Supplementary Materials

Supplementary Methods and Materials

Brain Frozen Sections

Mice were perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). Brains were dissected and fixed overnight in 4% PFA, then dehydrated with 30% sucrose. The dehydrated brains were embedded in optimal cutting temperature compound (OCT) and sectioned for histological staining.

Antibodies

To explore cellular colocalization of FUNDC1, sections were incubated with antibodies against FUNDC1 (1:100; CST, 49240), the neuronal marker NeuN (1:500; Abcam, ab279297), the astrocytic marker GFAP (1:500; Invitrogen, 14-9892-82), the microglial marker Iba1 (1:500; Abcam, ab5076), and the endothelial marker CD31 (1:100; R&D, AF3628). To assess changes in phosphorylated FUNDC1 and Src in neurons, sections were incubated with antibodies against NeuN and p-FUNDC1 (1:100; courtesy of Prof. Quan Chen) or p-Src (1:100; ABclonal, AP0511). Brain sections were also subjected to analysis of neuronal mitophagy using antibodies against NeuN, LC3 (1:200; CST, 43566), and Tomm20 (1:100; Abcam, ab283317).

For western blotting, the used antibodies are shown below:

FUNDC1 (1:1000; CST, 49240), p-FUNDC1 (1:1000; courtesy of Prof. Quan Chen), Tomm20 (1:500; Abcam, ab283317), COX4I1 (1:2000; CST, 4850), LC3 (1:1000; CST, 43566), SQSTM1 (1:1000; Abcam, ab109012), Src (1:1000; ABclonal, A0324), p-Src (1:1000; ABclonal, AP0511), poly (ADP-ribose) polymerase (PARP; 1:1000; CST, 9532), cleaved PARP (1:1000; CST, 9548), caspase 3 (1:1000; CST, 14220), cleaved caspase 3 (1:1000; CST, 9664) caspase 9 (1:1000; CST, 9504) and cleaved caspase 9 (1:1000; CST, 9509).

Mitochondrial Quality

Mitochondrial quality was evaluated by fluorescence staining using a JC1 staining kit (Solarbio, M8650) and a reactive oxygen species (ROS) fluorescent probe (Beyotime, S0033); the samples were observed with a fluorescence microscope (DM550, Leica, Germany) and cytoFLEX

flow cytometer (Beckman Coulter, USA), regarding.

Culture of HeLa Cells and OGD Procedure

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum. For OGD treatment, Hela cells were treated with glucose-free DMEM and immediately placed into a sealed chamber filled with a gas mixture containing only 1% O_2 for 3, 6, 9h.

Transfection of siRNA

Neurons were transfected with siRNA targeting Src at 10-day after isolation. The siRNAs were transfected using Lipofectamine™ 3000 (Invitrogen, L3000001), and the transfected cells were utilized for subsequent analysis after 72-96 hours of transfection. Four siRNA sequences targeting Src were designed and their transfection efficiencies were assessed using western blot analysis. Subsequently, the optimal sequence was selected for further experimentation (Figure S5A). The sequences are shown below (5'→3'):

si-Src-1:

Sense: GCCUAAAUGUGAAACACUATT

Antisense: UAGUGUUUCACAUUUAGGCTT

si-Src-2:

Sense: GGGCAAAUAUUUGCGGCUATT

Antisense: UAGCCGCAAAUAUUUGCCCTT

si-Src-3:

Sense: GAUCACUAGACGGGAAUCATT

Antisense: UGAUUCCCGUCUAGUGAUCTT

si-Src-4:

Sense: CAAGUCAUGAAGAAACUGATT

Antisense: UCAGUUUCUUCAUGACUUGTT

Supplementary Table. Study Design, Grouping of Mice, and Number of Mice Utilized.

Group	Survived	Dead	Excluded	Total	Usage
Experiment 1					1. TTC staining (n = 10/group), among whom 5 mice/group were used for CBF detection. 2. FJC staining (n = 6/group). 3. WB analysis for apoptosis (n = 6/group). 4. Neurological deficiency was assessed on all the survived mice (n = 22/group).
WT, (tMCAO)	22	3	2	27	
KO, (tMCAO)	22	5	2	29	
Experiment 2					All of the mice were used for assessment of long-term outcomes.
WT, (tMCAO)	6	4	1	11	
KO, (tMCAO)	5	5	1	11	
Experiment 3					1. WB analysis for temporal changes of mitophagy and phorsphorylation of FUNDC1 and Src (n = 3/time point). 2. Immunostaining for phosphorylated FUNDC1 and Src in sham, tMCAO 0 h and tMCAO 24 h groups (n = 6/group).
WT, (sham)	9	0	0	9	
WT, (tMCAO)	27	7	3	37	
Experiment 4					1. WB analysis for mitophagy (n = 6/group). 2. Confocal microscopy for mitophagy (n = 6/group). Brain sections from WT mice were shared with Experiment 3.
WT, (sham)	6	0	0	6	
KO, (sham)	12	0	0	12	
WT, (tMCAO)	6	0	1	7	
KO, (tMCAO)	12	2	0	14	
Experiment 5					1. WB analysis for mitophagy (n = 6/group). 2. TTC staining (n = 5 for WT or KO mice treated with vehicle, n = 6 for WT or KO mice treated with PP1). 3. Neurological deficiency was assessed on all the survived mice subjected to tMCAO (n = 11 for WT or KO mice treated with vehicle, n = 12 for WT or KO mice treated with PP1).
WT, (sham)	6	0	0	6	
KO, (sham)	6	0	0	6	
WT, (tMCAO+Vehicle)	11	2	0	13	
KO, (tMCAO+Vehicle)	11	3	0	14	
WT, (tMCAO+PP1)	12	1	1	14	
KO, (tMCAO+PP1)	12	0	0	12	
Experiment 6					All of the mice were used for assessment of long-term outcomes.
WT, (tMCAO+Vehicle)	7	8	3	18	
KO, (tMCAO+Vehicle)	10	5	0	15	
WT, (tMCAO+PP1)	8	7	0	15	

KO, (tMCAO+PP1)	6	9	2	17	
Total	216	61	11	293	

Supplementary Figures

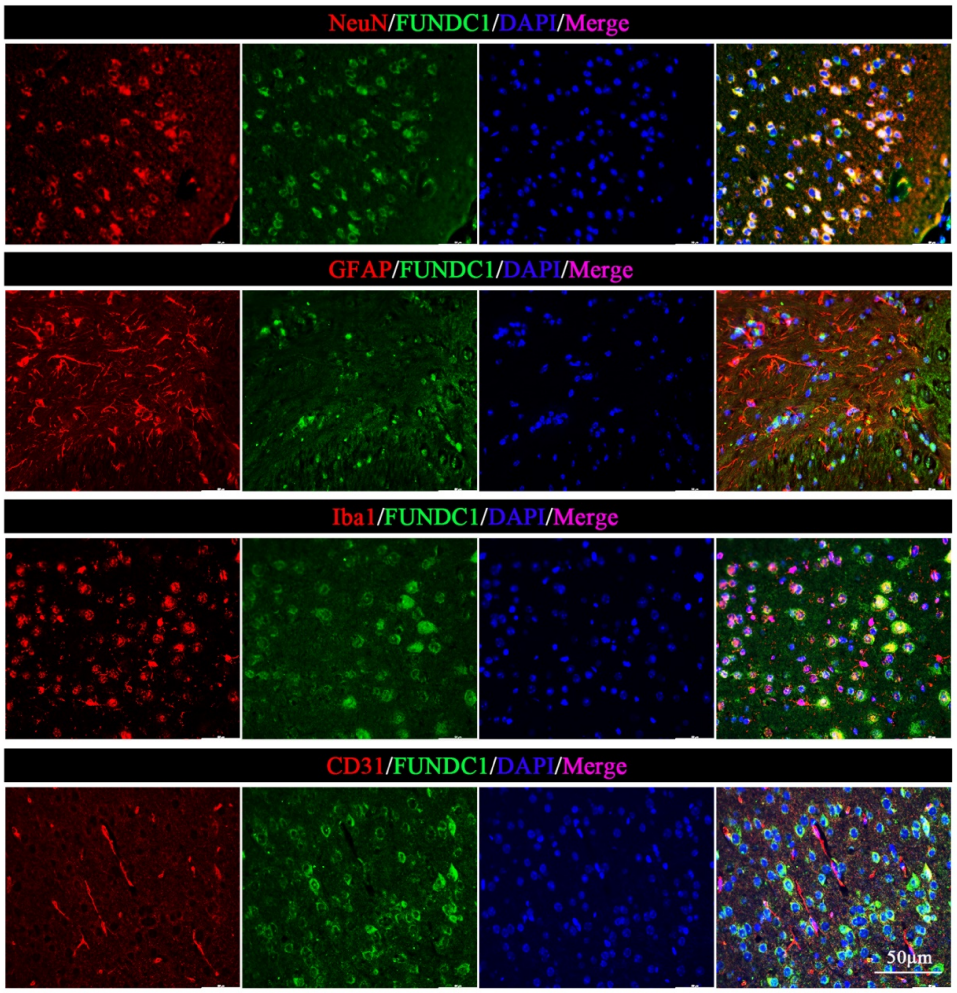

Figure S1. FUNDC1 is mainly located in neurons. FUNDC1 (green) was co-stained with cellular markers (red) including NeuN (neuron), GFAP (astrocyte), Iba1 (microglia) and CD31 (endothelium).

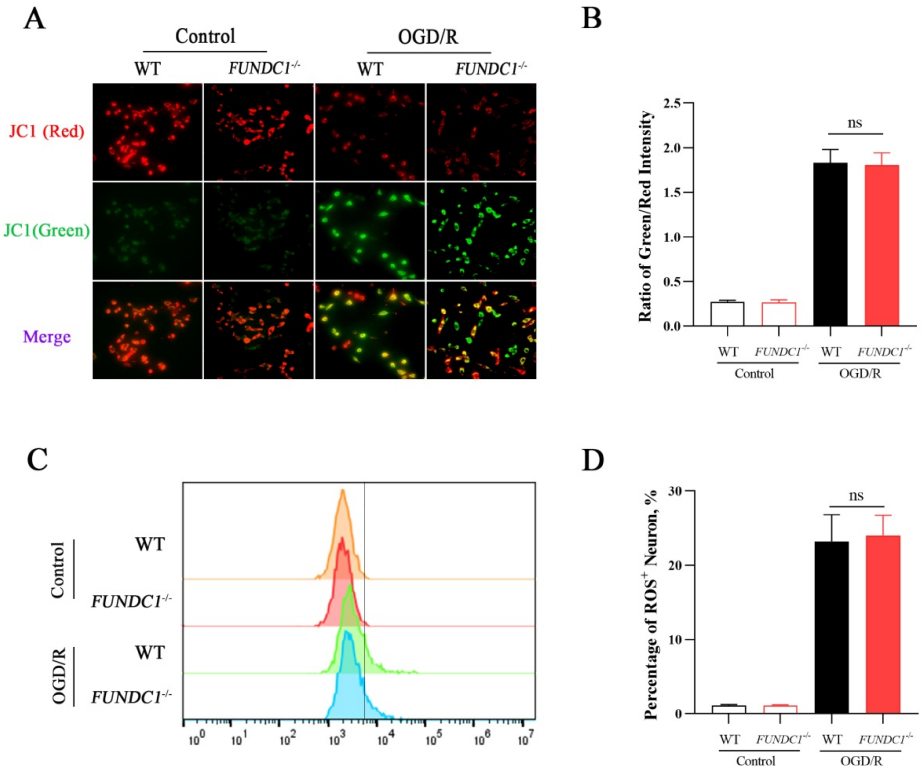

Figure S2. FUNDC1 knockout has no effects on mitochondrial quality in neurons suffered from I/R injury. A. Cortical neurons isolated from WT and *FUNDC1*$^{-/-}$ mice were subjected to control or OGD/R for 9 h. Mitochondrial membrane potential (MMP) was detected by JC1 staining. B. Quantification for ratio of green and red fluorescent intensity. n = 3 for independent experiments. C. Reactive oxygen species (ROS) generated from WT and *FUNDC1*$^{-/-}$ neuron subjected to control or OGD/R were analyzed by flow cytometry. D. Quantification for proportion of positive cells. n = 3 for independent experiments. n = 2 for control-treated cells and n = 3 for OGD/R-treated cells.

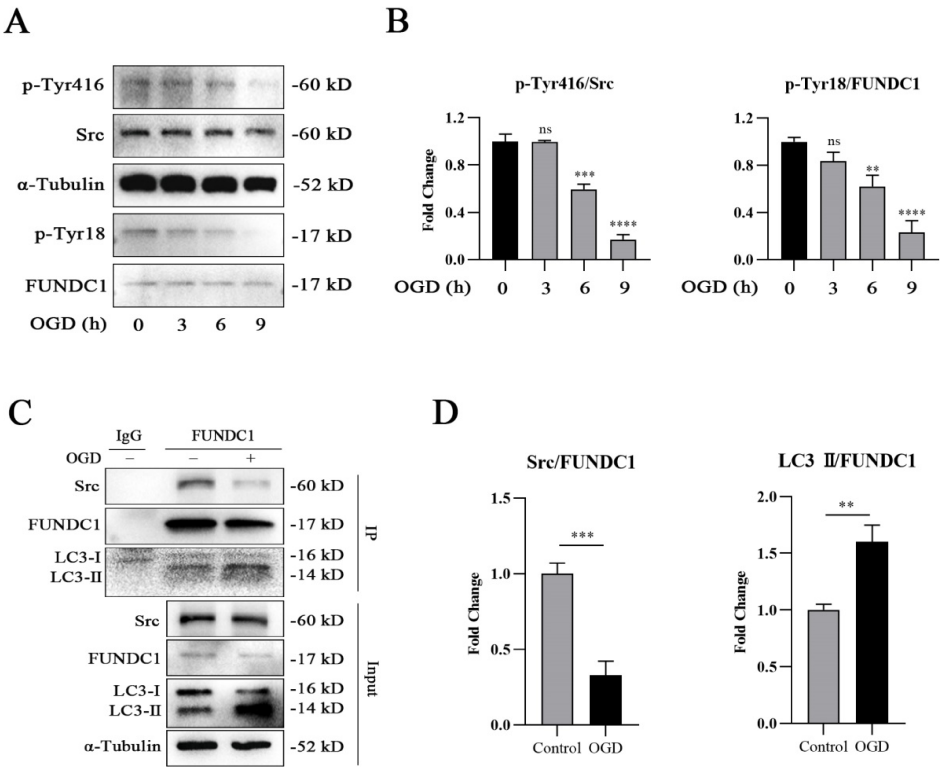

Figure S3. FUNDC1 is activated in response to hypoxia in HeLa cells. A. HeLa cells were subjected to OGD for 0 h, 3 h, 6 h, 9 h. Phosphorylation of FUNDC1 (Tyr18) and Src (Tyr416) at each time point after reperfusion were detected by western blotting. B. Semi-quantification for p-Tyr416 of Src and p-Tyr18 of FUNDC1. n = 3 for independent experiments. C. HeLa cells were subjected to control or OGD 9 h. Interactions of FUNDC1 with Src and LC3 were analyzed by co-immunoprecipitation. D. Semi-quantification for Src and LC3 II interacted with FUNDC1. n = 3 for independent experiments. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

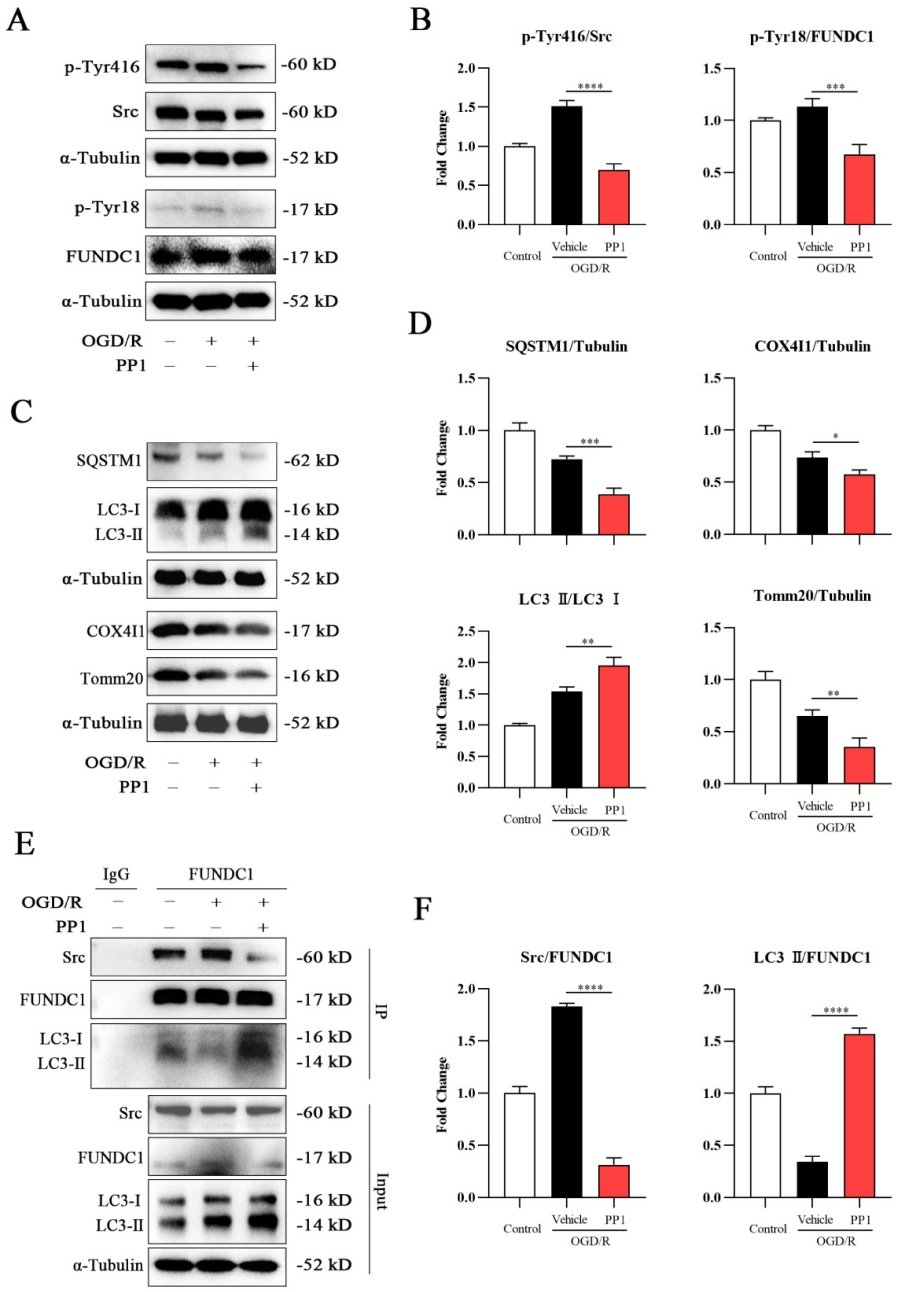

Figure S4. Inhibitions of Src activates FUNDC1 in OGD/R-treated neurons. A. Neurons subjected to OGD/R were treated with vehicle or PP1. The phosphorylation of FUNDC1 and Src was detected by western blotting. B. Semi-quantification for p-Tyr416 of Src and p-Tyr18 of FUNDC1. n = 3 for independent experiment. C. Mitophagy in PP1-treated neurons was detected by western blotting. D. Semi-quantification for mitophagy shown in panel C. n = 3 for independent experiment. E. Interactions of FUNDC1 with Src and LC3 in PP1-treated neurons were analyzed by co-immunoprecipitation. F. Semi-quantification for Src and LC3 II interacted with

FUNDC1. n = 3 for independent experiment. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

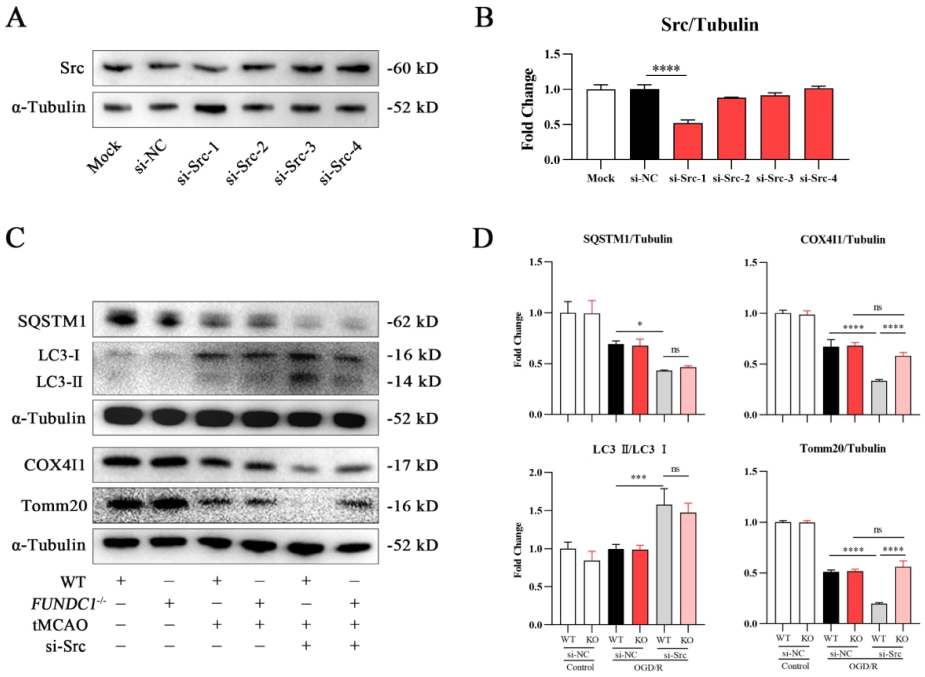

Figure S5. Knockdown of Src rescues FUNDC1-mediated mitophagy in OGD/R-treated neurons. A. Transfection efficiencies of the four siRNA sequences were assessed by western blotting. B. Semi-quantification for Src level, and the first sequence was selected for further experimentation. n = 3 for independent experiment. C. Neuronal mitophagy was detected by western blotting. D. Semi-quantification for mitophagy shown in panel C. n = 3 for independent experiment. * p < 0.05, *** p < 0.001, **** p < 0.0001.

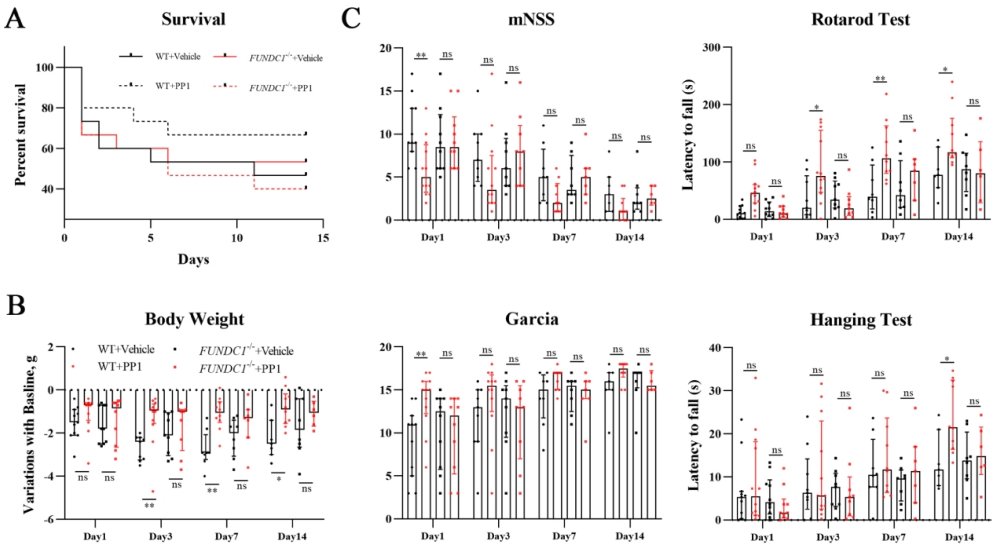

Figure S6. PP1 treatment improves long-term outcomes of WT mice rather than *FUNDC1*$^{-/-}$ mice. A. 14-day survival rate after tMCAO. n = 15 mice for group. B-C. Body weight recovery (B) and behavioral tests (C) including Garcia score, mNSS score, Rotarod test, Hanging test were assessed at 1 day (WT + Vehicle, n = 11; WT + PP1, n = 12; *FUNDC1*$^{-/-}$ + Vehicle, n = 10; *FUNDC1*$^{-/-}$ + PP1, n = 10), 3 day (WT + Vehicle, n = 9; WT + PP1, n = 12; *FUNDC1*$^{-/-}$ + Vehicle, n = 9; *FUNDC1*$^{-/-}$ + PP1, n = 9), 7 day (WT + Vehicle, n = 8; WT + PP1, n = 10; *FUNDC1*$^{-/-}$ + Vehicle, n = 8; *FUNDC1*$^{-/-}$ + PP1, n = 7), 14 day (WT + Vehicle, n = 7; WT + PP1, n = 10; *FUNDC1*$^{-/-}$ + Vehicle, n = 8; *FUNDC1*$^{-/-}$ + PP1, n = 6) after tMCAO. * $p < 0.05$, ** $p < 0.01$.