Retraction: The generation and characterization of novel Col1a1<sup>FRT-Cre-ER-T2-FRT</sup> and Col1a1<sup>FRT-STOP-FRT-Cre-ER-T2</sup> mice for sequential mutagenesis

Minsi Zhang and David G. Kirsch


The authors write:

In this paper, we reported two strains of novel mice: Col1a1<sup>FRT-STOP-FRT-Cre-ER-T2</sup> and Col1a1<sup>FRT-Cre-ER-T2-FRT</sup>. For figure 7, we generated primary sarcomas in Col1a1<sup>FRT-STOP-FRT-Cre-ER-T2; Kras<sup>FRT-STOP-FRT-G12D/+; p53<sup>FRT/FRT; Rosa26<sup>mTmG/+</sup></sup></sup> mice with intramuscular Adeno-FlipO and attempted to activate eGFP expression in tumors by injecting the mice with intraperitoneal (IP) tamoxifen. We observed that, following conventional IP tamoxifen administration, the tumors did not express eGFP. We also injected sarcomas directly with 4-hydroxy-tamoxifen (4-OHT). After multiple doses of 4-OHT injected directly into the tumor, we noted varying degrees of eGFP expression. Based on these results, we concluded that the low recombination efficiency of the mTmG allele in vivo after IP injection was due to limited penetration of the tamoxifen metabolite into the tumor in this sarcoma model.

Members of our lab have performed additional characterization of Col1a1<sup>FRT-Cre-ER-T2-FRT</sup> and Col1a1<sup>FRT-STOP-FRT-Cre-ER-T2</sup> mice. Although the description of our characterization of the Col1a1<sup>FRT-Cre-ER-T2-FRT</sup> allele remains valid, additional characterization of FlpO-activated sarcoma cell lines and mouse embryo fibroblasts (MEFs) from the Col1a1<sup>FRT-STOP-FRT-Cre-ER-T2</sup> mice show inefficient recombination of FLOX alleles when exposed to 4-OHT in vitro. This seems to be due to low expression of CreER following activation by FlpO recombinase. These new results indicate that our original conclusion that the poor uptake of tamoxifen by sarcomas impaired recombination from the FlpO-activated CreER in Col1a1<sup>FRT-STOP-FRT-Cre-ER-T2; Kras<sup>FRT-STOP-FRT-G12D/+; p53<sup>FRT/FRT; Rosa26<sup>mTmG/+</sup></sup></sup> mice is incorrect. Instead, low expression of CreER after FlpO activation in Col1a1<sup>FRT-STOP-FRT-Cre-ER-T2</sup> mice likely contributed to this phenotype. We apologize to readers for this error. Because the follow-up experiments using FlpO-activated cells derived from Col1a1<sup>FRT-STOP-FRT-Cre-ER-T2</sup> mice do not show efficient recombination of FLOX alleles in vitro, we would like to retract our paper.
The generation and characterization of novel Col1a1\textsuperscript{FRT-Cre-ER-T2-FRT} and Col1a1\textsuperscript{FRT-STOP-FRT-Cre-ER-T2} mice for sequential mutagenesis

Minsi Zhang\textsuperscript{1} and David G. Kirsch\textsuperscript{1,2,*}

ABSTRACT

Novel genetically engineered mouse models using the Cre-loxP or the Flp-FRT systems have generated useful reagents to manipulate the mouse genome in a temporally-regulated and tissue-specific manner. By incorporating a constitutive Cre driver line into a mouse model in which FRT-regulated genes in other cell types are regulated by Flp-FRT recombinase, gene expression can be manipulated simultaneously in separate tissue compartments. This application of dual recombinase technology can be used to dissect the role of stromal cells in tumor development and cancer therapy. Generating mice in which Cre-ERT\textsuperscript{2} is expressed under Flp-FRT-mediated regulation would enable step-wise manipulation of the mouse genome using dual recombinase technology. Such next-generation mouse models would enable sequential mutagenesis to better model cancer and define genes required for tumor maintenance. Here, we generated novel genetically engineered mice that activate or delete Cre-ERT\textsuperscript{2} in response to Flp recombinase. To potentially utilize the large number of Cre-loxP-regulated transgenic alleles that have already been targeted into the Rosa26 locus, such as ROSA\textsuperscript{26} reporter and mutant genes, we targeted the two novel Cre-FRT alleles into the endogenous Col1a1 locus for ubiquitous expression. In the Col1a1\textsuperscript{FRT-Cre-ER-T2-FRT} mice, Flp deletes Cre-ERT\textsuperscript{2}, so that Cre-ERT\textsuperscript{2} is only expressed in cells that have never expressed Flp. In contrast, in the Col1a1\textsuperscript{FRT-STOP-FRT-Cre-ER-T2} mice, Flp removes the STOP cassette to allow Cre-ERT\textsuperscript{2} expression so that Cre-ERT\textsuperscript{2} is only expressed in cells that previously expressed Flp. These two novel mouse strains will be complementary to each other and will enable the exploration of complex biological interactions in development, normal tissue homeostasis and cancer.

KEY WORDS: Mouse models, Sequential mutagenesis, Dual recombinase technology

INTRODUCTION

Genetically engineered mouse models are ideal for studying complex mammalian biological processes in vivo. Advances in genetic engineering have enabled the development of increasingly more precise, controlled manipulation of the mouse genome so that more intricate biological questions can be explored. Site-specific recombinase systems, such as the Cre-loxP and the Flp-FRT systems, were developed to allow for temporally-regulated and tissue-specific gene modification (Denekamp, 1996; O’Gorman et al., 1991; Orban et al., 1992; Saam and Henderson, 1988). A large number of conditional mouse alleles utilizing the Cre-loxP system have been generated. These include various loxP-flanked genes and numerous endogenous-promoter-driven Cre-recombinase alleles that are constitutively expressed. Cre recombinase, expressed from endogenous promoters enables tissue-specific deletion of DNA flanked by loxP sites to alter gene expression. The resulting tissue-specificity for recombination of the loxP sites decreases the likelihood of embryonic lethality. For example, Villin-Cre (El Marjou et al., 2004), which is expressed in the entire intestinal and colonic epithelium, and Fap\textsuperscript{Gr}x\textsuperscript{132-Cre} (Saam and Gordon, 1999), which is expressed in the distal two thirds of the small intestine and the entire colonic epithelium, can be used to express the embryonically lethal oncogenic KRAS\textsuperscript{G12D} by combining Kras\textsuperscript{loxP-STOP-loxP-G12D} (Jackson et al., 2001) to generate diffuse colonic hyperplasia in mice (Haigis et al., 2008). However, gene modification in the embryo might not fully recapitulate gene manipulation in the adult to model somatic gene mutations that lead to the development of sporadic cancers. One way to circumvent this limitation is to exogenously deliver Cre recombinase to the adult mice via infection with replicative-deficient adenoviruses (Adeno-Cre) (Wang et al., 1996). For example, intratracheal delivery of Adeno-Cre to adult mice with Kras\textsuperscript{F90L/F90L} and p53\textsuperscript{FL/FL} mutations (Marino et al., 2000) generated primarily small cell lung tumors that closely resembled human small cell lung cancer (Meuwissen et al., 2003). In addition to small cell lung tumors, this method also generated lung adenocarcinomas as well as medullary thyroid carcinomas both in mice that also developed small cell lung tumors and in mice that did not. This drawback to using viral vectors, particularly in cancer biology, is due to the fact that many cell types can be infected by the adenovirus. Thus, the cancer-initiating cell (i.e. cell of origin) can vary between tumors from different animals as well as different tumors within the same animal. This heterogeneity might lead to phenotypic differences in these mouse models of cancer. To further limit the expression of Cre to specific cell types within an organ, Cre can be expressed from the adenovirus using a cell-type-specific promoter (Sutherland et al., 2011, 2014).

Innovations in site-specific recombinase systems continue to improve tissue specificity and temporal regulation of gene expressions. For example, fusion proteins consisting of Cre recombinase and a mutated estrogen receptor that preferentially binds to the metabolites of the estrogen analog tamoxifen, such as Cre-ERT\textsuperscript{2}, have been generated (Indra et al., 1999). These fusion proteins allow for tamoxifen-inducible Cre recombinase to translocate into the nucleus and modify the genome. These Cre-fusion proteins allow temporal activation of oncogenes and/or deletion of tumor suppressor genes in the adult mouse in a cell-type-specific manner to generate cancer (Blum et al., 2013).
Recently, a second site-specific recombination system, Flp-FRT, which originated from *Saccharomyces cerevisiae*, has been applied to study cancer. Similar to Cre, Flp recombinase recognizes 34-bp sequences of DNA termed FRT sites. Like Cre, investigators have generated mice in which *KrasG12D* is regulated by a transcriptional terminator sequence flanked by FRT sites in the endogenous promoter (*KrasFRT-STOP-Flp*; *KrasG12D*) (Young et al., 2011). Similar to p53FL mice, we previously generated mice with the DNA-binding domain of p53 flanked by FRT sites (*p53FRT*) (Lee et al., 2012). The *p53FRT* alleles are combined with *KrasFRT-STOP-Flp* mice to generate primary sarcomas, lung cancer and pancreatic cancer (Lee et al., 2012; Moding et al., 2015, Schönhuber et al., 2014).

The use of only one recombinase system to mutate multiple genes limits the scope of research because all genetic manipulations are performed at the same time. In the case of mutations that drive cancer, the use of a single recombinase system prohibits the study of sequential mutagenesis to determine how the order of different driver gene mutations affects tumor phenotypes. Moreover, the consequence of a gene mutation at tumor initiation could be different from a mutation that is acquired later during tumor development. Indeed, when a single recombinase system is used to mutate multiple genes simultaneously, it is possible that cells might adapt to the loss of a gene at tumor initiation in ways that are different than if the same gene had been mutated later during tumor development. This might be particularly important for genetic experiments designed to identify therapeutic targets that are required for cancer maintenance because, in certain cases, inhibition of a target at the time of cancer initiation might lead to a different outcome than when a target is inhibited in an established tumor.

Others have demonstrated that the Flp-FRT and the Cre-loxP systems can be used sequentially. For example, lung adenocarcinoma has been generated by sequentially delivering Flp recombinase by adenovirus and Cre recombinase either by adenovirus or by tamoxifen to activate Cre-ER in mice harboring *BrafFRT-STOP-FRT* and either *p53FL* or *Cdkn2aFRT* mutations (Shai et al., 2015). Likewise, pancreatic adenocarcinoma has been generated by sequential mutagenesis, where first *KrasFRT-STOP-Flp* and then *Rosa26FRT-STOP-Cre-ErT2* are recombined by *Pdx1-Flp* to generate pancreatic tumors harboring oncogenic KRAS and the Cre-ER fusion protein, then tamoxifen is delivered to the animal to activate Cre-ER12 to recombine *p53FRT* in the pancreatic tumors, resulting in pancreatic tumors with both KRAS and p53 mutations (Schönhuber et al., 2014).

If we generated novel genetically engineered mice that activate or delete Cre-ER12 in response to Flp recombinase. Specifically, we generated two new mouse strains in which Cre-ER12 is knocked into the endogenous Coll1a1 locus so that the Rosa26 locus can be used for other genes or reporters of interest. In one allele, *Coll1a1FRT-Cre-ER-T2*; Cre-ER12 is flanked by FRT sites in the other allele, *Coll1a1FRT-STOP-FRT-Cre-ErT2*; Cre-ER12 is regulated by a FRT-SSTOP-FRT cassette. In *Coll1a1FRT-Cre-ER-T2*; Cre-ER12 mice, cells will only express Cre-ER12 and have the capacity for Flp-mediated recombination of *loxP* sites if they have never had prior exposure to Flp recombinase. Therefore, this strain will be useful for studying genes in stromal cells of established tumors that are initiated by Flp. In contrast, in *Coll1a1FRT-STOP-FRT-Cre-ErT2*; Cre-ER12 mice, in which Cre-ER12 is downstream from a FRT-SSTOP-FRT cassette, cells will only express Cre-ER12 and have the capacity for tamoxifen-mediated recombination of *loxP* sites after Flp-mediated removal of the STOP cassette. Therefore, this strain will be useful for sequential mutagenesis within tumor cells or for modifying the tumor cell genome in established cancers. Thus, these two new mouse strains will be complementary to each other and will enable the exploration of complex biological questions in cancer, normal development and tissue homeostasis.

RESULTS
Generation of Flp-FRT-regulated Cre-ER12 alleles
In order to develop technology for sequential mutagenesis in vivo using two site-specific recombinase systems, we generated *Coll1a1FRT-Cre-ER-T2-FRT* and *Coll1a1FRT-STOP-FRT-Cre-ErT2* mice. The rationale to generate *Coll1a1FRT-Cre-ER-T2-FRT* mice was to enable whole animal ubiquitous expression of Cre-ER12 until exposure to Flp recombinase (Fig. 1A). After Flp-mediated recombination of the FRT sites, cells are no longer able to express Cre-ER12 and therefore lose the ability to delete DNA flanked by *loxP* sites following exposure to tamoxifen. In this way, different mutations can be introduced in adjacent cells *in vivo* so that the consequences for intercellular interactions, such as those between cancer cells and stromal cells, can be studied. The rationale to generate *Coll1a1FRT-STOP-FRT-Cre-ErT2* was that initially no cell expresses Cre-ER12 because transcription of Cre-ER12 is terminated by an upstream FRT-site-flanked transcription STOP cassette (Fig. 1B). However, after Flp-mediated recombination, the STOP
cassette is excised. Therefore, these cells can initiate transcription of the Cre-ER T2 fusion protein, which, in response to subsequent exposure to tamoxifen, translocates into the nucleus to recombine DNA flanked by loxP sites. Cells without exposure to Flp will not be able to undergo Cre-mediated DNA recombination. In this way, the Col1a1FRT-STOP-FRT-Cre-ER T2 allele enables sequential mutations within the same cell over time. First, one mutation occurs in the cell from Flp recombinase, and then tamoxifen activates Cre recombinase in the same cell to mutate a second gene to study how the order of gene mutations affects cellular outcome. In addition, multiple genes can be mutated by Flp recombinase to initiate tumor development. Then, the role of a loxP-flanked gene in tumor maintenance can be studied because only the tumor cell will express Cre-ER T2. This allele can therefore be used to identify potential therapeutic targets.

To generate mice in which the expression of Cre-ER T2 is regulated by the Flp-FRT recombinase system and to fully utilize the large number of Cre-loxP-regulated transgenic alleles in the Rosa26 locus, such as fluorescent reporters and mutant genes, the two novel Cre-ER T2 alleles were targeted into the endogenous Col1a1 locus for ubiquitous expression (Fig. 2A,B). Targeting constructs for the generation of Col1a1FRT-Cre-ER T2-FRT and Col1a1FRT-STOP-FRT-Cre-ER T2 mice consisted of the sequence from the Col1a1 genomic DNA, CAG promoter, Cre-ER T2 regulated by FRT sites, and a neomycin selection cassette flanked by attP and attB sites. Therefore, transcription of the targeted Cre-ER T2 recombinase will be driven from the endogenous Col1a1 locus and enhanced by the addition of a CAG promoter. Constructs of the two Cre-ER T2 alleles were electroporated into 129/SVJae embryonic stem (ES) cells and successfully targeted ES cells were selected by neomycin (G418) treatment. Positively selected ES cells were analyzed for successful homologous recombination by Southern blot of genomic DNA (Fig. 2C,D). Correctly targeted ES clones were injected into C57BL/6 blastocysts and male high-percentage chimeras were selected to breed with C57BL/6 females to identify germline
transmission of the Cre-ER<sup>T2</sup> alleles. The neomycin selection cassettes were removed by PhiC31-integrase-mediated recombination of the att<sup>P</sup> and att<sup>B</sup> sites in vivo by crossing the mice to the pre-existing Rosa26PhiC31 strain (Raymond and Soriano, 2007).

Germline transmission of Col1a1<sup>FRT-Cre-ER<sup>T2</sup>-FRT-NEO</sup> was verified by PCR for the construct-specific neomycin selection cassette on tail-tip DNA (Fig. 3A). Heterozygosity for the knock-in allele was demonstrated by PCR for the wild-type Col1a1 locus (Fig. 3A). Successful PhiC31-mediated excision of the neomycin selection cassette was verified by PCR for gene products specific to the recombined neomycin selection cassette (excised NEO), the unrecombined neomycin selection cassette (NEO), the wild-type Col1a1 locus and the mutant PhiC31 allele (Fig. 3B).

Germline transmission of a single copy of Col1a1<sup>FRT-STOP-FRT-Cre-ER<sup>T2</sup>-NEO</sup> was verified by PCR for the construct-specific STOP cassette and the wild-type Col1a1 locus on tail-tip DNA (Fig. 3C). Deletion of the neomycin selection cassette by PhiC31 was verified by PCR for gene products specific for the recombined neomycin selection cassette (excised NEO), the unrecombined neomycin selection cassette (NEO), the STOP cassette, the wild-type Col1a1 locus and the mutant PhiC31 allele (Fig. 3D). One founder mouse for each of the novel Cre-ER<sup>T2</sup> strains was used to propagate the colony.

Characterization of Flp-FRT-regulated Cre-ER<sup>T2</sup> mice

To examine Cre-mediated recombination at a cellular level, tissues from Col1a1<sup>FRT-Cre-ER<sup>T2</sup>-FRT</sup>, Rosa26<sup>Neo<sup>TNG</sup></sup> mice were examined by immunofluorescence with and without tamoxifen treatment (Fig. 4). Without Cre-mediated excision of tdTomato, eGFP is expressed from the same locus. Because these mice are heterozygous for the Rosa26<sup>Neo<sup>TNG</sup></sup> allele, any expression of eGFP indicates Cre-mediated excision of tdTomato. In tissues taken from 6-week-old mice (n=2) without tamoxifen treatment (Fig. 4A), tamoxifen treatment (Fig. 4A), expression of tdTomato was widespread, and there was minimal tissue-specific eGFP expression. Tissues with some eGFP expression included the pancreas (Fig. 4Avii) and, to a lesser extent, a few skeletal muscle fibers (Fig. 4Aiii). When tissues from 6-month-old mice (n=3) obtained 30 days after treatment with a single dose of 75 mg
Fig. 3. Germline transmission, and successful PhiC- and FlpO-mediated recombination of attB/attP and FRT sites. (A) PCR of tail DNA for the neomycin cassette of the Col1a1 Crt-Cre-ER-T2-FRT-NEO and wild-type Col1a1 locus indicate germline transmission of a single copy of the Col1a1 Crt-Cre-ER-T2-FRT-NEO allele. (B) PCR for the recombinant neomycin cassette (excised NEO), unrecombined neomycin cassette (NEO), wild-type Col1a1 locus and mutant PhiC31 allele indicate successful PhiC31-mediated deletion of the neomycin selection cassette in the Col1a1 Crt-Cre-ER-T2-FRT-NEO allele. (C) PCR of tail DNA for the neomycin cassette of the Col1a1 Crt-Cre-ER-T2-FRT-NEO and wild-type Col1a1 locus indicate germline transmission of a single copy of the Col1a1 Crt-Cre-ER-T2-FRT-NEO allele. (D) PCR for the recombinant neomycin cassette (excised NEO), unrecombined neomycin cassette (NEO), presence of STOP cassette, wild-type Col1a1 locus and mutant PhiC31 allele indicate successful PhiC31-mediated deletion of the neomycin selection cassette in the Col1a1 Crt-Cre-ER-T2-FRT-NEO allele, generating the Col1a1 Crt-Cre-ER-T2-FRT-NEO allele owing to the presence of the FRT site in between the FWD1 and REV1 primers (see Fig. 2 for schematics with the location of the FRT sites relative to the primers).

tamoxifen/kg body weight in corn oil by intraperitoneal injection were examined (Fig. 4B), there was widespread eGFP expression and minimal tdTomato retention. The tissue with highest tdTomato retention was the brain (Fig. 4Bxxxii), where there was diffuse low tdTomato expression and regions of high eGFP expression (Fig. 4Bxxxiii). This might reflect diffusion limitations of tamoxifen across the blood-brain barrier, or brain parenchyma seems to have lower tdTomato expression (Fig. 4Axii) than other tissues such as the brain (Fig. 4Axxii), the liver, and the skeletal muscle fibers in the 4-month-old mice (Fig. 5Aiii) had less eGFP expression than those in the 6-month-old mice (Fig. 5Bvii). Additionally, the pancreas of the 4-month-old mice (Fig. 5Avii) had less eGFP expression than the pancreas of the 6-month-old mice (Fig. 5Bvii). The liver of both the 4-month-old (Fig. 5Aiv) and the 6-month-old (Fig. 5Bv) mice had similar eGFP expression. In contrast, the brain had no eGFP expression with the exception of a subset of the vasculature in both the 4-month-old mice (Fig. 5Axxxii) and the 6-month-old mice (Fig. 5Bxxxi). Taken together, these results demonstrate that there is time-dependent and tissue-specific Cre-ERT2 activity in Col1a1 Crt-Cre-ER-T2-FRT-NEO mice in the absence of tamoxifen, with the brain tissue being the least affected organ, and the pancreas, liver and skeletal muscle fibers being the most affected organs. In addition, tamoxifen potently induces Cre-ERT2-mediated recombination of unrecombined loxp sites.

To examine the leakiness of Cre-ERT2 in Col1a1 Crt-Cre-ER-T2-FRT-NEO mice, tissues from 6-month-old Col1a1 Crt-Cre-ER-T2-FRT-NEO mice treated with tamoxifen as compared to untreated controls (Fig. 4Axxii) and without tamoxifen treatment (Fig. 6). In the absence of tamoxifen, the pancreas was examined by immunofluorescence with (n=2) tamoxifen treatment (Fig. 6). In the absence of tamoxifen, there was widespread tdTomato expression without evidence of eGFP expression in any of the tissues examined (Fig. 6A). When tissues were collected 30 days after intraperitoneal delivery of one dose of 75 mg tamoxifen/kg body weight in corn oil, there continued to be widespread tdTomato expression without evidence of eGFP expression in any of the tissues examined (Fig. 6B). These results indicate that the STOP cassette is functional and does not allow for
Cre-ERT2-mediated transcription in the absence of Flp-mediated recombination and excision of the STOP cassette.

To determine whether the STOP cassette for the Col1a1FRT-STOP-FRT-Cre-ER-T2 allele can be excised by Flp-mediated recombination in vivo, mice with the Col1a1FRT-STOP-FRT-Cre-ER-T2 allele were crossed to KrasFRT-STOP-FRT-G12D/+;p53FRT/FRT mice (Lee et al., 2012) to generate Col1a1FRT-STOP-FRT-Cre-ER-T2/+;KrasFRT-STOP-FRT-G12D/+;p53FRT/FRT mice. Adenovirus expressing mammalian optimized Flp recombinase (Adeno-FlpO) was injected into the hindlimb of these mice to generate primary soft-tissue sarcomas. Once sarcomas developed, the bulk tumor tissues were excised and dissociated into single-cell suspensions and cultured in vitro. Genomic DNA was isolated from these cells and recombination of the STOP cassette was verified by PCR (Fig. 7A). Tail DNA taken at the time of genotyping from a Col1a1FRT-Cre-ER-T2-FRT mouse was used as a positive control for the absence of STOP cassette, and tail DNA taken at the time of genotyping from a Col1a1FRT-STOP-FRT-Cre-ER-T2 mouse was used as a negative control for the recombined STOP cassette.

To examine the functionality of the Col1a1FRT-STOP-FRT-Cre-ER-T2 allele in a tumor model, these mice were crossed to KrasFRT-STOP-FRT-G12D/+;p53FRT/FRT; Rosa26mTmG/+ mice to generate Col1a1FRT-STOP-FRT-Cre-ER-T2;KrasFRT-STOP-FRT-G12D/+;p53FRT/FRT; Rosa26mTmG/+ mice. Primary sarcomas were generated in the hindlimb of these mice by intramuscular injection of Adeno-FlpO. After the initial tumor was palpated in the Col1a1FRT-STOP-FRT-Cre-ER-T2;KrasFRT-STOP-FRT-G12D/+;p53FRT/FRT; Rosa26mTmG/+ mice, a single dose of 75 mg tamoxifen/kg body weight in corn oil was delivered via intraperitoneal injection. Tumors (n=2) were collected 10 days after the tamoxifen delivery, and immunofluorescence was used to evaluate tdTomato and eGFP expression (Fig. 7B). Unexpectedly, the whole tumor tissue remained tdTomato-positive without any evidence of eGFP expression. Because the STOP cassette can be appropriately removed by FlpO, as shown in Fig. 7A, the lack of tdTomato deletion and eGFP expression might be due to inadequate levels and/or penetration of the active tamoxifen metabolite into the tumor tissue. Therefore, alternative approaches of tamoxifen delivery were explored in subsequent cohorts. After the initial tumor was...
palpated in a second cohort \((n=2)\) of \(Col1a1^{FRT-STOP-FRT-Cre-ER-T2}\); \(Kras^{FRT-STOP-FRT-G12D/+}\); \(p53^{FRT/FRT}\); \(Rosa26^{mTmG/+}\) mice, a single dose of 0.75 mg 4-hydroxytamoxifen in DMSO was delivered via intratumoral injection. Tumors were collected 10 days after the 4-hydroxytamoxifen delivery, and tdTomato and eGFP expression were evaluated by immunofluorescence (Fig. 7C). In contrast to tumors from mice that were given intraperitoneal tamoxifen injection, the bulk tumor from mice that received intratumoral 4-hydroxytamoxifen were mostly eGFP-positive with scattered tdTomato expression. For another set \((n=2)\) of \(Col1a1^{FRT-STOP-FRT-Cre-ER-T2}\); \(Kras^{FRT-STOP-FRT-G12D/+}\); \(p53^{FRT/FRT}\); \(Rosa26^{mTmG/+}\) mice, a single dose of 2 mg 4-hydroxytamoxifen in PBS was delivered via subcutaneous injection between the scapula. Twenty-four hours following the single injection, 2 mg of 4-hydroxytamoxifen in PBS was delivered via subcutaneous injection between the scapula. Two more subcutaneous injections of 4-hydroxytamoxifen were delivered at the same site every 24 h for a total of four doses (one intraperitoneal and three subcutaneous). This tumor was collected 1 day after the last dose of 4-hydroxytamoxifen, and tdTomato and eGFP expression were evaluated by immunofluorescence (Fig. 7E). This tumor demonstrated scattered areas with high eGFP expression and larger areas with diffusely lower eGFP expression, and scattered tdTomato expression. This suggests that administration of multiple high doses of systemic 4-hydroxytamoxifen is capable of penetrating...
the tumor tissue to enable Cre-ERT2-mediated recombination of loxP sites.

Taken together, these results show that the Cre-ERT2 in both Col1a1FRT-Cre-ER-T2-FRT and Col1a1FRT-STOP-FRT-Cre-ER-T2 mice is functional and that the recombinase activity in both mice can be potently induced by the active tamoxifen metabolite. Additionally, the expression of Cre-ER T2 in Col1a1FRT-STOP-FRT-Cre-ER-T2 mice is tightly controlled by the STOP cassette. However, in this primary sarcoma model, penetration of the active tamoxifen metabolite into the tumor following a single intraperitoneal tamoxifen injection is insufficient to activate the Col1a1FRT-STOP-FRT-Cre-ER-T2 allele. Limited penetration of the active tamoxifen metabolite after a single intraperitoneal injection of tamoxifen might be due to the large size (approximately 200 mm^3) of the sarcomas at the time of palpation and thus might not be a limitation for other tumor models in which the tumors are smaller. Even in the primary sarcoma model, the limited penetration following a single intraperitoneal injection of tamoxifen can be overcome by several doses of 4-hydroxytamoxifen delivered by intraperitoneal and subcutaneous routes. Finally, in the Col1a1FRT-Cre-ER-T2-FRT mice there is a time-dependent recombinase activity of Cre-ERT2 in the absence of tamoxifen exposure, which might limit its utility in certain tissues for experiments that would require waiting several months before the administration of tamoxifen.

DISCUSSION

The primary mouse model is an important tool to study biological processes in vivo; therefore, novel mouse strains and mouse models provide an opportunity for scientific advancement. Here, we have generated two novel mouse strains to facilitate more precise manipulation of the mouse genome to answer complex questions. The two novel mouse strains take advantage of different site-specific recombinase systems to enable sequential mutagenesis separated in time and cellular location. These novel mouse strains will facilitate the study of complex questions in development, normal tissue homeostasis and disease processes, such as cancer. For example, these strains have a number of advantages for studying the role of genes in either tumor cells or stroma. First, the knock-in alleles of both novel mouse strains are targeted to...
the ubiquitously expressed Col1a1 locus instead of the frequently used Rosa26 locus. This Col1a1 site was selected with the available Rosa26 knock-in alleles in mind. An advantage of knock-in alleles over other transgenic approaches that generate alleles by random integration into the mouse genome is that the precise location of the knock-in allele is known. However, each mouse can only harbor two different copies of a mutant gene at one locus. Because there are many Rosa26 knock-in alleles, ranging from mutant genes to fluorescent reporters, the placement of the two novel Flp-regulated Cre-ERT2 alleles at the Col1a1 locus retains the availability of both alleles of the Rosa26 locus for other genes of interest.

Our results indicate that the Col1a1 locus is a good alternative to the Rosa26 locus for ubiquitous gene expression, as noted by diffuse eGFP expression after tamoxifen-mediated deletion of tdTomato in Col1a1FRT-Cre-ER-T2; Rosa26mTmG/+ mice. In addition, Cre-ERT2 is tightly regulated by the STOP cassette in Col1a1FRT-STOP-FRT-Cre-ER-T2; Rosa26mTmG/+ mice and, after successful removal of the STOP cassette by Adeno-FlpO, Cre-ERT2 can be efficiently activated by 4-hydroxytamoxifen in primary soft-tissue sarcomas via intratumoral injection or multiple systemic injections to express eGFP in the tumor parenchyma. A limitation of the Col1a1FRT-Cre-ER-T2 mice is the age-dependent tissue-specific Cre-ER T2 activity independent of
exogenous tamoxifen administration, which is most pronounced in the pancreas and the liver. This could result from a number of factors, including tissue-specific basal expression from the Col1a1 locus, tissue-specific exposure to endogenous estrogens that might activate Cre-ER\textsuperscript{T2}, and the rate of tissue-specific protein turnover. Tissues that are less affected include the bone marrow and the brain. This might limit the scope of experiments that can be performed with the Col1a1\textsuperscript{FRT-Cre-ER-T2-FRT} allele to younger mice, to a shorter timeframe for sequential mutagenesis, and/or to specific organs, especially in older mice. In contrast, we did not observe any leakiness from Cre-ER\textsuperscript{T2} in the Col1a1\textsuperscript{FRT-Cre-ER-T2-FRT} mice. Thus, the age of mice for experiments with this allele does not seem to be a critical experimental variable. In primary sarcomas initiated by Adeno-FlpO from Col1a1\textsuperscript{FRT-Cre-ER-T2-FRT-NEO}, p3\textsuperscript{3\textasciitilde{FRT}/FRT}; Rosa26\textsuperscript{mTmG/+} mice, we were able to activate Cre-ER\textsuperscript{T2} in tumor cells by intratumoral administration of 4-hydroxytamoxifen or multiple doses of systemic 4-hydroxytamoxifen. However, experiments with intraperitoneal injection of a single dose of tamoxifen failed to activate Cre-ER\textsuperscript{T2} in primary sarcomas when they were approximately 200 mm\textsuperscript{3}. Therefore, the delivery of an adequate level of active tamoxifen metabolites into the tumor will be necessary to apply this system in established sarcomas. In addition to injecting 4-hydroxytamoxifen into the tumor or systemically, another approach that could be utilized is delivery of tamoxifen in the food (Schönhuber et al., 2014).

Recently, in vivo manipulations of the mouse genome have been achieved using the CRISPR/Cas9 system to generate tumors, for example in the liver and lung, via: hydrodynamic injections (Xue et al., 2014), adenoviral delivery of both sgRNA and Cas9 (Sánchez-Rivera et al., 2014), or adenoviral delivery of sgRNA and Cre into Rosa26\textsuperscript{loxP-stop-loxP-Cas9} mice (Platt et al., 2014). There are distinct advantages and disadvantages to using either the CRISPR/Cas9 or the Cre-loxP recombine systems to model cancer depending on the application. For example, the CRISPR/Cas9 system does not require the generation of mice with multiple mutant alleles. Therefore, the CRISPR/Cas9 system enables testing of the impact of different gene mutations on a cancer model more quickly. However, the Cre-loxP recombine system can be much more efficient in generating a specific gene mutation. One existing application of the CRISPR/Cas9 system would be to combine the same efficiency of the CRISPR/Cas9 system, with the spatial and temporal control of stepwise genomic manipulations with the Flp-FRT-mediated Cre-ER\textsuperscript{T2} mouse strain. Specifically, the Col1a1\textsuperscript{FRT-STOP-loxP-Cas9} mouse strain could be used in combination with Rosa26\textsuperscript{loxP-stop-loxP-Cas9} mice such that in vivo genome-scale screening would be possible to evaluate cooperating mutations for cancer initiation and maintenance.

In summary, we have generated two complementary novel Flp-FRT-regulated Cre-ER\textsuperscript{T2} mouse lines that can be used for sequential mutagenesis with the FlpO system and the Cre-loxP systems. The Col1a1\textsuperscript{FRT-STOP-loxP-Cas9} allele can be combined with FlpO, FRT-flanked, or loxP-regulated genes to generate mice where distinct mutations can be expressed in neighboring cells to study intercellular communications such as those between tumor parenchymal and stromal cells. Furthermore, the Col1a1\textsuperscript{FRT-STOP-loxP-Cas9} allele can be combined with FlpO, FRT-flanked, or loxP-regulated genes to generate mice in which two distinct mutations can be expressed in a single cell sequentially. We anticipate that this allele will provide a tightly regulated tool to study sequential mutagenesis in cancer development and to test the role of genes in tumor maintenance by mimicking therapeutic target inhibition or activation during cancer therapy.

**MATERIALS AND METHODS**

All animal experiments were performed according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

**Generation of novel mouse models**

Knock-in alleles Col1a1\textsuperscript{FRT-STOP-loxP-Cre-ER-T2}; Kras\textsuperscript{FRT-STOP-FRT-NEO} were generated by targeting Col1a1\textsuperscript{FRT-Cre-ER-T2-FRT-NEO} with loxP-regulated Cre from the Col1a1 locus in 129/ SVjae embryonic stem cells (ES cells). 129/SVjae ES cells were selected in neomycin (G418), and screened for homologous recombination at the Col1a1 locus by PCR and Southern blot. ES cells that were successfully targeted at the Col1a1 locus were injected into C57BL/6 blastocysts, which were then implanted into pseudo-pregnant mice. Male chimeras were bred to female C57BL/6 mice and the resulting progeny with brown-colored coat (i.e. 129/SVj-verified) were utilized to generate germline transmission of the targeted alleles. Mice with germine transmission were crossed to the Rosa26\textsuperscript{loxP-stop-loxP-Cre-ER-T2} (Raymond and Soriano, 2007), which removed the attB/attP-flanked loxP cassette.

**Adenovirus-mediated sarcomagenesis**

Mouse models of soft-tissue sarcoma were generated in a mixed 129/SVJae and C57BL/6 background using a combination of previously described alleles: Kras\textsuperscript{FRT-STOP-FRT-NEO} (Young et al., 2011), p53\textsuperscript{R172H} (Lee et al., 2012) and Rosa26\textsuperscript{mTmG} (Muzumdar et al., 2007). A working preparation of mutation-deficient adenovirus expressing FlpO recombinase (Adeno-FlpO) was generated by precipitating 25 µl of Adeno-FlpO stock (University of Iowa Gene Transfer Vector Core, Iowa City, IA) with 3 µl 2-M CaCl\textsubscript{2} (Sigma-Aldrich, St Louis, MO) and 600 µl MEM (Sigma-Aldrich, St Louis, MO) as previously described (Kirsch et al., 2007). The working solution was then incubated at room temperature for 15 min prior to use. Soft-tissue sarcomas in the mouse hindlimb were generated by intramuscular (IM) injection of 50 µl Adeno-FlpO working solution via a 28.5-gauge insulin syringe (BD Biosciences, Franklin Lakes, NJ).

**4-hydroxytamoxifen and tamoxifen injections**

(Z)-4-hydroxytamoxifen (Sigma-Aldrich, St Louis, MO), or 4-OHT, was first dissolved in 100% ethanol at a concentration of 250 µg/ml, and then diluted with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO) to a final working solution concentration of 25 µg/ml. Soft-tissue sarcoma in the mouse hindlimb was then infiltrated with a single intratumoral injection of 30 µl 4-OHT (0.75 mg) working solution via a 28.5-gauge insulin syringe. Alternatively, 4-OHT was dissolved in one-to-one ratio of 100% ethanol and Kolliphor\textsuperscript{\textregistered} EL (Sigma-Aldrich, St Louis, MO) at a concentration of 20 µg/ml. Tumor cell isolation and culture

**Tumor cell isolation and culture**

Sarcomas were removed after animals were sacrificed and the tumors were digested with tissue digestion buffer consisting of 5 mg/ml collagenase IV (Sigma-Aldrich, St Louis, MO), 2.5 U/ml dispase (BD Biosciences, Franklin Lakes, NJ) and 0.05% trypsin (Life Technologies, Carlsbad, CA) at 37°C for 40 min. The resulting mixture was strained through a 70-µm filter (BD Biosciences, Franklin Lakes, NJ), and filtered cells were re-suspended in culture medium consisting of DMEM/ F12 (Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO) and 1× penicillin/streptomycin (Life Technologies, Carlsbad, CA).
Polymerase chain reactions
DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, Limburg) and PCR experiments were performed with a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA). Nucleic acid concentrations were measured using NanoDrop 1000 (Thermofinconcert, Waltham, MA). The location of the primer sequences relative to the targeted alleles are shown in Fig. 3 and the sequences are as follows: 5'-GGGCTCGACGACTAGTTAA-3' (FWD1), 5'-GAGATCTGGGATGAAGTAGTGTCT-3' (FWD2), 5'-ATCATGTCTGATTCCACC-3' (REV3), 5'-GCAAGTGTGGCTTTACGGTATCGCC-3' (FWD3), 5'-GCCCTAGATTTATCATGTCTGGATCCCCATC-3' (REV1), 5'-CCAGCATCCACATTCTCCTTT-3' (REV2). FWD1 and REV1 amplify a 395-bp product for Col1a1FRT-Cre-ER-T2-STOP and a 360-bp product for Col1a1FRT-Cre-ER-T2-FRT-STOP cassette is excised for if the STOP cassette is not excised for Col1a1FRT-Cre-ER-T2-NEO or if the Cre-ERT2 cassette is excised. PCR for the NEO cassette amplifies a 540-bp product if the NEO cassette is present in the Col1a1FRT-Cre-ER-T2-STOP and Col1a1FRT-Cre-ER-T2-STOP-NEO mice using the following two primers: 5'-CCCTGTTTACGGATTCGCC-3' (FWD5), 5'-GCCCTAGATTTATCATGTCTGGATCCCCATC-3' (REV5). The Rosa26*PCR1 PCR was performed using primers as previously described (Raymond and Soriano, 2007).

One touchdown PCR program was used for all reactions: (1) melting at 94°C for 180 s; (2) for 15 cycles, melting at 94°C for 45 s, annealing at 64°C for 45 s with −0.5°C per cycle, elongating at 72°C for 60 s; (3) for 25 cycles, melting at 94°C for 45 s, annealing at 57°C for 45 s, elongating at 72°C for 60 s; (4) elongating at 72°C for 600 s.

Frozen sectioning and immunofluorescence
For frozen sectioning, tumor and tissue specimens were dissected, embedded in OCT (Tissue-Tek, Torrance, CA) and frozen over dry ice. Frozen sections and 10-μm sections generated via a cryostat (Leica Microsystems, Wetzlar, Germany) were stored unfixed at −80°C. For immunofluorescence, slides were incubated with Alexa-Fluor-488-conjugated rabbit anti-GFP antibody (Life Technologies, Carlsbad, CA) at a concentration of 1:500 for 1 h at room temperature. Slides were then counterstained with 10 μg/ml 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St Louis, MO). Images were captured with a DM5500B microscope (Leica Microsystems, Wetzlar, Germany) using Leica Application Suite software.

Resource sharing
The Col1a1FRT-Cre-ER-T2-FRT and Col1a1FRT-Cre-ER-T2-STOP mouse strains in the mixed C57BL/6 and 129S/JVae background will be available at the Jackson Laboratory Repository with the JAX Stock Nos. 027750 and 027751. The strains are also available upon written request to D.G.K.

Acknowledgements
We acknowledge Ute Hochgeschwender and the Duke Neurotransgenic Laboratory for assistance with generating the Col1a1FRT-Cre-ER-T2-STOP and Col1a1FRT-Cre-ER-T2-STOP-NEO constructs, ES cell targeting and blastocyst injection. We thank Lixia Luo for assistance with generating the mouse colony. We thank Tyler Jacobs for providing the Rosa26*PCR1 mice.

Competing interests
The authors declare no conflict of financial interest.

Author contributions
M.Z. and D.G.K. conceived and designed experiments. M.Z. performed the experiments. M.Z. and D.G.K. analyzed the data and wrote the manuscript.

Funding
This work was supported by NIH grants P01 CA180880 (M.Z.) and R01 CA169220 (D.G.K.), and partially by the Cancer Center Support Grant CA014236.

References


