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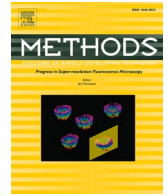
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Droplet digital PCR or quantitative PCR for in-depth genomic and functional validation of genetically altered rodents

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ABSTRACT

Gene targeting and additive (random) transgenesis have proven to be powerful technologies with which to decipher the mammalian genome. With the advent of CRISPR/Cas9 genome editing, the ability to inactivate or modify the function of a gene has become even more accessible. However, the impact of each generated modification may be different from what was initially desired. Minimal validation of mutant alleles from genetically altered (GA) rodents remains essential to guarantee the interpretation of experimental results. The protocol described here combines design strategies for genomic and functional validation of genetically modified alleles with droplet digital PCR (ddPCR) or quantitative PCR (qPCR) for target DNA or mRNA quantification. In-depth analysis of the results obtained with GA models through the analysis of target DNA and mRNA quantification is also provided, to evaluate which pitfalls can be detected using these two methods, and we propose recommendations for the characterization of different type of mutant allele (knock-out, knock-in, conditional knock-out, FLEEx, IKMC model or transgenic). Our results also highlight the possibility that mRNA expression of any mutated allele can be different from what might be expected in theory or according to common assumptions. For example, mRNA analyses on knock-out lines showed that nonsense-mediated mRNA decay is generally not achieved with a critical-exon approach. Likewise, comparison of multiple conditional lines crossed with the same CreER^{T2} deleter showed that the inactivation outcome was very different for each conditional model. DNA quantification by ddPCR of G0 to G2 generations of transgenic rodents generated by pronuclear injection showed an unexpected variability, demonstrating that G1 generation rodents cannot be considered as established lines.

1. Introduction

Modification of embryonic stem cells and now CRISPR/Cas9 genome editing [1] have enabled the generation of approximately a hundred thousand of genetically altered (GA) rodents with a variety of genes, markers or genomic region modifications. The impact of the model creation method, i.e. CRISPR/Cas9 genome editing, pronuclear injection for additive transgenesis (PNI) or homologous recombination in embryonic stem (ES) cells, must first be carefully assessed. For example, unexpected CRISPR/Cas9 genome-editing events are well described and may lead to the generation of a different mutation than that which is

expected [2,3]. However, many studies do not assess whether the verified mutation(s) then result in the expected mutant mRNA or protein. Recent publications show that improper validation of the consequence of a mutation has a strong impact on data interpretation and research reproducibility. Lindeboom *et al.* [4] showed that in cell knock-out (KO) models engineered by CRISPR/Cas9, the induction of frameshift mutations is not sufficient to induce degradation of messenger RNAs by nonsense-mediated mRNA decay (NMD). Partial rescue and gain-of-function effects may result from the production of truncated proteins. Likewise, Smith *et al.* also showed that cell KO models engineered through CRISPR/Cas9-induced frameshift mutations do not result in the

Abbreviations: GA, genetically altered; G0, founder generation; G1, first generation; G2, second generation; G3, third generation; NMD, nonsense-mediated mRNA decay; TAM, tamoxifen; WT, wild type; ddPCR, droplet digital PCR; qPCR, quantitative PCR; RT, reverse transcriptase; PNI, pronuclear injection; ES, embryonic stem; KO, knock-out; cKO, conditional knock-out; PM, point mutation; cPM, conditional point mutation (FLEEx switch); IKMC, International Knock-out Mouse Consortium; TCN, transgene copy number.

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expected complete loss of protein expression and activity. Residual protein expression in about one third of the tested KO lines was observed [5]. Similarly, improper validation can be observed in different GA lines. The *RIP-Cre* line, named for the rat insulin promoter Cre, is a good example of the impact of incomplete characterization. This line was widely used for Cre-mediated deletion of different floxed genes in pancreatic β cells until it was found that the glucose intolerance or frank diabetes phenotypes were in fact due to the *RIP-Cre* expression itself [6]. Finally, imported GA lines are not systematically controlled by scientists; indeed the Mutant Mouse Resource & Research Centers (MMRRC) showed that 15% of lines deposited to public repositories from individual labs do not match researchers' descriptions [7]. Discordant genotype [8] and poor line maintenance [7] may in part explain this issue. This emphasizes the importance of careful characterization of each newly generated or imported GA model for both genomic and functional validation.

Functional validation of the protein expression in mutant animals is the gold standard by which to verify the result of a gene modification. However, immunoblotting techniques such as western blotting are not truly quantitative [9,10] and are strongly biased by the availability of specific antibodies [11]. Moreover, detection is limited to the epitopes recognized by antibodies and therefore some truncated proteins or isoforms can be missed. Quantitative mass spectrometry proteomics is less biased and measures not only the target protein but a fraction of all proteins in the sample; however, it is also expensive, may not be available and, most importantly, the target gene must be sufficiently expressed to be detected [5,12].

While protein validation is an essential control, especially as correlation between expression levels of mRNA and protein is known to be poor [13], we show here that genomic and mRNA functional assessments are also useful for in-depth validation of GA rodents. We describe the use of droplet digital PCR (ddPCR) or quantitative PCR (qPCR) as an easy tool for the first level of evaluation of the functionality of genetic modifications. We present detailed strategies for assay designs and outcomes observed for different categories of alleles. A careful validation of the outcome of a genetic modification at the mRNA level reveals important information on the functional consequences of the generated genetic alteration.

2. Materials and methods

2.1. Sampling and extraction of genomic DNA or total mRNA

Ear or tail biopsies used for genomic validation were sampled following the protocol described in Jacquot et al. [8]. DNA lysis was then performed using the DNA Extract All Reagents kit (Applied Biosystems). Samples were lysed by addition of 25 μ l of lysis solution followed by heating at 95 °C for 10 min. After a quick centrifugation, 25 μ l of stabilizing solution was added and samples were diluted twenty times and stored at 4 °C.

All other organs used for genomic and functional validation from GA rodents were harvested and extracted (genomic DNA and/or total mRNA extraction) as described in Lindner et al. [14].

2.2. qPCR and RT-qPCR

qPCR and RT-qPCR were conducted using the LightCycler® 480 detection system, based on the LightCycler® 480 SYBR Green I master kit (Roche, Basel, Switzerland). The PCR reaction mixture contained 2 μ l cDNA (unknown concentration) or DNA (25 ng/ μ l), 0.085 μ l (0.7 μ M) of each primer, 6 μ l LightCycler® 480 SYBR Green I master mix and PCR-grade H₂O up to a total volume of 12 μ l. After initial enzyme activation (one cycle at 95 °C for 10 min), 45 amplification cycles (95 °C for 10 s, 60 °C for 10 s and 72 °C 5 s) were performed in 384-well optical reaction plates (Roche, Basel, Switzerland). To verify that the primer pair used produced only a single product, a dissociation protocol was added after

thermocycling, in which the PCR products were dissociated from 65 to 95 °C by increasing 2.5 °C per second. In all negative control samples, no amplification signal was detected. A standard curve was carried out to check efficiency of qPCR designs as recommended by [15]. Relative quantities were thus corrected for efficiency of amplification. For qPCR analyses, a normalization assay was done for each sample to correct the estimated DNA concentration.

2.3. ddPCR and RT-ddPCR

All ddPCR and RT-ddPCR was performed following the protocol described in Lindner et al. [14].

2.4. Statistical analyses and graphs

GraphPad Prism 9.0 (www.graphpad.com) was used for the graphical representations and statistical analyses. Because, for each gene altered, the normal level of the mRNA expression is not known and the sample size is small, non-parametric Mann-Whitney analyses were performed to compare experimental and control conditions. For additive transgenesis experiments, Mendelian distribution of transgenic offspring was evaluated using a chi-squared test. Two different hypotheses were tested for each generation: comparison of observed ratio with the expected ratio as predicted by Mendel's rules of inheritance assuming a single transgene integration site in a non-mosaic parent (i.e. 50% of the pups should be transgenic) or assuming two different transgene integration sites in a non-mosaic parent (i.e. 75% of the pups should be transgenic).

3. Considerations for assay design for each allele type

3.1. General considerations for mRNA quantification

Target organs is selected according to the known pattern of expression of the genes altered and relevant biological systems for the study and, as much as possible, for strong target gene expression (using BiGPS as described in Lindner et al. [14]). The sequence data resource available in Ensembl genome browser (www.ensembl.org) is used to determine the existing splice variants of the gene of interest [16]. Assays are designed to quantify as many as possible protein coding transcripts and always include the main protein coding transcript proposed by Ensembl. When possible, we take into consideration the exon-intron structure of the gene of interest to reduce the risk of false positives from amplification of any contaminating genomic DNA. For each category of alleles, DNA and the resulting anticipated mRNA maps are built using SnapGene v5 software (www.snapgene.com).

3.2. Quantification of nonsense-mediated mRNA decay in knock-out

The mRNA target assay is designed downstream of the floxed exon(s) (Fig. 1A), ideally spanning the two exons that are found in most of the protein coding alternative transcripts and always including the main transcript proposed by Ensembl. Experiments are performed on wild-type and knock-out homozygous littermates harvested at the same age.

3.3. Conditional knock-out mRNA quantification

In a conditional knock-out, the conditional allele should produce a wild-type mRNA, i.e. an mRNA that shows both the sequence and the level expressed in wild-type controls. Expected expression of a conditional allele should result in production of physiological levels of wild-type protein. The comparison between mRNA expression of the conditional and the wild-type control thus aims to evaluate that the conditional mRNA is expressed as a wild-type mRNA. The design used for knock-out mRNA functional validation should be used on wild-type controls and homozygous conditional knock-out mice (Fig. 1A).



Fig. 1. Assay design strategy for each allele type. The mRNA (and DNA when relevant) quantification assay designs are presented for each type of targeted (A-D, F, G) or additive (E) transgenic rodent model described in this article. Grey boxes represent the DNA regions that are identical between each allele structure. The position of each mRNA quantification assay is indicated by a dashed line; the position of a DNA quantification assay is indicated by a full line. The main mRNA(s) that are observed by the expression of each allele are indicated below each genetic map. (A) Design for conditional knock-out (cKO) and knock-out (KO) allele mRNA evaluation. (B) Design for point mutation (PM) allele mRNA evaluation. Red star represents the generated point mutation that can be an insertion, a deletion or a modification of one or few base pairs. (C) cDNA or whole gene humanization allele is represented by blue boxes. (D) IKMC *tm1a* allele is a knockout-first design including a *FRT-LacZ-loxP-Neo-FRT* cassette (purple box) before the critical exon. (E) Additive transgenesis by pronuclear injection of a plasmid or BAC sequence. The transgene is randomly inserted into the animal genome. (F) Conditional knock-out model crossed with a Cre or CreERT2 deleter. The animals generated are mosaics with cells that carry either the cKO allele or the KO allele (G) Conditional FLEEx model crossed with a Cre or CreERT2 deleter. The animals generated are mosaics with cells that carry either the FLEEx allele or the PM allele. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. FLEEx point mutation models

Two assays are designed to quantify the level of mRNA in FLEEx mutant animals before crossing or after crossing with a Cre deleter line, respectively. In most of the FLEEx designs (with an exception shown in [Supplementary Fig. 3A](#)), the FLEEx allele before Cre recombination should be a conditional allele and thus produce physiological levels of wild-type protein. The mRNA RT-qPCR or RT-ddPCR assay is therefore designed in such a way that at least one of the two primers is located in the repeated exon that does not contain the point mutation ([Fig. 1G](#)). The FLEEx allele after Cre recombination should express a point mutation mRNA: the mRNA RT-qPCR or RT-ddPCR assay is therefore designed with one of the two primers in the repeated exon that contains the point

mutation. mRNA expression is compared to that of wild-type control animals.

3.5. Humanized line mRNA quantification

3.5.1. Single nucleotide polymorphism animal model

A primer and probe hybridization arrangement in a competing duplex reaction is used for mutant mRNA quantification for comparison with that of the wild-type sequence as described in Lindner *et al* [[14](#)] and [Fig. 1B](#).

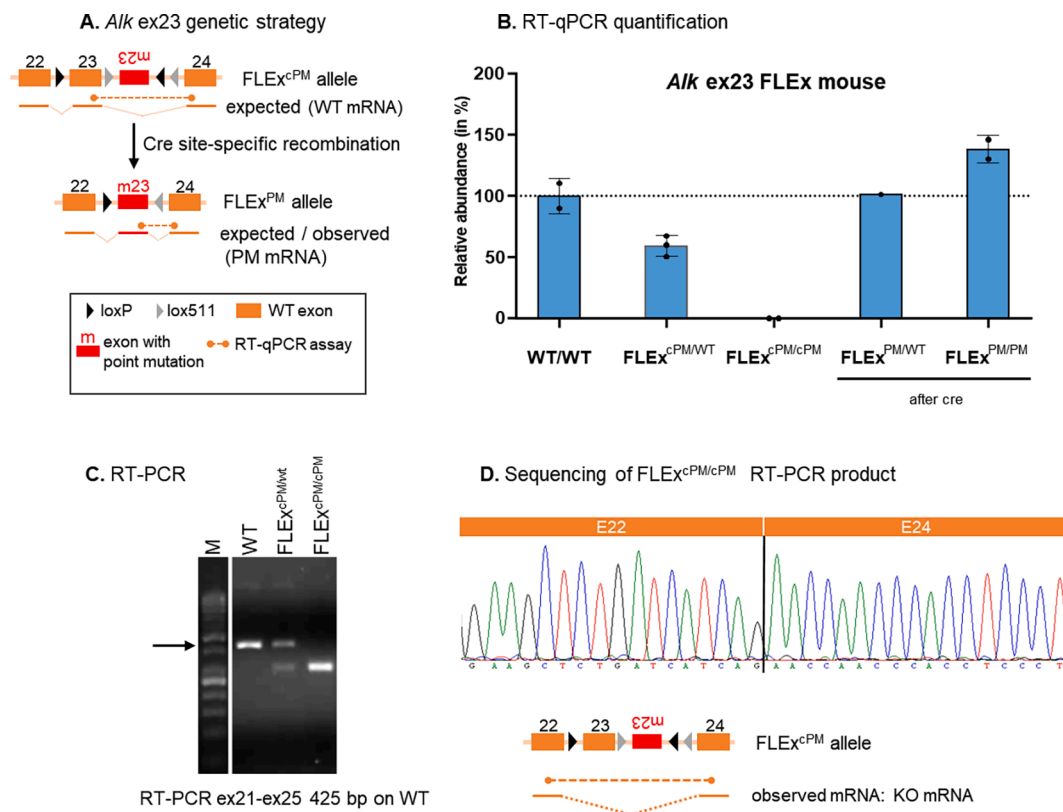


Fig. 2. Molecular characterization of *Alk* ex23 FLEEx model. (A) The genetic strategy for *Alk* ex23 FLEEx model is presented. Exons are displayed as orange boxes. The *Alk* ex23 is a conditional point mutation model (spatiotemporal expression of point mutation after Cre recombination). The mutated exon that contains the point mutation is displayed as a red box. *LoxP* and *lox511* are shown by blue and light grey arrows respectively. The position of each RT-qPCR assay is displayed by a blue dash. Anticipated and observed mRNA levels are displayed for each allele. The wild-type, conditional and point mutation alleles are labelled FLEEx^{WT}, FLEEx^{cPM} and FLEEx^{PM} respectively. (B) mRNA expression was evaluated by RT-qPCR in FLEEx heterozygous and homozygous mice before and after crossing with a Cre deleter. The mRNA expression is expressed as a percentage of that of the wild-type controls. Error bars represent one standard deviation. (C) RT-PCR for wild-type and FLEEx heterozygous and homozygous mice before Cre recombination. (D) Sequencing of RT-PCR product of FLEEx homozygous mice before Cre deletion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5.2. Insertion of the human cDNA and humanization of whole genomic fragment

An assay that is specific to the humanized region is designed and compared to a wild-type assay designed on the same region (Fig. 1C). Humanized heterozygote or homozygote animals are compared to wild-type controls.

3.6. Validation of *tm1a* allele mRNA expression in IKMC mouse lines

The International Knock-out Mouse Consortium (IKMC) has generated more than 5,000 multipurpose mouse models from targeted embryonic stem cells on the C57BL/6N genetic background [17]. The 'knock-out-first' allele (*tm1a*) generates a null allele through splicing to a *lacZ* trapping element contained in the targeting cassette [18]. By crossing with a Cre or Flp deleter, respectively, a reporter null (*tm1b*) or a conditional allele (*tm1c*) can be generated. In order to avoid wild-type mRNA production, the *tm1a* cassette includes the mouse *En2* splice acceptor and the SV40 polyadenylation sequences. However, from our experience, an SV40 polyadenylation sequence is not sufficient to terminate transcription from a strongly expressed promoter (data not shown). Two assays are designed to validate *tm1a* allele mRNA expression. The first assay is designed to only detect wild-type mRNA. One primer is designed in the exon upstream of the trapping targeting cassette; the other primer is designed in the downstream exon, i.e. the critical exon that is deleted in the *tm1d* knock-out allele (Fig. 1D). The second assay is designed to evaluate the leakiness of the trapping targeting cassette and is thus positioned in coding sequence downstream of

the trapping cassette. Homozygote *tm1a* mice are compared to wild-type controls.

3.7. Conditional knock-out crossed with Cre or CreER^{T2} deleter lines DNA and mRNA validations

After Cre mediated recombination, each analysed tissue can be a mosaic of conditional and knock-out cells. Quantifying the level of DNA recombination can therefore only be done using an assay that is specific to the knock-out allele or to the conditional allele (Fig. 1F). Designing the assay on the knock-out allele allows detection of a low level of recombination but because the corresponding qPCR product contains a palindromic *loxP* sequence, we systematically observed very poor efficiency of qPCR, which did not allow a precise quantification of the knock-out allele (data not shown). We did not encounter this issue when using ddPCR (data not shown). Designing a qPCR assay that detects the conditional allele but not the knock-out allele is thus advised even if it will not permit the detection of a low level of target gene inactivation (Fig. 1F).

For ddPCR experiments, we observed that the reference assay used for normalization of DNA target in the vicinity of the target gene provides more accurate quantification than a reference assay on another chromosome (data not shown).

Careful design is also required for mRNA target quantification. Stability of the knock-out mRNA is very variable between genes with knock-outs ranging from very low levels to overexpressed levels (compared to those of wild-type controls; see [5] and chapter 3.1). An assay that will

detect the knock-out mRNA, will not allow for an accurate quantification of the products of the target gene (Fig. 3A and data not shown). However, this assay remains useful to detect target gene inactivation leakiness when only a small proportion of expressing cells are inactivated, but does not quantify it (data not shown). Designing an assay that is specific to the wild-type mRNA (Fig. 1F) is generally more efficient to properly evaluate the efficiency of target gene mRNA reduction (see chapter 4.6).

3.8. Verification of transgenic line segregation by ddPCR and selection of established transgenic lines by mRNA analysis

Because the number of transgenic copies per line can be highly variable (see chapter 5.1) when generating a line by additive transgenesis, quantification is done by ddPCR to improve the precision of copy number quantification. A ddPCR assay is thus designed to be specific to the transgene (Fig. 1E). Additional ddPCR assays can also be designed to cover the length of the transgene sequence, to evaluate insertion of fragmented transgenes and determine transgene integrity [19]. Because a transgene integrates most frequently as head-to-tail and/or head-to-head array concatemers [20,21], a positive droplet may contain multiple transgene copies if the DNA extraction protocol does not fragment the genomic DNA enough. In particular for short transgenes, digestion of genomic DNA prior to ddPCR is recommended, as described by the manufacturer (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf).

mRNA expression analysis is then performed on confirmed established lines using an RT-qPCR or RT-ddPCR assay that is specific to the transgenic mRNA and is then compared to a second assay that is specific to the corresponding endogenous gene (Fig. 1E). It allows validation of the specificity of the transgene expression and its corresponding level compared to that of its mouse wild-type counterpart. Both assays are designed, as far as possible, to detect the same isoforms.

4. Controls for targeted mutagenesis

4.1. Validation of null alleles and nonsense-mediated mRNA decay

4.1.1. Principle and pitfalls

In animals, the most common strategy for generating a knock-out mutation is the deletion of a “critical” exon or of sequences that encode functionally essential protein domains. A critical exon can be defined as an exon that is common to all known isoforms (if possible), is contained in the first half of the coding region (or in exon(s) that encode a well-defined domain), and which upon removal causes a frame shift [22]. In this approach, one or more exons is fully deleted and a premature stop codon is created, which should result in mRNA NMD and degradation of the aberrant peptide produced [23]. Recently, Lindeboom *et al.* and Smith *et al.* showed that premature STOP codons are not sufficient to guarantee mRNA NMD in non-homologous end joining cell culture models [4,5]. In knock-out models achieved through a critical exon approach, stability of the knock-out mRNA is usually not known. We used RT-ddPCR or RT-qPCR to evaluate the presence of target gene mRNA in knock-out homozygotes from 28 GA lines in different tissues

Table 1

Knock-out mRNA expression level evaluated from 28 knock-out homozygote models. Data for each of the GA lines are presented in Supplementary Fig. 1.

Knock-out mRNA expression level ¹	Percent of GA lines
Not detected	0% (0)
<10%	7% (2)
10–30%	24% (7)
30–100%	59% (17)
>100%	10% (3)

¹ by comparison to wild-type controls

for each line (Table 1). Details for each GA lines (target gene and tested organs, level of mRNA expression, modification strategy for each line, position of the assays, etc.) are provided in Supplementary Fig. 1. The analysis of these 28 lines revealed that knock-out mRNAs were detected at variable levels, from very low to higher than mRNA levels in wild-type controls, for most mutated genes regardless of the deleted gene region and its size (Table 1 and Supplementary Fig. 1). Only 7% and 31% of the lines showed knock-out mRNA levels below 10% or below 30%, respectively (Table 1). Deletion of one critical exon led to levels of mRNA expression that varied between that of a very good knock-down to that of no reduction in knock-out mRNA level compared to wild-type controls (Supplementary Fig. 1). Deletions of multiple exons or large deletions including the 3' UTR did not guarantee better NMD (Supplementary Fig. 1).

4.1.2. Process for validation

In critical exon deletion models, knock-out mRNA is expressed at variable levels from low to overexpression (compared to those of wild-type controls) in the various GA lines tested. Even if most of the critical(s) exon(s) lines are likely to be complete knock-outs [24], it cannot be entirely excluded that for some lines, the knock-out mRNA may encode partially functional or unexpected gain-of-function proteins. No line in which promoter sequences were removed was evaluated in this study. However, this alternative type of design could avoid production of truncated protein from the knock-out allele but may have other unintended consequences on the transcriptional landscape of the mutated locus [25].

As the final goal of a KO model is the complete inactivation of a specific gene function, it is always desirable to interrogate the model for the presence of any protein product, for example by using Western blotting [26,27] or by a proteomics approach using mass spectrometry [5].

4.2. Validation of floxed alleles

4.2.1. Principle and pitfalls

Conditional knock-out alleles are widely used in mouse genetics as these enable generation of a cell type-, tissue type- or time-restricted gene inactivation. In the vast majority of conditional lines, *loxP* sequences are integrated into an intronic region, in the same orientation upstream and downstream of the gene sequence to be deleted. The entire region between the two *loxP* sites will be deleted as the result of a site-specific recombination event only in cell lineages in which the Cre protein is expressed and translocated into the nucleus (for CreER^{T2} models). This approach is thus based on the hypothesis that the insertion of *loxP* sequences into an intron does not impact the expression of the targeted, or any other, gene. However, some intronic mutations can have a severe impact on gene expression and are involved in multiple human diseases [28]. Floxed alleles that retain a neomycin-resistance (*neo*) selection cassette in an intronic sequence are not equivalent to conditional alleles as resulting mice may show some phenotypic defects [29]. Hypomorphic phenotypes [30–40] or those that result in an increase in mRNA expression [41] have been frequently observed with the use of *neo* cassettes. Selection markers are thus nowadays generally surrounded by attachment sites (*loxP*, *FRT*, *rox* or similar sites) that can be removed by site-specific recombination (SSR), e.g. using Cre, Flp or Dre recombinases [42]. In many available models, diagnostic restriction sites and *FRT* recombination sites are integrated in the upstream and downstream introns in addition to the two 34 bps *loxP* sequences (Supplementary Fig. 2B, [18]). In the vast majority of papers published, it has not been verified that the conditional allele expression actually results in a wild-type mRNA expressed at normal levels. Using RT-qPCR on 24 conditional mutant lines, no hypomorphic mRNA expression was observed (Supplementary Fig. 2A). RT-PCR experiments did not show alternative splicing or intron retention (Supplementary Fig. 2C).

4.2.2. Recommendations

A careful positioning of the SSR recognition sites (i.e. *loxP*) at more than 100 bps (ideally about 200 bps, depending on studies) from the targeted exons therefore seems to be sufficient to avoid hypomorphic mRNA expression of the conditional allele and absence of unwanted splicing in the vicinity of the floxed sequence. Conditional homozygote animals are important controls in addition to wild-type controls in any experiment.

4.3. FLEx models

4.3.1. Principle and pitfalls

FLEx technology (Fig. 1G) was developed using a combination of *lox* sequences for spatiotemporal inversion of a reporter gene [43,44] or production of conditional gene trap inactivation [45]. As proposed by Schnütgen *et al.*, one possible application of the FLEx switch is the generation of conditional point mutation models (cPM) [43]. Similar to a standard conditional approach, the cPM FLEx allele before Cre recombination should result in the production of physiological levels of wild-type mRNA and protein. We analysed mRNA expression by RT-qPCR before and after Cre mediated recombination on four FLEx mutant lines *Alk* (two models: one cPM model in exon 23 and PM to wild-type switch in exon 25), *Idh* (cPM in exon 3) and *Spg11* (cPM in exon 32). In all four models, we observed a very low level or no wild-type mRNA in mutant animals before Cre mediated switching (Fig. 2 & Supplementary Fig. 3). Additional RT-PCR experiments followed by sequencing were performed on all these models and systematically showed alternative splicing leading to deletion of the repeated and inverted exon(s) of the mRNA product (Fig. 2 & Supplementary Fig. 3). Capulli *et al.* used the same FLEx cPM approach for two genes (*Impd1* and *Cln7*) and also found abnormal splicing leading to the synthesis of non-functional proteins [46].

In all of these cases, the FLEx allele before Cre recombination does not produce the anticipated mRNA and cannot be used to achieve conditional point mutation models.

4.3.2. Strategy for validation

In our hands the FLEx approach for a conditional point mutation did not yield the anticipated mRNA before Cre mediated excision/inversion. To overcome this limitation, we hypothesized that degeneration of one of the repeated exonic sequences coupled with a humanization of one of the duplicated intronic sequences may avoid abnormal splicing (Supplementary Fig. 3M). This FLEx 2.0 method was successfully used for conditional point mutation ([47] and US patent PCT/EP2018/066015). Another alternative developed in the lab is applicable when the desired mutation(s) is/are located in the 3' end of the gene (Supplementary Fig. 3N). In this case, the insertion of a floxed region containing one or more modified exons and followed by an efficient polyA cassette (usually a 3X SV40 polyA cassette) has proven to be effective and nicely shows that a switch in the expression of the wild-type mRNA and protein to the mutated mRNA and protein are therefore valuable alternatives [48]. Systematic evaluation of mRNA or protein level before Cre-mediated deletion with these two alternative approaches is of course recommended. Ideally, homozygous animals without Cre-mediated deletion should be phenotyped in addition to wild-type control animals in any experiment.

4.4. Humanized mouse models

4.4.1. Principle and pitfalls

A large variety of genetic strategies are employed to develop genetically humanized mouse models. Creation of a single-nucleotide polymorphism animal model of human disease by the introduction of single or multi-nucleotide orthologous (causal) mutation is frequently used and is easy to achieve using CRISPR/Cas9 genome editing [1]. Insertion of the human cDNA at the ATG of the mouse orthologue is

another way to humanize a mouse gene using ES cells. Humanization of whole genomic fragments including both exonic and intronic regions can be achieved by pronuclear injection of a human BAC. In recent years, by combining embryonic stem cell and CRISPR/Cas9 approaches, replacement of a mouse gene by the whole human gene has also become achievable [1]. All of these approaches have both advantages and disadvantages. Whatever the allele design [1], both genomic and functional validation of genetic alterations are highly desirable before phenotyping.

For example, RT-qPCR was performed on eight different humanized models generated by insertion of the human cDNA in its mouse counterpart at the ATG position, and it was shown that for one of these eight lines the knock-in allele was not expressed, resulting in the generation of a knock-out model (data not shown).

When whole genomic fragments are inserted (pronuclear injection of a human BAC) or are used to replace their mouse counterparts (replacement by homologous recombination), not only exonic regions but also introns and underlying regulatory elements are humanized. Gene expression patterns and their underlying regulatory processes have substantially diverged between mouse and human, with the exception of a subset of core regulatory programs that are largely conserved [49,50]. The pattern of expression of humanized lines should therefore be systematically evaluated, especially if different isoforms are expected (Supplementary Fig. 4). For example, humanization of *Mrc1* by replacing the mouse gene sequence by its human version leads to different expression levels, but only in specific organs (Supplementary Fig. 4A).

4.4.2. Functional validation of humanized alleles

Generation of humanized mouse alleles remains complex as it is not possible to predict how a human sequence or gene will behave in a mouse context. Target mRNA quantification is an easy first step to evaluate which humanized lines are more likely to be hypomorphic models. Western blotting and functional assays will allow verification of the expression at the protein level. Ideally, a humanized model should be compared to the corresponding knock-out line to verify that the humanized protein can interact with its protein interactors in a mouse context.

4.5. Knock-out first and derivative alleles

4.5.1. Principle and pitfalls

We performed RT-qPCR on nine *tm1a* homozygote models using two assays (Supplementary Figure 5). The first assay (named WT RT-qPCR assay) was designed to detect wild-type mRNA, whereas the second design (named 3' RT-qPCR assay) is anchored downstream of the cassette and should detect both wild-type and chimeric mRNAs (if present). Wild-type mRNA was observed in homozygous *tm1a/tm1a* mice in four out of the nine models tested using the WT RT-qPCR assay, whereas the 3' RT-qPCR assay detected mRNA in seven of the nine lines. Similarly, Hanstein *et al.* detected 30% of wild-type RNA in *Panx1^{tm1a/tm1a}* mice and showed that this model presented a hypomorphic phenotype [51]. Likewise, *Slc25a21* RNA expression represented 29% of wild-type expression in the *Slc25a21^{tm1a/tm1a}* line using a 3' RT-qPCR assay [52]. In the *Cenpj^{tm1a/tm1a}* mutant, a small amount of wild-type RNA was detected by RT-qPCR, and western blotting confirmed the production of low levels of apparently full-length CENPJ protein [53]. Western blotting revealed 5–6% of ANKS1B wild-type protein expression in *Anks1b^{tm1a/tm1a}* mice [54]. *Mcp1^{tm1a/tm1a}* and *Tmen18^{tm1a/tm1a}* mice show very low residual wild-type transcript levels [55,56]. The *tm1a* allele can in some cases produce hypomorphic rather than complete knock-out mutants.

4.5.2. Functional validation

Our experience is that it is desirable to perform RNA quantification and/or protein analyses as control experiments before using *tm1a* lines. *Tm1a* is not expected to be a null allele as the critical region is still

present and may be spliced into transcripts. *Tm1b* is the allele that should be used for all knock-out experiments, as no complete protein can be expressed even if the RNA decay does not occur. Most of the phenotyped alleles in the International Mouse Phenotyping Consortium (IMPC) are *Tm1b* alleles.

4.6. Cre-dependent conditional knock-out (conditional knock-out crossed with Cre and CreER^{T2} deleter lines)

4.6.1. Principle and pitfalls

The Cre-*loxP* system allows cell-specific manipulation of gene expression by recombination of a floxed conditional allele into a knock-out allele only in cells expressing the Cre recombinase and their progeny (spatial control). Following tamoxifen injection, the inducible CreER^{T2} system provides both spatial and temporal control of *loxP* recombination. More details on Cre/*loxP* principles and applications can be found in many reviews (e.g. [57-60]). This technology is very powerful for studying the function of a gene in a particular biological system or cell-type context. However, to avoid misinterpreting conditional knock-out experiments, unexpected complexities resulting from use of the Cre/*loxP* system should not be neglected. Appropriate control groups will permit evaluation of phenotypes induced by the Cre-driver alone [6,58,60] or by toxicity of tamoxifen treatment (e.g. [61,62]). However, ectopic (or unreported) activity of Cre recombinase (e.g. [51,63-66]), leakiness in CreER^{T2} experiments (recombination in the absence of tamoxifen induction or cross-contamination, e.g. [67-70]), low efficiency of recombination and mosaicism (e.g. [71]) are not only linked to the Cre or CreER^{T2} driver but may also be different for each floxed gene [58] and even between littermates [66].

We analysed the outcome of Cre or CreER^{T2} mediated recombination for 20 conditional models crossed with different deleters. Because the knock-out mRNA is often not degraded by NMD (chapter 4.1), designing an assay that only detects wild-type mRNA is required to accurately evaluate the efficiency of mRNA reduction with any Cre deleter (Fig. 3). Using this design strategy, we found no correlation between mRNA reduction (shown as remaining wild-type target mRNA in all corresponding figures) and level of Cre recombination at the genomic level (shown as remaining level of floxed allele; Fig. 3 and Supplementary Figure 6). In particular, four and ten floxed alleles were crossed with the *Alb-CreER^{T2}* deleter and *ROSA26-CreER^{T2}*, respectively (Fig. 3 & Supplementary Figure 6). We found that the efficiency of Cre-mediated recombination was different from one floxed allele to another when using the same deleter in the different organs tested (Fig. 3 & Supplementary Figure 6). This difference is probably not linked to the tamoxifen treatment as the same tamoxifen injection protocol was used systematically (http://mousecre.phenomin.fr/static/pdf/Instruction_Tamoxifen.docx). All mice were injected at the same time point (9 weeks of age). The duration of the experimental procedure may explain differences in mRNA expression in a few cases. For example, with the in *Scap*^{cKO/cKO} line crossed with the *Alb-CreER^{T2}*, deletion is observed after 2 weeks but restoration of *Scap* wild-type mRNA is observed 9 weeks after the tamoxifen treatment (Fig. 3B). The timeline of the experiment therefore needs to be taken in account when analysing phenotyping results.

The size of the floxed region is another factor that limits efficient Cre-mediated excision [72-74]. We did not encounter poor Cre deletion with floxed loci ranging from 0.6 to 4.5 kb using multiple Cre or CreER^{T2} deleters (20 floxed alleles). However, recombination of a floxed 89 kb region was not observed in any of the tissues tested when crossing with the *ROSA26-CreER^{T2}* deleter (data not shown), which confirms that efficient spatial or spatio-temporal Cre/CreER^{T2} deletion is restricted to the use of smaller floxed regions. One hypothesis that remains to be confirmed could be that there is variability in the accessibility of the floxed locus to the Cre recombinase, which may be linked to variable chromatin compaction according to the cell type (Supplementary Figure 6D). Under this hypothesis, cells expressing the target gene are

more likely to be in an open chromatin structure than cells that do not express the target gene [75]. This could result in a lower recombination efficiency in cells expressing the Cre deleter but not expressing the target gene.

4.6.2. Validation of Cre-induced deletion

Cre- or CreER^{T2}-mediated gene inactivation should be verified in all animals used during an experiment. Ectopic (or unreported) activity of Cre recombinase, leakiness in CreER^{T2} experiments, mosaicism and restoration of floxed cells can be different for each floxed line and/or between animals (Fig. 3 & Supplementary Figure 6). This will also allow confirmation of the accuracy of the genotype of each animal (to prevent genotyping errors or animal switches after the initial genotyping). This is in keeping with previous observations that, even in a very standardized platform, more than 5% of mice genotyped twice show discordant genotypes [8]. Moreover, 30% of experimental cohorts show at least one animal with a discordant genotype if unique genotyping characterization is carried out before weaning [8].

Moreover, DNA quantification of the floxed allele is not sufficient to properly determine the efficiency of Cre-mediated recombination. Designing an mRNA assay on the floxed exon(s) allows a better estimation of the efficiency of the knock-down in each tissue (Fig. 3 & Supplementary Figure 6).

When CreER^{T2} lines are used, leakiness can be observed on mutant animals that are not treated with tamoxifen (Supplementary Figure 6C). Recombination of the floxed allele occurs without addition of tamoxifen. A high level of leakiness can be easily detected with assays designed to detect the reduction of the floxed DNA sequence or its corresponding wild-type mRNA (Fig. 1F & Supplementary Figure 6C). However, the use of a design that specifically detects the knock-out allele as a means to detect a low level of leakiness affords higher sensitivity (chapter 3.7). In addition, systematic analysis of a control organ in which the target gene is expressed while the Cre or CreER^{T2} is not, is a valuable method for the detection of ectopic, and in particular germline, Cre activity (see an example of the brain as a good negative control for an experiment with the *ROSA26-CreER^{T2}* line; Supplementary Figure 6 and [71]). Ectopic Cre deletion will be detected, for example, by the unexpected expression of the Cre protein in the germline cells of some animals. Furthermore, control animals from the various genotypes and treatments, i.e. cKO/cKO, Cre or CreERT2 line, WT/WT + tamoxifen (for CreER^{T2} experiments), cKO/cKO × CreER^{T2} deleter + OIL (for CreER^{T2} experiments) should be analysed, at least by using those tests that show a phenotype in the mutant group.

5. Establishing mouse lines produced by additive transgenesis

5.1. Establishment of transgenic lines

Pronuclear injection (PNI) of foreign DNA in one-cell embryos has been widely applied in transgenesis for gene addition. This DNA randomly integrates in the genome [76] most frequently as head-to-tail and/or head-to-head array concatemers [20,21] with nucleotide deletion or insertion at some ends [77,78]. It has been shown that the majority of founder transgenic mice are derived from mosaic embryos [79]. We used ddPCR on ear biopsies to evaluate mosaicism in founders and to validate the establishment of segregated G1 mice (G1 animals are obtained by crossing a G0 founder with wild-type mice). The G1 progeny of 20 different G0 founders were analysed to evaluate the number of transgenic copies in each pup and this was compared to the G0 transgene copy number (TCN, Fig. 4 & Supplementary Figure 8). The data show that only 20% of the lines show correlation between the G0 and G1 generation TCN, whereas for 80% of the lines no correlation is observed (Supplementary Table 1). For 25% of the lines this result could be explained by mosaicism in embryos, i.e. the DNA transgene integrates later than the one-cell embryo stage (Supplementary Table 1 and Supplementary Figure 8C, E, I and O). Result for an additional 20% of the

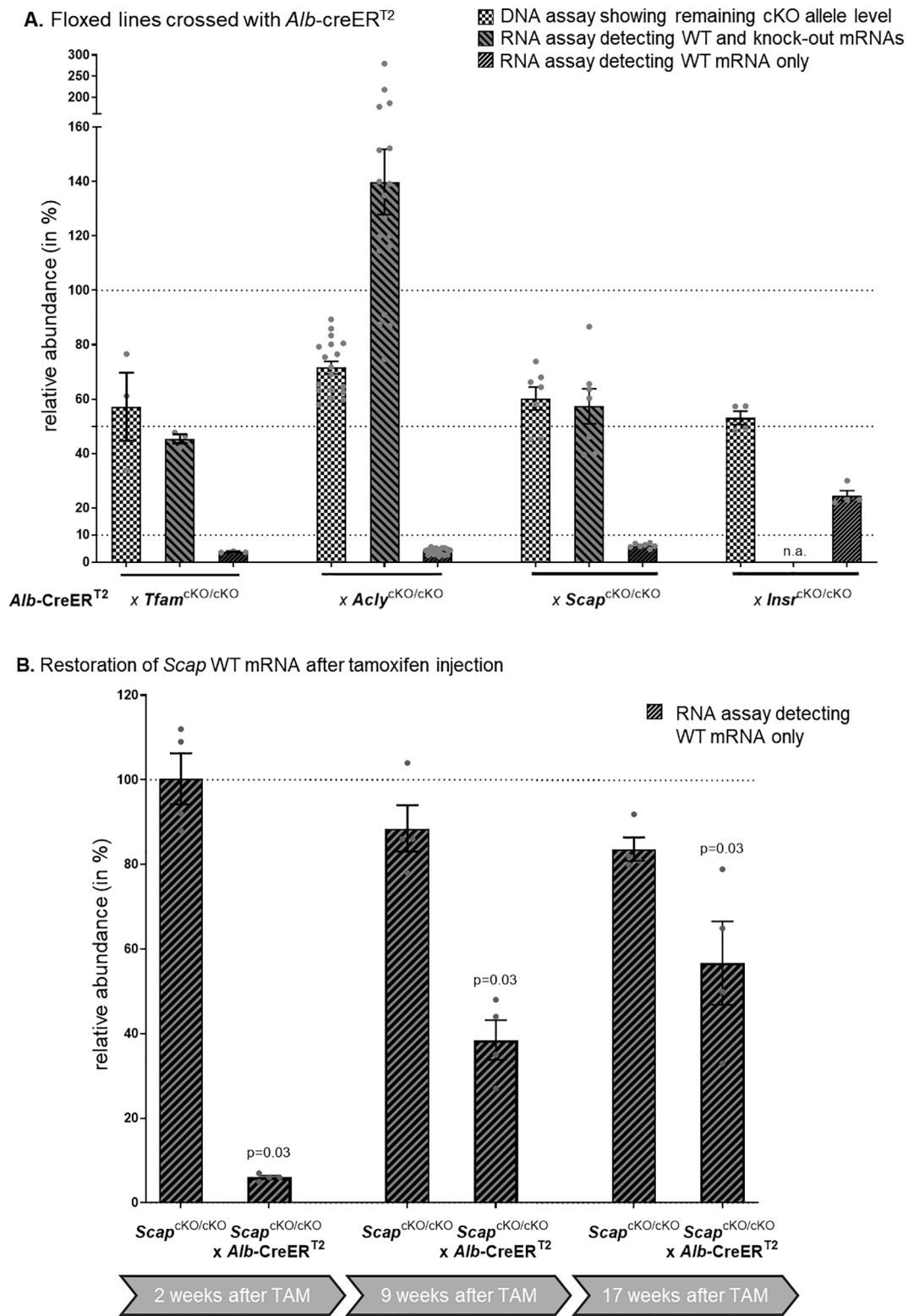


Fig. 3. mRNA and DNA evaluation of floxed allele inactivation after crossing with *Alb-CreER^{T2}* deleter. (A) DNA and remaining mRNA level were analysed 18 weeks after the last tamoxifen (TAM) injection for the *Acly*^{cKO/cKO} line or 2 weeks after for the 3 other floxed lines. mRNA expression was evaluated using two different designs: an assay that can detect both wild-type or knock-out RNA and an assay that can only detect endogenous WT mRNA. The DNA assay only detects WT or floxed alleles. DNA and mRNA levels are expressed as a percentage of those of the wild-type controls. All tamoxifen injections were performed as described in http://mousec.re.phenomix.fr/static/pdf/Instruction_Tamoxifen.docx. n.a.: not applicable (B) The *Scap* WT mRNA level was evaluated 2, 9 or 17 weeks after tamoxifen induction in the *Scap*^{cKO/cKO} × *Alb-CreER^{T2}* model. The experiment was done two times. Restoration of *Scap* wild-type mRNA is observed over time.

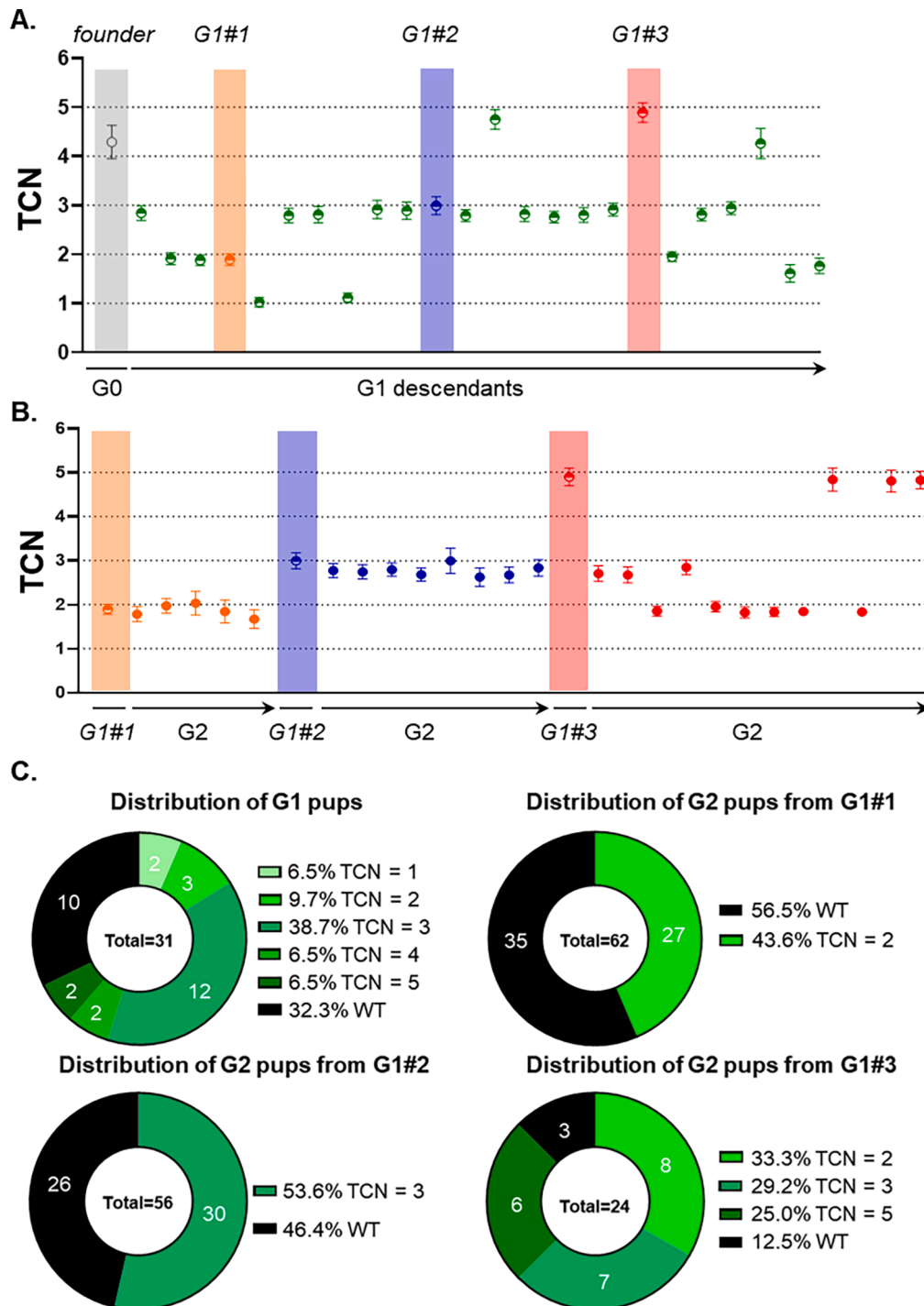


Fig. 4. Multiple sites of transgene integration evaluated by ddPCR and Mendelian inheritance. DNA from lysed biopsies was quantified by ddPCR to evaluate the transgene copy number (TCN) in G0, G1 and G2 resulting mice. In all breeding strategies, transgenic animals are crossed with wild-type mice of the same background to generate the next generation. G0: transgenic founder; G1: pups born from crossing a transgenic G0 with wild-type mice; G2: pups born from crossing a transgenic G1 with wild-type mice. For more clarity, the G1 and G2 WT animals are not shown in (A) or (B). Poisson error bars are indicated for each animal to estimate ddPCR partitioning variability. (A) G0 and corresponding G1 pups TCN. (B) Three G1 mice were selected to produce G2 descendants by crossing with WT mice. G1#1 line (TCN = 2) descendants are wild-type mice or transgenic with TCN = 2 (orange circles). G1#2 line (TCN = 3) descendants are wild-type or transgenic with TCN = 3 (blue circles). G1#3 line (TCN = 5) descendants are wild-type or transgenic with TCN = 2, 3 or 5 (red circles). (C) Distribution of G1 and G2 generations is shown in a donut chart. A chi-squared test was used to evaluate whether the observed ratio of genotypes is statistically different to the expected ratio. The distribution of G1 pups is statistically different from a 50% WT / 50% G1 transgenic mice ratio, i.e. one unique transgene integration that occurred at the one-cell stage in the PNI injected embryo. The distribution of G2 pups generated from the G1#1 or G1#2 line is not statistically different from a 50% WT / 50% G2 transgenic mice ratio, i.e. the expected ratio of crossing a transgenic heterozygote with WT animals. The distribution of G2 pups generated from G1#3 is statistically different from a 50% WT / 50% G2 transgenic mice ratio but not different from a 25% WT / 25% TCN = 2 / mice / 25% TCN = 3 / 25% TCN = 2 + 3, i.e. two sites of transgene integration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lines cannot be explained because of the limit of ddPCR quantification when more than 7 transgene copies per genome are to be detected (Supplementary Table 1 and Supplementary Figure 8 K, L, M & Q). In particular, as transgenes integrate as concatemers, these transgenic head-to-tail or head-to-head arrays are physically bound to each other and are unlikely to be dispersed in the process of generating an emulsion. A positive droplet can contain multiple transgene copies (e.g. two linked copies will be counted as one copy) resulting in poor quantification. More interestingly, 5 out of 20 G0 lines (25%) did not contain a single concatemer but rather contained at least 2 transgene integration sites (Fig. 4, Supplementary Table 1 and Supplementary Figure 8B, D, J, N and P). This result was also confirmed by evaluating the distribution of transgenic G1 versus wild-type G1 animals (Fig. 4 and Supplementary Figure 8). The expected ratio is 50% G1 wild-type and 50% G1 transgenic if the transgene is integrated in a unique site and at the one-cell embryo stage (Supplementary Table 1). A higher transgenic percentage can only be explained by more than one integration site. The last two founders (10%) showed a higher TCN in the G0 founders than in the G1 descendants, with a unique TCN in all G1 positive mice (Supplementary Table 1 and Supplementary Figure 8 A and G). This suggests that some expressing integration events were not transmitted to the progeny, while other non-expressing events were (e.g. integrations in a gene leading to lethality or mosaicism in the tail but not in the germ cells).

The loss of a transgene-induced phenotype in a transgenic line is classically explained by repeat-induced transgene silencing [80–82]. Our data show that a relatively high number of G1 transgenic animals do not have only a single insertion site, but can have two or more insertion sites. A typical breeding scheme for additive transgenesis is to establish one or two different sub-lines by breeding G1 animals with the assumption that each carries only one insertion site. Our findings illustrate that this assumption is not correct and it offers an alternative explanation as to why transgene expression could appear to be lost. We therefore do not recommend that different G1 animals generated from the same G0 founder should be considered as one transgenic line. A careful genomic validation by a precise quantification method such as ddPCR is highly recommended. Alternatively, a characterization of the insertion site in the line founder, e.g. using targeted locus amplification [83], is also a good solution. In G1 and later generations, evaluation of Mendelian distribution can also be used to verify the number of transgene integration events, i.e. when crossing a transgenic mutant with wild-type mice, 50% or 75% of pups should be detected as transgenic for one or two integration events respectively. In G0 founders, Mendelian distribution cannot be used to evaluate the number of integrations as founders can be mosaics. In conclusion, a transgenic line should only be considered as established after crossing with wild type when 50% of the resulting pups are transgenic and show the same TCN. A chi-squared test can be performed to verify whether the observed transgenic distribution is statistically different from the expected Mendelian distribution.

5.2. Selection of established transgenic lines

In addition to segregation of the various concatemer integrations, PNI additive transgenesis is also subject to positional effects, even if these effects can be reduced by using a large genomic transgene [84]. Genome sequences surrounding the transgene integration site can modify its expression resulting in ectopic, weak or even undetectable expression. These integration mechanisms imply that the level of over-expression cannot be controlled and must be evaluated in each established transgenic line. Digital droplet RT-PCR or RT-qPCR can be employed for selection of transgenic lines. In most transgenic lines, the promoter sequence is of mouse origin and in a smaller proportion, the promoter originates from another species (e.g. rat or human) but has an orthologue in mice. Transgenic mRNA expression can therefore be compared to the mouse endogenous gene corresponding to the transgenic promoter. This makes it possible to evaluate the specificity of expression of the transgenic line and to compare the level of transgene

mRNA expression to that of the endogenous gene. This permits selection of the most promising transgenic lines for further phenotypic characterization (Supplementary Figure 9 and [85] as examples).

We also compared the TCN obtained when small (plasmid) and big (BAC) constructs are used. A common belief in the field is that plasmid constructs lead to a higher TCN compared to BACs. Using ddPCR on ear DNA, we did not find a significant difference between plasmid (mean: 5.97, standard deviation: 13.21) and BAC (mean: 8.17, standard deviation: 12.78) PNI founders generated on the inbred C57BL/6N background (Supplementary Figure 7).

5.3. Concluding remarks

DNA and mRNA quantification assays can be highly informative in the validation of GA animals: with appropriate design, a wide variety of artefacts can be detected from mutated alleles that are not expressed as anticipated, or from transgenic lines that are not established. These approaches do not, however, allow detection of the full extent of unexpected consequences of animal model targeted mutagenesis or transgenesis. In particular, prior to validating the functionality of the generated mutation, it is essential to verify that the generated mutation is the expected one, i.e. the mutant allele shows the designed sequence and structure. For example, CRISPR/Cas9 genome editing may lead to a variety of unwanted mutations that need to be discarded [3]. A gene mutation can also impact its genetic environment; for example, by perturbing the expression of a neighbouring gene, which could explain the observed phenotype [52,86]. Validation of the product(s) from a modified allele is also essential to detect expression of residual or truncated proteins. To assure the relevance of a scientific result, the use of a GA model should ideally be complemented by other biological models, such as cellular models or those from different species, or by human data, as these can provide additional evidence for or against the integrity of the model.

CRedit authorship contribution statement

Loic Lindner: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Pauline Cayrou:** Methodology, Validation, Investigation. **Thomas W. Rosahl:** Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition. **Heather H. Zhou:** Conceptualization, Writing - original draft, Writing - review & editing. **Marie-Christine Birling:** Conceptualization, Writing - original draft, Writing - review & editing. **Yann Hérault:** Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition. **Guillaume Pavlovic:** Conceptualization, Methodology, Validation, Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ymeth.2021.04.001>.

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