Supplementary Material

SUPPLEMENTARY METHODS

Food Consumption

Chow was weighed prior to being added to each cage. During cage change (once per week) leftover food was weighed and the difference was calculated and divided by the number of mice in the cage to determine average individual food consumption. Data were recorded weekly for the duration of experiments.

Measurement of Rapamycin (RAPA) Using HPLC-tandem MS

All analytical standards and reagents used for this analysis were HPLC grade or above. Milli-Q water was used for the preparation of all solutions. The HPLC system consisted of a Shimadzu SCL-10A Controller, LC-10AD pump with a FCV-10AL mixing chamber, SIL-10AD autosampler, and an AB Sciex API 3200 tandem mass spectrometer with turbo ion spray. The analytical column was a Grace Alltima C18 (4.6 x 150 mm, 5 μ; Alltech (Deerfield, IL)) and was maintained at 60°C during the chromatographic runs in a Shimadzu CTO-10A column oven. Mobile phase A was 10 mM ammonium formate and 0.1% formic acid dissolved in HPLC grade methanol. Mobile phase B was 10 mM ammonium formate and 0.1% formic acid dissolved in 90% HPLC grade methanol. The flow rate of the mobile phase was 0.5 ml/min. The column was equilibrated with 100% mobile phase B. A step gradient was used to elute RAPA from the analytical column. RAPA was quantified with fragments of RAPA and ASCO with the following transitions: RAPA 931.6 864.5, ASCO 809.574 756.34. RAPA was quantified in mouse liver tissue as follows: 100 mg of calibrator and unknown tissue samples were mixed by sonication with 10 μL of 0.5 μg/mL ASCO (internal standard) and 300 ul of a solution containing 0.1% formic acid and 10 mM ammonium formate dissolved in 95% HPLC grade methanol. After sonication, the samples were vortexed vigorously for 2 min and centrifuged at 15,000 g for 5 min at 23°C. Supernatants were

transferred to 1.5 ml microfilterfuge tubes and centrifuged as above. Forty μL of the final extracts were injected into the HPLC/MS/MS. The ratio of the peak area of RAPA to that of the internal standard ASCO (response ratio) for each unknown sample was compared against a linear regression of calibrator response ratios at 0, 1.78, 3.13, 6.25, 12.5, 50, and 100 μg/g to quantify RAPA. The concentration of RAPA was expressed as μg/g of tissue (parts per million).

Total RNA extraction

RNA extracts were prepared from microvessel-depleted brain samples using the RNAqueous-4PCR Kit (ThermoFisher) under RNase-free conditions. Briefly, tissue was homogenized using pestles in lysis/binding solution and then mixed with an equivalent volume of 64% ethanol. This mixture was then applied to a filter cartridge and centrifuged at 10,000 x g for 1 minute, followed by sequential washes in wash solutions. The filter was then eluted with buffer pre-heated to 80C. DNA was removed from eluted samples by incubation at $37^{o}C$ with DNase I Buffer and DNase I, followed by a final incubation with DNase inactivation reagent.

cDNA synthesis and real-time quantitative PCR

cDNA synthesis and real-time quantitative PCR were performed as described previously [23]. Briefly, total RNA (125 ng) was converted to cDNA using the Applied Biosystems High Capacity cDNA RT Kit (Foster City, CA). "No ReverseTranscriptase" (No RT) negative controls were used in q-PCR to confirm effective removal of genomic DNA from RNA samples. Real-time quantifications of diluted cDNA and No RT controls were performed in triplicate reactions containing sample, SYBR green fluorescence (SsoFast EvaGreen Supermix, BioRad) and 400 nM of each forward and reverse primers in a BioRad CFX384 Real Time System. IL-6 and GAPDH cycling conditions consisted of one cycle at 95°C for 2 min followed by 40 cycles of denaturation (95°C, 5 s), annealing and elongation (60°C, 10 s). Relative gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method. The sequence of the primers were: IL-6 (Genebank acc.#

NM_012589): forward primer, (5'->3') ATGGATGCTTCCAAACTGGAT; reverse primer (5'-> 3'), TGAATGACTCTGGCTTTGTCT. GAPDH (Genebank acc. # X02231): forward primer, (5'->3') AATGCATCCTGCACCACCAAC; reverse primer (5'-> 3'), TGATGGCATGGACTGTGGTCAT.

Immunoblotting

Samples were resolved by SDS-PAGE (Bio-Rad, 10% Mini-PROTEAN TGX) and electroblotted onto nitrocellulose membrane (GE Healthcare). Each membrane was then blocked (2% Advanced ECL blocking solution, GE Healthcare), and incubated with primary and secondary antibodies. Samples were visualized via chemiluminescence using the GE Healthcare ECL Western blotting reagent system. Exposure to GE Healthcare Hyperfilm ECL was performed to obtain band intensities within the linear range of the antibody combinations used. Films were scanned at 300 dpi, and numeric band density and background values were acquired using ImageJ software [National Institutes of Health (NIH)].

Analysis of atherosclerosis

Atherosclerosis was analyzed as previously described [25, 26]. Briefly, mice were euthanized and hearts and aortas were perfused with PBS through the left ventricle. Aortas were dissected from the proximal ascending aorta to the bifurcation of the iliac artery and fixed with 4% paraformaldehyde in PBS. Hearts were embedded in OTC and frozen on dry ice. En face analysis was conducted by removing adventitial fat, opening the aortas longitudinally, and digitally photographing the samples at a fixed magnification. Total aortic area and lesion areas were calculated using ImagePro Plus 6.0 (Media Cybernetics) and expressed as percent lesion area. Atherosclerotic lesions of the aortic root were analyzed by serially sectioning through an 800μm segment. Eight 10μm sections separated by 80μm were stained with oil red O (ORO), counterstained with hematoxylin (Vector Labs), and digitized from each mouse. Lesion area was measured using ImagePro Plus 6.0 (Media Cybernetics) and expressed as millimeters squared.

Non-invasive measurement of cardiopulmonary parameters.

Mice were temporally anesthetized (1-4% inhaled isoflurane), and left ventricle hypertrophy was measured using the VEVO 2100 digital ultrasound microimaging system (VisualSonics) as previously described [27].

Morris water maze

The Morris water maze [28] was used to test spatial memory as described [24]. Mice were pre-screened using a battery of neurobehavioral tasks to ensure no sensorimotor deficits or deficiencies in swimming/climbing ability. This process includes assessment of righting reflex, ability to swim, ability to pull onto a visible platform, and visual capability to ensure that all animals were able to see and utilize visual cues. Experimenters were blind with respect to genotype and treatment. Briefly, mice were subjected to a series of four trials/day in a white tank filled with opaque water whitened by addition of non-toxic paint at 24.0±1.0°C. The water tank was surrounded by opaque dark panels with geometric designs approximately 30 cm from the edge of the pool to serve as distal cues. Mice were trained to find a circular platform (12cm diameter, 1 cm below water surface) in a fixed location using the spatial cues around the pool. Animals were released at randomized locations for each 60-sec trial. If mice did not find the platform in 60 seconds, they were gently guided to it. After remaining on the platform for 15-20 seconds, the animals were removed and placed in a dry cage under a warm heating lamp. Eight to ten minutes later, each animal completed a second trial using a different release position. This process was repeated for 4 trials/day for 4 consecutive days. After training day four, a 45-second probe trial was administered in which the platform was removed from the pool. The number of times that each animal crossed the previous platform location was determined as a measure of platform location retention. Animals were monitored daily and their weights were recorded weekly for the duration of experiments. Performance in all tasks was recorded by a

computer-based video tracking system (Water2020, HVS Image, Buckingham, UK). Animals that spent more than 70% of trial time in thigmotaxic swim or floating were removed from the study. Data were analyzed offline by using HVS Image and processed with Microsoft Excel before statistical analyses.

Spatial Novelty

The spatial novelty task was performed in an opaque white chamber measuring 11x11x12 in (W x D x H). This task is comprised of three phases: habituation, training, and testing. *Habituation:* mice were allowed to freely explore the empty chamber for one 5-minute session and were then returned to their home cages. *Training*: Spatial cues were placed on opposing walls of the chamber to provide orienting landmarks. Three visually distinct but comparably sized objects (approx. 1.5 cubic in) were placed in the NW, NE, and SE corners approximately 2.5in from the closest chamber walls. 24 hours after habituation, mice were returned to the chamber for one five-minute session during which exploration of each object, defined as the animal orienting toward an object and within 1cm, was recorded using stopwatches by an observer watching via webcam. Grooming behavior near or climbing on top of an object was excluded. Exploration of each object should be comparable as subjects should not display a preference for any particular object. At the conclusion of the session, mice were returned to their home cages. *Testing*: 3 hours after training, mice were returned to the chamber where one of the objects was moved to a new location (e.g. the opposite corner from its locaton during training), creating a spatially 'novel' environment. Object exploration was recorded by a human observer during a single five-minute session.

SUPPLEMENTARY FIGURES
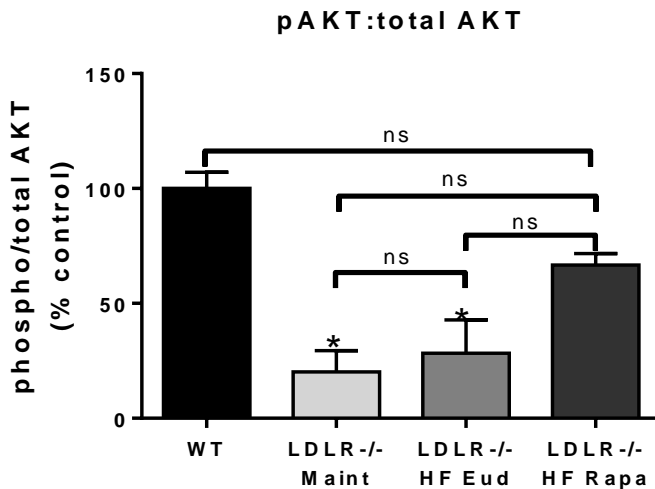
Supplementary Figure 1. mTORC2 activity in brain vasculature depends on LDLR genotype but is unaffected by chronic rapamycin treatment in LDLR$^{-/-}$ mice. Phosphorylation of Akt at Ser473 was significantly reduced in maintenance diet- and Eudragit-fed LDLR$^{-/-}$ mice on a HFD as compared to WT mice (One-way ANOVA, p=0.0124 and p=0.0133, respectively. Tukey's test applied to a significant effect of group, p=0.008). No differences in phosphorylation of Akt at Ser473 were observed between LDLR$^{-/-}$ mice fed control- or rapamycin-supplemented HFD, nor in the post-hoc comparisons between other LDLR$^{-/-}$ groups (n=3/group). Data are means ± SEM.
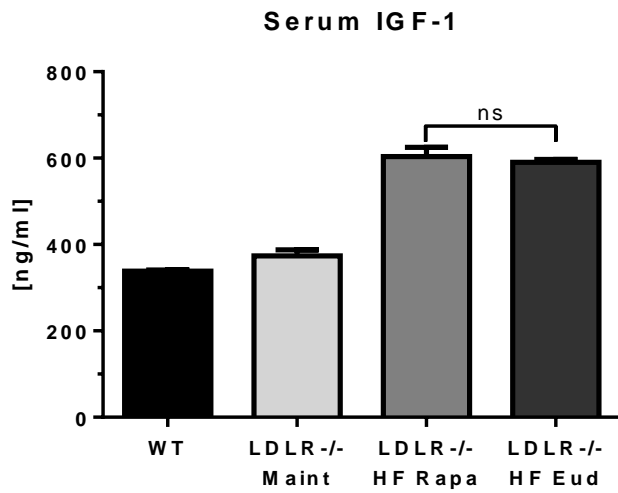
Serum IGF-1

Supplementary Figure 2. Chronic mTOR attenuation does not impact serum IGF-1 levels in LDLR-/- mice fed HFD. Serum levels of IGF-1 are significantly increased by HFD feeding, but chronic mTOR attenuation with rapamycin has no effect on IGF-1 levels. (One-way ANOVA, Tukey's multiple comparison test between LDLR^{-/-} HF Rapa and LDLR^{-/-} HF Eud p=0.900). n=4/group.