

*Supplemental Material for*

**TUMOR SUPPRESSOR RAS-ASSOCIATION DOMAIN FAMILY 5  
(RASSF5/NORE1) MEDIATES DEATH RECEPTOR LIGAND-  
INDUCED APOPTOSIS**

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Running Head: Rassf5 mediates TNF- $\alpha$  induced apoptosis.

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## Supplemental Figure Legend

**Supplement Fig. 1.** siRNA knockdown of *RASSF5*. U2OS cells were transfected with either control or *RASSF5* siRNA using Lipofectamine 2000 (Invitrogen), and total RNA isolated at 48 hrs post-transfection was analyzed for *RASSF5* and *GAPDH* transcripts by TaqMan RT-PCR analysis. (Applied Biosystems, Foster City, CA). Data were analyzed by comparative Ct method using *GAPDH* as an endogenous control and the *RASSF5* transcript levels were expressed as relative to the *RASSF5* transcript levels in untransfected U2OS cells (set to 1).

**Supplement Fig. 2.** U2OS cells cotransfected with Flag-*RASSF5* and MST1 were immunoprecipitated with either anti-FLAG (M2, Sigma) or anti-MST1 (Cell Signaling) antibodies and analyzed by Western blotting. Anti-IKK and anti-p65 (NFkB subunit) antibodies (Cell Signaling) were used as negative controls.

**Supplement Fig. 3.** U2OS cells cotransfected with Flag-*RASSF5* and TNF-R1 were immunoprecipitated with anti-TNF-R1 antibody (Cell Signaling) and analyzed by Western blotting using antibodies against *RASSF5*, MST1, WW45, YAP1 and LATS1.

**Supplement Fig. 4.** U2OS cells were transfected with control or *MST1*, *WW45*, *LATS1* or *YAP1* siRNA and cell lysates were analyzed by Western blotting with MST1, WW45, LATS1, YAP1 and  $\beta$ -actin antibodies.

**Supplement Fig. 5.** (A) *Rassf5*<sup>+/+</sup> or *Rassf5*<sup>-/-</sup> MEFs were treated with varying concentration of TNF- $\alpha$  and cycloheximide (10 $\mu$ g/ml) for 18 hr, and cells were stained with Annexin V and propidium iodide (PI) using Annexin V-FLOUS Staining kit (Roche). Stained cells were analyzed by FACSCalibur (BD Biosciences). A representative result from three independent experiments is shown. The numbers in each quadrant indicates percentage of cells positive for Annexin V, PI or both. Quadrant **I**: early-apoptosis population (Annexin V<sup>+</sup>; PI<sup>-</sup>), **II**: late apoptosis population (Annexin V<sup>+</sup>; PI<sup>+</sup>), **III**: necrosis population (Annexin V<sup>-</sup>; PI<sup>+</sup>). (B) Quantitation of three independent experiments performed as described in (A).

**Supplement Fig. 6.** (A) Total RNAs were isolated from *Rassf5*<sup>+/+</sup> or *Rassf5*<sup>-/-</sup> MEFs and expression of FADD, TRADD, TRAF2 or TNF-R1 was analyzed by real-time RT-PCR using TaqMan Probes (Applied Biosystems). Data were analyzed by comparative Ct method using *GAPDH* as an endogenous control and represent results from three independent experiments. (B) Cell lysates from U2OS cells transfected with control or *RASSF5* siRNA and *Rassf5*<sup>+/+</sup> or *Rassf5*<sup>-/-</sup> MEFs were analyzed by Western blotting with antibodies against TRAF2 (Cell Signaling), TNF-R1 (Cell Signaling) and  $\beta$ -actin.

**Supplement Fig. 7.** *Rassf5*<sup>+/+</sup> and *Rassf5*<sup>-/-</sup> mice were injected via tail vein with TNF- $\alpha$  (20 $\mu$ g/kg in PBS) and livers were dissected at 6 hr. Representative images of the livers are shown.

**Supplement Fig. 8.** Activation of Mst1 requires Rassf5. **(A)** *Rassf5* wildtype and mutant mice were injected via tail vein with TNF- $\alpha$  (20 $\mu$ g/kg in PBS) and at 6 hrs post-injection, livers were dissected, fixed in formalin and paraffin-embedded. Sectioned livers were then immunostained with anti-MST1 antibody (Bethyl Laboratories) and mounted with DAPI containing mounting medium (Vector Laboratories). Arrowheads indicate cells with nuclear Mst1. Images were taken with confocal LSM 5 Live/Axio Observer.Z1 microscope (Carl Zeiss MicroImaing. Inc). Bar= 20 $\mu$ m. **(B)** Liver extracts from *Rassf5* wildtype and mutant mice injected via tail vein with TNF- $\alpha$  (20 $\mu$ g/kg in PBS) were analyzed by Western blotting with anti-phospho-Mst1/2 (Ser183/180) and  $\alpha$ -tubulin antibodies.

**Supplement Fig. 9.** U2OS cells transfected with either control or *RASSF5* siRNA were treated with varying concentration of TNF- $\alpha$  and at indicated times, cell lysates were analyzed for Western blotting with antibodies against, I $\kappa$ B, phospho-I $\kappa$ B, IKK, and phospho-IKK (Cell Signaling). Anti-  $\beta$ -actin was used as loading control.

**Supplement Fig. 10.** *Rassf5*<sup>+/+</sup> and *Rassf5*<sup>-/-</sup> MEFs were treated with different doses of tamoxifen, staurosporine, nocodazole or methyl methanesulfonate (MMS) and cell survival was measured using Cell Counting kit-8 (Dojindo).

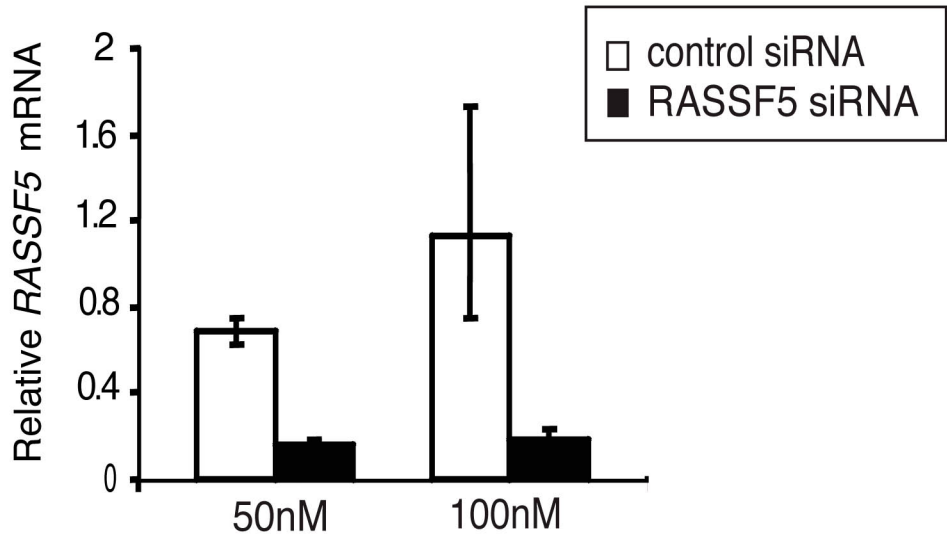
**Supplement Table 1.** Re-introduction of *Rassf5* in *Rassf5*-null MEFs modestly enhances TNF- $\alpha$  induced apoptosis.

	MEFs	Caspase 3 activity
Untransfected	<i>Rassf5</i> +/+	211260.1 $\pm$ 14024.3
	<i>Rassf5</i> -/-	17335.4 $\pm$ 966.9
Empty vector	<i>Rassf5</i> +/+	254312.4 $\pm$ 19999.7
	<i>Rassf5</i> -/-	15902.7 $\pm$ 2030.0
CMV- <i>Rassf5</i>	<i>Rassf5</i> +/+	278770.0 $\pm$ 36473.4
	<i>Rassf5</i> -/-	23223.7 $\pm$ 2880.4

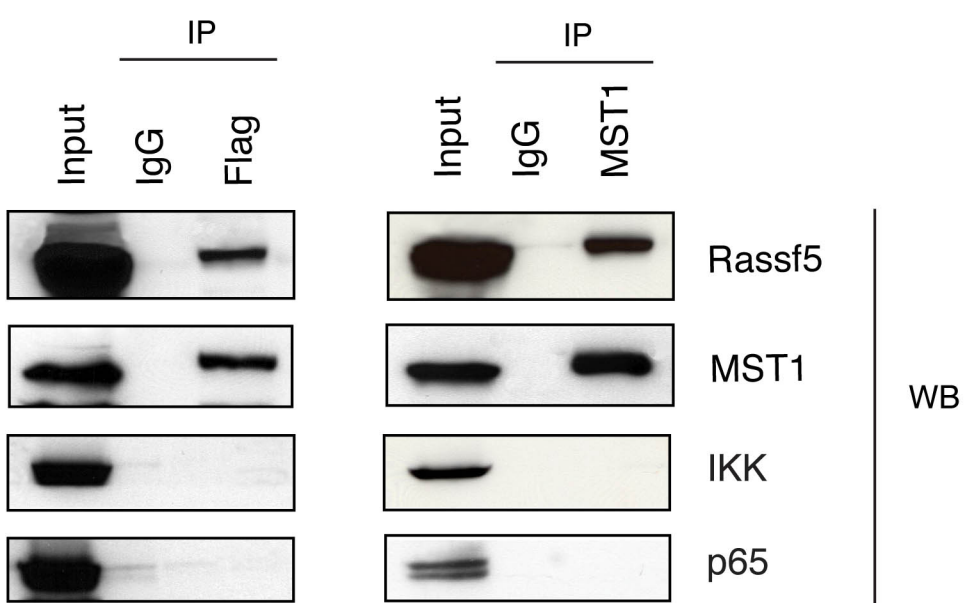
**Supplement Table 2.** Spontaneous tumors in *Rassf5* mice

Genotype	Tumor	Cases
<i>Rassf5</i> <sup>+/+</sup> (n=21)	Lymphoma	6/21
	Lung adenoma	2/21
<i>Rassf5</i> <sup>+/-</sup> (n=28)	Lymphoma	8/28
	Lung adenoma	3/28
	Hepatic adenoma	2/28
<i>Rassf5</i> <sup>-/-</sup> (n=28)	Lymphoma	6/28
	Lung adenoma	3/28
	Hepatic adenoma	1/28

n=total number of animals

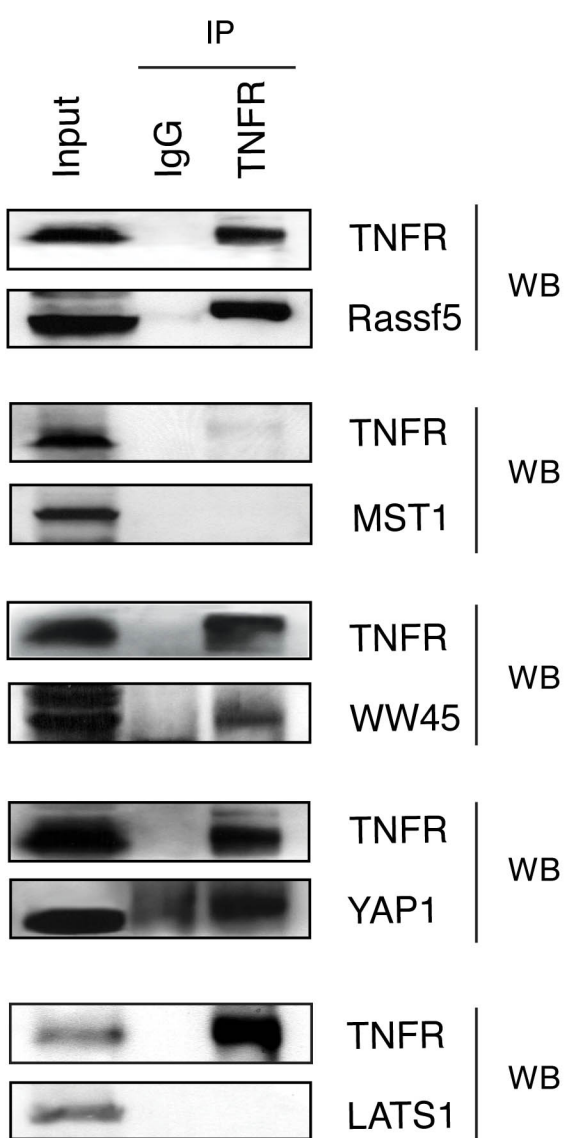


Supplement Figure 1

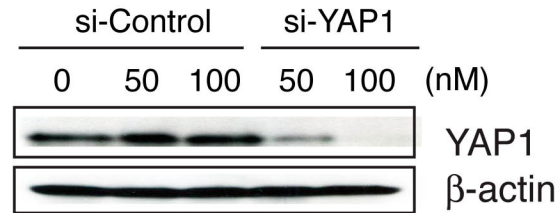
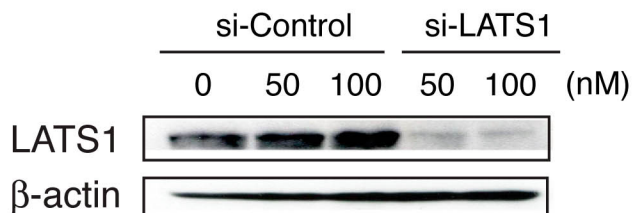
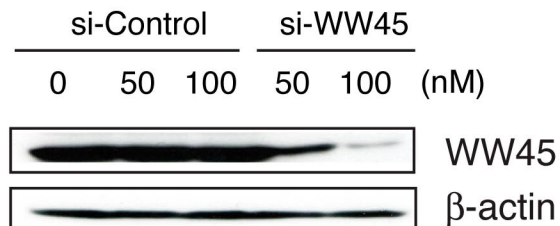
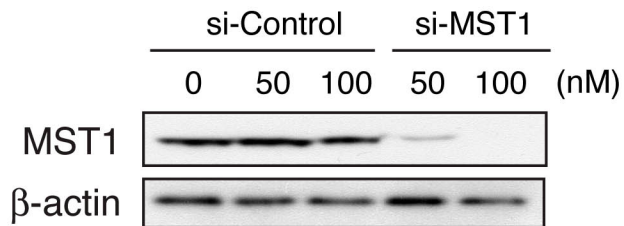


Supplemental Figure 2



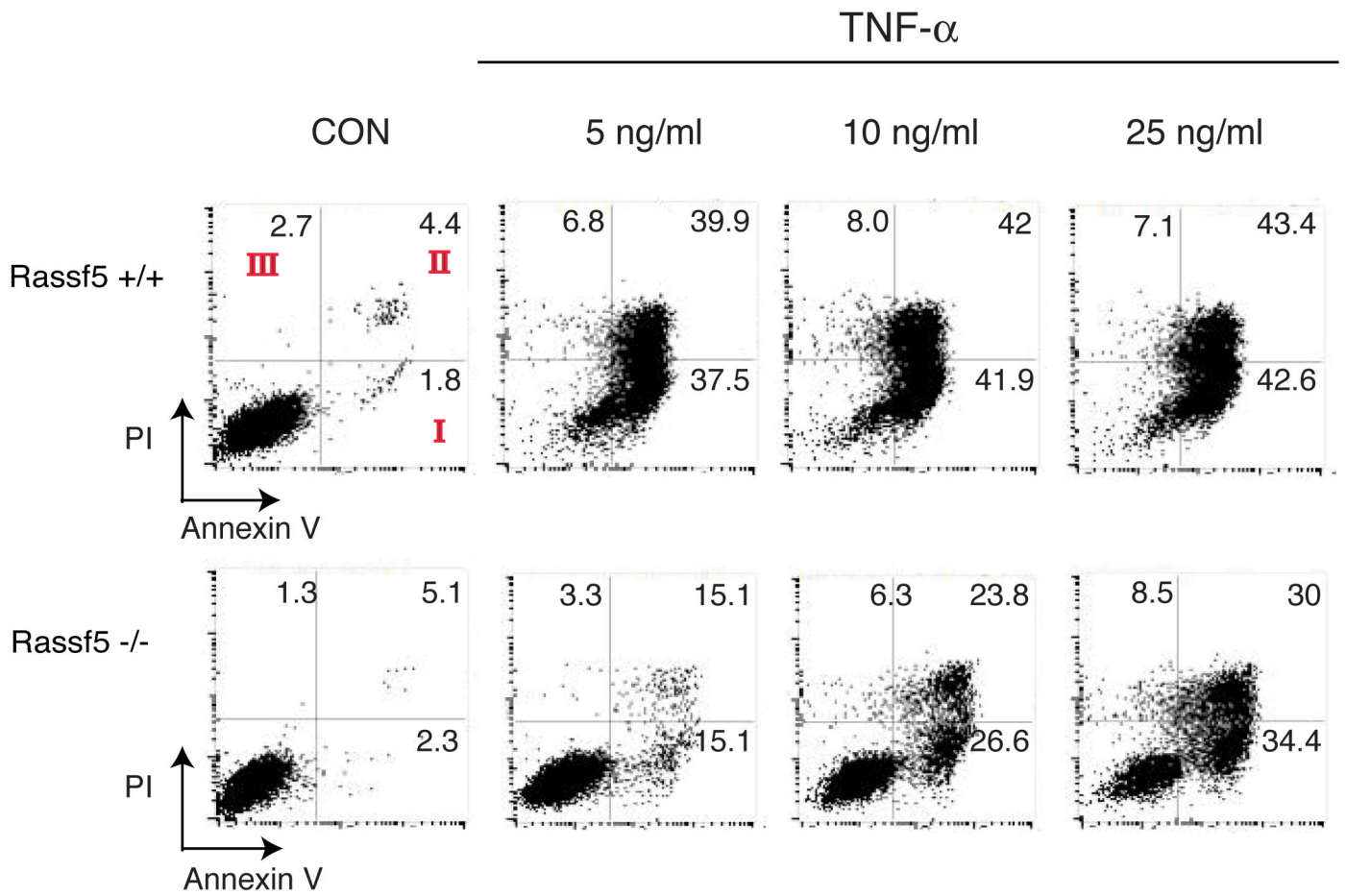


Supplemental Figure 3

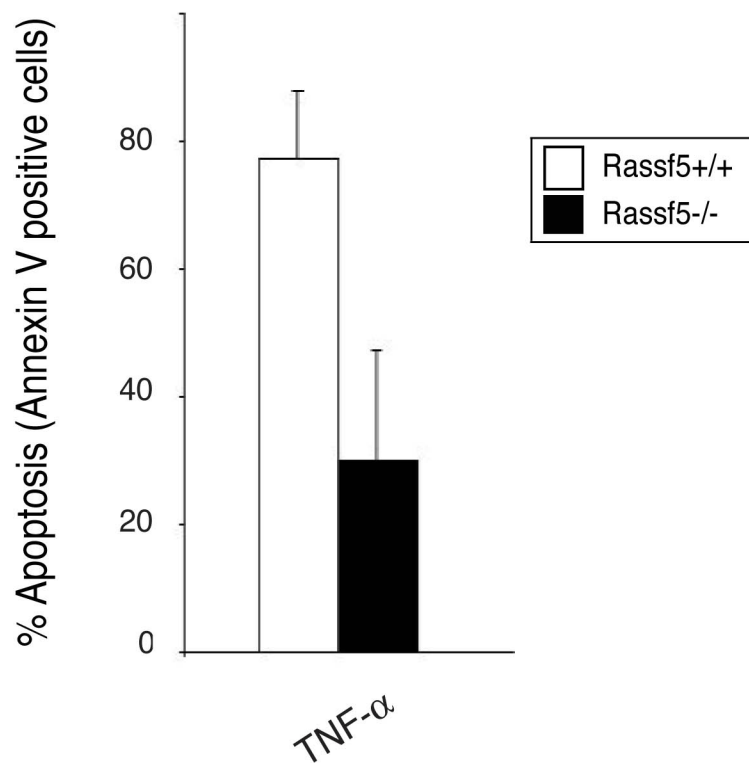


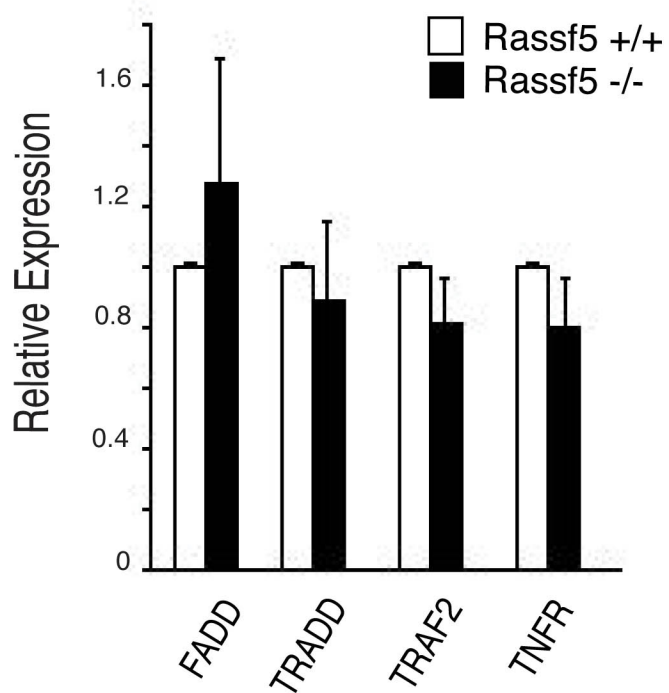
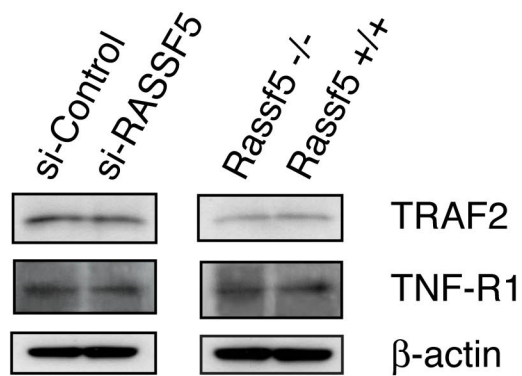
Supplement Figure 4

A



B



**A****B**

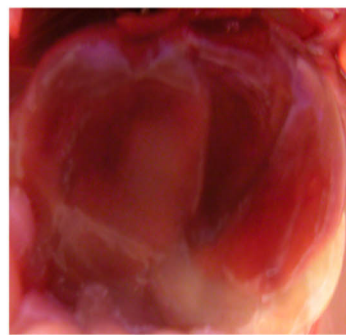
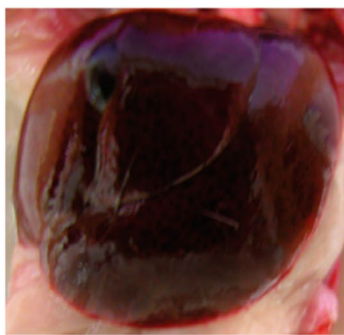
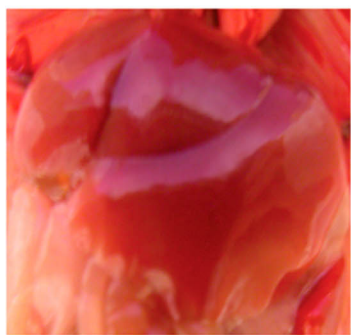
+ TNF- $\alpha$  (6 hrs)

No TNF- $\alpha$

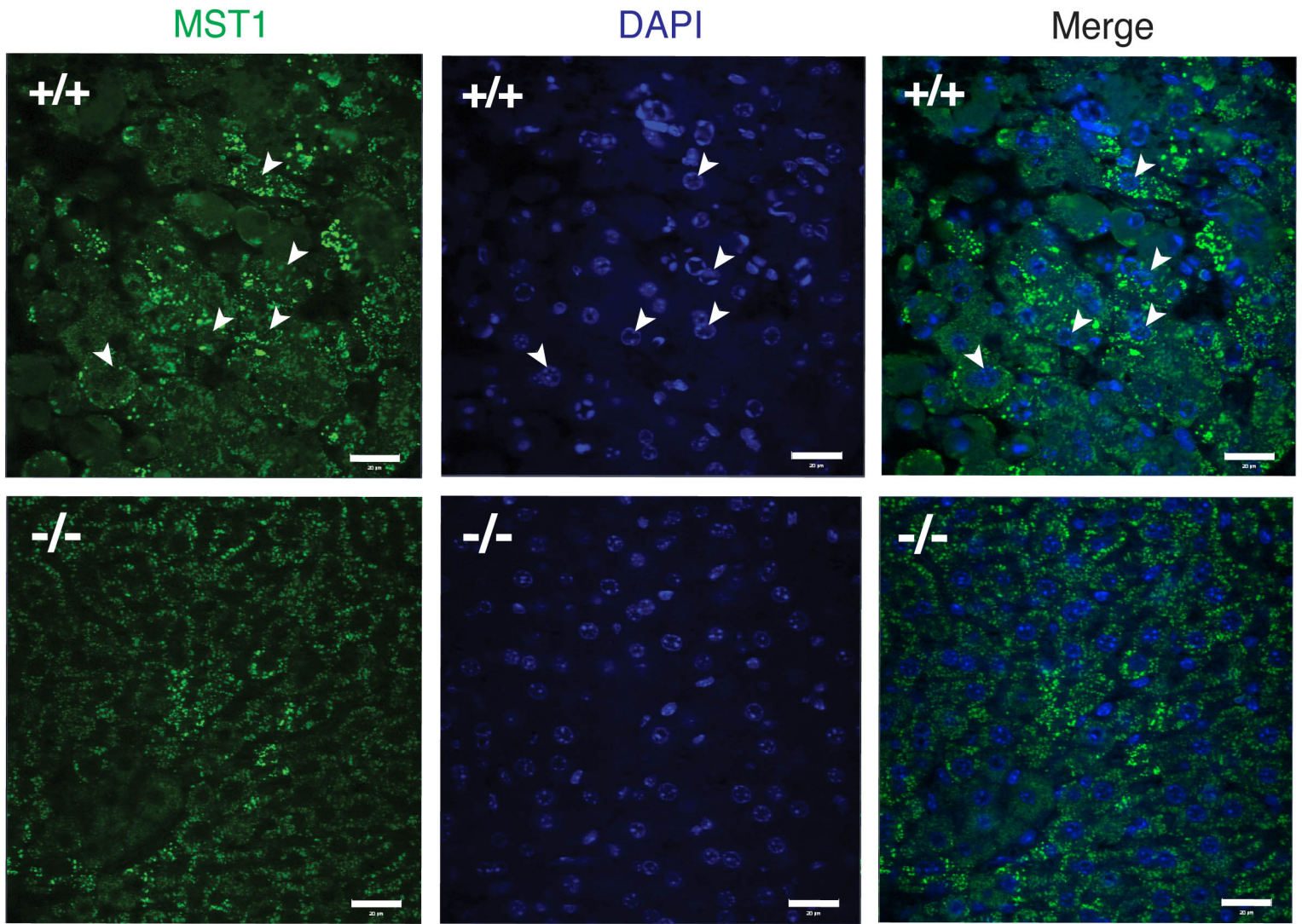
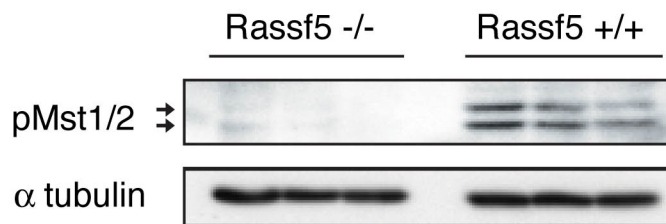
Rassf5 -/-

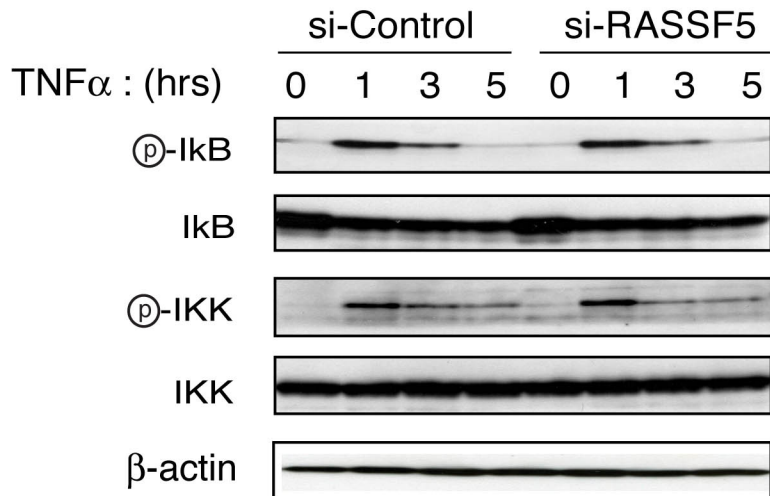
Rassf5 +/+

Rassf5 +/-



Supplement Figure 7

**A****B**



Supplemental Figure 9

