



Epigenetic regulation of the intestinal epithelium

Ellen N. Elliott¹ · Klaus H. Kaestner¹

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Abstract The intestinal epithelium is an ideal model system for the study of normal and pathological differentiation processes. The mammalian intestinal epithelium is a single cell layer comprising proliferative crypts and differentiated villi. The crypts contain both proliferating and quiescent stem cell populations that self-renew and produce all the differentiated cell types, which are replaced every 3–5 days. The genetics of intestinal development, homeostasis, and disease are well defined, but less is known about the contribution of epigenetics in modulating these processes. Epigenetics refers to heritable phenotypic traits, including gene expression, which are independent of mutations in the DNA sequence. We have known for several decades that human colorectal cancers contain hypomethylated DNA, but the causes and consequences of this phenomenon are not fully understood. In contrast, tumor suppressor gene promoters are often hypermethylated in colorectal cancer, resulting in decreased expression of the associated gene. In this review, we describe the role that epigenetics plays in intestinal homeostasis and disease, with an emphasis on results from mouse models. We highlight the importance of producing and analyzing next-generation sequencing data detailing the epigenome from intestinal stem cell to differentiated intestinal villus cell.

Keywords Intestinal epithelium · Epigenetics · DNA methylation · Colorectal cancer

Introduction

Waddington coined the term “epigenetics” in 1942 to describe the burgeoning field of developmental biology, and the mechanisms underlying development from the undifferentiated embryo to the mature adult organism [1]. In the modern context, epigenetics is defined as mitotically heritable phenotypes that are mediated by mechanisms other than alteration of DNA sequences [2]. These mechanisms affect chromatin organization, i.e., the three-dimensional structure of DNA within the nucleus, which in turn influences gene expression patterns and resulting phenotypic traits among distinct cell types.

Epigenetic modifications are classified into three general categories: DNA methylation, histone modifications, and nucleosome positioning. Although the term “epigenetic” refers to the inheritance of the mark through at least one mitotic cycle, all of the described epigenetic modifications are dynamic to some extent [3]. We know that DNA methylation patterns are first erased and then re-established during early embryonic development, and we understand the molecular mechanism of their *trans*-mitotic inheritance. In contrast, the processes underlying the maintenance of histone modifications and nucleosome arrangements through the cell cycle are not well understood. In these instances, the marks are termed “epigenetic” due to their capabilities to alter gene expression patterns, which are maintained in specific cell lineages. The definition of epigenetics is occasionally expanded to include non-coding RNAs, such as microRNAs [4, 5], which will not be reviewed here. For a thorough review of microRNAs in intestinal homeostasis and disease, we refer to [6–10].

✉ Klaus H. Kaestner
kaestner@mail.med.upenn.edu

¹ Department of Genetics and Institute for Diabetes, Obesity and Metabolism, University of Pennsylvania Perelman School of Medicine, 12-126 Translational Research Center, 3400 Civic Center Boulevard, Philadelphia, PA 19104, USA

Histone modifications

Histones are the core proteins that comprise nucleosomes, and form the structural basis underlying chromatin architecture. Nucleosomes consist of 147 base pairs of DNA wrapped around a histone octamer, which contains two copies of each core histone protein (H2A, H2B, H3, and H4) [11]. The placement and density of nucleosomes determines the relative accessibility of DNA to different transcription factors and enzymes, and thus can directly impact gene expression [12].

Histone proteins can be altered by covalent posttranslational modifications to their charged tails, which protrude from the histone and are thus accessible to various modifying enzymes. These modifications alter the electrostatic charge of the histone tails to induce changes in the chromatin structure. Repressive histone modifications create a more compact chromatin environment, while activating modifications allow DNA-binding factors and other proteins to interact with DNA and increase gene expression [13]. In addition, specific histone modifications allow binding of ‘reader’ proteins, which can transmit the charged histone state into altered gene actions. There are many types of histone modifications and corresponding enzymes. For the purpose of this review, we have chosen the most commonly profiled modifications described in the current literature.

Activating histone modifications

There are multiple histone modifications that distinguish active areas of chromatin, but the most commonly found at actively transcribed genes are tri-methylation of H3K4 (H3K4me3) at their transcription start sites [14], and H3K36 (H3K36me3) within gene bodies [15] (Table 1). The H3K4me3 mark is established by SET-domain proteins, including the well-characterized MLL protein [16]. MLL is a mammalian homologue of the *Drosophila* Trithorax complex, and is a target for translocations in

acute myeloid leukemia [16, 17]. MLL contains a SET domain responsible for methylation of H3K4 to the mono-, di-, and trimethylated state (H3K4me1, me2, me3), and associates with the WDR5, RBPB5, and ASH2L proteins, which are necessary for MLL targeting and function [16]. H3K36me3 marks exons within actively transcribed genes, and specifically functions to prevent aberrant RNA polymerase activity within active genes [18]. In addition, the H3K36me3 intergenic mark contributes to DNA mismatch repair [19] and alternative splicing [20].

Enhancers are distal *cis*-regulatory elements that can be bound by sequence-specific DNA-binding transcription factors to activate a given target gene, and are also associated with multiple histone modifications. Active enhancers are characterized by H3K27 acetylation (H3K27Ac) [21], which is established by both the CREB-binding protein (CBP) and p300 acetyltransferases [22] (Table 1). H3K4me1 and H3K4me2 also mark *cis*-regulatory elements such as enhancers and promoters, but do not necessarily denote active transcription [23]. Interestingly, enhancers are also frequently sites of reduced DNA methylation [24–27], indicative of cross-talk between histone modifications and DNA methylation machinery (see below).

Repressive histone modifications

The two most commonly profiled repressive histone methylation marks are H3K27me3 and H3K9me3 (Table 1). H3K27me3 is established by the Polycomb repressive complex 2 (PRC2), comprising the catalytic subunits EZH1 and EZH2, in addition to core subunits SUZ12, EED and RBBP7/4 [28]. H3K27me3 is regarded as a stable histone modification [29], and is widely distributed in ES cell chromatin [30]. PRC2 and H3K27me3 are responsible for silencing key lineage-specific regulators in mouse ES cells to repress differentiation processes and maintain pluripotency [31]. Depletion of EZH2 and EED in mouse ES cells impairs mesendoderm differentiation [32],

Table 1 Histone modifications and their mediators

Modification	Complex which catalyzes mark	Localization in chromatin and/or genome
Activating histone modifications		
H3K4me3	MLL1-4, SETD1A, SETD1B, ASH1L, PRMD9	Transcription start sites of active genes
H3K4me1, me2	MLL1-4, SETD1A, SETD1B, ASH1L, PRMD9	<i>Cis</i> -regulatory regions (enhancers), transcription start sites
H3K36me3	SETD2	Exons within actively transcribed genes
H3K27ac	CBP, p300	Active regulatory elements (enhancers)
Repressive histone modifications		
H3K27me3	PRC2 (EZH1, EZH2)	Silenced developmental and lineage-specific genes
H3K9me3	SUV39H1-2, SETDB1	Constitutive heterochromatin and repetitive elements

and deletion of *SUZ12*, *EZH2*, or *EE* causes severe gastrulation defects during embryogenesis [33–35], demonstrating the critical function of PRC2 in developmental and differentiation processes.

The H3K9me3 mark is considered a hallmark of constitutive heterochromatin and is regulated by the SUV39H family of methyltransferases, including G9a, SUV39H1/2, and SETDB1/2. SUV39H1/2 establish the H3K9me3 mark in heterochromatin [30], where its interaction with heterochromatin protein 1 (HP1) recruits additional SUV39H1/2 methyltransferases to promote gene silencing [36]. SETDB1 is highly expressed in ES cells, and is required to maintain pluripotency and self-renewal capabilities in vitro [37].

DNA methylation

DNA methylation refers to the covalent addition of a methyl group to a cytosine base, referred to as 5-methylcytosine (5mC), and commonly occurs in the context of a CpG dinucleotide. CpG dinucleotides are underrepresented in the mammalian genome due to the spontaneous deamination of 5mC to thymine. As a result of these processes, only 1 % of the human genome consists of CpG sites as opposed to the ~4 % expected by chance, and approximately 60–80 % of these CpG sites are methylated, depending on the cell type [38]. The remaining unmethylated CpGs are predominantly located in regions of increased CpG frequency, termed CpG islands (CGIs). CGIs, as defined by Takai and Jones [39], are ≥ 500 base pairs in length, have a CpG observed/expected ratio ≥ 0.65 , and have at least 55 % GC content. CGIs are generally unmethylated, and are located at 72 % of annotated promoters in the human genome [40]. Interestingly, regions of low CpG methylation can also be found for distal CGIs in enhancers [25–27].

The enzymes that establish DNA methylation patterns are DNA methyltransferases (DNMT). There are three DNMTs encoded in the mammalian genome, divided into two categories based on their sequence similarities and DNA methyltransferase activities in vitro. The “de novo” methyltransferases, DNMT3A and DNMT3B, have low affinity for hemi-methylated compared to unmethylated DNA in vitro [41], and can establish novel patterns of DNA methylation. The second type of DNMT is the “maintenance” methyltransferase DNMT1. DNMT1 associates with factors at the replication fork to copy patterns of methylation onto newly synthesized strands of DNA, thereby faithfully maintaining the pattern of DNA methylation across multiple cell divisions [42]. It has recently been suggested that the categories of “maintenance” and “de novo” are too simplistic to describe the complex

interplay of DNMT activity in vivo [43]. For example, deletion of *Dnmt1* in mouse ES cells causes only a 66 % decrease in DNA methylation levels [44], suggesting that DNMT3A and DNMT3B have some maintenance methyltransferase activity.

DNA methylation and therefore DNMTs are crucial for mammalian development. *Dnmt1*- or *Dnmt3b*-null mouse embryos arrest at E.9.5, and *Dnmt3a*-null newborn mice are runted and die within the first 2 weeks of life [44, 45]. Intriguingly, triple *Dnmt*-knockout (TKO) mouse ES cells have normal morphology and survival in vitro, but undergo apoptosis when induced to differentiate [46, 47]. In contrast, recent studies have shown that human ES cells require DNMT1 even during maintenance culture [48]. These results support the notion that mouse and human ES cells denote distinct pluripotent states, with human ES cells representing a later stage of epiblast development relative to mouse ES cells [49]. Overall, these data strongly implicate a critical role for DNA methylation in early embryonic development and differentiation processes.

Differential methylation patterns distinguish specific tissue and cells types, with differential methylation occurring at CGI shores and enhancer elements [50]. DNA methylation acts primarily as a repressive epigenetic modification in chromatin, downregulating expression of genes associated with regions of increased DNA methylation. There are two broad means by which DNA methylation can repress gene expression: direct inhibition of DNA-binding transcription factors, and interactions with other chromatin-remodeling enzymes to promote a repressive chromatin environment. Although several examples of direct inhibition of transcription factor binding to methylated DNA are known, including for CREB, AP-2, and E2F [51], the majority of studies have focused on how DNA methylation patterns influence global chromatin organization. The effects of DNA methylation on chromatin may be indirect, such as through the methyl CpG binding proteins MeCP2, MBD2, or MBD3. MeCP2 recruits histone deacetylases to methylated DNA, which promotes heterochromatin formation and stable gene repression [52, 53]. Additionally, the DNMTs directly interact with several chromatin-remodeling complexes, such as Polycomb group protein EZH2, to facilitate heterochromatin formation [54].

DNA methylation has long been considered the most “stable” of the epigenetic modifications. The earliest studies of DNA methylation and DNMT function described the processes of imprinting and X-chromosome inactivation during development [55–57]. Imprinting refers to the phenomenon of monoallelic gene expression in a parent-of-origin specific manner. One of the best-understood examples of imprinting occurs at the *H19/IGF2* locus, in which differential methylation at the imprinting control region

(ICR) located between the two genes determines monoallelic *H19* and *IGF2* expression. The ICR is methylated on the paternal allele, which results in *IGF2* expression from the paternal chromosome and *H19* expression from the maternal chromosome [58]. Imprints are maintained in all mature somatic cells types, and are only erased during primordial germ cell development in embryogenesis [59].

Nucleosome positioning and chromatin organization

Nucleosome remodeling complexes can completely alter the three-dimensional structure of chromatin by the addition, subtraction, or remodeling of nucleosome subunits. These dynamic activities are regulated by four families of proteins: SWI/SNF, ISWI, CHD, and INO80 [60]. These families of proteins assemble into large complexes, allowing them to alter nucleosome position within the larger context of histone modifications and DNA methylation patterns. All four types of complexes interact with multiple proteins, including nucleosomes, specific histone modifications, chromatin-remodeling enzymes, and transcription factors. In addition, these remodelers all contain a catalytic subunit with a DNA-dependent ATPase domain, which uses the energy from ATP hydrolysis to remodel nucleosomes [60].

The ATPases BRM and BRG1 are members of the SWI/SNF family, and share sequence homology with the *Drosophila* Trithorax genes [61]. BRM and BRG1 form multi-protein BAF remodeling complexes, which show a surprising amount of tissue and cell-type specificity via differential inclusion of BAF protein subunits [62]. Neural progenitors require BAF45a/53a subunits to support proliferation, while differentiation to postmitotic neurons necessitates BAF45b/45c/53b [63]. The SWI/SNF family is widely known as a master regulator of gene expression, having roles in various pathways related to cell adhesion, alternative splicing, cell cycle regulation and differentiation [64]. Several members, including BRG1 and SNF5, are frequently mutated or silenced in various types of cancers, pointing to a possible function as a tumor suppressor [65].

The CHD family of nucleosome remodelers is defined by ATPase proteins with chromodomains, which bind methylated lysine residues in histone tails [63]. CHD3/4 ATPases are essential for nucleosome remodeling activity in the Mi-2/NuRD complex. Mi-2/NuRD also contains the MBD3 protein, which binds methylated DNA, and histone deacetylases HDAC1 and HDAC2, which deacetylate histones and tighten local chromatin structure [66–68]. In ES cells, Mi-2/NuRD deacetylates H3K27 to promote PRC2-mediated transcriptional repression, which is required for ES cell pluripotency and differentiation [69, 70]. Overall, Mi-2/NuRD regulates transcriptional activity by coupling

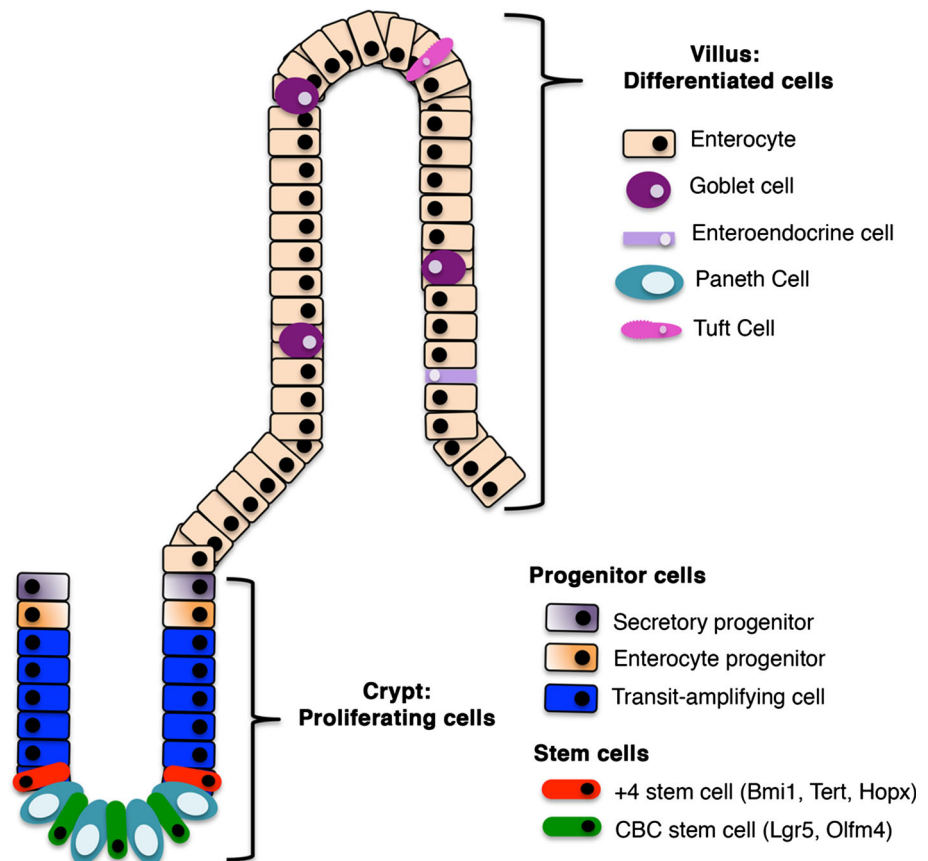
histone deacetylation with increased nucleosome density, which alters interactions with other histone modifying proteins.

The position of chromatin within the nucleus also plays a critical role in determining gene activity. Gene-rich chromatin accumulates within the center of interphase nuclei [71], and correlates with increased transcription and higher levels of the H3K4me2 mark [72]. In contrast, chromatin localized at the nuclear periphery displays low gene density [71] and transcription levels, and is enriched for heterochromatin histone modifications, such as H3K9me2 and H3K27me3 [72, 73]. H3K9me2 contributes to nuclear positioning of chromatin through interactions with the nuclear lamina [74, 75]; these interacting regions of chromatin are termed lamina-associated domains (LADs). H3K9me2 in LADs interacts with the lamin B receptor via heterochromatin protein 1 (HP1), tethering chromatin to the nuclear lamina [74]. H3K9me2 is essential for LAD localization, as loss of the H3K9 methyltransferase G9a impairs LAD formation in human cells in vitro [76]. Genome-wide studies have shown that LADs comprise approximately 40 % of the human genome [72], suggesting that these domains are important in gene regulation during different cellular processes, and may define chromatin structure in distinct cell types [73, 77]. Indeed, tethering of reporter genes to the nuclear periphery silences their expression, demonstrating a causal role for nuclear lamina association in gene regulation [78–80]. LADs are dynamic during directed differentiation of mouse ES cells to astrocyte fate in vitro, in which chromatin reorganization to the nuclear lamina correlates with gene repression [77].

In addition, LADs correlate with DNA methylation changes in several types of cancer [81–83]. Berman and colleagues profiled DNA methylation levels in primary colon tumors compared to adjacent normal colonic epithelium, and found that hypermethylated promoter CpG islands were located within large regions of hypomethylation [81]. Interestingly, they found that the boundaries of these colon-cancer partially methylated domains coincided with LADs identified in human cell lines. These results indicate that DNA methylation, histone modifications, and nucleosome positioning cooperate to regulate gene expression.

Histone modifications, such as the repressive H3K9me, can be recognized by DNMTs to influence heterochromatin formation and nucleosome remodeling. G9a dimethylates H3K9, which creates a binding site for heterochromatin protein 1 (HP1). HP1 then recruits DNMT1, which methylates CpGs to support permanent transcriptional repression [84, 85]. Interestingly, binding to DNMT1 also stabilizes localization of HP1, allowing HP1 to recruit other chromatin-remodeling complexes to form highly structured heterochromatin [84, 85]. In cancer, both HP1

Fig. 1 The mammalian intestinal epithelium. Longitudinal cross section of the adult intestinal epithelium. The intestinal epithelium is a single cell layer lining the lumen of the intestine, and is structured into proliferative crypts and differentiated villi. Crypts contain both proliferative CBC stem cells and quiescent +4 stem cells. These stem cells give rise to rapidly dividing transit-amplifying cells, which begin differentiation as they migrate out of the crypt. Secretory progenitors differentiated into goblet cells, enteroendocrine cells, Paneth cells. Enterocyte progenitors develop into absorptive enterocytes, which comprise ~90 % of the epithelium



and DNA methylation levels are globally reduced, signifying the importance of epigenetic cross-talk in disease progression [86–88].

The mammalian intestinal epithelium

One of the aims of epigenetic research is to elucidate how the various marks interact with one another, and which components are essential to disease progression and prevention. To study chromatin dynamics *in vivo*, during development as well as homeostasis and disease progression, requires a model system that closely parallels its human counterpart but is still genetically tractable. The mouse intestinal epithelium fits these requirements, and is an excellent model for the study of chromatin in modulating gene expression and disease. The structural components of the mouse intestinal epithelium are strikingly similar to those of the human intestine. Models for human intestinal disease, including colorectal cancer and inflammatory bowel disease, are well established in the mouse, which permits studies of chromatin marks and complexes in disease progression. The precise mechanisms that regulate intestinal epithelial homeostasis and disease

have remained elusive, and it is possible that epigenetic marks play a crucial role in such processes.

The adult intestinal epithelium consists of a single layer of columnar cells lining the lumen of the intestine. The epithelium is structured into crypts that invaginate into the underlying mesenchyme, and villi that project into the intestinal lumen (Fig. 1). In intestinal homeostasis, the crypt-based columnar (CBC) stem cells give rise to rapidly dividing transit-amplifying crypt cells. As the transit-amplifying cells exit the crypt, they differentiate into one of five major cell types: absorptive cells, called enterocytes, goblet cells, enteroendocrine cells, Paneth cells [89], and Tuft cells [90]. Paneth cells are retained at the base of the crypt, while the other types of differentiated cells migrate up the villi. In contrast, the colonic epithelium comprises entirely of crypts [91], which contain proliferative stem cells that are analogous to the intestinal CBC stem cells [92]. Colon stem cells also produce transit amplifying cells, which ultimately give rise to absorptive colonocytes, goblet cells, and enteroendocrine cells [91]. The colon does not contain Paneth cells, an important distinction between colonic and small intestinal crypts.

In both small intestine and colon, differentiated cells migrate and reach the top of the respective villus or crypt,

respectively, after 3–5 days, at which point the cells apoptose and are shed into the intestinal lumen. This high rate of cellular turnover indicates tight regulation of cell proliferation and differentiation processes. Indeed, misregulation of intestinal crypt proliferation is the hallmark of intestinal and colorectal cancer [93]. In the next sections, we will review the crucial roles of Wnt and Notch signaling in intestinal proliferation and differentiation processes. Then, we will discuss the prevailing models of epigenetic regulation in intestinal homeostasis.

The intestinal stem cell niche and Wnt signaling

Crypts form the intestinal stem cell niche, and harbor two well-characterized populations of stem cells (Fig. 1). The first are the crypt-based-columnar (CBC) stem cells, which express the markers *Lgr5* and *Olfm4*, and give rise to all cell types in the intestinal epithelium [92, 94]. CBCs are considered the active population of intestinal stem cells, and divide approximately once every 24 h. The second population of stem cells are the quiescent “+4 stem cells”, marked by *Bmi1*, *Tert*, and *Hopx* [95]. The +4 stem cells constitute a reserve population; upon ablation of the CBC population in mice, +4 stem cells can give rise to new CBC stem cells and repopulate the intestinal epithelium [96]. In separate studies, it was also shown that the CBC cells can give rise to +4 stem cells [97]. As a result, it is difficult to make a clear distinction between the two interconverting stem cell populations. It is possible that the crypt base contains a equipotent population of intestinal stem cells, and that the cell position within the crypt determines whether the cell is a fast- or slow-dividing stem cell. Evidence supporting this equipotent stem cell hypothesis comes from studies of irradiated mice, in which *Dll1*⁺ secretory precursors can convert to *Lgr5*⁺ CBCs to compensate for epithelial loss [98].

The main signaling pathway that supports proliferation in the crypt, both in vivo and in vitro, is the canonical Wnt signaling pathway. Briefly, canonical Wnt signaling depends on cytoplasmic stabilization of β -catenin, via disassociation of the Gsk3 β -APC-Axin destruction complex. Accumulation of stable β -catenin protein in the cytoplasm results in its translocation to the nucleus, where it interacts with Tcf/Lef DNA-binding effector proteins at target genes to activate transcription. Wnt activity is required to maintain proliferation in the adult intestinal crypt; overexpression of the secreted Wnt antagonist *Dkk1* in mouse intestinal epithelium inhibits proliferation, and blocks formation and maintenance of crypts [99, 100]. Deletion of *Tcf4*, the main β -catenin nuclear effector in mouse intestinal epithelium, causes complete loss of proliferation and stem cell identity in the adult intestine, and mice die ~9 days following ablation [101].

Paneth cells are proposed to have an active role in maintaining the CBC stem cells through their secretion of Wnt ligands [102]. This hypothesis was supported by observations that single *Lgr5*⁺ cells grow more efficiently in vitro when paired with a Paneth cell [102]. However, ablation of Paneth cells has no deleterious effects on CBC homeostasis or crypt architecture in adult mice [103], suggesting there are redundant mechanisms to maintain proliferation and Wnt signaling in vivo. Inhibiting Wnt ligand secretion concurrently in Paneth cells and subepithelial myofibroblasts did not alter crypt proliferation or crypt–villus architecture in adult mice [104]. However, there is some evidence that supports a critical role for the mesenchyme in maintaining the intestinal stem cell niche. Kabiri et al. inhibited epithelial Wnt secretion, and demonstrated that epithelial Wnt ligands are not required for crypt maintenance during development, homeostasis, or following injury [105]. Interestingly, ex vivo organoid cultures from these mice illustrated a dependence on exogenous Wnt sources. The authors reported that co-culture with wild-type intestinal stromal cells negated the requirement for supplementation with Wnt factors, strongly suggesting that the niche supporting cells exist within the intestinal stroma [105]. Thus, there are many populations of distinct mesenchymal cells surrounding the intestinal epithelium, and it is likely that one of these cell types is supporting the intestinal crypt.

Differentiation in the intestinal epithelium

There are five main types of differentiated cells in the intestinal epithelium: absorptive enterocytes, goblet cells, Paneth cells, enteroendocrine cells, and Tuft cells (Fig. 1). Although enterocytes comprise ~90 % of the intestinal villi cell population [89], the other types of differentiated cells secrete proteins that are crucial to intestinal function and survival. Goblet cells secrete mucin, the main component of mucus and the intestinal epithelial barrier, and are important to protect against potential pathogens in the lumen. Mucus coats the entire intestinal epithelium, acting as a lubricant to promote digestion, and preserves the structure of the epithelium upon physical force or injury [106]. Enteroendocrine cells secrete various hormones, including glucagon-like peptides 1 and 2 (GLP-1, -2), cholecystokinin (CCK), Glucose-dependent insulinotropic peptide (GIP), and somatostatin (SST) [107]. There are ~15 types of enteroendocrine cells, and their secreted hormones have important functions in feeding behavior, satiation, and glucose homeostasis [108]. For instance, GLP-1 is released in the small intestine following food intake, and promotes glucose uptake by stimulating pancreatic β -cell insulin secretion [109, 110].

Paneth cells are distinct from the other types of differentiated cells. As they differentiate from the transit-amplifying pool, they migrate to the base of the crypt and reside interspersed between the CBC stem cells. Paneth cells are long-lived relative to villus cell populations, surviving for approximately 1 month in the crypt epithelium [111]. Their main function is to secrete lysozyme and other defensin proteins that protect against bacterial infection in the epithelium [112].

Another type of endocrine cell, the Tuft cell, comprises 0.4 % of the intestinal epithelium [90] and has garnered much attention over the past 5 years due to the discovery of their distinct cell lineage. Although they require the pan-endocrine transcription factor *Atoh1* for differentiation, Tuft cells do not require the other factors necessary for enteroendocrine, goblet, or Paneth cell specification [113]. Tuft cells are marked by expression of *Dclk1*, and have been shown to contribute to intestinal recovery following injury [114, 115]. *Dclk1* is also a putative cancer stem cell marker, and loss of *Dclk1*⁺ cells abrogates tumorigenesis on the *Apc*^{Min/+} colorectal cancer mouse model [116, 117].

Notch signaling regulates differentiation and stem cells

Notch is one of the most important signaling pathways in the intestinal epithelium, and has crucial roles in regulating both intestinal proliferation and differentiation processes. The Notch signaling pathway works via lateral inhibition, in which one cell expressing the Delta ligand activates the Notch receptor on an adjacent cell. Activation of the Notch receptor causes cleavage of its intracellular domain, which subsequently translocates to the nucleus and forms a complex with the DNA-binding transcription factor CSL (CBF-1/RBP-Jκ, Su(H), Lag-1). CSL normally acts to repress gene expression, but binding of the Notch intracellular domain (NICD) converts CSL to a transcriptional activator. In the intestinal epithelium, secretory precursors expressing the Delta ligand (*Dll*) activate Notch signaling in neighboring cells. NICD activity increases expression of the bHLH transcription factor *Hes1*, which supports enterocyte differentiation via its repression of *Atoh1* [118, 119]. *Atoh1* is a bHLH transcription factor necessary [120, 121] and sufficient [122] for all secretory lineages in the intestinal epithelium. Notch signaling opposes *Atoh1* activation to direct cell fate into the enterocyte lineage. Loss of Notch signaling causes increased secretory cell differentiation along the crypt–villus axis, either through use of γ -secretase inhibitors [123, 124], or by ablation of Notch receptors [125, 126], *Dll* ligands [127], or CSL/RBP-Jκ [123]. Combined loss of Notch signaling and *Atoh1* expression blocks secretory cell fate conversion [124, 128–130] and induces global enterocyte

differentiation [130], establishing that Notch acts specifically through that *Atoh1* to regulate secretory cell fate, and that active Notch is not required for enterocyte differentiation. Conversely, mice with forced over-expression of Notch ICD display severe reduction of secretory cell types and increased enterocyte differentiation [131, 132].

Interestingly, over-expression of the NICD causes increased crypt cell proliferation [131, 132], whereas loss of Notch signaling blocks proliferation and converts crypt cells to secretory cell fates [123–125, 127]. Notch pathway inhibition decreases expression of CBC stem cell marker genes, including *Olfm4* and *Lgr5* [124, 126, 127]. Van-Dussen and colleagues found that the NICD directly activates expression of *Olfm4*, demonstrating the crucial function of Notch signaling in maintaining the CBC stem cell population [124].

Notch signaling components are expressed in the crypt, and interact with Wnt signaling to support stem cell renewal, proliferation, and differentiation processes [133]. In fact, Wnt activation strongly favors a secretory cell fate by its positive regulation of *Atoh1* and *Sox9* [100, 134], important for Paneth cell fate, and mouse models with decreased Wnt signaling display reduced numbers of secretory cells [100, 101]. Activation of Notch in the *Tcf4*-null, non-proliferative developing intestinal epithelium fails to restore progenitor cell division [135]. A recent report from Tian et al. [136] used Notch-inhibiting antibodies to illustrate that Notch inhibition allows increased Wnt activation of secretory genes, such as *lysozyme*, at the expense of crypt cell proliferation. Concurrent treatment with both Wnt- and Notch-blocking antibodies restored CBC proliferation and normal cell differentiation, suggesting that Notch mediates its effects in part through Wnt pathway inhibition [136]. These experiments demonstrate that cooperation between Notch and Wnt signaling is crucial to maintain proliferation and differentiation processes in the intestinal epithelium.

Transcriptional and epigenetic regulation of the intestinal epithelium

The genetics of key DNA-binding transcription factors in the intestinal epithelium has been extensively studied, but less is known about the role of chromatin marks and mediators in the processes of intestinal homeostasis. Two conflicting viewpoints have emerged over the past decade (Fig. 2). The first is the concept that the chromatin of the intestinal epithelium is largely permissive, and that transcription factor activity is the defining characteristic that alters gene expression patterns (Fig. 2a, b). The second posits that the chromatin itself plays an important role in regulating gene expression, and that chromatin states are

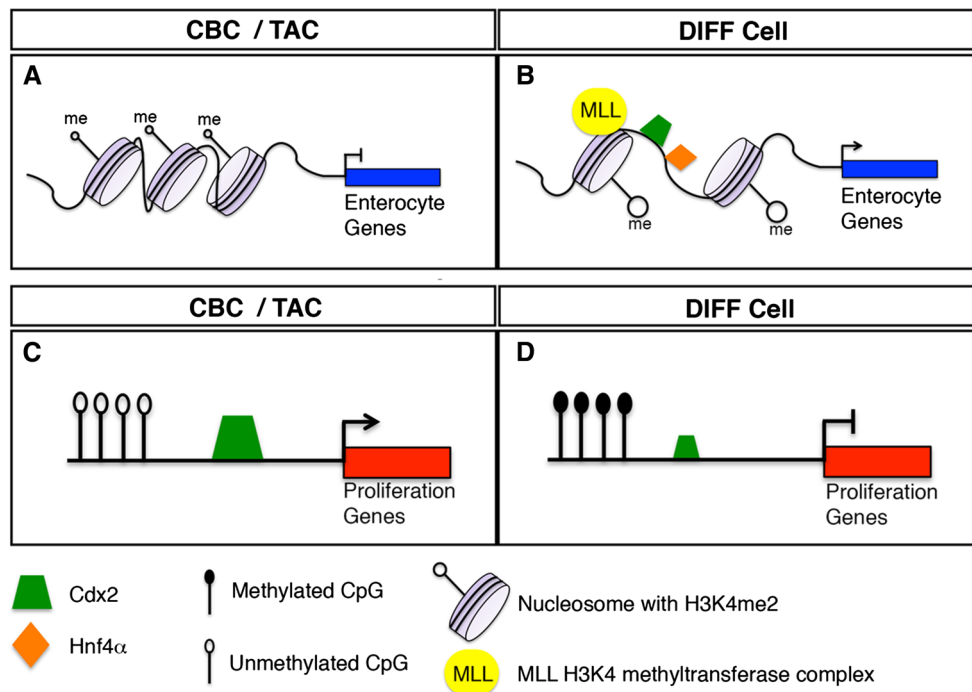


Fig. 2 Oposing roles for epigenetic modifications in the intestinal epithelium. **a, b** Intestinal chromatin is maintained in a permissive state by the transcription factor Cdx2 during intestinal differentiation [140]. **a** In proliferating CBC stem and transit amplifying (TAC) cells, Cdx2 is not bound at enterocyte-specific enhancers. As a result, these regulatory regions display reduced H3K4me2 (denoted by reduced size of the methylation circles, relative to **b**). Thus, the chromatin at these enhancers is in a repressive state, and the corresponding genes are not highly expressed. **b** In differentiating (DIFF) cells, Cdx2 binding supports H3K4me2 di-methylation at enterocyte-specific enhancers [141], presumably by MLL methyltransferase complex

activity. Co-binding of Cdx2 and Hnf4 α to these active enhancers causes expression of the associated enterocyte-lineage genes. **c, d** DNA methylation at enhancers upstream of proliferation genes regulates cell division in the intestinal epithelium [24]. **c** In dividing intestinal cells, enhancers are unmethylated, allowing Cdx2 to bind and activate progenitor gene expression. Size of Cdx2 protein (green) indicates amount of Cdx2 binding, relative to **d**. **d** In differentiated cells, enhancers associated with proliferation genes are methylated. DNA methylation restricts access by Cdx2 transcription factors, and genes are silenced. **a, b** adapted from [140]. **c, d** adapted from [24]

not necessarily dependent on transcription factor activity (Fig. 2c, d).

Transcription factors regulate open chromatin and intestinal homeostasis

There are several transcription factors that are globally important for intestinal epithelial development, maintenance, and proliferation. Cdx2 is master-regulator of intestinal epithelial differentiation, and is essential for the specification of all intestinal epithelia during mouse endoderm development [137]. Cdx2 ablation in mouse endoderm prevents colon and rectum morphogenesis and causes global defects in differentiation and proliferation transcriptional programs [137]. Cdx2 is also required for normal activation of pro-intestine transcription factors, including Hnf1 α , Hnf4 α , and Cdx1, and regulates Hedgehog ligand secretion from the epithelium to the underlying mesenchyme [137]. Loss of Cdx2 at mid-gestation perturbs apical–basolateral polarity of the developing epithelium,

causing deficient enterocyte development and maintenance [138]. In addition, inducible deletion of Cdx2 in the adult gut reduces villus length and the numbers of enterocytes, causing nutrient malabsorption and death within 3 weeks [139, 140]. Each of these studies demonstrated that Cdx2 is capable of both transcriptional repression and activation, based on gene expression changes following Cdx2 loss [137–139].

To study the CDX2 binding dynamics during intestinal cell differentiation, Verzi and colleagues first utilized the Caco-2 human cell line, which can be manipulated to produce homogeneous populations of proliferative and differentiated intestinal cells. They performed H3K4me2, H3K27ac, and CDX2 ChIP-Seq to demonstrate that CDX2 binds distinct *cis*-regulatory sites in differentiated and proliferative cell states (Fig. 2a, b) [140]. In the same model system, they showed that CDX2 preferentially co-localizes with GATA6 at enhancer-marked chromatin in proliferative cells, and HNF4 α at different enhancer elements in differentiated cell populations (Fig. 2b) [140]. In

differentiated mouse villi, Verzi and colleagues found that *Cdx2* co-localized with *Hnf4 α* at many distal enhancer elements, which they defined as 450–600 bp regions flanked by H3K4me2-marked nucleosomes (Fig. 2b) [141]. Loss of *Cdx2* in mouse intestinal villi reduced HNF4 α binding at co-bound sites [141], in agreement with previous data showing that *Cdx2* regulates *Hnf4 α* expression [137]. Interestingly, they also discovered that *Cdx2*-bound sites displayed reduced H3K4me2 nucleosome occupancy in *Cdx2*-deficient villi, and conclude that *Cdx2* is required to maintain open chromatin in differentiated cells [140, 141]. These findings build upon previous work that established *Cdx2* as the master-regulator of intestinal epithelial differentiation [137, 138], and suggest that *Cdx2* exerts this function both by its ability to control chromatin compaction and through its interactions with multiple transcription factors.

Based on the above data, it appears that *Cdx2* maintains the intestinal epithelium in a largely active chromatin state (Fig. 2a, b). These conclusions are supported by additional studies that more thoroughly analyzed histone marks during *in vivo* differentiation. Through advanced genetic and cell sorting techniques it is possible to isolate various populations of cells from the adult mouse intestine, including the Lgr5⁺ stem cells, secretory progenitors, and enterocyte progenitors. Kim and colleagues performed H3K4me2 and H3K27ac ChIP-Seq, DNaseI mapping, and RNA-Seq to define the different cell populations by their open chromatin states and associated gene expression [142]. Although the stem cells, secretory progenitors, and enterocytes display distinct transcript profiles, the enterocyte and secretory progenitor populations have remarkably similar H3K27ac, H3K4me2, and DNaseI profiles, which indicate active enhancer elements and globally open chromatin structure [142]. The authors propose that DNA-binding factors influence chromatin activity during the differentiation process, and that the labile chromatin structure renders progenitors capable of reacting to the available set of transcription factors. To test this hypothesis, they attempted to direct progenitor cell fate *in vivo* by genetic manipulation of lineage-specific transcription programs. Acute inhibition of Notch signaling forces progenitors to adopt a secretory cell fate within 2 days. Following global conversion of progenitors to secretory cell fate, the authors induced loss of *Atoh1*, which is completely required for secretory lineages. As the authors predicted, secretory progenitors present at the outset of *Atoh1* deletion convert to enterocyte fates [142]. They concluded that the dynamic abilities of progenitor cells to convert lineages is due to their similar chromatin profiles, and that expression of distinct transcription factors specifies cell fate.

Additional work has characterized the function of the H3K79 methyltransferase DOT1L in the mouse intestinal

epithelium. H3K79me2 is associated with both heterochromatin and euchromatin in model organisms and has known functions in transcriptional elongation, cell cycle checkpoints, and DNA repair [143]. DOT1L-mediated gene activation is commonly employed in human leukemias with MLL translocations, making DOT1L an attractive target in disease research [144]. Two recent studies analyzed the requirements for DOT1L and H3K79me, and reported strikingly different results.

Mahmoudi and colleagues originally identified Mllt10/Af10 in a screen for proteins that bind directly to the Tcf4/Wnt-signaling complex in the mouse intestinal epithelium [145]. MLLT10/AF10 directly interacts with the DOT1L methyltransferase to promote transcriptional activation, and is the primary mechanism by which DOT1L is activated in human leukemia [143]. The authors report that DOT1L and the Mllt10/Af10 mediators are essential for Wnt target activation in intestinal crypts [145]. However, these conclusions were only validated at *Axin* and *C-myc*, two canonical Wnt target genes.

A comparable study from an independent research group also evaluated the function of DOT1L in the intestinal epithelium, with different results [146]. They profiled H3K79me2 and RNA expression levels in villi and crypts isolated from adult mouse intestine. Surprisingly, the authors demonstrate similar levels of H3K79me2 at various Wnt targets in both crypt and villus compartments. They do note that H3K79me2 generally correlates with increased expression of associated genes, for both Wnt and non-Wnt target genes. Ablation of DOT1L in the intestinal epithelium caused global loss of H3K79me, but did not have any deleterious effects on differentiation or crypt–villus morphology. Indeed, Wnt targets were expressed at normal levels in the absence of DOT1L, indicating that the H3K79me2 mark is not essential for intestinal epithelial gene activation [146]. Ho and colleagues clearly demonstrate that DOT1L-mediated H3K79me2 associates with transcribed genes both in crypts and villi, and is not required for Wnt target gene activation in intestinal crypt cells [146].

Overall, these studies suggest that the chromatin landscape of the intestinal epithelium is largely accessible, particularly at enhancer and regulatory elements. This enhancer chromatin is amenable to changes induced by transcription factors, such as *Cdx2* and *Hnf4 α* , and allows progenitor cells to quickly adapt to specific cell fates. These statements align well with reports of plasticity within the intestinal epithelium upon tissue damage [98]. However, a comprehensive study comparing various repressive and activating histone modifications in intestinal epithelial cell sub-populations has not been reported. In the future, it will be important to profile multiple histone marks in the stem cell, progenitor, and differentiated cell types in

order to fully understand the function of chromatin during intestinal homeostasis and disease.

DNA methylation regulates enhancers and intestinal proliferation

The second view within the current literature introduced above is that chromatin and its associated modifications play a significant role in regulating proliferation and differentiation in the intestinal epithelium. This premise closely aligns with the two reports profiling genome-wide methylation levels in intestinal *Lgr5*⁺ CBC stem cells and differentiated villus cells. Both studies reported similar methylomes between stem and differentiated cells, and focused their analyses specifically on enhancer regions that have altered DNA methylation between the two cell populations.

In the first published study, the authors note low levels of DNA methylation at the majority of transcription start sites, which they suggest primes cells for the process of differentiation. Their data analysis required a 40 % change in DNA methylation between the two cell populations for identification as a differentially methylated region (DMRs), resulting in the discovery of only 50 DMRs [147]. The majority of these DMRs lost DNA methylation from stem to differentiated cell. Interestingly, these DMRs were positive for H3K4me1 and H3K27ac enhancer marks, and several of these putative enhancers loop and make contact with the transcription start site of differentially expressed genes [147]. Many DMRs were also located near Tcf4 binding sites, indicating possible regulation by Wnt signaling. Unfortunately, the authors provided only limited in vivo data testing of their hypothesis that loss of Tcf4 correlates with higher methylation levels at several DMRs in villi, and suggest that Wnt signaling interacts with chromatin to promote DNA demethylation during the differentiation process [147].

A second study employed vastly different bioinformatics approaches, resulting in identification of considerably more DMRs [24]. The authors note that modest DNA methylation levels of 13.9–50 % identify most enhancer regions [26], and suggest that a 40 % change in methylation for DMR identification is too strict a limitation. Sheaffer and colleagues report that the average change in methylation at DMRs was 15 %, and that these changes were enriched at CGIs and CGI shores [24]. These data align with the observations that alterations in CG methylation affecting tissue and cell-specific gene expression often occur in CGIs and shores [26, 27, 50]. They performed parallel H3K27ac ChIP-Seq and RNA-Seq, and also utilized available *Cdx2* and *Hnf4 α* ChIP-Seq data [141], to correlate DMRs with active enhancer elements during the differentiation process. Many DMRs that gained

methylation during differentiation associate with highly expressed ISC genes, and demonstrate increased H3K27ac and *Cdx2* binding in the *Lgr5*⁺ population relative to villus cells (Fig. 2c, d). DMRs that lost methylation from the stem to differentiated cell state corresponded with increased expression of the associated gene, which were enriched for metabolism and enterocyte-specific transcripts. Interestingly, they discovered that these differentiation-DMRs also gained H3K27ac, and displayed increased *Cdx2* and *Hnf4 α* binding in differentiated cells versus *Lgr5*⁺ ISCs [24]. These data indicate that DNA methylation works in combination with transcription factors to activate enhancers and modulate gene expression programs during intestinal differentiation.

The authors also employed genetic means to demonstrate that DNA methylation has significant effects on cell proliferation and differentiation in vivo. Inducible deletion of *Dnmt1* in the adult mouse intestine caused an approximately twofold expansion of the crypt compartment, and increased expression of *Lgr5* and *Olfm4*, markers of active CBC stem cells [24]. Targeted bisulfite sequencing upstream of these stem cell genes revealed demethylation at putative enhancer elements, implicating a role for *Dnmt1* and DNA methylation in promoting cell differentiation and restricting crypt cell proliferation (Fig. 2c, d) [24]. The above experimental results strongly suggest a role for DNA methylation in regulating enhancer activation and controlling processes relating to cell proliferation.

Epigenetic modifications and aberrations in intestinal disease

Colorectal cancer (CRC) is the third most common type of cancer in the United States, with a lifetime incidence of approximately 5 % in both men and women [148]. The risk of developing colorectal cancer increases with age, as 60 % of CRC diagnoses and 70 % of deaths relating to CRC occur in patients at 65 years or older [148]. The adenoma to carcinoma progression is well characterized in human CRC, and it may take several decades for a malignant tumor to fully form. Cancers begin as hyper-proliferative crypts that have accumulated mutations in tumor suppressor genes, such as *APC*, a Wnt inhibitor that is mutated in 80 % of sporadic CRCs [93]. Loss of *APC* causes constitutive Wnt activation and increased proliferation, resulting in dysplastic crypts. Over time, these hyper-proliferative foci develop into adenomatous polyps, or adenomas. Adenomas are generally benign, but a small percent progress to carcinoma, and adenomatous polyps are the main precursor to invasive colorectal cancers [149].

Development from adenoma to carcinoma requires multiple gene mutations; primary tumors harbor up to 80

distinct somatic mutations, and as many as 7 major genetic translocations or duplications [150, 151]. Tumor suppressor proteins, including *p53* and *PTEN* in addition to *APC*, accumulate inactivating mutations that allow unrestrained proliferative activity. Typically, both alleles of a tumor suppressor gene must be inactivated to cause a phenotype, following Knudson's two-hit rule [93]. Thus, germline inheritance of a heterozygous allele at these genes renders patients sensitive to cancer initiation by a loss-of-heterozygosity (LOH) mutation mechanism. Loss of tumor suppressor gene function is important in promoting genomic instability, a key feature of most invasive carcinomas. For instance, nearly 70 % of CRCs display mutations in chromosome 18q [152]. These mutations inactivate TGF- β signaling, which normally acts to restrict intestinal cell proliferation to the crypt. Oncogenic mutations are common in CRC as well, with approximately 40 % of CRCs demonstrating activating mutations at *KRAS* to support cell division via the EGFR pathway [93].

Sporadic CRCs are separated into two general categories: those with microsatellite instability and mismatch repair deficiencies, and those that are microsatellite stable but display chromosomal aneuploidy and large-scale genomic alterations [153]. Microsatellite instability (MSI) refers to changes in the length of microsatellite repeat elements, and is usually caused by defects in the DNA mismatch repair (MMR) machinery. MSI is significantly associated with the human heredity nonpolyposis colorectal cancer (HNPCC) syndrome [93]. The gene most commonly inactivated in MSI⁺ cancers is *Mlh1*, although other MMR proteins including *MSH2*, *PMS1*, and *PMS2* account for a significant subset of gene mutations [154]. The incidence of MSI is nearly 100 % in HNPCC patients, but occurs in only ~15 % of sporadic CRC [155].

The remaining 85 % of sporadic CRCs are microsatellite stable, but show a high degree of chromosomal instability (CIN) [156]. CIN tumors display increased rates of aneuploidy, such as the translocations frequently cited on chromosome 18q, and high levels of LOH at tumor suppressor genes [93]. The CIN phenotype is considered dominant; when CIN cells are fused with non-CIN cells, a CIN phenotype is transferred. When two non-CIN cells are fused, the mere presence of 4 copies of each chromosome does not induce CIN, indicating the cancer cells harbor a specific phenotype [157]. CIN tumors also associate with mutations in the genes encoding spindle-related proteins, such as *BUB1* and *MAD2* [158, 159]. However, there has been continued speculation that the CIN phenotype is simply an artifact of cancer progression, and this argument will likely continue until a molecular mechanism for CIN is found.

There are also several human genetic syndromes that predispose to microsatellite stable CRC, including familial adenomatous polyposis syndrome (FAP). FAP patients

have germ-line *APC* mutations, leaving them with only one functional copy of this crucial tumor suppressor protein [93]. Thus, the likelihood of loss of heterozygosity at the *APC* locus is dramatically increased in FAP patients, and nearly 100 % of patients or carriers develop CRCs by 36 years of age [160]. The genetic basis of FAP has made it attractive for use in model organisms. The *Apc*^{Min/+} mouse model is based on the human FAP syndrome, and is a commonly used paradigm for studies of CRC development and progression [161]. Since *APC* is also mutated in 80 % of sporadic CRC [93], the *Apc*^{Min/+} mouse is a representative model of human CRC initiation and progression.

The epigenetic contribution to CRC has been studied extensively over the past 30 years, but there is still much we do not understand about its role in carcinogenesis. The majority of these studies focused on the function of DNA methylation, because colorectal cancers generally display genome-wide hypomethylation that occurs early in the adenoma–carcinoma progression sequence [163, 164]. Below, we outline the proposed function of altered epigenetics in human CRC, and the information we have gleaned from studies of DNA methyltransferase mutations in mouse models of intestinal and colorectal cancer.

DNA hypomethylation and hypermethylation in human colorectal cancer

The precise function of hypomethylation in human CRC progression is not well understood. The earliest epigenetic profiling of cancer cells noted genome-wide hypomethylation in malignant human colorectal tumors [87, 88]. In later studies, it was discovered that even benign polyps and precancerous adenomas were substantially demethylated, with a reduction of 8–10 % compared to histologically normal adjacent tissue [163, 164]. These results were striking, and implicated a role for DNA methylation in colorectal tumorigenesis. Global genomic hypomethylation may cause activation of proliferation-associated genes, and lead to increased mutation rates based on in vitro data [165]. Analyses of human CRC cell lines indicates that hypomethylation causes expression of previously silenced genes [166], and correlated with increased genomic instability [167]. Ablation of *DNMT1* in a human CRC cell line caused hypomethylation, DNA replication defects, cell cycle arrest, and apoptosis [168, 169].

Notably, in CRC, tumor suppressor genes often display decreased expression, few genetic alterations, and increased promoter or CGI methylation. In sporadic MSI⁺ tumors, DNA mismatch repair (MMR) genes are often inactivated via promoter hyper-methylation [93]. MSI also correlates with loss of genomic imprinting [170], and mutations at the SWI/SNF chromatin-remodeling gene *ARID1A* [171]. Promoter hypermethylation also mediates inactivation of *SFRP*

Wnt signaling repressors, resulting in elevated Wnt signaling and proliferation in cultured CRC cells [172].

This promoter hypermethylation has been studied extensively, and is a defining characteristic of the CpG Island Methylator Phenotype, or CIMP [173]. CIMP-high (CIMP-H) tumors display increased DNA methylation at CGIs of tumor suppressors including *CDKN2A* (*p16*), *THBS1*, and *Mlh1*. CIMP-H tumors account for nearly all CRCs containing oncogenic *BRAF*/MAPK-pathway mutations [174], and is also common among tumors displaying MSI [175]. The association of MSI with CGI hypermethylation at multiple tumor suppressor genes strongly implicates CIMP as the underlying cause of genomic instability in MSI [174]. The Cancer Genome Atlas Network published an extensive study profiling DNA methylation and RNA expression from hyper-mutated versus non-hypermethylated categories of tumors [153]. In accordance with previous studies, they found that hyper-mutated cancers were enriched for hypermethylation and CIMP-H status, as well as *BRAF* mutations [153]. The mechanism underlying CIMP is not known. Several studies have reported that DNMT overexpression underlies this phenotype [176], but numerous reports have refuted this mechanism [177].

With the advent of next-generation sequencing, there have been multiple studies aiming to profile the epigenome of human CRC [50, 81, 83, 153, 178–184]. Enhancer chromatin is significantly altered in CRC, with tumors displaying distinct gains and losses of H3K4me1- and H3K27ac-marked enhancers compared to the normal crypt epithelium [179]. The authors termed these regions “variant enhancer loci” (VEL), and demonstrated that VELs correlate with altered transcription of nearby genes. VELs are also enriched for colon-cancer risk SNPs previously identified by genome-wide association studies [179]. CRCs display thousands of VELs [179], greatly outnumbering the genetic mutational load of a typical colorectal tumor [185], indicating that modification of enhancer chromatin is an essential step in CRC progression.

Several reports suggest that aberrant DNA methylation in CRC may involve the repressive Polycomb complex [180, 181, 183, 184]. Polycomb repressive complexes interact with DNMTs in human cell lines, and the EZH2 subunit of Polycomb is required to recruit DNMTs to H3K27-methylated CpGs [54]. Hypermethylated genes in CRC display both H3K27me3 and H3K4me3 marks reminiscent of ESCs, [180]. Hypermethylated CGIs are also marked by H3K27me3 prior to hypermethylation [181, 183], and exhibit a 12-fold enrichment for Polycomb target sequences [184]. Thus, in cancer, EZH2 activity may attract DNA methylation to permanently silence target genes, predisposing to cancer development.

Irizarry and colleagues performed genome-wide bisulfite sequencing in human colorectal tumors to assess exactly where hypermethylation occurs relative to CGIs and transcription start sites [50]. Interestingly, they found that hypermethylation in CRCs is enriched at CGI shores, defined as the regions 2 KB upstream and downstream of a respective CGI. The CGI enrichment data for CRCs are similar to the distribution they found for tissue-specific differentially methylated regions (DMRs) in normal tissues [50]. Furthermore, they found nearly equal amount of hypo- and hyper-methylated CGI shores in CRC compared to normal colon, suggesting that both types of methylation aberrations are involved in cancer formation. Many of these so-called cancer DMRs were enriched for tissue-specific DMRs, such as spleen, liver and brain, and gene ontology of the cancer-specific DMRs identified gene categories involved in pluripotency and development. Thus, many of the same pathways and tissues involved in development are altered in human CRC. This idea forms the basis for the epigenetic progenitor model of cancer, in which aberrant activity of differentiation pathways causes cancer [50].

DNA methylation in CRC: lessons from mouse models

As introduced above, the *Apc*^{Min/+} model, based on the human CRC syndrome FAP, forms the basis for many in vivo studies of intestinal carcinogenesis. *Apc*^{Min/+} mice develop tumors rapidly, with multiple tumors visible by 6 months of age throughout the small intestine and colon [161, 186]. Tumors in *Apc*^{Min/+} mice are *Apc*-negative, demonstrating that loss of heterozygosity (LOH) is required for tumor development [162]. Interestingly, *Apc*^{Min/+} mice form tumors predominantly in the small intestine, as opposed to human FAP patients, who develop adenomas in the colon [161]. The cause for this different anatomical site is not well understood, although it has been noted that several markers of human CRC, such as the CBC stem cell marker *Olfm4*, are not expressed in the murine colonic epithelium [94]. The structural similarities between colonic and small intestinal epithelium [91] allow comparison of *Apc*^{Min/+} intestinal tumor studies with human colon tumor progression. *Apc*^{Min/+} mice have proven to be a useful tool in understanding both intestinal and colorectal tumorigenesis, and is the model employed in the epigenetic studies outlined below.

Dnmt1

The earliest reports of the effects of DNA hypomethylation on tumorigenesis in *Apc*^{Min/+} mice utilized a combination of *Dnmt1* hypomorphic alleles and the pharmacological

Dnmt inhibitor 5-aza-deoxycytidine (5-aza). Laird and colleagues produced *Apc*^{Min/+}; *Dnmt1*^{S/+} hypomorphic mice, and treated them with 5-aza from 1 week of age to induce DNA hypomethylation. At 100 days, they observed that the *Apc*^{Min/+}; *Dnmt1*^{S/+} 5-aza treated mice had only two intestinal polyps, compared to 113 in the *Apc*^{Min/+} no 5-aza control. They concluded that DNA hypomethylation is not a causative factor in intestinal tumor development, and that DNA methylation is required for intestinal carcinogenesis.

Further studies enhanced this hypothesis, using varying *Dnmt1* hypomorphic alleles on the *Apc*^{Min/+} background. Cormier and Dove [187] used *Apc*^{Min/+}; *Dnmt1*^{N/+} mice to show that Dnmt1 regulates both intestinal tumor incidence and size, and noted that tumor suppression in this model was not dependent on p53. Eads and colleagues built on these data by constructing a *Apc*^{Min/+}; *Dnmt1*^{N/R} mouse line, which displayed severe hypomethylation and no polyp formation [188]. They also found that these mice display reduced CGI hypermethylation at tumor suppressor genes, and conclude that CGI hypermethylation is required for intestinal tumor development [188].

The most recent report more closely analyzes the varying stages of colonic tumor development in the absence of Dnmt1 [189]. *Apc*^{Min/+}; *Dnmt1*^{chip/c} mice exhibit reduced DNA methylation and colon macroadenoma formation, as expected. Interestingly, *Apc*^{Min/+}; *Dnmt1*^{chip/c} mice displayed increased numbers of colonic microadenomas, suggesting that Dnmt1 is not required to initiate tumors, but is required for their sustained growth. It should be noted that *Apc*^{Min/+} mice typically do not develop colonic tumors; unlike human FAP, *Apc*^{Min/+} mice develop primarily intestinal tumors. Yamada et al.'s *Apc*^{Min/+} colon controls only contain ~1 tumor per mouse, as opposed to the 100⁺ tumors an *Apc*^{Min/+} intestine would produce [189]. As a result, it is difficult to draw precise conclusions from this model.

Dnmt3a and Dnmt3b

Due to the impact of the CIMP phenotype in human CRC research, the function of de novo Dnmts in mouse tumor development has also been a significant area of research. Inducible overexpression of *Dnmt3a* had no effect on colon tumorigenesis on the *Apc*^{Min/+} background [190]. In the same study, inducible overexpression of Dnmt3b increased the number of colonic and intestinal macroscopic adenomas. Linhart and colleagues also report that Dnmt3b overexpression increased de novo methylation at *Sfrp2*, *Sfrp4*, and *Sfrp5*, which are endogenous suppressors of Wnt signaling [190]. *Sfrp* genes are common targets of promoter

hypermethylation in human CRCs [172]. In related studies in human colorectal cancer cell lines, demethylation of *Sfrp* gene promoters reactivated the associated genes, halting cell proliferation and inducing apoptosis [172].

Additionally, Dnmt3b overexpression in *Apc*^{Min/+} mice causes hypermethylation of similar gene sets in both colonic tumors and adjacent normal epithelium. Dnmt3b had no effect on the typical CIMP tumor suppressors, such as *Mlh1* or *Mgmt*, implying that Dnmt3b targets specific genes for hypermethylation [190]. Overall, these results suggest that Dnmt3b may promote the formation of colorectal cancers, and has a transformative effect on normal colonic epithelium.

The same group also published a study assessing the requirement for Dnmt3b in *Apc*^{Min/+} tumor development. They used the *Cre-loxP* recombination system to produce a colon-specific *Dnmt3b* deletion on the *Apc*^{Min/+} background. Lin and colleagues did not report any variation in normal wild-type mucosa in the absence of Dnmt3b [191]. In *Apc*^{Min/+} mice, loss of Dnmt3b led to a significant decrease in the number of macroscopic colonic tumors, but did not affect microadenoma initiation. The *Dnmt3b* deletion was fairly mosaic; only about half of microadenomas demonstrated loss of Dnmt3b protein. All mature tumors, however, contained patches of Dnmt3b⁺ cells, indicating an active selection against Dnmt3b-null microadenomas in tumor development [191]. These results suggest that Dnmt3b is crucial for progression to adenoma, but may not be essential to maintain a fully formed tumor.

Based on the above studies, one could conclude that DNA hypomethylation is unimportant for tumor development. However, there are several problems with these studies that remain to be addressed in the future. All of the above experimental models employed hypomorphic *Dnmt1* alleles, which caused hypomethylation in all tissues from early development onward. Our recent work demonstrates that loss of *Dnmt1* in mouse perinatal intestinal epithelium causes DNA damage and apoptosis of proliferative progenitor cells [192], while inducible ablation of *Dnmt1* in adult intestinal epithelium increases the crypt cell population [24]. These results clearly demonstrate differential requirements for Dnmt1 and DNA methylation in the developing versus mature intestine, and illustrate that *Dnmt1*-null intestinal phenotypes are dependent on age of ablation. Additionally, mesenchyme-specific transcription factors, such as *Foxl1*, are important modifiers of the *Apc*^{Min/+} phenotypes [193], and may be affected in *Dnmt1*-hypomorphic mice. Thus, it would be valuable to repeat the above experiments using epithelial specific and/or inducible *Cre-loxP* mouse models.

Summary and future perspectives

The mammalian intestinal epithelium is an exciting model system for the study of adult stem cell proliferation and renewal, as well as normal homeostasis and disease processes. We have made remarkable progress in understanding the genetics of the intestinal epithelium. However, there is a lack of information about the precise changes occurring in the epigenetic state during differentiation processes. Although many argue that transcription factors are the predominant factor in shaping the epigenome, there is evidence that epigenetic marks can directly impact gene expression patterns. In the future, it will be crucial to produce thorough epigenomic maps for the intestinal stem-cell and differentiated-cell populations, detailing repressive, activating, and regulatory epigenetic marks. Profiling similar epigenetic marks in human and mouse stem- and differentiated-intestinal epithelial cells is also essential to determine the overall accuracy of the mouse model system. Ultimately, a complete understanding of the genetic and epigenetic interactions during normal intestinal homeostasis progresses will help elucidate the mechanisms underlying intestinal disease.

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