Orthogonal CRISPR-Cas genome editing and efficient inhibition with anti-CRISPRs in zebrafish embryos

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Abstract

The CRISPR-Cas universe continues to expand. The type II CRISPR-Cas system from *Streptococcus pyogenes* (SpCas9) is most widely used for genome editing due to its high efficiency in cells and organisms. However, concentrating on a single CRISPR-Cas system limits options for multiplexed editing. We hypothesized that CRISPR-Cas systems originating from different bacterial species could operate simultaneously and independently due to their distinct single-guide RNAs (sgRNAs) or CRISPR-RNAs (crRNAs), and protospacer adjacent motifs (PAMs). Additionally, we hypothesized that CRISPR-Cas activity in zebrafish could be regulated through the expression of inhibitory anti-CRISPR (Acr) proteins. Here, we use a simple mutagenesis approach to demonstrate that CRISPR-Cas systems from *Streptococcus pyogenes (*SpCas9), *Streptococcus aureus* (SaCas9), and *Lachnospiraceae bacterium* (LbCas12a, previously known as LbCpf1) are highly effective, orthogonal systems capable of operating simultaneously in zebrafish. We also demonstrate that type II Acrs are effective inhibitors of SpCas9 in zebrafish. These results indicate that at least three orthogonal CRISPR-Cas systems and two anti-CRISPR proteins are functional in zebrafish embryos. These CRISPR-Cas systems and Acr proteins will enable combinatorial and intersectional strategies for spatiotemporal control of genome editing in zebrafish.

Introduction

The use of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated proteins (Cas) for genome editing has expanded significantly in recent years. CRISPR-Cas systems have several advantages over previous systems, such as zinc-finger nucleases and transcription activator-like effector nucleases, including the ease of design and use, low-cost, high efficiency, and customizability (Adli 2018; Knott and Doudna 2018; K. Liu et al. 2019). A large variety of CRISPR-Cas systems have been described, originating from different bacterial species. Currently, these systems are organized into two large classes, and further divided into six types based on the unique *cas* genes they contain (Makarova et al. 2019). Class 1 systems utilize multiple cas proteins in the effector complex, while Class 2 utilize a single protein for endonuclease activity. Class 2 systems are most commonly used for genome editing, usually type II and type V, due to the ease of delivering a single multi-domain protein in eukaryotes. Type II and type V systems have a few notable differences, including the enzyme and guide RNA structures, PAM and DNA target sequences, and the manner in which they create double-stranded breaks (**Figure 1A, B**). These characteristics can be significant for genome editing if they result in different editing outcomes or allow targeting to different regions of the genome.

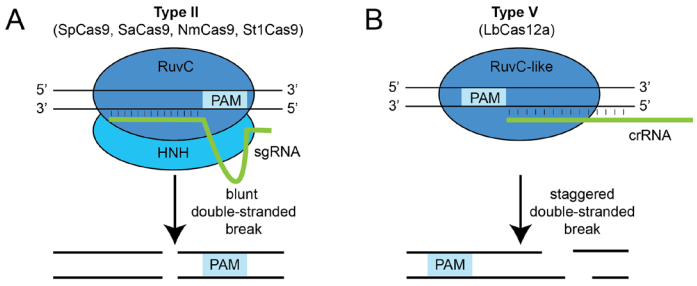

Figure 1: An overview of Type II and V CRISPR-Cas systems. A. Type II CRISPR-Cas systems employ a multidomain protein (Cas9) which complexes with CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) to cause target DNA cleavage. These two RNA molecules can be fused into a single-guide RNA (sgRNA), as shown. The Cas9 HNH domain cleaves the complementary strand, while the RuvC domain cleaves the non-complementary strand in the same position. This results in a blunt double-strand break. **B.** Type V CRISPR-Cas systems employ a distinct multidomain protein (Cas12a, previously known as Cpf1), which complexes with a crRNA to target DNA for cleavage. Cas12a does not require a tracrRNA. Type V enzymes contain a RuvC-like domain, but do not have an HNH nuclease domain (Zetsche et al. 2015; Makarova et al. 2019). This RuvC-like domain cleaves both DNA strands to create a staggered double-strand break. In either case, double-strand breaks can be repaired by the cells using a variety of DNA repair mechanisms, often resulting in insertions or deletions (indels).

The zebrafish has historically been a testbed for reverse genetic and RNA knockdown tools in animals, mainly due to regular access to large numbers of fertilized eggs that are easy to microinject (Nasevicius and Ekker 2000; Doyon et al. 2008; Huang et al. 2011; Bedell et al. 2012; Sander et al. 2011; Dahlem et al. 2012; Hwang et al. 2013; Jao, Wente, and Chen 2013; Gagnon et al. 2014; Feng et al. 2016; Moreno-Mateos et al. 2017; K. Liu et al. 2019). Indeed, the widely-used SpCas9 system was demonstrated first in zebrafish embryos, before applications to other organisms (Hwang et al. 2013; Chang et al. 2013). Since its introduction, notable improvements to CRISPR genome editing in zebrafish include computational prediction of active sgRNAs, methods for rapid sgRNA generation, multiplexed editing, and the use of concentrated SpCas9 protein and commercially-available crRNAs/tracrRNA (Gagnon et al. 2014; Moreno-Mateos et al. 2015; Shah et al. 2015; Varshney et al. 2015; Labun et al. 2016; Burger et al. 2016; Thyme et al. 2016; Wu et al. 2018; DiNapoli et al. 2018; Ata et al. 2018; Hoshijima et al. 2019; Kroll et al. 2020).

However, other CRISPR-Cas systems have been relatively underexplored in zebrafish. While SpCas9 has been widely used, a limited number of publications have described the activity of CRISPR-Cas systems from *Streptococcus aureus* (SaCas9), *Lachnospiraceae bacterium* (LbCas12a), *Francisella novicida* (FnCas12a), and *Acidaminococcus sp.* (AsCas12a) in zebrafish embryos (Feng et al. 2016; Moreno-Mateos et al. 2017; P. Liu et al. 2019). These systems expand the targetable space of the genome due to their distinct PAMs, and may empower intersectional strategies which employ multiple CRISPR-Cas systems. Additionally, CRISPR-Cas type II systems create blunt double-stranded breaks while type V systems generate a staggered cut, which has implications for both indel and knock-in mutagenesis in zebrafish (Moreno-Mateos et al. 2017).

Spatial and temporal control over genome editing in animals permit tissue-specific and developmental-stage specific mutagenesis for more sophisticated screens (Ablain et al. 2015; Yin et al. 2015; Shiraki and Kawakami 2018). These strategies generally rely on regulation of Cas enzyme expression, with ubiquitously-expressed guide RNA(s). However, these strategies can be leaky, have limited temporal control, and may require extensive molecular cloning and transgenesis. This limits their widespread adoption for medium- or large-scale genetic screens. A promising alternative strategy would employ anti-CRISPR (Acr) proteins (Marino et al. 2020). These proteins are capable of blocking CRISPR-Cas activity through direct

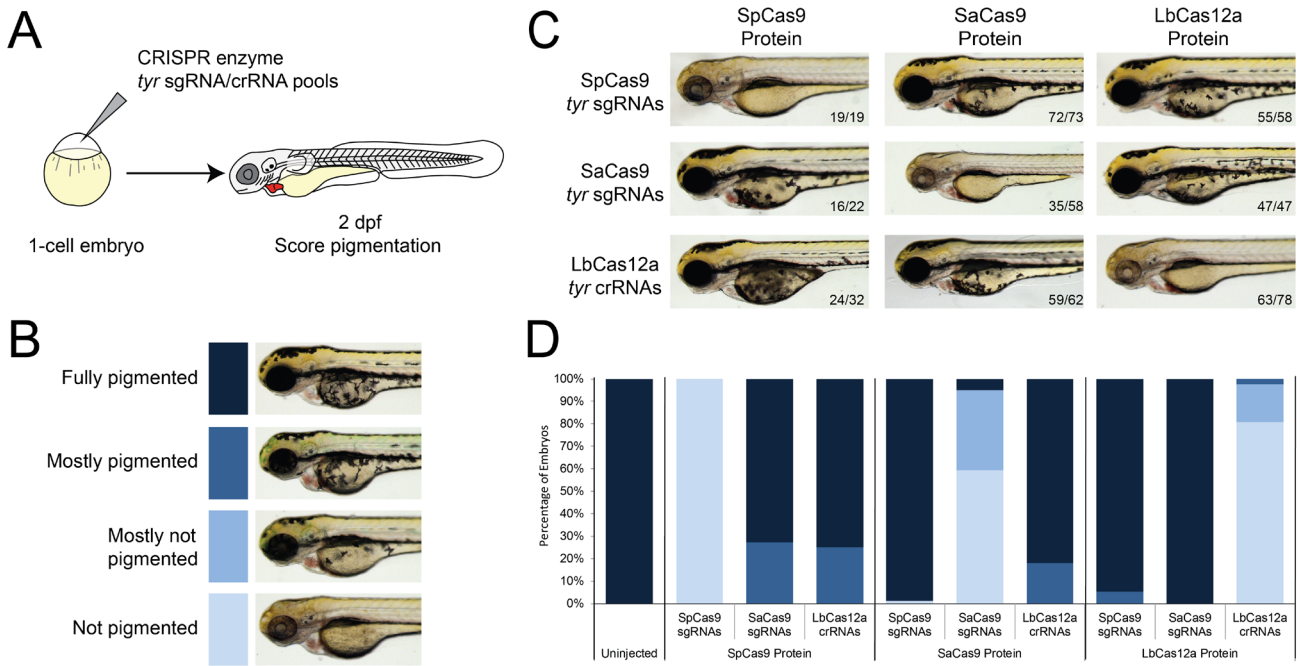

Figure 2: Highly efficient, orthogonal SpCas9, SaCas9 and LbCas12a genome editing in zebrafish embryos. A. Experimental design of the CRISPR screening method. A mix of CRISPR-Cas enzyme and *tyr* sgRNAs/crRNA pools is microinjected into the single-cell zebrafish embryo. Injected embryos are screened at 2 dpf for their level of pigmentation, an effective proxy for *tyr* mutagenesis. **B.** Example images of the four categories used to score pigmentation in embryos. Each category is roughly defined within a certain percentage of pigmentation: fully pigmented=100% pigmented, mostly pigmented=51-99% pigmented, mostly not pigmented=6-50% pigmented, not pigmented=0-5% pigmented. The associated colors act as the legend for panel D. **C.** Representative images of the phenotype of embryos after targeting the *tyr* gene with combinations of each CRISPR enzyme and pools of sgRNAs/crRNAs. Each CRISPR-Cas system is only functional when used with its corresponding sgRNAs/crRNAs. **D.** Quantification of pigmentation categories after microinjection as described in panel C. Minimal pigmentation loss in some cases was due to developmental delay. Raw data in Table S3. Panel B serves as the legend.

interaction with Cas proteins, preventing DNA target site recognition or preventing DNA cleavage. Tissue- or time-specific expression of Acr proteins could provide an alternative approach to control over CRISPR-Cas genome editing. Although many Acr proteins have been identified and tested in bacteria and mammalian cell lines (Pawluk et al. 2016; Rauch et al. 2017), none have been validated in zebrafish.

Here, we implemented a simple assay to screen the efficacy of CRISPR-Cas systems and the functionality of inhibitory Acr proteins in zebrafish embryos. We found that CRISPR-Cas systems from SpCas9, SaCas9 and LbCas12a were highly active for F0 mutagenesis and functionally orthogonal. We further demonstrated that they can be used for simultaneous genome editing in the same embryo. We showed that Acr proteins can be effective inhibitors of SpCas9 and SaCas9 in zebrafish. Together, these tools will enable sophisticated genome editing strategies in zebrafish.

Results

A simple assay for efficient CRISPR-Cas mutagenesis in zebrafish embryos

Many common quantitative assays exist to measure mutagenesis, such as the T7 Endonuclease 1 assay, RFLP mapping, Sanger sequencing or Illumina sequencing. These assays have many advantages, but can be expensive, require specialized equipment, and/or require a significant amount of molecular work. We implemented a simple phenotypic visual readout for CRISPR-Cas mutagenesis to allow screening of new candidate CRISPR systems. We designed single-guide RNAs (sgRNAs), or CRISPR RNAs (crRNAs) for Cas12a systems, targeting multiple sites of the gene *tyrosinase (tyr)*. *tyr* encodes an enzyme responsible for the conversion of tyrosine to melanin (Haffter et al. 1996; Camp and Lardelli 2001). Homozygous *tyr* mutant zebrafish embryos lack pigmentation, an easily observed phenotype at 2 dpf (days post-fertilization). For efficient F0 mutagenesis, we pooled together 3-5 sgRNAs or crRNAs, each targeting different sites in the *tyr* gene. A solution of CRISPR-Cas messenger RNA (mRNA) or protein, and a pool of sgRNAs or crRNAs, was microinjected into single-cell zebrafish embryos (**Figure 2A**). At 2 dpf healthy embryos were screened for pigmentation loss and sorted into one of four different categories (**Figure 2B**). While not as sensitive as alternative assays, our strategy is an easy, quick, and cost-effective test for CRISPR-Cas functionality that requires no specialized equipment.

Orthogonal and efficient CRISPR-Cas systems in zebrafish embryos

To screen for alternative CRISPR-Cas systems in zebrafish, we selected a variety of systems with unique protospacer adjacent motifs (PAMs). This included CRISPR-Cas systems from *Streptococcus pyogenes* (SpCas9), *Streptococcus aureus* (SaCas9), *Streptococcus thermophilus* (St1Cas9), *Neisseria meningitidis* (NmCas9), and *Lachnospiraceae bacterium (*LbCas12a). We did a side-by-side comparison of editing efficiencies between the various systems using our simple assay. The gene encoding each CRISPR enzyme was cloned into a common vector for *in vitro* transcription of mRNA. For each system, we designed an assay for generating sgRNAs or crRNAs using PCR extension of annealed DNA oligos followed by *in vitro* transcription. Next, we performed the mutagenesis assay described above. Following a microinjection of CRISPR-Cas mRNA and sgRNAs/crRNAs, fish were screened for pigmentation loss (**Figure 2A,B**). This screen demonstrated that SpCas9 and SaCas9 were functioning relatively efficiently, as expected (**Figure S1**). Injection of LbCas12a mRNA, however, did not result in efficient editing. We performed a similar mutagenesis assay utilizing commercially available enzymes instead of in vitro transcribed mRNA. LbCas12a enzyme was also non-functional with in vitro transcribed crRNAs. Next, we compared our in vitro transcribed crRNAs to commercially-synthesized crRNAs. We found that LbCas12a was highly functional with synthetic crRNAs (**Figure S2**), although we did not test a recently-published crRNA design shown to be compatible with in vitro transcription (P. Liu et al. 2019). By contrast, St1Cas9 mRNA and NmCas9 mRNA were not effective in inducing *tyr* mutant phenotypes. We attempted to troubleshoot their activity by re-synthesizing sgRNAs, using alternative sgRNA scaffolds, and growing the embryos at a higher incubation temperature; however, none of our attempts were able to induce robust *tyr* mutant phenotypes (**Figure S1**). We did not generate recombinant St1Cas9 or NmCas9 protein, and neither enzyme is commercially available. However, we confirmed that our constructs were likely expressed in the embryo by generating St1Cas9-t2a-GFP and NmCas9-t2a-GFP. GFP was expressed throughout the fish, implying that these enzymes were expressed but not functional in vivo (data not shown). Despite our best attempts, we never observed evidence of gene editing in our experiments with St1Cas9 or NmCas9. Since these systems are functional in mammalian cells, we conclude that further tests may ultimately demonstrate their activity in zebrafish (K. Liu et al. 2019).

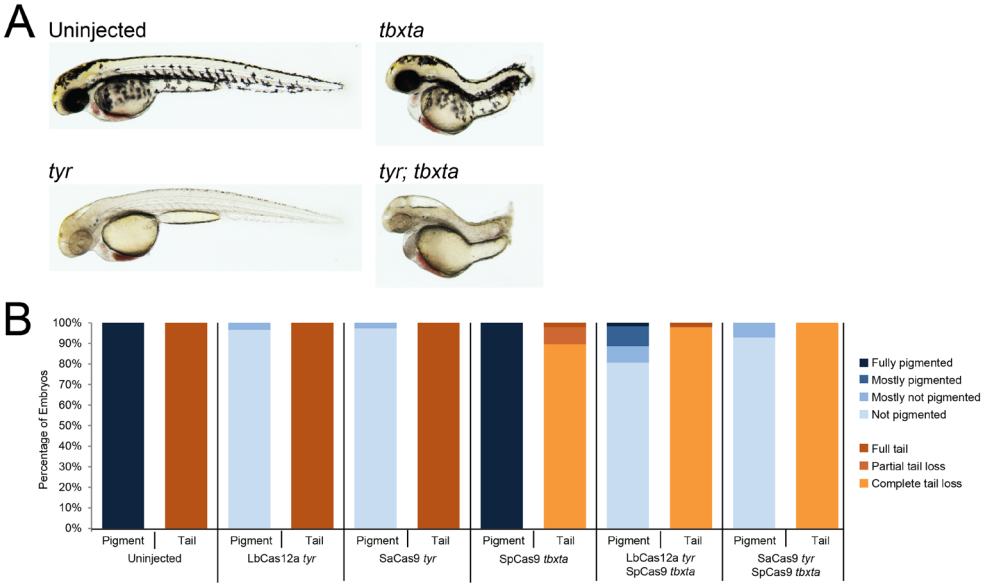

Figure 3. Multiplex mutagenesis with orthogonal CRISPR systems. A. Images of representative embryos from the multiplex mutagenesis experiment. Embryos are injected with CRISPR-Cas enzymes and their corresponding guide RNAs at the 1-cell stage. Negligible toxicity was observed at 24 hpf. Embryos representing uninjected and CRISPR-mutagenized embryos, as labeled, were mounted and imaged at 2 dpf, ideal for imaging both phenotypes. **B.** Quantification of embryonic pigmentation and tail phenotypes, in categories described by the legend. Pigmentation phenotypes were categorized as in Figure 2. Tail phenotypes were classified as full tail, partial tail loss, and complete tail loss. The latter is a complete phenocopy of the published *tbxta* mutant. Raw data in Table S3.

For our remaining experiments, we proceeded with commercially-available SpCas9, SaCas9, and LbCas12a protein for all microinjections. SpCas9, SaCas9, and LbCas12a all proved highly functional in disrupting the *tyr* gene (**Figure 2C,D**). Injections of SpCas9 resulted in 100% pigmentation loss in all embryos (19/19). Injections of SaCas9 resulted in complete loss of pigmentation in 59% of embryos (35/59) and nearly complete loss in another 35% of embryos (21/59). Injections of LbCas12a resulted in complete loss of pigmentation in 80% of embryos (63/78) and nearly complete loss in an additional 16% (13/78). Overall, we observed highly efficient homozygous mutation rates at the *tyr* gene for all three systems with direct injection of commercially-available Cas protein into zebrafish embryos.

Next, we tested whether these systems were orthogonal. We defined the term orthogonal to mean the CRISPR-Cas systems can only function by utilizing its corresponding sgRNAs/crRNAs and not the sgRNAs/crRNAs from other systems. We expected that all three systems would be fully orthogonal, given their evolutionary distance, distinct PAM motifs, and different mechanisms for DNA cleavage between Cas9 and Cas12a. To confirm this, we tested each of the three CRISPR-Cas systems with each of the three pools of sgRNAs/crRNAs targeting the *tyr* gene, and screened for pigmentation at 2 dpf. As expected, each of the CRISPR-Cas systems was only functional with its corresponding sgRNAs or crRNAs (**Figure 2B,D**). When injected with the sgRNA or crRNA pool from a different system, there was no evidence of gene editing. This confirms that SpCas9, SaCas9, and LbCas12a are fully orthogonal.

Multiplexed F0 mutagenesis with orthogonal CRISPR-Cas systems

Our results suggested that orthogonal CRISPR-Cas systems could be used simultaneously to edit different genomic regions in the same individual. To test this, we selected another gene with a distinctive mutant phenotype in zebrafish embryos - *tbxta* (also known as *ta* or *ntla*). *tbxta* encodes a T-box transcription factor required for mesoderm specification, and zebrafish *tbxta* mutants lack the notochord and tail mesoderm (Halpern et al. 1993; Schulte-Merker et al. 1994). We designed a set of SpCas9 guide RNAs targeting *tbxta*, and verified that injected embryos phenocopied the *tbxta* mutant with high penetrance (**Figure 3A,B**). Next, we generated double mutants using combinations of LbCas12a, SaCas9 and SpCas9 targeting *tyr* and *tbxta* simultaneously in the same embryos. We observed high rates of mutagenesis at both genes, with >90% of embryos exhibiting complete phenocopy of both mutants (**Figure 3A,B**). This experiment demonstrates that efficient multiplexed mutagenesis in zebrafish embryos is possible with orthogonal CRISPR-Cas systems.

Anti-CRISPR proteins can efficiently block CRISPR-Cas genome editing in zebrafish embryos

We hypothesized that anti-CRISPR proteins - small peptides that block the activity of Cas enzymes - would be

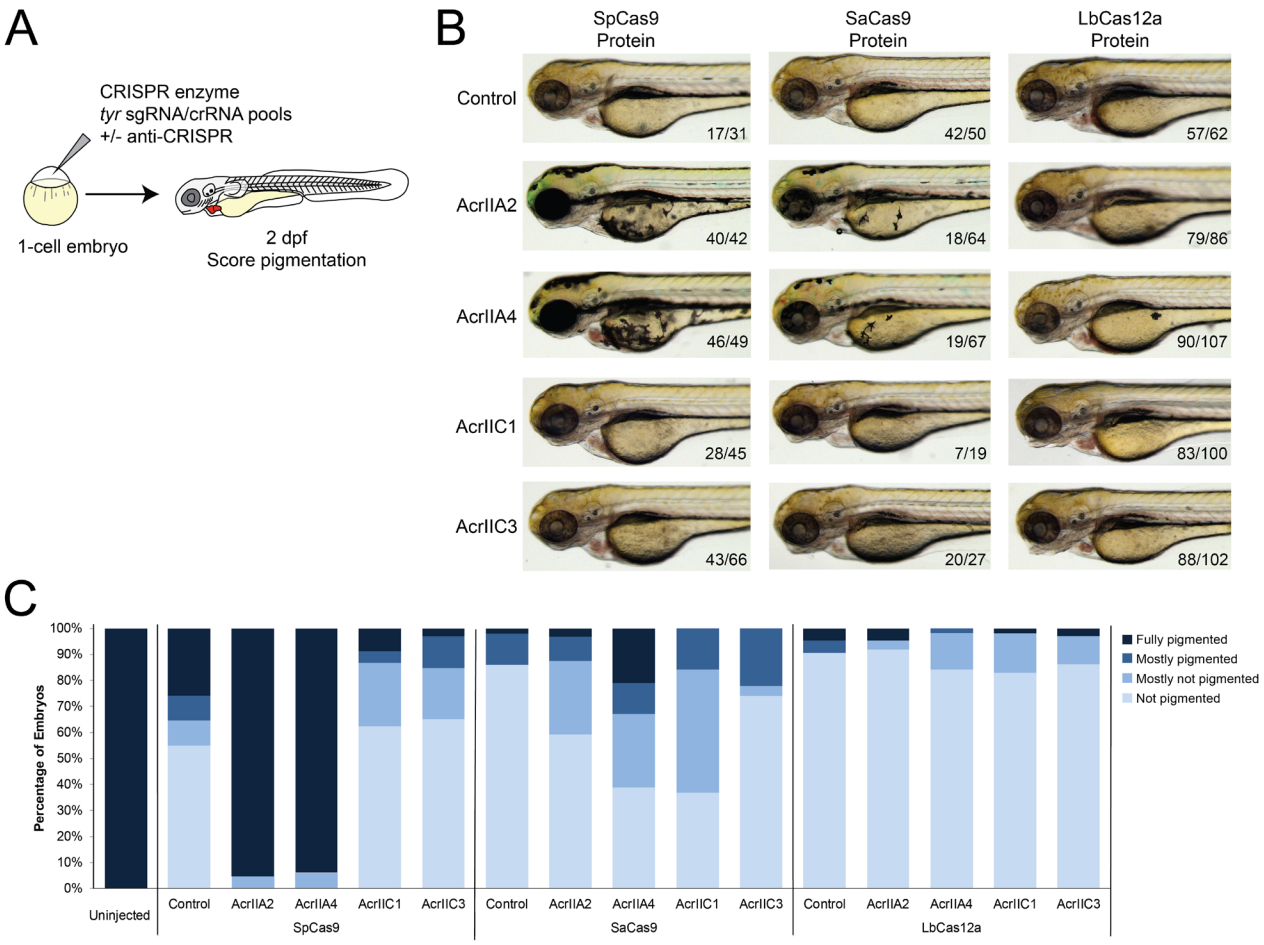

Figure 4: The activity of various anti-CRISPR proteins to inhibit SpCas9, SaCas9, and LbCas12a in zebrafish embryos. A. Experimental design of the assay for anti-CRISPR activity. A mix of CRISPR-Cas enzyme, *tyr* sgRNAs/crRNAs, and anti-CRISPR mRNA or water was microinjected into the single-cell zebrafish embryo. At 2 dpf the fish are screened for their level of pigmentation. **B.** Example images of embryos injected as described in panel A, for three CRISPR systems and four anti-CRISPR proteins. **C.** Quantification of pigmentation categories after microinjection as described. Raw data in Table S3.

effective inhibitors of CRISPR-Cas activity in zebrafish. We selected four type II Acr proteins that had previously been proven functional in bacterial and human cells: AcrIIA2, AcrIIA4, AcrIIC1, and AcrIIC3 (Rauch et al. 2017; Pawluk et al. 2016). We cloned each into a common vector for mRNA transcription. To test each Acr protein, they were co-injected as mRNAs alongside each CRISPR-Cas protein and its corresponding sgRNAs/crRNAs targeting *tyr*. Pigmentation was screened at 2 dpf (**Figure 4A**). We hypothesized that if the Acr was functional, it would block CRISPR-Cas editing of the *tyr* gene, resulting in the rescue of pigmentation. When injected with SpCas9, AcrIIA2 and AcrIIA4 blocked editing with a high efficiency (**Figure 4B,C**). As expected, AcrIIC1 and AcrIIC3, previously found to inhibit type II-C CRISPR-Cas systems, seemed to have little effect. When injected with SaCas9, only AcrIIA4 induced a moderate level of inhibition of editing, with 20% of the embryos fully pigmented. As expected, none of the Acr proteins were effective in blocking editing when co-injected with LbCas12a, as it is a type V CRISPR-Cas system.

We were initially surprised that anti-CRISPRs were so effective, since they were injected as mRNAs and needed to be translated in the embryo before they would be able to interfere with injected Cas protein activity. We hypothesized that Acr proteins would be more effective at inhibiting gene editing when co-injected with Cas9 mRNA. However, we found no difference in the level of CRISPR-Cas inhibition between injected SaCas9 mRNA or protein **(Figure S3**). We conclude that AcrIIA2 and AcrIIA4 are highly effective inhibitors of SpCas9, with moderate activity against SaCas9. More CRISPR inhibitor proteins have recently been described, and our simple and rapid assay for gene editing may serve as a platform for further screening for effective inhibitors of other CRISPR systems in zebrafish.

Discussion

In this study, we described a simple CRISPR-Cas mutagenesis screen and demonstrated the high efficiency of three orthogonal CRISPR-systems in zebrafish: SpCas9, SaCas9, and LbCas12a. We conclude that the lesser-used SaCas9 and LbCas12a systems are equally potent to SpCas9 when delivered as RNPs. In our hands, LbCas12a was only functional as a protein and with synthetic crRNAs; it is possible that using the full-length direct repeat sequence may permit in vitro transcription of effective crRNAs (P. Liu et al. 2019). It is possible that direct RNP injection of NmCas9 and St1Cas9 could also rescue their activity. We also showed that Acr proteins can be used for CRISPR-Cas inhibition in zebrafish. AcrIIA2 and AcrIIA4 were both effective inhibitors of SpCas9, and to a lesser extent AcrIIA4 inhibited SaCas9 activity. AcrIIC1 and AcrIIC3 have previously been shown to inhibit CRISPR-Cas activity in type II-C systems, such as NmCas9, so it is not surprising they were not effective against SpCas9, SaCas9 and LbCas12a. As exploration of different types of CRISPR-Cas systems and Acr proteins continue, their use for CRISPR regulation will grow.

Our findings expand the toolkit of CRISPR-Cas systems in zebrafish, and demonstrate some initial intersectional applications. We anticipate several areas in which simultaneous or intersectional applications of these systems could be beneficial. First, since each CRISPR-Cas system utilizes a unique PAM, more regions of the genome are now available for mutagenesis. Second, as LbCas12a generates staggered DSBs, this system may generate larger deletions or be more likely to drive homology-directed insertion events (Moreno-Mateos et al. 2017; P. Liu et al. 2019), giving potential advantages over SpCas9 or SaCas9 for certain applications. Third, the use of orthogonal CRISPR-Cas systems in the same individual opens the door for combining CRISPR modalities for more sophisticated screens, for example, combining F0 mutagenesis with simultaneous CRISPR activation, repression, base editing, or epigenetic modification (K. Liu et al. 2019; Thakore et al. 2016). Fourth, anti-CRISPR proteins offer a different strategy for regulated CRISPR activity in specific tissues or at particular developmental times, through the use of tissue-specific or inducible promoters. This spatiotemporal control will also enable more sophisticated genetic approaches. Fifth, orthogonal CRISPR systems can advance CRISPR cell lineage tracing and biological recording. Currently, the technology is often limited by the ability to induce recording at just one (McKenna et al. 2016; Spanjaard et al. 2018; Alemany et al. 2018) or two time points with some crosstalk (Raj et al. 2018). We anticipate that orthogonal systems can enable recording at more timepoints in the same individual, with higher confidence in the resulting lineage trees. There is similar potential for multi-channel recording with distinct CRISPR systems, for example by using one system to record lineage and another to record cell signaling.

Our study supports the continued exploration and application of emerging genome editing tools in zebrafish. The zebrafish offers immense practical advantages over nearly all other vertebrate model organisms. Here we have shown the functionality of several CRISPR-Cas systems and Acr proteins in zebrafish. It is likely that many more CRISPR-Cas and anti-CRISPR systems can be ported to zebrafish, offering further opportunities for targeting and multiplexing. We anticipate that sophisticated genetic strategies enabled by multiplexed CRISPR systems will have a large impact on how we study vertebrate development and model human diseases in zebrafish.

Materials and Methods

Zebrafish husbandry

All vertebrate animal work was performed at the facilities of the University of Utah, CBRZ. This study was approved by the Office of Institutional Animal Care & Use Committee (IACUC # 18-2008) of the University of Utah's animal care and use program.

Cloning and transcription of CRISPR-Cas systems and anti-CRISPR

CRISPR-Cas and anti-CRISPR plasmids were ordered from Addgene, thanks to gifts from many investigators (see **Table S1**). Open reading frames were amplified using PCR with primers that add overlapping ends corresponding to the pCS2 vector, and subcloned into the pCS2 vector using NEBuilder HiFi DNA Assembly (NEB). All oligo sequences are available in **Table S2**. Plasmids were miniprepped (Zymo) and confirmed via Sanger sequencing. Each vector was linearized using NotI restriction digest (NEB). Capped mRNA was synthesized using the HiScribe SP6 kit (NEB) and purified using the RNA Clean & Concentrator kit (Zymo). All constructs generated in this study will be made available on Addgene.

Generation of sgRNAs and crRNAs

SpCas9 *tyr* and *tbxta* sgRNAs were synthesized using EnGen sgRNA Synthesis Kit (NEB). SaCas9, LbCas12a, St1Cas9, and NmCas9 *tyr* sgRNAs were synthesized as previously described, with modifications (Gagnon et al. 2014). Briefly, gene-specific and constant oligos were designed for overlap extension. A reaction containing 2 ul constant oligo (5 uM), 2 ul gene-specific oligo (5 uM), 12.5 ul 2X Hotstart Taq mix, and 8.5 ul water was cycled on a thermocycler using this protocol - 95°C for 3 mins, then 30 cycles of (95°C 30 seconds, 45°C 30 seconds, 68°C 20 seconds), followed by 68°C for 5 minutes. Templates were run on a 1% TAE agarose gel to confirm correct band size, and purified using the DNA Clean and Concentrator kit

(Zymo). sgRNAs or crRNAs were transcribed using the HiScribe T7 High Yield RNA Synthesis kit (NEB), and purified using the RNA Clean & Concentrator kit (Zymo). Functional LbCas12a *tyr* crRNAs were synthesized (Synthego). The sgRNAs or crRNAs were then pooled into a single mix at equal molarities (~600 ng/ul). For SpCas9 we pooled 4 or 5 sgRNAs, SaCas9 we pooled 5 sgRNAs, and LbCas12a we pooled 3 crRNAs into the final pools we used to make injection mixes.

CRISPR-Cas injection mixes

We assembled microinjection mixes in the following order in 1.5 ml tubes: 1 ul of 1 M KCl (Sigma-Aldrich), 0.5 ul phenol red (Sigma-Aldrich), 1 ul of a mix of sgRNAs or crRNAs, generated as described above. This pre-mix was briefly vortexed and centrifuged to bring the solution to the bottom of the tube. Then 1 ul Cas mRNA (~300 ng/ul), 1 ul of 20 uM Cas protein (NEB), and/or 1 ul of anti-CRISPR mRNA (~500 ng/ul) was added to the tube, and the mix vortexed and centrifuged again. Injection mixes for multiplexed mutagenesis were generated by doubling the pre-mix and Cas protein volumes. Microinjection mixes were kept on ice until ready for injection. 1 nl was injected into the cell of a zebrafish zygote.

CRISPR mutagenesis assay

At 1 dpf, we screened to remove unfertilized or dead embryos. At 2 dpf, embryos were scored for pigmentation loss into one of four categories: fully pigmented (100% pigmentation), mostly pigmented (51-99% pigmentation), mostly not pigmented (6-50% pigmentation), and not pigmented (0-5% pigmentation) (**Figure 2B**).

Imaging

Larvae were imaged at 3 dpf, with the exception of the multiplexed mutagenesis embryos in **Figure 3**, which were imaged at 2 dpf. In cases where the larvae had not hatched from the chorion, they were manually dechorionated using tweezers. From a stock solution of 4 mg/ml of Tricaine (Sigma-Aldrich), we create a diluted solution of 0.0064 mg/ml in E3 buffer. The larvae were anesthetized in diluted Tricaine solution for 2 minutes. Once the larvae were immobile, they were moved onto a thin layer of 3% methylcellulose (Sigma-Aldrich) and oriented for a lateral view. Images of larvae were taken using a Leica M205FCA microscope with a Leica DFC7000T digital camera.

Author contributions

PRT led all experiments presented in the paper, including cloning CRISPR systems, microinjection, phenotyping, quantification and imaging, and led the writing of the manuscript. EPD assisted with cloning and various experiments. EPD and SNK conducted the multiplex mutagenesis experiments. MAH cloned the anti-CRISPR genes and conducted initial tests of their function. JAG conceived of and supervised the project, assisted with experiments, and assisted with writing the manuscript. The authors declare no conflict of interest.

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