Generation of primary tumors with Flp recombinase in 
FRT-flanked p53 mice

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SUMMARY
The site-specific recombinases Cre and Flp can mutate genes in a spatially and temporally restricted manner in mice. Conditional recombination of the tumor suppressor gene p53 using the Cre-loxP system has led to the development of multiple genetically engineered mouse models of human cancer. However, the use of Cre recombinase to initiate tumors in mouse models limits the utilization of Cre to genetically modify other genes in tumor stromal cells in these models. To overcome this limitation, we inserted FRT (flippase recognition target) sites flanking exons 2-6 of the endogenous p53 gene in mice to generate a p53FRT allele that can be deleted by Flp recombinase. We show that FlpO-mediated deletion of p53 in mouse embryonic fibroblasts impairs the p53-dependent response to genotoxic stress in vitro. In addition, using FSF-KrasG12D/+; p53FRT/FRT mice, we demonstrate that an adenovirus expressing FlpO recombinase can initiate primary lung cancers and sarcomas in mice. p53FRT mice will enable dual recombinase technology to study cancer biology because Cre is available to modify genes specifically in stromal cells to investigate their role in tumor development, progression and response to therapy.

INTRODUCTION
The transformation related protein p53 gene, Trp53, is the most frequently mutated gene in human cancer, altered in approximately 50% of human malignancies (Brosh and Rotter, 2009). The p53 nuclear phosphoprotein functions as a transcription factor that responds to cellular stress by initiating multiple signaling pathways. The p53 response varies across cell and tissue types, and involves a spectrum from transient cell cycle arrest to senescence and apoptosis (Stiewe, 2007). Mice deficient for p53 generally develop normally, but are predisposed to cancer at a young age (Donehower et al., 1992; Jacks et al., 1994).

The site-specific recombinases Cre and Flp allow for spatially and temporally regulated mutation of a target gene in the somatic tissues of mice (Branda and Dymecki, 2004). Conditional recombination of p53 using the Cre-loxP system has been utilized to delete or mutate p53 in a tissue-specific manner or to delete p53 at a specific time during development (Donehower and Lozano, 2009). This technology has led to the development of multiple mouse models of primary cancer (Marino et al., 2000; Lin et al., 2004; Jackson et al., 2005; Kirsch et al., 2007; Martinez-Cruz et al., 2008). Genetically engineered mouse models (GEMMs) might offer an advantage over xenograft and chemically induced cancer models by providing an opportunity to study mechanisms of autochthonous cancer development and response to treatment in an anatomically restricted manner in mice that are neither tumor-prone nor immunosuppressed (Sharpless and Depinho, 2006). However, most GEMMs use Cre-loxP technology to initiate cancer, limiting the availability of Cre recombinase to modify genes in tumor stromal cells.

Because Cre and Flp recombine distinct DNA target sites, loxP and FRT, respectively (Branda and Dymecki, 2004), these two highly efficient site-specific recombinase systems (Cre-loxP and Flp-FRT) have been used to create genetically engineered mice with a targeting construct with a removable positive selection cassette (Meyers et al., 1998). More recently, dual recombinase technology was used to sequentially delete p53 and activate the Kras oncogene, revealing the importance of timing of Kras and p53 mutations in tumorigenesis (Young et al., 2011). Despite the growing abundance of loxP-flanked (‘floxed’) alleles and tissue-specific Cre drivers, the Flp-FRT system has been utilized less frequently than Cre-loxP to modify genes in the somatic tissues in mice. Generating additional FRT-flanked (‘frted’) alleles will enable dual recombinase technology so that distinct gene mutations can be directed to different cell types by Cre and Flp recombinases. Here, we generated p53FRT mice in which the endogenous p53 allele is flanked by FRT sites so that it can be deleted by Flp recombinase.

RESULTS
Generation of p53FRT mice
To generate a frted p53 mouse, we constructed a targeting vector in which exons 2 through 6 of p53 genomic DNA are flanked by FRT sites (Fig. 1A). Exons 2-6 encode for the DNA-binding domain that is required for p53-dependent tumor suppression (Brady et al., 2011). The 5′ FRT site was inserted between exons 1 and 2, and a loxP-flanked (floxed) PGK-neo cassette (neo) followed by a 3′ FRT site was inserted between exons 6 and 7. A PGK-diphtheria toxin A (DTA) cassette was placed following exon 11 as a negative selectable marker. The targeting vector was linearized and electroporated into embryonic stem (ES) cells. Following selection

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Received 9 November 2011; Accepted 21 December 2011

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by G418, two out of 800 colonies had correctly undergone homologous recombination as demonstrated by PCR (Fig. 1B). Successful homologous recombination of the p53<sup>FRT</sup>-<sup>neo</sup> allele into the endogenous p53 locus was confirmed by Southern blot. As shown in Fig. 1A and 1C, a Scal-digested DNA fragment of 6.7 kb, which includes genomic DNA outside the targeting construct, was detected in these two ES cell clones using probes binding either neo or exon 11 of p53. ES cell line 7-9H and 8-8B was used to derive germline transmitting chimeric mice. Male chimeric mice were then bred to Meox2-Cre females to delete the floxed PGK-neo cassette in germline cells. Germline transmission of the targeted allele after deletion of the neo cassette (p53<sup>FRT</sup> allele as shown in Fig. 1A) was confirmed by PCR (Fig. 1D).

**Characterization of p53<sup>FRT</sup> MEFs**

To study FlpO-mediated recombination of the p53<sup>FRT</sup> allele, mouse embryonic fibroblasts (MEFs) were isolated from p53<sup>FRT</sup> mice and infected with an adenovirus expressing FlpO (Ad-FlpO) or eGFP (Ad-eGFP). FlpO is a Flp recombinase with codons optimized for mammalian systems (Raymond and Soriano, 2007). Successful recombination of the p53<sup>FRT</sup> allele was confirmed by PCR (Fig. 2A). Doxorubicin is a commonly used chemotherapeutic that is known to induce p53-mediated cell cycle arrest at the G1 checkpoint by increasing p21 protein levels (Attardi et al., 2004). To demonstrate that recombination of the p53<sup>FRT</sup> gene by FlpO impairs the p53 response to genotoxic stress, MEFs infected with Ad-FlpO or Ad-eGFP were treated with 0.5 µg/ml doxorubicin for 18 hours. Protein levels measured by western blot demonstrated decreased p53 in p53<sup>FRT</sup> mice infected with Ad-FlpO when compared with p53<sup>FRT</sup> MEFs infected with Ad-eGFP (Fig. 2B). In addition, FlpO-mediated recombination decreased p21 induction after exposure to doxorubicin. Levels of p53 and p21 in Ad-FlpO-infected p53<sup>FRT</sup> MEFs were slightly greater than control p53-null (p53<sup>−/−</sup>) MEFs, which probably reflects incomplete infection of the MEFs with adenovirus.

Primary mammalian cells such as MEFs have a limited life span in vitro owing to p53-mediated senescence and are transformed by loss of p53 (Harvey et al., 1993). We investigated whether cell-culture-induced senescence occurs in p53<sup>FRT</sup> MEFs after FlpO-mediated recombination by assessing population doubling of cells in vitro. Similar to p53<sup>−/−</sup> MEFs, p53<sup>FRT</sup> MEFs infected with Ad-FlpO did not show p53-mediated senescence and proliferated faster than p53<sup>FRT</sup> MEFs infected with Ad-eGFP (Fig. 2C). Additionally, FlpO-recombined p53<sup>FRT</sup> MEFs were found to be...
Flp-mediated deletion of p53

genetically unstable compared with p53WT MEFs, as demonstrated by their markedly increased DNA content at later passages (Fig. 2D).

**Generation of FlpO-driven tumors**

To study Flp-mediated recombination of the p53FRT allele in vivo, we crossed p53FRT mice with mice carrying a Flp-activated allele of oncogenic Kras to generate FSF-KrasG12D/+; p53FRT/FRT (KPFL) compound conditional mutant mice. It has been shown that activation of KrasG12D and deletion of p53 in LSL-KrasG12D/+; p53FRT/FL (KPFL) mice via intramuscular (IM) and intranasal (IN) infection with Ad-Cre is sufficient to initiate high-grade soft-tissue sarcomas and lung adenocarcinomas (Jackson et al., 2005; Kirsch et al., 2007). However, activation of KrasG12D via IM Ad-Cre infection does not initiate soft-tissue sarcomas and IN Ad-Cre infection generates only lung adenomas and low-grade adenocarcinomas. We infected KPFL mice with IM and IN Ad-FlpO, and extermity sarcomas and high-grade lung adenocarcinomas developed as early as 8 weeks after infection (Fig. 3A-D). The time frame of tumor development was similar to that of tumors generated in KPFL mice by Ad-Cre infection (Jackson et al., 2005; Kirsch et al., 2007). Although FSF-KrasG12D/+ mice can also develop lung tumors, the tumors are low grade (Young et al., 2011). KrasG12D/+ lung tumors have an average tumor volume doubling time of approximately 35 days (Oliver et al., 2010). By contrast, lung tumors from KPFL mice had a doubling time of approximately 2 weeks (Fig. 3E,F), which was similar to that of lung tumors generated in KPFL mice following Ad-Cre infection (Kirsch et al., 2010; Oliver et al., 2010).

**DISCUSSION**

Human cancers develop in a complex environment composed of blood vessels, fibroblasts and immune cells. The tumor microenvironment has been shown to contribute to all of the hallmarks of cancer (Hanahan and Weinberg, 2011). Primary mouse models of cancer driven by site-specific recombinases develop within the native microenvironment in immunocompetent mice. A number of studies have demonstrated that genetically engineered mouse models could more accurately recapitulate the tumor stroma and microenvironment of human cancer than xenograft models in immunocompromised mice (Olive et al., 2009; Graves et al., 2010; Maity and Koumenis, 2010). In addition, the response of these primary mouse cancer models to conventional and novel therapies has been shown to closely model the response of human cancers in clinical trials (Singh et al., 2010). Dual recombinase technology will enable further examination of the role of the tumor microenvironment in primary tumors (Fig. 4).

Interestingly, a mouse with deletion of the first six exons of p53 was previously reported to express a truncated RNA capable of coding for the C-terminus of the p53 protein that can be detected only after in vitro translation (Tyner et al., 2002). When combined with one wild-type copy of p53 (p53m/+), this mutant allele (m) leads to a gain of p53 function and early-aging associated phenotypes. However, p53m/m mice phenocopy p53−/− mice. We have looked for a truncated p53 protein in p53FRT/− MEFs infected with Ad-FlpO by western blot by using an antibody against the full-length p53 protein. However, we were unable to detect the truncated protein. This might be due to lack of antibody specificity for the C-terminal epitope or because the level of truncated p53 protein in mouse cells is below the detection limit by western blot, similar to that found in p53m/m mice (Tyner et al., 2002). Regardless of whether the truncated p53 protein is expressed, this recombinent allele lacks the ability to suppress tumor development. In addition, a FRT-flanked p53 mouse has been generated by Exelixis. In this mouse, exons 2-10 are flanked by FRT sites. This allele has been used to generate primary mouse lung tumors in combination with the LSL-KrasG12D allele using an adenovirus expressing both Cre and Flp (Singh et al., 2010). However, to our knowledge, our study is the first to use a p53FRT allele to generate lung adenocarcinomas and soft-tissue sarcomas with Flp-mediated recombination alone.

In summary, we have generated a conditional p53 mouse allele regulated by Flp recombinase. When used in combination with the
**METHODS**

**Mouse strains**

All animal procedures for this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Duke University. *FSF-Kras^G12D^* and *p53^-/-^* mice were kindly provided by Tyler Jacks and were described previously (Jacks et al., 1994; Young et al., 2011). *Meox2-Cre* mice were obtained from Jackson Laboratory (Tallquist and Soriano, 2000).

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The *FSF-Kras^G12D^* allele, the *p53^FRT^* mouse can be used to generate primary sarcomas and lung cancers with Flp recombinase. When combined with the growing number of *loxP*-flanked alleles and tissue-specific Cre drivers, this novel mouse model will enable dual recombinase technology to be employed to investigate the mechanism by which stromal cells contribute to cancer development, progression, and response to therapy.

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**Construction of the *p53^FRT-neo^* targeting vector and generation of mice**

Genomic DNA of the mouse *p53* gene was provided by Tyler Jacks and was used to make the targeting construct. We used an *Ndel* site in intron 1 and a *BamHI* site in intron 6 to insert a single *FRT* site before exon 2 and exon 7, respectively. A *loxP*-flanked PGK-*neo* cassette was inserted into intron 6 before the *FRT* site as a positive selectable marker and a PGK-**DTA** cassette was inserted into the targeting vector after exon 11 as a negative marker. The targeting vector was linearized with *PacI* and electroporated into ES cells using standard conditions. Diagnostic PCR was performed to identify ES clones with successful homologous recombination using primers flanking the PGK-*neo* cassette (sense primer 5’-TGCTCTGCGGAGGAAGTT-3’ and anti-sense primer 5’-CACCAGACAGGTTCTG-3’) and primers flanking the 5’ *FRT* site (sense 5’-GGAGACAGGTTCTG-3’ and anti-sense 5’-CTTCTAACAGCAAGGCAG-3’). Two out of 800 ES cells were positive at both sites. Genomic DNA of these two clones was digested by *ScaI* and successful homologous recombination of the *p53^FRT-neo^* allele was determined by Southern blot using probes binding to either *neo* or exon 11. One clone was used to derive male germline *p53^FRT-neo^* chimeras, which were bred with *Meox2-Cre* females to delete the floxed *neo* in the germ line. Deletion of *neo* was verified by PCR using primers flanking the recombinated *loxP* site and 3’ *FRT* site: sense 5’-CTGACGCCACCCGAGTCTG-3’ and anti-sense 5’-CTTGCGG-3’.

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**Fig. 3. Generation of primary cancers in *FSF-Kras^G12D^;/p53^FRT^* mice by Flp recombinase.** (A) Intramuscular injection of Ad-FlpO into *FSF-Kras^G12D^;/p53^FRT^* mice caused soft tissue sarcomas at the site of injection in the lower extremity 2 months post-injection. (B) Sections of the sarcomas were stained with hematoxylin and eosin, and show high-grade spindle cells. (C) Intranasal infection of Ad-FlpO into *FSF-Kras^G12D^;/p53^FRT^* mice caused lung adenocarcinomas 2 months post-infection. (D) Higher magnification of lung tumors demonstrates pleomorphic nuclei, prominent nucleoli and nuclear molding characteristic of high-grade adenocarcinoma. Scale bars: 100μm. (E) Relative tumor volume measured by micro-CT of lung cancers from *FSF-Kras^G12D^;/p53^FRT^* mice. A total of three tumors were contoured from two mice. Micro-CT scans were performed at 8 weeks and 10 weeks after infection with Ad-FlpO. (F) Doubling time in days for lung cancers in *FSF-Kras^G12D^;/p53^FRT^* mice. Data are presented as mean ± s.e.m.

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**Fig. 4. Rationale for dual recombinase technology.** (A) Ad-Cre infection generates tumors by expressing Cre recombinase in tumor-initiating cells. However, in this model, Cre recombinase cannot be utilized to selectively recombine additional floxed alleles in stromal cells. (B) Dual recombinase technology combines Ad-Flp infection with a tissue-specific Cre driver that recombines floxed alleles in stromal cells. For example, Tie2-Cre recombines floxed alleles in endothelial cells and macrophages. Tumors can be initiated by Flp-mediated activation of oncogenes and deletion of floted tumor suppressor genes. This approach enables recombination of floxed alleles in stromal cells expressing Cre recombinase only.
PCR genotyping
Tissue genotyping and amplification conditions were as follows: p53<sup>FRT</sup> allele, 5′ FRT sense primer 5′-CAAGAGAACTGTGCCCT-AAGAG-3′ and 5′ FRT anti-sense primer 5′-CTTTCTAA-CAGCAGAAAGCAGC-3′, cycling at 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 40 seconds; recombinant p53<sup>L2-6</sup> allele, 5′ FRT sense primer 5′-CAAGAGAACTGTGCCCTAAGAG-3′ and 3′ FRT anti-sense primer 5′-ACTCTGGAACAGAAACAGGCAGA-3′, cycling at 94°C for 30 seconds, 55°C for 45 seconds, 72°C for 2 minutes.

In vitro recombination in MEFs
Primary MEFs were isolated from pregnant female mice between 12.5 and 14.5 days of gestation. PCR genotyping was performed on DNA isolated from the embryo heads. MEFs were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 2-mercaptoethanol, glutamine and non-essential amino acids. Passage 4 MEFs were infected with 100 multiplicity of infection (MOI) Ad5CMVeGFP or Ad5CMVFlpO (University of Iowa Gene Transfer Vector Core, Iowa City, IA) in MEF media for 24 hours. Recombination was confirmed by PCR.

Immunoblotting
Induction of p53 and p21 in MEFs was achieved by treatment of passage 5 MEFs with 0.5 μg/ml doxorubicin (Sigma-Aldrich, St Louis, MO) for 18 hours. MEFs were washed with PBS and protein was harvested with RIPA buffer (Sigma-Aldrich, St Louis, MO). A total of 25 μg of total protein were loaded for electrophoresis into a 10% sodium dodecyl sulfate polyacrylamide gels. Separated proteins were transferred to a PVDF membrane. Membranes were blocked with 5% nonfat dry milk in TBS with 0.1% Tween 20. Protein levels were detected using antibodies against p53 (IMX25 clone, Vector Labs, Burlingame, CA; final dilution 1:1000), p21 (F-5 clone, Santa Cruz, Santa Cruz, CA; final dilution 1:1000) and actin (C4 clone, BD Biosciences, Franklin Lakes, NJ; final dilution 1:5000) followed by secondary goat anti-mouse IgG horseradish-peroxidase-conjugated antibody (Invitrogen, Carlsbad, CA; final dilution 1:2000). Bands were visualized using ECL. Plus western blotting detection reagents (Amersham, Pittsburgh, PA).

3T3 senescence
Primary MEFs were isolated from pregnant female mice at 300,000 cells per 6-cm dish and allowed to grow for 3 days. Cells were harvested, counted using a Z1 Coulter particle counter (Beckman Coulter, Brea, CA) and re-plated at 300,000 cells per 6-cm dish for a total of ten passages. DNA content of early and late passage MEFs was measured after staining with 50 μg/ml propidium iodide by flow cytometry using a FACSCanto analyzer (Becton Dickinson, UK).

Generation of primary tumors
Primary soft-tissue sarcomas and lung adenocarcinomas were generated as described previously by infecting Kp<sup>FRT</sup> mice with IM or IN Ad5CMVFipO (Kirsch et al., 2007; DuPage et al., 2009). Briefly, 25 μl Ad5CMVFipO (6×10<sup>10</sup> PFU/ml) was incubated in 600 μl minimum essential media (Sigma-Aldrich, St Louis, MO) with 3 μl 2 M CaCl<sub>2</sub> (Sigma-Aldrich, St Louis, MO) for 15 minutes to form calcium phosphate precipitates. A total of 50 μl precipitated virus per mouse was injected intramuscularly to generate sarcomas, or 30 μl precipitated virus followed by 30 μl media was administered via IN inhalation to initiate lung tumors.

Micro-CT scans
The computerized tomography (CT) data were acquired by X-RAD 225Cx (Precision X-ray, North Branford, CT) using 40 kVp X-rays with 2.5 mA current. Tumor volumes were calculated with Amira image analysis software (TGS, San Diego, CA).

ACKNOWLEDGEMENTS
We thank Ute Hochgeschwender and the Duke Neurotransgenic Laboratory for assistance with ES cell targeting and blastocyst injection. The Duke Neurotransgenic Laboratory is supported, in part, with funding from NIH-NINDS Center Core Grant SP30NS061789. We thank Tyler Jacks for providing p53<sup>FRT-Loxp</sup>-flanked alleles and tissue-specific Cre drivers to assess the effects of modifying specific genes in tumor stromal cells. Thus, this dual recombinase system will be a powerful tool for investigating the mechanisms by which the tumor microenvironment contributes to cancer development, progression and response to therapy.

RESOURCE IMPACT
Background
Cre recombinase has been used to develop many mouse models of primary cancer through enabling deletion of tumor suppressors and activation of oncogenes in somatic tissues of mice in a spatially and temporally restricted manner. The primary tumors in these models develop in the native microenvironment in immunocompetent mice and have been shown to faithfully mimic human tumorogenesis and responses to therapy. Despite the availability of other highly efficient recombinase-based approaches, mouse models of cancer initiated by recombinases have generally used Cre to mutate genes in tumor parenchymal cells, ruling out the possibility that Cre can be used to genetically modify tumor stromal cells.

Results
To develop a dual-recombinase system that would enable modification of different genes in tumor parenchymal cells and stromal cells, the authors of this study generated a FRT-flanked allele of the tumor suppressor p53 that can be deleted by Flp recombinase. They show that efficient deletion of p53 by FlpO impairs the ability of cells to respond to genotoxic stress and leads to genetic instability. To validate their system in vivo, the authors crossed mice carrying the FRT-flanked p53 allele with mice carrying a Flp-inducible allele of oncogenic Kras to create compound conditional mutant mice. Injection of an adenovirus expressing FlpO recombinase initiated primary soft-tissue sarcomas and lung adenocarcinomas after as little as 8 weeks.

Implications and future directions
This new mouse model, in which primary tumors can be induced using FlpO recombinase, can be used in combination with the growing number of loxP-flanked alleles and tissue-specific Cre drivers to assess the effects of modifying specific genes in tumor stromal cells. Thus, this dual recombinase system will be a powerful tool for investigating the mechanisms by which the tumor microenvironment contributes to cancer development, progression and response to therapy.


