Supplemental Materials

Null cyp1b1 activity in zebrafish leads to variable craniofacial defects associated with altered expression of extracellular matrix and lipid metabolism genes

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Figure S1. Amino acid sequence comparison of Human and Zebrafish cyp1b1. The alignment the human (ENSP00000478561.1) and zebrafish (ENSDARP00000107132.2) proteins was carried out with ClustalW (https://embnet.vital-it.ch/software/ClustalW.html). The asterisks indicate the positions where all the amino acids are identical, two vertical dots show amino acids with similar chemical properties and one dot denotes amino acid positions with weak chemical similarity. Dotted black square: Cytochrome P450 cysteine heme-iron ligand which is indicated according to the Prosite database (https://prosite.expasy.org/). The numbers correspond to amino acid positions.
Figure S2. Cyp1b1 expression analysis by fluorescent-whole mount in situ hybridization in zebrafish embryo eyes at 48 hpf. (A-D) Cyp1b1 antisense or (E and F) sense (control) RNA probes were labelled with Alexa Fluor-488 (green). The arrow indicates specific cyp1b1 expression in the optic fissure. The inset in (A) indicates the position of embryonic axes (D: dorsal; P: posterior; V: ventral; A: anterior). The image is representative of the result observed in 3 embryos of each genotype. Scale bar 50µm.
Figure S3. Phenotypic variability of *cyp1b1* F0 crispants (144 hpf) shown in Fig 3. One-cell embryos were microinjected with CRISPR/Cas9 ribonucleoprotein complexes targeting *cyp1b1* exon 1. Morphology of four crispant larvae with variable phenotypic manifestation was assessed microscopically at 144 hpf. **(WT)** Non injected wild type larvae were used as controls. **(Ctrl)** Control (no crRNA) microinjected embryos. White arrow: lower jaw underdevelopment. Red arrowhead: altered swim bladder development. Red dotted circle: wild type ocular periphery is indicated as a reference to show microphthalmia. Yellow arrowhead: pericardial edema. Scale bar = 250µm (A-D).
Figure S4. Phenotypic characterization of cyp1b1 F0 crispants (96 hpf) obtained by independent or simultaneous microinjection of two single crRNA guides. One-cell embryos were microinjected with CRISPR/Cas9 ribonucleoprotein complexes with the indicated crRNAs targeting cyp1b1 exon 1. (A) Crispants’ morphology was assessed microscopically at 96 hpf. Non microinjected (WT) and control microinjected (control) larvae were used as controls. White arrow: lower jaw underdevelopment. Red dotted circle: wild type ocular periphery is indicated as a reference to show microphthalmia. Yellow arrowhead: pericardial edema. Scale bar= 500 µm. (D) Proportion of F0 mutant phenotypes, n indicate the number of embryos analyzed.
Figure S5. Somite development of *cyp1b1* +/+ , +/- and +/- zebrafish larvae (24 hpf) shown in figure 4. Images in A, B, C, D correspond to panels B, F, J, N in figure 4, respectively. EG: embryo genotype. PG: parental genotype. ♀: female. ♂: male. Yellow lines indicate somite number. All images are representative of the results observed in 10 embryos of each treatment. Scale bar = 500µm.
Figure S6. Histology of the established cyp1b1-KO zebrafish line (168hpf). The histological phenotypes were analyzed in F4 zebrafish obtained by inbreeding of young (<6 months) F3 siblings. (A-K) Semi-thin (500 nm) tissue sections were stained with Toluidine blue. The squares and rectangles indicate the areas of the images magnified in the indicated panels. No significant differences were observed between the eyes of wild type and cyp1b1-KO zebrafish siblings. Scale bar in A and F: 100 μm. Scale bar in B-D and G-I: 25 μm. Scale bar in E and J: 5 μm. Ac: Anterior chamber. CEN: Corneal Endothelium. CEP: Corneal Epithelium. CS: Corneal Stroma. GCL: Ganglion Cell Layer. IPE: Iris Pigmented Epithelium. Sc: Sclera. INL: Inner Nuclear Layer. IPL: Inner Plexiform Layer. Ir: Iridophores. L: Lens. ONL: Outer Nuclear Layer. Xa: Xantophores. These images are representative of at least 10 histological sections of three different larvae of each genotype.
Figure S7. Differential expression analyses of RNA-seq data obtained from cyp1b1 knockout and wild type zebrafish larvae (168 hpf). (A) Correlation matrix of all samples, showing the similarity between samples. Pearson’s coefficient of sample’s normalized value (-1 ≤ r ≤ 1) is used to evaluate similarity between samples. The closer to zero the greater the similarity. (B) MA plot of logFC and LogCPM of all tested genes (wild type vs. cyp1b1 knockout). Dots above zero denote upregulated genes whereas red dots below zero indicate downregulated genes. Red dots correspond to significantly differentially expressed genes (|log2(FC)| > 1, p-value < 0.05). FC: fold change. CPM: counts per million. (C) Heatmap of hierarchical clustering showing significantly DEGs which are represented by red dots in B. Clustering was done using z-score for normalized values (log10 based, wild type vs. cyp1b1 knockout). The color key indicates the intensity associated with normalized expression values: Red, green and black represent the highest, the lowest and no different expression levels, respectively.
Table S1. List of 451 DEGs of the cyp1b1-KO with an absolute fold change (FC) of at least 2, and a p-value <0.05 (185 up- and 266 down-regulated). Upper case gene names correspond to human orthologues of zebrafish unnamed genes.

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Table S2. Biological process analysis of 451 DEGs in the cyp1b1-KO using the DAVID bioinformatic tool. Count indicates the number of genes included in each pathway.

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<th>Genes</th>
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<td>5</td>
<td>0.042</td>
<td>aporea, mfsd2aa, sidikey-192d15.2, osbpl3a, apoa4b.3</td>
</tr>
<tr>
<td>Homophilic cell adhesion via plasma membrane adhesion molecules</td>
<td>8</td>
<td>0.042</td>
<td>pcdh1g3, deg2.1, pcdh1g2, pcdh1g69, pcdh1g26, zgc:172122, dsc2l, pcdh1g30</td>
</tr>
<tr>
<td>Immune response</td>
<td>11</td>
<td>0.048</td>
<td>cxcl18b, zgc:153759, il1b, cts1, sidikey-11f4.14, loc101883994, ccl19a.1, sich211-261n11.7, prgb4, tnfsf13b, sich211-261n11.8</td>
</tr>
</tbody>
</table>
Table S3. Molecular Function analysis of 451 DEGs in the cyp1b1-KO using the DAVID bioinformatic tool. Count indicates the number of genes included in each pathway.

<table>
<thead>
<tr>
<th>Molecular Function Term</th>
<th>Count</th>
<th>P-Value</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidase activity</td>
<td>21</td>
<td>&lt;0.0001</td>
<td>cfd, casp8l2, mbtps1, tmprss13b, zgc:154142, c6ast3, c6ast4, zgc:92745, si:dkeyp-50d11.2, zgc:100868, psmb7, zgc:112160, casp7, ctsll, mmp11b, cts1, mmp28, npsn, pgep1, ctsd, furina</td>
</tr>
<tr>
<td>Iron ion binding</td>
<td>11</td>
<td>0.002</td>
<td>loc558816, zgc:194125, ch25h, cyp24a1, zgc:109934, cyp11c1, sc5d, cyp1b1, cyp4v8, cyp7a1, cyp8b1</td>
</tr>
<tr>
<td>Lipid binding</td>
<td>8</td>
<td>0.003</td>
<td>rbp2b, apoea, rbp1, acot12, ptilp, si:dkey-192d15.2, apoeb3, fabp11b</td>
</tr>
<tr>
<td>Oxidoreductase activity</td>
<td>20</td>
<td>0.004</td>
<td>cfd, c6ast3, c6ast4, ugt1ab, zgc:92745, ppm1f, alp3, zgc:100868, psmb7, zgc:112160, smpd5, casp7, ctsll, mmp28, cts1, ctsd, notum2, casp8l2, nudl3b, mbtps1, tmprss13b, zgc:154142, zgc:92137, si:dkeyp-50d11.2, psmc1b, mmp11b, ptrh2, npsn, furina, cel2</td>
</tr>
<tr>
<td>Hydrolase activity</td>
<td>30</td>
<td>0.005</td>
<td>cfd, c6ast3, c6ast4, ugt1ab, zgc:92745, ppm1f, alp3, zgc:100868, psmb7, zgc:112160, smpd5, casp7, ctsll, mmp28, cts1, ctsd, notum2, casp8l2, nudl3b, mbtps1, tmprss13b, zgc:154142, zgc:92137, si:dkeyp-50d11.2, psmc1b, mmp11b, ptrh2, npsn, furina, cel2</td>
</tr>
<tr>
<td>Monooxygenase activity</td>
<td>8</td>
<td>0.006</td>
<td>loc793236, ch25h, cyp24a1, cyp11c1, cyp1b1, cyp4v8, cyp7a1, cyp8b1</td>
</tr>
<tr>
<td>Serine-type peptidase activity</td>
<td>7</td>
<td>0.018</td>
<td>zgc:100868, cfd, zgc:112160, tmprss13b, zgc:154142, zgc:92745, furina</td>
</tr>
<tr>
<td>Heme binding</td>
<td>8</td>
<td>0.018</td>
<td>cyp24a1, cyp11c1, cyp1b1, duox, cyp4v8, cyp7a1, cyp8b1, ido1</td>
</tr>
<tr>
<td>Serine-typeendopeptidaseactivity</td>
<td>9</td>
<td>0.020</td>
<td>zgc:100868, cfd, mbtps1, zgc:112160, tmprss13b, zgc:154142, loc100149563, zgc:92745, furina</td>
</tr>
<tr>
<td>Ferric iron binding</td>
<td>3</td>
<td>0.024</td>
<td>loc558816, zgc:194125, zgc:109934</td>
</tr>
<tr>
<td>Transporter activity</td>
<td>9</td>
<td>0.038</td>
<td>aqu8a2, rbp2b, rbp1, slc15a1a, slc13a5b, aqu7, tipal, fabp11b, slc15a1b</td>
</tr>
<tr>
<td>Transcription factor activity, RNAStr assumed for core promoter proximal region sequence-specific binding</td>
<td>3</td>
<td>0.038</td>
<td>fosl1a, fosab, jdp2b</td>
</tr>
<tr>
<td>Metalloendopeptidase activity</td>
<td>6</td>
<td>0.048</td>
<td>lmln, mmp11b, mmp28, c6ast3, npsn, c6ast4</td>
</tr>
<tr>
<td>Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen</td>
<td>6</td>
<td>0.049</td>
<td>cyp24a1, cyp11c1, cyp1b1, cyp4v8, cyp7a1, cyp8b1</td>
</tr>
</tbody>
</table>
Table S4: Primer sequences and conditions used in RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5'→3' (F: forward; R: reverse)</th>
<th>Cycles</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon length (pb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyp1b1</td>
<td>F: ACAGCTCTCCAGTGGGATGAC&lt;br&gt;R: GTCTGCGATGGTGGGAGAC</td>
<td>40</td>
<td>60</td>
<td>114</td>
</tr>
<tr>
<td>ef1a</td>
<td>F: CTGGAGGCCAGCTCAAAAC&lt;br&gt;R: ATCAAGAAGAGTAGTACCGCTAC</td>
<td>40</td>
<td>60</td>
<td>87</td>
</tr>
<tr>
<td>igbp1b</td>
<td>F: GCCACAGGAGAGCAACAAGT&lt;br&gt;R: GGTGAACCTTCTCTCCCAACG</td>
<td>40</td>
<td>60</td>
<td>130</td>
</tr>
<tr>
<td>junbb</td>
<td>F: AAAATGGAGCAGCCGTTTA&lt;br&gt;R: CTCGGTCAGCCTCAAGTTCA</td>
<td>40</td>
<td>60</td>
<td>117</td>
</tr>
<tr>
<td>ubl7b</td>
<td>F: TTCGTACGCCGATCAATGCTG&lt;br&gt;R: GCCCTGAACCATCTCTGAACCTC</td>
<td>40</td>
<td>60</td>
<td>123</td>
</tr>
<tr>
<td>wdr35</td>
<td>F: TGCCGAAAAACCATAAAACTG&lt;br&gt;R: CTGCTTGGCCTGGGTACCAT</td>
<td>40</td>
<td>60</td>
<td>108</td>
</tr>
<tr>
<td>rbp1</td>
<td>F: CCATCACTTGATGGGAGAC&lt;br&gt;R: ATCTTTTGGCACAATCC</td>
<td>40</td>
<td>60</td>
<td>133</td>
</tr>
<tr>
<td>acta1b</td>
<td>F: ATCTGGCTGGATCGATCTTT&lt;br&gt;R: TCTCTTGGTACGCACAAAT</td>
<td>40</td>
<td>60</td>
<td>106</td>
</tr>
<tr>
<td>cyp24a1</td>
<td>F: CAGCCGAGCCCTGGAGAAGACT&lt;br&gt;R: CCAGCGCTCGGCTCTAC</td>
<td>40</td>
<td>60</td>
<td>139</td>
</tr>
</tbody>
</table>