Inducible Genome Editing with Conditional CRISPR/Cas9 Mice

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ABSTRACT Genetically engineered mouse models (GEMMs) are powerful tools by which to probe gene function in vivo, obtain insight into disease etiology, and identify modifiers of drug response. Increased sophistication of GEMMs has led to the design of tissue-specific and inducible models in which genes of interest are expressed or ablated in defined tissues or cellular subtypes. Here we describe the generation of a transgenic mouse harboring a doxycycline-regulated Cas9 allele for inducible genome engineering. This model provides a flexible platform for genome engineering since editing is achieved by exogenous delivery of sgRNAs and should allow for the modeling of a range of biological and pathological processes.

Rapid and facile genome editing has been enabled through the use of type II bacterial CRISPR (clustered, regularly interspaced, short palindromic repeats)/Cas9 (CRISPR-associated protein) systems. By taking advantage of RNA-directed targeting, the Cas9 endonuclease is used to induce DNA breaks at a given locus. These are subsequently repaired by either the mutagenic NHEJ (non-homologous end joining) pathway, by HDR (homology-directed recombination) if a repair template complementary to the mutagenic repair, while generating KrasG12D alleles by HDR (Platt et al. 2014). Another Cas9 transgenic mouse strain has been built in which a Cre/loxP-dependent conditional Cas9 allele was engineered into the Rosa26 locus (Gui et al. 2016). Using a different strategy, Dow et al. (Dow et al. 2015) produced GEMMs (genetically engineered mouse models) co-expressing DOX (doxycycline)-inducible Cas9 and a single guide (sg) RNAs in their germline. This latter platform illustrated the feasibility of inducible in vivo genome editing at multiple loci (p53 and Apc) to model cancer progression (Dow et al. 2015). These powerful models are enabling the application of Cas9 editing technology to a number of tissue- and embryo-based settings.

Here, we report on the generation of a DOX-inducible Cas9 mouse in which we placed a TRE (tetracycline responsive element)-inducible Cas9 into the Rosa26 locus. This mouse overcomes the challenges of Cas9, avoids potential genotoxicity associated with Cre recombinase (Leonistra et al. 2001), and maintains flexibility with respect to choice of sgRNA delivery.

MATERIALS AND METHODS

Generating Col1A1 Knock-in Cas9 Mice

A pUC57 derivative (pUC57a) with appropriate linker sequences tailored for multi-component assembly of the donor template was purchased from GenScript and contained the following adaptor sequence: 5′ CCATGTTGATCTATGGCCGTGAAAGAAGCCCCGGGCCACCAGAAGGCGCGCCCTTAATTAAACGGGTGGAGAATCTGCGGTGAGTGTTGTTGGGGGACCCCTTCTTGTTCTCTTTTTCGCTATTGAAATTCACTATTTATGAGGGGCAAAGTTTCAGGGTGTTGTTTAGAATGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGATGTCCCTTGTATCACCAACTGGGAAGAT
restriction sites, the Flag-Cas9-IREs-GFP fragment from pQCG2 (Malina et al. 2014) was cloned into pUC57a. The GFP ORF was transferred from pUC57a-Cas9-IREs-GFP into pCol-TGM-p53.1224 (Premririt et al. 2011) using NcoI (NEB). The resulting plasmid was partially cleaved with NcoI and the Cas9-IREs fragment from the parental pUC57a-Cas9-IREs-GFP vector transferred to generate pCol-Tre-Cas9-iG. Unique Ascl/XmnI restriction sites were then used to transfer the CAGs-rtTA3-SAdpA cassette (Dow et al. 2014) into pCol-Tre-Cas9-iG, downstream of GFP to generate the knock-in donor template, pCol-TGIG-rtTA3 (Figure S1). Plasmids are available from the authors upon request.

C10 ES cells were cultured in complete knock-out DMEM (15% ESC Qualified Serum, 1% Penicillin-Streptomycin, 1% Non-Essential Amino Acids, 1% L-Glutamine, 0.1% BME, 0.01% LIF) on gelatinized plates with PMEF-N Feeders (Millipore). Fifty micrograms of pCol-TGIG-rtTA3 was electroporated with 25 μg of Flpe recombinase expression plasmid (pCACs-Flpe) as previously described (Lin et al. 2012). After 2 days of recovery, recombinant clones were selected using 140 μg/mL hygromycin, assessed for DOX inducibility, and used to generate pCol-Tre-Cas9-iG. Unique AscI/XmnI restriction sites from the parental pUC57a-Cas9-IRES-GFP vector transferred to generate pQCi, which had been linearized with polymerase. This amplicon was then digested with AscI and cloned into the gutted pQCiG2 vector to produce pQCiG. Unique AscI/XmnI restriction sites were then used to transfer the CAGs-rtTA3-SAdpA cassette (Dow et al. 2014) into pCol-Tre-Cas9-iG, downstream of GFP to generate the knock-in donor template, pCol-TGIG-rtTA3 (Figure S1). Plasmids are available from the authors upon request.

**PCR and Genotyping**

Cas9 allele status was assessed with primers Col1A1-F: 5′AATCA TCCAGGTTGGACAGC3′, SAdpA-R (from Mirimus, NY): 5′CGTT AGGCGGTCTGATGACCCAGG3′, and Col1A1-R: 5′ACC GGAAAGATTGTTGTCTTCACA3′. Primers Col1A1-F and Col1A1-R provided a characteristic 379 bp band indicative of the presence of Cas9, whereas Col1A1-F and SAdpA-R generated a 239 bp band indicative of a wt Col1A1 locus. The Rosa26 locus status was assessed using the following primers: Rosa-A: 5′AAATGCGGCTCTGAG TTGTATA3′, Rosa-B: 5′GGGAAGAAGTTGGTTCTTCACA3′, Rosa-C: 5′GGAGGCGGGAGAAATGGATAG3′. Rosa-A and Rosa-B produce a ~500 bp band, indicative of a wild-type Rosa26 allele, while Rosa-A and Rosa-C produce a ~300 bp band, indicating the presence of the rtTA allele. Eμ-Myc allele status was assessed using primers 5′Eμ-Myc: 5′GGAGATTCGCTAGATCCAAGTGTA3′, and 3′Eμ-Myc: 5′GCT CTGTTCTCTCGCTTTGAAATT3′ which produces a 600 bp band when the Eμ-Myc allele is present. Recovery of the sgp53-3 sequences from tumor samples was achieved by PCR amplification using primers 5′GAAGATCTTTCTAGATCCG3′ and 3′AAAAAGCAG CGACTCGGGTG3′ (Cencic et al. 2014).

**Construction of the pUSPPC sgRNA-expression Vector**

To generate pUSPPC, we first replaced GFP in pQCG2 with mCherry by digesting with EcoRV/ClaI to remove the IRES-GFP sequence. This was replaced with the EMCV IRES which was PCR amplified from pQCG2 using primers “IRES-F” 5′ AGTAGCTGATATCCTCCAATTATCG ATTTGAATTCG3′ and “IRES-R” 5′ AGTAGCTAATGCATTAC ATGGGCGCATATTACG3′ and digested with ClaI/EcoRV and ligated into the gutted pQCG2 vector to produce ‘pQGC’. The mCherry coding region sequence was amplified using PCR with primers “mCherry-F” 5′ ATATCGCCCTAGGGTTTCTGAAAAAGC3′ and “mCherry-R” 5′ ATATCGCCCTAGGGTTTTCTTACACCTGCATCG3′ with Vent polymerase. This amplicon was then digested with AvrII and cloned into pQGC, which had been linearized with SpeI, to form ‘pQGC’. The Cas9 expression cassette was then removed from this vector using unique XhoI/EcoRV sites. The PGK-Puromycin cassette from pPrime-shRNA (Robert et al. 2014) was excised by first digesting with Pael, repairing with T4 DNA polymerase, and cleaving with XhoI. The resulting product was ligated into pQGC vector to generate pUSPPC (Figure S2).

**T7 Endonuclease I (T7EI) Cleavage Assay**

Genomic DNA from pUSPPC-transduced HSPCs was prepared using a Zymo Research Quick-gDNA MiniPrep kit (D3006). PCR amplification of the sgp53-3 targeted region of Trp53 was performed using Primer p53-3R, 5′ CCTGATCGTTACCTCGGCTTG3′ and Primer p53-3R: 5′ CAAGAAATAGTCAAGACCCGG3′ using Phusion High-Fidelity polymerase. The T7EI assay was then performed as previously described (Malina et al. 2013) and the entire reaction resolved on a 15% TBE polyacrylamide gel (29:1 acrylamide: bisacrylamide) before staining with SybrGold (ThermoFisher).

**HSPC Adoptive Transfers**

Low passage Phoenix-Eco packaging cells were cultured in complete DMEM (10% FBS, 1% Penicillin-Streptomycin, 1% L-Glutamine) and grown at 37°/5% CO2. Twenty four hours prior to transfection, 3.5 × 106 Phoenix-Eco cells were seeded in 10 cm plates in culture. Plasmids (10 μg) were co-transfected with 1 μg pCL-Eco replication-incompetent helper vector using calcium phosphate (Nahvi et al. 1996). Twenty-four hours after transfection and 12h before the first virus harvest, plates were washed with PBS and refreshed with 5 mL complete BCM (45% DMEM, 45% IMDM 10% FBS, 1% Penicillin-Streptomycin, 1% L-Glutamine). Twelve hours after refreshing media, virus was collected every 12h for a period of 48h.

R26-rtTA;TRE-CiG/rtTA;Eμ-Myc HSPCs were isolated from fetal livers at E13.5 days as previously described (McCubragh and Lowe 2001). Cells were placed in culture 12 hr before infection in BCM supplemented with 1 ng/mL IL-3, 10 ng/mL IL-6, 100 ng/mL SCF and incubated at 37°/5% CO2. Cultured HSPCs were infected four times at 12h intervals with viral supernatant from transduced Phoenix-Eco cells, supplemented with 1 ng/mL IL-3, 10 ng/mL IL-6, 100 ng/mL SCF and 4 μg/mL polybrene, and spinoculated at 950 xg for 1 hr at 32°. To induce Cas9 expression, cells were treated ex vivo with 1μg/mL doxycycline.

Transduction and GFP-induction efficiency was assessed by flow-cytometry prior to transplantation (Guaa EasyCye 8HT, Millipore).

For transplantations, 6-8 week old female C57BL/6 mice were placed on 0.125 mg/mL ciprofloxacin/2% sucrose two days before transplantation. Four hours before transplantation, mice were irradiated with 4 Gy γ radiation. Approximately 6 × 106 HSPCs were transplanted into irradiated mice by intravenous tail-vein injection 24 hr after the last transplantation. Mice were palpated bi-weekly to assess tumor status until the experimental end point at day 120.

**Western Blots**

Extracts were prepared from frozen cell pellets. Pellets were resuspended in RIPA buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 1 mM β-glycerophosphate, 1 mM PMSF, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 2.5 μM pepstatin) and incubated on ice for 10 min. Lysates were denatured in Laemmli bisacrylamide before staining with SybrGold (ThermoFisher).

**Southern Blot Analysis of the Col1A1 Locus**

Genomic DNA was isolated from ES cell clones, digested with EcoRI, and fractionated on a 0.8% TBE agarose gel (Sambrook and
After transfer to Hybond N+ membranes, the DNA was interrogated using a probe targeting the hygromycin gene outside of the region targeted by the donor template. The probe was generated by PCR amplification using primers A (ATGAAAAAGCCTGAACTCACCG) and B (GGAATTCGAGCTGACGCG) and labeled using ThermoFisher DecaLabel DNA Labeling Kit (K0662) with α-32P-dCTP (New England Nuclear, MA). Following hybridization, membranes were washed in 2x SSC/0.1% SDS once at 25°C, twice at 55°C, and then once with 1x SSC/0.1% SDS at 55°C.
Immunophenotyping and Immunohistochemical Analysis

Spleens were harvested from mice, macerated, and passed through a 40 μm cell strainer to create single-cell suspensions. Red blood cells were eliminated by lysis in ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) [pH 7.2] for 5 min on ice, before neutralizing with PBS. 300,000 cells were stained either with 0.06 μg PE-conjugated α-CD4 (BD Pharmigen 553652) or α-B220 (BD Pharmigen 553090) for 30 min before washing with PBS. PE+ and GFP+ populations were analyzed by flow cytometry (Guava EasyCyte 8HT, Millipore).

Tissues were harvested from mice and fixed in 10% buffered formalin for 48 h before embedding in paraffin. Sections (4 μm) were de-paraffinized using xylene and rehydrated through a series of decreasing concentration of ethanol washes, followed by a final water wash. Antigen retrieval was performed in a pressure cooker for 15 min in 10 mM sodium citrate [pH 6.0]/0.05% Tween-20. After washing, samples were blocked using TBS [pH 7.5]/10% FBS/1% BSA for 2h at RT and incubated with α-GFP antibodies (1:500, Cell Signaling 2555) overnight at 4°C. Slides were blocked with H₂O₂ for 10 min, incubated for 30 min with biotinylated goat anti-rabbit IgG and then streptavidin peroxidase (Anti-rabbit HRP/DAB detection kit, Abcam). Staining was performed using DAB chromogen and substrate from Abcam and counterstained using IHC-optimized hematoxylin (Vector Labs). Sections were dehydrated, mounted using permount, and slides scanned using an Aperio XT slide scanner with the resulting images analyzed using Aperio ScanScope.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS

Generation of a Doxycycline (DOX)-Inducible Cas9 Mouse

Recombinase-mediated cassette exchange (RMCE) is a rapid method by which to generate transgenic mice with DOX-inducible cDNAs or shRNAs. This approach is facilitated by the existence of pre-engineered

Figure 2 Assessing induction of Cas9 expression in vivo. A, Western blot of Cas9 from the indicated tissues harvested from TRE-CiG/rtTA or R26-rtTA;TRE-CiG/rtTA mice treated with vehicle (-) or DOX (+) for 1 week. S.I., small intestine; L.I., large intestine; s.e., short exposure; l.e., long exposure. B, Immunohistochemical staining for GFP expression in the small intestine, kidney, and liver from the indicated mice (+/− DOX). Magnification bars denote 50 μm. C. Quantitation of B220+ (pan B cell marker) and CD4+ (T) cells isolated from the spleen and thymus, respectively, of R26-rtTA;TRE-CiG/rtTA mice that had been treated with DOX for 1 week.
C10 ES cells containing a FRT-hygro-pA “homing” cassette downstream of the Col1A1 locus (Beard et al. 2006; Prembirit et al. 2011) (Figure 1A, top panel). We took advantage of the ease of manipulation of these cells and used FLPe recombinase to mediate recombination between the FRT sites at the Col1A1 locus and a site present in our pCol-TCiG-rtTA targeting vector (Beard et al. 2006; Prembirit et al. 2011). In this vector, we placed Cas9 and GFP under regulation of the tetracycline response elements (TRE) and positioned a second transcriptional unit downstream with the CAGs promoter (Okabe et al. 1997) driving reverse tet-transactivator (rtTA3) expression (Figure 1A, bottom panel). Following ES cell electroporation and RMCE, two hygromycin resistant cells, AK1 and AK2, were clonally expanded and characterized. We first ensured correct integration at the Col1A1 locus by Southern blotting, which revealed a diagnostic 8 kbp EcoRI fragment (Figures 1A, B). Both AK1 and AK2 ES cells showed DOX-dependent induction of Cas9 and GFP expression (Figures 1C, D). Expression of both Cas9 and GFP was reversible in AK1, with little Cas9 left 3 days after removal of DOX from the culture media (Figure 1E).

Characterization of Inducible Cas9 Expression in Mice

Transgenic mice were produced from AK1 ESCs, referred to henceforth as TRE-CiG/rtTA, and their preliminary characterization revealed weak global GFP induction in a large number of tissues following DOX treatment (data not shown). We reasoned that one possibility for this could be limiting rtTA3 activity and/or levels. Indeed, DOX induction of the TRE promoter in vivo can be restricted by limiting rtTA levels – as documented in GEMMs harboring conditional shRNAs (McJunkin et al. 2011). Specifically, McJunkin et al. (McJunkin et al. 2011) demonstrated that shRNA-mediated suppression of Replication Protein A (subunit 3) (Rpa3) in vivo is more potent when two rtTA expressing alleles are present in the germline of shRpa3-bearing mice, compared to mice expressing only one rtTA allele (McJunkin et al. 2011). To assess if rtTA was limiting in our system, we crossed Rosa26(R26)-rtTA mice to our TRE-CiG/rtTA GEMM and found that the resulting R26-rtTA; TRE-CiG/rtTA offspring displayed higher induced levels of Cas9 and GFP in a number of analyzed tissues compared to TRE-CiG/rtTA mice following DOX exposure (e.g., skin, spleen, thymus, small and...
Table 1 Blood Chemistry Analysis of R26-rtTA;TRE-CiG/rtTA Mice 6 Months Post-Induction

| Criterion                  | Units   | Normal Range       | R26-rtTA;TRE-CiG/rtTA (n = 2) |
|----------------------------|---------|--------------------|-------------------------------|
| Total Protein              | g/L     | 31-66              | 46                            |
| Albumin                    | g/L     | 25-48              | 22                            |
| Albumin/Globulin Ratio     |         |                    | 0.9                           |
| Glucose                    | mmol/L  | 5.0-10.7           | 9.9                           |
| BUN Urea                   | mmol/L  | 6.4-10.4           | 7.05                          |
| Creatinine                 | μmol/L  | 18-71              | 18                            |
| Total Bilirubin            | μmol/L  | 2-15               | 3                             |
| ALT                        | U/L     | 28-132             | 31.5                          |
| AST                        | U/L     | 59-247             | 78                            |
| Alkaline Phosphatase       | U/L     | 62-209             | 115                           |
| CK                         | U/L     | 68-1070            | 136                           |
| Cholesterol                | mmol/L  | 0.93-2.48          | 2.56                          |
| Sodium                     | mmol/L  | 124-174            | 50.5                          |
| Potassium                  | mmol/L  | 4.6-8.0            | 4.28                          |
| Chloride                   | mmol/L  | 92-120             | 115                           |
| Calcium                    | mmol/L  | 1.47-2.35          | 2.105                         |
| Phosphorus                 | mmol/L  | 1.97-3.26          | 2.205                         |
| Magnesium                  | mmol/L  | 0.33-1.60          | 1.06                          |

large intestine, liver) (Figures 2A, B). Induction of GFP was also observed in B cells isolated from spleen and thymus of R26-rtTA;TRE-CiG/rtTA mice (Figure 2C, 9%). The low level of induction in B cells may indicate that SpCas9 and GFP expression are uncoupled in this cell type.

We did not notice any evidence of toxicity associated with expression of the Cas9 transgene. Mice harboring the TRE-CiG/rtTA allele were fertile, had normal litters, and appeared morphologically normal. The TRE-CiG/rtTA allele was inherited at the expected Mendelian frequency with no significant associated sex effects in the inheritance pattern (Figure 3A). Long-term (6 months) treatment of R26-rtTA;TRE-CiG/rtTA mice with DOX did not affect weight gain (Figure 3B) nor overall general behavior. Cas9 was still expressed in tissues of mice continuously receiving DOX for 6 months and in most cases, levels appeared even higher than in tissues from R26-rtTA;TRE-CiG/rtTA mice that had been on DOX for 1 week (Figure 3C). Tissue analysis showed no discernible histological changes and we found no evidence of increased apoptosis (Figure 3D and data not shown). Additionally, blood chemistry from R26-rtTA;TRE-CiG/rtTA mice after 6 months of DOX treatment showed almost all values within normal range, with the values of blood cholesterol and potassium being the only parameters slightly outside the norm (Table 1). This is consistent with what has been reported for mice constitutively expressing Cas9 (PLATT et al. 2014) in that long-term sustained Cas9 expression is not associated with any overt detrimental phenotype or negative impact on the animal’s well being.

**Ex Vivo Genome Editing in Primary Hematopoietic Stem and Progenitor Cells (HSPCs)**

The Eµ-Myc mouse model is a robust and malleable model of non-Hodgkin’s lymphomas (HARRIS et al. 1988). Very powerful genetic screens for novel oncogenic drivers have utilized the Eµ-Myc model, capitalizing on the ability to manipulate HSPCs derived from embryos ex vivo coupled with in vivo selection. For example, the approach has identified novel oncogenic drivers by infecting Eµ-Myc HSPCs with libraries of shRNAs followed by transplantation into normal recipients and monitoring for tumor onset (SCUOPPO et al. 2012). Recently, we have taken advantage of this model and employed an in vivo CRISPR/Cas9 screen to identify and distinguish rare oncogenic modifier events from passenger or bystander mutations identified in human Burkitt’s lymphoma whole genome, exome, and transcriptome data (KATIGBAK et al. 2016).

As a prelude to future experiments in which sgRNA libraries could be used for in vivo screens in the Eµ-Myc model (WENDEL et al. 2007), we sought to assess if we could obtain Cas9-mediated editing in HSPCs that had been isolated from R26-rtTA;TRE-CiG/rtTA mice. For this, we tailored a retroviral delivery vector, pUSPPC, to constitutively express sgRNAs as well as puromycin and mCherry selectable markers (Figure 4A). Transduction of HSPCs derived from R26-rtTA;TRE-CiG/rtTA mice with pUSPPC lead to infection rates ranging from 38–48% (mCherry+ cells) (Figure 4B). Cas9 expression was induced upon exposure of HSPCs to DOX ex vivo (Figure 4C). Infection of these HSPCs with pUSPPC-sgp53-3 resulted in editing at the p53 locus, as assessed by the T7E1 cleavage assay (Figure 4D). This was not observed with HSPCs infected with pUSPPC-sgpTLR, a retrovirus expressing a neutral control sgRNA. Consistent with our finding that higher levels of Cas9 are induced in the presence of two rtTA alleles (Figure 2), higher levels of modification were observed in HSPCs derived from R26-rtTA;TRE-CiG/rtTA mice compared to HSPCs from TRE-CiG/rtTA mice (Figure 4D). These results demonstrate that Cas9 is inducible and functional in HSPCs and that conditional editing can be achieved ex vivo in HSPCs.

**Ex vivo manipulation of HSPCs and adoptive transfer experiment in the Eµ-Myc GEMM**

We have previously demonstrated that co-delivery of Cas9 with an sgRNA targeting p53 (pQCiG/sgp53-3) in Eµ-Myc HSPCs ex vivo accelerated tumor onset rates in transplanted normal, syngeneic recipients (MALINA et al. 2013). However, transduction efficiencies of HSPCs with the All-In-One pQCiG/sgp53-3 vector was low — likely a consequence of the large vector size required to deliver both Cas9 and the sgRNA, a feature known to negatively influence packaging and retroviral titers (GELINAS and TEMIN 1986). This limitation precludes using high complexity pools during genetic screens (KATIGBAK et al. 2016). In fact, in a previously reported screen involving HSPCs and pQCiG to identify oncogenic drivers in Burkitt’s lymphoma, we were restricted to using pools of only 5 sgRNAs since pools of 20 sgRNAs failed to identify p53 as an
oncogenic driver (Katigbak et al. 2016). We reasoned that the smaller size of pUSPPC would allow for higher pool complexities in such in vivo screens. Infection of R26-rtTA;TRE-CiG/rtTA;Eµ-Myc with pUSPPC driving an sgp53-3, following by stem cell transplant and monitoring for lymphomagenesis revealed that dilutions of pUSPPC/sgp53-3 up to 1:50 were still capable of yielding tumors (Figure 5A, B). Analysis of three tumors arising from pUSPPC/sgp53-3 infected R26-rtTA;TRE-CiG/rtTA HSCs revealed that we could recover the sgp53-3 sequences by PCR from the tumor sample (Figure 5C) and that mutations at the targeted locus had arisen (Figure 5D). These results demonstrate that engineered Cas9 expression in target cells of interest will facilitate in vivo genetic screens by enabling the use of higher complexity pools.

DISCUSSION
Here, we use RCME to generate a mouse model that expresses Cas9 in an inducible manner across a wide range of tissues. RCME is a powerful approach by which to generate Cas9 transgenic mice as it enables the insertion of Cas9 into a well-characterized locus, thus providing predictable and reproducible expression. The development of orthogonal Cas9 editing tools, as well as the extension of RNA-guided DNA targeting of catalytically inactive Cas9-fusions to study transcriptional activation, transcriptional repression, epigenetic modification, base editing, and locus imaging have greatly increased the range of applications of the CRISPR-Cas9 system (Sternberg and Doudna 2015). We envisage that RCME will be a powerful way to make additional transgenic models that allow expansion of the CRISPR-Cas9 tool set beyond genome engineering.

Although Cas9-expressing mice exist, our iteration distinguishes itself in several ways. Platt et al. (Platt et al. 2014) developed a Cre-dependent CRISPR-Cas9 mouse which constitutively expresses Cas9 in tissues expressing Cre-recombinase. Although in their model (as in ours) long-term Cas9 expression was not deleterious at an organismal level, Cas9 is a foreign antigen and one concern is the generation of an immune response that could result in elimination of cells from the edited pool. Indeed, experiments with adenovirus-mediated delivery of Cas9 and sgRNA to the liver uncovered a Cas9-specific immune response (Wang et al. 2015). Similar findings were also reported following delivery of split-Cas9 moieties via adeno-associated virus (AAV) vectors (Chew et al. 2016). As well, given that off-target effects...
are an ever-present concern when it comes to genome editing, constitutive Cas9 expression is less desirable since longer-term Cas9 expression is associated with more off-target damage than transient Cas9 expression (Do et al. 2014; ZURIS et al. 2015). Our system allows for controlled, short-term induction of Cas9 expression, which should decrease off-target mutagenesis and mitigate host immune responses against Cas9. There are several features of our system that could improve editing efficiency which would need to be systematically assessed. For example, editing in adoptive transplant experiments may benefit from extending the DOX treatment to recipient mice to sustain prolonged Cas9 expression. Dow et al. (DOW et al. 2015) have successfully generated transgenic mice that demonstrate inducible CRISPR/Cas9 editing upon DOX administration. This inducible system is quite powerful but limited in that the sgRNA expression cassette is co-integrated with Cas9, therefore necessitating generation of a new strain for every target. The TRE-CIG/rtTA mice we describe here allows for greater flexibility to study genotype-phenotype relationships in vivo.

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Figure 5 Assessing sgp53-3 oncogenic activity in vivo of different sgRNA pools. A. Schematic representation of stem cell transplant experiments involving Eμ-Myc HSPCs infected with pQCiG/sgp53-3 or Eμ-Myc; R26-rTA; TRE-CIG/rtTA HSPCs infected with pUSPPC/sgp53-3. B. Kaplan-Meier curve illustrating tumor-free survival of C57Bl/6 mice transplanted with Cas9-modified HSPCs. Eμ-Myc HSPCs were transduced with pQCiG/sgTLR or a 1:5 dilution of pQCiG/sgp53-3 with pQCiG/sgTLR. Eμ-Myc; R26-rTA; TRE-CIG/rtTA HSPCs were transduced with pUSPPC/sgTLR or 1:5 and 1:50 dilutions of pUSPPC/sgp53-3 with pUSPPC/sgTLR. N = 5 mice for each cohort. C. Recovery of sg53-3 sequences from tumors (T-15-3, T-15-4, T-15-6) arising from pUSPPC/sgp53-3 infected Eμ-Myc; R26-rTA; TRE-CIG/rtTA HSCs. pUSPPC-p53-3 plasmid served as positive control and liver genomic DNA and water input served as negative controls. PCR products were analyzed on a 0.8% agarose gel. Arrowhead indicates expected location of migration of PCR products. The presence of undigested product is likely the consequence of contaminating normal cells during tissue extraction.
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