Supplementary Figure 1. WW45 interacts with NEDD4

(a) WW45 interacts with NEDD4 in vivo. HEK293T cells were transfected as indicated and treated with 20 μM MG132 for 4 h. Cell lysates were immunoprecipitated with anti-FLAG M2 resin and immunoblotted with indicated antibodies.

(b) Amino acids 1-100 region of NEDD4 is necessary for binding to WW45. (Left panel) MYC9-WW45 was co-transfected with various FLAG-NEDD4 DNA plasmids (WT, wild-type; ΔN100, a.a. 101-1000; ΔN280, a.a. 281-1000, respectively) in HEK293T cells. At 24 h post-transfection, cells were treated with 20 μM MG132 for 4 h. Cell lysates were immunoprecipitated with anti-FLAG M2 resin and immunoblotted with indicated antibodies. (Right panel) Multiple amino acid sequence alignment among NEDD4 family members (i.e., NEDD4, NEDD4L, ITCH, WWP1, and WWP2) was performed using ClustalW1.83. Identical or similar amino acids are box-shaded in black or grey, respectively. Gaps are represented by dashes to optimize the alignment.

(c-e) ITCH does not interact with either WW45 or LATS2. FLAG-ITCH was co-transfected with indicated plasmids, (c) MYC9-WW45, (d) MYC-LATS1, (e) HA-LATS2. Cell lysates were immunoprecipitated with anti-FLAG M2 resin and immunoblotted with indicated antibodies.
**Supplementary Figure 2 (Seol et al.)**

(a) FLAG-NEDD4

(b) CHX

(c) siScr, siNEDD4, siITCH

(d) Lysates

Supplementary Figure 2 (Seol et al.)
Supplementary Figure 2. NEDD4 negatively regulates WW45
(a) NEDD4 overexpression results in the decrease of endogenous WW45. FLAG-NEDD4 (0, 0.5, 2 μg) was transfected in HEK293T cells as indicated.
(b) WW45 protein levels are rapidly declined in the presence of NEDD4. Cycloheximide (CHX) chase experiments were performed to determine the half-life of WW45 protein. HEK293T cells were transfected with either control or FLAG-NEDD4 plasmids as described in Methods. Cells were treated with CHX (200 μg ml⁻¹) and harvested at indicated times. WW45 protein levels were quantitated by ImageJ and normalized to GAPDH levels. Data are plotted as the mean ± SEM of three independent experiments.
(c) Knockdown of NEDD4, but not ITCH inhibits the degradation of WW45. CHX chase experiments were performed in the presence of indicated siRNAs in HEK293T. WW45 protein levels were quantitated by ImageJ and normalized to GAPDH levels. Data are plotted as the mean ± SEM of three independent experiments.
(d) Depletion of NEDD4 reduces ubiquitylation of WW45. HEK293T cells were transfected with siScrambled, siNEDD4 or siITCH. At 48 h post-transfection, cells were treated with 20 μM MG132 for 4 h. Cell lysates were immunoprecipitated with anti-WW45 antibody and immunoblotted with indicated antibodies.
Supplementary Figure 3 (Seol et al.)
**Supplementary Figure 3. LATS1/2 kinases are substrates of NEDD4**

(a) WW45 shRNA reduces WW45 protein and mRNA expression levels. HEK293T cells were transfected with shControl, shWW45 #1 or #2. At 48 h post-transfection, total RNA was subjected to RT-PCR analyses for WW45 and GAPDH expression, and cell lysates were subjected to immunoblotting with indicated antibodies.

(b) Knockdown of NEDD4 increases STS-induced apoptosis. HEK293T cells were transfected with siNEDD4 alone or in conjunction with WW45 shRNA as indicated. At 48 h post-transfection, 0.5 μM STS was treated for 12 h before harvest. Cell lysates were then collected and subjected to immunoblotting.

(c) Depletion of NEDD4 promotes STS-induced cell death. HEK293T cells were transfected with siScrambled, siNEDD4 alone or siNEDD4 in conjunction with shWW45 #1 as indicated. At 48 h post-transfection, cells were treated with 0.5 μM STS for 12 h. Cells were then harvested and subjected to FACS analysis. Data are plotted as the mean ± SEM of three independent experiments.

(d) Protein levels of MST1, WW45, and LATS2 are evaluated upon MG132 treatment. HEK293T cells were transfected with following plasmid constructs: HisMax-MST1, FLAG-WW45, and HisMax-LATS2. Cells were treated with 20 μM MG132 for 4 h as indicated.

(e) LATS2 binds to WW domains-containing N-terminal region of NEDD4. Xpress-LATS2 was transfected with various FLAG-NEDD4 constructs (WT, wild-type; N280, a.a. 1-280; WW, a.a. 281-620; HECT, a.a. 621-1000) in HEK293T cells. At 24 h post-transfection, cells were treated with 20 μM MG132 for 4 h. Cell lysates were immunoprecipitated with anti-FLAG M2 resin and immunoblotted with indicated antibodies.

(f) Endogenous LATS2 is destabilized, as more NEDD4 is expressed in HEK293T cells.

(g) LATS2 protein stability is reduced in the presence of NEDD4. CHX chase experiment was performed using either control or FLAG-NEDD4-transfected HEK293T cells. LATS2 protein levels were quantitated by ImageJ and normalized to GAPDH levels. Values represent mean ± SEM and each experiment was done in triplicate.

(h) NEDD4 ubiquitylates LATS2 in vitro. Purified FLAG-NEDD4 was used to ubiquitylate purified His-LATS2 in vitro as described in Methods. After in vitro ubiquitylation reaction, samples were analyzed by immunoblotting with anti-LATS2 antibody.

(i) LATS1 is also destabilized by NEDD4. MYC-LATS1 was transfected with either FLAG-NEDD4 WT or C967S (0, 0.5, 2 μg). Immunoblotting was performed with indicated antibodies.
Supplementary Figure 4. MST1 inhibits NEDD4-mediated WW45 down-regulation
(a) WW45 protein levels are increased in the presence of MST1/2. FLAG-WW45 was transfected with either HisMax-MST1 or HisMax-MST2 in HEK293T cells. Immunoblotting was performed with indicated antibodies. (b) MST1 inhibits the interaction between WW45 and NEDD4. Cells were transfected with HisMax-MST1, MYC9-WW45, and FLAG-NEDD4 as indicated. Cell lysates were immunoprecipitated with anti-MYC resin and immunoblotted with indicated antibodies. (c) MST1 interacts with the SARAH domain of WW45. HisMax-MST1 was co-transfected with FLAG-WW45 (WT, 199C, N198, and N321) in HEK293T cells. Cell lysates were immunoprecipitated with anti-FLAG M2 resin and immunoblotted with indicated antibodies. (d) MST1 protects WW45WT but not WW45N198 mutant from NEDD4-mediated down-regulation. FLAG-WW45 (WT and N198 mutant), HisMax-MST1, and HisMax-NEDD4 were transfected in HEK293T cells as indicated.
Supplementary Figure 5. NEDD4 controls the subcellular localization and the activity of YAP by regulating WW45 and LATS2
(a) NEDD4 retains YAP in the nucleus. GFP-YAP, HA-LATS2, and FLAG-NEDD4 were transfected in HEK293T cells as indicated. Cells were subjected to immunofluorescence staining and the subcellular localization of YAP, LATS2, and NEDD4 was visualized under a LSM710 multi-photon confocal laser scanning microscope (Zeiss). The scale bar represents 10 μm.
**Supplementary Figure 6 (Seol et al.)**

### a) Immunofluorescence Images

- **Cell Density**
  - Low
  - High

- **GFP-YAP**
  - Low
  - High

- **FLAG-NEDD4 (Wild-type)**
  - Low
  - High

- **FLAG-NEDD4 (C967S)**
  - Low
  - High

### b) RT-PCR

- **Cell Density**
  - L (Low)
  - M (Medium)
  - H (High)

- **Markers**
  - NEDD4
  - LATS2
  - WW45
  - GAPDH

### c) Western Blotting

- **Cell Density**
  - L (Low)
  - H (High)

- **IP: α-NEDD4**
  - α-Ub
  - α-NEDD4

- **Lysates**
  - M(K) 130
  - 95
  - 43

### d) Western Blotting for Remaining Protein Levels

- **CHX**
  - Low
  - High

- **Time (h)**
  - 0, 3, 6, 9

- **Markers**
  - α-NEDD4
  - α-β-actin
Supplementary Figure 6 (Seol et al.)
Supplementary Figure 6. NEDD4 is involved in the cell density-dependent activation of the Hippo pathway

(a) NEDD4 blocks the cell density-dependent cytoplasmic localization of YAP. GFP-YAP was transfected alone or in combination with either FLAG-NEDD4 WT or C967S mutant in NIH3T3 cells as indicated. At 24 h post-transfection, cells were replated as either sparse subconfluent or dense confluent monolayers. Cells were subjected to immunofluorescence staining and the subcellular localization of YAP and NEDD4 was visualized under a LSM710 multi-photon confocal laser scanning microscope (Zeiss). The scale bar represents 10 μm.

(b) \textit{NEDD4}, \textit{LATS2}, and \textit{WW45} mRNA levels remain unchanged regardless of differences in cell density. NIH3T3 cells were seeded at low, medium, and high cell densities. RNA was purified for conventional and quantitative real-time RT-PCR analyses of \textit{NEDD4}, \textit{LATS2}, \textit{WW45}, and \textit{GAPDH} expression (n=3).

(c) NEDD4 is inactivated at high cell density. NIH3T3 cells were cultured sparsely or at a dense confluent monolayer for 24 h. After MG132 (20 μM) treatment for 4 h, cells were harvested and lysates were immunoprecipitated with anti-NEDD4 antibody and immunoblotted with indicated antibodies.

(d) NEDD4 proteins are relatively stable at high cell density. Cycloheximide (CHX) chase experiments were performed to determine the half-life of NEDD4 protein. NIH3T3 cells were treated with CHX (200 μg ml⁻¹) and harvested at indicated times. NEDD4 protein levels were quantitated by ImageJ and normalized to β-actin levels (n=3).

(e) Ubiquitylation of WW45 and LATS2 are reduced at high cell density in a NEDD4-dependent manner. NIH3T3 stable cells were cultured as either sparse or dense monolayer for 24 h. Cells were harvested after 4 h treatment of 20 μM MG132. Immunoprecipitates with either anti-WW45 or anti-LATS2 antibody were immunoblotted with indicated antibodies.

(f) Cell density does not affect the interaction between WW45 and NEDD4. NIH3T3 cells were cultured either sparsely or densely and harvested after 24 h incubation. Cell lysates were immunoprecipitated with either anti-WW45 or anti-NEDD4 antibody and immunoblotted with indicated antibodies.

(g) Expression of YAP-controlled genes is inhibited in \textit{NEDD4} KD cells. Total RNA isolated from either control or \textit{NEDD4} KD-stable NIH3T3 cells was subjected to quantitative real-time RT-PCR analyses and normalized to the mean values of β-actin and HPRT. Data are plotted as the mean ± SEM of five independent experiments and analyzed by one-way ANOVA with Bonferroni's multiple comparison test (**P < 0.0001).
Supplementary Figure 7. NEDD4 regulates Hippo pathway in Drosophila

The exact genotypes of flies were described in Supplementary Table 1. (a) Reduced Wts protein level in the intestine of Wts-KD animal can be augmented in Wts/NEDD4 double KD animals. Conditional gene KD in enterocytes was performed by using Myo1A<sup>+/-</sup>-GAL4 (combining Myo1A-GAL4 and a temperature sensitive GAL4 inhibitor, tub-GAL80<sup>t</sup>). Approximately 25 midguts from each genotype were used for immunoblot analysis. (b) NEDD4 is required for host survival against pathogen infection. Adult male flies (5-day-old) were subjected to gut infection with the <i>E. carotovora</i> and their survival rates were measured as described previously 1. Survival in three or more independent cohorts comprising approximately 25 flies each was monitored over time. Data were analyzed by the Kaplan-Meier log-rank test. (c) NEDD4 is involved in determining adult gut size by regulating the Hippo pathway. The size of midguts (from the proventriculus to the junction between posterior midgut and hindgut) of adult animals (5-6-day-old female, n > 30 per genotype) was measured. Data are analyzed by ANOVA with Bonferroni’s post hoc analysis (*<i>P</i> < 0.05, ***<i>P</i> < 0.001) and presented as a scatter plot where horizontal line denotes median value. (d) NEDD4-KD is sufficient to decrease adult wing area. Representative adult wings of control and NEDD4-KD animals are shown. Scale bar, 100 μm. Quantification of wing area was performed exactly as describe previously<sup>2</sup>. Data were analyzed by using Mann-Whitney <i>U</i> test (**<i>P</i> < 0.001).
Supplementary Figure 8. WW45 and LATS1/2 are the substrates of NEDD4.
Relative WW45 and LATS2 protein levels in the main immunoblot figures were quantitated by ImageJ and normalized to β-actin levels. Values are plotted as the mean ± SEM of three independent experiments. Each corresponding figure number is shown above the graph.
Full blots of all the immunoblot slices in main Figures

Figure 1d

Figure 1e

Figure 2a

Figure 2b

Supplementary Figure 9 (Seol et al.)
Supplementary Figure 9 (Seol et al.)
Supplementary Figure 9. Full blots of all the immunoblot slices in main Figures are shown. Each corresponding figure number and used antibody information are indicated.
<table>
<thead>
<tr>
<th>Figures</th>
<th>Abbreviations</th>
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<tbody>
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<td>Control</td>
<td>hs-flp/++;ex-LacZ/++; FRT2A/FRT2A ubi-GFP</td>
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SUPPLEMENTARY REFERENCES
