

Supporting information for

The ubiquitin-specific protease USP8 deubiquitinates and stabilizes Cx43
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SUPPORTING EXPERIMENTAL PROCEDURES

PLASMIDS-USP8 expression vector was described (1). Site-directed mutagenesis was performed to generate the C786A mutant using wild-type USP8 plasmid as a template. To construct full-length Cx43 expression vectors, the coding sequence of Cx43 was amplified by PCR and cloned into pcDNA3.1. All Flag-tagged and HA-tagged Cx43 and USP8 were constructed by sub-cloning Cx43 and USP8 cDNA into pcDNA3-Flag and pcDNA3-HA (Invitrogen) respectively. His-tagged Cx43 expression vector was generated by sub-cloning Cx43 coding fragment into pcDNA3-His (Invitrogen). All GFP-tagged Cx43 and USP8 deletion mutants were constructed by inserting PCR products into the pEGFP-C1 vector (Clone tech). The cloning primers used in this study are listed in **supplemental Table 1**. All expression constructs were verified by Sanger sequencing. Plasmids expressing HA-tagged wild-type and mutant Ub in pcDNA-HA vector were acquired from Addgene.

REGEANTS-Cycloheximide, bafilomycin A1, chloroquine, HA and Flag peptide were purchased from Sigma-Aldrich (St.Louis, MO), bortezomib from Selleck, protein A/G-Plus-agarose beads from Santa Cruz Biotechnology (Santa Cruz, CA).

SUPPORTING REFERENCES

1. Ma, Z. Y., Song, Z. J., Chen, J. H., Wang, Y. F., Li, S. Q., Zhou, L. F., Mao, Y., Li, Y. M., Hu, R. G., Zhang, Z. Y., Ye, H. Y., Shen, M., Shou, X. F., Li, Z. Q., Peng, H., Wang, Q. Z., Zhou, D. Z., Qin, X. L., Ji, J., Zheng, J., Chen, H., Wang, Y., Geng, D. Y., Tang, W. J., Fu, C. W., Shi, Z. F., Zhang, Y. C., Ye, Z., He, W. Q., Zhang, Q. L., Tang, Q. S., Xie, R., Shen, J. W., Wen, Z. J., Zhou, J., Wang, T., Huang, S., Qiu, H. J., Qiao, N. D., Zhang, Y., Pan, L., Bao, W. M., Liu, Y. C., Huang, C. X., Shi, Y. Y., and Zhao, Y. (2015) Recurrent gain-of-function USP8 mutations in Cushing's disease. *Cell research* **25**, 306-317

Supporting Table S1

Plasmid		Sequence (5'-3')
GFP-USP8	Forward	GCGGATCCggacttcgtaacttaggaaa
	Reverse	GCCTCGAGtgtggctacatcagttactcg
GFP-MITRhod	Forward	GCGGATCCAtgcctgctgtggcttcagtt
	Reverse	GCCTCGAGgtgtgtatactggggataac
GFP-DUP	Forward	GCGGATCC ggacttcgtaacttaggaaa
	Reverse	GCCTCGAGtgtggctacatcagttactcg
GFP- ΔMITRhodDUP	Forward	GCGGATCC aatgctaaggctcactccacc
	Reverse	GCCTCGAGagtaagagctggccagaacc
GFP-Cx43	Forward	GCGGATCCatgggtgactggagcgcctta
	Reverse	GCCTCGAG gatctccaggtcatcaggcc
GFP-Cx43 ΔCTD	Forward	GCGGATCCatgggtgactggagcgcctta
	Reverse	GCCTCGAGcctgaagaaaacatagaagag
GFP-Cx43 ΔM1-2	Forward	GCGGATCC gttgccaaactgatgggtgt
	Reverse	GCCTCGAG gatctccaggtcatcaggcc
GFP-Cx43 M3-4	Forward	GCGGATCC gttgccaaactgatgggtgt
	Reverse	GCCTCGAGcctgaagaaaacatagaagag
GFP-Cx43 CTD	Forward	GCGGATCC ggcgttaaggatcgggttaag
	Reverse	GCCTCGAG gatctccaggtcatcaggcc
His-Cx43	Forward	GCGGATCC atgggtgactggagcgcctta
	Reverse	GCGCGGCCGCGTGATGATGATGGTGATGgatctccaggtcatcaggccg

Supp Figure 1

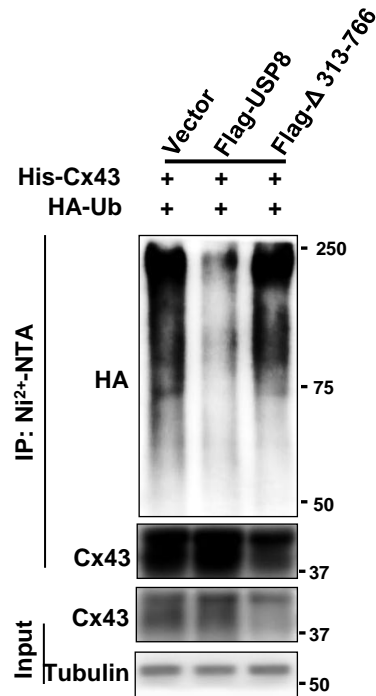


Fig. S1. USP8 mutant which lacks its central region (313-766 aa) failed to deubiquitinate Cx43 and increase its protein level. HEK293T cells were co-transfected with His-Cx43, HA-ubiquitin (Ub) and Flag-USP8 or Flag-USP8 mutant. After 48h, the cells were subjected to pull down using the Ni²⁺-NTA beads under denaturation conditions, followed by immunoblotting.

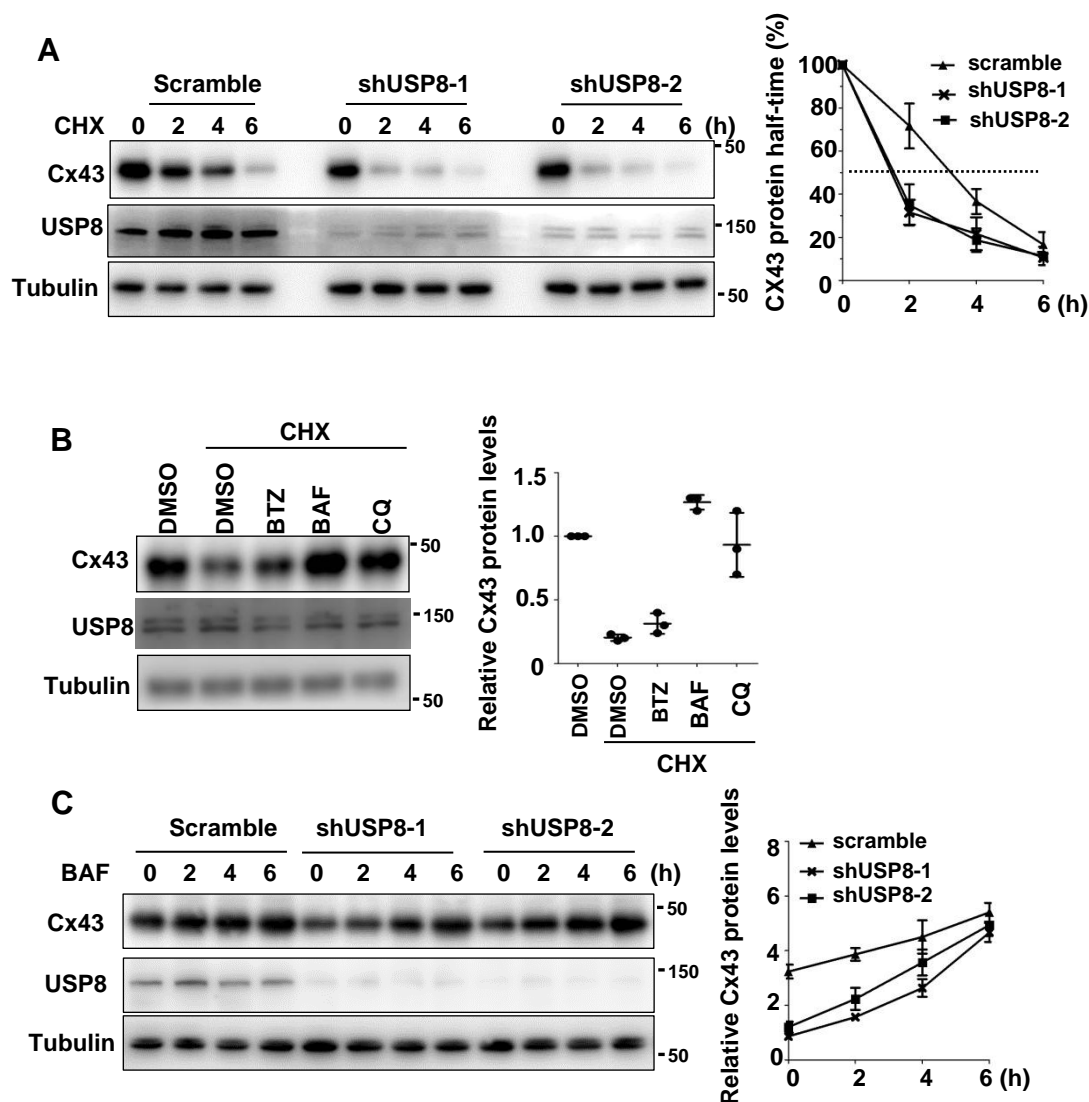


Fig. S2. USP8 protects Cx43 from autophagy-mediated degradation in MDA-MB-231. *A*, Immunoblotting of Cx43 and USP8 protein in scramble or shUSP8-infected MDA-MB-231 cells in the presence of cycloheximide (CHX, 50mg/ml). *B*, Immunoblotting of Cx43 protein in MDA-MB-231 cells treated with BTZ (50nM), BAF (200nM) or CQ (50 μ m) for 6 hours in the presence of CHX (50mg/ml). *C*, Immunoblotting of Cx43 protein in scramble or shUSP8-infected MDA-MB-231 cells treated with BAF (200nM) for indicated times. In A-C, quantification of the immunoblots comes from three independent experiments and shown at the right.

Supp Figure 3

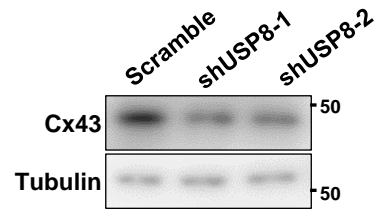


Fig. S3. USP8 knockdown decreased the protein level of Cx43 in Triton X-100 insoluble fraction in MEFs.