GATA6 is predicted to regulate DNA methylation in an in vitro model of human hepatocyte differentiation

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Hepatocytes are the dominant cell type in the human liver, with functions in metabolism, detoxification, and producing secreted proteins. Although gene regulation and master transcription factors involved in the hepatocyte differentiation have been extensively investigated, little is known about how the epigenome is regulated, particularly the dynamics of DNA methylation and the critical upstream factors. Here, by examining changes in the transcriptome and the methylome using an in vitro hepatocyte differentiation model, we show putative DNA methylation-regulating transcription factors, which are likely involved in DNA demethylation and maintenance of hypo-methylation in a differentiation stage-specific manner. Of these factors, we further reveal that GATA6 induces DNA demethylation together with chromatin activation in a binding-site-specific manner during endoderm differentiation. These results provide an insight into the spatiotemporal regulatory mechanisms exerted on the DNA methylation landscape by transcription factors and uncover an epigenetic role for transcription factors in early liver development.
Hepatocytes, the major parenchymal cells in the liver, are responsible for key liver functions such as metabolism and detoxification. In embryogenesis, the first fate decision to the hepatocyte lineage is the differentiation of primitive streak cells to definitive endoderm (DE) cells, which are a common precursor of endodermal tissues such as the liver, pancreas, and gut. Hepatoblasts are hepatic progenitor cells derived from the DE cells, which then sequentially differentiate into fetal-like hepatocytes and mature hepatocytes. Thus, hepatocytes emerge from pluripotent stem cells through several progenitor cell types.

Several transcription factors (TFs), including c-Jun, members of the Hepatocyte Nuclear Factor (HNF), and GATA family genes, are known to play important roles in liver development and hepatocyte differentiation. Notably, GATA6 knock-out mice die around E5.5 due to a deficiency of extra-embryonic endoderm development, which can be rescued by tetraploid embryo complementation assays, indicating that GATA6 is required for liver development and hepatic specification. Thus, multiple TFs sequentially and coordinately regulate peripheral genes necessary for hepatocyte differentiation.

Gene expression dynamics are regulated not only by the action of transcription factors but also by epigenetic modifications such as DNA methylation. DNA methylation of gene regulatory regions appears to be associated with silencing the expression of the downstream gene. Therefore, the DNA methylation profile is dramatically altered during embryogenesis and cellular differentiation, with roles in tightly regulating the expression of downstream genes. Indeed, DNA methylation plays a crucial role in the expression of numerous liver-specific genes, and DNA methyltransferase (DNMT) inhibitors facilitate trans-differentiation of adipose tissue-derived stem cells or mesenchymal stem cells to hepatocyte-like cells (HLCs). Collectively, these findings show that DNA methylation is a crucial factor for hepatic differentiation.

The gain of DNA methylation is directly achieved by de novo DNMTs and is maintained during cell divisions by a maintenance DNMT. On the other hand, DNA demethylation is achieved by cell proliferation dependent passive DNA demethylation or active DNA demethylation based on sequential oxidative processes by ten-eleven translocase (TET) enzymes, followed by base-excision repair. In addition, the oxidized forms of methylated cytosine (5-hydroxymethyl cytosines (5hmC), 5-formyl cytosine (5fC), and 5-carboxy cytosine (5caC)) are also depleted by passive demethylation mechanisms as these bases are not recognized by the maintenance DNA methylation mechanism. Thus, DNA methylation is a balance between the gain and loss of methylated bases.

In addition to the mechanisms by which DNA methylation is gained and lost, mechanisms underlying spatiotemporal regulation of DNA methylation are also critical in understanding the overall dynamics of DNA methylation. We and other groups recently reported that some TFs regulate the timing and tissue-specificity of DNA demethylation. Thus, a growing body of evidence suggests critical roles for TFs in the regulation of DNA methylation. However, the epigenetic roles of TFs specific for hepatocyte differentiation are yet to be identified.

In the present study, we combine TF binding motif (TFBM) over-representation analysis for differentially methylated regions with transcriptome analysis. We identified TFs with putative roles in regulating DNA methylation during hepatocyte differentiation by studying in vitro process of hepatocyte differentiation from human induced pluripotent stem cells (iPSCs). Of these TFs, we demonstrated that GATA6 is a master regulator for DNA demethylation and chromatin activation during the differentiation of the DE. Our data provide important insights into the regulatory mechanisms shaping the DNA methylation landscape during hepatocyte differentiation.

### Results

**Evaluation of an in vitro hepatocyte differentiation model.** We induced HLCs from human iPSCs in vitro using the Cellartis hepatocyte differentiation system (Takara bio), which is composed of three differentiation steps: iPSC to DE-like cell, DE-like cell to hepatoblast-like cell, and hepatoblast-like cell to HLC, followed by a maintenance culture (Fig. 1a, b). It has been reported that although this hepatic differentiation protocol does not activate several hepatic function-related genes, the efficiency of the hepatic specification is relatively high.

Indeed, over 96% of the cells in days 21 and 28 cultures were HNF4a positive without pancreatic or cardiac cell marker expressing cells (Fig. 1c, d and Supplementary Fig. 1a). Tissue-specific gene enrichment analysis for the upregulated genes between day 0 and day 28 revealed enrichments of endodermal tissue, such as the liver, supporting the hepatic differentiation (Supplementary Fig. 1b). To evaluate the differentiation in detail, we analyzed the mRNA expression of differentiation markers. Concurrent with the decrease of pluripotent markers, DE markers peaked at day 7, indicating the DE-like cell stage (Supplementary Fig. 1c). On day 14, hepatoblast markers were upregulated, indicating the hepatoblast-like cell stage (Supplementary Fig. 1c). Between day 14 and day 21, hepatic markers were upregulated, and a part of them kept the high expression during day 21 and 28, although others were decreased (Supplementary Fig. 1c). Alpha-fetoprotein (AFP), produced by fetal livers but not by adult livers, increased to 4.6-fold greater expression than the published fetal liver CAGE expression data between day 7 and day 21. Then AFP expression decreased to 49.2% of the fetal liver level from day 21 to day 28 (Supplementary Figs. 1c, 2a). AFP protein was also detected by immunocytochemistry of day 21 cells and Enzyme-linked immunosorbent assay in the culture medium (Fig. 1e and Supplementary Fig. 2b).

Whereas, although expression of Albumin (ALB), which is expressed in mature hepatocytes, was increased upon differentiation from day 14, it was much lower than that of the published hepatocyte, fetal liver, and adult liver (Supplementary Figs. 1c, 2a). Thus, these results suggest that day 21 and 28 cultures correspond to the fetal or immature hepatocyte stage, consistent with the earlier report.

Collectively, although the day 28 cells have the drawback of several mature hepatic gene expressions, the in vitro hepatocyte differentiation recapitulates the in vivo liver development until the stage of fetal or immature hepatocytes.

**DNA methylation dynamics throughout hepatocyte differentiation.** To investigate changes in DNA methylation during the in vitro hepatocyte differentiation model, we performed a methylome analysis of the time-course samples. Hierarchical clustering showed that iPSCs and DE-like cells were segregated from the differentiated cells that followed in the time-course, consistent with a commitment to the hepatocyte lineage (Fig. 1f). Comparing adjacent time points, we identified 3088, 446, 38, and 54 methylated CpGs and 3809, 11652, 7383, and 864 demethylated CpGs in each interval, indicating the bias toward demethylation (loss of methylation) (Fig. 1g). The expression of DNA methyltransferases tended to decrease with differentiation, in line with the decline in the number of methylated probes (Supplementary Fig. 2c). While the expression of TET1 decreased with differentiation, that of TET2 spiked on day 14, when the number of identified demethylated probes was the highest. Thus, these data suggest that the bias toward demethylation depends on the balance of methylation and demethylation enzymatic activities.

We associated biological functions to the differentially methylated regions using the Genomic Region Enrichment of Annotations Tool (GREAT) and summarized the results based...
on semantic similarity\textsuperscript{23}. This analysis revealed an enrichment in development and morphogenesis-related Gene Ontologies (GOs), including "pattern specification process", "anatomical structure development", "radial pattern formation", "developmental process", and "regulation of developmental process" (Supplementary Fig. 3). Overall, these results imply that DNA methylation mainly regulates genes related to the developmental process, consistent with specifying the cells into the hepatocyte lineage.

**Prediction of DNA methylation-regulating transcription factors throughout hepatocyte differentiation.** To identify TFs that regulate binding site-directed DNA methylation (hereafter referred to as DNA methylation-regulating TFs), we performed TFBM over-representation analysis for the differentially methylated CpG regions between two adjacent time points of the differentiation time-course. Because some TFs, such as TFs in the same family, share the same or similar binding motif, the results of TFBM over-representation analysis often include false positives. Therefore, to reduce the possibility of false positives, we further narrowed down the overrepresented TFBMs by considering TF expression (CAGE tag-per-million (TPM) ≥50) in either of the two adjacent time points of an interval (Fig. 2a). Comparing each adjacent time point, we identified in total 16 putative DNA methylation-regulating TFs in the methylated regions. Of these, 13 TFs, including POU5F1, a pluripotent cell-specific TF, were identified between Day 0 and Day 7 (Fig. 2b). In addition, GATA6, GATA3, and GATA4 were identified between day 7 and day 14 (Fig. 2b). Interestingly, these putative DNA methylation-regulating TFs for the methylated regions were prone to being highly expressed in the earlier time point of the intervals and then declined with the progress of differentiation (Fig. 2b, c and Supplementary Fig. 4a).

On the other hand, we identified 50 putative DNA methylation-regulating TFs in demethylated regions. Of these, HNF4A, an essential TF for liver development, was identified between day 7 and day 14, and between day 14 and day 21 (Fig. 2d). In addition, the over-representation of TFBMs for activator protein 1 (AP-1) components, such as JUN and FOS, which are involved in stress response and regeneration in the liver\textsuperscript{24}, increased from day 14 to day 21 (Fig. 2d). Importantly, GATA6, GATA4, and GATA3, which were overrepresented in the regions methylated between day 14 to day 21, were first overrepresented in the DE-like cell differentiation stage, and the over-representation of these binding motifs declined as differentiation proceeded (Fig. 2d). Contrary to the putative DNA methylation-regulating TFs for the methylated regions, expression of the putative DNA methylation-regulating TFs for the demethylated regions tends to be upregulated in later time points of the intervals (Fig. 2e and Supplementary Fig. 4b). Taken together, these results suggest that diverse TFs cooperatively regulate the DNA methylation landscape. In particular, GATA transcription factors appear to be the major factors for the regulation of DNA methylation, participating in both methylation and demethylation changes.

**GATA6 regulates binding site-directed DNA demethylation.** Of the GATA proteins, GATA4 and GATA6 are known to be essential TFs for the DE differentiation\textsuperscript{25,26}. Therefore, we focused
GATA6 is reported to be an upstream factor of GATA425. Indeed, greater than 1000-fold at 60 h compared with 48 h, whereas (Fig. 3a). Furthermore, GATA6 expression increased drastically, expression increased only fourfold at 66 h compared with 48 h, whereas GATA4 expression increased increased at 48 and 54 h, respectively, after the induction of differentiation and continued to increase with differentiation from four CpGs which were demethylated by GATA6 overexpression-induced DNA demethylation (Fig. 3b and Supplementary Fig. 5a). A pull-down assay between HaloTag-fused TET proteins and GATA6 revealed an association between TET proteins and GATA6, suggesting that GATA6 recruits TET proteins to their binding sites (Fig. 3c and Supplementary Fig. 5b). Thus, these results suggest that GATA6 induces DNA demethylation, recruiting the TET proteins.

DNA demethylation accompanies GATA6 binding during iPS-DE-like cell differentiation. To investigate the dynamics by which GATA6 regulates DNA demethylation, we performed finer time-course transcriptome and methylome analyses during the time window of GATA6 emergence (after 0, 48, 54, 60, 66, and 72 h of the differentiation process) (Fig. 4a). T, a marker of the primitive streak, was upregulated at 48 h and downregulated after 54 h (Supplementary Fig. 6a). DE markers were upregulated during the period of 48 to 72 h (Supplementary Fig. 6a). In agreement with the qRT-PCR analysis (Fig. 3a), the expression of GATA6 was slightly upregulated at 48 h and drastically increased after 48 h (Supplementary Fig. 6a). Hence, our data indicate that DE commitment occurs during the period of 48 to 72 h.

By comparing adjacent time points, we identified 120 (0 to 48 h), 94 (48 to 54 h), 26 (54 to 60 h), 19 (60 to 66 h), and 50 (66 to 72 h) methylated CpGs and 220 (0 to 48 h), 226 (48 to 54 h), 33 (54 to 60 h), 27 (60 to 66 h), and 27 (66 to 72 h) demethylated CpGs, respectively (Fig. 4b). However, we did not find the GATA6 binding motif overrepresented in those demethylated regions during any interval (Supplementary Fig. 6B). Because the time intervals between adjacent time points are 6 h except for the initial period (0 to 48 h), the changes in methylation levels may not be enough to be detected as demethylation (△M > 2). Indeed, the GATA6 binding motif was overrepresented at the regions demethylated between 0 and 72 h, and these demethylated regions the following analysis on possible epigenetic functions of GATA4 and GATA6 in DE differentiation. First, we performed qRT-PCR to confirm the expression changes of GATA4 and GATA6 during the DE-like cell differentiation. GATA6 and GATA4 expression increased at 48 and 54 h, respectively, after the induction of differentiation and continued to increase with differentiation (Fig. 3a). Furthermore, GATA6 expression increased drastically, greater than 1000-fold at 60 h compared with 48 h, whereas GATA4 expression increased increased fourfold at 66 h compared with 48 h, indicating the dominant impact of GATA6 (Fig. 3a). Indeed, GATA6 is reported to be an upstream factor of GATA425.

Because our recent screening study identified GATA6 as a candidate for DNA demethylation-regulating TF26, we performed Cloning-based bisulfite sequencing to validate the screening result. Cloning-based bisulfite sequencing for ±100 bp regions from four CpGs which were demethylated by GATA6 overexpression in the earlier report26 showed that turning the GATA6 overexpression over tended to partially recover the GATA6 overexpression-induced DNA demethylation (Fig. 3b and Supplementary Fig. 5a). A pull-down assay between HaloTag-fused TET proteins and GATA6 revealed an association between TET proteins and GATA6, suggesting that GATA6 recruits TET proteins to their binding sites (Fig. 3c and Supplementary Fig. 5b). Thus, these results suggest that GATA6 induces DNA demethylation, recruiting the TET proteins.

Fig. 2 Prediction of DNA methylation-regulating TFs. a The workflow of DNA methylation-regulating TF prediction. b, d Heatmap showing the P value of the one-sided (greater) exact Poisson test of overrepresented TF binding motifs at methylated (b) and demethylated (d) regions. Each column is an interval of adjacent time points. Each row is a putative methylation-regulating TF. The dendrogram of hierarchical clustering is shown at the left of the heatmap, and clusters are shown at the right of the heatmap as colors. c mRNA expression profile of the cluster 1 and 2 putative DNA methylation-regulating TFs for methylated regions. X- and Y-axes show time points of differentiation (hours from differentiation initiation) and tag-per-million (TPM) of CAGE, respectively. The color of each line and the error bar represent the maximum TPM and s.d, respectively. The experiment was performed in three biological replicates. e mRNA expression profile of the GATA3, GATA4, and GATA6. X- and Y-axes show time points of differentiation (hours from differentiation initiation) and tag-per-million (TPM) of CAGE, respectively. The color of each line and the error bar represent the maximum TPM and s.d, respectively. The experiment was performed in three biological replicates.
tend to be continuously demethylated from 0 h (Supplementary Fig. 6c, d). Therefore, to investigate whether the GATA6 binding motif is overrepresented for the cumulative changes in methylation, we compared the regions demethylated at each time point with that at 0 h. We identified 220 (0 to 48 h), 236 (0 to 54 h), 416 (0 to 60 h), 876 (0 to 66 h), and 620 (0 to 72 h) demethylated CpGs (Fig. 4c). Because these demethylated CpGs include those that were demethylated in the earlier time point and maintained the hypomethylated status, we only selected the demethylated CpGs that were newly detected as demethylated CpGs at each time point (referred to as uninherited demethylated CpGs) to clarify the effects of each additional period. GATA6 motif over-representation analysis in the vicinity of these uninherited demethylated CpG (uninherited demethylated regions: UDRs) revealed the GATA6 binding motif was overrepresented at 0 to 60 h and 0 to 66 h (Fig. 4d). To further substantiate the over-representation of the GATA6 binding motif at the UDRs, we performed ChiPmentation, which can provide evidence for actual physical interactions between genomic regions and GATA6. Consistent with the expression pattern of GATA6, GATA6 binding was not enriched at UDRs during the period 0 to 48 h, indicating the irrelevance of GATA6 during this period (Fig. 4e). In contrast, unlike binding motif over-representation, ChiPmentation showed interactions between GATA6 protein and most of the UDRs of all comparisons apart from the 0 to 48 h, consistent with the expression pattern of the GATA6 (Fig. 4e and Supplementary Fig. 6a). The GATA6 ChiPmentation peaks at the 72 h significantly overlapped with the regions demethylated during DE-like cell differentiation (486 regions: \( P \) value = 0.001, one-sided permutation test). Furthermore, of the overlapped regions, 48.8% (237 regions: \( P \) value = 0.001, one-sided permutation test) overlapped with the demethylated region by GATA6 overexpression (Supplementary Fig. 6e and Supplementary Data 1). Thus, our results indicate a correlation between GATA6 binding and DNA demethylation during DE-like cell differentiation.

The interrelation between DNA demethylation and chromatin status during iPS-DE differentiation. The majority of the demethylated regions were not promoters but other types of regulatory regions such as enhancers and non-annotated regulatory regions (Supplementary Fig. 7a). Therefore, we investigated the chromatin status of the demethylated regions. Active regulatory regions transcribe several classes of transcripts, including mRNA, promoter-upstream transcripts (PROMPTs), and enhancer RNAs (eRNAs), which are typically transcribed within ± 250 bp from the center of the regulatory region. Thus, the transcription level serves as an indicator of chromatin activity. To investigate the chromatin activity of the demethylated regions, we measured the average TPM of the UDRs (± 250 bp regions from the uninherited demethylated CpGs) by CAGE. The average TPMs of the UDRs were prone to increase as differentiation proceeds in all comparisons except for the 0 to 48 h, indicating the activation of gene regulatory regions (Fig. 5a).

To further analyze the interrelation between GATA6-mediated DNA demethylation and chromatin status, we measured chromatin accessibility by Omni-ATAC-seq. Chromatin accessibility at the UDRs increased between 0 and 48 h and was maintained over the following time points at most of the demethylated regions (Fig. 5b), in agreement with the transcription pattern and GATA6 binding (Fig. 4e and Supplementary Fig. 6a). These demethylated regions coincident with chromatin...
opening during the DE differentiation stage included regulatory regions of known GATA6 targets, such as SOX17 and GATA6 (autoregulation) (Fig. 5c and Supplementary Fig. 7b). Notably, the demethylated regions noted during DE-like cell differentiation were only marginally accessible in iPS cells (0 h), although GATA6 is not expressed at that time, suggesting that target regions of the GATA6-mediated DNA demethylation are pre-defined by chromatin accessibility (Fig. 5b). We also investigated the change in chromatin accessibility of ATAC-seq peaks, which opened in the period 0 to 48 h, dividing the peaks into
demethylated and hyper-methylation-maintaining regions. Mean accessibility level was higher in peaks at the demethylated regions than those at the hyper-methylation-maintaining regions at all time points even before chromatin opening (iPSCs), and the deviation of chromatin accessibility tended to be higher in the hyper-methylation-maintaining regions, suggesting that hypo-methylation stabilizes the open chromatin status (Supplementary Fig. 7c).

Taking advantage of our time-course multi-omics dataset, we compared the kinetics of GATA6 expression, GATA6 binding to the genome (ChiPmentation), methylation change (M-value), and chromatin status (ATAC-seq and Transcript) (Fig. 6a). Overall, the kinetics of GATA6 binding, chromatin accessibility, and transcription observed the same trends, regardless of the UDRs. While transcription levels and $-\Delta M$ value at the UDRs tended to increase after 48 h in accordance with GATA6 expression, GATA6 binding dramatically increased between 48 to 54 h and plateaued at 54 h, with a transient decrease at 66 h. Of note, chromatin accessibility increased in the period 0 to 48 h or 54 h and then decreased after the peaking, although DNA continued to be demethylated.

**Discussion**

In the present study, by applying transcriptome and TFBM over-representation analyses for differentially methylated regions, we comprehensively identified putative DNA methylation-regulating TFs during hepatocyte differentiation. Of these TFs, our results provide multiple strands of evidence that GATA6 is a primary epigenome regulator for the iPSC to DE-like cell differentiation.

Expressions of several hepatic function-related genes, such as Albumin, were low or not even on the day 28 HLCs (Supplementary Figs. 1c, 2a). Furthermore, several hepatic markers were decreased between day 21 and day 28, when the cells were cultured in the maintenance medium. This expression decline is likely the same phenomenon as the rapid loss of hepatic functionality when primary hepatocytes is cultured ex vivo. These drawbacks were already pointed out in the previous reports19,20.

Thus, because the liability must be due to the differentiation protocol, the functionality may be enhanced by recently reported improved protocol or 3D organoid culture. Nevertheless, because hepatic differentiation was high efficiency and near-homogeneous (Fig. 1c, d), the protocol used in this study seems to recapitulate the key molecular events of hepatic differentiation, which fulfill the purpose of this study.

We found enrichment of many TFBMs at demethylated regions during hepatocyte differentiation correlated with the expression of corresponding TFs (Fig. 2d, e and Supplementary Fig. 4b). In contrast, some TFBMs, such as POUSF1, GATA4, and GATA6, were overrepresented mainly in the methylated regions, and the expression of the corresponding TFs was inversely correlated with methylation change (Fig. 2b, c, e and Supplementary Fig. 4a), suggesting that gain of methylation may result from the loss of hypo-methylation maintenance by DNA demethylating-TFs. Interestingly, GATA4 and GATA6 binding motifs are also overrepresented in the demethylated regions at the DE-like cell differentiation, showing the dual roles of GATA4 and GATA6. To summarize, our data suggest that TF-mediated regulation of DNA methylation acts in both the gain and loss of methylation.

HNF4A is required during liver development for the establishment of 5hmC via interactions with TET333. Although the methylation array analyses used in the present study do not distinguish between methylated cytosine and 5hmC, HNF4A binding motifs were overrepresented in the demethylated regions during the hepatoblast-like cell differentiation (Fig. 2d). Since 5hmC has a short half-life34, our results suggest that HNF4A-induced 5hmC is immediately converted to 5fC, 5caC, or unmodified cytosine. Thus, these issues are unresolved at present and require further investigations. Concomitantly with the mRNA expression, GATA6 protein bound to the vast majority of demethylated regions, which was maintained through differentiation (Fig. 4e and Supplementary Fig. 6a). Ectopic expression of GATA6 in HEK 293 T cells supported the GATA6-mediated binding site-directed DNA demethylation (Fig. 3b). As GATA6 protein is associated with TET proteins, recruitment of TET protein to the GATA6 binding sites may be one of the mechanisms underlying the demethylation of RUNX118. Thus, these results demonstrate that GATA6 is a crucial regulator of DNA demethylation for early hepatic development.

GATA6 motif over-representation in the demethylated regions was not completely consistent with ChiPmentation results. Since GATA-binding proteins can bind various non-canonical motifs with comparable affinities to the canonical GATA-binding motif35, our TFBM over-representation analysis, in which we used the canonical GATA6 motif, may underestimate the TF binding. In addition, ChiPmentation may include indirect binding of GATA6 via their co-factors such as Friend Of GATA (FOG) proteins36. Nevertheless, TFBM over-representation has a value in predicting TF binding, because it does not depend on experimental difficulties such as antibody quality.

GATA6 is reported to be a pioneer factor that directly binds to non-permissive heterochromatin and primes the opening of chromatin and histone modifications by interacting with the chromatin remodeling complex37. DNA demethylation by GATA6 may be a step toward pioneering. On the other hand, how the pioneer factors recognize their target regions is not clear yet38. Our finding that GATA6 binding regions were already slightly accessible in iPSCs may explain the mechanism (Fig. 5b).

Chromatin accessibilities at the DNA demethylated regions increased from 0 to 48 h or 54 h and then declined, although DNA methylation kept decreasing, inconsistent with the notion that DNA methylation is correlated with closed chromatin. As the chromatin accessibility assay reflects not only the presence of open chromatin or nucleosome density but also TF binding, this data may be due to the TFs binding.

Although the underlying molecular mechanisms have not been investigated in this study, our analysis proposes a sequential reaction coordinated with the expression pattern of TFs. DNA demethylating-TFs first bind to the permissive heterochromatin sites where the TFBM are located. Then they open
and activate the chromatin at the binding sites, and finally complete DNA demethylation (Fig. 6b). This sequential reaction may be merely due to differences in reaction times between chromatin remodeling and DNA demethylation. While chromatin remodeling is an enzymatic reaction, DNA demethylation is achieved by several mechanisms, including cell division-dependent passive DNA demethylation that takes more time than a single enzymatic reaction.

GATA6 plays pivotal roles in endoderm cell development and pancreas and lung formations \(^{39-42}\). Therefore, GATA6...
Fig. 5 Chromatin status at demethylated regions. a Change in average TPM of demethylated regions during DE differentiation. X- and Y-axis represents time point and relative TPM (vs. TPM of 0 h), respectively. The black and gray circles represent average and individual data points, respectively. The light-green shade is the standard deviation. b Heatmaps showing mean Omni-ATAC-seq read coverage of 100 bp window at a range of ±5 kbp from demethylated CpGs. Each time point is horizontally aligned, and each of the UDRs is vertically aligned. Red is higher coverage of Omni-ATAC-seq reads. c A screenshot of the genome browser showing DNA demethylated regions during the DE differentiation stage, Omni-ATAC-read coverage, GATA6 ChIPmentation read coverage, and CAGE read coverage at GATA6 upstream region. The scale of each dataset is coverage of 10 million 100 nt reads. Red translucent rectangles represent demethylated regions. The enhancer track is based on the GeneHancer database, and enhancers overlapped with the demethylated region are shown as dark green. The genome version is hg19. M-value profiles of each demethylated probe are shown above (x-axis: time point (hour), y-axis: M-value).

Fig. 6 Multi-omics kinetic analysis. a Line plots showing changes in each demethylated region’s omics data. Y-axis is log2 fold-change (FC) for read coverages of ATAC-seq and ChIPmentation for GATA6 (left scale), and -∆M-value (left scale); TPM for GATA6 expression (right scale). X-axis represents the time points of the differentiation. CAGE-based GATA6 expression profiles are identical among the panels. b A schematic illustrating showing a model of interrelation between GATA6-mediated DNA demethylation and chromatin status. Haploinsufficiency causes several diseases, such as pancreatic agenesis. Because the epigenetic function of GATA6 was also confirmed in HEK293T cells, which are non-endodermal, by the artificial ectopic expression system, GATA6-mediated DNA demethylation may be associated with the other biological system and pathology of these diseases.

Methods

Cell culture and in vitro differentiation. The 201B7 human iPSC line was acquired from the RIKEN BioResource Center (BRC) and was cultured in a Cellartis® DEF-CS® Culture System (Takara Bio Inc., Shiga, Japan). For in vitro hepatocyte differentiation and DE differentiation, we used the Cellartis® Hepatocyte Differentiation Kit (Takara Bio Inc.) and the Cellartis® DE Differentiation Kit (Takara Bio Inc.), respectively, according to the manufacturers’ instructions. The culture conditions are shown in Fig. 1a.

Immunocytochemistry. The cells cultured on a cover glass were fixed in 4% formaldehyde for 15 min, followed by blocking using 5% skim milk. The cells were then incubated with primary antibodies diluted by the antibody reaction buffer (1% BSA 0.2% Triton-X100 containing D-PBS(+/+)) for 12 h at 4 °C. After washing in D-PBS(+/+) twice, the cells were incubated with secondary antibodies diluted by the antibody reaction buffer for 1 h at RT. The cells were mounted in slow-fade (Thermo Fisher Scientific Inc., Waltham, MA, USA) and analyzed by a BX-810 fluorescent microscope (Keyence Corporation, Osaka, Japan). Cell number measurements based on the immunocytochemistry images were performed using ImageJ. The antibodies used for the immunocytochemistry are shown in Supplementary Table 2.

Methylation array analysis. Genomic DNA was isolated using a Nucleospin® Tissue Kit (Macherey-Nagel, Düren, Germany). The methylation array used an Infinium Human methylationEPIC BeadChip (Illumina, San Diego, CA, USA), according to the manufacturer’s instructions. Data normalization and M-value computation were performed using lumiMethyNorm implemented in the Infini- niumDiffMetMotR R package. Differentially methylated probes were identified as those with an M-value difference (ΔM) > 2.

Cap analysis gene expression. Total RNA was extracted using Nucleospin® RNA (Macherey-Nagel) and 3 μg of the total RNA were reverse-transcribed using superscript III (Thermo Fisher Scientific Inc.). Cap structure of the RNA was biotinylated, followed by treatment of RNase ONE ribonuclease (Promega Corporation, Madison, WI, USA). RNA-cDNA hybrids were captured using streptavidin-coated magnetic beads (Thermo Fisher Scientific Inc.), and only single-stranded cDNAs were released from the beads. The released cDNAs were ligated to linkers and the second strand were synthesized using Deep Vent (exo-) DNA polymerase (New England BioLabs, Ipswich, MA, USA). The CAGE libraries were sequenced using single-end reads of 50 bp on the Illumina HiSeq 2500 (Illumina). The extracted CAGE tags were then mapped to the human hg19 genome by STAR. The tags per million (TPM) were calculated for each FANTOM5 TSS peak and regions extended ±250 bp from each differentially methylated CpG. Gene expression levels of each gene were computed as the sum of multiple TSS peaks associated with a single gene. The CAGE analysis was performed in three biological replicates.

Omni-ATAC-seq. Ten southland cells were stored at −80 °C in STEM CELL BANKER® (Takara Bio Inc.) until use. The cells were washed with PBS and nuclei were extracted. The extracted nuclei were resuspended in 50 μl of transposition mix (100 nM TED1 (Illumina), 0.01% digitonin, and 0.1% Tween-20, in TD buffer
Genomic DNA was isolated using a NucleoSpin Expression, the overexpression of GATA6 was shut off by removing the Dox. Transduced cells by culturing with 3% tors were transduced to HEK293T cells (BRC). After selection of the successfully Cloning-based bisul

Promega Corporation) was co-transfected with pCDNA3.2-GATA6 to

Table 1.

performed in two biological replicates.

ChIPmentation were shown in Supplementary Table 2. The ChIPmentation was

Western Blotting (Nakarai Tesque Inc., Kyoto, Japan), incubated with an anti-

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extracted from the reaction mixture with DNA Clean and Concentrator (Zymo

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percentages, which is with the regions extended ±100 bp for all methylation array probes. Log2, FDR and the ratio between the numbers of hit regions and all differentially methylated regions of the Top10 overrepresented GOs (Biological Process) were visualized.

Annotation of differentially methylated regions. Gene promoters were defined as 1 kbp upstream and 200 bp downstream regions of genes in geneid human release version 19. The enhancers used in this study were FANTOM5 human phase 1 and 2 permissive enhancers. Non-promoter and non-enhancer regions were defined as unannotated regions. The complete overlap between uninhherited demethylated CpGs and each regulatory region was counted.

Coverage analysis of GATA6 ChlPmentation and Omni-ATAC-seq. Bigwig Coverage files of CAGE ChlPmentation and Omni-ATAC-seq were computed using bam2wig.py with 1,000,000,000 wigsum (equals to coverage of 10 million 100 nt reads). The read coverage was visualized in the range between ±5 kbp from the demethylated CpGs using the EnrichedHeatmap function with the w6 mean model implemented in the EnrichedHeatmap R package.

Permutation test between GATA6 ChlPmentation peaks and the demethy-

labeled regions of DE differentiation stage. Differentially methylated CpGs that were identified as ΔM ≥ 2 and ±200 bp extended regions from the differentially methylated CpGs were used as differentially methylated regions. The differentially methylated regions were subjected to GREAT analysis using the submitGREATjob function implemented in the rGREAT R package with background data, which is with the regions extended ±100 bp for all methylation array probes. Log2, FDR and the ratio between the numbers of hit regions and all differentially methylated regions of the Top10 overrepresented GOs (Biological Process) were visualized.

Statistics and reproducibility. The numbers of biological and technical replicates are indicated for each experiment. The statistical tests used are shown in each analysis.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The datasets generated and analyzed during the current study are available in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE163331. Source data underlying the main figures are presented in
Supplementary Data 1. The M-value data of GATA6-overexpressing HEK293T cells that support the findings of this study are available in Figshare (https://doi.org/10.5084/m9.figshare.18576592.v1).

Code availability
Analysis codes and data are available in the Zenodo repository at the https://doi.org/10.5281/zenodo.6337878.

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Author contributions
T.S. participated in the study’s design, devised the methodology, performed the statistical analyses, carried out the molecular biology studies, acquired the funding, and drafted the manuscript. S.M., E.F., M.K., Y.M., Y.T., J.L., H.N., Y.N., and A.S. carried out the molecular biology experiments. H.S. helped to draft the manuscript, acquired the funding, and supervised the study. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.
