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Supplementary Materials for

MIC-DROP: A platform for large-scale *in vivo* CRISPR screen

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This PDF file includes:

Materials and Methods
Supplementary Text
Figs. S1 to S7
Tables S1 to S5
Captions for Movies S1 to S6

Other Supplementary Materials for this manuscript include the following:

Movies S1 to S6

Materials and Methods

Zebrafish husbandry and breeding: All protocols related to zebrafish (*Danio rerio*) were approved by the Institutional Animal Care and Use Committee at the University of Utah (Protocol # 19-09011). Adult TuAB strain zebrafish and *Tg*(*cmlc2*:NdsRed) were maintained in the Centralized Zebrafish Animal Resource (CZAR) core at 28–29 °C with a 14/10 light/dark cycle. *Tg*(*cmlc2*:eGFP) zebrafish were maintained in HJY lab (Eccles Institute of Human Genetics). To produce embryos, adult zebrafish in a 1:1 male:female ratio were placed in a breeding tank and separated by a divider overnight. Embryos were collected after removing the divider in the morning.

Guide RNA (gRNA) design and selection criteria: All gRNAs were designed using CHOPCHOP version 3.0.0 (https://chopchop.cbu.uib.no/). The targets were specified using the Gene ID or the ENSEMBL ID. "danRer10/GRCz10" was used as the reference sequence. The single gRNAs (sgRNAs) were designed for "knock-out" using "CRISPR/Cas9" from *Streptococcus pyogenes* with "NGG" as the PAM sequence. The sgRNA length without PAM was specified as "20" except in certain circumstances (see below) when "19" bases length was used. The default methods for determining off-targets in the genome "Off-targets with up to 3 mismatched in protospacer (Hsu et al., 2013)"; and an efficiency score calculation based on "Doench et al 2016- only for NGG PAM" were used. The 5' requirement for sgRNA was changed to "GN or NG" and the software used Thyme et. al. to "Check for self-complementarity" and to "Check for self-complementarity versus a Standard backbone (AGGCTAGTCCGT)". All other functions were kept at default options. The following criteria was followed to select 4 targets per gene: (1) Targets of 20 bp length in the early to middle exons that start with "GA" and had no off-targets with fewer than 3 bp mismatches were prioritized. (2) If guides that met criterion 1 could not be found, guides that started with "GA" and were 19 bp in length were used. (3) If criterion 1 and 2 were not met, gRNAs that started with "GN" were picked [Even though guides starting with "GA" are preferred by SP6 polymerase, we saw good transcription efficiency of guides starting with "GN"]. If it was not possible to design gRNA with no off-targets, guides with at least 3-bp mismatches of which at least 1 mismatch was in seed region were selected. For genes with ohnologs, 2 gRNA per ohnolog was designed. All gRNAs had 45-80% GC content. The gRNA sequences are listed in **Table S1** and **Table S5**. No unique gRNAs could be designed for six of the candidate genes.

***In vitro* transcription**: The DNA templates for *in vitro* transcription (IVT) were generated using fill in PCR of a target-specific forward oligo and a constant reverse oligo as reported in Gagnon *et al* (*14*). Target-specific forward oligos ATTTAGGTGACACTATA$(N)_{19/20}$GTTTTAGAG CTAGAAATAGCAAG containing a SP6 RNA polymerase site followed by 19 or 20 bp of the gRNA sequences were ordered from IDT as 25 nmol desalted and lyophilized powder. The constant reverse oligo AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGA CTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC was synthesized at the University of Utah DNA synthesis core and HPLC purified. Both the forward and reverse oligos were dissolved in nuclease free H_2O (Invitrogen; cat # AM9906) to a 100 µM concentration. Oligos for the screen were ordered in 96-well plate as 500 pmol desalted and lyophilized powder and reconstituted in water to a concentration of 10 µM. Here is the step-by-step protocol for generating the multiplexed gRNAs targeting a gene-of-interest:

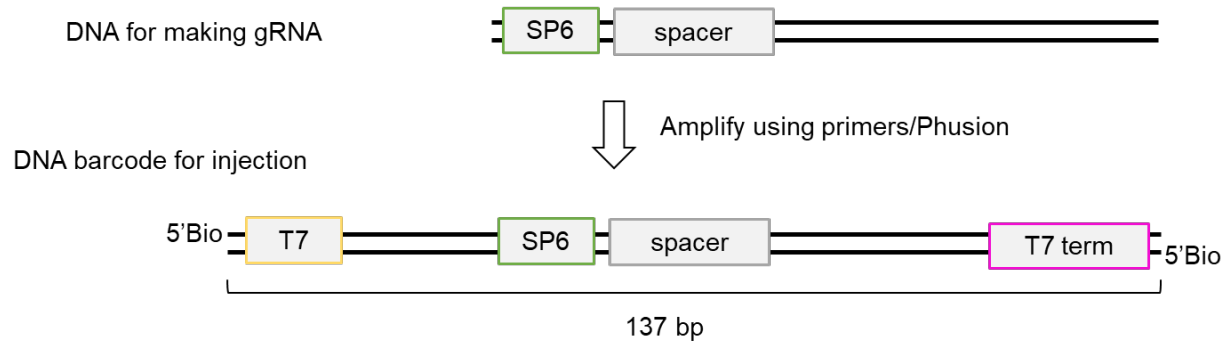
(1) In a 50 µL PCR reaction mix, add 1x HF buffer (NEB; cat # B0518S), 1 µM each of forward oligo and the constant reverse oligo, 200 µM dNTPs (Fisher Scientific; cat # R0194), 3% DMSO (v/v), and 1U of Phusion HS Flex DNA polymerase (NEB, cat # M0535L).
(2) Perform a fill-in PCR in a thermal cycler (BioRad) by incubating the sample at 98 °C for 2 min, 50 °C for 10 min, 72 °C for 10 min, and 12 °C for ∞.
(3) Cleanup the samples using a Zymo DNA Clean and Concentrator-5 kit (Zymo Research, cat # D4013) according to manufacturer's protocol. Use a ZR96 DNA Clean and Concentrator-5 clean up kit (Zymo Research, cat # D4024) if handling a large number of samples.
(4) Elute the double stranded DNA in 15 µL nuclease free water. Determine concentration using a Nanodrop (Thermo Scientific) and then store at −20 °C.
(5) Pool DNA templates for all four guides targeting a gene-of-interest in a 1:1:1:1 ratio.
(6) Performed IVT in RNAse free condition using a MEGAscript™ SP6 Transcription kit (Thermo Fisher Scientific, cat # AM1330). For each IVT reaction of 10 µL, add 1µL each of ATP, CTP, GTP, and UTP, 1 µL 10x reaction buffer, 0.125 µL of RNAse inhibitor (Thermo Fisher Scientific; cat # EO0382), 1 µL of SP6 enzyme mix, and 200 ng of total multiplexed DNA (50 ng each DNA).
(7) Incubate the IVT reaction at 37 °C overnight (~16 h).
(8) Bring the volume to 20 µL with nuclease-free water and then add 1 µL Turbo DNAse. Mix and incubate for 15 min at 37 °C.
(9) Isolate RNA using an RNA Clean and Concentrator-5 (Zymo Research, cat # R1013) or a ZR96 RNA Clean and Concentrator-5 (Zymo Research, cat # R1080) and elute in 12 µL nuclease free water.
(10) Determine RNA concentration using a Nanodrop (Thermo Scientific), assess RNA integrity using gel electrophoresis, and store the RNA samples at −80 °C. Since the gRNAs are *in vitro* transcribed from a pooled mix of DNA templates, the exact ratio of each gRNA sequence need not be determined. The ratios will depend on the transcription efficiencies of each template.

Barcode Generation: The DNA barcodes were generated by extending and putting a 5'-Biotin modification on the DNA template used for IVT according to the following protocol (also see schematic below). The 5'-Biotin modification was done to enable enrichment of the barcode for more efficient recovery in case of barcode degradation (see Supplementary Text for more details on barcoding).

(1) In a 50 µL PCR mix, add 1x HF buffer (NEB; cat # B0518S), 0.5 µM each of forward /5BiosG/CGTAATACGACTCACTATAGGGCTTCAGCCAAGGAAGCTACATTTAG GTGCACTATAG (IDT) and reverse primers /5BiosG/GCTAGTTATTGCTCAGCGGGTCTTGTTT CTCGGTGTGCTTGCTATTTCTAGCTCTAAAAC (IDT), 200 µM dNTPs (Fisher Scientific; cat # R0194), 3% DMSO (v/v), 50 ng of any one of the four DNA templates used for gRNA generation), and 1U of Phusion HS Flex DNA polymerase (NEB, cat # M0535L).
(2) PCR amplify the DNA barcode using the following conditions: at 98 °C for 30s, [98 °C for 10s, 53 °C for 30s, 72 °C for 10s] ×34 cycles, 72 °C for 5 min, 12 °C for ∞.
(3) Cleanup the DNA barcode using a Zymo DNA Clean and Concentrator-5 kit (Zymo Research, cat # D4013) and elute in 20 µL nuclease free water. To process a larger number of samples,

use a ZR96 DNA Clean and Concentrator-5 clean up kit instead (Zymo Research, cat # D4024).

(4) Determine DNA barcode concentration using a Nanodrop (Thermo Scientific) and then store samples at −20 °C.



Droplet generation: The CRISPR droplets were generated using a QX200 Droplet generator (BioRad, cat # 1864002) using 3% 008-Surfactant (w/v) (Ran Biotechnologies; cat # 008-FluoroSurfactant-1G) in Novec-7500 oil (Gallade Chemical, cat # HFE-7500) (3% HFE from here on) according to the following protocol.

(1) Make a 19 µL ribonucleoprotein (RNP) mix by adding 5000 ng of total gRNAs (4 gRNA/gene) targeting a gene-of-interest, 4.2 µL of 20 µM EnGen Cas9 (NEB, cat # M0646M), 2.5 µL of 10x Buffer 3.1 in nuclease free water.

(2) Incubate at room temperature for 10 min.

(3). Add 250 ng of DNA barcode (2.5 µL of 100 ng/ µL solution) and 3.5 µL of 0.5% Phenol Red dye in PBS (Sigma, cat # P0290) to the RNP mix. The final volume of the RNP mix should be 25 µL with final concentrations of 200 ng/µL gRNAs, 3.36 µM EnGen Cas9 nuclease, 1x Buffer 3.1, 10 ng/µL DNA barcode, and 0.07% Phenol Red.

(4) Mix the sample gently and transfer 20µL of it to a QX200 cartridge (BioRad, cat # 1864007) using a 20 µL multichannel pipet (Rainin). If preparing droplets for less than 8 samples, fill the remaining wells with 20 µL of 1x Droplet generation buffer (BioRad, cat # 1863052). Make sure no bubbles are formed in the cartridge during sample transfer.

(5) Load 70 µL of 3% HFE in the designated wells of the cartridge.

(6) Load the cartridge in a cartridge holder (BioRad), seal using a rubber gasket (BioRad, cat # 1864007), place it in the QX200 Droplet generator, and close the instrument. Droplet generation should begin once the instrument closes securely.

(7) Once droplet generation is complete (~2.5 min/8 samples), transfer the droplets immediately using a 200 µL multichannel pipet (Rainin) from the cartridge to PCR strip tubes (Fisher Scientific) containing 50 µL 3% HFE. The droplets float on the oil surface because of higher density of the oil than the aqueous droplets.

(8) If not using immediately, store the droplets at 4 °C for up to a month in capped PCR strip tubes.

(9) If intermixing droplets from different samples, combine 2 µL droplets from each sample into a separate PCR tube containing 3% HFE. For our screen, we intermixed droplets from 49-50 different samples (48 candidate genes + 1-2 positive controls).

(10) Mix the samples gently for even distribution. Avoid droplet coalescing during droplet transfer and mixing. Use P-20 and P-200 tips, because of their wider tip width, for transfer and mixing, respectively.

Droplet injection: All injections were performed in embryos at the 1-cell stage using a Microinjection system Pico-injector (Harvard Apparatus) fitted with a dissecting microscope (Leica Microsystems) according to the following protocol.

(1) Pull needles (Sutter Instrument, cat # TW100F-3) for microinjection using a P-1000 Micropipette puller (Sutter Instrument) at the following setting: Heat: 565, Pull: 64, Velocity: 77, Time: 80, and Pressure: 500. Use freshly pulled needles for droplet injection as any dust/debris in the needle can cause blockage during injection or may cause droplets to split.
(2) Use a P-20 µL pipette set at 3–4 µL pipette volume and a micro-loader tip (Eppendorf; cat # 5242956.003) to transfer around 300–500 droplets (along with the 3% HFE carrier oil) into a microinjection needle.
(3) Flick the needle gently to get rid of any trapped air bubbles. Avoid vigorous shaking during transfer or flicking.
(4) Attach the injection needle to the injector and trim the needle such that the opening width is around 10–20 microns.
(5) Because of the density difference between the oil and the aqueous droplets, the droplets collect at the top in the injection needle. Use the "Clear" setting to gently push out the excess 3% HFE carrier oil before injection.
(6) Once the droplets move near the tip, proceed with injection.
(7) Arrange embryos on an injection mold and inject one droplet. Adjust the settings for the time (40–70 msec) and pressure (6–9 psi) on the pico-injector such that each droplet is injected in 2–3 taps of the foot switch.
(8) Push out the oil between two consecutive droplets into the buffer surrounding the embryos until the next droplet approaches the needle tip.
(9) Inject the subsequent droplet in the next embryo (see **Movie S1**). To avoid injecting any oil (even though accidental injection of a small amount of oil is inconsequential), push out the oil between two adjacent droplets till a small amount of aqueous droplet comes out. Insert the needle into the embryo and inject the aqueous droplet into an embryo and pull the needle out of the embryo just before the subsequent oil reaches the tip of the needle.
(10) Inject 300–500 droplets from a single injection needle in one morning.
(11) Inject the same pool of intermixed droplets in at least 3 clutches of embryos on different days to account for batch variability.
(12) Transfer the injected embryos to a petri dish, wash once with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM MgSO4) to get rid of any carrier oil and residual RNP mix, split into multiple dishes (50–60 embryos per dish) to avoid overcrowding, and raise the embryos at 28.5 °C in E3 medium with methylene blue.

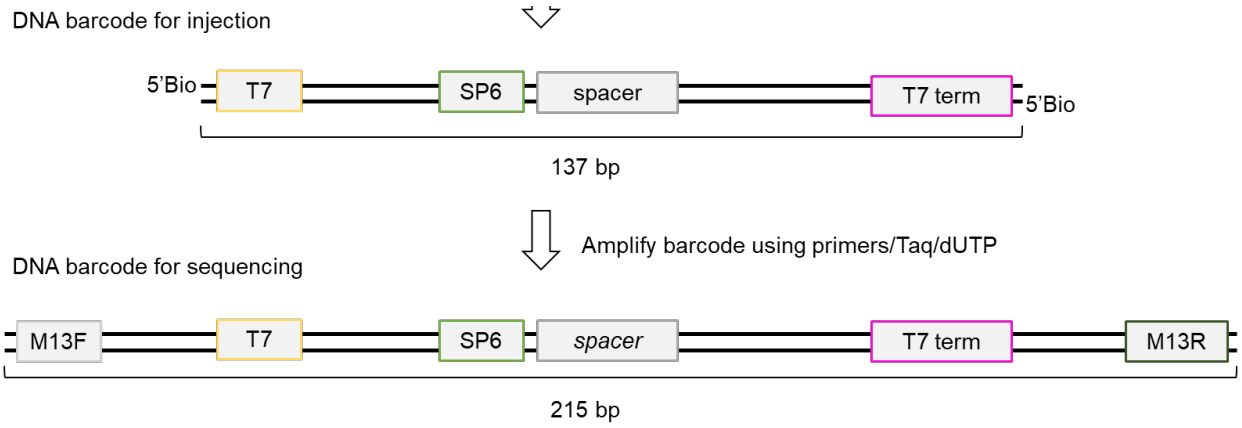
For the MIC-Drop screen described here, a total of ~3000 embryos were injected with a frequency of ~15 droplets/gene.

Phenotype screening: 24 hours post injection embryos were screened for any morphological phenotypes using a SteREO Discovery V8 dissecting microscope (Zeiss). Dead embryos were removed, and the old media was replaced with fresh E3 media. Embryos showing gross morphological defects caused by general nucleic acid toxicity (~10–15%) were also removed. The embryos were screened at multiple different time points – 24 hours post fertilization (hpf), 30 hpf,

48 hpf, 72 hpf – and any embryos showing cardiovascular phenotypes including arrhythmia, heart block, cardiac edema, heart perforation, blood pooling in the pericardial sac, enlarged/ diminutive atrium or ventricle, reduced blood flow in the caudal artery/vein, and heart looping defects were isolated.

Barcode retrieval and sequencing: To identify the target gene responsible for a phenotype-of-interest, retrieve the barcode using the following steps (see schematic below):

(1) Isolate any embryos showing phenotype(s)-of-interest.
(2) Wash 1x with E3, transfer the embryos to a new plate, and wash again 3x in E3 media to get rid of any residual DNA barcodes sticking to embryos.
(3) Transfer the embryos along with 10 µL E3 and add to 10 µL of a 2x lysis buffer (20 mM Tris (pH 8), 4 mM EDTA, 0.4% Triton X-100) with freshly added Proteinase K (Sigma, cat #3115828001) at a concentration of 0.2 mg/mL.
(4) Incubate the 20 µL sample overnight at 50 °C for complete lysis.
(5) Inactivate Proteinase K the following morning by heating at 95 °C for 10 min.
(6) Mix the lysate, centrifuge at 3000×g for 5 min to pellet the debris, and use the supernatant for PCR amplification of the DNA barcode.
(7) Assemble a 15 µL PCR reaction mix containing 1x GoTaq buffer, 0.5 µM each of forward (GTGTAAAACGACGGCCAGTA TGGCACCAACTCGATGACGTAATACGACTCACTATAGGGC) and reverse (CAGGAAACAGCTATGACATAGTCCTGCTGTACCAGGCGTCTGCTAGTTATTG CTCA GCGG) primers, 200 µM dNTPs (70:30 of dTTP:dUTP), 0.375 U UDG (NEB, cat # M0280S), 0.375 U GoTaq polymerase (Promega, cat #M3008) in a clean area. We assembled the PCR master mix inside a tissue-culture hood to minimize carryover DNA contamination. Additionally, UDG and 70:30 of dTTP:dUTP was used in the PCR reaction to degrade any possible carryover contamination product prior to amplification.
(8) Add 2 µL of embryo lysate (from step 6) to the PCR mix.
(9) Amplify the DNA barcode using the following conditions: 37 °C for 10 min, 95 °C for 10 min, [95 °C for 30s, 55 °C for 30s, 72 °C for 15s] ×34 cycles, 72 °C for 5 min, 12 °C for ∞. The initial incubation at 37 °C is performed to degrade carryover contamination by UDG followed by heat inactivation of the enzyme at 95 °C.
(10) Perform enzymatic cleanup by adding 0.5 µL (10U) of Exonuclease I (NEB, M0293) and 1 µL (1U) of shrimp alkaline phosphatase (NEB # M0371) to 5 µL of the PCR amplified product.
(11) Mix the solution from step 10 and incubate at 37 °C for 15 min and then heat-inactivate at 80 °C for 15 min.
(12) Dilute the sample 3x–5x with water and sequence using M13F or M13R primers.



Validation of editing efficiency: Editing efficiency was analyzed using either a T7 endonuclease (T7E1) assay or Amplicon sequencing. For T7E1 assay, the targeted region was amplified using Q5 high fidelity polymerase (NEB, cat # M0493S) and a set of primers flanking the cut site. 200 ng of the cleaned amplified product was first denatured and then reannealed by gradual cooling according to the manufacturer's protocol. The sample was treated with 10 U of T7E1 enzyme (NEB, cat # M0302S) in a total volume of 20 µL and incubated at 37 °C for 15 min. EDTA at a final concentration of 25 mM was added to quench the reaction. The samples were resolved on a 2% agarose gel. For Amplicon sequencing, 150-500bp amplicons from the targeted regions were sequenced on an Illumina platform using paired reading at a depth of 50,000 reads (Genewiz, Amplicon-EZ). Amplicon sequencing data were analyzed using Cas-Analyzer (http://www.rgenome.net/cas-analyzer/#!).

Light- and Optovin- induced motor response assay: Zebrafish larvae at 3dpf were arrayed in 96-well plates and treated with 10 µM optovin (Fisher Scientific, cat # 490110) in a total volume of 200 µL E3 media. Treated larvae were incubated at 37 °C for 1 h in dark. Subsequently, light-dependent motor response was assayed using a Zebrabox platform (ViewPoint Behavior Technology) fitted with an Optogenetic addon. Movement of the larvae was tracked and quantitated following 5× 1s pulse of violet light after 10s interval in the dark.

Computational pipeline to identify high-confidence genes for CRISPR screen: Raw RNA-seq data files (paired Fastq) were downloaded from the Gene Expression Omnibus (Accession # GSE85416) (*19,20*). Transcript abundances were quantified using kallisto and genome build GRCz10 release 89 (may2017.archive.ensembl.org) for all samples. Estimated counts for all transcripts per gene were summed to give a gene-level abundance estimation. Estimated counts were rounded to the nearest integer and subset to perform two separate differential expression analyses, the first comparing zebrafish larval heart samples (SRR4017367, SRR4017368, SRR4017369) to zebrafish adult heart samples (SRR4017370, SRR4017371, SRR4017372) and the second comparing the aforementioned adult samples to zebrafish adult muscle samples (SRR4017373, SRR4017374, SRR4017375). Genes with less than 10 counts across all samples (n=6803) were removed from the matrix prior to performing differential expression analysis. DESeq2 was run on each comparison using a negative binomial LRT model correcting for replicate (counts~ replicate + tissue). To find genes that are in enriched in larval cardiac tissue, the data was filtered by fold change and by adjusted p-value (false discovery rate < 1%). Genes that were significantly enriched in adult heart as compared to adult muscle (n=3488) and genes that were

significantly enriched in larval heart as compared to adult heart (n=4150) were carried forward in the analyses. Out of these datasets, 465 genes were found to be overlapping in each filtered comparison. The gene list was manually curated to remove any genes that were already known to have cardiac phenotypes in various animal models or predicted gene models that have not been characterized/validated. The final gene list contained 188 genes found to be enriched in larval cardiac tissue without known phenotypes, and 6 control genes with expected outcomes.

Rescue assay: Codon-optimized gene sequences were ordered as gene fragments (Genewiz), amplified, and cloned in a pcs2+ vector using restriction enzymes. The gene sequences were amplified using RNA-fwd (GACGTAAATGGGCGGTAGGCG) and RNA-Rev primers (CATGATTACGCCAAGCGCGC). mRNA was generated using a SP6 mMessage mMachine transcription kit (Thermo Fisher Scientific, cat # AM1340) per manufacturer's protocol. 1–1.5 nL of RNP containing 100 ng/µL gRNA, 2 µM Cas9, and 300 ng/µL mRNA was injected in embryos at 1-cell stage. Phenotype was analyzed at 3 dpf.

***o*-dianisidine staining:** Zebrafish embryos at 3 dpf were stained in the dark for 30 min with a solution containing 0.6 mg/mL *o*-dianisidine, 0.01 M sodium acetate (pH 4.5), 0.65% H_2O_2, and 40% EtOH (v/v). Stained embryos were washed with water and then fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 1 h. Next, embryos were treated for 30 min with a solution containing 0.8% KOH, 0.9% H_2O_2, and 0.1% Tween-20 to remove the pigments. Finally, the depigmented embryos were washed in 0.1% Tween-20 in PBS and then fixed with 4% PFA for at least 3 hours. All procedures were performed at room temperature. Embryos were stored in PBS at 4 °C and imaged using a Leica M205 FA Stereoscope.

Alcian blue stain: 5 dpf embryos were fixed in 4% PFA for 2 hours at room temperature. Embryos were dehydrated in 50% EtOH for 10 min at room temperature and then treated with a solution containing 0.04% alcian blue 8 GX (Sigma-Aldrich, cat # A5268), 0.005% alizarin red S (Sigma, cat # A5533), and 50 mM $MgCl_2$ in 70% EtOH and incubated overnight with at 4 °C. The embryos were washed with water once before depigmented using a solution containing 1% KOH and 1.5% H_2O_2 and treated for 20 min at room temperature. Next, tissues were cleared by washing with 0.25% KOH and 20% glycerol for 30 min at room temperature followed by another wash with 0.25% KOH and 50% glycerol. Samples were stored in 0.25% KOH and 50% glycerol at 4 °C and imaged using a Leica M205 FA Stereoscope.

Imaging: *Tg*(*cmlc2*:NdsRed) or *Tg*(*cmlc2*:eGFP) were euthanized by placing in 1% PFA for 5 min, embedded in agarose and imaged using a Zeiss LSM 700 confocal microscope. For live imaging, zebrafish larvae were anesthetized in 0.016% Tricaine in E3. Low magnification brightfield images were collected using a Leica M205 FA stereoscope. High magnification videos of zebrafish were collected using a Zeiss AXIO Observer. A1 microscope using a Metamorph software (Molecular Devices) at 10fps. All images were processed and analyzed using ImageJ (NIH).

Voltage mapping: Optical mapping was performed as previously described (*31*). Briefly, hearts from 72 hpf zebrafish embryos were isolated in Tyrode's buffer and loaded with the transmembrane potential-sensitive dye, FluoVolt™ (Life Technologies, cat # F10488) for 20 min to measure the action potentials. After transferring the stained hearts to fresh Tyrode's buffer to

remove excess dye, individual hearts were placed in chamber containing 0.05 mg/mL of the mechanical uncoupler Cytochalasin D (ThermoFisher Scientific, cat # PHZ1063) to inhibit contraction. Fluorescence intensities were recorded with an inverted microscope (TE-2000, Nikon) equipped with a high-speed CCD camera (RedShirtImaging) at a maximum frame rate of 2000 Hz. Propagation velocities and depolarization waves were extracted using custom scripts in MATLAB 9.5 software (Mathworks, version R2018b) as previously described (*31*). Briefly, activation times were defined as the time for 80% depolarization and isochronal maps representing the wavefront at fixed time intervals (10 ms) were calculated from the activation data using the contour-plotting function in MATLAB. Local conduction velocities of regions-of-interest (40 mm^2 in size) were defined as previously described (*31*).

Supplementary Text:

Barcoding: We generated the barcodes by adding 5'-Biotin modifications to any one of the four DNA templates used for *in vitro* transcription. The DNA barcode sequences were also extended on both ends such that the unique identifier sequence (same as the spacer sequence of the gRNA) is at a sufficient distance from the primer binding site for unambiguous Sanger sequencing (see Materials & Methods). The barcode itself does not get integrated in the zebrafish genome. The 5' biotin modification was added in anticipation that the DNA barcode will degrade over time and may require enrichment and subsequent PCR amplification prior to sequencing. However, our data suggest that PCR amplification of the modified barcode directly from up to 7 dpf embryo lysate is efficient and does not require prior enrichment (**Figure S3**). Thus, non-biotinylated DNA barcodes might also possibly be used, however we have not performed a side-by-side comparison to assess whether 5'-biotin modification enhances the barcode's stability. We further acknowledge that barcoding can be performed in multiple alternative ways, some of which are currently being tested in the lab. For example, a set of standard DNA barcodes can be used in lieu of amplifying the guide DNA sequences each time. Alternatively, visual barcodes or chemical tags that do not require sequencing may be a possibility.

Droplet generation and Scalability of MIC-Drop: We repurposed a BioRad QX200 instrument for droplet generation. The instrument generates ~20,000 droplets from a 20 µL sample of RNP mix targeting a gene-of-interest. Processing 8 samples at a time takes ~2.5 minutes thereby requiring ~30 minutes to generate droplets for 96 samples. The sample handling process could potentially be automated further using an Automated Droplet Generator (BioRad, cat # 17864101). Alternatively, a custom-made microfluidics-based droplet generator with an automated liquid handling system might be used for high-throughput sample processing. We anticipate that the MIC-Drop platform can be scaled-up to target thousands of genes. **Cost**: There is an initial cost to the gRNA library generation and purchasing of the BioRad QX200 instrument. However, once generated, the gRNA library can be shared or used tens of times (similar to a small- molecule library). Similarly, once purchased the same instrument can be used for droplet generation for multiple different gRNA libraries. The cost of droplet generation per sample can be significantly reduced by using user-made recombinant Cas9 protein. Moreover, a MIC-Drop screen uses only 20–50 of the 20,000 droplets targeting each gene generated by the droplet generator. Because the droplets are stable for months at 4 °C once generated, one could envision distributing the droplets to the scientific community to split the cost of library generation, thus, bringing down the cost to a few cents per gene per screen. **Time and labor**: The screen of ~200 genes by a single user was

completed within 6 weeks, including confirmation and follow up of hits. Up to 500 embryos were typically injected in one morning from a single injection needle containing a library of droplets targeting 50 genes. Each set of droplets was injected in at least 3 different clutches of embryos and on 2 different days to account for batch to batch variability. The MIC-Drop platform can be easily scaled-up. The time to complete a screen will depend on the number of genes targeted by the library, the number of users or instruments used for injection, as well as the day at which the phenotype is being scored. Certain phenotypes may only be obvious at later stages of development requiring longer screening time.

Translatability: We anticipate the MIC-Drop platform to be highly versatile with the potential to be used for a variety of functional genetic screens. Technically, the cargo inside the droplets can be changed without a significant modification to the protocol to enable CRISPR activation and inactivation screens, gene knockdown screens using Cas13, as well as knockout screens of non-coding genetic elements in zebrafish. In addition, the MIC-Drop platform may be translatable to other organisms where CRISPR micro-injection have been demonstrated successfully such as in *Xenopus*, *Drosophila*, sea urchins, and in mice. The droplet content will need to be optimized for each model organism being studied. **Challenges and considerations**: MIC-Drop screens are well-suited for identifying genes responsible for specific developmental and behavioral phenotypes. It is advisable to pick phenotypes that are distinct from gross morphological defects observed in embryos due to general nucleic acid toxicity and injection artifacts (**Figure S1E**). This will minimize false positives in the screen. Additionally, for gain-of-function behavior screens it will be important to pick robust behaviors that can be measured in a single mutant animal. Behavioral phenotypes that require an ensemble of mutant animals are not ideal for MIC-Drop screen. Moreover, for suppressor screens, the phenotype to be suppressed needs to be highly penetrant to minimize false positives.

Phenotyping in F0 "crispants": This and several studies from multiple independent labs have now shown that though mosaic, F0 "crispants" with high biallelic mutation efficiencies often successfully recapitulate germline mutant phenotypes *(10–13)*. Injection of multiplexed gRNAs targeting a gene frequently results in high on-target editing efficiency with no/minimal off-targets. **Challenges of F0 phenotyping**: Because phenotyping in F0 requires high biallelic mutation efficiency, good gRNA design is key. We have prioritized gRNAs that are predicted to have high on-target efficiencies. Additionally, multiplexing gRNAs targeting multiple exons ensures higher likelihood of indel generation. Despite high editing efficiency, however, there are multiple factors that may result in a lack of phenotype in "crispants", including alternative splicing and genetic compensation. Alternative splicing is especially relevant for genes with large number of exons such that inclusion of a premature termination codon in an earlier exon can still result in an alternatively spliced functional isoform. Multiplexing gRNAs targeting multiple different exons or targeting a functional domain (if known) may help circumvent this issue. Genetic compensation in a "crispant" can also result in lack of a phenotype *(28)*. gRNAs targeting the promoter site or gRNAs flanking a gene (for small genes) that result in complete gene deletion may alleviate this issue, although this may not be possible for all genes (genes with multiple promoters and genes spanning tens of kilobases). Simultaneous targeting of paralogs and/or ohnologs of a gene is an alternative strategy to circumvent genetic compensation. We observed high editing efficiency irrespective of whether a single gene or both of the ohnologs of a gene were targeted using MIC-Drop. Another point of consideration is the degree of mosaicism observed in F0 "crispants".

Although we observed high phenotype penetrance in all the test genes, mosaicism in “crispants” may result in the lack of a phenotype. In our assay to identify the target of optovin, ~50% of trpa1b mutant animals were still responsive (with reduced sensitivity) to light-triggered motion response likely due to mosaic knock out of the gene. Finally, it is critical to pick gRNAs with no/minimal off-targets for F0 phenotyping. In our screen, ~80% of the gRNAs had no predicted off-targets. Those with 1-2 off-targets had at least one mismatch in the seed region, which is predicted to abrogate editing at the off-target site. We also validated that the phenotype observed in the “crispants” is due to on-target gene knockout by performing rescue with mRNA injection. Nonetheless, generation of germline mutant lines to validate the “crispant” phenotype remains the gold standard. We plan on generating germline mutants of the “hit” genes to validate the “crispant” phenotypes.

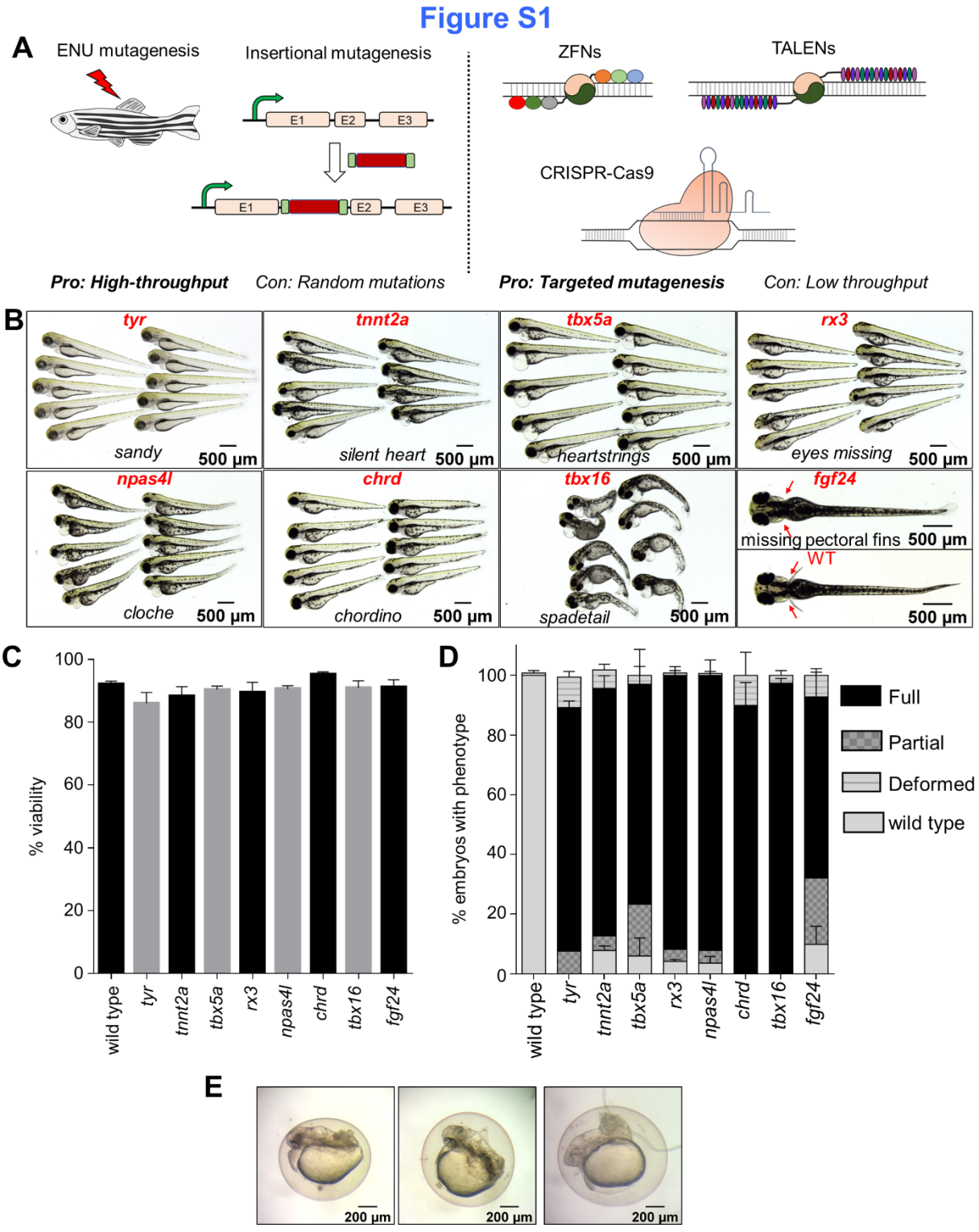
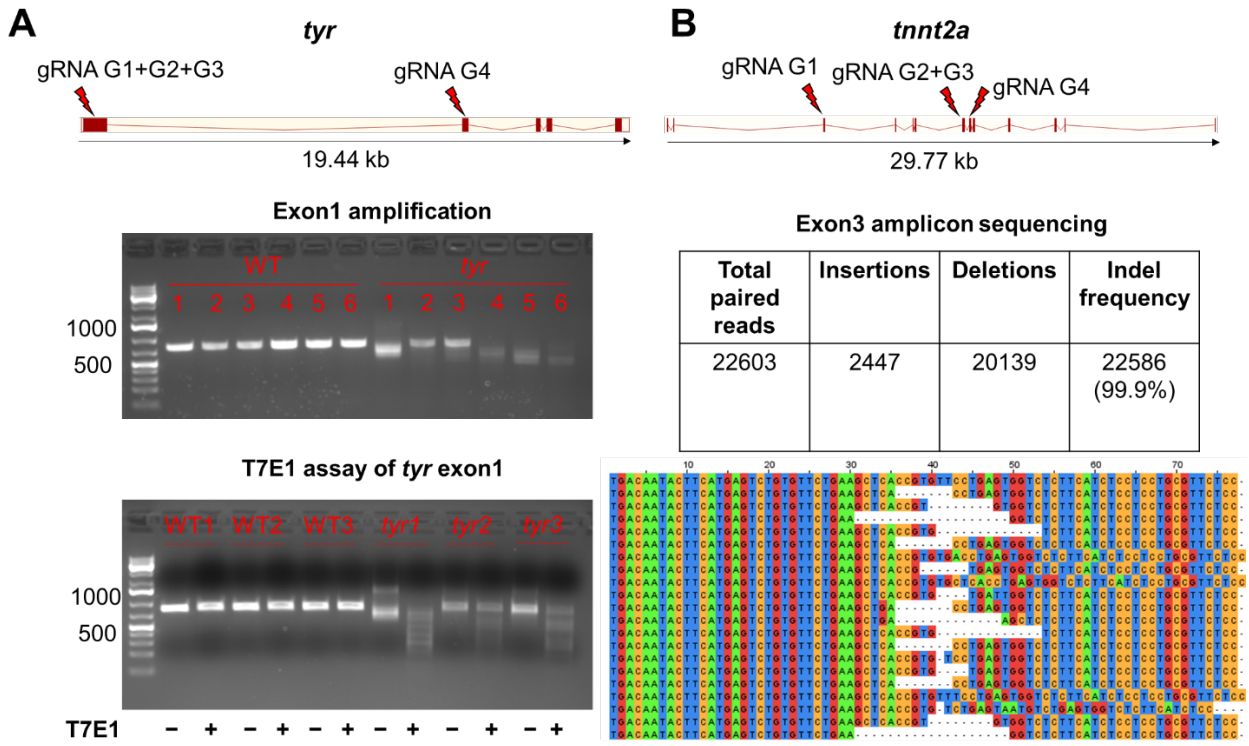


Fig. S1. Multiplexed gRNA injection recapitulates mutant phenotypes in F0 embryos. (**A**) Comparison of the advantages and disadvantages of forward-genetics *vs* reverse-genetics in zebrafish. MIC-Drop enables the targeted mutagenesis of reverse-genetics and the scalability of forward-genetics. (**B-D**) Injection of Cas9 and 4 gRNAs targeting each gene-of-interest

recapitulates known mutant phenotypes in F0 embryos with high efficiency (**D**) and with no significant toxicity (**C**). (**C**) N = (wild type-311; *tyr*-221; *tnnt2a*-191; *tbx5a*-224; *rx3*- 132; *npas4l*-235; *chrd*-149; *tbx16*-135; *fgf24*-99). (**D**) N = (wild type-287; *tyr*-190; *tnnt2a*-169; *tbx5a*-202; *rx3*-118; *npas4l*-213; *chrd*-142; *tbx16*-123; *fgf24*-91). (**E**) Representative images of gross morphological defects observed in ~10% of MIC-Drop injected embryos.

Figure S2



Total paired reads	Insertions	Deletions	Indel frequency
22603	2447	20139	22586 (99.9%)

Fig. S2. Multiplexed gRNA injection results in high targeted editing. (**A**) T7E1 assay in embryos injected with multiplexed gRNAs targeting *tyr* gene reveals high editing efficiency. Amplicons from the targeted site show large deletions (top gel; *tyr* samples 1-6). Treatment of the amplicons with T7 endonuclease shows multiple bands (bottom gel) suggesting high indel frequencies in the injected embryos. (**B**) Amplicon sequencing of *tnnt2a* exon 3 shows mosaicism with near complete editing efficiency, with a high frequency of 5-20bp deletions in the targeted site.

Figure S3

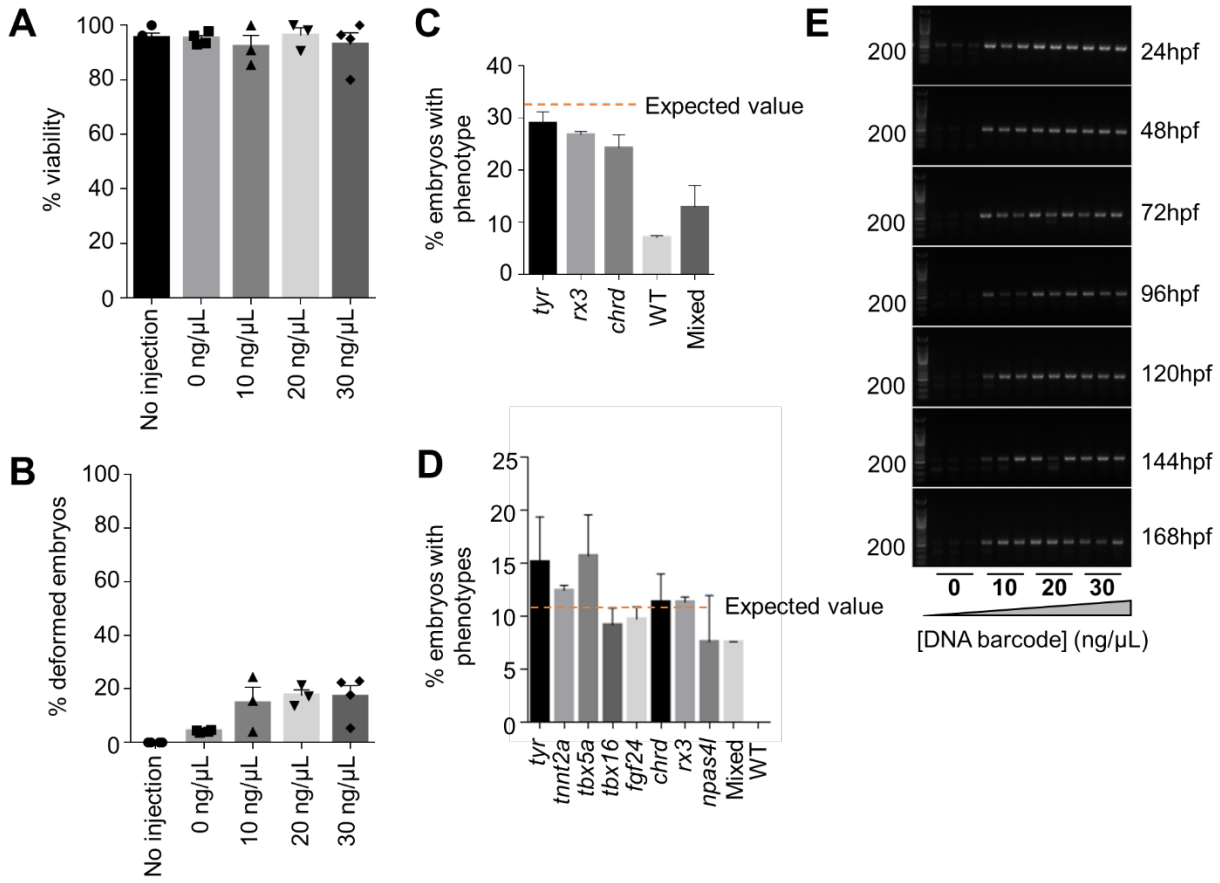


Fig. S3. MIC-Drop enables single-needle injection of droplets targeting multiple genes. (**A-B**) Incorporation of DNA barcodes in the droplets does not alter viability of the injected embryos (**A**) but causes slight increase in deformities resulting from nucleic acid toxicity (**B**) N = (no injection-315; 0 ng/µL-203; 10 ng/µL-152; 20 ng/µL-175; 30 ng/µL-225. (**C-D**) Single-needle injection of intermixed droplets targeting 3 genes (Total N = 239) (**C**) or 8 genes (Total N = 192)(**D**) and subsequent phenotyping and barcode sequencing reveal a proportionate representation of the droplets, with most embryos showing one of the unique phenotypes. ~5% of embryos show mixed phenotype and consequent mixed barcode sequencing results likely due to unintended co-injection of more than one droplet. (**E**) The DNA barcodes are stable after injection in embryos and can be successfully retrieved and sequenced at 168 hpf (7dpf).

Figure S4

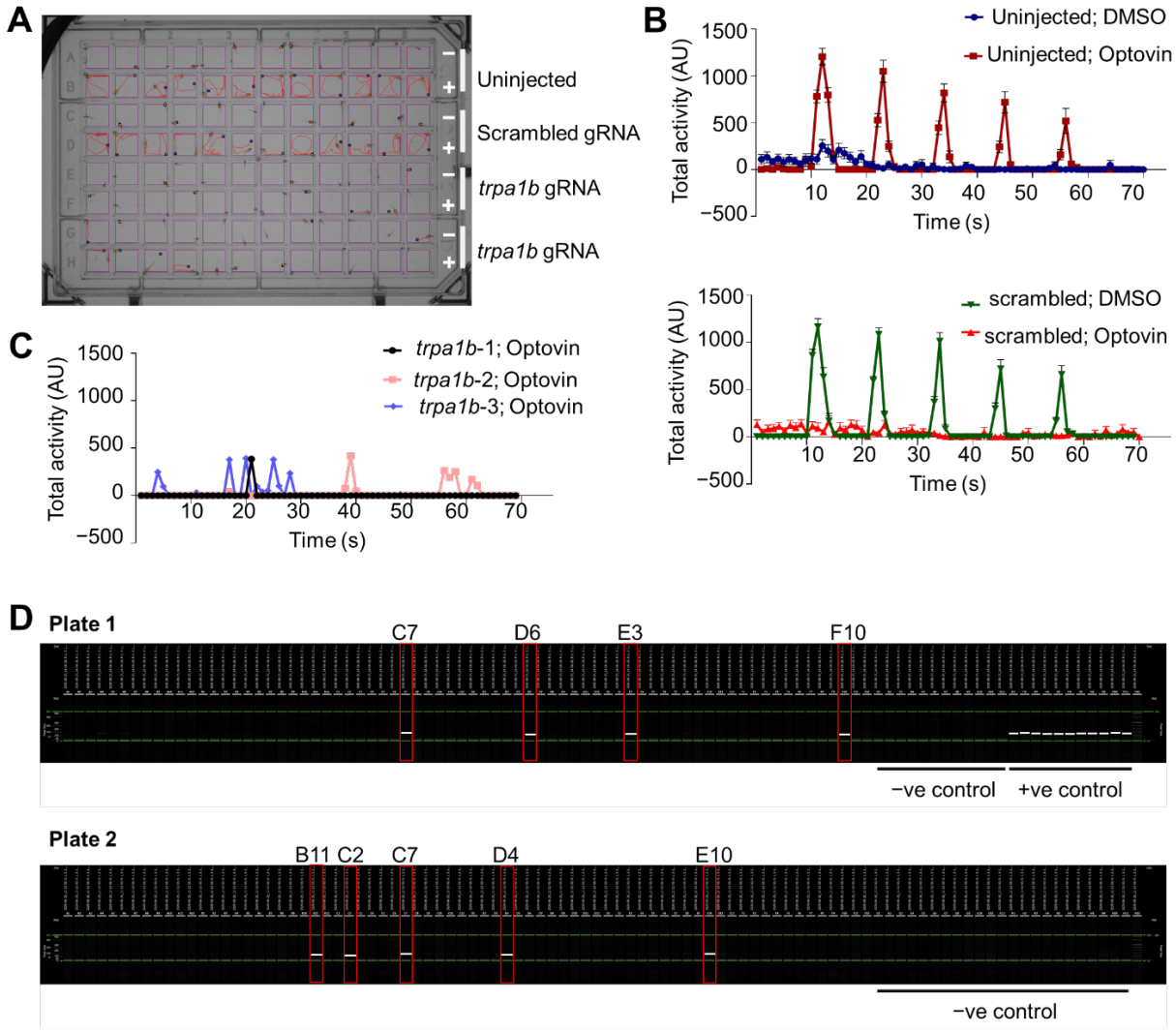


Fig. S4. MIC-Drop enables identification of gene targets of small-molecules. (**A-C**) Treatment of zebrafish embryos with optovin (+) results in a light-dependent motion response. Embryo tracking (**A**) and quantitation of movement (**B-C**) shows increased zebrafish activity triggered by pulsed violet light (also see Movie S2). Embryos injected with a set of non-targeting scrambled gRNAs (bottom) behave the same as uninjected controls (top) (**B**). Embryos injected with gRNAs targeting *trpa1b* are refractory and show no light-triggered movement (**A**). Optovin- and light-triggered activity quantitation of three sample embryos injected with *trpa1b*-targeting gRNAs. (**D**) Diagnostic PCR to test the barcode identities of embryos injected with 20:1 mix of droplets targeting scrambled: *trpa1b* (also see Fig. 2C). 6.25% of the intermixed droplet-injected embryos (9/144) have the *trpa1b* barcode. Uninjected embryos are used as negative controls. Lines are drawn on top of gel bands for ease of viewing**.**

Figure S5

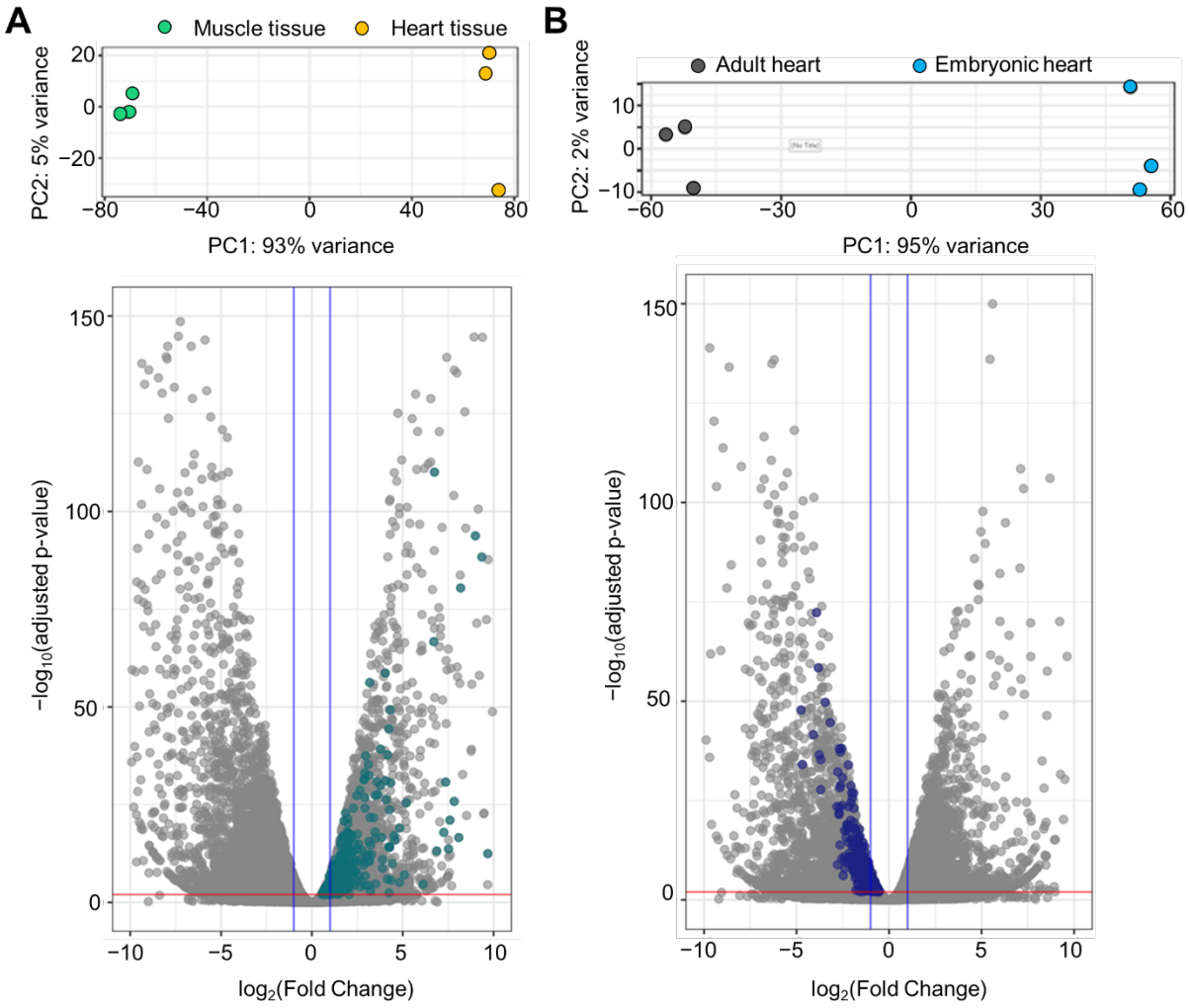


Fig. S5. RNAseq data analysis to curate a list of candidate genes important in vertebrate heart development. (**A**) Principle-component analysis (PCA) and volcano plot of differentially expressed genes in the zebrafish heart *vs.* zebrafish muscle tissue and (**B**) adult heart *vs*. embryonic heart. PCA analysis shows high sample-to-sample concordance (3 samples of each). Highlighted dots on volcano plots show genes enriched in the heart relative to muscle and embryonic heart relative to adult heart. Red line (5% FDR); blue line (2-fold differential expression).

Figure S6

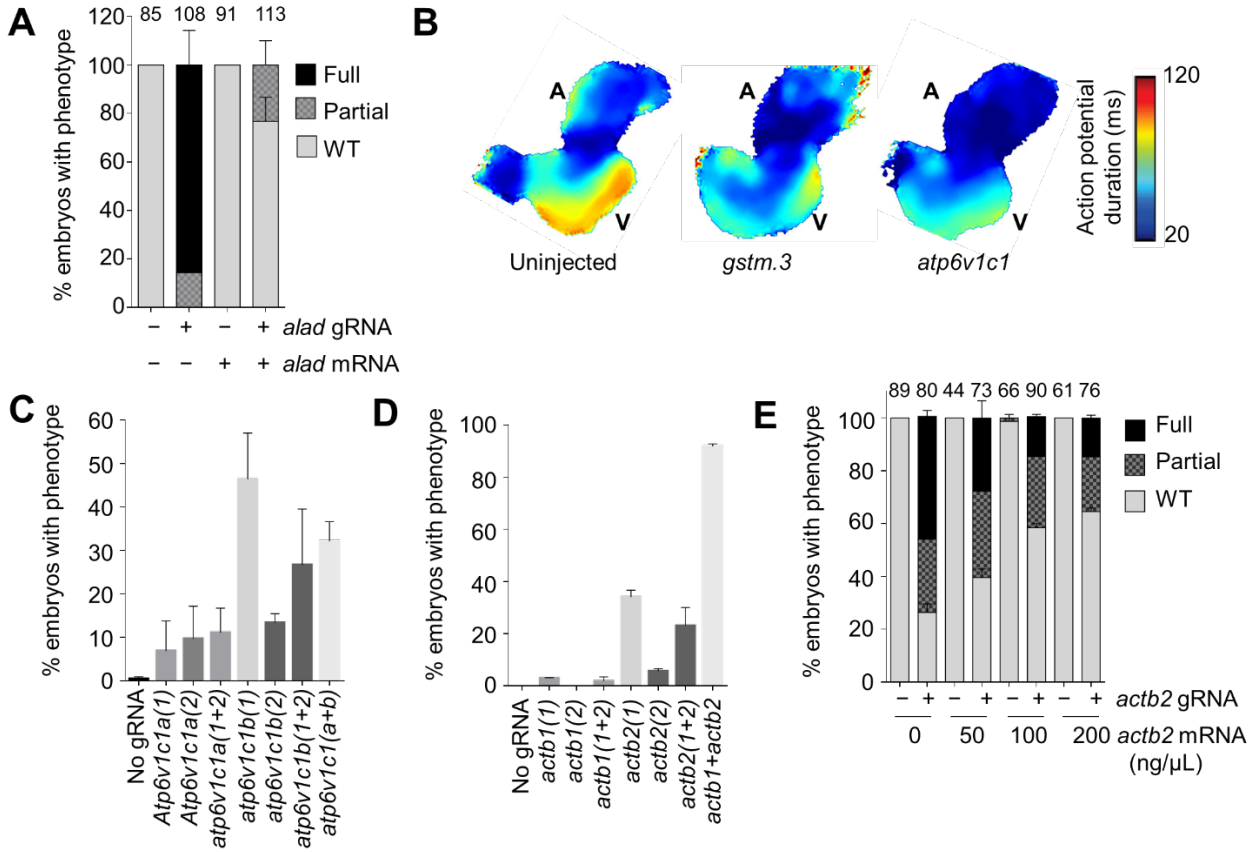


Fig. S6. CRISPR screen using MIC-Drop discovers novel genes responsible for vertebrate heart and blood development. (**A**) Injection of *alad* mRNA rescues the porphyria phenotype of *alad* crispants (also see Fig. 4A). Number of embryos counted is reported above each bar. (**B**) Representative action potential duration graphs of *gstm.3* and *atp6v1c1* crispants show shorter delay between atrium and ventricle beats compared to uninjected controls. (**C**) Loss of *atp6v1c1b* alone recapitulates the phenotypes observed in crispants injected with gRNAs targeting both *atp6v1c1a* and *atp6v1c1b* ohnologs. Two gRNAs (1 and 2) are used per ohnolog. N = (no gRNA-156; *atp6v1c1a*(1)-83; *atp6v1c1a*(2)-72; *atp6v1c1a*(1+2)-113; *atp6v1c1b*(1)-103; *atp6v1c1b*(2)-106; *atp6v1c1b*(1+2)-147; *atp6v1c1(a+b)*-149 (**D**) Similarly, loss of *actb2* alone results in cardiac defects. (**E**) The cardiac phenotype resulting from *actb2* loss can be rescued with injection of *actb2* mRNA. N = (no gRNA-249; *actb1*(1)-137; *actb1*(2)-130; *actb1*(1+2)-75; *actb2*(1)-138; *actb2*(2)-126; *actb2*(1+2)-80; *actb1+actb2*-95.

Figure S7

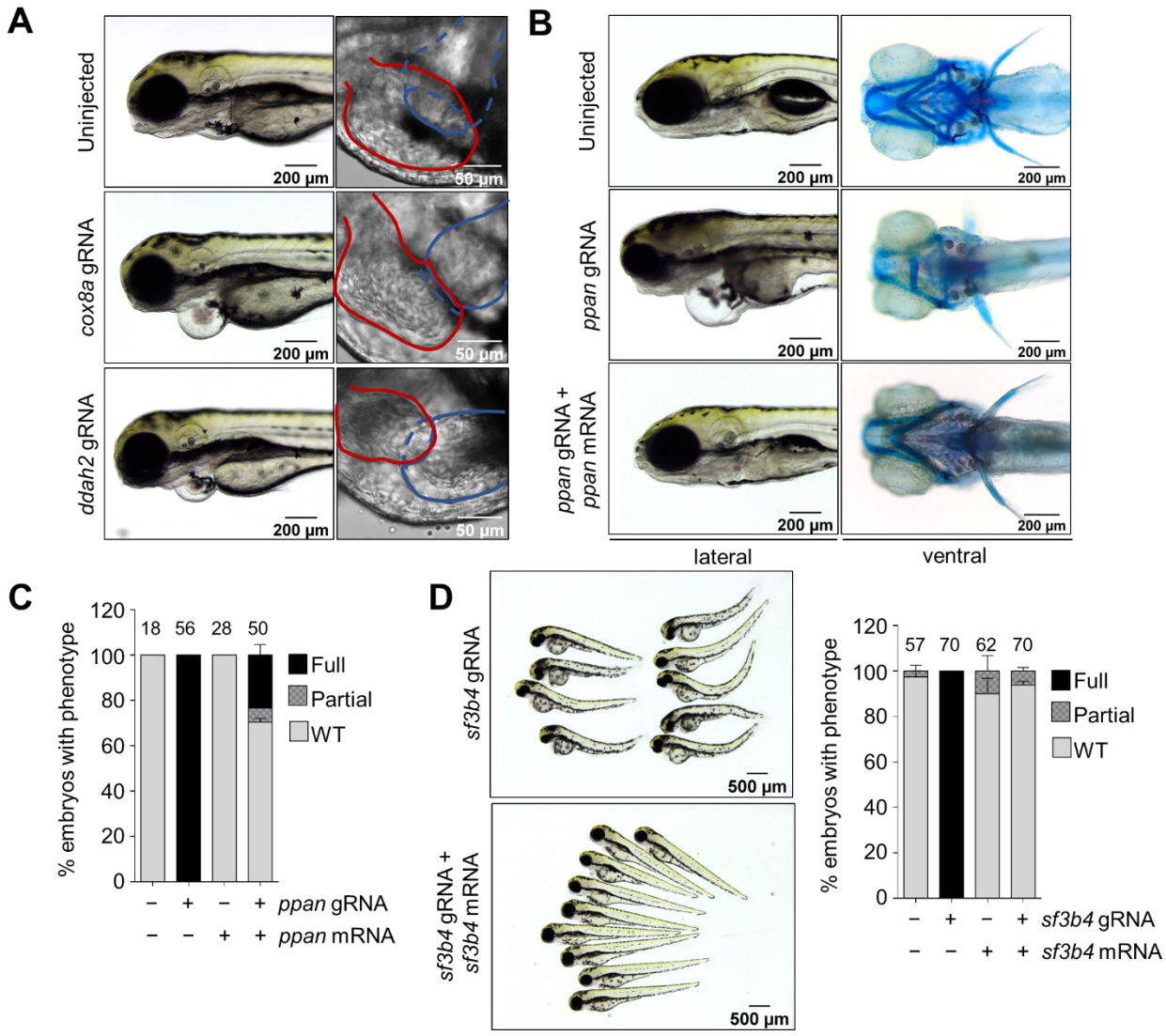


Fig. S7. CRISPR screen identifies novel genes responsible for cardiac development and function. (**A**) *cox8a* and *ddah2* crispants display cardiac edema and incomplete cardiac looping. Red outline: ventricle; Blue outline: atrium; atrium in the wild type (blue dashed line) is looped properly and therefore out of focus from the ventricle. (**B-C**) Loss of *ppan* results in cardiac edema, a silent ventricle, as well as jaw and craniofacial deformities. Alcian blue staining of 5 dpf embryos and quantitation (**C**) shows the deformities can be rescued by injection of *ppan* mRNA.
(**D**) Similarly, various phenotypes including a bent trunk, head and eye deformities, and a silent ventricle in *sf3b4* crispants can be completely rescued with *sf3b4* mRNA injection.

Table S1. gRNA sequences targeting *chrd*, *fgf24*, *npas4l*, *rx3*, *tbx5a*, *tbx16*, *tnnt2a, trpa1b* and *tyr***.**

Table S2. Genes differentially expressed in zebrafish heart relative to muscle tissue.

Table S3. Genes differentially expressed in embryonic heart relative to adult heart**.**

Table S4. Genes enriched in zebrafish embryonic heart.

Table S5. gRNA sequences targeting candidate genes responsible for vertebrate heart development, barcode retrieval frequency of gene from the screen, and cardiac and blood phenotypes observed in the droplet injected embryos.

Movie S1. Injection of droplets in zebrafish embryos.

Movie S2. *trpa1b* gRNA injected embryos recapitulate *trpa1b* loss of function (*trpa1b* -/-) phenotype and are refractory to optovin- and light- induced motion response.

Movie S3. Loss of *actb2* and *actb1* results in a linear heart with a small and silent ventricle.

Movie S4. *clec19a* crispants have defects in the atrioventricular valve formation resulting in blood regurgitation from the ventricle back to the atrium.

Movie S5. *ppan* cripants show cardiac edema as well as a silent ventricle in addition to gross defects in the head and the jaw at 5 dpf.

Movie S6. Hearts of *sf3b4* crispants show abnormal contraction compared to wildtype hearts.