STATE-OF-THE-ART REVIEW

Ptf1a function and transcriptional cis-regulation, a cornerstone in vertebrate pancreas development

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Vertebrate pancreas organogenesis is a stepwise process regulated by a complex network of signaling and transcriptional events, progressively steering the early endoderm toward pancreatic fate. Many crucial players of this process have been identified, including signaling pathways, cis-regulatory elements, and transcription factors (TFs). Pancreas-associated transcription factor 1a (PTF1A) is one such TF, crucial for pancreas development. *PTF1A* mutations result in dramatic pancreatic phenotypes associated with severe complications, such as neonatal diabetes and impaired food digestion due to exocrine pancreatic insufficiency. Here, we present a brief overview of vertebrate pancreas development, centered on *Ptf1a* function and transcriptional regulation, covering similarities and divergences in three broadly studied organisms: human, mouse and zebrafish.

Introduction

The pancreas of most vertebrates consists of two compartments with distinct functions. The exocrine pancreas is composed of enzyme-secreting acinar cells and duct cells, which transport these enzymes into the gastrointestinal tract. The endocrine pancreas comprises numerous discrete islets, embedded in the exocrine tissue, made up of different hormone-producing cell types, including α-cells (glucagon), β-cells (insulin), δ-cells (somatostatin), PP cells (pancreatic polypeptide), and ε-cells (ghrelin) [1,2]. Pancreas development in vertebrates is an intricate process through which various cell lineages develop from common endodermal progenitors and converge to form a single organ [3,4]. During early development, the endodermal epithelium evaginates and through signaling pathways based on diffusible molecules or cell–cell interactions, cis-regulatory elements (CREs) and transcription factors (TFs), gives rise to the dorsal and ventral buds [5,6] that contain multipotent pancreatic progenitor cells (MPCs). One important player is the pancreas-associated TF 1a (Ptf1a), a basic helix-loop-helix (bHLH) TF that forms the trimeric pancreas TF 1 complex (PTF1) with two other proteins: an E protein and Rbpj, or its pancreas-restricted paralogue Rbpjl

Abbreviations

BAC, bacterial artificial chromosome; bHLH, basic helix-loop-helix; CRE, cis-regulatory element; CS, Carnegie stage; DNT, dorsal neural tube; EPC, endocrine progenitor cell; MO, morpholino; MPC, multipotent progenitor cell; PNC, pancreatic Notch-responsive cell; PTF1, pancreas transcription factor 1; PTF1A, pancreas-associated transcription factor 1a; RA, retinoic acid; TF, transcription factor; TSS, transcriptional start site.

[7]. During vertebrate pancreas development, *Ptf1a* is thought to be required for pancreatic specification [8], exocrine versus endocrine fate decision [9,10], and maintenance of acinar cell identity [8,11], in addition to neurodevelopmental roles [12]. Furthermore, pancreatic MPCs have been characterized by co-expression of *Pdx1* and *Ptf1a* [8,13]. Loss-of-function mutations in the human *PTF1A* gene result in developmental defects such as cerebellar and pancreatic agenesis [14]. Importantly, mutations in CREs that regulate its expression in the pancreas also result in pancreatic agenesis, associated with permanent neonatal diabetes mellitus and exocrine insufficiency [15]. Moreover, loss of *Ptf1a* results in pancreatic MPCs switching to alternative cell fates in mice [13]. In zebrafish, *ptf1a* morphants display impaired development of the ventral pancreatic bud [16]. Thus, the vital role of *Ptf1a* in pancreas organogenesis is indisputable. Still, the precise mechanisms by which *Ptf1a* controls pancreatic development are not yet fully understood.

Vertebrate pancreas patterning and commitment

Vertebrate pancreas patterning and specification

During vertebrate pancreas development, several gradients of signaling molecules are established, many emanating from adjacent tissues, providing positional cues for the developing pancreas. By binding to cell surface receptors, these ligands restrict the field of MPCs, culminating in the expression of specific TF-encoding genes such as *Pdx1*, *Nkx6.1* and, more importantly in the context of the present review, *Ptf1a* [3,17,18].

In mouse, the endoderm folds to form the primitive gut tube, divided into foregut, midgut, and hindgut. Pancreas specification occurs in the foregut–midgut boundary, starting around embryonic day 7.5 (E7.5) (Fig. 1) [19,20]. In most vertebrates, the foregut endoderm is located adjacently to the notochord, an organ that plays a crucial role in pancreatic development [21–23]. Activin (bB) and FGF2, both emanating from the notochord repress endodermal *Shh*, thus allowing the expression of *Pdx1* [24]. This pro-*Pdx1* role is reinforced by the diffusion of Nog2 from the notochord [23], antagonizing the *Pdx1* repressive signal of BMP that emanates from the lateral plate mesoderm [25], controlling pancreas size [23] (Fig. 1). Pdx1 is one of the best characterized MPC TFs, and it is required for *Ptf1a* expression in the ventral pancreatic bud and partially in the dorsal bud of the mouse [26]. Additionally, Ptf1a binds to the promoter of *Pdx1* [27] and is likely

required for its expression, the two TFs being required for proper MPC identity [8,13].

Mesenchymal cells are other nonautonomous factors, crucial for pancreatic development. Among the signals produced by mesenchymal cells are BMPs, Fgf10, retinoic acid (RA), and Wnt [25,28–30]. Fgf10 is not required for initial bud formation but is important for the proliferation of $Pdx1^+$ MPC [28,31]. In *Xenopus*, exposure to soluble Wnt5a induced MPC gene expression, specifically *Pdx1* and *Ptf1a* [30]. Further supporting the important role of Wnt signaling in the identity of MPCs, it was observed that cis-regulatory modules active in human MPCs are associated with Wnt signaling target genes [32], in agreement with the pro-pancreatic role observed in mouse [30].
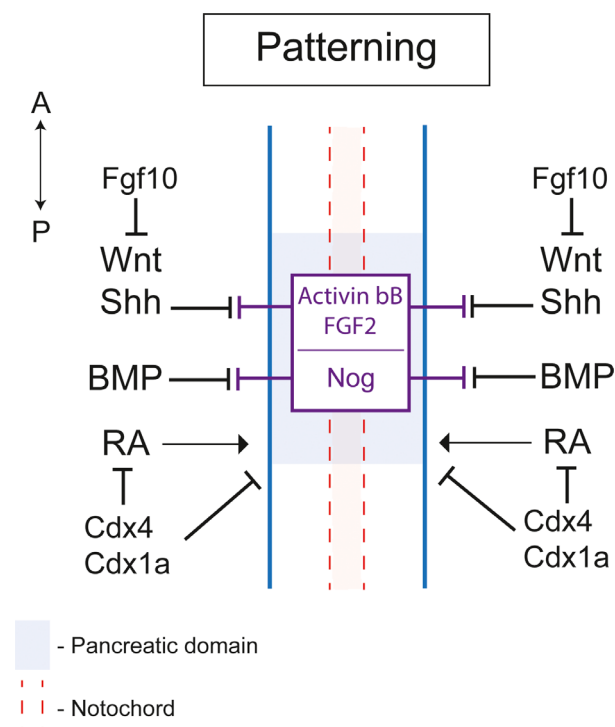

Fig. 1. Signaling pathways involved in vertebrate pancreas patterning and specification. Fgf10 is important for the proliferation of pancreatic progenitor cells [28] and interacts with both Wnt and Shh signaling, establishing a cross-regulation by feedback loops [86]. Wnt signaling has been observed to promote pancreatic fate, by expression of progenitor markers *Pdx1* and *Ptf1a* [30]. Activin (bB) and FGF2 are released from the notochord and regulate pancreas development, by repression of endodermal Shh [87]. Another signal from the notochord, Nog, inhibits BMPs emanating from the lateral plate mesoderm, controlling the pancreas size and location [23]. RA signaling is known to be crucial for pancreatic development [33] and the interaction between RA and Cdx factors is thought to be important for correct AP patterning [34].

The RA signaling pathway is required for proper pancreatic specification in zebrafish [33], being repressed by Cdx4 in the posterior endoderm [34] (Fig. 1).

Two temporal waves of embryonic endocrine differentiation have been observed in mouse embryos, the primary and secondary transitions [6]. However, in human embryos, an early phase of pancreatic endocrine differentiation, corresponding to the mouse primary transition, has not been detected [22] (Fig. 2). Human MPC shows expression of *PTF1A* [32], and its function is crucial for pancreas development [15]. MPCs in human ventral and dorsal pancreatic buds are also marked by expression of *PDX1, NKX6.1, SOX9,* and *GATA4* encoding TFs [35], essential for pancreatic specification, defining the MPC cell population. These cells are responsible for the formation of the dorsal and ventral pancreatic buds, during initial steps of pancreatic morphogenesis. After this, as differentiation starts, subgroups of progenitor cells appear at different locations. Central duct-like structures (composed of trunk cells) are involved in the formation of duct and endocrine cells, whereas more peripheral clustered cells (tip cells) differentiate to form acinar cells of the exocrine pancreas [35]. This regionalization is observable by patterns of gene expression. Trunk cells are known to express *NKX6.1* and *SOX9* and to have less *GATA4*, while tip cells remain *NKX6.1$^+$/SOX9$^+$/GATA4$^+$* [35] (Fig. 2). Shortly after, tip cells lose *NKX6.1* expression [22], suggesting an early segregation of the *PTF1A$^+$* acinar compartment [18,36,37], which initiates prior to the major wave of endocrine differentiation [35]. The segregation of *PTF1A* and *NKX6* expression into mutually exclusive domains suggests that a mutual repression mechanism is triggered just previous to endocrine and exocrine specification. Indeed, it has been suggested that through co-repression, *Nkx6.1* and *Ptf1a* function as antagonistic lineage determinants in MPCs, in an equilibrium that governs endocrine versus exocrine fate decision [18] (Fig. 2).

In mouse, MPCs have been characterized by the expression of several TF-encoding genes: *Ptf1a, Pdx1, Nkx6.1, Sox9, Nkx2.2, Hnf1β,* and *Cpa1* [3,37]. During secondary transition, similarly to human, tip cells of the branching epithelium adopt an acinar fate, being marked by the expression of *Ptf1a* and *Cpa1* [3,37], while cells in the trunk become restricted to a ductal or endocrine fate and are characterized by the expression of *Nkx6.1* and *Hnf1β* [3,37].

Endocrine pancreas determination in mammals

As MPCs segregate into endocrine and exocrine cellular compartments, they acquire expression of specific TF-encoding genes. The dynamics of this process is controlled by Notch signaling [38]. MPCs are under high levels of Notch signaling; however, when this pathway is inactivated, they become committed to an endocrine fate, becoming endocrine progenitor cells (EPCs) and activating genes encoding TFs required for endocrine differentiation [38,39]. In mouse, progenitor cells within the central duct-like structures acquire expression of *Ngn3* [40,41], marking the EPC population. Importantly, in human development, *NEUROG3* expression coincides with the appearance of the first fetal β-cells [35]. *SOX9* is one other important player in pancreas development. Its expression in MPCs of the human dorsal and ventral pancreatic buds has been observed to start around Carnegie stage 12 (CS12) [22]. It is absent from EPCs expressing *NEUROG3* and from differentiated endocrine cells. However, its expression persists in pancreatic duct cells [22].

Finally, EPCs differentiate into several hormone-producing cells, forming the pancreatic islets: α-cells—expressing glucagon, β-cells—expressing insulin, δ-cells—expressing somatostatin, and ε-cells—expressing ghrelin. Preceding differentiation, intermediate states exist where specific TFs control specific endocrine cell fates. Examples of such TFs are Pax4, Arx, Mafa, Mafb, Foxa2, or Pou3f4 [42]. Loss of either *Pax4* or *Arx* in mouse has been observed to not affect the total number of endocrine cells but instead changing the relative distribution of endocrine subtypes [43,44].

Divergent paths in zebrafish pancreas development

As in mouse, the zebrafish pancreas develops from two anlagen arising from foregut endoderm and containing MPCs, called dorsal and ventral buds [45]. The dorsal bud generates the first wave of endocrine cells, clustering at 24 hours postfertilization (hpf) to form the principal islet [46–48]. This first wave of differentiation only originates endocrine cells, contrasting with mammals, amphibians, and birds [49], leading to the hypothesis that the zebrafish dorsal bud is not truly analogous to the mammal one [10]. Indeed, whereas in mammals *Ptf1a* is expressed in both buds [8], the zebrafish protein is only required for the later developing ventral bud [16]. This most likely explains why the zebrafish dorsal bud only gives rise to endocrine cells. Moreover, while *Ngn3* is expressed in mouse EPCs, this is not the case in zebrafish, where its role is replaced by two other genes: *ascl1b* and *neurod1* [50]. The double knockdown of *ascl1b* and *neurod1* impairs endocrine differentiation, resulting in an almost
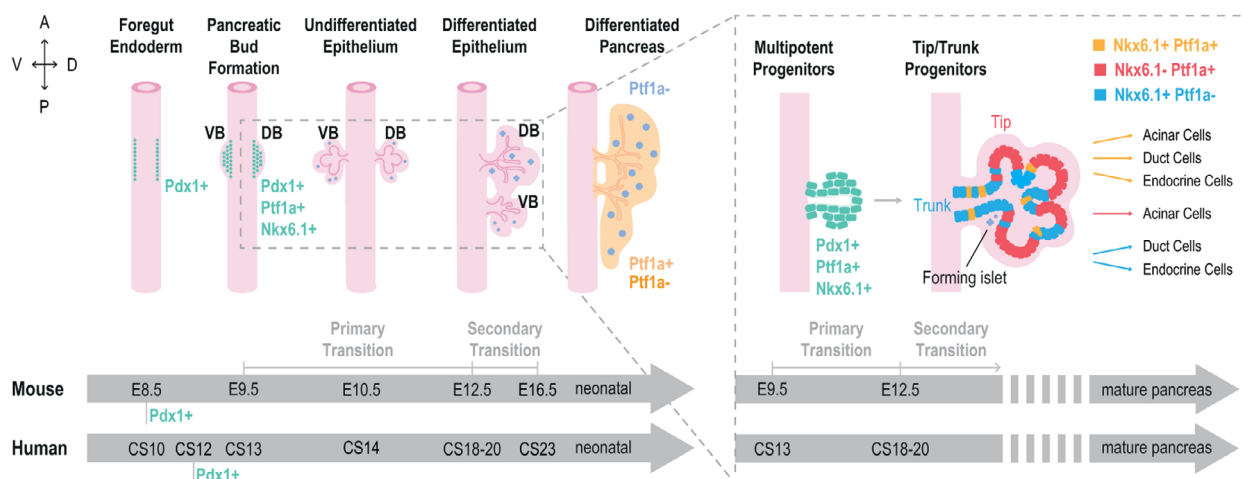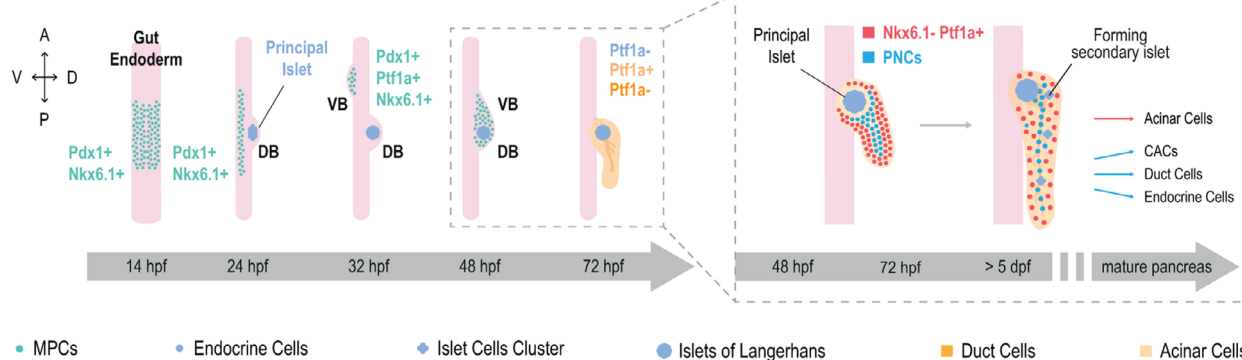
Fig. 2. Representation of the major morphogenetic events of pancreatic development in human [35,89], mouse [18,90,91], and zebrafish [51,53,92] , with corresponding developmental times (gray arrows). Primary and secondary transitions [90,91] are annotated, as well as the second wave of islet differentiation in zebrafish ('Forming secondary islets'; [93]). Expression of Ptf1a, as well as other selected key MPCs TFs encoding genes, as Pdx1 and Nkx6.1, is annotated. In the top right corner, orange cells represent the progressively restricted domain of Nkx6.1- and Ptf1a-positive cells that will give rise to mutually exclusive domains. Dashed lines highlight an overview of the main developmental processes during primary and secondary transitions in mouse and human, and during the second wave of islet differentiation in zebrafish. A, anterior; CACs, centroacinar cells; D, dorsal; DB, dorsal pancreatic bud; E, embryonic day; P, posterior; V, ventral; VB, ventral pancreatic duct.

complete absence of endocrine cell types [50]. In zebrafish, expression of *ascl1b* starts at 10 hpf [50], suggesting that the first cells in the pancreatic domain acquire an endocrine identity, even before the expression of key MPC markers such as *pdx1* (14 hpf) [50]. This species specificity of the zebrafish dorsal bud development in absence of *ptf1a* expression might help to better understand the potential role of *ptf1a* in pancreas development. Later, at 32hpf, the zebrafish ventral bud emerges anteriorly to the dorsal bud and gives rise to acinar, ductal, and a second wave of endocrine cells [16,45,51]. Differentiation of these later-appearing endocrine cells has been proposed to be equivalent to the secondary transition in mammals [52]. After this,

secondary islets appear along the pancreatic tail, which forms by growing in a posterior direction, after the envelopment of the principal islet (Fig. 2). Similar to what is seen in mouse, TF-encoding genes such as *pdx1*, *nkx6.1*, and *ptf1a* are expressed in zebrafish MPCs of the dorsal bud [13,53] (Fig. 3), arguing in favor of conserved pancreatic developmental genetic networks.

The role of Ptf1a in pancreas development

Ptf1a cDNA was first isolated from a rat exocrine pancreatic cell line and was considered as a
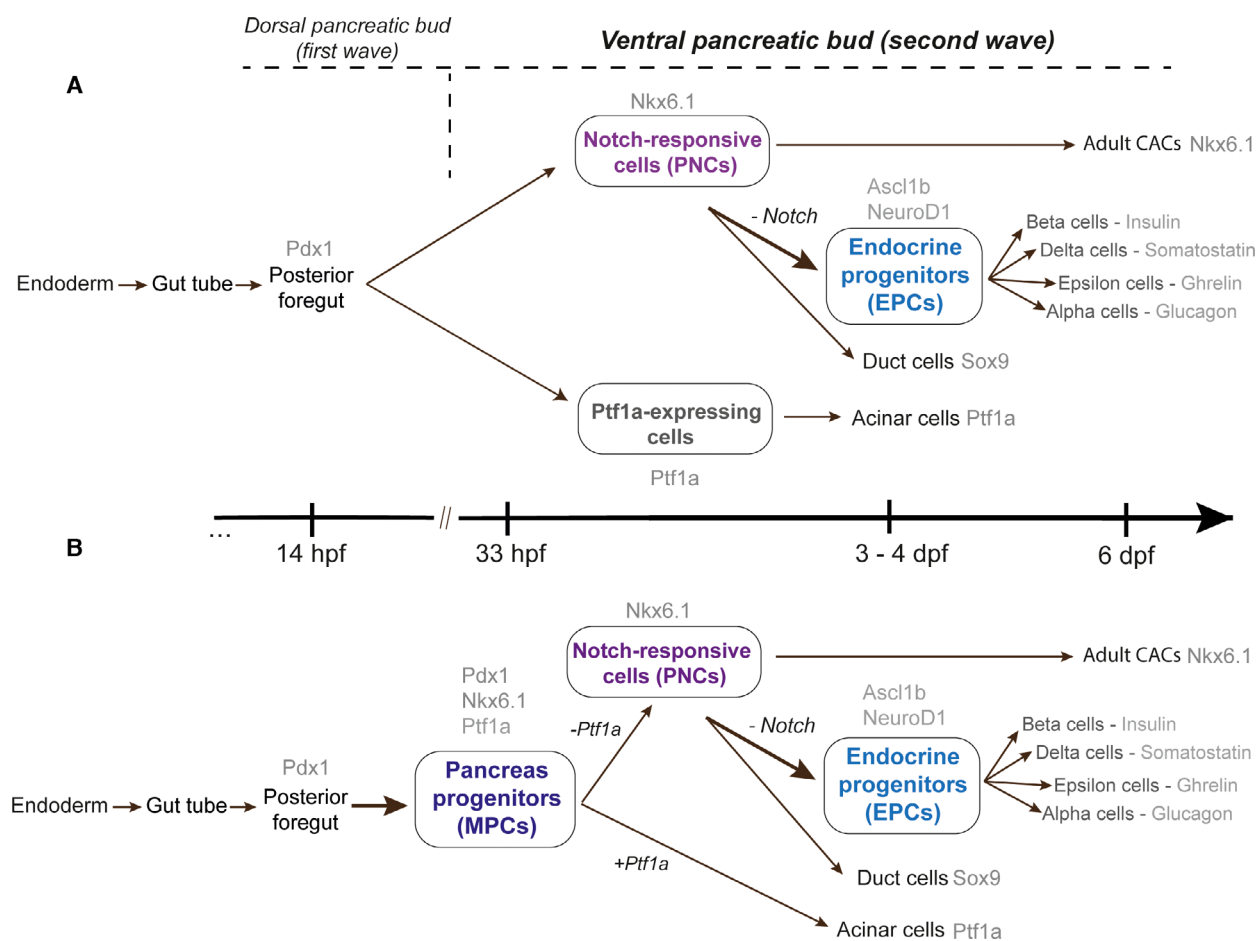
Fig. 3. Pancreatic development of the zebrafish, showing two suggested models. Key TFs involved in this process are shown. (A) In Wang *et al.* [51], the authors suggest that there is an early segregation of Ptf1a-positive cells and PNCs that differentiate to form separated domains of the pancreas (exocrine versus endocrine/ductal, respectively). (B) Another view, supported by the data in Ghaye *et al.*, suggests that multipotent progenitors expressing both nkx6.1 and ptf1a might progressively become responsive to Notch signaling, then starting to repress ptf1a expression, leading cell differentiation to a specific fate—endocrine/ductal [53]. In both, the progenitor cells that are known to be maintained in the adult zebrafish pancreas are also represented. These are called CACs—centroacinar cells [88].

transcriptional regulator of digestive enzymes such as amylase and elastase, in adult acinar cells [54]. In mouse, *Ptf1a* is expressed in early pancreatic MPCs during the primary transition [8] and later at the secondary transition, where it is restricted to MPC pro-acinar progenitors in the tip epithelium [3]. It is also expressed in differentiated acinar cells but not in ductal or endocrine tissue [54]. Ptf1a is a bHLH TF that forms the trimeric PTF1 complex with two other proteins: an E protein and Rbpj, or its pancreas-restricted paralogue Rbpjl [7] (Fig. 4). In mouse early development, *Ptf1a* is expressed in the MPC domain, along with other TF-encoding genes, as described above, such as *Pdx1* and *Nkx6.1* [3,8,18,55]. Rbpj/Ptf1a functions at these earlier stages, activating Rbpjl expression that, later on during acinar cell development, replaces

Rbpj function favoring the Rbpjl/Ptf1a complex [56,57] (Fig. 4). Unsurprisingly, mice encoding the Ptf1a[w298a] mutant protein that does not bind to Rbpj [7], but does to Rbpjl, phenocopy null mutants for Ptf1a, including absence of pancreatic ventral bud and delay of dorsal bud growth [56]. Additionally, in Rbpj-deficient pancreata, amylase-expressing acini and islets are formed during late embryonic and postnatal development, suggesting an essential role of Rbpj in early but not late development.

Rbpj can function by a Notch-dependent and Notch-independent way, in the latter case co-binding to Ptf1a [56]. These functions are nonoverlapping, being the Notch dependent related with the maintenance of a progenitor state, inhibiting ectopic endocrine progression [38,58], while the Ptf1a-bound Notch-

independent function is required for the growth and morphogenesis of pancreatic epithelia [56]. Moreover, the inactivation of Notch1 and Notch2 did not inhibit pancreatic development, suggesting that these are not essential for pancreatogenesis, contrary to Rbpj [59]. These results suggest that the early function of Rbpj is more linked with the activity of Ptf1a than to Notch signaling.

Ventral and dorsal buds have different requirements for early development. In mouse, loss-of-function of *Ptf1a*, *Pdx1*, or double loss-of-function, ablates ventral bud development [13]; however, the dorsal bud is yet able to develop alpha and beta cells [13]. In mutants for *Ptf1a*, the dorsal bud generates an extremely reduced endocrine pancreas with a reduced beta cell mass [8,13,60] but acinar cells are never observed
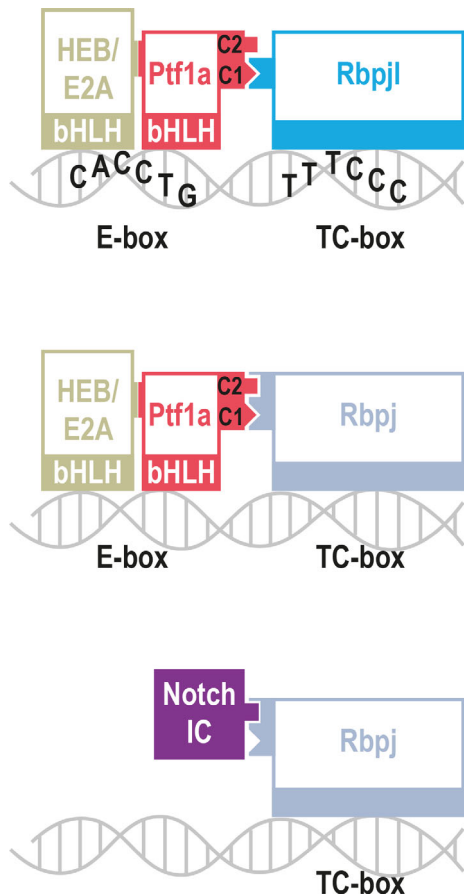

Fig. 4. The PTF1 complex. Ptf1a interacts in a trimer complex with an E protein (HEB/E2A) and Rbpjl (top panel) or Rbpj (middle panel), binding to a DNA E-box and a TC-box. Rbpj can operate in a Notch-independent way, by interacting with Ptf1a (middle panel), or in a Notch-dependent way, by interacting with the Notch intracellular domain (NotchIC; bottom panel).

[8,61]. Regarding the ventral pancreas, lineage tracing in *Ptf1a* mutant background shows labeling in gut cells, where normally *Ptf1a* is not expressed, suggesting that *Ptf1a* is required for the ventral pancreatic bud identity [8]. Additionally, independent double lineage tracing for *Ptf1a*- and *Pdx1*-expressing cells showed that the vast majority of pancreatic cells are derived from double *Ptf1a*^{+} and *Pdx1*^{+} cells. Although some *Pdx1*^{+} Ptf1a- derived beta cells were found, these had lower levels of insulin, suggestive of a lesser mature state, compared to the *Pdx1*^{+}/*Ptf1a*^{+} derived beta cells, highlighting the importance of *Ptf1a* in the development of mature beta cells [62]. This role in MPC identity is also corroborated by experiments of ectopic transient expression of *Ptf1a* in mouse embryonic stem cells, moderately inducing all lineages of pancreas development, including mature beta cells [63]. Additionally, Ptf1a likely contributes to the precise specification of pancreas, since *Ptf1a*-derived cells are detected only in the pancreas, contrasting with *Pdx1*-derived cells, found in the mouse pancreas, gastric antrum, and duodenum [62].

After this important early contribution to pancreas development, *Ptf1a* gets restricted to the tip of pancreatic branches formed during the secondary transition (Fig. 2). Lineage tracing experiments of *Ptf1a*-expressing cells show labeling in all acinar and most of duct and islets cells [8]. Conditional lineage tracing shows that at the primary transition, *Ptf1a*^{+} cells give rise to even parts of endocrine, duct, and acinar pancreas. At E13, already in the secondary transition, tip MPCs give rise mainly to acinar cells, with increasing proportions at E14 and E15, becoming almost fully committed to acinar progenitors [11]. This change in the multipotent potential of MPCs coincide with the mutually exclusive domains of expression of *Ptf1a* and *Nkx6.1*. Although at the primary transition (E10.5), there is co-expression of *Nkx6.1* with *Ptf1a* [18], and at E12.5, *Nkx6.1* is more restricted to the trunk and *Ptf1a* to the tip [18] (Fig. 2). By E14.5, when tip cells have fully committed to an acinar fate [3], *Nkx6.1* is almost completely excluded from the tip forming a sharp boundary with *Ptf1a* [18]. This is possible by a mutual repression of *Nkx6.1* and *Ptf1a* that becomes effective in the secondary transition [18]. NR5A2 is a member of the nuclear hormone receptor family [64], and its expression is controlled by the Ptf1 complex, being important for the formation and maintenance of the MPCs of the secondary transition, converting MPCs to an acinar lineage, and for expansion and differentiation of pre-acinar cells [65]. After differentiation of acinar cells, Ptf1a is required to maintain acinar cell identity in adult mice, since its conditional

loss-of-function in acinar cells results in loss of expression of digestive enzymes. Furthermore, the transcriptome of these cells is more similar to prenatal pancreas than to adult pancreas [66–68].

Observations in human genetic alterations have highlighted that *PTF1A* might have similar functions as described in mouse. A mutation in the *PTF1A* gene, resulting in a protein truncation, leads to cerebellar and pancreatic agenesis, that causes neonatal diabetes [14]. Additionally, alterations in pancreas-specific cis-regulatory regions of *PTF1A* induce pancreatic agenesis and neonatal diabetes, without clear cerebellar phenotypes [15,69–71]. These observations suggest that *PTF1A* might have an early role in endocrine and exocrine pancreas development, followed by a strong later requirement for exocrine proper differentiation. This is reinforced by the fact that single-cell RNA-seq in human pancreatic cells shows that $PTF1A^+/SOX9^+$ of the tip have a MPC-like profile [72].

In zebrafish, as in mammals, *ptf1a* plays an important role in acinar cell fate [16,73]. The dorsal bud never shows expression of *ptf1a*, giving rise only to the endocrine cells composing the principal islet [46]. This first wave of endocrine differentiation has some similarities to the mice primary transition. Interestingly, mouse embryos mutant for *Ptf1a* show a similar potential for early endocrine differentiation arising from the dorsal bud, in a *Ptf1a*-independent manner [13]. In zebrafish, *ptf1a* expression starts at 32hpf in the ventral bud, after endocrine differentiation of the principal islet from the dorsal bud [16], in cells that are $pdx1^+$ and $nkx6.1^+$ [9] (Figs 2 and 3). Later in development, ventral bud cells migrate in a dorsal and posterior direction enveloping the principal islet (Fig. 2). Similar to the mammal secondary transition, ventral bud cells give rise to endocrine cells that contribute to the principal islet between 48 and 120 hpf [74]. After 5–6 days of development, more endocrine cells appear along the pancreatic duct, forming secondary islets. In *ptf1a* loss-of-function, acinar cells and secondary islets are not detected, being only present the principal islet, while *ptf1a* lineage-labeled cells were converted into gall bladder and other nonpancreatic cell types [10]. Additionally, reduced levels of *ptf1a* present in a hypomorphic condition show a delay in ventral pancreas specification, accompanied by an exocrine to endocrine fate switch, suggesting that lower levels of Ptf1a can function, in a cell autonomous manner, to promote endocrine fate, whereas high levels repress it [9]. Accordingly, reduced Ptf1a dosage has been observed to promote a greater contribution toward nonacinar lineages [10].

It has been suggested that the zebrafish ventral bud contains two distinct progenitor cell populations: a population of Pancreatic Notch-responsive cells (PNCs) and a *ptf1a*-expressing population [51]. Ghaye and colleagues [53] hypothesized that Notch signaling responsiveness could be the key factor in the segregation of these cells into endocrine/ductal ($nkx6.1^+/ptf1a^+$/Notch on) or acinar cells ($nkx6.1^+/ptf1a^+$/Notch off). In this study, it was hypothesized that $nkx6.1^+$ cells progressively become PNCs, since initiation of *nkx6.1* expression is independent of Notch signaling [53]. Moreover, after transient co-expression of *nkx6.1* and *ascl1b* in the pancreatic anlagen, these cells segregate in two different domains, one expressing both genes and the other only *nkx6.1*. Through loss and gain-of-function experiments, the same authors have observed that Notch signaling works to maintain *nkx6.1* expression in PNCs, while repressing *ascl1b*. Therefore, only when Notch is inactivated, Notch-responsive cells transit into an endocrine progenitor state. Altogether, these results allow two different interpretations for how ventral MPCs are defined (Fig. 3). The first is that there is an early segregation of *ptf1a*+ cells and PNCs that differentiate to form separate domains (exocrine versus endocrine/ductal, respectively), supported by the fact that a unique progenitor domain has not been detected in the zebrafish [51]. Another view is that MPCs expressing both *nkx6.1* and *ptf1a* might progressively become responsive to Notch signaling, consequently repressing *ptf1a* expression, leading cells to a specific fate—endocrine/ductal [53]. On the other hand, cells retaining high Ptf1a levels (and Notch-unresponsive) differentiate over time to generate the acinar compartment.

The vital role of *Ptf1a* in vertebrate pancreas organogenesis is indisputable. *Ptf1a* has an early role in endocrine and exocrine pancreas development, followed by a later requirement for exocrine proper differentiation and maintenance of acinar fate. Importantly, these roles are dependent on the levels of expression of *Ptf1a* in specific cell types of the developing pancreas. The mechanisms regulating the expression of *Ptf1a* will be further discussed below.

Mechanisms of regulation of Ptf1a expression

Enhancers regulating expression of Ptf1a in vertebrates

The tissue-specific functions of *Ptf1a* require precise spatiotemporal regulation of its expression levels by the activity of multiple noncoding CREs. In mice,

Ptf1a levels are maintained by an autoregulatory enhancer (m5′-AR) located 13.4kb upstream of the *Ptf1a* transcriptional start site (TSS) and conserved among mammals [57,75] (Fig. 5A). m5′-AR contains two consensus binding sites for PTF1 validated *in vitro* [57,75] and Ptf1a protein binds to this sequence in mouse embryonic neural tube and adult pancreas [57,75,76]. In mouse transgenic reporter assays, m5′-AR drives reporter expression exclusively in *Ptf1a*+ cells [57,75,77]. These results strongly suggest that m5′-AR maintains *Ptf1a* levels through a positive autoregulatory loop. Additionally, m5′-AR fails to be activated in *Ptf1a* loss-of-function mice [75], showing that its activity requires pre-existing Ptf1a protein. Indeed, in luciferase assays, the enhancer is inactive in Ptf1a-negative cells but is activated when cells are co-transfected with plasmids expressing *Ptf1a* [57]. Likewise, in chick electroporation assays, reporter expression is restricted to Ptf1a-expressing domains but is induced in other domains when *Ptf1a* is ectopically expressed [75,77]. Thus, pre-existing Ptf1a is required and sufficient to activate m5′-AR. A similar CRE is present in zebrafish (z5′-AR; Fig. 5A). The z5′-AR is located 3 to 5kb upstream of *ptf1a* TSS, containing three consensus PTF1 binding sites, where the two most proximal are necessary and sufficient to drive reporter expression, suggesting that its activity is PTF1-dependent [78]. A bacterial artificial chromosome (BAC) spanning the *ptf1a* locus [79] and expressing morpholino (MO)-resistant *ptf1a* rescues pancreas development in zebrafish *ptf1a* morphants, but rescue fails when the z5′-AR is deleted [78], further illustrating how the z5′-AR is necessary to maintain the *ptf1a* levels required for pancreas development.

During neural development, *Ptf1a* expression gradually ceases following inhibitory neural fate specification. Therefore, there must exist a mechanism that overturns the m5′-AR-mediated autoregulatory loop. This mechanism likely involves *PR domain containing 13* (*Prdm13*), a direct downstream target of Ptf1a that can bind the Ptf1a-bound m5′-AR, blocking its activity [77]. Given the dependence of PTF1 for m5′-AR activity, the interaction of Prdm13 with Ptf1a is thought to disrupt the PTF1 complex. However, the precise manner through which Prdm13 represses m5′-AR activity and whether this mechanism is present or not in other tissues is currently unknown.

While the elements responsible for the trigger of *Ptf1a* expression in mice are unknown, an early-acting enhancer was identified in zebrafish (z3′-EA). z3′-EA is located 1–6 kb downstream of the TSS and drives reporter expression in the cerebellum and pancreas during early development [78]. In the pancreas,

reporter expression is first detectable at 34 hpf [78], coinciding with the onset of *ptf1a* expression in pancreatic progenitor cells [78,79]. In contrast, z5′-AR-driven reporter expression is only detectable at 42 hpf [78] (Fig. 5B,C). Moreover, z3′-EA activity decreases as acinar cell differentiation begins [78], suggesting that z3′-EA is the early trigger of *ptf1a* expression, which is subsequently maintained by the z5′-AR-mediated positive autoregulatory loop. Deletion of z3′-EA from a BAC encoding MO-resistant *ptf1a* fails to rescue normal pancreas development in *ptf1a* morphants. However, the fish still form a hypoplastic pancreas [78]. Therefore, while the z3′-EA is required for proper pancreas development, there may be other elements capable of triggering *ptf1a* expression.

In mice, a large fragment that spans from 2.4 to 14.8kb downstream of *Ptf1a* (m3′-12.4 kb region; Fig. 5A) drives reporter expression in the Ptf1a-expressing regions of the hindbrain, spinal cord, and retina [57,75]. However, reporter expression precedes *Ptf1a* expression. This, along with the fact that m3′-12.4kb region activity is still observed in *Ptf1a*-null mice [75], suggests that this enhancer has a role in the initial activation of *Ptf1a* expression in neural development. The m3′-12.4kb region also contains a 1.1kb fragment (m5′-DNT) that drives reporter expression in *Ptf1a*+ cells of the dorsal neural tube (DNT) from E10.5 to E12.5 [80], but in a broader pattern than the intact m3′-12.4 kb region, suggesting that the latter contains elements that spatially restrict the activity of the m5′-DNT [80]. Surprisingly, mice homozygous for m5′-DNT deletion reach adulthood with only a minor decrease of *Ptf1a* mRNA levels in the neural tube (E11.5) [81]. Thus, m5′-DNT may be only one of several enhancers responsible for early *Ptf1a* expression in neural development. For instance, there is evidence for the existence of a mouse cerebellar-specific enhancer (m3′-DcE). The cerebelless (*cbll*) mutant mice harbor a 313 kb deletion, 60 kb downstream of *Ptf1a* that results in cerebellar agenesis while their pancreas develops normally [82]. In fact, *Ptf1a* expression is lost in the cerebellum of *cbll* mice but maintained in other *Ptf1a*-expressing domains, including the pancreas. Therefore, the region likely contains one or more cerebellar-specific enhancers required for *Ptf1a* expression during cerebellar development.

Like in mouse, multiple sequences downstream of the zebrafish *ptf1a* gene display enhancer activity in the developing nervous system [78] (Fig. 5A) but, more recently, a zebrafish enhancer crucial for pancreas development was uncovered, 39kb downstream of the *ptf1a* TSS [83]. This *ptf1a* distal pancreatic enhancer (z3′-DpE; Fig. 5A) is active in MPCs and
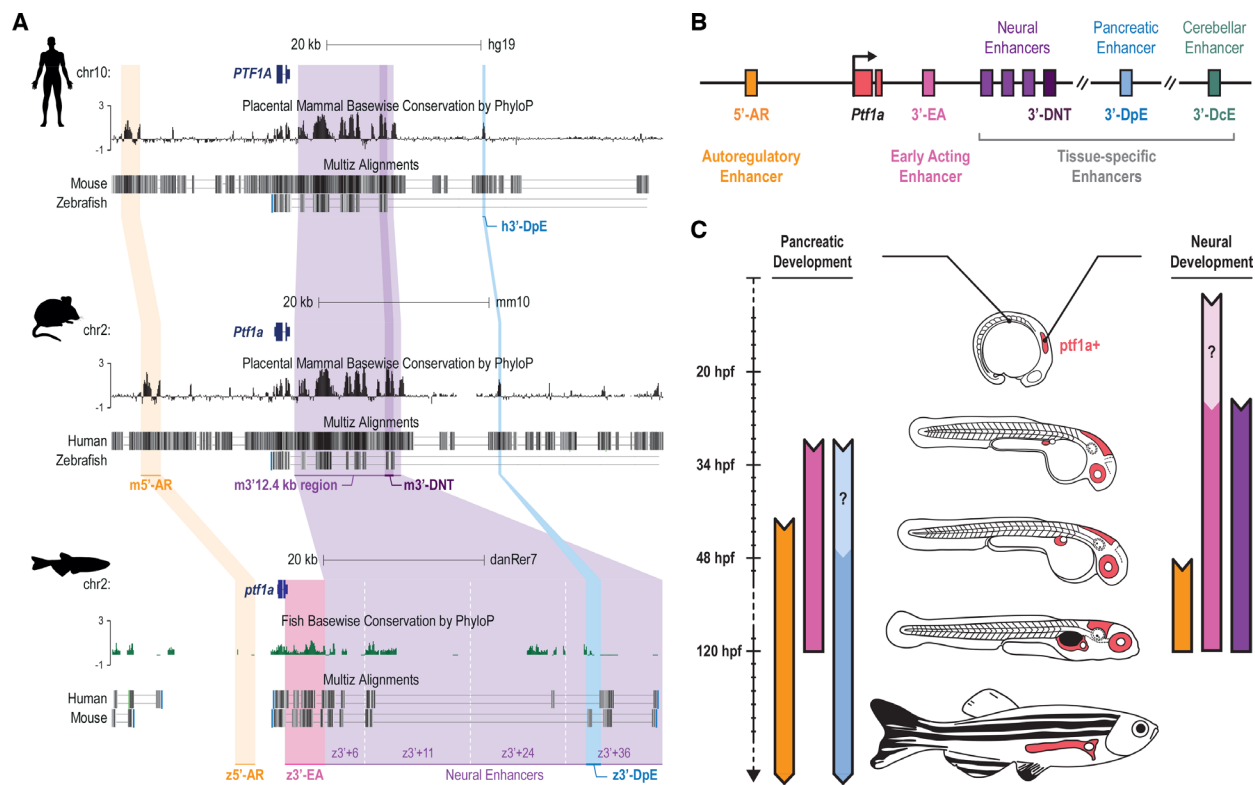
Fig. 5. (A) Regulatory landscape of *Ptf1a*. (Top) Human *PTF1A* distal pancreatic enhancer (h3′-DpE, blue). (Middle) Mouse autoregulatory enhancer (m5′-AR, orange), neural enhancers cluster region (m3′-12.4 kb region, light purple), and DNT-specific enhancer (m3′-DNT, dark purple). (Bottom) Zebrafish autoregulatory enhancer (z5′-AR), early-acting enhancer (z3′-EA), neural enhancers (z3′ + 6, z3′ + 11, z3′ + 24, and z3′ + 36), and distal pancreatic enhancer (z3′-DpE, blue). Sequence conservation across mammals (black) or fish (green) is shown for each panel and putative ortholog sequences between species are indicated by the colored boxes. z3′-DpE shows no sequence conservation with the human h3′-DpE enhancer; however, they likely represent functional equivalents, as assessed by functional assays. In mouse, there is a fragment with high degree of conservation with the h3′-DpE downstream of Ptf1a that could correspond to its functional equivalent, although this hypothesis has not been tested by functional assays. B) Summary of reported *Ptf1a* enhancers. *Ptf1a* expression during vertebrate development is regulated by multiple CREs, including an upstream autoregulatory enhancer (5′-AR), a proximal downstream early-acting enhancer (3′-EA) and multiple distal cell type-specific enhancers spread across a large genomic region. 5′-DpE, distal pancreatic enhancer; 5′-DcE, distal cerebellar enhancer (Adapted from [80]). (C) Reported activity of the z5′-AR (orange), z3′-EA (pink), z3′-DpE (blue), and neural enhancers (purple) throughout zebrafish embryonic development. Endogenous *ptf1a* expression is presented in red at 16hpf (14-somites), 35hpf (prim-22), 42hpf (high pec), 120hpf (larva), and adult zebrafish.

differentiated acinar and duct cells. Similar to the 5′-AR, z3′-DpE-driven reporter expression is weaker in progenitors and stronger in differentiated exocrine cells and the sequence interacts with the *ptf1a* promoter, suggesting that the z3′-DpE also regulates *ptf1a* expression directly [83]. However, unlike the z5′-AR, the z3′-DpE does not contain PTF1 consensus binding sequences.

A similar element is present in the human genome (Fig. 5A). Recessive mutations within a 400 bp non-coding sequence, 25 kb downstream of *PTF1A*, were identified in families with pancreatic agenesis. Moreover, chromatin conformation capture experiments in hESC-derived human MPCs revealed that the human

PTF1A distal enhancer (h3′-DpE) also interacts directly with the *PTF1A* promoter [15]. In contrast with *PTF1A* coding mutations, which result in pancreatic and cerebellar agenesis, neurological features are absent in reported cases of h3′-DpE mutations [15]. This illustrates how cis-regulatory mutations can have far greater tissue specificity than their disease-associated coding mutation counterparts.

Despite showing no sequence conservation with the human enhancer (Fig. 5A), z3′-DpE is likely functionally equivalent to h3′-DpE, with deletion of z3′-DpE resulting in pancreatic agenesis [83]. Interestingly, h3′-DpE is active in human MPCs but inactive in adult exocrine pancreatic cell lines [15], while z3′-DpE shows

activity in zebrafish MPCs but remains active in the adult zebrafish exocrine pancreas [83]. Therefore, although the two enhancers have equivalent functions during early pancreas development, the zebrafish sequence also contains cis-regulatory information relevant for adult pancreas function.

Collectively, the activity of the zebrafish *ptf1a* downstream enhancers recapitulates the reporter expression pattern observed for the mice 3′-12.4 kb region, but also exhibit activity in cerebellum and pancreas, which was absent for the mouse sequence [57,75]. To date, the presence of pancreas-specific enhancers downstream of the mouse *Ptf1a* gene has not yet been demonstrated. However, there is a fragment with high degree of conservation with the h3′-DpE downstream of the mouse *Ptf1a* that may correspond to its functional equivalent (Fig. 5A).

The CRE network controlling Ptf1a expression

A comparison of the findings from humans, mice, and zebrafish reveals an intricate network of CREs that regulate *Ptf1a* transcription during development (Fig. 5A), as well as direct links between noncoding mutations and adverse phenotypic effects resulting from disruption of progenitor cell expansion and cell fate decision. Yet, these findings are only a glimpse into the complex enhancer interactions underlying the spatiotemporal control of *Ptf1a* levels and several pivotal questions remain to be answered.

Firstly, what elements trigger initial *Ptf1a* expression? A good candidate is the 3′-EA, whose activity coincides with the onset of *Ptf1a* expression [78]. However, the 3′-EA is not strictly required for the induction of *Ptf1a* expression, at least in the pancreas [78], and the existence of other downstream enhancers, active early in development and inactive in adult tissues, may indicate that additional regulatory elements are required to establish the *Ptf1a* levels needed for the activation of 5′-AR (Fig. 6). In support of this theory, in humans, the distal h3′-DpE shows enhancer activity in early MPCs and its deletion affects very early events of pancreas development, causing pancreatic agenesis [15], a phenotype recapitulated in zebrafish by the deletion of z3′-DpE [83]. These results suggest that these CREs may aid 3′-EA to establish initial proper *Ptf1a* levels to activate the 5′-AR-mediated autofeedback loop. The identification of other CREs, within the regulatory landscape of *Ptf1a*, active early in development and inactive in adult tissues, will help to identify additional functional CREs that may contribute to the activation of 5′-AR. In addition to this, the TFs that bind to the 3′-EA and are responsible initiate its activity are still unknown.

Secondly, the activity of the *Ptf1a* autoregulatory 5′-AR may be more nuanced than described, with discrepancies between species and cell types. For instance, the two PTF1 binding sites of the m5′-AR display different properties, with the PTF1-J/PTF1-L complex binding slightly more effectively to the proximal binding site (P-site, 13.6 kb upstream of Ptf1a) than to the distal site (D-site, 14.8 kb upstream) *in vitro* [57]. Moreover, there is greater enrichment for Ptf1a ChIP-seq signal at the P-site than the D-site in adult mouse pancreas tissue [57]. Conversely, no significant differences in enrichment were reported in mouse neural tube (E11.5) [75]. In a rat pancreatic acinar cell line (AR4-2J cells), the ablation of just one of the two

Fig. 6. (A) Model of enhancer functions during acinar cell differentiation. *Ptf1a* expression in pancreatic precursors is initiated by the downstream proximal early-acting enhancer (3′-EA) in combination with the distal pancreatic enhancer (3′-DpE). The resulting Ptf1a protein forms the PTF1-J complex with an E protein and Rbpj. PTF1-J binds the upstream autoregulatory enhancer (5′-AR) directly, initiating a positive autoregulatory loop that maintains Ptf1a levels after the activity of the early-acting enhancers subsides (in the zebrafish, exceptionally, 5′-DpE remains active). During the secondary transition, *Ptf1a* expression is superinduced by an unknown mechanism in combination with the activity of 5′-AR, and cells begin the acinar developmental program. Increased Ptf1a levels lead to the expression of Rbpjl that gradually replaces Rbpj in the PTF1 complex (PTF1-L). PTF1-L binds and maintains the activity of the 5′-AR and the Rbpjl promoter resulting in two positive loops that sustain the elevated levels of PTF1-L required for transcription of downstream regulators of acinar development and genes encoding secretory digestive enzymes (Adapted from [57]). (B) Transient *Ptf1a* expression during the specification of spinal cord neurons. In this model, *Ptf1a* expression in the neural progenitors is initiated by the downstream proximal early-acting enhancer (3′-EA) in combination with one or more neural enhancers. The Ptf1a protein, in the PTF1-J complex, binds to the upstream autoregulatory enhancer (5′-AR) to regulate its own transcription. Ptf1a also activates transcription of inhibitory-neuron-specifier genes, such as *Pax2*, and indirectly suppresses the excitatory neuronal gene program by activating transcription of *Prdm13*. Prdm13 inhibits gene expression programs for excitatory neuronal lineages through multiple mechanisms, including interaction with the bHLH factor *Ascl1* to repress Ascl1-dependent activation of *Tlx3* expression [77]. *Prdm13* negatively regulates its own expression through a negative retro-control of its activator, *Ptf1a*, by interrupting the autoregulatory loop, possibly through the displacement of Rbpj in the PTF1-J complex [77,85]. Additionally, *Prdm13* may directly suppress its own expression through an unknown mechanism [85].
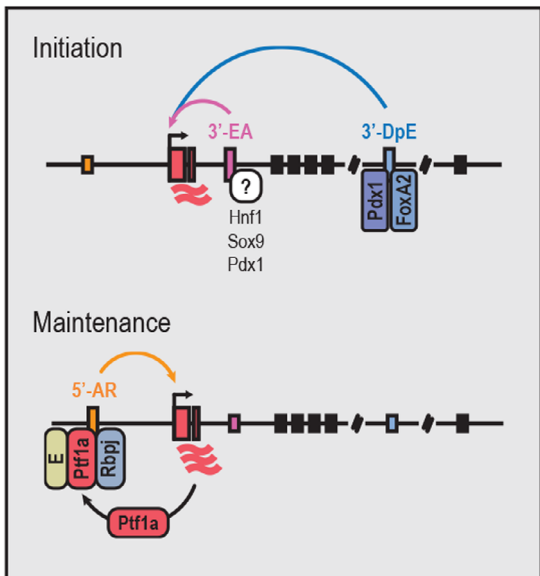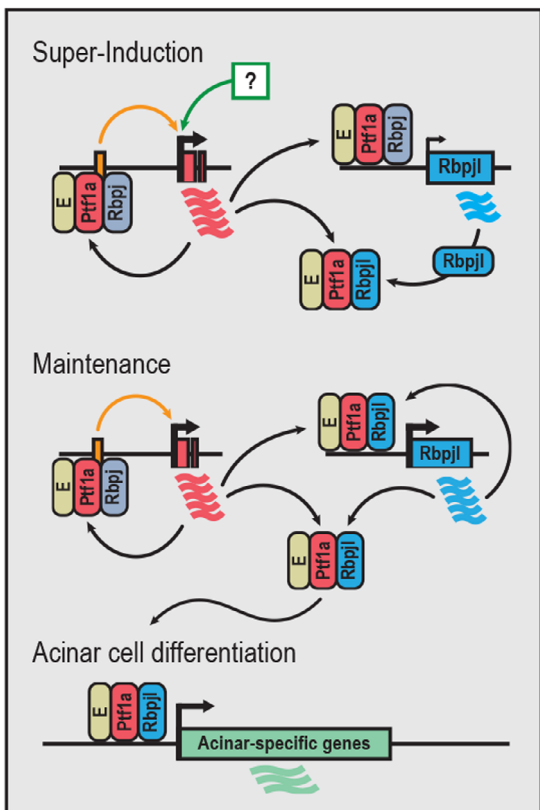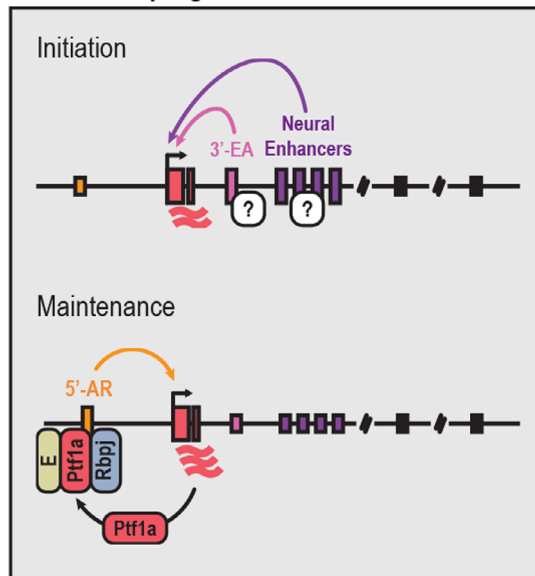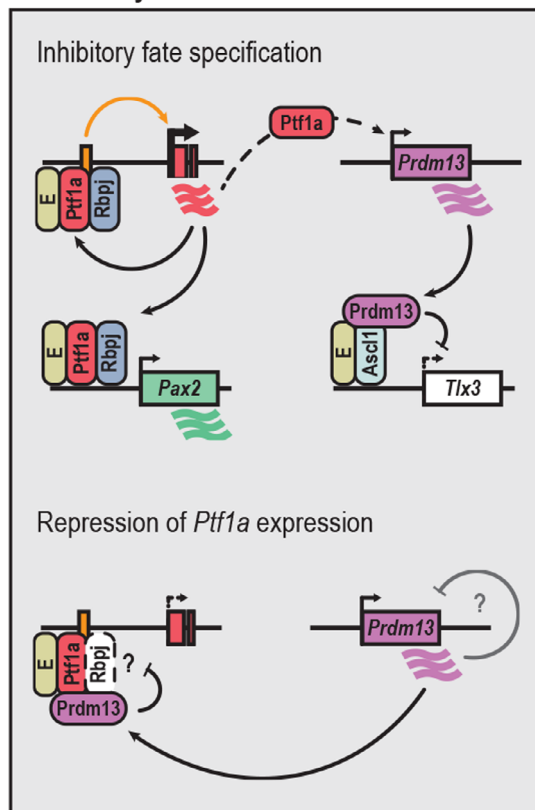
PTF1 binding sites has a dramatic impact on enhancer activity [57]. In contrast, while adult mice with homozygous deletions spanning the two PTF1 binding sites have adverse somatosensory phenotypes, mice that retain at least one of the sites develop normally [81]. To date, there are no studies documenting the phenotypic outcome in the pancreas of ablating only one PTF1 binding site. Additionally, in zebrafish, a third PTF1 binding site of unknown importance exists, although seemingly unnecessary for enhancer activity in the pancreas [78]. The selective use of PTF1 binding sites with slightly different binding properties may translate into variations in enhancer activity between tissues or species. However, as of yet, it remains unexplored.

Thirdly, what are the elements that super-induce *Ptf1a* expression? The 5′-AR subsequently maintains the elevated *Ptf1a* levels, but there is no evidence to suggest that it is responsible for the super-induction step, and none of the reported *ptf1a* enhancers seem to fulfill that function.

Finally, what elements halt the positive autoregulatory loop during neural development? Despite the growing understanding of how *Ptf1a* expression can be induced and sustained, to date, only one mechanism has been suggested to counteract the activity of the 5′-AR: an incoherent feedforward loop [84], in which *Ptf1a* upregulates both its expression and the expression of its repressor *Prdm13* [77,85] (Fig. 6). This interplay between *Ptf1a* and *Prdm13* in m5′-AR observed in neural development can help explain how the activity of 5′-AR is modulated in other tissues where *Ptf1a* is transiently expressed.

Concluding remarks

Ptf1a is a key player in pancreas specification. Although its function is not completely understood, it has been shown that Ptf1a has an early role in MPCs, followed by a later requirement for exocrine proper differentiation and maintenance of acinar fate. Loss-of-function mutations in the human PTF1A gene have long been associated with developmental defects of the pancreas and nervous system, including cerebellar and pancreatic agenesis associated with neonatal diabetes. More recently, the finding that cis-regulatory mutations are sufficient to reproduce equivalent defects, in a tissue-specific manner, highlights the importance of understanding the regulatory networks controlling Ptf1a levels during development.

Studies in mouse, human, and zebrafish show that *Ptf1a* expression is regulated by a set of functionally equivalent CREs, scattered throughout the *Ptf1a* regulatory landscape, including a highly conserved autoregulatory enhancer, a proximal downstream early-acting enhancer, and a series of tissue-specific distal downstream enhancers (Fig. 5B,C). Initial *Ptf1a* expression likely depends upon tissue-specific early-acting enhancers. After Ptf1a levels reach a certain threshold, the autoregulatory enhancer is activated and maintains proper *Ptf1a* expression through a positive autoregulatory loop. These complex dynamics seemingly coordinate *Ptf1a* expression across vertebrate species. However, most of the TFs and molecular mechanisms responsible for the initiation of *Ptf1a* expression are yet unknown, opening new horizons for the identification of top hierarchical components of pancreas developmental gene networks. The identification of such factors will be of great relevance as these are essential for the identity of MPCs. Finally, expanding our knowledge of the cis-regulatory machinery that controls *Ptf1a* expression to the molecular targets of Ptf1a should prove invaluable for better understanding pancreatic diseases such as pancreatic cancer and diabetes and improve protocols for *in vitro* pancreas differentiation.

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Conflict of interest

The authors declares no conflict of interest.

Author contributions

MD, JA and JB writing the original draft, review, and editing. JB supervision and funding acquisition.

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