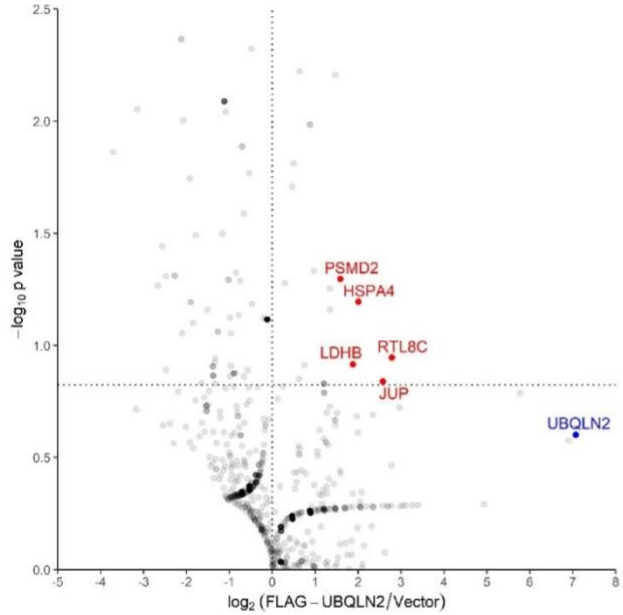
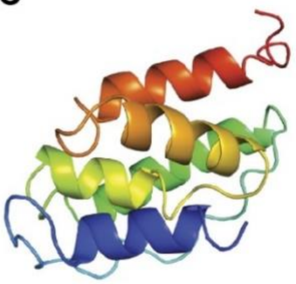
A



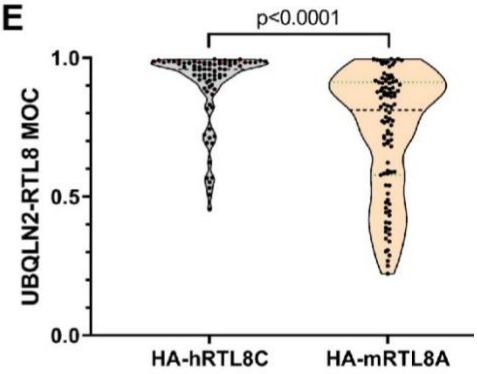
B

% Identity	hRTL8A	hRTL8B	hRTL8C	mRTL8A/B	mRTL8C
hRTL8A	100.00	96.46	93.81	67.26	63.39
hRTL8B	96.46	100.00	92.92	68.14	61.61
hRTL8C	93.81	92.92	100.00	69.03	64.29
mRTL8A/B	67.26	68.14	69.03	100.00	66.96
mRTL8C	63.39	61.61	64.29	66.96	100.00

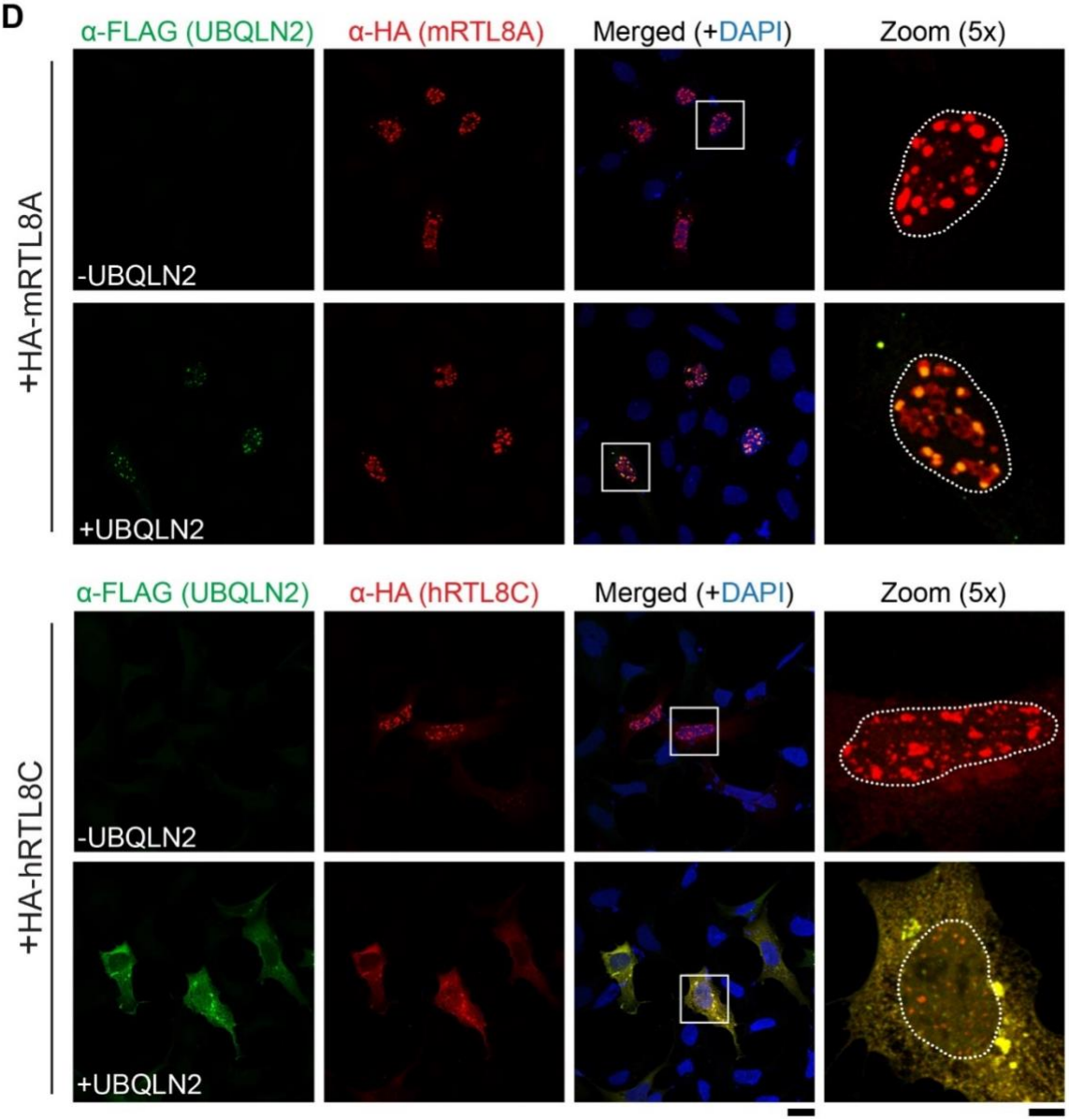
C



E



D



Supplementary Figure 1.

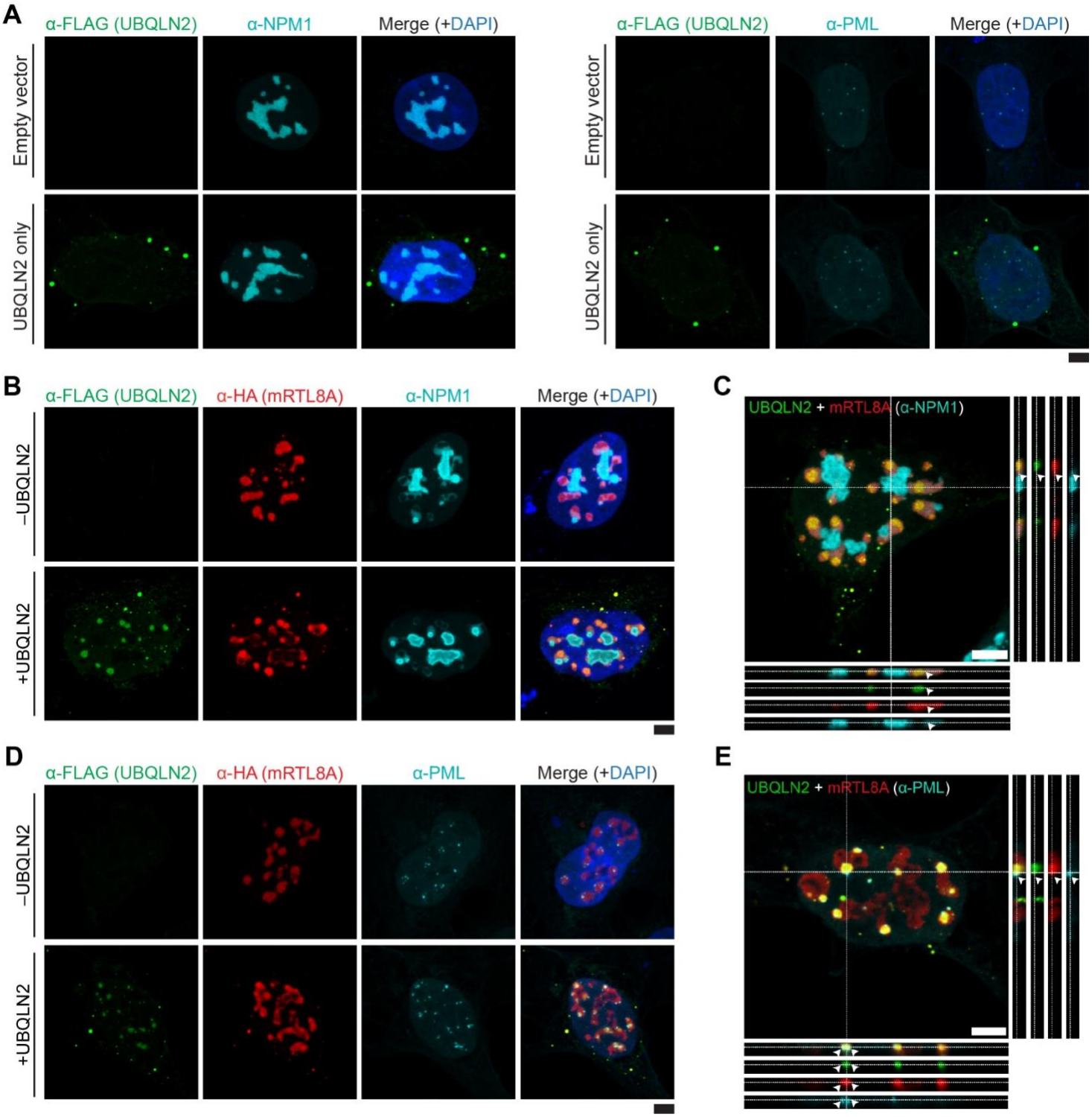
A) Volcano plot depicting enrichment of RTL8C pulled down from an immunoprecipitation-mass spectrometry screen in HEK293 cells identifying novel UBQLN2 interactors. A Student's t-test was used, $-\log_{10}$ p-value versus $\log_2$ fold enrichment values over control plotted on the x-axis. Values with a $-\log_{10}$ p-value greater than 0.8239 (corresponding to a p-value of 0.15) and $\log_2$ fold enrichment greater than 1.5 are shown in red. The p-value threshold was lowered so as not to omit possible interactors since only two replicates were used for plot generation. UBQLN2 is shown in blue.

B) Percentage Identity Matrix showing the RTL8 amino acid sequence percentage similarities for human (hRTL8) and mouse (mRTL8) proteins.

(C) Predicted 3D structure for human RTL8C using C-I-TASSER. hRTL8C is shown in spectrum color, corresponding with blue N-terminus to red C-terminus gradient. The predicted structure consists mainly of alpha-helical regions.

(D) Representative images showing co-localization between UBQLN2 and HA-mRTL8A or HA-hRTL8C. HEK293 cells were transfected with HA-mRTL8A or HA-hRTL8C (both *red*) and empty vector or FLAG-UBQLN2 (*green*). DAPI (*blue*) marks the nucleus. White dotted lines in the 5x magnified images outline the nucleus. (Scale bar for merged images = 20 µm and zoomed images= 5 µm).

(E) Quantification of MOC between UBQLN2 and mRTL8A or hRTL8C. UBQLN2 showed significantly higher overlap with hRTL8C. Outliers as determined by a Grubbs' test were excluded, following which an unpaired tow-tailed Student's t-test was performed to test for statistical significance. N=78 and 98 cells for mRTL8A and hRTL8C, respectively, from 3 biological replicates.



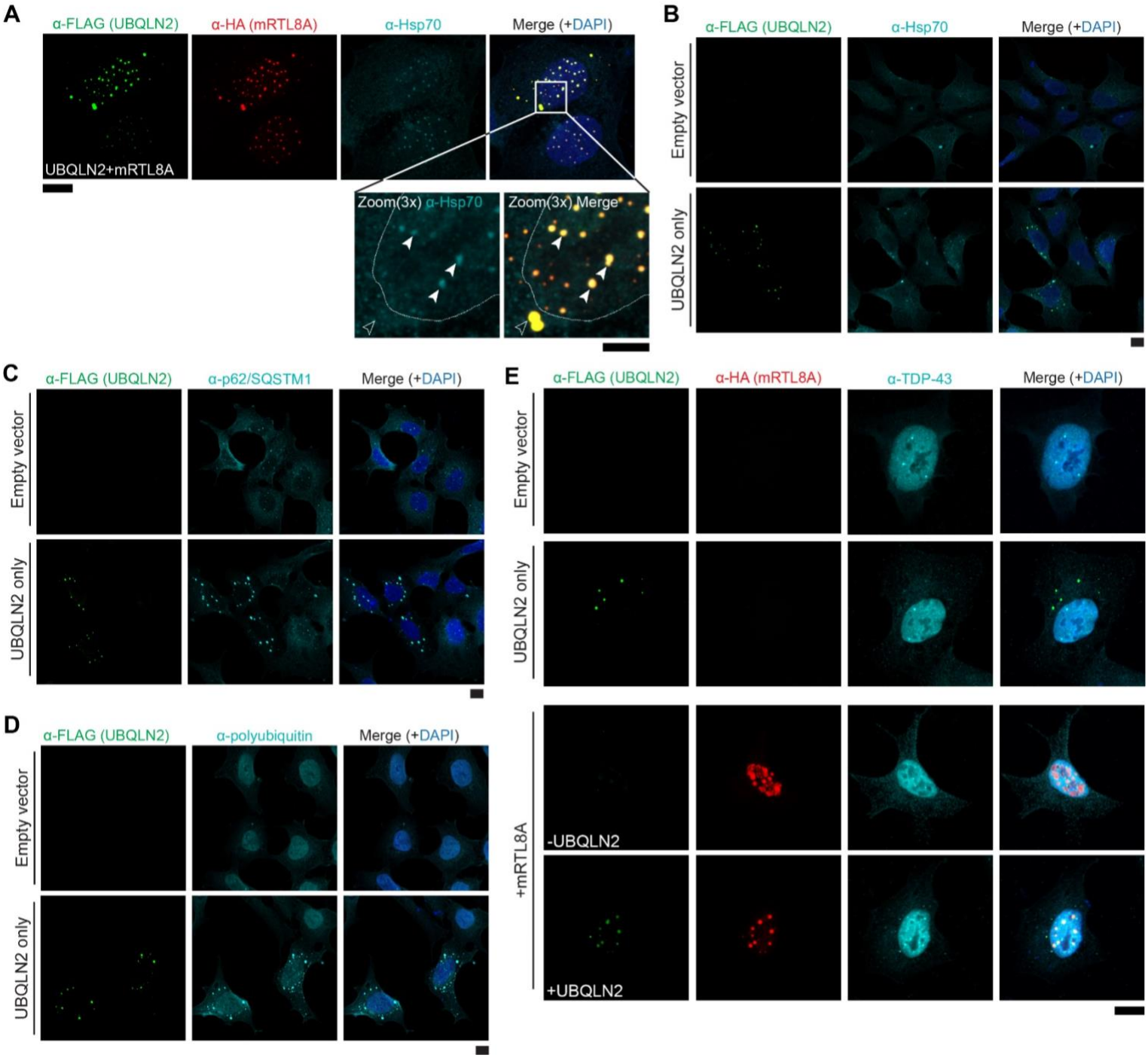
Supplementary Figure 2.

(A) Representative images of HEK293 cells transfected with empty vector or vector encoding FLAG-UBLQN2 (*green*) and stained for NPM1 or PML (*cyan*). (Scale bar = 5 µm).

(B) and **(D)** Representative images in addition to Figures 3C and 3D of co-localization between mRTL8A and nucleoli (**B**) or PML bodies (**D**) in the presence or absence of overexpressed UBQLN2. Cells were transfected

with mRTL8A (*red*) and an empty-vector or FLAG-UBQLN2 (*green*) and stained for NPM1 (**B**) or PML (**D**). DAPI marks the nucleus. (Scale bar = 5 µm).

(C) and **(E)** Individual channels' x-z and y-z projections for representative z stacks of cells transfected with both UBQLN2 and mRTL8A, shown in Figure 3C and 3D, respectively. White arrows indicate UBQLN2-mRTL8A subnuclear structures that are distinct from nucleoli (NPM1) and are in close proximity (but not completely colocalizing) with PML bodies. (Scale bar = 5 µm).

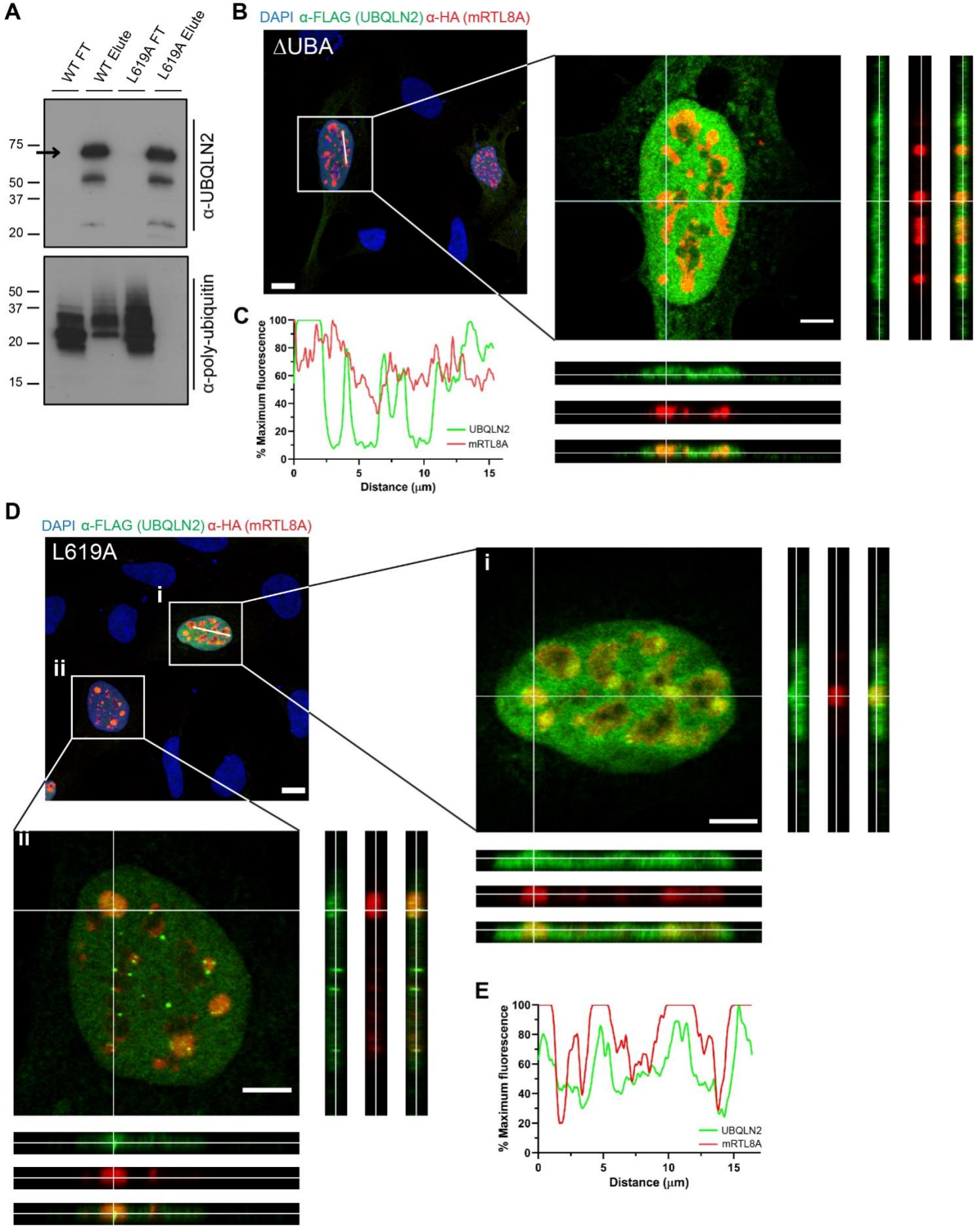


Supplementary Figure 3.

(A) Representative images of HEK293 cells co-transfected with FLAG-UBLQN2 and HA-mRTL8A, stained for Hsp70. In these images, nuclear mRTL8A structures (shown by filled arrows in the zoomed images) are not coalesced as they are in Figure 3A yet co-localize with UBQLN2 and Hsp70. Unfilled arrows indicate cytoplasmic UBQLN2-mRTL8A structures that do not co-localize with Hsp70. White outlines in the zoomed images represent the nucleus as determined by the extent of DAPI staining (blue in merged images). (Scale bar for merged images = 10 µm and zoomed images= 5 µm).

(B), **(C)** and **(D)** Representative images of HEK293 cells transfected with empty vector or vector encoding FLAG-UBLQN2 (*green*) and stained for Hsp70 (**B**), p62 (**C**) or poly-ubiquitin (**D**). (Scale bar = 10 µm).

(E) Representative images of HEK293 cells transfected with empty vector, FLAG-UBQLN2 (*green*) or mRTL8A (*red*) only, or FLAG-UBQLN2 and mRTL8A. Cells were stained for TDP-43 (*cyan*) with DAPI (*blue*) marking the nucleus. No co-localization was observed between TDP-43 and UBQLN2 or mRTL8A. (Scale bar = 10 µm).

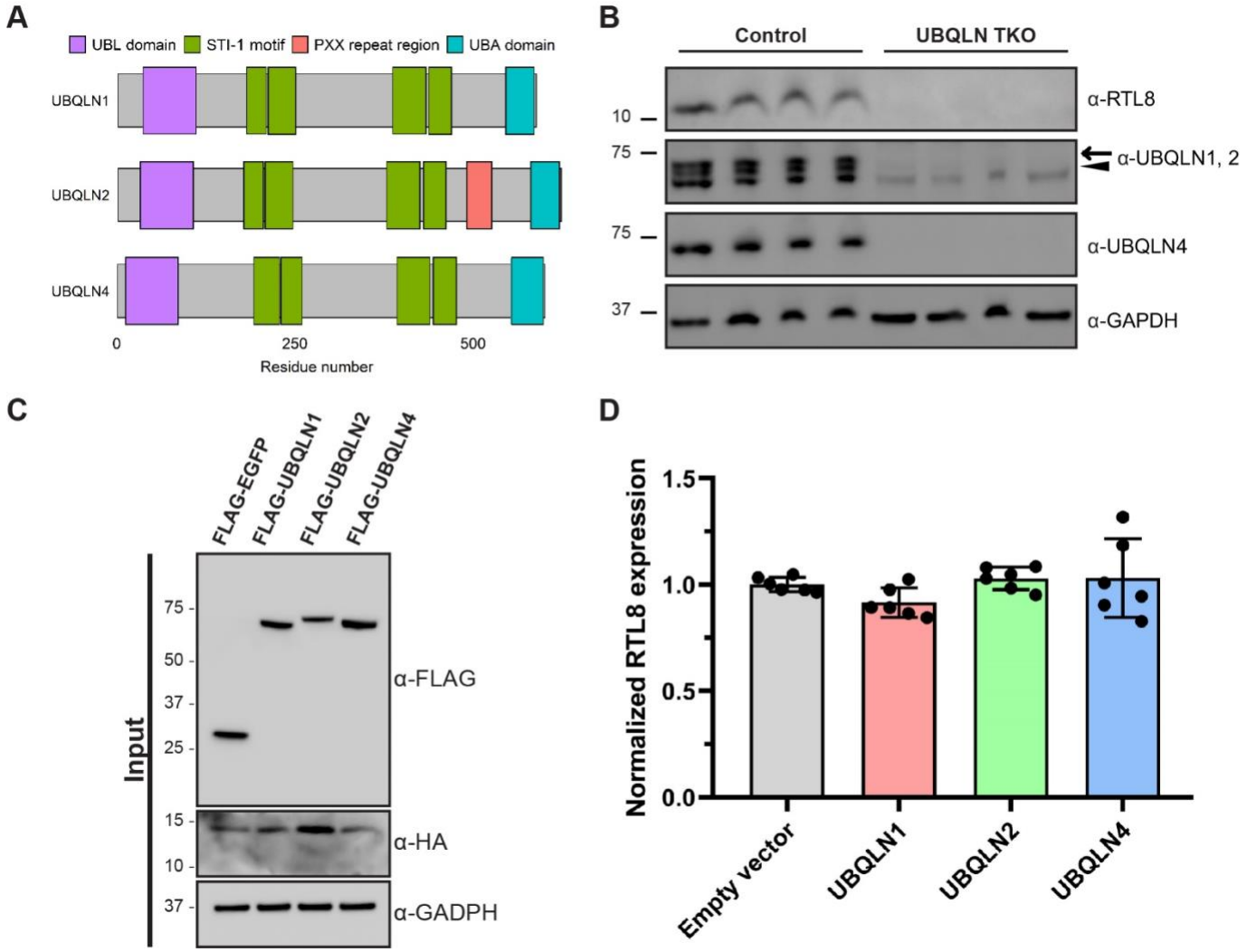

A
WT FT
WT Elute
L619A FT
L619A Elute
75
50
37
20
α-UBQLN2
50
37
20
15
α-poly-ubiquitin
B
DAPI α-FLAG (UBQLN2) α-HA (mRTL8A)
ΔUBA
C
% Maximum fluorescence
Distance (μm)
UBQLN2
mRTL8A
D
DAPI α-FLAG (UBQLN2) α-HA (mRTL8A)
L619A
i
ii
E
% Maximum fluorescence
Distance (μm)
UBQLN2
mRTL8A

Supplementary Figure 4.

(A) Representative immunoblot assessing pulldown of polyubiquitin by recombinant His-tagged WT or L619A UBQLN2. L619A was unable to pulldown polyubiquitin confirming loss of polyubiquitin binding. The flow-through (FT) and eluate were visualized by immunoblot with α-UBQLN2 (top) or α-polyubiquitin antibody (bottom). Arrow= UBQLN2 band.

(B) and **(D)** Individual channel's x-z and y-z projections for representative z stacks of cells transfected with mRTL8A and UBQLN2 ΔUBA or L619A, as shown in Figure 5C. ΔUBA UBQLN2 surrounded mRTL8A puncta but is not present within them, while L619A UBQLN2 is incorporated into mRTL8A puncta. (Scale bar for merged images = 10 µm and zoomed images= 5 µm).

(C) and **(E)** Pixel intensity plots for FLAG-UBQLN2 (*green*) and HA-mRTL8A (*red*) measured across the white lines overlaid on (**B**) and (**D**), respectively. L619A signal, but not ΔUBA UBQLN2 signal, overlaps with the mRTL8A signal. Raw values were normalized to the maximum fluorescence value for each channel and plotted as a function of distance.



Supplementary Figure 5.

(A) Schematic of the three brain-expressed ubiquilin proteins (UBQLNs 1,2 and 4) showing common structural motifs and the PXX repeat region that distinguishes UBQLN2.

(B) Representative immunoblot of lysates from HEK293 T-rex control (containing UBQLN1, 2 and 4) and UBQLN TKO HEK293 cells visualizing the loss of endogenous RTL8 proteins in TKO cells. Arrow and arrowhead denote bands corresponding to UBQLN2 and UBQLN1, respectively. GAPDH was used as a loading control.

(C) Lysate inputs corresponding to the representative co-immunoprecipitation immunoblot shown in Figure 6C. GAPDH was used as a loading control.

(D) Quantitative PCR showing no significant change in endogenous RTL8 transcript levels in TKO cells transfected with empty vector, FLAG-UBQLN1, UBQLN2 or UBQLN4 as shown in Figure 6F. Data are represented as mean ± SD normalized to empty vector. N=2 technical replicates from 3 biological replicates.