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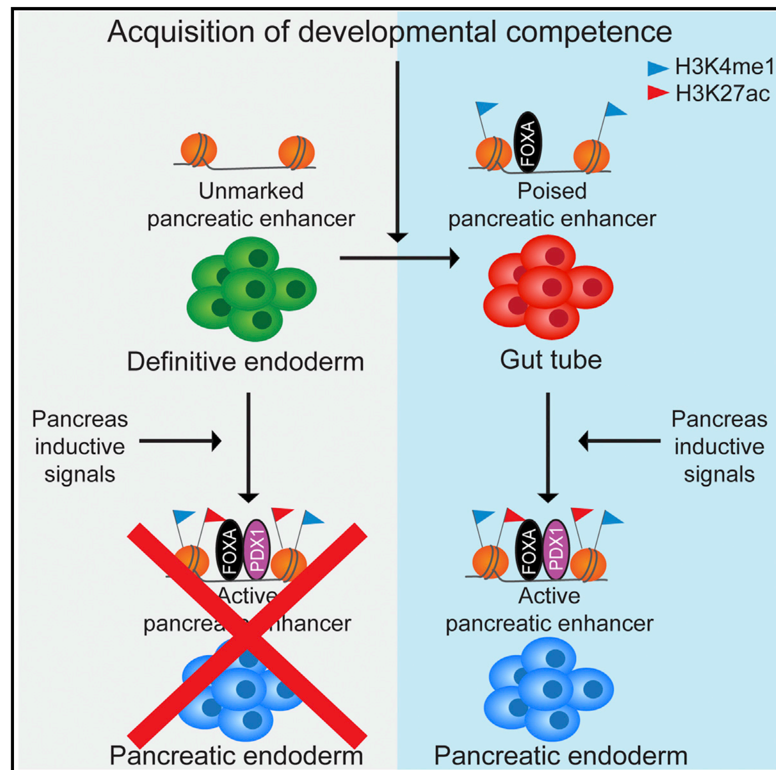
Epigenetic Priming of Enhancers Predicts Developmental Competence of hESC-Derived Endodermal Lineage Intermediates

Graphical Abstract



Highlights

- A poised enhancer landscape for endodermal organ lineages is established in gut tube
- Select enhancers involved in cellular identity are activated in descendent lineages
- Poised chromatin at lineage-specific enhancers indicates developmental competence
- Pioneer TFs associate with poised enhancers prior to activation by pro-lineage TFs

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In Brief

Embryonic development relies on the capacity of progenitor cells to respond appropriately to inductive signals, an ability termed developmental competence. By mapping enhancer-related histone modifications during pancreatic differentiation of human embryonic stem cells, Wang et al. identify a poised state at enhancers as predictive of developmental competence.

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Epigenetic Priming of Enhancers Predicts Developmental Competence of hESC-Derived Endodermal Lineage Intermediates

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SUMMARY

Embryonic development relies on the capacity of progenitor cells to appropriately respond to inductive cues, a cellular property known as developmental competence. Here, we report that epigenetic priming of enhancers signifies developmental competence during endodermal lineage diversification. Chromatin mapping during pancreatic and hepatic differentiation of human embryonic stem cells revealed the en masse acquisition of a poised chromatin state at enhancers specific to endoderm-derived cell lineages in gut tube intermediates. Experimentally, the acquisition of this poised enhancer state predicts the ability of endodermal intermediates to respond to inductive signals. Furthermore, these enhancers are first recognized by the pioneer transcription factors FOXA1 and FOXA2 when competence is acquired, while subsequent recruitment of lineage-inductive transcription factors, such as PDX1, leads to enhancer and target gene activation. Together, our results identify the acquisition of a poised chromatin state at enhancers as a mechanism by which progenitor cells acquire developmental competence.

INTRODUCTION

Embryonic development is a forward-moving process during which pluripotent cells become increasingly specialized as they develop toward a terminally differentiated state. The stepwise progression toward specific cell lineages occurs as a result of a series of inductive events. The ability of lineage intermediates to appropriately interpret inductive signals from their environment is referred to as developmental competence. A classic example of this is the induction of the neuronal lineage by mesodermal cells at the time of gastrulation. Signals from the mesoderm act on the ectoderm, causing it to form neural tissue (Linker and Stern, 2004). However, only ectoderm of a certain developmental age is capable of appropriately responding to the inductive signal (Storey et al., 1992). Thus, developmental competence is a cell-intrinsic property of the responder tissue. Furthermore, competence is not inherent to the pluripotent state but actively acquired during development. What mechanisms operate to render cells competent to respond to inductive cues with precise timing is currently unknown.

While transcription factors (TFs) are important contributors to cellular competence, they are not sufficient to explain the highly cell-type-specific responses to inductive cues during development. TFs typically occupy only a small fraction of their consensus binding motifs in the genome (Carr and Biggin, 1999; Iyer et al., 2001; Yang et al., 2006), suggesting that determinants beyond DNA sequence must dictate where and when TFs bind potential targets. Emerging evidence suggests that chromatin structure represents an inherent and important determinant of accessibility of DNA to TFs (Martino et al., 2009; Shogren-Knaak et al., 2006). Of particular interest is the chromatin state at enhancers, which plays a prominent role in spatiotemporal gene regulation during development (Creyghton et al., 2010; Heintzman et al., 2009; Koch et al., 2007; Rada-Iglesias et al., 2011; Visel et al., 2009). A central feature of enhancers is their ability to function as integrated TF binding platforms, where environmental signaling cues are interpreted in a context-dependent manner (Buecker and Wysocka, 2012; Jin et al., 2011). How enhancers acquire the ability to translate signals from the extracellular environment into cell-type-specific transcriptional responses during development is poorly understood.

In this study, we examined the possibility that the epigenetic state of enhancers could determine developmental competence in the context of endodermal and pancreatic development. We

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explored this question by generating comprehensive maps of enhancer-related chromatin modifications over a time course of human embryonic stem cell (hESC) differentiation through multiple developmental intermediates into pancreatic and hepatic cells. Through integrative analysis of these maps and further experimentation, we reveal previously insufficiently appreciated links between enhancer chromatin, TF recruitment, and developmental competence. First, we show that developmental competence is encoded at the level of enhancers and is established en masse in embryonic intermediates prior to lineage induction via acquisition of poised chromatin at enhancers specific to descendant lineages. Second, we find that TF complexes assemble at lineage-specific enhancers in a stepwise fashion. TFs involved in chromatin priming are recruited early when lineage intermediates acquire competence followed by the recruitment of lineage-inductive TFs to mediate activation. Together, these findings establish a functional link between the gain of a poised enhancer chromatin state and the temporal acquisition of competence during developmental progression.

RESULTS

Global Identification of Enhancers during Pancreatic Differentiation of hESCs

During early embryogenesis, the pancreas, liver, and lung develop from the endodermal gut tube (GT) (Wang and Sander, 2012). How and when organ-specific transcriptional programs are initiated during this developmental progression remains poorly understood. Because enhancers are important regulators of cell-type-specific gene expression (Heintzman et al., 2009; Koch et al., 2007; Rada-Iglesias et al., 2011; Visel et al., 2009), we reasoned that mapping enhancers during endodermal lineage progression could provide mechanistic insight into these questions. Thus, we comprehensively mapped putative enhancers during the stepwise progression of hESCs toward the pancreatic fate, using a system that accurately models early developmental processes, including GT formation and pancreatic lineage induction (Figure 1A). Specifically, we analyzed enhancer-associated histone modifications genome-wide at five defined stages of differentiation: hESCs, definitive endoderm (DE), primitive GT, posterior foregut (FG), and pancreatic endoderm (PE). These cell populations were each produced with >90% purity (Xie et al., 2013). We identified a total of 119,795 enhancers across these five stages of pancreatic differentiation (Figure 1B). The majority of marked enhancers at each stage are marked by H3K4me1 only, with only a small fraction also marked by H3K27ac (Figure 1C; Figures S1A and S1C; Table S1).

It has been suggested that H3K27ac can distinguish active from poised enhancers and that the poised state could facilitate enhancer activation (Creyghton et al., 2010; Rada-Iglesias et al., 2011). To obtain a direct readout of enhancer activity, we performed global nuclear run-on sequencing (GRO-seq) of nascent transcripts. We observed a significant enrichment of transcriptionally engaged RNA polymerases indicative of enhancer RNA (eRNA) production at active enhancers (defined by H3K4me1 and H3K27ac deposition) compared to poised enhancers (defined by only H3K4me1 deposition) (Figures 1D, S1B, and S1D). Combined with previous studies linking eRNA production to enhancer activity (Hah et al., 2013; Kim et al., 2010; Wang et al., 2011), our results suggest that enrichment of H3K4me1 alone likely constitutes an inactive enhancer state. We also examined the transcriptional activity of putative target genes by quantifying their GRO-seq and RNA-sequencing (RNA-seq) counts. Consistent with higher eRNA production at active enhancers, target promoters of active enhancers exhibited higher GRO-seq and RNA-seq counts than promoters linked to poised enhancers (Figures 1E, 1F, S1B, and S1D). These results define two functionally distinct classes of putative developmental enhancers based on chromatin features.

Identification of Enhancers that Drive Pancreatic Lineage Induction

Having globally predicted both poised and active enhancers across all five stages, we next sought to isolate the specific sets of enhancers that regulate developmental transitions and, in particular, pancreas induction. To do this, we clustered enhancers based on their predicted activity across all differentiation stages. This revealed three major categories: (I) active in a stage-restricted manner, (II) poised yet never active, and (III) constitutively active (Figure 2A). As expected, when tested in enhancer-reporter assays, category I enhancers exhibited stage-specific activity according to their H3K27ac status (Figures 2B, 2C, and S2A–S2C). Illustrating their importance in developmental gene regulation, category I enhancers were predicted to regulate developmental processes specific to the embryonic stage of their activity (Figures 2D and S2D). Of particular interest were enhancers that acquired H3K27ac during pancreatic lineage induction (FG/PE- and PE-specific clusters) (Figure 2A). These enhancers associated with genes involved in pancreas and endocrine systems development, which include the master regulators of pancreas development *PDX1*, *SOX9*, *PTF1A*, and *NKX6.1* (Shih et al., 2013) (Figures 2A, 2B, and 2D). It is likely that these enhancers have roles in regulating the induction of pancreatic gene expression programs when the pancreas develops from the GT.

Pancreatic Enhancers Acquire a Poised State in GT Intermediates prior to Activation

To investigate chromatin dynamics at enhancers associated with pancreatic lineage induction, we next examined H3K4me1 and H3K27ac intensities at pancreas-specific enhancers. In both the FG/PE- and PE-specific clusters, H3K4me1 levels accumulated prior to H3K27ac (Figure 2E), suggesting that pancreatic enhancers are poised prior to activation. It is interesting that pancreas-specific enhancers (FG/PE and PE clusters) exhibited relatively little H3K4me1 enrichment in hESCs (Figure 2E), indicating that the poised state is actively acquired during development. By examining the average fold change in H3K4me1 levels at each transition, we observed the largest fold increase in H3K4me1 at pancreatic enhancers during the DE-to-GT transition (Figure 2F), when these enhancers are still inactive as judged by H3K27ac levels and enhancer-reporter assays (Figures 2C, 2E, S2B, and S2C). Thus, enhancers associated with pancreatic lineage programs acquire a poised state during GT formation independent of pancreas-inductive signaling cues. These findings suggest that epigenetic bookmarking of pancreatic enhancers can pre-program GT intermediates to activate pancreas-specific genes when exposed to pro-pancreatic signaling cues.

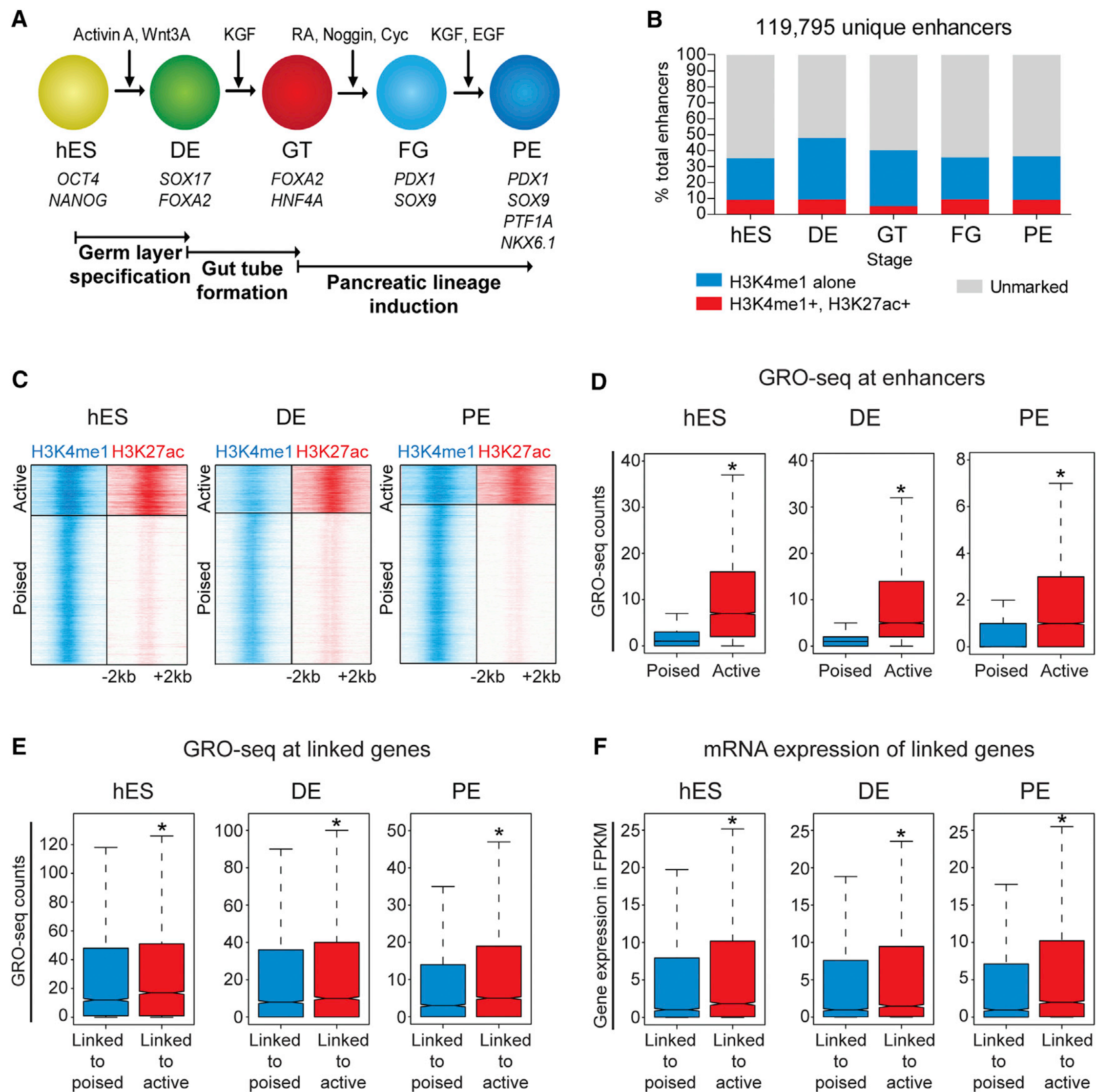

Figure 1. Global Identification of Poised and Active Enhancers during Pancreatic Differentiation of hESCs

(A) hESC (hES) differentiation strategy.

(B) Total number of candidate enhancers identified during pancreatic differentiation categorized by H3K27ac and H3K4me1 deposition.

(C) Density of ChIP-seq reads for H3K4me1 and H3K27ac relative to midpoint at putative poised and active enhancers.

(D) Box plots of GRO-seq counts at poised (H3K4me1 only) and active (H3K4me1 and H3K27ac) enhancers.

(E) Box plots of GRO-seq counts at linked genes of poised and active enhancers. Avg., average.

(F) Box plots of mRNA expression, measured in fragments per kilobase of exon per million fragments mapped (FPKM), at linked genes of poised and active enhancers. *$p < 2.2e-16$, Wilcoxon rank-sum test.

See also Figure S1 and Table S1.

A Poised Enhancer Landscape for Multiple Endodermal Lineages Is Established in GT Intermediates

One surprising result of the enhancer cluster analysis was that the majority (66%) of predicted enhancers during pancreatic lineage progression belong to category II (Figure 2A), meaning that they acquire a poised state yet never become active. Category II enhancers comprise four clusters: poised in hESCs (P1), poised at the DE stage (P2), poised with intermediate H3K4me1 levels at the GT, FG, and PE stages (P3), and poised with high H3K4me1 levels at the GT, FG, and PE stages (P4) (Figure 2A; Figure 3A). Intriguingly, H3K4me1 levels in clusters P3 and P4 exhibit a dramatic gain during the DE-to-GT transition (Figure 3A), as seen for pancreas-specific enhancers (Figures 2E and 2F).

This raises the question of why P3 and P4 enhancers acquire a poised state (H3K4me1) during GT formation. Given that the GT gives rise to multiple organs, including pancreas, lung, and liver, we hypothesized that these poised enhancers could become active either later during terminal differentiation into mature pancreatic cells or in alternate gut-tube-derived lineages. In

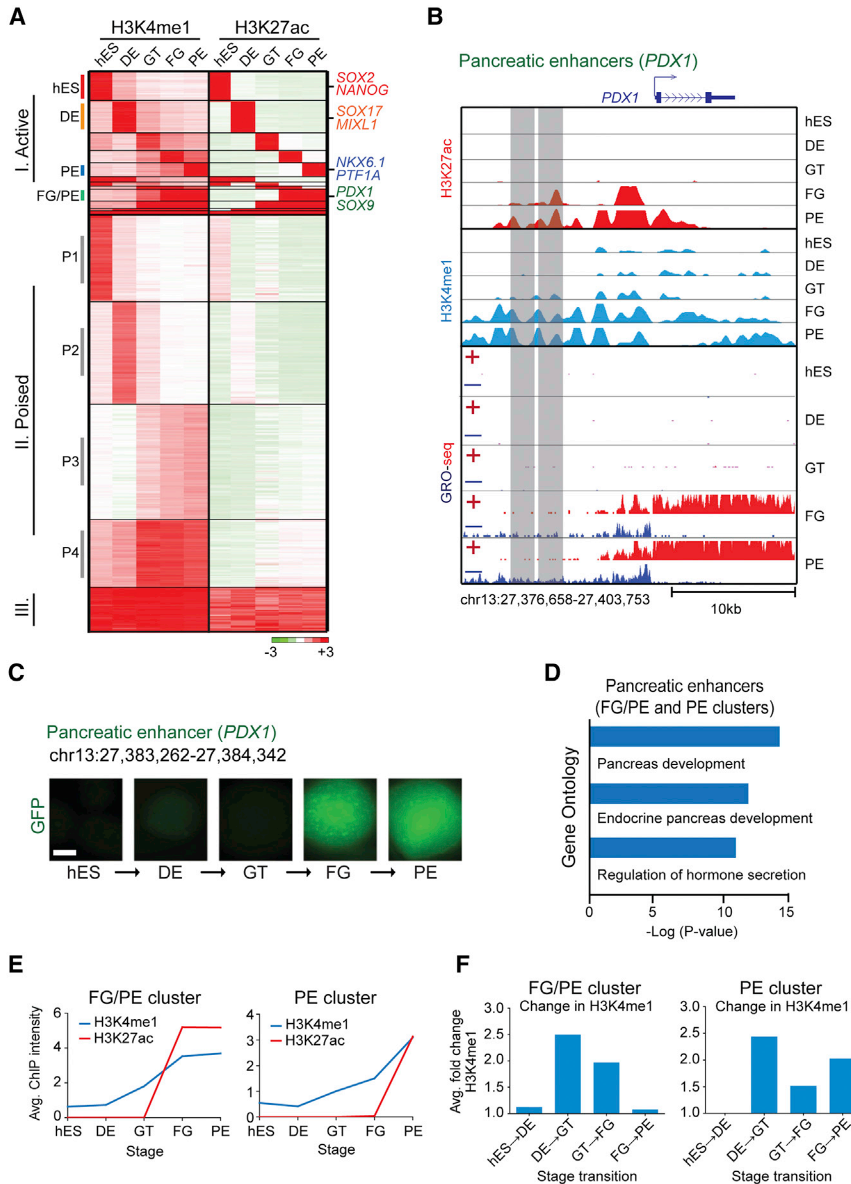

Figure 2. Identification and Characterization of Enhancers Important for Pancreatic Lineage Induction

(A) K-means clustering of putative enhancers during pancreatic differentiation based on H3K4me1 and H3K27ac signal intensity. Individual genes associated with enhancer clusters are listed on the right. hES, hESCs.

(B) ChIP-seq H3K4me1, H3K27ac, and GRO-seq profiles of representative FG/PE-specific enhancers near *PDX1*.

(C) In vitro GFP reporter assay for FG/PE-specific enhancer shown in (B, right side). GFP images of cell aggregates during pancreatic differentiation are shown. Scale bar, 100 μm.

(D) Enriched Gene Ontology terms for pancreatic enhancers.

(E) Average (Avg.) H3K4me1 and H3K27ac ChIP-seq signal intensity for pancreatic (FG/PE- and PE-specific clusters) enhancers during pancreatic differentiation.

(F) Average fold change in H3K4me1 signal intensity during each stage transition for pancreatic (FG/PE- and PE-specific clusters) enhancers.

See also Figure S2.

support of this hypothesis, enhancers from clusters P3 and P4 associated with genes involved in the development and function of pancreatic islets, lung, and liver (Figure 3B). Furthermore, poised enhancers in clusters P3 and P4 were enriched for binding motifs of TFs known to regulate the development of endodermal organs (Figure 3C), including FOXA, GATA, HNF4A, and HNF1 (Boj et al., 2010; Gao et al., 2008; Lango Allen et al., 2012; Lee et al., 2005; Li et al., 2000; Watt et al., 2007).

If these enhancers are indeed activated during terminal pancreatic differentiation or in alternate endodermal organs, one would expect enhancers in clusters P3 and P4 to selectively acquire H3K27ac in cells of gut-tube-derived organs. To test this in an unbiased fashion, we analyzed the H3K27ac status of enhancers from clusters P3 and P4 in differentiated tissues and cells originating from all three germ layers (Figure 3D). First, we identified distal tissue-specific H3K27ac peaks by querying data from the Roadmap Epigenomics Project as well as the Encyclopedia of DNA Elements (ENCODE) Consortium (Figure 3E). As expected, genes linked to tissue-specific H3K27ac peaks associated with biological processes characteristic of the respective tissue (Table S2). Next, we determined the extent to which these tissue-specific active enhancers were represented in each of the poised enhancer clusters. Enhancers in clusters P1 and P2, which are exclusively poised at the hESC or DE stage, showed no consistent overrepresentation in tissues from a specific germ layer (Figures S3A and S3B). By contrast, enhancers from clusters P3 and P4, which acquire a poised state at the GT stage, were significantly enriched among active enhancers in endoderm-derived tissues, including islet, liver, lung, pancreas, and small bowel (Figures 3F and 3G). Furthermore, endodermal active enhancers were only enriched in poised clusters P3 and P4 when compared to clusters P1 and P2 (Figures 3H–3J and S3C–S3N). Altogether, these results demonstrate that a poised chromatin landscape for enhancers linked to genes of multiple gut-tube-derived lineages is gained en masse during the DE-to-GT transition. This suggests that instructive information for gene expression programs of multiple descendant lineages is actively acquired and becomes encoded into enhancer chromatin at this time.

Acquisition of a Poised State at Lineage-Specific Enhancers Is Indicative of Developmental Competence

Developmental competence refers to a cell's ability to respond to inductive signals during development (Waddington, 1940).

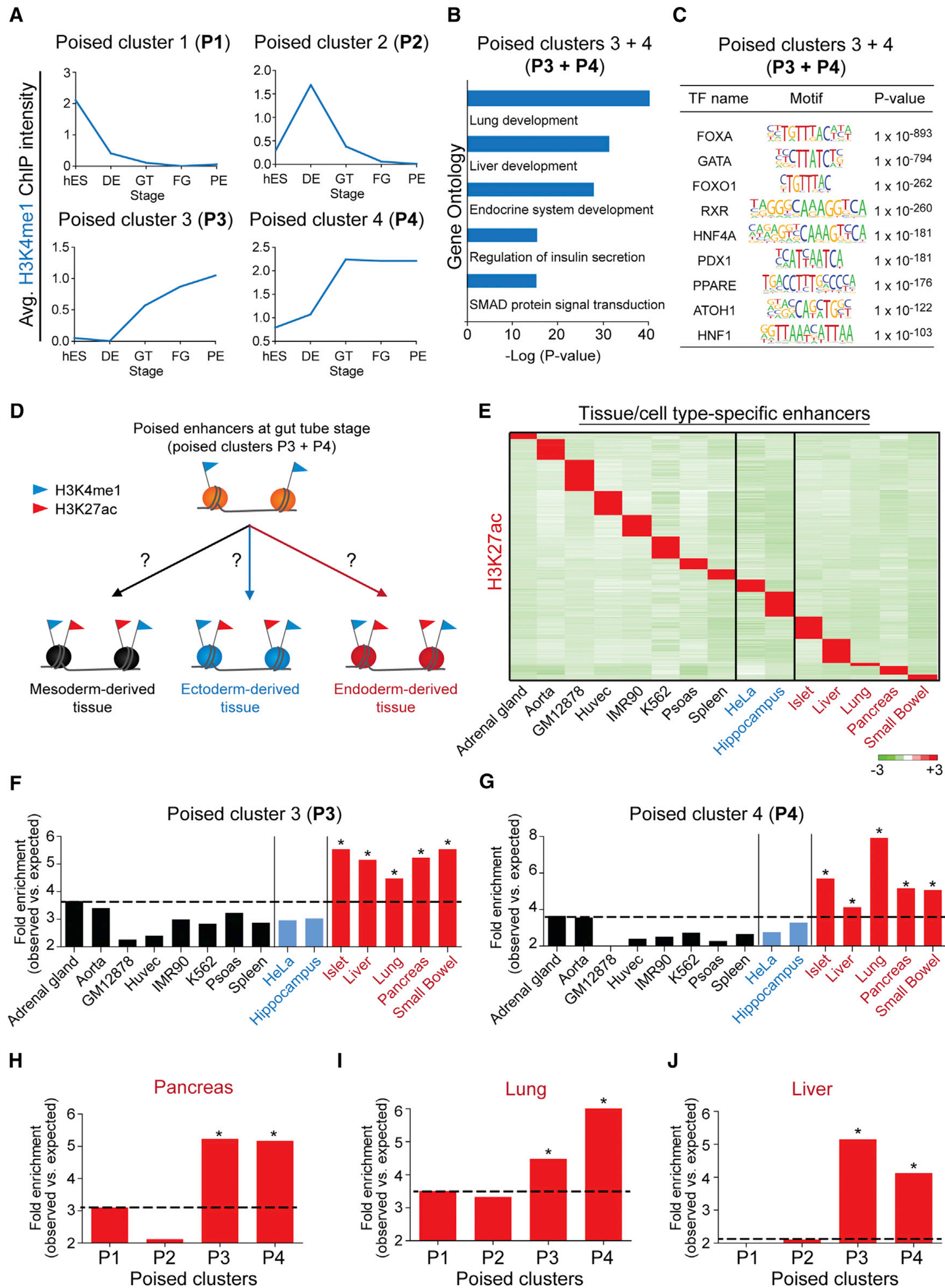

Figure 3. A Poised Enhancer Landscape for Multiple Endodermal Lineages Is Established in GT Lineage Intermediates

(A) Average (Avg.) H3K4me1 ChIP-seq signal intensity during pancreatic differentiation for enhancers in poised clusters P1, P2, P3, and P4 (see Figure 2A). hES, hESCs.

(B) Enriched Gene Ontology terms for enhancers in poised clusters P3 and P4.

(C) Enriched TF binding motifs with associated p values for enhancers in poised clusters P3 and P4.

(D) Experimental strategy to determine the activity of poised enhancers in tissues and cell types derived from mesoderm, ectoderm, and endoderm.

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We hypothesized that "poising" lineage-specific enhancers in GT intermediates could be a mechanism by which cells become competent to adopt endodermal organ fates in response to organ-inductive signals. To test this idea, we exposed DE- and GT-stage cells briefly to pancreas-, lung-, or liver-inductive cues and determined whether early lineage markers are induced (Figure 4A). Because pancreas, lung, and liver enhancers become poised in GT intermediates, we predicted that GT-stage cells, but not DE-stage cells, will readily respond to appropriate organ-inductive cues. First, to test pancreatic competence, we exposed DE and GT cells to pancreas-inductive factors (retinoic acid [RA], cyclopamine [Cyc], and noggin [Nog]) for 2 days. We found that expression of early pancreatic lineage markers, including PDX1, PROX1, and SOX9, was readily induced from GT cells (Figures 4B and S4A). In contrast, this was not the case for DE cells (Figures 4B and S4A). Second, to test lung competence, we cultured DE and GT cells for 3 days in the presence of lung-inductive factors (CHIR99021 [CHIR], fibroblast growth factor [FGF], keratinocyte growth factor [KGF], bone morphogenetic protein [BMP], and RA) (Huang et al., 2014). Analogous to our findings for pancreas, only GT-stage cells responded by activating expression of the lung markers SOX2 and NKX2.1 (Figure 4C). Finally, a similar experiment testing hepatic competence showed that GT-stage cells, but not DE-stage cells, quickly responded by expressing hepatic markers such as AAT, Transferrin, and AFP when exposed to the liver-inductive factors BMP and FGF (Wang and Sander, 2012) for 3 days (Figures 4D and S4B). The result that lung and liver could be induced from GT-stage cells was unexpected because the differentiation protocol has been specifically developed for production of pancreatic cells. Together, these findings establish a clear temporal connection between the acquisition of a poised enhancer state at organ-specific enhancers and the gain of developmental competence for the respective lineage.

Given the rapid induction of hepatic genes from GT-stage cells after exposure to BMP and FGF, we postulated that liver-inductive cues convert poised enhancers into an active state. To test this contention, we mapped active enhancers specific to GT-derived hepatic cells (Table S3). Consistent with the cells' hepatic identity, enhancers activated upon hepatic induction were proximal to key hepatic regulators (i.e., *C/EBP-α*), associated with genes for liver-specific biological processes and were enriched for recognition motifs of known hepatic TFs (Figures 4E, S4C, and S4D). Strikingly, 55% of active enhancers specific to GT-derived hepatic cells belonged to poised clusters P3 and P4, which gain H3K4me1 in GT intermediates (Figure 4F). A similar enrichment was not seen for poised clusters P1 and P2, which gain H3K4me1 at earlier stages (Figure 4G). These results demonstrate that the majority of enhancers that are activated in response to liver-inductive cues acquire a poised state at the transition from endoderm to GT.

Identification of TFs that Regulate Enhancers during Competence Establishment and Lineage Induction

Because enhancers contain clusters of TF binding sites (Buecker and Wysocka, 2012), we reasoned that important transcriptional regulators of developmental competence and lineage induction could be identified by analyzing lineage-specific enhancers. Therefore, to identify potential regulators of pancreatic competence and lineage induction, we performed MOTIF enrichment analysis for pancreatic enhancers (FG/PE and PE clusters). This analysis revealed enrichment for FOXA, GATA, PDX1, HNF4A, HNF1, and RFX motifs (Figure 5A), which bind TFs with documented functions in pancreas development (Boj et al., 2010; Gao et al., 2008; Jonsson et al., 1994; Lango Allen et al., 2012; Smith et al., 2010).

To begin to define the specific roles of candidate TFs at pancreas-specific enhancers, we examined mRNA levels of TFs corresponding to the enriched binding motifs. Our analysis revealed two general patterns: (1) TF expression coincides with pancreatic gene induction at the FG stage, and (2) TF expression is initiated in GT intermediates or prior (Figure S5A). As expected, the regulator of early pancreatic development *PDX1* (Ahlgren et al., 1996; Jonsson et al., 1994) belonged to the group of TFs that was first expressed at the point of pancreas induction (Figure 5B). It is interesting that expression of the FOXA family of TFs was initiated prior to pancreas induction (Figure 5B). FOXAs are known to function as pioneer TFs, which can actively open chromatin and facilitate binding of other TFs (Zaret and Carroll, 2011). Based on their temporal expression pattern, we speculated that FOXAs might have a role in establishing competence at poised enhancers, whereas PDX1 might be involved in activating poised enhancers. To test these predictions, we first examined occupancy of pancreatic enhancers by FOXAs and PDX1 using chromatin immunoprecipitation sequencing (ChIP-seq) (Tables S4, S5, and S6). We found that 34.8% (1,943/5,581; compared to an expected 2.8% by random chance) of pancreatic enhancers were indeed occupied by FOXA1 or FOXA2 at the GT stage prior to enhancer activation (Figure 5C). After pancreatic induction, 44.7% (2,497/5,581; compared to an expected 3.6% by random chance) of pancreatic enhancers showed binding of PDX1 (Figure 5D). Among the enhancers that were sequentially occupied by FOXAs and PDX1 was an enhancer for the early pancreatic regulator *PTF1A* (Figure 5E). Mutations in this enhancer have been linked to familial pancreatic agenesis (Weedon et al., 2014).

Next, to investigate whether FOXAs have a regulatory role at poised enhancers, we delivered short hairpin RNAs (shRNAs) targeting *FOXA1* to hESCs and differentiated these cells toward the pancreatic lineage. shRNA-mediated *FOXA1* knockdown caused an ~2-fold reduction in *FOXA1* transcript levels at the GT stage (Figure S5B). When *FOXA1*-depleted GT intermediates were further differentiated toward pancreas, we observed a reduction in mRNA levels for early pancreatic markers, including

(E) Heatmap showing H3K27ac ChIP-seq signal intensity for H3K27ac peaks specific to each listed tissue or cell type. Mesoderm-derived cells are depicted in black, ectoderm-derived cells are depicted in blue, and endoderm-derived cells are depicted in red.

(F and G) Enrichment of tissue- or cell-type-specific H3K27ac peaks in poised enhancer clusters P3 (F) (*p < 2.7e-8, chi-square test) and P4 (G) (*p < 0.03, chi-square test).

(H–J) Enrichment of pancreas-specific (H), lung-specific (I), and liver-specific (J) H3K27ac peaks in poised enhancer clusters P1, P2, P3, and P4. *p < 0.02, chi-square test.

See Figure S3 and Table S2.

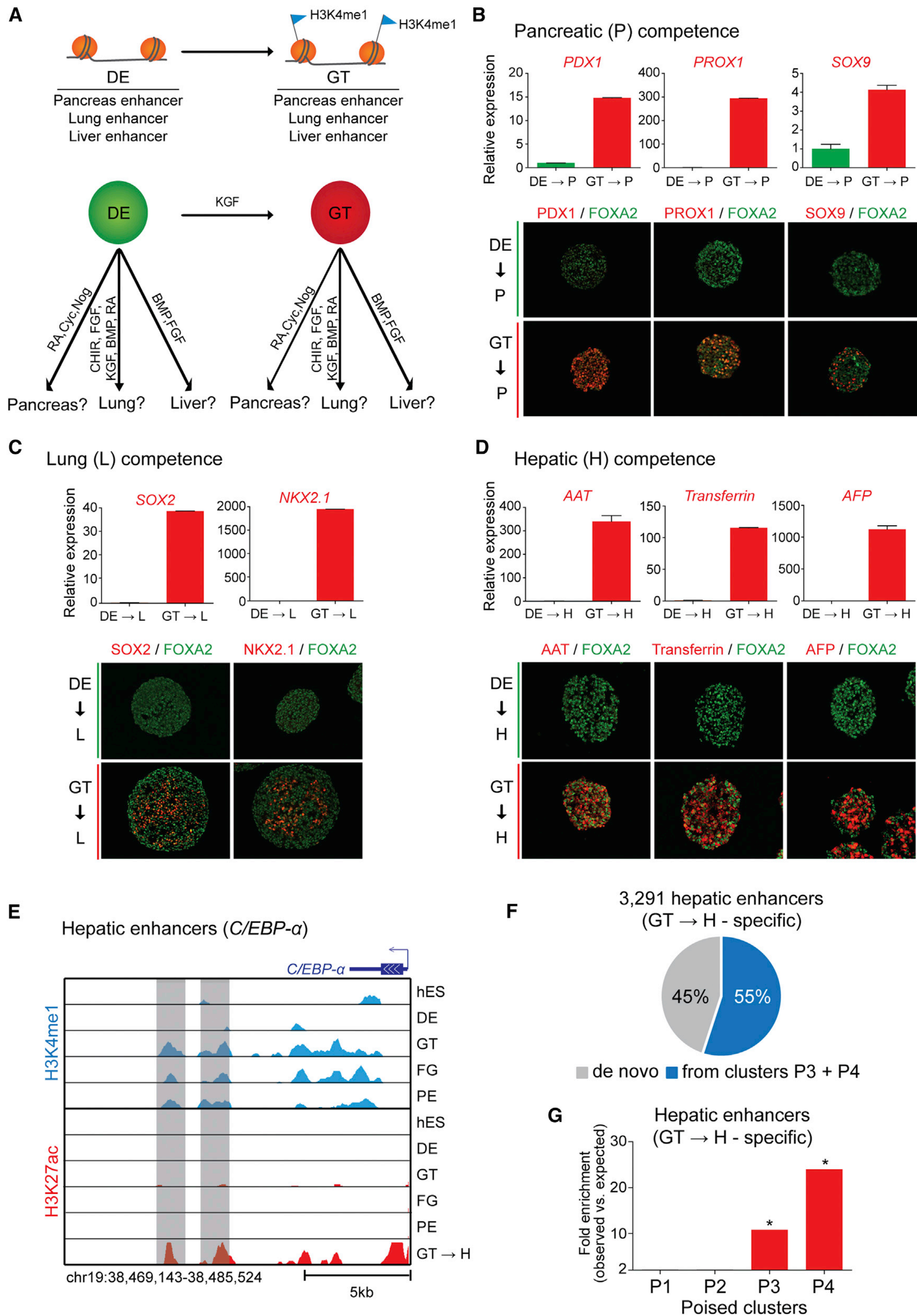


Figure 4. The Acquisition of a Poised Enhancer State Coincides with Gain of Developmental Competence

(A) Experimental strategy to test the competence of endodermal intermediates to activate pancreatic (P), lung (L), or hepatic (H) genes in response to their respective organ inductive signals.

(legend continued on next page)

PDX1, *SOX9*, *NKX6.1*, and *PTF1A* (Figure S5B), showing that *FOXA1* is necessary for the proper expression of early pancreatic genes. Given that the temporal pattern of FOXA recruitment to pancreatic enhancers mirrors H3K4me1 levels (Figures 2E and 5C), we tested whether *FOXA1* is required for H3K4me1 deposition at pancreatic enhancers. Examination of H3K4me1 levels by ChIP-qPCR analysis at multiple FOXA1-bound pancreatic enhancers did not reveal a noticeable difference in H3K4me1 enrichment between *FOXA1* knockdown and control cells at the GT stage (Figure 5F). These results suggest that FOXA1 is not directly involved in methylating histone 3 at lysine 4 but, instead, might recognize a poised enhancer state and help facilitate subsequent activation. This result is consistent with prior findings in different cell lines (Lupien et al., 2008).

We next investigated whether PDX1 is necessary for the activation of pancreatic enhancers by examining the effect of *PDX1* inhibition on expression of pancreatic genes and histone acetylation at enhancers. Consistent with the role of PDX1 in mice and humans (Jonsson et al., 1994; Stoffers et al., 1997), *PDX1* knockdown cells failed to initiate the expression of important early pancreatic genes upon directed pancreatic differentiation (Figures S5C and S5D). ChIP-seq analysis of H3K27ac in *PDX1* knockdown cells after pancreas induction revealed a significant decrease in H3K27ac intensity at PDX1-bound pancreatic enhancers (Figure 5G). For example, PDX1-bound enhancers near the genes encoding the pancreatic TFs SOX9 and NKX6.1 exhibited a drastic reduction in H3K27ac signal (Figure 5H). Thus, PDX1 occupies pancreatic enhancers and is required for their activation. Together, our findings suggest a model whereby pancreatic enhancers assemble sequentially. In the primitive GT, pancreatic enhancers acquire a poised state and become occupied by FOXA TFs. Exposure to pancreas-inductive signaling cues subsequently leads to PDX1 induction, its recruitment to pre-marked pancreatic enhancers, and histone acetylation (Figure 5I).

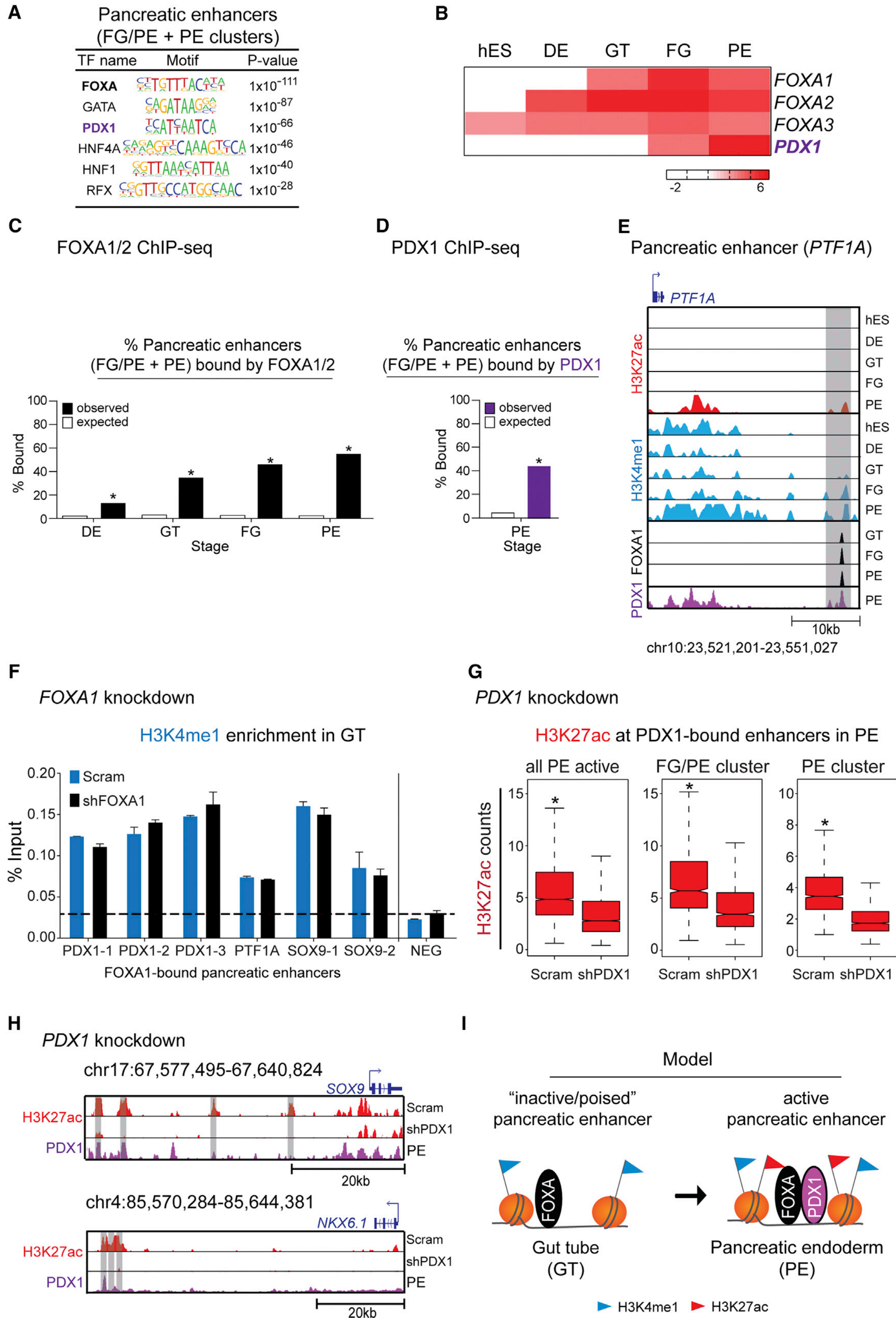
To examine whether this model of stepwise enhancer assembly applies more generally to endodermal lineages, we analyzed motifs of hepatic enhancers to make predictions about which TFs could poise and which could activate hepatic enhancers during liver differentiation. We reasoned that, by comparing motifs at hepatic enhancers that emerge from poised enhancers at the GT stage to motifs at de novo active hepatic enhancers, we could identify TFs with potential roles in "poising" or "activating" enhancers. This analysis revealed a specific enrichment for FOXA motifs at those hepatic enhancers that are poised in GT intermediates (clusters P3 and P4) (Figure S5E). 54.2% of active hepatic enhancers (981/1810; compared to an expected 3.0% by random chance) that are poised in GT were indeed occupied by either FOXA1 or FOXA2 prior to hepatic induction (Figures S5F and S5G). Thus, FOXAs associate with a poised enhancer landscape for multiple endodermal organ lineages prior to lineage induction. The requirement of FOXAs for pancreas (Figure S5B) and liver (Lee et al., 2005) development suggests that this early association of FOXAs with lineage-specific enhancers helps prepare poised enhancers for future activation. Similar to PDX1 in pancreas, enhancer activation in liver likely requires additional TFs. Comparison of TF recognition motifs at enhancers in poised clusters P3 and P4 that become active in GT-derived hepatic cells to motifs at enhancers in clusters P3 and P4 not active in hepatic cells revealed overrepresentation of binding motifs for HNF4A, HNF1, and TEAD (Figure S5H), suggesting that these TFs could be involved in the activation of hepatic enhancers.

Enhancers for Islet Cell Functional Genes Are Poised prior to Terminal Differentiation

An important characteristic of hESC-derived PE is its competency to differentiate into functional endocrine cells after subcutaneous engraftment into mice (Kroon et al., 2008; Xie et al., 2013). Based on our observation that a poised enhancer state indicates developmental competence during endodermal organ lineage induction, we postulated that a similar priming of enhancer chromatin could render pancreatic progenitor cells competent to activate endocrine functional genes in response to extrinsic signals. To test this, we first identified active enhancers in human cadaveric islets (Figures S6A and S6B; Table S7) and examined the extent to which these active islet enhancers are poised in PE prior to terminal differentiation. Strikingly, 25.5% (3,427/13,422) of all active islet enhancers are already poised in PE (Figure 6A). This is remarkable, considering that the differentiation of pancreatic progenitor cells into functional islet cells requires multiple developmental steps and considerable time. Furthermore, the select group of poised enhancers is relevant for the regulation of genes associated with vital islet-specific cellular properties, such as hormone biosynthesis and insulin secretion (Figure 6B). One such example is the type-2-diabetes-associated gene *SLC30A8* (van Hoek et al., 2008), for which we identified six associated enhancers that are poised in PE and active in islets (Figure 6C). This analysis shows that enhancers for important genes involved in the regulation of endocrine cell function are pre-marked in pancreatic progenitor cells. Thus, instructive information for gene expression programs of islet function appears to be programmed into enhancer chromatin prior to terminal differentiation.

To examine whether TFs are also sequentially recruited to islet enhancers as observed during pancreatic and hepatic lineage induction, we analyzed binding motifs at enhancers that are

(B–D) qRT-PCR analysis and immunofluorescence staining for the early pancreas markers PDX1, PROX1, and SOX9 in DE and GT cells treated with RA, Cyc, and Nog for 2 days (B); the early lung markers SOX2 and NKX2.1 in DE and GT cells treated with CHIR, FGF, KGF, BMP, and RA for 3 days (C); and the early liver markers alpha 1-antitrypsin (AAT), transferrin, and alpha-fetoprotein (AFP) in DE and GT cells treated with BMP and FGF for 3 days (D). Data are shown as average ± SEM.
(E) H3K27ac and H3K4me1 ChIP-seq profiles of enhancers in poised clusters P3 and P4 near the hepatic gene *C/EBP-α*. hES, hESCs.
(F) Percentage of hepatic enhancers (specific H3K27ac peaks in GT cells treated with BMP and FGF for 3 days; GT → H cells) that are poised in GT intermediates (clusters P3 + P4 in Figure 2A).
(G) Enrichment of hepatic enhancers (GT → H-cell-specific H3K27ac peaks) in poised enhancer clusters P1, P2, P3, and P4 (see Figure 2A). *$p < 2.2e-16$, chi-square test. RA, retinoic acid; Cyc, cyclopamine; Nog, noggin; CHIR, CHIR99021; FGF, fibroblast growth factor; KGF, keratinocyte growth factor; BMP, bone morphogenetic protein; hES, human embryonic stem cells; DE, definitive endoderm; GT, primitive gut tube; FG, posterior foregut; PE, pancreatic endoderm.
See also Figure S4 and Table S3.



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poised in PE and active in islets. Specifically, to identify TFs with potential roles at poised enhancers, we compared motifs at active islet enhancers, which are poised at the PE stage, to motifs at active islet enhancers, which are not poised at the PE stage. This analysis revealed enrichment for FOXA motifs at poised islet enhancers (Figure 6D). 39.3% (1,348/3,427; compared to an expected 2.5% by random chance) of these enhancers were occupied by either FOXA1 or FOXA2 in PE prior to endocrine differentiation (Figures 6C and 6E), showing that FOXAs indeed associate with islet enhancers prior to their activation. When we compared the motifs at poised enhancers that become active in islets to motifs at poised PE enhancers that do not become active in islets, we found enrichment for RFX and FOXO1 motifs (Figure 6F), indicating that these TFs could be important for activating islet enhancers during endocrine cell differentiation. In summary, these findings suggest a potential role for FOXA factors at poised islet enhancers in early embryonic intermediates and for FOXO1 and RFX family TFs in the subsequent activation of islet enhancers during terminal differentiation. These predictions bear a striking resemblance to our analysis identifying TFs regulating the stepwise activation of enhancers during pancreatic and hepatic lineage induction from the GT. Altogether, these observations support a model whereby FOXAs at poised enhancers play a critical role in establishing competence throughout development, including terminal differentiation into functional cell types.

DISCUSSION

Acquisition of Developmental Competence through De Novo Poising of Lineage-Specific Enhancers

An unanswered question in developmental biology is, which cell-intrinsic mechanisms enable developmental intermediates to specifically activate cell identity genes in response to extrinsic signaling cues? Here, we demonstrate that this cell-intrinsic property, referred to as developmental competence, is functionally linked to a poised chromatin state at cell-type-specific enhancers. Our findings suggest that bookmarking cell identity genes at the level of enhancers endows cells with the ability to interpret environmental differentiation cues correctly. Thus, the annotation of the poised enhancer repertoire during developmental progression can provide prescient information about future cellular states.

A key characteristic of developmental competence is that it is not a passive state but is actively acquired during differentiation. For example, endodermal cells activate pancreatic genes in response to co-culture with notochord. However, notochord is only capable of acting on endoderm after the endoderm has received prior instruction from mesoderm/ectoderm (Wells and Melton, 2000). Analogous to these findings in primary embryonic tissues, we observed that the competence to activate pancreas, lung, and liver genes in response to extrinsic signaling cues is actively acquired at the transition from DE to GT during the in vitro differentiation of hESCs. Because the de novo poising of lineage-specific enhancers coincides with the acquisition of developmental competence for pancreas, lung, and liver induction, our findings strongly suggest a functional link. We similarly find that enhancers associated with genes controlling endocrine cell function are poised in pancreatic progenitor cells, suggesting that poising cell-type-specific enhancers is relevant at multiple developmental steps, including terminal differentiation. Certainly, other transcriptional priming mechanisms, such as a bivalent chromatin state at the level of promoters (Bernstein et al., 2006; Mikkelsen et al., 2007; Xie et al., 2013), may also be biologically important for a cell's developmental potential. However, in contrast to the poised enhancer state, which is acquired during development with precise timing, promoters of organ-specific genes often exhibit a bivalent state already in pluripotent stem cells. Thus, bivalent domains alone cannot explain the acquisition of developmental competence during lineage progression. Our findings help explain why developmental intermediates respond to signaling cues with high precision in time.

Stepwise Enhancer Assembly during Lineage Progression

Our data show that the stepwise developmental transition at lineage-specific enhancers from unmarked chromatin to poised chromatin and then to histone H3K27 acetylation is associated with the sequential assembly of distinct classes of TFs at these enhancers. Specifically, our findings suggest that pioneer TFs—in particular, FOXAs—play a role at poised enhancers, while lineage-specifying TFs promote the transition from a poised to an active enhancer state. Whereas our work experimentally demonstrates a role for PDX1 in activating poised pancreatic enhancers, motifs overrepresented at hepatic and islet enhancers indicate similar functions for HNF4A and RFX

Figure 5. FOXA TFs and PDX1 Are Sequentially Recruited to Pancreatic Enhancers

(A) Enriched TF binding motifs with associated p values for pancreatic enhancers (FG/PE- and PE-specific clusters).

(B) Heatmap showing mRNA expression levels, measured in FPKM, of *FOXA* family TFs and *PDX1* during pancreatic differentiation. hES, hESCs.

(C) Percentage of pancreatic enhancers versus random genomic regions bound by FOXA1 or FOXA2 at the DE, GT, FG, and PE stages. *$p < 2.2e-16$, chi-square test.

(D) Percentage of pancreatic enhancers versus random genomic regions bound by PDX1 in PE. *$p < 2.2e-16$, chi-square test.

(E) H3K27ac, H3K4me1, FOXA1, and PDX1 ChIP-seq profiles at a candidate enhancer near *PTF1A* during pancreatic differentiation.

(F) H3K4me1 enrichment by ChIP-qPCR at FOXA1-bound pancreatic enhancers in scrambled control (Scram) and *FOXA1* knockdown (shFOXA1) cells differentiated to GT.

(G) Box plots of H3K27ac ChIP-seq counts at all enhancers active in PE, FG/PE-specific, and PE-specific enhancers in scrambled control (Scram) and *PDX1* knockdown (shPDX1) cells differentiated to PE. *$p < 2.2e-16$, Wilcoxon rank-sum test.

(H) H3K27ac ChIP-seq profiles at enhancers near *SOX9* and *NKX6.1* in Scram and shPDX1 cells differentiated to PE as well as the PDX1 ChIP-seq profile at the same enhancers in PE.

(I) Model for the stepwise activation of pancreatic enhancers. Pioneer TFs, such as FOXAs, associate with poised enhancers in GT intermediates. Lineage-specific TFs, such as PDX1, subsequently regulate the transition from a poised to an active enhancer state.

See also Figure S5 and Tables S4, S5, and S6.

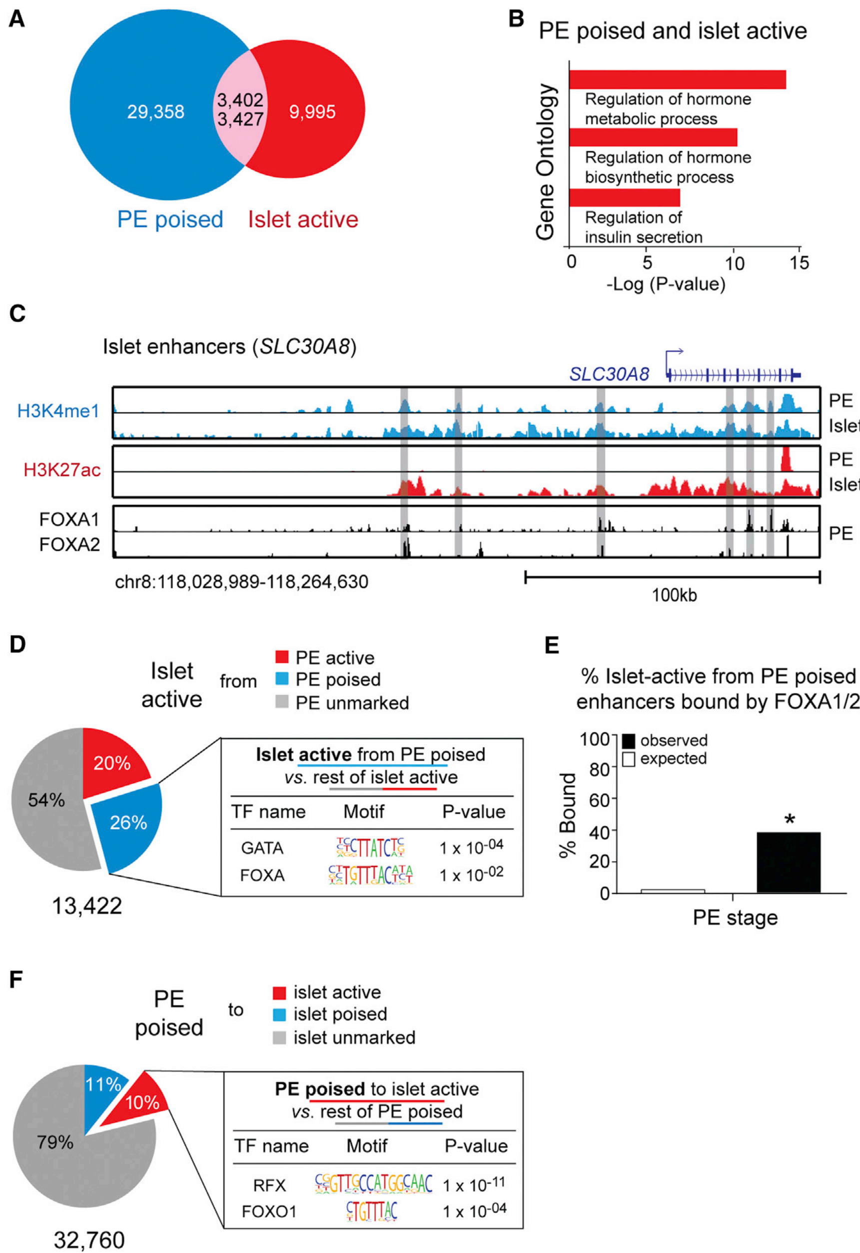

Figure 6. Enhancers Linked to Important Islet Cell Genes Are Poised in Pancreatic Progenitor Cells

(A) Overlap of poised enhancers in PE and active enhancers in cadaveric human islets. 3,402 out of 32,760 poised enhancers in PE become active in islets. 3,427 out of 13,422 active enhancers in islets are poised in PE.

(B) Enriched Gene Ontology terms for enhancers that are poised in PE and become active in islets.

(C) H3K4me1, H3K27ac, FOXA1, and FOXA2 ChIP-seq profiles in PE and islets at enhancers near *SLC30A8*.

(D) Enriched TF binding motifs with associated p values for the 26% of active islet enhancers that are poised in PE versus the remaining 74% of active islet enhancers.

(E) Percentage of enhancers, which are poised in PE and active in islets, versus random genomic regions bound by FOXA1 or FOXA2 in PE. *p < 2.2e-16, chi-square test.

(F) Enriched TF binding motifs with associated p values for the 10% of poised enhancers in PE that become active in islets versus the remaining 90% of poised enhancers in PE.

See also Figure S6 and Tables S5, S6, and S7.

factors during hepatic and islet cell differentiation, respectively. Such a notion is consistent with reported phenotypes of *Hnf4a* and *Rfx6* knockout mice (Li et al., 2000; Smith et al., 2010). In contrast to *PDX1* knockdown, which prevented H3K27ac deposition at pancreatic enhancers, *FOXA1* knockdown did not affect H3K4me1 levels. As reported in cell lines (Lupien et al., 2008), it appears that, although necessary for target gene activation, FOXA1 activity is not required for H3K4 methylation in the context of endoderm development. Given the known property of FOXAs to displace nucleosomes (Li et al., 2012), a likely mechanism by which FOXAs regulate enhancer activity is by establishing a transcriptionally permissive enhancer chromatin state.

This raises the question as to which, if any, TFs are responsible for the deposition of H3K4me1 at lineage-specific enhancers. Although our data suggest that FOXA1 is not required, it is possible that other FOXA TFs compensate for FOXA1 and mediate H3K4me1 deposition in FOXA1-deficient cells. Furthermore, other TFs not studied here could likewise play this role. Also unclear is the functional role of H3K4me1 in priming enhancers for future activation. While our data suggest that H3K4me1 deposition alone is not sufficient for future gene activation in a *FOXA1*-depleted state, it remains to be investigated whether enhancer activation requires H3K4 methylation and how TFs and epigenetic modifications cooperatively shape a transcriptionally permissive enhancer landscape prior to gene activation. Recent studies in the context of adipogenesis suggest that H3K4me1 deposition is indeed necessary for enhancer activation (Lee et al., 2013).

Implications for Cellular Reprogramming and Stem Cell Differentiation

The transcriptional priming of lineage-specific enhancers in early lineage intermediates prior to gene activation helps explain the highly context-dependent activity of lineage-specific TFs in cellular programming. Our findings suggest that effective cellular reprogramming of somatic cells requires a combination of both pioneer and lineage-specific TFs. Recent studies show that the reprogramming of fibroblasts into liver, neurons, or pluripotent cells indeed requires the inclusion of pioneer factors (Huang et al., 2011; Soufi et al., 2012; Wapinski et al., 2013). Similarly,

pioneer and lineage-specific TFs cooperatively regulate macrophage and B-cell gene transcription at the levels of enhancers (Heinz et al., 2010, 2013). We speculate that this regulatory logic is pervasive throughout development and relevant for reprogramming lineages from all germ layers.

As stem cell differentiation protocols to derive various terminal differentiated cell types continue to be developed, scaled, and optimized, assessing the acquisition of competence may be an important consideration. For example, the design of large-scale screens to identify molecules or factors that promote a specific differentiation step may be ineffective if the responder cell population has not yet acquired the transcriptional competence to appropriately respond. In addition, one emerging strategy for cell replacement therapy is the transplantation of stem-cell-derived lineage-specific progenitor cells. Inquiring whether these progenitors have acquired the competency or poised chromatin to form the desired therapeutic cell type may be beneficial in the assessment of suitability for transplantation.

EXPERIMENTAL PROCEDURES

hESC Culture

CyT49 hESCs were maintained and differentiated as previously described with minor modifications (Kroon et al., 2008; Schulz et al., 2012). hESC research was approved by the University of California, San Diego, Institutional Review Board and Embryonic Stem Cell Research Oversight Committee. For further details, see the Supplemental Experimental Procedures.

ChIP-Seq and Data Analysis

ChIP-seq was performed as previously described with minor modifications (Hawkins et al., 2010). All the sequencing experiments were performed using Illumina Hi-Seq 2000 instruments. Each read was aligned to the human genome build hg18 with Bowtie (Langmead et al., 2009). We used the first 36 base pairs (bp) for the alignment and only kept reads with up to two mismatches. Duplicated reads from the same library were removed. Datasets from highly correlated biological replicates were pooled for subsequent analysis. MACS (Zhang et al., 2008) was used for peak calling. Peaks were further filtered as described previously (Shen et al., 2012). For further details, see the Supplemental Experimental Procedures.

Enhancer Predictions

Enhancers were predicted as described previously, using H3K4me1, H3K4me3, and H3K27ac (Rajagopal et al., 2013). We first divided the human genome into 100-bp bins and counted the number of reads that fell within each bin. Then, the tag counts in each bin were normalized against the total number of reads and input as described previously (Shen et al., 2012). The normalized signals for each mark were merged as one input file for the enhancer prediction pipeline. To compute the false discovery rate (FDR), we first shuffled the rows and columns of the input data. Second, we ran the enhancer prediction pipeline on this simulated data. The FDR was computed as the ratio of the number of predicted enhancers from simulated data over the real data. We required that predicted enhancers have an FDR of $<2\%$ and are at least 3 kb away from a known transcriptional start site.

ACCESSION NUMBERS

All ChIP-seq and GRO-seq datasets have been deposited into GEO under accession number GSE54471.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2015.02.013.

AUTHOR CONTRIBUTIONS

A.W. and M.S. conceived the project. A.W., F.Y., Y.L., B.R., and M.S. designed the experiments and the data analysis. A.W., Y.L., R.X., T.H., N.P., K.M., J.P., J.W., D.L., and J.R. performed the experiments. F.Y., A.W., and Y.Q. performed the data analysis. A.W., F.Y., and M.S. wrote the manuscript.

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