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Sequence-Based Mapping and Genome Editing Reveal Mutations in Stickleback Hps5 Cause Oculocutaneous Albinism and the casper Phenotype

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ABSTRACT Here, we present and characterize the spontaneous X-linked recessive mutation casper, which causes oculocutaneous albinism in threespine sticklebacks (Gasterosteus aculeatus). In humans, Hermansky-Pudlak syndrome results in pigmentation defects due to disrupted formation of the melanin-containing lysosomal-related organelle (LRO), the melanosome. casper mutants display not only reduced pigmentation of melanosomes in melanophores, but also reductions in the iridescent silver color from iridophores, while the yellow pigmentation from xanthophores appears unaffected. We mapped casper using high-throughput sequencing of genomic DNA from bulked casper mutants to a region of the stickleback X chromosome (chromosome 19) near the stickleback ortholog of Hermansky-Pudlak syndrome 5 (Hps5). casper mutants have an insertion of a single nucleotide in the sixth exon of Hps5, predicted to generate an early frameshift. Genome editing using CRISPR/Cas9 induced lesions in Hps5 and phenocopied the casper mutation. Injecting single or paired Hps5 guide RNAs revealed higher incidences of genomic deletions from paired guide RNAs compared to single gRNAs. Stickleback Hps5 provides a genetic system where a hemizygous locus in XY males and a diploid locus in XX females can be used to generate an easily scored visible phenotype, facilitating quantitative studies of different genome editing approaches. Lastly, we show the ability to better visualize patterns of fluorescent transgenic reporters in Hps5 mutant fish. Thus, Hps5 mutations present an opportunity to study pigmented LROs in the emerging stickleback model system, as well as a tool to aid in assaying genome editing and visualizing enhancer activity in transgenic fish.

KEYWORDS genome editing stickleback pigmentation albinism Hermansky-Pudlak syndrome

The combination of new genome editing methods and next-generation sequencing technologies has ushered in a new era in genetics. High-throughput DNA sequencing enables rapid forward genetic mapping of Mendelian (Schneeberger et al. 2009; Cuperus et al. 2010; Doitsidou et al. 2010; Zuryn et al. 2010; Bowen et al. 2012; Obholzer et al. 2012) and quantitative (Glazer et al. 2014, 2015; Jamann et al. 2015) loci. The remarkably high efficiency of the CRISPR/Cas9 system across diverse species (Friedland et al. 2013; Hwang et al. 2013; Jinek et al. 2013; Guo et al. 2014; Square et al. 2015; Martin et al. 2016) now allows for functional reverse genetic analysis in any species competent for delivery of genome editing reagents. However, the optimization of genome editing protocols is still in its infancy, with the efficiency of DNA double-strand break formation and repair still requiring characterization in many species. Particularly powerful loci for this characterization are those that mutate to cause an obvious viable visible phenotype, such as loci that affect pigmentation (Dahlem et al. 2012; Guo et al. 2014; Irion et al. 2014; Square et al. 2015; Burger et al. 2016; Hoshijima et al. 2016).

The diversity of vertebrate skin pigmentation is due to an interplay between four main groups of pigment-containing cells known as chromatophores (Fujii 2000; Kelsh 2004; Mills and Patterson 2009; Irion et al. 2016). Chromatophores originate from the neural crest, which migrate and then differentiate into pigment cell types during embryonic development (Fujii 2000; Kelsh et al. 2009). Melanophores possess black or dark brown melanin-containing organelles, melanosomes, which are...
also present in the retinal pigment epithelium (RPE) (Marks and Seabra 2001; Wassemer et al. 2008). Iridophores appear iridescent and typically silvery due to the presence of light-reflective guanine-containing platelets, the size and orientation of which determine the reflective color (Cooper et al. 1990; Oshima 2001). Xanthophores appear yellow-red due to the presence of pteridine within pterinosomes (Ziegler 2003).

Erythrophores contain vesicles with red carotenoids obtained through diet, and are a critical mating cue in sticklebacks (Milinski and Seabra 2001; Wasmeier 2000; Dell Angelica 2004). LROs are required for iridophore development in zebrafish (Daly et al. 2013). The pink phenotype in Drosophila (Falcón-Pérez et al. 2007; Syrzycka et al. 2007), and the translucent phenotype in silkworms (Fujii et al. 2012) are similar biogenesis pathways (Marks and Seabra 2001). In a wide range of vertebrates and invertebrates, a highly conserved set of genes including members of LRO complexes (BLOC) 1, 2, and 3 (Helip- Wooley et al. 2007; Wei et al. 2013) regulate the formation and maturation of LROs, including pigment producing LROs, and are required for wild-type pigmentation (Helip-Wooley et al. 2007; Wei et al. 2013). In humans, mutations in the BLOC-2 complex member Hps5 result in Hermansky-Pudlak Syndrome type 5, which is characterized by oculocutaneous albinism, and bleeding diathesis (Huizing et al. 2004). Mutations in orthologs of Hps5 result in oculocutaneous albinism in a wide variety of model organisms, including the ruby-eyed 2 phenotype in mice (Zhang et al. 2003), the snow white phenotype in zebrafish (Daly et al. 2013), the pink phenotype in Drosophila (Falcón-Pérez et al. 2007; Syrzycka et al. 2007), and the translucent phenotype in silkworms (Fujii et al. 2012). In vertebrates, Hps5 is required for the maturation of type I to type II melanosomes (Nguyen et al. 2002), and also binds to and stabilizes other HPS proteins including Hps3 and Hps6 (Daly et al. 2013). In addition to conserved roles in melanosome maturation, Hps5 and Hps6 are required for iridophore development in zebrafish and Xenopus, respectively (Daly et al. 2013; Nakayama et al. 2016).

In zebrafish, the snow white mutant phenotype, oculocutaneous albinism, was shown to result from an I76N point mutation in the WD40 domain of Hps5 (Daly et al. 2013). This mutation results in relatively mild reductions in RPE and melanophore melanization due to fewer and smaller melanosomes, and loss of iridophores. snow white mutants display early larval lethality, representing the only lethal Hps5 allele reported, as well as the only mutation within the N-terminal WD40 domain (Daly et al. 2013).

Here, we report the discovery and characterization of a spontaneous threespine stickleback X-linked recessive albino mutation casper. A mapping-by-sequencing approach revealed casper to be tightly linked to the stickleback ortholog of Hps5. casper mutants have a 1 bp insertion, resulting in a predicted frame-shift mutation in Hps5 that results in an early truncation of the highly conserved protein product. Inducing mutations in Hps5 using the CRISPR/Cas9 system phenocopied the casper mutation. Lastly, we show the usefulness of casper embryos as a tool to visualize fluorescent reporters in adult fish. Together these data provide a new locus in an emergent genetic supermodel (Gibson 2005) to facilitate studies of genome editing, transgene expression, and pigmentation biology.

**MATERIALS AND METHODS**

**Animal husbandry and imaging**

Fish were raised in brackish water (3.5 g/L Instant Ocean salt, 0.217 ml/L 10% sodium bicarbonate) at 18°C in 8-hr of light per day in 110 L aquaria. Fry with standard length (SL) less than ~10 mm were fed a diet of live Artemia, with frozen Daphnia added as fish reached ~10 mm SL. Adults with SL greater than ~20 mm were fed a combination of frozen bloodworms and Mysis shrimp. To map the casper mutation, six crosses were generated by crossing four different marine males to heterozygous casper mutant females (Supplemental Material, Table S1). Experiments were approved by the Institutional Animal Care and Use Committees of the University of California-Berkeley (protocol # AUP-2015-01-7117). Embryos were visualized using Montage z-stacks on a Leica M165 FC dissecting microscope, using a GFP2 filter to visualize xanthophores, or on a Keyence VHX-5000 microscope. Adult fish were imaged using a Cannon Powershot S95 digital camera.

**DNA purification and sequencing**

For sequencing and genotyping, DNA was extracted from caudal fin tissue of the original casper male, the F1 female he was cross to, as well as 47 individual casper mutant F2 embryos. Caudal fin tissue or embryos were digested for 12 hr at 55°C in 600 μl of tail digestion buffer (10 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.05% SDS, 2.5 μl Proteinase K (Ambion AM2546)).

DNA from whole casper mutant F2 embryos was diluted to ~10 ng/μl for each fish and pooled. Barcoded Nextera libraries (Illumina FC-121-1031) were created from 50 ng of genomic DNA from the pooled embryos, as well as 50 ng of genomic DNA from the original casper male and F0 female mate following the manufacturer’s instructions. Quality was assayed on an Agilent bioanalyzer, and the resulting libraries were sequenced on a single lane of an Illumina HiSeq 4000 to generate 100 bp single-end reads.

**Bulk segregant analysis**

Reads were mapped to a revised assembly of the stickleback genome (Jones et al. 2012; Glazer et al. 2015), using bowtie2 (Langmead and Salzberg 2012) with parameters “–q-sensitive” (Table S2). Resulting SAM files were converted to BAM files, and sorted using Samtools version 0.1.18 (Li et al. 2009). Read groups were added, CIGAR strings fixed, mate pair information was fixed, and PCR duplicates were removed using picard tools v 1.51 (http://broadinstitute.github.io/picard). The Genome Analysis Tool Kit (McKenna et al. 2010; DePristo et al. 2011; Van der Auwera et al. 2013) (GATK’s (v3.2-2) IndelRealigner (parameter: “–LOD 0.4”), BaseRecalibrator, and PrintReads were used to finalize preprocessing of BAM files. Finally, Unified Genotyper was used to call variants, with parameters “–genotype_likelihoods_model BOTH -stand_call_conf 50.” The resulting VCF file was filtered for variants which had qual score >40, had more than five reads covering the variant, and were not found in the F0 female mated to the original casper male. Analysis was performed using a custom ipython notebook (see Data availability section below for link to custom scripts). Briefly, we computed the proportion of each variant that matched the casper allele, and results were smoothed by plotting the proportion of casper reads within a 50 variant sliding window, advancing five variants at a time. As we found casper to be X-linked, we reasoned that the
hemizygous XY casper animals should all be identical in sequence around the causative locus. We computed, as an additional measure, the proportion of variants within the window with reads matching more than one allele, which is expected to be 0 at the causative locus.

**PCR, cloning, and Sanger sequencing validation**

PCR primers (Table S3) were designed using Primer3 (Rozen and Skaletsky 2000) and ordered from IDT. PCR was performed using Phusion DNA polymerase (Macro laboratory, University of California [UC]-Berkeley) and Phusion Buffer (NEB B0518S). To obtain sequence of single clones, PCR products were purified using a Qiagen PCR purification kit, and digested with XhoI (NEB R0146L) and XbaI (NEB R0145L) in cutsmart buffer for 1 hr at 37°C. Digested products were ligated into a pBluescript II SK+ vector cut with XbaI and XhoI, transformed, and plated onto LB agar plates with ampicillin, IPTG, and X-gal. White colonies were picked and used as input to PCR as described above, adding a 5 min incubation at 95°C before thermocycling. Resulting reactions were purified using a Qiagen PCR purification kit. Purified PCR product (~20 ng) was Sanger sequenced by the UC-Berkeley DNA sequencing facility and results visualized using abiview.

**Genome editing of Hps5**

Genome editing reagents were designed as previously described (Talbot and Amacher 2014). Briefly, pCS2-nCas9n (Addgene 7929) was linearized following digestion with NotI. Linearized plasmid (~600 ng) was used as input to the mMessage SP6 kit, following the manufacturer’s instructions. mRNA quality was verified by running 0.5 μl of the reaction in 0.1% SDS on a Tris acetic acid EDTA (TAE) gel.

Guide RNAs (gRNAs) were designed using ZiFiT (Sander et al. 2010). DNA oligos were ordered from IDT, and gRNA templates were created with T7 promoters by PCR using Phusion polymerase. Resulting PCR products were gel purified, and 100 ng of the resulting elution was used as input to the MaxiScript T7 kit (Ambion), and guide RNA quality verified by running 0.5 μl of the reaction in a 50% formamide buffer on a TAE gel. Resulting gRNAs were precipitated using lithium chloride, incubated at ~80°C following addition of 75 μl 100% ethanol, and centrifuged at 15,000 rpm for 1 hr at 4°C. Following a wash with 200 μl of 75% ethanol and an additional centrifugation at 15,000 rpm for 10 min at 4°C, RNAs were resuspended in 20 μl of DEPC-treated water.

Stickleback embryos at the one cell stage were microinjected as described (Erickson et al. 2016) with some modifications. First, the concentration of gRNA in the 0.2 M KCl injection mixture was increased to 50 ng/μl. The Cas9 mRNA concentration was also doubled to 160 ng/μl, with 0.025% phenol red used as a tracking dye. Embryos were scored for casper-like phenotypes at 4 dpf postfertilization (dpf). Embryos were scored by eye, with embryos with any sign of mosaic albinoism in their RPE classified as “mosaic,” and embryos with ~75% albino RPE classified as “severe.” DNA from un.injected, wild-type injected, mosaic injected, and casper-like injected embryos was purified at 4–6 dpf, and deletions in Hps5 were validated using Sanger sequencing of PCR products as described above.

**Data availability**

Scripts used for bulk segregant analysis are available at https://github.com/trahsemaj/CASPER. The original casper male, the F0 mated to the casper male, and bulk segregant sequencing data are available from the SRA (accession number SRP111743).

**RESULTS**

**caser mutants display severely reduced pigmentation early during embryogenesis**

We discovered a single spontaneous mutant male stickleback displaying severe pigmentation defects. We named this mutation casper, and
recovered the mutation in subsequent generations (see below). casper mutants display oculocutaneous hypopigmentation in unhatched embryos when pigment first becomes apparent, becoming readily apparent by 7 dpf (Figure 1). Mutants appear fully viable and fertile (see below). casper mutants display severely reduced melanization of their RPE, the most obvious visible phenotype (Figure 1, A–D). Additionally, sexually mature casper males displayed severely reduced pigmentation in their testes relative to their wild-type siblings (Figure S1). Chromatophores are differentially affected in casper mutants. Beginning at the time of their first appearance (4 dpf), melanophores in casper mutants are present, but display severe reductions in melanization relative to their wild-type siblings (Figure 1, E–H). The silver pigmentation from iridophores appears absent from older casper fish (Figure 1, I and J).

The red erythrophores, which contain diet-supplied carotenoids (Wedekind et al. 1998), were never observed in the throats of sexually mature casper mutant males. However, the yellow xanthophores ( autofluorescent in sticklebacks) appear unaffected by the casper mutation in 10 dpf casper mutants (Figure S2).

casper mutants appear to initially inflate their swim bladder (Figure 1, E–H). Older mutants display variably penetrant minor difficulties in maintaining their position in the water column, suggesting possible swim bladder defects (14/15 1-month-old juvenile casper mutants were found within 1 cm of the bottom of their tank compared to 2/21 wild-type siblings, \( P < 0.01 \), Fisher’s exact test). Lastly, casper mutants display a bleeding phenotype, possibly due to a decreased clotting rate, following euthanization in 0.04% tricaine relative to their wild-type siblings (Figure 1, I–J).

casper is a spontaneous, X-linked recessive albino mutation

The original casper mutant fish was a single male, and first appeared in a clutch of 80 fish from a cross between a marine male from the Rabbit Slough, Alaska population, and a freshwater creek female from the Cerrito Creek, California population (Figure 2A). To map the locus responsible for the casper phenotype, the original casper male was outcrossed to a female fish from a different marine population (Table S1).
All the resulting F1 progeny were phenotypically wild-type, suggesting the casper mutation was either recessive or mosaic, with the germline of the original mutant fish not containing the casper mutation. As sticklebacks have a simple XY sex determination mechanism (Peichel et al. 2004), a spontaneous X-linked recessive mutation would be displayed in the original hemizygous male, but not in F1 progeny. We thus hypothesized that casper was X-linked. To test this hypothesis, we generated six outcrosses of F1 females to four males from two other populations and observed the F2s. Consistent with an X-linked mutation, 103/419 (24.6%) of the F2 offspring were casper mutants (Table S1), and molecular genotyping (Table S3) of 47 of the casper mutants showed that they were all male, confirming the sex-linked nature of casper ($P < 1.4 \times 10^{-14}$, two-tailed binomial test).

We next mapped the casper locus using a bulk segregant approach (Schneeberger et al. 2009; Cuperus et al. 2010; Doitsidou et al. 2010; Zuryn et al. 2010; Bowen et al. 2012; Obholzer et al. 2012). A barcoded Illumina sequencing library was created using genomic DNA pooled from 47 F2 casper males from six different F1 crosses. Additional barcoded libraries were created using RNA from the original casper fish as well as the F0 female crossed to casper, and all libraries were sequenced to moderate (~10–14x) coverage (Table S2). We mapped casper by examining the proportion of reads at each variant position that matched the casper male’s allele. As an additional measure, we also looked for a loss of variants with mixed mapped reads (reflecting positions where most, or all, mutant male fish have the same X-chromosome genotype), as measured by the proportion of variants which only have reads supporting more than one allele within a 50 variant genomic window (Figure 2B). Both measures had similar peaks along stickleback chromosome 19, the stickleback X chromosome (Peichel et al. 2004), near the stickleback ortholog of a human oculocutaneous albinism gene, Hermansky-Pudlak syndrome 5 (Hps5) (Figure 2B).

casper is the result of the insertion of a single base-pair into the coding sequencing of Hps5
We next sought to determine the mutation responsible for the casper phenotype. Genome-wide variant discovery using high-throughput sequencing data from the original casper male and the F0 wild-type female he was crossed to revealed 14 total variants within the predicted Hps5 coding frame. Of these, 10 were synonymous point mutations, and two were substitutions found in both the affected casper male and unaffected female. The unaffected female had a mutation that results in a substitution from alanine to valine, which appears neutral (score of 0) in the BLOSUM62 matrix (Henikoff and Henikoff 1992), and which is also found in the orthologous mouse Hps5 sequence (Figure 3A). The only remaining, and highest, impact variant was the insertion of a G in the sixth exon of Hps5, resulting in a frameshift and predicted early stop appearing seven codons following the novel insertion (Figure 3A and Figure S3). This variant is present only within the casper male, and not in his unaffected female mate. Sanger sequencing in both the wild-type female (Figure 3B) and casper male (Figure 3C) validated this insertion.

Genome editing of Hps5 phenocopies casper
We next tested whether other predicted loss-of-function mutations in stickleback Hps5 could phenocopy casper mutants. The CRISPR/Cas9 system has been shown to be effective in genome editing in a wide range of model organisms, including another teleost, zebrafish (Hwang et al. 2013; Talbot and Amacher 2014). Two guide RNAs (gRNAs) were designed to target the sixth exon of stickleback Hps5 (Figure 4A). Injection of either of the two gRNAs alone as well as Cas9 mRNA at the one-cell stage resulted in a wide range of insertions/deletions (indels) in the injected zebrafish embryos. However, when both gRNAs and Cas9 mRNA were coinjected, only one of the embryos developed pigmentation reductions, with 14/32 (44%) displaying pigmentations, with 14/32 (44%) displaying a mosaic loss of pigment in melanophores and RPE, and 15/32 (47% of F0 injected fish) displayed >75% loss of RPE (severe loss). (D) Adult Hps5 gRNA injected sticklebacks show a mosaic loss of pigmented melanophores and iridophores, and are partially translucent. Bars, 100 μm (C), 1 mm (D).
within the Hps5 coding region of representative injected embryos (Figure 4B). Coinjection of the two gRNAs with Cas9 mRNA resulted in local indels around each gRNA target, but also larger deletions between the two, with an overall increase in indel size over single guides (P < 0.05, 1-tailed Mann-Whitney U, Figure 4B).

Hps5 gRNA injected embryos phenocopied casper mutants, with severely reduced melanization in both the RPE and melanophores (Figure 4C). Coinjection of these two gRNAs resulted in highly efficient induction of pigmentation phenotypes. Only 3/32 (9%) of the surviving embryos had fully wild-type pigmentation at 6 dpf, while 15/32 (46.9%) displayed a severe loss of pigment, and 14/32 (44%) appeared mosaic (Figure 4C). Embryos injected with only a single gRNA displayed a similar loss of pigmentation, though with decreased efficiency (P < 0.01, binomial test, Table S4). Overall, we observed severe or mosaic casper-like pigmentation phenotypes in 29/32 (91%) of injected embryos, and 8/9 (90%) of sequenced target regions contained indels near a protospacer adjacent motif (PAM) (Figure 4B). Hps5 injected embryos were viable, and displayed mosaic reduced RPE and melanophore melanization into adulthood, as well as a mosaic loss of iridophore pigmentation (Figure 4D).

**Visualizing fluorescent transgenic reporters in casper mutants**

Fish embryos are highly transparent and develop externally, allowing for easy visualization of early embryogenesis. Combined with efficient transgene incorporation using Tol2 transgenesis (Kawakami 2005; Erickson et al. 2016), sticklebacks represent a powerful system for assaying the activity of developmental enhancers using fluorescent reporter constructs (Erickson et al. 2015). However, as fish develop and become more pigmented, imaging becomes extremely difficult, and investigations into late-acting enhancer elements require microdissection.

We next sought to test whether the reduced pigmentation of casper embryos would allow for easier imaging of enhancer patterns in juvenile fish.

We crossed a heterozygous casper female to a male carrying a single copy of a fluorescent reporter of a previously described 190 bp Bmp6 enhancer (Erickson et al. 2015), previously described to be active in the fins and teeth. Two months postfertilization, wild-type males showed reported green fluorescent protein (GFP) expression in the lens of the eye—a known internal positive control domain of expression of the zebrafish heat-shock 70-like (Hsp70) promoter used (Erickson et al. 2015)—with other expression domains obscured by pigmented cells (Figure 5A). casper mutants carrying the transgene showed similar robust lens expression, but also better revealed other visible juvenile expression domains in oral and pharyngeal teeth, as well as revealed a previously unreported major expression pattern in the liver (Figure 5, B–D).

**DISCUSSION**

Here, we present the first stickleback model of Hermansky-Pudlak syndrome, the spontaneous X-linked recessive casper phenotype, resulting from a frame-shifting insertion in Hps5. Hps5 mutants display oculocutaneous albinism, with severely decreased pigmentation in melanophores, iridophores, and erythrophores, though, interestingly, not xanthophores. These pigment phenotypes suggest that pterinosomes in xanthophores develop in a Hps5-independent manner, while the melanins, carotenoids, or guanine crystal containing organelles in melano- phores, erythrophores, and iridophores develop in a Hps5-dependent manner. Pigment was also drastically reduced in the RPE of Hps5 mutants, suggesting that defects in LRO biogenesis are not restricted to chromatophores, as the RPE does not contain chromatophores (Schaerermeyer and Heimann 1999). Additionally, we observed a bleeding phenotype in casper mutants, potentially similar to the bleeding
Hps5 underlies the casper phenotype

Our mapping-by-sequencing approach using bulk segregant analysis of casper mutants revealed a peak genetic signal near Hps5 on chromosome 19, the stickleback X chromosome. By Sanger sequencing, we found the insertion of a single guanine to a heptaguanine run in the coding frame of Hps5 in casper mutants. This spontaneous insertion might be due to the presence of this homopolymer repeat, as the coding frame of Hps5 is able to induce biallelic hits in stickleback embryos. We also see notypes were seen in both XY males and XX females, implying that mosaic pigmentation phenotypes. Furthermore, strong pigment phenotypes similar to casper phenotype. Although our approach using 47 pooled mutants identified a strong candidate gene, future mapping-by-sequencing of other mutations could generate improved genetic resolution by pooling even more mutant DNAs.

Live imaging of fluorescent reporters in casper embryos

casper and Hps5 mutant embryos are both semitransparent even as adults, unlike their wild-type siblings. This transparency allows for better live imaging of fluorescent transgenic reporters in Hps5 mutant fish. Imaging a previously characterized enhancer of Bnp6 (Erickson et al. 2015) in Hps5 mutants, we discovered a major unreported expression domain in the liver. We also found that imaging the previously reported dynamic tooth expression domains (Erickson et al. 2015) to be greatly facilitated by the depigmented phenotype of Hps5 mutants. The viable nature of the casper mutation allows for the creation of stable transgenic lines to more easily visualize reporter gene expression, especially at older postembryonic stages. The X-linked nature of the casper mutation allows for the recovery of casper males from outcrosses to different stable lines within a single generation.

Genome editing with CRISPR/Cas9

We report the first successful generation of loss-of-function mutations using the CRISPR/Cas9 system in sticklebacks. Coinjection of Cas9 mRNA along with either of two guide RNAs (gRNAs) targeted to exon 6 of Hps5 resulted in a high frequency (≥90%) of embryos with severe or mosaic pigmentation phenotypes. Furthermore, strong pigment phenotypes were seen in both XY males and XX females, implying that Cas9 is able to induce biallelic hits in stickleback embryos. We also see evidence for high efficiency in our single clone Sanger sequencing, as most (8/9) of our sequenced clones contained induced mutations. This high rate suggests that Hps5 guide RNAs could be used as a marker for other guide RNAs in a co-CRISPR approach (Kim et al. 2014; Kane et al. 2017), with more albino embryos representing embryos with high levels of nuclear Cas9 activity. As stickleback testes are pigmented,
Falcón-Pérez, J. M., R. Romero-Calderón, E. S. Brooks, D. E. Krantz, and E. C. Dell’Angelica, 2007 The Drosophila pigmentation gene pink (p) encodes a homologue of human Hermansky–Pudlak syndrome 5 (HPSS5). Traffic 8: 154–168.

Friedland, A. E., Y. B. Tzur, K. M. Esvelt, M. P. Colaiácovo, G. M. Church et al., 2013 Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nat. Methods 10: 741–743.

Fujii, R., 2000 The regulation of motile activity in fish chromatophores. Pigment Cell Res. 13: 300–319.

Fujii, T., Y. Banno, H. Abe, S. Katsuma, and T. Shimada, 2012 A homolog of the human Hermansky-Pudlak syndrome-5 (HPSS5) gene is responsible for the ou larval translucent mutants in the silkworm, Bombyx mori. Genetica 140: 463–468.

Friedman, A. E., Y. B. Tzur, K. M. Esvelt, M. P. Colaiácovo, G. M. Church et al., 2013 Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nat. Methods 10: 741–743.

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Fujii, R., 2000 The regulation of motile activity in fish chromatophores. Pigment Cell Res. 13: 300–319.

Friedman, A. E., Y. B. Tzur, K. M. Esvelt, M. P. Colaiácovo, G. M. Church et al., 2013 Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nat. Methods 10: 741–743.

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Fujii, R., 2000 The regulation of motile activity in fish chromatophores. Pigment Cell Res. 13: 300–319.

Friedman, A. E., Y. B. Tzur, K. M. Esvelt, M. P. Colaiácovo, G. M. Church et al., 2013 Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nat. Methods 10: 741–743.
Van der Auwera, G. A., M. O. Carneiro, C. Hartl, R. Poplin, G. Del Angel et al., 2013 From FastQ data to high confidence variant calls: the genome analysis toolkit best practices pipeline. Curr. Protoc. Bioinformatics 43: 11.10.1–11.10.33.

Wasmeier, C., A. N. Hume, G. Bolasco, and M. C. Seabra, 2008 Melanosomes at a glance. J. Cell Sci. 121: 3995–3999.

Wedekind, C., P. Meyer, M. Frischknecht, U. A. Niggli, and H. Pfander, 1998 Different carotenoids and potential information content of red coloration of male three-spined stickleback. J. Chem. Ecol. 24: 787–801.

Wei, A.-H., X. He, and W. Li, 2013 Hypopigmentation in Hermansky–Pudlak syndrome. J. Dermatol. 40: 325–329.

Zhang, Q., B. Zhao, W. Li, N. Oiso, E. K. Novak et al., 2003 Ru2 and Ru encode mouse orthologs of the genes mutated in human Hermansky-Pudlak syndrome types 5 and 6. Nat. Genet. 33: 145–153.

Ziegler, I., 2003 The pteridine pathway in zebrafish: regulation and specification during the determination of neural crest cell-fate. Pigment Cell Res. 16: 172–182.

Zuryn, S., S. Le Gras, K. Jamet, and S. Jarriault, 2010 A strategy for direct mapping and identification of mutations by whole-genome sequencing. Genetics 186: 427–430.

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