RESEARCH REPORT

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

Alberto Stolfi*, Shashank Gandhi, Farhana Salek and Lionel Christiaen*

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.

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 Ebf 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 Ecfla (EF1α) promoter (Sasakura et al., 2010). Fertilized eggs (>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...
Fig. 1. CRISPR/Cas9 components in Ciona intestinalis. (A) Top: nls::dCas9::eGFP protein used to assay nuclear localization in Ciona. dCas9 is Cas9 with two point mutations that render it catalytically dead. Bottom: nls::Cas9::Cas9 used for targeted mutagenesis. (B) Tail bud-stage embryo electroporated with Mesp>nls::dCas9::eGFP, confirming proper nuclear localization in B7.5 lineage cells. Scale bar: 25 μm. (C) Ebf.T74 sgRNA (F+E) version. The protospacer (red) is paired with its target 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 magnetically-activated cell sorting (MACS)-selected Ebf-expressing cells from pooled, dissociated embryos electroporated with Ebf→hCD4::mCherry, EFlα→nls::Cas9::nls and U6→Ebf.T74. 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 EFlα→nls::Cas9::nls and U6→Ebf.T74. 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 EFlα→hCD4::mCherry, EFlα→nls::Cas9::nls and U6→Ebf.T74. 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 EFlα→hCD4::mCherry, 25 μg EFlα→nls::Cas9::nls and 75 μg U6→Ebf.T74. 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-terminal 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 EFlα→nls::Cas9::nls, 10 μg U6→Ebf.T74 and 10 μg U6→Ebf.T83. 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 EFlα→hCD4::mCherry, 25 μg EFlα→nls::Cas9::nls and 75 μg U6→Ebf.T74 (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.
located upstream of the conserved motor neuron (MN) regulatory
larva (Horie et al., 2010). Previous studies suggested that Ebf is
neurons that innervate the tail to drive swimming behavior in the
expression in motor neurons
increase substantially from 8 to 12 hpf. Electroporated
CRISPR/Cas9-targeted mutagenesis of
embryos. The
increase between 8 and 12 hpf, suggesting that CRISPR/Cas9 is
limited by the onset of
4 hpf, but appeared at 8 hpf (Fig. 3D). Cleavage efficiency did not
samples extracted at different time points from the same pool of
dressed by CRISPR/Cas9-mediated targeted mutations be detected. We
tested how early in the development of
embryos could
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
to express Cas9 in B7.5 blastomeres. The B7.5 lineage
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 EF1α 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%. Cleavage of exon 9 amplicon from embryos electroporated with 10 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, EF1α 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.

**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≈Ebf5772). As predicted, co-electroporation of Ebf≈Ebf5772 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. 3. Genomic cleavage assays. (A) Cleavage assay of EF1α 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 EF1α 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 with 10 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.
38% of larvae (mutagenesis of 25 μg Isl (26 hpf, 18°C) electroporated with 50 expression in ASMPs is downregulated upon CRISPR/Cas9 targeting of markers Mrf precursors and express Isl Ebf is crucial for ASM specification and is also upstream of 4118 MnRs and pharyngeal muscles. We believe that the efficiency of Using the CRISPR/Cas9 system to generate targeted mutations, Conclusions gives rise to three distinct muscle cells: tail, heart and atrial siphon (pharyngeal) muscles (ASMs) (Hirano and Nishida, 1997). Gt gives rise to three distinct muscle cells: tail, heart and atrial siphon (pharyngeal) muscles (ASMs) (Hirano and Nishida, 1997). Moreover, 19 out of 30 such larvae raised to juveniles completely lacked ASMs (supplementary material Fig. S8). (C) CRISPR/Cas9-targeted expression can be rescued by a CRISPR/Cas9-resistant form of Ebf (45 μg Ebf/Ebfm774), 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 ASM migration (seen in 96% of larvae, n=100) and Isl>mCherry reporter expression in ASMPs (filled arrowheads; observed 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 only 22% of larvae. (F) Larva (22 hpf, 18°C) shows 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) Histograms indicating the fraction of electroporated embryos displaying the phenotypes represented in A-G. 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 Bael, 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.

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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 (EF1a) (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/)
Cloning of Ebf>Ep<sup>m774</sup> rescue construct

Quikchange (Agilent) was used to generate a synonymous mutation in the target ‘seed’ of Ebf<sup>m774</sup> from ACAGG to ACCGG to create Ebf<sup>p</sup>, 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 <i>C. intestinalis</i> (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, Z23781), rabbit polyclonal anti-GFP (Abcam, AB6556), Alexa Fluor-conjugated secondary antibodies (Molecular Probes, A21248, 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 by 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 migration in the simple chordate Ciona intestinalis.

References


PROTOCOL FOR CLONING SINGLE GUIDE RNA (sgRNA) EXPRESSION VECTORS
By A. Stolfi, S. Gandhi, F. Salek, and L. Christiaen

1 - Search gene or region of interest for potential PAM’s: “NGG” or “CCN” if targeting minus strand. Select 19 nucleotides 5’ to the PAM. This is the target.

```
TCAACCCAACTGAGGGTTGGACACAACAGC
```

2 - If the target contains too many T’s near the 3’ end (four T’s or more in a row will terminate transcription), or if it spans many known naturally-occurring SNPs or indels, discard it.

3- BLAST can be used to search for potential off-targets. BLAST 15 nt at 3’-most position. Search for adjacent PAM in all off-targets. If there is no PAM in the off-target, then Cas9 will not bind. If there is a PAM but only the seed or a few nucleotides match with the target, then this may be suitable as long as you are using Cas9 for generating DSBs, not dCas9 for CRISRPi.

4- For transcription initiation from U6 promoter, append “G” to the 5’ end of this to give a G(N)20 sequence. This is your protospacer.

```
GCTGAGGGTTGGACACAACAGC
```

5- Append “AGAT” to the 5’ end of the sequence. These are the overhangs needed for cloning. This is now your top oligo for cloning.

```
agatGCTGAGGGTTGGACACAACAGC
```

6- Copy reverse complement of G(N)20, append “AAAC” to this. This is the bottom oligo needed to anneal to your top oligo for cloning.

```
aaacGCTGTGTGTCACACCCTCAGC
```

7- Anneal oligos at 10 μM (boil for 5 minutes in 10mM Tris, pH7.5, 50mM NaCl, let cool to room temperature).
8- Dilute annealed oligos 1:1000 and ligate this into U6>sgRNA(F+E) linearized with Bsal. Bsal is an enzyme that cuts away from its recognition site, leaving sticky ends that depend on the context sequence:

![Diagram of Bsal cleavage and ligation](image)

Digestion + ligation with annealed oligos:

5’...atccgatgtatatagagagagagaggtctcaGTTTAAG...3’
3’...taggctaccatatctactctctctccagactCAAATC...5’

9- Transform into competent cells, pick colonies for mini cultures, screening by colony PCR using U6 promoter forward sequencing primer + bottom oligo used for annealing step above. Prep and sequence before using in electroporation.
Supplementary Figure S1. Testing of CRISPR/Cas9 components. (A,B) Immunohistostaining for HA-tagged Cas9 and dCas9 variants in electroporated embryos. Diffuse signal excluded from nuclei suggested these constructs were not properly localized to the nucleus. Thus they were not used in subsequent experiments. (C) Immunostaining for HA-tagged nls::β-Gal revealed similar cytoplasmic staining, suggesting HA tags may generally interfere with nuclear localization sequences (nls) in Ciona embryos. (D) In situ hybridization for eGFP in embryos electroporated with U6>Ebf.34 (F+E)TERM plasmid sequence, with terminator sequence (‘TERM’) in between the Ebf.34 sgRNA sequence and eGFP. Lack of signal was expected, due to termination of transcription before eGFP. A similar, but initially unexpected, lack of eGFP in situ hybridization signal in embryos electroporated with U6>Ebf.34.
**Supplementary Figure S2.** CRISPR/Cas9-mediated targeted mutagenesis in non-coding sequences upstream of *Foxf* and *Hand-related* genes. Alignment of wildtype and mutagenized CRISPR/Cas9-targeted sequences. 

**Top:** -1182 to -618 bp 5’ from the translation start of Foxf. 

**Bottom:** -2052 to -1489 bp 5’ from the translation start of Hand-related. Target sequences indicated in blue, or purple if sgRNA pairs with minus strand. Insertions and substitutions are indicated in red. Deletions are indicated by red dashes.
A

Ebf wildtype:

```
PAEGWTTGGGATVVENFDFGLQVV
CCAGCTGAGGGTTGGACAACAGGTGGAGCAACAGTTGTTATAGTTGGAGAAAATTTCTTTGACGGGCTTCAAGTTGTG
```

Ebf mutant cDNA clone 1:

```
PAGGWTT-----RLGLLVKV
CCAGCTGAGGGTTGGACA----------------AGAGGGTTGTGTTTGGTTCTGAGG----------------GTTGTG
```

Ebf mutant cDNA clone 2:

```
PAGSSGSAGATVVVG
CCAGCTGAGGGCTCCTCAGAGGAGCACAGTTGTTATAGTTGGAGAAAATTTCTTTGACGGGCTTCAAGTTGTG
```

B

**Wildtype**

**Ebf mutant cDNA clone 4**

**Supplementary Figure S3. Aberrant Ebf transcripts a result of CRISPR/Cas9 targeting of exon 9.** (A) Alignment of wildtype Ebf exon 9 cDNA sequence with that from two mutant cDNAs obtained from MACS-purified cells from manipulated embryos. Embryos were electroporated with EF1α>nls::Cas9::nls, U6>Ebf.744, and Ebf>hCD4::mCherry. Substitutions/indels are in red font and resulting amino acid sequence changes are in black font highlighted in red. (B) Schematic of mutant cDNA clone 4, in which it appears that a 61 bp deletion in exon 9, 5' to the PAM of Ebf.774 target, eliminated the normal splice acceptor site at the start of exon 9. In turn, the splicing machinery appeared to use an alternative site 111 nt 5' upstream, in intron 8. This results in the cDNA carrying some intronic sequences and a frameshift that results in a premature termination codon (PTC) in exon 9.
**Supplementary Figure S4. Gel image analysis** Calculations of cleavage efficiency as measured from gel analysis. (A) Lane 1 from Figure 3A, showing parental band (p) and two cleavage products (c1 and c2), as a result of incubation with detection enzyme. (B) Plot of pixel intensities along length of lane A. Cleavage efficiency calculations based on To calculate cleavage efficiency we measured the ratio of cleaved band and the sum of cleaved and parental band intensities (‘fraction cleaved’). Gel images were imported into ImageJ (National Institutes of Health). The lane is analyzed as a plot of pixel intensity along the length of the lane. This allows the user to measure the proportion of area under a specific peak on the plot relative to the total area of the plot. These values can be obtained by selecting the ‘label Peaks’ option under ‘gel analysis’. The ratio of cleaved band to the sum of cleaved and parental peaks (cleaved/cleaved + parental) = ‘fraction cleaved’ is used to calculate the cleavage efficiency based on the formula : 1 – [(1-fraction cleaved)^1/2] (Guschin et al. 2010). Cleavage efficiency of control amplicon from a 1:1 mix of mutant: wild-type template supplied by the assay kit is equal to the fraction cleaved. (C) Lane 2 from Figure 3A, showing same parental amplicon as in A, but without incubation with detection enzyme. As a result, no cleavage peaks appeared. (D) Plot from C showing no peaks in intensity around where cleavage bands were expected.
Supplementary Figure S5. Polymorphisms in the Mesp locus result in cleavage assay false positives. (A) Screen capture from GHOST C. intestinalis gbrowse (Satou et al. 2005) view of Mesp -280/+408 region, showing many different SNPs and indels sequenced from wild populations of Ciona in USA and Japan. (B) Cleavage assay on Mesp -280/+408 PCR products amplified from gDNA extracted from pooled, control embryos showing numerous cleavage bands, probably a result of detection of naturally occurring SNPs and indels, since the source of gametes used in laboratory are animals collected from highly polymorphic wild populations (Tsagkogeorga et al. 2012).
Supplementary Figure S6. Gel analyses of MACS-sorted and unsorted cells from embryos electroporated with low (25 μg) and high (75 μg) amounts of sgRNA vector. (A) Diagrams of ImageJ analysis performed on gel images shown in Figure 3C representing cleavage assays of amplicon from hCD4+ MACS-sorted and unsorted cells from embryos electroporated with 10 μg EF1α>hCD4::mCherry, 25 μg EF1α>nls::Cas9::nls and 75 μg U6>Ebf.774. (B) Diagrams of ImageJ analysis performed on gel images (not shown) representing cleavage assays of amplicon from hCD4+ MACS-sorted and unsorted cells from embryos electroporated with 10 μg EF1α>hCD4::mCherry, 25 μg EF1α>nls::Cas9::nls and 25 μg U6>Ebf.774. The relative signal intensities (y-axis) were plotted along the length of each gel lane (x-axis). The areas under the curves representing parental and cleaved band intensities were calculated by ImageJ. Lower
threshold of each curve was defined by its lowest signal intensity at the bottom of the gel image. Regions shaded in gray were excluded from the analysis due to non-specific band visible in reactions lacking detection enzyme, and were defined in the each enzyme lane and applied to its sister enzyme* lane as well. Cleavage efficiencies were calculated using the formula suggested by the GeneArt manual: \(1 - \left(1 - \text{fraction cleaved}\right)^{1/2}\) (Guschin et al. 2010). Higher cleavage efficiencies in embryos electroporated with 75 μg sgRNA vector suggest that increased mutagenesis rates result from lower mosaicism and higher sgRNA concentrations per transfected cell.
**EF1α>nls::dCas9::eGFP (eGFP IHC)**

4 hpf  5 hpf  6 hpf  7 hpf

Supplementary Figure S7. Time-series of EF1α promoter-driven Cas9 expression. eGFP immunohistostaining showing temporal dynamics of nls::dCas9::nls::eGFP expression and accumulation when driven by the EF1α promoter in *Ciona* embryos reared at 18°C. Expression is only clearly visible at 5 hpf, although it is still relatively weak. Strong expression is seen at 6 hpf and continues to build up at 7 hpf. We conclude that the EF1α promoter is not active much earlier than 4 hpf (~64 cell stage).
Supplementary Figure S8. Loss of NCAM expression in presumptive ASMPs upon mutation of Ebf in B7.5. *In situ* hybridization for ASMP marker NCAM in larvae electroporated with 70 μg *Mesp>nls::Cas9::nls* and 25 μg *U6>Control* (left) and in larvae electroporated with 70 μg *Mesp>nls::Cas9::nls* and 25 μg *U6>Ebf.774* (right). In 60% (*n*=100) of control embryos, NCAM is normally expressed in ASMPs (filled arrowhead), but is seen in presumptive TVCS (empty arrowhead) only in 32% of embryos (*n*=56) subjected to CRISPR/Cas9-targeting of *Ebf* exon 9. Larvae were fixed at 22 hpf 18°C.
**Supplementary Figure S9. CRISPR/Cas9-targeting of Ebf results in loss of ASMs in juveniles.**

(A) Juvenile (89 hpf) raised from embryos electroporated with 60 μg Mesp>nls::Cas9::nls + 40 μg U6>control and Mesp>H2B::mCherry (to visualize the lineage late in development). mCherry⁺ atrial siphon muscles (ASMs) or longitudinal muscles (LOMs) were visible in 11 out of 11 transgenic juveniles analyzed, indicating normal development. (B) Juvenile raised from embryos electroporated with 60 μg Mesp>nls::Cas9::nls + 40 μg U6>Ebf.774 + Mesp>H2B::mCherry. mCherry⁺ ASMs/LOMs were abolished in 19 out of 30 transgenic juveniles analyzed, suggesting that Ebf is required for their specification and differentiation, as suggested in previous studies (Stolfi et al. 2010; Wang et al. 2013; Razy-Krajka et al. 2014). h, heart. Asterisk indicates reabsorbed B7.5-derived anterior tail muscles.
**Supplementary Table S1.**

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

sgRNA targeting sequences used in this study:

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<td>Hand-r -1851</td>
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<tr>
<td>Control (sequence not present in <em>Ciona</em> genome)</td>
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Primers used for cloning of *cis*-regulatory sequences (5’ to 3’):

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Primers used for amplifying CRISPR/Cas9-targeted regions for cleavage assay or cloning (5’ to 3’):
### References for published plasmids used in this study

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