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RESEARCH ARTICLE

# The histone methyltransferase Setd7 promotes pancreatic progenitor identity

**Julia Kofent, Juan Zhang and Francesca M. Spagnoli***

## ABSTRACT

Cell fate specification depends on transcriptional activation driven by lineage-specific transcription factors as well as changes in chromatin organization. To date, the interplay between transcription factors and chromatin modifiers during development is not well understood. We focus here on the initiation of the pancreatic program from multipotent endodermal progenitors. Transcription factors that play key roles in regulating pancreatic progenitor state have been identified, but the chromatin regulators that help to establish and maintain pancreatic fate are less well known. Using a comparative approach, we identify a crucial role for the histone methyltransferase Setd7 in establishing pancreatic cell identity. We show that *Setd7* is expressed in the prospective pancreatic endoderm of *Xenopus* and mouse embryos prior to *Pdx1* induction. Importantly, we demonstrate that *setd7* is sufficient and required for pancreatic cell fate specification in *Xenopus*. Functional and biochemical approaches in *Xenopus* and mouse endoderm support that Setd7 modulates methylation marks at pancreatic regulatory regions, possibly through interaction with the transcription factor Foxa2. Together, these results demonstrate that Setd7 acts as a central component of the transcription complex initiating the pancreatic program.

**KEY WORDS: Fate specification, Pancreas, Setd7, *Xenopus*, ESC**

## INTRODUCTION

Establishment of cell identity during embryonic development requires the concerted action of transcription factors (TFs) and chromatin regulators in order to activate unique gene expression programs (Chen and Dent, 2014). Distinct epigenetic marks are established during acquisition of developmental competences and certain TFs can promote chromatin modification events required for gene activation by recruiting chromatin-remodelling enzymes to promoters and enhancers (Chen and Dent, 2014; Iwafuchi-Doi and Zaret, 2014; Spitz and Furlong, 2012). It is indeed likely that cell type-specific TFs confer specificity and context-dependent activities to ubiquitous histone-modifying enzymes by recruiting them to selected loci during differentiation. However, the interplay between chromatin modifiers, such as histone methyltransferases and demethylases, and TF networks in early lineage specification remains poorly understood.

The initiation of the pancreatic program from multipotent endodermal progenitors affords an excellent model to study the molecular mechanisms underlying such interplay in vertebrate embryos. Establishment of the pancreatic lineage is orchestrated by a network of TFs, including Pdx1, Ptf1a and Sox9 (Pan and Wright, 2011; Stanger and Hebrok, 2013). Chromatin states at lineage-specific regulatory regions in embryonic endoderm progenitors and in different stages of their descendants to pancreatic fate have been recently mapped (Avrahami and Kaestner, 2012; Wang et al., 2015; Xie et al., 2013; Xu et al., 2011). These studies also enabled the identification of some relevant histone-modifying enzymes that establish chromatin states and control cell fate choices within the endoderm (Avrahami and Kaestner, 2012; Xu et al., 2011). For instance, the histone acetyltransferase P300 and histone methyltransferase EZH2 enzymes have been reported to play a modulatory role within the endoderm, controlling liver versus ventral pancreas fate decisions (Xu et al., 2011). Moreover, histone modifiers are known to work in conjunction with endoderm TFs to drive differentiation within the endoderm (Kartikasari et al., 2013; Wang et al., 2015). Recent observations identified a conserved mechanism in mouse and human during endoderm differentiation whereby two T-box TFs, Tbx3 and Eomes, sequentially team up with the epigenetic modifier Jmjd3 (also known as Kdm6b) to drive stem cell differentiation towards definitive endoderm lineage (Kartikasari et al., 2013). In addition, FOXA factors are well-known endodermal TFs endowed with the remarkable ability to directly affect chromatin states (Cirillo et al., 2002; Iwafuchi-Doi and Zaret, 2014). Specifically, in the anterior endoderm, FOXA factors function as 'pioneer factors', opening the chromatin and providing a recruitment platform for the subsequent binding of other TFs, cofactors, chromatin-modifying and -remodelling enzymes, that culminate in the establishment of cell type-specific transcriptional programs (Cirillo et al., 2002; Iwafuchi-Doi and Zaret, 2014; Wang et al., 2015; Zaret et al., 2008). To date, it is unclear which are the epigenetic modifiers that cooperatively work with these TFs upstream of Pdx1 in endoderm progenitors and how they instruct progenitor cells to activate pancreatic cell fate.

Lysine methylation of histones plays a central role in transcriptional regulation and epigenetic inheritance (Bannister and Kouzarides, 2011; Chen and Dent, 2014; Zhang and Reinberg, 2001). We previously identified the SET domain containing (lysine methyltransferase) 7 (Setd7) (also known as SET9, SET7/9, KMT7) in two independent transcriptome analyses of mouse and *Xenopus* prospective pancreatic progenitors for being abundantly expressed before the induction of *Pdx1* expression (Rodríguez-Seguel et al., 2013; Spagnoli and Brivanlou, 2008). SETD7 was originally characterized as a monomethyltransferase of lysine 4 on histone H3 (H3K4me1) in human cells (Nishioka et al., 2002; Wang et al., 2001). Subsequently, in addition to its chromatin-modifying role, numerous non-histone substrates, including TFs, have been described for Setd7 (Chen et al., 2012; Francis et al., 2005; Keating and El-Osta, 2013; Miller et al., 2008; Zhang and Reinberg, 2001). Importantly, Setd7 has been shown to directly regulate

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transcription through interaction with crucial developmental TFs, such as MyoD or Pdx1, at the promoter or enhancer of target genes (Blum et al., 2012; Deering et al., 2009; Francis et al., 2005; Tao et al., 2011). For instance, *Setd7* is required for MyoD to bind to regulatory regions of muscle genes to activate gene expression and initiate the myogenic differentiation process (Blum et al., 2012; Tao et al., 2011). Consistently, knockdown of *setd7* in zebrafish embryos leads to defects in skeletal muscle formation and myofibril structures (Tao et al., 2011). In adult mammalian pancreatic islets, Setd7 is recruited to a complex with Pdx1 to mediate histone H3 methylation at the insulin gene promoter and recruitment of RNA polymerase II (Deering et al., 2009; Francis et al., 2005), suggesting an important role in normal β-cell function. Despite these diverse substrates and very broad targets, *Setd7* knockout mouse models have no obvious developmental defects and the exact *in vivo* function of *Setd7* remains largely undetermined (Campaner et al., 2011; Kurash et al., 2008; Lehnertz et al., 2011; Maganti et al., 2015).

In this study, we examine the possibility that the epigenetic regulator Setd7 plays a role in establishing pancreatic cell identity during embryonic development, before the β-cells are specified. We show that *Setd7* is expressed in the prospective pancreatic endoderm of *Xenopus* and mouse embryos prior to *Pdx1* induction. Importantly, we demonstrate that *setd7* is sufficient and required for induction of pancreatic genes in *Xenopus* endoderm. Using functional and biochemical approaches in *Xenopus* and mouse endoderm, we show that this developmental function depends on Setd7 histone methyltransferase activity. Consistently, Setd7 modulates methylation marks at pancreatic regulatory regions, possibly through interaction with the endodermal TF Foxa2. Taken together, our data suggest that Setd7 acts as a central component of the TF/chromatin complex initiating the pancreatic program.

## RESULTS

### Expression of Setd7 in the pancreatic endoderm is conserved across species

To start investigating the function of Setd7 in early pancreas development, we first analysed its expression pattern in both *Xenopus laevis* and mouse embryos (Fig. 1, Fig. S1A). The early developmental processes and molecular pathways that culminate in the induction of the pancreas are highly conserved across vertebrate species (Afelik et al., 2006; Horb and Slack, 2001; Kelly and Melton, 2000; Pearl et al., 2009). Fate map experiments in *Xenopus* have previously shown that pancreas progenitors arise from the anterior endoderm (AE), while posterior endoderm (PE) forms mainly intestine (Chalmers and Slack, 2000; Horb and Slack, 2001; Kelly and Melton, 2000). Importantly, we found higher levels of *setd7* expression in *Xenopus* AE compared with PE at all stages analysed, which mirrors the expression pattern of well-known AE TFs, such as *foxa2*, and of the pancreatic genes *pdx1* and *ptf1a* (Fig. 1A, Figs S1C-E and S2B). Furthermore, *in situ* hybridization analysis showed expression of *setd7* in the pancreatic endoderm at early tadpole stage, overlapping with the expression of *pdx1* and *insulin* (Fig. 1B-D). Subsequently, *setd7* continued to be expressed in the pancreas after fusion of the two buds, displaying a punctate staining throughout the whole tissue (Fig. 1E). Similarly, in the mouse embryo, *Setd7* was detected in E8.5 foregut endoderm progenitors and its expression levels increased with time in pancreatic progenitors (Fig. S1B), which is consistent with our previous RNA-seq analysis (Rodríguez-Seguel et al., 2013). Immunofluorescence (IF) analysis showed that Setd7 colocalizes with Foxa2 in the foregut endoderm of E8.5 mouse embryos (Fig. 1F) and with Pdx1 in pancreatic progenitors at E10.5 (Fig. 1G). Subsequently, starting from E12.5, Setd7 exhibited distinct subcellular localizations within the pancreatic epithelium (Fig. 1H,I). Specifically, Setd7 nuclear localization was maintained only in delaminating endocrine progenitor cells, which have low levels of E-cadherin and Pdx1 (Pan and Wright, 2011) (Fig. 1H,H′), whereas in the other pancreatic progenitor cells it displayed a cytoplasmic localization (Fig. 1H,I) that resembled the

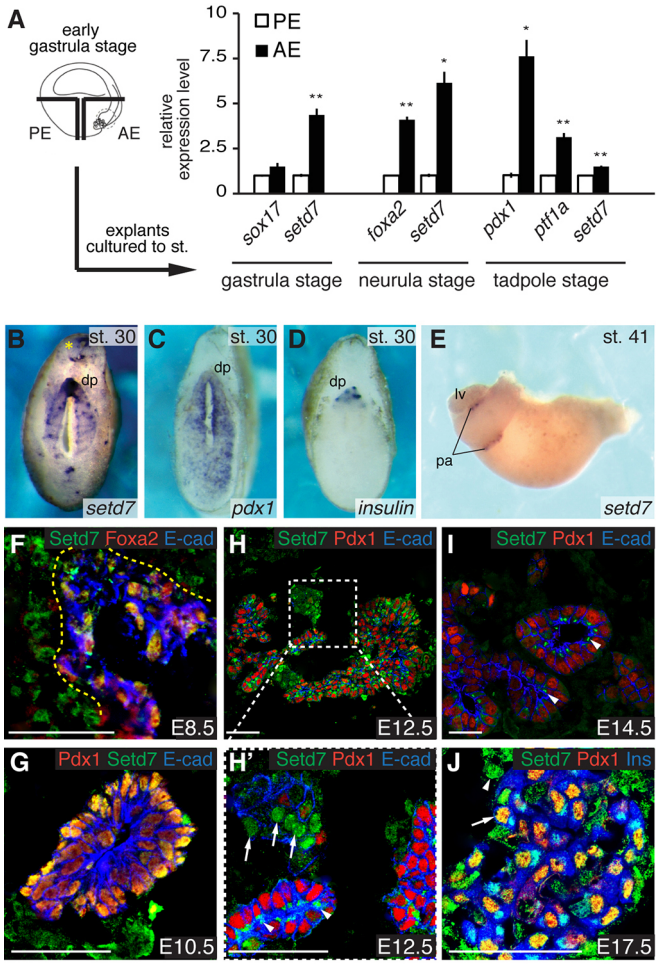


**Fig. 1. Expression of *Setd7* in the developing pancreas is conserved across vertebrate species.** (A) RT-qPCR analysis of indicated transcription factors in anterior (AE) and posterior (PE) endodermal explants isolated from gastrula stage (st.) *Xenopus* embryos and cultured *ex vivo* until indicated stages. AE but not PE gives rise to pancreatic endoderm that expresses *foxa2*, *pdx1* and *ptf1a* (Fig. S2) (Kelly and Melton, 2000; Horb and Slack, 2001; Spagnoli and Brivanlou, 2008). Data were normalized to that of *odc* and represented as fold change compared with PE sample (set to 1). *n*=4; error bars represent ±s.e.m. **P*<0.05, ***P*<0.01. (B-E) *In situ* hybridization analysis of *setd7* (B,E), *pdx1* (C) and *insulin* (D) expression in cross-sectioned *Xenopus* tadpole (st. 30) embryos and dissected gut at st. 41 (E). Yellow star (*) indicates *setd7* expression in the neural tube. dp, dorsal pancreatic bud; lv, liver bud; pa, pancreas. (F-J) Immunofluorescence analysis of Setd7 in the mouse endoderm and pancreas. Setd7 colocalizes with Foxa2 in the foregut endoderm at E8.5 (8- to 9-somite stages) (F; dotted line demarcates the ventral foregut epithelium) and with Pdx1 in pancreatic progenitors at E10.5 (G). At E12.5 and 14.5 (H,H′,I), Setd7 is retained in the cytoplasm of pancreatic epithelium (arrowheads) and nuclear localization is detected only in delaminating cells (arrows). At E17.5 (J), Setd7 protein colocalizes with Pdx1 and insulin (Ins) in β-cell progenitors (arrows) clustering into islets. Arrowheads indicate Setd7 nuclear staining in neighbouring islet cells. Scale bars: 50 µm.

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localization pattern of the apical Golgi marker GM130 (Fig. S1G). At E17.5, when endocrine cells begin to cluster into islets, nuclear Setd7 staining was detected in β-cells positive for insulin and Pdx1 as well as in adjacent islet cells that were negative for both insulin and Pdx1 (Fig. 1J), but absent in the acini (Fig. S1H). This is in line with previous observations showing Setd7 nuclear localization in adult mouse and human pancreatic islet cells (Deering et al., 2009). Taken together, our results indicate that Setd7 is expressed in the developing pancreas of both mouse and *Xenopus* embryos from very early stages, suggesting a potential evolutionarily conserved function in pancreatic progenitor cells.

### Setd7 is required to activate the pancreatic transcriptional cascade in the endoderm

To date, no specific function has been assigned to Setd7 in the endoderm and early pancreas development. To address this open question, we first performed gain-of-function experiments using *Xenopus* endodermal explants as an embryological assay (Fig. 2, Fig. S2). Injection of *Xenopus setd7* mRNA into PE induced the expression of typical AE TFs, such as *foxa2* and *prox1*, and expression of the pancreatic genes *pdx1*, *ptf1a*, *sox9* and *insulin* in PE cells that are normally devoid of pancreatic markers (Fig. 2A, Fig. S2B) (Kelly and Melton, 2000). These changes were accompanied by a slight induction of the gut surrounding mesodermal gene *tbx5* (Fig. 2A, Fig. S2E) (Horb and Slack, 2001). By contrast, the level of expression of the hepatic markers *hhex* and *for1* was not changed by ectopic *setd7* expression (Fig. 2A). Moreover, the mouse *Setd7* homologue displayed a similar activity when injected in PE explants, inducing AE and pancreatic genes at levels that appear even higher than that of *Xenopus* mRNA (Fig. 2D).

Notably, ectopic expression of *pdx1* and *ptf1a* was also detected along the gut of *setd7*-injected embryos in addition to their endogenous expression by whole-mount *in situ* hybridization analysis (Fig. 2B,C). These results suggest that *setd7* exerts a modifier activity within the endoderm, promoting pancreatic fate.

Next, to address the *in vivo* function of *setd7* in pancreas formation, we designed a knockdown strategy based on a specific antisense morpholino oligonucleotide against the *Xenopus laevis* gene (referred to as setd7-MO). To block *setd7* mRNA translation specifically within the territory where the pancreas is formed, we injected setd7-MO into the AE of *Xenopus* embryos at the 4- to 8-cell stage (Fig. S3). By whole-mount *in situ* hybridization, we observed severe reduction of *pdx1* and *ptf1a* expression in both pancreatic rudiments of setd7-MO-injected embryos compared with control embryos, whereas expression of *ptf1a* in the eye and hindbrain was not affected (Fig. 3A-D, Fig. S3). Similarly, the expression levels of the AE TFs *foxa2*, *prox1*, *hnf1b* as well as of the pancreatic genes *pdx1*, *ptf1a* and *sox9* were reduced in endodermal explants depleted of *setd7* in a dose-dependent manner (Fig. 3G). By contrast, the *hhex* expression domain was unchanged in setd7-MO-injected embryos and a reduction in expression was measured by RT-qPCR only at high MO dose, which also led to altered gut regionalization, as evidenced by reduction of expression of *intestinal fatty acid binding protein* (*ifabp*), and concomitant induction of the anterior stomach marker *sox2* (Fig. 3E-G, Fig. S3G) (Chalmers and Slack, 2000; Horb and Slack, 2001). Finally, at high dose of setd7-MO, a reduction in *insulin* expression also became apparent (Fig. 3G). Injection of the same dose of a 5 bp mismatch setd7-5MM-MO did not perturb pancreas formation, providing evidence of morpholino specificity (Fig. S4A) (Eisen and Smith,

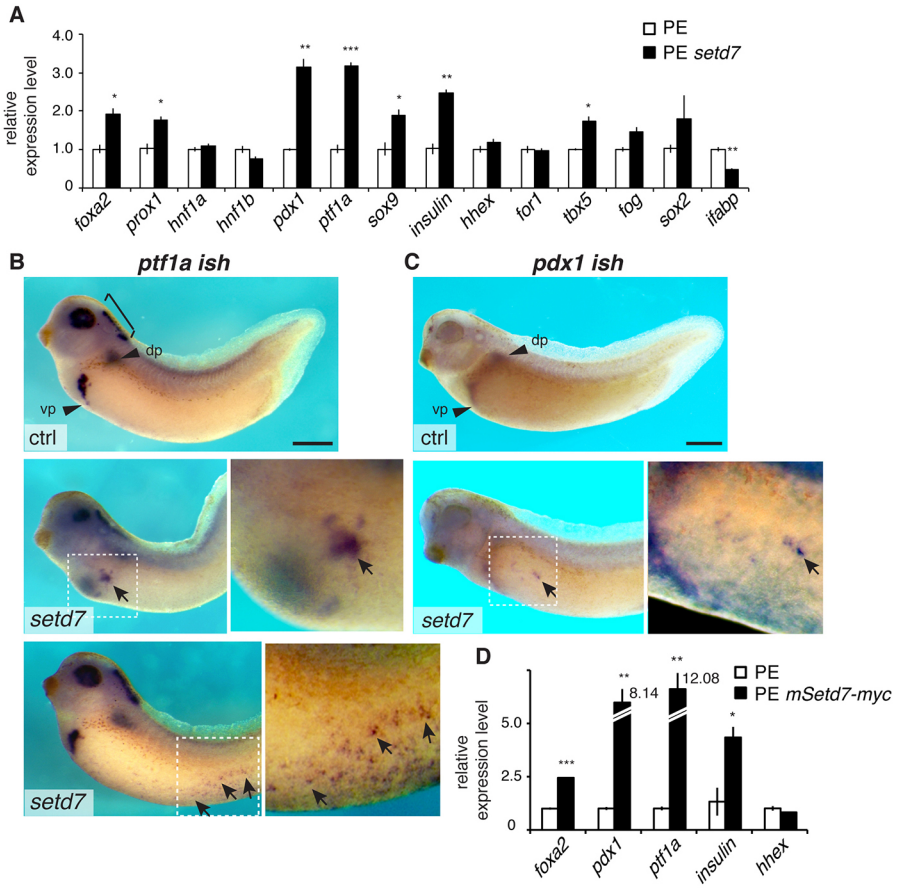


**Fig. 2. *Setd7* promotes pancreatic fate in the endoderm.** (A) RT-qPCR analysis of indicated genes in *Xenopus* PE explants injected with *Xenopus setd7* mRNA (1 ng). Embryos were injected into PE region at 4- to 8-cell stage, dissected at early gastrula stage, cultured until early tadpole stage and assayed for indicated genes; *n*=10. (B,C) Whole-mount ISH analysis of *ptf1a* (B) and *pdx1* (C) in uninjected (ctrl) and *setd7* mRNA-injected (1 ng) *Xenopus* embryos. Arrowheads indicate dorsal (dp) and ventral pancreatic (vp) buds. Brackets indicate *ptf1a* expression in the eye and hindbrain. Arrows indicate ectopic *ptf1a* and *pdx1* expression in the endoderm of *setd7*-injected embryos. Insets show higher magnification of regions indicated by dashed frame. Scale bars: 1 mm. (D) RT-qPCR analysis of indicated genes in *Xenopus* PE explants injected with mouse *Setd7*-Myc mRNA. All explants were assayed at tadpole stage; *n*=6. In A and D, data were normalized to that of *odc* and represented as fold changes compared with uninjected PE sample (set to 1). Error bars represent ±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001.

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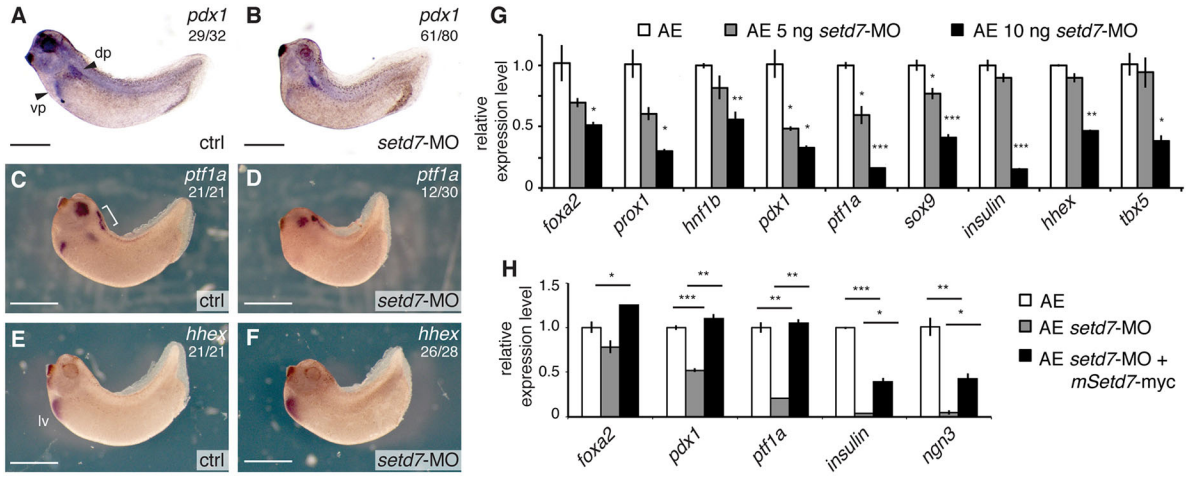


**Fig. 3. *Setd7* is required for specification of pancreatic progenitor cells.** (A-F) Whole-mount ISH analysis of *pdx1* (A,B), *ptf1a* (C,D) and *hhex* (E,F) in uninjected (ctrl) and setd7-MO-injected (5 ng) *Xenopus* embryos. Arrowheads indicate dorsal (dp) and ventral pancreatic (vp) buds. Brackets indicate *ptf1a* expression in the eye and hindbrain. Scale bars: 1 mm. (G) RT-qPCR analysis of indicated genes in *Xenopus* AE explants injected with increasing doses of setd7-MO. *n*=10. (H) RT-qPCR analysis shows that co-injection of mouse *Setd7* mRNA (1 ng) together with setd7-MO in AE restores the expression of indicated endodermal and pancreatic genes; *n*=4. Data were normalized to that of *odc* and represented as fold changes compared with AE samples (set to 1). Error bars represent ±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001.

2008). Furthermore, we performed rescue experiments that provide the most stringent test of specificity for MO-based knockdown experiments (Blum et al., 2015; Eisen and Smith, 2008). We used mouse *Setd7* mRNA, which is not targeted by setd7-MO, and showed that its ectopic expression fully rescued the expression of AE and pancreatic genes, such as *foxa2*, *pdx1*, *ptf1a*, upon setd7-MO knockdown, whereas *insulin* and *ngn3* expression was restored only to some extent (Fig. 3H, Fig. S4B). Overall, these results suggest that *setd7* is not only sufficient to induce pancreatic gene expression but is also required for establishment of pancreatic identity in the endoderm.

### Setd7 histone methyltransferase activity is required for pancreatic fate specification

Next, to address if Setd7 pancreatic fate induction is dependent on its histone methyltransferase activity, we used a Setd7 mutant variant, in which substitution of a single amino acid (H297A) within the SET domain results in the loss of enzymatic activity (Nishioka et al., 2002). This variant was previously reported to function as a dominant-negative mutant (Nishioka et al., 2002). In line with the setd7-MO phenotype, we observed reduction of the expression domain of *pdx1* and *ptf1a*, but no changes in *hhex* expression in *setd7*-H297A-injected embryos compared with uninjected embryos (Fig. 4A, Fig. S5). Moreover, RT-qPCR of AE explants isolated from *setd7*-H297A-injected *Xenopus* embryos confirmed the reduction in expression of pancreatic genes, such as *pdx1*, *ptf1a* and *insulin*, while *hhex* and *for1* expression levels were unchanged (Fig. 4B). Consistent with the setd7-MO knockdown phenotype, we found altered regional gene expression of well-known gut markers, such as *ifabp* and *sox2*, upon setd7-H297A injection (Fig. 4B). Together, these data corroborate the functional requirement of Setd7 for pancreas induction within the endoderm and suggest that its methyltransferase activity is crucial for this role.

### Setd7 affects epigenetic marks at pancreatic regulatory regions

Setd7 has been reported to act as a H3K4 methyltransferase, regulating transcriptional activation by interacting with tissue-specific TFs (Blum et al., 2012; Deering et al., 2009; Francis et al., 2005; Keating and El-Osta, 2013; Tao et al., 2011). We first examined the global cellular levels of H3K4me1, H3K4me3 and H3K27ac by western blot (WB) analysis of lysates from *setd7* mRNA- and setd7-MO-injected *Xenopus* embryos (Fig. 5A). Overall, we detected no change in global H3K4 methylation levels after setd7-MO knockdown but only a slight increase in H3K4me1 levels upon *setd7* ectopic expression (Fig. 5A). Next, to address if specific histone H3 modifications occurred at regulatory regions of essential pancreatic developmental genes, we carried out chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) experiments in *Xenopus* and mouse embryonic stem cell (ESC) model systems. *Xenopus laevis* pancreatic promoter and enhancer regions were identified based on: (1) sequence homology with well-characterized zebrafish, mouse and human regulatory regions and (2) published ChIP-seq datasets for active promoter chromatin marks, such as H3K4me3, and enhancer-related marks H3K4me1 and H3K27ac (Akkers et al., 2012; Gupta et al., 2014; Wills et al., 2014) (Table S1). Regulatory regions of hepatic genes, such as the *hhex* promoter, were previously characterized in *Xenopus laevis* (Rankin et al., 2011) and used here as control. Briefly, endoderm explants were dissected from embryos injected with setd7-MO, cultured until early tadpole stage and processed for ChIP experiments (Fig. 5B). Chromatin prepared from endoderm explants was incubated with antibodies against H3K4me1, H3K4me3, H3K27ac and GFP, as negative control, and immunoprecipitates were analysed by PCR with primers specific for *Xenopus* pancreatic enhancer and promoter regions. As expected, H3K4me3 occupancy was primarily localized to promoter regions but absent at enhancer regions in all samples analysed (Fig. S6A). By contrast, H3K4me1 and H3K27ac were enriched on both pancreatic enhancer and promoter regions (Fig. S6). Notably, we did not observe any enrichment of GFP at any tested region (Fig. S6A,C). Upon *setd7* knockdown, H3K4me1 occupancy remained mostly unchanged, except for a slight increase in the context of the *pdx1* and *ptf1a* enhancer sequences compared with the control (Fig. 5C). By contrast, a strong decrease in H3K4me3 was detected specifically at pancreatic promoter regions upon depletion of Setd7, but not at pancreatic enhancers or hepatic promoter (Fig. 5C). Finally, the H3K27ac signal was decreased at

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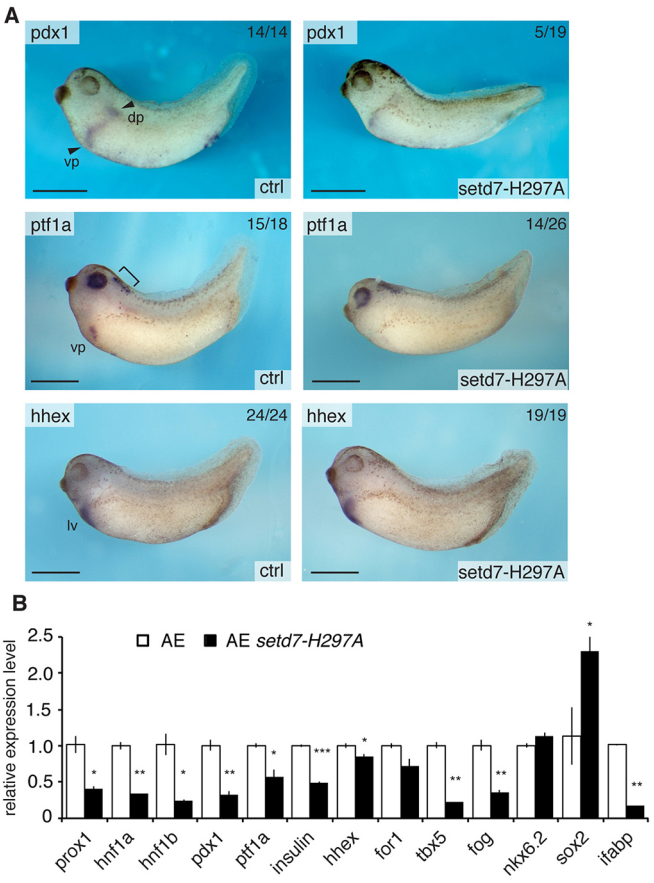


**Fig. 4. Setd7-H297A histone methyltransferase mutant lacks pancreatic fate-inducing activity.** (A) Whole-mount ISH of *pdx1*, *ptf1a* and *hhex* in uninjected (ctrl) and *setd7*-H297A-injected (1 ng) *Xenopus* embryos. Arrowheads indicate dorsal (dp) and ventral pancreatic (vp) buds. Brackets indicate *ptf1a* expression in the eye and hindbrain. Scale bar: 1 mm. lv, liver. (B) RT-qPCR analysis of *setd7*-H297A-injected AE explants for indicated markers. Data were normalized to that of *odc* and represented as fold changes compared with uninjected AE sample (set to 1). $n$=8; error bars represent ±s.e.m. *$P$<0.05, **$P$<0.01, ***$P$<0.001.

most of the tested pancreatic regulatory regions (Fig. 5C). Together, these results suggest that Setd7 affects histone H3 modifications in *Xenopus* pancreatic endoderm. Enrichment of H3K4me1, H3K4me3 and H3K27ac at regulatory regions is associated with active gene transcription (Heintzman et al., 2007). Thus, the decrease of H3K4me3 and H3K27ac at pancreatic promoters in setd7-MO endoderm is in line and might explain the reduction of pancreatic gene expression observed in *setd7* loss-of-function experiments (Figs 3 and 4).

To analyse whether the mammalian homolog of Setd7 has a conserved function and associates with chromatin at regulatory regions of pancreatic-specific genes, we performed ChIP analysis in mouse ESCs differentiated into pancreatic progenitor cells. We used the ESC system to model *ex vivo* endoderm and pancreas development. In particular, to induce pancreatic specification, mESCs were stimulated using a step-wise protocol adapted from previously published studies (D'Amour et al., 2006; Nostro et al., 2011; Rodríguez-Seguel et al., 2013). RT-qPCR analysis showed downregulation of pluripotency genes and upregulation of *Foxa2* and *Pdx1* in pancreatic endoderm (PaE) differentiated cells when compared with undifferentiated mESCs (Fig. 5D). Importantly, the levels of *Setd7* gradually increased when mESCs were induced to differentiate into definitive endoderm (DE) and PaE (Fig. 5D). ChIP analysis showed increased RNA polymerase II (PoI II) binding at the promoter regions of *Foxa2*, *Eomes* and *Pdx1* in PaE differentiated cells compared with mESCs, indicating active gene transcription, which is in line with the RT-qPCR result (Fig. 5D,E). By contrast, IgG control ChIP did not show any enrichment at those regulatory regions after differentiation (Fig. 5E). Moreover, promoters of key endodermal and pancreatic genes, such as *Eomes*, *Foxa2*, *Pdx1* and *Sox9*, displayed a gain in the H3K4me3 active mark upon ES differentiation into PaE (Fig. 5E). In parallel, we examined Setd7 binding on the same promoter regions by performing ChIP experiments on chromatin isolated from mESCs and PaE progenitors. Previous observations reported selective occupancy of Setd7 at *Foxa2* and *Eomes* promoters in mouse and human ESCs differentiated to DE (Kartikasari et al., 2013). We found that Setd7 not only occupies *Foxa2* and *Eomes* promoters but also the pancreatic *Pdx1* promoter in differentiated PaE, while it is absent from *Sox9* promoter (Fig. 5E). Taken together, these results indicate that the histone methyltransferase Setd7 binds to pancreatic promoters and exerts a conserved epigenetic regulatory activity during early pancreatic specification.

### Setd7 interacts with the TF Foxa2

Epigenetic modifiers are known to team up with lineage-specific TFs to drive cell differentiation (Chen and Dent, 2014; Kartikasari et al., 2013; Spitz and Furlong, 2012). Specifically, Setd7 was reported to interact with MyoD in differentiating myoblasts to activate the myogenic differentiation process (Blum et al., 2012; Tao et al., 2011) as well as with T-box TFs in cardiac progenitors (Chen et al., 2012; Miller et al., 2008). In the anterior endoderm, *Foxa* genes are expressed prior to *Pdx1* and disruption of *Foxa1* and *Foxa2* causes loss of *Pdx1* expression in the developing pancreas and concomitant reduction of other key transcriptional regulators (Fujitani et al., 2006; Gao et al., 2008; Melloul et al., 2002; Wu et al., 1997). We showed that Setd7 colocalizes with Foxa2 in the foregut endoderm and binds to regulatory regions of endodermal and pancreatic genes (Fig. 1, Fig. S1, Fig. 5). We then asked if Setd7 directly interacts with Foxa2 TF during early pancreatic lineage specification. Extracts of *Xenopus* embryos injected with Myc-tagged *setd7* and *foxa2* mRNAs were immunoprecipitated (Fig. 5B). Precipitation with anti-Myc antibody resulted in co-precipitation of Foxa2, indicating that Setd7 does indeed bind to Foxa2 in endodermal cells (Fig. 5F). Notably, anti-Myc antibody was also able to co-precipitate endogenous Foxa2, even though to a lesser extent (Fig. 5F). Moreover, we found that endogenous Setd7 and Foxa2 proteins interact in mESCs induced to differentiate into DE and PaE (Fig. 5F). In conclusion, these data indicate that Setd7 and Foxa2 physically interact in *Xenopus* and mouse endoderm, suggesting that this interaction contributes to the regulation of pancreatic gene expression and pancreas specification.

## DISCUSSION

In this study, we identify an early function for the histone methyltransferase Setd7 in pancreatic fate specification, prior to Pdx1. Conserved expression pattern of *Setd7* in vertebrate endoderm together with functional assays suggest that *Setd7* regulates the activation of a pancreatic-specific transcriptional program in the endoderm. *Setd7* is required for the establishment of active histone marks at pancreatic promoters and occupies H3K4me3-enriched endodermal and pancreatic promoters in ES cells differentiated into pancreatic progenitors. Collectively, our findings support a model in which interaction of Setd7 with the TF

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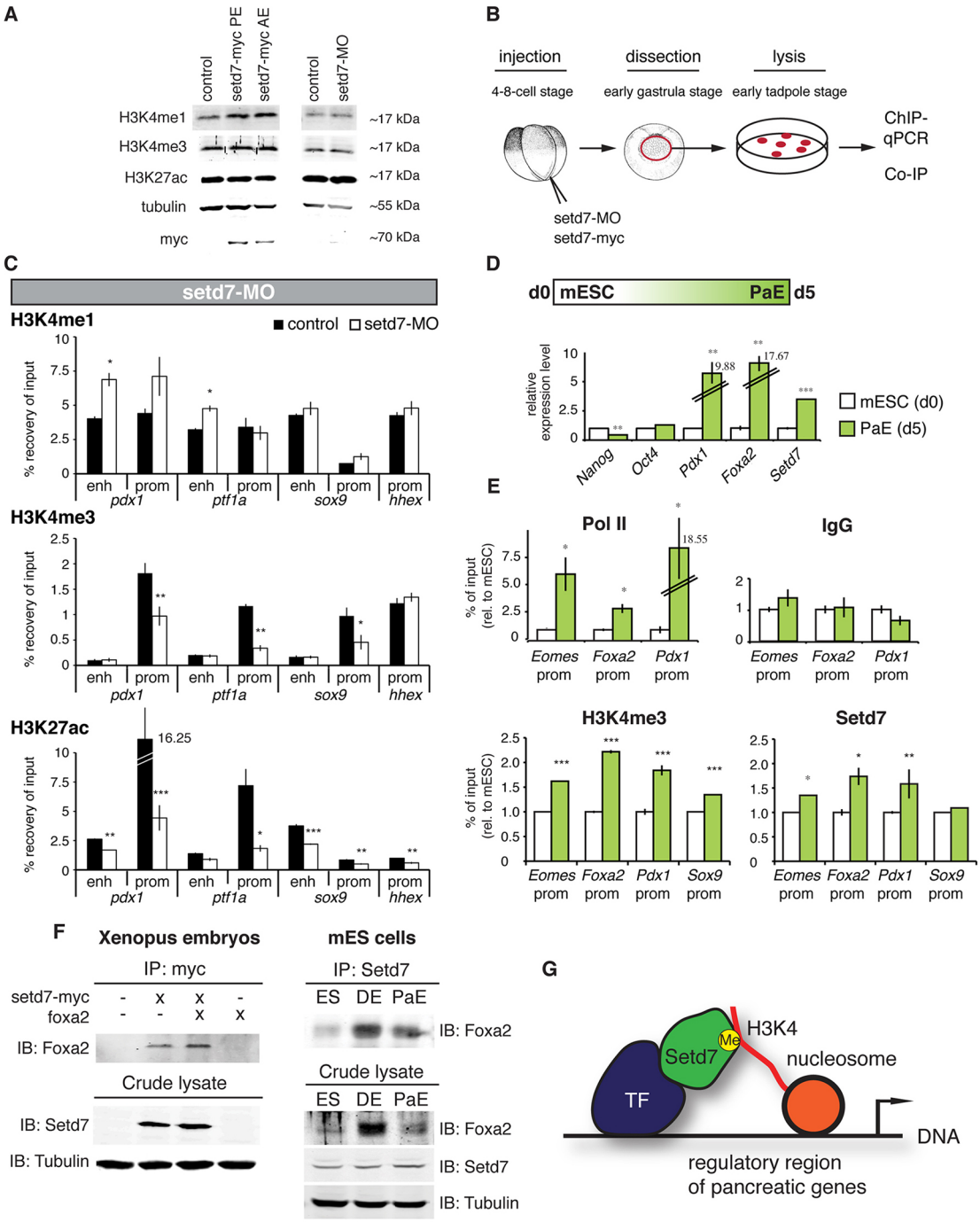


**Fig. 5. Epigenetic changes upon modulation of Setd7 levels.** (A) Global protein levels of H3K4me1, H3K4me3 and H3K27ac in *setd7-myc* (1 ng)- or setd7-MO (5 ng)-injected *Xenopus* lysates were determined by western blot. (B) Schematic illustration of sample preparation for ChIP-qPCR or Co-IP using *setd7* mRNA- or setd7-MO-injected endodermal explants. (C) ChIP experiments were performed from setd7-MO-injected and uninjected (control) *Xenopus* explants with the indicated histone modification antibodies. ChIP material was analysed by qPCR for selected pancreatic and hepatic regulatory regions. Data were normalized to % of input (*y*-axis). *n*=6, error bars represent ±s.d. **P*<0.05, ***P*<0.01, ****P*<0.001. (D) *Ex vivo* differentiation of mESCs into PaE. RT-qPCR showing expression levels of *Setd7*, pluripotency markers and pancreatic genes in PaE cells at day (d) 5 relative to undifferentiated mESCs at d0. Data were normalized to *Sdha* and represented as fold changes compared with mESC samples (set to 1). Error bars represent ±s.e.m. ***P*<0.01, ****P*<0.001. PaE, pancreatic endoderm. (E) ChIP experiments were performed on mESCs chromatin with the indicated antibodies. ChIP material was analysed by qPCR for selected endodermal and pancreatic regulatory regions. Data were normalized to % of input (*y* axis) and represented as fold changes compared with mESC undifferentiated samples (set to 1). *n*=4; error bars represent ±s.d. **P*<0.05, ***P*<0.01, ****P*<0.001. (F) Immunoprecipitation (IP) of Setd7 and Foxa2. Left panel, 4-cell st. embryos were injected with *Xenopus setd7-myc*, lysates were prepared at tadpole st., immunoprecipitated with anti-Myc antibody and analysed by immunoblot (IB) with anti-Foxa2 antibodies. Ectopic Setd7-Myc was checked by IB on the crude extracts used for the IP reaction. Right panel, IP of endogenous Setd7 and Foxa2 in differentiated mESCs. Lysates were prepared from undifferentiated mESCs and definitive endoderm (DE) and PaE cells, immunoprecipitated with anti-Setd7 antibody and analysed by IB with the indicated antibodies. Expression of Setd7 and Foxa2 was checked by IB on the crude extracts used for the IP reaction. As a loading control, all membranes were stripped and reprobed with anti-α-tubulin. (G) Model for Setd7-mediated activation of pancreatic genes. The model suggests that Setd7 is co-recruited by a cell-specific TF, such as Foxa2, to promoters of pancreatic genes, such as Pdx1, where it is required for the establishment of H3K4me3.

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Foxa2 might mediate chromatin recruitment of Setd7 to the *Pdx1* promoter, which, in turn, results in H3K4me3 modification and activation of *Pdx1* gene expression (Fig. 5G). Importantly, similar TF-mediated co-recruitment of Setd7 to lineage-specific promoters has been shown in various cell types (Blum et al., 2012; Francis et al., 2005; Keating and El-Osta, 2013; Tao et al., 2011), representing a unifying mechanism by which this epigenetic modifier instructs lineage choices and differentiation. A similar interaction might also be required for auto-regulation of the *Foxa2* promoter and other Foxa2-dependent genes. Finally, co-recruitment of Setd7 by TFs to specific promoters might explain how Setd7, which has neither a canonical nuclear localization signal nor nuclear export signals and has been reported to localize to both cytoplasm and nucleus by us and others (Keating and El-Osta, 2013) (Fig. 1), regulates gene expression and determines lineage specification. Conversely, because Foxa TFs cannot directly methylate histones (Iwafuchi-Doi and Zaret, 2014; Lupien et al., 2008; Wang et al., 2015), it is likely that its interaction with epigenetic regulators, such as Setd7, enables histone modification events in the context of pancreatic lineage specification.

Mono-methylation of H3K4 at enhancers can be used to determine global cell type-specific gene expression, whereas tri-methylation of H3K4 in association with H3K27ac are characteristics of active promoters (Creyghton et al., 2010; Heintzman et al., 2007, 2009; Rada-Iglesias et al., 2011). Our results suggest that Setd7 is required for the establishment of H3K4me3 modification at promoters instead of H3K4me1 at enhancers in the *Xenopus* endoderm. Enrichment of Setd7 at promoter regions of various endodermal genes (Kartikasari et al., 2013), at the *insulin* promoter (Francis et al., 2005) and MyoD-dependent promoters (Tao et al., 2011) has been previously reported. In addition, several studies suggest that Setd7 acts as monomethylase of H3K4 only *in vitro*, but little or no enzymatic activity has been shown when nucleosomal H3 is used as substrate (Keating and El-Osta, 2013; Lezina et al., 2014; Nishioka et al., 2002; Wang et al., 2001). Finally, depletion or knockdown of Setd7 does not affect the global cellular levels of H3K4me1 marks in mouse (Campaner et al., 2011; Keating and El-Osta, 2013; Lehnertz et al., 2011) or frog embryos (Fig. 5), respectively. Thus, these observations have raised the possibility that another lysine methyltransferase, instead of Setd7, might maintain nucleosomal H3K4me1 patterns at enhancers or, maybe, works synergistically with Setd7. An alternative explanation might be that Setd7 exerts different catalytic activities in a cell type- or cell context-dependent manner. It is also conceivable that Setd7-mediated H3K4me1 at promoters is required for subsequent di- or tri-methylation by other lysine methyltransferase (KMT) enzymes. For instance, Setd7 appears to function synergistically with Smyd3, a KMT that uses H3K4me1 and H3K4me2 as substrate to establish H3K4me3, during heart development in zebrafish (Kim et al., 2015). On the other hand, other studies associated Setd7 itself with the establishment of di- and tri-methylation (Dhayalan et al., 2011; Francis et al., 2005; Maganti et al., 2015). Finally, acetylation of H3K27 by p300 is facilitated by Setd7-mediated H3K4 methylation (Wang et al., 2001), which might explain the observed reduction of H3K27ac upon setd7-MO knockdown.

We show that *setd7* is required for induction of pancreatic fate and pancreatic gene expression in *Xenopus* endoderm. Yet, the requirement and possible function(s) of *Setd7* during mammalian pancreatic development have to be assessed. *Setd7*-knockout mice are viable and without any overt phenotypes (Campaner et al., 2011; Kurash et al., 2008; Lehnertz et al., 2011), although neither the embryonic pancreas nor adult pancreas has been analysed in these mutants. In addition, there are overall major discrepancies among the phenotypes reported in these three different *Setd7* mouse models (Campaner et al., 2011; Kurash et al., 2008; Lehnertz et al., 2011) that might be due to differences in the mouse genetic background or targeting strategies used. More recently, a tamoxifen-inducible β-cell-specific *Setd7* knockout mouse was established using a gene targeting strategy that was different to those used before (Maganti et al., 2015). In this study, specific ablation of *Setd7* in adult pancreatic β-cells results in glucose intolerance and β-cell dysfunction that is reminiscent of *Pdx1* heterozygous mouse mutation and parallels previous *in vitro* observations in isolated islets (Brissova et al., 2002; Deering et al., 2009; Maganti et al., 2015). Importantly, these findings suggest a late role for *Setd7* in the maintenance of key *Pdx1* target genes and *Pdx1* itself (Maganti et al., 2015); whether *Pdx1* is also dependent upon the action of *Setd7* at earlier stages in the mouse developing pancreas, as we observed in the *Xenopus*, deserves further investigation. Interestingly, upon depletion of Setd7 in the frog endoderm, the reduction of pancreatic gene expression appears concomitant with changes in regional gene expression of well-known gut markers, such as *sox2*, and of some gut-type mesoderm, marked by *tbx5* (Chalmers and Slack, 2000; Horb and Slack, 2001), suggesting a broader role for Setd7 in regional specification and patterning of the endoderm. Ultimately, future studies are also required to address if there are differences or discrepancies between mutant and morphant phenotypes and the reasons underlying potential phenotypic differences. For instance, in zebrafish, it has been shown that the activation of compensatory networks can buffer against some deleterious mutations, which instead does not occur after translational or transcriptional knockdown (Rossi et al., 2015).

Both TFs and epigenetic modifications, and their interplays regulate cell fate decisions and define cell identity (Chen and Dent, 2014). Our study provides insights into the mechanisms governing these interactions to control pancreatic gene expression. Further understanding of these processes and elucidation of the function of chromatin-modifying enzymes are key for acquiring precise control of the mechanisms driving cell fate transitions. Indeed, relevant chromatin-modifying enzymes might serve as novel pharmacological targets to enhance stem cell programming or reprogramming approaches to pancreas cell fate. Their addition to current strategies might prove tremendously beneficial for the ultimate goal of generating a renewable source of pancreatic β-cells.

## MATERIALS AND METHODS

### *Xenopus* embryos experiments

*Xenopus* embryo manipulations and dissections were performed as described (Spagnoli and Brivanlou, 2008). *Mus musculus Setd7* clone was purchased from Source BioScience and its ORF cloned into pCS2+MT and pGEM-T easy. Full-length *Xenopus laevis setd7* was cloned based on homology sequence to the *X. tropicalis* cDNA (NM_001032339) and its ORF sub-cloned into pCS2++, pCS2+MT and pGEM-T easy plasmids. *Xenopus laevis setd7* NCBI accession number is KX495235. *Xenopus setd7* locus is on chromosome 1, alloallele 1L:53930670-53952383. A sequence with multiple mismatches (79% similarity, 14.5% gaps) was found on alloallele 1S: 49194482-49201531. *Xenopus* setd7-H297A was generated by site-directed mutagenesis (Quick-Change II kit, Agilent). mRNAs for all these constructs were synthesized on linearized templates (using *Asc*I or *Bst*XI) with SP6 RNA polymerase using the mMessage mMachine Kit (Ambion). Morpholino antisense oligonucleotides used in this study for *X. laevis* setd7 (5′-CCACTGTTTCATCCTCGCTGTCCAT-3′) and 5-mispair control (5′-CCAgTcTTTgATCCTCcCTcTCCAT-3′) were purchased from GeneTools LLC.

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### RT-qPCR analysis

RT-PCR was performed as described (Spagnoli and Brivanlou, 2008). Real-time PCR reactions were carried out using the SYBR Green Master Mix (Roche) on ABI StepOnePlus. A standard of 40 cycles was performed. Ornithine decarboxylase (*odc*) was used as a reference gene for *Xenopus* samples. Succinate dehydrogenase (*Sdha*) was used as reference gene for mouse samples. All the values were normalized to the reference gene and calculated using the $2^{-\Delta\Delta Ct}$ method. Data were determined in triplicate. For primer sequences, see Table S1.

### Immunohistochemistry and *in situ* hybridization

Mouse embryos and pancreata were fixed in 4% paraformaldehyde at 4°C from 2 h to overnight. Subsequently, samples were equilibrated in 20% sucrose solution and embedded in OCT compound (Sakura). Cryosections (10 µm) were incubated with TSA (Perkin Elmer) blocking buffer for 1 h at room temperature (RT) and afterwards with primary antibodies at appropriate dilution (Table S2). All confocal images were acquired with an LSM 700 confocal laser-scanning microscope (Zeiss). Whole-mount *in situ* hybridizations in *Xenopus* embryos were performed according to Harland (1991). *In situ* probes were prepared as follow: for *setd7*-pGEM-T (*Xba*I/T3); *pdx1/Xlhbox8*-pGEM-T (*Sca*I/SP6); *hHex*-pKSII (*Bam*HI/T7); *insulin*-pGEM-T (*Sal*I/T7); *ptf1a*-pCRscript (*Not*I/T7) (gift from Marko Horb, Marine Biological Laboratory, Woods Hole, MA, USA).

### Cell culture experiments

Mouse ES cells (R1 mESC line) were maintained on gelatin-coated plates with mouse embryonic fibroblasts (MEFs) in standard mESC medium: DMEM medium (Invitrogen), 2 mM Glutamax (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 15% fetal bovine serum (FBS; PAN), 0.1 mM β-mercaptoethanol (Sigma) and 1000 U/ml leukaemia inhibitory factor (ESGRO). For differentiation, cultures were MEF depleted and seeded in mESC medium at high confluency on gelatin-coated dishes. Monolayer differentiation was carried out as described previously (D'Amour et al., 2006; Nostro et al., 2011; Rodríguez-Seguel et al., 2013). Briefly, DE medium to day 2 consisted of RPMI medium (Invitrogen) and 0.2% FBS supplemented with activin A (50 ng/ml) and Wnt3a (25 ng/ml) at day 1, and activin A at day 2. PE medium to day 5 consisted of RPMI medium and 2% FBS supplemented with Wnt3a (3 ng/ml) and FGF10 (50 ng/ml). All recombinant proteins were purchased from (R&D Systems), unless otherwise stated.

### Immunoprecipitation and western blot analysis

Immunoprecipitation was performed as previously described (Spagnoli and Brivanlou, 2008) with mouse anti-Myc agarose beads (Sigma) and anti-Setd7 (Abcam). Immunoblots were incubated with the following antibodies [anti-Myc (Millipore, 06-549), 1:1000; anti-tubulin (Sigma, T9026), 1:5000; anti-Foxa2 (Santa Cruz, sc-6554), 1:250; anti-Setd7 (Abcam, ab14820), 1:100; anti-H3K4me1 (Abcam, ab8895), 1:1000; anti-H3K4me3 (Abcam, ab8580), 1:1000; anti H3K27ac (Abcam, ab4729), 1:1000; Table S3] and analysed using the LI-COR Odyssey system.

### ChIP-qPCR analysis

ChIP analysis of *Xenopus* whole embryos and explants was done as described (Akkers et al., 2012; Wills et al., 2014). Briefly, endoderm from uninjected and setd7-MO injected *Xenopus* embryos was dissected at early gastrula stage (st. 10) and cultured until early tadpole stage (st. 25), lysed, crosslinked and sonicated using Diagenode Bioruptor (high intensity, for 25 min, each cycle 15 s ON and 15 s OFF, at 4°C). Chromatin of 200 explants (or 100 embryos) was precipitated with the following antibodies: anti-H3K4me1, anti-H3K4me3, anti H3K27ac, anti-GFP (Abcam, ab290) and anti-RNA polymerase II (Diagenode, c15200004) (Table S3). Chromatin extraction was done using phenol-chloroform or Chelex-100 methods. ChIP on differentiated mESCs was done according to Kartikasari et al. (2013). Briefly, cells were crosslinked in 1% formaldehyde for 10 min followed by incubation in 0.125 mM glycine and after lysis sonicated using a Diagenode Bioruptor (high intensity, 27 cycles, each cycle 30 s ON and 30 s OFF). Sonicated chromatin was precipitated with the following antibodies: anti-Setd7, anti-H3K4me3, anti-Foxa2, anti-Eomes, anti-IgG, anti-GFP (Abcam) and anti-RNA polymerase II (Diagenode). Real-time PCR using the SYBR Green Master Mix (Roche) was carried out on an ABI StepOnePlus. Analysis of ChIP-qPCR samples was based on the '% of input' method, relative to control samples (set to 1). Unpaired two-tailed Student's *t*-tests were used to assess statistical significance.

### Statistical tests

All results are expressed as mean±s.d. or s.e.m., as indicated. Each experiment was repeated multiple (≥3) times independently. The significance of differences between groups was evaluated with Student's *t*-test. $P<0.05$ was considered statistically significant.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

F.M.S. conceived and directed the study. J.K. performed most of the experiments. J.Z. assisted with IP and Western blots, ES experiments. F.M.S. and J.K. wrote the manuscript and generated figures. All authors proofread and approved the final manuscript.

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#### Supplementary information

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