Characterization and Validation of Cre-Driver Mouse Lines

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ABSTRACT

Conditional gene manipulations in mice are increasingly popular strategies in biomedical research. These approaches rely on the production of conditional genetically engineered mutant mouse (GEMM) lines with mutations in protein-encoding genes. These conditional GEMMs are then bred with one or several transgenic mouse lines expressing a site-specific recombinase, most often the Cre recombinase, in a tissue-specific manner. Conditional GEMMs can only be exploited if Cre transgenic mouse lines are available to generate somatic mutations, and thus the number of Cre transgenic lines has significantly increased over the last 15 years. Once produced, these transgenic lines must be validated for reliable, efficient, and specific Cre expression and Cre-mediated recombination. In this overview, the minimum level of information that is ideally required to validate a Cre-driver transgenic line is first discussed. The vagaries associated with validation procedures are considered next, and some solutions are proposed to assess the expression and activity of constitutive or inducible Cre recombinase before undertaking extensive breeding experiments and exhaustive phenotyping. *Curr. Protoc. Mouse Biol.* 1:1-15 © 2011 by John Wiley & Sons, Inc.

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INTRODUCTION

Much of the recent progress in mammalian functional genomics has been driven by the use of genetically engineered mutant mouse (GEMM) lines. Informative mutations can now be generated in almost any mouse gene, either through classic gene targeting (conventional germline knockouts) or, increasingly, through conditional gene targeting, a strategy that allows temporal and spatial control of the onset of gene ablation/modification (Lewandoski, 2001; Metzger and Chambon, 2001; Branda and Dymecki, 2004; Argmann et al., 2005). The most successful approach for conditional gene targeting is based on the Cre-*loxP* system (Sauer and Henderson, 1989; Lakso et al., 1992; Rajewsky et al., 1996; Nagy, 2000; Collins et al., 2007; Birling et al., 2009), in which the allele of interest is flanked by recognition sites for the Cre DNA recombinase, the *loxP* sites. When such "floxed" mice are bred with transgenic mice expressing the Cre recombinase in a tissue-specific fashion, the gene of interest is knocked out/altered only in this particular tissue. An added sophistication is the inclusion of temporal control, which can be achieved using ligand-activated chimeric recombinases composed, for instance, of the fusion of the Cre recombinase with the ligand-binding domain of a mutated form of the estrogen receptor (ER), which can be activated only by synthetic ER ligands (e.g., tamoxifen), but not by natural estrogen-like compounds (Feil et al., 1996; Danielian et al., 1998; Schwenk et al., 1998; Vasioukhin et al., 1999; Metzger and Chambon, 2001; Hayashi and McMahon, 2002). This strategy avoids problems with early lethality, developmental effects, and compensatory mechanisms, which are often apparent in classical germline or somatic knockout models.

In 2006 large collaborative research efforts were launched by the European Commission, the U.S. National Institutes of Health (NIH), and Genome Canada to establish libraries of mutant mouse ES cell lines, each of which

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carries an altered or "floxed" allele of a single gene (Austin et al., 2004; Auwerx et al., 2004; Collins et al., 2007). These mutant ES cell mutations can be readily transformed into mice using blastocyst injection, and the mutation activated by crossing the mouse bearing the floxed allele with a Cre-driver strain to induce the mutation in spatially and temporally determined patterns. The full power of conditional GEMMs, however, can only be exploited if transgenic mouse lines expressing the Cre recombinase in a tissue-, organ-, and cell-type-specific manner are available to allow the creation of somatic mutations.

When searching the literature, investigators will find many Cre-driver transgenic lines that have been used successfully. However, for multiple reasons, data available for mouse Cre lines are often incomplete. Ideally, a minimum level of information should be available to users to allow selection of appropriate Cre transgenic lines for gene-deletion experiments–specifically: (1) specificity and efficiency of Cre expression and Cre-mediated deletion; (2) reproducibility of the deletion from animal to animal for the same floxed allele; (3) reproducibility of the deletion with different floxed alleles; (4) timing of Cre expression and Cre-mediated deletion for noninducible Cre mouse lines; (5) kinetics and efficiency of Cre induction and absence of leakage for inducible Cre mouse lines; and (6) phenotypes caused by either integration-mediated mutagenesis, by Cre "toxicity," or by passenger genes in the construct. Although community efforts are underway to fully characterize newly produced Cre-driver mice (e.g., CREATE European Project, *http://dev.creline.org/home*; Mouse Clinical Institute, *http://www.ics-mci.fr/crezoo.html*), this ideal level of information is typically not known for currently available mice. To ensure the correct interpretation of resulting phenotypes, it is thus up to investigators to carefully verify the most critical parameters before setting up their experiment.

In that context, this overview discusses critical parameters associated with production, validation, and use of Cre-driver transgenic lines, and presents some simple assays that can be used for characterization of these mouse lines. These assays can be combined to characterize newly produced strains, ultimately streamlining the establishment of

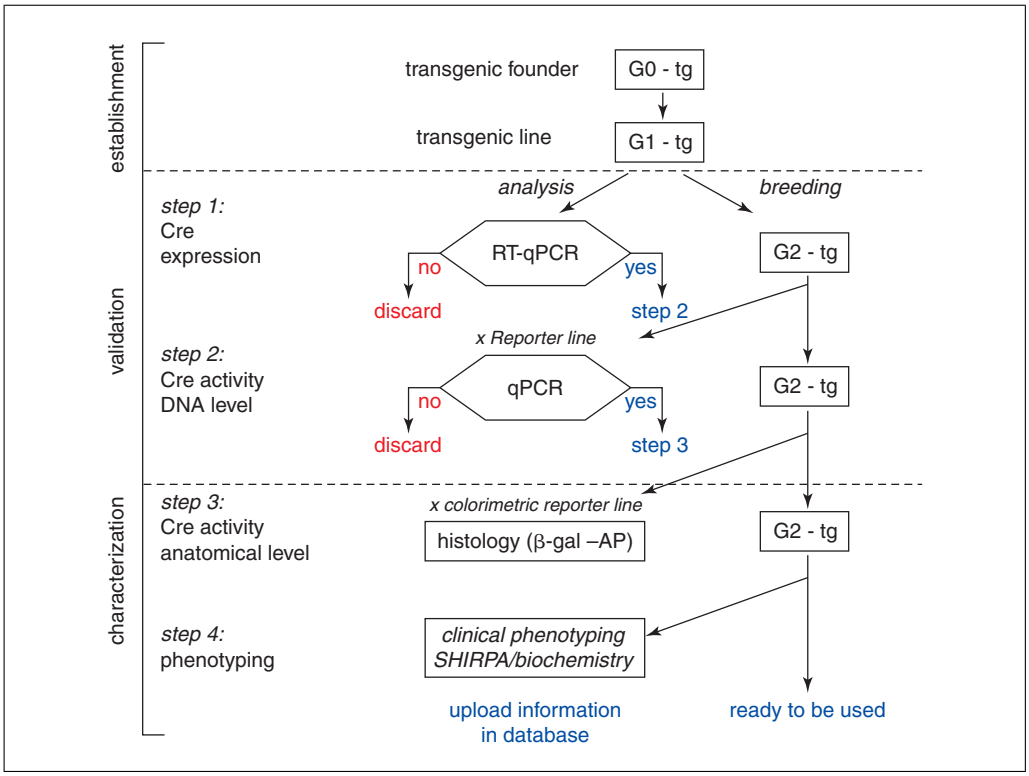

Figure 1 Flow chart combining simple assays allowing one to characterize Cre recombinase expression and activity in transgenic Cre lines from the genomic to the cellular level. Each step can also be applied to complete or confirm available data on Cre-driver lines. G, generation; tg, transgenic; β–gal, β-galactosidase; AP, alkaline phosphatase; RT-qPCR, quantitative real-time reverse transcriptase PCR; qPCR, quantitative PCR.

homogeneously validated Cre lines, or individual assays can be applied to existing lines for which available information is incomplete and/or requires confirmation.

A flow chart summarizing the appropriate application of the strategies described in this article is shown in Figure 1.

SPECIFICITY AND EFFICIENCY OF Cre EXPRESSION

Because the key feature of conditional gene targeting is its spatial or temporal restriction, the first parameter to control is the fidelity of Cre recombinase expression. Indeed, all systems used to generate tissue-specific Cre-expressing mice rely on appropriate promoters and/or enhancers to control the expression of Cre in a specific cell lineage. However, recombination could occur in cells outside of the desired target tissue due to unexpected or inappropriate expression of the transgene. This can be due to incomplete information about the gene whose promoter is used to drive recombinase expression, a transgene-insertion effect, or, in the case of inducible Cre, a "leakage effect" of the construct in other tissues. A broad, rapid, and cost-effective screen to verify the full expression pattern of the Cre recombinase in the selected transgenic lines can be performed by quantitative real-time reverse transcriptase PCR (RT-qPCR). By testing a large range of organs, information on ectopic or unexpected expression can be obtained rapidly before starting crosses with the floxed-allele transgenic mice.

Table 1 List of 25 Samples for Analysis of Cre Expression by RT-qPCR

System	Organ
Vascular	Aorta
	Heart
Digestive	Jejunum
	Colon
	Liver
	Pancreas
	Stomach
Skeletal	Bone
Metabolism	BAT[a]
	WAT[b]
	Muscle
Respiratory	Lung
Hematopoietic	Spleen
	Skin
Nervous	Olfactory bulbs
	Cortical and subcortical area
	Hypothalamus
	Hippocampus and thalamus
	Cerebellum
	Brainstem
	Spinal Cord
Urogenital	Ovary/Testis
	Kidney
Sensory	Eye

[a]BAT, brown adipose tissue.
[b]WAT, white adipose tissue.

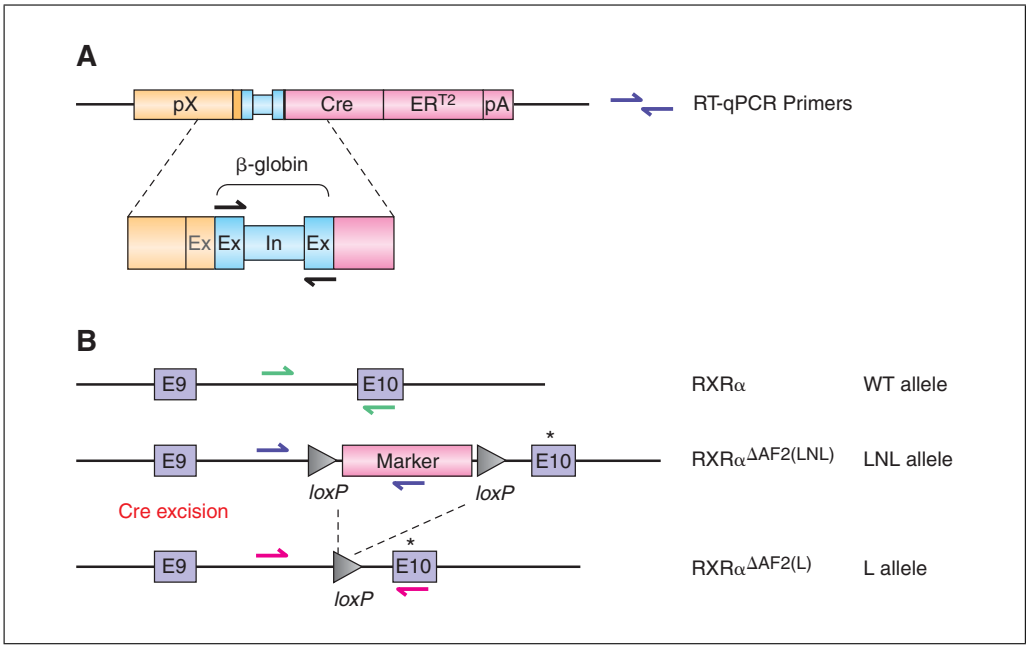

Figure 2 (**A**) Schematic representation of the CreER^{T2} transgene used for pronuculei injection (Feil et al., 1997; Indra et al., 1999). The pair of primers used for RT-qPCR amplification of Cre transcripts is located in the β-globin region between a tissue-specific promoter X (pX) and the CreER^{T2} gene. (**B**) RXRα$^{\Delta AF2(LNL)}$ mouse line. This line is used as a floxed reporter line for determination of Cre recombinase activity by qPCR. A floxed Neo cassette is inserted between exon 9 and 10 of the RXRα gene, and a mutation (*) is present in exon 10 (Mascrez et al., 1998). Three pairs of primers have been designed that allow specific amplification of the wild-type (WT, green), floxed (LNL, blue), and excised (L, pink) alleles. Ex, exon; In, Intron; pA, polyadenylation site; pX, promoter of the gene X.

Experiments can be performed on one male and one female per line, with a minimum number of 25 samples, as suggested in Table 1, in order to cover the major body systems. However, depending on the expected tissue specificity of Cre recombinase expression, additional samples can be added. According to the Cre transgene used to generate the selected Cre-driver line, a set of primers is designed in a common part of the Cre cassette. In the case of the CreER^{T2} cassette, which has been largely used to produce tamoxifen-inducible Cre expression (Feil et al., 1997; Indra et al., 1999), specific primers can be designed in the β-globin intron (Fig. 2A). As a control of sensitivity, the expression of the endogenous gene whose promoter is used to drive Cre expression should also be measured. Standard validated procedures for RT-qPCR can be applied, as described elsewhere (Bookout et al., 2006; Gofflot et al., 2007; *http://empress.har.mrc.ac.uk/*), and in accordance with Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009; *http://www.gene-quantification.de/miqe.html*).

As an example of the predictive value of this assay, analysis in the Ppm1a-CreER^{T2} line is described here, in which CreER^{T2} expression is driven by the promoter of the ubiquitous "protein phosphatase 1A, magnesium-dependent, alpha isoform" gene (Lifschitz-Mercer et al., 2001). Also described is the Vil1-CreER^{T2} mouse line, in which the recombinase is targeted to the epithelial cells of the intestinal crypts (Meseguer and Catterall, 1987; Pinto et al., 1999; Robine et al., 1997). Both lines are produced and available at the Mouse Clinical Institute (*http://www.ics-mci.fr/crezoo.html*). Several lines generated after microinjection of the same construct were analyzed, and both the expression pattern and expression level were compared. In the Ppm1a-CreER^{T2} line, Cre transcripts were

Figure 3 *(figure appears on next page)* Characterization of Cre expression in the ubiquitous Ppm1a-CreER^{T2} (**A, B**) and digestive tract-specific Vil1-CreER^{T2} (**C**) mouse lines. (A, C) Comparison of relative Cre expression between different transgenic lines (*N* is at least 2 for each line) determined by RT-qPCR in 25 tissue samples. (B) Comparison of relative expression of *Cre* versus the endogenous *Ppm1a* mRNAs ($N = 2$).

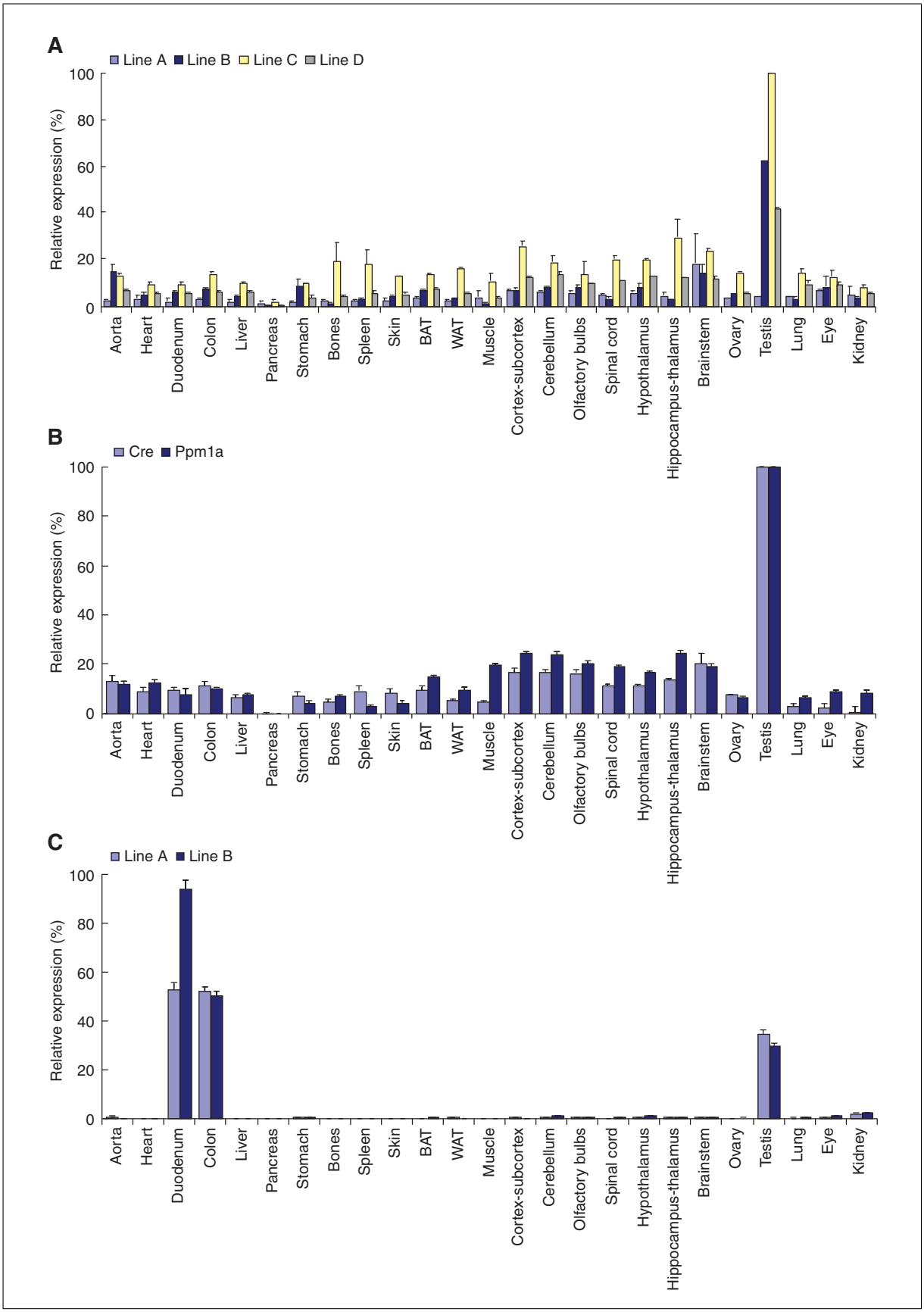

Figure 3 *(legend appears on previous page)*

amplified in 25 RNA samples obtained from two animals per line. Relative Cre expression was easily detected in most of the 25 samples analyzed, confirming the ubiquitous nature of the promoter selected (Fig. 3A). One transgenic line (line C) expressed the transgene at a higher level, with little variation between individuals and organs. In these lines, the expression level of *Cre* mRNA was close to that of the endogenous *Ppm1a* mRNA, as illustrated in Figure 3B. Notably the testis, an organ expressing high levels of the *Ppm1a* mRNA, expressed the highest amount of CreER^{T2} mRNA. *Cre*, and *Ppm1a* mRNAs were detected at very low levels in the pancreas, a tissue notorious for its high content of RNase, making RNA extraction challenging (Chirgwin et al., 1979). In contrast, amplification of *Cre* transcripts in 24 organs from two different Vil1-CreER^{T2} mouse lines revealed significant *Cre* mRNA expression only in the jejunum, colon, and testis (Fig. 3C), with relative levels of *Cre* mRNA being higher in line B. For both lines, the level of *Cre* expression was in the same range as the level of the endogenous *Vil1* mRNA (data not shown). Importantly, this analysis revealed the presence of *Cre* transcripts in the testis, a site of expression that was not expected for the *Vil1* promoter. Infidelity of *Cre* expression and recombination in the germline has previously been reported by others (Schmidt-Supprian and Rajewsky, 2007). This example emphasizes the importance of verifying ectopic/unexpected expression to avoid misinterpretation of phenotype due to recombination in other cells than the desired target.

SPECIFICITY AND EFFICIENCY OF Cre-MEDIATED DELETION

Although RT-qPCR can provide easy and sensitive detection of *Cre* expression, the information most needed by investigators is about Cre-mediated recombination. As opposed to *Cre* expression analysis, Cre activity can only be tested in animals that have been crossed with mouse lines harboring a floxed allele and, for inducible Cre lines, that have been injected either with the inducer or vehicle. Although for most published Cre lines recombination properties have been validated by reporter gene studies, users should be aware that the efficiency of recombination can be locus dependent, and, therefore, the recombination pattern obtained with a particular reporter gene does not necessarily predict that of other floxed genes (see Vooijs et al., 2001; J. Becker and B. Kieffer, pers. comm.). Indeed, the chromatin structure at the locus of interest, the state of DNA methylation, and the transcriptional activity seem to affect the efficiency of recombination. In addition, it has been reported that the ability of a floxed target gene to be recombined could also vary between cell types (Kellendonk et al., 1999). This was potentially explained by differential accessibility of Cre to *loxP* sites due to cell type- and development-specific chromatin conformations. Before starting an extensive phenotypic analysis, it is thus mandatory to monitor recombination at the target locus.

The most common procedures used to examine the pattern of Cre-mediated recombination in various tissues are Southern blot analysis and simple PCR. Although these procedures are robust, they do not provide a quantitative evaluation of the recombination and are of restricted sensitivity, especially when limited samples are available. An alternative to score for both the efficiency and specificity of Cre recombinase deletion at the DNA level is quantitative PCR. This procedure can (i) provide information on Cre excision efficiency with high sensitivity and reproducibility, (ii) evaluate the reproducibility of deletion from animal to animal on the same floxed alleles, (iii) evaluate the reproducibility of deletion between different floxed lines and, (iv) for inducible Cre lines, verify the efficiency of the tamoxifen induction on the CreER^{T2} transgene activity.

This analysis of Cre recombination activity is performed in animals that have been crossed with any mouse lines harboring a floxed allele, either a so-called "reporter line" or the transgenic line with the floxed allele of interest. As an example, at the Mouse Clinical Institute, the reporter line used to test the recombinase excision activity by qPCR is a floxed RXRα transgenic line, the RXR$\alpha^{\Delta AF2\ (LNL)}$ (Mascrez et al., 1998; Fig. 2B). Crosses between Cre lines and this reporter line are set up to obtain double transgenic mice, and three pairs of primers are used to specifically amplify the wild-type (WT), floxed (LNL), and excised (L) alleles (Figure 2B). In the case of CreER^{T2} transgenic animals, bigenic mice are injected either with tamoxifen or vehicle before analysis. For this step, the selection of samples can be made on the basis of the RT-qPCR data, if available—i.e., only positive Cre expression samples are analyzed. Additional samples of one positive system may also be analyzed in-depth, e.g., more segments of the digestive tract can be analyzed as illustrated below in the case of the Vil1-CreER^{T2}.

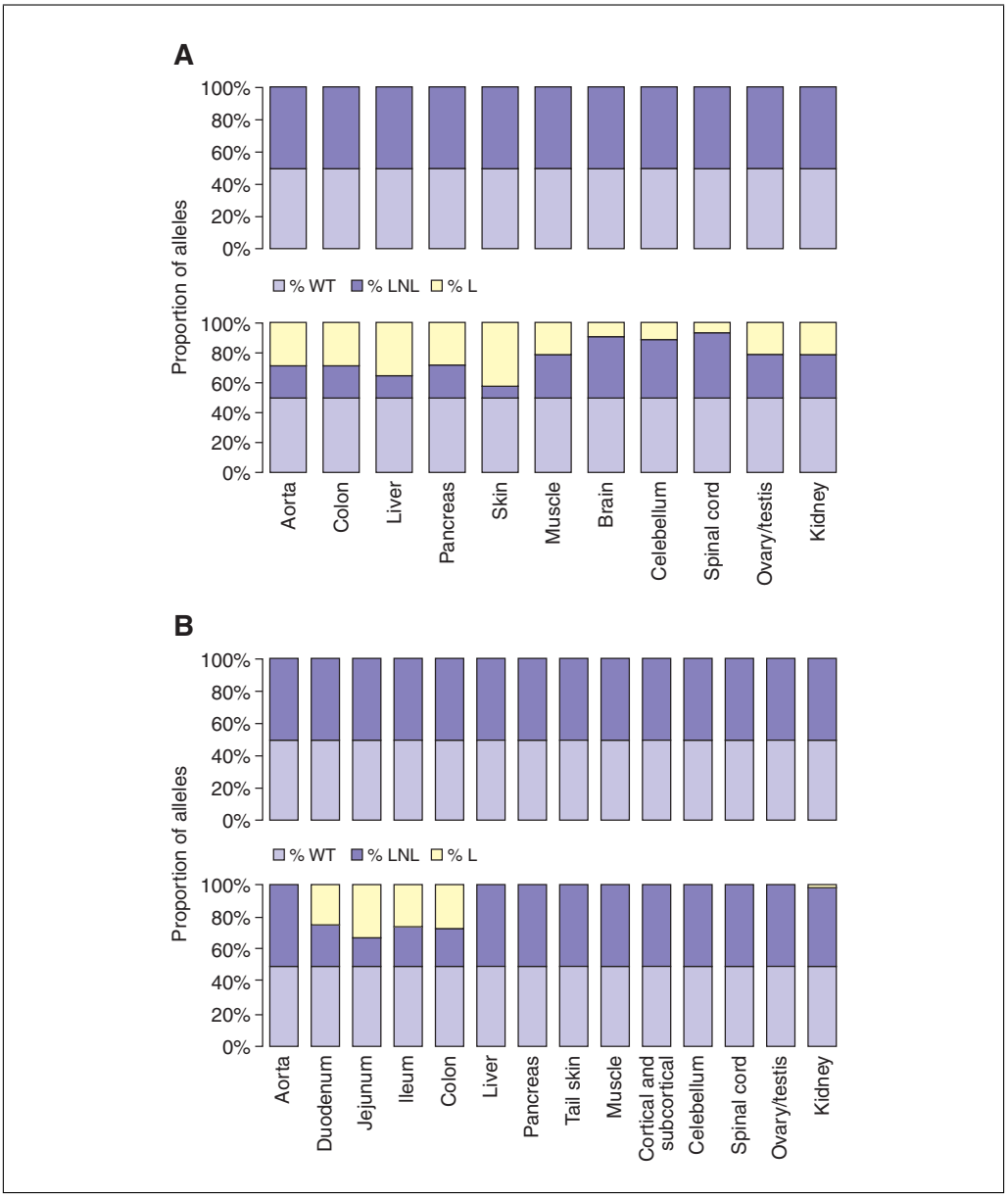

Figure 4 Determination of Cre-mediated excision by qPCR in the ubiquitous Ppm1a-CreER^{T2} (**A**) and digestive tract-specific Vil1-CreER^{T2} (**B**) mouse lines. Comparison of the percentage of excised allele (L) versus floxed (LNL) and wild type (WT) allele in double transgenic animals Ppm1a-CreER^{T2}/RXR$\alpha^{\Delta AF2(LNL)}$ and Vil1-CreER^{T2}/RXR$\alpha^{\Delta AF2(LNL)}$ mice injected with vehicle (top) or with tamoxifen (bottom). For tamoxifen injections, tamoxifen (Sigma, cat. no. T56648) was prepared at 10 mg/ml in sunflower seed oil (Sigma, cat. no. S5007). Intraperitoneal injection of 100 μl of this solution was performed for 5 consecutive days (1 mg/mouse/day) with mice aged 10 weeks old, and whose weight was >20 g. Identical amounts of sunflower seed oil (vehicle) were administered following the same protocol to control mice.

To illustrate the predictive value and sensitivity of this test, Figure 4 shows the analysis of the Ppm1a-CreER^{T2} and Vil1-CreER^{T2} mouse lines. In samples dissected from Ppm1a-CreER^{T2}/RXR$\alpha^{\Delta AF2\ (LNL)}$ mice injected with vehicle, only the WT and floxed alleles were present, while the excised allele was detected in all the 11 tissues analyzed from tamoxifen-injected double transgenic animals (Fig. 4A). The proportion of excision ranged from 5% to 43%, the WT allele being 50%. The highest level of excision was observed in the skin and liver, and the lowest levels in the three samples from the central nervous system (CNS). The relatively low level of excision in the brain of the Ppm1a-CreER^{T2} mice, with regard to Cre mRNA expression levels, is most likely due to insufficient entrance of tamoxifen into brain cells. Indeed, induction of Cre activity in the brain seems to be slower than in other organs,

perhaps due to the blood-brain barrier and/or to the slower renewal of cells. For brain targeting, it is thus recommended to perform analysis 1 month, instead of 1 week, after the last injection of tamoxifen (Metzger and Chambon, 2001; Weber et al., 2001). In comparison, in bigenic Vil1-CreER^{T2}/ RXR$\alpha^{\Delta AF2\,(LNL)}$ mice injected with tamoxifen, the excised allele was detected only in the digestive tract, at a relative proportion of 15% to 30% (Fig. 4B). No excised allele was detected in the reproductive organs of the selected line, despite the fact that *Cre* expression was measured by RT-qPCR in the testis. This could be due either to the sensitivity of this test or to lower tamoxifen access to this tissue.

ANATOMICAL PATTERN OF Cre-MEDIATED DELETION

As most transgenic Cre lines are driven by cell-specific promoters, the required level of information for validation is the location of Cre excision activity within specific functional or cellular compartments of an organ. The classic way to characterize Cre activity at the cellular level is to cross Cre-driver lines with colorimetric reporter lines, such as the ROSA26, ACZL, and ZAP reporter mouse lines (Fig. 5). In the ROSA26 reporter line, the ROSA26 allele is targeted with a Cre excision–conditional *lacZ* reporter (Soriano, 1999). In the ACZL reporter line, a floxed CAT transcription unit prevents *lacZ* expression in absence of Cre-mediated recombination (Akagi et al., 1997). The Z/AP reporter line (Lobe et al., 1999) utilizes two reporters: the *lacZ* reporter marks cells before excision occurs, while the heat-resistant human placental alkaline phosphatase (hAP) marks cells after Cre-mediated DNA excision. As for the qPCR test, the analysis is performed on samples dissected from double transgenic animals. However, samples need first to be embedded, sectioned, and stained for either Xgal and/or hAP.

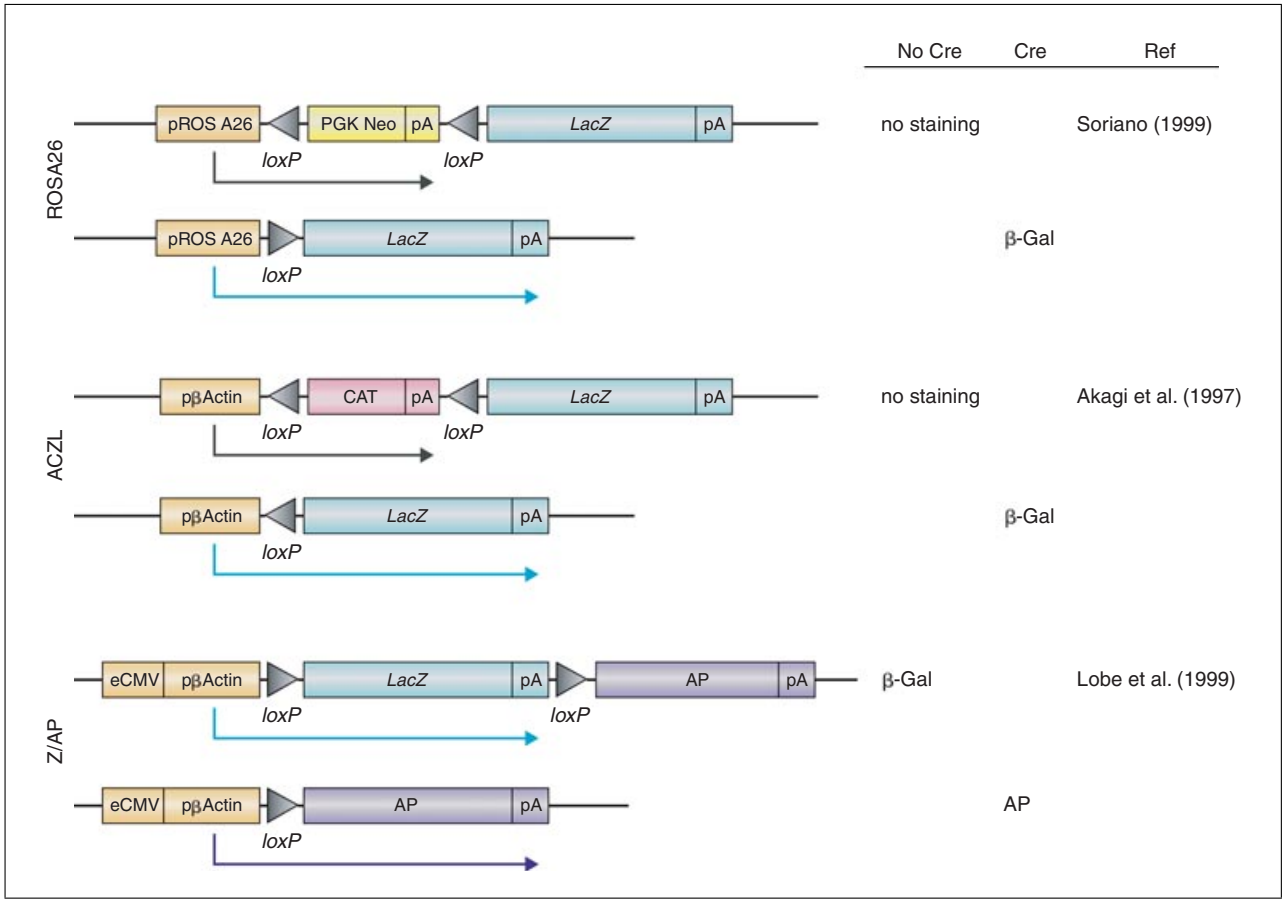

Figure 5 Schematic representation of the construction and activity of the three most popular colorimetric reporter lines used to test for Cre activity in Cre-driver transgenic lines. β-gal, β-galactosidase; AP, alkaline phosphatase; CMV, cytomegalovirus; PGK, phosphoglycerate kinase; CAT, chloramphenicol acetyltransferase; pA, polyadenylation site.

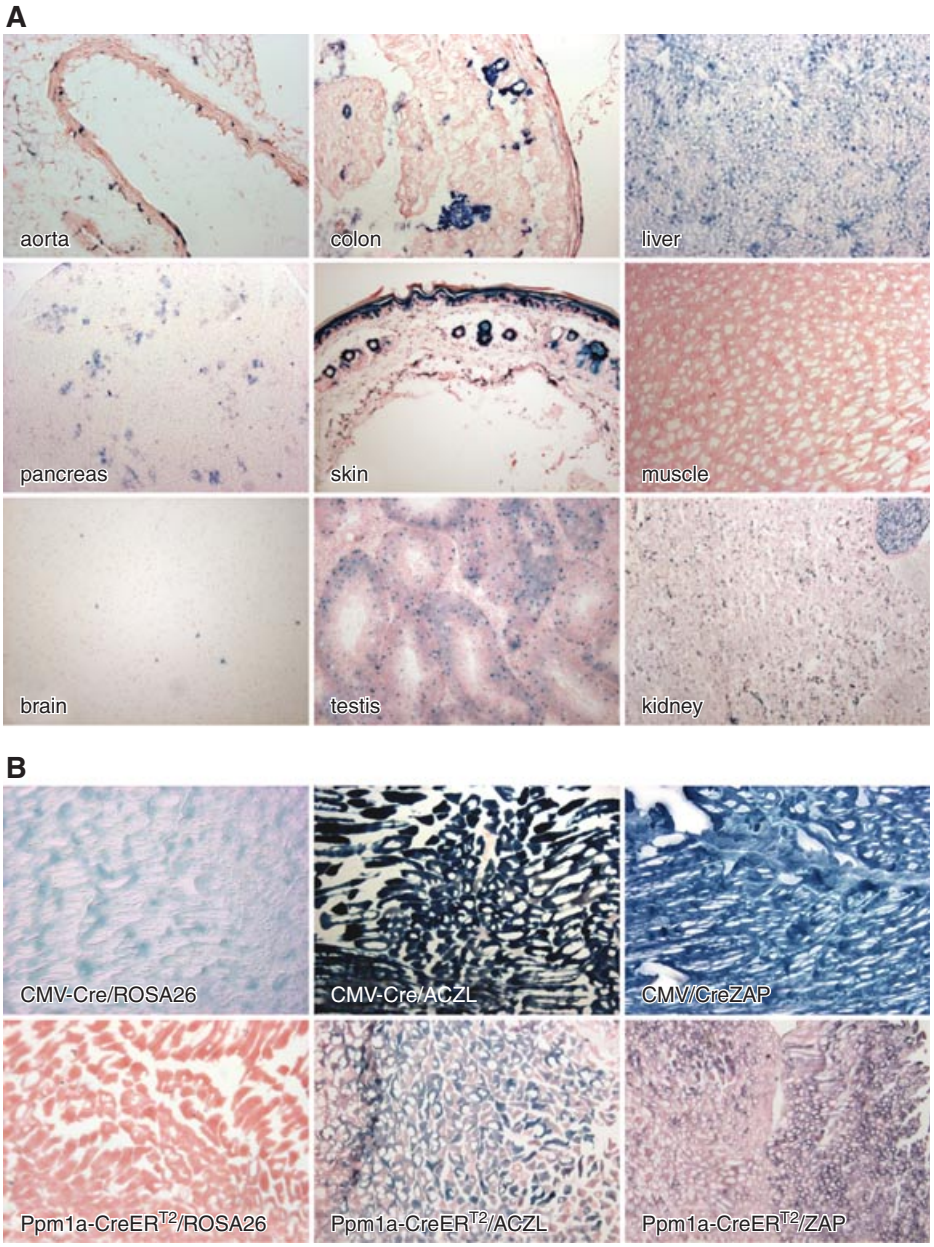

Figure 6 Characterization of Cre activity at histological level in the Ppm1a-CreER^{T2} mice line. (**A**) Cre-mediated expression of the reporter gene β-galactosidase in nine organs dissected from double-transgenic Ppm1a-CreER^{T2}/ROSA26 mice injected with tamoxifen. (**B**) XGal (ROSA, ACZL) and hAP (Z/AP) staining of muscle sections revealing (i) the reporter expression pattern in three different colorimetric reporter lines crossed with a CMV-Cre deleter mice (bottom row) (Dupe et al., 1997), and (ii) the localization of Cre activity in Ppm1a-CreER^{T2} mice crossed with each of these reporter lines (top row).

In the case of CreER^{T2} transgenic animals, bigenic mice are also injected either with tamoxifen or with vehicle 1 week or 1 month before sample collection. The selection of samples could be made on the basis of the RT-qPCR and/or qPCR analysis; in that case, only tissues in which positive Cre expression and/or recombination activity have been observed are further analyzed, limiting the number of samples to be processed. However, for mouse lines in which Cre is targeted not only to a particular tissue but to a specific cell type that may represent only a very small proportion of the organ cellular population, the sensitivity of RT-qPCR or qPCR may be limited, and it is advisable to confirm negative results at the

histological level to avoid discarding potentially valuable lines.

A matter of concern with this procedure is the potential lack of ubiquitous expression of the reporter in existing reporter lines, especially at adult stages. In addition, tissue-specific Cre lines generated by pronuclear injection often exhibit mosaic expression of the recombinase (Schwenk et al., 1998), meaning that only sub-regions of the tissue/organ show active recombination. To illustrate these points, Cre activity was characterized in detail at the cellular level in Ppm1a-CreER^{T2} mice crossed with the ROSA26 reporter line. Bigenic Ppm1a-CreER^{T2}/ROSA26 mice were injected with either tamoxifen or vehicle. Xgal staining of sections revealed the activity of the Cre recombinase in all but one organ, the muscle, analyzed in tamoxifen-injected double transgenic mice (Fig. 6A). In all organs positively labeled, the area of excision within an organ was mosaic and the cell excision within a tissue was not complete. The liver and skin displayed the highest level of excision, while the lowest level was observed in the brain, in line with the qPCR analysis. No significant Cre activity was detected in mice injected with vehicle (data not shown). The absence of staining observed in the muscle was unexpected in light of the Cre expression and activity data. To confirm this observation, Cre recombination activity was evaluated, and detected, in the muscle after crosses with the ACZL and ZAP reporter mouse lines (Fig. 6B, bottom row). This analysis revealed that absence of Xgal staining in the muscle of Ppm1a-CreER^{T2}/ROSA26 mice was associated with the ROSA26 reporter line and not with absence of Cre recombination activity. This could be due to the absence or weak expression of the ROSA26 reporter in this tissue, a hypothesis supported by the higher Cre excision level observed in the ACZL and ZAP reporter mouse lines, which express higher levels of the reporter gene in skeletal muscle, as evaluated after crossing with a ubiquitous deleter, the CMV-Cre mouse line (Dupe et al., 1997; Fig. 6B, top row).

The cellular characterization of Cre activity in the Ppm1a-CreER^{T2} underscores the value of using different reporter mouse lines according to the targeted organ/tissue. Among the colorimetric reporter lines classically used in the literature, a preliminary comparison indicated that the ROSA26 line seems the most adequate for the majority of promoters, as it showed reporter activity in the larger number of organs evaluated after crossing with a ubiquitous Cre-deleter mouse line, while the ACZL and Z/AP displayed expression in a more restricted number of tissues (O. Wendling and D. Metzger, unpub. observ.). Our study, however, revealed the usefulness of these two lines for tissue-specific analysis, e.g., in the skeletal muscle. To address the problem of the lack of ubiquitousness of reporter lines, detection of Cre mRNA or Cre protein at the anatomical level by in situ hybridization (ISH) or immunohistochemistry (IHC) can be used. These two procedures are discussed below.

ADDITIONAL OR ALTERNATIVE PROCEDURES TO DETECT Cre EXPRESSION

To map *Cre* expression at the anatomical level, nonradioactive in situ hybridization (ISH) using digoxigenin-labeled probes can be performed in a standard 5-step procedure: hybridization of the probe to pretreated tissue at 65°C; stringent post-hybridization washes; blocking steps to prepare for the immunodetection; primary antibody anti-DIG-AP incubation; and colorimetric alkaline phosphatase detection (Chotteau-Lelievre et al., 2006; Gofflot et al., 2007; Knoll et al., 2007). Although this procedure may require a higher level of expertise, it also has several advantages. It can be used in parallel with, or in place of, the RT-qPCR, as it scores for *Cre* expression and does not require crosses with a transgenic floxed line. As such, it is thus totally independent of any reporter activity, and also independent with respect to cellular access of inducer such as tamoxifen. It can be combined with other detection procedures, either by double in situ hybridization or immunohistochemistry, allowing precise characterization of the cellular population targeted by Cre expression. For those less familiar with ISH, immunodetection procedures for Cre protein have been previously described (Kaelin et al., 2006; Knoll et al., 2007).

To illustrate these two procedures, two inducible tissue-specific Cre lines were used: the Tph2-CreER^{T2} (gift of P. Chambon and D. Metzger) and the Ins1-CreER^{T2}, in which *Cre* expression is targeted to highly restricted cell populations, the raphe serotonergic neurons and the β-cells of pancreatic islets, respectively. As illustrated in Figure 7, ISH was successfully used to detect *Cre* mRNA in the raphe nuclei of the Tph2-CreER^{T2} mice (Fig. 7A). *Cre* expression was similar to *lacZ* staining on brain sections from tamoxifen-injected

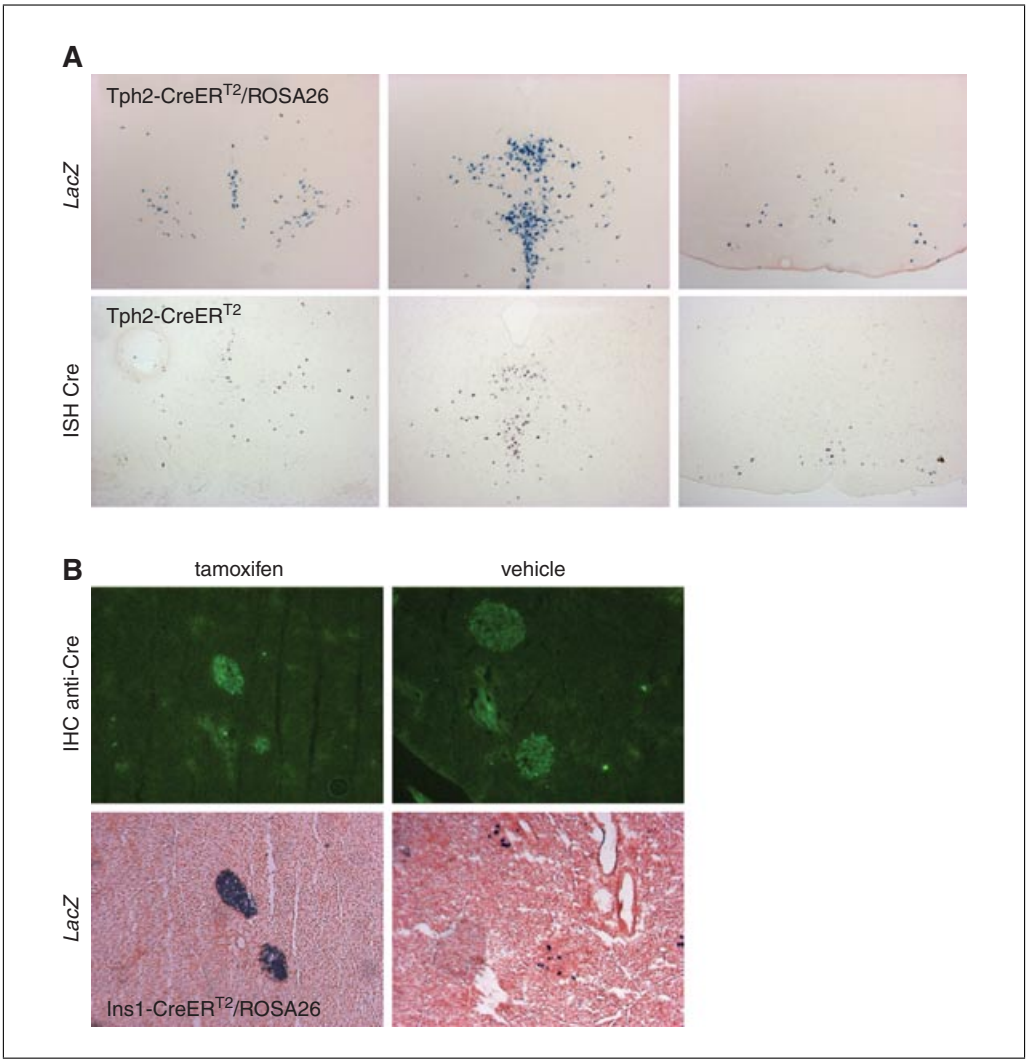

Figure 7 Characterization of Cre activity at histological level in the Tph2-CreER^{T2} and Ins1-CreER^{T2} mouse lines. (**A**) Sections through the pons, midbrain, and medulla revealing Cre activity in the raphe nuclei through Cre-mediated expression of the reporter gene β-galactosidase (Tph2-CreER^{T2}/ROSA26 mice injected with tamoxifen, top row) and Cre expression through ISH with a specific Cre probe (Tph2-CreER^{T2} mice, bottom row). (**B**) IHC detection of Cre protein and Xgal staining of pancreas sections of tamoxifen or vehicle-injected Ins1-CreER^{T2}/ROSA26 mice revealed specific Cre protein content and Cre activity restricted to the islets of Langerhans. ISH procedures have been described elsewhere (Chotteau-Lelievre et al., 2006; Gofflot et al., 2007). For IHC, primary rabbit anti-Cre (1:8000 dilution, VWR, cat. no. 69050-3) was used with goat anti-rabbit antibody coupled to horseradish peroxidase (1:100 dilution; Invitrogen, cat. no. G-21234) as secondary antibody. After washing, Cre was visualized by FITC-tyramide amplification (1/50, 30-min incubation) (PerkinElmer, cat. no. SAT701B).

Tph2-CreER^{T2}/ROSA26 mice, revealing that all 9 nuclei of the raphe in the midbrain and medulla were specifically labeled.

As demonstrated by the analysis of the Ppm1a-CreER^{T2} line, the pancreas is an organ for which the isolation of RNA is particularly challenging (Chirgwin et al., 1979) and *Cre* mRNA could not be detected reliably in pancreas samples of Ins1-CreER^{T2} mice. In that particular case, IHC was used and allowed the detection of Cre protein in the islets of Langerhans of the Ins1-CreER^{T2} mice (Fig. 7B). This result was further confirmed at the histological level in Ins1-CreER^{T2}/ROSA26 mice, in which specific Cre-mediated excision was detected by *lacZ* staining in the islets of Langerhans of the pancreas.

PHENOTYPIC CHARACTERIZATION OF Cre LINES

Transgenic lines produced by conventional transgenesis can develop unexpected

phenotypes due to integration-mediated mutagenesis or passenger genes in the construct (Lusis et al., 2007). In addition, a high level of Cre protein expression can result in cellular toxicity (Forni et al., 2006; Schmidt-Supprian and Rajewsky, 2007). In that context, successfully characterized lines should also be subjected to a final functional test. Indeed, functional abnormalities in Cre mice could be a confounding factor for the interpretation of the phenotype observed when that Cre line is used to delete a gene of interest. When producing a new Cre-driver transgenic line, a standard and simple behavioral, biochemical, and metabolic phenotyping procedure would allow one to discard Cre mouse lines with interfering phenotypes before distribution and archiving. First, to evaluate the general health and basic neurological status, the modified SHIRPA protocol (*http://empress.har.mrc.ac.uk/browser/*) can be used, as it is a rapid, high-throughput non-invasive and non-stressful test suited for a global evaluation of the phenotype (Mandillo et al., 2008). Second, clinical and basal metabolic parameters in G2 mice maintained under basal chow–fed conditions should be monitored (Champy et al., 2004). Finally, some specific tests could be used to evaluate the functions of the organ(s) to which Cre expression is specifically targeted. For example, blood pressure, heart rate, heart weight, and histology could be specifically evaluated in mice with Cre targeted to cardiac muscle, while rotarod test, grip strength, endurance running, and muscle histology could be investigated in mice with Cre targeted to skeletal muscle (see *http://empress.har.mrc.ac.uk/browser/* for detailed procedures).

Instructive of the importance of such a phenotypic characterization, the Vil1-CreER^{T2} mice have a severe functional abnormality which is due to the presence of a passenger gene, the G-protein coupled receptor *Tgr5* (Thomas et al., 2008) in the BAC construct, precluding its use for metabolic studies (Fig. 8A). *Tgr5* mRNA is 5-fold overexpressed in the Vil1-CreER^{T2} mouse line

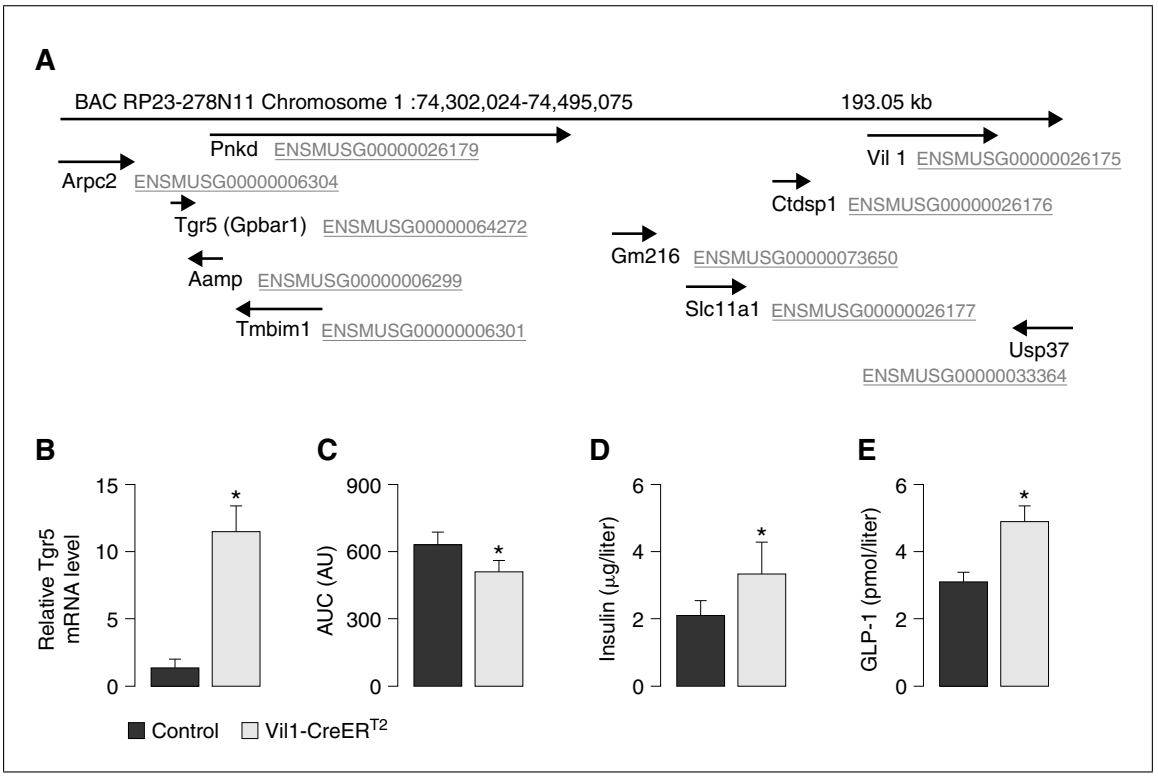

Figure 8 Phenotypic analysis of the Vil1-CreER^{T2} transgenic line. (**A**) Schematic representation of the mouse BAC used for the construction of the Vil1-CreER^{T2} transgene. (**B**) Expression of mRNA levels of the GPCR *Tgr5* in the ileum of control and Vil1-CreER^{T2} transgenic mice. (**C**) Mean ± SD of the area under the curve during an oral glucose tolerance test in control ($N = 8$) and Vil1-CreER^{T2} ($N = 8$) mice fed a high-fat, high-sucrose diet for 12 weeks. (**D**-**E**) Insulin and GLP-1 levels measured in the serum 15 min after the administration of the oral glucose load in the control and Vil1-CreER^{T2} transgenic mice of panel (B).

(Fig. 8B). As *Tgr5* controls the expression of GLP-1 in entero-endocrine cells (Thomas et al., 2009), which in turn improves insulin secretion, Vil1-CreER^{T2} mice have improved glucose tolerance with a reduced area under the curve (AUC) in oral glucose tolerance tests (Fig. 8C). The higher insulin release as a consequence of the increase of GLP-1 levels after a glucose challenge explains the improved glucose tolerance (Fig. 8D-E). In other cases, the phenotype could be the consequence of insertional mutagenesis. In such conditions, it can be worth testing Cre lines derived from another founder.

SUMMARY

Advances in the sophisticated manipulation of the mouse genome now allow the generation of mutant mice for disease modeling and functional analysis by conditional mutagenesis. In the coming years, site-specific recombination transgenic mice will become necessary tools for most scientists to generate conditional mutations. Although a few initiatives attempt to establish and/or index a range of transgenic mouse lines that express Cre recombinase in different tissues (e.g., Gensat, *http://www.gensat.org/index.html*; CreXMice, *http://nagy.mshri.on.ca/cre/*; MUGEN Mutant Mice database, *http://bioit.fleming.gr/mugen/mde.jsp*; CREATE, *http://dev.creline.org/home*), most of the current data are disparate, heterogenous, and incomplete. It will thus be up to Cre users to thoroughly characterize and/or validate the appropriate Cre-driver mouse lines before undertaking extensive breeding experiments and exhaustive mutant analyses that could otherwise lead to inconclusive or incorrect conclusions. As already mentioned, investigators will have to test their selected Cre lines on their own conditional mutants, as efficiency of Cre-mediated excision varies from one allele to another. When bigenic mice are generated and phenotyped, it is mandatory to check in parallel that Cre-mediated excision has indeed occurred in the organ of interest.

In this overview, a set of basic assays is described that can be used either to confirm existing data or as a streamlined and standardized characterization scheme for newly established Cre lines. Some of the artifacts and problems that can be associated with Cre-driver mouse lines are also discussed, with emphasis on the importance of the characterization steps. Finally, the more a given Cre line is used, the more of these issues will be evaluated. Investigators using Cre mouse lines are therefore encouraged to upload their information onto databases where all information is centralized and available to the scientific community, as exemplified by the Cre-X-Mice database (Nagy et al., 2009).

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