Developmental Biology 423 (2017) 111–125

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Germ cell regeneration-mediated, enhanced mutagenesis in the ascidian *Ciona intestinalis* reveals flexible germ cell formation from different somatic cells

Keita Yoshida[a,1], Akiko Hozumi[a,1], Nicholas Treen[a,2], Tetsushi Sakuma[b], Takashi Yamamoto[b], Maki Shirae-Kurabayashi[c], Yasunori Sasakura[a,*]

[a] Shimoda Marine Research Center, University of Tsukuba, Shimoda, Shizuoka 415-0025, Japan
[b] Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8526, Japan
[c] Sugashima Marine Biological Laboratory, Graduate School of Science, Nagoya University, Japan

ARTICLE INFO

Keywords:
Ciona
Ascidian
Primordial germ cell
Regeneration
TALEN
Mutagenesis

ABSTRACT

The ascidian *Ciona intestinalis* has a high regeneration capacity that enables the regeneration of artificially removed primordial germ cells (PGCs) from somatic cells. We utilized PGC regeneration to establish efficient methods of germ line mutagenesis with transcription activator-like effector nucleases (TALENs). When PGCs were artificially removed from animals in which a TALEN pair was expressed, somatic cells harboring mutations in the target gene were converted into germ cells, this germ cell population exhibited higher mutation rates than animals not subjected to PGC removal. PGC regeneration enables us to use TALEN expression vectors of specific somatic tissues for germ cell mutagenesis. Unexpectedly, *cis* elements for epidermis, neural tissue and muscle could be used for germ cell mutagenesis, indicating there are multiple sources of regenerated PGCs, suggesting a flexibility of differentiated *Ciona* somatic cells to regain totipotency. Sperm and eggs of a single hermaphroditic, PGC regenerated animal typically have different mutations, suggesting they arise from different cells. PGCs can be generated from somatic cells even though the maternal PGCs are not removed, suggesting that the PGC regeneration is not solely an artificial event but could have an endogenous function in *Ciona*. This study provides a technical innovation in the genome-editing methods, including easy establishment of mutant lines. Moreover, this study suggests cellular mechanisms and the potential evolutionary significance of PGC regeneration in *Ciona*.

1. Introduction

Regeneration is an important biological process that can address mechanisms how multicellular organisms repair wounds and create lost tissues and organs (Sehring et al., 2016). In addition, regeneration is useful for advancing genetic technologies. A well-known example is the clone technologies in plants and animals (Rink, 2013). With the aid of these technologies we can easily increase the number of organisms that have identical genomes, useful for homogenous conditioning of experiments as well as maintaining commercial availability of valuable strains. In this study, we attempted to add another application of regeneration for the study of genetic functions.

Knockout of genes with engineered nucleases has enabled us to address gene functions quickly and easily. Researchers can build a set of nucleases that specifically target an arbitrarily selected gene (Meng et al., 2008; Cermak et al., 2011). The constructed nucleases, when expressed in a cell, bind to their targeted DNA in the genome and induce double strand breaks near the binding site. The double strand breaks are repaired through endogenous systems, and during repair errors are introduced that could disrupt gene functions. Gene knockout with engineered nucleases has been introduced in various organisms because engineered nucleases do not demand special conditions that are required for the conventional gene knockout methods such as embryonic stem cells or very quick generation time (Capecchi, 1989; Wienholds et al., 2003; Cuppen et al., 2007). The efficiencies of knockouts with engineered nucleases, however, differ among organ-

* Corresponding author.
E-mail address: sasakura@shimoda.tsukuba.ac.jp (Y. Sasakura).
[1] These authors equally contributed to this work.
[2] Present address: Lewis-Sigler Institute for Integrative Genomics, Princeton University, NJ 08544, USA.

http://dx.doi.org/10.1016/j.ydbio.2017.01.022
Received 31 October 2016; Received in revised form 12 January 2017; Accepted 31 January 2017
Available online 01 February 2017
0012-1606/

isms and tissue/cells, and customization and improvements of the methods in each organism may be necessary to achieve highly efficient knockouts. Moreover, establishment of mutant lines of the genes essential for development is not easy with engineered nucleases because of the lethal effect of introduced nucleases. The issue remains a significant hurdle for mutagenesis to solve (Blitz et al., 2016).

The chordate ascidian *Ciona intestinalis* is an important organism for understanding developmental mechanisms and evolution of chordates (Satoh, 2003; Lemaire, 2009). In this ascidian, gene knockouts with engineered nucleases and CRISPR/Cas9 have been recently reported (Kawai et al., 2012; Treen et al., 2014; Yoshida et al., 2014; Sasaki et al., 2014; Stolfi et al., 2014). Knockout with TALE nucleases (TALENs) is particularly splendid because it achieves efficient *Ciona* gene knockouts. When TALENs are expressed in *Ciona* somatic cells in a tissue-specific manner with an enhancer responsible for the tissue, the animals exhibit phenotypes according to the functions of genes even in the G0 generation (Treen et al., 2014; Kawai et al., 2015). TALENs can introduce mutations in germ-line cells as well as somatic cells via microinjection of TALEN mRNAs (Yoshida et al., 2014). This indicates that investigation of gene functions in the germ cells is probable with TALENs and the capability of establishment of mutant lines, because *Ciona* has relatively short generation time that is comparable to that of zebrafish, and because in-land culture systems have been built in *Ciona* (Joly et al., 2007).

Although TALEN-mediated germ cell mutagenesis has been achieved in *Ciona*, some issues remain in the current method. First, mutant lines for genes essential for *Ciona* development are difficult to be established using the current microinjection-meditated method, since introduced TALEN mRNA is transmitted to almost all blastomeres by dispersion that results in the disruption of its target gene in the ubiquitous fashion; thus the experiment will have a fatal effect on *Ciona* if the target gene has a pivotal role during development. Second, the mutation frequency of germ cells is not constantly high; the mutation frequencies are quite variable among specimens and some animals show very low mutation frequencies in germ cells. For this reason, we need to screen many G0 animals in order to find a founder animal with high mutation rate in germ cells after 2–3 months of culturing. Third, microinjection of TALEN mRNA into eggs is necessary for germ cell mutations. Microinjection is a relatively difficult and time-consuming technique compared to electroporation that has been used for mutating *Ciona* somatic cells (Treen et al., 2014; Stolfi et al., 2014). A considerable way to circumvent these issues would be using a *cis* element of a gene active in germ cells to drive TALENs in a germ cell-specific manner, as was reported in a number of organisms (Ren et al., 2013; Dong et al., 2015; Wang et al., 2015). For this purpose, a *cis* element that could drive genes in the early stage of germ cell formation is desirable; however, a *cis* element that is suitable for this purpose has not been isolated in *Ciona*. Therefore, an alternative approach is necessary for improving germ cell knockouts in this animal. We can solve most issues of germ cell mutagenesis if we could convert somatic cells to germ cells, firstly because the mutation frequency of somatic cells is constantly high (Yoshida et al., 2014), and secondary because somatic cells can be mutated by electroporation of expression vectors as mentioned above (Treen et al., 2014).

For overcoming the issues associated with germ cell mutations in *Ciona*, we focused on its high regeneration capacity. For example, the adult central nervous system of *Ciona* can be completely regenerated after removal (Dahlberg et al., 2009). Another famous regeneration event is seen in germ cells (Takamura et al., 2002; Shirae-Kurabayashi et al., 2006). Primordial germ cells (PGCs) of *Ciona* can be initially recognized as the posterior most blastomeres that accumulate various maternal RNAs (Prodon et al., 2007). After embryogenesis, PGCs are located in the ventral side of the tail at the larval stage. The PGCs migrate to the trunk during metamorphosis, then further migrate into the gonad. PGCs can be easily removed at the larval stage by surgically cutting the tail. The adults grown from the tail-cut larvae again possess Vasa-positive PGCs (Vasa is a conserved marker protein for PGCs; Takamura et al., 2002). This phenomenon suggests that *Ciona* PGCs can be regenerated from cells that may be otherwise destined to a somatic fate. In this study, we showed first that germ cell mutagenesis can be achieved by mutating somatic cells and following conversion to germ cells via PGC regeneration. Second, by using regeneration-mediated germ cell mutagenesis, we established a more reproducible method for *Ciona* germ cell mutagenesis than previously reported (Yoshida et al., 2014). The method does not have the major issues of germ-line mutagenesis described above. Hereafter we name the PGCs specified by maternal factors "mPGCs" after maternal PGCs, and regenerated PGCs "rPGCs".

2. Materials and methods

2.1. Animals and transgenic lines

Wild type *Ciona intestinalis* was cultivated at Maizuru (Kyoto), Misaki (Kanagawa), Mukaishima (Hiroshima) and Usa (Kochi). TALEN-introduced animals were cultured by an inland culture system (Joly et al., 2007). Transgenic lines for observing R3 and R4 of the intestine and the stomach are Tg[MiCiTSAdTPOG]101, Tg[MiTSAdTPOG]75 and Tg[MiTSAdTPOG]87, respectively (Yoshida and Sasakura, 2012). Transgenic lines for observing fluorescent proteins in testes are Tg[MiCiTnIG]2, Tg[MiCiTnIG]3, Tg[MiCiNutG]3, Tg[MiCiNutK]4 and Tg[MiCiNutK]8 (Joly et al., 2007; Sasakura et al., 2010; Iitsuka et al., 2014). Tg[MiCiNutK]4 and Tg[MiCiNutK]8 were created via a previously described method (Matsuoka et al., 2005). All transgenic lines are available from the CITRES database of the National BioResource Project, Japan (http://marinebio.nbrp.jp/ciona/).

2.2. Constructs

The TALENs targeting *Hox4*, *Hox5* and *Hox12* were described previously (Treen et al., 2014; Yoshida et al., 2014). The left and right *Hox4* TALEN assemblies were re-created by 4-module golden gate method (Cermak et al., 2011; Sakuma et al., 2013). The previously described TALEN structure (Treen et al., 2014) was simplified by putting the TALEN and mCherry on a single ORF separated by a 2A peptide sequence (GSGEGRGSLLTCGDVEENPGP) (Szymczak et al., 2004) by amplifying the backbone TALEN and 2A::mCherry insert by PCR with 15 bp overlapping regions and recombining them using an In-Fusion HD cloning kit (Clontech). The activity of the constructed TALENs was estimated by expressing under the control of the *EF1α* promoter according to the previous method (Treen et al., 2014). The *EF1α* promoter was replaced with the promoters of *AKR*, *EpiI*, *Nut*, and *TnI* (Davidson et al., 2003; Joly et al., 2007; Kitaura et al., 2007; Hozumi et al., 2010; Sasakura et al., 2010) using an In-Fusion HD cloning kit. The replacement was carried out through two ways. One was that removing the *EF1α* promoter using *Not*I, and another promoter was inserted into the *Not*I site of the TALEN vector. The other was that the TALEN repeats were isolated from *EF1α* expression vector with *Sal*I and *Bam*HI, then the fragment was subcloned into the inverse PCR product of an arbitral promoter and TALEN fusion vector by In-Fusion. The primers for the inverse PCR were 5′-accaacagaaggatccccgagaggacatcacat-3′ and 5′-ctgcgcgactgtcgacctcactttgggcttgatc-3′. The official names of the vectors and transgenic lines according to the nomenclature rules for tunicates (Stolfi et al., 2015) were listed in Table 8. *Hox12* TALEN expression vectors with *EpiI* and *Titf cis* elements used the old TALEN expression system described in our previous report (Treen et al., 2014). *Hox12* cDNA was amplified by PCR and was subcloned into pBS-HTB vector (Akanuma et al., 2002).

2.3. Microinjection and electroporation

pBS-HTB vectors with coding region of TALENs or *Hox12* open reading frame were linearized with *Xho*I. mRNAs of TALENs were synthesized using the Megascript T3 kit (Ambion), the poly (A) tailing kit (Ambion), and Cap structure analog (New England Biolabs). Microinjection of mRNA was performed according to a previous report (Hikosaka et al., 1992). The concentration of mRNA in the injection medium was adjusted to 100 ng/μl or 10 ng/μl for each L and R TALENs. The concentration of *Hox12* mRNA in the injection medium was 2.5 ng/μl. Electroporation was performed according to the previous report (Treen et al., 2014). 30 μg of expression vectors of L and R TALENs were electroporated for each electroporation. After electroporation, the embryos were washed in filtered seawater several times to remove excess plasmid DNA. Animals with strong RFP fluorescence were selected at the tailbud stage for further culturing.

When animals reached the larval stage (approximately 20–24 h after fertilization at 18 °C), about a half of the tail was removed using a scalpel. Our previous studies (Nakazawa et al., 2013; Kawai et al., 2015) showed that the endodermal strand starts to migrate into the trunk at about 24 h after fertilization; this processes essential to form the intestine during metamorphosis. Our tail cut surgery therefore removes a portion of the endodermal strand. This removal does not seem to cause a serious negative effect on *Ciona* survival after metamorphosis (Table 1). This is probably because the lost endodermal cells can be regenerated.

2.4. Detection of mutations and genotyping

Sperm was collected from TALEN-introduced animals and genomic DNA was isolated using a Wizard genomic DNA purification kit (Promega), the genomic region including the target site of the TALEN pair was amplified by PCR with ExTaq hot start version (Takara-bio). The PCR bands were analyzed by the surveyor nuclease assay (Kawai et al., 2012) or heteroduplex mobility shift assay with polyacrylamide gel electrophoresis (Ota et al., 2013) to examine their heterogeneity of the sequence that reflects the presence of mutated gene. The PCR bands were subcloned into a conventional vector, and sequences of some clones were determined. The frequency of mutated fragments among sequenced clones indicates the frequency of the sperm from an animal with mutated target gene. Sperm or eggs from TALEN-introduced animals were crossed with wild type counterparts to generate G1 progeny. A single G1 juvenile was dissolved in 50 μl TE with Proteinase K according to the previous method (Sasakura et al., 2003; Yoshida et al., 2014) to extract its genomic DNA. The genomic DNA solution was used to amplify the target site of a TALEN pair and analyzed as mentioned above. Some PCR fragments of *Hox4* were digested with *Psi*I to see the presence of mutations as described previously (Yoshida et al., 2014). Two G1 *Hox12* mutant carriers derived from *Hox12* TC8 in Table 3 were crossed to generate hetero-allelic *Hox12* mutants.

3. Results

3.1. Generation of mutated germ cells through mutated somatic cells

We first addressed whether somatic cells mutated by TALENs could be transdifferentiated into mutated germ cells. For this purpose, a pair of TALEN mRNAs targeting *Hox5* was chosen (Yoshida et al., 2014) since knockout of this gene does not cause a detrimental effect on development. *Hox5* TALEN mRNA was microinjected into unfertilized eggs, and these eggs were fertilized with wild type sperm. The TALEN-introduced embryos were allowed to develop until the larval stage, their mPGCs were removed by cutting tails, and then the animals were further cultured until the reproductive stage. At this stage, animals had sperm in their sperm duct and the sperm possessed fertility when it was used to inseminate wild type eggs. We named the animals developed from tail-cut larvae “TC animals” for simplicity.

We investigated whether *Hox5* was mutated in the sperm genome of the TC animals introduced with *Hox5* TALEN mRNAs at the 1-cell stage. As shown in Fig. 1A and Table 2, the TC animals had mutated *Hox5* in the sperm genome. Four animals were investigated, and the averaged frequency of mutated *Hox5* clones among sequenced PCR clones was 69.8%. When compared to the mutation frequency of *Hox5* in sperm of tail un-cut animals (Yoshida et al., 2014 and this study), *Hox5* mutation frequency in TC animals increased, but the increase was not supported by the statistical analysis (Fig. 1B). This is probably because *Hox5* TALEN can mutate *Hox5* gene so efficiently that the effect on tail cut treatment could be masked due to high mutation efficiencies in tail un-cut animals. The experiments with *Hox5* TALENs suggest that tail cut treatments could be used for germ cell mutagenesis and this method has the potential to increase mutation frequency of *Ciona* germ cells.

3.2. Hox12 is essential for proper intestine formation

Before continuing the story of tail cut treatment, we need to explain the phenotypes seen in *Hox12*-deficient *Ciona*. *Hox12* is expressed in the posterior part of the epidermis at the tailbud stage (Ikuta et al., 2010), and the expression is necessary for proper formation of the posterior taper of the tail (Ikuta et al., 2010; Treen et al., 2014). After metamorphosis, *Hox12* starts to be expressed in the posterior part of the intestine (Ikuta et al., 2004); however, the function of *Hox12* in the intestine remains unknown. We designed a pair of TALENs targeting exon of *Hox12* encoding homeodomain. When *Hox12* was knocked out by microinjecting mRNAs of the TALENs, the *Hox12*-knockout animals exhibited malformation in the intestine after metamorphosis (Fig. 2A and B). The intestine in the *Hox12*-knockout animals was expanded (40%, n=45) compared to that in the control (uninjected) animals (0%, n=51). This morphological defect in the juvenile intestine was ameliorated by introducing *Hox12* mRNA together with *Hox12* TALENs (16%, n=73; Fig. 2C), suggesting that the phenotype was specifically caused by the knockout of *Hox12*. *Hox12*-knockout animals died in several days after metamorphosis, suggesting an essential role of *Hox12* after metamorphosis.

Table 1
Tail cut does not significantly affect growth after metamorphosis.

		7 days after fertilization				14 days after fertilization			
		Juvenile with good growth	Juvenile with bad growth	Larvae	Total number	Juvenile with good growth	Juvenile with bad growth	Larvae	Total number
Exp. 1	Control	36	3	1	40	32	6	0	38
	Tail cut	19	9	5	33	26	4	0	30
Exp. 2	Control	38	5	1	44	33	4	0	37
	Tail cut	28	6	1	37	24	6	0	30

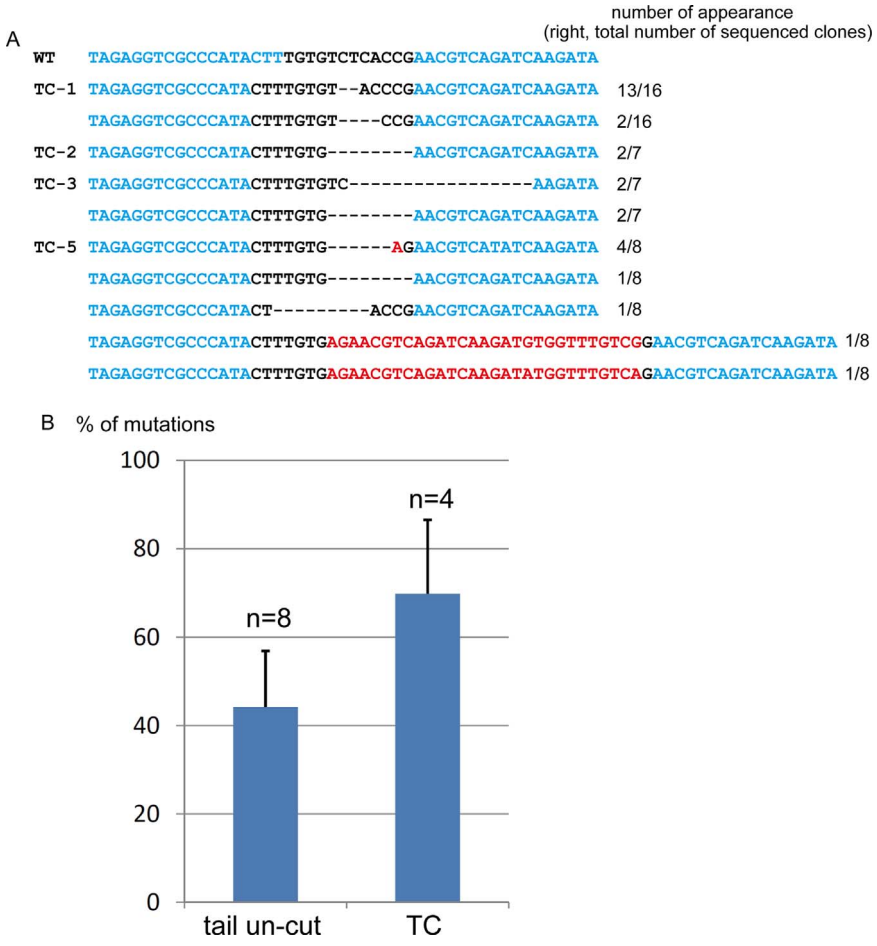

Fig. 1. Sperm of PGC-removed animals possesses mutated *Hox5* in its genome. (A) Mutations of *Hox5* detected from the sperm of *Hox5* TALEN mRNA microinjected and PGC removed animals. The left and right TALEN binding sites were shown in blue color. A deleted nucleotide was shown by a bar. The inserted nucleotides were shown in red. The left-side numbers indicate the corresponding individuals in Table 2. WT, wild type. (B) Comparison of mutation frequencies of *Hox5* in sperm from PGC remaining (tail un-cut) and removed (TC) animals. Their significance of the scores were not supported by the Student's *t*-test comparing each other. Bars represent standard errors. N indicates the number of examined animals.

Table 2
Mutation frequency of *Hox5* in sperm of tail cut animals.

Animal ID	Mutation frequency	Number of sequenced clones	Number of mutation patterns	Note
Non tail-cut-1	62.5	16	1	Reported in Yoshida et al. (2014)
Non tail-cut-2	12.5	16	1	Reported in Yoshida et al. (2014)
Non tail-cut-3	43.7	16	1	Reported in Yoshida et al. (2014)
Non tail-cut-4	6.25	16	1	Reported in Yoshida et al. (2014)
Non tail-cut-5	0	16	NE	Reported in Yoshida et al. (2014)
Non tail-cut-6	78.5	14	1	Reported in Yoshida et al. (2014)
Non tail-cut-7	100	7	2	
Non tail-cut-8	50	8	1	
TC-1	93.7	16	2	
TC-2	28.5	7	1	
TC-3	57.1	7	2	
TC-5	100	8	5	

A GFP-marker transgenic line of the intestine suggested that the part of the intestine named region 3 (R3 in Yoshida and Sasakura 2012) was particularly expanded in the *Hox12*-deficient animals (100%, n=9; Fig. 2D and E). Because food seemed to be clogged in the expanded intestine (Fig. 2B), we assumed that the posterior opening of the intestine might have been absent in *Hox12*-deficient animals. When we observed differentiation of the posterior end of the intestine with a GFP-marker line of the region (R4 in Yoshida and Sasakura (2012)), the GFP marker could be observed in *Hox12*-deficient animals (100%, n=18; Fig. 2F and G), suggesting that expansion of the intestine was not caused by the failure in the differentiation of the posterior end of the intestine. Moreover, we observed whether an anterior structure of the gut, namely the stomach, was expanded in *Hox12*-knockout juveniles. Using a transgenic line expressing GFP in the stomach (Yoshida and Sasakura, 2012), we showed that the stomach region was not expanded in *Hox12*-knockout juveniles (0%, n=24; Fig. 2H and I). Taken together, we concluded that the intestinal deficiency seen in *Hox12*-knockout animals is not due to the mis-specification of the regions of the digestive tube.

As mentioned above, *Hox12* is expressed at the posterior part of the tail epidermis at the tailbud stage (Ikuta et al., 2004). In order to investigate whether the function of *Hox12* at the embryonic epidermis

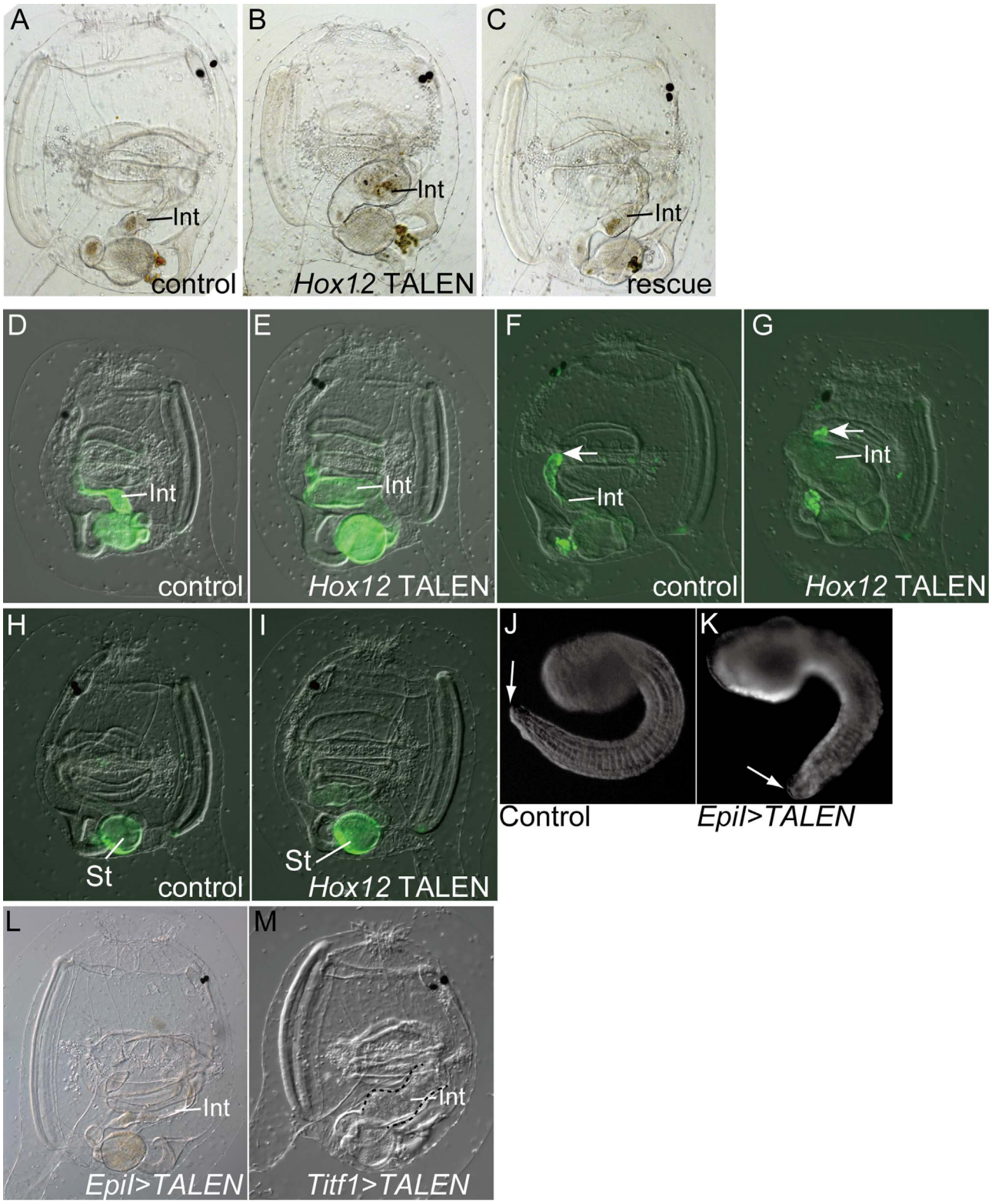

Fig. 2. *Hox12* is necessary for proper intestine formation. (A) An uninjected control juvenile. Int, intestine. (B) A juvenile developed from an egg introduced with the *Hox12* TALEN mRNA. Its intestine was expanded. (C) A juvenile developed from an egg introduced with the *Hox12* TALEN and *Hox12* mRNAs. Its intestine was not expanded. (D) A juvenile of the transgenic line Tg[MiCiTSAdTPOG]101 expressing GFP in the stomach and region R3 of the intestine. The green pseudocolor indicates GFP expression. (E) A Tg[MiCiTSAdTPOG]101 juvenile introduced with *Hox12* TALEN expression vector driving TALEN with *Titf cis* element. (F) A juvenile of the transgenic line Tg[MiCiTSAdTPOG]87 expressing GFP in the region R4 of the intestine. The arrow indicates the posterior end of the intestine. (G) A Tg[MiCiTSAdTPOG]87 juvenile introduced with *Titf1* > *Hox12* TALEN expression vector. Its posterior end emits green fluorescence, indicating the occurrence of R4 specification. (H) A juvenile of the transgenic line Tg[MiCiTSAdTPOG]75 expressing GFP in the stomach (St). (I) A Tg[MiCiTSAdTPOG]75 juvenile introduced with *Titf1* > *Hox12* TALEN expression vector. No ectopic expression of GFP is seen. The faint green pseudocolor in the intestine is background signal derived from autofluorescence of digested food. (J) A control tailbud embryo. The arrow indicates the taper of the tail. (K) A tailbud embryo introduced with a pair of *EpiI* > *Hox12* TALEN expression vectors by electroporation. The photograph is the merged image of the mCherry fluorescence and differential image contrast images. Its tail taper is somewhat rounded. (L) A juvenile introduced with a pair of *EpiI* > *Hox12* TALEN expression vectors by electroporation at the 1-cell stage. Its intestine was not expanded. (M) A juvenile introduced with a pair of *Titf1* > *Hox12* TALEN expression vectors by electroporation at the 1-cell stage. Its intestine was expanded (dotted lines).

is responsible for the "posterior" structure of the intestine after metamorphosis, we carried out tissue-specific knockout of *Hox12*. When *Hox12* TALENs were expressed specifically in the epidermis with the *cis* element of *EpiI*, a maker gene for the epidermis, the animals showed the rounded tail phenotype (Fig. 2J and K), as previous studies described (Ikuta et al., 2010; Treen et al., 2014), while no effect was seen in the intestine (0%, n=7; Fig. 2L). By contrast, the expanded intestine phenotype was observed when *Hox12* TALENs were expressed with the endodermal *cis* element of *Titf1* (Ristratore et al., 1999), a transcription factor gene expressed majorly in the endoderm and in a few neural cells (78%, n=47; Fig. 2M; Kawai et al., 2015). These results suggest that the expanded intestine phenotype is independent of the function of *Hox12* in the epidermis, and *Hox12* is necessary in the endoderm for its proper formation.

3.3. Enhanced generation of Hox12 mutants by PGC regeneration

A problem with TALEN-mediated mutagenesis is the difficulty to obtain mutated germ cells if the gene has an essential role during development, simply because we cannot culture TALEN-introduced animals until the reproductive stage due to lethal phenotypes. This issue is true not only of *Ciona* but also true of various animals in which a controllable expression method of genome editing is unavailable (Blitz et al., 2016). We addressed this issue with *Hox12* TALENs. A simple countermeasure is to reduce the mutation efficiency so as to decrease the chance to mutate at most one of the two *Hox12* copies in cells, because heterozygous mutants of genes are generally viable. In order to achieve this, we reduced the quantity of TALEN mRNA introduced into an egg. When one tenth the conventional amount of *Hox12* TALEN mRNAs than the conventional method was introduced into *Ciona* (10 ng/μl vs 100 ng/μl each of TALEN mRNAs in the injection media), many animals seemed healthy at the time when almost all of *Hox12*-mutated animals failed to survive in the conventional method (Table 3).

We collected sperm from animals introduced with 1/10 *Hox12* TALEN mRNAs, and observed mutation frequency of *Hox12* in their sperm. 44.4% (n=9) animals had mutated *Hox12* in the sperm genome (non tail-cut in Table 4), but the mutation frequencies were not high (12.7% in average) compared to the results of *Hox5* knockout (50.5% in average when mutation-positive animals are scored). We investigated the inheritance of the mutated *Hox12* to the next generation. A founder animal inherited mutated *Hox12*, but the rate was low, as expected from mutation frequency in the sperm (Table 4, non tail-cut-8). These results suggest that reducing the quantity of TALEN mRNA would be a good approach for producing a mutant line of a gene with an essential role during development, but the low mutation rate requires laborious screening.

To overcome this issue, we applied the tail cut approach to *Hox12* mutagenesis. TC animals exhibited a higher and more consistent mutation frequency of *Hox12* in their sperm genome compared with tail un-cut animals (Fig. 3A and B; Table 4). The tail cut method also achieved high rate of inheritance of mutated *Hox12* to the next generation (Fig. 3C and Table 4). We obtained homozygous mutants of *Hox12* by crossing two heterozygous *Hox12* mutant carriers. The homozygous mutants exhibited expansion of their intestines (Fig. 3D–F). These results first suggest that the tail cut method significantly enhances mutation frequency in germ cells, and second that we can efficiently establish a mutant line for a gene essential for development through TALEN-mediated mutagenesis coupled with the tail cut operation.

3.4. Germ cell mutagenesis by electroporation

As mentioned in the introduction section, germ cell mutagenesis of *Ciona* cannot currently be done by electroporation of a TALEN expression vector. Because electroporation is much easier than microinjection, electroporation-mediated germ cell mutagenesis would be advantageous over microinjection-mediated methods. The difficulty of germ cell mutagenesis via electroporation is due to the lack of a *cis* element that can effectively promote gene expression in mPGCs. By contrast, we could express genes in somatic cells with various *cis* elements in *Ciona* (Corbo et al., 1997). Our tail cut method uses transdifferentiation of somatic cells into germ cells, suggesting that we could express TALENs with a *cis* element for a somatic tissue by electroporation and then could convert the mutated somatic cells into rPGCs via tail cut.

In order to address the above strategy, we expressed TALENs that target *Hox4* with some tissue-specific *cis* elements. We chose *Hox4* TALENs because disruption of *Hox4* does not cause an opposing effect on development (Yoshida et al., 2014), and because we successfully converted *Hox4* TALENs into the compatible version for our tissue-specific expression system (modified version of Treen et al. (2014); Fig. 4A), while we failed the conversion for *Hox5* TALENs. We used the *cis* elements of *AKR*, *CesA*, *Nut* and *TnI* that respectively express genes in the mesenchyme, epidermis, neural tissues and muscle from the embryonic stage (Fig. 4B–E; Davidson and Levine, 2003; Hozumi et al., 2010; Sasakura et al., 2010). When TALEN vector-electroporated animals developed into the larvae, the animals were subjected to tail cut in order to induce conversion of somatic cells into rPGCs. The animals were further cultured until the reproductive stage, and their sperm genome were analyzed to see mutations in *Hox4*.

Hox4 mutations were detected in the sperm genomes when *Hox4* TALENs were expressed in the epidermis, neural tissue and muscle (Fig. 4F and Table 5). The presence of mutations in the sperm genome was confirmed by the inheritance of mutated *Hox4* to the progeny (Fig. 4G). These results suggest that *CesA*, *Nut* and *TnI* promoters can be used for mutating germ cells as well as somatic cells. In order to see the frequency of mutated sperm, we examined the frequency of *Hox4* mutant carriers in the progeny obtained by the cross of a wild type and sperm of a *Hox4* TALEN electroporated TC animal. *TnI* showed the best results among these promoters and *Nut* promoter exhibited a comparable result to that of *TnI* promoter (Table 5), suggesting that

Table 3
Survival rate of *Hox12*-TALEN injected juveniles.

Trial	Quantity of TALENs	% Survival						
		0 week[a]	1 week	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks
Exp. 1	Uninjected	100 (n=31)	90.3	83.8	67.7	67.7	NE[b]	NE
	100 ng/μl	100 (n=90)	76.6	21.1	2.2	0		
	10 ng/μl	100 (n=85)	100	43.5	15.2	10.5	NE	NE
Exp. 2	Uninjected	100 (n=16)	93.5	93.5	93.5	87.5	87.5	87.5
	100 ng/μl	100 (n=54)	75.9	48.1	27.7	20.3	12.9	1.8
	10 ng/μl	100 (n=81)	88.8	81.4	67.9	59.2	56.7	55.5

[a] The day when animals completed metamorphosis (4 days after fertilization).
[b] Not examined.

Table 4
Mutation of *Hox12* in the sperm genome by the tail cut method.

Animal ID	Mutation frequency (%)[a]	Number of sequenced clones	Number of mutation patterns	Frequency of progeny with *Hox12* mutation[b]	Number of examined progeny
Non tail-cut-1	Mutation not indicated by the surveyor assay	NE[c]	NE	NE	
Non tail-cut-2	6.3	16	1	NE	
Non tail-cut-3	6.3	16	1	0	15
Non tail-cut-4	Mutation not indicated by the surveyor assay	NE	NE	NE	
Non tail-cut-5	Mutation not indicated by the surveyor assay	NE	NE	NE	
Non tail-cut-6	Mutation not indicated by the surveyor assay	NE	NE	NE	
Non tail-cut-7	Mutation not indicated by the surveyor assay	NE	NE	NE	
Non tail-cut-8	13.3	15	2	13.3	48
Non tail-cut-9	25	12	1	NE	
TC1	83.3	12	2	81.25	16
TC2	20	10	2	6.66	15
TC3	100	15	4	43.75	16
TC4	6.6	15	1	12.5	16
TC5	81.25	16	6	56.25	16
TC6	20	15	1	7.14	14
TC7	12	25	1	4.16	24
TC8	68	25	1	81.2	16
TC9	44.4	9	1	NE	

[a] Mutation frequencies were analyzed when the presence of mutation was suggested by the surveyor assay.
[b] Progeny derived from sperm of TALEN-introduced animals and wild type eggs.
[c] NE, not examined.

these promoters could be primarily selected for routine germ cell mutagenesis if target genes do not have a significant function in one of the two tissues. *CesA* promoter could be an alternative choice when targeting genes that may have an important role in both muscle and neural tissue.

Intact *Ciona* are thought to generate their mPGCs by accumulating a series of maternal mRNAs to the posterior blastomeres through the relocation of the egg cytoplasm after fertilization (Fujimura and Takamura, 2000; Shirae-Kurabayashi et al., 2006; Prodon et al., 2007). Tail cut experiments suggest *Ciona* can produce PGCs without accumulation of maternal RNAs. We are interested in how *Ciona* uses two different pathways for PGC formation. Particularly, it is intriguing whether *Ciona* uses the pathway of somatic-to-germ line conversions even if mPGC remains, or the pathway is only triggered by the loss of mPGC through our artificial operation. To address this question, we investigated whether PGC generation from somatic cells occur in *Ciona* whose primary PGCs remained intact. We examined the presence of sperm mutations in tail un-cut animals whose somatic cells were mutated with the *Hox4* TALEN pair. Among seven animals into which *Nut > Hox4* TALEN vector was electroporated, four had sperm mutated with *Hox4* even though the animals' mPGCs were not removed by tail cutting (Fig. 4H and Table 6). Likewise, five among seven animals into which *TnI > Hox4* TALEN vector electroporated and tail remaining animals generated germ cells whose *Hox4* was mutated, suggesting that these TALEN expression vectors can introduce mutations in germ lines without tail cutting. The tail cut operation is however desirable to obtain animals with good mutation rate of a target gene in germ lines, since the progeny of TC animals exhibited significantly higher rate of possessing mutated *Hox4* than those of tail un-cut animals (Fig. 4I, $p < 0.0006$).

The results of PGC-remaining animals raised a possibility that the mechanism mutating *Hox4* in the germ line with TALEN expression vectors might be through expression of TALENs in the mPGCs by these vectors rather than through PGC regeneration from mutated somatic cells. We provide supporting evidence that opposes the possibility as follows. We could not observe mCherry expression in the larval PGCs and young gonads of animals into which *Nut > Hox4-TALEN::2A::mCherry* vector was electroporated (n=30; Fig. 5A–C), suggesting that this promoter does not have the activity to induce gene expression at earlier stage of PGC specification (we did not investigate the *TnI* promoter because its expression in the larval tail muscle made it difficult to examine expression in the PGCs in the tail). Our previous study suggested that plasmid DNAs introduced into *Ciona* in a transient fashion rarely remain at later developmental stage (Sasakura, 2007), suggesting that TALEN expression vectors need to mutate gene at relatively earlier developmental stage before the vectors are lost. This could oppose the fact that *Nut* promoter does not express TALENs at the early stage of PGC formation. Moreover, transgenic lines suggest that *TnI* promoter does not have the activity to express genes in germ lines after metamorphosis (Fig. 5D). The *Nut* promoter, by contrast, has the activity to express genes in the maternal fashion (Iitsuka et al., 2015). This is in accordance with maternal expression of *Ci-Nut* mRNA (Etani and Nishikata, 2002). However, observation of *Nut > gfp* and *Nut > kaede* transgenic lines showed that the *Nut* promoter does not have the activity to express genes in the sperm and testis (Fig. 5E), suggesting that the activity of *Nut* promoter could not explain the presence of mutations in sperm.

The use of tissue-specific expression vectors could be advantageous to establish mutant lines of genes essential for development, because we can mutate the genes in tissues in which the genes are not expressed or do not have a pivotal role in order to obtain healthy animals that could be viable until germ cell maturation. We examined the feasibility of the method by establishing mutant lines of *Hox12*. Using this method progeny inheriting mutated *Hox12* from their founders generated by introducing *Nut > Hox12* TALEN vectors followed by tail cutting (Fig. 6A and B). The progeny possesses deletions of 1–97 bases of nucleotides from the *Hox12* exon encoding the homeodomain (Fig. 6C), suggesting that the mutated *Hox12* alleles could not produce functional *Hox12* proteins due to frame-shift mutations in this essential domain. This data eliminated the possibility that tissue-specific expression vector mediated germ cell mutagenesis method could generate only weak or neutral mutations in genes essential for development, strengthening the feasibility of the use of TALEN expression vectors for germ cell mutagenesis.

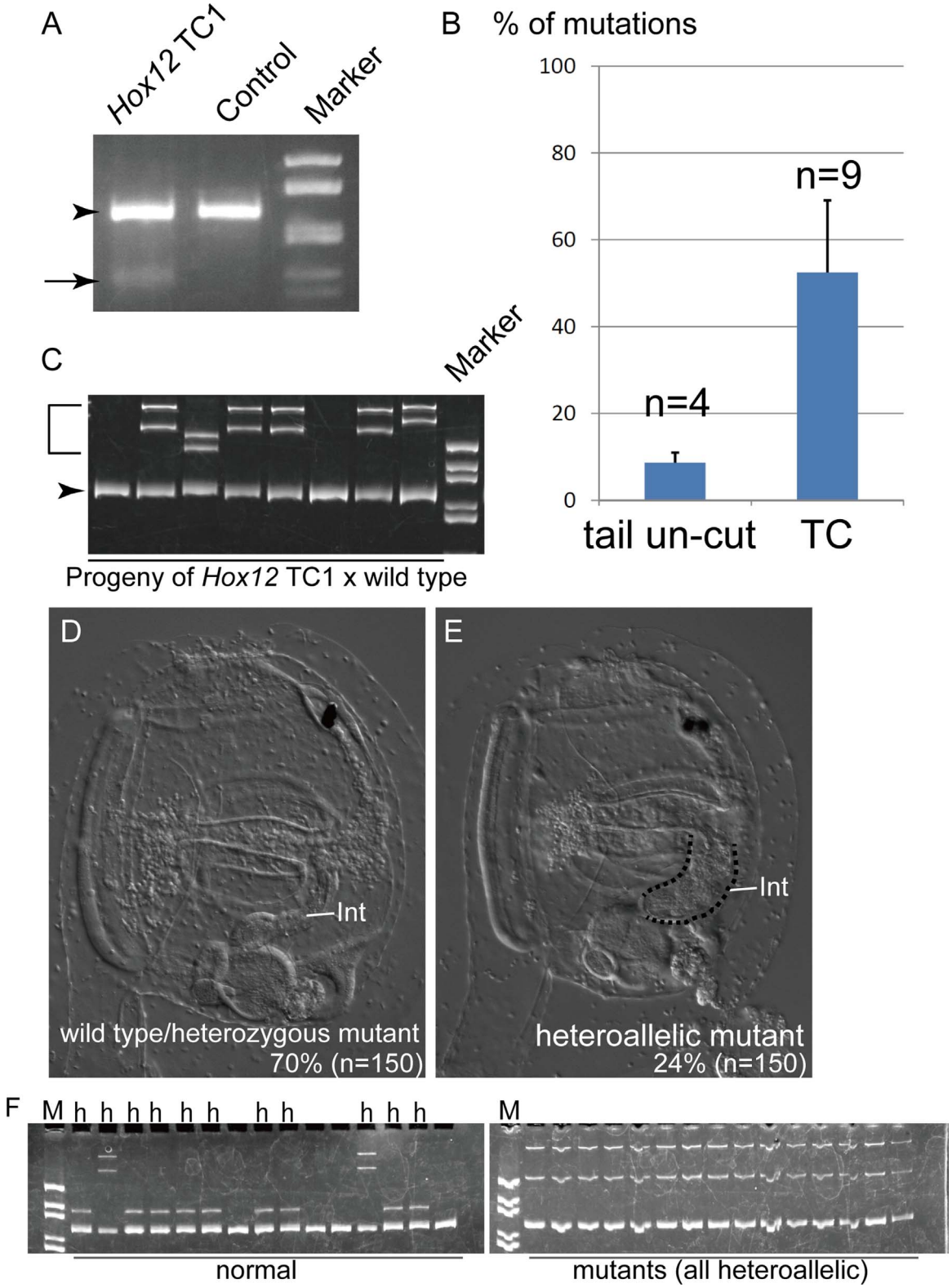

Fig. 3. Enhanced germ cell mutation of *Hox12* with TALENs and PGC removal. (A) Surveyor nuclease assay of *Hox12* amplicons. The left-side lane is the result of sperm genome from an animal into which reduced amount of *Hox12* mRNA was injected at 1-cell stage followed by PGC removal (TC1 in Table 4). The positions of the Cel-I un-digested band and digested band are respectively shown by an arrowhead and arrow. The middle lane is the result of sperm of an un-injected control animal. (B) Comparison of mutation frequencies of *Hox12* in sperm from PGC remaining (tail un-cut) and removed (TC) animals. Their significance of the scores were supported by the Student's *t*-test comparing each other ($p < 0.02$). Bars represent standard errors. N indicates the number of examined animals. (C) *Hox12* mutations detected in progeny generated by the cross of *Hox12* TC1 (sperm) and a wild type (egg) animals. The PCR bands were analyzed by heteroduplex mobility shift assays. Each lane indicates the result of a single juvenile. The presence of the shifted bands (parenthesis) indicate the formation of heteroduplexes with mismatched nucleotides, indicating the presence of mutations. The arrowhead indicates the position of PCR bands without mismatches. The animals in lanes 2–5 and 7, 8 possess mutated *Hox12*. (D-F) Analyses of the juveniles generated by the cross of two *Hox12* heterozygous mutants. (D) A wild type or heterozygous *Hox12* mutant juvenile whose intestine is not expanded. (E) A homozygous *Hox12* mutant exhibiting expanded intestine (dotted lines). (F) Genotyping of the juveniles. Left and right panel respectively illustrate the results of normal and intestine-expanded juveniles. In the left electrophoresis, the band patterns indicate that animals were heterozygous mutants (their parents have different *Hox12* mutations) or homozygotes of normal *Hox12* alleles that do not exhibit shifted bands. "h" indicates the lanes of heterozygous animals. In the right electrophoresis, all animals exhibit the same band pattern that is not seen in the left gel, suggesting that they are homozygous (strictly speaking heteroallelic combined) mutants.

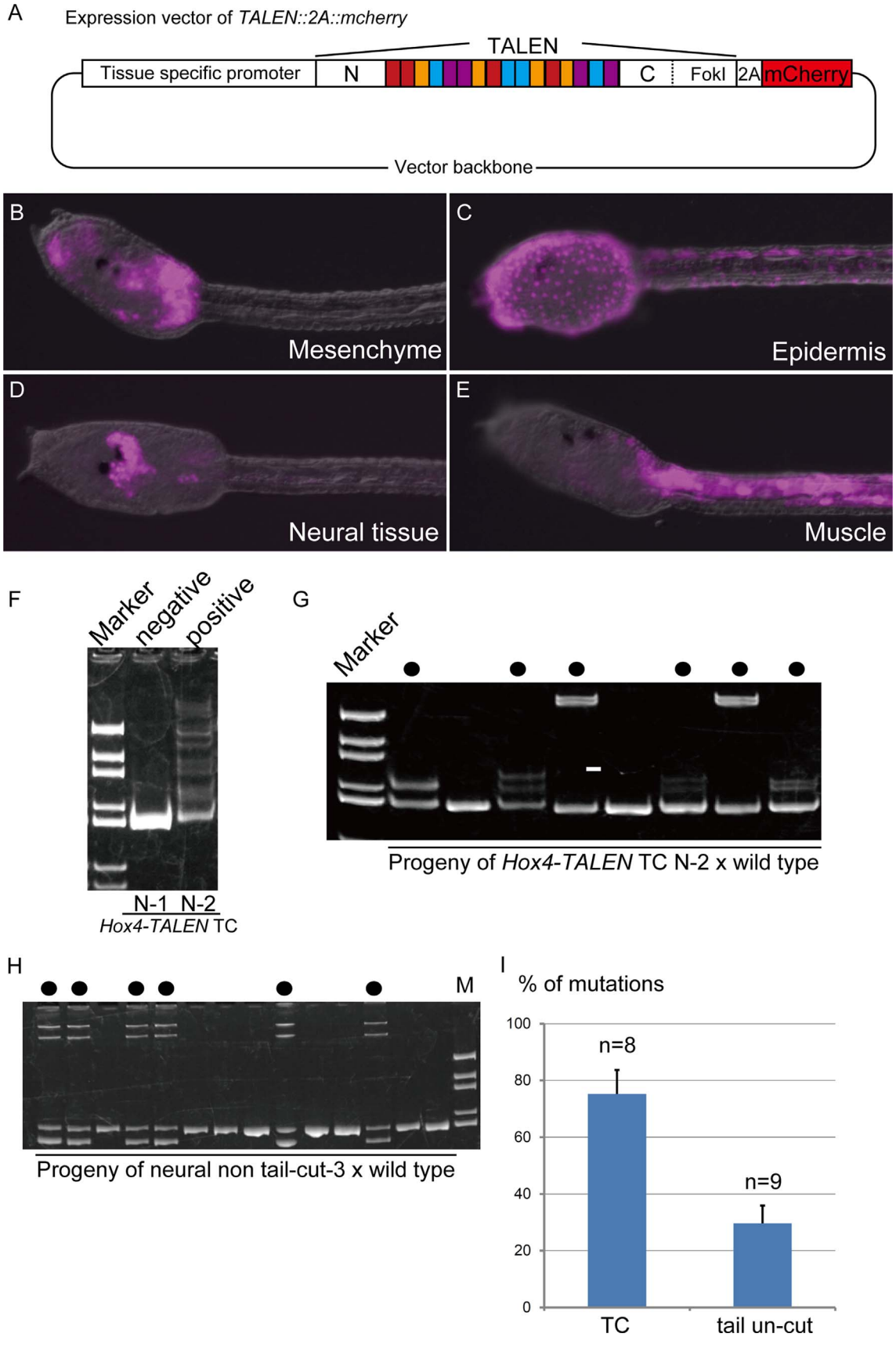
A
Expression vector of TALEN::2A::mcherry
TALEN
Tissue specific promoter
N
C
FokI
2A
mCherry
Vector backbone
B
Mesenchyme
C
Epidermis
D
Neural tissue
E
Muscle
F
Marker
negative
positive
N-1 N-2
Hox4-TALEN TC
G
Marker
Progeny of Hox4-TALEN TC N-2 x wild type
H
M
Progeny of neural non tail-cut-3 x wild type
I
% of mutations
100
80
60
40
20
0
n=8
n=9
TC
tail un-cut

Fig. 4. Tissue-specific expression vector yields mutated germ cells. (A) The design of an improved tissue-specific TALEN expression vector. (B) mCherry fluorescence in the mesenchyme (pseudocolored in magenta) derived from *AKR > Hox4* TALEN vector. (C) mCherry fluorescence in the epidermis derived from *EpiI > Hox4* TALEN. (D) mCherry fluorescence in the neural tissue derived from *Nut > Hox4* TALEN. (E) mCherry fluorescence in the muscle derived from *TnI > Hox4* TALEN. (F) Surveyor nuclease assay of *Hox4* amplicons. The result of sperm genome from two animals into which *Nut > Hox4* TALEN vector was electroporated at 1-cell stage followed by PGC removal (N-1 and N-2 in Table 5). The position of Cel-I endonuclease-digested band is shown by an arrow. The middle lane is the result of the animal whose sperm did not possess mutated *Hox4*, and the right lane is the result of the animal whose sperm possessed mutated *Hox4*. (G) Inheritance of mutated *Hox4* to the next generation. The electroporation exhibited the results of eight progeny from sperm of N-2 and wild type eggs. The dots indicate juveniles that inherited mutated *Hox4* from N-2. (H) Progeny of a *Nut > Hox4* TALEN electroporated that did not experience PGC removal (neural non tail-cut-3 in Table 6) inherited mutated *Hox4*. (I) Comparison of mutation frequencies of *Hox4* in sperm from PGC removed (TC) and remaining (tail un-cut) animals. The graphs include the results of *Nut > Hox4* TALEN and *TnI > Hox4* TALEN expression vectors. The significance of these scores was supported by a Student's *t*-test comparing each other ($p < 0.0004$). Bars represent standard errors. N indicates the number of examined animals.

Table 5
Knockout of *Hox4* in sperm genome with tissue-specific TALEN expression vectors.

Tissue	Animal ID	Mutation frequency (%)	Examined number[a]	Number of mutation patterns[b]	Note
Mesenchyme	A-1	0	(11)		Sperm negative[c]
	A-2	0	45		
Epidermis	C-1	0	(11)		Sperm negative
	C-2	25	20	5	
	C-3	0	44		
	C-4	0	41		
	C-5	80	45	3	
	C-6	40	45	1	
Neural	N-1	0	(12)		Sperm negative
	N-2	56.2	16	3	
	N-3	100	23	3	
	N-5	0	(11)		Sperm negative
	N-6	57.1	14	1	
	N-7	50	14	2	
Muscle	T-1	100	16	2	
	T-2	50	16	2	
	T-4	100	42	3	
	T-5	88.8	45	4	

[a] The numbers in parentheses indicate the number of sequenced PCR clones amplified from their sperm genomes. The numbers without parenthesis indicate the number of progeny examined by electrophoresis of PCR bands.
[b] Larger number of either sequence patterns of PCR clones or band patterns of electrophoresis are shown.
[c] We did not examine the mutation in their progeny because sperm of these animals was negative for mutated *Hox4*.

Table 6
Knockout of *Hox4* in sperm genome with tissue-specific TALEN expression vectors without tail cut.

Tissue	Animal ID	Mutation freq (%)	N[a]	Number of mutation patterns[b]
Neural	non tail-cut-1	0	44	
	non tail-cut-2	0	45	
	non tail-cut-3	48.8	43	1
	non tail-cut-4	0	45	
	non tail-cut-5	31.1	45	2
	non tail-cut-6	19.5	41	1
	non tail-cut-7	2.2	44	1
Muscle	non tail-cut-1	15.5	45	1
	non tail-cut-2	48.8	43	4
	non tail-cut-3	31.1	45	3
	non tail-cut-4	0	45	
	non tail-cut-5	0	22	
	non tail-cut-6	13.3	45	1
	non tail-cut-7	56.8	25	1

[a] The numbers indicate the number of progeny examined by electrophoresis of PCR bands.
[b] Number of band patterns exhibited by electrophoresis are shown.

3.5. Different mutations in sperm and eggs

The above experiments and our previous study showed that TALEN can induce mutations in the sperm genome (Yoshida et al., 2014), so far it has not been reported that TALENs can mutate the genome in ovaries. In order to examine this possibility, we analyzed genomes of the progeny derived from eggs of TALEN-introduced animals and wild type sperm.

We found that progeny derived from the eggs of TALEN-introduced TC animals and wild type sperm had mutations in the target genes (Fig. 7 and Table 7). By comparing the estimated mutation rate in the egg genome to those of the sperm genome of single animals (this experiment could be done because *Ciona* is hermaphroditic), we found that their rates were substantially different in three out of four cases (Table 7). Moreover, the mutations seen in the egg genome were different from those in the sperm genome in two cases (Fig. 7). These results suggest that sperm and eggs derived from rPGCs can have independent origins of their mother somatic cells, or that TALENs introduce mutations after the lineages of eggs and sperm were separated from the same PGCs. A previous study showed that regeneration of Vasa-positive PGCs occurs at least 9 days after settlement (Takamura et al., 2002), suggesting that it may take more than 9 days for the rPGCs to separate into egg and sperm lineages. It is unlikely that microinjected TALEN mRNA could be stable in animals more than 9 days and still induce efficient mutagenesis in sperm and egg progenitor cells. Because *Hox12* TALEN mRNA microinjections exhibited different mutations of sperm and eggs (Fig. 7A–C and Table 7), the former possibility is more feasible: primordial cells of sperm and eggs could be regenerated from different somatic cells.

4. Discussion

In the present study, we showed that somatic cells mutated by TALENs can be converted into mutated germ lines by the removal of maternal primordial germ cells (mPGCs) in the ascidian *Ciona intestinalis*. This PGC regeneration enables us to generate mutant lines of genes that are essential for development and for this reason it is difficult to establish mutant lines by conventional methods. Moreover, electroporation-mediated germ line mutagenesis can be achieved by the PGC regeneration: the method eases the performance of mutating germ cells that previously demanded microinjections. From these points of view, PGC regeneration approach facilitates germ cell mutagenesis in *Ciona*, and these kind of experiments can routinely be done in future studies for addressing gene functions by establishing mutant lines with TALEN expression vectors (Table 8).

4.1. Utilization of regeneration-mediated germ cell mutagenesis

Generally speaking, a serious obstacle for establishing mutant lines is that target genes with essential roles during development cause the death of knockout animals in the G0 generation before the reproductive stage. This study provides two countermeasures for this obstacle. First, reducing the amount of introduced TALENs can increase the surviving rate of animals. Reducing the amount of TALENs decreases the chance for their target gene to be mutated. This simple modification could be easily carried out in various organisms and for genes that have

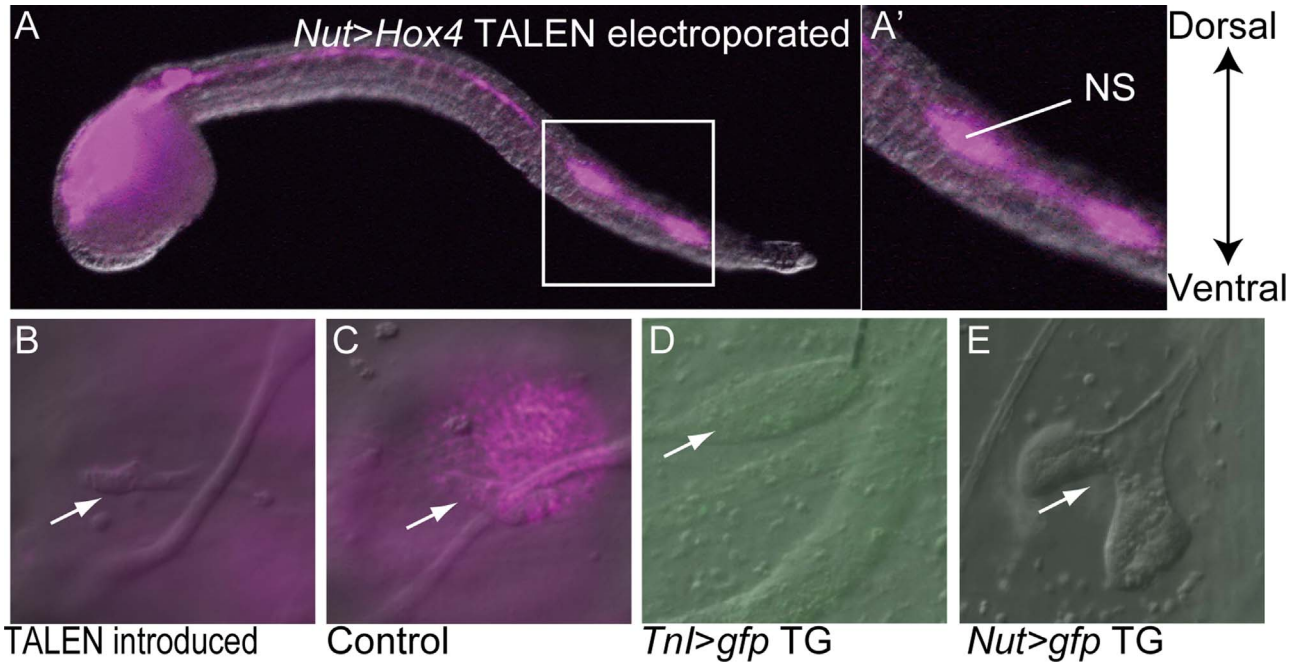

Fig. 5. *Nut* and *TnI cis* elements do not have the activity to express genes in PGC and male germ line. (A) A late tailbud embryo into which *Nut* > *Hox4* TALEN was electroporated at the 1-cell stage. Although mCherry expression was visible in the nervous system (NS) at the dorsal side, the fluorescence could not be seen in the ventral side of the tail (A′) where PGCs are located. (B) The developing gonad (arrow) of a juvenile into which *Nut* > *Hox4* TALEN was electroporated at the 1-cell stage. No mCherry fluorescence is seen. (C) The gonad of a control (*un*-electroporated) juvenile. The background magenta pseudocolor derives from autofluorescence. (D) The testis of an adult of *TnI* > *gfp* transgenic line (TG). No GFP fluorescence is seen. Very faint green pseudocolor is due to autofluorescence. (E) The testis of an adult of *Nut* > *gfp* transgenic line.

housekeeping functions. An inferiority of this method is the decreased mutation rate in germ cells; low mutation frequencies demand screening of multiple G1 animals for finding mutant carriers. Therefore, reduced TALEN introduction method is especially useful for organisms that can generate many progeny in laboratory conditions. The other countermeasure is using tissue-specific expression of TALENs coupled with PGC regeneration. We showed that several somatic tissues can reproduce germ cells in *Ciona*, indicating that in order to achieve germ cell mutagenesis, we can select a *cis* element that expresses TALENs in a tissue where their target gene does not have an essential role for development. Among the *cis* elements we tested in this study, we recommend the use of *TnI* and *Nut* promoters as we find they have the most reproducible ability to mutate germ cells. In both countermeasures, the PGC regeneration approach enhanced their usability by increased mutation rates in germ cells. The regeneration-mediated system could be applied to organisms that have a regeneration system similar to *Ciona*. Moreover, the principle of this method, using regeneration for accumulating mutated cells, could be used in any cell population that has a regeneration capacity, by inducing regeneration from mutated cells after removal of un-mutated cells.

The other application of the regeneration-mediated method is the quick isolation of germ cell populations with high mutation rates as early as the G0 generation (the generation introduced with TALENs). In the conventional genetic screenings, we first need to establish G1

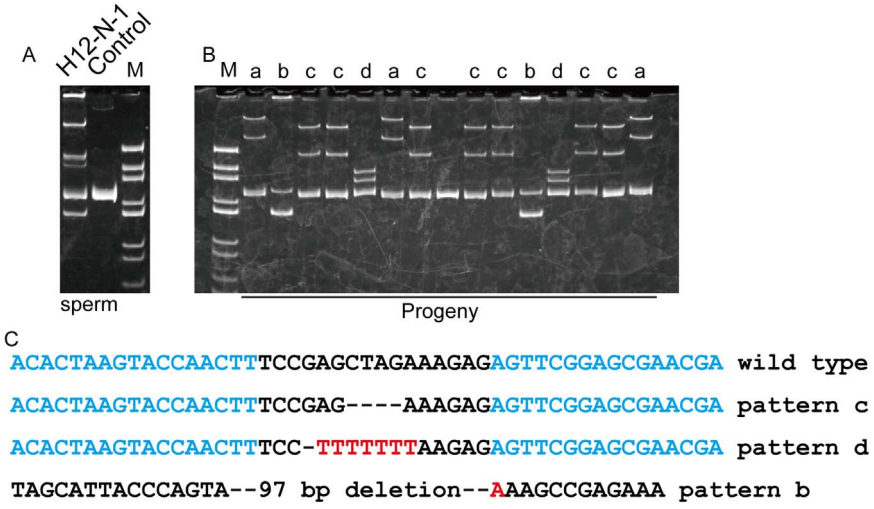

Fig. 6. *Hox12*-mutated germ cells can be generated by tissue-specific expression vector of TALENs coupled with mPGC regeneration. (A) The result sperm genomic DNA isolated from two animals into which *Nut* > *Hox12* TALEN (H12-N-1, left) or a control vector (Control, middle) were electroporated at 1-cell stage followed by PGC removal. Sperm of H12-N-1 possessed mutated *Hox12*, as shown by the presence of shifted bands. M, size marker. (B) Progeny from sperm of H12-N-1 and eggs of a wild type inherited mutated sperm. All but one progeny exhibited shifted bands, suggesting that they are heterozygous mutants. The lanes with shifted bands are labeled with lower characters that correspond to the mutations shown in (C). (C) Sequencing of mutated *Hox12* in progeny of H12-N-1. The left and right TALEN binding sites were shown in blue. A deleted nucleotide was shown by a bar. The nucleotides that are not found in the wild type allele are shown in red. Pattern b had a long deletion that deleted all of the binding sites of *Hox12* TALENs.

Fig. 7. Sperm and eggs from an TALEN-introduced and mPGC removed animals exhibit different mutation patterns. (A) The heteroduplex mobility shift assay of the progeny of *Hox12* TALEN TC-8 in Tables 4 and 7. The left four lanes indicate the results of juveniles from eggs of *Hox12* TALEN TC-8 and wild type sperm, and the right 11 lanes indicate the results of juveniles from sperm of *Hox12* TALEN TC-8 and wild type eggs. M, size marker. The positions of the shifted bands are different, suggesting that *Hox12* has different mutations between sperm and eggs. (B) Sequencing of the PCR fragments confirmed the presence of different mutations in progeny derived from sperm and eggs of *Hox12* TALEN TC-8. The left and right TALEN binding sites were shown in blue. The positions of deleted nucleotides are shown by a bar. The inserted nucleotides are shown in red. (C) Sequencing of the PCR fragments confirmed the presence of different mutations in progeny derived from sperm and eggs of *Hox12* TALEN TC-7. The left and right TALEN binding sites were shown in blue. The positions of deleted nucleotides are shown by a bar. Because the number of juveniles with mutated *Hox12* was very small (see Table 7), we sequenced the target site of *Hox12* in sperm genome of TC-7 and confirmed that sperm has a different mutation pattern with that in eggs. (D) Sequencing of the PCR fragments confirmed the presence of the same mutation in progeny derived from sperm and eggs of *Hox4* TALEN N-6. The left and right TALEN binding sites were shown in blue. The positions of deleted nucleotides are shown by a bar.

heterozygous mutant carriers of genes, and then two G1 carriers are crossed to create homozygous mutants at G2 generation. The germ cells of the G2 homozygous mutants could be observed to see phenotypes. The crossing procedures can be skipped by the regeneration-mediated method in order to see phenotypes in germ cells. Besides the quickness to approach phenotypes of genes-of-interest, the advantage of G0 animals to see genetic functions in germ cells is unnecessary to consider dominant lethal effects that may cause sterility of germ cells. At the same time, examining phenotypes of G0 germ cells has a disadvantage that the mosaicism of mutations could weaken, or make it difficult to analyze, phenotypes. For reducing the effect of mosaicism, higher mutation frequency in the germ cell genome is desirable. The sperm from tail cut animals exhibit high mutation frequency compared to the TALEN-mediated sperm mutagenesis that does not use PGC regeneration (Yoshida et al., 2014 and this study). High mutation rates are beneficial for reducing complexity of identifying phenotypes.

Table 7
Comparison of mutation frequency in sperm and eggs.

Target gene	Animal ID	Mutation frequency[a] in sperm (N[b])	Number[c] of detected mutation patterns in sperm	Mutation frequency[a] in eggs (N[b])	Number[c] of detected mutation patterns in eggs	Method
Hox4	C-2	25% (20)	3	0% (24)	0	Expression vector electroporation
Hox4	N-6	57% (14)	1	62.5% (16)	1	Expression vector electroporation
Hox12	TC-7	4.16% (24)	1	75% (24)	1	mRNA injection
Hox12	TC-8	81.2% (16)	1	37.5% (16)	1	mRNA injection

[a] The scores were examined by genotyping of the progeny isolated from a cross with wild type animals.
[b] The numbers in parentheses indicate the number of examined juveniles.
[c] The scores are derived from the patterns of heteroduplex mobility shift assay. Therefore, the number could be different from the sequencing of PCR products shown in Tables 3 and 4.

Table 8
Official names of vectors and transgenic lines used in this study.

Abbreviated name or aim of experiment in the manuscript	Full name according to the nomenclature rule	Reference
Tg[MiCiTSAdTPOG]75	*Ciinte.* E[pMi-*Ciinte.* REG.KH2012.L3.178445-177583|*TPO* > *GFP*]75	Yoshida and Sasakura 2012
Tg[MiCiTSAdTPOG]87	*Ciinte.* E[pMi-*Ciinte.* REG.KH2012.L3.178445-177583|*TPO* > *GFP*]87	Yoshida and Sasakura 2012
Tg[MiCiTSAdTPOG]101	*Ciinte.* E[pMi-*Ciinte*.REG.KH2012.L3.178445-177583|*TPO* > *GFP*]101	Yoshida and Sasakura 2012
Tg[MiCiTnIG]2	*Ciinte*.Tg[pMi-*Ciinte*.REG.KH2012.C11.1684372-1685258|*TnI* > *GFP*]2	Joly et al. (2007)
Tg[MiCiTnIG]3	*Ciinte*.Tg[pMi-*Ciinte*.REG.KH2012.C11.1684372-1685258|*TnI* > *GFP*]3	Hozumi et al. (2010)
Tg[MiCiNutG]3	*Ciinte*.Tg[pMi-*Ciinte*.REG.KH2012.C14.1414843-1413824|*Nut* > *GFP*]3	Joly et al. (2007)
Tg[MiCiNutK]4	*Ciinte*.Tg[pMi-*Ciinte*.REG.KH2012.C14.1414843-1413824|*Nut* > *Kaede*]4	
Tg[MiCiNutK]8	*Ciinte*.Tg[pMi-*Ciinte*.REG.KH2012.C14.1414843-1413824|*Nut* > *Kaede*]8	
EF1α > TALEN::2A::mCherry[a]	p*Ciinte*.REG.KH2012.C14.789808-786328|*EF1α > TALEN::2A::mCherry*	
AKR > TALEN::2A::mCherry[a]	p*Ciinte*.REG.KH2012.C1.8707588-8706567|*AKR > TALEN::2A::mCherry*	
CesA > TALEN::2A::mCherry[a]	p*Ciinte*.REG.KH2012.C7.1308813-1306649|*CesA > TALEN::2A::mCherry*	
EpiI > TALEN::2A::mCherry[a]	p*Ciinte*.REG.KH2012.C1.9315249-9313208|*EpiI > TALEN::2A::mCherry*	
Nut > TALEN::2A::mCherry[a]	p*Ciinte*.REG.KH2012.C14.1414843-1413824|*Nut > TALEN::2A::mCherry*	
TnI > TALEN::2A::mCherry[a]	p*Ciinte*.REG.KH2012.C11.1684372-1685258|*TnI > TALEN::2A::mCherry*	
pBSCiEpiImCherryCiEpiICiHox12 TALEN L/R	p(*Ciinte*.REG.KH2012.C1.9315249-9313208|*EpiI > mCherry:Ciinte.* REG.KH2012. C1.9315249-9313208|*EpiI > Hox12 TALEN L/R)*	Treen et al. (2014)
pBSCiEpiImCherryCiTiTF1CiHox12 TALEN L/R	p(*Ciinte.* REG.KH2012. C1.9315249-9313208|*EpiI > mCherry:Ciinte.* REG.KH2012. C10.3638397-3636215|*Titf1 > Hox12 TALEN L/R)*	

[a] Arbitrary TALEN repeat can be inserted in the vectors.

4.2. The origin and nature of regenerated PGCs in Ciona

This study had two purposes. One was establishment of a method of high efficient germ-line mutagenesis, and the other was addressing mechanisms of *Ciona* PGC regeneration by genetic labeling of cells. When designing this research, we expected that we could determine the source somatic tissue of regenerated PGCs. However, our results suggest that germ cells could be regenerated from several tissues, suggesting that the source of the PGCs is not fixed to a specific tissue. This implies that many somatic cells of *Ciona* remain totipotent after differentiation that could achieve casual dedifferentiation of cells to be germ cells. This unexpected feature might be related to the high regeneration capacity of this organism (Dahlberg et al., 2010; Auger et al., 2010; Jeffery, 2015). Some ascidians exhibit colonial life style in which animals (called zooids) grow through a clonal manner (Oka and Watanabe, 1957; Kurn et al., 2011). These colonial ascidians possess very high regenerative capabilities that enable the production of an entire body from a small fraction of somatic cells (Voskoboynik et al., 2008; Rinkevich et al., 2013). In colonial ascidians, a kind of migrating cells are the precursors of regenerated germ cells (Sunanaga et al., 2006, 2010; Brown and Swalla, 2007; Brown et al., 2009; Kassmer et al., 2015). It is possible that these precursor cells originate from various somatic cells, utilizing a similar mechanism to *Ciona*. Colonial ascidians are thought to have been evolved from solitary ascidians (Zeng et al., 2006; Lemaire and Piette, 2015). It is possible that the high regeneration capacity in solitary ascidians might have functioned as the driving force for the evolution of colonial ascidians.

In the regeneration-mediated method, mutations in sperm and eggs can be different. As stated in the result section, this fact suggests different origins of sperm and egg primordial cells given by regeneration. The specification of ovary and testis of *Ciona* occurs at 11–12 days after metamorphosis (Okada and Yamamoto, 1999). The ovary and testis, during PGC regeneration, could accept independent somatic cells as germ cell precursors that could substitute for lost PGCs. It is not known how the differentiation of male and female germ cells is specified in the hermaphroditic body of ascidians, and our regeneration-mediated system would be valuable for investigating this mechanism by the genetic labeling and mutagenesis of PGCs and their precursor somatic cells.

We found that somatic cell mutagenesis can generate mutated germ cells even if the tail was not cut. In this case, the mutation frequency of germ cells decreased compared to mPGC removed animals. The lower mutation frequency is probably because the majority of the germ cells of tail un-cut animals may have derived from the maternal PGCs specified during embryogenesis and thus they are not mutated by TALENs, in addition to some incorporation of new PGCs that are originated from mutated or un-mutated somatic cells. The presence of mutated germ cells in non tail-cut animals whose somatic cells are mutated suggests that transdifferentiation of somatic cells into PGCs occurs even without artificial removal of maternal PGCs. This would somewhat contradict the fact that *Ciona* produces mPGCs by a specific mechanism through accumulated maternal mRNAs (Fujimura and Takamura, 2000; Prodon et al., 2007): the acquisition of this trait might not be necessary if *Ciona* could produce PGCs through somatic cells. Perhaps PGC regeneration is a countermeasure for *Ciona* to produce germ cells when the adults encounter an accident such as maternal PGCs are lost or could not produce sufficient amount of germ cell progenitors. *Ciona* may have developed the robust system of PGC regeneration to assure its reproduction.

Engineered nucleases have provided breakthroughs for biologists. With the aid of these tools, knockout of genes can be achieved in many organisms. Moreover, many applications of the nucleases have been reported to improve the currently available methods and establishing new approaches. Our study provides a new strategy to make use of regeneration for knockouts. Adopting this strategy, easy and efficient knockouts of specific cell types could be achieved in various organisms, and the innovation will enable us to address gene functions that are difficult with previous approaches.

4.3. Hox12 is essential for forming proper intestine

Although this is a side story of this paper, we showed that *Ciona Hox12* is necessary for proper formation of the intestine. Without *Hox12*, the intestine was expanded. By contrast, several markers suggest that specification of the intestinal regions are normal in *Hox12* mutants. Therefore, the defect observed in the *Hox12* mutants can be classified into a morphological defect rather than mis-specification of the digestive tube. *Hox* genes are famous in causing "homeotic" alternations of tissues when mutated. This may not be true of *Ciona Hox12*. Rather, the function of *Ciona Hox12* seems similar to the *Hox* genes whose targets are the realizator genes that would directly influence the morphology of segments in *Drosophila* (Hueber and

Lohmann, 2008). We are interested in this phenotype, since another posterior-class of *Hox* gene of *Ciona*, namely *Hox10*, also functions in the morphogenetic movement of the posterior endoderm but the gene is unnecessary for specifying its regional identity (Kawai et al., 2015). It is interesting whether the endodermal expressions of *Hox* genes (Ikuta et al., 2004; Nakayama et al., 2016) share the function responsible for morphogenesis in *Ciona*, and whether this feature is conserved among animals. Deepening the study of *Ciona Hox* genes will be beneficial to deduce the basal role(s) of *Hox* in the formation of multicellular animal bodies.

Acknowledgments

We thank Drs. Shigeki Fujiwara, Kunifumi Tagawa, Nobuo Yamaguchi and all members of the Department of Zoology, Kyoto University, the Misaki Marine Biological Station, the University of Tokyo, and the Maizuru Fishery Research Station of Kyoto University for the cultivation and provision of *Ciona* adults. This study was supported by Grants-in-Aid for the Scientific Research from JSPS and MEXT to YS (23681039, 15K14520, 16H04815). This study was further supported by grants from the National BioResource Project (NBRP).

References

Akanuma, T., Hori, S., Darras, S., Nishida, H., 2002. Notch signaling is involved in nervous system formation in ascidian embryos. Dev. Genes Evol. 212, 459–472.
Auger, H., Sasakura, Y., Joly, J.S., Jeffery, W.R., 2010. Regeneration of oral siphon pigment organs in the ascidian *Ciona intestinalis*. Dev. Biol. 339, 374–389.
Blitz, I.L., Fish, M.B., Cho, K.W., 2016. Leapfrogging: primordial germ cell transplanation permits recovery of CRISPR/Cas9-induced mutations in essential genes. Development 143, 2868–2875.
Brown, F.D., Swalla, B.J., 2007. Vasa expression in a colonial ascidian, *Botrylloides violaceus*. Evol. Dev. 9, 165–177.
Brown, F.D., Tiozzo, S., Roux, M.M., Ishizuka, K., Swalla, B.J., De Tomaso, A.W., 2009. Early lineage specification of long-lived germline precursors in the colonial ascidian *Botryllus schlosseri*. Development 136, 3485–3494.
Capecchi, M.R., 1989. Altering the genome by homologous recombination. Science 244, 1288–1292.
Corbo, J.C., Levine, M., Zeller, R.W., 1997. Characterization of a notochord-specific enhancer from the *Brachyury* promoter region of the ascidian, *Ciona intestinalis*. Development 124, 589–602.
Cermak, T., Doyle, E.L., Christian, M., Wang, L., Zhang, Y., Schmidt, C., Baller, J.A., Somia, N.V., Bogdanove, A.J., Voytas, D.F., 2011. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res. 39, e82.
Cuppen, E., Gort, E., Hazendonk, E., Mudde, J., van de Belt, J., Nijman, I.J., Guryev, V., Plasterk, R.H., 2007. Efficient target-selected mutagenesis in *Caenorhabditis elegans*: toward a knockout for every gene. Genome Res. 17, 649–658.
Dahlberg, C., Auger, H., Dupont, S., Sasakura, Y., Thorndyke, M., Joly, J.S., 2009. Refining the *Ciona intestinalis* model of central nervous system regeneration. PLoS One 4, e4458.
Davidson, B., Levine, M., 2003. Evolutionary origins of the vertebrate heart: specification of the cardiac lineage in *Ciona intestinalis*. Proc. Natl. Acad. Sci. USA 100, 11469–11473.
Dong, S., Lin, J., Held, N.L., Clem, R.J., Passarelli, A.L., Franz, A.W.E., 2015. Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, *Aedes aegypti*. PLoS One 10, e0122353.
Etani, K., Nishikata, T., 2002. Novel G-protein-coupled receptor gene expressed specifically in the entire neural tube of the ascidian *Ciona intestinalis*. Dev. Genes Evol. 212, 447–451.
Fujimura, M., Takamura, K., 2000. Characterization of an ascidian DEAD-box gene, *Ci-DEAD1*: specific expression in the germ cells and its mRNA localization in the posterior-most blastomeres in early embryos. Dev. Genes Evol. 210, 64–72.
Hikosaka, A., Kusakabe, T., Satoh, N., Makabe, K.W., 1992. Introduction and expression of recombinant genes in ascidian embryos. Dev. Growth Differ. 34, 627–634.
Hozumi, A., Kawai, N., Yoshida, R., Ogura, Y., Ohta, N., Satake, H., Satoh, N., Sasakura, Y., 2010. Efficient transposition of a single *Minos* transposon copy in the genome of the ascidian *Ciona intestinalis* with a transgenic line expressing transposase in eggs. Dev. Dyn. 239, 1076–1088.
Hueber, S.D., Lohmann, I., 2008. Shaping segments: *Hox* gene function in the genomic age. Bioessays 30, 965–979.
Iitsuka, T., Mita, K., Hozumi, A., Hamada, M., Satoh, N., Sasakura, Y., 2014. Transposon-mediated targeted and specific knockdown of maternally expressed transcripts in the ascidian *Ciona intestinalis*. Sci. Rep. 4, 5050.
Ikuta, T., Satoh, N., Saiga, H., 2010. Limited functions of *Hox* genes in the larval development of the ascidian *Ciona intestinalis*. Development 137, 1505–1513.
Ikuta, T., Yoshida, N., Satoh, N., Saiga, H., 2004. *Ciona intestinalis Hox* gene cluster: its dispersed structure and residual colinear expression in development. Proc. Natl. Acad. Sci. USA 101, 15118–15123.
Jeffery, W.R., 2015. Closing the wounds: one hundred and twenty five years of regenerative biology in the ascidian *Ciona intestinalis*. Genesis 53, 48–65.
Joly, J.S., Kano, S., Matsuoka, T., Auger, H., Hirayama, K., Satoh, N., Awazu, S., Legendre, L., Sasakura, Y., 2007. Culture of *Ciona intestinalis* in closed systems. Dev. Dyn. 236, 1832–1840.
Kassmer, S.H., Rodriguez, D., Langenbacher, A.D., Bui, C., De Tomaso, A.W., 2015. Migration of germline progenitor cells is directed by sphingosine-1 phosphate signaling in a basal chordate. Nat. Commun. 6, 8565.
Kawai, N., Ochiai, H., Sakuma, T., Yamada, L., Sawada, H., Yamamoto, T., Sasakura, Y., 2012. Efficient targeted mutagenesis of the chordate *Ciona intestinalis* genome with zinc-finger nucleases. Dev. Growth Differ. 54, 535–545.
Kawai, N., Ogura, Y., Ikuta, T., Saiga, H., Hamada, M., Sakuma, T., Yamamoto, T., Satoh, N., Sasakura, Y., 2015. *Hox10*-regulated endodermal strand cell migration is essential for development of the ascidian intestine. Dev. Biol. 403, 43–56.
Kitaura, Y., Sonobe, H., Tanaka, O., Honda, D., Watanabe, Y., Makabe, K.W., Takamura, K., Nishikata, T., 2007. Neural-tube specific paralogus genes and their upstream regulatory sequences. Mem. Konan Univ. Sci. & Eng. Ser. 54, 75–88.
Kurn, U., Rendulic, S., Tiozzo, S., Lauzon, R.J., 2011. Asexual propagation and regeneration in colonial ascidians. Biol. Bull. 221, 43–61.
Lemaire, P., 2009. Unfolding a chordate developmental program, one cell at a time: invariant cell lineages, short-range inductions and evolutionary plasticity in ascidians. Dev. Biol. 332, 48–60.
Lemaire, P., Piette, J., 2015. Tunicates: exploring the sea shores and roaming the open ocean. A tribute to Thomas Huxley. Open Biol. 5, 150053.
Matsuoka, T., Awazu, S., Shoguchi, E., Satoh, N., Sasakura, Y., 2005. Germline transgenesis of the ascidian *Ciona intestinalis* by electroporation. Genesis 41, 61–72.
Meng, X., Noyes, M.B., Zhu, L.J., Lawson, N.D., Wolfe, S.A., 2008. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. Nat. Biotechnol. 26, 695–701.
Nakayama, S., Satou, K., Orito, W., Ogasawara, M., 2016. Ordered expression pattern of *Hox* and *ParaHox* genes along the alimentary canal in the ascidian juvenile. Cell Tissue Res. 365, 65–75.
Nakazawa, K., Yamazawa, T., Moriyama, Y., Ogura, Y., Kawai, N., Sasakura, Y., Saiga, H., 2013. Formation of the digestive tract in *Ciona intestinalis* includes two distinct morphogenic processes between its anterior and posterior parts. Dev. Dyn. 242, 1172–1183.
Oka, K., Watanabe, H., 1957. Vascular budding, a new type of budding in *Botryllus*. Biol. Bull. 112, 225–240.
Okada, T., Yamamoto, M., 1999. Differentiation of the gonad rudiment into ovary and testis in the solitary ascidian, *Ciona intestinalis*. Dev. Growth Differ. 41, 759–768.
Ota, S., Hisano, Y., Muraki, M., Hoshijima, K., Dahlem, T.J., Grunwald, D.J., Okada, Y., Kawahara, A., 2013. Efficient identification of TALEN-mediated genome modifications using heteroduplex mobility assays. Genes Cells 18, 450–458.
Prodon, F., Yamada, L., Shirae-Kurabayashi, M., Nakamura, Y., Sasakura, Y., 2007. *Postplasmic/PEM* RNAs: a class of localized maternal mRNAs with multiple roles in cell polarity and development in ascidian embryos. Dev. Dyn. 236, 1698–1715.
Ren, X., et al., 2013. Optimized gene editing technology for *Drosophila melanogaster* using germ line-specific Cas9. Proc. Natl. Acad. Sci. USA 110, 19012–19017.
Rink, J.C., 2013. Stem cell systems and regeneration in planaria. Dev. Genes Evol. 223, 67–84.
Rinkevich, Y., et al., 2013. Repeated, long-term cycling of putative stem cells between niches in a basal chordate. Dev. Cell. 24, 76–88.
Ristratore, F., Spagnuolo, A., Aniello, F., Branno, M., Fabbrini, F., Di Lauro, R., 1999. Expression and functional analysis of *Cititf1*, an ascidian NK-2 class gene, suggest its role in endoderm development. Development 126, 5149–5159.
Sakuma, T., et al., 2013. Efficient TALEN construction and evaluation methods for human cell and animal applications. Genes Cells 18, 315–326.
Sasaki, H., Yoshida, K., Hozumi, A., Sasakura, Y., 2014. CRISPR/Cas9-mediated gene knockout in the ascidian *Ciona intestinalis*. Dev. Growth Differ. 56, 499–510.
Sasakura, Y., 2007. Germline transgenesis and insertional mutagenesis in the ascidian *Ciona intestinalis*. Dev. Dyn. 236, 1758–1767.
Sasakura, Y., Awazu, S., Chiba, S., Satoh, N., 2003. Germ-line transgenesis of the Tc1/*mariner* superfamily transposon *Minos* in *Ciona intestinalis*. Proc. Natl. Acad. Sci. USA 100, 7726–7730.
Sasakura, Y., Suzuki, M.M., Hozumi, A., Inaba, K., Satoh, N., 2010. Maternal factor-mediated epigenetic gene silencing in the ascidian *Ciona intestinalis*. Mol. Genet. Genom. 283, 99–110.
Satoh, N., 2003. The ascidian tadpole larva: comparative molecular development and genomics. Nat. Rev. Genet. 4, 285–295.
Sehring, I.M., Jahn, C., Weidinger, G., 2016. Zebrafish fin and heart: what's special about regeneration? Curr. Opin. Genet. Dev. 40, 48–56.
Shirae-Kurabayashi, M., Nishikata, T., Takamura, K., Tanaka, K.J., Nakamoto, C., Nakamura, A., 2006. Dynamic redistribution of vasa homolog and exclusion of somatic cell determinants during germ cell specification in *Ciona intestinalis*. Development 133, 2683–2693.
Stolfi, A., et al., 2015. Guideline for the nomenclature of genetic elements in tunicate genomes. Genesis 53, 1–14.
Stolfi, A., Gandhi, S., Salek, F., Christiaen, L., 2014. Tissue-specific genome editing in *Ciona* embryos by CRISPR/Cas9. Development 141, 4115–4120.
Sunanaga, T., Saito, Y., Kawamura, K., 2006. Postembryonic epigenesis of Vasa-positive germ cells from aggregated hemoblasts in the colonial ascidian, *Botryllus primigenus*. Dev. Growth Differ. 48, 87–100.
Sunanaga, T., Inubushi, H., Kawamura, K., 2010. Piwi-expressing hemoblasts serve as germline stem cells during postembryonic germ cell specification in colonial

ascidian, *Botryllus primigenus*. Dev. Growth Differ. 52, 603–614.
Szymczak, A.L., Workman, C.J., Wnag, Y., Vignali, K.M., Dilioglou, S., Vanin, E.F., Vignali, D.A.A., 2004. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide–based retroviral vector. Nat. Biotechnol. 22, 589–594.
Takamura, K., Fujimura, M., Yamaguchi, Y., 2002. Primordial germ cells originate from the endodermal strand cells in the ascidian *Ciona intestinalis*. Dev. Genes Evol. 212, 11–18.
Treen, N., Yoshida, K., Sakuma, T., Sasaki, H., Kawai, N., Yamamoto, T., 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.
Voskoboynik, A., et al., 2008. Identification of the endostyle as a stem cell niche in a colonial chordate. Cell Stem Cell 3, 456–464.
Wang, Z., Xing, H., Dong, L., Zhang, H., Han, C., Wang, X., Chen, Q., 2015. Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in *Arabidopsis* in a single generation. Genome Biol. 16, 144.
Wienholds, E., van Eeden, F., Kosters, M., Mudde, J., Plasterk, R.H., Cuppen, E., 2003. Efficient target-selected mutagenesis in zebrafish. Genome Res. 13, 2700–2707.
Yoshida, R., Sasakura, Y., 2012. Establishment of enhancer detection lines expressing GFP in the gut of the ascidian Ciona intestinalis. Zoolog. Sci. 29, 11–20.
Yoshida, K., Treen, N., Hozumi, A., Sakuma, T., Yamamoto, T., Sasakura, Y., 2014. Germ cell mutations of the ascidian *Ciona intestinalis* with TALE nucleases. Genesis 52, 431–439.
Zeng, L., Jacobs, M.W., Swalla, B.J., 2006. Coloniality has evolved once in Stolidobranch ascidians. Integr. Comp. Biol. 46, 255–268.