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Knockout of Endospanin 1 via CRISPR in Zebrafish, Danio rerio

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By: Jared Kittinger

Abstract:

I made endospanin 1 knockout (KO) zebrafish to examine its effects on lipid and bone metabolism. Endospanin 1, or leptin receptor overlapping transcript (leprot), is a cytosolic protein linked to the protein hormone leptin that influences the trafficking of leptin receptors to the plasma membrane of cells. Genes for endospanin and tyrosinase (a pigmentation enzyme) were targeted via a microinjection of guide RNAs and CRISPR Cas9 into zebrafish embryos at 2-4 cell stages. I was able to disrupt the endospanin 1 gene (based upon the disruption of tyrosinase), but very few mutant zebrafish fully developed into adults. Only low KO mosaic zebrafish survived to early adulthood. If the assumption that endospanin 1 affects bone metabolism is supported, the results would suggest that endospanin 1's elimination may prevent bones from properly forming during zebrafish development.

Introduction:

Leptin is a protein hormone discovered in 1994 (Zhang et al., 1994) that is released by most tissues (primarily adipocytes in mammals) and binds to leptin receptors expressed on most cells (Münzberg & Morrison, 2015). The effects of leptin are numerous. It induces satiety similarly to CCK and regulates lipid metabolism if it binds to its receptors on the arcuate nucleus of the hypothalamus in mammals (Roujeau et al., 2019). It has a profound effect on the metabolism of bones such as stimulating fibroblasts and osteoblasts (Tsuji et al., 2010). Studies in which leptin was knocked down in zebrafish demonstrate decreases in bone density in important structures like the vertebral column (Liu et al., 2012), but it is unknown if this was due

to osteoclast activity increasing or just osteoblast activity decreasing. Blood concentrations (titer) of leptin in mammals rise as adipocytes accumulate, but continued high leptin titer leads to the organism becoming insensitive/resistant to leptin (Izquierdo et al., 2019). This resistance may be mediated by the transcription and translation of another gene, endospanin 1.

Originally discovered in 1997, endospanin 1 encodes a 14 kDa protein initially named OB-RGRP (leptin receptor gene-related protein) to denote the protein's relation to leptin receptors (Bailleul et al., 1997). Later studies would rename it to leptin receptor overlapping transcript (leprot) and finally to endospanin 1 (Séron et al., 2011). Endospanin 1 regulates leptin receptors by reducing their expression on the cell surface (Bailleul et al., 1997). It achieves this by catching and arresting leptin receptors beneath the plasma membrane of cells (Séron et al., 2011; Roujeau et al., 2019). Interestingly, these withheld leptin receptors are not destroyed as western blots performed on lysed cells showed no leptin receptor concentration difference between cells with and without endospanin 1 (Séron et al., 2011).

In obese mice where endospanin 1 was knocked out via a microinjection of lentiviruses into their arcuate nucleuses' neurons (Couturier et al., 2007; Vauthier et al., 2014), they lost weight post operation despite no change in their available food. The researchers were unable to make the mice obese again. While these studies heavily emphasized the roles of endospanin 1 and leptin in lipid metabolism, the more important takeaway was that knocking out endospanin 1 in organisms leads to a mechanism to modulate leptin sensitivity. Furthermore, the knockout of endospanin 1 shortly after the conception of an organism may reveal the influence that leptin-resistance and endospanin 1 has on the development (like bone formation) of that organism. I hypothesize that if endospanin 1 is knocked out via a microinjection of CRISPR Cas9 into *Danio*

rerio, then they will never lose leptin sensitivity throughout their lives and bone metabolism may be affected.

Endospanin has homologs across many species consisting of at least two to three distinct translated regions (exons) with no more than five (as in humans) (Kent et al., 2002). Given how endospanin is found in many species from teleosts (bony fish) to mammals with conserved primary sequence and gene order (synteny) (Gorissen et al., 2009; Londraville et al., 2017), it most likely has a similar and important function across all those species (Londraville et al., 2017). Zebrafish, a species of tropical teleost fish native to the rivers of India (Parichy, 2015), have endospanin paralogs (Kent et al., 2002; Howe et al., 2013). These are leprot (endospanin 1) and leprotL1 with two and three main exons respectively (Kent et al., 2002; Howe et al., 2013; Cunningham et al., 2022). Both are found on the second zebrafish chromosome (Cunningham et al., 2022). LeprotL1 is a product of the recent teleost whole-genome duplication event with subsequent mutations (Gorissen et al., 2009; Howe et al., 2013). As these exon's sequences are like the human version of endospanin, zebrafish likely have a version of endospanin with a very similar function and importance to the human version. Knocking out endospanin 1 in zebrafish embryos will therefore shed light on how endospanin may affect humans throughout life.

Endospanin 1 (Leprot, ENSDARG00000110105) is found on the reverse strand of DNA on the second zebrafish chromosome from 13,691,834 to 13,688,624 base pairs (Cunningham et al., 2022) and it is the target of this study. It exists in two primary exons coding for 102 amino acids (Cunningham et al., 2022). I designed guide RNAs to cut in both exons to interrupt any potential protein product if transcription and translation were to occur. CRISPR gene editing class II type II technology in which a Cas9 protein was used to perform the double-stranded breaks within the zebrafish's DNA to deactivate endospanin 1 (Pickar-Oliver & Gersbach, 2019).

Tyrosinase, a pigmentation gene, was also knocked out to give mutants an easily identifiable abnormal phenotype.

Methodology:

Animal Husbandry: Zebrafish were maintained under the conditions outlined by *The Zebrafish Book* (Westerfield, 2000) and the 20-20-05 LFD University of Akron Institutional Animal Care and Use Committee (IACUC) protocol. The zebrafish were fed fresh brine shrimp and zebrafish meal from (Skretting USA) daily and held in zebrafish modular unit tanks of 9.5 liters maintained at a constant temperature of 28°C. Low ammonia levels (<0.02 ppm) and a light/dark cycle of 12:12 hours respectively were maintained. The tanks were cleaned every week in addition to the water constantly being purified and treated with water conditioner (Seachem Prime) to dechlorinate the tap water used. Zebrafish were crossed by putting 3 males and 3 females into a 6L tank with 2 layers of marbles and a heater set to 28°C. Artificial grass was floated on the water surface, and the zebrafish were left overnight. They crossed upon being exposed to light with the zygotes falling between the marbles to avoid the zebrafish accidentally eating them. The zebrafish juveniles were fed Gemma 75 (Skretting USA) for the first 10 to 14 days post fertilization (dpf). After the second week, the zebrafish juveniles were fed Gemma 150 (Skretting, USA) and fresh brine shrimp until they were older than 30 dpf. After which they were transferred to bigger zebrafish modular unit tanks and remained on a once-a-day feed of brine shrimp for the remainder of the experiment.

Microinjection Solution: The Ohio State University Department of Molecular Genetics CRISPR pipeline for zebrafish procedure (Talbot & Amacher, 2014) was followed to make the

CRISPR Cas9 compounds. Recombinant *Streptococcus pyogenes* Cas9 (Thermofisher Scientific, Oakwood Village, Ohio) was used as the Cas9 endonuclease. The *S. pyogenes* Cas9 cut the first exon in this target sequence, 5'-GGATGTGCACTGGAACAGTT[TGG]-3' (PAM site indicated by brackets). This sequence was determined to be the best site by ZiFiT (Sander et al., 2010) due to it having a very low chance of off-target cuts and a high affinity for the Cas9 endonuclease. A 20 bp target sequence within the gRNA (5'-GGAUGUGCACUGGAACAGUU -3') bound to the zebrafish's DNA to allow the Cas9 to make a cut. The cut broke both strands of DNA resulting in distorted ends that the organism tried to repair. This stimulated non-homologous end joining (NHEJ) repair which removes single-stranded nucleotides on either side of the cut until double-stranded nucleotides are found resulting in a deletion (Chang et al., 2017). The number of base pairs deleted is variable but likely lead to a frameshift mutation and an early stop codon. To ensure the gene was completely silenced, a second cut site (on the second exon) was targeted. This second target site was 5'-GGTATCGTGGTGTCAGCTTA[NGG]-3' with N being a thymine (gRNA is 5'-GGUAUCGUGGUCAGCUUA -3'). **Figure 1** is an annotated chart from Ensembl that shows where the cuts occurred (Cunningham et al., 2022). Tyrosinase target sequence guides were incorporated into the main injection mixture to knock out pigmentation in affected cells.

Microinjections: Microinjection of CRISPR Cas9 endonucleases were performed by transferring the zygotes/embryos to a grooved, sterile, and moist agarose gel. On the microinjection apparatus (Narishige IM-300 microinjector with microscope), a small diameter glass capillary needle (Fisher Scientific) created from a micropipette puller (Sutter P-30 micropipette puller) assumed the metal placeholder's position. The tip was broken on a glass

petri dish with a scalpel, and then the injection solution was made. The CRISPR Cas9 injection solution comprised of 7 ul of gRNA total for endospanin 1 and tyrosinase (50-75 ng/ul), 1ul of 2M KCl, 1 ul of phenol red, and 1ul of recombinant *S. pyogenes* Cas9. This solution was diluted to half with pure molecular grade water before being loaded into the capillary needle. 58 zygotes/embryos (2-4 cell stage) were injected with the multiplex CRISPR compound. The cut-off time for injections was 45 minutes post-fertilization with the bulk being injected at around 15 minutes post-fertilization. After this time, the remaining non-injected zygotes/embryos were collected to be used as a control not subjected to the injection stress. The injected zygotes/embryos were collected and sorted into nursery cups of their respective treatment groups. Methylene blue (0.1 ppm) was added to the nursery water cups as an antifungal. The cups were maintained at constant conditions like the adult zebrafish except being fed Gemma 75 between 3-14 dpf and Gemma 150 14-30 dpf. The DNAs of the injected and not-injected wild-type zebrafish were obtained by soaking the juveniles/adults in a small amount of water (<5 ml of water for adults, <2 ml for juveniles) and 10 ng/ml of proteinase K solution to disrupt the plasma membrane of the shed cells (Zhang et al., 2020). The DNA from the shed cells was used for PCR, and the products were run on an agarose gel. The primers used were forward 5'-TGTGATTCAATGGGGTGCCG-3' and reverse 5'-TCACCAGAAAGATGCAGCCAT-3' to make an expected amplicon size of 265 base pairs (from nucleotides 313 to 557). The PCR products were sequenced and analysed via a HRMA analysis (a quick test for detecting a size change in PCR products). Bands of the expected size were excised from the gel with a sterile scalpel blade. The excised gel was melted using a sand bath, and the sample was transferred to new PCR tubes so that PCR can be done again. This ensures that the target sequence is amplified greatly.

5’ Upstream Sequence	.....tcagattttagtggaaacgcctaattcattctttcttctcttttattta
Exon 1 ENSDARE00000844567	GCTCTTGTTTCGTTATCCTTTAGTGGTGCACTTGGACTGACATTTCTCCTTTTGGGATGT GCACTGGAACACGTTTGG
Intron 2-3	gtatgtctgttatttgcaaacttta.....tttgtgtgcttctatctatctacag
Exon 2 ENSDARE00001283065	ACAGTATTGGCCCATGTTTGTTCTGATATTCTACATCTTATCACCTATACCAAATCTAAT AGCCAGGCGGCATGCGGATGACACTGAGTCAAGCAATGCATGCCGGGAACTTGCATACT TTTTAACCACAGGTATCGTGGTGTCAGCTTATGGTCTCCCTGTTGTGCTGGCTCGAAAAG CTGTGGTAAGTAGGAGTTTATGTCCCTCTTTAAATAAATTTTGGTTACTGTAAACTACTTG CATGGCTGATGATATAGTTTTCTTGGTTAATCAGTAAAATTAGTAACACCTTAAGGTCTC ATTTGCTAACATTTGTTGATAAATTATTCATTCATGGGGTACATGCCTCGCCGTTAATAA ATTATTATATATTAAATTATGTTAATTATATATTAAACTGACATACAACAGCTTTGGTGG CATAAGACTTTTTCATATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCT ATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCT ATATATATATATATATATATATATATATATATATATATATATATATATATATATATATAT ATATAGTTGCCTTGAACCGGGCCAAAGCACGCTTGTCCCCCTCCTGTCTCCCCTGACGGC CCGCACTCACATGACATTCAGAGCACTCACACTTCTCTGGGCACGGTTCGGATAGCATAG TGTGAGTAGGCCCTTAGGCCCCGTTTACACTAATACGTCTTAGTATTAAAATGACGTTTA AAAAACTAAAATGATCCACATCCACACTGTTTTACCTAGCTTTTCTGAACAGCCCTCCGT CCACTGAAAACGCACATCACGTGATCAGACACAGCCACACACACTGTCATGCGCTTCTCT GAGCTCCAGAGAGCAGTGCACGTCAGACAGTTATCAAGAATGTACCGCTGGATCGCGTC TCCAGGGCCGGCCCAAGCCTTCAGGGGGCCCTAAGCAGGATTGTATTTGGGGCCCCTTTG GTGCAAACAATATGACAAATTATCATCAATCCTTGATAATTCACACACTATAAATTTAAA ACACACATTATCGATTTTATTCGCTGTAGCTGTGTTGCTTACATCATAAAATATATGTTTT AAGACAATTCTAAATATTATTTTGCAAAAACAACTTCCTATTGATCCAACTTTTTATATG ACTATTTGCCACAAATCTAAATAATTAGACACAAAACATTAATATGGGCTGGCAGAAAC AAAAGCAGCTGGAAGTAGCCAATAACATACTTTAAGACGCTGCCAAACAGTCAAACATA GTAACAATAAAGATACATTTTTTTAATTGGATATAAATGTTAATACTGAAAAAAATTCAT ATTGTATTTTGTAGTTCAAATAACTTAAAACATCTATTTAAATTAAAATCAAACTAAATC AGGAAGCATTTTCTATACTCGCATATCAATCAAATTAATAAATATGTACAAAAATTTTCA GTTTTCAGAAATAATTCTAAATTAAGTGAATTTTTTTTTGTTAAACAAATTAATCTGCCAAT GAGATAAGTAAAATAATCTTATTTAAAACCTGAAACAATCTTTTTATTTATTTACCCTAAT TGGCAGATTATTGAGCTTTTTTTTAAATGAAAATTCACATAATTTTGACTAAAAATCTTT CTAAAAAATGTTTATTAGTTTAAGAATTTTTAGATATTTGGACTAAAAATCACACAAAAA CTATAAGAAAGAAAATCATTTTTGCAGTGTATTTACCAACTGTCAATTGTATAAATCAGA AGTTTATTTTTTTAATCACCAAGTTCATGAAAAAGCATTTTGCTGCTGTTCCAGGCACTG CTGCTTCAATCACGATATCAGTGATTCTGCGATATATTGATAGATCACAAGAAAACCAGC CACAATATATCACAATTTTGGTAAATGAAGGGGGAAAATGCATCAAAAATGTATGATGA ATTTCTTTTATAAACACGCATTTATATAATTGCAGTAATGTAAAACAAGTGCACATCTGT CAAACAAGCTTAAAAATGCTGATGTGCAGAGAGGAGTTTATGACTGAAGAGAGCACATC TCTATCACAGCAGCAAAATCTGTTAATAGTGGAATATGCATTTAAGAAAAAACTGCCAA AATAGTAATTTATTGCTTTCTAATTTTCTGTAAACTTAAAACACTTATTACAACTAATTTT ATGTAAACATGGAAACATTTTATGCTTAGCCCTGGTTTATTTTTAATAAAAAATATGTATC CCCACTATTTTTTCAACTGATCCGAATGCTCTGAGAGGTGAGCACAGACTCCTCTAAGCA CGTATGTTTTCAGCAGTGGAGAACATTAGTTATTTACTCATCTAAGTATTTTATTTCCCCA ATTTATTTATTTTTTTGCCAAAATACCATCTACAAGACTTTGACAAGGTCAGTAAGAGTC AAATAAATGCAAGGCCCTTCTAATTTAATGTTCATGTCCTATTGATATCTTTATATATTT TGGCGGAGTTCAAAAGTTTGAGCACATAAGAAGTTGCACTGGCCGCGGGCACGCGCGAG CGTCATAGTGAAGTGAAAGCGTGTCTGTAAGAAAGAAACTAGTGGCAAATTCGGGGGAG

	CTGGATGTCTGAAACGCACACAAATACATTGGCCTAGCTTGGACAGAAGAAAGTGTCTG TATGCCTGAGATAGTCACCATTTCCATATGGCTCATCTCTCAGTGGTTTACATGACGCTG GTGTATATCTAATGATCCGCCATTACGGGAAGCGCCACCTGGTGGCGGCTGGGTGTACTG TAGCTAAAGCGGTTATCTGCTGCAGTCGGTGTTGAAAACTGATTAATTAAATCAGATCTT TTGTAAATTTAGGTAAATATCTTGGCGCCTGGGTATGTATCGCGGTCAGAAATAACACTA TAAATGGGCATATATTTTGTCCCACCACAAATCCACAACTTAACAAACTAAAACGAAAG TCACACAATAATTTCAAGCACTCTTGCGGGCCCCCTGGTGGCCACGGGGCCCTA
3’ Downstream sequence	agcagccgcttagtttcgcttatgccttgggccggctctgcatcatcatg.....

Figure 1: Complete DNA sequence of endospanin 1 with exons labelled within zebrafish according to Ensembl (Cunningham et al., 2022). Cyan highlight indicates PAM the sites that will activate the CRISPR Cas9 endonuclease upon it binding to the hybridized guide with target sequence. Yellow highlight is the target sequence picked on ZiFiT (Sander et al., 2010), and the red highlight denotes the base directly to the right of the cut. Red lettering is untranslated regions of the exons while blue lettering is the translated regions.

Results:

Endospanin 1 was successfully knocked out via the microinjection of CRISPR Cas 9 endonucleases based on the disruption of tyrosinase in tissues. 58 embryos were injected with either an endospanin-only (18) or an Endospanin-tyrosinase (40) CRISPR mixture containing the endospanin 1 guides (**Figure 1**). Of the 18 endospanin-only injected zebrafish, two embryos (11%) developed with both surviving past four weeks. Of the 40 endospanin 1 and tyrosinase injected embryos, ten embryos (25%) developed post injection. Six of these ten endospanin-tyrosinase injected embryos developed abnormal pigmentation (**Figures 2-3**) consistent with a KO of tyrosinase in their eye (and body) three days post fertilization. Two of these tyrosinase KO zebrafish juveniles passed away within two weeks. The remaining four had a wild-type phenotype (normal eye pigmentation). Of the remaining four zebrafish juveniles with mutant phenotypes, two died in early adulthood shortly after being genotyped and presented abnormal behavior like refusing to eat. HRMA analysis of PCR products of their DNA had shown a decrease in melting temperature from 79.23 ℃ in wild-type samples to 75.885 ℃ in the

presumed endospanin 1 mutant samples (Δ3.345 ℃) (**Figure 4**). Another three mutant phenotype mosaic zebrafish died the following month (>four months post fertilization) leaving just one which was sacrificed later for definitive results. This longest living zebrafish mutant had three distinct bands on its back (**Figure 5**) indicative of a tyrosinase K.O. and presumably an endospanin 1 K.O. though sequencing data from the performed PCR was inconclusive. Two of the endospanin-tyrosinase K.O. wild-type phenotype zebrafish were sacrificed for a western blot against endospanin 1 that used antibodies previously designed by a former student, but the blot was unsuccessful. As for the wild-type control of the study, only ten of the 50 non-injected zebrafish embryos (20%) developed and survived past four weeks. None of the mutant phenotype mosaics were crossed with other zebrafish as they died before they were big enough to breed and before their mutation sequences were known.

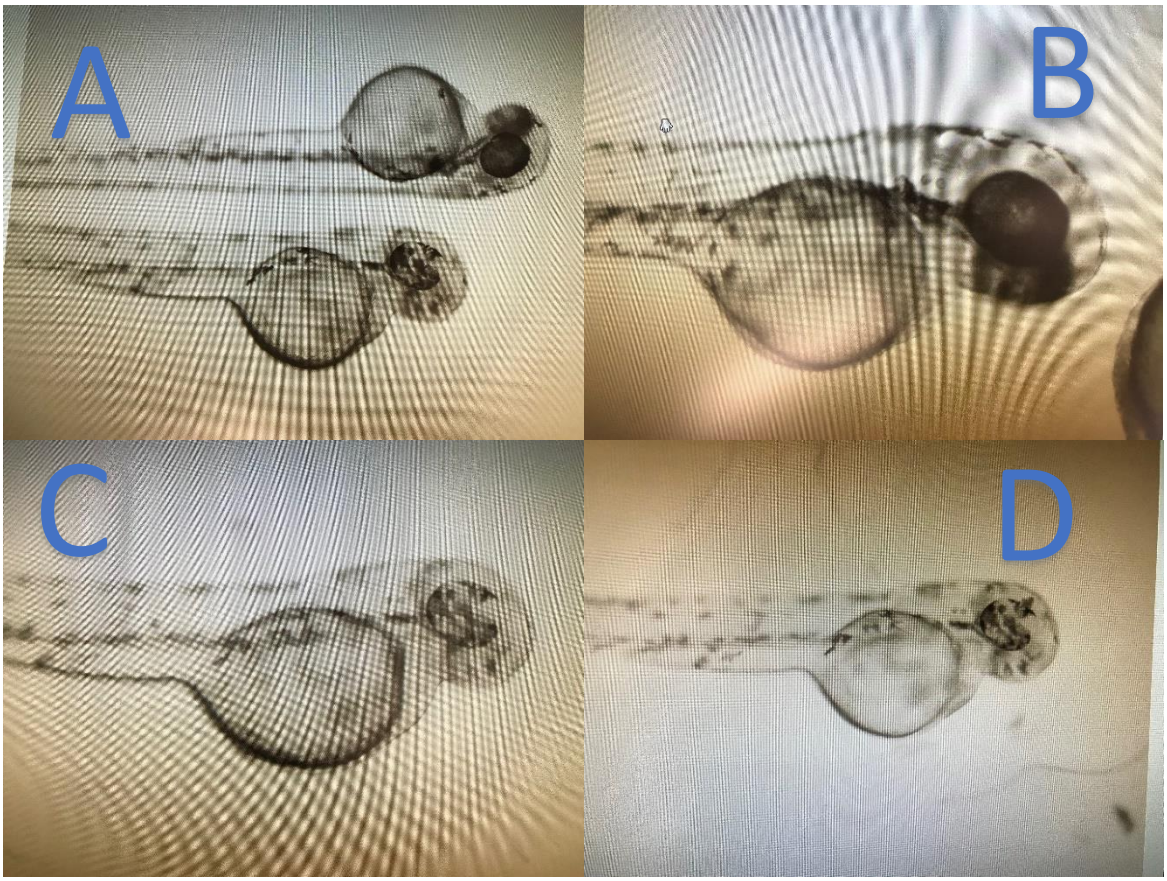

Figure 2: Picture A is a dorsolateral profile of a wild-type zebrafish (on top) and an endospanin-tyrosinase injected zebrafish (on bottom). Picture B is a closeup of a wild-type zebrafish. Pictures C and D are closeups of endospanin-tyrosinase injected zebrafish with reduced pigmentation primarily in their eyes. All pictures were taken three days post fertilization.

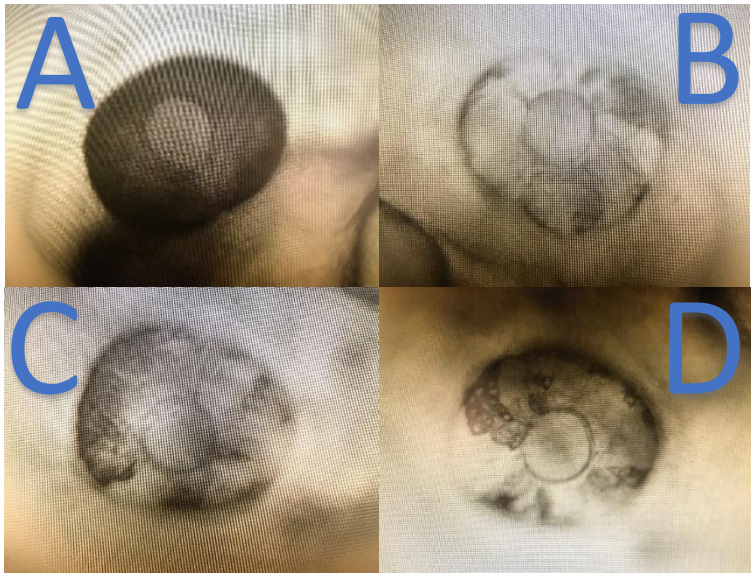

Figure 3: Picture A is a zoomed in view of a wild-type zebrafish eye displaying normal pigmentation. Pictures B, C, and D are closeup views of eyes of endospanin-tyrosinase injected zebrafish all displaying reduced pigmentation. All pictures were taken three days post fertilization.

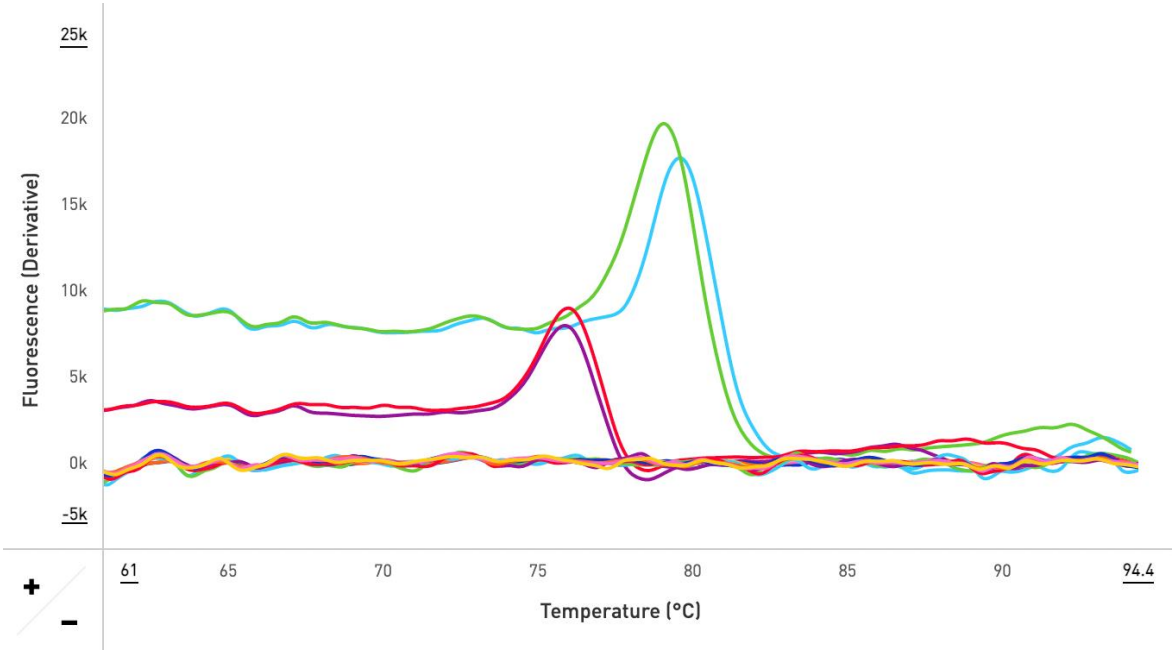

Figure 4: High Resolution Melting Analysis (HRMA) of four PCR samples performed on four different zebrafish. Blue and Green were from wild-type zebrafish. Blue melted at 79.49 ℃, and green melted at 78.97 ℃. Red and Purple were PCR products from two individual presumed mutants treated with the same primers. Purple melted at 75.82 ℃ while red melted at 75.95 ℃.

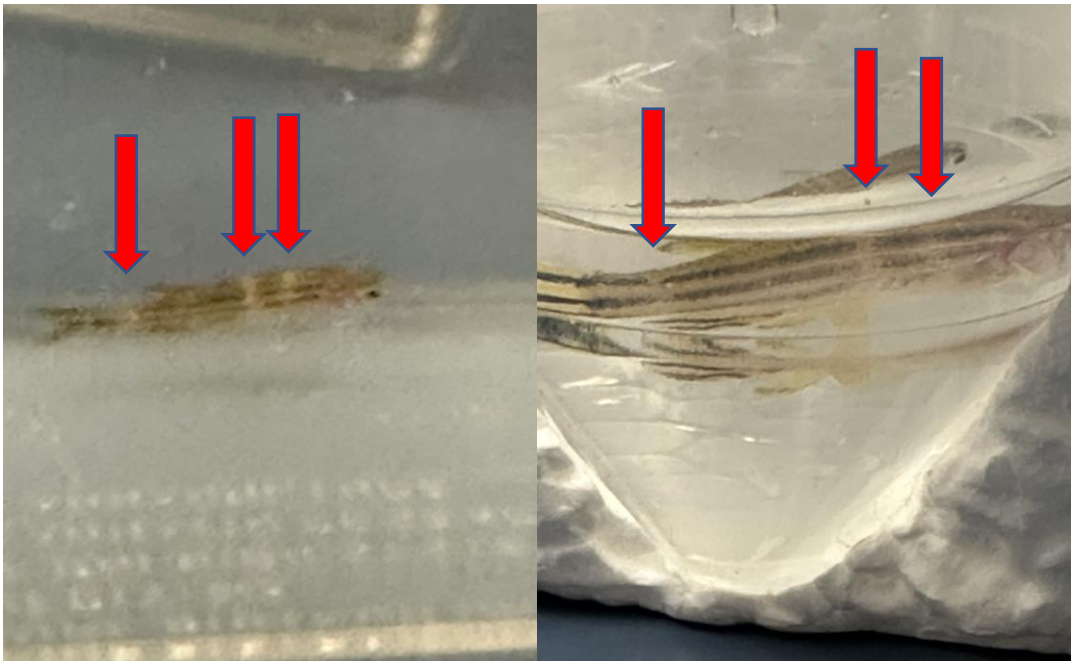

Figure 5. Both pictures are dorsolateral profiles of the oldest endospanin-tyrosinase injected mutant. This zebrafish displays three prominent vertical colorless bands where tyrosinase was knocked out (denoted in each picture by red arrows). The picture on the left was the mutant swimming in its tank, while the picture on the right was from when an environmental DNA sample was being collected.

Discussion:

Upon the discovery and subsequent cloning of the blood protein hormone leptin in 1994 (Zhang et al., 1994), leptin has attracted much attention primarily for its role in influencing lipid metabolism. In mammals, leptin is primarily made by and secreted into the blood stream by adipocytes (Münzberg & Morrison, 2015) to work like cholecystokinin (CCK) and induce satiety in the brain (Peikin, 1989). Leptin works similarly in that it must bind to one of its receptors on the plasma membrane of neurons in the arcuate nucleus of the hypothalamus to provoke feelings of satiety (Roujeau et al., 2019). However, leptin receptors are not just found in the arcuate nucleus, but rather in most tissues which allows leptin to have significantly more functions than just appetite regulation (Münzberg & Morrison, 2015).

One of these main functions is bone metabolism; the primary mechanism by which leptin affects bone metabolism is through stimulating fibroblasts to lay the foundations of bone matrix and differentiate into osteoblasts (Tsuji et al., 2010). Whenever leptin is knocked down in an organism, the resulting skeleton tends to be smaller and more fragile, especially with regards to important bones like vertebrae (Liu et al., 2012). If an organism's leptin titer was significantly increased, they would be expected to feel more satiated and have more dense/bigger skeletons. However, this is not the case as individuals who are exposed to a significantly higher leptin titer become resistant to leptin signaling in time (Izquierdo et al., 2019). This leptin resistance is the work of another gene, endospanin 1.

Endospanin 1 was discovered 3 years after leptin in 1997 (Bailleul et al., 1997) and was found to have an interaction with leptin receptors. This led the researchers to naming the 14 kDa cytosolic protein OB-RGRP (Obese (leptin) receptor gene-related protein) which was later renamed to leprot (leptin receptor overlapping transcript) and finally to endospanin 1 (Séron et al., 2011). Endospanin 1 does not destroy leptin receptors but rather keeps them from being transported to the cell surface (Séron et al., 2011; Roujeau et al., 2019). The action of endospanin 1 arresting these receptors within the cell prevents leptin from working (especially as a lipid metabolism regulator), which I hypothesize prevents vertebrates from limiting their production of fat storages when food is abundant. It also has the effect of limiting bone formation from continuing to rise as weight increases as this may have adverse effects on the organism. This protein also seems to be why exogenous leptin supplements cannot be used to properly treat obesity (El-Haschimi et al., 2000), as adding leptin will not greatly affect leptin signaling when leptin's receptors are the limiting reagent of the pathway. Therefore, if endospanin 1 could be

knocked out, that organism may be immune to both obesity and potentially osteoporosis especially where food is accessible and the need for fat storages decreases.

My study sought to achieve this through using zebrafish (*Danio rerio*), a teleost native to tropical streams in India (Parichy, 2015), as they have an endospanin 1 gene and are good model organisms when it comes to knocking out genes (Howe et al., 2013). The main function of leptin within mammals is lipid metabolism and bone metabolism, but this is altered in zebrafish leptin as fish do not typically build up significant fat reserves to where they would be considered obese (Londraville et al., 2017). Therefore, zebrafish would be a great model for observing how the loss of endospanin 1 effects leptin's role in bone metabolism more than satiety and lipid metabolism (Londraville et al., 2022).

I assert that endospanin 1 was knocked out via the microinjection of second generation CRISPR Cas9 in this study. Using the primers designed for endospanin 1, its sequence was consistently found in wild-type zebrafish while the sequences for the presumed mutants came back as unreadable. The multiple cut sites (target sequences) selected for endospanin 1 might have resulted in a significantly bigger cut than expected. Furthermore, zebrafish embryos co-injected with endospanin 1 and tyrosinase KO solutions showed disrupted pigmentation in their eyes and body. I claim the cells in which tyrosinase was knocked out, endospanin 1 was also cut at least in one target sequence. Overall, while I was not able to obtain solid evidence to the disruption of the endospanin 1 gene (leprot, ENSDARG00000110105), I believe that a definitive answer as to what mutation was induced would be found if I was provided additional funding to afford whole chromosome sequencing of the presumed zebrafish mutants.

Loss of endospanin 1 seemed to result in lower survivability, especially in the endospanin-only injected zebrafish, but poor embryo quality may have contributed (80% of the

not-injected embryos did not develop). A preliminary study in which only a tyrosinase guide RNA was injected (with CRISPR Cas9) showed that tyrosinase did not affect the survivability of embryos. This means, aside from poor embryo quality due to a lack of crossing zebrafish regularly, the only other factor that could have resulted in lower survivability within the embryos was the loss of endospanin 1.

Overall, I assert that endospanin 1 can be knocked out within a zebrafish embryo, though it may affect the survivability of the embryo it was injected into. Future studies in which bone formation is analysed throughout the development of endospanin 1 knockout mutants may shed light on why loss of endospanin 1 reduces survivability. Knockout of this gene may also affect feeding habits that result in poor juvenile nutrition, though this is speculation as leptin does not function heavily as a satiety regulatory in teleosts (like zebrafish) (Liu et al., 2012; Londraville et al., 2017). Given the opportunity to redo the experiment, electroporation would be used instead of microinjections for the delivery of the CRISPR Cas9 and target sequences solution as that would almost guarantee the complete knockout of endospanin 1 in all cells leaving few mosaic zebrafish. This, along with sequencing the entire second zebrafish chromosome after CRISPR would definitively prove if endospanin 1 is essential (as no mutants would survive if it were), and allow for the study of unimpeded leptin signaling if endospanin 1 turns out to not be essential.

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