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Insulin-like genes in ascidians: findings in *Ciona* and hypotheses on the evolutionary origins of the pancreas

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Abstract

Insulin plays an extensively characterized role in the control of sugar metabolism, growth and homeostasis in a wide range of organisms. In vertebrate chordates, insulin is mainly produced by the beta cells of the endocrine pancreas, while in non-chordate animals insulin-producing cells are mainly found in the nervous system and/or scattered along the digestive tract. However, recent studies have indicated the notochord, the defining feature of the chordate phylum, as an additional site of expression of insulin-like peptides. Here we show that two of the three *insulin-like* genes identified in *Ciona intestinalis*, an invertebrate chordate with a dual life cycle, are first expressed in the developing notochord during embryogenesis and transition to distinct areas of the adult digestive tract after metamorphosis. In addition, we present data suggesting that the transcription factor *Ciona* Brachyury is involved in the control of notochord expression of at least one of these genes, *Ciona insulin-like 2*. Lastly, we review the information currently available on insulin-producing cells in ascidians and on pancreas-related transcription factors that might control their expression.

Keywords

ascidian; *Brachyury*; *Ciona*; *cis*-regulatory module; insulin; notochord; pancreas; transcription factor

INTRODUCTION

Insulin, insulin-like growth factors (IGFs), insulin-like (INSLs) and relaxin (RLN) genes compose an evolutionarily conserved superfamily and encode a variety of regulatory polypeptides related by shared amino acid subdomains (e.g., Conlon, 2001). Among the main conserved residues are a few cysteines that establish intra- and inter-chain disulfide bonds. Disulfide bonds are necessary for the stability and the biological activity of insulin and its related peptides (Vinther et al., 2013), and enable them to control a wide range of cellular processes, including nutrient homeostasis and regulation of cell growth and survival in a variety of tissues, as well as aging (Fulzele and Clemens, 2012; Werner and Leroith, 2014; Edmondson et al., 2003; Kenyon, 2011). In widely different organisms, cells

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producing insulin and related peptides have been described as specialized neurons in the nervous system, both central and peripheral, or as endocrine cells that can be either disseminated in various regions of the digestive tract or concentrated in the parenchyma of the pancreas or its evolutionary equivalents. Collectively, insulin- and other hormone-producing cells belong to the neuroendocrine system (NES) and are crucial components of the brain-gut axis (Conlon et al., 1988; Konturek et al., 2004). In invertebrate protostomes, the neuronal fraction of the NES is usually predominant and most insulin-producing cells are found in the brain, as is the case in insects and molluscs (Luo et al., 2012; Smit et al., 1998). However, in echinoderms, non-chordate deuterostomes, immunolocalization experiments have shown that the gut of the pluteus larva contains cells expressing insulin-related peptides (de Pablo et al., 1988). Subsequent studies confirmed the presence of two genes of the insulin/IGF/INSL/RLN superfamily in the *S. purpuratus* genome, *Sp-IGF1* and *Sp-IGF2* (Burke et al., 2006). Related analyses in another echinoderm, the sea star *Pisaster ochraceous*, also pointed at the existence of cells expressing *insulin*-related genes in the developing gut (Wilson and Falkmer, 1965). In chordates, the main group of deuterostomes, insulin-producing cells increase in number in various areas of the digestive tracts, and ultimately, in vertebrates, are found grouped in the islets of Langerhans of the pancreas alongside other endocrine cell types (Madsen, 2007). Interestingly, in addition to being expressed in cells of the NES, in a few chordates *insulin* and *insulin-like* genes have been found in the notochord. This is the case for two of the three *insulin-like* genes in a urochordate, the ascidian *Ciona intestinalis*. Below we summarize the early reports of insulin-producing cells in the ascidian digestive organs, we describe in detail the expression of the three *Ciona insulin-like* genes before and after metamorphosis, and suggest the possible involvement of the transcription factor Brachyury in the regulation of the expression of one of these genes in the developing notochord. We conclude by reviewing the current knowledge on the expression of pancreas-related transcription factors and co-factors in *Ciona*.

LARVAL AND POST-METAMORPHOSIS ENDODERMAL DERIVATIVES, DIGESTIVE STRUCTURES AND INSULIN-PRODUCING CELLS IN *CIONA*

Cell fate and function of endoderm precursors in ascidians

The organization of the larval body plan of solitary ascidians is remarkably similar to a simplified version of the embryos of higher chordates. The roughly 2,600 cells that are found in a larva of the ascidian genera *Halocynthia* or *Ciona* are organized into a few main tissues, including epidermis, notochord, muscle, endoderm, nervous tissue and sensory organs, and in the mesenchyme and its specialized regions, namely trunk lateral cells and trunk ventral cells (Passamaneck and Di Gregorio, 2005; Munro et al., 2006). In particular, the notochord invariantly contains 40 cells, is overlaid by a dorsal nerve cord composed of ~400 cells, and is flanked on each side by muscle cells, amounting to a total of 36 cells in *Ciona* and 40 cells in *Halocynthia* (Satoh, 2014). Ventral to the notochord, and occupying a large part of the larval trunk, which is also regarded as the ascidian “head”, are ~500 endodermal cells, which are topologically subdivided into trunk endoderm and endodermal strand and have been compared to a primordial non-functional pharynx and to the hypochord of fish and amphibians, respectively (Lofberg and Collazo, 1997). Interestingly, the

endodermal cells of the larvae of many ascidian species do not form a functional digestive tract, although they express evolutionarily conserved markers of endoderm differentiation, such as alkaline phosphatase (Whittaker, 1977; Nishida and Kumano, 1997), and orthologs of the essential endodermal transcription factors TTF-1 and Foxa2 (Ristoratore et al., 1999; Di Gregorio et al., 2001). Thus, differently from larvae of non-chordate marine invertebrates such as the sea urchin pluteus, which is able to feed on algae and diatoms and can survive up to several weeks without metamorphosing (Hinegardner, 1969), most ascidian larvae are unable to feed themselves and are usually able to swim for only a day or two before settling to undergo metamorphosis (Satoh, 2014). During early embryonic development, between the 32-cell and the 64-cell stage, the endodermal precursors, mostly vegetal blastomeres, become fate-restricted. The endoderm fate determination is coordinated by maternal determinants that are pre-localized in the egg cytoplasm (Nishida, 1996). As a consequence, presumptive endodermal blastomeres are capable of developing into endoderm even when isolated from the remainder of the embryo (Reverberi and Minganti, 1946). A key trigger of the early determination of the ascidian endoderm is the transactivator beta-catenin, the main mediator of the canonical Wnt signaling pathway (Imai et al., 2000). The nuclear translocation of beta-catenin in the vegetally-located endodermal precursors is also required to initiate the induction of the notochord cell fate in adjacent blastomeres (Nakatani and Nishida, 1994). The consequent segregation of the three germ layers, ectoderm, mesoderm and endoderm, unravels as the effect of a combinatorial code of nuclear beta-catenin activation; blastomeres with no nuclear beta-catenin activity will become part of the ectoderm, blastomeres that maintain stable high levels of nuclear beta-catenin activity will give rise to endoderm, and cells that change their nuclear levels of beta-catenin from high to low will form the notochord (Hudson et al., 2013). In addition, high levels of activation of the FGF signaling pathway are required for the endoderm induction in A-line cells (Shi and Levine, 2008; Lemaire, 2009). *Ci-FoxA-a*, which encodes an ortholog of the Foxa2 transcription factor, is one of the first genes expressed in early endodermal precursors (Di Gregorio et al., 2001) under the regulation of beta-catenin; morpholino-mediated ablation of its expression results in loss of endodermal markers (Imai et al., 2006). Ci-FoxA-a in turn regulates expression of two additional transcription factors, Ci-Otx and Ci-Lhx3; the latter activates genes required for endoderm differentiation in the larva, and along with Ci-FoxA-a activates Ci-Otx, which is believed to be required for the activation of Ci-GATA-a and other genes that control differentiation of the adult endoderm (Imai et al., 2006). After the specification of the presumptive endoderm is finalized, endodermal precursors divide at a steady rate until their final number of ~500 is reached, around the tailbud stages. Endodermal cells remain yolky and maintain an undifferentiated appearance until metamorphosis (Hirano and Nishida, 2000).

Metamorphosis and formation of digestive organs

At the time of metamorphosis extensive changes begin for the body plan as a whole, and for the endodermal cells as well, as the organogenesis of the juvenile digestive tract commences and the animal begins to feed. Metamorphosis is a complex, multi-step process and its timing is tightly regulated (Nakayama-Ishimura et al., 2009). At metamorphosis, the main chordate features of the larval body plan, *i.e.* the notochord, dorsal neural tube and ventral endoderm, are either lost or extensively rearranged, and are replaced by other chordate

hallmarks, such as the endostyle, which is regarded as a primordial thyroid gland (Roche et al., 1962; Ristoratore et al., 1999; Sasaki et al., 2003), and pharyngeal gill slits (Tanaka et al., 1996). In addition, a rudimentary heart and different kinds of blood cells begin to develop, as well as the gonad and its annexes, oviduct and spermiduct (Satoh, 2014). Neither the notochord nor the tail muscles contribute to the formation of the adult body, and both these tissues and the tail are largely reabsorbed. As the tail degenerates and the rotation of the body axis is completed the notochord and muscle cells move to the trunk, and eventually their remnants are found near the holdfast (Sato et al., 1997). Remarkably, however, most cells of the larval nervous system, with the exception of those located in the nerve cord, become part of the adult CNS, which is composed of neural ganglion and neural gland, and are incorporated into structures that develop from the neural gland, including a ciliated duct and funnel, and a dorsal strand (Manni et al., 2005). In particular, some of the ependymal (non-nervous) cells of the larva can transdifferentiate into neurons in the adult, thus acting as stem-like cells (Horie et al., 2011). On the surface of the embryo, epidermal cells begin to express the cellulose synthase gene *Ci-CesA* beginning at the neurula stage, and at metamorphosis these cells become part of the cellulose-based tunic (Nakashima et al., 2004; Sasakura et al., 2005), along with part of the mesenchymal cells (Hirano and Nishida, 1997). Trunk ventral cells divide after metamorphosis to generate the heart (Davidson and Levine, 2003; Satou et al., 2004) and the atrial siphon muscles (Stolfi et al., 2010). Trunk lateral cells originate blood cells, part of the body wall muscle and the cells that surround the gill slits (Hirano and Nishida, 1997), and act as neural crest-like migratory cells (Jeffery et al., 2008). In the case of the endoderm, it is noteworthy that two cells of the endodermal strand, expressing the conserved marker *vasa* (*CiVH*), migrate from the tail to the gonad anlage to originate the primordial germ cells in the adult (Takamura et al., 2002). The remaining cells of the endodermal strand and their descendants give rise to the intestine, while the trunk endoderm folds into tubular structures that will develop into esophagus, stomach and endostyle (Nakazawa et al., 2013). Therefore, altogether the larval endoderm participates in the formation of a number of adult structures, including endostyle, pharyngeal band, dorsal tubercle, peribranchial epithelium, as well as the digestive organs: the pharynx, also indicated as branchial (or pharyingeal) sac or branchial basket, esophagus, stomach, intestine and pyloric gland (Chiba et al., 2004). A small hepatopancreas has been described in *Halocynthia* (Hirano and Nishida, 2000) and its existence has been more recently implied in *Ciona* (Hammond et al., 2005).

Detection of peptide- and insulin-producing cells in Ciona: early studies

In adult ascidians, sea water incoming from the oral siphon is conveyed to the pharynx, where it is moved through the gill slits, also called stigmata, by the ciliary activity of the cells of the pharyngeal wall. As the water moves through the slits, it oxygenates the blood by coming in close contact with small blood vessels that run inside the thin pharyngeal wall (Satoh, 2014). At the same time, food particles contained in the sea water, such as algal fragments, are trapped by the abundant mucus secreted by the endostyle and moved to the esophagus by the cilia of the cells bordering the gill slits. The fully developed esophagus is about one centimeter in length and is lined by a columnar epithelium rich in mucus and ciliated cells. In the esophagus, the food particles are conveyed towards the stomach, while they begin to be compacted into thin, mucus-laden strings, or “ropes”, that in transparent

ascidians can be seen transiting through the digestive organ and being expelled from the atrial siphon (Yonge, 1925; Millar, 1953). For this reason, the esophagus was believed to mainly serve as a conduit and a compactor for food particles, until insulin-immunoreactive cells were described in the esophageal epithelium in *Styela clava*, along with a second type of predicted endocrine cells (Bevis and Thorndyke, 1978). These observations suggested that this part of the digestive tract might also serve a secretory function. This hypothesis is corroborated by our recent results, presented below.

After moving through the esophagus, the food reaches the stomach, a sac-like ovoidal organ containing in its cavity about 40 ridges that increase its inner surface, similar in function to the rugae found in the stomach of vertebrates (Millar, 1953). Three main cell types have been described in the ascidian stomach: vacuolated cells, which are believed to be equivalent to the absorptive cells of the vertebrate intestine, ciliated mucous cells, and secreting cells, which have been tentatively indicated in the past as the site of accumulation of digestive enzymes (Thorndyke, 1977) and were recently proven to express a trypsin-type protease and a chymotrypsin-like protease (gene models: KH.L97.6 and KH.S425.3, respectively; Dehal et al., 2002; Satou et al., 2008; Ogasawara et al., 2002; http://bioinfo.s.chiba-u.jp/ciaid/). An early comparative study on the physiology of digestion tested the ability of extracts prepared from the *Ciona* gut to digest different carbohydrates, and it revealed that the only carbohydrates that were processed were starch, glycogen and sucrose (Yonge, 1925). In support of these results, later studies showed the presence of alpha-amylases in the gills of *Ciona intestinalis* and other ascidians (Fiala-Medioni and Pequignat, 1975), and currently at least one gene encoding a putative alpha-amylase has been annotated in the *Ciona* genome (gene model: KH.C5.659). Additional genes encoding predicted enzymes involved in carbohydrate metabolism have been found in *Ciona*, including glucosidases, maltase-glycoamylases and sucrase-isomaltase; in particular, expressed sequence tags (ESTs) counts indicate expression of a glucosidase (gene model: KH.L170.27) gene in the pyloric gland of the adult ascidian (Satou et al., 2002, 2005; http://ghost.zool.kyoto-u.ac.jp/reference_kh.html). Extensive cytochemical and immunohistological studies in *Ciona* have revealed the presence of several gastroenteric and neurohormonal peptides in the gut and in the nervous system of adult ascidians. Positive immunolocalization signals were obtained in both the neural complex and the digestive tract of *Ciona* for bombesin (whose mammalian homologs are gastrin-releasing peptide and neuromedin B), pancreatic polypeptide, substance P, somatostatin, secretin and neurotensin, while beta-endorphin, prolactin, and vasoactive intestinal peptide and motilin appeared restricted to the nervous system; serotonin was originally reported only in the digestive tract (Fritsch et al., 1980), but more recent studies have shown its presence in the visceral ganglion of metamorphosed *Ciona* larvae (De Bernardi et al., 2006). Together, these early immunolocalization experiments allowed the creation of a precise map that pinpoints the location of cells that are immunoreactive for each peptide in both gut and neural complex (Fritsch et al., 1982). Recent post-genomic studies have confirmed some of these early finding by identifying two *Ciona* orthologs of neurotensin, *Ci-ntlp-A* and *Ci-ntlp-B*, which are both expressed in the neural complex; *Ci-ntlp-B* is also expressed in the digestive tract (Kawada et al., 2011). A hybrid of gastrin and cholecystokinin, called cionin, has also been identified in the neural ganglion of *Ciona* (Johnsen and Rehfeld, 1990). However, genes for

somatostatin, pancreatic polypeptide, ghrelin, amylin and glucagon seem to be missing from the *Ciona* genome, although in the latter case members of the glucagon superfamily have been identified in the solitary ascidian *Chelyosoma productum* (McRory and Sherwood, 1997a). Collectively, these findings fueled the hypothesis that *Ciona* and other ascidians occupy an intermediate position between invertebrates, which predominantly rely on neurosecretion to regulate homeostasis, and vertebrates, which perform these functions through endocrine cells that are either scattered along the intestine or aggregated into large glands. In agreement with this scenario, reactivity to ox and chicken insulin was detected in extracts prepared from both the stomach and the intestine of *Ciona* (Davidson et al., 1971) and subsequent studies reported immunoreactivity to members of the insulin superfamily in cells of the digestive tract of this species (Reinecke et al., 1993, 1999). In addition, insulin-immunoreactive cells and a network of insulin-immunoreactive fibers were reported in the *Ciona* neural ganglion (O'Neil et al., 1986). Within the digestive tract, double immunofluorescence experiments showed that polyclonal antisera against human IGF1 and human insulin both reacted with epithelial cells of the gastrointestinal mucosa, showing overlap in ~95% of the cells, while no cells that were exclusively reactive to the insulin antiserum were found (Reinecke et al., 1993). Similar overlap was seen in the neural ganglion and nerve fibers, although insulin reactivity was preferentially observed in the fibers rather than in the neuronal bodies (Reinecke et al., 1993). Follow-up studies that compared the distribution of IGF1 and insulin immunoreactivity to that for relaxin, another member of the insulin/IGF/INSL/RLN superfamily, revealed the partial coexistence of reactivity to IGF1 and relaxin in cells of the *Ciona* ovary, whereas no insulin-reactive cells were detected in this tissue. Conversely, no positive signal for relaxin was detected in the intestine (Reinecke et al., 1999). These results resemble those obtained in the amphioxus *Branchiostoma lanceolatum*, where cells positive to both IGF1 and insulin antisera were detected in the gastrointestinal tract as well as in the CNS (Reinecke et al., 1999).

INSULIN-LIKE *GENES IN* CIONA INTESTINALIS

Genomic organization

Insulin is synthesized as a multi-domain pre-pro-peptide in which the A- and B- chains are connected by a C-peptide, and an N-terminal signal peptide is present (Conlon, 2001). Post-translational processing by a signal peptidase removes the signal peptide and converts pre-proinsulin into proinsulin as it moves to the endoplasmic reticulum; subsequently, endoproteolytical convertases cleave the intervening C-peptide, leaving the A and B chains linked by disulfide bonds in the mature insulin chain. Instead, IGFs retain the C-peptide and contain additional domains, namely chains D and E (Reinecke and Collet, 1998). Invertebrate insulin-like genes constitute large and divergent families that share the basic insulin structure and contain a number of members that vary among different species; for example, seven insulin-like genes have been described in *Drosophila* (Nässel, 2002) and three in *Hydra* (Fujisawa and Hayakawa, 2012). After the first assembly of the *Ciona intestinalis* genome was completed (Dehal et al., 2002), an *insulin-like* gene was identified and named *Ci-insulin* (Satou et al., 2003); however, reports from another ascidian, *Chelyosoma productum*, in which two *insulin-like* genes had been found (McRory and Sherwood, 1997b), prompted further searches for members of the insulin/IGF/INSL/RLN

superfamily in *Ciona*. Gene clustering and phylogenetic analyses revealed two additional *insulin-like* genes in *Ciona intestinalis*, which were named *Ci-INS-L1* (the former *Ci-insulin*), *Ci-INS-L2* and *Ci-INS-L3* (Olinski et al., 2006a, 2006b). All three genes have been mapped to chromosome 2q, one of the largest *Ciona* chromosomes (Shoguchi et al., 2006). *Ci-INS-L2* (gene model: KH.C2.313) and *Ci-INS-L3* (gene model: KH.C2.1012) are tightly clustered in tandem orientation within ~7.7 kb, while *Ci-INS-L1* (gene model: KH.C2.75) is found ~4.4 Mb away (Fig. 1a). Both genomic regions have been shown to display conserved synteny with related gene clusters in human and other vertebrate chromosomes (Olinski et al., 2006a). *Ci-INS-L1* is an ortholog of human INSLs and RLNs, and a cleavage site for a signal peptide, along with a putative processing site, were predicted within its putative amino acid sequence (Olinski et al., 2006b). A similar structure was predicted for the product of *Ci-INS-L3*, which is expected to retain the C-peptide and mature into a single-chained IGF-like peptide. Instead, the predicted Ci-INS-L2 peptide contains, in addition to a signal peptide cleavage sequence, two processing sites flanking the C-peptide and a suboptimal target sequence for proprotein convertase, which together make *Ci-INS-L2* more related to *insulin* genes (Olinski et al., 2006b).

Expression patterns of the Ciona insulin-like genes

Whole-mount *in situ* hybridization (WMISH) data had indicated that *Ci-INS-L1* is expressed in numerous territories, and in particular in endodermal strand, notochord, nerve cord and peripheral nervous system (Satou et al., 2001). Expression of *Ci-INS-L2* had been reported in muscle, notochord, epidermis, endoderm, nerve cord, mesenchyme, presumptive palps and other territories at the initial and early tailbud stages, however no expression was detected in the notochord of middle and late tailbuds (Satou et al., 2001), and in larvae expression *Ci-INS-L2* had only been observed in the epidermis (Kusakabe et al., 2002). No *in situ* expression data were available for *Ci-INS-L3*. The lack of expression data for *Ci-INS-L3* and our laboratory's long-standing interest in notochord gene expression prompted us to further investigate the expression of this gene family, in both the pre- and post-metamorphosis body plan. Our results confirmed the widespread early expression of *Ci-INS-L1* and highlighted the strong signal in notochord, trunk endoderm and mesenchyme cells throughout the tailbud stages, as well as the down-regulation of notochord expression in late tailbuds (Fig. 1b, top row). In the case of *Ci-INS-L2*, after detecting a transient signal in muscle precursors at the 110-cell stage, we observed sustained expression in notochord cells and their progenitors beginning at gastrulation, accompanied by transient expression in the primordium of the adhesive organ (Fig. 1b, middle row). We also observed expression of *Ci-INS-L3* in muscle, mesenchyme and neural precursors during gastrulation, and, during the tailbud stages, in mesenchyme cells and a few areas of the presumptive adhesive organ (Fig. 1b, bottom row). Occasionally, what seemed a very faint, transient signal was visible in the notochord at the tailbud stage when relaxed hybridization conditions were used (data not shown).

On the basis of its closer similarity to *insulin* genes, *Ci-INS-L2* was selected for further *in situ* hybridization studies in juveniles. Using the same probe employed for the *in situs* on embryos, we detected expression of *Ci-INS-L2* in esophagus and stomach of early juveniles (Fig. 2a,b; inset in b). The expression of *Ci-INS-L2* in the stomach partially overlaps with

the expression of *Ci-Na$^+$/K$^+$-transporting ATPase alpha3-1* (aka *ATP1A1/2/3/4*, gene model: KH.L157.5), which we used as a positive control for the *in situs* on juveniles (Fig. 2c,d) on the basis of its strong expression in the stomach (Ogasawara et al., 2002) and for its reported presence in pancreatic beta cells of vertebrates, where it is believed that inhibition of this ion pump by glucose triggers insulin secretion (Owada et al., 1999). In juveniles, we observed expression of *Ci-Na$^+$/K$^+$-transporting ATPase alpha3-1* in the neural complex (CNS), which is formed in part by cells that originally belonged to the sensory vesicle (see above; blue asterisks in Fig. 2c,d), in most of the surface of the stomach, and in a small part of the esophagus (Fig. 2d, and inset).

As a next step, expression of all three genes was analyzed in juveniles and adults by RT-PCR. All three genes were found to be expressed in juveniles (data not shown). The results of representative RT-PCR experiments on adults are shown in Fig. 2f. Transcripts for *Ci-INS-L2* were present, although at variable levels, in all the adult tissues that were analyzed (Fig. 2f); interestingly, expression of this gene varied in different tracts of the adult intestine (Fig. 2e, f). Differently from what was reported in early immunofluorescence studies (Reinecke et al., 1993), *Ci-INSL-2* is the only one of the three genes that we could detect in section 2 of the adult intestine (Fig. 2f). Expression of *Ci-INS-L3* peaked in stomach and neural complex, but was not detected in the middle section of the intestine (Fig. 2e, f). *Ci-INS-L1* was only detected in the stomach, at seemingly low levels (Fig. 2f).

Other components of the insulin pathway in Ciona

The *Ciona* genome contains an *insulin receptor* gene, *Ci-insrr* (gene model: KH.L8.8), which was reported as weakly and transiently expressed in the notochord of initial tailbud embryos and seemed to switch from the notochord to the nervous system at later stages (Satou et al., 2001, 2003). Together with our results on the notochord expression of *Ci-INS-L2*, these data suggest that, at least during the initial tailbud stage, *Ciona* insulin and/or IGF-like proteins might work in an autocrine fashion. It is known that upon binding its receptor, insulin induces, through its receptor tyrosine kinase, the phosphorylation of insulin receptor substrate proteins (IRSs); in turn, IRSs can associate with the phosphatidylinositol 3-kinase (PI 3-kinase) to activate it, as well as with several other substrates, thus triggering a variety of cascading intracellular signals (White, 1997). The *Ciona* genome contains a single *irs* gene, annotated as *Ci-irs1/2/4*, whose expression is still unknown.

In addition to genes directly related to insulin and its receptors, recent genome annotations indicate that *Ciona* possesses three insulin-like growth factor binding protein (*igfbp*) genes, *igfbp1/2*, *igfbp1/3/4/5/6* and *igfbp2/3/4/5* (http://www.aniseed.cnrs.fr), whose expression is still uncharacterized. On the other hand, it is noteworthy that in addition to seemingly lacking evident *glucagon* orthologs, *Ciona* appears to be devoid of adipokinetic hormone, which in *Drosophila* and other organisms is structurally and functionally related to glucagon. Together, these observations suggest the hypothesis that *insulin*-like genes in *Ciona* might predominantly regulate growth and longevity, and play a rather minor role in glucose metabolism.

EVOLUTIONARILY CONSERVED MARKERS OF PANCREAS DEVELOPMENT AND ACTIVATORS OF *INSULIN* GENE EXPRESSION ACROSS CHORDATES

The regulation of *Ci-INSL-2* expression after metamorphosis remains to be investigated. This provides us with the opportunity to concisely review the current knowledge gathered in *Ciona* on the main evolutionarily conserved transcription factors required for pancreas formation, endocrine pancreas specification, and insulin secretion, and to hypothesize possible regulatory mechanisms. A list of the main transcription factors that control pancreas development and insulin expression across chordates is provided in Table 1, and some of the most relevant information on candidate activators of *insulin/IGF* gene expression in *Ciona* is illustrated in Fig. 4. As we mentioned above, Ci-FoxA-a is one of the earliest transcription factors expressed in all endoderm precursors during embryogenesis (Di Gregorio et al., 2001); however, in juveniles this gene is exclusively detected in the endostyle (Hiruta et al., 2005); while no expression has been reported in other endodermal derivatives, EST counts indicate that some transcripts are found in the neural complex (http://www.aniseed.cnrs.fr) (Table 1). These observations suggest that even though Ci-FoxA-a plays a conserved role in endoderm differentiation in ascidian embryos, it may not be involved in the direct control of *insulin/IGF* gene expression, at least in juveniles. If this hypothesis were confirmed, it would pinpoint an important difference between *Ciona* and vertebrates, where Foxa2, to which Ci-FoxA-a is closely related, controls expression of pancreatic duodenal homeobox 1 (Pdx1) in pancreatic beta-cells (Lee et al., 2002).

In turn, Pdx1, aka insulin promoter factor 1 (Ipf1), a ParaHox homeodomain transcription factor, is a well-characterized and conserved activator of *insulin* expression in vertebrates (e.g., Madsen et al., 1997). This transcription factor, which is required for the formation of the mature pancreas (Stoffers et al., 1997), directly binds a CTAATG site in the proximal promoter region of the human *insulin* gene; a point mutation that changes this sequence to CTgATG reduces binding of IPF1/PDX1 *in vitro* and has been suggested to be a predisposing factor to type 2 diabetes (Malecki et al., 2006). *Ciona* possesses one ortholog of *Ipf1*, among numerous other homeobox genes (Di Gregorio et al., 1995). This gene is expressed weakly and ubiquitously in early embryos (Imai et al., 2004) and its product was detected *via* antibody staining in various regions of the CNS, in mesenchyme and in the adhesive organ of larvae (Corrado et al., 2001) (Fig. 4); the expression in the *Ciona* CNS is consistent with the reported expression of the orthologous gene in the developing rat CNS (Perez-Villamil et al., 1999). However, expression of *Ciona Ipf1* in either juveniles or adults is yet to be analyzed, and the available EST counts do not indicate expression in any of the adult tissues that were sampled.

In rat, the *insulin 1* promoter is bound by two homeodomain proteins, cdx-3 and lmx-1; both proteins can activate insulin transcription in transgenic assays (German et al., 1992). *Ciona* possesses one *Cdx* homeobox gene, which is expressed in various embryonic tissues, including endodermal strand, and seems weakly expressed in a few regions of stomach and intestine in juveniles (Table 1). Two *Lmx* genes are found in *Ciona*, likely as a result of a lineage-specific duplication, *Ci-Lmx* and *Ci-Lmx-like* (Jose'-Edwards et al., 2011). Both

genes are expressed in the sensory vesicle, although in different domains; however, *Ci-Lmx-like* is also expressed in notochord cells beginning at gastrulation, and persists in this tissue until the mid-tailbud stage (Jose'-Edwards et al., 2011). This suggests the possibility that Ci-Lmx-like might cooperate with Ci-Bra in activating *Ci-INSL-2* and/or *Ci-INSL-1*.

A key regulator of the specification of all pancreas endocrine precursors, neurogenin-3 (Gradwohl et al., 2000; Rukstalis and Habener, 2009), which in *Ciona* is present as a single-copy gene annotated as *Ci-neurogenin*, is expressed in vertebrates in small regions of the nervous system and later on in the precursors of pancreatic endocrine cells; interestingly, *Ci-neurogenin* is also expressed in small segments of the embryonic CNS (Imai et al., 2009) (Table 1), but is restricted to the ectoderm of the atria in juveniles and seems absent from both the digestive tract and the neural complex (Mazet et al., 2005). In the embryonic nervous system, Ci-neurogenin has been shown to control expression of *Ci-Onecut* (Pezzotti et al., 2014); interestingly, HNF-6/OC-1, one of the mouse orthologs of *Ci-Onecut*, is required for the differentiation of endocrine pancreatic cells, being responsible for the proper onset of Ipf1/Pdx1 expression in endoderm, and for the maintenance of neurogenin-3 (Jacquemin et al., 2000). However, expression of *Ci-Onecut* after metamorphosis has not been analyzed, and the EST counts indicate little or no expression in adults. In addition to controlling expression of *Ci-Onecut*, Ci-neurogenin has also been found to act upstream of *Ci-Mnx* in the developing nervous system of *Ciona* (Imai et al., 2009; Hudson et al., 2003; Ogasawara et al., 2002). Ci-Mnx is considered an ortholog of Mnx/Hlxb9/HB9, another vertebrate transcription factor with a dual neural/pancreatic function (Harrison et al., 1999; William et al., 2003).

Ci-isl1, the *Ciona* ortholog of *islet-1*, the LIM-homeodomain transcription factor required for the formation of all endocrine cells of the pancreatic islets (Ahlgren et al., 1997), is initially expressed in the precursors of the adhesive organ and sensory vesicle, and is transiently expressed in the notochord in tailbud-stage embryos (Giuliano et al., 1998). However, in juveniles this gene has only been detected in the epidermis (Ogasawara et al., 2002) and its expression in fully developed adult animals is still unknown. The *Ciona* ortholog of *Nkx6.1* (Table 1), another regulator of insulin secretion found predominantly in mature pancreatic endocrine cells (Schisler et al., 2005), is weakly expressed in the embryonic epidermis and is predominant in the visceral ganglion and lateral nerve cord (Imai et al., 2009), but has not been analyzed by *in situ* hybridization in either juveniles or adults, and is not detected by EST counts in adult tissues.

Orthologs of additional markers of pancreatic development that are found in the *Ciona* genome but whose expression is still unknown include *Arx*, which in mammals is required for the development of pancreatic alpha-cells (Wilcox et al., 2013). Limited information is available on the expression of the *Ciona* orthologs of *Lrh-1* and *PTF-1L*, which in vertebrates cooperate in controlling exocrine pancreas-specific expression of lipases, proteases and other digestive enzymes (Holmstrom et al., 2011); *Ciona Lrh-1/NR5A1/2/5* (aka *FTZF*) (Table 1) is not detectable during early embryogenesis (Imai et al., 2004) and *Ciona PTF1A* is yet to be characterized. Neither gene has been analyzed after metamorphosis.

It is noteworthy that a few relevant pancreatic transcription factors appear to be absent or highly divergent in ascidians. For example, *Ciona* seems to lack a *bona fide* ortholog of *Pax4*, which is required for the development of both beta- and delta-cells and in particular for the differentiation of beta-cells in mammals (Sosa-Pineda et al., 1997). In addition, despite the presence of several *Nkx* genes, including the early endodermal marker *TTF-1/Nkx2.1* (Ristoratore et al., 1999), a clear ortholog of *Nkx2.2* could not be found in *Ciona* (Wada et al., 2003). In mouse, *Nkx2.2* is expressed predominantly in CNS, islets and intestinal endocrine cells, where it controls cell-type specification. Knock-out mice lacking *Nkx2.2* are hyperglycemic due to the lack of insulin-producing beta-cells, which are replaced by ghrelin-producing epsilon-cells, and they also have a reduced number of the glucagon-producing alpha-cells (Sussel et al., 1998). The situation is similar in the case of *Mafb*, which in vertebrates is required for beta-cell maturation (Artner et al., 2007), as neither of the two *Ciona Maf*-related genes can be assigned to the *Mafb* class. Similarly, in the case of the *Ciona Pou* genes, none seems to belong to the Pou3 class, which includes Pou3f4/Brn4, a transcription factor involved in both the control of glucagon expression in alpha-cells and the acquisition of alpha-cell identity (Hussain et al., 2002).

Of note, *Ci-GATAa*, aka *GATA4/5/6*, which is first expressed in trunk endoderm and is found in the digestive organs after metamorphosis, is equally related to *GATA-4* and *GATA-6*, which are both required for endocrine and exocrine pancreas formation, respectively (Table 1), and have been reported to play functionally redundant roles (Carrasco et al., 2012).

NOTOCHORD EXPRESSION OF *INSULIN-LIKE* GENES: BEYOND ASCIDIANS

After the discovery of a hybrid *insulin/IGF* from *Branchiostoma californiensis* (Chan et al., 1990), the amphioxus genome project identified five additional *insulin/relaxin* genes from *B. floridae*, as well as their putative receptors (Holland et al., 2008). Four of these genes display a clustered organization and conserved synteny with the clustered *insulin-like* genes in humans and in *Ciona* (Holland et al., 2008). Thus far, expression in notochord is yet to be reported for any of these genes. However, in vertebrates, we found reports of the notochord expression of insulin-like peptides and their receptors in animals representative of different classes. Both IGF1 and its receptor, IGF1-R, were found to be expressed in the notochord of the Chilean flounder, *Paralichtys adspersus* and in numerous other tissues (Escobar et al., 2011). Similar results were reported in the grass carp, *Ctenopharyngodon idellus*, where the *igf2b* gene was transcribed in notochord and brain of the embryos and in multiple tissues of the adult fish (Yuan et al., 2011). In zebrafish, four *IGF* genes, *igf-1a*, *igf-1b*, *igf-2a* and *igf-2b*, have been reported; of these, *igf-1b* is exclusively detected in the gonad and *igf-2b* is liver-specific (Zou et al., 2009). *igf-1a* and *igf-2a* were found in multiple tissues, including the notochord (Zou et al., 2009), although another study published in the same year described *igf-2a* as notochord-specific (White et al., 2009). Morpholino-mediated knockdown of *igf-2a* led to various defects, including notochord undulations (White et al., 2009). Over-expression of this gene by mRNA injection affected notochord formation and increased the distance between contralateral somites, thus impairing midline development;

in addition, 24% of the *igf-2a* mRNA-injected embryos had partially or fully duplicated notochords (Zou et al., 2009).

In amphibians, expression of a member of the IGFBP superfamily, *Xenopus* xIGFBP-rP10, has been reported in the notochord and other tissues (Kuerner and Steinbeisser, 2006). In birds, the *igf1r* mRNA was found at high levels in the notochord and somites in the 20-somite stage chick embryo (Holzenberger et al., 1996), confirming earlier immunolocalization studies (Ralphs et al., 1990). In mammals, expression of both insulin and IGF1 was detected in the rat notochord by immunostaining (Korgun et al., 2003). Remarkably, in humans, both IGF1 and IGF1R have been reported in chordoma, a notochord-derived tumor (Nibu et al., 2013); interestingly, 41% of the chordomas analyzed in a subsequent study contained phosphorylated IGF1R/IR, which was absent in benign notochordal tumors and in fetal notochord (Sommer et al., 2010).

Together, these results suggest that *insulin-like* genes, their receptors and other members of the insulin pathway are evolutionarily conserved components of the basic notochord toolkit.

CIS-REGULATORY SEQUENCES CONTROLLING NOTOCHORD EXPRESSION OF *CI-INSL-2*

Despite the recurring presence of *insulin* and/or *IGF* gene(s) expression in the notochord in different chordates, neither the transcription factors nor the structure of the *cis*-regulatory sequences responsible for it are known. Therefore, we attempted the identification of *cis*-regulatory modules (CRMs) in the *Ci-INSL-1* and *Ci-INSL-2* genomic loci, and focused in particular on *Ci-INSL-2* since this gene displays the most specific and sustained notochord expression among the two (Fig. 1b) and it has been described as the most closely related to vertebrate *insulin* genes (Olinski et al., 2006b). Tiled genomic fragments, covering approximately 6 kb, were cloned from the *Ci-INSL-2* locus and tested for *cis*-regulatory activity in *Ciona* embryos (Fig. 3a and data not shown). This analysis led to the identification of a 1.645-kb genomic region active in notochord cells, located downstream of the *Ci-INSL-2* transcription unit (Frg. 1, Fig. 3); this region was reduced, through serial truncations (e.g., the 1.26-kb Frg. 2, Fig. 3a,c), to a minimal 172-bp CRM (Frg. 8, Fig. 3a,b,d), which recapitulated the notochord activity of the longer fragments. Through site-directed point mutations, this minimal CRM was found to require for its activity two putative binding sites for *Ciona* Brachyury (Ci-Bra), a well-characterized notochord-specific transcription factor (e.g., Di Gregorio and Levine, 1999; Dunn and Di Gregorio, 2009; Katikala et al., 2013) (Fig. 3b,d,e). Moreover, testing of genomic fragments located within and upstream of the *Ci-INSL-2* transcription unit revealed the existence of multiple *cis*-regulatory elements disseminated along this locus (Fig. 3a, f-i). In particular, additional regions able to direct notochord gene expression were uncovered, including a 418-bp sequence encompassing the first intron (Frg. 9) that directs expression in secondary notochord (the posterior-most eight notochord cells near the tip of the tail), among other tissues (Fig. 3g), and a 1.34-kb region located directly upstream of *Ci-INSL-2* that directs occasional staining in primary notochord (data not shown). The latter region, along with sequences located further upstream, recapitulated the expression of *Ci-INSL-2* in the primordium of the adhesive organ (Fig. 3h). In addition, one 248-bp fragment nested within

the main notochord CRM was active in ventral tail epidermal neurons (Frg. 6, Fig. 3f), while the 1.34-kb region located directly upstream of *Ci-INSL-2* (Frg. 10) was also active in sensory vesicle and nerve cord (Fig. 3h). A few of the fragments tested were also, or predominantly, active in muscle and mesenchyme cells (Fig. 3g-i); however, *Ci-INSL-2* is not detected in larval muscle or CNS, indicating that these regulatory elements might be either inactive or repressed within their genomic context. We also tested two genomic fragments totaling ~2 kb from the *Ci-INSL-1* upstream region, which were active in muscle, mesenchyme and nerve cord (data not shown). Published chromatin immunoprecipitation (ChIP) profiles (Kubo et al., 2010) indicate that the *Ci-INSL-1* locus is bound *in vivo* by Ci-Bra, as is *Ci-INSL-2*, which shows high peaks of occupancy by Ci-Bra particularly in its 3′-end and downstream regions, where the notochord CRM that we have identified is found (Fig. 3). Both loci are occupied in early embryos by Ci-FoxA-a, while the *Ci-INSL-3* locus is not occupied by either transcription factor (Kubo et al., 2010). These data seem consistent with the expression patterns of these three genes (Fig. 1).

CONCLUDING REMARKS

BRACHYURY AND OTHER POSSIBLE ACTIVATORS OF *INSULIN* EXPRESSION IN ASCIDIANS

In ascidian embryos, endodermal cells induce notochord formation through the activation of the BMP and FGF signaling pathways (Darras and Nishida, 2001; Imai et al., 2002a). In particular, in *Ciona*, the nuclear translocation of beta-catenin activates expression of the transcription factor FoxD by binding three Tcf/LEF binding sites in its 5′-flanking region (Imai et al., 2002b). In turn, FoxD induces transcription of Ci-ZicL, a zinc-finger transcription factor that acts as a main activator of *Ci-Bra* expression in Aline notochord precursors (Yagi et al., 2004), possibly by working in concert with the maternally expressed Ci-p53/p73-a and Ci-p53/p73-b (Noda, 2011). Expression of Ci-Bra in B-line notochord precursors is activated by two signaling pathways, Nodal and Notch/Su(H) (Hudson and Yasuo, 2006). These pathways are complemented by the action of two FGF ligands, Ci-FGF9/16/20 and Ci-FGF8/17/18 (Yasuo and Hudson, 2007). Finally, the activation of Ci-Bra expression in notochord precursors at the 64-cell stage (Corbo et al., 1997) triggers the deployment of an extensive notochord gene battery, which is responsible for the following steps of notochord formation and includes a large number of structural genes, as well as a suite of transcription factors that likely act as Ci-Bra intermediaries (Takahashi et al., 1999; Di Gregorio and Levine, 1999; Hotta et al., 2008; Dunn and Di Gregorio, 2009; Jose'-Edwards et al., 2011, 2013; Katikala et al., 2013). These studies clearly show the causal role of the endoderm in the formation of the ascidian notochord; however, it remains unclear whether any feedback of the notochord on the endoderm is required for endoderm differentiation in ascidians. The situation is quite different in higher chordates; for example, studies in chick embryos show that during early development the notochord is in direct contact with the endoderm that will give rise to the dorsal pancreatic bud, and its removal eliminates expression of markers of pancreas development, including insulin, glucagon and carboxypeptidase A (Kim et al., 1997). Related studies in human embryos confirm that the dorsal foregut endoderm is directly adjacent to the notochord before the onset of PDX1 (Jennings et al., 2013). Together, these results indicate that the notochord plays a permissive

role in the specification of pancreatic cells, and highlight the involvement of the notochord in promoting insulin expression in adjacent endodermal cells (Kim et al., 1997). Nevertheless, it remained unclear how expression of *insulin* genes is controlled within the notochord itself. The results that we presented here suggest that in *Ciona* the single-copy, notochord-specific Brachyury is involved in the control of notochord expression of at least one *insulin-like* gene, *Ci-INSL-2*. The absence of *Ci-INSL-2* expression in the endoderm during embryogenesis might seem puzzling, however it is noteworthy that before metamorphosis the endoderm of *Ciona* and other solitary ascidians is not functional, and that in juveniles, at a time when the intestine is fully functional and the notochord is no longer present, a strong expression of *Ci-INSL-2* is detected in esophagus and stomach and other regions of the digestive system. These findings raise questions on the identity of the activator(s) of *Ci-INSL-2* expression in these post-metamorphosis structures, and might suggest a continued role of Ci-Bra in the activation of *Ci-INSL-2* transcription after metamorphosis. Interestingly, ENU-induced *Ci-Bra* mutants display, in addition to the predictable disruption of notochord formation, remarkable post-metamorphosis defects in the development of the digestive organs of the juvenile (Chiba et al., 2009). It could have been simply hypothesized that these defects were due to a role of Ci-Bra in the formation of endodermal structures after metamorphosis, which would be reminiscent of the role of *Brachyury* orthologs in non-chordate invertebrates (Hamaguchi et al., 2012). However, the lack of *Ci-Bra* transcripts in hatched larvae and in juveniles (Chiba et al., 2009) makes this scenario quite unlikely. Rather, it has been proposed that the lack of functional Ci-Bra protein in *Ci-Bra* mutants might cause some notochord cells to stochastically transfate to endoderm, instead of undergoing apoptosis at the time of metamorphosis, with the result of generating supernumerary endodermal cells that are not properly incorporated within the developing digestive organs (Chiba et al., 2009). These findings indicate that insulin expression in the digestive tract of *Ciona* is controlled by transcription factor(s) other than Ci-Bra. Hence, we can use the information that is currently available on the post-metamorphosis expression of *Ciona* orthologs of known activators of insulin expression in other chordates, summarized in Table 1 and Fig. 4, to begin to delineate the identity of some of the putative activators of *Ci-INSL-2* transcription in the digestive organs. As we discussed above, the expression of the *Ciona* ortholog of Ipf1/Pdx1, one of the most relevant transcription factors required for pancreas formation and an activator of insulin expression (Stoffers et al., 1997; Malecki et al., 2006) is yet to be analyzed in either juveniles or adults. Therefore, the possibility that *Ciona* Ipf1/Pdx1 might be activating expression of the *insulin-like* genes in the digestive tract remains open. *Ci-isl*, the *Ciona* ortholog of Islet-1, a transcription factor required for the development of all pancreatic endocrine cells, is detected in CNS, adhesive organ, trunk ventral cells and, transiently, in the notochord of tailbud embryos (Giuliano et al., 1998; Stolfi et al., 2010). The expression of *Ci-isl* in the notochord suggests that this factor might be co-regulating expression of *Ci-INSL1* or *Ci-INSL2* together with Ci-Bra; however, after metamorphosis its expression has only been reported in the precursors of the atrial siphon muscle and in the epidermis (Ogasawara et al., 2002; Wang et al., 2013); these results minimize the possibility that Ci-islet might be activating *insulin-like* expression in the digestive organs. Nevertheless, other known activators of *insulin* transcription, namely Ci-MafA, Ci-Mist1, Ci-GATA-4, Ci-Cdx1/2/4 and Ci-CREB (Table 1, Fig. 4), are expressed, in juveniles, in territories fully or partially

overlapping with that of *Ci-INSL2* expression, and might therefore contribute to the activation of *Ci-INSL2* expression in the digestive tract.

EVOLUTIONARY ORIGINS OF THE ENDOCRINE PANCREAS

Lampreys and hagfish, collectively known as cyclostomes or agnathans, are considered the most primitive living vertebrates (Shimeld and Donoghue, 2012). During their larval stage, lampreys possess an “islet organ” that mainly consists of insulin-producing beta-cells accompanied by at least four different types of granular cells that might function as different types of endocrine cells (Brinn and Epple, 1976). In adult lampreys, the islet organ acquires additional cell types that are indicated as B, D, and F cells, each producing a different hormone (Youson and Al-Mahrouki, 1999), thus making this structure more closely related to the pancreas of jawed vertebrates.

The general consensus on the evolutionary origins of the pancreas is that insulin-producing beta-cells are the essential and most primitive components of its endocrine division (e.g., Madsen, 2007). This hypothesis is supported by the findings of insulin and IGF in the hepatopancreas of molluscs (Gallardo et al., 2003; Chung, 2014), and by our findings in *Ciona*, where the existence of glucagon, amylin, pancreatic peptide, ghrelin and somatostatin has not been confirmed. The diffused expression of *Ci-INSL-2* argues against the existence of a discrete endocrine pancreatic region in *Ciona*. Such region is somewhat more refined in amphioxus, where the expression patterns of *AmphiXlox*, the ortholog of *Ipf1/Pdx1*, and *AmphiCdx* (Table 1) overlap in the posterior gut (Brooke et al., 1998), while the localization of *AmphiNeurogenin* and of insulin-like peptides suggest that the presumptive pancreas might lie in the mid-gut (Holland et al., 2000; Reinecke, 1981). In ascidians other than *Ciona*, particularly *Halocynthia*, a so-called hepatopancreas is anatomically distinguishable as an outpocketing of the stomach, and is believed to release digestive enzymes into the lumen of the stomach, thus acting as a primitive exocrine pancreas (Hirano and Nishida, 2000). Even though a hepatopancreas has been described in *Ciona* (Hammond et al., 2005), its anatomical bearings and its functional role in this species are less clear. Therefore, it would be of interest to assess the expression of genes encoding digestive enzymes and additional markers of the exocrine pancreas in other regions of the digestive tract in both juveniles and adults, in order to determine whether a primordial exocrine pancreas-like structure exists in *Ciona* and other ascidians. With respect to the endocrine pancreas, we could hypothesize that in *Ciona* the transcription factors that in vertebrates are coexpressed in pancreatic beta-cells might instead control insulin expression in different regions of the digestive system, by acting either individually or combinatorially, as opposed to being colocalized within a small organ-like structure. In conclusion, our experimental results, combined with information found in the literature that we have reviewed here, indicate that a significant fraction of the components of the pancreatic gene regulatory network, along with the molecular infrastructure required for the formation of insulin-producing cells, are present and might be operational in ascidians. Moreover, several pancreatic transcription factors are deployed in the digestive organs after metamorphosis; however, it should be remembered that some of the factors that in vertebrates control pancreatic organogenesis and differentiation might be playing derived, lineage-specific roles in ascidians.

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Abbreviations

bp	base pair(s)
CNS	central nervous system
kb	kilobase(s), or 1000 base pairs
EST	Expressed Sequence Tag
mut	mutant
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
wt	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

References

Ahlgren U, Pfaff SL, Jessell TM, Edlund T, Edlund H. Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. Nature. 1997; 385:257–260. [PubMed: 9000074]

Artner I, Blanchi B, Raum JC, Guo M, Kaneko T, Cordes S, Sieweke M, Stein R. MafB is required for islet beta cell maturation. Proc Natl Acad Sci U S A. 2007; 104:3853–3858. [PubMed: 17360442]

Baquié M, St-Onge L, Kerr-Conte J, Cobo-Vuilleumier N, Lorenzo PI, Jimenez Moreno CM, Cederroth CR, Nef S, Borot S, Bosco D, Wang H, Marchetti P, Pattou F, Wollheim CB, Gauthier BR. The liver receptor homolog-1 (LRH-1) is expressed in human islets and protects {beta}-cells against stress-induced apoptosis. Hum Mol Genet. 2011; 20:2823–2833. [PubMed: 21536586]

Bevis PJ, Thorndyke MC. Endocrine cells in the oesophagus of the ascidian *Styela clava*, a cytochemical and immunofluorescence study. Cell Tissue Res. 1978; 187:153–158. [PubMed: 343918]

Bort R, Martinez-Barbera JP, Beddington RS, Zaret KS. Hex homeobox gene-dependent tissue positioning is required for organogenesis of the ventral pancreas. Development. 2004; 131:797–806. [PubMed: 14736744]

Brinn JE, Epple A. New types of islet cells in a cyclostome, *Petromyzon marinus* L. Cell Tissue Res. 1976; 171:317–329. [PubMed: 61816]

Brooke NM, Garcia-Fernàndez J, Holland PW. The ParaHox gene cluster is an evolutionary sister of the Hox gene cluster. Nature. 1998; 392:920–922. [PubMed: 9582071]

Burke RD, Angerer LM, Elphick MR, Humphrey GW, Yaguchi S, Kiyama T, Liang S, Mu X, Agca C, Klein WH, Brandhorst BP, Rowe M, Wilson K, Churcher AM, Taylor JS, Chen N, Murray G, Wang D, Mellott D, Olinski R, Hallböök F, Thorndyke MC. A genomic view of the sea urchin nervous system. Dev Biol. 2006; 300:434–460. [PubMed: 16965768]

Buteau J, Accili D. Regulation of pancreatic beta-cell function by the forkhead protein FoxO1. Diabetes Obes Metab. 2007; 9(Suppl 2):140–146. [PubMed: 17919188]

Carrasco M, Delgado I, Soria B, Martín F, Rojas A. GATA4 and GATA6 control mouse pancreas organogenesis. J Clin Invest. 2012; 122:3504–3415. [PubMed: 23006330]

Chan SJ, Cao QP, Steiner DF. Evolution of the insulin superfamily: cloning of a hybrid insulin/insulin-like growth factor cDNA from amphioxus. Proc Natl Acad Sci U S A. 1990; 87:9319–9323. [PubMed: 1701257]

Chiba S, Sasaki A, Nakayama A, Takamura K, Satoh N. Development of *Ciona intestinalis* juveniles (through 2nd ascidian stage). Zoolog Sci. 2004; 21:285–298. [PubMed: 15056923]

Chiba S, Jiang D, Satoh N, Smith WC. Brachyury null mutant-induced defects in juvenile ascidian endodermal organs. Development. 2009; 136:35–39. [PubMed: 19019990]

Chung JS. An insulin-like growth factor found in hepatopancreas implicates carbohydrate metabolism of the blue crab *Callinectes sapidus*. Gen Comp Endocrinol. 2014; 199:56–64. [PubMed: 24503150]

Conlon JM, Reinecke M, Thorndyke MC, Falkmer S. Insulin and other islet hormones (somatostatin, glucagon and PP) in the neuroendocrine system of some lower vertebrates and that of invertebrates--a minireview. Horm Metab Res. 1988; 20:406–410. [PubMed: 2901999]

Conlon JM. Evolution of the insulin molecule: insights into structure-activity and phylogenetic relationships. Peptides. 2001; 22:1183–1193. [PubMed: 11445250]

Corbo JC, Levine M, Zeller RW. Characterization of a notochord-specific enhancer from the *Brachyury* promoter region of the ascidian, *Ciona intestinalis*. Development. 1997; 124:589–602. [PubMed: 9043074]

Corrado M, Aniello F, Fucci L, Branno M. Ci-IPF1, the pancreatic homeodomain transcription factor, is expressed in neural cells of *Ciona intestinalis* larva. Mech Dev. 2001; 102:271–274. [PubMed: 11287209]

Dalle S, Quoyer J, Varin E, Costes S. Roles and regulation of the transcription factor CREB in pancreatic β-cells. Curr Mol Pharmacol. 2011; 4:187–195. [PubMed: 21488836]

Darras S, Nishida H. The BMP signaling pathway is required together with the FGF pathway for notochord induction in the ascidian embryo. Development. 2001; 128:2629–2638. [PubMed: 11526070]

Davidson B, Levine M. Evolutionary origins of the vertebrate heart: Specification of the cardiac lineage in *Ciona intestinalis*. Proc Natl Acad Sci U S A. 2003; 100:11469–11473. [PubMed: 14500781]

Davidson JK, Falkmer S, Mehrotra BK, Wilson S. Insulin assays and light microscopical studies of digestive organs in protostomian and deuterostomian species and in coelenterates. Gen Comp Endocrinol. 1971; 17:388–401. [PubMed: 4106218]

De Bernardi F, Pennati R, Candiani S, Zega G, Groppelli S, Pestarino M. Serotonin in the morphogenesis of ascidian nervous system. Caryologia. 2006; 59:375–379.

Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, De Tomaso A, Davidson B, Di Gregorio A, Gelpke M, Goodstein DM, et al. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science. 2002; 298:2157–2167. [PubMed: 12481130]

de Pablo F, Chambers SA, Ota A. Insulin-related molecules and insulin effects in the sea urchin embryo. Dev Biol. 1988; 130:304–310. [PubMed: 3053295]

Di Gregorio A, Spagnuolo A, Ristoratore F, Pischetola M, Aniello F, Branno M, Cariello L, Di Lauro R. Cloning of ascidian homeobox genes provides evidence for a primordial chordate cluster. Gene. 1995; 156:253–257. [PubMed: 7758964]

Di Gregorio A, Levine M. Regulation of *Ci-tropomyosin-like*, a Brachyury target gene in the ascidian, *Ciona intestinalis*. Development. 1999; 126:5599–5609. [PubMed: 10572037]

Di Gregorio A, Corbo JC, Levine M. The regulation of forkhead/HNF-3beta expression in the *Ciona* embryo. Dev Biol. 2001; 229:31–43. [PubMed: 11133152]

Di Gregorio A, Levine M. Analyzing gene regulation in ascidian embryos: new tools for new perspectives. Differentiation. 2002; 70:132–139. [PubMed: 12147132]

Dufort D, Schwartz L, Harpal K, Rossant J. The transcription factor HNF3beta is required in visceral endoderm for normal primitive streak morphogenesis. Development. 1998; 125:3015–3025. [PubMed: 9671576]

Dunn MP, Di Gregorio A. The evolutionarily conserved *leprecan* gene: its regulation by Brachyury and its role in the developing *Ciona* notochord. Dev Biol. 2009; 328:561–774. [PubMed: 19217895]

Edmondson SR, Thumiger SP, Werther GA, Wraight CJ. Epidermal homeostasis: the role of the growth hormone and insulin-like growth factor systems. Endocr Rev. 2003; 24:737–764. [PubMed: 14671001]

Escobar S, Fuentes EN, Poblete E, Valdés JA, Safian D, Reyes AE, Alvarez M, Molina A. Molecular cloning of IGF-1 and IGF-1 receptor and their expression pattern in the Chilean flounder (Paralichthys adspersus). Comp Biochem Physiol B Biochem Mol Biol. 2011; 159:140–147. [PubMed: 21447399]

Fiala-Medioni A, Pequignat E. Enzyme activities in the gills of benthic filter feeders (ascidia). C R Acad Sci Hebd Seances Acad Sci D. 1975; 281:1123–1126. [PubMed: 813884]

Fritsch HA, Van Noorden S, Pearse AG. Substance P-, neurotensin- and bombesin-like immunoreactivities in the gill epithelium of *Ciona intestinalis* L. Cell Tissue Res. 1980; 208:467–473. [PubMed: 6156765]

Fritsch HA, Van Noorden S, Pearse AG. Gastro-intestinal and neurohormonal peptides in the alimentary tract and cerebral complex of *Ciona intestinalis* (Ascidiaceae). Cell Tissue Res. 1982; 223:369–402. [PubMed: 6279305]

Fujisawa T, Hayakawa E. Peptide signaling in *Hydra*. Int J Dev Biol. 2012; 56:543–550. [PubMed: 22689368]

Fulzele K, Clemens TL. Novel functions for insulin in bone. Bone. 2012; 50:452–456. [PubMed: 21723973]

Gallardo N, Carrillo O, Moltó E, Deás M, González-Suárez R, Carrascosa JM, Ros M, Andrés A. Isolation and biological characterization of a 6-kDa protein from hepatopancreas of lobster *Panulirus argus* with insulin-like effects. Gen Comp Endocrinol. 2003; 131:284–290. [PubMed: 12714010]

German MS, Wang J, Chadwick RB, Rutter WJ. Synergistic activation of the insulin gene by a LIM-homeo domain protein and a basic helix-loop-helix protein: building a functional insulin minienhancer complex. Genes Dev. 1992; 6:2165–2176. [PubMed: 1358758]

Gierl MS, Karoulias N, Wende H, Strehle M, Birchmeier C. The zinc-finger factor Insm1 (IA-1) is essential for the development of pancreatic beta cells and intestinal endocrine cells. Genes Dev. 2006; 20:2465–2478. [PubMed: 16951258]

Giuliano P, Marino R, Pinto MR, De Santis R. Identification and developmental expression of *Ci-isl*, a homologue of vertebrate islet genes, in the ascidian *Ciona intestinalis*. Mech Dev. 1998; 78:199–202. [PubMed: 9858732]

Goldsworthy M, Hugill A, Freeman H, Horner E, Shimomura K, Bogani D, Pieles G, Mijat V, Arkell R, Bhattacharya S, Ashcroft FM, Cox RD. Role of the transcription factor sox4 in insulin secretion and impaired glucose tolerance. Diabetes. 2008; 57:2234–2244. [PubMed: 18477811]

Gradwohl G, Dierich A, LeMeur M, Guillemot F. neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A. 2000; 97:1607–1611. [PubMed: 10677506]

Hamaguchi T, Takashima S, Okamoto A, Imaoka M, Okumura T, Murakami R. Dorsoventral patterning of the *Drosophila* hindgut is determined by interaction of genes under the control of two independent gene regulatory systems, the dorsal and terminal systems. Mech Dev. 2012; 129:236–243. [PubMed: 22898294]

Hammond JA, Nakao M, Smith VJ. Cloning of a glycosylphosphatidylinositol-anchored alpha-2-macroglobulin cDNA from the ascidian, *Ciona intestinalis*, and its possible role in immunity. Mol Immunol. 2005; 42:683–694. [PubMed: 15781112]

Harrison KA, Thaler J, Pfaff SL, Gu H, Kehrl JH. Pancreas dorsal lobe agenesis and abnormal islets of Langerhans in Hlxb9-deficient mice. Nat Genet. 1999; 23:71–75. [PubMed: 10471502]

Hinegardner RT. Growth and development of the laboratory cultured sea urchin. Biol Bull. 1969; 137:465–475. [PubMed: 5407180]

Hirano T, Nishida H. Developmental fates of larval tissues after metamorphosis in ascidian *Halocynthia roretzi* I. Origin of mesodermal tissues of the juvenile. Dev Biol. 1997; 192:199–210. [PubMed: 9441662]

Hirano T, Nishida H. Developmental fates of larval tissues after metamorphosis in the ascidian, *Halocynthia roretzi*II. Origin of endodermal tissues of the juvenile. Dev Genes Evol. 2000; 210:55–63. [PubMed: 10664148]

Hiruta J, Mazet F, Yasui K, Zhang P, Ogasawara M. Comparative expression analysis of transcription factor genes in the endostyle of invertebrate chordates. Dev Dyn. 2005; 233:1031–1037. [PubMed: 15861404]

Holland LZ, Schubert M, Holland ND, Neuman T. Evolutionary conservation of the presumptive neural plate markers AmphiSox1/2/3 and AmphiNeurogenin in the invertebrate chordate amphioxus. Dev Biol. 2000; 226:18–33. [PubMed: 10993671]

Holland LZ, Albalat R, Azumi K, Benito-Gutiérrez E, Blow MJ, Bronner-Fraser M, Brunet F, Butts T, Candiani S, Dishaw LJ, et al. The amphioxus genome illuminates vertebrate origins and cephalochordate biology. Genome Res. 2008; 18:1100–1111. [PubMed: 18562680]

Holmstrom SR, Deering T, Swift GH, Poelwijk FJ, Mangelsdorf DJ, Kliewer SA, MacDonald RJ. LRH-1 and PTF1-L coregulate an exocrine pancreas-specific transcriptional network for digestive function. Genes Dev. 2011; 25:1674–1679. [PubMed: 21852532]

Holzenberger M, Lapointe F, Leibovici M, Lièvre CA. The avian IGF type 1 receptor: cDNA analysis and in situ hybridization reveal conserved sequence elements and expression patterns relevant for the development of the nervous system. Brain Res Dev Brain Res. 1996; 97:76–87.

Horie T, Shinki R, Ogura Y, Kusakabe TG, Satoh N, Sasakura Y. Ependymal cells of chordate larvae are stem-like cells that form the adult nervous system. Nature. 2011; 469:525–528. [PubMed: 21196932]

Hotta K, Takahashi H, Satoh N, Gojobori T. Brachyury-downstream gene sets in a chordate, *Ciona intestinalis*: integrating notochord specification, morphogenesis and chordate evolution. Evol Dev. 2008; 10:37–51. [PubMed: 18184356]

Hudson C, Darras S, Caillol D, Yasuo H, Lemaire P. A conserved role for the MEK signalling pathway in neural tissue specification and posteriorisation in the invertebrate chordate, the ascidian *Ciona intestinalis*. Development. 2003; 130:147–159. [PubMed: 12441299]

Hudson C, Yasuo H. A signalling relay involving Nodal and Delta ligands acts during secondary notochord induction in *Ciona* embryos. Development. 2006; 133:2855–2864. [PubMed: 16835438]

Hudson C, Kawai N, Negishi T, Yasuo H. β-Catenin-driven binary fate specification segregates germ layers in ascidian embryos. Curr Biol. 2013; 23:491–495. [PubMed: 23453950]

Hunter CS, Dixit S, Cohen T, Ediger B, Wilcox C, Ferreira M, Westphal H, Stein R, May CL. Islet α-, β-, and δ-cell development is controlled by the Ldb1 coregulator, acting primarily with the islet-1 transcription factor. Diabetes. 2013; 62:875–886. [PubMed: 23193182]

Hussain MA, Miller CP, Habener JF. Brn-4 transcription factor expression targeted to the early developing mouse pancreas induces ectopic glucagon gene expression in insulin-producing beta cells. J Biol Chem. 2002; 277:16028–16032. [PubMed: 11834725]

Imai K, Takada N, Satoh N, Satou Y. (beta)-catenin mediates the specification of endoderm cells in ascidian embryos. Development. 2000; 127:3009–3020. [PubMed: 10862739]

Imai KS, Satoh N, Satou Y. Early embryonic expression of FGF4/6/9 gene and its role in the induction of mesenchyme and notochord in *Ciona savignyi* embryos. Development. 2002; 129:1729–1738. [PubMed: 11923208]

Imai KS, Satou Y, Satoh N. Multiple functions of a Zic-like gene in the differentiation of notochord, central nervous system and muscle in *Ciona savignyi* embryos. Development. 2002; 129:2723–2732. [PubMed: 12015299]

Imai KS, Hino K, Yagi K, Satoh N, Satou Y. Gene expression profiles of transcription factors and signaling molecules in the ascidian embryo: towards a comprehensive understanding of gene networks. Development. 2004; 131:4047–4058. [PubMed: 15269171]

Imai KS, Levine M, Satoh N, Satou Y. Regulatory blueprint for a chordate embryo. Science. 2006; 312:1183–1187. [PubMed: 16728634]

Imai KS, Stolfi A, Levine M, Satou Y. Gene regulatory networks underlying the compartmentalization of the *Ciona* central nervous system. Development. 2009; 136:285–293. [PubMed: 19088089]

Jacquemin P, Durviaux SM, Jensen J, Godfraind C, Gradwohl G, Guillemot F, Madsen OD, Carmeliet P, Dewerchin M, Collen D, Rousseau GG, Lemaigre FP. Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3. Mol Cell Biol. 2000; 20:4445–4454. [PubMed: 10825208]

Jeffery WR, Chiba T, Krajka FR, Deyts C, Satoh N, Joly JS. Trunk lateral cells are neural crest-like cells in the ascidian *Ciona intestinalis*: insights into the ancestry and evolution of the neural crest. Dev Biol. 2008; 324:152–160. [PubMed: 18801357]

Jennings RE, Berry AA, Kirkwood-Wilson R, Roberts NA, Hearn T, Salisbury RJ, Blaylock J, Piper Hanley K, Hanley NA. Development of the human pancreas from foregut to endocrine commitment. Diabetes. 2013; 62:3514–3522. [PubMed: 23630303]

Jensen J, Pedersen EE, Galante P, Hald J, Heller RS, Ishibashi M, Kageyama R, Guillemot F, Serup P, Madsen OD. Control of endodermal endocrine development by Hes-1. Nat Genet. 2000; 24:36–44. [PubMed: 10615124]

Johnsen AH, Rehfeld JF. Cionin: a disulfotyrosyl hybrid of cholecystokinin and gastrin from the neural ganglion of the protochordate *Ciona intestinalis*. J Biol Chem. 1990; 265:3054–3058. [PubMed: 2303439]

José-Edwards DS, Kerner P, Kugler JE, Deng W, Jiang D, Di Gregorio A. The identification of transcription factors expressed in the notochord of *Ciona intestinalis* adds new potential players to the brachyury gene regulatory network. Dev Dyn. 2011; 240:1793–1805. [PubMed: 21594950]

José-Edwards DS, Oda-Ishii I, Nibu Y, Di Gregorio A. Tbx2/3 is an essential mediator within the Brachyury gene network during *Ciona* notochord development. Development. 2013; 140:2422–2433. [PubMed: 23674602]

Katikala L, Aihara H, Passamaneck YJ, Gazdoiu S, José-Edwards DS, Kugler JE, Oda-Ishii I, Imai JH, Nibu Y, Di Gregorio A. Functional Brachyury binding sites establish a temporal read-out of gene expression in the *Ciona* notochord. PLoS Biol. 2013; 11(10):e1001697. [PubMed: 24204212]

Kawada T, Ogasawara M, Sekiguchi T, Aoyama M, Hotta K, Oka K, Satake H. Peptidomic analysis of the central nervous system of the protochordate, *Ciona intestinalis*: homologs and prototypes of vertebrate peptides and novel peptides. Endocrinology. 2011; 152:2416–2427. [PubMed: 21467196]

Kenyon C. The first long-lived mutants: discovery of the insulin/IGF-1 pathway for ageing. Philos Trans R Soc Lond B Biol Sci. 2011; 366:9–16. [PubMed: 21115525]

Kim SK, Hebrok M, Melton DA. Notochord to endoderm signaling is required for pancreas development. Development. 1997; 124:4243–4252. [PubMed: 9334273]

Kinkel MD, Eames SC, Alonzo MR, Prince VE. Cdx4 is required in the endoderm to localize the pancreas and limit beta-cell number. Development. 2008; 135:919–929. [PubMed: 18234725]

Konturek SJ, Konturek JW, Pawlik T, Brzozowski T. Brain-gut axis and its role in the control of food intake. J Physiol Pharmacol. 2004; 55:137–154. [PubMed: 15082874]

Korgun ET, Dohr G, Desoye G, Demir R, Kayisli UA, Hahn T. Expression of insulin, insulin-like growth factor I and glucocorticoid receptor in rat uterus and embryo during decidualization, implantation and organogenesis. Reproduction. 2003; 125:75–84. [PubMed: 12622698]

Krapp A, Knöfler M, Ledermann B, Bürki K, Berney C, Zoerkler N, Hagenbüchle O, Wellauer PK. The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. Genes Dev. 1998; 12:3752–3763. [PubMed: 9851981]

Kubo A, Suzuki N, Yuan X, Nakai K, Satoh N, Imai KS, Satou Y. Genomic cis-regulatory networks in the early *Ciona intestinalis* embryo. Development. 2010; 137:1613–1623. [PubMed: 20392745]

Kuerner KM, Steinbeisser H. Expression analysis of IGFBP-rP10, IGFBP-like and Mig30 in early *Xenopus* development. Dev Dyn. 2006; 235:2861–2867. [PubMed: 16894599]

Kusakabe T, Yoshida R, Kawakami I, Kusakabe R, Mochizuki Y, Yamada L, Shin-I T, Kohara Y, Satoh N, Tsuda M, Satou Y. Gene expression profiles in tadpole larvae of *Ciona intestinalis*. Dev Biol. 2002; 242:188–203. [PubMed: 11820815]

Laser B, Meda P, Constant I, Philippe J. The caudal-related homeodomain protein Cdx-2/3 regulates glucagon gene expression in islet cells. J Biol Chem. 1996; 271:28984–28994. [PubMed: 8910549]

Lee CS, Sund NJ, Vatamaniuk MZ, Matschinsky FM, Stoffers DA, Kaestner KH. Foxa2 controls Pdx1 gene expression in pancreatic beta-cells in vivo. Diabetes. 2002; 51:2546–2451. [PubMed: 12145169]

Lemaire P. Unfolding a chordate developmental program, one cell at a time: invariant cell lineages, short-range inductions and evolutionary plasticity in ascidians. Dev Biol. 2009; 332:48–60. [PubMed: 19433085]

Liu WD, Wang HW, Muguira M, Breslin MB, Lan MS. INSM1 functions as a transcriptional repressor of the neuroD/beta2 gene through the recruitment of cyclin D1 and histone deacetylases. Biochem J. 2006; 397:169–177. [PubMed: 16569215]

Luo J, Becnel J, Nichols CD, Nässel DR. Insulin-producing cells in the brain of adult *Drosophila* are regulated by the serotonin 5-HT1A receptor. Cell Mol Life Sci. 2012; 69:471–484. [PubMed: 21818550]

Madsen OD, Jensen J, Petersen HV, Pedersen EE, Oster A, Andersen FG, Jørgensen MC, Jensen PB, Larsson LI, Serup P. Transcription factors contributing to the pancreatic beta-cell phenotype. Horm Metab Res. 1997; 29:265–270. [PubMed: 9230347]

Madsen OD. Pancreas phylogeny and ontogeny in relation to a 'pancreatic stem cell'. C R Biol. 2007; 330:534–537. [PubMed: 17631449]

Malecki MT, Lebrun P, Pezzolesi M, Warram JH, Krolewski AS, Jhala US. A newly identified mutation in an IPF1 binding site of the insulin gene promoter may predispose to type 2 diabetes mellitus. Diabetologia. 2006; 49:1985–1987. [PubMed: 16741735]

Manni L, Agnoletto A, Zaniolo G, Burighel P. Stomodeal and neurohypophysial placodes in *Ciona intestinalis*: insights into the origin of the pituitary gland. J Exp Zool B Mol Dev Evol. 2005; 304:324–339. [PubMed: 15887241]

Martinelli P, Cañamero M, del Pozo N, Madriles F, Zapata A, Real FX. Gata6 is required for complete acinar differentiation and maintenance of the exocrine pancreas in adult mice. Gut. 2013; 62:1481–1488. [PubMed: 23002247]

Mavropoulos A, Devos N, Biemar F, Zecchin E, Argenton F, Edlund H, Motte P, Martial JA, Peers B. sox4b is a key player of pancreatic alpha cell differentiation in zebrafish. Dev Biol. 2005; 285:211–223. [PubMed: 16055112]

Mazet F, Hutt JA, Milloz J, Millard J, Graham A, Shimeld SM. Molecular evidence from *Ciona intestinalis* for the evolutionary origin of vertebrate sensory placodes. Dev Biol. 2005; 282:494–508. [PubMed: 15950613]

McRory J, Sherwood NM. Two protochordate genes encode pituitary adenylate cyclase-activating polypeptide and related family members. Endocrinology. 1997; 138:2380–2390. [PubMed: 9165026]

McRory JE, Sherwood NM. Ancient divergence of insulin and insulin-like growth factor. DNA Cell Biol. 1997; 16:939–949. [PubMed: 9303436]

Metzger DE, Gasperowicz M, Otto F, Cross JC, Gradwohl G, Zaret KS. The transcriptional co-repressor Grg3/Tle3 promotes pancreatic endocrine progenitor delamination and β-cell differentiation. Development. 2012; 139:1447–1456. [PubMed: 22434868]

Millar, RH. Ciona. Liverpool: University of Liverpool Press; 1953.

Munro E, Robin F, Lemaire P. Cellular morphogenesis in ascidians: how to shape a simple tadpole. Curr Opin Genet Dev. 2006; 16:399–405. [PubMed: 16782323]

Nakatani Y, Nishida H. Induction of notochord during ascidian embryogenesis. Dev Biol. 1994; 166:289–299. [PubMed: 7958453]

Nakayama-Ishimura A, Chambon JP, Horie T, Satoh N, Sasakura Y. Delineating metamorphic pathways in the ascidian *Ciona intestinalis*. Dev Biol. 2009; 326:357–367. [PubMed: 19100250]

Nakashima K, Yamada L, Satou Y, Azuma J, Satoh N. The evolutionary origin of animal cellulose synthase. Dev Genes Evol. 2004; 214:81–88. [PubMed: 14740209]

Nakazawa K, Yamazawa T, Moriyama Y, Ogura Y, Kawai N, Sasakura Y, Saiga H. Formation of the digestive tract in *Ciona intestinalis* includes two distinct morphogenic processes between its anterior and posterior parts. Dev Dyn. 2013; 242:1172–1183. [PubMed: 23813578]

Nässel DR. Neuropeptides in the nervous system of *Drosophila* and other insects: multiple roles as neuromodulators and neurohormones. Prog Neurobiol. 2002; 68:1–84. [PubMed: 12427481]

Naya FJ, Huang HP, Qiu Y, Mutoh H, DeMayo FJ, Leiter AB, Tsai MJ. Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. Genes Dev. 1997; 11:2323–2334. [PubMed: 9308961]

Nibu Y, José-Edwards DS, Di Gregorio A. From notochord formation to hereditary chordoma: the many roles of Brachyury. Biomed Res Int. 2013; 2013:826435. [PubMed: 23662285]

Nishida H. Vegetal egg cytoplasm promotes gastrulation and is responsible for specification of vegetal blastomeres in embryos of the ascidian *Halocynthia roretzi*. Development. 1996; 122:1271–1279. [PubMed: 8620854]

Nishida H, Kumano G. Analysis of the temporal expression of endoderm-specific alkaline phosphatase during development of the ascidian *Halocynthia roretzi*. Dev Growth Differ. 1997; 39:199–205. [PubMed: 9108333]

Noda T. The maternal genes Ci-p53/p73-a and Ci-p53/p73-b regulate zygotic ZicL expression and notochord differentiation in *Ciona intestinalis* embryos. Dev Biol. 2011; 360:216–229. [PubMed: 21925489]

Oda-Ishii I, Di Gregorio A. Lineage-independent mosaic expression and regulation of the *Ciona multidom* gene in the ancestral notochord. Dev Dyn. 2007; 236:1806–1819. [PubMed: 17576134]

Ogasawara M, Sasaki A, Metoki H, Shin-i T, Kohara Y, Satoh N, Satou Y. Gene expression profiles in young adult *Ciona intestinalis*. Dev Genes Evol. 2002; 212:173–185. [PubMed: 12012232]

Olinski RP, Dahlberg C, Thorndyke M, Hallböök F. Three insulin-relaxin-like genes in *Ciona intestinalis*. Peptides. 2006b; 27:2535–2546. [PubMed: 16920224]

Olinski RP, Lundin LG, Hallböök F. Conserved synteny between the *Ciona* genome and human paralogons identifies large duplication events in the molecular evolution of the insulin-relaxin gene family. Mol Biol Evol. 2006a; 23:10–22. [PubMed: 16135778]

O'Neil GS, Falkmer S, Thorndyke MC. Insulin-like immunoreactivity in the neural ganglion of the ascidian *Ciona intestinalis*. Acta Zool. 1986; 67:147–153.

Owada S, Larsson O, Arkhammar P, Katz AI, Chibalin AV, Berggren PO, Bertorello AM. Glucose decreases Na+,K+-ATPase activity in pancreatic beta-cells. An effect mediated via Ca2+-independent phospholipase A2 and protein kinase C-dependent phosphorylation of the alpha-subunit. J Biol Chem. 1999; 274:2000–2008. [PubMed: 9890957]

Passamaneck YJ, Di Gregorio A. *Ciona intestinalis*: chordate development made simple. Dev Dyn. 2005; 233:1–19. [PubMed: 15765512]

Passamaneck YJ, Katikala L, Perrone L, Dunn MP, Oda-Ishii I, Di Gregorio A. Direct activation of a notochord cis-regulatory module by Brachyury and FoxA in the ascidian *Ciona intestinalis*. Development. 2009; 136:3679–3689. [PubMed: 19820186]

Perez-Villamil B, Schwartz PT, Vallejo M. The pancreatic homeodomain transcription factor IDX1/IPF1 is expressed in neural cells during brain development. Endocrinology. 1999; 140:3857–3860. [PubMed: 10433248]

Pezzotti MR, Locascio A, Racioppi C, Fucci L, Branno M. Auto and cross regulatory elements control Onecut expression in the ascidian nervous system. Dev Biol. 2014; 390:273–287. [PubMed: 24680893]

Pin CL, Rukstalis JM, Johnson C, Konieczny SF. The bHLH transcription factor Mist1 is required to maintain exocrine pancreas cell organization and acinar cell identity. J Cell Biol. 2001; 155:519–530. [PubMed: 11696558]

Pontoglio M, Sreenan S, Roe M, Pugh W, Ostrega D, Doyen A, Pick AJ, Baldwin A, Velho G, Froguel P, Levisetti M, Bonner-Weir S, Bell GI, Yaniv M, Polonsky KS. Defective insulin secretion in hepatocyte nuclear factor 1alpha-deficient mice. J Clin Invest. 1998; 101:2215–2222. [PubMed: 9593777]

Ralphs JR, Wylie L, Hill DJ. Distribution of insulin-like growth factor peptides in the developing chick embryo. Development. 1990; 109:51–58. [PubMed: 2209470]

Reinecke M. Immunohistochemical localization of polypeptide hormones in endocrine cells of the digestive tract of *Branchiostoma lanceolatum*. Cell Tissue Res. 1981; 219:445–456. [PubMed: 7023687]

Reinecke M, Betzler D, Drakenberg K, Falkmer S, Sara VR. Occurrence of members of the insulin superfamily in central nervous system and digestive tract of protochordates. Histochemistry. 1993; 99:277–285. [PubMed: 8500991]

Reinecke M, Collet C. The phylogeny of the insulin-like growth factors. Int Rev Cytol. 1998; 183:1–94. [PubMed: 9666565]

Reinecke M, Eppler E, David I, Georges D. Immunohistochemical evidence for the presence, localization and partial coexistence of insulin, insulin-like growth factor I and relaxin in the protochordate *Ciona intestinalis*. Cell Tissue Res. 1999; 295:331–338. [PubMed: 9931379]

Reverberi G, Minganti A. Fenomeni di evocazione nello sviluppo dell'uovo di Ascidie. Risultati dell'indagine sperimentale sull'uovo di *Ascidiella aspersa* e *Ascidiella malaca* allo stadio di 8 blastomeri. Pubbl Stn Zool Napoli. 1946; 20:199–252.

Ristoratore F, Spagnuolo A, Aniello F, Branno M, Fabbrini F, Di Lauro R. Expression and functional analysis of Cititf1, an ascidian NK-2 class gene, suggest its role in endoderm development. Development. 1999; 126:5149–5159. [PubMed: 10529431]

Ritz-Laser B, Mamin A, Brun T, Avril I, Schwitzgebel VM, Philippe J. The zinc finger-containing transcription factor Gata-4 is expressed in the developing endocrine pancreas and activates glucagon gene expression. Mol Endocrinol. 2005; 19:759–770. [PubMed: 15539431]

Roche J, Salvatore G, Rametta G. On the presence and the biosynthesis of thyroid hormones in a tunicate, *Ciona intestinalis* L. Biochim Biophys Acta. 1962; 63:154–165. [PubMed: 13974546]

Rukstalis JM, Habener JF. Neurogenin3: a master regulator of pancreatic islet differentiation and regeneration. Islets. 2009; 1:177–184. [PubMed: 21099270]

Sasaki A, Miyamoto Y, Satou Y, Satoh N, Ogasawara M. Novel endostyle-specific genes in the ascidian *Ciona intestinalis*. Zoolog Sci. 2003; 20:1025–1030. [PubMed: 12951410]

Sasakura Y, Nakashima K, Awazu S, Matsuoka T, Nakayama A, Azuma J, Satoh N. Transposon-mediated insertional mutagenesis revealed the functions of animal cellulose synthase in the ascidian *Ciona intestinalis*. Proc Natl Acad Sci U S A. 2005; 102:15134–15139. [PubMed: 16214891]

Sato Y, Terakado K, Morisawa M. Test cell migration and tunic formation during post-hatching development of the larva of the ascidian, *Ciona intestinalis*. Dev Growth Differ. 1997; 39:117–126. [PubMed: 9079041]

Satoh, N. Developmental genomics of ascidians. New Jersey: Wiley-Blackwell; 2014.

Satou Y, Takatori N, Yamada L, Mochizuki Y, Hamaguchi M, Ishikawa H, Chiba S, Imai K, Kano S, Murakami SD, Nakayama A, Nishino A, Sasakura Y, Satoh G, Shimotori T, Shin-I T, Shoguchi E, Suzuki MM, Takada N, Utsumi N, Yoshida N, Saiga H, Kohara Y, Satoh N. Gene expression profiles in *Ciona intestinalis* tailbud embryos. Development. 2001; 128:2893–2904. [PubMed: 11532913]

Satou Y, Yamada L, Mochizuki Y, Takatori N, Kawashima T, Sasaki A, Hamaguchi M, Awazu S, Yagi K, Sasakura Y, Nakayama A, Ishikawa H, Inaba K, Satoh N. A cDNA resource from the basal chordate *Ciona intestinalis*. Genesis. 2002; 33:153–154. [PubMed: 12203911]

Satou Y, Sasakura Y, Yamada L, Imai KS, Satoh N, Degnan B. A genomewide survey of developmentally relevant genes in *Ciona intestinalis* V. Genes for receptor tyrosine kinase pathway and Notch signaling pathway. Dev Genes Evol. 2003; 213:254–263. [PubMed: 12739141]

Satou Y, Imai KS, Satoh N. The ascidian Mesp gene specifies heart precursor cells. Development. 2004; 131:2533–2541. [PubMed: 15115756]

Satou Y, Kawashima T, Shoguchi E, Nakayama A, Satoh N. An integrated database of the ascidian, *Ciona intestinalis*: towards functional genomics. Zoolog Sci. 2005; 22:837–843. [PubMed: 16141696]

Satou Y, Mineta K, Ogasawara M, Sasakura Y, Shoguchi E, Ueno K, Yamada L, Matsumoto J, Wasserscheid J, Dewar K, Wiley GB, Macmil SL, Roe BA, Zeller RW, Hastings KE, Lemaire P, Lindquist E, Endo T, Hotta K, Inaba K. Improved genome assembly and evidence-based global

gene model set for the chordate *Ciona intestinalis*: new insight into intron and operon populations. Genome Biol. 2008; 9:R152. [PubMed: 18854010]

Schisler JC, Jensen PB, Taylor DG, Becker TC, Knop FK, Takekawa S, German M, Weir GC, Lu D, Mirmira RG, Newgard CB. The Nkx6.1 homeodomain transcription factor suppresses glucagon expression and regulates glucose-stimulated insulin secretion in islet beta cells. Proc Natl Acad Sci U S A. 2005; 102:7297–7302. [PubMed: 15883383]

Sander M, Neubüser A, Kalamaras J, Ee HC, Martin GR, German MS. Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. Genes Dev. 1997; 11:1662–1673. [PubMed: 9224716]

Seymour PA, Freude KK, Tran MN, Mayes EE, Jensen J, Kist R, Scherer G, Sander M. SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proc Natl Acad Sci U S A. 2007; 104:1865–1870. [PubMed: 17267606]

Shi W, Levine M. Ephrin signaling establishes asymmetric cell fates in an endomesoderm lineage of the *Ciona* embryo. Development. 2008; 135:931–940. [PubMed: 18234724]

Shih DQ, Navas MA, Kuwajima S, Duncan SA, Stoffel M. Impaired glucose homeostasis and neonatal mortality in hepatocyte nuclear factor 3alpha-deficient mice. Proc Natl Acad Sci U S A. 1999; 96:10152–10157. [PubMed: 10468578]

Shimeld SM, Donoghue PC. Evolutionary crossroads in developmental biology: cyclostomes (lamprey and hagfish). Development. 2012; 139:2091–2099. [PubMed: 22619386]

Shoguchi E, Kawashima T, Satou Y, Hamaguchi M, Sin-I T, Kohara Y, Putnam N, Rokhsar DS, Satoh N. Chromosomal mapping of 170 BAC clones in the ascidian *Ciona intestinalis*. Genome Res. 2006; 16:297–303. [PubMed: 16354750]

Smit AB, van Kesteren RE, Li KW, Van Minnen J, Spijker S, Van Heerikhuizen H, Geraerts WP. Towards understanding the role of insulin in the brain: lessons from insulin-related signaling systems in the invertebrate brain. Prog Neurobiol. 1998; 54:35–54. [PubMed: 9460792]

Sommer J, Itani DM, Homlar KC, Keedy VL, Halpern JL, Holt GE, Schwartz HS, Coffin CM, Kelley MJ, Cates JM. Methylthioadenosine phosphorylase and activated insulin-like growth factor-1 receptor/insulin receptor: potential therapeutic targets in chordoma. J Pathol. 2010; 220:608–617. [PubMed: 20140939]

Sosa-Pineda B, Chowdhury K, Torres M, Oliver G, Gruss P. The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. Nature. 1997; 386:399–402. [PubMed: 9121556]

Spence JR, Lange AW, Lin SC, Kaestner KH, Lowy AM, Kim I, Whitsett JA, Wells JM. Sox17 regulates organ lineage segregation of ventral foregut progenitor cells. Dev Cell. 2009; 17:62–74. [PubMed: 19619492]

Stoffers DA, Thomas MK, Habener JF. Homeodomain protein IDX-1: a master regulator of pancreas development and insulin gene expression. Trends Endocrinol Metab. 1997; 8:145–151. [PubMed: 18406800]

Stolfi A, Gainous TB, Young JJ, Mori A, Levine M, Christiaen L. Early chordate origins of the vertebrate second heart field. Science. 2010; 329:565–568. [PubMed: 20671188]

Sussel L, Kalamaras J, Hartigan-O'Connor DJ, Meneses JJ, Pedersen RA, Rubenstein JL, German MS. Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. Development. 1998; 125:2213–2221. [PubMed: 9584121]

Takahashi H, Hotta K, Erives A, Di Gregorio A, Zeller RW, Levine M, Satoh N. Brachyury downstream notochord differentiation in the ascidian embryo. Genes Dev. 1999; 13:1519–1523. [PubMed: 10385620]

Takamura K, Fujimura M, Yamaguchi Y. Primordial germ cells originate from the endodermal strand cells in the ascidian *Ciona intestinalis*. Dev Genes Evol. 2002; 212:11–18. [PubMed: 11875652]

Taleb N, Polychronakos C. RFX6 is needed for the development and maintenance of the β-cell phenotype. Islets. 2011; 3:291–293. [PubMed: 21750414]

Tanaka KJ, Ogasawara M, Makabe KW, Satoh N. Expression of pharyngeal gill-specific genes in the ascidian *Halocynthia roretzi*. Dev Genes Evol. 1996; 206:218–226. [PubMed: 24173523]

Taylor BL, Liu FF, Sander M. Nkx6.1 is essential for maintaining the functional state of pancreatic beta cells. Cell Rep. 2013; 4:1262–1275. [PubMed: 24035389]

Thorndyke MC. Observations on the gastric epithelium of ascidians with special reference to *Styela clava*. Cell Tissue Res. 1977; 184:539–550. [PubMed: 589659]

Vinther TN, Norrman M, Ribel U, Huus K, Schlein M, Steensgaard DB, Pedersen TÅ, Pettersson I, Ludvigsen S, Kjeldsen T, Jensen KJ, Hubálek F. Insulin analog with additional disulfide bond has increased stability and preserved activity. Protein Sci. 2013; 22:296–305. [PubMed: 23281053]

Wada S, Tokuoka M, Shoguchi E, Kobayashi K, Di Gregorio A, Spagnuolo A, Branno M, Kohara Y, Rokhsar D, Levine M, Saiga H, Satoh N, Satou Y. A genomewide survey of developmentally relevant genes in *Ciona intestinalis* II. Genes for homeobox transcription factors. Dev Genes Evol. 2003; 213:222–234. [PubMed: 12736825]

Wada H. Origin and genetic evolution of the vertebrate skeleton. Zoolog Sci. 2010; 27:119–123. [PubMed: 20141417]

Wang H, Maechler P, Antinozzi PA, Hagenfeldt KA, Wollheim CB. Hepatocyte nuclear factor 4alpha regulates the expression of pancreatic beta -cell genes implicated in glucose metabolism and nutrient-induced insulin secretion. J Biol Chem. 2000; 275:35953–35959. [PubMed: 10967120]

Wang W, Razy-Krajka F, Siu E, Ketcham A, Christiaen L. NK4 antagonizes Tbx1/10 to promote cardiac versus pharyngeal muscle fate in the ascidian second heart field. PLoS Biol. 2013; 11(12):e1001725. [PubMed: 24311985]

Werner H, Leroith D. Insulin and insulin-like growth factor receptors in the brain: Physiological and pathological aspects. Eur Neuropsychopharmacol. 2014 Jan 31. pii: S0924-977X(14)00041-8.

Westmoreland JJ, Kilic G, Sartain C, Sirma S, Blain J, Rehg J, Harvey N, Sosa-Pineda B. Pancreas-specific deletion of Prox1 affects development and disrupts homeostasis of the exocrine pancreas. Gastroenterology. 2012; 142:999–1009.e6. [PubMed: 22178591]

Wilcox CL, Terry NA, Walp ER, Lee RA, May CL. Pancreatic β-cell specific deletion of mouse Arx leads to β-cell identity loss. PLoS One. 2013; 13;8(6):e66214.

William CM, Tanabe Y, Jessell TM. Regulation of motor neuron subtype identity by repressor activity of Mnx class homeodomain proteins. Development. 2003; 130:1523–1536. [PubMed: 12620979]

Wilson S, Falkmer S. Starfish insulin. Can J Biochem. 1965; 43:1615–1624. [PubMed: 5864477]

White MF. The insulin signalling system and the IRS proteins. Diabetologia. 1997; 40(Suppl 2):S2–17. [PubMed: 9248696]

White YA, Kyle JT, Wood AW. Targeted gene knockdown in zebrafish reveals distinct intraembryonic functions for insulin-like growth factor II signaling. Endocrinology. 2009; 150:4366–4375. [PubMed: 19443571]

Whittaker JR. Segregation during cleavage of a factor determining endodermal alkaline phosphatase development in ascidian embryos. J Exp Zool. 1977; 202:139–153. [PubMed: 411883]

Yagi K, Satou Y, Satoh N. A zinc finger transcription factor, ZicL, is a direct activator of Brachyury in the notochord specification of *Ciona intestinalis*. Development. 2004; 131:1279–1288. [PubMed: 14993185]

Yang Y, Chang BH, Samson SL, Li MV, Chan L. The Krüppel-like zinc finger protein Glis3 directly and indirectly activates insulin gene transcription. Nucleic Acids Res. 2009; 37:2529–2538. [PubMed: 19264802]

Yasuo H, Hudson C. FGF8/17/18 functions together with FGF9/16/20 during formation of the notochord in *Ciona* embryos. Dev Biol. 2007; 302:92–103. [PubMed: 17022960]

Yonge CM. Studies on the comparative phisiology of digestion. III. Secretion, digestion, and assimilation in the gut of *Ciona intestinalis*. British J Exp Biol. 1925; 2:373–388.

Youson JH, Al-Mahrouki AA. Ontogenetic and phylogenetic development of the endocrine pancreas (islet organ) in fish. Gen Comp Endocrinol. 1999; 116:303–335. [PubMed: 10603271]

Yuan XN, Jiang XY, Pu JW, Li ZR, Zou SM. Functional conservation and divergence of duplicated insulin-like growth factor 2 genes in grass carp (*Ctenopharyngodon idellus*). Gene. 2011; 470:46–52. [PubMed: 20951190]

Zeller RW, Weldon DS, Pellatiro MA, Cone AC. Optimized green fluorescent protein variants provide improved single cell resolution of transgene expression in ascidian embryos. Dev Dyn. 2006; 235:456–467. [PubMed: 16287050]

Zhang C, Moriguchi T, Kajihara M, Esaki R, Harada A, Shimohata H, Oishi H, Hamada M, Morito N, Hasegawa K, Kudo T, Engel JD, Yamamoto M, Takahashi S. MafA is a key regulator of glucose-stimulated insulin secretion. Mol Cell Biol. 2005; 25:4969–4976. [PubMed: 15923615]

Zou S, Kamei H, Modi Z, Duan C. Zebrafish IGF genes: gene duplication, conservation and divergence, and novel roles in midline and notochord development. PLoS One. 2009; 4(9):e7026. [PubMed: 19759899]

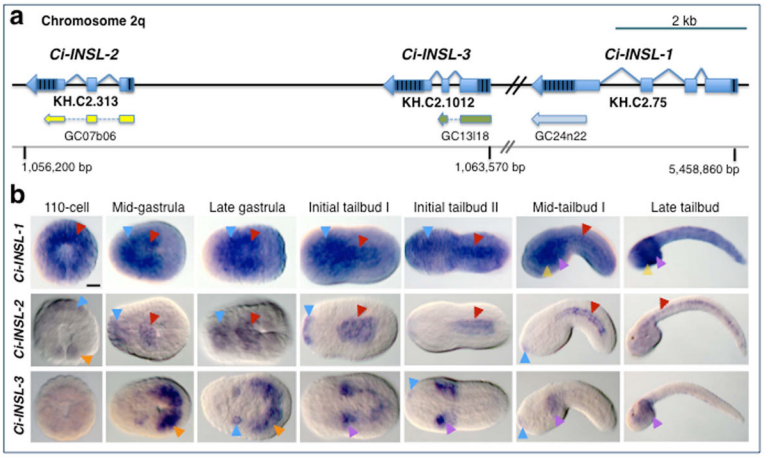

Figure 1. Genomic organization and embryonic expression of *Ciona insulin-like* genes

a: Schematic representation of the regions of *Ciona intestinalis* chromosome 02q that contain the genomic loci of *Ci-INSL-2*, *Ci-INSL-3* and *Ci-INSL-1*. Genes are represented as light-blue bars (exons) and angled lines (introns); striped areas indicate untranslated regions. Current gene model identifiers are indicated underneath each gene. The ESTs used for *in situ* hybridizations are depicted below each gene model in yellow, green, and light blue, respectively, along with the coordinates that allow their identification within the *Ciona intestinalis* Gene Collection (Satou et al., 2002). A grey horizontal line symbolizes the chromosomal DNA and reports the location of essential mapping landmarks in base pairs (bp). **b:** Microphotographs of *Ciona intestinalis* whole-mount embryos at the stages indicated on top of each column, hybridized *in situ* with digoxygenin-labeled antisense RNA probes synthesized from the EST clones shown in **a**, essentially as previously described (Oda-Ishii and Di Gregorio, 2007). Gene names are indicated on the left side of each row. 110-cell embryos are shown in vegetal views, the remaining embryos are shown with anterior to the left. Arrowheads point at representative regions of larger structures, and are color-coded as follows: red, notochord; blue, nervous system and primordium of the adhesive organ, which represents the anterior-most structure in tailbud embryos; orange, muscle precursors; purple, mesenchyme; yellow, endoderm. Scale bar: ~25 microns.

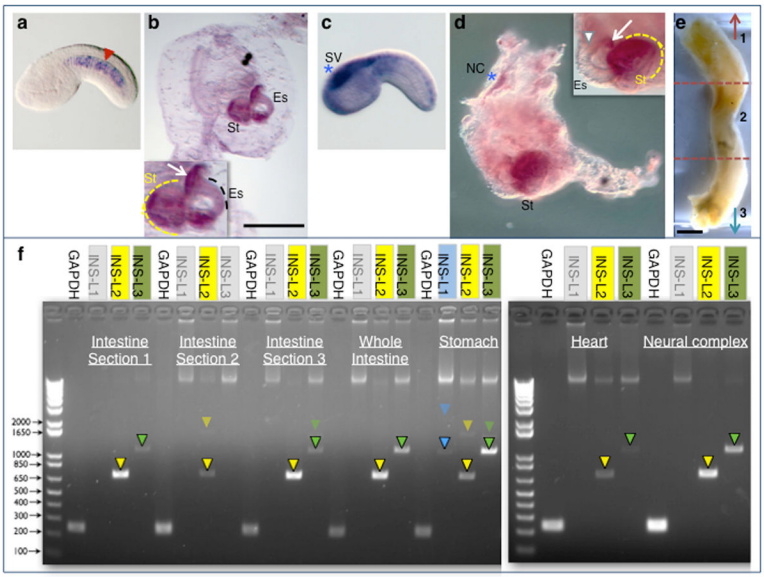

Figure 2. Expression of *Ciona insulin-like* genes after metamorphosis

a: Mid-tailbud I *Ciona* whole-mount embryo hybridized *in situ* with the *Ci-INSL-2* digoxygenin-labeled antisense RNA probe. Red arrowhead: notochord, showing expression. **b:** Whole-mount *Ciona* stage 4 juvenile (Chiba et al., 2004) hybridized *in situ* with the *Ci-INSL-2* digoxygenin-labeled antisense RNA probe, as in **a**. Expression is seen in esophagus (Es) and stomach (St) (both magnified in inset). The stomach is partially encircled by a yellow dashed line; the esophagus is contoured by a dashed black line. **c:** Mid-tailbud I *Ciona* whole-mount embryo hybridized *in situ* with the *Ci-Na*$^+$*/K*$^+$*-transporting ATPase alpha3-1* (aka *ATP1A1/2/3/4*, gene model: KH.L157.5) digoxygenin-labeled antisense RNA probe. Blue asterisk: sensory vesicle (SV), showing expression. **d:** Whole-mount *Ciona* juvenile, around stage 6 (Chiba et al., 2004), hybridized *in situ* with the *Ci-Na*$^+$*/K*$^+$*-transporting ATPase alpha3-1* probe. Expression is seen in part of the stomach and possibly in part of the rim of the esophagus (inset), as well as in the neural complex (NC). The blue asterisks in **c** and **d** refer to the persistence of cells of the sensory vesicle in the adult neural complex. Inset shows a higher magnification view of the esophagus and stomach, as seen on a different plane of focus. The white arrow points at the rim of the esophagus; the white arrowhead highlights the ventral groove of the esophagus, an anatomical point of reference. The stomach is partially encircled by a yellow dashed line. **e:** Dissected intestine of an adult *Ciona*, which was used for the detection of the expression of *Ci-INSL-1*, *Ci-INSL-2* and *Ci-INSL-3* by RT-PCR. The intestine was arbitrarily subdivided into three sections of similar length, indicated as 1-3 and separated by dashed lines. The red arrow points towards the stomach, the teal arrow towards the anus. Scale bars: in **b**, 200 microns; in **e**, 2 millimiters. **f:** Gel electrophoresis of RT-PCR products obtained from RNAs extracted from different regions of the intestine and the stomach of an adult *Ciona* individual, and from hearts and neural complexes pooled from multiple animals. Gel lanes are labeled on top with the names of the genes that were monitored. Gene names were colored in transparent grey to symbolize lack of expression. Unlabeled lanes contain a molecular weight marker; the sizes of its most informative bands are indicated on the left side. Expression of the housekeeping gene *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) was monitored in each experiment as

a positive control. Expected sizes of PCR products: GAPDH, 250 bp; INS-L1, 1,074 bp for spliced transcript (blue arrowhead) and ~2.2 kb for genomic (faded blue arrowhead); INS-L2, 710 bp for spliced transcript (yellow arrowheads) and ~1.55 kb for genomic (faded yellow arrowheads); INS-L3, 1,069 bp, for spliced transcript (green arrowheads) and ~1.47 kb for genomic (faded green arrowheads). Conditions for RT-PCR: RNA was extracted by dissecting adult animals and by processing organ tissues as recommended by the Qiagen RNeasy kit (QIAGEN). DNAse treatment was used to minimize genomic contamination, according to the manufacturer's instructions. cDNA synthesis was then conducted for each sample using the designed primers and standard RT-PCR protocol with Superscript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. PCR conditions included an initial denaturation step at 94°C for 2.5 minutes, and 27 cycles as follows: 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds. After a last step at 72°C for 10 minutes, samples were cooled at 16°C and then stored at −20°C. cDNA products were subsequently used to amplify specific regions of the *GAPDH* control and of each *Ci-INSL* gene. Forward and reverse primers for *GAPDH* and each *Ci-INSL* gene were designed in the first and in the last exons.

The sequences of the primers were:

GAPDH.F 5′-GCAGGAGCTGGCATTGCAC-3′ and GAPDH.R 5′-ACAGTAAGATTGCATGCAACAG-3′ for *GAPDH*; IL-1.V1.F 5′-TCTTTTATAGGACACAGCGCATTA-3′ and IL-1.V2.R 5′-TAAACGACCATCACAGAACAATG-3′ for *Ci-INSL-1*; IL-2.V1.F 5′-CAGCTTTGTAACAGGACAGTGAAT-3′ and IL-2.Int.R1 5′-TGTGCAAAGCTGTGTTTTCCAAGC-3′ for *Ci-INSL-2*; IL-3.V1.F 5′-AGATAAAGGGATTAGGCAGAAGTG-3′ and IL-3.V2.R 5′-GCTGTCGTACTGGGGTGTAAATA-3′ for *Ci-INSL-3*.

For the PCR amplification of *GAPDH* and *Ci-INS-L* genes, an initial 2.5-minute cycle at 94°C preceded 30 cycles as follows: 94°C for 30 seconds, 55°C for 30 seconds and 68°C for 3 minutes. After a last step at 68°C for 30 minutes, samples were cooled at 16°C for at least 15 minutes before gel electrophoresis.

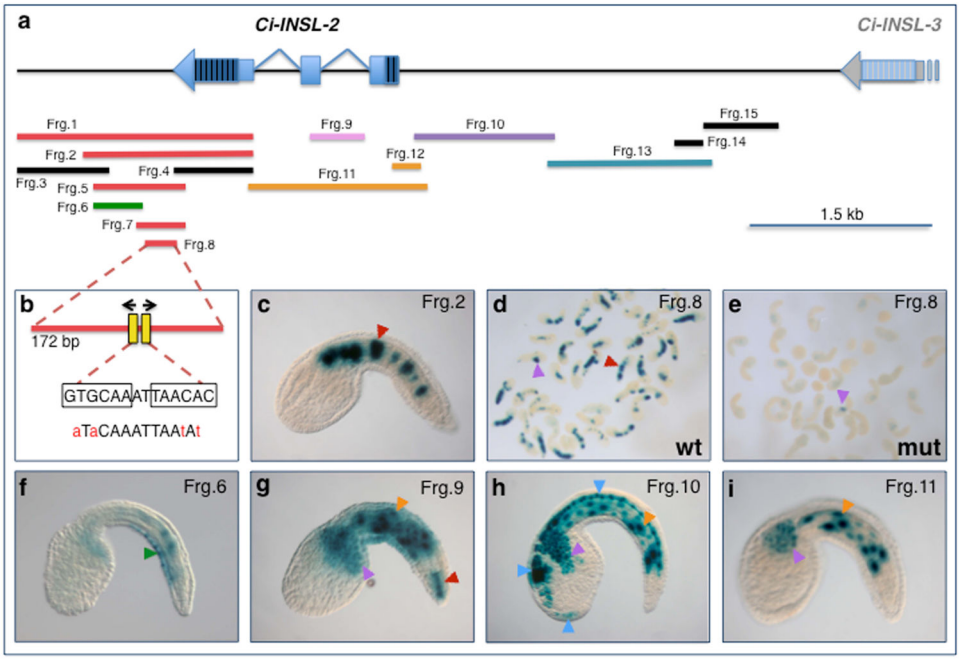

Figure 3. Identification and characterization of a notochord CRM and various *cis*-regulatory elements in the *Ci-INSL-2* genomic locus

a: Schematic representation of the chromosomal region encompassing the *Ci-INSL-2* gene and part of *Ci-INSL-3*. Genes are represented as light-blue bars (exons) and lines (introns); striped areas indicate untranslated regions. Part of the *Ci-INSL-3* coding region is depicted for reference (grey box). Colored horizontal bars symbolize genomic fragments, abbreviated as "Frg." followed by a number, that were individually cloned upstream of the *Ci-fkh/FoxA-a* basal promoter region (Oda-Ishii and Di Gregorio, 2007) and tested *in vivo* in *Ciona* embryos by electroporation (Di Gregorio and Levine, 2002). The bars are color-coded as follows: red, fragments that predominantly show activity in the notochord; pink, fragments active in secondary notochord and additional tissues; orange, fragments predominantly active in muscle; light purple, fragments active in muscle, nervous system and mesenchyme; green, fragments mainly active in epidermis; teal, fragments mainly active in muscle and nervous system; black, fragments with no detectable activity. **b:** schematic structure of the minimal 172-bp notochord CRM isolated from the 3′-region of *Ci-INSL-2* (Frg. 8). Two putative Ci-Bra binding sites (Passamaneck et al., 2009) with inverted orientation were mutagenized as shown below the diagram; mutations are indicated in red and lower case. These mutations were sufficient to completely abolish notochord activity of the CRM. **c:** Representative transgenic embryo carrying one of the truncations of the notochord CRM (Frg. 2, 1.26 kb). **d:** Low-magnification microphotograph of a group of transgenic embryos electroporated with the wild-type (wt) 172-bp *Ci-INSL-2* notochord CRM (Frg. 8). Notochord staining (red arrowhead) is seen in most embryos and is occasionally associated with mesenchyme staining (purple arrowhead). **e:** Low-magnification microphotograph of a group of transgenic embryos from the same clutch as the embryos shown in **d**, electroporated with the 172-bp *Ci-INSL-2* notochord CRM carrying the mutations (mut) in the putative Ci-Bra binding sites illustrated in **b** and reared, fixed and stained in parallel with the embryos shown in **d**. Staining is only detected in mesenchyme cells (purple arrowhead).

f–i: Representative transgenic embryos carrying the fragments indicated on the top right corner of each panel, photographed after X-gal staining. Arrowheads: red, notochord; green, epidermis and tail epidermal neurons; orange: muscle; purple, mesenchyme; blue, nervous system.

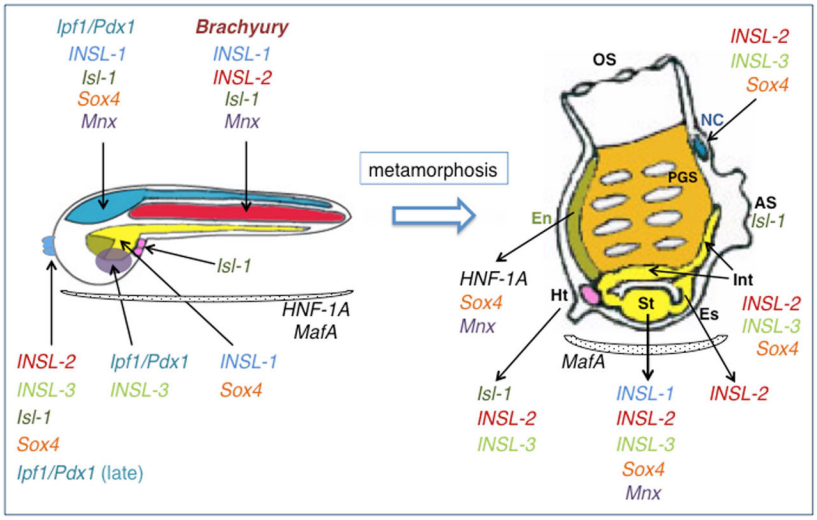

Figure 4. Graphic summary of the pre- and post-metamorphosis expression territories of *Ciona* orthologs of evolutionarily conserved pancreatic transcription factors

Simplified drawings of a *Ciona* tailbud embryo (left) and a *Ciona* juvenile (right), not drawn to scale. For simplicity, the only tissues depicted in the tailbud are the notochord (red), CNS and adhesive organ (blue), trunk endoderm and endodermal strand (yellow), presumptive endostyle (green), trunk ventral cells (pink), mesenchyme (purple). In the juvenile, the only organs depicted are the endostyle (green), pharynx (orange) and pharyngeal gill slits, heart (pink), neural complex (blue), esophagus, stomach and intestine (all yellow). Gene names are abbreviated as in Table 1. Black arrows connect the genes to the territories where their expression has been reported (Table 1 and references therein). For the sake of completeness, we have tentatively included in this figure the expression patterns of *Ci-INSL-1* and *Ci-INSL-3*; however, these patterns were detected in adults and might not be fully preserved in juveniles. Dotted semi-circles neighboring gene names symbolize ubiquitous or semi-ubiquitous expression of that particular gene. Abbreviations: OS, oral siphon; NC, neural complex; AS, atrial siphon; Int, intestine; Es, esophagus; St, stomach; Ht, heart; En, endostyle; PGS, pharyngeal gill slits.

Table 1

Transcription factors and co-factors involved in pancreas formation and/or insulin gene expression, and their *Ciona* orthologs.

Vertebrate transcription factor/cofactor, name and family	**Function in endoderm and/or pancreas**	**References**	***Cionaintestinalis* ortholog**	***Ciona* gene model**	**Expression in *Ciona* embryos**	**Expression in *Ciona* aftermetamorphosis**
Sox17 (SRY-box, HMG)	Endoderm formation and specification; organ lineage segregation	Spence et al., 2009	*Ci-SoxF, Ci-Sox15/17/18*	KH.C7.407	Epidermis, mesenchyme (Imai et al., 2004)	Stomach, other regions of the digestive tract (Ogasawara et al., 2002)
HNF-3β/Foxa2 (Forkhead box)	Endoderm specification, gut formation	Dufort et al., 1998	*Ci-fkh/HNF-3β/FoxA-a*	KH.C11.313	Notochord, CNS, endoderm (Di Gregorio et al., 2001)	Endostyle (Hiruta et al., 2005)
Ipf1/Pdx1 aka Idx1, Xlox (ParaHoxhomeodomain)	Pancreas formation, endocrine cells differentiation, insulin expression	Stoffers et al., 1997; Malecki et al., 2006	*Ci-Ipf1*	KH.C14.554	Ubiquitous in early embryos (Imai et al., 2004); CNS, mesenchyme, adhesive organ in larvae (Corrado et al., 2001)	Not analyzed
Neurogenin-3/HLH-4 (bHLH)	Specification of all endocrine cells	Gradwohl et al., 2000	*Ci-neurogenin*	KH.C6.129	Neural precursors, tail epidermis, tail neurons, visceral ganglion, nerve cord (Imai et al., 2004, 2009)	Ectoderm of the atria (Mazet et al., 2005)
HNF-6/OC-1 (Cut homeodomain)	Endocrine cells differentiation	Jacquemin et al., 2000	*Ci-Onecut*	KH.C6.185	Sensory vesicle, visceral ganglion, nerve cord (Moret et al., 2005; Imai et al., 2009)	Not analyzed
Mnx/Hlxb9/HB9 (homeodomain)	Development of dorsal pancreatic lobe; beta-cells formation	Harrison et al., 1999	*Ci-Mnx/Ci-HB9*	KH.L128.12	Notochord andneural precursors, muscle, visceral ganglion, tail neurons (Hudson et al., 2003)	Endostyle, stomach (Ogasawara et al., 2002)
Islet-1 (LIM-homeodomain)	Formation of pancreatic mesenchyme, dorsal exocrine cells differentiation, development of all endocrine cells	Ahlgren et al., 1997	*Ci-isl1*	KH.L152.2	Nerve cord, visceral ganglion, primordium of adhesive organ, notochord, sensory organs (Giuliano et al., 1998). Trunk ventral cells and their descendants (Stolfi et al., 2010)	Epidermis (Ogasawara et al., 2002) Atrial siphon muscle precursors, heart(Wang et al., 2013)
Ldb1 (co-factor) (LIM-domain binding protein)	Co-regulator of Islet-1 target genes, including insulin, MafA, Arx	Hunter et al., 2013	*Ci-Ldb1/2*	KH.C14.544	Not analyzed	Not analyzed
Sox9 (SRY-box, HMG)	Maintenance of pancreatic progenitors	Seymour et al., 2007	*Ci-SoxE, Ci-Sox10/15/8/9*	KH.L154.42	Whole embryo, stronger in nervous system and mesenchyme (Imai et al., 2004)	Pyloric gland (Wada et al., 2010)
Hex (homeodomain)	Specification of ventral pancreas	Bort et al., 2004	*Ci-Hex*	KH.L171.10	Mesenchyme, trunk lateral cells (Imai et al., 2006)	Not detected http://bioinfo.s.chibau.jp/ciaid/
Hes-1 (bHLH)	Endocrine cells differentiation	Jensen et al., 2000	*Ci-Hes, Ci-HER, Ci-E(spl)/hairy-c*	KH.L34.9	Whole embryo (Imai et al., 2004)	Not analyzed
Beta2/NeuroD1 (bHLH)	Islets maturation, control of beta-cell number	Naya et al., 1997	*Ci-atonal, Ci-ath5, Ci-HAND1/2*	KH.C8.175	Epidermal neurons (Imai et al., 2004)	Not analyzed

Vertebrate transcription factor/cofactor, name and family	Function in endoderm and/or pancreas	References	*Cionaintestinalis* ortholog	*Ciona* gene model	Expression in *Ciona* embryos	Expression in *Ciona* aftermetamorphosis
Pax6 (Paired-box)	Differentiation of alpha-and beta-cells	Sander et al., 1997	*Ci-Pax6*	KH.C9.68	Neural precursors, nerve cord, sensory vesicle, visceral ganglion (Imai et al., 2004, 2009; Irvine et al., 2008)	Not analyzed
Nkx6.1 (NK homeodomain)	Control of islet size and beta-cell number	Taylor et al., 2013	*Ci-Nkx6.1, NK6-1/2/3*	KH.C5.502	Epidermis, visceral ganglion, nerve cord (Imai et al., 2009)	Not analyzed
HNF-3α/Foxa1 (Forkhead box)	Glucose homeostasis, beta-cell and pancreatic islet function	Shih et al., 1999	*Ci-FoxA-b/FoxA1/2/3*	KH.C11.718	Whole embryo, stronger in nervous system and mesenchyme (Imai et al., 2004)	Not detected (Ogasawara et al., 2002)
Arx (Aristalesshomeo domain)	Acquisition and maintenance of alpha-cells identity	Wilcox et al., 2013	*Ci-Arx, Ci-aristaless*	KH.L119.26	Not analyzed	Not analyzed
Ptf1a-p48 (bHLH)	Regulation of exocrine cells differentiation	Krapp et al., 1998	*Ci-Ptf1a*	KH.C3.967	Not analyzed	Not analyzed
MafA (basicLeucine zipper)	Control of insulin transcription	Zhang et al., 2005	*Ci-Maf-like, Ci-L-maf*	KH.C12.675	Maternal ubiquitous signal (Imai et al., 2004)	Endostyle, esophagus, stomach, intestine, branchial pharynx, body wall muscle, neural complex, epidermis, heart, hemocytes (Ogasawara et al., 2002)
Mist1 (bHLH)	Maintenance of exocrine pancreas organization, acinar cell identity	Pin et al., 2001	*Ci-Mist, Ci-bHLHA15*	KH.C3.308	Mesenchyme (Imai et al., 2004)	Stomach and intestine http://bioinfo.s.chibau.jp/ciaid/
GATA-4 (Zinc-finger)	Activation of glucagon expression	Ritz-Laser et al., 2005	*Ci-GATAa, Ci-GATA4/5/6*	KH.L20.1	Trunk endoderm, trunk ventral cells (Hudson et al., 2006); in larvae, it switches to sensory vesicle, mesenchyme, trunk lateral cells (D'Ambrosio et al., 2003)	Stomach and part of the intestine http://bioinfo.s.chibau.jp/ciaid/ Heart (Wang et al., 2013)
GATA-6 (Zinc-finger)	Acinar differentiation, maintenance of exocrine pancreas	Martinelli et al., 2013	Same as above	Same as above	Same as above	Same as above
Prox1 (homeodomain)	Pancreas morphogenesis and exocrine pancreas homeostasis	Westmoreland et al., 2012	*Ci-Prox1*	KH.C4.430	Whole embryo (Imai et al., 2004)	Not analyzed
Same as above	Same as above	Same as above	*Ci-Prox1, Ci-prox, Ci-Prox-a*	KH.C4.500	Not analyzed	Not analyzed
Rfx6 (winged-helix)	Development and maintenance of beta-cells phenotype	Taleb and Polychronakos, 2011	*Ci-Rfx4/6, Ci-Rfx4b*	KH.S600.1	Not analyzed	Not analyzed
Cdx2/3, Cdx4 (ParaHoxhomeod omain)	Cdx2/3: Regulation of glucagon expression Cdx4: Control of number and movements of beta-cells	Cdx2/3: Laser et al., 1996 Cdx4: Kinkel et al., 2008	*Ci-Cdx, Ci-Cdx1/2/4*	KH.C14.408	Nerve cord precursors, secondary muscle lineage, tail epidermis, trunk lateral cells, endodermal strand (Satou et al., 2001; Imai et al., 2006)	Seemingly small areas of stomach and intestine http://bioinfo.s.chibau.jp/ciaid/

Vertebrate transcription factor/cofactor, name and family	Function in endoderm and/or pancreas	References	*Cionaintestinalis* ortholog	*Ciona* gene model	Expression in *Ciona* embryos	Expression in *Ciona* aftermetamorphosis
Lmx-1 (homeodomain)	Activation of insulin expression	German et al., 1992	*Ci-Lmx-like, Ci-Lmx1A*	KH.C9.485	Notochord, sensory vesicle (Jose'-Edwards et al., 2011)	Not analyzed
Lrh-1/NR5A2 (nuclear receptor)	Protection of beta-cells against apoptosis	Baquie' et al., 2011	*Ci-Lrh-1, Ci-NR5A1/2/5, Ci-FTZF*	KH.C6.10	Not expressed (Imai et al., 2004)	Not analyzed
HNF-1A/HNF-1alpha (homeodomain)	Control of insulin secretion	Pontoglio et al., 1998	*Ci-HNF-1A*	KH.C12.461	Ubiquitous, likely maternal (Imai et al., 2004)	Endostyle http://bioinfo.s.chibau.jp/ciaid/
HNF-4A/HNF-4alpha/NR2A1 (nuclear receptor)	Control of the expression of beta-cell genes involved in insulin secretory response to glucose andnutrients	Wang et al., 2000	*Ci-HNF-4A*	KH.C3.84	Ubiquitous, likely maternal; trunk endoderm (transient); a pair of tail epidermal neurons (Imai et al., 2004)	Not analyzed
Sox4 (SRY-box, HMG)	Insulin secretion, endocrine pancreas development, alpha-cells differentiation	Goldsworthy et al., 2008; Mavropoulos et al., 2005	*Ci-SoxC, Ci-Sox11/12/4*	KH.C7.523	Neural precursors, trunk endoderm, CNS, adhesive organ, tail epidermis, epidermal neurons (Imai et al., 2004)	Stomach, part of the intestine, endostyle, branchial pharynx, neural complex http://bioinfo.s.chibau.jp/ciaid/
Glis3 (Zinc-finger, Krüppel-like)	Direct and indirect activator of insulin expression	Yang et al., 2009	*Ci-Glis1/3, Ci-ZF137*	KH.C5.53	Not expressed until the late tailbud stage; in late tailbuds, expressed in a small area of the anterior sensory vesicle (Miwata et al., 2006)	Not analyzed
Insm1 (Zinc-finger)	Transcriptional repression of BETA2/NeuroD1; development of beta-cells and intestinal endocrine cells	Liu et al., 2006 Gierl et al., 2006	*Ci-Insm1b, Ci-ZF041*	KH.C4.378	Narrow area in the nerve cord encompassing two symmetric small groups of cells (Miwata et al., 2006)	Not analyzed
Grg3/Tle3 (co-factor)	Stimulation of beta-cell differentiation	Metzger et al., 2012	*Ci-Groucho, Ci-Tle1/2/3/4*	KH.L96.11	Whole embryo, later mesenchyme (Imai et al., 2004)	Endostyle, branchial pharynx, body wall muscle, possibly part of the intestine (Ogasawara et al., 2002)
FoxO1 (Forkhead box)	Regulation of beta-cell proliferation, positive regulator of Beta2/NeuroD1 and MafA	Buteau and Accili, 2007	*Ci-FoxO, Ci-FoxO1/3/4/6*	KH.L20.26	Epidermis of late tailbuds (Imai et al., 2004)	Neural complex, possibly some faint signal in stomach and intestine http://bioinfo.s.chibau.jp/ciaid/
CREB (Leucine zipper)	Regulation of insulin, cyclinD1, cyclinA2 and other targets in response to different stimuli	Dalle et al., 2011	*Ci-CREB1, Ci-ATF1, Ci-CREB/ATFc*	KH.C1.26	Transient notochord, endoderm, epidermis, nervous system, mesenchyme (Imai et al., 2004)	Endostyle, possibly part of the stomach http://bioinfo.s.chibau.jp/ciaid/

Abbreviations: SRY, sex determining region Y-box; HMG, high mobility group; bHLH, basic helix-loop-helix. Prox1 appears twice because *Ciona* possesses two orthologs of this gene.