LEGENDS TO SUPPLEMENTAL FIGURES

FIGURE S1. **Defect of large lysosome formation revealed by LAMP-1 staining in *Enpp2*$^{-/-}$ VE cells.** Confocal microscopic images of VE cells stained with anti-LAMP-1 antibody (red) and Alexa488-phalloidin (green) are shown. LAMP-1-positive lysosomes were smaller in *Enpp2*$^{-/-}$ VE cells than in wild-type VE cells. Scale bar represents 6 μm.

FIGURE S2. **Ex vivo whole embryo culture faithfully mimics the in vivo development.** A, gross appearance of the yolk sac (top), embryo proper (middle), and LysoTracker Red staining of yolk sac VE cells (bottom). E8.5 wild-type embryos grown in utero, wild-type embryos cultured from E7.5 for 1 day (E7.5 + 1 DIV), E8.5 *Enpp2*$^{-/-}$ embryos grown in utero, and *Enpp2*$^{-/-}$ embryos cultured from E7.5 for 1 day are shown. Arrows indicate the head cavity in *Enpp2*$^{-/-}$ embryos. Ex vivo cultured embryos (wild-type and *Enpp2*$^{-/-}$) appear to be essentially the same as in-utero-grown embryos. Scale bar represents 350 μm (top, middle) and 6 μm (bottom). B, quantitation of the size and number of lysosomes in VE cells. In the *Enpp2*$^{-/-}$ embryos cultured for 1 day, the size of lysosomes was significantly decreased and the number of lysosomes was significantly increased in VE cells compared with those in the controls. The values are the means ± S.E.M. (*$P < 0.05$, **$P < 0.01$; unpaired t-test). The number of embryos examined is shown in each column.

FIGURE S3. **Effect of the inhibitors of the Rho-ROCK-LIMK pathway on lysosomes.** A-B, confocal microscopic images of the yolk sac stained with LysoTracker Red. After E7.5 whole embryos were cultured with the indicated inhibitors for 1 day, LysoTracker Red staining was performed. Treatment with C3 exoenzyme (C3), H1152, or S3 peptide resulted in the size reduction of lysosomes. Scale bars represent 10 μm.

FIGURE S4. **Effects of ROCK inhibitors on lysosomes.** A, confocal microscopic images of the yolk sac stained with LysoTracker Red. After E7.5 whole embryos were cultured in the presence of the indicated ROCK inhibitors for 1 day, LysoTracker Red staining was performed. Scale bar represents 10 μm. B, quantitation of the lysosome size. Treatment with ROCK inhibitors, hydroxyfasudil or Y-27632, resulted in the size reduction of lysosomes. The values are means ± S.E.M. (*$P < 0.05$, **$P < 0.01$; ANOVA with a Tukey-Kramer post-hoc test). The number of embryos examined is shown in each column.

FIGURE S5. **A LIMK inhibitor peptide suppresses cofilin phosphorylation.** Confocal microscopic images of VE cells. After E7.5 whole embryos were cultured in the presence of S3 peptide (15 μg/ml) or RV peptide (15 μg/ml) for 1 day, VE cells were stained with anti-phospho-cofilin or anti-cofilin antibody. Treatment with S3 peptide reduced cofilin phosphorylation without affecting cofilin protein levels. Scale bar represents 5 μm.

FIGURE S6. **Electroporation-mediated activation of the Rho-ROCK-LIMK pathway induces lysosome defects in VE cells.** A-B, confocal microscopic images of the VE cells stained with Alexa546-phalloidin (A) or LysoTracker Red (B). After whole embryos were electroporated with the expression constructs for the indicated molecules and cultured for 1 day, they were stained with Alexa546-phalloidin or LysoTracker Red. To identify electroporated cells, EGFP was co-expressed by subcloning into a pEGFP-IRES2 vector or making EGFP (or YFP) fusion proteins. Asterisks indicate electroporated cells. CA-Rho or CA-LIMK induced the increase of phalloidin staining, whereas cofilin (S3E)

had no apparent effect (A). In the VE cells expressing CA-Rho, CA-LIMK, or cofilin (S3E), lysosomes became smaller. Scale bars indicate 10 μm. C, summary of the effects of CA-Rho, CA-LIMK, and cofilin (S3E) on lysosome size. The VE cells possessing at least 1 lysosome with an area more than 40 μm^2 were categorized as "normal", whereas those possessing only small lysosomes (area smaller than 40 μm^2) were categorized as "small." The cells showing no visible LysoTracker Red staining were categorized as having "no signal." The numbers of the VE cells examined are shown on the right.

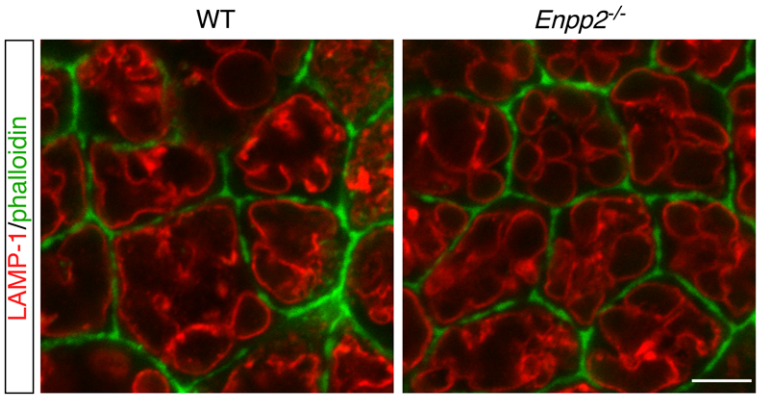

Figure S1

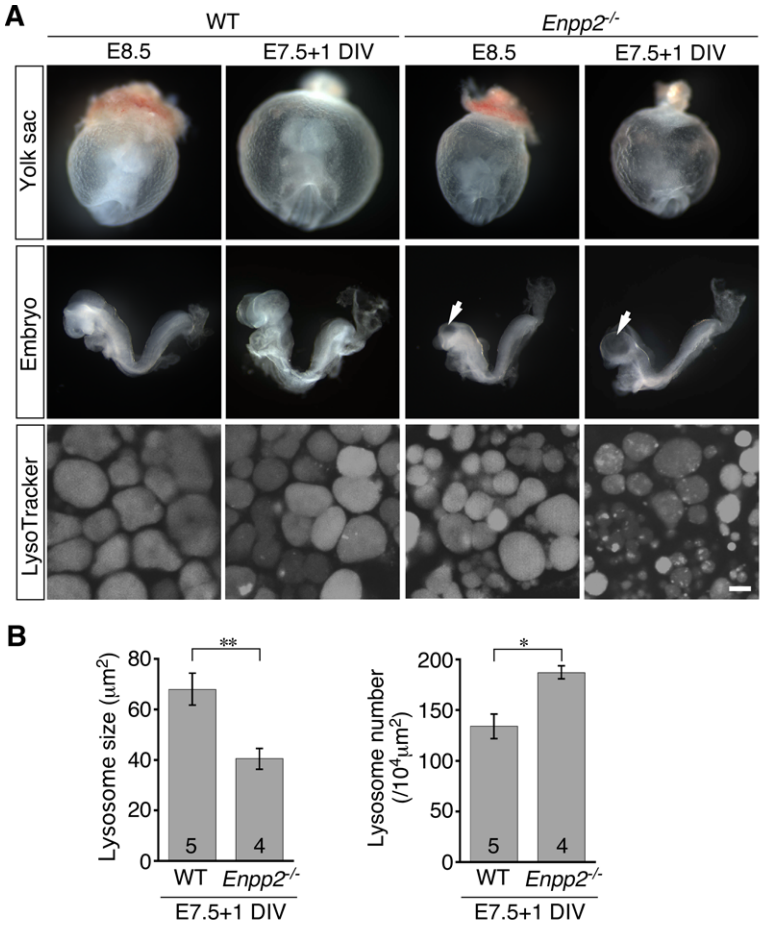
A
WT
Enpp2-/-
E8.5
E7.5+1 DIV
E8.5
E7.5+1 DIV
Yolk sac
Embryo
LysoTracker
B
Lysosome size (μm2)
80
60
40
20
0
**
5
4
WT
Enpp2-/-
E7.5+1 DIV
Lysosome number (/10⁴μm²)
200
150
100
50
0
*
5
4
WT
Enpp2-/-
E7.5+1 DIV

Figure S2

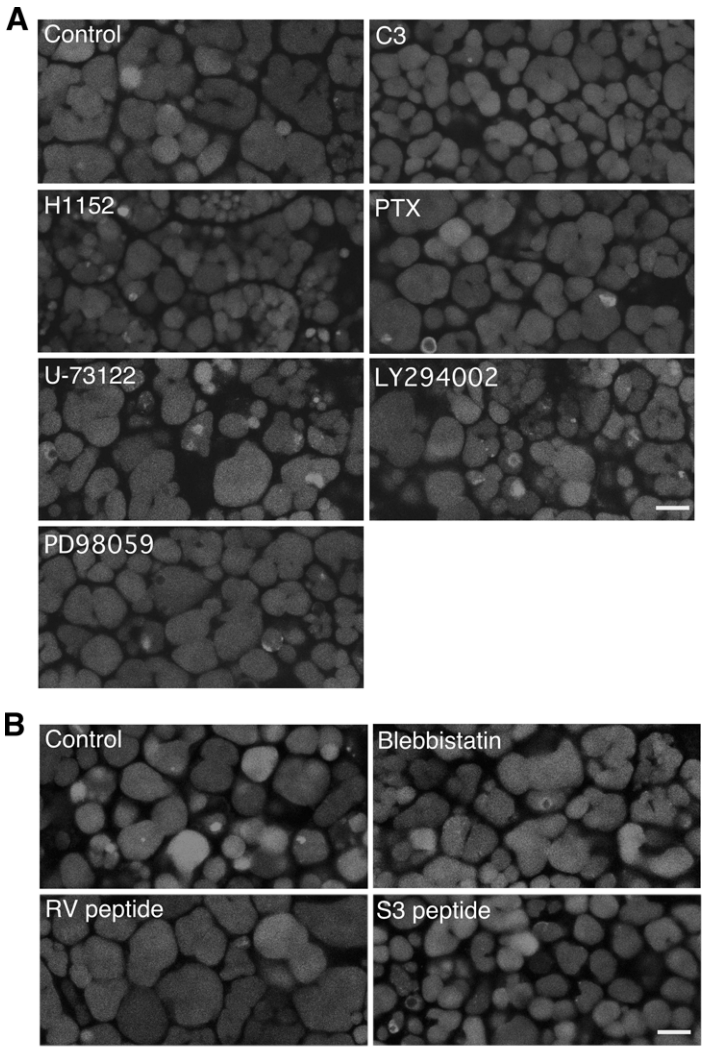
A
Control
C3
H1152
PTX
U-73122
LY294002
PD98059
B
Control
Blebbistatin
RV peptide
S3 peptide

Figure S3

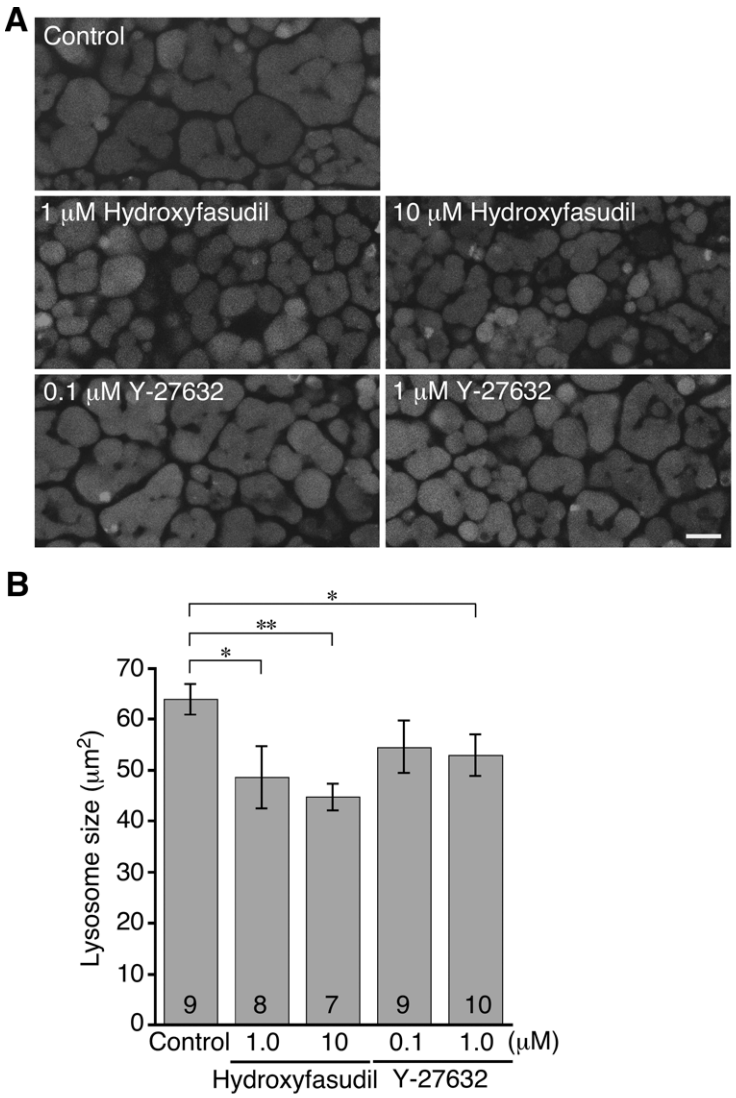
A
Control
1 μM Hydroxyfasudil
10 μM Hydroxyfasudil
0.1 μM Y-27632
1 μM Y-27632
B
*
**
*
Lysosome size (μm2)
70
60
50
40
30
20
10
0
9
8
7
9
10
Control
1.0
10
0.1
1.0
(μM)
Hydroxyfasudil
Y-27632

Figure S4

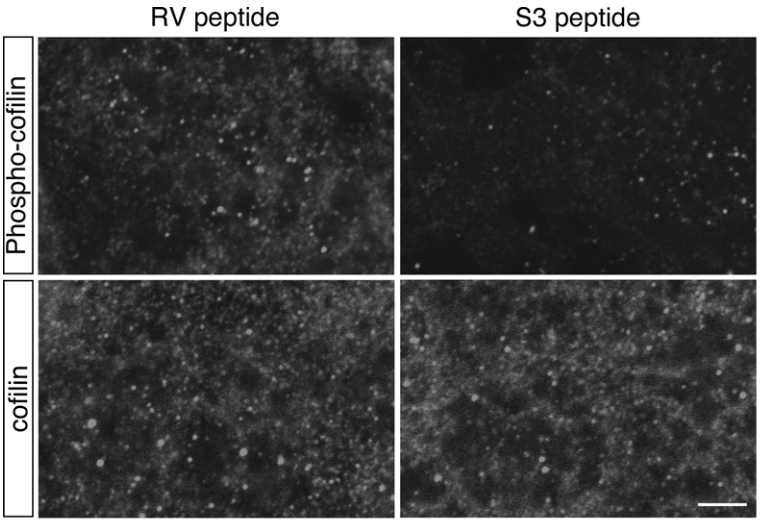

Figure S5

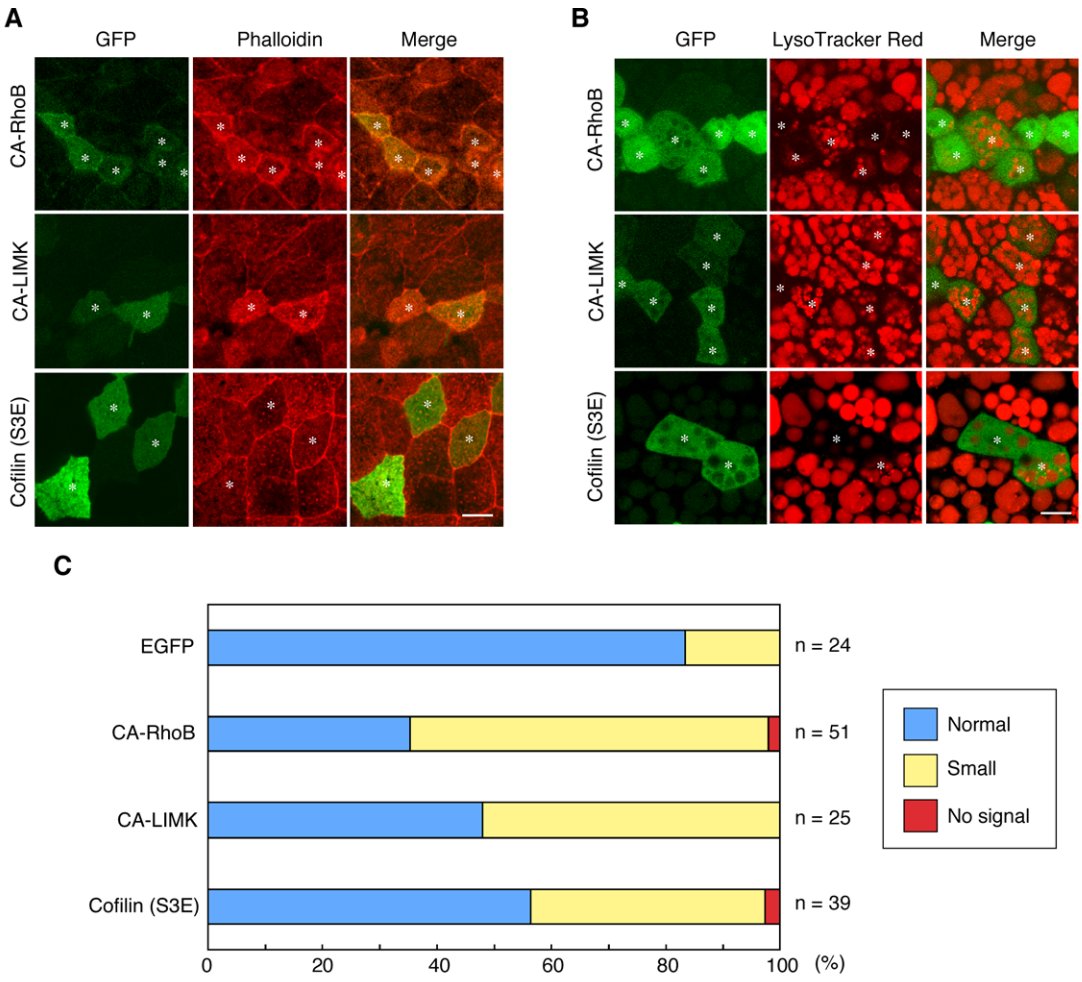
A
GFP
Phalloidin
Merge
CA-RhoB
CA-LIMK
Cofilin (S3E)
B
GFP
LysoTracker Red
Merge
CA-RhoB
CA-LIMK
Cofilin (S3E)
C
EGFP
CA-RhoB
CA-LIMK
Cofilin (S3E)
n = 24
n = 51
n = 25
n = 39
0
20
40
60
80
100
(%)
Normal
Small
No signal

Figure S6