JBC/2009/082347

SUPPLEMENTAL MATERIAL

The ubiquitin ligase Nedd4-1 is required for heart development and is a suppressor of Thrombospondin-1

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Supplementary Methods:

Co-immunoprecipitation (Co-IP): WT, Nedd4-1$^{+/-,trap}$ and Nedd4-1$^{-/-trap}$ MEF cells were lysed with 1 ml of lysis buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 1% Triton X-100 and 5mM EDTA, 10μg/ml leupeptin, aprotinin and pepstatin each, plus 1mM PMSF, 50mM NaF, 1mM $NaVO_4$, 50μM LnLL(*N*-acetyl-Leu-Leu-norleucinal) and 0.4mM chloroquine), cleared by centrifugation and used for immunoblotting or Co-IP experiments. For Co-IP, 1 mg of cell lysates were pre-cleared with Protein G-Sepharose beads overnight at 4°C, incubated with 2 μg of antibody (anti-Tsp-1) per mg of protein overnight (4°C), then incubated for 1.5 h with Protein G–Sepharose beads at 4°C. Beads were then washed with lysis buffer (2x) and 3 times with HNTG (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100). Bound proteins were resolved on SDS-PAGE, and immunoblotted with anti Nedd4-1 antibodies. For Co-IP in transfected Hek293T cells, a similar procedure was used, only Flag-Tsp-1 was immunoprecipitated with anti-Flag M2 agarose beads, and immunoblotted with anti GFP antibodies to recognize the transfected GFP-tagged Nedd4-1, as below.

Ubiquitination and Pulse-chase assays: For analysis of Tsp-1 ubiquitination, Hek293T cells were transfected with HA-ubiquitin (HA-Ub), Flag-Tsp-1 (human) and GFP-Nedd4-1(human). Cells were treated with MG132 (20 μM) and chloroquine (100μM) for 3h and then lysed in lysis buffer supplemented with 50 μM LLnL (*N*-acetyl-Leu-Leu-norleucinal; Sigma-Aldrich). To ensure ubiquitination of Tsp-1 and not of associated proteins, the cell lysate (1mg protein) was boiled for 5 min in 1% SDS. The boiled lysates were then diluted 11 times with lysis buffer to dilute the SDS before their incubation with 30μl anti Flag antibodies (M2-agarose, Sigma) per mg protein. Beads were washed twice with lysis buffer and twice with HNTG and immunoblotted with anti-HA antibody to detect ubiquinitated Tsp-1. For analysis of ubiquitination of endogenous Tsp-1, WT and Nedd4-1$^{-/-trap}$ MEFs were treated as above, and cell lysates incubated with 25μl Protein G–Sepharose beads bound to 2-4μg anti Tsp-1 antibody per mg protein. Anti ubiquitin antibodies were used to detect ubiquitinated Tsp-1.

For pulse-chase studies, WT and Nedd4-1$^{-/-trap}$ MEFs were treated with cycloheximide (CHX, 50 μg/ml) for 0, 30, 60 and 90 min., and 40 μg protein per sample separated on SDS-PAGE, immunoblotted for Tsp-1 and quantified by densitometry.

Supplementary Figure Legends

Supplementary Figure S1: Thoracic sections through hearts of Nedd4-1 knockout (-/-) and WT (+/+) E13.5 embryos. Serial (5 μm) sections of the heart stained with H&E are shown, revealing heart defects in the Nedd4-1 knockout mice. (some sections, which reveal the same pattern as the preceding ones, are not shown).

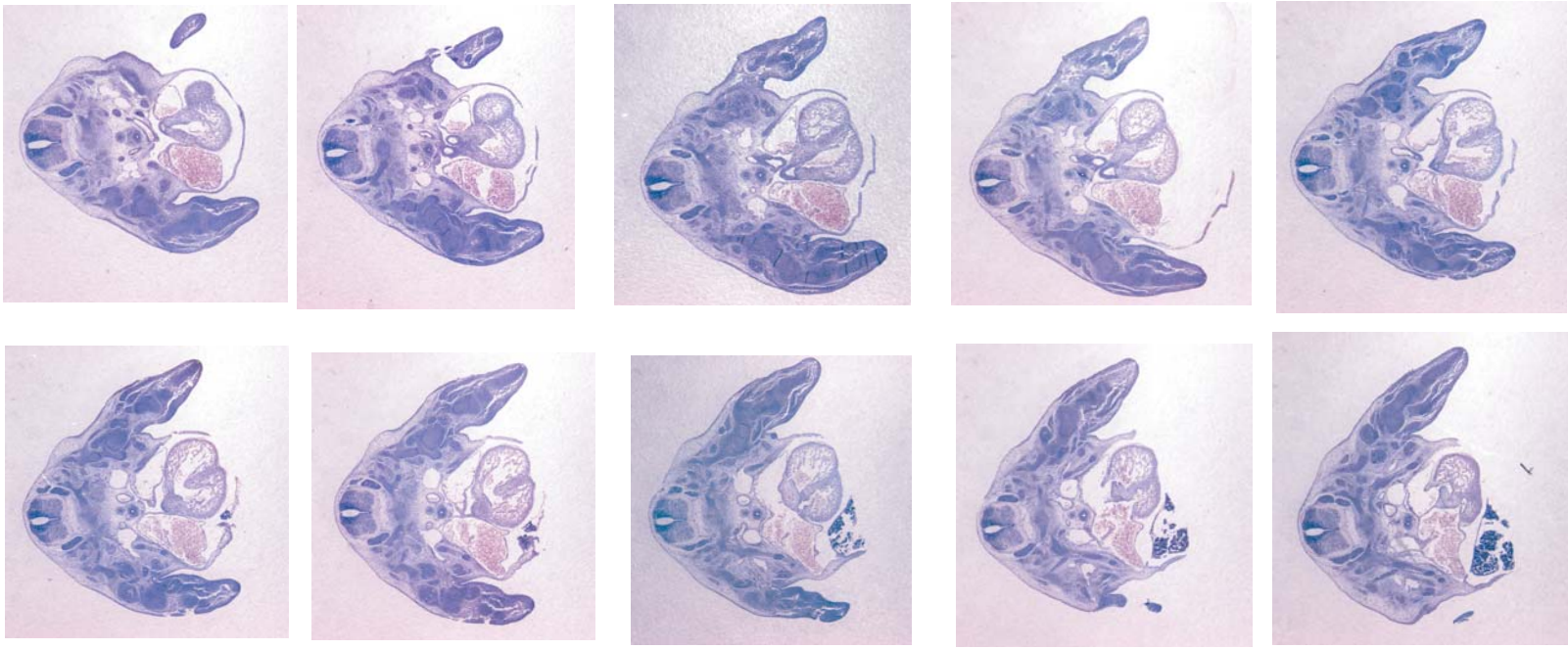
Supplementary Figure S2. **Mass spectrometric analysis of the cysteine-containing peptides in the WT and Nedd4-1$^{-/-,trap}$ MEF cells using cleavable ^{13}C-ICAT**.
The MS spectra show the comparison of light (^{13}C0) and heavy (^{13}C9) labeled peptide elution profiles of triply-charged Nedd4-1 peptide (LAVCGNPATSQPVTSSNHSSR) ion at m/z 780.721 (light) and doubly-charged Tsp-1 peptide (CEGSSVQTR and LCNNPTPQFGGK) ions at m/z 597.275 (light), 601.782 (heavy), 751.860(light) and 756.376 (heavy). No heavy labeled Nedd4-1 peptide (expected m/z 783.74) was observed in the Nedd4-1 knockout MEF cells (panel A). A total of five peptides were identified by MS/MS measurements in Tsp-1, two of them illustrated in panels B and C. The Tsp-1 protein was determined to be up-regulated by 8.1-fold based on the MS quantitative H/L ratios of the cleavable ICAT labeled peptides.

Supplementary Figure S3: Weak biochemical interaction between Tsp-1 and Nedd4-1, and lack of Tsp-1 stabilization in the Nedd4-1$^{-/-,trap}$ MEFs
A). Co-immunoprecipitation (Co-IP) of Nedd4-1 and Tsp-1: Flag-tagged Tsp-1 (WT or a PY motif mutant in which the Tyr was mutated to Ala) and GFP-tagged Nedd4-1 were co-transfected into Hek293T cells. After cell lysis, Tsp-1 was immunoprecipitated with antibodies to the Flag tag, and proteins immunoblotted for Nedd4-1 (upper panel) or Tsp-1 (lower panel). Note the Co-Ip of both WT Tsp-1 and its PY motif mutant with Nedd4-1. (B) Co-IP of endogenous Nedd4-1 and Tsp-1. Tsp-1 was immunoprecipitated from the indicated MEFs and immunoblotted for endogenous Nedd4-1. (C). Nedd4-1 can ubiquitinate Tsp-1: Hek293T cells were co-transfected with Flag-Tsp-1 (WT or PY motif mutant), GFP-Nedd4-1 (WT or a catalytically-inactive CS mutant) and HA-ubiquitin (HA-Ub). Cells were then lysed, lysate boiled in SDS to remove putative Tsp-1 associated proteins, the SDS diluted and Tsp-1 immunoprecipitated from the lysates, resolved on SDS-PAGE and immunoblotted with anti HA antibodies to detect ubiquitinated Tsp-1, as well as with anti Flag (Tsp-1), anti GFP (Nedd4-1) and anti actin, for controls. (D) Pulse-chase analysis to determine protein stability of Tsp-1 in cells lacking Nedd4-1: WT (+/+) and Nedd4-1$^{-/-,trap}$ (-/-) MEFs were treated with cycloheximide (CHX) to block protein synthesis, and levels of endogenous Tsp-1 analyzed by immunoblotting at the indicated times (upper panel). Lower panel summarizes the results of 4 independent pulse-chase experiments (mean± SD, normalized for actin).

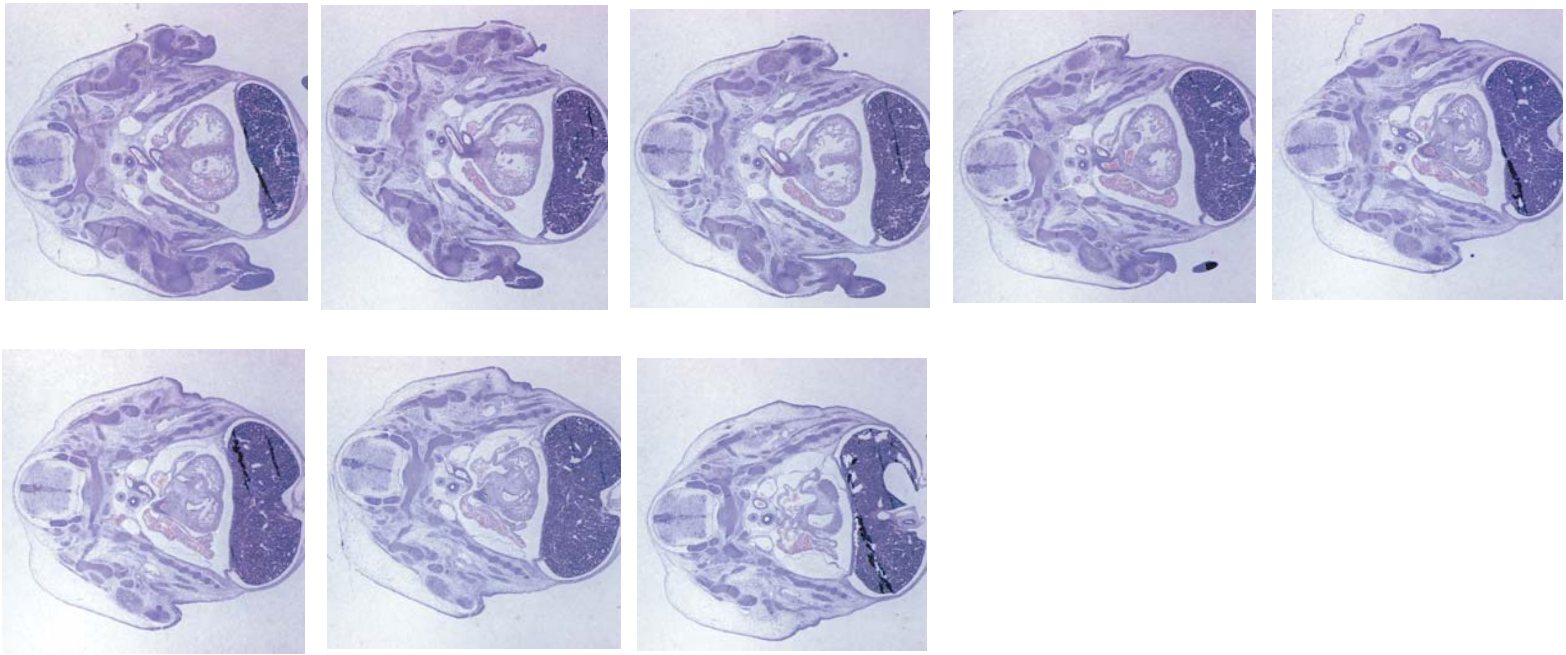
Supplementary Figure S4: Elevated levels of annexin A7, annexin A2 and Grb10 in the Nedd4-1$^{-/-,trap}$ MEFs are not reduced by aspirin treatment.
WT (+/+) and Nedd4-1$^{-/-,trap}$ (-/-) MEFs were treated with the indicated amounts of aspirin for 4 or 12 hrs and annexin A2, annexin A7 and Grb10 levels analyzed by immunoblotting using their respective antibodies. Note the increase in amounts of these three proteins in the knockout MEFs, and the lack of reduction in their levels by aspirin treatment. The same results were obtained after aspirin treatment for 4 or 12 hrs.

Supplementary Movies 1 and 2:
PECAM-1 staining of the vasculature of E10.5 WT ((WT), movie 1) and Nedd4-1$^{-/-,trap}$ (KO), movie 2) embryos followed by optical projection tomography (OPT) analysis and 3D reconstruction.

E13.5, Nedd4-1 (-/-)

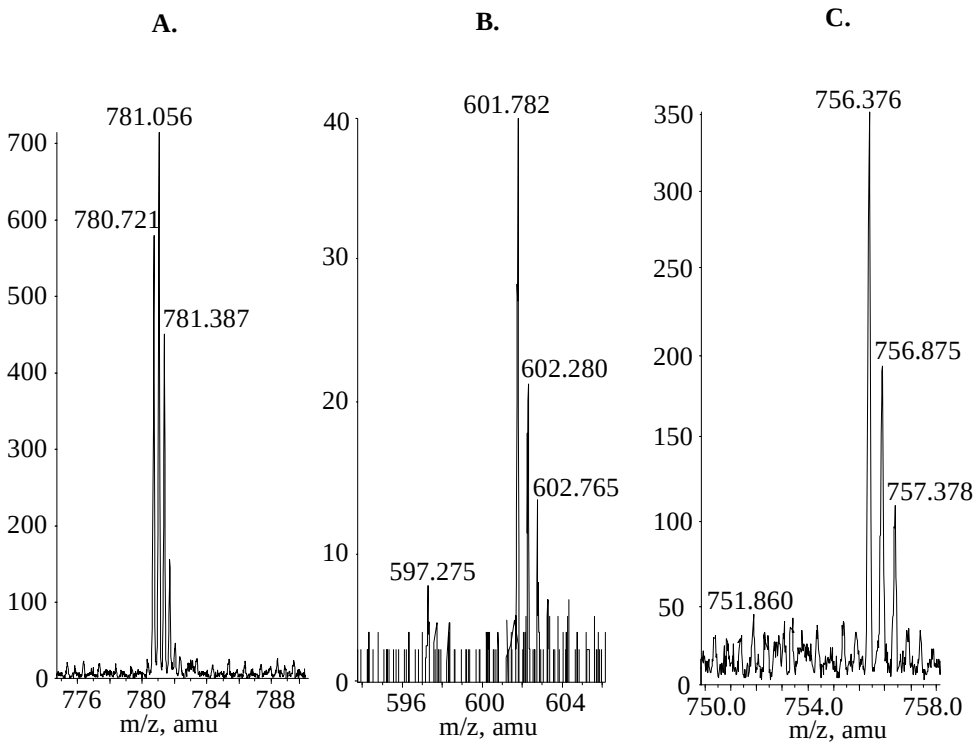


E13.5, Nedd4-1 (+/+)

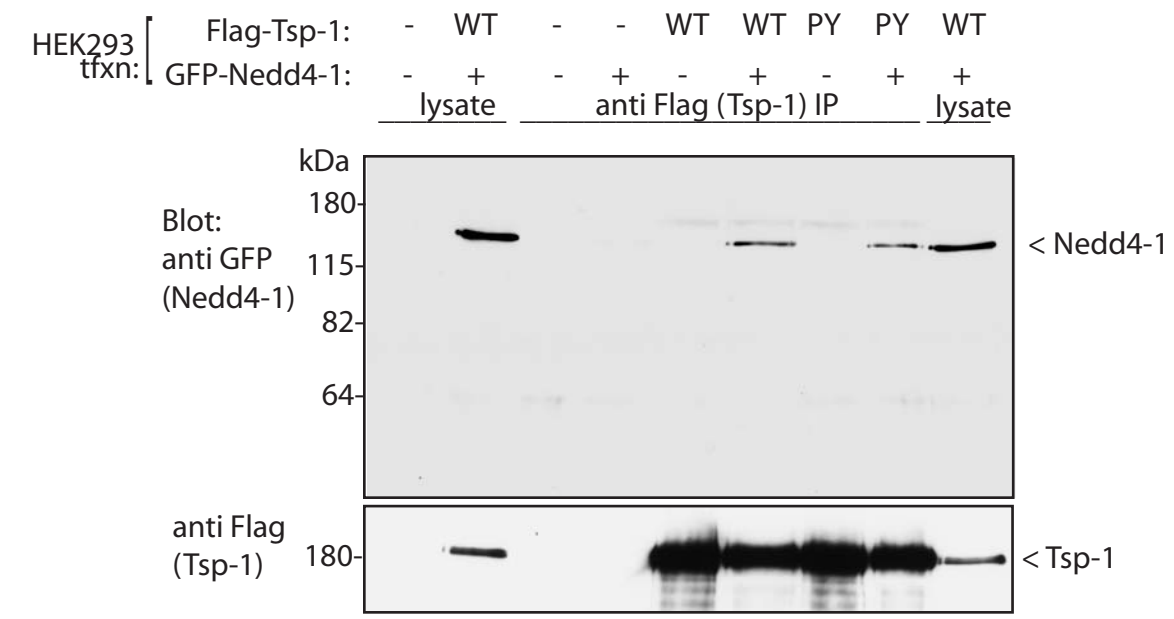


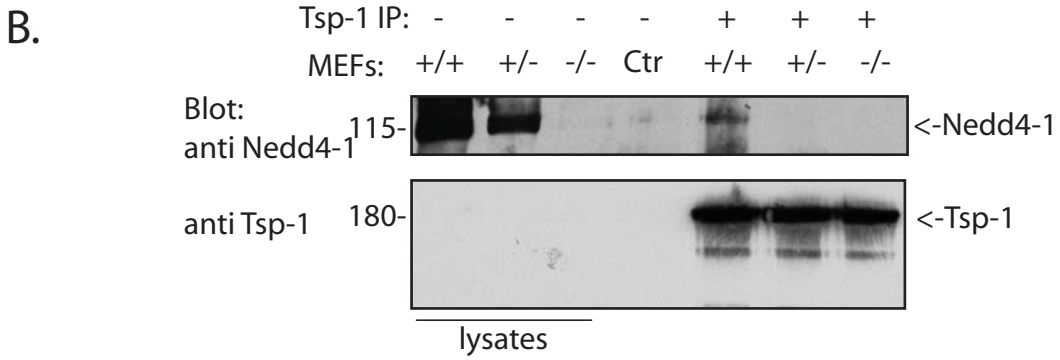
Supplementary Figure S1

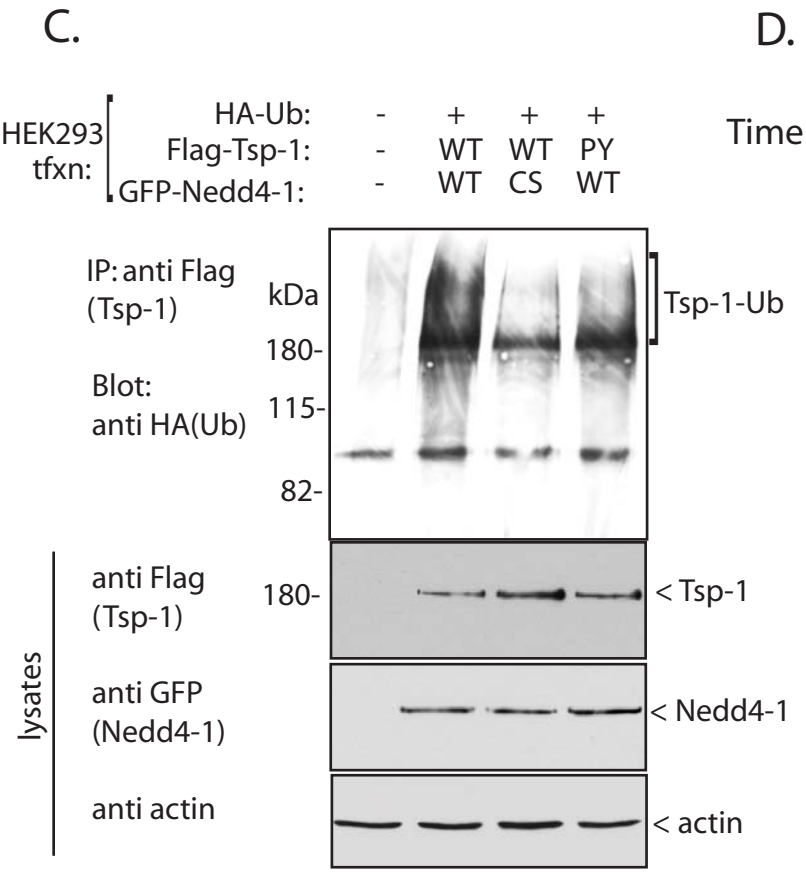
Supplementary Figure S2

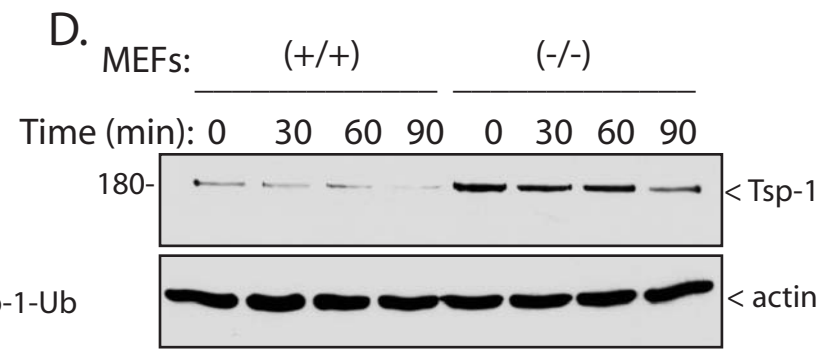


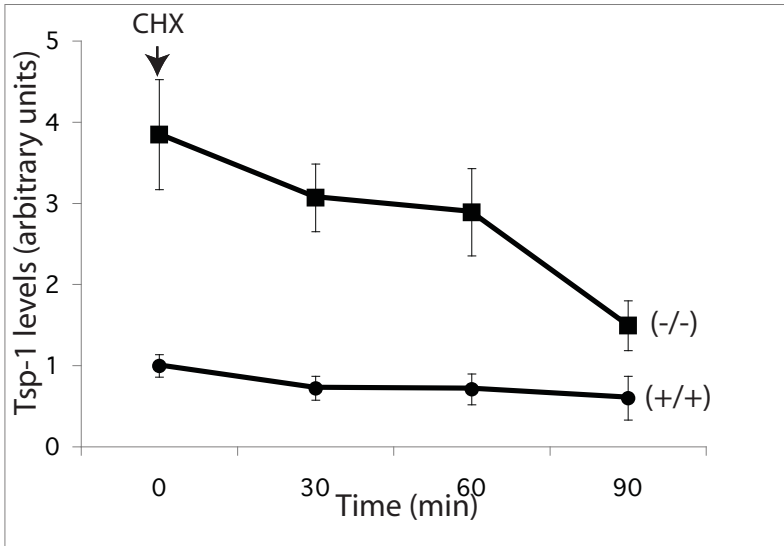
Supplementary Figure S3





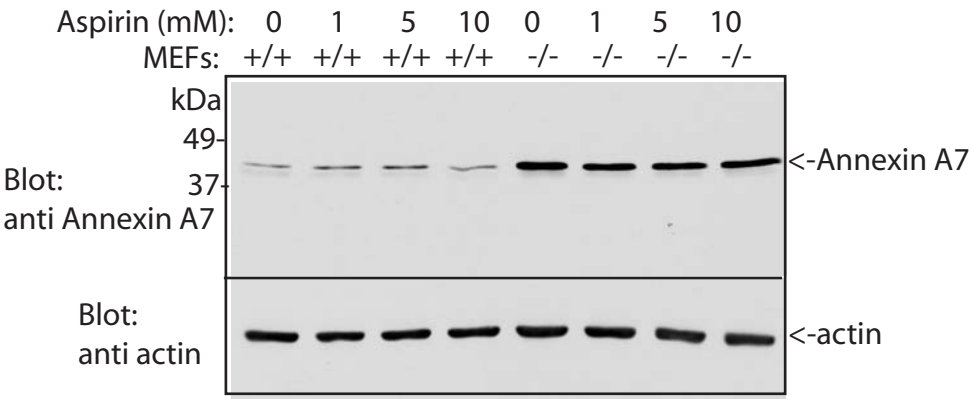




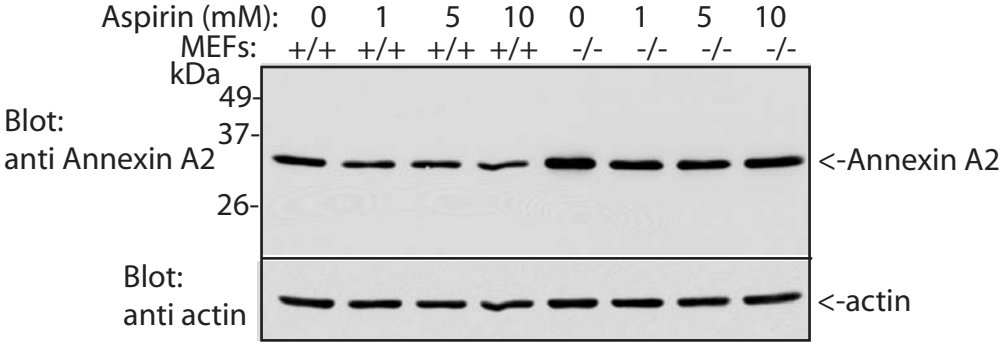


Supplementary Figure S4

A.



B.



C.