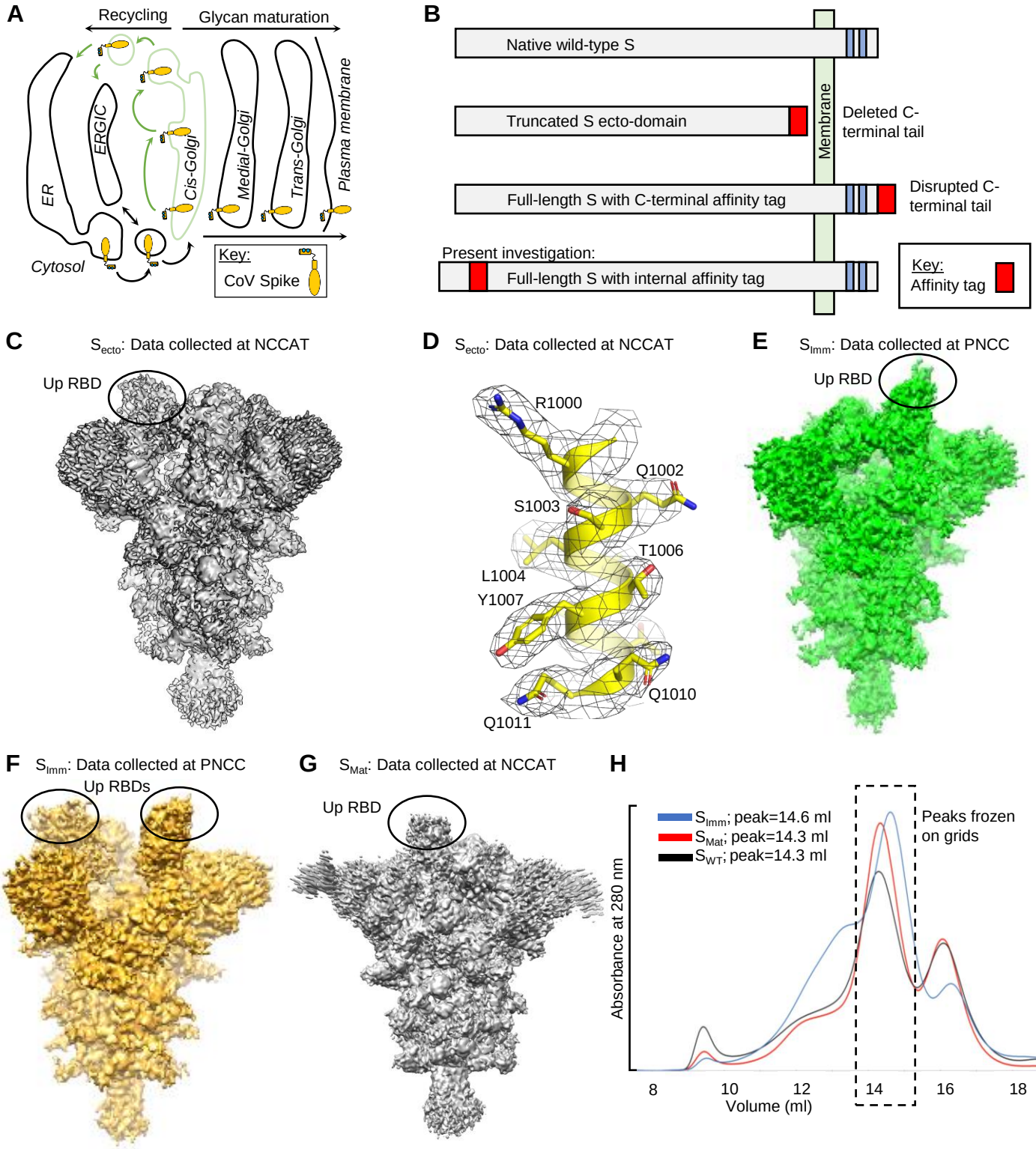

Figure: Preliminary data. (A) S recycling and export pathway. **(B)** S construct designs contrasting our strategy of internal tagging with prior C-terminal truncation and C-terminal tagging strategies. **(C and D)** Unsharpened 2.5Å resolution cryoEM map of S_{ecto}. **(E and F)** CryoEM reconstructions of S_{Imm} showing two different RBD conformations. **(G)** Preliminary cryoEM reconstruction of our recently collected S_{Mat} dataset from NCCAT. **(H)** Chromatograms showing purification of S_{Imm}, S_{Mat}, and S_{WT} in detergent on a superose 6 size-exclusion chromatography column.