Tissue-specific and ubiquitous gene knockouts by TALEN electroporation provide new approaches to investigating gene function in Ciona

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ABSTRACT
Custom designed nucleases can simplify gene targeting experiments and have the potential to allow these techniques to be performed in a wide range of organisms. Transcriptional activator-like effector nucleases (TALENs) are starting to fulfill this potential with the advantages of low cost and fast construction times. Here, we report that TALENs are highly effective at inducing mutations in specific genomic loci in the ascidian chordate Ciona intestinalis. In Ciona there are well-established methods to introduce exogenous DNA by electroporation, and we show that this method can be used to introduce constructs that can express TALENs ubiquitously or in specific tissues. Our current protocols enable the rapid analysis of hundreds of TALEN-induced mutants. TALEN electroporations result in a high rate of mutations. These mutations can result in gene knockouts that recapitulate previously described functions of Fgf3 and Hox12. We show that TALENs can work efficiently to cause tissue-specific knockouts and demonstrate this by knocking out Hox12 in the epidermis and Fgf3 in neural tissues. We also use tissue-specific knockouts to reveal a new function of Fgf3 during ascidian larval metamorphosis.

KEY WORDS: Gene targeting, TALENs, Reverse genetics

INTRODUCTION
Gene targeting is well established in mice (Capecchi, 1989), making available a range of experimental approaches not possible in most other model organisms (Abzhanov et al., 2008). Custom designed nucleases have the potential to 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 demonstrated (Meng et al., 2008; Ochiai et al., 2010; Miller et al., 2011; Carlson et al., 2012; Lei et al., 2012). TALENs, in particular, have the potential to be exploited 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 ligation 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., 2013a; Sakuma et al., 2013b; Bedell et al., 2012). Ascidian tunicates are attractive as 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 available experimental techniques including transposon transgenesis (Sakuma 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 is then reliably expressed by hundreds of synchronously developing embryos (Corbo et al., 1997). Ciona electroporations are useful in cis-regulatory analysis (e.g. Roure et al., 2007; Johnson et al., 2004; Khoeiry et al., 2010; Imai et al., 2012). However, a reliable method for gene knockout does not exist in this organism and most data on gene function are based on knockdowns by morpholino antisense oligonucleotides. 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. We have previously reported the possibility of gene targeting in Ciona by microinjecting ZFN mRNA to reliably knockout transgenes (Kawai et al., 2012), but to date there have 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. Here, we show that TALENs can be used reliably in Ciona to introduce mutations at high frequency into embryos by electroporation, and that these TALENs can be used to perform tissue-specific gene knockouts.

RESULTS
TALEN construction
The TALENs used in this paper have a 136 amino acid N-terminal region that contains a FLAG tag, either 15.5 or 16.5 TAL repeats with the final repeat targeting a T nucleotide, and a 63 amino acid C-terminal region (Fig. 1A). The spacer region between the TALEN binding sites is 16-18 nucleotides long (Fig. 1B). The destination vector for the final ligation step during construction was modified...
for use in Ciona electroporation experiments with the Ci-EF1α (eukaryotic transcription elongation factor alpha – a ubiquitous promoter) (Fig. 1C,D) cis-regulatory elements driving TALEN expression. In the same construct we included a fluorescent reporter expressing Venus for the L-TALEN and mCherry for the R-TALEN, driven by the Ci-EpiI (epidermis-specific) cis-regulatory element (Fig. 1C). The constructs were simultaneously electroporated into fertilized eggs and if both reporters were expressed it was assumed that the TALEN pair was also expressed (Fig. 1D). These embryos could then be further analyzed to confirm that the TALENs had induced a DSB, and subsequent mutations at the targeted genomic loci could be detected by amplifying the targeted region by PCR and sequencing the products. The mutations induced by TALENs can result in disruption of the targeted genes.

Ubiquitous TALEN knockouts

We constructed TALENs targeting the genes Fgf11, Fgf3 and Hox12 by Golden Gate ligations and inserted them into Ci-EF1α TALEN vectors. These constructs (40-80 μg DNA) were electroporated into fertilized Ciona eggs. 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 isolated during the tailbud stage and the TALEN-targeted region amplified by PCR. Mutations could be detected by a SURVEYOR assay (Fig. 2A), in which DNA is cut if any mismatched base-pairing lesions are present. In all three cases, PCR products from TALEN-electroporated embryos showed additional bands after melting, reannealing and incubation with the SURVEYOR nuclease. PCR products from these electroporations were sequenced and mutations were detected (Fig. 2B; supplementary material Fig. S1). The mutation rate from the sequenced DNA was between 71% and 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 inducing mutations in Ciona embryos. Recent large-scale studies have reported TALEN mutation efficiencies between 3% and 95% (Reyon et al., 2012; Kim et al., 2013), and the TALEN knockouts we have performed fall at the top end of this range.

Ciona has several genes that are considered orthologous to vertebrate Fgfs, of which Fgf11 has no detectable expression during development and no known function in Ciona (Satou et al., 2002); also, any functions that the vertebrate equivalent of this gene might have are not well understood as the Fgf11 protein 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, no knockout phenotypic effects should be 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, ~100 early to mid-gastrula embryos were isolated and left to develop to a stage when 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 (supplementary material Fig. S2), even when 40 μg, a relatively low amount of DNA, was electroporated. Therefore, there is some toxicity associated with Ci-EF1α-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 (Fig. 3A; supplementary material Fig. S2). If the abnormal development observed 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, whereas the opposite was observed. It has been shown in human cells that TALEN cytotoxicity appears to be related to TALEN protein length and has no correlation to the mutational capabilities of the TALEN pair (Reyon et al., 2012). The Ci-EF1α promoter shows some degree of toxicity when it is used to drive mCherry expression by itself, possibly owing to the high volume of protein produced by this promoter. Most of these defects appear at 11 hours post-fertilization (hpf), when neurulation and notochord intercalation have just finished. Therefore, it is probable that the high level of defects seen from the EF1α-driven Fgf11 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 is cell cycle defects caused by the need to repair broken DNA strands.

Hox genes in Ciona have remained poorly described, with the exception of Hox1 (Sasakura et al., 2012) and Hox12 (Ikuta et al., 2010). In Ciona, Hox12 is expressed in the posterior tail tip, and when Hox12 is knocked down using morpholinos a rounding of the tail tip is observed. When Ci-EF1α–Hox12 TALENs were electroporated, a high level of defects was observed (supplementary material Fig. S2C), including the previously described tail tip rounding (Fig. 3B).

Fgf3 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 Fgf3 have been shown to disrupt the cell convergent extension of notochord intercalation (Shi et al., 2009). The explanation for this is that 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 larval tail. Ci-EF1α–Fgf3 TALEN electroporations were capable of reproducing the function of Fgf3 described from morpholino knockdowns. A high proportion of Fgf3 TALEN embryos showed defects at the tailbud stage. We replaced the Venus reporter in the Fgf3 L-TALEN with mCherry and co-electroporated Ci-Bra-GFP (notochord promoter) in order to clearly visualize the notochord precursor cells. These embryos demonstrated a failure to intercalate the notochord cells (Fig. 3C).

The mutation rate and relative intensity of the Cel-I assay bands was evaluated in greater detail (supplementary material Fig. S3). Those embryos displaying the highest and lowest levels of mCherry fluorescence from an individual electroporation were isolated and the mutation rate estimated by Cel-I assay and sequencing of Hox12. A correlation could be seen between the intensity of reporter gene expression and the mutation rate, with high mCherry expression resulting in a higher mutation rate.

Based on these results, the electroporation of constructs driving ubiquitously expressed TALENs is very effective at inducing specific mutations. These mutations are capable of recreating previously described phenotypes induced using morpholinos. However, at least with the Ci-EF1α promoter driving TALEN expression, a high level of side effects can be seen. One concern with this methodology is the potential for electroporated DNA to show mosaic expression in Ciona. This is a common concern with all Ciona electroporations and we consider it not to be a serious problem provided there is appropriate screening of the electroporated embryos. The Ci-EF1α TALENs are very good at demonstrating the efficiency of TALENs to cause DSBs and subsequent mutations and for confirming the functions of genes, but to investigate new functions a conditional knockout strategy might be more useful.

Tissue-specific TALEN knockouts

Many genes involved in embryonic development show a range of pleiotropic functions, some of which are lethal when disrupted (Duffy, 2002); a conditional knockout strategy could be of value in understanding these processes in greater detail. Fgf3 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 Ci-EF1α–expressed TALENs were very efficient at causing mutations, we 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 (Fig. 4A). We used cis-regulatory elements driving TALEN expression in the epidermis (Ci-Epi1), early neural lineage (Ci-Nut) and in mature neurons (Ci-PC2) to perform tissue-specific knockouts (Fig. 4A, A″). The TALENs we used contain an N-terminal FLAG tag. A western blot for this tag from embryos electroporated with the left or right TALEN arms for all TALENs used in the tissue-specific knockout experiments
showed high levels of TALEN protein to be present (supplementary material Fig. S4).

When *Hox12* TALENs were specifically expressed in the epidermis a high level of tail rounding was seen (Fig. 4B). This was as high as 78% when 100 μg DNA was electroporated (supplementary material Fig. S5A). 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. *Ci-Fgf8/17/18* is expressed at the tip of the tail, and *Hox12* morphants show a failure to maintain expression of this gene during tail growth (Ikuta et al., 2010). When *Ci-Epi1>Hox12* TALENs were electroporated the *Ci-Fgf8/17/18* transcript could not be observed by WISH (supplementary material Fig. S6), confirming that the phenotypes caused by *Hox12* TALENs reflect the endogenous function of this gene. The proportion of embryos showing tail rounding and reduced *Ci-Fgf8/17/18* expression was less than 100%. This might 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.

*Fgf3* TALENs were specifically expressed in neural tissues with the early neural promoter of *Ci-Nut* (Fig. 4C). Electroporation of *Fgf3* TALENs using this promoter is expected to cause mutations and disrupt the function of *Fgf3* from an early stage. A high level of defects was seen in the majority of embryos electroporated with *Fgf3* TALENs in neural tissues (supplementary material Fig. S5B). These embryos showed a similar range of phenotypes to the *Ci-EF1α* TALEN electroporations (Fig. 4C) and previously described morpholino knockdowns, with a range of major defects varying in severity and timing (Shi et al., 2009).

When *Fgf3* TALENs were expressed using the *PC2* promoter that drives downstream genes after convergent extension of the notochord, normal development was seen and the swimming larvae 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 *Fgf3* TALENs were expressed throughout the nervous system before and during metamorphosis, tail absorption was arrested (Fig. 4D; supplementary material Fig. S5C). *Fgf11* TALENs expressed through the same promoter did not result in an arrest of tail absorption (supplementary material Fig. S5C). When larvae were treated with bFGF after hatching, but prior to attachment, tail metamorphosis proceeded normally (supplementary material Fig. S5C), 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 needed for tail absorption. Another possible explanation is 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.

**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 employed mRNA microinjections (Carlson et al., 2012; Lei et al., 2012; Kawai et al., 2012), which 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 a knowledge of cis-regulatory regions to drive the expression of TALENs. TALEN mRNA microinjections should also be useful in *Ciona*, but *Ciona* microinjections are difficult and time consuming compared with electroporation. For other non-standard model organisms, mRNA injection will remain the most feasible method for the foreseeable future, but for model organisms in which reporter constructs are routinely used, such as zebrafish and *Xenopus*, the techniques that we have described will be immediately applicable for tissue-specific knockouts. For *Ciona*, the combination of TALEN...
knockouts with well-established electroporation protocols allows mutants to be generated and quickly screened, involving numbers of embryos not possible with other model organisms, allowing greater confidence in the functional effects of the mutations. A major concern with TALENs is toxicity. Our results show that this still remains a serious concern for ubiquitous knockouts using the Ci-EF1α promoter, but using tissue-specific promoters the level of toxicity appears to be minimal.

There are numerous roles for Fgf signaling at many stages of Ciona development (e.g. Stolfi et al., 2011; Davidson et al., 2006; Kourakis and Smith, 2007). We have revealed 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 conditional knockout 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 presented in this paper provide a demonstration of how this could be done. TALEN technology has the potential to overcome existing barriers to achieving gene knockout and allows a range of gene functional studies to be performed in ways not previously possible.
to remove any traces of plasmid DNA. Where necessary, BSA or human basic FGF (bFGF or FGF2; Invitrogen) was added to the developing embryos at 18 hpf.

**Detection of TALEN mutations**

TALEN-electroporated embryos were observed for expression of Venus and/or mCherry fluorescence. Then, 50-100 embryos with strong expression were isolated and genomic DNA was extracted using a the Wizard Genomic DNA Isolation Kit (Promega) according to the manufacturer’s instructions. TALEN-targeted regions were amplified by PCR using a proofreading polymerase (PrimeSTAR HS DNA polymerase, Takara). The primers used for PCR were: 5′-GATCCATGACCTGACGGATTTAGC-3′ and 5′-CTACCTCTAACCCTTCAATGGCCC-3′ for Fgf11; 5′-CTTCAACTAGTAAGATCGGGGACCAG-3′ and 5′-CTACATTCCGGGATCTTGATGTC-3′ for Fgf5; and 5′-TGGAGCTACGACCATGTA-3′ and 5′-ATCTGGTCTCTTACGACAGTG-3′ for Ci-β-catenin.

For each sample, 20 electroporated embryos showing high levels of mCherry fluorescence were isolated, placed in an SDS/2-mercaptoethanol loading buffer and separated by electrophoresis on a 15% polyacrylamide gel and treated with anti-FLAG IgG (1:5000; clone M2; Sigma-Aldrich, F3165) as primary antibody, followed by HRP-labeled anti-mouse IgG (1:20,000; Invitrogen, 62-6520) as secondary antibody. Reactive bands were visualized by treatment with ECL-Plus (GE Healthcare).

**Assessment of TALEN mutant phenotypes**

Images of TALEN mutants were taken with an Axio Imager Z.1 fluorescence microscope (Carl Zeiss). To assess TALEN mutations at the tailbud stage, ~100 embryos were isolated during the early to mid-gastrula stage, before any defects were expected. These embryos were then allowed to develop to the tailbud stages, when morphological defects were observed. For assessing 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, ~100 hatched larvae were isolated in a clean Petri dish and left to attach. Any treatments were added to the seawater in the Petri dish at 18 hpf, and larvae were then assessed at 42 hpf for tail absorption.

**In situ hybridization**

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

**Western blot**

For each sample, 20 electroporated Ciona embryos showing high levels of mCherry fluorescence were isolated, placed in an SDS/2-mercaptoethanol loading buffer and separated by electrophoresis on a 15% polyacrylamide gel. The gel was then transferred to a polyvinylidene fluoride membrane and treated with anti-FLAG IgG (1:5000; clone M2; Sigma-Aldrich, F3165) as primary antibody, followed by HRP-labeled anti-mouse IgG (1:20,000; Invitrogen, 62-6520) as secondary antibody. Reactive bands were visualized by treatment with ECL-Plus (GE Healthcare).

**References**


