DNA methylation is required for the control of stem cell differentiation in the small intestine

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The mammalian intestinal epithelium has a unique organization in which crypts harboring stem cells produce progenitors and finally clonal populations of differentiated cells. Remarkably, the epithelium is replaced every 3–5 d throughout adult life. Disrupted maintenance of the intricate balance of proliferation and differentiation leads to loss of epithelial integrity or barrier function or to cancer. There is a tight correlation between the epigenetic status of genes and expression changes during differentiation; however, the mechanism of how changes in DNA methylation direct gene expression and the progression from stem cells to their differentiated descendants is unclear. Using conditional gene ablation of the maintenance methyltransferase Dnmt1, we demonstrate that reducing DNA methylation causes intestinal crypt expansion in vivo. Determination of the base-resolution DNA methylome in intestinal stem cells and their differentiated descendants shows that DNA methylation is dynamic at enhancers, which are often associated with genes important for both stem cell maintenance and differentiation. We establish that the loss of DNA methylation at intestinal stem cell gene enhancers causes inappropriate gene expression and delayed differentiation.

[Keywords: DNA methylation; intestinal stem cell; Dnmt1]

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The intestinal epithelium tightly regulates proliferation and differentiation in space and time and must maintain this control over thousands of cell divisions throughout life for organismal health. The small intestinal epithelium has a unique organization, with crypts harboring stem cells, which produce progenitors (transit-amplifying cells), and finally clonal populations of differentiated cells that migrate in ordered cohorts up the crypt–villus axis (Sheaffer and Kaestner 2012). The intestinal epithelium maintains this “self-renewal” capacity through the function of both quiescent and fast-cycling stem cells (Yeung et al. 2011). Crypt base columnar (CBC) stem cells are fast cycling, express LGR5, and are responsive to Wnt signaling (Barker et al. 2007). These cells are bona fide stem cells that can be isolated and form intestinal enteroids in vitro that self-renew and differentiate into all functional intestinal cell types (Barker et al. 2007). Excessive activation of Wnt signaling by disruption of APC specifically in CBC stem cells increases proliferation and causes formation of adenomas (Barker et al. 2009).

DNA methylation is critical for the regulation of gene expression during differentiation in many self-renewing tissues, including the germline and embryonic, hematopoietic, and epidermal stem cells (Sen et al. 2010; Smith and Meissner 2013). DNA methylation patterns are established and maintained by the de novo DNA

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methyltransferases Dnmt3a and Dnmt3b and the main-
tenance methyltransferase Dnmt1, respectively. Dnmt1
is required for both differentiation and self-renewal in
embryonic and adult stem cells [Lei et al. 1996; Lee et al.
2001; Broske et al. 2009; Trowbridge et al. 2009; Sen et al.
2010]. Comparison of DNA methylation patterns in hu-
man embryonic stem cells [ESCs] and differentiated fetal
fibroblasts at the genome-wide level revealed differential
methylation at genes important for stem cell maintenance
and differentiation processes [Lister et al. 2009]. DNA
demethylation at enhancers and promoters is correlated
with priming and activation of lineage-specific genes at the
appropriate time during development [Palacios et al. 2010].
Conversely, promoters involved in stem cell gene expres-
sion frequently become more methylated as cells differen-
tiate [Kim et al. 2010a; Polo et al. 2010; Schmidt et al.
2012]. These studies show that DNA methylation is
important in multiple differentiation systems; however,
the function of DNA methylation in intestinal epithelial
differentiation has not been evaluated.

We hypothesized that differential DNA methylation
contributes to intestinal epithelial differentiation and
function and therefore set out to determine the contribu-
tion of DNA methylation during adult intestinal stem
cell [ISC] differentiation. Reduction of DNA methylation
maintenance by acute deletion of Dnmt1 in the intestinal
epithelium caused crypt expansion and decreased differ-
etiation. Using whole-genome shotgun bisulfite sequenc-
ing [WGBS], we show that DNA methylation is dynamic
during the rapid transition from stem to the fully mature,
differentiated epithelial cells. Our study reveals that the
expression of important intestine-specific genes depends
on methylation status and that Dnmt1 contributes to the
timely repression of ISC genes during differentiation in
vivo.

Results

As the first step in our investigation of the potential
contribution of DNA methylation to intestinal prolifera-
tion and differentiation, we determined the expression
patterns of all three DNA methyltransferases in the adult
mouse intestine. Dnmt1 was restricted to the crypts
[Supplemental Fig. 1A; Suetake et al. 2001], while Dnmt3a
was expressed throughout the epithelium, with higher
expression in crypts [Supplemental Fig. 1B]. Overall,
Dnmt1 and Dnmt3a mRNA expression levels in the
intestinal epithelium were even higher than those found
in ESCs, where methyltransferases are known to be
required for the establishment and preservation of DNA
methylation of imprinted loci, repetitive elements, and
tissue-specific CpG islands [Supplemental Fig. 1D; Li et al.
1992; Okano et al. 1999; Liang et al. 2002; Hattori et al.
2004]. In contrast, only minimal levels of Dnmt3b protein
were present in the intestine, confirming previous obser-
vations in colonic crypts [Supplemental Fig. 1C; Steine
et al. 2011]. In addition, Dnmt3b mRNA levels were
fivefold lower in the intestinal epithelium than in ESCs
[Supplemental Fig. 1D]. We conclude that cells in the crypt
zone, including stem and progenitor cells, express high
levels of Dnmt1 and Dnmt3a, suggesting that both main-
tenance and de novo DNA methylation might be required
in the proliferative compartment of the gut.

Next, we tested the hypothesis that methylation plays
a role in the timing of differentiation using genetic
means. Germline deletion of Dnmt1 in mice causes a
66% decrease in global methylation levels and embry-
onic lethality [Li et al. 1992]. To avoid developmental
defects, we used Dnmt1lox/lox; Villin-Cre-ERT2 mice to
inducibly delete Dnmt1 in the adult gut epithelium. Six
days after intraperitoneal tamoxifen administration, all
Dnmt1 gene expression was efficiently extinguished in the
adult mouse small intestinal epithelium of Dnmt1lox/lox;
Villin-Cre-ERT2 (mutant) compared with tamoxifen-
treated Dnmt1lox/lox [control] mice [Fig. 1A,B]. Deletion
was achieved throughout the entire length of the intestine
and was confirmed by quantitative RT–PCR [qRT–PCR],
with mutants showing a 90% reduction of Dnmt1 mRNA
levels [Fig. 1C].

The acute deletion of Dnmt1 caused a modest but
statistically significant expansion of the small intestinal
crypt zone. The crypt zone, designated by the prolifera-
tion marker Ki67, was expanded twofold in mutant mice
[Fig. 1D–F] and exhibited increased expression of the Wnt-
responsive ISC genes Sox9 and Msi1 [Fig. 1G–I; Supple-
mental Fig. 2G,H; Potten et al. 2003; Formeister et al.
2009]. In addition, we observed a corresponding decrease
in steady-state mRNA levels of the differentiated enter-
cyte markers alkaline phosphatase [AP] and lactase [Lct]
[Stegmann et al. 2006] as well as a decreased AP-positive
domain in the crypt–villus axis [Fig. 1J–L]. Interestingly,
cell fate decisions among differentiating cells were largely
unaffected by the loss of Dnmt1, as the proportion of the
differentiated cell types, including enteroendocrine, gob-
let, and Paneth cells, stayed constant [Supplemental Fig.
2A–F]. However, increased numbers of secretory progen-
itor cells were found above the crypt base with goblet cell
morphology and lysozyme expression, suggesting a partial
disruption in secretory cell specification [Supplemental
Fig. 2F; Schneider et al. 2010].

Having established that DNA methylation is required
for intestinal crypt homeostasis, we determined base-
resolution DNA methylomes of ISCs and differentiated
cells in the small intestine in order to begin to investigate
the molecular defects underlying the observed mutant
phenotype. We separated stem cells and their immediate,
proliferating daughters using fluorescent-activated cell
sorting [FACS] for green fluorescent protein [GFP] in Lgr5-
EGFP-ires-CreERT2 transgenic mice, as previously de-
scribed [van der Flier and Clevers 2009; Munoz et al.
2012]. Highly enriched differentiated villous epithelial
cell fractions were collected by EDTA disassociation and
gentle scraping. The villous cell fractions contained termi-
nally differentiated intestinal epithelial cells, the ma-
majority of which are enterocytes, as well as goblet and
enteroendocrine cells [van der Flier and Clevers 2009].
Confirmation of cell purity was performed by qRT–PCR
for the stem cell-specific marker Lgr5, the proliferation
marker Ki67, and the enterocyte marker Lct [Supplemen-
tal Fig. 3A]. DNA extracted from the LGR5+/ stem and
differentiated cell populations from a pooled cohort of five or two mice, respectively, were used for genome-wide analysis of DNA methylation, and three independent biological replicates from each of the two cell populations were used for mRNA expression analysis.

To obtain single-base-pair resolution of DNA methylation in intestinal LGR5\(^+\) stem and differentiated epithelial cells, we used WGSBS. Genomic sequencing cumulative coverage for LGR5\(^+\) and differentiated cells was an average of 26-fold and 20-fold, respectively [Supplemental Fig. 4A]. We observed little non-CpG methylation across the genome for both cell populations [Supplemental Fig. 4B]. Similar to observations from the first published base-resolution methylome of mouse ESCs, promoters were hypomethylated, while the remainder of the genome was highly methylated in the intestinal genome [Supplemental Fig. 4C; Stadler et al. 2011].

We segmented both intestinal methylomes into three classes: fully methylated regions (FMRs; >9 Mb), low-methylated regions (LMRs; 13.9%–50%), and unmethylated regions (UMRs; <13.9%), as previously described [Stadler et al. 2011]. We found an increased number of LMRs in differentiated cells compared with UMRs and containing 13.9%–50% methylation levels, were undetectable in their analysis. The average change in methylation state at DMRs during intestinal differentiation was previously, CpG islands across the genome were highly enriched for UMRs in both LGR5\(^+\) stem and differentiated cells [Fig. 2A; Supplemental Fig. 5A,B; Stadler et al. 2011]. While only 0.4% of the mouse genome is located in CpG islands (“Genome” in Fig. 2A), ~20% of UMRs in both LGR5 stem and differentiated cells are at CpG islands [Fig. 2A]. Likewise, CpG shores make up only 2.4% of the mouse genome but represent 29.8% and 34.4% of LGR5 stem and differentiated cell UMRs. In contrast, the fraction of LMRs [13.9%–50% CpG methylation] that mapped to CpG islands and CpG shores in both cell types is lower than expected from the genome-wide distribution of these features [Fig. 2A].

Comparison of LGR5\(^+\) stem and differentiated cell UMRs and LMRs using stringent statistical criteria [for details, see the Materials and Methods] revealed a total of 4240 regions covering 4.58 Mb, identified as differentially methylated regions (DMRs). Recently, Kaaij et al. 2013] reported very few DNA methylation changes during adult intestinal differentiation using a cutoff of a minimal differential methylation of 40%. However, the majority of enhancer regions, defined by Stadler et al. 2011] as LMRs and containing 13.9%–50% methylation levels, were undetectable in their analysis. The average change in methylation state at DMRs during intestinal differentiation was
~15%. Interestingly, regions of differential methylation were overrepresented at both CpG islands (4.4% vs. 0.4% in the genome) and CpG shores (10.1% vs. 2.4% in the genome), suggesting that methylation changes frequently occur in genomic elements relevant to gene regulation [Fig. 2A].

A more detailed analysis of these methylome data revealed additional interesting points. When plotting the methylation classes UMR, LMR, and DMR relative to the center of CpG islands, as expected, >80% of CpG islands coincide with UMRs [Fig. 2B]. Conversely, LMRs are depleted at CpG islands, with increasing representation at CpG shores [Fig. 2C]. Regions of differential methylation are enriched at CpG islands but to a much lesser extent than UMRs [Fig. 2D]. A similar profile emerges when all transcriptional start sites [TSSs] are considered. The representation of UMRs is near 60% at TSSs, reflecting the fact that not all promoters are associated with CpG islands and not all UMRs are at TSSs [Fig. 2E]. LMRs, in contrast to UMRs, are found mainly either upstream of the TSSs or within gene bodies, often within the first intron [Fig. 2F]. Finally, DMRs show a peak at TSSs but also a strong shoulder within the first intron, where enhancers are frequently found in mammalian genes [Fig. 2G]. When segmenting the entire genome into regions of interest, such as proximal and distal promoters, introns, and coding regions, one can visualize the fact that the vast majority of the genome is made up of intergenic and intronic regions [Fig. 2K]. However, UMRs and LMRs in both the LGR5* and differentiated cells are concentrated in likely regulatory regions, including introns [40%], proximal [4%] and distal promoters [6%], and caudal regions [7.5%], defined as the 5 kb 3’ to the end of each gene [Fig. 2H,I]. Locations of DMRs were found to be enriched within introns [47.3%] and distal promoters [7.5%] [Fig. 2J], suggesting that DNA methylation changes are concentrated in regulatory regions, while other regions of the genome exhibit stable methylation patterns.

Global analysis of the DMRs identified above established that most UMRs gain methylation during differentiation [Fig. 3A], whereas LMRs show both gain and loss of methylation [Fig. 3B]. Importantly, loss of methylation at DMRs located in regulatory regions, such as promoters, introns, and 3’ untranslated regions (UTRs), correlates with higher expression of the associated gene in differentiated cells as compared with LGR5* cells [Fig. 3C; Supplemental Fig. 6]. Several lines of evidence suggest that intestine-specific DMRs are functionally relevant. First, DMRs located in regulatory regions such as distal promoters and introns showed average methylation gains of...
13.3% and 14.2%, respectively (data not shown). Conversely, DMRs located at promoters had a significantly lower mean gain of 8.5% methylation (data not shown). These data reveal that methylation gain during differentiation is not a general phenomenon across all UMRs of the genome but is targeted to likely enhancer regions.

Second, DMRs that lose methylation during differentiation were associated with genes involved in important aspects of small intestine enterocyte metabolism, including "triglyceride biosynthesis," "serotonin metabolism," and "retinol biosynthesis" (Fig. 3D; see Supplemental Fig. 3C for full list of statistically significant pathways; Gershon 2004; Harrison 2005; Pan and Hussain 2012). Strikingly, motif finding analysis revealed that these regions are enriched for binding sites of multiple factors required for intestinal epithelial differentiation, including HNF-4α (Garrison et al. 2006), Onecut (Dusing et al. 2010), and GATA (Fig. 3F; Beuling et al. 2011). In order to further investigate the possible role for dynamic methylation at relevant regulatory elements, we performed chromatin immunoprecipitation (ChIP) combined with deep sequencing (ChIP-seq) for the active enhancer mark H3K27ac in both LGR5+ stem and differentiated cell populations (Supplemental Fig. 4A). DMRs that lost methylation during differentiation showed a dramatic gain of H3K27ac in differentiated cells [Fig. 3H], suggesting that loss of DNA methylation is associated with activation of specific enhancers during the differentiation process.

In contrast, DMRs that gained methylation during differentiation were associated with genes involved in signaling pathways of established importance in ISCs, including the Wnt/b-catenin and Rho family GTPase pathways (Fig. 3E; see Supplemental Fig. 3C for full list of statistically significant pathways; Stappenbeck and Gordon 2000; Sakamori et al. 2012; Schepers and Clevers 2012). Interestingly, these latter regions are enriched for ISC transcription factor motifs. Percentage reflects the fraction of regions that were found with each motif within the specific group of DMRs. [H, I] Percentage of DMRs that lose DNA methylation [H] or gain methylation [I] that show overlap with regions that contain H3K27ac in either LGR5 or DIFF cells.

Next, we used mRNA sequencing to determine the gene expression profile of LGR5+ stem and differentiated cell populations. We observed high levels of expression for the expected stem cell and enterocyte-specific genes in LGR5+ stem and differentiated cell populations, re-
spectively (Supplemental Fig. 3B). Genes that were highly expressed in the villous cell population were designated as “differentiation-induced genes.” Because the LGR5+ cell population is made up of both stem cells and their immediate daughter cells, we used the previously published ISC RNA signature (Munoz et al. 2012) to separate “ISC genes” from “progenitor genes” in our data set. Using this approach, we identified 1093 genes that were highly expressed in the LGR5+ population that have not been previously characterized as ISC-specific genes, which we therefore defined as “progenitor genes” (Supplemental Table 1).

To investigate the relationship between differential expression and differential DNA methylation of nearby regulatory elements in the intestinal epithelium differentiation process, we integrated our DNA methylation and gene expression data sets. We found that 128 of the 384 ISC-specific expressed genes are associated with regions that gained methylation during differentiation, suggesting that extinction of the ISC signature in differentiated cells is at least partially driven by de novo methylation (Supplemental Table 2). Strikingly, the vast majority of these DMRs (73.7%) were located in introns. These genes include well-characterized CBC stem cell genes such as Lgr5, Ascl2, and Olfm4, which exhibited one to three DMRs [Fig. 4A]. We verified important DMRs identified by WGSBS using targeted bisulfite sequencing in three biological replicates each of differentiated and LGR5+ stem cells. Methylation levels of individual CpGs contained within DMRs associated with Olfm4 [Fig. 4B], Hes1 [Fig. 4C], and Lct [Fig. 4D], while not significantly increased at each individual CpG, on average showed methylation gains similar to those that we had observed by WGSBS. As a control, we analyzed the imprinted locus H19 by pyrosequencing, which exhibited constant methylation during intestinal differentiation, demonstrating that the methylation changes between the LGR5+ and differentiated cells are specific to

**Figure 4.** DMRs are associated with intestinal-specific genes and correlate with expression during differentiation. (A) Partial list of genes with differential mRNA expression and associated DMRs in LGR5+ stem cells compared with differentiated cells. Transcripts with increased expression in LGR5+ stem cells were categorized as ISC genes or progenitor genes as described in the text. Transcripts with increased expression in villous cells were categorized as differentiation-induced genes. Genes are listed with the number of associated DMRs, average change in methylation [DIFF−LGR5] and corresponding P-value, and mRNA fold change [DIFF−LGR5] and corresponding P-value. (B–D) Verification of DMRs by bisulfite sequencing. Three biological replicates of LGR5+ and DIFF cells were used to verify identified DMRs by targeted bisulfite sequencing to determine methylation at the single-CpG level and regional average. DIFF cells show increased regional average methylation at the DMRs associated with Olfm4 [LGR5: 2.2% ± 1.4%; DIFF: 5.1% ± 1.2%] [B] and Hes1 [LGR5: 3.7% ± 2.9%; DIFF: 9.5% ± 2.1%] [C]. (D) DIFF cells show decreased regional average methylation at the DMR associated with Lct [LGR5: 49.8% ± 10.5%; DIFF: 18.9% ± 5.9%]. (*) P < 0.05; (**) P < 0.01; (*** ) P < 0.001 by t-test.
DMRs (Supplemental Fig. 7A). Pathway analysis showed this group of 128 ISC signature genes to be enriched for Wnt/b-catenin and TGFβ signaling, supporting the notion that these genes maintain a low-methylation status in ISCs and progenitor cells (Fig. 5A, Supplemental Fig. 3D shows all significant gene categories).

Of the 1093 "progenitor" genes defined above, we found that 140 gained methylation during differentiation (Supplemental Table 3). Among this group were genes important for directing differentiation, such as Bmp7, Ephb2, and Hes1 (Fig. 4A). More globally, the 140 progenitor genes with increased methylation after differentiation were enriched for functional categories such as "translation initiation" and "Notch signaling," pathways known to be critical for proliferation and differentiation (Fig. 5C; Supplemental Fig. 3D shows all significant gene categories; Duranton et al. 1998; Wang et al. 2012; Noah and Shroyer 2013). Finally, 523 out of 3758 "differentiation-induced" genes, highly expressed in villous cells, showed decreased methylation after differentiation and included enterocyte-specific genes such as Lct, Alpi, and Krt20 (Fig. 4A). The majority of DMRs (61%) that lost methylation were found located in introns. This group was enriched for genes important for enterocyte metabolic functions and immune response such as "triacylglycerol biosynthesis" and "iNOS signaling" (Fig. 5E; Supplemental Fig. 3D shows all significant gene categories; Supplemental Table 4; Cavicchi and Whittle 1999; Pan and Hussain 2012).

Strikingly, DMRs associated with differential gene expression showed dynamic association with H3K27ac during differentiation. A proportion of ISC genes that gained DNA methylation during differentiation also lost H3K27ac (Fig. 5B). In contrast, progenitor genes that gained methylation had no change in H3K27ac (Fig. 5D). Strikingly, the vast majority of DMRs that lost methylation during differentiation acquired H3K27ac (Fig. 5E). To determine which regions were indeed active enhancers bound by intestinal transcription factors, we compared the differentiation-specific regions that lost methylation with previously published binding profiles for Cdx2 and Hnf4a from villous cells (Verzi et al. 2013). Remarkably, we found that 70% of these regions are bound by one or both of these factors (Fig. 5G). Interestingly, genes with increased methylation in differentiated cells cover a spectrum of ISC factors, suggesting that DNA methylation coordinates the shutdown of multiple ISC-specific pathways during differentiation. To explore this notion further, we examined two genes that are important for stem cell maintenance, Olfm4 and Hes1, in more detail.

Olfm4 was exclusively expressed in LGR5+ ISCs and gained methylation at multiple sites during differentiation. Olfm4 has DMRs located at 19 kb and 14 kb upstream of the TSS that gained 14% and 10% methylation, respectively, as LGR5+ stem cells differentiate (Fig. 6A). A third large DMR gained 12% methylation, is located over the Olfm4 proximal promoter, and extends into the first half of the coding region of the gene (Fig. 6A). To our knowledge, nothing is currently known about the role of distal enhancer elements in the regulation of Olfm4 expression. However, there are multiple transcription factor-binding sites in the proximal promoter important for Olfm4 expression, including nuclear factor-κB (NF-κB) [Huang et al. 2010; Kim et al. 2010b] and RBP-J, the main transcriptional mediator of Notch signaling [VanDussen et al. 2012]. In the DMR at the proximal promoter, CpGs are directly adjacent to the NFκB- and predicted RBP-J-
binding sites. Additionally, we found that the H3K27ac chromatin mark of active enhancers associated with one of the distal DMRs in LGR5\(^+\) cells [Fig. 6A]. The H3K27ac mark is present at this enhancer in LGR5\(^+\) stem cells but is lost after differentiation. Previously, DNA methylation had also been correlated with OLFM4 expression in human cancer, and the proximal DMR that we identified in the mouse Olfm4 gene contains six of the eight corresponding CpGs of the human promoter that are important for cancer-induced expression through retinoic acid signaling [Liu et al. 2010].

We found Hes1, a gene critical for both proliferation and differentiation of intestinal epithelia, to be highly expressed in both stem and progenitor cells [Ueo et al. 2012]. We identified DMRs with methylation gain after differentiation located at 17 kb, 13 kb, and 9 kb upstream of the Hes1 TSS [Fig. 6B]. These distal DMRs were highly associated with the H3K27ac chromatin mark in both LGR5\(^+\) and differentiated cells [Fig. 6B]. Hes1 is well known as a target of Notch signaling through RBP-J sites found in the proximal promoter [Jarriault et al. 1995]. Additionally, cis-regulatory modules found in likely Hes1 enhancers have also been shown to be important in the regulation of Hes1 expression and contain predicted RBP-J-binding sites [Jeziorska et al. 2012]. Interestingly, one of these cis-regulatory modules is located within our \(-9\)-kb DMR and is bound by Cdx2 and Hnf4\(\alpha\) in villous cells, suggesting that this region is actively recruiting intestinal transcription factors [Fig. 6B].

Having established that DNA methylation is indeed dynamic at specific and relevant regulatory regions in the genome during the transition from stem to fully mature, differentiated epithelial cells, we investigated ISC genes that showed methylation differences between LGR5\(^+\) stem and differentiated cells in the Dnmt1\(^{lox/lox}\); Villin-Cre-ERT2 intestine. As predicted, Olfm4 and Hes1 showed an expanded expression domain in the mutant intestine [Fig. 7A,B,D,E]. These data prompted us to examine the methylation status at the DMRs associated with Olfm4 and Hes1. Due to the temporal delay between altered DNA methylation on gene expression, we examined DNA from mutant and control mice 2 d before the most severe crypt expansion was manifested. At this time point, we already detected increased mRNA levels of both Hes1 and Olfm4 [Fig. 7C,F]. Strikingly, mutants showed a 10% decrease of methylation at both genes [Fig. 7G,H], while methylation at the H19 imprinted locus was not affected in Dnmt1 mutant mice [Supplemental Fig. 7B]. These data demonstrate that Dnmt1 is required in intestinal crypts for the increase in methylation between ISCs and differentiated cells at intestinal specific loci and not just for maintenance of global DNA methylation patterns in the adult intestine.

**Discussion**

Our study shows that DNA methylation changes significantly and at relevant sites during the 3- to 5-d transition from cycling stem to differentiated intestinal epithelial cells and is required for the appropriate timing of differentiation. Our genome-wide approaches, integrating data on DNA methylation, histone marks, transcription factor binding, and mRNA expression profiling, allowed us to define new regulatory regions that are differentially methylated during intestinal epithelial differentiation and are associated with the control of critical intestinal genes. These genome and genetic data demonstrate that gene expression changes of essential intestinal genes...
during differentiation are in part controlled by DNA methylation. Many of the DMRs identified by this study are located outside of proximal gene promoters or CpG islands and have not been previously implicated in the regulation of gene expression. Based on the H3K27ac chromatin modification dynamics and the association of multiple intestinal-specific transcription factors, many of these DMRs are likely active intestinal enhancers. During differentiation, enhancer regions associated with genes important for enterocyte differentiation, such as Alpi and Lct, are demethylated, coordinating the binding of transcription factors, such as Cdx2 and Hnf4α, with the activation of gene expression [Fig. 7I]. Concurrently, DNA methylation increases at ISC enhancers, such as those of Olfm4 and Hes1, correlating with repression of the corresponding genes [Fig. 7I]. Loss of maintenance methylation by Dnmt1 causes increased ISC gene expression due to enhancer hypomethylation, which may allow for inappropriate binding of transcription factors, such as Cdx2, thus blocking progression through differentiation [Fig. 7I]. ISC gene expression is eventually attenuated, presumably due to loss of exposure to signaling factors critical for stem cell maintenance, such as Wnt and Notch, as cells move up the crypt–villus axis.

ISC gene expression depends critically on complex combinations of signaling pathways and transcription factors [Sheaffer and Kaestner 2012]. Deletion of Wnt or Notch signaling components causes loss of the intestinal epithelium through failure of self-renewal of ISCs and appropriate differentiation [Korinek et al. 1998; Pellegrinet et al. 2011; Ueo et al. 2012; van Es et al. 2012; VanDussen et al. 2012]. Conversely, increased activation of Wnt and Notch signaling causes expansion of the ISC niche and disrupted differentiation [Moser et al. 1990; Stanger et al. 2005; Zecchini et al. 2005]. In coordination with these signaling pathways, transcription factors such as the Cdx proteins are required for expression of ISC genes, and genetic ablation of Cdx1 and Cdx2 causes total loss of the epithelium [Gao et al. 2009; Gao and Kaestner 2010; Verzi et al. 2011]. Furthermore, in human intestinal Caco-2 cells, Cdx2 binding is associated with ISC genes in the undifferentiated state, and its binding profile overlaps with Tcf4, the major Wnt effector [Verzi et al. 2010]. Our DMRs show high overlap with the Cdx2 ChIP-seq binding profile [Verzi et al. 2013]. Our data set will be a useful resource for further work to determine which role DNA methylation plays in the coordination of signaling pathways and transcription factor binding, such as Wnt and Cdx2, to activate gene expression during normal intestinal differentiation.

Changes in DNA methylation during intestinal differentiation may be important for the combinational control of gene expression by modulating chromatin accessibility for multiple transcription factors. DMRs were enriched for the predicted binding sites of multiple transcription factors, including Cdx2, GATA, Hnf1α, and Hnf4α, which act in combination to control gene activation during intestinal differentiation [Benoit et al. 2010; Verzi et al. 2013]. Transcription factor binding is important to main-
tain DNA hypomethylation and chromatin accessibility at enhancers in both mouse and human cells (Palacios et al. 2010; Stadler et al. 2011; Thurman et al. 2012). In support of these findings, we show that after intestinal differentiation, regions that have reduced DNA methylation are occupied by multiple transcription factors. However, our data also suggest that DNA methylation is critical for appropriate timing of gene repression and may not simply play a passive role in response to transcription factor binding. Similarly, genetic deletions of Dnmt3a and Dnmt1 in the hematopoietic stem cell reveal that DNA methylation is required for silencing of hematopoietic stem cell genes (Trowbridge et al. 2009; Challen et al. 2012). In addition, our data establish a role for DNA methylation in the terminal repression of ISC genes. Thus, DNA methylation may initiate gene repression by imposing changes in chromatin that antagonize transcription factor binding to enhancers. More studies are needed to show whether DNA methylation controls accessibility of direct effectors of Wnt and Notch signaling, such as Tcf4 and RBP-J, to enhancers after intestinal differentiation in vivo.

In summary, we demonstrate that maintenance of DNA methylation by Dnmt1 is required for the repression of ISC genes to allow appropriate timing of differentiation. DNA methylation is dynamic at many relevant regulatory elements during intestinal epithelial differentiation. Our data suggest that DNA methylation plays an active role in the regulation of gene expression by controlling accessibility of enhancers during intestinal epithelial differentiation.

Materials and methods

Mice

Dnmt1lox/lox mice were provided by Rudolf Jaenisch (Jackson-Grusby et al. 2001), and Villin-Cre-ERT2 mice were received from Sylvia Robine (El Marjou et al. 2004). For genome-wide analysis, cells were isolated from mice on the C57Bl/6 background and Lgr5-EGFP-ires-CreERT2 transgenic mice (Barker et al. 2007). For Dnmt1 deletion experiments, Cre recombination was induced by three daily oral administrations of 1.6 mg of Dnmt1 deletion experiments, Cre recombination was induced by three daily oral administrations of 1.6 mg of tamoxifen [Sigma] in an ethanol/sunflower oil mixture and littermate controls without the Cre-ERT2 transgene plus tamoxifen treatment. All procedures involving mice were conducted in accordance with approved Institutional Animal Care and Use Committee protocols.

Intestinal epithelium isolation and FACS

Small intestines were opened longitudinally and washed in PBS. Villous epithelial cells were removed by gentle scraping with a coverslip. The intestine was cut into 1-mm pieces and incubated in 5 mM EDTA in HBSS for 35 min, and the epithelial cell layer was isolated by manual pipetting and collected for whole-intestine analysis or passed through a 70-μm filter to isolate crypts. Crypts were dissociated into single cells using 5 mM EDTA in HBSS for 35 min, and the epithelial cells were isolated from mice on the C57Bl/6 background and Lgr5-EGFP-ires-CreERT2 transgenic mice (Barker et al. 2007). For Dnmt1 deletion experiments, Cre recombination was induced by three daily oral administrations of 1.6 mg of tamoxifen [Sigma] in an ethanol/sunflower oil mixture and littermate controls without the Cre-ERT2 transgene plus tamoxifen treatment. All procedures involving mice were conducted in accordance with approved Institutional Animal Care and Use Committee protocols.

mRNA expression analysis

mRNA expression was measured using qRT–PCR, as previously described (Gupta et al. 2007). Primer sets can be found in the Supplemental Material [Supplemental Table 5]. RNA sequencing libraries were constructed from ~200 μg of total RNA isolated using the TruSeq RNA sample prep kit [Illumina]. Single-end sequencing was performed on an Illumina HiSeq2000 (100-bp reads). Reads were aligned to the mouse reference genome [NCBI build 37, mm9] using TopHat (Trapnell et al. 2012). TopHat was run with the University of California at Santa Cruz reFlate
annotation file (GTF format) and the “–no-novel-juncs” option to map reads only in the reference annotation. With three biological replicates, gene expression levels were calculated by Cuffdiff [Trapnell et al. 2012] using the same reference annotation file. mRNA levels were expressed in reads per kilobase of transcript per million mapped reads (RPKM).

Pathway and motif analysis

The pathway analyses were generated through the use of Ingenuity Pathway Analysis (IPA; Ingenuity Systems, http://www.ingenuity.com). De novo motif analyses were performed using HOMER [Heinz et al. 2010].

Histological procedures and in situ hybridization

Tissues were isolated and fixed using 4% paraformaldehyde and then embedded in paraffin. Antigen retrieval was performed using the 2100 Antigen Retriever in buffer A [Electron Microscopy Sciences], and standard immunostaining procedures were performed for Dnmt1 [Santa Cruz Biotechnology], Chga [Immunostar], Dnmt3a [Santa Cruz Biotechnology], Dnmt3b [Abcam], E-Cadherin [BD Transduction Laboratories], Sox9 [Millipore], Lyz [Dako], Ki67 [BD Pharmingen], and Muc2 [Santa Cruz Biotechnology]. Immunohistochemical procedures were modified for Hes-1 [Ben Stanger, University of Pennsylvania], including antigen retrieval in high-pH antigen unmasking solution [Vector Laboratories] and signal amplification with the TSA Fluorescein system [Perkin Elmer]. AP staining was performed using NBT and BCIP (Boehringer Ingelheim). In situ hybridization for Olfm4 was performed as described previously [Barker et al. 2007]. All microscopy was performed on the Nikon Eclipse 80i.

Pyrosequencing and bisulfite sequencing

One-hundred nanograms of DNA was bisulfite-converted using the Epitect bisulfite kit [Qiagen]. Pyrosequencing assays were designed using Pyromark assay design software [Qiagen]. For pyrosequencing, template DNA was amplified using the Pyromark PCR kit [Qiagen], and sequencing was performed using the PyroMark Q96 ID [Qiagen]. For bisulfite sequencing, template DNA was amplified using KAPA HIFI Uracil PyroMark Q96 ID (Qiagen). For bisulfite sequencing, template DNA was amplified using the PyroMark assay design software (Qiagen). DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. Nat Genet 41: 1207–1215.

ChIP analysis

LGR5+ and differentiated cells were isolated and incubated in 1.11% formaldehyde for 10 min at room temperature. Chromatin was sonicated into 200- to 300-bp fragments using the BioRuptor (Diagenode). Immunoprecipitation was performed using 2 μg of H3K27ac antibody [Active Motif] and 2 μg of chromatin for two biological replicates for each cell type. ChIP-seq libraries were made using the ChIP-seq Library Prep reagent set [New England Biolabs]. Paired-end sequencing was performed on the hSeq2000 [50-bp reads]. Multiplexed libraries for H2K27ac chromatin-immunoprecipitated chromatin and input DNA were sequenced to 50-bp single reads on an Illumina hSeq2500 in rapid-run mode. Reads were aligned to the genome using Bowtie [-k 1 -m 1–best–strata]. Peaks were called using HOMER in histone mode and with PCR duplicates discarded. Regions were selected with a false discovery rate cutoff of 1%.

Data access

All WGSBS, RNA sequencing, and ChIP-seq data generated in this study have been deposited in ArrayExpress under accession number E-MTAB-2350.

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