Recombinase-based conditional and reversible gene regulation via XTR alleles

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Synthetic biological tools that enable precise regulation of gene function within in vivo systems have enormous potential to discern gene function in diverse physiological settings. Here we report the development and characterization of a synthetic gene switch that, when targeted in the mouse germline, enables conditional inactivation, reports gene expression and allows inducible restoration of the targeted gene. Gene inactivation and reporter expression is achieved through Cre-mediated stable inversion of an integrated gene-trap reporter, whereas inducible gene restoration is afforded by Flp-dependent deletion of the inverted gene trap. We validate our approach by targeting the p53 and Rb genes and establishing cell line and in vivo cancer model systems, to study the impact of p53 or Rb inactivation and restoration. We term this allele system XTR, to denote each of the allelic states and the associated expression patterns of the targeted gene: eXpressed (XTR), Trapped (TR) and Restored (R).
Identifying causal relationships between gene function and the physiological programmes they control is a central goal of biological research and an unmet challenge in cancer. Although loss-of-function mutations in tumour suppressor genes are the most common type of genetic alteration in cancer, identifying how these genes function within physiologically relevant in vivo settings remains difficult. Restoration of tumour suppressor gene function has the potential to identify relevant programmes of tumour suppression in physiologically diverse settings. This strategy rests on the premise that latent tumour suppressive programmes are poised to react to the reintroduction of tumour suppressor genes, and that the ensuing changes they orchestrate will expose relevant mechanisms of tumour suppression. In addition, experimental restoration of tumour suppressor genes highlights the potential of future therapies aimed at restoring tumour suppressive pathways to treat cancer. Several different approaches have been used to restore endogenous gene expression in vivo and these have identified tumour suppression programmes that are tissue and context specific.1–6. Despite the power of these approaches, their widespread application to in vivo biological systems has been constrained by multiple technological hurdles and limitations inherent to each method (see Discussion).

Conditional approaches to inactivate gene expression in the mouse commonly rely on expression of specialized site-specific recombinases such as Cre and Flp. These recombinases facilitate deletion of DNA sequences that are flanked by similarly oriented DNA elements called loxP or FRT, respectively. Cre and Flp can also facilitate the reversible inversion of DNA sequences that intervene inversely oriented loxP or FRT sites. However, the inherent reversibility of the inversion reaction is often problematic due to the mosaic pattern of DNA rearrangements that result. To drive reproducible and stable DNA inversions, mutant loxP and FRT sites have been used to facilitate the permanent inversion of DNA sequences containing gene traps, to create conditional alleles in mouse embryonic stem (ES) cells and adult mice.2–10. Here we developed a synthetic gene switch, XTR, that uses mutant loxP sites to invert a synthetic fluorescent reporter trap element to drive conditional inactivation of endogenous genes. In addition, we engineered the capability to restore accurate endogenous gene expression via Flp-dependent deletion of the synthetic gene switch in a temporally controlled manner. Designed for broad use and compatibility with established recombinase-based tools, XTR alleles provide a powerful method to establish causal relationships between genes and the greater physiological programmes they regulate in specific contexts.

Results

XTR allele design. Our goal was to develop a single unifying allelic system that works in concert with existing genetically engineered mouse models of human cancer using the site-specific DNA recombinases Cre and Flp, and would enable temporally controlled gene inactivation followed by accurate and inducible gene restoration (Fig. 1a). To take advantage of the large array of methods to deliver Cre and the diverse model systems that rely on Cre-driven cancer initiation, we developed a ‘double-floxed’ gene trap that can be stably inverted by Cre, to conditionally inactivate virtually any gene of interest. The gene trap consists of a strong adenoviral 40 splice acceptor (SA), followed by the coding sequence for green fluorescent protein (GFP) and the SV40 polyadenylation transcription termination sequence (Fig. 1b). We chose this DNA element because of its small size, ability to report expression from the endogenous host gene once trapped and its proven ability to recapitulate gene knockout phenotypes in cells.11–16. To enable stable inversion of the gene trap we oriented inverted pairs of Lox5171 and Lox2722 sites in an alternating manner such that each pair flanked the gene trap.17. This double-floxed organization results in the permanent inversion of the gene trap after two successive Cre-mediated recombination reactions (Fig. 1b)7,8. In addition, we flanked the entire gene trap with FRT sites to enable deletion of the gene trap by Flp recombinase and restoration of the targeted gene (Fig. 1b). The GFP reporter feature of the XTR system requires in-frame splicing from the upstream exon in instances where XTR is inserted downstream of the translation initiation site of the host gene. To allow general applicability, we developed independent XTR allele targeting vectors with the SA–GFP in each of the three reading frames (Supplementary Fig. 1).

Targeting and validation of XTR alleles at Rb1 and Trp53. To validate this system and generate alleles that will be of widespread use to the cancer biology community, we targeted the XTR cassette to evolutionarily non-conserved regions of the first intron of the Trp53 and Rb1 genes (p53 and Rb hereafter). Correctly, targeted ES cells gave rise to multiple independent mouse lines harbouring XTR alleles (Supplementary Figs 2 and 3). To validate each feature of the XTR system, we established murine embryonic fibroblast (MEF) lines from XTR/XTR, XTR/+ and +/+ littermates for both Rb and p53. MEF lines were sequentially exposed to Cre and then Flp recombinase via adenoviral infection or through the tamoxifen-inducible Cre-ER fusion protein. In both p53XTR/XTR and RbXTR/XTR MEFs, Cre efficiently converted the XTR alleles to the TR conformation and Flp subsequently converted the TR alleles to the R conformation (Fig. 1c,d). A key feature of the XTR system is the ability of TR alleles to report host gene expression patterns through GFP expression, while simultaneously eliminating host gene expression. After exposing p53XTR/XTR or RbXTR/XTR MEF lines to Cre, these cells expressed GFP (Fig. 1e). As designed, the pattern of GFP expression reports the activity of the respective promoters for each gene: p53XTR/TR cells expressed robust levels of GFP after Cre-mediated recombination, whereas RbXTR/TR cells expressed lower yet detectable levels of GFP. Importantly, the induction of GFP in both p53XTR/TR and RbXTR/TR MEFs coincided with a respective loss of p53 and Rb protein expression (Fig. 1f,g). The major goal of the XTR system is to allow a previously inactivated gene to be restored on exposure to Flp recombinase. Infection of p53XTR/TR or RbXTR/TR MEFs with adenoviral FlpO (mammalian codon-optimized Flp) effectively restored p53 and Rb expression to levels indistinguishable from those in p53XTR/XTR or RbXTR/XTR MEF lines (Fig. 1f,g).

MEFs from wild-type mice lose proliferative potential after multiple serial passages and enter into a senescent state.18. Consistent with their normal regulation of p53, p53XTR/XTR MEFs ceased to divide after an initial proliferative phase and entered senescence. To determine whether TR alleles phenocopy null alleles, we converted pre-senescent p53XTR/XTR MEFs to p53XTR/TR MEFs with AdCre. p53XTR/TR MEFs bypassed the proliferative arrest and proliferated indefinitely. Consistent with restoration of p53 in MEFs using other methods, restoration of p53 expression via AdFlpO treatment in immortalized p53XTR/TR MEF cultures completely arrested cell proliferation, demonstrating that sustained inactivation of p53 is required for cellular immortalization of MEFs (Fig. 1h)19. Collectively, these results demonstrate that the XTR allele allows normal p53 regulation, the TR allele is equivalent to null and the R allele restores physiological gene expression.
**RbTR alleles are functionally null.** To assess the ability of RbTR alleles to functionally inactivate Rb, we crossed Meox2-Cre transgenic mice that express Cre in the germline with RbXTR animals, to generate RbTR+/+ mice. As Rb is an essential gene, we would not expect live-born RbTR/+/ mice if the TR allele phenocopied a knockout allele. Indeed, out of 51 pups born through these crosses, zero RbTR/+/ pups were generated, whereas both Rb+/+ and RbTR/+ pups were observed at the expected frequency (Fig. 2a). Analysis of embryonic day 13.5 mice, a time point before the onset of lethality-causing phenotypes, revealed the presence of grossly normal RbTR/TR embryos that expressed GFP. As anticipated, RbTR/TR embryos had twofold higher levels of GFP than RbTR/+ embryos (Fig. 2b). Taken together, these analyses suggest that Rb gene function is lost in RbTR/TR mice.

**p53TR alleles accelerate Myc-driven lymphomagenesis.** Loss of p53 expression is causally associated with tumour progression in human cancers and multiple mouse models exist where loss of p53 exacerbates cancer phenotypes. To determine whether TR alleles could be used to functionally inactivate tumour suppressor genes in diverse tumour models, we crossed the p53XTR allele to

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*Figure 1* | **XTR alleles facilitate Cre-mediated inactivation and subsequent Flp-dependent restoration of endogenous genes.** (a) Cre converts XTR alleles to the TR allele, thereby inactivating gene function. Flp restores gene function by conversion to R. (b) Schematic of the XTR allele. Cre drives irreversible inversion of a double-floxed gene trap consisting of a splice acceptor (SA) enhanced GFP complementary DNA and the polyadenylation transcriptional terminator sequence (pA). Inversion can proceed either through sequential action of Cre on Lox2272 sites then Lox5171 sites (2272 intermediate) or Lox5171 then Lox2272 sites (5171 intermediate). Stable inversion accepts splicing from upstream exons in the host gene, reads out GFP expression and then terminates transcription, leading to functional inactivation of the host gene’s expression. Flp drives deletion of the gene trap (SA-GFP-pA), thereby restoring normal splicing of the host gene. AdCre and AdCre followed by AdFlpO treatment is indicated (c,d). PCR-based detection of Rb (c) and p53 (d) XTR, TR, R and wild-type (+) alleles in MEFs of the indicated genotype. (e) Detection of GFP reporter expression from TR alleles in RbTR/TR and p53TR/TR MEFs by flow cytometry analysis. Representative of ≥3 cell lines. (f) Immunoblot analysis of Rb and GFP expression in RbXTR/TR MEFs treated sequentially with AdCre and/or AdFlpO as indicated. β-Tubulin is a loading control. (g) Immunoblot analysis of p53 and GFP expression in p53XTR/XTR MEFs treated sequentially with AdCre and/or AdFlpO as indicated. Hsp90 is a loading control. (h) 3T3 proliferation assay of p53XTR/XTR MEFs treated sequentially with AdCre (day 3) then AdFlpO (day 21) as indicated. Representative of two p53XTR/XTR cell lines.
three different well-characterized cancer models. The Eμ-Myc transgenic mouse models Burkitt’s type lymphoma, a B-cell malignancy driven by high levels of Myc expression from the immunoglobulin heavy chain enhancer23. Lymphomagenesis in this model is limited by a p53-dependent apoptotic programme where additional Cre-dependent alleles are required for tumour development in p53TR/TR mice after inhalation of AdCre. (j) Representative lungs from KpXTR/XTR and KpXTRfloxed/floxed mice shown by whole-mount bright-field and fluorescent (GFP) microscopy and also haematolxin and eosin (H&E) staining of histological sections. (k,l,m) Comparison of tumour burden (% of lung area), tumour number and tumour grade between KpXTR/XTR and KpXTRfloxed/floxed mice. Scale bars, 25 μm.

p53TR alleles phenocopy p53floxed alleles in cancer models. The XTR system was designed to be compatible with cancer models where additional Cre-dependent alleles are required for tumour development in p53TR/TR mice after inhalation of AdCre.
formation. To assess this potential, we crossed p53XTR mouse to 
KrasLSL-G12D/+ mice, which harbour a conditional oncogenic 
KrasG12D allele whose expression is dependent on exposure to 
Cre.26,27. KrasLSL-G12D/+ and traditional p53flox alleles have been 
used to model several human tumour types including soft tissue 
sarcoma and lung adenocarcinoma.28,29. In each of these models, 
Cre-expressing viruses are delivered directly to the site of tumour 
formation by direct injection into the muscle to generate sarcomas 
or inhalation to generate lung adenocarcinoma. To determine 
whether p53XTR could work in concert with the KrasLSL-G12D/+ 
allele and phenocopy p53flox alleles in these tumour models, we 
initiated tumours in KrasLSL-G12D/+ p53XTR/+(KPXTR/XTR) and 
KrasLSL-G12D/+ p53flox/flox(KPflox/flox) mice and compared the 
frequency of tumour initiation and degree of histological 
progression in models of sarcoma and lung adenocarcinoma 
(Fig. 2e–m). Intramuscular injection of AdCre initiated sarcomas 
in KpXTR/XTR and Kpflx/flox mice with similar penetrance 
and median time to sarcoma onset (Fig. 2f). KpXTR/XTR and 
Kpflx/flox sarcomas were histologically similar, containing 
high-grade spindle cell lesions with atypical nuclei and frequent 
miotic figures, and consistent with conversion to p53TR/+ sarcomas 
caused by KpXTR/XTR mice were GFP positive (Fig. 2g).

Inhalation of AdCre into KpXTR/XTR and Kpflx/flox mice 
resulted in robust lung tumour formation that led to declines in 
survival with similar rates in each cohort. KpXTR/XTR mice had 
tumours with bright GFP signal consistent with conversion to 
p53flox/+ (Fig. 2j). Tumour number, grade and the extent of 
tumour burden in Kpflx/flox and KpXTR/XTR mice were also 
distinguishable (Fig. 2k–m). Taken together, these data 
demonstrate that XTR is a robust method to inactivate tumour 
suppressor gene function in diverse tumour types with similar 
effectiveness to conventional floxed alleles. In addition, the bright 
GFP signal from the p53XTR/+ allele affords a convenient method 
to identify, isolate and track tumour cells.

Discussion
Restoration of tumour suppressor gene function in cancer cells 
in vivo has proven to be a powerful means to identify context-
specific programmes of tumour suppression. However, the 
widely practiced approach of restoring gene function in established 
tumours within their natural setting has been greatly limited by 
previous approaches that are incompatible with specific genes of 
interest or by strategies that require multiple technically 
challenging steps to implementation.1,3,8

We and others have used genetically engineered alleles in 
which a loxp-flanked transcription/translation stop cassette 
(loXP-STOP-loxP; LSL) is inserted into the first intron of a gene 
of interest.1,2. An LSL allele is a null allele until Cre-mediated 
recombination deletes the STOP cassette, thus allowing normal 
expression of the targeted genes. For example, the p53LSL allele is 
a functionally null allele of p53 and p53SL/SL mice develop 
spontaneous lymphomas and sarcomas at the same frequency and 
rate as p53 KO mice. Initially, p53SL/SL mice were used to 
study the consequences of p53 restoration in T-cell lymphomas 
and soft tissue sarcomas that naturally arise in p53-deficient 
mice. Most genetically engineered mouse cancer models rely on 
Cre to activate or inactivate genes of interest, but because LSL 
approaches require Cre for gene restoration they are not 
compatible with these existing models. Because of this major 
limitation, we made use of a lung cancer model (KrasLSL-G12D/+ 
where KrasG12D is activated spontaneously due to a stochastic 
recombination event32). These mice develop lung adenocarcinomas with 100% penetrance at an early age. This afforded 
us the opportunity to generate KrasLSL-G12D/+ p53LSL/SL mice and to 
restore p53 in these early-to-moderate stage lung cancers. 
Performing this restoration in large cohorts of mice was 
frustrating, owing to the mortality associated with the frequent 
and rapid development of sarcomas and lymphomas in p53-deficient mice. Extensive ageing of the mice was not 
possible and we were unable to assess the effects of p53 
restoration on the most clinically relevant advanced stages of 
primary lung tumours and metastases.

The most critical limitation of the LSL system is that LSL alleles 
cause germline deficiency. Thus, LSL alleles cannot be used to 
study genes that are required for embryonic development. 
Unfortunately, the vast majority of tumour suppressor genes are 
embryonic lethal (for example, Rb1, Pten, Apc, Nf1, Nf2, Ptc, Vhl, 
Smad4, Atr, Smarca4, Atrid1a, Snf5, Nkx2-1, Nkx3-1, Tsc1, Tsc2 
and so on), thus leaving very few tumour suppressor genes with 
which to use this system (for example, p53, Cdkn2a and Atm). 
This fact, together with its incompatibility with other Cre-based 
systems, has severely limited the utility of this approach.

Fusion of an ER fragment to proteins may, in some instances, 
allow for tamoxifen-dependent activity of the fused protein. 
In the limited cases where this has proven effective, it has been a
robust method. A p53ERTam knock-in allele has been used to model p53 restoration in Myc-induced lymphomas and in KrasG12D-induced lung adenocarcinomas. Although, unlike the LSL approach, the ER-fusion alleles are compatible with Cre-based cancer models, ER fusions are still limited to non-essential genes, as mice with homozygous knock-in would still be expected to recapitulate the embryonic lethality of null mice. In addition, not all proteins will tolerate carboxy and/or amino-terminal fusions with the ER and there can be concern of unknown alterations in function of the ER fusion protein. Finally, this approach is limited to proteins that carry out their functions in the nucleus, as the mechanism of induction is based on tamoxifen-induced nuclear translocation of the fusion protein.

Regulatable small hairpin RNA (shRNA) is a very different method that overcomes several of the limitations associated with LSL and ER fusion-based approaches. However, the techniques involved are challenging and, to date, the generation of regulatable RNA interference transgenic mice has been employed by few laboratories. This approach has been used to regulate the expression of three tumour suppressor genes (Apc, Pten and p53).
in relevant cancer models\textsuperscript{5,6,3,1,33,34}. To effectuate potent knockdown of the target gene and recreate phenotypes equivalent to null alleles, multiple specialized techniques, mouse strains and ES cell lines are required\textsuperscript{35}. The success of the regulatable shRNA to recapitulate phenotypes associated with gene loss requires the ability to generate a sufficiently potent shRNA. Screening of dozens of shRNAs is therefore required and specialized protocols to ascertain whether an shRNA is likely to be effective as a single-copy integrated transgene is necessary\textsuperscript{35,36}. Despite this, potent shRNAs have been identified that approximate null alleles in certain experimental systems\textsuperscript{3,5,31,37,38}.

Off-target effects associated with shRNA expression are also potentially problematic\textsuperscript{39}. High expression of a heterologous shRNA could obscure biological readouts by knocking down the expression of unintended messenger RNA targets or by overwhelming the RNA interference processing machinery to such an extent that naturally expressed micro RNAs are not overwhelming the RNA interference processing machinery to such an extent that naturally expressed micro RNAs are not overwhelming the RNA interference processing machinery to such an extent that naturally expressed micro RNAs are not overwhelming the RNA interference processing machinery to such an extent that naturally expressed micro RNAs are not overwhelming the RNA interference processing machinery to such an extent that naturally expressed micro RNAs are not overwhelming the RNA interference processing machinery to such an extent that naturally expressed micro RNAs are not overwhelming the RNA interference processing machinery to such an extent that naturally expressed micro RNAs are not overwhelming the RNA interference processing machinery to such an extent that naturally expressed micro RNAs are not.

ShRNA could obscure biological readouts by knocking down the expression of unintended messenger RNA targets or by overwhelming the RNA interference processing machinery to such an extent that naturally expressed micro RNAs are not normally produced\textsuperscript{40–42}. Each of these technical issues may have profound consequences on the biology of cells in question that will have an impact on data interpretation. Currently, regulatable shRNA strategies rely on tet-based systems which limit their functionality within other systems that also utilize tet-regulated transgenes. For example, multiple cancer models rely on tet-inducible oncogenes to drive tumour formation and these models rely on continual expression of the oncogenic driver to maintain the cancer\textsuperscript{43–46}. Although the regulatable shRNA can be easily added into this approach to ascertain the added effect of target gene knockdown, the ability to clearly determine the effect of target gene restoration is not possible due to the simultaneous loss of oncogene expression on doxycyclin removal.

The simplicity and functionality of the XTR allele system offers several significant advantages over existing strategies, to interrogate gene function in the mouse. Similar to conventional approaches to create conditional alleles in the mouse, XTR integration relies on gene targeting in ES cells. Creation of XTR alleles uses the same methods that are standard protocols in academic and commercial ES cell/transgenic mouse facilities and thus requires no specialized technical hurdles. As outlined in Supplementary Fig. 3, generation of XTR alleles requires a neoXTR allele intermediate that necessitates secondary selection of either ES cell clones or plasmids that lost the FRT-Neo-FRT. For p53 and Rb, this was achieved by either electroporation with Flp-expressing plasmids or by crossing with germline Flp-expressing mice\textsuperscript{47}. Further use of the XTR system at other loci will be necessary to determine whether locus-dependent effects exist that could limit this strategy. CRISPR-based approaches to generate conditional alleles by directly injecting zygotes with modified XTR vectors lacking the FRT-Neo-FRT cassette could obviate the need for this step\textsuperscript{38,49}.

XTR combines three separate tools into one discrete genetic element to conditionally inactivate a gene of interest, accurately report host gene expression once inactivated and facilitate precise gene restoration in an inducible manner. Bringing these tools together into one strategy offers unparalleled functionality to a single genetically engineered allele. With the increased functionality to mark gene inactivation and report accurate gene expression levels of the targeted gene, as well as the ability to rescue gene function, XTR is positioned to greatly expand current capabilities to interrogate gene function within \textit{in vivo} systems. Thus far, our experience targeting XTR to p53 and Rb loci suggest a ‘plug and play’ simplicity that abrogates the need for development, testing and optimization that is associated with ER fusion and regulatable shRNA strategies. However, targeting of additional loci will be required to affirm the generalizable nature of the XTR approach with respect to preserving proper host gene regulation and robustness of gene inhibition. Although the XTR system would not be compatible with the few examples of Flp-dependent alleles that exist\textsuperscript{30,31}, its seamless integration into the numerous Cre-based model systems available should facilitate its widespread utility. Similar to most conditional approaches to inactivate gene function in the mouse, XTR alleles require specialized methods or mouse strains to deliver Cre recombinase to specific cell types of interest. Our data suggest that either promoter-specific transgenes or viral based approaches to deliver Cre to tissues of interest is a robust strategy to inactivate genes of interest in the mouse with XTR. Finally, restoration of gene function using XTR alleles requires strategies to regulate Flp activity. As demonstrated, we used a tamoxifen-inducible Rosa26\textsuperscript{FpO-ER} allele that is widely expressed and therefore suitable for a broad range of applications using the XTR system\textsuperscript{30}. However, additional strategies to regulate FlpO may augment the utility of the XTR system in specialized scenarios.

Here we have targeted the XTR cassette to two important tumour suppressor genes to address cancer-relevant questions. However, we envision XTR as a powerful approach to investigate gene function in diverse biological settings to gain important insight into mechanisms at the tissue, cellular or molecular level. In addition, XTR alleles have the potential to model therapeutic interventions in disease settings, where temporally inactivating a putative drug target through the genetic means intrinsic to XTR could predict efficacy or identify unforeseen complications of future therapies. More broadly, the ability to restore gene function using the XTR system offers a major opportunity in conditional genetic methods to facilitate the widespread application of \textit{in vivo} gene restoration approaches.

**Methods**

**Creation of base targeting vectors.** The XTR allele requires targeting to the desired gene loci, to carry out inducible gene trapping via splice acceptance from upstream exons; therefore, pNeoXTR plasmids were created to accept splicing from each of the three reading frames (Supplementary Fig. 1; pNeoXTR f0 (Addgene \#69137), pNeoXTR f1 (Addgene \#69158) and pNeoXTR f2 (Addgene \#69159)). Components were assembled from pl451 (Addgene \#22687), pFLP-F2 (a gift from Patrick Stern) and pSA-GFP-pA1\textsuperscript{11} (gift from Jan Carette). Alternative reading frames were established by Quickchange method (Agilent) following the manufacturer’s instructions. Full sequence of each targeting plasmid is available at Addgene.

Targeting arms were generated by PCR and cloned into base targeting vectors. Amplification of the p53 left arm was accomplished with forward primer 5’-ttggcgcggctcagctactgcttgtaag-3’ and reverse primer 5’-ttgacaacagcaggtttagtaa-3’. Amplification of the p53 right targeting arm was accomplished with forward primer 5’-gggtattaagcagctagctgct-3’and reverse primer 5’-ctcgaccaaaaggtcagtcg-3’. Amplification of the Rb left arm was accomplished with forward primer 5’-caggggcgcgcaacagtagctgacagt-3’ and reverse primer 5’-ccagctgatcctgcgaacagtagctgacagt-3’. Amplification of the Rb right arm was accomplished with forward primer 5’-gggtattaagcagctagctgct-3’ and reverse primer 5’-gggtattaagcagctagctgct-3’.

**ES cell culturing and electroporation.** Targeting constructs were linearized and electroporated independently into F1 C57BL/6 × 129S4 hybrid v6.5 ES cells (gift from Rudolph Jaenisch) using standard conditions. Neomycin (300 \textmu g/mL) G418-resistant colonies were isolated, expanded and screened by PCR and Southern blotting. Correctly targeted clones were either directly injected into C57BL/6J blastocysts or first electroporated with supercoiled pCAGGS-FLP (gift from R. Jaenisch) and plated, to generate subclones. ES cell subclones lacking the NeoR cassette after FLP recombination were then injected into C57BL/6J blastocysts. Both strategies yielded several high-percent chimera and the establishment of the in vivo system.

**Identification of targeted ES clones.** PCR was used to screen for probable targeted clones for both p53 and Rb NeoXTR alleles. Reactions spanning the left targeting arm for p53NeoXTR-targeted clones identified positive clones with primers 5’-ttgcagagcagagctgagttg-3’ and 5’-ttgatggagattgtgagcagtc-3’. These were subsequently screened via Southern blotting. PCR screening strategies for Rb were successful and failed to detect even those subsequently identified by Southern blotting. For Southern blotting, genomic DNA was digested overnight at 37 °C with 20 Units of each restriction enzyme. The next day, another 10 Units were added...
and incubated at 37 °C for an additional 4–6 h. DNA fragments were resolved on 0.7% tris-acetate-EDTA gels at low voltage overnight, stained with ethidium bromide, depurinated, denatured and neutralized before transfer to Hybond X membranes. Ultraviolet cross-linked DNA was hybridized with radioactive probes suspended in Express Hyb solution (Clontech) as per the manufacturer’s instructions. Radiolabelled probes were generated by random primer methods using PrimeIt II Kits (Agilent) and purified with Quick Spin Spin Columns (Roche). DNA probes were generated by PCR of mouse genomic DNA, Rb 5'-probe was amplified with 5’-attaagctctgactcag-3’ and 5’-gcagccgagagcacttg-3’ primers. The 53°-probe was amplified with 5’-atatttggacacctgcggc-3’ and 5’-cagggagacagcctttag-3’ primers. The GFP probe was generated using PSA-GFP-PA as a template with 5’-agggcagggagtggc-3’ and 5’ttctggagacatagccacactg-3’ primers. PCA DNA from ES cell clones following Flpe electroporation was screened by PCR with 5’-cttggagacatagccacactg-3’ and 5’-ggcagaggcagtaaaacagagagc-3’.

Germline deletion of NeoR, p53flox/flox, RbneoXTR/- and Rosa26Fbpl mice were crossed to Rosa26Meox2-Cre mice. RbneoXTR/-; Rosa26Fbpl mice and RbneoXTR/- mice were then crossed to wild-type mice and p53 mice to lost the Rosa26Fbpl allele were screened for retention of XTR and loss of NeoR. See also Supplementary Fig. 3.

Generation and analysis of MEFs. MEFs were generated by timed matings from E13.5 to E15.5 embryos. Adenoviral infections to express Cre or Fp0 recombinases (purchased from the Transfer Vector Core at the University of Pennsylvania) were carried out on sub-confluent cultures using 10^5–10^6 viral particles in 6- to 10-cm dishes. Quantification of GFP in MEFs was performed on an LSR II cytometer (BD Biosciences) at the PennFlow Core. Proliferation assays were carried out in 3T3 dishes. Quantification of GFP in MEFs was performed on an LSR II cytometer (BD Biosciences). Immunoprecipitation was carried out in RIPA buffer with standard techniques using Protein G beads. Actin was detected with a monoclonal antibody (Cell Signaling Technology, #2146 1:5,000), Actin (Sigma, A2066, 1:1,000). Immunoprecipitation was carried out using Protein A/G beads and 4-Hydroxytamoxifen dissolved in ethanol was administered once at the time of cell injection. DNA from ES cell clones following Flpe electroporation was screened by PCR with 5’-cttgaagaagatgacacttatag-3’ and 5’-ggcagaggcagtaaaacagagagc-3’.

Histology and microscopy. Fluorescent signals from tumours were imaged with the IVIS Spectrum (Caliper Life Sciences) or a fluorescence stereo dissection microscope (Leica). Tissues were dissected into 10% Neutral buffered formalin and fixed 16–20 h at 4 °C before being dehydrated in a graded alcohol series. Paraﬃn-embedded histological sections were produced at the Abramson Family Cancer Research Institute Histopathology Core as 4 μm sections before staining. Immunostaining was carried out after citrate-based antigen retrieval with 0.05% antibody (Abcam ab19790; 1:1,000) and ﬂuorescent secondary detection (Thermo Fisher, 7-Chicken Alexa Fluor 594, 1:200). Microscopy was performed on a Leica DMi6000B inverted light and ﬂuorescent microscope.

References
1. Ventura, A. et al. Restoration of p53 function leads to tumour regression in vivo. Nature 445, 661–665 (2007).
2. Feldser, D. M. et al. Stage-specific sensitivity to p53 restoration during lung cancer progression. Nature 468, 572–575 (2010).
3. Martins, C. P., Brown-Swiggart, L. & Evan, G. I. Modeling the therapeutic efﬁcacy of p53 restoration in tumors. Cell 127, 1323–1334 (2006).
4. Jurrutia, M. R. et al. Selective activation of p53-mediated tumour suppression in high-grade tumours. Nature 468, 567–571 (2010).
5. Xue, W. et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature 454, 656–660 (2007).
6. Miething, C. et al. PTEN action in leukaemia dictated by the tissue microenvironment. Nature 510, 402–406 (2014).
7. Schnutgen, F. et al. A direct single-cell algorithm for monitoring Cre-mediated recombination at the cellular level in the mouse. Nat Biotechnol 21, 562–565 (2003).
8. Schnutgen, F. et al. Genomewide production of multipurpose alleles for the functional analysis of the mouse genome. Proc Natl Acad Sci USA 102, 7221–7226 (2005).
9. Stern, P. et al. A system for Cre-regulated RNA interference in vivo. Proc Natl Acad Sci USA 105, 13895–13900 (2008).
10. Economides, A. N. et al. Conditions by inversion provide a universal method for the generation of conditional alleles. Proc Natl Acad Sci USA 110, E1379–E1388 (2013).
11. Carette, J. E. et al. Haploid genetic screens in human cells identify host factors used by pathogens. Science 326, 1231–1235 (2009).
12. Carette, J. E. et al. Ebola virus entry requires the cholesterol transporter Niemann-Pick Cl. Nature 477, 340–341 (2011).
13. Lee, C. C., Carette, J. E., Brummelkamp, T. R. & Ploegh, H. L. A reporter screen in a human haploid cell line identifies CYLD as a constitutive inhibitor of NF-kappaB. PLoS ONE 6, e17317 (2011).
14. Jae, L. T. et al. Deciphering the glycosylome of dystroglycanopathies using haploid cell lines for lassa virus entry. Science 340, 479–483 (2013).
15. Reiling, J. H. et al. A haploid genetic screen identifies the major facilitator domain containing 2A (MFSD2A) transporter as a key mediator in the response to tunicamycin. Proc Natl Acad Sci USA 108, 11756–11765 (2011).
16. Reiling, J. H. et al. CREB3-ARF4 signalling pathway mediates the response to Golgi stress and susceptibility to pathogens. Nat Cell Biol 15, 1473–1485 (2013).
17. Chiu, S. H. et al. A conditional system to specifically link disruption of protein-coding function with reporter expression in mice. Cell Rep 7, 2078–2084 (2016).
18. Sherr, C. J. & DePinho, R. A. Cellular senescence: mitotic clock or culture emergency? Cell 102, 1053–1056 (2000).
19. Christophorou, M. A. et al. Temporal dissection of p53 function in vitro and in vivo. Nat Genet. 37, 718–726 (2005).
20. Tallquist, M. D. & Soriano, P. Epiblast-restricted Cre expression in MORE mice: a tool to distinguish embryonic vs. extra-embryonic gene function. Genesis 46, 113–115 (2000).
21. Jacks, T. et al. Effects of an Rb mutation in the mouse. Nature 359, 295–300 (1992).
22. Lee, E. Y. et al. Mice deﬁcient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature 359, 288–294 (1992).
23. Adams, J. M. & Cory, S. Myc oncogene activation in B and T lymphoid tumours. Proc. R. Soc. Lond. B. Biol. Sci. 226, 59–72 (1985).
24. Schmitt, C. A., McCurrach, M. E., de Stanchina, E., Wallace-Brodeur, R. R. & Ploegh, H. L. A reporter screen for lassa virus entry. Science 340, 479–483 (2013).
25. Christophorou, M. A. et al. Temporal dissection of p53 tumour suppressor functions in vivo. Cancer Cell 15, 289–292 (2009).
26. Jackson, E. L. et al. Analysis of lung tumour initiation and progression using conditional expression of oncogenic K-ras. Genes Dev 15, 3243–3248 (2001).
27. Tuveson, D. A. et al. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. Cancer Cell 5, 375–387 (2004).
28. Jackson, E. L. et al. The differential effects of mutant p53 alleles on advanced murine lung cancer. Cancer Res. 65, 10280–10288 (2005).
29. Kirsch, D. G. et al. A spatially and temporally restricted mouse model of soft tissue sarcoma. Nat Med. 13, 992–997 (2007).
30. Lao, Z., Raju, G. P., Bai, C. B. & Joyner, A. L. MASTR: a technique for mosaic mutant analysis with spatial and temporal control of recombination using conditionally ﬂoxed alleles in mice. Cell 182, 386–396 (2012).
31. Premiersruit, P. K. et al. A rapid and scalable system for studying gene function in mice using conditional RNA interference. Cell 145, 145–158 (2011).
32. Johnson, L. et al. Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. Nature 410, 1111–1116 (2001).
33. Dickins, R. A. et al. Tissue-specific and reversible RNA interference in transgenic mice. Nat. Genet. 39, 914–921 (2007).
34. Dow, L. E. et al. Apc restoration promotes cellular differentiation and reestablishes crypt homeostasis in colorectal cancer. Cell 161, 1539–1552 (2015).
35. Dow, L. E. et al. A pipeline for the generation of shRNA transgenic mice. Nat. Protoc. 7, 374–393 (2012).
36. Fellmann, C. et al. Functional identification of optimized RNAi triggers using a massively parallel sensor assay. Mol. Cell 41, 733–746 (2011).
37. Dickins, R. A. et al. Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. Nat. Genet. 37, 1289–1295 (2005).
38. Hemann, M. T. et al. An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes in vivo. Nat. Genet. 33, 396–400 (2003).
39. Kaelin, Jr. W. G. Molecular biology. Use and abuse of RNAi to study mammalian gene function. Science 337, 421–422 (2012).
40. Jackson, A. L. et al. Widespread siRNA “off-target” transcript silencing mediated by seed region sequence complementarity. RNA 12, 1179–1187 (2006).
41. Echeverri, C. J. et al. Minimizing the risk of reporting false positives in large-scale RNAi screens. Nat. Methods 3, 777–779 (2006).
42. Grimm, D. et al. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441, 537–541 (2006).
43. Fisher, G. H. et al. Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. Genes Dev. 15, 3249–3262 (2001).
44. Politi, K. et al. Lung adenocarcinomas induced in mice by mutant EGFR receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors. Genes Dev. 20, 1496–1510 (2006).
45. Ji, H. et al. The impact of human EGFR kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies. Cancer Cell 9, 485–495 (2006).
46. Moody, S. E. et al. Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. Cancer Cell 2, 451–461 (2002).
47. Farley, F. W., Soriano, P., Steffen, L. S. & Dymecki, S. M. Widespread recombinase expression using FLPeR (flipper) mice. Genesis 28, 106–110 (2000).
48. Yang, H. et al. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 154, 1370–1379 (2013).
49. Yang, H., Wang, H. & Jaenisch, R. Generating genetically modified mice using CRISPR/Cas-mediated genome engineering. Nat. Protoc. 9, 1956–1968 (2014).
50. Young, N. P. & Jacks, T. Tissue-specific p19Arf regulation dictates the response to oncogenic K-ras. Proc. Natl Acad. Sci. USA 107, 10184–10189 (2010).
51. Lee, C. L. et al. Generation of primary tumors with Flp recombinase in FRT-flanked p53 mice. Dis. Model Mech. 5, 397–402 (2012).
52. Jonkers, J. et al. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat. Genet. 29, 418–425 (2001).
53. DuPage, M., Dooley, A. L. & Jacks, T. Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. Nat. Protoc. 4, 1064–1072 (2009).

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Author contributions

D.M.F. conceived the project and designed the XTR system. D.M.F. and C.R.C. cloned XTR targeting vectors. A.A.B. performed ES cell work. D.M.F. and S.E.T. established mouse lines. C.R.O., S.E.T., Y.Y., M.C., B.L. and D.M.F. performed experiments. M.M.W. and T.J. provided conceptual advice. C.R.O. and D.M.F. wrote the manuscript with comments from all authors.

Additional information

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