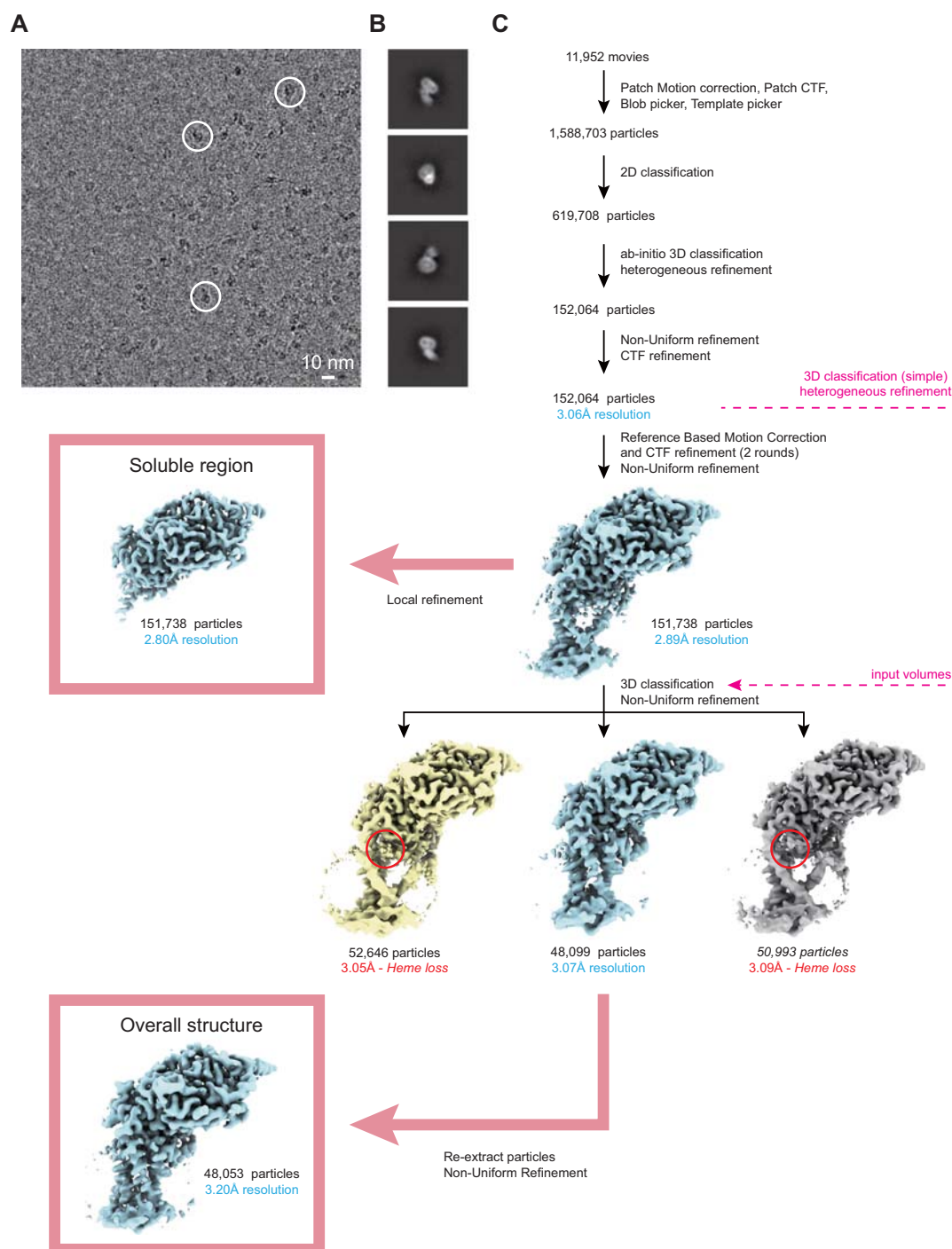
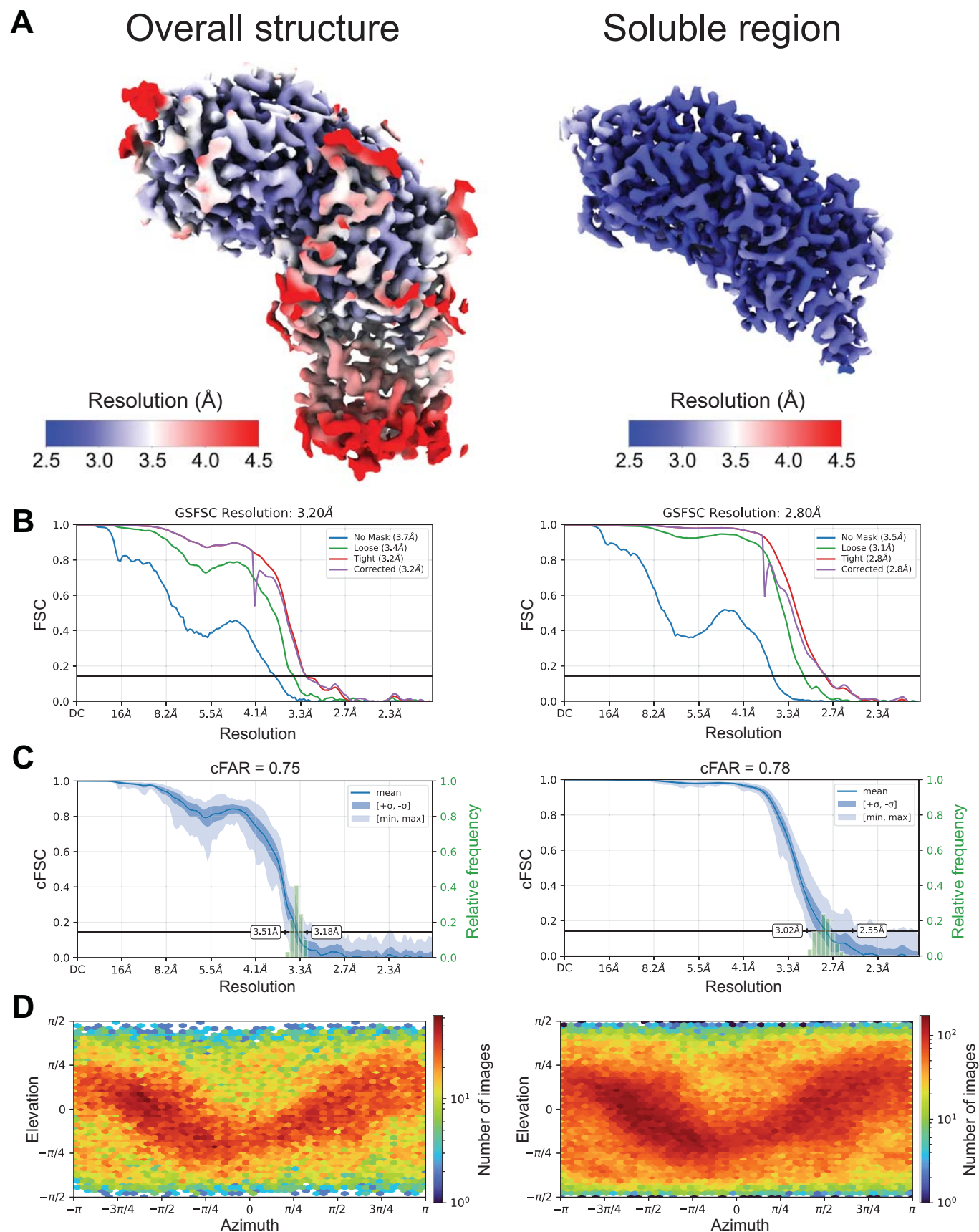


Expanded View Figures

**Figure EV1. Cryo-EM workflow.**

(A) Representative micrograph. Example particle images are circled. (B) 2D class averages of EtfD. (C) Simplified cryo-EM workflow. Red circles indicate the region in EtfD where the heme was lost.



**Figure EV2. Cryo-EM map validation.**

(A) Local resolution map for the full EtFD structure (*left*) and the locally refined soluble region of EtFD (*right*). (B) Fourier Shell Correlation (FSC) curves obtained after gold-standard refinement. (C) Conical FSC (cFSC) plots and their associated cFSC area ratio (cFAR) scores. (D) Particle orientation distribution plots.

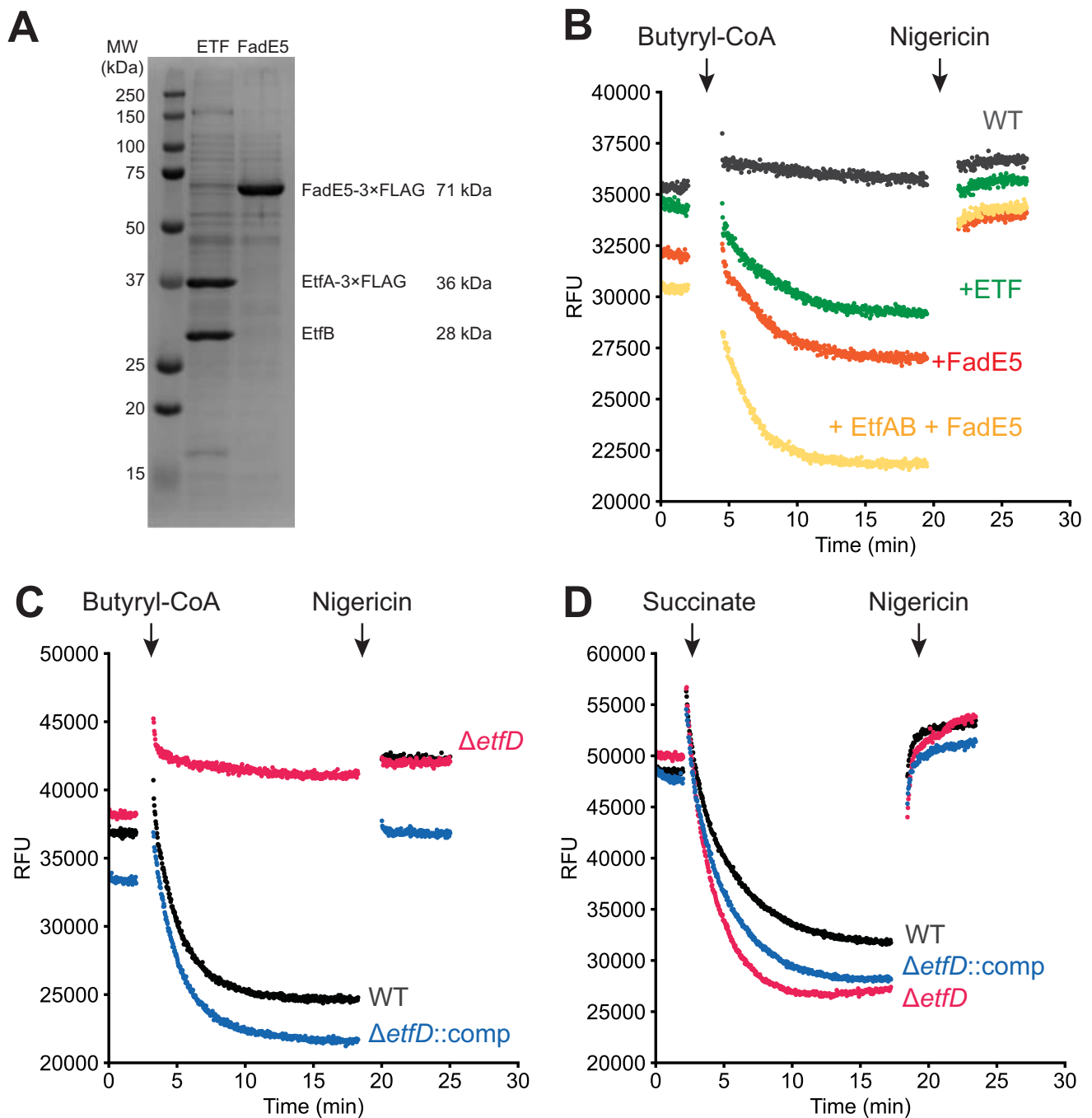


Figure EV3. Assay replicates.

(A) SDS-PAGE gels of purified ETF and FadE5. (B) Butyryl-CoA-driven proton pumping activity in IMVs prepared from wild-type *M. smegmatis* (GMC_MSM1) supplemented with either buffer (black), ETF (green), FadE5 (orange), or ETF and FadE5 (yellow). (C) Butyryl-CoA-driven proton pumping activity in the presence of ETF and FadE5 for IMVs prepared from *M. smegmatis* that is either wild-type (black), Δ etfD (red) (GMC_MSM9), or Δ etfD complemented with *M. tuberculosis* EtfD (GMC_MSM10). (D) Succinate-driven proton pumping activity in IMVs prepared from *M. smegmatis* that is either wild-type (black), Δ etfD (red), or Δ etfD complemented with *M. tuberculosis* EtfD (blue).

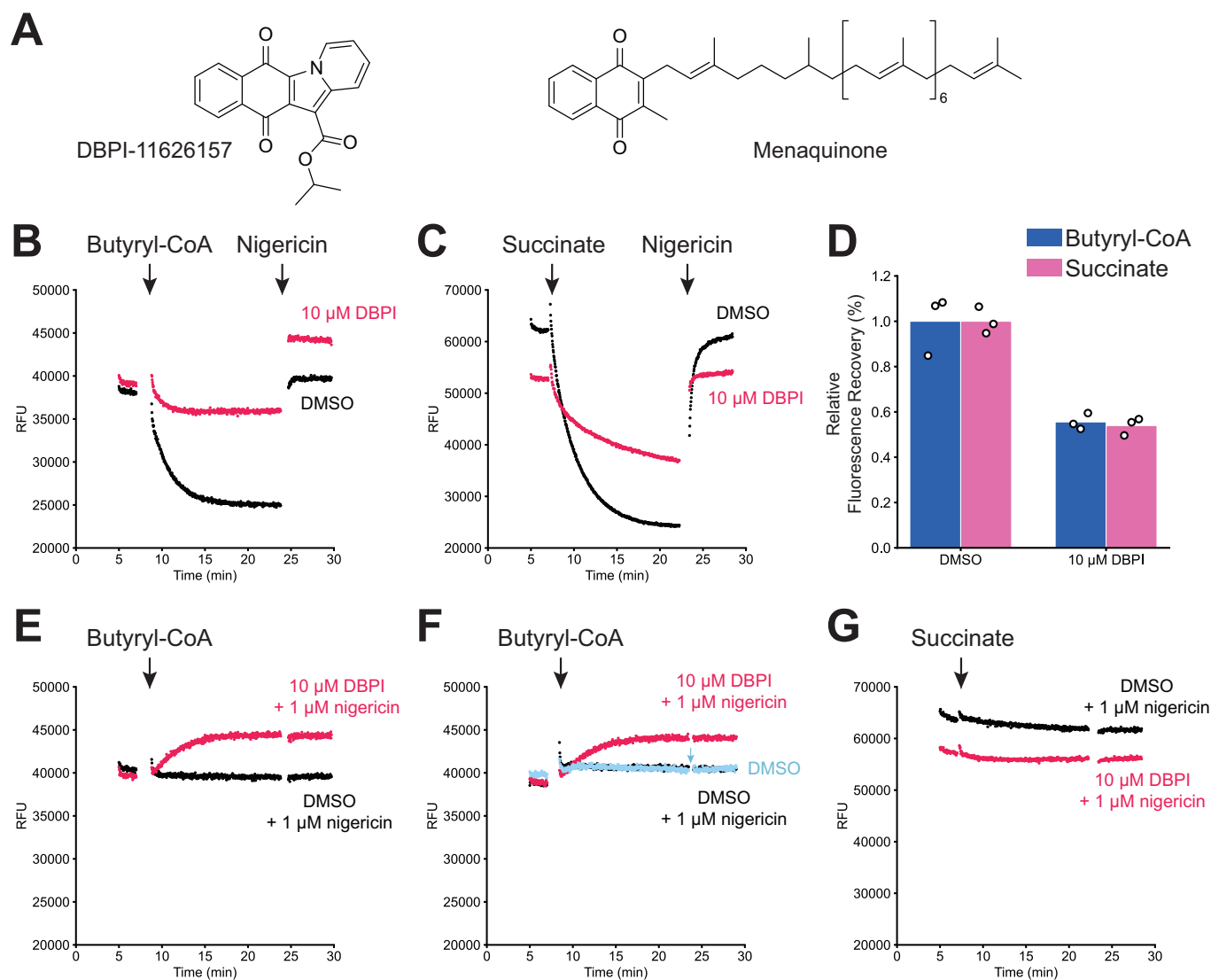


Figure EV4. Effect of DBPI-11626157 on butyryl-CoA-driven and succinate-driven IMV acidification.

(A) Chemical structure of DBPI-11626157 (left) and menaquinone (right). (B) Butyryl-CoA-driven acidification of $\Delta\text{etfD}::\text{rv0338c}$ IMVs in the presence of 2 μM ETF and 1 μM FadE5, with 10 μM DBPI-11626157 (red) or DMSO (black). Data is representative of three replicates. (C) Succinate-driven acidification of $\Delta\text{etfD}::\text{rv0338c}$ IMVs with 10 μM DBPI-11626157 (red) or DMSO (black). Data is representative of three replicates. (D) Quantification of butyryl-CoA-driven and succinate-driven IMV acidification assays. (E) Addition of butyryl-CoA to $\Delta\text{etfD}::\text{rv0338c}$ IMVs in the presence of 1 μM nigericin, 2 μM ETF, and 1 μM FadE5, with 10 μM DBPI-11626157 (red) or DMSO (black). Data is representative of three replicates. (F) Addition of butyryl-CoA to $\Delta\text{etfD}::\text{rv0338c}$ IMVs without FadE5 or ETF with 10 μM DBPI-11626157 and 1 μM nigericin (red), DMSO and 1 μM nigericin (black), or DMSO alone (blue). Nigericin (1 μM) was added to the DMSO-only well during the assay (blue arrow). Data is representative of two replicates. (G) Addition of succinate to $\Delta\text{etfD}::\text{rv0338c}$ IMVs in the presence of 1 μM nigericin with 10 μM DBPI-11626157 (red) or 1 μM nigericin and DMSO (black). Data is representative of three replicates.

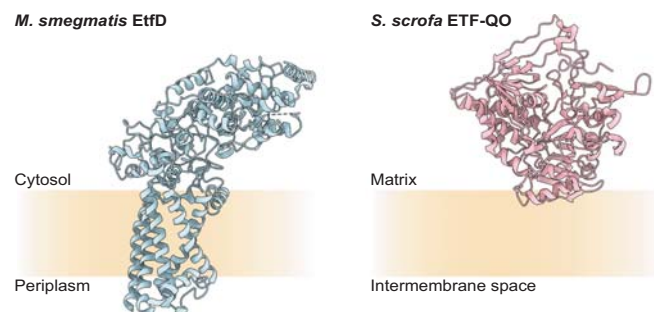
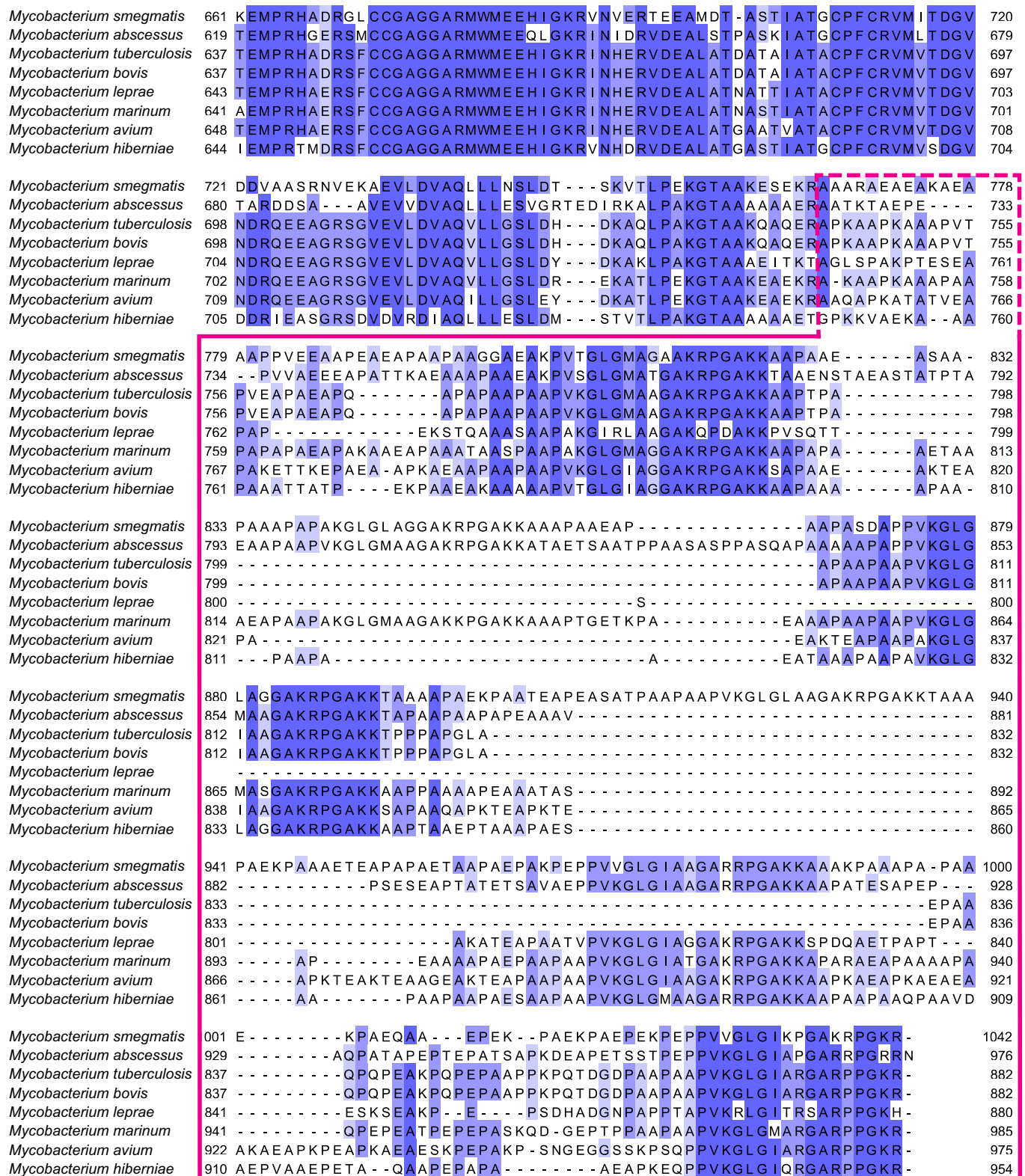


Figure EV5. Structure of mycobacterial and mammalian ETF dehydrogenases.

Comparison of EtfD from *M. smegmatis* with ETF-QO oxidoreductase from *Sus scrofa* (PDB: 2GMH).



Disordered region

Figure EV6. Sequence alignment of the disordered region of mycobacterial EtF domain homologs.

Sequences are colored by conservation. The disordered region is indicated by a pink box. The start of the disordered region differs between species (dashed line).

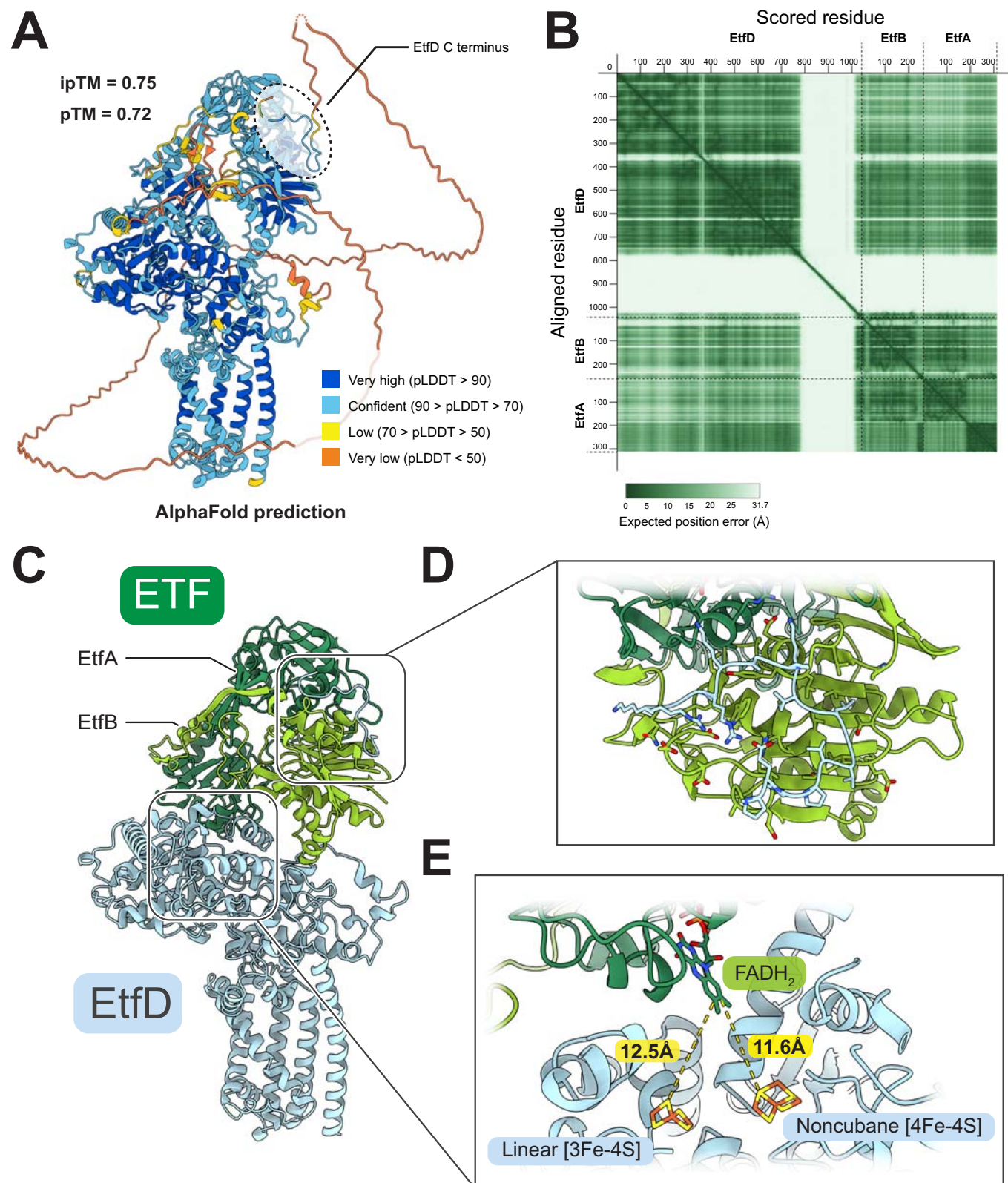
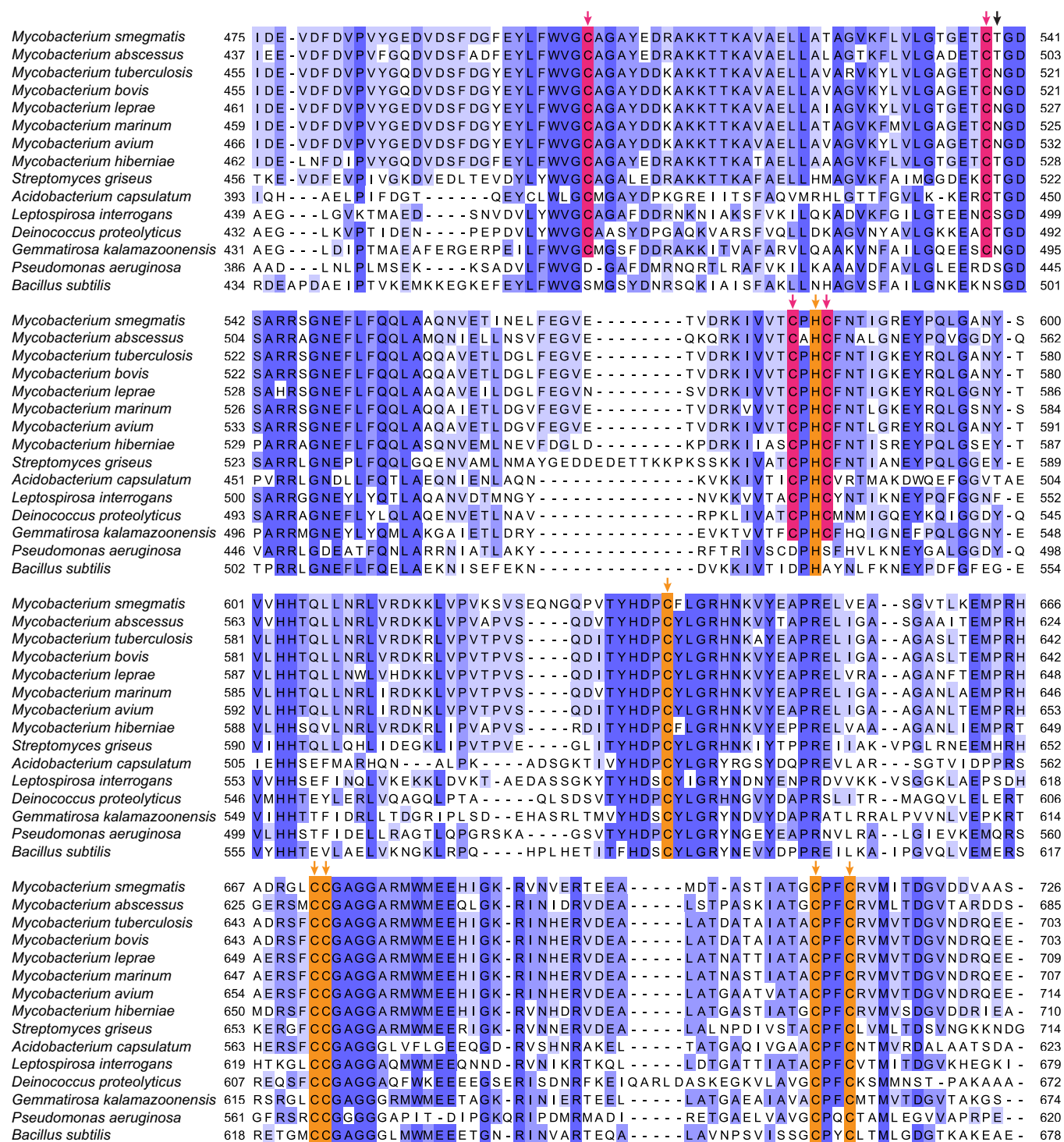


Figure EV7. AlphaFold prediction of the ETF:EtFD complex.

(A) AlphaFold 3 prediction of the ETF-EtFD complex structure, colored by pLDDT score. (B) Predicted aligned error plot (Elfmann and Stülke, 2023). (C) Overview of the interaction between ETF (green) and EtFD (blue). The ~30 kDa disordered region of EtFD is not shown for clarity. (D) Close-up view of the interaction predicted by AlphaFold 3 between the disordered region of EtFD and ETF. (E) Location of the docked FAD cofactor of ETF from PDB 1EFV, relative to cluster D1 and D2 of EtFD.



linear [3Fe-4S] **noncubane [4Fe-4S]**

Figure EV8. Sequence alignment of the CCG domains of selected EtFD homologs.

Coordinating residues are shown with red (linear [3Fe-4S] cluster) and orange (noncubane [4Fe-4S] cluster) arrows. The black arrow shows the position of the supernumerary cysteine in Hdr. Sequences are colored by conservation.