In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system

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Chromosomal rearrangements have a central role in the pathogenesis of human cancers and often result in the expression of therapeutically actionable gene fusions1. A recently discovered example is a fusion between the genes echinoderm microtubule-associated protein like 4 (EML4) and anaplastic lymphoma kinase (ALK), generated by an inversion on the short arm of chromosome 2: inv(2)(p21p23). The EML4–ALK oncogene is detected in a subset of human non-small cell lung cancers (NSCLC)2 and is clinically relevant because it confers sensitivity to ALK inhibitors3. Despite their importance, modelling such genetic events in mice has proven challenging and requires complex manipulation of the germ line. Here we describe an efficient method to induce specific chromosomal rearrangements in vivo using viral-mediated delivery of the CRISPR/Cas9 system to somatic cells of adult animals. We apply it to generate a mouse model of Eml4–Alk-driven lung cancer. The resulting tumours invariably harbour the Eml4–Alk inversion, express the Eml4–Alk fusion gene, display histopathological and molecular features typical of ALK+ human NSCLCs, and respond to treatment with ALK inhibitors. The general strategy described here substantially expands our ability to model human cancers in mice and potentially in other organisms.

Genetically engineered mouse models of human cancers have proven indispensable to dissect the molecular mechanisms underlying tumorigenesis4 and provide powerful preclinical platforms for studying drug sensitivity5 and resistance6–8. Although many gain- and loss-of-function mutations observed in human cancers can be modelled using current gene-targeting technologies, chromosomal rearrangements leading to oncogenic gene fusions have proven challenging to faithfully recapitulate.

Figure 1 | Induction of Eml4–Alk rearrangement in murine cells using the CRISPR-Cas9 system. a, Schematic of the In(17) involving the Eml4 and Alk loci. Red arrows indicate the sites recognized by the sgRNAs. b, A schematic of the loci before and after the inversion with the location of the primers used (top panel). PCRs were performed on genomic DNA extracted from NIH/3T3 cells transfected with the indicated pX330 constructs (middle panels). The PCR bands were sub-cloned and the sequences of four independent clones and a representative chromatogram are shown in the lower panels. c, Schematic of the Eml4–Alk fusion transcript (top panel). Detection of the Eml4–Alk fusion transcript by RT–PCR on total RNAs extracted from NIH/3T3 cells transfected with the indicated pX330 constructs (bottom left panel). Sequence of the PCR product showing the correct Eml4–Alk junction (bottom right panel). d, Schematic of the break-apart interphase FISH strategy. In cells with the Eml4–Alk inversion, the red and green probes become separated, and the green and orange probes become juxtaposed. e, Break-apart interphase FISH assay on a NIH/3T3 clone selected from cells co-transfected with pX330-Eml4 and pX330-Alk. Both wild type (white arrows) and the In(17) Eml4–Alk allele (red arrow) are detected.

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in mice. Ectopic expression of fusion oncoproteins from transgenes is widely used to study their oncogenic properties\(^1\)--\(^3\), though with this approach the fusion protein is invariably expressed at non-physiologic levels and neither the role of reduced dosage of the wild-type alleles nor the contribution of the reciprocal product of the translocation can be examined. Strategies that express the fusion transcript from the endogenous locus of the S' element\(^1\) only partially address these limitations, whereas approaches that engineer \(loxP\) sites at each breakpoint and produce rearrangements in the presence of Cre recombinase\(^4\)--\(^6\) are laborious and have limited applications. Novel genome-editing technologies provide a potentially more flexible strategy to produce precise genomic changes including oncogenic chromosomal rearrangements\(^7\)--\(^10\), but they have not yet been adapted to model such rearrangements \textit{in vivo}.

In the mouse genome, \(Eml4\) and \(Alk\) are located on chromosome 17, approximately 11 megabases (Mb) apart, in a region that is syntenic to human chromosome 2 (p21–p23) (Fig. 1a). We attempted to model the most common \(EML4–ALK\) variant in human NSCLCs\(^1\) by introducing concomitant double-strand DNA breaks at intron 14 of \(Eml4\) (which corresponds to intron 13 of \(EML4\)) and at intron 19 of \(Alk\) (Fig. 1a, b and Extended Data Fig. 1). To induce the DNA breaks we chose the CRISPR system\(^2\) because it only requires co-expression of Cas9 and an appropriately designed single-guide RNA molecule (sgRNA)\(^3\).

We cloned sgRNAs targeting the \(Eml4\) and \(Alk\) sites into the Cas9-expressing plasmid pX330 (ref. 24) and co-transfected the resulting constructs in NIH/3T3. PCR analysis demonstrated the induction of the \(Eml4–Alk\) inversion and of a large deletion of the region between the two cut sites in the transfected cell population (Fig. 1b). The presence of the desired \(Eml4–Alk\) inversion was confirmed by sequencing the corresponding \(Eml4–Alk\) fusion transcript (Fig. 1c) and directly visualized by interphase FISH (Fig. 1d, e). Using a similar strategy, we also modelled the \(Npm1–Alk\) rearrangement, a reciprocal chromosomal translocation commonly observed in anaplastic large cell lymphomas\(^3\) (Extended Data Fig. 2). These results confirm that the CRISPR system can be adapted to engineer large deletions, inversions, and chromosomal translocations in eukaryotic cells.

Although appropriate for cell-based experiments, expression of two sgRNAs from separate constructs would be impractical \textit{in vivo}. We therefore engineered plasmids to simultaneously express Cas9 and two distinct sgRNAs from tandem U6 promoters (Extended Data Fig. 3a). Their transfection in NIH/3T3 cells resulted in comparable levels of the two

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**Figure 2** | Intratracheal delivery of Ad-EA leads to lung cancer formation in mice. a, Haematoxylin–eosin staining of lungs from mice at the indicated times after intratracheal instillation of Ad-EA. b, Representative \(\mu\)CT scans (top) and macroscopic appearance (bottom) of lungs from mice at 8 weeks post-infection with Ad-Cre or Ad-EA. Numerous neoplastic lesions are evident in the Ad-EA-infected lung. c, Representative immunostainings of Ad-EA-induced lung tumours with the indicated antibodies. d–j, Tumour architecture and cytology of Ad-EA-induced tumours. Representative micrographs showing: papillary (d) or acinar (e) tumours, lesions originating in proximity of intrabronchial hyperplasia (f), atypical adenomatous hyperplasia (g), mild to moderate nuclear atypia (h, top and bottom images), cells with large cytoplasmic vacuole and eccentric nuclei (i, top and bottom images), and PAS-positive tumours (j, top and bottom images).
sgRNAs, efficient cleavage of the targeted sites, and accumulation of the Eml4–Alk inversion (Extended Data Fig. 3b–d).

To deliver Cas9 and sgRNAs targeting the Alk and Eml4 loci to the lungs of adult mice, we next transferred the dual sgRNA/Cas9 cassette into an adenoviral shuttle vector (Extended Data Fig. 4a) and produced recombinant adenoviruses (hereafter referred to as ‘Ad-EA’). Adenoviruses are ideal because they efficiently infect the lung epithelium of adult mice and do not integrate into the host genome. Infection of mouse embryos with Ad-EA led to the expression of Cas9 and both sgRNAs, and to the rapid generation of the desired Eml4–Alk inversion (Extended Data Fig. 4b–d). We estimated that the Eml4–Alk inversion was produced in approximately 3–4% of infected MEFs (Extended Data Fig. 4e, f).

To induce the Eml4–Alk rearrangement in vivo we next infected a cohort of adult CD1 and C57BL/6J (B6) mice by intratracheal instillation of Ad-EA (n = 52: 22 B6, 30 CD1) or control adenoviruses expressing either the Cre recombinase (Ad-Cre, n = 15: 6 B6, 9 CD1) or Cas9 alone (Ad-Cas9, Fig. 2a–c, n = 19: 9 B6, 10 CD1). An annotated list of all infected animals is provided in Extended Data Table 1.

At two days, and at one week post-infection, the lungs appeared histologically normal with no obvious signs of cytoxicity except for the presence of occasional inflammatory infiltrates (Fig. 2a and data not shown). However, one month after Ad-EA infection, the lungs of mice of both strains presented multiple small lesions that upon histopathological examination appeared to be papillary intrabronchiolar epithelial hyperplasia, atypical adenomatous hyperplasia (AAH) or early well-differentiated adenocarcinomas. By 6–8 weeks post-infection, larger tumours were easily detectable by micro-computed tomography (μCT) and macroscopically visible at necropsy (Fig. 2b). At 12–14 weeks post-infection, the lungs of Ad-EA-infected mice invariably contained multiple large lesions histologically classified as lung adenocarcinomas.

In Ad-EA-infected animals, multiple bilateral lung tumours were frequently detected by 4–7 weeks post-infection (n = 23/26 mice), and invariably after 8 weeks post-infection (n = 34). In contrast, Ad-Cre-infected mice remained tumour-free at all time points examined (n = 14 mice, range 4–18 weeks), with the exception of two CD1 mice in each of which we observed a single small adenoma. Analogously, even at the latest time point examined (9 weeks post-infection), none of the Ad-Cas9 infected mice was tumour-free at all time points examined (n = 14 mice, range 4–18 weeks).

Figure 3 | Lung tumours induced by Ad-EA infection harbour the Eml4–Alk inversion. a, b, Bright field images and merge fluorescent images at increasing magnification of break-apart interphase FISH showing the presence of the Eml4–Alk inversion in a tumour from an Ad-EA-infected mouse (8 weeks post-infection) (a) and wild-type configuration of the Eml4 and Alk loci in a control tumour from a conditional K-RasG12D mouse (b). c, Detection of the wild-type Eml4 locus and Eml4–Alk inversion in micro-dissected tumours from Ad-EA-infected mice using a three-primer PCR strategy. d, RNAs extracted from the same tumours shown in c were reverse-transcribed and amplified using a three-primer strategy to detect the Eml4 and Eml4–Alk transcripts. e, RT–PCR detection (left) of the full-length Eml4–Alk complementary DNA (~3.2 kilobases (kb)) in the tumours shown in c. The full-length PCR products were sequenced on both strands. A chromatogram of the Eml4–Alk junction is shown (right). f, Representative immunohistochemistry of Ad-EA-induced lung tumours stained with antibodies against the indicated phospho-proteins. A bar-plot of staining intensity for the indicated phospho-proteins is also shown. Tumours from two mice for each group were scored.

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mice presented lung tumours \((n = 8\) mice\), whereas at same time point all Ad-EA infected mice had developed multiple tumours \((P < 0.0001,\ \text{Fisher's exact test})\). These results indicate that intratracheal delivery of Ad-EA can initiate lung tumorigenesis with high penetrance and low latency, and that this effect cannot be attributed to adenoviral infection or Cas9 expression alone.

All tumours examined were positive for the pneumocyte marker Nkx2-1 \((\text{also known as TTF1})\) and negative for p63 and Sox2, in agreement with the diagnosis of lung adenocarcinoma \((\text{Fig. 2c})\). The tumours were also strongly positive for the alveolar type II marker surfactant protein C \((\text{SpC})\), whereas the Clara cell marker CCSP \((\text{also known as CC10})\) was undetectable. The adenocarcinomas had a papillary or, less frequently, acinar architecture \((\text{Fig. 2a, f})\), and areas of AAH were frequently observed, especially at earlier time points \((\text{Fig. 2g})\). The majority of tumour cells appeared low-grade, with occasional instances of intermediate nuclear atypia with enlarged nuclei and prominent nucleoli \((\text{Fig. 2h, top and bottom images})\). Approximately 20% of tumours contained cells with a large cytoplasmic vacuole and a peripherally located nucleus \((\text{Fig. 2i, top and bottom images})\). These cells are reminiscent of signet ring cells, which are commonly observed in human ALK+ NSCLCs. Approximately 30% of adenocarcinomas displayed areas of intense positivity at the periodic acid-Schiff \((\text{PAS})\) staining \((\text{Fig. 2j, top and bottom images})\).

Interphase FISH analysis demonstrated the presence of a mono- or bi-allelic \(E\text{ml}4–\text{Alk}\) inversion in every Ad-EA-induced tumour examined \((n = 4\) animals\) \((\text{Fig. 3a})\), but not in control K-Ras\(G^{12D}\)-driven tumours \((\text{Fig. 3b})\). We further confirmed the presence of the \(E\text{ml}4–\text{Alk}\) rearrangement and expression of the full-length \(E\text{ml}4–\text{Alk}\) transcript in microdissected tumours by performing genomic PCR and reverse transcription PCR followed by sequencing \((\text{Fig. 3c–e})\).

Activation of the human ALK oncogene via deregulation, translocation, or amplification has been shown to lead to constitutive phosphorylation of ERK, STAT3, and AKT. At 12–14 weeks post-infection, all lung tumours derived from Ad-EA-injected mice showed phosphorylation and nuclear localization of Stat3. Phosphorylation of Akt and Erk1/2 were also frequently, but not invariably, observed \((\text{Fig. 3f})\).

Finally, we examined the sensitivity of Ad-EA-induced lung tumours to crizotinib, a dual ALK/MET inhibitor used in the clinic to treat patients affected by ALK+ NSCLCs. Ten Ad-EA-infected CD1 mice were monitored by \(\mu\text{CT}\) scans starting at 9 weeks post-infection until the appearance of multiple large lung tumours, at which point the animals were randomly assigned to receive a daily dose of crizotinib \((n = 7)\) or vehicle \((n = 3)\) \((\text{Fig. 4a})\). After two weeks of treatment the animals in the crizotinib group displayed complete \((6/7)\) or partial \((1/7)\) tumour regression, as indicated by \(\mu\text{CT}\) scans and confirmed at necropsy, whereas all control animals showed signs of disease progression \((\text{Fig. 4b, c, Extended Data Fig. 5, Extended Data Table 2 and Supplementary Videos 1–10})\). Histological analysis showed that in the crizotinib group the tumours had undergone marked atrophy or were replaced by areas of intense inflammatory necrosis \((\text{Fig. 4d, e})\).

Collectively, these results demonstrate that the CRISPR technology can be adapted to engineer oncogenic chromosomal rearrangements in mice. The new mouse model of \(E\text{ml}4–\text{Alk}\)-driven lung cancer we have generated to validate this approach faithfully recapitulates the molecular and biological properties of human ALK+ NSCLCs, including a marked sensitivity to the ALK-inhibitor crizotinib. This model provides unique opportunities to dissect the molecular mechanisms through which \(E\text{ml}–\text{Alk}\) drives tumour formation, to test the efficacy of targeted therapies, and to investigate the mechanisms of drug resistance \(\text{in vivo}\).

The CRISPR-based strategy described here offers several advantages over germline engineering via transgenesis or homologous recombination.
By inducing the rearrangement in only a subset of somatic cells, the resulting lesions more closely recapitulate the stochastic nature of tumour formation in humans. In addition, by modifying the endogenous loci, expression of the resulting fusion genes is subjected to physiologic transcriptional and post-transcriptional regulation, accurately modeling the reduced dosage of the wild-type alleles and the expression of the reciprocal product of the translocation/inversion. Finally, because our method requires only the generation of an appropriate viral vector and no germline manipulations, it can be readily adapted to model chromosomal rearrangements in other species, including non-human pri- mates, and as such will facilitate the study of species-specific differences in tumour progression and therapy response in vivo.

Despite these key advantages, some caveats of the CRISPR technology must also be considered. The efficiency with which the rearrange- ments are induced is relatively low and is likely to be affected by the distance between the cut sites and their accessibility to Cas9. Although a low efficiency may be desirable when inducing oncogenic rearrangements, it is a concern if the goal is to generate chromosomal rearrangements in the majority of cells. Furthermore, every possible allele combination of the two target loci (indels, inversions, deletions) will be induced by the dual sgRNA/Cas9 system, potentially complicating the interpreta- tion of these studies.

In summary, the general strategy we have developed substantially expands our ability to model cancers driven by chromosomal rearrange- ments and will facilitate the development of pre-clinical models to study the mechanisms of drug resistance and test novel therapies.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS

Plasmids and adenoviral vectors. The pX330 vector expressing Cas9 (Addgene plasmid 42233) was digested with BbsI and ligated to annealed and phosphorylated sgRNA oligonucleotides targeting Eml4, Alk and Npm1. For cloning of tandem U6-sgRNA-Cas9 constructs, the second U6-sgRNA cassette was amplified using primers containing the XbaI and KpnI sites and cloned into the pX330 construct containing the appropriate sgRNA. For Adeno-Eml4-Alk cloning, pX330-Alk-Eml4 vector was modified by adding an XhoI site upstream the first U6 promoter. An EcoRI/Xhol fragment containing the double U6-sgRNA cassette and the Flag-tagged Cas9 was then ligated into the EcoRI/XhoI-digested pacAd5 shuttle vector. NIH/3T3 cells were transfected in 6-well plates with 3 μg of total plasmid DNA per well using lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. To enrich for transfected cells, transfections included 1 μg of a plasmid expressing the Puromycin-resistance gene (pSico) and cells were incubated with 2 μg/ml of puromycin for 2 days. Recombinant adenoviruses were generated by Virquest (Ad-EA and Ad-Cas9) or purchased from the University of Iowa (Ad-Cre). MEFs infections were performed by adding adenovirus (3 × 10^6 plaque-forming units (p.f.u.)) to each well of a 6-well plate.

PCR and RT–PCR analysis. For PCR analysis of genomic DNA, cells were collected in lysis buffer (100 nM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl supplemented with fresh protease K at final concentration of 100 ng/ml). Genomic DNA was extracted with phenol-chloroform-isooamyl alcohol and precipitated in ethanol. The DNA pellet was dried and resuspended in double-distilled water. For RT–PCR, total RNAs were extracted with TRIzol (Life Technologies) following manufacturer’s instructions. cDNAs were prepared using the Superscript III kit, following the manufacturer’s instructions. The primers and the primer pairs used in the various PCR reactions are provided in Extended Data Tables 3 and 4.

Quantification of inversion efficiency in MEFs. We first isolated an NIH/3T3 subclone carrying a mono-allelic Eml4–Alk inversion validated by interphase FISH. Genomic DNA extracted from this clone was mixed with increasing amounts of genomic DNA from parental NIH/3T3 cells to generate a series of standards containing known percentage of Eml4–Alk alleles. The standards and the test samples were then subjected to quantitative PCR (Applied Biosystem) using primers amplifying the Eml4–Alk junction (Eml4-for and Alk-rev, see Extended Data Table 3) or a control gene (miR-17-92-gDNA-for and miR-17-92-gDNA-rev) and the fraction of Eml4-Alk alleles in the test was calculated by plotting the AAG values on the standard curve. qPCR analysis was performed using SYBR Green (Life Technology).

Cell lines. MEFs were generated from E14.5 wild-type embryos following standard procedures. NIH/3T3 were purchased from ATCC.

Mouse husbandry and adenoviral infection. Mice were purchased from The Jackson Laboratory (C57BL/6J) or from Charles River (CD1) and housed in the SPF MSKCC animal facility, where the health status of the colony is constantly monitored by the veterinary staff and by a sentinel program. For adenoviral infection, MSKCC animal facility, where the health status of the colony is constantly monitored by the veterinary staff and by a sentinel program. For adenoviral infection, MSKCC animal facility, where the health status of the colony is constantly monitored by the veterinary staff and by a sentinel program.

Interphase fluorescent in situ hybridization. Interphase FISH experiments were performed and interpreted by the MSKCC cytogenetic core using a 3-colour probe mix designed to detect and discriminate between Alk–Eml4 fusion and other rearrangements of Alk. The probe comprised mouse BAC clones mapping to: 3’ Alk (17qE1.3, RP23-306H20, RP23-397M18 labelled with green dUTP), 5’ Alk (17qE1.3, RP23-12H17, RP23-403F20 labelled with red dUTP), and 5’ Eml4 (17qE4, RP23-193B15 labelled with orange dUTP). Probe labelling, hybridization, washing, and fluorescence detection were done according to standard procedures. Cell line collection and metaphase spreads were prepared according to standard cytogenetics procedures. For NIH/3T3, FISH signals were enumerated in a minimum of 20 metaphases to determine locus specificity, and 100 interphase cells to determine Alk–Eml4 fusion status. Each paraffin section was first scanned under ×100 objective to assess signal pattern and select representative regions for analysis. At least three images per representative region were captured (each image was a compressed stack of 12 z-sections at 0.5 micron intervals). Signal counts were performed on the captured images and a minimum of 50 interphase nuclei was analysed to determine the Alk–Eml4 fusion status. Based on the observed distance between the green (3’ Alk), red (5’ Alk), and orange (5’ Eml4) signal in the negative controls (parental cell line and Ad-Cre-infected cells), interphase cells were classified as normal, Eml4–Alk positive, or other.

Surveyor assay. The genomic region flanking the CRISPR/Cas9 target site was first amplified by PCR. After a cycle of melting and re-annealing to allow heteroduplex formation, the ampolion was digested with the surveyor nuclease (Transgenomic) for 1 h at 42 °C according to the manufacturer’s directions and the digestion products were separated on a 2% agarose gel.

Northern blot analysis. 10 μg of RNA previously extracted with TRIzol (Life Technologies) were run on a 15% denaturing polyacrylamide gel and blotted onto a nitrocellulose membrane for 1 h at 100 V at room temperature. The membranes were then hybridized to radiolabelled oligonucleotides complementary to the Alk (5'-TACAGATAGACATGCCAGGAC), Eml4 (5'-TCTTCTAGTAGACCCCCGACAAA C) sgRNAs, or mU6 (5'-GCAGGGGCGATGCTAATCTTCTCTCCTTGATCGG) dissolved in ExpressHyb (Clontech) at 42 °C overnight. Washes were performed at room temperature in 2x SSC and 0.2 SSC.

Lung processing and antibodies for immunohistochemistry. Lungs were inflated by intratracheal injection of 4% paraformaldehyde (PFA), incubated for 18–24 h in 4% PFA, and then transferred to 70% ethanol for at least 24 h before further processing. The following antibodies were used: phospho-Stat3 (Tyr705, Cell Signaling Technology #9135, 0.1 μg/ml 1); phospho-Erk1/2 (Thr202/Tyr204, Cell Signaling Technology #4370 1 μg/ml 1); phospho-Akt (Ser473, Cell Signaling Technology #4060 1 μg/ml 1); Nck2-1 (Epitomics, EP1584Y 1:1,200); Flag (Sigma, M2 1:1,000); P63 (Santa Cruz H-137) sc8343, 1:1,000); Sox2 (Cell Signaling Technology, C70B1 #3728, 1:1,000); CC10/CCSP (Millipore, 07-623, 1:2,000); SpC (Millipore, AB3786, 1:1,000).

µCT imaging. µCT Scans were performed on the Mediso Nano SPECT/CT System covering only the lung fields of each mouse. Each scan averaged covering 240 projections with an exposure time of 1,000 ms set at a pitch of 1 degree. The tube energy of the X-ray was 55 kVp and 145 μA. The in-plane voxel sizes chosen were small and thin creating a voxel size of 73 × 73 × 73 μm. The final reconstructed image consisted of 368 × 368 × 1,897 voxels. Scans were analyses with the Osirix software.

Crizotinib treatment. Mice were randomized to receive either control vehicle (water) or crizotinib at 100 mg per kg per os daily for at least 14 consecutive days. Mice were monitored daily for weight and clinical signs. Investigators were not blind with respect to treatment.

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Extended Data Figure 1 | Human and murine Eml4–Alk. a, Alignment of human EML4 exon 13 and mouse Eml4 exon 14. b, Alignment of the junction between the human EML4–ALK (variant 1) and the predicted murine Eml4–Alk proteins.
Extended Data Figure 2 | Induction of the Npm1–Alk translocation in NIH/3T3 cells. a, Schematic of the Npm1–Alk translocation. Red arrows indicate the sites recognized by the sgRNAs. b, Sequences recognized by the sgRNAs and location of primers used to detect the Npm1–Alk and Alk–Npm1 rearrangement (top panel). PCR on genomic DNA extracted from NIH/3T3 co-transfected with pX330 constructs expressing the indicated sgRNAs (middle panel). Sequences of four independent subclones obtained from the PCR products and representative chromatogram (bottom panel). c, Detection of the Npm1–Alk fusion transcript by RT–PCR on total RNAs extracted from NIH/3T3 cells co-transfected with the indicated pX330 constructs (left panel). The PCR band was extracted and sequenced to confirm the presence of the correct Npm1–Alk junction (bottom-right panel). Representative results from two independent experiments.
Extended Data Figure 3 | Comparison of dual and single sgRNA-expressing plasmids. a, Schematic of pX330 (A) and its derivatives (B–E) used in these experiments. NIH/3T3 were transfected with these constructs and lysed to extract total RNA and genomic DNA. b, RNAs were analysed by northern blotting with probes against the Alk (left) or Eml4 (right) sgRNAs. c, d. The DNA samples were subjected to surveyor assays (c), or amplified by PCR to detect the Eml4-Alk inversion (d).
Extended Data Figure 4 | Induction of the Eml4–Alk inversion in primary MEFs using an adenoviral vector expressing Flag–Cas and tandem sgRNAs. 

**a,** Schematic of the adenoviral vectors. **b,** Immunoblot using an anti-Flag antibody on lysates from MEFs infected with the indicated adenoviruses. **c,** Small-RNA northerns using probes against sgEml4 and sgAlk on total RNAs from cells infected with Ad-Cas9 or Ad-EA. **d,** PCR-mediated detection of the Eml4–Alk inversion in MEFs infected with Ad-Cas9 or Ad-EA for the indicated number of days. **e,** Standard curve generated performing quantitative PCR analysis on genomic DNA containing a known fraction of Eml4–Alk alleles. Average of two independent experiments. **f,** Quantification of the fraction of MEFs harbouring the Eml4–Alk inversion at the indicated time points after infection with Ad-EA or Ad-Cas9. Values are mean of three independent infections ± s.d.
Extended Data Figure 5 | Radiologic response of Ad-EA-induced tumours to crizotinib treatment. μCT images from crizotinib- or vehicle-treated mice at day 0 and after 2 weeks of treatment.
Extended Data Table 1 | Mouse cohorts

| Mouse Sex | Virus Group | Notes | Time since infection (weeks) |
|-----------|-------------|-------|----------------------------|
|           |             |       | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
| V         | M           | NA-CE | NO  | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES | YES |
| I         | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 1         | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 2         | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 3         | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 4         | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 5         | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 6         | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 7         | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 8         | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 9         | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 10        | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 11        | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 12        | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 13        | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 14        | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 15        | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 16        | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 17        | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |
| 18        | F           | NA-CE | YES | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  | NO  |

Figure Legend
- YES: one or more tumours detected
- NO: no tumours detected
- 4: evaluation by µCT
- 1: evaluation by necropsy and histopathology
- V: mouse treated with vehicle (water)
- C: mouse treated with crizotinib (100 mg per kg per day)

This spreadsheet contains an annotated list of every mouse used in this study and the virus used for the intratracheal infection. The interval (in weeks) since infection is shown as a coloured horizontal bar. The time, outcome, and method of tumour detection are also reported. Symbols used are: YES = one or more tumours detected; NO = no tumours detected; 4 = evaluation by µCT; 1 = evaluation by necropsy and histopathology; V = mouse treated with vehicle (water); C = mouse treated with crizotinib (100 mg per kg per day).
## Extended Data Table 2 | Response to crizotinib treatment

| Mouse ID | Sex | Time (weeks) since infection at the start of treatment | Weeks treated | Treatment   | Outcome at 2 weeks   | Notes                      |
|----------|-----|------------------------------------------------------|---------------|-------------|----------------------|----------------------------|
| OP1300   | F   | 9.7                                                  | 2             | Crizotinib  | Complete Response    | Suppl. Videos 3 and 4      |
| OP1290   | F   | 12.3                                                 | 2             | Crizotinib  | Complete Response    | Suppl. Videos 7 and 8      |
| OP1283   | F   | 12.3                                                 | 2             | Crizotinib  | Complete Response    |                            |
| OP1258   | F   | 11.0                                                 | 2             | Crizotinib  | Complete Response    |                            |
| OP1293   | F   | 13.3                                                 | 2             | Crizotinib  | Partial Response     | Suppl. Videos 9 and 10     |
| OP1295   | F   | 12.0                                                 | 2             | Crizotinib  | Complete Response    |                            |
| OP1298   | F   | 12.0                                                 | 2             | Crizotinib  | Complete Response    |                            |
| OP1280   | F   | 11.0                                                 | 2             | Vehicle     | Progression          | Suppl. Videos 5 and 6      |
| OP1259   | F   | 12.0                                                 | 2             | Vehicle     | Progression          | Suppl. Videos 1 and 2      |
| OP1292   | F   | 13.3                                                 | 2             | Vehicle     | Progression          |                            |

Table showing the response to crizotinib or vehicle treatment as judged by μCT.
## Extended Data Table 3 | Oligonucleotides used in this study

| Name                        | Sequence                                    |
|-----------------------------|---------------------------------------------|
| Alk_cDNA-rev                | GGTACATGATGTTGCGAGGTCC                     |
| Alk_Exon29_rev              | GCTAGTGGAGTACAGGGCTC                      |
| Alk_gDNA-for (primer D Fig1b and Supp Fig1b) | GCAGCGGGGCTTCGAGAGGGC                   |
| Alk_gDNA-rev (primer C Fig1b and Supp Fig1b) | GTTTTACTGTGTCAGAAAGGG                  |
| Alk-rev                     | CAAGGCGATGGAACACCTGAA                     |
| Eml4_cDNA-for               | TGGAGTGCAACCTCACCACAA                     |
| Eml4_cDNA-rev               | GCAAAGCTCTAATGGGGGC                      |
| Eml4_Exon1_for             | TAGAACTGAGGCAAGATGGAGCGGGTTTCCGC         |
| Eml4_gDNA-for (primer A Fig1b) | GCTCAAGAGGGTGGGTTGTTG                      |
| Eml4_gDNA-rev (primer B Fig1b) | CAGGGCTGTGCCATAGATAC                |
| Eml4-for                    | GAGGCTTTGTGATACATCGGT                     |
| Eml4-rev                    | TAGGAGCGAGTGGGGCTAC                      |
| GAPDH_cDNA-for             | ACCACAGTCCATGGCCTACATGCC                 |
| GAPDH_cDNA-rev             | GTCTCGCTCGTGGAAAGATGG                    |
| miR17-92_gDNA-for          | TCGAGTATCGAGAATGG                        |
| miR17-92_gDNA-rev          | TAGCCAGAAGTTGCGAAATTGG                   |
| Npm1_cDNA-for              | ACTACGTTGCGCGCTGAAC                      |
| Npm1_gDNA-for (primer A Supp Fig1b) | GTCCTCTGGCTACATTGGG                  |
| Npm1_gDNA-rev (primer B Supp Fig1b) | CTCAGGAGCGATCGCTTT              |

This table lists the names and sequences of each DNA oligonucleotide used in this study.
Extended Data Table 4 | Primer pairs and PCR reactions

| Name                | Description                  | Expected size (bp) |
|---------------------|------------------------------|--------------------|
| Alk_gDNA-for        | Surveyor assay               | 961                |
| Alk_gDNA-rev        |                              |                    |
| Eml4_gDNA-for       | Surveyor assay               | 602                |
| Eml4_gDNA-rev       |                              |                    |
| Eml4_gDNA-for       | Eml4-Alk genomic             | 527                |
| Alk_gDNA-rev        |                              |                    |
| Alk_gDNA-for        | Alk-Eml4 genomic             | 1036               |
| Eml4_gDNA-rev       |                              |                    |
| Alk_gDNA-for        | Deletion                      | 1044               |
| miR17-92_gDNA-for   | Control (gDNA)               | 255                |
| miR17-92_gDNA-rev   |                              |                    |
| GAPDH_cDNA-for      | Control (cDNA)               | 237                |
| GAPDH_cDNA-rev      |                              |                    |
| Eml4-for            | Eml4-Alk genomic (three primers) | Eml4: 240          |
| Eml4-rev            |                               | Eml4-Alk: 190      |
| Alk-rev             |                              |                    |
| Eml4_cDNA-for       | Eml4-Alk transcript (three primers) | Eml4: 336          |
| Alk_cDNA-rev        |                               | Eml4-Alk: 276      |
| Eml4_cDNA-for       | Eml4-Alk transcript (junction) |                   |
| Alk_cDNA-rev        |                               | 276                |
| Eml4_Exon1_for      | Eml4-Alk transcript (full length) |                   |
| Alk_Exon29_re        |                               | 3238               |
| Npm1_gDNA-for       | Npm1-Alk genomic             | 581                |
| Alk_gDNA-rev        |                              |                    |
| Npm1_gDNA-for       | Alk-Npm1 genomic             | 1036               |
| Alk_cDNA-rev        | Npm1-Alk transcript          | 404                |

This table lists the primer pairs and the sizes of the expected products for each PCR reaction described in this study.
Corrigendum: In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system

Danilo Maddalo, Eusebio Manchado, Carla P. Concepcion, Ciro Bonetti, Joana A. Vidigal, Yoon-Chi Han, Paul Ogrodowski, Alessandra Crippa, Natasha Rekhtman, Elisa de Stanchina, Scott W. Lowe & Andrea Ventura

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During the assembly of Fig. 1b of this Letter, the sequences of the targeted regions in Eml4 and Alk, shown in the schematic in the top panel, were inadvertently swapped. In addition, the legend for the top panel of Fig. 1b should have read: “b, A schematic of the loci before and after the inversion with the location of the primers used (top panel). The sequences recognised by the guide RNAs are shown in italics, the protospacer adjacent motif (PAM) sequences are in bold and underlined. Asterisks indicate the predicted Cas9 cleavage sites (positions −3 and −4 from the PAM).” The corrected panel is shown in Fig. 1 of this Corrigendum. We apologize for the confusion this may have caused.

Figure 1 | This is the corrected top panel of Fig. 1b of the original Letter.