

## Src inhibition rescues FUNDC1-mediated neuronal mitophagy in ischaemic stroke

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**ABSTRACT**

**Background** Ischaemic stroke triggers neuronal mitophagy, while the involvement of mitophagy receptors in ischaemia/reperfusion (I/R) injury-induced neuronal mitophagy remain not fully elucidated. Here, we aimed to investigate the involvement of mitophagy receptor FUN14 domain-containing 1 (FUNDC1) and its modulation in neuronal mitophagy induced by I/R injury.

**Methods** Wild-type and FUNDC1 knockout mice were generated to establish models of neuronal I/R injury, including transient middle cerebral artery occlusion (tMCAO) in vivo and oxygen glucose deprivation/reperfusion (tMCAO) in vitro. Stroke outcomes of mice with two genotypes were assessed. Neuronal mitophagy was analysed both in vivo and in vitro. Activities of FUNDC1 and its regulator Src were evaluated. The impact of Src on FUNDC1-mediated mitophagy was assessed through administration of Src antagonist PP1.

**Results** To our surprise, FUNDC1 knockout mice subjected to tMCAO showed stroke outcomes comparable to those of their wild-type littermates. Although neuronal mitophagy could be activated by I/R injury, FUNDC1 deletion did not disrupt neuronal mitophagy. Transient activation of FUNDC1, represented by dephosphorylation of Tyr18, was detected in the early stages (within 3 hours) of neuronal I/R injury; however, phosphorylated Tyr18 reappeared and even surpassed baseline levels in later stages (after 6 hours), accompanied by a decrease in FUNDC1-light chain 3 interactions. Spontaneous inactivation of FUNDC1 was associated with Src activation, represented by phosphorylation of Tyr416, which changed in parallel with the level of phosphorylated FUNDC1 (Tyr18) during neuronal I/R injury. Finally, FUNDC1-mediated mitophagy in neurons under I/R conditions can be rescued by pharmacological inhibition of Src.

**Conclusions** FUNDC1 is inactivated by Src during the later stage (after 6 hours) of neuronal I/R injury, and rescue of FUNDC1-mediated mitophagy may serve as a potential therapeutic strategy for treating ischaemic stroke.

**INTRODUCTION**

Ischaemic stroke injury disrupts neuronal mitochondrial homeostasis, leading to oxidative stress and subsequent neuronal death.<sup>1 2</sup> Mitophagy, an evolutionarily conserved process that selectively removes unwanted or superfluous mitochondria

**WHAT IS ALREADY KNOWN ON THIS TOPIC**

⇒ Ischaemic stroke triggers neuronal mitophagy, and reinforcement of mitophagy is a promising therapeutic strategy for neuroprotection. FUN14 domain-containing 1 (FUNDC1) is a mitophagy receptor that has been validated as a therapeutic target for ischaemic diseases in other systems. However, the roles of FUNDC1 in neuronal mitophagy induced by ischaemic stroke remain poorly elucidated.

**WHAT THIS STUDY ADDS**

⇒ To our surprise, loss of FUNDC1 has no significant effect on stroke outcomes and neuronal mitophagy during ischaemia stroke. FUNDC1 is phosphorylated and inactivated in the later stage of neuronal ischaemia/reperfusion injury, thereby losing its ability to regulate mitophagy. The hyperactivation of Src in neurons during ischaemic stroke impedes the interaction between FUNDC1 and light chain 3, resulting in the dysfunction of FUNDC1.

**HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY**

⇒ This study has revealed a novel pathophysiological process during ischaemic stroke, in which mitophagy receptor FUNDC1 is inactivated in neurons. Although neuronal mitophagy can be triggered by ischaemic stroke, this process is restricted by the inactivation of FUNDC1. Restoration of FUNDC1-mediated mitophagy in neurons represents a promising neuroprotective strategy for ischaemic stroke that reinforces neuronal mitophagy.

via autophagy, plays a crucial role in maintaining mitochondrial quality.<sup>3 4</sup> Neuronal ischaemia/reperfusion (I/R) injury triggers mitophagy as a self-protective process to prevent massive cellular death by eliminating damaged mitochondria.<sup>5</sup> Studies have shown that the enhancement of mitophagy is considered a promising neuroprotective strategy for ischaemic stroke,<sup>5–10</sup> indicating the insufficiency and restraint of neuronal mitophagy during I/R injury.

Nevertheless, the regulatory mechanisms underlying I/R-induced neuronal mitophagy

remain unclear. Previous investigations have clarified the roles of PINK1/Parkin<sup>5</sup> and BNIP3L/Nix<sup>8,10</sup> in neuronal mitophagy during ischaemic stroke, but the involvements of mitophagy receptors during neuronal I/R injury remain need further exploration. FUN14 domain-containing 1 (FUNDC1) is a mitophagy receptor located on the outer mitochondrial membrane,<sup>11–14</sup> which contains an LC3-interacting region, by which FUNDC1 interacts with microtubule light chain 3 (LC3), the major component of autophagosomes<sup>15</sup>; thus, FUNDC1 recruits autophagosomes and clears damaged mitochondria.<sup>16</sup> Researches on ischaemic diseases outside of the central nervous system have revealed that disruptions of FUNDC1-mediated mitophagy can negatively impact cellular survival and function during I/R injury.<sup>17,18</sup> However, the specific contributions of FUNDC1 to neuronal mitophagy and stroke pathogenesis remain underestimated. Further studies regarding FUNDC1-mediated regulation of neuronal I/R-induced mitophagy are needed to establish a biological and molecular basis for mitophagy-targeted interventions.

In this study, our initial hypothesis was that FUNDC1 played a significant role in neuronal mitophagy induced by I/R. However, we discovered that the actual involvement of FUNDC1 in neuronal I/R-induced mitophagy was impaired. The dysfunction of FUNDC1 in neurons induced by I/R injury was found to restrain mitophagy, whereas the restoration of FUNDC1 promoted neuroprotection by facilitating mitophagy.

## MATERIALS AND METHODS

### Animals

A total of 293 C57BL/6J mice were used in this study. All animal procedures were approved by the Institutional Ethics Committee of the Second Affiliated Hospital, Zhejiang University of Medicine (Approval no. AIRB-2022-0862). Wild-type (WT) mice were supplied by SLAC (Shanghai, China), and whole genome FUNDC1 knockout (*FUNDC1*<sup>-/-</sup>) mice were provided by Professor CQ and LL. Mice were bred and housed in individual, well-ventilated cages with access to food and water ad libitum. The mice were maintained on a 12 hours light/dark cycle. The study design, mice grouping and application of mice used are delineated in online supplemental table.

### Mouse transient middle cerebral artery occlusion model and PP1 treatment

Mice weighing 20–25 g (8–10 weeks old) were given ear labels and randomly allocated to an operator who was blinded to genotype during the surgical procedure by using a random number table. To exclude potential effects of female hormones on autophagy,<sup>19</sup> only male mice were used. Mice were anaesthetised by isoflurane inhalation (4% for induction and 1% for maintenance). Silicone-coated monofilament (RWD Life Science, MSMC21B007PK50) was inserted into the left external carotid artery, passed through the internal carotid artery

and used to occlude middle cerebral artery. The filament was withdrawn 60 min after occlusion. The rectal temperature was maintained at 37±0.5°C during surgery. For mice treated with the Src antagonist PP1 (Aladdin, P126623), the drug was intraperitoneally injected immediately after reperfusion, using a dose of 1.5 mg/kg.<sup>20</sup> Mice that could not eat or drink normally or fell into coma after surgery were euthanised and excluded. All surviving mice were included in the experimental analysis if they met the following criteria: obvious neurological deficits on recovery from anaesthesia; no findings of subarachnoid haemorrhage, haematoma in skull base, or haematoma in brain parenchyma during autopsy and sample collection; and obvious infarcts present during sample collection.

### Monitoring of cerebral blood flow

Laser speckle contrast imaging (LSCI) was used to detect cerebral blood flow (CBF) at each time point during transient middle cerebral artery occlusion (tMCAO) modelling. The skull surface was exposed to LSCI equipment (RWD Life Science China) with an 80 mW laser. Images were dynamically and continuously captured for 10 s (10 ms exposure time), then analysed using custom software.

### Assessment of stroke outcomes

Short-term outcomes were manifested by assessment of 24 hours mortality, infarcted volume, neurological deficits, apoptosis and neuronal degeneration. Infarcted volume was assessed by staining with triphenyltetrazolium chloride monohydrate (TTC). One-millimetre-thick coronal brain sections were immersed in 1–2% TTC solution for 20 min at 37°C. Infarcted volume was calculated using the formula established by Swanson *et al.*<sup>21</sup> To evaluate neurological deficits, the Bederson score and Grip test were analysed at 24 hours after surgery.<sup>22</sup> Assessment of 24 hours mortality, apoptosis and neuronal degeneration are described in the following paragraph. To assess long-term outcomes, the number of survivors in each group was recorded daily for 14 days. Body weight measurements and behavioural tests (Modified Neurological Severity Score, Garcia score, rotarod test and hanging test)<sup>22,23</sup> were conducted at 1, 3, 7 and 14 days after surgery in live mice. All assessments were performed in a blinded manner.

### Histology

Brain frozen sections were prepared for histological staining (online supplemental materials). To detect neuronal degeneration, sections were stained with Fluoro-Jade C (FJC; Biosensis, TR-100-FJT). Immunofluorescence was performed to explore FUNDC1 cellular localisation, changes in phosphorylated FUNDC1 and Src in neurons. These samples were visualised and photographed using a fluorescence microscope (DM550, Leica, Germany). Immunofluorescence was also conducted for analysis of neuronal mitophagy, and these samples were visualised and photographed using a laser confocal microscope (STELLARIS 5, Leica, Germany). The antibodies

used for immunofluorescence are described in online supplemental materials.

### Neuronal culture, oxygen glucose deprivation/reperfusion and PP1 treatment

Cortexes were dissected from E17 fetal mice and digested with 0.25% trypsin. Cells were seeded at a density of  $2 \times 10^5$  cells/cm<sup>2</sup> onto plates or dishes coated with poly-L-lysine. Neurons were grown in a neurobasal medium containing 1% B27 and 0.5 mmol/L glutamine. Neurons were used for experiments 10 days after seeding.

For oxygen glucose deprivation/reperfusion (OGD/R) treatment, cells were treated with glucose-free DMEM (Dulbecco's Modified Eagle Medium) and immediately placed into a sealed chamber filled with a gas mixture containing only 1% O<sub>2</sub> for 2 hours; they were subsequently immersed in normal culture medium and placed into an incubator under normoxia conditions. For cells treated with PP1, the drug was added to the medium immediately after reperfusion; its final concentration was 5  $\mu$ M.<sup>24</sup>

### Cellular fluorescence staining

To detect mitophagy in primary neurons, cells were seeded onto cell culture imaging dishes. At 48 hours before OGD, cells were infected with adenovirus expressing GFP-LC3B fusion protein (Ad-GFP-LC3B, 20 MOI; Beyotime, C3006). At the end of reperfusion, cells were labelled with MitoTracker Deep Red FM (Beyotime, C1032) and fixed with 4% paraformaldehyde (PFA). To assess colocalisation of LC3 puncta and FUNDC1, cells were infected with GFP-LC3B. At the end of reperfusion, cells were fixed with 4% PFA and incubated with an antibody against FUNDC1 (1:100; CST, 49240). A laser confocal microscope was used to capture images.

### Western blotting and co-immunoprecipitation

Western blotting was performed to analyse apoptosis, mitophagy, FUNDC1 and Src phosphorylation and precipitates of co-immunoprecipitation. Proteins were extracted from brain tissues and cells using the RIPA (Radio Immunoprecipitation Assay) lysis buffer. Proteins were separated by polyacrylamide gel electrophoresis, then electrotransferred to PVDF (Polyvinylidene Fluoride) membranes. The membranes were subsequently blocked with 10% skim milk at room temperature for 1 hour, incubated with primary antibodies at 4°C overnight and secondary antibodies at room temperature for 1 hour and finally exposed with ECL (Enhanced Chemiluminescence) reagent. The antibodies used are shown in online supplemental materials.

For immunoprecipitation, cells were lysed with 500  $\mu$ L of NP-40 lysis buffer on ice for 30 min, then centrifuged at 12 000 $\times$ g for 15 min at 4°C. Lysates were immunoprecipitated for 2 hours at room temperature, using an antibody against FUNDC1 (1:50) and Pierce Protein A/G Magnetic Beads (Invitrogen, 88803). Precipitates were eluted from beads by incubation in the lysis buffer for 5 min at 95°C; they were subsequently analysed by western blotting.

### Statistical analysis

The Shapiro-Wilk test was used to assess data normality. Normally distributed data were expressed as means with SD; skewed data are shown as medians with IQRs. For comparisons between two groups, normal data were compared using Student's t-test; skewed data were compared using the Mann-Whitney U test. For comparisons among  $\geq 3$  groups, normal data were compared using one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test; skewed data were compared using the Kruskal-Wallis test. Two-way ANOVA was performed for comparisons among time points. The log-rank test was conducted to compare long-term survival. The  $\chi^2$  test was used for the comparison of the 24-hour death rate. ImageJ was used for image analysis. GraphPad Prism V.8.0.2 was used for all statistical analysis; p values < 0.05 were considered statistically significant.

## RESULTS

### Genetic deletion of FUNDC1 does not influence stroke outcomes

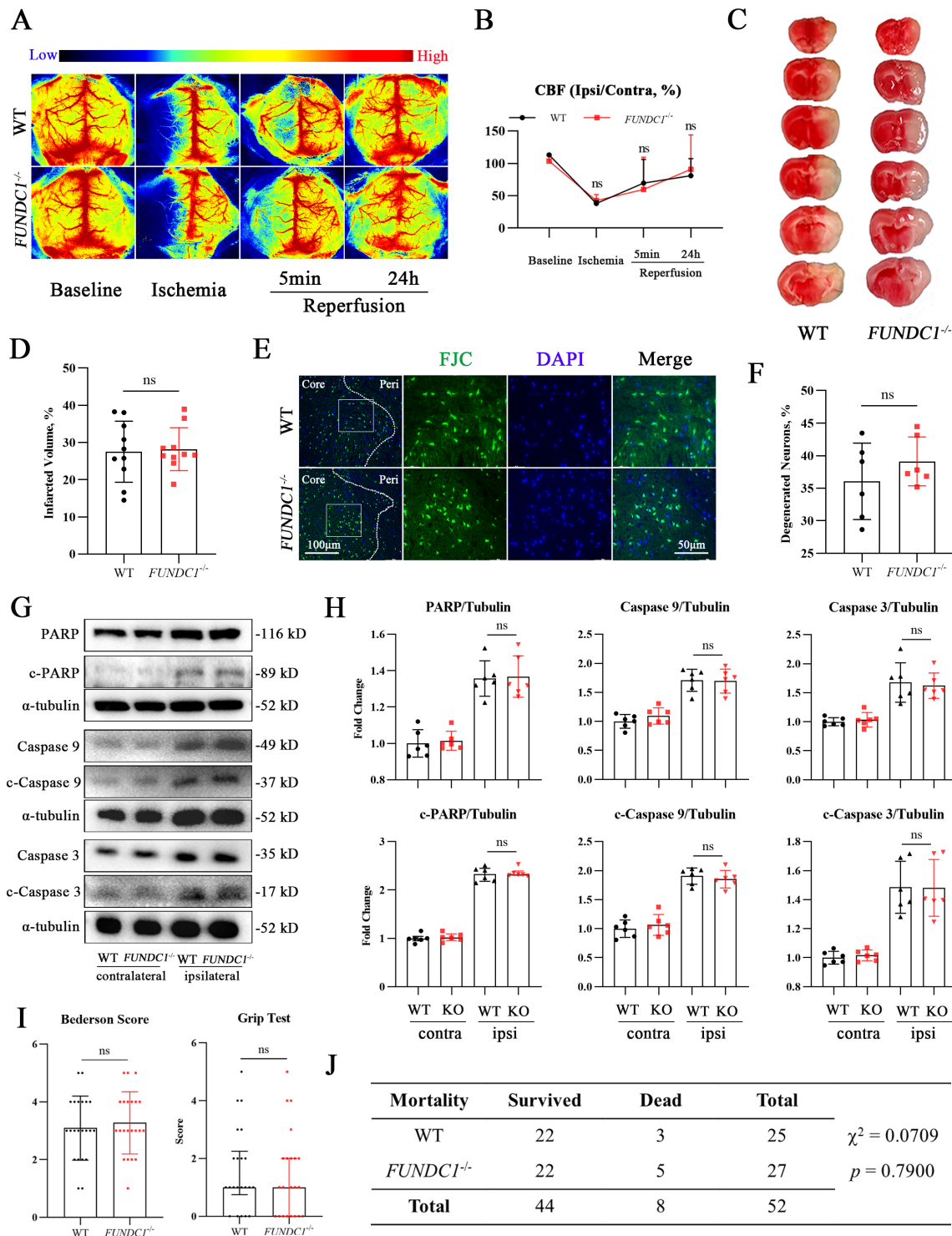
To determine the direct contributions of FUNDC1 to ischaemic stroke pathogenesis, *FUNDC1*<sup>-/-</sup> mice were applied. During tMCAO surgery, CBF was monitored (figure 1A,B) to exclude potential effects of FUNDC1 on platelet aggregation and angiogenesis.<sup>25 26</sup> Surprisingly, at 24 hours after tMCAO surgery, *FUNDC1*<sup>-/-</sup> mice (28.18 $\pm$ 5.78%) showed similar infarcted volumes compared with WT mice (27.52 $\pm$ 8.20%, Student's t-test p > 0.05; figure 1C,D). Percentages of degenerated neurons in the infarcted areas were comparable between two genotypes (figure 1E,F), and loss of FUNDC1 did not affect cellular apoptosis in the infarcted hemisphere (figure 1G,H). Meanwhile, all surviving mice in the two groups showed similar neurological deficiency (figure 1I). Furthermore, *FUNDC1*<sup>-/-</sup> mice (22.73%, 5/22) showed similar mortality rate to that of WT mice (13.64%, 3/22, Fisher's p > 0.05; figure 1J). These results suggested that FUNDC1 knockout had no effects on short-term outcomes of stroke.

To further confirm the influence of FUNDC1 on ischaemic stroke, long-term outcomes were also evaluated. The 14-day survival rate showed no difference (WT 60%, 6/10 vs *FUNDC1*<sup>-/-</sup> 50%, 5/10, log-rank p > 0.05; figure 2A). FUNDC1 deletion also had no effect on body weight recovery (figure 2B) or behavioural performance (figure 2C) at any time point. These results suggested that FUNDC1 knockout also had no influence on long-term outcomes of stroke.

### Loss of FUNDC1 does not disrupt I/R-induced neuronal mitophagy

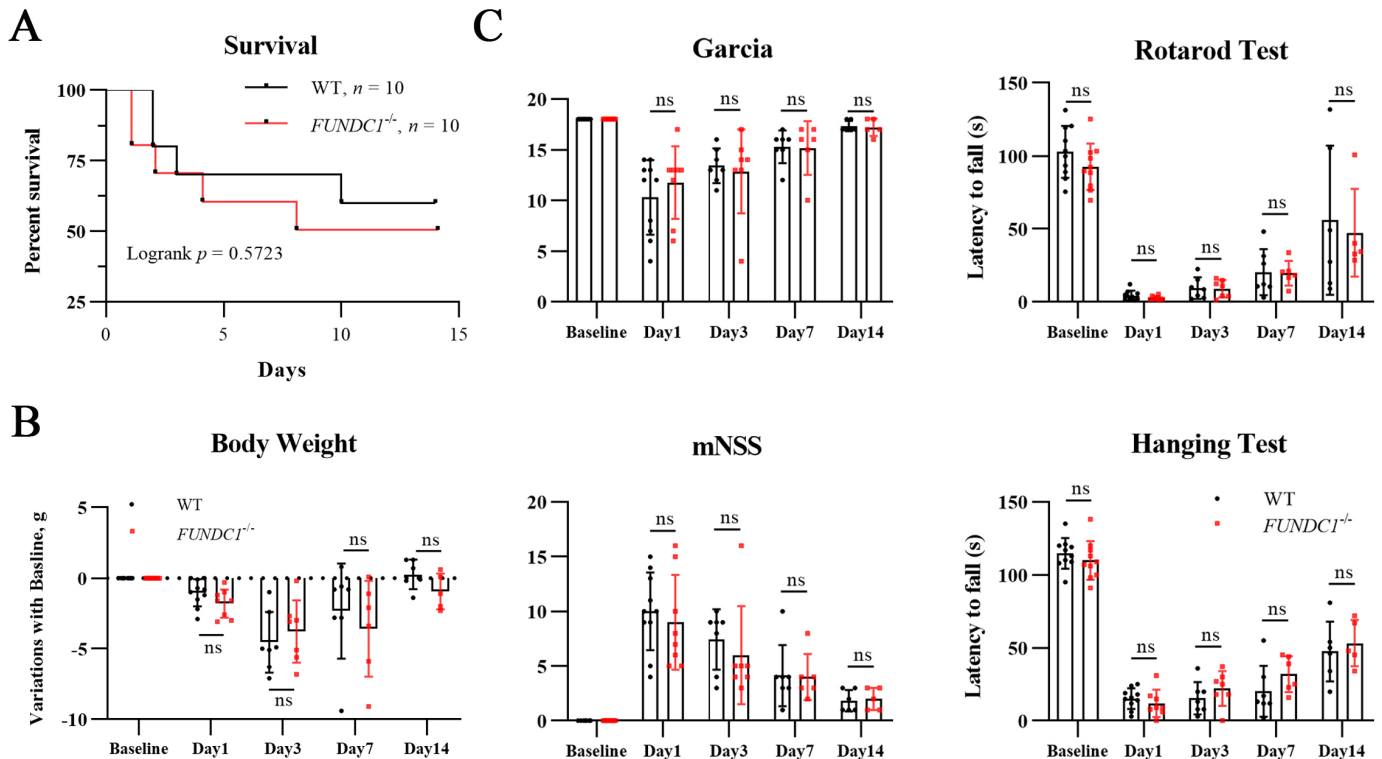
The above findings indicated that FUNDC1 does not affect ischaemic stroke pathogenesis on the systematic level. Therefore, we explored neuronal mitophagy to determine whether it was influenced by FUNDC1. Analysis of temporal variations in mitophagy after tMCAO





**Figure 1** Knockout of FUNDC1 has no effect on short-term outcomes of ischaemic stroke. (A) Cerebral blood flow (CBF) of WT and *FUNDC1*<sup>-/-</sup> mice. (B) Quantification for the variation of CBF at each time point of the perioperative period. n=5 mice for each genotype. (C) Representative TTC staining of brains from WT and *FUNDC1*<sup>-/-</sup> mice subjected to tMCAO at 24 hours after surgery. (D) Quantification for the infarcted volumes. n=10 mice for each genotype. (E) Degenerated neurons in the infarcted brains at 24 hours after reperfusion. (F) Quantification for the percentage of degenerated neurons. n=6 mice for each genotype. (G) Apoptosis in ipsilateral and contralateral brain tissues from WT and *FUNDC1*<sup>-/-</sup> mice subjected to tMCAO were detected by western blotting at 24 hours after reperfusion. (H) Semi-quantification for apoptosis-associated protein PARP, cleaved PARP, caspase 9, cleaved caspase 9, caspase 3 and cleaved caspase 3. n=6 mice for each group. (I) Comparison for neurological deficits including Bederson score (left) and Grip test (right). n=22 mice for each genotype. (J)  $\chi^2$  test fourfold tables for 24-hour death events in WT and *FUNDC1*<sup>-/-</sup> mice. DAPI: 4',6-diamidino-2-phenylindole; FJC, Fluore Jade C; FUNDC1, FUN14 domain-containing 1; KO: Knockout; PARP: poly ADP-ribose polymerase; tMCAO, transient middle cerebral artery occlusion; TTC, triphenyltetrazolium chloride; WT, wild-type.





**Figure 2** Absence of FUNDC1 does not influence long-term outcomes of ischaemic stroke. (A) 14-day survival rate after tMCAO.  $n=10$  mice for each genotype. (B–C) Body weight recovery (B) and behavioural tests (C) including Garcia score, mNSS score, rotarod test, hanging test were assessed before the surgery (WT,  $n=10$ ;  $FUNDC1^{-/-}$ ,  $n=10$ ) and at 1 day (WT,  $n=10$ ;  $FUNDC1^{-/-}$ ,  $n=8$ ), 3-day (WT,  $n=7$ ;  $FUNDC1^{-/-}$ ,  $n=7$ ), 7-day (WT,  $n=6$ ;  $FUNDC1^{-/-}$ ,  $n=6$ ), 14-day (WT,  $n=5$ ;  $FUNDC1^{-/-}$ ,  $n=5$ ) after tMCAO. FUNDC1, FUN14 domain-containing 1; mNSS, Modified Neurological Severity Score; tMCAO, transient middle cerebral artery occlusion; WT, wild-type.

revealed that cerebral I/R-induced sustained mitophagy in the first 24 hours, characterised by a progressive reduction in mitochondrial markers Tomm20 and COX4I1, as well as an increase in the LC3 II/I ratio and a decrease in SQSTM1 (Sequestosome 1) (figure 3A,B). Subsequently, we used  $FUNDC1^{-/-}$  mice to clarify the involvement of FUNDC1 in neuronal mitophagy. Baseline mitophagy did not differ between WT and  $FUNDC1^{-/-}$  mice (figure 3C,D). However, at 24 hours after tMCAO, mitophagy process also showed no differences (one-way ANOVA  $p>0.05$ ; figure 3C,D) between WT and  $FUNDC1^{-/-}$  mice. Similarly, among neurons at sites of infarction, FUNDC1 did not affect the overlap of LC3 (autophagosome) and Tomm20 (mitochondria; one-way ANOVA  $p>0.05$ ; figure 3E,F).

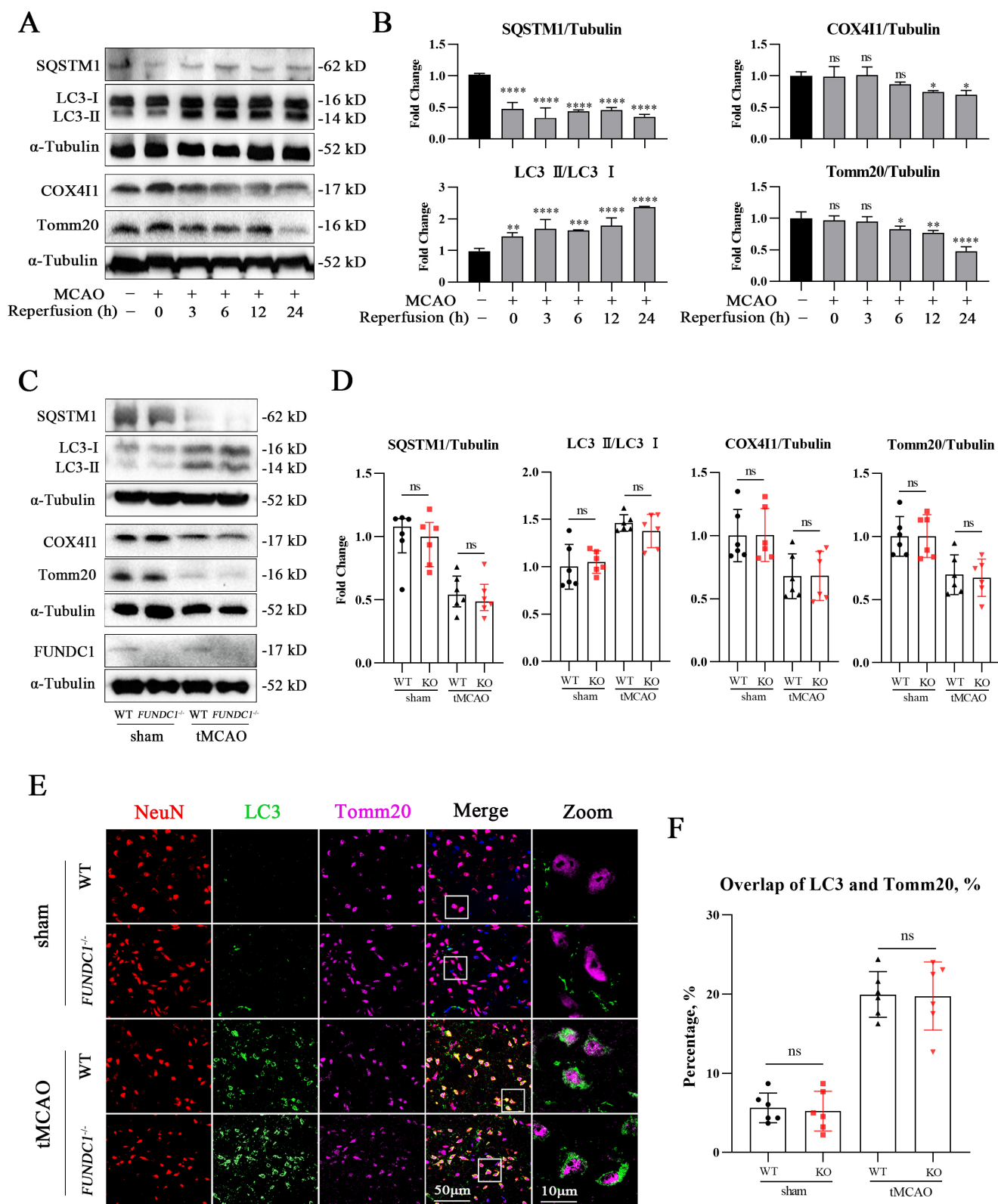
We also confirmed the above findings by in vitro analysis. FUNDC1 was predominantly localised in neurons (online supplemental figure S1), thus in vitro analysis was conducted on isolated neurons. As expected, isolated cortical neurons under OGD/R conditions exhibited mitophagy, and mitochondrial clearance peaked at 9 hours after reperfusion (figure 4A,B). However, consistent with the in vivo findings,  $FUNDC1^{-/-}$  neurons showed no changes in mitophagy compared with WT neurons, regardless of culture conditions (normal or 9 hours after OGD/R; one-way ANOVA  $p>0.05$ ; figure 4C,D). Additionally,  $FUNDC1^{-/-}$  neurons displayed similar numbers of LC3 puncta or overlap coefficient of LC3 and mitochondria

(one-way ANOVA  $p>0.05$ ; figure 4E,F), regardless of culture conditions. Analysis of mitochondrial quality in  $FUNDC1^{-/-}$  neurons also showed no changes in mitochondrial membrane potential (online supplemental figure S2A,B) or reactive oxygen species production (online supplemental figure S2C,D).

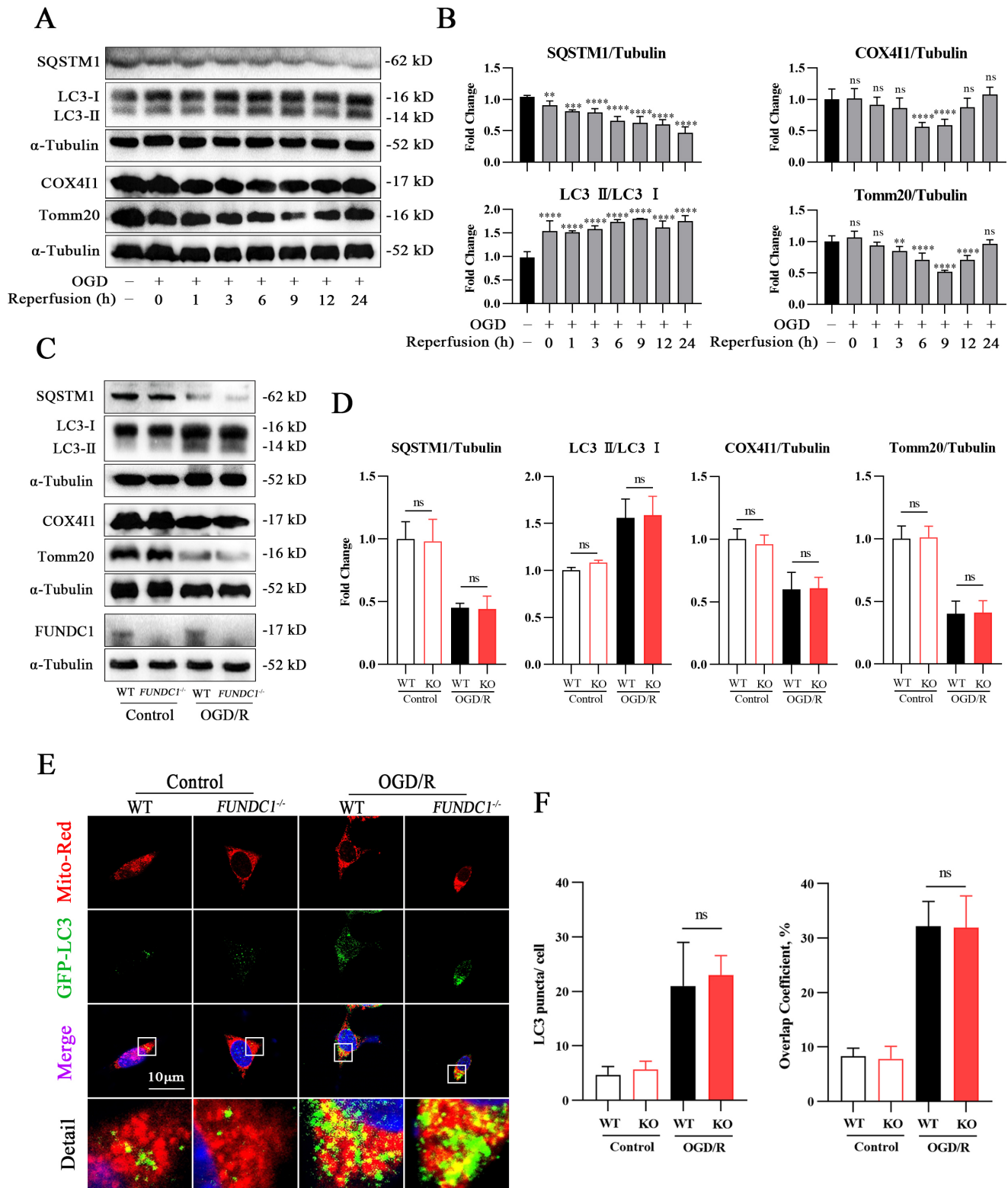
These findings suggested that although ischaemic stroke triggers neuronal mitophagy, FUNDC1 is not involved in this process. The loss of FUNDC1 does not influence neuronal mitophagy during cerebral I/R injury.

### FUNDC1 is inactivated in later stages of neuronal I/R injury

The above results indicated that FUNDC1 activation, represented by dephosphorylation of Tyr18,<sup>11 16</sup> may be impaired during neuronal I/R injury. Thus, we analysed temporal variations in FUNDC1 phosphorylation of Tyr18 in neurons during ischaemic stroke. In the in vivo analysis, the level of total FUNDC1 did not substantially change at any time point during I/R injury (figure 5A,B). However, p-Tyr18 dephosphorylation was only observed within 3 hours after reperfusion (two-way ANOVA  $p<0.05$ ); it returned to baseline at 6 hours and was subsequently detected at levels above baseline (two-way ANOVA  $p<0.05$ ; figure 5A,B). Immunostaining also showed that Tyr18 on FUNDC1 was dephosphorylated on reperfusion but hyperphosphorylated at 24 hours after reperfusion (figure 5C,D) in mice subjected to tMCAO.

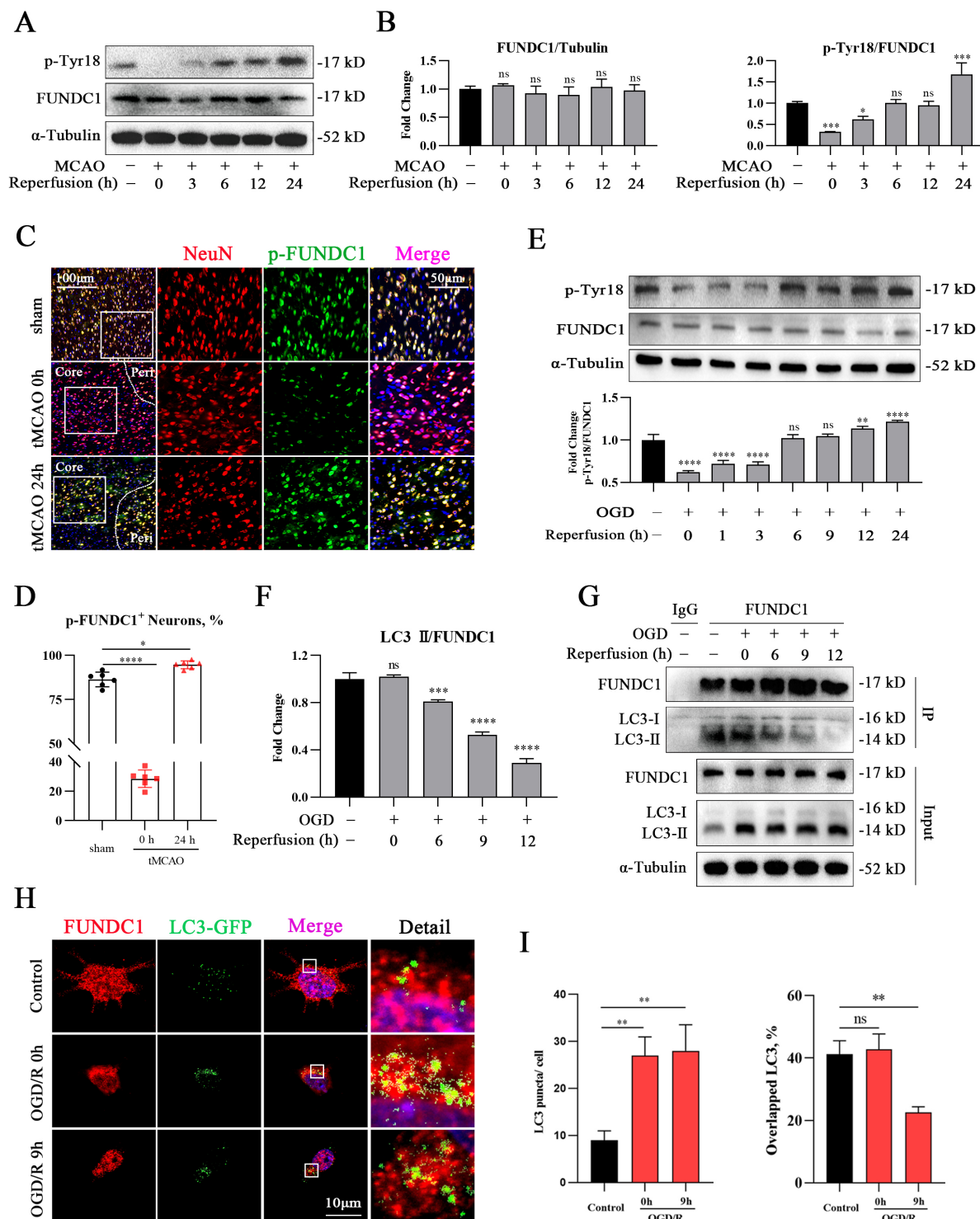


**Figure 3** Deletion of FUNDC1 does not affect neuronal mitophagy in vivo. (A) Time course of mitophagy in vivo was detected by western blotting. (B) Semi-quantification for western blotting detection in panel A.  $n=3$  mice for each time point. (C) WT and  $FUNDC1^{-/-}$  mice were subjected to sham surgery or tMCAO for 24 hours. Mitophagy was detected by western blotting. (D) Semi-quantification for western blotting detection in panel C.  $n=6$  mice per group. (E) Brain sections from both genotypes of normal mice or mice with stroke were labelled with NeuN (red), LC3 (green), and Tomm20 (magenta). (F) Quantification of the overlap coefficient of LC3 and Tomm20.  $n=6$  mice per group. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , \*\*\*\* $p<0.0001$ . FUNDC1, FUN14 domain-containing 1; KO: Knockout; LC3, light chain 3; SQSTM1: Sequestosome 1; tMCAO, transient middle cerebral artery occlusion; WT, wild-type.



**Figure 4** Loss of FUNDC1 does not affect neuronal mitophagy and mitochondrial qualities in vitro. (A) Time course of neuronal mitophagy in vitro was detected by western blotting. (B) Semi-quantification for western blotting detection in panel A.  $n=3$  for independent experiments. (C) Cortical neurons isolated from WT and *FUNDC1*<sup>-/-</sup> mice were subjected to control or OGD/R treatment for 9 hours. Mitophagy was detected by western blotting. (D) Semi-quantification for western blotting detection in panel C.  $n=3$  per independent experiment. (E) Normal-cultured or OGD/R-treated neurons with two genotypes were labelled with MitoTracker (red) and GFP-LC3 (green), then visualised by confocal microscopy. (F) Quantification of the number of LC3 puncta per cell (left) and colocalisation coefficient of mitochondria and LC3 puncta (right).  $n=3$  for independent experiment. \*\* $p<0.01$ , \*\*\* $p<0.001$ , \*\*\*\* $p<0.0001$ . FUNDC1, FUN14 domain-containing 1; GFP: Green Fluorescent Protein; KO: Knockout; LC3, light chain 3; OGD/R, oxygen glucose deprivation/reperfusion; SQSTM1: Sequestosome 1; WT, wild-type.





**Figure 5** FUNDC1 is inactivated in later stages of neuronal I/R injury. (A) Time course of FUNDC1 phosphorylation was detected by western blotting in vivo. (B) Semi-quantification for p-Tyr18 and total FUNDC1 in panel A.  $n=3$  mice per time point. (C) Immunostaining of p-FUNDC1 (green) in neurons (red). (D) Quantification for proportion of p-FUNDC1 positive neurons.  $n=6$  mice per time point. (E) Time course of FUNDC1 phosphorylation was detected by western blotting in vitro.  $n=3$  for independent experiments. (F) Semi-quantification for LC3 II interacted with FUNDC1 as is shown in panel G.  $n=3$  for independent experiments. (G) Isolated cortical neurons were subjected to 2 hours of OGD, followed by 0 hours, 6 hours, 9 hours or 12 hours of reperfusion. Interactions between FUNDC1 and LC3 were detected by co-immunoprecipitation. (H) Fluorescent staining of FUNDC1 (red) and GFP-LC3 (green) in isolated neurons subjected to control treatment, OGD/R 0 hours, or OGD/R 9 hours. (I) Quantification of the number of LC3 puncta per cell (left) and percentage of LC3 puncta colocalised with FUNDC1 (right).  $N=3$  for independent experiment. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . FUNDC1, FUN14 domain-containing 1; Green Fluorescent Protein; I/R, ischaemia/reperfusion; LC3, light chain 3; OGD/R, oxygen glucose deprivation/reperfusion; tMCAO, transient middle cerebral artery occlusion; WT, wild-type.

In vitro analysis showed a similar variation process of p-Tyr18 (figure 5E).

These results differ from previous findings in HeLa cells,<sup>11 16</sup> where cellular hypoxia consistently activated FUNDC1 (online supplemental figure S3A,B), indicating that interactions between FUNDC1 and LC3 in neurons may be different from that in HeLa cells. Co-immunoprecipitation revealed that, in contrast to HeLa cells (online supplemental figure S3C,D), I/R injury lead to a continuous decrease in LC3–FUNDC1 interactions in neurons after reperfusion, despite the increase in total LC3 in neurons (figure 5F,G). Furthermore, morphological assessment showed that, although OGD/R treatment increased the number of LC3 puncta, the proportion of LC3 puncta overlapped with FUNDC1 had decreased by 9 hours after reperfusion (figure 5H,I). These data indicated that FUNDC1 was activated in early stages (within 3 hours) but inactivated in later stages (after 6 hours) of neuronal I/R, which conflicted with mitophagy progression.

### Src is hyperactivated during neuronal I/R injury

The phosphorylation of Tyr18 on FUNDC1 is regulated by the tyrosine kinase Src,<sup>11 16</sup> and Src is activated by phosphorylation of its Tyr416 residue.<sup>27</sup> To explore potential triggers of FUNDC1 inactivation, we analysed temporal variations in phosphorylated Src (Tyr416). In brain samples, total Src levels were not altered at any time point after I/R injury (figure 6A,B). Similarly, p-Src (Tyr416) showed synchronous spatial-temporal variations with p-FUNDC1 (Tyr18), represented by Tyr416 dephosphorylation at a very early stage (two-way ANOVA  $p < 0.05$ ) within 3 hours but hyperphosphorylation at later stages (two-way ANOVA  $p < 0.05$ ) of I/R injury (figure 6A–D). Similar results were also found in cell samples (figure 6E). Src activation in neurons also differed from the findings in HeLa cells, where cellular hypoxia continuously inactivated Src (online supplemental figure S3A,B), as previously reported.<sup>11</sup> Moreover, in contrast to the results in HeLa cells (online supplemental figure S3C,D), FUNDC1 exhibited continuously increasing interactions with Src in neurons after reperfusion (figure 6F,G).

These results suggested that Src is inactivated in early stages (within 3 hours) but activated in later stages (after 6 hours) of neuronal I/R injury, consistent with changes in FUNDC1 activity; thus, the spontaneous inactivation of neuronal FUNDC1 during I/R-induced mitophagy may be related to hyperactivation of Src.

### Pharmacological inhibition of Src rescues FUNDC1-mediated mitophagy in neurons

To further confirm that FUNDC1 inactivation is caused by Src activation in neurons subjected to I/R injury, we examined whether inhibition of Src could rescue FUNDC1-mediated mitophagy. In neurons subjected to OGD/R injury, treatment with PP1 (5  $\mu$ M) reduced phosphorylation of Src at Tyr416; this effect was accompanied by dephosphorylation of FUNDC1 at Tyr18 (online

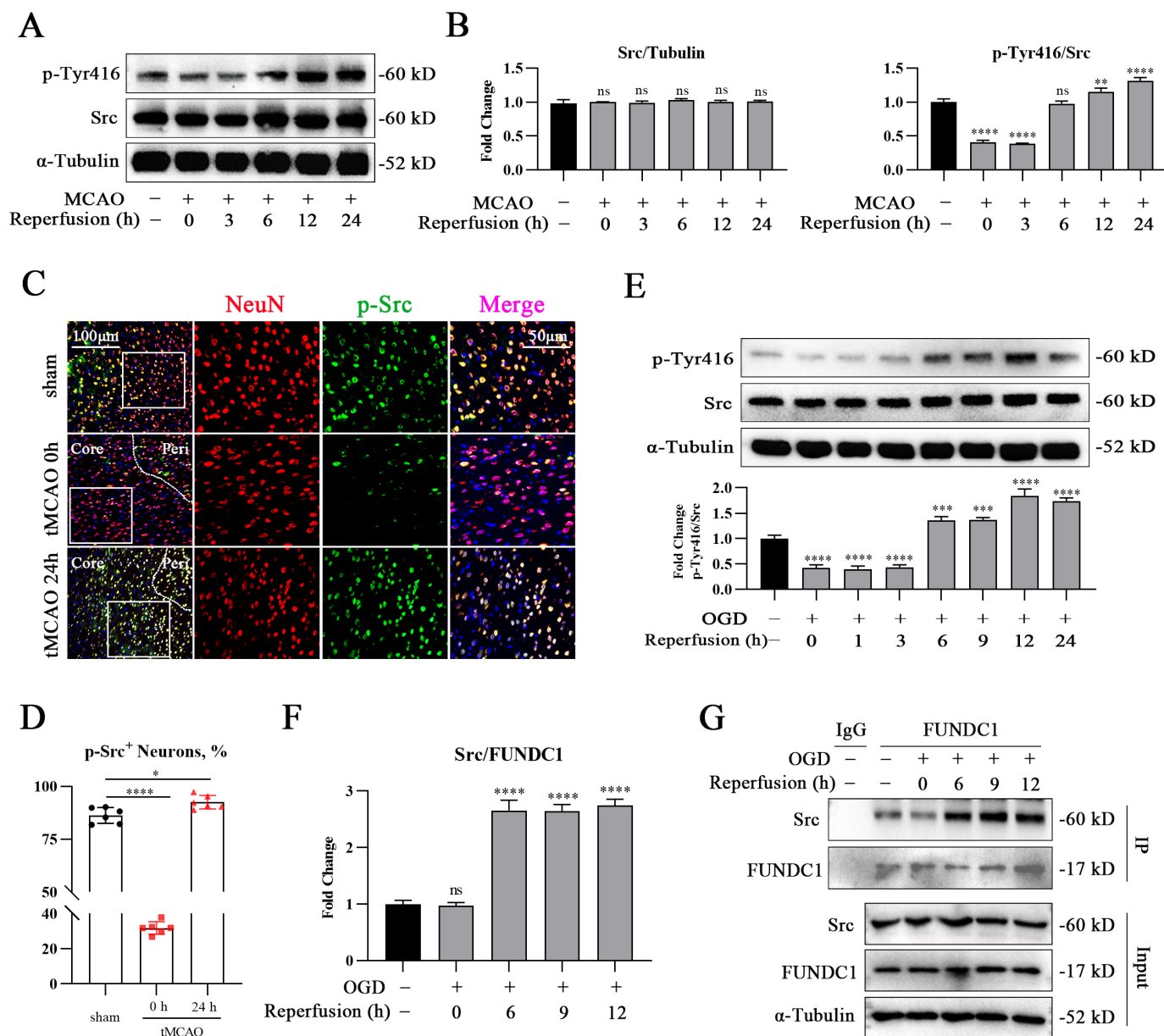
supplemental figure S4A,B), suggesting that Src inhibition could rescue FUNDC1 function. Next, PP1 treatment promoted mitophagy in OGD/R-treated neurons (online supplemental figure S4C,D). To confirm that the PP1-induced mitophagy enhancement was mediated by FUNDC1, we analysed interactions between FUNDC1 and autophagosomes. Co-immunoprecipitation suggested that PP1 treatment decreased interactions between FUNDC1 and Src, while increasing interactions between FUNDC1 and LC3 (online supplemental figure S4E,F). Morphological assessment revealed that PP1 treatment increased the number of LC3 puncta in OGD/R-treated neurons; it also increased the proportion of LC3 puncta overlapped with FUNDC1 (figure 7A,B).

Subsequently, we analysed the involvement of FUNDC1 in mice with stroke that had received intraperitoneal PP1 (1.5 mg/kg).<sup>20</sup> As expected, Src inhibition promoted mitophagy in WT mice (one-way ANOVA  $p < 0.05$ ; figure 7C,D). In contrast, in *FUNDC1*<sup>−/−</sup> mice, although PP1 treatment promoted macroautophagy (decreased SQSTM1 and increased LC3-II/I) as observed in WT mice, it failed to promote mitochondrial clearance (one-way ANOVA  $p > 0.05$ ; figure 7C,D). To eliminate potential non-specific effects of chemical inhibitors, these findings were verified by employing small interfering RNA targeting Src in the in vitro analysis (online supplemental figure S5B). At the systemic level, we evaluated the effects of Src inhibition on ischaemic stroke outcomes. We found that PP1 treatment reduced infarct volume (one-way ANOVA  $p < 0.05$ ) and alleviated neurological deficiency in WT mice, but it did not substantially affect those in *FUNDC1*<sup>−/−</sup> mice (one-way ANOVA  $p > 0.05$ ; figure 7E,F,G). For the long-term observation, PP1 cannot improve survival rate but significantly promoted recovery of body weight and neurological functions in WT mice rather than in *FUNDC1*<sup>−/−</sup> mice (online supplemental figure S6). These findings suggested that pharmacological inhibition of Src promotes the activation of FUNDC1-mediated mitophagy during neuronal I/R injury, thereby improving ischaemic stroke outcomes.

Taken together, the results of this study indicate that FUNDC1 is spontaneously inactivated in neurons in later stages of I/R injury; it is not required in I/R-induced mitophagy. Mechanistic analysis showed that FUNDC1 inactivation in ischaemic neurons is partially associated with the presence of hyperactivated Src.

## DISCUSSION

Neuroprotective therapies are urgently required for the treatment of ischaemic stroke<sup>28</sup>; because mitophagy plays a key role in the maintenance of neuronal mitochondrial homeostasis,<sup>1 3 4</sup> it is a promising target for neuroprotection. However, current knowledge of mitophagy and its modulatory mechanisms has been established through studies involving immortal cells.<sup>29</sup> Neurons are highly differentiated and polarised cells; thus, mitophagy in neurons proceeds through unique mechanisms that differ from the pathways involved in immortal cells.



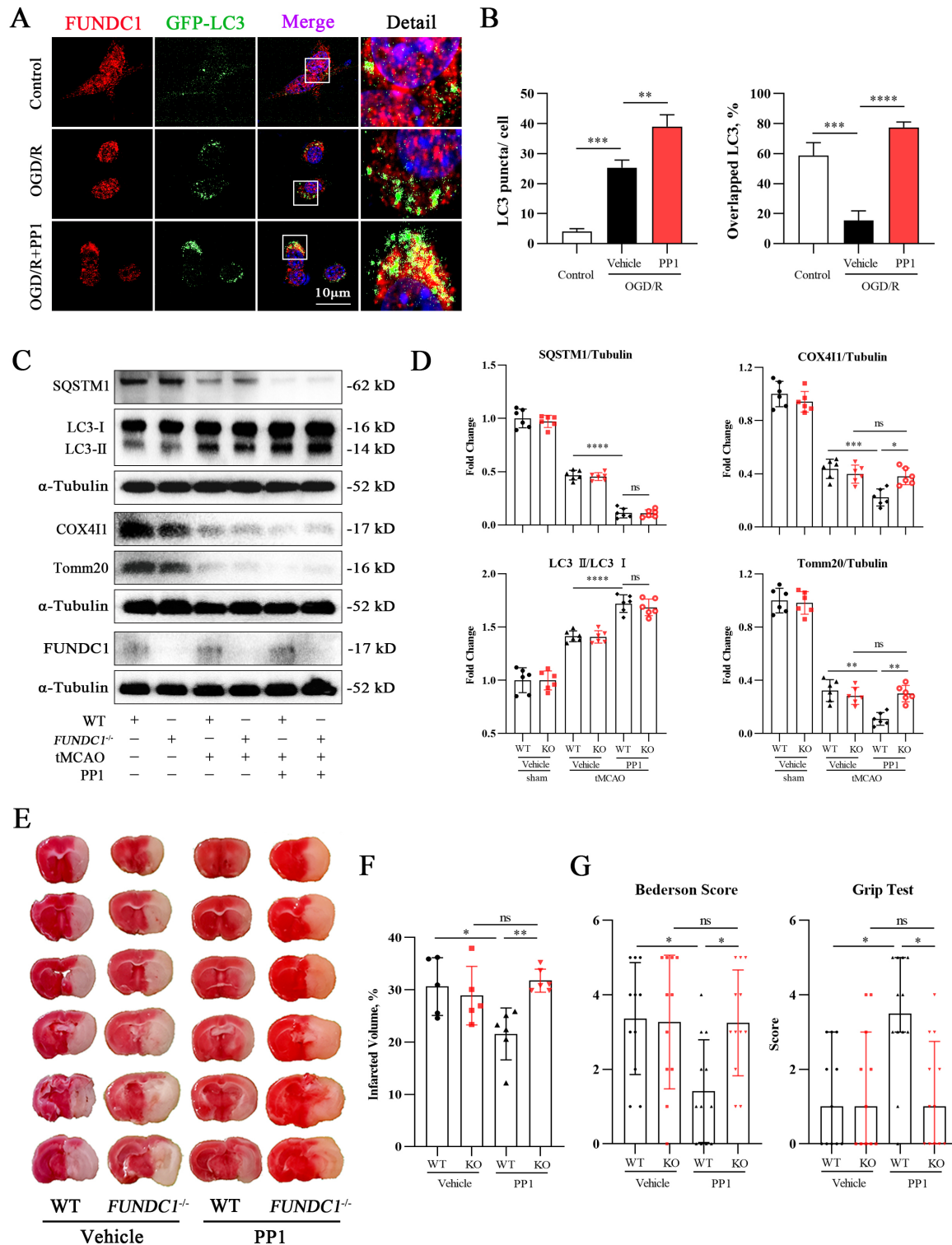
**Figure 6** Src is activated in later stages of neuronal ischaemia/reperfusion injury. (A) Src phosphorylation in vivo at different time points was detected by western blotting. (B) Semi-quantification for p-Tyr416 and total Src in panel A.  $n=3$  mice per time point. (C) Immunostaining of p-Src (green) in neurons (red). (D) Quantification for proportion of p-Src positive neurons.  $n=6$  mice per time point. (E) Src phosphorylation in vitro at different time points was detected by western blotting.  $n=3$  for independent experiments. (F) Semi-quantification for Src interacted with FUNDC1 as is shown in panel G.  $n=3$  for independent experiments. (G) Time course of FUNDC1-Src interaction in isolated neurons were detected by co-immunoprecipitation. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , \*\*\*\* $p<0.0001$ . FUNDC1, FUN14 domain-containing 1; OGD, oxygen glucose deprivation; tMCAO, transient middle cerebral artery occlusion.

In the present study, we have demonstrated that neurons exhibit impaired FUNDC1-mediated mitophagy during I/R injury. Unlike immortal cells, ischaemic insults in neurons cause excessive activation of Src, which subsequently phosphorylates Tyr18 on FUNDC1; FUNDC1 then loses its ability to interact with LC3, which ultimately prevents FUNDC1 involvement in neuronal mitophagy (figure 8). Previous studies explored the involvement of PINK1/Parkin and BNIP3L/Nix in I/R-induced mitophagy in neurons,<sup>58</sup> revealing that I/R injury can induce

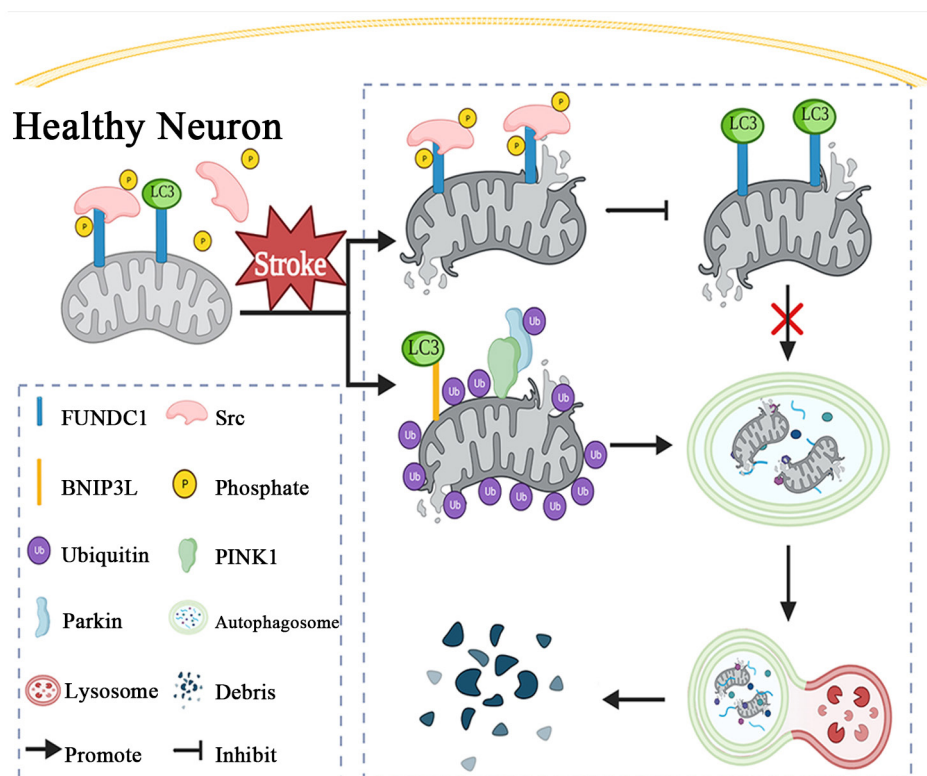
mitophagy through the PINK1/Parkin and BNIP3L/Nix pathways in neurons, even in the absence of FUNDC1 (figure 8).

It is important to consider whether these findings indicate that FUNDC1 cannot serve as a therapeutic target for ischaemic stroke. Our data show that Src inhibition rescues FUNDC1 activity and elicits neuroprotective effects via FUNDC1-mediated mitophagy. Thus, treatments that restore FUNDC1 activity may be useful therapies for stroke. Research involving other organs may





**Figure 7** Pharmacological inhibition of Src rescues FUNDC1-mediated mitophagy in neurons subjected to ischaemia/reperfusion injury. (A) Neurons subjected to OGD/R were treated with vehicle or PP1, and were labelled using antibodies against FUNDC1 (red) and GFP-LC3 (green). (B) Quantification of the number of LC3 puncta per cell (left) and percentage of LC3 puncta colocalised with FUNDC1 (right).  $n=3$  for independent experiment. (C) Mitophagy in mice with both genotypes treated by PP1 in brain samples was detected by western blotting. (D) Semi-quantification for western blotting detection in panel C.  $n=6$  mice per group. (E) Representative TTC staining of brains from vehicle-treated WT mice ( $n=5$ ), *FUNDC1*<sup>-/-</sup> mice ( $n=5$ ), PP1-treated WT mice ( $n=6$ ), and PP1-treated *FUNDC1*<sup>-/-</sup> mice ( $n=6$ ). (F) Quantification of infarcted volume. (G) Comparison of neurological deficits among vehicle-treated WT mice ( $n=11$ ), *FUNDC1*<sup>-/-</sup> mice ( $n=11$ ), PP1-treated WT mice ( $n=12$ ), and PP1-treated *FUNDC1*<sup>-/-</sup> mice ( $n=12$ ). \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , \*\*\*\* $p<0.0001$ . FUNDC1, FUN14 domain-containing 1; GFP: Green Fluorescent Protein; OGD/R, oxygen glucose deprivation/reperfusion; KO: Knockout; LC3, light chain 3; SQSTM1: Sequestosome 1; tMCAO, transient middle cerebral artery occlusion; WT, wild-type.



**Figure 8** Working model of FUNDC1 inactivation by Src during neuronal I/R injury (created with BioRender.com). Although neuronal I/R induces mitophagy, hyperactivated Src inactivates FUNDC1, leading to FUNDC1 exclusion from mitophagy. Even in the absence of FUNDC1, neurons can initiate mitophagy through the BNIP3L/Nix and PINK1/Parkin pathways. FUNDC1, FUN14 domain-containing 1; I/R, ischaemia/reperfusion.

support this hypothesis. With respect to renal I/R injury, ischaemic preconditioning of renal tubular cells activates FUNDC1-mediated mitophagy by phosphorylating Ser17 on FUNDC1.<sup>18</sup> In myocardial infarction, the dephosphorylation of Ser13 on FUNDC1 through CK2 inactivation may protect cardiomyocytes against ischaemic injury.<sup>30</sup> A recent research suggests that tPA (tissue Plasminogen Activator) treatment during ischaemic stroke activates FUNDC1-mediated mitophagy in neurons.<sup>9</sup> Coupled with our results, the previous findings imply that tPA might regulate FUNDC1 phosphorylation through various pathways, thereby enhancing FUNDC1 activity. Unfortunately, the previous work did not investigate the mechanisms by which FUNDC1 is involved in tPA treatment.

In contrast to neurons, immortal cells (eg, HeLa cells) subjected to hypoxia exhibit Src inhibition and the onset of FUNDC1-mediated mitophagy.<sup>11</sup> Hypoxia is a well-known hallmark of the tumour microenvironment, associated with increases in malignancy and treatment resistance.<sup>31</sup> Analysis using HeLa cells, a representative cervical cancer cell line, have suggested that FUNDC1 is a useful target for controlling hypoxia-related tumour progression. Importantly, FUNDC1 regulates malignancy in various cancers (eg, cervix and breast).<sup>30 32 33</sup> However, conclusions based on analysis of immortal cells are not always relevant to other cell types.

There were some limitations in this study. Whole genome knockout of FUNDC1 was applied in this work. However,

despite its primary localisation in neurons, FUNDC1 also exhibits high expression levels in microglia (~50%; online supplemental figure S1), suggesting that further investigation is necessary to determine whether FUNDC1 regulates the immune microenvironment in response to I/R injury. Meanwhile, neurons are highly polarised cells, with long axons and dendritic processes. The mitochondria in neurons display a wide distribution and considerable mobility; they have diverse functions across neuronal compartments in both resting and stressed conditions.<sup>34</sup> For example, soma and axons exhibit different mitophagy dynamics in response to OGD/R; neuronal I/R injury contributes to mitochondrial redistribution.<sup>29 35</sup> This study did not explore FUNDC1-driven changes in mitochondrial distribution or activity in different neuronal compartments. Furthermore, the effects of phosphorylation at other locations were not fully explored. Further studies are needed to determine how Ser13 and Ser17 on FUNDC1 (and their regulators) participate in I/R injury-mediated neuronal mitophagy.

In conclusion, although neuronal I/R injury triggers mitophagy, FUNDC1 is inactivated by Src at later stages of I/R injury and does not significantly influence mitophagy progression. FUNDC1-mediated mitophagy can be rescued by the inhibition of hyperactivated Src, suggesting that FUNDC1 rescue can be used to enhance neuronal mitophagy and protect neurons against ischaemic stroke.

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**Contributors** TT: Experimental Design, Experimental Operations (Blinded to Group Allocation), Data Collection, Manuscript Draft. L-bH: Randomisation, Blinding (Aware of the Group Allocation), Experimental Operations, Data Analysis. CD: Experimental Operations, Validation, Data Curation. ZZ, NW, CD and TW: Validation. HZ, SX and LF: Data Curation. X-jF: Copyright for Biorender. FY: Supervision, Funding Acquisition. XZ: Conceptualisation, Experimental Facilities and Laboratory Space. GC and JL: Review, Supervision, Project administration, Funding acquisition. TT and JL: Guarantor.

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