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# Supplemental Material to:

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## FOXO3 induces FOXO1-dependent autophagy by activating the AKT1 signaling pathway

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**Figure S1**

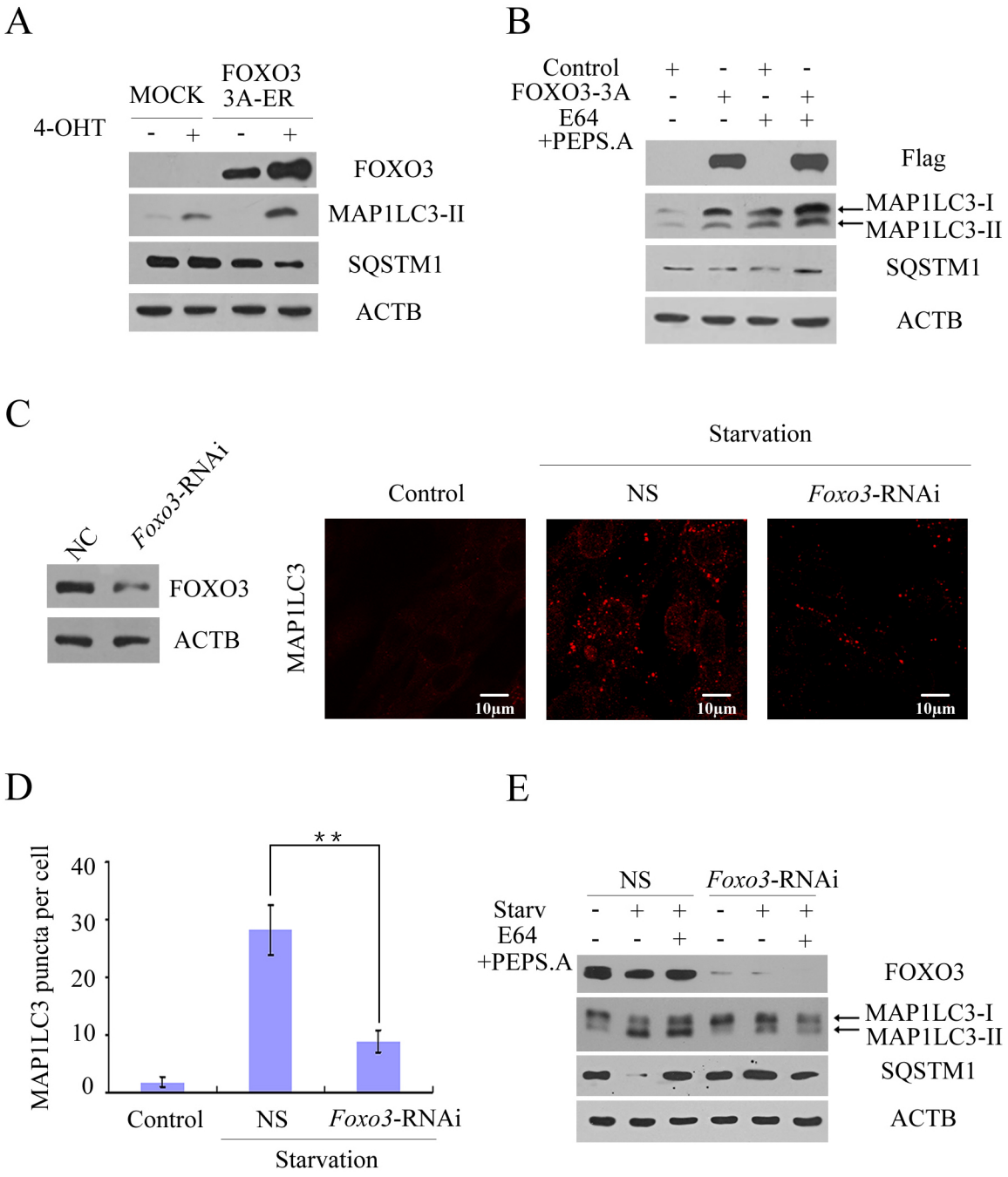
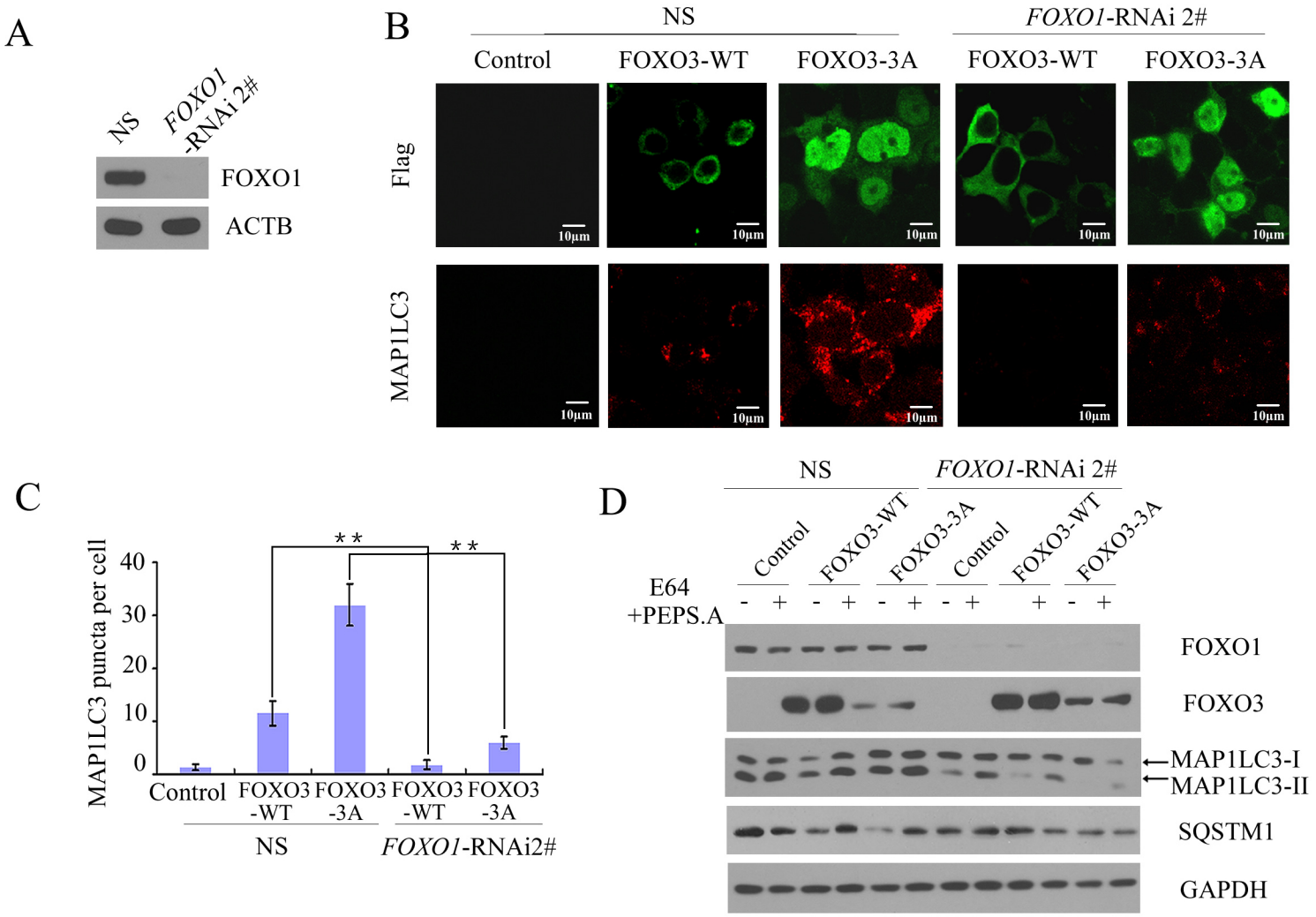

A
MOCK
FOXO3 3A-ER
4-OHT
- + - +
FOXO3
MAP1LC3-II
SQSTM1
ACTB
B
Control + - + -
FOXO3-3A - + - +
E64 - - + +
+PEPS.A
Flag
MAP1LC3-I
MAP1LC3-II
SQSTM1
ACTB
C
NC
Foxo3-RNAi
FOXO3
ACTB
Starvation
Control
NS
Foxo3-RNAi
MAP1LC3
10μm
10μm
10μm
D
MAP1LC3 puncta per cell
40
30
20
10
0
**
Control
NS
Foxo3-RNAi
Starvation
E
NS
Foxo3-RNAi
Starv - + + - + +
E64 - - + - - +
+PEPS.A
FOXO3
MAP1LC3-I
MAP1LC3-II
SQSTM1
ACTB

**Figure S2**


A
NS
FOXO1 -RNAi 2#
FOXO1
ACTB
B
NS
FOXO1-RNAi 2#
Control
FOXO3-WT
FOXO3-3A
FOXO3-WT
FOXO3-3A
Flag
MAP1LC3
10μm
C
MAP1LC3 puncta per cell
40
30
20
10
0
**
**
Control
FOXO3 -WT
FOXO3 -3A
FOXO3 -WT
FOXO3 -3A
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FOXO1-RNAi2#
D
NS
FOXO1-RNAi 2#
Control
FOXO3-WT
FOXO3-3A
Control
FOXO3-WT
FOXO3-3A
E64
+PEPS.A
- + - + - + - + + - +
FOXO1
FOXO3
MAP1LC3-I
MAP1LC3-II
SQSTM1
GAPDH

**Figure S1.** FOXO3 triggered autophagy in MEF cells and C2C12 cells. (A) Wild-type MEF cells or TM-ER MEF cells were treated with or without 4-OHT (20 mg/ml). 6 h post-transfection, cell lysates were extracted and were then analyzed by western blot with anti-FOXO3, anti-MAP1LC3, anti-SQSTM1 or anti-ACTB antibody. (B) C2C12 cells were transfected with empty control plasmid or Flag-FOXO3 (3A) plasmid in the presence or absence of E64 and pepstatin A. 48 h post-transfection, cell lysates were extracted and analyzed by western blot with anti-Flag, anti-MAP1LC3, anti-SQSTM1 or anti-ACTB antibody. (C) C2C12 cells were transfected with nonspecific siRNA or *Foxo3* siRNA. 48 h post-transfection, cells were HBSS-starved or further cultured in a complete medium for 2 h. A western blot was performed to detect the expression of FOXO3 (left panels). Endogenous MAP1LC3 puncta (right panels) were observed by confocal microscopy. Scale bars, 10 μm. (D) Quantification of endogenous MAP1LC3 puncta per cell. Error bars represent the standard deviation (n=50 for three independent experiments). **$p<0.01. (E) Nonspecific siRNA or *Foxo3* siRNA was transfected into C2C12 cells. 48 h post-transfection, cells were HBSS-starved or further cultured in complete medium for 2 h with or without E64 and pepstatin A. Cell lysates were extracted and immunoblotted with anti-FOXO3, anti-MAP1LC3, anti-SQSTM1 or anti-GAPDH antibody.

**Figure S2.** FOXO1 is required for FOXO3-induced autophagy (using a different *FOXO1* siRNA sequence). (A) Stable *FOXO1*-RNAi HEK293T cell lines transfected with different *FOXO1* RNAi oligonucleotides were established. Western blot analysis of FOXO1 expression in stable nonspecific-RNAi-expressing HEK293T cells (NS) and stable *FOXO1*-siRNA HEK293T cells (*FOXO1*-RNAi). (B) Empty control, Flag-FOXO3 (WT) or Flag-FOXO3 (3A) plasmid was transfected into NS cells or *FOXO1*-RNAi cells. Expression of Flag-FOXO3 (upper panels) or endogenous MAP1LC3 puncta (lower panels) was analyzed by confocal microscopy. Scale bars, 10 μm. (C) Quantification of endogenous MAP1LC3 puncta per cell. Error bars represent the standard deviation (n=50 for three independent experiments). **$p<0.01. (D) HEK293T (NS) cells or *FOXO1*-RNAi cells were transfected with control plasmid, Flag-FOXO3 (WT) or Flag-FOXO3 (3A) plasmid and were then treated with or without E64 and pepstatin A. 48 h post-transfection, cell lysates were extracted and were immunoblotted with anti-FOXO1, anti-FOXO3, anti-MAP1LC3, anti-SQSTM1 or anti-GAPDH antibody.