Supplementary Materials for

C9orf72 is required for proper macrophage and microglial function in mice

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This PDF file includes:

Other Supplementary Materials for this manuscript includes the following:

Excel files GSEA_17m_C9orf72_null
GSEA_human_C9ALS_sALS

Materials and Methods

Mouse production and Genotyping:

KOMP 3110043O21Rik mice: Hemizygous *311004O21Rik* knock-in mice were purchased from the UC Davis KOMP Repository (www.komp.org). Mice were generated using the 3110043O21Rik$^{tm1(KOMP)Mbp}$ targeting vector designed in the National Institutes of Health Knockout Mouse project (**https://www.komp.org/alleles.php#deletion-csd**). All experimental protocols and procedures were approved by the Animal Committee at Cedars-Sinai Medical Center. Genotyping primers for KOMP mice: Endogenous Forward Primer A: 5'- TCCCCGCTCCTCTTAAGCAC-3', endogenous reverse primer B: 5'-AAGCAAAGGTAGCCGCCAACA-3', and KO reverse primer C: 5'-TGATATCGTGGTATCGTTATGCG-3'. Primer A and primer B amplify the endogenous mouse sequence and give a PCR product of 481 bp. Primer A and primer C amplify the KO cassette insertion site and give a PCR product of 267 bp.

The 3110043O21Rik mouse strain used for this research project was created from ES cell clone (DEPD00552_3_B01) generated by the Mouse Biology Program (www.mousebiology.org), and made into mice by the KOMP Repository (WWW.KOMP.org) at the University of California Davis (U42RR024244). Targeting vectors used were, generated by the Wellcome Trust Sanger Institute and Children's Hospital Oakland Research Institute as part of the Knockout Mouse Project (3U01HG004080).

Zinc finger allele mice: Zinc Finger Nuclease (ZFN)-mediated deletion in *C9orf72*. Two CompoZr^{TM} Custom Zinc Finger Nucleases (Sigma-Aldrich, CSTZFN-1KT) were designed to target the sequence TGTTGC located immediately downstream of the exon 2 ATG (GRCm38:4:35,218,827). Both ZFNs were micro-injected in fertilized C57BL6/J oocytes and the live born were screened for deletions by loss-of-allele (LOA) assays designed with probes covering the respective cutting sites. Founders positive in the LOA screens were mated with wild-type C57BL6/J and the progeny was sequenced at the locus targeted by the ZFN to map the deletions. The deletion for the exon 2 F12 line encompassed 73 bp in total, 38 bp upstream of the start codon and 32 bp downstream. This mapped to mm10/GRCm38:4:35,218,896-35,218,822.

RNA isolation and real-time qRT-PCR and Western blot analysis:

RNA isolation using TRIzol® and a tissue grinder according to the protocol (Life Technologies). RNA was reverse-transcribed to cDNA with oligo(dT) with the Promega Reverse Transcriptase System and analyzed using SYBR Green Master Mix (Applied Biosystems). C9orf72 (Forward- 5'-GCAA GCGTTCGGATAATGTGAGACC-3' and Reverse- 5'AGCCGCCAACAAGGGTGATTCA-3'), TREM2 (Forward: 5'-GACCTCTCCACCAGTTTCTCC-3' and Reverse: 5'-TACATGACACCCTCAAGGACTG-3'), IL-6 (Forward: 5'-GCCAGAGTCCTTCAGAGAGATACA-3' and 5'-CTTGGTCCTTAGCCACTCCTTC-3'), IL-10 (Forward- 5'-AAGGACCAGCTGGACAACAT-3' and 5'-TCATTTCCGATAAGGCTTGG-3'), IL-1 beta (Forward- 5'-TGTAATGAAAGACGGCACACC-3' and 5'-TCTTCTTTGGGTATTGCTTGG-3'), mouse C9, normalized expression was normalized to mouse beta-actin (Forward: 5'-AGGTATCCTGACCCTGAAG-3' and Reverse: 5'-GCTCATTGTAGAAGGTGTGG-

3') or 18S (Forward: 5'-GATGGTAGTCGCCGTGCC -3' and Reverse: 5'-GCCTGCTGCCTTCCTTGG -3'). For F12 mice: IDT PrimeTime Mm.PT.56a.28998869 FAM Taqman assay, spanning the exon 8-9 junction. GAPDH (Life Tech TaqMan Mm03302249_g1 primer limited VIC) was used as reference gene. 10 ng of spinal cord cDNA was used per reaction, multiplexed, with 3 technical replicates. For Western blot analysis, tissue was lysed RIPA buffer and quantitated using BCA assay (Pierce). C9orf72 was detected with anti-C9orf72 (ProteinTech), p62, and mouse beta-actin (Sigma) were detected using fluorescent secondary antibodies and the Odyssey imager from LI-COR. For LC3, protein lysates were transferred to PVDF membranes and detected with chemiluminescence via the BioRad imager.

RNA Seq Analysis and Pathway analysis:

RNA was purified and sequencing libraries were generated for Iontorrent (spinal cord) sequencing per manufacturer's instructions. The resulting reads were aligned to mouse genome build mm10 using TOPHAT. BAM files were imported to Partek software to generate RPKM values and for differential expression analysis. Gene Set Enrichment Analysis (GSEA) was performed following recommendations detailed on the GSEA website (Mootha et al., 2003; Subramanian et al., 2005) with 1000 permutations of the gene sets and a false discovery rate p-value of less than 0.05 was accepted as significant. DAVID (Database for Annotation, Visualization and Integrated Discovery) pathway analysis was performed using gene lists imported into the online functional annotation tool (http://david.niaid.nih.gov).

For the human GSEA, the raw count table was obtained from (Prudencio et al, 2015) series GSE67196 for the control, sporadic, and *C9orf72* patient frontal cortex and cerebellum samples. RPKM values were derived for these samples using the edgR package in R Bioconductor. Total raw counts in each sample were used as the library size, and the transcript lengths for each HGNC identifier were obtained from Ensembl BioMart. Calculating the median RPKM values for each gene and discarding all genes with a median value below 1 filtered for 11912 genes used in GSEA. The signal-to-noise metric (difference in mean expression divided by the sum of standard deviations, $(\mu_A - \mu_B)/(\sigma_A + \sigma_B)$) was calculated for each gene when comparing frontal cortices or cerebella between sporadic or *C9orf72* ALS cases to control groups. These gene values were ranked from highest to lowest, indicating which genes are most up-regulated and down-regulated with the least variation. GSEA was performed on these ranked gene lists similarly to the mouse data set, with 1000 permutations of the gene sets and a false discovery rate P-value of less than 0.05 was accepted as significant. For sporadic ALS compared to control samples, 17 pathways were significantly up-regulated in cerebellum and 183 pathways were significantly up-regulated in frontal cortex. The union of these two sets resulted in a set of 191 pathways significantly up-regulated in sporadic ALS samples. For *C9orf72* ALS compared to control samples, 148 pathways were significantly up-regulated in cerebellum, and 48 pathways were significantly up-regulated in frontal cortex. The union of these two sets resulted in a set of 168 pathways significantly up-regulated in *C9orf72* ALS samples. These 191 and 168 enriched pathways were intersected against the significantly enriched pathways in the mouse GSEA to find overlapping pathways. Intersect analysis of enriched pathways was performed using the Vennerables package in R.

Mouse electrophysiology:

Compound muscular action potentials (CMAPs) were recorded using subdermal scalp electrodes and a VikingQuest EMG machine. The recording and reference electrodes were placed between the 2nd and 3rd, and 3rd and 4th digits. A ground electrode was placed at the base of the tail. The distal stimulation site was at the ankle, and the proximal stimulation site at the sciatic notch. CMAP amplitudes were calculated from baseline to peak from the distal stimulation site. For electromyography, reference electrode was placed subdermally under the skin of the foot pad, ground at the base of the tail, and recording electrode into the gastrocnemius or tibialis anterior muscle on each side (four recorded muscles per mouse). Recordings of insertional and spontaneous activity were performed by a blinded observer on 17 month old mice.

Mouse behavioral analysis:

Rotarod: The rotarod was used to measure motor function/coordination. Mice were placed on an accelerating rod elevated 18 inches above a soft surface. For each rotarod session, the mice were first habituated to the rotating rod for 60 seconds at a low constant speed of 3 rpm then immediately tested in a 210-second trial. For the first 30 seconds, the rod was set at a constant speed of 3 rpm. Then from 30 to 210 seconds, the rod gradually accelerated from 3 rpm to 30 rpm over a 3-minute period. The trial was repeated 3 times with a 30-minute inter-trial period where mice were placed back in their homecage. The latency for the mice to fall off the rod was averaged across the 3 trials. *Grip strength*: This test measures grip force ability of the forelimbs and hindlimbs. For measuring tensile force, the mouse is held by the base of the tail and allowed to grip a metal grid either with its forepaws or with its four paws. The mouse was then gently pulled horizontally to exert the tensile force. The peak force (in g) at the point the mouse released its grip was recorded through a digital force gauge attached to the metal grid. Three successive measurements were averaged for both the forelimbs and the 4-paws grip strengths. *Open field*: Spontaneous motor activity of the mice was measured in the open field test. Mice were individually placed into a clear Plexiglas chamber (16" L x 16" W x 15" H) surrounded by two bands of photobeams and optical sensors that measure horizontal locomotor and vertical rearing activity. Animals moved freely within the enclosure for 60 minutes. Movement was detected as breaks within the photobeam matrices and automatically recorded using PAS software (www.sandiegoinstruments.com).

Immunohistochemistry:

Spinal cords, brains and spleens were fixed in 4% paraformaldehyde (PFA; pH 7.4) for 5 days, embedded in paraffin and 4 µm thick serial coronal sections were cut. Sets of sections were deparaffinized using xylenes and then hydrated with decreasing concentrations of ethanol followed by either immunohistochemical processing or Hematoxylin and Eosin (Cat #HHS16 and HT110332, Sigma-Aldrich, St. Louis, MO, USA) staining. For immunohistochemical processing of tissue sections, antigen retrieval was performed by submerging the sections in citrate buffer (pH 6.0) and microwave at 800 W for 10 min. The M.O.M. immunodetection kit (Cat #BMK-2202, Vector Lab, Inc, Burlingame, CA, USA) was used when primary antibody raised in mouse (anti-ubiquitin,

Cat #MAB1510, Millipore; anti-NeuN, Cat #MAB377, Millipore) was employed. The sections were treated with 0.3% hydrogen peroxide (H_2O_2)/phosphate buffered saline (PBS) to inactivate endogenous peroxidase and blocked with working M.O.M. mouse Ig blocking solution, followed by incubation with the primary antibody for overnight at 4°C in a humid chamber. While using other primary antibodies (anti-glial fibrillary acidic protein, Cat #Z0334, Dako; anti-Iba1, Cat #019-19741; anti-CD11b, Cat #ab75476, Abcam; anti-p62, Cat #BML-PW9860-0100, Enzo Life Sciences, or Cat #ab56416, Abcam; anti-N-terminal TDP-43, Cat #10782-2-AP, ProteinTech; anti-D20, Cat #14-201, e Bioscience; anti-CD3, Cat #ab5690, Abcam ; anti-F4/80, Cat #ab6640, Abcam; and anti LAMP1, Cat #sc-17768 or sc-19992, Santa Cruz Biotech, the sections were treated with 1% H_2O_2/Methanol to inactivate endogenous peroxidase and blocked with 10% horse serum followed by incubation with the primary antibodies diluted in Tris-buffered saline (TBS, pH 7.4) containing 1% horse serum and 0.5% Triton X-100. The sections were then incubated for 1 h with either M.O.M. biotinylated anti-mouse IgG or biotin-conjugated anti-rabbit goat IgG (1:800; Cat #E0432, Dako, Carpinteria, CA, USA), followed by 30 min additional incubation with horseradish peroxide avidin-biotin complex (Vectastain Elite ABC Kit, Cat #PK-6100, Vector Lab, Inc., Burlingame, CA, USA). 3,3′-diaminobenzidine (0.6% solution in TBS) was used as the chromogen and counterstaining was performed with hematoxylin. Finally, the sections were dehydrated through graded ethanol solutions, and cover slipped using DPX mountant for histology (Cat #06522, Sigma-Aldrich, St. Louis, MO, USA). Additional set of sections were processed with the relevant fluorescent secondary antibodies (Molecular Probes, Carlsbad, CA, USA 1:1000)- goat anti-rabbit Alexa Fluor 594 (Cat #A11037), goat anti-mouse Alexa Fluor 488 (Cat #A11029), goat anti-mouse Alexa Fluor 594 (Cat #A11032) or goat anti-rat Alexa Fluor 488 (Cat #A11106). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Cat #D1306, Molecular Probes/Invitrogen, Carlsbad, CA, USA) and mounted with prolong gold antifade mountant (Cat #P36930, Molecular Probes/Invitrogen, Carlsbad, CA, USA). Positive and negative controls were run along each batch of the sections processed.

Peripheral axon counts and neuromuscular junction staining:

For peripheral axon counts, the sensory and motor branch of the femoral nerve were dissected above the medial quadriceps femoris and fixed in 2% glutaraldehyde, 2% paraformaldehyde, in PBS buffer. Nerves were embedded in plastic and 0.5-µm sections were cut and stained with toluidine blue. Axon counts and axon area measurements were quantified as previously described in detail previously (Bogdanik et al., 2013). Briefly, with the ImageJ software, the Threshold function was used to highlight axoplasm only on whole nerve sections; the Analyze Particle function was then used to count the number of myelinated axons and their cross-sectional areas in each nerve.

Flow cytometry and FACS sorting:

Antibodies used for flow cytometric analysis were: CD19 (Clone ID3), CD8 (Clone 53-6.7), CD11b (Clone H1/70), Ly6C (Clone A1-21) and Ly6G (Clone1A8) from BD Biosciences; CD3 (Clone 145-2C11), CD4 (Clone GK1.5), F4/80 (Clone BM8), CD11c and (Clone N418) from BioLegend. Bone marrow and spleen single-cell suspensions were removed of red blood cells (RBC lysis buffer, Sigma). Nonspecific

antibody binding was prevented by prior incubation with Fc block (anti-CD16/anti-CD32). An LSRFortessa (BD Biosciences) was used for flow cytometry and data were analyzed with FlowJo. B cells ($CD19^+$), T cells ($CD3^+$) and $CD11b^+$ cells were purified from splenocytes by fluorescence-activated cell sorting (FACS) using a FACSAria III cell sorter (BD Biosciences).

Phagocytosis, reactive oxygen species (ROS), and Cytokine assays:

Bone marrow cells collected from the femurs of mice were cultured in RPMI medium with hM-CSF for 6 days to derive macrophages (BMDM). For the phagocytosis assay, BMDM were treated with FITC-labeled zymosan particles for 15 minutes and then analyzed by flow cytometry. For ROS analysis, BMDM were primed with 20 U/ml IFN gamma overnight and then incubated with luminol and depleted zymosan and analyzed for 60 minutes. Cytokine production: Bone marrow derived macrophages were plated at 50,000 cells per well in 96-well plate and stimulated with LPS (100 ng/ml), Pam3CSK4 (1μg/ml), peptidoglycan (PGN, 20 μg/ml), or CpG (1 μg/ml) for 18 hrs. Supernatants were assayed for TNF-α by ELISA (Biolegend). For IL-1β, cells were plated at 100,000 cells per well and primed with LPS for 4h, followed by stimulation with 125 μg/ml Silica for 6 hours.

Microglia and Neuron purification:

The meninges were removed from the C9 wild-type and null mouse brains and dissociated using the Neural dissociation kit (Miltenyi Biotech) and GentleMACS dissociator (NTDK Brain setting). The lysate was collected and passed through a 70 μm strainer to obtain a single cell suspension. Next, myelin was removed from the cells by incubating with magnetic myelin beads (Myelin Removal Beads II, human, mouse, rat, Miltenyi Biotech) and removed using the AutoMACs. Cells were then incubated with CD11b+ magnetic beads (CD11b (Microglia) MicroBeads, human and mouse, Miltenyi Biotech) and sorted using the AutoMACs. The CD11b+ cells were counted and plated in Microglia complete media containing DMEM/F-12, GlutaMAX™ with HEPES (Invitrogen), 10% fetal calf serum, 100μg/ml Penicillin/ Streptomycin/0.25 μg/ml fungizone, with 10 ng/ml of the following growth factors: Recombinant mouse M-CSF, Recombinant mouse GM-CSF (R&D systems) and TGF-β1 (Miltenyi Biotech) for 6 days before analysis. For isolation of neurons, brains were isolated from wild-type mice, their meninges removed and dissociated using Adult brain dissociation kit and GentleMACS dissociator with heaters (Miltenyi Biotech). Neurons were isolated using Neuron isolation kit protocol (Miltenyi Biotec). After sorting, cells were pelleted and stored at -80°C until further analysis.

Bone Marrow derived macrophages and microglia immunocytochemistry:

BMDM and microglia were incubated with LysoTracker Red for 90 minutes and then imaged. Next, cells were fixed with 4% PFA for ~20 minutes and incubated with Lamp1 (Santa Cruz Biotechnologies, Cat # sc-19992), Rab5a (Novus Biological, Cat # NF120-13253), Rab7 (GeneTex, Cat # GTX 16196, and GFP (Clontech, Cat # 632381) antibodies. Cell counts were performed blinded on at least 5 random images per animal and the percent of cells with LysoTracker accumulation was recorded.

C9orf72 Lentivirus:

C9orf72-TV1 FCIV 9 (hC9-iso1) and C9orf72-TV2 FCIV (hC9-iso2) were generated from C9orf72 TV1 or TV2 (Sareen et al, *Sci Trans Med*, 2013). The C9orf72 TV1 or TV2 constructs were cut with BglII and BamHI, cloned into the BamHI site of a Lentiviral backbone containing a Ubiquitin promoter, a multiclonal site, and an IRES-Venus-Fluorescent-Protein, and PCR screened for directionality. Lentiviruses were produced according to standard methods. Briefly, HEK 293FT cells were plated onto 10 cm dishes and transfected using Lipofectamine 2000 reagent (Life Technologies) with a packaging vector (delta 8.9), envelope vector (vesicular stomatitis virus-glycoprotein), and transfer vector encoding the gene to be expressed. Medium was changed one hour after transfection and collected and pooled at 24 h, 48 h and 72 h. Pooled Lentiviral stocks were concentrated using Lenti-X Concentrator (Clontech) according to the supplied protocol.

Primary cortical neuron cultures:

Primary cortical neurons cultures were prepared from P1 pups and cultured in B27-Neurobasal medium for 7 days. On day 7 in vitro, primary cortical neurons were incubated with LysoTracker Red for 90 minutes, imaged, and then fixed and stained with Lamp1 as described above.

Human tissues:

Autopsy tissues from *C9orf72* expansion carriers and sporadic ALS patients were collected under an IRB approved protocol at Washington University in St. Louis, MO.

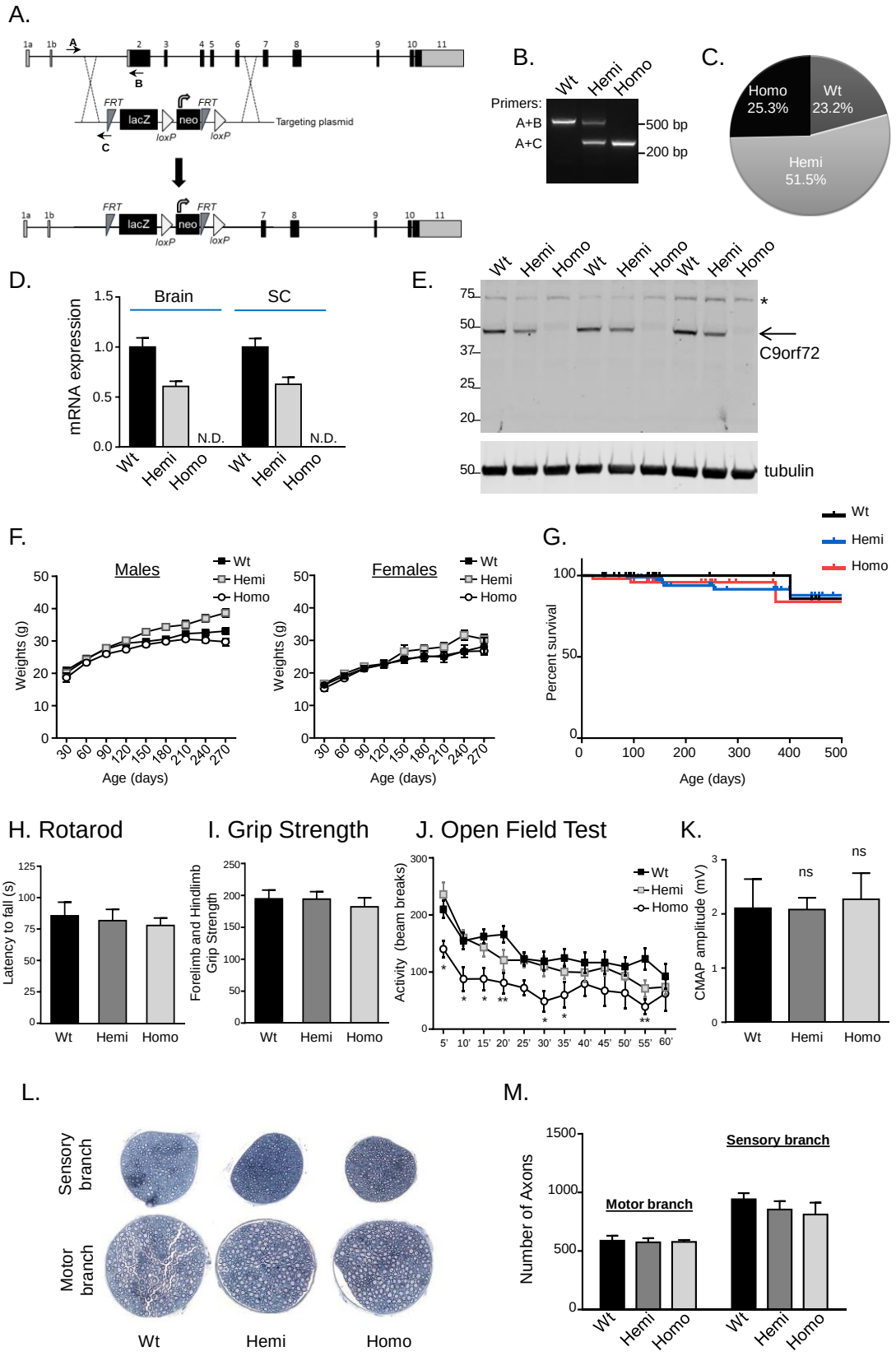
Statistical Analyses:

Statistical tests used are indicated in figure legends. For one-way ANOVAs the posthoc test Dunnett was used and for two-way ANOVAs the posthoc test Tukey was used. All data are shown as mean ± SEM, and all analyses were conducted using Prism software (Graphpad).

Author Contributions

J.G.O. coordinated the project, was involved in all experiments, as well as data collection and statistical analysis. L.B. generated and characterized the F12 C9 null mice, performed axon counts, and assisted with histological evaluation. A.Y. and H.S.G. performed flow cytometry and were involved in BMDM experiments and analysis. D.L. and J.G.O were involved in microglia experiments and performed qRT-PCR analysis. A.W. and D.M.U. performed BMDM cytokine stimulation and inflammatory marker analysis. A.K.M.G.M. performed immunohistochemistry experiments. S.C. established and maintained mouse colonies. A.K.M.G.M., S.C., J.G.O. performed tissue collections and analysis. S.B. and T.M.M. obtained, embedded and processed human tissue. J.P.V. performed mouse behavioral analysis. J.Z. assisted with cell counts and quantitation. R.H.B. performed electrophysiology experiments. R.H.B. and R.H. analyzed RNA-seq data. J.G.O, M.H., K.K. and R.H.B. planned, designed, and interpreted the experiments. J.G.O and R.H.B. wrote the manuscript.

Figure S1


A.
B.
C.
Primers:
A+B
A+C
500 bp
200 bp
Wt
Hemi
Homo
Homo 25.3%
Wt 23.2%
Hemi 51.5%
Targeting plasmid
FRT
lacZ
neo
loxP
D.
E.
Brain
SC
mRNA expression
N.D.
C9orf72
tubulin
F.
G.
Males
Females
Weights (g)
Age (days)
Percent survival
H. Rotarod
I. Grip Strength
J. Open Field Test
K.
Latency to fall (s)
Forelimb and Hindlimb Grip Strength
Activity (beam breaks)
CMAP amplitude (mV)
ns
L.
M.
Sensory branch
Motor branch
Number of Axons

N.

		L-Gas		L-TA		R-Gas		R-TA	
Mouse #	Genotype	Fibrilation and positive sharp waves	Fasiculations	Fibrilation and positive sharp waves	Fasiculations	Fibrilation and positive sharp waves	Fasiculations	Fibrilation and positive sharp waves	Fasiculations
6357	*C9orf72* $^{+/+}$	0	0	0	0	0	0	0	0
6403	*C9orf72* $^{+/+}$	0	+	0	occasional	0	0	0	0
6404	*C9orf72* $^{+/+}$	0	occasional	0	+	0	occasional/CRD (1)	0	0
6448	*C9orf72* $^{+/-}$	0	occasional	0	0	0	0	0	0
6449	*C9orf72* $^{+/-}$	0	0	0	+	0	0	0	0
6458	*C9orf72* $^{+/-}$	0	0	0	occasional/CRD (1)	0	0	0	0
6445	*C9orf72* $^{-/-}$	0	0	0	0	+	+	+	occasional
6447	*C9orf72* $^{-/-}$	0	occasional	0	0	0	0	0	0
6450	*C9orf72* $^{-/-}$	0	occasional	0	+	+	occasional	0	+
6455	*C9orf72* $^{-/-}$	0	0	0	0	0	0	0	+
6456	*C9orf72* $^{-/-}$	0	occasional	occasional	0	0	0	0	0

Figure S1. (**A**) Schematic diagram of homologous recombination construct from the NIH KOMP project with arrows denoting primer locations. (**B**) Genotyping results for wild-type (Wt), hemizygous (Hemi) and homozygous (Homo) mice. (**C**) Mendelian ratios (n=154 pups). (**D**) RNA levels by quantitative RT-PCR of C9orf72 in brain and spinal cord (SC) from indicated genotypes. N.D. – not detected. (**E**) Immunoblot of C9orf72 protein levels in brain (top – arrow), with tubulin shown below as a loading control, and quantitation normalized to wild-type control. The asterisk (*) indicates a nonspecific band. (**F**) Body weights of males and females over time. (**G**) Survival curve of hemizygous and homozygous C9orf72 null mice did not differ from wild-type mice out to 500 days. (**H,I**) Rotarod and grip strength showed no significant difference between Wt, Hemi, and Homo *C9orf72* null mice at 9 months (n= 15,19,8 respectively). (**J**) Homo showed diminished total activity vs. Wt and hemi starting at 5 minutes (p=0.02, two-way ANOVA) in 9 month old animals, presumably due to their enlarged spleens. (**K**) Compound motor action potential (CMAP) amplitudes from foot flexor muscles after stimulation of the distal tibial nerve in 12 month old animals (n=3). (**L**) Toluidine blue stained plastic sections of femoral motor and sensory nerves of 12 month mice. (**M**) 12 month old mice showed no difference in axon numbers in either the motor or sensory branches of the femoral nerve (n=3). (**N**) Table of electromyography findings in the gastrocnemius (Gas) or tibialis anterior (TA) muscles on the right (R) and left (L) performed blinded by an experienced electromyographer (R.H.B.) on 17 month old animals. Occasional fasciculation potentials, fibrillations and positive sharp waves, and complex repetitive discharges were seen in aged animals of all genotypes. There were no findings suggestive of motor neuron disease at 17 months in any genotype.

Figure S2

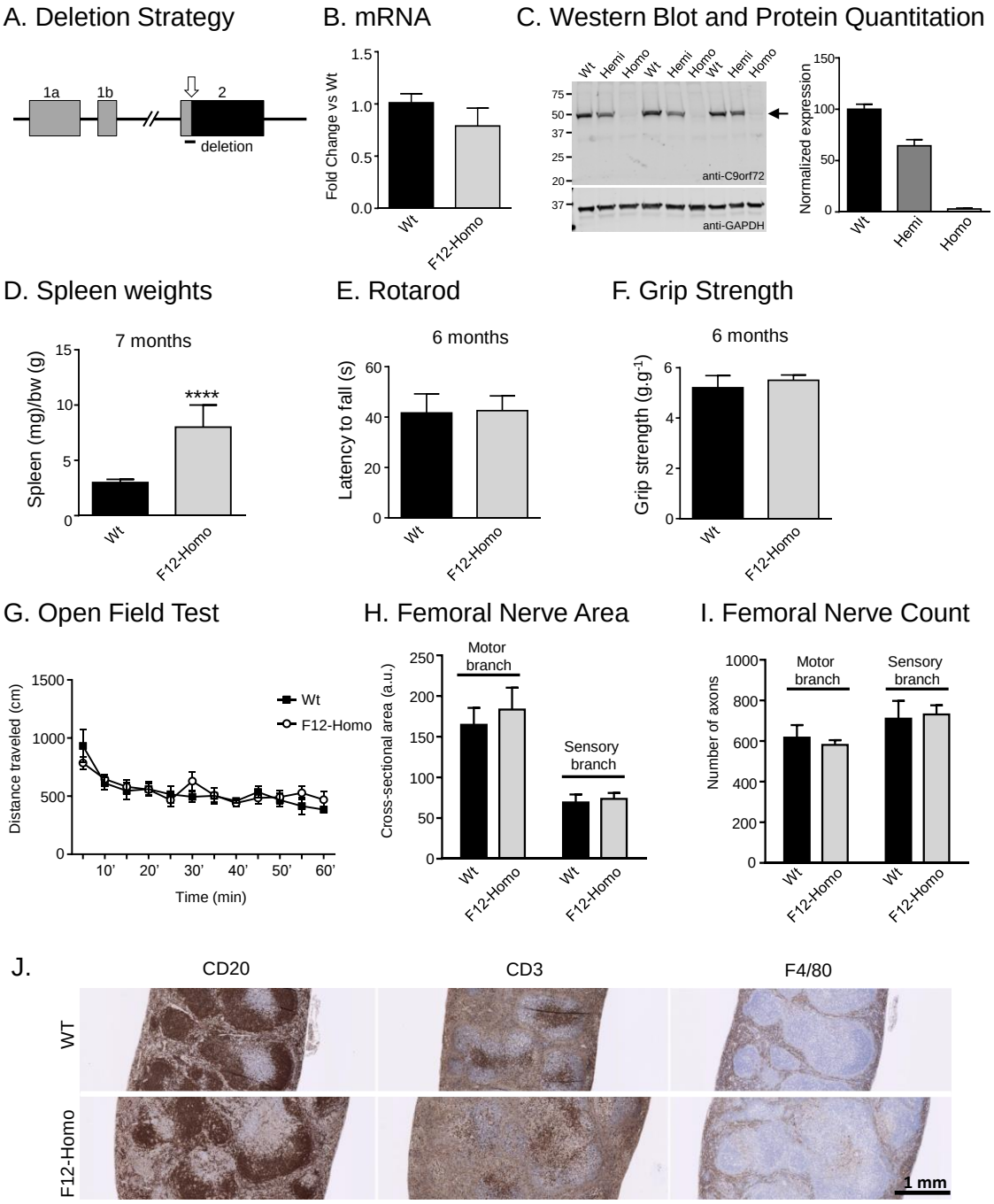


Figure S2. (**A**) Schematic diagram of the deletion in exon 2 of *3110043O21Rik* (*C9orf72*) for F12 C9 null homozygous mice. Cut-sites of the ZFNs are indicated with downward arrow. Coding sequence is in black, starting at the ATG in exon 2 which is removed by the deletion. (**B**) Quantitative RT-PCR analysis of mRNA expression of mouse *C9orf72* showing relatively well preserved RNA level compared to wild-type (Wt). (**C**) Western blot and quantification for C9orf72 (top) and loading control GAPDH

(bottom) on brain lysates from wild-type and homozygous null F12 mice showing complete loss of C9orf72 protein from deletion of the start codon. The faint band remaining in the Exon 2 F12 deletion line is a cross-reacting protein. Molecular weights in KDa are indicated on the left. (**D**) Spleen weights (mg) normalized to body weight (g) at 7 months (n=8) show F12 homozygotes have significantly larger spleens than wild-type controls (****p=<0.0001, two-tailed t test). (**E**) Rotarod latency to fall in seconds for homozygous male F12 mice versus wild-type at 6 months. (n= 7/8 per group) (**F**) Forelimb grip strength normalized to body weight for homozygous F12 males compared to wild-type at 6 months (n= 8 per group). (**G**) Distance run in the open field per 5 min interval over a 60 minutes observation for F12 males and controls at 6 months (n=8 per group). (**H**) Cross-sectional area in pixels (arbitrary units, a.u.) and axon counts (**I**) of the motor and sensory branches of the femoral nerves at 9 months (n=8 per group). **(J)** Immunocytochemistry for B cells (CD20), T cells (CD3) and red pulp macrophages (F4/80) on spleen sections of 5 months old F12 homozygous mice compared to wild-type controls. The F12 homozygous *C9orf72* null line also had enlarged spleens and lymph nodes with altered follicular architecture filled with engorged macrophages similar to the changes seen in the KOMP targeted allele (Fig. 2 of main text). Scale bar = 1mm.

Figure S3

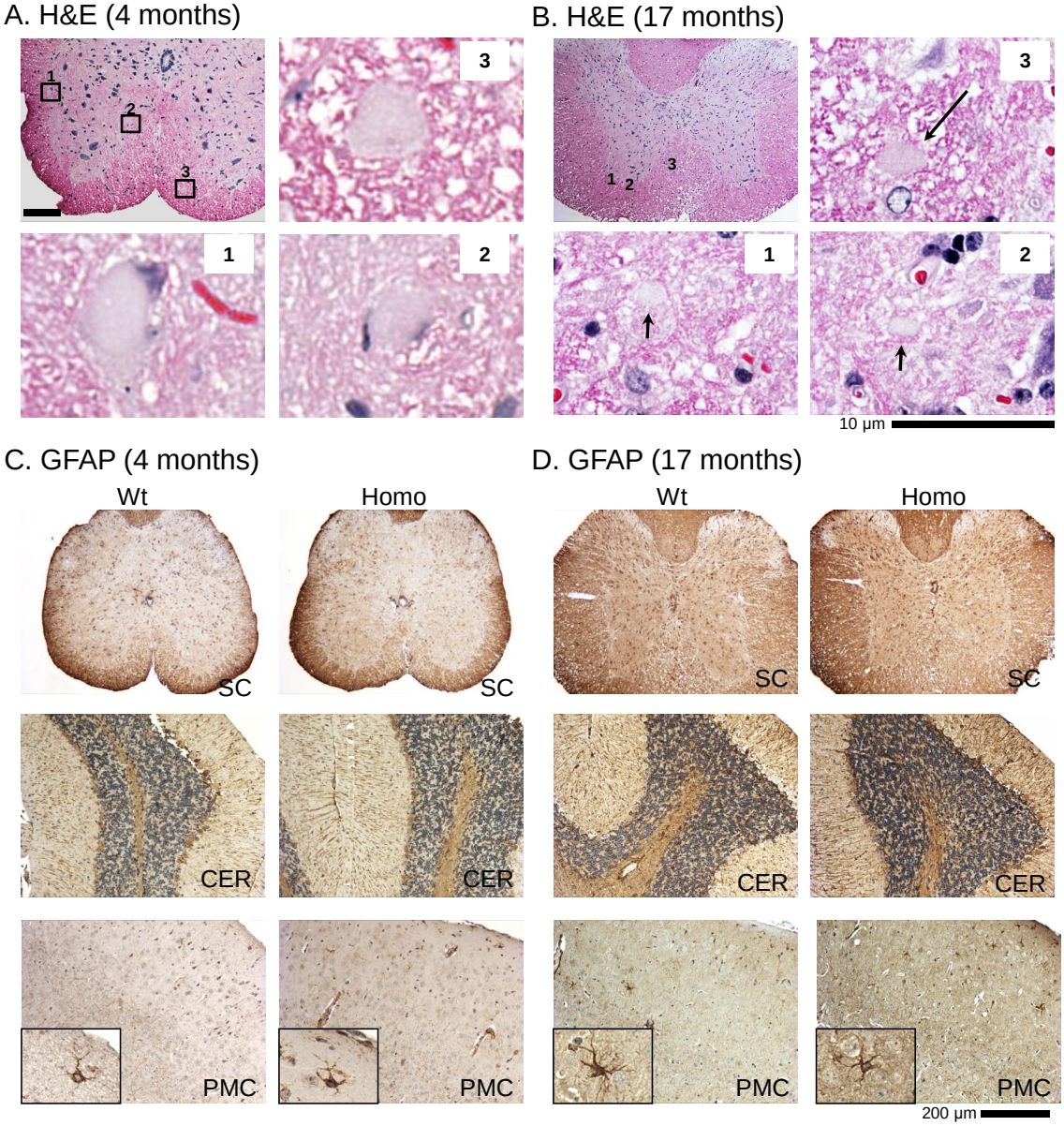

A. H&E (4 months)
B. H&E (17 months)
1
2
3
10 µm
C. GFAP (4 months)
D. GFAP (17 months)
Wt
Homo
SC
CER
PMC
200 µm

Figure S3 cont'd

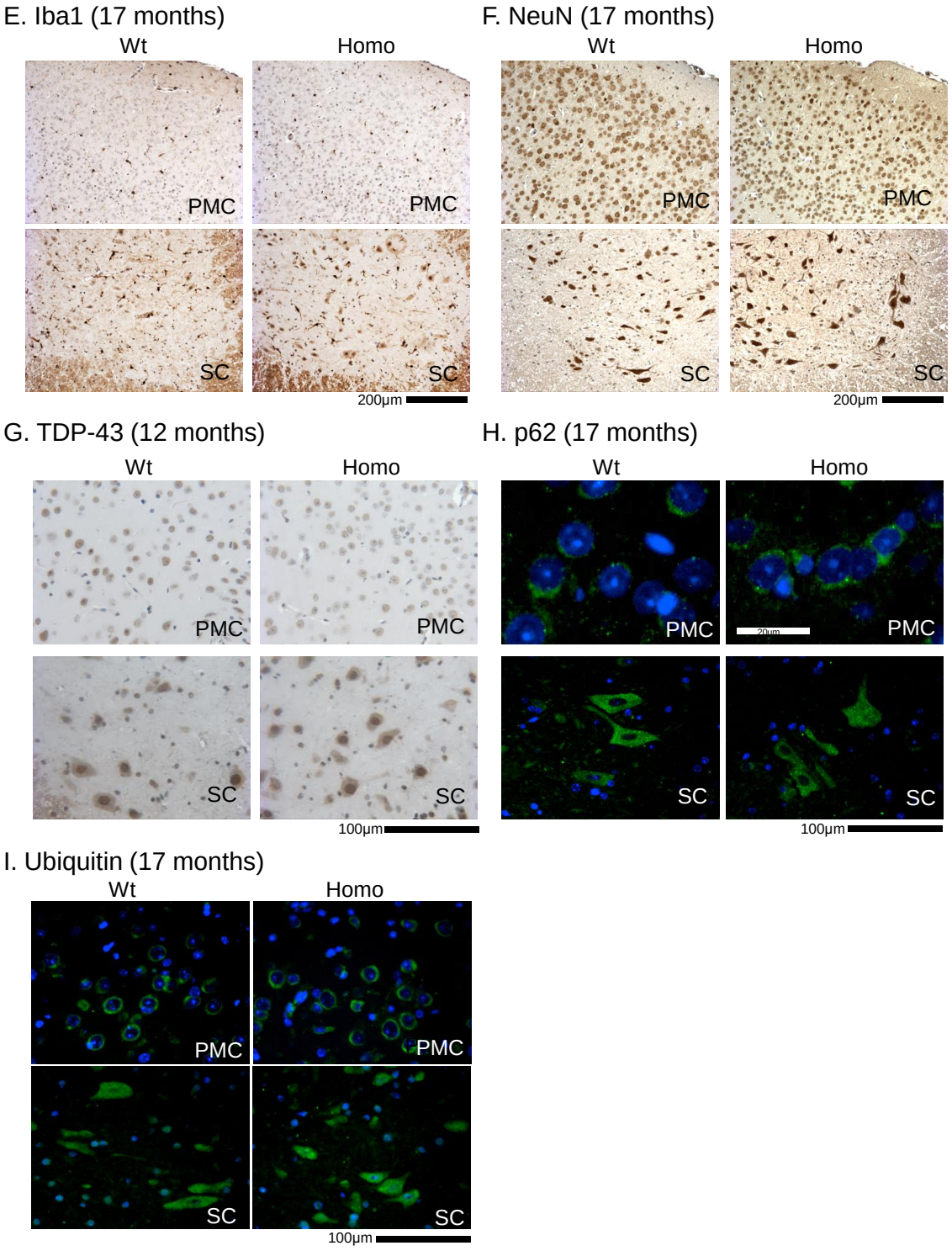


Figure S3. Histopathologic analyses in the spinal cord and brain of *C9orf72* null mice. (**A,B**) H&E staining of homozygous spinal cord at 4 months (**A**) and 17 months (**B**). Rare chromatolytic structures (A1-3 and B1-3) were detected in both the grey and white matter in spinal cords of *C9orf72* null mice, which did not change in frequency with age. (**C,D**)

Immunohistochemical staining of GFAP in spinal cord (SC, top), cerebellum (CER, middle), and primary motor cortex (PMC, bottom) at 4 months (**C**) and 17 months (**D**) in wild-type and homozygous *C9orf72* null mice. (**E**) Iba1 staining in primary motor cortex (PMC, top) and spinal cord (SC, bottom) in wild-type and in homozygous nulls at 17 months (**F**). NeuN staining at 17 months in primary motor cortex (PMC, top) and spinal cord (SC, bottom) in wild-type and homozygous null mice . (**G**) TDP-43 staining in primary motor cortex (PMC, top) and spinal cord (SC, bottom) in wild-type and homozygous null mice at 12 months. (**H,I**) Immunofluorescent staining of p62 (**H**) and ubiquitin (**I**) in primary motor cortex (PMC, top) and spinal cord (SC, bottom) in wild-type and homozygous null mice at 17 months.

Figure S4

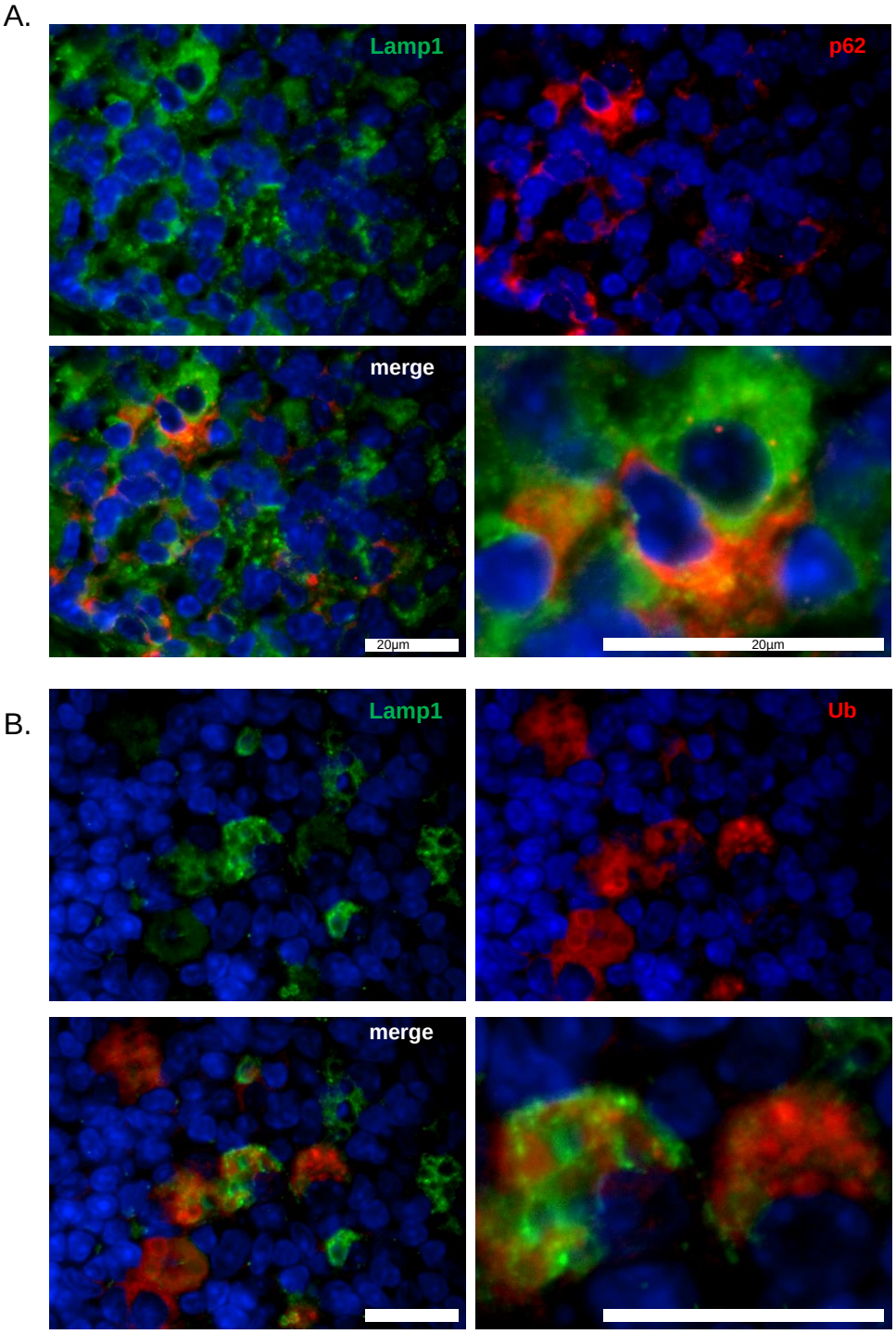
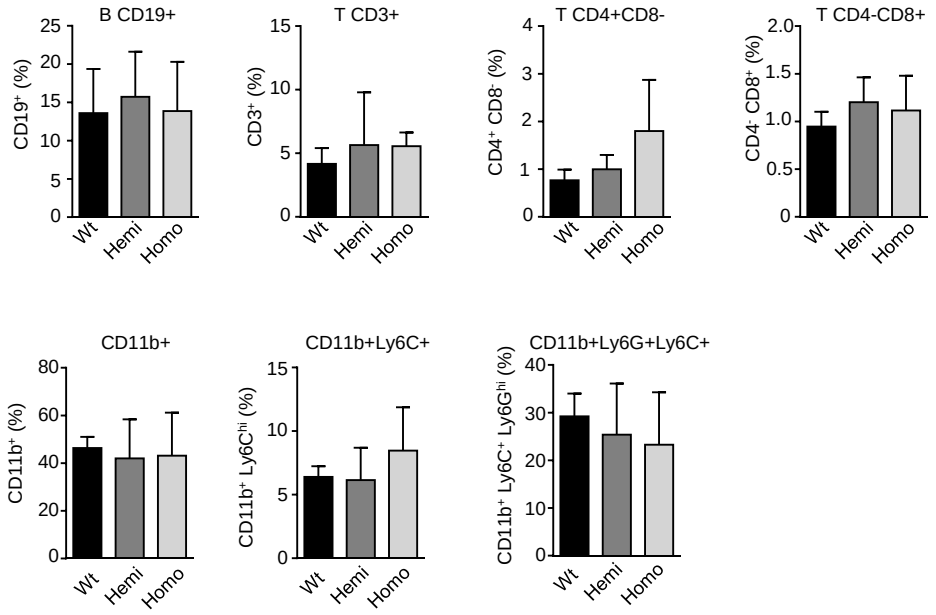


Figure S4. (**A**) Enlarged cells in C9orf72-/- spleens double-labelled with Lamp1 (green) and p62 (red). The cytoplasmic debris stained positive for both Lamp1 and p62, with occasional overlap. (**B**) C9orf72-/- spleen co-stained with Lamp1 (green) and ubiquitin (Ub) (red). The infiltrating macrophages appearing cells showed positive cytoplasmic reactivity for both Lamp1 and ubiquitin.

Figure S5

A.


B CD19+
CD19+ (%)
T CD3+
CD3+ (%)
T CD4+CD8-
CD4+ CD8- (%)
T CD4-CD8+
CD4- CD8+ (%)
CD11b+
CD11b+ (%)
CD11b+Ly6C+
CD11b+ Ly6Chi (%)
CD11b+Ly6G+Ly6C+
CD11b+ Ly6C+ Ly6Ghi (%)
Wt
Hemi
Homo

B.

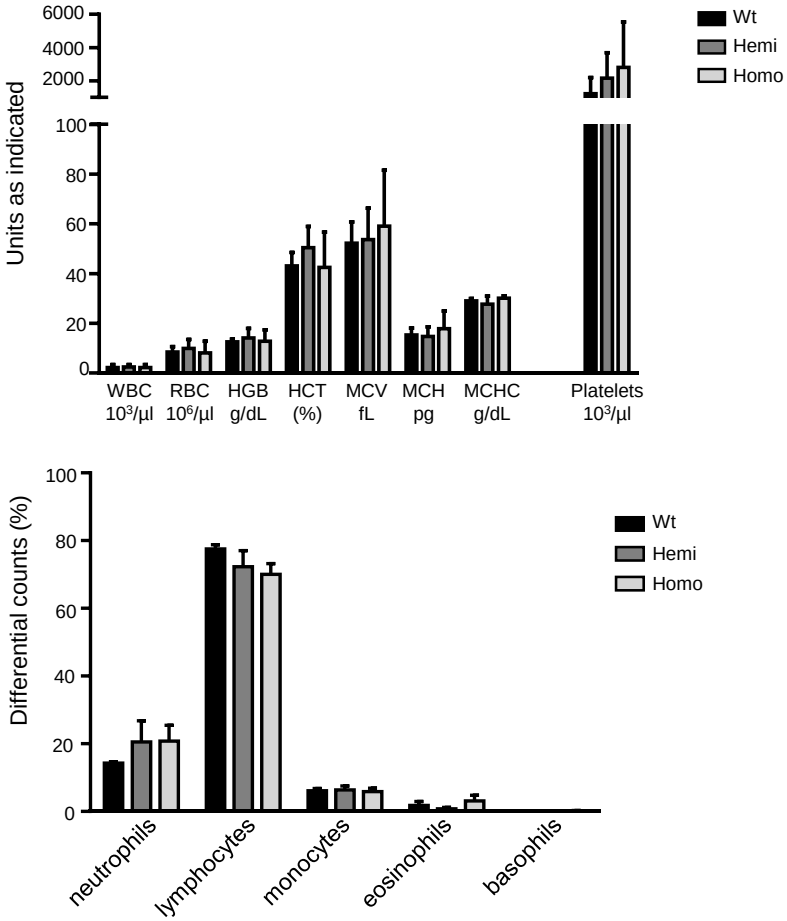
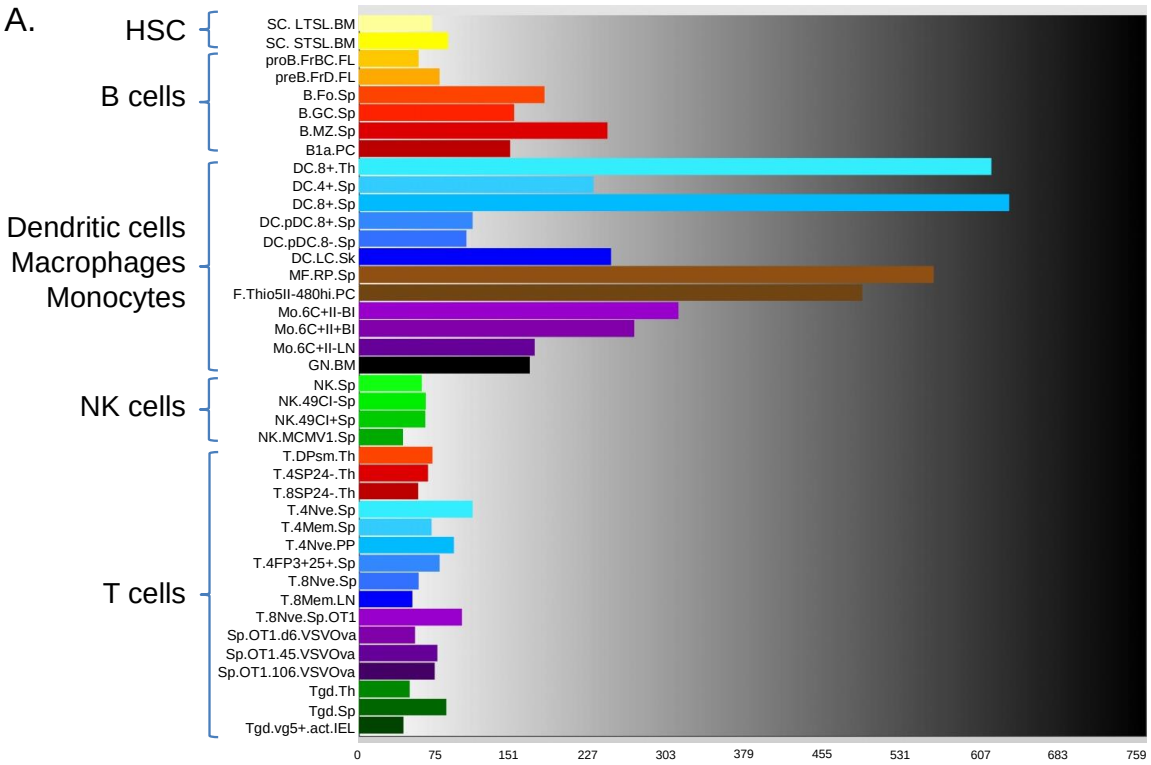

Units as indicated
Wt
Hemi
Homo
WBC 10³/µl
RBC 10⁶/µl
HGB g/dL
HCT (%)
MCV fL
MCH pg
MCHC g/dL
Platelets 10³/µl
Differential counts (%)
neutrophils
lymphocytes
monocytes
eosinophils
basophils

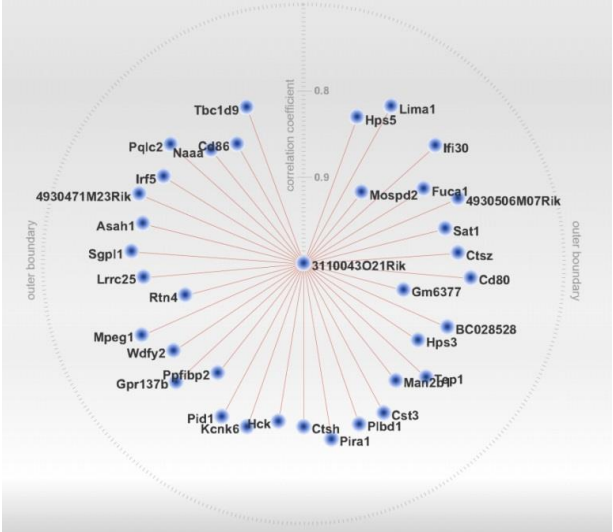
Figure S5. (**A**) Flow cytometric analyses of B cells (CD19), T cells (CD3, CD4, CD8), and monocyte populations (CD11b, Ly6C, Ly6G) in bone marrow from wild-type (Wt), hemizygous (Hemi) and homozygous (Homo) *C9orf72* null mice. No significant differences were observed in the different cell populations in bone marrow at 5 months of age (n=6). (**B**) Complete blood counts on Wt, Hemi and Homo *C9orf72* null mice at 5 months of age (n=4). No differences in the number of white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), or platelets were observed between the different genotypes. Differential counts of WBC subsets also showed no difference in the number of circulating neutrophils, lymphocytes, monocytes, eosinophils, or basophils.

Figure S6

A.



B.



C.

Annotation Cluster 1	**Enrichment Score: 6.507683992341271**							
Category	Term	PValue	Genes	List Total	Fold Enrichment	Bonferroni	Benjamini	FDR
GOTERM_CC_FAT	GO:0005764~**lysosome**	4.08E-08	CTSZ, NAAA, IFI30, MAN2B1, FUCA1, CTSH, 4930471M23RIK, ASAH1	26	21.614	2.32E-06	2.32E-06	4.04E-05

Figure S6. (**A**) Gene skyline graph from the immunological genome project (www.immgen.org) of *3110043O21Rik* (*C9orf72*) showing relative expression levels in the "Key Populations" data group including hematopoietic stem cell (HSC), B-cell, dendritic cell, macrophage, monocyte, natural killer (NK), and T-cell populations. Highest expression of *C9orf72* is present in monocyte derivative cells including splenic macrophages and dendritic cells. (**B**) Gene constellation analysis showing the top 35 genes most closely correlated in expression with *3110043O21Rik* (*C9orf72*) across the "key populations" data group of the immunological genome database. (**C**) Database for Annotation Visualization and Integrated Discovery (DAVID) pathway analysis output for top 35 genes co-expressed with *C9orf72* revealed highly significant enrichment for only one pathway, GOTERM GO:0005764~lysosome with a corrected p-value of 2e-6.

Figure S7

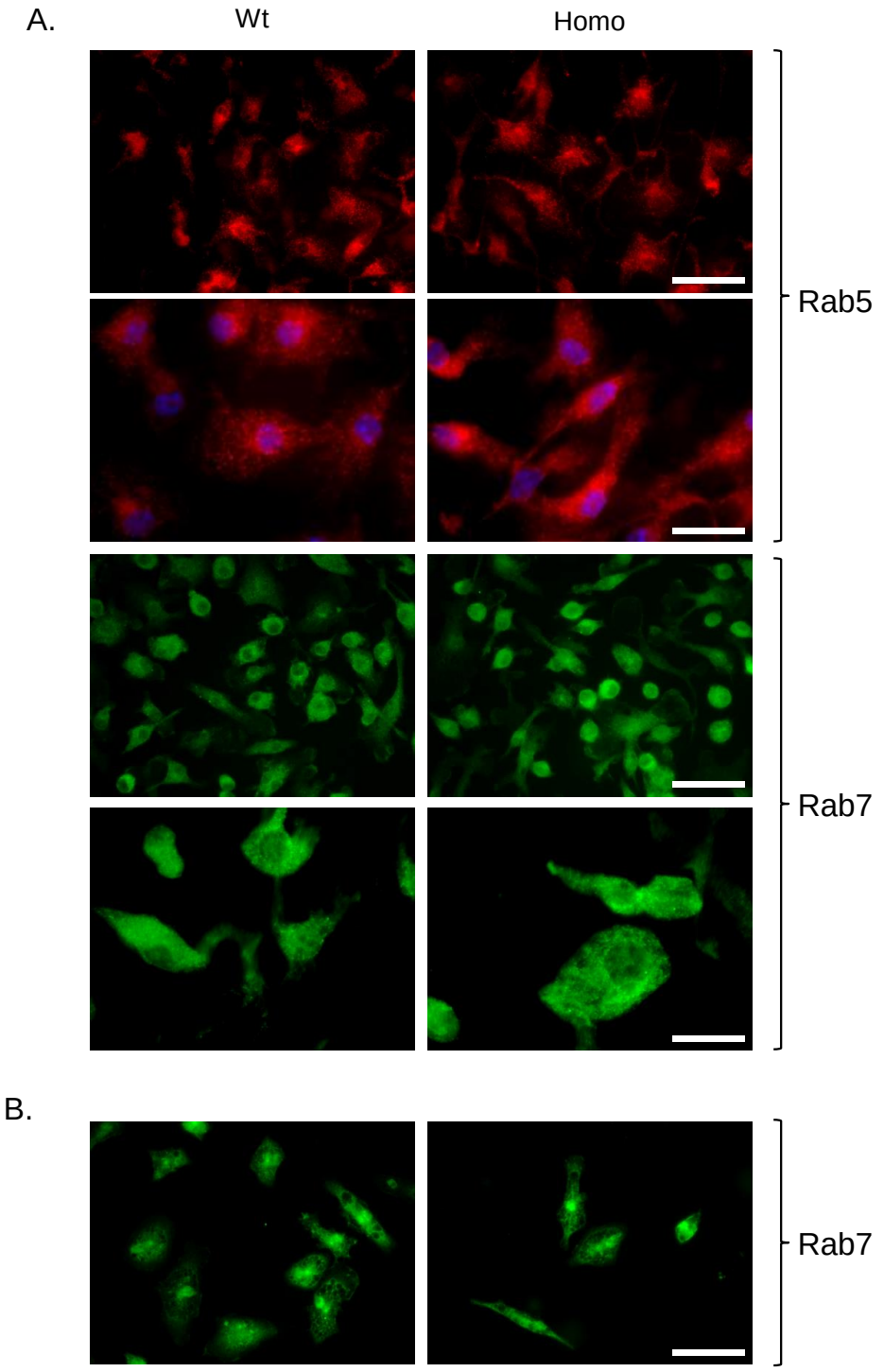


Figure S7. (**A**) Immunocytochemistry of Rab5 and Rab7 in BMDM from wild-type (Wt) and homozygous C9orf72-nulls (homo). Scale bar = 50 µm and 20 µm. (**B**) Purified microglia from Wt and homo mice show no detectable difference in Rab7 staining. Scale bar = 50 µm

Figure S8

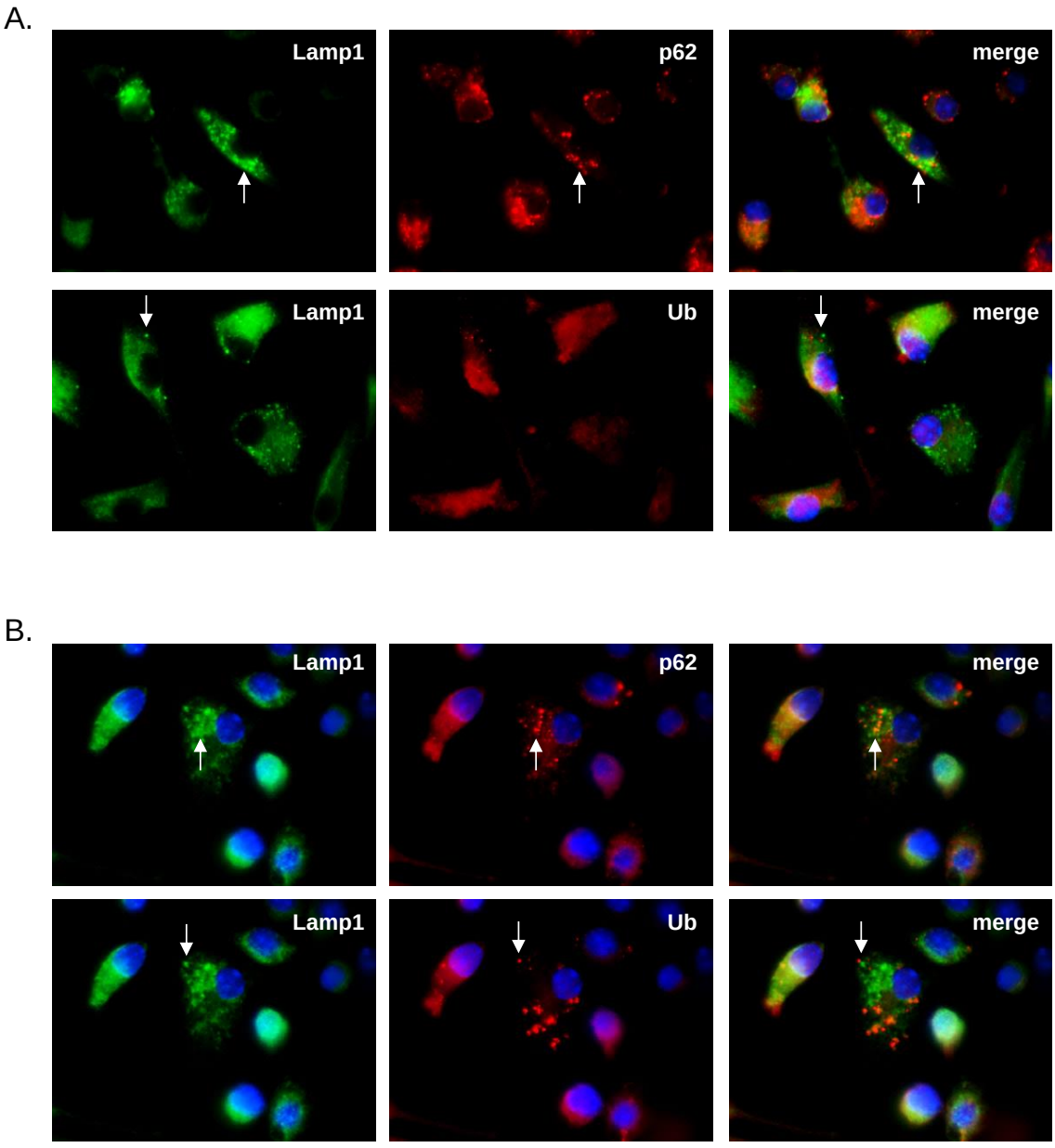


Figure S8. (**A**) Immunocytochemistry co-staining of Lamp1/p62 (top) and Lamp1/ubiquitin (Ub, bottom) in BMDM from *C9orf72* null mice. Lamp1 and p62 staining patterns were distinct with occasional overlap, where Lamp1 and ubiquitin were present with little to no overlap. (**B**) Co-staining of Lamp1 (green), p62 (red) and ubiquitin (red, bottom) in purified microglia from homozygous C9orf72 nulls. Similar to BMDM, Lamp1 and p62 have distinct staining patterns with occasional overlap, whereas Lamp1 and Ub stain structures in the same cell, but had little to no overlap.

Figure S9

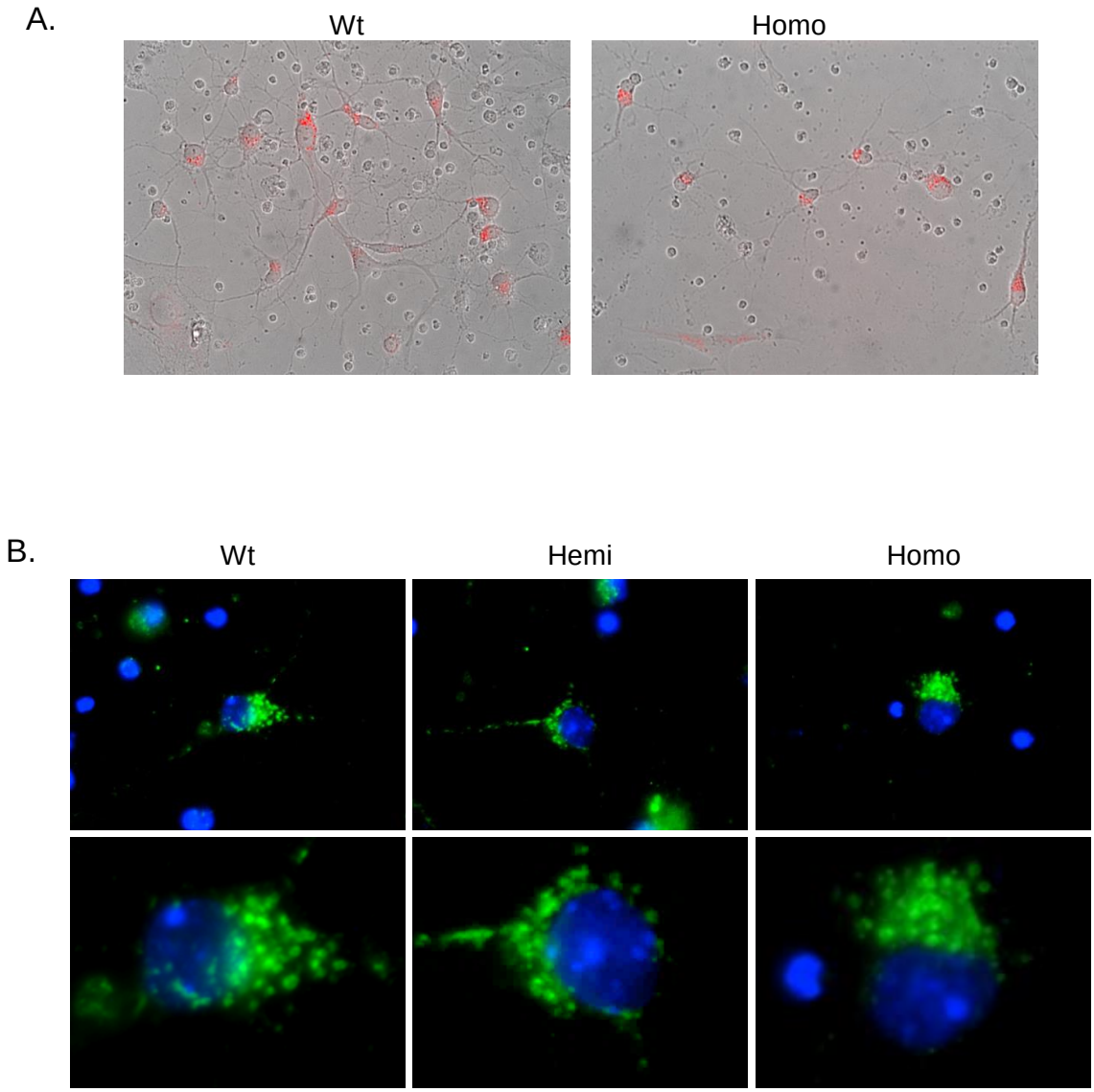


Figure S9. (**A**) Primary cortical cultures from wild-type (Wt) and C9orf72 null (Homo) mice incubated with LysoTracker and imaged on day 7 in vitro. Image analysis of bright-field image overlaid with the LysoTracker image showed no detectable difference in LysoTracker staining between C9orf72 null wild-type neurons. (**B**) Primary cortical cultures from wild-type, hemizygous, and homozygous C9orf72 null mice were stained with Lamp1 antibody. No detectable difference in number or size of lysosomes was observed.

Figure S10

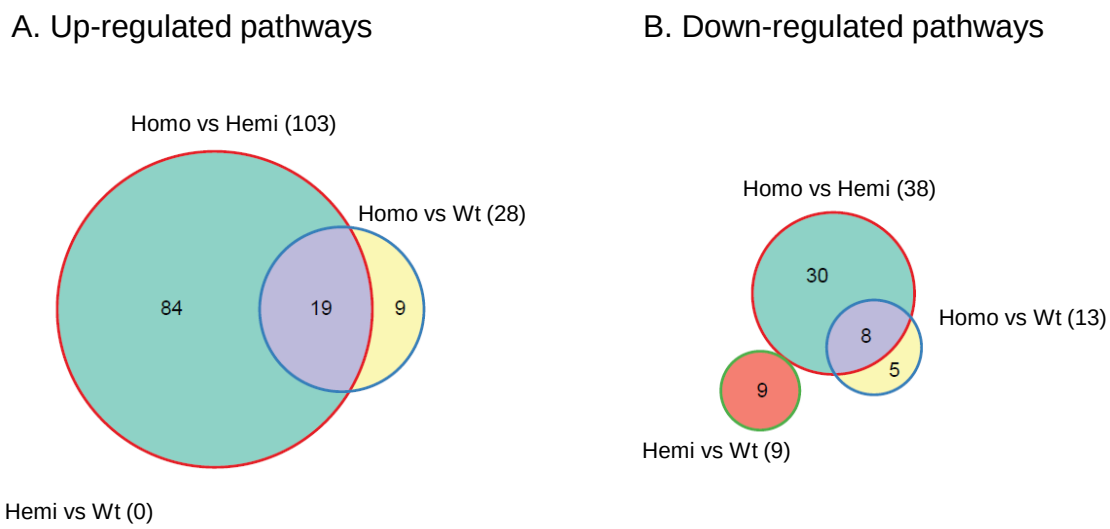


Figure S10. (**A**)Venn diagram of up-regulated pathways between the different genotypes in the spinal cord of C9orf72 null mice at 17 months. There are 19 pathways up-regulated in the C9orf72 null homozygotes compared to hemizygous and wild-type spinal cord tissue. (**B**) Venn diagram of the down-regulated pathways in the spinal cord at 17 months.

Figure S11

A. Human Motor Cortex

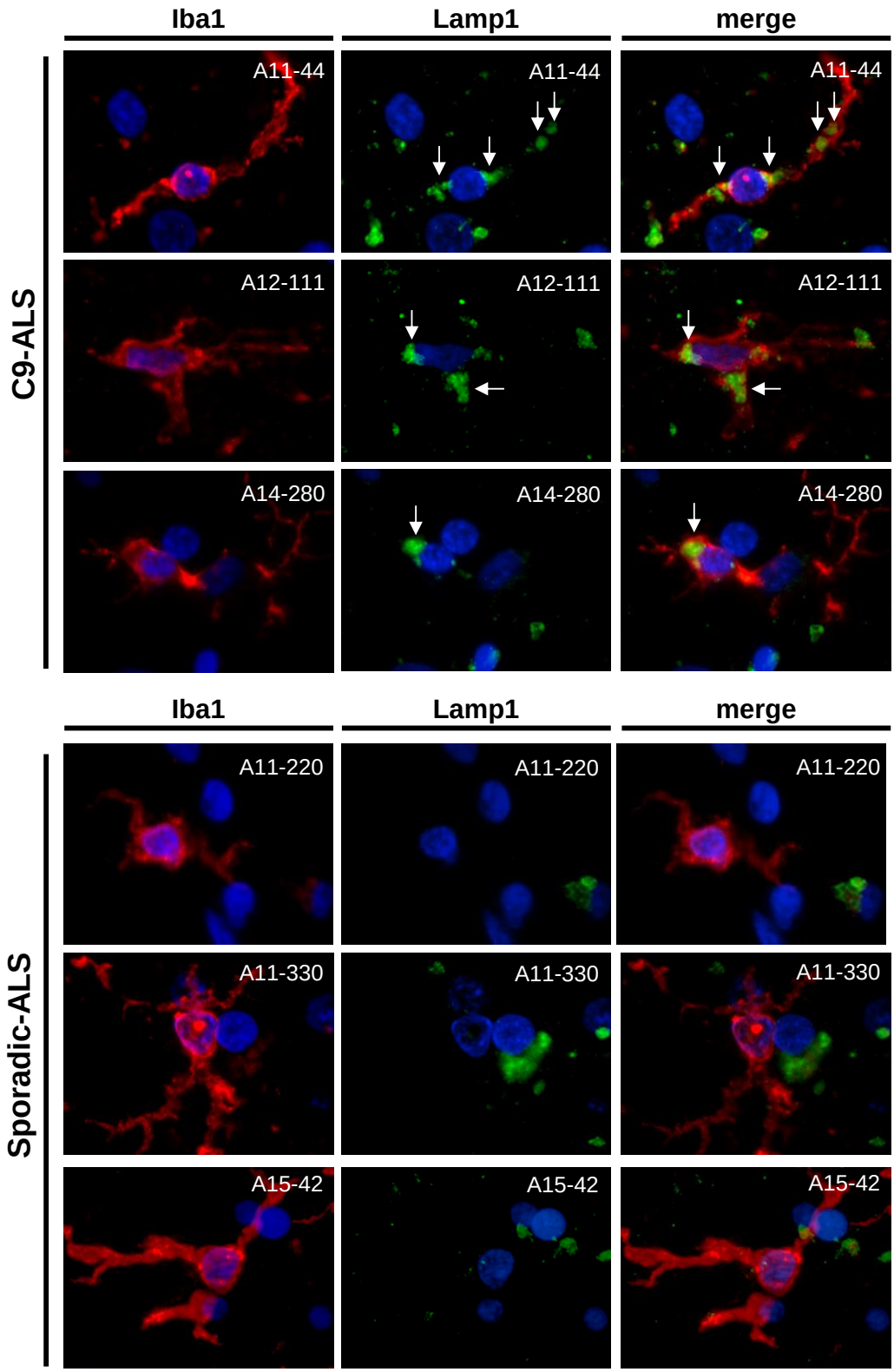
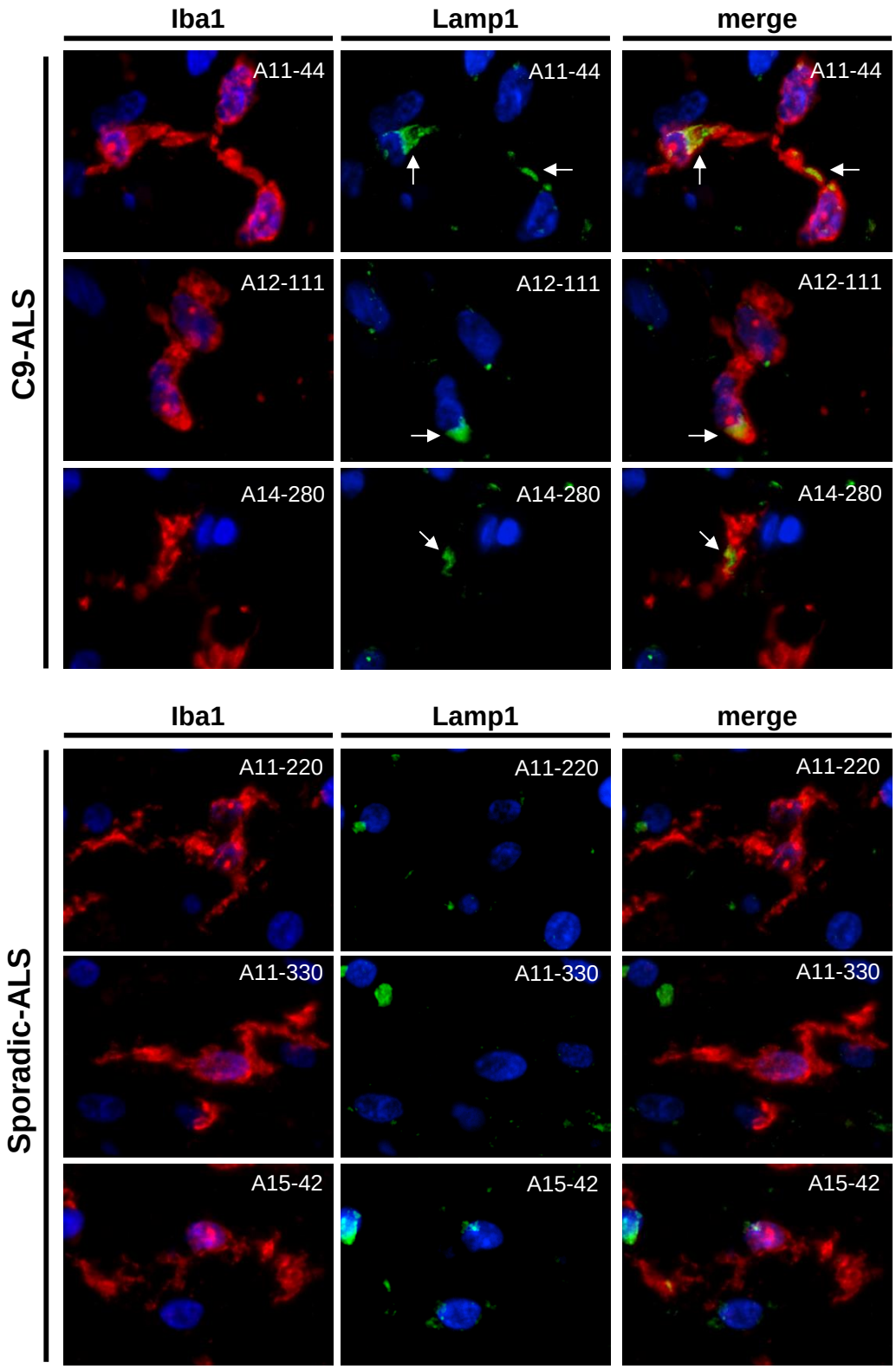


Figure S11 continued

B. Human Spinal Cord



C.

Immune pathways upregulated in C9-ALS vs. sporadic ALS	FDR q-value
REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_AND_A_NON_LYMPHOID_CELL	6.89E-04
REACTOME_INTERFERON_ALPHA_BETA_SIGNALING	0.005879
KEGG_COMPLEMENT_AND_COAGULATION_CASCADES	0.006209
KEGG_SYSTEMIC_LUPUS_ERYTHEMATOSUS	0.012784
KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION	0.027705
CYTOKINE_BINDING	0.033769

Figure S11. Postmortem human motor cortex (**A**) and spinal cord (**B**) from 3 C9orf72 expansion ALS cases, and 3 sporadic ALS cases double-labelled with Iba1 to identify microglia (red), and Lamp1(green) to identify lysosomes. Large accumulations of Lamp1 immunoreactivity (white arrows) were detected in activated microglia of C9-ALS patients, but not sporadic ALS subjects in both the motor cortex (**A**) and in the spinal cord (**B**). (**C**) Table listing immune pathways that were significantly up-regulated on gene set enrichment analysis (FDR<0.05) of cerebellar tissue from C9orf72 expansion patients (C9-ALS) vs. sporadic ALS patients (primary data from reference *21*).