

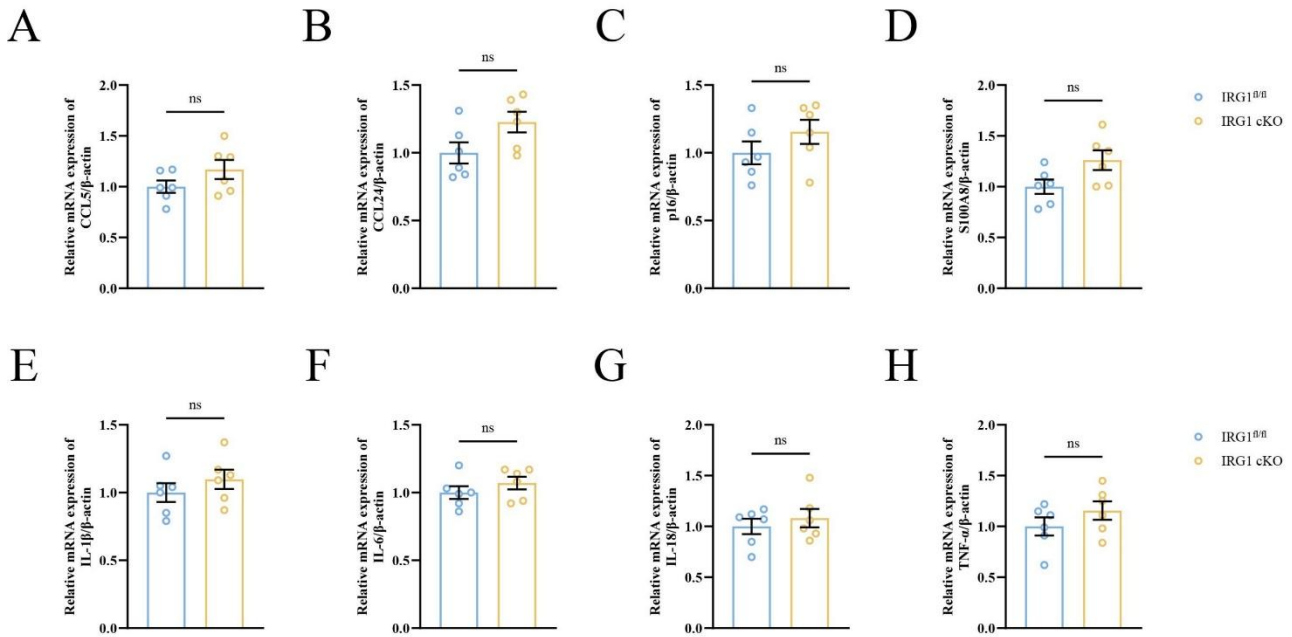
SUPPLEMENTARY DATA

**IRG1/Itaconate Inhibits Microglial Senescence-Like  
Transition by Modulating Mitochondrial Dynamics  
through Rhoa Alkylation in Subarachnoid Hemorrhage**

**Zeyu Zhang, Yanlin Chen, Weizhen Gao, Fengchen Zhang, Yichao Jin, Jie Li, Jiaqi Wang, Tao  
Lv, Feng Jia, Xiaohua Zhang**

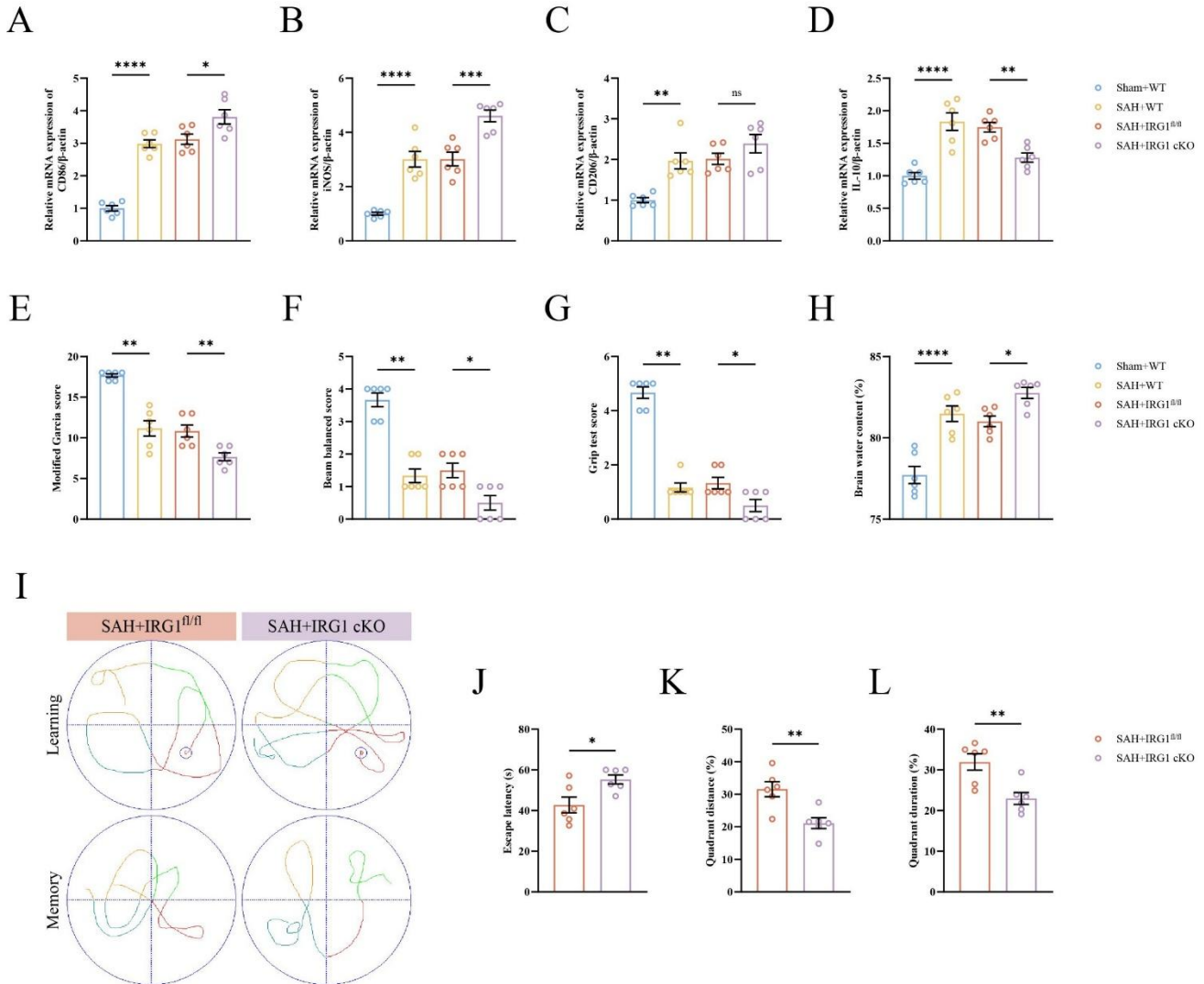
# SUPPLEMENTARY DATA

## Supplementary Figures



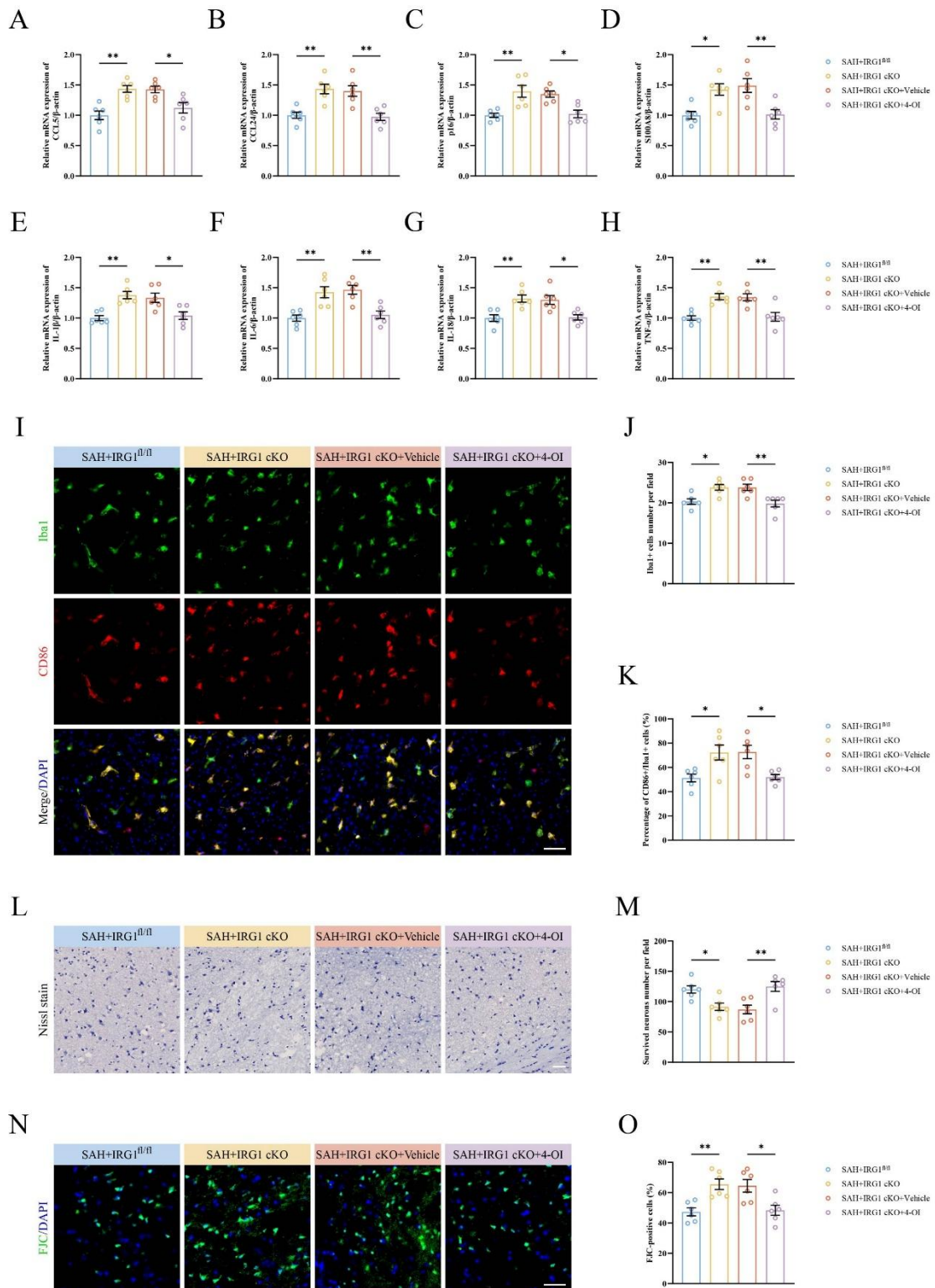
**Supplementary Fig. 1. Effect of IRG1 deficiency on microglial senescence-like transition under physiological conditions.** A-H. qPCR to evaluate CCL5, CCL24, p16, S100A8, IL-1 $\beta$ , IL-6, IL-18, and TNF- $\alpha$  expressions in mice of IRG1<sup>fl/fl</sup> and IRG1 cKO groups (n=6 mice per group). Relative mRNA levels were calculated using the  $2^{-\Delta\Delta C_t}$  method. Data are presented as mean  $\pm$  SEM, normalized to the first group, and analyzed by Student's t-test (A-H).

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**Supplementary Fig. 2. IRG1 deficiency exacerbates microglial inflammatory activation and neurological deficits after SAH.** **A-D.** qPCR to evaluate CD86, iNOS, CD206, and IL-10 expressions in mice of Sham+WT, SAH+WT, SAH+IRG1<sup>fl/fl</sup>, and SAH+IRG1 cKO groups (n=6 mice per group). **E-G.** Short-term neurological function at 24 h post-SAH (n=6 mice per group). Assessments include the modified Garcia score (evaluating 6 parameters: activity, symmetry, extension, climbing, proprioception, and reflex), beam balance, and grip tests. Higher scores indicate superior neurological performance. **H.** Measurement of brain water content (n=6 mice per group). **I-L.** Long-term neurological function from days 22–26 post-SAH in mice of SAH+IRG1<sup>fl/fl</sup> and SAH+IRG1 cKO groups (n=6 mice per group). Spatial learning was assessed via escape latency in the Morris water maze (days 22–25). Memory retention was evaluated on day 26 via a probe trial, recording swim trajectories and the percentage of time and distance spent in the target quadrant. Relative mRNA levels were calculated using the  $2^{-\Delta\Delta C_t}$  method. All behavioral assessments were performed by two investigators blinded to the experimental groups. Data are presented as mean  $\pm$  SEM, normalized to the first group, and analyzed by One-way ANOVA (**A-D, H**), Kruskal-Wallis H test (**E-G**), or Student's t-test (**J-L**).

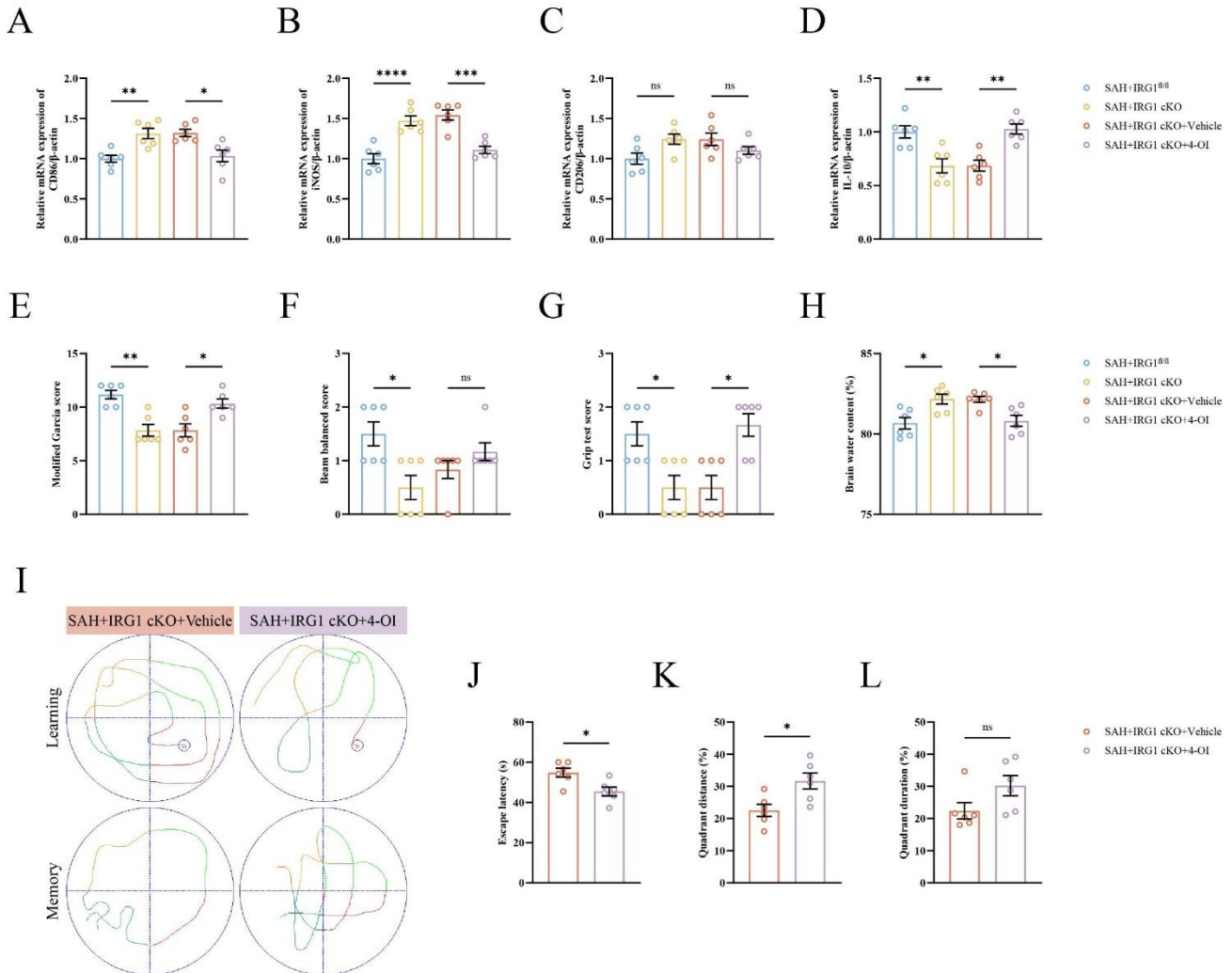
# SUPPLEMENTARY DATA



**Supplementary Fig. 3. 4-OI rescues the exacerbation of microglial senescence-like transition and brain injury caused by IRG1 deficiency.** A-H. qPCR to evaluate CCL5, CCL24, p16, S100A8, IL-1 $\beta$ , IL-6, IL-18, and TNF- $\alpha$  expressions in mice of SAH+IRG1<sup>fl/fl</sup>, SAH+IRG1 cKO, SAH+IRG1 cKO+Vehicle, and SAH+IRG1 cKO+4-OI groups (n=6 mice per group). I-K. Immunofluorescence staining to evaluate microglial number and polarization (scale bar=60 $\mu$ m; n=6 mice per group). L-M. Nissl staining to assess neuronal survival (scale bar=40 $\mu$ m; n=6 mice per group). N-O. FJC staining to assess neuronal degeneration and death (scale bar=40 $\mu$ m; n=6 mice per group). Relative mRNA levels were calculated using the

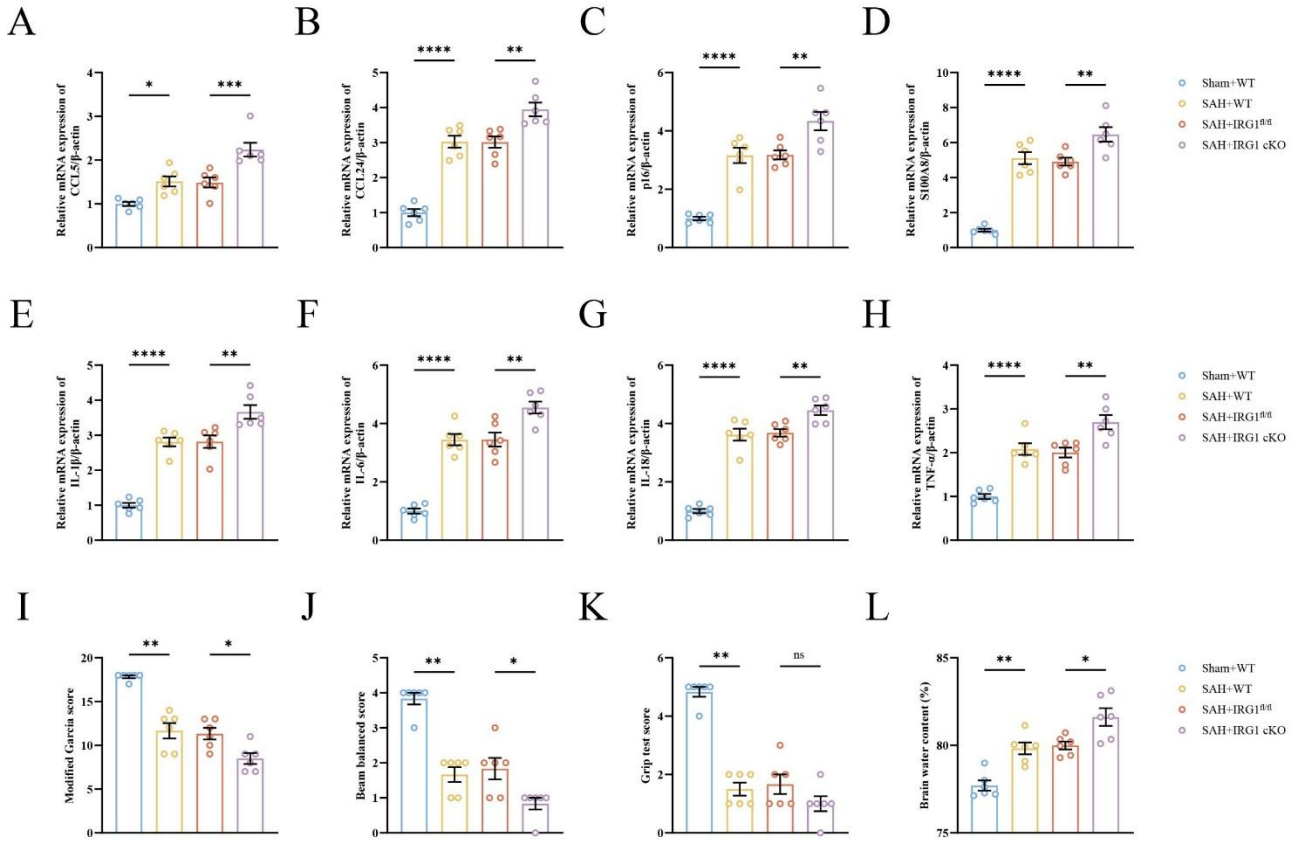
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$2^{-\Delta\Delta Ct}$  method. Image analysis was performed in a blinded manner. Data are presented as mean  $\pm$  SEM, normalized to the first group, and analyzed by One-way ANOVA (A-H, J-K, M, O).



**Supplementary Fig. 4. 4-OI rescues the exacerbation of microglial inflammatory activation and neurological deficits caused by IRG1 deficiency.** A-D. qPCR to evaluate CD86, iNOS, CD206, and IL-10 expressions in mice of SAH+IRG1<sup>fl/fl</sup>, SAH+IRG1 cKO, SAH+IRG1 cKO+Vehicle, and SAH+IRG1 cKO+4-OI groups (n=6 mice per group). E-G. Short-term neurological function at 24 h post-SAH (n=6 mice per group). Assessments include the modified Garcia score (evaluating 6 parameters: activity, symmetry, extension, climbing, proprioception, and reflex), beam balance, and grip tests. Higher scores indicate superior neurological performance. H. Measurement of brain water content (n=6 mice per group). I-L. Long-term neurological function from days 22–26 post-SAH in mice of SAH+IRG1 cKO+Vehicle and SAH+IRG1 cKO+4-OI groups (n=6 mice per group). Spatial learning was assessed via escape latency in the Morris water maze (days 22–25). Memory retention was evaluated on day 26 via a probe trial, recording swim trajectories and the percentage of time and distance spent in the target quadrant. Relative mRNA levels were calculated using the  $2^{-\Delta\Delta Ct}$  method. All behavioral assessments were performed by two investigators blinded to the experimental groups. Data are presented as mean  $\pm$  SEM, normalized to the first group, and analyzed by One-way ANOVA (A-D, H), Kruskal-Wallis H test (E-G), or Student's t-test (J-L).

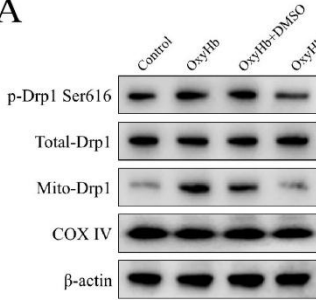
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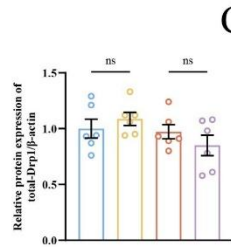
**Supplementary Fig. 5. Effects of IRG1 deficiency on microglial senescence-like transition, inflammation, and neurological function after SAH in female mice.** **A-H.** qPCR to evaluate CCL5, CCL24, p16, S100A8, IL-1β, IL-6, IL-18, and TNF-α expressions in mice of Sham+WT, SAH+WT, SAH+IRG1<sup>fl/fl</sup>, and SAH+IRG1 cKO groups (n=6 mice per group). **I-K.** Modified Garcia score, beam balance score, and grip test score to evaluate short-term neurological function (n=6 mice per group). **L.** Measurement of brain water content (n=6 mice per group). Relative mRNA levels were calculated using the 2<sup>-ΔΔCt</sup> method. All behavioral assessments were performed by two investigators blinded to the experimental groups. Data are presented as mean ± SEM, normalized to the first group, and analyzed by One-way ANOVA (**A-H, L**) or Kruskal-Wallis H test (**I-K**).

# SUPPLEMENTARY DATA

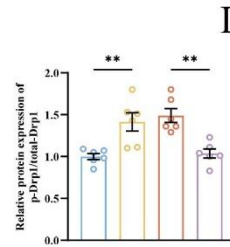
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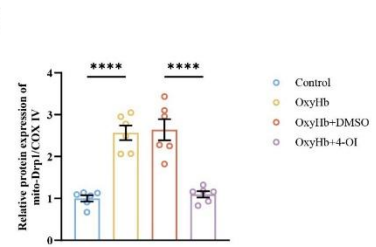
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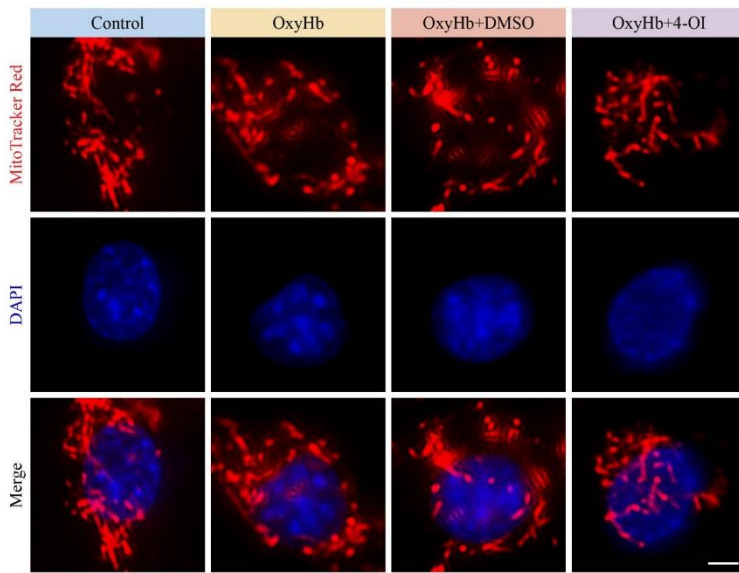
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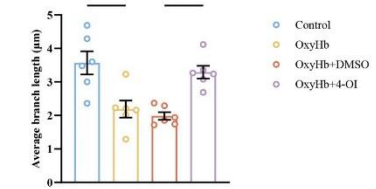
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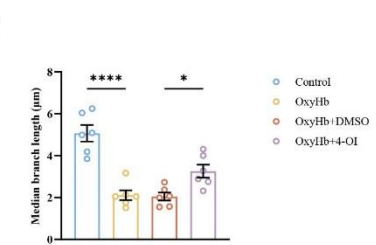
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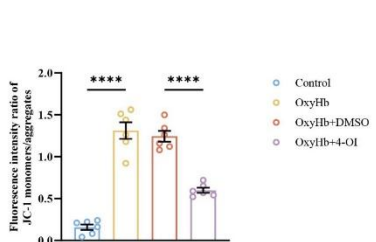
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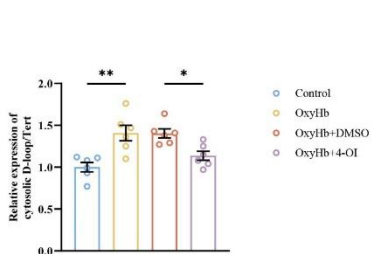
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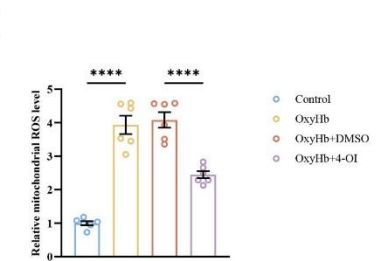
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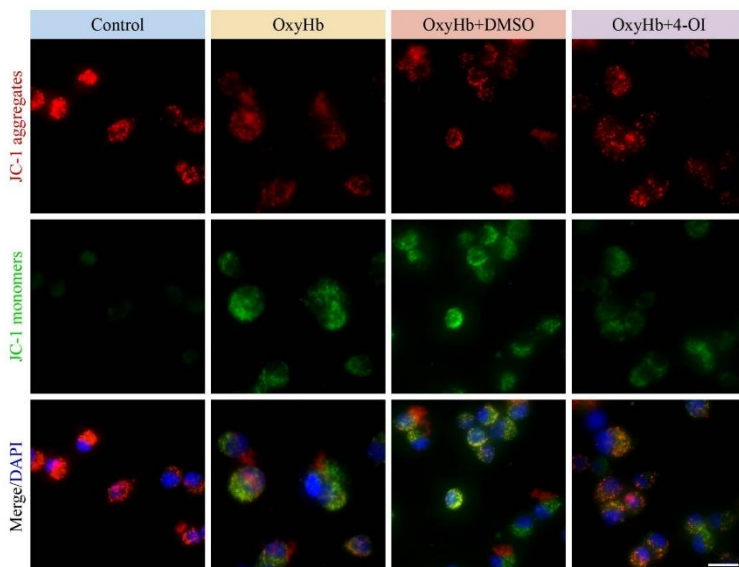
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K



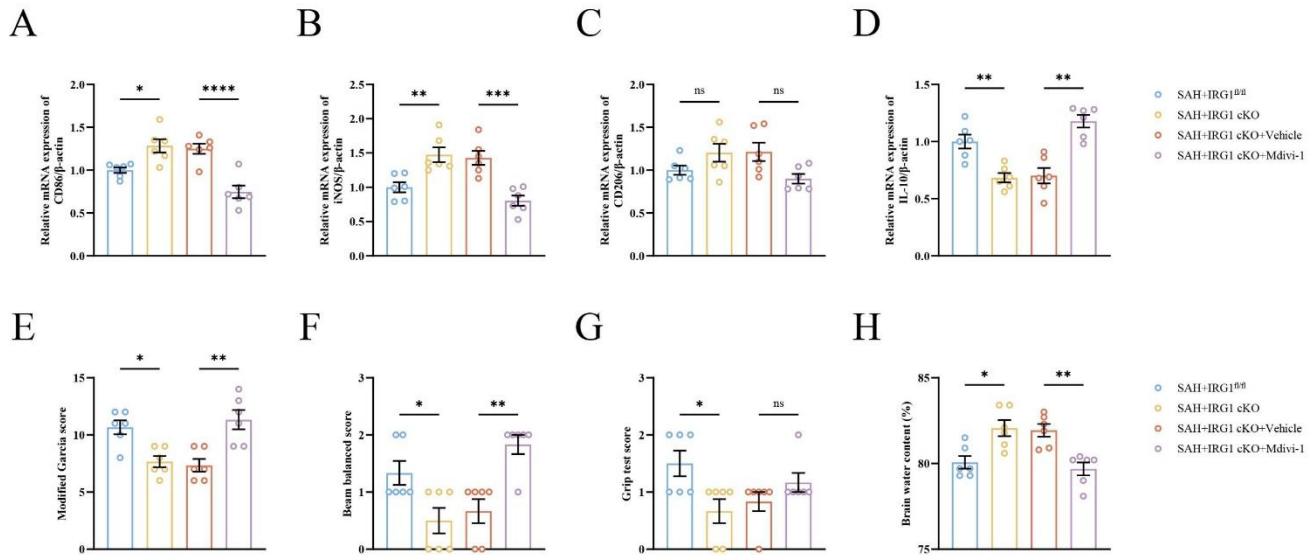
H



**Supplementary Fig. 6. 4-OI inhibits Drp1-dependent mitochondrial fission and improves mitochondrial function in OxyHb-stimulated microglia. A-D.** Western blot to evaluate the phosphorylation activation and mitochondrial translocation of Drp1 in BV2 cells treated with Control, OxyHb, OxyHb+DMSO, and OxyHb+4-OI (n=6 independent biological replicates)

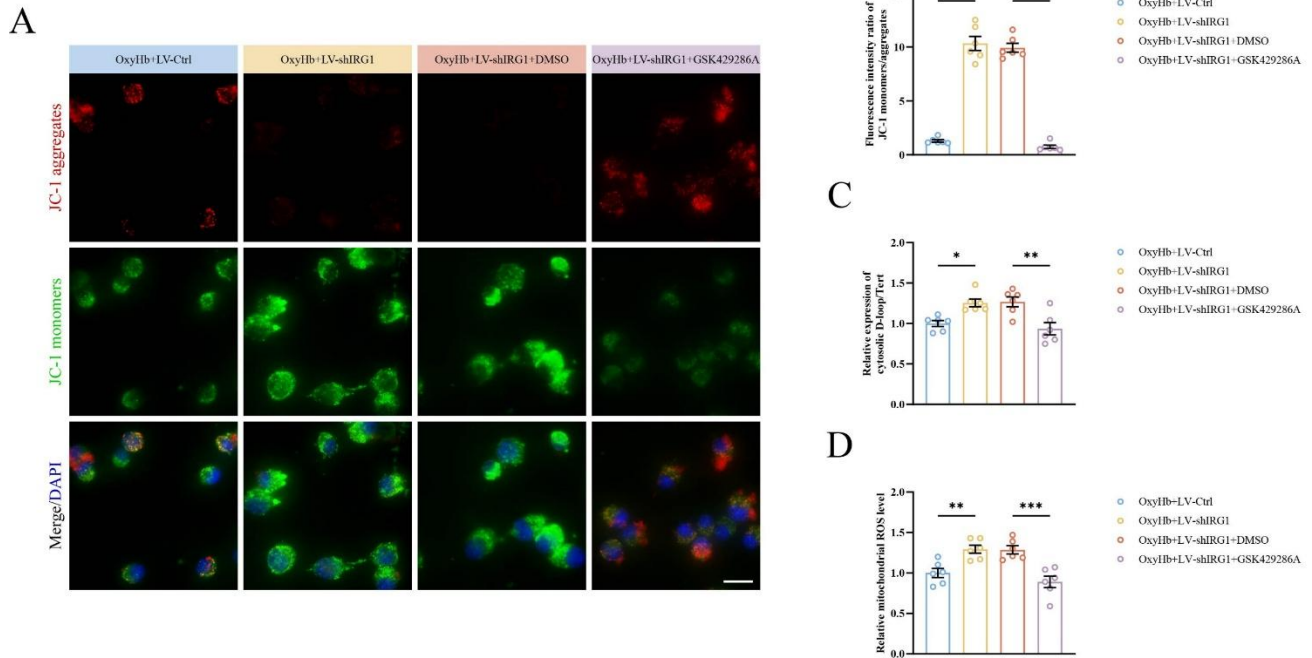
# SUPPLEMENTARY DATA

per group). **E-G**. Mito-Tracker Red CMXRos fluorescent probe to evaluate mitochondrial mean and median branch lengths (scale bar=5 $\mu$ m; n=6 independent biological replicates per group). Mitochondrial morphology was analyzed using the ImageJ mitochondrial morphology macro. **H-I**. JC-1 fluorescent probe to evaluate mitochondrial membrane potential level (scale bar=20 $\mu$ m; n=6 independent biological replicates per group). Fluorescence ratios (Green/Red) were calculated to determine membrane potential. **J**. qPCR to evaluate cytoplasmic D-loop level (n=6 independent biological replicates per group). **K**. MitoSOX Red to evaluate mitochondrial ROS level (n=6 independent biological replicates per group). Band intensities were quantified using ImageJ and normalized to the loading control. Image analysis was performed in a blinded manner. Data are presented as mean  $\pm$  SEM, normalized to the first group, and analyzed by One-way ANOVA (**B-D, F-G, I-K**).



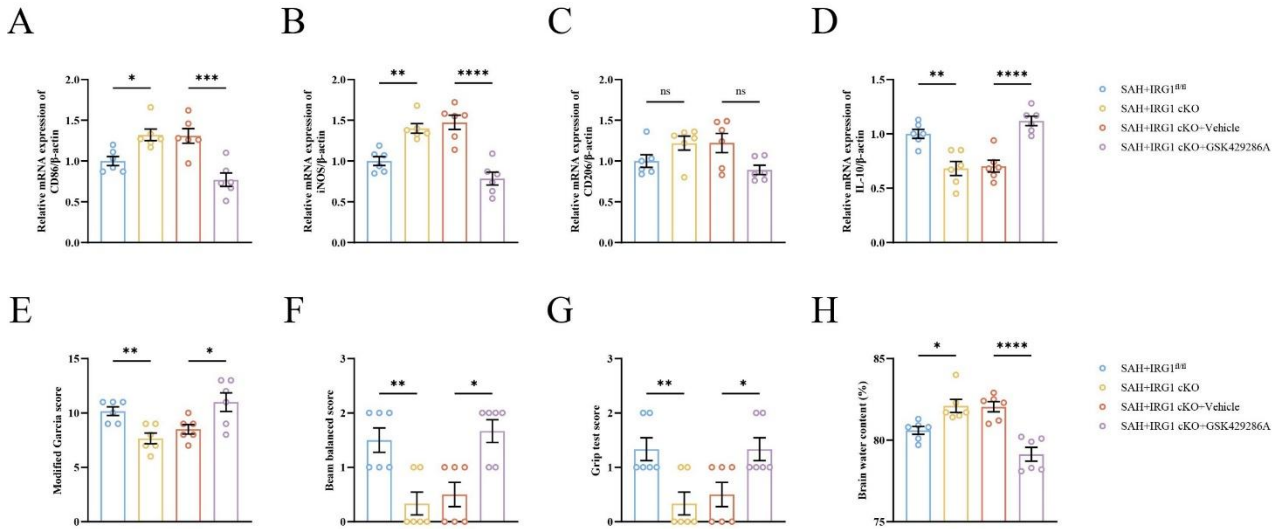
**Supplementary Fig. 7. Mdivi-1 rescues the exacerbation of microglial inflammatory activation and neurological deficits caused by IRG1 deficiency.** **A-D**. qPCR to evaluate CD86, iNOS, CD206, and IL-10 expressions in mice of SAH+IRG1<sup>fl/fl</sup>, SAH+IRG1 cKO, SAH+IRG1 cKO+Vehicle, and SAH+IRG1 cKO+Mdivi-1 groups (n=6 mice per group). **E-G**. Short-term neurological function at 24 h post-SAH (n=6 mice per group). Assessments include the modified Garcia score (evaluating 6 parameters: activity, symmetry, extension, climbing, proprioception, and reflex), beam balance, and grip tests. Higher scores indicate superior neurological performance. **H**. Measurement of brain water content (n=6 mice per group). Relative mRNA levels were calculated using the  $2^{-\Delta\Delta C_t}$  method. All behavioral assessments were performed by two investigators blinded to the experimental groups. Data are presented as mean  $\pm$  SEM, normalized to the first group, and analyzed by One-way ANOVA (**A-D, H**) or Kruskal-Wallis H test (**E-G**).

# SUPPLEMENTARY DATA



**Supplementary Fig. 8. GSK429286A rescues the exacerbated mitochondrial dysfunction caused by IRG1 deficiency.** **A-B.** JC-1 fluorescent probe to evaluate mitochondrial membrane potential level in BV2 cells treated with OxyHb+LV-Ctrl, OxyHb+LV-shIRG1, OxyHb+LV-shIRG1+DMSO, and OxyHb+LV-shIRG1+GSK429286A (scale bar=20 $\mu$ m; n=6 independent biological replicates per group). Fluorescence ratios (Green/Red) were calculated to determine membrane potential. **C.** qPCR to evaluate cytoplasmic D-loop level (n=6 independent biological replicates per group). **D.** MitoSOX Red to evaluate mitochondrial ROS level (n=6 independent biological replicates per group). Image analysis was performed in a blinded manner. Data are presented as mean  $\pm$  SEM, normalized to the first group, and analyzed by One-way ANOVA (**B-D**).

# SUPPLEMENTARY DATA



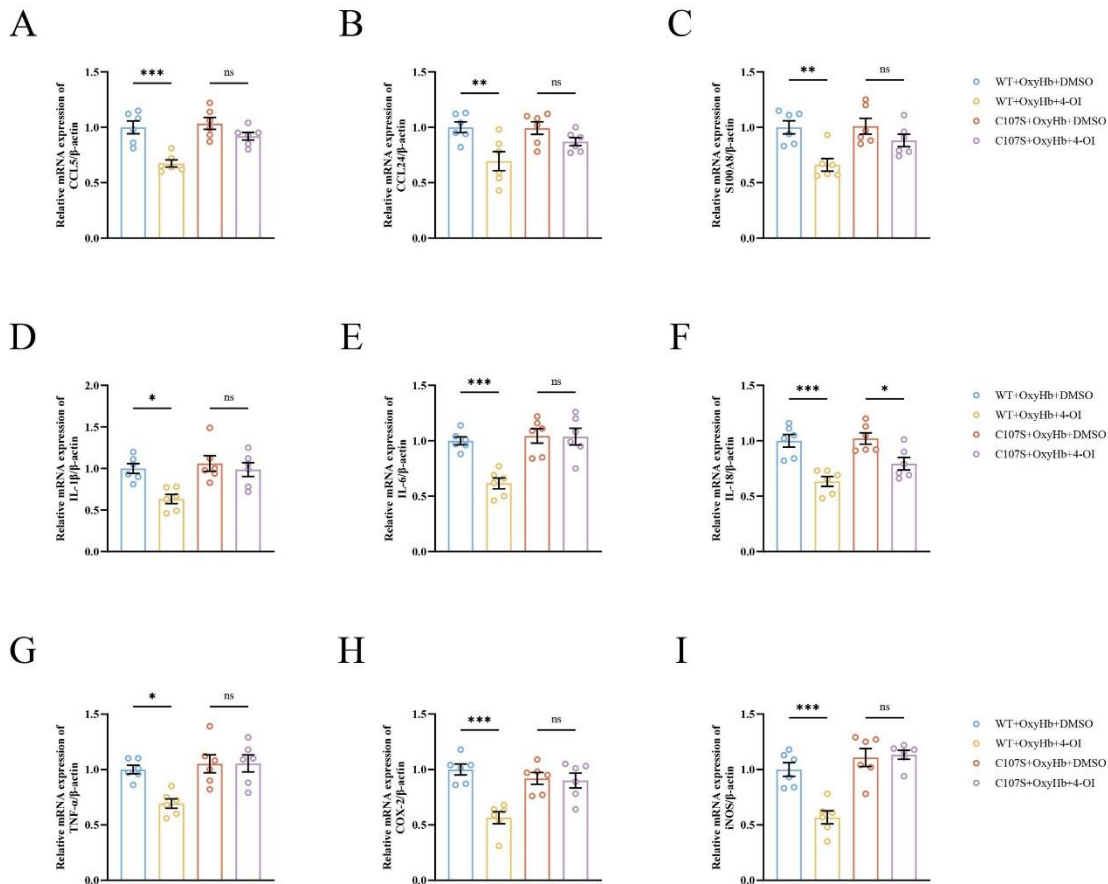
**Supplementary Fig. 9. GSK429286A rescues the exacerbation of microglial inflammatory activation and neurological deficits caused by IRG1 deficiency.** A-D. qPCR to evaluate CD86, iNOS, CD206, and IL-10 expressions in mice of SAH+IRG1<sup>fl/fl</sup>, SAH+IRG1 cKO, SAH+IRG1 cKO+Vehicle, and SAH+IRG1 cKO+GSK429286A groups (n=6 mice per group). E-G. Short-term neurological function at 24 h post-SAH (n=6 mice per group). Assessments include the modified Garcia score (evaluating 6 parameters: activity, symmetry, extension, climbing, proprioception, and reflex), beam balance, and grip tests. Higher scores indicate superior neurological performance. H. Measurement of brain water content (n=6 mice per group). Relative mRNA levels were calculated using the  $2^{-\Delta\Delta C_t}$  method. All behavioral assessments were performed by two investigators blinded to the experimental groups. Data are presented as mean  $\pm$  SEM, normalized to the first group, and analyzed by One-way ANOVA (A-D, H) or Kruskal-Wallis H test (E-G).

## A

*Homo sapiens* 80-LMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKDLRNDEHTRRE-131  
*Mus musculus* 80-LMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKDLRNDEHTRRE-131  
*Rattus norvegicus* 80-LMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKDLRNDEHTRRE-131  
*Pan troglodytes* 80-LMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKDLRNDEHTRRE-131  
*Bos taurus* 80-LMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKDLRNDEHTRRE-131  
*Canis lupus familiaris* 80-LMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKDLRNDEHTRRE-131  
*Sus scrofa* 80-LMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKDLRNDEHTRRE-131  
*Danio rerio* 80-LMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKDLRNDEHTRRE-131  
*Oryctolagus cuniculus* 80-LMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILVGNKKDLRNDEHTRRE-131  
*Caenorhabditis elegans* 80-LMCFSIDSPDSLENIPEKWTPEVRHFCPNVPIILVGNKRDLRSDPQTVRE-131

**Supplementary Fig. 10. Analysis of species conservation of the C107 site of RhoA.** A. Sequence alignment of the C107 site of RhoA among *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Pan troglodytes*, *Bos taurus*, *Canis lupus familiaris*, *Sus scrofa*, *Danio rerio*, *Oryctolagus cuniculus*, and *Caenorhabditis elegans*.

# SUPPLEMENTARY DATA



**Supplementary Fig. 11. C107S mutation of RhoA reverses the inhibitory effect of 4-OI on senescence-like transition and inflammatory response. A-I.** qPCR to evaluate CCL5, CCL24, S100A8, IL-1 $\beta$ , IL-6, IL-18, TNF- $\alpha$ , COX-2, and iNOS expressions in BV2 cells of WT+OxyHb+DMSO, WT+OxyHb+4-OI, C107S+OxyHb+DMSO, and C107S+OxyHb+4-OI groups (n=6 independent biological replicates per group). Relative mRNA levels were calculated using the  $2^{-\Delta\Delta C_t}$  method. Data are presented as mean  $\pm$  SEM, normalized to the first group, and analyzed by One-way ANOVA (A-I).

# SUPPLEMENTARY DATA

## Supplementary Tables

**Supplementary Table 1. Primer sequence for qPCR**

<b>Gene</b>	<b>5' to 3' Forward Primer Sequence</b>	<b>5' to 3' Reverse Primer Sequence</b>
CCL5	GCAGCAAGTGCTCCAATCTTGC	TCTTCTCTGGGTTGGCACACAC
CCL24	AGCCGGAGGTGTAACCTCAGATG	GCTGTGGTTGAAACCCAATGCC
p16	TCAGGTGATGATGATGGGCAACG	TCGAATCTGCACCGTAGTTGAGC
S100A8	TTCGAGGAGTTCCTTGCGATGG	TGTGGCTGTCTTTGTGAGATGCC
IRG1	AGCAACCACGCTGAATGTCTTC	GGCAACATCTGGGACTTAGAACCC
IL-1 $\beta$	GCAACTGTTCCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	ACGGCCTTCCCTACTTCACAAG	ACAGGTCTGTTGGGAGTGGTATCC
IL-18	ACAGGCCTGACATCTTCTGCAAC	TGGAAGGTTTGGGGCGGCTTTC
TNF- $\alpha$	AAGCCTGTAGCCCACGTCGTA	GGCACCAGTAGTTGGTTGTCTTTG
COX-2	ACTGCGAGCTAAGAGCTTCAGG	TGCAGTGCTGAGTTCCTTCGTG
iNOS	ATTTGCGGAGATGTGCGCATGG	TGGCCACCAGCTTCTTCAATGTG
CD86	GCCACAGATACTGGAAGAATCCAC	CAGTGCTGCCTGCTAAGTTGTG
CD206	TGAGTCCAGGAACAAGTGACACG	TCGCATGCTCATTCTGCTCGATG
IL-10	TAAGAGCAAGGCAGTGGAGCAG	CATGGCCTTGTAGACACCTTGG
$\beta$ -actin	GTCGTACCACAGGCATTGTGATGG	GCAATGCCTGGGTACATGGTGG
D-loop	AATCTACCATCCTCCGTGAAACC	TCAGTTTAGCTACCCCAAGTTTAA
TERT	CTAGCTCATGTGTCAAGACCCTCTT	GCCAGCACGTTTCTCTCGTT

## SUPPLEMENTARY DATA

**Supplementary Table 2. Antibody information for western blot and immunofluorescence**

<b>Antibody</b>	<b>Company</b>	<b>Item number</b>	<b>Application</b>	<b>Dilution</b>
IRG1 antibody	ZEN-BIOSCIENCE	163860	Western Blot	1/1000
p-Drp1 antibody	Abcam	ab314755	Western Blot	1/1000
Drp1 antibody	Bioss	bs-4100R	Western Blot	1/1000
COX IV antibody	ZEN-BIOSCIENCE	250135	Western Blot	1/1000
RhoA antibody	Bioss	bsm-54572R	Western Blot	1/1000
ROCK1 antibody	Bioss	bs-1166R	Western Blot	1/1000
p-ROCK1 antibody	Bioss	bs-4630R	Western Blot	1/1000
ROCK2 antibody	Bioss	bs-1205R	Western Blot	1/1000
$\beta$ -actin antibody	ZEN-BIOSCIENCE	250132	Western Blot	1/5000
Iba1 antibody	Abcam	ab283319	Immunofluorescence	1/100
CD86 antibody	Proteintech	83213-1-RR	Immunofluorescence	1/200
IRG1 antibody	Bioss	bs-16705R	Immunofluorescence	1/200