

SUPPLEMENTARY INFORMATION

N4BP1 uses tandem KH domains to associate with EDC4 and mRNA decapping factors in P-bodies

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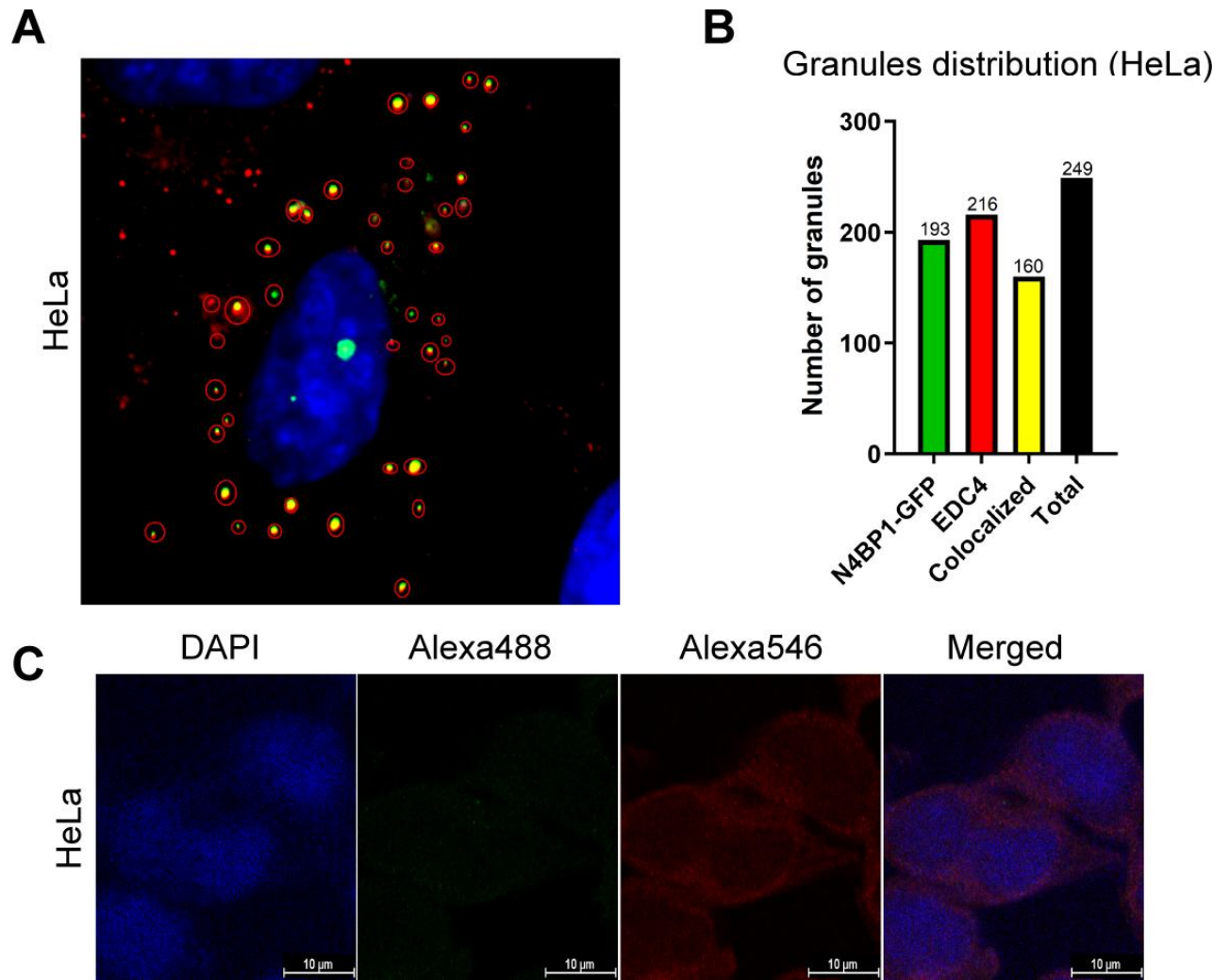


Figure S1. N4BP1-GFP, EDC4 and colocalizing granules. **A.** Circled granules that were counted on each picture. **B.** Granule distribution counted from N4BP1-GFP expressing HeLa cells, sum from 5 pictures for each separate replicate, n=3. **C.** Confocal images of secondary antibodies only control in HeLa cells.

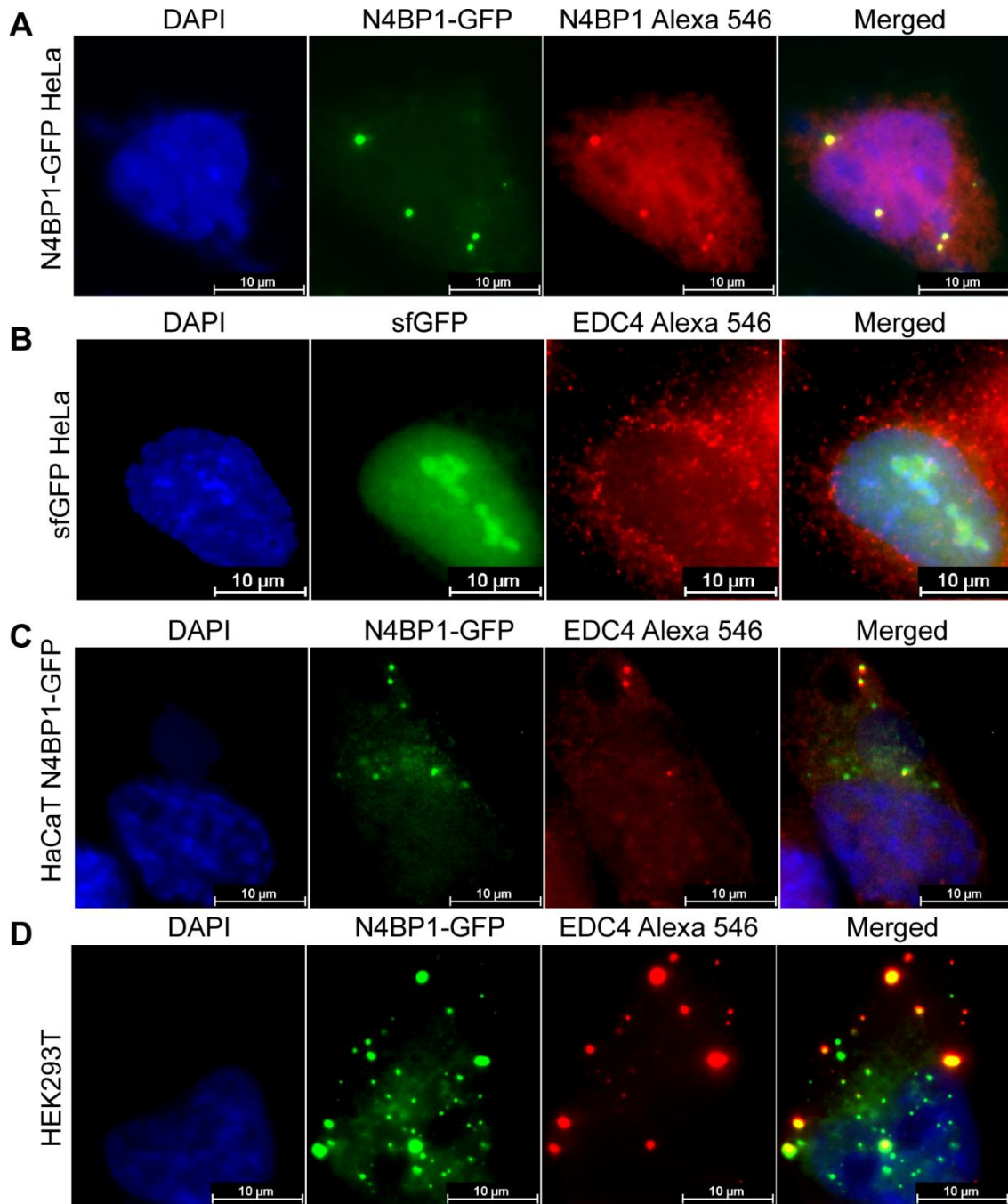


Figure S2. Control of immunofluorescent staining. **A.** Immunofluorescent staining of N4BP1 with anti-N4BP1 Ab in HeLa cells transfected with N4BP1-GFP pcDNA3.0. **B.** Immunofluorescent staining of EDC4 in HeLa cells transfected with GFP pcDNA3.0. GFP alone does not colocalize with EDC4. **C.** Immunofluorescent staining of EDC4 in HaCaT cells transfected with N4BP1-GFP pcDNA3.0. **D.** Immunofluorescent staining of EDC4 in N4BP1-GFP expressing HEK293T cells.

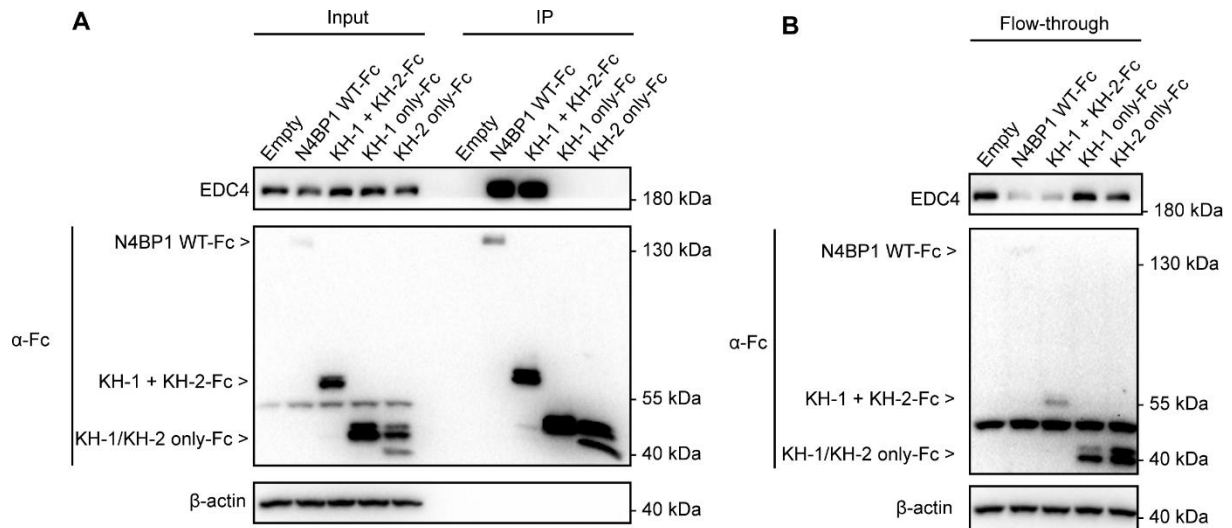


Figure S3. Analysis of the impact of the KH domains of N4BP1 on its association with the decapping complex, represented by EDC4. A. Pull-down assay of full length N4BP1 and its N-terminal fragments (tandem KH domains, KH1 and KH2), followed by Western-blot analysis. **B.** Western blot analysis of flow-through fractions from the pull-down assay of N4BP1s fragments. Presented results are representative of 3 biological replicates.

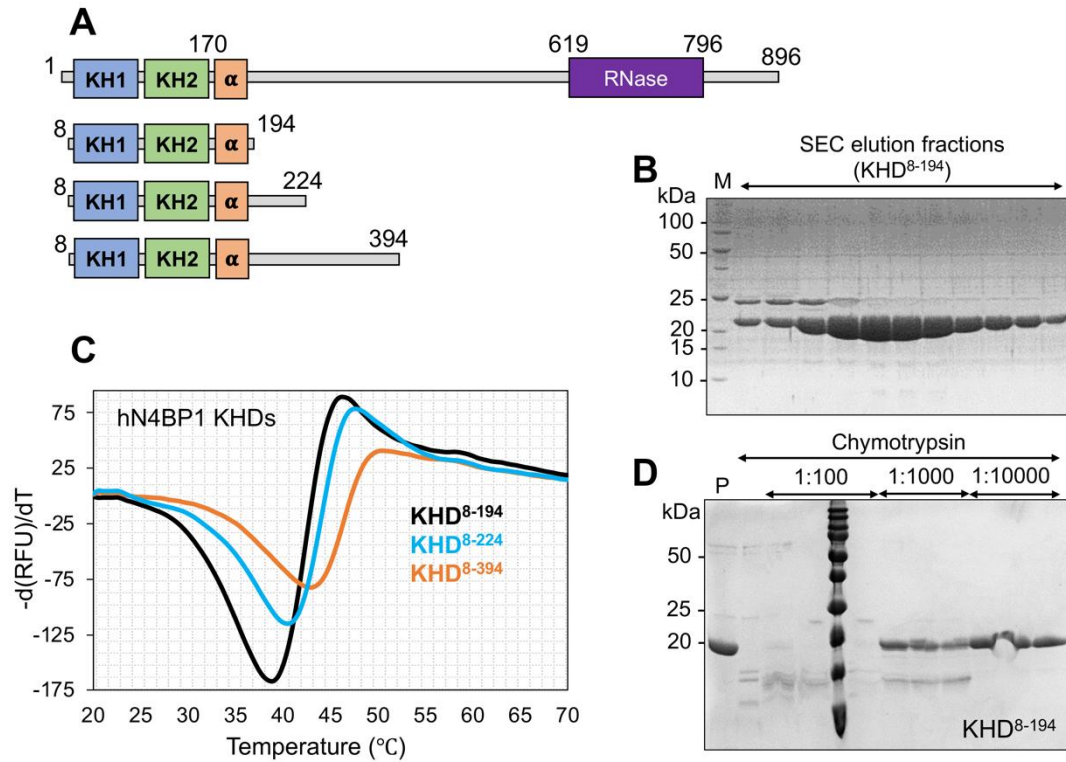


Figure S4. Purification and biophysical characterization of N4BP1 KH domains. **A.** Domain architecture of the human N4BP1 and different N4BP1 constructs harboring KHDs used in the study. **B.** SDS-PAGE showing size exclusion chromatography (SEC) fractions for N4BP1 KHD⁸⁻¹⁹⁴ protein. Only the fractions containing pure KHD⁸⁻¹⁹⁴ protein were further used. **C.** Thermal-melting transitions for different KHD proteins. **D.** Limited proteolysis of KHD⁸⁻¹⁹⁴ with chymotrypsin. KHD⁸⁻¹⁹⁴ shows minimal degradation at 1:10,000 w/w ratio and was used for crystallization.

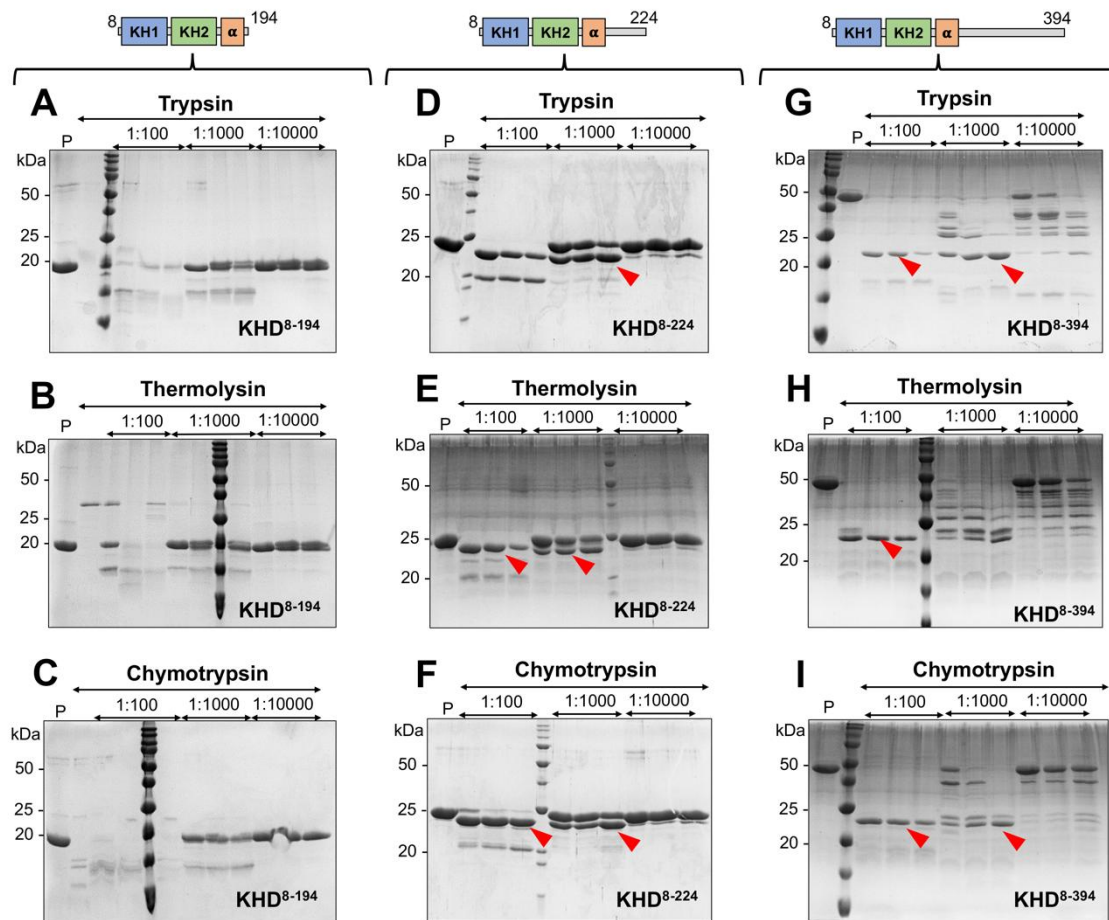


Figure S5. Limited proteolysis of N4BP1 KHDs. Limited proteolysis of KHD⁸⁻¹⁹⁴ with **A.** trypsin, **B.** thermolysin and **C.** chymotrypsin. Limited proteolysis of KHD⁸⁻²²⁴ with **D.** trypsin, **E.** thermolysin and **F.** chymotrypsin. Limited proteolysis of KHD⁸⁻³⁹⁴ with **G.** trypsin, **H.** thermolysin and **I.** chymotrypsin. KHD⁸⁻¹⁹⁴ shows only slight degradation with the proteases. The stable products formed after proteolysis of KHD⁸⁻²²⁴ and KHD⁸⁻³⁹⁴ are marked with red arrowheads.

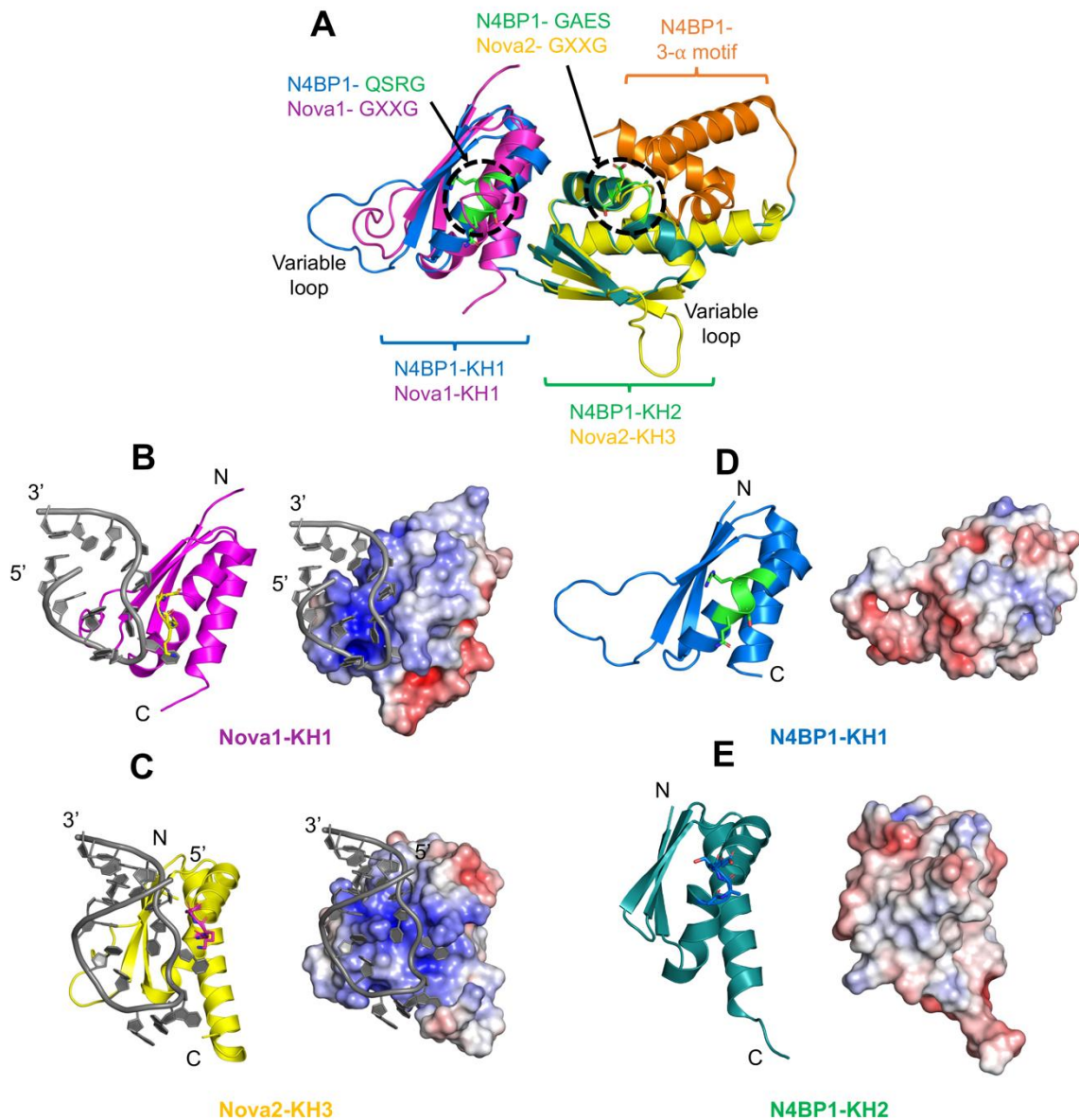


Figure S6. N4BP1 exhibits non-canonical features in its KH domains. **A.** Structural superposition of N4BP1 KHD⁸⁻¹⁹⁴ with the Nova1 KH1 (PDBid 2ANN, magenta) and Nova2 KH3 (PDBid 1EC6, yellow) structures. The signature invariable GXXG motif of KH domains is mutated to QSRG (shown as sticks in green) in N4BP1 KH1 and to GAES (shown as sticks in green) in KH2. Both sites are highlighted with black dotted ellipses. Cartoon and electrostatic surface representation of **B.** the Nova1 KH1-RNA structure (PDBid 2ANN) and **C.** the Nova2 KH3-RNA structure (PDBid 1EC6). The GXXG motif (shown as sticks) and the positively charged grooves on Nova1/2 KHDs support the RNA interaction. Cartoon and electrostatic surface view of the N4BP1 KH1 **D.** and KH2 **E.** domains. The N4BP1 KHDs lack a positive groove for RNA binding and their GXXG motifs (shown as sticks) are mutated. The electrostatic surface potential is displayed in a range from -5 (red) to $+5$ kT/e (blue).

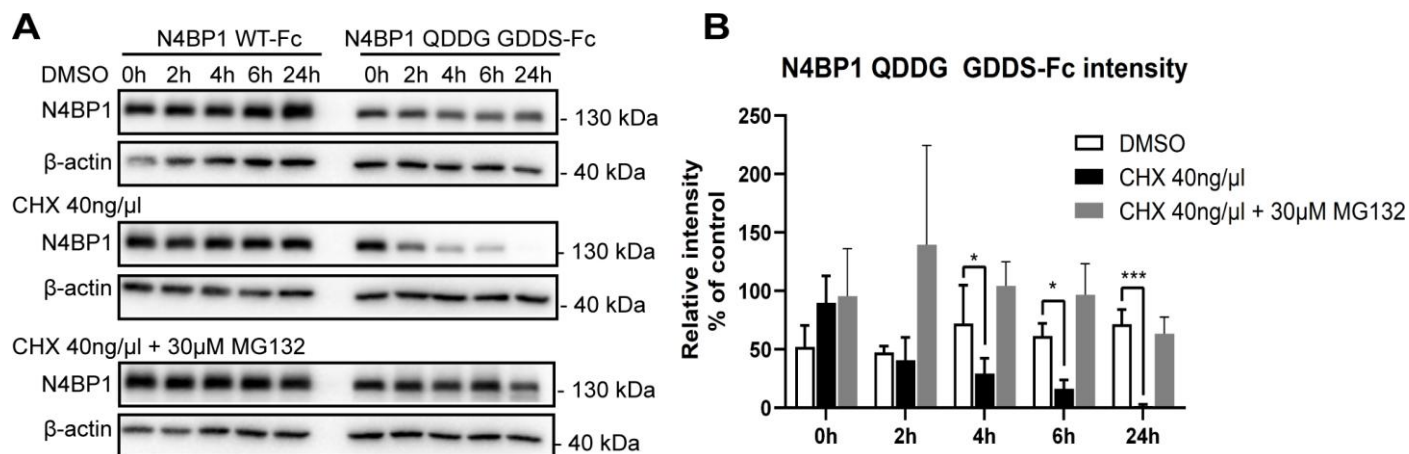


Figure S7. QDDG GDDS N4BP1 mutant undergoes proteasomal degradation. **A.** Western blot of WT and QDDG GDDS N4BP1-Fc mutant after stimulation with cycloheximide (CHX, 40 ng/μl) and cycloheximide with 30 μM MG132. DMSO was used as a control. **B.** Densitometric analysis of Western Blot shown of Fig. S6A. Values presented are mean ± SD counted as % of control (N4BP1 WT-Fc), relative to β-actin. Two way ANOVA was used for statistical analysis. Data were collected from 3 independent experiments. * $p < 0,05$, *** $p < 0,001$.

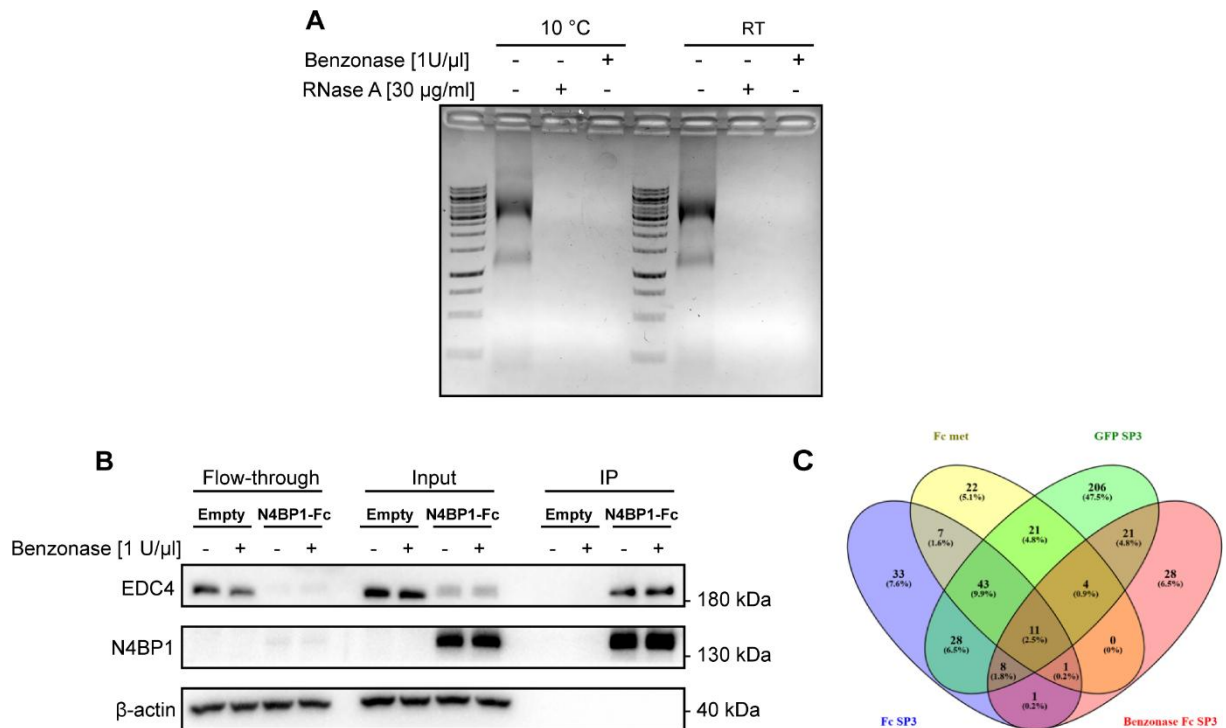


Figure S8. A. Electrophoresis of 2 μg RNA samples untreated or treated with RNase A or benzonase at 10 °C or room temperature. All samples were supplemented with 2 mM MgCl₂ in order to ensure optimal conditions for benzonase activity. **B.** Western blot analysis of samples subjected to immunoprecipitation following benzonase treatment for 2h at 4 °C. **C.** Venn diagram illustrating number of proteins identified by mass spectrometry under all tested conditions including benzonase treated samples.

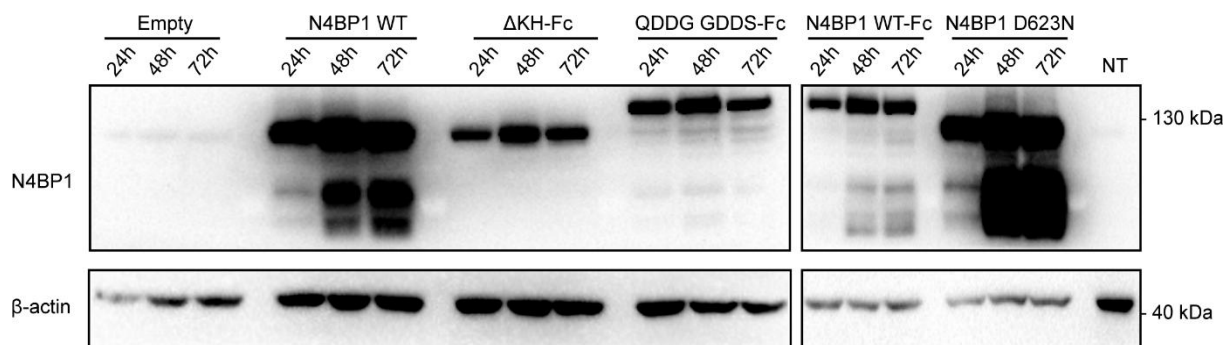


Figure S9. Western blot analysis at 48 hours post-transfection confirming robust expression of wild-type (WT) N4BP1 as well as the D623N, ΔKH-Fc, QDDG GDDS-Fc mutants, along with Fc-tagged WT N4BP1 in HEK293T cells.

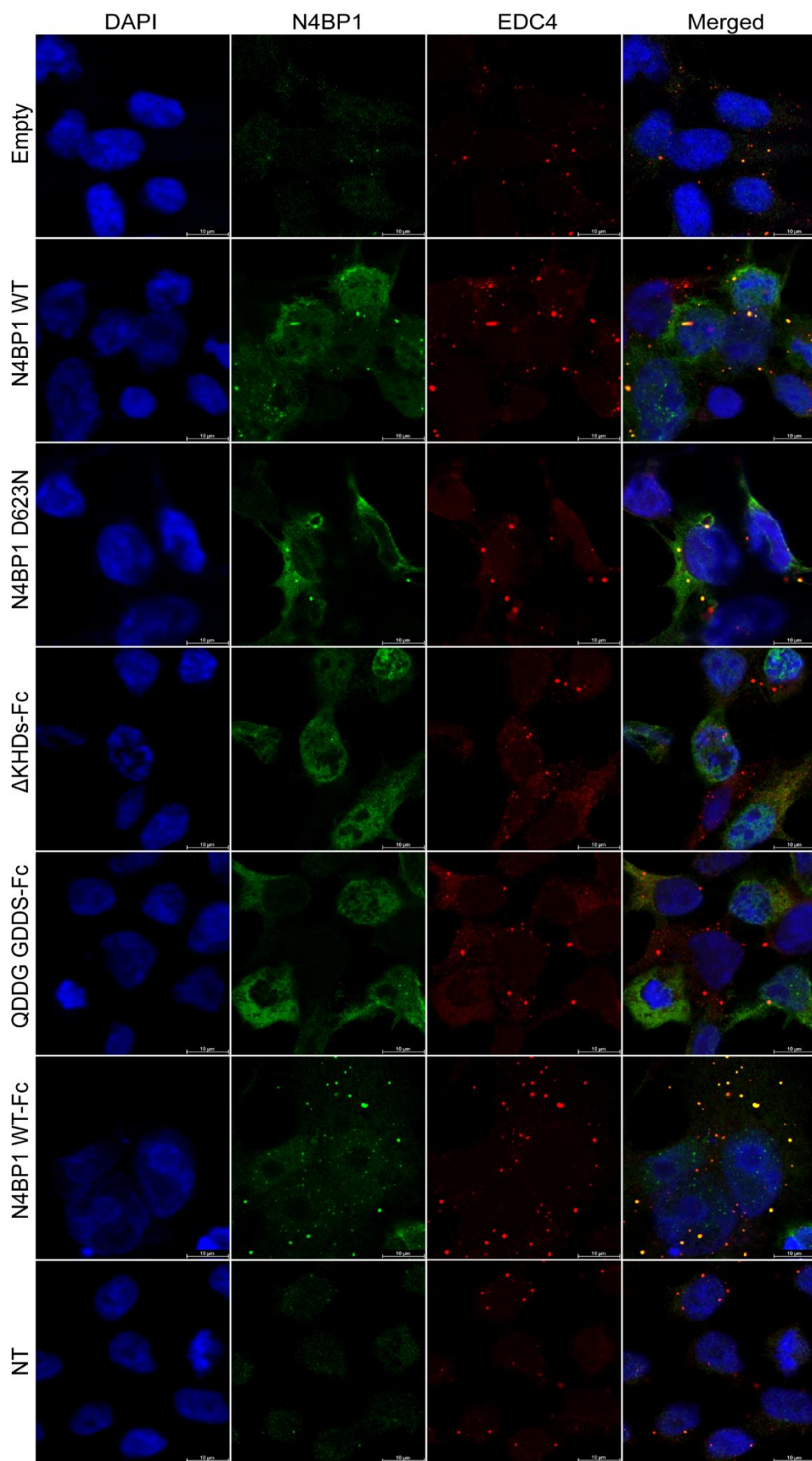


Figure S10. Immunofluorescent staining of N4BP1 and EDC4 in HEK293T cells after co-transfection with empty vector or N4BP1 expression vector and pspax2. Cells were fixed 48 hours post-transfection. In the case of empty and non-transfected (NT) samples endogenous N4BP1 can be observed.

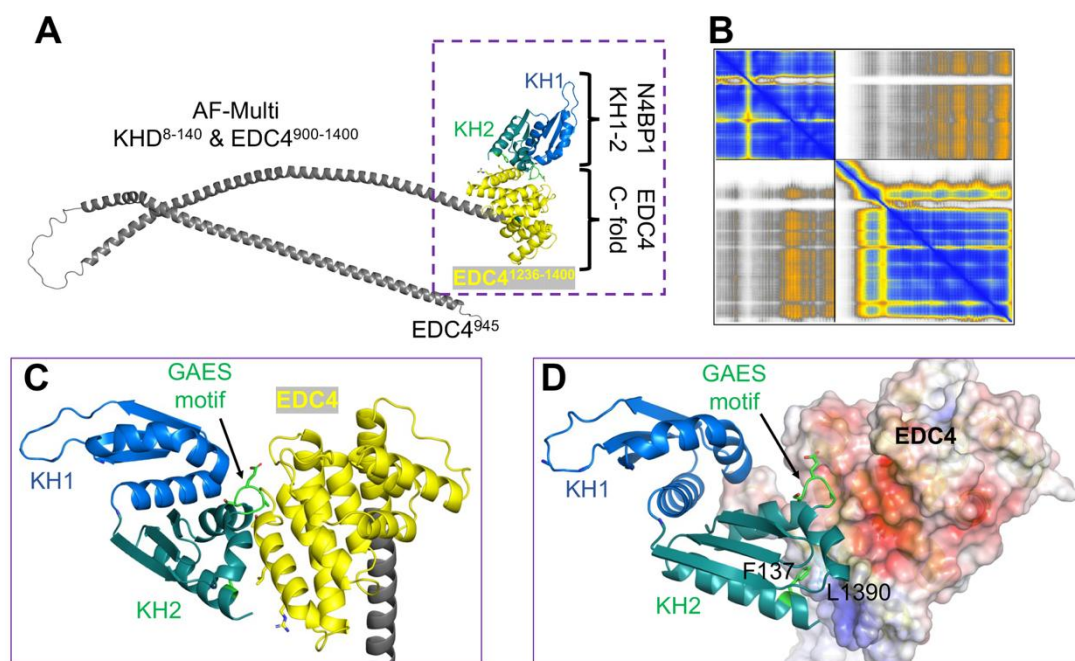


Figure S11. Model of the N4BP1-EDC4 complex generated using AlphaFold-Multi illustrates interaction between tandem KHDs of N4BP1 and the C-terminal α -helical fold of EDC4. **A.** Cartoon representation of modeled structure of EDC4 (depicted as gray and yellow) shows interaction with tandem KHDs of N4BP1 (depicted as blue and teal). **B.** The PAE plot for the predicted structure model. **C.** The cartoon representation of the interface between the N4BP1 KHD and the EDC4 C-terminal fold (C-fold). The KH2 GAES motif (shown in green sticks) seems to directly interact with the EDC4 C-fold. **D.** N4BP1 KH2 GAES motif docks onto the surface of EDC4 C-fold. The KHD is shown in cartoon and the C-fold is shown as electrostatic surface with potential range from -5 (red) to +5 kT/e (blue).

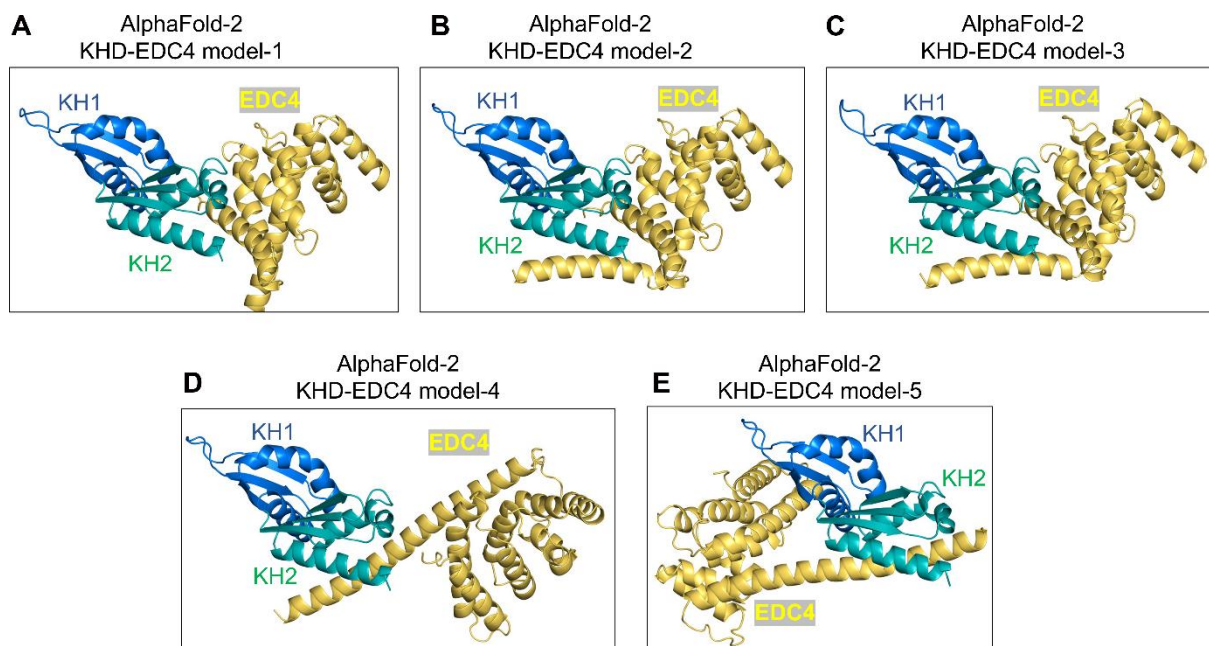
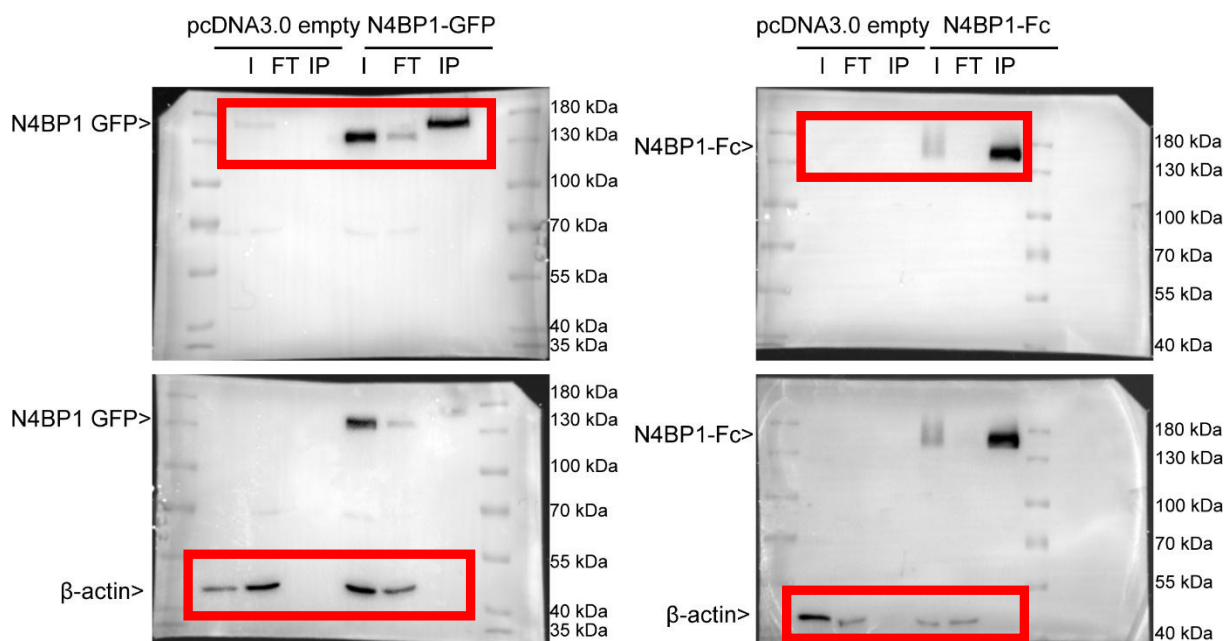
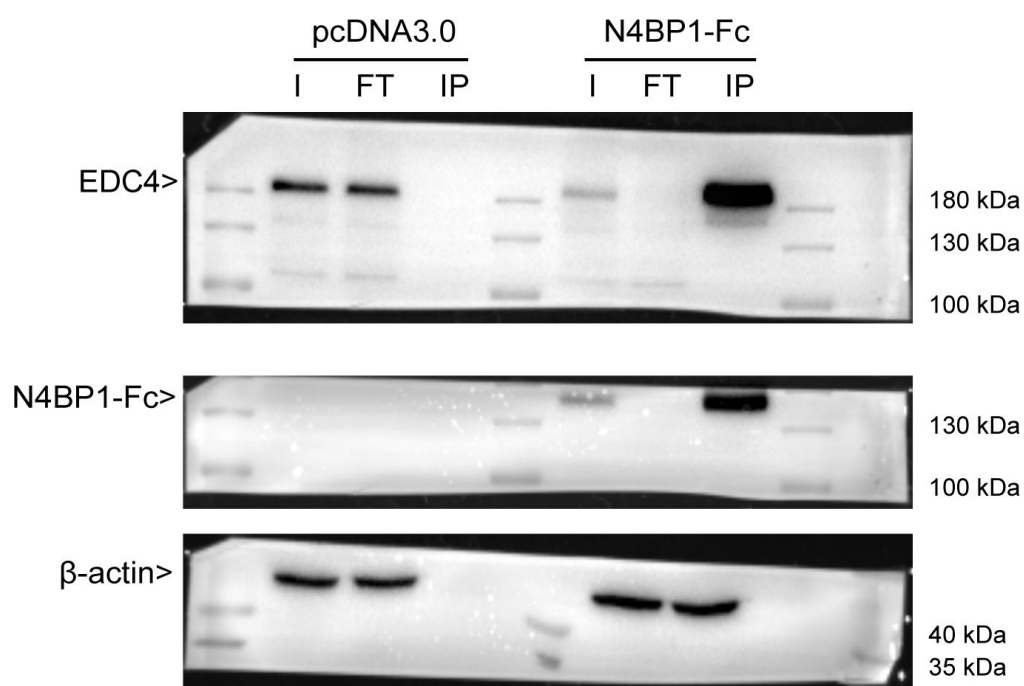


Figure S12. Cartoon representation of all 5 structure models for N4BP1 KHD and EDC4 C-fold as predicted by AlphaFold 2.

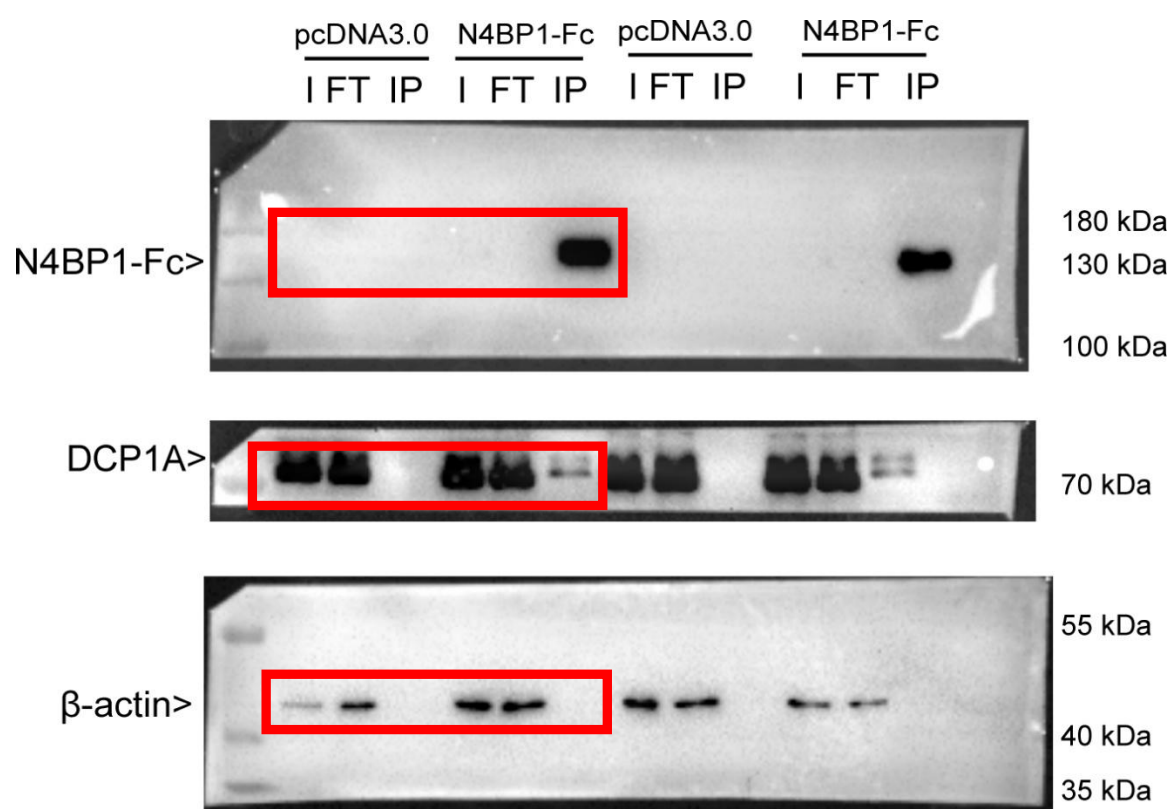
Raw blot images (merged with MW marker) for Fig. 1A, 1B



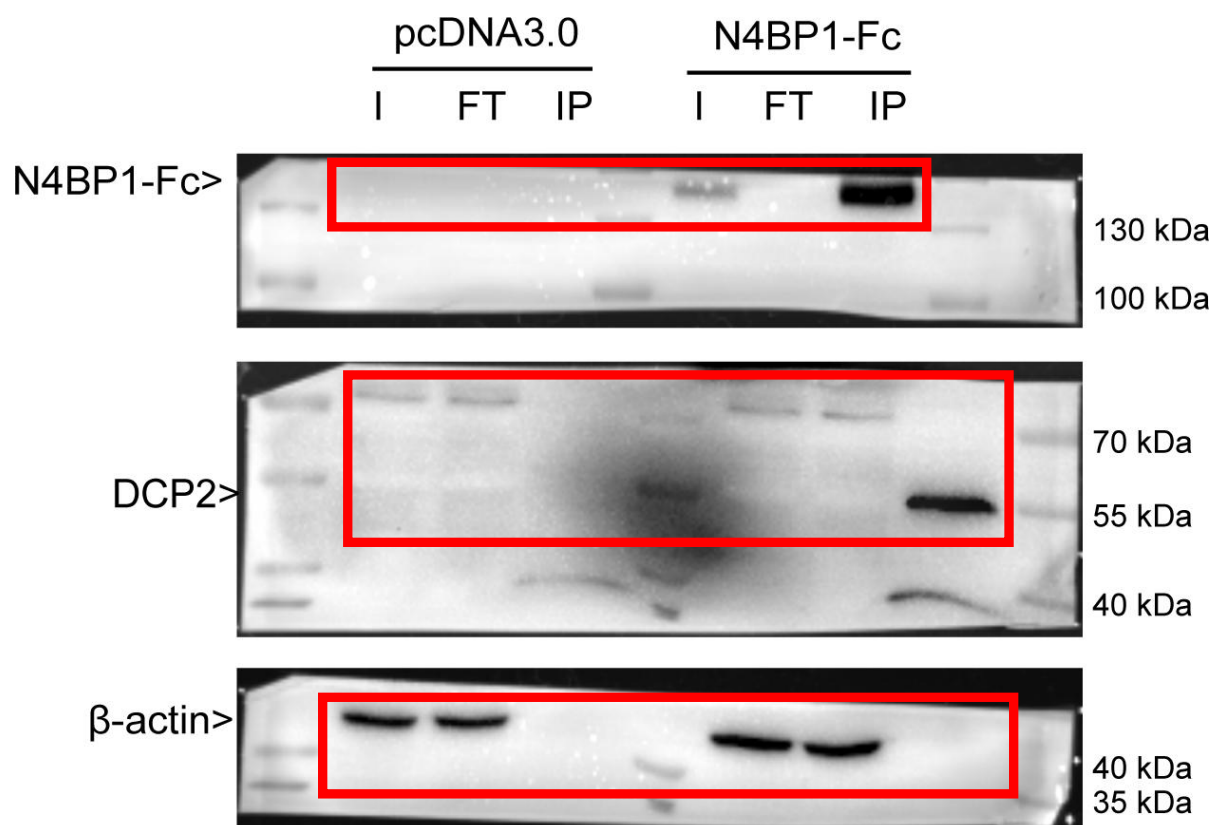
Raw blot images (merged with MW marker) for **Fig. 2F**



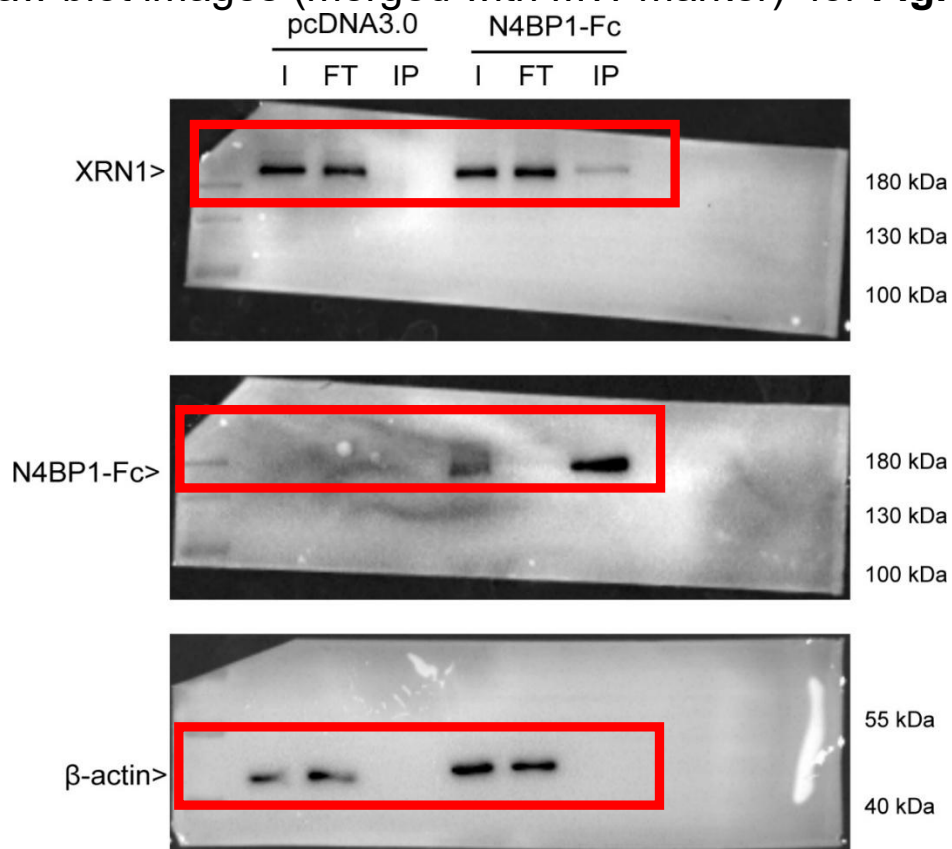
Raw blot images (merged with MW marker) for **Fig. 2G**



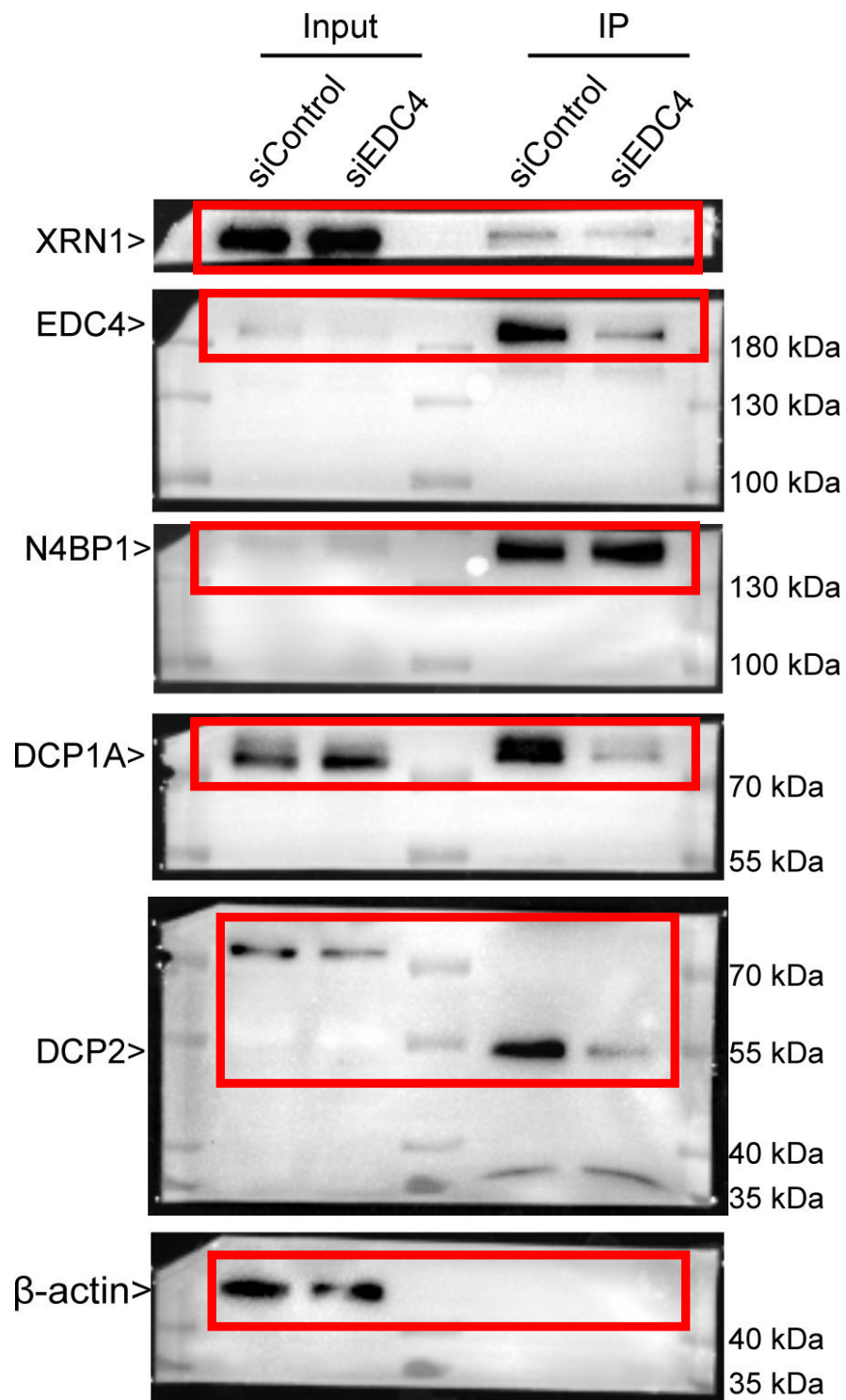
Raw blot images (merged with MW marker) for **Fig. 2H**



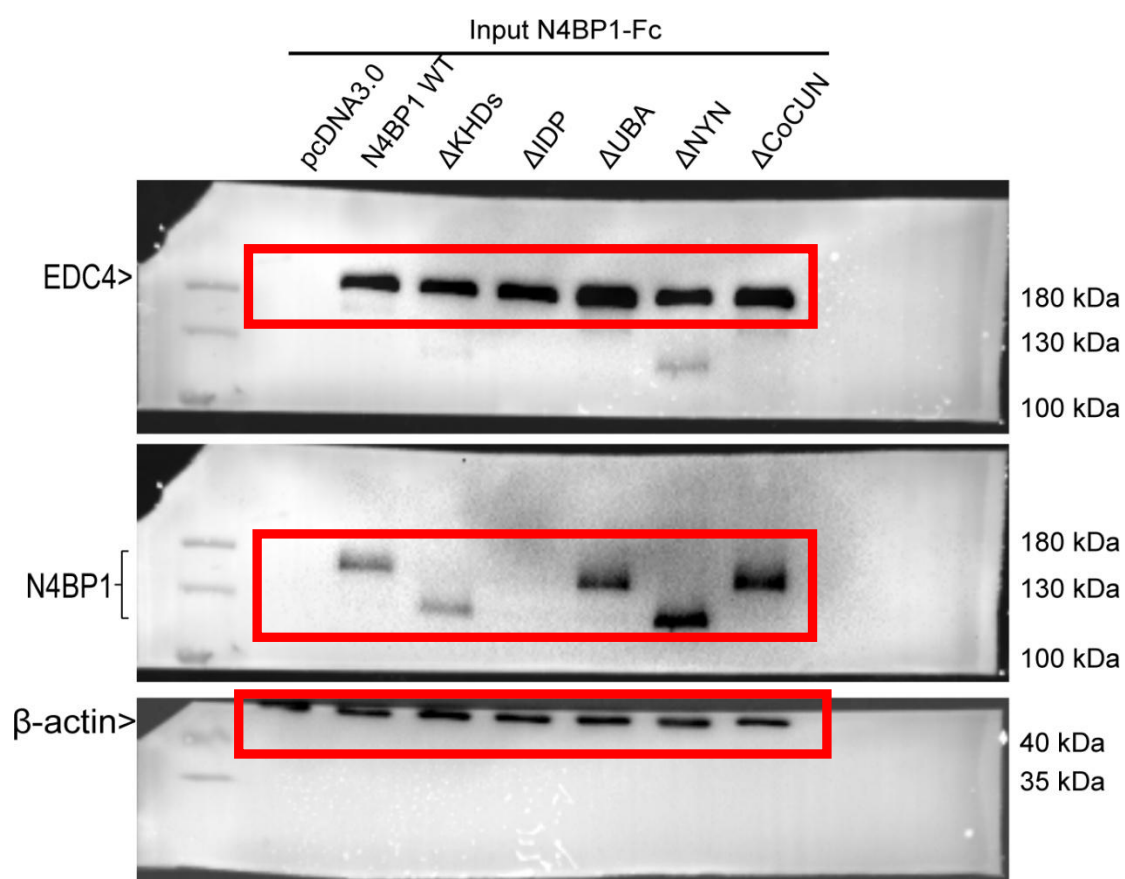
Raw blot images (merged with MW marker) for **Fig. 2I**



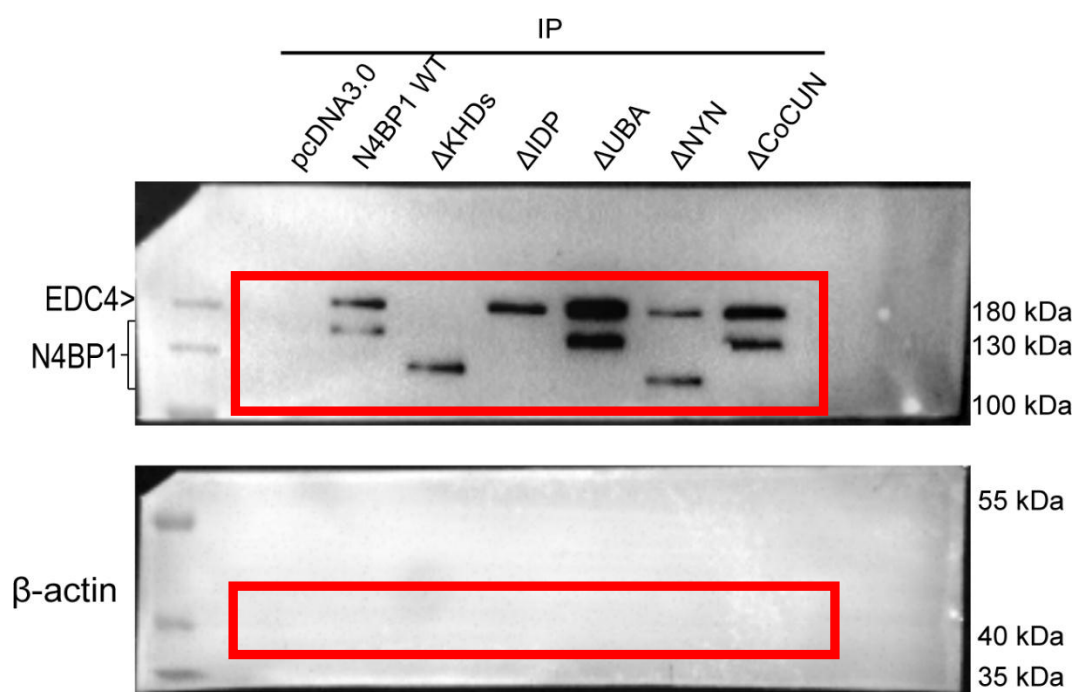
Raw blot images (merged with MW marker) for **Fig. 2K**



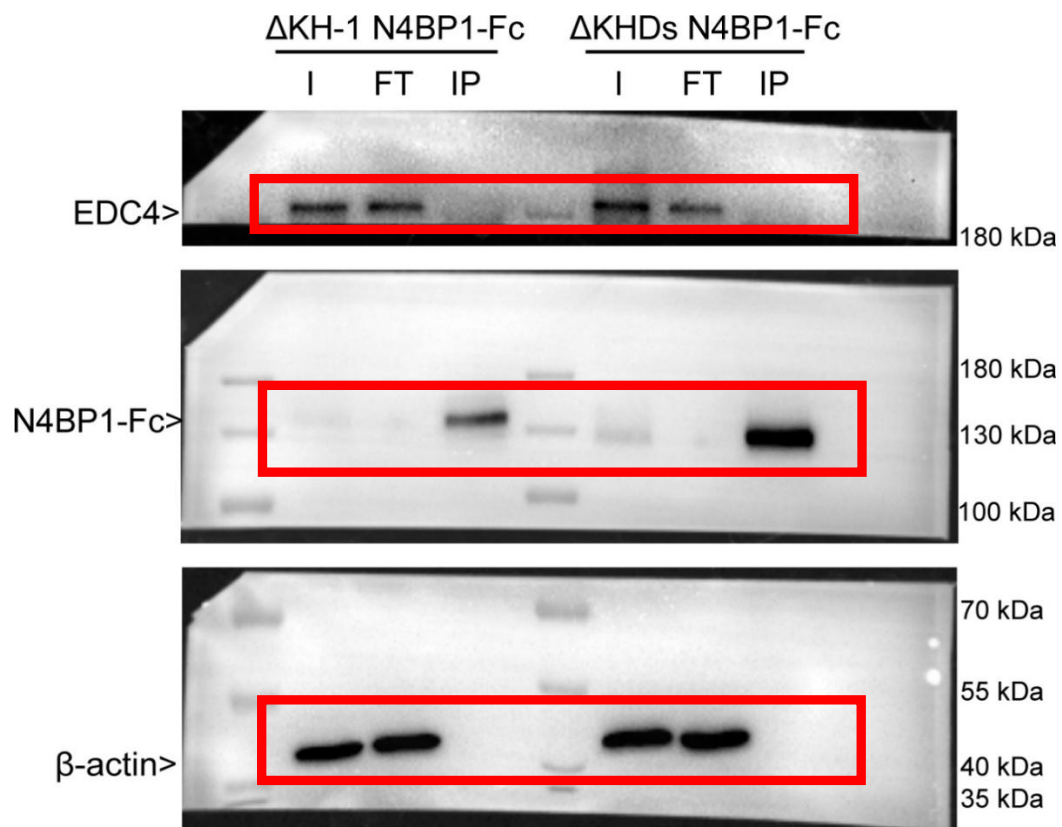
Raw blot images (merged with MW marker) for **Fig. 3B**



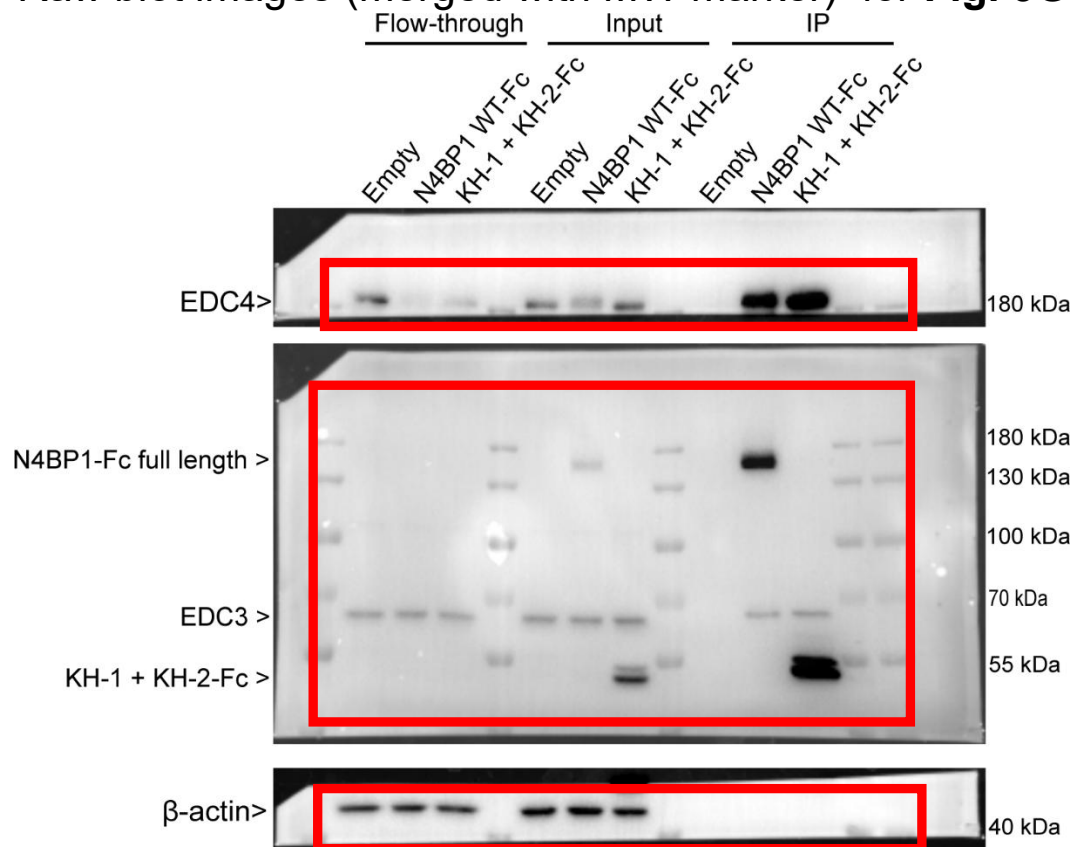
Raw blot images (merged with MW marker) for **Fig. 3C**



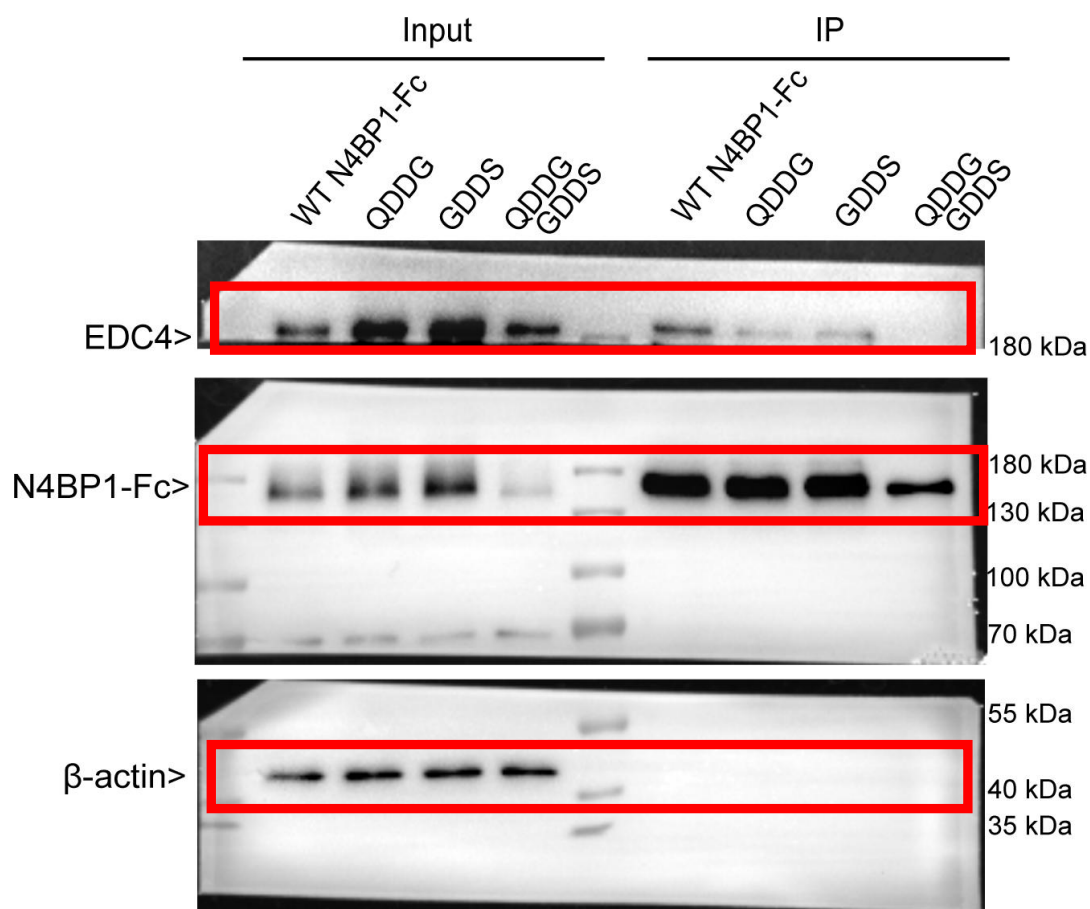
Raw blot images (merged with MW marker) for **Fig. 3D**



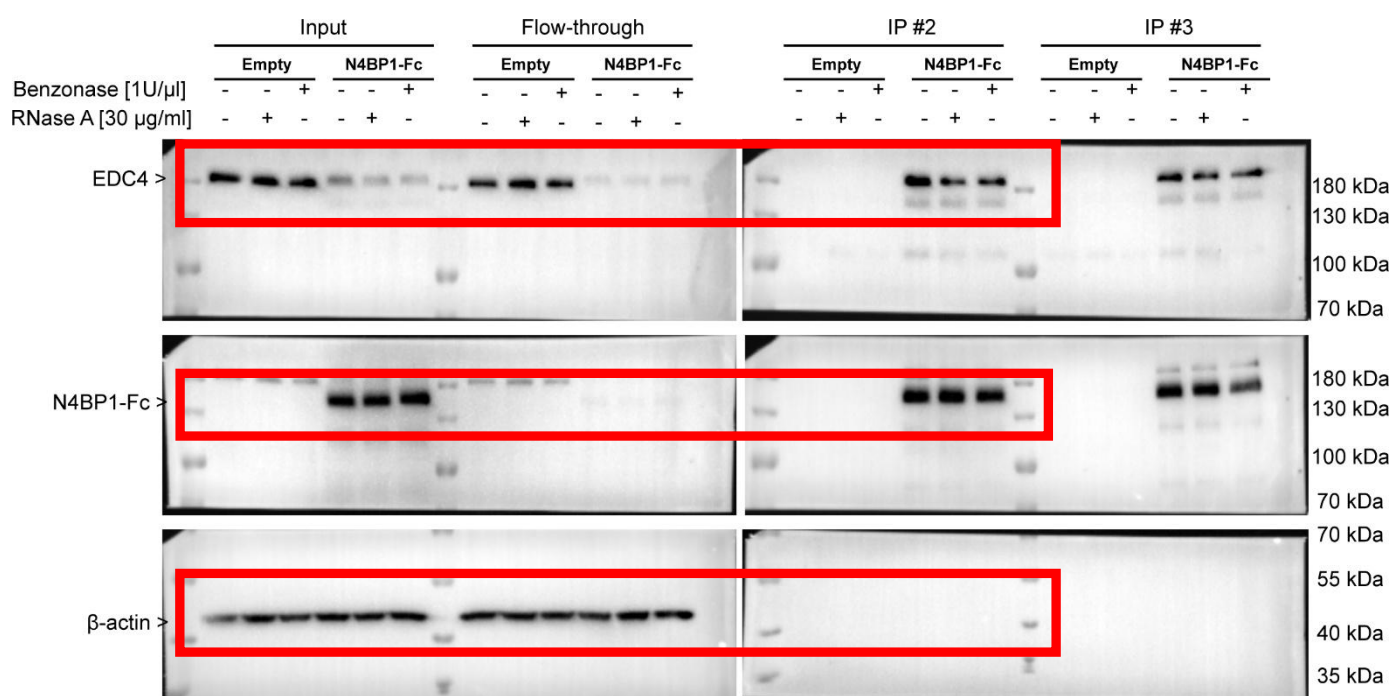
Raw blot images (merged with MW marker) for **Fig. 3G**



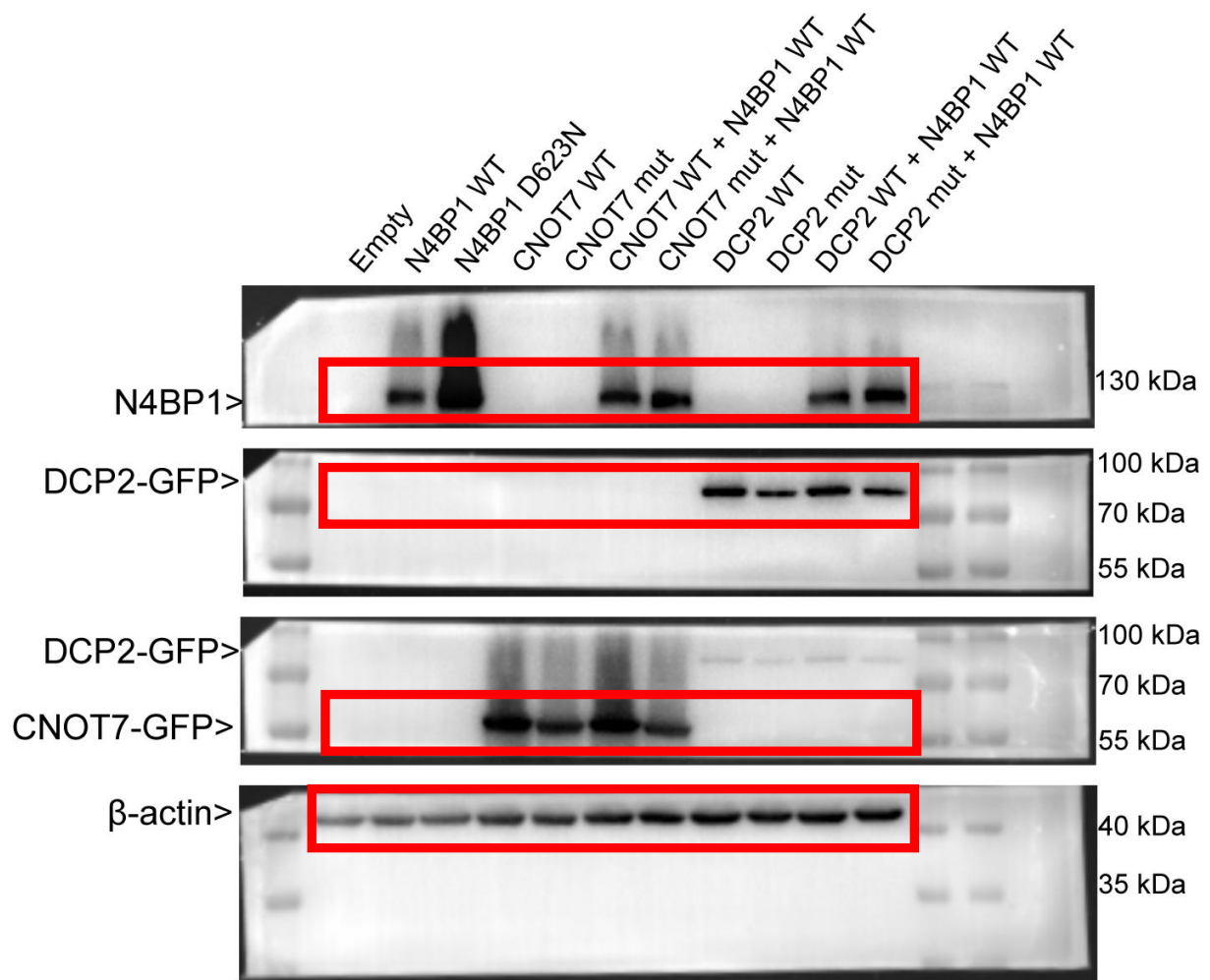
Raw blot images (merged with MW marker) for **Fig. 4D**



Raw blot images (merged with MW marker) for **Fig. 5A**



Raw blot images (merged with MW marker) for **Fig. 6D**



Raw blot images (merged with MW marker) for Fig. S8B

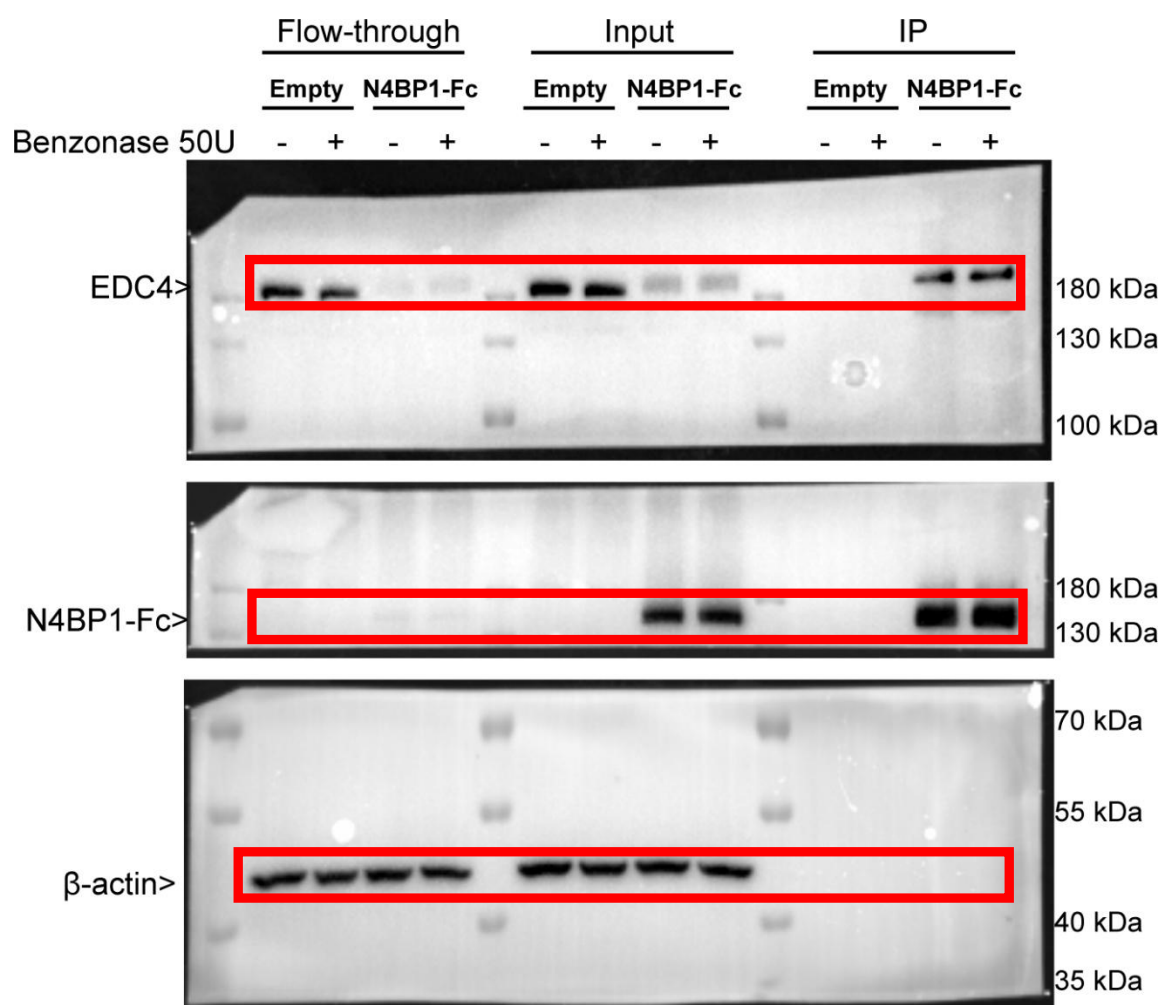


Figure S13. Raw, uncut Western blot membranes of the blots shown in the main manuscript. The corresponding main manuscript figure number for each membrane is indicated above the respective membrane in this figure. Red fields mark the parts included in the main manuscript figures.

Supplementary Table 1. Sequences of primers used to amplify mutations of N4BP1 in PCR and Q-RT-PCR targets and target sequences of used siRNAs.

Primer name:	Primer sequence 5'-3'
ΔKH-1 1-57 Forward	GCGCAGGAGGCGGTGCACAGC
ΔKH-1 1-57 Reverse	CATGGCGGGCGCGGCCTC
ΔKHDs 1-191 Forward	GGTGAGGAGAATCTCTTTGAAACAGG
ΔKHDs 1-191 Reverse	CATGGCGGGCGCGG
ΔIDP 192-612 Forward	GGGAGAACGGATTTGAAACACATTGTTATAGATGG
ΔIDP 192-612 Reverse	TTGTGTGAGTGTCAAAAGTTCTTTTTTCAAGG

ΔUBA 344-392 Forward	GAAGACAGAGAATTTTCAGCTG
ΔUBA 344-392 Reverse	CTCAGTAGTTTCTTTTATATCTGGAC
ΔNYN 616-896 Forward	GGATCCGACAAAACACACATGC
ΔNYN 616-896 Reverse	ATCCGTTCTCCCTGGTTCA
ΔCoCUN 849-896 Forward	GGATCCGACAAAACACACATGC
ΔCoCUN 849-896 Reverse	CTGAGCTGGCATGGGCAGG
QDDG Forward	GACGACGGCCGTATCGAGGGCCTG
QDDG Reverse	CTGCTCCAGCAGCTCCG
GDDS Forward	GACGACAGCCTGTTTCTGAAAAGCTTG
GDDS Reverse	CCCAACAAAAATGCAGTGCATG
N4BP1 D623N Forward	TTGTTATAAATGGGAGCAATGTTGC
N4BP1 D623N Reverse	TGTGTTTCAAATCCGTTCT
HIV-1 <i>pol</i> Forward	ACATGGGAAGCATGGTGGACA
HIV-1 <i>pol</i> Reverse	TTTCCCTATTGGCTGCCCA
HIV-1 <i>Tat/rev</i> Forward	ATGGCAGGAAGAAGCGGAG
HIV-1 <i>Tat/rev</i> Reverse	ATTCCTTCGGGCCTGTCG
28s <i>rRNA</i> Forward	CACCCACTAATAGGGAACGTG
28s <i>rRNA</i> Reverse	CTGACTTAGAGGCGTTCAGTC
<i>N4BP1</i> Forward	CAGTGGAGAACAAGGCGTGA
<i>N4BP1</i> Reverse	GCCACCAGTTTTGTCCGCTA
<i>FREM2</i> Forward	TGGACAAAGGCAACCACAGC
<i>FREM2</i> Reverse	CGTTAACTTGTTTCCACCACCATCT
<i>NOTCH3</i> Forward	TGGCAGTAGAGGGCATGGTG
<i>NOTCH3</i> Reverse	GGGGGTCTCCTCCTTGCTAT
<i>ETV1</i> Forward	TACATGGTCACCAATAGTCAGCG
<i>ETV1</i> Reverse	CTTCTGCAAGCCATGTTTCCTG
KH-1/2only Forward	GGAGGAGGATCCGACAAAACACACATGC
ΔKH-1 1-76 Forward	CTAGAAGAAAGAGAATGTTACCCCAAGG
KH-1only Reverse	AGGTTACAGATTTCCTTTAATATATTCCTTG
KH-2only Reverse	TTTATTTTCAAAGAGCTTTACAAATTGTTGAATGTGACTCC
siRNA:	siRNA target sequence:
siGENOME Non-targeting	UAGCGACUAAACACAUCAA
siRNA pool D-001206-13-05	UAAGGCUAUGAAGAGAUAC
(siControl)	AUGUAUUGGCCUGUAUUAG
	AUGAACGUGAAUUGCUCAA

siGENOME Human <i>EDC4</i> siRNA SMART pool D- 016635 (siEDC4)	GGACCAUGCCACCCAUUA
	GAUGGAGAUCGGCAUAAUA
	UGACCAGAACCGAGAGUUA
	GCCCACAGCACCUGACAUU