Conditional Mutagenesis in Oligodendrocyte Lineage Cells

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Abstract

Cell-type-specific gene targeting with the Cre/loxP system has become an indispensable technique in experimental neuroscience, particularly for the study of late-born glial cells that make myelin. A plethora of conditional mutants and Cre-expressing mouse lines is now available to the research community that allows laboratories to readily engage in *in vivo* analyses of oligodendrocytes and their precursor cells. This chapter summarizes concepts and strategies in targeting myelinating glial cells in mice for mutagenesis or imaging, and provides an overview of the most important Cre driver lines successfully used in this rapidly growing field.

Key words Transgenic mice, Cre, Cell-type-specific targeting, Oligodendrocyte, Myelin

1 Introduction

1.1 Genetics of Oligodendrocyte Lineage Cells

Mouse genetics has become a powerful technique to study oligodendrocyte function and myelination *in vivo*. Building on a rich body of work from the 1960s on naturally occurring mouse mutants with characteristic dysmyelinating phenotypes and illustrious names, such as *jimpy*, *rumpshaker*, *shiverer*, and *quaking* mice, oligodendrocyte and myelin research made early use of cDNA and genomic cloning techniques. This allowed researchers to work with genetically defined mouse mutants of cloned genes well before most other fields in the neuroscience. In the 1990s, the generation of targeted mutations in mice, by homologous genomic recombination and selection of correctly targeted embryonic stem (ES) cells [1] (schematically depicted in Fig. 1), became a revolutionary technique throughout biology, allowing the functional analysis of virtually any gene *in vivo*. Thus, a few years after its introduction as a technique [2], a plethora of newly cloned genes, predicted to be important for the development of oligodendrocyte lineage cells (OLC, in the following for oligodendrocyte precursor cells (OPC) and mature oligodendrocytes) and Schwann cells were successfully inactivated and their phenotypes dissected. Targeted genes

Fig. 1 Scheme for conditional targeting of a gene in mice. **(a)** Simplified genomic structure of the wild-type allele. The locus comprises two exons (blue boxes E1 and E2) with the entire coding region (ORF) located within E2. **(b)** The targeting construct harbors a frt-flanked neo^R selection cassette in intron 1 and the loxP-flanked exon 2. Frt sites are depicted as green rectangles, loxP sites as orange triangles. **(c)** Modified allele after homologous recombination in ES cells. Homologous recombinants are identified by nested PCR using primers #1 and #2, and #1' and #2', respectively. Integration of the 3' loxP site is independently demonstrated by PCR with primers #3 and #4. **(d)** Allele after deletion of the neo^R selection cassette. Breeding of targeted mice with Flp-deleter mice can lead to mosaic offspring. Presence of the selection cassette may be addressed with primers #2 and #5, absence of the neo^R cassette with primers #5 and #6. **(e)** Cell-type-specific ablation of the modified allele in mice is demonstrated by PCR using primers #7 and #8

included those for myelin-specific structural proteins, such as MPZ [3], PLP1 [4], or CNP [5], and OLC-specific transcription factors, such as Olig 1 [6] or MYRF [7]. These added to the natural mouse mutants, often point mutations, for which gain- and loss-of-function effects were sometimes difficult to separate. Protocols for the positive/negative selection of mutant ES cells for germline transmission have been optimized [8] and the standard work flow in the authors' lab is detailed in [4, 9].

1.2 Principles of Conditional Mutagenesis in Mice

The ubiquitous inactivation of any widely expressed gene with a role in OLC can cause strong developmental defects and even early lethality in mice, which limits the value of "conventional" null mutants in the analysis of oligodendrocytes of the postnatal or adult nervous system. This is particularly true for genes whose knockouts die prematurely, such as the null mutants of the transcription factor (TF) genes *Olig2* [10], *Mrf* [7], or *Sox10* [11] and in the majority of cases, in which the targeted gene has vital roles outside the oligodendroglial cell lineage. This is also the case for TF

genes, such as *Zfhx1b/Sip1* [12], *Yy1* [13], or *Hif1alpha* [14], for specific micro-RNAs and their processing enzymes, and for genes encoding growth factors, their receptors, and downstream effectors in signaling cascades.

To circumvent these problems, sequence-specific recombination systems, such as the “Flp/frt” system (derived from *Saccharomyces cerevisiae* [15]) or the more widely utilized “Cre/loxP” system (derived from the bacteriophage P1 [16]), have been established that enable a spatially and temporally controlled modification of target genes. Cre, a 38 kD cyclization recombinase, belongs to the integrase family of site-specific recombinases and catalyzes recombination at specific recognition sites, termed “loxP” sequences that comprise 2 palindromic 13 bp binding sites flanking a central asymmetric 8 bp “spacer region” (Fig. 2). The Cre/loxP technology can be used to carry out deletions, integrations, inversions, or translocations of double-stranded DNA fragments. However, the most widespread application of the Cre/loxP technology in mice is the cell-type-specific (“conditional”) deletion of loxP-flanked (“floxed”) target genes. For this purpose, two mouse lines are required that work in combination: (1) a Cre “driver” line that expresses Cre (or the CreER^{T2} variant, *see* below) under control of a cell-type-specific promoter and (2) a mouse line, in which the gene of interest has been “floxed,” mostly by homologous recombination in ES cells. In the floxed line, an essential part of the targeted gene is flanked by two equally oriented, 34 bp long loxP sites (these sequences are absent from the normal murine genome). The position of both loxP sites is chosen such that their presence does not interfere with normal gene expression, typically in an intronic position. After crossbreeding the two mouse lines, all Cre-expressing cells in the double-transgenic offspring (as defined by the cell-type-specific promoter/enhancer driving the Cre transgene) will, in principle, recombine the floxed target gene, leaving a single loxP site behind. In mice homozygous for the floxed gene, this leads to the irreversible inactivation of gene function in a cell-type-specific manner.

Since the first demonstration of a cell-type-specific gene knock-out in living mice, the ablation of a ubiquitously expressed DNA polymerase selectively from T-cells [17], a long list of conditional mouse mutants has demonstrated the usefulness of this approach, including for oligodendroglial and myelin-related research. For

Fig. 2 Sequence of a wild-type loxP site. Please note that the bases in the 8 bp “spacer region” (except for the two in the middle) may differ in genetically engineered loxP variants

example, by inactivation of two ubiquitously expressed enzymes, PTEN and mTOR, selectively in cells of the oligodendroglial lineage, we and others have identified the PI3K/AKT/mTOR pathway as a physiological regulator of myelin membrane growth [18–20].

The Cre-loxP technology can also be used to turn on (or to turn off) regular transgenes generated by pronuclear injection. This approach has been helpful in mouse models of diseases that are caused by the overexpression of genes or transgenes with gain-of-function mutations. For example, a cell-type-specific “turn-off” strategy has been used to selectively remove an entire loxP-flanked transgene (encoding a mutant form of superoxide dismutase-1, SOD1), which is ubiquitously expressed, from OPC in the CNS. In this mouse model of amyotrophic lateral sclerosis (ALS), the loss of SOD1 toxicity from OPC was sufficient to delay disease onset [21], proving the role of OLC in a neurodegenerative disease. For “turning on” transgenes, most often a floxed “stop” cassette is placed upstream of a transgene that prevents functional translation of the RNA transcript. However, once the stop cassette is excised by Cre-mediated recombination, transgene expression, driven by a ubiquitously active promoter, is induced (*see* for example [22]). Importantly, upon Cre recombination the mouse genome is irreversibly changed. Experimental systems, in which transgene expression is switched “on” or “off” in a reversible manner, e.g., by the tetracycline regulated transactivator system [23, 24], have been discussed elsewhere [25].

1.3 Floxed Mice: Modifying Target Genes by Introducing loxP Sites

To generate mouse mutants with a Cre-modifiable target gene, essential regions of this gene need to be “floxed” in a way that the newly introduced loxP sequences do not interfere with gene regulation, mRNA splicing, or the protein coding region. Thus, loxP sites are typically placed into introns. However, also loxP insertions into 5′ or 3′ untranslated regions (UTRs) have been reported that did not interfere with wild-type-like expression of the target genes [26]. Since truncated proteins may still be functional or exert dominant-negative effects, floxing the first exon (s) of a gene with the ATG start codon is more likely to create a functional null allele. The generation of loxP-flanked alleles is commonly achieved by homologous recombination of a cloned genomic targeting construct that is transfected into embryonic stem cells (ESC) (Fig. 1). This “knock-in” strategy will be increasingly replaced by CRISPR/Cas9 nuclease guided gene editing of ESC or fertilized oocytes. Numerous protocols of Crispr/Cas9-based genome editing have been published recently, including the generation of conditional alleles [27–29].

Homologous recombination requires the cloning of a genomic targeting vector with a selection cassette, most commonly a neomycin resistance gene. In contrast to a conventional gene targeting construct, this neo^R cassette should be subsequently removed (also

from intronic sites) because cryptic splice sites may affect gene expression and turn the floxed allele into a hypomorph or even into a null allele [26, 30]. Thus, strategies have been developed to remove such a cassette, either *in vitro* or *in vivo*, once the correctly targeted allele has been identified. To do so, the selection cassette is often flanked by “*frt*” sites (for ‘flip recognition target’), the palindromic 34 bp sequence for the yeast Flp (“flippase”) recombinase [15]. Subsequent breeding to “Flp-deleter” mice [15, 31], in which Flp is active in germ cells or at the zygote stage, results in the removal of a selection cassette *in vivo* and in the germline [30], as schematically shown in Fig. 1.

Alternatively, the selection cassette in a targeting construct can be made removable by flanking it with a third loxP sequence. Recombination would require crossbreeding to Cre-deleter mice, such as EIIa-Cre [32, 33], aiming for partial recombination of specifically those loxP sites that flank the selection cassette.

We note that these intercrosses produce often mosaic offspring. *Frt* sites will not be necessarily recombined in all cells of a mouse that has inherited both the *frt*-targeted allele and the Flp-deleter allele [30]. Dependent on the recombination status of the germline cells, its offspring will (or will not) transmit the desired loxP-flanked allele, i.e., the allele specifically lacking the *frt*-flanked selection cassette (*see also* Fig. 1). Likewise, crossing floxed mice with a mutant allele harboring 3 loxP sites to a Cre-deleter mouse, such as EIIa-Cre males, will produce mosaic offspring, in which the moderately efficient expression of Cre leads to different recombination events (i.e., recombination between loxP1/2, loxP2/3, loxP1/3, and no recombination at all) to a variable degree. Depending on the recombination status of the individual germ cells, further breeding of mosaic mice to wild-type mice may yield all four types of targeted alleles in the next generation, and those mice that inherited the desired “selection cassette-removed-only allele” can be chosen for establishing the intended floxed mouse line. For this reason, mosaic offspring and all mice in the subsequent generation(s) have to be carefully checked for the segregation of the different loxP-flanked alleles and for the loss of the Cre or Flp genes. This requires a PCR analysis of genomic DNA using different sets of primers designed to detect all possible recombination events (*see* Fig. 1 for *frt*-flanked selection cassettes and [26, 34, 35] for the work flow of turning 3-loxP into 2-loxP mouse mutants).

The need for custom-made, self-designed floxed mutants has substantially decreased in recent years as a growing number of targeted ES cell clones and floxed mouse lines is made available to the scientific community by the International Knock-out Consortium (IKMC, www.knockoutmouse.org), which includes the European and the North American Conditional Mouse Mutagenesis Programs (EUCOMM, NorCOMM) [36]. In addition, more than 1270 floxed mouse strains are currently listed and made

available by the Jackson Laboratory (<https://www.jax.org/mouse-search>). See **Notes 1** and **2** for additional transgenic mice of relevance to study oligodendrocyte biology.

1.4 Cre and Inducible CreER^T Mouse Lines for Oligodendrocyte Lineage Cells

Many genes have been identified that are predominantly expressed in oligodendrocytes and/or their precursor cells. Based on their spatiotemporal expression profile, the corresponding Cre (or CreER^T, *see* below) driver lines can be categorized by their expression in OPC, mature oligodendrocytes, or the entire oligodendroglial cell lineage. For example, OPC are most often targeted with Cre expressed under control of the Ng2 or Pdgfr alpha regulatory region. Oligodendrocytes can be targeted by Cnp, Plp1, or Tmem10 promoter-driven Cre-transgenes, and the entire lineage can be targeted with Sox10-Cre driver lines (*see* Table 1). Most mouse lines are also listed online by the Network Glia (<https://www.networkglia.eu/tiermodelle>) and by the Jackson Laboratory (<https://www.jax.org/research-and-faculty/tools/cre-repository>), including purchasing options.

Oligodendrocytes in the CNS are of different spatial origin, which suggests some heterogeneity also with respect to gene expression, development, and adult function [54, 66, 67]. Moreover, cortical and subcortical oligodendrocyte defects have been associated with diseases of the CNS, including schizophrenia, depression, autism, and Alzheimer's disease [68]. Thus, it may be desirable to target oligodendroglial genes in cortical and subcortical oligodendrocytes and spare oligodendrocytes in brain areas essential for basic motor functions (cerebellum, spinal cord). We thus added also forebrain-specific Cre driver lines into Table 1, which can be used to target relevant oligodendroglial subsets, for example, when the target gene itself is expressed only in oligodendrocyte lineage cells, or if the targeted transgene (which shall be turned on or off) is driven by an OLC-specific promoter.

A better temporal control over Cre-mediated genomic recombination has been achieved with an inducible system, in which Cre is expressed as a chimeric fusion protein harboring the ligand-binding domain of a modified estrogen receptor that is activatable by the administration of tamoxifen. Specifically, Cre (when fused to the G521R-mutated hormone-binding domain of the human estrogen receptor) is still responsive to the synthetic estrogen receptor ligand 4-hydroxytamoxifen (4-OHT) but not to endogenous 17 β -estradiol (E2) [69, 70]. In the absence of tamoxifen, CreER^T is sequestered in the cytosol by binding to a heat-shock protein (Hsp90) and cannot enter the nucleus. However, upon binding of Tamoxifen (the prodrug) or 4-OHT (the active metabolite), CreER^T translocates into the nucleus and catalyzes recombination (Fig. 3). By this mechanism an extra level of temporal control is achieved in cell-type-specific gene targeting. The functionally improved version CreER^{T2} [71] is a triple mutant G400V/

Table 1
Selected genetically engineered mouse lines expressing Cre or CreER^T in the oligodendroglial lineage

Mouse line	Laboratory contact	Transgenic strategy	Side of recombination	References
<i>OPC</i>				
<i>Pdgfra</i> -CreER ^T	W.D. Richardson w.richardson@ucl.ac.uk	PAC transgenic mice expressing a TM-inducible CreER ^T under the control of the murine <i>Pdgfra</i> promoter/enhancer. The PAC comprises the entire <i>Pdgfra</i> gene (modified by fusion of the CreER ^T coding sequence into the <i>Pdgfra</i> start codon), with 55 kb of 5'- and 74 kb of 3' flanking sequences	OPC, few projection neurons in the forebrain (tested with TM application >P45)	[37]
<i>Pdgfra</i> -CreER ^T	D. Bergles dbergles@jhmi.edu	BAC transgenic mice that express CreER ^T under control of the murine <i>Pdgfra</i> promoter/enhancer. The BAC comprises the entire <i>Pdgfra</i> gene (with CreER ^T being inserted behind the 5'UTR), with 71 kb of 5'- and 41 kb of 3' flanking sequences	OPC, a subset of perivascular pericytes, ependymal cells in the choroid plexus (tested with TM application >P30)	[38]
<i>Ng2</i> -Cre	A. Nishiyama akiko.nishiyama@uconn.edu	BAC transgenic mice that express Cre under the control of the murine <i>Ng2</i> (<i>Cygp4</i>) promoter/enhancer. The BAC comprises the entire <i>Ng2</i> gene (with exon 1 being modified by the Cre coding sequence), with 60 kb of 5'- and 114 kb of 3' flanking sequences	<i>Ng2</i> -expressing cells (incl. OPCs) in the CNS and other organs	[39]
<i>Ng2</i> -CreER ^T BAC	A. Nishiyama akiko.nishiyama@uconn.edu	BAC transgenic mice that express a TM-inducible CreER ^T under the control of the murine <i>Ng2</i> (<i>Cygp4</i>) promoter/enhancer. The BAC comprises the entire <i>Ng2</i> gene (with exon 1 being modified by the CreER ^T coding sequence), with 60 kb of 5'- and 114 kb of 3' flanking sequences	<i>Ng2</i> -expressing cells (incl. OPCs) in the CNS and other organs	[40]
<i>Ng2</i> -CreER ^T	F. Kirchhoff frank.kirchhoff@uks.eu	Targeted insertion of CreER ^T into the <i>Ng2</i> gene locus (by homologous recombination in ESC)	<i>Ng2</i> -expressing cells (incl. OPCs) in the CNS and other organs, few neurons (when tested with TM application >P30)	[41]
<i>Ase1</i> -CreER ^T	J.E. Johnson jane.johnson@outsouthwestern.edu	Random integration of a BAC (comprising the <i>Ase1</i> gene and approx. 100 kb of 5'- and 200 kb of 3'-flanking sequences). The <i>Ase1</i> coding sequence is replaced by CreER ^T	Ventricular zone cells in certain domains of the developing nervous system (restricted to OL precursors when tested with TM application at E16)	[42]
<i>OL</i>				
<i>Cnp</i> -Cre ^a	K.-A. Nave nave@em.mpg.de	Targeted replacement of the <i>Cnp</i> coding region by Cre (by homologous recombination in ESC)		[5]

(continued)

Table 1
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Mouse line	Laboratory contact	Transgenic strategy	Side of recombination	References
			OL, SC, some OPC (also spinal MN and other CNS neurons when highly sensitive reporters are used)	
<i>Mbp-Cre</i>	M. Giovannini marco.giovannini@cphb.fr	Random integration of a transgenic construct harboring 1.3 kb of the 5' flanking sequence of the murine <i>Mbp</i> gene directing Cre expression	Two lines (<i>Mbp-Cre6</i> ; <i>Mbp-Cre9</i>) OL, testis, pituitary gland, heart and other organs. Recombination specificity in the brain not assessed at the cellular level	[43]
<i>Mbp-Cre</i>	M. Miura mmiura@hana.med.osaka-u.ac.jp	Random integration of a transgenic construct harboring 6.3 kb of the 5' flanking sequence of the murine <i>Mbp</i> gene directing Cre expression	OL, mainly in white matter of the spinal cord	[44]
<i>Mbp-CreER^T</i>	A. Gow agow@med.wayne.edu	Random integration of a transgenic construct harboring 1.9 kb of the 5' flanking sequence of the murine <i>Mbp</i> gene directing CreER ^T expression	TM-induced recombination of floxed genes in OL	[45]
<i>Plp-Cre</i>	R. Kothary rkothary@ohri.ca	Random integration of a transgenic construct harboring 2.4 kb of 5' flanking sequence, exon 1 (ATG mutated to CAG) and intron 1 of the murine <i>Plp1</i> gene to direct Cre expression	Widespread recombination in CNS, non-recombined cells are limited to vasculature	[46]
<i>Plp-Cre</i>	K.-A. Nave nave@ern.mpg.de	Random integration of a transgenic construct harboring 3.7 kb of 5' flanking sequence, exon 1 and intron 1 of the murine <i>Plp1</i> gene to direct Cre expression (Cre coding region (lacking the start ATG) was inserted in PLP exon 2; endogenous start ATG of PLP in exon 1 serves as an initiation codon)	Six lines with varying expression in OL, Purkinje cells and other neurons, and expression in other organs	[47, 48]
<i>Plp-CreER^{T,b}</i>	U. Suter usuter@cell.biol.ethz.ch	Random integration of a transgenic construct harboring 2.4 kb of 5' flanking sequence, exon 1 (ATG mutated to CAG) and intron 1 of the murine <i>Plp1</i> gene to direct Cre expression	TM-induced recombination of floxed genes in OL, (OPCs), SC	[49]
<i>Plp-CreER^{T,b}</i>	B. Popko bpopko@neurology.bsd.uchicago.edu	Random integration of a transgenic construct harboring 2.4 kb of 5' flanking sequence, exon 1 and intron 1 of the murine <i>Plp1</i> gene and the sequence of CreER ^T	TM-induced recombination of floxed genes in OL and SC (virtually no recombination in OPCs) (proven for TM application > P30)	[50]
<i>Mog-i-Cre</i>	A. Waisman Waisman@uni-mainz.de	Targeted insertion of Cre into the first exon of the <i>Mog</i> gene (by homologous recombination in ESC)	TM-induced recombination of floxed genes in OL (and certain neuronal populations [51])	[52]

<i>Tmem10/Opalin-Cre</i>	B. Xiao bxiao365@yahoo.com	Targeted insertion of Cre into the ORF of the <i>Tmem10</i> gene (by homologous recombination in ESC)	OL; not in OPCs, astrocytes or microglia	[53]
<i>OLC (OPC and OL)</i>				
<i>Olig2-Cre</i>	R.J. Franklin rj1000@cam.ac.uk W.D. Richardson w.richardson@ucl.ac.uk	Targeted introduction of Cre into the single exon of the <i>Olig2</i> gene (by homologous recombination in ESC)	OLC (close to 100% at OPC stage), motor neurons, subset of astrocytes	[54, 55]
<i>Sox10-Cre^a</i>	W.D. Richardson w.richardson@ucl.ac.uk G. Koentges g.koentges@ucl.ac.uk	Random integration of a 170 kb PAC transgene	OLC and neural crest derived cell lineages	[56]
<i>Sox10-iCreER^T</i>	W.D. Richardson w.richardson@ucl.ac.uk	Random integration of a 127 kb PAC (harboring the entire <i>Sox10</i> gene) in which the <i>Sox10</i> ORF was replaced by an iCreER ^T fusion gene	TM-induced recombination of floxed genes in OLC and neural crest derived cell lineages	[57]
<i>Sox10-iCreER^T BAC</i>	L. Dimou leda.dimou@uni-ulm.de	Random integration of a >210 kb BAC (harboring the entire <i>Sox10</i> gene) in which <i>Sox10</i> exon 3 was replaced by an iCreER ^T fusion gene	TM-induced recombination of floxed genes in OLC and neural crest derived cell lineages	[58]
<i>ShF:Cre^a</i>	A. McCallion andy@jhmi.edu	Random integration of a transgenic construct with the <i>Sox10</i> distal enhancer directing Cre expression	OLC and neural crest derived cell lineages	[59]
<i>Olig1-Cre</i>	Q.R. Lu Richard.Lu@cchmc.org C. Stiles stiles@dfci.harvard.edu D. Rowitch mailto:avid.Rowitch@ucsf.edu	Targeted replacement of the majority of the <i>Olig1</i> ORF by Cre (by homologous recombination in ESC). Two variants of the mouse line are available: frt-flanked neo cassette included [10] or removed (by breeding to a Flp-deleter mouse) [6]	Olig1-expressing cells of the CNS (incl. OL and >30% of GABAergic cortical interneurons)	[6, 10, 60]
<i>Regional Cre drivers (for OLC-specific genes)</i>				
<i>FoxG1-IRES-Cre</i>	D. Kawaguchi dkawaguchi@mol.f.u-tokyo.ac.jp	Targeted insertion of an IRES-Cre cassette into the 3'UTR of the <i>Foxg1</i> gene locus	Telencephalon	[61]
<i>Emx-Cre^c</i>	K.R. Jones krjones@stripe.colorado.edu	Targeted insertion of an IRES-Cre cassette into the 3'UTR of the <i>Emx1</i> locus	Dorsal telencephalic pallium	[62]

(continued)

Table 1
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Mouse line	Laboratory contact	Transgenic strategy	Side of recombination	References
<i>Emx</i> -CreER ^T	W.D. Richardson w.richardson@ucl.ac.uk	Random integration of a PAC transgene (approx. 180 kb in size), in which iCreER ^T was fused into the translation initiation codon of the <i>Emx1</i> gene	Dorsal telencephalic pallium	[54]
<i>Gsh2</i> -iCre	W.D. Richardson w.richardson@ucl.ac.uk	Random integration of a PAC transgene (approx. 110 kb in size) in which iCre was fused into the translation initiation codon of the <i>Gsh2</i> -iCre gene	Lateral and/or caudal ganglionic eminences	[54]

Abbreviations used (in addition to gene names): *ATG* start codon, *BAC* bacterial artificial chromosome, *CreER^T* tamoxifen-inducible Cre recombinase, *ESC* embryonic stem cells, *iCre* optimized, codon improved version of Cre recombinase [63], *IRE5* internal ribosomal entry site, *kilobases* DNA, *MN* motor neurons, *OLC* oligodendrocytes, *OLC* oligodendrocyte lineage cells, *OPC* oligodendrocyte precursor cells, *ORF* open reading frame, *PAC* P1-derived artificial chromosome, *SC* Schwann cells, *TM* tamoxifen^aCre expression in a variable subset of male germline cells reported: offspring of a Cre: floxed male may carry a ubiquitously recombined (i.e., deleted) floxed allele. It is suggested to pass the Cre-allele through the female germline

^bCre expression in a subset of male and (possibly) female germline cells reported: some offspring of a Cre::floxed male or female may carry the recombined floxed allele

^cA modest level of TM-independent recombination of loxP-flanked genes has been reported [64, 65]: Vehicle-injected fl/fl::CreER⁺ mice should be used as controls when utilizing this mouse line

Fig. 3 Schematic depiction of the conditional inactivation of a loxP-flanked (floxed) target gene (orange) in oligodendrocyte lineage cells using Tamoxifen-dependent activation of CreER^{T2}. Note that the CreER^{T2} gene (red) is expressed only in oligodendrocytes (on top) but the encoded protein (blue) prevented from entering the cell nucleus by being bound to Hsp90, a heat-shock protein. Upon administration of tamoxifen (yellow), occupation of the hormone-binding site of CreER^{T2} causes a conformational change and the release of Hsp90, followed by nuclear entry of CreER^{T2} and genomic recombination

M543A/L544A of the ligand-binding domain that exhibits decreased recombinase activity in the nonactivated state and increased efficacy upon tamoxifen binding. Today, it is the predominantly used variant for the generation of tamoxifen-inducible Cre driver lines (Table 1).

1.5 Breeding Conditional Mouse Mutants

On average, and depending on strain, the life span of mice is about 2 years. Females become sexually mature at the age of 6–8 weeks and remain fertile for about 6 months. During that time, they can give rise to approx. 2–4 litters of 5–10 pups each. With 19 days of gestation and 3 weeks until weaning (and routine genotyping), the generation time of mice is about 3 months. Males should be used for breeding not before 8 weeks of age and remain fertile for approximately 12 months. Breedings are ideally set up with 1 male and 2 female mice that are only separated 1–2 days before delivery.

For an efficient and cost-saving breeding strategy, the first generation of mice, which are heterozygous for a floxed gene X (termed X^{fl/+} in the following), should be intercrossed by brother/

sister matings and to a $Y^{Cre/+}$ (or $Y^{CreER/+}$) mouse that expresses Cre (or $CreER^{T2}$) under the desired cell-type-specific promoter from gene “Y.” By simple Mendelian rules, the crossbreeding of heterozygous $X^{fl/+}$ mice to each other (in the “F0 generation”) will generate 25% homozygous $X^{fl/fl}$ offspring in F1. Breeding of the same $X^{fl/+}$ heterozygotes to the $Y^{Cre/+}$ (or $Y^{CreER/+}$) driver mice will generate 25% $X^{fl/+}::Y^{Cre/+}$ (or $X^{fl/+}::Y^{CreER/+}$) offspring in F1 that are heterozygous for both mutant alleles. The subsequent mating of unaffected $X^{fl/+}::Y^{Cre/+}$ (or $X^{fl/+}::Y^{CreER/+}$) mice (F1) to the homozygous $X^{fl/fl}$ mice (F1, also unaffected) will already produce a F2 generation, in which 25% of the mice are “mutants” and possibly phenotypically affected, i.e., homozygous for two floxed alleles and heterozygous for the Cre driver ($X^{fl/fl}::Y^{Cre/+}$ or $X^{fl/fl}::Y^{CreER/+}$). Another 25% of mice are “controls” that are homozygous for the floxed gene but harbor no Cre allele ($X^{fl/fl}::Y^{+/+}$). However, by maintaining these F2 mice as future parental animals and mating them to each other, the generation of experimental mice and controls becomes more efficient: conditional mutants ($X^{fl/fl}::Y^{Cre/+}$ or $X^{fl/fl}::Y^{CreER/+}$) and controls ($X^{fl/fl}::Y^{+/+}$) will always be born in a 50/50 ratio. This breeding strategy can generally be applied if a $CreER^{T2}$ driver line is used, because the mutant animals ($X^{fl/fl}::Y^{CreER/+}$) are not burdened prior to Tamoxifen injection (Fig. 4). Obviously, for constitutive Cre driver lines this efficient breeding strategy is only suitable if the mutants ($X^{fl/fl}::Y^{Cre/+}$) are not burdened with a disabling phenotype or even infertile. In that case the parental $X^{fl/fl}$ mice should be intercrossed with unaffected double heterozygotes ($X^{fl/+}::Y^{Cre/+}$), as suggested before for the generation of F2 mice.

The efficient breeding strategy will also not suffice if (1) Cre is a knock-in and haploinsufficiency of this locus matters, (2) when cytotoxic effects of Cre are suspected [72, 73] or (3) when the conditional deletion of only one of the floxed target alleles is already of phenotypic relevance. In these cases additional breedings have to be included in order to generate control genotypes, such as $X^{+/+}::Y^{Cre/+}$ (for toxicity and haploinsufficiency of the Cre-expressing locus) or $X^{fl/+}::Y^{Cre/+}$ ($X^{fl/+}::Y^{CreER/+}$) (for haploinsufficiency of the target locus).

In the unlikely event that conditional mutants ($X^{fl/fl}::Y^{Cre/+}$ or $X^{fl/fl}::Y^{CreER/+}$) are never born and embryonic lethality can be ruled out, one should consider that Cre and the floxed gene are located on the same chromosome. This can make it difficult, or virtually impossible, to obtain the required genotype. Here, using a different Cre driver line may be the simplest solution to the problem.

Floxed alleles can be easily converted to null alleles by crossbreeding to Cre-deleter strains, such as EIIa-Cre [32, 33] that recombine in the germline. Thus, two strategies for working with conditional mutants are followed by utilizing either homozygously floxed mice ($X^{fl/fl}$) or heterozygously floxed mice ($X^{fl/-}$) on the null mutant background. Both strategies have advantages and

Fig. 4 Breeding scheme for the generation of conditional null mutants and controls. Having ensured that “floxed” mice are normally developed, the most convenient strategy is to generate and intercross parental animals of the genotypes $fl/fl::+/+$ and $fl/fl::CreER^{T2}/+$. This results in an equal number of offspring with the genotypes $fl/fl::+/+$ and $fl/fl::CreER^{T2}/+$. Injection of tamoxifen to the conditional mutants induces Cre-mediated recombination and thus cell-type-specific ablation of the gene of interest (orange frame). Experimental results from these animals should be carefully controlled by including three control conditions (black frames). First, vehicle treated $fl/fl::CreER^{T2}/+$ mice are used to control for effects of the Cre mutant allele, such as haploinsufficiency effects, Cre toxicity related issues or potential background recombination by non-induced Cre (in comparison to vehicle treated $fl/fl::+/+$ controls). Second, tamoxifen-treated $fl/fl::+/+$ animals without the Cre allele are used to determine the influence of Tamoxifen on the experimental paradigm

disadvantages. Since recombination of one allele is faster than of two, it may be more efficient to work on a heterozygous null mutant background, especially when Cre expression is low and/or transient. However, mice heterozygous for myelin genes can have relevant phenotypes [74, 75]. Thus, phenotypes caused by haploinsufficiency of the target gene may mask the conditional full mutant phenotype.

It is worth considering that Cre expression can sometimes be ectopically activated in the early embryo, which can give rise to fully recombined target genes in the germline. This has been reported for Sox10-Cre males [56] and is also sporadically evident in Cnp-Cre male mice [5] (Fig. 5, Table 1).

Fig. 5 Genomic PCR analysis of floxed mice by using a “two-Primer” strategy for the detection of short loxP-flanked gene modifications. **(a)** Primers #1 and #2 are positioned 5′ and 3′ from an essential exon of gene “X” that is flanked by loxP sites and detect the wild-type (+), the loxP-flanked (fl), and the recombined (fl-rec) allele. **(b)** Agarose gel electrophoresis on PCR products amplified from genomic tail tip DNA of mice with the genotypes *geneX^{fl/+}::Cnp^{+/+}* (left panels); *geneX^{fl/+}::Cnp^{Cre/+}* (middle panels); and *geneX^{fl-rec/+}::Cnp^{Cre/+}* (right panels). The top row represents PCR products obtained from the targeted gene “X,” the bottom row represents PCR products obtained from the *Cnp* allele. Please note that *Cnp^{Cre}* recombines in Schwann cells present in the tail tips. Thus, presence of the fl-rec allele is expected in all floxed mice that carry an additional *Cnp^{Cre}* allele (middle panels). In contrast, germline recombination, which fully converts the floxed allele into a ubiquitously present null allele, is indicated when (next to the wild-type allele) the floxed allele is not detectable, while the fl-rec product is present in high amounts (right panel). Please note that longer loxP-flanked sequences require a “three primer strategy” with, for example, an additional antisense primer being included that is located in the targeted exon. Here, this primer in combination with primer #1 would amplify a product from the wild-type and the floxed allele and primers #1 and #2 (more widely spaced from each other in this case) would only amplify a product after Cre-mediated recombination, since they would be spaced too much apart to amplify from the regular wild-type and the non-recombined floxed allele, respectively

1.6 Treatment Regimen for Tamoxifen-Induced CreER^{T2}-Mediated Recombination

Optimal protocols for tamoxifen-based CreER^{T2}-mediated recombination have to be empirically determined because the recombination efficiency depends on different variables, such as (1) the specifics of the “floxed” gene, including the methylation/acetylation state of the floxed gene (which defines the accessibility of loxP sites) and the length of the floxed DNA segment, (2) the type of applied drug, i.e., tamoxifen or 4-OHT, (3) the route of administration (i.p. injection, gavage) and the frequency and length of treatment, and (4) the variable of age, sex, and genetic background of the recipient mouse. We suggest the protocol in Subheading 1.8 as a starting point to assess the efficiency of tamoxifen-induced CreER^{T2}-mediated recombination in adult animals. An alternative

is the application protocol proposed by the Jackson labs (<https://www.jax.org/research-and-faculty/tools/cre-repository/tamoxifen>).

1.7 Utilizing *Cre-Indicator Mice*

To assess cellular recombination specificity and efficiency of any Cre driver line, a plethora of “indicator” mice have been generated, in which (upon crossbreeding) Cre activates permanently the expression of reporter genes encoding fluorescent proteins or histochemically detectable enzymes. Note that the strength of the cellular signal does not reflect Cre expression, but rather the strength of the promoter that drives the reporter. Widely used are knock-ins into the constitutively active ROSA26 locus or transgenes expressed under control of ubiquitously active promoters, such as that of the chicken beta-actin gene. The Jackson laboratory alone lists more than 280 Cre “reporter” strains (<https://www.jax.org/mouse-search>). The general idea of this strategy is that upon Cre-mediated excision of a loxP-flanked “stop”-cassette, cells will express easily identifiable reporter genes encoding, for example, LacZ [76, 77], alkaline phosphatase [78], or fluorescent proteins such as EGFP [79, 80], EYFP [81, 82], ECFP [82], ZsGreen [81], an EGFP-tandem version of dimeric DsRed (=tdsRed [83]), or tdTomato [81]. Since Cre-mediated recombination is irreversible, reporter genes become permanently activated in Cre-expressing cells and all their descendants. This allows this strategy to be applied for cell lineage tracing and fate mapping analyses, which have been heavily used in the analysis of oligodendrocytes and the spatial and temporal analysis of their precursor cells [37, 84–88] (see **Note 3**).

In dual reporter lines, the excised “stop” sequence is by itself a reporter gene, encoding a different fluorophore. Upon Cre recombination, this leads to a “switch” of color, including a variable time in which both reporters may be detectable. Weakly expressed fluorescent reporters can sometimes be made visible by more sensitive immunostaining. An X-Gal staining protocol to analyze the expression of beta-galactosidase (lacZ) as a reporter is given in Subheadings 2 and 3.

For myelin research, membrane-tagged reporters are of special interest. The ROSA^{mT/mG} reporter mouse line [89] expresses a ubiquitous cell membrane-localized tdTomato (mT) protein but, upon Cre-mediated recombination, a membrane-localized EGFP (mG). Similarly, ROSA^{nT/nG} reporter mice switch the same reporter proteins, which are directed to the cell nucleus [90]. Other reporter mice express fluorescent proteins that are targeted to membranes by a GPI anchor (CAG::GFP-GPI) or myristoylation (CAG::myr-Venus) [91]. Note that in myelinating glial cells, fluorescent proteins are too bulky to be wrapped into a compacted myelin sheath [92], but rather demarcate cytosolic tubes, paranodes, and other non-compacted regions of the myelin sheath.

For the sparse-labeling of individual oligodendrocytes, any of the indicator mice discussed above can be used in combination with a CreER^{T2} driver line and a single low-dose injection (i.p.) of tamoxifen. Here, suggested doses range from 25 to 200 mg/kg [64]. Another application is the simultaneous activation of single reporter genes in multicolor fluorescent (“brain-bow”) reporter mice that enable the single cell analysis of oligodendrocytes and their interactions in white matter tracts that are crowded with OLC [64, 93].

1.8 Technical Caveats

The Cre/loxP system is a powerful yet not unproblematic approach. One concern is that high-level expression of Cre itself may have off-target and thus unintended side effects on genomic integrity [94]. Thus, as a control group, the inclusion of mice with the Cre driver but without floxed alleles is highly recommended. Moreover, a randomly integrated Cre transgene may interfere with the expression of neighboring genes, a problem that can be reduced when only working with heterozygous Cre mice. While for targeted Cre “knock-in” mice the insertion site is well defined, haploinsufficiency may still occur and should be controlled for (by including non-Tamoxifen-induced CreER^{T2} mice or Cre mice without floxed alleles). We have, for example, replaced the coding region of *Cnp* by Cre, which has created a *Cnp* null allele, and heterozygous mice are fully normal and useful for the conditional deletion of floxed genes in oligodendrocytes. However, *Cnp*^{Cre/+} mice develop a mild behavioral phenotype after 18 months of age with signs of neuroinflammation [74], which should be controlled for in any genetic experiment addressing brain aging. Expressing Cre downstream of an IRES (internal ribosomal entry site) may solve such problems [61].

Another problem, mostly affecting transgenic Cre lines, is the fact that promoters and regulatory elements are not always as cell-type-specific in development as anticipated from gene expression in the adult. For example, PLP1 is a highly cell-type-specifically expressed myelin protein, but 4 out of 6 Plp1-Cre driver lines generated in our lab showed a variable degree of recombination in neuronal subpopulations [47, 48]. This is most likely due to a low level of (transient) Cre expression already in neural stem cells. The same is true for *Cnp*-Cre mice, which are used to target oligodendrocytes, but also mark certain neuronal subtypes when used in combination with specific reporters [95, 96]. These off-target effects require a highly sensitive reporter line, in which the single target gene is very accessible and rapidly recombined. Thus, activation of a single reporter gene does not yet prove that any two floxed alleles of real target genes are likewise recombined.

However, off-target recombination illustrates a Cre-specific problem: the enzyme is effective in trace amounts and any recombination is irreversible. On the other hand, Cre-mediated recombination is often not fully penetrant, i.e., less than 100% of the envisioned target cell population can be recombined. In this case, recombination efficiency is not necessarily limited by the Cre driver, but by the floxed alleles, whose chromatin state and accessibility for Cre may prohibit recombination in some but not all cells. What defines the epigenetic state of these target cells is unknown.

As mentioned above, for some OLC-specific Cre driver lines (not only those generated by pronuclear injection) at least sporadic Cre expression has been detected in the male germline (less frequent in female germline cells), and is likely underdiagnosed (or not reported) in other lines. When unaware of this, the resulting transmission of unexpected null alleles can severely perturb the experiment. A suitable PCR strategy to identify (1) wild-type, (2) floxed, and (3) fully recombined (fl-rec) alleles is presented in Fig. 5. Note that if Cre is also expressed in Schwann cells, the fully recombined allele can always be detected in the genomic DNA obtained from tail biopsies (independent of germline recombination).

In experiments with CreER^{T2} driver lines one should keep in mind that a low level of recombination may occur in the absence of tamoxifen, because the fusion protein is not 100% excluded from the nucleus. This has been reported, e.g., for two widely used Plp1-CreER^{T2} lines [64, 65], and is possibly due to the strong activity of the Plp1 promoter in combination with an easily accessible target gene.

Improvements of the Cre/loxP technique hold the potential of enhancing the cellular specificity of Cre or speeding up conditional mutagenesis. One is based on a “Split-Cre” complementation system, in which two inactive Split-Cre fragments are expressed under different promoters. Here, Cre recombinase activity is only regained when both halfmers are expressed in the same cell [97, 98].

Alternatively, it may suffice and be much faster than cross-breeding to delete genes in mature oligodendrocytes of homozygously floxed mice by stereotactically injecting Cre-expressing viruses into the brain. For oligodendrocytes, adeno-associated viruses (AAV) of various serotypes that express Cre (or CreER^{T2}) under control of a 1.3 kb promoter fragment from the mouse Mbp gene are commercially available (Vector Biolabs).

2 Materials

2.1 X-Gal Staining

2.1.1 Avertin Solution

1. Dissolve 2 g 2,2,2-tribromoethanol in 2 mL *tert*-amyl alcohol and add 96 mL of warm ddH₂O (40 °C). Stir for 30 min on a magnetic stirrer.
2. Sterile filtrate through a 0.2 µm filter.
3. Store in the dark at 4 °C for up to 4 weeks.
4. Use 200 µL per 10 g of mouse weight.

2.1.2 4%

Paraformaldehyde (PFA)
in 0.1 M Phosphate
Buffer Mix

1. 400 mL Na₂HPO₄ (0.2 M).
2. 100 mL NaH₂PO₄ (0.2 M).
3. 100 mL Formol (37%, filtered).
4. 400 mL ddH₂O.

2.1.3 X-Gal Solutions

1. X-Gal Solution A: 0.01% Sodium deoxycholate, 0.02% 4-Nonylphenyl-polyethylene glycol, 2 mM MgCl₂, 5 mM K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆] mix and keep it the dark at room temperature.
2. X-Gal Solution B (X-Gal stock solution): dissolve 20 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside in 1 mL DMSO.

2.2 Extraction of Genomic DNA and Genotyping of Cnp-Cre Mutant Mice

1. 10× Modified Gitschier Buffer (MGB): 6.7 mL 1 M Tris-HCl pH 8.8 (f.c.: 670 mM), 1.66 mL 1 M (NH₄)₂SO₄ (f.c. 166 mM), 650 µL 1 M MgCl₂ (f.c. 65 mM), add ddH₂O to 10 mL.
2. Proteinase K stock solution: 10 mg/mL (in ddH₂O).
3. Triton X-100 diluted 1:10 in PCR grade ddH₂O.

2.3 Intraperitoneal Injection of Adult Mice with Tamoxifen

1. Tamoxifen.
2. Corn Oil.
3. Ethanol.
4. 1 mL syringe graded in 100 µL intervals.
5. 22–27 gauge needle.
6. Sharp container.
7. Chemical fume hood or biological safety cabinet.
8. Tissue lyser.

3 Methods

3.1 X-Gal Staining

1. Anesthetize mouse with Avertin-solution.
2. Perfuse mice through the left ventricle with 15 mL of Hank's balanced salt solution, followed by 50 mL of 4% paraformaldehyde in PBS.

3. Post-fix brain and/or spinal cord in 4% paraformaldehyde for 2 h.
4. Section in phosphate-buffered saline (PBS) on a vibratome at 20–200 μm .
5. Incubate sections in freshly prepared X-gal solution (600 μL of solution “B” added to 10 mL solution “A”) in a humidified chamber (cell culture incubator) at 37 °C for up to 16 h in the dark. Check for staining under a microscope.
6. Post-fix in 4% paraformaldehyde for 30 min.
7. Wash sections two times in 1 $\times$ PBS for 10 min each, and mount them in AquaPolymount or other suitable mounting media.

3.2 Extraction of Genomic DNA and Genotyping PCR for *Cnp-Cre* Mutant Mice

3.2.1 DNA Extraction

Quick DNA Prep Protocol

Extract genomic DNA from tail tips, mouths swaps, or ear punches by using a kit according to the manufacturer’s protocols or by the following cheap “Quick DNA prep protocol.”

All of the following steps are carried out in a 96 well plate using a shaking incubator and a water bath.

1. Prepare fresh 1 $\times$ MGB (sufficient for 96 samples):
 - (a) 2.2 mL 10 $\times$ MGB.
 - (b) 220 μL β -Mercaptoethanol (final conc. 1%).
 - (c) 1.1 mL 10% Triton X-100 (final conc. 0.5%).
 - (d) 18.48 mL PCR-grade ddH₂O.
2. Add 80 μL of 1 $\times$ MGB to each tail/ear-punch/mouth swap.
3. Add 20 μL of proteinase K stock solution.
4. Digest tissues/cells for 1–2 h at 55°C while gently shaking.
5. Vortex samples briefly.
6. Quick spin down to collect all liquid.
7. Heat inactivate proteinase K by heating samples at 95°C for 5 min.
(Important: traces of proteinase K suffice to inactivate Taq polymerase).
8. Spin down debris (5–10 min at 2000 $\times g$).
9. Use 1–2 μL of supernatant for PCR.

3.2.2 Standard-PCR- Protocol for Genotyping *Cnp-Cre* Mice

1. Prepare PCR reaction mix:
 - (a) 1 μL DNA (100–400 ng).
 - (b) 0.5 μL Puro3 s primer (10 pmol/ μL).

- (c) 0.5 μL Cnp-E3 s primer (10 pmol/ μL).
 - (d) 1.0 μL Cnp-E as (10 pmol/ μL).
 - (e) 2 μL dNTP mix (2 mM).
 - (f) 2 μL 10 $\times$ RedTaq buffer (Sigma).
 - (g) 1 μL Red Taq polymerase (1 U/ μL , Sigma).
 - (h) Add ddH₂O.

 - (i) 20 μL .
2. Use Thermocycling program:
 - (a) 95 °C 3 min.
 - (b) 50 °C 30 s.
 - (c) 72 °C 60 s
 - (d) 95 °C 30 s.
 - (e) Back to 2 for 35 cycles.
 - (f) 50 °C 60 s.
 - (g) 72 °C 10 min.
 - (h) Store at 4 °C.
 3. Separate PCR products on agarose gels for visualization. A 700 bp product from the wild-type allele is amplified by Cnp-E3 s (5'-GCCTTCAAACCTGTCCATCTC-3') and Cnp-E as (5'-CCCAGCCCTTTTATTACCAC-3'). The targeted Cre-allele is positively identified with primers puro3 s (5'-CATAGCCTGAAGAACGAGA-3') and Cnp-E as, yielding a 400 bp fragment.

3.3 Tamoxifen Induction Regimen

1. In a chemical fume hood: dissolve tamoxifen in corn oil at a concentration of 10 mg/mL by high speed shaking (e.g., in a tissue lyser) at 37 °C for 20 min. Tamoxifen is not soluble in water.
2. A Tamoxifen solution is sensitive to UV light and, similar to crystalline Tamoxifen powder, must be stored in the dark (foil-wrapped vial) at 4 °C.
3. Sanitize injection site with 70% ethanol.
4. Administer Tamoxifen on 2 $\times$ 5 consecutive days (with a pause of 2 days in between) with one intraperitoneal injection a day. A standard dose of 100 μL Tamoxifen solution contains 1 mg Tamoxifen per adult mouse and day, and is effective to induce Cre-mediated recombination (*see* **Notes 4–10**).

4 Notes

1. Several mouse lines have been generated that harbor floxed transgenes for experiments in optogenetics, i.e., the light-induced opening or closing of ion channels in Cre-expressing cells [99]. Cre-dependent optogenetic tool-mice will aid, e.g., in studies on activity-dependent myelination [100] when expressed in genetically defined neuronal subsets. This includes principal neurons and parvalbumin-positive interneurons, both of which can be myelinated within the cortex of mice [101].
2. Other floxed indicator strains expressing effective sensors or effectors that may be useful for research on OLC include:
 - (a) Ai95D mice, expressing a Cre-dependent calcium indicator and fast variant of GCaMP6 [102].
 - (b) R26:lacZbpA(flox)DTA and R26:eGFP(flox)DTA mice that can be used for Cre-mediated cell ablation [103, 104].
 - (c) Floxed miRAP and floxed Ribo-Tag mice, which allow cell-type-specific profiling of miRNAs and translated mRNAs, respectively [105, 106].
 - (d) PhAM^{flox} (photo-activatable mitochondria) mice expressing the photo-convertible fluorescent protein Dendra2 for labeling and analysis of mitochondria, their fusion/fission and transport [107].
 - (e) ROSA26-LSL-Cas9 mice for in vivo gene editing when combined with single guide RNAs and a source of Cre [108].
 - (f) ATP-sensor ATeam1.03^{YEMK} mice to uncover energy deficits in axons with pathological myelination [114].
3. Recombination efficiencies of different reporter genes vary when tested in combination with the same Cre driver mouse (see for example [96]). Thus, reporter gene expression can be no proof that recombination of a floxed target gene has been efficient. Recombination must be additionally confirmed by other methods, such as PCR amplification of genomic DNA, Western blotting, in situ hybridization, or immunostaining.

Tamoxifen-induced recombination of target genes

4. Tamoxifen is a human carcinogen, teratogen, and mutagen. Review and follow the material safety data sheet and wear appropriate personal protective equipment. Pregnant women should not be exposed to or handle Tamoxifen in any form.
5. Tamoxifen is excreted by mice for up to 72 h after the last injection. Considered hazardous waste, bedding has to be

changed and adequately disposed 72 h after the last tamoxifen administration.

6. Tamoxifen-treated mice should not be housed with untreated animals, because unintended recombination can occur by the contamination with traces of tamoxifen during activities such as licking, grooming, or coprophagy.
7. Tamoxifen is transmitted transplacentally and via lactation, which can lead to CreER^{T2}-mediated recombination in embryos or early postnatal pups (*see* for example [49]).
8. Tamoxifen has considerable off-target effects that may confound the phenotypical analysis of floxed CreER^{T2} mutants [109]. It has, for example, been reported to impair consolidation and retrieval of memory in mice at doses relevant for inducing Cre-mediated recombination of floxed target genes [110]. Thus, inclusion of a tamoxifen-injected control group in the behavioral analysis of mice is highly recommended (Fig. 4).
9. Tamoxifen promotes the differentiation of OPC in vitro and in demyelinated lesions in vivo [111, 112] which again demonstrates the importance of including tamoxifen-injected experimental control groups (Fig. 4).
10. Ivermectin, a drug used to treat laboratory mice against ectoparasites, such as pinworms and fur mites, can cause unintended CreER^{T2}-mediated recombination of loxP sites [113]. Here, oral ivermectin treatment of parental mice expressing CreER^{T2} under the control of an ubiquitin promoter and YFP under Rosa26 regulatory elements induced CreER^{T2}-mediated recombination at loxP sites in T-cells of the offspring. Because of the blood brain barrier, the relevance of this finding to research on oligodendroglial cell lineages is yet unclear.

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