Qi et al., Proteolytic Processing Regulates Toll-like Receptor 3 Stability and Endosomal Localization.

SUPPLEMENTAL METHOD

Plasmid constructions - S-344-C-HA encodes amino acids 1-28 of TLR3, fused to amino acids 344-906 and a C-terminal HA tag (YPYDVPDYA). M-344-C-HA encodes amino acid methionine fused to amino acids 344-906 of TLR3, and a C-terminal HA tag. The cDNAs in S-344-C-HA and M-344-C-HA were constructed by PCR from TLR3-HA using the forward primer 5'-phosphate-CCCAAGATTGATGATTTTTCTTTTCAGTGGCTAAAATGTTTG-3' and reverse primers: 5'-Phosphate-CTTGGTGGTGGAGGATGCACACAGCATCCCAAAGG-3' or 5'-Phos-CATGATGGCCCTCCTACCGGTGATCTCAGGTAGGC-3'. Each PCR product was ligated using T4 ligase to allow circularization. All cDNAs sequences were determined to ensure that the constructs contained no unintended changes.

Construction and characterization of mutant Δ64 that lacked residues 289 to 352 of TLR3 was described in Qi et al. (2010).

Knockdown by siRNA and q-RT-PCR- Knockdown of Unc93b1 in human cells used the protocol described in Qi et al. (23). 72 h after transfection, total RNA was extracted using an RNEasy Mini kit from Qiagen. The first strand of cDNA synthesis used 1 μg of total RNA, 4 μM randomized 9-nt primer and M-MuLV reverse transcriptase (New England Biolabs). q-PCR was performed using Ambion *SYBR® Green* Real-Time PCR Master Mixes. The primer pair specific for GAPDH had the sequences of 5'GAGTCAACGGATTTGTGCGT3' and 5'-TGGGATTTCCATTGATGACA-3'. The primer pair for Unc93b1 was 5'-TGATCCTGCACTACGACGAG-3' and 5'-GCGAGGAACATCATCCACTT-3'. All reaction mixtures were heated to 95°C for 10 min, followed by 40 cycles of amplification consisting of 15 sec at 95°C, 20 sec at 55°C, and 30 sec at 72°C. The data were analyzed using the $\Delta\Delta C_T$ method as described by Livak and Schmittgen (2001).

siRNA to murine TLR3 was from Santa Cruz Biotechnology (Cat. # sc-40259). siRNA was transfected to RAW264.7 cells using Lipofectamine RNAiMax (Invitrogen). 24 h after transfection, the cells were induced with ligands for 18 h, followed by collection of the culture media for quantification of IL-6 concentration by ELISA as described in Lai et al. (28). Aliquots of the transfected cells were also lysed and extracted for total RNA to quantify TLR3 message relative to β-actin was quantified as described above. Amplification of TLR3 cDNA used the primer pair with the sequences of 5'-CCC CCT TTG AAC TCC TCT TC-3' and 5'-TTT CGG CTT CTT TTG ATG CT-3'. The primer pair specific for β-Actin contained the sequences of 5'- GGC ATT GTT ACC AAC TGG GAC GAC-3' and 5'-CCA GAG GCA TAC AGG GAC AGC ACA G 3'.

SUPPLEMENTAL TABLE 1.

Supplement Table 1. Quantification of the effects of inhibitors on TLR signaling.

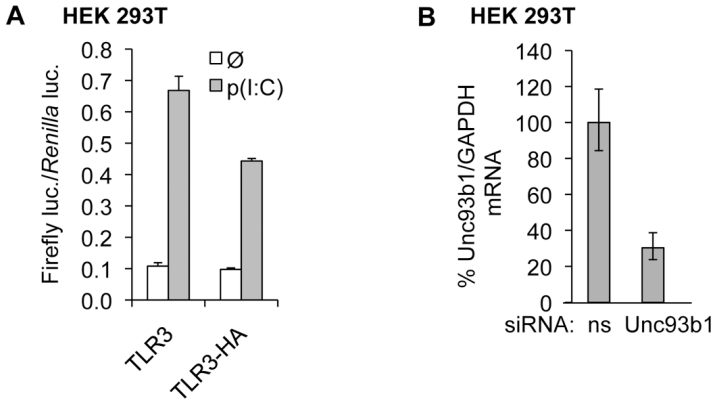
	RAW264.7 (IL-6)			HEK293T (Luc.)			Huh7.5 (Luc.)			BEAS-2B (IL-6)		
	DMSO	BAF	Z-FA-FMK	DMSO	BAF	Z-FA-FMK	DMSO	BAF	Z-FA-FMK	DMSO	BAF	Z-FA-FMK
TLR3	100±4 [1]	23±1	80±2	100±11	43±8	107±8	100±4	21±7	86±6	100±12	3±0	110±12
p-value[2]		**0.001**	0.239		**0.015**	0.332		**0**	0.25		**0.021**	0.965
TLR4	100±3	80±34	60±1	100±9	97±5	76±16	100±9	95±23	160±21			
p-value		**0.011**	**0**		0.789	0.316		0.959	0.469			
TLR9	100±16	54±1	63±3	100±1	43±3	53±7.5	100±9	26±3	31±2			
p-value		**0.001**	**0.001**		**0.007**	**0.036**		**0.009**	**0.014**			
TLR7	100±6	14±3	15±1									
p-value		**0**	**0**									

[1]All data are normalized to the the amount of IL6 or luciferase levels from cells expressing a TLR but treated with only DMSO.
[2] p-values are calculated by student T-test. P-values <0.05 are highlighted in bold.

SUPPLEMENTAL FIGURES AND LEGENDS

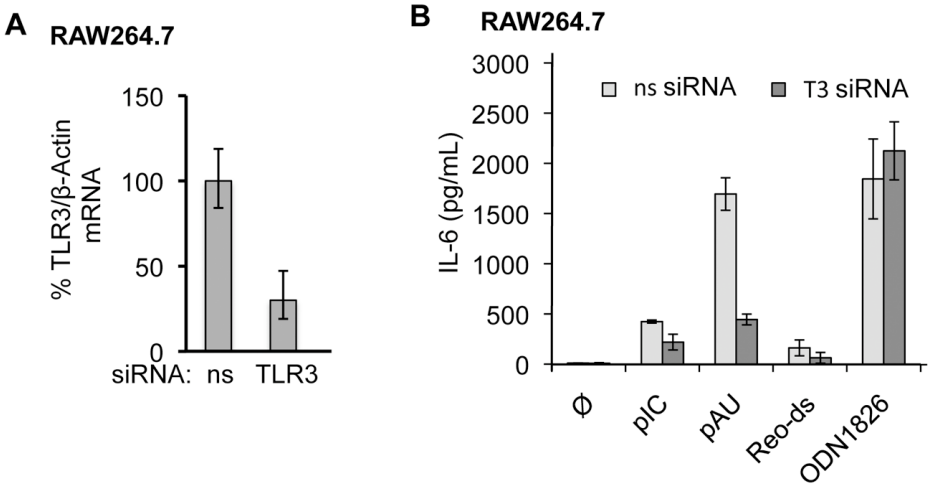
sFig. 1. **A)** TLR3-HA activates signal transduction in luciferase reporter assay in HEK293T cells. TLR3 and TLR3-HA were both transfected to HEK293T cells along with luciferase reporter controlled by the ISRE promoter. Cells were induced with poly(I:C) for 18 h and quantified for luciferase activity. **B)** Knockdown of Unc93b1 message by siRNA reduced Unc93b1 mRNA levels. HEK293T cells transfected with non-specific siRNA (ns) or siRNA specific to Unc93b1. The level of Unc93b1 message relative to the GAPDH message was quantified by RT-qPCR. The values in the samples treated with the ns siRNA were normalized to 100%.

Supplement Fig. 1

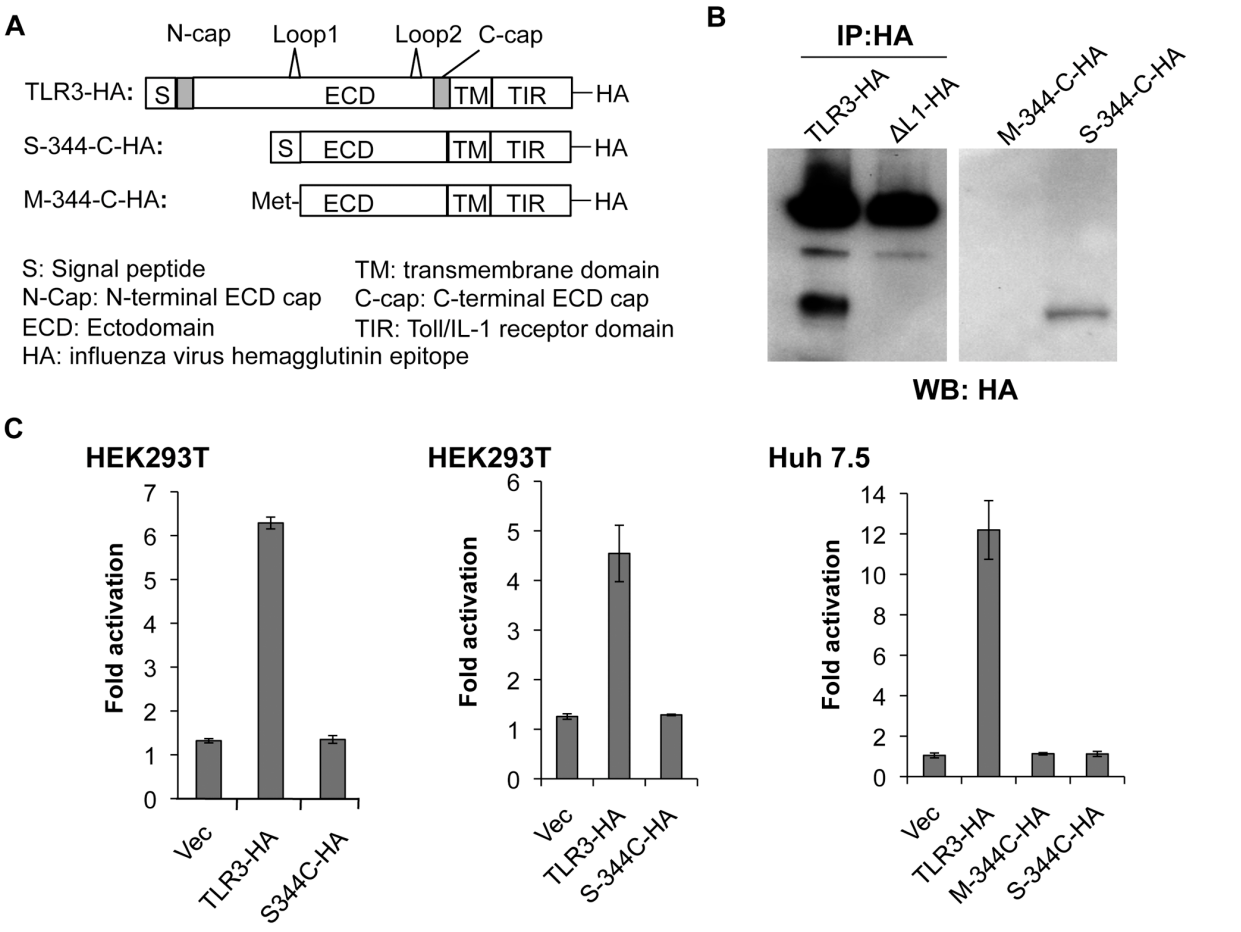


sFig. 2. IL-6 production in RAW264.7 cells in response to dsRNA dsRNA ligands required TLR3. **A)** Knockdown of TLR3 mRNAs by siRNA. RAW264.7 cells transfected with non-specific siRNA (ns) or siRNA specific to TLR3 using RT-qPCR. The value with ns treatment relative to the β-Actin mRNA was normalized as 100%. **B)** SiRNA knockdown of TLR3 reduced IL6 production in response to RNA ligands. RAW264.7 cells transfected with non-specific (NS) siRNA or siRNA to TLR3 were induced with poly(I:C) (50 ng/ml), poly(A:U) (5 ng/ml), Reovirus double-stranded RNA (Reo-ds) (50 ng/ml), or ODN1826 (1 μM). Culture media were collected and the secreted IL-6 was quantified by ELISA.

Supplement Fig. 2



sFig. 3. Truncations that mimic the CTF of TLR3 are deficient in signal transduction. **A)** Schematic of the epitope-tagged TLR3 truncations. The domains in TLR3 are labeled and their names shown below the schema. **B)** Plasmids expressing TLR3 truncations were transfected to HEK293T cells. The cells were lysed and probed to detect the HA tag using Western blots. All images were from the same exposure of the same blot at the same scale, but samples unrelated to the results were cropped. Cells expressing TLR3-HA immunoprecipitated by HA to enhance the detection of the CTF were also included as a reference of the molecular size of CTF. **C)** Signaling activity of the truncations as assayed by luciferase reporter assay.



Reference cited:
Livak, K. J., Schmittgen, T. D. (2001). Methods **25**. 402-408.