# Efficient *in vitro* and *in vivo* self-repression of SpCas9 gene using a molecular Hara-Kiri method.

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# Abstract

The CRISPR/Cas9 system is currently a major revolution in the field of biology. Because of its simplicity compared to other endonucleases, this system is being experimented in diverse fields. However, a major disadvantage is the toxicity linked to sustained Cas9 expression. In the present study, we present an approach to effectively suppress the expression of the Streptococcus pyogenes Cas9 (SpCas9) gene. This approach that we call the molecular Hara-Kiri method, involves two sgRNAs targeting two sequences in the SpCas9 gene. The SpCas9 enzyme binds to the Protospacer Adjacent Motifs following the two sequences targeted by the sgRNAs and induces two Double Strand Breaks (DSBs) in its own gene (Hara-Kiri). The sequence located between the DSBs is then deleted. Most of the time, the SpCas9 gene is repaired by Non-Homologous End Joining without INDELs. By adequately selecting the targeted sequences, the junction of the SpCas9 gene residues generates a TAA type stop codon within this truncated gene to effectively suppress its expression. This results in dramatic decrease of the SpCas9 protein *in vitro* and *in vivo*.


# Introduction

The CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeat / CRISPR-associated 9) system is a real revolution in genome editing (Horvath and Barrangou 2010, Jinek, Chylinski et al. 2012, Mali, Esvelt et al. 2013, Hsu, Lander et al. 2014, Sander and Joung 2014). The great interest in this approach among scientists is explained by its simplicity compared to other genome editing methods such as Meganucleases (MGNs) (Gouble, Smith et al. 2006), Zinc Finger Nucleases (ZFNs) (Kim, Cha et al. 1996) and Transcription Activator-Like Effector Nucleases (TALENs) (Joung and Sander 2013). The first and most often used CRISPR/Cas enzyme for editing the genome in several organisms is type II *Streptococcus pyogenes* (SpCas9). The CRISPR/Cas9 system requires a single guided RNA (sgRNA), which matches an 18 to 20 nucleotides target sequence in DNA, which is followed by an adequate NGG Protospacer Adjacent Motif (PAM). This PAM recruits the SpCas9 nuclease to generate a double-strand break (DSB) at exactly at three nucleotides from it towards the 5' end of the target sequence (Cong, Ran et al. 2013, Doudna and Charpentier 2014). Other Cas enzymes, derived from other bacteria, require different PAMs and induce a DSB at different sites relative to the PAM (Ran, Cong et al. 2015, Zetsche, Gootenberg et al. 2015, Kim, Koo et al. 2017).

Several studies have shown the high efficacy of the CRISPR/Cas9 system *in vitro and in vivo* (Cho, Kim et al. 2013, Zheng, Cai et al. 2014, Long, Amoasii et al. 2015, Nelson, Hakim et al. 2015, Ousterout, Kabadi et al. 2015, Tabebordbar, Zhu et al. 2015, Bengtsson, Hall et al. 2017). However, one disadvantage of this system is the toxicity linked to the sustained expression of the SpCas9 gene (Fu, Foden et al. 2013, Hsu, Scott et al. 2013, Cho, Kim et al. 2014). It has unfortunately been confirmed that SpCas9 generates unwanted DSBs in the coding and non-coding regions of the genome (Montague, Cruz et al. 2014, O'Geen, Henry et al. 2015, Tsai, Zheng et al. 2015). This difficulty considerably limits the possible use of using SpCas9 in therapeutic approaches in humans. To overcome this difficulty, several avenues have been explored. One of them aims to improve the specificity of the SpCas9 nuclease. Some authors have indeed

shown that modifying a few SpCas9 amino acids reduced the non-specific interactions of the Cas9 protein with DNA and thus reduced the number of off-target DSBs (Ran, Hsu et al. 2013, Tsai, Wyvekens et al. 2014, Kleinstiver, Pattanayak et al. 2016, Slaymaker, Gao et al. 2016). The sustained expression of SpCas9 favors off-target DSBs and thus increases its toxicity. Thus other methods aim to reduce the duration of its expression, one of them is to use the SpCas9 mRNA or the SpCas9 protein rather than the SpCas9 gene inserted in a plasmid or viral vector (Kim, Kim et al. 2014, Ramakrishna, Kwaku Dad et al. 2014, Hashimoto and Takemoto 2015). By using the SpCas9 mRNA, the synthesis of the protein is limited in time and thus it is possible to reduce the off-target cuts.

The use of SpCas9 mRNA or protein is however not possible for *in vivo* gene editing requiring a systemic delivery. This is a serious handicap for the treatment of diseases such as Duchenne Muscular Dystrophy (DMD), which affects all skeletal muscles and the heart. Paradoxically, among the solutions proposed above to resolve the problem posed by SpCas9 toxicity, there is very little research on how to limit the duration of expression of the SpCas9 gene *in vitro* or *in vivo* (Chen, Liu et al. 2016, Petris, Casini et al. 2017, Ruan, Barry et al. 2017, Shin, Jiang et al. 2017, Tu, Yang et al. 2017). A previous study has shown that DSBs generated by SpCas9, given its high efficacy, occur within only a few hours after transfection (Chen, Liu et al. 2016). This means that the expression beyond this time of the SpCas9 gene inserted in plasmid or viral vector is not necessary for efficient genome editing. It has also been demonstrated, as noted above, that the toxicity was related to prolonged expression of the SpCas9 gene.

Our results show that the transient expression of the SpCas9 gene inserted in a plasmid or a viral vector is sufficient to generate DSBs in the intended target sequence but also in the SpCas9 gene itself to prevent additional unwanted DSBs occurring during a sustained expression. To this end, we have built two sgRNAs that target the SpCas9 gene. The SpCas9 protein was recruited at these two PAMs to generate two DSBs resulting in the deletion of part of the SpCas9 gene sequence located between the two cleavage sites. The junction of the SpCas9 gene residues by Non-Homologous End Joining (NHEJ), most of the time without micro-insertions or micro-deletions (INDELs), generated a frame shift resulting in a stop TAA codon within the truncated SpCas9 gene, thus repressing its expression.

# Results

### *In vitro* repression of the SpCas9-FLAG gene with a pair of sgRNAs

We initially identified the PAMs in the SpCas9-FLAG gene present in the pX458 plasmid and selected a pair of sgRNAs ($sgRNA_{1SpCas9}$ and $sgRNA_{2SpCas9}$), which permitted to not only delete a part of the SpCas9-FLAG gene but also to generate a TAA stop codon at the junction site of the remaining fragments (Figure 1 and supplementary Figure 1). To repress the expression of the SpCas9-FLAG gene, HEK 293T cells were thus transfected with the pX458 plasmid and co-transfected either immediately or with a delay with the $pBSU6_{1\text{-}2\text{-}SpCas9}$ plasmid coding for this pair of sgRNAs.

#### *a) Early in vitro repression of the SpCas9-FLAG gene*

*Detection of the truncated SpCas9 gene*

HEK 293T cells were transfected with two plasmids: the pX458 and the $pBSU6_{1\text{-}50/5\text{-}54}$ coding for two sgRNAs targeting exons 50 ($sgRNA_{DMD1\text{-}50}$) and 54 ($sgRNA_{DMD5\text{-}54}$) of the DMD gene. Some cells were also simultaneously transfected with the $pBSU6_{1\text{-}2\text{-}SpCas9}$ plasmid. The cell DNA was extracted 12, 24, and 48 and 72 hours after the transfections and the SpCas9-FLAG gene was PCR amplified with the primers $FW_{SpCas9}$ and $RV_{SpCas9}$. In the cells transfected only with pX458 and the $pBSU6\text{-}_{1\text{-}50/5\text{-}54}$, a 1633 bp amplicon was detected (Figure 2A, lines 2, 4, 6 and 8) corresponding to the expected non-truncated SpCas9 gene. However, a 658 bp amplicon was detected in cells transfected with the 3 plasmids (Figure 2A, lines 3, 5, 7 and 9) corresponding with the expected SpCas9-FLAG gene truncated following cuts by the two sgRNAs. At short intervals after transfection (i.e., 12 and 24 h), only some of the SpCas9-FLAG genes were truncated, thus two different SpCas9-FLAG gene amplicons were obtained (1633 bp and of 658 bp (Figure 2A, lines 3 and 5) whereas only the 658 bp amplicon was detected after 48 or 72 hours showing that the SpCas9-FLAG gene was completely repressed (Figure 2A, lines 7 and 9).

The amplicons illustrated in the figure 2A lines 6 (1633 bp) and 7 (658 bp) were cloned into pMiniT and sequenced. The full and truncated SpCas9-FLAG sequences obtained were identical to the expected theoretical sequences (Supplementary Figures 2A and 2B), except for one clone in which there was an insertion of one nucleotide resulting in a frame shift of the SpCas9-FLAG gene (Supplementary Figure 2C).

*Detection of the hybrid exon 50-54 in the DMD gene*

In this experiment, the DMD gene was also targeted by two sgRNA reacting with exons 50 and 54 to produce the hybrid exon 50-54. This hybrid exon was detected by PCR amplification with primers $FW_{int49DMD}$ and $RV_{int54DMD}$ targeting respectively the end of intron 49 and the beginning of intron 54. The expected amplicon of 540 bp was obtained. This band was stronger in the samples from unrepressed SpCas9 control (Figure 2B, lines 3, 5, 7 and 9) and lower in those with a repression of the SpCas9-FLAG gene (Figure 2B, lines 2, 4, 6 and 8).

*Demonstration of repression of the SpCas9-FLAG gene in vitro*

The deletion of a part of SpCas9-FLAG gene and the generation of the stop codon resulted in a strong reduction of the SpCas9-FLAG protein band. The PAGE-Coomassie results showed a dramatically decrease of the SpCas9-FLAG protein in the cell wells transfected with the plasmids $pBSU6_{\text{-}1\text{-}50/5\text{-}54}$, pX458 and $pBSU6_{1\text{-}2SpCas9}$ (Figure 2C, lines 3, 5, 7 and 9). There was a stronger expression of the SpCas9-FLAG gene in the cell wells transfected with only the plasmids $pBSU6_{\text{–}50/5\text{-}54}$ and pX458 (unrepressed SpCas9-FLAG gene) (Figure 2C, lines 4, 6 and 8). A western blot with an anti-FLAG mouse monoclonal antibody coupled to HRP confirmed this result (Figure 2D).

### *b) Delayed in vitro repression of the SpCas9-FLAG gene*

*Detection of the truncated SpCas9 gene*

The previous experiment was repeated with the modification that the third plasmid pBSU61-2-SpCas9 was transfected in the cells either simultaneously or 12 or 24 hours later. DNA was extracted from the cells at different times (24, 48 and 72 hours) after the initial transfection and the SpCas9-FLAG gene was PCR amplified as above. Two amplicons (1633 and 658 bp) were present (Figure 3A). The truncated SpCas9-FLAG band (658 bp) was initially present at low intensity in the sample obtained 24 hours after the initial transfection. In these samples, the full-length band (1633 bp) was reduced in intensity in the samples with a delayed administration of the pBSU61-2SpCas9. However, 72 hours after the initial transfection, only truncated SpCas9-FLAG amplicon was detected in the samples in which the pBSU61-2SpCas9 plasmid was transfected at time 0. No truncated SpCas9-FLAG gene band (i.e., 658 bp) was detected in the samples not transfected with pBSU61-2SpCas9. The results showed that the band intensity of the truncated SpCas9-FLAG gene depends on the length of time since the targeting of this gene with the two sgRNAs.

*Demonstration of repression of the SpCas9 gene*

A western blot for the SpCas9-FLAG protein showed a dramatic decrease of this protein in the samples from the cell wells with simultaneous transfection of the sgRNAs targeting that gene (i.e., transfected with $pBSU6_{1\text{-}2SpCas9}$ at time 0). There was a higher expression of the SpCas9-FLAG protein in the samples transfected with $pBSU6_{1\text{-}2SpCas9}$ with a 12 or 24 hours delay and the strongest expression of the SpCas9-FLAG protein was observed in the samples that were not transfected with $pBSU6_{1\text{-}2SpCas9}$ (Figure 3B lines 5 and 12).

*Detection of the hybrid DMD exon 50-54*

This second experiment also aimed to determine the effects of the delayed transfection of $pBSU6_{1\text{-}2SpCas9}$ on the formation of the hybrid *DMD* exon 50-54. The hybrid DMD exon 50-54 band (at 540 bp) intensity was strong, medium and low in the samples respectively not transfected, transfected with a delay or transfected simultaneously with $pBSU6_{1\text{-}2SpCas9}$ (Figure 3C).

### *1. In vivo* experiments

**a)** *In vivo detection of the truncated SpCas9 gene*

Transgenic hDMDD52/mdx mice containing the human DMD gene with a deletion of exon 52 were used for the *in vivo* experiments. This deletion changes the reading frame of the DMD gene and thus prevents the expression of the human dystrophin protein under the sarcolemma. Some hDMDD52/mdx mice were injected i.v. simultaneously with $AAV9_{SpCas9}$ (coding for the SpCas9 gene under the CMV promoter) and with $AAV9_{sgRNA1\text{-}50/5\text{-}54}$ (coding for two sgRNAs targeting exons 50 and 54 of the DMD gene) (Figure 4A). Twenty-four hours later the mouse M2 was injected i.v. with the $AAV9_{sgRNA1\text{-}2\text{-}SpCas9}$. All mice were sacrificed four weeks later. To detect the wild type and the truncated SpCas9, PCR amplifications were

performed with DNA samples from the TA, heart and diaphragm. No SpCas9 amplicon was detected in the organs of the negative control mouse (Figure 4B, M1). In the mouse M3 treated with $AAV9_{SpCas9}$ and $AAV9_{sgRNA1\text{-}50/5\text{-}54}$, only the wild type SpCas9 amplicon (1633 bp) was detected in the different organs (Figure 4B). As expected, the truncated SpCas9 amplicon (658 bp) was amplified in all organs of only the mouse M2 treated with the three AAV9.

*b) Repression of the SpCas9 gene*

To detect the repression of the SpCas9 gene, western blots were performed with the proteins extracted from the TA, heart and diaphragm. No 150 kDa band was detected in the proteins extracted from the negative control mouse M1 that was not infected with an AAV9SpCas9 (Figure 4C). There was a very low intensity 150 kDa band in the proteins extracted from the mouse M2 treated with three AAV9 (AAV9SpCas9, AAV9sgRNA1-50/5-54 and AAV9sgRNA1-2SpCas9) but a very strong band was present in the mouse M3 treated with only two AAV9 (AAV9SpCas9 and AAV9sgRNA1-50/5-54) (Figure 4C)

*c) Detection of the hybrid exon 50-54 in the DMD gene in vivo*

PCR amplification of genomic DNA extracted from TA muscle, the heart and the diaphragm of all three mice was performed by using $FW_{int49DMD}$ and $RV_{int54DMD}$ primers. In the negative control mouse (M1), no hybrid DMD exon 50-54 band of 540 bp was detected in the all DNA samples. The 540 bp amplicon due to the presence of the hybrid DMD exon 50-54 was detected in the samples from the mice threated with $AAV9_{sgRNA1\text{-}50/5\text{-}54}$, $AAV9_{SpCas9}$ and $AAV9_{sgRNA1\text{-}2SpCas9}$ (M2) or with $AAV9_{sgRNA1\text{-}50/5\text{-}54}$ and $AAV9_{SpCas9}$ (M3). The intensity of the band due to DMD hybrid exon 50-54 was slightly stronger in the samples from the mouse M3 than in those from the mouse M2 (Figures 4D).

## 2. Insertion of a short oligonucleotide at the DSB sites in SpCas9-FLAG and DMD genes

We used Guide-Seq method to detect the presence of off-target mutations. HEK 239T cells were thus transfected with plasmids coding for the SpCas9-FLAG, the sgRNAs targeting exons 50 and 54 of the DMD gene and with a 34 bp double stranded oligonucleotide (dsODN) with phosphothiorate linkages at the 5' and 3' ends of both strands. For the PCR amplification, we used a forward primer binding to the DMD intron 49 ($FW_{int49DMD}$) and a reverse primer reacting with the dsODN ($RV_{dsODN}$). A second PCR used a forward primer binding to the dsODN ($FW_{dsODN}$) and a reverse primer binding to DMD intron 50 antisense strand ($RV_{int50DMD}$). Both PCR produced amplicons with the expected theoretical size thus showing the insertion of the dsODN into the cleavage site located in exon 50 of DMD gene. These amplicons were not amplified in the negative control (Figure 5A line 1). Similar results were also obtained using primers to amplify DMD exon 54 using a forward primer reacting with intron 53 ($FW_{int53DMD}$) and a reverse primer (RVdsODN) reacting with the dsODN producing a 824 nucleotides amplicon and with a second PCR using forward primers reacting with the dsODN ($FW_{dsODN}$) and a reverse primer reacting with DMD intron 54 ($RV_{int54DMD}$) producing a 414 nucleotides amplicon. Both PCRs demonstrated the insertion of the dsODN into the cleavage site of the exon 54 DMD (Figure 5B).

To detect the insertion of the dsODN into the cleavage sites of the SpCas9-FLAG gene, two different PCR were done. The first PCR used a forward primer reacting with the SpCas9 gene ($FW_{SpCas9}$) and a reverse primer reacting with the dsODN ($RV_{dsODN}$). The second PCR used a forward primer reacting with the dsODN ($FW_{dsOND}$) and a reverse primer reacting with the Cas9 gene ($RV_{SpCas9}$). As for genomic DNA, the amplicons obtained confirm the insertion of the dsODN into the cleavage sites generated in the SpCas9-FLAG gene by Hara-Kiri (Figure 5C1).

### 4. Amplification of truncated SpCas9-FLAG gene after insertion of the dsODN

The SpCas9-FLAG gene was amplified with primers $FW_{SpCas9}$ and $RV_{SpCas9}$ from DNA extracted from HEK 293T cells that received different treatments: transfected or not with the plasmids inducing Hara-Kiri ($pBSU6_{1\text{-}2\text{-}SpCas9}$) and co-transfected or not with the dsODN. Paradoxically, the intensity of the truncated SpCas9-FLAG amplicon bands in the wells co-transfected with the Hara-Kiri plasmids and the dsODN was lower than in the wells transfected with the Hara-Kiri plasmids without the dsODN (Figure 5C1, lines 7, 7 and 11). To find out if there has been any repression of SpCas9-FLAG in the wells transfected with the dsODN, a western blot with anti-FLAG was performed. The western blot confirmed the repression of the SpCas9 expression with Hara-Kiri despite the low intensity of the truncated SpCas9-FLAG gene amplicon band (Figure 5C4, lines 3, 7 and 11)

### 3. Guide-Seq data

4. The results of high-throughput sequencing are summarized in Table 5. They include the percentages on target and off target reads obtained following the transfection of 293T cells as indicated in materials and methods. The on target reads include insertion of the dsODN in the SpCas9 gene in the R samples transfected with the gRNAs targeting that gene (data not shown). The off-target reads were higher percentages of total reads in the NR samples (lines 1, 3 and 5 respectively 25%, 26% and 18% of all reads) in which the SpCas9 gene was not repressed as in the R samples (lines 2, 4 and 6 respectively 4%, 5% and 6% of all reads) in which the SpCas9 gene was repressed by two sgRNAs (Hara-Kiri).

It is important to note that all off target sites identified by the Guide-Seq procedure were those that have been identified *in silico* with the mit.educ platform. Off targets with a high score *in silico* were also those for which we have identified a larger number of reads. This is particularly true for sgRNA1-50 and off target at chromosome 12. More than half of the off target mutations for this sgRNA are located at that predicted site. Similar results were also obtained with sgRNAs5-54 and 1-50 / 5-54.

The Hara-Kiri repression drastically reduced the percentage of off target mutations. The remains of reads that were highlighted by deep sequencing are those that were most represented in the NR samples. Transfection of plasmid $pBSU6_{1\text{-}2SpCas9}$ coding for two sgRNAs targeting the SpCas9 gene did not induced mutations at new off-target sites.

# Discussion

The molecular Hara-Kiri method aims to suppress the expression of SpCas9 using the CRISPR induced deletion (CinDel) approach, that we developed previously (Iyombe-Engembe, Ouellet et al. 2016). Here, instead of creating a new in frame codon, this approach used two sgRNAs targeting two sites of the SpCas9-FLAG gene located either in the pX458 expression plasmid (Fig. 1A, 1B) or the SpCas9 gene present in the AAVSpCas9 vector (Fig. 4B). DSBs occurred at two selected sites of the SpCas9 gene when the Cas9 protein was recruited at the PAM sites of its own gene (Hara-Kiri). The sequence located between the two cleavage sites was then deleted. The remains of the SpCas9 gene were joined by NHEJ most of the times without INDELs, generating a TAA type stop codon (Fig. 1A and 1C). To assess the effectiveness of the approach, we used two types of repression: the immediate and delayed repression of the SpCas9-FLAG gene *in vitro* and of the SpCas9 gene *in vivo*.

The results obtained with Hara-Kiri method confirmed the high precision of the nucleotide deletion obtained by using two sgRNAs and the SpCas9 targeting its own SpCas9 gene *in vitro* and *in vivo*. The precision that characterizes the use of two cleavage sites with two sgRNAs and which resulted in a large deletion, resulting most of the time in a perfect junction of the remains of the targeted gene and very few INDELs has been described by other authors (Zheng, Cai et al. 2014) and by our team (Iyombe-Engembe, Ouellet et al. 2016). However, the mechanism that governs this type of repair is not yet fully elucidated.

Our results also showed a progressive accumulation of the truncated SpCas9 gene *in vitro.* There is also a dramatic decrease of the SpCas9-FLAG protein when the coding gene was repressed *in vitro* and of the SpCas9 protein when that gene was repressed *in vivo.* Finally, our results showed that despite the self-destruction of the SpCas9 gene, an effective genome editing of the DMD gene was obtained *in vitro* and *in vivo* as shown by the generation of the hybrid DMD exon 50–54.

Preliminary results obtained with the Guide-Seq method showed that the 34 bp dsODN used with in this technique was correctly inserted into the cleavage sites of the DMD gene and the SpCas9 present in a plasmid. However, the insertion of this dsODN frequently prevented the deletion of the sequence located between the two cleavage sites in the SpCas9-FLAG and the formation of the TAA stop codon. Since the insertion of 1 or 2 dsODN (34 or 68 bp) changed the SpCas9-FLAG reading frame, these insertions induced the repression of the SpCas9-FLAG gene as shown by western blot (Fig. 5C4).

Instead of using two sgRNAs to suppress the SpCas9 gene, some authors (Chen, Liu et al. 2016, Petris, Casini et al. 2017) used a single sgRNA to generate a DSB. The joining of the extremities by NHEJ induced INDELs, which changed the reading frame roughly 2 out of 3 times. This implies that the reading frame would remain intact following 1 out of 3 repairs. Thus, this approach does not completely prevent the sustained expression of SpCas9. The results obtained in the present study show that even a small amount of SpCas9 is sufficient to induce genome editing of the target DMD gene (Fig. 2B, lane 2). Thus, we believe that a remaining 33% expression of the SpCas9 gene over a long period would be sufficient to generate continuously off-target mutations in the genome, which is a non-satisfactory outcome.

Other researchers fused SpCas9 and ubiquitin genes to allow the degradation of Ub-SpCas9 protein with the Ubiquitin Proteasome System (UPS) (Tu, Yang et al. 2017), thus preventing the accumulation of

SpCas9 protein and reducing off-target mutations. The degradation of Ub-SpCas9 is totally dependent on activation of the proteasome. It will be difficult to predict the activation of the proteasome in different cell types at different phases of the cell cycle when the SpCas9 gene is administered systemically *in vivo* with an AAV vector. Moreover, the Ub-SpCas9 protein would continuously be produced and would thus produce off-target mutations and in an immune response against this foreign protein also resulting in a non-satisfactory outcomes.

Ruan et al. (Ruan, Barry et al. 2017) have used two sgRNAs to delete completely the SpCas9 gene of the plasmid vector. The deletion of the entire coding sequence of SpCas9 still fused with the nuclear localization sequence entails the risk of random insertion at another site of the genome and its probable expression. Since the deleted gene remains fused to the nuclear localization sequence, there is a possible expression of a SpCas9 protein capable of being imported into the nucleus by nuclear importins and causing off-target mutations.

Recently, another team used an anti-CRIPSR protein, the AcrIIA4 protein, to reduce off-target mutations (Shin, Jiang et al. 2017). Their experiments were done *in vitro*, however, an *in vivo* application of this approach seems to be difficult, especially for a clinical application. The presence of a foreign protein such as AcrIIA4 in a patient can be dangerous (stimulation of the immune system, probable interaction with other ribonucleic complexes). Moreover, since the repression is stoichiometric, the amount of AcrIIA4 must be at least equal to that of the sgRNA-Cas9 complexes for the repression to be effective. This equality could be difficult to achieve *in vivo*.

Our *in vitro* and *in vivo* results with the molecular Hara-Kiri method on the repression of the SpCas9 gene are very encouraging in comparison with these other approaches. The Hara-Kiri method permits the expression of SpCas9 during an adequate period to obtain the desired mutation of the target gene but allows an effective repression of the nuclease after the desired target mutations has been obtained.

The goal pursued by the Molecular Hara-Kiri method is to repress the SpCas9 gene to prevent its prolonged expression responsible for off-target mutations using the CinDel method previously developed by our team (Iyombe-Engembe, Ouellet et al. 2016). The results of the Guide-Seq method showed a very significant reduction of the percentage of off-target reads when the cells were transfected with two gRNAs targeting the SpCas9 gene to repress its expression. Indeed, repression of the SpCas9 gene allowed off-target reduction from 25–5% when only DMD exon 50 was targeted by a sgRNA, from 26–4% when only DMD exon 54 was targeted by a gRNA and from 18–5% when DMD exons 50 and 54 were targeted by two sgRNAs (Table 5). It is interesting to note that transfection of 293T with plasmid $pBSU6_{1\text{-}2SpCas9}$ coding for two gRNAs targeting the SpCas9 gene did not increase the percentage of off-target mutations and did not induce mutation at new off-target sites. This data once confirmed the effectiveness of the molecular Hara-Kiri method in the repression of the SpCas9 gene.

This control of the exact timing of Cas9 expression opens the possibility of using the CRISPR/Cas9 gene editing technology for therapeutic applications.

## Materials And Methods

# Expression vectors

The expression plasmid pX458 (pSpCas9-FLAG (BB)-2A-GFP) (Table 1) (gift from Feng Zhang, Addgene, Cambridge, MA) encodes the SpCas9-FLAG gene under CBh promoter. The SpCas9 gene is fused in its N-terminus with a sequence coding for the triple FLAG peptide (Fig. 1A).

# Construction of sgRNAs targeting SpCas9-FLAG and the DMD genes

Several PAMs were identified in the SpCas9 gene, however two of them were selected because they were in adequate positions to produce a TAA stop codon after the deletion of the sequence located between their cut sites. The sequences preceding these PAMs were thus targeted by $sgRNA_{1\text{-}SpCas9}$ and $sgRNA_{2\text{-}SpCas9}$ (Supplementary Fig. 2A, Table 2). Two double stranded oligonucleotides were synthetized by Integrated DNA Technologies (IDT, Coralville, IA) and inserted separately into the pBSU6 expression vector (pBSU6_FE_ScaffoldRSV_GFP, gift from Dirk Grimm Lab, Heidelberg University, Germany) after the digestion with BbsI and BsmBI (NEB, Inc., Ipswich, MA) to produce the $pBSU6_{1\text{-}2SpCas9}$plasmid.

Our group has previously identified $sgRNA_{DMD1\text{-}50}$ and $sgRNA_{DMD5\text{-}54}$ (Table 2) targeting exons 50 and 54 of the human *DMD* gene to produce the hybrid exon 50–54 (Iyombe-Engembe, Ouellet et al. 2016). Two double-stranded oligonucleotides coding for these sgRNA were therefore synthesized by IDT and inserted into the pBSU6 plasmid to produce the pBSU61-50/5–54 plasmid (Table 1).

# AAV9 viral vectors

The viral vectors $AAV9_{sgRNA1\text{-}50/5\text{-}54}$, $AAV9_{SpCas9}$ and $AAV9_{sgRNA1\text{-}2\text{-}SpCas9}$ (Table 4) containing respectively two sgRNAs targeting DMD exons 50 and 54, the SpCas9 gene and two sgRNAs targeting the SpCas9 gene were produced by Molecular Tools Platform, CRIUSMQ, Québec, Canada.

# The hDMD / mdx mouse and the hDMDΔ52/mdx mouse

The hDMD/mdx and the hDMDΔ52/mdx mice (gifts from Dr. A Aartsma-Rus, Leiden University Medical Center, Leiden, Netherlands) contain respectively the full human DMD gene (2.4 Mb) (coding for dystrophin) and the human DMD gene with the deletion of the exon 52. These hDMD genes (WT and mutated) are stably integrated into the mouse chromosome 5. The production of the hDMD/mdx and the hDMDΔ52/mdx mice was authorized by Dutch Ministry of Agriculture (’t Hoen, de Meijer et al. 2008). All mouse experiments were performed in accordance with the relevant animal research guidelines and regulations. For the sacrifice the mice were anesthetised with Isoflurane and their blood was collected by a cardiac puncture.

# Cell culture and Hara-Kiri in vitro assay

On day 1 (D1), 200,000 HEK 293T cells / well (24 well plate) were cultured in Dulbecco Modified Eagle Medium (DMEM) with 10% FBS (Fetal Bovine Serum) and 1% penicillin / streptomycin and incubated at 37°C under 5% $CO_2$. The cells were transfected when they reached a 70–80% confluence. This transfection was performed in Opti-Mem (Invitrogen Inc., Waltham, MA) with Transfex (American Type Culture Collection (ATCC, Manassas, VA). Two transfection protocols were used: the first one called early repression and the second called delayed repression of the SpCas9-FLAG gene.

# Early repression

To demonstrate the formation of the truncated SpCas9-FLAG gene containing the TAA stop codon and hybrid DMD exon 50–54, we used two series of HEK 293T cell wells. The first series of wells was transfected with plasmids pX458 (coding for SpCas9-FLAG), pBSU6-1-50/5–54 (coding for 2 sgRNAs targeting the human DMD gene) and with pBSU61-2-SpCas9 (coding for 2 sgRNAs targeting the SpCas9 gene) (Table 1). This third plasmid permitted to inactivate the SpCas9-FLAG gene. The second series of wells were transfected only with plasmids pBSU6-1-50/5–54 and pX458 and thus the SpCas9-FLAG expression was not repressed. Well 1 was not transfected with any plasmid and was used as negative control. Cells were detached with trypsin collected 12, 24, 48 and 72 hours after transfection. The cells of each well were divided in two Eppendorfs (one for DNA extraction and one other for protein extraction).
*Delayed repression*

For the delayed repression *in vitro* experiment, the HEK 293T cells were initially transfected only with the plasmids pBSU6-1-50/5–54 and pX458 (Table 1), allowing the DMD gene edition to occur during a few hours. The cells were subsequently transfected at various times with the pBSU6-1-2SpCas9 plasmid (Table 1) to repress SpCas9-FLAG gene. Four series of cell wells were used for this experiment. Well 1 was not transfected with any plasmid. At the beginning (time 0 h) of the experiment, wells 2 to 14 were transfected with the pBSU6-1-50/5–54 and pX458 plasmids. The pBSU61-2SpCas9 plasmid was transfected at time 0 h in wells 2, 4, 7 and 11 for early repression of the SpCas9-FLAG gene. For the delayed Cas9 repression experiment, the plasmid pBSU61-2SpCas9 was transfected at time 12 h in the cells of wells 6, 9 and 13. This plasmid was also transfected in cells of wells 10 and 14 at time 24 h. The pBSU6-1-2 SpCas9 plasmid was not transfected in the cells of the wells 3, 5, 8 and 12 (unrepressed SpCas9-FLAG gene control). Cells were collected at times 12, 24, 48 and 72 h.

# IV injection of AAV9 in the hDMDΔ52/mdx

Two hDMDΔ52/mdx (M2 and M3) mice were injected IV with $AAV9_{SpCas9}$ ($9.6 \times 10^{13}$ gc/kg) (coding for SpCas9) and with $AAV9_{sgRNA1\text{-}50/5\text{-}54}$ ($1.2 \times 10^{14}$ gc/kg) (coding for 2 sgRNAs targeting exons 50 and 54 of the DMD gene) (Table 4). Twenty-four hours later, the mouse M2 was injected IV with $AAV9_{sgRNA1\text{-}2SpCas9}$ ($4 \times 10^{13}$ gc/kg). Mouse M1 (hDMD/mdx) was not threated and was used as a control. The mice were sacrificed four weeks later and the TA muscles, the heart and the diaphragm of each mouse were divided in three parts. One part was used to extract DNA (genomic and viral). Proteins were extracted from the second part.

# Extraction of genomic, plasmid and viral DNA

To extract the genomic and plasmid DNAs from HEK 293T cells, the cells were lysed with the mixture containing sarcosyl 10%, EDTA 0.5 M pH 8 ad proteinase K (20 mg/ml) (Wisent Inc., St Bruno, Quebec, Canada). The genomic and plasmid DNA were extracted from the cell lysate with the phenol-chloroform method, quantified with Biodrop (Harvard Bioscience, Inc., Holliston, MA) and were used for PCR reactions.

TA muscle, heart and diaphragm from the sacrificed mice were treated with proteinase K (20 mg/ml) in Tris-EDTA-NaCl-SDS buffer at 56°C overnight. Genomic and viral DNAs were then extracted with the phenol-chloroform method and were used for PCR reactions.

# Protein extraction

Proteins were extracted from the cell samples by the methanol-chloroform method (Wessel and Flugge 1984). The proteins were labeled with the Amino-black and quantified with ChemiDocTM SRS (Bio Rad, Mississauga, ON, Canada).

# Genomic DNA PCR

The transfection of the pBSU61-50/5–54 and pX458 plasmids (Table 1) allowed the pairing of sgRNADMD1-50 and sgRNADMD5-54 (Table 2) on their target sequences respectively in exons 50 and 54 of the DMD gene and the recruitment of SpCas9 proteins. The latter then generated two DSBs, which induced the deletion of the exonic and intronic sequences located between the two cuts. The PCR amplification of the hybrid exon 50–54 was performed with a forward primer ($FW_{int49DMD}$) targeting the end of intron 49 of the DMD gene and a reverse primer ($RV_{int54DMD}$) targeting the beginning of the intron 54 (Table 3).

# PCR on plasmid DNA and on viral DNA (pX458 or $AAV_{SpCas9}$)

A PCR amplification of the SpCas9 gene located in the plasmid pX458 and in the $AAV9_{SpCas9}$ was done with forward primer $FW_{SpCas9}$ and reverse primer $RV_{SpCas9}$ (Table 3). For the wild type SpCas9 gene, this produced a 1633 bp while a 658 bp amplicon was obtained for the truncated SpCas9 gene.

# PCR amplicons cloning and sequencing

The 1633 bp amplicon from the unrepressed SpCas9 gene and the 658 bp amplicon from the truncated SpCas9 gene were cloned into the pMiniT plasmid (NEB Inc., Ipswich, MA). The competent Top10 (Thermo-Fisher Scientific Baltics UAB, Vilnius, Lithuania) bacteria were transformed. The plasmids were then extracted with Miniprep (Bio Basic Canada, Markham, ON, Canada) and sequenced (Plateforme de séquençage, CHUL, QC, Canada).

# PAGE-SDS

The proteins extracted from samples were reduced with β-mercaptoethanol in a water bath at 95°C for 5 min. The samples were then centrifuged. Polyacrylamide gel electrophoresis was performed with the supernatants.

# Western blot

For the *in vitro* experiments, the SpCas9 protein was fused at its N-terminus with the FLAG peptide, a western-type immunoblotting was thus performed with an anti-FLAG mouse monoclonal antibody (Sigma Aldrich Inc., Saint Louis, MO) coupled to HRP (Horseradish peroxidase).

The *in vivo* experiments, since no FLAG epitope was fused with the Cas9 nuclease, the western type immunoblotting was done with an anti-SpCas9 mouse monoclonal antibody (Abcam, Toronto, ON, Canada). The second antibody was an anti-mouse coupled to HRP (Sigma Aldrich, Oakville, ON, Canada). The HRP activity of the second antibody was revealed by chemiluminescence (Bio-Rad Inc., Philadelphia, PA) using a Hyblot CL film (Denville Scientific Inc., Holliston, MA).

# Guide-Seq experiment

a) *In vitro experiment*

The Guide-Seq method developed by Tsai et al. (26) was used to detect the off-target mutations. The phosphothioate oligonucleotides as well as the different primers were produced by Integrated DNA Technologies (IDT, Coralville, IA). The HEK 293T cells were transfected with plasmids $pBSU6_{1\text{-}50/5\text{-}54}$ (sgRNAs targeting exons 50 and 54 of DMD gene), $pBSU6_{1\text{-}50}$ (sgRNA targeting only exon 50), $pBSU6_{5\text{-}54}$ (sgRNA targeting only exon 54 of DMD gene), $pBSU6_{1\text{-}2SpCas9}$ (sgRNAs targeting two sequences in the SpCas9-FLAG gene) and pX458 (coding for SpCas9 gene). The phosphothioate 34 bp oligonucleotide (10 pmol) was also added in some wells. These transfections were performed in the 24-well plates containing about 100,000 HEK 293T cells per well (70–80% confluence) following growth in DMEM medium with 1% of penicillin/streptomycin and 10% of the FBS (fetal bovine serum) at 37°C in 5% $CO_2$ under 10% moisture. For these transfections, Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used at 2 µl per 500 ng of plasmid in Opti-Mem (Life Technologies Corporation, Grand Island, NY). The following plasmids were transfected in some of the cells $pBSU6_{1\text{-}50}$ (600 ng), $pBSU6_{5\text{-}54}$ (600 ng), $pBSU6_{1\text{-}50/5\text{-}54}$ (600 ng) and/or pX458 (600 ng) in presence or not of the phosphorylated dsODN (10 pmol). A total of 12 wells were transfected with the different plasmid mixes. The first well was not transfected and served as a negative control. Wells 3 and 4 were used as negative controls for transfection without and with repression of SpCas9-FLAG respectively. Forty-eight hours later, the cells of each well were divided into two parts, one to extract the DNAs with the phenol-chloroform method and the other to extract the proteins with the methanol-chloroform method. The genomic DNA was quantified with the Biodrop Harvard Bioscience Inc. (Montreal Biotech Inc., QC, Canada) and the proteins were stained with amino-black dye, reduced with β-mercapto-ethanol five minutes at 95°C and quantified with Image (Bio Rad,

Mississauga, ON, Canada). To confirm the insertion of the dsODN, three PCR reactions were conducted in genomic DNA and plasmid DNA.

To detect the insertion of the 34 bp dsODN in DMD exon 50, two different PCR amplifications were done (Fig. 5A). The first PCR used a forward primer reacting with the end of DMD intron 49 ($FW_{int49}$) and a reverse primer reacting with the dsODN ($RV_{dsODN}$). The second PCR used a forward primer reacting with the dsODN ($FW_{dsOND}$) and a reverse primer reacting with the beginning of DMD intron 50 ($RV_{int50DMD}$). Two different PCR reactions also detected the insertion of the dsODN in DMD exon 54 (Fig. 5B). The first PCR used a forward primer reacting with the end of DMD intron 53 ($FW_{int53DMD}$) and a reverse primer reacting with the dsODN ($RV_{dsODN}$). The second PCR used a forward primer reacting with the dsODN ($FW_{dsOND}$) and a reverse primer reacting with the beginning of DMD intron 54 ($RV_{int54DMD}$). The insertion of the dsODN in the SpCas9 gene was finally detected by two different PCR (Fig. 5C2). The first PCR used a forward primer reacting with the SpCas9 gene ($FW_{SpCas9}$) and the $RV_{dsODN}$ primer. The second PCR used the $FW_{dsOND}$ primer and a reverse primer reacting with the Cas9 gene ($RV_{SpCas9}$).

To perform Guide-Seq protocol, the 400 ng of the genomic DNA were fragmented into average length of 500 pb segments in a total volume of 120 µl TE 1X with Branson 500S Sonifer (Branson ultrasonics, Lawrenceville, MA) according to the followed program: total time 20 seconds, amplitude: 10, pulse on: 10 seconds and pulse off: 5 seconds. The sheared samples were purified by Ampure XP (Beckman Coulter, Inc., Brea, CA) in 1:1 ratio and eluted with 15 µl TE 1X. 14 µl of the eluent were used to end-repair, A-tail and adapter ligation. The ligation product was purified with Ampure XP in 9:10 ratio and eluted in 10 µl TE 1X and allowed to perform sense and antisense PCR1 reactions. The PCR1 products were purified with Ampure XP in 12:10 and eluted in 15 µl TE 1X. The PCR2 reactions were performed with PCR1 cleaned product and allowed to incorporate the P7# Illumina adapter. The PCR2 products were purified with Ampure XP in 7:10 ratio and eluted in 30 µl TE 1X.

The library quantification was done by qPCR following Tsai et al. (26) protocol using the Kapa Biosystems (Wilmington, MA) Kit for Illumina technology. The deep sequencing was performed at McGill University Genome Center using Illumina (San Diego, CA) Miseq Reagent Kit V2-300.

# b) In vivo experiment

Two hDMDΔ52/mdx (MI and MII) mice were injected IV with $AAV9_{SpCas9}$ ($9.6 \times 10^{13}$ gc/kg) (coding for SpCas9) and with $AAV9_{sgRNA1\text{-}50/5\text{-}54}$ ($1.2 \times 10^{14}$ gc/kg) (coding for 2 sgRNAs targeting exons 50 and 54 of the DMD gene) (Table 4). In addition to these two AAV9, 1mM of the 34 nucleotides oligonucleotide were also injected IV to these mice. Twenty-four hours later, the mouse MII was injected IV with $AAV9_{sgRNA1\text{-}2SpCas9}$ ($4 \times 10^{13}$ gc/kg) (coding for 2 sgRNAs targeting SpCas9 gene). The mice were sacrificed four weeks later and the liver, the heart and the diaphragm of each mouse were extracted. The genomic DNA was extracted from these organs using phenol-chloroform method previously described.

The DNA extracted from these different samples was then fragmented using the method previously described.

# Identification of on-target and off-target reads

Deep sequencing results were generated in Business Activity Monitoring (BAM) format (ngs-service@genomequebec.com, McGill University, Montreal, Canada). These results have been converted to FASTQ with Galaxy.org software. The FASTQ file was then opened with Sublime Text.

The 293Tcells were transfected with either plasmids $pBSU6_{1\text{-}50}$ and pX458 (Table 5, line 1), $pBSU6_{5\text{-}54}$ and pX458 (Table 5, line 3), or $pBSU6_{1\text{-}50\ /\ 5\text{-}54}$ and pX458 (Table 5, line 5) to target respectively the exons 50, 54 and 50–54 without repressing the SpCas9 gene to obtain the non-repressed (NR) reads. The repressed reads (R) were obtained from 293 T transfected with the same plasmids but also transfected 12 hours later with the plasmid $pBSU6_{1\text{-}2SpCas9}$ containing two sgRNAs targeting the SpCas9 gene (Table 5, lines 2, 4 and 6). The total number of reads is the sum of reads (+) and (-) to the dsODN (data not shown). The identification of on-target reads was done from the Sublime Text file. The first identification operation consisted in highlighting in each read the barcode (unique for each sample), which could be a sense or an antisense sequence. The second identification was the dsODN insertion, which could again be sense or antisense. The third identification was the sequence of the molecular barcode, which again could be sense or antisense. The fourth identification operation made it possible to locate the sequences of exons 50 and 54 or of the SpCas9 gene linked to the dsODN, which confirmed the on-target insertion. Indeed since the expected insertion sites at the dsODN in exons 50 and 54 and in the SpCas9 gene were known, we made two different BLAST analysis one using a 30 nucleotides sequence located 5’ of the expected dsODN insertion site and the other located 3’ of this expected insertion site. The number of "matches" (i.e., the correct matches between the selected DMD or SpCas9 sequence and the sequence obtained by deep sequencing and linked to the dsODN) appears at the bottom left of the Sublime text screen and was noted.

The off-target reads are sequences containing the barcode, the dsODN, the molecular index but which contained a DNA sequence with no similarity with the target sequences of the exons 50 and 54, or of the SpCas9 gene. To locate these DNA sequences linked to the dsODN in the genome, we selected about 150 nucleotides and searched these sequences with BLAST to determine their degree of similarity with the human genomic sequences. Sequences having a similarity to a coding or non-coding region of the genome were thus identified. These sequences were then searched in the Sublime Text file containing deep sequencing results to determine the frequency of mutations at this off-target site. Table 5 summarizes the percentages of on-target and off-target mutations.

# Declarations

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### Author contributions

JPIE and BD conducted the *in vitro* experiments; JR and KC conducted the *in vivo* experiments. AG contributed to the deep-sequencing data analysis and DO and JPT contributed to the writing of the article.

### Data availability

Data will be made available following a reasonable request.

### Statements and Declarations

This research was supported by grants from the Canadian Institute of Health Research and from the Cell and Gene Foundation (Jesse Journey 125794). JPIE had a fellowship from the Programme Canadien de Bourses de la Francophonie (PCBF). The authors have no relevant financial or non-financial interests to disclose. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. *All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by* Jean-Paul Iyombe-Engembe, Benjamin Duchêne, Joël Rousseau, Dominique Ouellet, Khadija Cherif, Antoine Guyon and Jacques P. Tremblay. *The first draft of the manuscript was written by* Jean-Paul Iyombe-Engembe, *and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Centre de recherche du Chu de Québec Université Laval (#*2018-3937, 2017-10-12).

### Ethical statement

The animal experiments conducted for this article were approved by the Comité de Protection des Animaux de l'université Laval (CPAUL).

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# Tables

Table 1
plasmid names

| Plasmid name | Sequence included |
|---|---|
| pX458 | SpCas9-FLAG and GFP |
| $pBSU6_{1-2-SpCas9}$ | $sgRNA_{1-SpCas9}$ and $sgRNA_{2-SpCas9}$ |
| $pBSU6_{1-50/5-54}$ | $sgRNA_{DMD1-50}$ and $sgRNA_{DMD5-54}$ |
| $pBSU6_{1-50}$ | $sgRNA_{DMD1-50}$ |
| $pBSU6_{5-54}$ | $sgRNA_{DMD5-54}$ |

Table 2
sgRNA

| sgRNA name | Targeted sequence |
|---|---|
| $sgRNA_{1-SpCas9}$ | 5' CGACCTGCGGCTGATCTATCTGG 3' |
| $sgRNA_{2-SpCas9}$ | 5'CCCCTGGAACTTCGAGGAAGTGG 3' |
| $sgRNA_{DMD1-50}$ | 5'TAGAAGATCTGAGCTCTGAGTGG 3' |
| $sgRNA_{DMD5-54}$ | 5'ACTTGGCCCTGAAACTTCTCCGG 3' |

Table 3
primers

| Primer name | Primer sequence |
|---|---|
| $FW_{SpCas9}$ | 5' ACAAAGACGATGACGATAAGATGG 3' |
| $RV_{SpCas9}$ | 5' TTATACACGGTGAAGTACTCGTAC 3' |
| $FW_{int49DMD}$ | 5' TTCACCAAATGGATTAAGATGTTC 3' |
| $RV_{int54DMD}$ | 5' TAGGATGAGACCATGTACAGCTAT 3' |
| $RV_{int50DMD}$ | 5’ ACTCCCCATATCCCGTTGTC 3’ |
| $FW_{int53DMD}$ | 5’ GTTTCAAGTGATGAGATAGCAAGT 3’ |
| $RV_{dsODN}$ | 5’GGATCTCGACGCTCTCCCTATACCGTTATTAACATATGACA 3’ |
| $FW_{dsOND}$ | 5’GGATCTCGACGCTCTCCCTGTTTAATTGAGTTGTCATATGTTAATAAC 3’ |

Table 4
virus names

| Virus name | Sequence included |
|---|---|
| $AAV9_{SpCas9}$ | SpCas9 gene under the CMV promoter |
| $AAV9_{sgRNA1-50/5-54}$ | $sgRNA_{DMD1-50}$ and $sgRNA_{DMD5-54}$ |
| $AAV9_{sgRNA1-2-SpCas9}$ | $sgRNA_{1-SpCas9}$ and $sgRNA_{2-SpCas9}$ |

**Table 5: the percentages of the on target and of the off target**

| Line # | Sample name | At time 0 | At time 12 hrs | On target % | Off- target % |
|---|---|---|---|---|---|
| 1 | Exon 50-NR | $sgRNA_{1\text{-}50}$ | | 75 | 25 |
| 2 | Exon 50-R | $sgRNA_{1\text{-}50}$ | $gRNA_{1\text{-}2SpCas9}$ | 96 | 4 |
| 3 | Exon 54-NR | $sgRNA_{5\text{-}54}$ | | 74 | 26 |
| 4 | Exon 54-R | $sgRNA_{5\text{-}54}$ | $gRNA_{1\text{-}2SpCas9}$ | 95 | 5 |
| 5 | Exon50-54-NR | $sgRNA_{1\text{-}50/5\text{-}54}$ | | 82 | 18 |
| 6 | Exon 50-54-R | $sgRNA_{1\text{-}50/5\text{-}54}$ | $gRNA_{1\text{-}2SpCas9}$ | 96 | 4 |

# Figures

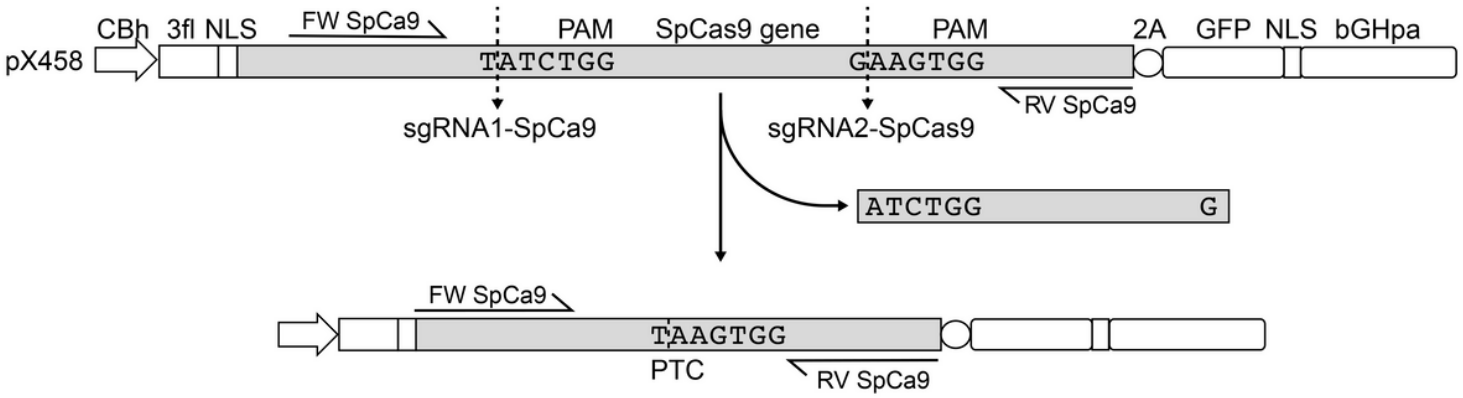
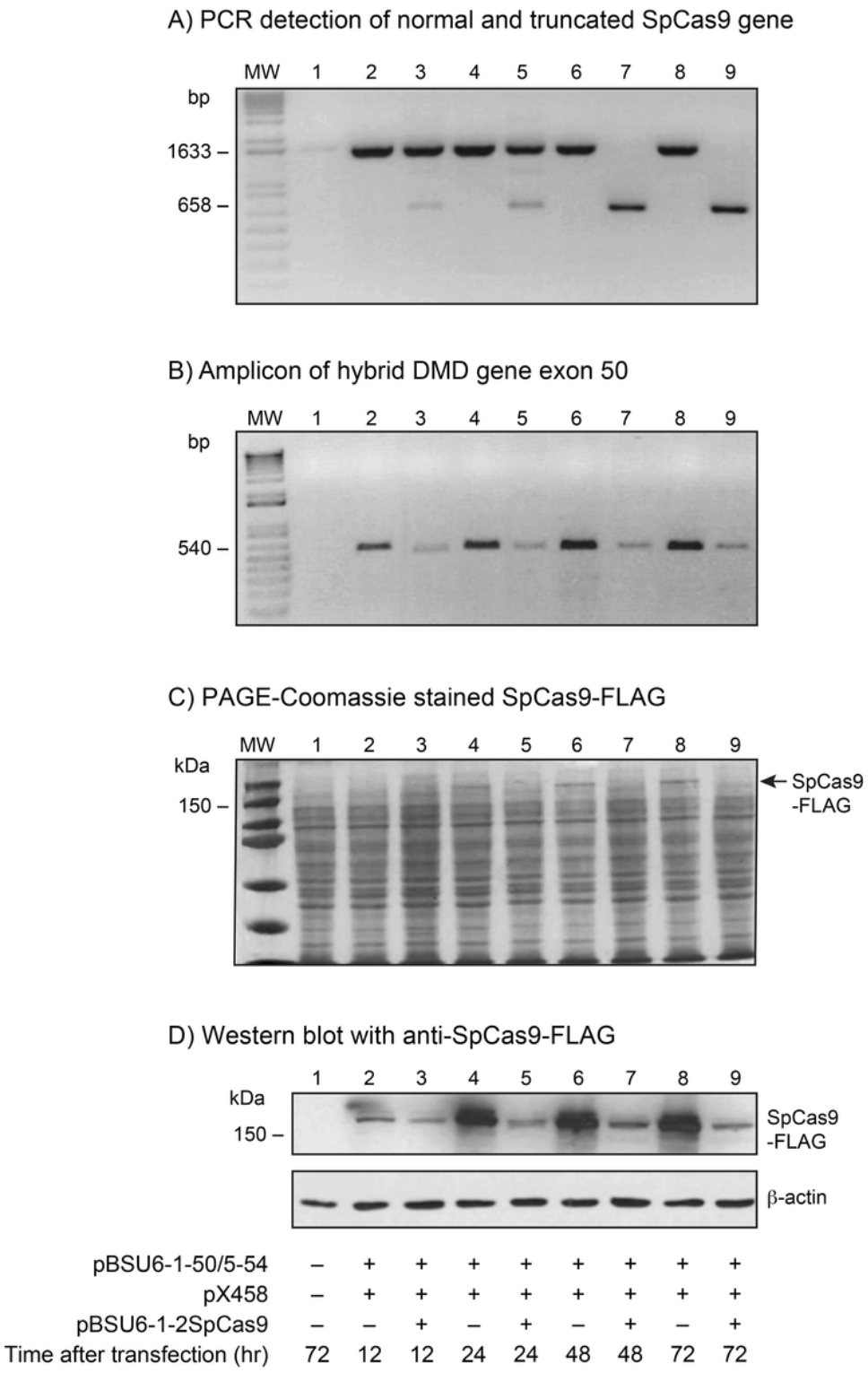



**Figure 1**

**Original and truncated SpCas9 gene**. Scheme of the pX458 plasmid containing the SpCas9 gene under a chicken b-actin promoter (CBh). The SpCas9 gene is preceded by a triple FLAG (3fl). The plasmid also includes two nuclear localization signals (NLS), a sequence coding for a 2A peptide, the green fluorescent protein (GFP) and a bovine growth hormone polyA (bGHpa). The cleavage sites of the Hara-Kiri method are indicated by double arrows. After the deletion of the fragment of the SpCas9 gene located between the two cut sites, there is the formation of a TAA stop codon. The positions of the $FW_{SpCas9}$ and $RV_{SpCas9}$ primers are indicated.




## Figure 2

**Immediate SpCas9 repression *in vitro*:** For A, B, C and D, cells in line 1 are from a negative control well (i.e., untreated cells). Cells in wells 2, 4, 6 and 8 were transfected only with pBSU61-50/5-54 and pX458 while cells in wells 3, 5, 7 and 9 simultaneously transfected in addition with pBSU61-2-SpCas9. Cells were collected 12, 24, 48 and 72 hours later. **A) PCR detection of normal and truncated SpCas9 gene:** Wells 2, 4, 6 and 8 showed the presence of the normal SpCas9 gene (1633 bp), wells 3 and 5 showed the presence

of the normal SpCas9 gene and of the truncated SpCas9 gene (658 bp) due to a partial cleavage of the SpCas9 gene 12 or 24 hours after the transfection. Only the truncated SpCas9 amplicon was observed in the wells 7 and 9 due to a total cleavage of that gene 48 or 72 hours after the transfection. **B) Amplicon of hybrid DMD gene exon 50-54:** PCR amplification of the hybrid DMD exon 50-54 produced a 540 bp band, which slightly increased progressively in intensity with increasing incubation times in the wells 3, 5, 7 and 9. This band remained at low intensity in wells 2, 4, 6 and 8 even with increasing incubation times. MW: Fragment size marker. **C) PAGE-Coomassie stained SpCas9-FLAG:** Proteins were extracted 12, 24, 48 and 72 hours after the transfection. A band of approximately 200 kDa corresponding to SpCas9-FLAG (arrow) was observed in the wells 4, 6 and 8. No SpCas9 band was detected in wells 3, 5, 7 and 9. **D) Western blot with anti-SpCas9-FLAG:** A band of approximately 200 kDa is present in wells 2, 4, 6 and 8. Only a minimal band of SpCas9-FLAG was present in wells 3, 5, 7 and 9.

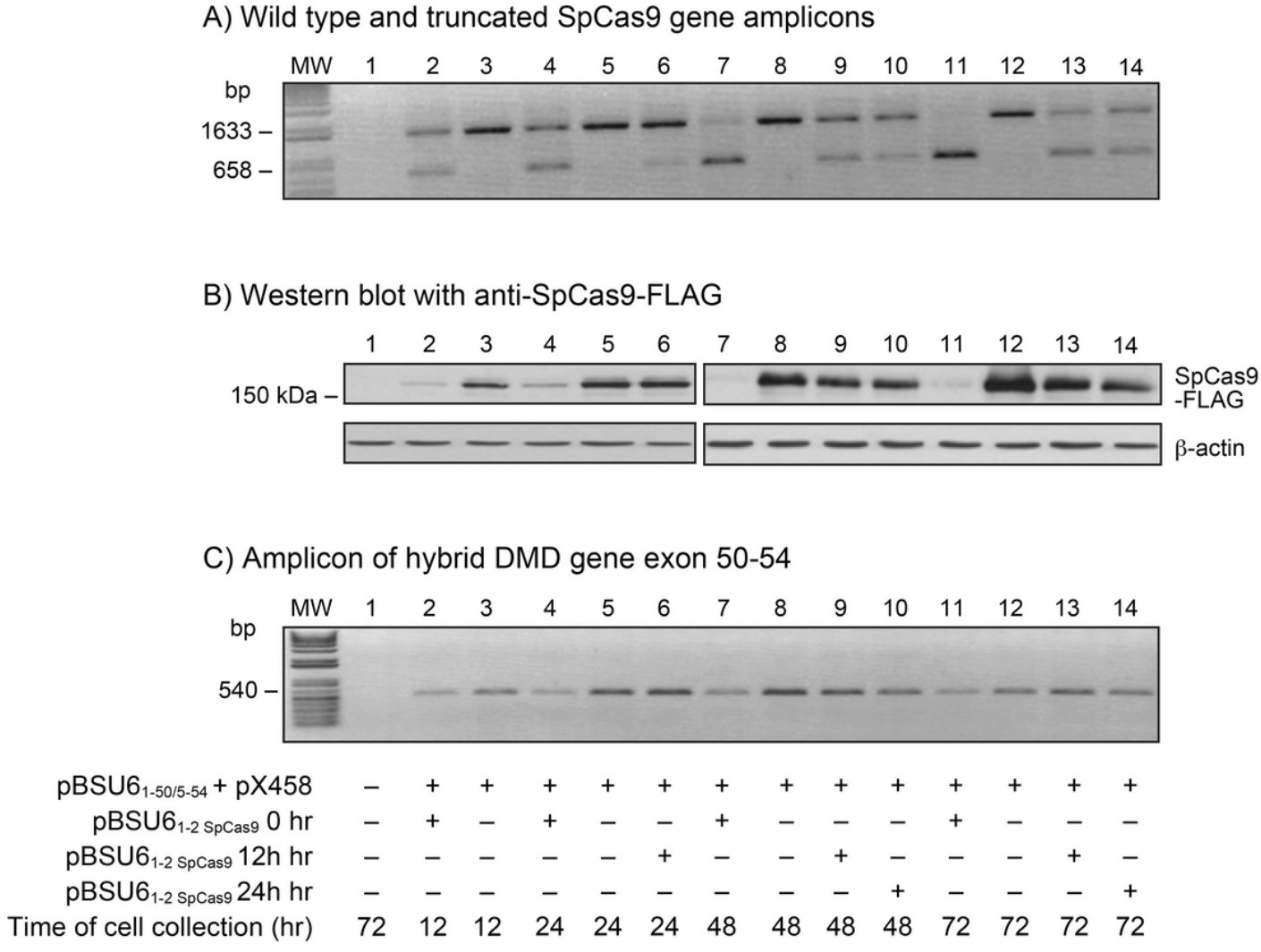
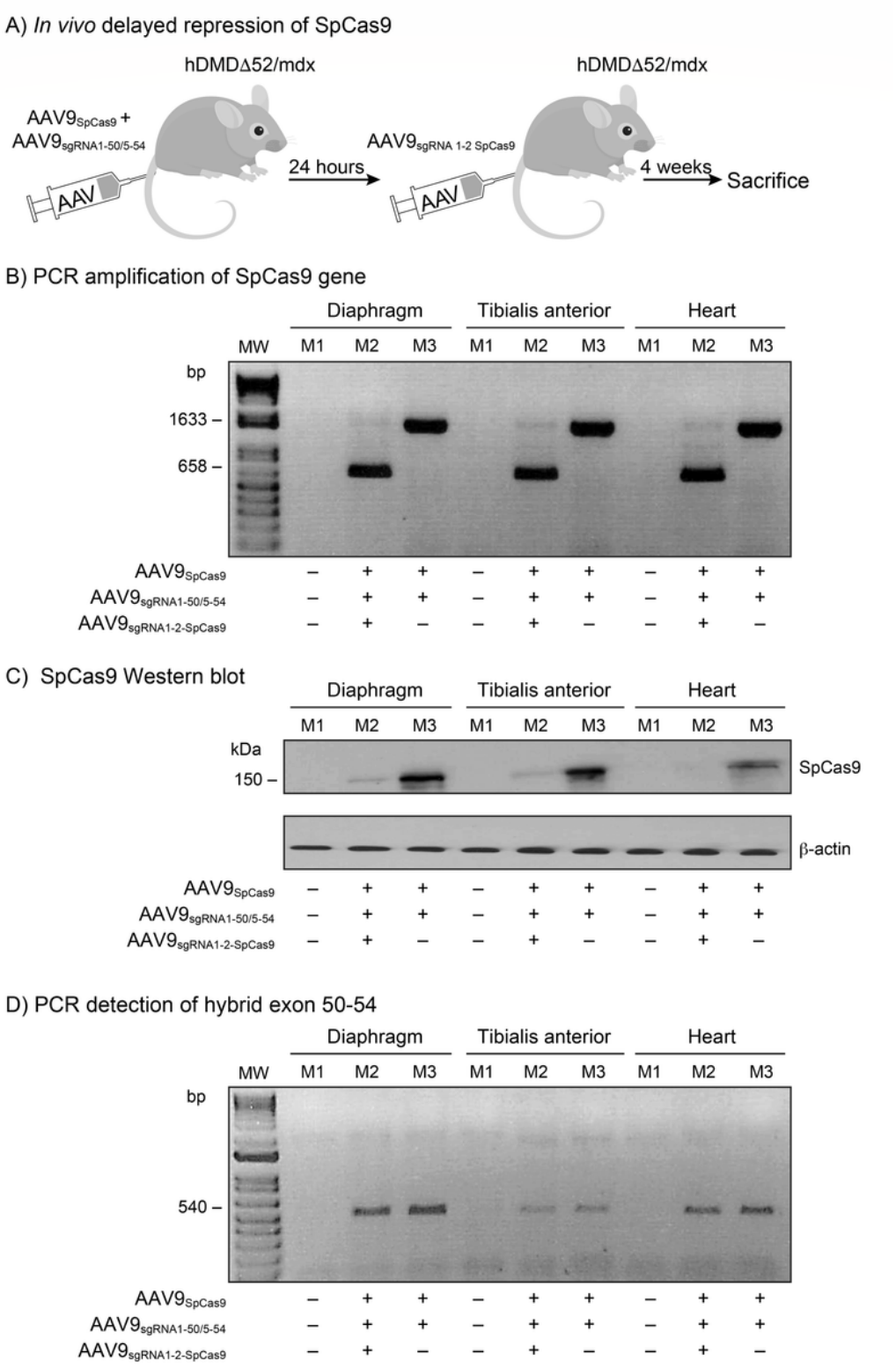



**Figure 3**

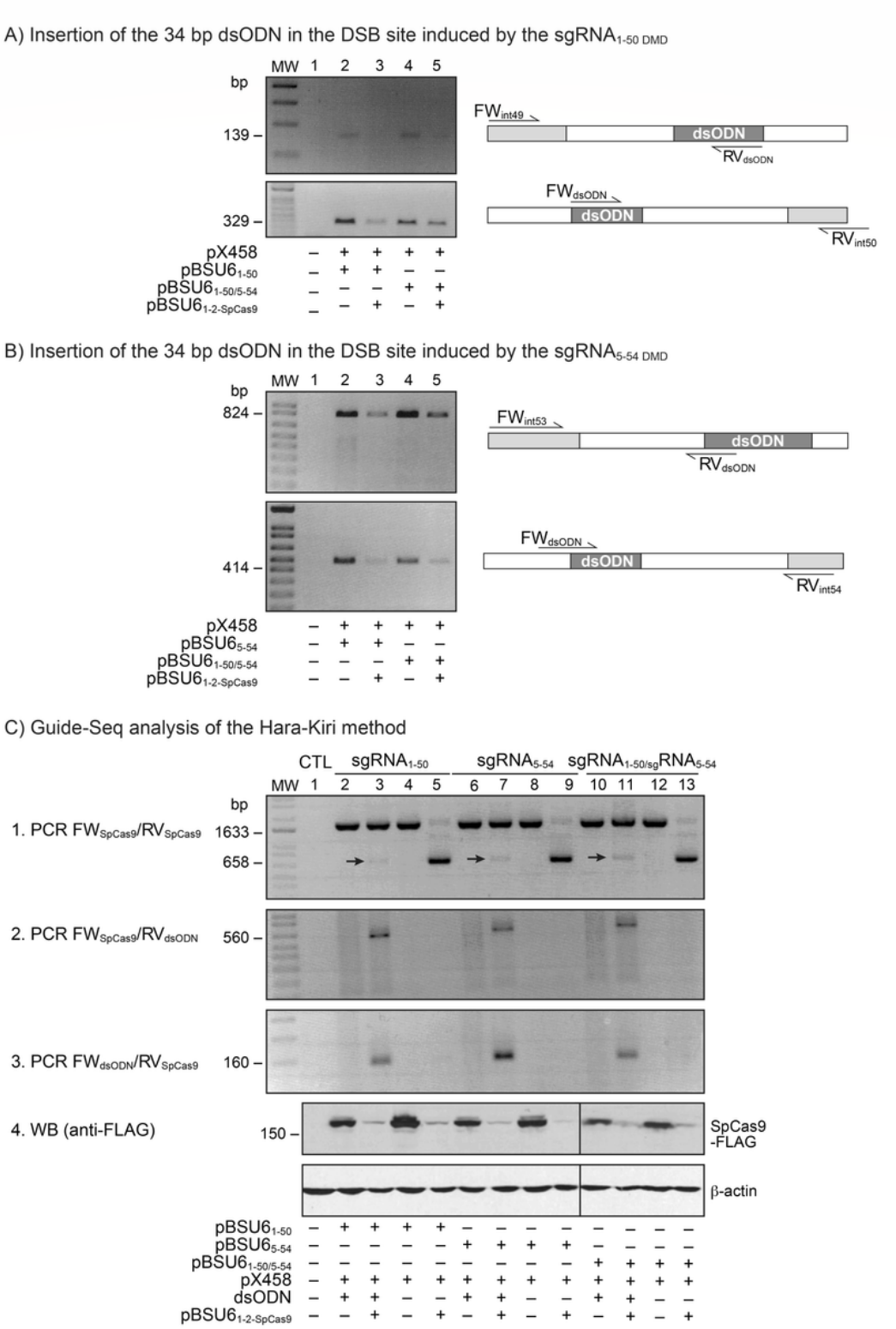
**Delayed repression *in vitro:*** Well 1: negative control (untreated cells). Cells of wells 3, 5, 8 and 12 were transfected only with pBSU61-50/5-54 and pX458 while wells 2, 4, 7 and 11 were simultaneously transfected in addition with pBSU61-2SpCas9. Cells of the wells 6, 9 and 13 were transfected initially with pBSU61-50/5-54 and pX458 and 12 hrs later pBSU61-2SpCas9 was transfected in the cells (delayed repression after 12 hrs). Cells of the wells 10 and 14 were transfected initially with pBSU61-50/5-54 and pX458 and 24 hrs later pBSU61-2SpCas9 was transfected in the cells (delayed repression after 24 hrs). Cells were collected 12, 24, 48 and 72 hours after the initial transfection. A) **Wild type (WT) and truncated SpCas9 gene amplicons:** Two SpCas9 amplicons (1633 bp and 658 bp) are present in wells 2, 4, 6, 7, 9, 10, 13 and 14 corresponding respectively to the WT SpCas9 gene and of the truncated SpCas9 gene due to a partial cleavage of SpCas9 gene 12 to 48 hours after the transfection with the sgRNAs targeting the SpCas9 gene (plasmid pBSU61-2SpCas9). A single band of 658 bp was observed in the well 11 due to the complete cleavage of the SpCas9 gene 72 hours after the transfection of plasmid pBSU61-2SpCas9. A single band of 1633 bp was also observed in wells 3, 5, 8 and 12 because the cells were not transfected with plasmid pBSU61-2SpCas9. **B) Western blot with anti-SpCas9-FLAG:** A band of approximately 200 kDa corresponding to SpCas9-FLAG is present in the wells 3, 5, 8 and 12. The intensity of this band increases with incubation time after the transfection (unrepressed SpCas9 gene control)). In the wells 2, 4, 7 and 11, only a low intensity SpCas9-FLAG band is present (immediate repression). In the cells of the wells 6, 9, 10, 13 and 14, the intensity of this band is reduced in comparison with that of unrepressed SpCas9 gene. **C) Amplicon of hybrid DMD gene exon 50-54:** The hybrid DMD exon 50-54 was amplified by PCR (540 bp band). The intensity of this band increased progressively in the wells 3, 5 and 8 with increasing incubation time. In wells 2, 4, 7 and 11, the cells were transfected with plasmids pBSU61-50/5-54, pX458 and pBSU61-2SpCas9 respectively for 12, 24, 48 and 72 hours after the first transfection, the intensity of the hybrid exon 50-54 band is thus lower. MW: Fragment size marker.




## Figure 4

**A: *In vivo* delayed repression of SpCas9.** The figure illustrates a scheme of the experiment. Mouse 1 (M1) was a negative control and was not injected with any AAV. Mice 2 and 3 (M2 and M3) were injected i.v. with AAV9SpCas9 and with AAV9sgRNA1-50/5-54 at time 0 hr. Twenty-four (24) hours later mouse 3 (M3) were injected i.v. with AAV9sgRNA1-2-SpCas9. The mice were sacrificed 4 weeks later. **B) PCR amplification of SpCas9 gene.** The SpCas9 gene was PCR amplified from the DNA extracted from the

diaphragm, Tibialis anterior and heart of the 3 mice. No SpCas9 band was detected in M1. A strong truncated SpCas9 band (658 bp) was detected in the 3 tissues of M2 but a very weak full size SpCas9 band (1633 bp) was still present. Only the full size SpCas9 amplicon (1633 bp) was detected in M3. **C) SpCas9 Western blot.** No SpCas9 band was detected in tissues obtained in M1. However, a strong SpCas9 band was detected in M3 and this band was reduced in intensity in the M2 mouse tissues due to the repression of the SpCas9 gene by the Hara-Kiri treatment. **D) PCR detection of hybrid exon 50-54.** The hybrid exon 50-54 was not detected in the tissues extracted from mouse M1. It was however present in all the tissues extracted from mouse M2 and M3.




## Figure 5

**Hara-Kiri results.** HEK 293T were transfected with plasmids pX458, pBSU61-50, pBSU6-5-54, pBSU61-50/5-54 and/or pBSU61-2-SpCas9 in presence of a 34 bp phosphorylated dsODN. **A)** The insertion of the 34 bp dsODN in the DSB site induced by the sgRNA1-50 DMD was detected by PCR amplification using primers FWint49DMD and RVdsODN producing a 139 bp band (top figure). The insertion of the dsODN in exon 50 was also confirmed with primers $FW_{dsODN}$ and $RV_{int50DMD}$ producing a 329 bp band (bottom

figure). **B)** The insertion of the 34 bp dsODN in the DSB site induced by the sgRNA5-54 DMD was detected by PCR using primer $FW_{int53DMD}$ and $RV_{dsODN}$, producing a 824 bp band (Top figure). The insertion of the dsODN in DMD exon 54 was also detected by PCR with primers $FW_{dsODN}$ and $RV_{int54DMD}$ producing a 414 band (Bottom figure). **C**) HEK 293T cells were transfected with pBSU61-50 (600 ng), pBSU65-54 (600 ng), pBSU61-50/5-54 (600 ng), pBSU61-2-SpCas9 (600 ng) and/or pX458 (600 ng) in presence or not of the phosphorylated dsODN (10 pmol). **C1)** The truncation of the SpCas9 gene by the two sgRNAs targeting that gene present in $pBSU6_{1\text{-}2SpCas9}$ in the Hara-Kiri method was detected by PCR amplification with the $FW_{SpCas9}$ and $RV_{SpCas9}$ primers. The 1633 bp amplicon corresponded with the non-truncated SpCas9 gene while the 658 bp amplicon was due to the internally deleted SpCas9 gene. Paradoxically, the intensity the 658 bp amplicon was very low in the well where SpCas9 was repressed with the pBSU6-1-2SpCas9 and in which dsODN was added (arrow, wells 3, 7 and 11)

**C2).** The insertion of dsODN at the DSB sites in SpCas9 gene was detected by PCR with primers $FW_{SpCas9}$ / $RV_{dsODN}$ producing a 560 bp amplicon. **C3)**. The insertion of dsODN at the DSB sites in SpCas9 gene was also detected by PCR with primers $FW_{dsODN}$ and $RV_{SpCas9}$ producing a 160 bp amplicon. **C4)**. The expression of the SpCas9-FLAG gene was detected by western blot using a mAb against the FLAG epitope. The expression of that protein was strong when the cells were not transfected with plasmid pBSU61-2-SpCas9 (lines 2, 4, 6, 8, 10 and 12) and was weak in cells transfected with that plasmid (lines 3, 5, 7, 9, 11 and 13).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfigure1andlegend.docx
- Supplementaryfigure2andlegend.docx