

Lrp5 Mutant and Crispr Zebrafish Faithfully Model Human Osteoporosis, Establishing the Zebrafish as a Platform for CRISPR-Based Functional Screening of Osteoporosis Candidate Genes

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ABSTRACT

Genomewide association studies (GWAS) have improved our understanding of the genetic architecture of common complex diseases such as osteoporosis. Nevertheless, to attribute functional skeletal contributions of candidate genes to osteoporosis-related traits, there is a need for efficient and cost-effective in vivo functional testing. This can be achieved through CRISPR-based reverse genetic screens, where phenotyping is traditionally performed in stable germline knockout (KO) mutants. Recently it was shown that first-generation (F0) mosaic mutant zebrafish (so-called crisprants) recapitulate the phenotype of germline KOs. To demonstrate feasibility of functional validation of osteoporosis candidate genes through crisprant screening, we compared a crisprant to a stable KO zebrafish model for the *Lrp5* gene. In humans, recessive loss-of-function mutations in *LRP5*, a co-receptor in the Wnt signaling pathway, cause osteoporosis-pseudoglioma syndrome. In addition, several GWAS studies identified *LRP5* as a major risk locus for osteoporosis-related phenotypes. In this study, we showed that early stage *Lrp5* KO larvae display decreased notochord mineralization and malformations of the head cartilage. Quantitative micro-computed tomography (micro-CT) scanning and mass-spectrometry element analysis of the adult skeleton revealed decreased vertebral bone volume and bone mineralization, hallmark features of osteoporosis. Furthermore, regenerating fin tissue displayed reduced Wnt signaling activity in *Lrp5* KO adults. We next compared *Lrp5* mutants with crisprants. Next-generation sequencing analysis of adult crisprant tissue revealed a mean out-of-frame mutation rate of 76%, resulting in strongly reduced levels of Lrp5 protein. These crisprants generally showed a milder but nonetheless highly comparable skeletal phenotype and a similarly reduced Wnt pathway response compared with *Lrp5* KO mutants. In conclusion, we show through faithful modeling of LRP5-related primary osteoporosis that crisprant screening in zebrafish is a promising approach for rapid functional screening of osteoporosis candidate genes. © 2021 American Society for Bone and Mineral Research. © 2021 The Authors. *Journal of Bone and Mineral Research* published by Wiley Periodicals LLC on behalf of American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: GENETIC ANIMAL MODELS; WNT/B-CATENIN/LRPS; OSTEOPOROSIS; BONE QCT/μCT

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Osteoporosis is a highly prevalent systemic skeletal disease characterized by loss of bone mass and low bone mineral density (BMD) that results in bone fragility and increased fracture risk.⁽¹⁾ Osteoporosis is a complex disorder, influenced by both environmental and genetic factors. BMD and osteoporotic fractures are highly heritable with heritability estimated between 0.5 and 0.8, meaning that a substantial part of their variation is determined by genetic factors.⁽²⁾ Genomewide association studies (GWAS) in large cohorts from consortia such as GEFOS/GENOMOS and UK Biobank have identified hundreds of susceptibility loci for osteoporosis-related traits.^(3–5) For example, BMD in lumbar spine and femoral neck was linked to 56 loci, of which 50 were replicated in an independent study that additionally revealed 153 previously unknown loci.^(4,6) Major risk loci, such as *ESR1* and *LRP5*, have been verified in different GWAS studies.^(6–9) However, for the vast majority of identified loci, both causal variants and perturbed genes and their molecular functional consequences remain largely unknown. Therefore, the next step is to validate their functional roles in the pathogenesis of osteoporosis.

Different model systems for bone diseases have been developed. In vitro cellular models are useful for studying intra- and intercellular signaling and to determine relatively simple phenotypic readouts such as cell survival and mineralization capabilities. For example, CRISPR/Cas9-mediated KO of *DAAM2*, which was identified as one of the top candidate osteoporosis genes in a UK Biobank GWAS, in osteoblast cell lines resulted in a marked reduction in inducible mineralization.⁽⁵⁾ Nevertheless, these 2D in vitro models are not able to mimic the complex nature of the vertebral skeletal system, rendering their relevance as an osteoporosis model rather limited. Small animal models—namely rodents—are well-established tools for osteoporosis studies.^(10–12) However, since large-scale generation and phenotyping of KO mouse models is a slow and costly approach,^(13,14) the role of most genes residing at GWAS loci still remains unknown. This indicates the need for alternative methods to accelerate validation of candidate osteoporosis genes.

The zebrafish (*Danio rerio*) has become a valuable animal model in the field of biomedical research because of its high fecundity, short development time, genetic similarity to humans, small size, and low husbandry costs.⁽¹⁵⁾ Zebrafish have also proved to be a relevant genetic model for the study of different skeletal diseases.^(16–21) The molecular basis and the key regulators of skeletogenesis appear to be highly conserved between zebrafish and mammals,^(22–24) which is further corroborated by studies where it was shown that zebrafish and mammals respond similarly to treatment with known osteogenic compounds such as calcitriol, parathyroid hormone (PTH), and bisphosphonates.^(25,26) As zebrafish are aquatic animals, the weight-bearing demand on the skeleton is reduced, enabling many skeletal mutant zebrafish lines to survive into adulthood far longer than their orthologous mouse counterparts.⁽²⁷⁾

With the advent of CRISPR/Cas9 technology, reverse genetic screening of candidate genes has seen a significant increase in zebrafish by studying germline mutants.^(28,29) However, recently a new approach has been developed in which phenotyping is performed directly in F0 (mosaic) founder zebrafish, called “crispants”.⁽³⁰⁾ CRISPR/Cas9 mutagenesis usually produces high out-of-frame mutation rates, some of which copy the loss-of-function phenotype of a gene. This approach reduces the time and

resources needed to obtain stable knockout (KO) lines and enables rapid, reverse genetic screens of candidate genes, contributing to skeletal disease. In a study by Watson and colleagues, the comparison between somatic, CRISPR-generated F0 mutants and homozygous germline mutants for *plod2* and *bmp1*, two genes implicated in recessive forms of human osteogenesis imperfecta, revealed phenotypic convergence, suggesting that CRISPR screens of F0 animals can faithfully recapitulate the phenotype of skeletal disease models.⁽³¹⁾

In the current study, we aimed to assess the feasibility of CRISPR-based F0 (crispant) screens for functional validation of osteoporosis candidate genes by comparing a crispant to a stable KO zebrafish model for the *lrp5* gene. In human, loss-of-function mutations in *LRP5*, a co-receptor in the Wnt signaling pathway, cause osteoporosis-pseudoglioma syndrome (OPPG), an autosomal recessive disorder characterized by severe osteoporosis and congenital blindness.⁽³²⁾ Loss-of-function mutations result in the inability of Wnt ligands to bind to *LRP5* and its co-receptor Frizzled, resulting in continuous degradation of β -catenin and impaired osteoblast differentiation.⁽³³⁾ In addition, *LRP5* has been identified as a major risk locus in several GWAS studies for osteoporosis and common polymorphisms in the *LRP5* gene have been shown to impact BMD.⁽³⁴⁾ Rodent *Lrp5* KO models show a reduced BMD, bone volume, and bone strength and have primarily been used to study the impact of Wnt signaling inhibition on skeletal development.^(35–37) At the same time, we wanted to establish the *lrp5* KO and crispant models as genetically induced osteoporosis zebrafish models that can be useful to increase fundamental knowledge of genetic forms of osteoporosis and can be used as a tool for osteogenic compound screening.

Materials and Methods

Zebrafish maintenance

Semi-closed recirculating housing systems (ZebTec, Tecniplast, Buguggiate, Italy) were used for zebrafish (*Danio rerio*) housing. They were kept at a temperature between 27°C and 28°C, pH 7.5, conductivity ± 500 μ S, and a 14/10 light/dark cycle (Ghent University, Ghent zebrafish facility). Dry food (Gemma Micro, Skretting, Stavanger, Norway) was provided once a day, and Micro Artemia (Ocean Nutrition, Essen, Belgium) was additionally fed once a day. Breeding and the collecting of embryos were performed according to previously described protocols.⁽³⁸⁾ Anesthesia was induced by exposure to 160 mg/L MS-222 (ethyl 3-aminobenzoate methane sulfonate, cat no. E10521, Sigma-Aldrich, Overijse, Belgium) for 2 minutes, and euthanasia was performed by an overdose of 1 g/L MS-222 for 10 minutes. Mutant zebrafish (*lrp5* sa11097) in AB background were acquired from the European Zebrafish Resource Center (EZRC; Eggenstein-Leopoldshafen, Germany). *lrp5*^{+/-} zebrafish were in-crossed to obtain *lrp5*^{-/-} mutants. Subsequently, *lrp5*^{+/-} and *lrp5*^{-/-} zebrafish were each in-crossed to obtain progeny for experiments. Additionally, *lrp5*^{+/-} zebrafish were outcrossed with transgenic reporters, Tg(*Ola.Sp7*:Kaede), referred to as *osx*:Kaede and Tg(*7xTCF-Xla.Sia*:GFP^{la4}) and subsequently in-crossed to obtain transgenic *lrp5*^{-/-} or *lrp5*^{+/-} zebrafish.^(39,40) To reduce mortality in *lrp5*^{-/-} zebrafish larvae, a special protocol was developed to grow *lrp5*^{-/-} and matched control zebrafish (siblings) to adulthood. Larvae were housed individually in a 24-well plate from 3 days post fertilization (dpf) until 21 dpf in an incubator at 28°C. At 5 dpf, E3 medium was replaced by a

filtered rotifer solution, which was refreshed on alternating days until 21 dpf. At 21 dpf, zebrafish were placed in zebrafish tanks in the zebrafish facility and grown to adulthood. All animal studies were conducted in agreement with EU Directive 2010/63/EU for animals, permit no. ECD 19/09 (Ghent University). All efforts were made to minimize pain, distress, and discomfort.

Genotyping *lrp5* mutant

Genotyping of zebrafish carrying the *lrp5* sa11097 allele (c.851 C < A) was performed by Sanger sequencing. DNA was extracted by heating caudal fin tissue, obtained by fin-clipping, in 50 μ L 50 mM sodium hydroxide (NaOH) at 95°C for 25 minutes, followed by adding of 5 μ L 1 M Tris-HCL (pH = 8). PCR amplification was performed using the following primers: Forward: 5'-CATC-TATGTGTTGACGTTTAC-3', Reverse: 5'-GATGTAAGCCAACACC-TAAAC-3'. Thermocycling conditions were 95°C for 4 minutes, followed by 12 cycles of 94°C for 20 seconds, 58°C for 15 seconds, and 72°C for 1 minute and 24 cycles of 94°C for 40 seconds, 46°C for 40 seconds, 72°C for 30 seconds, and a final extension of 72°C for 10 minutes. Finally, Sanger-sequencing was performed on amplified *lrp5* fragments, using the same primer set as for the PCR amplification.

Guide RNA design and injection

For the generation of *lrp5* crispants, we used two-part guide RNAs, which are duplexes made of a trans-activating CRISPR-RNA (tracrRNA) to which the CRISPR nuclease binds and a CRISPR-RNA (crRNA) that is target-specific (<https://eu.idtdna.com/>). Benchling was used to design crRNAs targeted at the gene region where the KO mutation in the *sa11097* allele is residing (<https://benchling.com>). These were subsequently checked for predicted out-of-frame efficiency via InDelphi (<https://indelfi.giffordlab.mit.edu/>). The crRNA predicted to be most efficient by InDelphi was subsequently used for experiments: TTACTCGCCGATGGACATCC. A non-specific crRNA was used to generate control-injected larvae. This “scrambled” sequence, GCAGGCAAAGAATCCCTGCC, was previously described,⁽⁴¹⁾ and zebrafish injected with this gRNA are referred to as Sham-injected controls throughout the article. The final gRNA duplexes were generated by creating a mix of 200 μ M crRNA and 200 μ M tracrRNA, heating to 95°C for 5 minutes before storing them at -20°C. After fertilization, we micro-injected one-cell-stage wild-type (WT) AB, Tg(*osx:Kaede*) or Tg(7xTCF-*Xla.Sia:GFP*^{ia4}) zebrafish embryos with a 1.4 nL injection mix, containing RNase-free water, Phenol Red sodium salt indicator (P4758, Sigma-Aldrich), and ribonucleoproteins (RNPs) consisting of 800 pg gRNA duplex and 800 pg Cas9 protein (ToolGen, Seoul, South Korea; Cas9 wild-type nuclease protein with nuclear localization signal).

Next-generation sequencing analysis of *lrp5* crispants

DNA was extracted from caudal fins obtained from adult *lrp5* crispant zebrafish and from 2 dpf whole *lrp5* crispant embryos (pools of 10) by adding 50 μ L 50 mM NaOH and incubation at 95°C for 25 minutes. PCR amplicons were generated as described above (genotyping *lrp5* mutant), prepared for sequencing using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and subsequently deep sequenced on a MiSeq instrument (Illumina) using 2 $\times$ 250 bp cycles.⁽⁴²⁾ BATCH-GE was used to analyze the raw NGS data to determine the presence of sequence aberrations.⁽⁴²⁾ These data were subsequently used to determine the percentage of out-of-frame mutations.

Western blotting

Protein was extracted from caudal fins of 5-month-old zebrafish. The tissue of five pooled samples was homogenized in a lysis buffer containing 375 μ L radio-immune precipitation assay (Sigma-Aldrich) and 15 μ L 25x stock protease inhibitor (Sigma-Aldrich). Pestles were used for homogenization before centrifugation at 12,000g for 10 minutes at 4°C. Supernatant was transferred to fresh tubes before concentration measurements using Pierce BCA protein assay kit (Thermo Fischer Scientific, Merelbeke, Belgium) in a GloMax-Multi Detection System (Promega, Leiden, Belgium). Protein concentrations were equalized between samples in a final volume of 20 μ L and mixed with 3 μ L marker (non-reducing lane markers sample buffer, Thermo Fisher Scientific) and 2 μ L dithiothreitol (Thermo Fisher Scientific) and incubated for 5 minutes at 98°C. Samples were run on 4% to 12% Bis-Tris Protein Gels (Thermo Fischer Scientific) and proteins were blotted onto a nitrocellulose membrane by an iBlot 2 Dry Blotting System (Life Technologies Europe, Merelbeke, Belgium) and immunolabeled with anti-LRP5 rabbit monoclonal antibodies (1:1000, D80F2, Cell Signaling Technology, Bioké, The Netherlands) or anti- α -tubulin mouse monoclonal IgM antibodies (1:1200, T5168, Sigma-Aldrich, Benelux). Secondary antibodies (1:1500, ab97240, Goat Anti-Mouse, and 1:1500, ab97051, Goat Anti-Rabbit, Abcam, Cambridge, UK) were incubated for 1 hour at room temperature. Finally membranes were developed via a chemiluminescent reaction using the Super Signal West Dura Extended Duration Substrate (Life Technologies Europe), followed by fluorescent imaging using the Amersham Imager 680 (GE Healthcare, Machelen, Belgium).

RNA extraction and RT-qPCR

RNA was extracted from amputated caudal fins of adult zebrafish. After the initial fin-clip, zebrafish were housed individually for 4 days before the second fin-clip was performed. RNA extraction was performed by using Trizol (Life Technologies Europe), followed by purification using the RNeasy Mini Kit (Qiagen, Hilden, Germany) in combination with on-column DNase I treatment (Qiagen) according to the manufacturer's guidelines. Concentrations of RNA were measured using a DropSense96 (Trinean, Pleasanton, CA, USA) device and cDNA synthesis was carried out using 150 ng RNA in a 20 μ L reaction with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). RT-qPCR primers for the genes were designed using NCBI Primer-BLAST (Supplemental Table S1), while primers used for the reference genes *elfa* and *bactin2* were previously published.⁽⁴³⁾ qPCR reaction mixtures were composed of 2.5 μ L SsaAdvanced Universal SYBR Green Supermix (Bio-Rad), 5 ng cDNA and 250 nM of both forward and reverse primers were run on a LightCycler 480 (Roche, Basel, Switzerland) according to a previously published protocol.⁽⁴³⁾ The qBase+ software package (v3.0, Biogazelle, Gent, Belgium) was used for data analysis. A generalized linear mixed model with a Gaussian error structure and a logarithmic link function was used to regress each gene's normalized relative quantities (NRQ-values) on the effect of group, time, and their interaction. Models were fit using the lme4 package and *p* values were calculated with lmerTest.^(44,45) Multiple testing correction was performed using the Benjamini-Hochberg procedure for the false discovery rate.⁽⁴⁶⁾ A *p* value was calculated for the moderating effect of fin type on the effect of time. A significant *p* value therefore implies a significant difference in the extent to which the expression of normal fin tissue changes with

respect to regenerating fin tissue. For ease of understanding, Fig. 5B-B' displays a difference in fold change instead of a difference-in-differences.

Alizarin red and alcian blue staining

Zebrafish larvae (7 and 13 dpf) were euthanized by an overdose of 1 g/L MS-222 and fixed for 1 hour at room temperature (RT) in 4% paraformaldehyde (PFA) (Sigma Aldrich) in phosphate buffered saline (PBS) (Cell Signaling Technology, Bioké, The Netherlands). Samples were dehydrated gradually to 70% ethanol for storage and rehydrated for processing. Zebrafish were bleached to remove pigment by incubation in 3% H₂O₂ (hydrogen peroxide), 2% potassium hydroxide (KOH) (Sigma-Aldrich) for 30 minutes at RT. Alizarin red S (AR, Sigma-Aldrich) was dissolved to 0.005% in a 1% KOH, 2% Triton (Sigma-Aldrich) solution for staining. After staining for 3 hours at RT, larvae were washed in PBS and transferred to a glycerol (Life Technologies Europe) for storage. Alcian blue (Sigma-Aldrich) was dissolved to 0.1% in 70% ethanol and 1% HCl by sonication. Animals were stained at RT overnight, followed by destaining in 70% ethanol, 5% HCl for 4 hours. After washing with PBS, larvae were transferred to 100% glycerol for storage. Images were taken using a Leica microscope M165FC and DFC450C camera operated by LAS V4.3 software (Leica Microsystems, Wetzlar, Germany). Fiji software (ImageJ 1.52i)⁽⁴⁷⁾ was used to take all measurements. Standard length was measured by converting pixels to mm via the scalebar and the angle tool was used to determine the angle of the ceratohyal. Red stain of the head elements was quantified by applying a universal threshold across all images followed by measuring the remaining positive area. Stained notochord area was measured by hand using the polygon selection tool. One-way ANOVA with Tukey's honest significant difference, Mann-Whitney *U* test ($n < 30$), and unpaired *t* test ($n > 30$) were used in GraphPad Prism (v8.2.1; GraphPad, La Jolla, CA, USA) where corrected *p* values were considered significant when ≤ 0.05 .

Adult zebrafish (5 months) were euthanized by an overdose of 1 g/L MS-222 and stained with AR as previously described.⁽⁴⁸⁾ In short, zebrafish were fixated for a week in 0.2% PFA (Sigma-Aldrich), 5% Triton-X (Sigma-Aldrich, T8787) and 1% KOH (Sigma-Aldrich). The zebrafish were subsequently cleared and permeabilized in enhancement solution, 20% ethylene glycol (Sigma Aldrich, 324558), 5% Triton-X (Sigma Aldrich, T8787), and 1% KOH (Sigma Aldrich) for 24 hours before being incubated in bone staining solution containing 0.05% ARS (AR, Sigma-Aldrich) for 24 hours. Next, the specimens were destained for 72 hours in 20% Tween20 (Sigma-Aldrich, P1379) and gradually transferred to 100% glycerol (Life Technologies Europe) in 1-hour steps of 0%, 20%, 40%, 60%, 80%, and finally 100% glycerol. A Leica M165FC microscope and DFC450C camera was used to capture images using LAS V4.3 software (Leica Microsystems).

Osteoblast imaging and analysis in Tg(*osx:Kaede*) and visualization of Wnt signaling in Tg(7xTCF-Xla.Sia:GFP^{ia4})

Tg(*osx:Kaede*) zebrafish larvae (7 dpf) were euthanized by an overdose of 1 g/L MS-222 and subsequently imaged with a Leica microscope M165FC and mounted camera (DFC450C) operated by LAS V4.3 software (Leica Microsystems). Osteoblast-positive area was measure by applying a universal threshold on all images by using Fiji software (ImageJ 1.52i).⁽⁴⁷⁾ Mann-Whitney *U* tests were performed in GraphPad Prism (v8.2.1).

Tg(7xTCF-Xla.Sia:GFP^{ia4}) zebrafish larvae were euthanized at 7 dpf by an overdose of 1 g/L MS-222 and were subsequently imaged using a Zeiss (Jena, Germany) observer Z1 with a Colibri light source and Axiocam 506 camera with the AF580 filter settings.

Micro-computed tomography scanning

Zebrafish were euthanized at 5 months post fertilization (mpf) by an overdose of 1 g/L MS-222 and fixed in 4% formaldehyde (47608-1L-F, Sigma-Aldrich) in PBS for 24 hours. Length and sex were recorded, and zebrafish were stored in 70% ethanol until scanning. The whole body was imaged using a micro-computed tomography (microCT) scanner SKYSCAN 1172 (Bruker Corporation, Kontich, Belgium) at a voxel size of 21 and 5 μ m. Images of the vertebral column of the zebrafish, scanned at 5 μ m, were generated using CT vox (Bruker, v3.3.0). Detailed analysis of the 21 μ m scans was performed on single DCM files, generated by NRecon (Bruker Corporation) in FishCuT software.⁽⁴⁹⁾ FishCuT output data were subjected to statistical analysis in R (version 3.5.1) using a custom-made script.⁽⁴⁹⁾ Two repeated-measures ANOVA models were used to account for the dependent measures of multiple vertebrae per zebrafish (one for centrum volume, one for centrum tissue mineral density [TMD]). The outcome (volume or TMD) was scaled to the mean value in the respective control group ("control" for the mutant experiment and "sham" for the crispant experiment) to account for batch and age differences. A fixed effect of vertebra (5 to 27) and genotype (mutant, crispant) was included and a random effect for each fish. The R package lme4 was used to fit the model.⁽⁴⁴⁾ Diagnostic plots of the residuals, including a QQ-plot from the car package, revealed no severe violations of model assumptions.⁽⁵⁰⁾ From this model, a 95% confidence interval was constructed using the profile likelihood method.

Inductively coupled plasma mass spectrometry

First, standard length and body weight of 5 mpf euthanized zebrafish was measured. Next, the complete vertebral column posterior to the Weberian apparatus and anterior to the ural vertebrae was dissected under a stereomicroscope (Leica M60) and was subsequently weighed and measured. Two hundred microliters of nitric acid (65%) was used to dissolve the tissue before total calcium, phosphorus, and magnesium levels were measured by inductively coupled plasma mass spectrometry (ICP-MS, Thermo Fisher Scientific) according to a previously published protocol.⁽⁵¹⁾

Fin regeneration

Amputation of the ventral lobe of the caudal fin in adult zebrafish was performed under general anesthesia (160 mg/L MS-222). The Zeiss observer Z1 with a Colibri 2 light source and an Axiocam 506 camera was used to take images of Tg(7xTCF-Xla.Sia:GFP^{ia4}) fin rays in the green channel by using AF480 filter settings. The Zeiss observer was used to photoconvert native green Kaede fluorescence in *osx:Kaede* fins at 0 days post amputation (dpa) by 30 seconds 405 nm UV exposure. Subsequent images (GFP3 and dsRed filters) were taken with a Leica microscope M165FC and DFC450C camera operated by LAS V4.3 software (Leica Microsystems). In vivo AR staining was performed sequentially at 0, -5, -10, -15, and 20 dpa for 15 minutes with 0.01% AR according to a previously published protocol.⁽⁵²⁾ Stereomicroscope images and Zeiss observer images were processed, ie,

rotated, scaled, cropped, aligned, and merged using Fiji software (ImageJ 1.52i).⁽⁴⁷⁾

Statistical analysis

Statistical analysis was performed using GraphPad (version 8.2.1) and R (version 3.5.1).

Results

Mutant and crispant *lrp5* zebrafish models

The zebrafish *lrp5* gene (ENSDF0000006921) resides on chromosome 25 and contains 24 exons compared with 23 exons in the human *LRP5* gene (ENSG00000162337) (Fig. 1A). Amino acid sequence alignment between human and zebrafish *lrp5* reveals an overall sequence identity of 72% (Supplemental Fig. S1). The zebrafish mutant that we report here (EZRC, sa11097) harbors a c.851C > A mutation in *lrp5*, generating a premature stop codon in exon 5 at AA position 284 (p.Y284X) (Fig. 1B) resulting in a complete absence of Lrp5 protein in homozygous mutants (Fig. 1C).

Guide RNAs (gRNAs) were designed via Benchling to target the *lrp5* gene region where the c.851C > A (sa11093) mutation is located. The gRNA with the highest out-of-frame (OOF) efficiency, 71.7%, as predicted by the InDelphi-mESC prediction model,^(53,54) was selected for further analysis. This gRNA was co-injected with Cas9 into one-cell stage embryos. We hypothesized that high rates of OOF mutations generated by CRISPR/Cas9 mutagenesis in zebrafish embryos (crispants) would result in levels of Lrp5 protein that are sufficiently reduced to lead to a phenocopy of the *lrp5*^{-/-} mutant. At 2 dpf, DNA was extracted from different pools ($n = 12$), each containing 10 embryos, and subjected to next-generation sequencing (NGS) analysis of *lrp5* exon 5 fragments, which were analyzed by the in-house developed BATCH-GE tool.⁽⁴²⁾ The mean fraction of reads containing indels in these pools was 91.2% (confidence interval [CI] $\pm$ 3.98%) with a mean OOF rate of 60.1% (CI $\pm$ 3.10%). NGS analysis of pools ($n = 5$) containing sham-injected embryos did not reveal any indel mutations in exon 5 amplicons. Crispants were subsequently grown to adulthood and caudal fin tissue was collected. NGS analysis on DNA extracted from these fins ($n = 15$), revealed indels in 95% (CI $\pm$ 4.39%) of the reads with an OOF rate of 76.6% (CI $\pm$ 5.16%). Theoretically, with an OOF efficiency of 76.6%, 59% (0.766²) of the cells should contain OOF *lrp5* indels on both alleles, resulting in a complete *lrp5* KO in these cells. For the vast majority of the remaining cells, although also carrying in-frame indels, there is still a relatively high probability of Lrp5 dysfunctionality (considering the mean indel rate of 95%). We next assessed Lrp5 protein levels in fin tissue from adult *lrp5* crispants (Fig. 1C). We found that the amount of Lrp5 protein is significantly reduced in *lrp5* crispants, thereby closely mimicking the situation in the *lrp5*^{-/-} mutant. The remaining Lrp5 protein shown on Western blot most likely derived from the small fraction of wild-type or in-frame mutated *lrp5* alleles.

Lrp5^{-/-} mutant and *lrp5* crispant zebrafish larvae show malformations of the head cartilage and reduced notochord mineralization

A high mortality was noted in *lrp5*^{-/-} mutant larvae, where only 10% of the larvae could be raised past 13 dpf (Supplemental Fig. S2) when placed individually in 24-well plates and fed with rotifers on a daily basis. In *lrp5* crispants, on the other hand, using

a similar husbandry protocol, about 50% of larvae survived beyond 13 dpf (Supplemental Fig. S2), making it feasible to acquire larger sample sizes with crispants.

To determine the effect of *lrp5* loss-of-function on the formation of the early skeletal elements in both mutants and crispants, the cartilage and mineralized bone were visualized by alcian blue (AB) and alizarin red (AR), staining respectively (Figs. 2A-A' and 3A-A'). AB staining at 7 dpf revealed that, although there was no difference in standard length, ruling out any general developmental delay in these models, the head was significantly shorter in both the *lrp5*^{-/-} mutants ($p < 0.0001$) and *lrp5* crispants ($p < 0.005$) (Fig. 2B-B'). A detailed analysis of cartilage structures revealed a decreased length of Meckel's cartilage, ceratohyal, and the first ceratobranchials in *lrp5*^{-/-} mutants ($p < 0.0001$, < 0.0001 , < 0.0001) and *lrp5* crispants ($p < 0.005$, < 0.0001 , < 0.0001), respectively. The left and right ceratohyal in both mutant and *lrp5* crispants are bended in the middle. The resulting angle between the left and right ceratohyal and also the first ceratobranchials was significantly wider in both the *lrp5*^{-/-} mutants ($p < 0.005$, < 0.005) and *lrp5* crispants ($p < 0.005$, < 0.0001) than in the corresponding controls. Next, AR staining (Fig. 3A-A'), revealed a strongly decreased mineralization of the notochord tip at 7 dpf in both the *lrp5*^{-/-} mutant ($p < 0.0001$) and *lrp5* crispant ($p < 0.0001$) larvae compared with corresponding controls (Fig. 3B-B'). The notochord is a flexible rod-shaped structure that is considered as the earliest form of the vertebral column in teleosts and is surrounded by a collagen type II sheath secreted and mineralized by chordoblasts, thereby forming the chordacentra.^(55,56) The mature vertebral column develops on top of the mineralized notochord sheath by osteoblast-dependent intramembranous ossification. Quantification of total head mineralization, without inclusion of the anterior notochord sheath of the anterior tip, did not reveal any differences in mutant and *lrp5* crispant larvae, indicating normal mineralization of head elements at 7 dpf (Fig. 3B-B'). Highly similar results were obtained in 13 dpf larvae, where the head length was significantly reduced in the *lrp5*^{-/-} mutants ($p < 0.005$) and *lrp5* crispants ($p < 0.05$), while there was no difference in craniofacial mineralization (Supplemental Fig. S3). At 13 dpf, the mineralization of the anterior notochord tip was still significantly reduced in *lrp5*^{-/-} mutants ($p < 0.0001$) and *lrp5* crispants ($p < 0.0001$), suggesting that in both models the mineralization at this location is impaired rather than delayed, while craniofacial mineralization is not affected throughout early life stages.

To explore the suitability of using the *lrp5* mutant and crispant larvae for osteogenic compound screening, a proof-of-principle experiment with the known osteogenic compound calcitriol (vitamin D, 1 α ,25-dihydroxyvitamin D3) was performed.^(25,57) The decreased mineralization of the anterior tip of the notochord sheath could be partially rescued by administration of 30 pg/mL calcitriol from 3 to 7 dpf in both mutant ($p < 0.05$) and *lrp5* crispant ($p < 0.005$) larvae (Fig. 3B-B'). Finally, immature osteoblasts were imaged by using an osteoblast-specific reporter, Tg(*osx*:Kaede) (Fig. 3A-A'). However, no differences in surface area covered by osteoblasts could be observed for *lrp5*^{-/-} mutants nor *lrp5* crispants (Fig. 3B-B').

MicroCT and inductively coupled plasma mass spectrometry of adult *lrp5* knockout and *lrp5* crispant zebrafish

The adult skeleton in *lrp5*^{-/-} mutant, *lrp5* crispant, and control siblings was visualized by microCT scanning (Fig. 4A-A').

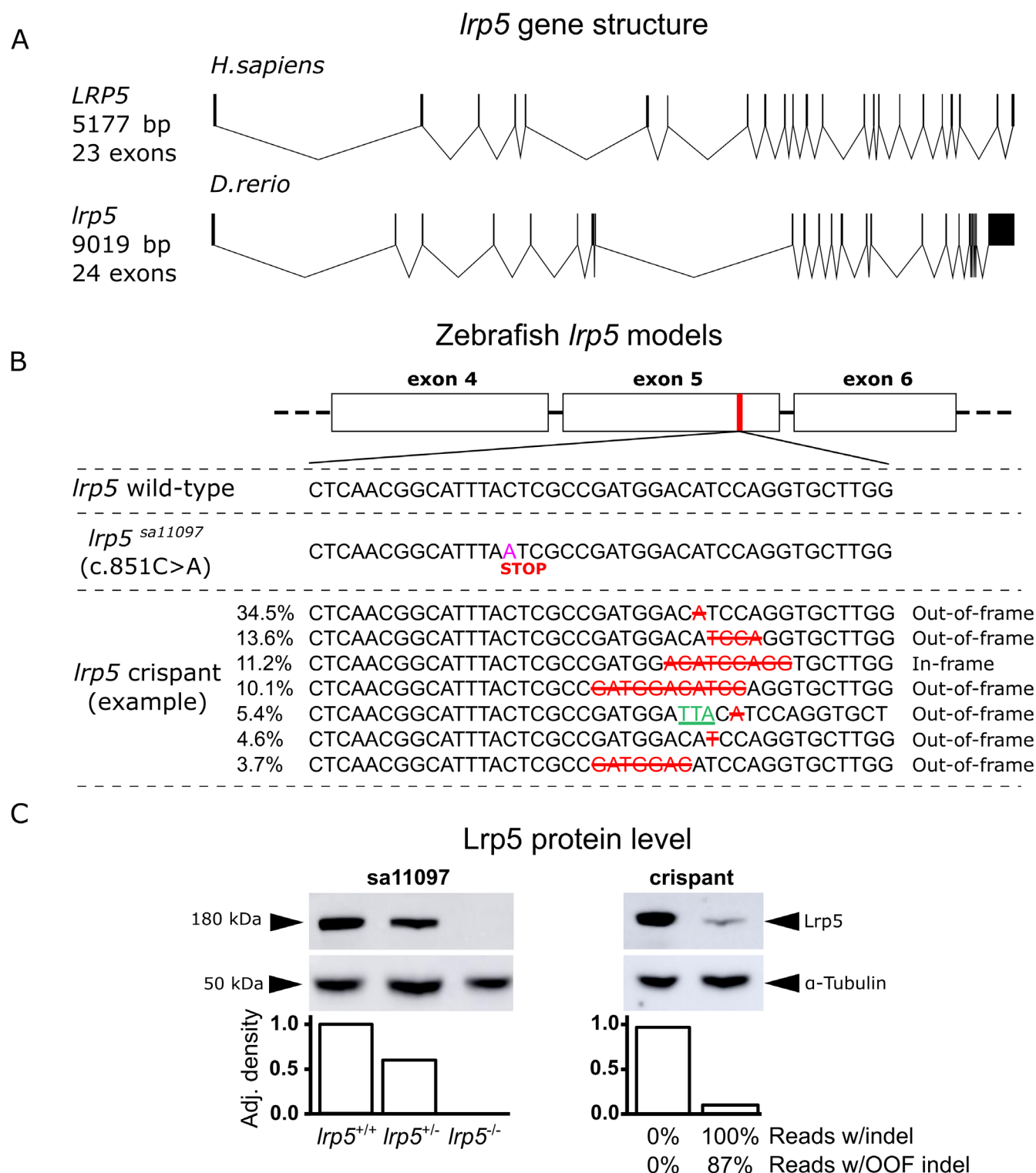


Fig 1. Germline and somatic (crispant) *lrp5* mutations. (A) Schematic representation of the gene structure of human *LRP5* (ENSG00000162337) located on chromosome 11 and zebrafish *lrp5* (ENSARG00000006921) located on chromosome 25. Boxes and lines represent exons and introns, respectively. (B) Exon 5 base pair sequence in wild-type zebrafish (top), in mutant *lrp5*^{sa11097} zebrafish (middle) indicating the location of the c.851C > a mutation resulting in a premature stop-codon (p.Y284X) and in *lrp5* crispant zebrafish (bottom) with representation of next-generation sequencing (NGS) reads (>2%) containing different out-of-frame and in-frame indel mutations. Substitution mutations are represented in pink, deletions by red strikethroughs, and insertions by green underscores. (C) Western blots for Lrp5, in representative samples obtained from fin tissue, show a loss of Lrp5 protein in *lrp5*^{-/-} and a 90% reduction in a *lrp5* crispant with 100% mosaicism with 87% out-of-frame mutations. OOF = out-of-frame.

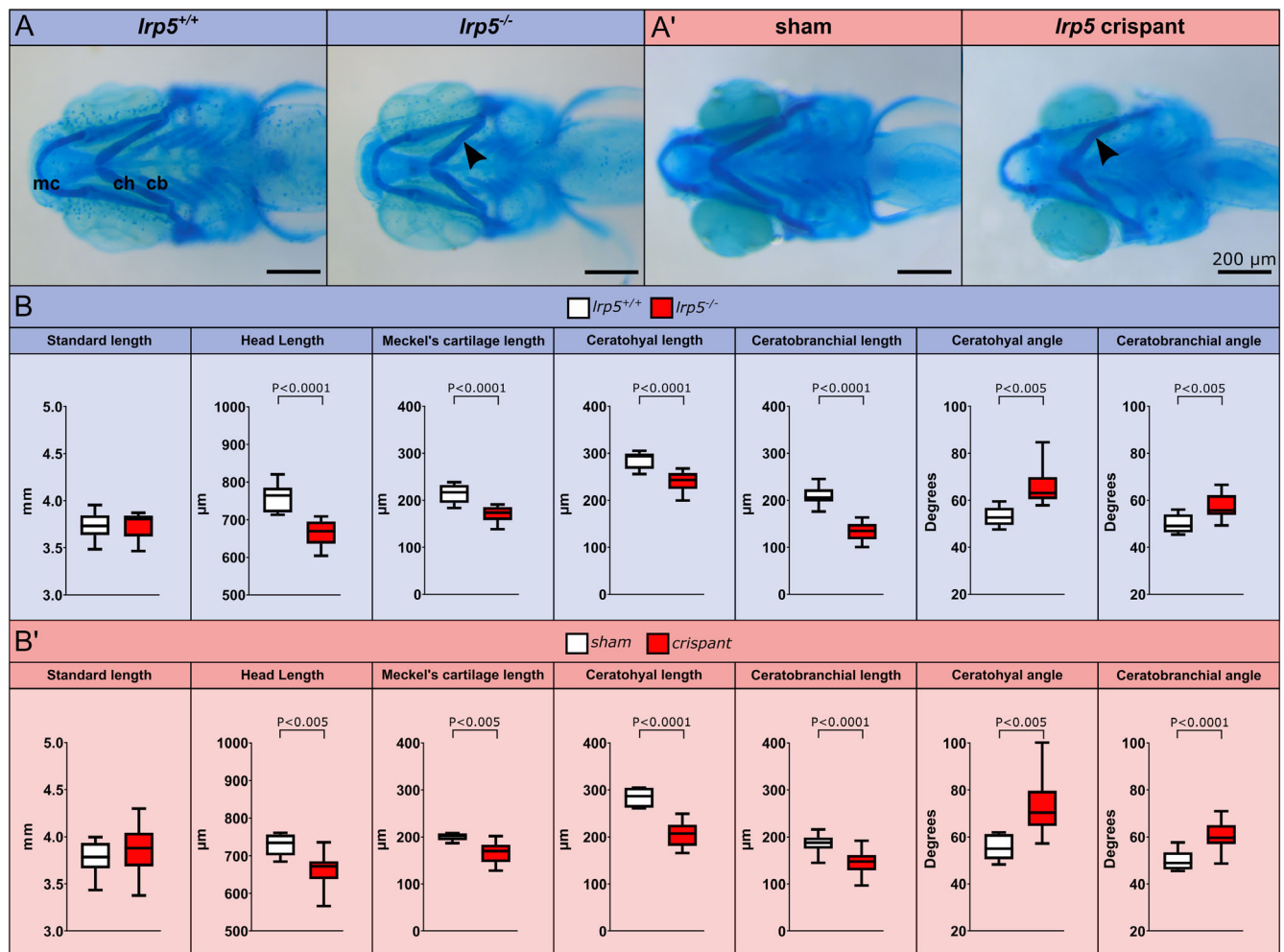


Fig 2. Alcian blue cartilage staining in *lrp5* mutants and crispants. (A-A') Alcian blue cartilage staining of representative 7 dpf *lrp5*^{-/-} (A) and *lrp5* crispant (A') larvae (arrowhead indicates kink in the ceratohyal). (B-B') Boxplots of analyses of different head cartilage structures in *lrp5*^{-/-} (*n* = 26) (B) and *lrp5* crispants (*n* = 33) (B') including length of the head, meckel's cartilage (mc), ceratohyals (ch), and ceratobranchials (cb) and the angle between the left and right ceratohyal and ceratobranchials. Standard length was measured from the anterior tip of the snout to the most posterior tip of the notochord. Head length was measured from the anterior tip of the dentary to the most posterior part of the occipital arch. Larvae are oriented with anterior to the left from a ventral perspective. Scale bars = 200 μm.

Although standard length was not found to be different (Fig. 4C-C'), maximal projections demonstrate malformations of the head skeleton, including a significantly shorter head in *lrp5*^{-/-} mutants (*p* < 0.005) and *lrp5* crispants (*p* < 0.0001). Additional measurements showed a significantly more obtuse angle between the left and right ceratohyal in both the *lrp5*^{-/-} mutant (*p* < 0.0001) and *lrp5* crispants (*p* < 0.05) (Supplemental Fig. S4), thereby showing similar morphological abnormalities of the head skeleton between larval and adult stages. High-resolution (5-μm voxel size) microCT scanning and whole mount AR staining revealed no obvious morphological abnormalities of the vertebral column (Supplemental Fig. S5). Quantitative analysis of microCT data (21 μm voxel size) was done using FishCuT software.⁽⁴⁹⁾ Each vertebral body posterior to the Weberian apparatus (first four vertebrae) and anterior to the pereural and uveal vertebrae was segmented into neural arch, centrum, and haemal arch. For each of these segments, the length, TMD, volume, and thickness were determined and tested for statistical

differences between groups via a regression-based statistical test. Vertebral body centra showed a decreased TMD (*p* < 0.001), volume (*p* < 0.05), and thickness (*p* < 0.05), but a similar length along the vertebral column of *lrp5*^{-/-} mutants compared with control siblings (Fig. 4B). *lrp5* crispants revealed a similar phenotype in the vertebral column, with a decreased centrum TMD (*p* < 0.01) and centrum volume (*p* < 0.001) but no difference in centrum thickness and length (Figure 4B'). Additional analysis revealed decreases in TMD, volume, and thickness of the neural and haemal arches in both the *lrp5*^{-/-} mutant and *lrp5* crispants (Supplemental Fig. S6).

The mean centrum TMD in *lrp5*^{-/-} mutants and *lrp5* crispants was reduced by 8% and 7%, respectively, while centrum volume was reduced by 17% and 21% in *lrp5*^{-/-} mutants and *lrp5* crispants, respectively, indicating similarly decreased TMD and centrum volume in *lrp5* mutants and crispants.

To further assess the similarity of the vertebral phenotype between *lrp5*^{-/-} mutants and *lrp5* crispants, centrum TMD and

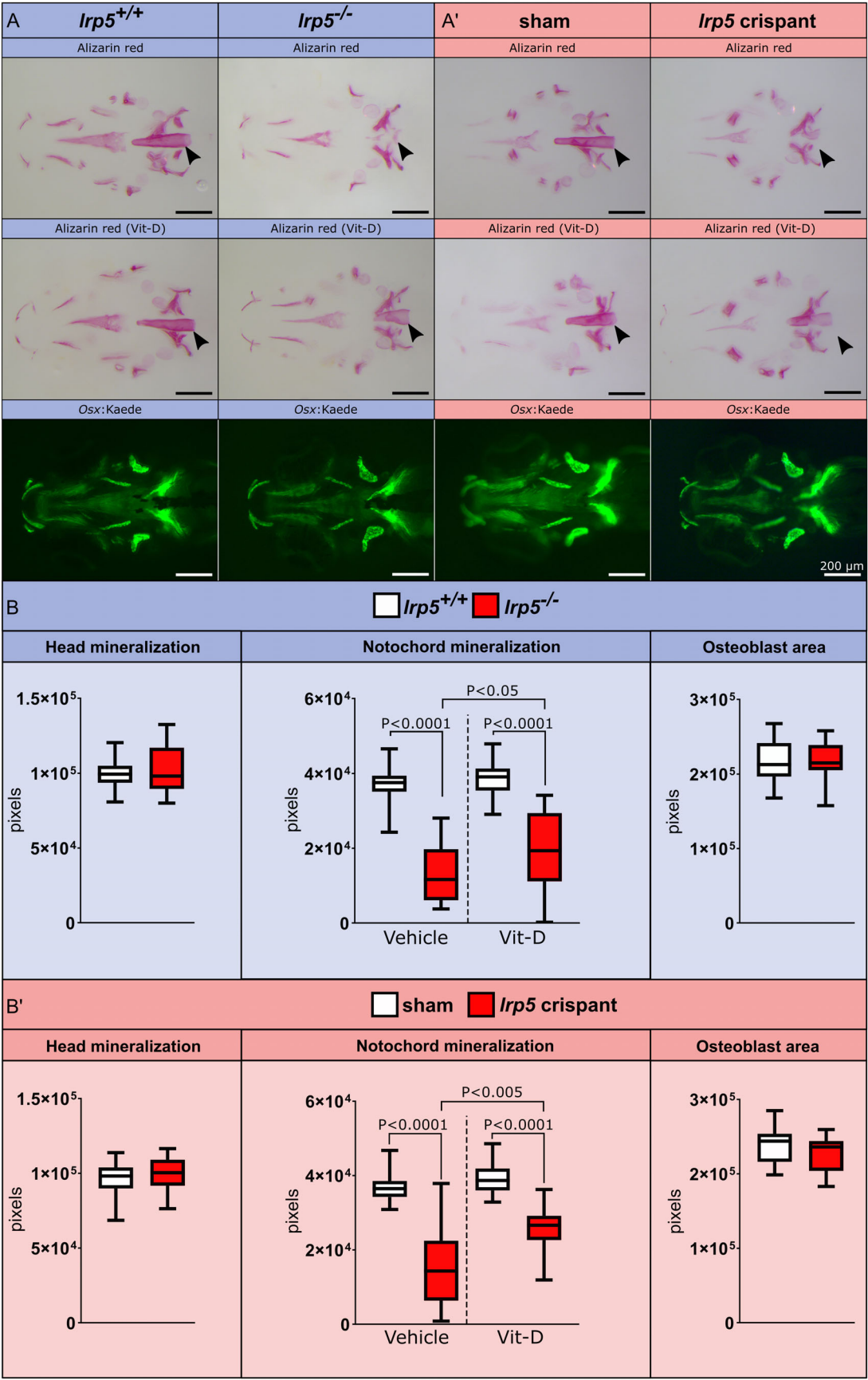


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volume of the different centra were scaled to the respective control groups and tested using two repeated-measures ANOVA (Supplemental Fig. S7A, Supplemental Data S1). This analysis demonstrates that there is no appreciable difference between the TMD and centrum volume phenotypes of *lrp5*^{-/-} mutant and *lrp5* crispant zebrafish.

In *lrp5* crispants, the variability in mean centrum TMD (SD = 83 mgHA/cm³) and volume (SD = 3.5 × 10⁶ μm³) was higher than in *lrp5*^{-/-} mutant TMD (SD = 44 mgHA/cm³) and volume (SD = 1.80 × 10⁶ μm³). We hypothesized that the higher variability of skeletal parameters in crispants could be attributed to variable OOF mutation efficiencies throughout the different crispant zebrafish used for μCT analysis. However, most samples (5/6) displayed limited phenotypic variability despite variability in OOF mutation load (Supplemental Fig. S7B, C). The increased variability in centrum TMD and volume in this set of crispants was mainly attributable to one sample with the highest OOF mutation efficiency, resulting in a noticeably more severe phenotype.

In addition to variability between samples, it is also possible that crispants have a patchy/mosaic phenotype throughout the vertebral column due to local variability in loss-of-function as previously demonstrated by Watson and colleagues.⁽³¹⁾ Therefore, the phenotypic variability throughout the vertebral column was visualized by plotting the centrum TMD and volume per vertebrae per sample (Supplemental Fig. S7D, E). However, *lrp5* crispants did not show vertebral regions with obvious differences in centrum TMD and volume compared with surrounding vertebral regions, similar to *lrp5*^{-/-} mutants. These results are indicative of efficient loss-of-function mutagenesis throughout the vertebral column in *lrp5* crispants.

Finally, to confirm decreased TMD in both the *lrp5*^{-/-} mutants and *lrp5* crispants, we applied inductively coupled plasma mass spectrometry (ICP-MS) on dissected vertebral columns to determine the abundance of calcium, phosphate, and magnesium, which are the main components of hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂). The obtained data were normalized for vertebral column weight and length. A significant reduction in calcium (*p* < 0.05), phosphate (*p* < 0.05), and magnesium (*p* < 0.05) was found in the *lrp5*^{-/-} mutant vertebral bodies, confirming the microCT results (Fig. 4D) (Supplemental Data S2). A less pronounced but significant reduction of calcium (*p* < 0.05), phosphate (*p* < 0.05), and magnesium (*p* < 0.05) was also found in the vertebral bodies of *lrp5* crispants (Supplemental Data S3), again demonstrating high similarity between full KO and *lrp5* crispant phenotypes (Fig. 4D'). Additionally, the calcium/phosphate ratio was not found to be different from matched controls in both the *lrp5*^{-/-} mutants (*lrp5*^{+/+} 1.94 [CI ± 0.028] versus *lrp5*^{-/-} 1.93 [CI ± 0.020]) and crispants (sham 1.78 [CI ± 0.022] versus *lrp5* crispant 1.71 [CI ± 0.047]).

Decreased response of Wnt signaling during fin regeneration in *lrp5*^{-/-} mutant and *lrp5* crispant zebrafish

Next, we aimed to study the impact of loss-of-function of the *lrp5* gene on the Wnt signaling pathway in zebrafish. First, we investigated levels of canonical Wnt activity at the age of 7 dpf by using the β-catenin responsive transgenic line Tg(7xTCF-Xla.Sia:GFP^{lacZ}). This revealed canonical Wnt activity mainly in the central nervous system, cardiac tissue, and oral region but not in cartilage or notochord elements, which are the most obviously affected structures in *lrp5*^{-/-} mutants and crispants at this stage (Supplemental Fig. S8). Next, we examined Wnt signaling in adult zebrafish. Pronounced and well-described Wnt signaling in adult zebrafish occurs during fin regeneration, as this pathway is crucially important for different regeneration processes, including the differentiation and proliferation of osteoblasts, which are essential for the regeneration of the bony fin rays.^(58,59)

¹ Therefore, we primarily focused on caudal fin regeneration to monitor the consequences of loss of *lrp5* in both *lrp5*^{-/-} mutants and *lrp5* crispants on canonical Wnt signaling. First, we investigated levels of canonical Wnt activity post-amputation using the β-catenin responsive transgenic line Tg(7xTCF-Xla.Sia:GFP^{lacZ}) (Fig. 5A-A'). We observed a significantly reduced area of canonical Wnt responsive cells in *lrp5*^{-/-} mutants and *lrp5* crispants compared with controls in the regenerating tissue of the blastema at 4 days post amputation (dpa) (*n* = 5). However, follow-up at 10 dpa revealed similar levels of Wnt signaling in fin tissue between *lrp5*^{-/-} mutants and controls (*n* = 5) (Supplemental Fig. S9). In addition, we performed RT-qPCR expression analysis in pre-amputation (intact) and regenerating post-amputation adult fin tissue of the commonly used Wnt signaling markers *axin2* (which is part of the destruction complex of β-catenin), the paralogues *dkk1a* and *dkk1b* (which encode the Wnt signaling inhibitor *Dkk1*), and *tcf7* and *lef1* (which encode the TCF/LEF transcriptional complex downstream of Wnt).⁽⁶⁰⁾ *AXIN2*, *TCF7*, and *LEF1* are key components of the canonical Wnt pathway that are known to be induced upon Wnt activation,^(61,62) while *DKK1* expression can be upregulated via β-catenin/TCF and functions as a component of a negative feedback loop that inhibits Wnt signaling.⁽⁶³⁾ At two time points, 0 dpa (intact tissue) and 4 dpa (during regeneration), RNA was extracted from the caudal fin of the same zebrafish and used for expression analysis. A generalized linear mixed model was used to determine significant effects of genotype, time, and their interaction (Supplemental Data S4 and Data S5). Overall, as expected, the expression level of the different Wnt signaling markers was markedly upregulated during regeneration (fold change >1) in both *lrp5*^{+/+} and sham controls, indicating the validity of the assays (Fig. 5B-B'). Importantly, this upregulation was decreased for all tested markers in both the *lrp5*^{-/-} mutants and *lrp5* crispants, although not always significantly in both models. While *Axin2* and *dkk1b*

Fig. 3 Alizarin red mineral staining in *lrp5* mutants and crispants. (A-A') Alizarin red stain of representative *lrp5*^{-/-} (*n* = 24) (A) and *lrp5* crispants (*n* = 32) (A'), which display a marked reduction in the mineralization of the anterior notochord sheath, which is indicated by arrowheads (top row). Vitamin D treatment was able to partially rescue mineralization of the notochord sheath, which is indicated by arrowheads (middle row) compared with vehicle-treated controls. Osteoblasts in the head skeleton visualized in *lrp5*^{-/-} (*n* = 31) and *lrp5* crispants (*n* = 27) by using the *osx*:Kaede transgenic line (bottom panel), does not reveal any apparent differences. (B-B') *lrp5*^{-/-} (B) and *lrp5* crispants (B') do not show a difference in the mineralization of the head skeleton. Notochord sheath mineralization was significantly reduced in *lrp5*^{-/-} and *lrp5* crispants but significantly increased upon vitamin D treatment. No significant differences were found in osteoblast-positive area. Alizarin red-stained larvae are oriented with anterior to the left from a dorsal perspective, and Tg(*osx*:Kaede) larvae are visualized from a ventral perspective with anterior to the left side. Scale bars = 200 μm.

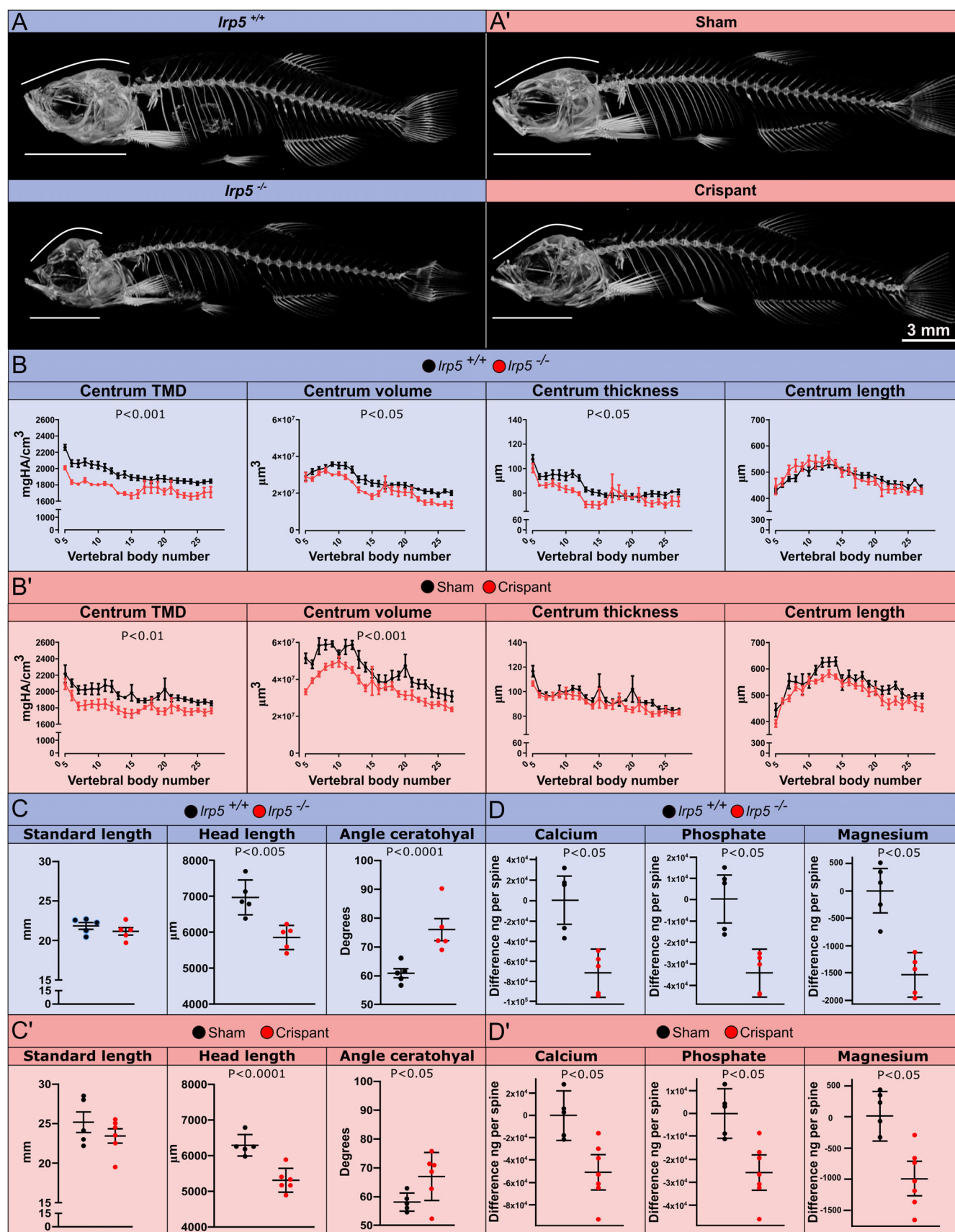


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were found to be significantly less upregulated in both the *lrp5*^{-/-} mutants ($p < 0.001$, < 0.05) and *lrp5* crispants ($p < 0.0001$, < 0.0001) compared with the respective controls (Fig. 5B-B'), *dkkb1a*, *tcf7*, and *lef1* upregulation was only significantly decreased in *lrp5* crispants ($p < 0.0001$) (Fig. 5B'). Overall, these results demonstrate that the *lrp5*^{-/-} mutant and *lrp5* crispants show impaired Wnt signaling.

Since LRP5 mediates the differentiation and maturation of osteoblasts through the Wnt signaling pathway, we visualized immature osteoblasts at 4 dpa by using the transgenic Tg(*osx*:Kaede) background (Fig. 5C-C'). Kaede-positive osteoblasts in the fin rays were photoconverted from green to red at 0 dpa, whereas newly differentiated *osx*:Kaede positive osteoblasts at the blastema appeared in green.⁽³⁹⁾ At 4 dpa, the green signal was quantified per fin ray and revealed a significantly decreased signal in both the *lrp5*^{-/-} mutants ($p < 0.0001$) and *lrp5* crispants ($p < 0.0001$) (Fig. 5C-C').

Finally, to investigate the impact of impaired Wnt signaling on the regenerative capacity of the caudal fin, we performed sequential in vivo AR staining at 0, 5, 10, 15, and 20 dpa. No differences were found in the regenerative capacity of the fin rays when measuring the length of the newly regenerated mineralized fin rays (Supplemental Fig. S10), but an irregular morphology of the newly regenerated fin was observed at 20 dpa in both *lrp5*^{-/-} mutants and *lrp5* crispants (Fig. 5D-D'); segmentation of the fin rays was not affected, but the direction of growth, caudal to the amputation plane, was focused inwards, resulting in narrower fins.

Discussion

In vivo functional testing of candidate genes in complex human diseases has recently been revolutionized through the development of novel CRISPR/Cas9-based methods, enabling rapid and cost-effective screening.⁽⁶⁴⁾ In this work, we assessed the feasibility of CRISPR/Cas9-based F0 (crispant) screens in the zebrafish model system for functional validation of candidate genes for osteoporosis, a common multifactorial low-bone-mass disorder with which more than 200 candidate genes/loci have been associated.⁽⁶⁵⁾ As a proof-of-concept, we generated a crispant zebrafish model for the *lrp5* gene, one of the top candidate genes consistently replicated in GWAS for osteoporosis, and compared these *lrp5* crispants to a full *lrp5* KO zebrafish model, both in larval and adult stages.

In larval stages, we noted highly similar abnormalities in the head skeleton of *lrp5* mutants and crispants. The head was shorter and cartilage structures such as the ceratohyal and ceratobranchials showed wider angles, abnormalities that further persisted into adult stages. In a previously established

morpholino-based transient *lrp5* knock-down model, similar although much more severe malformations of the lower jaw and pharyngeal skeleton were found with reverse-oriented ceratohyals and a complete loss of ceratobranchials 1–4 in early life stages, while expression of *lrp5* in wild-type zebrafish was found mainly in these cartilage elements.⁽⁶⁶⁾ The *lrp5* KO and crispant zebrafish models presented in this study therefore provide additional evidence that support the role of *lrp5* in cartilage development of the head skeleton. Indeed, although not a common phenotype, OPPG patients, carrying recessive loss-of-function mutations in the LRP5 gene, have also been described with developmental defects in the head, including microcephaly and enophthalmos.⁽⁶⁷⁾

Remarkably, we did not observe any differences in the mineralization of early skeletal elements nor in the number of osteoblasts present in the head skeleton. However, the mineralization of the anterior tip of the notochord sheath was found to be substantially delayed in both crispants and mutants. In teleosts, the notochord consists of large vacuolated chordocytes, surrounded by chordoblasts that deposit the notochord sheath and induce its mineralization, without the involvement of osteoblasts.⁽⁵⁵⁾ This suggests that, at least in zebrafish, Lrp5 also plays a role in the regulation of mineralization by other cell types, besides osteoblasts. Exposure to vitamin D, a known mediator of calcium and phosphate homeostasis in osteoblasts,⁽⁶⁸⁾ was able to partially rescue the reduced notochord mineralization, suggesting that vitamin D can also have an impact on chordoblast functioning. Vitamin D receptors can be found in chondrocytes, osteoblasts, osteocytes, and osteoclasts,⁽⁶⁹⁾ and therefore probably also in chordoblasts. Overall, these results point to the therapeutic potential of vitamin D in LRP5 deficiency syndromes.

In adult life stages, a similarly reduced mineral density and volume of the vertebral bone was found in *lrp5* mutants and crispants. The zebrafish vertebral column is similar to the human vertebral column but is formed by intramembranous rather than endochondral ossification.⁽⁷⁰⁾ Nonetheless, zebrafish vertebral bodies are composed of a combination of compact and trabecular bone⁽⁷¹⁾ that is remodeled throughout life,⁽²⁴⁾ making them a relevant structure for the study of human bone disorders. To demonstrate the translational value of the bone phenotype observed in the *lrp5* models for the human condition, osteoporosis, bone parameter values can be converted to standardized scores. Standardized scores are obtained by subtracting the reference sample mean from affected sample's divided by the reference sample standard deviation. This is analogous to Z-scoring, although the current sample size of $n = 5$ is insufficient to establish real Z-scores at this time, which are calculated based on a much larger reference population. The *lrp5* mutants and crispants show a standardized score of -3.8 and -4.6 for centrum TMD and -2.0 and -2.0 for centrum volume, respectively. In

Fig. 4 MicroCT (21 μ m) and inductively coupled plasma mass spectrometry of the vertebral column in adult *lrp5* mutants and crispants. (A-A') Maximal projections of microCT scans (21 μ m) of 5-month-old *lrp5*^{-/-} (A) and *lrp5* crispant (A') with matched controls display head malformations. Horizontal lines ventral to the skull represent head length that is shorter in *lrp5*^{-/-} mutants and crispants, while curved lines dorsal to the skull represent skull shape that is more rounded in *lrp5*^{-/-} mutants and crispants. (B-B') Detailed analysis of individual vertebra (5–27 anterior–posterior) reveals a significant reduction in tissue mineral density (TMD), volume, and thickness in the centra along the vertebral column in *lrp5*^{-/-} ($n = 5$) (B) and in TMD and volume in *lrp5* crispants ($n = 6$) (B'). (C-C') Standard length was not found to be different between *lrp5*^{-/-} mutants ($n = 5$) (C) and *lrp5* crispants ($n = 6$) (C') with matched controls but a significant reduction in head length and a wider angle between the ceratohyals was observed (Supplemental Fig. SS4). (D-D') Element analysis of the mineral content by inductively coupled plasma mass spectrometry ($n = 5$ for both *lrp5*^{-/-} and *lrp5* crispants) revealed a significant reduction in calcium, phosphate, and magnesium. Maximal projections are portrayed from a lateral perspective with anterior to the left side. Error bars represent standard deviation. Scale bars = 3 mm.

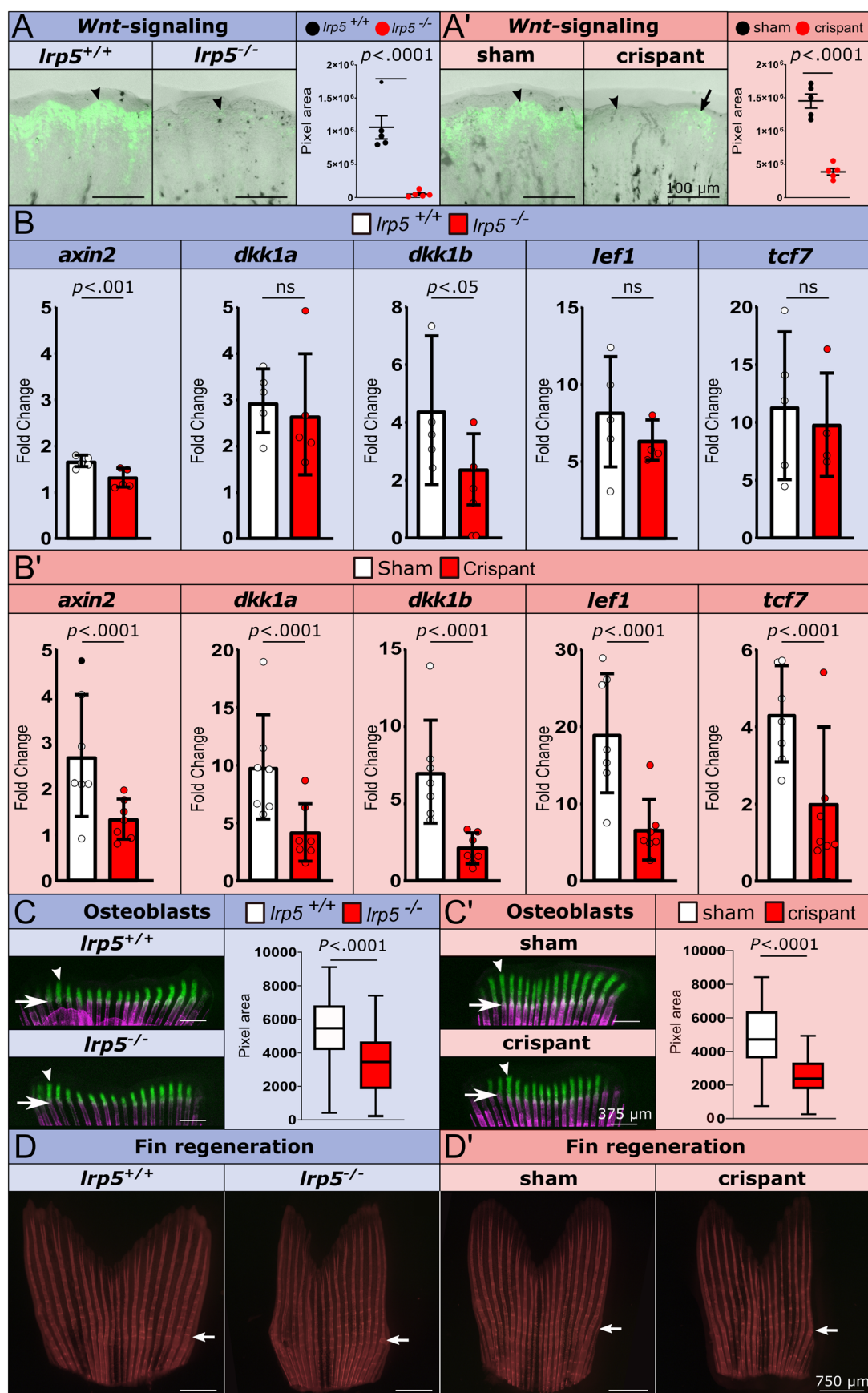


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human subjects, DXA- or pQCT-measured areal BMD, and volumetric BMD of cortical and trabecular bone, are typically expressed as Z-scores, where Z-scores lower than -2 are indicative for osteoporosis.^(72–74) Previous studies using DXA and pQCT scanning in OPPG patients, carrying recessive *LRP5* mutations, revealed decreased bone density (vBMD Z-score -1.57) and cortical bone area of the tibia (Z-score -2.19)⁽⁷⁵⁾ and decreased BMD of the lumbar spine (Z-scores between -1.2 and -7.8).⁽⁷⁶⁾ In addition, dominantly inherited *LRP5* mutations were described to result in low bone mass phenotypes in children and adults.^(34,77,78) Therefore, considering that the degree of reduction in bone density is similar, the skeletal phenotype in the zebrafish *lrp5* mutants and crispants is highly reminiscent of the osteoporotic phenotype found in human patients carrying inactivating *LRP5* mutations. Element analysis of the vertebral column via ICP-MS confirmed the decreased abundance of bone minerals (hydroxyapatite) in our *lrp5* zebrafish models. Therefore, we show here that, in addition to being applied to analyze mineral content in zebrafish scales,⁽⁵¹⁾ this technique can also be applied for the analysis of mineral elements in the zebrafish vertebral column, as an alternative approach to measure the mineralization status. In human, there have been reports of a link between fragility fractures and an increased calcium to phosphorus ratio, which is an important factor in bone strength.⁽⁷⁹⁾ However, using element analysis, we could not detect differences in calcium to phosphate ratios between the *lrp5* zebrafish models and matched controls.

To our knowledge, no other genetically modified zebrafish model with an osteoporotic phenotype characterized by a decreased bone density and volume in adult life stages has been described, because previously described zebrafish osteoporosis models were induced by administration of compounds such as metals and glucocorticoids rather than genetic modification.^(51,80) Several mouse models for *Lrp5* deficiency have already been described, each of them presenting relevant skeletal phenotypes including a reduction of BMD, vertebral bone volume, and bone strength.^(35–37) The strong similarity of the skeletal phenotype between these models and the zebrafish models described here suggests a conserved role for LRP5 in vertebrate species. In mice, loss of *Lrp5* function results in impaired Wnt/ β -catenin signaling, thereby reducing osteoblast activity as well as the number and proliferation of osteoblasts.⁽³⁷⁾ To assess the activity of the Wnt signaling pathway in *lrp5* zebrafish mutants and crispants, we studied fin regeneration, a process in which the Wnt signaling pathway is known to be strongly induced in WT.⁽⁸¹⁾ Using the Wnt/ β -catenin signaling reporter *7xTCF-Xla.Sia:GFP^{ia4}*, a significant reduction in canonical Wnt signaling was observed in the blastema of *lrp5* mutants and crispants.

These observations were further corroborated by a reduction in expression of Wnt signaling inhibitors and the *tcf/lef* transcriptional complex in blastema tissue. Therefore, in both the *lrp5* mutant and crispant, the loss of function of *lrp5* results in a reduction of canonical Wnt signaling, thus resembling the pathogenetic molecular mechanisms described in previously published *lrp5* models.^(35,37)

Differentiation of new functional osteoblasts in the blastema is dependent on the generation of an osteoblastogenic progenitor population in the most distal blastema tip by localized Wnt/ β -catenin signaling.⁽⁵⁹⁾ In accordance with this, the number of newly differentiated *osx/sp7*-positive osteoblasts was reduced in the blastema of *lrp5* mutants and crispants. The phenotypic consequences of the impaired Wnt signaling and osteoblast differentiation in the regenerating tail fin involved aberrations in the directional growth of the long fin rays of the lobe region, resulting in narrower fins. These abnormalities can be expected because Wnt/ β -catenin signaling is not only responsible for osteoblast differentiation but also orchestrates growth, differentiation, and patterning in the blastema via secreted factors such as Fgfs and Bmps.⁽⁶⁰⁾ In contrast, no difference in osteoblast numbers was found in *lrp5* mutants and crispants during early larval stages. This is in agreement with previous work by Willems and colleagues that demonstrated early larval *lrp5* expression and Wnt signaling in ventral head cartilage rather than in bone elements containing osteoblasts. In addition, by using the *Tg(7xTCF.XlaSiam:nlsGFP^{ia4})* transgenic background, Brunt and colleagues showed that Wnt signaling is specifically present in the craniofacial cartilage at 5 dpf, and by using the same transgenic line at 7 dpf, we could also not detect any Wnt activity in osteoblast-containing elements. Overall, together with the absence of mineralization defects of early skeletal elements and the observed malformation of the head cartilage in *lrp5* mutants and crispants, this suggests that in early stages, *lrp5*-mediated Wnt signaling is active in cartilage but not in the developing bone.^(66,82)

In this study, we demonstrated that a crispant zebrafish model for the *lrp5* gene strongly mimics the full *lrp5* KO zebrafish model, both in larval and adult stages and both at the phenotypic and molecular level. Since the analysis of crispant models is performed directly in F0 mosaic founder zebrafish, the workload, time, and resources usually needed to generate a stable KO model is significantly reduced. The generation of a full dominant/recessive KO zebrafish model requires at least an additional 6 to 9 months compared with the creation of crispants, when an average generation time of 3 months to reach adulthood is considered. Additionally, we observed a significantly increased survival in crispants compared with the KO model, thereby

Fig. 5 Investigation of *wnt*-signaling, osteoblast differentiation, and fin ray morphology during caudal fin regeneration. (A–A') *Wnt*-signaling activity visualized in the blastema at 4 days post amputation (dpa), as indicated by black arrowheads in *Tg(7xTCF-Xla.Sia:GFP^{ia4})*. Wnt activity was absent in the blastema of *lrp5*^{−/−} (A) and significantly reduced in the *lrp5* crispants (A'). However, several fin rays did show some *wnt* activity, albeit reduced, as indicated by a black arrow. The GFP-positive area was quantified for the entire blastema ($n = 5$). Error bars represent the standard deviation. Scale bars = 100 μ m. (B–B') Fold changes in gene expression of *wnt*-signaling markers (post-amputation relative expression level/pre-amputation relative expression level) in *lrp5*^{−/−} versus *lrp5*^{+/+} and *lrp5* crispant versus sham. Reduced upregulation after fin-amputation was found for *axin2*, *dkk1a*, *dkk1b*, *tcf7*, and *lef1* for both *lrp5*^{−/−} mutants and crispants versus their respective controls. Error bars represent the standard deviation (Supplemental Data S4 and Data S5). (C–C') *Tg(osx:Kaede)* was used to image the osteoblast response in fin rays during fin regeneration in *lrp5*^{−/−} (A) and *lrp5* crispants (A') at 4 dpa. The new green *osx:Kaede* signal, representative of new osteoblasts posterior to the amputation zone (white arrow), was quantified per fin ray, indicated by white arrowheads (fin ray number 2–7 and 12–17 counting dorsal to ventral) and revealed a reduced signal in both *lrp5*^{−/−} and *lrp5* crispants. Scale bars = 375 μ m. (D–D') Alizarin red-stained fins at 20 dpa reveal inward-directed growth of the fin rays in the fin lobes in both *lrp5*^{−/−} ($n = 5$) (A) and *lrp5* crispant ($n = 5$) (A') models. White arrows represent the location of amputation. Scale bars = 750 μ m.

increasing the number of samples available for experimentation. Consequently, the crisprant approach allows a more rapid and easier reverse genetic screen of candidate genes, contributing to osteoporosis and other skeletal diseases. Furthermore, crisprants are able to overcome a number of issues that often arise with the use of antisense morpholino (MO) knock-down,⁽⁸³⁾ a commonly used reverse genetics (screening) approach in zebrafish to assess gene function. First, MO effects are transient and limited to embryonic and early larval stages (up to 5 dpf), excluding the full modeling of skeletal disorders of which many aspects only appear beyond 5 dpf. Second, upon MO injection, a frequent occurrence of p53-dependent apoptosis⁽⁸⁴⁾ and off-target effects resulting in so-called “pseudophenotypes”⁽⁸⁵⁾ have been noted. The latter phenomenon could also be an explanation for the generally far more severe phenotypes observed in *lrp5*-morphants described by Willems and colleagues compared with the crisprant (and mutant) models generated in our study. Conversely, the milder phenotype in *lrp5* crisprant and mutant larvae compared with morphants may also be explained by the activation of genetic compensation, a mechanism that is known to reduce the impact of mutations resulting in nonsense-mediated decay by upregulating other related genes.⁽⁸⁶⁾

The success of a crisprant screen highly depends on the efficiency of the CRISPR/Cas9-mediated gene disruption and more specifically on the rate of OOF-indel mutations. In theory, when mutagenesis is maximally efficient, only two-thirds of indels are expected to be OOF, which means that only 4 of 9 cells carry bi-allelic OOF mutations.⁽³⁰⁾ In this case, phenotypes may be absent because of a substantial number of cells in the mosaic mutant animal that carry in-frame mutations that still result in functional protein. Therefore, in our study, we used the InDelphi machine learning model⁽⁵³⁾ to predict CRISPR/Cas9 editing outcomes and to select the most potent *lrp5* gRNA design, in this way favoring frameshift gene editing outcomes prone to nonsense-mediated decay. The selected gRNA resulted in experimental OOF rates of 76.6%, close to the InDelphi-predicted OOF rates of 71.7%. This confirms previous work that showed that InDelphi can accurately predict CRISPR/Cas9 gene editing outcomes in zebrafish and *Xenopus* embryos,⁽⁵⁴⁾ and is therefore an important tool for the generation of crisprant models. Importantly, when targeting a gene of interest by crisprant analysis, it is important to take into account the possible existence of paralogues, that originate from an additional genome duplication in the zebrafish genome.⁽⁸⁷⁾ As paralogous genes can have either redundant or different functions, it is therefore advisable to target these paralogues both independently and simultaneously.

Additionally, understanding the distribution of different clonal cell populations containing different indels throughout the adult body is an important aspect in studying CRISPR/Cas9-induced phenotypes. It was shown by Watson and colleagues⁽³²⁾ that single clonal populations are not responsible for forming individual bony elements, suggesting that skeletal structures contain heterogeneous populations of cells, whereby the different populations contain different indels.⁽³¹⁾ It is likely that the high OOF-indel rate obtained in the *lrp5* crisprants results in absence of phenotypic mosaicism by creating a high enough fraction of loss-of-function (LOF) cells per vertebral body, resulting in a uniform phenotype reminiscent of a full KO phenotype. On the other hand, it was previously shown that Wnt/ β -catenin signaling, mediated by LRP5, has both autocrine and paracrine actions,⁽⁸⁸⁾ which can also explain the uniform phenotypic expressivity in the *lrp5* crisprants. For crisprant analysis of genes that function cell-autonomously, phenotypic expressivity may

be less uniform, although Watson and colleagues developed statistical methods for decoding spatially variable skeletal phenotypes in crisprants.

Because of the phenotypic uniformity, the *lrp5* mutant and crisprant had a similarly significant skeletal phenotype, when analyzing similar numbers of animals. Future experiments aimed at understanding the distribution of mosaic cell clusters in adult zebrafish can be based upon new single-cell sequencing approaches that have recently been used to reveal cell lineages in multicellular organisms.^(89,90)

Taken together, we demonstrated that *lrp5* mutant and crisprant zebrafish models faithfully recapitulate the osteoporotic phenotype found in human patients carrying LRP5 loss-of-function mutations. Therefore, these models can provide new insights into the biological role of LRP5 in skeletal development and maintenance, have the potential to reveal new pathogenetic mechanisms involved in human osteoporosis, and can be used as a tool for osteogenic compound screening. In addition, we showed that crisprant screening in zebrafish is a promising approach for rapid functional screening of osteoporosis candidate genes, which are increasingly identified through large-scale genomics analyses.

Disclosures

All authors state that they have no conflicts of interest.

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PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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