Ac/Ds transposition for CRISPR/dCas9-SID4x epigenome modulation in zebrafish

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ABSTRACT

Due to its genetic amenability coupled with advances in genome editing, zebrafish is an excellent model to examine the function of (epi)genomic elements. Here, we repurposed the Ac/Ds maize transposition system to efficiently characterise zebrafish cis-regulated elements, also known as enhancers, in F₀-microinjected embryos. We further used the system to stably guide RNAs enabling CRISPR/dCas9-interference (CRISPRi) perturbation of enhancers without disrupting the underlying genetic sequence. In addition, we probed the phenomenon of antisense transcription at two neural crest gene loci. Our study highlights the utility of Ac/Ds transposition as a new tool for transient epigenome modulation in zebrafish.

KEY WORDS: Zebrafish, Ac/Ds, CRISPRi, Enhancer, Antisense

INTRODUCTION

Genomic transposition is an established approach for somatic and germline integration of DNA constructs in the zebrafish model. Tol2-mediated transposition is a reliable method for generating transgenic reporter lines in zebrafish (Kawakami, 2004), while Ac/Ds and MMLV (Vrljicak et al., 2016) are used less. The maize Ac/Ds system (McClintock, 1950) consists of two Ds (Dissociation) genetic elements and an Ac (Activator) transposase (Fedoroff et al., 1983). Ac/Ds transposition led to highly efficient integration of reporter constructs with a remarkable germline transmission rate in the zebrafish (Emelyanov et al., 2006). It was subsequently used to generate the chemically inducible LexPR transactivation system (Emelyanov and Parinov, 2008; Kenyon et al., 2018), and perform systematic mutagenesis gene-trapping screens (Quach et al., 2015). While these studies demonstrated the utility of Ac/Ds as an efficient method for the propagation of transgenes through the germline, they overlooked its strong potential for transient expression of DNA elements in F₀ embryos. Regardless of the integration method, transposition in F₀ zebrafish embryos produces variable somatic results with high rates of non-specific background and mosaic expression, hence limiting the potential of this model for transient studies. Therefore, the analysis of exogenous features often relies on generation of transgenic F₁ offspring, which is time- and resource-consuming for medium to high throughput screening of transgenes. We sought to develop a flexible molecular toolkit by repurposing the Ac/Ds system to transiently screen reporter constructs and target gene expression in the zebrafish with functional and quantifiable output.

Perturbation approaches in F₀ zebrafish embryos currently exist, such as morpholino-mediated obstruction of protein synthesis or RNA-silencing or gene editing using TALENs or CRISPR/Cas tools. CRISPRi/dCas9-based interference (CRISPRi) uses nuclease-deficient Cas9 (dCas9) targeted to specific genomic regions using guide RNAs (sgRNAs). dCas9 is targeted to transcription start sites (TSS) of genes to inhibit RNA Polymerase II by steric hindrance (Qi et al., 2013), or fused to effector domains such as Kruppel-associated box (KRAB) (Gilbert et al., 2013) or four concatenated mSin3 repressive domains (SID4x) (Konermann et al., 2013) to induce chromatin changes inhibitive of transcription. Crucially, this allows the tuning of gene expression without modifying the endogenous locus sequences (Liu et al., 2017; Thakore et al., 2015; Williams et al., 2018; Dong et al., 2017; Long et al., 2015). Unlike the creation of indels in Cas9 F₀ mutants, where genome editing occurs early in development and is propagated through subsequent cell divisions, CRISPRi in F₀ embryos requires extended expression of sgRNAs for the duration of an experiment. This is limited by the current gold standard approach of injecting in vitro-transcribed sgRNAs, which degrade quickly in the absence of Cas9 protein (Burger et al., 2016; Hendel et al., 2015). Reliable assessment of CRISPRi effects will therefore be limited to early development in F₀ embryos and require germline propagation of sgRNA constructs for later-stage analyses.

In this study, we demonstrated that Ac/Ds transposition is an efficient method for the transient propagation of transgenes with sustained expression of sgRNAs to 5 dpf. As a result, Ac/Ds-integrated sgRNAs in microinjected transgenic embryos expressing sox10-specific dCas9-SID4x enabled tissue-specific perturbation of epigenomic features in the zebrafish with robust and detectable effects from 24 hpf. Our approach broadens the utility of the zebrafish embryo for rapid studies of non-coding genomic elements, including enhancers, and complements current methods for targeting protein-coding genes.

RESULTS AND DISCUSSION

Ac/Ds transposition enables efficient expression of transgenes in F₀ embryos

To repurpose the maize Ac/Ds transposition system for zebrafish, we first generated a new enhancer–reporter construct pVC-Ds-E1b:
eGFP-Ds (‘Ac/Ds-enh’). A multiple cloning site for testing enhancer sequences was placed upstream of an E1b minimal promoter (Becker et al., 2016), driving the expression of eGFP. The entire cassette was flanked by Ds elements for integration into the genome (Emelyanov and Parinov, 2008; Emelyanov et al., 2006) (Fig. 1A). We compared the activity of Ac/Ds- and Tol2-mediated transposition (Kawakami, 2004) by transiently expressing previously identified zebrafish enhancers for pax3a, ets1 and sox10 (Lukoseviciute et al., 2018). Embryos were microinjected with the same amounts of nucleic acid (30 pg Ac/Ds or Tol2 vector DNA, 24 pg Ac or Tol2 mRNA), and those with the same expression patterns in both conditions were counted. We found that Ac/Ds is similar or better than Tol2 in producing embryos with specific expression patterns (45.2 to 88.0% versus 26.3 to 75.0%) for the six different enhancers we have tested (Fig. 1A; Table S1). Tol2 integrations consistently exhibited a fainter eGFP signal which can be bypassed by injecting higher amounts of nucleic acid (150 pg vector DNA with 80 pg mRNA) (Fig. 1B).

The high efficiency of Ac/Ds integrations with lower amounts of DNA and mRNA renders it an excellent transient DNA expression system in zebrafish. Not only can the toxicity issues be avoided (those are observed when higher injected levels are required for somatic integration), but ectopic activity from the episomal expression of the non-integrated plasmid is also minimised. This places the zebrafish embryo on par with the chick embryo as an excellent model for testing enhancer activity transiently in F0 embryos (Streit et al., 2013; Weinberger et al., 2021 preprint). We conclude that our Ac/Ds transposition approach enables consistent tissue-specific expression patterns and is an excellent binary tool for transient screening of enhancer activity in zebrafish.

**Ac/Ds-sgRNA expression system for transient CRISPR/Cas**

Next, we capitalised on robust Ac/Ds transposition in transient to generate a constitutive sgRNA expression system for CRISPR/Cas experiments in F0 embryos. We cloned a sgRNA cassette containing a zebrafish-specific U6a promoter (Yin et al., 2015) into a custom-made Ac/Ds mini-vector (‘Ac/Ds-sgRNA’) (Fig. 2A). The 20 bp spacer region within the cassette was flanked by BsmBI restriction sites to facilitate Golden Gate-like cloning (Clarke et al., 2012) of different sgRNAs. We compared the expression of a

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**Fig. 1. Evaluation of Ac/Ds and Tol2 transposition in F0 embryos.** (A) Schematic of enhancer construct containing enhancers (‘enhancer’) upstream of E1b minimal promoter (‘E1b-mp’) driving eGFP expression. Two versions of each enhancer construct (with Ds- or Tol2-integration arms) were tested by microinjection into one-cell-stage embryos. Ac or Tol2 clutches of the same enhancer were imaged using the same settings on a fluorescent stereomicroscope. Embryos with the same expression pattern (Table S1) were counted. Scale bars: 500 µm. (B) Ds- or Tol2-armed pax3a enh5 was microinjected with Ac or Tol2 mRNA, respectively, into Gt(FoxD3:mCherry)ct110aR embryos to visualise the neural tube (mCherry, cyan). Live confocal imaging highlighted similar levels of neural crest cell labelling (eGFP, yellow) between 30 pg Ds and 150 pg Tol2 vector DNA, but 30 pg Tol2 vector DNA yielded a visually weaker signal. Scale bars: 50 µm.
scrambled sgRNA sequence (AGCGCCTACGCGCATGGCCT) from integrated Ac/Ds-sgRNA versus in vitro-transcribed transcript(s). The vector (50 pg) with Ac mRNA (24 pg), were microinjected into wild-type embryos and sgRNA expression was assayed 5 and 24 h post injection (hpi) and 5 days post injection (dpi). We found that sgRNA expression at 5 dpi could only be detected using Ac/Ds (Fig. 2A’). Consequently, we demonstrated in a separate study that our approach enabled efficient F0 mutant studies up to 5 dpf via co-injection with Cas9 mRNA (Weinberger et al., 2020).

To achieve tissue-specific CRISPRi, we generated a BAC transgenic line TgBAC(sox10:dCas9-SID4x-2a-Citrine)ox117 (‘ox117’) using a previously validated sox10 BAC allele TgBAC(sox10:cytoBirA-2a-mCherry)ox104 (‘ox104’) (Trinh et al., 2017). Ox117 resulted from the replacement of sox10’s first exon with a cassette containing dCas9 fused to four tandem mSin3 repressive domains (SID4x) (Konermann et al., 2013), T2A ribosome-skipping element, and Citrine fluorescent protein (‘Citrine’). Homology arms (red lines) enabled replacement of sox10’s first exon in BAC clone DKEY-201F15 with the dCas9-SID4x cassette. Transgenic offspring displayed largely overlapping expression with a different allele made from the same BAC (TgBAC(sox10:cytoBirA-2a-mCherry), ‘ox104’) in cranial and trunk neural crest cells, as well as the otic vesicle (ov) (Fig. 2B). However, unlike the ox104 line, Citrine is not expressed in ox117’s neural tube (B’’). Scale bars: 50 µm.
CRISPRi of endogenous enhancers induces detectable changes in gene expression

Previous studies have demonstrated the requirement for multiple sgRNAs to elicit successful CRISPRi effects (Qi et al., 2013; Williams et al., 2018). We reasoned that the small size of the Ac/Ds-sgRNA vector (<4.5 kb), coupled with the small load required for activity, would permit using multiple sgRNAs for CRISPRi microinjections. High-quality pools of individually cloned sgRNAs were prepared using a simplified pooled transformation approach reminiscent of the generation of large scale sgRNA libraries. *Ox117* transgenic embryos were microinjected with experimental (targeting putative enhancers) or scrambled sgRNA pools. Microinjection of 200 pg of sgRNA pool (consisting of 4 to 15 sgRNAs) per embryo yielded survival rates of ~50% or higher. 24 hpf embryos were dissociated and FAC-sorted to obtain Citrine-positive cells expressing sox10 BAC-driven dCas9-SID4x. Gene expression was measured by TaqMan qPCR chemistry and qpcR analysis in R (Ritz and Spiess, 2008) (Fig. 3A).

We evaluated putative enhancers for cadherin 7a (*cdh7a*) gene. In chicken, Cdh7 function was first described in delaminating neural crest forming the melanocyte precursor subpopulation (Nakagawa and Takeichi, 1995, 1998), and later demonstrated to be important for trigeminal ganglia assembly (Wu and Taneyhill, 2019). We assessed four candidate zebrafish *cdh7a* enhancers (Up-1 and Up-2 i.e. ‘Group Upstream’; Int-1 and Int-2 i.e. ‘Group Intronic’) identified using chromatin accessibility data from zebrafish neural crest (Lukoseviciute et al., 2018; Trinh et al., 2017) (Fig. 3B). Using our Ac/Ds-enh vector we validated their activity in F0 embryos, where sporadic co-labelling with endogenous sox10 expression could be detected in the trunk neural crest and cranial mesenchyme (Group Upstream only) or otic vesicle (Group Intronic only) (Fig. 3B′-B″). As these enhancers displayed detectable differences in chromatin accessibility in *foxd3*-mutants compared to wild type (Lukoseviciute et al., 2018), we reasoned that they were ideal for demonstrating utility of sox10:CRISPRi in investigating the function of novel enhancers. Targeting all enhancers simultaneously with a pool of 15 sgRNAs led to ~50% downregulation (median±s.d.=−0.48±0.089; P=0; n=4) of *cdh7a* expression compared to scrambled control (Fig. 3C, green plot). This was consistent with the additive action of multiple enhancers in regulating gene expression of their target genes (Choi et al., 2021; Fulco et al., 2016). Furthermore, this effect was reversed, with ~50% upregulation of *cdh7a* when sgRNAs targeting intrinsic enhancers were excluded from the pool (Fig. 3C, orange plot). Given that both groups of enhancers functioned cooperatively to fine-tune and maintain appropriate levels of *cdh7a* expression in scrambled sgRNAs/unperturbed condition, two plausible scenarios could explain our observation: either intrinsic elements acted as activator enhancers for *cdh7a* while upstream elements were repressive, or targeting dCas9-SID4x to Group Intronic may have inadvertently blocked transcriptional elongation of *cdh7a* itself. However, a previous study assessing steric hindrance effect of dCas9/dCas9-KRAB highlighted that this occurred when sgRNAs targeted ~50 and +300 bp from the TSS of endogenous genes (Gilbert et al., 2014). Given that our enhancers were ~20-72 kbp up or downstream of *cdh7a*’s TSS, we reasoned that the latter scenario was unlikely.

We further assessed two upstream enhancers of *pdgfra*, a previously identified gene with differential chromatin accessibility in vagal neural crest (Ling and Sauka-Spengler, 2019). We observed a similar effect to the *cdh7a* Group Upstream-only CRISPRi, with an upregulation of *pdgfra* expression following CRISPRi using a pool of 7 sgRNAs (median±s.d.=2.82±1.419; P=0.01; n=4) (Fig. 3C′). As these enhancers were proximal (both within ~6.5 kb of the TSS), we speculate the presence of yet unidentified distal enhancer(s) with strong activating capacity akin to *cdh7a* distal Group Intronic enhancers (41 and 72 kbp downstream from TSS). Taken together, these results demonstrated the utility of our sox10: CRISPRi/Ac/Ds-sgRNA approach as an exploratory tool to transiently probe enhancer function in *vivo* in a cell-specific manner.

CRISPRi at 5′ site of antisense transcription affects protein and chromatin homeostasis

CRISPRi with dCas9 alone was initially reported to have strand-specific activity when targeting transcriptional elongation, but not initiation, when using control sgRNAs that bind the non-template strand (Qi et al., 2013). However, this effect was less clear in later studies and was lost when dCas9 was fused to a repressor or activator domain (Gilbert et al., 2013, 2014; Howe et al., 2017). In light of these findings, we adapted sox10:CRISPRi/Ac/Ds-sgRNA to query antisense transcripts at two neural crest genes (*sox9a-AS* and *foxd3-AS*) (Fig. 4A). SgRNAs were selected irrespective of strand to target the initiation of antisense transcription (Fig. 4A, star). Wild-type staining and visualisation of *sox9a-AS* and *foxd3-AS* transcripts using hybridization chain reaction (Choi et al., 2018) indicated broad, low-level expression throughout the embryo, which partially overlapped their cognate genes, *sox9a* and *foxd3* (Fig. 4A′).

As before, *ox117* transgenic embryos were microinjected with a pool of 10 sgRNAs (five per locus) or four scrambled sgRNAs and FAC-sorted to obtain Citrine-positive cells expressing sox10 BAC-driven dCas9-SID4x. To assess local and global effects following CRISPRi, strand-specific RNAseq libraries were prepared, followed by unbiased transcript quantification and differential expression (Fig. 4B). Bootstrapped estimates of *sox9a-AS* and *foxd3-AS* indicated ~2.3- and 1.7-fold upregulation (average, n=2), respectively, although neither was statistically significant (Fig. 4C). This lack of significance for the observed effect was unsurprising given their low levels of expression: *sox9a-AS* expression was detected ~8- and 30-fold lower compared to its two cognate isoforms, while *foxd3-AS* expression was ~50-fold lower. This result illustrated inherent challenges of capturing and studying lowly/dynamically expressed antisense transcripts in general. We also observed misregulation (up- or downregulation) of the sense transcripts, indicating a potential secondary effect on gene expression due to chromatin changes at their 3′UTR region resulting from dCas9-SID4x recruitment (Howe et al., 2017; Murray et al., 2015), which may have contributed to the net upregulation of *sox9a* and *foxd3-AS* following CRISPRi.

Next, we asked if global cellular changes could be detected resulting from the observed misregulation in sense/antisense transcription of *sox9a* and *foxd3* (Fig. 4C′). Concomitant or concurrent with local destabilisation of *sox9a*/*foxd3* sense/antisense transcripts, we observed an overrepresentation of downregulated
genes (qval<0.05) involved in nucleosome assembly and organisation, namely histone genes such as h2ax, h2az2b, hist1h2a3, hist1h2a5, hist1h2a6, hist1h2a10, hist1h2a11, h3f3b.1, H3C15, h3f3d, and hist1h4 l. This was consistent with reported dynamic histone turnover rates being correlated with the level of antisense, rather than sense, transcription (Murray et al., 2015).

**METHODS & TECHNIQUES**

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<th>Pooled transformation Min/midprep</th>
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<td>3 - 5 sgRNAs per target</td>
<td>Guide pool + Ac transposase mRNA in/out cross</td>
<td>24h</td>
<td>Collect Citrine+ cells</td>
<td>qRT-PCR target gene</td>
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**Fig. 3. CRISPRi of neural crest enhancers.** (A) Sox10:CRISPRi workflow to investigate function of enhancers in microinjected zebrafish embryos. 3-5 spacer sequences per enhancer for 2-4 enhancers per target gene were selected and individually cloned into Ac/Ds U6 vector. Cloned guides were combined according to experimental design and transformed to obtain a single prep per pool with sufficient quality and concentration. Scrambled guide pools were prepared in parallel as control. Guide pools (enhancer or scrambled) were microinjected into one-cell-stage embryos and allowed to develop for 24 h. Embryos were dissociated and FAC-sorted to collect sox10:Citrine+ cells expressing dCas9-SID4x. RNA was extracted and expression of endogenous target controlled by the enhancers tested was measured by quantitative PCR. (B) UCSC Genome Browser snapshot of the cdh7a locus showing ATAC-seq from sox10+ cells (black track). Four cdh7a putative enhancer regions were investigated: two upstream (rectangles) and two within introns (diamonds). (B’) Combined readout of ‘upstream’ or ‘intronic’ cdh7a enhancer activity in F0 embryos. Each enhancer was cloned into Ac/Ds enhancer:GFP construct (Fig. 1). ‘Upstream’ or ‘intronic’ enhancers were pooled and microinjected into one-cell-stage embryos. Enhancer(s) activity (‘GFP’ in relation to endogenous sox10 (‘sox10’) expression was detected by immunohistochemistry at 24 hpf. (B”) Sporadic overlap (white arrows) of enhancer(s) activity were detected in the trunk neural crest in both cases, in the cranial mesenchyme only for ‘upstream’, and in the otic vesicle for ‘intronic’ enhancers. Maximum intensity Z-stack projections are shown in (B’), single plane confocal images are shown in (B”). Scale bars: 50 µm. (C) Quantitative PCR of cdh7a following CRISPRi of its putative enhancers (n=4 per condition). Downregulation of cdh7a (medians+s.d.=0.48±0.089; P=0) was observed when all four enhancers were targeted, compared to scrambled control. This effect was reversed (median+s.d.=1.42±0.155; P=0) when the upstream enhancers only were targeted. (C’) A similar upregulation effect (median+s.d.=2.82±1.419; P=0.01) when upstream enhancers were targeted was observed at the pdgfra locus.
However, in our study it was not clear whether the effect was due to disruption of either or both. We also observed an overrepresentation of downregulated genes related to the translational machinery, including ribosomal genes such as rpl15, rpl35a, rpl12, rps23, rpl14, rpl39, rpl18, rpl8, rpl21, and rps18. On the other hand, protein-refolding genes, including chaperones, were upregulated (qval < 0.05), as were genes involved in development of pigment cells, which are neural crest derivatives. Given the 3' UTR region’s classical role in the translational regulation of mRNA transcripts, this was consistent with misregulation at the sense 3' UTR regions of sox9 and foxd3. Taken together, we concluded that CRISPRi/Ac/Ds-sgRNA is a useful approach in zebrafish to study context-specific epigenome features and their impact on gene regulatory processes.

Fig. 4. See next page for legend.
Fig. 4. CRISPRi of antisense transcription initiation at neural crest genes. (A) UCSC Genome Browser snapshot of the sox9a and foxd3 loci with ATAC-seq (black track) and strand-specific RNA-seq (red track, sense; blue track, antisense) from sox10+ cells (ATAC-seq) or nuclei (RNA-seq). Selected guide RNAs target the initiation of antisense transcription (star) of sox9a-AS or foxd3-AS. (A') Hybridisation Chain Reaction detection of coding/sense (sox9a, foxd3) and antisense (sox9a-AS, foxd3-AS) transcripts in 24 hpf embryos. Antisense transcripts/puncta (cyan) were expressed in a ubiquitous, basal-like fashion. A similar basal-like expression of sense transcripts/puncta (red channel) was observed, along with spatially restricted regions of higher expression (white arrows). Single plane confocal images of the otic vesicle region are shown. Scale bars: 50 µm. (B) Sox10: CRISPRi workflow to investigate effect of sense/antisense transcription in microinjected zebrafish embryos. Ac/Ds U6 cloned guides were pooled according to experimental design (10 guides in total, five per locus) and transformed into bacteria to obtain a single prep per pool of high quality. Guide pools (foxd3-sox9a; or scrambled) were microinjected into one-cell-stage ax117 embryos and allowed to develop for 24 h. Embryos were dissociated and FAC-sorted to collect sox10:Citrine+ cells expressing dCas9-SID4x. RNA was extracted and RNAseq libraries were prepared using rRNA-depletion followed by strand-specific dUTP method with two replicates per condition. Transcript quantification and differential expression were performed using the kallisto/sleuth statistical pipeline. (C) Quantification of transcripts (transcripts per million, TPM) following sox10:CRISPRi. Bootstrapped estimates of sense and antisense sox9a/foxd3 transcripts in each biological replicate. Down/upregulation could be detected for sense and antisense foxd3/sox9a transcripts in experimental versus scrambled condition although not statistically significant, with the exception of foxd3 (FC=1.261, qval=0.007). (C') PANTHER Gene Ontology (GO) overrepresentation statistical analysis of differentially expressed genes (qval <0.05). Top 10 GO terms (Fisher’s exact test, with false discovery rate <0.01) shown.

MATERIALS AND METHODS
Zebrafish husbandry
Zebrafish experiments were conducted according to regulated procedures authorised by the UK Home Office within the Animals (Scientific Procedures) Act 1986 framework. Wild-type and transgenic embryos were derived from AB or AB/TL mixed strains.

Plasmids and oligo sequences
Plasmids (including Addgene IDs where applicable) and oligo sequences are available as Tables S2 and S3, respectively.

Cloning of Ac/Ds-enh reporter vectors
Putative enhancer elements were amplified from genome DNA by PCR and cloned into pVC-Ds-Elbe:GFP-Ds (Addgene ID 102417) linearised with NheI. Cloning was performed using In-Fusion HD Cloning Plus (Takara) or Gibson Assembly cloning (Gibson et al., 2009). 5′ overhangs were appended to enhancer-specific primers (Table S3) as follows: TCGAGTT-TACGTACCGCTAG on forward and TATCGCCGCAAGCTTGCTAG on reverse. Cloning reactions were transformed into chemo-competent cells and plasmids were prepared using QIaprep Spin Miniprep kit (27104, Qiagen), with the remaining ∼20 ml pelleted, ∼20 ml supernatant removed and frozen as back-up for Midiprep. Final pools were resuspended in RNase-free water. mRNA quality was assessed by gel electrophoresis (sharp intact band without degradation) and quantified using Qubit™ RNA HS Assay kit (Q32852, ThermoFisher). For long term storage in −80°C the purified mRNA was prepared as 1 µl aliquots and limited to one freeze-thaw cycle. Prior to use, an aliquot is freshly diluted with nuclear-free water.

Ac/Ds microinjections
Preparation of Ac/Ds constructs/pools for microinjections
For long term storage, all stock preps were eluted in Elution Buffer (Qiagen). To minimise toxicity for microinjections, preps were diluted at least fivefold with nuclease-free water. Therefore, transformation efficiencies and elution volumes must be considered to maximise concentration of final preps.

Preparation of sgRNA-Ac/Ds pools
An equal amount of every U6 guide in the desired pool (we have tested four to 15 guides; in theory this can be higher if necessary) was combined to obtain a pool at least 40 ng/µl in concentration measured on Nanodrop. This usually required at least 200 ng per guide. If necessary, the pool was adjusted to 40–50 ng/µl with nuclease-free water: 10 µl was transformed into 100 µl Stellar™ Competent Cells (636763, Takara), 1 ml prewarmed SOC medium was added, then incubated at 37°C with shaking for 1 h. For each transformation, two ampicillin agar plates were prewarmed prior to plating. One plate was plated with 100 µl of 1:100 dilution (10 µl of transformation plus 990 µl pre-warmed SOC). The remaining undiluted transformation was pelleted at 4000 g for 10 min at RT and excess SOC removed until ∼200 µl remained for resuspension following by plating. Following overnight incubation at 37°C, the Total Colony Yield (no.of colonies×100÷0.1) was determined using the 1:100 plate. To ensure sufficient transformation efficiency, the Total Colony Yield must be at least 1000X greater than the number of guides in the pool (e.g. at least 15 colonies for a pool of 15 guides, in our hands using Stellar cells, we consistently achieved >10 times this rate). To prepare preps, 12 ml of precultured plain LB broth was dispensed onto the undiluted plate and the bacteria gently scraped into the media followed by careful collection of the slurry into a precultured Falcon tube. This step was repeated once to ensure thorough collection of colonies. 5 ml of the slurry was used for Miniprep preparation of the final pool (Qiaprep Spin Miniprep kit (27104, Qiagen), with the remaining ∼20 ml pelleted, supernatant removed and frozen as back-up for Midiprep. Final pools were quantified using Qubit™ dsDNA HS Assay kit (Q32851, ThermoFisher) and representation of guides assessed by PCR.

Microinjection conditions
The following serves as a starting guide and should be adjusted depending on user and microinjector. For Ac/Ds vectors, each embryo was injected

et al., 2016), ensuring that they do not contain a U6 termination sequence (TTT). Complementary oligo pairs were ordered as follows: (1) TCTG-5′[20 bp spacer without PAM]3′ and (2) AAAC-5′[20 bp spacer without PAM in reverse complement]3′ (Table S3). 50 µM of each oligo was combined in a 50 µl reaction and annealed in a thermocycler (94°C 5 mins, decrease to 22°C at 1°C/min, 4°C hold). Cloning reaction was prepared by combining 70 ng pVC-Ds-DrU6a:sgRNA-Ds vector, 5 ng annealed sgRNA, 10 U of BsmBI and 20 U of T4 DNA ligase (M0202, NEB) in 1X T4 DNA Ligase Buffer with final volume 20 µl. GoldenGate-like cycling conditions were used as follows: 10X(37°C 5 mins, 16°C 10 min), 50°C 5 mins, 80°C 5 mins. 2 µl of the reaction was transformed into chemosensitive cells and plated onto Ampicillin plates. One or two colonies per sgRNA were screened by Sanger sequencing using U6a promoter primer (TCATCTACCACCTCCAAA) and quantified using Nanodrop.

Ac transposase mRNA synthesis
To prepare Ac transposase mRNA, pAC-SP6 (Addgene ID 102418) (Emelyanov et al., 2006) was linearised with BsmBI and purified under RNase-free conditions. In vitro transcription was performed using mMESSAGE mMACHINE™ SP6 Transcription Kit (AM1340, ThermoFisher). mRNA was purified under RNase-free conditions using phenol-chloroform followed by ethanol precipitation and the pellet resuspended in RNase-free water. mRNA quality was assessed by gel electrophoresis (sharp intact band without degradation) and quantified using Qubit™ RNA HS Assay kit (Q32852, ThermoFisher). For long term storage in −80°C the purified mRNA was prepared as 1 µl aliquots and limited to one freeze-thaw cycle. Prior to use, an aliquot is freshly diluted with nuclear-free water.
with 30 pg of DNA and 24 pg Ac mRNA. For comparison with Tol2 vectors, each embryo was injected with 30 pg of DNA and 24 pg Tol2 mRNA, or 150 pg of DNA and 50 pg Tol2 mRNA (letality ∼50%). For CRISPRi experiments, 200 pg of sgRNA pool per condition (experimental and scrambled) and 24 pg Ac mRNA were injected per embryo. All microinjections were performed by injecting ∼2 nl into the blastula of one-cell-stage embryos within 5 to 20 min post fertilisation.

Ac/Ds-U6 as sgRNA vector versus in vitro-transcribed sgRNA RT-PCR
Total RNA was extracted from pools of 11 microinjected embryos per condition (vector, or IVT) using RNAqueous-Micro Total RNA Isolation Kit (AM1931, ThermoFisher). Reverse transcription (RT) was performed using 0.5 µM of R_s_gRNA_scaffold_tail (Table S3) in a 10 µl reaction (1 µg starting RNA) with SuperScript III Reverse Transcriptase (18080903, ThermoFisher) at 55°C for 60 min. Primary PCR was performed using the following primers: R_s_gRNA and F_s_scrambled1_spacer (Table S3). In a 20 µl reaction, 0.1 µM primer per primer was combined with 1 µl of template (reverse transcription reaction) in 1X standard Taq polymerase PCR reaction. Cycling was performed as follows: 95°C 5 min, 35X (95°C 30 s, 55°C 30 s), 68°C 30 s, 12°C hold. Next, secondary PCR was performed using the following primers: R_nested_sgRNA and F_nested_scrambled1_spacer (Table S3). In a 20 µl reaction, 0.1 µM primer per primer was combined with 1 µl of template (primary PCR reaction) in 1X standard Taq polymerase PCR reaction. Cycling was performed as follows: 95°C 5 min, 23X (95°C 30 s, 55°C 30 s), 68°C 30 s, 12°C hold. Results were analysed on a single 2% agarose gel with 100 bp ladder.

Generation of CRISPRi transgenic line
TgBAC(sox10::dCas9-SID4x-2a-Citrine) in vitro
Generation of CRISPRi transgenic line

Embryo dissociation and FACTS
24 hpf CRISPRi-microinjected embryos (experimental and scrambled) were dechorionated and collected into low-binding microcentrifuge tubes. E3 medium was removed, and embryos washed once with 0.22 µM filter-sterilised Hank’s buffer: 1X HBSS (14185052, ThermoFisher), 2.5 mg/ml BSA (A3059, Sigma-Aldrich), 10 mM HEPES (15630056, ThermoFisher). For dissociation, up to 200 embryos were incubated in 600 µl of Dissociation Solution: Papain 0.02 U/µl (10108014001, Sigma-Aldrich), ROCK inhibitor 10 µg/ml (A3008, Generon UK), DNasel 1 mg/ml (10104159001, Sigma-Aldrich) in sterile Hank’s buffer. Samples were heatblock-incubated at 37°C and triturated every 5 min using a p200 low-binding tip for no longer than 20 min in total. Once dissociation was complete, reactions were stopped by adding each sample to 4 ml of Stop Solution: DNasel 1 mg/ml, ROCK inhibitor 10 µg/ml in sterile Hank’s buffer. Each sample was triturated gently with a glass serological pipette and passed through a 40 µM cell strainer into a fresh 50 ml Falcon tube. Cells were pelleted at 500 g for 7 min at 4°C. Supernatants were carefully removed until 500 µl remained. 0.5 µl e Bioscience™ Fixable Viability Dye eFluor™ 780 (65086514, ThermoFisher) was added, cells gently resuspended with a low-binding tip, and transferred into 5 ml polystyrene round tubes (7340443, VWR) for FACTS. Cell sorting was performed on BD Aria III or Fusion with 100 µM nozzle. Citrine+ cells were collected into 100 µl Hank’s buffer in normal binding microcentrifuge tubes and placed on ice. Collected cells were immediately pelleted at 500 g for 5 min at 4°C and Hank’s buffer carefully removed. Total RNA was extracted and DNasel-treated using RNAqueous-Micro Total RNA Isolation Kit (AM1931, ThermoFisher) under strict RNase-free conditions. Yield was quantified using Qubit RNA HS Assay Kit (Q32852, ThermoFisher) for downstream applications.

cdh7a and pdgfra qRT-PCR
Equal amounts of RNA per condition (experimental and scrambled) were used as input for cDNA synthesis. cDNA synthesis was performed according to manufacturer’s protocol using GoScript Reverse Transcription Mix, Oligo(dT) (A2790, Promega) with -RT controls included. For qPCR, 1 µl of cDNA was used in 10 µl reactions on StepOnePlus Real-Time PCR System and Software v2.3 (ThermoFisher). TaqMan probes (ThermoFisher) used were Dr03130102_m1 (cdh7a), Dr03086868_m1 (pdgfra), and Dr03436842_m1 (gapdh) as endogenous control. Probes were used in combination with TaqMan Fast Advanced Master Mix (444456, ThermoFisher). Results were analysed using R library qpcr version 1.4-0 by fitting to sigmoidal model (Ritz and Spiess, 2008). Code walkthrough of the analysis is available on https://vchongmorrison.github.io/zhCRISPRi.

Whole-mount immunohistochemistry
For fixation, 4% formaldehyde was freshly prepared using Pierce™ 16% Formaldehyde (w/v), Methanol-free (28906, ThermoFisher) and calcium-/magnesium-free PBS pH 7.4 (10010001, ThermoFisher). Embryos were fixed for 1 h at RT on a nutator. Fixed embryos were dehydrated by incubation in 1:1 ratio methanol/PBS±0.8% Triton X-100 (PBSTR), followed by storage (at least overnight up to 6 months) in 100% methanol at −20°C. After rehydration from 100% methanol to PBSTR, embryos were blocked in 10% normal goat serum, 1% DMSO in PBSTR for at least 1 h at RT. Primary antibodies were mixed with fresh blocking solution and added to embryos for overnight incubation on nutator at 4°C: chicken anti-GFP (ab13970, Abcam) and rabbit anti-zfSsox10 (GTX128374, GeneTex) at 1:200 dilution each. Following extensive washing with PBSTR, secondary antibodies were mixed with fresh PBSTR and added to embryos for incubation on nutator at RT for 4 h: donkey anti-rabbit 568 nm (A10042, ThermoFisher) and goat anti-chicken 647 nm (A21449, ThermoFisher) at 1:400 dilution each. Embryos were washed extensively in PBSTRs and Hoechst 33258 (H3569, ThermoFisher) added at 1:1000 dilution (to label nuclei) prior to confocal imaging.

Hybridization Chain Reaction v3.0
HCR v3.0 (Choi et al., 2018) DNA probes to detect sense and antisense sox9a and foxd3 transcripts, as well as Citrine, were designed using an in-house script (Trivedi and Powell, unpublished). Oligos (Table S3) were ordered desalted and resuspended to 100 µM in RNase-free water from Integrated DNA Technologies. Eight probe pairs and 10 probe pairs were selected to detect sense and antisense transcripts, respectively. Probes were pooled by target and adjusted with RNase-free water to 1 µM working concentration. HCR was performed according to official source modified protocol (Bruce et al., 2021) with the following experimental conditions: HCR incubation with Detection Solution for 30 min (no Proteinase K treatment), hybridisation with 16 µl of 1 µM probe cocktail in 500 µl Probe Hybridisation Buffer. RNA detection and amplification were performed with the following initiator-fluorophore combination: foxd3 coding B3-Alexa488, foxd3 antisense B4-Alexa488, sox9a coding B1-Alexa647, sox9a antisense B2-Alexa488. Hoechst 33258 (H3569, ThermoFisher) was added at a 1:1000 dilution (to label nuclei) prior to confocal imaging.

Confocal microscopy imaging
Live embryos were mounted in 1% low melting point agarose with 0.5 g/L tricine and kept hydrated with E3 medium containing 0.5 g/L tricine throughout. Fixed and immunostained samples were mounted in a similar fashion with PBS instead of E3/tricaine. Imaging was performed on Zeiss LSM 780 Upright using W Plan-Apo 20×/1.0 DIC VIS-IR WD=1.8 (cover glass corrected) objective. All images were acquired using ZEN 2011 (Black Edition) software.

Strand-specific RNAseq
25 to 50 ng of total RNA (depending on yield and quality) were used as input for ribo-depletion followed by strand-specific RNAseq library preparation according to manufacturer’s protocol using KAPA RNA
HyperPrep Kit with RiboErase (HMR) (KK8560, Roche). RNA fragmentation was performed at 94°C for 6 min. Libraries were ligated with 1.5 μM NEBNext Adaptor (E7337A, NEB) followed by amplification/indexing with 10 μM per oligo from NEBNext Multiplex Oligos for Illumina Set 1 (E7335, NEB) for 15 cycles. Fragment sizes of final libraries were assessed using D1000 ScreenTape assay and reagents (5067-5582/3, Agilent). Libraries were quantified by qPCR, with size-adjustment, using NEBNext Library Quant Kit for Illumina (E7630, NEB). For sequencing, equal amounts of each library were pooled followed by resequencing by qPCR without size-adjustment. 4 nM of the pool with 1% PhiX spike-in was sequenced using NextSeq 500/550 High Output Kit v2.5 (150 Cycles) (20024907, Illumina) on NextSeq500 platform in paired-end mode (2:80 cycles). Reads were trimmed by quality and for presence of adaptors using cutadapt v2.10 (Martin, 2011). Transcript quantification was performed with kallisto v0.46.1 (Bray et al., 2016) and differential transcript expression analysis performed with sleuth v3.0.30 (Pimentel et al., 2017). Gene Ontology (database released 2022-07-01) overrepresentation was performed using PANTHER release 17.0 on rhiobai v0.7.7 (GO Biological Process Complete, Fisher’s exact test, FDR correction) (Mi et al., 2019; Rezvani et al., 2022; Thomas et al., 2022). Code walkthrough of the analysis is available on https://vchongmorrison.github.io/zfCRISPRi.

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Competing interests

P.R.R. is co-founder and equity holder in OxStem Cardio. All the other authors declare no competing interests.

Author contributions


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Data availability

Raw data and code walkthrough of analyses in this study are available on https://vchongmorrison.github.io/zfCRISPRi. Lab protocols are available on https://vchongmorrison.github.io.

First person

This article has an associated First Person interview with the first author of the paper.

References


Table S1. Ac/Ds and Tol2 enhancer screening in F0 microinjected embryos.

<table>
<thead>
<tr>
<th>Enhancers</th>
<th>Expression pattern, stage</th>
<th>Percentage of embryos (+/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ac/Ds</td>
</tr>
<tr>
<td>pax3a_E2</td>
<td>Neural (mid- and hindbrain), 24 hpf</td>
<td>81.9 (68/83)</td>
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<tr>
<td>pax3a_E5</td>
<td>Dorsal neural tube, 8-10 ss</td>
<td>90.4 (104/115)</td>
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<tr>
<td>ets1_E5</td>
<td>Cranial and trunk neural crest, 12-14 ss</td>
<td>56.7 (85/150)</td>
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<td>ets1_E4</td>
<td>Cranial and trunk neural crest, 10-12 ss</td>
<td>65.6 (61/93)</td>
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<td>sox10_E2</td>
<td>Cranial neural crest, 26 ss</td>
<td>47.3 (43/91)</td>
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<tr>
<td>sox10_E5</td>
<td>Otic vesicle ± trunk, 24 hpf</td>
<td>45.2 (57/126)</td>
</tr>
</tbody>
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Table S2. Antisense transcription RNAseq - differentially expressed transcripts

Click here to download Table S2

Table S3. (A) Plasmids featured in the main study. (B) Plasmids NOT featured in the main study.

Click here to download Table S3

Table S4. (A) Oligo sequences (sgRNAs excluded) (B) Oligo sequences (sgRNAs).

Click here to download Table S4

References
