METHODS PAPER

Establishment of a conditional *Nomo1* mouse model by CRISPR/Cas9 technology

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Abstract

The *Nomo1* gene mediates a wide range of biological processes of importance in embryonic development. Accordingly, constitutive perturbation of *Nomo1* function may result in myriad developmental defects that trigger embryonic lethality. To extend our understanding of *Nomo1* function in postnatal stages and in a tissue-specific manner, we generated a conditional knockout mouse model of *Nomo1*. To achieve this, we used clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology in C57Bl/6J mouse zygotes to generate a new mouse model in which exon 3 of the *Nomo1* gene is specifically flanked (or floxed) by LoxP sites (*Nomo1*^f/f). *Nomo1*^f/f mouse embryonic fibroblasts were transduced with a Cre adenovirus and efficiently recombined between LoxP sites. Genomic and expression studies in *Nomo1*-transduced MEFs demonstrated that the *Nomo1* exon 3 is ablated. Western blot assay showed that no protein or early truncated protein is produced. In vivo assay crossing *Nomo1*^f/f mouse with a Msi1-CRE transgenic mouse corroborated the previous findings and it showed Nomo1 exon 3 deletion at msi1+ cell compartment. This short technical report demonstrates that CRISPR/Cas9 technology is a simple and easy method for creating conditional mouse models. The *Nomo1*^f/f mouse will be useful to researchers who wish to explore the role of *Nomo1* in any developmental stage or in a tissue-specific manner.

Keywords CRISPR/Cas9 · Knock out · Conditional Mouse Model · NOMO1

Introduction

The Human Genome Project (HPG) was an international, collaborative research program whose goal was the complete mapping of all the genes of human beings. The HPG revealed that there are probably about 20,500 human genes [7] and subsequently much effort has been expended on understanding their functions and on studying how gene mutations affect the occurrence of human diseases. Since human and mouse genes are 95% homologous, it soon became apparent that mouse genes could serve as tools for understanding human gene function. In this context, researchers and various consortia initially used gene targeting or gene trapping in mouse stem cells to knock out the corresponding murine homologous loci [1, 10]. Phenotypic characterization of these mutations is the most accurate and widely used method for elucidating in vivo gene functions and the genetics of diseases. Nevertheless, customary knockout methods can give rise to embryonic lethality from a gene mutation, which prevents researchers from studying the gene in postnatal stages [35]. Tissue-specific gene functions could remain unknown when the null mutation cannot be exclusively produced in a particular tissue. To overcome these obstacles, conditional gene knockout with the Cre-loxP recombination system [31]

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is widely used to eliminate a specific gene in a particular tissue at a certain development stage [15]. Many mouse stem cell clones have been developed by the International Knockout Mouse Consortium with putative conditional null mutations that are being used to generate conditional mice [1, 8]. However, the process of generating the mouse model from the ES cell clone is not free of problems: it is laborious, time-consuming, and can only be carried out in certain mouse strains. Fortunately, the recently developed clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system has changed the gene targeting landscape, making the use of transgenesis and mutagenesis technology more feasible, not only in zebrafish or mice as was first reported [28], but in all organisms [14, 19, 32]. This report describes in detail the generation by CRISPR/Cas9 technology of a novel conditional *Nomo1* mouse, which will allow *Nomo1* gene function to be studied in a rigorous and specific way that avoids embryonic lethality.

The Nodal subfamily belongs to the group of TGFβ proteins that play a key role in the embryonic development of vertebrates. Nodal signaling is essential for the patterning of the early embryo during mesoderm and endoderm induction as well as the specification of left–right asymmetry [26, 27]. Gene expression studies showed that Nodal proteins act before gastrulation to specify the mesodermal and endodermal progenitors, and that the patterning depends on graded signaling with participation of Nodal inhibitors [5, 9, 29]. These inhibitors can directly bind and inhibit Nodal proteins or compete with them to bind to their coreceptors [3, 6]. Nomo (Nodal modulator) and its binding partner, Nicalin, are components of a novel protein complex involved in Nodal signaling. This complex has been revealed as an antagonist of Nodal signaling during mesendodermal patterning in zebrafish. The evolutionary conservation of Nicalin and Nomo in metazoans and plants indicates that the Nomo/Nicalin complex could have a similarly important role. Nomo/Nicalin transcripts were detected in all postnatal human tissues, and an overlapping subcellular distribution was found in endoplasmic reticulum membranes. In zebrafish embryos, Nomo expression was detected in the endoderm and anterior mesendoderm, and its overexpression was found to impair forebrain patterning [11]. Other studies showed that the *Nomo1* gene positively regulates the expression of the master regulator and other key development genes involved in bone formation and cartilage development, and that it is essential for cartilage development in zebrafish [2]. The *NOMO1* gene is also known to be substantially downregulated in human ventricular septal defect myocardium and, thus, may be an important molecular pathway in human heart development [34]. In humans, tumors from the early-onset subgroup of colorectal cancers showed a homozygous deletion in *NOMO1* and were associated with a microsatellite-stable feature and better prognosis, although the molecular mechanism was not identified [21].

Despite the study of *Nomo* in embryogenesis and pattern expression in embryo and adult tissues, its biological functions in mammalian postnatal tissues remain unknown. Since *Nomo* is required to produce proper axial mesoderm patterning, bone and heart formation, complete *Nomo* inhibition could impair embryo development. To study its functions during postnatal stages we decided to create a new mouse model to conditionally interfere with its functions.

Material and methods

Ethics statement

This study followed Spanish and European Union guidelines for animal experimentation (RD 1201/05, RD 53/2013 and 86/609/CEE). The study was approved by the Bioethics Committee of the University of Salamanca.

In silico analysis and CRISPR/Cas9 system design

The mouse *Nomo1* gene contains 31 coding exons that produce 5 different transcripts (annotated as *Nomo1*-201 to *Nomo1*-205). *Nomo1*-201, with a length of 4257 bp, is the only one capable of translating into a protein, comprising 1214 residues. *Nomo1* exon 3 is the first exon with a residue overlapping the splicing site. In silico analysis showed that an early truncated Nomo1 protein could be produced when exon 3 is removed. Two sgRNAs (*Nomo1*-sgRNA1 and *Nomo1*-sgRNA2), targeting the coding sequence of *Nomo1* exon 3 and the adjacent intron sequence, were designed with the Spanish National Biotechnology Centre (CNB)-CSIC web tool (http://bioinfogp.cnb.csic.es/tools/breakingcas/) (Fig. 1). Two complementary oligos corresponding to sgRNAs were designed, including two 4-bp overhang sequences (*Nomo1* UP and LO oligos, Table 1).

As earlier papers described, a long single-strand oligonucleotide of DNA (long ssDNA) (Integrated DNA Technologies, IDT) was considered as donor for homologous recombination to create the conditional KO allele [17, 18, 24]. A *Nomo1* long ssDNA containing LoxP sequences in the same orientation flanking exon 3 was designed, adding silent mutations that destroy PAM sequences. This had an expanded total length of 789 bp (Supplementary Fig. S1).

Generation of CRISPR/Cas9 reagents

Cas9 mRNA and sgRNA were generated by cloning into a plasmid vector and followed by in vitro transcription [12]. To clone the sgRNAs into the pX458 vector it was previously linearized with BpiI (NEB). The two complementary
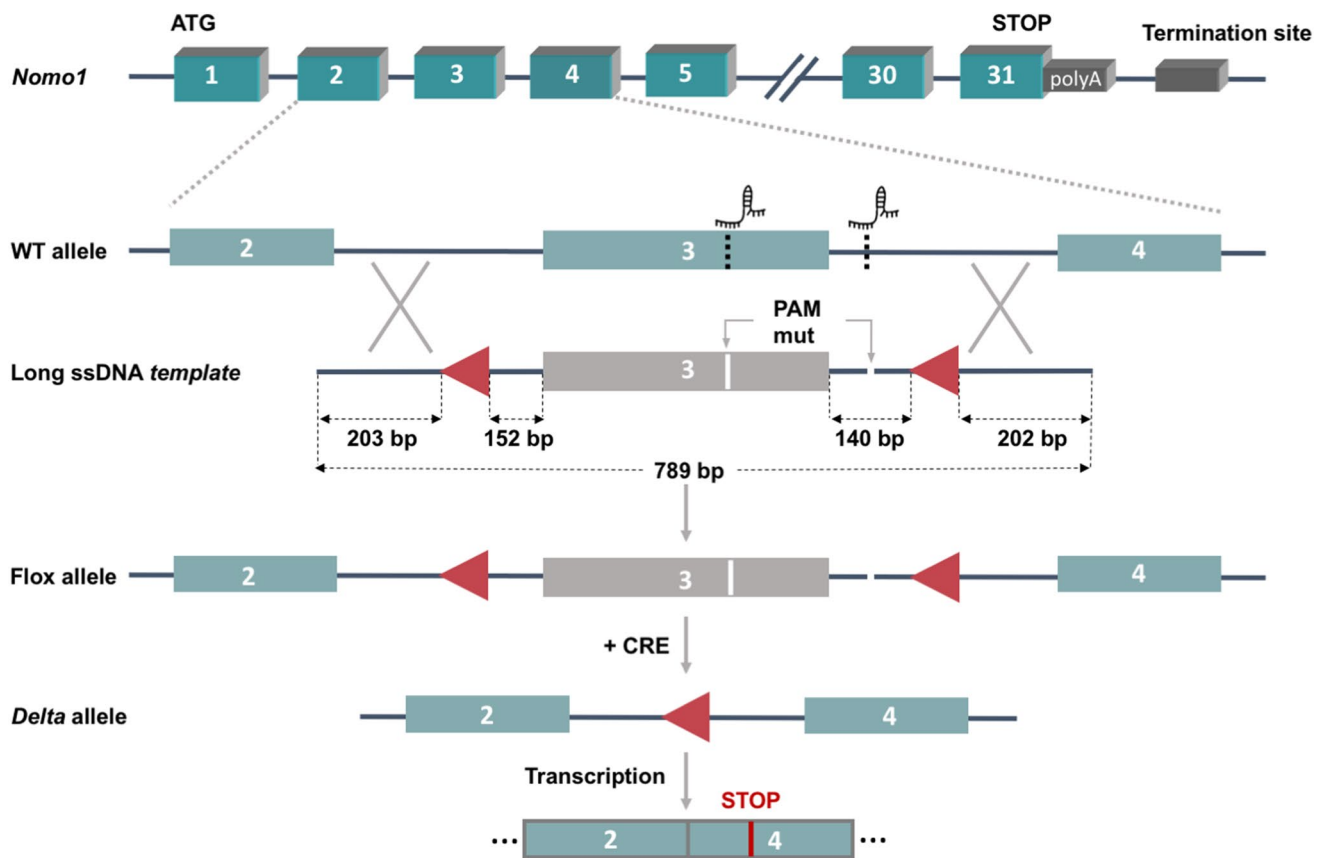
Fig. 1 Schematic representation of *Nomo1* locus and targeting strategy. Detail of wild-type *Nomo1* exon 3 with expected cleavage points of two sgRNAs and the long ssDNA template used for HDR, carrying two silent mutations in the PAM sequences of sgRNAs (Loxp sites are represented by red arrowhead). The flox allele represents the expected result of homology recombination between wt allele and the template. Finally, the delta allele arises from Cre recombinase action, eliminating exon 3 in mRNA and generating a stop codon in Nomo1 exon 4

Oligo	Sequence
*Nomo*1_1UP	caccgCCTGAAGATCGAACCTCCTC
*Nomo*1_1LO	aaacGAGGAGGTTCGATCTTCAGGc
*Nomo*1_2UP	caccgGAGTAACGGGGCGCTTCGCA
*Nomo*1_2LO	aaacTGCGAAGCGCCCCGTTACTCc
*Nomo*1_1T7 F	<u>TAATACGACTCACTATAGG</u>CCTGAAGATCGAACCTCCTC
*Nomo*1_2 T7 F	<u>TAATACGACTCACTATAGG</u>GAGTAACGGGGCGCTTCGCA
gRNA R	GCACCGACTCGGTGCCACT
T7 Cas9 F	<u>TAATACGACTCACTATAGG</u>GATGGCCCCAAAGAAGAAGCGGA
T7 Cas9 R	CTTTTTCTTTTTTGCCTGGCC
*Nomo*1 F	AGGGAGAAAGAGGATCAGCAG
*Nomo*1 R	CCACACAGATTAGGACTCTACT
*Nomo*1Ex2F	AGTTATACACCAAGCATGGGAC
*Nomo*1Ex4R	CTTGCCATTCACAGAGAAGCC
*Nomo1*Ex5qPCR F	TGCTGACTCGAAGATCCAGTC
*Nomo1*Ex6qPCR R	TCCTTCAGCGCCCAGGTCG
Gapdh qPCR F	TGCACCACCAACTGCTTAGC
Gapdh qPCR R	CACCACCTTCTTGATGTCATCA

Table 1 Oligos used for in vitro transcription of CRISPR/Cas9 system and analysis

oligos corresponding to each *Nomo1* sgRNA were denatured at 95 °C for 5 min, ramp-cooled to 25 °C over 45 min to allow annealing, and they were ligated with the linearized pX458 (Addgene plasmid # 48138) [25]. Competent cells were transformed with 2 µl of the ligated plasmid, and single colonies were grown before plasmid extraction using a QIAprep Spin Maxiprep kit (Qiagen). The correct insertion of the sgRNA sequences was confirmed by Sanger sequencing. sgRNA sequences were PCR-amplified from px458-based vector with primers carrying the T7 RNA polymerase promoter at the 5′ ends (Nomo1 T7 F and gRNA R, Table 1). PCR products were purified (NZYGelpure, NZYTech) and used as a template for in vitro T7 RNA polymerase transcription (MEGAshortscript T7 Transcription Kit, Thermo Fisher).

The Cas9 nuclease ORF, including NLS, was also PCR-amplified from pX458 vector [25] with primers carrying the T7 RNA polymerase promoter at the 5′ ends (T7 Cas9 F and R, Table 1). The Cas9 PCR product was purified and used as a template for in vitro transcription, 5′ capping (mMESSAGE mMACHINE T7 Transcription Kit, Thermo Fisher), and 3′ poly(A) tailing [Poly(A) Tailing Kit, Thermo Fisher]. Transcription products were purified with RNeasy Mini Kit (Qiagen) and eluted in nuclease-free EmbryoMax microinjection buffer (Millipore).

Nowadays, all this reagents and methodology can be replaced by injecting embryos with CRISPR ribonucleoproteins (RNPs), acquiring commercial sgRNAs and Cas9 protein [4, 13, 20, 24].

In vitro test to analyze Nomo1-sgRNAs and Cas9 activity

Ten surplus C57BL/6J zygotes from routine processes carried out in the Transgenesis Facility of University of Salamanca were microinjected with 20 ng/µl of Nomo1 sgRNAs, 20 ng/µl of Cas9 mRNA, and 20 ng/µl of Cas9 protein into the cytoplasm and pronucleus. Microinjected embryos were grown on KSOM with non-essential amino acids (Millipore), 37 °C and 5% CO_2 atmosphere at the blastocyst stage. Genomic DNA from single blastocyst-staged embryos were extracted in 10 µl of lysis buffer (50 mM KCl, 10 mM Tris–HCl pH 8.5, 0.1% Triton X-100, and 4 mg/ml of proteinase K) at 55 °C overnight, then heated at 95 °C for 10 min. 2 µl of this DNA solution was used as a template for two rounds of PCR (30 cycles + 20 cycles) to amplify the target sequences using a specific primer for the *Nomo1* target region (Nomo1 F and R oligos, Table 1). PCR products were purified using a High Pure PCR Product Purification Kit (Roche) and sequenced by the Sanger method using forward and reverse PCR primers.

Mice and embryo microinjection

Sixty-seven 1-cell-stage embryos from 6 superovulated C57BL/6J females were harvested and microinjected with 20 ng/µl of sgRNA, 20 ng/µl of Cas9 mRNA, 20 ng/µl of Cas9 protein and 25 ng/µl of long ssDNA template solution into the cytoplasm and pronucleus. Microinjected embryos were grown overnight on KSOM with non-essential amino acids (Millipore), 37 °C and 5% CO_2 atmosphere. Fifty-six two-cell-stage embryos were recovered and transferred to two pseudopregnant females. Embryo donor females were euthanized by cervical dislocation and given humanitarian care in accordance with the standards of the Bioethical Committee of University of Salamanca and with Spanish and European Union guidelines for animal experimentation.

Msi1-Cre transgenic mice (MGI:5296021) [22] (see Supplementary Data) was used to express CRE protein at brain and intestinal crypt cells when they were crossed with the *Nomo1* conditional model (*Nomo1*[f/f] F2). Cre expression location was tested crossing Msi1-Cre with a Rosa26 βgal Cre-reporter mouse [30] (Supplementary Fig. S2).

Mouse embryonic fibroblast extraction, culture and viral transduction

Mouse embryonic fibroblasts (MEFs) were extracted 13.5 days *post coitum* (dpc) following procedures described at Bio Protoc. 2013 Sep 20; 3(18): e908. MEFs were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% of penicillin/streptomycin (Life Technologies) at 37 °C in a 5% CO_2 atmosphere.

Cre recombinase was expressed in MEFs by virally transducing 300 MOI of Ad5CMVCre-eGFP virus (Viral Vector Core, University of Iowa). $1 \times 10e5$ MEFs were incubated overnight with the virus in the absence of FBS and 48 h after infection. DNA and RNA were extracted with a QIAamp DNA Micro Kit and an RNAeasy Mini Kit, respectively (Qiagen).

Analysis of Cas9-system targets sites

Mouse genomic DNA was extracted from tail biopsy though proteinase K digestion, followed by phenol/chloroform purification. Genomic DNA from MEFs was extracted using the QIAamp DNA Micro Kit (Qiagen) following the manufacturer's protocol. To amplify target regions of mouse *Nomo1* gene, PCR was performed with *Nomo1* F and *Nomo1* R oligos (Table 1). PCR products were purified using a High Pure

PCR Product Purification Kit (Roche) and sequenced by the Sanger method using forward and reverse PCR primers.

To characterize the various alleles generated in mouse pups or in MEFs, PCR products were subcloned and transformed in bacteria. DNA from single clones was extracted with a QIAprep Spin Miniprep Kit (Qiagen) and Sanger-sequenced.

RT-PCR and quantitative PCR

To test *Nomo1* mRNA expression, 150 ng of total mRNA from MEFs was in vitro-retrotranscribed using a SuperScript III First-Strand Synthesis Super Mix kit (Thermo Fisher). MEF cDNA was used as a template to PCR-amplify the specific sequence between exons 2 and 4 with oligos *Nomo-1*Ex2F and *Nomo1*Ex4R (Table 1). PCR product was purified and Sanger-sequenced.

Nomo1 mRNA expression was quantified by qPCR (Applied Biosystems) using oligos targeting *Nomo1* exon 5 *Nomo1*Ex5qPCRF 5 and *Nomo1* exon 6 *Nomo1*Ex6qPCRR. *GAPDH* mRNA expression was used as a control (oligos *Gapdh* qPCR F and R, Table 1).

Western blot

Nomo1 protein expression was assessed by SDS-PAGE and western blotting using a rabbit anti-mouse Nomo1 antibody (1:200; Thermo Fisher). Horseradish peroxidase-conjugated anti-rabbit antibody (1:1000; Cell Signaling) was used as a secondary antibody. Antibodies were detected using ECLTM Western Blotting Detection Reagents (RPN2209, GE Healthcare). As a control, β-actin expression was measured using mouse anti-β-actin (1:20,000, Sigma Aldrich).

Statistical analysis

Statistical analysis of annexin V expression was performed using GraphPad Prism version 6.00 for Mac OS X (Graph-Pad Software, La Jolla, California, USA, www.graphpad.com). Differences in Nomo1 expression were tested by two-way ANOVA, followed by Tukey's post hoc multiple comparison test. Experimental results were expressed as the mean ± standard deviation (SD). Statistical significance was concluded for values of $p < 0.05$.

Results

CRISPR/Cas9 reagents efficiently induce mutations at the target site of the Nomo1 sgRNAs

Before injecting embryos with CRISPR/Cas9 reagents, we tested the activity of *Nomo1* sgRNAs by injecting 10 C57BL/6J zygotes with a mixture of mRNA Cas9, Cas9 protein and *Nomo1* sgRNAs. A nested PCR of material obtained from single blastocyst stage embryos was performed. Genomic DNA from all the embryos was analyzed by Sanger sequencing. A large number of mutations at the expected cut site for both *Nomo1* guides was detected in most of the single embryos. A high proportion of mosaic embryos with different alleles was detected (Fig. 2A); in most of them, the alleles exhibited a specific deletion corresponding to the target area (43 bp) flanked by the cut sites between *Nomo1* guides (Fig. 2B) and other mutated (Fig. 2C) and wild-type (wt) alleles.

CRISPR/Cas9 technology produces *Nomo1* mosaic mice with a wide variety of mutated alleles

Two pseudopregnant females were transferred, each with 28 two-cell embryos. An offspring of 5 live (#1 to #5) and 5 dead (#6 to #10) pups were born from them. The target region of *Nomo1* locus was analyzed by PCR in all of them. All pups, except #3, showed the 786-bp band corresponding to the wt allele. Additionally, six of them displayed secondary bands with higher or lower molecular weights (Fig. 3A), indicating the presence of mosaic pups. Sanger sequencing of all pups confirmed the presence of mosaic animals with three or more edited alleles in most of them. Sanger sequencing revealed a high percentage of null alleles in dead pups due to frameshift mutations (Fig. 3B).

CRISPR/Cas9 technology efficiently produces a *Nomo1* conditional allele

Three of the pups analyzed (#1, #9 and # 10) displayed 1 higher secondary band with the expected weight (854 bp), a result that was compatible with the conditional designed allele (Fig. 3A). Sanger sequencing revealed the presence of LoxP sites in two of the pups. In silico analysis showed that pups #9 and #10 contained 1 partially recombined allele with a single LoxP site and the mutated PAM sequence. However, pup #1 had four distinct alleles: one wt, two with insertions or deletions (indels), and one allele edited with the conditional design (Fig. 3C).

Mosaic *Nomo1* conditional founder F0 establishes the colony and effectively segregates the conditional allele

Mosaic male #1 was viable and was selected as the founder (F0) of the colony. The F0 was mated with a C57Bl6/J
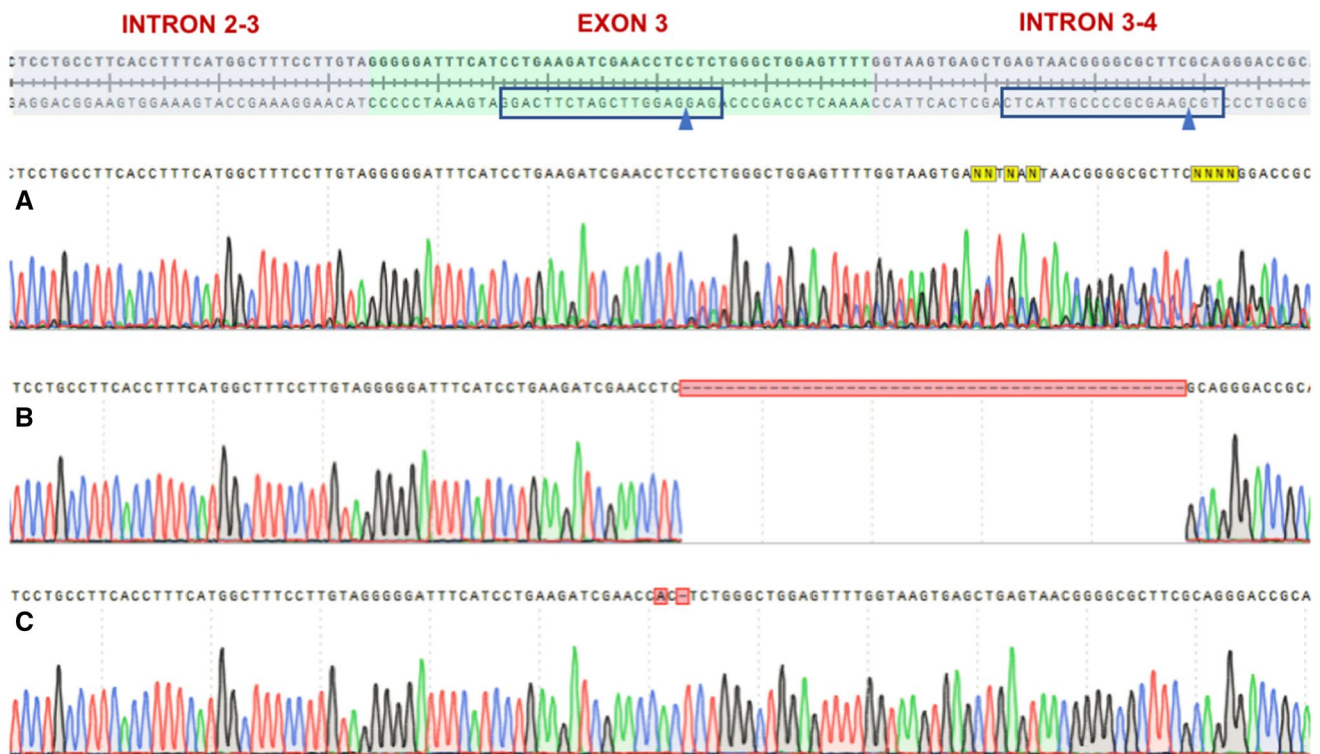
Fig. 2 Analysis of SgRNAs and CRISPR/Cas9 activity in *Nomo1* locus. **A** Sanger sequencing of *Nomo1* exon 3 amplified from a single edited embryo with both sgRNAs. A mixed sequence is noted between the expected cleavage point (blue arrowhead) of the two sgRNAs (blue box). Subcloning and Sanger sequencing revealed **B** a 46-bp deletion in the sequence between cleavage points of the two sgRNAs, and **C** some indels due to the Cas9 action of just one cleavage point

female to segregate the conditional allele into an inbred strain. F1 litter sizes varied from 4 to 9 pups per delivery, and the conditional allele was segregated in a 1/3–1/4 ratio (Fig. 3D). Heterozygous (*Nomo1*^f/+) F1 were fertile and mated to generate homozygous mutants (*Nomo1*^f/f) F2. The *Nomo1*^f/f F2 mice born had a pattern Mendelian inheritance; they were viable and fertile, and displayed no obvious alterations in size or behavior.

Cre recombinase efficiently removes *Nomo1* exon 3 in vitro and in vivo

To test whether Cre activity effectively removes the LoxP exon 3 flanked region, we transduced E12.5-derived MEFs from *Nomo1*^+/+, *Nomo1*^f/+ and *Nomo1*^f/f mice with GFP-Cre adenovirus. *Nomo1*^+/+, *Nomo1*^f/+ and *Nomo1*^f/f MEFs expressed a high level of GFP after adenoviral infection, indicating an efficient expression of Cre recombinase (Fig. 4A). Genotyping of MEFs after adenoviral infection showed no differences in *Nomo1*^+/+ or non-transduced MEFs. However, all mutant MEFs (*Nomo1*^f/+ and *Nomo1*^f/f) showed a lower band corresponding to the molecular weight expected after Cre activity (482 bp) and exon 3 removal (Fig. 4B). In order

to corroborate these findings, we crossed a female *Nomo1*^f/f with a male Msi1-Cre transgenic mouse (Msi1-CRE^tg/tg) [22]. Intestinal and brain tissues from *Nomo1*^+/f; Msi1-CRE^+/tg double mutants were collected and genomic DNA was isolated. A specific lower band due to Cre activity at Msi1+ cells at *Nomo1* floxed locus was obtained (Fig. 4C).

Cre recombinase activity in *Nomo1*^f/f cells generates a *Nomo1* null allele

Expression of *Nomo1* mRNA and protein was studied in adeno-CRE transduced and non-transduced MEFs from *Nomo1*^+/+, *Nomo1*^f/+ and *Nomo1*^f/f mice. After adenoviral infection, *Nomo1* mRNA expression was checked by RT-PCR in all types of MEFs. All MEFs showed an mRNA of 233 bp, corresponding to the specific amplified *Nomo1* cDNA region containing exon 3. Nevertheless, *Nomo1*^f/+ and *Nomo1*^f/f MEFs showed an additional 187 bp lower band (Fig. 5A). PCR subcloning and Sanger sequencing of this lower band demonstrated the correct excision of exon 3. In silico analysis of the *Nomo1*^f/f allele after Cre activity revealed the presence of an early stop codon that
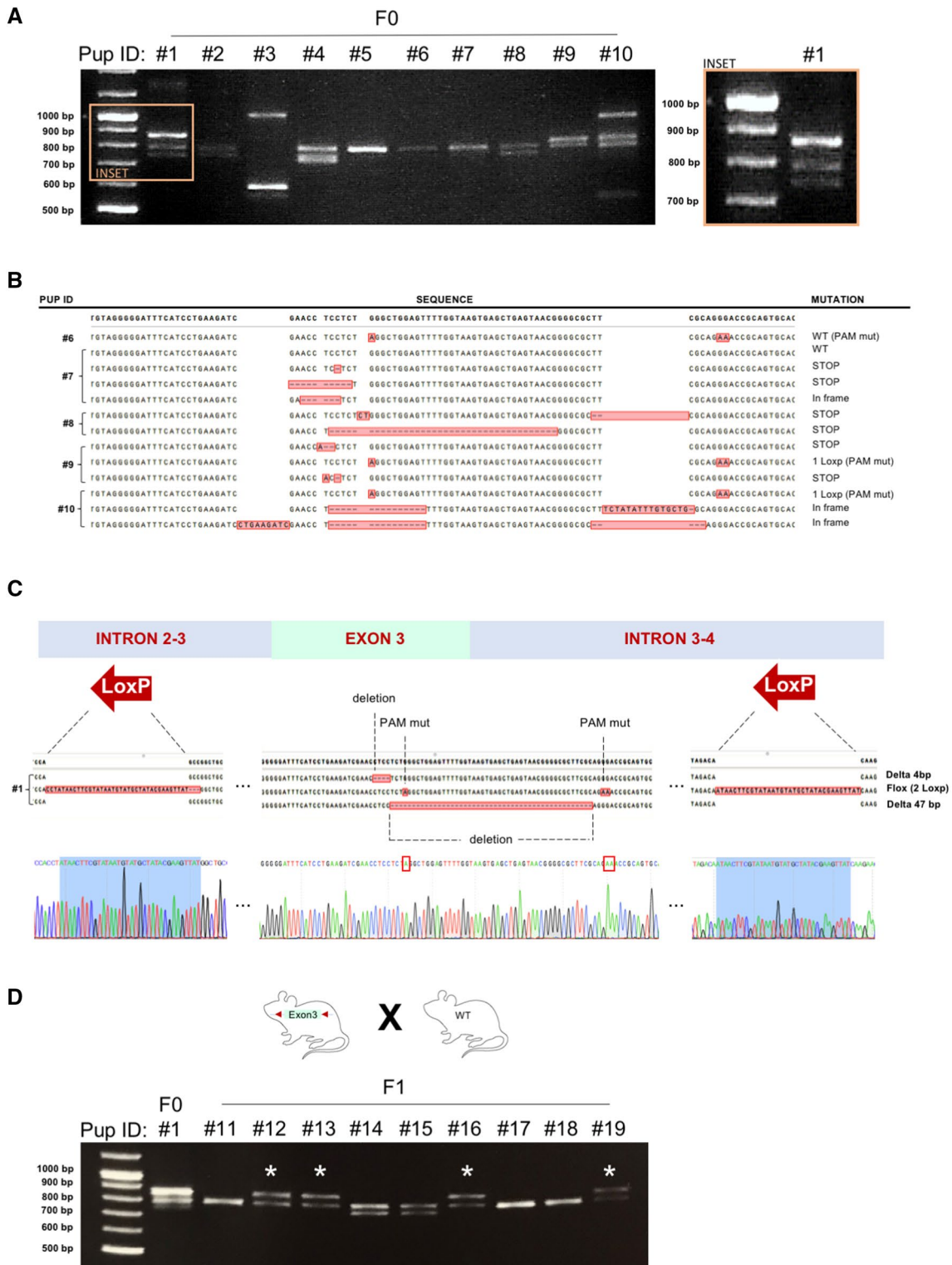
Fig. 3 Offspring analysis. **A** Genotyping of F0 (ten pups) by PCR of the *Nomo1* locus. Several modifications were observed. Inset: pup #1 shows three alleles, two of which correspond to the expected molecular weight of the WT and flox alleles. **B** Subcloning and sequencing of some alleles carried by offspring. **C** Subcloning and sequencing of alleles observed in pup ID #1. Two alleles showed deletions (4 bp and 47 bp), and the third allele (flox allele) corresponds to an insertion of two Loxp sites flanking the exon 3 and silent PAM mut at the expected points. **D** Breeding scheme and flox allele segregation. PCR analysis of F1 offspring detected 4 of 9 pups carrying the flox allele (asterisk)

Fig. 4 Functional analysis of the *Nomo1* flox allele. **A** Fluorescent microscopy of MEFs extracted from *Nomo1*^{+/+}, *Nomo1*[+/f] and *Nomo1*[f/f] 13.5 d.p.c. embryos transduced with adeno-Cre-GFP virus. **B** PCR amplification of the *Nomo1* locus of non-transduced and transduced MEFs with adeno-Cre-GFP virus. *Nomo1*[+/f] and *Nomo1*[f/f] transduced MEFs showed 854 bp and 482 bp bands corresponding to the flox and delta alleles, respectively. *Nomo1*[+/f] also featured a wt allele (786 bp). Whereas *Nomo1*^{+/+}-transduced MEFs showed a single band (786 bp) corresponding to the wt allele. **C** Msi1-Cre[tg/tg] transgenic mice were crossed with *Nomo1*[f/f] mice. PCR amplification of the *Nomo1* locus detected heterozygous double mutants at F1 pups (upper gel). Intestine and Brain genomic DNA were isolated from double mutant Msi1-Cre[+/tg] *Nomo1*[+/f] mice and PCR amplification of the *Nomo1* locus detected a 482 bp band due to Cre activity on Nomo1 floxed locus (lower gel)
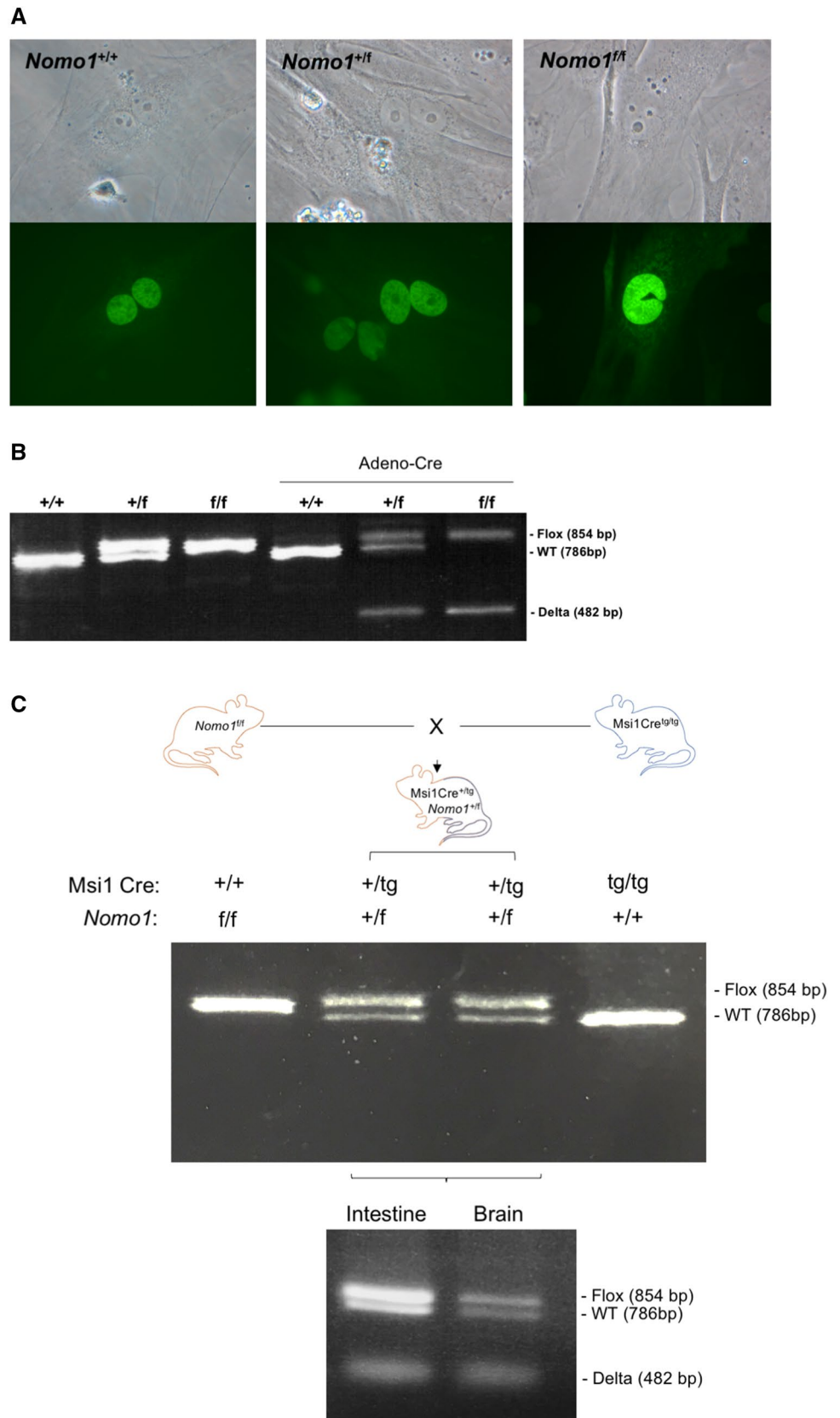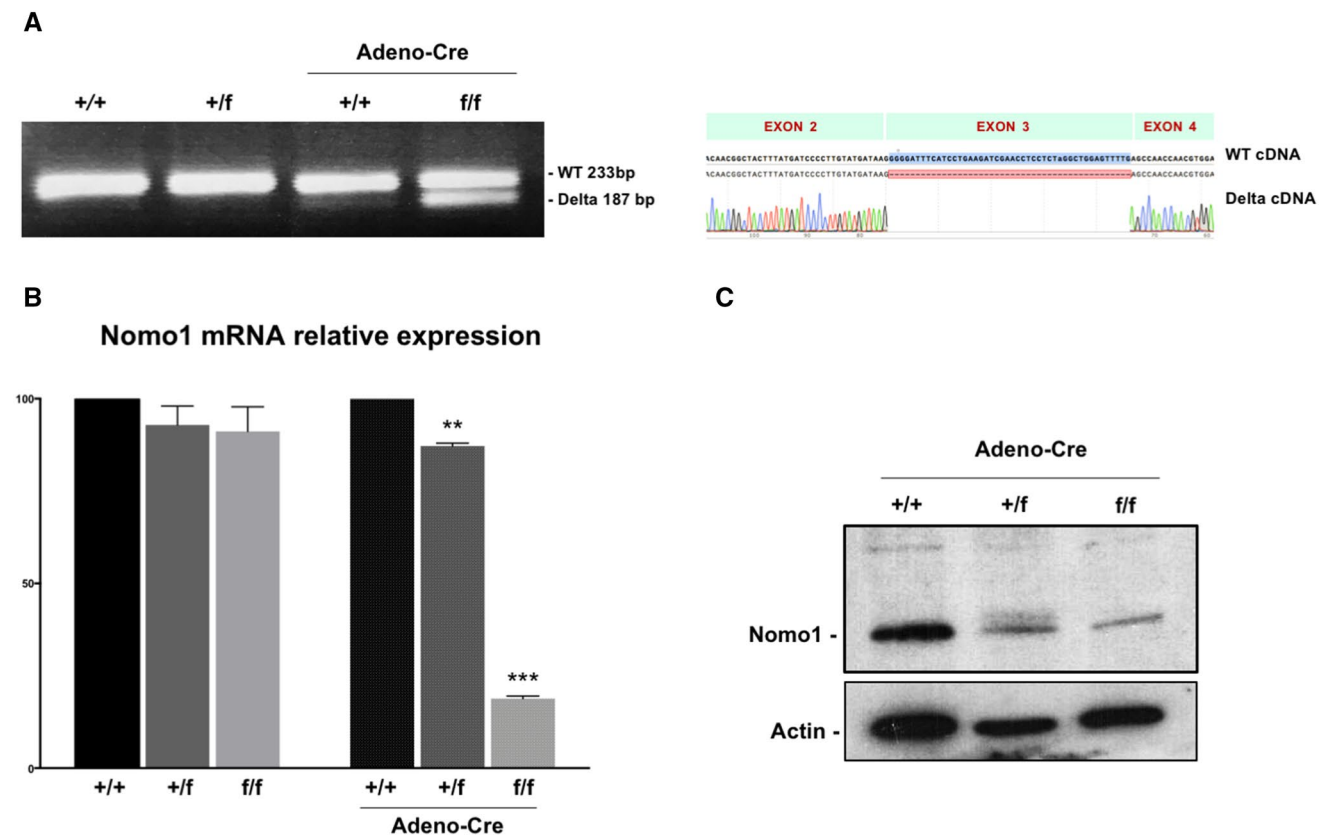
Fig. 5 Effect of Nomo 1 exon 3 excision. **A** cDNA from transduced MEFs were obtained and amplified to analyze the mRNA expression of *Nomo1*. MEFs with the floxed allele express wild type (233 bp) and Delta (187 bp) transcripts (left). Sanger sequencing revealed that Delta transcript corresponds to mRNA without exon 3 (right). **B** Q-PCR to quantify *Nomo1* mRNA. *Nomo1*$^{f/f}$ and *Nomo1*$^{f/+}$ MEFs showed a lower expression of *Nomo1*$^{+/+}$ after adeno-Cre transduction. *Nomo1*$^{f/f}$ transduced MEFs showed the lowest level of *Nomo1* expression (80% lower level, ***p < 0.001). **C** Western blot analysis of Nomo1 protein expression. After adeno-Cre transduction, *Nomo1*$^{+/f}$ and *Nomo1*$^{f/f}$ MEFs showed a dramatic reduction of Nomo1 protein, more evident in *Nomo1*$^{f/f}$ transduced MEFs

triggered a truncated Nomo1 protein with a different translating frame due to absence of the exon 3 (Fig. 5A).

To study the effect of *Nomo1* exon 3 excision by Cre recombinase, *Nomo1* expression was measured by real-time PCR and western blot. *Nomo1* mRNA expression was quantified in *Nomo1*$^{+/+}$, *Nomo1*$^{f/+}$ and *Nomo1*$^{f/f}$ MEFs before and after adeno-Cre transduction. We observed a substantial reduction (> 80%) of *Nomo1* mRNA in *Nomo1*$^{f/f}$-transduced MEFs compared with *Nomo1*$^{+/+}$ (Fig. 5B). These results were corroborated at the protein level by western blot assay, which showed a lower level of *Nomo1* expression in *Nomo1*$^{f/+}$ and minimal expression in *Nomo1*$^{f/f}$ MEFs due to the presence of a minimal percentage of non-transduced *Nomo1*$^{f/f}$ MEFs (Fig. 5C).

Discussion

Genetically engineered mouse models are the best tool for studying gene function and human diseases. In particular, the Cre-LoxP system is widely used to investigate gene

functions in specific cellular compartments (spatial control) and/or at specific ages (temporal control). Hundreds of gene-floxed lines and constitutive or tissue-specific Cre-driver mouse lines have been generated to date and more continue to be developed. They are key to understanding the gene function in a specific cell context. In mouse models of human cancer, instead of using tissue-specific Cre mouse lines, a more real approximation might be to turn-off/turn-on the tumor suppressor gene/oncogene in a small group of specific cells using Cre-viral vectors.

Until recently, the process of generating conditional alleles in mice involved manipulating embryonic stem cells, which is a laborious, time consuming and inefficient process. Nowadays, CRISPR/Cas9 technology has improved and simplified this process, allowing us to generate a conditional model that is quicker to use with a reduction in time and number of animals.

This technical report shows that is possible to create a conditional mouse model using a small group of females as embryo donors. However, a careful study of the gene sequence is always necessary. At this point, short exons in

early positions with overlapping codons should be chosen to guarantee an effective null allele when they are removed by Cre activity. Other aspects to consider are the LoxP positions flanking the key exons and the distance between them. In order not to alter the branch site, the LoxP site should not be placed within the first 100 nucleotides upstream of the 3′ end of the intron to ensure that the splicing process is not affected. The first intron of a gene should be avoided because it could contain regulatory DNA sequences and disrupting a key transcription factor binding site could affect the expression of the gene. Regarding the size of the fragment floxed, although big deletions have been achieved with the Cre-*LoxP*-system flanking the full length of a gene, LoxP positions could certainly affect the efficiency of recombination if they are separated by a long distance.

Despite all these general instructions, an in vitro study to test sgRNAs and Cas9 activity is always recommended before use. In this paper we have used surplus embryos with an identical genetic background. However, mouse cell lines could be used for this purpose as long as they have the same sgRNA target sequence as the mouse embryo. Thus, the target areas would have to be sequenced in order to rule out polymorphisms and mutations that could interfere with the CRISPR/Cas9 activity. Although the CRISPR/Cas9 system is a rapid, simple, and often extremely efficient gene-editing method, the main obstacle to producing gene-edited animals is mosaicism in the founders [16, 23, 33]. As our results show, most mice had three or more alleles and, fortunately, the conditional allele was segregated. However, in general, the major challenge of mosaicism is that the mutated allele will not be transmitted to the offspring. In any case, mosaicism also complicates genotype analysis and PCR cloning, and genome sequencing approaches are always required. Nevertheless, not all are disadvantages regarding mosaicism, and it can provide useful advantages, such as the rapid estimation of candidate gene functions in vivo, and the direct comparison of mutant and wt cells in the same organ of mosaic animals. Furthermore, the presence of null alleles in a small proportion of cells enables animals to survive beyond the lethal phase, thus enabling study of the null phenotype in specific groups of cells [36]. Additionally, a mosaic founder mouse can segregate different alleles, generating several mutants in one step.

Given all these challenges and strengths, CRISPR-Cas technology allows the genome mouse embryo to be modified in an easy and conditional manner. We have used this technology to generate a conditional mouse model to study *Nomo1* gene function and have demonstrated that Cre activity removes exon 3 to produce a non-functional allele.

Compliance with ethical standards

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