Screening for insulin-independent pathways that modulate glucose homeostasis identifies androgen receptor antagonists

Sri Teja Mullapudi\textsuperscript{1}, Christian S. M. Helker\textsuperscript{1}, Giulia L.M. Boezio\textsuperscript{1}, Hans-Martin Maischein\textsuperscript{1}, Anna M. Sokol\textsuperscript{2}, Johannes Graumann\textsuperscript{2,3}, Stefan Guenther\textsuperscript{4}, Hiroki Matsuda\textsuperscript{1,5}, Stefan Kubicek\textsuperscript{6}, Yu Hsuan Carol Yang\textsuperscript{1}, Didier Y.R. Stainier\textsuperscript{1*}

1. Max Planck Institute for Heart and Lung Research, Department of Developmental Genetics, Bad Nauheim, Germany
2. Biomolecular Mass Spectrometry, Max-Planck-Institute for Heart and Lung Research, Bad Nauheim, Germany
3. German Centre for Cardiovascular Research (DZHK), Partner Site - Rhine-Main, Germany
4. Max Planck Institute for Heart and Lung Research, ECCPS Bioinformatics and Deep Sequencing Platform, Bad Nauheim, Germany
5. Department of Biomedical Sciences, College of Life Sciences, Ritsumeikan University, Kusatsu, Japan
6. CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria

*Correspondence to:
Didier Stainier (Didier.Stainier@mpi-bn.mpg.de)
Department of Developmental Genetics
Max Planck Institute for Heart and Lung Research.
Ludwigstrasse 43, 61231 Bad Nauheim, Germany
Phone: +49 (0) 6032 705-1333
Fax:+49 (0) 6032 705-1304
Abstract

Pathways modulating glucose homeostasis independently of insulin would open new avenues to combat insulin resistance and diabetes. Here, we report the establishment, characterization and employment of a vertebrate ‘insulin-free’ model to identify insulin-independent modulators of glucose metabolism. *insulin* knockout zebrafish recapitulate core characteristics of diabetes and survive only up to larval stages. Utilizing a highly efficient endoderm transplant technique, we generated viable chimeric adults that provide the large numbers of *insulin* mutant larvae required for our screening platform. Using glucose as a disease-relevant readout, we screened 2233 molecules and identified 3 that consistently reduced glucose levels in *insulin* mutants. Most significantly, we uncovered an insulin-independent beneficial role for androgen receptor antagonism in hyperglycemia, mostly by reducing fasting glucose levels. Our study proposes therapeutic roles for androgen signaling in diabetes and, more broadly, offers a novel in vivo model for rapid screening and decoupling of insulin-dependent and -independent mechanisms.
Introduction

Characterized by the inability to control blood glucose levels, diabetes is a metabolic disease of major socio-economic concern. Blood glucose levels are regulated by multiple tissues including the pancreas, muscle, liver, adipocytes, gut and kidney (DeFronzo, 2009). Signals from endocrine hormones are integrated at each tissue to effectively maintain glucose homeostasis, and aberrations in this interplay underlie the pathogenesis of diabetes. Currently, seven classes of antidiabetic drugs exist, of which only three function without increasing circulating insulin levels and only one that definitively functions independently of insulin (Chaudhury et al., 2017). Restoring normoglycemia independently of insulin secretion or action could delay disease progression as an improved glycemic status can restore β-cell mass and function (Wang et al., 2014). Lower dependence on insulin-stimulating therapies can also prevent hyperinsulinemia-driven insulin resistance (Shanik et al., 2008) and obesity (Mehran et al., 2012). In contrast to insulin stimulators, Biguanides (E.g.: Metformin) and Thiazolidinediones (E.g.: Pioglitazone) are effective antidiabetic agents that primarily sensitize tissues to insulin (reviewed by (Soccio et al., 2014; Rena et al., 2017)). Likewise, sodium-glucose transporter 2 inhibitors (E.g.: Dapagliflozin) have a complementary mechanism of reducing glucose reabsorption in the kidney (Bailey et al., 2013). Increasing evidence points to additional molecular pathways that can improve metabolic homeostasis independently of insulin, for instance, using leptin therapy (Neumann et al., 2016) or during exercise (Stanford & Goodyear, 2014). Interestingly, currently prescribed drugs were discovered from their historical use in herbal medicine (Ehrenkranz et al., 2004; Bailey, 2017) or from screens directed against hyperlipidemia (Fujita et al., 1983). However, so far, an unbiased search for insulin-independent pathways controlling glucose homeostasis has remained elusive, primarily due to the lack of a disease-relevant animal model for rapid screening. Due to its high fecundity and amenability to chemical screening, the zebrafish serves as an excellent platform to study diabetes, and it has been successfully used to study β-cell mass and activity, as well as glucose metabolism (Andersson et al., 2012; Gut et al., 2013; Tsuji et al., 2014; Nath et al., 2015; Li et al., 2016; White et al., 2016; Matsuda et al., 2018). Here, using the zebrafish model, we generated an innovative drug discovery strategy, screened chemical libraries and specifically identified insulin-independent effects of androgen signaling on glucose homeostasis.

Results and Discussion

Insulin is crucial for zebrafish metabolic homeostasis

Insulin plays a central role in glucose homeostasis by increasing glucose uptake in peripheral tissues, promoting glycogenesis in the liver and decreasing glucose production by inhibiting glucagon secretion (Aronoff et al., 2004). We generated zebrafish devoid of insulin signaling
and determined the degree to which these mutants recapitulate core features of diabetic metabolism observed in mammals. The zebrafish genome contains two insulin genes – *insulin (ins)* and *insulinb (insb)*. Using CRISPR/Cas9 mutagenesis, we generated a 16 bp deletion allele for *ins* (Figure 1A) and a 10 bp insertion allele for *insb*. Although *ins* and *insb* mutant embryos appear morphologically unaffected (Figure 1 - figure supplement 1A), Insulin was entirely absent in pancreatic islets from *ins* mutants (Figure 1B), whereas, there was no observable change in *insb* mutant islets (Figure 1 - figure supplement 1B, 1C). Deletion of *ins* led to a drastic increase in total glucose levels (up to 10-fold), measured from 1 to 6 days post fertilization (dpf) (Figure 1C). Additionally, staining for lipid content using Nile Red revealed large unused yolk reserves (Figure 1D), suggesting defects in lipid absorption and processing. Due to a combination of these metabolic defects, *ins* mutants do not survive beyond 12 dpf (Figure 1E). Moreover, although 3-month old (adult) *ins +/-* animals are normoglycemic (Figure 1 - figure supplement 1D), 50 dpf (juvenile) *ins +/-* animals are noticeably smaller (Figure 1 - figure supplement 1E), consistent with a role for Insulin in growth control (Nakae et al., 2001). *insb* mutants, on the other hand, are viable and fertile. As *insb* expression is negligible beyond 48 hpf (Papasani et al., 2006; White et al., 2017) (Figure 1 - figure supplement 1F), we overexpressed *insb* under the *ins* promoter to assess if *insb* was functional. Under the hyperglycemic conditions resulting upon morpholino-mediated *ins* knockdown, *insb* overexpression successfully lowered glucose levels, thus indicating that *insb* is functional (Figure 1 - figure supplement 1G). However, due to the post-embryonic expression of *ins*, survival and metabolic homeostasis in zebrafish depends primarily on *ins*. This predominant role of *ins* distinguishes the zebrafish insulins from the redundant metabolic roles of mouse *Ins1* and *Ins2* (Duvillie et al., 1997). To further explore the nature of metabolic defects in zebrafish *ins* mutants, we probed the proteome of 108 hpf *ins* mutants and compared it to seven other proteomes from diabetic tissues of murine or human origin (Figure 1F) (Hwang et al., 2010; Mullen & Ohlendieck, 2010; Giebelstein et al., 2012; Valle et al., 2012; S.-J. Kim et al., 2014; Capuani et al., 2015; Braga et al., 2016; Zabielski et al., 2016). Strikingly, pathways like gluconeogenesis, mitochondrial dysfunction, Sirtuin signaling, and oxidative phosphorylation, which were affected in diabetic conditions across these studies, were also similarly disrupted in zebrafish *ins* mutants (Supplementary file 1). Together, these findings indicate that zebrafish *ins* is crucial for metabolic homeostasis and survival and that its absence recapitulates core features of diabetic metabolism already at larval stages.

**Highly efficient endoderm transplant technique rescues *ins* mutants to adulthood**

Screening of small molecules in *ins* mutants requires large numbers of mutant embryos. However, the early lethality of *ins* mutants did not allow the generation of adult animals that could be incrossed. To overcome this obstacle, we used an efficient endoderm induction (Kikuchi et al., 2001) and transplantation technique (Stafford et al., 2006) (Figure 2A) to
selectively modify endodermal tissues without altering the germline. \textit{ins} +/-; \textit{Tg(ins:DsRed)} embryos were injected with \textit{sox32} mRNA at the one-cell stage, conferring an endodermal fate on all cells. Between 3 to 4 hpf, cells were transplanted from these embryos to the mesendoderm of similarly staged embryos from \textit{ins} +/-; \textit{Tg(ins:RasGFP)} incrosses. This transplantation procedure was remarkably efficient at contributing to host endoderm (Figure 2 - figure supplement 1C’’), and nearly every transplanted embryo contained pancreatic islets with both transplanted \textit{ins} +/- and host \(\beta\)-cells (Figure 2B-B’’). These chimeric animals were grown to adulthood and genotyping (Figure 2 - figure supplement 1A-B) revealed a near Mendelian ratio of mutant animals (Figure 2C). These chimeric mutant animals contain \textit{ins} +/- endodermal tissues but retain an \textit{ins} -/- germline, and allowed an all-mutant progeny to be obtained by crossing.

\textbf{Small molecule screen in} \textit{ins} \textbf{mutants reveals insulin-independent modulators of glucose metabolism}

With the ability to obtain large numbers of \textit{ins} mutant embryos, we next aimed to analyze the effect of known glucose homeostasis modulators and also to screen for novel ones. We tested the effects of molecules that have been proposed to exert insulin-independent modulation of glucose homeostasis, amongst other effects. Anti-diabetics such as Metformin, Pioglitazone and Dapagliflozin, as well as the Lyn kinase activator MLR1023 (Saporito et al., 2012), were tested. We also tested Fraxidin, identified in a screen for molecules that increase glucose uptake in zebrafish (Lee et al., 2013). Surprisingly, Metformin and MLR1023 exhibited no glucose-lowering effect in \textit{ins} mutants suggesting that they act more as sensitizers of insulin signaling rather than acting independently of insulin. On the other hand, Pioglitazone, Dapagliflozin and Fraxidin reduced glucose levels by 11, 12 and 5\% respectively (Figure 3A), thus attributing part of their glucose lowering effect to an insulin-independent mechanism. Based on these data, we decided to screen chemical libraries using our model to identify molecules that could reduce glucose levels by more than 10\%. To rapidly measure glucose levels in a 96-well plate format, we adapted a glucose measuring kit to be sensitive to endogenous changes in larval glucose levels (Figure 3 - figure supplement 1A-C), and established a screening pipeline (Figure 3B). We screened 2233 molecules in 2 replicates at 10 \(\mu\)M concentration and found 3 hits (Figure 3C) that reproducibly reduced glucose levels upon retesting with independent chemical stocks and the unmodified standard glucose measurement kit. These 3 hits - Flutamide (androgen receptor antagonist), ODQ (soluble guanylyl cyclase inhibitor (Boulton et al., 1995)) and Vorinostat (broad HDAC inhibitor (Finnin et al., 1999)) were found upon retesting multiple times to reduce glucose levels by 40, 22 and 19\% respectively (Figure 3D, Figure 3 - figure supplement 1D).
Androgen Receptor (AR) antagonism regulates glucose homeostasis in hyperglycemic larval and adult animals

Given the strong reduction in glucose levels observed after Flutamide treatments, we further tested the hypothesis that glucose levels in ins mutants were being reduced through androgen receptor antagonism. First, Flutamide caused a dose-dependent decrease in glucose levels in ins mutants (Figure 4A - figure supplement 1A). Second, we treated ins mutants with AR antagonists of two types: (i) steroidal (Cyproterone) and (ii) non-steroidal (Nilutamide, Hydroxyflutamide, Bicalutamide, Enzalutamide). We observed consistent decrease in glucose levels across all treatments, albeit at varying efficiency (Figure 4A), possibly reflecting the specificity of these antagonists towards zebrafish AR (Raynaud et al., 1979; Teutsch et al., 1994; Tran et al., 2009). Finally, to modulate AR protein levels, we injected 1 ng of control or ar morpholino (MO) in one-cell stage embryos and observed a reduction of glucose levels in ar MO injected ins mutants (Figure 4B) but not in ar MO injected wild-type animals (Figure 4C). These data support a role for antagonizing AR specifically in hyperglycemic conditions.

A number of mechanisms have been proposed to explain the predisposition of women with androgen excess to diabetes, including insulin resistance, visceral adiposity and β-cell dysfunction (Navarro et al., 2015). Under high fat diet, a combination of neuronal and pancreatic β-cell specific roles for AR have been proposed to predispose female mice with androgen excess to diabetes (Navarro et al., 2018). Supporting this role, ar gene expression was observed in the zebrafish central nervous system and, additionally, in the liver (Gorelick et al., 2008) (Figure 4A - figure supplement 1C). To investigate how AR antagonism mediates glucose level reduction in ins mutants, we evaluated the effects of antagonist treatment using transcriptomic studies. RNA-Seq analyses on 120 hpf ins mutants treated with Flutamide or Cyproterone revealed 504 and 476 differentially expressed genes (DEGs) compared to vehicle treated mutants (Figure 4D), respectively. Of these DEGs, 40 were regulated in parallel (both up or both down) for both AR antagonists tested, likely highlighting the common AR-specific effects. Cross-referencing these candidates with a transcriptomic comparison of ins mutants to phenotypically wild-type animals, led to 12 genes (Figure 4E) that were differentially expressed upon loss of ins, and were partially or fully restored to wild-type levels upon treatment with AR antagonists (Figure 4 - figure supplement 1B). Genes such as btg2 and insig1 have been reported to play crucial roles in controlling liver gluconeogenesis (Carobbio et al., 2013; Y. D. Kim et al., 2014), and they also contain two androgen response elements (AREs) close to their transcription start site (Figure 4A - figure supplement 1D). Additionally, upon intraperitoneal injections of Flutamide in hyperglycemic adult animals (Figure 4A - figure supplement 1E), we observed up to 19% lower fasting plasma glucose levels were observed (Figure 4F), likely due to reduced hepatic glucose production. Our findings corroborate the observations of better anthropometric indices previously observed with Flutamide (Sahin et al., 2004) or Metformin +...
Flutamide combination therapies (Gambineri et al., 2004; Amiri et al., 2014) and attribute a part of this beneficial effect to Flutamide’s insulin-independent action through AR antagonism.

In conclusion, ours is the first study reporting the generation and use of a rapid screening strategy to identify insulin-independent pathways modulating metabolism in vertebrates. Given the recent success of SGLT2 inhibitors as combination therapy in diabetes (Bailey et al., 2013), our study is an important step towards identifying more insulin-independent mechanisms governing glucose homeostasis. One of the limitations in our screen is the relatively low size of the chemical library screened. However, as the endoderm transplant technique reported here can be combined with several genetic or metabolic readouts, future studies with larger chemical libraries should unveil mechanisms governing other disease-relevant phenomena as well. Such comprehensive insight into insulin-independent mechanisms and their interactions with insulin signaling in homeostasis and disease will open new avenues for targeting therapies to treat metabolic disorders.

Materials and Methods

Zebrafish lines

Zebrafish husbandry was performed under standard conditions in accordance with institutional (MPG) and national ethical and animal welfare guidelines. The transgenic and mutant lines used in the study are Tg(ins:DsRed)$^{m1018}$ (Anderson et al., 2009), Tg(-4.0ins:GFP)$^{yT5}$ (Huang et al., 2001), Tg(sox17:GFP)$^{s870}$ (Sakaguchi et al., 2006), Tg(ins:EGFP-HRas, cryaa:mCherry)$^{bns294}$, Tg(ins:Flag-NTR, cryaa:mCherry)$^{s950}$ (Andersson et al., 2012), Tg(ins:TagRFPt-P2A-insB)$^{bns285}$, ins$^{bns102}$ (ins mutants), and insb$^{bns295}$ (insb mutants).

1, 2, 3, 4, 5 and 6 days post fertilization (dpf) correspond to 24, 48, 72, 96, 120, and 144 hours post fertilization (hpf).

CRISPR/Cas9 mutagenesis

CRISPR design platform (http://crispr.mit.edu) was used to design sgRNAs against ins (targeting sequence: TCCAGTGTAAGCACTAACCCAGG) and insb (targeting sequence: GGATCGCAGTCTTCTCC) genes and constructs were assembled as described previously (Jao et al., 2013; Varshney et al., 2015). Briefly, a mixture of 25 pg gRNA with 300 pg Cas9 mRNA was injected into 1-cell stage wild-type embryos. High-resolution melt analysis (HRMA) (Eco-Illumina) was used to determine efficiency of sgRNAs and genotype animals with ins primers 5’-GTGCTCTGGTGGCCTTGGG-3’ and 5’-CATCGACCAGATGAGATCCACAC-3’, and insb primers: accordingly, 5’-AGTATTAAATCCTGCTGCGGCG-3’ and 5’-GTGTAGAAGAACTCCTAGGC-3’.
Immunostaining, Nile Red staining and imaging

Immunostaining and imaging was performed as described previously (Yang et al., 2018). Briefly, zebrafish larvae were euthanized with incubation on ice and fixed overnight at 4°C with 4% PFA (dissolved in buffer with composition: 22.6 mM NaH$_2$PO$_4$, 77 mM Na$_2$HPO$_4$, 120 μM CaCl$_2$, 117 mM sucrose, pH 7.35). After two PBS washes, the larvae were deskinned, and permeabilized using PBS containing 0.5% TritonX-100 and 1% DMSO for 1 hr. Larvae were then incubated in blocking buffer (Dako) containing 5% goat serum for 2 hrs, and incubated with primary antibody overnight at 4°C. Next, samples were washed 3 x 10 min with PBS containing 0.1% TritonX-100, incubated overnight at 4°C with secondary antibody and DAPI (10 µg/ml), washed 3 x 10 min and mounted in agarose. Antibody dilutions used are as follows: guinea pig anti-Insulin polyclonal (1:100, Thermo), mouse anti-Glucagon (1:300, Sigma), chicken anti-GFP (1:300, Aves), goat anti-guinea pig AlexaFluor568 (1:300, Thermo), goat anti-mouse AlexaFluor647 (1:300, Thermo), goat anti-chicken AlexaFluor488 (1:500, Thermo). Zeiss LSM700 (10X) and LSM800 (25X) was used to acquire data, and Imaris (Bitplane) was used for analyzing data and creating maximum intensity projection images.

Neutral lipid staining using Nile Red dye was performed at a working concentration of 0.5 μg/mL for 30 minutes in the dark, followed by acquisition of fluorescent images using an LP490 filter on a Nikon SMZ25 stereomicroscope.

Morpholino and intraperitoneal injections

For knockdown of gene expression, the following splice-blocking antisense morpholinos (Gene Tools, LLC) were injected into one-cell embryos at the indicated dosage per embryo:

insa MO (4 ng, 5′-CCTCTACTTGACTTTCTTACCCAGA-3′) (Ye et al., 2016)
ar MO (1 ng, 5′-AGCAGAGCCGCTCTTACCTGCCAT-3′) (Peal et al., 2011)
Standard control MO (5′-CCTCTTACCTCAGTTACAATTTA-3′)

Intraperitoneal injections and glucose measurement in 6 month-old adult zebrafish was performed as described previously (Moss et al., 2009; Eames et al., 2010). Briefly, ablation of β-cells in Tg(ins:NTR) animals was performed by injecting 0.25 gm MTZ/kg body weight twice – on Day 0 and Day 1 – injecting twice improved the consistency of ablation. Flutamide (10 mg/kg) or vehicle (DMSO) was injected on Day 2, 3 and 4. For injections, animals were anaesthetized using 0.02% Tricaine. On Day 4, animals were euthanized and blood glucose was measured using FreeStyle Freedom Lite Glucose Meter (Abbott). Non-parametric Student’s t-test was used for statistical analyses and $P$ values are shown in the figures.

Larval glucose measurement

Glucose measurements were performed as described previously (Jurczyk et al., 2011), with minor modifications. After desired treatment conditions, pools of 10 animals were collected in 1.5 mL Eppendorf tubes, water removed, and frozen at -80 °C. For analysis, pools of wild-type
embryos were resuspended in PBS. Samples were homogenized using a tissue homogenizer (Bullet Blender Gold, Next Advance). Glucose Assay Kit (CBA086, Merck) was used for glucose detection. Different volumes were used for resuspension and glucose detection between wild types and ins mutants: wild-type samples were resuspended in volume corresponding to 8 μl/animal and 8 μl was used for the glucose detection reaction. ins mutant embryos, due to their higher glucose content, were resuspended in volumes corresponding to 16 μl/animal and only 2 μl was used for glucose detection. Non-parametric Student’s t-test was used for statistical analyses and P values are shown in the figures.

Transplantations

For the endoderm transplant experiment, sox32 mRNA was transcribed using Sp6 mMessage mMachine kit (Ambion). Using a micro-injector, 100 pg/embryo of sox32 mRNA was injected in donor homozygous Tg(ins:DsRed) embryos. Embryos from an ins +/- incross served as host embryos. Between the 1k-cell and sphere stage (3-4 hpf), 15-20 cells from the donor were transplanted to host embryos, targeting the host mesendoderm, at the margin of the blastoderm.

Wholemount in situ hybridization

Larvae were collected at 120 hpf and fixed with 4% paraformaldehyde in PBS overnight at 4 °C. In situ hybridization was performed as described previously (Thisse & Thisse, 2008). ar Digoxigenin-labelled anti-sense probe was synthesized using T7 polymerase (Roche) and DIG RNA labelling kit (Roche). The probe template was amplified using the following primers: ar ISH-forward 5’-TGGAGTTTTCTTCTCCA-3’ and ar ISH-reverse 5’-TAATACGACTCACTATAGGGTCATTTGTGGAACAGGATT-3’, obtaining a 1100 bp probe as described previously (Gorelick et al., 2008). Embryos were imaged on a Nikon SMZ25 stereomicroscope. Mutant and wild-type larvae were processed in the same tube and genotyped after the images were taken.

Drug screening

At 72 hpf, 3 ins mutant larvae were pooled in each well of a 96-well plate in 200 μl of egg water buffered with 10mM HEPES. All drug treatments were performed at 10 μM with 1% DMSO, unless otherwise stated. Drug treatment was performed from 84 hpf to 120 hpf, after which each well was visually analyzed to assess toxicity. Subsequently, 100 μl of egg water was removed and 25 μl of 5X Cell Culture Lysis Buffer (Promega) was added. The plate was left shaking for 1 min at 750 rpm, and after gentle shaking at 150 rpm for 30 min, another round of vigorous shaking was performed for 1 min at 750 rpm. 8 μl from each well was used for the glucose detection reaction in a new 96-well plate using the Glucose Assay Kit (CBA086, Merck).
All drug treatments were performed at 10 μM, unless indicated otherwise. Drugs with an average glucose-lowering effect of over 10% were retested.

Drug libraries used in this screen are:

a. 1440 molecules from Edelris Keymical Collection (Edelris) (0 hits).
b. 285 molecules from the CLOUD collection (Licciardello et al., 2017) (2 hits).
c. 156 molecules identified as insulin stimulators (Matsuda et al., 2018) (1 hit).
d. 352 kinase inhibitors (SelleckChem) (0 hits)

Transcriptomic analyses

For RNA-seq analysis, total RNA was isolated from 120 hpf zebrafish using the RNA Clean & Concentrator kit (Zymo Research) combined with DNase digestion (RNase-free DNase Set, Promega) to avoid contamination by genomic DNA. RNA and library preparation integrity were verified with LabChip Gx Touch 24 (Perkin Elmer). 3μg of total RNA was used as input for Truseq Stranded mRNA Library preparation following manufacture’s low sample protocol (Illumina). Sequencing was performed on NextSeq500 instrument (Illumina) using v2 chemistry, resulting in minimum of 23M reads per library with 1x75bp single end setup. The resulting raw reads were assessed for quality, adapter content and duplication rates with FastQC (Andrews, 2010). Trimmomatic version 0.33 was employed to trim reads after a quality drop below a mean of Q20 in a window of 5 nucleotides (Bolger et al., 2014). Only reads between 30 and 150 nucleotides were cleared for further analyses. Trimmed and filtered reads were aligned versus the Ensembl Zebrafish genome version DanRer10 (GRCz10.90) using STAR 2.4.0a with the parameter “--outFilterMismatchNoverLmax 0.1” to increase the maximum ratio of mismatches to mapped length to 10% (Dobin et al., 2013). The number of reads aligning to genes was counted with featureCounts 1.4.5-p1 tool from the Subread package (Liao et al., 2014). Only reads mapping at least partially inside exons were admitted and aggregated per gene. Reads overlapping multiple genes or aligning to multiple regions were excluded. Differentially expressed genes were identified using DESeq2 version 1.62 (Love et al., 2014). Maximum Benjamini-Hochberg corrected p-value of 0.05, and a minimum combined mean of 5 reads were set as inclusion criteria. The Ensemble annotation was enriched with UniProt data (release 06.06.2014) based on Ensembl gene identifiers ("Activities at the Universal Protein Resource (UniProt)," 2014). RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7283.
For microarray expression profiling, RNA was isolated from pooled 108 hpf zebrafish larvae using the RNA Clean & Concentrator kit (Zymo Research) combined with DNase digestion (RNase-free DNase Set, Promega). 10 animals were used for each pooled sample. Sample quality was tested using a Bioanalyzer and microarray analysis was performed by Oak Labs (Germany). Microarray data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7282.

Mass spectrometric analysis

For each of the three biological replicates within a genotype, protein was extracted from pools of 600 larvae at 5 dpf using 4% SDS in 0.1 M Tris/HCl, pH 7.6 and a tissue disrupting sterile pestle (Axygen) for lysis. After heating to 70°C at 800 rpm for 10 min and DNA shearing by sonication, cell debris was removed by centrifugation at 14,000 x g for 10 min and retaining the supernatant. Using DC protein assay (BioRad), 7 mg of solubilized proteins per sample were Acetone precipitated at -20°C, overnight, followed by centrifugation at 14,000 x g for 10 min and washing the pellet washing using 90% acetone. After evaporation of residual acetone, samples were dissolved in urea buffer (6 M urea, 2 M thiourea, 10 mM HEPES, pH 8.0), followed by enzymatic peptidolysis as described (Graumann et al., 2008) with the following adaptations: 10 mM dithiothreitol, 55 mM iodoacetamide and 100:1 protein to enzyme ratio of the proteolytic enzymes were used. The resulting peptide concentration was estimated using the Fluorimetric Peptide Assay (Pierce) and peptides were labelled by reductive demethylation as described (Boersema et al., 2009) using light (L) and heavy (H) labels for wild type and mutants respectively. Differentially labelled peptides were mixed at 1:1 and subjected to a high pH reversed-phase peptide fractionation kit (Pierce). Full proteome analysis from the resulting eight fractions was performed using an EASY-nLC 1000 UHPLC system (ThermoFisher Scientific) and 20 cm in-house packed C18 capillary emitter columns (70 µm inner column diameter, 1.9 µm C18 beads, Dr. Maisch GmbH) coupled to a QExactive HF orbitrap mass spectrometer (ThermoFisher Scientific) using an electrospray ionization source. The gradient employed consisted of linearly increasing concentrations of solvent B (90% acetonitrile, 1% formic acid) over solvent A (5% acetonitrile, 1% formic acid) from 5% to 30% over 215 min, from 30% to 60%, from 60% to 95% and from 95% to 5% for 5 min each, followed by re-equilibration with 5% of solvent B. The constant flow rate was set to 400 nl/min.

Full MS spectra were collected for a mass range of 300 to 1750 m/z with a resolution of 60,000 at 200 m/z. The ion injection target was set to 3 x 10^6 and the maximum injection time limited to 20 ms. Ions were fragmented by higher energy collision dissociation (HCD) using a normalized collision energy of 27, an isolation window width of 2.2 m/z and an ion injection target of 5 x 10^5 with a maximum injection time of 20 ms. Precursors characterized with unassigned charge state and a charge state of 1 were excluded from selection for fragmentation. Precursor resequencing was prevented using dynamic exclusion time of 20 s.
Resulting tandem mass spectra (MS/MS) were acquired with a resolution of 15,000 in a top 15 loop. Data analysis: MS raw data were processed by MaxQuant (1.6.0.1) (Cox & Mann, 2008) using the Uniprot zebrafish database (as of 20.04.2017) containing 59066 entries and the following parameters: a maximum of two missed cleavages, mass tolerance of 4.5 ppm for the main search, trypsin as the digesting enzyme, carbamidomethylation of cysteines as a fixed modification and oxidation of methionine as well as acetylation of the protein N-terminus as variable modifications. For the dimethyl-labeled protein quantification, isotope labels were configured for peptide N-termini and lysine residues with a monoisotopic mass increase of 28.0313 and 36.0757 Da for the light and heavy labels, respectively. Peptides with a minimum of seven amino acids were included in the analysis. MaxQuant was set to filter for 1% false discovery rate on the peptide and protein levels, both. Only proteins with at least two peptides and one unique peptide were considered identified and included in further data analysis.

Canonical Pathway Analysis was performed using Ingenuity Pathway Analysis (IPA) (Qiagen). Differentially expressed proteins from our study (log2FC+1.5) and from previously published datasets were subjected to a Comparison Analysis in IPA. $P$-value maximum cut-off was set at 0.05 and the processes are listed according to those affected across the most studies.

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**Ethics Statement**

Zebrafish husbandry was performed under standard conditions in accordance with institutional (MPG) and national ethical and animal welfare guidelines approved by the ethics committee for animal experiments at the Regierungspräsidium Darmstadt, Germany.

**Competing Interests**

The authors declare no competing interests.
References

Activities at the Universal Protein Resource (UniProt). (2014). *Nucleic Acids Res*, 42(Database issue), D191-198. doi:10.1093/nar/gkt1140

Amiri, M., Golsorkhtabarami, M., Esmailizadeh, S., Ghofrani, F., Bijani, A., Ghorbani, L., & Delavar, M. A. (2014). Effect of Metformin and Flutamide on Anthropometric Indices and Laboratory Tests in Obese/Overweight PCOS Women under Hypocaloric Diet. *J Reprod Infertil*, 15(4), 205-213.

Anderson, R. M., Bosch, J. A., Goll, M. G., Hesselson, D., Dong, P. D., Shin, D., Chi, N. C., Shin, C. H., Schlegel, A., Halpern, M., & Stainier, D. Y. (2009). Loss of Dnmt1 catalytic activity reveals multiple roles for DNA methylation during pancreas development and regeneration. *Dev Biol*, 334(1), 213-223. doi:10.1016/j.ydbio.2009.07.017

Andersson, O., Adams, B. A., Yoo, D., Ellis, G. C., Gut, P., Anderson, R. M., German, M. S., & Stainier, D. Y. (2012). Adenosine signaling promotes regeneration of pancreatic beta cells in vivo. *Cell Metab*, 15(6), 885-894. doi:10.1016/j.cmet.2012.04.018

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data.

Aronoff, S. L., Berkowitz, K., Shreiner, B., & Want, E. D. (2010). Metformin: historical overview. *Diabetologia*, 60(9), 1566-1576. doi:10.1007/s00125-017-4318-z

Bailey, C. J., Gross, J. L., Hennicken, D., Iqbal, N., Mansfield, T. A., & List, J. F. (2013). Dapagliflozin add-on to metformin in type 2 diabetes inadequately controlled with metformin: a randomized, double-blind, placebo-controlled 102-week trial. *BMJ Medicine*, 11(1), 1043. doi:10.1186/1741-7015-11-43

Boersema, P. J., Raijmakers, R., Lemeer, S., Mohammed, S., & Heck, A. J. (2009). Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat Protoc*, 4(11), 205-212. doi:10.1038/nprot.2009.21

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30(15), 2114-2120. doi:10.1093/bioinformatics/btu170

Boulton, C. L., Southam, E., & Garthwaite, J. (1995). Nitric oxide-dependent long-term potentiation is blocked by a specific inhibitor of soluble guanylyl cyclase. *Neuroscience*, 69(3), 699-703.

doi:https://doi.org/10.1016/0306-4522(95)00349-N

Braga, C. P., Boone, C. H. T., Grove, R. A., Adamcova, D., Fernandes, A. A. H., Adamec, J., & de Magalhães Padilha, P. (2016). Liver Proteome in Diabetes Type 1 Rat Model: Insulin-Dependent and -Independent Changes. *OMICs: A Journal of Integrative Biology*, 20(12), 711-726.

doi:10.1089/omi.2016.0135

Capuani, B., Della-Morte, D., Donadel, G., Caratelli, S., Bova, L., Pastore, D., De Canio, M., D'Aguanno, S., Coppola, A., Pacifici, F., Arriga, R., Bellia, A., Ferrelli, F., Tesasoero, M., Federici, M., Neri, A., Bernardini, S., Sbraccia, P., Di Daniele, N., Sconocchia, G., Orlandi, A., Urbani, A., & Lauro, D. (2015). Liver protein profiles in insulin receptor-knockout mice reveal novel molecules involved in the diabetes pathophysiology. *American Journal of Physiology-Endocrinology and Metabolism*, 308(9), E744-E755. doi:10.1152/ajpendo.00447.2014

Carobbio, S., Hagen, R. M., Lelliott, C. J., Slawik, M., Medina-Gomez, G., Tan, C.-Y., Sicard, A., Atherton, H. J., Barbarroja, N., Bjursell, M., Bohlooly-Y, M., Virtue, S., Tuthill, A., Lefai, E., Laville, M., Wu, T., Considine, R. V., Vidal, H., Langin, D., Oresic, M., Tjahones, F. J., Fernandez-Real, J. M., Griffin, J. L., Sethi, J. K., López, M., & Vidal-Puig, A. (2013). Adaptive Changes of the Insig1/SREBP1/SCD1 Set Point Help Adipose Tissue to Cope With Increased Storage Demands of Obesity. *Diabetes*, 62(11), 3697.
Chaudhury, A., Duvoor, C., Reddy Dendi, V. S., Kraleti, S., Chada, A., Ravilla, R., Marco, A., Shekhawat, N. S., Montales, M. T., Kuriakose, K., Sasapu, A., Beebe, A., Patil, N., Musham, C. K., Lohani, G. P., & Mirza, W. (2017). Clinical Review of Antidiabetic Drugs: Implications for Type 2 Diabetes Mellitus Management. *Front Endocrinol (Lausanne)*, 8, 6. doi:10.3389/fendo.2017.00006

Cox, J., & Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology*, 26, 1367. doi:10.1038/nbt.1511

DeFronzo, R. A. (2009). From the Triumvirate to the Ominous Octet: A New Paradigm for the Treatment of Type 2 Diabetes Mellitus. *Diabetes*, 58(4), 773.

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, 29(1), 15-21. doi:10.1093/bioinformatics/bts635

Duvillie, B., Cordonnier, N., Deltour, L., Dandoy, Giebelstein, J., Poschmann, G., Hojlund, K., Schechinger, W., Dietrich, J. W., Levin, K., Beck, N. P. (1999). Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature*, 401(6749), 188-193. doi:10.1038/43710

Fujita, T., Sugiyama, Y., Taketomi, S., Sohda, T., Kawamatsu, Y., Iwatsuka, H., & Suzuoki, Z. (1983). Reduction of Insulin Resistence in Obese and/or Diabetic Animals by 5-[(4-[1-Methylcyclohexylmethoxy]benzyl]-thiazolidine-2,4-dione (ADD-3878, U-63,287, Ciglitazone), a New Antidiabetic Agent. *Diabetes*, 32(9), 804.

Gambineri, A., Pelusi, C., Genghini, S., Morselli-Labate, A. M., Cacciari, M., Pagotto, U., & Pasquali, R. (2004). Effect of flutamide and metformin administered alone or in combination in dieting obese women with polycystic ovary syndrome. *Clinical Endocrinology*, 60(2), 241-249. doi:10.1111/j.1365-2265.2004.01973.x

Giebelstein, J., Poschmann, G., Hojlund, K., Schechinger, W., Dietrich, J. W., Levin, K., Beck-Nielsen, H., Podwojski, K., Stuhler, K., Meyer, H. E., & Klein, H. H. (2012). The proteomic signature of insulin-resistant human skeletal muscle reveals increased glycolytic and decreased mitochondrial enzymes. *Diabetologia*, 55(4), 1114-1127. doi:10.1007/s00125-012-2456-x

Gorelick, D. A., Watson, W., & Halpern, M. E. (2008). Androgen receptor gene expression in the developing and adult zebrafish brain. *Developmental Dynamics*, 237(10), 2987-2995. doi:10.1002/dvdy.21700

Graumann, J., Hubner, N. C., Kim, J. B., Ko, K., Moser, M., Kumar, C., Cox, J., Scholer, H., & Mann, M. (2008). Stable isotope labeling by amino acids in cell culture (SILAC) and proteome quantitation of mouse embryonic stem cells to a depth of 5,111 proteins. *Mol Cell Proteomics*, 7(4), 672-683. doi:10.1074/mcp.M700460-MCP200

Gut, P., Baeza-Raja, B., Andersson, O., Hasenkamp, L., Hsiao, J., Hessler, D., Akassoglou, K., Verdin, E., Hirschey, M. D., & Stainier, D. Y. (2013). Whole-organism screening for gluconeogenesis identifies activators of fasting metabolism. *Nat Chem Biol*, 9(2), 97-104. doi:10.1038/nchembio.1136

Huang, H., Vogel, S. S., Liu, N., Melton, D. A., & Lin, S. (2001). Analysis of pancreatic development in living transgenic zebrafish embryos. *Mol Cell Endocrinol*, 177(1-2), 117-124.
Hwang, H., Bowen, B. P., Lefort, N., Flynn, C. R., De Filippis, E. A., Roberts, C., Smoke, C. C., Meyer, C., Højlund, K., Yi, Z., & Mandarino, L. J. (2010). Proteomics Analysis of Human Skeletal Muscle Reveals Novel Abnormalities in Obesity and Type 2 Diabetes. *Diabetes, 59*(1), 33.

Jao, L.-E., Wente, S. R., & Chen, W. (2013). Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proceedings of the National Academy of Sciences, 110*(34), 13904.

Jurczyk, A., Roy, N., Bajwa, R., Gut, P., Lipson, K., Yang, C., Covassini, L., Racki, W. J., Rossini, A. A., Phillips, N., Stainier, D. Y., Greiner, D. L., Brehm, M. A., Bortell, R., & dilorio, P. (2011). Dynamic glucoregulation and mammalian-like responses to metabolic and developmental disruption in zebrafish. *Gen Comp Endocrinol, 170*(2), 334-345. doi:10.1016/j.ygcen.2010.10.010

Kikuchi, Y., Agathon, A., Alexander, J., Thisse, C., Waldron, S., Yelon, D., Thisse, B., & Stainier, D. Y. (2001). casanova encodes a novel Sox-related protein necessary and sufficient for early endoderm formation in zebrafish. *Genes Dev, 15*(12), 1493-1505. doi:10.1101/gad.892301

Kim, S.-J., Chae, S., Kim, H., Mun, D.-G., Back, S., Choi, H. Y., Park, K. S., Hwang, D., Choi, S. H., & Lee, S.-W. (2014). A Protein Profile of Visceral Adipose Tissues Linked to Early Pathogenesis of Type 2 Diabetes Mellitus. *Molecular &amp;amp; Cellular Proteomics, 13*(3), 811.

Kim, Y. D., Kim, S. G., Hwang, S. L., Choi, H. S., Bae, J. H., Song, D. K., & Im, S. S. (2014). B-cell translocation gene 2 regulates hepatic glucose homeostasis via induction of orphan nuclear receptor Nur77 in diabetic mouse model. *Diabetes, 63*(6), 1870-1880. doi:10.2337/db13-1368

Lee, J., Jung, D. W., Kim, W. H., Um, J. I., Yim, S. H., Oh, W. K., & Williams, D. R. (2013). Development of a highly visual, simple, and rapid test for the discovery of novel insulin mimetics in living vertebrates. *ACS Chem Biol, 8*(8), 1803-1814. doi:10.1021/cb4000162

Li, M., Page-McCaw, P., & Chen, W. (2016). FGF1 Mediates Overnutrition-Induced Compensatory beta-Cell Differentiation. *Diabetes, 65*(1), 96-109. doi:10.2337/db15-0085

Liao, Y., Smyth, G. K., & Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics, 30*(7), 923-930. doi:10.1093/bioinformatics/btt656

Licciardello, M. P., Ringler, A., Markt, P., Klepsch, F., Lardeau, C.-H., Sdelci, S., Schirghuber, E., Müller, A. C., Caldera, M., Wagner, A., Herzog, R., Penz, T., Schuster, M., Boidol, B., Dünrberger, G., Folkvaljon, Y., Stattin, P., Ivanov, V., Colinge, J., Bock, C., Kratochwill, K., Menche, J., Bennett, K. L., & Kubicek, S. (2017). A combinatorial screen of the CLOUD uncovers a synergy targeting the androgen receptor. *Nature Chemical Biology, 13*, 771. doi:10.1038/nchembio.2382

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol, 15*(12), 550. doi:10.1186/s13059-014-0550-8

Matsuda, H., Mullapudi, S. T., Carol Yang, Y. H., Masaki, H., Hesselson, D., & Stainier, D. Y. R. (2018). Whole Organism Chemical Screening Identifies Modulators of Pancreatic beta Cell Function. *Diabetes*. doi:10.2337/db17-1223

Mehran, A. E., Templeman, N. M., Brigidi, G. S., Lim, G. E., Chu, K. Y., Hu, X., Botezelli, J. D., Asadi, A., Hoffman, B. G., Kieffer, T. J., Bamji, S. X., Clee, S. M., & Johnson, J. D. (2012). Hyperinsulinemia drives diet-induced obesity independently of brain insulin production. *Cell Metab, 16*(6), 723-737. doi:10.1016/j.cmet.2012.10.019

Moss, J. B., Koustubhan, P., Greenman, M., Parsons, M. J., Walter, I., & Moss, L. G. (2009). Regeneration of the pancreas in adult zebrafish. *Diabetes, 58*(8), 1844-1851. doi:10.2337/db08-0628

Mullen, E., & Ohlendieck, K. (2010). Proteomic profiling of non-obese type 2 diabetic skeletal muscle. *Int J Mol Med, 25*(3), 445-458.

Nakae, J., Kido, Y., & Accili, D. (2001). Distinct and Overlapping Functions of Insulin and IGF-I Receptors. *Endocrine Reviews, 22*(6), 818-835. doi:10.1210/edrv.22.6.0452

Nath, Anjali K., Ryu, Justine H., Jin, Youngnam N., Roberts, Lee D., Dejam, A., Gerszten, Robert E., & Peterson, Randall T. (2015). PTPMT1 Inhibition Lowers Glucose through Succinate
Dehydrogenase Phosphorylation. *Cell Reports*, 10(5), 694-701. doi:https://doi.org/10.1016/j.celrep.2015.01.010

Navarro, G., Allard, C., Morford, J. J., Xu, W., Liu, S., Molinas, A. J., Butcher, S. M., Fine, N. H. F., Blandino-Rosano, M., Sure, V. N., Yu, S., Zhang, R., Munzberg, H., Jacobson, D. A., Katakam, P. V., Hodson, D. J., Bernal-Mizrachi, E., Zsombok, A., & Mauvais-Jarvis, F. (2018). Androgen excess in pancreatic beta cells and neurons predisposes female mice to type 2 diabetes. *JCI Insight*, 3(12). doi:10.1172/jci.insight.98607

Navarro, G., Allard, C., Xu, W., & Mauvais-Jarvis, F. (2015). The role of androgens in metabolism, obesity, and diabetes in males and females. *Obesity (Silver Spring)*, 23(4), 713-719. doi:10.1002/oby.21033

Neumann, U. H., Denroche, H. C., Mojibian, M., Covey, S. D., & Kieffer, T. J. (2016). Insulin Knockout Mice Have Extended Survival but Volatile Blood Glucose Levels on Leptin Therapy. *Endocrinology*, 157(3), 1007-1012. doi:10.1210/en.2015-1890

Papasani, M. R., Robison, B. D., Hardy, R. W., & Hill, R. A. (2006). Early developmental expression of two insulin ligands in zebrafish (Danio rerio). *Physiol Genomics*, 27(1), 79-85. doi:10.1152/physiogenomics.00012.2006

Peal, D. S., Mills, R. W., Lynch, S. N., Mosley, J. M., Lim, E., Ellinor, P. T., January, C. T., Peterson, R. T., & Milan, D. J. (2011). Novel chemical suppressors of long QT syndrome identified by an in vivo functional screen. *Circulation*, 123(1), 23-30. doi:10.1161/CIRCULATIONAHA.110.003731

Raynaud, J.-P., Bonne, C., Bouton, M.-M., Lagace, L., & Labrie, F. (1979). Action of a non-steroid antiandrogen, RU 23908, in peripheral and central tissues. *Journal of Steroid Biochemistry*, 11(1, Part 1), 93-99. doi:https://doi.org/10.1016/0022-4731(79)90281-4

Rena, G., Hardie, D. G., & Pearson, E. R. (2017). The mechanisms of action of metformin. *Diabetologia*, 60(9), 1577-1585. doi:10.1007/s00125-017-4342-z

Sahin, I., Serter, R., Karakurt, F., Demirbas, B., Culha, C., Taskapan, C., Kosar, F., & Aral, Y. (2004). Metformin versus flutamide in the treatment of metabolic consequences of non-obese young women with polycystic ovary syndrome: a randomized prospective study. *Gynecol Endocrinol*, 19(3), 115-124.

Sakaguchi, T., Kikuchi, Y., Kuroiwa, A., Takeda, H., & Stainier, D. Y. (2006). The yolk syncytial layer regulates myocardial migration by influencing extracellular matrix assembly in zebrafish. *Development*, 133(20), 4063-4072. doi:10.1242/dev.02581

Saporito, M. S., Ochman, A. R., Lipinski, C. A., Handler, J. A., & Reaume, A. G. (2012). MLR-1023 Is a Potent and Selective Allosteric Activator of Lyn Kinase In Vitro That Improves Glucose Tolerance In Vivo. *Journal of Pharmacology and Experimental Therapeutics*, 342(1), 15.

Shanik, M. H., Xu, Y., Škrha, J., Dankner, R., Zick, Y., & Roth, J. (2008). Insulin Resistance and Hyperinsulinemia. *Diabetes Care*, 31(Supplement 2), S262.

Soccio, Raymond E., Chen, Eric R., & Lazar, Mitchell A. (2014). Thiazolidinediones and the Promise of Insulin Sensitization in Type 2 Diabetes. *Cell Metab*, 20(4), 573-591. doi:https://doi.org/10.1016/j.cmet.2014.08.005

Stafford, D., White, R. J., Kinkel, M. D., Linville, A., Schilling, T. F., & Prince, V. E. (2006). Retinoids signal directly to zebrafish endoderm to specify &lt;em&gt;insulin&lt;/em&gt;-expressing β-cells. *Development*, 133(5), 949.

Stanford, K. I., & Goodyear, L. J. (2014). Exercise and type 2 diabetes: molecular mechanisms regulating glucose uptake in skeletal muscle. *Adv Physiol Educ*, 38(4), 308-314. doi:10.1152/advan.00080.2014

Teutsch, G., Goubet, F., Battmann, T., Bonfils, A., Bouchoux, F., Cerede, E., Gofflo, D., Gaillard-Kelly, M., & Philibert, D. (1994). Non-steroidal antiandrogens: Synthesis and biological profile of high-affinity ligands for the androgen receptor. *The Journal of Steroid Biochemistry and Molecular Biology*, 48(1), 111-119. doi:https://doi.org/10.1016/0960-0760(94)90257-7
Thisse, C., & Thisse, B. (2008). High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc*, 3(1), 59-69. doi:10.1038/nprot.2007.514

Tran, C., Ouk, S., Clegg, N. J., Chen, Y., Watson, P. A., Arora, V., Wongvipat, J., Smith-Jones, P. M., Yoo, D., Kwon, A., Wasielewska, T., Welsbie, D., Chen, C. D., Higano, C. S., Beer, T. M., Hung, D. T., Scher, H. I., Jung, M. E., & Sawyers, C. L. (2009). Development of a Second-Generation Antiandrogen for Treatment of Advanced Prostate Cancer. *Science*, 324(5928), 787.

Tsuji, N., Ninov, N., Delawary, M., Osman, S., Roh, A. S., Gut, P., & Stainier, D. Y. (2014). Whole organism high content screening identifies stimulators of pancreatic beta-cell proliferation. *PLoS One*, 9(8), e104112. doi:10.1371/journal.pone.0104112

Valle, A., Catalán, V., Rodríguez, A., Rotellar, F., Valentí, V., Silva, C., Salvador, J., Frühbeck, G., Gómez-Ambrosi, J., Roca, P., & Oliver, J. (2012). Identification of liver proteins altered by type 2 diabetes mellitus in obese subjects. *Liver International*, 32(6), 951-961. doi:10.1111/j.1478-3231.2012.02765.x

Varshney, G. K., Pei, W., LaFave, M. C., Idol, J., Xu, L., Gallardo, V., Carrington, B., Bishop, K., Jones, M., Li, M., Harper, U., Huang, S. C., Prakash, A., Chen, W., Sood, R., Ledin, J., & Burgess, S. M. (2015). High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. *Genome Res*, 25(7), 1030-1042. doi:10.1101/gr.186379.114

Wang, Z., York, N. W., Nichols, C. G., & Remedi, M. S. (2014). Pancreatic beta cell dedifferentiation in diabetes and redifferentiation following insulin therapy. *Cell Metab*, 19(5), 872-882. doi:10.1016/j.cmet.2014.03.010

White, D. T., Eroglu, A. U., Wang, G., Zhang, L., Sengupta, S., Ding, D., Rajpurohit, S. K., Walker, S. L., Ji, H., Qian, J., & Mumm, J. S. (2016). ARQiv-HTS, a versatile whole-organism screening platform enabling in vivo drug discovery at high-throughput rates. *Nature Protocols*, 11, 2432. doi:10.1038/nprot.2016.142

White, R. J., Collins, J. E., Sealy, I. M., Wali, N., Dooley, C. M., Digby, Z., Stemple, D. L., Murphy, D. N., Billis, K., Hourlier, T., Füllgrabe, A., Davis, M. P., Enright, A. J., & Busch-Nentwich, E. M. (2017). A high-resolution mRNA expression time course of embryonic development in zebrafish. *Elife*, 6, e30860. doi:10.7554/eLife.30860

Yang, Y. H. C., Kawakami, K., & Stainier, D. Y. R. (2018). A new mode of pancreatic islet innervation revealed by live imaging in zebrafish. *Elife*, 7, e34519. doi:10.7554/eLife.34519

Ye, L., Robertson, M. A., Mastracci, T. L., & Anderson, R. M. (2016). An insulin signaling feedback loop regulates pancreas progenitor cell differentiation during islet development and regeneration. *Dev Biol*, 409(2), 354-369. doi:10.1016/j.ydbio.2015.12.003

Zabielski, P., Lanza, I. R., Gopala, S., Heppelmann, C. J., Bergen, H. R., 3rd, Dasari, S., & Nair, K. S. (2016). Altered Skeletal Muscle Mitochondrial Proteome As the Basis of Disruption of Mitochondrial Function in Diabetic Mice. *Diabetes*, 65(3), 561-573. doi:10.2337/db15-0823
Figures

Figure 1

A

CRISPR target site

ins

\[
\ldots\text{TGCAGGATGCAACACGGGGAGCAGGAGCAAGCA\ldots}
\]

\(\text{ins}^{631/632}\)

\[
\ldots\text{TGCAGGATGCAACACGGGGAGCAGGAGCAAGCA\ldots}
\]

16 bp deletion

A'  Signal Peptide  IL Chain  A Chain  C - Peptide

Ins protein

B

\(\text{ins}^{+/-}\)  \(\text{ins}^{-/-}\)

ins-GFP

96 hpf

Insulin

Glucagon

C

Glucose levels

\[
\begin{align*}
\text{Glucose levels (pmol/larva)} \\
10000 & \quad \text{ins}^{-/-} \\
1000 & \quad \text{ins}^{+/-}
\end{align*}
\]

Days post fertilization (dpf)

D

Lipid Staining

\(\text{ins}^{+/-}\)  \(\text{ins}^{-/-}\)

120 hpf

E

ins \(+/-\)  X  ins \(+/-\)

\[
\begin{align*}
\% \text{ of animals} \\
9 & \quad \text{ins}^{+/-} \\
10 & \quad \text{ins}^{+/-} \\
11 & \quad \text{ins}^{+/-} \\
12 & \quad \text{ins}^{-/-}
\end{align*}
\]

days post fertilization (dpf)

F

Comparative analysis of proteomes

Diabetic proteome

- Sirtuin Signaling
- Clathrin-mediated Endocytosis
- Glucosemetabolism
- Oxidative Phosphorylation
- Mitochondrial Dysfunction
- Aldosterone Signaling
- Glycolysis I
- NRF2-mediated Oxidative Stress
- Cardiac Hypertrophy Signaling
- Cellular Effects of Sildenafil
- ILK Signaling
- Signaling by Rho Family GTPases
- RhoGDI Signaling
- Fatty Acid ß-oxidation I
- EIF2 Signaling
- LXIV/ITXR Activation
- Protein Ubiquitination
- Xenobiotic Metabolism Signaling
- Aryl Hydrocarbon Receptor
Figure 1 - figure supplement 1

A  72 hpf

WT

ins -/-

insb -/-

B 72 hpf

WT

insb -/-

C

Ins 4/4

6/6

D Glucose levels (3 mpf)

Glucose levels (mg/dL)

0 50 100 150

inis +/+ inis +/-

P = 0.729

E Animal Size (50 dpf)

Body length (cm)

inis +/+ inis +/-

P = 0.0059

F

Log2 Expression

0 2 4 6

inisb

inis

0 hpf 3 hpf 6 hpf 16 hpf 30 hpf 72 hpf 120 hpf

G Glucose levels (96 hpf)

Glucose levels (pmol/larva)

ctrl MO insa MO + insb OE

P = 0.0035

ctrl MO insa MO + insb OE
**Figure 2**

(A) **ins**+/+ *Tg(ins:Dsred) X** ins+/+ *Tg(ins:Dsred)*

- **ins**+/+ *Tg(ins:RasGFP) X** ins+/− *Tg(ins:RasGFP)*

25% progeny ins−/−

Transplant endoderm-fated cells

Raise to adulthood

(b) **48 hpf**

ins:DsRed

ins:GFP

Merge

(c) 

Endoderm Transplanted Hosts

% of animals

|                | 3 mpf |
|----------------|-------|
| ins+/+        |       |
| ins+/−        |       |
| ins−/−        |       |

Endoderm Transplanted Hosts

% of animals

|                | 3 mpf |
|----------------|-------|
| ins+/+        |       |
| ins+/−        |       |
| ins−/−        |       |
Figure 2 - figure supplement 1

| Genotype     | Melting Temp | Melting Temp II |
|--------------|--------------|-----------------|
| Wild-type    | 82.2         |                 |
| Heterozygous | 82.1         | 76.5            |
| Mutant       | 81.1         |                 |

A

B

C

C'  

C''  

H2B - mCherry  

sox17;GFP  

Merge  

48 hpf
Figure 3 - figure supplement 1

A. Glucose standard curve

B. Protocol:
- Remove 100 μL medium
- Check larval health
- Add 25 μL 5X Cell Culture Lysis Buffer
- Shaking 750 rpm, 1 min
- Shaking 150 rpm, 30 min
- Shaking 750 rpm, 1 min
- Use 8 μL for glucose measurement

C. Glucose levels in lysis buffer

D. Chemical structures:
- Flutamide
- ODQ
- Vorinostat

Relative glucose levels

Control  Iso  Iso + Met

*p < 0.0001  *p = 0.0296
Figure 4 - figure supplement 1

A

Dose Response - Flutamide

B

| Gene    | Mutant vs Hets or WT (Log2FC) | Flutamide vs DMSO (Log2FC) | Cypromeone vs DMSO (Log2FC) | Expression levels (baseMean) |
|---------|-------------------------------|-----------------------------|----------------------------|-------------------------------|
| abraa   | 1.00                          | -0.61                       | -0.71                      | 2183                          |
| fosab   | 1.21                          | -0.56                       | -1.12                      | 592                           |
| slc2a12 | 1.20                          | -0.55                       | -0.60                      | 1209                          |
| insq1   | 1.25                          | -0.40                       | -0.79                      | 2485                          |
| btg2    | 1.05                          | -0.40                       | -0.98                      | 1573                          |
| vim     | -0.32                         | 0.37                        | 0.41                       | 1242                          |
| abcb4   | -0.51                         | 0.42                        | 0.51                       | 1647                          |
| col9a1b | -0.32                         | 0.48                        | 0.47                       | 1319                          |
| bfsp2   | -0.32                         | 0.49                        | 0.39                       | 1063                          |
| abcc12  | -0.32                         | 0.50                        | 0.94                       | 702                           |
| alas1   | -0.74                         | 0.69                        | 2.03                       | 1952                          |
| tnnt2d  | -1.00                         | 0.74                        | 0.48                       | 607                           |

C

D

instq1

btg2

200 bp

ARE

E

Inject MTZ

Inject MTZ

Inject Vehicle/Flutamide (i.p.)

Day 0

Day 1

Day 2

Day 3

Day 4

Fasting Glucose Measurement

Tq(ins:NTR) adults
Figure Legends

Figure 1. **insulin is crucial for zebrafish metabolic homeostasis.** A) CRISPR target site in the *insulin* gene, with PAM sequence highlighted, and the resulting 16 bp deletion allele (below). A') Schematic of wild-type insulin protein and the predicted mutant protein which contains novel sequence (black) and a premature stop (red). B) Confocal projection images of pancreatic islets in 96 hpf *Tg(ins:GFP) ins+/+* and *ins-/-* animals immunostained for Insulin (red), Glucagon (cyan). C) Free glucose levels measured in wild-type and mutant animals from 1 to 6 dpf; mean ± SEM, n=2-4 replicates. D) Nile Red staining (green) for neutral lipids in 120 hpf wild-type (top) and mutant (bottom) larvae, with yolk lipid content outlined (yellow dots). E) Genotype distribution from *ins +/-* incross, calculated as the percentage of total animals at each stage; mean ± SEM, n = 32 animals. F) Heat map of the proteomic signature of zebrafish *ins* mutants at 120 hpf compared to signatures from rodent (R) and human (H) diabetic proteome studies. Canonical pathways implicated in most studies are listed first. P-value cut-off set at < 0.05.

Scale bars: 10 μm (B), 500 μm (D).

Figure 1 – figure supplement 1. **ins, and not insb, is the predominant paralog expressed in zebrafish pancreatic islets.** A) Brightfield images of 72 hpf wild-type, *ins* mutant and *insb* mutant larvae. B-C) Confocal planes of pancreatic islets in 72 hpf wild-type and *insb* mutant larvae stained for Insulin (white). D) Blood glucose levels from wild-type and *ins +/-* 3 mpf adult animals; mean ± SEM, n=11 animals. E) Body length measurements from wild-type and *ins +/-* 50 dpf juvenile animals; mean ± SEM, n=5-6 animals. F) mRNA expression time course of *ins* and *insb* transcripts during zebrafish development from 0 to 120 hpf. G) 4 ng of control (ctrl) or *ins* morpholino (MO) was injected in non-transgenic or *Tg(ins:insb)* expressing (*insb* OE) embryos at the 1-cell stage and glucose levels were measured at 96 hpf; mean ± SEM, n=3 replicates. Scale bars: 250 μm (A), 5 μm (C, D).

Figure 2. **Highly efficient endoderm transplant technique rescues ins mutants to adulthood.** A) Schematic depicting the endoderm transplantation protocol; *sox32*-injected *ins +/-* donor cells (orange) were transplanted into host embryos (blue) to form chimeric animals. B-B‘) Confocal projection images of pancreatic islets from 48 hpf chimeric animals show β-cells from the host (green, B‘) and the transplanted *ins +/-* cells (magenta, B). C) Quantification of genotype abundance in the raised 3 mpf chimeric animals, determined by genotyping fin tissue; mean ± SEM, n = 3 transplant experiments, 18 – 32 animals per experiment. Scale bar: 10 μm.

Figure 2 – figure supplement 1. **sox32 mRNA-injected cells contribute to host endoderm upon transplantation.** A) High-resolution melt analysis patterns for genotyping *ins +/-* (blue), *ins +/-* (green) and *ins -/-* (brown) animals. B) Representative example of genotyping 31 chimeric adults from fin tissue, revealing 8 of 31 animals to be mutant (brown). C-C‘) Confocal images of a 48 hpf host embryo injected with *H2B-mCherry* mRNA (magenta) and transplanted with donor...
Figure 3. Small molecule screen in ins mutants reveals insulin-independent modulators of glucose metabolism. A) Relative glucose levels in 120 hpf ins mutant larvae after 36 hrs of treatment with anti-diabetic drugs (250μM Metformin, Pioglitazone, Dapagliflozin) or reported insulin mimetics (MLR1023, Fraxdin), mean ± SEM, n = 3-7 replicates. B) Schematic representation of the screening pipeline: ins mutant larvae were treated with small molecules starting from 84 hpf and total glucose levels measured at 120 hpf. C) Scatter-plot showing relative change in glucose levels upon treatment with 2233 small molecules. X and Y axes represent two replicates performed for each drug, with the dotted purple lines marking 0.9 on each axis. D) Relative glucose levels at 120 hpf upon treatment of ins mutants with the 3 hits – Flutamide, Vorinostat and ODQ, mean ± SEM, n = 6-7 replicates.

Figure 4 – figure supplement 1. A 96-well plate-adapted protocol to measure glucose levels is suitable for small molecule screening. A) Plotting the measured glucose interpolated from the regular standard curve (blue) shows a shifted curve in the presence of lysis buffer (orange) with a dynamic range still suitable for measuring glucose. B) Schematic showing the plate-adapted glucose readout assay. C) Relative glucose levels upon treatment of wild-type larvae from 4 to 6 dpf with beta-adrenergic agonist Isoprenaline (Iso) or both Isoprenaline and 250μM Metformin (Iso + Met); mean ± SEM, n = 4-5 replicates. D) Structures of the hit molecules - Flutamide, ODQ and Vorinostat.

Figure 4. Androgen Receptor (AR) antagonism improves glucose homeostasis in hyperglycemic larvae and adults. A) Relative glucose levels in ins mutants at 120 hpf upon treatment with various AR antagonists, mean ± SEM, n = 3-7 replicates. Enzalutamide and Bicalutamide treatments were performed at 20 μM concentration. B) Glucose levels in 72 hpf ins mutants after injection with 1 ng of ctrl or ar MO, mean ± SEM, n = 3 replicates. C) Glucose levels in 72 hpf wild types after injection with 1 ng of ctrl or ar MO, mean ± SEM, n = 4 replicates. D) RNA-seq analysis of ins mutant larvae treated with Flutamide or Cyproterone, showing differentially expressed genes (DEGs) compared to DMSO-treated larvae in blue and green, respectively. The red dots indicate DEGs common to both treatments. E) Workflow used for filtering candidate genes: 40 DEGs modulated in the same direction (both up or both down) were analyzed in relation to the microarray dataset (mutant vs phenotypically wild-type 108 hpf larvae). F) Glucose levels measured in adult Tg(ins:NTR) animals after β-cell ablation and intraperitoneal injection with vehicle (DMSO) or Flutamide; mean ± SEM, n = 5-6 animals.

Figure 4 – figure supplement 1. Flutamide reduces glucose in a dose-dependent manner, possibly exerting its effects through liver gluconeogenic enzymes. A) Dose response curve of the glucose lowering effect of Flutamide on ins mutant larvae, treated from 84 to 120 hpf; mean
± SEM, n = 3 replicates. B) The 12 genes differentially regulated in ins mutants compared to non-mutant siblings and modulated in the opposite direction upon treatment with Flutamide or Cyproterone, compared to DMSO; listed with their fold change (Log₂ scale) and expression levels under control condition (base mean); genes are ordered by the fold change under Flutamide vs DMSO condition. C) Wholemount in situ hybridization for ar transcripts in 120 hpf wild-type and ins mutant larvae showing expression in the brain and liver (red arrowheads). D) Schematic of insig1 and btg2 gene loci, with locations of Transcription Start Site (TSS) and Androgen Response Elements (ARE, blue arrowheads) indicated. E) Schematic of vehicle vs Flutamide treatment of Tg(ins:NTR) adult animals following β-cell ablation with Metronidazole (MTZ) injection to induce hyperglycemia. Scale bar: 250 μm.
**Titles for Supplementary Files**

**Supplementary file 1**: List of proteins with Log2FC > 1 or Log2FC < -1 from proteomic analyses comparing 120 hpf *ins* mutant and wild-type animals.