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# A new mouse line for cell ablation by diphtheria toxin subunit A controlled by a Cre-dependent FLEx switch

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## Abstract

Recombinase responsive mouse lines expressing diphtheria toxin subunit A (DTA) are well established tools for targeted ablation of genetically defined cell populations. Here we describe a new knock-in allele at the *Gt(Rosa)26Sor* locus that retains the best features of previously described DTA alleles—including a CAG promoter, attenuated mutant DTA cDNA, and ubiquitous EGFP labeling—with the addition of a Cre-dependent FLEx switch for tight control of expression. The FLEx switch consists of two pairs of antiparallel lox sites requiring Cre-mediated recombination for inversion of the DTA to the proper orientation for transcription. We demonstrate its utility by Cre-dependent ablation of both a broad domain in the embryonic nervous system and a discrete population of cells in the fetal gonads. We conclude that this new DTA line is useful for targeted ablation of genetically-defined cell populations.

### Keywords

DTA; Rosa26; loxP; DIO; En1; Dppa3

## Introduction

Targeted ablation is a well-established method for investigating the role of individual cells and cell populations in the development and function of complex organs or whole organisms. In laboratory mice, expression of genetically encoded diphtheria toxin subunit A (DTA) permits efficient ablation of genetically defined cell lineages by inactivation of elongation factor 2 (EEF2), preventing protein synthesis and rapidly leading to cell death (Breitman *et al.*, 1987; Collier, 2001; Palmiter *et al.*, 1987). This ablation is highly specific, because mice lack a functional receptor for diphtheria toxin (Mitamura *et al.*, 1995), and DTA lacks the B subunit required for penetration of cell membranes (Collier, 2001).

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Therefore, ablation is restricted to cells that actively synthesize DTA protein, and cells that have not expressed DTA are unaffected by toxin released from adjacent apoptotic cells.

Numerous transgenic and knock-in lines have been generated to take advantage of the efficiency and specificity of ablation offered by DTA in the mouse. Of these, the most experimentally flexible are several lines in which expression of DTA is dependent on the activity of Cre recombinase (Brockschnieder *et al.*, 2004; Brockschnieder *et al.*, 2006; Ivanova *et al.*, 2005; Marques *et al.*, 2009; Voehringer *et al.*, 2008; Wu *et al.*, 2006; Yoshikawa *et al.*, 2011). These alleles can be used to ablate any cell lineage for which a Cre recombinase driver line is available, and if an inducible Cre driver is used, ablation can be temporally controlled

Given the extreme toxicity of DTA, expression of even an attenuated mutant form of the protein (Maxwell *et al.*, 1987) must be very tightly regulated. In Cre-dependent DTA alleles, this control is achieved using loxP-flanked transcriptional stop cassettes placed upstream of the DTA cDNA to terminate transcription and block expression in the absence of Cre recombination. Unfortunately, comparison of several different stop cassettes has demonstrated that they vary in their ability to tightly control marker expression (Dymecki *et al.*, 2010), and even the most efficient permits low-level “leaky” expression of fluorescent proteins under certain conditions (Plummer *et al.*, 2015). Leaky expression of DTA is more difficult to detect, but some evidence suggests that it can be a problem with significant experimental implications. For example, when expression of Cre-dependent DTA transgenes was driven by the highly active CAG promoter (Niwa *et al.*, 1991), very few founder animals survived (Matsumura *et al.*, 2004; Sato and Tanigawa, 2005). The established transgenic lines exhibited unexpectedly low levels of expression (Sato and Tanigawa, 2005) or incomplete ablation of Cre-expressing cells (Matsumura *et al.*, 2004), consistent with selection against those founders exhibiting the expected robust CAG promoter activity and survival of mice carrying the transgene in a genomic context that suppresses the promoter. These results suggest that low-level leak through the stop cassette is lethal when combined with a highly active promoter. In a moderately expressed DTA allele, leak through a stop cassette could cause sporadic loss of cells at any developmental time point and in any cell-type. Such sporadic ablation would be very difficult to detect in the absence of a gross phenotype, but the loss of relatively few cells could complicate interpretation of more subtle phenotypes.

An alternative to a transcriptional stop cassette is the Cre-dependent flip-excision (FLEx) switch (Schnutgen *et al.*, 2003) which uses two pairs of antiparallel lox sites to permanently invert a DNA segment. Initial characterization *in vitro* and in cell culture demonstrated that recombination of a FLEx switch is highly efficient, and subsequent testing in mice showed it to be functionally indistinguishable from simple recombination of a single pair of loxP sites (Schnutgen *et al.*, 2003). Although originally designed to reveal Cre recombination by turning on expression of a reporter gene while a second is simultaneously turned off, FLEx switches (also called DIO, Double-floxed Inverted Open reading frame) are now widely used in viral constructs for tight control of gene expression (Atasoy *et al.*, 2008; Sohal *et al.*, 2009). The FLEx switch offers a similar advantage in a transgenic DTA construct, because

the DTA cDNA can be placed in an antisense orientation relative to the promoter, providing tighter control of DTA expression relative to stop cassettes in the absence of Cre activity.

Here, we describe a new Cre-responsive DTA allele that is the first to utilize a FLEx switch to minimize the possibility of off-target ablation. In addition, this new allele combines features that are found individually in the most frequently utilized of the previously published DTA alleles. As in the *Gt(ROSA)26Sor*$^{tm1(DTA)Mrc}$ allele (Wu *et al.*, 2006), we use the attenuated Tox176 mutant cDNA (Maxwell *et al.*, 1987) to further reduce the impact of off-target expression. An enhanced green fluorescent protein (EGFP) cDNA expressed in the absence of Cre recombination enables rapid visual genotyping and confirmation that the allele is expressed in the target cell population, similar to the *Gt(ROSA)26Sor*$^{tm1(DTA)Jpmb}$ allele (Ivanova *et al.*, 2005). To confirm that our new allele permits efficient ablation of targeted cell populations, we have tested it using two different Cre lines: An *En1*$^{Cre}$ knock in allele (Kimmel *et al.*, 2000) expressed in the embryonic midbrain and rostral hindbrain (Li *et al.*, 2002), and a tamoxifeninducible *Dppa3-cre/Esr1** transgene that is expressed in germ cells (Hirota *et al.*, 2011). These results demonstrate that this new DTA allele is useful for ablation of genetically defined cell populations within and outside the nervous system.

## Results and Discussion

To ensure that the new Cre-responsive DTA allele, *Gt(ROSA)26Sor*$^{tm2.1(CAG-EGFP,-DTA*G128D)Pjen}$ (hereafter designated *RC::L-DTA*; **R**osa-**C**AG-**l**ox-**D**TA), is expressed in the widest range of cells types throughout development, we targeted the *Gt(ROSA)26Sor* locus (Friedrich and Soriano, 1991) and used a synthetic CAG promoter (Niwa *et al.*, 1991) to drive expression. Following the CAG promoter is a FLEx switch with EGFP between the first two lox sites and DTA between the second and third, a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (Zufferey *et al.*, 1999), and a bovine growth hormone poly(A) cassette (Fig. 1a). The Cre-responsive FLEx switch (Schnutgen *et al.*, 2003) controls DTA expression. In the absence of Cre activity, EGFP is expressed and the DTA cDNA is in an antisense orientation relative to the CAG and Gt(ROSA)26Sor promoters, greatly reducing the likelihood of off-target expression. *RC::L-DTA* homozygotes are viable and born at Mendelian ratios (13/53 offspring of heterozygote intercrosses). Male homozygotes appear to be infertile. The cause of this infertility is unknown, but females are fully fertile and neither males nor females exhibit any other abnormal phenotypes. These results confirm tight control of DTA expression in the presence of the highly active CAG promoter. We observed bright, ubiquitous EGFP fluorescence in heterozygous embryos (Fig. 1b), demonstrating that the allele is broadly expressed and will be useful for ablating cells of any type for which a Cre driver line is available.

To confirm that Cre recombination of *RC::L-DTA* mediates a switch from EGFP to DTA expression (Fig. 2a) and to test its ability to ablate cells within a relatively broad expression domain in the central nervous system, we crossed *RC::L-DTA* heterozygotes with *En1*$^{Cre}$ heterozygous mice (Kimmel *et al.*, 2000). In *En1*$^{Cre}$ mice, Cre activity is observed starting at ~E8 and by E9.5 is detected in virtually all cells of the midbrain and anterior hindbrain (Li *et al.*, 2002). In *En1*$^{cre}$; *RC::L-DTA* double heterozygotes, ablation of this *En1*-expressing domain was obvious at E9.5 (Fig. 2b). At E10.5, development of forebrain structures such as

the telencephalic vesicles appeared to be stunted relative to wild-type controls, perhaps due to loss of signaling from neurons in the midbrain and hindbrain (Fig. 2b). To examine the efficiency of ablation, we performed RNA in situ hybridization using a probe against *En1*. At E9.5, we detected En1 transcripts in the midbrain/hindbrain of control embryos but not *$En1^{Cre}$; RC:L-DTA* double heterozygotes (Fig. 3, top), suggesting complete ablation of the *En1* domain. Further analysis using a probe against Cre confirmed complete loss of Cre-expressing cells in the midbrain/hindbrain (Fig. 3, bottom). Thus, *RC::L-DTA* can ablate a broad domain of cells approximately one day after initiation of Cre expression. This result is in line with the time course of ablation observed for other Cre-responsive DTA alleles (Ivanova *et al.*, 2005; Wu *et al.*, 2006).

As a further test of *RC::L-DTA*, we examined its ability to ablate a small, unipotent cell population using the *Dppa3-cre/Esr1** transgene (Hirota *et al.*, 2011) to target embryonic germ cells. A tamoxifen-inducible transgene was required due to early, ubiquitous expression of *Dppa3* (PGC7) in the pre-implantation mouse embryo (Sato *et al.*, 2002). After dosing pregnant dams with tamoxifen (5 mg/kg i.p. daily) between E10.5 and E12.5, we observed an approximate 70-90% reduction in the number of TRA98-positive germ cells in the testes and ovaries of *Dppa3-cre/Esr1*; RC::L-DTA* double heterozygous embryos at E18.5, relative to littermate controls (Fig. 4). The recombination rate is consistent with previously published results using this Cre transgene (Hirota *et al.*, 2011)

Taken together, these results demonstrate that this new allele represents an improvement over existing Cre-responsive DTA alleles. *RC::L-DTA* retains the best features of previously published alleles—including CAG promoter, EGFP, and attenuated DTA—with the addition of a FLEx switch for tight control of DTA expression. Our successful production of DTA mice with a CAG promoter, along with WPRE sequence which may further enhance DTA expression, inserted into a locus known to be permissive of robust CAG activity is strong evidence that the FLEx switch efficiently suppresses DTA expression in the absence of Cre. We conclude that *RC::L-DTA* will be valuable for ablation of genetically defined cell populations and will make it available to other researchers through the Jackson Laboratory mouse repository (Stock# 026944).

## Methods

### Generation of *RC::L-DTA* mice

A targeting vector for homologous recombination in embryonic stem cells was generated as previously described (Sciolino *et al.*, 2016). This targeting vector contains 5′ and 3′ homology to the *Gt(ROSA)26Sor* locus, CAG promoter (Niwa *et al.*, 1991), rox-flanked His3-SV40 transcriptional stop cassette (Sauer, 1993), FRT-flanked His3-SV40 cassette, modified FLEx switch (Schnutgen *et al.*, 2003) containing an EGFP cDNA, woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (Zufferey *et al.*, 1999), bovine growth hormone poly(A) cassette, and attB/attP-flanked Neomycin resistance cassette. A cDNA encoding the Tox176 (G128D) mutant DTA (Maxwell *et al.*, 1987) was cloned into the center of the FLEx switch, in antisense orientation relative to the CAG promoter and EGFP cDNA to produce the final targeting vector (Fig. 5).

Linearized vector was electroporated into G4 embryonic stem cells (B6129 F1 genetic background), and homologous recombinants were identified by long range PCR and Southern blotting. A recombinant clone was transiently transfected with pPGFKPhiC31obpa (Raymond and Soriano, 2007) to remove the attB/attP-flanked Neo cassette before cells were injected into B6(Cg)-*Tyr*$^{c-2J}$/J blastocysts to produce chimeric mice. This new DTA allele was designed to be responsive to Dre (rox), Flp (FRT), and Cre (lox) recombinases, allowing intersectional genetic control of DTA expression similar to a previously described hM3Dq allele (Sciolino et al., 2016). However, when these mice were crossed to a ubiquitous Cre driver, placing the DTA in sense orientation relative to the CAG promoter, we observed no live-born Cre+; DTA+ double heterozygotes (0/21 offspring of heterozygote intercross, p=0.0104 $\chi^2$). Furthermore, when using tissue-specific Dre, Flp, and Cre drivers for intersectional crosses, we observed phenotypes consistent with sporadic DTA expression in cells that had expressed Cre but not Dre or Flp (data not shown). These results are consistent with leaky expression of DTA through the transcriptional stop cassettes. Therefore, we decided to remove the two stop cassettes, generating a new Cre-responsive allele in which DTA expression is controlled solely by the FLEx switch. The original chimeras were bred with B6;129-*Tg(CAG-dre)1Afst* mice (Anastassiadis *et al.*, 2009) and B6.Cg-*Tg(ACTFlpe)9205Dym*/J mice (Rodriguez *et al.*, 2000) to excise the rox- and FRT-flanked transcriptional stop cassettes and generate the *RC::L-DTA* line. This new strain was maintained by backcrossing to C57BL/6J mice and intercrossed to confirm that homozygotes are viable. *RC::L-DTA* mice will be available to the research community through the Jackson Laboratory (Stock# 026944).

## Experimental crosses and tamoxifen treatment

All mouse experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory animals of the National Institutes of Health. The protocols were approved by the Animal Care and Use Committee (ACUC) of the National Institute of Environmental Health Sciences. Mice were maintained on a 12-hour light/dark cycle at 72±2 °F and given water and food *ad libitum*. All mice used in experiments were bred in-house at NIEHS.

*RC::L-DTA* mice were intercrossed with *En1*$^{tm2(cre)Wrst}$ (Kimmel *et al.*, 2000) and *Tg(Dppa3-cre/Esr1*)9Sait* (Hirota *et al.*, 2011) mice. For timed matings and embryo collection, noon of the day on which plugs were observed was taken to be E0.5, and pregnant dams were euthanized by CO2 inhalation before embryo dissection. Pregnant dams from the cross with *Tg(Dppa3-cre/Esr1*)9Sait* were treated with tamoxifen (Sigma-Aldrich) diluted in corn oil (Sigma-Aldrich) injected intraperitoneally (i.p.) at 5 mg/kg once daily at E10.5. E11.5, and E12.5. Tamoxifen treated embryos at E18.5 were euthanized by decapitation after removal from the uterus.

## In Situ Hybridization

Mouse embryos were fixed overnight at 4 °C in 4% paraformaldehyde (PFA) diluted in 0.01M phosphate buffered saline (PBS). After fixation, embryos were equilibrated in 10%, 20%, and finally 30% sucrose diluted in PBS. The cryoprotected embryos were embedded in Tissue Freezing Medium (General Data Company) and sectioned on a Leica CM305 S

cryostat (Leica Biosystems, Buffalo Grove, IL). 14-μm thick sections were mounted on Superfrost Plus microscope slides (Thermo Scientific, Waltham, MA), and stored at −80 °C after air drying. RNA *in situ* hybridization was performed using RNAscope technology (Wang *et al.*, 2012) according to manufacturer's instructions for the RNAscope 2.5 HD Reagent Kit-Red (Advanced Cell Diagnostics, Hayward, CA). Sections were hybridized with probes against En1 (Cat# 442651, Advanced Cell Diagnostics) and Cre (Cat# 312281, Advanced Cell Diagnostics). Hybridized RNA was detected with Fast Red dye, and the tissue was counterstained with hematoxylin.

### Immunohistochemistry

For immunohistochemistry on frozen sections, gonads were fixed in 4% PFA in PBS overnight at 4 °C, dehydrated through a sucrose gradient, embedded in O.C.T. Compound (Sakura Finetek, Torrance, CA) and cryosectioned at 10 μm increments. Tissue sections were preincubated with 5% normal donkey serum in PBS for 1 hour, then incubated with anti-TRA98 (1:1000, MBL International, Woburn, MA) in PBS-Triton X-100 (0.1%) solution with 5% normal donkey serum at 4 °C overnight. The antibody-labeled tissue sections were washed three times in PBS and incubated in Alexa 594 goat anti-rat secondary antibody (1:500; Invitrogen, Carlsbad, CA) for one hour at room temperature, before a final three washes in PBS and mounting in Vector Mount with DAPI (Vector Laboratories, Burlingame, CA).

### Imaging

Fluorescent and bright-field images of PFA-fixed whole-mount embryos were collected on a SteREO Lumar.V12 stereomicroscope (Carl Zeiss Microscopy, Thornwood, NY). Bright-field images of embryo sections labeled by in situ hybridization were collected on an Olympus IX70 inverted microscope (Olympus Corporation, Center Valley, PA). Immunolabeled slides of embryonic gonads were imaged on a Leica DMI4000 confocal microscope. Images were modified only by using Photoshop software (Adobe Systems, San Jose, CA) to adjust brightness and contrast across the entire image.

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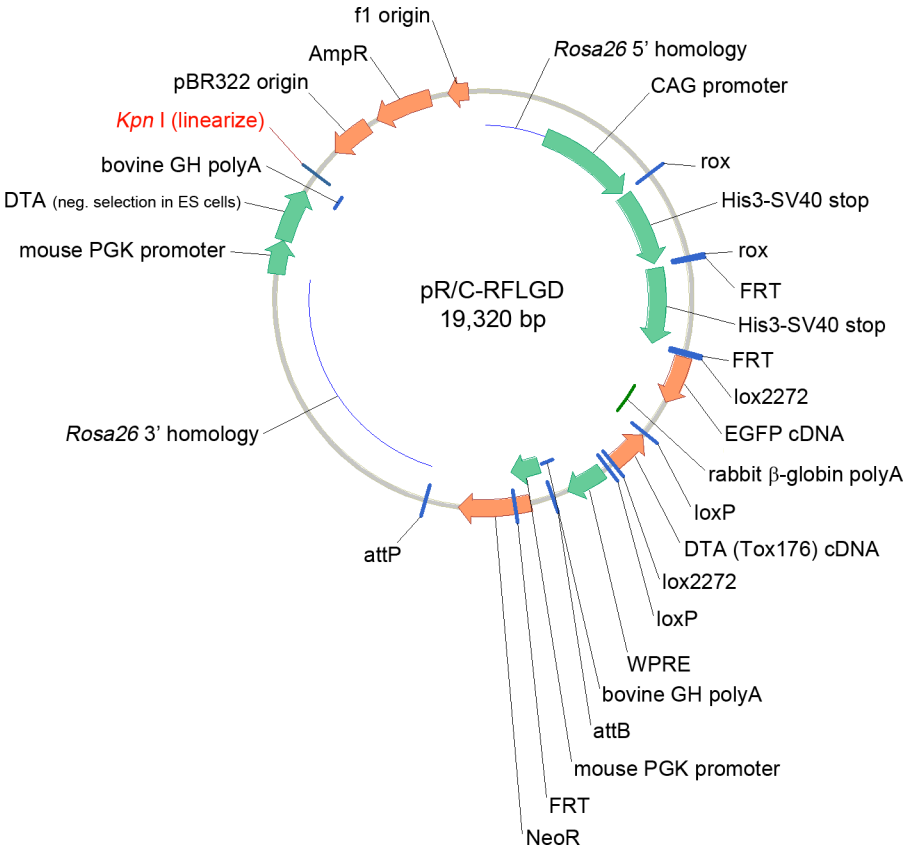


**Figure 1. *RC::L-DTA* allele**

(a) Schematic diagram of the *Gt(ROSA)26Sor* locus showing insertion of the CAG promoter, EGFP, and DTA cDNA. Open rectangles represent noncoding exons of *Gt(ROSA)26Sor*, and solid lines represent introns. DTA is oriented antisense to the CAG promoter and is located between two pairs of antiparallel lox sites (wildtype loxP and variant lox2272) which form the Cre-dependent FLEx Switch. WPRE, woodchuck hepatitis virus post-transcriptional regulatory element (Zufferey *et al.*, 1999); poly(A), rabbit β-globin polyadenylation cassette (following EGFP) or bovine growth hormone polyadenylation cassette (following WPRE). (b) In the absence of Cre recombinase, ubiquitous EGFP expression is observed in *RC::L-DTA* heterozygous embryos at E11.5. Image shows native fluorescence in a fixed embryo. Scale bar, 2 mm.

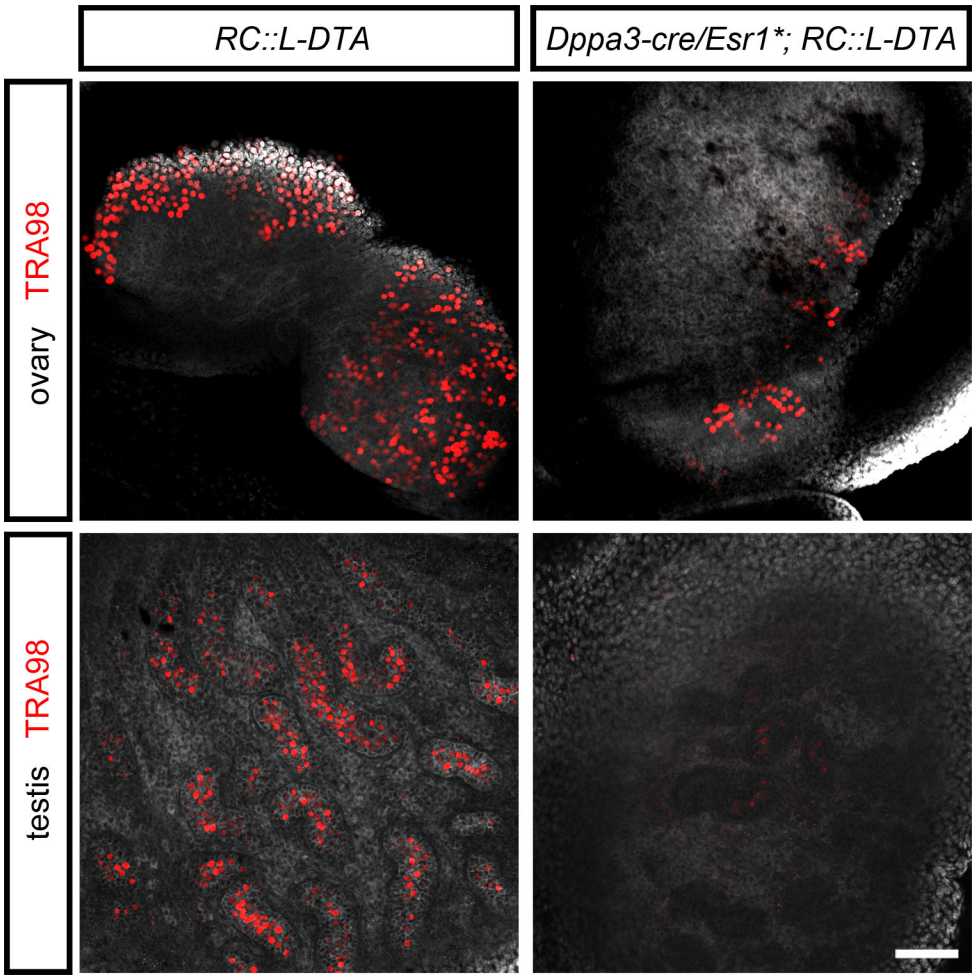


**Figure 2. Ablation of embryonic midbrain and hindbrain tissue following Cre-mediated recombination of *RC::L-DTA* in the *En1* expression domain**

(a) Cre-mediated recombination of *RC:L-DTA* results in sequential inversion and deletion events which delete EGFP and invert DTA to the correct orientation for transcription. (b) Ablation of cells in mesencephalon (Me) and anterior rhombencephalon (Rh), including the embryonic cerebellum (Ce), is observed in whole-mount $En1^{Cre}$; *RC::L-DTA* double heterozygous embryos at E9.5 and E10.5. Cells outside the midbrain/hindbrain $En1^{Cre}$ expression domain are not ablated, but expansion of the telencephalic vesicles (Te) appears to be delayed at E10.5. Di, diencephalon; OV, otic vesicle; 4V, fourth ventricle. Scale bar, 500 μm (E9.5) or 961 μm (E10.5).

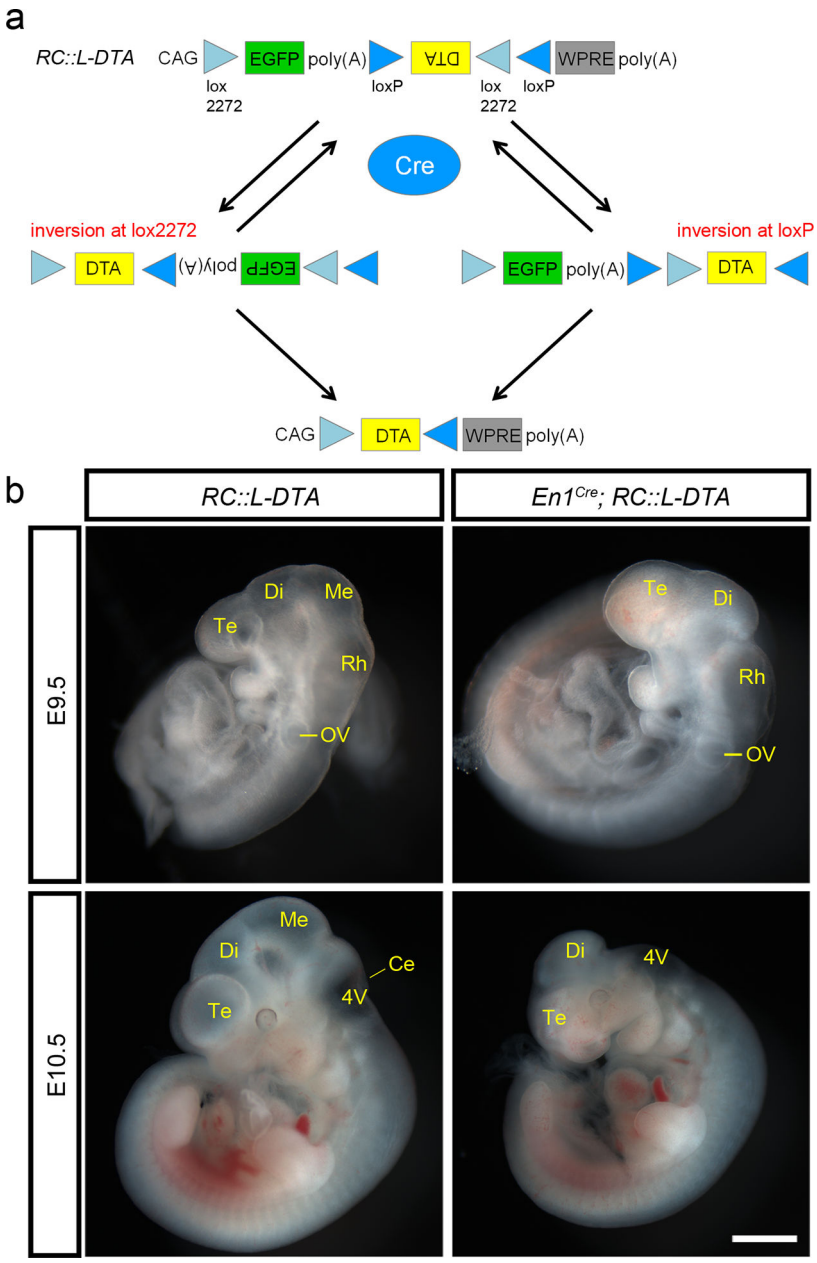


**Figure 3. Loss of Cre expressing cells in midbrain and hindbrain following *En1*$^{Cre}$-mediated expression of DTA**

*In situ* hybridization demonstrates overlapping expression of *En1* (top) and *Cre* (bottom) transcripts in heterozygous *En1*$^{Cre}$ embryos at E9.5 (left), but expression is absent in *En1*$^{Cre}$; *RC::L-DTA* double heterozygotes (right). Scale bar, 500 μm

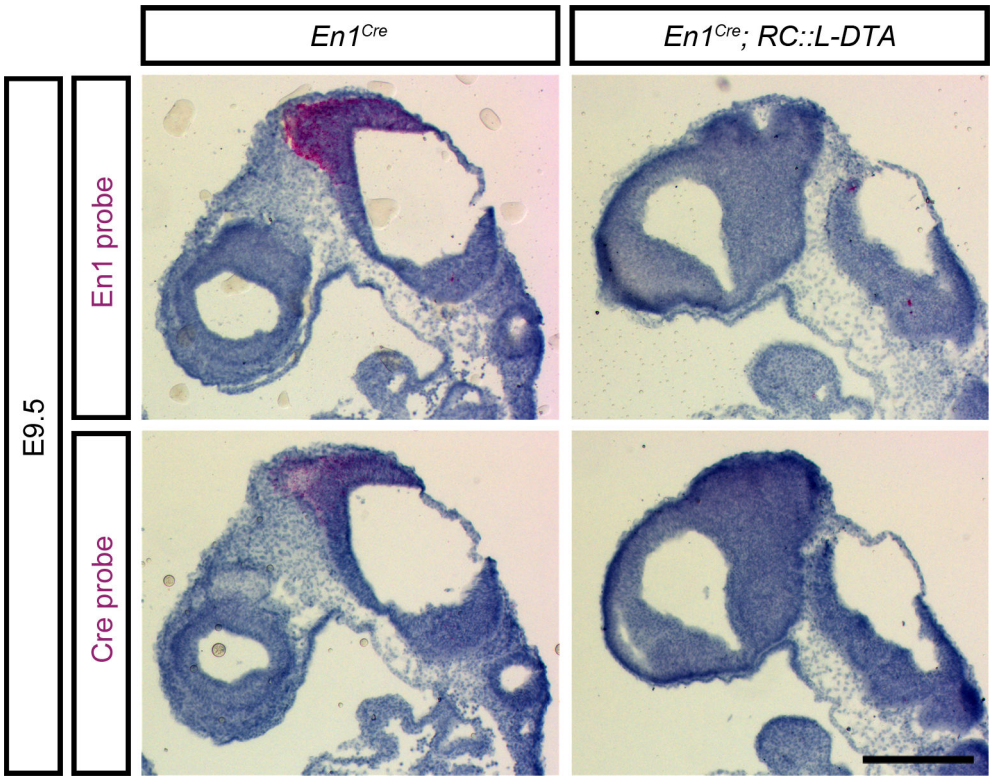


**Figure 4. Ablation of germ cells in *Dppa3-cre/Esr1*; RC::L-DTA* mice after prenatal treatment with tamoxifen**

In embryonic ovary (top left) and testis (bottom left) of tamoxifen-treated *RC::L-DTA* mice at E18.5, nuclei of germ cells (red) are labeled by an antibody against TRA98 (*Gcna*) (Carmell *et al.*, 2016). The number of surviving germ cells is greatly reduced in ovary and testis tamoxifen-treated *Dppa3-cre/Esr1*; RC::L-DTA* double heterozygotes (right, top and bottom). DAPI staining (gray) shows tissue not stained by the TRA98 antibody. Scale bar, 100 μm.

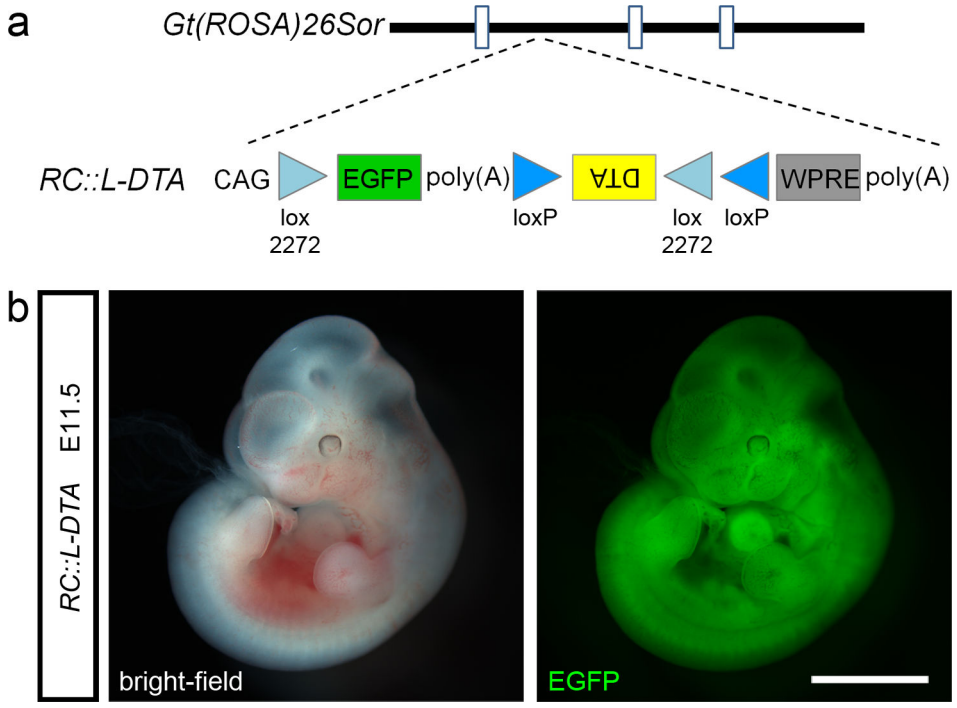


**Figure 5. Targeting vector for generation of recombinase-responsive DTA allele at the *Gt(ROSA)26Sor* locus**

Prior to electroporation of embryonic stem cells, the vector was linearized with *Kpn* I restriction enzyme. Two segments of genomic DNA (*Rosa26* 5′ homology and *Rosa26* 3′ homology) target the intervening expression construct to intron 1 of *Gt(ROSA)26Sor*. The vector backbone, including a PGK-DTA-bGHpolyA cassette for negative selection, was eliminated during homologous recombination.