Supplementary materials for

Evaluation of the Binding Kinetics of RHEB with mTORC1 by In-cell and In vitro Assays

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Content:

Note 1. Human mTOR gene sequence (7647 bp)
(http://www.kazusa.or.jp/kop/vd/FHC01207/)

(https://www.uniprot.org/uniprot/P42345)

Red highlights indicate gene sequences of the selected fragments of RHEB binding site

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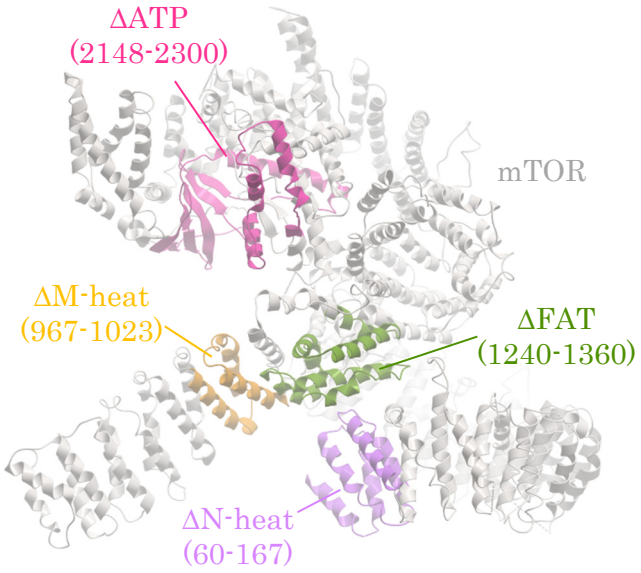

Note 2. Human RHEB gene sequence (507 bp)

(https://www.uniprot.org/uniprot/Q15382)

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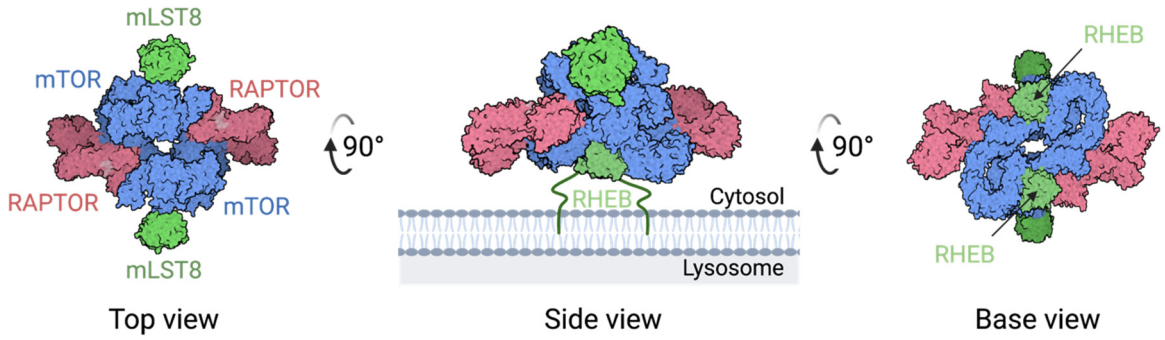


Figure S1: On-lysosome activation of mTORC1 by RHEB. Illustration created by BioRender (https://app.biorender.com/).

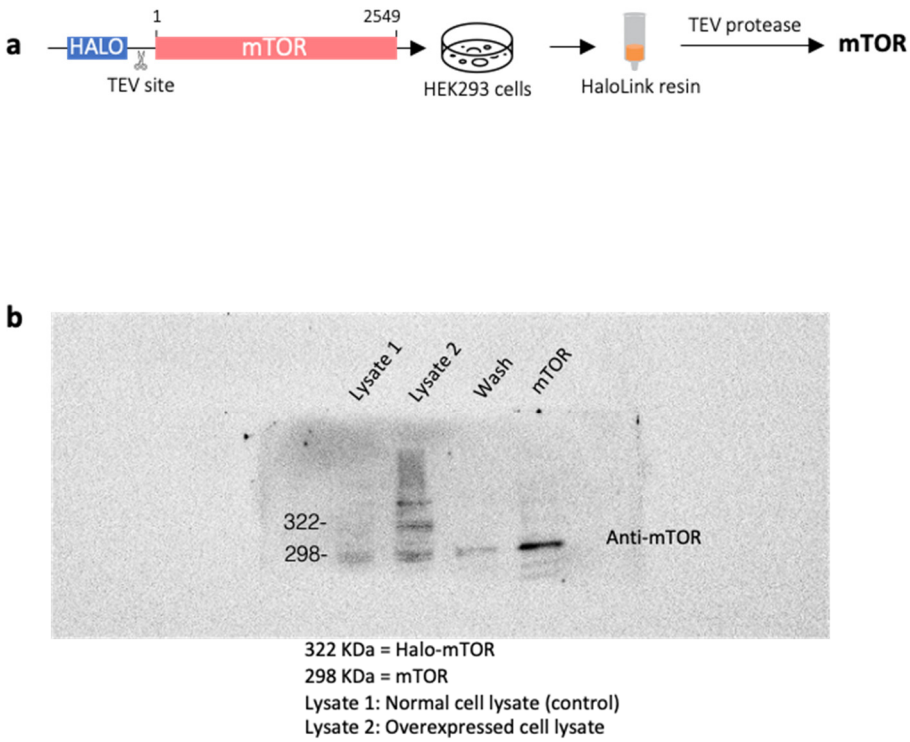


Figure S2. Construction and purification of overexpressed mTOR. (**a**) Schematic illustration showing the Halo-tagged mTOR construct in pFN21A vector which was transfected into HEK293 cells and purified by HaloLink resin. TEV site (EDLYFQ ↓ S) is a cleavage site for TEV protease. (**b**) Whole view of Western blot analysis of the different stages of mTOR purification showing the expression of the Halo-tagged mTOR.

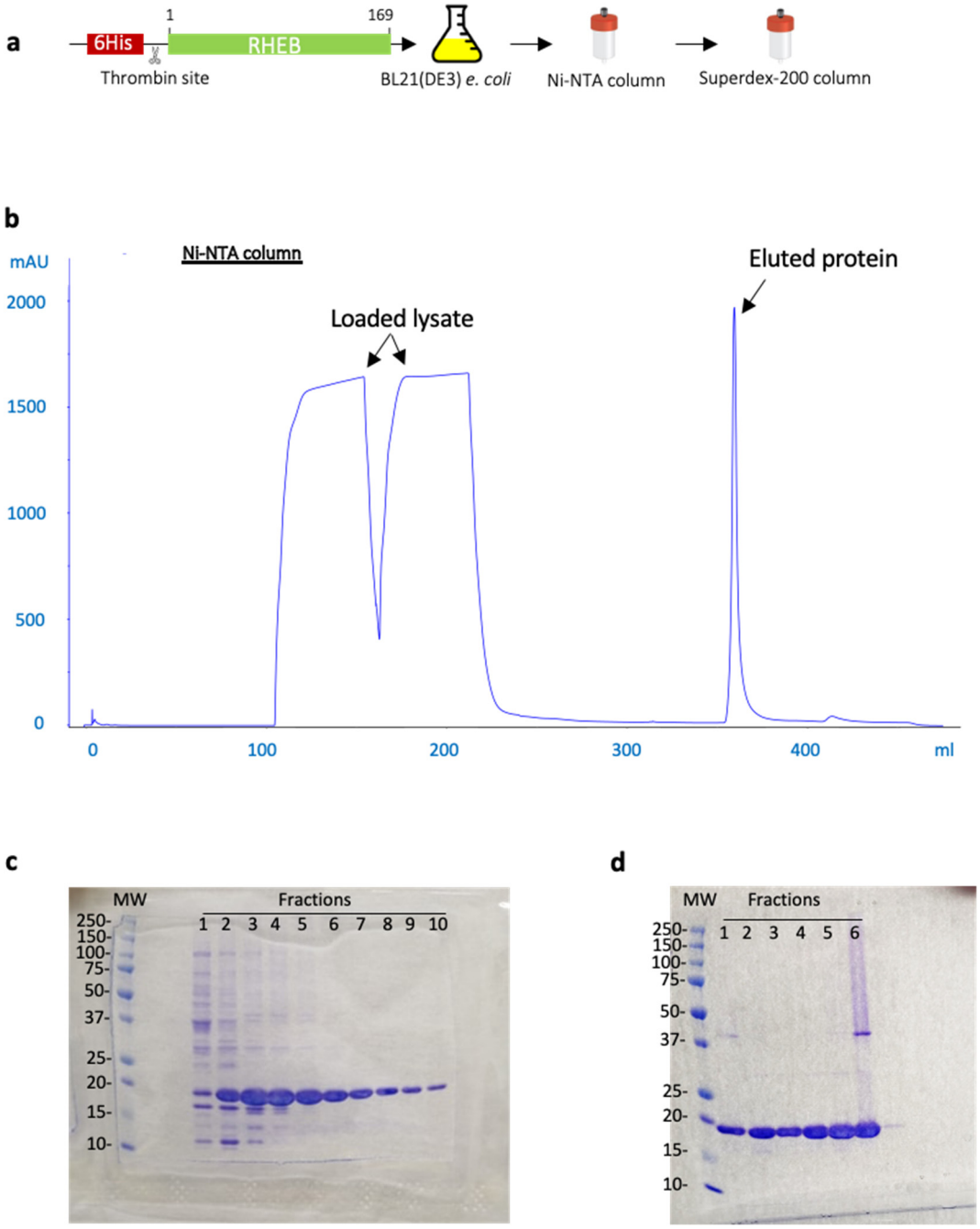


Figure S3. Construction and purification of overexpressed RHEB (aa 1-169). (**a**) Schematic illustration showing the 6His-tagged RHEB construct in pET15b vector which was transformed into BL21(DE3) E. coli and purified by Ni-NTA and Superdex-200 columns. (**b**) FPLC chromatogram of RHEB purification by Ni-NTA column. (**c**) Coomassie brilliant blue R-250 stained SDS-PAGE of the eluted fractions of RHEB (~20 kDa) from Ni-NTA column. The contaminating bands of fractions 1-4 were due to the molecules nonspecifically bound on the Ni-NTA column. Fractions 5-10 were used for Superdex-200 purification. (**d**) Full image SDS-PAGE (stained with Coomassie brilliant blue R-250) of the eluted fractions of RHEB protein from Superdex-200 column. Fractions 1 and 6 contain mixture of RHEB monomer and dimer. Fractions 2-5 were used as a final RHEB product.

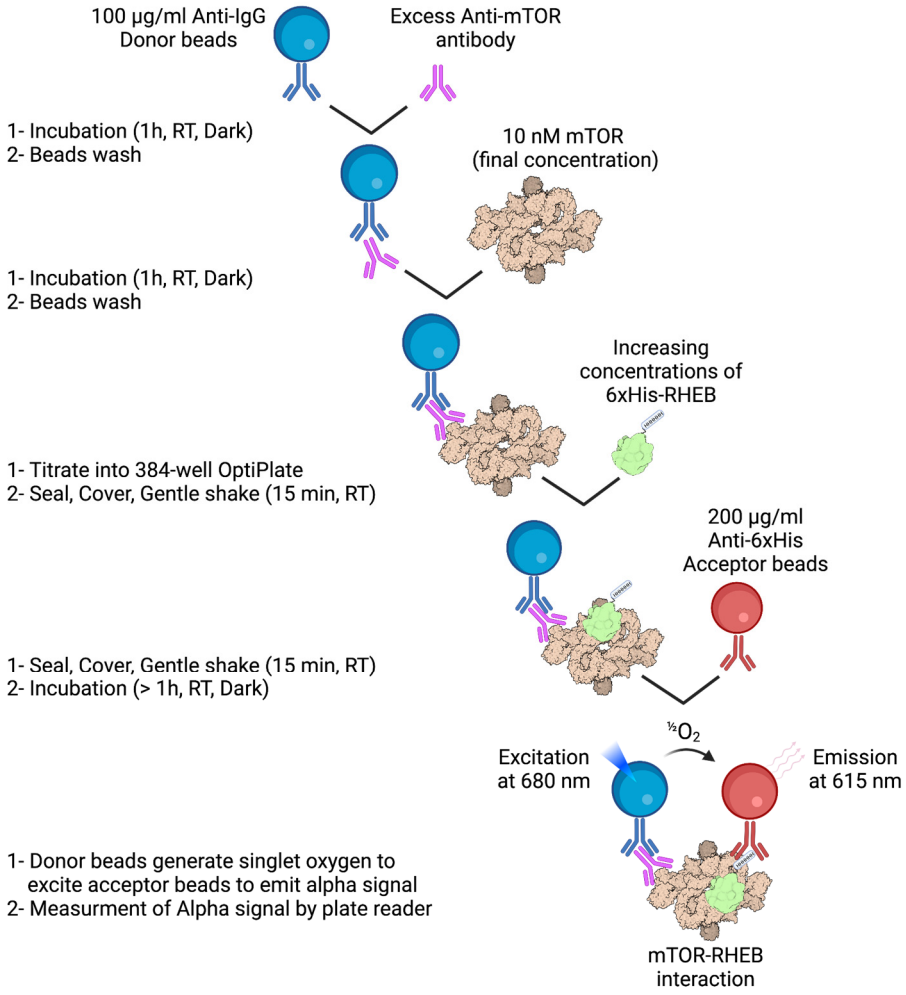


Figure S4. The established AlphaLISA method used to determine the binding kinetics of RHEB-mTOR interaction. RT, room temperature. Illustration created by BioRender (https://app.biorender.com/).

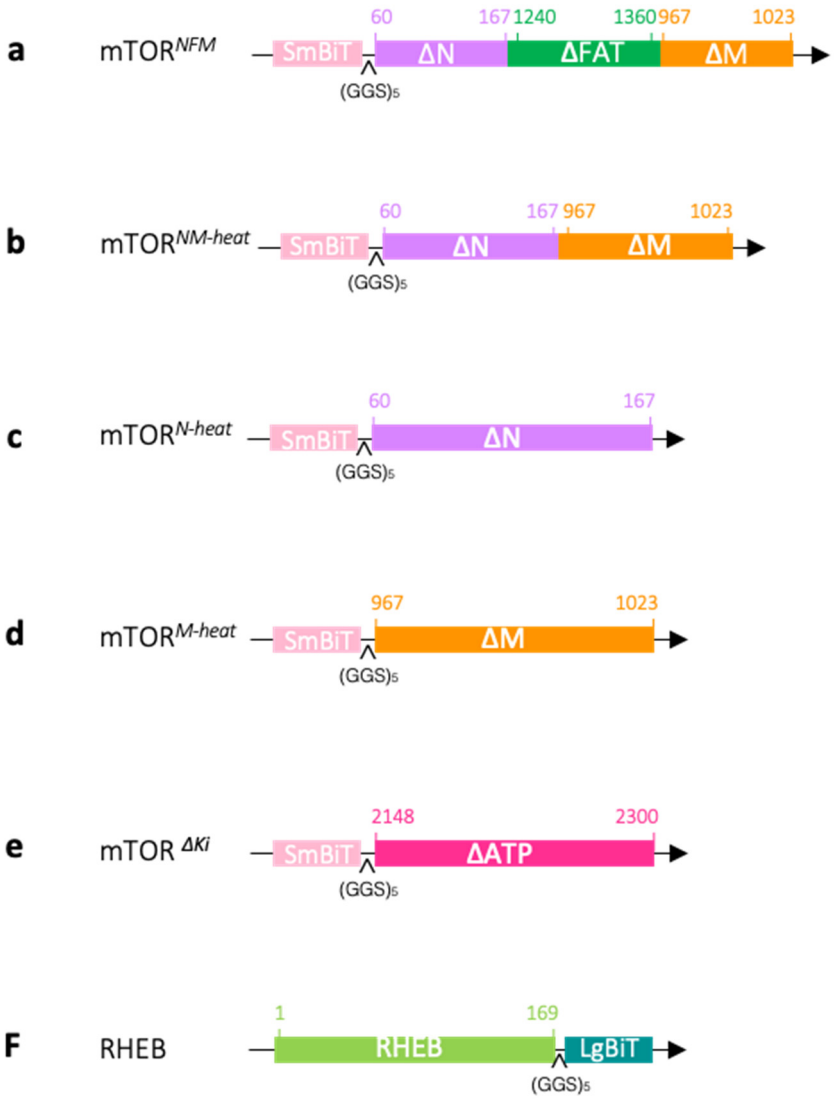


Figure S5. Plasmids of the indicated constructs for NanoBiT assay showing the SmBiT subunit at the N-terminus and the LgBiT subunit of RHEB at the C-terminus. $(GGS)_5$ is five repeats of GGS amino acid sequence as a linker between the protein of interest and the SmBiT or LgBiT.

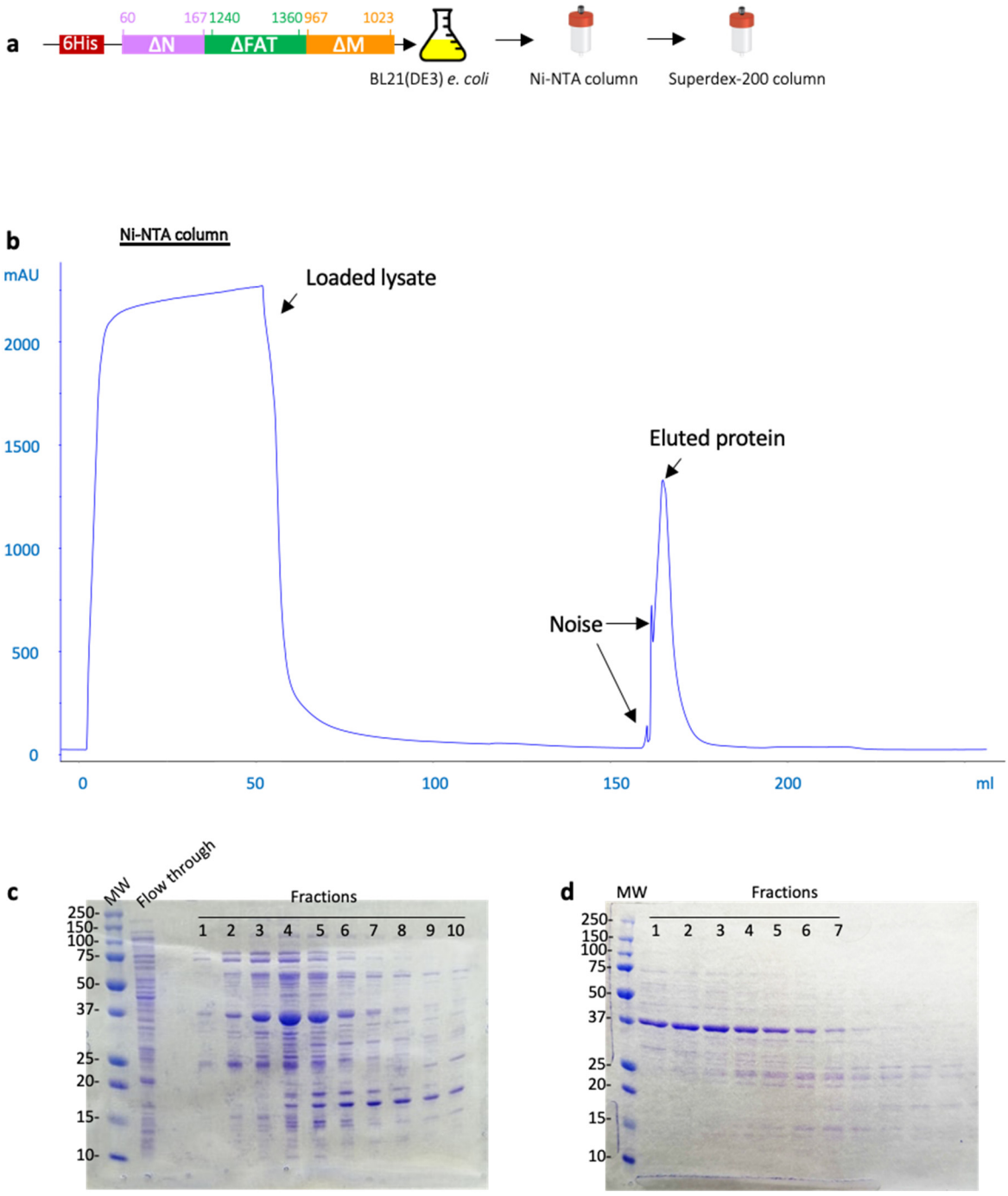
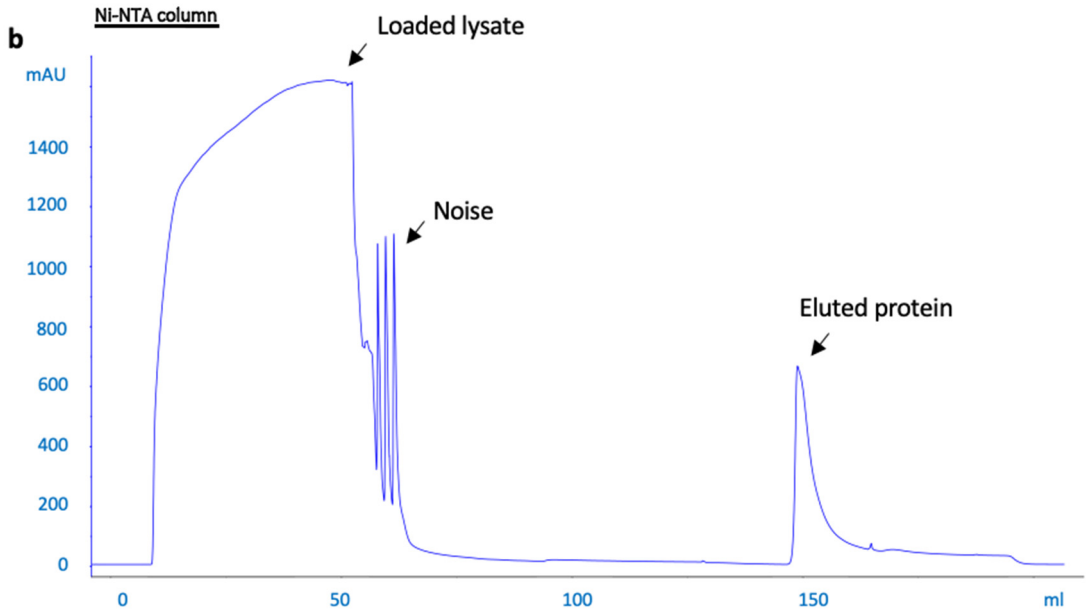


Figure S6. Construction and purification of overexpressed ΔN-FAT-M (aa 60-167, aa 1240-1360, aa 967-1023). (**a**) Schematic illustration showing the 6His-tagged ΔN-FAT-M construct in pET15b vector which was transformed into BL21(DE3) E. coli and purified by Ni-NTA and Superdex-200 columns. (**b**) FPLC chromatogram of ΔN-FAT-M purification by Ni-NTA column. Loaded lysate is a flow through without binding. (**c**) Coomassie brilliant blue R-250 stained SDS-PAGE of the eluted fractions of ΔN-FAT-M (~37.5 kDa) from Ni-NTA column. Fractions 1-6 were used for Superdex-200 purification. (**d**) Full image SDS-PAGE (stained with Coomassie brilliant blue R-250) of the eluted fractions of ΔN-FAT-M protein from Superdex-200 column. Fractions 1-4 were used as a final ΔN-FAT-M product. The contaminating bands were probably derived from protein oligomerization.


a
6His
60
167
ΔN
BL21(DE3) e. coli
Ni-NTA column
Superdex-200 column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Ni-NTA column
Loaded lysate
Noise
Eluted protein
mAU
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1000
800
600
400
200
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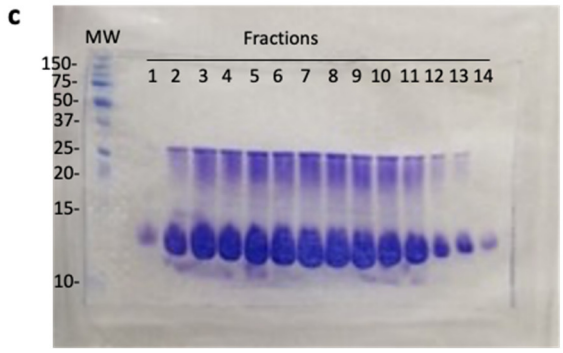
0
50
100
150
ml


c
MW
Fractions
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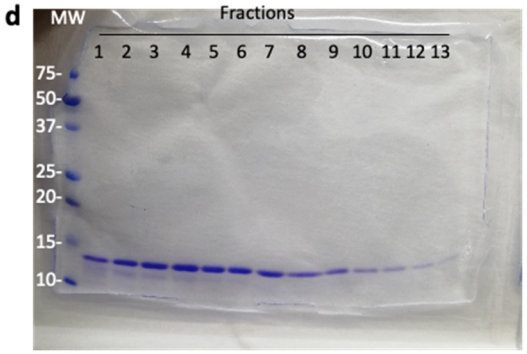
75-
50-
37-
25-
20-
15-
10-


d
MW
Fractions
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50-
37-
25-
20-
15-
10-

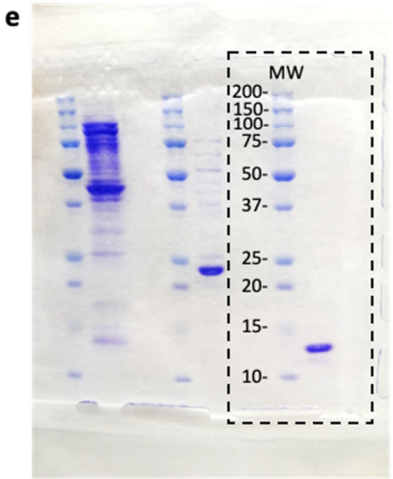
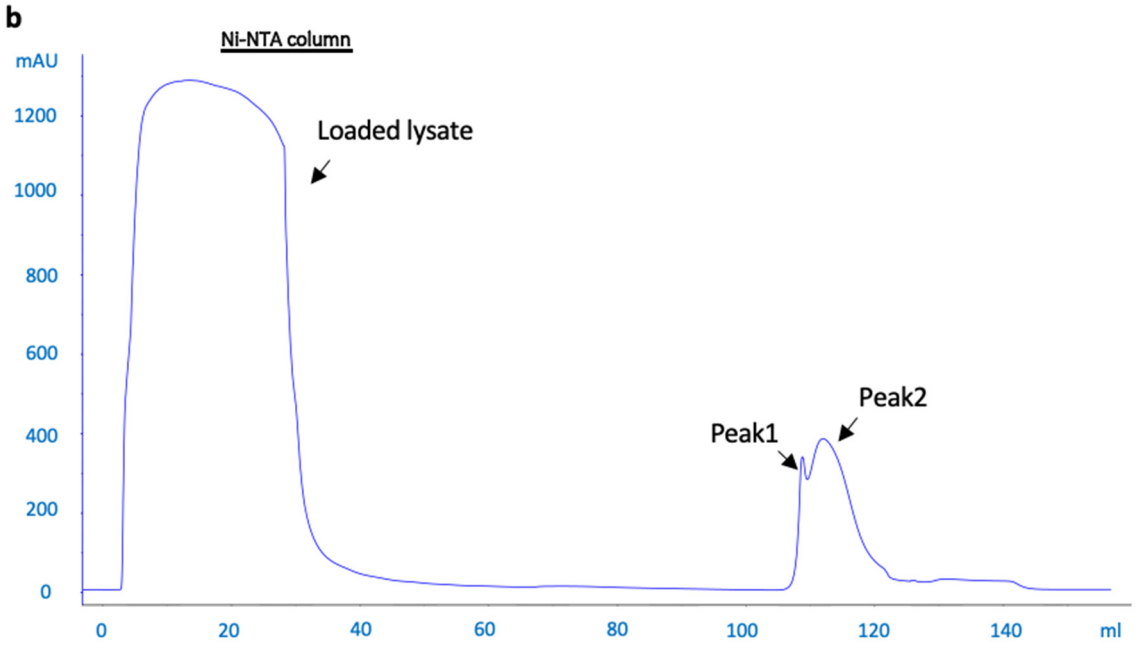
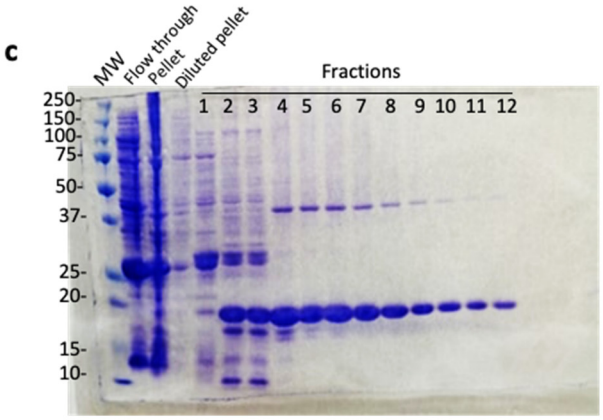
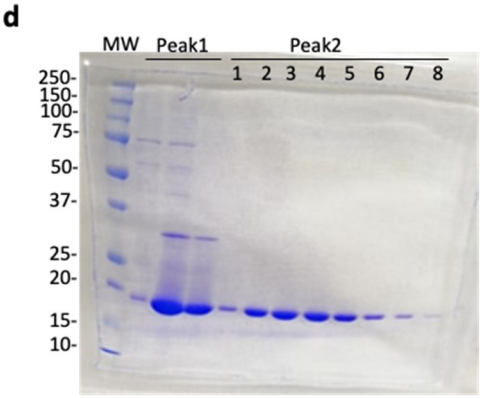

e
MW
200-
150-
100-
75-
50-
37-
25-
20-
15-
10-

Figure S7. Construction and purification of overexpressed ΔN (60-167). (**a**) Schematic illustration showing the 6His-tagged ΔN construct in pET15b vector which was transformed into BL21(DE3) E. coli and purified by Ni-NTA and Superdex-200 columns. (**b**) FPLC chromatogram of ΔN purification by Ni-NTA column. Loaded lysate is a flow through without binding. (**c**) Coomassie brilliant blue R-250 stained SDS-PAGE of the eluted fractions of ΔN (~13.3 kDa) from Ni-NTA column. Fractions 1-14 containing mixture of monomer and dimer were used for Superdex-200 purification. Loaded lysate is a flow through without binding. (**d**) Full image of SDS-PAGE (stained with Coomassie brilliant blue R-250) of the eluted fractions of ΔN protein eluted from Superdex-200 column. (**e**) Full image of SDS-PAGE showing the final product of ΔN highlighted by the dotted square. The other bands are for another studies.


a
2148
2300
6His
ΔATP
BL21(DE3) e. coli
Ni-NTA column
Superdex-200 column


b
Ni-NTA column
mAU
1200
1000
800
600
400
200
0
Loaded lysate
Peak1
Peak2
0
20
40
60
80
100
120
140
ml


c
MW
Flow through
Pellet
Diluted pellet
Fractions
1 2 3 4 5 6 7 8 9 10 11 12
250-
150-
100-
75-
50-
37-
25-
20-
15-
10-


d
MW
Peak1
Peak2
1 2 3 4 5 6 7 8
250-
150-
100-
75-
50-
37-
25-
20-
15-
10-

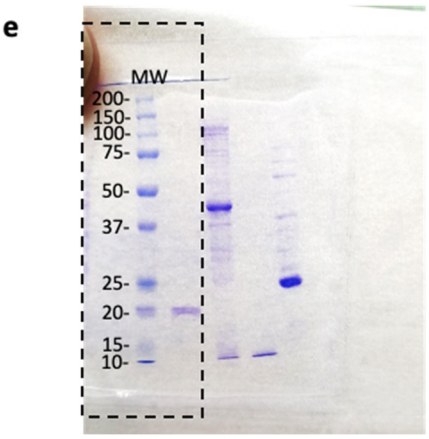

e
MW
200-
150-
100-
75-
50-
37-
25-
20-
15-
10-

Figure S8. Construction and purification of overexpressed ΔATP (2148-2300). (**a**) Schematic illustration showing the 6His-tagged ΔATP construct in pET15b vector which was transformed into BL21(DE3) E. coli and purified by Ni-NTA and Superdex-200 columns. (**b**) FPLC chromatogram of ΔATP purification by Ni-NTA column. Loaded lysate is a flow through without binding. (**c**) Full image of SDS-PGE (stained with Coomassie brilliant blue R-250) of the fractions of ΔATP (~20 kDa) eluted from Ni-NTA column. The contaminating bands of fractions 1-3 were due to the nonspecific binding on Ni-NTA column. Fractions 4-12 contain mixture of monomer and dimer were used for Superdex-200 purification. (**d**) Full image of SDS-PAGE (stained with Coomassie brilliant blue R-250) of the eluted fractions of ΔATP protein from Superdex-200 column. Peak1 fractions were for protein oligomers while peak2 fraction were for ΔATP monomer. (**e**) Full image of SDS-PAGE showing the final product of ΔATP highlighted by the dotted square. The other bands are for another studies.

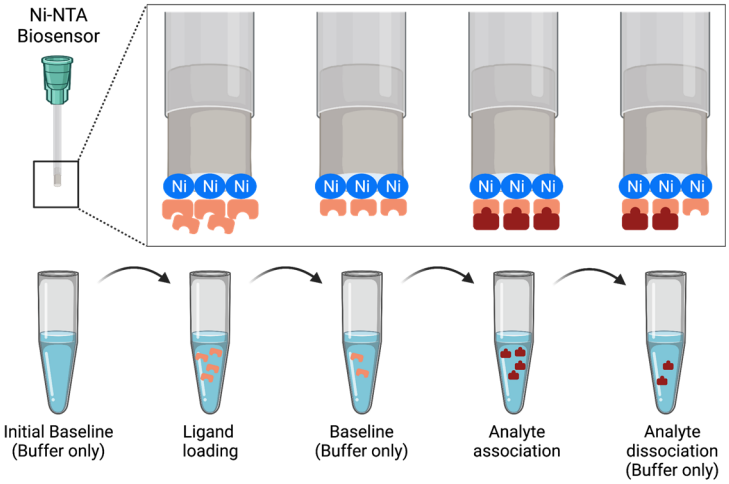


Figure S9. Schematic illustration showing the BLItz measurement steps of protein-protein interactions. The experiments were performed with shaking at 2000 rpm at 25 °C. The illustration was created by BioRender (https://app.biorender.com/).