Supporting Information

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SI Materials and Methods

Cloning and Expression of Zebrafish (zf)12-LO in *Pichia pastoris*. The coding sequence of *alox12* was amplified via RT-PCR using total RNA from embryos [two days post fertilization (dpf)], Pfx-Polymerase and the following primers: zf*alox12* coding sequence for: 5′-CGACAACTTGAGAAGAT**CAAA**ATG*CACCATCATCACCACCAT*GAGTACAAAGTGACAGTGGC-3′, zf*alox12* coding sequence rev: 5′-GCAAGACCGGTCTTCTCTCAAATAGTGATGCTGTTTTCCA-3′. The amplified cDNA was cloned into the pPICZ A vector (Invitrogen, Sweden) via in vivo recombination. Transformed bacteria were selected with Zeocine. Positive colonies were amplified and the resulting plasmid was purified, sequenced, and restricted with *PmeI* for transformation of *Pichia pastoris*. Yeast cells were transformed following Invitrogen manual of the EasySelect™ *Pichia* Expression Kit. For expression, an overnight culture was diluted 1:1,000 in Buffered Minimal Glycerol medium (Invitrogen formula), pH 6.8, and incubated 72 h at 26 °C. The OD_{600}-value of the preculture was measured, cells were harvested (3,000 × *g*, 4 °C, 7 min), the pellet was resuspended in Buffered Minimal Methanol medium (Invitrogen formula) to reach an OD_{600} 20–30, and cells were incubated 48 h at 26 °C. To extract the protein, cells were harvested (3,000 × *g*, 4 °C, 7 min) and resuspended in 1/100 expression volume breaking buffer (100 mM Tris/HCl, pH 8, 100 mM KCl, 10% glycerol, 5 mM mercaptoethanol). An equal volume of glass beads was added and cells were broken by stirring the mixture with a bead beater (Biospec Products, Bartlesville, USA) (6 × 1 min, 10 min break). Glass beads were removed by filtration and the resulting solution was centrifuged (10,000 × *g*, 4 °C, 7 min) to remove the broken cells. To precipitate DNA, 10% streptomycin sulfate (Sigma, Sweden) was added drop wise followed by incubation on ice for 30 min and centrifugation (10,000 × *g*, 10 min, 4 °C). Imidazol was added to the supernatant to a final concentration of 20 mM. Recombinant zf12-LO was purified by affinity chromatography on a Ni^{2+}-nitrilotriacetic acid column. Equilibration of the column was done with 25 mM Tris/HCl, pH 7.8/20 mM Imidazol. The enzyme containing supernatant was applied, followed by a washing step with 25 mM Tris/HCl, pH 7.8/20 mM Imidazol and 25 mM Tris/HCl, pH 7.8/1 M KCl/40 mM Imidazol, respectively. Finally, the protein was eluated with 25 mM Tris/HCl, pH 7.8/100 mM Imidazol.

Antibody Production and IgG Purification

Polyclonal rabbit anti-zf12-LO antiserum was raised by subcutaneous injections of 30 μg of purified enzyme mixed with complete Freund's adjuvant (Sigma) in the neck skin of a rabbit. Booster injections using the same amount of enzyme and adjuvant were given after 4 and 6 w. Blood (30 mL) was drawn week 8 and allowed to coagulate at room temperature followed by centrifugation (2,000 rpm, 10 min, Hereus Sepatech rotor #2705). The IgG fraction of the antiserum was obtained by chromatography on a Protein A Hi Trap column (GE Healthcare) according to the manufacture's instructions. After elution of IgG with 4 mL of glycine buffer (0.1 M pH 3.0) the buffer was changed on a PD-10 column (GE Healthcare) eluted with 75 mM Tris/HCl, pH 7.0, and frozen in aliquots. The polyclonal rabbit anti-zf12-LO antiserum detected exogenously expressed zf12-LO as a band migrating at approximately 77 kDa on immunoblots in agreement with the expected molecular weight.

RT-PCR. To verify the knock-down of zf12-LO, RT-PCR analyses were performed. Total RNA was isolated from 20 embryos (two dpf) using the TRIzol reagent method (Invitrogen, Sweden) and used for first-strand cDNA synthesis using reverse transcription with Superscript II (Invitrogen, Sweden). PCR analyses were performed using Taq-Polymerase in a 2720 Thermal Cycler (Applied Biosystems) according to the manufacturer's instructions. (Primers: PCRzf*alox12* forward primer: 5′-ATCCTGGCCTGGACTTCTG-3′, PCRzf*alox12* reverse primer: 5′-GGTCACTCC AGGACTTTCCA-3′). For controlling the sequence of the PCR products an automated sequencing was performed (SEQLAB, Göttingen, Germany).

Plastic Embedding, Sectioning of Embryos, and Staining. Representative embryos of every injection (10 of each) were fixed in Bouin's fixative (Polysciences, Warrington, PA) overnight at 4 °C. Afterwards embryos were washed with 70% ethanol at room temperature in 30 min periods until no yellow color was seen in the washing solution, followed by an ethanol dehydration serial at room temperature, 30 min for each step (1 × 80% EtOH, 1 × 95% EtOH, 2 × 100% EtOH). For embedding, embryos were infiltrated with catalyzed JB4 resin (Polysciences, Warrington, PA) overnight at 4 °C. The embryos were then transferred to activated JB-4 and embedded in a mould. Sections (10 μm) were obtained using the Leica RM2155 microtome and counterstained with Hematoxylin/Eosin using standard procedures.

Analysis of LO Activity in zf Embryos. Approximately 100 embryos were washed two times with PBS followed by an incubation in 1× PBS without Ca/1 mM EDTA, for 30 min at 37 °C. Embryos were homogenized using syringes with 22G needles followed by centrifugation (16,100 × *g*, 15 min, 4 °C). The supernatant was incubated with substrate mix (final concentration: 5 mM ATP, 1.3 mM $CaCl_2$, 1.2 mM EDTA, 100 μM arachidonic acid, 10 μM 13-hydroperoxy-octadecadienoic acid, 77 mM Tris/HCl pH 7.5) for 30 min at 37 °C. Incubations were terminated by adding three reaction volumes of cold stop solution (60% acetonitrile, 40% H_2O, 0.1% acetic acid vol/vol) including 3.3 μM 17(*S*)-hydroxy-(13*Z*, 19*Z*, 15*E*)-docosatrienoic acid as internal standard. Aliquots were injected onto a C18 HPLC column (Waters Nova Radial Pak), and arachidonic acid metabolites were isocratically eluted with acetonitrile/H_2O/acetic acid (60:40:0.1, vol/vol) at a flow rate of 1 mL/min. The eluate was monitored at 235 nm for detection of hydroperoxy-and hydroxy-eicosatetraenoic acids (H(P)ETEs).

Results

Confirmation of the Knock-Down of zf12-LO in Embryos via RT-PCR and 12-HETE Measurement. To prove that the incorrect splicing of the zf12-LO mRNA caused by MO-2 led to improper translation, RT-PCR analyses with total RNA isolated from wild type (WT) and morphants were performed. Analyses of the WT, MO-1, MO-1, and -2 mis PCR products showed an amplification of the corresponding product (497 bp) of the correctly spliced 12-LO mRNA. On the other hand, in the sample of MO-2 treated embryos four PCR products were amplified (Fig. S5*A*). Sequence analyses demonstrated that these products resulted from different splice variants of the 12-LO mRNA (Fig S5*B*).

The expected PCR product that corresponds to the splice variant where exon 1 was directly linked to exon 3 after blockage of the second donor splice site by MO-2, was seen as a 292 bps fragment (Fig. S5 *A* and *B*, band 4). The 582 bps and 377 bps

fragments correspond to splice variants where exon 2 and/or intron 2 were not spliced out correctly (Fig. S5 *A* and *B*, band 1 and 3). These three isoforms contain a stop codon, 150 to 360 nucleotides downstream of the ATG, which leads to translation of peptides that comprise only the first 50 to 120 amino acids of the whole protein and therefore lack most of the known catalytic domain of the enzyme. In addition, a weak 497 bps fragment, corresponding to the correctly spliced mRNA, appeared (Fig. S5 *A* and *B*, band 2).

To further confirm the specific knock-down of the enzyme in the embryos, the enzymatic activity of zf12-LO was investigated. For this purpose WT, MO-1 and -2 mis and MO-1 and -2 treated embryos at two dpf were homogenized and incubated with arachidonic acid. The resulting metabolites were then analyzed by RP-HPLC. The analyses showed a formation of 12-HETE in WT (Fig. S6*B*) and MO-1 and -2 mis treated embryo homogenates (Fig. S6 *D* and *F*), whereas homogenates of embryos treated with MO-1 or MO-2 exhibited negligible 12-HETE production (Fig. S6 *C* and *E*). In homogenates of WT embryos, which were not incubated with exogenous arachidonic acid, no formation of 12-HETE could be detected (Fig. S6*A*). To define the product as 12-HETE cochromatography with 12-HETE synthetic standards was performed, which showed coelution of the zf product with the 12-HETE standard. This result shows that the observed phenotype of the morphants arose through a specific knock-down of the zf12-LO.

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359 DFHV.....NTKAREQLI 416   human 5(S)-LO
351 DFQL.....NTRARTQLI 408   human 12(S)-LO
351 DFQL.....NVRARTGLV 408   human 15(S)-LO-1
364 EFSF.....NTLARELLI 421   human 15(S)-LO-2
359 DFHV.....NTKAREQLI 416   mouse 5(S)-LO
351 DFQL.....NTLARNNLV 408   mouse ep 12(S)-LO
352 DLQL.....NVRARSDLI 409   mouse 15(S)-LO
351 DFQL.....NTRSRTQLI 408   mouse pl 12(S)-LO
365 EFYI.....NTLARELLV 422   mouse 8(S)-LO
352 DFQV.....NVRARNGLV 409   rabbit 15(S)-LO
510 DSGN.....NALARQILI 567   arabidopsis LO-1
545 DAGY.....NARARQSLV 602   arabidopsis LO-2
375 EFQV.....NTLARQTLI 432   xenopus 12(S)-LO
389 EFYS.....NSIGRAVLL 446   human 12(R)-LO
389 EFYS.....NSIGRALLL 446   mouse 12(R)-LO
358 EFQV.....NCRGRTQLL 415   zebrafish 12-LO
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Fig. S1. Amino acids involved in stereospecificity, alignments of S- and R-LOs: Alignment of residues implicated in the control of LO stereospecificity from a representative selection of S-LOs and all known vertebrate R-LOs. According to the "Coffa site" rule the amino acid residue marked with an asterisk is conserved as an Ala in S-LOs. An exception is the mouse platelet 12S-LO with a Ser at this position. The equivalent residue in R-LOs is conserved as a Gly. The additionally highlighted residue Asp/Glu plays an important role in substrate entry and thus chirality of the product. In most of S-LOs an Asp is found at this position whereas in R-LOs Glu is predominant.

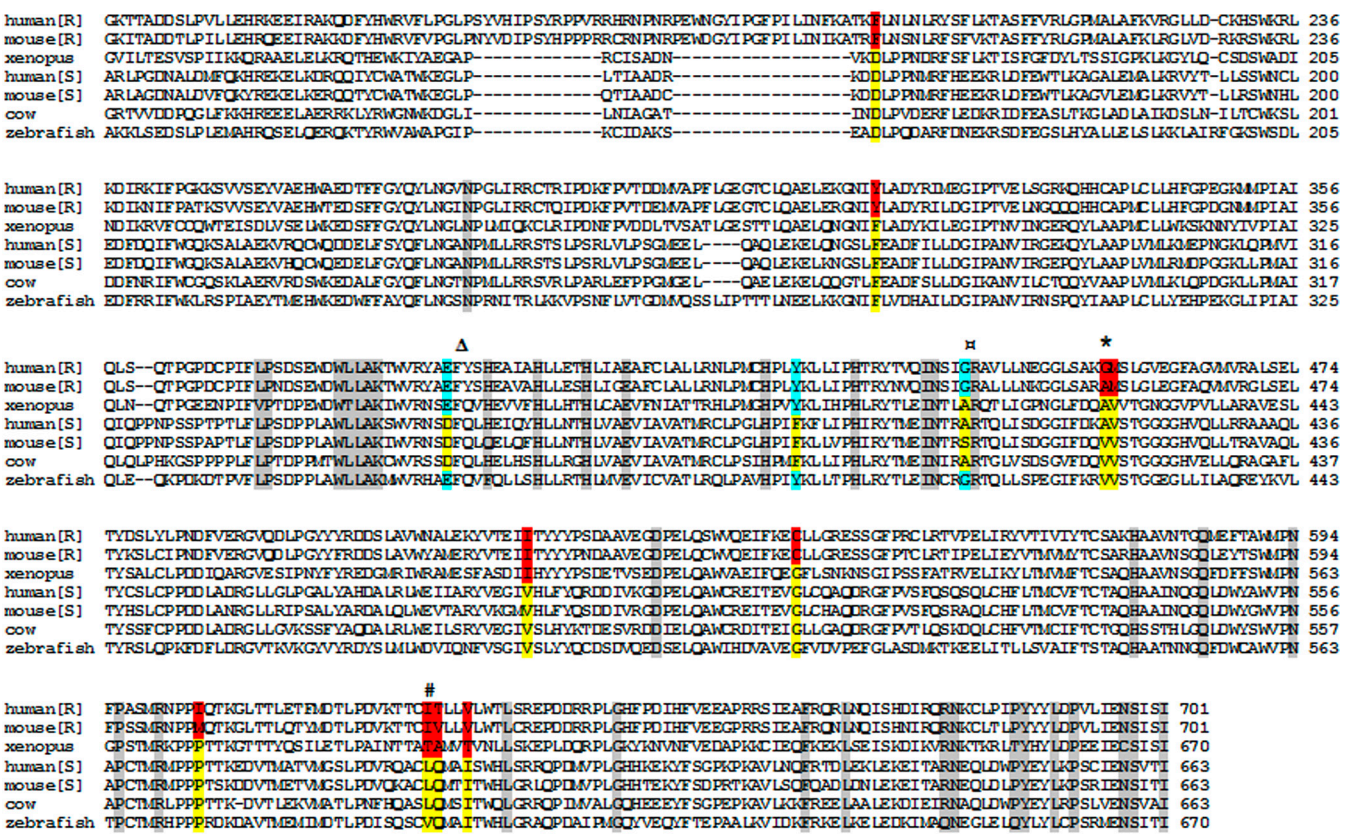

Fig. S2. Alignment of zf12-LO with other 12S- and 12R-LOs. A representative selection of 12S- and 12R-LOs are aligned with zf12-LO. Amino acids depicted in red and in blue indicates zf enzyme sequence similar to S-LOs and R-LOs, respectively. Concerved amino acids are depicted in gray. Borngräber I determinant (empty triangle), Coffa determinant (empty circle), Sloane determinant (asterisk) and Jisaka/Borngräber II determinant (number symbol) are pointed out.

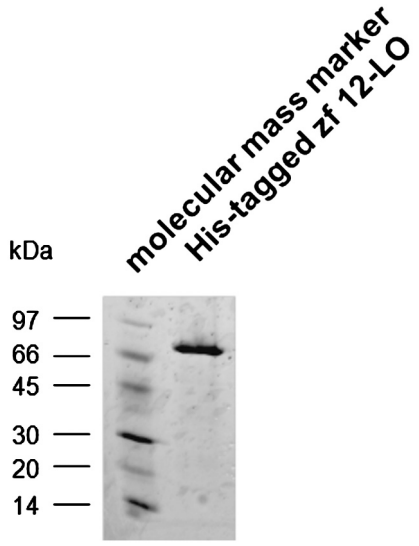

Fig. S3. SDS-PAGE of purified recombinant zf12-LO Ni_2+-column purified recombinant protein was loaded onto a 8–15% SDS-Phast-gel (Amersham Pharmacia Biotech). Gel was stained with Comassie Brilliant blue.

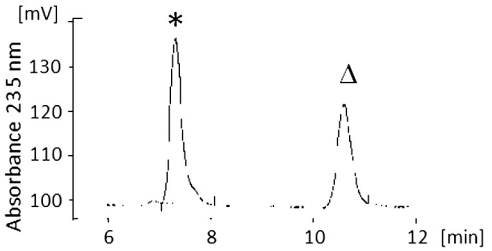

Fig. S4. RP-HPLC analysis of 12 HETE formation by zf12-LO. Asterisk: zf12-LO products; triangle: internal standard 17(*S*)-hydroxy-(13*Z*, 19*Z*, 15*E*)-docosatrienoic acid.

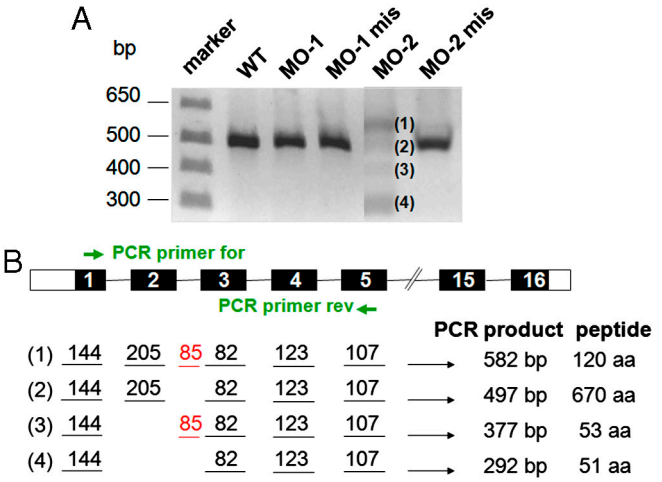

Fig. S5. Confirmation of the knock-down of zf12-LO via RT-PCR. (*A*) The efficiency of the knock-down by MOs was verified by RT-PCR using total RNA isolated from 20 embryos at two dpf. Electrophoretical analyses showed an amplification of the expected product from the correctly spliced mRNA (497 bp) for WT, MO-1 (250 μM) as well as for MO-1 mis (250 μM) and MO-2 mis (500 μM) treated embryos. Contrary, in the sample of MO-2 (500 μM) treated embryos four PCR products were amplified (band 1–4), which resulted from different splice variants of the mRNA. (*B*) The formation of the four different splice variants is illustrated. The 292 bp product corresponds to the expected splice variant, where exon 1 was directly linked to exon 3 (band 4). Additionally the PCR product (497 bp) corresponding to the correct spliced mRNA was detectable (band 2). The 582 bp (band 1) and 377 bp (band 3) products are related to variants where exon 2 and/or intron 2/3 were not spliced out. The length of the peptides translated from the different splice variants is denoted. The primers used for RT-PCR are depicted in green.

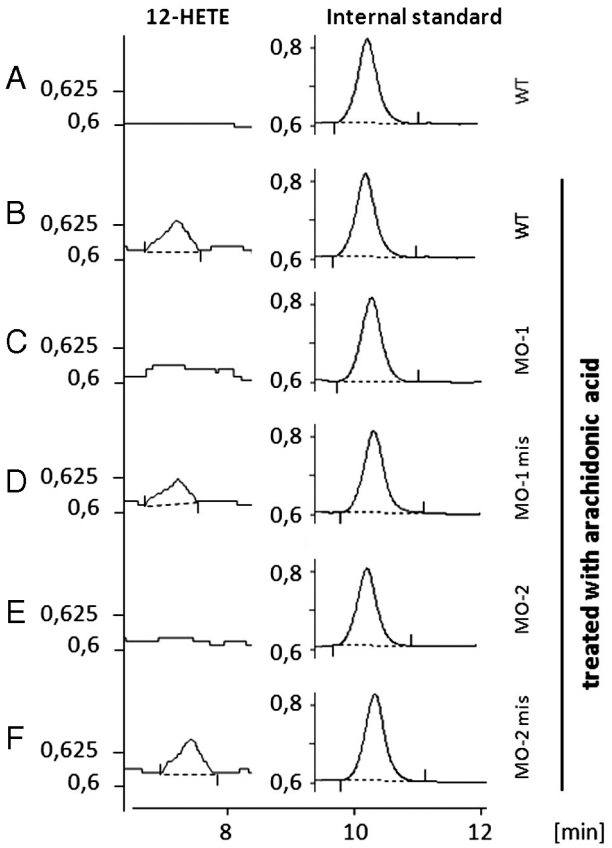

Fig. S6. Knock-down of zf12-LO, loss of 12-HETE production. The enzymatic activity of zf12-LO was investigated by incubation of arachidonic acid with homogenates from WT, MO-1 and -2 treated and MO-1 and -2 mis treated embryos (100 embryos each) at two dpf. The obtained LO products were analyzed by Reverse Phase (RP)-HPLC. (*A*) In homogenates of WT embryos, not incubated with arachidonic acid, no HETE production was detected. The incubation of homogenates from WT (*B*), MO-1 mis (*D*) and MO-2 mis (*F*) treated embryos led to a 12-HETE formation. In contrast, homogenates from MO-1 (*C*) and MO-2 (*E*) morphants showed a negligible 12-HETE production after incubation. Internal standard: 17(*S*)-hydroxy-(13*Z*, 19*Z*, 15*E*)-docosatrienoic acid.

Table S1. Protein sequence identity of zf12-LO with other animal lipoxygenases

LO isoform	Human 12(R)-LO	Human 5-LO	Human 15-LO-1	Mouse ep12-LO	Mouse 8-LO	Xenopus 12-LO	Zebrafish 12-LO
Human p12-LO	38%	42%	65%	59%	37%	38%	45%
Human 12(R)-LO		41%	35%	35%	50%	45%	41%
Human 5-LO			40%	39%	42%	42%	45%
Human 15-LO-1				66%	37%	35%	44%
Mouse ep12-LO					36%	35%	42%
Mouse 8-LO						47%	41%
Xenopus 12-LO							40%