



Development of Donor DNA Construct for RNA-Guided Recombinase (RGR) Genome Editing Platform-mediated Knockout of Myostatin b (*mstnb*) Gene in Zebrafish

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ABSTRACT

Background: The RNA-guided recombinase (RGR) platform is a next-generation genome editing platform functioning on safer and cell-independent recombinase action instead of nuclease action. It is a fusion of nuclease-null or dead Cas9 (dCas9) and hyperactivated recombinase. RGR platform recombines user-modified donor DNA and targeted genomic DNA to carry out genome editing. RGR platform can be used to 'create' upstream STOP codon by recombining donor DNA so modified to knockout a targeted gene. The present study reports the development of such a donor DNA construct for RGR platform-mediated knockout of zebrafish myostatin b (*mstnb*) gene.

Methods: Zebrafish myostatin gene sequence was retrieved and it was screened for the presence of target sites for RGR platform based on Sin hyperactivated recombinase. A gene locus flanked by two RGR platform target sites was selected to create the upstream STOP codon using RGR platform. The gene fragment containing the locus and the RGR target sites was PCR amplified and a substitution mutation was introduced using site-directed mutagenesis. The mutated sequence was, confirmed using Sanger DNA sequencing. Structural analysis was performed to assess the impact of the mutation using SWISS-MODEL.

Result: The donor DNA construct to create an upstream STOP codon in the exon 2 of zebrafish *mstnb* gene was developed. It was further characterized using Sanger DNA sequencing, which revealed precise mutation and formation of the upstream STOP codon at the desired locus flanked by two RGR target sites. *In silico* analysis of the mutated gene sequence revealed the loss of the functional domains and reduced structural integrity, supporting the loss of myostatin function.

Key words: Hyperactivated recombinase, Myostatin b, RGR platform, Site-directed mutagenesis.

INTRODUCTION

Over the past 20 years, genome editing has made substantial progress. 'Mega nucleases', or homing endonucleases, are the first class of genome editing enzymes. These proteins identify dsDNA of 20-30 bp motifs through specific protein-DNA interactions (Stoddard, 2011). The use of zinc finger proteins for gene editing is a significant advancement. Subsequent advancements led to the development of zinc finger nucleases with dsDNA recognition ability. Subsequently, successful modification of eukaryotic genomes was achieved using synthetic fusion proteins that combined the specific dsDNA recognition ability of zinc finger domains (3 bp per ZF domain) with the dsDNA cleavage function of the FokI catalytic domain (a type IIS restriction enzyme) (Kim *et al.*, 2012). These zinc finger nucleases (ZFNs) have been effectively utilized for targeted cleavage and subsequent editing of genomic sites in eukaryotic model organisms (animals, plants) and human stem cells (Urnov *et al.*, 2010). A similar approach involves the adaptation of transcription activator-like effector nucleases (TALENs), where the same FokI nuclease domain is fused to the TALE protein, typically featuring an 11-domain dsDNA recognition structure (1 bp per domain). Since the FokI domain generates dsDNA cleavage as a dimer, ZFNs and TALENs operate as dimers with a central

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cleavage site. A practical challenge with ZFNs, TALENs and homing endonucleases is that modifying their target specificity is relatively labor-intensive, as it necessitates varying degrees of protein engineering (Mehta *et al.*, 2017). The CRISPR/Cas9 system, on the other hand, enabled the development of a number of approaches that allow for precise and site-specific DNA editing, broadening the possibilities for creating complex genetic models in any species, including zebrafish (De Santis *et al.*, 2020). However, nuclease-based genome editing techniques

pose problems since they require a DSB, which can lead to genotoxicity and they depend on cellular repair machinery for genome editing (Sancar *et al.*, 2004; Gabriel *et al.*, 2011; Fu *et al.*, 2014; Boutin *et al.*, 2022). Recombinases, such site-specific recombinases (SSRs), offer a single, accurate DNA modification without DSB and lower genotoxicity, making them a safer alternative to nuclease-based genome editing (Grindley *et al.*, 2006). Tyrosine recombinases (like Flp and Cre) and serine recombinases (like Hin, Sin and Gin) are two types of SSRs; serine recombinases provide greater versatility for genome engineering (Grainge and Jayaram, 1999; Grindley *et al.*, 2006; Sarkar *et al.*, 2007; Pathak *et al.*, 2020). Its strict target specificity, which necessitates either rare pre-existing sites in higher eukaryotic genomes or the laborious pre-introduction of target sites therein, has restricted the use of wild site-specific recombinases (SSRs) in genome editing (GE) (Thyagarajan *et al.*, 2000; Chalberg *et al.* 2006; Grindley *et al.* 2006; Quyyoom *et al.*, 2025). Using strategies like directed evolution, “hyperactivated” SSR variations with relaxed target specificity and no need for auxiliary proteins have been created to circumvent these issues. By recombining minimum recognition sequences, these modifications improve their application in GE (Buchholz and Stewart, 2001; Gaj *et al.*, 2013; Sirk *et al.*, 2014).

The RGR platform is a fusion of hyperactivated SSR and dead Cas9 (dCas9) that can precisely catalyze strand cleavage, exchange and re-ligation of two double-stranded DNA sequences. Moreover, these are independent of cellular machinery and produce a single well-defined product, which makes genome editing simple and programmable (Mali *et al.* 2013; Sonwane, 2014; Chaikind *et al.* 2016; Standage-Beier *et al.* 2019; Sonwane *et al.*, 2021; Sonwane *et al.*, 2022; Quyyoom *et al.* 2025). RGR platform can be used to add or remove targeted nucleotide(s) using RGR-mediated cassette exchange (RGR-MCE) involving double recombination between modified donor DNA and genomic DNA utilizing a unique pair of RGR target sites flanking the modified locus. Using this, an upstream STOP codon to knock out the targeted gene can also be created.

Myostatin (*mstnb*), 26 kDa homodimeric protein of the TGF- β superfamily, is a well-known negative regulator of skeletal muscle development (Mcpherron *et al.* 1997). It was first discovered in breeds of cattle, *Bos taurus* such as Belgian Blue and Piedmontese (Mcpherron and Lee, 1997) and later identified in sheep, dogs and humans, highlighting its evolutionary conservative role. In mammals, it is expressed in skeletal muscle and in teleost fish, its primary expression is in skeletal muscle but the additional expression is in different tissues such as the brain, ovary, gill, spleen, gut and to a lesser extent in the testes (Mehra and Kumar, 2022). In mammals, it is encoded by a single gene, while teleosts possess two paralogs and salmonids carry four copies due to genome duplication events (Østbye *et al.*, 2007). Structurally, *mstnb* contains three exons and

two introns, encoding a protein with 374 amino acids with conserved features such as an N terminal signal peptide, an RSRR hydrolysis site and a cysteine-rich C terminal domain. It gets activated by proteolytic cleavage of the NH₂-terminal or pro-domain, resulting in the formation of an active COOH terminal dimer. In order to recruit the alk-3 or alk-4 coreceptor, it interacts with the activin type II receptor. This co-receptor initiates a cell signaling cascade in the muscle, activating the SMAD family to regulate myostatin gene expression (Rodriguez *et al.* 2014). According to Wang *et al.* (2018), myostatin b (*mstnb*) is involved in muscle development and myostatin a (*mstna*) is associated with immune function.

Over the last few decades, aquaculture has experienced enormous growth in terms of both production volume and value, which has coincided with a rise in demand for aquaculture products, notably fish. (Action 2020). In order to improve the growth rate of aquatic species, several genetic techniques have been utilized (Osmond and Colombo 2019). Since myostatin negatively regulates skeletal muscle growth, it has been a target for genetic manipulation in aquaculture species to improve growth rates.

Zebrafish serve as a crucial model for genetic and biological research owing to their significant genetic resemblance to humans, simplicity of manipulation and applicability for wide range of studies (Teame *et al.*, 2019; Espino-Saldaña *et al.*, 2020; Choi *et al.*, 2021). The present study aimed to develop a donor DNA construct with an upstream STOP codon to knockout *mstnb* gene using RGR platform-mediated genome editing in zebrafish.

MATERIALS AND METHODS

The experiment was conducted between 2021 and 2024 at the Fish Genetics and Biotechnology Laboratory, ICAR-Central Institute of Fisheries Education (CIFE), Mumbai, India, as part of doctoral research work. The Zebrafish myostatin b (*mstnb*) gene sequence was retrieved from NCBI database (NCBI Reference Sequence: NC_007120.7). The search pattern (5' - CCN₍₇₄₎GG - 3') corresponding to the hyperactivated Sin recombinase-based RGR platform was entered into the DNA pattern find tool (http://bioinformatics.org/sms2/dna_pattern.html) and the RGR target sites were identified in the *mstnb* sequence. With the intention of creating substitution mutation, the sequence was carefully analyzed where a single nucleotide substitution would lead to an upstream stop codon. It was ensured that this locus was flanked by a pair of RGR target sites. PCR primers flanking the pair of RGR target sites were designed using the EditSeq (DNASTAR) software and their quality was verified with IDT's OligoAnalyzer tool. Primers were then synthesized commercially (Forward primer- 5'-GAATAAACCTACAACCTGAAAACCATATTCC-3' and the Reverse primer- 5'-GGTAACAACAGACCAACTCACC-3') to amplify the region.

Genomic DNA was extracted from zebrafish muscle using the phenol-chloroform method. The *mstnb* target region was amplified using Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific™ Cat. No.: F530S) and the amplicons were purified using QIAquick Gel Extraction Kit (Qiagen, Cat. No. ID: 28704). The amplicon was cloned using pJET1.2 blunt cloning vector (Thermo Scientific Cat. No. K1231). The recombinants were screened using colony PCR with DreamTaq DNA polymerase (Thermo Scientific Cat. No: EP0701). Positive colonies were cultured and the plasmid DNAs were isolated using QIAprep Spin Miniprep Kit (Qiagen, Cat. No.: 27104). The insert was confirmed using Sanger DNA sequencing.

The Q5® Site-Directed Mutagenesis Kit (NEB, Cat. No.: E0554S) was used to carry out site-directed mutagenesis following the manufacturer protocol. Mutagenic primer was designed (using the NEBase changer tool) in such a way that it incorporated a single nucleotide mismatch at the desired site with 10 complementary nucleotides at the 3' end of the primer targeting the template plasmid. The second primer was designed fully complementary to the plasmid sequence and oriented in the opposite direction, enabling exponential amplification. After PCR amplification, the reaction was treated with a KLD (Kinase, Ligase and *DpnI*) enzyme mix to remove template DNA and circularize the mutated plasmid. The resulting product was transformed into *E. coli*. Transformed cultures were plated on LB agar containing ampicillin and were incubated overnight at 37°C. Proper insertion of the substitution mutation was confirmed using Sanger DNA sequencing. Furthermore, the sequence of mutated *mstnb* was subjected to *in silico* analysis. The InterPro database (<https://www.ebi.ac.uk/interpro/>) provides an integrative classification of protein sequences into families and identifies functionally important domains and conserved sites. It combines 13 protein signature databases into one central resource (Paysan-Lafosse *et al.*, 2023). Both the wild-type zebrafish *mstnb* sequence and the mutated nucleotide sequences were analyzed for functional domains to determine whether the mutations resulted in a defective or absent C-terminal domain. After analysing the functional domain, the structure analysis was done in SWISS-MODEL.

RESULTS AND DISCUSSION

The myostatin gene is present on chromosome 9 of zebrafish and its size is 3.8 Kb [NC_007120.7]. Using DNA star EditSeq software, the target region at 1390 bp position in exon 2 was identified. Here, if the guanine nucleotide was substituted with thymine, it results in the conversion of glycine amino acid into an upstream stop codon (TGA), thereby creating a nonsense mutation. This would lead to functional disruption of the *mstnb* gene.

The template DNA was successfully amplified, yielding a clear band of the expected size (469 bp) visualized on a 1% agarose gel (Fig 1). After purification of the eluted

template DNA, the amplicon was cloned into pJET1.2 blunt vector and transformed into *E. coli* (DH5α strain). Following overnight incubation, multiple colonies were observed. Of the four colonies tested by colony PCR, all exhibited the expected band size of 469 bp, with no amplification in the non-template control (NTC) (Fig 2). Then the plasmid was isolated (Fig 3) and checked on the gel for its quality and was sequence analysed for the insert sequence. Sequencing analysis confirmed that the target region was successfully cloned into the vector (Fig 5A).

The plasmid was further used to introduce a site-directed mutation, *i.e.*, single nucleotide substitution. For each plasmid (Fig 4), glycerol stocks were prepared and stored at -80°C for future use. Sequencing of the forward

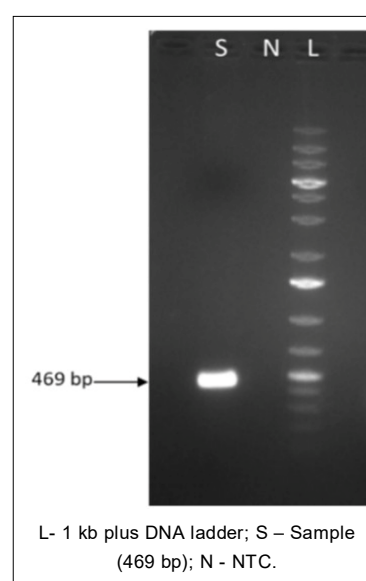


Fig 1: PCR amplification of *mstnb*.

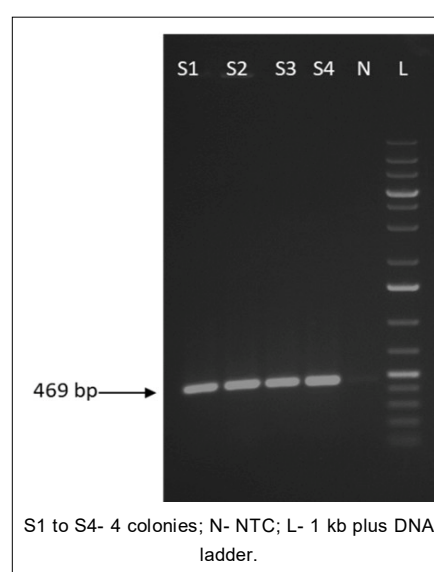


Fig 2: Colony PCR of putative recombinants.

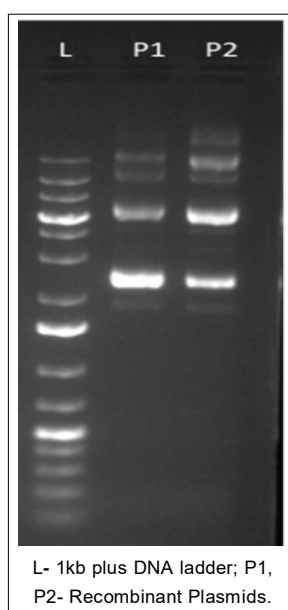


Fig 3: Plasmid isolation of recombinant plasmids.

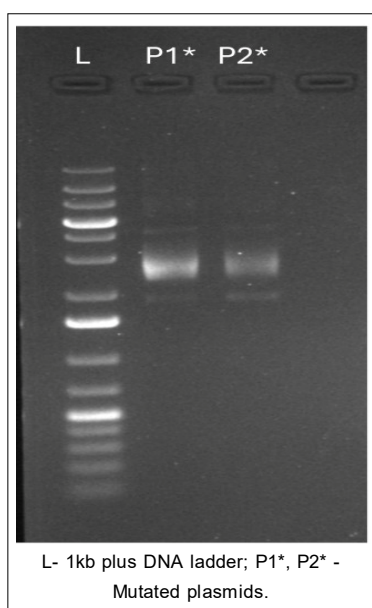


Fig 4: Plasmid isolation of mutated plasmids.

strand confirmed the successful incorporation of the intended mutation, specifically the substitution of thymine for guanine at the target site, confirming the accuracy of the designed modifications. The sequencing chromatograms exhibited clear and distinct peaks at the mutation site, with no additional alterations detected in the surrounding regions, confirming the specificity and accuracy of the mutagenesis process (Fig 5B). Furthermore, no off-target mutations were observed, validating the precision of the experimental approach.

The *in silico* analysis in the InterPro revealed that wild sequences have the TGF-beta propeptide domain and the cytokine activity domain that is conserved in nature. It identified the growth factor activity (GO:0008083) and cytokine activity (GO:0005125) with cellular localization in the extracellular region (GO:0005576) and space (GO:0005615) (Fig 6A). These features align well with the role of negative myostatin regulation. In the truncated sequence results, the cytokine activity (GO:0005125) is disrupted and loss of TGF-beta propeptide domain (Fig 6B). This suggests that it may lead to loss of function of that gene. Since this propeptide domain will cleave and activate the functional C terminal domain.

Then, we analyzed the protein structure using the SWISS MODEL for both the wild and truncated proteins. (Fig 7A and B). The structural validation of wild-type and truncated *mstnb* models reveals a notable decline in model quality following truncation. The QMEANDisCo global score for the wild-type model is 0.62 ± 0.05 , indicating a moderately reliable structure, while the truncated model exhibits a lower score of 0.49 ± 0.06 , reflecting a low-quality model. The Ramachandran plot analysis shows that the wild-type *mstnb* has 88.71% of residues in the favored region and 1.88% as outliers, compared to the truncated model, which has 84.53% favored residues and 3.87% outliers suggesting increased backbone strain post-truncation (Fig 8A and B). MolProbity scores further support this trend (<1.5 indicates good geometry), with the wild-type scoring 1.29 and the truncated version at 1.54, where higher values indicate less accurate geometry. The QMEAN Z score analysis (Qualitative model energy analysis) evaluates the structural quality of the predicted model by comparing it with a non-redundant set of high-resolution PDB structures. The wild-type *mstnb* model's red star aligning closer to the central

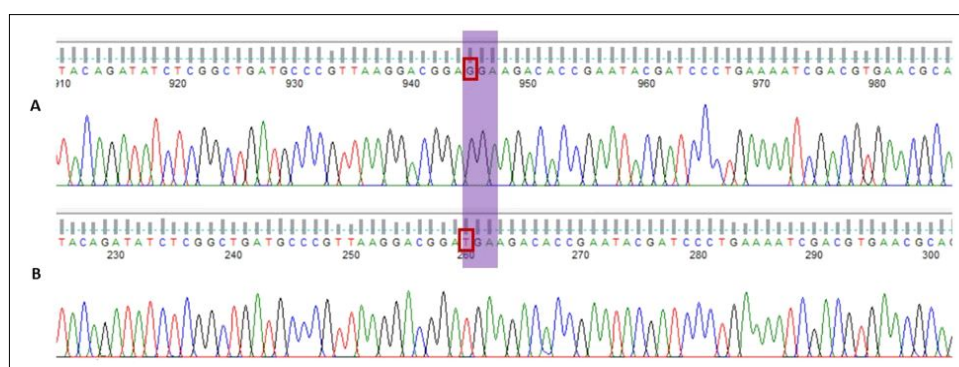


Fig 5: Incorporation of desired mutation in the plasmid (A) Non-modified sequence (B) Modified sequence (G>T).

cluster of PDB structures ($1 < |Z \text{ score}| < 2$) suggests a good-quality model. In contrast, the truncated model's star is shifted downward ($|Z \text{ score}| > 2$), signifying greater structural deviation from experimentally resolved proteins (Fig 9A and B). Collectively, these results highlight that truncation likely disrupts key secondary structures required for dimerization, weakening the interface necessary for forming functional dimers. The destabilization showed by the truncated model of *mstnb* supports the hypothesis that introducing an upstream stop codon in exon 2 can abolish myostatin activity.

The work described here defines a strategy to construct a donor DNA by incorporating single nucleotide substitution. To accomplish precise gene editing, three primary methods are currently used: Prime editor (PE),

base editor (BE) and HDR (Zhang *et al.* 2022; Jin *et al.* 2024; Pacesa *et al.* 2024). Among these, HDR is the most adaptable; however, it relies on programmable endonucleases to generate DSBs, which could lead to undesirable modifications *via* end-joining pathways. Thus, enhancing HDR efficiency has been a significant focus, with strategies falling into two categories: Optimizing donor DNA design and directing the DSB repair pathway toward HDR (Jin *et al.* 2024). HDR results in either knock-in events or point variant repair by recombination using a donor DNA template. Additionally, the applicability of HDR in post-mitotic cells is limited because it is mostly active during the S phase of the cell cycle (Chapman *et al.* 2012; Cox *et al.* 2015; Croci *et al.* 2020). However, recent works have demonstrated that HDR is effective in non-dividing cells,

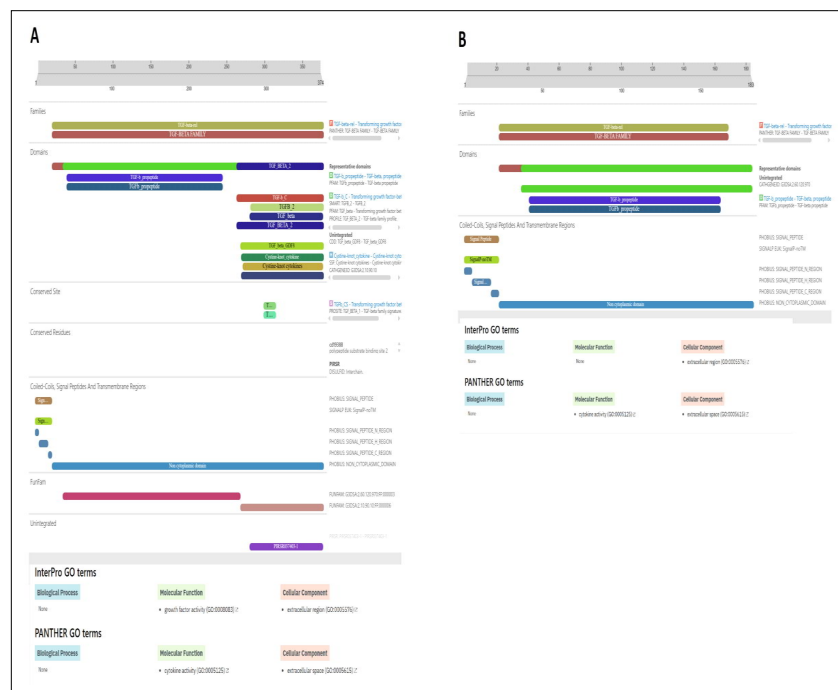


Fig 6: (A) InterPro results of wild *mstnb* sequence and (B) The truncated sequence of *mstnb*.

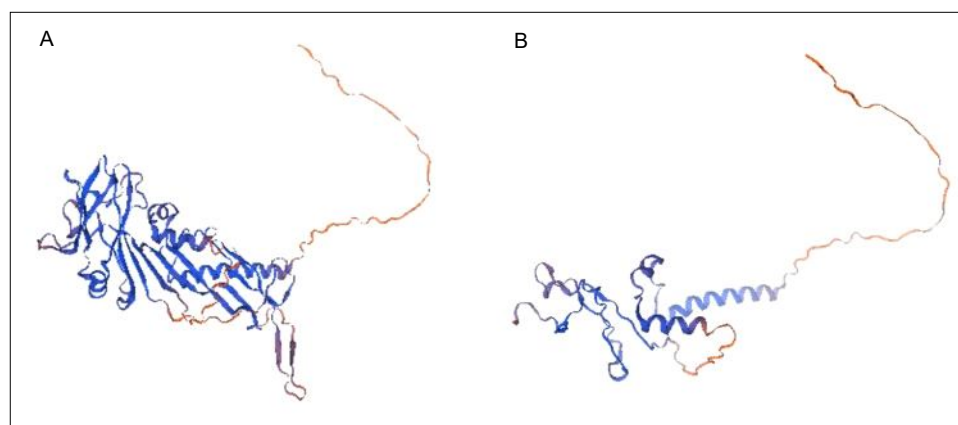


Fig 7: Three-dimensional protein structure of (A) Wild *mstnb* (B) Truncated *mstnb* using SWISS MODEL- homology modelling.

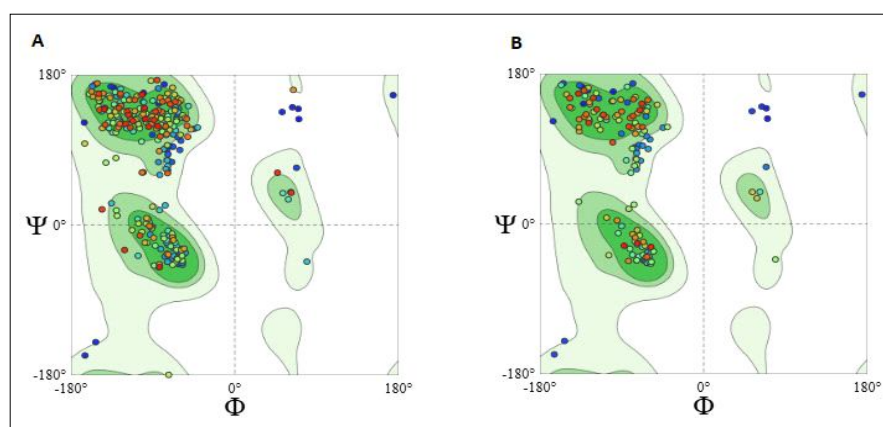


Fig 8: Ramachandra plot for the (A) Wild *mstnb* protein structure (B) Truncated *mstnb* protein structure.

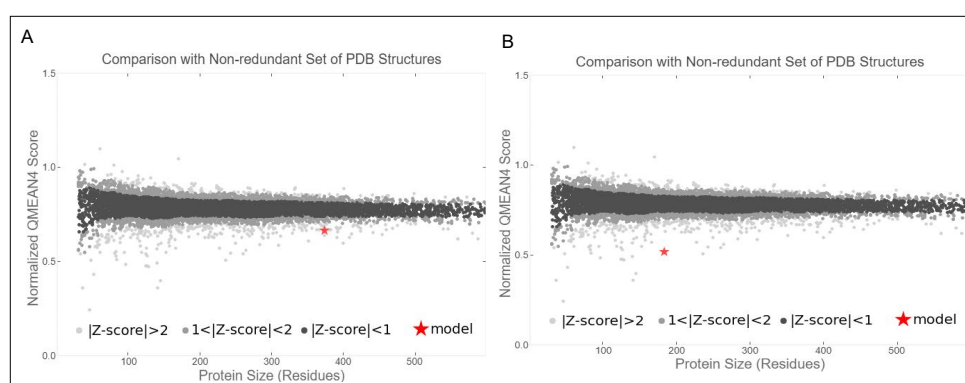


Fig 9: Normalized QMEAN Z score analysis for (A) Wild *mstnb* and (B) Truncated *mstnb*.

such as terminally differentiated neurons, allowing its application to disorders affecting tissues with limited regeneration/renewal capacity, including the CNS (Ishizu *et al.* 2017; Nishiyama *et al.* 2017; Croci *et al.* 2020). Cas9-induced HDR stimulation increases the frequency of targeted transgene integration by at least twofold, which makes HDR-accurate genome editing possible (Charpentier *et al.* 2018; Tang *et al.* 2019). The length of the homology arm is also a significant factor in raising the HDR rate since longer homology arms result in higher recombination efficiency (Li *et al.* 2014). Song and Stieger (2017) discovered that in HEK cells linearised plasmids are more efficient than plasmid donors, implying that circular DNAs can be randomly disrupted at undesired sites. However, in *Drosophila* embryos the plasmid donor was found to be more efficient than the linearised donor, most likely due to the degradation of linear DNA by exonucleases or the conversion of long concatemers. The delivery strategy may also influence the fate of the plasmid donor and linearised donor because the length of duration time of donor in the cytoplasm increases the possibility of degradation (Song and Stieger, 2017). Xu *et al.* (2013) used TALEN to precisely target exon 2 of human *mstnb* locus and suggested that targeting exon 2 is a viable approach to disable the function of myostatin.

Homology modeling (or comparative protein structure modeling) approaches developed to build three-dimensional models of a protein from its amino acid sequence as an input and align with a similar known protein structure (template) (Topham *et al.* 1990; Šali and Blundell 1993; Bordoli *et al.* 2009). Homology models are widely used in many applications, such as virtual screening, designing site-directed mutagenesis experiments or rationalizing the effects of sequence variations (Bordoli *et al.*, 2009). Accurate prediction of protein stability changes resulting from single amino acid mutations is important for understanding protein structures and designing new proteins (Cheng *et al.*, 2006). By using homology modelling, the loss of domains in truncated protein was identified in this study. In most studies (Khalil *et al.* 2017; Coogan *et al.* 2022; Yan *et al.* 2022) they targeted mostly *mstnb* gene, exon 1. Here, we targeted exon 2 and wanted to know its effects. Since *in silico* analysis confirmed that even if we target exon 2, the C-terminal domain is affected, which is the active terminal for *mstnb* gene function.

CONCLUSION

The study successfully created a nonsense mutation in the exon2 of *mstnb* gene. Sequencing confirmed the substitution of nucleotide (G>T) in the DNA sequences.

The mutation led to premature truncation of the protein and loss of functional domains confirmed by *in silico* analysis. These findings provide a basis for further *in vivo* validation and highlight the potential of this approach for successful gene editing strategies.

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Disclaimers

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Conflict of interest

The author declares that they are no conflict of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish or preparation of the manuscript.

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