

1 **The balance between Cathepsin C and Cystatin F**
2 **controls remyelination in the brain of *Plp1*-**
3 **overexpressing mouse, a chronic demyelinating**
4 **disease model.**

5 Takahiro Shimizu^{1,2,8}, Wilaiwan Wisessmith^{1,2,5}, Jiayi Li^{2,3}, Manabu Abe⁴, Kenji
6 Sakimura⁴, Bantit Chetsawang⁵, Yoshinori Sahara⁹, Koujiro Tohyama^{7,9}, Kenji F.
7 Tanaka^{2,6}, and Kazuhiro Ikenaka^{2,3}

8 1 These authors contributed equally.

9 2 Division of Neurobiology and Bioinformatics, National Institute for Physiological
10 Sciences, Okazaki, Japan.

11 3 Department of Physiological Sciences, Graduate University for Advanced Studies
12 (SOKENDAI), Okazaki, Japan.

13 4 Brain Research Institute, Niigata University, Niigata, Japan.

14 5 Research Center for Neuroscience, Institute of Molecular Biosciences, Mahidol
15 University, Salaya, Nakhonpathom, Thailand.

16 6 Department of Neuropsychiatry, Keio University, Tokyo, Japan.

17 7 Center for Electron Microscopy and Bio-Imaging Research, Iwate Medical
18 University, Iwate, Japan

19 8 (Current affiliation): Wolfson Institute for Biomedical Research, University College
20 London, London, United Kingdom.

21 9 Department of Physiology, Iwate Medical University School of Dentistry, Iwate,
22 Japan.

23

1 Correspondence should be addressed to: Kazuhiro Ikenaka, 5-1 Aza-Higashiyama, Myodaiji,
2 Okazaki, Aichi, Japan 444-8787. Telephone number: +81-564-59-5245.

3 E-mail address: ikenaka@nips.ac.jp

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5 **Running title**

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1 **Main points**

2 -- The balance between cystatin F (CysF) and cathepsin C (CatC) regulates remyelination in a
3 mouse model of demyelinating disease.

4 -- CysF and CatC knockdown, and CatC overexpression in microglia were generated using a
5 versatile gene manipulation system.

6

7 **Keywords**

8 Demyelination, remyelination, microglia, cysteine protease inhibitor

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10 **The authors have no conflicts of interest to declare.**

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1 **Abstract**

2 In demyelinating diseases such as multiple sclerosis (MS), an imbalance between the
3 demyelination and remyelination rates underlies the degenerative processes. Microglial
4 activation is observed in demyelinating lesions; however, the molecular mechanism
5 responsible for the homeostatic/environmental change remains elusive. We previously found
6 that cystatin F (CysF), a cysteine protease inhibitor, is selectively expressed in microglia only
7 in actively demyelinating/remyelinating lesions but ceases expression in chronic lesions,
8 suggesting its role in remyelination. Here we report the effects of manipulating the expression
9 of CysF and cathepsin C (CatC), a key target of CysF, in a murine model of transgenic
10 demyelinating disease, *Plp^{4e/-}*. During the active remyelinating phase, both CysF knockdown
11 (CysFKD) and microglial-selective CatC overexpression (CatCOE) showed a worsening of
12 the demyelination in *Plp^{4e/-}* transgenic mice. Conversely, during the chronic demyelinating
13 phase, CatC knockdown (CatCKD) ameliorated the demyelination. Our results suggest that
14 the balance between CatC and CysF expression controls the demyelination and remyelination
15 process.

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1 **Introduction**

2 In multiple sclerosis (MS), the immune system attacks the myelin sheath causing
3 inflammation and injury to the sheath, and ultimately to the nerve fibers that it surrounds.
4 This results in numerous sclerotic regions and the appearance of naked axons where
5 premyelinating oligodendrocytes failed to remyelinate the axons. It has become increasingly
6 important to elucidate the causes of this impaired remyelination in order to aid the
7 development of a treatment for chronic MS lesions. An imbalance in the
8 demyelination/remyelination rate underlies the degenerative processes in demyelinating
9 lesions. By investigating the environmental cues associated with this imbalance, insights into
10 means of limiting demyelination and promoting remyelination may be obtained. It was
11 originally proposed that the main cause of impaired remyelination was the lack of
12 oligodendrocyte progenitor cells (OPCs). However, premyelinating oligodendrocytes and
13 OPCs have been found in demyelinated MS lesions (Chang et al., 2000; Chang et al., 2002)
14 and in mice with experimental autoimmune encephalomyelitis (EAE) (Girolamo et al., 2011).
15 Moreover, we have shown that additional oligodendrocyte lineage cells are produced during
16 repair, but in chronic demyelinated lesions their processes degenerate after maturation
17 (Shimizu et al., 2013).

18 Thus, in chronic demyelinated lesions the environment surrounding oligodendrocytes
19 and OPCs likely changes from being one that is permissive to remyelination to one that is
20 non-permissive. The significance of the environment surrounding oligodendrocytes and
21 OPCs has been gaining increased attention due to the identification of factors such as PSA-
22 NCAM (Charles et al., 2002) and hyaluronan (Back et al., 2005) that inhibit remyelination.

23 Microglia are well known to act as innate resident immune cells and active surveyors
24 of the extracellular environment in the central nervous system (CNS) (Arnoux and Audinat,
25 2015; Kreutzberg, 1996; Ransohoff and Perry, 2009; Schafer et al., 2012; Shemer et al.,

1 2015). During the remyelination process, microglia play a critical role in myelin removal
2 (Kotter et al., 2006). However, it has been reported that microglial paralysis can repress
3 experimental autoimmune encephalomyelitis (EAE) (Heppner et al., 2005). Moreover, it is
4 known that macrophages laden with myelin are anti-inflammatory in MS (Boven et al., 2006),
5 and that myelin-phagocytosing macrophages in the CNS and the peripheral nervous system
6 (PNS) show both inflammatory and anti-inflammatory responses (van Rossum et al., 2008).
7 Also, CCR5 expression on microglia is associated with early remyelination (Trebst et al.,
8 2008). Thus, the comprehensive role of microglia in remyelination remains elusive.

9 We have utilized the hemizygous proteolipid protein 1 (*Plp1*) transgenic mouse strain,
10 4e (*Plp^{4e/-}*), which has two extra *Plp1* genes, and demonstrated its use as an ideal model for
11 studying demyelination and remyelination (Kagawa et al., 1994; Shimizu et al., 2013). The
12 use of appropriate animal models is essential for studying the mechanisms underlying these
13 environmental changes. Transgenic *Plp^{4e/-}* mice appear to undergo normal myelination with
14 no signs of myelin degeneration until two months of age. However, at two months of age they
15 show spontaneous demyelination accompanied by remyelination in the early phase but which
16 terminates after 6 months of age (during the chronic demyelinating phase), resulting in the
17 appearance of chronic lesions throughout the central nervous system and naked axons
18 (Kagawa et al., 1994). During the demyelinating phase, *Plp^{4e/-}* mice do not show T-cell
19 infiltration in the lesions. However, an upregulation of *IL-1 β* and *TNF- α* mRNA was detected,
20 although their protein levels were below detection (unpublished data). While the
21 oligodendrocytes extended processes parallel with the axons, they failed to form myelin
22 structure, indicative of the presence of premyelinating oligodendrocytes (Ma et al., 2006).
23 The presence of premyelinating oligodendrocytes in chronic demyelinated lesions is also
24 associated with MS (Chang et al., 2002). Thus, we used the *Plp^{4e/-}* mouse as a model to study
25 the mechanisms behind remyelination impairment in chronic lesions.

1 In a previous study, we performed cDNA microarray analysis using *Plp^{4e/-}* mice to
2 search for changes in gene expression (Ma et al., 2007). We identified that microglial CysF
3 expression is indicative of ongoing demyelination/remyelination and the absence of CysF
4 expression indicates the cessation of remyelination in demyelinating lesions (Ma et al., 2011).
5 Cystatin F is a cysteine protease inhibitor expressed selectively in immune cells (Halfon et al.,
6 1998). The official symbol of cystatin F is Cst7, also known as CMAP (cystatin-like
7 metastasis-associated protein) and leukocystatin; in this study we will use CysF for the
8 abbreviation of cystatin F. A major target of CysF is the lysosomal cysteine protease
9 cathepsin C (Hamilton et al., 2008), which is known to regulate the activation of effector
10 granule-associated serine proteases in T cells, natural killer cells, neutrophils and mast cells
11 that are related to immune and inflammatory processes (Adkison et al., 2002; Eyles et al.,
12 2006). The official symbol of cathepsin C is Ctsc, also known as DPP1, DPPI and AI047818,
13 but here we will use CatC as the abbreviation for cathepsin C. In our previous study, CysF
14 was shown to be expressed by activated microglia primarily in the white matter during acute
15 demyelination and active remyelination, but its expression was terminated during the chronic
16 demyelinating phase (at 8 months of age) except in regions where the myelin remained intact.
17 Moreover, in MS, CysF expression is limited to the remyelinating plaques but not found
18 within the chronic lesions, consistent with the pattern in the *Plp^{4e/-}* mouse model of
19 demyelinating disease (Ma et al., 2011).

20 In this study, we investigated the roles of CatC and CysF in *Plp^{4e/-}* mice. We
21 generated novel transgenic mouse lines in which the levels of CatC or CysF can be
22 manipulated by use of the Flexible Accelerated STOP Tetracycline Operator knockin
23 (FAST) system (Tanaka et al., 2010). We show that the balance between CatC and CysF
24 expression in the brain controls remyelination in chronic demyelinating lesions. These results

1 should aid in the development of novel and effective treatments for MS and other
2 demyelinating diseases.

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1 **Materials and Methods**

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3 **Ethics statement**

4 All animal procedures were conducted in accordance with the guidelines described by the
5 National Institutes of Health Guide for the Care and Use of Laboratory Animals, and
6 approved by the National Institute for Physiological Sciences Animal Care and Use
7 Committee. The generation of STOP-tetO mice was in accordance with the guidelines set by
8 the animal welfare committee and the ethics committee of Niigata University.

9

10 **Generation of STOP-tetO knockin mice**

11 The targeting vector containing the 10 kb 3'-homology arm, Neo STOP-tetO cassette, 1.8 kb
12 5'-homology arm, and diphtheria toxin A subunit (DTA) was designed to insert the Neo
13 STOP-tetO cassette just upstream of the cathepsin C or cystatin F translation initiation site
14 (Tanaka et al., 2010). The C57BL/6N ES cell line RENKA (Mishina and Sakimura, 2007)
15 was used for the recombination. Chimeric mice were mated with C57BL/6N mice, and
16 germline-transmitted offspring were established as CatC STOP-tetO and CysF STOP-tetO
17 heterozygous knockin mice (CatC^{STOP-tetO/+} and CysF^{STOP-tetO/+}, respectively).

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19 **Southern blotting**

20 Genomic DNA was digested with *SpeI* or with *PstI* and *EcoRI* for 5'- and 3'-homology arms
21 of CatC STOP-tetO mice, and with *AflIII* or with *XbaI* and *EcoRV* for the 5'- or 3'-homology
22 arms of CysF STOP-tetO mice, respectively. The fragments were separated on a 0.8%
23 agarose gel, and blotted onto a nylon membrane. ³²P-random-prime-labeled probe (Prime-it II,
24 Stratagene, La Jolla, CA) was hybridized. The probe positions for the 5'- and 3'-homology
25 arms were from -4048 to -3029 or from -3036 to -2077 bases upstream, and from 5373 to

1 6103 or from 4615 to 5664 bases downstream from the translation initiation site of CatC or
2 CysF, respectively. The probes were located outside of and within the 5'- and 3'-homology
3 arms, respectively. Predicted sizes were: 10 kb and 6.8 kb (*SpeI* digestion) for the 5'-
4 homology arm in the wild type allele and the CatC STOP-tetO allele; and 10 kb (*PstI*
5 digestion) or 5 kb (*EcoRI* digestion) in the wild type allele and 8 kb (*PstI* digestion), 5 kb
6 (*EcoRI* digestion) in the CatC STOP-tetO allele for the 3'-homology arm, and 8.5 kb and 5.7
7 kb (*AflIII* digestion) for the 5'- homology arm in the wild type allele and the CysF STOP-tetO
8 allele; and 11 kb (*XbaI* digestion) and 12 kb (*EcoRV* digestion) in the wild type allele and 7.5
9 kb (*XbaI* digestion), 12 kb (*EcoRV* digestion) in the CysF STOP-tetO allele for the 3'-
10 homology arm, respectively.

11

12 **Animals**

13 We used the hemizygous *Plp1* transgenic mouse strain 4e (*Plp^{4e/-}*; C57Bl6/DBA2 mixed
14 background; Kagawa et al., 1994), CysF STOP-tetO mouse (*CysF^{STOP-tetO/+}*), CatC STOP-
15 tetO mouse (*CatC^{STOP-tetO/+}*) (both C57BL/6N background) and Iba1-tTA mouse line 75
16 (initial BDF1 background backcrossed to C57BL/6 background; Tanaka et al., 2012).
17 Genotyping of the *Plp^{4e/-}* and Iba1-tTA mice was performed as described previously (Kagawa
18 et al., 1994; Tanaka et al., 2012). For CysF STOP-tetO mice, we used CysF 791L: 5'-
19 GCTGCTGTTATGCTTGATCCC-3', CysF 708L: 5'-TCTCAGGGTTCCAAGAGTGTCC-
20 3', CysF 202U: 5'-TTTCTTCACATCAGCATCCC-3' and tetOup: 5'-
21 AGCAGAGCTCGTTTAGTGAACCGT-3' primers. For CatC STOP-tetO mice,
22 CatC205L:5'-AAGGCAAGGACTCAGGGACAGAAA-3', CatC361U: 5'-
23 TTTGGCGTTCCTTGAAAGGCAGAG-3' and tetOup primers. To detect the CysF or CatC
24 WT allele, we used CysF202U and CysF708L (PCR product size: 910 bp), or CatC361U and
25 CatC205L (PCR product size: 566 bp), respectively. To detect the CysF STOP-tetO or CatC

1 STOP-tetO KI allele, we used tetOup and CysF791L (PCR product size: approximately 930
2 bp) or CatC205L (PCR product size: approximately 340 bp), respectively.

3

4 **Preparation of primary cultured microglia and immunocytochemistry**

5 The previously reported method for rat primary microglial cultures (Sawada et al., 1990;
6 Suzumura et al., 1987) was applied to mouse. Microglial cells were harvested from primary
7 mixed glial cell cultures prepared from neonatal CD1 mouse brains (Charles River,
8 Kanagawa, Japan). In brief, after carefully removing the meninges, neonatal brain was
9 dissociated by gentle pipetting. The cell suspension was plated in 75 cm² culture flasks
10 (Falcon 3024: Becton-Dickinson Japan, Tokyo, Japan) at a density of five brains per 12 flasks
11 in 10 ml Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 5
12 mg/ml bovine insulin, and 0.2% glucose. Microglial cells were isolated on days 14-21 on a
13 rotary shaker as previously reported (Suzumura et al., 1987) and cultured in the medium for
14 two days. Two days after microglial separation, immunocytochemistry was performed as
15 described previously (Shimizu et al., 2013). The following primary antibodies were used:
16 rabbit anti-Iba1 polyclonal antibody (1:1000; Wako, Osaka, Japan), rabbit anti-cystatin F
17 polyclonal antibody (1:1000; Gift from Prof. Colin Watts, University of Dundee), mouse
18 anti-cathepsin C polyclonal antibody (1:100; R&D system, Minneapolis, MN).

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20 **Cathepsin C activity assay**

21 Fresh brain tissues from CatC^{STOP-tetO/+} and CatCOE mice were homogenized in 100 µl of
22 lysis buffer (1% Triton-X-100 in PBS) per 10 mg of brain tissue two times for 10 seconds on
23 ice using a potter homogenizer and centrifuged at 10,500 g at 4°C for 15 min. Supernatants
24 were collected and used in the activity assay to measure CatC activity in the transgenic mice.
25 Reaction mixtures containing 50 mM NaAc pH 5.5, 30 mM NaCl, 1 mM DTT, 0.5 mM

1 EDTA, 10 mM *o*-phenanthroline, 10 µg/ml Pepstatin A and sample were prepared. After pre-
2 incubation of the reaction mixtures for 2 min at room temperature, 100 µM substrate (H-
3 GlyArg-β-naphthylamide in DMSO, BACHEM, Torrance, CA) was added and incubated for
4 15 min at 37°C. Fluorescence intensity (Excitation 335 nm, Emission 405 nm) was monitored
5 for 15 min at 30 sec intervals to determine the initial rates based on initial velocity (v) using
6 4-Methoxy-β-naphthylamine as a reference compound.

7

8 ***In Situ* hybridization and immunohistochemistry**

9 Mice were perfused intracardially through the left ventricle with 4 %
10 paraformaldehyde (PFA), and brains and spinal cords were dissected out. The tissues were
11 post-fixed with 4 % PFA overnight and cryoprotected with 20 % sucrose-PBS buffer
12 overnight and frozen. Cryosections were prepared at 20 µm thickness with a cryostat and
13 used for in situ hybridization (ISH) and immunohistochemistry (IHC). ISH was performed as
14 described (Ma et al., 2011). The following plasmids containing mouse cDNA were used to
15 generate the ISH probes: *c-fms* (Ma et al., 2011), *cystatin F* (Ma et al., 2011), and *cathepsin*
16 *C* (nucleotides 193-1324 corresponding to NM_009982.4).

17 Immunohistochemistry was performed as described (Shimizu et al., 2013). The
18 following primary antibodies were used: rabbit anti-Iba1 polyclonal antibody (1:1000; Wako,
19 Osaka, Japan), rabbit anti-cystatin F polyclonal antibody (1:1000; Gift from Prof. Colin
20 Watts, University of Dundee), mouse anti-cathepsin C polyclonal antibody (1:100; R&D
21 system, Minneapolis, MN), mouse anti-CNPase monoclonal antibody (1:1000; Sigma, St.
22 Louis, MO), goat anti-arginase-1 polyclonal antibody and rat anti-CD16/32 monoclonal
23 antibody (1:200; Santa Cruz, CA, USA). Secondary antibodies were labelled with biotin
24 (Vectastain) or were FITC-conjugated (Invitrogen, Carlsbad, CA). DAB coloring was

1 performed as described previously (Ma et al., 2011). Images were captured using an Olympus
2 digital camera system (DP70) in combination with a microscope (Olympus BX51).

3

4 **Image analysis and statistical analyses**

5 The myelin-stained region (area of CNPase labelling) was quantified by measuring the
6 density using the Image J program for densitometric analysis (National Institutes of Health,
7 Bethesda, MD, USA). Data were expressed as the mean \pm S.E.M. Significance was assessed
8 by an unpaired two-tailed Student's *t*-test using GraphPad Prism version 5.0b statistics
9 software (GraphPad Software, Inc., La Jolla, CA, USA). *P*-values of less than 0.05 were
10 considered significant.

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12 **Electron microscopy**

13 Mice were perfused intracardially through the left ventricle with a fixative containing 2.5 %
14 (v/v) glutaraldehyde and 2.0 % (w/v) PFA in 0.1 M cacodylate buffer (pH 7.4). Brains were
15 dissected and immersed in the same fixative for a couple of days at 4 °C, then 0.5 mm-thick
16 para-sagittal slices were cut with a razor blade and 2 mm x 1.5 mm blocks from the cerebellar
17 lobes were processed for electron microscopy. After osmification in a 1 % (w/v) osmium
18 tetroxide solution for 2 h at 4 °C, the specimens were dehydrated through a graded alcohol
19 series and embedded in Epon 812 (TAAB Laboratories, Aldermaston, UK). Semi-thin
20 sections at 1 μ m thickness were collected, stained with 0.5% (w/v) Toluidine blue in 0.1 M
21 PB and imaged with a light microscope (Olympus) equipped with an auto-digitizer to
22 generate “virtual slides” (NanoZoomer-RS, Hamamatsu Photonics). For whole area analysis
23 of the same specimens by EM, ultrathin sections were cut on an ultramicrotome (Ultracut
24 UCT, Leica, Germany) without trimming, collected on platinum-coated glass slides, stained
25 with uranyl acetate and lead citrate and imaged with a scanning electron microscope

1 equipped with a back-scattered electron beam detector (SU8010, Hitachi, Japan) at 1.5 KV
2 accelerating voltage. From each section, a digitized image with an area of about 200 μm by
3 300 μm was captured at a resolution of 24.8 nm/pixel.

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1 **Results**

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3 **Spatial and temporal expression pattern differences of cathepsin C and cystatin F**

4 **between active remyelinating and chronic demyelinated phases**

5 In *Plp^{4e/-}* mice, while spontaneous myelin degeneration starts at 2 months of age,
6 rapid axon remyelination occurs until 6 months of age. After 6 months, the maturation of
7 oligodendrocytes is arrested and remyelination ceases (Ma et al., 2006; Shimizu et al., 2013).
8 We previously found that *CysF* gene expression was greatly reduced at 8 months of age
9 compared to 4 and 6 months in *Plp^{4e/-}* mice (Ma et al., 2007; Ma et al., 2011). It has been
10 reported that a major target of CysF is a cysteine protease, CatC (Hamilton et al., 2008). Thus
11 we examined *CatC* mRNA expression in *Plp^{4e/-}* mice at different time points. At the onset of
12 myelin degeneration in *Plp^{4e/-}* mice at 2 months of age, *CysF* mRNA expression began (Fig.
13 1A, C), however, *CatC* mRNA was not yet detected (Fig. 1B, D). During the subsequent
14 demyelination/remyelination phase at 4 months of age, the *CysF* expression pattern was
15 similar to that of *CatC* (Fig. 1E-H). At 7 months of age, the expression of both *CysF* and
16 *CatC* was reduced. However, the reduction in *CysF* mRNA expression seemed more rapid
17 than that of *CatC* (Fig. 1I-N); thus, in some regions, *CysF* expression had mostly disappeared
18 (Fig. 1M) while *CatC* expression continued (Fig. 1N). From these results we concluded that
19 *CysF* expression starts earlier than *CatC*, when demyelination is ongoing, but terminates its
20 expression earlier than *CatC* in *Plp^{4e/-}* mice. CysF and CatC were shown to be lysosomal
21 proteins in a cell line of human leukemic monocyte lymphoma, U937, and in hypoxic–
22 ischemic brains (Hamilton et al., 2008; Koike et al., 2013). We attempted to co-stain the same
23 section for both CatC and CysF, which are expressed in lysosomes, but *PLP^{4e/-}* transgenic
24 mice have lipofuscin granules which auto-fluoresce in demyelinating lesions (data not

1 shown). This strong fluorescence prevented us from detecting proteins in demyelinating brain
2 tissue from *Plp^{4e/-}* mice by immuno-fluorescence.

3 Thus, in order to determine if CysF protein colocalizes with CatC protein in microglia,
4 we utilized mouse primary cultured microglia. We found that CysF did colocalize with CatC
5 (Fig. 1O), suggesting that CatC activity can be inhibited by CysF in microglia.

6

7 **Generation of STOP-tetO knockin mice to manipulate *CatC* and *CysF* gene expression**

8 To study the role of CatC and CysF in demyelinating diseases, we generated
9 transgenic mouse lines in which CatC or CysF expression can be manipulated using the
10 FAST system we established previously (Tanaka et al., 2010). We crossed those mouse
11 strains with *Plp^{4e/-}* mice to examine the roles of CatC and CysF during the
12 remyelinating/demyelinating stages using the FAST system. The STOP sequence in the
13 FAST system forces termination of transcription (Supplement 1, 2); thus, homozygous mice
14 bearing the STOP-tetO knockin alleles in the absence of the tTA allele (*CatC^{STOP-tetO/STOP-tetO}*,
15 *CysF^{STOP-tetO/STOP-tetO}*) should behave as knockdown (KD)/knockout (KO) mice because of the
16 STOP sequence. In this study, we refer to *CatC^{STOP-tetO/STOP-tetO}* and *CysF^{STOP-tetO/STOP-tetO}* as
17 CatCKD and CysFKD, respectively.

18 In adult wild type mice, CatC protein and mRNA were found only in the CA2 region
19 of the hippocampus and choroid plexus (Fig. 2A, B), and were not found in any other regions
20 of the brain. In the CatCKD mouse, *CatC* mRNA and protein were suppressed by the STOP
21 sequence, as expected (Fig 2C, D). CysF was not found in any area of the brain at either
22 postnatal developmental stages or in adults (data not shown, Ma et al., 2011) but *CysF*
23 mRNA and protein were induced during the acute demyelination and active remyelination
24 process in *Plp^{4e/-}* mice at 4 months of age (Fig. 2E, F, H, I). CysFKD mice on the *Plp^{4e/-}*
25 background at 4 months of age were devoid of *CysF* mRNA, as expected (Fig. 2G, J).

1

2 **Generation of Cathepsin C or Cystatin F overexpression in mouse microglia using the** 3 **FAST system**

4 Since we found that CatC and CysF colocalized within microglia (Fig. 1O), we
5 generated microglia-selective CatC and CysF overexpressing mice as a gain-of-function
6 study. The overexpression of CatC (CatCOE; CatC^{STOP-tetO/+}:: Iba1-tTA) or CysF (CysFOE;
7 CysF^{STOP-tetO/+}:: Iba1-tTA) was achieved by crossing respective STOP-tetO knockin mice
8 with the microglia-selective tTA-expressing line Iba1-tTA.

9 To confirm the tTA-mediated CysF overexpression, brains from postnatal day 21
10 (P21) mice were examined. Iba1-tTA mice did not show any CysF expression (Fig. 3A, E),
11 whereas CysFOE mice showed abundant *CysF* mRNA (Fig. 3B) and protein (Fig. 3F)
12 without increasing the number of microglia (Fig. 3C, D). In these mice, all *CysF* mRNA-
13 positive cells were also Iba1-positive ($[CysF^+Iba1^+] / [total\ Iba1^+] \approx 90\%$; Fig. 3G).

14 CysF is known to be glycosylated (Ni et al., 1998), and both the monomeric and
15 slowly migrating glycosylated forms were detected (Fig. 3I), consistent with a report by
16 Hamilton et al. (Hamilton et al., 2008). However, the ratio of *CysF* mRNA and Iba1 double-
17 positive cells to the total number of Iba1-positive cells in 11-week-old CysFOE mice
18 ($[CysF^+Iba1^+] / [total\ Iba1^+] \approx 50\%$; Fig. 3H) was greatly decreased compared to that in P21
19 mice (Fig. 3G). Thus, either CysF expression driven by the tTA-tetO system was shut down,
20 or the level of *CysF* mRNA derived from this system was below the limit of detection by ISH
21 through an unknown mechanism.

22 In wild type mice, CatC is expressed mainly in the CA2 region of the hippocampus
23 and choroid plexus (Fig. 2A, B). In CatCOE mice, CatC-overexpressing Iba1-positive cells
24 were observed throughout the entire brain (about 70% of cells were CatC⁺Iba1⁺; Fig. 4A-C).
25 The enzymatic activity of CatC in the brain of CatCOE mice was examined using a specific

1 substrate (H-Gly-Arg- β -naphthylamide) and was found to be greatly increased compared to
2 controls, indicating that induced CatC protein has enzymatic activity (Fig. 4D).

3 From these crosses, we succeeded in obtaining CysFKD, CatCKD and CatCOE mice.
4 Unfortunately, the CysFOE mice generated using this recombination system did not show the
5 gene expression expected to be derived from the tTA-tetO system. Therefore, the use of the
6 CysFOE mice was omitted from this study.

7

8 **Phenotypic evaluation in mice with *CysF* or *CatC* genetic manipulations**

9 In these mice in which we manipulated the expression of microglial genes, it was
10 necessary to evaluate the differences between WT and CysFKD, CatCKD or CatCOE mice
11 during adult stages. None of these transgenic lines showed any overt phenotypes or clinical
12 symptoms, therefore we examined the specific microglial morphology in these mice. Given
13 that adult mice are needed to evaluate demyelinated lesions, we used two-month-old mice for
14 this experiment. In wild type mice, the microglial morphology, as detected by Iba-1 IHC,
15 showed a normal “resting” shape, i.e. small cell body and numerous very thin and highly
16 branched processes (Fig. 5A). In CatCKD mice, the morphology of the microglia remained in
17 the resting stage, similar to wild type mice (Fig. 5A, B). In contrast, in both the CysFKD (Fig.
18 5C) and CatCOE mice (Fig. 5D), the “activated” microglia phenotype was found,
19 characterized by shorter and thicker processes with a swollen cell body. Previous studies
20 showed that the *CysF* knockout mice do not show any clinical symptoms or behavioral
21 abnormalities (Mouse genomic informatics, MGI, 2008). However, our results from this
22 histological examination suggested abnormal microglial activation in adult CysFKD mice,
23 and that *CysF* is necessary for the resting microglial phenotype at two months of age, even if
24 the mRNA expression may somehow be below the detection limit by ISH in WT mice.

25

1 **CysF deprivation enhanced demyelination in *Plp*^{4e/-} mice.**

2 In *Plp*^{4e/-} mice at 4 months of age, when simultaneous myelin degeneration and
3 regeneration is occurring, both CysF and CatC are expressed in the white matter of
4 demyelinating lesions (Fig. 1E-H and O). To explore the role of CysF during this active
5 remyelinating phase in *Plp*^{4e/-} mice, we crossed *Plp*^{4e/-} and CysF^{STOP-tetO} mice to generate
6 CysFKD::*Plp*^{4e/-} mice. We stained brain tissues from 4-month-old CysFKD::*Plp*^{4e/-} and
7 control (CysF^{STOP-tetO/+}::*Plp*^{4e/-}) mice for CNPase and quantified the CNPase-positive area.
8 The CNPase staining from a single cerebellar folium in parasagittal sections is shown in Fig.6
9 (D), and was measured as described in the Materials and Methods. CysFKD::*Plp*^{4e/-} mice
10 showed more severe myelin loss compared to control mice (Fig. 6 A-C). The percentage of
11 the CNPase-stained area in a single cerebellar folium from CysFKD::*Plp*^{4e/-} mice was
12 significantly decreased ($5.15 \pm 0.86\%$, n = 6) compared to the control group ($10.63 \pm 0.49\%$,
13 n = 5; Fig. 6C). We also examined the olfactory tracts and found that 4-month-old
14 CysFKD::*Plp*^{4e/-} mice also showed a significant decrease ($37.78 \pm 1.52\%$, n=6) compared to
15 the control group ($50.05 \pm 1.42\%$, n = 5). These results demonstrated that genetic
16 manipulation to eliminate CysF results in aggravated early phase demyelination in the *Plp*^{4e/-}
17 mouse model of chronic demyelination. This suggests that CysF is important for maintaining
18 myelin regeneration.

19

20 **CatC overexpression also enhanced demyelination in *Plp*^{4e/-} mice.**

21 Given that the increase in CatC activity resulting from CysF deprivation exacerbated
22 demyelination (Fig. 1I-N, Fig. 5 A, C, and Fig. 6A-C), the overexpression of the *CatC* gene
23 in *Plp*^{4e/-} mice should result in a similar phenotype to CysFKD mice, i.e. enhanced
24 demyelination. Thus, CatCOE::*Plp*^{4e/-} mice were analyzed to examine the effect of microglial
25 CatC overexpression on demyelination. CatCOE::*Plp*^{4e/-} mice showed more severe myelin

1 loss at 4 months of age (when we examined the effect of CysFKD on demyelination in
2 CysFKD::*Plp*^{4e/-} mice), and many CatCOE::*Plp*^{4e/-} mice died by 4 months of age (data not
3 shown). Thus, we chose an earlier time point at 2.5 months of age to analyze CatCOE::*Plp*^{4e/-}
4 and *Plp*^{4e/-} mice for the effects of CatCOE on demyelination. We found more severe myelin
5 loss in CatCOE::*Plp*^{4e/-} mice compared to the *Plp*^{4e/-} mice used here as controls (Fig. 7A-B).
6 We quantified the CNPase-stained area in a single cerebellar folium in parasagittal sections,
7 as shown in Fig. 6D. The percentage of the CNPase-stained area in the CatCOE::*Plp*^{4e/-} mice
8 was significantly decreased ($5.10 \pm 0.36\%$, n = 8) compared to the control group ($7.86 \pm$
9 0.64% , n = 4; Fig. 7C). We also examined the olfactory tracts and found that, at 2.5 months
10 of age, CatCOE::*Plp*^{4e/-} mice showed a significant decrease ($43.42 \pm 3.09\%$, n = 8) compared
11 to control *Plp*^{4e/-} mice ($63.65 \pm 2.54\%$, n = 4). Furthermore, we performed electron
12 microscopic (EM) imaging of the cerebellum from CatCOE::*Plp*^{4e/-} and *Plp*^{4e/-} mice (Fig. 7D
13 and E). We observed massive myelin loss in CatCOE::*Plp*^{4e/-} mice at 2.5 months of age,
14 while myelin was maintained in *Plp*^{4e/-} mice of the same age (Fig. 7A and B). This is
15 consistent with the results obtained using the CysFKD mice. These results indicate that the
16 balance between CatC and CysF is very important for maintaining myelin regeneration
17 during the early phase of demyelinating diseases.

18

19 **CatC knockdown rescued the chronic demyelinating lesions in *Plp*^{4e/-} mice.**

20 Given that CatCOE and CysFKD in *Plp*^{4e/-} mice showed exaggerated demyelination,
21 we sought to reverse the balance of CatC/CysF expression using CatCKD mice. To examine
22 the impact of CatC knockdown on demyelination, we used CatCKD::*Plp*^{4e/-} mice and
23 CatC^{STOP-tetO/+}::*Plp*^{4e/-} mice as controls, and analyzed the phenotypes of these mice at 4, 6 and
24 8 months of age. There were no significant differences in the CNPase-positive areas between
25 CatCKD::*Plp*^{4e/-} and CatC^{STOP-tetO/+}::*Plp*^{4e/-} mice at 4 months of age when active

1 remyelination is ongoing (data not shown). CatC^{STOP-tetO/+}::*Plp*^{4e/-} mice gradually lost myelin
2 in this region at 6 months of age (Fig. 8A) and clearly showed severe myelin loss at 8 months
3 of age (Fig. 8C). However, CatCKD::*Plp*^{4e/-} mice had substantially more remaining myelin
4 compared to CatC^{STOP-tetO/+}::*Plp*^{4e/-} mice (Fig. 8A-D). We quantified the CNPase-stained area
5 in a single cerebellar folium from parasagittal sections as shown in Fig. 6 and 7. The
6 percentage of the CNPase-stained areas in CatCKD::*Plp*^{4e/-} mice ($14.11 \pm 1.25\%$, n = 9) was
7 significantly higher than in CatC^{STOP-tetO/+}::*Plp*^{4e/-} mice ($7.90 \pm 2.97\%$, n = 3) at 8 months of
8 age (Fig. 8F), while no significant differences were found at 6 months of age (Fig. 8E; n=3
9 mice each per group). We also examined the olfactory tracts and found that CatCKD::*Plp*^{4e/-}
10 mice at 8 months of age showed a significantly higher percentage of stained area ($47.62 \pm$
11 5.27% , n= 9) compared to control CatC^{STOP-tetO/+}::*Plp*^{4e/-} mice ($21.80 \pm 1.37\%$, n=3). To
12 confirm this result, we performed EM imaging of these mice. More myelinated axons were
13 observed in CatCKD::*Plp*^{4e/-} mice (Fig. 8 H) compared to controls (Fig. 8 G). We also
14 quantified the number of myelinated axons for each diameter range. We found that
15 CatCKD::*Plp*^{4e/-} mice at 8 months of age had an increased number of myelinated axons with
16 larger diameters (myelinated axon diameter between 1 and 3.5 μm) compared to WT mice of
17 the same age (Fig. 9A). We also found that CatCKD::*Plp*^{4e/-} mice had a higher average g-ratio
18 compared to WT mice, indicating that CatCKD::*Plp*^{4e/-} mice had thinner myelin sheaths and
19 that CatCKD promoted remyelination in *Plp*^{4e/-} mice (Fig. 9B). These results show that the
20 balance between CatC and CysF is an important factor controlling the myelin regeneration
21 process even during late stages of chronic demyelinating diseases. During the chronic
22 demyelinating phase in which CatC expression dominates CysF expression, CatC deprivation
23 can rescue *Plp*^{4e/-} mice from severe demyelination.

24

1 **Discussion**

2 In chronic MS lesions, the remyelination process is impaired resulting in
3 demyelinated lesions (Chang et al., 2002; Kuhlmann et al., 2008; Noseworthy, 1999). The
4 most crucial question is what factors govern the oligodendrocyte precursor recruitment and
5 oligodendrocyte differentiation phases of remyelination, and whether misregulation of any of
6 these factors can account for remyelination failure. Previous studies have identified several
7 pathways with the potential to have therapeutic efficacy (Fancy et al., 2010). We had
8 previously reported that microglia express CysF only during the active remyelinating stage
9 (at 4-6 months) and that downregulation of CysF expression indicates the cessation of
10 remyelination in demyelinating regions (Ma et al., 2011). CysF is a specific inhibitor of CatC,
11 and CatC mediates the generation of pro-inflammatory cytokines such as tumor necrosis
12 factor- α (TNF- α) which is highly toxic to oligodendrocytes, and interleukin 1- β (IL-1 β) a
13 remyelination promoting factor (Adkison et al., 2002; Hamilton et al., 2008; Mason et al.
14 2001, McLaurin et al., 1995; Ye and D'Ercole, 1999). Thus, CysF could be one of the factors
15 specifically influencing differentiation and/or survival. Here, we studied the roles of CatC
16 and CysF in mouse models in which it was possible to manipulate the expression of CatC and
17 CysF.

18 This study demonstrated that the CysF/CatC system is one possible mechanism that
19 may inhibit myelin regeneration. We found activated microglia in adult CysFKD and
20 CatCOE mice. However, CatC knockdown mice did not show any overt phenotypes. In
21 CatCOE and CysFKD mice, the increase in CatC and deprivation of its inhibitor CysF might
22 increase CatC activity in microglia causing the microglia themselves to become activated.
23 Maintenance of the brain environment is very important for the recovery process from injury,
24 diseases or infection; this includes the phagocytosis of debris and secretion of growth signals,
25 cytokines and other factors. Microglial cells monitor the wellbeing of their environment and

1 are able to respond to signs of homeostatic disturbance with a program of supportive and
2 protective activity, to safeguard innate defense mechanisms, or to assist in specific immune
3 responses (Hanisch, 2002; Kotter et al, 2006; Lampron et al., 2015; Stollg and Jander, 1999).
4 Activated microglia have been found to persist in chronic demyelinating lesions (Tanuma et
5 al., 2006; Taupin et al., 1997). It seems that changes in the normal levels of CatC or CysF
6 beyond physiological levels cause a disturbance in the brain homeostasis and the activation of
7 microglia. It is possible that CatC and CysF could send crucial signals that alert the CNS to
8 prepare microglia which can respond to any type of CNS disturbance.

9 In *Plp^{4e/-}* mice, *CysF* and *CatC* had similar expression patterns during the active
10 remyelinating phase at 4 months of age, shown in Fig. 1E-H. However, CysF was found to be
11 expressed earlier than CatC during active demyelination and remyelination (Fig.1 A-D) when
12 it dominated CatC expression prior to the emergence of chronic lesions (Fig. 1A-H). During
13 this phase, the myelin regeneration process is very important for maintaining the myelin
14 sheath; this is similar to the early phase of MS which shows relapse-remission symptoms
15 (Niehaus et al., 2000). At 2 months of age, *Plp^{4e/-}* mice show neither paralysis nor muscular
16 dystrophy defects, but do show signs of nerve conduction disturbance (Tanaka et al., 2006)
17 and tonic seizures similar to the early symptoms of MS (Kelley and Rodriguez, 2009). CysF
18 induction in microglia is dependent on the phagocytosis of compact myelin membranes (Ma
19 et al., 2011), thus CysF might have a role in supporting the myelin regeneration process. Here
20 we showed that *CysF* knockdown in *Plp^{4e/-}* mice resulted in exaggerated demyelination at
21 earlier phases of demyelination. CysF inhibits CatC protease activity and disrupts
22 downstream signals that can cause disturbance in the myelin regeneration process. (Hamilton
23 et al., 2008; Irmeler et al., 1995; Pham and Ley, 1999). The balance between CatC and CysF
24 expression also seems to play a critical role in myelin regeneration during the early phase of
25 chronic demyelinating diseases. This conclusion was confirmed by CatC overexpression in

1 microglia showing severe demyelination at a very early phase of chronic demyelination. In
2 the early phase of chronic demyelinating disease, CysF should play a dominant role in
3 supporting myelin regeneration by interfering with CatC function. In this study, we showed
4 only a lobe of the cerebellum and olfactory tracts for statistical analysis, although *Plp^{4e/-}* mice
5 undergo demyelination throughout the entire CNS. While demyelination does not typically
6 proceed uniformly and shows some regional variation, even among individuals, the
7 demyelination we observed in the cerebellum and olfactory tracts was relatively constant.
8 Therefore, we chose these regions for our statistical analysis.

9 During the late phase of chronic demyelinating disease, CatC was found to dominate
10 CysF and full activation of microglia is expected. This could cause impaired myelin
11 regeneration and the appearance of naked axons in the *Plp^{4e/-}* mice (Kagawa et al., 1994). We
12 found that CatC knockdown resulted in more intact myelin in the late phase of chronic
13 demyelination. This provides strong evidence that CatC is one of the key factors that induces
14 demyelination. The conclusions drawn from this study demonstrated that the balance between
15 CatC and CysF expression plays an important role in chronic demyelinating disease.

16

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1 **Legends**

2 **Figure 1. The expression patterns of *cystatin F* and *cathepsin C* are similar during the**
3 **active remyelinating phase but not in chronic lesions.**

4 *In situ* hybridization for *cystatin F* (A, C, E, G, I, K, M) and *cathepsin C* (B, D, F, H, J, L, N)
5 was performed on brain tissues from *Plp^{4e/-}* mice at 2 (A-D), 4 (E-H) and 7 (I-N) months of
6 age. (A), (B), (E), (F), (I) and (J) are magnified images of the areas outlined in black from (C),
7 (D), (G), (H), (K) and (L), respectively. (M) and (N) are magnified images of the areas
8 outlined in red in (I) and (J), respectively. Immunocytochemistry demonstrated that CatC
9 (shown in red) and CysF (shown in green) were co-localized in primary cultured microglial
10 cells (O). Scale bars: A, 200 μm ; C, 600 μm ; N, 100 μm ; O, 20 μm .

11

12 **Figure 2. Successful knockdown of Cathepsin C and Cystatin F in homozygous STOP-**
13 **tetO mice.**

14 Adult wild type and CatCKD mice were analyzed for *CatC* mRNA (A, C) and CatC protein
15 expression (B, D). Four-month-old *Plp^{4e/-}* mice were analyzed for *CysF* mRNA (E, H) and
16 *CysF* protein expression (F, I). Four-month-old *CysFKD::Plp^{4e/-}* mice were analyzed for
17 *CysF* mRNA expression (G, J). H, I and J are magnified images of the corpus callosum
18 region in E, F and G, respectively. Scale bars: 100 μm for A, C, E, G; 50 μm for B, D, F, H, I,
19 J.

20

21 **Figure 3. Successful overexpression of Cystatin F in Iba1-positive microglia.**

22 *In situ* hybridization for *CysF* and *c-fms* mRNA, and immunohistochemistry for CysF were
23 performed on spinal cord sections from Iba1-tTA (A, C, E) and CysFOE mice (B, D, F). The
24 inset in (F) is a magnified image of the area outlined in black and indicated by a red
25 arrowhead. Double staining of *CysF* mRNA by *in situ* hybridization and Iba1 by

1 immunohistochemistry was performed on thoracic spinal cords from CysFOE mice at
2 postnatal day 21 (P21) (G) and at 11 weeks (H). The inset in (G) is a magnified image of the
3 cell outlined in black. Brain lysates from wild type and CysFOE mice were used for cystatin
4 F immunoblotting (I). The arrow indicates monomers of cystatin F and the asterisk indicates
5 the glycosylated slow migrating forms. Scale bars: D, 500 μm ; F, 50 μm ; H, 200 μm .

6

7 **Figure 4. Successful overexpression of Cathepsin C in Iba1-positive microglia.**

8 (A-C) CatC and Iba1 are shown in red and green, respectively, in the brain of a CysFOE
9 (CatC^{STOP-tetO/+::Iba1-tTA}) mouse. CatCOE and CatC^{STOP-tetO/+} mice were analyzed for CatC
10 activity using a specific substrate for cathepsin C (D). The control wells contained buffer and
11 protease substrates only. The fluorescence signal was measured from time 0 when brain lysate
12 supernatants from CatCOE and CatC^{STOP-tetO/+} mice were added. Scale bars: A-C, 20 μm .

13

14 **Figure 5. Morphological analysis of microglia in *CatC* and *CysF* genetically manipulated**
15 **mice.**

16 Wild type (A), CatCKD (B), CysFKD (C) and CatCOE (D) mice at two months of age were
17 analyzed for microglial morphology in the cerebellum. Parasagittal brain cryosections were
18 used for Iba1 immunohistochemistry to visualize microglial morphology. Scale bar in D, 20
19 μm .

20

21 **Figure 6. CysF knockdown worsened the demyelination in *Plp*^{4e/-} mice.**

22 CysF^{STOP-tetO/+::Plp}4e/- (A) and CysFKD::Plp^{4e/-} (B) mice at 4 months of age were analyzed for
23 myelin loss in the brain by staining for CNPase. CysF^{STOP-tetO/+::Plp}4e/- was used as the
24 control. (C) The percentage of the myelin stained area was quantified by densitometry
25 analysis and the differences between the groups are represented in the graph: CysF^{STOP-}

1 $\text{tetO}^{+/+}::Plp^{4e/-}$ ($10.63 \pm 0.49\%$, $n=5$), $\text{CysFKD}::Plp^{4e/-}$ ($5.15 \pm 0.86\%$, $n=6$); $*p < 0.05$ compared
2 to the control group. (D) Schematic of mouse parasagittal sections. The region indicated by
3 the black dashed line represents the area that was quantified. Scale bars: A and B, $50 \mu\text{m}$.

4

5 **Figure 7. CatC overexpression induced earlier demyelination in $Plp^{4e/-}$ mice.**

6 Two-month-old $Plp^{4e/-}$ (A) and $\text{CatCOE}::Plp^{4e/-}$ (B) transgenic mice were assayed for myelin
7 in the brain. The percentage of the CNPase-stained area was quantified by densitometry
8 analysis and differences between the groups are represented in the graph (C): $Plp^{4e/-}$ ($7.86 \pm$
9 0.64% , $n=4$), $\text{CatCOE}::Plp^{4e/-}$ ($5.10 \pm 0.36\%$, $n=8$). (D, E) Electron microscopic images of
10 the cerebellar lobe region shown in Fig. 6D from $Plp^{4e/-}$ (D) and $\text{CatCOE}::Plp^{4e/-}$ (E) mice at
11 2.5 months of age ($*p < 0.05$ compared to the control group). Scale bars: A-B, $50 \mu\text{m}$; D-E,
12 $10 \mu\text{m}$.

13

14 **Figure 8. CatC knockdown reduced demyelination in $Plp^{4e/-}$ mice.**

15 $\text{CatC}^{\text{STOP-tetO}/+}::Plp^{4e/-}$ (A and C) and $\text{CatCKD}::Plp^{4e/-}$ (B and D) mice were analyzed for the
16 amount of myelin in the brain at 6 and 8 months of age. $\text{CatC}^{\text{STOPtetO}/+}::Plp^{4e/-}$ mice were used
17 as controls. The percentage of the myelin stained area was quantified by densitometry
18 analysis and the differences between the groups are represented in the graphs (E and F):
19 $\text{CatC}^{\text{STOP-tetO}/+}::Plp^{4e/-}$ ($7.90 \pm 2.97\%$, $n=3$), $\text{CatCKD}::Plp^{4e/-}$ ($14.11 \pm 1.25\%$, $n=9$). (G, H)
20 Electron microscopic images of the cerebellar lobe region shown in Fig. 6D from $\text{CatC}^{\text{STOP-}}$
21 $\text{tetO}/+::Plp^{4e/-}$ and $\text{CatCKD}::Plp^{4e/-}$ mice at 8 months of age ($*p < 0.05$ compared to the control
22 group). Scale bars: A-D, $200 \mu\text{m}$; G-H, $10 \mu\text{m}$.

23

24 **Figure. 9 Cathepsin C knockdown promoted remyelination in $Plp^{4e/-}$ mice.**

1 (A) Distribution of myelinated axon diameters measured at the cerebellar lobe region shown
2 in Fig. 6D from WT and CatCKD::*Plp*^{4e/-} mice at 8 months of age. The vertical axis indicates
3 the number of myelinated axons and the horizontal axis indicates the myelinated axon
4 diameter range. (B) Axon diameter and g-ratio distribution measured at the cerebellar lobe
5 from WT and CatCKD::*Plp*^{4e/-} mice at 8 months of age. The vertical axis indicates axon
6 diameter and the horizontal axis indicates the g-ratio.

7

8 **Supplementary 1. Construction of CysF STOP-tetO mice and Southern blotting.**

9 (A) The upper figure shows a schematic of the wild type genome, the targeting vector and
10 CysF STOP-tetO allele structures, and the *Afl*III restriction sites; the 5' probe position is
11 indicated by a black bar. Black arrows represent primer positions. Open triangles represent
12 loxP sites. Filled triangles represent FRT sites. Neo is the PGK-EM7-NEO minigene. STOP
13 is the cassette containing elements designed to terminate both transcription and translation.
14 ATG represents the translation initiation site. tetO is the cassette containing the tetracycline
15 operon site and CMV minimal promoter. The lower figure shows the Southern blotting
16 results from genomic DNA of wild type, heterozygote and homozygote CysF STOP-tetO
17 mice. (B) The upper figure shows a schematic of the wild type, targeting vector and CysF
18 STOP-tetO allele structures, as well as the 3' probe position, and *Xba*I and *Eco*RV restriction
19 sites. The lower figure shows the Southern blotting results from genomic DNA of wild type,
20 heterozygote and homozygote CysF STOP-tetO mice.

21

22 **Supplementary 2. Construction of CatC STOP-tetO mice and Southern blotting.**

23 (A) The upper figure shows a schematic of the wild type genome, the targeting vector and
24 CatC STOP-tetO allele structures, as well as the 5' probe position and *Spe*I restriction sites.
25 The symbols are the same as in Supplementary 1. The lower figure shows the Southern

1 blotting results from genomic DNA of wild type, heterozygote and homozygote CatC STOP-
2 tetO mice. (B) The upper figure shows a schematic of the wild type genome, the targeting
3 vector and CatC STOP-tetO allele structures, as well as the 3' probe position, and *Pst*I and
4 *Eco*RI restriction sites. The lower figure shows the Southern blotting results from genomic
5 DNA of wild type, heterozygote and homozygote CatC STOP-tetO mice.

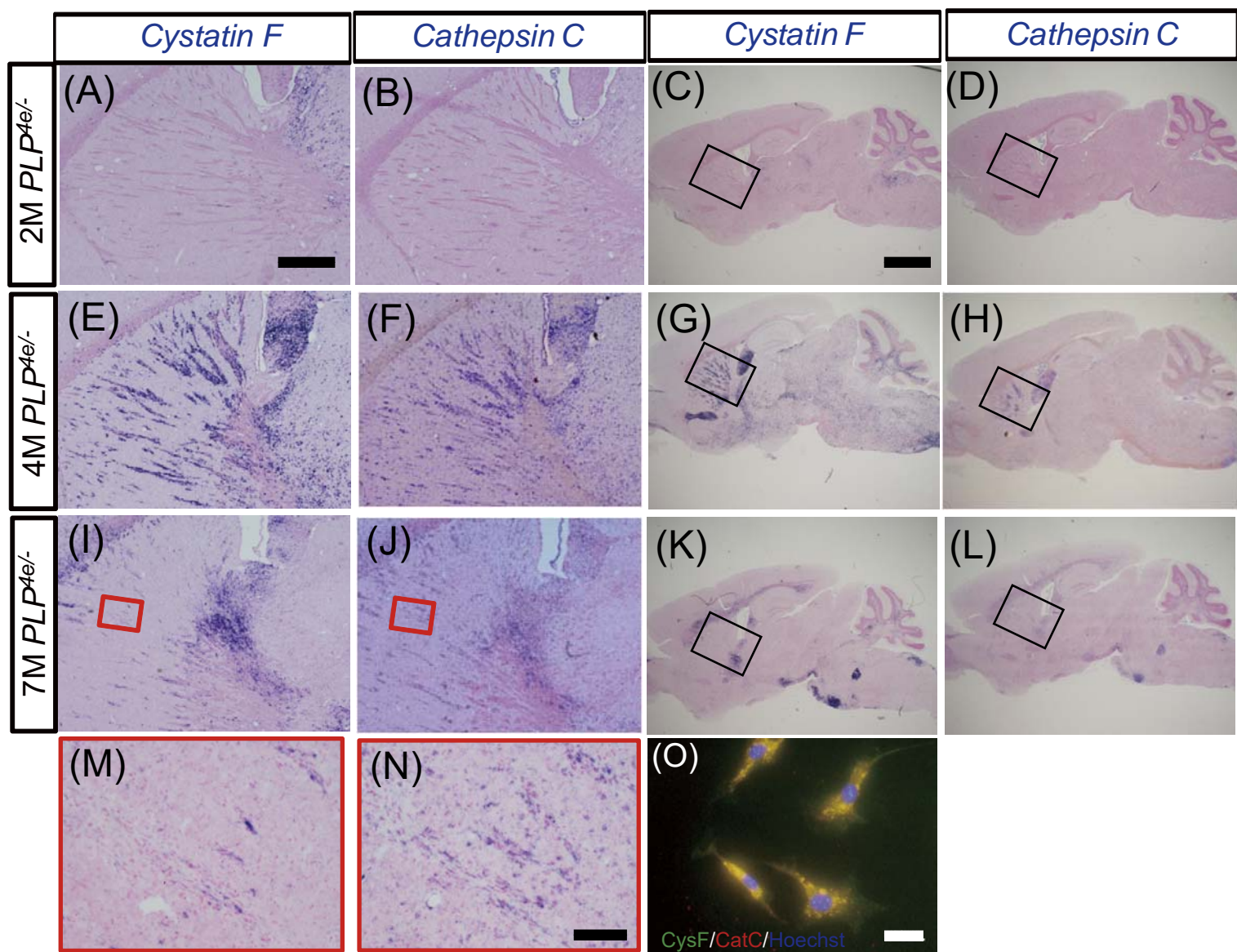
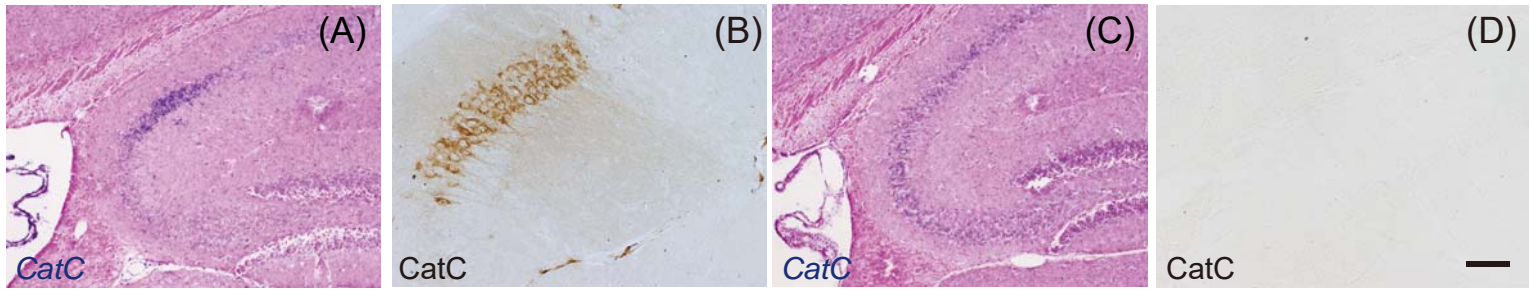


Figure 1

Wild type

CatCKD



Plp^{4e/-}

CysFKD::Plp^{4e/-}

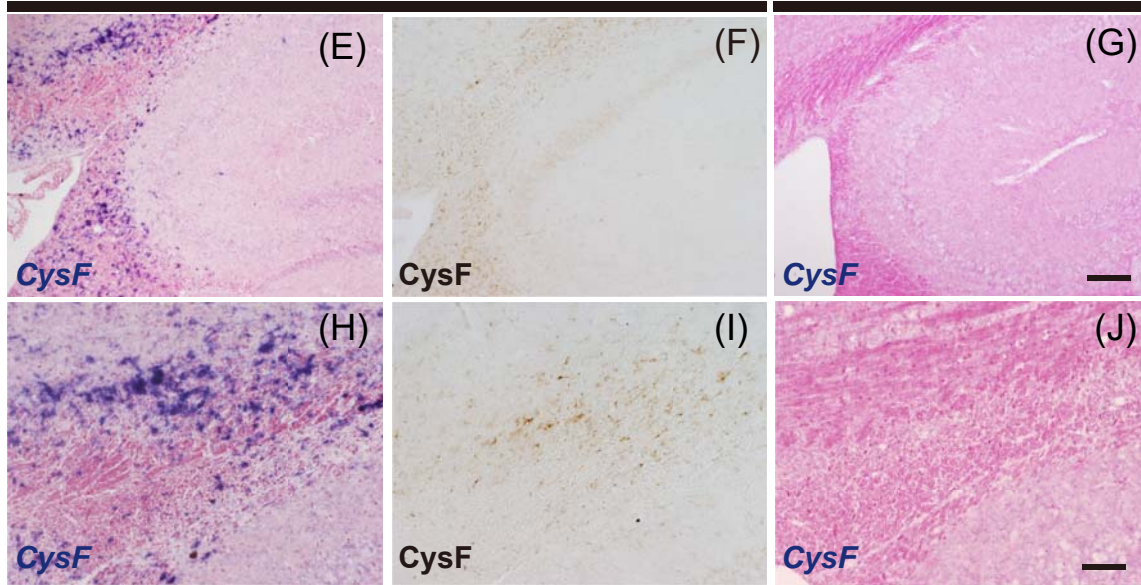


Figure 2

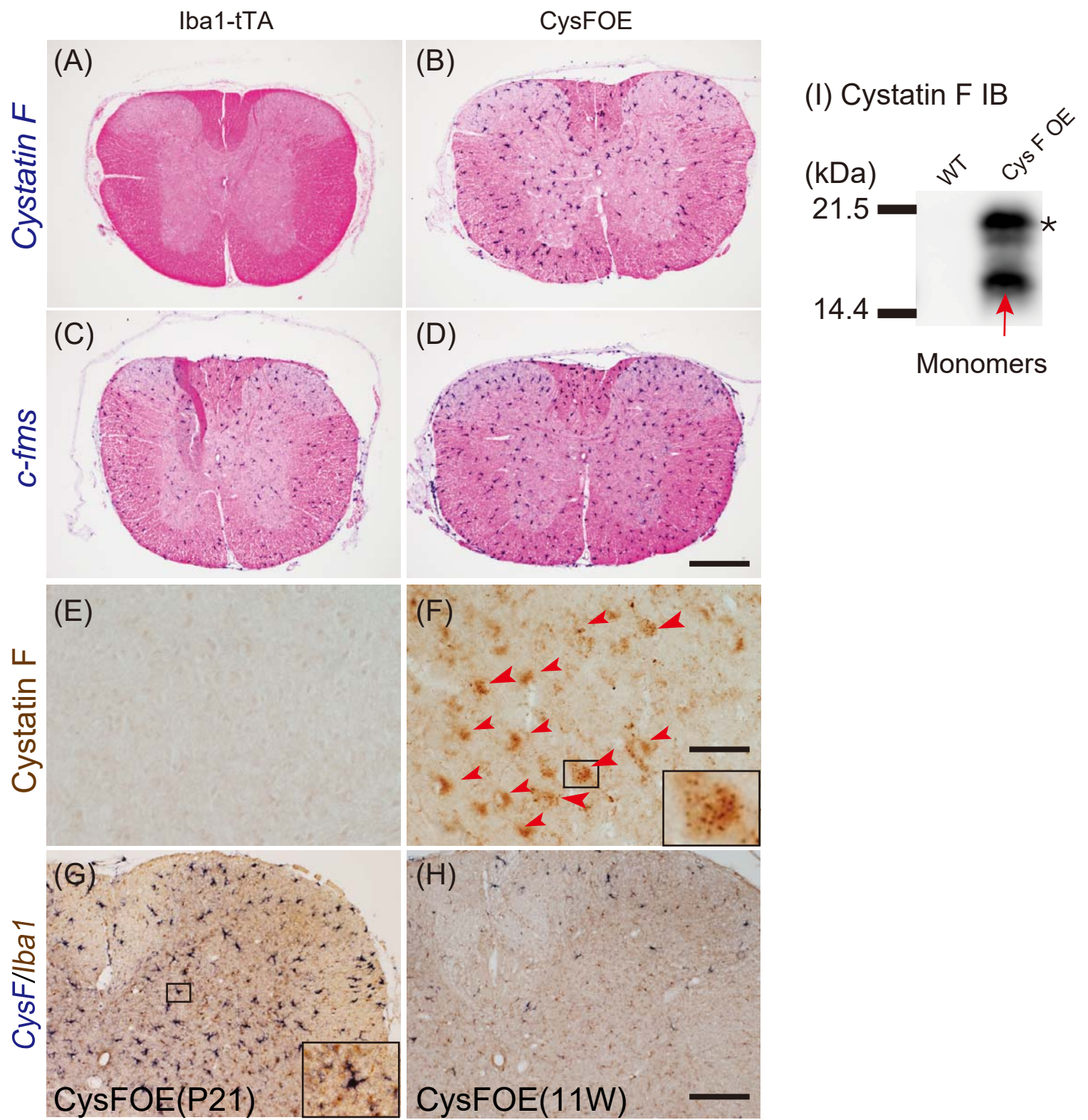


Figure 3

CatCOE

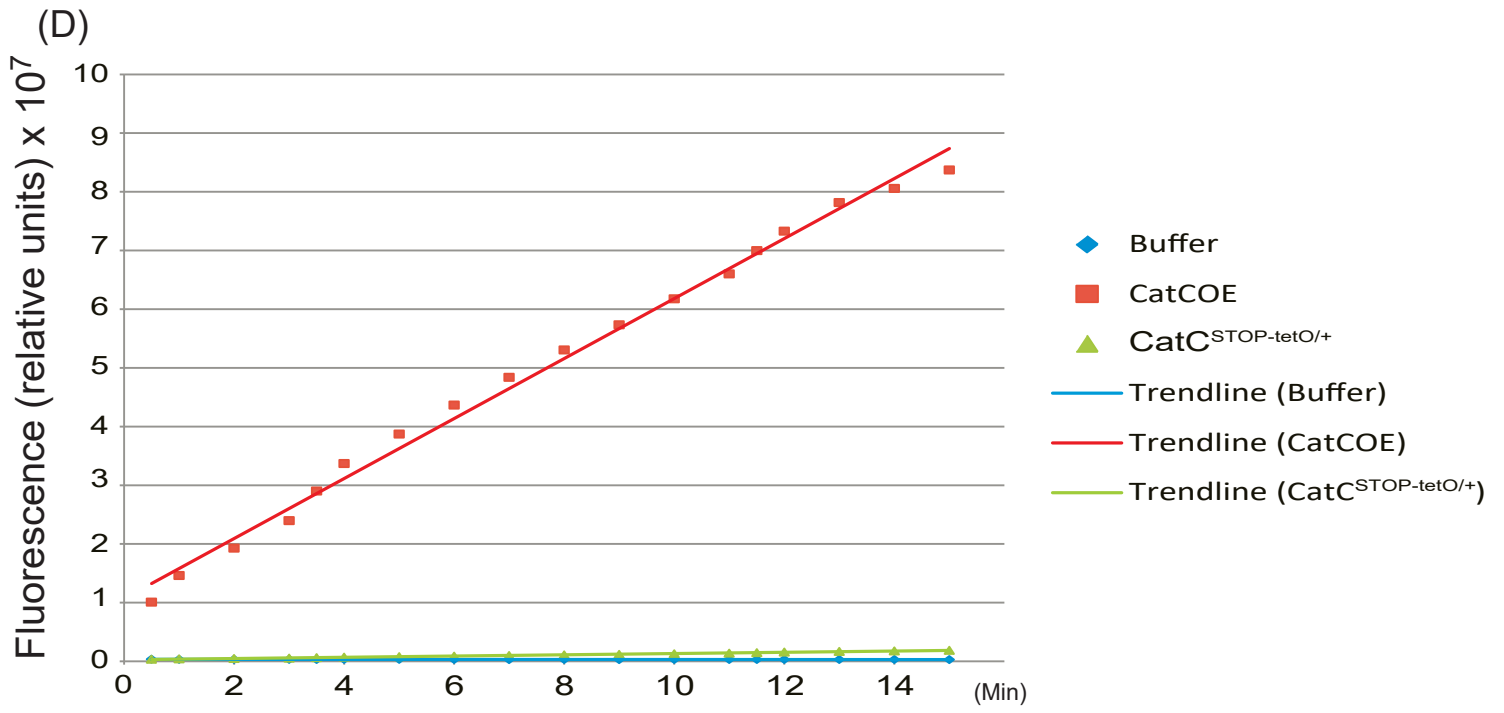
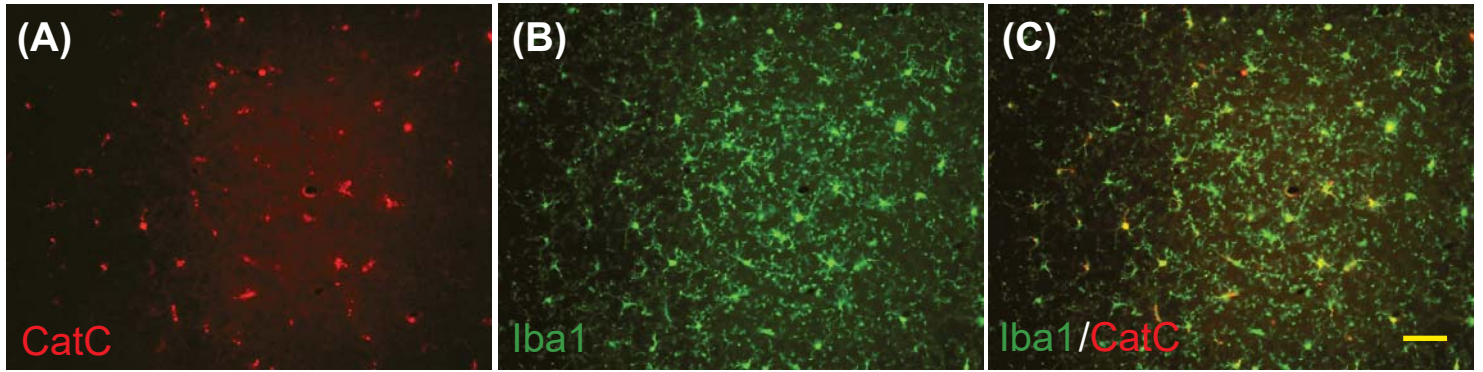


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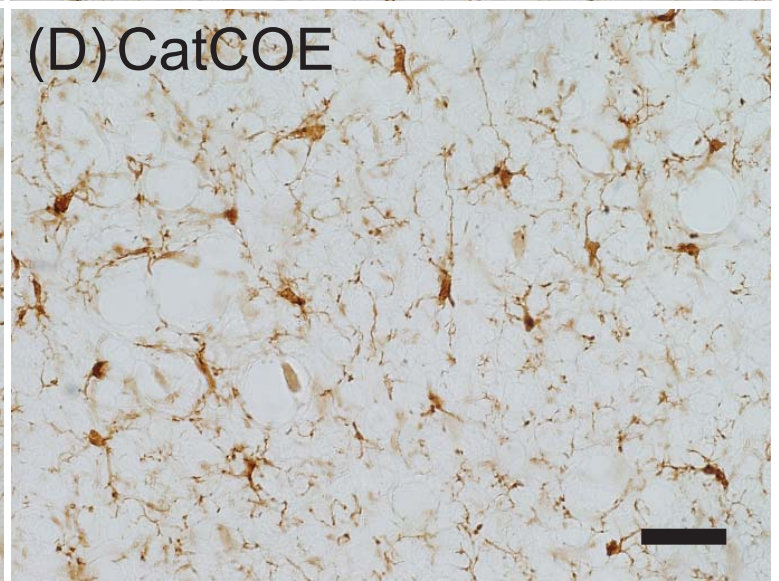
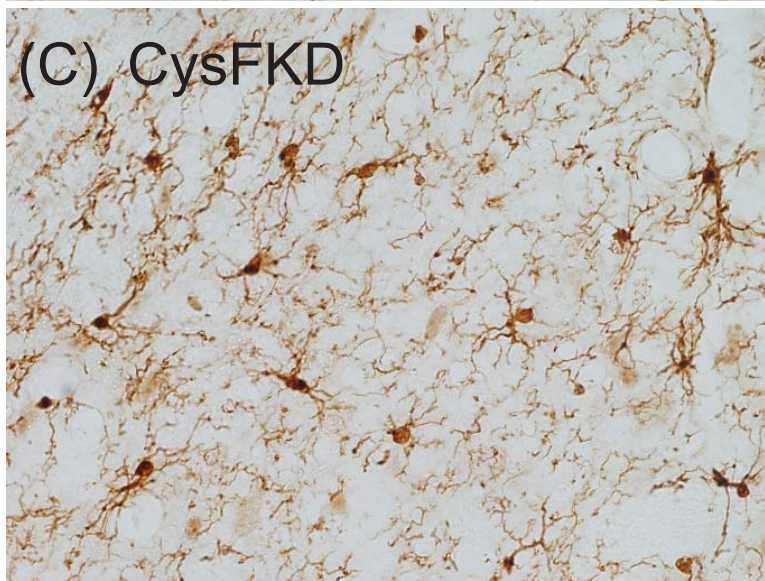
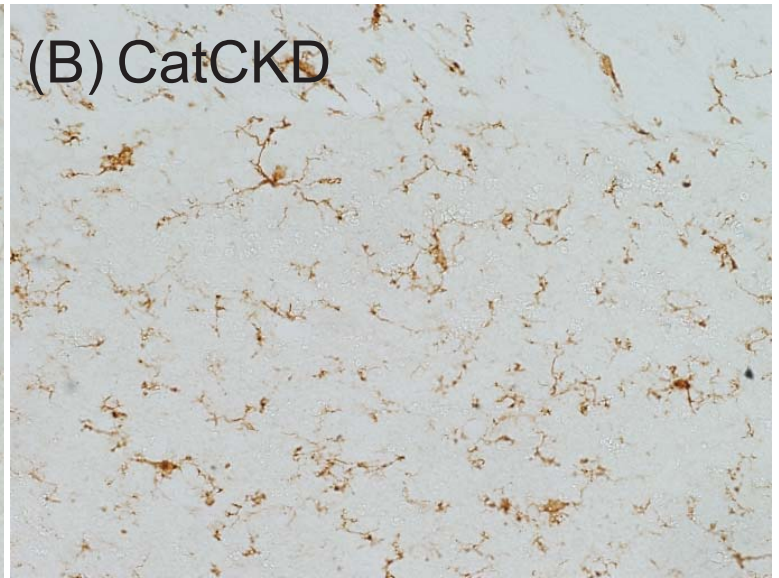
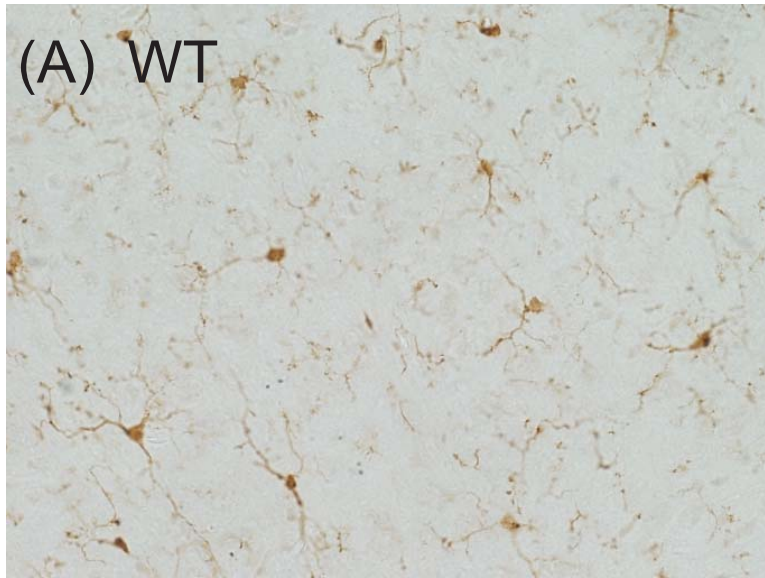
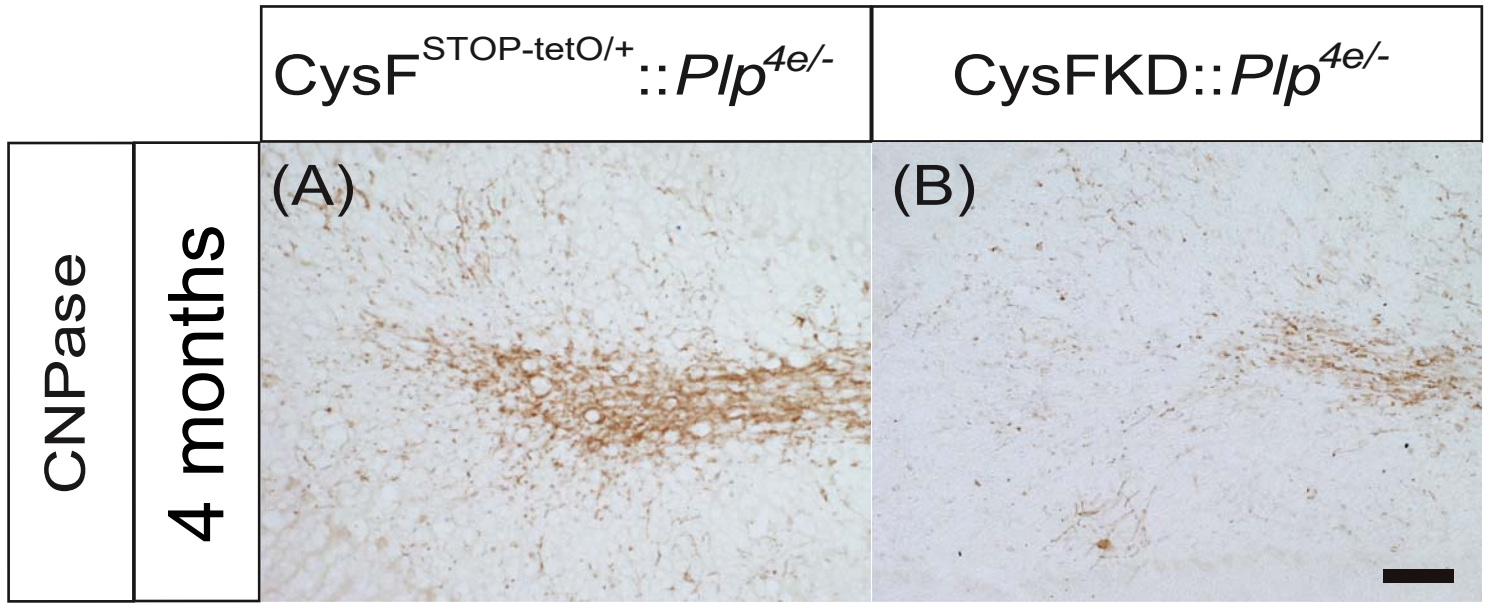
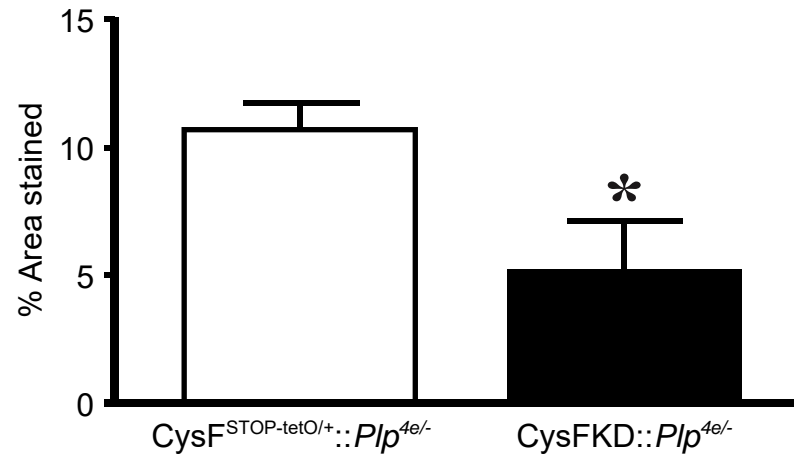


Figure 5



(C)



(D)

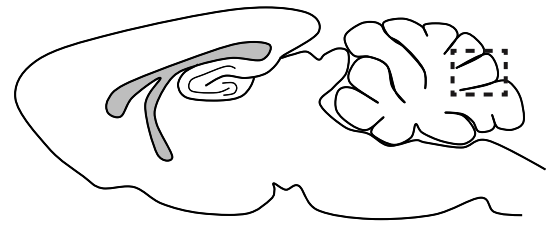


Figure 6

2.5 Months

Plp^{4e/-}

CatCOE::*Plp*^{4e/-}

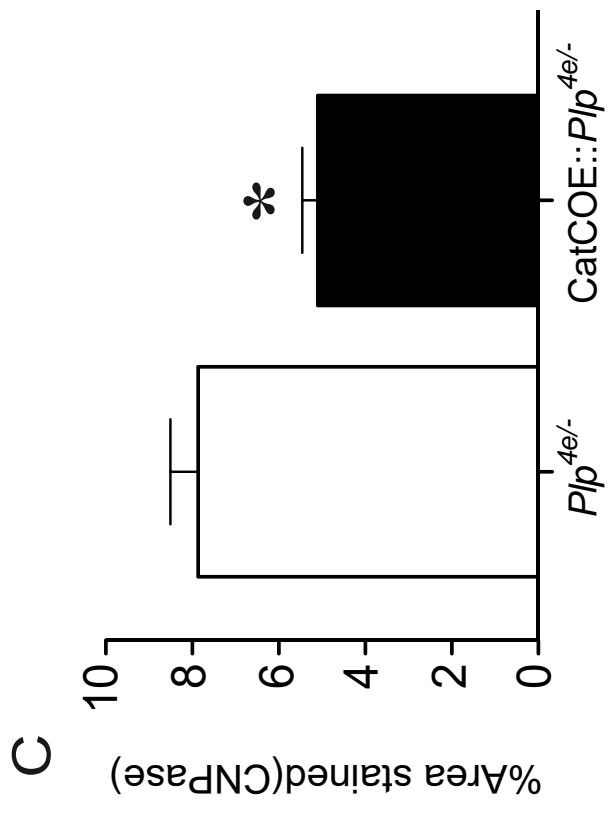
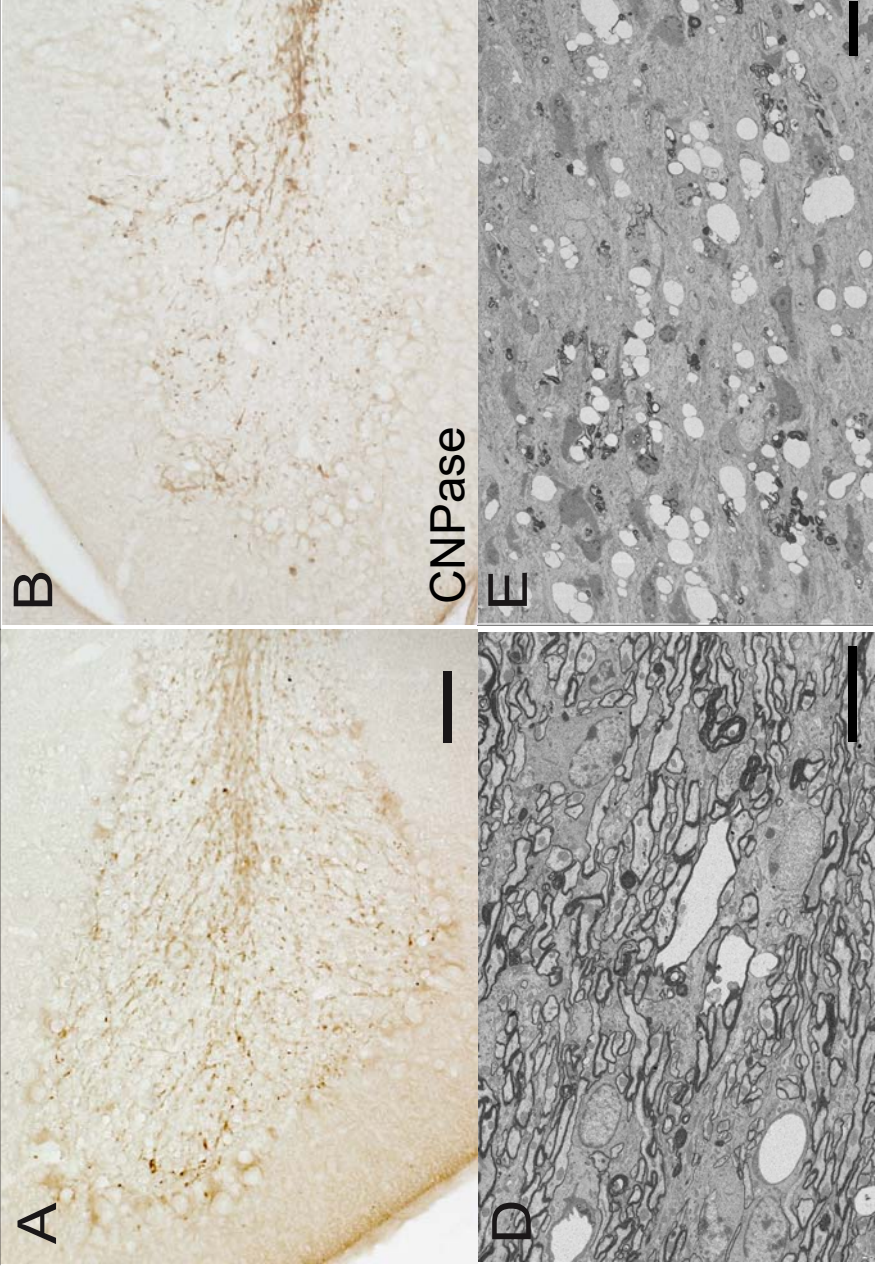


Figure 7

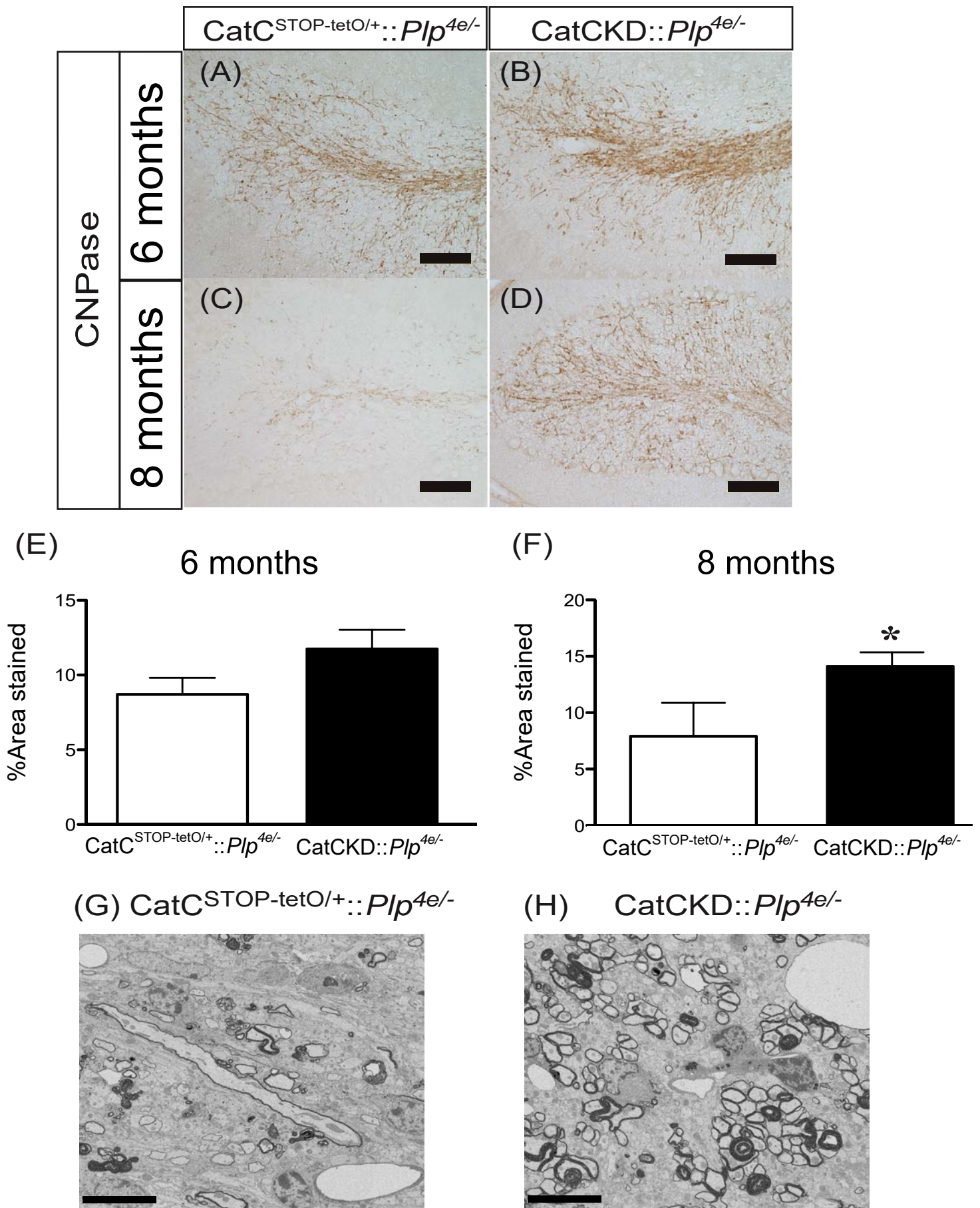


Figure 8

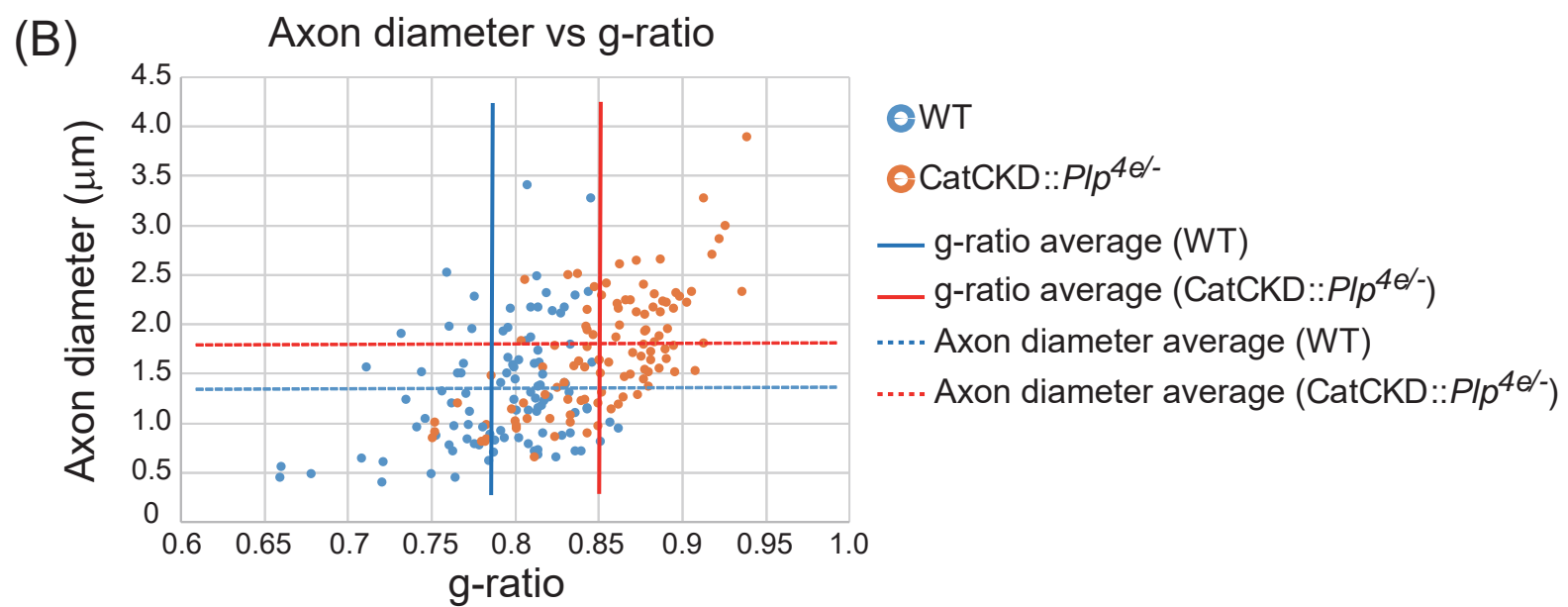
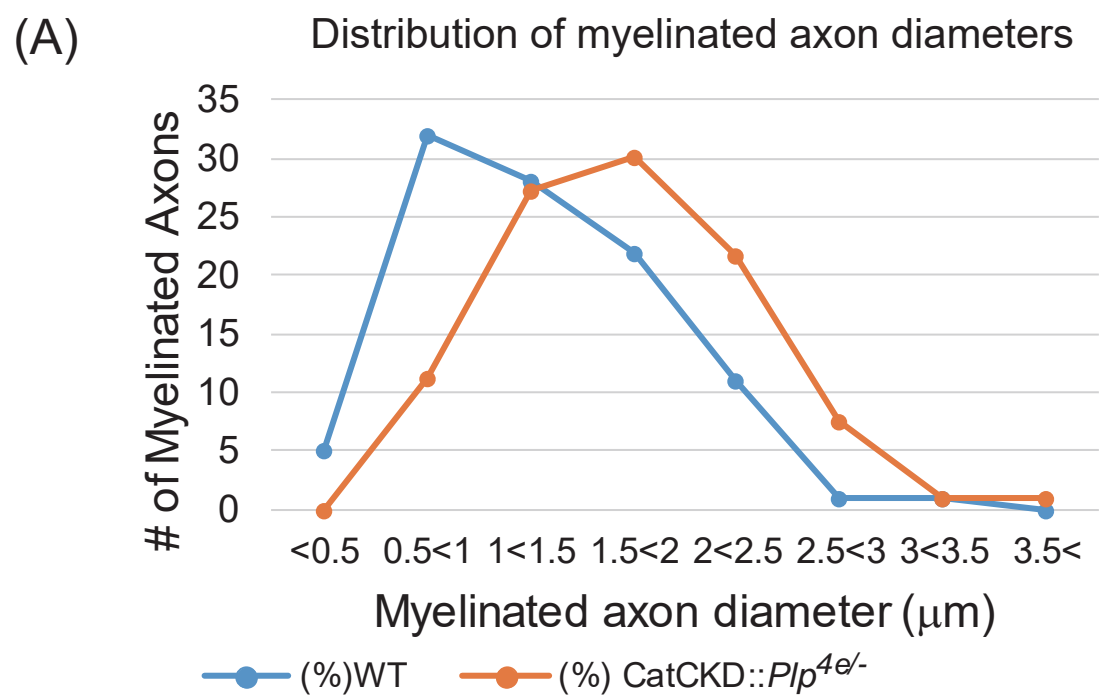
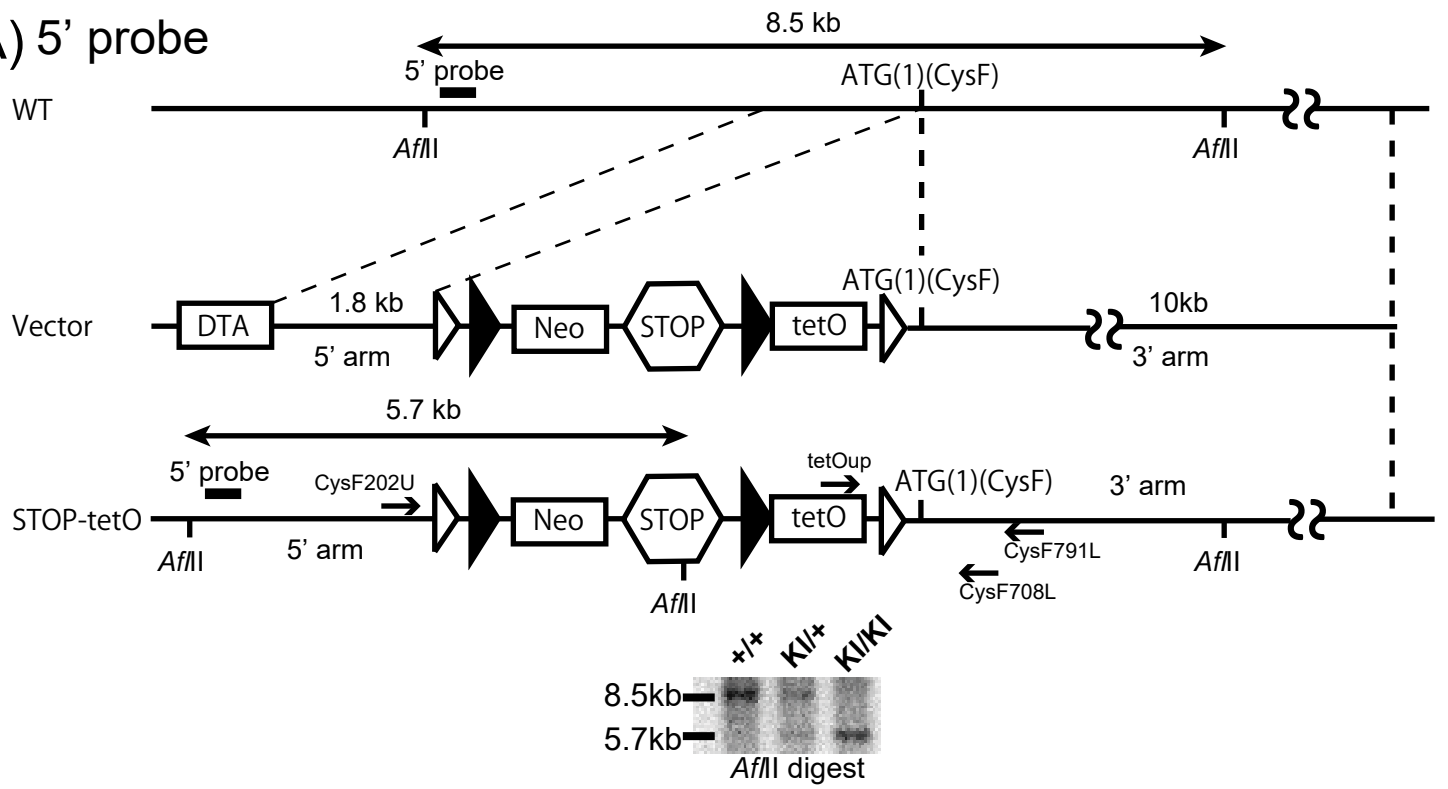


Figure 9

(A) 5' probe



(B) 3' probe

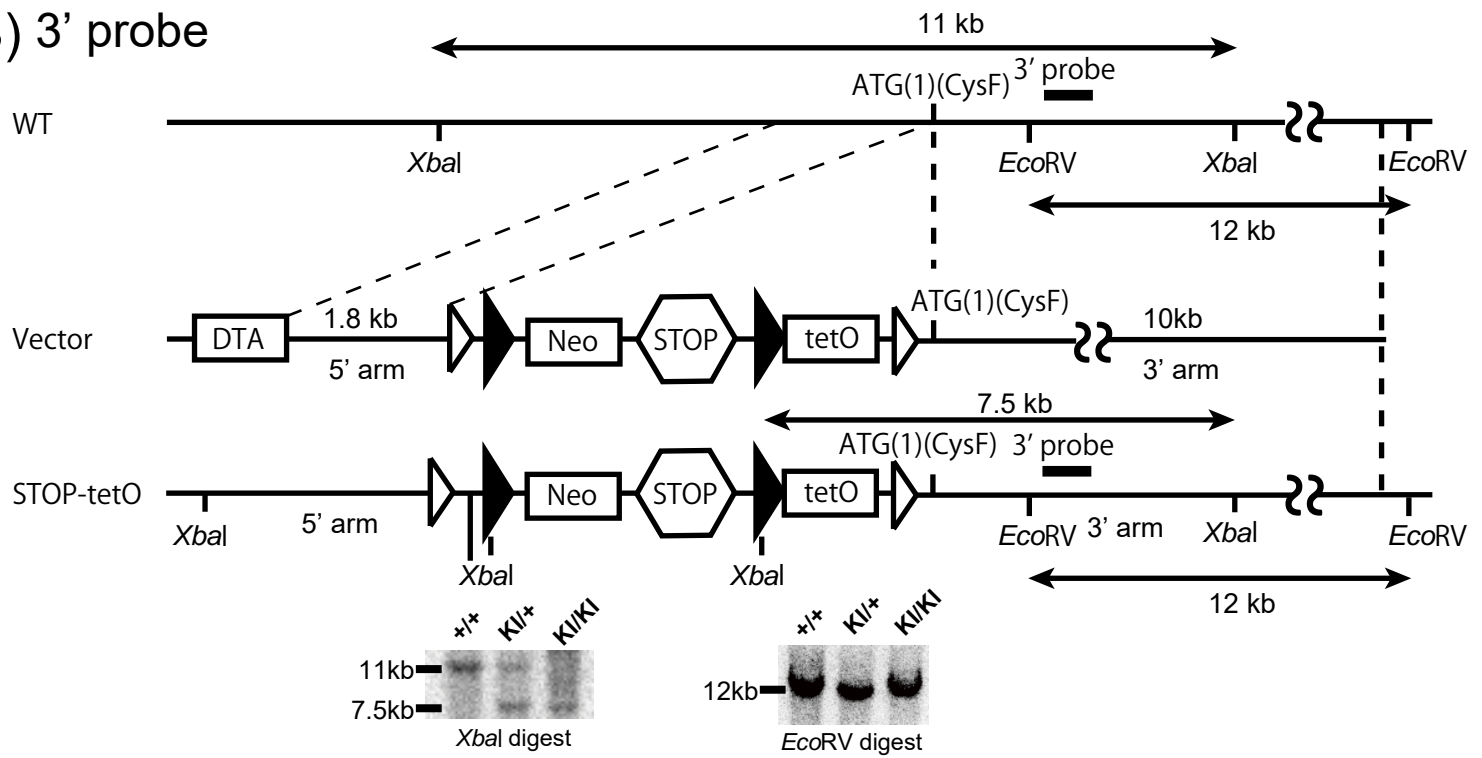
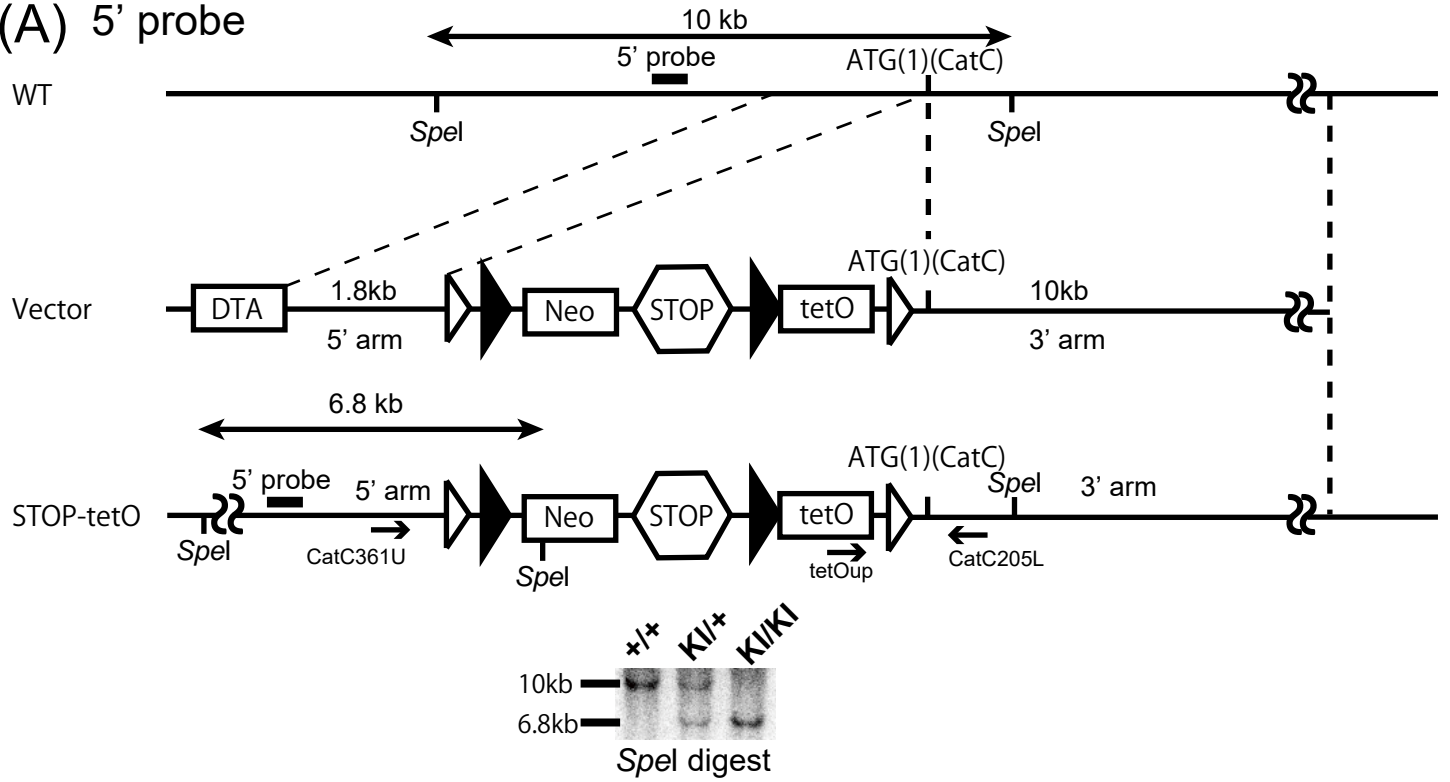


Figure S1

(A) 5' probe



(B) 3' probe

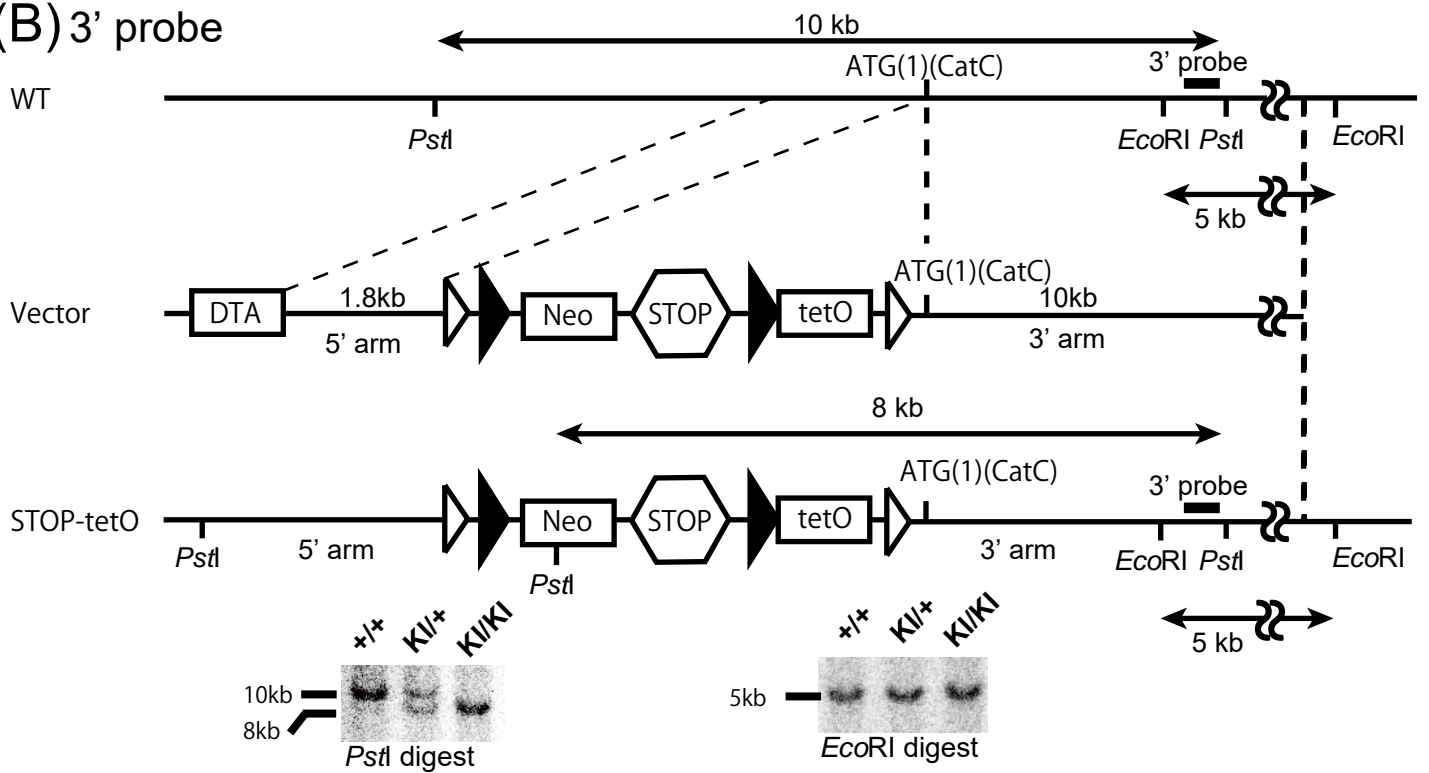


Figure S2