Supporting Information

The Antiviral Enzyme, Viperin, Activates Protein Ubiquitination by the E3 Ubiquitin Ligase, TRAF6

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Experimental Protocols and Supplemental Figures

Plasmids, reagents, and antibodies:

Human TRAF6-N (aa 50-211) construct in pET21c was a kind gift of Prof. Hao Wu (Harvard University). Ubc13 and Uev1A constructs in pGEX6P3 were a kind gift of Prof. Catherine Day (University of Otago, New Zealand). The truncated human viperin-ΔN50 (aa 51-361) was cloned into pRSF-duet vector with an N-terminal His tag. The human E1 enzyme (E-304-050) was purchased from R&D systems. Ubiquitin was purchased from Sigma Aldrich. Pierce protein A/G plus agarose resin and control agarose resin (Pierce classic IP kit 26146) were purchased from Thermo-Fisher Scientific. The rabbit polyclonal viperin antibody (11833-1-AP), rabbit polyclonal ubiquitin antibody (10201-2-AP) both were obtained from ProteinTech. The rabbit polyclonal TRAF6 antibody (sc-7221) was obtained from Santa Cruz Biotechnology. Goat anti-rabbit (170-6515)) Ig secondary antibody was purchased from BioRad.

Protein Expression and Purification:

TRAF6-N Human TRAF6-N[1] containing a C-terminal His tag was expressed in *E. coli* BL21 (DE3). Cultures were grown to OD_{600} 0.6-0.8 before cold shocking the cells in ice bath for 30 min. The protein was induced with 0.5 mM IPTG and 0.1 mM $ZnCl_2$ and incubated at 20°C overnight. Cells were lysed by sonication in buffer A (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole and 10% glycerol) with protease inhibitor cocktail and clarified by centrifugation at 18,000 rpm for 1 h at 4°C. The cleared lysate was loaded onto the HisTrap prepacked column. The column was washed with buffer A (10 mM imidazole) until no further protein eleuted. The column was then washed with 90% buffer A and 10% buffer B (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 500 mM imidazole and 10% glycerol) followed by washing with 80% buffer A and 20% buffer B. Finally, the protein was eluted with 60% buffer A and 40% buffer B (200 mM imidazole). The fractions containing TRAF6-N were pooled, concentrated, and further purified by size-exclusion chromatography. The concentrated protein was loaded on to Superdex 200 16/120 column equilibrated in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, and 10% glycerol. Fractions containing TRAF6-N were pooled, concentrated and stored at – 20 C.

Ubc13 and Uev1A The expression, purification, and GST tag cleavage protocols of Ubc13 and Uev1A were performed as described previously.[2]

Viperin-ΔN50 Human viperin-ΔN50 was cloned into the expression vector pRSF-duet, and transformed into *E. coli* BL21 (DE3) using standard methods. The expression of viperin-ΔN50, purification of the protein and reconstitution of the iron-sulfur cluster under anaerobic conditions were performed as described previously.[3]

Preparation of reagents for anaerobic experiments

All buffers and stock solutions used in experiments were thoroughly degassed before introducing them into the anaerobic chamber. The buffer solutions were allowed to equilibrate in open bottles for at least 24 h before use to allow residual oxygen to defuse from the solution. Sensitive reagents such as ATP and protein solutions were introduced into the anaerobic chamber as concentrated stock solutions in small volumes in Eppendorf tubes. These were allowed to equilibrate uncapped for several minutes in the anaerobic chamber to allow oxygen to diffuse from the solution and used at dilutions of 50 – 100-fold so that the concentration of oxygen introduced into enzymes assays was minimized.

Co-immunoprecipitation of viperin and TRAF6-N

In an anaerobic environment (Coy anaerobic chamber), 1 µM viperin-ΔN50 and or 1 µM TRAF6-N were mixed in a buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM DTT, and 10% glycerol and incubated at 4 °C for 1 h. Then, 0.5 µg of anti-viperin antibody was added to each reaction and incubated at 4 °C for further 1 h. Next, 10 uL of protein A/G Agarose beads (20 uL slurry) equilibrated in the same buffer, were added and the mixture was then incubated with end-to-end mixing outside the Coy chamber in 4 °C for 1 h. The beads were washed three times with excess buffer before incubating with 2X SDS loading buffer containing 5% 2-mercaptoethanol. The mixture was then agitated for 20 min, and heated at 95 °C for 10 min, and the beads removed by centrifugation. The proteins were analyzed by SDS-PAGE (4 – 20 % gradient gels) and immunoblotted with appropriate antibodies using standard protocols.

Ubiquitination assay:

All assays containing viperin-ΔN50 were conducted inside a Coy anaerobic chamber.

For ubiquitination assays, 0.1 µM E1, 2 µM Ubc13, 2 µM Uev1A, 2 µM TRAF6-N, and/or 2 µM viperin-ΔN50 were mixed in a buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM DTT, 2 mM ATP, and 5 mM $MgCl_2$ and incubated at 37°C. Reactions were initiated by the addition of ubiquitin, 35 µM final concentration, and at various times, aliquots of the assay mixture were removed and quenched by adding equal volumes of 2X SDS loading buffer containing 2-mercaptoethanol. Proteins were analyzed by SDS-PAGE on 4-20% gels. Gels were stained with Coomassie brilliant blue and ubiquitin bands quantified with reference to known standards.

To examine which bands represented ubiquitinated forms of TRAF6, samples were analyzed using by SDS-PAGE on 4-20% gels that were then subjected to immunoblot analysis using standard techniques with antibodies to ubiquitin, viperin or the N-terminal domain of TRAF6.

References

1. Yin, Q.; Lin, S. C.; Lamothe, B.; Lu, M.; Lo, Y. C.; Hura, G.; Zheng, L. X.; Rich, R. L.; Campos, A. D.; Myszka, D. G.; Lenardo, M. J.; Darnay, B. G.; Wu, H., E2 interaction and dimerization in the crystal structure of TRAF6. *Nat. Struct. Mol. Biol.* **2009,** *16*, 658-662.

2. Middleton, A. J.; Budhidarmo, R.; Das, A.; Zhu, J. Y.; Foglizzo, M.; Mace, P. D.; Day, C. L., The activity of TRAF RING homo- and heterodimers is regulated by zinc finger 1. *Nat Commun* **2017,** *8,* article # 1788.

3. Ghosh, S.; Patel, A. M.; Grunkemeyer, T. J.; Dumbrepatil, A. B.; Zegalia, K.; Kennedy, R. T.; Marsh, E. N. G., Interactions between Viperin, Vesicle-Associated Membrane Protein A, and Hepatitis C Virus Protein NS5A Modulate Viperin Activity and NS5A Degradation. *Biochemistry* **2020,** *59*, 780-789.

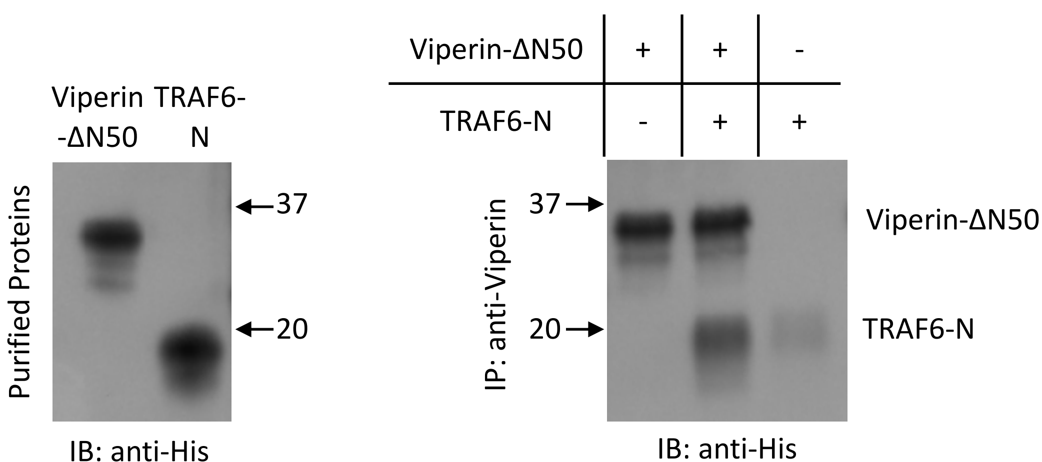

Figure S1

Co-immunoprecipitation of TRAF6-N by viperin-ΔN50. Pull-down assays were performed as described above. Purified viperin-ΔN50 and TRAF6-N were incubated together and anti-viperin antibodies used to precipitate the complex. Pulled-down proteins were analyzed by immunoblotting and visualized by immunostaining with antibodies to the His-tags present on both proteins.

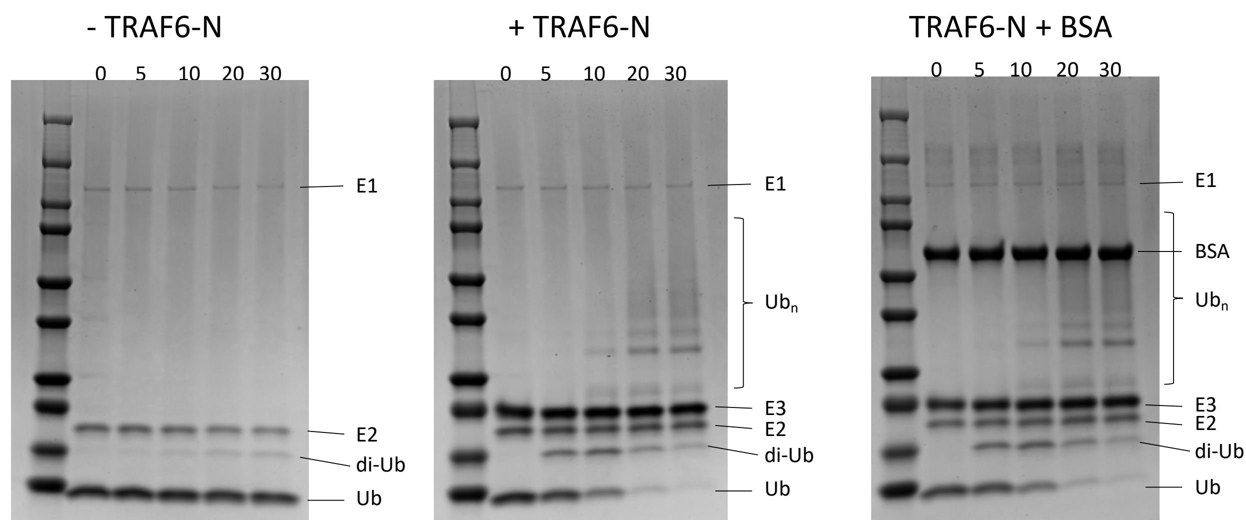

Figure S2 Control experiments to establish that viperin specifically activates TRAF6-N: **A:** Ubiquitination system reconstituted without TRAF6-N as the E3-ligase component; only a small amount of di-ubiquitin is formed after 30 min. **B:** Ubiquitination system reconstituted including TRAF6-N as the E3-ligase component; TRAF6-N catalyzes the formation of ubiquitin oligomers. **C:** Addition of bovine serum albumin as a control for non-specific TRAF6-N activation (1:1 molar ratio with TRAF6-N) has no effect on the rate of TRAF6-N-catalyzed ubiquitin ligation.

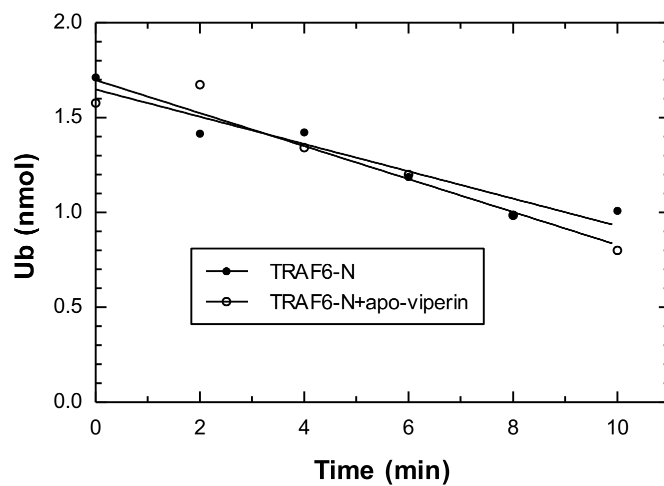

Figure S3 Comparison of the initial rates of ubiquitin ligation catalyzed by TRAF6-N in the presence and absence of viperin-ΔN50-C83A (apo-viperin). The viperin-ΔN50-C83A mutation removes one of the sulfur ligands to the [4Fe4S] cluster, resulting in apo-enzyme. Viperin-ΔN50-C83A has no significant effect on the rate of ubiquitin ligation.

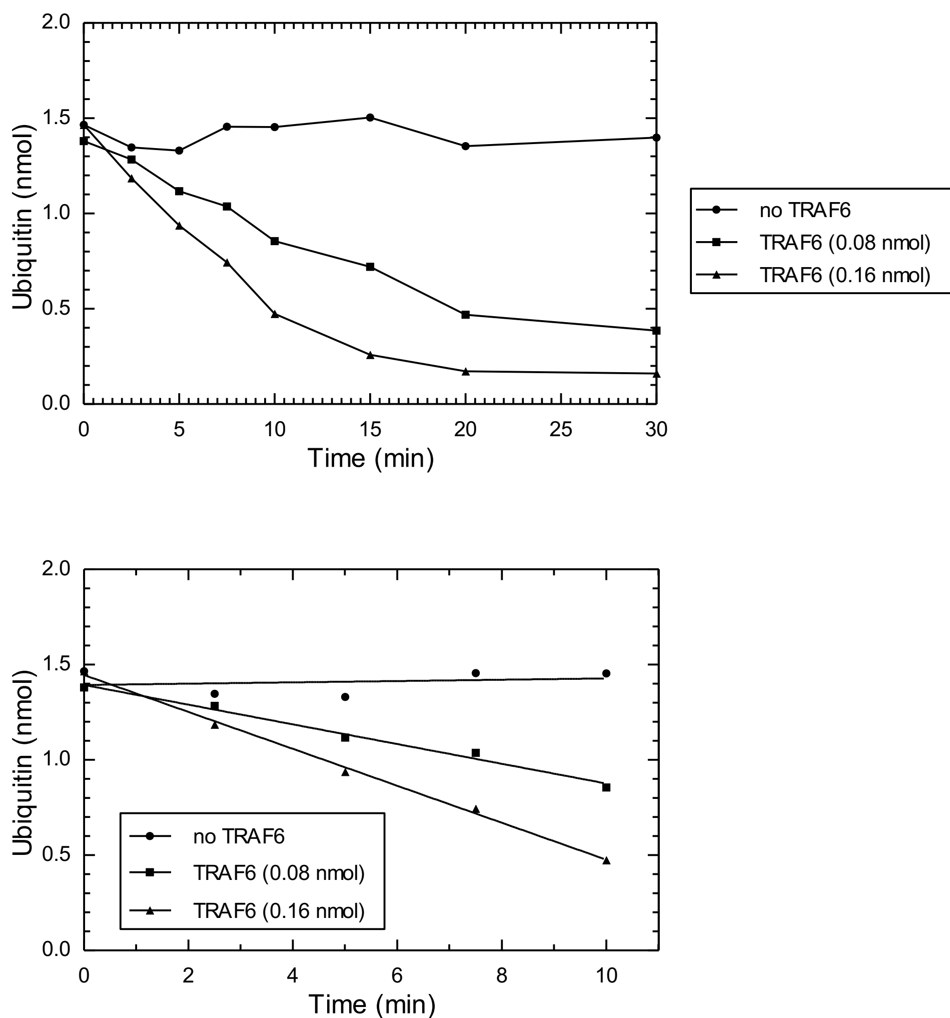

Figure S4

Top Time course for a typical ubiquitination reaction followed by monitoring the disappearance of the band due to unreacted ubiquitin. *Bottom* During the initial period of the reaction, the rate of ubiquitin consumption is linear with TRAF6-N concentration.

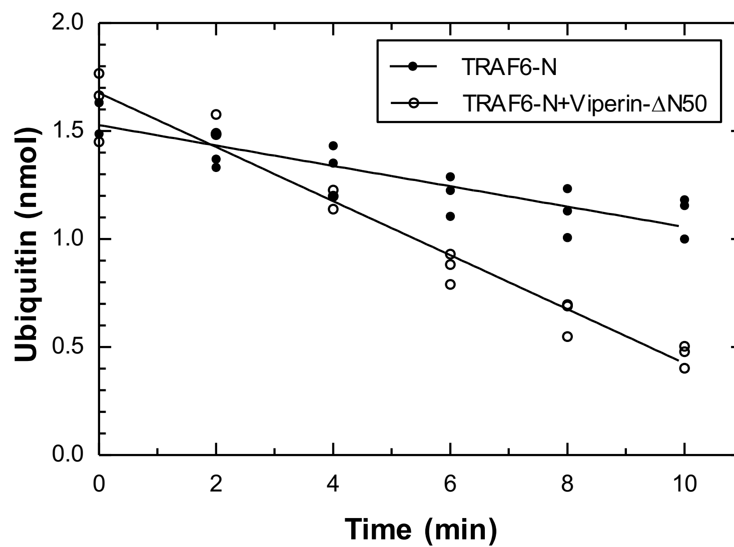

Figure S5

Comparison of the initial rates of ubiquitin ligation catalyzed by TRAF6-N in the presence and absence of viperin-ΔN50. The presence of viperin results in a ~2.5-fold increase in the rate of ubiquitin ligation; average of 3 independent experiments.