# Novel Approaches for Determining Gene Functions in Ascidian Development and Evolution

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Science

(Doctoral Program in Biological Sciences)

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**Table of Contents**

## 1. Abstract

It is well established that a limited set of transcription factors and signaling ligands have a central role in animal development. This occurs through the timing and location of the expression of these genes within the developing embryo and the interactions of those genes with their *cis*-regulatory targets. These interactions can be summarized as gene regulatory networks. In early ascidian development a lot is know about how the different embryonic territories are established. But the current understanding of these networks, and the technologies available to perturb them are starting to provide diminishing returns. I have created a predicative model based on the gene expression patterns and embryo morphology of urodele ascidians compared with anural ascidians. This model immediately suggests on unexpected role of the transcription factor ZicL in controlling the number of cell divisions in mesodermal cell lineages. A range of experimental manipulations was performed that suggest this model is correct and can provide a complete explanation for the evolution of anural ascidians. Many genes, such as *ZicL,* involved in early development are also involved in later developmental processes. The knockout of genes by established methods is only suitable to observe a gene's earliest function. To overcome this problem I have developed a novel form of gene targeting taking advantage of unique gene transformation techniques available for ascidians to create a novel conditional knockdown approach that makes later stages of ascidian development available for experimental investigations. This is expected to lead to a range of insights in the future to improve our understanding of gene functions in development and evolution.

## 2. General Introduction

"*The Ascidians, for instance, and the like so far resemble plants as they never live free and unattached, but, on the other hand, inasmuch as they have a certain flesh-like substance, they must be supposed to possess some degree of sensibility*"

The above quotation from *On the Parts of Animals* (Aristotle, ~350BC) marks the start of scientific studies on ascidians. As the quotation asserts ascidians are exclusively marine, sessile, filter-feeding animals with the most distinctive features being excurrent (atrial) and incurrent (oral) siphons. As well as a body covered in a thick tunic. There are 2874 described ascidian species (Appeltans et al., 2013). As well as appearing superficially plant-like, the ascidian tunic has the protective functions that along with the filter feeding sessile lifestyle could also lead to comparisons with bivalve molluscs (Cuvier, 1815). However the comparisons made from adult morphologies are illusory, observations of ascidian development clearly show that the embryo has a notochord and a dorsal neural tube (Kowalevsky, 1866; 1871). This means ascidians, along with the other tunicates: larvaceans and salps are chordates, members of the same superphylum that includes amphioxus and vertebrates (Satoh et al., 2014).

After fertilization all animals undergo cleavage into a 2, and 4-cell embryo. In one of the pioneering early experiments of embryology, Hans Driesch showed that for sea urchins, when individual blastomeres from the 2 or 4-cell stage were isolated from the whole animal a small individual sea urchin would faithfully develop, despite being deprived of up to three quarters of its embryonic material (Driesch 1891). Interestingly, despite Driesch getting full credit for this work in the canonical history of developmental biology, Ernst Haeckel had performed a similar experiment showing

the pluripotency of sipohonophore blastomeres over 20 years earlier (Haeckel 1869, Sanchez-Alvarado and Yamanaka, 2014) Driesch's work sits somewhat uncomfortably next to the findings on ascidians by his contemporary Laurent Chabry. Chabry showed that a 2-cell ascidian embryo, where 1 of the cells was ablated, develops into a half embryo. The individual cells of the 4-cell embryo were also shown to develop in a comparable manner (Chabry, 1887). The simple embryos of 2 marine invertebrates appeared to develop according to completely different principles. Supplemental work from other organisms, most notably amphibians, led to the establishment of the regulative and mosaic theories of development, where cells within a sea urchin are specified according to signals from the other cells, and where cells in an ascidian are specified according to unequal inheritance of cytoplasmic components. Edwin Conklin observed the mosaic development of ascidians first-hand (Conklin 1905). The eggs of *Styela* ascidians contain dense pigmented granules. Upon fertilization these pigments accumulate in the vegetal pole of the zygote and their fate can be tracked by simple observation. Using the optics available in the early 20$^{th}$ century Conklin followed the fate of this pigmented region to its eventual terminal state as muscle cells. This landmark study in developmental biology was revisited in the late 20$^{th}$ century using intracellular tracers to more accurately track the fate of cells (Nishida and Satoh., 1983; 1985; Nishida., 1987), and by the start of the 21$^{st}$ century the molecular nature of the muscle determinant that was speculated to exist within the segregated cytoplasm was identified (Nishida and Sawada., 2001). As developmental biology matured, the boundaries between regulative and mosaic development began to disappear, for example the sea urchin oral-aboral axis is specified by a redox gradient that is established by the unequal segregation of mitochondria (Coffman and Davidson., 2001). In ascidians numerous examples where signaling induces cell fate will be referenced within this thesis.

The presence of a notochord allows tunicates to be unambiguously classified as chordates. Other chordates include the vertebrates as well as the cephalochordates such as amphioxus. Hemichordates are deuterostomes that have morphologies that immediately suggest some relationship with the chordates, but hemichordates do not have a clear notochord. Throughout the 20$^{th}$ century many scholars attempted to explain the evolution of chordates by whatever comparative approaches have been fashionable for the time. With the advent of DNA sequencing, comparative studies could take advantage of unambiguous approaches to construct phylogenies. Wada and Satoh (1994) used 18S rDNA sequences to conclude that tunicates were basal chordates. But subsequent, more in-depth studies taking advantage of the massive quantities of data that genome sequencing has provided (Dehal et al., 2002; Delsuc et al., 2006; Putnal et al., 2008) reversed this and placed cephalochordates at the base of the chordate lineage with tunicates being the invertebrates that have the most recent common ancestry with vertebrates. Hemichordates are placed as a non-chordate out-group. Despite some sporadic dissent this account of chordate evolutionary history is currently the general consensus and I will consider it correct in this thesis, but as it has changed before I cannot exclude the possibility that it will change again.

A separate, but related argument to what is the phylogeny of chordates, is what extant non-vertebrate is the best representative of a primitive chordate. This argument can be quite sensitive as it is the justification of many individual's research programs and careers. I do not address this question in this thesis; in fact I have deliberately ignored it. But my work is based off the assumption that amphioxus most likely shares the closest body-plan to the ancestral chordate (Holland 2013). However in terms of gene expression patterns, hemichordates may be more similar similar to vertebrates (Pani et al., 2012). As for ascidians, of course there are

similarities between tunicate embryo body plans, and conserved gene expression patterns, between these organisms and vertebrates, but I feel that evolutionary developmental biology has been too concerned with finding comparisons between one organism and a vertebrate. There is little scientific advancement to be made from such comparisons currently. The work in this thesis is based on a concept of attempting to understand the fundamental mechanisms by which organisms work. In our current understanding of the natural world this is how information encoded in the genome can lead to the physical properties of organisms. Developmental biology attempts to answer these questions by establishing rules for an organism's development. This has led to an exceptional level of understanding of the mechanisms that govern how a small handful of anointed organisms. While there is undoubtedly deep conservation of genes, and mechanisms of development, every time an organism has evolved a novel trait, it has done so by breaking an established rule of the organism that preceded it. Organisms are complicated and there are many variables that must be taken into account if one wishes to investigate a mechanism. One way to overcome this problem is to look at how another organism has modified a process. Instead of using development to understand evolution, this way of thinking allows us to use evolution to understand development. This has been the way of thinking that I have attempted to use in my studies of ascidians in this thesis.

The decision to limit mechanistic studies of animal development was not done out of ignorance or laziness. It was essential to establish a robust set of experimental tools. Recent advancements, mostly in genome editing have the potential to unleash the past few decade's worth of advancements in research techniques upon whatever organism an experimenter chooses. It is my hope that these advancements will allow us to return to an attitude common to the authors of the earlier studies I have cited in this introduction, where a wide range of species are used to understand the

processes of life, but that this is done without sacrificing any of the experimental robustness that can be found in more recent work.

## 4. Gene Targeting in *Ciona* using TALENs

Portions of this chapter have been previously published as:
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.

### 4.1 Introduction

Gene targeting is well-established in mice (Capecchi, 1989), making possible a range of experimental approaches not possible in most other model organisms (Abzhanov et al., 2008). Custom designed nucleases can potentially allow gene targeting to become routine in a wide range of organisms (Porteus and Carroll, 2005). The mutational activity of zinc-finger nucleases (ZFNs) and Transcriptional activator-like effector nucleases (TALENs) has been shown (Meng et al., 2008; Ochiai et al., 2010; Miller et al., 2011; Carlson et al., 2012; Lei et al., 2012). TALENs in particular have potential to be embraced by researchers as they have short construction times and can be made with standard molecular cloning procedures (Cermak et al., 2011). Construction of TALENs can be achieved by making a DNA construct through multiple simultaneous ligations with unique DNA overhangs for each TAL repeat. When a TALEN induces a double-strand break (DSB) a mutation can occur when the cellular DNA repair mechanisms fail (Cermak et al., 2011). Recently a range of improvements has been made to the basic TALEN architecture to improve the mutational efficiency, mostly by reducing the length of the N and C terminal regions (Sakuma et al., 2013; Bedell et al., 2012).

Ascidian tunicates are attractive model organisms for developmental biology as they develop quickly and have a low number of cells in early embryonic development, making highly accurate lineage analysis possible (Kumano and Nishida, 2007). As the invertebrates that have the most recent common ancestry to vertebrates (Delsuc et al., 2006), experimental findings from tunicates can provide insights into developmental and evolutionary biology (Lemaire, 2011). The ascidian *Ciona intestinalis* is particularly well suited to genetic approaches, with experimental techniques including transposon transgenesis (Sasakura et al., 2003) and chemical mutagenesis (Chiba et al., 2009). The *Ciona* genome was sequenced in 2002 (Dehal et al., 2002), has been well annotated and a range of web-based tools are available. Established electroporation protocols allow the introduction of exogenous DNA or RNA that will be reliably expressed by thousands of synchronously developing embryos (Corbo et al., 1997). *Ciona* electroporations are useful to perform *cis*-regulatory analysis (for example: Roure et al., 2007; Johnson et al., 2004; Khoueiry et al., 2010; Imai et al., 2012). However a reliable method to knockout genes does not exist in this organism and most data on gene function is based on knockdowns by morpholino anti-sense oligos. Morpholinos can be very useful to knockdown mRNA translation early in development (Imai et al., 2006) but there are several limitations to injecting morpholinos and interpreting the experimental results (Eisen and Smith, 2008). Use of RNAi in *Ciona* is currently very limited. Kawai et al (2012) previously reported the possibility for gene targeting in *Ciona* by microinjecting ZFN mRNA to reliably knockout transgenes. But prior to the publication of results contained within this thesis there had been no reports describing the use of ZFNs or TALENs to disrupt endogenous genes in *Ciona* by electroporation or microinjection. Furthermore there have been no reports using ZFNs or TALENs to perform tissue specific knockouts in any organism. In this thesis I show that TALENs can be used

reliably in *Ciona* and that TALEN mutations can be introduced with a high frequency into embryos by electroporation and that those TALENs can be used to perform tissue-specific gene knockouts.

## 4.2 Materials and Methods

### 4.2.1 TALEN construction

TALE repeats were synthesized, cloned and assembled using Golden Gate cloning methods. The TALEN framework including N- and C-terminal domains of TALE and the FokI nuclease domain was taken from pTALEN_v2 vectors (Sanjana et al., 2012; Addgene, Massachusetts, USA). The full TALEN was ligated into a pBluescript based vector and promoters for TALEN expression were then inserted upstream of the TALENs by ligation into restriction sites. The upstream *cis*-regulatory region for the gene encoding prohormone convertase 2 (*Ci-PC2)* was amplified from wild *Ciona* genomic DNA using the oligonucleotide primers 5'-tggatccgTAACACCACGATATTAAAT-3' and 5'-tgcggccgcCATTCAAATAAAATGCTGCT-3' (restriction sites in lower case). The TALEN targeting sites were identified using TALE-NT software (Doyale et al., 2012; https://tale-nt.cac.cornell.edu/). *Ci-Epi1*>mCherry and Venus expression cassettes (Ogura et al., 2011) were ligated into a *Sac*I site upstream of the TALEN gene and promoter.

For the construction of TALENs incorporating a 2A peptide sequence between the TALEN and mCherry protein sequences, the 2A peptide sequence (GSGEGRGSLLTCGDVEENPGP, Szymczak et al., 2004) was inserted between the end of a TALEN open reading frame (without a stop codon) and the start of an mCherry open reading frame (without the starting methionine codon). This was done by performing PCR on the TALEN pBluescript destination vector using the primers:GAATTCCAGCTGAGCGCCGGTCG and TGAGCGGAAATTGATCTCGCCA. This PCR fragment was used as a vector to insert a 2A-mCherry fusion that was

made by PCR amplification of mCherry using the primers:

actgggtctctGTGAGCAAGGGCGAGGAGGATA

TTACTTGTACAGCTCGTCCATGCCG (restriction site in lower case), the PCR product was digested with BsaI and ligated to an adaptor sequence made by annealing the oligonucleotides:

GGATCAGGAGAAGGAAGAGGATCACTTCTTACATGTGGAGATGTTGAAGAAAAC

CCAGGACCA

and

tcacTGGTCCTGGGTTTTCTTCAACATCTCCACATGTAAGAAGTGATCCTCTTCCTT

CTCCTGATCC

(uncomplimentary site annealing to BsaI restriction site in lower case). The ligation was purified and the TALEN vector and the 2A-TALEN insert were fused together using the In-Fusion cloning kit (Takara, Tokyo Japan) according to the manufacturers instructions. After TALEN construction, promoters were inserted into a NotI sequence upstream of the open reading frame.

**4.2.2 Electroporation**

DNA electroporations were performed on dechorionated, fertilized eggs using standard procedures (Corbo et al., 1997). Electroporations were performed at 15-25 minutes after fertilization and 40 – 100 $\mu$g of DNA was electroporated in 800 $\mu$l of mannitol seawater. For experiments where TALEN mutations would be sequenced, eggs were fertilized with sperm from the same individual to minimize any potential polymorphisms and after electroporation the embryos were washed in filtered seawater 6 times to remove any traces of plasmid DNA. Where necessary BSA or human basic FGF (bFGF; Invitrogen, California, USA) was added to the developing embryos at 18 hour post fertilization (hpf).

### 4.2.3 Detection of TALEN mutations

TALEN electroporated embryos were observed for expression of Venus and/or mCherry fluorescence. 50-100 embryos with strong expression were isolated and genomic DNA was extracted using a Promega Wizard Genomic DNA isolation kit (Promega, Wisconsin, USA.) according to the manufacturer's instructions. TALEN targeted regions were amplified by PCR using a proofreading polymerase (PrimeSTAR HS DNA polymerase, Takara, Tokyo, Japan). The primers used for PCR were 5'-GTATCCATGACGTCAGCAGTTTATGC-3' and 5'-CTACCTCTTACTCCTTTCAATTGCCC-3' for *Fgf11*, 5'-CTTAACTAAGTAAGATCGGGGGACAC-3' and 5'-CTACATTCACCGGTACTGTTACGTC-3' for *Fgf3* and 5'-TTGTAGCTCACGACCATGTAG-3' and 5'-ATCTTCGTCCTCTACAGACTG-3' for *Hox12*. 400 ng of purified PCR product was heated to 95$^{o}$C and gradually cooled to 25$^{o}$C over 90 minutes and then treated with SURVEYOR Cel-I nuclease (Transgenomic, Nebraska, USA) at 42$^{o}$C for 30 minutes. Treated PCR products were then visualized by gel electrophoresis. Purified PCR products were sequenced using standard procedures and mutation rates were estimated by counting the number of mutated sequences compared to the total number of sequences.

### 4.2.3 Assessment of TALEN mutant phenotypes

Images of TALEN mutants were taken with an Axio Imager Z.1 fluorescent microscope (Carl Zeiss, Oberkochen, Germany). To assess TALEN mutations at the tailbud stage, approximately 100 embryos were isolated during the early-mid gastrula stage, before any defects were expected. These embryos were then allowed to develop to the tailbud stages, where morphological defects were observed. For assessing the development at 19 hpf for *Ci- EF1α>Fgf11* TALEN electroporations the

same procedure was carried out, but only those embryos showing normal development at 11 hpf were assessed. To assess metamorphosis approximately 100 hatched larvae were isolated in a clean petri dish and left to attach. Any treatments made were added to the seawater in the petri dish at 18 hpf, and larvae were then assessed at 42 hpf for tail absorption.

### 4.2.4 *In situ* hybridization

Whole-mount *in situ* hybridization (WISH) was performed according to standard procedures as previously described (Yasuo and Satoh, 1994).

### 4.2.5 Western Blotting

For each sample 20 electorporated *Ciona* embryos showing high levels of mCherry fluorescence were isolated, placed in an SDS/2-mercaptoethanol buffer and separated by gel electrophoresis on a 15% polyacrylamide gel. After electrophoresis the gel was transferred to a polyvinylidene fluoride membrane and treated with anti-flag IgG (clone M2; Sigma-Aldrich, Mo, USA) as a primary antibody, followed by HRP labeled anti-mouse IgG (Invitrogen) as a secondary antibody. Reactive bands were visualized by treatment with ECL-Plus (GE Healthcare, Buckinghamshire, UK).

## 4.3 Results

### 4.3.1 TALEN Construction

The TALENs used in this thesis have an N-terminal 136 amino acids long containing a FLAG-tag, either 15.5 or 16.5 TAL repeats with the final repeat targeting a T nucleotide and a C-terminal 63 amino acids long (Figure 10A). The spacer region between the TALEN binding sites was 16-18 nucleotides long (Figure 10B). The destination vector for the final ligation step during construction was modified for use in *Ciona* electroporation experiments with the *CiEF1α* (eukaryotic transcription elongation factor alpha - a ubiquitous promoter; Figure 1C,D) *cis*-regulatory element driving TALEN expression. On the same construct we included a fluorescent reporter expressing Venus for the L-TALEN and mCherry for the R-TALEN, driven by the *CiEpiI* (epidermis specific) *cis*-regulatory element (Figure 10C). The constructs were simultaneously electroporated into fertilized eggs and if both reporters were expressed it was assumed that the TALEN pair was also expressed (Figure 10D). These embryos could then be further analyzed to confirm the TALENs have induced a DSB and subsequent mutations at the targeted genomic loci can be detected by amplifying the targeted region by PCR and sequencing the products. The mutations induced by TALENs can result in a disruption to the targeted genes.

### 4.3.2 Ubiquitous TALEN knockouts

I constructed TALENs targeting the genes *CiFgf11*, *CiFgf3* and *CiHox12* by Golden Gate ligations. TALENs targeting these sequences were designed and ligated into *CiEF1α* TALEN vectors. These constructs were electroporated into fertilized *Ciona* eggs. 40-80 μg of DNA was electroporated. In all electroporations, strong expression of both Venus and mCherry could be seen. Genomic DNA from TALEN-

electroporated embryos showing a high level of reporter gene expression was taken during the tailbud stage and the TALEN targeted region was amplified by PCR. Mutations could be detected by a SURVEYOR assay (Figure 11A) where DNA is cut if any mismatched base-pairing lesions are present. In all 3 cases PCR products from TALEN-electroporated embryos showed additional bands after melting, re-annealing and incubation with the SURVEYOR nuclease. PCR products from these electroporatons were sequenced and mutations were detected (Figs 11B). The mutation rate from the sequenced DNA was between 71%-95%. The mutations observed ranged from a 111 bp (22.3 bp average) insertion to a 13 bp (7.4 bp average) deletion. Based on these results TALENs appear to be highly effective at efficiently inducing mutations in *Ciona* embryos. Recent large-scale studies of TALEN mutation efficiency shows mutation efficiency varies between 3%-95% (Reyon et al., 2012; Kim et al., 2013), the TALEN knockouts we have performed fall within this range.

*Ciona* has several genes considered orthologous to vertebrate *Fgf*s, of these *Fgf11* has no detectable expression during development and no known functions in *Ciona* (Satou et al., 2002), also any functions the vertebrate equivalent of this gene may have are not well understood as it has been shown to be incapable of binding to Fgf receptors (Guillemot and Zimmer, 2011). A TALEN targeting this gene was chosen to act as a control. Potential off target mutagenic effects, or other forms of toxicity are a serious concern when using TALENs (Moore et al., 2012). When mutations are introduced to target *Fgf11* there should be no knockout phenotypic effects seen. Therefore any defects in embryos expressing the highly active *Fgf11* TALEN can be considered to be due to the presence of this TALEN. To assess the expression of TALEN phenotypes approximately 100 early-mid gastrula embryos were isolated and left to develop to a stage where the defects could be observed.

When TALENs targeting *Fgf11* were ubiquitously expressed, a high level of defects could be seen at the early tailbud stage (Figure 12), even when 40 *μ*g, a relatively low amount of DNA, was electroporated. Therefore there is some toxicity associated with *CiEF1α* expressed TALENs. However when the embryos showing high levels of reporter gene expression that showed no apparent defects at the early tailbud stage were isolated and left to develop, over 90% of these successfully developed to swimming larva (Figure 12,13). If the abnormal development seen was mostly due to off-target effects of TALENs, these would be expected to accumulate during development leading to an increase in abnormalities over time; the opposite was observed. It has been shown in human cells that TALEN cytotoxicity appears to be related to the TALEN protein length and has no correlation to the mutational capabilities of the TALEN pair (Reyon et al., 2012). The *CiEF1α* promoter shows some degree of toxicity when it is used to drive mCherry expression by itself, possibly due to the high volume of protein produced by this promoter. Most of these defects appear at 11 hours post fertilization (hpf), the point where neurulation and notochord intercalation have just finished. Therefore it is probable that the high level of defects seen from the *CiEF1α* driven *CiFgf11* TALENs are the result of an accumulation of toxic effects of the TALEN proteins at a crucial and easily disrupted stage in *Ciona* development. Another possibility could be due to cell-cycle defects caused by the need to repair broken DNA strands.

Hox genes in *Ciona* have remained poorly described with the exception of *CiHox1* (Sasakura et al., 2012) and *CiHox12* (Ikuta et al., 2010). In *Ciona Hox12* is expressed in the posterior tail tip and when *CiHox12* is knocked down using morpholinos a rounding of the tail tip is observed. When *CiEF1α >CiHox12* TALENs were electroporated, a high level of defects could be observed (Figure 12C), including the tail tip rounding that has been previously described (Figure 13B).

*CiFgf3* is expressed in the CNS including the neural tube throughout development and shows some later weak expression in the trunk mesenchyme during the tailbud stage. Morpholino knockdowns of *CiFgf3* have been shown to disrupt the cell convergent extension of notochord intercalation (Shi et al., 2009). The explanation for this is that the signaling from the ventral neural tube acts as a cue for the dorsal notochord cells to intercalate and form the embryonic notochord, a major component of the *Ciona* larva tail. *CiEF1α>CiFgf3* TALEN electroporations were capable of reproducing the function of Fgf3 described from morpholino knockdowns. A high proportion of *CiFgf3*-TALEN embryos showed defects at the tailbud stage. I replaced the Venus reporter in the *CiFgf3*-L-TALEN with mCherry and co-electroporated *CiBra>GFP* (notochord promoter) in order to clearly visualize the notochord precursor cells. These embryos demonstrated a failure to intercalate the notochord cells (Figure 13C).

The mutation rate and relative intensity of the Cel-I assay bands was evaluated in greater detail (Figure 14). Those embryos displaying the highest and lowest levels of mCherry fluorescence from an individual electroporation were isolated and the mutation rate was estimated by a Cel-I assay and sequencing for *CiFgf3 and CiHox12.* A correlation could be seen between the intensity of the reporter gene expression and mutation rate with high mCherry expression resulting in a higher mutation rate.

Based on these results, the electroporation of constructs driving ubiquitously expressing TALENs are very effective at inducing specific mutations. These mutations are capable of recreating previously described phenotypes induced using morpholinos. However, at least with the *CiEF1α* promoter driving TALEN expression, a high level of side effects can be seen. One concern with this methodology is the potential for electroporated DNA in *Ciona* to show mosaic expression. This is a

common concern with all *Ciona* electroporations and we feel is not a serious problem with appropriate screening of the electroporated embryos. The *CiEF1α* TALENs are very good at demonstrating the efficiency of the TALENs to cause DSBs and subsequent mutations, and for confirming the functions of genes, but to investigate new functions, a conditional knockout strategy may be more useful.

### 4.3.3 Tissue specific TALEN knockouts

Many genes involved in embryonic development show a range of pleiotropic functions, some of these are lethal when disrupted (Duffy, 2002), a conditional knockout strategy could be valuable to understand these processes in greater detail. *CiFgf3* continues to be expressed after notochord intercalation has finished and into the later tailbud stages. Morpholinos or ubiquitous TALEN knockouts are of little use to understand these functions, as only the earliest defect will be observable. Even if the embryo survives and continues to develop, any further mutant phenotypes observed could be secondary consequences of the first phenotype. As the *CiEF1α* expressed TALENs were very efficient at causing mutations I attempted to switch the promoters driving TALEN expression to tissue specific variants. Using these constructs we can expect the active TALENs to be expressed in specific tissues when we see reporter gene expression (Figure 15A). I used *cis*-regulatory elements driving TALEN expression in the epidermis (*CiEpi1*), early neural lineage (*CiNut*), and in the mature neurons (*CiPC2*) to perform tissue-specific TALEN knockouts (Figure 15A' ,A''). The TALENs I have used contain an N-terminal FLAG-tag.

When *CiHox12* TALENs were specifically expressed in the epidermis a high level of tail rounding was seen (Figure 16B). This was as high as 78% when 100 μg of DNA was electroporated (Figure 17A). A western blot for the TALEN FLAG tag from embryos electroporated with the left or right TALEN arms for all TALENs used in

the tissue specific knockout experiments all showed high levels of TALEN protein present (Figure 17D).

TALENs expressed in the epidermis did not show severe defects apart from the expected tail rounding even when high amounts of DNA (100 μg) were electroporated. *CiFgf8/17/18* is expressed at the tip of the tail, and *CiHox12* morphants show a failure to maintain expression of this gene during tail growth (Ikuta et al., 2010). When *CiEpi1>CiHox12* TALENs were electroporated the *CiFgf8/17/18* transcript could not be observed by WISH (Figure 18), confirming the phenotypes caused by *CiHox12* TALENs reflects the endogenous function of this gene. The percent of embryos showing tail rounding and reduced *CiFgf8/17/18* was less than 100%. This could possibly be due to the tail tip being formed from a very small number of the total tail cells providing them with some protection from electroporation transfection.

*CiFgf3* TALENs were specifically expressed in neural tissues with the early neural promoter of *Ci-Nut* (Figure 16C,D,E). Electroporations of *CiFgf3* TALENs using this promoter is expected to cause mutations and disrupt the function of Fgf3 from an early stage. A high level of defects could be seen in the majority of embryos electroporated with *CiFgf3* TALENs in neural tissues (Figure 17B). These embryos showed a similar range of phenotypes to the *CiEF1α* TALEN electroporatons (Figure 16E) and previously described morpholino results with a range of major defects seen varying in severity and timing (Shi et al., 2009).

When *CiFgf3* TALENs were expressed using the *CiPC2* promoter that drives downstream genes after convergent extension of the notochord, normal development was seen and the swimming larva attached to the bottom of petri dishes and underwent metamorphosis. *Ciona* metamorphosis involves a series of major changes to the body plan including absorption of the larval tail and rotation of the body axis

(Nakayama-Ishimura et al., 2009). When *CiFgf3* TALENs were expressed throughout the nervous system before and during metamorphosis, tail absorption was arrested (Figures 16G and 17C). *CiFgf11* TALENs expressed through the same promoter did not result in an arrest of tail absorption (Figure 16F). When larvae were treated with basic FGF after hatching, but prior to attachment, tail metamorphosis proceeded normally (Figure 17C), confirming that FGF is necessary for tail absorption. A possible explanation for this is that Fgf3 signaling from the neural tube is one of several inductive signals that are needed for tail absorption. Another possible explanation could be due to the absence of Fgf3 in the nervous system, and that Fgf3 is needed to correctly program the nervous system to allow normal tail absorption to take place. Little is known about the inductive cues for metamorphosis in *Ciona* but it is likely that Fgf3 has some role to play based on these results.

### 4.3.4 TALEN-2A-mCherry Ubiquitous and Tissue Specific Genome Editing

The TALEN constructs described above are very successful at introducing mutations, both ubiquitously and in a tissue specific manner. Nevertheless the plasmid constructs used could be refined to make the construction process more simple, and for an easier interpretation of results. The viral 2A peptide has previously been utilized in cells to produce a TALEN and fluorescent protein on a single transcript (Ding et al., 2013). The TALEN constructs were redesigned based on this principle so that the promoter would drive the simultaneous expression of a TALEN and mCherry (Figure 19 A,B) This led to high levels of mutations (Figure 19 C) The mutation rates were not as high as the previously described rates for *CiHox12, CiFgf11* and *CiFgf3*, but 3 out of 4 are above 50% and overall the mutation rates are acceptable. When the promoter was switched to a tissue specific promoter, high

levels of fluorescence could be seen specifically in the appropriate tissues (Figure 19 D,E,F), indicating that the TALEN-2A-mCherry system is a simple and robust tool for genome editing in *Ciona*

### 4.4 Discussion

ZFN/TALEN gene targeting is a powerful new addition to the molecular geneticist's repertoire of tools. So far the descriptions of TALEN knockouts in animals have used mRNA microinjections (Carlson et al., 2012; Lei et al., 2012; Kawai et al., 2012). mRNA microinjections have some advantages over the TALEN electroporations we have described as they are likely to act earlier, and only require knowledge of the DNA sequence of the target region. Electroporations as we have described require the knowledge of *cis*-regulatory regions to drive expression of TALENs. TALEN mRNA microinjections should also be useful in *Ciona,* but *Ciona* microinjections are difficult and time consuming. By comparison *Ciona* electroporation experiments are very fast. For other non-standard model organisms, mRNA injection will remain the most feasible method for the foreseeable future, but for model organisms where reporter constructs are routinely used, such as zebrafish or *Xenopus*, the techniques we have described will be immediately to perform tissue specific knockouts. For *Ciona* the combination of TALEN knockouts with well-established electroporation protocols allows mutants to be generated and quickly screened with numbers of embryos not possible with other model organisms allowing greater confidence in the results of functions of these mutations. A major concern with TALENs is the toxicity. My results show that this still remains a serious concern for ubiquitous knockouts using the *CiEF1α* promoter, but using tissue specific promoters the level of toxicity appears to be minimal, the TALEN-2A-mCherry system also appears to provide a minimal level of toxicity.

There are numerous roles for Fgf signaling in many stages of *Ciona* development (for example: Stolfi et al., 2011, Davidson et al., 2006 Kourakis et al., 2007). We have shown a new role for Fgf3 signaling during metamorphosis. Our

conditional knockout of Fgf3 throughout the nervous system in larvae results in a clear arrest to the normal process of tail absorption. Therefore tail absorption is dependent on Fgf signaling.

The ability to quickly and easily perform the conditional knockouts of genes in *Ciona* has the potential to lead to an improved understanding of how a chordate body plan is formed, and how it can be drastically changed through metamorphosis. The results in this paper provide a demonstration of how this could be done. TALEN technology has the potential to overcome previous barriers to gene knockouts and allow a range of gene function studies to be done in ways previously not possible.

## 5. Conclusions and Future Directions

For the work presented in Chapter 3, there are several experiments that can be done to elaborate on the novel finding that ZicL has an essential role in the number of cell divisions in the early ascidian embryo. Some of these experiments can be made more feasible by the technical advances made in Chapter 4. The most immediate concern is the expression of ZicL in other ascidians. The work was inspired by an attempt to explain the evolution of the anural ascidian larval form, or at least the number of cells in the anural ascidian larval embryo. The predictions have been valid upon experimental testing in *Ciona*. However there is a difference between what mechanisms can work through the perturbation analysis I have performed, and those mechanisms that do work through the process of evolution to give rise to novelties. My results immediately suggest that ZicL should be expressed in the 16-cell stage of all anural ascidians. Since the larvacean tunicate *Oikopleura dioica* has 20 notochord cells and 20 muscle cells (Nishida 2008) ZicL could also be expressed from the 16-cell stage in these organisms, but as *Oikopleura* is highly derived from standard ascidian development this is not as likely as it is for anural ascidians. Investigating the expression of ZicL in these organisms is an immediate priority. If my predictions for these experiments are correct then further functional and *cis*-regulatory analysis of ZicL can be done in this species that should result in the complete explanation of the cause of evolution of a novel larval form.

Another question that demands immediate attention is exactly how ZicL performs its effect to control the number of cell divisions. The work in this thesis strongly indicates that it works combinatorially with tissue specific transcription factors to stop cell division. Brachyury has been shown to be necessary for the expression of the cyclin dependent kinase inhibitor (Cki)-B (Kuwajima et al., 2014).

And ZicL is essential for expression of *Brachyury* (Yagi et al., 2004). Therefore ZicL should act, at least indirectly to activate the expression of *Cki-B*. The ability for ZicL to bring forward the number of cell divisions, and the inability of Brachyury to do the same indicates that this process is not indirect. A recently developed concept is that of a "pioneer transcription factor" (Zaret and Carroll., 2011) where a transcription factor acts to re-order chromatin for later transcription factors to perform their functions. The data in this thesis strongly indicates that ZicL acts as a pioneer transcription factor in this manner.

One further way that the function of ZicL could be investigated could be by performing a *cis*-regulatory analysis of cell cycle genes, specifically cell cycle inhibitory genes. This could be done in conjunction with tissue specific transcription factors. If both Brachyury and ZicL were shown to bind to enhancers of the *Cki-B* gene then this would support the arguments for the function of ZicL as a pioneer transcription factor. Another factor that should be taken into account is the actual function of the ZicL (and other transcription factors) protein. In the gene regulatory networks upon which this project is based, we are considering the presence of an mRNA as an indication that the mRNA is being translated into a protein, and that the protein is having an authentic effect on the cell. These have all not been confirmed. A range of immunological and chromatin works as well as other biochemical assays could be established in the simple, limited number of cells of an early *Ciona* embryo, one limitation to this is that since early ascidian embryos have a low number of cells, therefore they have a low quantity of chromatin. Nevertheless this limitation is likely to be overcome in the next few years.

The genome editing tools presented in Chapter 4 could be used to answer some of the questions that are presented in Chapter 3. One major limitation is that the decisive test of a gene's function comes from knocking out that gene. Therefore

we could knock out ZicL using TALENs. A potential problem is that, for example, we want to count the number of notochord cells, but without ZicL, there will be no notochord cells, as the fate of the lineage has not been specified. This is why the experiments on ZicL have primarily relied on misexpressions. The field of genome editing is moving at an unprecedentedly fast pace. A very recent advance is the development of the bacterial defense system CRISPR to be utilized for genome editing (Shalem et al., 2014). Shalem et al was published in January 2014, by November 2014 the number of research papers successfully utilizing this technique has been so great that it would be impractical to cite them. Very recently 2 reports have been published using CRISPR/Cas in *Ciona* (Sasaki et al., 2014; Stolfi et al., 2014) In both these reports CRISPR/Cas could induce mutations, but the mutation rate was not as high as my TALEN data. However CRISPR/Cas was able to effectively mutate genomic sequences that TALENs could not, and construction sgRNAs for CRISPR/Cas is extremely simple. So at the very least CRISPR/Cas is useful as an alternative genome editing strategy in *Ciona*, and it is not inconceivable that it will eventually become the preferred one.

It is inevitable that in the future we will consider the science done in the past to be experimentally primitive. Therefore 20 years into the future the current state of knowledge about how animal development works will be seen as inadequate. It is impossible to predict what these inadequacies will be, but I think it is likely that the current style of only looking at an extremely small subset of living things and then extrapolating that information to cover all the other living things will be seen as being grossly inadequate. Genome editing and genome sequencing technologies will allow the advancements of the late 20$^{th}$ and early 21$^{st}$ century to be unleashed upon myriad organisms. Perhaps some combination of the observational (Wilson 1925) and

experimental (Gilbert 2013) approaches will result in new advances that will provide a more robust set of general rules for how living things work.

## 6. Acknowledgements

This work was done under the supervision of Professor Yasunori Sasakura, The support that he has given me during the three years I have been a member of his laboratory is too great to appropriately acknowledge in this paragraph. The work on genome editing was done as a substantial collaboration with Hiroshima University, and I thank Professor Takashi Yamamoto and Dr Tetsushi Sakuma for their invaluable assistance to get my genome editing projects working. Dr Keita Yoshida and Ms Haruka Sasaki of the University of Tsukuba, as well as Dr Narudo Kawai of Keio University also contributed to the TALEN work. Culturing of wild-type ascidians was done with the support of Maizuru Fishery Research Center, Kyoto University; Misaki Marine Research Center, University of Tokyo; Usa Marine Biological Institute, Kochi University. My thanks are also given to all members of the Sasakura Laboratory and Shimoda Marine Research Center from 2012-2015.

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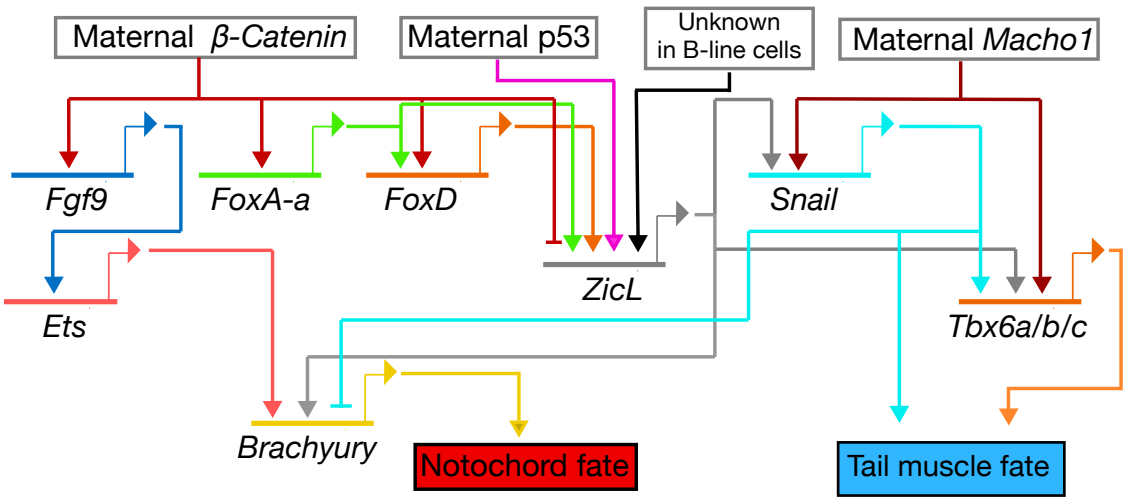



**Figure 1. A combined gene regulatory network for the establishment of notochord and tail muscle fate in *Ciona.***

A representation of regulatory interactions in early *Ciona* mesoderm development leading to the establishment of clonal restriction to either notochord or tail muscle cell fate. Maternal genes acting before the 16-cell stage at the start of the network are boxed in gray. B-line notochord cells and A-line muscle cells have notable differences in the early interactions from the network in the figure. The network was constructed from publically available, experimentally validated datasets cited in the main text.

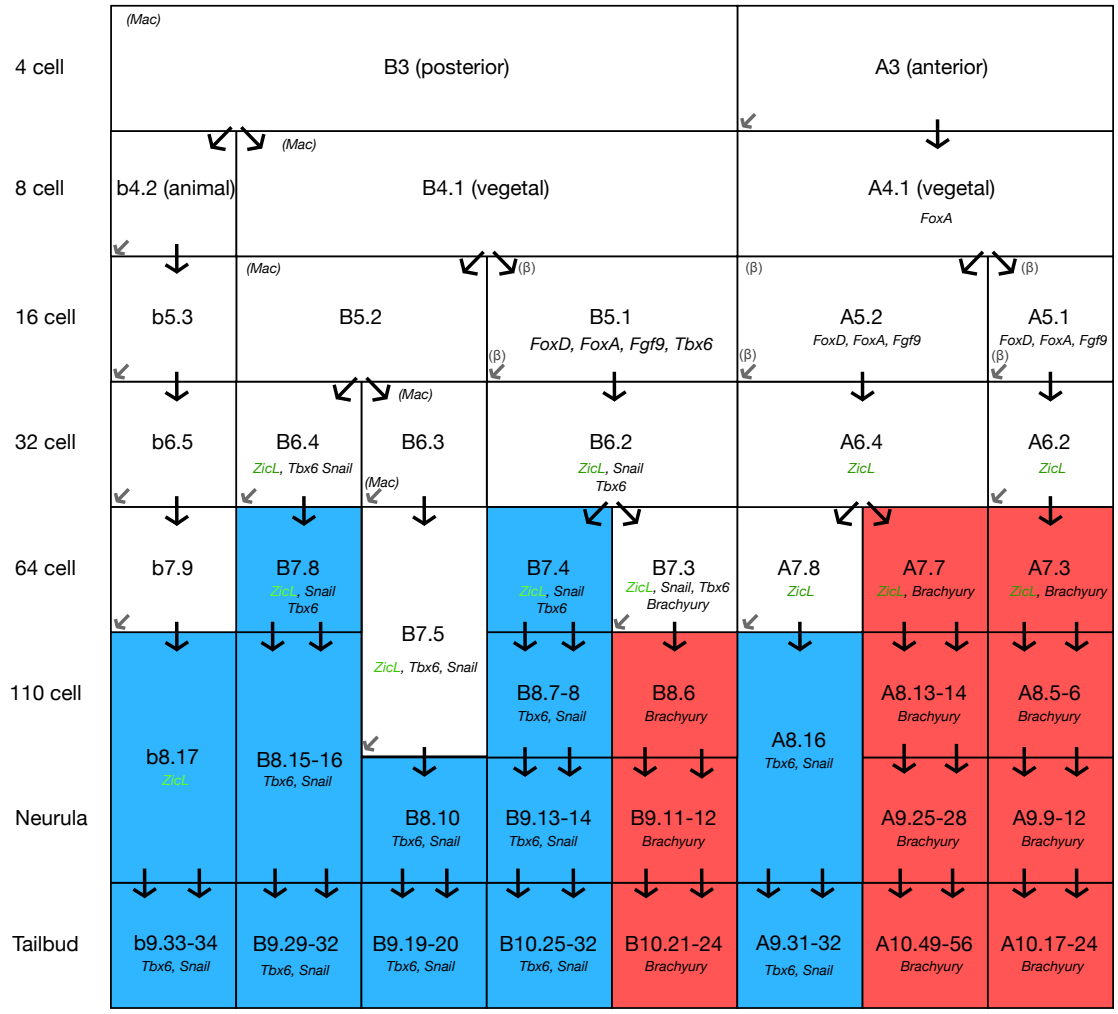



**Figure 2. The notochord and tail muscle cell lineages in *Ciona*.**

Cell lineages relevant to notochord and tail muscle development in *Ciona*. Each box represents a single cell, or groups of similar sister-cells in the later 3 levels. Each cell is named (e.g. B3) based on Conklin's nomenclature (Conklin, 1905). Grey arrows represent a cell lineage descending from the boxed cell that does not contribute to either the notochord or muscle. Muscle cells are represented in blue and notochord cells in red. Genes expressed that are relevant to the establishment of the lineage fate are written in italic. The cells that inherit nuclear-localized β-catenin or *CiMacho1* mRNA are indicated by (β) and (*Mac)* respectively. Cell division timings and developmental stages are only a rough approximation and are mostly unequal after the 32-cell stage. The figure was constructed from publically available datasets cited in the main text.

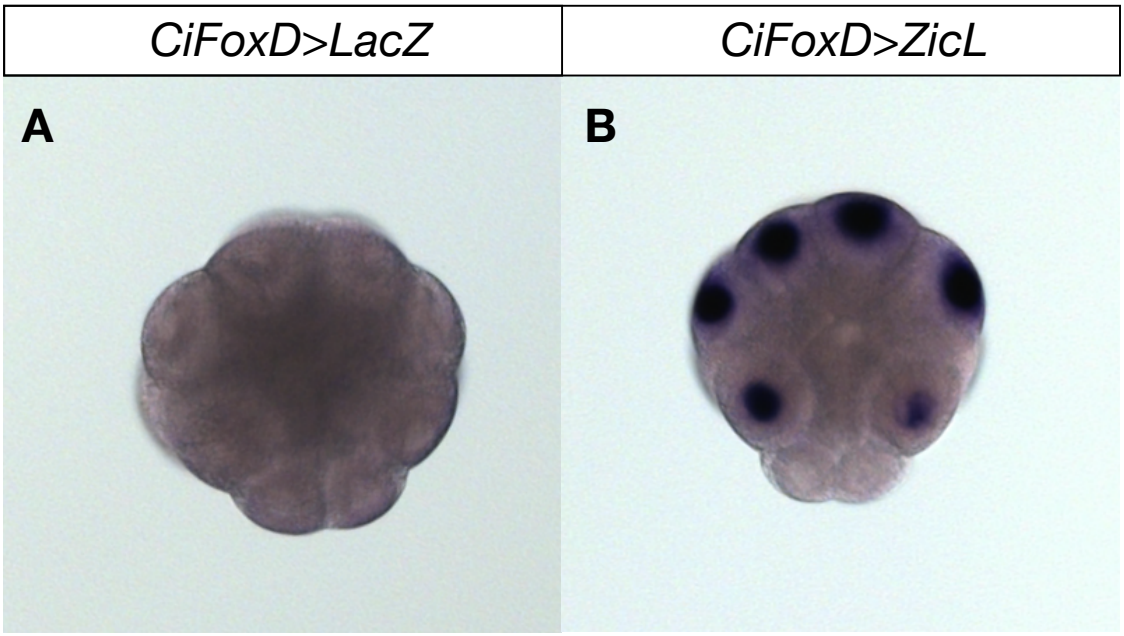



**Figure 3. Early misexpression of *CiZicL.***

*In-situ* hybridization of *CiZicL* transcripts in 16-cell *Ciona* embryos electroporated with a control (A) plasmid, or a plasmid designed to misexpress *CiZicL* one cell cycle early (B) All embryos are oriented with the vegetal hemisphere facing the camera.

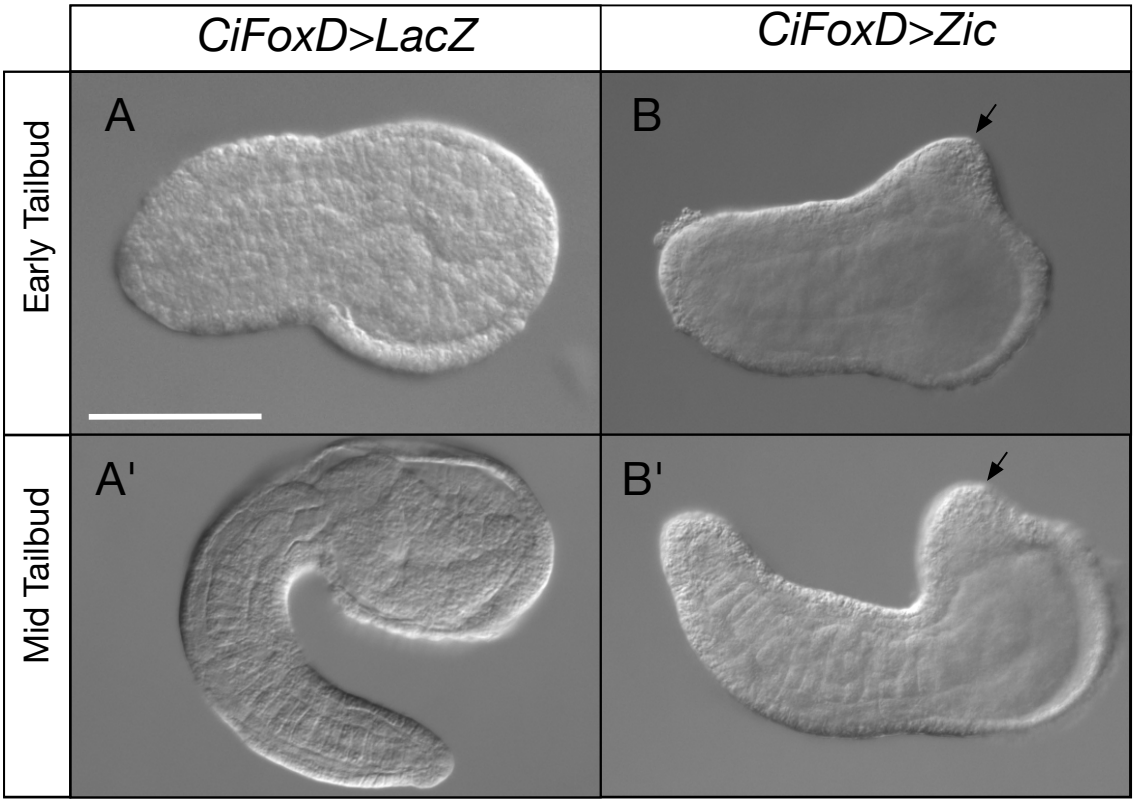



**Figure 4. Consequences of *CiFoxD>CiZicL* misexpression**

(A,A',B,B') Differential interference contrast images of early and mid tailbud *Ciona* embryos electroporated with a control (*CiFoxD>LacZ*) plasmid, showing normal development, or a plasmid designed to misexpress *CiZicL* one cell cycle early (*CiFoxD>CiZicL*), showing severe defects. Bulges formed by the altered position of neural folds are shown with arrows. Scale bar = 100 μm. All embryos in this figure are oriented dorsal up, anterior left.

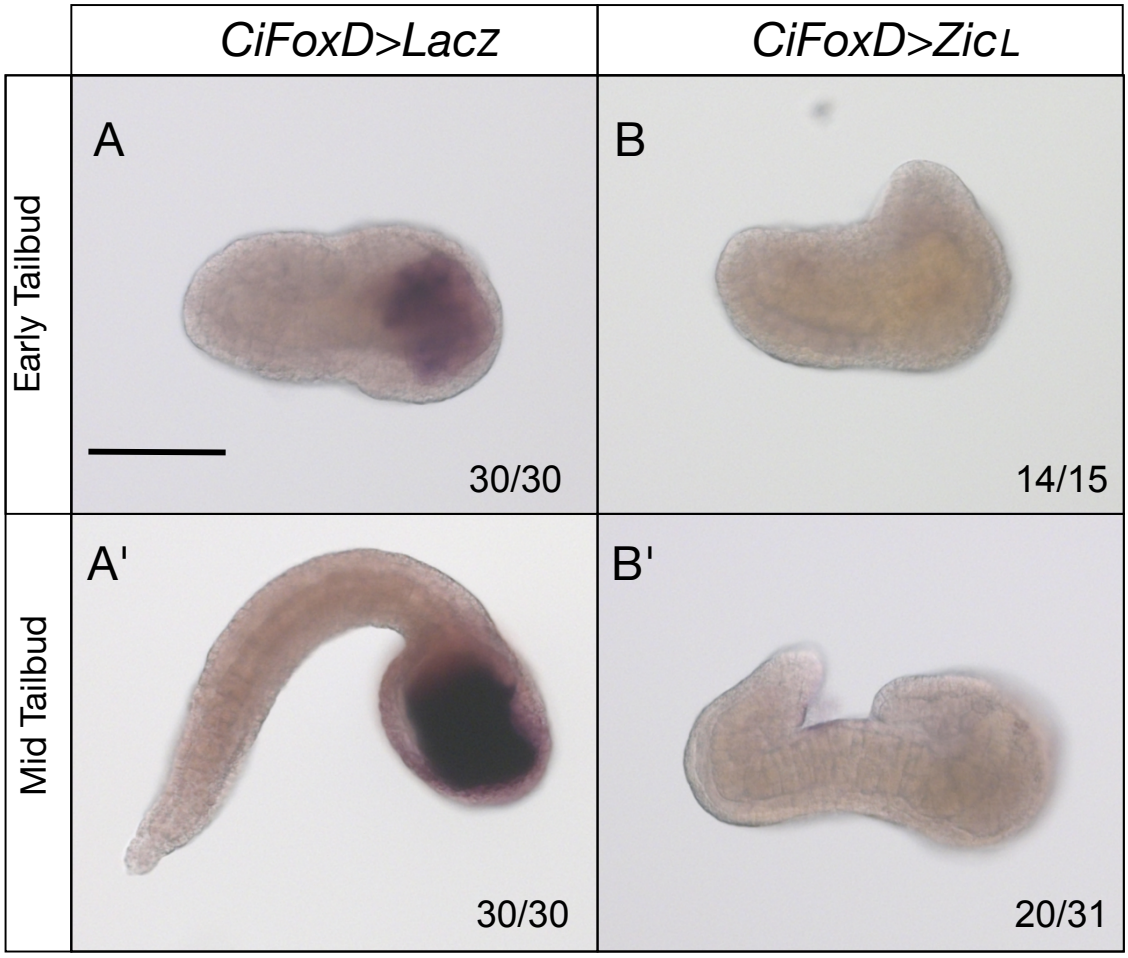



**Figure 5. *CiFoxD>CiZicL* completely converts the endoderm**

(A,A',B,B') Bright-field images of *Ciona* embryos after chromogenic staining with NBT/ BCIP to visualize endogenous alkaline phosphatase activity in the endoderm. In *CiFoxD>CiZicL* electroporations alkaline phosphatase activity is completely abolished. Scale bar = 100 μm. All embryos in this figure are oriented dorsal up, anterior left.

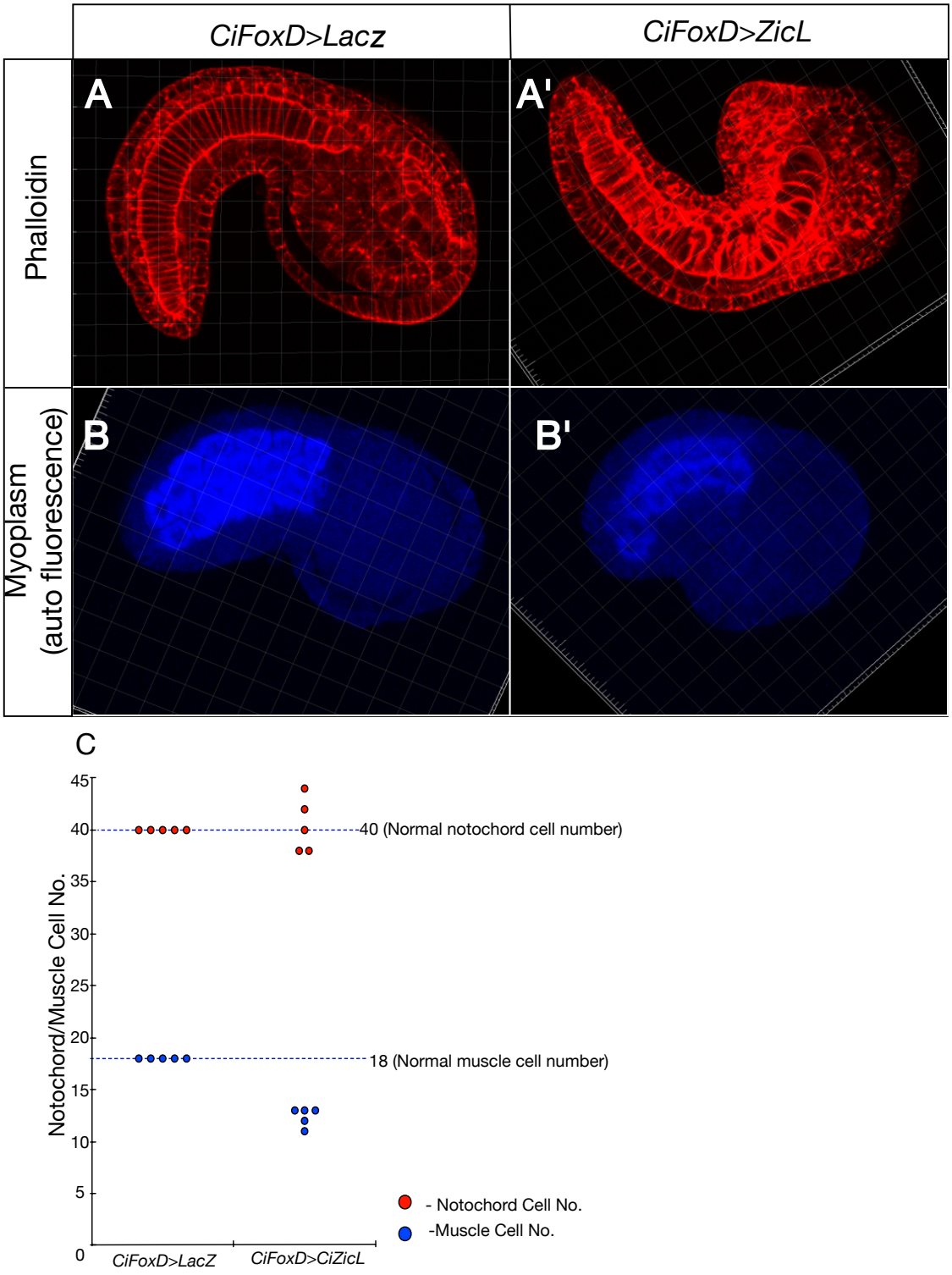



**Figure 6. Early ZicL expression leads to a reduced number of notochord and tail muscle cells.**

(A,A',B,B') 3D representations of Z-stacked confocal sections of tailbud stage embryos. (A,A') Embryos where cell membranes are visualized by staining F-actin with phalloidin. Individual notochord cells can be seen. (B,B') Fixed embryos where the myoplasm rich muscle cells are visualized by auto fluorescence under high exposure to confocal lasers. In these images a single square on the background grid has an area of 400 $\mu m^2$. (C) Numbers of notochord and tail muscle cells counted in 5

control (*CiFoxD*>LacZ) and 5 *CiZicL* misexpressed (*CiFoxD>CiZicL*) embryos. Number of notochord cells counted in an individual embryo is represented by a red circle and number of muscle cells in one half of an individual embryo by a blue circle. Counted notochord cells include any that may have been converted from another fate.

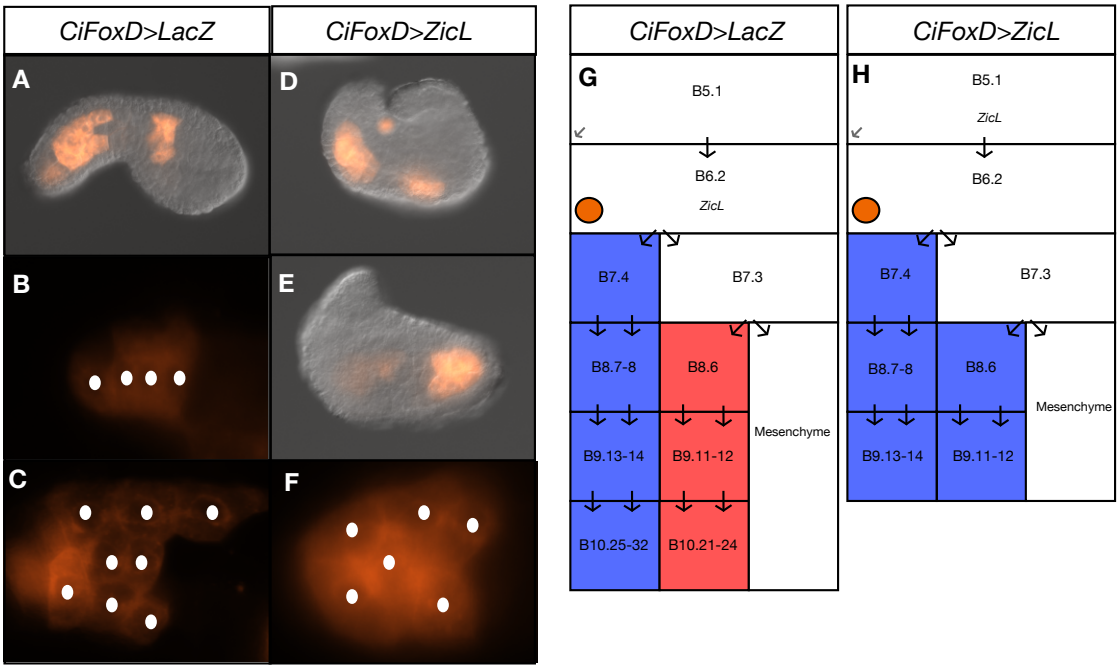



**Figure 7. Tracing the B6.2 lineage after early expression of *CiZicL*.**

B6.2 blastomeres were labeled with DiI and the decendents were visualized in control electroporated embryos (*CiFoxD>LacZ*, A, B, C) and electroporated embryos with early *CiZicL* expression (*CiFoxD>ZicL,* D, E, F). (A) Tailbud embryo from control electroporation, DiI labeling can be seen in tail muscle and mesenchyme cells. Labeling in the posterior notochord cells can only be faintly seen as it is out of focus. (B) DiI labeling of notochord cells, individual nuclei have been marked with a white dot. (C) DiI labeling of muscle cells, individual nuclei have been marked with a white dot. (D, E,) Tailbud embryos from *CiFoxD>ZicL* misexpression. The 2 distinct forms shown in the image were present in approximately equal proportions of the electroporated population. In both forms, DiI staining can be seen in tail muscle and presumptive mesenchyme cells. The single dot of DiI staining at the most dorsal point in (D) is from excess DiI that is pushed to the embryos exterior during gastrulation. (F) DiI labeling of muscle cells, individual nuclei have been marked with a white dot. (G) Cell lineage of the B5.1 decedents based on previous studies and confirmed in this figure. (H) Cell lineage of B5.1 decedents based on an interpretation of the data in this figure. Orange dots represent cells labeled with DiI.

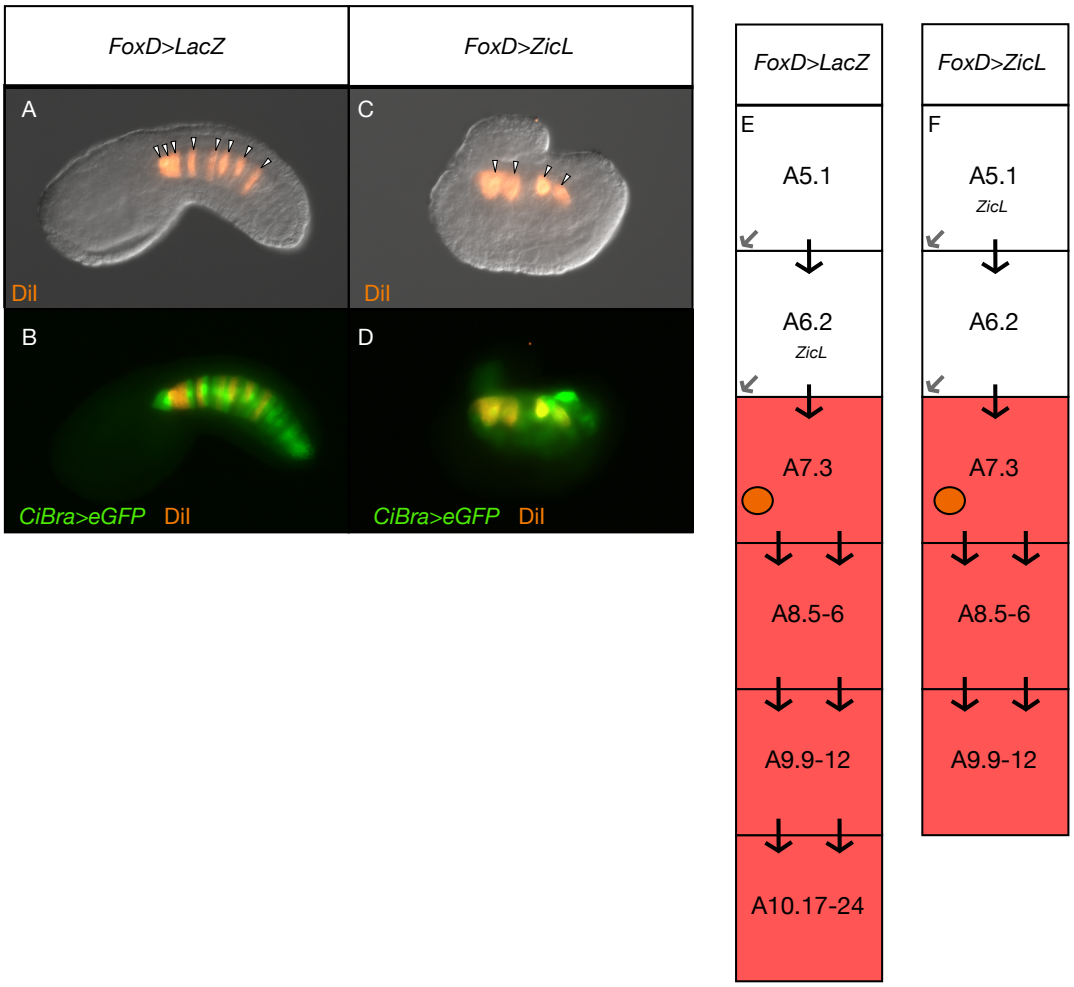



**Figure 8. Tracing the A7.3 lineage after early expression of *CiZicL*.**

A7.3 blastomeres were labeled with DiI and the descendants were visualized in control electroporated embryos (*CiFoxD>LacZ*, A, B) and electroporated embryos with early CiZicL expression (*CiFoxD>ZicL*, C,D). (A) Tailbud embryo from control electroporation, DiI labeling can be seen in 8 notochord cells. (B) Fluorescence micrograph of the embryo in (A) eGFP expression can be seen in notochord cells. (C,D) Tailbud embryos from *CiFoxD>ZicL* misexpression. DiI labeling can be seen in only 4 cells. (E) Cell lineage of the A5.1 decedents based on previous studies and confirmed in this figure. (F) Cell lineage of A5.1 decedents based on an interpretation of the data in this figure. Orange dots represent cells labeled with DiI.

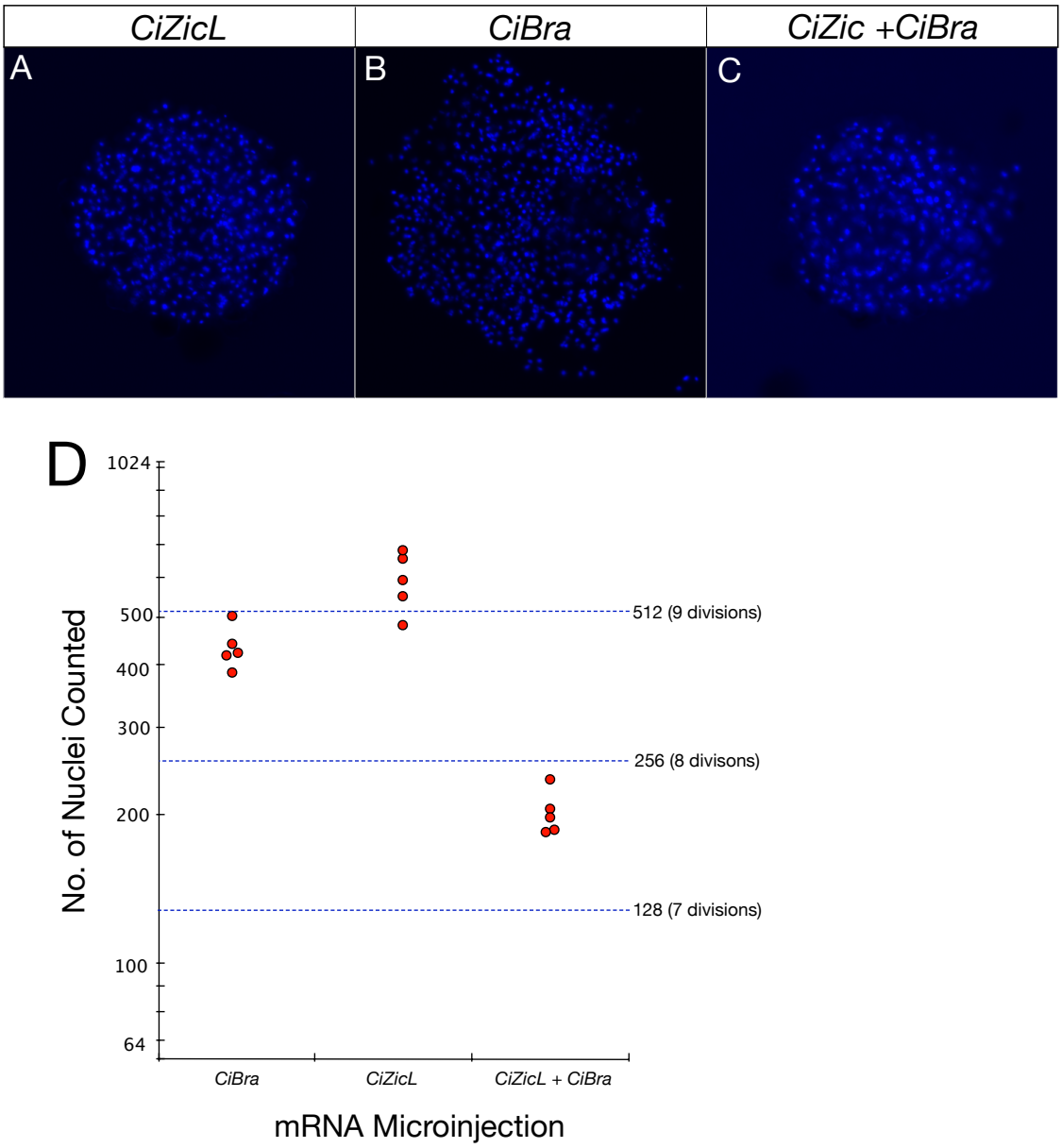



**Figure 9. *ZicL* induces cell cycle arrest through combinatorial interactions with tissue-specific transcription factors.**

(A,B,C) Fluorescence micrographs of compressed ascidians embryos stained with DAPI. All embryos were at 9 HPF when the preparation for imaging began. Embryos were microinjected with mRNAs indicated. (D) Number of stained nuclei counted from 5 individual embryos from each microinjection.

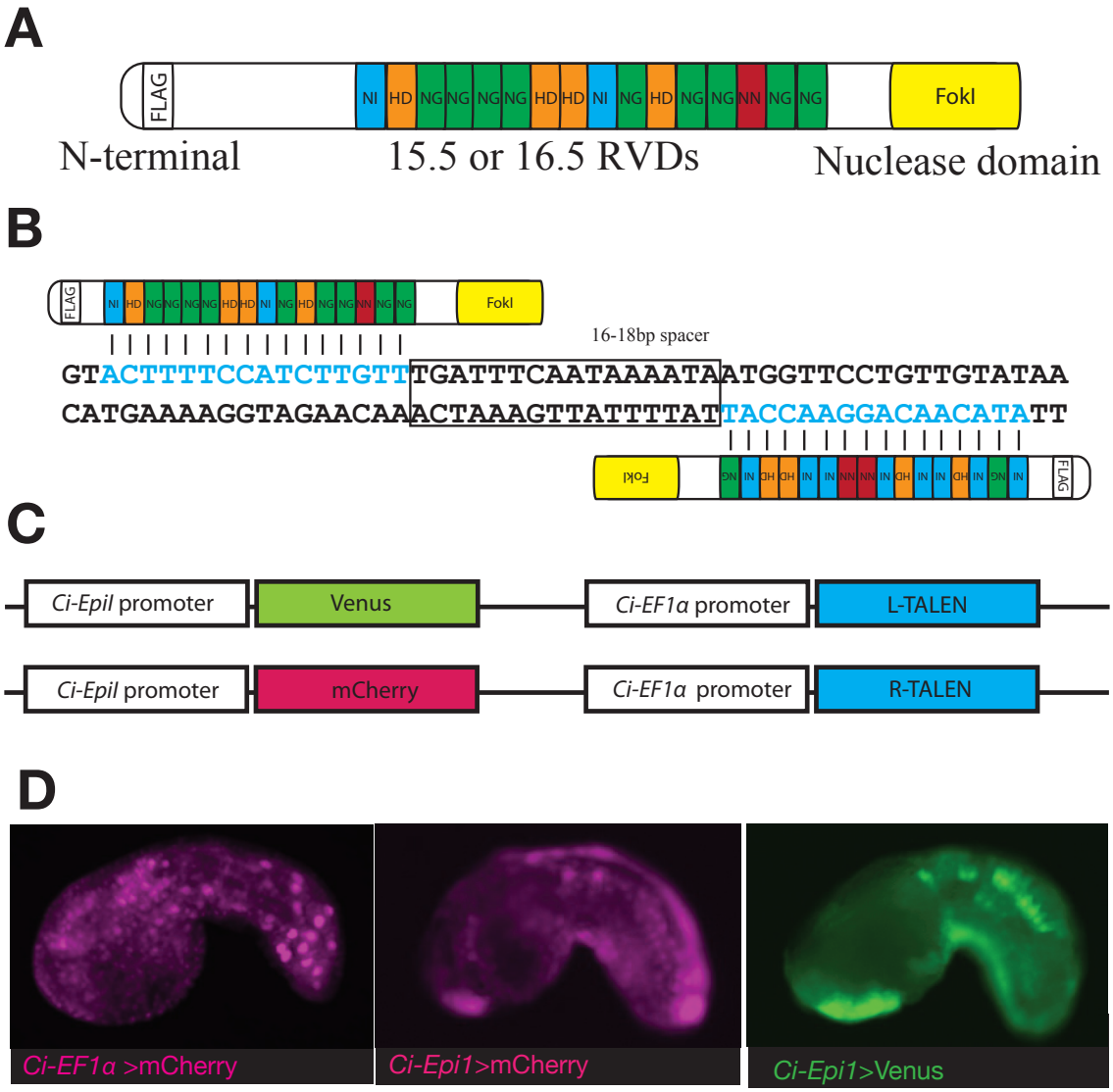
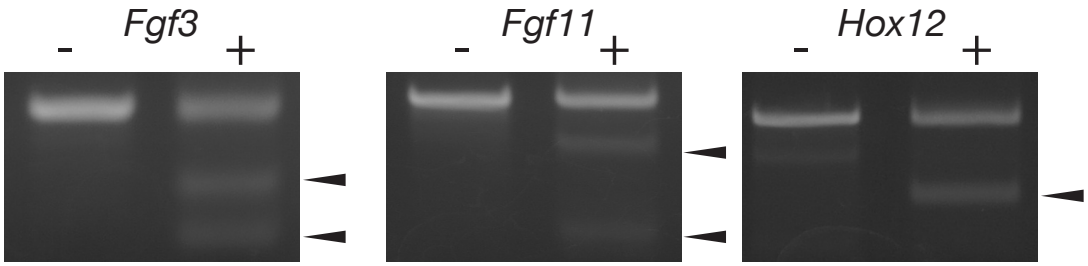



Figure 10. **Electroporation constructs used to drive ubiquitous TALEN expression in *Ciona* embryos.**

(A) Overview of the general architecture of a TALEN incorporating the design principles used in this thesis. Repeat variable diresidues (RVDs) are color coded with variable amino acids indicated for this representative TALEN. FLAG represents the presence of a FLAG epitope tag. (B) Schematic example of a TALEN pair binding to a specific DNA sequence (DNA sequence and TALENs depicted are the targets for *CiFgf3*). RVD's are shown binding to specific nucleotides. A spacer region where a double-stranded break will occur is indicated. (C) Description of the pair of TALEN constructs used to drive expression in *Ciona*. The promoters that drive specific expression in the epidermis (*CiEpi1*) or ubiquitous expression throughout the embryo (*CiEF1α*) are shown upstream of the gene whose expression they will drive. (D) Examples of reporter gene expression using the promoters shown in (C) in 9 hpf *Ciona* embryos.

# A




# B

*Fgf3* (16/19)

ACTCCCACGTACTTTTCCATCTTGTTTGATTTCAATAAAATAATGGTTCCTGTTGTATAATCGATACA wt (3)
ACTCCCACGTACTTTTCCATCTTGTTTGATTTCAA----ATAATGGTTCCTGTTGTATAATCGATACA -4 (1)
ACTCCCACGTACTTTTCCATCCTGTTTGATTTC----AAATAATGGTTCCTGTTGTATAATCGATACA -4 (1)
ACTCCCACGTACTTTTCCATCTTGTTTGA-------AAAATAATGGTTCCTGTTGTATAATCGATACA -7 (2)
ACTCCCACGTACTTTTCCATCTTGTTTGA--------AAATAATGGTTCCTGTTGTATAATCGATACA -8 (2)
ACTCCCACGTACTTTTCCATCTTGTTTGATTT---------AATGGTTCCTGTTGTATAATCGATACA -9 (1)
ACTCCCACGTACTTTTCCATCTTGTTTGA---------AATAATGGTTCCTGTTGTATAATCGATACA -9 (2)
ACTCCCACGTACTTTTCCATCTTGTTTGA----------ATAATGGTTCCTGTTGTATAATCGATACA -10 (1)
ACTCCCACGTACTTTTCCATCTTGTTTGATT----------AATGGTTCCTGTTGTATAATCGATACA -10 (2)
ACTCCCACGTATTTTTCCATATTGTAAGAT----------TGGTTCCTGTTGTATAATCGATACA -13 (1)
ACTCCCACGTACTTTTTCCATCTTGTTTG---------AAATAATGGTTCCTGTTGTATAATCGATACA -9 +1 (1)
ACTCCCACGTACTTTTCCATCTTGTTTGATTTGAATTTGAAAATAATGGTTCCTGTTGTATAATCGATACA +3 (1)
ACTCCCACGTACTTTTCCATCTTGTTTGATTTAAACGTAAATAAAAACCTTGACCGTCTTACCCATAACTC
CCACGTACTTTTTCCATCTTGTTTGAACGGTAAACGTAAATAAAAACCTTGACCGTCTTACCCAATTATATA
AAAACACAATAATGGTTCCTGTTGTATAATCGATACA +111 (1)

*Fgf11* (10/14)

AGGGTGGCATAAATATCTGGTGATTTTCGCACACGGCAGGTTATTAGTGTGCAGTATTCTGTAGAAAGAAGA wt (4)
AGGGTGGCATAAATATCTGGTGATTTTCGCACACGG---GTTATTAGTGTGCAGTATTCTGTAGAAAGAAGA -3 (1)
AGGGTGGCATAAATATCTGGTGATTTTCGCACA-----GGTTATTAGTGTGCAGTATTCTGTAGAAAGAAGA -5 (1)
AGGGTGGCATAAATATCTGGTGATTTTCGCACAC------TTATTAGTGTGCAGTATTCTGTAGAAAGAAGA -6 (1)
AGGGTGGCATAAATATCTGGTGATTTTCGCA-------GGTTATTAGTGTGCAGTATTCTGTATAAAGAAGA -7 (1)
AGGGTGGCATAAATATCTGGTGATTTTCG----------GTTATTAGTGTGCAGTATTCTGTAGAAAGAAGA -10 (1)
AGGGTGGCATAAATATCTGGTGATTTTCGCA----------TATTAGTGTGCATTATTCTGTAGAAAGAAGA -10 (1)
AGGGTGGCATAAATATCTGGTGATTTTCGCACA-------TTATTTAGTGTGCAGTATTCTGTAGAAAGAAGA -7+1 (1)
AGGGTGGCATAAATATCTGGTGATTTTCGCACACGGCTGATTTTCGCACAGGTTATTAGTGTGCAGTATTCTG
TAGAAAGAAGA +12 (1)
AGGGTGGCATAAATATCTGGTGATTTTCGCACAGGTAAATATCTGGTGATTTTCGGCAGGTTATTAGTGTGCA
GTATTCTGTAGAAAGAAGA +20 (2)

*Hox12* (20/21)

TCGAAGGCCATACACTAAGTACCAACTTTCCGAGCTAGAAAGAGAGTTCGGAGCGAACGAATTCATAAGCC wt (1)
TCGAAGGCCATACACTAAGTACCAACTTTCCGAG----AAAGAGAGTTCGGAGCGAACGAATTCATAAGCC -4 (3)
TCGAAGGCCATACACTAAGTACCAACTTTCCGAG-----AAGAGAGTTCGGAGCGAACGAATTCATAAGCC -5 (4)
TCGAAGGCCATACACTAAGTACCAACTTTCCGAG------AGAGAGTTCGGAGCGAACGAATTCATAAGCC -6 (1)
TCGAAGGCCATACACTAAGTACCAACTTTCCGA-------AGAGAGTTCGGAGCGAACGAATTCATAAGCC -7 (2)
TCGAAGGCCATACACCAAGTACCAACTTTCCGAG--------AGAGTTCGGAGCGAACGAATTCATAAGCC -8 (2)
TCGAAGGCCATACACTAAGTACCAACTTT--------TTAAGAGAGTTCGGAGCGAACGAATTCATAAGCC -8 (1)
TCGAAGGCCATACACTAAGTACCAACTTTCCGAGC----------GTTCGGAGCGAACGAATTCATAAGCC -10 (1)
TCGAAGGCCATACACTAAGTACCAACTTTCCGAG----------AGTTCGGAGCGAACGAATTCATAAGCC -10 (2)
TCGAAGGCCATACACTAAGTACCAACTTTCCGAGT----------GTTCGGAGCGAACGAATTCATAAGCC -10(1)
TCGAAGGCCATACACTAAGTACCAACTTTCCGAGC------GAGAGAATTCGGAGCGAACGAATTCATAAGCC +2 -6 (1)
TCGAAGGCCATACACTAAGTACCAACTTTCCGAGCGAAGGCCATACACTAAGTACCAAGAGAGTTCGGAGC
GAACGAATTCATAAGCC +17 (1)
TCGAAGGCCATACACTAAGTACCAACTTTCCGAGCCATACACTAAGTACCAACAGCCATACACTAAGTACC
AACAAGAGAGTTCGGAGCGAACGAATTCATAAGCC +34 (1)

Figure 11. **TALEN induced mutations in *Ciona* embryos.**

(A) Gel electrophoresis of PCR amplifications of *Ciona* genomic DNA electroporations. The PCR product has been treated with SURVEYOR nuclease prior to electrophoresis. PCR products came from untreated embryos (-) or TALEN electroporated embryos (+). Bands specific to the TALEN mutated DNA are shown by arrowheads. (B) Sequenced mutations detected by sequencing the PCR products shown in (A). The un-mutated sequence is shown on the first line followed by mutated sequences. The numbers in parenthesis after the gene name is the number of mutated sequences observed. TALEN binding regions are highlighted in blue.

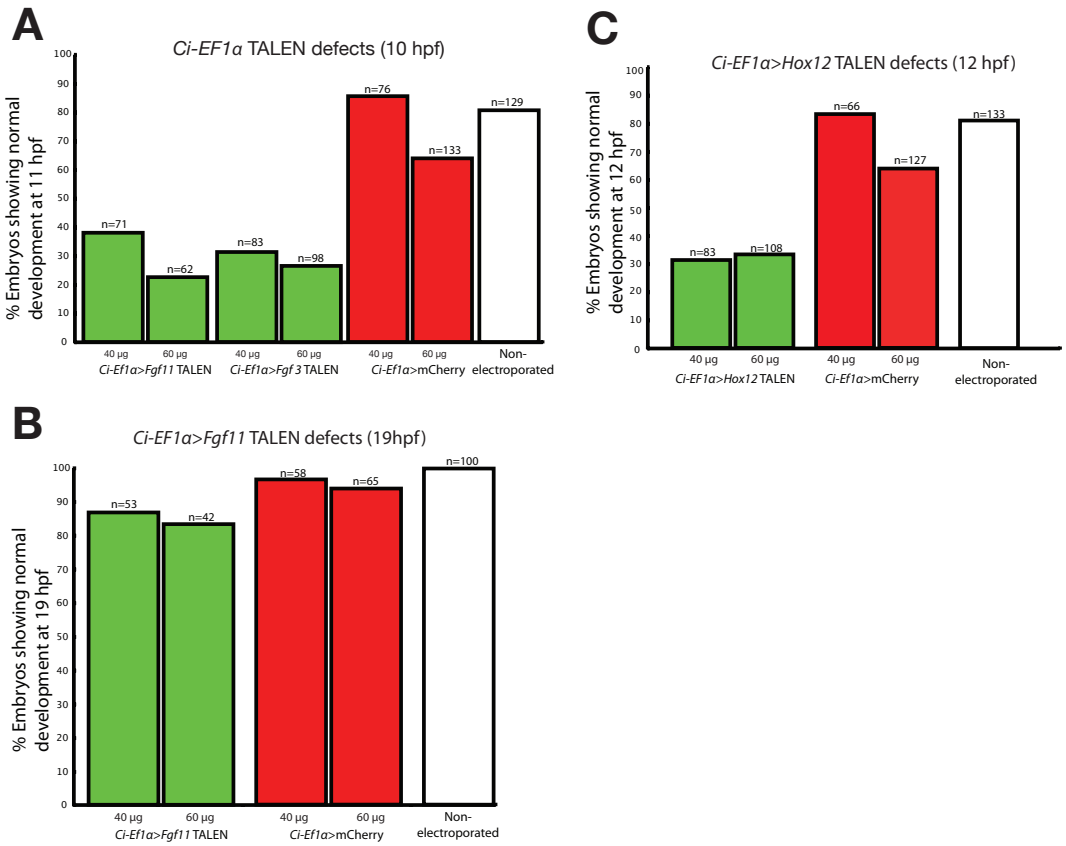



**Figure 12. Defects observed in embryos ubiquitous TALEN electroporations.** (A) Percentage of embryos showing normal development at 11 hpf after electroporation with constructs driving *CiFgf11* and *CiFgf3* TALEN expression using the *CiEF1a* promoter. (B) Percentage of embryos showing normal development at 19 hpf after electroporation with constructs driving *CiFgf11* TALEN expression using the *CiEF1a* promoter. Only embryos that showed normal development by 11 hpf were counted. (C) Percentage of embryos showing normal development at 12 hpf after electroporation with constructs driving *CiHox12* TALEN expression using the *CiEF1a* promoter. Amount of DNA present in the electroporation cuvette is indicated in µg.

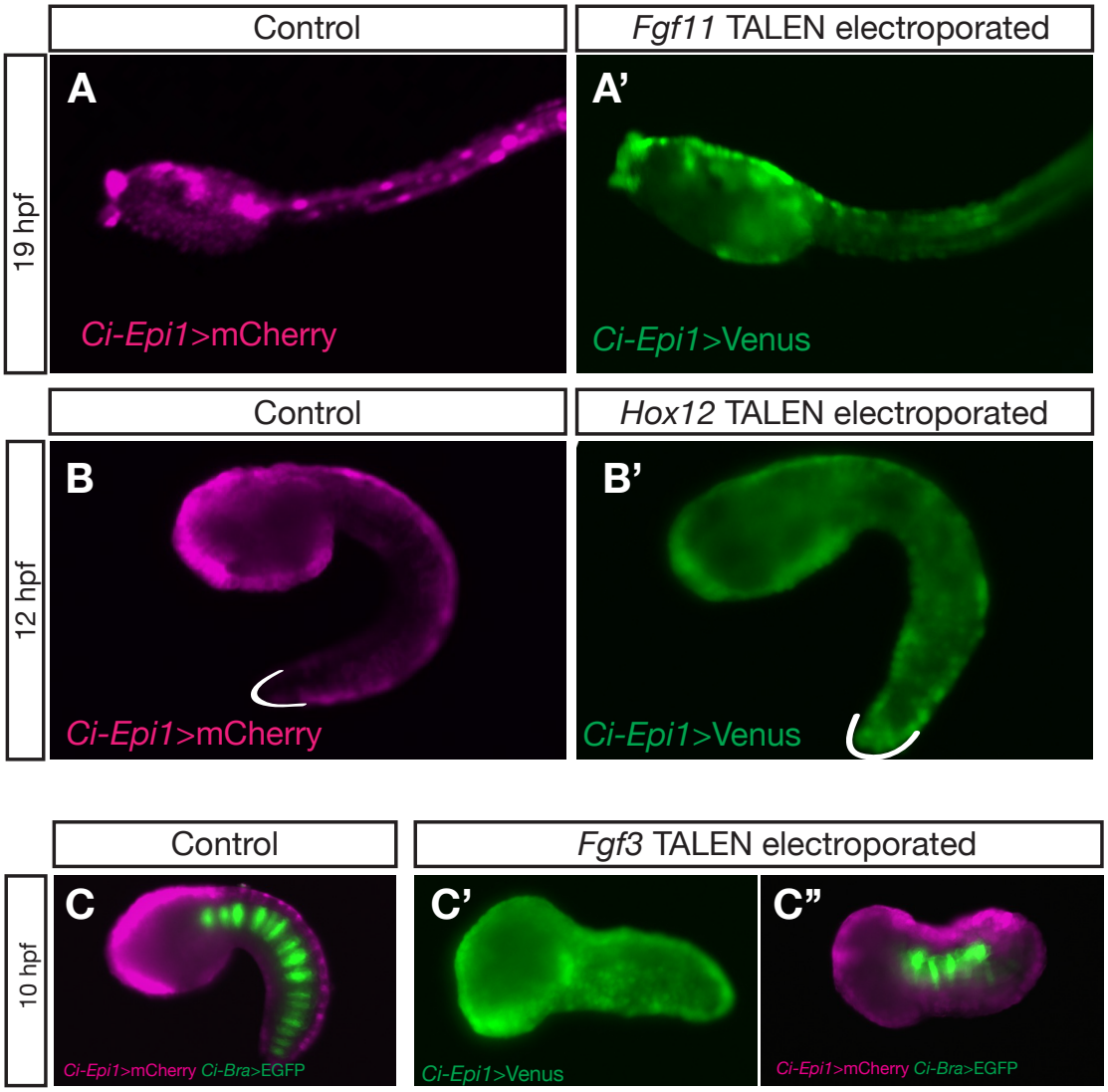
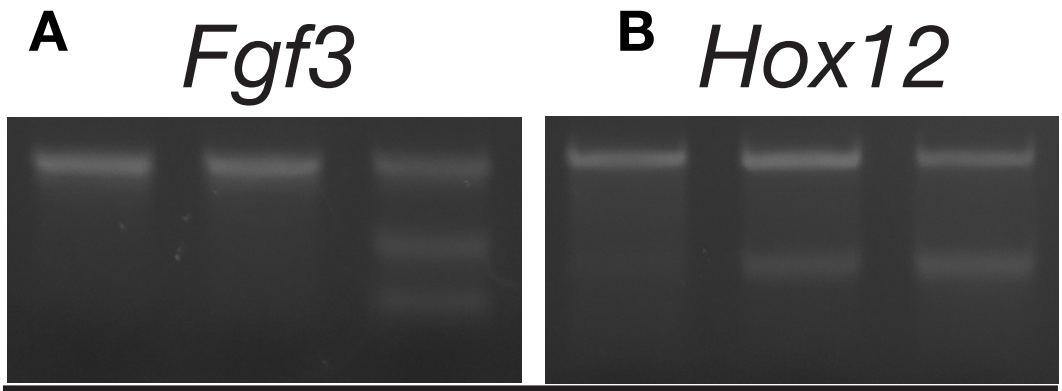



**Figure 13. Ubiquitous TALEN expressions in *Ciona*.**

Reporter constructs were electroporated without TALENs to demonstrate normal development (A, B, C). Fluorescent proteins were expressed in the epidermis (*CiEpi1*>mCherry) and the notochord (*CiBra*>EGFP). (A') *CiFgf 11* TALEN electroporated larva at 19 hpf showing normal development. (B') *CiHox12* TALEN electroporated embryo at 12 hpf. The shape of the tail tip is indicated with a white line (36/80 embryos with abnormal development had rounded tails). (C') *CiFgf3* TALEN electroporated embryo at 10 hpf showing multiple defects. (C") *CiFgf3* TALEN electroporated embryo at 10 hpf. Venus has been removed from the R-TALEN construct to observe failure of notochord intercalation using *CiBra*>EGFP (63/83 embryos had notochord defects).




| C | mCherry fluorescence intensity | | | | |
|---|---|---|---|---|---|
| Not electroporated | Low | High | Not electroporated | Low | High |
| Mutation rate | | | | | |
| - | 11% | 67% | - | 48% | 88% |

**Figure 14. Mutation rate relative to reporter gene expression level.**

Gel electrophoresis of PCR amplifications of *Ciona* genomic DNA electroporations. The PCR product has been treated with SURVEYOR nuclease prior to electrophoresis. Directly below each lane is displayed the mCherry fluorescent intensity for the embryos the genomic DNA was isolated from as well as the mutation rate detected from sequencing the PCR products. Number of samples sequenced were: *CiFgf3* low fluorescence - 19; *CiFgf3* high fluorescence - 18; *CiHox12* low fluorescence - 21; *CiHox12* high fluorescence – 17.

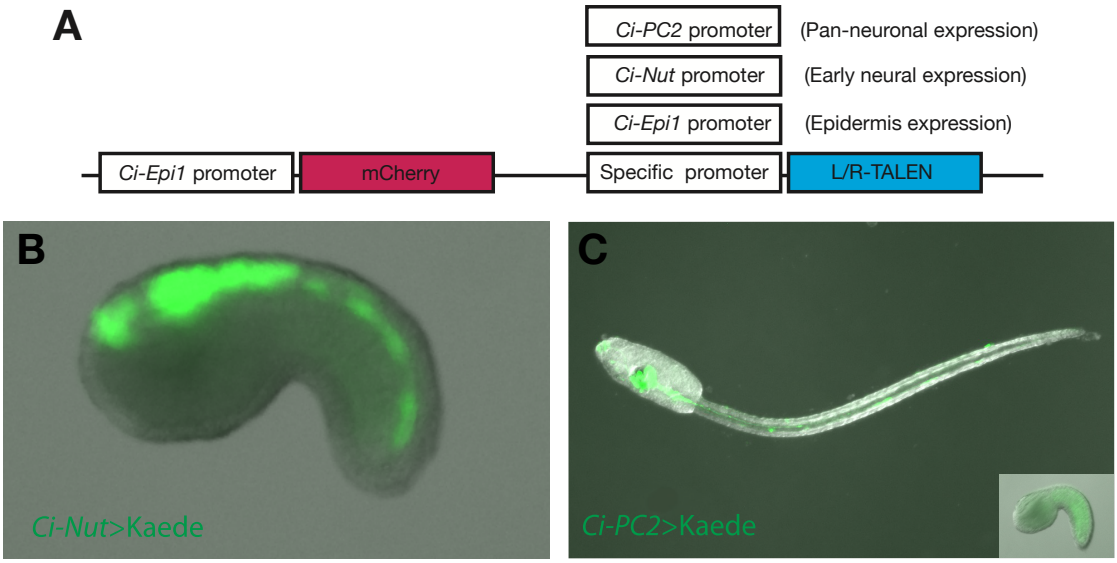



**Figure 15 Tissue specific TALEN expressions in *Ciona*.**

(A) A diagram showing the constructs used to drive tissue specific TALEN expression. mCherry is expressed in the epidermis (*CiEpi1*). TALENs are expressed in early neural cell lineages (*CiNut*, B), mature neuronal tissue (*C-PC2*, C), and epidermis (*CiEpi1*) C is a dorsal view of the larvae; inset image shows only autofluorescence at 9 hpf.

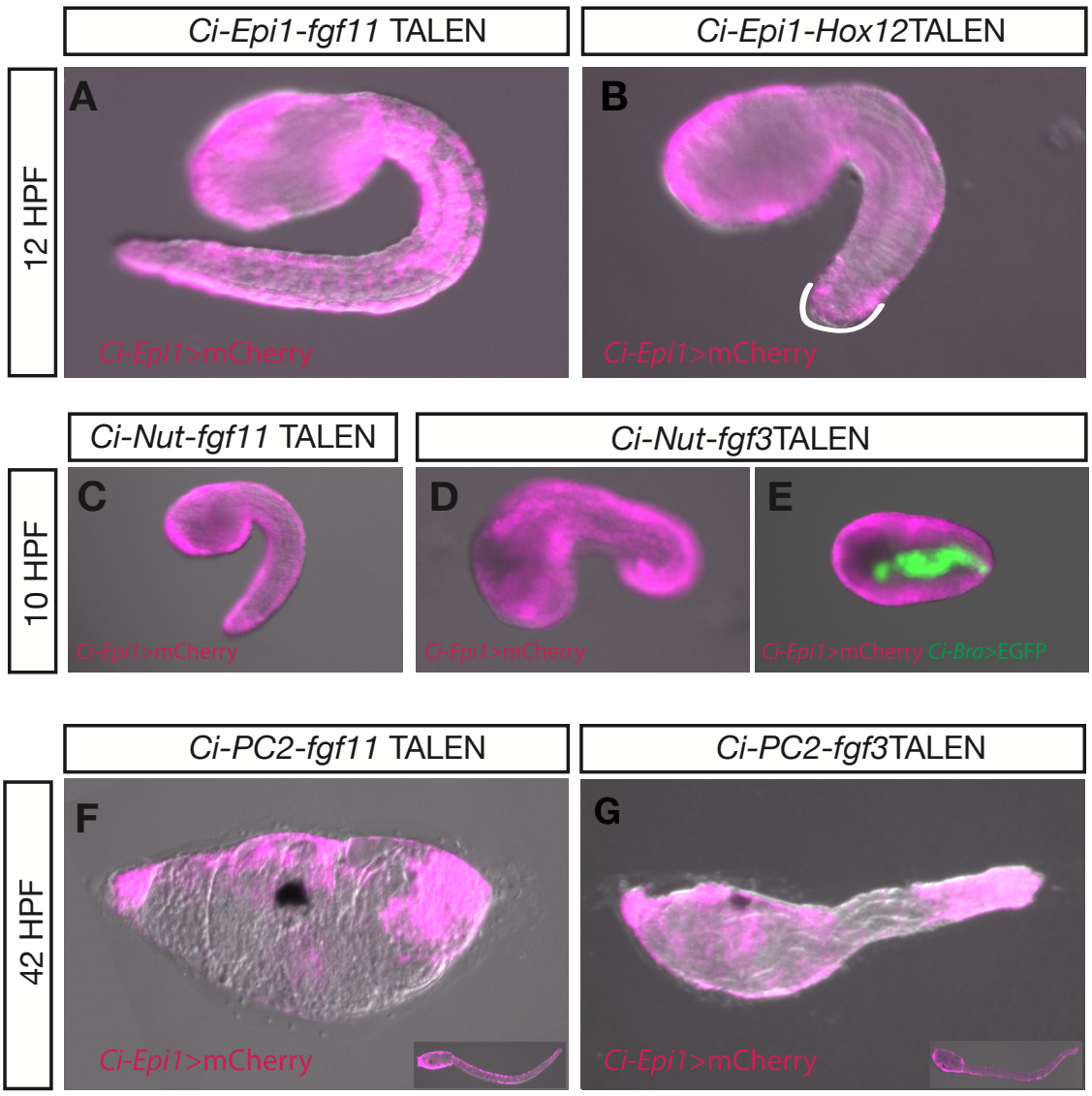



**Figure 16. Tissue specific TALEN gene knockouts in Ciona.**

Reporter constructs were electroporated with active TALENs targeting *CiFgf11* to demonstrate normal development at 12 hpf (A), 10 hpf (C) and 42 hpf (F), fluorescent proteins were expressed in the epidermis (*CiEpi1*>mCherry). (B) *CiHox12* TALENs expressed specifically in the epidermis. Tail rounding is indicated with a white line. (D) *CiFgf3* TALENs expressed specifically in neural tissue (E) *CiFgf3* TALENs expressed specifically in neural tissue. Notochord precursor cells are indicated with *CiBra>EGFP*. (G) *CiFgf3* TALENs expressed specifically in mature neuronal tissue in larvae undergoing metamorphosis. Inset images show normal development at 18 hpf.

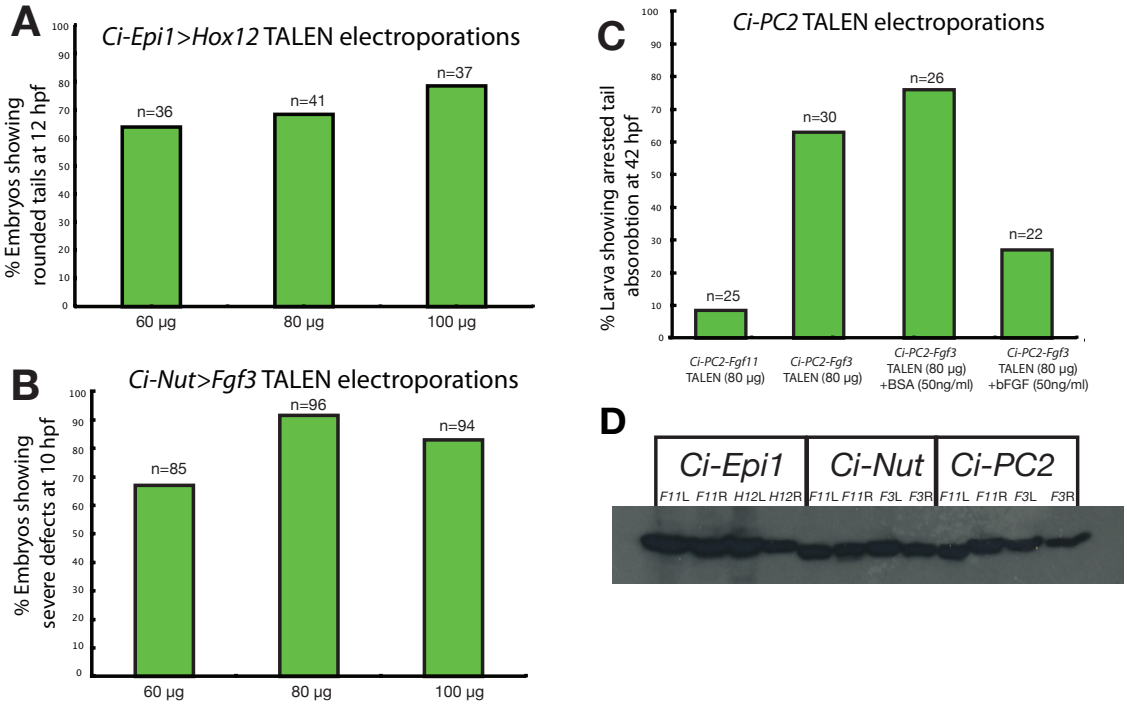



**Figure 17. Defects observed in embryos after tissue-specific TALEN electroporations.**

(A) Percent of embryos showing rounded tails at 12 hpf after electroporations with TALENs targeting *CiHox12* expressed in the epidermis. (B) Percent of embryos showing severe developmental defects at 10 hpf after expression with TALENs targeting *CiFgf3* expressed in neural tissues. (C) Percent of attached larva that failed to undergo complete tail absorption at 42 hpf after electroporation with TALENs targeting *CiFgf3* in mature nervous tissue. Amount of DNA present in the electroporation cuvette is indicated in µg. (D) Western Blot detecting FLAG-tag immunoreactivity of single TALEN electroporated embryos. *CiEpi1/Nut/PC2* indicates the *Cis*-regulatory regions used to drive TALEN expression. TALENs abbreviations are *F11L – Fgf11* left TALEN, *F11R – Fgf11* right TALEN, *H12L – Hox12* left TALEN, *H12R – Hox12* right TALEN, *F3L - Fgf3* left TALEN, *F3R – Fgf3* right TALEN.

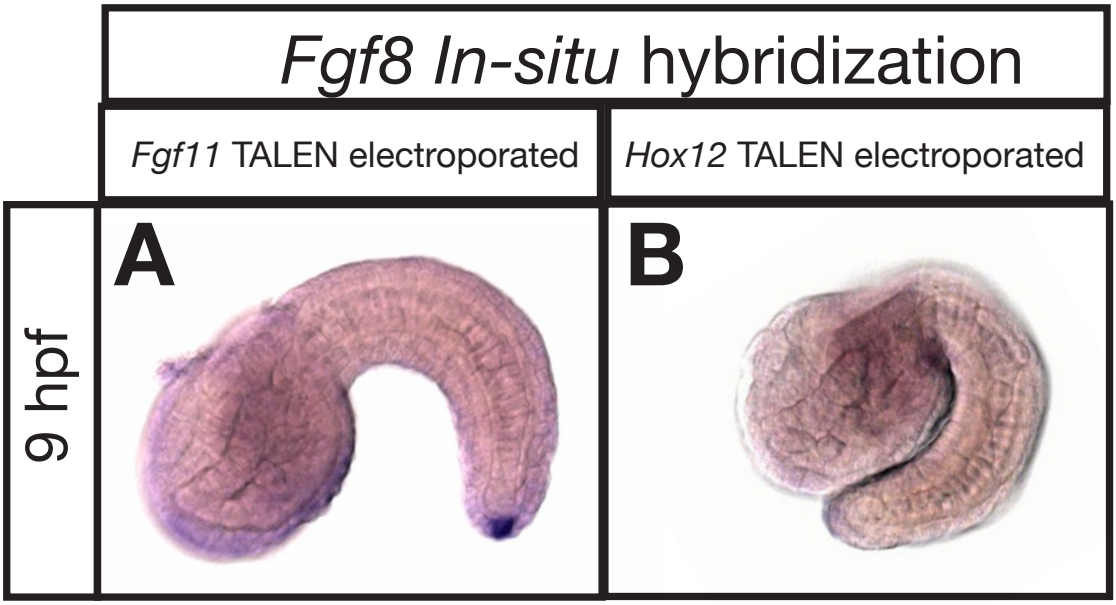



**Figure 18. Verification of TALEN gene disruption.**

*In-situ* hybridization of *Fgf8* expression in embryos electroporated with *CiFgf11* (A) and *CiHox12* (B) TALENs expressed in the epidermis using the *CiEpi1 cis*-regulatory regions. Observed expressions were seen in 19/19 embryos for *CiFgf11* mutants and 11/17 embryos for *CiHox12* mutants.

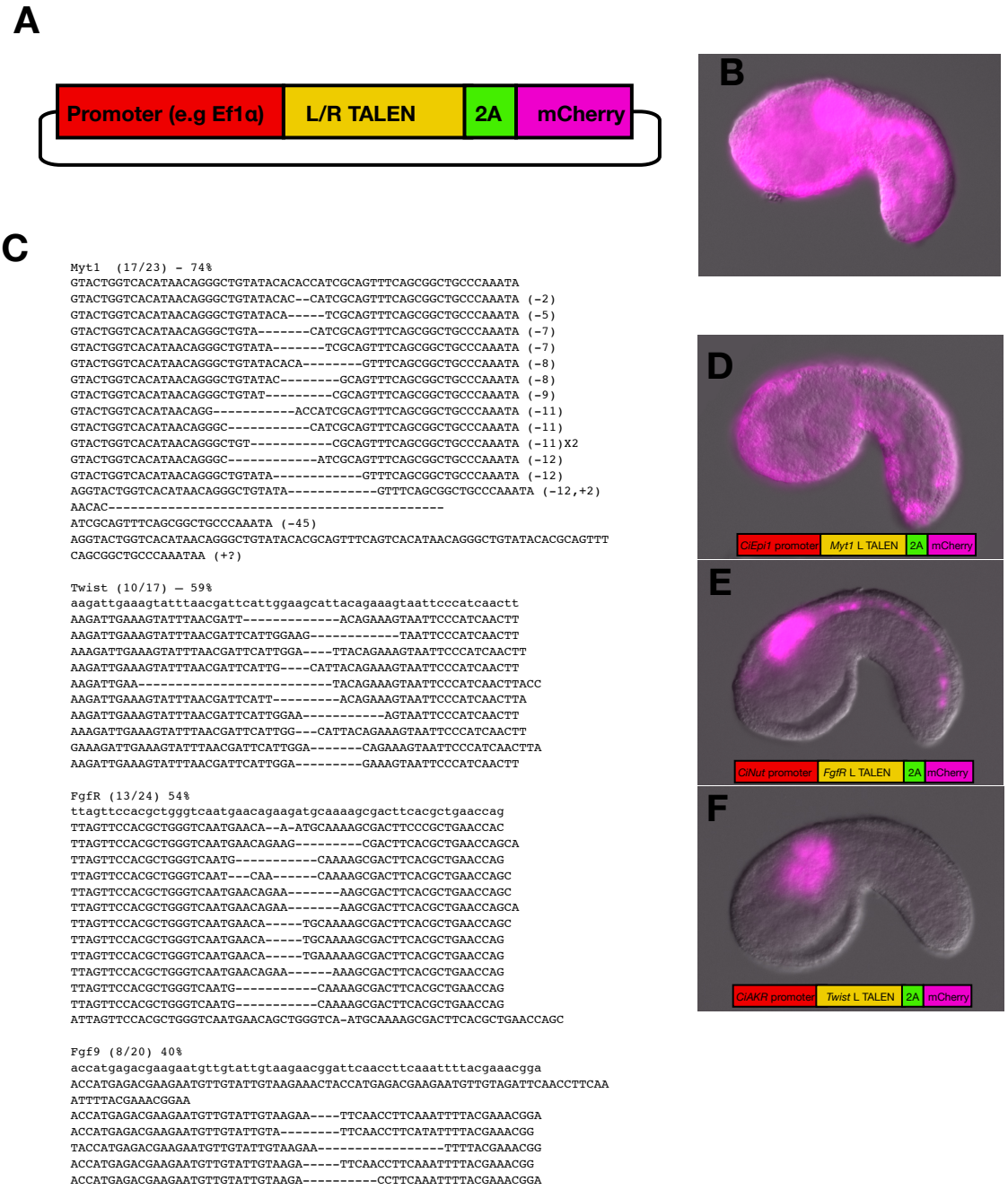



**Figure 19. An upgraded TALEN construct incorporating the TALEN and fluorescent reporter on a single transcript**

(A) Design of a TALEN construct driven by a ubiquitous (*CiEF1α*) promoter. The TALEN and mCherry portions of the cassette's open reading frame are flanked by a 2A peptide sequence that causes the ribosome to fail joining amino acids upon protein synthesis at that region. (B) *Ciona* embryo electroporated with a single TALEN (*CiMyt1* L TALEN-2A-mCherry) ubiquitous mCherry fluorescence can be seen. (C) Mutation rates

for sequenced PCR products of genes mutated by *CiEF1α* >TALEN-2AmCherry electroporations. (D-F) *Ciona* embryos electroporated with single TALEN-2A-mCherry constructs with tissue specific promoters for the epidermis (D, *CiEpiI* promoter), Neural cells (E, *CiNut* Promoter), and trunk mesenchyme (F, *CiAKR* promoter).