Cre Recombinase Activity Is Inhibited *In Vivo* but not *Ex Vivo* by a Mutation In the Asymmetric Spacer Region of the Distal LoxP Site

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Abstract

The cre/loxP recombination system is a valuable tool used to generate tissue specific genomic rearrangements in mouse models. The deletion of a region of interest flanked by two loxP sites is accomplished by the recombinase (cre) enzyme, which binds to the inverted repeat segments of two loxP sites and recognition of a conserved TA sequence in the asymmetric central spacer region "ATAACTTCGTATA -NNNTANNN-TATACGAAGTTAT. *In vivo*, we found that a single T to C mutation at position 4 of the central spacer region in the distal (3′) loxP site, completely inhibited the recombination reaction in two conditional mouse models. These mice were generated using a mitochondrial methionyl-tRNA formyltransferase (*Mtfmt*) gene targeted construct and cre transgene under the control of tissue-specific promoters: calcium/calmodulin-dependent kinase II alpha (*Camk2a*-cre) and myosin light polypeptide 1 (*Myl1*-cre). Surprisingly, transient transfection of a plasmid expressing cre in dermal fibroblasts derived from the same mutant floxed *Mtfmt*(loxP/loxP) mice line, successfully deleted the region of interest. This study demonstrates the sequence specificity required *in vivo*, the possibility of bypassing this limitation by expressing high levels of cre recombinase *ex vivo* and raises concerns related to the quality control of large scale production of gene targeted constructs and mice.

Keywords

mitochondria; cre-lox; recombination

RESULTS AND DISCUSSION

The mouse mitochondrial methionyl-tRNA formyltransferase (*Mtfmt*) is a nuclear gene encoding for a protein with crucial role in mitochondrial protein synthesis in mammals. Homozygous disruption of the *Mtfmt* gene in mice has been reported to be embryonic/ preweaning lethal (as described in the Mouse Genome Informatics [MGI] web site; http://

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www.informatics.jax.org/allele/key/570424). In humans, mutations in this gene have been associated with Leigh syndrome, a severe neurometabolic disorder combined with oxidative phosphorylation deficiency (Haack *et al.*, 2013). We used an *Mtfmt* targeting construct with conditional potential to create and characterize tissue specific mouse models of *Mtfmt* deficiency in central nervous system and skeletal muscle. The conditional potential approach is offered using the cre/loxP recombination technology to produce an *Mtfmt* conditional knockout allele in mice (Skarnes *et al.*, 2011).

The "Knockout first" (initially a non-expressive form) allele with conditional potential (*Mtfmt* tm1a(KOMP)Wtsi) mouse was acquired as cryopreserved embryo from the international Knockout Mouse Consortium University of California (UC) Davis, project KOMP-CSD (ID: 25662). The mouse *Mtfmt* is a nuclear gene of 9 exons and 17,273 bp located in chromosome 9 (9qC). The targeted gene with conditional potential contained a 5′ homology arm of 4651 bp which includes the first 3 exons followed by a trapped cassette (flanked by flippase recognition target –frt- sites) inserted in the intronic region between exons 3 and 4, and a 3′ arm of 5240 bp containing exons 5 and 6. The conditional targeting construct was designed inserting loxP sites flanking exon 4 identified as a critical region. This exon is common to all transcript variants and when deleted predicted to result in frame shift and a null phenotype.

To generate *Mtfmt* conditional knockout (KO) mice, a founder heterozygous female was recovered from the cryopreserved *Mtfmt* embryos. The integrity of the construct was confirmed by short PCR amplification of different regions of the allele (Fig. 1), which included the proximal (5′) loxP and distal (3′) loxP sites flanking critical exon 4 and the wild type allele. In order to convert the non-expressing form of the construct and use the conditional potential, mice heterozygous for the conditional *Mtfmt* allele were crossed to homozygous flippase (Flp) transgenic mice (not shown). Successful deletion of the trapped cassette was confirmed by the presence of a diagnostic 539 bp band (postFlp) (Fig 1) or a 396 bp band confirming the wild type allele. Integrity of the distal loxP site resulted in a 274 bp band also used to determine zygosity (Fig 1). Homozygous *Mtfmt*(LoxP/LoxP) floxed mice, which were required for the last crossing were obtained by intercrossing heterozygous *Mtfmt*(+/LoxP). Deletion of the critical exon was generated initially by crossing heterozygous *Mtfmt*(+/LoxP) mice with a transgenic line expressing recombinase (cre) under the control of two different tissue-specific promoters: calcium/calmodulin-dependent kinase II alpha (Tg(*Camka*-cre)3Szi or *Camk2a*-cre) (Dragatsis & Zeitlin, 2000) and myosin light polypeptide 1 (*Myl1tm2(*cre*)Sjb* or *Myl1*-cre)(Bothe *et al.*, 2000).

Positive *Camk2a-cre* and *Myl1-cre* transgene mice were confirmed by PCR amplification of the *cre* transgene region and the presence of a fragment of 281 bp along with the amplification of a gene control of 420 bp (Fig 1). Final tissue specific *Mtfmt* knockout in central nervous system (CNS) and skeletal muscle were generated by crossing homozygous *Mtfmt*(LoxP/LoxP) with double-transgenic heterozygous *Mtfmt*(+/LoxP) *Myl1*-cre(+/−) for the muscle model and *Mtfmt*(+/LoxP) *Camk2a*-cre (+/−) in the case of the CNS model.

Both models, CNS KO *Mtfmt* and Skeletal Muscle KO *Mtfmt* conditional mice were then characterized phenotypically during the first 4 months of age with no indication of

dysfunctional phenotype using different behavioral tests (not shown). None of the models showed any significant difference in body weight when compared to littermate controls. We did not notice any difference in their locomotor activity using spontaneous cage activity or treadmill test and no apparent defects in motor coordination and balance using the rotarod test (not shown). The lack of the expected disease phenotype in our conditional KO mice led us further test whether cre/loxP recombination occurred successfully in both conditional KO models (Fig 2). We evaluated tissues from cortex and hippocampus in the CNS KO mice (Fig 2a) where expression of the cre recombinase transgene driven by the *Camk2a*-cre promoter has been shown to start at postnatal day 1 and has worked successfully in several models developed in our laboratory (Diaz *et al.*, 2012). We did not detect any recombinants in the CNS KO mice using the specific primers that amplify the putative deleted region in the *Mtfmt* gene after cre recombinase activity. Same results were observed in the skeletal muscle KO mice model (Fig 2b). In this case, we tested quadriceps and gastrocnemius where cre expression driven by the *Myl1*-cre promoter has been shown to start at embryonic day 13.5 (Bothe *et al.*, 2000; Diaz *et al.*, 2005) In both KO mice models we also confirmed the retention of the proximal and distal loxP sites and the presence of the cre transgene in the experimental KO DNA samples which confirmed the absence of recombination despite the presence of the loxP/cre elements *in vivo*.

Parallel to our *in vivo* studies we also generated a stable *Mtfmt* KO cell model *ex vivo* using dermal fibroblasts derived from a 3 month adult homozygous *Mtfmt*(LoxP/LoxP) mouse followed by immortalization and monoclonal expansion (Fig 2c). In this case, transfection of a plasmid expressing cre recombinase enzyme under the CMV promoter, generated recombinants that showed amplification of the putative deleted region by PCR with the same set of primers used in the *in vivo* models (red arrows, Fig 2a, 2b, 2c). This confirmed cre mediated recombination *ex vivo* and the successful generation of *Mtfmt* KO clones from these fibroblasts.

The absence of recombination *in vivo* and successful recombination *ex vivo* led us to sequence the (5′) proximal (not shown) and (3′) distal loxP specific sites in both models using cortex from the CNS *Mtfmt* KO mice and quadriceps samples in the skeletal muscle *Mtfmt* KO mice. (Fig 3). Sequencing analysis detected a T>C mutation at position 4 of the asymmetric central spacer region in the distal loxP site in both models which blocked the recombination reaction. We confirmed that this mutation was also present in the *Mtfmt* KO dermal fibroblasts *ex vivo* model, which surprisingly did not inhibit the recombination reaction and deleted the region of interest (Fig 3).

We wondered whether the mutation originated as a *de novo* event during the breeding strategy used to generate conditional mice in our facility. In order to clarify this issue, we used DNA from the founder heterozygous female acquired from the original KOMP embryos to amplify and sequence the loxP site regions of the *Mtfmt* modified allele and found the same T>C mutation in the distal loxP site. This finding and further analysis by KOMP confirmed the mutation was present not only in the Embryonic Stem cell line used to derive this KOMP *Mtfmt* mouse, but also in other ES lines made with the same *Mtfmt* construct. According to KOMP, they do not know of other ES clones, altering different genes, containing this mutation, suggesting that it happened in the specific targeting plasmid

for *Mtfmt*. After we contacted KOMP with this information, they offered to correct the mutation of the distal LoxP *in vivo* by CRISPR editing in oocytes. However, repeated attempts were not successful.

The deletion of the region of interest flanked by two loxP sites by the recombinase (cre) enzyme involves a precise binding of cre protein to the 13 bp inverted repeat segments of both loxP sites and recognition of an 8bp sequence in the asymmetric spacer region "ATAACTTCGTATA -NNNNNNNN-TATACGAAGTTAT". The role of the nucleotide sequence promiscuity in the spacer region and the effects of homology between both loxP sites in the efficiency of cre recombination have been investigated previously by other groups (Lee & Saito, 1998; Missirlis *et al.*, 2006; Sheren *et al.*, 2007). These studies have found that sequence changes in the 8 bp spacer region reduce recombination efficiency at different degrees (40 to 70%) and in some cases efficiency decreases further if there is a mismatch homology in spacer sequences between both, proximal and distal loxP sites.

When evaluating the wild type asymmetric spacer region ATG**TA**TGC, *in vitro* studies have reported the central nucleotides TA in positions 4–5, to be conserved and essential for the recombination reaction (Missirlis et al., 2006). Further studies on the cre-mediated recombination mechanism by Lee et al (Lee & Saito, 1998) demonstrated that a change in position 4 resulting in a ATG**CA**TGC sequence, blocks the resolution of the Holliday junction intermediate strand exchange step in an *in vitro* recombination assay. However, when using loxP sites with matching mutated spacer sequences, recombination efficiency improved and reached up to 75% efficiency. Requirements for the central TA sequence are still controversial. Studies *in vitro* and *in vivo* using a cre-expressing E.coli by Sheren (Sheren et al., 2007) have reported the central "TA" sequence not to be required for cre mediated recombination.

Our *in vivo* studies generating a conditional mouse model using two different established cre drivers, showed that T>C change in position 4 of the asymmetric spacer region of the distal (3′) loxP site prevents the recombination reaction. This finding indicates that promiscuity in position 4 is not permissive during the cre-mediated recombination *in vivo*. Although the possibility of inhibition due not only to conserved base specificity requirements in the central "TA" sequence, but as result of the mismatch created between the proximal loxP (ATG**TA**TGC) and mutated distal loxP (ATG**CA**TGC) could be considered, this rationale does not explain our findings *ex vivo*, where the mismatch created by this mutation resulted in successful recombination in fibroblasts derived from the same loxP/loxP mouse. Therefore, we speculate that high cre expression levels in our *ex vivo* model was higher than the transgenic expression *in vivo*. These differences could affect the DNA-binding affinities as previously proposed by Hippel (von Hippel & Berg, 1986). We attempted to compare the levels of cre transcripts in the expressing tissues of *Myl1*-cre (muscle) and *Camk2a*-cre (cortex) with the transfected immortalized fibroblast cells in culture, but these results were not informative for two reasons: 1) Only a few fibroblast are transfected with lipid based reagents; 2) These few transfected cells express very high levels during the first 72 hours but these levels reduce dramatically once the cre plasmid integrates and stable clones are analyzed. This decrease is particularly strong for genes controlled by the CMV promoter (Xia *et al.*, 2006; Liew *et al.*, 2007).

Our observations demonstrate the relevance of changes in the asymmetric spacer region of the loxP sites and suggest an alternative regulation mechanism of recombinase activity both *in vivo* and *ex vivo*. It also highlights potential pitfalls in quality control of high-throughput production of genetically modified mice. Complete sequencing of loxP sites may be required in addition to PCR-based validation.

METHODS

Mtfmt Construct: A Knockout first allele with conditional potential (*Mtfmt*^{tm1a(KOMP)Wtsi}) construct was acquired as cryopreserved embryo from the international Knockout Mouse Consortium University of California (UC) Davis, project KOMP-CSD (ID: 25662). Cryopreserved Mtfmt embryos were recovered in the Specific pathogen free (SPF) barrier facility at the transgenic core facility at the University of Miami. The EUCOMM-CSD knockout first allele initially a non-expressive form harbors a promoterless selection cassette and can be converted to a conditional allele using flippase recombination.

Generation of the *In vivo Mtfmt* knockout mouse model

Conditional Mtfmt knockout generation—Initially the reporter–gene trapped cassette which disrupts the entire targeted gene function was removed crossing these mice with a flippase (Flp) recombinant transgenic line. After removal, the deletion of the critical exon (exon 4) flanked by loxP sites was generated crossing these mice with a transgenic line expressing the recombinase (cre) under the control of two different tissue-specific promoters: calcium/calmodulin-dependent kinase II alpha (Tg(Camka-cre)3Szi or *Camk2a*-cre) and myosin light polypeptide 1 (*Myl1*^{tm2(cre)Sjb} or *Myl1*-cre). To generate the tissue-specific knockouts (KO), double heterozygous mice *Mtfmt* ^{(+/loxP),} cre ^(+/−) were mated to homozygous Mtfmt^(loxP/loxP) and the resulting progeny with the desired genotype selected for phenotypic studies.

Genotype confirmation of conditional knockout mice—The genotype was performed using the KOMP short-PCR (design ID:41729) strategy to confirm the integrity of the construct and to identify mice during the generation of conditional targeted mice. DNA extracted using NAOH from mice tails were used to identify KO mice as those carrying both *Mtfmt* floxed alleles and the cre transgene (*Mtfmt*^(loxP/loxP) cre^(+/−)) and littermate controls as those in which the cre transgene was absent (*Mtfmt*^(loxP/loxP) cre^(−/−)).

PCR amplification—DNA was isolated from tail samples and amplified by PCR. Reactions were standardized in a 10μl final reaction using dNTPs (0.20mM) primers (1μM) Taq (0.3U/μl) 1X buffer, 25 mM MgCl$_2$. A touchdown cycling temperature protocol was standardized as follows: Denaturation at 94°C for 5 min follow by 10 cycles of 94°C 15 sec, annealing temperature 65°C for 30 sec (decreasing 1°C/cycle) and 72°C extension for 40 sec and final 30 cycles at 55°C for 30 sec. Final extension cycle of 72°C for 5 min. The set of primers and fragments expected from the project KOMP-CSD (ID: 25662) design ID: 41729 were used to confirm the pre and post recombination with cre and/or Flp deleters. Primers to confirm the cre recombinase transgene were used following the ICS genotyping protocol from Jackson laboratories.

Phenotypic Studies

Mice identified as *Camk2a*-cre KO and *Myl1*-cre KO were characterized to evaluate the effects of *Mtfmt* ablation in physical and behavioral phenotypes. All mice procedures were performed according to the protocol approved by the University of Miami Institutional Animal Care and Use Committee.

Body weight changes—Body weight was monitored on a monthly basis during the first 4 months of age in knockouts and control littermates.

Spontaneous home-cage activity: was performed once a month starting at 2 month of age during the 12h dark cycle. In this test, mice were housed individually in their regular cage environment (Columbus Instruments, Inc) 30 min prior to their dark cycle (from 6 pm to 6 am). Cages were placed in the monitor sensor system which consists of a series of horizontal beams created by Infrared beams and detectors located laterally on opposite sides of the cage. General locomotor activity was measure as breaks of the beams and recorded as ambulatory counts.

Rota-rod (IITC Life Sciences) test: was used on a monthly base. In this test, mice are placed in an accelerating moving cylinder with acceleration increments of 0.07 RPM per second. Balance was tested as the ability of mice to maintain themselves in the rotating cylinder during the first 3 minutes. Mice were trained two times in consecutive days for habituation and data recorded at the third day. Final values were expressed as averaged latency to fall.

Performance on the treadmill—The running maximal capacity was tested using a treadmill system with shock grids as electrical stimulus. Mice were habituated to the treadmill during two consecutive days for 5 minutes before the recording on the third day using a speed of 8 meter/minute-steps per second.

Generation of the *Ex vivo Mtfmt* knockout model

Cells and culture conditions—Dermal fibroblasts were derived from skin tissue explants from 3 month adult homozygous *Mtfmt*$^{(loxP/loxP)}$ mice. After euthanizing, small skin fragments of approximately 1 cm^2 were removed from the limb area and transferred to a plate with sterile PBS. Tissue was minced in small pieces and stretched in a gelatin (0.1%) coated culture plates with complete medium (DMEM, 10% FBS, 1mM Sodium Pyruvate (Gibco), 0.5 μg/ml Fungizone, 20 μg/ml Gentamicin). Fibroblast starting to exit tissue fragments within 2–5 days were then trypsinized and collected for expansion in 10 cm dishes.

Immortalization-Retroviral transduction—Efficient immortalization of primary dermal fibroblasts was done using a retrovirus vector encoding HPV-16 E6/E7 genes packed in PAC 317 cells. Fibroblasts at passage 1 grown to 80% confluency were infected with high titers of retrovirus in medium containing 4g/ml Polybrene (Sigma, St. Louis, MO). Selection of clonal cells was performed in medium containing Geneticin® (G418 sulfate) (GIBCO, NY) at 500ug/ml for 15 days. Several clones were then isolated with cloning rings, and individual clones were continuously expanded in selection media

Transfection with plasmid-CMV-cre—Immortalized loxP/loxP (parental) clones were grown in a 10 cm dish and transfected with a plasmid expressing cre-recombinase driven by the CMV promoter. Parental fibroblasts were grown to 70% confluency and transfected using a lipid based reagent (GenJet™) (Signagen, Rockville, MD) with 1 μg of plasmid. Selection of stable transfected cells and expansion of *Mtfmt* knockout clones was performed using 500 μg/ml of hygromycin B (Invitrogen, Grand Island, NY) followed by isolation with cloning rings for monoclonal expansion.

Confirmation of Mtfmt mutant recombinants—Confirmation of successful recombination using cre transgene expression *in vivo* and ex vivo was done by amplifying the putative deleted region in the *Mtfmt* gene after cre recombinase activity by short PCR using the same set of primers described above. DNA was extracted from hippocampus and cortex in the *Camk2a*-cre KO mice model and gastrocnemius and quadriceps in the *Myl1*-cre KO mice. *Ex vivo*, *Mtfmt* KO clones cells were used for DNA extraction and the parental *Mtfmt* (loxP/loxP) clone was used as control.

Acknowledgments

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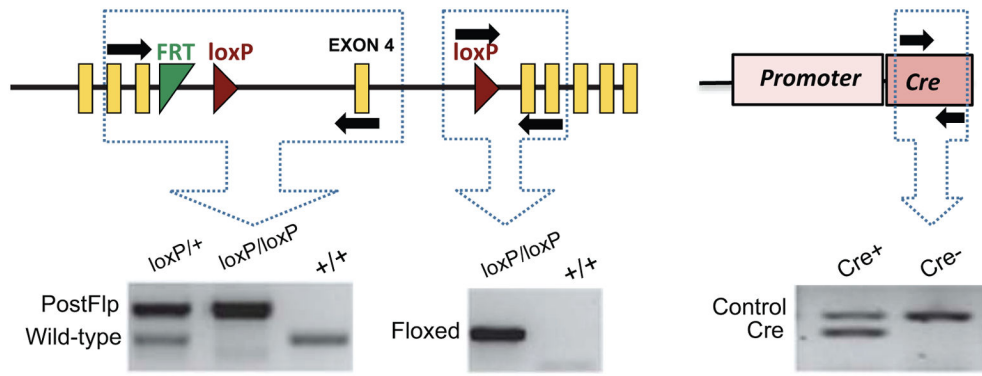
Figure 1. *Mtfmt* floxed allele structure
Confirmation of the *Mtfmt* targeted gene with conditional allele potential showing 9 exons (yellow squares), the Flippase Recognition unique site (FRT) after removal of the gene trap cassette by Flp recombinase (green arrow), followed by the proximal (5′) and distal (3′) loxP site (red arrows) flanking exon 4. Integrity of the loxP sites and confirmation of zygosity (heterozygous loxP/+, homozygous LoxP/loxP, and wild type (+/+)) was confirmed by short PCR amplification with specific primers (black arrows). Cre recombinase gene was confirmed by amplification of the common cre region in both promoters *Camk2a*-cre and *Myl1*-cre using a wild type gene amplification as control.
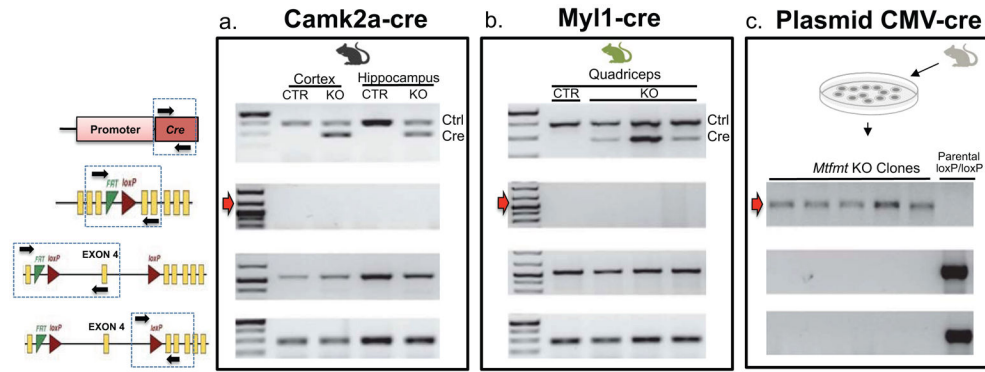
Figure 2. *Mtfmt* loxP allele did not recombine *in vivo*

Absence of cre mediated recombination in both tissue specific KO mice models was confirmed in cortex and hippocampus tissues for the *Camk2a*-cre model (panel a) and quadriceps in the *Myl1*-cre mouse model (panel b) by absence of the putative deleted region amplification (red arrow) and retention of the proximal, distal loxP sites and cre recombinase transgene. *Ex vivo*, using *Mtfmt* $^{(loxP/loxP)}$ mice derived fibroblasts and a plasmid expressing cre recombinase driven by an CMV promoter (panel c), successful recombination shows a positive band corresponding to the putative deleted region after cre recombinase expression in the *Mtfmt* knockout clones (red arrow) and absence of amplification corresponding to the proximal and distal loxP sites compared to parental *Mtfmt* $^{(loxP/loxP)}$ fibroblasts.
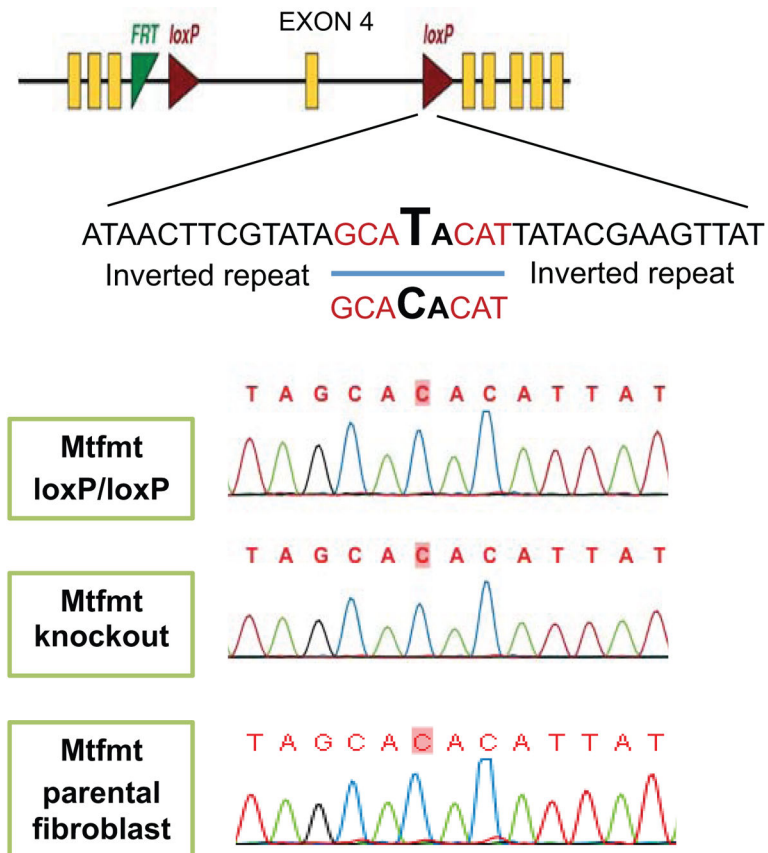
Figure 3. Detection of a central spacer mutation in the distal loxP

Sequencing chromatograms show the sequence of the distal (3′) loxP site and location of the mutation T>C at position 4 of the asymmetric central spacer region (underlined). This mutation was present in both CNS (*Camk2a*-cre) and skeletal muscle (*Myl1*-cre) models *in vivo* and in the *Mtfmt* (loxP/loxP) parental fibroblasts *ex vivo*. The proximal (5′) loxP site chromatogram was normal (not shown).