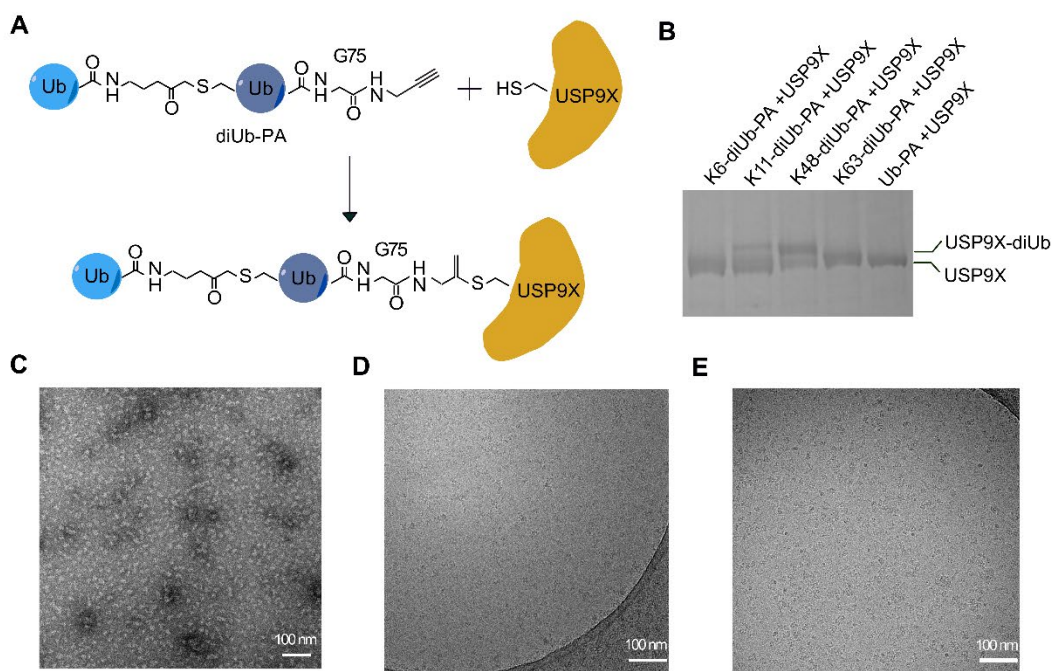


## Feasibility & Data:

Ubiquitin-specific peptidase 9X (USP9X) is a 230 kDa deubiquitinase (DUB) that plays important roles in cellular processes and signaling pathways by modulating the deubiquitination of several known protein substrates in apoptosis, cell cycle control, protein trafficking, and DNA damage repair. In addition, USP9X has been linked to cancer, neurodevelopment disorders and neurodegenerative diseases, including autism, epilepsy, and Alzheimer's disease. However, the mechanistic details of USP9X's function in various biological processes have not been extensively studied. To date, a complex structure of the full-length (FL) USP9X with its native substrates has not been reported. We recently showed that the K48-diubiquitin activity-based probe can label the FL USP9X efficiently by capturing the catalytic cysteine in the USP9X active site (**Figure 1A, B**). Notably, diubiquitin probes of other linkages (K6, K11, and K63) showed either no labeling or much reduced labeling of the FL USP9X, suggesting exquisite ubiquitin chain-linkage specificity of the FL USP9X. The structural basis of this remarkable observation is not yet known. A cryo-EM structure determination of the USP9X-K48-diUb complex structure will provide the much-needed information in understanding the molecular basis of the ubiquitin chain linkage specificities of USP9X and DUBs in general.

The negative stain (**Figure 1C**) and cryo-EM images (**Figure 1D, E**) of USP9X collected using a Talos L120C microscope at the University of Delaware Bioimaging Center exhibit regular-shaped particles of good density and homogeneity. We are encouraged by the preliminary results and hope to solve the complex structure of USP9X-K48-diubiquitin by accessing the more advanced microscopes and other resources at NCCAT while learning the various aspects of cryo-EM structure determination.



**Figure 1.** Complex of USP9X-K48 diubiquitin. (A) Illustration of the USP9X-K48 diubiquitin complex. (B) USP9X labeling by diubiquitin (diUb) activity-based probes of different linkages. 0.45  $\mu$ M USP9X was labeled by 1  $\mu$ M probe and characterized by SDS-PAGE. (C) Negative stain EM of USP9X imaged at 0.1 mg/ml. (D) Cryo-EM image of USP9X imaged at 1 mg/ml. (E) Cryo-EM image of USP9X-K48 diUb probe complex. The complex was formed by mixing 4.5  $\mu$ M USP9X and 20  $\mu$ M K48- diUb probe.