Supplementary Information

Figure Legends

S1. CagA is required for *H. pylori*-induced NF-κB-driven gene expression. RT-PCR as in Fig 1A was performed using *H. pylori* strains WT NCTC11637, 7.13 and their ΔCagA mutants strains. Expression levels of IL-8 were measured by RT-PCR at 0, 2, 4 and 8 hr infection.

S2. *H. pylori* activates NF-κB-driven luciferase reporters in a CagA-dependent manner. 5XκB-luciferase (5XκB-Luc), IL-8-luciferase (IL-8-luc) or E-selectin-luciferase (E-selectin-luc) reporter plasmids were transfected into AGS cells followed by infection with *H. pylori*. Luciferase activity was measured 6 h after infection.

S3. RelA and p50 make up the NF-κB complex induced by *H. pylori* to bind an NF-κB DNA probe. Super-shift assay using anti-RelA or anti-p50 antibodies was performed using WT 60 min timepoint sample from Fig 1B. Super-shifts are indicated by arrows.

S4. TAK1 is required for activation of NF-κB by *H. pylori* in MEFs. WT and TAK1-/- MEFs were uninfected or infected for 8 hrs with *H. pylori*. Quantitative RT-PCR was performed to analyze the *H. pylori*-induced expression of NF-κB target genes.

S5. TAK1 is required for *H. pylori*-induced phosphorylation of RelA at serine 536. Control or TAK1 siRNA was transfected into AGS cells, and levels of S536 phosphorylated RelA, RelA and TAK1 were detected by immunoblotting after WT *H. pylori* infection for indicated time points.

S6. CagA enhances the ubiquitination of TAK1 in the denatured condition. Flag-TAK1, HA-ubiquitin and T7-CagA were transfected into HEK293T cells. Cells were harvested in lysis

buffer containing 1% SDS, boiled for 5 min, diluted to 0.1% SDS and immunoprecipitated with anti-Flag beads for 2 hrs. Immunoprecipitates were washed 3 times and separated on an SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted with indicated antibodies.

S7. Phosphorylation of CagA is not required for the CagA-mediated enhanced ubiquitination of TAK1. WT (HA-CagA) or phosphorylation-deficient mutant CagA (HA-CagA-PR) were transfected with HA-ubiquitin and Flag-TAK1 into HEK293T cells. Ubiquitination of TAK1 was detected by immunoblotting Flag immunoprecipitates with anti-HA antibodies.

S8. CagA colocalizes with TAK1 *in vivo.* AGS cells were transfected with HA-CagA and Flag-TAK1, and immunofluorescent staining was performed to show the cellular localization of both proteins.

S9. CagA interacts with TAK1 *in vitro*. GST or GST-CagA was incubated with *in vitro*-translated S35-labeled Flag-TAK1 and precipitated with glutathione beads. The recovered materials were detected by radiography.

S10. N- and C-terminal regions of CagA interact with TAK1. GST-CagA deletion mutants were used in pull-down *in vitro* translated TAK1. Pull-downs and inputs were separated by SDS-PAGE and TAK1 was detected by autoradiography. Coomassie staining shows GST-CagA deletion mutant input.

S11. N-terminal region of TAK1 associates with CagA. GST-CagA was used to pull-down *in vitro*-translated Flag-TAK1 deletion mutants. Pull-downs and inputs were separated by SDS-PAGE and immunoblotted for Flag. Coomassie staining shows GST-CagA input.

S12. CagA is required for the *H. pylori*-induced phosphorylation of TAK1 at threonine 187. TAK1 immunoprecipitates from WT and ΔCagA *H. pylori* infected AGS cells was immunoblotted for phospho-TAK1 (T187).

S13. *H. pylori* infection stimulates the activation of IKK. IKK immunoprecipitates from WT *H. pylori*-infected cells were subjected to *in vitro* IKK kinase assay was performed as in Fig 4F. WT or SSAA GST-IκBα were used as substrates.

S14. Oligomerization-defective mutant of CagA fails to enhance the ubiquitination of TAK1. Flag-TAK1, His-ubiquitin, and either full-length (FL), ΔABCC or ΔABCC-16AA (oligomerization mutant) CagA were transfected into HEK293T cells. Flag-TAK1 was immunoprecipitated, and immunoblotted as indicated.

S15. Oligomerization-defective mutant of CagA fails to activate NF-κB. IL-8-luciferase reporter was transfected along with full-length (FL), ΔABCC or ΔABCC-16AA into HEK293T cells. Luciferase activity was measured 30 hrs post-transfection.

S16. Lysine 34 is not involved in the CagA-mediated ubiquitination of TAK1. HEK293T cells were transfected with Flag-TAK1 WT or K34R, HA-ubiquitin and T7-CagA as indicated. Flag-TAK1 was immunoprecipitated and blotted for HA-ubiquitin. Levels of immunoprecipitated TAK1 and CagA are also shown.

S17. Mutation of lysine 34 of TAK1 to arginine does not affect its ability to activate NF-κB. HEK293T cells were transfected with IL-8-luciferase reporter and Flag-TAK1 WT or K34R and increasing amounts of T7-CagA. Luciferase activity was measured 30 hrs post-transfection.

S18. TRAF6 is important for the *H. pylori*-induced expression of TNF-α. AGS cells were treated with control or TRAF6 siRNA. Treated cell were infected with *H. pylori* for the indicated time points and mRNA expressions of IL-8 and TNF-α were measured by RT-PCR.

S19. CagA does not affect the interaction between TAK1 and TRAF6. HEK293T cells were transfected as indicated. T7-TRAF6 was immunoprecipitated, and immunoprecipitates were immunoblotted as indicated. Expression levels of each protein are shown in the lower three panels.

S20. CagA interacts with TRAF6 *in vitro*. GST and GST-CagA were used to pull-down *in vitro*-translated TRAF6. Pull-downs were separated by SDS-PAGE and TRAF6 was detected by autoradiography.

S21. CagA enhances the ubiquitination of TRAF6. HEK293T cells were transfected as indicated. T7-TRAF6 immunoprecipitates were immunoblotted for HA-ubiquitin and T7-TRAF6 as indicated. Levels of Flag-CagA are shown in the lower panel.

S22. TRAF6 does not affect the interaction between CagA and TAK1. GST and GST-CagA were used to pull-down *in vitro*-translated TAK1 in the presence or absence of *in vitro*-translated TRAF6. Pull-downs and inputs were separated by SDS-PAGE and TAK1 and TRAF6 were detected by immunoblotting or autoradiography, repectively.

Materials and Methods

Plasmids. CagA was amplified by PCR from the genomic DNA of *H. pylori* strain G27 and subcloned into the pcDNA3.1-Flag or pcDNA3.1-T7 vector. CagA and deletion mutants 1-454, 438-890 and 885-1216 were subcloned from Flag-CagA into the pcDNA3.1-Flag or pGEX-4T-1

vector. TAK1 deletion mutants 1-193, 194-386 and 387-579 were subcloned into the pcDNA3.1-Flag vector. TRAF6 was subcloned into the pcDNA3.1-T7 vector. HA-CagA, HA-CagA-PR, HA-CagA ΔABCC and HA-CagA ΔABCC-16AA from *H. pylori* strain NCTC11637 were described previously (Higashi et al, 2005; Saadat et al 2007).

Antibodies. Normal rabbit IgG, antibodies against CagA, TAK1, TRAF6, IKK1, IκBα, RelA, Flag, HA, ubiquitin, and GST were from Santa Cruz Bio. Anti-T7 antibody was from Covance. Phospho-S536 RelA and Phospho-TAK1 (T187) were from Cell Signaling.

TAK1 siRNA knockdown and rescue. TAK1 siRNA from Ambion was transfected into AGS cells using Lipofectamine 2000 according to the manufacturer's protocol. 36 hrs post-transfection, cells were split, and 36 hr later were infected with *H. pylori*. For rescue, cells were transfected with 40ng Flag-TAK1 per well of a 12-well plate using Lipofectamine 2000 24 hrs before infection.

Establishment of cell lines stably expressing TRAF6 shRNA

The TRAF6-specific short hairpin RNA oligomer (5'-GGAGAAACCTGTTGTGATT-3') was subcloned into the pSUPERretro-neo vector (Oligoengine). The vector was transfected into Phoenix-Ampho packaging cells and the supernatants were collected 48 h after transfection and used to infect AGS cells in the presence of 10 μg/ml polybrene (Sigma) for 24 h. After changing the medium, cells were cultured for 16 h and then selected with 0.5 mg/ml G418 (Sigma) for 7 days. The knock-down efficiency was assessed by immunoblotting.

***In vitro* kinase assay.** IKK1 or TAK1 immunoprecipitated from transfected HEK293T cells or *H. pylori* infected AGS cells, was washed 3 times with IP buffer (Chen et al, 2001) and once with 1X kinase assay buffer (10mM HEPES pH7.4, 1mM $MnCl_2$, 5mM $MgCl_2$, 12.5mM

glycerol-2-phosphate, 50μM Na_3VO_4, 2mM NaF, 0.5mM DTT), and incubated with either recombinant IκBα (0.3μg) for IKK or alone (TAK1) in kinase assay buffer with 70μCi [γ-^{32}P] ATP (3,000 Ci/mmol) for 30 min at 30˚C. Samples were separated by 10% SDS-PAGE and visualized by autoradiography.

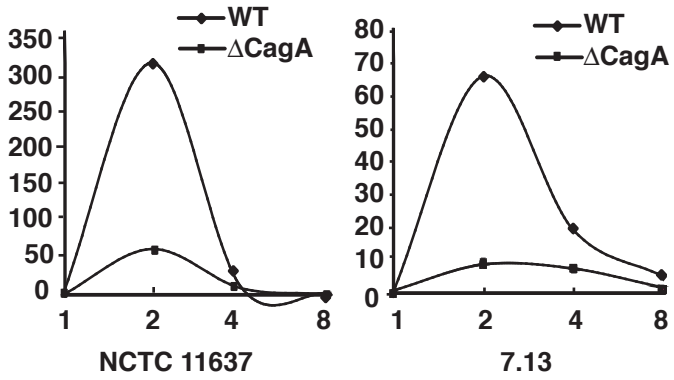
Indirect immunofluorescence staining. AGS cells grown on cover-slips were transfected with Flag-TAK1 and HA-CagA for 36 hrs, washed twice in PBS, fixed in 3.7% formaldehyde (Sigma) in PBS for 20 min, and permeabilized in 0.1% (vol/vol) NP-40 in PBS for 10 min. After washes with PBS, cells were incubated in blocking buffer (5% normal goat serum and 1 mg/ml BSA in PBS) for 1 h and then incubated for 1 h with rabbit anti-HA antibodies (1:100, Santa Cruz) and mouse anti-Flag antibodies (1:100, Sigma) at room temperature. After 2 15-min washes in PBS, the cells were incubated with FITC-conjugated goat anti-rabbit IgG (1:200) and Texas Red-conjugated anti-mouse IgG (1:200, Invitrogen) for 60 minutes in blocking buffer at room temperature. Cells were washed twice for 15 min each in PBS. Images were acquired by AXIOVERT 200 motorized inverted microscopy using an Axiovert 200M (Zeiss).

Supplemental References

Higashi H, Yokoyama K, Fujii Y, Ren S, Yuasa H, Saadat I, Murata-Kamiya N, Azuma T, Hatakeyama M (2005) EPIYA Motif Is a Membrane-targeting Signal of Helicobacter pylori Virulence Factor CagA in Mammalian Cells. *J Biol Chem* **280**(24): 23130-23137

Saadat I, Higashi H, Obuse C, Umeda M, Murata-Kamiya N, Saito Y, Lu H, Ohnishi N, Azuma T, Suzuki A, Ohno S, Hatakeyama M (2007) Helicobacter pylori CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. *Nature* **447**: 330-333.

S1


WT
ΔCagA
350
300
250
200
150
100
50
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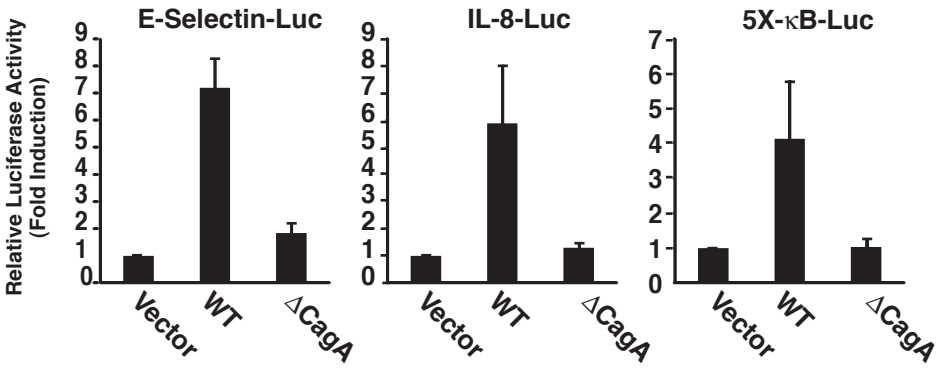
1
2
4
8
NCTC 11637
80
70
60
50
40
30
20
10
0
7.13

S2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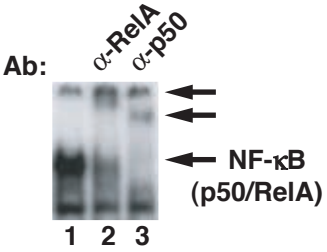
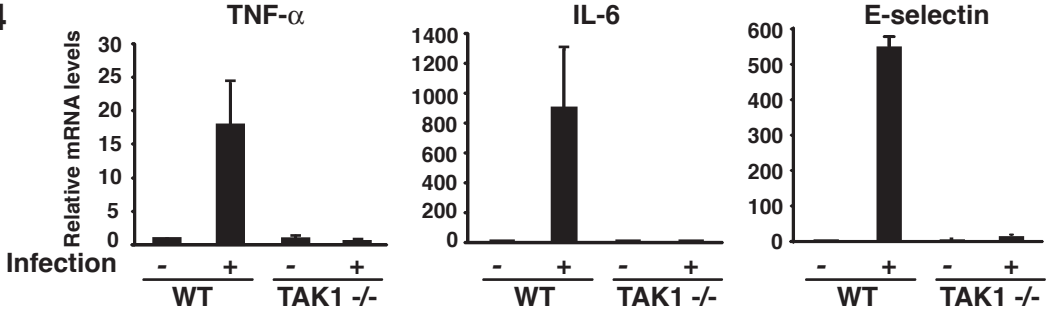
E-Selectin-Luc
IL-8-Luc
5X-κB-Luc
Vector
WT
ΔCagA

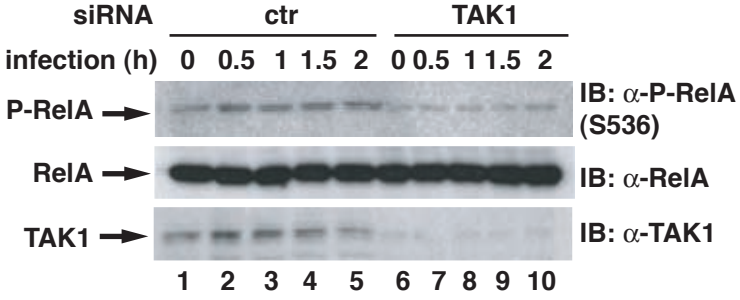
S3


Ab:
α-RelA
α-p50
NF-κB
(p50/RelA)
1 2 3

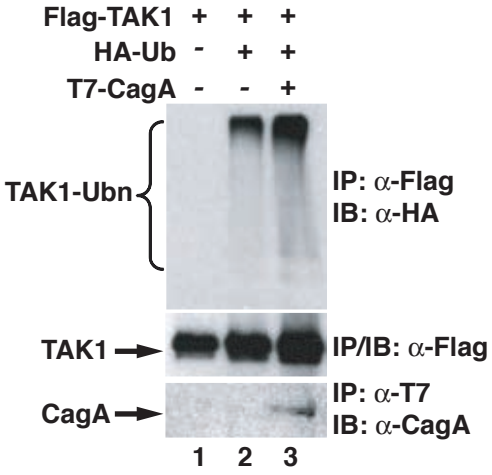
S4


TNF-α
IL-6
E-selectin
Relative mRNA levels
Infection
WT
TAK1 -/-

S5


siRNA
ctr
TAK1
infection (h) 0 0.5 1 1.5 2 0 0.5 1 1.5 2
P-RelA
IB: α-P-RelA (S536)
RelA
IB: α-RelA
TAK1
IB: α-TAK1
1 2 3 4 5 6 7 8 9 10

S6



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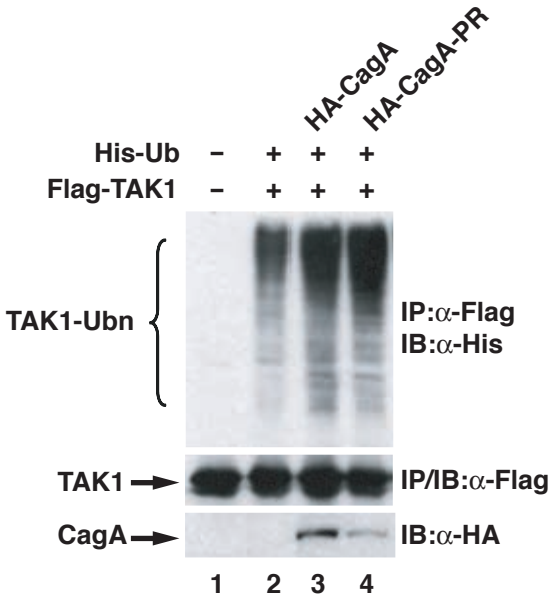

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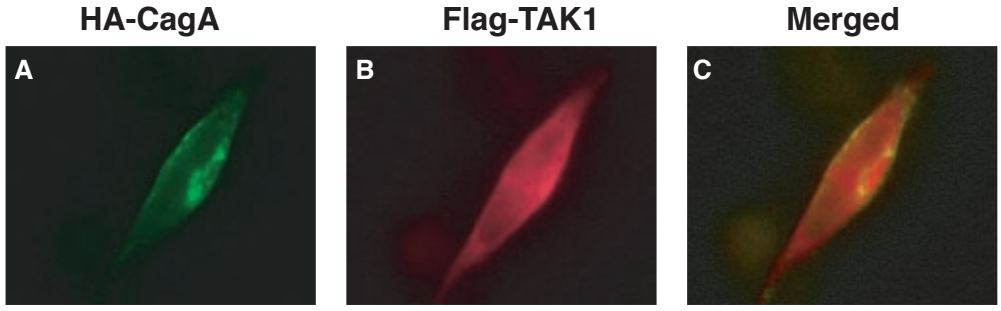

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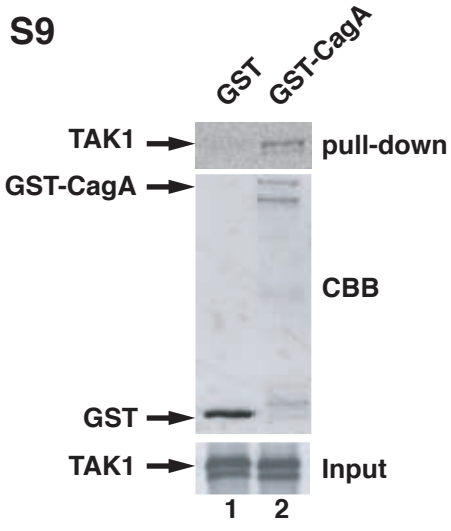

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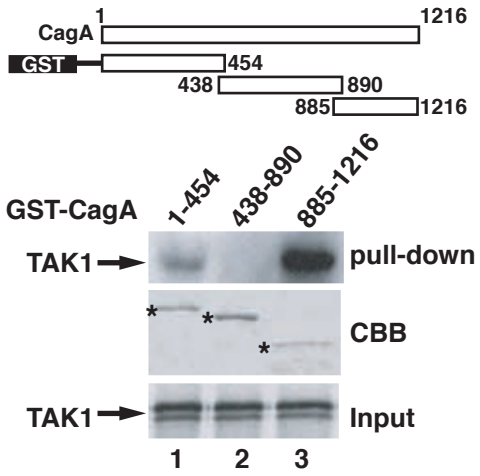

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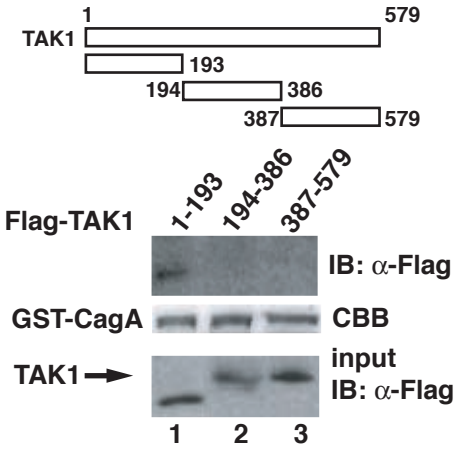

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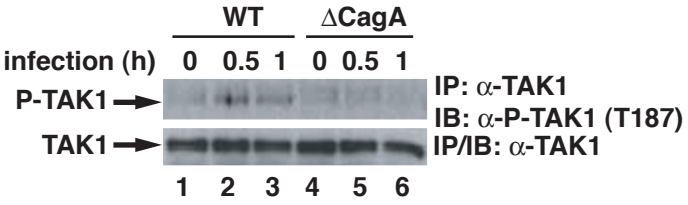

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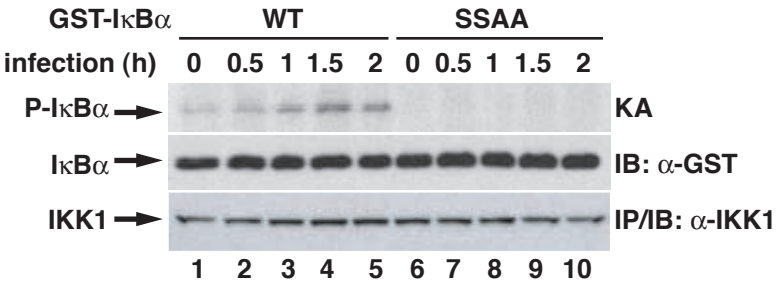

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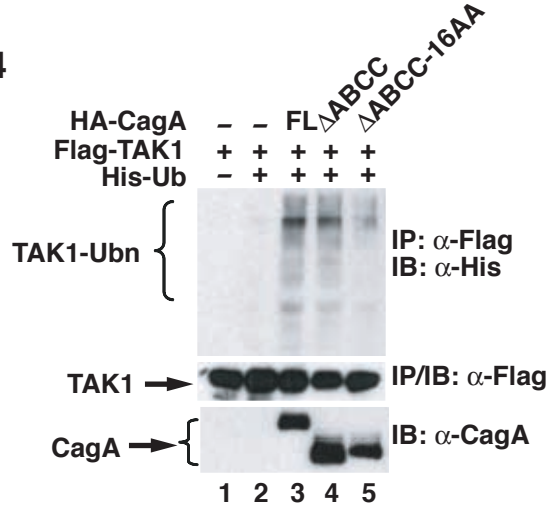

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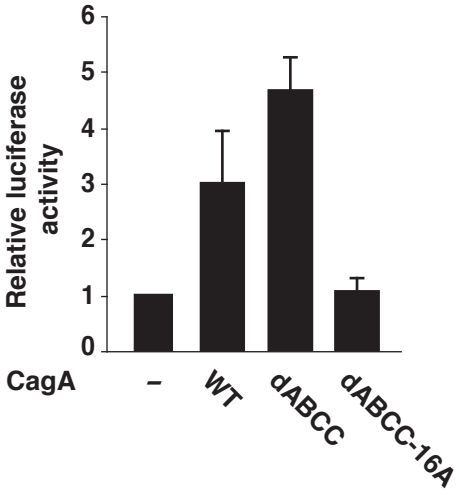

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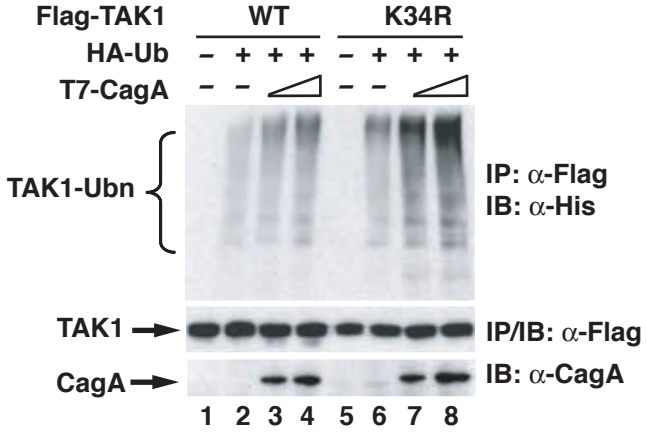

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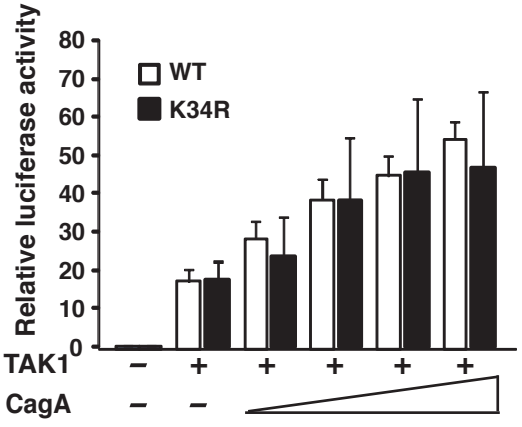

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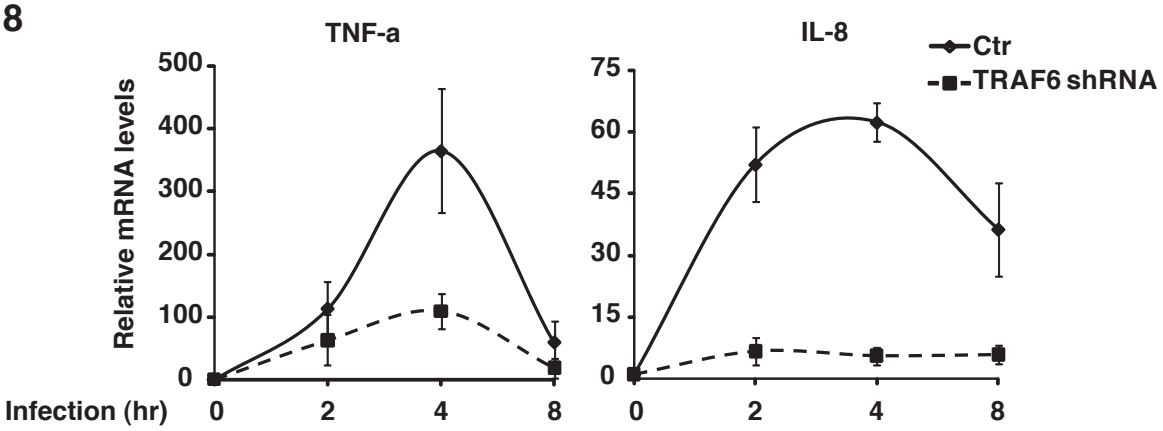

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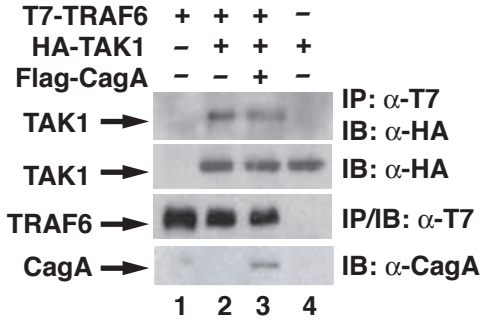

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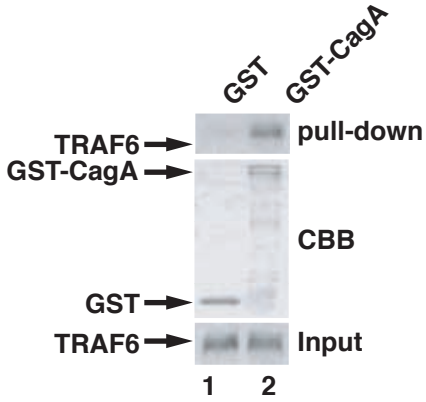

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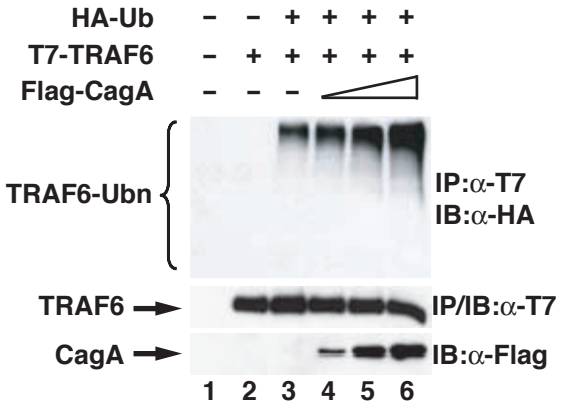

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