Expanded View Figures

Figure EV1. The UFL1-DDRGK1 complex.

A Model confidence of the UFL1-DDRGK1 AlphaFold2 complex prediction. Left: Predicted local distance difference test (pLDDT); Right: predicted Align Error (pAE) plots of model 1, and models 2–5. For visualization purposes, only rank_1 was used in Fig 1 (accompanies Fig 1A).

B Model of the DDRGK1-UFL1 complex, colored according to pLDDT, highlighting the lower confidence in the structure of the N-terminal helix.

C SDS–PAGE showing the purity of the indicated fusion proteins.

D–H Loading controls of *in vitro* ufmylation assays (accompanies Fig 1E–H). (D, E) fusion constructs, (F, G) ternary complex. (H) Presence of UFL1/UFL1ΔN in the membrane: Since we hardly see UFL1/UFL1ΔN in the Ponceau staining (F), we performed Western blot analysis with anti-Myc. All reactions were loaded on bis-Tris-PAGE (except the reaction of Fig 1G, which was loaded on 8–16% Tris gel).

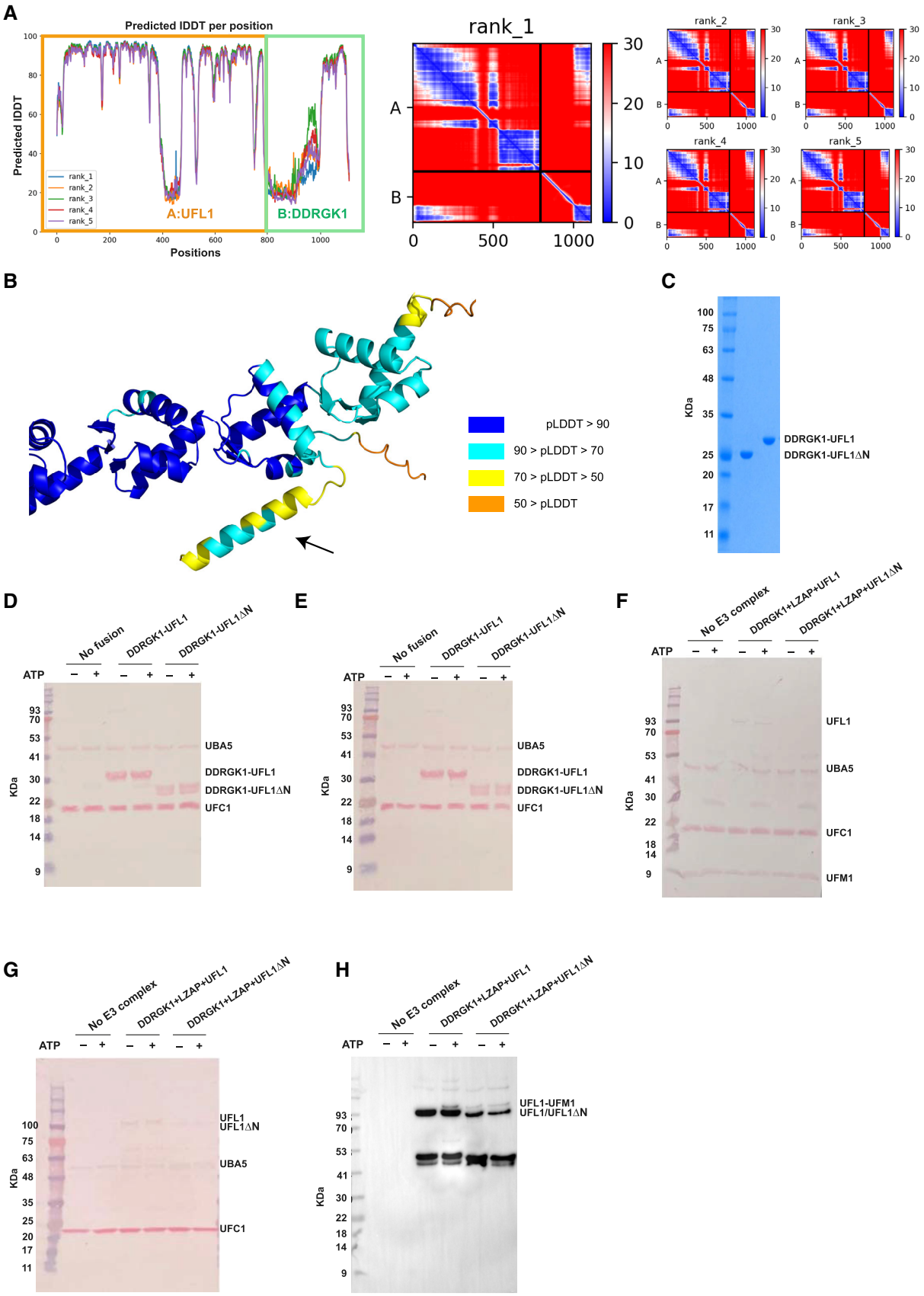
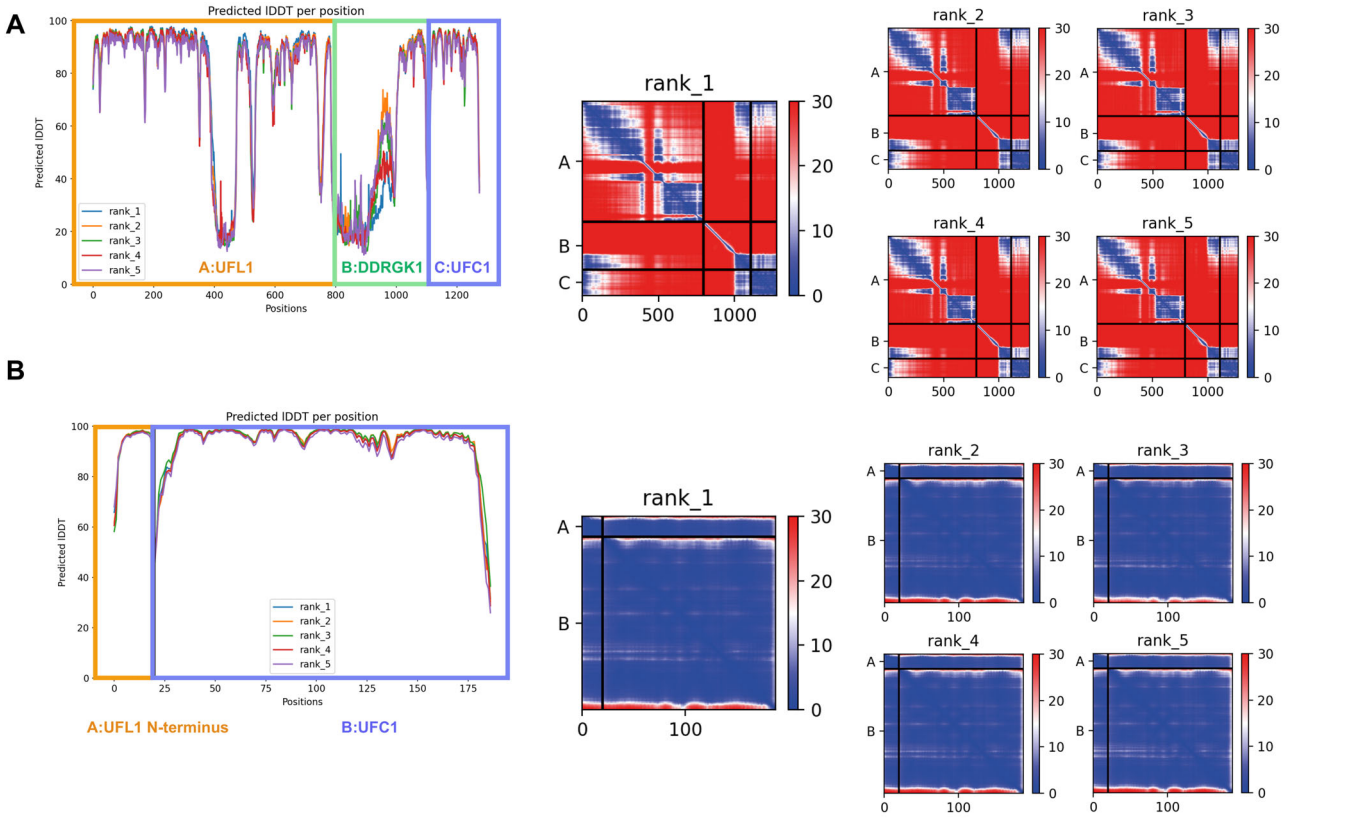


Figure EV1.



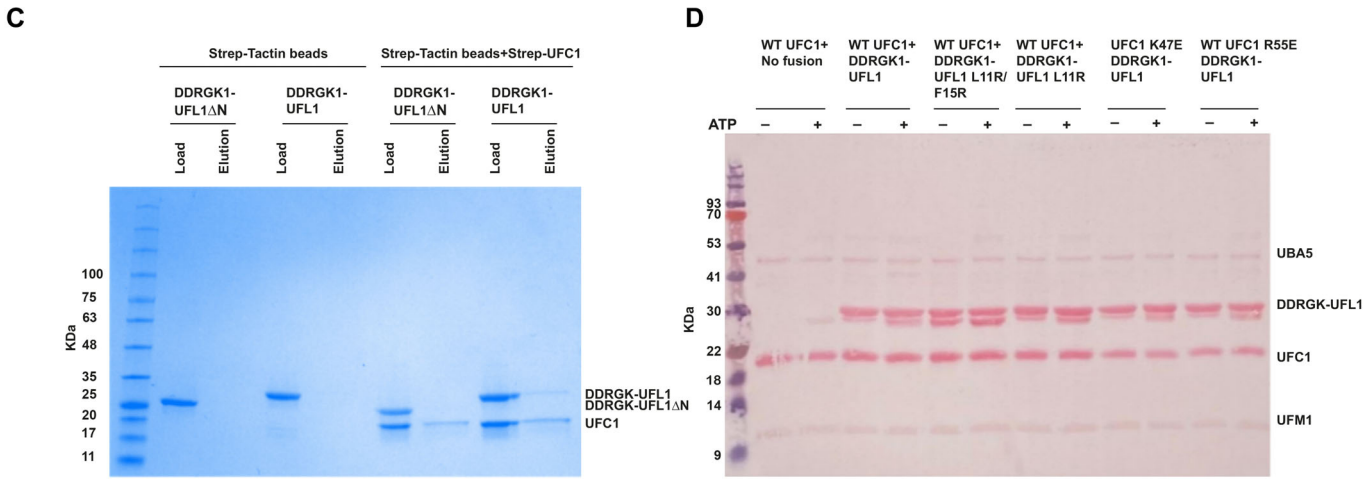


Figure EV2. UFC1 interaction with UFL1 N-terminal helix.

A, B Model confidence of the AlphaFold2 complex prediction of A UFL1-DDRGK1-UFC1 and B UFL1 N-terminal helix-UFC1. Left: Predicted local distance difference test (pLDDT); Right: predicted Align Error (pAE) plots of model ranked 1, and models ranked 2–5.

C Coomassie stain gel shows that UFC1 binds to DDRGK1-UFL1 but not DDRGK1-UFL1ΔN, demonstrating that this interaction depends on the presence of the N-terminal helix of UFL1.

D Loading control of *in vitro* ufmylation assay with mutants of DDRGK1-UFL1 and UFC1 (accompanies Fig 2I).

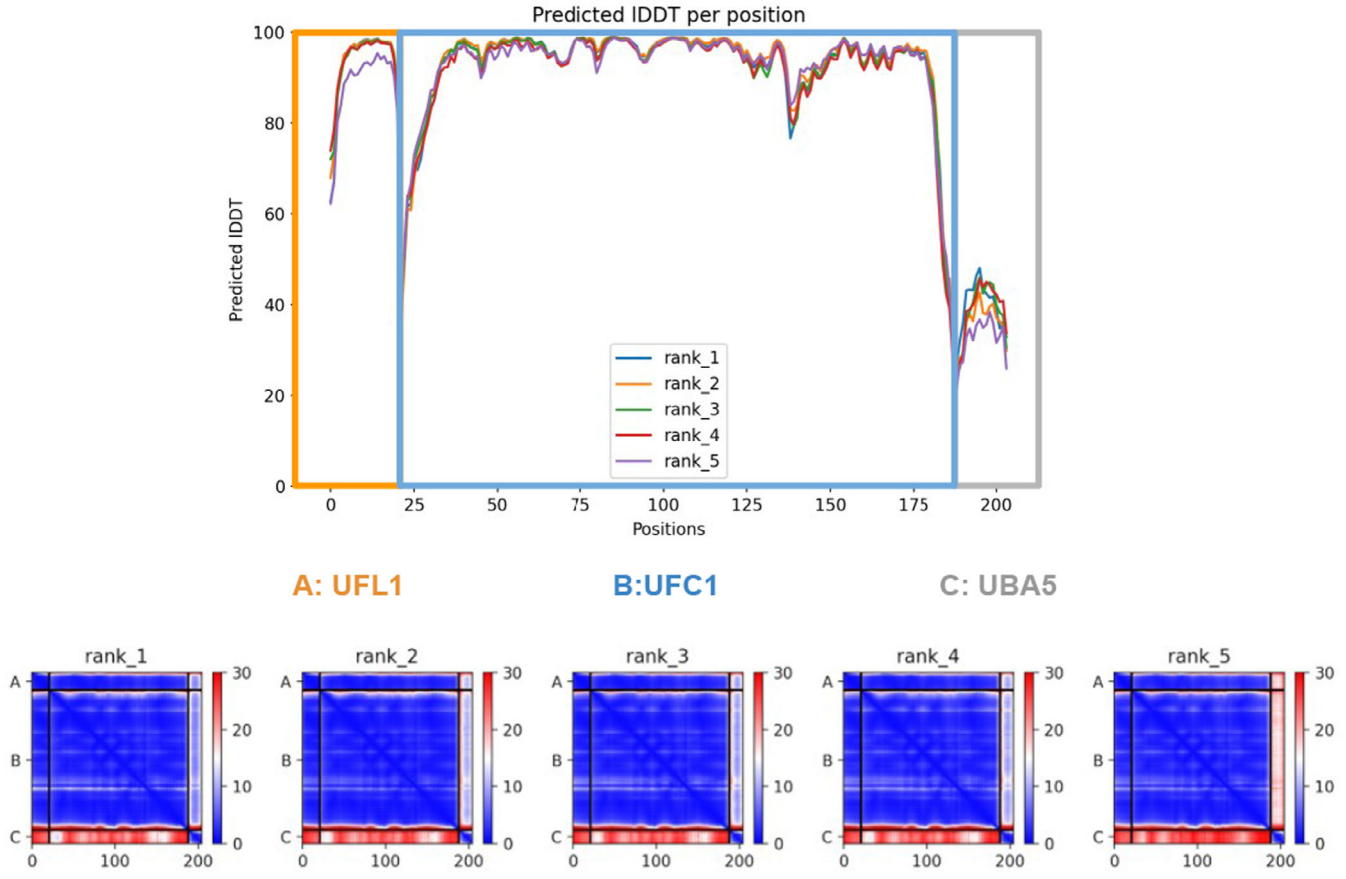


Figure EV3. UBA5 versus UFL1 computational competition assay.

Model confidence of the AlphaFold2 complex prediction of UFL1 N-terminal-UFC1-UBA5 C-terminal. Top: predicted local distance difference test (pLDDT); Bottom: predicted Align Error (pAE) plots of all models (accompanies Fig 4C).

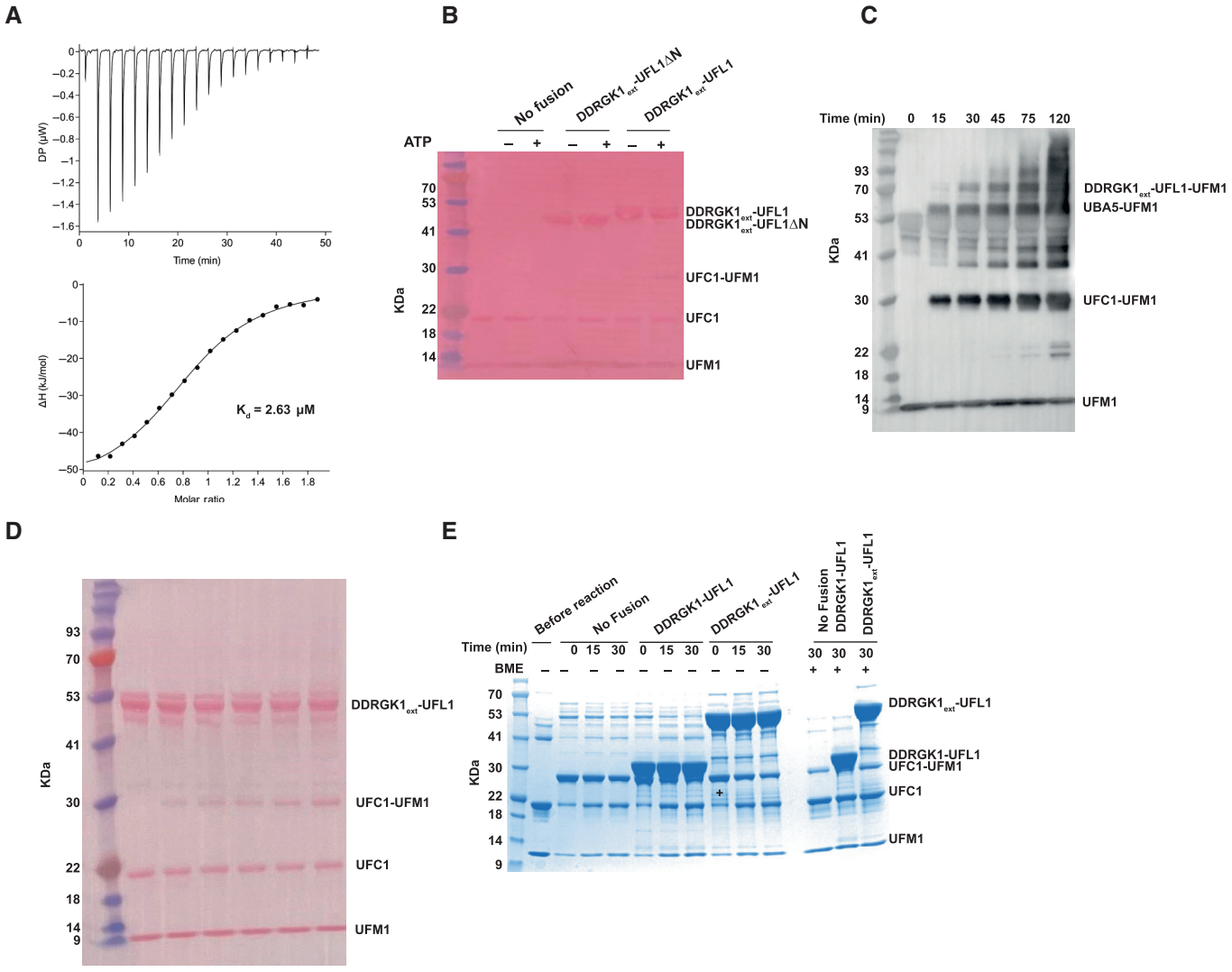


Figure EV4. Binding and activity characterization of DDRGK1$_{ext}$-UFL1.

A ITC experiment of UFC1 binding to DDRGK1$_{ext}$-UFL1. The top graph represents raw data of heat flow versus time. The area under the peaks of the upper panel was integrated and plotted as kJ per mole of UFC1 as a function of binding stoichiometry in the bottom panel. Thermodynamic parameters are summarized in Table EV3.

B Loading control of *in vitro* ufmylation assay (accompanies Fig 5D).

C *In vitro* ufmylation assay in the presence of DDRGK1$_{ext}$-UFL1. Western blot analysis of ufmylated products as function of time (blotting with anti-FLAG, since UFM1 has an FLAG-tag).

D Loading control of (C).

E Single turnover lysine discharge assay showing the effect of DDRGK1-UFL1 or DDRGK1$_{ext}$-UFL1 on discharge of UFC1 by free lysine.