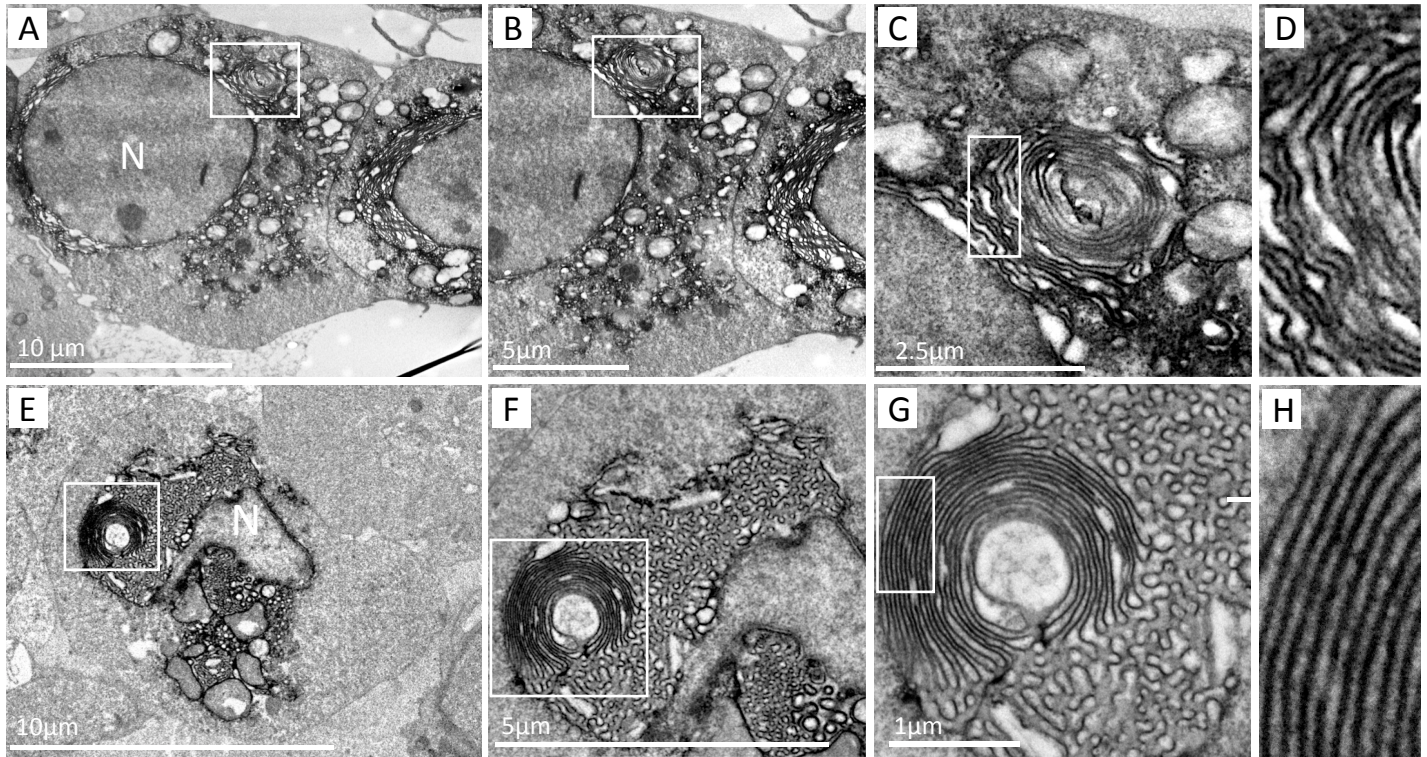
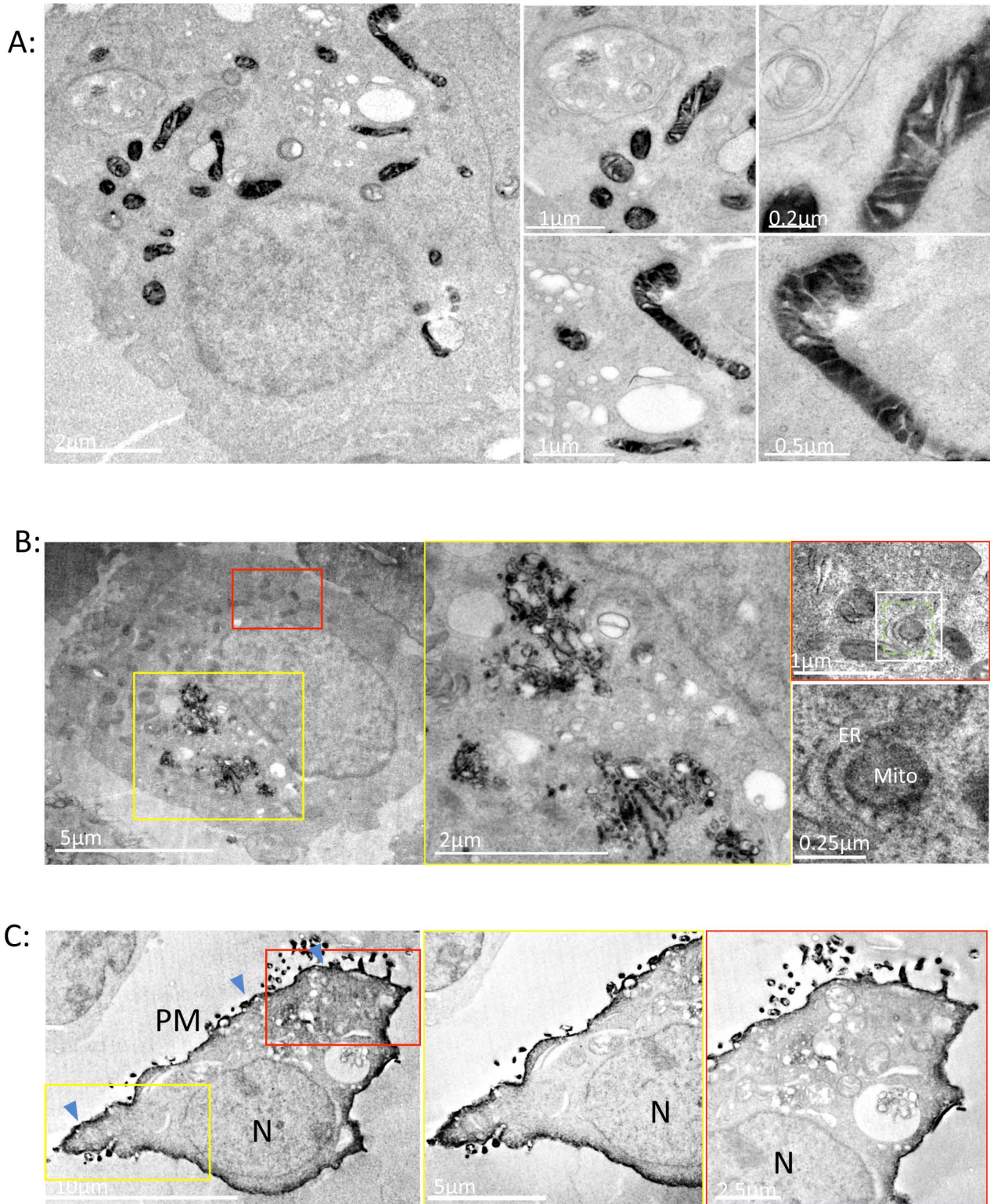


**Figure S1. A combination of chemical fixation and cryofixation exhibits superior ultrastructure preservation and membrane staining over traditional methods in HEK-293T cells:** Cells prepared by conventional glutaraldehyde fixation and dehydration methods (A, B and G); or glutaraldehyde followed by cryofixation (C, D and H); or cryofixation alone (E, F and I) were examined by thin-section EM. Panels G, H and I represent magnified views from panels B, D and F, respectively. Cells prepared by glutaraldehyde/cryofixation (Glut+HPF+FS) display dense homogenous staining of the cytosol that resembles that seen in cells preserved by cryofixation (HPF+FS), while cells prepared by conventional means (Glut+Alcohol) do not. Glut = glutaraldehyde. HPF = high pressure freezing. FS = freeze substitution.

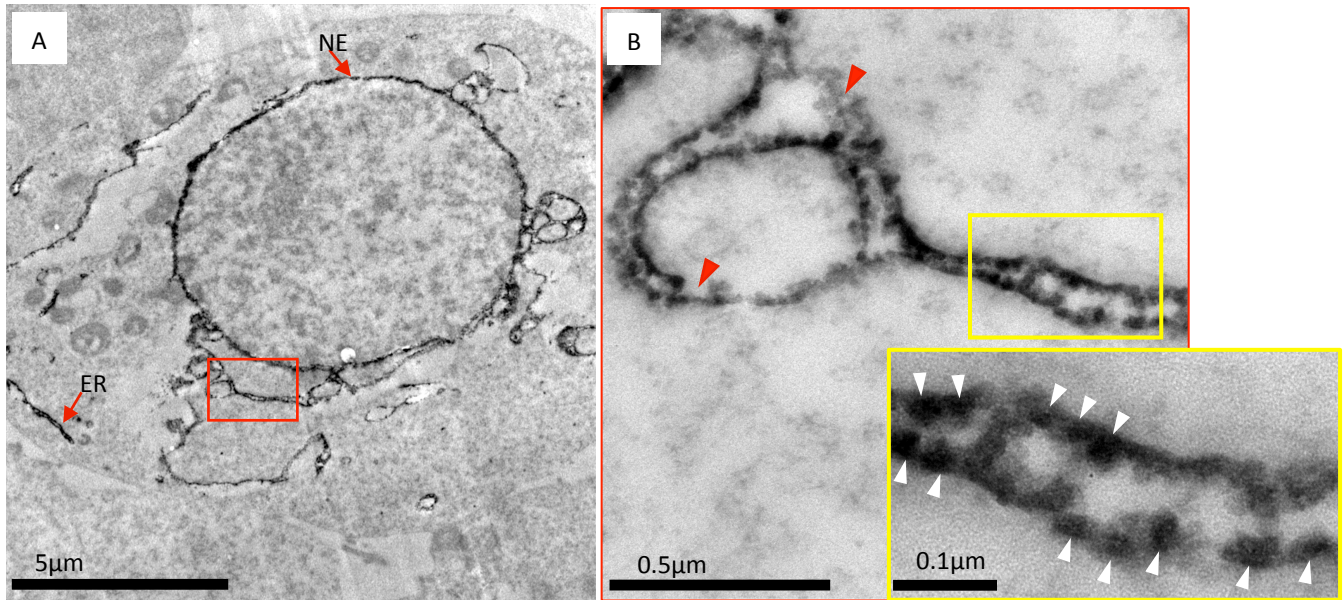


**Figure S2. Comparison of OSER membrane preservation using traditional chemical fixation versus cryoAPEX.** The reorganized ER morphology in chemically fixed, DAB reacted ERM-APEX2 expressing HEK293 cells that were processed via traditional chemical fixation/alcohol dehydration (A-D) or by cryoAPEX (E-H) was compared following post staining with uranyl acetate and Sato's lead solution. The evenly-spaced parallel lamellar stacking of the ER derived membranes obtained by cryoAPEX (exemplified in panels G and H), as opposed to the ruffled membranes obtained by traditional methods (panels C and D), highlights the superior membrane preservation obtained by cryoAPEX.



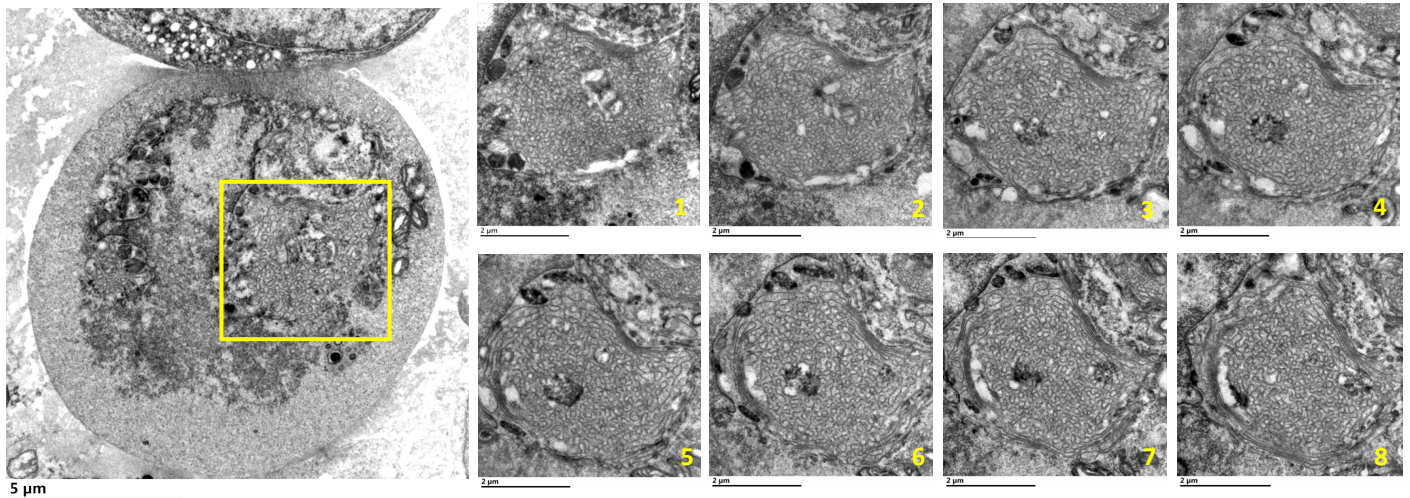


**Figure S3. Organellar controls show specificity of the signal obtained from APEX2-tagged proteins.** APEX2-tagged protein constructs designed to localize to the mitochondrial matrix (mito-V5-APEX2; shown in A), or the golgi lumen ( $\alpha$ -mannII-APEX2; shown in B), or the plasma membrane (CAAX-APEX2; shown in C) were transiently expressed in HEK293 cells and the samples processed by cryoAPEX. Each construct yielded organelle specific densities. Magnified views of two sections (yellow or red boxes) from the cells expressing  $\alpha$ -mannII-APEX2 (panel B) or CAAX-APEX2 (panel C) are shown. The red and white boxes in panel B further indicate the presence of organelles like the mitochondria and ER that can be visualized even in the presence of a golgi-specific DAB density associated with  $\alpha$ -mannII-APEX2; however, these mitochondria and ER membranes do not display DAB staining, highlighting that we do not observe mislocalized or artefactual signals.



**Figure S4. Localization of HYPE using the traditional chemical fixation/dehydration method: A)** Image of a thin section of a cell expressing HYPE-APEX2 shows specific staining of the cortical ER and the nuclear envelope (red arrows). **B)** At a higher magnification, periodic HYPE-specific foci were apparent within stretches of the ER (yellow box and white arrow heads in the inset), despite extensive membrane disruption, indicated by red arrows. NE = nuclear envelope.





Section thickness: 90nm

**Figure S5. Superior ultrastructural preservation enables the tracking of ERM's subcellular localization via serial sectioning:** To demonstrate the consistency of the membrane ultrastructure preservation obtained by cryoAPEX, cells expressing ERM-APEX2 were serially sectioned and a specific area (panel A, yellow box) was imaged. Multiple such ribbons containing between 10 and 20 serial sections of 90nm thickness were collected, screened and imaged. Representative images of 8 serial sections showing ER localization of ERM are presented (images serially numbered 1 though 8). Sections exhibit well-preserved OSERs. Thus, we can follow ERM localization and associated ER morphology changes throughout the volume of the cell without loss of contextual information.

**Table S1.**

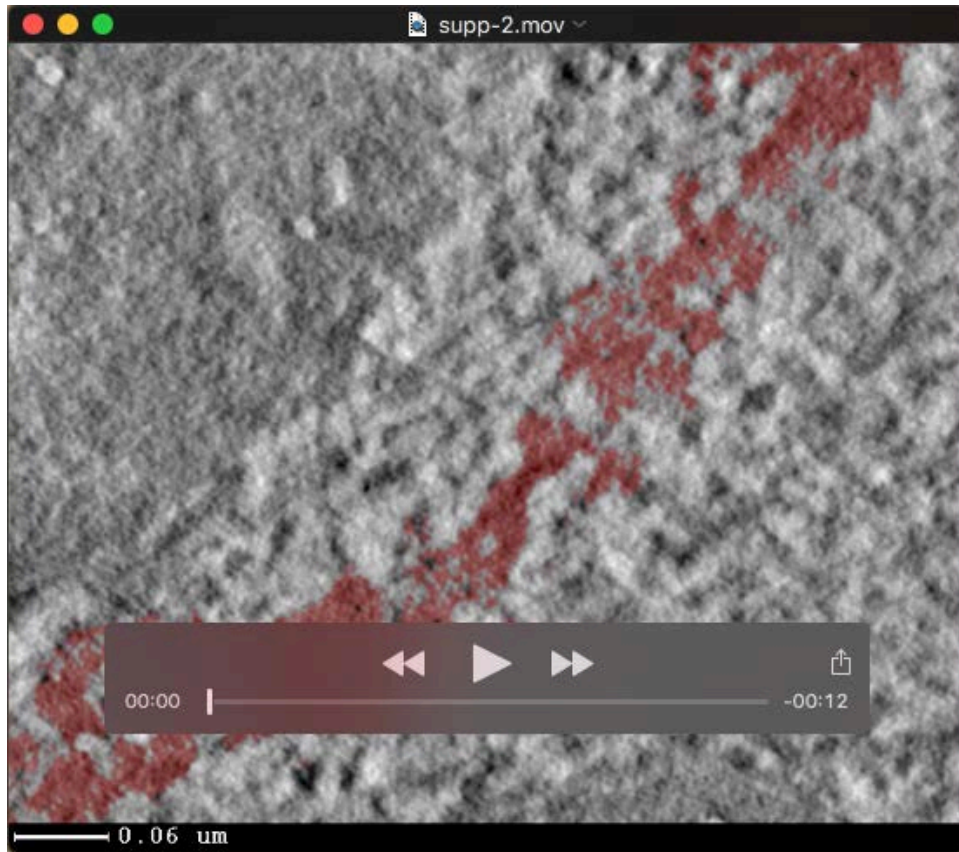
**Sequence for  $\alpha$ -MannII-APEX2:**

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ctcacaaaagcttccgagcttgggttgctgatgcctaa





**Movie 1.** Tomogram of HYPE density within the ER lumen



**Movie 2.** Tomogram of the thresholded HYPE density in the ER lumen.