Islet is a key determinant of ascidian palp morphogenesis

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ABSTRACT

The anterior-most ectoderm of ascidian larvae contains the adhesive papillae, or palps, which play an important role in triggering the metamorphosis of swimming tadpoles. In Ciona intestinalis, the palps consist of three conical protrusions within a field of thickened epithelium that form late in embryogenesis, as tailbuds mature into larvae. The palp protrusions express the LIM-homeodomain transcription factor Islet. Protrusion occurs through differential cell elongation, probably mediated by Islet, as we find that ectopic expression of Islet is sufficient to promote cell lengthening. FGF signaling is required for both Islet expression and palp morphogenesis. Importantly, we show that Islet expression can rescue the palp-deficient phenotype that results from inhibition of FGF signaling. We conclude that Islet is a key regulatory factor governing morphogenesis of the palps. It is conceivable that Islet is also essential for the cellular morphogenesis of placode-derived sensory neurons in vertebrates.

KEY WORDS: Islet, LIM domain, Ascidian, Cell shape, Palps, Placode

INTRODUCTION

Ascidians belong to the Subphylum Tunicata, and, as such, represent the sister group to the vertebrates (Delsuc et al., 2006). The experimental tractability, genomic simplicity and phylogenetic placement of ascidians allow for analysis of evolutionary origins of key vertebrate innovations, such as the second heart field and cranial placodes (Mazet et al., 2005; Stolfi et al., 2010). The vertebrate placodes are transient, focal ectodermal thickenings that arise within a horseshoe-shaped territory flanking the anterior neural plate in developing embryos. They contribute to the paired sense organs of the head as well as sensory components of cranial ganglia (Schlosser, 2010; Streit, 2008), and are believed to have been an important factor in the radiation of vertebrates (Gans and Northcutt, 1983).

The adhesive papillae, or palps, of ascidian tadpoles are a specialized region of the anterior-most ectoderm that might represent an ancestral placode. They develop from an ectodermal thickening that arises at the anterior border of the neural plate, and give rise to peripheral neurons, the axons of which project into the sensory vesicle (simple brain) (Imai and Meinertzhagen, 2007; Takamura, 1998). The cell lineage that gives rise to the palps expresses a variety of transcription factors that function in vertebrate placode development, including eyes-absent, DMRT, FoxG, Emx, COE, Dlx-c and Islet (Caracciolo et al., 2000; Giuliano et al., 1998; Mazet et al., 2005; Park and Saint-Jeannet, 2010; Tassy et al., 2010; Tresser et al., 2010). Papillae of various ascidian species are reported to consist of three cell types: secretory cells and two types of neurons (Dolcemascolo et al., 2009; Imai and Meinertzhagen, 2007). The palp neurons have been proposed to function in both chemo- and mechanosensory.

The palps perform two crucial and related functions for the tadpole. First, they secrete the adhesive substance(s) and thus serve as the attachment site when larvae settle upon a solid substrate. Second, this attachment event serves as the trigger for the complex, multistep process of metamorphosis, in which the motile larval body plan is reorganized into a sessile, filter-feeding form (Nakayama-Ishimura et al., 2009; Sasakura et al., 2012). Arguably, the choice of settlement site is the single most important event in the life history of ascidians, as it will directly impact on opportunities for feeding and reproduction, which for sessile animals are limited by the immediate environment. There is evidence that ascidian larvae discriminate among possible settlement sites, responding to both biotic and abiotic factors (Groppelli et al., 2003; Pennati et al., 2009; Svane and Young, 1989; Torrence and Cloney, 1983), and it seems probable that neural activity in the palps controls both settlement and metamorphosis.

Despite the importance of the palps for ascidian biology, their development and physiology remained poorly defined. To better understand palp development, we performed expression profiling on sorted palp cells and identified Emx as enriched in the palp lineage. At the late tailbud stage, Emx is expressed in a striking ring-shaped pattern, and this discovery prompted our investigation of Islet, which is expressed in the center of the Emx rings. We show that expression of Islet correlates with protrusion of the palps in maturing tadpoles and that protrusion occurs by differential cell lengthening within the placode epithelium. Additionally, we find that Islet misexpression throughout the palp ectoderm promotes the protrusion of a single, large palp. Furthermore, ectopic expression of Islet in non-placodal ectoderm is sufficient to promote cell elongation. The fibroblast growth factor-MAP kinase (FGF-MAPK) signaling cascade is required for Islet expression in the palp primordia of tailbud stage embryos. Whereas perturbation of FGF signaling in the anterior neural tissue inhibits development of the palps, expression of Islet can rescue this defect. Thus, we conclude that Islet is a key regulatory factor governing morphogenesis of the palps.

RESULTS

Emx, Buttonhead and Islet mark the presumptive palps

In an effort to identify new genes involved in development of the palps, we performed expression profiling on isolated cells from the palp lineage. The mouse cell surface antigens CD4 and CD8 were expressed in various tissues of the embryo, which allows enrichment of specific cell populations with antibody-coupled magnetic beads. CD4 was expressed in the palp lineage using the FoxC enhancer. CD8 was expressed with the ZicL enhancer and used for negative selection of the central nervous system (CNS), muscles and mesenchyme. Negative selection was important because the FoxC enhancer sometimes drives low-level ectopic expression in parts of the CNS.
RNA was isolated from the sorted palp cells and from the CD8-expressing cell population and processed for hybridization to Affymetrix GeneChip microarray (GEO number GSE57920; see Methods in the supplementary material for details of the cell sorting and expression profiling protocols).

We found that the homeodomain transcription factor Emx (empty spiracles, ems in Drosophila) was enriched in the palp lineage of neurula stage embryos (5.1-fold enrichment, P=4.4×10⁻⁴). Interestingly, a previously published in situ pattern for Emx showed it to be expressed in an arc-shaped pattern in the anterior neural plate (Imai et al., 2004). This pattern appears similar to that of another homeodomain transcription factor, Six1/2 (Imai et al., 2004), so we performed double in situ hybridization (ISH) to characterize these patterns in more detail. We found that Emx and Six1/2 are expressed in the anterior-most region of the neural plate in sequential arc-shaped stripes (Fig. 1A-A‴). Emx is mainly expressed more anteriorly, but is also detected in some of the adjacent posterior Six1/2-expressing cells. In tailbud stage embryos, we found that Emx is expressed in a ring-shaped pattern corresponding to the three presumptive palps and also in the epidermis overlying the sensory vesicle (Fig. 1B,B‴).

We next compared the ring-shaped Emx pattern to those of two known palp markers, Buttonhead (Btd) and Islet. Btd (also known as Sp8 or ZF220) is expressed in the palp lineage at the early tailbud stage, downstream of FoxC, the earliest marker of the palp lineage (Ikeda et al., 2013; Imai et al., 2004, 2006). In late tailbud embryos, Btd appears to be co-expressed with Emx in the rings, and also occurs in the intervening anterior-most ectoderm, but is specifically excluded from the center of the rings (Fig. 1C-C‴). The LIM-homeodomain (LIM-HD) transcription factor Islet marks a number of vertebrate placodes and, in Ciona, is expressed in three discrete foci marking the presumptive palps (Giuliano et al., 1998; Park and Saint-Jeannet, 2010). We found that Islet is expressed in the center of the Emx rings, and might also be co-expressed with Emx in the rings, but not in the intervening ectoderm (Fig. 1D-D‴).

**Islet expression correlates with palp protrusion**

Upon closer inspection, we found that Islet expression correlates with protrusion of the palps during maturation of tailbud embryos into larvae. Islet transcripts are detected in the three presumptive palps of late tailbud embryos (Fig. 2A). A close-up view of an individual palp reveals that the Islet transcript is specifically detected in the protrusion, as revealed by Hoechst counterstaining (Fig. 2B-C‴). We have isolated an enhancer for Islet located in the first intron that drives reporter (mCherry) expression in the palps, and also sometimes in the notochord and the pigmented otolith, which are also normal sites of Islet expression (Fig. 2D). The anterior ectoderm of the late tailbud adopts a thickened, columnar epithelial shape, characteristic of ectodermal placodes. The means by which the palps protrude from the anterior surface of the embryo appears to be a simple cell shape change within this thickened epithelium (Manni et al., 2004). The cells expressing the Islet reporter are elongated in comparison to the neighboring, non-expressing cells (Fig. 2E,E‴). In mature larvae, the tips of the palps bear fine, fingerlike projections, as revealed by phalloidin staining (Fig. 2F,G). Of note, we find that these projections derive from the Islet⁺ cells in the palps (Fig. 2G‴).

We then measured the correlation between protrusion of the palps and expression of the Islet reporter in late tailbud embryos. Tailbuds that develop three detectable palp protrusions express the Islet reporter to the greatest extent (Fig. 2H). Sometimes the orientation of the embryo on the slide makes it difficult to clearly discern all three palps, and sometimes, even under control conditions, the palps do not develop normally. The trend is clear, however: as the number of detectable palp protrusions decreases, so does expression of the

**Fig. 1. Emx, Btd and Islet mark the presumptive palps.**

(A) Double ISH of late neurula stage embryo. Emx transcripts detected with fluorescein-labeled probe (green) are expressed in the anterior-most neural plate, as well as the posterior (tail) ectoderm. Six1/2 transcripts detected with DIG-labeled probe (red) are expressed in anterior neural plate posterior to the Emx expression domain. (B) Late tailbud stage embryo hybridized with Emx probe (DIG-labeled). Emx is expressed in three rings that delimit the presumptive palp protrusions, and also weakly in the dorsoanterior ectoderm. (C) Double ISH, late tailbud embryo. Emx transcript is detected with fluorescein-labeled probe (green); two of three presumptive palps are shown. Btd transcripts are detected with DIG-labeled probe (red) and occur with Emx in the rings and also in ectoderm between the rings. (D) Double ISH of late tailbud embryo. Islet probe (DIG-labeled, red) is detected in spots corresponding to the presumptive palp protrusions and is flanked by rings of Emx expression (fluorescein-labeled, green). (A′,A″,C″,D″) Close-up views of corresponding panels, focused on palp region. Nuclei are stained with Hoechst 33342. (A′,A″,B″,C″,D″) In situ signal of respective single probes shown for clarity. Scale bars: 10 μm.
lineage at the 112-cell stage, the time at which palps become specified. Expression of the control transgene FoxC>lacZ does not alter palp morphogenesis, as visualized by FoxC>H2B:Cherry and Islet>YFP-caax (membrane-targeted YFP) reporter genes (Fig. 3A). However, upon misexpression of full-length Emx (FoxC>Emx), or a constitutive repressor form consisting of the DNA-binding domain fused to the WRPW repressor motif (FoxC>Emx:WRPW), loss of Islet reporter expression occurs in the palps (Fig. 3B,C,E). Mosaic misexpression of the FoxC>Emx and FoxC>Emx:WRPW transgenes results in corresponding losses of individual palp protrusions, whereas the unelectroporated halves develop a normal palp (Fig. 3B,C).

These results suggest that Emx acts as a transcriptional repressor, which is consistent with the presence of a conserved engulfed homology domain in the N-terminus (Jackman et al., 2000). Thus, it is possible that the ring-shaped pattern of Emx expression (Fig. 1B) in the presumptive palps functions to limit the expression of Islet to discrete foci, but it is currently unknown whether the endogenous Emx repressor regulates Islet in vivo.

**Islet misexpression promotes protrusion of a single, large palp**

We next tested the effect of Islet misexpression on palp development. We hypothesized that ectopic Islet expression throughout the palp ectoderm might drive the protrusion of a single palp rather than three distinct entities. Indeed, we found that expression of FoxC>Islet led to the formation of a single, large palp (Fig. 3D, compare with Fig. 3A). We further found that embryos expressing a repressor form of Islet, FoxC>Islet:WRPW, fail to express the Islet reporter and to protrude palps (Fig. 3F). This suggests that Islet acts as a transcriptional activator, and that Islet target genes are required to drive protrusion of the palps.

Islet expression in the palps might be subject to autoregulatory feedback. Expression of the Islet>YFP-caax reporter gene is restricted to discrete foci in control embryos (Fig. 3A), but is ectopically activated throughout the palp ectoderm upon expression of the Islet-coding sequence using the FoxC enhancer (Fig. 3D). FoxC>Islet:WRPW not only repressed palp protrusion but also eliminated expression of the Islet>YFP-caax reporter gene, although expression persisted in the notochord cells, which did not express Islet:WRPW (Fig. 3F). Thus, a positive autoregulatory mechanism might help to ensure maintenance of Islet expression following onset. In zebrafish embryos, a similar autoregulatory mechanism serves to maintain Isl2 expression in Rohon–Beard neurons and sensory neurons of the trigeminal placode (Segawa et al., 2001).

We next asked whether Islet misexpression could repress Emx, to determine whether a mechanism of mutual repression might be responsible for their complementary expression patterns in the palps. We found that expression of FoxC>Islet did not affect Emx expression in the palps (supplementary material Fig. S2). We then tested the effect of simultaneous co-expression of Emx and Islet on palp development, to determine whether Islet expression could overcome the repressive effect mediated by Emx. Indeed, a protrusive single palp forms in the presence of FoxC>Emx and FoxC>Islet (Fig. 3G), similar to that develops in the presence of FoxC>Islet alone. This suggests that Islet activity is epistatic to Emx in the regulation of target genes required for palp protrusion.

**Islet promotes cell elongation**

The single large palp that forms upon FoxC>Islet expression appeared to have cells elongated beyond the range seen under
normal conditions. The palps, however, might be biased toward cell elongation because they derive from a placode. We therefore expressed Islet in a region of non-placodal ectoderm to assay its effect on cell shape. An enhancer for the FoxF gene has been described (Beh et al., 2007), which drives expression in the trunk and tail ectoderm. We examined mosaic embryos and found that expression of FoxF>Islet results in trunk ectodermal cells that are elongated in comparison to cells on the unelectroporated side of the embryo (Fig. 4B-B”). By contrast, trunk ectoderm cells of mosaic embryos expressing the FoxF>lacZ control plasmid are of similar size (Fig. 4A-A”).

To better characterize this effect, we analyzed 20 mosaic embryos, each expressing either FoxF>lacZ or FoxF>Islet, and normalized cell lengths on the perturbed side to cell lengths on the unelectroporated side. We found that cells of control embryos show little variation in size (Fig. 4C; supplementary material Fig. S3). Expression of FoxF>Islet, however, reproducibly promotes cell lengthening, with effects ranging from ~1.3-fold to 2.2-fold as compared with unelectroporated cells in the same embryo (Fig. 4C; supplementary material Fig. S4). These results are highly significant, with \(P=6.8 \times 10^{-8}\), according to the Wilcoxon two-sample test. We conclude that Islet expression is sufficient to promote cell shape changes in an ectopic context. Islet might thus function both as a determinant of cell identity and a regulator of cell shape (see Discussion).

**FGF-MAPK signaling is required for proper Islet patterning**

Previous work identified the FGF-MAPK signaling pathway as an important regulator of both specification and subsequent morphogenesis of the palps (Hudson et al., 2003; Wagner and Levine, 2012). Specifically, we showed that perturbation of FGF-MAPK signaling led to ectopic FoxC expression, but larvae that developed under these conditions failed to develop palps. A recent study revealed that, whereas FoxC is a marker of the palp lineage, it is not a marker of palp fate; FoxC expression in the palp lineage persists under conditions that inhibit palp development (Ikeda et al., 2013). Moreover, Islet expression is lost upon MAPK inhibition from the 8-cell stage onward, and MAPK signaling through the neurula stage is reported to be required for normal palp development (Hudson et al., 2003). We therefore examined the timing of the FGF-MAPK signaling requirement for Islet expression in the palps.

We expressed a dominant-negative FGF receptor (DN FGFR) in the palp lineage using the FoxC enhancer and found that Islet reporter activity in the palps was lost (Fig. 5A-C). We next used the MEK inhibitor U0126 to block MAPK signaling at later developmental time points, mid-gastrula and mid-neurula. We found that treatment at mid-gastrula stage led to loss of Islet transcripts in the palp region, although expression in the notochord and A10.57 motoneuron persisted (Fig. 5E, compare with control Fig. 5D; and see Discussion). Interestingly, MAPK inhibition at the mid-neurula stage led to ectopic Islet expression in a U-shaped pattern, appearing as though the three discrete foci seen in the wild-type condition are fused in the treated tailbuds (Fig. 5F). This result suggests that localized repressors might delimit Islet expression, although it is unlikely that Emx functions in this capacity, as its expression is unaffected by U0126 treatment (supplementary material Fig. S5). We conclude that sustained MAPK signaling is required for proper patterning of Islet in the presumptive palps.

**Islet rescues palp development upon inhibition of FGF signaling**

We next asked whether Islet expression could rescue the palpless phenotype that results from inhibition of FGF signaling. We previously used the DMRT enhancer to misexpress DN FGFR in the anterior neural plate, which gives rise to the palps and the anterior sensory vesicle (brain) of larvae. This treatment leads to ectopic FoxC expression, a truncated sensory vesicle and impaired palp development (Fig. 6B, compare with control larva in Fig. 6A) (Wagner and Levine, 2012). Combining the DMRT>DN FGFR perturbation with FoxC>Islet, however, led to a rescue of palp development. Cells misexpressing Islet become elongated, to an even greater extent than in wild-type palps (Fig. 6C, compare with Fig. 6A). This treatment leads to a larger palp than that obtained with the FoxC>Islet transgene (Fig. 3D), as inhibition of FGF signaling results in an increase in the number of FoxC-expressing cells, and a corresponding expansion in the misexpression of Islet.

Fig. 3. Emx and Islet exert differential effects on both Islet expression and palp development. (A-D,F,G) Embryos were electroporated with FoxC>H2B:Cherry and Islet-YFP-CAAX reporter plasmids and stained with phallolin. (A) Control embryo expressing FoxC>lacZ shows Islet reporter expression in protrusive palps. (B) Mosaic embryo shows expression of the Islet reporter and protrusion of the palps are repressed in the presence of full-length Emx, whereas protrusion occurs normally on the unelectroporated side. (C) Mosaic embryo expressing FoxC>Emx:WRPW (constitutive repressor) does not express Islet reporter in the palps and does not protrude palps on the electroporated side. (D) Expression of FoxC>Islet leads to a single palp protrusion and ectopic activation of Islet reporter. (E) Quantification of results shown in A-C. To the right (3 palps, 2 palps, etc.) is shown the number of protrusion and ectopic activation of Islet reporter. (F) Mosaic embryo expressing FoxC>Islet DBD:WRPW (constitutive repressor) shows repression of Islet reporter in the palps and loss of protrusion on the perturbed side. (G) Co-expression of FoxC>Emx and FoxC>Islet leads to a single, protrusive palp and ectopic activation of Islet reporter. Scale bars: 10 μm.
We have presented evidence that the LIM-HD transcription factor Islet directs the cell elongation that results in the palp protrusions of Ciona tadpoles. Islet expression in the palps occurs precisely in the regions of protrusion (Fig. 2B–C′), and we rarely observe activation of the Islet>lacZ reporter in the absence of protrusions (Fig. 2H). Perturbations that inhibit Islet expression (misexpression of either Emx, Fig. 3B, or DN FGFR, Fig. 6B) also inhibit palp development. Notably, however, co-expression of Islet with either Emx (Fig. 3G) or DN FGFR (Fig. 6C,E) rescues the palp-deficient phenotype observed when either Emx or DN FGFR is expressed alone. Palps that develop under the rescue condition (DN FGFR+Islet) express the differentiation marker β-crystallin (Fig. 6E).

The palps arise from the anterior-most ectoderm, which exhibits discrete and complementary patterns of Islet, Btd and Emx expression (e.g. Fig. 1C″,C‴,D″). Ultrastructural and neuroanatomical studies have reported three distinct cell types in the Ciona palps, although there are probably at least four (Dolcemascolo et al., 2009; Imai and Meinertzhagen, 2007). The interpapillary area marked by Btd might contain the adhesive-secreting cells, as the corresponding region in a related ascidian, Botryllus schlosseri, consists of secretory cells (Caicci et al., 2010). Small, round neurons called basal cells have been observed at the base of the papillae in Ciona; these basal cells might correspond to the Emx+ rings that delimit the protrusions (Imai and Meinertzhagen, 2007). Within the palp protrusions, there appear to be two distinct cell types. Spindle-shaped neurons (called anchor cells) with axons projecting into the brain are well-documented and believed to have a sensory function (Dolcemascolo et al., 2009; Imai and Meinertzhagen, 2007; Torrence and Cloney, 1983). The Islet+ cells we have described are marked by apical digitiform protrusions (Fig. 2G), similar to the axial columnar cells previously reported (Dolcemascolo et al., 2009). We have never observed axons extending from these cells, and their function remains uncertain. It is possible that the Islet-expressing cells are secondary sensory cells that are innervated by trunk epidermal or other neurons.

It is noteworthy that in Ciona, Islet is expressed in additional cell types with unique and characteristic shapes: the otolith, the notochord and the A10.57 motoneuron (Giuliano et al., 1998; Stolfi and Levine, 2011). The otolith is a gravity-sensing organ, consisting of five types with unique and characteristic shapes: the otolith, the notochord and the A10.57 motoneuron (Giuliano et al., 1998; Stolfi and Levine, 2011). The otolith is a gravity-sensing organ, consisting of five different types with unique and characteristic shapes: the otolith, which protrudes from the cell body, is elaborated into a broad ‘foot’ that inserts into the membrane of the sensory vesicle (Sakurai et al., 2004). The notochord cells, by contrast, are arranged as a flattened stack of coins at the early tailbud stage. Later, as the tadpole matures, the notochord cells undergo a dramatic cell shape change, becoming elongated and cylindrical, followed by vacuolation and tubulogenesis (Denker and Jiang, 2012). The motor ganglion controls the swimming behavior of the tadpole and consists of five different types with unique and characteristic shapes: the otolith, which protrudes from the cell body, is elaborated into a broad ‘foot’ that inserts into the membrane of the sensory vesicle (Sakurai et al., 2004). The notochord cells, by contrast, are arranged as a flattened stack of coins at the early tailbud stage. Later, as the tadpole matures, the notochord cells undergo a dramatic cell shape change, becoming elongated and cylindrical, followed by vacuolation and tubulogenesis (Denker and Jiang, 2012). The motor ganglion controls the swimming behavior of the tadpole and consists of five
pairs of neurons. Only the posterior-most pair, A10.57, expresses Islet, and its cell body is markedly elongated in comparison to the other motoneurons that do not express Islet (Stolfi and Levine, 2011). The identification of Islet target genes in the palps, notochord and otolith could reveal important cellular effector genes contributing to their distinctive elongated morphologies.

Islet belongs to a subclass of homeodomain transcription factors (together with Lhx and Lmx) distinguished by the presence of two LIM domains in the N-terminus. The LIM domain is a type of zinc finger that functions as a protein-binding platform with diverse functions (Kadrmas and Beckerle, 2004; Zheng and Zhao, 2007). LIM-HD proteins require the nuclear cofactor LIM-domain-binding protein-1 (Ldb-1, also known as NLI or CLIM) for activity (Matthews and Visvader, 2003). Ldb-1 has a LIM-interaction domain (LID) that directly interacts with the LIM domains of Islet, as well as a dimerization domain, which enables the formation of complex multimeric protein assemblies that can activate or repress transcription (Jurata and Gill, 1997; Jurata et al., 1998). Expression of isolated protein domains (either the Ldb-1 LID, or the LIM domains from Islet) produces a dominant-negative phenotype by disrupting the native Ldb-1-Islet complexes. This approach produces cellular phenotypes similar to those obtained by DNA- and RNA-based loss-of-function assays (Becker et al., 2002; Segawa et al., 2001). The LIM domains are thus essential for the biological function of Islet. LIM domains are also found in a variety of proteins that associate with the actin cytoskeleton, many of which, although primarily cytosolic, have been shown to shuttle into and out of the nucleus. An emerging hypothesis is that LIM domains act as biosensors, communicating across cellular compartments to coordinate nuclear regulatory states with cytoskeletal activity (Kadrmas and Beckerle, 2004). LIM-HD transcription factors may thus be uniquely well-suited for the genetic control of cell morphology.

Islet is expressed in a wide variety of neurons across metazoans (Jackman et al., 2000; Nomaksteinsky et al., 2013; Simmons et al., 2012; Voutev et al., 2009). Though widely known for its role in...
Fig. 7. Summary of gene regulatory network underlying palp development. Based on results of current and previous studies (Ikeda et al., 2013; Imai et al., 2006; Wagner and Levine, 2012). Genes in bold black font are expressed in indicated region; gray font indicates genes repressed in those areas; blue font denotes untested regulatory interactions; parentheses indicate genes that were expressed in progenitors of that region but have since been downregulated. (A) A subset of the neural progenitors at indicated stages. Dotted vertical line denotes midline. Blastomere names (according to ascidian nomenclature) indicated on right side. Lowercase and uppercase letters (e.g. a7 and A7) refer to cells of animal and vegetal hemispheres, respectively. (A) At 64-cell stage, the bipotent palp/CNS progenitors express DMRT1 and Otx in response to FGF signal from vegetal neural precursors. Bipotency requires active repression of ZicL by BZ1/BZ2. (B) At 112-cell stage, the palp and anterior CNS fates have segregated. Anterior CNS fate requires FGF signaling to induce ZicL, and restrict FoxC to the palp lineage. (C) In late tailbud embryos, Six3/6 is expressed in anterior brain, downstream of ZicL. (We speculate that Islet and Btd might be actively repressed in the anterior brain by Six3/6. This is because ectopic expression of ZicL in palp progenitors leads to repression of Islet and Btd, and this effect might be mediated by the Six3/6 repressor.) The palps are the anterior-most region of specialized ectoderm (derived from FoxC+ population shown in B) expressing Btd and Emx, as well as the protrusions expressing Islet. Emx might function to limit Islet to discrete foci. Islet is expressed in response to FGF signaling (but see Fig. 5F) and is maintained by positive autoregulation.

motor neuron specification, it also functions in motor axon outgrowth in both Drosophila and vertebrates (Liang et al., 2011; Segawa et al., 2001; Thaler et al., 2004; Thor and Thomas, 1997). Islet orthologs are widely expressed in sensory neurons as well, where they also influence cell shape. Mouse Isl2, for example, is expressed in a subset of retinal ganglion cells that possess a distinctive morphology, with characteristic dendritic lamination patterns and axonal projection targets (Triplet et al., 2014). In zebrafish embryos, inhibition of Isl2 results in aberrant axon positioning, as well as defective axon outgrowth and branching of Rohon-Beard and trigeminal sensory neurons (Andersen et al., 2011; Segawa et al., 2001). Similar defects have been reported upon inhibition of Isl1 in both mouse and zebrafish (Liang et al., 2011; Tanaka et al., 2011). Neuron-specific Islet target genes have been identified, and they are important for neural morphology (Aoki et al., 2014). Slit-mediated axon branching, for example, relies on Isl2 target genes such as PlexinA4 (Miyashita et al., 2004; Yeo et al., 2004). The Islet-dependent outgrowth and branching of axons and dendrites seen in vertebrates might have evolved from a simpler, ancestral regulatory network controlling cell shape, such as that featured in Ciona.

In summary, we have provided evidence that Islet functions downstream of FGF signaling to regulate target genes required for palp morphogenesis, including cellular effectors underlying elongation. Given the correlation between Islet expression and cell shape in both Ciona and vertebrates, it is conceivable that a detailed understanding of Islet function may help to illuminate mechanisms by which transcriptional regulation directs the process of cellular morphogenesis.

MATERIALS AND METHODS

Cell sorting and expression profiling

Cells of the specified palp lineage were isolated from dissociated embryos expressing the cell surface marker CD4:GFP using antibody-coupled magnetic beads. RNA was isolated from these isolated cells and hybridized to Affymetrix GeneChip microarray. See Methods in the supplementary material for details.

Molecular cloning

Cloning of Islet- and DN FGF-coding sequences, and of enhancers for DMRT, FoxC, FoxF, ZicL and βγ-crystallin, has been described (Beh et al., 2007; Shimeld et al., 2005; Stolfi and Levine, 2011; Wagner and Levine, 2012). Emx-coding sequence (gene model KHL142.14.v1.A.ND1-1) was PCR-amplified from mixed stage cDNA with the oligos Emx cds NF: TAATGCGGCCGCAACCAGTATTCTTAACCAATCC and Emx cds Target2: TAATGCTAGCTCTCAATAGAGCCTGTTGAC, and cloned into a plasmid downstream of the FoxC enhancer by standard methods. The DNA-binding domain of Emx was amplified with the oligos Emx DBD SpeR: TAATCTAGTGCTACCCCTCTTTGCTTGTATCAC, and cloned upstream into a plasmid downstream of the FoxC enhancer by standard methods. The DNA-binding domain of Emx was amplified with the oligos Emx DBD NheF: TAATGCTAGCTTATTGATGGCGAATCCATTTC and Emx DBD SpeR: TAATCTAGTGCTACCTCTTTCTCGTATCAC, and cloned upstream of the WRPW repression domain as described (Stolfi et al., 2011). Islet cis-regulatory DNA was pieced together from a 500 bp promoter region and the partial sequence of the first intron cloned upstream. The Islet promoter was amplified with Isl pro XhoF: TAATCTCGAGTAACTCAGTATGCGCGTGTCG and Isl pro NR: TAATGCGGCCGCTTCTGAGTATAAAACCTAGGAAC. The Islet intrinsic enhancer was amplified with Islet int1a AscF: TAATGGCGGCCGCCTTCTGAGCTTATTAAG and Islet int1a XhoR: TAATCTGAGTGCCGACAAAACCTACCTATTTTATTCC. See supplementary material Fig. S1 for the complete sequence. The Islet DNA-binding domain was amplified with Isl DBD NheF: TAATGCTAGCTAAAGATGCGAAGACGACGCGAG and DBD NgoMIV R: TAATGCGGCCCGCCGCGCTTCTGAGATTG, and cloned upstream of the
WRP repressor motif to create Isl-WRPW. For details of molecular cloning of CD4:GFP and CD8:mCherry constructs see Methods in the supplementary material.

Embryo manipulation
Adult Ciona intestinalis animals were obtained from M-REP. Protocols for fertilization, dechorionation and electroporation have been described (Christiaen et al., 2009a,b). Plasmid concentration for electroporation varied between 40 and 80 μg per replicate; all experiments were performed at least twice. U0126 (Promega) was resuspended in DMSO and diluted to 10 μM in filtered artificial seawater (FASW). Phalloidin (Molecular Probes) was diluted 1:500 in PBT (PBS+0.1% Tween 20) and incubated with embryos overnight. Hoechst 33342 (Life Technologies) staining was performed at 2 μg/ml in PBT for 2-4 h. Imaging was performed on a Zeiss 700 laser scanning confocal microscope or Zeiss AxioImager.A2 upright microscope.

In situ hybridization (ISH)
DNA templates for Ifest, Ski1/2 and Bld probe synthesis were obtained from Ciona Gene Collection Release 1 (clone numbers GC01d01, GC05e01 and GC02k02, respectively). The Eux probe was transcribed from the antisense strand of the coding sequence. The probe synthesis (Wagner and Levine, 2012) and double ISH protocol have been described (Stolfi et al., 2011). Briefly, antisense RNA probes were transcribed in the presence of either fluorescein-UTP (Roche) or digoxigenin-UTP (Roche). Probes were detected with peroxidase-conjugated antibodies (anti-DIG-POD, Roche 11207733910, 1:1000; or anti-Fluorescein-POD, Roche 14126346910, 1:1000) combined with tyramide signal amplification (Cy3 TSA kit or Fluorescein TSA kit, PerkinElmer). When present, GFP was detected with primary antibody rabbit anti-GFP (Invitrogen A-11122, 1:1000) and secondary antibody donkey anti-rabbit Alexa Fluor 488 (Invitrogen A-21206, 1:1000).

Cell length analysis
Confocal microscopy was used to image a total of 20 mosaic embryos each for the control (FoxF^lacZ) and the experiment (FoxF^Islet). A single section from each embryo was chosen for analysis. We chose sections in which the surface ectoderm of both the control and perturbed halves were clearly visible and free of distortions. A total of ten measurements were taken by hand with ImageJ software on each side (electroporated and unelectroporated), from similar positions along the anterior-posterior axis. The average measured length was used to generate the normalized value (electroporated/unelectroporated) for each embryo. We sought to avoid making measurements very near the pals, which are thickened, and near the trunk-tail junction, because sometimes this region appears pinched or distorted due to variation in the shape/orientation of embryos. P-values were calculated using the Wilcoxon two-sample test.

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Competing interests
The authors declare no competing financial interests.

Author contributions
E.W. designed and performed the experiments and wrote the manuscript in consultation with A.S. and M.L. A.S. cloned Ifest enhancer and Ifest-coding sequences (full length and WRPW fusion), Y.G.C. prepared and analyzed microarray samples.

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Supplementary material
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