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Designing and Construction of Donor DNA for RNA-Guided Recombinase (RGR) Platform-Mediated Knockout of Leptin B (*Lepb*) Gene in Zebrafish

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ABSTRACT

Background: The RNA-guided recombinase (RGR) platform, combining a hyper-activated sin recombinase with catalytically inactive Cas9 (dCas9), offers a programmable, cell-independent and potentially safer alternative to CRISPR/Cas9 for targeted genome editing. This study was conducted to design and construct a donor DNA plasmid enabling targeted knockout of the *lepb* gene in zebrafish (*Danio rerio*) using the RGR platform.

Methods: The *lepb* sequence was retrieved from the NCBI database and sin recombinase-based RGR recognition motifs were identified and analyzed functionally and structurally using CYC_REC, SOPMA and NetSurfP-3.0. SWISS-MODEL generated 3D structures of *lepb,* which were validated for quality. An experiment was conducted to build a donor DNA plasmid containing a mutated *lepb* fragment through site-directed mutagenesis (SDM) and verified by sequencing.

Result: A nonsense mutation (GAG to TAG) was introduced at 1738 bp, disrupting a critical glutamate residue, leading to loss of *lepb* protein function. *In silico* analysis revealed the loss of key structural elements (partial helix-C, complete helix-CD loop and helix-D residues) and disulfide bridge-forming cysteine residues (C112 and C157), which are crucial for *lepb-lepr* binding, stability and folding of the *lepb* protein. The homology modeling confirmed substantial alteration in the *lepb* conformation due to truncation. The donor DNA plasmid containing a 1808 bp-long *lepb* fragment with the targeted mutation (T) was constructed and validated. No off-target effects were detected. This study demonstrates the precision and feasibility of RGR-mediated *lepb* knockout and provides a validated donor DNA plasmid with potential applications in growth trait enhancement and functional genomics in zebrafish.

Key words: Genome editing, Hyper-activated sin recombinase, *Lepb* knockout, RGR platform, Zebrafish.

INTRODUCTION

Genome editing enables the precise alterations of DNA in living organisms via the addition, deletion and substitution of desired genetic material (Yang *et al*., 2025). Conventional tools, including CRISPR/Cas9 (Reddy *et al*., 2024), have advanced genetic engineering in gene manipulation and disease treatment (Ferdous *et al*., 2022). However, these methods face challenges such as error-prone cellular repair mechanisms (Silva *et al*., 2011) and unintended mutations, which can lead to genotoxicity (Boutin *et al*., 2022). In contrast, recombinase-mediated genome editing offers precise and consistent DNA manipulations (Pathak *et al*., 2020, 2022).

Site-specific recombinases (SSRs) are unique enzymes that rearrange the DNA between specified target sites. 'Sin resolvase' enzyme is a member of the serine recombinases family, isolated initially from *Staphylococcus aureus* multi-resistance plasmid pI9789 (Smith and Thorpe 2002). Wild Sin recombinase requires a synaptosome with non-specific DNA-binding Hbsu protein to organize the recombination of two 86-bp resH sites with two binding sites (Rowland *et al*., 2009). Then, the experimentally developed mutant forms of Sin recombinases, capable of detecting distinct target sites from their prior targets, were isolated (Gaj *et al*., 2011; Sirk *et al*., 2014). Furthermore, an evolved site-specific sin recombinase-based RNA-guided recombinase (RGR) platform has also been developed (Gaj *et al*., 2013) and optimized (Amrutham, 2021; Sonwane, 2021) for genome engineering. This sin recombinases-based RGR platform functions on a complex 78 bp target sites comprised of central core recombinase recognition sites (32 bp) flanked by gRNA binding sites (20 bp) that are flanked by protospacer adjacent motif (PAM) sequences (3bp). The sequence of this RGR platform target site is 5′-CCN$_{(74)}$GG- 3′ where N = [ATGC]. It has a high target

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specificity compared to TALE recombinases (TALER) and zinc-finger recombinases (ZFR) due to the requirement of complex and lengthy target sequences and its dimeric nature (Sonwane, 2014; Chaikind *et al*., 2016; Standage-Beier *et al*., 2019; Sonwane *et al*., 2022). CRISPR/Cas9 creates DSBs and repairs them primarily via NHEJ in non-dividing cells like neurons and specialized epithelial cells, yielding unpredictable indels rather than precise edits, as HDR is suppressed outside S/G2 phases. Repair outcomes vary by cell type, with non-dividing cells exhibiting slower resolution and lower sequence diversity compared to dividing cells, increasing off-target risks and genotoxicity (Nami *et al*., 2018; Ramadoss *et al*., 2025). The RGR platform directly cleaves the DNA strands before promoting the strand exchange and re-ligation of two DNA sequences. It does not rely on error-prone cellular repair mechanisms (Quyoom *et al*., 2025). It leads to producing a single well-defined product through stable and precise genome manipulations effectively via dual gRNAs in non-dividing cells and cells like a zygote (Turan *et al*., 2013; Standage-Beier *et al.,* 2019). The higher targeting capacity of evolved sin recombinase action-based RGR platform has been evaluated for targeted DNA integration at several pre-determined genomic loci in the bovine genome *in vitro* (Sonwane, 2021) and also has been assessed for targeted transgenesis of the EGFP transgene at a pre-determined locus of enah-srp9 intergenic region of zebrafish genome *in vivo* (Padhan, 2022; Nidarshan, 2023).

In aquaculture, feeding is crucial for the optimal development and well-being of cultured species, with aquafeeds comprising 50-80% of production costs (Hasan, 2017). To reduce feed cost, understanding the energy balance and appetite regulation is essential for enhancing aquaculture profitability by increasing the food conversion Ratio (FCR). Generally, fish regulate feed intake through complex interactions among hormonal and neural mechanisms, notably via the hypothalamic-pituitary axis. During satiation or fasting, hormones such as leptin (*lep*) and ghrelin, respectively, influence appetite regulation by sending signals to the hypothalamus to control or stimulate hunger (Martins *et al*., 2022). Especially in the anorexigenic pathway, during periods of high fuel stores (glucose or lipids) or low plasma leptin levels, the liver produces and secretes leptin, which circulates to the hypothalamus and binds to the LepR receptor in cell membranes. When leptin binds to LepR, it stimulates the Jak/STAT signalling system, leading to the downregulation of orexigens (NPY and AGRP) and the overexpression of anorexigens (POMC and CART) in the nucleus. Changes in hypothalamic neuropeptides lead to lower food intake levels (Blanco and Soengas, 2021). Thus, the *lep* gene knockout enhances the feed intake with increased FCR for improving aquaculture profitability by lowering feed costs while ensuring better growth performance. Leptin (*lep*) is generally a non-glycosylated peptide hormone encoded by the obese gene (*ob*)/*lep* gene. Zebrafish have two *lep* paralogues, leptin a (*lepa*) and leptin b (*lepb*), which are located on chromosomes 18 and 4, respectively (Gorissen *et al*., 2009). The *lepa* has a limited effect on food intake (Yuan *et al*., 2020), whereas *lepb* significantly reduces food intake in many fishes (Volkoff, 2016), similar to its role in mammals. Zebrafish (*Danio rerio*) serve as a model organism (Chia *et al*., 2022) for *lep*-related studies, due to favourable genetic characteristics. Studies indicate that food deprivation has significantly reduced *lep* mRNA levels, suggesting its role in appetite control (Garcia-Suarez *et al*., 2018; Butt *et al*., 2019). Genome editing using CRISPR/Cas9 has linked *lep* to obesity in zebrafish, with gene knockdowns resulting in an obese phenotype (Audira *et al*., 2018; Hu *et al*., 2022). This study focuses on developing a donor DNA construct to knock out the zebrafish *lepb* gene using a hyper-activated sin recombinase-based RGR platform.

MATERIALS AND METHODS

This study was carried out at the Fish Genetics and Biotechnology Laboratory of ICAR-Central Institute of Fisheries Education, Mumbai, as part of PhD research work from 2021 to 2024. The zebrafish *lepb* gene sequence [(Gene ID: 564348 and accession no. : NC_007115.7 (c19033832-19031768)] and its translated protein sequence (Protein ID: NP_001025357.2) were retrieved in the FASTA format from the NCBI database (https://www.ncbi.nlm.nih.gov). The hyperactivated Sin recombinase-based RGR target (5′-CCN$_{(74)}$GG- 3′) sites were identified in the zebrafish *lepb* gene sequence via DNA Pattern Find (https://www.bioinformatics.org/sms2/dna-pattern.html). The target locus to be mutated was screened by obtaining flanked RGR target sites using the EditSeq (DNA STAR) software. The amino acid (aa) relation with a selected locus to be mutated was identified using Gene Runner (https://gene-runner.software.informer.com/6.5/) and based on the predicted size of the amino acids pre- and post-truncation, referred to as wild *lepb* and truncated *lepb*, respectively.

In this study, widely used computational tools were employed to analyse the truncated *lepb* protein structure, as they offer complementary, high-accuracy predictions across key structural properties, enabling a robust *in silico* pipeline that helps determine the effect of mutation. The nature of both *lepb* proteins was identified using the SOSUI server (https://harrier.nagahama-i-bio.ac.jp/sosui/mobile/). The CYS_REC tool was employed to estimate the existence and absence of disulfide bonds and the number of cysteine residues (https://www.softberry.com/berry.phtml? topic=cys_recand group=programsandsubgroup=propt). The secondary structure of both *lepb* proteins was acquired using the SOPMA tool (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma_f.html) and the NetSurfP-3.0 tool (https://services.healthtech.dtu.dk/services/NetSurfP-3.0/). The wild *lepb* and truncated *lepb* protein three-dimensional (3D) structures were predicted using Expasy web server SWISS-MODEL (https://swissmodel.expasy.org/) and their quality was evaluated

by MolProbity (Williams *et al*., 2018), ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php)and Ramachandran plot (Ramachandran, 1963).

An experiment using site-directed mutagenesis was planned to create an upstream stop codon in a selected locus of the *lepb* gene utilizing the NEBase changer tool (https://nebasechanger.neb.com/). For this, the genomic DNA (gDNA) was isolated from the wild zebrafish, *D. rerio*, using the Phenol-chloroform method. The specific set of primers [i.e. (*Lepb*-F)- 5′-ATTGCTCGAACCACCATCAG-3′, (*Lepb*-R)- 5′-ATGTTGAGGCAGAGCTTCTC-3′] was designed using Editseq DNA Star tool and its quality was checked by using Oligoanalyzer™ tool (https://sg.idtdna.com/pages/tools/oligoanalyzer).

The target region of *lepb* was amplified from zebrafish gDNA using Phusion™ High–Fidelity DNA Polymerase (Thermo Scientific™ Cat. No.: F530S) with specific primers (*Lepb*-F and *Lepb*-R) and the amplicons were purified using a QIAquick Gel Extraction Kit (Qiagen, Cat. No. 28706). The amplicon was cloned into the pJET1.2 blunt vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Cat. No. #K1231) protocol. It transformed into the competent DH5α strain, *E. coli* cells, following the heat shock transformation protocol suggested by Froger and Hall (2007) with minor modifications. The recombinants were screened using the colony PCR with DreamTaq DNA polymerase (ThermoScientific Cat. No: EP0701). The recombinant plasmids were isolated using the QIAprep Spin Miniprep Kit (QIAGEN, Cat no. / ID. 27104) and the presence of insert-DNA was confirmed by Sanger DNA sequencing.

The Q5® Site-directed mutagenesis (Q5SDM) kit (NEB, Cat. No. E0554S) protocol was followed to create the upstream stop codon using specific mutagenic primers [i.e. (Q5SDM_*Lepb*-F) - 5′-GATAGACTTAtAGACTCT CCTGA GGACAC-3′, (Q5SDM_*Lepb*-R) - 5′-TGGACCTGCTGTAG GT GC-3′] designed using the NEBase changer tool. The mutated recombinant plasmid was transformed into high-efficiency competent *E. coli* cells. Further, two putative mutated recombinants were cultured in 5 ml of LB broth with ampicillin (100 µg/ml) at 37°C, overnight @ 200 rpm in a shaking incubator. The mutagenic plasmid was isolated using the QIAprep Spin Miniprep Kit and the existence of mutated insert-DNA was ascertained *via* Sanger DNA sequencing using specific primers (pJet1.2-F - CGACTCAC TATAGGGAGAGCGGC; pJet1.2-R- AAGAACATCGATTTTCC ATGGCAG). The acquired sequences were examined to validate the accuracy of the mutation and confirm the absence of off-target mutations using software such as Chromas and EditSeq (DNA STAR), which searched the target locus along with a 30-nucleotide region of the insert-DNA (Porkodi *et al*., 2025).

RESULTS AND DISCUSSION

Donor DNA designing

This study discovered four target sites in the 2065 bp-long zebrafish *lepb* gene sequence for the sin hyperactivated recombinase-based RGR platform. Two target sites in the direct strand of the *lepb* gene were utilized to locate the target locus to be mutated at the 1738th position, in exon 1. A guanine (G) to thymine (T) substitution was designed to create a nonsense mutation, enabling a premature upstream stop codon (GAG to TAG). The corresponding amino acid relation with the target locus revealed glutamate (E97) in the 168 aa-long zebrafish *lepb* protein. The truncated *lepb* protein was created by substituting glutamate ('E97') with '#' (Stop codon), resulting in a shortened protein size from 168 aa to 96 aa.

The wild and truncated *lepb* of *D. rerio* were considered membrane proteins due to their transmembrane region (residues 2-24, KSSMIFCLLISSLVAVSISRPTA) of the protein (Fig 1a and 1b). The wild *lepb* contains four cysteine residues (C8, C112, C148 and C157) (Fig 1a) and the C8 only remained in the truncated *lepb* (Fig1b). An intramolecular disulfide bridge formed by two cysteine residues (especially from the CD loop and at the C-terminal end) is crucial for the 3D structure, stability and bioactivity of the *lep* protein (Bakshi *et al*., 2022). Like other vertebrates (Cai *et al*., 2024), wild *lepb* of zebrafish also has a disulfide bridge forming two cysteine residues, such as C112 in the CD loop and C157 at the C-terminal end, which are absent in the truncated *lepb* protein, resulting in reduced stability, improper folding and impaired bioactivity of the *lepb* protein.

The wild *lepb* contains 52.98% alpha helix and 3.57% beta-turn, while the truncated *lepb* has 51.04% alpha helix and 2.08% beta-turn. The alpha helix and beta sheets are crucial for the secondary and tertiary structures of the protein (Rajeshwar and Abraham 2024). The truncation results in a reduction of amino acids in these critical regions, affecting the protein's secondary and tertiary structures. Key features such as a conserved disulfide bridge and hydrophobic amino acids are crucial for the stable four-helix packing required for proper protein folding and function across vertebrates (Londraville *et al*., 2017; Al Mughram *et al*., 2023). Zebrafish wild *lepb* also consists of four anti-parallel alpha helices denoted as helix-A (residues 25-47, PEDRIRIIARTTISRIKKIKDEH), helix-B (residues 63-80, PIDGLSSVLSYLSYLQLR), helix-C (residues 86-109, AQHLQQVQIDLETLLRTLEELAVS) and helix-D (residues 133-153, NYLHLLELQRFLEKLCLNIDK), linked with two long AB (residues 48-62, FQMSPEIDFGPDIDN) and CD (residues 110-132, QGCPLPNPETPVHKEETAFPVTS) loops and one short BC (residues 81-85, LHVPP) loop (Fig 1a) like *lep* of other vertebrates (Bakshi *et al*., 2022; Casado *et al*., 2023).

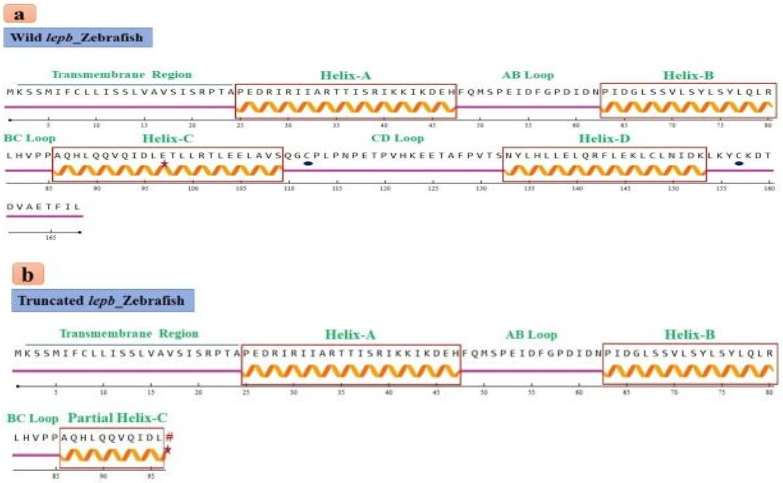
Like other vertebrates' *lep* (Greco *et al*., 2021), the zebrafish *lepb* interacts with its receptor (*lepr*) via three binding sites. Site-I forms the hexameric *lep-lepr* complex through residues of the C-terminus of helix-D and AB loop, which are essential for receptor activation (Peelman *et al*., 2006). Site-II contains residues of helix-A and helix-C, facilitating the high-affinity interaction with the CRH2 domain of *lepr* (Iserentant *et al*., 2005), while site-III connects the N-terminus

of helix-D with the Ig-like domain of *lepr* (Rock *et al*., 1996), enabling the conformational changes to activate the *lepr*. However, the truncated *lepb* fails to activate *lepr* due to the lack of partial helix-C, the complete CD loop, helix-D and the C-terminal end (Fig 1b), which disrupts complex formation and receptor dimerization, thereby inhibiting signalling pathways (JAK2/STAT3, JAK2/STAT5, PI3K/IRS/AKT and SHP2/ERK pathways). Especially, the anorexigenic action of *lepb* will be restricted, allowing fish to take a feed effectively and gain more body weight. Therefore, the fish growth rate will be increased by enhanced FCR and will quickly attain the marketable size compared to non-edited fish (Del Vecchio *et al*., 2021).

Additionally, the 3D models of wild *lepb* and truncated *lepb* proteins of *D. rerio* were constructed using the SWISS-MODEL server with the A0A671LBE5.1.A template further confirmed the structural modifications resulting from the mutation. The global model quality estimate (GMQE) scores were 0.80 and 0.82 for wild *lepb* and truncated *lepb* proteins, respectively, indicating satisfactory alignment quality, ranging from 0 to 1 (Waterhouse *et al*., 2024). High GMQE scores supported the model validation. The Qualitative Model Energy ANalysis (QMEAN) scores were -0.84 for wild *lepb* and -1.17 for truncated *lepb*, indicating a slight decline in model quality after truncation. However, scores were near zero, exhibiting strong alignment with experimental structures (Benkert *et al*., 2011). In MolProbity analysis, the residues in the favoured region of wild *lepb* and truncated *lepb* proteins of *D. rerio* were 90.30% and 94.68%, respectively. The 1.82% outliers were in wild *lepb*, while truncated *lepb* had none. Both models showed over 90% of residues in the favoured region and less than 2% in the outlier region, signifying reliable model quality (Sobolev *et al*., 2020). The molprobity scores (Williams *et al*., 2018) were 1.12 for wild *lepb* and 1.34 for truncated *lepb* with the latter suggesting decreased quality due to structural alterations. The Ramachandran plots ensured exceptional quality, with most residues in the favoured (green) and allowed (light green) regions. The ProSA-web Z-scores were -7.02 for wild *lepb* and -3.53 for truncated *lepb*, further confirming the proteins' validity, as negative Z-scores (Elengoe *et al*., 2014; Singh *et al*., 2018) indicate good quality. The various analyses revealed that the nonsense mutation at the 1738th nucleotide base (G) (locus) in the zebrafish *lepb* can effectively knock out its expression.

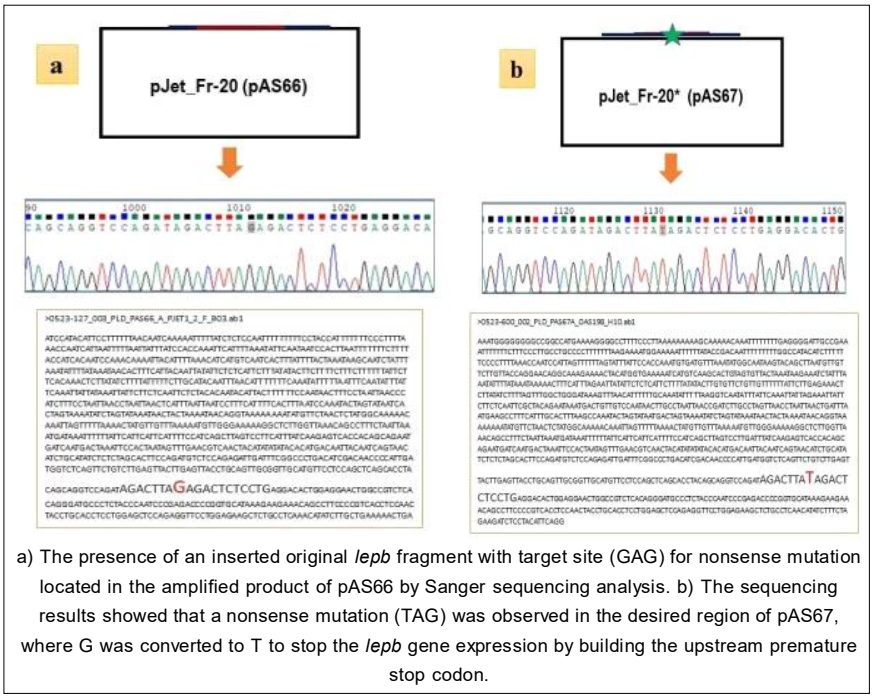
Construction of Donor DNA

To construct the donor DNA (pAS67), gDNA was isolated from zebrafish tissue and pectoral fin and the *lepb* gene fragment (Fr-20) was amplified through PCR with a targeted amplicon length of 1808 bp. The Fr-20 contains the potential locus flanked by RGR target sites. After gel elution, the purified Fr-20 was cloned into the pJET1.2 blunt vector (plasmid pAS66) and transformed into the competent DH5α strain, *E. coli* cells, where six well-separated colonies were screened using Colony PCR to confirm the existence of Fr-20. The recombinant plasmid DNAs (pAS66 A and B) were isolated from the two putative transformants and Sanger DNA sequencing verified the presence of insert-DNA (Fr-20) with a potential locus, guanine (G) (Fig 2a). Subsequently, Donor DNA (pAS67) containing mutagenic amplified product (Fr-20*) was constructed using the recombinant



The red-coloured star denotes a corresponding amino acid of the potential target. E97 (black colour) and # (red colour) represent the glutamate and stop codon, respectively. The Circle (dark blue colour) indicates the disulfide bond cresting cysteine residues (C112 and C157). The wild *lepb* consists of four anti-parallel alpha helices, namely helix-A, helix-B, helix-C and helix-D, linked with two long AB and CD loops and one short BC loop. But, the truncated *lepb* lacks partial helix-C, the complete CD loop, helix-D and the C-terminal end due to nonsense mutation.

Fig 1: Comparison of predicted modifications in alpha helices of (a) wild *lepb* and (b) truncated *lepb* of *D. rerio*.



a) The presence of an inserted original *lepb* fragment with target site (GAG) for nonsense mutation located in the amplified product of pAS66 by Sanger sequencing analysis. b) The sequencing results showed that a nonsense mutation (TAG) was observed in the desired region of pAS67, where G was converted to T to stop the *lepb* gene expression by building the upstream premature stop codon.

Fig 2: Characterization of the constructed donor DNA plasmid for *lepb* gene knockout in zebrafish.

plasmid (pAS66) as a template according to the Q5 SDM kit protocol, resulting in a premature upstream stop codon (GAG to TAG) mutation by substituting the guanine (G) with thymine (T) at the 2016th nucleotide position of recombinant plasmid pAS66. The mutagenic recombinant plasmids (pAS67 A and B) were isolated from the two putative mutated recombinants. The success of the mutagenesis was confirmed through sequencing, ensuring a guanine (G) substituted precisely by thymine (T) (Fig 2b) at the expected nucleotide position in the mutagenic donor DNA plasmids (pAS67 A and B). Successful donor DNA construction was achieved through site-directed mutagenesis, with sequencing verifying the intended mutation, thymine (T) substituting guanine (G) at the target site. The sequencing chromatograms showed distinct peaks confirming the presence of the mutation at the target site, with no additional alterations detected in the surrounding regions, confirming the specificity and accuracy of the mutagenesis process (Fig 2b). Additionally, no off-target mutations were observed, validating the precision of the experimental approach. It supports that the anorexigenic function of the zebrafish *lepb* can effectively be knocked out by using the generated donor DNA in this study.

Generally, zebrafish *lep* knocked out mutants exhibit increased feed intake and an obese phenotype (Audira *et al*., 2018; Hu *et al*., 2022). In future *in vivo* validation studies, the genotyping, multi-omics integration, body length-weight measurement, 3D locomotor activity, aggressiveness test, circadian rhythm test, passive avoidance test (Short-term memory test), colour preferences assay, predator avoidance test, biochemical assays (to determine the appetite-controlling hormone, oxidative, neurotransmitter, anti-oxidative capacity, lipid peroxidation, stress hormone, DNA damage and inflammation markers) and proximate analysis can be used further to examine the changes occurred in the *lepb* knocked out mutants physiological, phenotypical and behavioural outcomes (Audira *et al*., 2018; Del Vecchio *et al*., 2021).

CONCLUSION

The study effectively developed a donor DNA construct with a specific nonsense (G→T) mutation in the exon 1 of the zebrafish *lepb* gene. The nonsense mutation results in premature truncation and functional disruption of the protein through structural modifications, as confirmed by *in silico* analysis and a 3D homology model. These findings offer a reliable and scalable platform for subsequent in *vivo* validation, establishing a robust strategy for precise and safe genome modifications across aquatic species.

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Disclaimers

The perspectives and insights presented in this paper are exclusively those of the authors and do not reflect the views of their affiliated institutions. We are responsible for the precision and comprehensiveness of the information accessible, but we assume no responsibility for any direct or indirect losses resulting from the use of this content.

Informed consent

The animal and bacterial models were utilized in this study, following the guidelines and recommendations of the institute's ethical committee.

Conflict of interest

All authors declare no conflicts of interest and have approved the manuscript for publication.

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