Inducible in vivo genome editing with CRISPR-Cas9

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CRISPR-Cas9-based genome editing enables the rapid genetic manipulation of any genomic locus without the need for gene targeting by homologous recombination. Here we describe a conditional transgenic approach that allows temporal control of CRISPR-Cas9 activity for inducible genome editing in adult mice. We show that doxycycline-regulated Cas9 induction enables widespread gene disruption in multiple tissues and that limiting the duration of Cas9 expression or using a Cas9D10A (Cas9n) variant can regulate the frequency and size of target gene modifications, respectively. Further, we show that this inducible CRISPR (iCRISPR) system can be used effectively to create biallelic mutation in multiple target loci and, thus, provides a flexible and fast platform to study loss-of-function phenotypes in vivo.

The type II bacterial CRISPR (clustered, regularly interspaced, short palindromic repeats)-Cas9 (CRISPR-associated protein) system can be engineered to induce RNA-directed, double-strand DNA breaks or single-strand ‘nicks’ using a mutated form of Cas9 (Cas9D10A or Cas9n)1–3. CRISPR-Cas9 technology has been used to create heritable changes in the mouse genome, dramatically decreasing the time required to develop genetically engineered mouse models (GEMMs)4–6. However, homozygous germline mutations often result in embryonic lethality or developmental defects and are not tissue specific, limiting the utility of such models for studying gene function in adult tissues. Additionally, although CRISPR-Cas9–directed mutagenesis in zygotes enables rapid generation of compound mutants, genetic mosaicism in the founders and allele segregation limits the ability to produce large cohorts of experimental mice without further intercrossing. Here, we describe a rapid tetracycline (doxycycline)-regulated approach that enables inducible in vivo genetic manipulation of multiple loci from a single transgene.

We and others have previously used a recombinase-mediated cassette exchange approach to deliver tetracycline-inducible cDNAs and short hairpin (sh)RNAs to a defined genomic locus, thus reducing founder effects associated with random integration7,8. As proof-of-principle demonstration, we chose to target the well-characterized adenomatous polyposis coli (Apc) tumor suppressor, as germline loss of Apc is embryonic lethal9, whereas disruption of Apc in the intestine adenomatous polyposis coli (Apc) tumor suppressor, as germline loss directly demonstrates, we chose to target the well-characterized adenomatous polyposis coli (Apc) tumor suppressor, as germline loss of Apc is embryonic lethal9, whereas disruption of Apc in the intestine adenomatous polyposis coli (Apc) tumor suppressor, as germline loss often occurs in APC mutant colon cancer. Analysis of transfected mouse embryonic stem cells (ESCs) using Surveyor assays showed the expected modifications (Supplementary Fig. 1a).

We next cloned each U6-sgRNA cassette into a new ‘col1a1-targeting construct, upstream of TRE3G-regulated GFP-IRES-Cas9’ (c3GIC9; Fig. 1a), which facilitates the generation of constructs carrying multiple U6-sgRNA cassettes in series. As a control for sgRNA expression and Cas9-mediated DNA cleavage, we generated an sgRNA targeting a nongenic region on mouse chromosome 8 (CR8). We produced KH2 ESC clones targeting CR8, Apc and Trp53 and tested for the presence of insertions and deletions (indels) by Surveyor assay. Both GFP induction (linked to Cas9; Fig. 1b) and target modification occurred within 2 d of doxycycline treatment (Fig. 1c) and increased with time (Fig. 1d). In addition, targeted ESC clones carrying both Apc and Trp53 sgRNAs in tandem showed equivalent indel frequency (Fig. 1c), indicating that the presence of two sgRNAs in series does not influence the efficiency of Cas9-mediated cleavage.

To directly assess the capability of our system to induce biallelic modification in multiple genes, we treated two independent c3GIC9-Apc/Trp53 parental ESC clones with doxycycline for 10 d, removed doxycycline and allowed single clones to expand in the absence of Cas9 expression. Of 154 individual ESC clones analyzed by Sanger sequencing, greater than 94% displayed biallelic disruption at a given locus (Fig. 1e). Both alleles showed a frameshift/in-frame indel ratio of close to 2:1 (expected by chance; Fig. 1f), implying that selection for loss-of-function mutations did not account for the high frequency of indels observed. Most importantly, more than 90% of all clones assessed showed biallelic modification at both Apc and Trp53 (Fig. 1g, left) and nearly half of all clones contained frameshift disruptions at each of the four alleles (Fig. 1g, right). These data suggest that the iCRISPR system can be used to induce disruption of multiple targets from a single transgene.

In some cases we identified ESC clones that showed evidence of indels in the absence of doxycycline (see asterisk, Fig. 1c). Of 39 clones analyzed, 13 (33%) displayed some level of off-doxycycline mutagenesis. However, the frequency of ‘off-dox’ mutations did not

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increase with continuous culture\(^{11}\) (Fig. 1d), suggesting they arise due to leaky expression of Cas9 during transfection. Thus, we screened up to six clones for each targeting event, to identify non-mutated clones for mouse production.

CRISPR-Cas9 genome editing can also cause ‘off-target’ mutations\(^{12}\). We identified potential off-target loci (Supplementary Table 1) and assessed their mutation in doxycycline-treated c3GIC9-\(\text{Apc}/\text{Trp53}\) ESCs. Of all off-target predictions for sg-Apc,\(\text{Apc}/\text{Trp53}\) ESCs treated with doxycycline for 10 d, then expanded as individual clones in the absence of doxycycline observed in some clones. (d) Indel frequency at the Apc locus in transgenic c3GIC9-\(\text{Apc}/\text{Trp53}\) ESC culture with or without 1 \(\mu\)g/ml doxycycline as indicated (error bars are s.e.m., \(n = 2\)). (e) Frequency of wild-type, monoallelic or biallelic indels at the Apc and Trp53 loci in c3GIC9-\(\text{Apc}/\text{Trp53}\) ESCs treated with doxycycline for 10 d, then expanded as individual clones in the absence of doxycycline (clone A, \(n = 83\); clone B, \(n = 71\)). (f) Frequency of frameshift (blue) and in-frame mutations (green) at the Apc and Trp53 loci in ESC clones described in e. (g) Pie charts representing the frequency of indels (left) and frameshift mutations (right) at both Apc and Trp53, showing 91–94% of clones carry biallelic indels at both loci and 40–49% carry frameshift mutations in all four alleles.

of founders confirmed that each of the targeted alleles was transmitted in Mendelian ratios suggesting no toxicity during embryonic development (Supplementary Table 2). Still, even small amounts of leaky Cas9 expression would be expected to induce germline mutation events. Sequencing revealed that only 16/166 loci (9.6%) of the off-spring carried heterozygous mutations in CR8, Apc or Trp53. Within each strain, these heterozygous mutations were identical, suggesting they arose from rare events in ESCs that contribute to the germline of the founder, rather than independent, spontaneous events during breeding. Thus, leaky Cas9 expression during ESC clone generation can occasionally induce doxycycline-independent mutagenesis, but it is readily manageable by selecting appropriate F\(_1\) progeny for strain propagation.

Conditional deletion of Apc in the mouse intestine induces hyperplastic proliferation and blocks differentiation, leading to rapid intestinal dysfunction and death\(^{13}\). To determine whether the CRISPR-Cas9 system could recapitulate conditional Apc knockout phenotypes in vivo, we treated 4- to 5-week-old R26-rtTA/c3GCI9 bitransgenic animals with doxycycline and monitored them over time. Surveyor analysis of intestinal villi after 10 d of doxycycline showed expected gene alterations (Supplementary Fig. 2a) and immunostaining confirmed Apc and p53 protein loss (Fig. 2a). Of note, we observed some p53-positive cells and rare instances of increased staining, perhaps reflecting deletion of a region that regulates protein stability (Fig. 2a, arrow). Those animals carrying Apc-targeted sgRNAs showed a 40- to 60-fold induction of the Wnt target genes Axin2 and Lgr5 and a dramatic (130-fold) increase in the proto-oncogene cMyc (Supplementary Fig. 2b). Mirroring the molecular response, histology revealed dramatic hyperproliferation, crypt expansion (K67), a marked reduction in differentiation (Keratin 20) and ectopic production of lysozyme-positive
Paneth cells (Fig. 2b and Supplementary Fig. 2c), as expected following acute deletion of Apc\(^{13}\). Surprisingly, the Cas9n–paired sgRNA approach was as efficient at producing Apc mutations as Cas9 (Supplementary Fig. 2a), promoted a similar or greater increase in Wnt target gene expression (Supplementary Fig. 2b) and produced an identical intestinal phenotype (Fig. 2b).

To more closely evaluate iCRISPR function over time, we exploited the fact that Apc inactivation induces a measurable morphological (spheroid) change in cultured intestinal organoids\(^{14}\). We isolated intestinal crypts from bitransgenic mice and assessed organoid morphology at different times following doxycycline addition. In vitro CRISPR induction for as little as 2 d led to the production of undifferentiated spheroids (Fig. 2c, not shown) whose frequency increased with time (Fig. 2d). D7 doxycycline-treated cultures were virtually 100% spheroid and showed complete loss of full-length Apc protein with a corresponding increase in nonphosphorylated β-catenin, a downstream consequence of Apc loss (Fig. 2e and Supplementary Fig. 3b). Cultures derived from doxycycline-treated c3GIC9-Apc/Trp53 tandem mice showed near complete loss of p53 protein only in c3GIC9-Apc/Trp53 cells. An uncropped, full-length image of the Apc blot is presented in Supplementary Figure 3, showing expression of the truncated protein after CRISPR-Cas9-mediated editing.

Interestingly, p53 expression in organoid cultures and intestine was mosaic, potentially reflecting in-frame deletions that do not cause loss of protein expression or cells that did not modify the Trp53 locus (Fig. 2a,e and Supplementary Fig. 3). To comprehensively assess the frequency of indels produced following Cas9 or Cas9n induction, we sequenced each target locus in gDNA from the intestine and thymus of doxycycline-treated mice at an average depth of 55,000 reads/sample. Both tissues showed a high frequency (50–85%) of target gene modification following 10 d of doxycycline (Fig. 3a), although the sizes and types of indels varied between each locus and tissue (Fig. 3b and Supplementary Fig. 4). Cas9n in combination with paired Apc-targeted sgRNAs showed a bias toward deletion events compared to the single Apc-targeted sgRNA (Supplementary Fig. 4c), and a significant increase in deletion size (Fig. 3b and Supplementary Fig. 4a,d; intestine, \(P = 0.0002\); thymus, \(P < 0.0001\)). Consistent with observations from ESCs, c3GIC9 tandem mice showed concordant mutation of both Apc and Trp53 genes (Fig. 3c, \(R^2 = 0.972\)).

Cas9 mutagenesis can produce both frameshift and in-frame indels that should, in principle, occur at a ratio of 2:1. Because in-frame deletions can produce functional proteins, the relative abundance of frameshift mutations in the tissue can indicate whether loss of gene function has a positive, negative or neutral impact on cell fitness. Consistent with this idea, Apc and Trp53 inactivation produce no selective advantage in ESCs, and both loci showed a predicted 2:1 ratio of frameshift to in-frame deletions (Fig. 1f). Conversely, Apc inactivation produces a substantial selective advantage in the intestine, and intestinal tissue from sgApc/Cas9 mice displayed a strong bias for Apc truncations (97% frameshift mutations; Fig. 3d). In these same animals, p53 inactivation produces little additional advantage in the time frame of analysis\(^{15}\), and, consequently, the frequency of frameshift indels remained near the predicted 2/3 frequency (72%). Remarkably, although disruption of Apc conferred a strong selective advantage and disruption of p53 did not, both loci showed equivalent CRISPR-mediated modification (Fig. 3a), implying that the system can be used to study ‘fitness neutral’ mutations.

As seen in ESCs and organoids, Cas9-induced mutagenesis in vivo was time dependent, as Cas9 expression for 4 d induced significantly fewer mutations and reduced overall disease burden in the intestine (Supplementary Fig. 5; intestine, \(P < 0.0001\); thymus, \(P = 0.03\)). Thus, varying the duration of Cas9 expression by timing doxycycline exposure provides a further level of control for altering gene function in vivo. Moreover, the ability to limit Cas9 expression may reduce toxicity and off-target effects associated with constitutive expression of sgRNA-Cas9 complexes, which can associate with thousands of genomic sequences\(^{16}\).

In our system the spatial induction of mutagenesis is dictated by where the rTA allele, and thus Cas9, is expressed. Hence, strategies...
that restrict rtTA to defined tissues provide a means to produce tissue-specific gene disruption. To explore the potential for Cas9-induced genome editing in other tissues we used hydrodynamic transfection to express rtTA in a restricted and mosaic fashion in the liver of c3GIC9-Apc mice. Histological analyses showed expected cytoplasmic and nuclear accumulation of total and nonphosphorylated β-catenin specifically within GFP-positive hepatocytes (Supplementary Fig. 6). Thus, by controlling the expression of rtTA by either focused delivery (above) or using Cre-dependent systems\(^\text{17}\), the CRISPR system can be adapted to producing tissue-specific and conditional gene deletions \textit{in vivo}. Taken together, our data show that doxycycline-dependent Cas9 or Cas9n induction \textit{in vivo} induces target gene alterations that can recapitulate the effects of traditional conditional knockout approaches.

Recent studies have described the application of CRISPR-Cas9–mediated genome editing \textit{in vivo} using viral delivery or DNA transfection\(^\text{18–21}\). Although they enable rapid CRISPR-based editing to produce gene modifications and chromosomal rearrangements, such methodologies are restricted to those tissues that can be efficiently accessed by exogenous constructs (i.e., liver, lung, brain). From a mouse-modeling standpoint, the iCRISPR platform is conceptually analogous to inducible Cre-based systems (i.e., CreER) though the effectiveness of each is difficult to directly compare. First, unlike the deletions created by loxP-based alleles, CRISPR-Cas9 relies on imprecise DNA repair by nonhomologous end joining (NHEJ), and thus generates a heterogeneous population of genetic mutants. In theory, the frequency of ‘null’ alleles can be increased through the use of sgRNAs targeting the coding region of an essential protein domain and/or using paired sgRNAs to increase deletion size (Fig. 3b). Second, the efficiency of Cre-mediated deletion varies between genomic regions and the size of the recombination event, whereas sgRNA sequence or variation in NHEJ efficiency may influence CRISPR-Cas9 mutagenesis. Importantly, we do not see the iCRISPR approach as replacing Cre/loxP-based conditional knockouts, but rather providing a flexible and complementary alternative to study gene function \textit{in vivo}, either when traditional methods are not applicable (e.g., for creating specific genetic truncations), or sufficiently rapid or scalable.

In R26-rtTA mice, TRE\(^\text{3G}\)-mediated Cas9 induction is strong in the intestine, skin and thymus, and although the TRE\(^\text{3G}\) promoter shows some mosaicism in adult mice, given appropriate rtTA expression (e.g., by direct delivery or alternate transgenic strains), it can induce gene disruption in other tissues, such as the liver (Supplementary Fig. 6). The incorporation of alternate TRE promoters or Cre-dependent TRE-LSL-Cas9 transgenes, analogous to those produced for tissue-specific shRNA expression\(^\text{17}\), should enable inducible mutagenesis in a wider range of tissues, thereby allowing tissue-restricted conditional gene disruption during development or in adults. Furthermore, the iCRISPR platform is adaptable to Cas9-mediated gene regulation via transcriptional repression (CRISPRi) or activation (CRISPRa) using dCas9-KRAB and dCas9-VP64 variants, respectively\(^{22,23}\). These systems offer a powerful approach to simultaneously regulate the expression of multiple genes without mutational heterogeneity, however, they are yet to be fully evaluated \textit{in vivo} and, unlike Cas9, do not induce the permanent genetic changes frequently associated with disease.

Finally, our work suggests that use of paired sgRNAs in combination with Cas9n can drive robust loss-of-function phenotypes \textit{in vivo} while reducing off-target mutagenesis, making it the system of choice for most applications. Although this approach requires the expression of two sgRNAs per target, the c3GIC9n targeting vector can accommodate at least six U6-sgRNA cassettes without loss of on-target efficiency (Supplementary Fig. 7). Of course, increasing the number of genomic targets will also increase the complexity and heterogeneity of mutations, and thus, the analysis of strains that target many genes is likely to be complicated and best suited to cancer studies where such changes are positively selected. Regardless, inducible CRISPR-Cas9–mediated genome editing provides a simple strategy to develop conditional, genetic ‘deletion’ models in less than 6 months, providing a flexible, fast and low-cost platform to study gene function \textit{in vivo}.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*
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AUTHOR CONTRIBUTIONS

L.E.D. conceived of the project, performed and analyzed experiments and wrote the paper. K.P.O.R., A.M., G.L. and D.F.T. performed and analyzed experiments. E.R.K. and N.D.S. developed informatics pipelines and analyzed data. S.W.L. supervised experiments, analyzed data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Isolation and culture of small intestinal organoids. **Isolation.** 15 cm of the proximal small intestine was removed and flushed with cold PBS. After opening longitudinally it was washed in cold PBS until the supernatant was clear. The intestine was then cut into 5 mm pieces and placed into 10 ml cold 5 mM EDTA-PBS and vigorously resuspended using a 10 ml pipette. The supernatant was aspirated and replaced with 10 ml EDTA and placed at 4°C on a benchtop roller for 10 min. This was then repeated for a second time for 30 min. The supernatant was aspirated and then 10 ml of cold PBS was added to the intestine and resuspended with a 10 ml pipette. After collecting this 10 ml fraction of PBS containing crypts, this was repeated and each successive fraction was collected and examined underneath the microscope for the presence of intact intestinal crypts and lack of villi. The 10 ml fraction was then mixed with 10 ml DMEM Basal Media (Advanced DMEM F/12 containing Pen/Strep, Glutamine, B27 (Invitrogen 15750-044), 1 mM Na-ascorbate (Sigma-Aldrich A9165-SG)) containing 10 U/ml DNase I (Roche, 04716728001) and filtered through a 100 μm filter into a BSA-coated (1%) tube. It was then filtered through a 70 μm filter into a BSA-coated (1%) tube and spun at 1,200 r.p.m. for 3 min. The supernatant was aspirated and the cell pellet mixed with 5 ml basal media containing 5% FBS and centrifuged at 650 r.p.m. for 5 min. The purified crypts were then resuspended in basal media and mixed 1:10 with Growth Factor Reduced Matrigel (BD, 354230). 40 μl of the resuspension was plated in a 48-well plate and placed in a 37°C incubator to polymerize for 10 min. 250 μl of small intestinal organoid growth media (basal media containing 50 ng/ml EGF (Invitrogen PMG8043), 100 ng/ml Noggin (Peprotech 250-38), and 500 ng/ml R-spondin (R&D Systems, 3474-RS-050)) was then laid on top of the Matrigel. Where appropriate, media was supplemented with 0.5 μg/ml doxycycline (higher concentrations of doxycycline are toxic to organoids).

**Maintenance.** Media was changed on organoids every 2 d, and they were passaged 1:4 every 5–7 d. To passage, the growth media was removed and the Matrigel was resuspended in cold PBS and transferred to a 15 ml falcon tube. The organoids were mechanically dissociated using a p1000 or a p200 pipette and pipetting 50–100 times. 7 ml of cold PBS was added to the tube and pipetted 20 times to fully wash the cells. The cells were then centrifuged at 1,000 r.p.m. for 5 min and the supernatant was aspirated. They were then resuspended in GFR Matrigel and replated as above. For freezing, after spinning the cells were resuspended in Basal Media containing 10% FBS and 10% DMSO and stored in liquid nitrogen indefinitely.

**Protein analysis.** Small intestine organoids were grown in 300 μl of Matrigel in one well each of a 6-well dish containing 3 ml of growth media for 4 d post-passage, then treated with 125 ng/ml adriamycin for 4 h to induce p33 expression. Organoids were then recovered from the Matrigel using several rinses with cold PBS. Organoid pellets were lysed with Lamelli buffer. Antibodies used for western blot analysis were: anti-APC (1:400, FE9 clone, Millipore #MABC202), anti-nonphosphorylated β-catenin (1:1,000, #8814, Cell Signaling Technology), anti-p33 (1:500, #NCL-p33-505, Novoceastra) and anti-actin-HRP (1:10,000, #ab49900, Abcam).

**RNA isolation and qPCR.** For small intestine samples, villi and crypts were harvested from the proximal duodenum (~1 inch) by scraping with a glass slide. Villi/crypt pellets were lysed with a wand homogenizer in 1 ml of Trizol (Invitrogen, 15596-026) RNA was extracted according to the manufacturer’s instructions and contaminating DNA was removed by DNase treatment for 10 min and column purification (Qiagen RNAeasy). cDNA was prepared from 1 μg total RNA using Taqman reverse transcription kit (Applied Biosystems, #N808-0234) with random hexamers. Quantitative PCR detection was performed using SYBR green reagents (Applied Biosystems) using primers specific to Axin2, Lgr5, Myc (Supplementary Table 3).

**Mutation detection by SURVEYOR assay.** Cas9-induced mutations were detected using the SURVEYOR Mutation Detection Kit (Transgenicom/IDT). Briefly, an approximately 500 bp region surrounding the expected mutation site was PCR-amplified using Hercule II (600675, Agilent Technologies). PCR products were column purified (Qiagen) and subjected to a series of melt-anneling temperature cycles with annealing temperatures gradually

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**ONLINE METHODS**

**Animals.** Production of mice and all treatments described were approved by the Institutional Animal Care and Use Committee (IACUC) at Memorial Sloan Kettering Cancer Center (NY), under protocol number 11-06-012. ESC-derived mice were produced by blastocyst injection by the MSKCC transgenic core facility. Animals were maintained on a mixed C57Bi6/129 and progeny from breeding were genotyped for specific alleles (R26-rtTA and col1A1) using primers and protocols previously described23,24. Doxycycline was administered via food pellets (625 mg/kg) (Harlan Teklad). Animal studies were not blinded. For detection of p53 protein, animals were euthanized 4 h following 6Gy whole body irradiation. For hydrodynamic plasmid delivery 12.5 μg of pCAGs-rtTA3 plasmid was mixed with sterile 0.9% sodium chloride solution. Mice were injected with a total amount of sodium chloride/plasmid mix corresponding to 10% of the body weight into the lateral tail vein within 5–7 s.

**Cloning.** hCas9 (from pX330) or hCas9n (pX335) was cloned into the col1A1-targeting vector downstream of the TRE3G-GFP-IRES. Sequences encoding guide RNAs were cloned into pX330 and pX335 for initial validation1. U6 promoter + guide RNA pairs were PCR amplified (sequences in Supplementary Table 3) and cloned (NsiI/SfiI) into an NsiI site upstream of the TRE3G promoter. The NsiI site re-forms upstream of the U6-sgRNA cassette and can be used for sequential addition of sgRNAs. The col1A1-TRE3G-GFP-IRES-Cas9 (c3GIC9) and c3GIC9n targeting vectors cannot be deposited1.

**ESC targeting.** All ESCs were maintained on irradiated feeders in M15 media containing LIF as previously outlined24. Two days following transfection cells were treated with media containing 150 μg/ml hygromycin and individual surviving clones were picked after 9–10 d of selection. Two days after clones were picked, hygromycin was removed from the media and cells were cultures in standard M15 thereafter. To confirm single copy integration at the col1A1 locus, we first validated expected integration by multiplex col1A1 PCR24, and second, confirmed the presence of a single GFP cassette using the Taqman copy number assay, according to the manufacturer’s instructions (Invitrogen).

**Immunohistochemistry and immunofluorescence.** Tissue, fixed in freshly prepared 4% paraformaldehyde for 24 h, was embedded in paraffin and sectioned by IDEXX RADIL (Columbia, MO). Sections were rehydrated and counterstained with Harris’ hematoxylin. For immunofluorescent stains, secondary antibodies used were: anti-chicken 488 (1:500, DyLight IgG, #ab96947) and anti-rabbit 568 (1:100, anti-rabbit ImmPRESS HRP-conjugated secondary antibodies (1:150, Abcam #15270). For immunohistochemistry, sections were incubated with anti-rabbit ImmPRESS HRP-conjugated secondary antibodies (Vector Laboratories, #MP7401) and chromagen development performed using ImmPact DAB (Vector Laboratories, #SK4105). Stained slides were counterstained with Harris’ hematoxylin. For immunofluorescent stains, secondary antibodies were applied in TBS for 1 h at room temperature in the dark, washed twice with TBS, counterstained for 3 min with DAPI and mounted in ProLong Gold (Life Technologies, #P36930). Images of fluorescent sections stained by means of in situ hybridization and immunohistochemistry were acquired on a Zeiss Axioscope Imager Z.1 using a 10× (Zeiss NA 0.3) or 20× (Zeiss NA 0.17) objective and an ORCA/ER CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). Raw .tiff files were processed using Photoshop CS5 software (Adobe Systems Inc., San Jose, CA) to adjust levels and/or apply false coloring.
lowered in each successive cycle. SURVEYOR nuclease was then added to selectively digest heteroduplex DNA. Digest products were visualized on a 2% agarose gel.

**Sequence analysis.** Sanger. c3GIC9-Apc/Trp53 ESCs clones were treated with doxycycline (1 µg/ml) for 10 d, removed from doxycycline for 2 d, then plated as single cells at low density and allowed to expand over 6 d. Individual ESC clones were picked directly into 15 µl DNA lysis buffer (70 mM Tris pH8.8, 160 mM (NH4)2SO4, 6.5 mM MgCl2, proteinase K (400 µg/ml)) and incubated at 55 °C for 2 h followed by 95 °C for 20 min. Target regions were amplified from 1 µl of crude gDNA using specific PCR primers (Supplementary Table 3) and sequenced by Dye terminator sequencing (Genewiz Inc., South Plainfield, NJ). In the event of nonhomozgyous indel events, overlapping sequences traces were deconvoluted using a ‘CRISPR-Caller’ algorithm (https://github.com/shackett/CRISPR-Caller/blob/master/CRISPR-Caller.R), modified to map to the mouse genome. Any traces that could not be deconvoluted to unambiguously identify two unique alleles (e.g., more than two peaks per position or unmappable regions) were excluded from further analysis.

**MiSeq.** Target regions were amplified from genomic DNA using specific PCR primers (Supplementary Table 3), amplicons were pooled in equimolar amounts and sequencing libraries were prepared using the Kapa Biosystems HTP library Preparation kit (cat# kk8234), strictly following the manufacturer’s instructions. Barcoded libraries were pooled into a 150 bp/150 bp paired-end MiSeq run, using the TruSeq SBS Kit v3 (Illumina). To look for insertion/deletion events, the sequence data (FASTQ files) were mapped to the mouse (MM10) genome using BWA version 0.7.10 (bwa mem -M). The output SAM files were then directly processed to filter for one of the reads in the pair and also filtered for mapq ≥ 50. In this read set we directly scanned the cigar string for either (i) reads that were predicted length (150 bp) or (ii) reads that contained one, and only one insertion (I) or deletion (D) event. 150 bp reads that were counted as WT and for reads containing an indel, we extracted the length of the event and stored a histogram of indel lengths. The final output was a count of the total number of reads that passed filtering broken down in those with and without indels. Due to the relatively high error rate inherent to the sequencing throughout the length of the reads, single-base substitutions were ignored and scored as WT. This may have resulted in a slight overestimation of the frequency of WT reads.

**Off-target predictions.** We identified 20 mer sequences in the mouse genome that showed complete or partial identity to the primary Apc- and Trp53-targeted sgRNAs according to the following criteria: up to 1 mismatch in the 12 bp seed region immediately adjacent to the 3′ NGG PAM sequence, and up to 5 mismatches in the 8 bp region at the 5′ end of the sgRNA. To do this we used the SHRiMP mapper (gmapper-ls) searching for all gapless alignments with 30% alignment score against the full mouse genome. This list was then post-processed to filter matches for the above mismatch criteria. For each predicted region we designed PCR primers surrounding the site and assessed indel formation by Surveyor assay.

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