Cell Reports, Volume *42*

Supplemental information

A comparative analysis of microglial inducible Cre lines

Travis E. Faust, Philip A. Feinberg, Ciara O'Connor, Riki Kawaguchi, Andrew Chan, Hayley Strasburger, Maximilian Frosch, Margaret A. Boyle, Takahiro Masuda, Lukas Amann, Klaus-Peter Knobeloch, Marco Prinz, Anne Schaefer, and Dorothy P. Schafer

Supplementary Figure 1: Flow cytometry of *Rosa26*^{mTmG} microglia

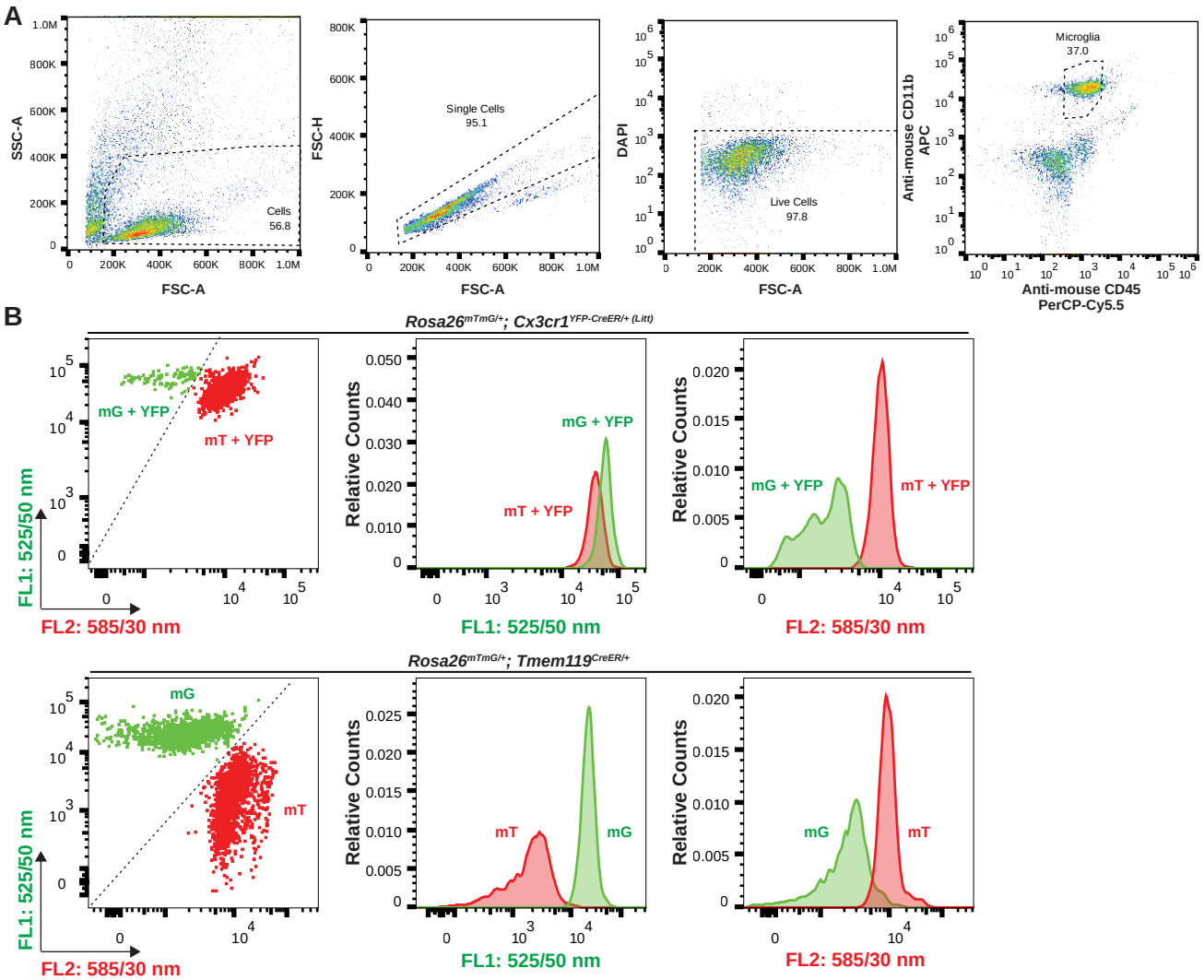

Figure S1. Flow cytometry of *Rosa26*^{mTmG} microglia.

Related to Figure 1

(A) Plots of flow cytometry gates used for fluorescence-activated cell sorting (FACS) of microglia.

(B) Flow cytometry analysis of recombined mGFP^{+} (mG) vs. non-recombined mTomato^{+} (mT) microglia in *Rosa26*$^{mTmG/+}$*; Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ mice expressing YFP and *Rosa26*$^{mTmG/+}$*; Tmem119*$^{CreER/+}$ mice with no YFP. In both CreER lines, the recombined microglia form a distinct population identified by reduced fluorescence in the FL2 585/30 nm channel and increased fluorescence in FL1 525/50 nm channel.

Supplementary Figure 2: Homozygosity for CreER increases recombination efficiency

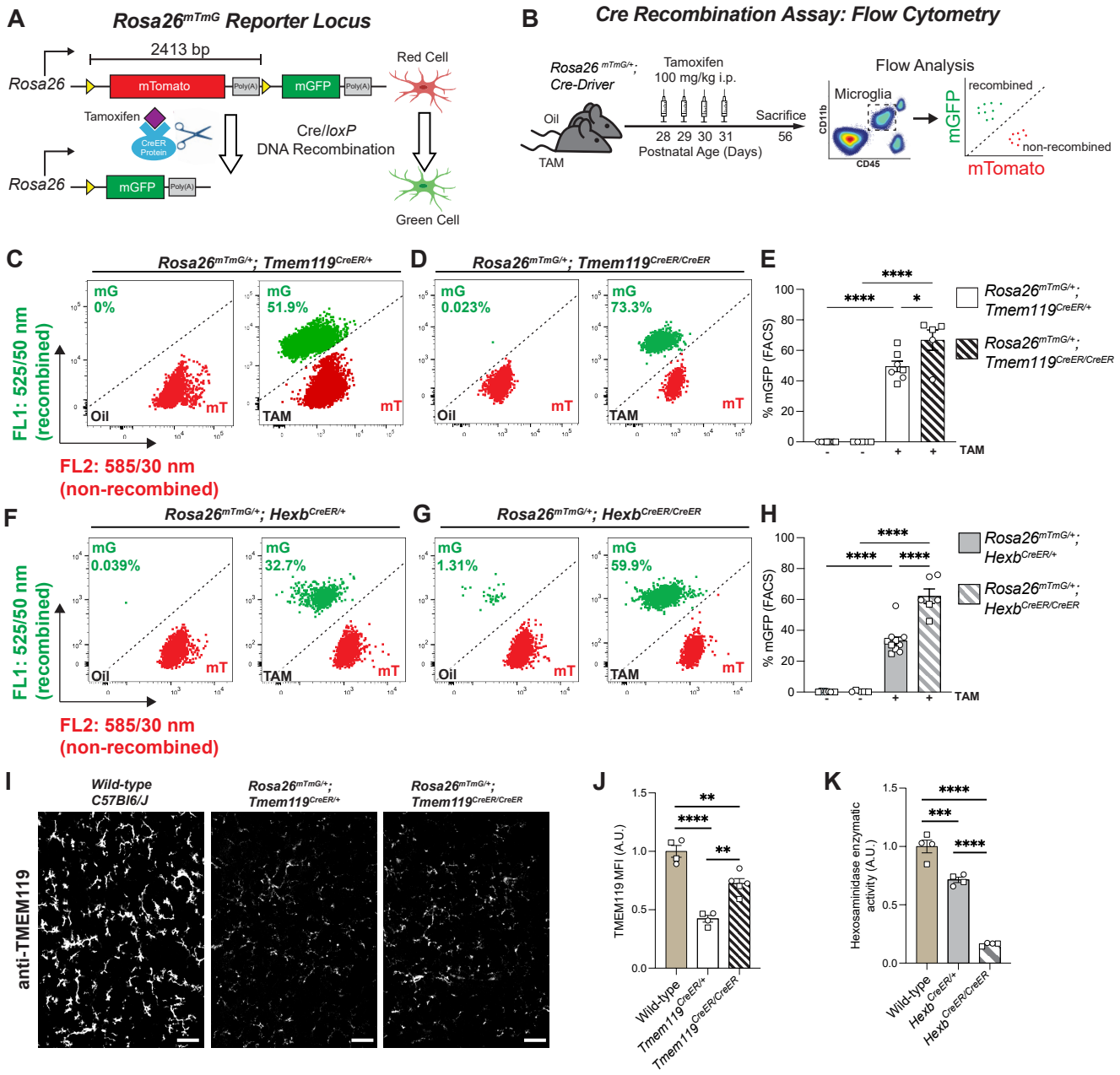

Figure S2. Homozygosity for CreER increases recombination efficiency.

Related to Figure 1

(A) Diagram of the *Rosa26*^{mTmG} allele and corresponding cellular fluorescence before and after Cre/*loxP* DNA recombination. *loxP* sites are indicated by yellow triangles.

(B) Diagram of experimental protocol used to assess tamoxifen (TAM) induced Cre/*loxP* recombination of *Rosa26*$^{mTmG/+}$ in microglia by flow cytometry.

(C, D, F, and G) Representative flow cytometry results show the percentage of mGFP^{+} (mG^{+}) and mTomato^{+} (mT^{+}) microglia from mice heterozygous for *CreER* used for flow cytometry in Figure 1 and mice homozygous for *CreER*.

(E and H) Quantification of the percentage of recombined mGFP^{+} microglia in mice heterozygous vs. homozygous for (E) *Tmem119*^{CreER} and (H) *Hexb*^{CreER} showed increased recombination in homozygous CreER mice compared to heterozygous CreER mice for both CreER lines (2-way ANOVA with Tukey's post hoc test. *Tmem119*^{CreER}: n = 8 oil Cre/+, 5 oil Cre/Cre, 7 TAM Cre/+, 5 TAM Cre/Cre mice. *Hexb*^{CreER}: n = 9 oil Cre/+, 5 oil Cre/Cre, 9 TAM Cre/+, 6 TAM Cre/Cre mice. * $p < 0.05$, **** $p < 0.0001$).

(I) Representative immunofluorescent images of brain sections from *Tmem119*$^{CreER/+}$, *Tmem119*$^{CreER/CreER}$, and wild-type mice immunolabeled with anti-TMEM119. Scale bars 20 μm.

(J) Quantification of anti-TMEM119 mean fluorescence intensity (MFI) shows reduced levels in *Tmem119*$^{CreER/+}$ and *Tmem119*$^{CreER/CreER}$ mice compared to wild-type (1-way ANOVA with Tukey's post hoc. n = 4, 4, 5 mice. ** $p < 0.01$, **** $p < 0.0001$).

(K) Quantification of hexosaminidase enzymatic activity shows reduced activity in microglia isolated by fluorescence-activated cell sorting from *Hexb*$^{CreER/+}$ and *Hexb*$^{CreER/CreER}$ mice compared to wild-type, indicating reduced levels of HEXB protein (1-way ANOVA with Tukey's post hoc test. n = 4 mice per genotype. *** $p < 0.001$, **** $p < 0.0001$).

All data are presented as mean ± SEM. Individual datapoints indicate males (squares) and females (circles).

Supplementary Figure 3: Analysis of *Rosa26*^{mTmG} recombination by immunofluorescence

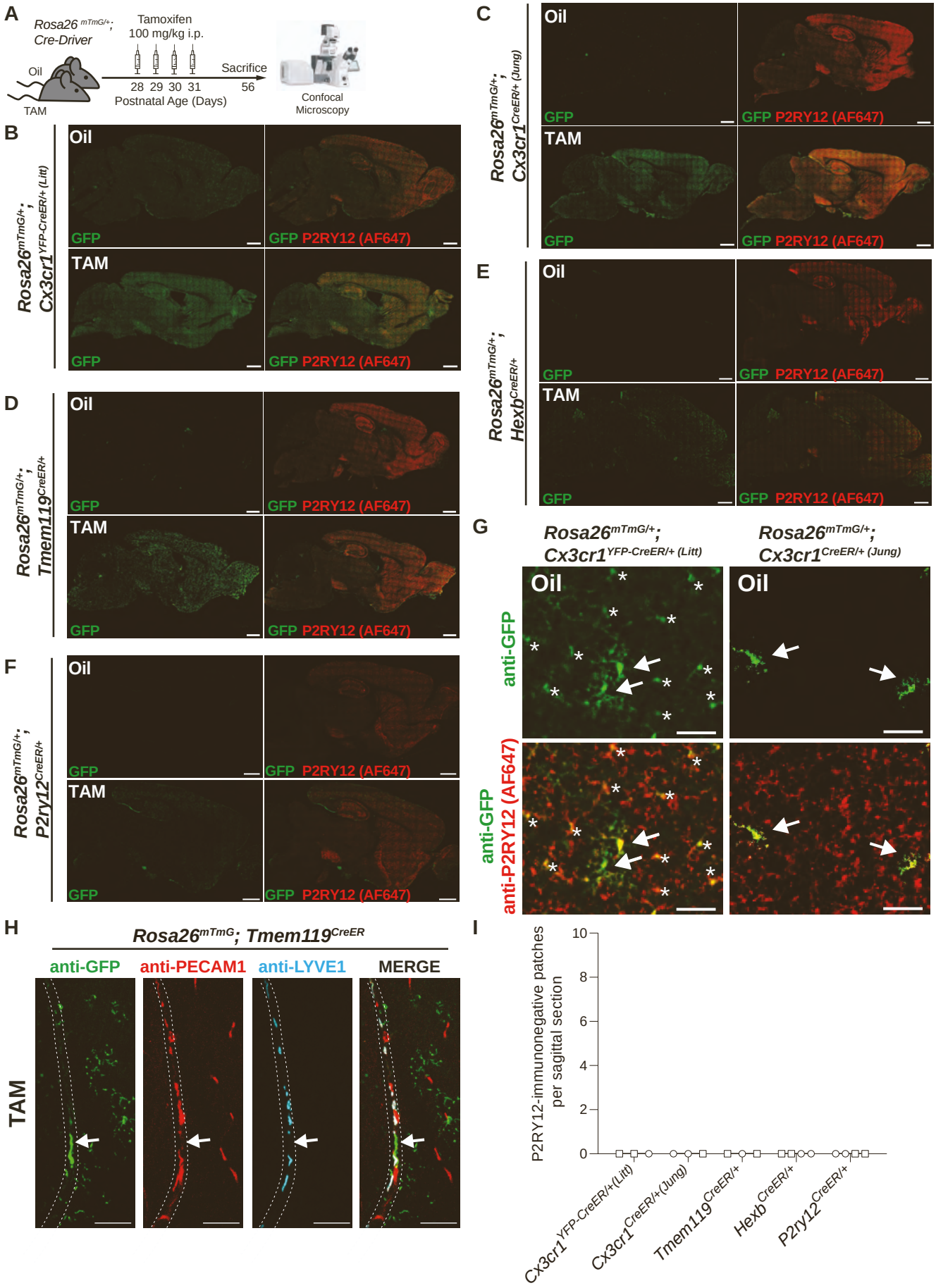

Figure S3. Analysis of *Rosa26*^{mTmG} recombination by immunofluorescence.

Related to Figure 2

(A) Diagram of experimental protocol used to assess tamoxifen (TAM) induced Cre/*loxP* recombination of *Rosa26*$^{mTmG/+}$ in microglia by immunofluorescence.

(B-F) Representative immunofluorescent images of brain sections from right hemisphere of oil and TAM injected mice used for flow cytometry in Figure 1 (see also Figs. 1-3, 5). Sections were immunolabeled for anti-P2RY12 (AlexaFluor647 (AF647) pseudo-colored red) to identify microglia and anti-GFP (green) to identify recombined cells. Scale bars 1 mm.

(G) High magnification representative immunofluorescent images of brain sections from right hemispheres of oil injected *Rosa26*$^{mTmG/+}$*; Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ and *Rosa26*$^{mTmG/+}$*; Cx3cr1*$^{CreER/+\ (Jung)}$ mice (see also Figure 5D). Sections were immunolabeled for anti-P2RY12 (AlexaFluor647 (AF647) pseudo-colored red) to identify microglia and anti-GFP (green) to identify recombined mGFP^{+} microglia (white arrows). In the *Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ line, the soma of unrecombined microglia are also immunolabeled by anti-GFP due to the constitutive expression of YFP (asterisks), but can be distinguished from recombined mGFP^{+} microglia by fluorescence intensity and membrane labeling. Scale bars 50 μm.

(H) Representative immunofluorescent image of a brain section from a TAM injected *Rosa26*$^{mTmG/+}$*; Tmem119*$^{CreER/+}$ mouse, immunolabeled with anti-GFP (green) to label recombined cells, anti-PECAM1 (red) to label endothelial cells, and anti-LYVE1 (cyan) to label border-associated macrophages. Before immunostaining, the section was photobleached to reduce endogenous mTomato signal. White arrow indicates an mGFP^{+} cell within the meningeal layer (white outline) that is immunonegative for both PECAM1 and LYVE1. Scale bar 50 μm.

(I) Quantification of the number of cortical patches devoid of anti-P2RY12 immunofluorescence in microglial CreER lines exposed to TAM at P28 (*n* = 3, 3, 3, 4, 4 mice).

Data are presented as mean ± SEM. Individual datapoints indicate males (squares) and females (circles).

Supplementary Figure 4: RNA sequencing of microglia after tamoxifen exposure

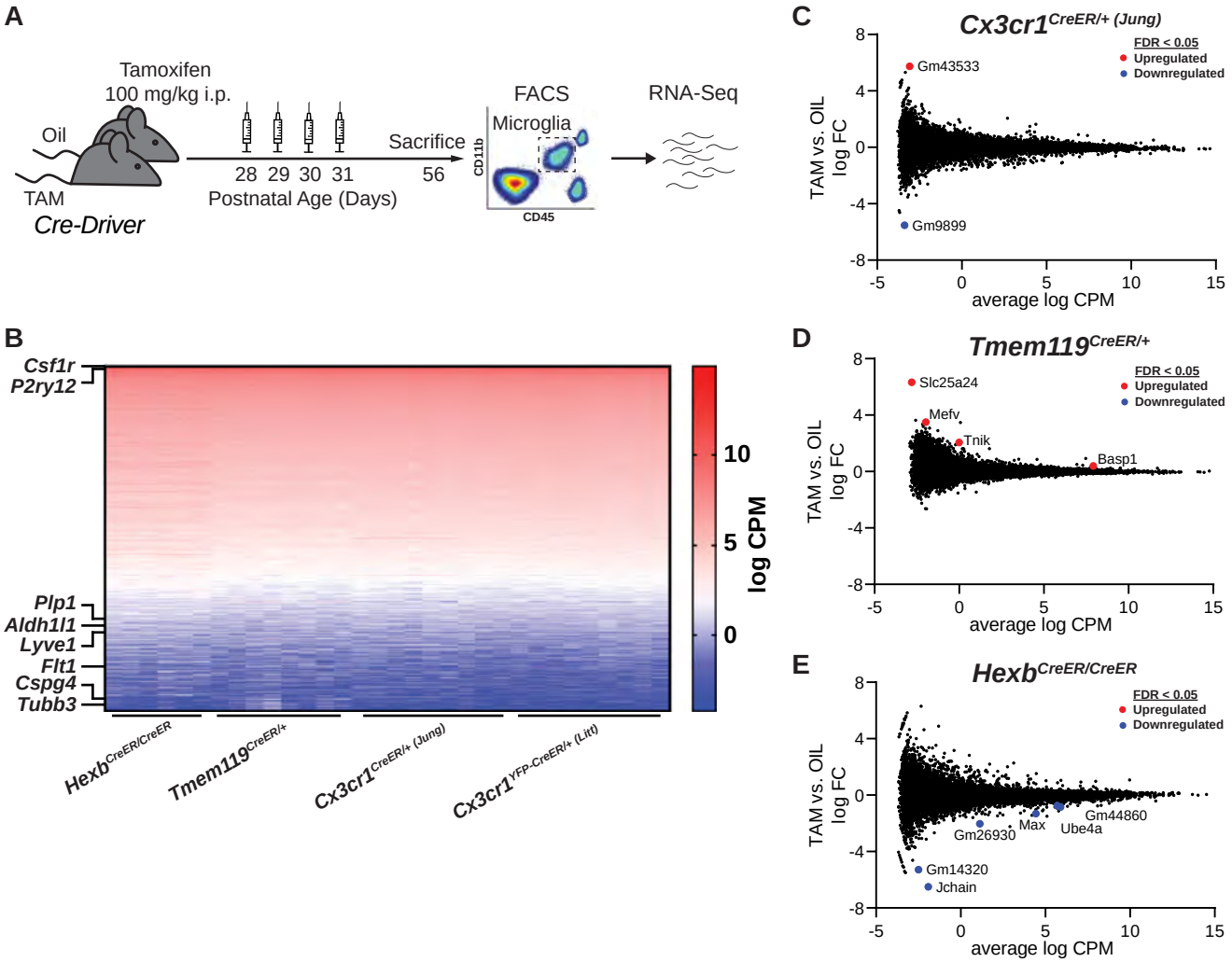

Figure S4. RNA sequencing of microglia after tamoxifen exposure

Related to Figure 4.

(A) Diagram of experimental protocol used to perform RNA sequencing on microglia from CreER lines injected with tamoxifen (TAM) or oil.

(B) Heatmap of gene expression values (log counts per million (CPM)) across all samples. Rows corresponding to cell-type specific genes markers for microglia (*Csf1r, P2ry12*), oligodendrocytes (*Plp1*), astrocytes (*Aldh1l1*), border-associated macrophages (*Lyve1*), endothelial cells (*Flt1*), oligodendrocyte precursor cells (*Cspg4*), and neurons (*Tubb3*) are annotated.

(C-E) Smear plots of TAM vs. oil injected (C) $Cx3cr1^{CreER/+\ (Jung)}$, (D) $Tmem119^{CreER/+}$, and (E) $Hexb^{CreER/CreER}$ mice depicting log fold change (FC) on the y-axis against log CPM on the x-axis ($Cx3cr1^{YFP\text{-}CreER/+\ (Jung)}$: n = 4, 5 mice; $Tmem119^{CreER/+}$: n = 4, 4 mice; $Hexb^{CreER/CreER}$: n = 3, 3 mice). Differentially expressed genes with false discovery rate (FDR) < 0.05 are annotated in red (upregulated by TAM) or blue (downregulated by TAM).

Supplementary Figure 5: The number of spontaneously recombined cells increases with age in *Rosa26*^{mTmG};*Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ mice

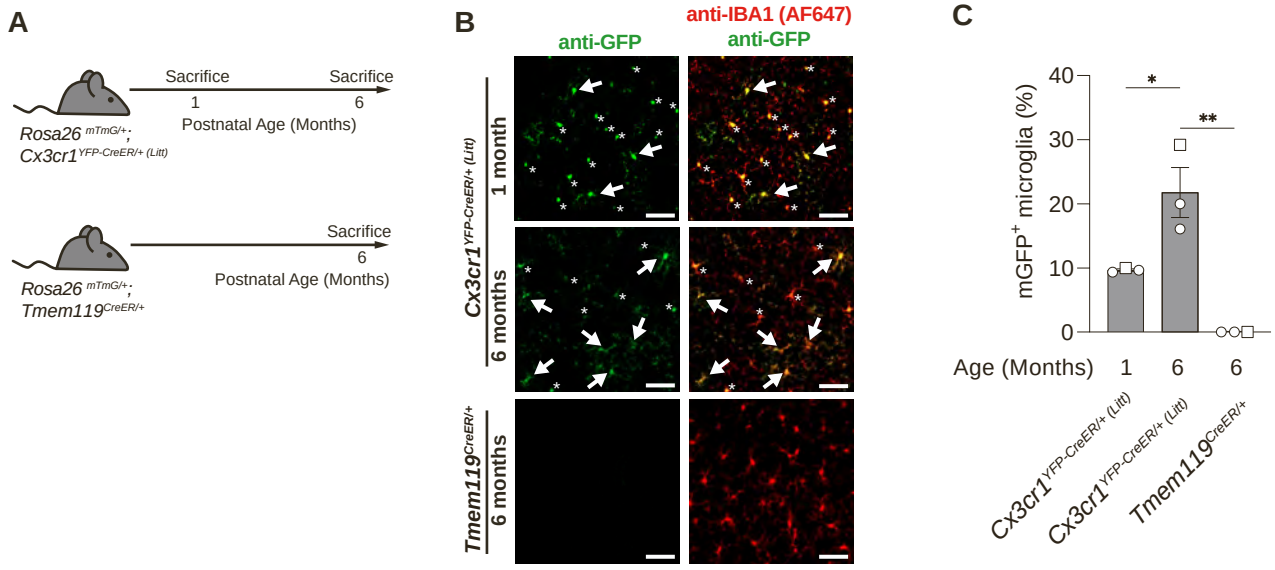

Figure S5. **The number of spontaneously recombined cells increases with age in *Rosa26*$^{mTmG/+}$; *Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ mice.**

Related to Figure 5.

(A) Diagram of genotypes and ages used for assessment of spontaneous Cre/*loxP* recombination of *Rosa26*$^{mTmG/+}$ mice with no injection.

(B) Representative immunofluorescent images of brain sections immunolabeled for anti-GFP (green) and anti-IBA1 (AlexaFluor647 (AF647) pseudo-colored red) to identify recombined mGFP^{+} microglia (white arrows). In the *Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ line, the soma of unrecombined microglia are also immunolabeled by anti-GFP due to the constitutive expression of YFP (asterisks), but can be distinguished from recombined mGFP^{+} microglia by fluorescence intensity and membrane labeling. Scale bars 50 μm.

(C) Quantification of the percentage of recombined mGFP^{+} microglia in the cortex shows increased recombination of *Rosa26*$^{mTmG/+}$ in 6 month old *Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ mice vs. 1 month old *Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ mice and 6 month old *Tmem119*$^{CreER/+}$ mice (1-way ANOVA with Tukey's post hoc; n = 3 mice per group. * $p < 0.05$, ** $p < 0.01$).

All data are presented as mean ± SEM. Individual datapoints indicate males (squares) and females (circles).

Supplementary Figure 6: *Tmem119*^{CreER} efficiently recombines short inter-*loxP* distances

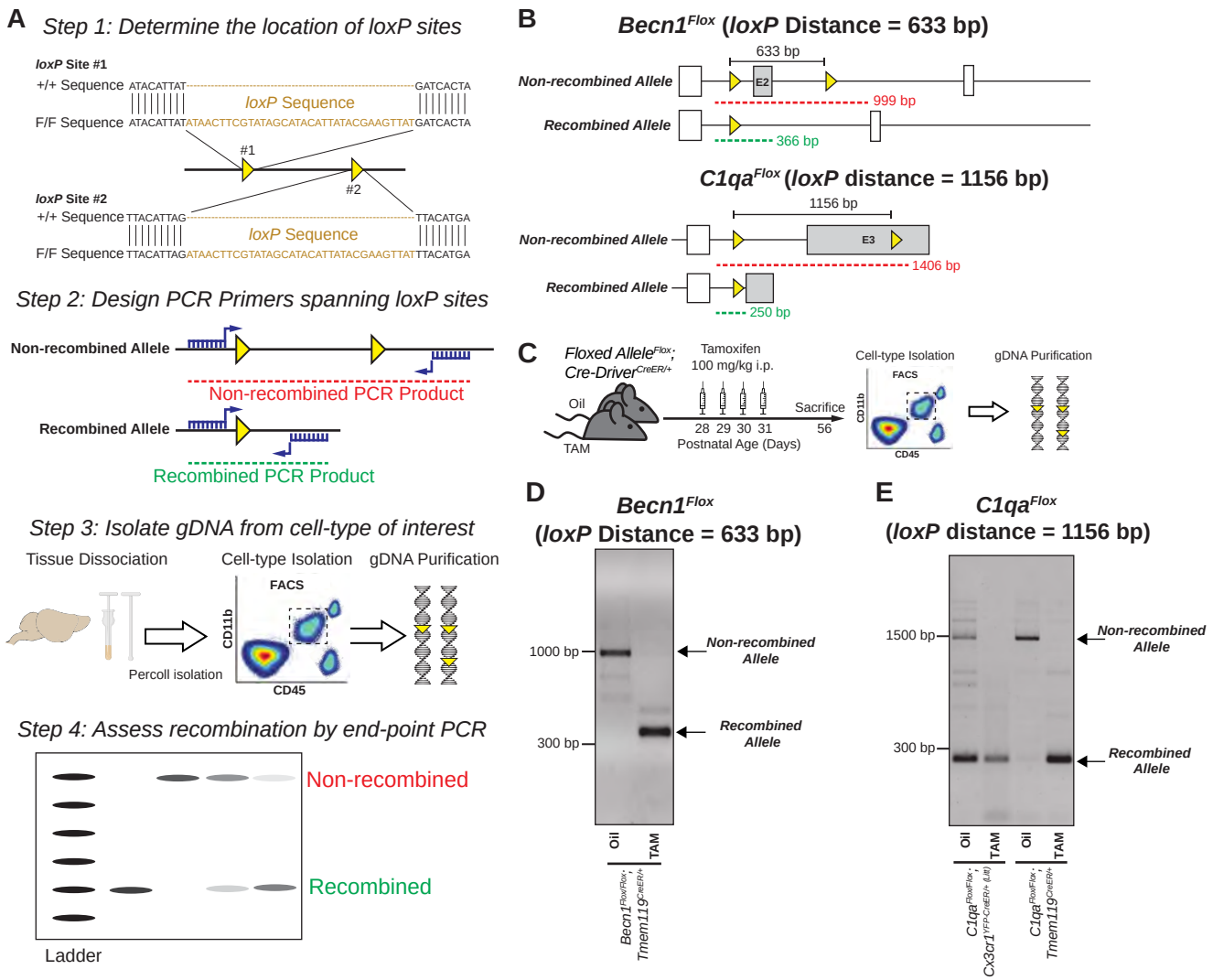

Figure S6. *Tmem119*^{CreER} efficiently recombines short inter-*loxP* distances

Related to Figure 6

(A) Diagram of protocol to assess Cre/*loxP* recombination of genomic DNA (gDNA) from microglia isolated by fluorescence-activated cell sorting (FACS).

(B) Diagram of *Becn1*^{Flox} allele and *C1qa*^{Flox} allele before and after Cre/*loxP* recombination showing the locations of the *loxP* sites (yellow triangles), the inter-*loxP* distance, and the endpoint PCR products for non-recombined (red) and recombined (green) gDNA.

(C) Diagram of experimental protocol used to obtain microglial gDNA from CreER lines injected with tamoxifen (TAM) or oil.

(D) Gel image of endpoint PCR products from oil-injected samples from *Becn1*$^{Flox/Flox}$; *Tmem119*$^{CreER/+}$ mice only show the non-recombined allele and TAM-injected samples from *Becn1*$^{Flox/Flox}$; *Tmem119*$^{CreER/+}$ mice only show the recombined allele, indicating no spontaneous recombination without TAM and efficient recombination with TAM.

(E) Gel image of endpoint PCR products from oil-injected samples from *C1qa*$^{Flox/Flox}$; *Tmem119*$^{CreER/+}$ mice only show the non-recombined allele and TAM-injected samples from *C1qa*$^{Flox/Flox}$; *Tmem119*$^{CreER/+}$ mice only show the recombined allele, indicating no spontaneous recombination without TAM and efficient recombination with TAM. In contrast, the gel image of endpoint PCR products from oil-injected *C1qa*$^{Flox/Flox}$; *Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ shows both recombined and non-recombined alleles, indicative of spontaneous recombination.

Supplementary Figure 7: Analysis of recombination efficiency in homozygous *Rosa26*$^{mTmG/mTmG}$ microglia and heterozygous *Rosa26*$^{mTmG/+}$ primary microglia

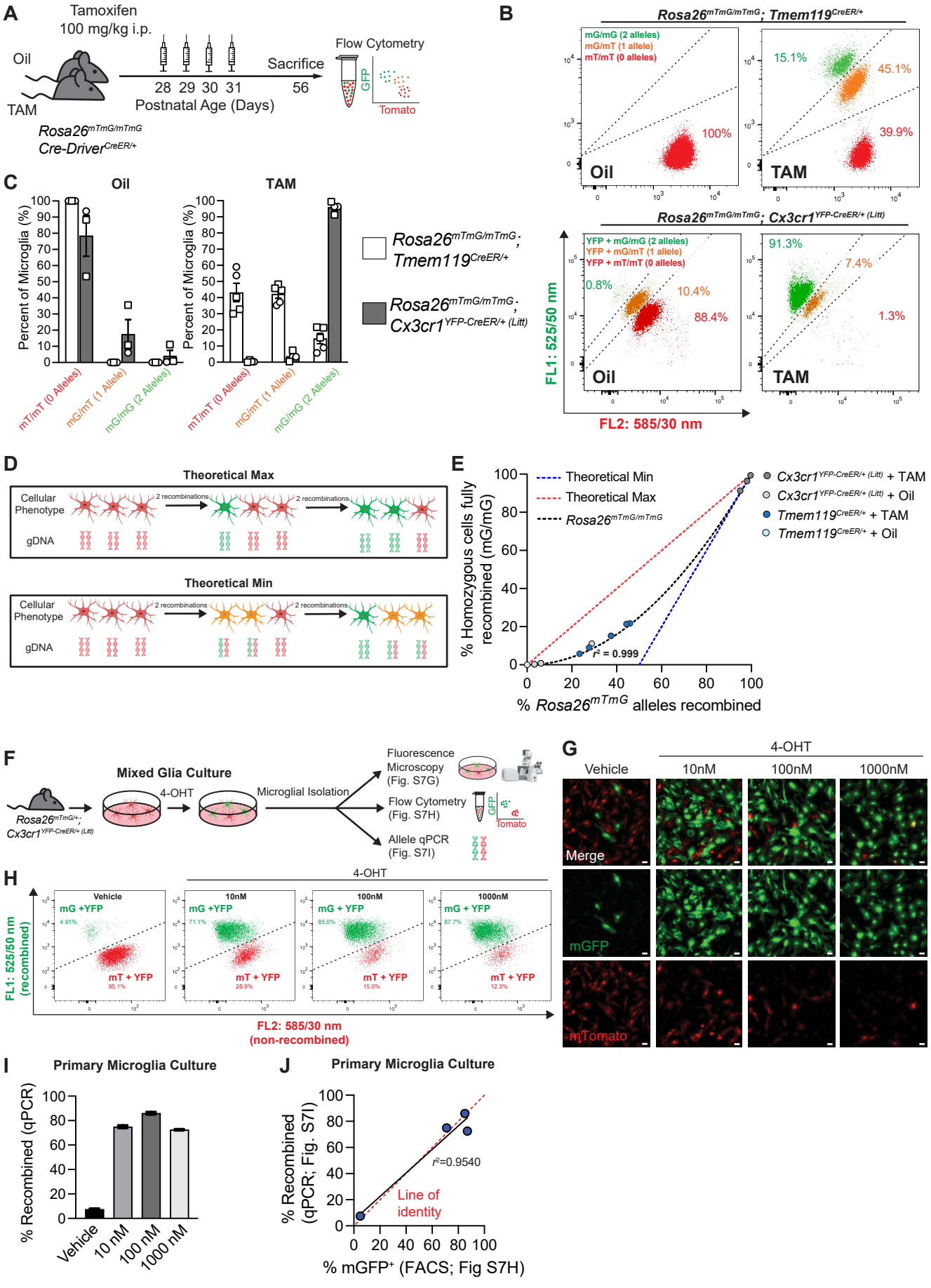

Figure S7. Analysis of recombination efficiency in homozygous *Rosa26*$^{mTmG/mTmG}$ microglia and heterozygous *Rosa26*$^{mTmG/+}$ primary microglia

Related to Figure 7

(A) Diagram of protocol to assess Cre/*loxP* recombination of homozygous *Rosa26*$^{mTmG/mTmG}$ microglia isolated by flow cytometry from mice injected with oil or tamoxifen (TAM).

(B) Flow cytometry analysis of doubly recombined mGFP$^+$/mGFP$^+$ (mG/mG) vs. singly recombined mGFP$^+$/mTomato$^+$ (mG/mT) microglia vs. non-recombined mTomato$^+$/mTomato$^+$ (mT/mT) microglia in *Rosa26*$^{mTmG/mTmG}$*; Tmem119*$^{CreER/+}$ mice with no YFP and *Rosa26*$^{mTmG/mTmG}$*; Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ mice expressing YFP.

(C) Bar graph of the number of non-recombined mT/mT, singly recombined mG/mT, and doubly recombined mG/mG microglia per animal after injection with oil or TAM.

(D) Diagram of the number of non-recombined (red), singly recombined (orange), and doubly recombined cells (green) obtained after recombination of genomic DNA (gDNA). Diagrams show the theoretical maximum (max; top) and theoretical minimum (min; bottom) of doubly recombined cells for a given number of recombined alleles of gDNA.

(E) Plot of the percentage of alleles recombined vs. the number of homozygous cells fully recombined for the theoretical min (blue dashed line), theoretical max (red dashed line), and observed data of *Rosa26*$^{mTmG/mTmG}$ recombination in oil-treated *Tmem119*$^{CreER/+}$ mice (light blue dots), tamoxifen (TAM)-treated *Tmem119*$^{CreER/+}$ mice (dark blue dots), oil-treated *Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ mice (light grey dots), and TAM-treated *Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ mice (dark grey dots). The observed data from *Rosa26*$^{mTmG/mTmG}$ mice fit to a second-order polynomial curve (black dashed line; $y = x^2$; $r^2 = 0.999$).

(F) Diagram of experiment to assess Cre/*loxP* recombination in primary microglia cultures from *Rosa26*$^{mTmG/+}$*; Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ mice after exposure to 4-hydroxytamoxifen (4-OHT).

(G) Fluorescent images of endogenous mGFP (green) and endogenous mTomato (red) in primary microglia cultures from *Rosa26*$^{mTmG/+}$*; Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ mice after exposure to 4-OHT. Scale bars 25 μm.

(H) Flow cytometry analysis of recombined mGFP^{+} (mG) vs. non-recombined mTomato^{+} (mT) microglia in primary microglia cultures from *Rosa26*$^{mTmG/+}$*; Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ mice after exposure to 4-OHT.

(I) Quantification of the percentage of recombined gDNA by quantitative PCR (qPCR) in primary microglia exposed to vehicle (ethanol) or 10 nM, 100 nM, or 1000 nM of 4-OHT (n = 2 experiments, each with 3 technical replicates).

(J) Graph of percent recombination of *Rosa26*^{mTmG} in primary microglia from *Rosa26*$^{mTmG/mTmG}$; *Cx3cr1*$^{YFP\text{-}CreER/+\ (Litt)}$ mice after exposure to 4-OHT as measured by flow cytometry analysis vs. the recombination rate as measured by qPCR of microglial gDNA isolated by fluorescence-activated cell sorting (FACS). Data points fit to a linear curve (black line; r^2 = 0.9540), closely aligned with the line of identity (red dashed line), indicating that qPCR provides a linear, quantitative measurement of *Rosa26*^{mTmG} recombination in in vitro samples.

Data in (C) and (I) are presented as mean ± SEM. Individual datapoints in (C) indicate males (squares) and females (circles).

Table S1. Comparison of microglia CreER lines

Related to Figure 1

Cre/*loxP* considerations	***Cx3cr1*$^{YFP\text{-}CreER\ (Litt)}$**	***Cx3cr1*$^{CreER\ (Jung)}$**	***Tmem119*^{CreER}**	***Hexb*^{CreER}**	***P2ry12*^{CreER}**
Strength of CreER activity	++++	++++	++	++	+
Retained expression of gene driving CreER	++	++	+++	+++	++++[a]
Specificity for microglia vs. parenchymal CNS cell types	++++	++++	++++	++++	++++
Specificity for microglia vs. border-associated macrophages	+	+	++++	++++	++++
Off-target effects of tamoxifen at P28	-	-	-	-	-
Tamoxifen-independent recombination	+	+	-	-	-

Table comparing microglia CreER lines. Each consideration is rated on a scale from low (-) to high (++++).

a. McKinsey et al. [S1]

Table S2. DNA Primers

Related to STAR Methods

Primer	**Sequence**
Rosa26^{Ai9} endpoint PCR, forward primer	*GCGGGCCCTAAGAAGTTCCTAT*
Rosa26^{Ai9} endpoint PCR, reverse primer	*TTGCACTTAACGCGTACAAGGC*
C1qa^{Flox} endpoint PCR, forward primer	*TGACCCTCCCAGTCTCCTGCAG*
C1qa^{Flox} endpoint PCR, reverse primer	*CCCCAGGGTGCTAAAGCCCCAT*
Rosa26^{mTmG} endpoint PCR, forward primer	*GCAACGTGCTGGTTATTGTG*
Rosa26^{mTmG} endpoint PCR, reverse primer	*TTCTGCTGGTAGTGGTCGGCGA*
Becn1^{Flox} endpoint PCR, forward primer	*GGTAGCCGCGGCCGCATTTAAA*
Becn1^{Flox} endpoint PCR, reverse primer	*TGACGCCCTCTTCTGGCCTCTC*
Rosa26^{mTmG} qPCR, control, forward primer	*GCAACATCCTGGGGCACAAGCT*
Rosa26^{mTmG} qPCR, control, reverse primer	*TTCTGCTGGTAGTGGTCGGCGA*
Rosa26^{mTmG} qPCR, floxed, forward primer	*GACCGCCAAGCTGAAGGTGACC*
Rosa26^{mTmG} qPCR, floxed, reverse primer	*TGAAGCCCTCGGGGAAGGACAG*
Rosa26^{mTmG} qPCR, recombined, forward primer	*GCAACGTGCTGGTTATTGTG*
Rosa26^{mTmG} qPCR, recombined reverse primer	*GGCCATTCTCCTGTCCGTTCGC*

Supplemental references

S1. McKinsey, G.L., Lizama, C.O., Keown-Lang, A.E., Niu, A., Santander, N., Larpthaveesarp, A., Chee, E., Gonzalez, F.F., and Arnold, T.D. (2020). A new genetic strategy for targeting microglia in development and disease. eLife *9*. 10.7554/eLife.54590.