Expanded View Figures

Figure EV1. Merlin ubiquitination associates with activation of the Hippo pathway.

A Total lysates from indicated cell lines cultured in regular conditions at a steady state were subjected to Western blotting. The same lysates were also used in Fig 1A.

B FH-912 cells treated with DMSO or thapsigargin and subjected to Western blotting. The ratio of mono-ubiquitinated to native Merlin in each lane was quantified by ImageJ and is shown under the blot. Native Merlin (asterisk); ubiquitinated Merlin (arrow).

C Met5-A cells stably transduced with indicated shRNAs against Merlin or a scrambled shRNA were detached (Sus., suspension) by trypsinization using the procedure described in Materials and Methods and reseeded (Attach, attached) for 4 h. Total lysates from these cells were subjected to Western blotting.

D Longer exposure of the blot in Fig 1H. Ubiquitinated Merlin (arrows and arrowheads).

E LN229 cells transduced with vector or EGFP-RAC1(Q61L) were treated with DMSO or thapsigargin (TG) and subjected to immunofluorescent staining. Scale bar = 20 μm.

F The ratio of the relative amount of YAP/TAZ in the nucleus (N) compared to the cytoplasm (C) from panel (E) was quantified. Mean ± s.e.m, two-way ANOVA. ****$P < 0.0001$. Each data point represents one cell. N_{Vector} = 16 cells in each condition, $N_{RAC1(Q61L)}$ = 17 cells in each condition. All cells were from multiple random images collected from one experiment. Two independent experiments were performed and gave similar results.

Source data are available online for this figure.

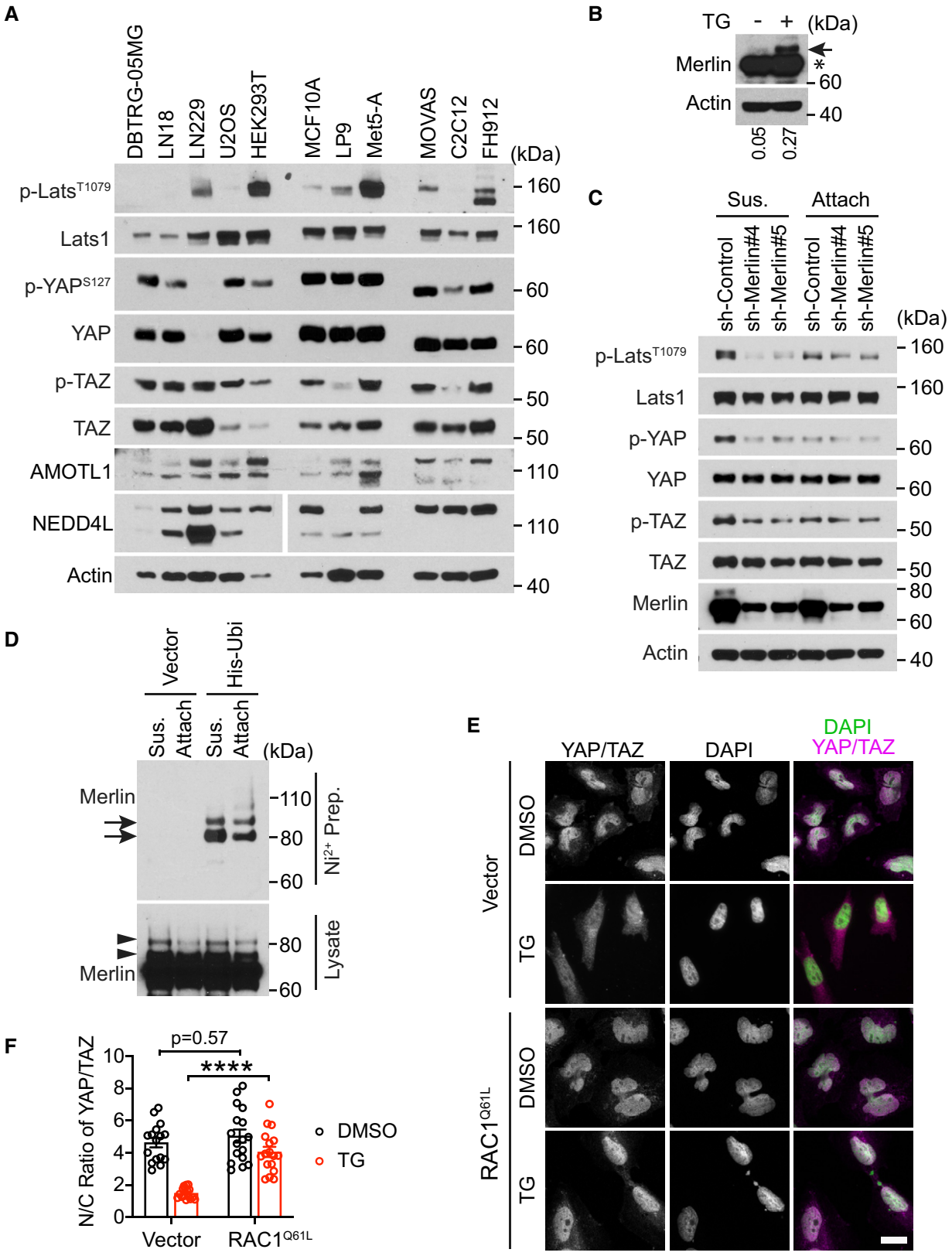

Figure EV1.

A

Protein name	Flag-Merlin IP		
	Normalized unique peptides		TG/DMSO ratio
	TG	DMSO	
Merlin	100	100	1.0
DCAF1	9.8	8.0	1.2
DDB1	4.8	4.1	1.2
AMOTL1	3.9	3.2	1.2
AMOT	0.6	0.3	1.9
NEDD4L	0.6	0.3	1.8
AMOTL2	0.5	0.6	0.9
CUL4B	0.1	0.1	0.7
HUWE1	0.0	0.1	0.5

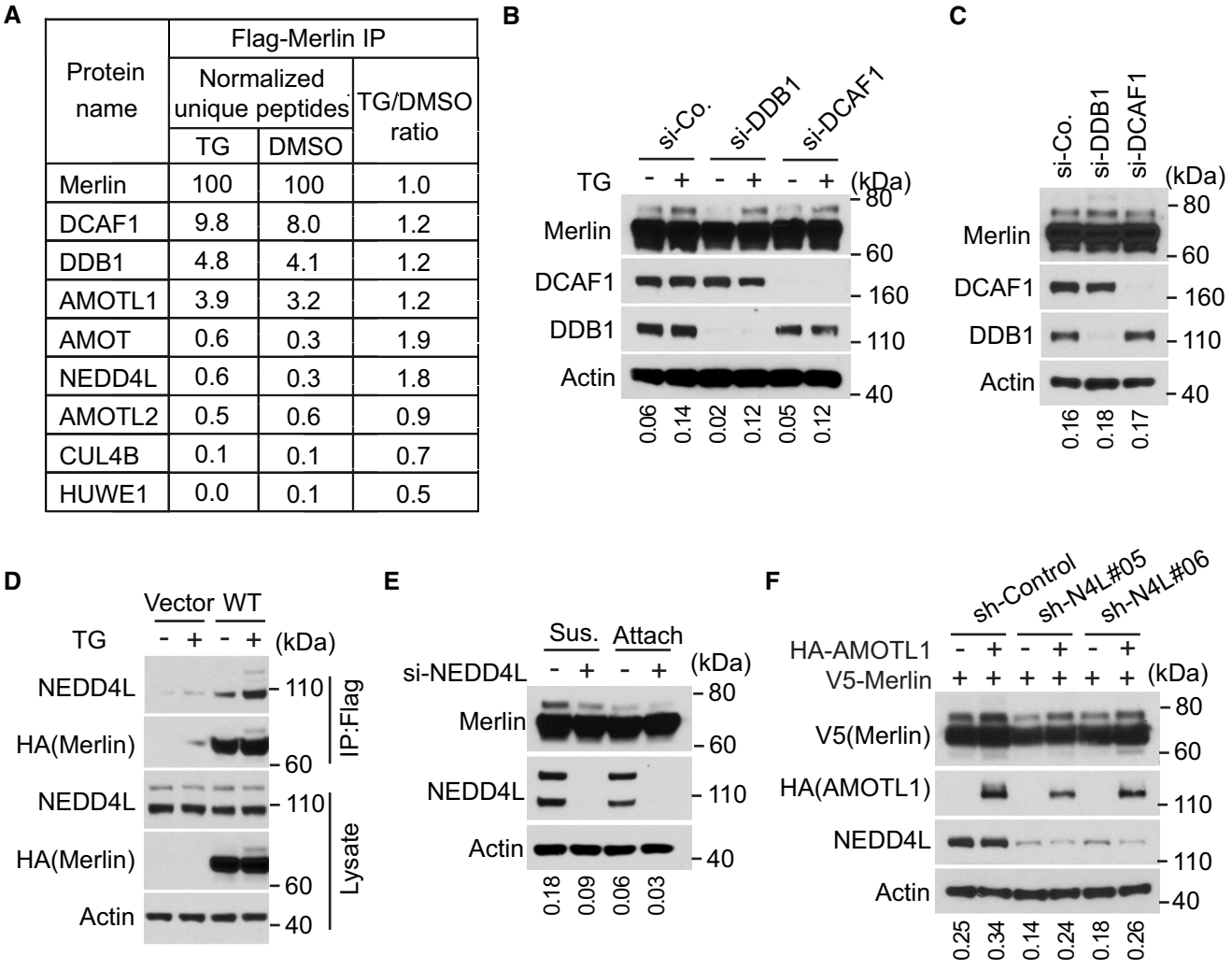

Figure EV2.

Figure EV2. NEDD4L is required for Merlin ubiquitination.

A Numbers of unique peptides of each identified protein shown in Fig 3A were normalized to those of Merlin from the indicated treatment condition. The ratio of these normalized peptides from TG-treated cells to those from DMSO-treated cells is shown.

B LN229 cells transfected with indicated siRNAs were treated with DMSO or thapsigargin (TG) and subjected to Western blotting. The ratio of mono-ubiquitinated to native Merlin in each lane was quantified by ImageJ and is shown under the blot.

C LN229 cells transfected with indicated siRNAs were detached using the procedure described in Materials and Methods and subjected to Western blotting. The ratio of mono-ubiquitinated to native Merlin in each lane was quantified by ImageJ and is shown under the blot.

D Merlin-depleted LN229 cells stably transduced with Flag-tagged wild-type Merlin were treated with DMSO or thapsigargin (TG). The cells were lysed and subjected to immunoprecipitation with a Flag antibody. The lysate and immunoprecipitated products were subjected to Western blotting. The ratio of mono-ubiquitinated to native Merlin in each lane of the lysate blot was quantified by ImageJ and is shown under the blot.

E Met5-A cells transfected with a pool of four siRNAs targeting NEDD4L (+) or a scrambled siRNA (−) were detached (denoted by Sus., referring to suspension) by using the procedure described in Materials and Methods and reseeded (Attach) for 4 h. Total lysates from these cells were subjected to Western blotting. The ratio of mono-ubiquitinated to native Merlin in each lane was quantified by ImageJ and is shown under the blot.

F HEK293T cells stably transduced with indicated shRNAs targeting NEDD4L or a scrambled shRNA were transfected with indicated genes. The cells were then lysed and subjected to Western blotting. The ratio of mono-ubiquitinated to native Merlin in each lane was quantified by ImageJ and is shown under the blot.

G, H HEK293T cells were transfected with indicated genes and subjected to Western blotting. The ratio of mono-ubiquitinated to native Merlin in each lane was quantified by ImageJ and is shown under the blot.

Source data are available online for this figure.

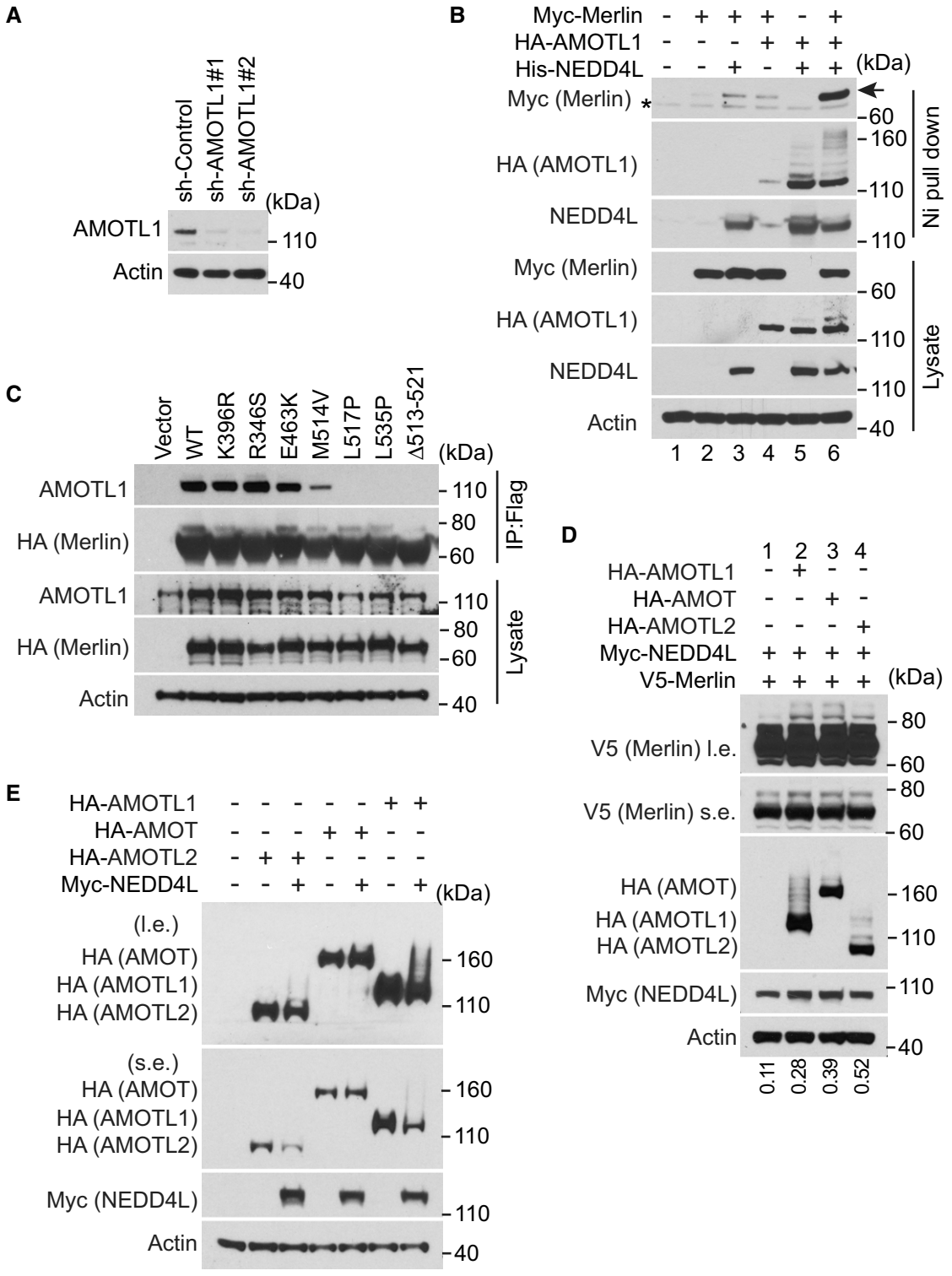
A
sh-Control
sh-AMOTL1#1
sh-AMOTL1#2
(kDa)
AMOTL1
110
Actin
40
B
Myc-Merlin - + + + - +
HA-AMOTL1 - - - + + +
His-NEDD4L - - + - + +
(kDa)
Myc (Merlin) *
60
HA (AMOTL1)
160
110
NEDD4L
110
Ni pull down
Myc (Merlin)
60
HA (AMOTL1)
110
NEDD4L
110
Lysate
Actin
40
1 2 3 4 5 6
C
Vector
WT
K396R
R346S
E463K
M514V
L517P
L535P
Δ513-521
(kDa)
AMOTL1
110
HA (Merlin)
80
60
IP:Flag
AMOTL1
110
HA (Merlin)
80
60
Lysate
Actin
40
D
1 2 3 4
HA-AMOTL1 - + - -
HA-AMOT - - + -
HA-AMOTL2 - - - +
Myc-NEDD4L + + + +
V5-Merlin + + + +
(kDa)
V5 (Merlin) l.e.
80
60
V5 (Merlin) s.e.
80
60
HA (AMOT)
HA (AMOTL1)
HA (AMOTL2)
160
110
Myc (NEDD4L)
110
Actin
40
0.11 0.28 0.39 0.52
E
HA-AMOTL1 - - - - - + +
HA-AMOT - - - + + - -
HA-AMOTL2 - + + - - - -
Myc-NEDD4L - - + - + - +
(kDa)
(l.e.)
HA (AMOT)
HA (AMOTL1)
HA (AMOTL2)
160
110
(s.e.)
HA (AMOT)
HA (AMOTL1)
HA (AMOTL2)
160
110
Myc (NEDD4L)
110
Actin
40

Figure EV3.

Figure EV3. AMOTL1 promotes Merlin ubiquitination.

A Merlin-depleted LN229 cells stably expressing Flag-HA-tagged Merlin used in Fig 4D were stably transduced with indicated shRNAs targeting AMOTL1 or a scrambled shRNA and subjected to Western blotting.

B HEK293T cells were transfected with indicated genes and subjected to nickel pull-down in a native condition followed by Western blotting. Myc-Merlin (arrow); non-specific bands (asterisk).

C Merlin-depleted LN229 cells stably transduced with Flag-tagged wild-type Merlin or the indicated mutants were subjected to immunoprecipitation with a Flag antibody. The lysate and immunoprecipitated products were subjected to Western blotting.

D, E HEK293T cells were transfected with indicated genes and subjected to Western blotting. The ratio of mono-ubiquitinated to native Merlin in each lane was quantified by ImageJ and is shown under the blot in (D).

Source data are available online for this figure.

Figure EV4. Merlin ubiquitination associates with Lats1 activation.

A Met5-A cells were detached by using the procedure described in Materials and Methods and reseeded (Attach) for 4 h. Total lysates from these cells were subjected to Western blotting. The ratio of mono-ubiquitinated to native Merlin in each lane was quantified by ImageJ and is shown under the blot.

B Met5-A cells transfected with a pool of four siRNAs against NEDD4L (+) or a scrambled siRNA (−) were detached (Sus., suspension) by using the procedure described in Materials and Methods and reseeded (Attach, attached) for 4 h. Total lysates from these cells were subjected to Western blotting.

C Merlin-depleted LN229 cells were stably transduced with HA-tagged wild-type (WT) Merlin or its K396R mutant. These cells were treated with thapsigargin (TG) and subjected to PLA using HA and EBP50 antibodies. Scale bar = 20 μm.

D PLA signals (dots) in each cell from the results in (C) were quantified. Mean ± s.e.m, two-way ANOVA. Each data point represents an image field containing averagely 10 cells. $N_{Vector/DMSO}$ = 12, $N_{Vector/TG}$ = 12, $N_{WT/DMSO}$ = 20, $N_{WT/TG}$ = 16, $N_{K396R/DMSO}$ = 20, $N_{K396R/TG}$ = 20 images in each condition. All images were collected from one experiment. Two independent experiments were performed and showed similar results.

E Merlin-depleted LN229 cells were stably transduced with HA-tagged Merlin. These cells were then transduced with a pool of four siRNAs against NEDD4L or a scrambled siRNA and treated with DMSO or thapsigargin (TG). PLA was performed in these cells using HA and Lats1 antibodies. Scale bar = 20 μm.

F LN229 cells stably transduced with Flag-HA (FH)-tagged wild-type Lats1 or its UBA domain deletion (ΔUBA) mutant were treated with DMSO or thapsigargin (TG). The cells were lysed and subjected to immunoprecipitation with a Flag antibody. The lysate and immunoprecipitated products were subjected to Western blotting. The ratio of mono-ubiquitinated to native Merlin in lane 5 of IP and lysate blots was quantified by ImageJ and is shown under the blot, respectively. Native Merlin (asterisk); ubiquitinated Merlin (arrowhead).

G, H Merlin-depleted LN229 cells stably transduced with Flag-tagged wild-type Merlin or its mutants were treated with DMSO or thapsigargin (TG). The cells were subjected to cytosolic (C)/membrane (M) fractionation followed by Western blotting.

Source data are available online for this figure.

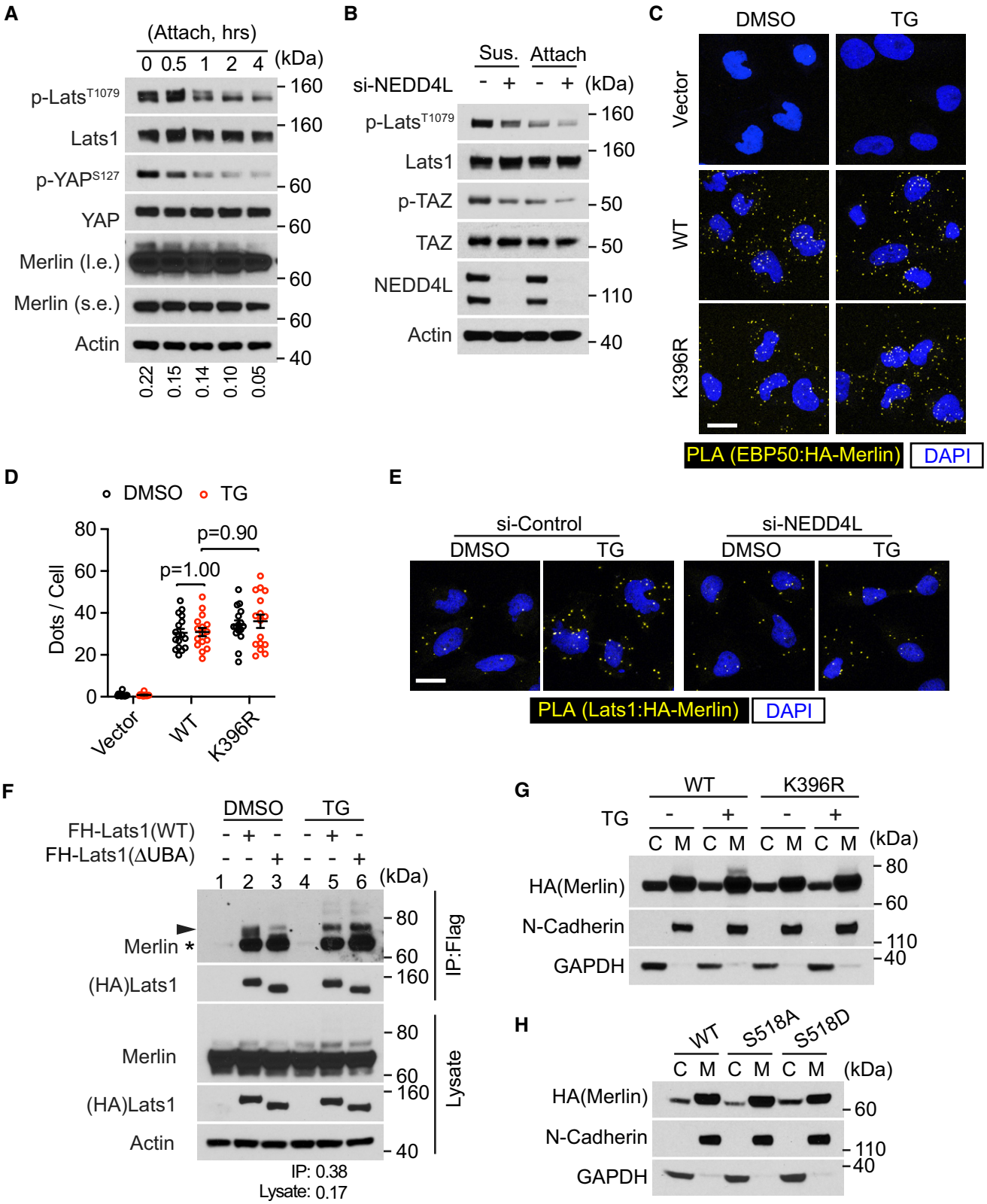
A
(Attach, hrs)
0 0.5 1 2 4 (kDa)
p-Lats^T1079
Lats1
p-YAP^S127
YAP
Merlin (l.e.)
Merlin (s.e.)
Actin
160
160
60
60
60
60
40
0.22 0.15 0.14 0.10 0.05
B
Sus. Attach
si-NEDD4L - + - + (kDa)
p-Lats^T1079
Lats1
p-TAZ
TAZ
NEDD4L
Actin
160
160
50
50
110
40
C
DMSO TG
Vector
WT
K396R
PLA (EBP50:HA-Merlin) DAPI
D
DMSO TG
Dots / Cell
p=0.90
p=1.00
Vector WT K396R
E
si-Control si-NEDD4L
DMSO TG DMSO TG
PLA (Lats1:HA-Merlin) DAPI
F
DMSO TG
FH-Lats1(WT) - + - - + -
FH-Lats1(ΔUBA) - - + - - +
1 2 3 4 5 6 (kDa)
Merlin *
(HA)Lats1
IP:Flag
Merlin
(HA)Lats1
Actin
Lysate
80
60
160
80
60
160
40
IP: 0.38
Lysate: 0.17
G
WT K396R
TG - + - +
C M C M C M C M (kDa)
HA(Merlin)
N-Cadherin
GAPDH
80
60
110
40
H
WT S518A S518D
C M C M C M (kDa)
HA(Merlin)
N-Cadherin
GAPDH
60
110
40

Figure EV4.

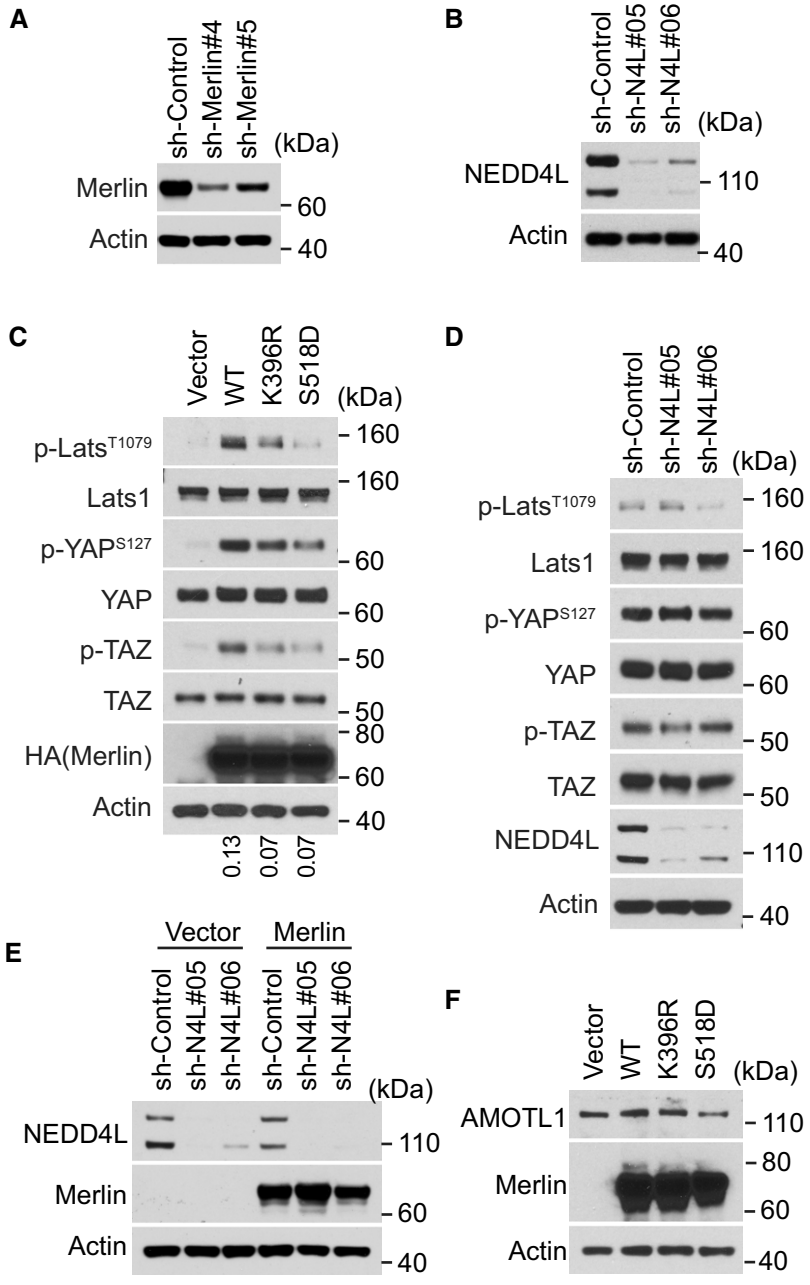

Figure EV5. Depletion and overexpression of Merlin in various cells.

A Met5-A cells stably transduced with the indicated shRNAs against Merlin or a scrambled shRNA were subjected to Western blotting.

B Met5-A cells stably transduced with the indicated shRNAs against NEDD4L or a scrambled shRNA were subjected to Western blotting.

C Meso-33 cells transduced with vector or the indicated Merlin forms were subjected to Western blotting. The ratio of mono-ubiquitinated to native Merlin in each lane was quantified by ImageJ and is shown under the blot.

D Meso-33 cells stably transduced with the indicated shRNAs targeting NEDD4L or a scrambled shRNA were detached by using the procedure described in the Materials and Methods and subjected to Western blotting.

E Meso-33 cells stably transduced with the indicated shRNAs against NEDD4L or a scrambled shRNA were then transduced with vector or Merlin prior to Western blotting.

F FC-1801 cells stably transduced with vector or the indicated Merlin forms were subjected to Western blotting.

Source data are available online for this figure.