Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to:

Elevated 4-hydroxynonenal induces hyperglycaemia via Aldh3a1 loss in zebrafish and associates with diabetes progression in humans

Bowen Lou[1,2], Mike Boger[1], Katrin Bennewitz[1], Carsten Sticht[3], Stefan Kopf[4,5], Jakob Morgenstern[4,5] ,

Thomas Fleming[4,5], Rüdiger Hell[6], Zuyi Yuan[2], Peter Paul Nawroth[4,5,7] and Jens Kroll[1]*

[1]Department of Vascular Biology and Tumor Angiogenesis, European Center for Angioscience (ECAS), Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany.

[2]Cardiovascular Department, the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, 710048, People's Republic of China.

[3]Center for Medical Research (ZMF), Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany.

[4]Department of Internal Medicine I and Clinical Chemistry, Heidelberg University Hospital, Heidelberg, Germany.

[5]German Center for Diabetes Research (DZD), Neuherberg, Germany.

[6]Metabolomics Core Technology Platform, Centre for Organismal Studies, Heidelberg University, Heidelberg, Germany.

[7]Joint Heidelberg-IDC Translational Diabetes Program, Helmholtz-Zentrum, München, Heidelberg, Germany.

*Correspondence: Prof. Dr. Jens Kroll, Email: jens.kroll@medma.uni-heidelberg.de.

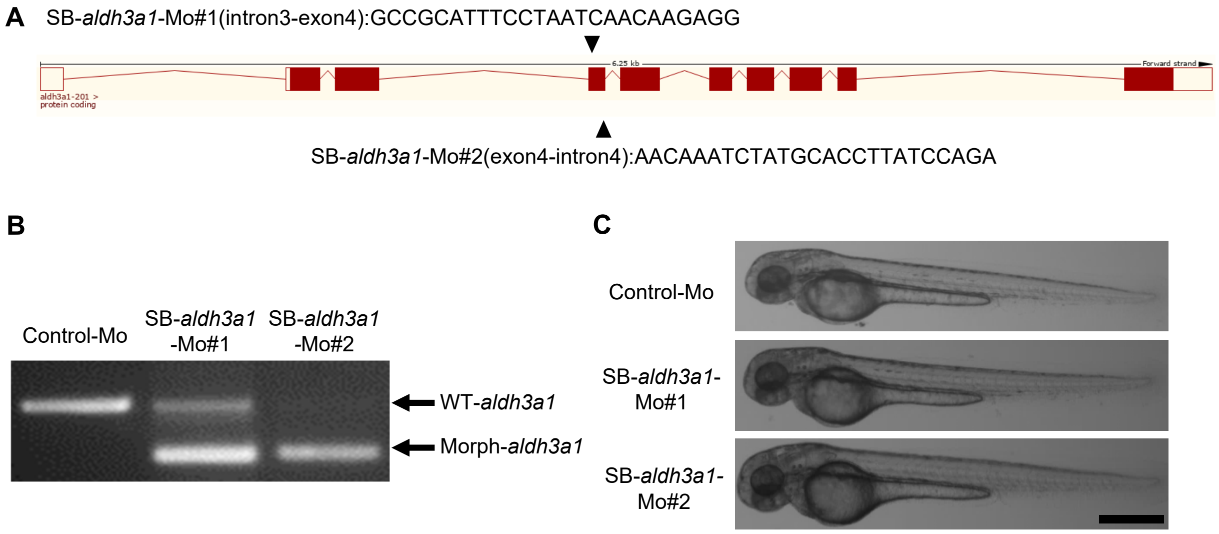

Figure S1. *Aldh3a1* morpholino design and validation.
(A). SB-*aldh3a1*-Mo#1 and SB-*aldh3a1*-Mo#2 target intron3-exon4 and exon4-intron4 junctions of *aldh3a1-201,* respectively. (B). Validation of splice-blocking morpholinos: SB-*aldh3a1*-Mo#1 and SB-*aldh3a1*-Mo#2. RT-PCR of Control-Mo, SB-*aldh3a1*-Mo#1 and SB-*aldh3a1*-Mo#2 injected larvae at 48 hpf show wild type and generation of morphant *aldh3a1* signals. 6 ng of morpholinos: Control-Mo, SB-*aldh3a*1-Mo#1 and SB-*aldh3a1*-Mo#2 were injected into the one-cell stage of zebrafish embryos, respectively. (C) Microscopic images showed normal gross morphology of zebrafish larvae at 48 hpf after morpholino injection. Black scale bar =500 µm. WT, wild type; Mo, morpholino; Morph: morpant.

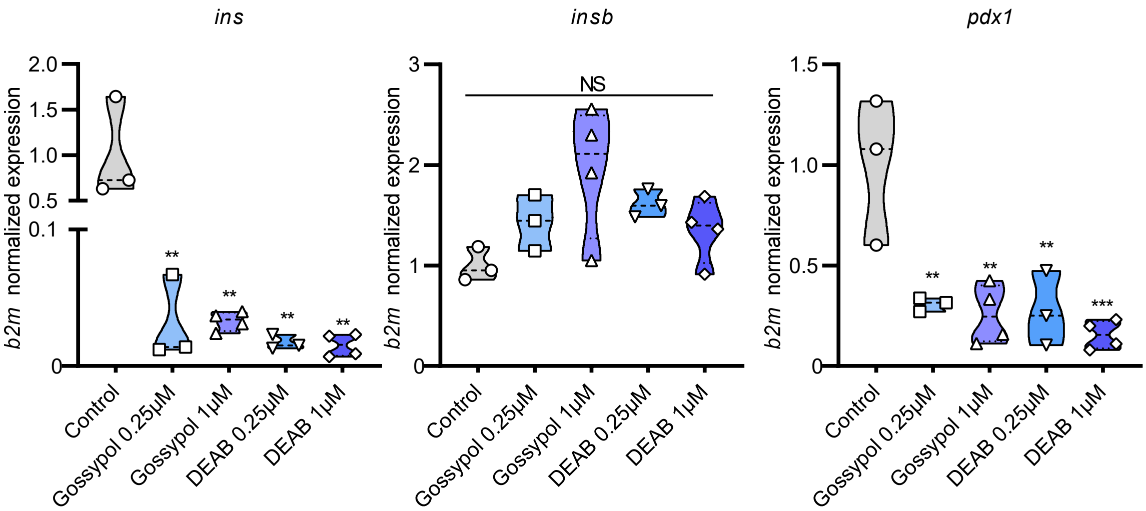

Figure S2. ***ins*** **and** ***pdx1*** **mRNA expression were decreased significantly while** ***insb*** **was unaltered in wild type zebrafish larvae at 48 hpf after ALDH inhibitor treatment.**

Expression of mRNA was analysed by RT-qPCR and was normalized to *b2m*. The average values of control group were standardized to 1, n = 3-4 clutches with 30 larvae per group. ALDH inhibitors, 0.25 μM and 1 μM Gossypol and DEAB treatment were started at 3 hpf and continued until end; Medium was changed every day. For statistical analysis one-way ANOVA followed by Sidak's multiple comparison test was applied, $^{**}p<0.01$ $^{***}p<0.001$. DEAB, N,N-diethylaminobenzaldehyde.

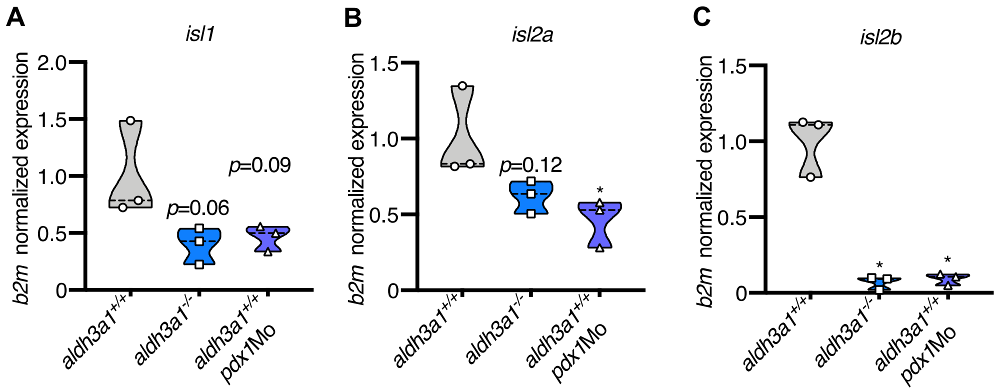

Figure S3. Aldh3a1 knockout larvae exhibited decreased insulin-related gene expression.
(A-C). Similar to *pdx1* morpholino mediated silencing, pancreas development-related genes, *isl1* (A) and *isl2a* (B) mRNA showed decreased trends while *isl2b* (C) mRNA reduced strongly in *aldh3a1*$^{-/-}$ larvae compared to *aldh3a1*$^{+/+}$ larvae at 48 hpf. 6 ng of morpholino: SB-*pdx1*-Mo was injected into the one-cell stage of zebrafish embryos. Expression of mRNA was analysed by RT-qPCR and expression was normalized to *b2m*. The average values of *aldh3a1*$^{+/+}$ larvae were standardized to 1, n = 3 clutches with 30 larvae per group. For statistical analysis one-way ANOVA followed by Sidak's multiple comparison test was applied, *p<0.05. *b2m, β2 microglobulin; isl1, ISL LIM homeobox 1*; *isl2a, ISL LIM homeobox 2a*; *isl2b, ISL LIM homeobox 2b*;NS, not significant;Mo, morpholino.

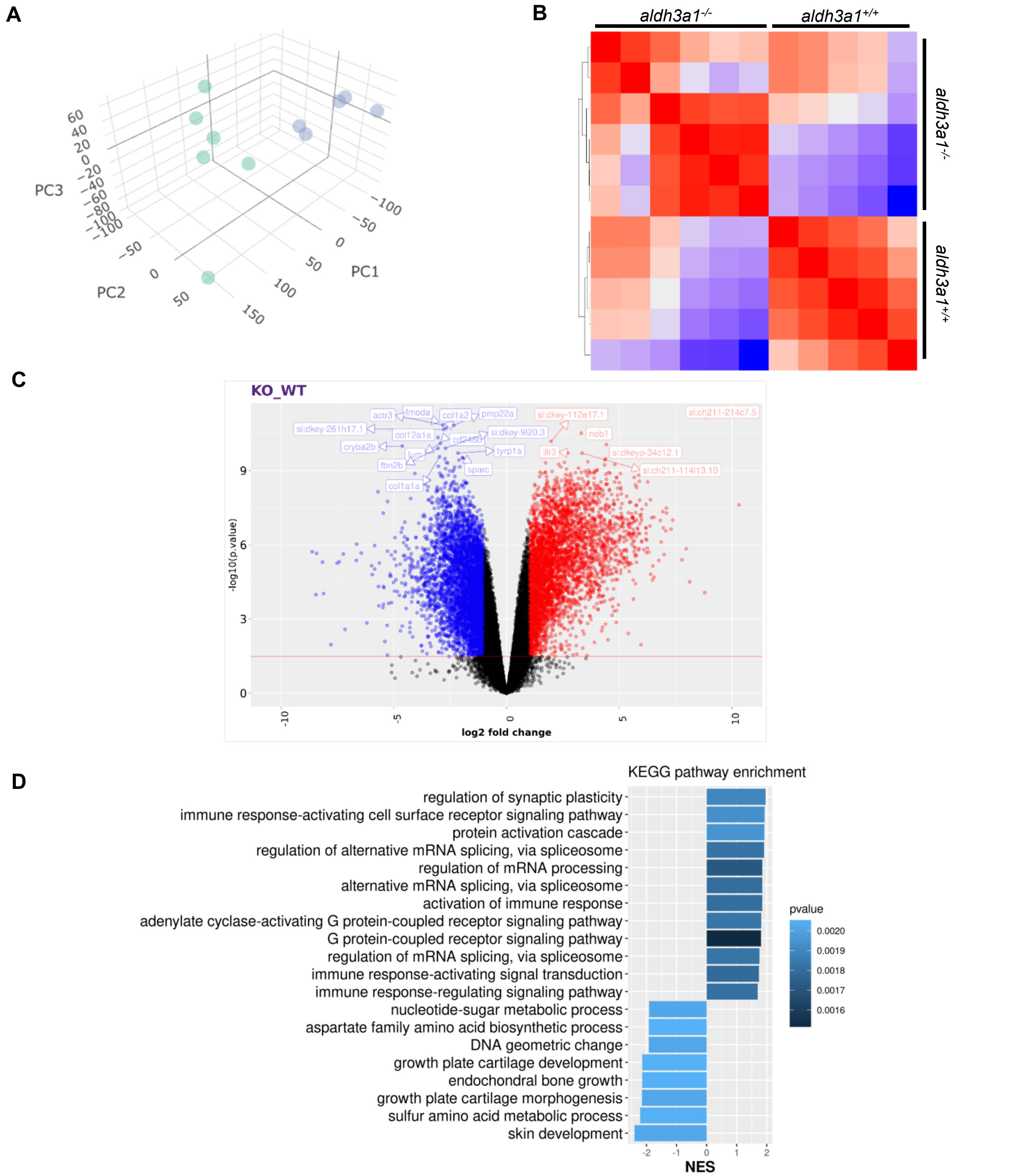

Figure S4. An overview of RNA Sequencing Results.
(A). Results of the quality control in gene expression analysis between *aldh3a1*$^{-/-}$ and *aldh3a1*$^{+/+}$ zebrafish larvae at 48 hpf. Principal component 1,2 and 3 are on the axis. The plots showed the *aldh3a1*$^{-/-}$ (n = 6) in green and *aldh3a1*$^{+/+}$ (n = 5) in blue. (B). Heatmaps of each samples showed comparable property between *aldh3a1*$^{-/-}$ and *aldh3a1*$^{+/+}$ zebrafish larvae. (C). Volcano plot showed significant down-regulated (blue dots) and up-regulated (red dots) genes between *aldh3a1*$^{-/-}$ and *aldh3a1*$^{+/+}$ zebrafish larvae. (D). Top 20 KEGG pathway enrichment of RNA Sequencing results between *aldh3a1*$^{-/-}$ and *aldh3a1*$^{+/+}$ zebrafish larvae.

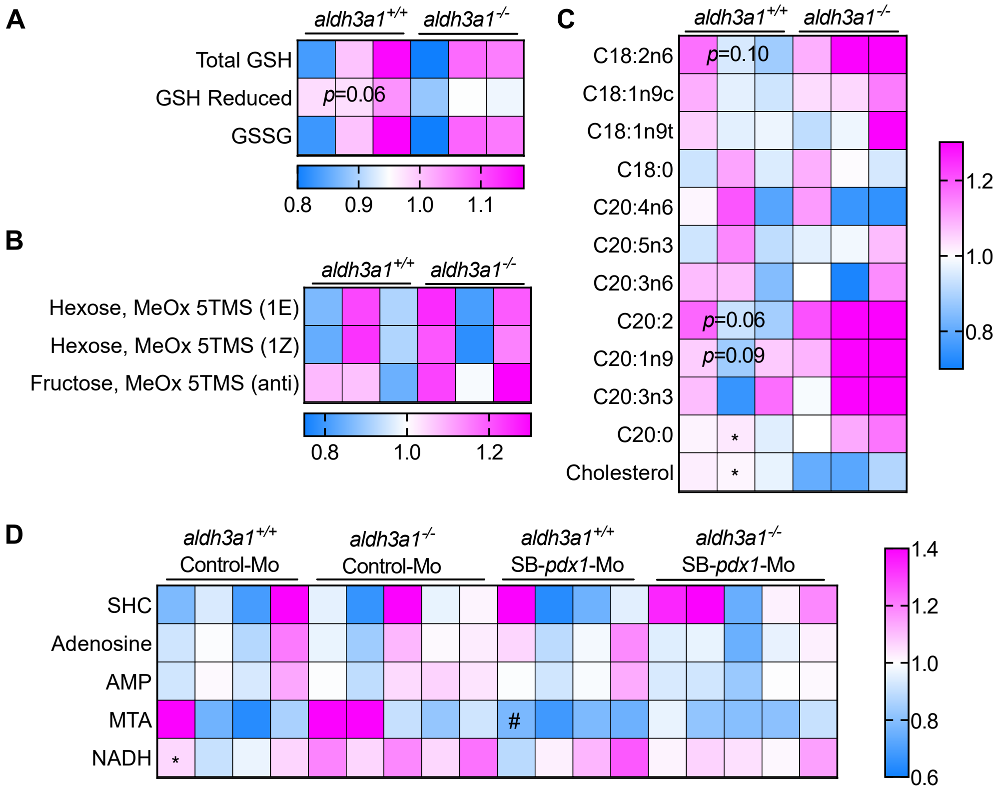

Figure S5. Metabolomic screening displayed several alternations between *aldh3a1*$^{-/-}$ and *aldh3a1*$^{+/+}$ *Tg(fli1:EGFP)* zebrafish larvae at 96 hpf.

(A-C).Heatmap showed the metabolomic screening of thiols (A), sugar level (B) and fatty acids (C) between *aldh3a1*$^{-/-}$ and *aldh3a1*$^{+/+}$ zebrafish larvae at 96 hpf: (A). GSH reduced showed decreased sign in *aldh3a1* mutants; (B). No changes in sugar level; (C). C20:0 was increased while cholesterol was decreased in *aldh3a1* mutants; n = 3 clutches with 50 larvae, for statistical analysis Student's t-test was applied; *p<0.05, **p<0.01, ***p<0.001 (D). Heatmap showed adenosines among each group of zebrafish larvae at 96 hpf. *: NADH was increased significantly in *aldh3a1*$^{-/-}$ zebrafish larvae compared to *aldh3a1*$^{+/+}$ larvae with control morpholino injection. $^{\#}$: MTA was decreased significantly in *aldh3a1*$^{-/-}$ zebrafish larvae compared to *aldh3a1*$^{+/+}$ larvae with *pdx1* morpholino injection. 6 ng of each morpholino was injected into the one-cell stage of zebrafish embryos respectively. n = 4 to 5 clutches with 50 larvae, for statistical analysis one-way ANOVA followed by Sidak's multiple comparison test was applied. In heatmap, the average values of *aldh3a1*$^{+/+}$ larvae/ *aldh3a1*$^{+/+}$ larvae with control morpholino injection were standardized to 1.

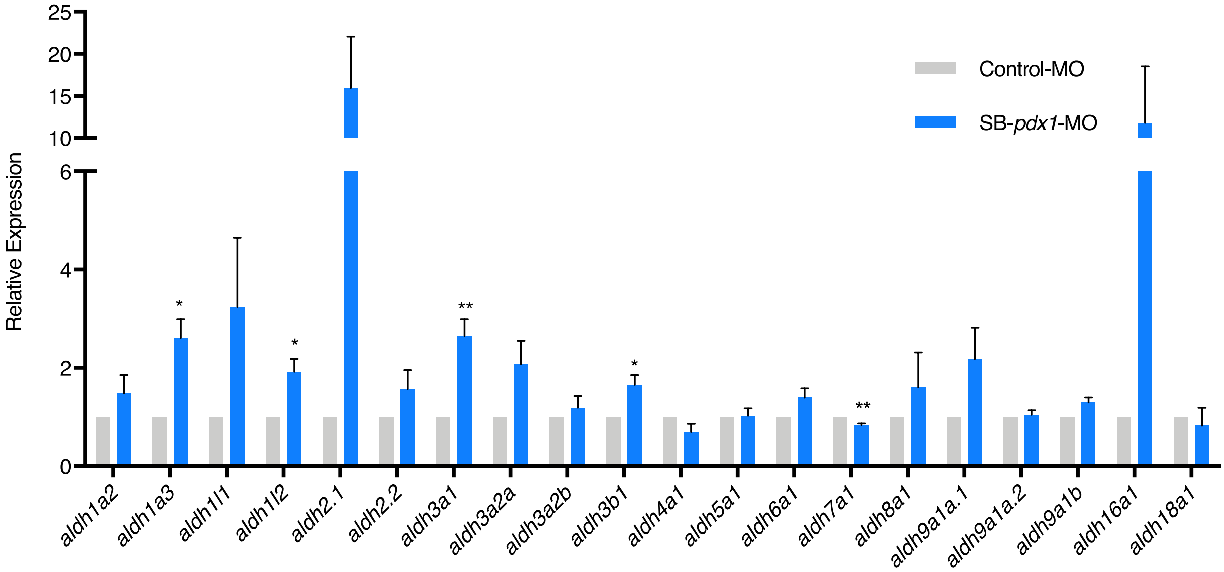

Figure S6. *Aldh* mRNA levels in hyperglycaemic zebrafish larvae.
Aldh1a3, aldh1l2, aldh3a1 aldh3b1 mRNA levels were raised and *aldh7a1* was decreased significantly in zebrafish larvae at 48 hpf after *pdx1* morpholino injection. 6 ng of morpholinos: Control-Mo and SB-*pdx1*-Mo were injected into the one-cell stage of zebrafish embryos, respectively. Expression of mRNA was analyzed by RT-qPCR at 48 hpf and expression was normalized to beta-actin. Values for Control-Mo injected zebrafish larvae were standardized to 1; n = 3 clutches with 30 larvae per group; Mean ± SEM, for statistical analysis Student's t-test was applied, *p<0.05, **p<0.01.

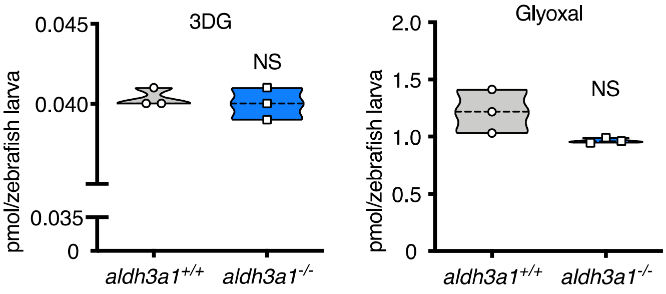

Figure S7. 3DG and glyoxal were unchanged at 96 hpf.
N = 3 clutches with around 50 larvae per group. For statistical analysis paired samples t-tests were applied. NS, not significant.

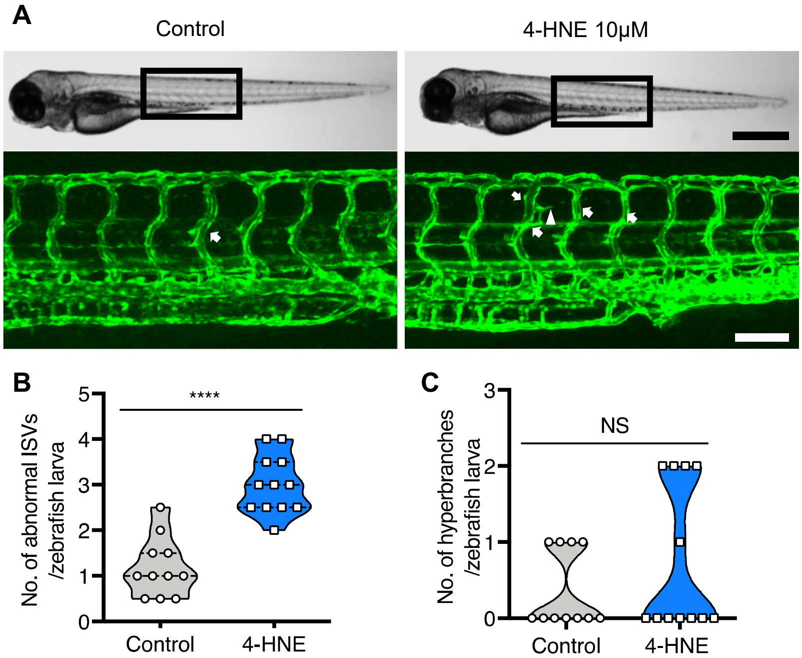

Figure S8. Exogenous 4-HNE partially altered trunk vasculature morphology in *Tg(fli1:EGFP)* zebrafish larvae.
Exogenous 4-HNE partially altered trunk vasculature morphology, leading to increased formation of abnormal ISVs (white arrows) in *Tg(fli1:EGFP)* zebrafish larvae at 96 hpf. 10 µM 4-HNE treatment were started at 3 hpf and continued until 96 hpf; Medium was changed every day. (A). Light microscopic images showed the gross morphology of zebrafish larvae and black boxes indicate region seen in the confocal images. White scale bar = 100 µm, black scale bar =500 µm. (B-C). Quantification of abnormal ISV and hyper branches formation in violin plots, n = 11-12 per group, for statistical analysis one-way ANOVA followed by Sidak's multiple comparison test was applied, ****p<0.0001. NS, not significant.

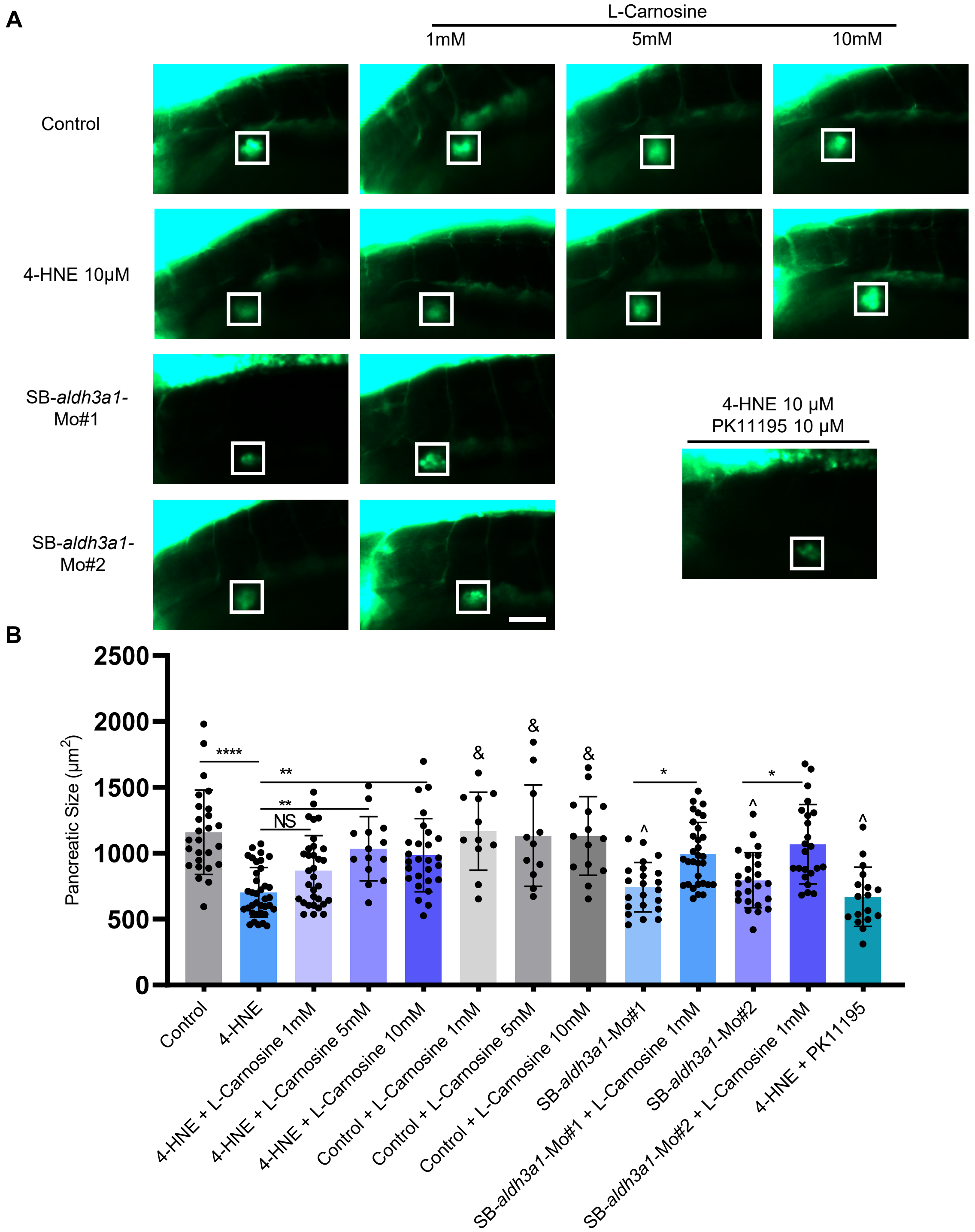

Figure S9. L-Carnosine can rescue the pancreas disruption caused by both *aldh3a1* silencing and 4-HNE incubation dose-dependently in *Tg(hb9:GFP)* zebrafish larvae.
(A).1mM L-Carnosine can rescue the pancreas disruption caused by both *aldh3a1* silencing morpholinos injection in *Tg(hb9:GFP)* zebrafish larvae at 72 hpf. But 1mM L-Carnosine just showed rescued trend in 10μM 4-HNE incubation caused pancreas disruption, and 5mM and 10mM can restore the pancreas morphology significantly. 10 μM PK11195 can not rescue the pancreas disruption caused by 4-HNE treatment. White box indicates the primary pancreas; White scale bar = 50 μm. (B). Quantification of area size of the early pancreas, n = 11-36 per group. For statistical analysis one-way ANOVA followed by Sidak's multiple comparison test was applied, *p<0.05, **p<0.01, ****p<0.0001. [&], p> 0.05 compared to Control group. ^, p<0,0001 compared to Control group. NS, not significant.

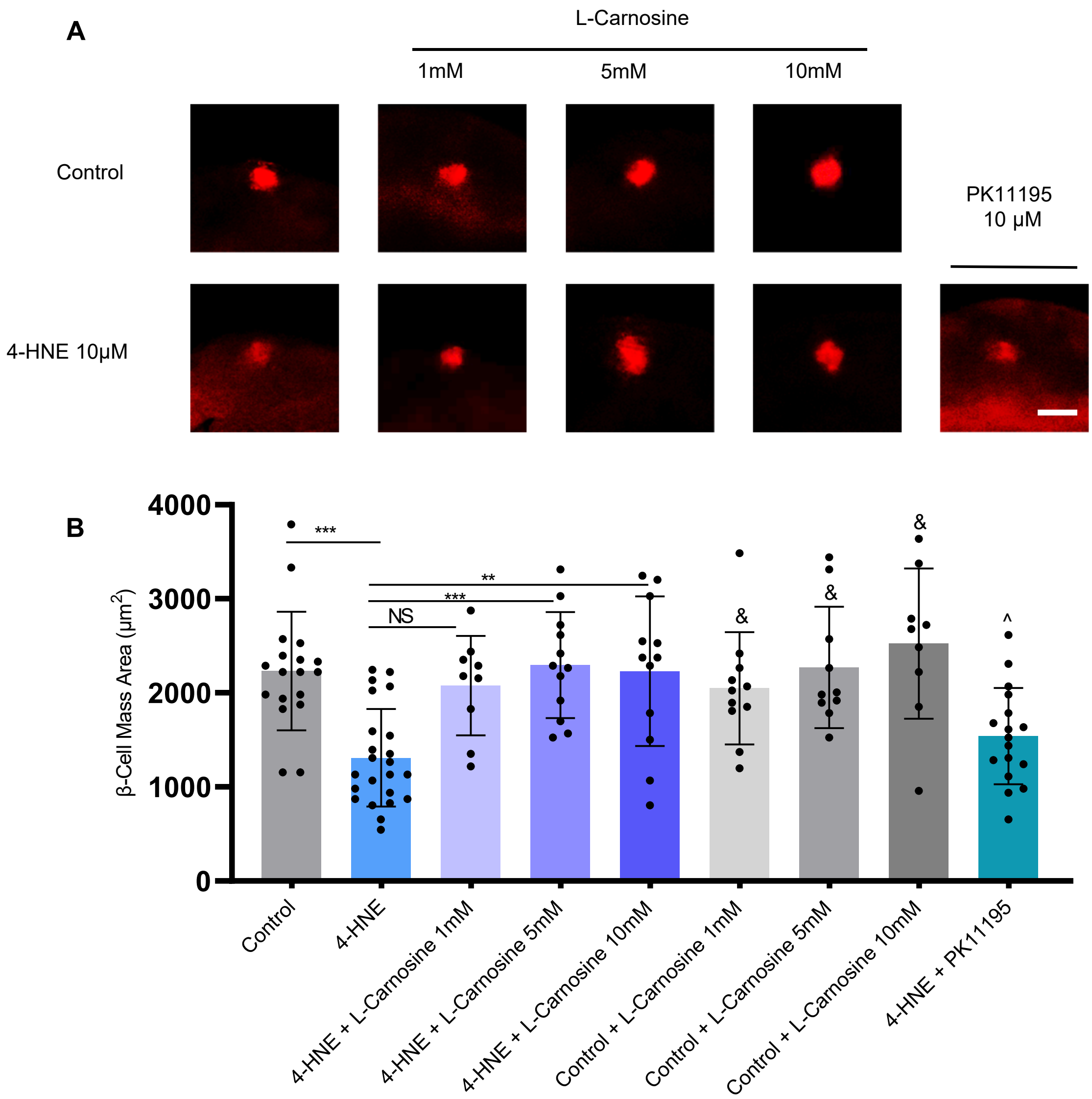

Figure S10. L-Carnosine can rescue the pancreas disruption caused by 4-HNE incubation dose-dependently in *Tg(ins:nfsB-mCherry)* zebrafish larvae.

(A). 1mM L-Carnosine showed rescued trend in pancreas disruption caused by 10µM 4-HNE incubation in *Tg(ins:nfsB-mCherry)* larvae; and 5mM and 10mM can restore the pancreas morphology significantly. 10 µM PK11195 can not rescue the pancreas disruption caused by 4-HNE treatment. White scale bar = 50 µm. (B). Quantification of area size of the early pancreas, n = 9-17 per group. For statistical analysis one-way ANOVA followed by Sidak's multiple comparison test was applied, **$p<0.01$, ***$p<0.0001$. $^{\&}$, $p> 0.05$ compared to Control group. ^, $p=0.039$ compared to Control group. NS, not significant.

Time/Concentration	0μM	5μM	10μM	25μM
24hpf	30	30	30	30
48hpf	29	27	26	29
72hpf	27	24	24	24
96hpf	27(1edema)	24(3edema)	24(6edema)	20(12edema)

Table S1. Survival condition of 4-HNE incubation in zebrafish larvae from 24 hpf to 96 hpf.

Group	Age	Sex	BMI	HbA1c(%)	Fasting Glucose (mg/ml)	LDL (mg/dl)	TG (mg/dl)	4-HNE (ng/ml)
Control	48	male	26.8	5.4	90	214	111	17.98
	70	female	24.9	5.5	96	149	66	7.28
	66	female	19.3	5.3	92	138	107	21.30
	59	female	29.3	5.1	89	153	102	21.70
	63	male	34.7	6.0	98	140	182	15.86
	45	female	23.6	5.3	87	116	76	12.84
	58	male	25.4	5.2	96	202	112	9.38
	40	female	24.3	5.3	91	81	37	20.64
	60	male	24.8	5.9	95	116	96	11.22
Mean(Control)	56.1		25.9	5.4	92.7	149.1	98.8	15.36
T2DM	65	female	30.5	7.4	195	96	189	31.78
	56	female	28.0	8.9	148	78	126	36.08
	68	male	24.6	7.3	147	108	108	25.48
	46	male	31.1	7.3	181	131	245	17.90
	64	female	23.3	6.5	136	157	105	12.10
	76	male	24.0	7.8	205	38	64	25.40
	64	female	28.4	9.9	225	173	181	36.90
	63	male	25.7	7.4	155	127	115	9.98
	54	male	31.0	7.7	148	115	116	18.34
	63	male	31.8	5.6	130	98	85	22.18
	49	female	28.1	5.8	115	126	112	18.98
Mean(T2DM)	60.7		27.9	7.4	162.3	113.4	131.5	23.19
p	0.333	0.673	0.247	<0.001	<0.001	0.085	0.142	0.0322

Table S2. Baseline characteristics of the control and T2DM patient cohorts. All parameters (4-HNE excluded) were determined prior to collection.

Morpholinos	Sequence
SB-*aldh3a1*-Mo#1	5'-GCCGCATTTCCTAATCAACAAGAGG-3'
(targets intron3 – exon4 junction)	
SB-*aldh3a1*-Mo#2	5'-AACAAATCTATGCACCTTATCCAGA-3'
(targets exon4 – intron4 junction)	
SB-*pdx1*-Mo	5'-GATAGTAATGCTCTTCCCGATTCAT-3'
(targets translation start site)	
Control-Mo	5'-CCTCTTACCTCAGTTACAATTTATA-3'

Genotyping primer name	Primer sequence (5' to 3')
*Aldh3a1*Mo-Genotyping-1.1*	AGGTGGCAGAGCGTGAGATG
*Aldh3a1*Mo-Genotyping-1.2*	GAACCCCTCCTGTCACCACC

*These primer pair can be used for both *aldh3a1* morpholinos as the PCR product spans both target sites.

Table S3. Morpholinos and the genotyping primers for zebrafish *aldh3a1* morpholinos.

CRISPR-construct name	Oligonucleotide sequence (5‘ to 3‘)
Aldh3a1-CRISPR-for	TAGGGGTCTGGATCTGCCTGAC
Aldh3a1-CRISPR-rev	AAACGTCAGGCAGATCCAGACC
Genotyping primer name	**Primer sequence (5‘ to 3‘)**
Aldh3a1-Genotyping#1.1	ACATGGACTGAACAGTGACCTTGG
Aldh3a1-Genotyping#1.2	CTCACGCTCTGCCACCTTGAT
Aldh3a1-Genotyping#1.3	CTCTTTGTGAAATCCTAAACCCT
Aldh3a1-Genotyping#1.4	TGTCTGCATGGCGTTCAGTGA

Table S4. CRISPR construct and genotyping primers for zebrafish *aldh3a1.*

qPCR primer name	Primer sequence	qPCR primer name	Primer sequence
β-actin-qPCR-for	ACGGTCAGGTCATCACCATC	aldh6a1-qPCR-rev	TCTTTGGCCTGAGGTGAGAT
β-actin-qPCR-rev	TGGATACCGCAAGATTCCAT	aldh7a1-qPCR-for	AACCGCAGCACCGAATATGT
b2m-qPCR-for	ACTGCTGAAGAACGGACAGG	aldh7a1-qPCR-rev	TCTGCTATGGTTGCCTGACG
b2m-qPCR-rev	GCAACGCTCTTTGTGAGGTG	aldh8a1-qPCR-for	CTCCAGCTTCTCCAATCAGG
aldh1a2-qPCR-for	AACCACTGAACACGGACCTC	aldh8a1-qPCR-rev	GTAAACGCTCCGCTCCAC
aldh1a2-qPCR-rev	ATGAGCTCCAGCACACGTC	aldh9a1a.1-qPCR-for	GCTCTGTTCGAAATCTGTGTTCC
aldh1a3-qPCR-for	CGTGTTTGCAGACTCAGACC	aldh9a1a.1-qPCR-rev	CGACCAGTTGCTGGCTCGTA
aldh1a3-qPCR-rev	TGAAGAAAGCCCCCTTCTG	aldh9a1a.2-qPCR-for	TCCCATGGTGGCTAAAGTGT
aldh1l1-qPCR-for	GCTGCCCAGACACAGAGG	aldh9a1a.2-qPCR-rev	TAGCTGCCATTTCCAAAACC
aldh1l1-qPCR-rev	AACCCTCCCTTCTTATCACCA	aldh9a1b-qPCR-for	GGAGCAAGCCAAGAACGA
aldh1l2-qPCR-for	AGCCGCTTCAATGGATGTAG	aldh9a1b-qPCR-rev	GGATCTGCAGGGCTGAAA
aldh1l2-qPCR-rev	GAACACCAGCGCATTTCTG	aldh16a1-qPCR-for	CCACAGGGTGTTGTGACGGT
aldh2.1-qPCR-for	CGCACTGTATATCGCCAGTTTA	aldh16a1-qPCR-rev	AGGAGGCCAGGAAGAGCAGT
aldh2.1-qPCR-rev	GGACCAAACCCTGGGATAAT	aldh18a1-qPCR-for	GCACAGGAAGCCCTGTCTAT
aldh2.2-qPCR-for	TGCAGTCTCCTTCAGTGTGG	aldh18a1-qPCR-rev	CTCTTCACGAGTGCTCACCA
aldh2.2-qPCR-rev	TGCCCAGCCAGCATAATAC	glo1-qPCR-for	AGCAGACAATGCTGCGGGTG
aldh3a1-qPCR-for	CACTGTTGATACTTTACCTTTTGGAG	glo1-qPCR-rev	CTACGGGAGAACGTCCAGGC
aldh3a1-qPCR-rev	CAAACGTGTGTTTCCCATGA	ins-qPCR-for	GGTCGTGTCCAGTGTAAGCA
aldh3a2a-qPCR-for	TGATGAATCTGAGTGTTACATTGC	ins-qPCR-rev	GGAAGGAAACCCAGAAGGGG
aldh3a2a-qPCR-rev	TGGCCCAAAGATCTCTTCC	insb-qPCR-for	CCTGGAGACCTTGCTGGCTTTG
aldh3a2b-qPCR-for	CACTTCTCTGTCAGCTCTCTGC	insb-qPCR-rev	CCAGGTGGTAGATGGTGCAGG
aldh3a2b-qPCR-rev	GATAGCGGCCCATACCACT	isl1-qPCR-for	AGGGTATGGCAGCCGAGGTC
aldh3b1-qPCR-for	CATGACTCTTCCTGGTTTACCC	isl1-qPCR-rev	GCTTGCATGCTTAGTACTTGGGC
aldh3b1-qPCR-rev	TGATAGTTGCCCATCCCACT	isl2a-qPCR-for	CATCCCAGAACCTGTGCCAGT
aldh4a1-qPCR-for	TGGACCAAAGACATCCGATT	isl2a-qPCR-rev	ACTCGTATGACCCGTGGGCT
aldh4a1-qPCR-rev	AGCAGAACTTTGCAACTTGGT	isl2b-qPCR-for	GCTGGGAGCGGGATACAAGG
aldh5a1-qPCR-for	GGGCCTCTTATCAACTCACG	isl2b-qPCR-rev	TCCGGACTTCTTTTTGGAATGATCC
aldh5a1-qPCR-rev	TCCATGATCCACAGCGTCT	pdx1-qPCR-for	ACACGCACGCATGGAAAGGACA
aldh6a1-qPCR-for	GTCTACGTGTCAATGCAGGTG	pdx1-qPCR-rev	GCGGGCGCGAGATGTATTTGTT

Table S5. qPCR primers.