The molecular basis of metabolic syndrome: studies in zebrafish

Nowik, N.

Chapter 4

Leptin is essential for insulin signaling during zebrafish embryogenesis

Natalia Nowik, Kongju Zhu, Yi Ding,

Fons Verbeek, Herman P. Spaink

Abstract

Leptin has been extensively studied in obesity and type 2 diabetes and has been shown to play a key role in whole-body energy homeostasis, however, its function in insulin signaling is still poorly understood. In this study, we show that leptin deficient zebrafish mutant embryos and larvae appear to be totally insulin resistant and show a diabetic phenotype at all stages of embryogenesis and larval development. This phenotype can be reversed by the injection of human leptin. Both metformin and the phosphatase inhibitor NSC-87877 are able to reverse this diabetic phenotype of the zebrafish larvae at the larval stage. In contrast to metformin, NSC-87877 was also active at early embryonic stages. Gene knockdown studies in the leptin mutant background indicate that the results are translatable to *Xenopus laevis* embryos and that the small non-receptor tyrosine phosphatase Ptpn6 is the most likely target responsible for the antidiabetic effect of NSC-87877.

Introduction

The metabolic syndrome is currently an increasing worldwide problem, affecting 425 million people of the world population (IDF Diabetes Atlas, 8th edn.). It includes type 2 diabetes mellitus (T2DM), which is increasing among the world population, reaching currently a pandemic form[1,2]. Current treatment methods of diabetes type 2 are still limited and fall in three categories, of which the insulin sensitizers such as metformin that remedy insulin resistance, the primary hallmark of diabetes 2, are the best studied and widely used[3]. Research on insulin resistance and type 2 diabetes has used human cell cultures and various animal models, mainly rodents. The mutant mice strains of the leptin and leptin receptor genes, the ob/ob, db/db strains, respectively have diabetic phenotypes and are therefore highly useful for diabetes research[4]. In addition, high fat diet mice, Zucker fatty rats and ZDF rats, have been widely used to study T2DM, obesity and the function of leptin signaling in metabolic syndrome[5,6].

Leptin is a cytokine produced mainly by mature adipocytes in white adipose tissue. In the brain it regulates food intake, appetite behavior and energy expenditure. Leptin mutations in rodents lead to hyper obesity and other abnormalities, which are described as main factors that influence development of diabetic symptoms[4]. The molecular mechanisms by which leptin controls insulin resistance in various target tissues are largely unknown[7]. Leptin's function is correlated with proteins tyrosine phosphatases that are key regulatory factors in many signal transduction pathways underlying vertebrate development[8]. Protein tyrosine phosphatase 1B (PTP1B) has emerged as a novel promising therapeutic target for the treatment of T2DM, as it plays an important role in the negative regulation of insulin signal transduction pathways[9]. Moreover, the expression of hypothalamic PTP1B is upregulated in leptin resistant animals[10]. It was recently shown that inhibition of low-molecular-weight

tyrosine phosphatase (LMPTP) in rodents results in attenuation of high-fat diet-induced diabetes[11].

In adult zebrafish leptin receptor and leptin genes have shown to have a conserved role in glucose homeostasis but, does not appear to play a role in adipose tissue homeostasis[12]. Zebrafish models have been proposed as alternative test systems for studying insulin resistance and T2DM that gives several opportunities to explore metabolic diseases, using numerous transgenic and knockout lines[13]. Although there are already established diabetic adult zebrafish models, which are based on a high fat feeding system[12,14], there is still lack of alternative early stages larval models, which provide the opportunity to perform fast and large scale screening assays, shortly after fertilization. As shown by Marin Juez *et al.* (2014)[15] zebrafish larvae are highly suited to study insulin resistance and are, therefore, a promising model system to study T2DM in a non-feeding situation. Marin Juez *et al.*, identified Shp-1 gene, also called *ptpn6*, in zebrafish larvae as a key factor in insulin resistance[15]. In this study we analyze the function of leptin and *ptpn6* in insulin resistance in zebrafish larvae. We have developed a novel high throughput method to test anti-diabetic drugs based on the fact that leptin deficient zebrafish larvae are totally insulin resistant and show as a result a diabetic phenotype already at very early stages of embryonic development. We show that metformin is highly effective for treating this diabetic phenotype in 4 days old zebrafish larvae and, using our high throughput test system, we have identified also the phosphatase inhibitor NSC-87877 as an alternative anti-diabetic drug that shows anti-diabetic effects at much earlier time points of development. Gene knockdown studies in the leptin mutant background indicate that Shp-1 is the most likely target responsible for the antidiabetic effect of NSC-8787.

Materials and methods

Zebrafish husbandry

Zebrafish lines were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (zfin.org). The breeding of adult fish was approved by the local animal welfare committee (DEC) of the University of Leiden (license number: 10612) and adhered to the international guidelines specified by the EU Animal Protection Directive 2010/63/EU. Adult zebrafish were not sacrificed for this study. All experiments in this study were performed on embryos/larvae before the free-feeding stage and did not fall under animal experimentation law according to the EU Animal Protection Directive 2010/63/EU.

Fish lines used in this work were the following: wild-type (WT) strain AB/TL, homozygous mutant (*lepb*$^{-/-}$) and WT siblings (*lepb*$^{+/+}$). Homozygous F1 carriers were outcrossed once against wild-type, and were subsequently incrossed, resulting in *lepb*$^{-/-}$ and *lepb*$^{+/+}$ siblings

that were used for experiments. For genotyping, genomic DNA was amplified using forward primer 5'-GAGACTCTCCTGAGGACACTGG-3' and reverse primer 5'-GCATGGCTTACACATTTCAGAG-3', amplifying a 201 base pair (bp) product containing the mutation, which can be detected using 2% agarose gel. Embryos were grown at 28.5 °C in egg water (60 µg/ml sea salt, Sera marin, Heinsberg, Germany). For live-imaging or injection assays, larvae were anesthetized in egg water medium containing 0.02% buffered Tricaine (3-aminobenzoic acid ethyl ester; Sigma-Aldrich, St Louis, MO, USA).

Insulin injection

1 nl of 100 nM human recombinant insulin (Sigma–Aldrich, the Netherlands) was injected into the caudal aorta of 4 days post fertilization (dpf) zebrafish larvae using a glass capillary as described in Juez *et al.*, 2014. 1 nl of PBS was used as a control injection.

Glucose treatment

Zebrafish larvae at 4 dpf were placed in 12 well plates (10 embryo per well) and immersed for two hours in 4 mL egg water, containing 250 mM of glucose (Sigma, USA, CAS. No. 50-99-7). After immersion first group was washed three times with egg water and collected for measurements, the rest were exposed to clean egg medium. Samples were taken after 120 min and after 240 min of washing period. As a control, larvae were exposed to mannitol (250 mM; Sigma, USA, CAS No. 69-65-8), instead of glucose, under the same conditions.

***In Vivo* Glucose Uptake Assay**

Lepb$^{+/+}$ and *lepb*$^{-/-}$ mutants were injected in the yolk with 2.5 mg/mL 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) at 4 hours post fertilization (hpf) and at 24 hpf, a fluorescent glucose analog (Life Technologies, Leiden, Netherlands), and incubated at 28.5 °C for 30-60 minutes. At the termination of the incubation period, seven embryos per condition from 1 day old group were anesthetized with 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma-Aldrich), both groups were analyzed under a fluorescence stereomicroscope and a confocal microscope.

Metformin treatment

Lepb$^{+/+}$ and *lepb*$^{-/-}$ mutants, used for the ELISA assays, were treated with 10 µM metformin Cayman Chemicals, Ann Arbor, MI, USA) added to egg water containing DMSO from 3 dpf for 24 hours, as the control group, larvae were incubated only in water with DMSO. Embryos used for the fluorescent glucose assay received 10 µM metformin at 2 hpf under the egg chorion or into the yolk and the second dose together with fluorescent glucose injection at 24 hpf.

NSC-87877 treatment

Lepb$^{+/+}$ and *lepb*$^{-/-}$ mutants were incubated from 3dpf for 24 hours in egg water that contained NSC-87877 and DMSO, as the control group, larvae were incubated, during the same period of time, only in water with DMSO. Embryos used for the fluorescent glucose assay received 10 µM NSC-87877 at 2 hpf under the egg chorion or into the yolk and the second dose together with fluorescent glucose injection into the yolk at 24 hpf, 4 hpf and 6 hpf.

Glucose measurements

Quantitative analysis of glucose levels was performed from whole body lysates using a glucose assay kit (Cayman Chemical, Ann Arbor, MI, USA). Briefly, 7 zebrafish larvae in each experimental group were sonicated in 30 µL Assay Buffer on ice. According to the instructions, standard curves were generated using glucose standard solution. A total of 25 µL assay Enzyme Mix (Cayman Chemical, Ann Arbor, MI, USA) was added and incubated for 10 min at 37°C. Fluorescence (514 nm) was measured using a BioTek plate reader equipped with GEN 5 software (v.2.04, BioTek, Winooski, VT, USA).

Morpholino injections

For knockdown of particular genes, morpholino oligonucleotides (Gene Tools, LLC, Philomath, OR, USA) were injected into 1-cell zebrafish embryo. The morpholinos were diluted to a concentration of 0.5 mM in 1× Danieau's buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, and 5.0 mM HEPES (pH 7.6)) and 1 nL was injected using a Femtojet injector (Eppendorf, Hamburg, Germany). The *ptpn6* morpholino was used at a concentration of 0.08 mM as published previously by Juez *et al.*, 2014. Morpholino sequences are presented in Supplementary table 1.

CRISPR/Cas9 mutagenesis

Site-specific CRISPR-Cas9 sgRNAs (actatagGGGGTCTCGGGATTGGGTAGgttttag) were generated using the online software CHOPCHOP according to Montague *et al*., 2014[16]. *Lepb*$^{-/-}$ mutant fish were generated using CRISPR-mediated gene knockout approach and described previously[17]. As described in Suppl.fig.1 two different deletion mutants were generated. However, in most experiments, larvae with a heterozygote combination of the two mutations were used because the fact that there are two mutations was discovered later than the experiments were performed.

Frog husbandry and microinjection

All frog (*Xenopus laevis*) procedures and care were approved by the animal experiments committee (dierexperimentencommissie, DEC) of Leiden university. Frog embryos were collected by natural mating of wild-type females with males. For morpholino injection, morpholinos against the long-form and the short-form (Suppl.tabel 1) transcripts of leptin were mixed together. 30 ng of each was then injected into the blastomeres of the embryo at 2-cell or 4-cell stage. After injection, the embryos were cultured to stage 26 (staged according to Nieuwkoop and Faber) for glucose injection. For rescue experiments, 10 µM NSC-87877 was co-injected with the two morpholinos at 2-cell or 4-cell stage.

Images quantification

Bright-field images were obtained using Leica M165C stereomicroscope equipped with a DFC420C digital color camera (Leica Microsystems, Wetzlar, Germany). For fluorescent image acquisition, Leica MZ16FA stereo fluorescence microscope equipped with a DFC420C digital color camera (Leica Microsystems, Wetzlar, Germany) with GFP filter settings. For confocal laser scanning microscopy (CLSM) we used a Leica TCS SPE (Leica Microsystems, Wetzlar, Germany). For each larva a bright field image and a fluorescent channel image were obtained. In 24 hours post fertilization analyses of the glucose values first the overall shape of the larva was extracted from the bright field channel – this gives the total area. The yolk and yolk-extension were extracted from the fluorescent channel as these had the highest fluorescence, from this the surface areas for yolk and yolk-extension were established. The body of the larva could be found by excluding the area of yolk and yolk-extension and within the body the otic vesicle is taken as a boundary for the head. In this manner the body is divided in areas for each of which the surface area was computed, and which sum to the total surface. The area of the head was determined from the bright field image and was used as a mask in the fluorescent channel image to obtain the fluorescence for only the head area. This was expressed as a numerical density, that was the sum of the total fluorescence in the head area divided by the surface area of the head. The images and computations were corrected for a specific background fluorescence.

To estimate the fluorescence ratio between zygotic cell mass and yolk in early zebrafish embryos using wide field stereo microscopy, a square of 4 micrometer in center of these two parts of the embryos was quantified for fluorescence intensity. Ratio between the two blocks was measured using pixel counting software as described in Stoop *et al*. (2011)[18]. In *Xenopus laevis* embryos we compared the total fluorescence in the larvae to the fluorescence at the injection site that was arbitrarily defined as a square of 10 micrometer. To measure the fluorescence ratio between cell mass and yolk in early zebrafish embryos with CLSM we measured the total volume of these parts of the embryo and quantified the fluorescence in these parts using FIJI software.

Statistical analyses

Statistical differences were analyzed with Prism 6.0 (GraphPad Software, San Diego, CA, USA) using t-test for comparisons between two groups and one-way ANOVA (with Tukey's post hoc test correction) for multiple group comparisons and considered to be significant at $P<0.05$.

Results

General characterization of leptin b mutant zebrafish larvae

The zebrafish genome contains two leptin genes, *lepa* and *lepb*, and one leptin receptor gene[19] which have been previously studied by gene knock down and knock out studies[20,12]. Since pilot morpholino studies indicated a possible function of *lepb* in glucose transport of zebrafish larvae (Suppl.fig.2), the CRISPR/CAS9 gene editing tool was used to generate a *lepb* knock out zebrafish mutant line. The sgRNA was designed to target exon 2 of the *lepb* gene where the target site was located (Suppl.fig.1A). This resulted in two different mutations of 7 and b bp respectively, both predicted to lead to frameshift mutations (Suppl.fig.1A). Adult F0 fish from sgRNA injections were incrossed to obtain the F1 generation, where germline transmission of mutant alleles was confirmed by genotyping of its offspring. After outcrossing with the wild type line, and two incrosses we selected knock out mutant lines which were used for this research. Two groups of $lepb^{-/-}$ and $lepb^{+/+}$ larvae from the third generation were compared under normal embryo raising conditions to test for differences in unchallenged survival during development (data not shown). The $lepb^{-/-}$ mutants reached adulthood in a normal time span leading to adults with a normal fertility rate at 4 month post fertilization (data not shown).

Mutation of the *lepb* gene causes insulin resistance in the larval stage

To study how the $lepb^{-/-}$ zebrafish mutant responds to hyperinsulinemia, we injected insulin into the caudal aorta of a zebrafish larvae at 4 dpf. Glucose measurements were performed at 0, 30, and 240 minutes after the injection (Fig.1A). The results (Fig.1B) show a significant decrease in glucose level after insulin injection in wild type fish, whereas the glucose level rather increases after insulin administration in the mutant. Moreover, glucose basal levels at the first time point were much higher than in the wild type controls. These results indicate that the $lepb^{-/-}$ mutant is insulin resistant even prior to 4 dpf.

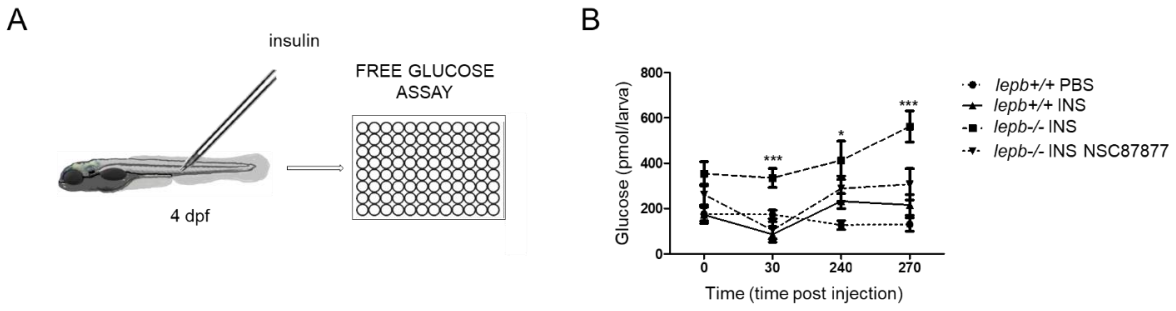


Figure 1. Insulin sensitivity and glucose levels of lepb mutant larvae at 4 dpf. (A) Insulin injection: Zebrafish larvae at 4 dpf receive 1nl of human recombinant insulin or PBS, into the caudal vein. The samples are collected at 0, 30 and 240 min post injections, to measure free insulin glucose level in the body, using ELISA Glucose Assay Kit. **(B)** Results of human recombinant insulin (INS) injection. Data, mean ± s.e.m, are combined from five biological replicates *P<0.05, ***P<0.001

We used the diabetic phenotype of our *lepb*$^{-/-}$ mutant to establish a method that corresponds to Oral Glucose Tolerance Test (OGTT) used in mice[21], to rapidly analyze glucose metabolism without injection procedures and stress-inducing anesthetic treatment. In this method, similar to the glucose tolerance test applied in mice, 4 dpf zebrafish larvae were immersed in egg water containing a 250 mM glucose concentration for two hours. Afterwards the larvae were incubated for 4 hours in glucose-free medium. The non-metabolizable compound mannitol was used as a control for osmotic effects. Samples were taken at 0, 120 and 240 minutes after washing by immediate homogenization of whole larvae in the buffer (Fig.2A). The results of the glucose measurement showed that in the control group, free glucose concentrations reached the basal level after 240 minutes post washing. In contrast, glucose levels remained at very high levels in the *lepb*$^{-/-}$ mutant after the washing step. These results show that the rapid glucose bathing method is highly efficient to demonstrate the diabetic characteristics of fish larvae at 4 days post fertilization (Fig.2B).

In order to further study glucose metabolism in the *lepb*$^{-/-}$ larvae, we used a previously published method based on the injection of 2-NBDG, a fluorescently labeled glucose analog in the yolk at 24 hours post fertilization embryos[22] (Fig. 3A). In agreement with this publication, we observed that in wild type larvae the fluorescent glucose is rapidly transported into the tissues of the embryo, with the brain as the most prominent destination (Fig. 3B). In contrast, in *lepb*$^{-/-}$ mutants there is no observable glucose uptake from injected yolk, where all the injected glucose remains.

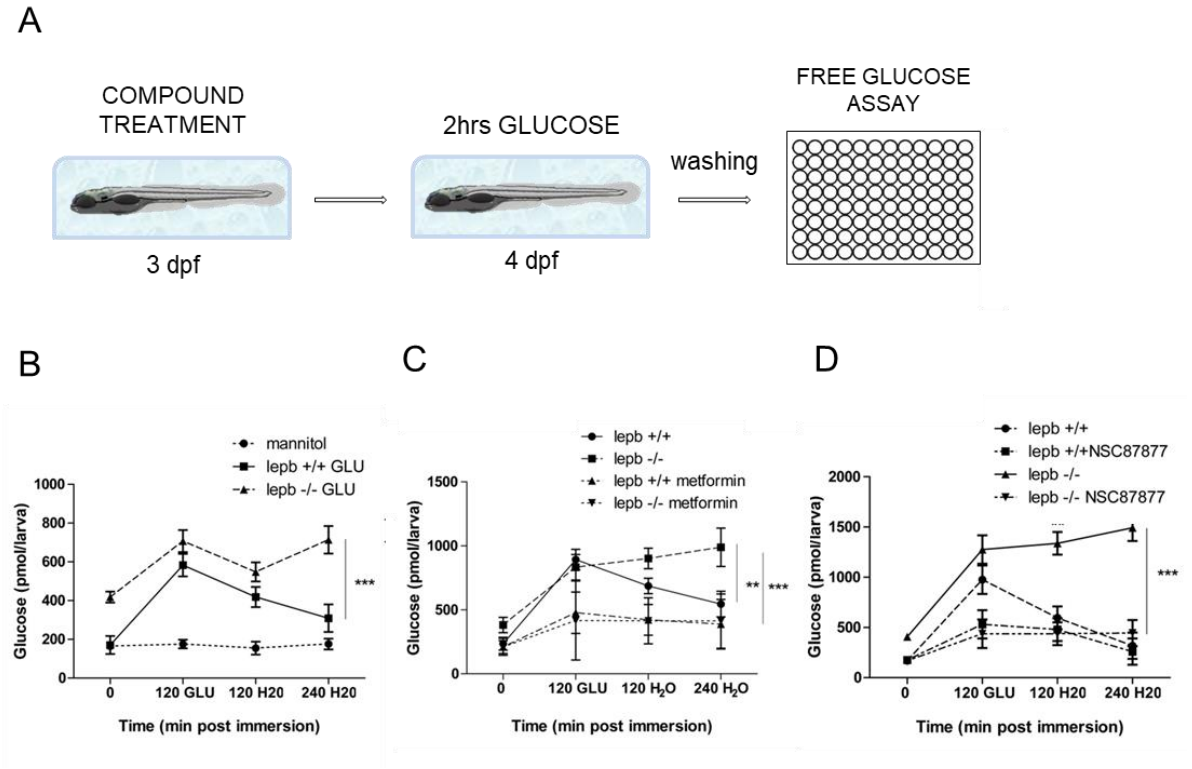
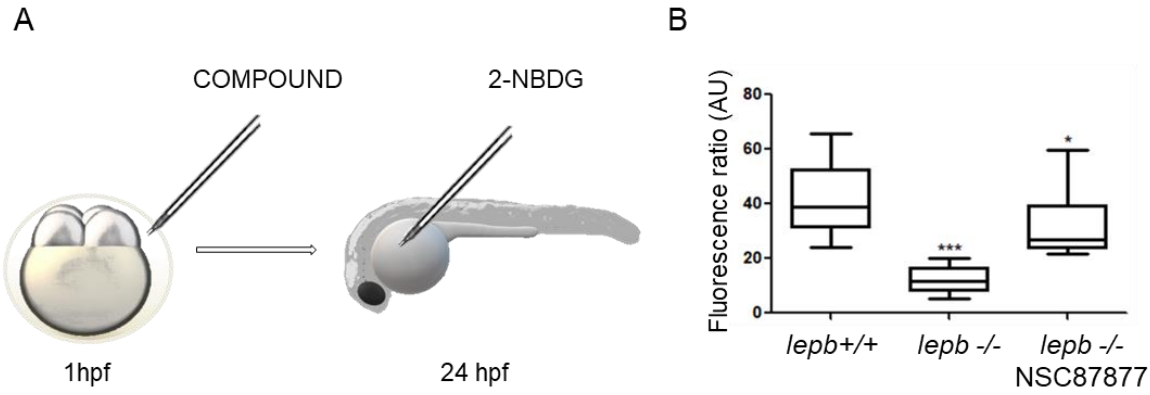


Figure 2. Glucose immersion. (A) Zebrafish larvae at 4 dpf are immersed in a medium containing 250 mM of glucose or mannitol. The samples are collected after 0 min,120 min of glucose immersion and 120 min and 240 min of washing in clean egg water, to measure free glucose level in the body, using ELISA Glucose Assay Kit. It is an alternative and less invasive method of testing insulin resistance in zebrafish larvae. **(B)** Glucose levels were determined after immersion in glucose using the ELISA method from Fig.7A **(C)** The effect of metformin on glucose levels **(D)** The effect of NSC-87877 on glucose levels. Data, mean ± s.e.m, are combined from five biological replicates (n=10 larvae/group), significance was measure between *lepb*$^{+/+}$ and *lepb*$^{-/-}$ groups unless indicated differently. **P<0.01, ***P<0.001

In conclusion, glucose transport in the *lepb*$^{-/-}$ mutant is completely blocked already at 24 hours post fertilization (Fig.3B).

Drug treatments of the *lepb*$^{-/-}$ mutant.

We have used our developed methods for measuring glucose uptake to test the effect of the antidiabetic drug metformin. Metformin was added to control and *lepb*$^{-/-}$ fish at 3 dpf and the glucose bathing assay was performed 24 hours later (Fig.2A). The results show that metformin at a concentration of 10 µM was highly effective in reverting the *lepb*$^{-/-}$ diabetic phenotype to the wild-type phenotype (Fig.2C). However, using the fluorescent glucose injection method we observed only a marginal effect of metformin at 24 hpf (Suppl.fig.3).



C

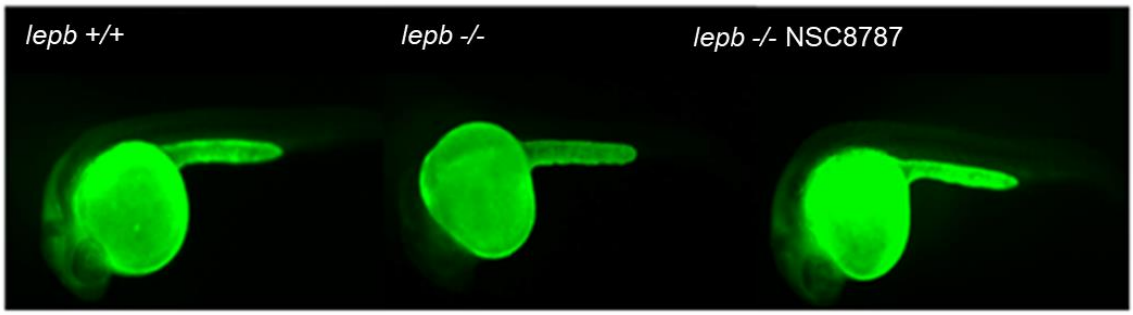


Figure 3. Quantification of glucose distribution in the brain after NSC-87877 treatment. (A) Injection of 2-NBDG in 24 hours embryos after NSC-87877 treatment **(B)** Glucose transport to the brain is decreased in *lepb*$^{-/-}$ mutant (**C)** Images were obtained by stereo fluorescence microscopy unless indicated otherwise. Data, mean ± s.e.m., are combined from three biological replicates (n=10 larvae/group). *P<0.05, ***P<0.001

We also tested other putative anti-diabetic drugs based on the published role of small non-receptor tyrosine phosphatases in insulin resistance[23]. One of the tested compounds, NSC-87877 has been published to inhibit Shp-2 and Shp-1[24,26] and also other targets such as the dual specificity phosphatase DUSP26[25,26]. NSC-87877 showed to be able to completely revert the diabetic phenotype of the *lepb*$^{-/-}$ at 4 dpf (Fig.2D). NSC-87877 was also able to significantly revert the glucose uptake deficiency at 24 hpf in the fluorescent glucose assay (Fig.3B,C). Moreover, we tested the effect of NSC-87877 on free glucose levels after insulin injection. The data show that NSC-87877 treatment of the *lepb*$^{-/-}$ mutant restores insulin sensitivity (Fig.1B).

***Lepb* controls glucose transport and insulin resistance during the early embryonic stages.**

Motivated by our results in the early larval stage we tested the function of the *lepb* gene during early embryonic stages. We found that knockout of *lepb* completely inhibited glucose transport between yolk and the developing zygotic cells even at very early stages, namely after 4 and 6 hpf. However, at earlier stages than the 64 cell stage, glucose transport was not influenced by the *lepb* mutation (data not shown). Moreover, we found that NSC-87877, injected under the chorion or in the yolk (Fig.4A), partially reversed glucose transport

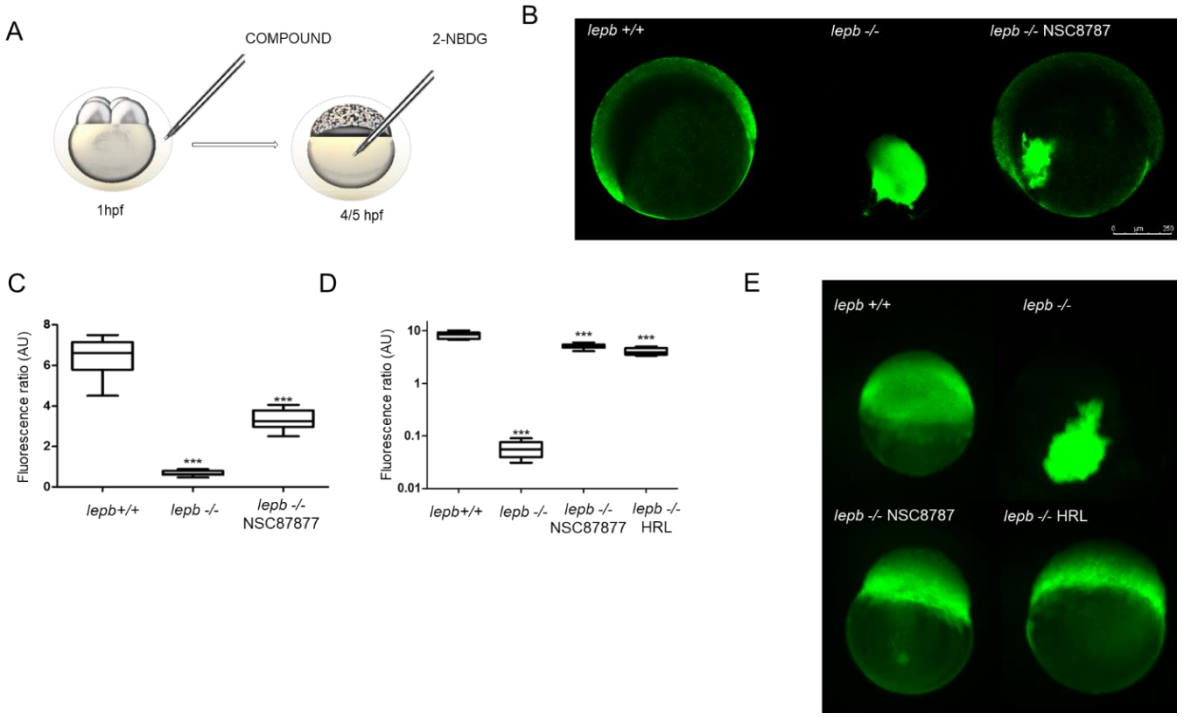


Figure 4. Early glucose injections. (A) Fluorescent glucose injection at 4-6 hpf: Drugs have been injected into the yolk of zebrafish embryos at early stages of embryogenesis (1 hpf). In the case of the 6 hpf analyses we made use of CLSM to distinguish the zygotic cells from the yolk. At 4 hpf stereo fluorescence microscopy was sufficient to discern the yolk from the zygotic cell mass. **(B, D)** Early stages (6 hpf) 2-NBDG injections according to method A. NSC-87877 was injected through the chorion or into the yolk sac at 1 hpf. Use was made of CLSM to discern the yolk form the zygotic cell mass. **(C, E)** Early stages (4 hpf) 2-NBDG injections according to method A. NSC-87877 or recombinant human leptin protein (HRL) was injected into the yolk sac at 2 hpf. Data, mean ± s.e.m., are combined from three biological replicates (n=10 larvae/group). ***P<0.001

inhibition at 4 and 8 hpf (Fig.4B,C). Interestingly, recombinant human leptin was also able to rescue glucose transport in the mutant (Fig.4D,E). In order to test whether the defect in glucose transport in early embryogenesis was related to insulin resistance we developed an assay for testing the effect of insulin at 4 hpf. This assay is based on the injection in the yolk of 1 nl glucose solution of 200 mg/ml in the yolk in the presence of the standard concentration of 2-NBDG (Fig.5A). In wild type embryos transport of the fluorescence glucose derivative is no longer observed due to competition with unlabeled glucose.

The apparent limitation of the glucose transport capacity at this glucose concentration could be overcome by the co-injection of human recombinant insulin showing the sensitivity of early embryos to insulin. In contrast glucose transport in the *lepb*$^{-/-}$ mutant was not significantly affected by injection of insulin, indicating insulin resistance (Fig.5B,C).

Gene knockdown studies for leptin signaling pathway analysis and translational studies in *Xenopus laevis*

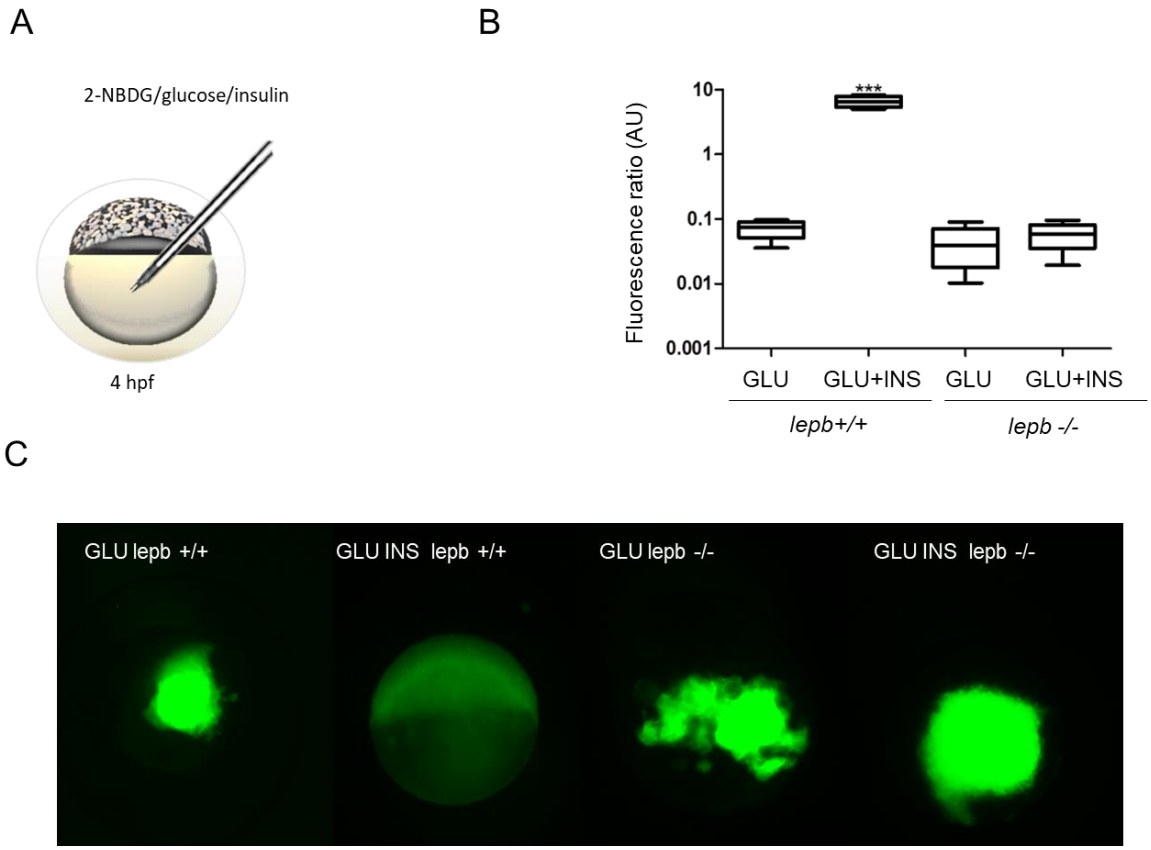


Figure 5. The effect of injection of human recombinant insulin into the yolk of 4 hpf zebrafish embryos. (A, B, C) Injections with 1 nl of glucose (200 mg/ml) in the presence of 2-NBDG results in accumulation of fluorescence in the yolk of 4 hpf embryos. 100 nM human recombinant insulin (INS) was co-injected with the 2-NBDG. Data, mean ± s.e.m) are combined from three biological replicates (n=10 larvae/group). ***P<0.001

We used morpholino anti sense technology to further study the signal transduction pathways underlying the identified function of leptin in early embryogenesis (Fig.6A). Firstly, we showed that the phenotype of knockdown of the *lepb* gene is glucose transport in early embryogenesis is also observed after injection of morpholino's against *lepb* (Fig.6B,C and Suppl.fig.2). Subsequently, we also tested morpholino's against the leptin receptor (*lepr*) and the second leptin gene (*lepa*). The results showed that *lepr* phenocopied accurately the effect of the *lepb* morpholino treatment. In contrast, *lepa* showed no significant effect on glucose transport in early embryogenesis (Fig.6B,C and Suppl.fig.2). These results indicated that a *lepb-lepr* signaling pathway was functionally similar to the function of leptin in humans. In order to get an indication on the target of NSC-87877, that was responsible for rescuing the *lepb* phenotype, we tested morpholino's against the most likely targets of this inhibitor[25,26]. Using the fluorescent glucose assay, we could demonstrate that knock down of the *ptpn6* gene completely reverted the *lepb*$^{-/-}$ mutant to the wild-type phenotype (Fig.6D,E). In contrast, knock down of other possible targets of NSC-87877, the closely related phosphatases *ptpn11a* (Shp-2a) and *ptpn11b* (Shp-2b), or the dual specificity phosphatase DUSP26 did not restore glucose transport (Suppl.fig.2B,2C). This indicates that *ptpn6* is the likely target of NSC-87877 responsible for the reversion of the *lepb*$^{-/-}$ phenotype. We used *Xenopus laevis* that has been used classically for embryogenic studies to show that the function of *lepb* in glucose transport is also relevant in embryos of other vertebrate organisms. Two morpholino's against the two leptin genes of *X. laevis* were designed and

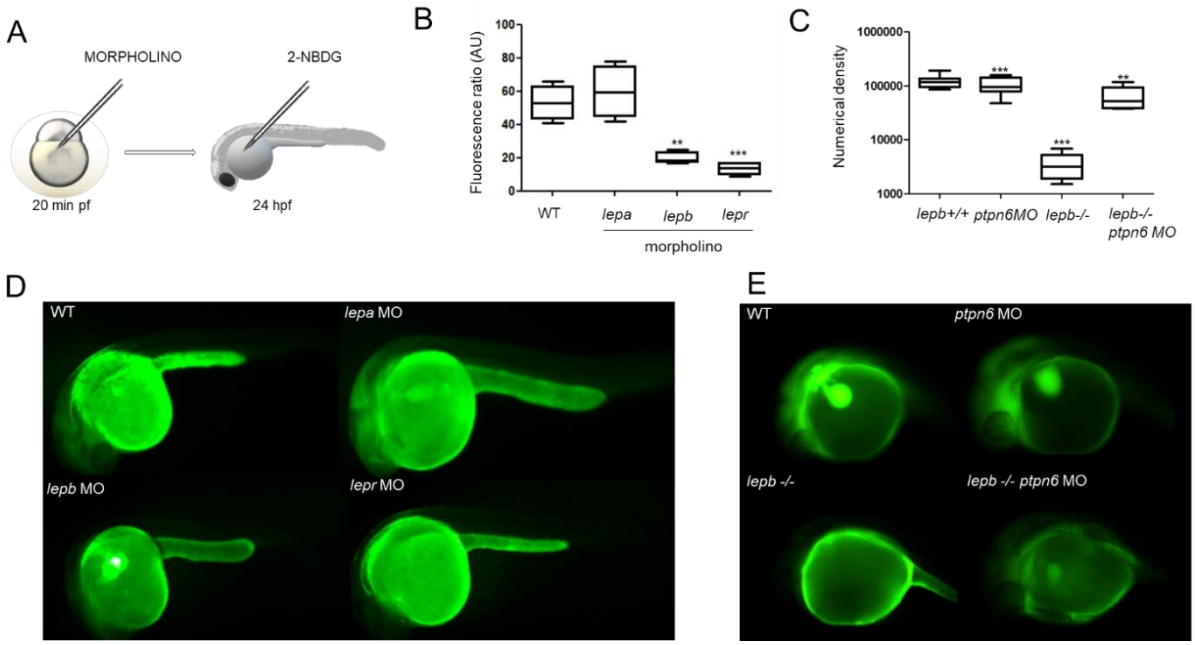


Figure 6. Late gene knockdown studies in zebrafish embryos. (**A**) Fluorescent glucose injection at 24 hpf: Gene expression was abolished in one cell stadium and at 24 hpf the embryos were injected into the yolk with fluorescent labelled glucose. One hour after injections, the accumulation of glucose in the brain of lepb$^{+/+}$ and lepb$^{-/-}$ zebrafish larvae can be observed with stereo fluorescence microscopy and has been quantified for the brain area using a custom written script **(B,D)** Knockdown of gene expression with morpholino's against *lepb, lepa* and *lepr* and imaging of glucose distribution at 24 hpf using method **(C,E**) Rescue of the *lepb* glucose transport deficiency by *ptpn6* morpholino at 24 hpf. Data, mean ± s.e.m., are combined from three biological replicates (n=10 larvae/group). **P<0.01, ***P<0.001

tested simultaneously as described in the material and methods (Fig.7A). Leptin knockdown results in inhibition of glucose transport after 24 hpf. Showing that lack of leptin expression in *X. laevis* leads to a similar glucose transport inhibition as in the zebrafish larvae. Moreover, morphant larvae show developmental abnormalities comparing to the control group. Importantly, injections with NSC-87877 together with morpholino not only rescue the developmental phenotype, but also glucose transport in the morphants (Fig.7B,C). These results clearly show that leptin plays a crucial role in glucose transport during early embryogenesis also in *X. laevis*.

Discussion

We show that a *lepb*$^{-/-}$ mutant zebrafish line is diabetic during larval development. This diabetic phenotype is characterized by insulin resistance and subsequent inhibition of glucose uptake at both systemic level and peripheral organs such as the brain. We also demonstrate that leptin b is essential for transport of glucose in the early stages of embryogenesis. Gene knockdown studies show that the leptin receptor is equally important to *lepb* but that *lepa* doesn't seem to play an important function. Knockdown of the *lepb* gene could be rescued by injection of human recombinant leptin even though this protein has only 18 percent of identity with the zebrafish leptin protein, showing the relevance of the results in our zebrafish test system for the function of mammalian leptin. In order to show that our findings are indeed relevant for the function of leptin in embryogenesis of other vertebrates models we tested the function of leptin in *Xenopus laevis* which is one of the few other vertebrate models

in which embryos can be easily handled. The results show that the two *X. laevis* leptins have a function in glucose transport during embryogenesis. Since the *X. laevis* leptins are distantly related in sequence to the zebrafish leptins, this indicates that the function of leptin in glucose transport is translatable to all vertebrates. The essential function of leptin in glucose transport during embryogenesis is surprising since in rodent models leptin is supposed to have a complex function in insulin resistance that involves systemic signaling via the blood stream[4], whereas at 4 hpf an organ system has not yet developed. This function of leptin b indicates a role of insulin receptors at the very early stages of embryogenesis that is confirmed by the effects of human recombinant insulin injected into the yolk sac. In previous work one of the zebrafish insulin receptors (*Insrb*) was reported to be expressed at 18 somite stage and both insulin receptors were maternally expressed in fertilized eggs[28]. In addition, two insulins have been described to be expressed during early zebrafish development. Of these two genes, *Insb*, was shown to be expressed at proliferating blastomeres at 3 and 4 hpf[29]. However, there is no knowledge which glucose transporters could be involved in glucose transport during embryogenesis. Considering the fact that glucose transport up to 64 cells stage was not dependent on leptin shows that such transporters and their control by leptin develops after the syncytial stage of embryogenesis. Our results suggested that also in adults, leptin was directly involved in glucose homeostasis, in line with the study of Michel *et al*, (2016)[12] who reported a diabetic phenotype in *lepr* knockdown zebrafish.

This supports the potential of the leptin signaling pathway for therapeutic purposes as reviewed by Coppari and Bjorbaek (2012)[30]. Although leptin has mainly been reported to be produced by adipocytes, and few other tissues such as the intestinal epithelium mainly during inflammatory conditions[31,32] there is also evidence that leptin is produced by human skeletal muscle in adults[33]. In a recent publication by Kang *et al.* (2016)[34] enhancer elements controlling *lepb* expression in zebrafish were shown to be triggered in injured tissues. Considering that these enhancer elements were also functional in mice tissue during wounding suggests a conserved function of leptin in wound repair. These and many other results indicate that in adults the function of leptin is much broader than the canonical adipocyte-brain axis[35, 36,37,38]. It has also been shown that in human placenta that there is an abundant production of the leptin protein[39]. Considering that leptin in mammalian cells can be transported by transcytosis to neighboring cells or tissues[40,41], it is an interesting idea that in mammalian embryogenesis placental leptin plays a role in glucose transport in the zygote.

In analogy with adult mammalian diabetes studies, the diabetic phenotype of zebrafish larvae can be reverted by external treatment with metformin at 3 days post fertilization. However, such treatment with metformin was not effective at earlier stages of development. The phosphatase inhibitor NSC-87877 can also revert the diabetic phenotype of the *lepb*$^{-/-}$ mutant at 3 dpf and even at much earlier stages of development. The fact that metformin was not active at earlier larval stages using fluorescent glucose uptake studies can be explained in several ways. A likely explanation is that there is no uptake of metformin through the skin and

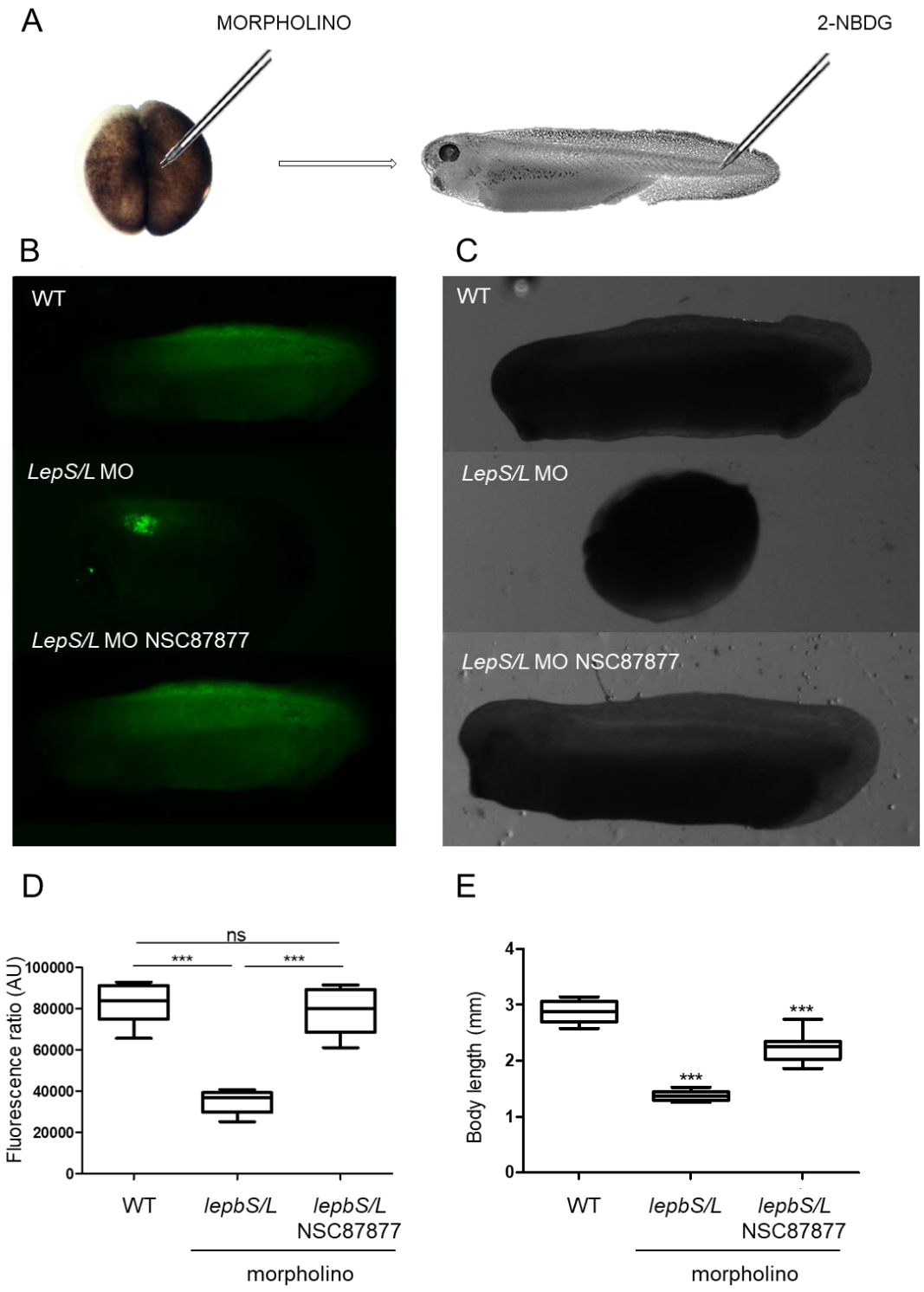


Figure 7. Gene knockdown studies in *Xenopus laevis* embryos. (**A**) Knock down of both the leptin S and leptin L genes of *Xenopus laevis* embryos. (**B, C**) 2-NBDG was injected at 48 hpf distribution was quantified using a custom script. Data, mean ± s.e.m., are combined from three biological replicates (n=10 larvae/group). ns = not significant, ***P<0.001

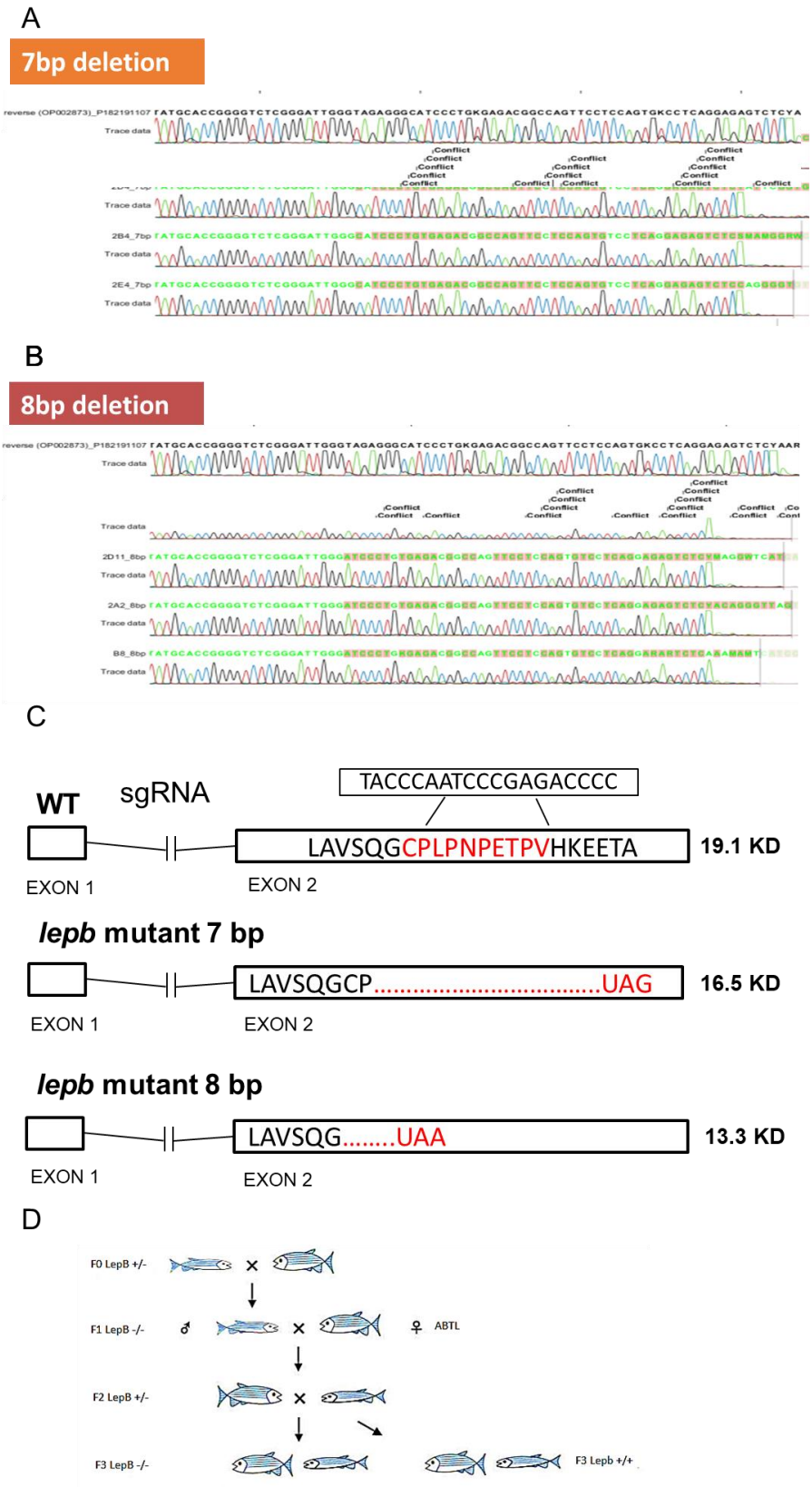
the observed effect a 4 dpf is through oral uptake when the mouth of the larvae has opened possibly because it might only function via ingestion in the intestinal track as observed in mammals[42]. In this respect NSC-87877 that is active after external treatment at 24 hpf, apparently is taken up through the skin and therefore could have applications in non-oral dosing systems.

Based on the reversal of the *lepb*$^{-/-}$ mutant by gene knockdown of *ptpn6*, the product of this gene is the most likely target of NSC-87877 underlying the anti-diabetic effect. Knockdown of other possible targets of NSC-87877, *ptpn11a* and *ptpn11b* and *dusp26* could not rescue the *lepb*$^{-/-}$ phenotype. *In vitro* inhibition studies showed that NSC-87877 has a similar inhibitory effect on truncated human PTPN6 and PTPN11 proteins at a five time higher IC50 than PTP1B[27]. It was later shown that NSC-87877 has a more potent inhibitory effect on the dual phosphatase DUSP26 than on full length human PTPN6 protein[26]. The expression pattern of *dusp26* that is restricted to neuroendocrine tissues in zebrafish larvae[43] supports our negative results in the rescue assay.

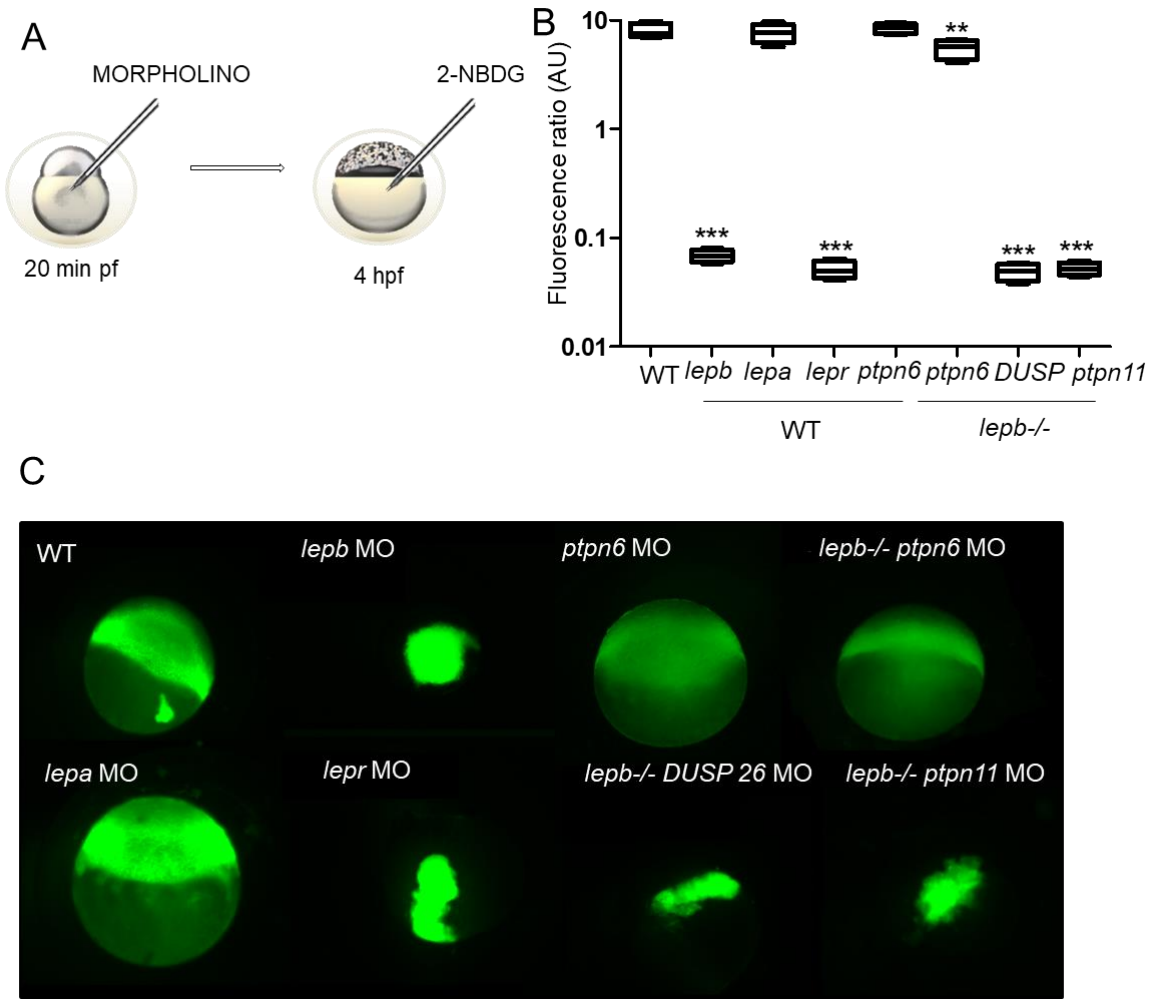
The direct effect of metformin and NSC-87877 in reversal of insulin resistance caused by a genetic defect shows the powerful action of these drugs at the level of insulin resistance. An effect of metformin on restoration of zebrafish larval beta cell development in a a *lepr* mutant was previously demonstrated by Michel *et al.* (2016)[12]. The drug metformin is already used for many years as first choice anti-diabetic drugs[44], however, its targets are multiple and therefore its function is still poorly understood[45,46]. In particular, the effect of metformin on insulin resistance, the hallmark of T2DM, needs further study[47,48].

The signaling pathway of insulin is highly conserved within all vertebrates. The positive effect of metformin in the zebrafish larval system shows that the results of mammalian diabetic test systems can be translated to lower vertebrate test systems. Considering that for treatment of T2DM it might be useful to overcome the very basic effect of insulin resistance indicates that our high throughput zebrafish model will be useful to identify new potentially antidiabetic drugs that can be further tested for their potential in mammalian studies.

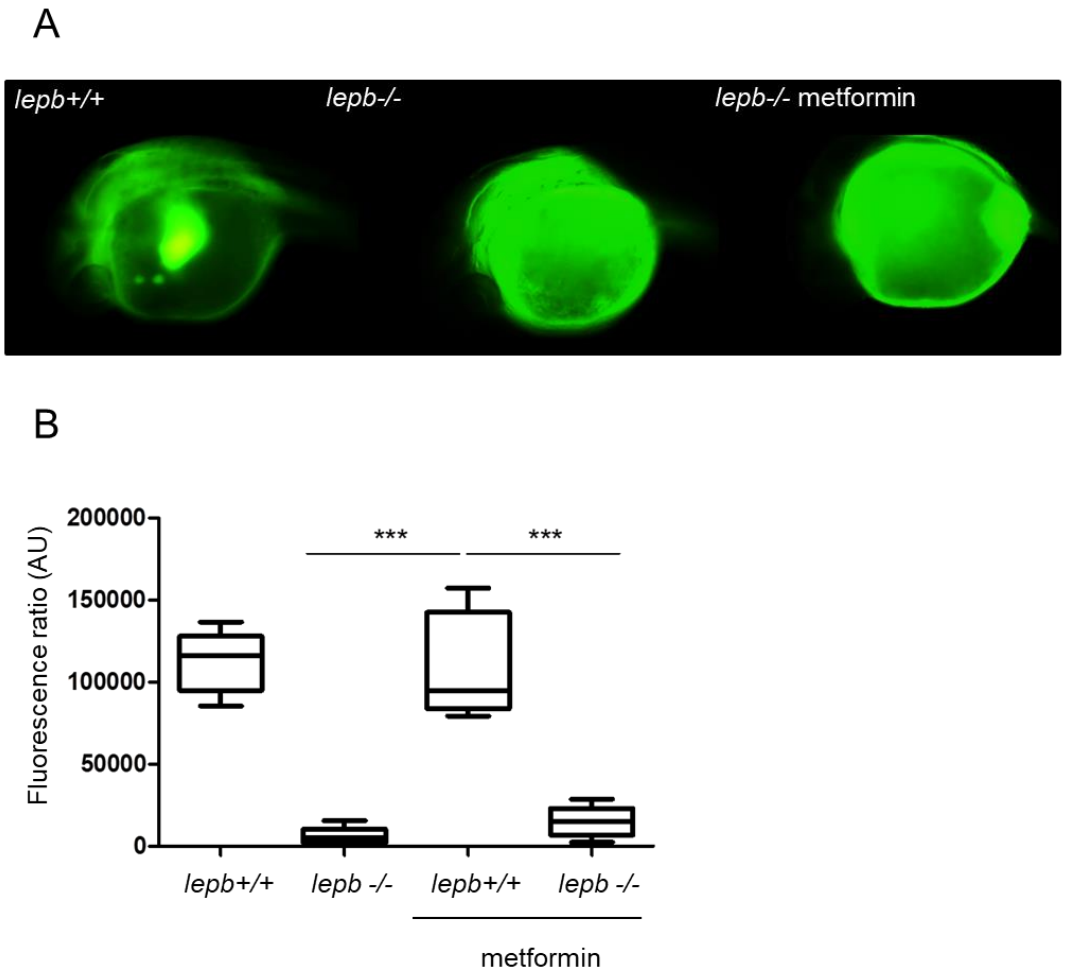
Supplementary materials



Supplementary Figure 1. An overview of the zebrafish leptin mutant. (A,B,C,D) We have found two leptin mutations 7bp and 8bp, which have the same phenotype and function.



Supplementary Figure. 2. Early gene knockdown studies in zebrafish embryos. (**A,B,C**) Knockdown of gene expression with morpholino's against *lepb, lepa*, *lepr, ptpn6* in lepb$^{+/+}$ embryos, as well as *ptpn6, DUSP 26* and *ptpn11*, in the lepb$^{-/-}$ embryos and imaging of glucose distribution at 4 hpf using method from Fig.2A. Data, mean ± s.e.m., are combined from three biological replicates (n=10 larvae/group). **P<0.01, ***P<0.001



Supplemental Figure 3. Effect of metformin. (**A**) We observe only a marginal effect of metformin in the *lepb*$^{-/-}$ larvae at 24 hpf. (**B**) After quantification of the fluorescent signal in the brain, there is no significant difference between *lepb* and metformin treated embryos. Cell to yolk fluorescent ratio, based on the confocal pictures, shows the difference in glucose uptake from the yolk between the three groups. Data, mean ± s.e.m., are combined from three biological replicates (n=10 larvae/group). ***P<0.001

Supplementary Table 1. List of morpholinos

Name	Sequence
lepb	5′-TTTTTTGCTTTGTTAATATCATCCCT-3′
lepa	5′-TTGAGCGGAGAGCTGGAAA-3′
lepr	5′-TCAAGACAGACATCATTTCACTTGC-3′
ptpn6	5′-ACTCATTCCTTACCCGATGCGGAGC-3′
DUSP26	5′-GAGACAATCTGGACATAAACGCCAT-3′
ptpn11 a and b	5′-GGTGGAACCACCTTCGGGATGTCAT-3′
control morpholino	5′- CCTCTTACCTCAGTTACAATTTATA-3′

References

1. Hu FB. Globalization of Diabetes. 2011; Diabetes Care 34: 1249–1257.
2. Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, Mullany EC, Biryukov S, Abbafati C, Abera SF, Abraham JP, Abu-Rmeileh NM, Achoki T, AlBuhairan FS, Alemu ZA, Alfonso R, Ali MK, Ali R, Guzman NA, Ammar W, Anwari P, Banerjee A, Barquera S, Basu S, Bennett DA, Bhutta Z, Blore J, Cabral N, Nonato IC, Chang JC, Chowdhury R, Courville KJ, Criqui MH, Cundiff DK, Dabhadkar KC, Dandona L, Davis A, Dayama A, Dharmaratne SD, Ding EL, Durrani AM, Esteghamati A, Farzadfar F, Fay DF, Feigin VL, Flaxman A, Forouzanfar MH, Goto A, Green MA, Gupta R, Hafezi-Nejad N, Hankey GJ, Harewood HC, Havmoeller R, Hay S, Hernandez L, Husseini A, Idrisov BT, Ikeda N, Islami F, Jahangir E, Jassal SK, Jee SH, Jeffreys M, Jonas JB, Kabagambe EK, Khalifa SE, Kengne AP, Khader YS, Khang YH, Kim D, Kimokoti RW, Kinge JM, Kokubo Y, Kosen S, Kwan G, Lai T, Leinsalu M, Li Y, Liang X, Liu S, Logroscino G, Lotufo PA, Lu Y, Ma J, Mainoo NK, Mensah GA, Merriman TR, Mokdad AH, Moschandreas J, Naghavi M, Naheed A, Nand D, Narayan KM, Nelson EL, Neuhouser ML, Nisar MI, Ohkubo T, Oti SO, Pedroza A, Prabhakaran D, Roy N, Sampson U, Seo H, Sepanlou SG, Shibuya K, Shiri R, Shiue I, Singh GM, Singh JA, Skirbekk V, Stapelberg NJ, Sturua L, Sykes BL, Tobias M, Tran BX, Trasande L, Toyoshima H, van de Vijver S, Vasankari TJ, Veerman JL, Velasquez-Melendez G, Vlassov VV, Vollset SE, Vos T, Wang C, Wang X, Weiderpass E, Werdecker A, Wright JL, Yang YC, Yatsuya H, Yoon J, Yoon SJ, Zhao Y, Zhou M, Zhu S, Lopez AD, Murray CJ, Gakidou E. 2014; Global, regional and national prevalence of overweight and obesity in children and adults 1980-2013: A systematic analysis. The Lancet 384 766 – 781.
3. Chen Y, Ma H, Zhu D, Zhao G, Wang L, Fu X, Chen W. 2017; Discovery of Novel Insulin Sensitizers: Promising Approaches and Targets. PPAR Res. 2017:8360919.
4. Wang B, Chandrasekera PC, Pippin JJ. 2014; Leptin- and leptin receptor-deficient rodent models: relevance for human type 2 diabetes. Curr Diabetes Rev. 10 (2):131-45.
5. Heydemann A, González-Vega M, Berhanu TK, Mull AJ, Sharma R, Holley-Cuthrell J. 2016; Hepatic Adaptations to a High Fat Diet in the MRL Mouse Strain are Associated with an Inefficient Oxidative Phosphorylation System. Jacobs J Diabetes Endocrinol. 2(1).
6. Reed MJ, Scribner KA. 1990; In-vivo and in-vitro models of type 2 diabetes in pharmaceutical drug discovery. Diabetes Obes Metab.;1(2):75-86.
7. Zhou Y, Rui L. 2013; Leptin signaling and leptin resistance. Front Med. 2013 Jun; 7(2): 207–222.
8. Ullrich, A. and Schlessinger, J. 1990; Signal transduction by receptors with tyrosine kinase activity. Cell 61, 203-21.
9. Tamrakar AK, Maurya CK, Rai AK. 2014; PTP1B inhibitors for type 2 diabetes treatment: a patent review (2011 - 2014). Expert Opin Ther Pat. 24(10):1101-15.

10. White CL, Whittington A, Barnes MJ, Wang Z, Bray GA, Morrison CD. 2009; HF diets increase hypothalamic PTP1B and induce leptin resistance through both leptin-dependent and -independent mechanisms. Am J Physiol Endocrinol Metab. 2 96(2):E291-9.
11. Stanford SM, Aleshin AE, Zhang V, Ardecky RJ, Hedrick MP, Zou J, Ganji SR, Bliss MR, Yamamoto F, Bobkov AA, Kiselar J, Liu Y, Cadwell GW, Khare S, Yu J, Barquilla A, Chung TDY, Mustelin T, Schenk S, Bankston LA, Liddington RC, Pinkerton AB, Bottini N. 2017; Diabetes reversal by inhibition of the low-molecular-weight tyrosine phosphatase. Nat Chem Biol. 13(6):624-632.
12. Michel M, Page-McCaw PS, Chen W, Cone RD. 2016; Leptin signaling regulates glucose homeostasis, but not adipostasis, in the zebrafish. Proc Natl Acad Sci USA. 113 (11):3084-9.
13. Gut P, Reischauer S, Stainier DYR, Arnaout R. 2017; Little fish, big data: zebrafish as a model for cardiovascular and metabolic disease. Physiol Rev. 97(3):889-938.
14. Zang L, Shimada Y, Nishimura N. 2017; Development of a Novel Zebrafish Model for Type 2 Diabetes Mellitus. Sci Rep. 7 (1):1461.
15. Marín-Juez R, Jong-Raadsen S, Yang S, Spaink HP. 2014; Hyperinsulinemia induces insulin resistance and immune suppression via Ptpn6/Shp1 in zebrafish. J Endocrinol. 222 :229-241.
16. Montague TG, Cruz JM, Gagnon JA, Church GM, Valen E. 2014; CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic Acids Res. 42(Web Server issue):W401–W407.
17. Chang N, Sun C, Gao L, Zhu D, Xu X, Zhu X, Xiong JW, Xi JJ. 2013; Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. Cell Res. 23(4):465-72.
18. Stoop EJ, Schipper T, Huber SK, Nezhinsky AE, Verbeek FJ, Gurcha SS, Besra GS, Vandenbroucke-Grauls CM, Bitter W, van der Sar AM. 2011; Zebrafish embryo screen for mycobacterial genes involved in the initiation of granuloma formation reveals a newly identified ESX-1 component. Dis Model Mech 4: 526–536.
19. Gorissen M, Bernier NJ, Nabuurs SB, Flik G, Huising MO. 2009; Two divergent leptin paralogues in zebrafish (Danio rerio) that originate early in teleostean evolution. J Endocrinol. 201(3):329-39.
20. Liu Q, Dalman M, Chen Y, Akhter M, Brahmandam S, Patel Y, Lowe J, Thakkar M, Gregory AV, Phelps D, Riley C, Londraville RL. 2012; Knockdown of leptin A expression dramatically alters zebrafish development. Gen Comp Endocrinol. 178(3):562-72.
21. Nagy C, Einwallner E. 2018; Study of In Vivo Glucose Metabolism in High-fat Diet-fed Mice Using Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT). J Vis Exp. 131.
22. Marín-Juez R, Rovira M, Crespo D, van der Vaart M, Spaink HP, Planas JV. 2015; GLUT2-mediated glucose uptake and availability are required for embryonic brain development in zebrafish. J Cereb Blood Flow Metab. 35(1):74-85.

23. Hale AJ, Ter Steege E, den Hertog J. 2017; Recent advances in understanding the role of protein-tyrosine phosphatases in development and disease. Dev Biol. 428(2):283-292.
24. Pérez-Fernández A, López-Ruano G, Prieto-Bermejo R, Ijurko C, Díez-Campelo M, Sánchez-Guijo F, Hernández-Hernández Á. 2019; SHP1 and SHP2 inhibition enhances the pro-differentiative effect of phorbol esters: an alternative approach against acute myeloid leukemia. J Exp Clin Cancer Res. 38(1):80.
25. Shi Y, Ma IT, Patel RH, Shang X, Chen Z, Zhao Y, Cheng J, Fan Y, Rojas Y, Barbieri E, Chen Z, Yu Y, Jin J, Kim ES, Shohet JM, Vasudevan SA, Yang J. 2015; NSC-87877 inhibits DUSP26 function in neuroblastoma resulting in p53-mediated apoptosis. Cell Death Dis. 6:e1841.
26. Song M, Park JE, Park SG, Lee DH, Choi HK, Park BC, Ryu SE, Kim JH, Cho S. 2009; NSC-87877, inhibitor of SHP-1/2 PTPs, inhibits dual-specificity phosphatase 26 (DUSP26). Biochem Biophys Res Commun. 381(4):491-5.
27. Chen L, Sung SS, Yip ML, Lawrence HR, Ren Y, Guida WC, Sebti SM, Lawrence NJ, Wu J. 2006; Discovery of a novel shp2 protein tyrosine phosphatase inhibitor. Mol Pharmacol. 70(2):562-70.
28. Toyoshima Y, Monson C, Duan C, Wu Y, Gao C, Yakar S, Sadler KC, LeRoith D. 2008; The role of insulin receptor signaling in zebrafish embryogenesis. Endocrinology. 149(12):5996-6005.
29. Papasani MR, Robison BD, Hardy RW, Hill RA. 2006; Early developmental expression of two insulins in zebrafish (Danio rerio). Physiol Genomics. 27(1):79-85.
30. Coppari R, Bjørbæk C. 2012; Leptin revisited: its mechanism of action and potential for treating diabetes. Nat Rev Drug Discov. 11(9):692-708.
31. Azuma T, Suto H, Ito Y, Ohtani M, Dojo M, Kuriyama M, Kato T. 2001; Gastric leptin and Helicobacter pylori infection. Gut. 49(3):324-9.
32. Mackey-Lawrence NM, Petri WA Jr. 2012; Leptin and mucosal immunity. Mucosal Immunol. 5(5):472-9.
33. Wolsk E, Mygind H, Grøndahl TS, Pedersen BK, van Hall G. 2012; Human skeletal muscle releases leptin in vivo. Cytokine. 60(3):667-73.
34. Kang J, Hu J, Karra R, Dickson AL, Tornini VA, Nachtrab G, Gemberling M, Goldman JA, Black BL, Poss KD. 2016; Modulation of tissue repair by regeneration enhancer elements. Nature. 532(7598):201-6.
35. Faggioni R, Feingold KR, Grunfeld C. 2001; Leptin regulation of the immune response and the immunodeficiency of malnutrition. FASEB J. 15(14):2565–2571.
36. Fantuzzi G, Faggioni R. 2000; Leptin in the regulation of immunity, inflammation, and hematopoiesis. J Leukoc Biol. 68(4):437–446.
37. Lago R, Gomez R, Lago F, Gomez-Reino J, Gualillo O. 2008; Leptin beyond body weight regulation--current concepts concerning its role in immune function and inflammation. Cell Immunol. 252(1-2):139–145.

38. Poeggeler B, Schulz C, Pappolla MA, Bodó E, Tiede S, Lehnert H, Paus R. 2010; Leptin and the skin: a new frontier. Exp Dermatol. 19(1):12-8. Review.
39. Zhao J, Townsend KL, Schulz LC, Kunz TH, Li C, Widmaier EP. 2004; Leptin receptor expression increases in placenta, but not hypothalamus, during gestation in Mus musculus and Myotis lucifugus. Placenta. 25(8-9):712-22.
40. Cammisotto PG, Bendayan M, Sané A, Dominguez M, Garofalo C, Levy E. 2010; Receptor-Mediated Transcytosis of Leptin through Human Intestinal Cells In Vitro. Int J Cell Biol. 2010:928169.
41. Tu H, Hsuchou H, Kastin AJ, Wu X, Pan W. 2010; Unique leptin trafficking by a tailless receptor. FASEB J. 24(7):2281-91.
42. Bailey CJ, Turner RC. 1996; Metformin. N Engl J Med. 334(9):574-9.
43. Yang CH, Yeh YJ, Wang JY, Liu YW, Chen YL, Cheng HW, Cheng CM, Chuang YJ, Yuh CH, Chen YR. 2017; NEAP/DUSP26 suppresses receptor tyrosine kinases and regulates neuronal development in zebrafish. Sci Rep. 7(1):5241.
44. Powers, Alvin C. "Chapter 344. Diabetes Mellitus." Harrison's Principles of Internal Medicine, 18e Eds. Dan L. Longo, et al. New York, NY: McGraw-Hill, 2012.
45. Foretz M, Guigas B, Bertrand L, Pollak M, Viollet B. 2014; Metformin: from mechanisms of action to therapies. Cell Metab. 20(6):953-66.
46. Florez JC. 2017; The pharmacogenetics of metformin. Diabetologia. 60:1648–1655.
47. Natali A, Ferrannini E. 2006; Effects of metformin and thiazolidinediones on suppression of hepatic glucose production and stimulation of glucose uptake in type 2 diabetes: a systematic review. Diabetologia. 49(3):434-41.
48. Fullerton MD, Galic S, Marcinko K, Sikkema S, Pulinilkunnil T, Chen ZP, O'Neill HM, Ford RJ, Palanivel R, O'Brien M, Hardie DG, Macaulay SL, Schertzer JD, Dyck JR, van Denderen BJ, Kemp BE, Steinberg GR. 2013; Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. Nat Med. 19(12):1649-54.