Specific Aim 3: Testing predictions of CoV inter-species transmission

In Year 3 we established an effective and economic reverse genetics system for bat SL-CoV which can be applied to efficiently rescue SL-CoVs that are difficult to culture. This can be used to explore the functions of newly identified SL-CoV genes, as well as to assess pathogenesis of novel bat SL-CoVs. Using this system, we demonstrated that the unique ORFx in WIV1 and WIV16 is a functional gene involving modulation of the host immune response but not essential for *in vitro* viral replication (Zeng et al, 2016, J Virol).

Identification of Three Novel SL-CoVs with Potential for Direct Transmission to Humans
In Y2, we conducted full-length genome sequencing of 11 novel SL-CoVs detected in a single bat habitat in Yunnan province, which included strains highly similar to human/civet SARS-CoV in the most variable genes (N-terminal domain and RBD in the S gene, ORF8 and ORF3) (under revision). Based on recombination analysis, we hypothesized that the direct progenitor of the pandemic SARS-CoV may originated from this location after sequential recombination events at multiple genomic positions.

Among the 11 newly identified SL-CoVs, three different strains namely Rs4874, Rs7327 and Rs4231 contained no deletions in the RBD region but their RBD sequences varied from each other. Rs4874 has an S gene almost identical to that of WIV16. Rs7327's S protein varies from that of WIV1 and WIV16 at three aa residues in the receptor-binding motif, including one contact residue (aa 484) with human ACE2. Rs4231 shares similar NTD sequence with WIV1 and WIV16, but has a distinct RBD sequence. In Year 3, we successfully isolated Rs4874 from the single fecal sample. Using the reverse genetic system we previously developed, we constructed two chimeric viruses with the WIV1 backbone replaced with the S gene of Rs7327 and Rs4231, respectively. Vero E6 cells were respectively infected with Rs4874, WIV1-Rs4231S and WIV1-Rs7327S, and efficient virus replication was detected by immunofluorescence assay in all infections. To assess the usage of human ACE2 by the three novel SL-CoVs, we conducted virus infectivity studies using HeLa cells with or without the expression of human ACE2. All viruses replicated efficiently in the human ACE2-expressing cells. The results were further confirmed by quantification of viral RNA using real-time RT-PCR (*Fig.11*).

<u>These finding suggests that diverse variants of SL-CoV S protein without deletions in their RBD are able to use human ACE2 as receptor for cell entry. Diverse SL-CoVs capable of direct transmission to humans are circulating in bats in southwestern China, which represents a potential risk of emergence given the opportunity to spillover to other animals and/or human populations</u>.