Inventory of supplemental information

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Figure S7. Rubicon plays distinctive roles in conventional and TLR signaling-mediated autophagy.	**Figure 7**

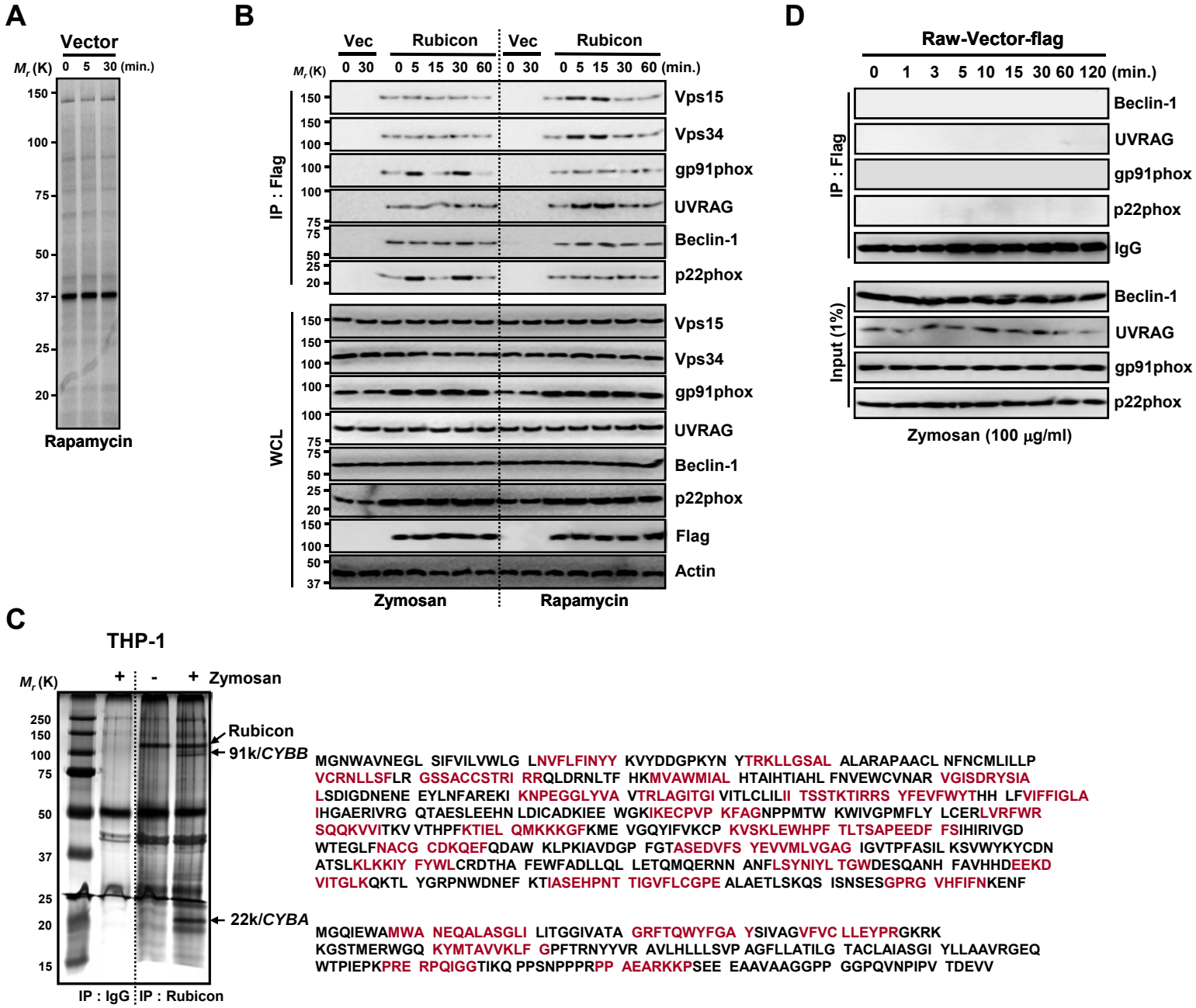

A
Vector
M_r (K) 0 5 30 (min.)
150
100
75
50
37
25
20
Rapamycin
B
Vec
Rubicon
Vec
Rubicon
M_r (K)
0 30
0 5 15 30 60
0 30
0 5 15 30 60 (min.)
IP : Flag
Vps15
Vps34
gp91phox
UVRAG
Beclin-1
p22phox
WCL
Vps15
Vps34
gp91phox
UVRAG
Beclin-1
p22phox
Flag
Actin
Zymosan
Rapamycin
D
Raw-Vector-flag
0 1 3 5 10 15 30 60 120 (min.)
IP : Flag
Beclin-1
UVRAG
gp91phox
p22phox
IgG
Input (1%)
Beclin-1
UVRAG
gp91phox
p22phox
Zymosan (100 μg/ml)
C
THP-1
M_r (K)
+ - + Zymosan
250
150
100
75
50
37
25
20
15
Rubicon
91k/CYBB
22k/CYBA
IP : IgG
IP : Rubicon
MGNWAVNEGL SIFVILVWLG LNVFLFINYY KVYDDGPKYN YTRKLLGSAL ALARAPAACL NFNCMLILLP
VCRNLLSFLR GSSACCSTRI RRQLDRNLTF HKMVAWMIAL HTAIHTIAHL FNVEWCVNAR VGISDRYSIA
LSDIGDNENE EYLNFAREKI KNPEGGLYVA VTRLAGITGI VITLCLILII TSSTKTIRRS YFEVFWYTHH LFVIFFIGLA
IHGAERIVRG QTAESLEEHN LDICADKIEE WGKIKECPVP KFAGNPPMTW KWIVGPMFLY LCERLVRFWR
SQQKVVITKV VTHPFKTIEL QMKKKGFKME VGQYIFVKCP KVSKLEWHPF TLTSAPEEDF FSIHIRIVGD
WTEGLFNACG CDKQEFQDAW KLPKIAVDGP FGTASEDVFS YEVVMLVGAG IGVTPFASIL KSVWYKYCDN
ATSLKLKKIY FYWLCRDTHA FEWFADLLQL LETQMQERNN ANFLSYNIYL TGWDESQANH FAVHHDEEKD
VITGLKQKTL YGRPNWDNEF KTIASEHPNT TIGVFLCGPE ALAETLSKQS ISNSESGPRG VHFIFNKENF
MGQIEWAMWA NEQALASGLI LITGGIVATA GRFTQWYFGA YSIVAGVFVC LLEYPRGKRK
KGSTMERWGQ KYMTAVVKLF GPFTRNYYVR AVLHLLLSVP AGFLLATILG TACLAIASGI YLLAAVRGEQ
WTPIEPKPRE RPQIGGTIKQ PPSNPPPRPP AEARKKPSEE EAAVAAGGPP GGPQVNPIPV TDEVV

Fig S1

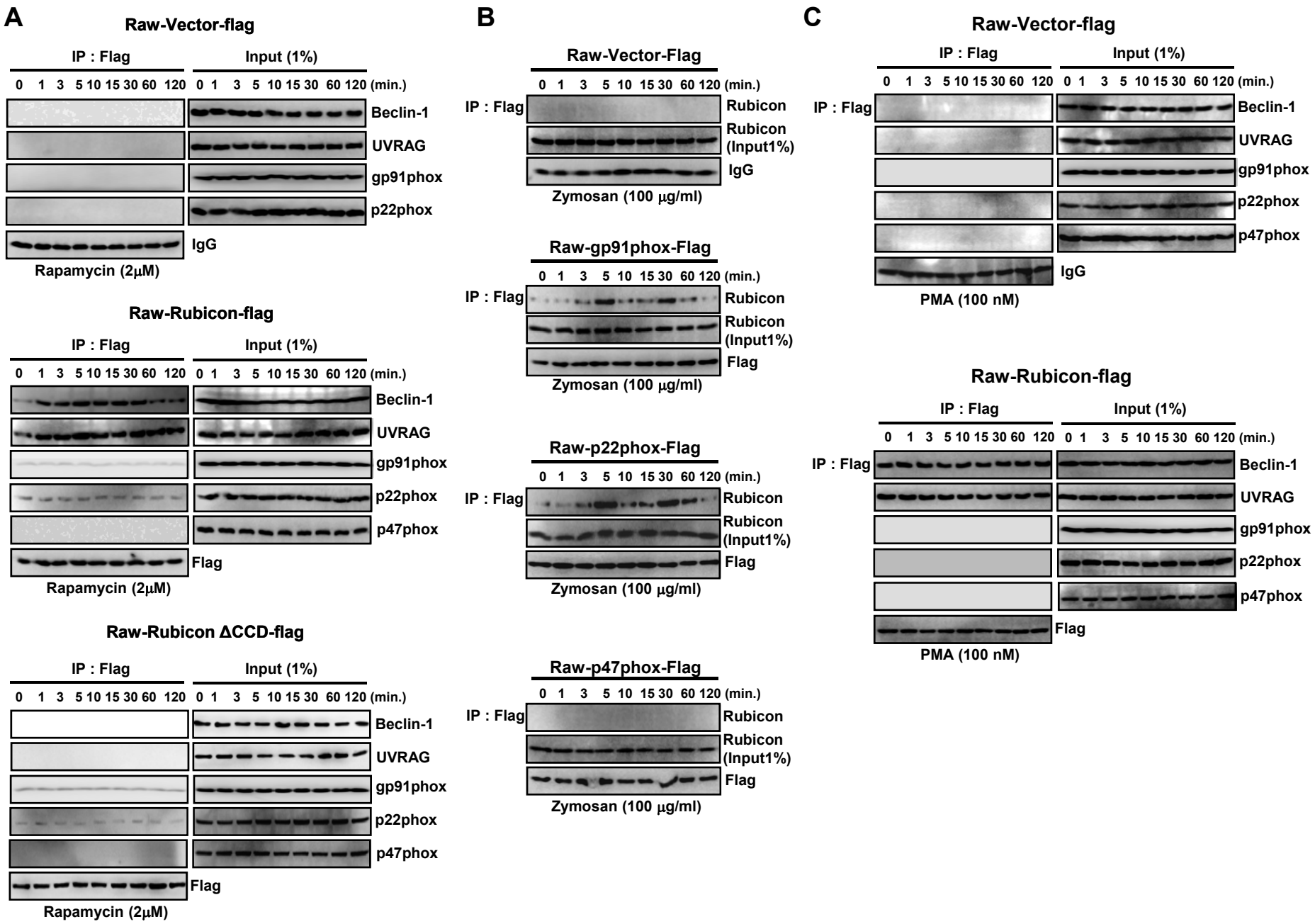

A
Raw-Vector-flag
IP : Flag
Input (1%)
0 1 3 5 10 15 30 60 120 0 1 3 5 10 15 30 60 120 (min.)
Beclin-1
UVRAG
gp91phox
p22phox
IgG
Rapamycin (2μM)
Raw-Rubicon-flag
IP : Flag
Input (1%)
0 1 3 5 10 15 30 60 120 0 1 3 5 10 15 30 60 120 (min.)
Beclin-1
UVRAG
gp91phox
p22phox
p47phox
Flag
Rapamycin (2μM)
Raw-Rubicon ΔCCD-flag
IP : Flag
Input (1%)
0 1 3 5 10 15 30 60 120 0 1 3 5 10 15 30 60 120 (min.)
Beclin-1
UVRAG
gp91phox
p22phox
p47phox
Flag
Rapamycin (2μM)
B
Raw-Vector-Flag
0 1 3 5 10 15 30 60 120 (min.)
IP : Flag
Rubicon
Rubicon (Input1%)
IgG
Zymosan (100 μg/ml)
Raw-gp91phox-Flag
0 1 3 5 10 15 30 60 120 (min.)
IP : Flag
Rubicon
Rubicon (Input1%)
Flag
Zymosan (100 μg/ml)
Raw-p22phox-Flag
0 1 3 5 10 15 30 60 120 (min.)
IP : Flag
Rubicon
Rubicon (Input1%)
Flag
Zymosan (100 μg/ml)
Raw-p47phox-Flag
0 1 3 5 10 15 30 60 120 (min.)
IP : Flag
Rubicon
Rubicon (Input1%)
Flag
Zymosan (100 μg/ml)
C
Raw-Vector-flag
IP : Flag
Input (1%)
0 1 3 5 10 15 30 60 120 0 1 3 5 10 15 30 60 120 (min.)
IP : Flag
Beclin-1
UVRAG
gp91phox
p22phox
p47phox
IgG
PMA (100 nM)
Raw-Rubicon-flag
IP : Flag
Input (1%)
0 1 3 5 10 15 30 60 120 0 1 3 5 10 15 30 60 120 (min.)
IP : Flag
Beclin-1
UVRAG
gp91phox
p22phox
p47phox
Flag
PMA (100 nM)

Fig S2

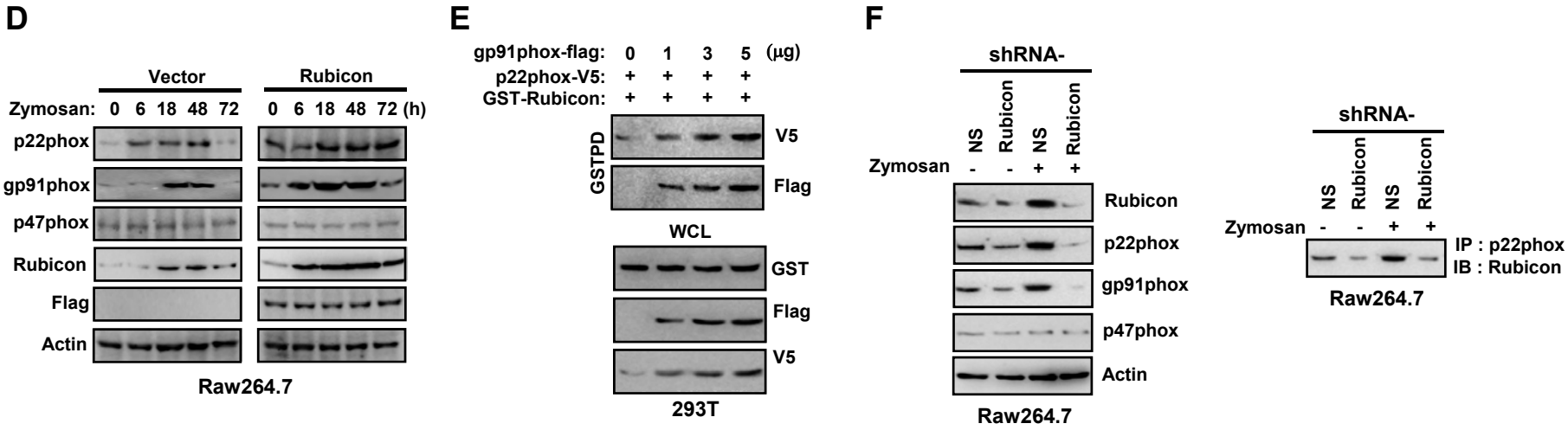

D
Vector
Rubicon
Zymosan: 0 6 18 48 72 0 6 18 48 72 (h)
p22phox
gp91phox
p47phox
Rubicon
Flag
Actin
Raw264.7
E
gp91phox-flag: 0 1 3 5 (μg)
p22phox-V5: + + + +
GST-Rubicon: + + + +
GSTPD
V5
Flag
WCL
GST
Flag
V5
293T
F
shRNA-
NS
Rubicon
NS
Rubicon
Zymosan - - + +
Rubicon
p22phox
gp91phox
p47phox
Actin
Raw264.7
shRNA-
NS
Rubicon
NS
Rubicon
Zymosan - - + +
IP : p22phox
IB : Rubicon
Raw264.7

Fig S2

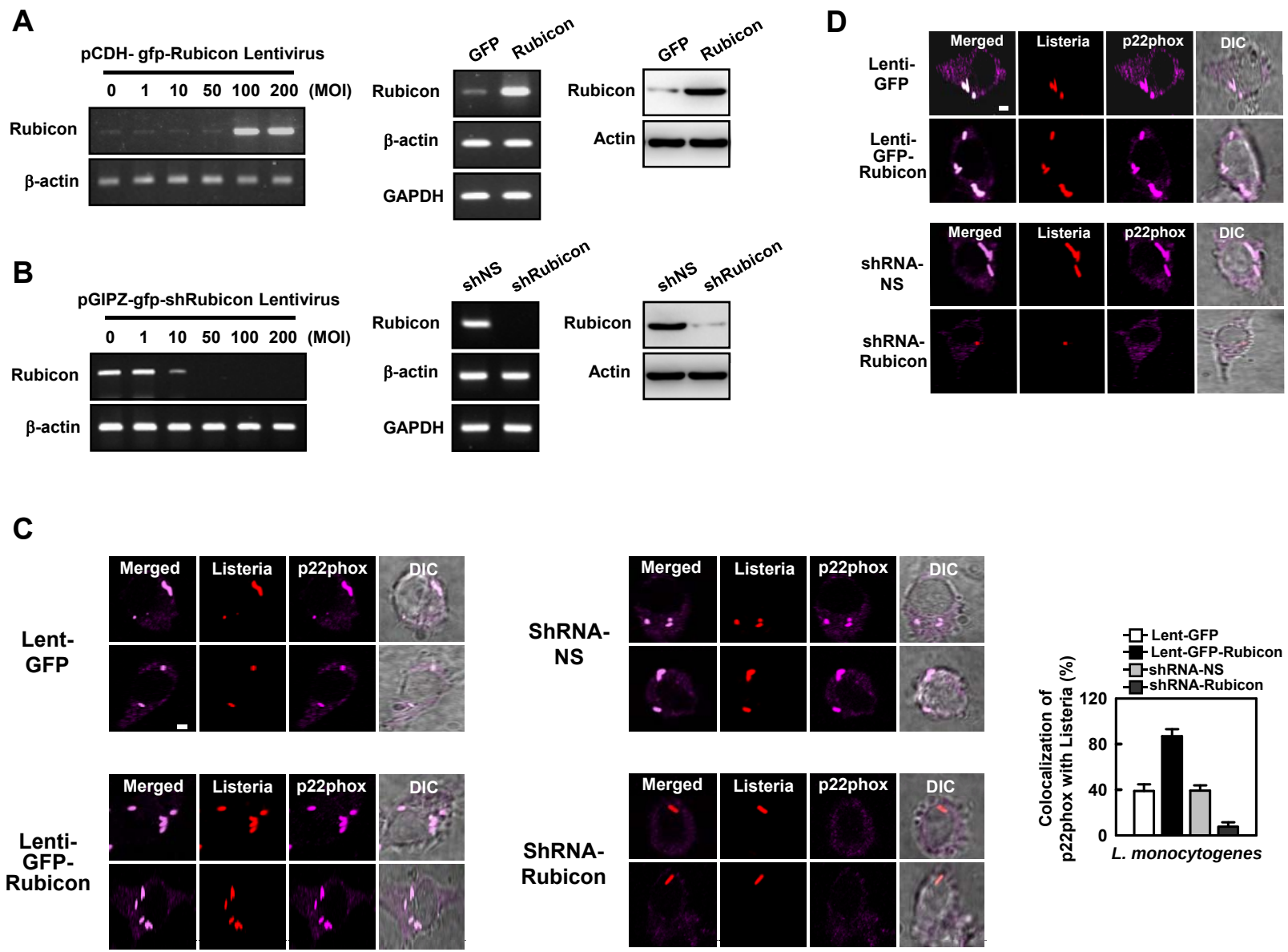

A
pCDH- gfp-Rubicon Lentivirus
0 1 10 50 100 200 (MOI)
Rubicon
β-actin
GFP Rubicon
Rubicon
β-actin
GAPDH
GFP Rubicon
Rubicon
Actin
B
pGIPZ-gfp-shRubicon Lentivirus
0 1 10 50 100 200 (MOI)
Rubicon
β-actin
shNS shRubicon
Rubicon
β-actin
GAPDH
shNS shRubicon
Rubicon
Actin
C
Lent-GFP
Lenti-GFP-Rubicon
ShRNA-NS
ShRNA-Rubicon
Merged
Listeria
p22phox
DIC
Colocalization of p22phox with Listeria (%)
Lent-GFP
Lent-GFP-Rubicon
shRNA-NS
shRNA-Rubicon
120
80
40
0
L. monocytogenes
D
Lenti-GFP
Lenti-GFP-Rubicon
shRNA-NS
shRNA-Rubicon
Merged
Listeria
p22phox
DIC

Fig S3

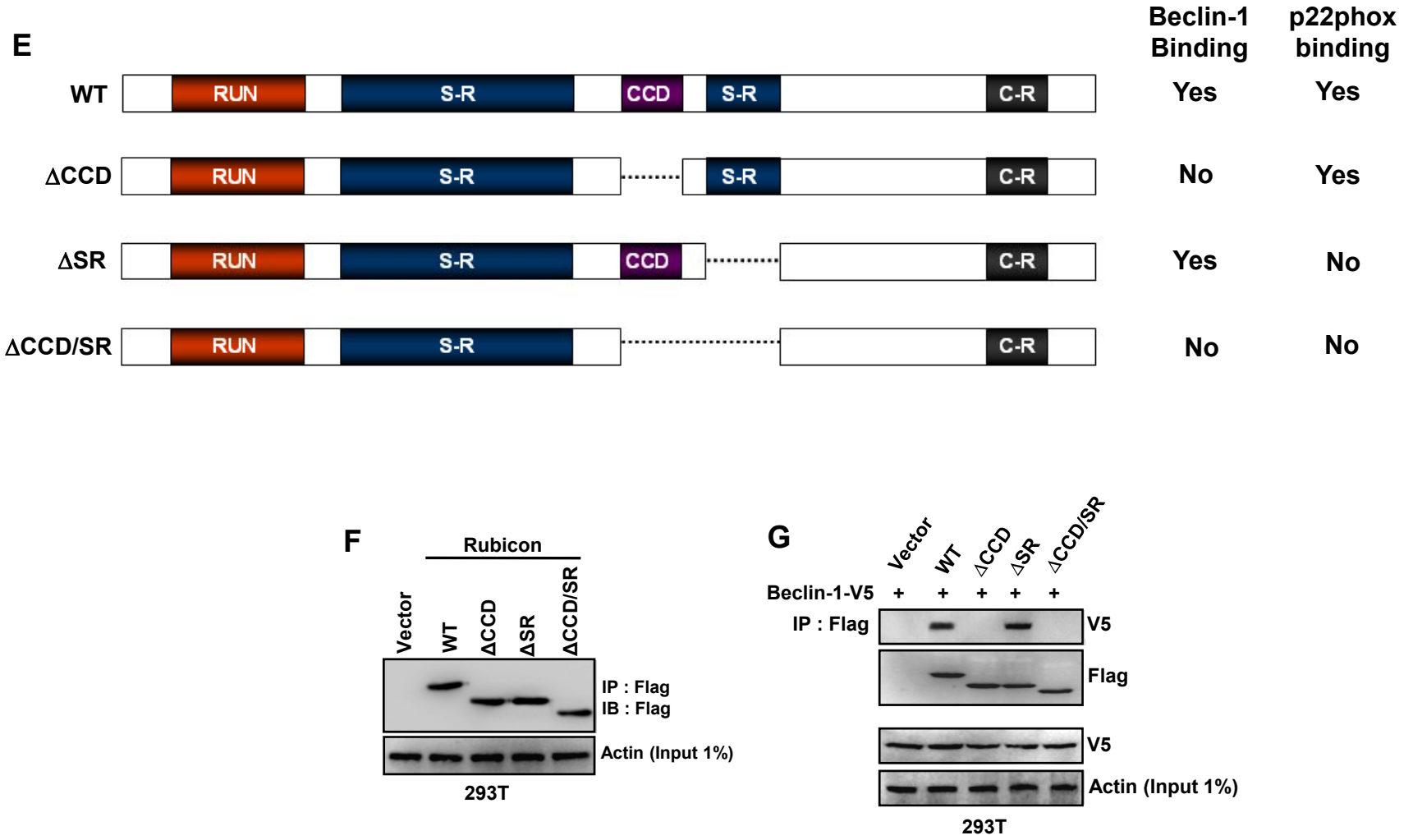

E
Beclin-1 Binding
p22phox binding
WT
RUN
S-R
CCD
S-R
C-R
Yes
Yes
ΔCCD
RUN
S-R
S-R
C-R
No
Yes
ΔSR
RUN
S-R
CCD
C-R
Yes
No
ΔCCD/SR
RUN
S-R
C-R
No
No
F
Rubicon
Vector
WT
ΔCCD
ΔSR
ΔCCD/SR
IP : Flag
IB : Flag
Actin (Input 1%)
293T
G
Vector
WT
ΔCCD
ΔSR
ΔCCD/SR
Beclin-1-V5 + + + + +
IP : Flag
V5
Flag
V5
Actin (Input 1%)
293T

Fig S3

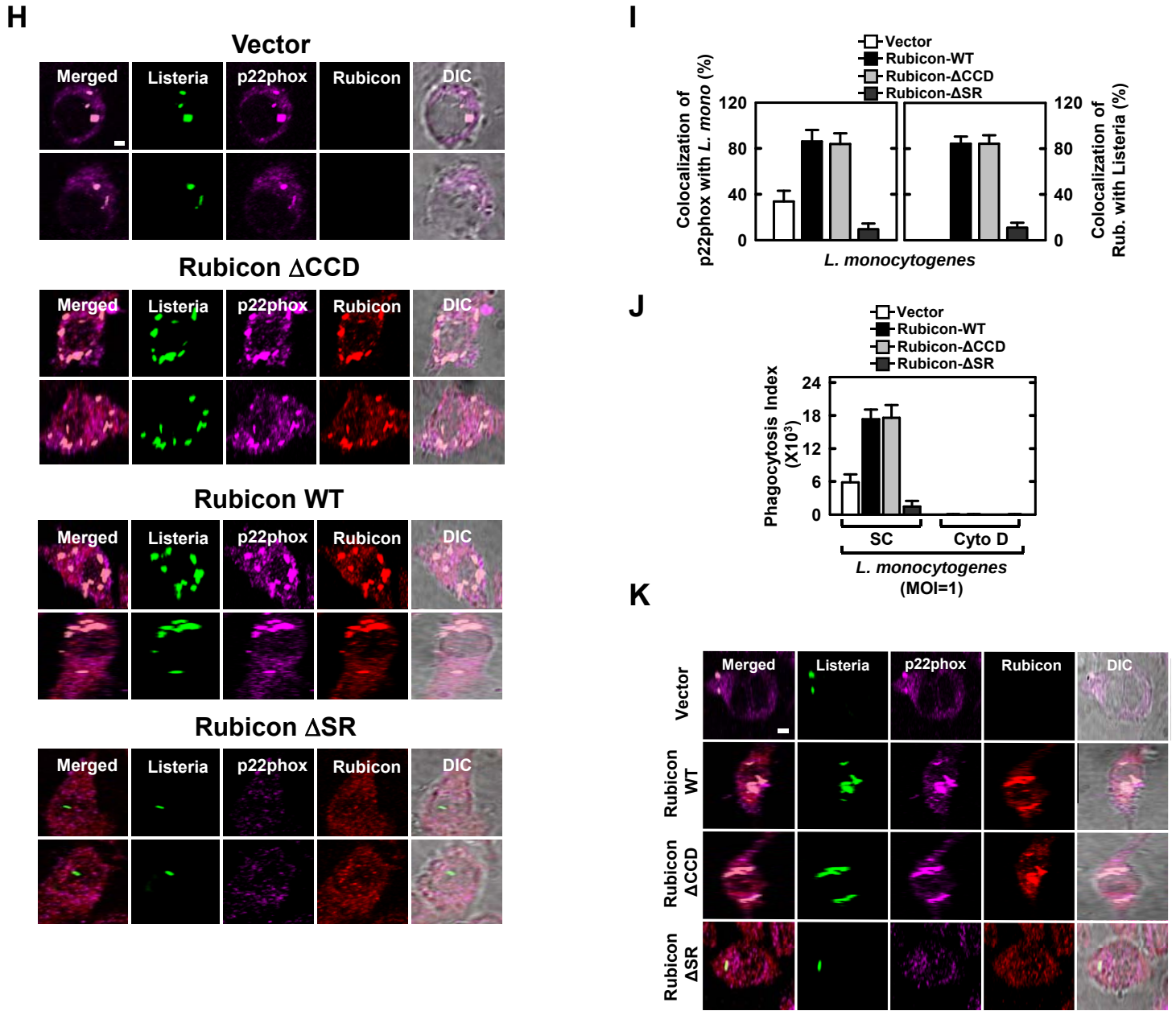
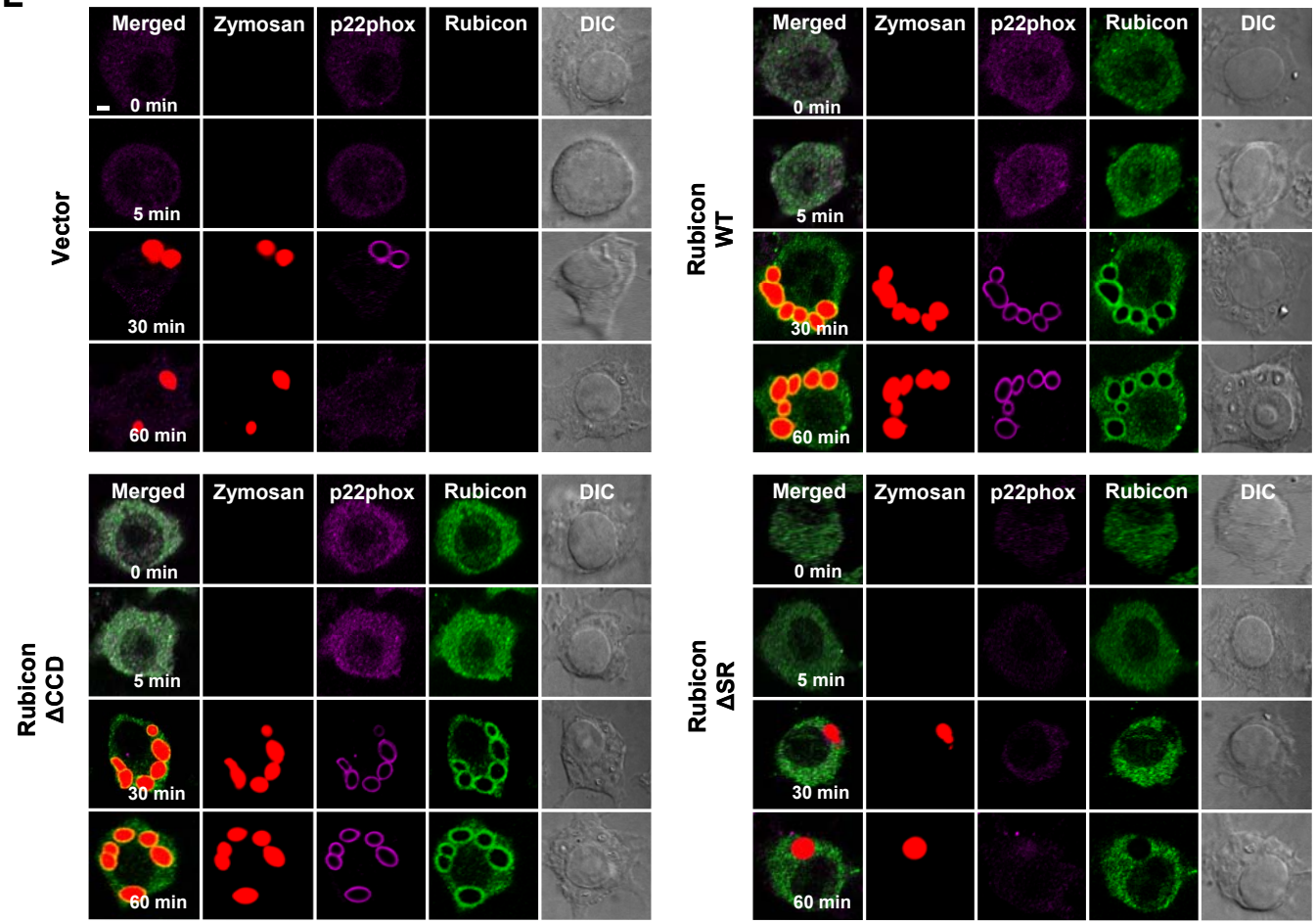

H
Vector
Merged
Listeria
p22phox
Rubicon
DIC
Rubicon ΔCCD
Rubicon WT
Rubicon ΔSR
I
Vector
Rubicon-WT
Rubicon-ΔCCD
Rubicon-ΔSR
Colocalization of p22phox with L. mono (%)
Colocalization of Rub. with Listeria (%)
120
80
40
0
L. monocytogenes
J
Phagocytosis Index (X10³)
24
18
12
6
0
SC
Cyto D
L. monocytogenes
(MOI=1)
K
Vector
Rubicon WT
Rubicon ΔCCD
Rubicon ΔSR

Fig S3

L


Merged
Zymosan
p22phox
Rubicon
DIC
0 min
5 min
30 min
60 min
Vector
Rubicon WT
Rubicon ΔCCD
Rubicon ΔSR

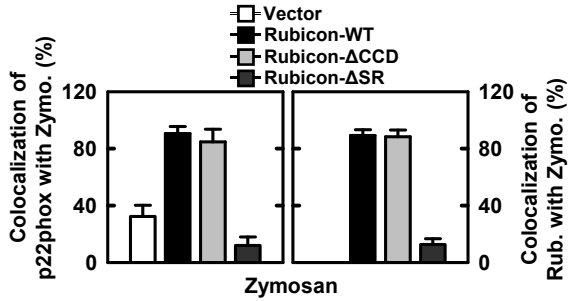

Vector
Rubicon-WT
Rubicon-ΔCCD
Rubicon-ΔSR
Colocalization of p22phox with Zymo. (%)
Colocalization of Rub. with Zymo. (%)
120
80
40
0
Zymosan

Fig S3

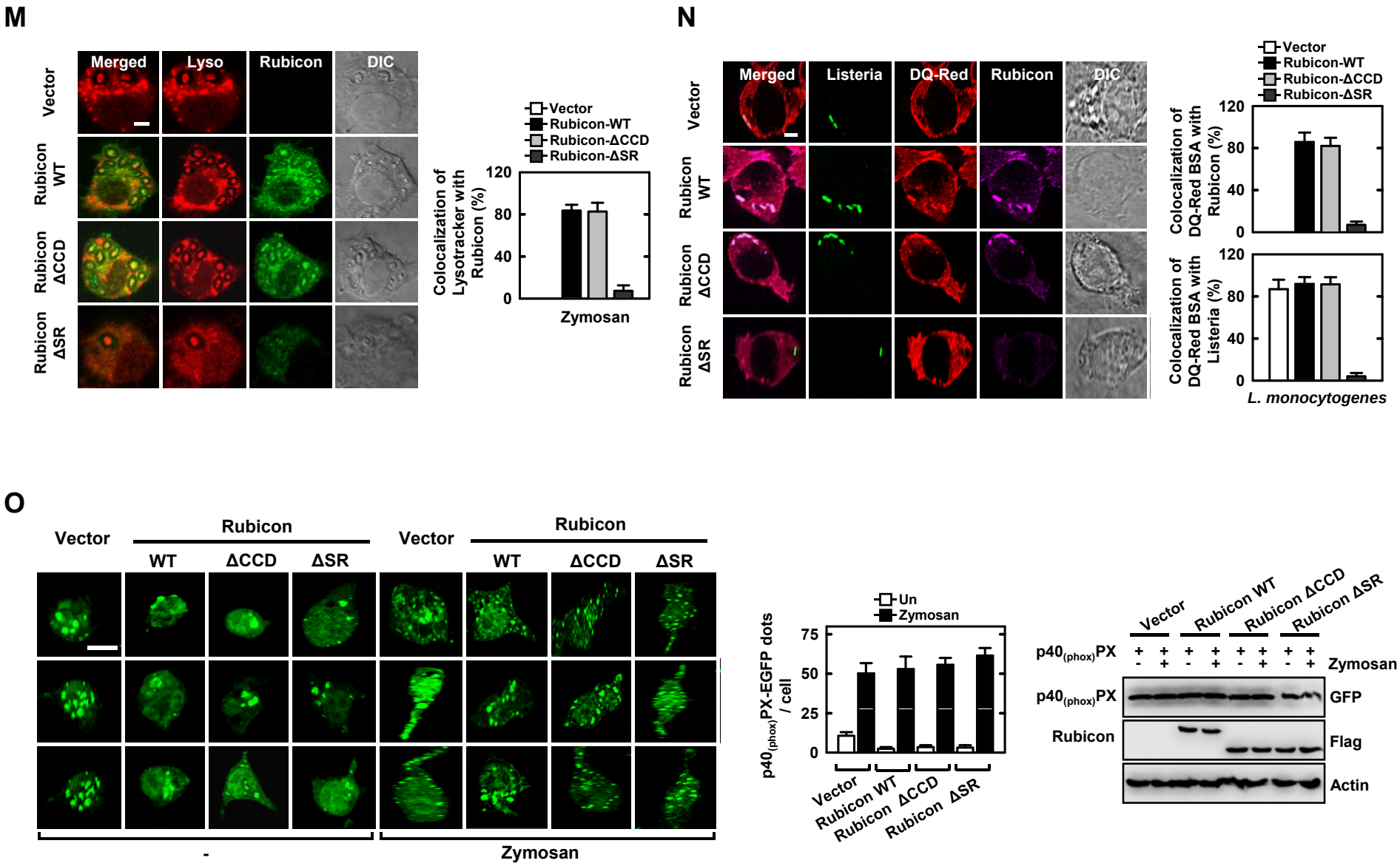

M
Merged
Lyso
Rubicon
DIC
Vector
Rubicon WT
Rubicon ΔCCD
Rubicon ΔSR
Vector
Rubicon-WT
Rubicon-ΔCCD
Rubicon-ΔSR
Colocalization of Lysotracker with Rubicon (%)
120
80
40
0
Zymosan
N
Merged
Listeria
DQ-Red
Rubicon
DIC
Vector
Rubicon WT
Rubicon ΔCCD
Rubicon ΔSR
Vector
Rubicon-WT
Rubicon-ΔCCD
Rubicon-ΔSR
Colocalization of DQ-Red BSA with Rubicon (%)
Colocalization of DQ-Red BSA with Listeria (%)
120
80
40
0
L. monocytogenes
O
Vector
Rubicon
WT
ΔCCD
ΔSR
Vector
Rubicon
WT
ΔCCD
ΔSR
-
Zymosan
Un
Zymosan
p40(phox)PX-EGFP dots / cell
75
50
25
0
Vector
Rubicon WT
Rubicon ΔCCD
Rubicon ΔSR
Vector
Rubicon WT
Rubicon ΔCCD
Rubicon ΔSR
p40(phox)PX
Zymosan
p40(phox)PX
GFP
Rubicon
Flag
Actin

Fig S3

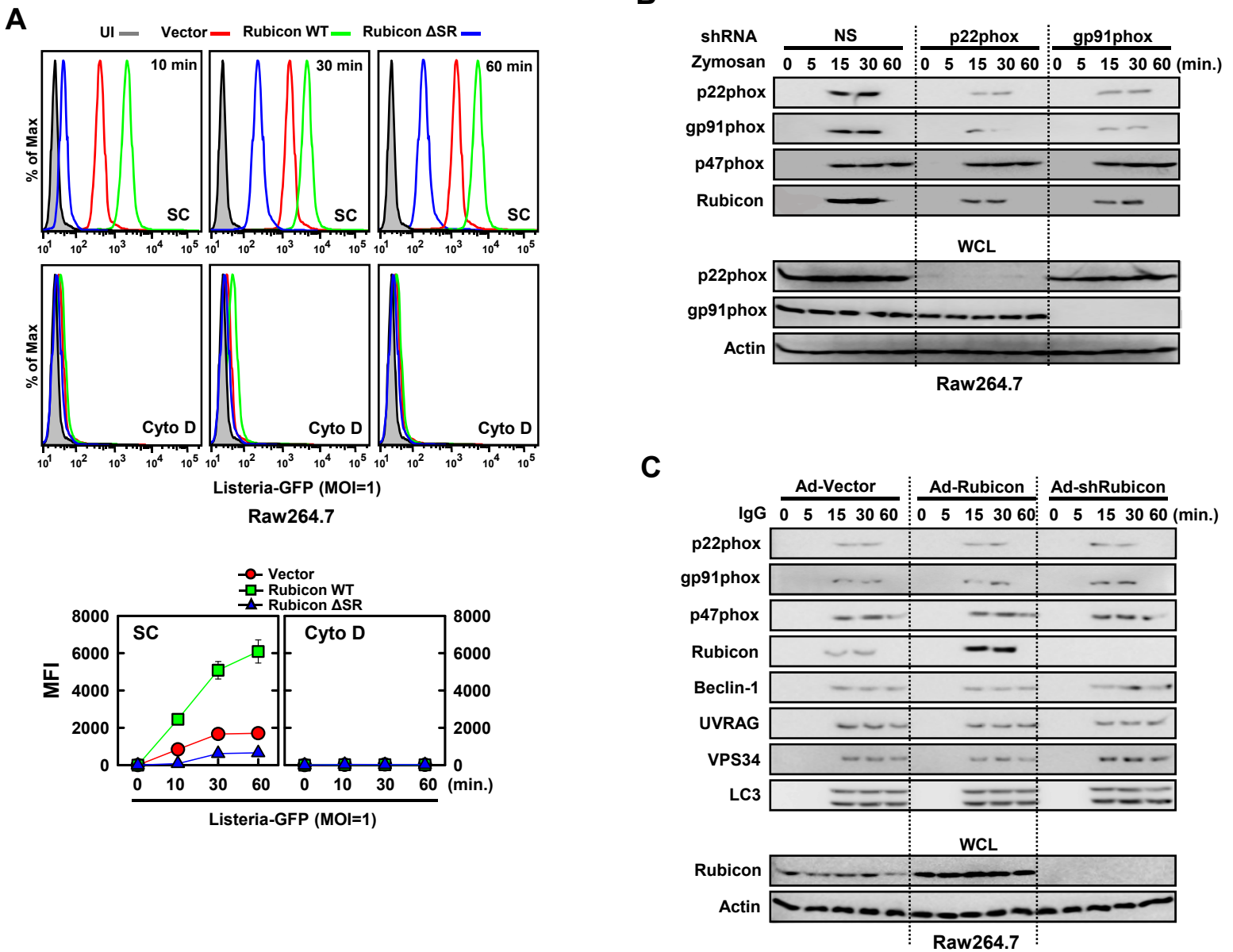

A
UI
Vector
Rubicon WT
Rubicon ΔSR
10 min
30 min
60 min
% of Max
SC
Cyto D
Listeria-GFP (MOI=1)
Raw264.7
MFI
(min.)
B
shRNA
NS
p22phox
gp91phox
Zymosan
0 5 15 30 60 (min.)
p47phox
Rubicon
WCL
Actin
C
Ad-Vector
Ad-Rubicon
Ad-shRubicon
IgG
Beclin-1
UVRAG
VPS34
LC3

Fig S4

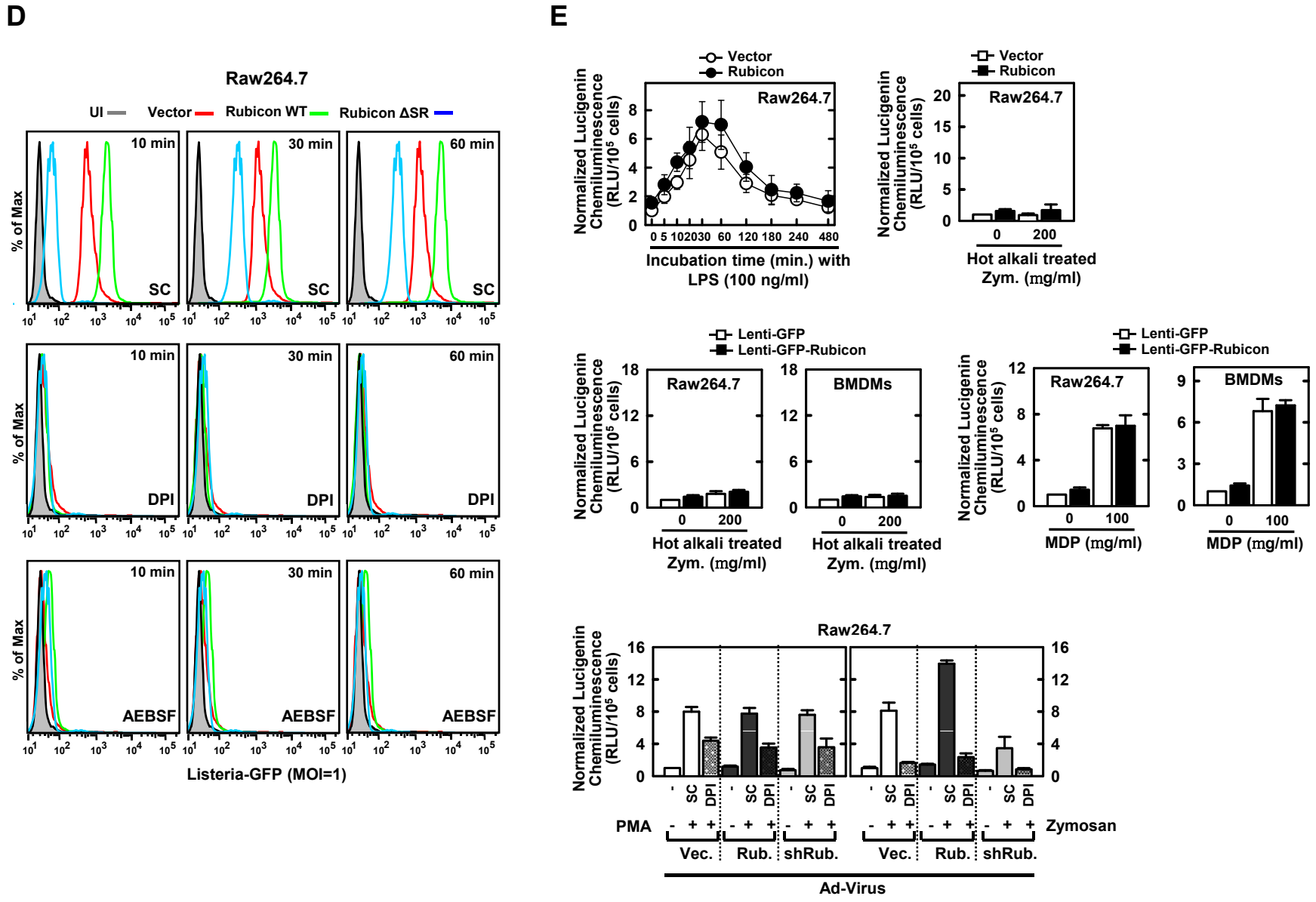

D
Raw264.7
UI
Vector
Rubicon WT
Rubicon ΔSR
10 min
30 min
60 min
% of Max
SC
DPI
AEBSF
Listeria-GFP (MOI=1)
E
Vector
Rubicon
Raw264.7
Normalized Lucigenin Chemiluminescence (RLU/10⁵ cells)
Incubation time (min.) with LPS (100 ng/ml)
Hot alkali treated Zym. (mg/ml)
Lenti-GFP
Lenti-GFP-Rubicon
BMDMs
MDP (mg/ml)
PMA
Zymosan
Vec.
Rub.
shRub.
Ad-Virus

Fig S4

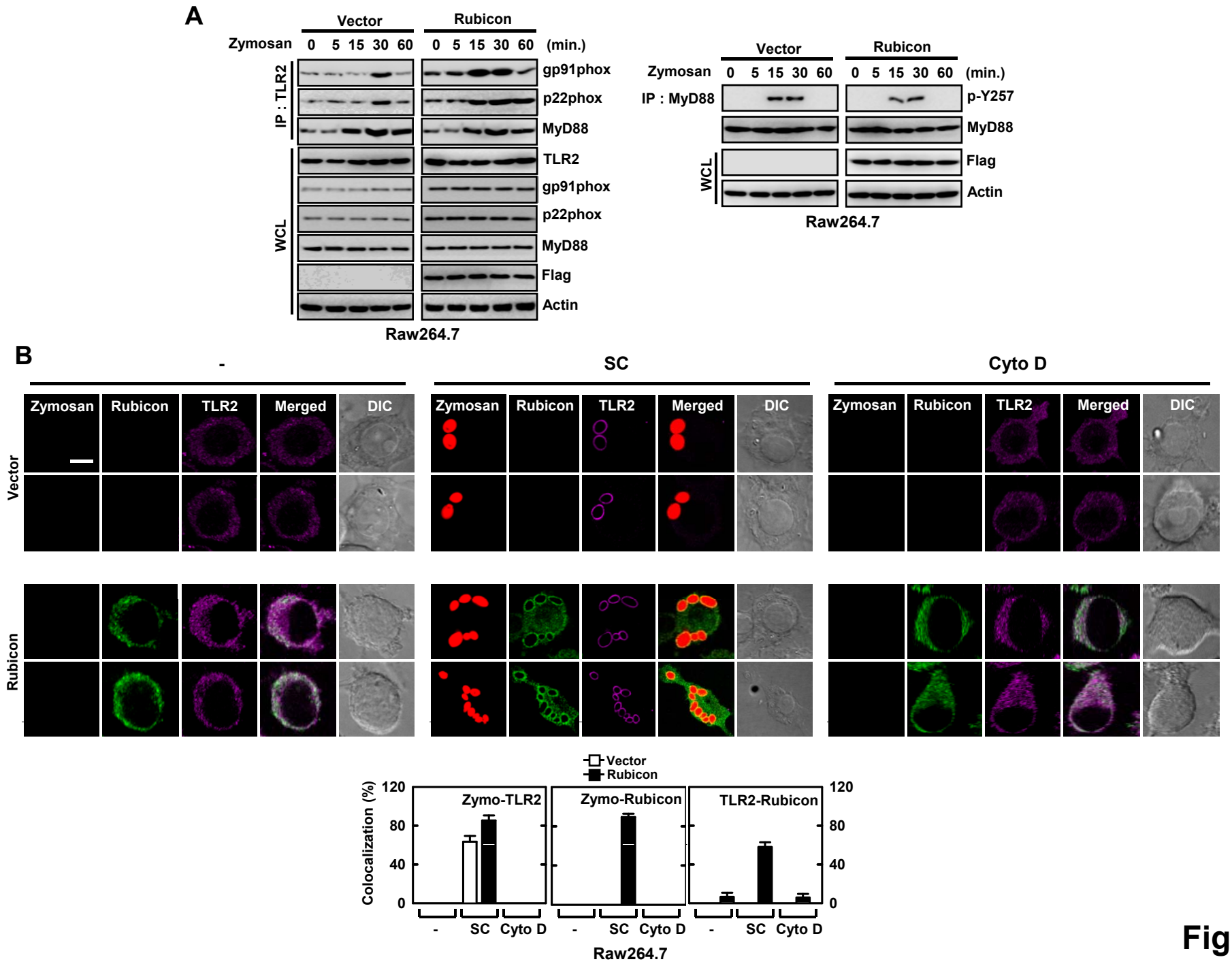

A
Vector
Rubicon
Zymosan
0 5 15 30 60 0 5 15 30 60 (min.)
IP : TLR2
gp91phox
p22phox
MyD88
WCL
TLR2
gp91phox
p22phox
MyD88
Flag
Actin
Raw264.7
Vector
Rubicon
Zymosan
0 5 15 30 60 0 5 15 30 60 (min.)
IP : MyD88
p-Y257
MyD88
WCL
Flag
Actin
Raw264.7
B
-
SC
Cyto D
Zymosan
Rubicon
TLR2
Merged
DIC
Vector
Rubicon
Vector
Rubicon
Colocalization (%)
120
80
40
0
Zymo-TLR2
Zymo-Rubicon
TLR2-Rubicon
- SC Cyto D
Raw264.7

Fig S5

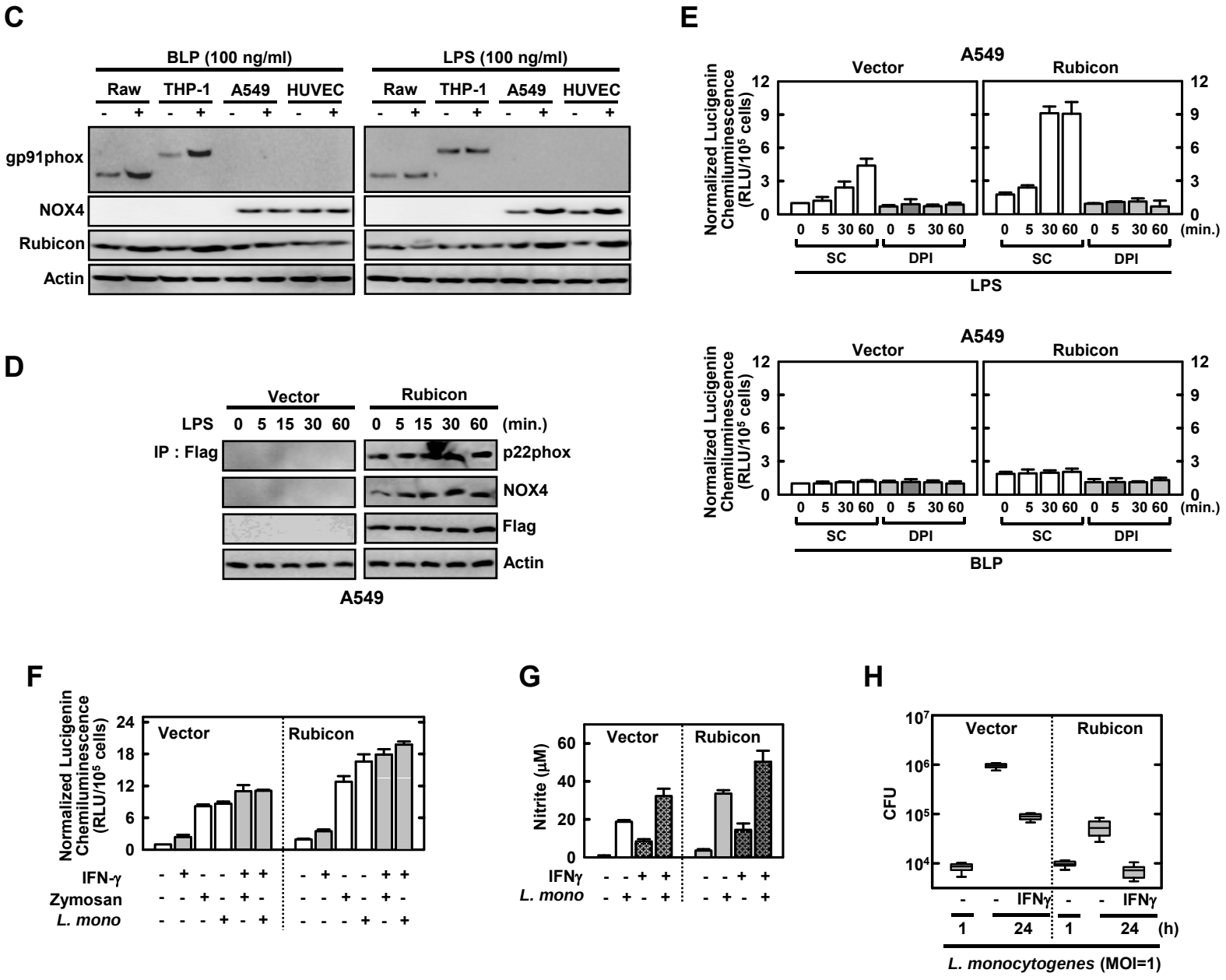

C
BLP (100 ng/ml)
LPS (100 ng/ml)
Raw
THP-1
A549
HUVEC
gp91phox
NOX4
Rubicon
Actin
D
Vector
Rubicon
LPS 0 5 15 30 60 (min.)
IP : Flag
p22phox
NOX4
Flag
Actin
A549
E
A549
Vector
Rubicon
Normalized Lucigenin Chemiluminescence (RLU/10⁵ cells)
SC
DPI
LPS
BLP
(min.)
F
IFN-γ
Zymosan
L. mono
G
Nitrite (μM)
IFNγ
H
CFU
L. monocytogenes (MOI=1)
(h)

Fig S5

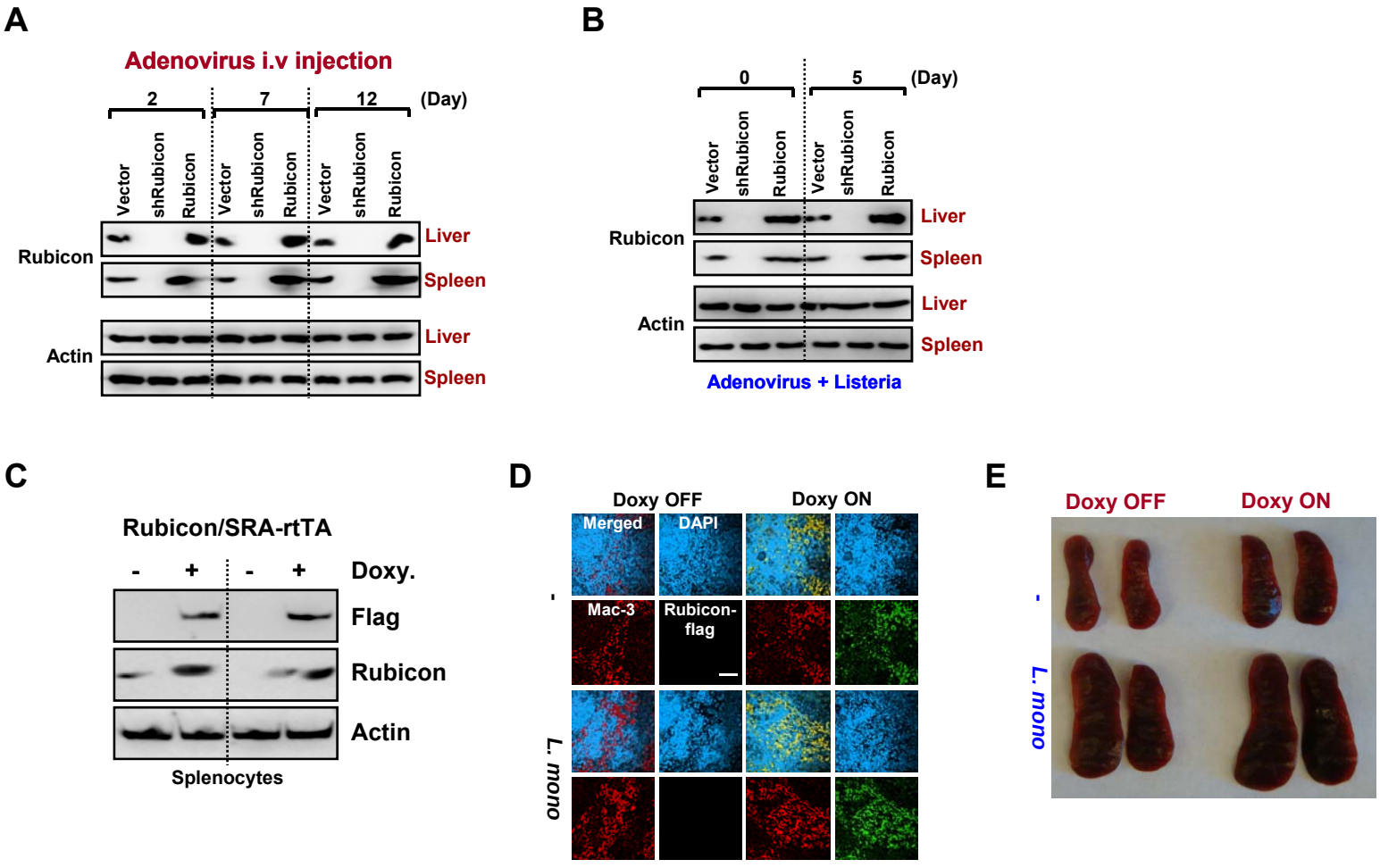

A
Adenovirus i.v injection
2
7
12
(Day)
Vector
shRubicon
Rubicon
Rubicon
Liver
Spleen
Actin
Liver
Spleen
B
0
5
(Day)
Vector
shRubicon
Rubicon
Rubicon
Liver
Spleen
Actin
Liver
Spleen
Adenovirus + Listeria
C
Rubicon/SRA-rtTA
- + - +
Doxy.
Flag
Rubicon
Actin
Splenocytes
D
Doxy OFF
Doxy ON
Merged
DAPI
Mac-3
Rubicon-flag
-
L. mono
E
Doxy OFF
Doxy ON
-
L. mono

Fig S6

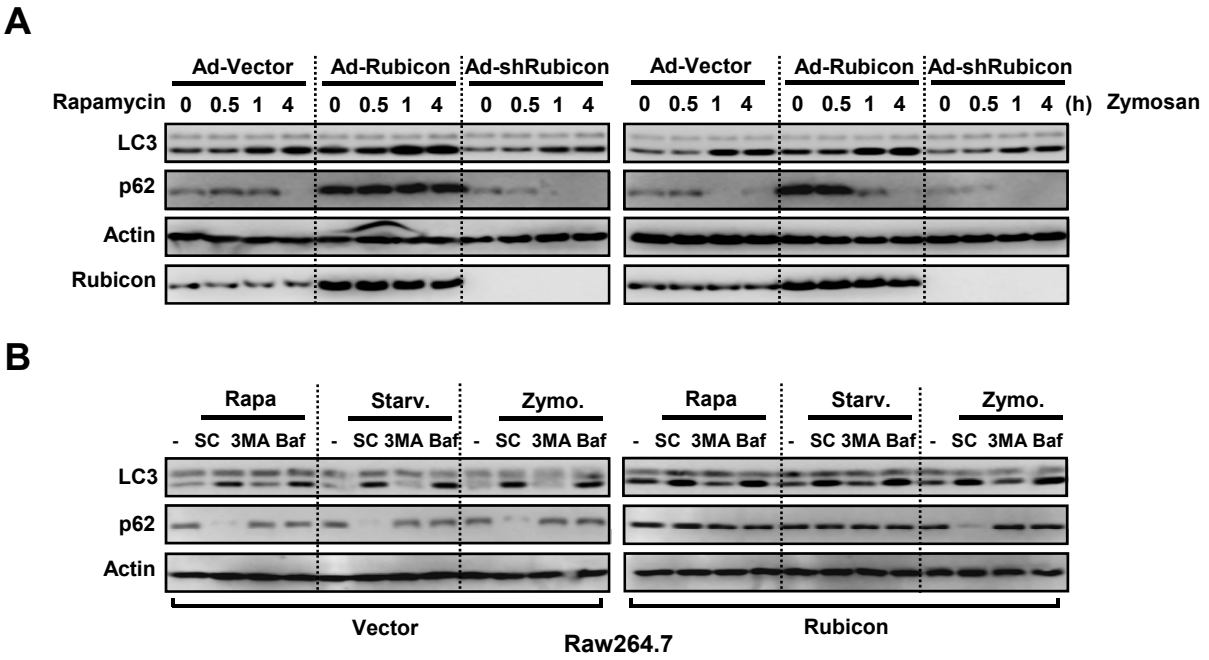

A
Ad-Vector
Ad-Rubicon
Ad-shRubicon
Ad-Vector
Ad-Rubicon
Ad-shRubicon
Rapamycin
0 0.5 1 4
0 0.5 1 4
0 0.5 1 4
0 0.5 1 4
0 0.5 1 4
0 0.5 1 4 (h)
Zymosan
LC3
p62
Actin
Rubicon
B
Rapa
Starv.
Zymo.
Rapa
Starv.
Zymo.
- SC 3MA Baf
- SC 3MA Baf
- SC 3MA Baf
- SC 3MA Baf
- SC 3MA Baf
- SC 3MA Baf
LC3
p62
Actin
Vector
Rubicon
Raw264.7

Fig S7

Supplemental Information

Supplementary Figure Legends

Figure S1. Rubicon interactions with p22*phox*-gp91*phox* complex.

(A) Radio-actively labeled THP-1 cells containing vector were stimulated with rapamycin (2μM) for the indicated times, followed by IP with αFlag for autoradiography. **(B)** Differential Rubicon interactions. Raw264.7 cells containing vector or Flag-Rubicon WT were stimulated with 100μg/ml zymosan or 2μM rapamycin for indicated times, followed by IP with αFlag and IB with αVps15, αVps34, αgp91*phox*, αUVRAG, αBeclin-1, αp22*phox*, αActin or αFlag. **(C)** Identification of p22*phox* (*CYBA*) and gp91*phox* (*CYBB*) as Rubicon-binding proteins. Rubicon complexes purified from THP-1 cells with or without zymosan stimulation were subjected to Mass Spectrometry analysis. The red-colored letters indicate the peptides identified from mass spectrometry analysis. **(D)** Raw264.7 cells containing vector were stimulated with zymosan for indicated times, followed by IP with αFlag and IB with αBeclin-1, αUVRAG, αgp91*phox*, αp22*phox* or αFlag.

Figure S2. Rubicon interactions with Beclin-1-UVRAG complex and p22*phox*-gp91*phox* complex.

(A) Rubicon interaction with Beclin-1-UVRAG complex. Raw264.7 cells containing vector, Flag-Rubicon WT or ΔCCD were stimulated with 2μM rapamycin for indicated times (min), followed by IP with αFlag and IB with αBeclin-1, αUVRAG, αgp91*phox*, αp22*phox*, αp47*phox*, or αFlag. **(B)** Rubicon interaction with p22*phox* and gp91*phox* complex. Raw264.7 cells containing vector, Flag-gp91*phox*, Flag-p22*phox* or Flag-p47*phox* were stimulated with 100μg/ml zymosan for indicated times, followed by IP with αFlag and IB with αRubicon or αFlag. **(C)** PMA stimulation

does not induce Rubicon interaction with p22*phox* and gp91*phox* complex. Raw264.7 cells containing vector or Flag-Rubicon WT were stimulated with 100nM PMA for indicated times, followed by IP with αFlag and IB with αBeclin-1, αUVRAG, αgp91*phox*, αp22*phox*, αp47*phox*, or αFlag. **(D)** Raw264.7 cells containing vector or Flag-Rubicon were stimulated with 100μg/ml zymosan for indicated times, followed by IB with αgp91*phox*, αp22*phox*, αp47*phox*, αRubicon, αFlag or αActin. **(E)** At 48h posttransfection with GST-Rubicon and V5-p22*phox* vector with increasing amount of Flag-gp91*phox* vector, 293T cell lysates were used for GST-PD, followed by IB with αV5 or αFlag. WCLs were used for IB with αGST, αFlag, or αV5. **(F)** Depletion of Rubicon expression affects the levels of p22*phox* and gp91*phox* expression. At 48h postinfection with shRNA knockdown vector (lenti-shRNA-NS or lenti-shRNA-Rubicon, MOI=50), Raw246.7 cells were stimulated with or without zymosan for 18h and WCLs were used for IB with indicated antibodies (left) or IP with αp22*phox* and IB with αRubicon (right).

Figure S3. Rubicon affects p22*phox* localization.

(A, B, C and **D)** Rubicon affects the p22*phox* localization. At 48 h postinfection with lenti-GFP-Rubicon **(A)** or lenti-shRNA-Rubicon **(B)** at various MOIs, Raw264.7 cells were used for RT-PCR for Rubicon and β-actin. At 48h postinfection with lenti-GFP or lenti-GFP-Rubicon at MOI=100 **(A)** or lenti-shRNA-NS or lenti-shRNA-Rubicon at MOI=50 **(B)**, Raw264.7 cells were used for RT-PCR for Rubicon, β-actin or GAPDH or IB with αRubicon or αActin. Raw264.7 cells were then infected with **(C)** heat-killed (HK)-TRITC-labeled *L. monocytogenes* (MOI=1) or **(D)** live-TRITC-labeled *L. monocytogenes* (MOI=1) for 30 min, followed by confocal microscopy with αp22*phox*. Bar, 2 μm. The right panel shows the colocalization index (%) between p22*phox* and GFP-*L. monocytogenes* determined by computer program.

(E, F, and **G)** Rubicon interactions with Beclin-1 and p22*phox*. **(E)** Schematic diagram of Rubicon structure and summary of Rubicon interactions with Beclin-1 and p22phox. **(F)** Flag-Rubicon WT and mutant expression in Raw264.7 cells. WCLs were used for IP with αFlag and IB with αFlag. **(G)** Beclin-1 interaction with Rubicon WT and mutants. At 48h posttransfection with indicated constructs, 293T cells lysates were used for IP with Flag**,** followed by IB with Flag or V5. WCLs were used for IB to show V5-Beclin-1 and Actin.

(H, I, J, K and **L)** Rubicon enhances the co-localization of p22*phox* with *L. monocytogenes*-containing phagosomes. **(H)** Raw264.7 cells containing vector, Flag-Rubicon WT, ΔCCD or ΔSR were infected with HK-GFP-*L. monocytogenes* (MOI=1) for 30 min, followed by confocal microscopy with αp22phox and αFlag. Bar, 2 μm. **(I)** Colocalization index (%) between p22*phox* and GFP-*L. monocytogenes*. **(J)** Phagocytosis index (%) of GFP-*L. monocytogenes*. Treatment with the Cytochalasin D (Cyto D, 10μM), phagocytosis inhibitor, was included as a negative control. SC, solvent control. **(K)** Raw264.7 cells containing vector, Flag-Rubicon WT, ΔCCD or ΔSR were infected with Live-GFP-*L. monocytogenes* (MOI=1) for 30 min, followed by confocal microscopy with αp22phox and αFlag. Bar, 2 μm. **(L)** Raw264.7 cells containing vector, Flag-Rubicon WT, ΔCCD or ΔSR were stimulated with 100μg/ml Texas-Red labeled opsonized-zymosan particles for indicated times, followed by confocal microscopy with αp22*phox* and αFlag. Bar, 2 μm. The bottom panel shows the colocalization index (%) between p22*phox* and zymosan-coated beads (left) or between Rubicon and zymosan-coated beads at 30 min.

(M, N, and O) Rubicon expression enhances the co-localization between lysosomes and phagosomes upon zymosan stimulation. Raw264.7 cells containing vector, Flag-Rubicon WT, ΔCCD or ΔSR were stimulated with zymosan (100μg/ml) for 30min and staining with lysosome dye LysoTracker (50 nM) (**M**) or infected with HK-GFP-*L. monocytogenes* (MOI=1) and a self-

quenched red BODIPY dye-conjugated bovine albumin serum (DQ-Red BSA) (10μg/ml) (**N**), followed by confocal microscopy with αRubicon. Bar, 2 μm. The right panels show the colocalization index (%) between Rubicon and LysoTracker (**M**) or between Rubicon/Listeria and DQ-Red BSA (**N**), respectively. (**O**) PI(3)KC3 activity upon zymosan treatment. At 24 h posttransfection with p40(phox) PX–EGFP fusion, Raw264.7 cells expressing vector, Rubicon WT, Rubicon ΔCCD mutant, and Rubicon ΔSR mutant were stimulated with zymosan (100μg/ml) for 30min and subjected to an inverted fluorescence microscope. p40(phox) PX–EGFP-positive vesicles were quantified as the mean ± SD of combined results from three independent experiments. Bar, 10 μm. WCLs were used for immunoblotting with αGFP, αFlag, or αActin.

Figure S4. Rubicon enhances phagocytosis in a p22*phox*-binding-dependent manner

(**A**) Raw264.7 cells containing vector, Rubicon WT, or ΔSR were infected with HK-GFP-*L. monocytogenes* (MOI=1) for the indicated times, followed by flow cytometry analysis to detect internalized HK-GFP-*L. monocytogenes*. The middle panel shows the treatment with Cytochalasin D (Cyto D, 10 μM), phagocytosis inhibitor as a negative control. SC, solvent control. The mean fluorescence intensity values of HK-GFP-*L. monocytogenes* from flow cytometry were used to generate phagocytosis rate kinetics (bottom). (**B**) At 48h postinfection with lentivirus-GFP (MOI=50), lentivirus-shRNA-p22*phox* (MOI=50) or lentivirus-shRNA-gp91*phox* (MOI=50), Raw264.7 cells were stimulated with 100μg/ml zymosan-coated particles for indicated times, followed by lysis and sucrose-gradient ultracentrifugation to isolate zymosan bead-containing phagosomal fractions. Phagosomal fractions were subjected to IB with αgp91*phox*, αp22*phox*, αp47*phox*, or αRubicon. WCLs were used for IB with αgp91*phox*, αp22*phox* or αActin. (**C**) At 48h postinfection with recombinant Ad-Vector (MOI=200), Ad-Rubicon (MOI=100), or Ad-shRubicon (MOI=200) virus, Raw264.7 cells were stimulated with

100μg/ml IgG-coated particles for indicated times, followed by lysis and sucrose-gradient ultracentrifugation to isolate the bead-containing phagosomal fractions (please see the Method section). Phagosomal fractions were subjected to IB with αBeclin-1, αUVRAG, αgp91*phox*, αp22*phox*, αp47*phox*, αVPS34, αLC3 or αRubicon. Whole cell lysates (WCL) were used for IB with αRubicon or αActin.

(D and **E)** NADPH oxidase activity is necessary for Rubicon-mediated increased of phagocytosis. **(D)** Raw264.7 cells containing vector, Rubicon WT, or ΔSR were infected with HK-GFP-*L. monocytogenes* (MOI=1) for the indicated times, followed by flow cytometry analysis to detect internalized HK-GFP-*L. monocytogenes*. The bottom panels show the treatment with 20 μM DPI or 0.5 mM AEBSF NADPH oxidase inhibitor as a negative control. SC, solvent control. **(E)** Rubicon shows no significant effect on the LPS-, hot-alkali-depleted zymosan or MDP-mediated release of ROS. Raw264.7 cells expressing vector or Flag-Rubicon or BMDMs infected with lenti-GFP or lenti-GFP-Rubicon (MOI=100) were analyzed for NOX activity upon stimulation with 100ng/ml LPS, 200μg/ml hot alkali-treated zymosan, 100μg/ml MDP or 100nM PMA. Quantitative data are the mean ± SD of values from three experiments.

Figure S5. Rubicon enhances TLR signal transduction

(A and **B)** Rubicon enhances TLR2 signal transduction. **(A)** Raw264.7 cells containing vector or Flag-Rubicon were stimulated with 100μg/ml zymosan for indicated times, followed (left panel) by IP with αTLR2 and IB with αgp91*phox*, αp22*phox*, and αMyD88 or (right panel) by IP with αMyD88 and IB with αY257-phospho-specific-MyD88 or αMyD88. WCL was used for IB with αTLR2, αFlag or αactin. **(B)** Raw264.7 cells containing vector or Flag-Rubicon WT were stimulated with 100μg/ml zymosan for 30min, followed by confocal microscopy with αTLR2 and αRubicon. Bar, 5 μm. The left panel, no Texas-Red labeled opsonized-zymosan particles; the

middle panel, Texas-Red labeled opsonized-zymosan particles with solvent control (SC); and the right panel, Texas-Red labeled opsonized-zymosan particles with the Cytochalasin D (Cyto D, 10 μM) treatment. The bottom panel shows the colocalization index (%) between zymosan-coated beads and TLR2 (left) or Rubicon (middle) and or between TLR2 and Rubicon (right).

(C, D, E, F, G, and H) Rubicon enhances TLR4 signal transduction and plays an important role in the TLR-NADPH oxidase-mediated anti-microbial pathway.

(C, D, and **E)** Rubicon enhances TLR4 signal transduction. (**C**) Raw264.7, THP-1, A549, and HUVEC cells were used for IB with αgp91*phox* (NOX2), αNOX4, and αRubicon or αActin. **(D)** A549 cells containing vector or Flag-Rubicon were stimulated with LPS for the indicated times and used for IP with αFlag and IB with αp22phox or αNOX4. WCLs were used for IB with αFlag or αActin. **(E)** A549 cells containing vector or Flag-Rubicon were stimulated with 100ng/ml LPS or 100ng/ml BLP for the indicated times in the presence or absence of 20 μM DPI NADPH oxidase inhibitor, followed by NOX enzymatic assay. Quantitative data are the mean ± SD of values from three experiments. (**F, G,** and **H**) Rubicon plays an important role in the TLR-NADPH oxidase-mediated anti-microbial pathway. Raw264.7 cells containing vector or Rubicon were stimulated with zymosan (100μg/ml), IFNγ (10ng/ml), and/or *L. monocytogenes* (MOI=1) and assayed for NADPH oxidase (**F**), NO synthase activity (**G**) and intracellular bacterial loads (**H**) as described in Figure 4.

Figure S6. *In vivo* Rubicon expression in infected mice.

(A) Rubicon expression in the spleen and liver of recombinant Ad-infected mice. At days 2, 7 and 12 post-injection with Ad-vector ($1x10^{13}$ pfu/kg), Ad-shRubicon ($1x10^{12}$ pfu/kg), or Ad-Rubicon ($1x10^{13}$ pfu/kg) *i.v* via tail vein, spleens and livers were taken out to show the expressions of Rubicon. IB with αRubicon or αActin. **(B)** At 48h post-injection with Ad-vector

($1x10^{13}$ pfu/kg), Ad-shRubicon ($1x10^{12}$ pfu/kg), or Ad-Rubicon ($1x10^{13}$ pfu/kg) twice *i.v* via tail vein, mice were infected with *L. monocytogenes* ($1x10^{6}$ CFU/mouse). At 5 days *p.i*. livers and spleens were harvested for IB with αRubicon. **(C, D** and **E)** Doxycycline-inducible, macrophage-specific expression of Rubicon in SRA-rtTA-Rubicon mice. After 7 days of doxycycline treatment, mice were infected with *L. monocytogenes* ($1x10^{7}$ CFU/mouse). At 4 days *p.i*. spleens were harvested for IB with αFlag, αRubicon or αactin **(C),** immune-stained with αMac-3, αFlag or DAPI **(D),** and for photograph **(E).** Bar, 20 μm.

Figure S7. Rubicon plays distinctive roles in conventional and TLR signaling-mediated autophagy. (A) At 48h postinfection with recombinant Ad-Vector (MOI=200), Ad-Rubicon (MOI=100), or Ad-shRubicon (MOI=200) virus, Raw246.7 cells were treated with 2μM rapamycin or 100μg/ml zymosan for indicated times and their cell lysates were used for IB with αLC3, αp62, αRubicon, or αActin. **(B)** Raw264.7 cells containing vector or Rubicon were stimulated with mock (-) or rapamycin (Rapa), starvation (Starv) or zymosan (Zymo) along with solvent control (SC), 10μM 3MA or 200nM Bafilomycin (Baf) pretreatment for 2 h, followed by IB with αLC3, αp62, or αActin.

Supplemental Experimental Procedures

Cell culture

Primary bone marrow-derived macrophages (BMDMs) from 6- to 8-week-old female C57BL/6 mice were prepared. All animals were maintained in a pathogen-free environment. Briefly, bone marrow cells from the femur and tibia were cultured for 4 days in 10% L929 culture media (as a source of M-CSF)-containing Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) containing 4 mM glutamine and 10% FBS. Mouse macrophage cell line RAW264.7 (ATCC TIB-71; American Type Culture Collection) and HEK293T (ATCC-11268) were maintained in DMEM (Gibco-BRL) containing 10% FBS (Gibco-BRL), sodium pyruvate, nonessential amino acids, penicillin G (100 IU/ml), and streptomycin (100 µg/ml). Human monocytic THP-1 (ATCC TIB-202) cells were grown in RPMI 1640/glutamax supplemented with 10% FBS and treated with 20 nM PMA (Sigma-Aldrich) for 24 h to induce their differentiation into macrophage-like cells, followed by washing three times with PBS. Transient transfections were performed with Lipofectamine 2000 (Invitrogen), or calcium phosphate (Clontech), according to the manufacturer's instructions. RAW264.7 and THP-1 stable cell lines were generated using a standard selection protocol with 4 µg/ml of puromycin.

Reagents

CHX, BSA, IgG, Cytochalasin D, 3-Methyladenine, Bafilomycin A1, and rapamycin were from Sigma; Zymosan and BLP (Pam2CSK4) were purchased from Invivogen; NAC, DPI, AEBSF, DHE and CM-H_2DCFDA were from Calbiochem; recombinant mIFN-γ were from eBioscience; Texas Red-conjugated zymosan A (*S. cerevisiae*) BioParticles, LysoTracker Red DND-99 and DQ-Red BSA were from Invitrogen.

Bacterial strains

L. monocytogenes and *L. monocytogenes* expressing GFP strains were provided by D. Portnoy (University of California-Berkely), and *M. bovis* BCG and *M. bovis* BCG-GFP strains were provided by Yi Luo (University of Iowa). *L. monocytogenes* and *M. bovis* BCG were grown at 37°C in brain-heart–infusion (BHI) broth medium (BD) and Middlebrook 7H9 medium supplemented with Tween 80, glycerol and OADC (Difco Laboratories), respectively. For all assays, mid-log-phase bacteria (absorbance, 0.9) were used. Batch cultures were aliquoted and stored at −80°C. Representative vials of *L. monocytogenes* and *M. bovis* BCG were thawed and enumerated for viable colony-forming unit (CFU) on BHI agar (BD) and Middlebrook 7H10 agar (Difco), respectively. The effective concentration of LPS was <50 pg/ml in those experiments with a bacterium-to-cell ratio of 10:1. Heat-killed *L. monocytogenes* were obtained by heating for 30 min at 60°C.

Autoradiography

Raw264.7 were labeled for 6h with S^{35} Met/Cys (10 μCi/ml; MP Biomedicals, Inc.) in Met/Cys-free DMEM containing dialyzed 10% FBS (Sigma) and 1% L-glutamine (Gibco-BRL), then were stimulated for indicated times at 37 °C with Zymosan or rapamycin and rinsed. 1μg of Flag antibody was added to 1ml of cell lysates and incubated at 4°C for 18h for immunoprecipitation. After addition of protein A/G agarose beads, incubation was continued for 2h. Immunoprecipitates were extensively washed with lysis buffer and eluted with SDS loading buffer by standing on RT for 30 min. After fixing and amplifying, gels were dried and radiolabeled gels were visualized by autoradiography for 18 h.

Plasmid construction

All constructs for transient and stable expression in mammalian cells were derived from the pEBG-GST mammalian fusion vector and the pEF-IRES-Puro expression vector. DNA fragments corresponding to the coding sequences of the human Rubicon, p22*phox*, gp91*phox*, and p47*phox* genes were amplified by polymerase chain reaction (PCR) and subcloned into pEF-IRES-puro between the *Afl*II and *Xba*I sites and selected for stable transfectants. AU1-tagged Rubicon and V5-tagged Beclin1 were cloned into the *Afl*II and *Xba*I sites in pEF-IRES-Puro. GST-tagged Rubicon, p22*phox*, and mutant genes were cloned into a pEBG derivative encoding an N-terminal GST epitope tag between the *BamH*I and *Not*I sites. Flag-tagged truncated mutant constructs of Rubicon were created by subcloning the PCR products of cDNA fragments containing each domain of the target genes into pEF-IRES-Puro. All constructs were sequenced using an ABI PRISM 377 automatic DNA sequencer to verify 100% correspondence with the original sequence.

Yeast two-hybrid screen

Yeast transformation with human leukocyte cDNA library was performed as recommended by the manufacturers. Briefly, Yeast strain Y187 bearing Gal4-Rubicon full length or C-terminal region fusion gene plasmid was grown overnight in synthetic dropout (SD)/-Trp medium to a density of approximately 10^7 cells/ml, then diluted in 1 liter of warmed YPD to an optical density (OD_{600}) of 0.2 - 0.3 and grown to exponential stage. Cells were harvested and washed with 100 ml of water twice and TE buffer (Clontech) once. The pellets were resuspended in 8 ml of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M Li-acetate (LiOAc) and the suspension was mixed with 1 mg of transforming DNA and 20 mg of single-stranded salmon sperm DNA, after which 60 ml of a solution of 40% polyethyleneglycol-4000 in Tris-EDTA-LiOAc was added and mixed thoroughly, followed by incubation at 30°C with agitation for 30 min. After a heat pulse at 42°C for 15 min, cells were pelleted, washed with 50 ml of Tris-EDTA, and plated on selective

medium. Library screening and recovery of plasmids were performed according to manufacturer's instructions (Clontech).

Confocal fluorescence microscopy

For immunostaining, RAW 264.7 and BMDMs cells were seeded on 12-well culture dishes that contained 18 mm diameter round glass coverslips (10^5 cells per well). Cells were fixed with 4% paraformaldehyde in PBS at 4°C for 10 min and permeabilized with 0.25% Triton X-100 in PBS for 15 min before being treated with 10% BSA, 3% FBS for 1 hr at 25°C. Fixed cells were then stained with primary antibodies, including goat anti-p22*phox* (C-17; Santa Cruz Biotech), mouse anti-gp91*phox* (54.1; Santa Cruz Biotechnology), rabbit anti-Flag (Sigma), and rabbit anti-Beclin1 (Cell Signaling) overnight at 4°C. After extensively washing to remove excess primary antibodies with PBS+0.5% Tween20, fixed cells were incubated for 1h at RT with fluorescently labeled secondary antibodies [anti-rabbit IgG-FITC or TRITC, anti-mouse IgG-FITC or TRITC, and anti-goat IgG-Cy5 (Molecular Probes)], followed by extensively washing with PBS+0.5% Tween20 twice and PBS once (each time for 25 min). Cells were imaged with a laser-scanning confocal microscopy (Nickon Eclipse C1)

Preparation of Latex Beads

Polystyrene beads (1.7 μm diameter, 2.5% suspension; Polysciences, Inc.) were coated with BSA (100 μg/ml), IgG (100 μg/ml), Zymosan (1 mg/ml) or PBS (control) for 1h at 37 °C. Beads were washed extensively in PBS before use.

Flow cytometry

Cells were analyzed by flow cytometry for the expression of p22phox and gp91phox using a FACSCanto II flow cytometer as indicated by the manufacturer (Becton Dickinson). After two washes with PBS, cells were fixed in 2% paraformaldehyde for 10 min at 37°C or permeabilized

by adding ice-cold 100% methanol for 30 min on ice. Cells were stained with primary mAb against p22phox-alexa fluor 488 (CS9, AbD Serotech) or gp91phox alexa fluor 488 (NL7, AbD Serotech) for 30 min at 4°C (1:200). After two washes with PBS, cells were fixed in 4% paraformaldehyde and immediately analysed. In each case, 10,000 cells were acquired. The data were analyzed using BD FACSCanto II (Becton Dickinson).

Immunoblotting and immunoprecipitation for membrane bound proteins

Cells were mechanically scraped in PBS, washed, and then resuspended in Tris buffer (20 mM Tris, 250 mM Sucrose, 2 mM EDTA, 10 mM EGTA, pH 7.5). Cell suspensions were then incubated for 15 min on ice during homogenization with syringe, and centrifuged at 4°C 100,000g for 1 h, to pellet membranes. Supernatants with cytosolic proteins were collected and stored at -20°C until used for immunoblotting or immunoprecipitation. Membrane pellets were repeatedly lysed with Tris-SDS buffer (25 mM Tris, 150 mM NaCl, 1% SDS, 1 mM EDTA, 1 mM EGTA, pH 7.5), and membrane extracts were then prepared by centrifugation at 20°C, 100,000*g* for 15 min and stored at -80°C until used for immunoblotting or immunoprecipitation. Combined cytosolic and membrane fractions were finally analyzed by immunoblotting or immunoprecipitation with the appropriate corresponding antibodies.

Immunoblot analysis and immunoprecipitation

For immunoblotting, polypeptides were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane (Bio-Rad). Immunodetection was achieved with V5 (Invitrogen), AU1 (Covance), LC3 (LC3.No.6, Cosmo Bio Co), 4G10 (Upstate), VPS34 (Abgent), and Rubicon (Bethyl Laboratories). Antibodies to Flag and UVRAG from Sigma, GST (Z-5), actin (C4), p22phox (FL-195, 44.1, and C-17), gp91phox (54.1 and H-60), p47phox (H-195), p62/SQSTM1 (D-3), IkBα (C-21), MyD88 (E-11), VPS15 (JK-13) and TLR2 (TL2.1) were purchased from Santa Cruz Biotechnology, Inc. Specific Abs to phospho-(Thr202/Tyr204)-

ERK1/2, phospho-(Thr180/Tyr182)-p38, phospho-(Thr183/Tyr185)-SAPK/ JNK, phospho-IkBα, and Beclin1 were purchased from Cell Signaling Technology. Proteins were visualized by a chemiluminescence reagent (Pierce) and detected by a Fuji Phosphor Imager.

For GST Pulldown, cells were harvested and then lysed in NP-40 buffer supplemented with a complete protease inhibitor cocktail (Roche). Post-centrifuged supernatants were pre-cleared with protein A/G beads at 4°C for 2 h. Pre-cleared lysates were mixed with 50% slurry of glutathione-conjugated Sepharose beads (Amersham Biosciences), and the binding reaction was incubated for 4 h at 4°C. Precipitates were washed extensively with lysis buffer. Proteins bound to glutathione beads were eluted with SDS loading buffer by boiling for 5 min.

For immunoprecipitation, cells were harvested and then lysed in NP-40 buffer supplemented with a complete protease inhibitor cocktail (Roche). After pre-clearing with protein A/G agarose beads for 1 h at 4°C, whole-cell lysates were used for immunoprecipitation with the indicated antibodies. Generally, 1-4 μg of commercial antibody was added to 1 ml of cell lysates and incubated at 4°C for 8 to 12 h. After the addition of protein A/G agarose beads for 1 h, immunoprecipitates were extensively washed with lysis buffer and eluted with SDS loading buffer by boiling for 5 min.

Protein Purification and Mass Spectrometry

To identify Rubicon-binding proteins, THP1 cells expressing Flag-Rubicon were stimulated with or without zymosan for 30 min, harvested and lysed with NP-40 buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP40) supplemented with a complete protease inhibitor cocktail (Roche). Post-centrifuged supernatants were pre-cleared with protein A/G beads at 4^0C for 2 h. Pre-cleared lysates were mixed with αFlag antibody-conjugated with agarose beads for 4 h at 4^0C. Precipitates were washed extensively with lysis buffer. Proteins bound to beads were eluted and separated on a Nupage 4-12% Bis-Tris gradient gel (Invitrogen). After silver staining

(Invitrogen), specific protein bands were excised and analyzed by ion-trap mass spectrometry at the Harvard Taplin Biological Mass Spectrometry facility, and amino acid sequences were determined by tandem mass spectrometry and database searches.

Autophagy analysis

For starvation, cells were washed three times with PBS and incubated in Hank's solution (Invitrogen) for 4 h at 37°C. Cells were treated with Hank's solution or complete medium containing 2 μM rapamycin (Sigma) for 4 h. For the LC3 mobility shift assay or p62 immunoblot, cells were treated for 30 min on ice, lysed with 1% Triton X-100, and then subjected to immunoblotting analysis with an antibody against LC3 or p62.

Enzyme-linked immunosorbent assay

Murine BMDMs, RAW264.7 cells, and THP-1 cells were treated as indicated and processed for analysis by sandwich ELISA. Cell culture supernatants and mice sera were analyzed for cytokine content using BD OptEIA ELISA set (BD Pharmingen) for the detection of TNF-α and IL-6. All assays were performed as recommended by the manufacturers.

Production of lentiviral shRubicon or Rubicon

For silencing of human Rubicon, Oligonucleotide sequences for shRNA interference with Rubicon expression are bp 513-536 of 5' -GAUCGAUGCGUCCAUGUUU- 3', followed by a 9-nucleotide non-complementary spacer (TTCAAGAGA) and the reverse complement of the initial 19-nucleotide sequence. These dsDNA oligonucleotides were cloned into the pGIPZ lentiviral vector (Open Biosystems). Lentiviruses were produced by transient transfection using packaging plasmids (psPAX2 and pMD2.VSV-G purchased from Addgene) after Lipofectamine 2000 mediated transient transfection into 293T cells. Control vector was constructed by

inserting sequences with limited homology to the Rubicon sequences. DNA fragments corresponding to the coding sequences of the Rubicon genes were amplified by PCR and subcloned into pCDH-CMV vector (System Biosciences).

Transduction of lentivirus-shRubicon or lentivirus-Rubicon

Viral-containing media were collected at 72 hr posttransfection and harvested for the viral particles by passing the supernatants through a 0.45 μm filter. The supernatants were used to infect 2×10^5 cells in six-well plates in presence of 8μg/ml Polybrene (Sigma). For lentivirus infection, cells (5×10^5 cells/ml) in DMEM + 10% FBS were seeded in 24-well plates. After 24h, cells were infected with recombinant lentiviruses at various MOIs in the presence of 8μg/ml Polybrene. On the following day, medium was freshly replaced, and cells were incubated for additional 4 days for Rubicon expression. A parallel experiment using a GFP encoding lentivirus indicated that a minimum of 80% of Raw264.7 and BMDMs cells were transduced by lentiviruses. Titration of lentiviral vectors was determined using 293T cells. Briefly, approximately 2×10^5 cells were plated in each well of a six-well plate. On the following day, cells were infected with viral supernatants in the presence of 8μg/ml Polybrene. After 24h, medium was removed and replaced with fresh medium containing 5μg/ml puromycin. On day 14, cells were stained with crystal violet for 15 min and colonies were counted using a cutoff of 50 viable cells.

Construction of adenoviral shRubicon or Rubicon

An adenoviruses expressing short hairpin RNA (shRNA) to the Rubicon gene was constructed using the AdEasy system (Stategene). The shRNA oligonucleotides sequences were as follows: 5'-gatcc cc gat aga cag tat atc aga a ttc aag aga t tct gat ata ctg tct atc ttttt a -3', 5'- g gg cta tct gtc ata tag tct t aag ttc tct a aga cta tat gac aga tag aaaaa ttcga-3'. These dsDNA oligonucleotides were cloned into the pSuper vector (OligoEngine) between the *BglII* and *HindIII*

restriction sites containing the human H1 promoter. The double-strand shRNA oligonucleotides containing the termination signal were inserted at the 3' end of the human H1 promoter and subcloned into the pShuttle vector (Stategene) *NotI* and *HindIII* restriction sites. Control vector was constructed by inserting a sequence that expresses an shRNA with limited homology to the Rubicon sequences. DNA fragments corresponding to the coding sequences of the Rubicon genes were amplified by PCR and subcloned into the pShuttle-CMV vector (Stategene) between the *NotI* and *EcoRV* restriction sites

Adenovirus production

Recombinant adenoviruses were constructed using AdEasy system (Stategene): digested adenovirus vectors with the *Pac I* were transfected into the AD-293 producer cells in a 6-well-plate and cultured with fresh media until cytopathic effect (CPE) was observed. When 80% CPE were observed, recombinant adenoviruses were harvested by repeatedly freezing at –80 °C and thawing at 37 °C four times. Cell lysates were then centrifuged at 2,000g for 30 min at 25 °C and the supernatants containing recombinant adenovirus particles were stored at –80 °C.

All adenoviruses were propagated in AD-293 cells, purified and concentrated by BD Adeno-X^{TM} purification kit (BD Biosciences Clontech). The typical titers were in the range of 10^{12}–10^{13} plaque-forming units (pfu)/mL as determined via plaque assay using 1.25 % SeaPlaque GTG agarose (BioWhittaker Molecular Applications) overlay. A sterile carrier solution [phosphate-buffered saline (PBS)] was used for control injections and dilution of the viruses.

Statistical analysis

All data were analyzed using Student's *t* test with a Bonferroni adjustment or ANOVA for multiple comparisons and are presented as the mean ± SD. Differences were considered significant at $p < 0.05$. Where indicated, Prism software (Graph Pad) was used for two-way

analysis of variance and Kaplan-Meier survival analyses.