Supplemental Fig s1. TNFα treatment increases the expression level of Daxx

HeLa cells were treated with TNFα (5 ng/ml) for indicated time period. The cell extracts were subjected to western blotting for Daxx and actin.

Supplemental Fig s2. Identification of the Daxx region responsible for ASK1-dependent protein stabilization

293 cells were transfected with expression plasmids for the indicated EGFP-Daxx deletion mutants (open area), EGFP together with empty vector (3.1), ASK1-HA (ASK1) or ASK1 KM-HA (ASK1-KM) and incubated for 2 days. Cell extracts were analyzed by western blotting with anti-EGFP antibody. Expression level of EGFP serves as a reference. Note that the Daxx deletion constructs, 1-625, 1-418, and 70-216, were stabilized by ASK1.

Supplemental Fig s3. Daxx is a direct substrate for ASK1 kinase

In vitro ASK1 kinase assay was performed with bacterially produced GST, GST-Daxx 70-216 and GST-MKK6 as substrates. MKK6 is a MAP2K and a known substrate for ASK1,

and was used as a positive control in the experiments. ASK1-HA or ASK1 KM-HA was expressed in cells and used as an enzyme source. (A) 293 cells were transfected with ASK1-HA or ASK1 KM-HA expression plasmid and subjected to immunoprecipitation with anti-HA antibody. GST, GST-Daxx (70-216) or GST-MKK6 fusion protein was incubated with ASK1-HA in the presence of [γ-^{32}P] ATP and ASK1 kinase activities were determined as described in Materials and Methods. The reaction mixtures were separated on SDS-PAGE, and phosphorylated proteins were visualized by PhosphorImager. Arrows indicate the positions of GST, GST-Daxx (70-216), GST-MKK6 and ASK1-HA proteins. The expression levels of ASK1-HA and ASK1 KM-HA (B), as well as GST, GST-Daxx (70-216), and GST-MKK6 (C) were determined by western blotting with anti-GST antibody. Consistent with the previous report (1), autophosphorylation of ASK1 was also observed (a: lanes 1, 3 and 5) in wild type ASK1 but not in ASK1 KM mutant.

Supplemental Fig s4. Mapping of ASK1 phosphorylation sites within the N-terminal region of Daxx

Cell extracts prepared from 293 cells overexpressing ASK1-HA were subjected to immunoprecipitation with anti-HA antibody. Various Ser and Thr within the amino acids position 70-216 of Daxx were substituted to Ala, as indicated. Recombinant wild type and mutated GST-Daxx (70-216) proteins were incubated with immunopurified ASK1-HA in the presence of [γ-^{32}P] ATP. IP-kinase assay was carried out as described in Materials and Methods. The reaction mixtures were separated by SDS-PAGE and phosphorylated proteins were detected by PhosphorImager. Arrowheads indicate the positions of phosphorylated GST-Daxx (70-216) and ASK1-HA proteins, respectively. Note that a substitution mutant with Ser/Thr at the positions of 174, 176, 178, 181, and 184 to Ala failed to be phosphorylated by ASK1 (lane 5).

Supplemental Fig s5. Ser 176 and Ser 184 in Daxx are phosphorylated by ASK1

Ser and Thr within amino acids 70-216 of Daxx were substituted to Ala as indicated. ASK1-HA was immunopurified from 293 cell extracts and IP-kinase assay was performed as described in Materials and Methods. The reaction mixtures were then separated by

SDS-PAGE and the phosphorylation of GST-Daxx protein was measured by PhosphorImager. Note that a substitution mutant of Ser 176 and Ser 184 almost completely lost the ability to be phosphorylated by ASK1.

Supplemental Fig s6. Phosphorylation deficient Daxx mutants still interact with ASK1.

293 cells were transfected with FLAG-Daxx (WT, ST0-176/184S, ST0, or S176/184A) and ASK1 ΔN-HA expression plasmids in the combinations as indicated. Two days after transfection, cell extracts were prepared and subjected to immunoprecipitation with anti-FLAG antibody followed by western blotting with anti-HA or anti-FLAG antibody. The expression levels of FLAG-Daxx and ASK1 ΔN-HA were identified by western blotting.

Supplemental Fig s7. The characterization of anti-phospho-Daxx specific antibody

293 cells were transfected with expression plasmids for ASK1 KM-HA and the phosphorylation of endogenous Daxx was determined (A). HeLa cells expressing FLAG-

Daxx were treated with TNF-α (1 ng/ml) for 18 h and cell lysates were prepared (B). The cell extracts were immunoprecipitated with anti-phospho-Daxx specific antibody or normal rabbit IgG followed by western blotting with anti-Daxx or FLAG antibodies. The expression level of endogenous Daxx or FLAG-Daxx in cells was determined by western blotting with the indicated antibodies. Since the expression level of Daxx or FLAG-Daxx was increased by the presence of ASK1, the amounts of cell lysate in the immunoprecipitation reactions were adjusted to contain equal amount of Daxx protein (WB: Daxx or FLAG-Daxx). (C) FLAG-Daxx S176/184A was expressed with ASK1-HA or ASK1 KM-HA and cell lysates were prepared 2 days after transfection. (D) HeLa cells expressing FLAG-Daxx S176/184A were treated with TNFα (1 ng/ml) for 18 h and cell lysates were prepared. The cell extracts were immunoprecipitated with anti-phospho-Daxx specific antibody followed by western blotting with anti-FLAG antibody. The expression level of FLAG-Daxx S176/184A in cells was determined by western blotting with anti-FLAG antibody. Note that the recovery of FLAG-Daxx S176/184A by anti- phospho-Daxx specific antibody was not increased by ASK1 and TNFα treatment. Both the ectopic expression of ASK1 and TNFα treatment

induce the phosphorylation at Ser 176 and Ser 184 in Daxx.

Supplemental Fig s8. Daxx plays a critical role on apoptosis induced by TNFα

ME180 cells were transfected with control, Daxx-1 or Daxx-2 siRNA. Twenty-four hours after transfection, cells were serum starved for 20 h and incubated with TNFα (0, 1 or 5 ng/ml) for 18 h. Cell killing effect was determined by the induction cleaved PARP fragment. The cell extracts were subjected to western blotting for PARP, Daxx and actin.

Supplemental Fig s9. Phosphorylation on Ser 176 and Ser 184 in Daxx plays a critical role on TNFα-induced apoptosis

FLAG-Daxx WT or FLAG-Daxx S176/184A mutant expression plasmid was transfected into ME180 cells and TNFα (0, 1 or 5 ng/ml) treatment was performed to induce apoptosis. Cell lysates were analyzed by western blotting for visualizing cleaved PARP as an apoptotic marker. Total (endogenous and ectopically expressed) amount of Daxx was determined by western blotting with anti-Daxx antibody. The amount of actin serves as a

loading control.

Supplemental Fig s10. Mutant p53 inhibits the interaction between ASK1 and Daxx

Expression plasmids for R175H p53 mutant, ASK1-HA or ASK1 KM-HA together with FLAG-Daxx were transfected into 293 cells in the indicated combinations. Two days after transfection, cells were harvested and immunoprecipitation was performed by using anti-FLAG antibody. The immune complexes were analyzed by western blotting with anti-HA antibody. The expression levels of FLAG-Daxx, ASK1-HA, p53 R175H (the lower bands indicated by asterisk are endogenous wild type p53 from 293 cells) and actin were identified by western blotting.

Supplemental Fig s11. The ASK1-Daxx positive feedback loop and the gain-of-function of mutant p53

This model illustrates that ASK1 directly binds to, phosphorylates and stabilizes Daxx. The fact that Daxx itself activates ASK1 suggests the existence of the positive feedback loop

between Daxx and ASK1, which will amplify the stress signals from TNFα to support sustained activation of MAP kinases. Tumorigenic mutant p53, which often accumulates at a large amount in cells function as negative regulators to this positive feedback loop by preventing the interaction between ASK1 and Daxx, and thus stimulating Daxx destabilization.

Reference

1. Tobiume K, Saitoh M, Ichijo H. Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of pre-formed oligomer. J Cell Physiol 2002; 191: 95-104.

Fig. s1

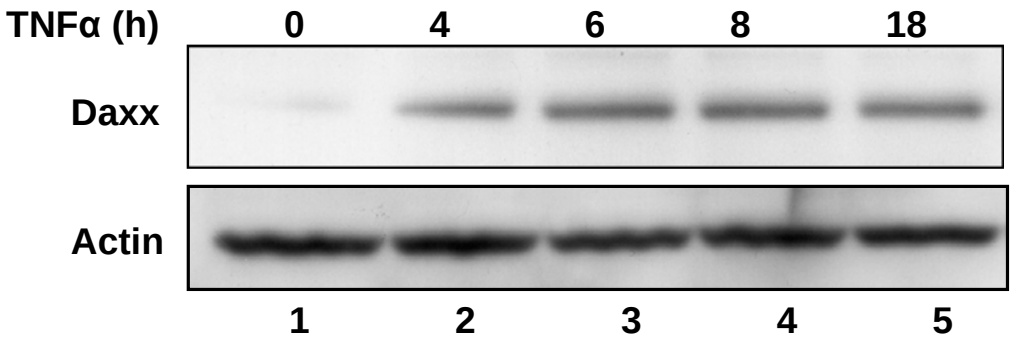
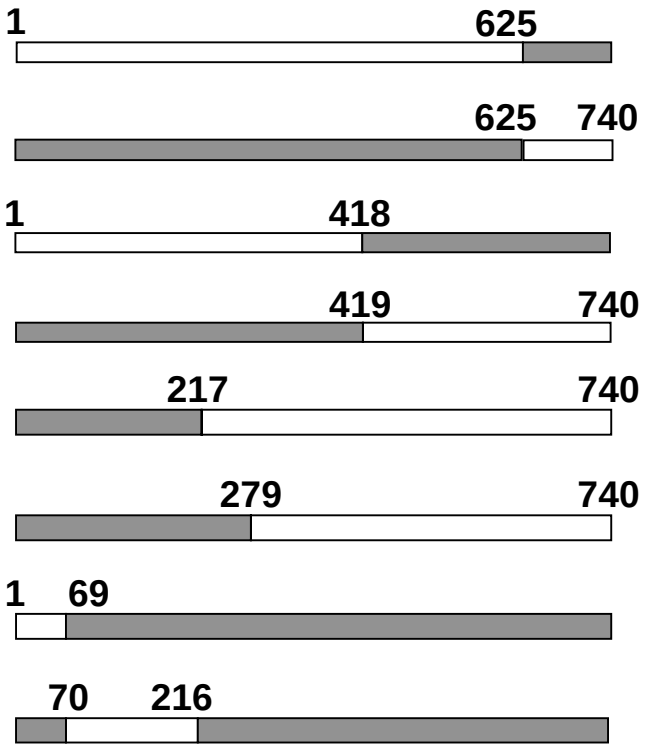
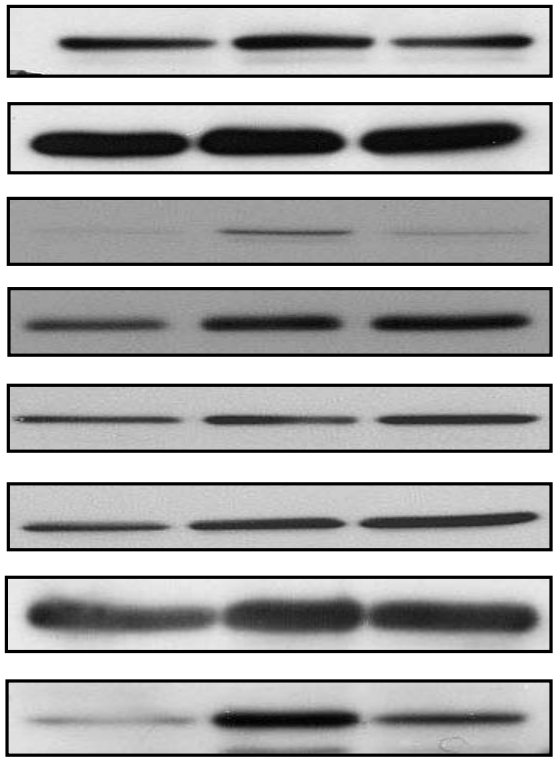


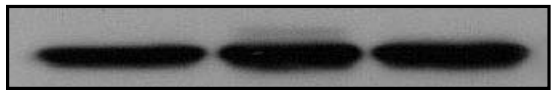
Fig. s2



Deletion mutants of Daxx

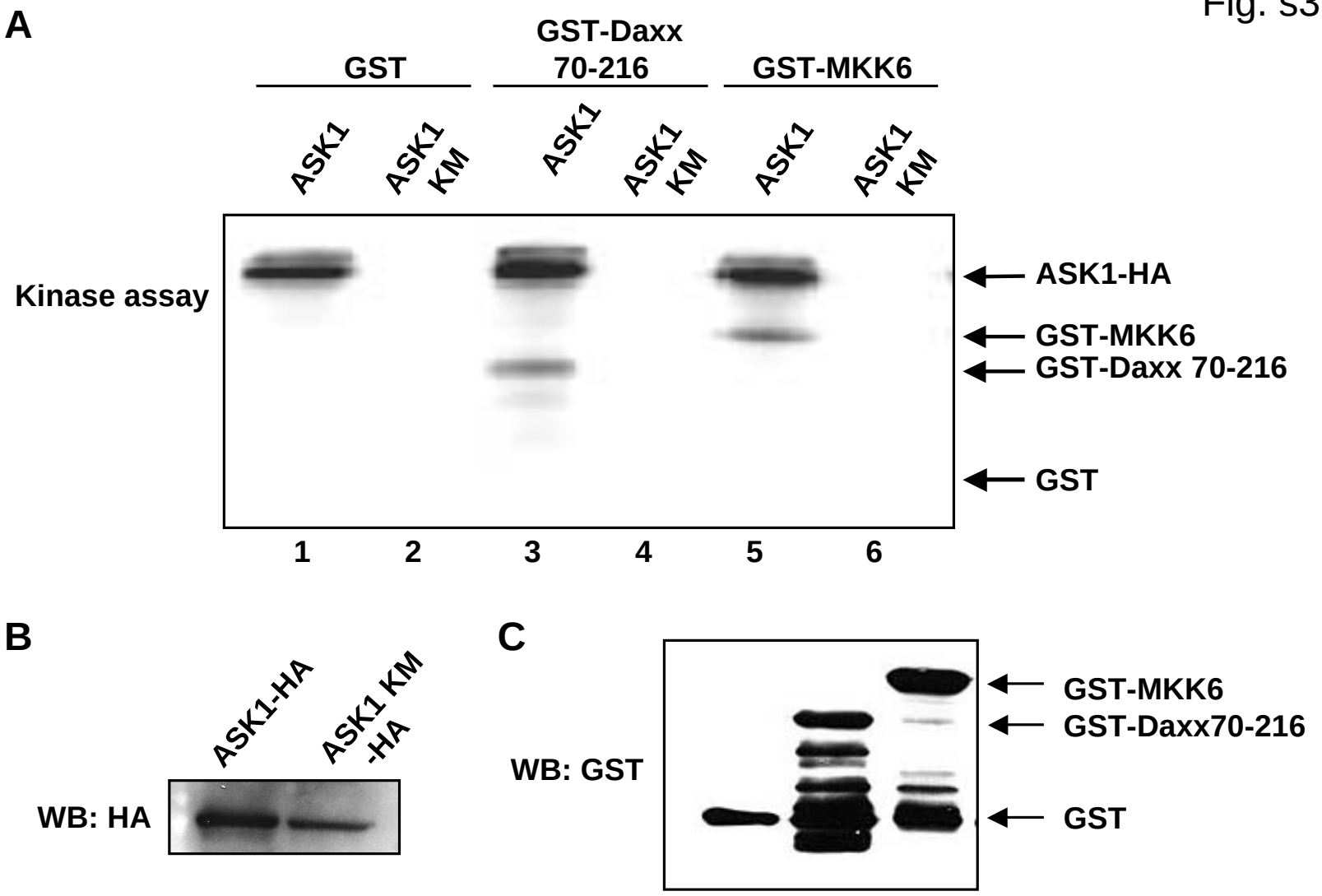


	Stabilization
1-625	○
625-740	x
1-418	○
419-740	x
217-740	x
279-740	x
1-69	x
70-216	○



Control EGFP

Fig. s3


A
GST
GST-Daxx 70-216
GST-MKK6
ASK1
ASK1 KM
ASK1
ASK1 KM
ASK1
ASK1 KM
Kinase assay
ASK1-HA
GST-MKK6
GST-Daxx 70-216
GST
1
2
3
4
5
6
B
ASK1-HA
ASK1 KM -HA
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C
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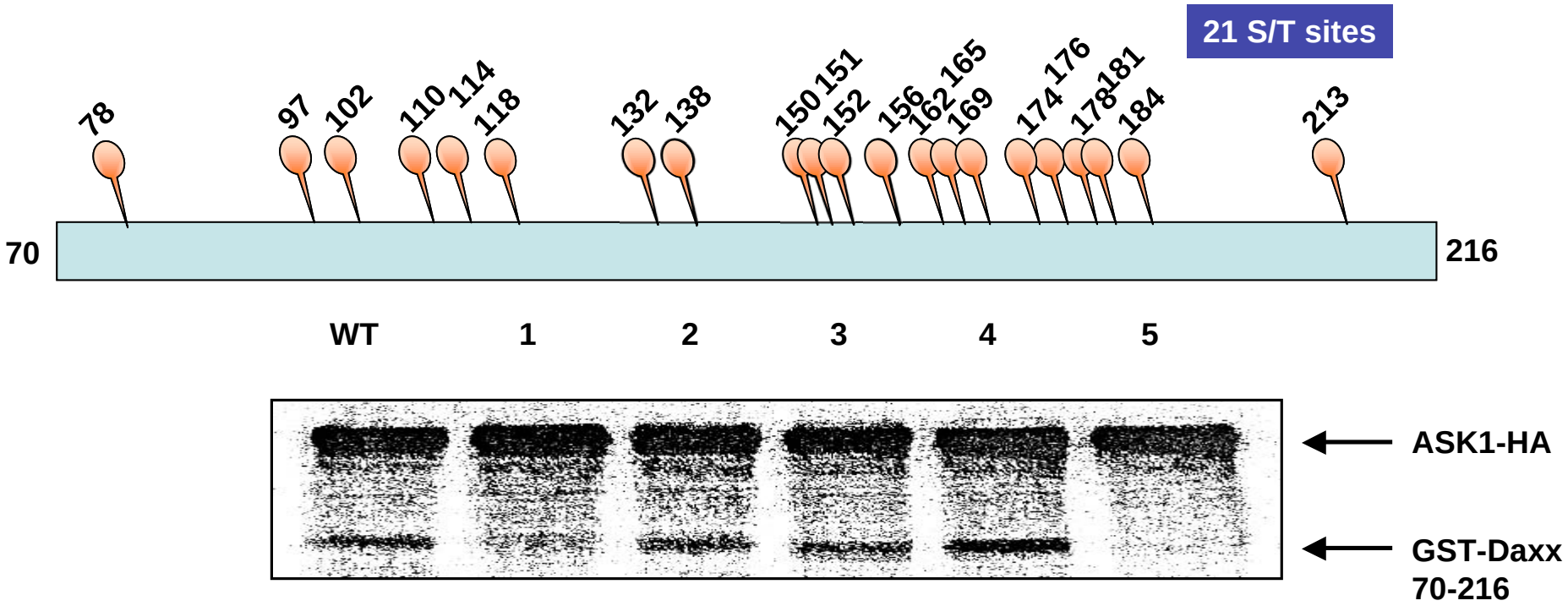
GST

Fig. s4



WT : Wild Type

Lane 1 : S/T 97, 102, 110, 114, 118, 132, 138 → A

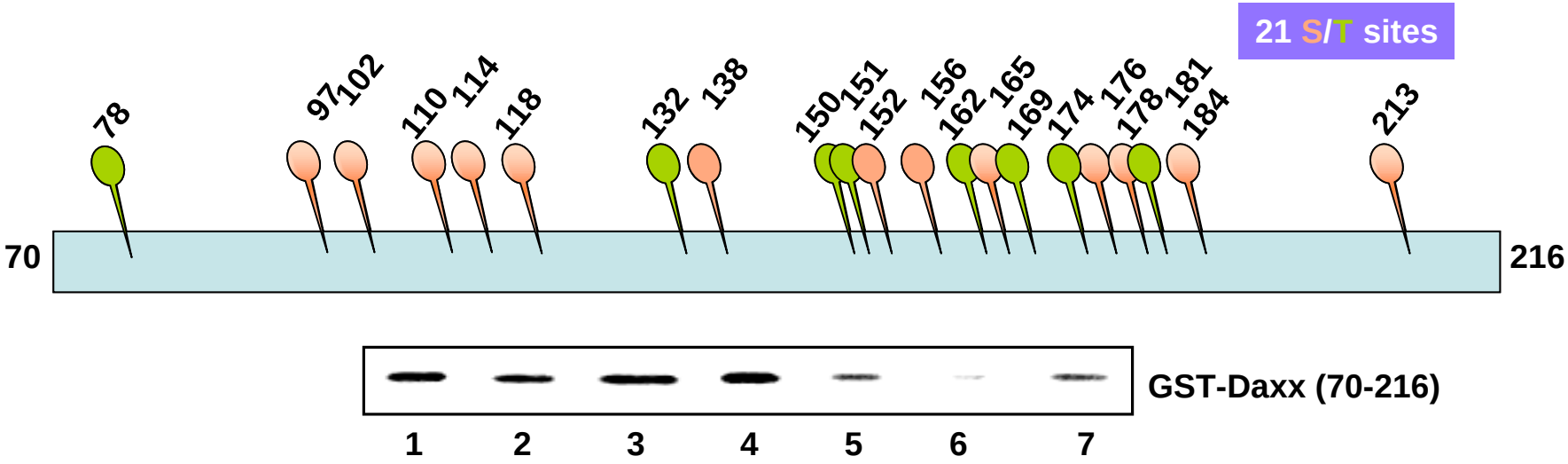
Lane 2 : S/T 132, 138 → A

Lane 3 : S/T 162, 165, 169 → A

Lane 4 : S/T 174, 176, 178 → A

Lane 5 : S/T 174, 176, 178, 181, 184 → A

Fig. s5



Lane 1 : S 110, 114, 118 → A

Lane 2 : S 152, 156 → A

Lane 3 : S 213 → A

Lane 4 : T 174, 181 → A

Lane 5 : S/T 176, 181 → A

Lane 6 : S 176, 184 → A

Lane 7 : S/T 181, 184 → A

Fig. s6

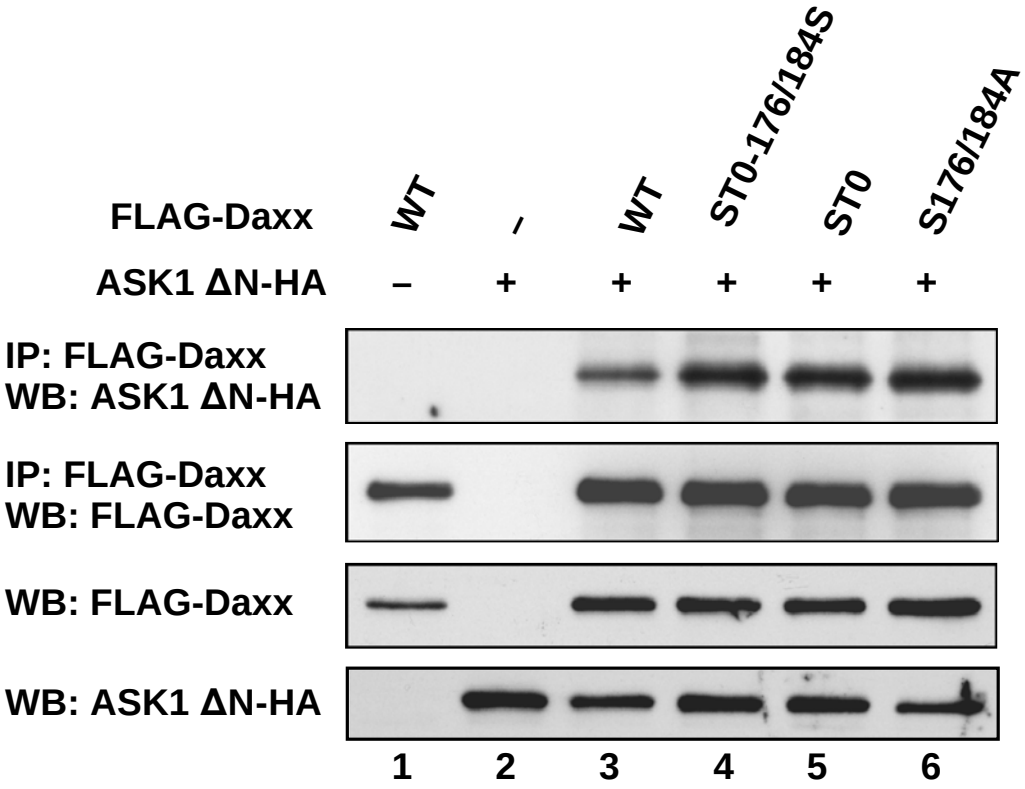
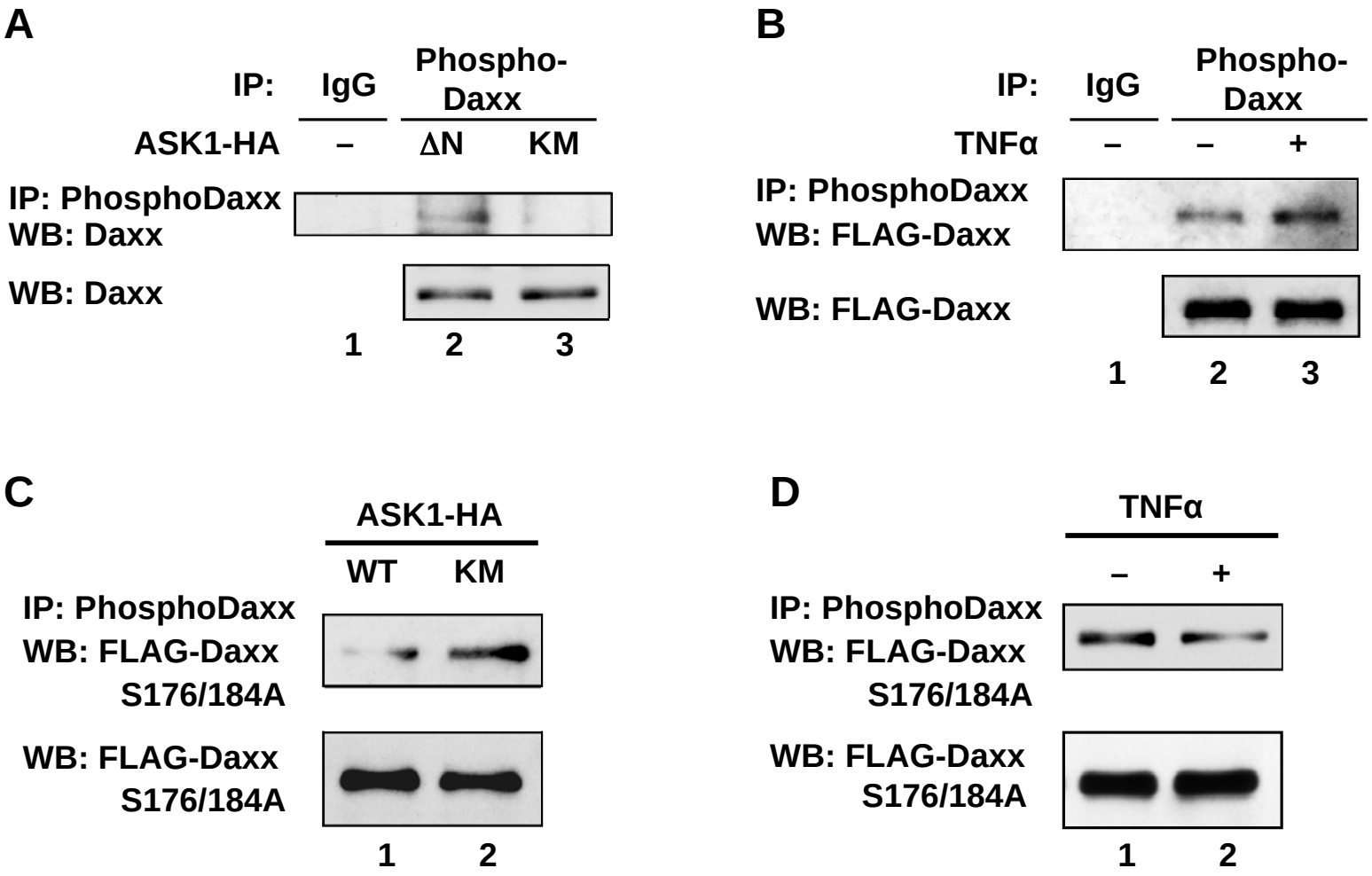

FLAG-Daxx
WT
-
WT
ST0-176/184S
ST0
S176/184A
ASK1 ΔN-HA
–
+
+
+
+
+
IP: FLAG-Daxx
WB: ASK1 ΔN-HA
IP: FLAG-Daxx
WB: FLAG-Daxx
WB: FLAG-Daxx
WB: ASK1 ΔN-HA
1
2
3
4
5
6

Fig. s7


A
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Phospho-Daxx
ASK1-HA
–
ΔN
KM
IP: PhosphoDaxx
WB: Daxx
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1
2
3
B
IP:
IgG
Phospho-Daxx
TNFα
–
–
+
IP: PhosphoDaxx
WB: FLAG-Daxx
WB: FLAG-Daxx
1
2
3
C
ASK1-HA
WT
KM
IP: PhosphoDaxx
WB: FLAG-Daxx S176/184A
WB: FLAG-Daxx S176/184A
1
2
D
TNFα
–
+
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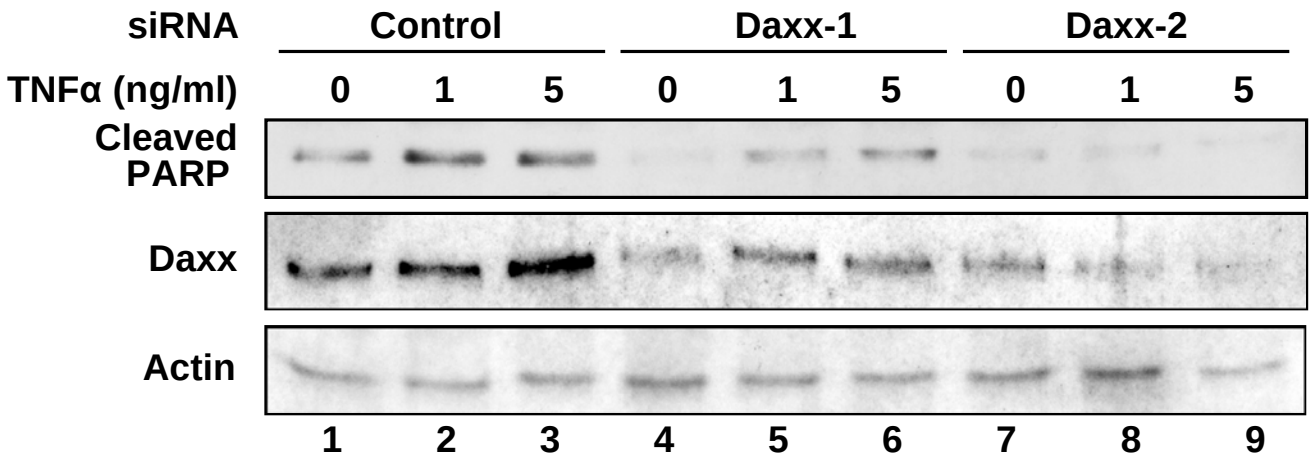
1
2

Fig. s8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Control
Daxx-1
Daxx-2
TNFα (ng/ml)
0 1 5 0 1 5 0 1 5
Cleaved PARP
Daxx
Actin
1 2 3 4 5 6 7 8 9

Fig. s9

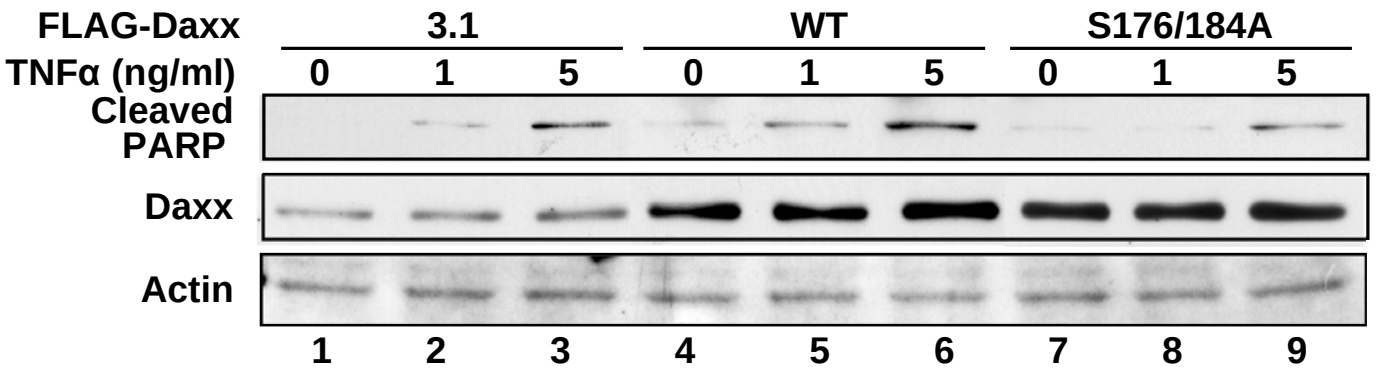
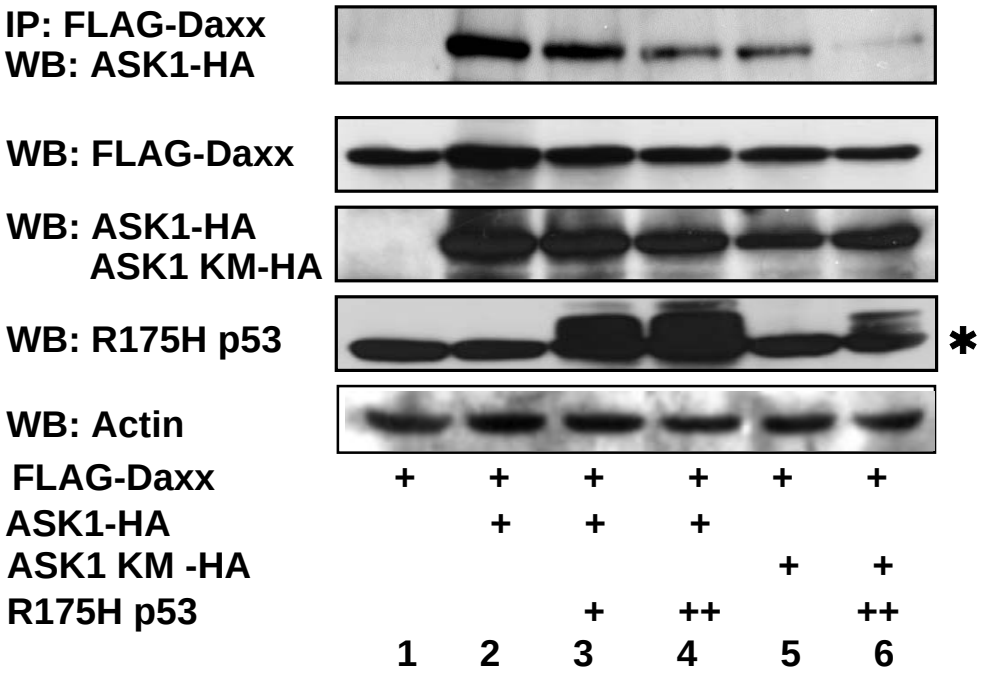

FLAG-Daxx
3.1
WT
S176/184A
TNFα (ng/ml)
0
1
5
0
1
5
0
1
5
Cleaved PARP
Daxx
Actin
1
2
3
4
5
6
7
8
9

Fig. s10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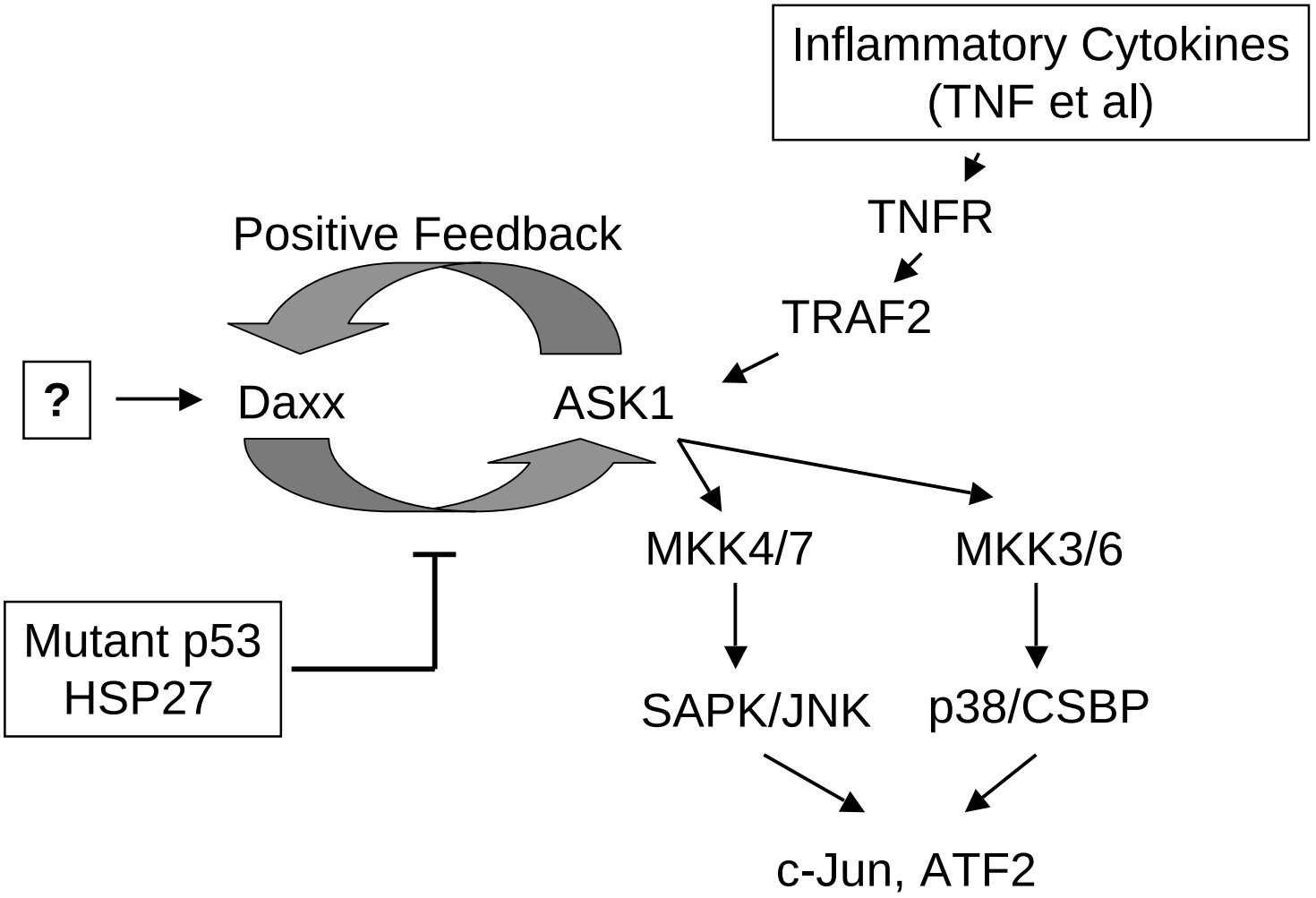
WB: ASK1-HA
WB: FLAG-Daxx
WB: ASK1-HA
ASK1 KM-HA
WB: R175H p53
*
WB: Actin
FLAG-Daxx + + + + + +
ASK1-HA + + +
ASK1 KM -HA + +
R175H p53 + ++ ++
1 2 3 4 5 6

Fig. s11


Inflammatory Cytokines
(TNF et al)
TNFR
TRAF2
Positive Feedback
?
Daxx
ASK1
MKK4/7
MKK3/6
Mutant p53
HSP27
SAPK/JNK
p38/CSBP
c-Jun, ATF2