

## RESEARCH REPORT

# GATA4 and GATA6 regulate pancreatic endoderm identity through inhibition of hedgehog signaling

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**ABSTRACT**

GATA4 and GATA6 are zinc finger transcription factors that have important functions in several mesodermal and endodermal organs, including heart, liver and pancreas. In humans, heterozygous mutations of either factor are associated with pancreatic agenesis; however, homozygous deletion of both *Gata4* and *Gata6* is necessary to disrupt pancreas development in mice. In this study, we demonstrate that arrested pancreatic development in *Gata4*<sup>fl/fl</sup>; *Gata6*<sup>fl/fl</sup>; *Pdx1:Cre* (pDKO) embryos is accompanied by the transition of ventral and dorsal pancreatic fates into intestinal or stomach lineages, respectively. These results indicate that GATA4 and GATA6 play essential roles in maintaining pancreas identity by regulating foregut endodermal fates. Remarkably, pancreatic anlagen derived from pDKO embryos also display a dramatic upregulation of hedgehog pathway components, which are normally absent from the presumptive pancreatic endoderm. Consistent with the erroneous activation of hedgehog signaling, we demonstrate that GATA4 and GATA6 are able to repress transcription through the sonic hedgehog (*Shh*) endoderm-specific enhancer *MACS1* and that GATA-binding sites within this enhancer are necessary for this repressive activity. These studies establish the importance of GATA4/6-mediated inhibition of hedgehog signaling as a major mechanism regulating pancreatic endoderm specification during patterning of the gut tube.

**KEY WORDS:** GATA4, GATA6, Pancreas, Foregut endoderm, Hedgehog, Mouse

**INTRODUCTION**

The pancreas is a vital organ that functions to regulate digestive processes and glucose homeostasis (Hegyí and Petersen, 2013; Mastracci and Sussel, 2012). In adults, the three major diseases associated with the pancreas are pancreatitis, pancreatic cancer and diabetes. In addition, congenital genetic defects contribute to defective organ development, such as annular pancreas, and pathological conditions, including pancreatic agenesis and neonatal diabetes (Etienne et al., 2012; Rubio-Cabezas and Ellard, 2013).

Pancreas development is initiated in two distinct regions of the foregut endoderm in response to signals derived from adjacent tissues (Chen et al., 2004; Kim and MacDonald, 2002; Kumar et al., 2003; Zaret and Grompe, 2008; Zaret et al., 2008). In particular, several studies have shown that a major function of these signals is to repress hedgehog signaling in the presumptive pancreatic endoderm. In the prospective dorsal pancreatic endoderm, notochord-derived activin and fibroblast growth factor (FGF)

signals are necessary for the inhibition of sonic hedgehog (*Shh*) to allow the induction of bud morphogenesis and the pancreatic transcriptional program (Apelqvist et al., 1997; Hebrok et al., 1998). In the prospective ventral pancreatic endoderm, signals secreted by the lateral plate mesoderm, cardiac mesoderm and septum transversum play important roles in inducing pancreatic versus liver fates; the inhibition of WNT and bone morphogenetic protein (BMP) signaling is necessary for ventral pancreas induction, and FGF induces the local expression of *Shh* to inhibit pancreatic fates in favor of liver lineages (Deutsch et al., 2001; Zaret and Grompe, 2008). As development proceeds, the dorsal and ventral pancreatic buds merge, pancreatic cell specification is initiated and the diverse pancreatic cell types differentiate and proliferate to form the mature functional organ (reviewed by Jorgensen et al., 2007; Pan and Wright, 2011; Pictet and Rutter, 1972).

The GATA regulatory proteins belong to a highly conserved six-member family of zinc finger transcription factors that play essential distinct and overlapping roles during embryonic development, including germ layer specification, organ formation and cell lineage determination. GATA1, GATA2 and GATA3 are important for hematopoiesis, whereas GATA4, GATA5 and GATA6 are important for the development of mesoderm- and endoderm-derived organs, including heart, liver and pancreas (Zhou et al., 2012). *Gata4* null mice die at around embryonic day (E) 9.5 owing to defects in heart morphogenesis (Kuo et al., 1997; Molkenin et al., 1997; Narita et al., 1997) and *Gata6* null mice die before E7.5 due primarily to defects in extra-embryonic endoderm (Koutsourakis et al., 1999; Morrissey et al., 1998). In the pancreas, *Gata4* and *Gata6* have overlapping expression in the pancreatic endoderm, but gradually become expressed in separate domains: *Gata4* becomes restricted to the exocrine compartment and *Gata6* is predominantly expressed in the endocrine compartment (Decker et al., 2006; Ketola et al., 2004). Using a Cre-lox approach, we previously demonstrated that simultaneous deletion of *Gata4* and *Gata6* from pancreatic progenitor cells leads to pancreatic agenesis in newborn mice (Carrasco et al., 2012; Xuan et al., 2012). The importance of GATA4 and GATA6 in human pancreas development has also been highlighted in reports of genetic cases of human pancreatic agenesis: *GATA6* haploinsufficiency contributes to the majority of pancreatic agenesis cases (Lango Allen et al., 2012), and *GATA4* haploinsufficiency has been documented in a small number of patients with pancreatic agenesis (Shaw-Smith et al., 2014).

To characterize the molecular changes underlying GATA4/6-mediated pancreas development, we performed global transcriptome analysis on pancreatic buds isolated from E12.5 embryos from control and *Gata4*<sup>fl/fl</sup>; *Gata6*<sup>fl/fl</sup>; *Pdx1:Cre* (pDKO); *R26R:Tomato* mice. Consistent with impaired pancreas development, the majority of downregulated genes were pancreatic progenitor markers. Surprisingly, however, many of the upregulated genes included a large number of intestinal and gastric

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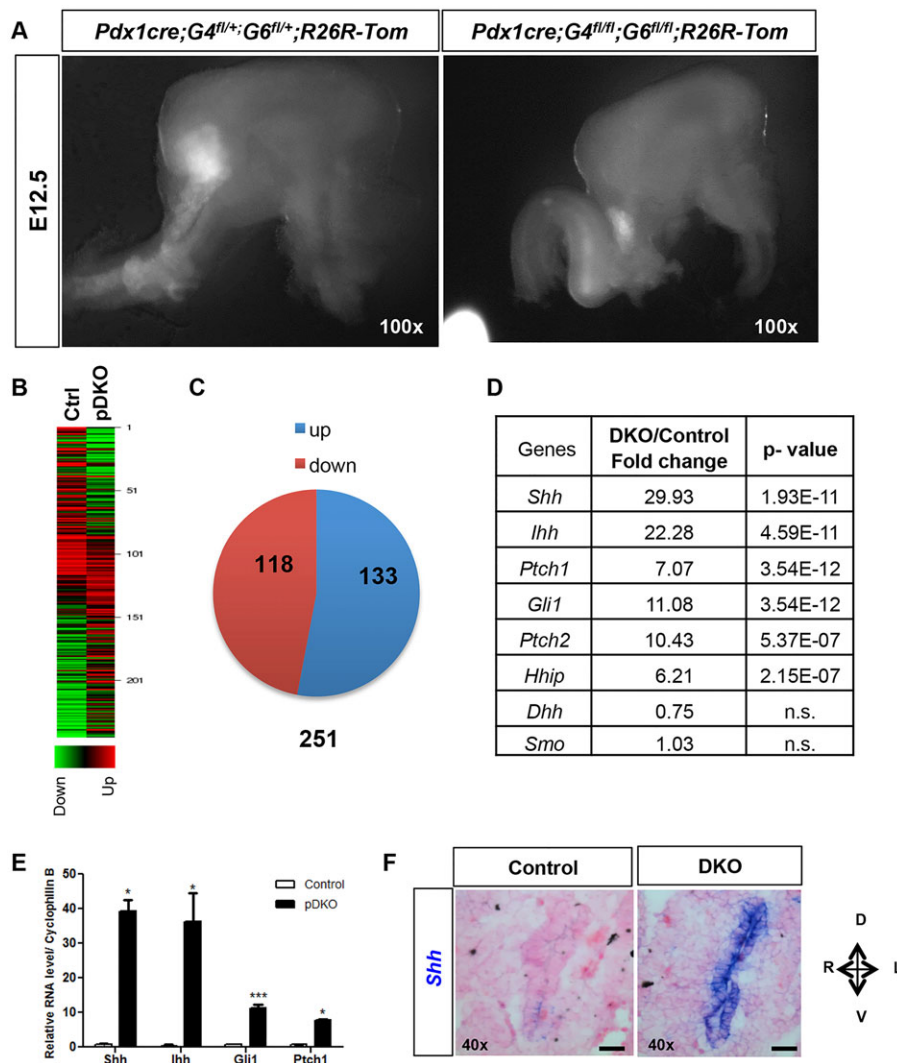
transcription factors. Furthermore, genetic lineage tracing of cells expressing the pancreatic progenitor marker *Pdx1* indicated that the pancreatic lineages were converted to stomach and intestinal cell fates. In addition, there was a notable upregulation of many components of the hedgehog pathway, suggesting that the major mechanism of GATA4/6-regulated pancreas development is through suppression of the hedgehog pathway. Previous studies have demonstrated that GATA factors can negatively regulate *Shh* expression in the stomach, limb bud, and somites (Daoud et al., 2014; Jacobsen et al., 2005; Kozhemyakina et al., 2014). Our studies suggest that GATA4 and GATA6 pattern the foregut endoderm through the repression of hedgehog signaling.

## RESULTS AND DISCUSSION

### Hedgehog signaling is highly upregulated in E12.5 pDKO pancreata

Simultaneous deletion of *Gata4* and *Gata6* in the pancreatic progenitor domain resulted in severely aplastic pancreatic buds (Carrasco et al., 2012; Xuan et al., 2012). To characterize the molecular pathways that function downstream of GATA4 and GATA6 to mediate the regulation of pancreatic differentiation and morphogenesis, we performed genome-wide transcriptome analysis of the arrested pancreatic rudiments from pDKO embryos compared with littermate control embryos. A *R26R;Tomato* fluorescent reporter

was introduced into the strain to facilitate purification of the PDX1-derived lineages from pooled pancreata of each genotype (Fig. 1A). Quantitative real-time PCR (qPCR) was used to confirm deletion of *Gata4* and *Gata6*, and validate the reduction in pancreatic progenitor markers *Pdx1* and *Ptf1a* (Fig. S1). RNA-Seq analysis of pooled wild-type versus pDKO Tomato+ pancreata identified ~251 genes that were significantly differentially expressed in the mutant embryos: 118 genes were downregulated and 133 genes were upregulated (Fig. 1B,C). Strikingly, hedgehog signaling was revealed to be the most affected pathway [using ingenuity pathway analysis (IPA) software], with many of the transcriptionally regulated components of the hedgehog pathway being dramatically upregulated (Table 1). qPCR confirmed a 30- to 50-fold upregulation of the two major hedgehog ligands *Shh* and Indian hedgehog (*Ihh*), and a 7- to 10-fold upregulation of the receptor patched homolog 1 (*Ptch1*) and the downstream transcriptional activator *Gli1* (Fig. 1D). *In situ* hybridization for *Shh* on E10.5 embryos revealed the erroneous expression of *Shh* expression throughout the pDKO pancreatic domain (Fig. 1E). As inhibition of *Shh* expression by notochord-derived activin and FGF or cardiac mesoderm-derived FGF has been postulated to be an early event in pancreas induction (Deutsch et al., 2001; Hebrok et al., 1998), our findings suggest that repression of hedgehog signaling downstream of these mesodermal signals is mediated by GATA4 and GATA6 activity.



**Table 1. Fold change and P-values from representative components of the hedgehog pathway**

Gene	Fold change in pDKO versus control embryos	P-value
<i>Shh</i>	29.93	$1.93 \times 10^{-11}$
<i>Ihh</i>	22.28	$4.59 \times 10^{-11}$
<i>Ptch1</i>	7.07	$3.54 \times 10^{-12}$
<i>Gli1</i>	11.08	$3.54 \times 10^{-12}$
<i>Ptch2</i>	10.43	$5.37 \times 10^{-07}$
<i>Hhip</i>	6.21	$2.15 \times 10^{-07}$
<i>Dhh</i>	0.75	n.s.
<i>Smo</i>	1.03	n.s.

n.s., not significant.

### Cell fate switching occurs in the pDKO pancreatic endoderm

Previous studies have demonstrated that the absence of *Shh* expression in the early pancreatic domain is required for normal pancreas development (Hebrok et al., 2000). Furthermore, ectopic upregulation of *Shh* (Apelqvist et al., 1997; Haumaitre et al., 2005) is associated with diminished pancreas formation and expanded stomach or gut regionalization. Consistent with the elevation of hedgehog signaling in the pDKO pancreatic endoderm domain, there was a large reduction of pancreatic progenitor markers in the pDKO, whereas there was a notable increase in genes encoding stomach progenitor markers, such as *Sox2* and *Nkx6-3* (5.8- and 19.7-fold, respectively), and intestinal progenitor marker genes, such as *Cdx2* and *Isx* (20-fold) (Table 2).

To determine whether the reciprocal changes in marker expression resulted from re-specification of the pancreatic endoderm in the pDKO embryos, we assessed pancreatic, stomach and intestinal lineage markers in the pDKO; R26R:Tomato mice using the lineage label to track the potentially re-specified pancreatic lineages. As expected, in control E10.5 pancreatic endoderm, Tomato-labeled pancreatic lineage cells in the dorsal pancreatic anlage express the pancreatic lineage marker *Pdx1*, but do not express the stomach marker gene *Sox2* (Fig. 2A-D'). However, in the pDKO embryos, Tomato-labeled pancreatic lineage cells begin to express *Sox2* in the place of *Pdx1* (Fig. 2E-H'), indicating that pancreatic lineage cells in the dorsal pancreas have

**Table 2. Representative list of selected pancreatic, intestinal and stomach genes that are differentially regulated in the pDKO pancreatic rudiments**

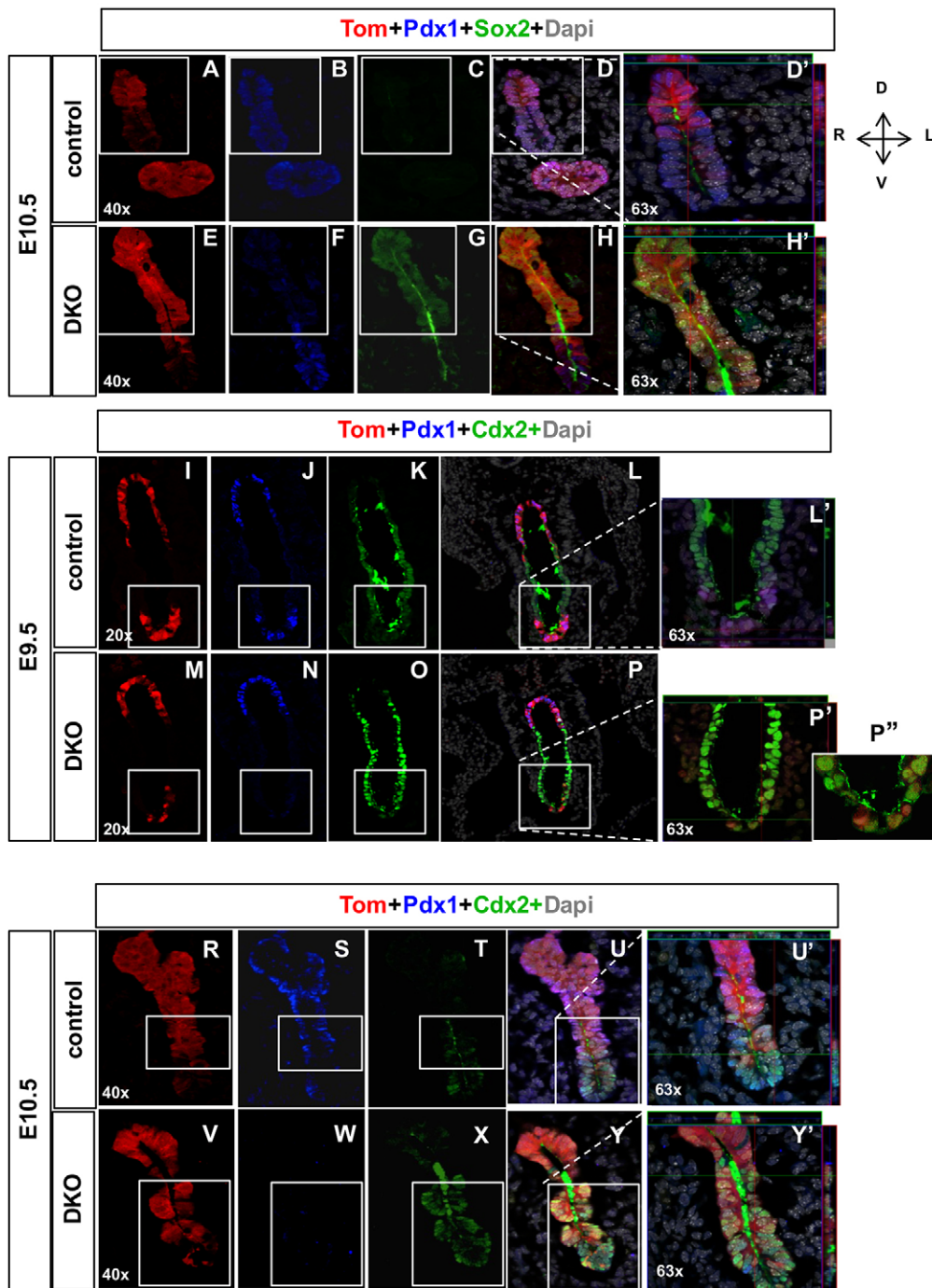
Gene	Fold change	Normal expression domain
<i>Pdx1</i>	↓ 1.7	Pancreas
<i>Nkx2-2</i>	↓ 5.8	Pancreas
<i>Nkx6-1</i>	↓ 2.5	Pancreas
<i>Cpa1</i>	↓ 70.0	Pancreas
<i>Neurog3</i>	↓ 45.0	Pancreas
<i>Prox1</i>	↓ 3.3	Liver and pancreas
<i>Cdx2</i>	↑ 20.8	Intestine
<i>Isx</i>	↑ 18.8	Intestine
<i>Nkx6-3</i>	↑ 19.7	Anterior duodenum/posterior stomach
<i>Sox2</i>	↑ 5.82	Stomach
<i>Foxa1</i>	↑ 17.4	Foregut endoderm
<i>Nepn</i>	↑ 8.0	Stomach/pancreas/small intestine
<i>Foxf2</i>	↑ 25.1	Distal gut mesenchyme (activated by hedgehog signaling)
<i>Foxf1</i>	↑ 9.1	Distal gut mesenchyme (activated by hedgehog signaling)
<i>Ins2</i>	↓ 2.8	Endocrine pancreas
<i>Gcg</i>	↓ 7.1	Endocrine pancreas
<i>Ghrl</i>	↓ 3.5	Endocrine pancreas

switched to a stomach identity. Similarly, in the ventral pancreatic endoderm, the pancreatic lineages appear to adopt intestinal cell fates (Fig. 2I-Y). In pDKO E9.5 embryos, a small number of pancreatic lineage cells begin to co-express *Pdx1* and *Cdx2* or express *Cdx2* in place of *Pdx1* (Fig. 2M-P'). By E10.5, there is an increasing number of *Pdx1* lineage-labeled cells that express *Cdx2* (Fig. 2V-Y'). At E18.5, conversion of the Pdx1 lineage to mature stomach fates is widespread and many of the Tomato lineage-labeled cells have become incorporated into the stomach epithelium as previously demonstrated (Xuan, et al., 2012) where they co-express mature stomach markers, including MUC5AC and ATP4B (Fig. S2). These results suggest that the erroneous upregulation of the hedgehog pathway in pDKO embryos results in re-specification of the dorsal and ventral pancreatic lineages to the adjacent stomach and intestinal fates, respectively.

### GATA4 and GATA6 suppress the activity of the *Shh* foregut endoderm enhancer *MACS1*

GATA regulation of *Shh* expression has been reported in several organ systems. During gastric development, *Gata4* and *Shh* expression is mutually exclusive, suggesting that GATA4 might inhibit the expression of *Shh* (Jacobsen et al., 2005). In the limb bud, GATA6 was shown to inhibit *Shh* gene expression through a limb bud-specific enhancer of the *Shh* gene (Kozhemyakina et al., 2014), and in the somitic system, GATA4/5/6 were found to inhibit *Shh* signaling by inhibiting *Gli1* expression, although direct inhibition of *Shh* was not required (Daoud et al., 2014). The upregulation of hedgehog pathway expression in the pDKO mice suggested that *Shh* might be repressed by GATA4 and/or GATA6. An 806-bp *MACS1* enhancer located ~740 kb upstream from the *Shh* transcriptional start site has been shown to be sufficient for driving *Shh* expression specifically in the foregut endoderm (Anderson et al., 2014; Sagai et al., 2009). Using position weight matrix analysis we identified four putative GATA consensus elements within the *MACS1* enhancer (Fig. 3A, red text). Chromatin immunoprecipitation (ChIP) analysis of these sites revealed strong binding of GATA4 and GATA6 to the two most upstream GATA consensus sites (Gata site 1 and Gata site 2) and minimal, if any, binding to the 3' sites (Gata site 3 and Gata site 4) (Fig. 3B,C).

To determine whether GATA4 and GATA6 could inhibit *Shh* expression through the *MACS1* enhancer element, we performed luciferase experiments in a *Shh*-expressing pancreatic  $\alpha$ TC6 cell line that lacks endogenous *Gata4* and *Gata6* (Fig. S3A). Transfection of pGL4.27-*MACS1* led to an approximately sixfold increase of luciferase activity compared with pGL4.27 vector alone, suggesting that *MACS1* has high activity in these cells (Fig. 3C), which is consistent with previous *in vivo* expression analysis (Kawahira et al., 2005). Furthermore, co-transfection with either *Gata4* or *Gata6* reduced *MACS1* activity by >50%, with GATA6 having the largest effect (~75%). Furthermore, simultaneous transfection of half the amount each of *Gata4* and *Gata6*, such that the total amount of GATA protein remained constant (Fig. S3B,C) demonstrated that GATA4 and GATA6 could function together to repress *MACS1* activity (Fig. 3D). Surprisingly, however, overexpression of *Gata4* and/or *Gata6* was still able to partially repress a *MACS1* fragment that contained mutations in the four putative Gata consensus sites (Fig. 3A, blue text; Fig. 3D, 'MACS1mut'). This might indicate that overexpression of GATA factors can bind and activate through cryptic Gata sites (Fig. 3A, green text) present within the *MACS1* enhancer or that GATA factors mediate *MACS1* repression through an indirect mechanism, as previously described in the heart (Rivera-Feliciano et al., 2006).

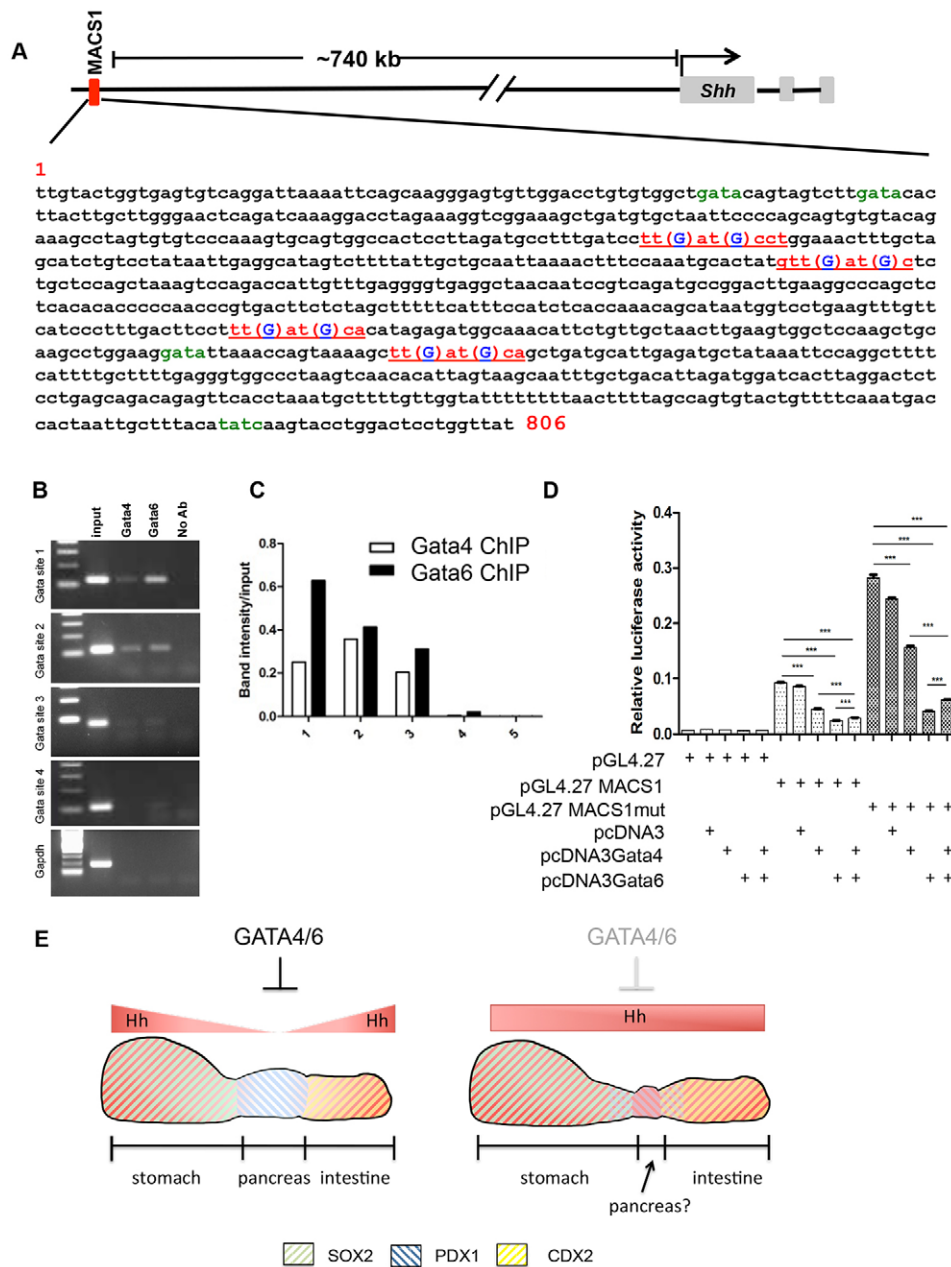


**Fig. 2. Pancreatic lineage cells in pDKO embryos switch cell fates.** (A-H') Representative sections of immunofluorescence-stained pDKO dorsal pancreatic lineage cells from E10.5 embryos. In control embryos, pancreatic lineage cells express Pdx1 (blue) (B) but do not express Sox2 (green) (C). Merged images (D, D') show overlapping Tomato-expressing (red) cells derived from the Pdx1 lineage and Pdx1-expressing cells (blue). In pDKO embryos (E-H'), Pdx1 lineage cells (E) do not express Pdx1 (F), but instead express Sox2 (G). Merged images (H, H') show overlapping Tomato- and Sox2-expressing cells, suggesting that pancreatic-derived lineages are converted to a stomach identity. (I-P'') Representative sections of E9.5 pDKO foregut endoderm showing that pancreatic lineage cells express intestinal markers. In control embryos, Tomato+ pancreatic-derived lineages (I) express Pdx1 (J), but not Cdx2 (K), confirming their pancreatic identity. Images from merged channels (L, L') show overlapping expression of Tomato+ cells and Pdx1+ cells. In the pDKO ventral pancreatic domain, a few pancreatic lineage cells (M) have lost their expression of Pdx1 (N) and start to express the intestinal cell marker Cdx2 (O), suggesting that these cells are converting into intestinal cells. Merged images are shown in P-P''. (R-Y') At E10.5, control pancreatic lineage cells (R) continue to express Pdx1 (S), but not Cdx2 (T). Merged images are shown in U and U'. In pDKO, increasing numbers of Pdx1-derived Tomato+ lineage cells (V) do not express Pdx1 (W) and instead express Cdx2 (X). Merged channels (Y, Y') demonstrate overlapping expression of Tomato and Cdx2, suggesting that these cells are switching to an intestinal cell fate. Boxes indicate regions shown at higher magnification in D', H, L', P', U' and Y'.

Repression of the hedgehog pathway in the presumptive ventral and dorsal pancreatic endoderm in response to signals from adjacent tissues has been well documented (Apelqvist et al., 1997; Chung and Stainier, 2008; dilorio et al., 2007; Haumaitre et al., 2005; Hebrok et al., 1998; Roy et al., 2001). However, the transcriptional regulators that mediate these mesodermal signals upstream of the hedgehog pathway have not yet been identified. In this study, we demonstrate the importance of GATA4 and GATA6 in repressing hedgehog pathway components within the pancreatic anlage. Furthermore, our *in vitro* data suggest GATA-mediated repression of *Shh* occurs partially through the endoderm-specific *MACS1* enhancer. Consistent with a crucial role for GATA4 and GATA6 in

repressing hedgehog signaling in the pancreatic endoderm, there is a conversion of pancreatic lineages to stomach or intestinal cell fates when GATA function is absent. The timing of *Shh* activation relative to the observed fate changes supports a model in which GATA-mediated repression of hedgehog signaling is necessary to delineate and maintain foregut endoderm fates (Fig. 3E). These findings also reinforce the proposed role for GATA4 and GATA6 as pioneer factors that are positioned at the top of the gene regulatory cascade that patterns tissue-specific gene expression pathways (Zaret et al., 2008).

Recently, it has been reported that haploinsufficiency of *GATA4* or *GATA6* genes account for more than half of all human pancreatic agenesis cases (Lango Allen et al., 2012), suggesting their important



**Fig. 3. GATA4 and GATA6 inhibit the activity of the *Shh* endoderm enhancer *MACS1*.** (A) Schematic of the *MACS1* enhancer element relative to the transcriptional start site of *Shh*. The *MACS1* element is located ~740 kb upstream of the *Shh* transcriptional start site. The *MACS1* element is 806 base pairs, and is predicted to have four consensus GATA-binding sites (red text). Mutations in the GATA-binding sites are indicated by blue text. Four potential cryptic GATA-binding sites are designated with green text. (B) PCR analysis of E14.5 pancreata ChIP samples on four putative GATA-binding sites. The first two sites have strong binding for GATA4 and GATA6, whereas the third site has very weak binding. The fourth site has no detectable binding for GATA4 and GATA6. (C) Quantification of band intensities relative to their own inputs. Lane 1=Gata site 1 (GATA4 and GATA6 binding is about 25% and 63%, respectively); Lane 2=Gata site 2 (36% for GATA4 and 41% for GATA6); Lane 3=Gata site 3 (20% for GATA4 and 31% for GATA6); Lane 4=Gata site 4 (0.4% for GATA4 and 2.1% for GATA6); Lane 5=CPA1 site (no measurable binding).  $n=3$ . (D) Luciferase assay in  $\alpha$ -TC6 cells. The pGL4.27-*MACS1* plasmid has high luciferase activity in  $\alpha$ -TC6 cells; this effect is suppressed by expression of GATA4 or GATA6. Co-transfection of GATA4 and GATA6 additively suppressed *MACS1* expression. pGL4.27-*MACS1mut* refers to the *MACS1* fragment that is mutated for the four GATA sites (red text in A). Error bars represent s.e.m. \*\*\* $P<0.001$ .  $n=5$ . (E) Model summarizing the downstream consequences of a lack of GATA4 and GATA6 in the pancreatic epithelium. GATA4 and GATA6 expression in the presumptive pancreatic foregut endoderm represses hedgehog signaling to allow for the induction of *Pdx1* expression (blue stripes) and initiation of pancreatic fates. In the absence of GATA4 and GATA6, hedgehog signaling is erroneously activated in the pre-pancreatic endoderm domain. As a result, the PDX1 domain is respecified into stomach fates expressing SOX2 (green stripes) and intestinal fates expressing CDX2 (yellow stripes). In the absence of GATA4 and GATA6, a small remnant of tissue co-expressing PDX1 and SOX2 or CDX2 can be detected. This domain transiently expresses pancreatic markers.

role in human pancreas development. Our discovery that GATA4 and GATA6 are essential for maintaining repression of hedgehog signaling provides important implications for the pathways

regulated by the GATA factors during human pancreas development and could lead to novel strategies to detect and/or prevent pancreatic agenesis before birth.

## MATERIALS AND METHODS

### RNA-Seq and bioinformatics analysis

Fluorescently labeled cells from E12.5 control and pDKO pancreata were manually dissected using a Leica MZ16F fluorescence dissecting microscope. Six controls and 12 pDKO pancreata were pooled for RNA-Seq ( $n=1$  pools per genotype). RNA was generated (RNeasy Micro kit; Qiagen) and tested for quality (RIN values  $>8$ ; Agilent Bioanalyzer 2100). RNA-Seq was performed on the Illumina HiSeq 2000V3 Instrument (Columbia Genome Center) at a depth of 25-30 million 100-bp single-end reads. FPKM values were used to measure RNA expression level and 23,700 genes were compared between control and mutant samples. DESeq analysis (DESeq2, R software package) was performed to identify differentially expressed genes ( $P<0.0008$ ). RNA-Seq data have been deposited in Gene Expression Omnibus under accession number GSE77083.

### Quantitative real-time PCR

RNA from E12.5 pancreata was isolated (RNeasy Micro, Qiagen) to generate cDNA (Invitrogen). qRT-PCR was performed using Taqman AOD probe sets or SYBR green Premix (Bio-Rad). Taqman AODs: *Pdx1*, Mm00435565-ml; *Ptf1a*, Mm04203788-gl; *Gata4*, Mm00484689-ml; *Gata6*, Mm00802636-ml; *Cdx2*, Mm01212280-ml; *Sox2*, Mm03053820-s1 (Open Biosystems-Thermo Scientific). Specific primers: *Shh* ex1 F, 5'-GGAGCAGACCGGCTGATGAC-3'; *Shh* ex2 R, 5'-TCGGTCACTCGCAGCTTAC-3'; *Ihh* ex1 F, 5'-TCTTCAAGGACG-AGGAGAACACG 3'; *Ihh* ex2 R, 5'-CACCCGAGTTTACACACAG-3'; *Gli1* F, 5'TGGTACCATGAGCCCTTCTT 3'; *Gli1* R, 5'-GTGGTACAC-AGGGCTGGACT-3'; *Ptch1* F, 5'ATCTCGAGACCAACGTGGAG 3'; *Ptch1* R, 5'-GCCTTCTCTCTATCTTCTGACG-3'; *Gata4* F, 5'-TAGTCTGGCAGTTGGCACAG-3'; *Gata4* R, 5'-ACGGGACACTACCTGTGCAA-3'; *Gata6* F, 5'-AGTTTCCGGCAGAGCAGTA-3'; *Gata6* R, 5'-AGTCAAGGCATCCACTGTC-3'.

### In situ hybridization and immunofluorescence analysis

RNA *in situ* hybridization was performed as previously described (Prado et al., 2004). *Shh* antisense riboprobe was prepared from a pBSK-*Shh* plasmid containing a full-length *Shh* cDNA. T3 RNA polymerase was used to transcribe a *Hind*III-linearized plasmid. Brightfield images were acquired using a Leica DM5500 microscope.

All immunofluorescence analysis was performed on frozen sections as previously described (Xuan et al., 2012). Primary antibodies were: rabbit anti-Pdx1 (1:1000; 07-696, Millipore), mouse anti-Sox2 (1:250; MAB4343, Santa Cruz), mouse anti-Cdx2 (1:80; CDX2-88, BioGenex), mouse anti-Muc5AC (1:500; ab3649, Abcam) and rabbit anti-ATP4b (1:1000; MA3-923, Thermo Fisher Scientific). Secondary antibodies were Alexa Fluor 488-, 594- or 697- conjugates (1:500; Jackson ImmunoResearch). Fluorescence and confocal images were acquired using a Zeiss LSM 710 confocal microscope. Images and z-stack images were analyzed using ZEN software (Zeiss).

### Western blot analysis

Cell lysates from a-TC cells were analyzed using mouse anti-Gata4 (1:1000; SC-25310, Santa Cruz), rabbit anti-Gata6 (1:200; SC-9055, Santa Cruz) and rabbit anti-Gapdh (1:1000; ab9845, Abcam). Quantification of the relative intensity was performed using ImageJ software.

### Chromatin immunoprecipitation

E14.5 pancreata were manually dissected. Chromatin was prepared from these tissues as reported previously (Xuan et al., 2012). The following primers were used for PCR analysis: *MACS1* 5' primers: forward 5'-GTGTACAGAAAGCCTAGTGTGC-3', reverse 5'-GCAGCAATAAAAGACTATGCCTC-3'; *MACS1* 3' primers: forward 5'-ACATTCTGTTGCTA-ACTTGAAGTG-3', reverse 5'-AAGCCTGGAATTTATGACTCTCA-3'; Gata site 2 primers: forward 5'-GAGGCATAGTCTTTTATTGCTGC-3', reverse 5'-GCATCTGACGGATTGTTAGCCT-3'; Gata site 3 primers: forward 5'-ATGGTCTGAAGTTTGTTCATCC-3', reverse 5'-CACTTC-AAGTTAGCAACAGAATGT-3'; CPA1exF, CGGAGCTAGTAGCAAC-CCCT; CPA1exR, CAGGAGCTGGTTCTGATGTG.

### Luciferase assay

The 806-bp *MACS1* genomic fragment was PCR amplified (5' GGTACC-TTGTACTGGTGAGTGT, 3' AGATCTATAACCAGGAGTCCAGG) and cloned into pGL4.27 luciferase reporter plasmid. A DNA fragment containing the 806-bp *MACS1* mut with point mutations in the four putative Gata sites was synthesized by Integrated DNA Technologies. Full-length cDNAs of mouse *Gata4* and *Gata6* were cloned into the pcDNA3 vector. These plasmids were transfected along with *Renilla* luciferase construct into  $\alpha$ -TC6 cells using X-treme (Roche) transfection reagent according to the manufacturer's instructions. Forty-eight hours after transfection, cell lysates were collected and luciferase samples were prepared using the Dual Luciferase Reporter Assay System (Promega). Luciferase activities were measured using an Orion II Luminometer and luciferase activity was normalized to *Renilla* activity.

### Image quantification

ImageJ software was used to quantify the band intensities in the images for PCR gel and western blotting.

### Statistical analysis

All values are expressed as mean  $\pm$  s.e.m. Statistical analysis was performed using a two-tailed Student's *t*-test. Results were considered significant at  $P<0.05$ .

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

L.S. and S.X. designed experiments and interpreted results. S.X. performed the experiments. L.S. and S.X. wrote the manuscript.

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### Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.127217/-DC1>

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