INTRODUCTION

The hormone leptin is known for its role in regulating food intake, autonomic outflow, and endocrine function to maintain energy balance. Although leptin is the primary adipostatic factor in mammals, it has been well established that leptin can affect glucose homeostasis, independent of its adipostatic actions.¹⁻⁵ This is supported by the following key observations. First, leptin is more potent at regulating glucose levels in the blood than it is at suppressing appetite.⁶ Second, acute disruption of leptin action in vivo raises blood glucose and plasma insulin levels before effects on

Abstract

Leptin is best known for its role in adipostasis, but it also regulates blood glucose levels. The molecular mechanism by which leptin controls glucose homeostasis remains largely unknown. Here, we use a zebrafish model to show that Wnt signaling mediates the glucoregulatory effects of leptin. Under normal feeding conditions, leptin regulates glucose homeostasis but not adipostasis in zebrafish. In times of nutrient excess, however, we found that leptin also regulates body weight and size. Using a Wnt signaling reporter fish, we show that leptin activates the canonical Wnt pathway in vivo. Utilizing two paradigms for hyperglycemia, it is revealed that leptin regulates glucose homeostasis via the Wnt pathway, as pharmacological inhibition of this pathway impairs the glucoregulatory actions of leptin. Our results may shed new light on the evolution of the physiological function of leptin.

KEYWORDS

CRISPR Cas9, glucose tolerance test, leptin receptor, leptin-a, leptin-b, lithium chloride
body weight become apparent, and treatment with leptin in leptin-deficient Lep⁶/⁶ mice corrects glucose levels before body mass.³ Third, Lep⁶/⁶ and leptin receptor-devoid LeprAH/db mice become hyperinsulinemic before they become obese.⁴ Fourth, humans who suffer from lipodystrophy and rodent models of this disease, characterized by very low body fat and leptin levels, exhibit hyperglycemia, hyperinsulinemia, and insulin resistance. All of these symptoms are corrected by leptin therapy,⁹,¹⁰ which received approval by the FDA for this treatment purpose.¹¹

Leptin signaling is evolutionarily well-conserved. Homologs for leptin and the leptin receptor are present even in invertebrate species like Drosophila melanogaster.¹² Although leptin from species of different animal classes has low primary sequence homology, the secondary, tertiary, and quaternary structure, as well as key amino acids required for leptin’s physiological activity, are evolutionarily conserved.¹³ Zebrafish (Danio rerio) express two leptin paralogs: leptin a and b.¹⁴ Both, like all vertebrate leptin paralogs, consist of four alpha-helices and contain a pair of cysteine residues that form a disulfide bridge. Three receptor interaction sites have been mapped, and each of these has at least some degree of amino acid sequence conservation.¹⁵

Remarkably, a single study reported that leptin does not mediate adipostasis in zebrafish, but instead has an essential role in the regulation of glucose homeostasis. Loss of the zebrafish leptin receptor leads to an increase in the number of pancreatic β-cells and elevated levels of insulin mRNA in larvae. However, adipostasis, food intake, and fertility remain unchanged.¹⁶

The most extensively studied leptin signaling pathway is the janus kinase 2—signal transducer and activator of transcription 3 (JAK2-STAT3) pathway, which mediates most of the metabolic effects of leptin. While clear evidence suggests regulation of body weight and food intake by leptin is mediated by the JAK2-STAT3 pathway,¹⁷,¹⁸ evidence for the involvement of this pathway in the glucoregulatory actions of leptin is scarce. Indeed, leptin has been shown to directly regulate insulin sensitivity via the insulin receptor substrate—phosphoinositide 3-kinase—protein kinase B (IRS-P13K-AKT) pathway.¹⁹ Furthermore, data from our laboratory²⁰–²² suggest that leptin can potentially regulate glucose homeostasis via the wingless/integration 1 (Wnt) pathway.

A genome-wide association study (GWAS) identified that polymorphisms in the transcription factor 7-like 2 (TCF7L2) gene, which encodes a transcription factor of the canonical Wnt pathway, increase the risk of type 2 diabetes.²³ The canonical Wnt pathway, known mostly for its role in embryogenesis and tumor formation, is activated when a Wnt ligand binds to the frizzled (Fzd) receptor, which subsequently forms a complex with the co-receptor lipoprotein related protein (LRP) 5/6. This causes disheveled (Dvl) to phosphorylate LRP, which then inactivates glycogen synthase kinase 3β (GSK3β) decreasing phosphorylation of the transcriptional co-activator β-catenin. Stabilized β-catenin then enters the nucleus where it associates with transcription factors of the lymphoid enhancer factor (LEF)/T cell factor (TCF) family, to ultimately regulate the transcription of downstream target genes such as CCND1 and AXIN2.

Glycogen synthase kinase 3β is an important intracellular inhibitor of the Wnt pathway. While some studies point towards a role for GSK3β in skeletal muscle as a mediator of glucose metabolism,²⁴ data from our laboratory show that GSK3β action specifically in the hypothalamus is essential for glucose homeostasis. Lep⁶/⁶ mice were found to have elevated levels of active hypothalamic GSK3β, and glucose intolerance in these mice was acutely ameliorated by intracerebroventricular injection of a GSK3β inhibitor. Furthermore, neuron-specific overexpression of GSK3β in the hypothalamus exacerbated the effects of a high-fat diet in wild-type mice compared with mice fed a standard diet, measured as increased hyperphagia, obesity, and glucose intolerance.²⁵ These studies suggest that canonical Wnt signaling might be the main driver for the glucoregulatory actions of leptin.

Utilizing CRISPR-mediated leptin- and leptin receptor deletion and a transgenic Wnt-reporter fish line, we identify that the action of leptin to solely regulate glucose homeostasis is limited to normal feeding conditions, whereas in times of nutrient excess leptin also regulates body size. This suggests that the glucoregulatory action of the hormone may be the first acquired during evolution. Intriguingly, in the transgenic Wnt-reporter zebrafish line, we provide the first direct evidence that leptin activates canonical Wnt signaling mediated via the leptin receptor and that the glucoregulatory action of leptin is mediated through this pathway. Furthermore, activation of this pathway by LiCl mimics the glucoregulatory action of leptin in its entirety, confirming the essential role of Wnt signaling in the regulation of glucose homeostasis.

2 | MATERIALS AND METHODS

2.1 | Zebrafish husbandry

Zebrafish (AB strain) were maintained in 3.5 L tanks on a Palletized Centralized Life Support System (Tecniplast). The water in this recirculating system was pumped through mechanical filtration, charcoal filtration, and UV treatment, and 10% of the water was replaced every hour. The water was kept at 26–30°C, with pH 7.6–8.0 and a conductivity of 300–600 μS. The facility environment maintained a 14-h light and 10-h dark cycle. Water quality parameters were automatically measured and adjusted.
and remained within acceptable limits for the duration of the study.

2.2 | CRISPR Cas9 mutagenesis

Single guide RNAs (sgRNAs) were synthesized in vitro. Cas9 mRNA was transcribed from a pT3TS-nCas9 plasmid (Addgene plasmid #46757). Offspring of AB or Tg(7xTCF/Xla, Siam:tlsmCherry)ia526 zebrafish were injected at the one-cell stage into the cell with ~1 nl of a solution containing zebrafish 212.2 ng/μl Cas9 mRNA and 35.4 ng/μl gRNA, based on. As a positive control, and to test the quality of Cas9 mRNA, we used a sgRNA targeting the tyrosinase gene. Mutagenic efficiency was analyzed using a three-primer fluorescence PCR method. Biallelic mutant founder fish (F0) were inbred, giving rise to stable mutant offspring. Target sequences were lepr GGAGCGCCAGTAAAGCCGTGTGG; lepa GGAATCTCTGGATAATGTCCTGG; lepb ACAGAAC TGAGACCATCAATGGG; tyr GGACTGGAGGACTTCTGGGG.

2.3 | Overfeeding

Three-month-old male leptin mutant fish and wild-type control fish were assigned to either a 6-week overfeeding regime, consisting of 6 daily feeds, or a standard diet of 2 feeds per day. Feeds alternated between 20 mg/fish of ZM-400© fish pellets and freshly hatched brine shrimp (Artemia nauplii, 30 mg cysts/fish). ZM dry pellets (Zebrafish Management Ltd.) consisting of 58% protein, 14.5% fat, 11.5% ash, 7.0% moisture, 30 000 I.U./kg vitamin A, 2500 I.U./kg vitamin D3, 400 mg/kg vitamin E, 2000 mg/kg vitamin C, 30 mg/g ω3 highly unsaturated fatty acids. Feeding times were Zeitgeber Time (ZT) 1:00 (with lights turning on at ZT 0:00), ZT 3:30, ZT 5:00, ZT 7:30, ZT 9:00, and ZT 11:30h under the overfeeding regime, and ZT 3:30 and ZT 7:30 in the normal-fed group. Feeding was done manually, and leftover food was removed by siphoning to prevent an effect of water quality on body weight. Body weights were measured weekly. Standard length (SL), defined as the length measured from the tip of the snout to the posterior end of the last vertebra, was measured at week 0, week 3, and week 6. Finally, glucose tolerance was measured at the end of the dietary intervention.

2.4 | Compound exposure

Metformin (Sigma) was dissolved in fish water to a final concentration of 20 μM. The metformin solution was freshly prepared and changed daily. PNU74654 (Abcam) and pyrvinium pamoate (Sigma) were dissolved in DMSO and added to tank water or E3 medium at a final concentration of 10 μM. LiCl (Sigma) was dissolved directly in tank water or E3 medium at a concentration of 10 μM.

2.5 | Image analysis

Images were taken using a Leica M205 FA epifluorescence microscope and analyzed using Fiji/ImageJ. Hypothalamic WNT pathway activation was quantified by manually drawing a region of interest around the part of the hypothalamus that is not obscured by the eye from a lateral perspective, followed by measurement of average fluorescent intensity in the region of interest.

2.6 | Blood sampling

Borosilicate glass micropipettes (Harvard Apparatus) were pulled on a Sutter p-97 Flaming Brown glass micropipette puller to create needles with a 1.0 outer diameter. Using scissors, the needle tips were cut obliquely to create a tip diameter of 100–300 μm. Next, needles were heparinized (5mg/ml heparin in saline) using an aspirator tube assembly. For blood collection, a heparinized needle was inserted in the nosepiece end of the aspirator tube assembly. Adult zebrafish were anesthetized with 0.13% tricaine (3-aminobenzoic acid ethyl ether methanesulfonate, MS222). Anesthetized fish were carefully transferred onto soft tissue paper soaked in tricaine solution. Another soaked tissue was used to cover the fish’s head. The needle was then carefully inserted at a 30°–45° angle into the dorsal aorta (DA), along the body axis and ventral to the spine. Generally, blood would rise into the needle in a pulsatile manner. If blood did not rise, gentle suction was applied through the mouthpiece, and the needle was moved gently by hand to encourage blood flow. The minimal required sample volume (0.6 μl) was collected. The needle was immediately removed, and gentle finger pressure with a soaked tissue was applied to the puncture site for ~15 s or until the bleeding stopped. Fish were then transferred to a recovery tank (28.5°C), and water was gently swirled towards the gills.

2.7 | Glucose immersion

The glucose immersion method was adapted from Ref. [27] Fish were placed in standard housing tanks containing a 1% glucose solution (55.5 mM). Because the tanks were not on the normal recirculation system, solutions
were renewed daily after feeding to prevent the growth of microorganisms. Blood samples were taken daily.

2.8 | Intraperitoneal glucose tolerance tests

Fish were fasted for 72 h to bring glucose levels down to baseline. Following anesthesia, fish were weighed and injected intraperitoneally with 0.5 mg glucose/g fish weight and allowed to recover for 30, 90, and 180 min after injection. Glucose concentrations were measured from blood samples of 0.6 μl using a commercially available glucometer (Accu-Chek Performa; Roche).

2.9 | Statistics

The data were analyzed by one- or two-way ANOVA followed by a Holm-Šidák comparison test, as appropriate, using GraphPad Prism 7 statistical software. The body weight data were analyzed by repeated-measures two-way ANOVA. The results are presented as mean ± SEM, and differences were considered significant if \( p < .05 \).

3 | RESULTS

3.1 | Leptin treatment ameliorates hyperglycemia in leptin-deficient and wild-type zebrafish

To study leptin function in zebrafish, we created CRISPR-mediated knockout zebrafish lines on an AB, background lacking leptin-a (lep\(a^{n2301}\)), leptin-b (lep\(b^{n2302}\)), or the leptin receptor (lep\(r^{n2303}\)) (Figure S1). Raising the fish at identical tank densities, we found that body weight and standard length did not differ between wild-type zebrafish and any of the knockout lines that were created (Figures S2 and S3), neither in males nor females at 4, 6, or 12 months of age. To investigate whether leptin ameliorates hyperglycemia in leptin- or leptin receptor-deficient zebrafish, we induced a hyperglycemic state by immersing male zebrafish of age. To investigate whether leptin ameliorates hyperglycemia in leptin- or leptin receptor- deficient zebrafish, we induced a hyperglycemic state by immersing male zebrafish of age. To investigate whether leptin ameliorates hyperglycemia in leptin- or leptin receptor- deficient zebrafish, we induced a hyperglycemic state by immersing male zebrafish of age. To investigate whether leptin ameliorates hyperglycemia in leptin- or leptin receptor- deficient zebrafish, we induced a hyperglycemic state by immersing male zebrafish of age.

28 Immersion in 1% glucose steadily elevated basal blood glucose levels at a rate of 15–20 mg/dl per day, whereas immersion in normal system water did not change basal blood glucose levels. On the third day of immersion, 1 h before blood sampling, fish were treated with either recombinant mouse leptin (2 mg/kg) or vehicle (Cortland salt solution). Leptin treatment prevented the artificially induced elevation of blood glucose levels in wild-type males (94.2 ± 3.7 mg/dl in leptin-treated fish vs. 111.8 ± 8.5 mg/dl in vehicle-treated fish, \( p < .05 \); Figure 1A) and females (95.4 ± 5.4 vs. 111.0 ± 5.1, \( p < .05 \); Figure 1B), lep\(a^{n2301}\) males (106.6 ± 6.8 vs. 128.8 ± 5.4, \( p < .05 \); Figure 1C), and females (103.4 ± 6.6 vs. 130.2 ± 6.1, \( p < .05 \); Figure 1D) and lep\(b^{n2302}\) males (98.2 ± 5.8 vs. 120.2 ± 4.4, \( p < .05 \); Figure 1E) and females (95.2 ± 3.5 vs. 121.2 ± 4.8, \( p < .05 \); Figure 1F). On the contrary, leptin treatment was unable to ameliorate hyperglycemia in lep\(r^{n2303}\) males 130.8 ± 4.6 vs. 135.0 ± 12.9; Figure 1G) and females (129.6 ± 8.0 vs. 132.6 ± 6.4; Figure 1H). The glucose-lowering effects of leptin were temporary, as blood glucose levels on day four no longer significantly differ between leptin-treated and vehicle-treated fish. The pattern of blood glucose elevation and the effect of leptin on hyperglycemia were identical between males and females. Female zebrafish have a more variable body weight compared with males, due to the fact that they continuously produce eggs, which can make up to 25% of their total mass. For these reasons, we performed all subsequent experiments in males.

3.2 | Overfeeding elicits an effect of leptin on body size regulation in zebrafish

Under normal feeding conditions, leptin regulates glucose homeostasis but not adipostasis in the zebrafish. We investigated whether leptin- and leptin receptor-deficient zebrafish are more prone to diet-induced obesity (DIO) and impaired glucose tolerance. To this end, we exposed lep\(a^{n2301}\) fish, lep\(b^{n2302}\) fish, lep\(r^{n2303}\) fish, or wild-type control fish (\( n = 12 \)) to an overfeeding regime or a normal diet for six weeks. Glucose tolerance was tested at the start and end of this period. Intraperitoneal glucose tolerance tests (ipGTts) revealed that although basal blood glucose levels were not significantly different (Figure 1A,B), glucose clearance in leptin receptor knockout fish was reduced by 26% compared with wild-type fish (Figure 2A,D). Surprisingly, we found that overfeeding elicits an effect of leptin on body weight. lep\(r^{n2303}\) fish (0.40 ± 0.01 g, \( p < .001 \) and lep\(a^{n2301}\) fish (0.38 ± 0.01 g, \( p < .001 \), but not lep\(b^{n2302}\) fish (0.35±0.01 g) have significantly increased body weight compared with overfed wild-type controls (0.34 ± 0.01 g; Figure 2E). There was also an increase in standard length from 28.4 ± 0.23 to 30.5 ± 0.31 mm (\( p < .05 \)) in lep\(a^{n2301}\) fish and 28.8 ± 0.25 to 31.7 ± 0.36 mm (\( p < .05 \)) in lep\(r^{n2303}\) fish (Figure 2F). Overfeeding increased basal blood glucose levels (from 64.1 ± 3.5 to 100.5 ± 1.8 mg/dl, \( p < .001 \); Figure 2B) and impaired glucose tolerance by 25%, independent of genotype (Figure 2C,D). We treated overfed fish with
metformin, the first-line treatment for T2DM in humans\textsuperscript{29} for the last week of the 6-week overfeeding regimen. Metformin reduced the area under the curve by 17\%, with levels no longer significantly different to levels in non-overfed wild-type fish (Figure S4). These results demonstrate that in zebrafish, under normal feeding conditions, leptin regulates glucose homeostasis but not body weight. Only in times of nutrient excess is leptin able to regulate both body weight, standard length, and glucose homeostasis.

FIGURE 1 Leptin treatment ameliorates hyperglycemia in male and female zebrafish. (A, B) Blood glucose values of male (right) and female (left) wild-type zebrafish over time following immersion in a 1% glucose solution. On the third day, 1 h before blood sampling, fish were injected intraperitoneally with recombinant mouse leptin (2 mg/kg) or vehicle. *\(p < .05\), repeated-measures one-way ANOVA. Data are displayed as mean ± SEM. (C, D) Male (right) and female (left) *lepa\textsuperscript{nc291} fish. (E, F) Male (right) and female (left) *lepb\textsuperscript{nc292} fish. (G, H) Male (right) and female (left) *lepr\textsuperscript{nc293} fish.
3.3 Leptin activates the canonical Wnt pathway in vivo

To investigate whether leptin activates the canonical Wnt pathway, we used a transgenic zebrafish line (Tg(7xTCF-Xla.Siam:nlsCherry)ia5) that sensitively detects translocation of the TCF7L2-β-catenin complex into the nucleus, thereby indicating canonical Wnt pathway activity. Because the Wnt pathway is markedly involved in embryonic patterning, we first established that at 5 days post fertilization (dpf), the developmental Wnt pathway activity has, with the microscope and imaging parameters we used, subsided to a level where it is mostly confined to the heart (Figure 2A). The canonical Wnt pathway can be pharmacologically activated with lithium chloride (LiCl), which inhibits GSK3β. The lowest effective dose of LiCl activating the Wnt pathway in 5 dpf Tg(7xTCF-Xla. Siam:nlsCherry)ia5 larvae was found to be 10 μM (Figure S5). We, therefore, conducted all subsequent experiments at 10 μM LiCl. The Wnt pathway can be inhibited with pyrvinium pamoate or PNU74654. Pyrvinium pamoate is an anthelmintic drug that potentiates the activity of casein kinase 1α (CK1α), leading to enhanced degradation of β-catenin. PNU74654 disrupts the interaction between β-catenin and TCF/LEF transcription factors. We demonstrated that pharmacological activation or inhibition of the canonical Wnt pathway reliably increases or decreases the fluorescent signal in Tg(7xTCF-Xla. Siam:nlsCherry)ia5 larvae (Figure S6). Strikingly, recombinant mouse leptin appeared to be efficacious in zebrafish and treating 5 dpf Tg(7xTCF-Xla. Siam:nlsCherry)ia5 larvae with leptin (100 nM) for 2 h led to robust activation of the fluorescent construct (Figure 3B).
FIGURE 3  Wnt pathway activation by leptin in Tg(7xTCF-Xla. Siam:nlsmCherry)ia5 larvae. (A) 5 dpf Tg(7xTCF-Xla. Siam:nlsm Cherry)ia5 larvae treated with vehicle (Cortland salt solution). Left: bright field image, with anatomical landmarks encircled; Middle: Epifluorescence image, with white arrow indicating teeth protrusion; Right: Magnification of yellow box in middle image, with hypothalamus and heart encircled. Scale bar = 500 μM. (B) 5 dpf Tg(7xTCF-Xla. Siam:nlsm Cherry)ia5 larvae treated with 100 nM recombinant leptin for 2 h. (C) 5 dpf CRISPR-mediated leptin receptor-deficient Tg(7xTCF-Xla. Siam:nlsm Cherry)ia5 larvae treated with 100 nM recombinant leptin for 2 h. (D) 5 dpf CRISPR-mediated leptin receptor-deficient Tg(7xTCF-Xla. Siam:nlsm Cherry)ia5 larvae treated with 10 μM LiCl for 2 h. (E) Fluorescence intensity in the hypothalamus of differentially treated 5 dpf Tg(7xTCF-Xla. Siam:nlsmCherry)ia5 larvae. A–B = p < .05, one-way ANOVA. (F) Fluorescence intensity in the heart of differentially treated 5 dpf Tg(7xTCF-Xla. Siam:nlsmCherry)ia5 larvae. A–B = p < .05, one-way ANOVA
To confirm that Wnt reporter activation is mediated through leptin signaling, we used CRISPR/Cas9 to create lepR crispants that are mosaic for leptin receptor knockout and incubated them with either recombinant leptin (Figure 3C) or 10 μM LiCl (Figure 3D) at 5 dpf. Mosaic knockout of the leptin receptor blocked the ability of leptin (22.23 ± 1.2 AU, p = .571), but not LiCl (40.2 ± 2.2 AU, p < .001), to activate WNT signaling. Quantitation of Wnt reporter read-out in the hypothalamus (Figure 3E), compared with the heart (Figure 3F) determined that leptin activates the Wnt pathway specifically in the hypothalamus. In the hypothalamus, leptin increased fluorescence intensity significantly compared with vehicle-treated larvae (33.52 ± 2.4 vs. 19.22 ± 0.9 AU, p < .001), whereas in the heart leptin did not significantly increase fluorescence intensity (32.2 ± 1.8 vs. 27.3 ± 0.8 AU, p = .091).

### 3.4 | Inhibition of the canonical Wnt pathway blocks the glucoregulatory effect of leptin

To investigate whether leptin regulates glucose homeostasis in the zebrafish, we performed ipGTTs to induce an acute glycemic challenge, and in a separate experiment, we immersed fish in a 1% glucose solution to induce a persistent hyperglycemic challenge. LiCl and leptin treatment reduced the AUC to a similar extent (−20%; p < .05), whereas combined application was not more effective (Figure 4A,B). Under artificially induced hyperglycemia (Figure 4C,J) these effects were replicated with a 10% reduction (LiCl; p < .05), and a 15% reduction (leptin and leptin+LiCl; p < .05) in glucose levels after 3 days. To test whether the glucose-lowering effect of leptin is dependent on Wnt pathway activation, we applied PNU74654 2 h before leptin treatment. PNU74654 pretreatment led to a return of glucose levels to that observed in control conditions. PNU74654 alone, on the contrary, lead to a 28% reduction in AUC (p < .05). During persistent hyperglycemia, Wnt pathway inhibition with PNU74654 did not significantly affect the rise of blood glucose (117 ± 5.7 mg/dl) when compared with control fish (113 ± 4.1 mg/dl). More importantly, however, PNU74654 prevented the ability of leptin to lower blood glucose levels (107 ± 7.9 mg/dl), compared with leptin-treated fish (97 ± 3.1 mg/dl; p < .05; Figure 4F). These findings demonstrate that intact canonical Wnt signaling is required for the ability of leptin to regulate blood glucose levels.

### 3.5 | Activation of the canonical Wnt pathway and glucose-lowering effects of leptin are dependent on a functional leptin receptor

We next investigated whether the glucoregulatory actions of canonical Wnt signaling are dependent on a functional leptin system. We revisited the experimental paradigm described above using lepa<sup>nz301</sup> fish, lepb<sup>nz302</sup> fish, and lep<sup>nz303</sup> fish. Leptin treatment improved glucose tolerance in lepa<sup>nz301</sup> fish (by 30%; p < .05, Figure 5A,D) and lepb<sup>nz302</sup> fish (by 20%; p < .05, Figure 5B,E). In lep<sup>nz303</sup> zebrafish, leptin was unable to improve glucose tolerance with levels identical to the control group (Figure 5C,F). LiCl treatment improved glucose tolerance in all groups (22% for lepa<sup>nz301</sup> fish, 15% for lepb<sup>nz302</sup> fish, and 28% for lep<sup>nz303</sup> fish; p < .05), the glucose-lowering effect of LiCl is mediated distal to the leptin receptor. No additive effect was found between leptin and LiCl.

In accordance, LiCl treatment attenuated persistent hyperglycemia in lepa<sup>nz301</sup> fish (126.0 ± 6.1 vs. 102.0 ± 6.5 mg/dl; Figure 5G), lepb<sup>nz302</sup> fish (121.5 ± 3.8 vs. 95.7 ± 7.8 mg/dl; Figure 5H), and lep<sup>nz303</sup> zebrafish (130.1 ± 6.6 vs. 102.0 ± 6.5 mg/dl; Figure 5I). The effect of LiCl on blood glucose levels appears to be longer lasting than the effect of leptin (Figure 1H–K). LiCl-treated fish had significantly lower blood glucose levels not only immediately after the treatment ended, but on the following day as well (155.8 ± 6.1 vs. 127.5 ± 6.7 mg/dl for lepa<sup>nz301</sup> fish; 146.7 ± 8.2 vs. 122.5 ± 9.4 mg/dl for lepb<sup>nz302</sup> fish; 178.2 ± 5.3 vs. 127.5 ± 7.2 mg/dl for lep<sup>nz303</sup> fish). Again, we did not identify any combined action of leptin or LiCl on glucose homeostasis. Together, these data demonstrate that canonical Wnt pathway activation via LiCl-mediated inhibition of GSK3β regulates glucose homeostasis distal to the leptin receptor.

### 4 | DISCUSSION

Most studies investigating the neuroendocrine regulation of body weight, energy, and glucose homeostasis have been performed in rodents, using powerful genetic tools that have been well-established in, for example, mice. The recent identification of leptin and leptin receptor gene homologs and the invention of the CRISPR-Cas9 gene-editing technology in other vertebrates such as zebrafish...
Activation of canonical Wnt signaling improves glucose tolerance in lepac2301 fish, lepb2302 fish, and lepr2303 zebrafish. (A) Glucose tolerance of adult male lepac2301 zebrafish (n = 6). Fish were treated 10 μM LiCl (3 h before glucose injection), 0.6 g/L of recombinant mouse leptin dissolved in Cortland salt solution (1 h before glucose injection), vehicle only, or a combination of LiCl and leptin. Following 0.5 mg/g glucose injection, blood samples were taken at 30, 90, and 180 min post-injection. (B) Same as (A), but for lepb2302 fish. (C) Same as (A) and (B), but for lepr2303 fish. (D) Area under the curve of (A). **p < .001, one-way ANOVA. (E) Area under the curve of (B). *p < .05, one-way ANOVA. (F) Area under the curve of (C). **p < .001, one-way ANOVA. (G) Blood glucose values of adult male lepac2301 zebrafish (n = 6) over the course of a 4-day immersion in a 1% glucose solution. On the third day, fish were exposed to 10 μM LiCl for three h before daily blood sampling. One hour before blood sampling, fish were injected intraperitoneally with 0.6 g/L of recombinant mouse leptin dissolved in Cortland salt solution, or with vehicle only. *p < .05, repeated-measures ANOVA. (H) Same as (G), but for lepb2302 fish. *p < .05, repeated-measures ANOVA. (I) Same as (G) and (H), but for lepr2303 fish. *p < .05, repeated-measures ANOVA. (J) Blood glucose levels of (G), comparing pre-treatment (day 2) and post-treatment (day 3). (K) Same as (J) but relating to (H). (L) Same as (J) and (K) but relating to (I).
allows a comparative analysis of leptin function. Here, we comprehensively studied which physiological role leptin plays in the zebrafish, a vertebrate animal model belonging to an evolutionarily older animal class than mammals.

While raising these fish, we took great care to prevent any tank density effects on body weight, which have been shown to affect postembryonic development, somatic growth, and fat accumulation. Previous studies on teleost leptin knockout models have yielded contradicting results, with one study convincingly disproving a role for leptin as an adipostat in the zebrafish, whereas others reported an effect of leptin on adipostasis in zebrafish and medaka.

Because of its ability to readily absorb molecules from the surrounding water, compound exposure is relatively straightforward in zebrafish. We took advantage of this by immersing fish in a 1% glucose solution to induce a persistent hyperglycemic challenge (Figure 1). This procedure has previously been shown to induce hyperglycemia over time. In line with previous observations,
glucose levels were significantly elevated after two days of immersion and leptin administration on the third day consistently reduced glucose levels in wild-type, lepa<sup>nz301</sup> fish and lepb<sup>nz302</sup> fish, but not in lepr<sup>nz303</sup> fish, confirming that the glucose-lowering properties of leptin are mediated by the long form of the leptin receptor. This is further substantiated by the fact that glucose levels in the 1% glucose-vehicle treated fish were higher after 3 days of immersion in lepr<sup>nz303</sup> fish compared not only to wild-type fish but also to lepa<sup>nz301</sup> fish and lepb<sup>nz302</sup> fish. The elevated glucose levels in lepr<sup>nz303</sup> fish compared with lepa<sup>nz301</sup> fish and lepb<sup>nz302</sup> fish suggests that loss of only one of the two leptin paralogs is not sufficient to induce hyperglycemia, but the loss of both variants at the leptin receptor level is required to reveal the full phenotype. The fact that recombinant mouse leptin was active in zebrafish suggests that leptin function is highly preserved across species. This finding is in line with other studies that previously demonstrated the anorexigenic effects of recombinant leptin in trout (Oncorhynchus mykiss)<sup>37</sup> and goldfish (Carassius auratus).<sup>38</sup>

Michel et al. demonstrated that leptin regulates glucose homeostasis but not adipostasis in zebrafish. We sought to investigate this further by exploring potential catabolic actions of leptin under overfeeding conditions. It has been shown that zebrafish become obese when they are exposed to an overfeeding regime, and they display metabolic alterations similar to DIO mammals, like hypertriglyceridemia, hepatic steatosis, and systemic inflammation.<sup>39</sup>

Performing GTTs, under normal feeding conditions, we could confirm the results by Michel et al. Knockout of the leptin receptor impaired glucose tolerance but had no effect on body weight regulation in the zebrafish, as neither did knockout of leptin-a or leptin-b. However, this was limited to fish fed normally. Intriguingly, overfeeding elicited an effect of leptin on body weight and standard length. Under these conditions, lepr<sup>nz303</sup> fish had elevated body weight compared with lepb<sup>nz302</sup> fish and wild-type zebrafish. Interestingly, lepa<sup>nz301</sup> fish also showed elevated body weight and standard length, suggesting a specific bodyweight regulatory and somatic effect of leptin-a. In silico binding simulation of zebrafish leptin-a and leptin-b predicts significantly lower binding energy to the leptin receptor for leptin-b.<sup>40</sup> Previous studies point toward a role for leptin-b in tissue regeneration rather than energy homeostasis.<sup>41</sup>

Further studies are required to delineate functional differences between the two leptin paralogues.

Overfeeding per se led to glucose intolerance in fish independent of genotype. This suggests that this treatment maximally impairs the glucoregulatory system as loss of leptin function in addition to overfeeding does not impair glucose tolerance any further. One recent study could show that overfeeding of zebrafish larvae leads to leptin resistance and reduced hypothalamic pomca levels, leading to activation of the melanocortin system, elevation of growth hormone levels, and enhanced somatic growth.<sup>42</sup> Previous studies all point toward conservation of both the intracellular JAK2/STAT3 signaling circuitry,<sup>15,37</sup> as well as the intercellular AgRP and POMC signaling circuitry.<sup>13</sup> We, therefore, envision these same pathways to be responsible for the body mass increase found in zebrafish upon overfeeding. However, our data point towards a fundamentally differential physiological role for leptin in the zebrafish, depending on nutrient availability: Under normal feeding conditions, leptin regulates glucose homeostasis. In times of nutrient excess, on the contrary, an effect of leptin to regulate body weight and somatic growth becomes apparent. From an evolutionary perspective, this suggests that the ancestral function of leptin is as a glucoregulatory hormone and that its adipostatic function in mammals may have been acquired at some point during evolution. Most aquatic species continue to grow somatically throughout life, whereas growth in terrestrial animals usually reaches a plateau due to gravity limitations. Because somatic

![Figure 5](image-url)  
**FIGURE 5** Glucose tolerance in wild-type zebrafish following Wnt pathway manipulation and leptin treatment. (A) Glucose tolerance of adult wild-type male zebrafish (n = 6). Fish were treated 10 μM LiCl (3 h before glucose injection), 0.6 g/L of recombinant mouse leptin dissolved in Cortland salt solution (1 h before glucose injection), vehicle only, or a combination of LiCl and leptin. Following 0.5 mg/g glucose injection, blood samples were taken at 30, 90, and 180 min post-injection. (B) Area under the curve of (A). A–B = p < .05, one-way ANOVA. (C) Blood glucose values of adult wild-type male zebrafish (n = 6) over the course of a 4-day immersion in a 1% glucose solution. On the third day, fish were exposed to 10 μM LiCl for 3 h before daily blood sampling. One hour before blood sampling, fish were injected intraperitoneally with 0.6 mg/L of recombinant mouse leptin dissolved in Cortland salt solution, or with vehicle only. (D) Glucose tolerance of adult wild-type male zebrafish (n = 6). Fish were treated 10 μM PNU74654 (3 h before glucose injection), 0.6 g/L of recombinant mouse leptin dissolved in Cortland salt solution (1 h before glucose injection), vehicle only, or a combination of PNU74654 and leptin. (E) Area under the curve of (D). A–B and A–C = p < .05, one-way ANOVA. (F) Blood glucose values of adult wild-type male zebrafish (n = 6) over the course of a 4-day immersion in a 1% glucose solution with exposure to 10 μM pyrvinium pamoate. (J) Comparison of blood glucose levels in (C), (F), and (I), pre-treatment (day 2), and post-treatment (day 3). *p < .05, repeated-measures ANOVA.
growth limits movement much less in the water than on land, an adipostatic role of leptin may not be as critical as in terrestrial species. This is in line with the recent discovery of the gravitostat in mammals.43 This system has been shown to regulate fat mass in obese mice independently of leptin, whereas leptin-mediated regulation of fat mass seems to be limited to healthy lean mice.44

The canonical Wnt pathway has been shown to be activated by glucose in a macrophage cell line.45 In mice, we demonstrated that Wnt signaling in the hypothalamus is impaired during obesity and reinstated by leptin treatment.20 In the present study, we take advantage of the optical transparency of zebrafish larvae to provide the first in vivo evidence of canonical Wnt pathway activation by leptin. Using LiCl as a positive control, we found that leptin-induced Wnt activation was especially prominent in the hypothalamic region in the brain of zebrafish larvae. Intriguingly, the knockout of the leptin receptor totally abolished this activation, suggesting that leptin activation of the Wnt pathway is solely mediated by the leptin receptor. Leptin receptor expression in the zebrafish is found not only in the hypothalamus, but also in a variety of peripheral organs, including the eye, gut, liver, pancreas, and heart.46 Another region that showed the high intensity of fluorescence after Wnt activation by LiCl was the heart. However, leptin did not significantly induce Wnt fluorescence in the heart.

We could demonstrate that pharmacological inhibition of the Wnt pathway blocks the ability of leptin to lower blood glucose levels both during acute and persistent hyperglycemia, suggesting that leptin regulates glucose homeostasis predominantly via the Wnt pathway. An antidiabetic action upon activation of the Wnt pathway has been confirmed for LiCl treatment, which has been shown to attenuate non-fasting blood glucose levels in diabetic Lepob/ob BTBR T+Itpr3tf/J (BTBR) mice.28 In accordance, we can show that LiCl treatment improves glucose tolerance and normalizes blood glucose levels during a persistent hyperglycemic challenge in zebrafish. Activation of the Wnt pathway with LiCl improved glucose homeostasis, even in leptin- or leptin receptor-deficient fish, suggesting that LiCl acts independently of leptin and that leptin acts upstream of the canonical Wnt signaling cascade. We could previously show that leptin induces phosphorylation of LRP6 in the arcuate nucleus of the Djungarian hamster (Phodopus sungorus).47 Together, these data provide solid evidence that the Wnt pathway is a newly identified leptin signal transduction pathway and that leptin regulates glucose homeostasis through this pathway.

The ability of Wnt signaling to regulate blood glucose levels is often ascribed to GSK3β being a site of convergence between canonical Wnt signaling and insulin signaling.22 We inhibited the Wnt pathway both upstream (using pyrvinium pamoate) and downstream (using PNU74654) of GSK3β, yet both manipulations impaired glucose tolerance and blocked the glucoregulatory effect of leptin. Oncological studies suggest that insulin and the Wnt ligands regulate GSK3β via different mechanisms, thus leading to distinct downstream cellular events. For example, in a number of cancer cell lines, a 2-h treatment with insulin leads to increased Ser9 GSK3β phosphorylation and decreased GSK3β enzymatic activity, but free cytoplasmic β-catenin levels remained unchanged.47 Wnt ligands and LiCl on the contrary induced accumulation of free cytoplasmic β-catenin but had no effect on GSK3β phosphorylation. Other studies have found that activation of Wnt target genes can be induced by IGF-1 and insulin.48

Genome-wide association studies indicated the Wnt transcription factor TCF7l2 to be the highest genetic risk factor for developing T2DM.23 Curiously, subsequent studies investigating the link between Wnt signaling and T2DM focused largely on the insulin-producing pancreatic β-cells, despite evidence that canonical Wnt signaling is not active in adult β-cells of mice49 and zebrafish.50 Our data suggest that the association between TCF7l2 and T2DM is mediated through the leptin system.

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DISCLOSURES
The authors have no conflict of interest to declare.

ETHICS STATEMENT
Procedures involving animals were performed in accordance with national animal ethics legislation and received approval by University of Otago Animal Ethics Committee.

AUTHOR CONTRIBUTIONS
Kaj Kamstra, Julia A. Horsfield, and Alexander Tups designed research. Kaj Kamstra and Mohammed Z. Rizwan performed research. Kaj Kamstra, Mohammed Z. Rizwan, and Alexander Tups analyzed data. Kaj Kamstra, Mohammed Z. Rizwan, David R. Grattan, Julia A. Horsfield, and Alexander Tups wrote the paper. All authors have approved the final version.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author, A.T., upon request.

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REFERENCES

1. Pelleymounter MA, Cullen MJ, Baker MB, et al. Effects of the obese gene product on body weight regulation in ob/ob mice. Science (New York, NY). 1995;269(5223):540-543.
2. Muzzin P, Eisensmith RC, Copeland KC, Woo SL. Correction of obesity and diabetes in genetically obese mice by leptin gene therapy. Proc Natl Acad Sci USA. 1996;93(25):14804-14808.
3. Schwartz MW, Baskin DG, Bukowski TR, et al. Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. Diabetes. 1996;45(4):531-535.
4. Farooqi IS, Jebb SA, Langmack G, et al. Effects of recombinant leptin therapy in a child with congenital leptin deficiency. New Engl J Med. 1999;341(12):879-884.
5. Yu X, Park BH, Wang MY, Wang ZV, Unger RR. Making MG Jr, Schwartz MW. Intracellular signalling. Key enzyme in leptin-induced anorexia. Science (New York, NY). 1996;274(5293):1358-1360.
6. Hedbacker K, Birsoy K, Wysocki RW, et al. Antidiabetic effects of IGFBP2, a leptin-regulated gene. Cell Metab. 2010;11(1):11-22.
7. Koch C, Augustine RA, Steger J, et al. Leptin rapidly improves glucose homeostasis in ob/ob mice by increasing hypothalamic insulin sensitivity. J Neurosci. 2010;30(48):16180-16187.
8. Genuith SM, Przybylski RJ, Rosenberg DM. Insulin resistance in genetically obese, hyperglycemic mice. Endocrinology. 1971;88(5):1230-1238.
9. Oral EA, Simha V, Ruiz E, et al. Leptin replacement therapy for insulin deficiency type 1 diabetic rodents thrive without insulin. Proc Natl Acad Sci USA. 2008;105(37):14070-14075.
10. Hedbacker K, Birsoy K, Wysocki RW, et al. Antidiabetic effects of IGFBP2, a leptin-regulated gene. Cell Metab. 2010;11(1):11-22.
11. Wang MY, Chen L, Clark GO, et al. Leptin therapy in a child with congenital leptin deficiency. New Engl J Med. 1999;341(12):879-884.
12. Rajan A, Perrimon N. Drosophila cytokine unpaired 2 regulates physiological homeostasis in ob/ob mice by increasing hypothalamic insulin sensitivity. J Neurosci. 2010;30(48):16180-16187.
13. Benzler J, Andrews ZB, Pracht C, et al. Hypothalamic WNT signalling is impaired during obesity and reinitiated by leptin treatment in male mice. Endocrinology. 2013;154(12):4737-4745.
14. Boucsein A, Benzler J, Hempp C, Stohr S, Helfer G, Tups A. Photoperiodic and diurnal regulation of WNT signaling in the arcuate nucleus of the female Djungarian hamster, Phodopus sungorus. Endocrinology. 2016;157(2):799-809.
15. Helfer G, Tups A. Hypothalamic Wnt signalling and its role in energy balance regulation. J Neuroendocrinol. 2016;28(3):12368.
16. Grant SFA, Thorleifsson G, Reynisdottir I, et al. Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. Nat Genet. 2006;38(3):320-323.
17. Nikouliina SE, Claraldi TP, Mudaliar S, Carter L, Johnson K, Henry RR. Inhibition of glycogen synthase kinase 3 promotes insulin action and glucose metabolism in human skeletal muscle. Diabetes. 2002;51(7):2190-2198.
18. Benzler J, Ganjgama K, Kruger M, et al. Hypothalamic glycogen synthase kinase 3β has a central role in the regulation of food intake and glucose metabolism. Biochem J. 2012;447(1):175-184.
19. Varshney GK, Carrington B, Pei W, et al. A high-throughput functional genomics workflow based on CRISPR/Cas9-mediated targeted mutagenesis in zebrafish. Nat Protoc. 2016;11(12):2357-2375.
20. Capiotti KM, Antonioli R Jr, Kist IW, Bogo MR, Bonan CD, Da Silva RS. Persistent impaired glucose metabolism in a zebrafish hyperglycaemia model. Comp Biochem Physiol B: Biochem Mol Biol. 2014;171:58-65.
21. Gleeson M, Connaughton V, Arneson LS. Induction of hyperglycaemia in zebrafish (Danio rerio) leads to morphological changes in the retina. Acta Diabetol. 2007;44(3):157-163.
22. Shaw RJ, Lamia KA, Vasquez D, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science (New York, NY). 2005;310(5754):1642-1646.
23. Moro E, Ozhan-Kizil G, Mongera A, et al. In vivo Wnt signaling tracing through a transgenic biosensor fish reveals novel activity domains. Dev Biol. 2012;366(2):327-340.
24. Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci USA. 1996;93(16):8455-8459.
25. Thorne CA, Hanson AJ, Schneider J, et al. Small-molecule inhibition of Wnt signaling through activation of casein kinase 1α. Nat Chem Biol. 2010;6(11):829-836.
26. Farooqi IS, Jebb SA, Langmack G, et al. Effects of recombinant leptin therapy in a child with congenital leptin deficiency. New Engl J Med. 1999;341(12):879-884.
27. Rajan A, Perrimon N. Drosophila cytokine unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion. Cell. 2012;151(1):123-137.
28. Denver RJ, Bonett RM, Boorse GC. Evolution of leptin structure and function. Neuroendocrinology. 2011;94(1):21-38.
29. Gorissen M, Bernier NJ, Naboors SB, Flik G, Huising MO. Two divergent lep tin paralogues in zebrafish (Danio rerio) that originate early in teleostean evolution. J Endocrinol. 2009;201(3):329-339.
30. Londraville RL, Prokop JW, Duff RJ, Liu Q, Tuttle M. On the molecular evolution of leptin, leptin receptor, and endospanin. Front Endocrinol. 2017;8:58.
31. Michel M, Page-McCaw PS, Chen W, Cone RD. Leptin signaling regulates glucose homeostasis, but not adipostasis, in the zebrafish. Proc Natl Acad Sci USA. 2016;113(11):3084-3089.
32. Hübschle T, Thom E, Watson A, Roth J, Klaus S, Meyerhof W. Leptin-induced nuclear translocation of STAT3 immunoreactivity in hypothalamic nuclei involved in body weight regulation. J Neurosci. 2001;21(7):2413-2424.
33. Bjorbaek C, El-Haschimi K, Frantz JD, Flier JS. The role of SOCS-3 in leptin signaling and leptin resistance. J Biol Chem. 1999;274(42):30059-30065.
34. Niswender KD, Morton GI, Stearns WH, Rhodes CJ, Myers MG Jr, Schwartz MW. Intracellular signalling. Key enzyme in leptin-induced anorexia. Nature. 2001;413(6858):794-795.
intake and stage specific effects on growth and fat allocation. *Gen Comp Endocrinol*. 2013;195:9-20.

37. Aguilar AJ, Conde-Sieira M, Polakof S, Miguez JM, Soengas JL. Central leptin treatment modulates brain glucosensing function and peripheral energy metabolism of rainbow trout. *Peptides*. 2010;31(6):1044-1054.

38. de Pedro N, Martinez-Alvarez R, Delgado MJ. Acute and chronic leptin reduces food intake and body weight in goldfish (*Carassius auratus*). *J Endocrinol*. 2006;188(3):513-520.

39. Oka T, Nishimura Y, Zang L, et al. Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. *BMC Physiol*. 2010;10:21.

40. Prokop JW, Duff RJ, Ball HC, Copeland DL, Londraville RL. Leptin and leptin receptor: analysis of a structure to function relationship in interaction and evolution from humans to fish. *Peptides*. 2012;38(2):326-336.

41. Kang J, Hu J, Karra R, et al. Modulation of tissue repair by regeneration enhancer elements. *Nature*. 2016;532(7598):201-206.

42. Löhr H, Hess S, Pereira MMA, et al. Diet-induced growth is regulated via acquired leptin resistance and engages a Pomc-somatostatin-growth hormone circuit. *Cell Rep*. 2018;23(6):1728-1741.

43. Jansson J-O, Palsdottir V, Hägg DA, et al. Body weight homeostat that regulates fat mass independently of leptin in rats and mice. *Proc Natl Acad Sci USA*. 2018;115(2):427-432.

44. Ohlsson C, Hägg DA, Hammarhjelm F, et al. The gravitostat regulates fat mass in obese male mice while leptin regulates fat mass in lean male mice. *Endocrinology*. 2018;159(7):2676-2682.

45. Anagnostou SH, Shepherd PR. Glucose induces an autocrine activation of the Wnt/beta-catenin pathway in macrophage cell lines. *Biochem J*. 2008;416(2):211-218.

46. Liu Q, Chen Y, Copeland D, et al. Expression of leptin receptor gene in developing and adult zebrafish. *Gen Comp Endocrinol*. 2010;166(2):346-355.

47. Ding VW, Chen RH, McCormick F. Differential regulation of glycogen synthase kinase 3beta by insulin and Wnt signaling. *J Biol Chem*. 2000;275(42):32475-32481.

48. Desbois-Mouthon C, Cadoret A, Blivet-Van Eggelpoël MJ, et al. Insulin and IGF-1 stimulate the beta-catenin pathway through two signalling cascades involving GSK-3beta inhibition and Ras activation. *Oncogene*. 2001;20(2):252-259.

49. Krutzfeldt J, Stoffel M. Regulation of wingless-type MMTV integration site family (WNT) signalling in pancreatic islets from wild-type and obese mice. *Diabetologia*. 2010;53(1):123-127.

50. Facchinello N, Tarifeño-Saldívar E, Grisan E, et al. Tcf7l2 plays pleiotropic roles in the control of glucose homeostasis, pancreas morphology, vascularization and regeneration. *Sci Rep*. 2017;7(1):9605.

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