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RESEARCH REPORT

Tissue-specific genome editing in *Ciona* embryos by CRISPR/Cas9

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ABSTRACT

The CRISPR/Cas9 system has ushered in a new era of targeted genetic manipulations. Here, we report the use of CRISPR/Cas9 to induce double-stranded breaks in the genome of the sea squirt *Ciona intestinalis*. We use electroporation to deliver CRISPR/Cas9 components for tissue-specific disruption of the *Ebf (Collier/Olf/EBF)* gene in hundreds of synchronized *Ciona* embryos. Phenotyping of transfected embryos in the 'F0' generation revealed that endogenous *Ebf* function is required for specification of *Islet*-expressing motor ganglion neurons and atrial siphon muscles. We demonstrate that CRISPR/Cas9 is sufficiently effective and specific to generate large numbers of embryos carrying mutations in a targeted gene of interest, which should allow for rapid screening of gene function in *Ciona*.

KEY WORDS: Ascidians, CRISPR/Cas9, Ciona, Ebf, Genome editing, Islet

INTRODUCTION

Recent advances have harnessed the CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated proteins) system for targeted genome editing (Ran et al., 2013). This prokaryotic immune system functions through short RNAs that guide Cas to foreign DNA (Barrangou et al., 2007; Brouns et al., 2008; Marraffini and Sontheimer, 2008). Modified variants of this system have been used for genome editing applications in various organisms (Cong et al., 2013; Dickinson et al., 2013; Friedland et al., 2013; Hwang et al., 2013; Mali et al., 2013; Qi et al., 2013; Wang et al., 2013a; Yang et al., 2013).

Ascidians of the genus *Ciona* are model organisms for chordate developmental genomics (Satoh, 2014). Hundreds of synchronized *Ciona* embryos can be simultaneously electroporated with plasmid DNA for high-throughput transgenesis (Corbo et al., 1997). This technique is used in gain-of-function experiments for overexpression of protein-coding genes (Stolfi and Christiaen, 2012). However, few options exist for scalable, tissue-specific loss-of-function experiments in *Ciona*.

Targeted mutagenesis in *Ciona* was reported using transcription activator-like effectors (TALEs) and zinc-finger nucleases (Kawai et al., 2012; Treen et al., 2014; Yoshida et al., 2014). Here, we report the use of CRISPR/Cas9 to induce site-specific double-stranded breaks (DSBs) in the *C. intestinalis* genome. By targeting a crucial region of the *Ebf* gene, we demonstrate the power of a simple electroporation-based transfection technique to deliver CRISPR/Cas9 components for tissue-specific, targeted mutagenesis in large batches of F0-generation *Ciona* embryos.

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RESULTS AND DISCUSSION

Optimization of CRISPR/Cas9 components for *Ciona*

To test Cas9 expression in *Ciona*, we found that Cas9 flanked by two nuclear localization signals (nls) and a C-terminal eGFP tag (Chen et al., 2013; Fig. 1A) was strongly expressed and localized to nuclei (Fig. 1B). We avoided the use of Cas9 variants bearing an N-terminal hemagglutinin (HA) epitope tag, as these were found to be excluded from nuclei (supplementary material Fig. S1A-C).

We used the U6 promoter (Nishiyama and Fujiwara, 2008) to drive RNA polymerase III-dependent constitutive expression of single guide RNAs (sgRNAs) (Jinek et al., 2012; Mali et al., 2013). A recent study indicated that four consecutive Ts in the sgRNA hairpin result in premature transcriptional termination of T-rich sgRNAs (Wu et al., 2014). We used a modified sgRNA scaffold ('F+E', Fig. 1C) (Chen et al., 2013) to circumvent this problem. Indeed, by *in situ* hybridization we were able to detect transcription through the F+E backbone (Fig. 1D), but not through the original backbone (supplementary material Fig. S1D,E). This suggests that sgRNA constructs using the original backbone might not be expressed efficiently in *Ciona* embryos due to early termination in the hairpin.

CRISPR/Cas9-induced DSBs in the *Ciona Ebf* gene

Collier/Olf/EBF genes are transcriptional regulators of cell fate and differentiation in diverse tissues (Dubois et al., 1998; Crozatier and Vincent, 1999). We used CRISPR/Cas9 to induce mutations in exon 9 of *Ebf* (previously known as *COE*), the sole *Ciona* homolog of mammalian *EBF1/2/3/4*. This gene plays crucial roles in specification of motor ganglion neurons (Kratsios et al., 2012) and pharyngeal muscle precursors (Razy-Krajka et al., 2014; Stolfi et al., 2010). *Ebf* exon 9 codes for part of the IPT domain (immunoglobulin-like, plexin, transcription factor), situated between the DNA-binding and helix-loop-helix (HLH) domains (Fig. 2A).

We designed sgRNA vectors targeting exon 9 (*Ebf.774* and *Ebf.813*) and a vector driving *nls::Cas9::nls* expression using the ubiquitous *Eef1a* (*EF1α*) promoter (Sasakura et al., 2010). Fertilized eggs (n>100) were pooled and electroporated with sgRNA/Cas9 plasmids. At 16 h postfertilization (hpf), genomic DNA extracted from the entire batch of transfected embryos (the 'F0' generation) was pooled. *Ebf* exon 9 was PCR-amplified and the resulting products TOPO-cloned. When individual clones were sequenced, three out of seven sequences carried deletions in exon 9 (Fig. 2B), suggesting that Cas9 had specifically induced DSBs that were then imperfectly repaired. Targeting with Cas9+*Ebf.774* sgRNA (not in combination with *Ebf.813* sgRNA) yielded six additional unique *Ebf* mutations (Fig. 2C, see below). We also generated targeted mutations in 5′ flanking regions of *Foxf* and *Hand-related* (supplementary material

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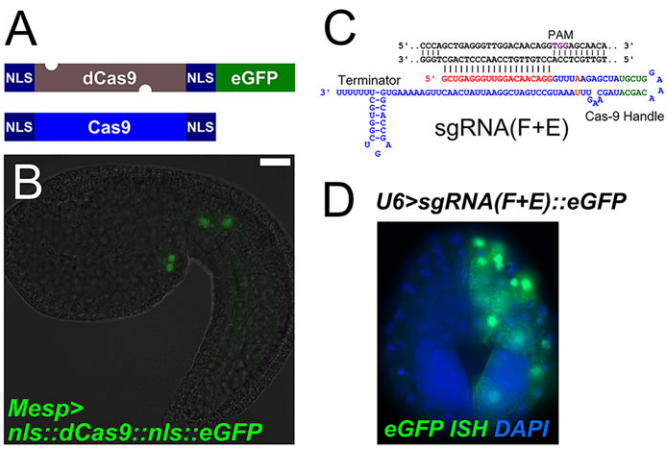

Fig. 1. CRISPR/Cas9 components in *Ciona intestinalis*. (A) Top: nls::dCas9::nls::eGFP protein used to assay nuclear localization in *Ciona*. dCas9 is Cas9 with two point mutations that render it catalytically dead. Bottom: nls::Cas9::nls used for targeted mutagenesis. (B) Tail bud-stage embryo electroporated with *Mesp>nls::dCas9::nls::eGFP*, confirming proper nuclear localization in B7.5 lineage cells. Scale bar: 25 µm. (C) *Ebf.774* sgRNA (F+E version). The protospacer (red) is paired with its target in *Ebf*. 'F' modification is in orange, 'E' modification is in green. (D) *In situ* hybridization using *eGFP* probe in gastrula-stage embryo electroporated, with *U6>sgRNA(F+E)::eGFP* in right hemisphere, indicating successful transcription of the sgRNA in 100% of embryos (*n*=100).

Fig. S2), suggesting that CRISPR/Cas9 can be harnessed for targeted mutagenesis of a variety of loci in the *Ciona* genome.

To verify that CRISPR/Cas9-targeted mutagenesis of the *Ebf* locus results in mutant *Ebf* transcripts, RNA was isolated from magnetic-activated cell sorting (MACS)-selected *Ebf*-expressing cells from pooled, dissociated embryos electroporated with *Ebf>hCD4::mCherry*, *EF1α>nls::Cas9::nls* and *U6>Ebf.774*. Partial *Ebf* cDNA fragments were amplified by RT-PCR and TOPO-cloned. Four out of eight sequenced clones had indels in the target sequence (supplementary material Fig. S3).

Estimates of CRISPR/Cas9 efficiency by genomic cleavage assay

We used GeneArt Genomic Cleavage Detection to evaluate CRISPR/Cas9-induced mutagenesis. Cleavage assays were performed on *Ebf* exon 9 PCR products from batches of embryos electroporated with *EF1α>nls::Cas9::nls* and *U6>Ebf.774*. This resulted in a cleavage efficiency of 31.5% according to the formula provided by the manufacturer (Fig. 3A; supplementary material Fig. S4).

It should be noted that we found that the cleavage assay detected a large number of naturally occurring polymorphisms at other loci (supplementary material Fig. S5), potentially limiting its usefulness for studies using highly polymorphic *Ciona* populations (Tsagkogeorga et al., 2012).

Cleavage assay of genomic DNA from MACS-enriched transfected cells

A cleavage assay is likely to underestimate actual mutagenesis efficiency due to mosaicism of electroporated plasmids in *Ciona* embryos (Zeller et al., 2006). Electroporated embryos contain both transfected and non-transfected cells. In order to enrich for transfected cells, we performed MACS on dissociated cells from pooled embryos co-electroporated with 10 µg each of *EF1α>hCD4::mCherry*, *EF1α>nls::Cas9::nls* and *U6>Ebf.774*. The cleavage assay indicated a 27.1% efficiency in hCD4^{+}-sorted cells, whereas cleavage bands from hCD4^{-} flow-through or unsorted cells were barely visible (Fig. 3B). These findings indicate that transfection mosaicism can mask mutagenesis efficiency, a problem that is overcome by MACS selection of transfected cells.

To test whether cleavage efficiencies could be improved by increasing CRISPR/Cas9 plasmid concentrations, we electroporated embryos with 10 µg *EF1α>hCD4::mCherry*, 25 µg *EF1α>nls::Cas9::nls* and 75 µg *U6>Ebf.774*. This resulted in a cleavage

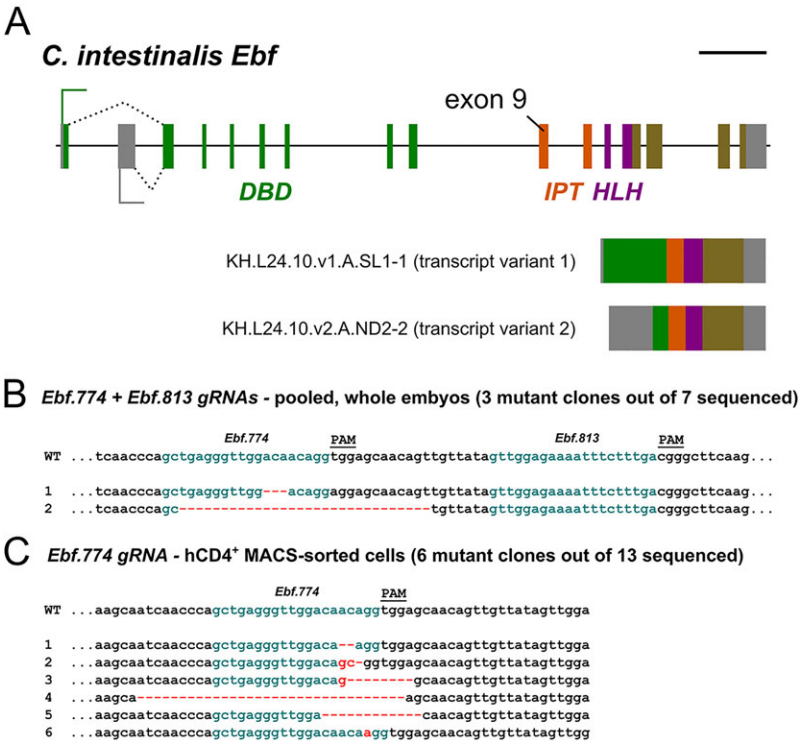

Fig. 2. CRISPR/Cas9-mediated mutagenesis of *Ebf*. (A) *C. intestinalis Ebf* gene, showing exons (solid boxes) and introns (scale bar: 1 kb). Exons are colored according to domains: green, N terminus+DNA-binding domain (DBD); orange, IPT; magenta, atypical HLH; brown, transactivation domain; gray, untranslated regions. Promoters (proximal and distal) are indicated by elbows. Alternative splicing is indicated by dotted line, giving rise to *Ebf* transcript variants shown below. Both contain the conserved zinc-coordinating motif of the DBD. (B) Alignment of wild-type and mutant *Ebf* alleles cloned from pooled embryos electroporated with 10 µg *EF1α>nls::Cas9::nls*, 10 µg *U6>Ebf.774* and 10 µg *U6>Ebf.813*. Three out of seven clones had a mutation (two unique mutations). (C) Mutant *Ebf* alleles cloned from MACS-sorted hCD4^{+} cells dissociated from embryos electroporated with 10 µg *EF1α>hCD4::mCherry*, 25 µg *EF1α>nls::Cas9::nls* and 75 µg *U6>Ebf.774* (see 'Cleavage assay of genomic DNA from MACS-enriched transfected cells' section for details). Six out of 13 clones had a mutation (all unique). Target sequences indicated in blue. Indels and substitutions are indicated in red.

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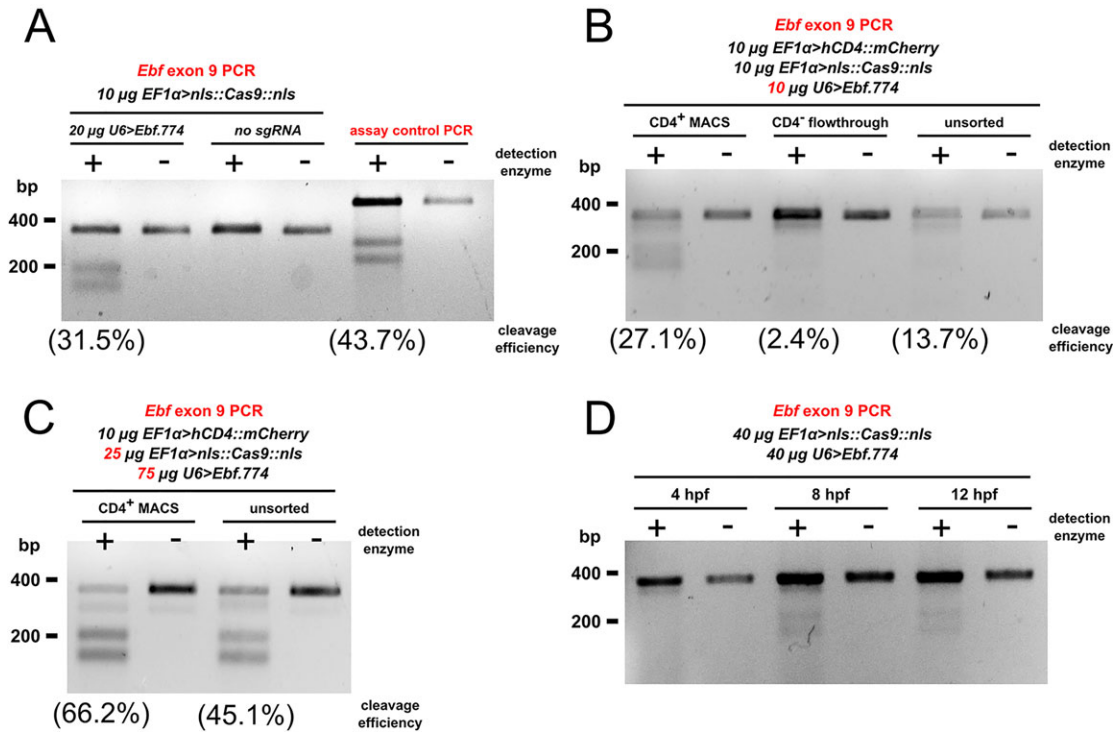

Fig. 3. Genomic cleavage assays. (A) Cleavage assay of *Ebf* exon 9 amplicon from pooled embryos electroporated with *EF1α>nls::Cas9::nls+U6>Ebf.774* or *EF1α>nls::Cas9::nls* alone. Cleavage efficiency was calculated at 31.5%. Cleavage of *Ebf* exon 9 amplicon from control (*Cas9*-alone) embryos was not detected. Cleavage of kit control amplicon (1:1 mix of wild-type and mutant sequences) was 43.7%. (B) Assay of amplicon from MACS-sorted cells from embryos electroporated with10 µg *EF1α>nls::Cas9::nls*, 10 µg *U6>Ebf.774* and 10 µg *EF1α>hCD4::mCherry* (for MACS selection). Cleavage efficiency in sorted hCD4$^+$ cells was 27.1%, versus 2.4% in hCD4$^-$ flow-through and 13.7% in unsorted cells from the same pool of dissociated embryos. (C) Assay of amplicon from MACS-sorted cells from embryos electroporated with 10 µg *EF1α>hCD4::mCherry*, 25 µg *EF1α>nls::Cas9::nls* and 75 µg *U6>Ebf.774*. Cleavage efficiency in sorted hCD4$^+$ cells was 66.2% and 45.1% in unsorted cells from the same embryo pool (see supplementary material Fig. S6). (D) Assay of pooled embryos electroporated with *EF1α>nls::Cas9::nls+U6>Ebf.774*, collected at 4, 8 and 12 hpf. Cleavage bands are visible at 8 hpf, but not at 4 hpf. Efficiency does not increase substantially from 8 to 12 hpf.

efficiency of 45.1% in unsorted cells and 66.2% in hCD4$^+$ cells (Fig. 3C; supplementary material Fig. S6). Moreover, when TOPO-cloned, *Ebf* exon 9 amplicons from hCD4$^+$ cells yielded six out of 13 sequences bearing novel mutations (Fig. 2C). Taken together, these data suggest that sgRNA vector distribution and concentration are limiting factors for efficient CRISPR/Cas9-mediated mutagenesis in electroporated embryos.

Temporal dynamics of CRISPR/Cas9 activity in *Ciona* embryos

We tested how early in the development of *Ciona* embryos could CRISPR/Cas9-mediated targeted mutations be detected. We performed cleavage assays on PCR products amplified from DNA samples extracted at different time points from the same pool of transfected embryos. We found that mutations were not detectable at 4 hpf, but appeared at 8 hpf (Fig. 3D). Cleavage efficiency did not increase between 8 and 12 hpf, suggesting that CRISPR/Cas9 is limited by the onset of *EF1α* promoter activity (~5 hpf, supplementary material Fig. S7), and that target sequence mutagenesis rapidly reaches saturation.

CRISPR/Cas9-targeted mutagenesis of *Ebf* abolishes *Islet* expression in motor neurons

We next tested whether CRISPR/Cas9 could disrupt Ebf function in electroporated embryos. The *Ciona* motor ganglion is composed of neurons that innervate the tail to drive swimming behavior in the larva (Horie et al., 2010). Previous studies suggested that Ebf is located upstream of the conserved motor neuron (MN) regulatory factor *Islet* (*Isl*) in A10.57 MNs (Imai et al., 2009). To express Cas9 in early ectoderm, we chose the *Sox1/2/3* (also known as *SoxB1*) promoter. We batch-electroporated embryos with *Sox1/2/3>nls::Cas9::nls+U6>Ebf.774* and assayed MN-specific *Isl* reporter expression (Stolfi et al., 2010) at the larval stage. *Isl* reporter expression in MNs was seen in only 23% (n=100) of larvae that had been electroporated with *Sox1/2/3>nls::Cas9::nls+U6>Ebf.774* (Fig. 4B,H), compared with 68% (n=100) of control larvae (Fig. 4A,H), suggesting that Ebf is required for *Isl* activation in MNs.

Rescue of targeted mutations in endogenous *Ebf* by CRISPR-resistant *Ebf*

Off-target effects of CRISPR/Cas9 in eukaryotic cells have raised concerns about the specificity of purported targeted mutations (Hsu et al., 2013). In order to verify that downregulation of *Isl* was specifically attributable to Ebf loss-of-function, we designed a rescue construct. It consisted of an *Ebf* promoter driving an *Ebf*-coding sequence that contained synonymous substitutions in the *Ebf.774*-targeted seed sequence (*Ebf>Ebf^{m774}*). As predicted, co-electroporation of *Ebf>Ebf^{m774}* rescued *Isl* reporter expression (Fig. 4C,H). We therefore conclude that the observed loss of *Isl* expression was not due to non-specific effects of CRISPR/Cas9, and that the majority of disrupted *Ebf* sequences are in fact loss-of-function alleles.

Mesoderm-specific targeting of *Ebf* abolishes pharyngeal muscle specification

To target *Ebf* specifically in the B7.5 lineage, we used the *Mesp* promoter to express Cas9 in B7.5 blastomeres. The B7.5 lineage

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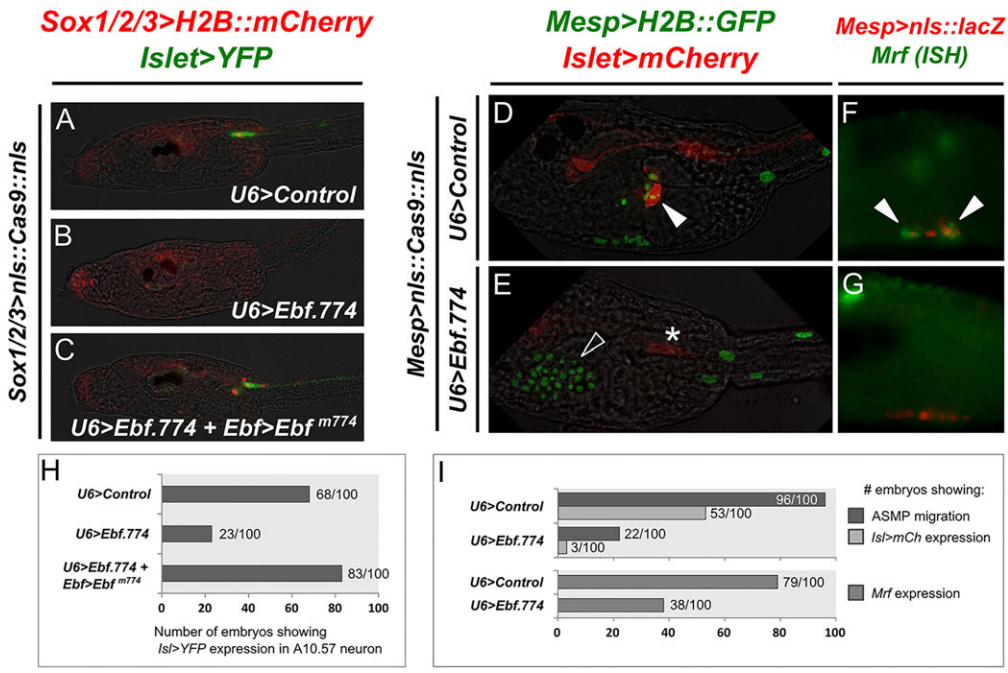

Fig. 4. Phenotypic assays for tissue-specific loss of *Ebf* in F0 embryos. (A) Larva (17.5 hpf, 22°C) electroporated with 35 µg *Sox1/2/3>nls::Cas9::nls* and 25 µg *U6>Control sgRNA*, showing normal expression of *Isl>YFP* reporter in A10.57 MN (observed in 68% of larvae, *n*=100). (B) CRISPR/Cas9-targeted mutagenesis of *Ebf* exon 9 (35 µg *Sox1/2/3>nls::Cas9::nls*, 25 µg *U6>Ebf.774*) results in only 23% of larvae showing MN-specific *Isl* reporter expression (*n*=100). (C) Lost *Isl* expression can be rescued by a CRISPR/Cas9-resistant form of *Ebf* (45 µg *Ebf>Ebf*^{m774}), as *Isl>YFP* is now seen in 83% of larvae (*n*=100). (D) Larva (26 hpf, 18°C) electroporated with 50 µg *Mesp>nls::Cas9::nls* and 25 µg *U6>Control*, showing normal ASMP migration (seen in 96% of larvae, *n*=100) and *Isl>mCherry* reporter expression in ASMPs (filled arrowhead; seen in 53% of larvae, *n*=100). (E) Upon electroporation with 50 µg *Mesp>nls::Cas9::nls* and 25 µg *U6>Ebf.774*, ASMP migration is observed in only 22% of larvae (*n*=100) and *Isl>mCherry* expression in ASMPs is only seen in 3% of larvae (*n*=100; empty arrowhead). Asterisk indicates *Isl*>mCherry expression in A10.57 neuron, confirming that disruption of *Ebf* was B7.5 lineage specific. (F) Control larvae (22 hpf, 18°C) show normal expression of *Mrf* (assayed by *in situ* mRNA hybridization) in ASMPs (filled arrowheads; observed in 79% of larvae, *n*=100). (G) *Mrf* expression in ASMPs is downregulated upon CRISPR/Cas9 targeting of *Ebf* (50 µg *Mesp>nls::Cas9::nls*+25 µg *U6>Ebf.774*), as *Mrf* expression is seen in only 38% of larvae (*n*=100; empty arrowheads). (H,I) Histograms indicating the fraction of electroporated embryos displaying the phenotypes represented in A-G.

gives rise to three distinct muscle cells: tail, heart and atrial siphon (pharyngeal) muscles (ASMs) (Hirano and Nishida, 1997). *Ciona* Ebf is crucial for ASM specification and is also upstream of *Isl* expression in these cells (Razy-Krajka et al., 2014; Stolfi et al., 2010).

Lateral trunk ventral cells (TVCs) are normally specified as ASM precursors and express *Isl* (Fig. 4D) and the pharyngeal muscle markers *Mrf* (also known as *MyoD*) and *Ncam* (Fig. 4F; supplementary material Fig. S8) (Razy-Krajka et al., 2014). They detach from their sister cells (the heart precursors), activate *Isl* and migrate dorsally to surround the atrial siphon primordia (Fig. 4D). In larvae electroporated with *Mesp>nls::Cas9::nls* and *U6>Ebf.774*, lateral TVCs migrate and activate the *Isl* reporter in only 22% and 3% of larvae, respectively (*n*=100 each, Fig. 4E,I). A similar loss of *Mrf* and *Ncam* expression was also observed in these larvae (Fig. 4F,G,I; supplementary material Fig. S8). Moreover, 19 out of 30 such larvae raised to juveniles completely lacked ASMs (supplementary material Fig. S9). We conclude that Ebf is necessary in the B7.5 lineage for ASMP specification and migration, consistent with previous results using dominant repressor forms of Ebf (Razy-Krajka et al., 2014; Stolfi et al., 2010).

Conclusions

Using the CRISRPR/Cas9 system to generate targeted mutations, we have demonstrated a requirement for *Ebf* in specification of *Isl*$^{+}$ MNs and pharyngeal muscles. We believe that the efficiency of CRISPR/Cas9, allied to the ease of transfection of *Ciona* embryos, promises for rapid scaling of genome editing in this model chordate.

Note added in proof

While our paper was in press, Sasaki et al. reported CRISPR/Cas9-mediated gene knockout in C. intestinalis embryos (Sasaki et al., 2014).

MATERIALS AND METHODS

Molecular cloning

Putative (N)21-GG targets were screened for polymorphisms and off-targets. sgRNA vectors were designed according to Mali et al. (2013) with scaffold modification ('F+E') (Chen et al., 2013). Annealed oligonucleotides were ligated into *U6>sgRNA(F+E)* linearized with *Bsa*I, downstream of the *Ciona* U6 promoter (Nishiyama and Fujiwara, 2008). See supplementary material protocol for further information. For sgRNA transcription assays, *eGFP* sequence was inserted downstream of Ebf.34 sgRNA in F+E or original vectors, with or without termination sequences.

nls::Cas9::nls and *nls::Cas9::nls::eGFP* were derived from *Sp-dCas9* (Qi et al., 2013). Cas9 variants were inserted downstream of the promoters *Mesp* (Davidson et al., 2005), *Eef1a* (*EF1α*) (Sasakura et al., 2010) and *Sox1/2/3* (Stolfi et al., 2014). *Isl* and *Ebf* drivers/reporters have previously been published (Stolfi and Levine, 2011; Stolfi et al., 2010; Wang et al., 2013b).

For RT-PCR, RNA was extracted using RNAqueous kit (Ambion) and first strand-synthesized by Sensiscript-RT (Qiagen) primed with (dT)15 oligonucleotides. All targeted genomic or cDNAs amplified were cloned into pCRII-TOPO Dual-Promoter (Invitrogen).

Detailed primer, sgRNA and probe sequence information can be found in supplementary material Table S1. Our sgRNA and Cas9 vectors can be obtained through Addgene (www.addgene.org/browse/article/9026/)

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Cloning of *Ebf>Ebf^{m774}* rescue construct

Quikchange (Agilent) was used to generate a synonymous mutation in the target 'seed' of *Ebf*.774 from ACAGG to ACCGG to create *Ebf^{m774}*, which was subcloned downstream of the *Ebf cis*-regulatory sequences (−3348/exon1b).

Embryo electroporation and imaging

DNA electroporation was performed on fertilized, dechorionated eggs from *C. intestinalis* (Type A) obtained commercially (M-REP) as described (Christiaen et al., 2009a,b). Electroporated plasmid amounts (e.g. 10 µg) were per 700 µl of total volume. *In situ* hybridization was carried out as described (Beh et al., 2007; Christiaen et al., 2009c). Probes were transcribed *in vitro* from linearized plasmid. PCR products with flanking T7 promoters were used as template for *eGFP* probes. Rat anti-HA (Roche, Cat#1867423), mouse monoclonal anti-β-Gal (Promega, Z3781), rabbit polyclonal anti-GFP (Abcam, AB6556), Alexa Fluor-conjugated secondary antibodies (Molecular Probes, A212428, A21422, A10037, A21434, A21042) were diluted 1:500-1:1000. Embryos were fixed in 4% (para) formaldehyde-MEM buffer and mounted in 2% DABCO/50% glycerol/PBS. Images were taken on a Leica inverted TCS SP8 X confocal microscope or a DM2500 epifluorescence microscope.

Cleavage detection assay

Pooled embryos or cells were lysed in buffer from GeneArt Genomic Cleavage Detection Assay Kit (Invitrogen). PCR was performed from lysate using AmpliTaq supplied with the kit, except for assays on MACS-selected cells, for which Pfx platinum polymerase (Invitrogen) was used. For AmpliTaq we used the kit protocol with 60°C annealing temperature. For Pfx, we used 30 cycles, 60°C annealing temperature and 68°C extension temperature. For experiments comparing sgRNA vector concentrations, 50-cycle reactions were required to amplify sufficient material for assays and cloning. PCR products were purified using QIAquick PCR purification kit (Qiagen) or Nucleospin gel cleanup kit (Macherey-Nagel). Denaturing, re-annealing and incubation with detection enzyme were performed according to the manual. Reactions were run on a 2% agarose gel at 70 V for 30 min and imaged using Molecular Imager Gel Doc XR+ (Bio-Rad). Analysis was performed with ImageJ (National Institutes of Health). Cleavage efficiency was determined according to kit guidelines (Guschin et al., 2010).

Embryo dissociation and MACS sorting

Embryos were dissociated as described (Christiaen et al., 2009d). OctoMACS starting kit (Miltenyi Biotec) was used according to the manufacturer's recommendations, with modifications. Prior to sorting, dissociated cells were resuspended in 90 µl 0.05% BSA in calcium/magnesium-free artificial sea water and incubated with 10 µl anti-hCD4 antibody-coated microbeads (Miltenyi Biotec) at 4°C for 1 h. After applying diluted cells to pre-filter and column, flow-through was collected as hCD4^{-} cells. Columns were washed three times with the same buffer and hCD4^{+} cells were flushed with the kit plunger.

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Competing interests

The authors declare no competing financial interests.

Author contributions

A.S. and L.C. conceived the study. A.S., S.G. and F.S. performed experiments and analyzed data under the supervision of L.C. A.S., S.G., F.S. and L.C. wrote the paper.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.114488/-/DC1

References

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A. and Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**, 1709-1712.

Beh, J., Shi, W., Levine, M., Davidson, B. and Christiaen, L. (2007). FoxF is essential for FGF-induced migration of heart progenitor cells in the ascidian Ciona intestinalis. *Development* **134**, 3297-3305.

Brouns, S. J. J., Jore, M. M., Lundgren, M., Westra, E. R., Slijkhuis, R. J. H., Snijders, A. P. L., Dickman, M. J., Makarova, K. S., Koonin, E. V. and van der Oost, J. (2008). Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* **321**, 960-964.

Chen, B., Gilbert, L. A., Cimini, B. A., Schnitzbauer, J., Zhang, W., Li, G.-W., Park, J., Blackburn, E. H., Weissman, J. S., Qi, L. S. et al. (2013). Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* **155**, 1479-1491.

Christiaen, L., Wagner, E., Shi, W. and Levine, M. (2009a). Isolation of sea squirt (Ciona) gametes, fertilization, dechorionation, and development. *Cold Spring Harb. Protoc.* **2009**, ppdb.prot5344.

Christiaen, L., Wagner, E., Shi, W. and Levine, M. (2009b). Electroporation of transgenic DNAs in the sea squirt Ciona. *Cold Spring Harb. Protoc.* **2009**, ppdb.prot5345.

Christiaen, L., Wagner, E., Shi, W. and Levine, M. (2009c). Whole-mount in situ hybridization on sea squirt (Ciona intestinalis) embryos. *Cold Spring Harb. Protoc.* **2009**, ppdb.prot5348.

Christiaen, L., Wagner, E., Shi, W. and Levine, M. (2009d). Isolation of individual cells and tissues from electroporated sea squirt (Ciona) embryos by fluorescence-activated cell sorting (FACS). *Cold Spring Harb. Protoc.* **2009**, ppdb.prot5349.

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A. et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823.

Corbo, J. C., Levine, M. and Zeller, R. W. (1997). Characterization of a notochord-specific enhancer from the Brachyury promoter region of the ascidian, Ciona intestinalis. *Development* **124**, 589-602.

Crozatier, M. and Vincent, A. (1999). Requirement for the Drosophila COE transcription factor Collier in formation of an embryonic muscle: transcriptional response to notch signalling. *Development* **126**, 1495-1504.

Davidson, B., Shi, W. and Levine, M. (2005). Uncoupling heart cell specification and migration in the simple chordate Ciona intestinalis. *Development* **132**, 4811-4818.

Dickinson, D. J., Ward, J. D., Reiner, D. J. and Goldstein, B. (2013). Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination. *Nat. Methods* **10**, 1028-1034.

Dubois, L., Bally-Cuif, L., Crozatier, M., Moreau, J., Paquereau, L. and Vincent, A. (1998). XCoe2, a transcription factor of the Col/Olf-1/EBF family involved in the specification of primary neurons in Xenopus. *Curr. Biol.* **8**, 199-209.

Friedland, A. E., Tzur, Y. B., Esvelt, K. M., Colaiácovo, M. P., Church, G. M. and Calarco, J. A. (2013). Heritable genome editing in C. elegans via a CRISPR-Cas9 system. *Nat. Methods* **10**, 741-743.

Guschin, D. Y., Waite, A. J., Katibah, G. E., Miller, J. C., Holmes, M. C. and Rebar, E. J. (2010). A rapid and general assay for monitoring endogenous gene modification. *Methods Mol. Biol.* **649**, 247-256.

Hirano, T. and Nishida, H. (1997). Developmental fates of larval tissues after metamorphosis in ascidian Halocynthia roretzi. I. Origin of mesodermal tissues of the juvenile. *Dev. Biol.* **192**, 199-210.

Horie, T., Nakagawa, M., Sasakura, Y., Kusakabe, T. G. and Tsuda, M. (2010). Simple motor system of the ascidian larva: neuronal complex comprising putative cholinergic and GABAergic/glycinergic neurons. *Zoolog. Sci.* **27**, 181-190.

Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., Li, Y., Fine, E. J., Wu, X., Shalem, O. et al. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31**, 827-832.

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., Peterson, R. T., Yeh, J.-R. J. and Joung, J. K. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.* **31**, 227-229.

Imai, K. S., Stolfi, A., Levine, M. and Satou, Y. (2009). Gene regulatory networks underlying the compartmentalization of the Ciona central nervous system. *Development* **136**, 285-293.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A. and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821.

Kawai, N., Ochiai, H., Sakuma, T., Yamada, L., Sawada, H., Yamamoto, T. and Sasakura, Y. (2012). Efficient targeted mutagenesis of the chordate Ciona intestinalis genome with zinc-finger nucleases. *Dev. Growth Differ.* **54**, 535-545.

Kratsios, P., Stolfi, A., Levine, M. and Hobert, O. (2012). Coordinated regulation of cholinergic motor neuron traits through a conserved terminal selector gene. *Nat. Neurosci.* **15**, 205-214.

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E. and Church, G. M. (2013). RNA-guided human genome engineering via Cas9. *Science* **339**, 823-826.

Development (2014) 141, 4115-4120 doi:10.1242/dev.114488

Marraffini, L. A. and Sontheimer, E. J. (2008). CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* **322**, 1843-1845.

Nishiyama, A. and Fujiwara, S. (2008). RNA interference by expressing short hairpin RNA in the Ciona intestinalis embryo. *Dev. Growth Differ.* **50**, 521-529.

Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P. and Lim, W. A. (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**, 1173-1183.

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A. and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281-2308.

Razy-Krajka, F., Lam, K., Wang, W., Stolfi, A., Joly, M., Bonneau, R. and Christiaen, L. (2014). Collier/OLF/EBF-dependent transcriptional dynamics control pharyngeal muscle specification from primed cardiopharyngeal progenitors. *Dev. Cell* **29**, 263-276.

Roure, A., Rothbächer, U., Robin, F., Kalmar, E., Ferone, G., Lamy, C., Missero, C., Mueller, F. and Lemaire, P. (2007). A multicassette Gateway vector set for high throughput and comparative analyses in ciona and vertebrate embryos. *PLoS ONE* **2**, e916.

Sasakura, Y., Suzuki, M. M., Hozumi, A., Inaba, K. and Satoh, N. (2010). Maternal factor-mediated epigenetic gene silencing in the ascidian Ciona intestinalis. *Mol. Genet. Genomics* **283**, 99-110.

Sasaki, H., Yoshida, K., Hozumi, A. and Sasakura, Y. (2014). CRISPR/Cas9-mediated gene knockout in the ascidian Ciona intestinalis. *Dev. Growth Differentiation* **56**, 499-510.

Satoh, N. (2014). *Developmental Genomics of Ascidians*. Hoboken, NJ: John Wiley & Sons.

Satou, Y., Yamada, L., Mochizuki, Y., Takatori, N., Kawashima, T., Sasaki, A., Hamaguchi, M., Awazu, S., Yagi, K., Sasakura, Y. et al. (2002). A cDNA resource from the basal chordate Ciona intestinalis. *Genesis* **33**, 153-154.

Stolfi, A. and Christiaen, L. (2012). Genetic and genomic toolbox of the chordate Ciona intestinalis. *Genetics* **192**, 55-66.

Stolfi, A., Lowe, E.K., Stolfi, A., Ristoratore, F., Brown, C.T., Swalla, B.J. and Christiaen, L. (2014). Divergent mechanisms regulate conserved cardiopharyngeal development and gene expression in distantly related ascidians. *eLife* 10.7554/eLife.03728.

Stolfi, A. and Levine, M. (2011). Neuronal subtype specification in the spinal cord of a protovertebrate. *Development* **138**, 995-1004.

Stolfi, A., Gainous, T. B., Young, J. J., Mori, A., Levine, M. and Christiaen, L. (2010). Early chordate origins of the vertebrate second heart field. *Science* **329**, 565-568.

Treen, N., Yoshida, K., Sakuma, T., Sasaki, H., Kawai, N., Yamamoto, T. and Sasakura, Y. (2014). Tissue-specific and ubiquitous gene knockouts by TALEN electroporation provide new approaches to investigating gene function in Ciona. *Development* **141**, 481-487.

Tsagkogeorga, G., Cahais, V. and Galtier, N. (2012). The population genomics of a fast evolver: high levels of diversity, functional constraint, and molecular adaptation in the tunicate Ciona intestinalis. *Genome Biol. Evol.* **4**, 740-749.

Wang, H., Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., Zhang, F. and Jaenisch, R. (2013a). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* **153**, 910-918.

Wang, W., Razy-Krajka, F., Siu, E., Ketcham, A. and Christiaen, L. (2013b). NK4 antagonizes Tbx1/10 to promote cardiac versus pharyngeal muscle fate in the ascidian second heart field. *PLoS Biol.* **11**, e1001725.

Wu, X., Scott, D. A., Kriz, A. J., Chiu, A. C., Hsu, P. D., Dadon, D. B., Cheng, A. W., Trevino, A. E., Konermann, S., Chen, S. et al. (2014). Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. *Nat. Biotechnol.* **32**, 670-676.

Yang, H., Wang, H., Shivalila, C. S., Cheng, A. W., Shi, L. and Jaenisch, R. (2013). One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* **154**, 1370-1379.

Yoshida, K., Treen, N., Hozumi, A., Sakuma, T., Yamamoto, T. and Sasakura, Y. (2014). Germ cell mutations of the ascidian Ciona intestinalis with TALE nucleases. *Genesis* **52**, 431-439.

Zeller, R. W., Virata, M. J. and Cone, A. C. (2006). Predictable mosaic transgene expression in ascidian embryos produced with a simple electroporation device. *Dev. Dyn.* **235**, 1921-1932.