A novel inducible von Willebrand Factor Cre Recombinase mouse strain to study microvascular endothelial cell-specific biological processes *in vivo*

Dinesh Yadav, Ph.D.[1], Jeremy A. Conner, B.S.[1], Yimin Wang, Ph.D.[1], Thomas L. Saunders, Ph.D.[2], Eroboghene E. Ubogu, M.D.[1]

[1]Neuromuscular Immunopathology Research Laboratory, Division of Neuromuscular Disease, Department of Neurology, University of Alabama at Birmingham, Birmingham, Alabama, United States of America.

[2]Transgenic Animal Model Core, University of Michigan, Ann Arbor, Michigan, United States of America.

Address all correspondence to:

Dr. Eroboghene E. Ubogu

Division of Neuromuscular Disease

Department of Neurology

University of Alabama at Birmingham

1720 7th Avenue South, Sparks Center Suite 200

Birmingham, AL 35294-0017

United States of America

Telephone Number: +1-205-934-2120, Facsimile Number: +1-205-975-6758

E-mail: ubogu@uab.edu

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Abstract

Mouse models are invaluable to understanding fundamental mechanisms in vascular biology during development, in health and different disease states. Several constitutive or inducible models that selectively knockout or knock in genes in vascular endothelial cells exist; however, functional and phenotypic differences exist between macrovascular and microvascular endothelial cells in different organs. In order to study microvascular endothelial cell-specific biological processes, we developed a Tamoxifen-inducible von Willebrand Factor (vWF) Cre recombinase mouse in the SJL background. The transgene consists of the human vWF promoter with the microvascular selective 734 base pair sequence to drive Cre recombinase fused to a mutant estrogen ligand-binding domain [ERT2] that requires Tamoxifen for activity (CreERT2) followed by a polyadenylation signal. We initially observed Tamoxifen-inducible restricted bone marrow megakaryocyte and sciatic nerve microvascular endothelial cell Cre recombinase expression in offspring of mixed strain hemizygous C57BL/6-SJL founder mice bred with mT/mG reporter mice, with >90% bone marrow megakaryocyte expression efficiency. We subsequently backcrossed founder mice to the SJL background using speed congenics, and intercrossed for multiple generations to develop hemizygous Tamoxifen-inducible vWF Cre recombinase (vWF-iCre/+) SJL mice. Microvascular endothelial cell-specific Cre recombinase expression occurred in the sciatic nerves, brains, spleens, kidneys and gastrocnemius muscles of adult vWF-iCre/+ SJL mice bred with Ai14 tdTomato reporter mice, with retained low level bone marrow and splenic megakaryocyte expression. This novel mouse model would support hypothesis-driven mechanistic studies to decipher the role(s) of specific genes transcribed by microvascular endothelial cells during development, as well as in physiologic and pathophysiologic states in an organ- and time-dependent manner.

Key Words: Cre recombinase, inducible, mice, microvascular endothelial cells, reporter mouse, sciatic nerves, Tamoxifen, transgenic mice, vascular biology, von Willebrand Factor

Introduction

Vascular biologists have generated multiple mouse strains in different genetic backgrounds to tightly regulate gene deletion, or express transgenic genes in the murine endothelium over the past 30 years. These strains facilitate *in vivo* modeling of biologically relevant molecular pathways in normal development or physiologic processes, or disease states, guided by human observational data (Cleaver, 2021). Some of these mouse models are germline knockouts, with possible adaptive changes that may occur in other associated genes or epigenetic factors, while others conditionally alter gene expression across all or different subsets of vascular or lymphatic endothelial cells. Cre recombinase, or tetracycline-controlled transactivator protein tTA are standard means to achieve conditional gene knockout or knockin, with a goal to better understand the role(s) of specific genes in murine vascular biology with human implications.

Cre recombinase is a 343 amino acid, 38 kDa bacteriophage P1 protein that catalyzes a site specific recombination reaction between two 34-base-pair “locus of crossing over in P1” DNA sequences (called loxP) sites that are absent from the mammalian genome (Alvarez-Aznar et al., 2020; Crouthamel et al., 2012; Semprini et al., 2007; Song and Palmiter, 2018; Sternberg and Hamilton, 1981; Thyagarajan et al., 2000). A gene containing two loxP sites flanking essential exons can be deleted in cell types that selectively express Cre driven by a specific promoter constitutively or inducibly (Alvarez-Aznar et al., 2020; Crouthamel et al., 2012; Nagy, 2000; Song and Palmiter, 2018). Conditional transgene activation is achieved by placing a flanked loxP (floxed) stop cassette between the promoter and its gene that is removed upon Cre-mediated excision, results in transgene expression (Alvarez-Aznar et al., 2020; Crouthamel et al., 2012; Lakso et al., 1992; Song and Palmiter, 2018).

Many of these murine models alter gene expression in both microvascular and microvascular endothelial cells in multiple or specific organs, with some also demonstrating altered gene

expression in non-endothelial cells such as in hematopoietic stem cells, yolk sac, placenta, osteoblasts, megakaryocytes, hepatocytes, macrophages, lymphocytes, adipocytes, keratinocytes and skeletal muscles, to mention a few (Cleaver, 2021).

Due to phenotypic and functional differences between micro- and macrovascular endothelial cells in the same tissues and organs (Aird, 2007a; Aird, 2007b; Yano et al., 2007), developing mouse strains that facilitate selective microvascular gene alternation is important. Such models are relevant to decipher key molecular determinants and signaling pathways in tight junction-forming microvascular endothelial cells that form the blood-brain, blood-nerve, blood-retina and blood-testis barriers. The most commonly used vascular endothelial cell models, based on tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie-1) or TEK receptor tyrosine kinase (Tek, also known Tie-2) alter gene expression in all endothelial cells (Carlson et al., 2005; Cleaver, 2021; Gustafsson et al., 2001; Kisanuki et al., 2001; Korhonen et al., 2009; Teng et al., 2002). Data from the mouse sciatic nerve transcriptome demonstrated more diffuse *Tie*- and *Tek* cellular transcript expression in adult (60 day old) mice by single cell whole exome shortgun sequencing (RNA Seq) (Gerber et al., 2021).

von Willebrand factor (vWF) gene encodes a glycoprotein transcribed and translated by microvascular endothelial cells and megakaryocytes that forms multimeric complexes required for platelet adhesion at sites of vascular injury. vWF is predicted to be involved in chaperone, identical protein and integrin binding activity and implicated in liver and placenta development . A Cre recombinase transgenic mouse model designed to specifically knockout genes in murine brain endothelial cells was developed and published over 10 years ago, driven by a human microvascular endothelial cell-specific promoter (Tg (VWF-Cre)^{1304Roho}) (Crouthamel et al., 2012); however, these mice are not commercially available or maintained in a known mutant mouse repository for scientific use, questioning strain adequacy or viability. In order to study

organ- and time-dependent microvascular endothelial cell signaling pathways in development, post-natal health, aging and disease, we developed a Tamoxifen-inducible vWF Cre recombinase transgenic mouse strain with a highly efficient Cre-mediated recombination, and show specific microvascular endothelial cell expression in multiple organs, including the brain and sciatic nerves, using validated fluorescent reporter mouse strains.

Materials and Methods

Development of Tamoxifen inducible von Willebrand Factor Cre recombinase transgenic mice

The University of Michigan Transgenic Animal Model Core synthesized of a human vWF genomic DNA fragment containing the 734 base pair microvascular specific sequence (Aird et al., 1995; Jahroudi and Lynch, 1994) to drive Cre recombinase fused to a mutant estrogen ligand-binding domain [ERT2] that requires Tamoxifen for activity (CreERT2) followed by a polyadenylation signal. The synthesized genomic fragment was subcloned into a CreERT2 plasmid (Matsuda and Cepko, 2004) that placed CreERT2 under the control of the synthesized human vWF promoter, and the cloned transgene was verified by DNA sequencing. The cloned transgene was subsequently removed from the plasmid cloning vector and purified for subsequent microinjection.

The purified transgene containing the human vWF promoter, inducible Cre (iCre) and polyadenylation signal sequences was microinjected into fertilized mouse eggs obtained by mating mixed strain C57BL/6 X SJL F1 male and female mice, as previously described (Becker and Jerchow, 2011), and transferred to pseudopregnant recipients to produce hemizygous transgenic (designated as vWF-iCre/+) N1 founder mice at the University of Michigan. All hemizygous vWF-iCre/+ mice were transferred to the University of Alabama at Birmingham after confirmatory tail snip genotyping.

Identification of inducible von Willebrand Factor Cre recombinase transgene

All experimental mice were housed in the Research Services Building animal facility, University of Alabama at Birmingham (AAALAC International approved). The studies described were conducted in accordance with all provisions of the Animal Welfare Act, the U.S. Government Principles Regarding the Care and Use of Animals, the Guide (Guide for the Care and Use of Laboratory Animals), National Research Council, Institute of Laboratory Animal Resources, the PHS Policy on Humane Care and Use of Laboratory Animals and the additional local policies of the Center of Comparative Medicine, University of Alabama at Birmingham under approved Institutional Animal Care and Use Committee Protocol # 141009960. Mice were adequately fed with irradiated chow and water provided *ad lib* and housed under micro-isolated, pathogen-free ventilated cages with a 12 hour light-dark cycle compliant with National Institute of Health (NIH) guidelines on animal care. Cages are changed in laminar flow hoods using aseptic micro-isolator techniques, with standard animal husbandry performed by certified veterinary technicians and research staff. All experiments performed were in compliance with the ARRIVE guidelines.

Tail snip genotyping was performed to identify hemizygous mixed strain C57BL/6-SJL vWF-iCre/+ N1 founder mice at 2-3 weeks of age. DNA was extracted using the Viagen Biotech DirectPCR Lysis reagent (catalog # 102-T) followed by DNA purification using isopropanol-ethanol, according to the manufacturer's instructions. Isolated DNA dissolved in ddH_2O was quantified and purity verified by absorbance at 260 nm and 260/280 nm absorbance ratios using a Thermo Scientific Nanodrop ND-1000 spectrophotometer with the NanoDrop 1000 3.8.1 software program, and reconstituted at a concentration of 50 ng/µL with sterile ddH_2O prior to storage at -20°C.

PCR amplification was performed using primers designed to detect both the human vWF promoter and the iCre sequence as follows: vWF-iCre Forward Primer 5'-AATCTTTTCTCCTGCTTTAAAGAAATGTT-3' and vWF-iCre Reverse Primer 5'-ATCTGTGACAGTTCTCCATCAGGGATCT-3'. Primers were produced by the UAB Heflin Center Genomics Core/ Integrated DNA technologies (www.idtdna.com). PCR amplification was performed by adding 1.0 µL purified tail snip genomic DNA to a mixture of 0.5 µL each of forward and reverse primers from a 10 µM stock concentration with 12.5 µL of 2X Promega GoTaq® Green Master Mix (commercially available premixed ready-to-use solution containing bacterially derived Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations, catalog # PRM5123) and 10.5 µL of sterile ddH_2O, with a total of 25 µL PCR reaction mixture. DNA amplification was performed using a Techne TC-412 Thermal cycler programmed as follows: Initiation/Melting 95°C for 5 minutes, 35 cycles for denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 30 seconds, followed by amplification at 72°C for 5 minutes and termination at 4°C.

PCR products were loaded on a 2% agarose gel in 1X TAE buffer (40 mM **T**ris base, 20 mM **A**cetic acid, and 1 mM **E**thylenediaminetetraacetic acid [EDTA]) containing 0.01% MilliporeSigma™ GelRed™ Nucleic Acid Stain (Fisher Scientific, catalog # SCT123) with electrophoresis performed at 200V for 30 minutes to detect the 604 base pair transgene amplicon. GeneRuler 100 bp DNA Ladder (ThermoFisher scientific, catalog # FERSM0242) was used to confirm band size. Direct or reverse digital images were generated using an AlphaImager HP image documentation system (Cell Biosciences) attached to a Sony ICX267AL 1.39 Megapixel CCD camera and processed using the AlphaView software program. This genotyping protocol was subsequently used to detect the vWF-iCre transgene from the tail snips of all mice when weaned at 3-4 weeks of age.

Verification of restricted expression using mT/mG reporter mice and determination of Cre recombinase efficiency in the bone marrow

In order to verify that Cre recombinase expression was restricted to vWF-expressing cells (megakaryocytes and microvascular endothelial cells), 8 week old male and female N1 founder hemizygous C57BL/6-SJL vWF-iCre/+ mice were mated with 8 week old male and female homozygous B6.129(Cg)-Gt(ROSA)26Sor$^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$/J mice (Jax mice Stock # 007676, kind gift from Dr. Jennifer Pollock, University of Alabama at Birmingham), designated as mT/mG$^{flox/flox}$. This is a mouse strain with a cell membrane-targeted, two-color fluorescent Cre recombinase reporter allele. Prior to Cre recombination with excision of loxP sites, the β-actin promoter driven cell membrane-localized tandem dimer Tomato (mT) red fluorescence protein expression is widely expressed in cells/tissues. The cell membrane-localized green fluorescent protein (mG) replaces mT in Cre-expressing cells/ tissues after recombination and excision (Hasegawa et al., 2013; Muzumdar et al., 2007). Genotyping was performed to detect the 128 base pair mutant and 212 base pair wildtype alleles according to the vendor's protocol (https://www.jax.org/Protocol?stockNumber=007676&protocolID=20368).

Three male and 2 female heterozyous mT/mG (mT/mG$^{flox/+}$) with (vWF-iCre/+) or without (+/+) the hemizygous transgene were injected with 100 mg/kg Tamoxifen in corn oil i.p. at 5 weeks of age to evaluate specificity and efficiency of Cre-mediated recombination 3 weeks after injection (Jahn et al., 2018). Bone marrow was isolated from the femurs and tibia of Tamoxifen-treated mT/mG$^{flox/+}$; vWF-iCre/+ and mT/mG$^{flox/+}$; +/+ mice immediately after euthanasia by cervical disarticulation under deep ketamine/xylazine anesthesia (100 mg/kg/10 mg/kg i.p.) (Amend et al., 2016). Four long bones were harvested per mouse and kept on ice prior to bulk processing.

Unfixed bone marrow smears were diffusely spread on Fisherbrand™ Superfrost™ glass slides (Fisher Scientific, catalog # 12-550-14G) without heparin treatment in the dark, carefully stained

with 0.45 µM 4′,6-diamidino-2-phenylindole (DAPI) in 1X phosphate buffered saline (PBS) for 5 minutes, washed once with 1X PBS for 5 minutes, and mounted using ProLong™ Gold Antifade aqueous mounting medium (ThermoFisher Scientific, catalog # P36930) with coverslips placed over the entire smear and sealed with nail polish. Slides were immediately visualized using a Nikon ECLIPSE Ci upright epifluorescent microscope attached to a Nikon DS-Qi2 monochromatic camera. Digital photomicrographs generated at 400X magnification to visualize bone marrow cells and detect megakaryocytes throughout each smear using the Nikon NIS Elements AR software program. The efficiency for Cre-mediated recombination was determined in each Tamoxifen-treated mT/mG$^{flox/+}$ mouse as follows: (total number of mG-expressing megakaryocytes divided by total number of mG or mT-expressing megakaryocytes) x 100%, with the mean recombination efficiency calculated.

Sciatic nerve indirect fluorescent immunohistochemistry reporter protein expression

Sciatic nerves were harvested from both hind limbs prior to removal of femurs and tibia in Tamoxifen-treated mT/mG$^{flox/+}$; vWF-iCre/+ and mT/mG$^{flox/+}$; +/+ mice as described above. Unfixed nerves were immediately embedded in Scigen Tissue Plus™ Optimum Cutting Temperature® (OCT) Compound (Fisher Scientific, catalog # 23-730-571) and stored at -80°C until processed. 10 µm axial cryostat sections were placed on Fisherbrand™ Superfrost™ slides (Fisher Scientific, catalog # 12-550-14G), air dried for 20 minutes at room temperature, fixed with 500 µL of 4% paraformaldehyde in 1X PBS for 30 minutes at room temperature and washed twice with 1X PBS for 10 minutes. Sections were blocked with 500 µL of 10% normal goat serum (NGS) in 1X PBS, followed by incubation with 1:100 dilution (5 µg/mL) of rabblt anti-red fluorescent protein (RFP) polyclonal IgG antibody (Abcam catalog # 62341) in 2% NGS in 1X PBS at room temperature for 1 hour. After washing with 2% NGS in 1X PBS for 10 minutes, sections were incubated with 1:500 dilution (4 µg/mL) of goat anti-rabbit IgG (H+L)

AlexaFluor®594 antibody (Invitrogen, catalog # A-32740) in 2% NGS in 1X PBS for one hour in the dark, with all subsequent processing performed in the dark to prevent photobleaching.

After washing with 2% NGS in 1X PBS for 10 minutes, sections were incubated with 1:500 dilution (10 µg/mL) of rabbit anti-green fluorescent protein (GFP) polyclonal IgG antibody (Abcam catalog # ab290) in 2% NGS in 1X PBS for 1 hour, followed by a wash as previously described, then incubated with 1:500 (4 µg/mL) dilution goat anti-rabbit IgG (H+L) AlexaFlour®488 antibody (Invitrogen, catalog # A-11008) in 2% NGS in 1X PBS for 1 hour. After washing, sections were stained with 0.45 µM DAPI in 1X phosphate buffered saline (PBS) for 5 minutes, washed once with 1X PBS for 5 minutes, mounted using ProLong™ Gold Antifade aqueous mounting medium (ThermoFisher Scientific, catalog # P36930) and coverslips placed and sealed with nail polish. Slides were stored at 4°C overnight and visualized with Nikon ECLIPSE Ci upright epifluorescent microscope attached to a Nikon DS-Qi2 monochromatic camera. Digital photomicrographs were generated using the auto-exposure feature (100 to < 1000 ms for the ultraviolet channel and 1s for the red and green channels) to acquire digital images with “look up table” (LUT) adjustments made to reflect observed signal intensities during microscopy in a non-destructive manner during image processing, taking into account variations in background signal and fluorescent intensities in different specimens. Images were merged using the Nikon NIS Elements AS software program.

Backcross to SJL using speed congenics

In order to study the role of microvascular endothelial cell-specific gene deletion in murine models of peripheral nerve inflammation (which are relatively mild in C57BL/6 mice), mixed strain hemizygous C57BL/6-SJL vWF-iCre/+ mice were backcrossed to the SJL background by speed congenics, performed at Charles River using their proprietary Marker-Assisted Accelerated Backcrossing (MAX-BAX®) protocol.

Briefly, a 384 single nucleotide polymorphism (SNP) panel was employed to generate mouse tail snip allelic profiles that were spread across the genome at 7 Mbp intervals for comparison to the wildtype SJL strain reference profile. Each SNP is dimorphic with one allele identified with fluorescent dye 6-Fluorescein amidite (FAM)-labeled complementary oligonucleotide (TaqMan) probe and the other allele detected using fluorescent dye modified *Aequorea victoria* Green Fluorescent Protein (VIC)-labeled probe. Homozygosity at each SNP markers was indicative of genetic alignment with SJL mice. The Percent Match of each mouse with the SJL background was determined by comparing the genotype at each SNP marker, with >99.9% match indicative of complete backcross.

To confirm Tamoxifen-induced specific Cre recombinase expression in microvascular endothelial cells in multiple organs, with higher expression relative to megakaryocytes in hemizygous vWF-iCre/+ SJL mice, 6-9 week old adult male and female mice were mated with age-matched homozygous B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG\text{-}tdTomato)Hze}$/J (Jax Mice Stock #007914, kind gift from Dr. Karen Gamble, University of Alabama at Birmingham), designated as Ai14 tdTomato$^{flox/flox}$. Prior to Cre-mediated recombination, expression of the CAG promoter driven tandem dimer Tomato (tdTomato) red fluorescent protein is prevented by a loxP-flanked stop cassette. Following Cre recombinase excision, transcription with subsequent translation of robust red fluorescent protein expression occurs in Cre-expressing cells/ tissues (Madisen et al., 2010). Genotyping was performed to detect the 196 base pair mutant and 297 base pair wildtype alleles according to the vendor's protocol (https://www.jax.org/Protocol?stockNumber=007914&protocolID=29436). Heterozygous mixed strain C57BL/6-SJL Ai14 tdTomato$^{flox/+}$; vWF-iCre/+ mice were intercrossed with homozygous C57BL/6 Ai14 tdTomato$^{flox/flox}$ mice to generate mixed strain C57BL/6-SJL Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ mice for subsequent reporter expression experiments.

Cohorts of 5-7 week old mixed strain C57BL/6-SJL Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ mice were injected with 100 mg/kg Tamoxifen in corn oil i.p. to evaluate specificity of Tamoxifen-induced Cre recombination, with mixed strain C57BL/6-SJL Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ injected with equivalent volumes of corn oil serving as controls for Tamoxifen-independent recombination (which has been described in Cre expressing Ai14 tdTomato$^{flox/flox}$ mice)(Alvarez-Aznar et al., 2020; Song and Palmiter, 2018), and mixed strain C57BL/6-SJL Ai14 tdTomato$^{flox/flox}$; +/+ (lacking the vWF-iCre transgene) injected with 100 mg/kg Tamoxifen in corn oil or corn oil alone serving as controls for non-specific or cell-independent red fluorescent protein expression.

24-25 days after injection, mice underwent whole body perfusion fixation via intracardiac infusion of 4% paraformaldehyde in 1X PBS at 37°C under deep ketamine-xylazine (100 mg/kg/ 10 mg/kg) anesthesia after a sterile 4°C 1X PBS infusion flush, as published (Gage et al., 2012; Wu et al., 2021) (courtesy of Dr. Scott Wilson, University of Alabama at Birmingham). The femur and tibial bones on both sides were harvested and processed for bone marrow smear analyses in the dark on the day of tissue harvest, as previously described. Both sciatic nerves, brain, spleen, kidneys and gastrocnemius muscles were harvested from each mouse, additionally fixed for 24 hours by immersion in 4% paraformaldehyde in 1X PBS at 4°C, washed three times in 1X PBS for 10 minutes and cryopreserved for 12-15 hours in 30% sucrose weight/volume in 1X PBS, washed as described above, and embedded in OCT Compound with storage at -80°C until further processing.

Direct fluorescent immunohistochemistry to evaluate selective microvascular endothelial cell Cre expression in reporter mice

Paraformaldehyde-fixed, sucrose cryoprotected frozen sciatic nerves, brain, spleen, kidneys and gastrocnemius muscles were evaluated to determine specific Tamoxifen-inducible Cre

recombinase expression in microvascular endothelial cells in the previously described cohort of mixed strain C57BL/6-SJL Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ mice and littermate controls injected with corn oil alone or tdTomato$^{flox/flox}$ mice lacking the vWF-iCre transgene.

10 µm axial or longitudinal cryostat sections were placed on Fisherbrand™ ColorFrost™ Plus slides (Fisher Scientific, catalog # 12-550-18) and air dried for 12-15 hours overnight in the dark at room temperature. Subsequent processing was performed in the dark to prevent endogenous tdTomato red fluorescence photobleaching. Sections were incubated with 500-1000 µL of 1:200 dilution (5 µg/mL) of *Lycoperiscon esculentum* agglutinin lectin-DyLight® 488 (fluorochrome-conjugated Tomato lectin (TL), which is the most sensitive marker for mouse vascular endothelial cells, binding to N-acetylglucosammine residues (Battistella et al., 2021)) in 1X PBS at room temperature for 1 hour. Following 1-2 washes with 1X PBS for 10 minutes each, autofluorescence quenching was performing using Vector® TrueVIEW® Autofluorescence Quenching Kit (Vector Laboratories, catalog # SP-8400) at room temperature, according to the vendor's protocol.

Following this process, sections were washed with 1X PBS 1-2 times for 10 minutes each, and treated with 500-1000 µL of 0.45 µM DAPI in 1X PBS for 5 minutes at room temperature. Following washes as described above, sections were mounted with VECTASHIELD Vibrance® Antifade Medium (provided with autofluorescence quenching kit) with coverslips placed over slides and sealed with nail polish. Slides were stored in the dark at 4°C for no longer than 48 hours, and viewed using a Nikon ECLIPSE Ci upright epifluorescent microscope attached to a Nikon DS-Qi2 monochromatic camera. Digital photomicrographs were generated and merged using the Nikon NIS Elements AR software program as previously described.

Paraformaldehyde-fixed bone marrow smears were generated on the day of whole animal fixation to verify retained megakaryocyte Cre expression in mixed strain C57BL/6-SJL Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ mice compared to littermate controls injected with corn oil alone or tdTomato$^{flox/flox}$ lacking the vWF-iCre transgene in the dark , as previously described. Splenic megakaryocytes were identified in paraformaldehyde-fixed, sucrose cryoprotected cryostat sections processed to detect Cre expression in microvascular endothelial cells, as described above, with comparative levels of expression qualitatively determined based on red fluorescence mean fluorescent intensity. Slides were viewed at 400X magnification using a Nikon ECLIPSE Ci upright epifluorescent microscope attached to a Nikon DS-Qi2 monochromatic camera, with digital photomicrographs generated and merged as previously described.

Results

The confirmed vWF-iCre transgene DNA sequence containing the human vWF promoter that includes the microvascular specific sequence (position -487 to +246 of the human vWF gene) (Aird et al., 1995; Jahroudi and Lynch, 1994) located at position 236 to 969 of the transgene sequence is shown in **Supplementary File 1**). Following microinjection into fertilized mouse eggs obtained by mating mixed strain C57BL/6 X SJL F1 male and female mice, and implantation into pseudopregnant females, 143 pups (77 males and 66 females) were born. Tail snip genotyping identified 7 male and 6 female mixed strain C57BL/6-SJL hemizygous vWF-iCre/+ founder mice (**Figure 1A**). 1 male and 2 female founder mice were mated with mT/mG$^{flox/flox}$ reporter mice to generate mT/mG$^{flox/+}$; vWF-iCre/+ reporter mice (**Figure 1B**) to evaluate Tamoxifen-dependent Cre recombination specificity and efficiency.

Bone marrow megakaryocyte specific Cre expression was observed in Tamoxifen-injected mT/mG$^{flox/+}$; vWF-iCre/+ mice with no Cre expression observed in Tamoxifen-injected

mT/mG$^{flox/+}$; +/+ littermate controls (**Figure 2**). Mean Cre recombination efficiency was 91.2% (range 75.9-100.0, N=5). Cre expression in structures morphologically consistent with microvessels was also observed in the sciatic nerves of Tamoxifen-injected mT/mG$^{flox/+}$; vWF-iCre/+ mice with Cre expression absent in Tamoxifen-injected mT/mG$^{flox/+}$; +/+ littermate controls (**Figure 3**). These data support the notion that the vWF-iCre transgene is specific and undergoes Tamoxifen-driven recombination with a high efficiency *in vivo*.

Male and female mixed strain C57BL/6-SJL hemizygous vWF-iCre/+ founder mice were backcrossed with wildtype SJL mice, and offspring genotyped to identify hemizygous vWF-iCre/+ mice (**Figure 4A**). The Percentage Match, indicative of alignment with wildtype SJL mice, was determined by tail snip genotyping and mice with highest SJL background were backcrossed with wildtype SJL mice until congenic SJL vWF-iCre/+ mice were obtained at N6. Following intercrossing wildtype SJL with hemizygous SJL vWF-iCre/+ for >10 generations, Ai14 tdTomato$^{flox/flox}$ reporter mice were mated with vWF-iCre/+ mice to generate Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ mice (**Figure 4B**) to directly evaluate specific Tamoxifen-induced microvascular endothelial cell Cre expression in multiple organs and evaluate for Tamoxifen-independent and non-specific Cre expression in these organs.

Tamoxifen-induced microvascular endothelial cell specific Cre expression was observed in the sciatic nerves (**Figure 5**), brains (**Figure 6**), spleens (**Figure 7**), kidneys (**Figure 8**) and gastrocnemius muscles (**Figure 9**) of Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ mice, with no Cre expression observed in Ai14 tdTomato$^{flox/flox}$; +/+ mice. Reduced red fluorescent protein expression was observed in splenic megakaryocytes compared to microvascular endothelial cells in Tamoxifen-induced Tomato$^{flox/flox}$; vWF-iCre/+ mice (**Figure 7**), supporting the notion that the transgene is preferentially transcribed in microvascular endothelial cells. However, variable Tamoxifen-independent microvascular endothelial cell Cre expression was observed in all

organs of some Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ mice evaluated (highest in the spleen and lowest in the brain), indicative of transient Cre recombinase expression during development or postnatally in corn oil-treated Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ mice or germline transmission of the excised tdTomato allele to these mice, as previously described as a limitation of the Ai14 tdTomato$^{flox/flox}$ reporter mouse strain (Alvarez-Aznar et al., 2020; Song and Palmiter, 2018).

Tamoxifen-dependent and -independent Cre recombination was also observed in the bone marrow of Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ mice with no Cre expression observed in Ai14 tdTomato$^{flox/flox}$; +/+ mice (**Figure 10**). These data show that that the vWF-iCre transgene is specifically expressed in microvascular endothelial cells and megakaryocytes in the SJL background; however independent recombination events preclude the use of Ai14 tdTomato reporter mice to study time-dependent microvascular endothelial cell specific gene deletion in SJL vWF-iCre/+ mice.

Discussion

We have successfully developed a Tamoxifen-inducible vWF Cre recombinase mouse strain with high recombination efficiency demonstrated in bone marrow megakaryocytes, and specific expression in microvascular endothelial cell in multiple organs, including peripheral nerves, using two reporter mouse strains. Detailed protocols are provided to allow independent verification of these results, including the vWF-iCre transgene sequence. Although mT/mG reporter mice are preferred over Ai14 tdTomato mice to determine cell and time-dependent Cre expression (Alvarez-Aznar et al., 2020), our inability to obtain these mice in a cost-effective and timely manner during the COVID-19 pandemic resulted in using Ai14 tdTomato reporter mouse to demonstrate microvascular endothelial cell specific Cre recombinase expression in congenic vWF-iCre/+ SJL mice.

The observed Tamoxifen-independent megakaryocyte and microvascular endothelial cell Cre expression in some Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ mice that was maximal in the spleen and least observed in the brain supports the notion that Cre expression is cell-specific, with potential transient Cre expression during development or postnatally accounting for these observations (Alvarez-Aznar et al., 2020; Song and Palmiter, 2018). No unexpected Cre expression occurred in the selected vWF-iCre/+ SJL mouse organs studied using the tdTomato reporter strain apart from gastrocnemius muscles that exhibited a variable mosaic pattern in fascicles in both Tamoxifen- and corn-oil treated mice. This also suggests germline or early development vWF gene transcription in some muscle fibers coupled with transient Cre-mediated recombination (Alvarez-Aznar et al., 2020; Song and Palmiter, 2018). These observations imply that the tdTomato reporter mice is inadequate to study the dynamics of vWF-iCre transgene expression in conditional gene knockout or knock in using this model.

Studies evaluating specific gene alterations in microvascular endothelial cells using the vWF-iCre/+ SJL mice should consider whether the gene(s) of interest is/are constitutively expressed in bone marrow megakaryocytes to ensure that platelet formation and function are not disrupted, as this could compromise wound healing or result in unexpected phenotypes. SJL mice are not great breeders, so these mice may be backcrossed to better breeder strains, such as the commonly used C57BL/6 background based on the experimental model studied. Tamoxifen-inducible vWF Cre recombinase mice would support ongoing studies aimed to understand the junctional complex and transporter biology of restrictive tight junction-forming microvascular endothelial barriers, such as the blood-brain, blood-nerve, blood-retina and blood-testis barriers during development, in health and normal aging, and in different disease states, guided by observational protein, proteome, transcript or transcriptome data from human specimens. Of particular relevance to peripheral nerves, the vWF-iCre model provides an avenue to study undetermined key molecular determinants and signaling pathways of unique

biologic networks ascertained from the published human blood-nerve barrier transcriptome, including microvascular endothelial cell-to-cell, and endothelial-glial, -pericyte, -perineurial and -immune cell crosstalk, to mention a few (Palladino et al., 2017).

Conclusions

A viable Tamoxifen-inducible vWF Cre recombinase SJL mouse strain has been developed with microvascular endothelial cell specific expression in multiple organs, including peripheral nerves. These mice are easily backcrossed to different mouse backgrounds without altering the transgene. This Cre recombinase strain would support hypothesis-driven mechanistic studies to decipher the role(s) of specific genes transcribed by microvascular endothelial cells during development, as well as in physiologic and pathophysiologic states in an organ- and time-dependent manner. Careful selection of reporter mouse strains that do not exhibit significant Tamoxifen-independent Cre expression is essential to track the dynamics of conditional gene deletion or expression *in vivo* or *in situ* using this model. Work is ongoing to decipher the essential or redundant role(s) of specific junctional complex genes transcribed by the restrictive tight-junction forming blood-nerve barrier endoneurial endothelial cells during development and following traumatic nerve injury using this model.

Acknowledgments

We acknowledge Wanda Filipiak and Galina Gavrilina for generating founder transgenic mice via pronuclear microinjection of fertilized eggs and the Transgenic Animal Model Core of the University of Michigan's Biomedical Research Core Facilities for their technical expertise. Special thanks to past members of the Neuromuscular Immunopathology Research Laboratory, particularly Dr. Chaoling Dong, for their essential technical assistance necessary for developing, characterizing and maintaining the Tamoxifen-inducible von Willebrand factor Cre recombinase SJL mouse strain colony at the University of Alabama at Birmingham.

Funding Sources

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Conflicts of Interest

The Tamoxifen-inducible von Willebrand factor Cre recombinase SJL mouse strain has been accepted for acquisition by the Mutant Mouse Resource & Research Center (MMRRC:071293-MU) for exclusive worldwide distribution to academic, non-profit and for-profit organizations and companies based on Materials Transfer Agreements and commercial licensing contracts

Data Statement

Data that support the study's findings are available to members of the scientific community on request from the corresponding author, E.E.U.

Figures with Captions

Figure 1. Mixed strain C57BL/6-SJL vWF-iCre/+ founder and mT/mG reporter mice genotyping. Reverse digital photomicrographs of 2% agarose electrophoresis gels in 1X TAE buffer show some results of tail snip genotyping of the vWF-iCre transgene in mixed strain C57BL/6-SJL founder mice following initial microinjection into pseudopregnant females, with the vWF-iCre transgene identified at 604 bp **(A)**. Following crossing with mT/mG$^{flox/flox}$ reporter mice, mT/mG$^{flox/+}$; vWF-iCre/+ F1 mice are generated to further determine Cre recombinase expression cell specificity and efficiency. The vWF-iCre transgene is identified at 604 bp, with the mutant and wildtype mT/mG genes identified at 128 bp and 212 bp respectively (**B**). C in the figures represents lanes with absent DNA (ddH_2O) template controls.

Figure 2. Tamoxifen-induced Cre recombinase expression in bone marrow megakaryocytes of mT/mG$^{flox/+}$; vWF-iCre/+ C57BL/6-SJL mice. Representative digital photomicrographs of unfixed bone marrow smears from mT/mG$^{flox/+}$; vWF-iCre/+ F1 mice show megakaryocyte-specific mT to mG expression following Tamoxifen-induced recombination (**A-L**) that does not occur in control mice lacking the vWF-iCre transgene (**M-P**). Scale bars: A-D and M-P = 100 µm, E-L = 50 µm.

Figure 3. Tamoxifen-induced Cre recombinase expression in sciatic nerve microvessels in mT/mG$^{flox/+}$; vWF-iCre/+ C57BL/6-SJL mice. Representative digital photomicrographs of paraformaldehyde-fixed cryostat axial sciatic nerve sections from mT/mG$^{flox/+}$; vWF-iCre/+ F1 mice show microvessel-like structure mT to mG expression following Tamoxifen-induced recombination, as detected by anti-RFP and anti-GFP antibodies (**A-D**, white arrows) that does not occur in control mice lacking the vWF-iCre transgene (**E-H**, white arrows). Higher magnification cropped digital photomicrographs of sciatic nerve endoneurial microvessel-like structures demonstrating mT to mG expression to support tissue-specific inducible Cre recombinase expression are shown in **I-P**, with vWF-iCre transgene absent littermate control

endoneurial microvessel-like structures without mT to mG expression shown in **Q-X** (white arrows). Scale bars: A-H = 100 µm, I-L and Q-T = 20 µm, M-P and U-X = 5 µm.

Figure 4. Mixed strain C57BL/6-SJL vWF-iCre/+ genotyping for speed congenics and mixed strain Ai14 tdTomato$^{flox/flox}$ SJL reporter mice genotyping. Representative reverse digital photomicrographs of 2% agarose electrophoresis gels in 1X TAE buffer show some results of tail snip genotyping of the vWF-iCre transgene in mixed strain C57BL/6-SJL mice during backcross to the SJL background, with the vWF-iCre transgene identified at 604 bp. Numbers above lanes indicate ear tag identification numbers required to identify preferred breeders following speed congenics **(A)**. Following development of the congenic SJL vWF-iCre/+ mouse strain with colony expansion, these mice were crossed with Ai14 tdTomato$^{flox/flox}$ reporter mice, and mixed strain Ai14 tdTomato$^{flox/+}$; vWF-iCre/+ F1 mice mated with Ai14 tdTomato$^{flox/flox}$ mice to generate Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ F2 mice to further determine Cre recombinase expression cell specificity. The vWF-iCre transgene is identified at 604 bp, with the mutant and wildtype Ai14 tdTomato genes identified at 196 bp and 297 bp respectively (**B**). C in the figure A represents lanes with absent DNA (ddH_2O) template controls.

Figure 5. Tamoxifen-induced Cre recombinase expression in sciatic nerve microvascular endothelial cells of Ai14 $^{flox/flox}$; vWF-iCre/+ SJL mice. Representative digital photomicrographs of paraformaldehyde-fixed, sucrose cryopreserved cryostat axial sciatic nerve sections from Ai14$^{flox/flox}$; vWF-iCre/+ SJL F2 mice show tdT expression co-localizing with Tomato Lectin (TL) green fluorescence expression, consistent with microvascular endothelial cell-specific Cre recombinase expression following Tamoxifen-induced recombination (**A-D**, white arrows). Cre expression was absent from the sciatic nerves in control mice lacking the vWF-iCre transgene (**E-H**). Higher magnification cropped digital photomicrographs of sciatic nerve endoneurial endothelial cells demonstrating tdTomato expression to support Tamoxifen-inducible Cre

recombinase expression are shown in **I-X**. Scale bars: A-H = 100 µm, I-K = 50 µm, M-P = 5 µm, Q-T = 20 µm, U-X = 10 µm.

Figure 6. Tamoxifen-induced Cre recombinase expression in brain microvascular endothelial cells of Ai14$^{flox/flox}$; vWF-iCre/+ SJL mice. Representative digital photomicrographs of paraformaldehyde-fixed, sucrose cryopreserved cryostat longitudinal brain sections from Ai14$^{flox/flox}$; vWF-iCre/+ SJL F2 mice show tdT expression co-localizing with Tomato Lectin (TL) green fluorescence expression, consistent with microvascular endothelial cell-specific Cre recombinase expression following Tamoxifen-induced recombination (**A-D**, white arrows). Cre expression was absent from the brains in control mice lacking the vWF-iCre transgene (**E-H**). Higher magnification cropped digital photomicrographs of brain microvascular endothelial cells demonstrating tdT expression to support Tamoxifen-inducible Cre recombinase expression are shown in **I-L** compared to absent tdT microvascular endothelial cell expression in the brains of mice that do not possess the vWf-iCre transgene, shown in **M-P**. Higher magnification brain microvessels with endothelial cell-specific tdT expression following Tamoxifen-induced recombination in Ai14$^{flox/flox}$; vWF-iCre/+ are also shown (**Q-T**). Scale bars: A-H = 200 µm, I-L = 50 µm, M-T = 25 µm.

Figure 7. Tamoxifen-induced Cre recombinase expression in spleen microvascular endothelial cells and splenic megakaryocytes of Ai14 $^{flox/flox}$; vWF-iCre/+ SJL mice. Representative digital photomicrographs of paraformaldehyde-fixed, sucrose cryopreserved cryostat longitudinal spleen sections from Ai14$^{flox/flox}$; vWF-iCre/+ SJL F2 mice show tdT expression co-localizing with Tomato Lectin (TL) green fluorescence expression, consistent with microvascular endothelial cell-specific Cre recombinase expression following Tamoxifen-induced recombination (**A-F**, white arrows). Cre expression was absent from the spleens in control mice lacking the vWF-iCre transgene (**E-H**). Representative digital photomicrographs of paraformaldehyde-fixed, sucrose

cryopreserved cryostat longitudinal spleen sections from Ai14$^{flox/flox}$; vWF-iCre/+ SJL F2 mice show low tdT expression in splenic megakaryocytes (**J-K**, white arrows) compared to high expression in microvascular endothelial cells seen with lower magnification images, consistent with Tamoxifen-induced Cre recombinase expression driven by a microvascular endothelial-cell specific vWF promoter. Scale bars: A-C and G-K = 200 µm, D-F = 100 µm.

Figure 8. Tamoxifen-induced Cre recombinase expression in kidney microvascular endothelial cells of Ai14 $^{flox/flox}$; vWF-iCre/+ SJL mice. Representative digital photomicrographs of paraformaldehyde-fixed, sucrose cryopreserved cryostat longitudinal kidney sections from Ai14$^{flox/flox}$; vWF-iCre/+ SJL F2 mice show tdT expression co-localizing with Tomato Lectin (TL) green fluorescence expression, consistent with microvascular endothelial cell-specific Cre recombinase expression following Tamoxifen-induced recombination in the cortex (**A-D**, white arrows). Cre expression was absent from the cortical microvessels in control mice lacking the vWF-iCre transgene (**E-H**). Medullary microvessels demonstrating tdT expression following Tamoxifen-induced recombination in Ai14$^{flox/flox}$; vWF-iCre/+ SJL F2 mice are shown (**I-L**, white arrows). Higher magnification cropped digital photomicrographs of the kidney cortex shows microvascular endothelial cell tdT expression in a glomerulus (G) and post-capillary venule (PCV) that is absent from a peritubular capillary (PTC) in a Ai14$^{flox/flox}$; vWF-iCre/+ SJL F2 mouse, supporting selective kidney microvascular endothelial cell Tamoxifen-inducible Cre recombinase expression (**M-P)**. Scale bars: A-H = 200 µm, I-L = 100 µm, M-P = 50 µm.

Figure 9. Tamoxifen-induced Cre recombinase expression in gastrocnemius muscle microvascular endothelial cells of Ai14 $^{flox/flox}$; vWF-iCre/+ SJL mice. Representative digital photomicrographs of paraformaldehyde-fixed, sucrose cryopreserved cryostat axial muscle sections from Ai14$^{flox/flox}$; vWF-iCre/+ SJL F2 mice show tdT expression co-localizing with Tomato Lectin (TL) green fluorescence expression, consistent with microvascular endothelial

cell-specific Cre recombinase expression following Tamoxifen-induced recombination (**A-D**, white arrows). A mosaic pattern of tdT expression is observed within muscle fascicles. Cre expression was absent from the gastrocnemius muscles in control mice lacking the vWF-iCre transgene (**E-H**). Higher magnification cropped digital photomicrographs of endomysial microvascular endothelial cells demonstrating tdT expression to support Tamoxifen-inducible Cre recombinase expression are shown in **I-L**. Scale bars: A-H = 100 µm, I-L = 10 µm.

Figure 10. Tamoxifen-induced Cre recombinase expression in bone marrow megakaryocytes of Ai14 $^{flox/flox}$; vWF-iCre/+ SJL mice. Representative digital photomicrographs of paraformaldehyde-fixed bone marrow smears from Ai14 tdTomato$^{flox/flox}$; vWF-iCre/+ F2 mice show high (yellow arrows) and low (white arrows) megakaryocyte-specific tdT expression following Tamoxifen-induced recombination (**A-F**) that does not occur in control mice lacking the vWF-iCre transgene (**G-L**). Scale bars: A-L = 50 µm.

Supplementary Figure 1. vWF-iCre transgene sequence. The confirmed transgene sequence containing the human vWF promoter, with the microvascular specific sequence located at position 236 to 969 (yellow highlight), followed by the inducible Cre recombinase sequence and the polyadenylation signal is shown.

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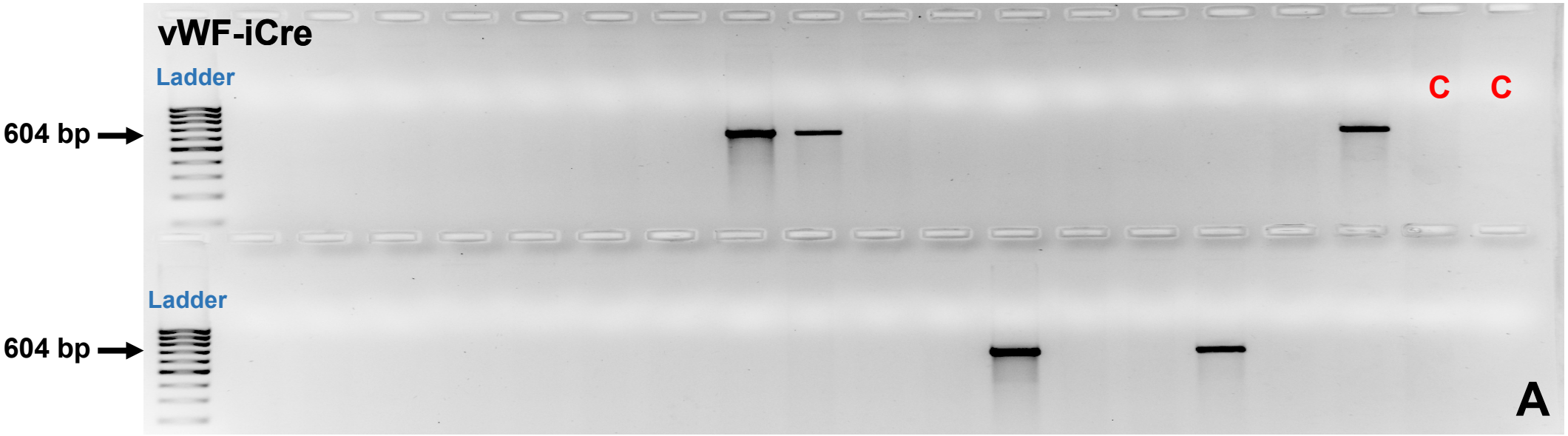
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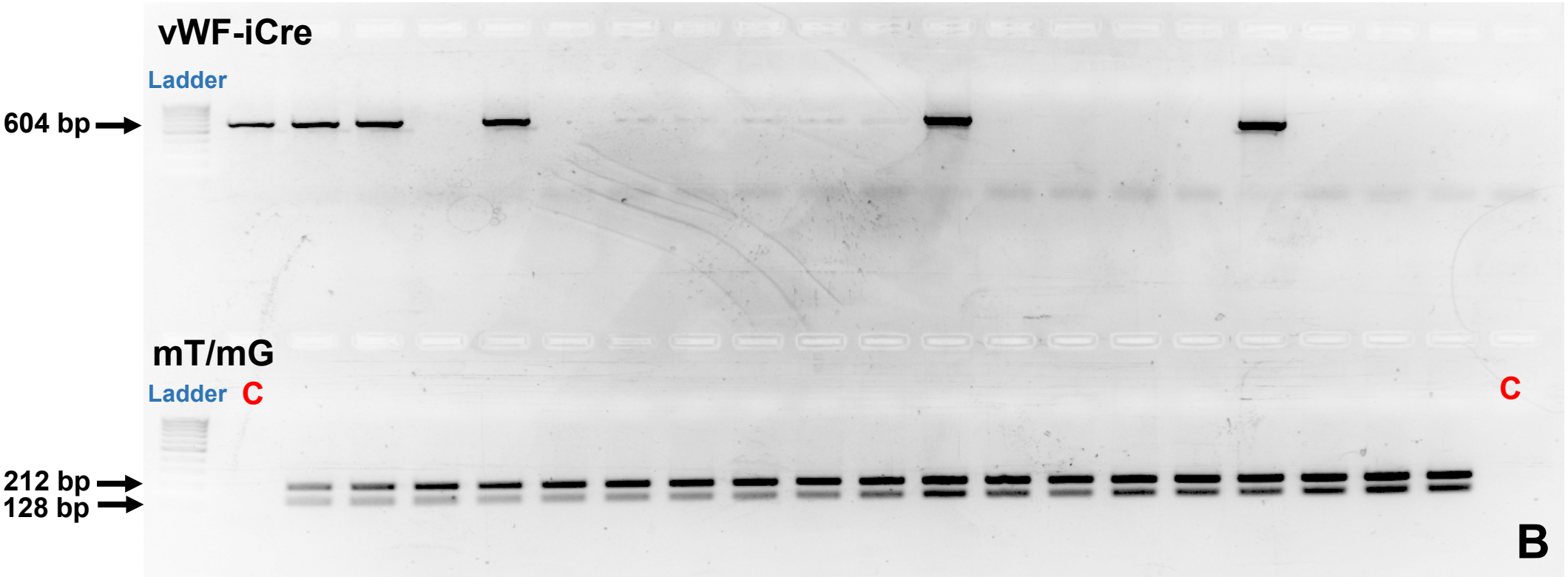
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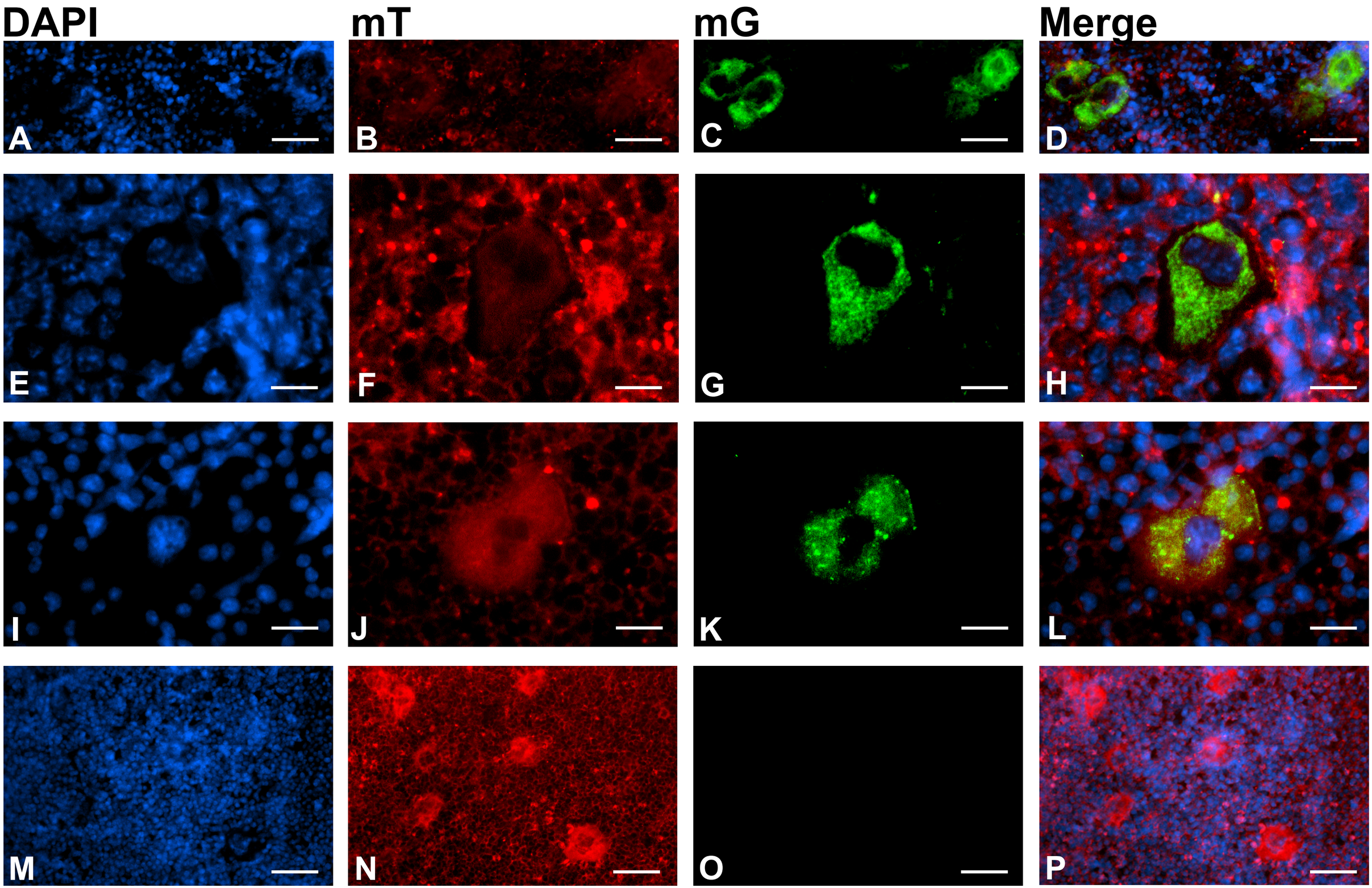
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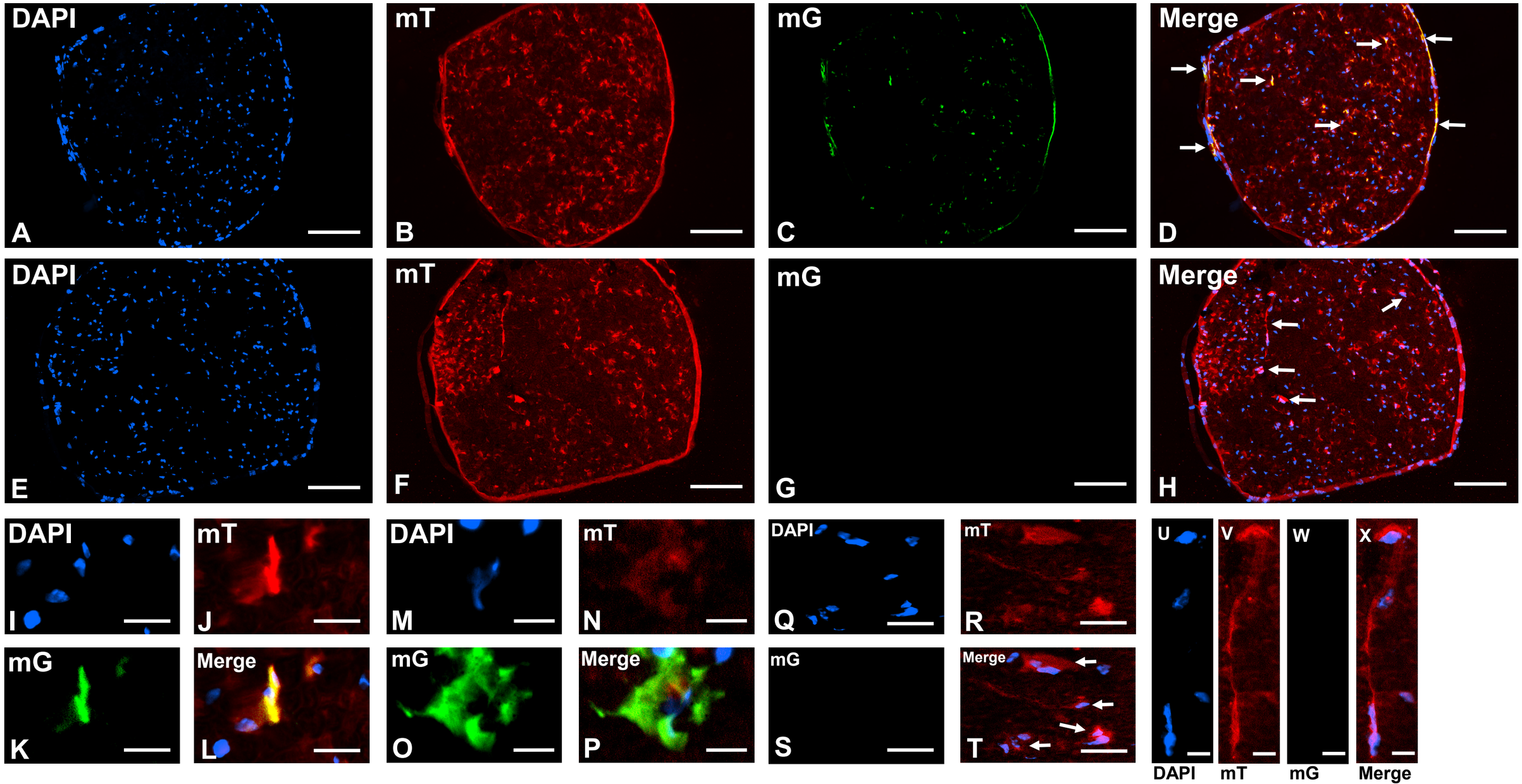
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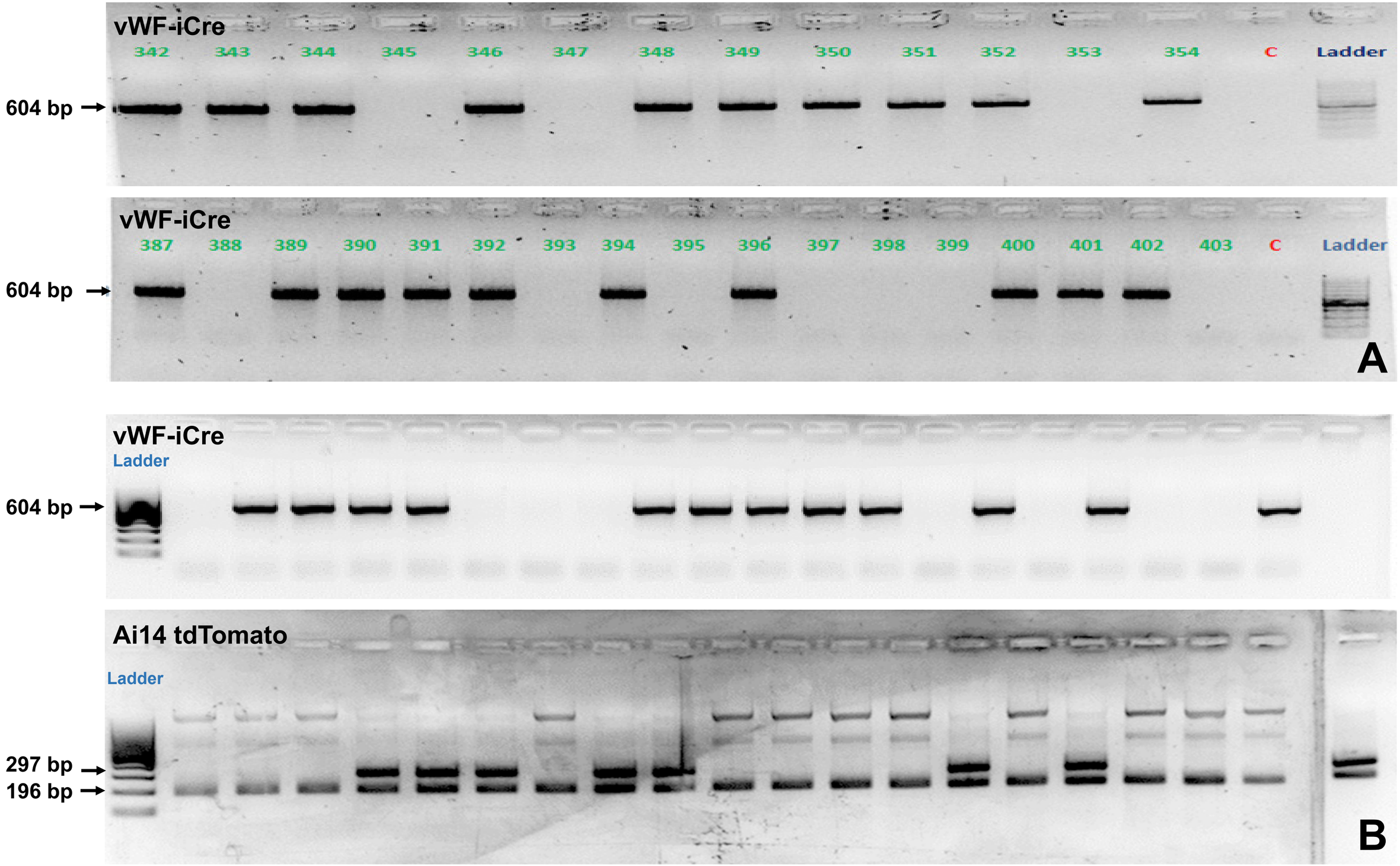
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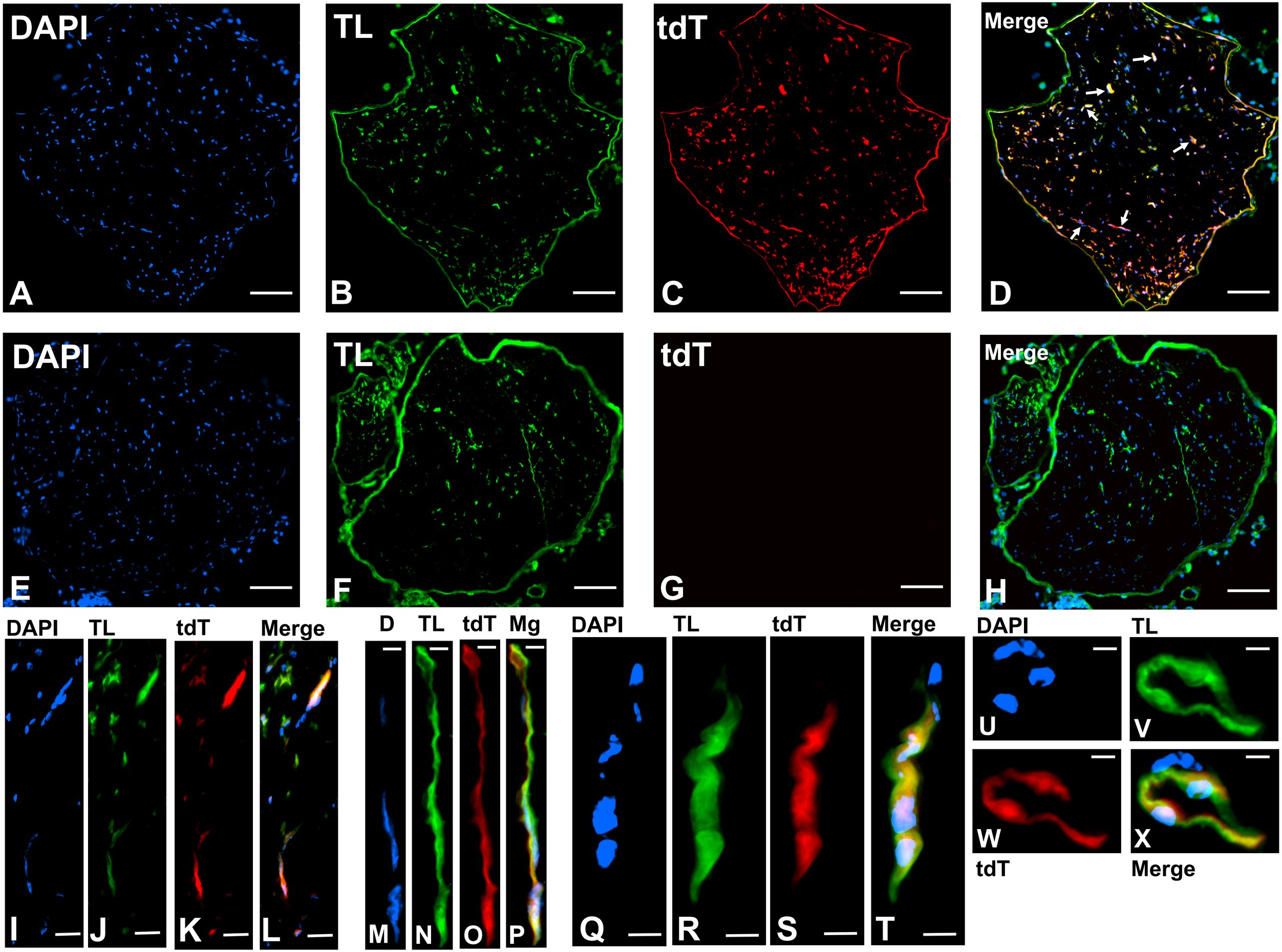
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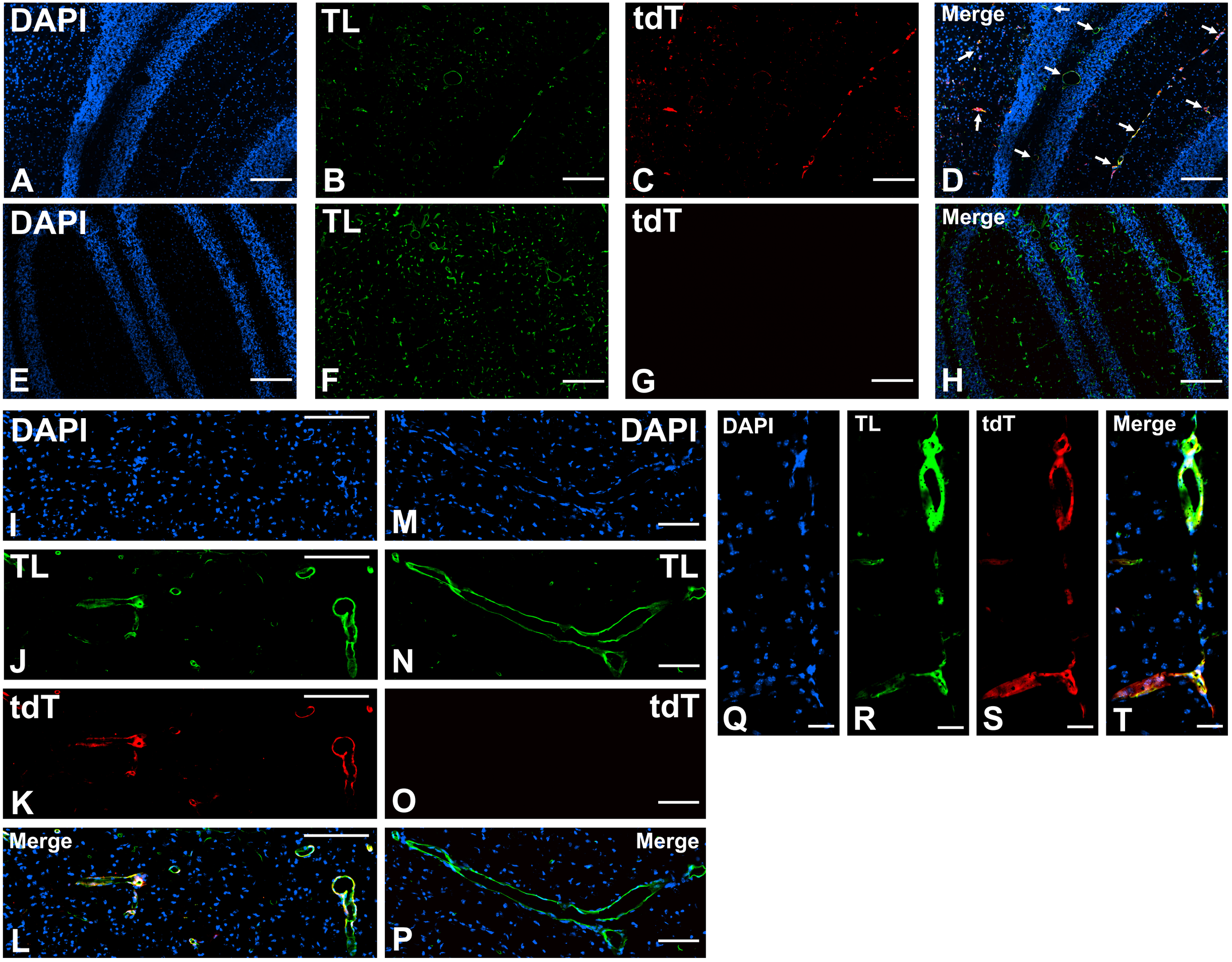
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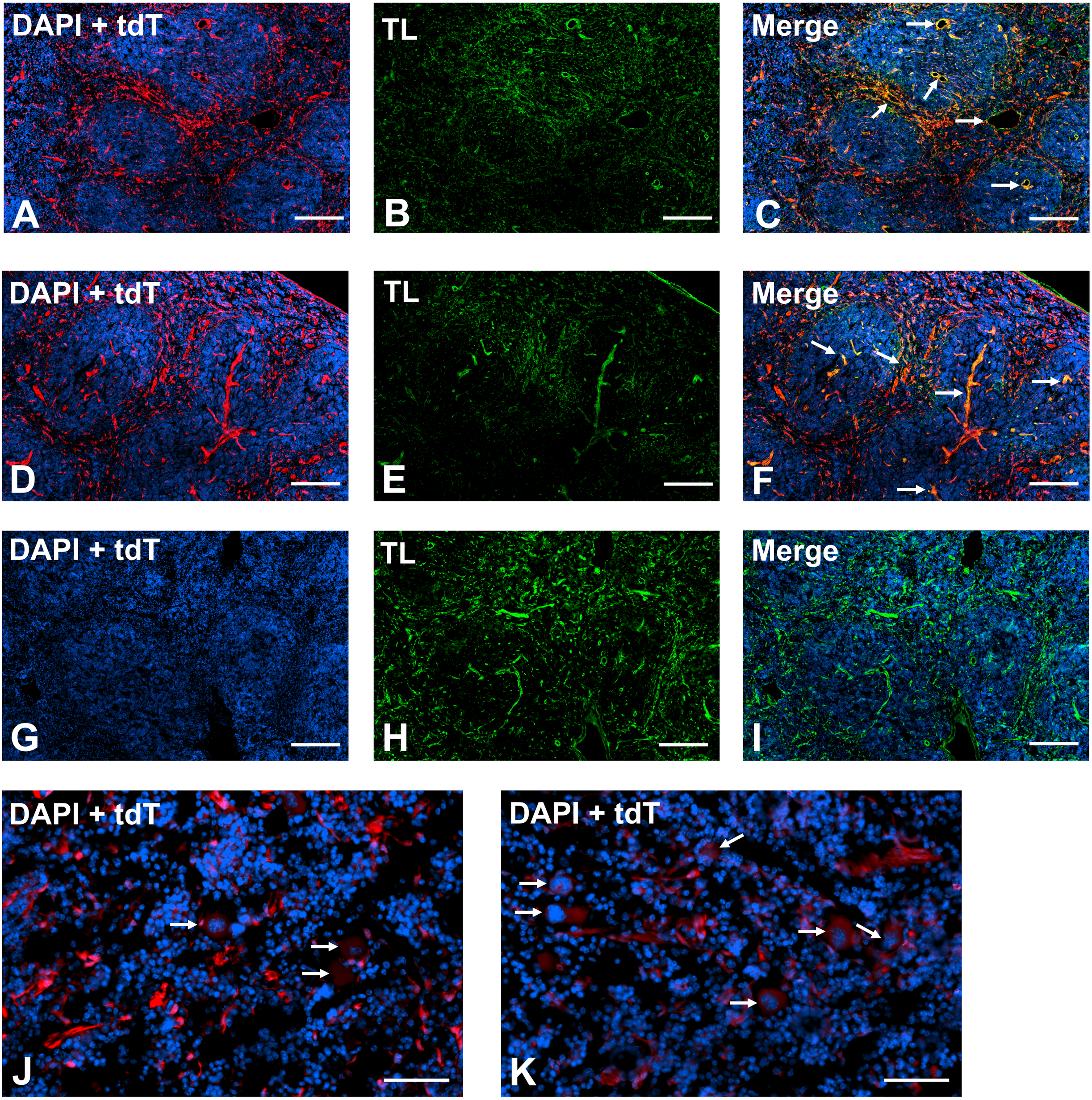
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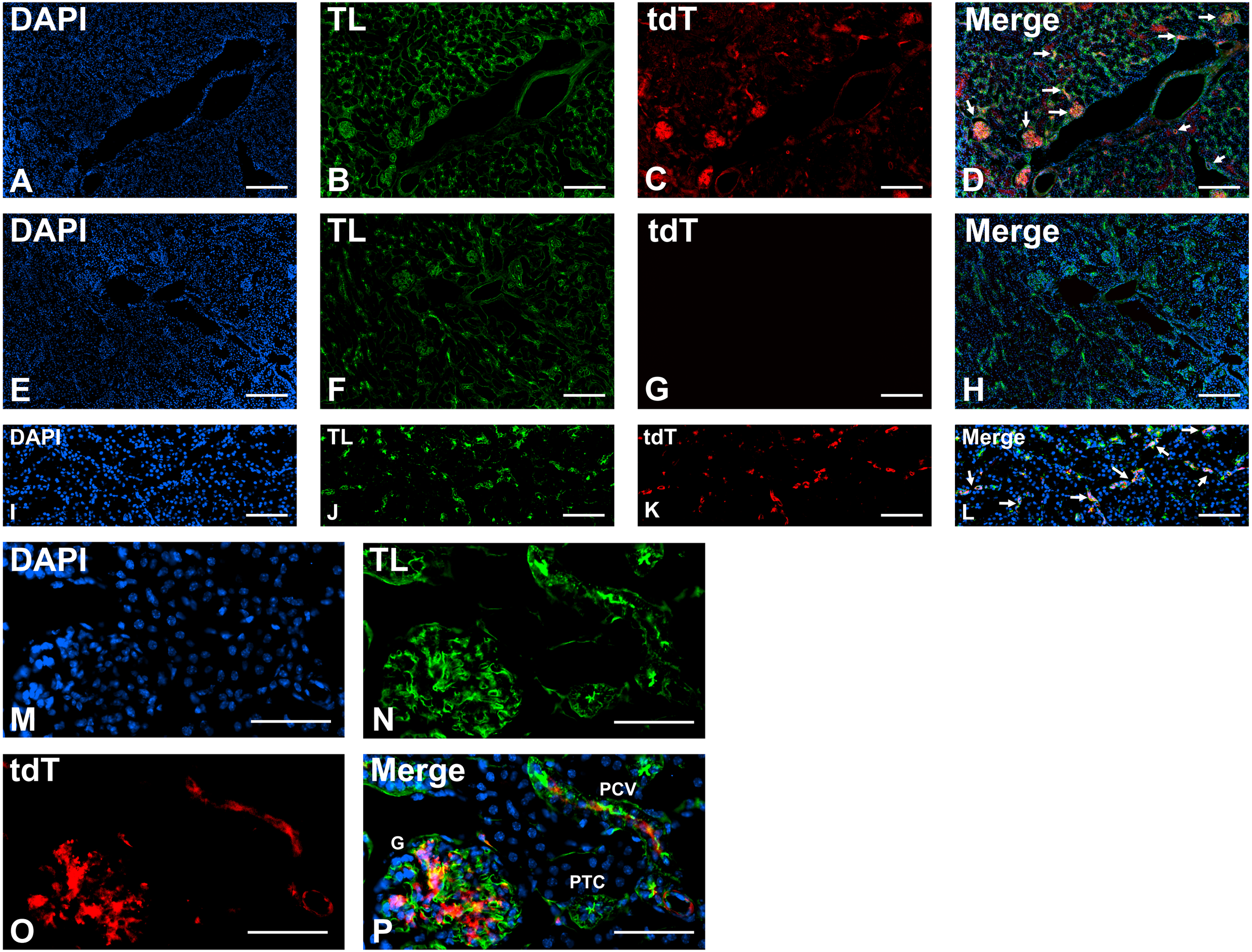
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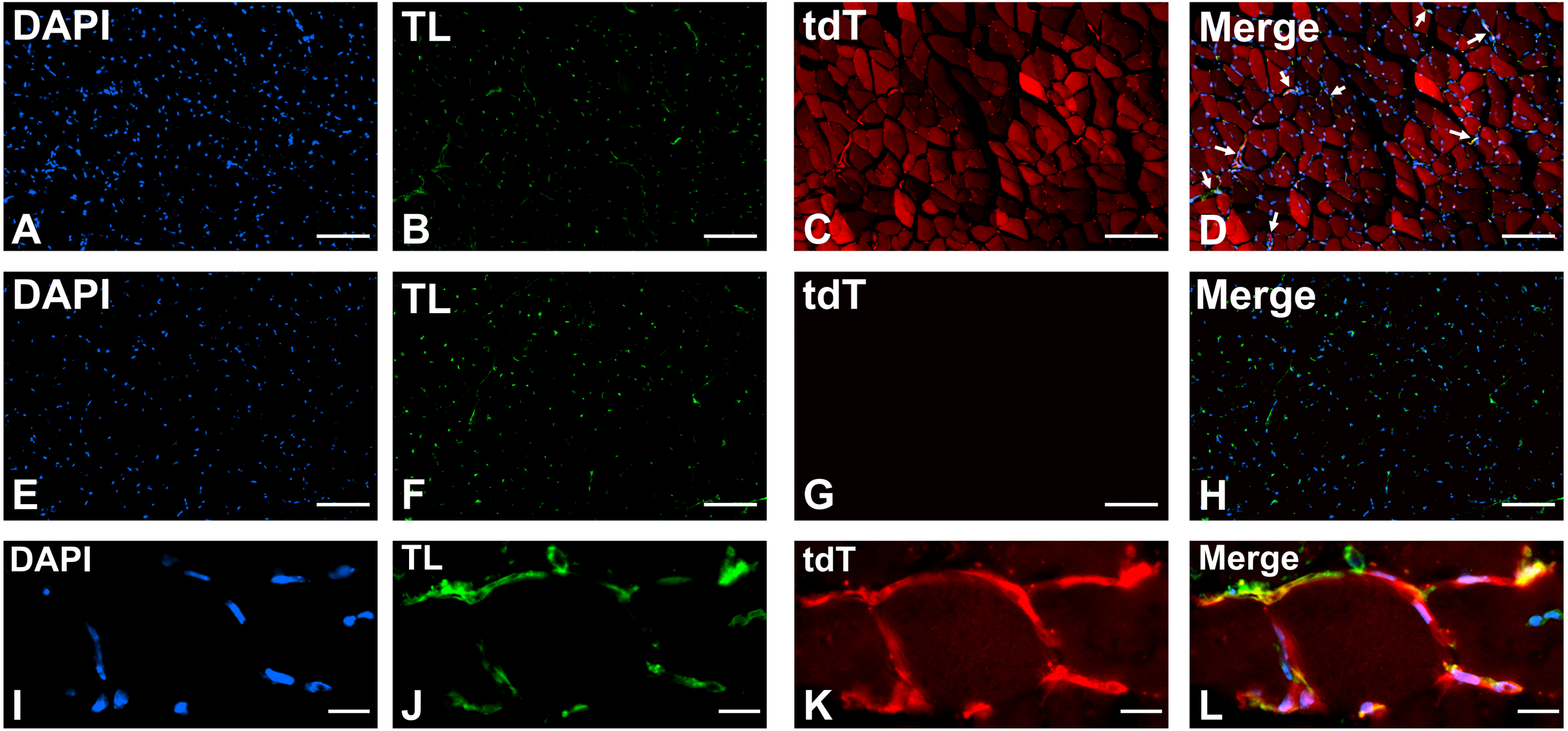
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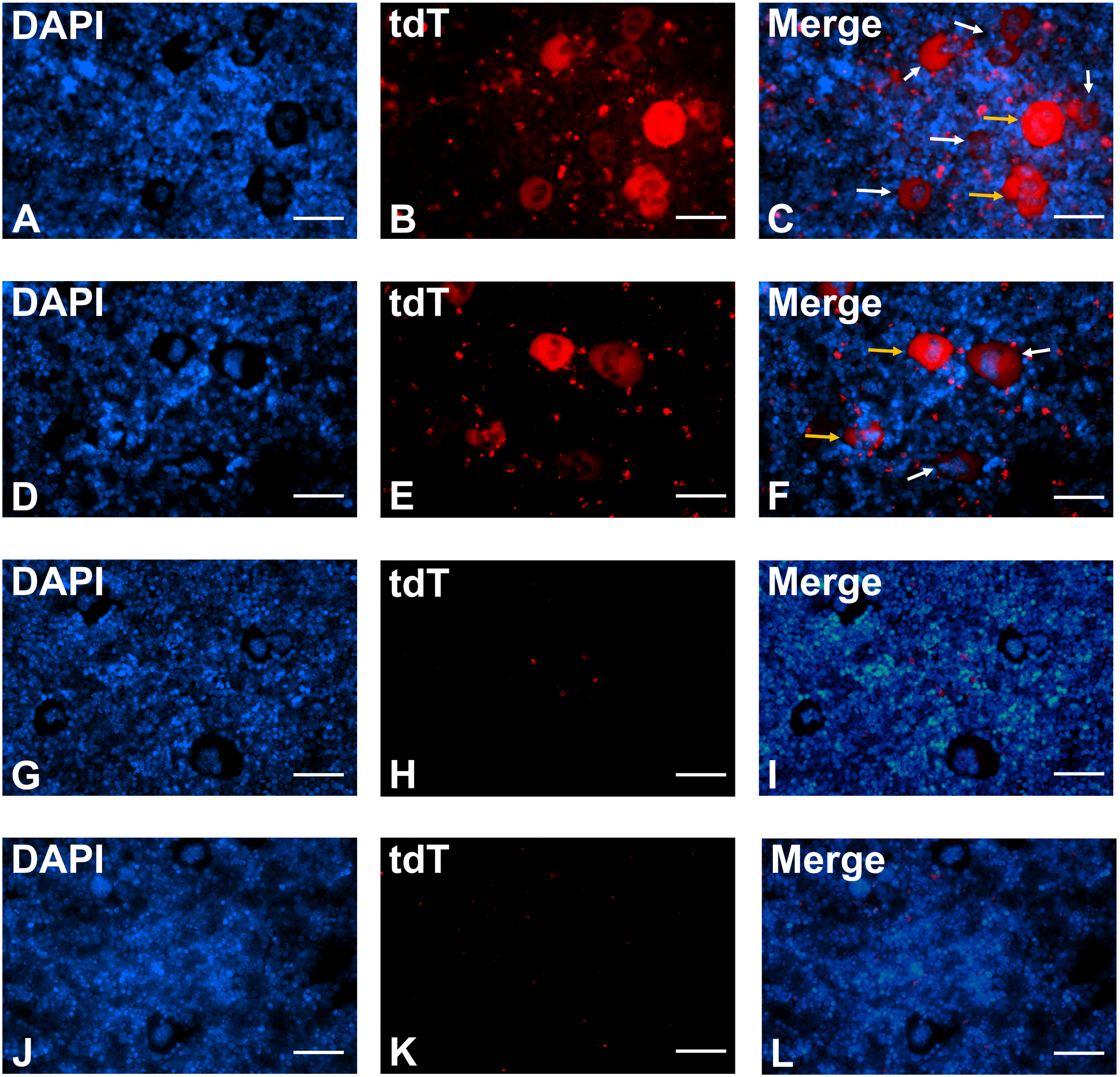
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