SUPPLEMENTAL MATERIALS

Detailed Methods

Animal Model

The experiments performed followed the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the protocol was approved by Institutional Animal Care and Use Committee (approval number: 20190115). A single mouse was considered an experimental unit. 6-month-old C57BL/6J x 129 mice harboring the H67D HFE (n = 25, male = 11, female = 14) and WT HFE (n = 25, male = 13, female = 12) were used in this study (The Jackson Laboratory, Bar Harbor, ME, USA; RRID: IMSR_JAX:023025). These mice were maintained at the Penn State College of Medicine and housed under 12-hour light cycles with free access to food and water. Mice of each genotype were used as available and not discriminated based on sex. Mice were anesthetized for the duration of the procedure via inhalation of 1-2% isoflurane prior to ICH induction. The appropriate level of anesthesia was assessed by toe-pinch before the start of the procedure after which blood was obtained from the animal's cheek vein. An autologous, two-step infusion model was followed to create a moderate-sized hematoma in the right striatum of each animal (2.0 mm lateral and 0.5 mm anterior to bregma at a depth of 3.5 mm). A total of 30 μL of blood was infused at a rate of 1μL/min. Following the infusion, the syringe remained in place for 20-minutes and was slowly removed to minimize the regurgitation of the blood out of the brain parenchyma. Bone wax was then used to seal the burr hole and the scalp was closed using three interrupted 3-0 vicryl sutures. Sham surgeries (H67D (n = 8, male = 4, female = 4) and WT (n = 8, male = 4, female = 4) followed a similar protocol without the 30 μL of blood being infused, instead the syringe was held in place for the duration of the procedure. The animals were then removed from the stereotactic frame and placed in a temperature-controlled incubator set at 37°C to recover.

Motor Coordination and Impairment

Each animal had their motor function assessed via rotarod testing. Animals underwent an initial training period three days before their scheduled ICH induction. During this period, mice were placed

on the rod for 30 seconds without rotation, after which the rod rotated at 4 revolutions per minute (RPM). Animals were considered trained once they were able to stay on the rod at 4 RPM for 60 seconds. The testing period followed a previously published ramp-up paradigm in which the rotation speed of the rod increased from 4 RPM to 20 RPM over the course of two minutes.[1] Following the ICH induction procedure, the animals were subjected to testing conditions each day until sacrifice.

Hematoxylin & Eosin Staining and Lesion Volume Analysis

A subset of animals (H67D (n =3, male = 2, female =1) and WT (n = 3 male = 2, female = 1)) were sacrificed at 3-days post ICH and perfused with lactated ringers followed by 4% paraformaldehyde to determine fundamental genotypic differences in lesion volume. Following sacrifice, the brains of each animal were rapidly harvested and stored in 4% paraformaldehyde for 24-hours and then dehydrated in 30% sucrose for 48-hours at 4ºC. Once the brains were no longer buoyant in the sucrose solution, they were frozen in optimal cutting temperature compound and sectioned into 30 µm serial, coronal sections through the entirety of the hematoma. Each serial section was then washed with phosphate-buffered saline and stained with Hematoxylin & Eosin according to the manufacturer's instructions (Abcam; ab245880). The images of each section were captured using a Nikon light microscope (Nikon H600L) and the cross-sectional area of the lesion was calculated blindly using ImageJ. Each cross-sectional area was then multiplied by the thickness of the section to obtain a lesioned volume of the section. Each lesioned volume of the section was then summed to determine the total lesion size.

Blood Iron and Hematocrit Analysis

100 µL of whole blood was drawn from the cheek vein of H67D (n = 4, male = 2, female = 2) and WT (n = 4, male = 2, female =2) mice. 50 µL from each animal was digested in nitric acid and 30% hydrogen peroxide. Whole blood iron concentrations were determined from these digested samples by ICP-MS against a standard. The remaining 50 µL were used for hematocrit levels using a Heska HT5 Veterinary Hematology Analyzer.

Fluorojade-B Staining

A subset of animals H67D (H67D (n = 7, male = 3, female = 4) and WT (n = 7, male = 4, female = 3)) was sacrificed at 3-days post ICH to determine differences in degenerated neurons by utilizing Fluorojade-B Staining (FJB). Brains fixed with paraformaldehyde as described above were frozen in optimal cutting temperature compound and sectioned into 15 µm coronal sections. Three sections within 100 µm of the needle track from each animal were taken, washed with PBS and stained with FJB according to the manufacturer's instructions (VWR 76459-462). The images of each section were captured using a Nikon Fluorescent microscope (Nikon H600L Intensilight C-HGFIE). Total perihematomal FJB-positive cells within the striatum were quantified blindly using ImageJ software.

Immunofluorescence and Analysis

Immunofluorescent staining was performed as previously described.[2] Briefly, another three sections within 100 µm of the needle track of each animal were rehydrated through graded alcohol changes and then 1xPBS and incubated with 0.5% H_2O_2 for 1h at room temperature. Again, sections were washed with 1xPBS and boiled at 80^0C in sodium citrate reagent (pH 6.0) for 30 minutes. After a few washes of 1X PBS, slides were treated with 0.5% triton/1xPBS for 30 minutes. Then slides were blocked with 0.3% triton/10% normal goat serum (NGS) for 1-hour at room temperature. Stains measuring CC1 and FTH1 were blocked with 10% NGS, 0.3% Tween, 1% BSA, 0.3M Glycine for 1-hour at room temperature. For CytC double immunostaining, tissue sections were then incubated with primary antibodies of NeuN (rabbit polyclonal, Millipore ABN78; 1:500) or GFAP (rabbit polyclonal, ab68428; 1:500) and CytC (mouse monoclonal, ab13575; 1:100) in 10% NGS/0.3% triton overnight at 4ºC. For Co-localization experiments, tissue sections were incubated with primary antibodies of NeuN (mouse monoclonal, milipore MAB377; 1:500), GFAP (mouse monoclonal, Milipore MAB360; 1:500), Iba1 (rabbit monoclonal, ab178846, 1:500), or CC1 (mouse monoclonal, sigma OP80, 1:50) and either FTH1 (rabbit monoclonal, Cell Signaling Technology, 1:100, 4393S), Nrf2 (rabbit monoclonal, Abcam, 1:1000, ab92946), or GPX4 (rabbit monoclonal, Abcam, 1:1000, ab125066). The next day, the slides were washed with 0.3% triton/1xPBS, 3x15 minutes at room temperature. Then sections were incubated with appropriate secondary antibody (Goat anti-rabbit 1:500, conjugated with Alexa-

flour -546 (red), Life Technologies A11010; and Goat anti-mouse 1:500, conjugated for Alexa flour -488 (green), Life Technologies A11029) in 10% normal goat serum and 0.3% triton for 1-hour. The slides were again rinsed with 0.3% triton/1xPBS, 3x15 minutes at rocker, then incubated with a Dapi stain (1:5000) for 10 minutes and washed with 1xPBS, 2x10 minutes, The slides were quickly dipped into water and cover slipped in poly aqua mounting media. Three images from each section were captured around perihematomal region of each section using confocal microscopy (using a Zeiss LSM 900 confocal microscope.). The number of CytC+ NeuN cells and CytC+ GFAP cells the perihematomal region were counted by a blinded researcher using NIH Image J software as described earlier and the average count of three images were used for the analysis.[3] Briefly, the double stained images were converted to a binary image and adjusted to a certain threshold to minimize the background to count the number of particles. The image size, particle size and DPI (dot/per inches) were kept constant for each image to minimize any variations in measurements.

Immunoblotting

Protein levels were detected via immunoblotting as previously described.[4] Animals were sacrificed at 3-days post ICH and perfused with lactated ringers. The brains were rapidly harvested and divided in the sagittal plane into two equal hemispheres. Each hemisphere was homogenized individually in NP40 buffer and protease inhibitor cocktail (PIC, Sigma), and total protein was quantified using a bicinchonic assay (Pierce). Then, 25 µg cellular protein was loaded onto a 4 to 20% Criterion TGX Precast Protein Gel (Bio-Rad). Proteins were transferred onto a PVDF membrane and probed for FTH1 (Cell Signaling Technology, 1:1000, 4393S), Nrf2 (Abcam, 1:1000, ab92946), GPX4 (Abcam, 1:1000, 125066), or beta-actin (Abcam, 1:1000, ab115777) which served as a loading control. PVDF membranes were then incubated with the appropriate secondary antibody conjugated to HRP (1:5000, GE Amersham), and bands were visualized using ECL reagents (PerkinElmer) on an Amersham Imager 600 (GE Amersham). Target proteins were normalized to beta-actin and expressed as a ratio.

Supplemental Figures and Figure Legends

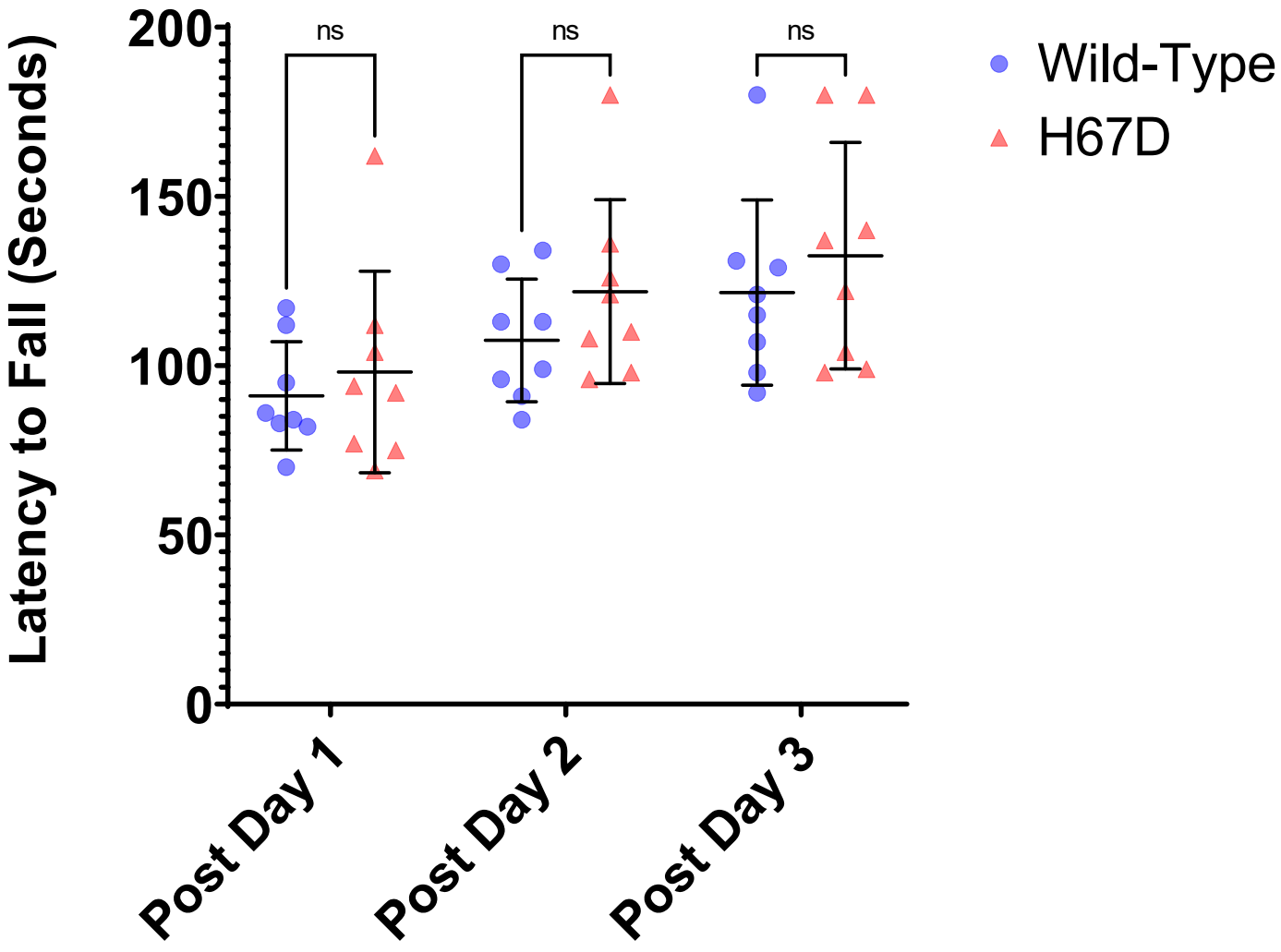


Figure S1: No Significant Difference in Rotarod Performance Between Sham H67D Mice and Sham WT Mice

Functional motor recovery was measured by latency to fall from rotarod. H67D mice (n = 8, males = 4, females = 4) and WT mice (n = 8, males = 4, females = 4) display no differences in functional recovery at day 1 post-sham (H67D: mean = 98.13, SD = 29.74; WT: Mean = 91.13, SD = 16.00; p = 0.567), day 2 post-sham (H67D: mean = 121.88, SD = 27.17; WT: Mean = 107.5, SD = 18.12; p = 0.234), or day 3 post-sham (H67D: mean = 132.5, SD = 33.43; WT: Mean = 121.63, SD = 27.37; p = 0.488).

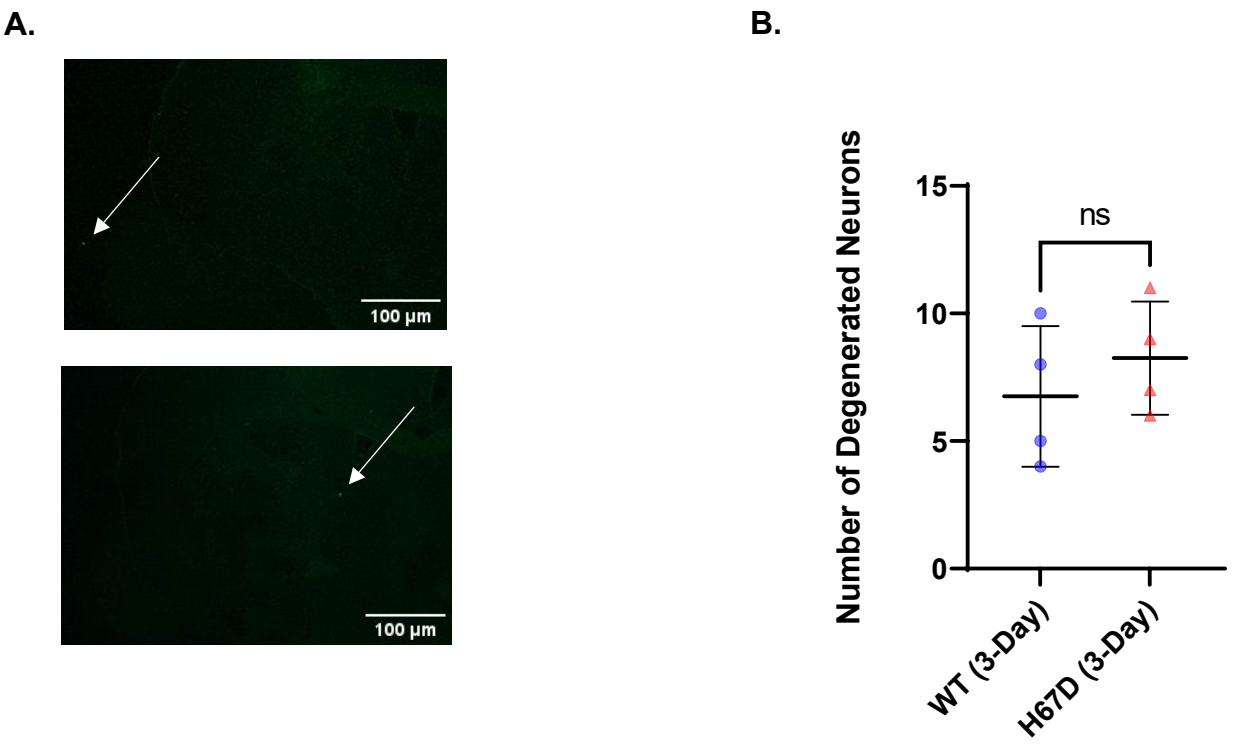


Figure S2: No Significant Difference in Number of Degenerated Neurons Between Sham H67D Mice and Sham WT Mice

Fluorojade-B staining was utilized to determine differences in the number of degenerated in the striatum of A) sham wild-type mice (top) and sham H67D mice (bottom) at 3-days following the sham procedure. B) Quantification of FJB-positive cells done using ImageJ. The sham H67D mice showed no significant differences in FJB-positive cells (n = 4, male = 2, female = 2, mean = 8.25, SD = 2.22) compared to sham wild-type mice (n = 4, male = 2, female = 2, mean = 6.75, SD = 2.75) ($p = 0.429$). Error reported as standard deviation, ns = not-significant.

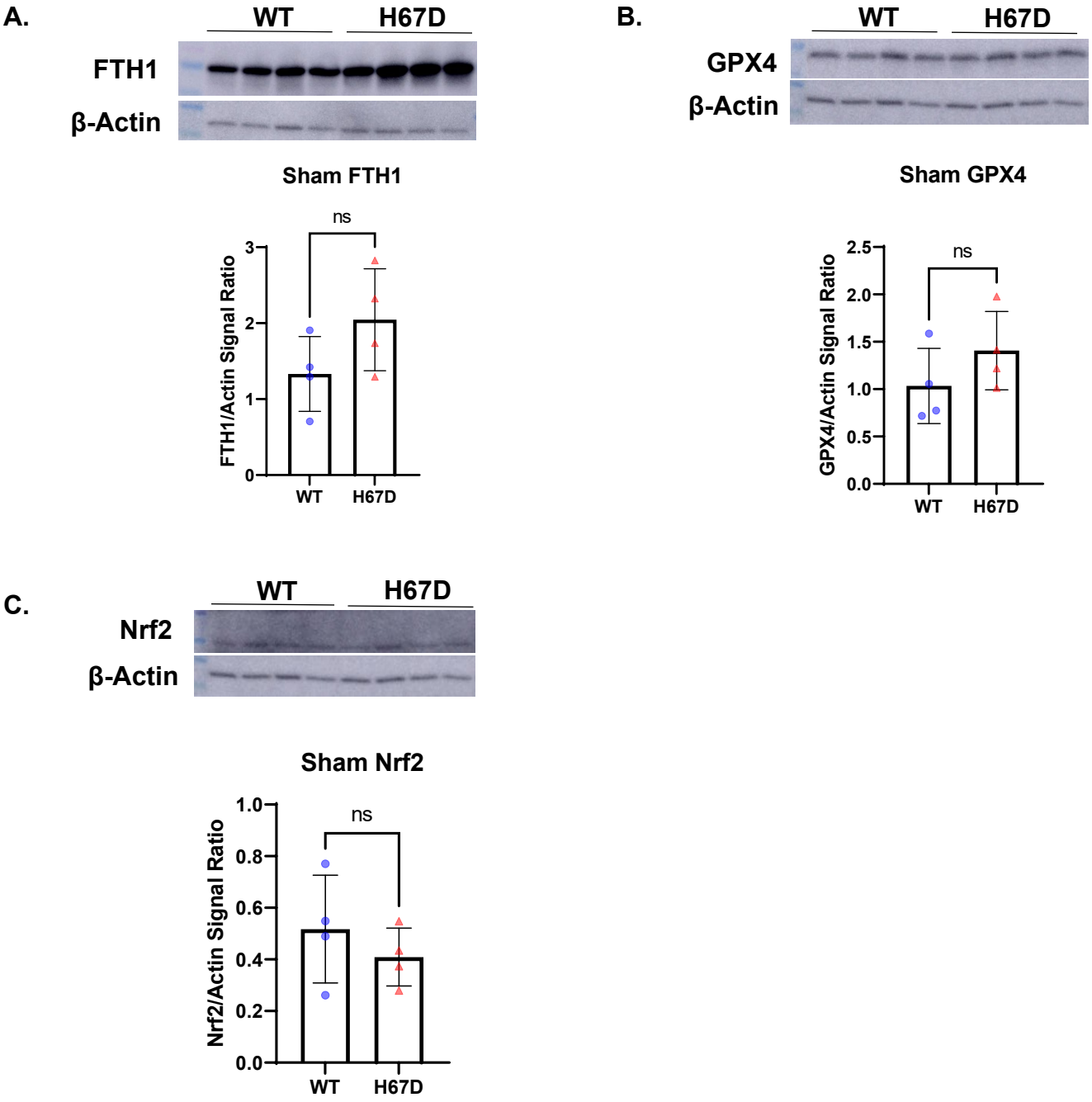
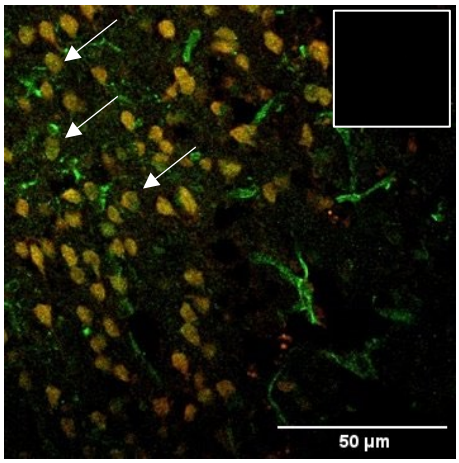


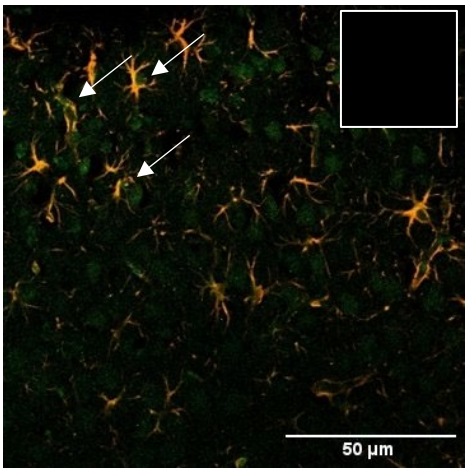
Figure S3: No Significant Difference in FTH1, Nrf2, or GPX4 Levels in The Sham-Affected Hemisphere Between H67D Mice and WT Mice

A) H-Ferritin levels in the sham-affected hemisphere of H67D mice (n = 4, males = 2, females = 2, mean = 2.045, SD = 0.671) are not significantly different to levels in the sham-affected hemisphere of WT mice (n = 4, males = 2, females = 2, mean = 1.332, SD = 0.493) (p = 0.137). B) GPX4 levels in the sham-affected hemisphere of H67D mice (n = 4, males = 2, females = 2, mean = 1.407, SD = 0.414) are not significantly different to levels in the sham-affected hemisphere of WT mice (n = 4, males = 2, females = 2, mean = 1.034, SD = 0.397) (p = 0.242). C) Nrf2 levels in the sham-affected hemisphere of H67D mice (n = 4, males = 2, females = 2, mean = 0.409, SD = 0.112) are not significantly different to levels in the sham-affected hemisphere of WT mice (n = 4, males = 2, females = 2, mean = 0.518, SD = 0.209) (p = 0.396). Error reported as standard deviation, ns = not-significant.

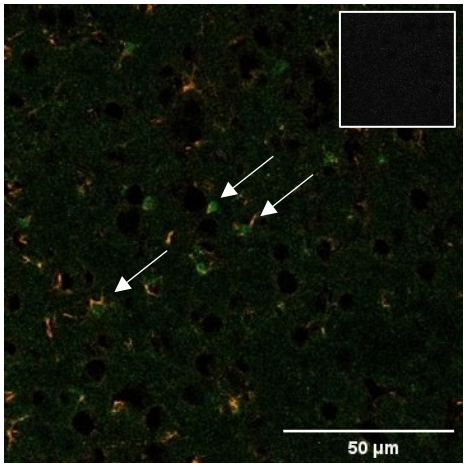
A. Nrf2/NeuN



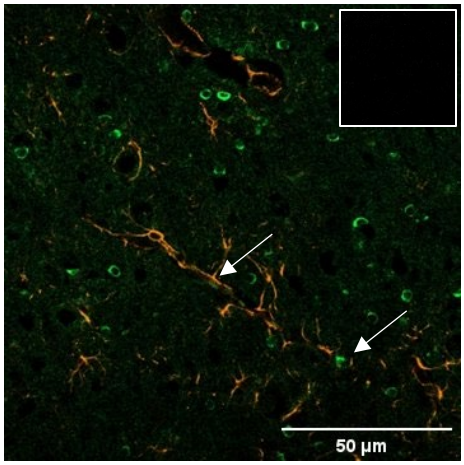
B. Nrf2/GFAP



C. GPX4/GFAP



D. FTH1/GFAP



E. FTH1/CC1

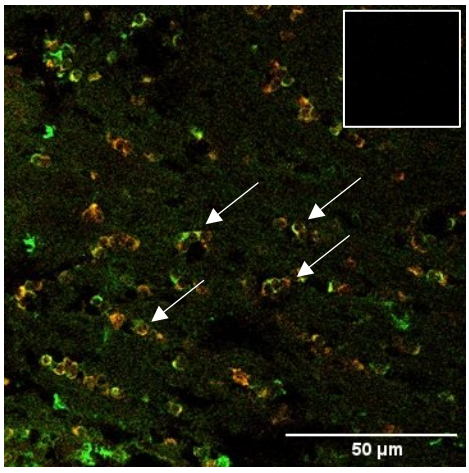


Figure S4: Immunofluorescent Co-Localization of Nrf2, GPX4, or FTH1 to Cell Type in the ICH-affected hemisphere

A) Co-Localization of Nrf2 (green) and Neurons/NeuN (red) in the ICH-affected hemisphere. B) Co-Localization of Nrf2 (green) and Astrocytes/GFAP (red) in the ICH-affected hemisphere. C) Co-Localization of GPX4 (green) and Astrocytes/GFAP (red) in the ICH-affected hemisphere. D) Co-Localization of FTH1 (green) and Astrocytes/GFAP (red) in the ICH-affected hemisphere. E) Co-Localization of FTH1 (green) and Oligodendrocytes/CC1 (red) in the ICH-affected hemisphere. Respective negative immunofluorescent staining displayed in upper right corners.