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Distinct enhancers of *ptf1a* mediate specification and expansion of ventral pancreas in zebrafish

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ABSTRACT

Development of the pancreas and cerebellum require *Pancreas-specific transcription factor-1a* (*Ptf1a*), which encodes a subunit of the transcription factor complex PTF1. *Ptf1a* is required in succession for specification of the pancreas, proper allocation of pancreatic progenitors to endocrine and exocrine fates, and the production of digestive enzymes from the exocrine acini. In several neuronal structures, including the cerebellum, hindbrain, retina and spinal cord, *Ptf1a* is transiently expressed and promotes inhibitory neuron fates at the expense of excitatory fates. Transcription of *Ptf1a* in mouse is maintained in part by PTF1 acting on an upstream autoregulatory enhancer. However, the transcription factors and enhancers that initially activate *Ptf1a* expression in the pancreas and in certain structures of the nervous system have not yet been identified. Here we describe a zebrafish autoregulatory element, conserved among teleosts, with activity similar to that described in mouse. In addition, we performed a comprehensive survey of all non-coding sequences in a 67 kb interval encompassing zebrafish *ptf1a*, and identified several neuronal enhancers, and an enhancer active in the ventral pancreas prior to activation of the autoregulatory enhancer. To test the requirement for autoregulatory control during pancreatic development, we restored *ptf1a* function through BAC transgenesis in *ptf1a* morphants, either with an intact BAC or one lacking the autoregulatory enhancer. We find that *ptf1a* autoregulation is required for development of the exocrine pancreas and full rescue of the *ptf1a* morphant phenotype. Similarly, we demonstrate that a *ptf1a* locus lacking the early enhancer region is also capable of rescue, but only supports formation of a hypoplastic exocrine pancreas. Through our dissection of the complex regulatory control of *ptf1a*, we identified separate cis-regulatory elements that underlie different aspects of its expression and function, and further demonstrated the requirement of maintained *ptf1a* expression for normal pancreatic morphogenesis. We also identified a novel enhancer that mediates initiation of *ptf1a* expression in the pancreas, through which the signals that specify the ventral pancreas are expected to exert their action.

Introduction

The vertebrate pancreas forms from evaginations of the endoderm, the dorsal and ventral pancreatic buds, that later fuse to produce a single organ composed of hormone-secreting endocrine cells, digestive enzyme-secreting exocrine cells, and duct cells. In the zebrafish, the dorsal bud forms by 24 h post fertilization (hpf) (Biemar et al., 2001) and exclusively produces endocrine cells of the principal islet. The ventral bud arises at 34 hpf and grows toward the dorsal bud-derived islet, with which it completely merges by 52 hpf (Field et al., 2003). The pancreas specific transcription factor 1a (*Ptf1a*) has a critical role in pancreatic development. Loss of *Ptf1a* in mice results in loss of the ventral pancreatic bud and early arrest in development of the dorsal bud (Krapp et al., 1998; Kawaguchi et al., 2002). In zebrafish, depletion of *ptf1a* through antisense morpholinos results in the absence of a ventral bud, but the dorsal bud is unaffected (Lin et al., 2004; Zecchin et al., 2004). Despite the critical requirement for *Ptf1a* in early pancreatic development, the signals that initiate its expression in the ventral pancreas are poorly characterized. *vHNF1* is an attractive candidate for the direct activation of *Ptf1a*, since loss of *vHNF1* in mouse results in loss of endodermal *Ptf1a* expression (Haumaitre et al., 2005) and similarly in zebrafish results in the

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loss of pancreatic marker expression (Sun and Hopkins, 2001). Identification of the *Ptf1a* regulatory elements that mediate the activation of its pancreatic expression is therefore an important step to determine the involvement of specific transcription factors in ventral pancreas specification.

In addition to its function in pancreas specification, *Ptf1a* has a dosage sensitive role in the allocation of pancreatic progenitors to the exocrine and endocrine compartments in both mouse (Fukuda et al., 2008; Schaffer et al., 2010) and zebrafish (Dong et al., 2008). A cross-repressive loop between the pro-exocrine *Ptf1a* and the pro-endocrine *Nkx6* operates to segregate endocrine and exocrine cells in the pancreas (Schaffer et al., 2010). Both *Ptf1a* and *Nkx6.1* are known to activate their own expression (Masui et al., 2008; Iype et al., 2004) and the autoregulatory function of both genes in the context of competing cell fate determinants has been proposed to either confer stability to the bipotential progenitor state (Huang et al., 2007) or to maintain stability of the commitment to a given lineage after the resolution of the bipotential fate choice.

Ptf1a is also important in the development of several populations of inhibitory neurons. Specifically, in the cerebellar primordium *Ptf1a* is expressed in the ventricular zone, a germinative zone that generates progenitors of the inhibitory Purkinje cells and interneurons (Pascual et al., 2007). Loss of *Ptf1a* leads to a loss of Purkinje cells and interneurons during embryonic development, which secondarily results in postnatal cerebellar agenesis in mice (Hoshino et al., 2005) and humans (Sellick et al., 2004). Within more caudal rhombomeres of the mouse hindbrain, *Ptf1a* expressing domains contribute inhibitory neurons to the cochlear nucleus (Yamada et al., 2007) and climbing fiber neurons of the precerebellar system (Fujiyama et al., 2009). Similarly, in zebrafish the *ptf1a* expressing domain contributes to the precerebellar system (Bae et al., 2009). Within the dorsal spinal cord, *Ptf1a* is required for the generation of two populations of inhibitory interneurons (Glasgow et al., 2005). Also, in the retina *Ptf1a* is required in both mouse (Fujitani et al., 2006) and zebrafish (Jusuf et al., 2011) for the generation of inhibitory interneurons that modulate the signals generated by the photoreceptor cells.

Despite the critical requirement for *Ptf1a* in the development of pancreas and cerebellum, the regulatory elements that initiate expression in these organs have remained elusive. A region of ~30 kb encompassing mouse *Ptf1a* was examined for cis-regulatory activity (Masui et al., 2008). This study identified sequences downstream of the gene that direct transcription to the hindbrain, spinal cord and retina, but are not active in cerebellum. The distal portion of the upstream sequence (−15.6 to −13.4 kb) harbors two conserved predicted PTF1 binding sites and directs transcription to the embryonic pancreas. The transcriptional activity in the pancreas is absent in *Ptf1a* mutants, and PTF1 complex binds directly to the sequence *in vitro*, consistent with autoregulatory function. The upstream enhancer is also active in the dorsal spinal cord, retina, hindbrain, and cerebellum (Meredith et al., 2009), where its activity is similarly autoregulatory. In the pancreas, the autoregulatory control has been hypothesized to effect an irreversible commitment to the acinar cell program, although its role has not been directly tested.

We have carried out a comprehensive survey of the genomic interval spanning zebrafish *ptf1a* for cis-regulatory activity, focusing specifically on elements regulating transcription in the developing pancreas. We identified an autoregulatory enhancer analogous to that in the mouse, and showed that the predicted PTF1 binding sites are both necessary and sufficient for its activity. We also identified a sequence capable of initiating early expression in the pancreas, in a region with high homology between teleosts and mammals. We describe additional sequences active in regions of the nervous system that normally express *ptf1a*, including the cerebellum. Finally, we tested specifically for the requirement for *ptf1a* autoregulation in pancreas development, and find that it is necessary for early expansion of the exocrine pancreas.

Table 1
Primers used to subclone candidate enhancer sequences and for BAC recombineering.

+1ptf1a F	GCAATGTTGCTATTAGTATCCCGTA
+1ptf1a R	TGCTTAAGCTCAACCAGCCTA
*att*B1-*17ptf1a* F	GGGGACAAGTTTGTACAAAAAAGCAGGCCACGTGATAACAGAAACAAGGCATTAACAG
*att*B2-*17ptf1a* R	GGGGACCACTTTGTACAAGAAAGCTGGGTATTCACAGCGATGAACAAATCAAGCACTC
*att*B1+*36ptf1a* F	GGGGACCACTTTGTACAAGAAAGCTGGGTTGCGGACTTTTAAGGGGAAAAACGTA
*att*B2+*36ptf1a* R	GGGACAAGTTTGTACAAAAAAGCAGGCTTTGGCAATTGGTGTCACGTCCTTTAAT
*att*B1-*3ptf1a* F	GGGGACAAGTTTGTACAAAAAAGCAGGCCAACAACCACTCACCAGACCCCTTTT
*att*B2-*3ptf1a* R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTGCTGTGTTGGTGTTGCCCAGTAAC
Autoreg2kb F	ACTAATCTGAAACAGCCTGCG
Autoreg1.3 kb F	TTTTAACGCGTCGGACATTG
Autoreg R	TCAATTGCCATTTTAAACGCT
Synth-S	ACCGGTCGCATGTGGCTGGATTCCCAGCACCGGTGTCCATGTGTTGCTATTCCCATCGCCGGCGCAAGTGGTGTGATTCCCACGCCGGC
Synth-AS	GCCGGCGTGGGAATCACACCACTTGCGCCGGCGATGGGAATAGCAACACATGGACACCGGTGCTGGGAATCCAGCCACATGCGACCGGT
35hom-FRT3-tet R	AGCGTGTGAACTCCCACAACGCATCATGAGGGTTTGAAGTTCCTATTCTTCAAATAGTATAGGAACTTCCTCTTGGGTTATCAAGAGGG
35hom-tet F	ACAGGTTTACAACCAATGTGCACTCGACTGTTAGATCCTAATTTTTGTTGACACTCTA
35hom-FRT3-Kan F	CTAATAAGTTTGGTCTAATAATTTTTACTTTTTCCGAAGTTCCTATTCTTCAAATAGTATAGGAACTTCTATGGACAGCAAGCGAACCG
35hom-Kan R	TTGTTCTGTTAATGCCTTGTTTCTGTTATCACGTGTCAGAAGAACTCGTCAAGAAG
5hom-F3-Kan F	AGCGCTCGGTGCGCTGTTGCTGCGCCTGTTGCTCCGAAGTTCCTATTCTTCAAATAGTATAGGAACTTCTATGGACAGCAAGCGAACCG
5hom-Kan R	GTTAGGAGCGCAAGCTGCAGAACACTGGGGATCCTTCAGAAGAACTCGTCAAGAAG
3hom-Spec F	CATTCAGTGTCGGCAGTGGGTCTCTTTGAGAGCAGCCAGCCAGGACAGAAATGCCT
3hom-F3-Spec R	TGCACCCCGCTGCAAGCTGCAACGCGAGGGACAATGAAGTTCCTATACTATTTGAAGAATAGGAACTTCTTATTTGCCGACTACCTTGGTGA
1hom-F3-Kan F	ATGATGATCCAATAAAATTTTTTTATTTCACTCACGAAGTTCCTATTCTTCAAATAGTATAGGAACTTCTATGGACAGCAAGCGAACCG
1hom-Kan R	TTTTTCATTAGTACGCTAAAAAATTCAAACAAACGTCAGAAGAACTCGTCAAGAAG
6hom-Spec F	GCCTAATTTTCCAGTAGGCTGGTTGAGCTTAAGCACCAGCCAGGACAGAAATGCCT
6hom-F3-Spec R	AATATGTAGCACTCTGTCGAGTCTTGTTGCAAGTTGAAGTTCCTATACTATTTGAAGAATAGGAACTTCTTATTTGCCGACTACCTTGGTGA
*Eco*RI-KzZPtf1a F	AAAAAGAATTCGCAGCCGCCATGGATACGGTCCTAGATCCATTCACAGGCTTAGACTCTT
*Xho*I-zPtf1a R	AAAAAACTCGAGTTAGGAAATGAAATTAAAGGG
*Pac*I-FRT-Kan F	CCCCCCTTAATTAAGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCTATGGACAGCAAGCGAACCG
*Pac*I-FRT-Kan R	CCCCCCTTAATTAAGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCTCAGAAGAACTCGTCAAGAAG
300prom F	AATATAGGCAGTCGCTTTGTCATCA
3′UTR R	AAAAAATTTTATTGGATCATCATATCAGT

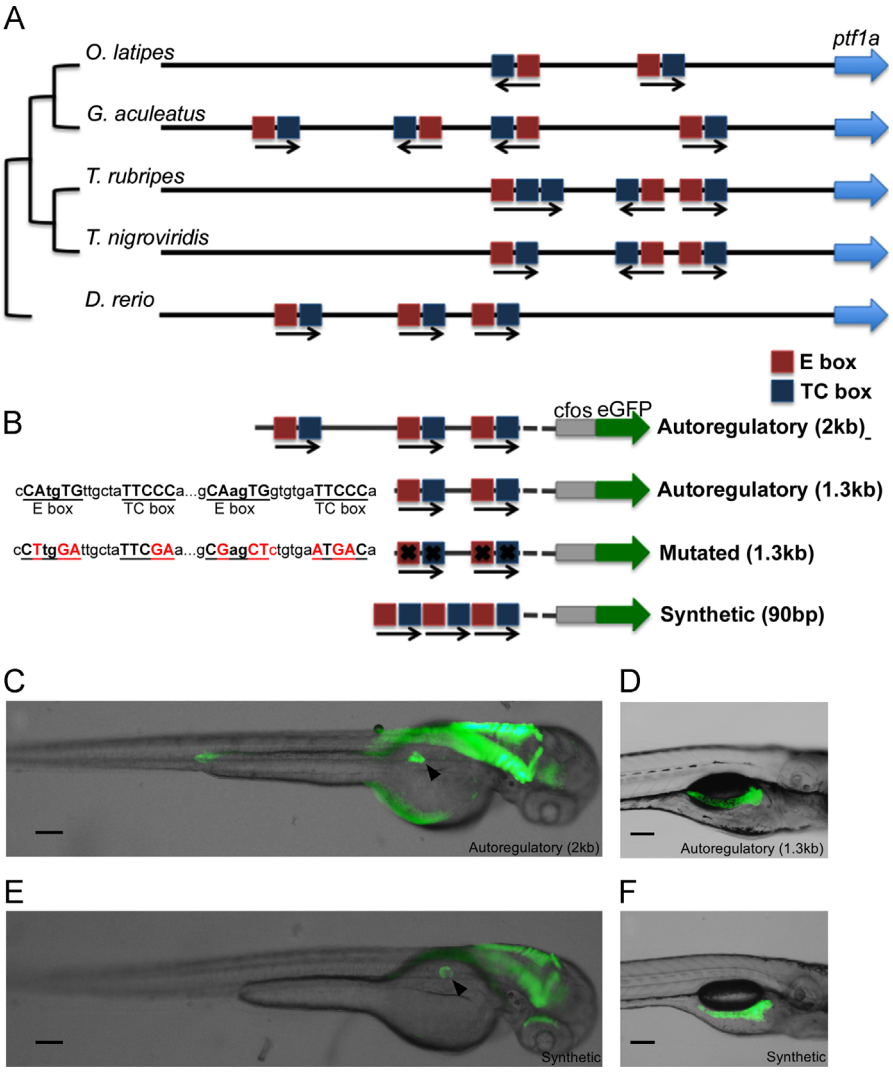

Fig. 1. Identification of teleost *ptf1a* autoregulatory enhancers. (A) The diagram represents the predicted PTF1 binding sites upstream of the *ptf1a* coding sequence from the indicated teleost species; the red squares indicate E boxes (CANNTG) and the blue squares TC-boxes (TTCCC). (B) The transgenes generated for analysis of the autoregulatory enhancer are diagramed, including the exact sequences of the E and TC boxes of the two proximal sites, and the mutations introduced into them. Transgenes containing all three (C) or only the two proximal (D) PTF1 binding sites, in conjunction with the heterologous *cFos* minimal promoter, have similar activity to the entire *ptf1a* locus at 2 dpf (C), where they are active in the nervous system and pancreas (arrowhead), and at 5 dpf (D), where they show robust activity in the exocrine pancreas. The indicated mutations of the E-box and TC-box sequences abolish this activity (Supplementary Fig. 1). (E, F) A 90 bp synthetic enhancer containing the 3 predicted binding sites recapitulates this expression pattern at 2 dpf (E) and 5 dpf (F), but in addition is active in the retina, a site of endogenous *ptf1a* expression. medaka: *O. latipes*, stickleback: *G. aculeatus*, fugu: *T. rubribes*, tetraodon: *T. nigroviridis*, zebrafish: *D. rerio*. Scale bars: 200 μm.

Materials and methods

Maintenance of zebrafish stocks

All fish were maintained under standard conditions, in accordance with regulations of the Institutional Animal Care and Use Committee of the University of Pennsylvania. Transgenic lines *ptf1a:egfp*^{jh} (Godinho et al., 2005) and *ins:mCherry*^{jh2} (Pisharath et al., 2007) have been previously described.

Transgenic analysis of non-coding sequences

The *+6ptf1a*, *+11ptf1a* and *+24ptf1a* fragments were produced by *Nco*I digest of the BAC CH73-141D4 and cloned into a Tol2 vector with a minimal mouse *cFos* promoter and an *egfp* reporter followed by the SV40 polyadenylation signal. The remaining non-coding sequences were amplified with the primers listed in Table 1.

Amplicons produced with primers containing *att* sites were cloned in the pDONR221 vector (Invitrogen) through Gateway BP recombination (Invitrogen). Amplicons without *att* sites were cloned in the Gateway entry vector pCR8-TOPO (Invitrogen). The synthetic autoregulatory enhancer was generated by annealing the complementary oligos Synth-S and Synth-AS (Table 1). The annealed double stranded product was then cloned in the pCR8-TOPO vector. All non-coding sequences were cloned into the pGW-cfosEGFP (Fisher et al., 2006a, 2006b) vector through Gateway LR recombination (Invitrogen).

BAC recombineering

BAC recombineering was performed basically as described (Sharan et al., 2009); primer sequences are listed in Table 1. To delete the region containing *dlgap1a*, FRT3 sites flanking the gene were introduced by recombination. To insert the site upstream of *dlgap1a*, the primers 35hom-FRT3-tetR and 35hom-tetF were used to generate a targeting cassette. Then the CH211-142H2 BAC,

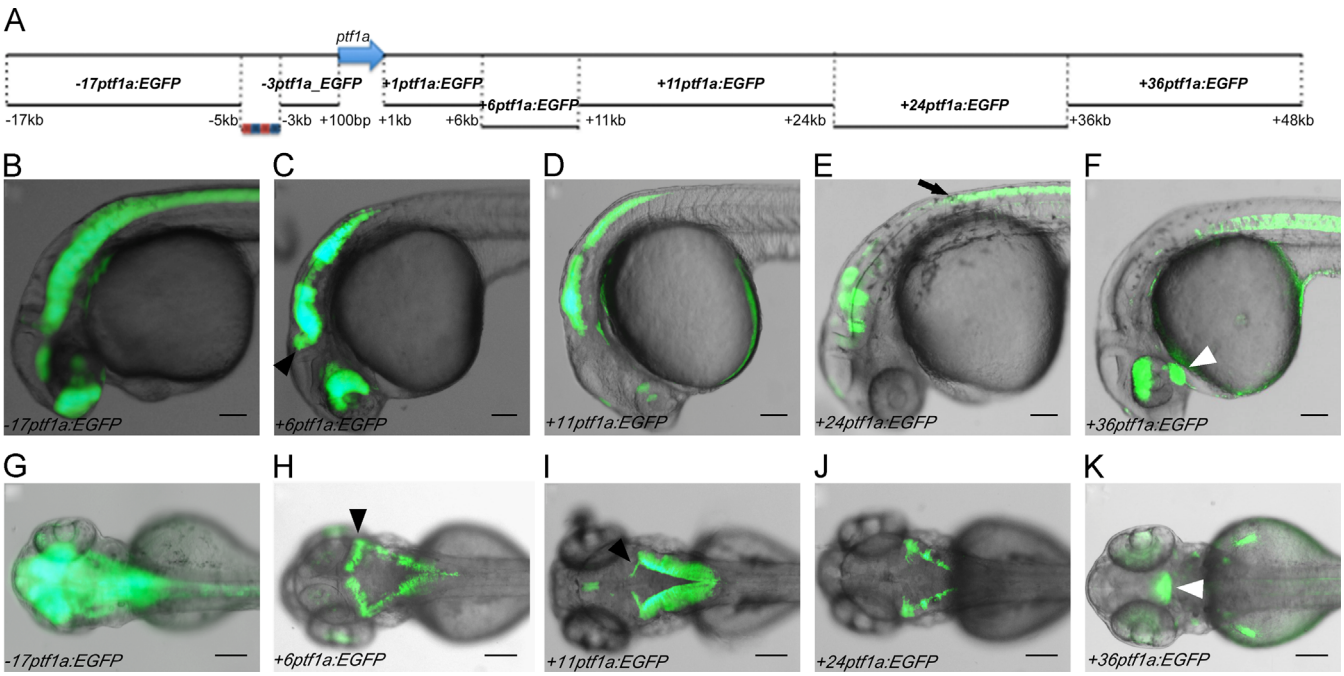

Fig. 2. Survey of *ptf1a* locus for regulatory activity. (A) The diagram indicates the non-coding sequences, ranging in size from 3 to13 kb and collectively spanning the region between −17 kb and +48 kb of the transcriptional start site of *ptf1a*, which were assayed for enhancer activity. The isolated sequences showed transcriptional activity at 1 dpf (B–F) and 3 dpf (G–K) in regions of the nervous system and retina overlapping endogenous *ptf1a* expression. The *−17ptf1a* element located upstream of the autoregulatory enhancer drives non-specific neuronal expression at 1 dpf (B) and 3 dpf (G). The *+6ptf1a* enhancer is active in the retina, cerebellum (black arrowhead), hindbrain and spinal cord at 1 dpf (C) and 3 dpf (H). The *+11ptf1a* enhancer is also active in the hindbrain, including the cerebellum (black arrowhead), the spinal cord and more weakly in the retina (D: 1 dpf, I: 3 dpf). The *+24ptf1a* element activates expression in certain rhombomeres of the hindbrain and in the spinal cord (black arrow) (E: 1 dpf, J: 3 dpf). The *+36ptf1a* element is active in the retina, hypothalamus (white arrowhead), fin buds and notochord (F: 1 dpf, K: 3 dpf). Scale bars: 200 μm.

modified by replacement of the *ptf1a* coding sequence with *mCherry*, was grown in the EL250 bacterial strain to $OD_{600}=0.6$ at 32 °C, followed by manual mixing in a 42 °C waterbath for 15 min to induce recombination proteins. Electrocompetent cells were prepared and transformed with the first targeting cassette following standard protocols with an Eppendorf Electroporator 2510. After a 1 h recovery, recombinant clones were selected with the tetracycline. The targeting cassette to introduce a second FRT3 site close to the 3′ end of *dlgap1a* was generated using the primers 35hom-FRT3-KanF and 35hom-KanR, and transformed as above; recombinants were selected with kanamycin. After the sequential recombination steps that introduced FRT3 sites flanking the *dlgap1a* region, recombination of the two sites and excision of the 93 kb sequence was achieved by FLPe induction with arabinose treatment (0.1% for 1 h at mid-log phase). Recombinant clones at each step were verified through PCR or sequencing.

Additional BAC modifications were made similarly, through sequential transformations and selections, followed by FLPe-mediated recombination. To generate a *ptf1a* coding sequence resistant to translation blocking MO, *ptf1a* cDNA was first amplified from the IMAGE clone using primers *Eco*RI-KzZPtf1a-F and *Xho*I-zPtf1a-R, and then flanked by 300 bp homology arms. An FRT-flanked selection cassette was amplified using primers *Pac*IFRTKanF and *Pac*IFRTKanR and added downstream of the *ptf1a* cDNA. The entire coding sequence targeting cassette was then amplified using the primers 300promF and 3′UTR-R, before transformation and antibiotic selection. To make the Δ-5-3 BAC, the primer pairs 5hom-F3-KanF/5hom-KanR and 3hom-SpecF/3hom-F3-SpecR were used to generate targeting cassettes to flank the autoregulatory enhancer with FRT sites. Similarly, to make the Δ+1+6 BAC, the primer pairs 1homF3-KanF/1hom-KanR and 6hom-SpecF/6hom-F3-SpecR were used to amplify the fragments used to flank the early enhancer with FRT sites. For efficient transgenesis, a cassette containing the inverted Tol2 arms was targeted to the chloramphenicol resistance gene of the BAC backbone, as previously described (Suster et al., 2011)

Zebrafish embryo injections

Transgenesis with Tol2-based plasmid constructs was performed as previously described (Fisher et al., 2006a, 2006b). Unless otherwise noted, consistent reporter gene expression was observed in at least three independent lines for each construct. For BAC transgenesis each embryo was injected with 100 pg BAC DNA and 40 pg Tol2 transposase RNA. To block *ptf1a* translation, embryos were injected with 0.5pmoles of *ptf1a*-MO:CCAACACAGTGTCCATTTTTTGTGC (Gene Tools) as previously described (Lin et al., 2004).

Photomicroscopy

Embryos were visualized by epifluorescence on a Zeiss V12 Stereomicroscope, and imaged with AxioVision 4.5 software. For confocal imaging, embryos were anesthetized in Tricaine and mounted in MatTek glass bottom culture dishes in 1% low melt SeaPlaque agarose. Images were acquired on an Olympus IX 81 microscope equipped with a Yokogawa CSU 10 scan head with a Hamamatsu EMCCD camera (model C9100-13, Bridgewater, NJ). Diode lasers for excitation (488 nm for eGFP and 561 nm for mCherry) were housed in a Spectral Applied Research launch (Richmond Hill, Ontario). Hardware was controlled by Slidebook version 5.0 (Intelligent Imaging Innovations), and confocal image stacks were processed with ImageJ (⟨http://rsbweb.nih.gov/ij⟩).

Results

Given the hypothesized importance of *Ptf1a* autoregulation in mouse pancreas (Masui et al., 2008) we sought to determine if

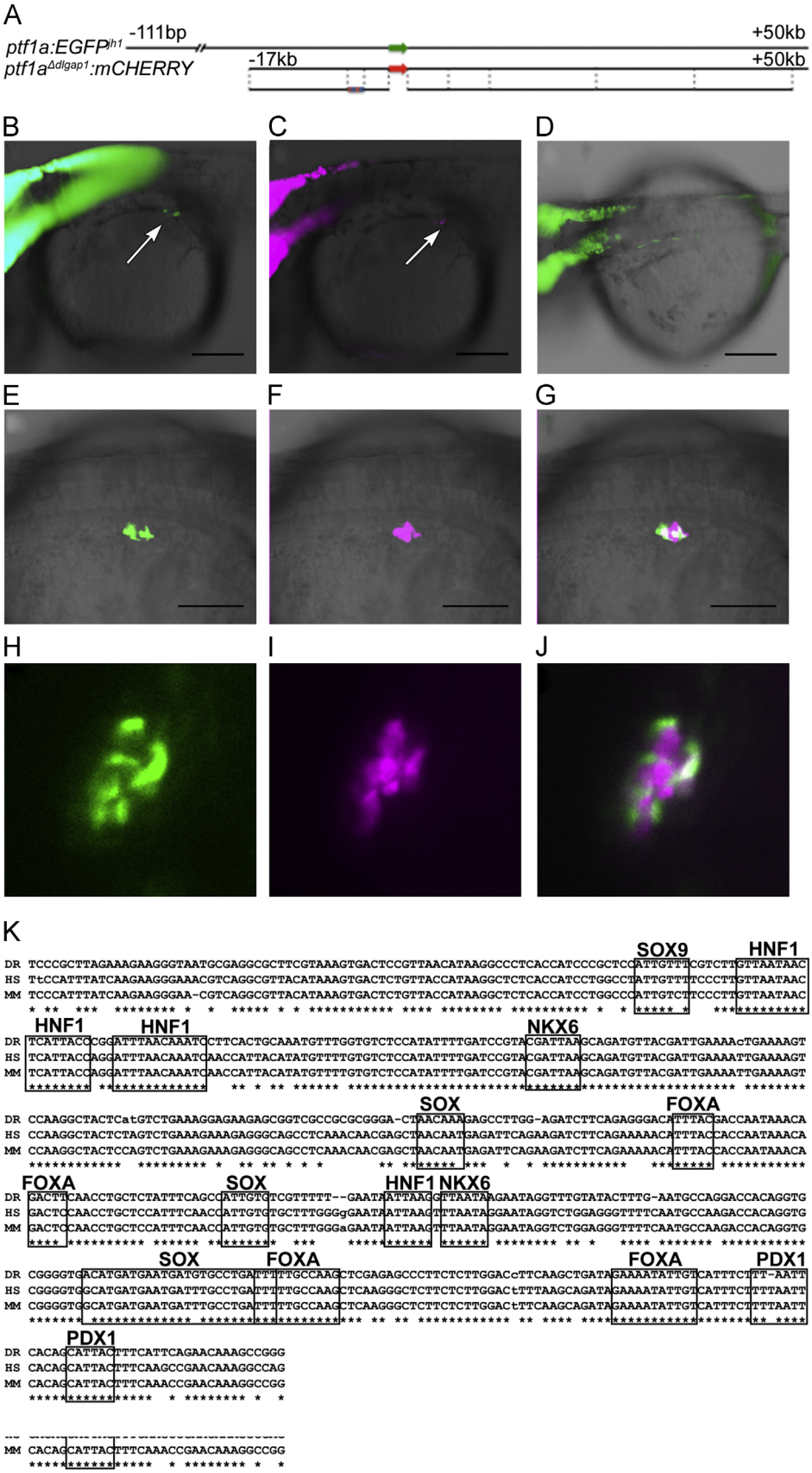

Fig. 3. The early pancreatic *ptf1a* enhancer. (A) The diagram compares the previously published −111 kb to +50 kb *ptf1a:egfp* BAC transgene, to the truncated -17 kb to +50 kb *ptf1a*$^{\Delta dlgap1}$*:mcherry* BAC, with only 17 kb of upstream sequence. (B) Expression of *egfp* from the longer BAC at 34 hpf completely overlaps *mCherry* expression from the shorter BAC (C), including early expression in the pancreas (arrows in B, C); this early pancreatic expression is not seen with the autoregulatory enhancer (D). (E) The *+1ptf1a* enhancer is active at 38 hpf in a cluster of cells surrounding the principal islet, marked by expression of the *ins:mCherry* transgene (F). (H–J) Confocal microscopy of embryos doubly transgenics for *+1ptf1a:EGFP* (H) and *ins:mCherry* (I) at 48 hpf show that the two cell populations are adjacent but do not overlap, as seen in a single 5 μM slice (J). (G) A 500 bp core of the *+1ptf1a* enhancer is highly conserved from mammals to teleosts, and contains predicted binding sites for a number of biologically relevant transcription factors, including Pdx1, Sox9, and Hnf1. DR: zebrafish, HS: human, MM: mouse. Scale bars: 200 μm.

Ptf1a autoregulation is a shared feature in teleosts. Precalculated multiple sequence alignments generated by the PHAST package did not identify sequences homologous to the characterized mouse autoregulatory enhancer in any of the teleost species with available genome sequences (zebrafish, medaka, stickleback, fugu and tetraodon). We hypothesized that *Ptf1a* autoregulation is conserved in teleosts, but that sequence homology is confined to the enhancer's transcription factor binding sites. Therefore, we searched for the motifs CANNTGN$_6$TTCCC (one helical turn spacer) and CANNTGN$_{16}$TTCCC (two helical turns spacer), which are based on the PTF1 binding sites in the *Rbpjl* promoter (Masui et al., 2007) and the *Ptf1a* autoregulatory enhancer (Masui et al., 2008). We identified 3 matches to these motifs in the region between 5 kb and 3 kb upstream of the zebrafish *ptf1a* transcriptional start site (Fig. 1). There were no other matches to the PTF1 consensus binding sequence in the 50 kb on either side of the zebrafish *ptf1a* gene, suggesting that this is the only element mediating autoregulation of *ptf1a* expression in zebrafish. Further, we identified 2–4 matches to the consensus PTF1 binding sequence within ~5 kb upstream of the other teleost *ptf1a* genes (Fig. 1A), strongly supporting a function for these sites in *ptf1a* autoregulation.

We tested the ability of sequences containing the predicted zebrafish PTF1 binding sites to regulate expression of a fluorescent reporter gene, in conjunction with a heterologous minimal promoter, in transgenic fish (Fig. 1C and D). A sequence containing all three predicted sites had activity in the pancreas and the hindbrain, consistent with function as an autoregulatory enhancer. We further determined that a shorter fragment containing the two most proximal predicted PTF1 binding sites was able to similarly recapitulate endogenous *ptf1a* expression. Site-directed mutagenesis of these two sites abolished enhancer activity (Supplementary Fig. 1). We also generated a 90 bp synthetic enhancer containing the three PTF1 sites. The synthetic construct regulated an expression pattern similar to the intact enhancer, indicating that the three PTF1 binding sites are sufficient for *in vivo* activity of the enhancer (Fig. 1E and F). The synthetic enhancer exhibited activity in the retina, which although it is an endogenous site of expression, is not observed with the full autoregulatory enhancer. This observation suggests that additional sequences in the autoregulatory enhancer function to suppress its activity in the retina.

We next sought to identify all additional cis-regulatory elements associated with *ptf1a*, in particular those that initiate expression in the pancreas and cerebellum prior to activation of the autoregulatory enhancer. We examined the non-coding sequence between the upstream *dlgap1a* gene (17 kb upstream) up to 48 kb downstream of *ptf1a* for regulatory activity. We divided the sequence of this interval into 7 consecutive fragments (Fig. 2A). These were tested as described above for their ability to direct reporter gene expression to sites of endogenous *ptf1a* expression in transgenic zebrafish. We identified several enhancers that regulated expression of the transgene in the nervous system, including the hindbrain, retina and spinal cord (Fig. 2). Three enhancers, *+6ptf1a*, *+11ptf1a* and *+24ptf1a*, organized consecutively downstream of *ptf1a*, regulated expression in different regions of the hindbrain (Fig. 2 C–E and H–J). Two of these elements, *+6ptf1a* and *+11ptf1a*, also exhibited robust activity in the anterior hindbrain, including the area corresponding to the cerebellar primordium (Fig. 2H and I). Three regulatory elements were active in the spinal cord, specifically *+6ptf1a*, *+11ptf1a* and *+24ptf1a* (Fig. 2E and data not shown). Finally, three regulatory elements (*+6ptf1a*, *+11ptf1*a, and *+36ptf1a*) were active in the retina (Fig. 2C, D, and F). We further compared the expression patterns regulated by several neuronal enhancers to that of the intact locus in double transgenic animals (Supplementary Fig. 2). We find that the hindbrain expression of *+11ptf1a:EGFP* and the spinal cord expression of *+24ptf1a:EGFP* extend to more dorsal domains than the expression from the intact locus. These results suggest that genomic context and interactions with surrounding regulatory elements are necessary to faithfully produce the correct expression pattern.

The farthest upstream sequence tested, *−17ptf1a*, directed expression ubiquitously in neural tissue (Fig. 2B and G), an activity that might be related to the upstream *dlgap1a* gene, which encodes for a synaptic protein (Kim et al., 1997). Additionally, the most downstream sequence tested, *+36ptf1a*, was active in the notochord (Fig. 2F), expression likely related to the downstream gene *si:ch211-207d6.2*, whose murine homolog *Sickle tail* is expressed in the notochord and required for proper vertebrae development (Semba et al., 2006).

To test whether the interval described above contains all necessary regulatory sequences, we utilized a bacterial artificial chromosome (BAC) transgene that faithfully recapitulates endogenous *ptf1a* expression (Park et al., 2008). The neighboring gene upstream of *ptf1a* differs between teleosts and mammals. We expect that regulatory elements conserved among vertebrates will remain syntenic to the gene throughout evolution and therefore we predicted that conserved regulatory elements would lie within the region bounded by the nearest upstream gene, *dlgap1a*. We deleted 93 kb of upstream sequence, including the *dlgap1a* gene (Fig. 3A). We showed that the truncated BAC (with 17 kb of direct upstream sequence and 50kb of downstream sequence) has activity identical to the original BAC (Fig. 3B–D) and therefore contains all necessary regulatory elements for proper *ptf1a* expression.

Within the tested interval, we identified a single region, *+1ptf1a*, which regulated expression in the early pancreas. The cells in which *+1ptf1a* is active are in close proximity to but do not overlap with the dorsal bud derived β-cells, marked by the expression of the *insulin:mCherry* transgene (Fig. 3E–J). This localization is consistent with the position of the ventral bud derived pancreatic progenitors that surround the earlier appearing cluster of endocrine cells of the principal islet. Upon expansion of the pancreas and cytodifferentiation of exocrine cells, *+1ptf1a:egfp* activity persisted in a limited region surrounding the principal islet at least until 5 dpf (Supplementary Fig. 3). The persistent fluorescence may represent GFP perdurance in slowly dividing cells, or perhaps correspond to ongoing newly generated exocrine precursors in which the early enhancer continues to be active. The *+1ptf1a* element spans 5 kb immediately downstream of the *ptf1a* gene and has a high degree of sequence conservation to other species, including human and mouse, suggesting a conserved functional role. Specifically, this interval contained a 527 bp sequence that is 79% identical between zebrafish and human, and 96% identical between mouse and human (Fig. 3K). We tested the highly conserved shorter sequence for its ability to direct expression to the pancreas, but found that, in isolation, it lacks enhancer activity.

To further characterize the spatial and temporal activities of the two pancreatic enhancers, we compared expression regulated by them to that of the intact *ptf1a* locus. Embryos transgenic for the early enhancer, the autoregulatory enhancer or the entire BAC were assayed by *in situ* hybridization for pancreatic *egfp* expression at 4 h intervals between 30 hpf and 46 hpf (Fig. 4). Consistent with previous reports, expression from the intact BAC was first seen at 34 hpf (24/24 embryos, Fig. 4A). While expression in the *+1ptf1a:egfp* transgenics was variable, a fraction of embryos had pancreatic expression at 34hpf (Fig. 4B) and expression was observed in the majority of embryos (28/36) by 38 hpf (Fig. 4D). At all time points, the expression regulated by the *+1ptf1a* enhancer was in a subset of cells in the domain in which the intact locus showed expression. In contrast, the autoregulatory enhancer did not regulate detectable pancreatic expression until

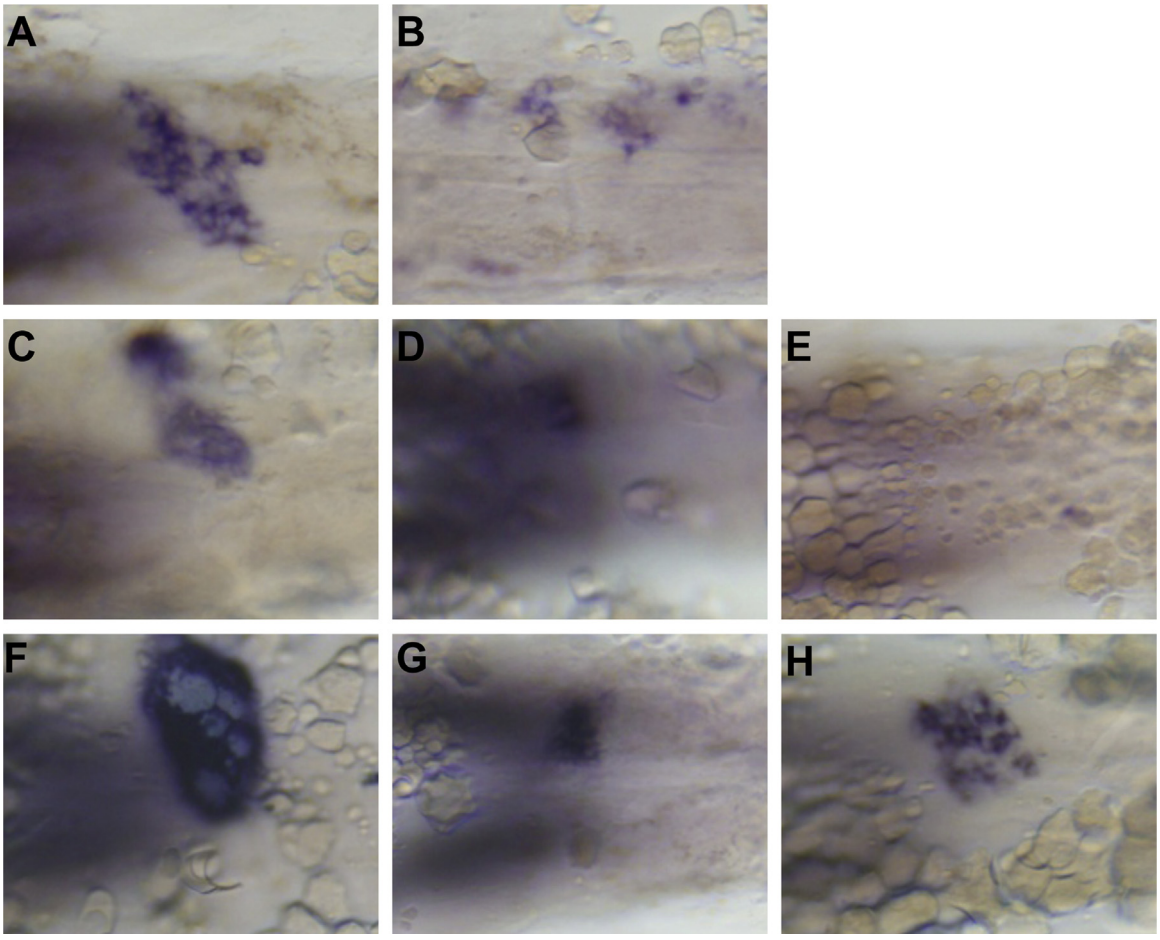

Fig. 4. Activity of pancreatic *ptf1a* enhancers relative to the intact locus. Activity of the early *+1ptf1a* (B, D, G) and the autoregulatory (E, H) enhancers was assayed in transgenic embryos by *in situ* hybridization for *egfp*. For comparison, *egfp* expression is also shown for the intact locus in *ptf1a:egfp*^{jh1} transgenic embryos (A, C, F). At 30 hpf, none of the embryos had detectable expression in pancreatic precursors (data not shown). At 34 hpf (A, B) and 38 hpf (C–E), all *ptf1a:EGFP*^{jh1} embryos (n=24 and 40) showed a group of positive cells stretched across the midline from left anterior to right posterior (A, C), a subset of embryos transgenic for the early enhancer showed smaller groups of positive cells, in the left anterior endoderm (B, D), while all embryos transgenic for the autoregulatory enhancer lacked expression, shown at 38 hpf (E). At 42 hpf, *ptf1a:egfp*^{jh1} embryos had larger, less scattered groups of positive cells (F), while both the early enhancer (G) and the autoregulatory enhancer (H) were active in a subset of cells in the same domain. For all photographs, yolk was dissected away and the endoderm is shown from the ventral side; embryos are oriented with anterior to the left and left to the top.

42 hpf (Fig. 4H), also in a smaller group of cells than those expressing the intact locus.

We identified two regulatory elements apparently controlling *ptf1a* expression in the pancreas: an early enhancer that initiates expression, and an autoregulatory enhancer that maintains or augments expression. Because the early enhancer has restricted spatial and temporal activity, and because we failed to identify additional enhancers that control *ptf1a* expression in the mature pancreas, we hypothesized that autoregulation has a major role in development of the pancreas following its initial specification. To test this hypothesis, we examined the effect of the lack of autoregulatory *ptf1a* control on pancreatic development. We altered a BAC containing *ptf1a* through the introduction of synonymous substitutions in the coding sequence, rendering the transcript resistant to a translation-blocking morpholino (MO) that efficiently depletes endogenous PTF1a and inhibits development of the ventral pancreatic bud (Lin et al., 2004). We further deleted the entire autoregulatory element from the BAC, generating the Δ-5-3 BAC transgene that expresses *ptf1a* under control of the full complement of characterized regulatory elements minus the autoregulatory enhancer. We subsequently established stable transgenic lines with the two BAC transgenes.

We tested the ability of the Δ-5-3 BAC to rescue pancreatic development, by MO injection into embryos transgenic for the *ptf1a:egfp* BAC (Fig. 5). In control embryos, MO injection almost completely abolished visible exocrine pancreas tissue at 3 or 5 dpf (4 partial pancreas/137 embryos; Fig. 5C). Presence of the BAC encoding MO-resistant *ptf1a* rescued pancreas development, wholly or partially, in a substantial fraction of morphants (22/88 embryos, or 50% of expected 44 BAC carriers; Fig. 5D, F). In contrast, the Δ-5-3 BAC failed to rescue pancreas development (1 partial pancreas/90 embryos, or 45 expected BAC carriers; Fig. 5E). The Δ-5-3 BAC rescues represent the combined results of rescues performed in 2 independent transgenic lines. These findings indicate that *ptf1a* autoregulation has a very critical role during early pancreatic development, and lack of the relevant transcriptional activity leads to an early failure of the pancreas to expand.

To address the role of the *+1ptf1a* enhancer in pancreas specification, we similarly engineered a BAC encoding for MO-resistant *ptf1a* transcript and lacking the 5 kb region corresponding to the *+1ptf1a* enhancer, the Δ+1+6 BAC. As above, we depleted endogenous *ptf1a* through MO injections in *ptf1a:egfp* transgenics. We then compared the abilities of the Δ+1+6 BAC and the intact BAC to rescue pancreas formation in 5 dpf mosaic transgenic larvae. In MO-injected embryos, none (0/63) had visible exocrine pancreas tissue at 5 dpf, In embryos injected with MO and the intact BAC, a fraction of larvae had pancreas tissue (16/196) at 5 dpf, consistent with the ability of the same BAC to

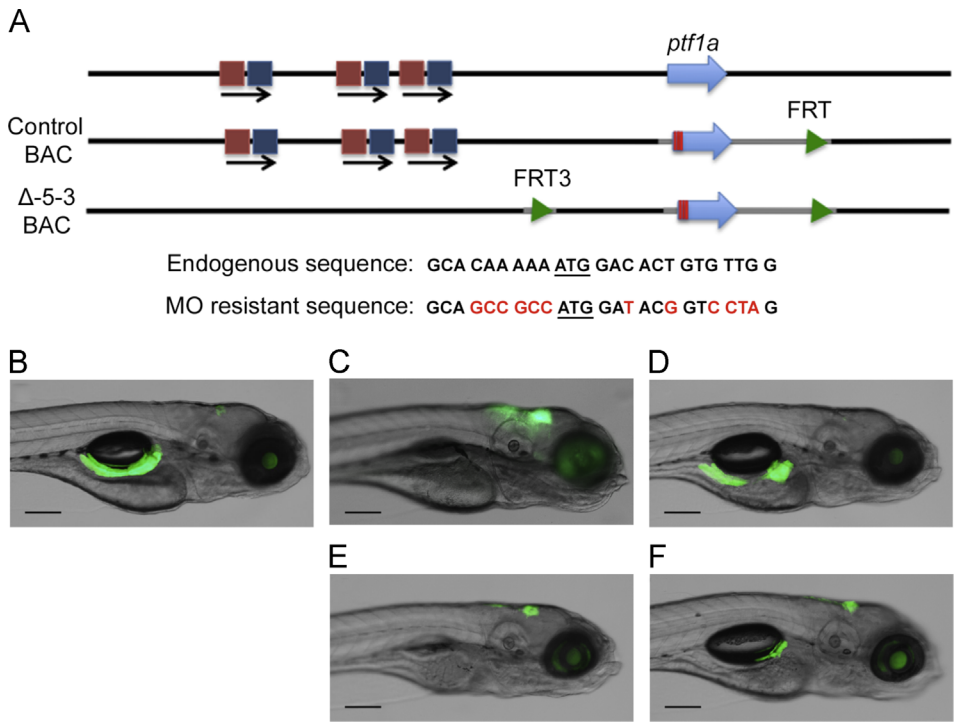

Fig. 5. *ptf1a* autoregulation is required for exocrine pancreas development. (A) The diagram indicates the endogenous *ptf1a* locus (top line). In the control BAC (middle line) the *ptf1a* coding sequence was modified by synonymous substitutions and the addition of a Kozak sequence (at bottom) resulting in a transcript resistant to the translation blocking MO. The Δ-5-3 BAC has an additional 2 kb deletion removing the autoregulatory enhancer. (B) At 5 dpf, the *ptf1a:egfp* BAC shows robust expression in exocrine pancreas, as well as some hindbrain expression. In fish without a rescuing BAC, MO injection abolished exocrine pancreas development at 5 dpf (C), while the presence of the control BAC expressing MO-resistant *ptf1a* allowed complete (D) or partial (F) pancreas development at the same stage. In contrast, the Δ-5-3 BAC failed to rescue ventral pancreas development (E), although hindbrain expression was apparently normal. Scale bars: 200 μm.

rescue the MO phenotype in established transgenic lines. Surprisingly, pancreas tissue was observed in a larger fraction (43/209) of embryos injected with MO and the Δ+1+6 BAC. While different degrees of mosaicism between embryos leads to variability in these transient assays, comparison of the best pancreas rescues seen for each BAC demonstrates that the control BAC was able to fully rescue pancreas formation (Fig. 6C, E, and G) while the BAC lacking the early enhancer was only able to promote the formation of a hypoplastic pancreas (Fig. 6D, F, and H); the mean pancreas size in the embryos receiving the BAC lacking the early enhancer was only 35% the average size of the pancreas rescued by the intact BAC (Fig. 6B).

Discussion

Ptf1a has demonstrated importance in neuronal specification and development of the exocrine pancreas. However, prior to our work, identified cis-regulatory elements could only account for part of the transcriptional regulation of the gene. Therefore, we undertook a comprehensive, unbiased analysis of the zebrafish *ptf1a* locus to fully characterize its regulatory context. Previous work had established the presence of an autoregulatory enhancer upstream of mouse *Ptf1a* (Masui et al., 2008; Meredith et al., 2009). Based on identification of consensus binding site sequences for PTF1, we identified a similarly functioning enhancer upstream of the zebrafish gene. The region we identified as the autoregulatory enhancer contains three potential PTF1 binding sites, which we showed are both necessary and sufficient for its function. We also computationally identified similar regions upstream of multiple teleost *ptf1a* genes, suggesting that autoregulation of *ptf1a* is a deeply conserved feature. It is important to note that sequence conservation tools did not identify the autoregulatory sequences in teleosts, and therefore the absence of recognizable sequence conservation does not preclude the presence of functional conservation.

Ptf1a has an important role in promoting inhibitory fates in several neuronal populations. Previous work in mice identified a downstream regulatory element with activity in the hindbrain, spinal cord and retina (Masui et al., 2008; Meredith et al., 2009), although it did not direct expression to the cerebellum and additionally showed ectopic activity in populations of excitatory neurons of the spinal cord. Here we report the identification of several independent enhancers in zebrafish that recapitulate different subsets of the endogenous expression pattern in the hindbrain, spinal cord, and the cerebellum. Surprisingly, although the *+6ptf1a* sequence is highly similar to the orthologous sequence in the mouse, a larger transgene encompassing the orthologous sequence showed no cerebellar activity in transgenic mice. This discrepancy could be due to divergence of the regulatory sequences in the two species or the presence of additional regulatory elements in the zebrafish sequence. We additionally identified multiple enhancers with activity in different regions of the hindbrain, suggesting the presence of diverse regulatory mechanisms activating *ptf1a* expression in different regions of the hindbrain. Whether these enhancers with partially overlapping expression patterns synergize *in vivo* to create exact borders of expression as suggested elsewhere (Perry et al., 2011) or whether these enhancers operate in a modular fashion will be interesting to determine in the future.

The region immediately downstream of zebrafish *ptf1a* was able to drive expression in the pancreatic progenitors, an activity not found for the orthologous mouse sequence (Masui et al., 2008). While this discrepancy could be due to divergence between the mouse and zebrafish sequences, other reasons could account for the differences. First, the mouse downstream regulatory sequence excluded 330 bp corresponding to the 3′UTR and a further 536bp of poorly conserved sequence directly downstream

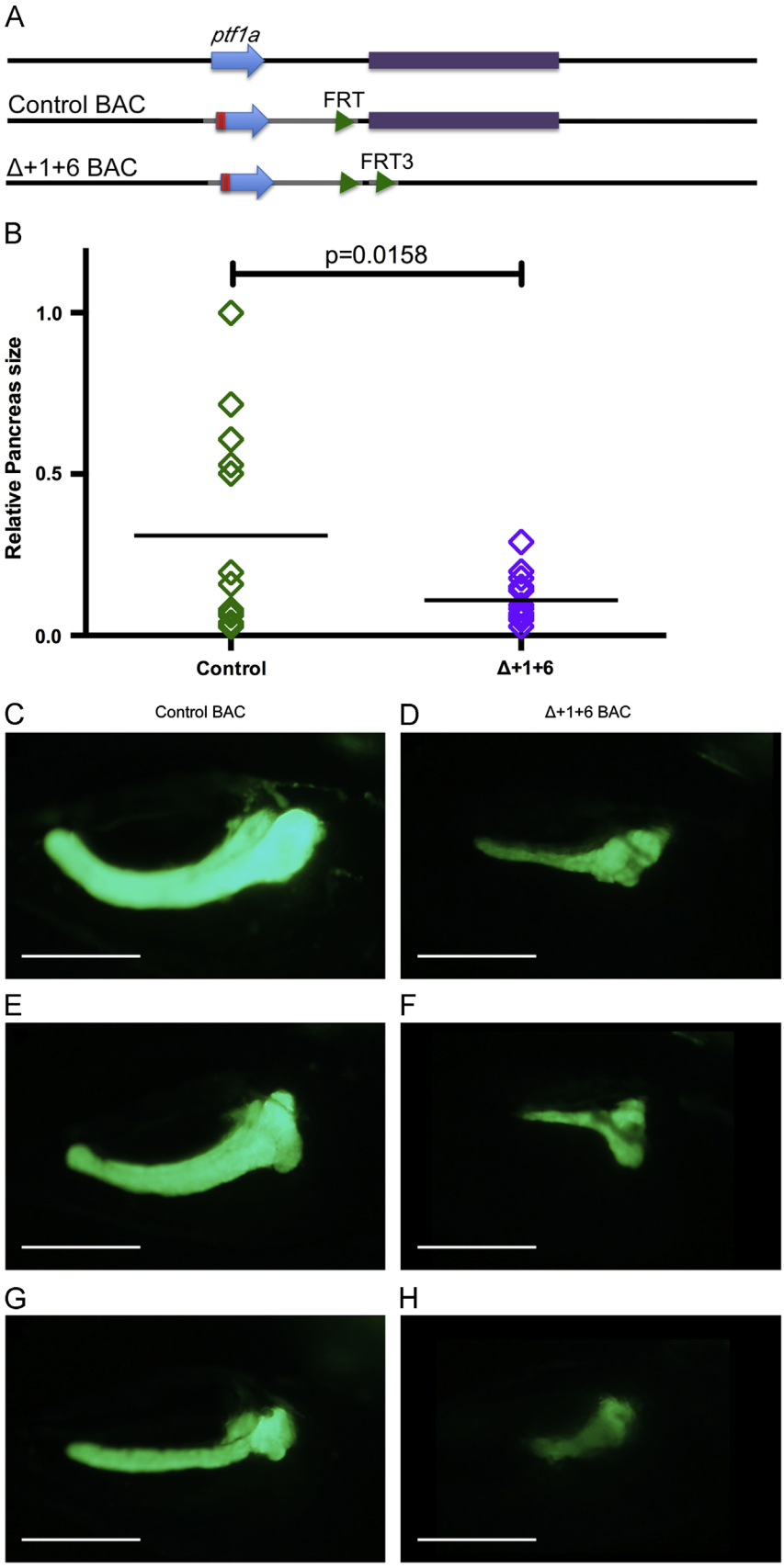

Fig. 6. Loss of activity of the early +1*ptf1a* enhancer results in the formation of a hypoplastic pancreas. (A) The diagram indicates the endogenous *ptf1a* locus (top line). In the control BAC (middle line) the *ptf1a* coding sequence was modified by synonymous substitutions and the addition of a Kozak sequence (as in Fig. 5) resulting in a transcript resistant to the translation blocking MO. The Δ+1+6 BAC has a 5 kb deletion removing the early enhancer. (B) Pancreas size was measured at 5 dpf following an injection of *ptf1a* MO and either the control BAC or the Δ+1+6 BAC. (B) Rescues with the Δ+1+6 BAC resulted in a significantly smaller pancreas size ($p=0.0158$). Three examples are shown of the maximum pancreata sizes measured at 5 dpf, after rescue with either the control BAC (C, E, G) or the BAC with the deletion of the early enhancer (D, F, H). Scale bars: 200 μm.

of the mouse 3′UTR. It is possible that the early pancreatic activity requires sequences in this region. We also found that the activity of this enhancer was sensitive to position effects, necessitating the screening of a relatively large number of founders to identify those with early pancreatic expression. The extremely high degree of conservation of this downstream region suggests that the mechanisms controlling initiation of *ptf1a* expression in the pancreas are conserved between teleosts and mammals. There are predicted binding sites for Hnf1, Sox9, and Pdx1 conserved in this region from teleosts to mammals, all attractive candidates for regulators of early *ptf1a* expression and specification of the exocrine progenitor cells. With the identification of this enhancer, the *in vivo* role for all of these factors in inducing *ptf1a* and subsequent pancreatic development can be addressed directly.

We tested the requirement for the identified early enhancer region in exocrine pancreas specification. Somewhat surprisingly, we found that a BAC lacking the early enhancer region was capable in mosaic injected larvae of rescuing the formation of exocrine pancreas tissue, and in fact did so at a higher frequency than the intact BAC, although the average size of rescued pancreatic tissue was significantly smaller. These data suggest that there are additional sequences outside of the deleted enhancer region that can lead to a low level of *ptf1a* expression; once the expression meets the threshold to activate the autoregulatory enhancer, it is self-sustaining. Interestingly, injection of the Δ+1+6 BAC into *ptf1a:egfp* transgenics in the absence of MO leads to ectopic GFP expression in a fraction of embryos, in some cases even apparent development of pancreatic tissue bilaterally (data not shown). We hypothesize that the deleted region contains both positive regulatory elements that increase the level of *ptf1a* in the early pancreas, but also negative regulatory sequences that help to prevent ectopic expression in adjacent non-pancreatic endoderm.

It has been hypothesized that *Ptf1a* autoregulation plays a role in late pancreatic development and in the mature pancreas to stabilize commitment to the exocrine lineage (Masui et al., 2008). As a direct test of this hypothesis, we removed the autoregulatory enhancer from a BAC transgene containing the entire regulatory region of *ptf1a*. In contrast to the previously described *ptf1a* hypomorphic mutant (Dong et al., 2008), Ptf1a encoded by the BAC lacking the autoregulatory enhancer is fully functional to activate all of its target genes. However, in the absence of expression from the endogenous *ptf1a* locus, as in a null mutant or morphant, the *ptf1a* locus on the BAC is incapable of regulating its own expression. In the absence of this autoregulatory loop, we find that *ptf1a* cannot support development of the exocrine pancreas. Our results thus reveal a requirement for *ptf1a* autoregulation much earlier during embryonic development than expected. These results demonstrate that *ptf1a* autoregulation has a critical role in pancreatic development and unlike other developmental regulatory elements, whose deletion might cause no effect or only produce effects under environmental stress (Perry et al., 2011; Frankel et al., 2010), the autoregulatory function is strictly required for ventral pancreas expansion. The presence of sequences with predicted autoregulatory function in the teleost loci described here and in other vertebrates (Masui et al., 2008) suggests that autoregulation is a well conserved and critical aspect of *ptf1a* expression, a hypothesis supported by our functional BAC rescue assays.

Our work has produced a more complete picture of *Ptf1a* regulation, including the first evidence of regions regulating initial expression in the pancreas and cerebellum, the two structures most severely affected in mutants. It will now be possible to test specific hypotheses about transcription factors and signaling pathways required for *Ptf1a* induction. In addition, we have directly tested the requirement for autoregulation at the locus, and shown that it is critical for early development of the exocrine pancreas. The transgenic model we have generated will also be useful in better understanding the role of *Ptf1a* autoregulation in the allocation and commitment of pancreatic cells to the exocrine and endocrine lineage. Previous studies have shown that maintaining *Ptf1a* expression during embryonic development blocks endocrine differentiation (Schaffer et al., 2010) and antagonizing *ptf1a* function postembryonically can lead to an exocrine-to-endocrine conversion (Hesselson et al., 2011). Our BACs lacking the autoregulatory enhancer can further be used to examine the requirement for *ptf1a* autoregulation in processes of pancreatic development, such as the expansion and maintenance of pancreatic progenitors and the balance of exocrine and endocrine progenitors. These efforts will ultimately help in understanding cell fate choices in the pancreas and enable manipulations of these processes toward the generation of desired cell types.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.07.011.

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