Dnmt3a and Dnmt3b in Epidermal Homeostasis and Cancer Progression

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Summary

Summary

DNA methylation is an essential epigenetic mechanism controlling stem cell fate and stem cell differentiation. Dnmt3a and Dnmt3b are *de novo* DNA methyltransferases, meaning they establish the genome-wide levels of DNA methylation. How they maintain tissue homeostasis, and how their function is disrupted in cancer, is still unknown. Here we have demonstrated that Dnmt3a and Dnmt3b are required for epidermal stem cell function, associating with the most active enhancers in a histone H3K36me3-dependent manner. Dnmt3a promotes enhancer DNA hydroxymethylation, whereas Dnmt3b maintains high levels of DNA methylation surrounding the enhancers. Using conditional knockout mouse models, we elucidated that Dnmt3a and Dnmt3b are dispensable for the formation of a functional epidermis. Nevertheless, the loss of Dnmt3a, but not Dnmt3b, produced a strong predisposition to skin cancer initiation. During skin tumorigenesis, Dnmt3a associates with its target enhancers to promote their DNA hydroxymethylation. The regulation of this mechanism might be essential for maintaining the homeostasis of human adult tissues and for repressing tumor formation.

Resumen

Resumen

La metilación del ADN es un mecanismo epigenético esencial para controlar el linage y la diferenciación de las células madre. Las dos *de novo* metiltransferasas del ADN, Dnmt3a y Dnmt3b establecen los niveles de metilación del ADN en todo el genoma. Hemos demostrado que Dnmt3a y Dnmt3b son necesarias para la función de las células madre de la epidermis y que esta función depende de su asociación con los enhancers transcripcionales más activos en una forma dependiente de la modificación de la histona H3K36me3. Dnmt3a promueve la hidroximetilación en el centro de los enhancers, mientras que Dnmt3b mantiene altos niveles de metilación alrededor de los enhancers. Modelos de knockout en la epidermis de ratón nos han indicado que Dnmt3a y Dnmt3b no son indispensables para la formación de una epidermis funcional. Sin embargo, la pérdida de Dnmt3a, y no de la Dnmt3b, predispone enormemente a la iniciación del cáncer de piel. Durante la formación de los tumores de la piel, Dnmt3a se asocia y promueve la hidroximetilación de los enhancers de sus genes diana para promover su expresión. El correcto mantenimiento de este mecanismo epigenético puede ser esencial para la funcionalidad de el tejidos humanos y para bloquear la formaciones de tumorales.

Preface

Preface

DNA methylation is an essential epigenetic mechanism that regulates gene expression. While DNA methylation dynamics during embryonic formation is fairly well understood, its role in adult tissues is still under investigation. Prior to starting my PhD work, we found that the two *de novo* DNA methyltransferases Dnmt3a and Dnmt3b were differentially expressed during epidermal stem cell differentiation. Interestingly, their expression patterns were completely opposite. We thus decided to further investigate the role of DNA methylation, and specifically of Dnmt3a and Dnmt3b, during the process of human epidermal stem cell differentiation. To characterize the epigenomic importance of Dnmt3a and Dnmt3b, we performed several next generation sequencing (NGS) experiments in primary human epidermal stem cells, which mimic faithfully the *in vivo* patterns of human epidermal cells. To gain insights into the epidermal roles of Dnmt3a and Dnmt3b in an *in vivo* setting, we generated a conditional epidermal knockout for either protein. We obtained interesting results that complemented and expanded the genomic data obtained from the human stem cell project, with Dnmt3a and Dnmt3b found to locate to several chromatin regions that are essential for both the self-renewal and the proper differentiation of adult stem cells

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I am so happy you came back to Barcelona, to finish what we had started. Now I am done with the thesis and we can fly away.

Where, we do not know. But we will be together.

Introduction

1. INTRODUCTION

1.1 Epigenetic mechanisms

The role of epigenetic mechanisms in establishing different cell fates during embryogenesis is fairly well understood. However, if and how epigenetic mechanisms are necessary to stably maintain the identity of adult stem cells and their progeny during tissue homeostasis and repair is still under intense investigation. In recent years, a considerable number of studies have shown that factors involved in chromatin regulation during embryogenesis have diverse roles in adult tissues.

Adult stem cells maintain adult tissue homeostasis by replenishing dying or damaged cells, or by regenerating injuries. However, since tissues significantly differ in their turnover rates, each imposes a different demand for cellular input on its resident stem cells. Intriguingly, various mechanisms have evolved in different adult stem cells that involve the interplay between quiescent and actively proliferating stem cell populations, stochastic regulation of symmetric or asymmetric stem cell divisions, neutral competition of the stem cell progeny, or combinations of the these, all of which efficiently ensure tissue homeostasis (Solanas and Benitah, 2013). In addition, some tissues, such as the keratinocyte compartment of skin, the mammary gland, or the prostate, are highly compartmentalized and rely on distinct populations of stem cells often located adjacent to each other, which display completely

different behavior (Rinaldi and Benitah, 2014). How different stem cells are stably established during embryogenesis, and how they retain or loose their identity while self-renewing or differentiating, respectively, is still largely unknown.

Epigenetic modifications are chemical modifications either of double-strand DNA or of the core nucleosome histones, catalyzed by different families of enzymes. These modifications alter the structure of chromatin either by facilitating or preventing the binding of transcription factors and other co-factors. Importantly, although the turnover of some of these modifications is far more rapid than previously thought, these are often stably transmitted after a cellular division and can therefore affect the identity of the progeny. In this sense, epigenetic mechanisms allow genetically identical cells to stably adopt different phenotypes by controlling the transcription availability of different parts of the genome packaging or by opening different parts of the chromatin. In fact, findings from several laboratories in recent years indicate that the combination of transcription factors and chromatin remodeling factors might be essential for different aspects of the biology of adult stem cells (Rinaldi and Benitah, 2014). Importantly, a number of epigenetic factors are mutated in human cancers and deregulated during ageing, suggesting that perturbations in their functions may be causative of disease (Cebria-Costa et al., 2014).

1.1.1 Modifications of double-stranded DNA

DNA methylation is the most abundant and well-studied epigenetic modification in mammalians. It was first identified in 1975 (Holliday and Pugh, 1975; Riggs, 1975) by two independent articles. Thousands of studies have followed this pioneer work in an attempt to elucidate the implications and mechanisms of DNA methylation. Until a decade ago, the role of DNA methylation was believed to be straight-forward and could be recapitulated in two words: stable and repressive, with DNA methylation believed to be a stable genomic modification related to gene repression. However, later studies and the advance of technologies brought new light to the field, with new results challenging the dogmas of the DNA methylation field.

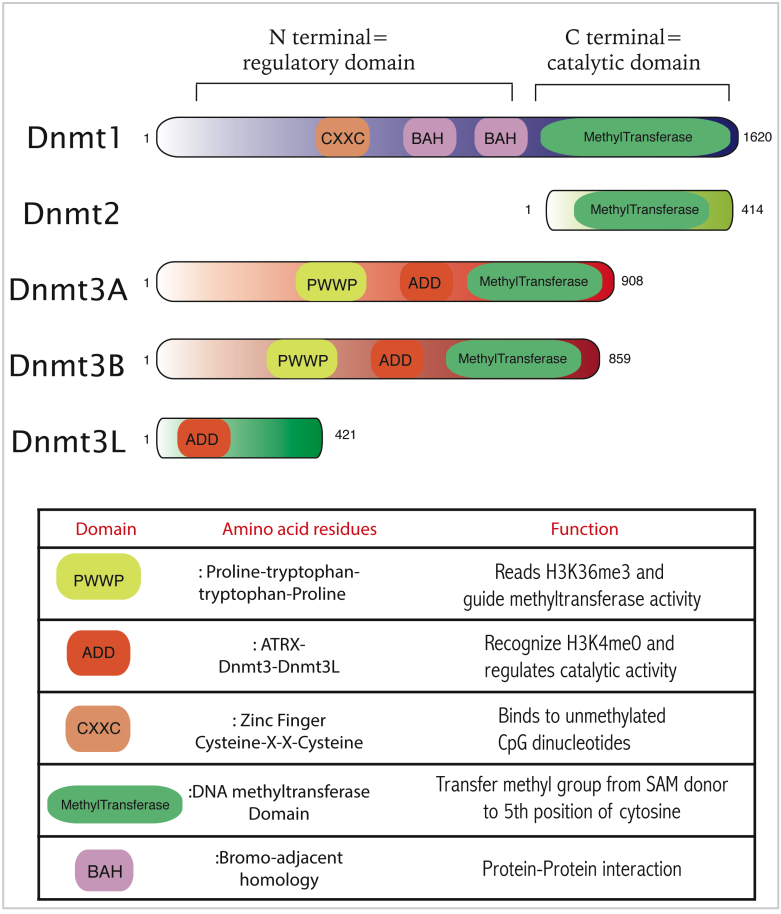
Briefly, DNA methylation occurs on the fifth position of the cytosine (5-mC), predominantly at CpG dinucleotides. 5-mC is frequent throughout the entire genome and is involved in regulating several aspects of gene expression, such as long-term gene silencing, transcriptional elongation, and maintenance of genomic stability (Smith and Meissner, 2013; S. C. Wu and Y. Zhang, 2010).

Generally, if a gene promoter is methylated, it will not be actively transcribed. Conversely, a high level of 5-mC in the gene exons correlates with an actively transcribed gene. In fact, 5-mC inhibits gene transcription initiation but does not inhibit transcriptional elongation.

Regions of compacted chromatin, such as heterochromatin sites, retrotransposons, microsatellites, and telomeres, normally have very high levels of methylation(Hon et al., 2013) However, it is still unclear whether changes in DNA methylation are causative of regulating gene expression *per se*, or rather important for the long-term stabilization of the 3D conformation and/or gene expression activity downstream of other regulatory proteins. Recent studies in adult stem cells support this last hypothesis (Bock et al., 2012; T.-H. Kim et al., 2014; Sheaffer et al., 2014).

1.1.2 Dnmts, players in the DNA methylation field

DNA methylation is established and maintained by a highly conserved family of nuclear enzymes called DNA methyltransferases (Dnmt). Of the five members identified to date, only three have been shown to be active DNA methyltransferases: Dnmt3a, Dnmt3b, and Dnmt1.



Domain	Amino acid residues	Function
PWWP	: Proline-tryptophan-tryptophan-Proline	Reads H3K36me3 and guide methyltransferase activity
ADD	: ATRX-Dnmt3-Dnmt3L	Recognize H3K4me0 and regulates catalytic activity
CXXC	: Zinc Finger Cysteine-X-X-Cysteine	Binds to unmethylated CpG dinucleotides
MethylTransferase	:DNA methyltransferase Domain	Transfer methyl group from SAM donor to 5th position of cytosine
BAH	:Bromo-adjacent homology	Protein-Protein interaction

Figure 1: Schematic representation of the mammalian DNA methyltransferase family

All active Dnmts consist of an N-terminal regulatory domain and a C-terminal catalytic part, which are highly conserved among the three (Figure 1). Active Dnmt catalyzes the transfer of a methylgroup from S-adenosyl L-methionine (SAM) to the 5′-position of a cytosine.

Dnmt3a and Dnmt3b are *de novo* DNA methyltransferases, since they have more affinity for unmethylated, as compared to hemimethylated, cytosines (Okano et al., 1999; Seisenberger et al., 2012; Watanabe et al., 2002). Additionally, they establish the DNA methylation pattern in the mammalian embryos.

In contrast, Dnmt1 primarily maintains the set pattern of DNA methylation during DNA replication, although this also requires the help of Dnmt3a and Dnmt3b (Figure 2). An accessory, inactive DNA methyltransferase (Dnmt3L) stimulates their activities but, interestingly, also competes for chromatin binding with others Dnmt3 (Suetake et al., 2004; Wienholz et al., 2010).

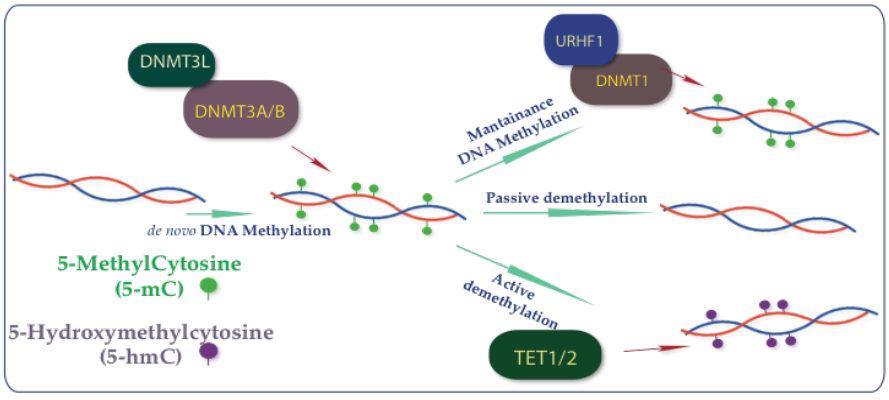


Figure 2: Schematic representation the major DNA methylation and demethylation pathways (adapted from Rinaldi L and Benitah SA, FEBS 2014)

Intriguingly, 5-mC not only occurs in double-stranded DNA but also in RNA, and especially in tRNA. This modification is catalyzed by two specific RNA methyltransferases: Dnmt2 and NSun2 (Goll et al., 2006; Tuorto et al., 2012).

1.1.3 DNA methylation and demethylation: 5-methylcytosine versus 5-hydroxymethylcytosine

DNA methylation is a stable but dynamic DNA modification. In fact, 5-mC is a reversible modification, since methylated cytosines can be either actively or passively demethylated (S. C. Wu and Y. Zhang, 2010) Passive demethylation occurs mainly through dilution during cell replication, when DNA methyltransferases might be inhibited or absent, resulting in locus-specific or global loss of DNA methylation (Bostick et al., 2007; Sharif et al., 2007; H. Wu and Y. Zhang, 2014). On the other hand, active demethylation relies on the ability of 5-mC to be oxidized into 5-hydroxymethylcytosine (5-hmC) by TET proteins, as reported in 2009 by the group of Anjana Rao (Tahiliani et al., 2009) (Figure 3).

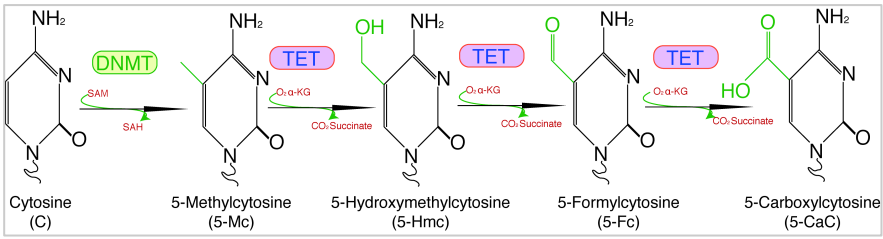


Figure 3: The step-wise cytosine modification pathway that includes cytosine methylation by Dnmts and oxidation of 5mC by TET proteins

The discovery of 5-hmC was perceived with great excitement from the epigenetic field, since it was not clear for many years how exactly 5-mC was re-transformed to an unmethylated cytosine (Figure 3). Therefore, a large number of researchers started focusing on the dynamics between DNA methylation and DNA hydroxymethylation.

The dynamics of DNA modifications are clearly exemplified during the very early stages of formation of the mammalian embryo. Here, maternal DNA is highly demethylated by Tet1/2, with a progressive and transient increase of hydroxymethylation. Subsequently, the *de novo* Dnmts re-establish the correct pattern of DNA methylation in the second week of murine embryonic stage (Meyenn et al., 2016; Seisenberger et al., 2012).

Initially, 5-hmC was thought to be only a transient DNA modification important for the demethylation of 5-mC, but a recent chemical study proved that 5-hmC is actually a stable DNA modification in mammalian genomes (Bachman et al., 2014).

In contrast to the repressive mark of 5-mC, 5-hmC is actually a permissive DNA modification. The finding that 5-hmC is in fact enriched at active regions, such as distal regulatory regions (also called enhancers) (Ficz et al., 2011; Sun et al., 2015; Yu et al., 2012) and bivalent promoters (Pastor et al., 2011), was highly surprising and stimulated further interest and research in the epigenetics field

1.1.4 Distal regulatory regions: enhancers

Recently, much attention has focused on studying whether and how distal regulatory regions (enhancers) regulate gene expression through modifications of the high-order three-dimensional conformation of chromatin. Enhancers are defined by a combination of distinct histone marks, including acetylation of lysine 27 of histone 3 (H3K27ac), and for having a high ratio of monomethylation at lysine 4 of histone H3 (H3K4me1) versus trimethylation of the same residue (H3K4me3) (Heintzman et al., 2007).

Enhancers are bound by a great number of transcriptional regulators such as transcription factors, epigenetic activators and repressors, RNA polymerases, microRNAs, and long non-coding RNAs. These interactors are essential to form the loop between the enhancer and the gene to be transcribed (T.-K. Kim and Shiekhattar, 2015). Overall, chromatin accessibility is primarily orchestrated by a high number of tissue-specific transcription factors (TFs) (Ballare et al., 2013; Beato and Eisfeld, 1997; Di Croce et al., 1999; van Oevelen et al., 2015). TFs are pioneer factors that bind to their target sequences within the regular nucleosome conformation and initiate events leading to chromatin remodeling and activation (Beato and Eisfeld, 1997). Upon external stimulus, TFs bind inactive chromatin and recruit epigenetic factors to remodel nucleosome positioning in order to recruit other factors and to establish the 3D loop that

enhances transcription of the targeted gene (Figure 4) (Ballare et al., 2013; Ceballos-Chavez et al., 2015; Filion and Beato, 2015).

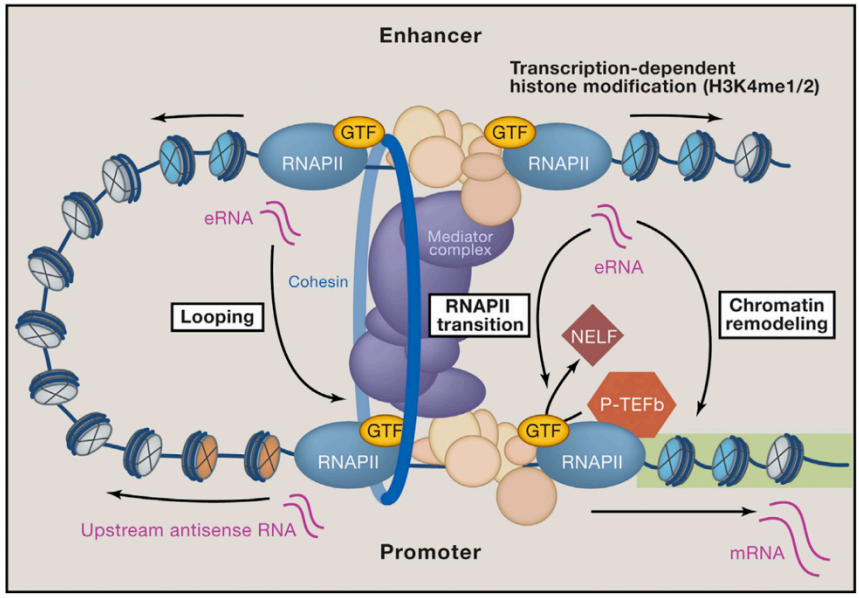


Figure 4: Enhancers boost genes expression through a 3D loop formed and maintained by a great number of chromatin remodeling factors, TFs, and member of the RNA polymerase machinery (taken from Kim and Shiekhattar, Cell 2015).

However, enhancers are very cell-type specific, and they change localization dramatically between cell types and even during differentiation of stem cells. Recently, important findings from the Richard Young laboratory have reported that two kinds of distal regulatory regions exist in the mammalian genome: the so-called typical enhancers and the super-enhancers (Figure 5). Typical enhancers have been classically defined as a single distant element that drives the expression of its target gene through one single 3D loop, and there are an estimated 10–30,000 active typical enhancers in the human genome. In contrast, there are far fewer super-enhancers, with typically only 300–800 per cell lineage. These super-enhancers are located in an agglomerate of chromatin organized in several 3D loops, serving as a gigantic genomic platform for a great number of transcriptional regulators: transcription factors, epigenetic activators and repressors, RNA polymerases, microRNAs, long non-coding RNAs, and mediator and integrator complexes (Kim and Shiekhattar, Cell 2015). This entire intricate structure serves to maintain the robust and continual transcription of the target gene, which is transcribed at very high levels. Indeed, genes regulated by super-enhancers are key genes for cell identity and for their cell-type–specific functions (Hnisz et al., 2013; Whyte et al., 2013).

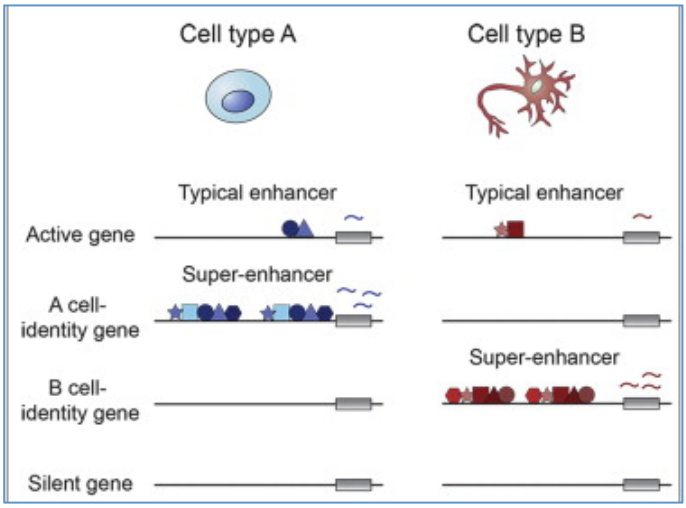


Figure 5: Super-enhancers are clusters of multiple enhancers that target and drive the expression of essential genes for specific cell-type identities, usually by targeting cell identity genes (taken from Whyte et al. Cell, 2013)

Typical enhancers are abundant in the mammalian genome and normally show low levels of DNA methylation (Hon et al., 2013; Rada-Iglesias et al., 2011). Intriguingly, a recent report has shown that a certain subset of enhancers are actually DNA methylated, suggesting a active role for 5-mC at enhancers (Charlet et al., 2016). In any case, the role of DNA modifications at enhancers and their contributions to enhancer looping and activity is still mostly unknown (Heintzman et al., 2007; Plank and Dean, 2014).

In this thesis, I addressed the relationship between DNA modifications and enhancer activity. Specifically, we show that the DNA modifications modulated by Dnmt3a and Dnmt3b are important for regulating enhancer activity in adult stem cell

1.2 Epigenetic factors regulating epidermal stem cell function

1.2.1 The epidermis

The keratinocyte compartment of the skin is formed by the epidermis and appendages, including hair follicles, sebaceous glands, and sweat glands (Solanas and Benitah, 2013). The epidermises, and the structures physically linking it to hair follicles, are under constant demand for cellular replenishment, imposing a strong pressure over its resident stem cells. Conversely, hair follicles undergo bouts of hair growth followed by long periods of dormancy. Each compartment is maintained by its own subset of stem cells that accordingly show very different behaviors (Solanas and Benitah, 2013). Although several populations of stem cells have been identified in each of these compartments, it was still not known, at the beginning of this work, how epigenetic mechanisms influence their establishment during development, or their maintenance in adulthood. Recent reports have provided much information about how chromatin-remodeling factors regulate the behavior of some of these stem cell subsets during homeostasis of their particular compartment in adulthood (Avgustinova and Benitah, 2016; Rinaldi and Benitah, 2014).

1.2.2 The choice of epidermal stem cells: self-renew or differentiate

Epidermal stem cells must cope with a constant demand of cellular supply due to the loss of cornified cells in the uppermost layer of the epidermis. Interestingly, the choice faced by actively dividing epidermal stem cells—to self-renew and/or to differentiate—is under stochastic regulation. Thus, it cannot be predicted whether a basal cell in the epidermis will divide symmetrically into two stem cells or two differentiated cells, or asymmetrically to produce a daughter stem cell and a differentiated one (Figure 6) (Clayton et al., 2007; Jones and Watt, 1993; Mascre et al., 2012).

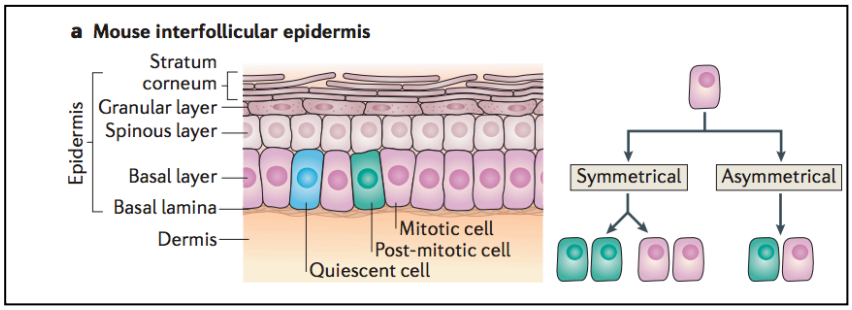


Figure 6: Quiescent and actively proliferating stem cell populations, regulated stochastically in a symmetric or asymmetric manner, ensure tissue homeostasis of the epidermis and of whole skin (taken from Solanas and Benitah. NMCB, 2013)

Whether these stochastic choices require the influence of chromatin remodeling factors to allow transcriptional noise and plasticity is not known(Avgustinova and Benitah, 2016; Luis et al., 2011; Rinaldi and Benitah, 2014). Furthermore, similar to hematopoietic stem cells, murine and human epidermises also contain a relatively

quiescent population of stem cells thought to contribute primarily to damage repair . In any case, epidermis is a high-turnover tissue, replenished constantly by epidermal stem cells (Blanpain and Simons, 2013; Solanas and Benitah, 2013). In contrast, other adult tissues do not rely on the constant turnover of stem cells. For example, muscle stem cells (also called satellite cells) are usually in a quiescent state, becoming activated first upon muscle damage (Sousa-Victor et al., 2015). Adult muscle stem cells are governed by other mechanism, such as autophagy that protect the satellite cells to enter into a geriatric irreversible senescence state (Garcia-Prat et al., 2016; Sousa-Victor et al., 2014). However, once the stochastic fate—quiescence, proliferation, or differentiation—of the epidermal stem cell has been determined, the concerted activity of different epigenetic complexes is required.

1.2.3 Role of DNA modifications in epidermal stem cells

The expression of the *maintenance* DNA methyltransferase Dnmt1 is high in the basal layer of the epidermis and decreases as the epidermis stratifies. DNA methylation follows the same pattern of Dnmt1 expression, and analysis of DNA methylation content (by MeDIP-ChIP) revealed that 5-mC decreases its amplitude in differentiated keratinocytes as compared to epidermal basal cells (Sen et al., 2010). Interestingly, Dnmt1-deficient human epidermal stem cells cannot fully maintain their pattern of DNA methylation and undergo premature and irreversible differentiation (Sen et al.,

2010), although it is unclear to what extent this effect is entirely dependent on the observed changes in DNA methylation. Intriguingly, conditional deletion of Dnmt1 in mouse epidermis does not affect the development of the epidermis or its appendages, and only shows signs of uneven epidermal thickness and a reduction of the length of the hair shafts upon ageing (Li et al., 2012). In accordance with this, genome-wide single-basepair resolution analysis revealed very similar patterns of DNA methylation between quiescent and actively proliferating hair follicle stem cells isolated from young mice (Bock et al., 2012). Nevertheless, this study reports more than 2,000 differential methylated regions, although these regions were not correlated with changes in gene expression. Considering that deletion of Dnmt1 results in hair follicle defects only upon ageing, it will be of interested to study whether changes in DNA methylation become prominent as hair follicle stem cells age, similar to what has been observed in hematopoietic stem cells.

Notably, no previous studies have focused on elucidating the role of Dnmt3a or Dnmt3b, neither in human nor in mouse epidermis.

In this thesis, I provide evidence for the role of Dnmt3a and Dnmt3b regulating the homeostasis of human epidermal stem cell functions. Dnmt3a is essential for maintaining the high levels of 5-hmC at active enhancers in human epidermal stem cells—a genomic regulation that we also identified in skin tumorigenesis using conditional knockout models. In contrast, Dnmt3b is important for maintaining the high levels of DNA methylation at active enhancers in human epidermal stem cells.

Importantly, we were also able to show that, in skin tumorigenesis, Dnmt3a (but not Dnmt3b) has a critical role in the suppression of tumor initiation, while both Dnmt3a and Dnmt3b prevent tumor progression.

Objectives

Objectives

1) To study the role of Dnmt3a and Dnmt3b during human epidermal stem cell homeostasis.

2) To investigate the function of Dnmt3a and Dnmt3b in murine epidermis during homeostatic conditions and skin tumorigenesis.

Results

First manuscript

Dnmt3a and Dnmt3b Associate to Enhancers to Regulate Human Epidermal Stem Cell Homeostasis

Lorenzo Rinaldi, Debayan Datta, Judit Serrat, Lluis Morey, Guiomar Solanas, Alexandra Avgustinova, Enrique Blanco, José Ignacio Pons, David Matallanas, Alex Von Kriegsheim, Luciano Di Croce and Salvador Aznar Benitah

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Second manuscript

Dnmt3a associates with promoters and enhancers to protect epidermal stem cells from cancer

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Manuscript under preparation

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Dnmt3a associates with promoters and enhancers to protect epidermal stem cells from cancer

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Short Title: Dnmt3a protects skin stem cells from cancer

Summary

Misregulation of DNA methylation is critical for oncogenic transformation, and one of the two *de novo* DNA methyltransferases, Dnmt3a, is mutated in human acute myeloid leukemia, and is a tumor suppressor in several types of murine models of cancer. However, the molecular mechanisms underlying the tumor-suppressive role of Dnmt3a remain poorly understood. Using conditional knockout mouse models, here we show that Dnmt3a—but not Dnmt3b—strongly protects epidermal stem cells from carcinogen-induced malignant transformation. However, progression from benign lesions to aggressive carcinomas was unaffected in Dnmt3a-depleted epidermis. Only upon combined deletion of Dnmt3a and Dnmt3b, squamous cell carcinomas acquired a more aggressive fate and even became metastatic. Mechanistically, Dnmt3a drives the expression of epidermal differentiation genes by interacting with their enhancers, and inhibits the expression of lipid metabolism and cell proliferation genes by directly methylating their promoters. Altogether, we demonstrate that Dnmt3a, but not Dnmt3b, is critical for suppressing tumor initiation, while both enzymes play roles (albeit redundant) in preventing tumor progression.

Introduction

DNA methylation is an epigenetic mechanism that regulates several aspects of gene expression, such as long-term gene silencing, transcriptional elongation, and maintenance of genomic stability (Allis and Jenuwein, 2016; Avgustinova and Benitah, 2016; Rinaldi and Benitah, 2014). It is found throughout the vertebrate genome and is deposited by DNA methyltransferases on the fifth position of cytosine (5-mC), predominantly at CpG dinucleotides. The role of DNA methylation in establishing different cell fates during embryogenesis is fairly well understood. However, if and how DNA methylation is necessary to stably maintain the identity of adult stem cells, and how this process is disrupted during oncogenic transformation, is under intense investigation (Shen and Laird, 2013).

Three DNA methyltransferases are encoded in the vertebrate genome. Dnmt1 is predominantly associated with the maintenance of DNA methylation following cell division due to its high affinity for hemimethylated DNA. Consequently, depletion of Dnmt1 leads to a significant reduction of the global levels of 5-mC (Lei et al., 1996; E. Li et al., 1992). Dnmt3a and Dnmt3b are *de novo* DNA methyltransferases that establish genome-wide DNA methylation during mammalian embryogenesis and adult stem cell homeostasis (Okano et al., 1999). In mouse embryonic stem cells, the combined loss of Dnmt3a and Dnmt3b leads to the progressive loss of DNA

methylation, suggesting that these enzymes are additionally involved in maintaining 5-mC levels (Chen et al., 2003).

Since Dnmt3a-null mice die perinatally, and ablation of Dnmt1 and Dnmt3b results in embryonic lethality around E14.5, conditional deletion mouse models have been necessary to study the functions of Dnmt3a and Dnmt3b in adulthood(Okano et al., 1999; Ueda et al., 2006). Hematopoietic stem cells (HSCs) lacking Dnmt3a cannot differentiate correctly upon serial transplantation, and end up developing a range of severe myeloid and lymphoid malignancies in aged animals (Challen et al., 2012; Mayle et al., 2015). Conversely, HSCs-depleted of Dnmt3b show no phenotypical differences with respect to wild-type controls, whereas combined ablation of Dnmt3a and Dnmt3b in HSCs result in an enhanced block of hematopoietic differentiation as compared to Dnmt3a loss alone (Challen et al., 2014). Interestingly, the observed phenotype seems specific to stem cells, as the fully differentiated cardiac myocytes carrying a combined deletion of Dnmt3a and Dnmt3b are indistinguishable from wild-type controls (Nuhrenberg et al., 2015). Similarly, to HSCs, purified murine neural stem cells (SCs) lacking Dnmt3a do not show problems with self-renewal but fail to differentiate properly (Wu et al., 2010). In addition, Dnmt3a acts as a potent tumor suppressor in lung tumorigenesis, to promote adenoma progression, but not initiation, downstream of oncogenic K-Ras (Gao et al., 2011). This is in contrast with the pro-tumorigenic activity of Dnmt3b, which at least in the colon

epithelium cooperates with the loss of APC to drive adenoma initiation and growth (Lin et al., 2006; Steine et al., 2011)

Recently, progress has been made in identifying the molecular mechanisms underlying the biological functions of Dnmt3a and Dnmt3b by studying their genome-wide localization. For instance, Dnmt3b associates with and methylates the gene bodies of actively transcribed genes in murine embryonic SCs and human embryonic carcinoma cells (Baubec et al., 2015; Jin et al., 2012; Morselli et al., 2015). Likewise, it has been proposed that gene body methylation is responsible of most of the transcriptional changes underlying the ability of Dnmt3a to promote neural SCs differentiation, and in protecting the lung epithelium from tumor progression (Gao et al., 2011; Wu et al., 2010). We have recently reported that Dnmt3a and Dnmt3b are indispensible for the self-renewal of human epidermal SCs, whereas Dnmt3a is also required for their proper differentiation (Rinaldi et al., 2016).

Mechanistically, Dnmt3a and Dnmt3b bind to and promote the activity of enhancers in both human epidermal stem cells and differentiated keratinocytes (although Dntm3a having a stronger affinity than Dnmt3b for enhancers in differentiated keratinocytes). Interestingly, both proteins preferentially associate to super-enhancers rather than typical enhancers. Nonetheless, they differ in their mechanism of action, since Dnmt3a (together with Tet2) is essential to maintain high levels of 5-hydroxymethylcytosine (5-hmC) at the center of its target enhancers, while Dnmt3b promotes 5-mC along the body of the enhancer. These regulatory regions

dictate the transcription of essential genes necessary for epidermal SCs identity and maintenance, such as *FOS, ITGA6, TP63, KRT5*. Similar to its role in mouse ES cells, Dnmt3b also binds to and methylates the gene bodies of these genes to reinforce their expression (Rinaldi et al., 2016). Dnmt3a also associates to the enhancers regulating the expression of genes such as *IVL, LOR, FLG2,* and *KRT1* which drive the differentiation of SCs into mature keratinocytes (Rinaldi et al., 2016).

However, to date, no *in vivo* studies have investigated the roles of Dnmt3a and Dnmt3b in adult epidermal stem cell function and malignant transformation. Using mouse models carrying an epidermis-specific ablation of either Dnmt3a or Dnmt3b, or both, we demonstrate that Dnmt3a and Dnmt3b are largely dispensable for skin homeostasis. However, Dnmt3a has a critical role in suppressing squamous tumor initiation, but not progression, while both Dnmt3a and Dnmt3b concertedly prevent tumor progression.

Results

We first studied the pattern of expression of Dnmt3a and Dnmt3b during epidermal development, and in the adult epidermis. At E14.5 Dntm3a was expressed in the entire Keratin-14+ compartment comprising the basal layer of the embryonic epidermis the hair placodes (Figure S1A). At P0, all Keratin-14+ basal cells were positive for Dnmt3a with the exception of the hair follicle bulb cells (Figure S1A-B). By the time animals reached adulthood, overall

Dnmt3a levels remained high in the hair follicle bulge where most hair follicle stem cells reside (Solanas and Benitah, 2014), and decreased in the interfollicular epidermis, although some basal IFE cells expressed high levels (arrows) (Figure S1A-B). On the other hand, we were not capable of detecting Dnmt3b by immunofluorescence staining in sections of developing or adult mouse epidermis (not shown), suggesting that Dnmt3a is the predominant *de novo* DNA methyltransferase in newborn and adult epidermis (Challen et al., 2014). Additionally, RNA-seq data confirmed that Dnmt3a was enriched almost 5-fold as compared to Dnmt3b in epidermal stem cells (Figure S1C). However, Dnmt1, the main DNA methyltransferase, was the most abundant DNA methyltransferase, both in interfollicular epidermis and in hair follicle stem cells (Figure S1C).

To gain insight to the roles of Dnmt3a and Dnmt3b in epidermal tissue function, we generated epidermis-specific conditional knockout (cKO) mice by crossing animals containing the Dnmt3a or Dnmt3b gene flanked by loxP sites with animals carrying the Keratin14-CRE-YFP-cassette (hereafter referred to as Dnmt3a/3b cKO) (Gao et al., 2011). Surprisingly, neither Dnmt3a- nor Dnmt3b cKO displayed noteworthy epidermal phenotypical differences as compared to their wild-type littermates at different postnatal ages (Figure S1D-E and Figure S2). Despite its strong staining in hair follicle stem cells, the loss of Dnmt3a did not result in evident changes in hair follicle cycling and pelage growth (Figure S1D).

Deregulation of DNA methylation can alter gene expression, leading to tumor suppressor silencing or oncogene activation (Witte et al., 2014), and mutation/deregulation of Dnmt3a and Dnmt3b has been observed in several tumor types (Leppert and Matarazzo, 2014; Subramaniam et al., 2014). Recently, Dnmt3a has attracted much attention, as it is one of the most frequently mutated genes in cancer (Kim et al., 2013), especially in acute myeloid leukemia samples (Garg et al., 2015; Ley et al., 2010). In fact, a recent report further showed that, in human acute myeloid leukemia samples, a loss-of-function mutation of Dnmt3a is one of the earliest mutations that occurs during tumorigenesis (Shlush et al., 2014). Importantly, these mutations are functional since knock-in mice that model it develop a range of severe myeloid and lymphoid malignancies (Challen et al., 2012; Mayle et al., 2015). In addition, HSCs harboring inactivating mutations of Dnmt3a are clonally selected in ageing humans (Shlush et al., 2014). However, much less is known about how deregulation of Dnmt3a and Dnmt3b affect tumorigenesis in epithelial tissues.

To elucidate the roles of Dnmt3a and Dnm3b in skin tumorigenesis, we first generated tumors from the epidermis using the chemically-induced carcinogenesis protocol based on DMBA/TPA (Ewing et al., 1988). Interestingly, after only 2 weeks of exposure to TPA, large patches of the treated skin in the majority of Dnmt3a cKO mice showed clear signs of hair follicle growth, compared to wild-type littermates, suggesting that in homeostatic conditions, Dnmt3a might act as a break to maintain hair follicles in a dormant state

(Figure 1A). Strikingly, the first epidermal squamous malignancies appeared significantly sooner in Dnmt3a cKO than in their wild-type littermates after two months of DMBA/TPA treatment, demonstrating that Dnmt3a acts as a barrier against tumor initiation (Figure 1B, C). Dnmt3a cKO animals also showed a significant increase in tumor burden, with an average of 17 tumors per animal compared to 3 tumors per wild-type animal after 6 months of initiating the experiment (Figure 1D).

Although Dnmt3a cKO animals showed a strong increase in tumor initiation and burden, they developed the same percentage of aggressive squamous cell carcinomas than wild-type mice (Figure 1E). Indeed, a detailed histological analysis of the tumors collected from Dnmt3a cKO and wild-type animals indicated that Dnmt3a-cKO mice developed the same percentage of benign tumors, such as keratoacanthomas and papillomas, as well as of malignant invasive papillomas and squamous cell carcinomas (SCCs) (Figure 1F). Dnmt3a-cKO mice only developed an increase in the percentage of sebaceous adenomas (Figure 1F). No metastases were scored in any of the animals, as expected using this protocol in mice with a C57/Bl6 genetic background (Sundberg et al., 1997). Altogether, these results indicate that loss of Dnmt3a dramatically increases tumor initiation and slightly skews the histology of tumors towards the sebaceous lineage, but does not affect tumor progression.

Figure 1

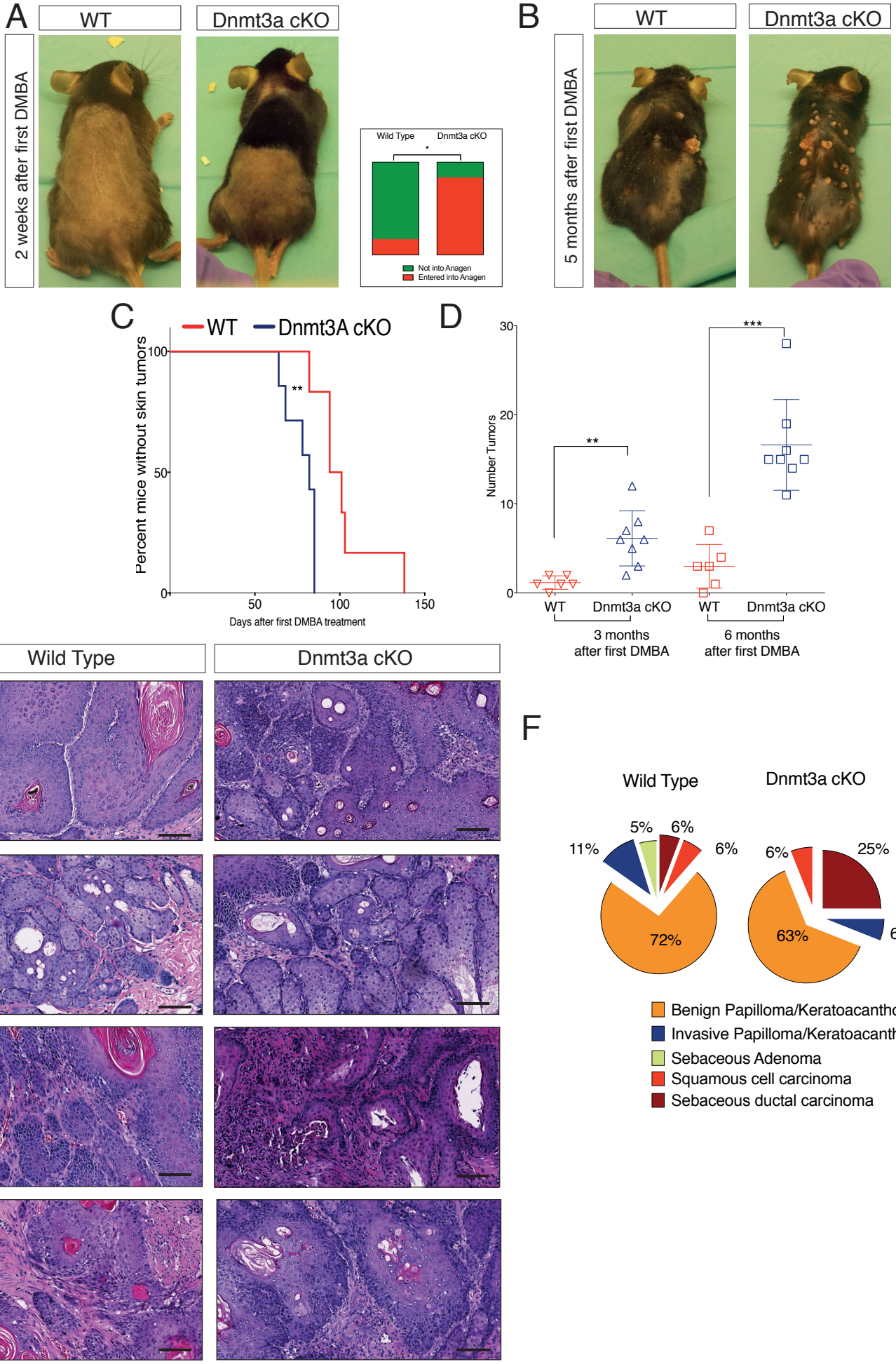


Figure 1: Dnmt3a loss results in massive increases in skin tumor initiation.
(A,B) Representative images of wild-type and Dnmt3a cKO animals treated once weekly with DMBA/TPA for two weeks (A)or for five months (B). Graph shows the percentage of animals WT (n=6) or Dnmt3a cKO (n=6) in anagen after two weeks of treatment, p=0.02, Chi-Square test.
(C) Time of appearance, expressed in percentages of skin tumors on wild-type or Dnmt3a cKO animals, p=0.005.
(D) Number of skin tumors after three or six months of DMBA/TPA treatment, p=0.001 and p=0.0007.
(E) Representative images (hematoxylin/eosin staining) of different subtypes of skin tumors.
(F) Histopathological analysis of the different subsets of skin tumors that appeared after DMBA/TPA treatment of wild-type or Dnmt3a cKO animals.

We next wanted to investigate whether the strong increase in tumor initiation in Dntm3a-cKO mice was specific to the deletion of Dnmt3a, or more generally related to reduced levels of DNA methylation. In addition, Dnmt3a suppresses K-Ras-driven lung tumor progression, whereas Dnmt3b is pro-tumorigenic in APC-deficient colorectal adenomas (Gao et al., 2011; Lin et al., 2006). Hence, we also wanted to test whether Dnmt3a and Dnmt3b also exert opposing effects regarding epidermal squamous tumorigenesis. To this end, we applied the same DMBA/TPA protocol in mice lacking Dnmt3b, the other principal *de novo* DNA methyltransferase, in the epidermis (Dnmt3b-cKO). We observed no differences between wild-type and Dnmt3b-cKO mice with respect to either the timing of tumor initiation or tumor burden (Figure S3A). These results show that Dnmt3a, rather than Dnmt3b, is the main *the novo* DNA methyltransferase having an effect towards epidermal tumorigenesis.

To assess whether Dnmt3a and Dnmt3b potentially play redundant roles during tumorigenesis, we also induced tumors using the DMBA/TPA protocol in animals carrying an epidermis-specific deletion for both Dnmt3a and Dnmt3b (DcKO). Strikingly, DcKO animals formed a morphologically normal skin with all its appendages and did not develop any epidermal abnormality even up to 70 weeks of age (Figure S2B). Thus, these results strongly suggest that *de novo* DNA methylation is dispensable for long-term tissue homeostasis. When subjected to tumorigenesis, DcKO animals displayed a significantly higher tumor burden than wt mice,

similar to the increase observed in Dnmt3a cKO mice (Figure 1 and Figure S3B–F). However, histopathological analysis revealed that the DcKO mice formed aggressive squamous cell carcinoma at a higher frequency as compared to the single cKOs of Dnmt3a or Dnmt3b, indicating that the loss of both Dnmt3a and Dnmt3b in the epidermis gives rise to more aggressive types of epidermal malignancies (Figure S3C). In addition, metastatic nodules in the lungs were observed in 30% of DcKO animals (2 out of six), but in none of the wild type, Dnmt3a cKO, or Dnmt3b cKO animals (Figure S3D). Taken together, these results suggest that Dnmt3a suppresses epidermal tumor initiation, while Dnmt3b is dispensable for both epidermal tissue homeostasis and skin tumorigenesis. However, both Dnmt3a and Dnmt3b repress the malignant transformation of epidermal cells into aggressive SCCs, since the deletion of both Dnmt3s resulted in the formation of a high number of aggressive skin tumors.

We were next interested in deciphering the molecular mechanisms underlying the tumor-suppressive function of Dnmt3a during tumor initiation. To this end, we isolated the basal integrin α6+ tumor cells from individual four wild-type and eight Dnmt3a cKO tumors by FACS-based cell sorting, and performed whole-genome expression profiling by RNA-seq, and studied the profiles of DNA methylation and hydroxymethylation by MeDIP-seq and hydroxyMeDIP-seq (hMeDIP-seq) with DNA from the same samples (Figure 2A).

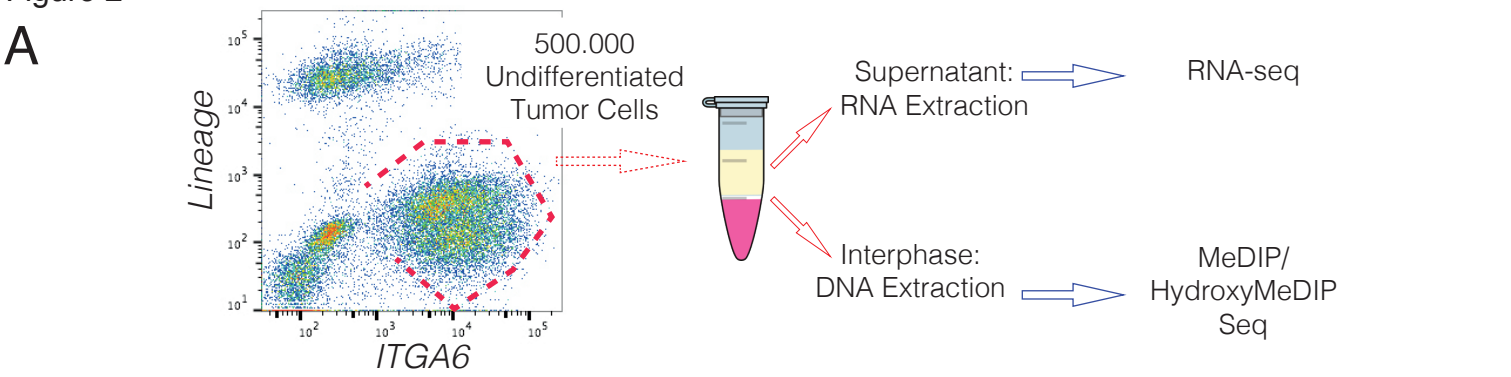
The PCA analysis of the RNA-seq samples showed that the four wild-type tumors clustered together, indicating that overall their transcriptomes were defined by common genes involved in processes of squamous neoplasia (Figure 2B, C). In contrast, the Dnmt3a cKO tumors were highly heterogeneous at the RNA level, suggesting that the loss of Dnmt3a could results in the deregulation of numerous different pathways in cancer cells, or that in the context of Dnmt3 loss, different cell of origins (i.e. basal IFE cells, hair follicle stem cells, or Lrig+ stem cells) might be more prone to generate more transcriptionally divergent tumors. Nevertheless, 391 genes were consistently differentially expressed between wild-type and Dnmt3a cKO tumors, of which 114 were downregulated and 277 were upregulated. The downregulated genes were mainly associated with apoptosis, suggesting that loss of Dnmt3a promotes cell survival and protects against programmed cell death; TUNEL staining confirmed that Dnmt3a cKO tumors had fewer apoptotic cells as compared to wild-type tumors (Figure S4).

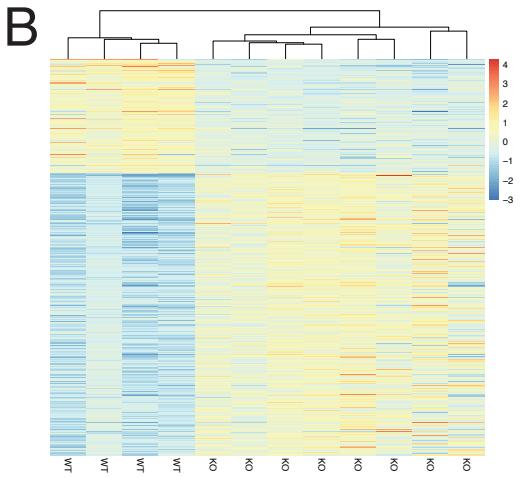
Gene ontology (GO) analysis of the 277 genes that were upregulated in Dnmt3a cKO basal tumor cells highlighted two principal pathways that were over-represented in all eight Dnmt3a cKO tumors: Wnt pathway signaling (with both ligands and receptors upregulated) and lipid metabolism (Figure 2D). As we did not observe any difference in immunohistochemical staining of the nuclear beta-catenin in Dnmt3a-cKO tumors compared to wt lesions, however, it is possible that the upregulation of the Wnt ligands and/or Wnt receptors did not reflect an activated canonical

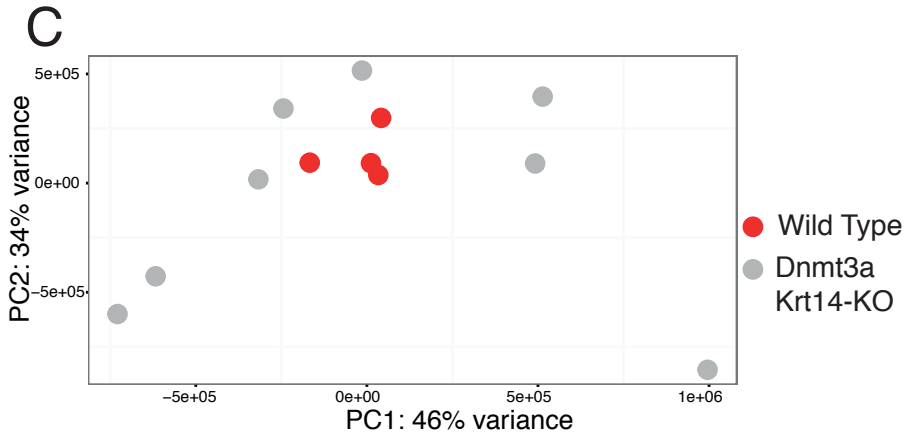
Wnt pathway (data not shown). Recently lipid metabolism has been associated with tumor initiation in colorectal and liver cancers (Beyaz et al., 2016; Luo and Puigserver, 2016; Ma et al., 2016).

We found a number of genes associated with fatty acid and lipid metabolism to be upregulated in Dnmt3a cKO tumors (Figure 2D). The most upregulated genes encoded the key pro-adipogenic transcription factors PPAR-α and PPAR-γ, which promote adipocyte differentiation and the expression of genes involved in fatty acid metabolism, and which are not normally expressed in homeostatic epithelial cells (Fajas et al., 2001). The role of these transcription factors in cancer is still poorly understood, although they tend to be upregulated in a large portion of human tumors (Fajas et al., 2001). Immunofluorescence staining confirmed that PPAR-γ was also upregulated at the protein level in Dnmt3a-cKO tumors (Figure 2E). Interestingly, the expression of PPAR-γ has been extensively reported to be under epigenetic control by repressive mechanisms such as H3K9 methylation, and including DNA methylation (Wang et al., 2013; Zhao et al., 2013).

Figure 2







D

Downregulated in Dnmt3a cKO tumors	Biological Processes	Upregulated in Dnmt3a cKO tumors
Apoptotic process	1.63E-03	Fatty acid metabolic process 1.38E-08
IL1B, FAIM3, CD24, HSPB1, ECSCR, NOL3, AIM2, DRAM1, S100A9, RNF152, MGMT, GPX1, HIGD2A, CDKN2A, TMEM173		*Mgll, Scd1, Faah, Adh7, Elovl3, Lpl, Fa2h, Nr1h3, Acot1, Acacb, Pdk4,Ppara, Ephx2, Cd36, Acot5, Avpr1a, Acox2, Pparg*
Locomotion	2.94E-03	Lipid storage 3.72E-06
SLIT1, IL1B, RAP2A, CD24, HSPB1, CNTN1, ECSCR, CELSR1, ST8SIA4, S100A9, PRCP, GPX1, CCL11		*Lpl, Nr1h3, Acacb, **Ppara**, Cd36, Cidea, **Pparg***
Cell-cell adhesion	6.69E-03	Positive regulation of cell proliferation 7.35E-04
PCDHB12, PCDH9, CELSR1, PCDHB13		*Kit, Tfap2b, Cdc7, Fgf1, Il31ra, Bex1, Cxcl12, Tgm2, Crip2, Plag1, Fgf21, Lifr, Aldh3a1, Clec11a, Igfbp2, Ell3, Avpr1a, Akr1c18, Ntrk2*
SIgnal Transduction Pathways		
Toll like Receptor	9.02E-04	PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA 5.45E-05
RAP2A, MTTP, S100A9, THBS2		*Scd1, Lpl, Nr1h3, Ldhb, Acot1, Fgf21, Ppara, Zfp423, Cd36, Lcn2, Cidea, G0s2, Pparg*
APOPTOSIS	1.59E-03	PEROXISOME PROLIFERATIVE ACTIVATED RECEPTOR ALPHA 2.45E-03
FAIM3, CD24, HSPB1, NOL3, AIM2, GLIPR1, DRAM1, MGMT, GPX1		*Scd1, Lpl, Nr1h3, Acot1, Fgf21, Pdk4, Ppara, Pparg*

E

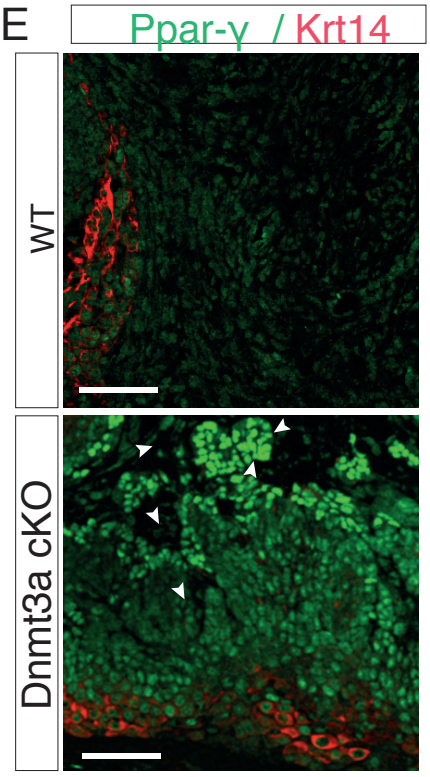


Figure 2: Deletion of Dnmt3a results in increased tumors heterogeneity and upregulation of genes related to lipid metabolism.
(A) Schematic representation of FACS sorting strategy to isolate both RNA and DNA from an undifferentiated (ITGA6+) part of the tumor.
(B) Heatmaps representing gene expression (rlog transformed values) of the 391 differentially expressed genes between wild-type and Dnmt3a cKO sorted tumor cells.
(C) Two-dimensional principal-component analysis (PCA) of wild-type and Dnmt3a cKO cells.
(D) Gene ontology analysis using Genomatix Online Software of the 114 downregulated and 277 upregulated genes in Dnmt3a cKO tumors, divided by biological processes and over-represented signal transduction pathways.
(E) Immunofluorescence staining for Krt14 and PPAR-γ of skin tumors from wild-type and Dnmt3a cKO animal, scale bar=100um

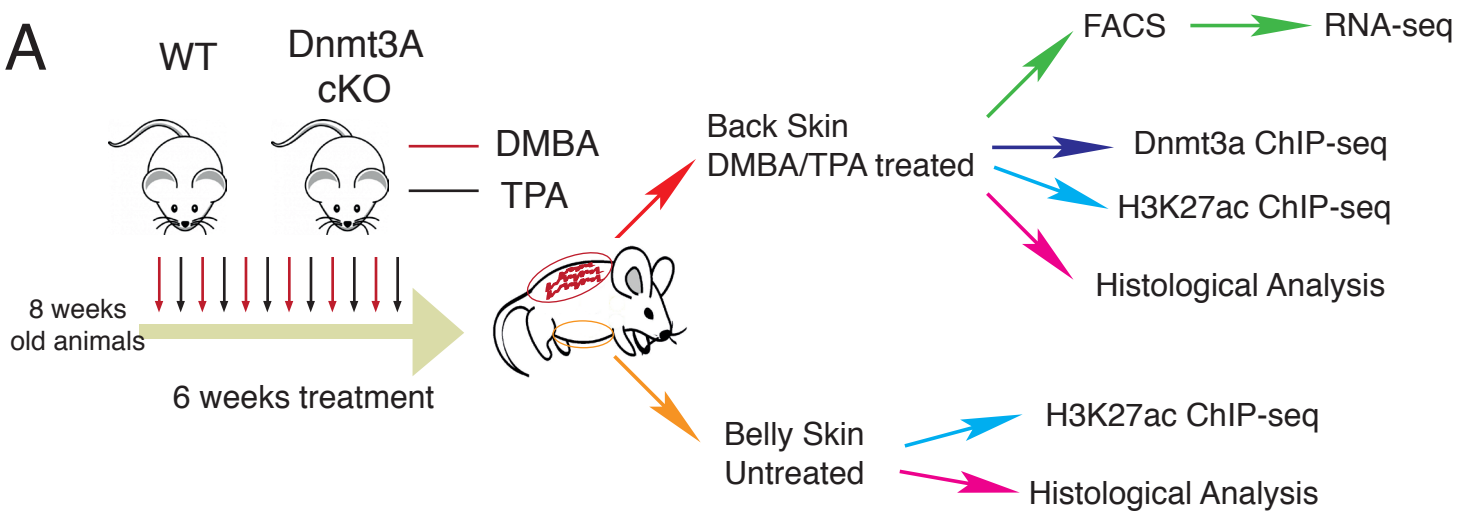
To assess if the loss of Dnmt3a also leads to a faster cellular turnover, we quantified cellular proliferation by Ki67 antigen staining. Notably, proliferation was increased only in pre-cancerous DMBA/TPA-treated Dnmt3a cKO epidermis (Figure S5A-middle panel), with no differences in proliferation found between wild-type and Dnmt3a cKO homeostatic epidermis and tumors (Figure S5A, B). These results suggest that the loss of Dnmt3a gives both a cell survival and proliferative advantage to mutagenized cells in the pre-cancerous epidermis, which could account for the increased tumor initiation. Once tumors are initiated, cell death and proliferation within the tumor are balanced in a Dnmt3a-independent manner

To further dissect the early molecular changes that result in the tumor-suppressing role of Dnmt3a in the epidermis, we did a short (6-week long) DMBA/TPA carcinogenesis treatment (Figure 3A). We then FACS-isolated interfollicular epidermal (IFE) stem cells (Itga6^{bright}CD34-), and hair follicle stem cells (Bulge; Itga6^{bright}CD34+) from the DMBA/TPA-treated pre-cancerous back skin of wild-type or Dnmt3a-cKO animals for RNA-seq analysis. After DMBA/TPA treatment, most of the differentially expressed genes were upregulated, similar to the Dnmt3a-cKO sorted tumor cells; interestingly, the genes were also linked to lipid metabolism and cell proliferation (Figure S6A, B).

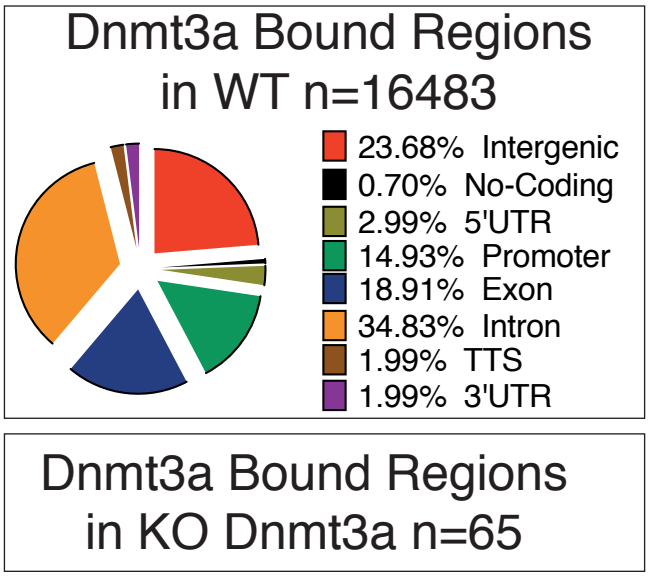
To study a possible role of Dnmt3a in active regulatory regions as we have previously shown in human epidermal stem cells (Rinaldi et al., 2016), we also performed ChIP-Seq for Dnmt3a in DMBA/TPA-treated pre-cancerous back skin epidermises from

wild-type or Dnmt3a-cKO animals. We also performed ChIP-Seq for H3K27ac in wild type animals both in back-skin treated and in the matched, untreated belly skin (Figure 3A). We detected 16,483 genomic locations bound by Dnmt3a in wild-type animals, but only 64 in Dnmt3a cKO. Of the bound regions in the wild-type epidermis, more than 20% corresponded to intergenic regions (Figure 3B). Using the same parameters for the H3K27ac ChIP-seq in wild-type cells, we identified 3,097 intergenic regions enriched for H3K27ac that corresponded to active enhancers, 10% of which were bound by Dnmt3a (Figure 3C). Interestingly, the active enhancers bound by Dnmt3a corresponded to genes essential for *keratinocyte differentiation* and *transcriptional regulation*, such as *Evpl*, *ppl*, *Fos, Myc, Cebpa,* and *Fosl2* (Figure 3C-D), indicating that Dnmt3a controls keratinocyte identity and differentiation by binding to associated enhancers in human and mouse epidermal basal cells (Rinaldi et al., 2016).

Figure 3



B



C

Enhancers bound by Dnmt3a (n=363)

GO Terms	P-value
Regulation of Transcription	9.96E-15
Epithelial cell differentiation	6.05E-06
Water homeostasis	6.68E-06
Apoptotic process	9.75E-06

D

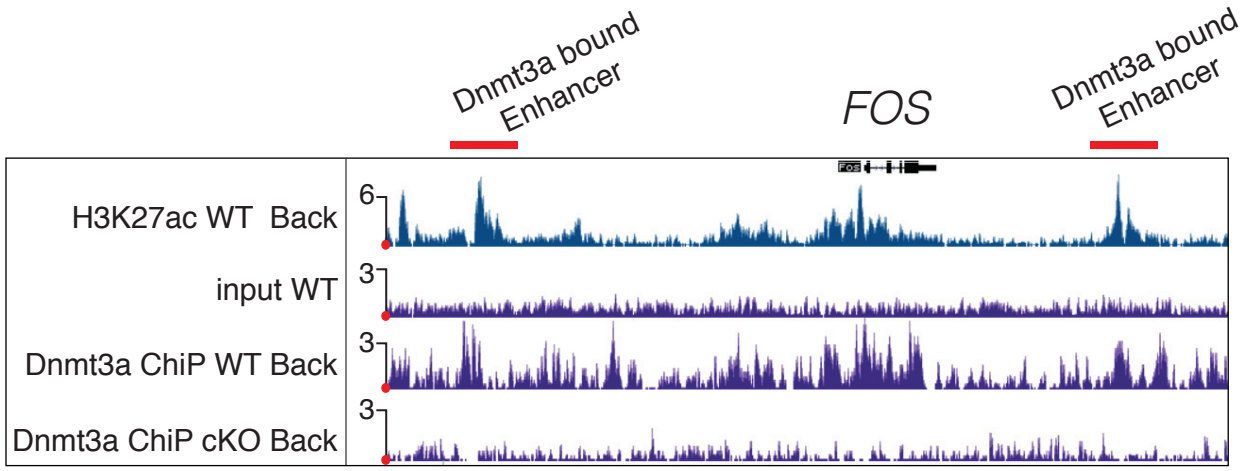


Figure 3: Dnmt3a binds a subset of enhancers in tumor-initiating cells.
(A) Schematic representation of a short treatment of DMBA/TPA, used to recapitulate skin tumor initiation in wild-type and Dnmt3a cKO animals. (B) Genomic localizations of peaks in Dnmt3a ChIP-seq in isolated epidermis from wild-type animals after six weeks of DMBA/TPA treatment. (C) Gene ontology analysis of the 363 H3K27ac-enriched regions (located at least 4 kb away from the TSS) bound by Dnmt3a in isolated epidermis from wild-type animals after six weeks of DMBA/TPA. (D) Screenshot of enhancers bound by Dnmt3a in DMBA/TPA-treated skin. All tracks are normalized to the number of mapped reads.

To understand if the loss of Dnmt3a was accompanied by changes in DNA modifications at these enhancers, we compared these data with MeDIP-seq and hMeDIP-seq performed on FACS-sorted tumor cells. The profiles of MeDIP-seq and hMeDIP-seq around transcription start sites (TSS) and enhancers agreed with published data (Figure S7A), and the CG content in our MeDIP-seq/hMeDIP-seq was highly enriched as compared to the input, both of which are measures of good quality data (Figure S7B).

Tumors lacking Dnmt3a showed a significant reduction in DNA methylation not only around the center of enhancers bound by Dnmt3a in wild-type conditions (Figure 4A) but, intriguingly, also in enhancers not bound by Dnmt3a (Figure 4B). These results are in contrast to our previous results in human epidermal stem cells were depletion of Dnmt3 reduced DNA methylation and hydroxymethylation only at its target enhancers (Rinaldi et al., 2016). Although we do not know at stage the reasons for this difference, this suggests either that the genomic localization of Dnmt3a in murine IFE stem cells might be much broader than what we observed by our ChIP-seq analysis, and that we are only detecting the strongest peaks because of technical reasons (i.e. low affinity of antibody, low ChIP-grade antibody). Alternatively, the binding of Dnmt3a might be very dynamic such that ChIP-seq only captures a snapshot/subset of the enhancers that are methylated by Dnmt3a. Therefore, some enhancers that are also critical for epidermal homeostasis may be permanently bound by Dnmt3a,

while others may be more transiently bound and hence can only be identified by careful MeDIP-seq analysis.

Dnmt3a has also been shown to be responsible for establishing and maintaining the levels of both 5-mC and 5-hmC around the targeted enhancers (Colquitt et al., 2014; Yang et al., 2016). In fact, in accordance with our previous observations (Rinaldi et al., 2016), our hMeDIP-seq data confirmed that enhancers targeted by Dnmt3a have higher 5-hmC as compared to the enhancers not bound by Dnmt3a (Figure 4C). In addition, the loss of Dnmt3a led to a significant reduction of 5-hmC in the enhancers normally bound by Dnmt3a in wild-type epidermis. Intriguingly, the enhancers not normally bound by Dnmt3a in wild-type epidermis also showed a reduction of 5-hmC levels (Figure 4C), further corroborating that some enhancers dynamically bound by Dnmt3a may not be identified by our ChIP-seq analysis. However, the ratio of 5-hmC levels at enhancers bound by Dnmt3a between wild-type and Dnmt3a cKO epidermal cells is significantly higher as compared to the ratio of 5-hmC levels between the enhancers that are not normally bound by Dnmt3a (Figure 4D). This indicates that the presence of Dnmt3a directly correlates with significantly higher 5-hmC levels, likely because Dnmt3a provides 5-mC as a substrate for generating 5-hmC, as we have previously shown (Rinaldi et al., 2016).

Figure 4

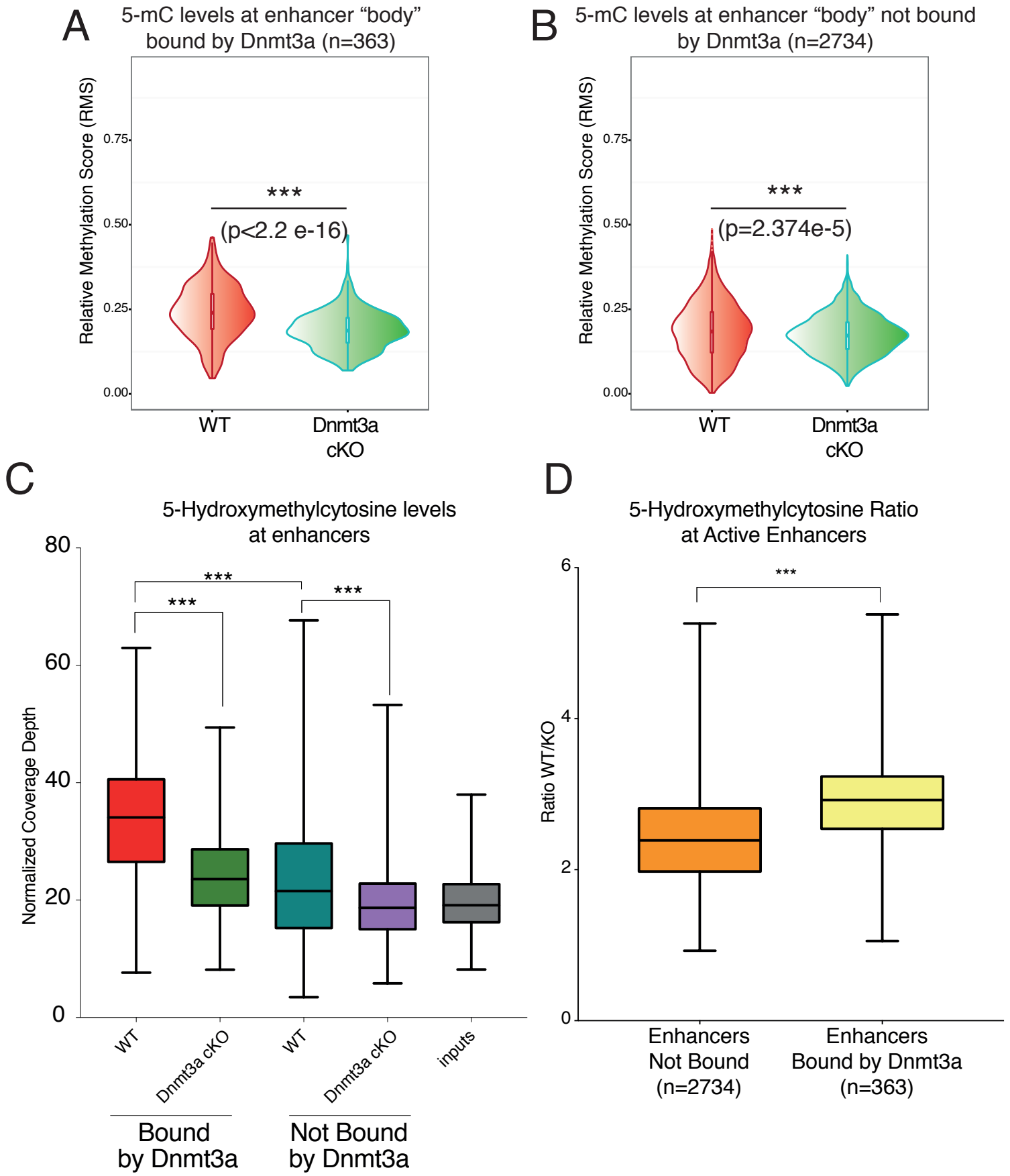


Figure 4: Depletion of Dnmt3a leads to loss of both DNA methylation and hydroxymethylation around the targeted enhancers.
(A) Relative methylation score (RMS) measured around 363 enhancers bound by Dnmt3a (–5 kb, +5 kb) from independent biological replicates of FACS sorted tumor cells from wild-type (n = 2) and Dnmt3a cKO (n = 2) ($p < 2.2 \times 10\text{-}16$).
(B) RMS measured around 2,734 enhancers not bound by Dnmt3a (–5 kb, +5 kb) from independent biological replicates of FACS-sorted tumor cells from wild-type (n = 2) and Dnmt3a cKO (n = 2) animals (p = 2.374e-5).
(C) Global levels of 5-hmC at enhancer center (–2Kb, +2Kb) were quantified using HOMER software in independent biological replicates of FACS sorted tumor cells from wild-type (n = 2) and Dnmt3a cKO (n = 2) at enhancers bound or not by Dnmt3a.
(D) Ratio between the 5-hmC levels at enhancers bound or not by Dnmt3a in wild-type and Dnmt3a cKO.

In addition to active enhancers, a large proportion (19%) of the enriched regions for Dnmt3a corresponded to promoters/TSSs (Figure 3B). To understand if Dnmt3a was methylating these promoters, we overlaid the Dnmt3a ChIP-seq with the MeDIP-seq data. Notably, the promoters bound by Dnmt3a showed a strong and statistically significant loss of DNA methylation around the corresponding TSS (Figure 5A). To understand if it was a consequence of a global hypo-methylation in Dnmt3a cKO cells, we have analyzed also the promoters not bound by Dnmt3a. Strikingly, Dnmt3a does not alter the levels of DNA methylation at the unbound promoters, in fact we found no statistical difference between the levels of DNA methylation in the Dnmt3a cKO cells as compared to wild-type tumor cells (Figure 5B). The loss of DNA methylation at the promoters/TSSs bound by Dnmt3a was also accompanied by an increase in the transcription of these genes, measured by RNA-seq in the tumors (Figure 5C). Altogether, these data suggest that the molecular role of Dnmt3a during skin tumorigenesis is to directly repress the expression of a specific subset of genes by methylating their promoters/TSSs; in addition, Dnmt3a modulates the activity of a subset of enhancers.

To assess if Dnmt3a directly regulates lipid metabolism during skin tumorigenesis, we checked for binding of Dnmt3a at genes of the PPAR family. Importantly, both *PPAR-α* and *PPAR-γ* promoters were bound by Dnmt3a in wild-type but not Dnmt3a cKO epidermis (Figure 5D). To understand if Dnmt3a directly maintains the low levels of transcription of PPAR-γ, we assessed DNA methylation

levels at the PPAR-γ TSS, which is bound by Dnmt3a in wild-type tumors. Indeed, 5-mC levels were lower in Dnmt3a cKO as compared to wild-type tumors, indicating a DNA methylation–dependent mechanism of transcriptional repression (Figure 5E).

Consistent with a transcriptional derepression of the locus following loss of DNA methylation, PPAR-γ mRNA levels were upregulated both in pre-cancerous interfollicular epidermis and in tumors lacking Dnmt3a, suggesting that the upregulation of PPAR-γ and the ability to metabolize lipids is acquired at the pre-cancerous stage, even before overt tumors appear (Figure 5F,G). *PPAR-α* was only differentially expressed in Dnmt3a cKO tumors, but not in the precancerous epidermis, demonstrating that Dnmt3a loss alone is not sufficient to de-repress this locus and suggesting that other epigenetic mechanisms may compensate the loss of Dnmt3a.

Figure 5

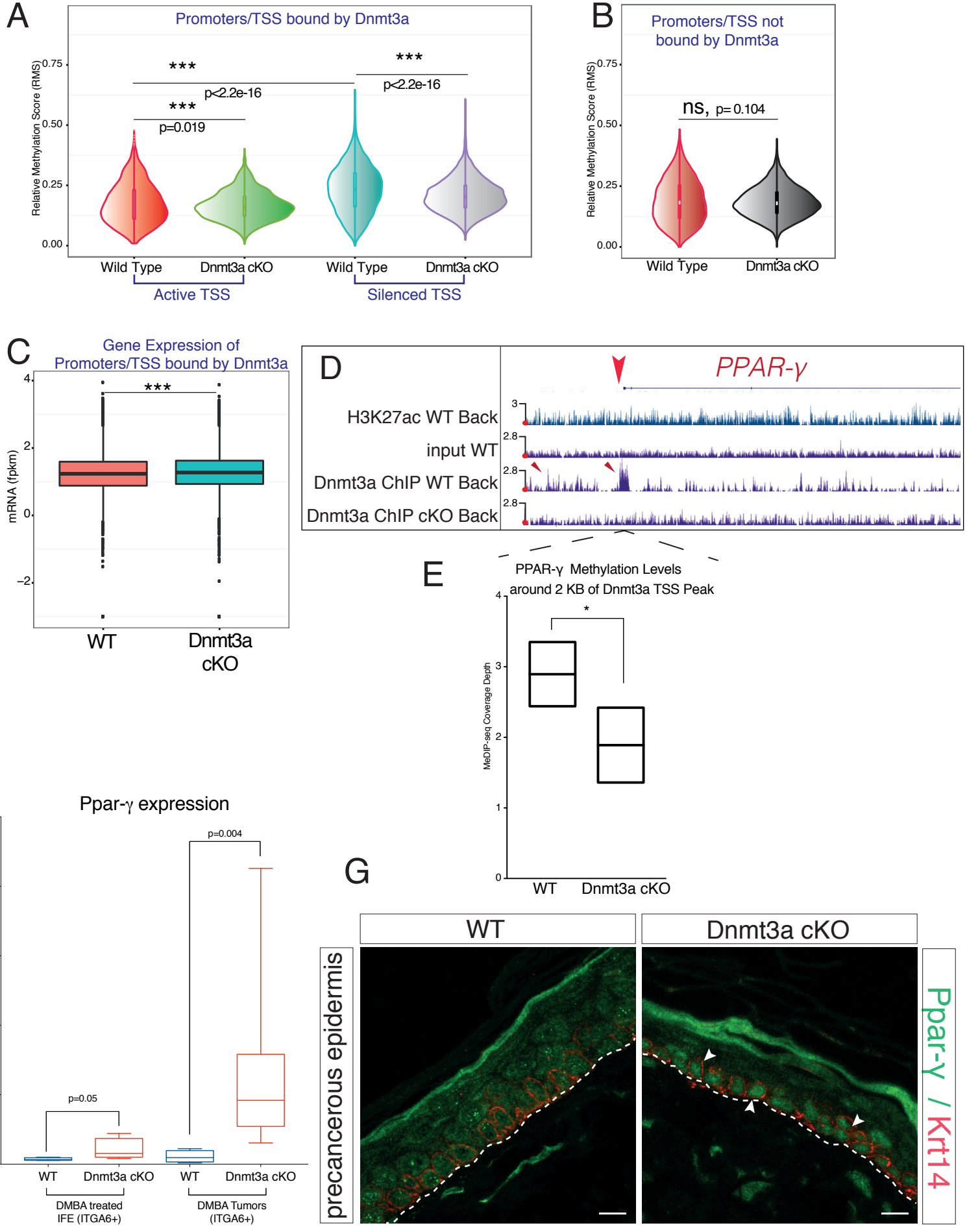


Figure 5: Dnmt3a binds and methylates a subset of promotes in DMBA/TPA-treated epidermal cells.
(A) RMS measured around promoters bound by Dnmt3a (–5 kb, +5 kb) from two biological replicates of FACS-sorted tumor cells. (B) RMS measured around promoters not bound by Dnmt3a (–5 kb, +5 kb) from two independent biological replicates of FACS-sorted tumor cells. (C) FPKM values of genes bound at the TSS by Dnmt3a in DMBA skin tumors from wild-type or Dnmt3a cKO animals. (D) Screenshot of PPAR-γ gene, all tracks normalized. (E) Normalized methylation score measured around TSS of PPAR-γ (–1 kb to + 1 kb) bound by Dnmt3a. (F) FPKM values of PPAR-γ expression in DMBA/TPA-treated IFE cells and in DMBA skin tumors. (G) Immunofluorescence staining for Krt14 and PPAR-γ of DMBA/TPA-treated skin and skin tumors from wild-type and Dnmt3a cKO animals.

Discussion

Dnmt3a modifies cytosine at CpG dinucleotides and is responsible for the proper differentiation of adult stem cells (Challen et al., 2012; Mayle et al., 2015; Shlush et al., 2014). Recently we and others have shown in human epidermal stem cells and murine olfactory sensory neurons, respectively, that Dnmt3a regulates gene expression by cooperating with Tet to maintain high levels of 5-hmC at transcriptional regulatory elements (Colquitt et al., 2014; Rinaldi et al., 2016). Using knock out mouse models, we now have demonstrated that this function of Dnmt3a is maintained in mouse epidermal homeostasis and is altered during skin tumorigenesis. Recent works have shown that the absence of Dnmt3a or Tet2 in hematopoietic stem cells predisposes to leukemia formation (Rasmussen et al., 2015; Yang et al., 2016). Notably, restoring the expression of Dnmt3a after the leukemia had been established did not revert the phenotype (Yang et al., 2016). These results are consistent with ours in the epidermis regarding a role for Dnmt3a in squamous tumor initiation rather than tumor progression. On the other hand, the role of Dnmt3a in tumorigenesis is tissue specific, since in the lung it does not affect tumor initiation but rather tumor progression (Gao et al., 2011). Interestingly, in the work of Gao et al., most of the changes in gene expression in Dnmt3a-depleted cells were attributed to alterations in gene body methylation, rather than at promoters. Conversely, in our model, we see significant changes at regulatory elements (i.e. promoters and enhancers) that

lead to changes in gene expression in Dnmt3a-depleted epidermal tumors.

Dnmt3a is frequently mutated in human tumors (Kim et al., 2013). Importantly, when looking at the timing of its inactivation, Dnmt3a is frequently found to be one of the first mutations to occur during tumorigenesis (Shlush et al., 2014), highlighting the potent tumor suppressor role of this enzyme. Dnmt3a/b are the *de novo* DNA methyltransferases. In contrast, Dnmt1 is the major DNA methyltransferase and is responsible for about 70% of DNA methylation levels (E. Li et al., 1992). In epidermal tissue, the loss of Dnmt1 leads to an increased proliferation and to a partial alopecia(J. Li et al., 2012). Anyhow the impact of Dnmt1 on skin tumors is not known and its role in other cancer types is controversial. Interestingly, the deletion of a functional Dnmt1 slightly promotes colon tumor initiation but strongly suppresses tumor growth (Morita et al., 2013; Sheaffer et al., 2016; Yamada et al., 2005), which is surprising in light of the strong impact that we observed for the Dnmt3a and Dnmt3b DcKO on tumor initiation and tumor progression. In addition, a global reduction of 5-hmC is a hallmark of several cancer types, including SCC, and is often correlated with poor prognosis (Ficz and Gribben, 2014; Lian et al., 2012; Liao et al., 2016; Shi et al., 2016; Zhang et al., 2016). Yet, it is unclear if the loss of 5-hmC is a cause or consequence of tumor progression. Currently, we cannot exclude that precisely the loss of DNA modifications at regulatory elements could play a role in cancer initiation. However, accumulating evidence demonstrates a

clear relationship between Dnmt3a–Tet–5-hmC levels: the inactivation of this axis in adult stem cells predisposes to the amplification of the stem cell pool, leading to higher chances of tumor initiation.

Others and we have shown that the deregulation of this epigenetic pathway in adult stem cells is essential for the development of several types of malignancies. A number of recent studies have highlighted the importance of a persistent lipid metabolism in promoting tumor transformation in the gut and liver, chemoresistance of leukemia stem cells, and for establishing and promoting the metastatic activity of metastatic-initiating cells in human neoplasias (Beyaz et al., 2016; Ma et al., 2016; Ye et al., 2016). Our results show that Dnmt3a directly represses the master regulators of lipid metabolism and adipogenesis PPAR-α and PPAR-γ. The upregulation of these transcription factors could therefore predispose Dnmt3a-deficient epidermis to develop a higher number of tumors. Further studies will be needed to determine if PPAR-γ inhibition is sufficient to block the tumor-initiation phenotype seen in Dnmt3a cKO. Altogether our results have revealed several intriguing results. First, the *de novo* DNA methyltransferases are dispensable for homeostasis in a tissue with a high turnover rate such as the epidermis. This is true even when we deleted both Dnmt3a and Dnmt3b where we have likely eliminated most of the DNA methylation in the tissue, especially in elderly animals. However, an epidermis devoid of *de novo* DNA-methyltransferase is highly sensitive to tumorigenesis. In this sense,

we show that squamous tumor initiation and burden, but not progression, is dramatically accelerated and increased upon loss of Dnmt3a. Intriguingly, although loss of Dnmt3b does not affect tumorigenesis on its own, when deleted in combination with Dnmt3a, it drives tumor progression resulting even the formation of metastases. Mechanistically, we show that Dnmt3a drives promoter methylation and enhancer hydroxymethylation (together with the Tet enzymes) to regulate gene expression. However, future studies will be necessary to study how the function of both Dnmt3a and Dnmt3b is deregulated in human squamous neoplasias, and why mechanistically their depletion results in such strong synergistic effect towards tumor progression.

Reference:

Allis, C.D., Jenuwein, T., 2016. The molecular hallmarks of epigenetic control. Nat Rev Genet.

Avgustinova, A., Benitah, S.A., 2016. Epigenetic control of adult stem cell function. Nat Rev Mol Cell Biol.

Baubec, T., Colombo, D.F., Wirbelauer, C., Schmidt, J., Burger, L., Krebs, A.R., Akalin, A., Schubeler, D., 2015. Genomic profiling of DNA methyltransferases reveals a role for DNMT3B in genic methylation. Nature.

Beyaz, S., Mana, M.D., Roper, J., Kedrin, D., Saadatpour, A., Hong, S.-J., Bauer-Rowe, K.E., Xifaras, M.E., Akkad, A., Arias, E., Pinello, L., Katz, Y., Shinagare, S., Abu-Remaileh, M., Mihaylova, M.M., Lamming, D.W., Dogum, R., Guo, G., Bell, G.W., Selig, M., Nielsen, G.P., Gupta, N., Ferrone, C.R., Deshpande, V., Yuan, G.-C., Orkin, S.H., Sabatini, D.M., Yilmaz, O.H., 2016. High-fat diet enhances stemness and tumorigenicity of intestinal progenitors. Nature 531, 53–58.

Challen, G.A., Sun, D., Jeong, M., Luo, M., Jelinek, J., Berg, J.S., Bock, C., Vasanthakumar, A., Gu, H., Xi, Y., Liang, S., Lu, Y.,

Darlington, G.J., Meissner, A., Issa, J.-P.J., Godley, L.A., Li, W., Goodell, M.A., 2012. Dnmt3a is essential for hematopoietic stem cell differentiation. Nat Genet 44, 23–31.

Challen, G.A., Sun, D., Mayle, A., Jeong, M., Luo, M., Rodriguez, B., Mallaney, C., Celik, H., Yang, L., Xia, Z., Cullen, S., Berg, J., Zheng, Y., Darlington, G.J., Li, W., Goodell, M.A., 2014. Dnmt3a and Dnmt3b have overlapping and distinct functions in hematopoietic stem cells. Cell Stem Cell 15, 350–364.

Chen, T., Ueda, Y., Dodge, J.E., Wang, Z., Li, E., 2003. Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. Mol Cell Biol 23, 5594–5605.

Colquitt, B.M., Markenscoff-Papadimitriou, E., Duffie, R., Lomvardas, S., 2014. Dnmt3a regulates global gene expression in olfactory sensory neurons and enables odorant-induced transcription. Neuron 83, 823–838.

Ewing, M.W., Conti, C.J., Kruszewski, F.H., Slaga, T.J., DiGiovanni, J., 1988. Tumor progression in Sencar mouse skin as a function of initiator dose and promoter dose, duration, and type. Cancer Res 48, 7048–7054.

Fajas, L., Debril, M.B., Auwerx, J., 2001. Peroxisome proliferator-activated receptor-gamma: from adipogenesis to carcinogenesis. J Mol Endocrinol 27, 1–9.

Ficz, G., Gribben, J.G., 2014. Loss of 5-hydroxymethylcytosine in cancer: cause or consequence? Genomics 104, 352–357.

Gao, Q., Steine, E.J., Barrasa, M.I., Hockemeyer, D., Pawlak, M., Fu, D., Reddy, S., Bell, G.W., Jaenisch, R., 2011. Deletion of the de novo DNA methyltransferase Dnmt3a promotes lung tumor progression. Proc Natl Acad Sci U S A 108, 18061–18066.

Garg, M., Nagata, Y., Kanojia, D., Mayakonda, A., Yoshida, K., Haridas Keloth, S., Zang, Z.J., Okuno, Y., Shiraishi, Y., Chiba, K., Tanaka, H., Miyano, S., Ding, L.-W., Alpermann, T., Sun, Q.-Y., Lin, D.-C., Chien, W., Madan, V., Liu, L.-Z., Tan, K.-T., Sampath, A., Venkatesan, S., Inokuchi, K., Wakita, S., Yamaguchi, H., Chng, W.J., Kham, S.-K.Y., Yeoh, A.E.-J., Sanada, M., Schiller, J., Kreuzer, K.-A., Kornblau, S.M., Kantarjian, H.M., Haferlach, T., Lill, M., Kuo, M.-C., Shih, L.-Y., Blau, I.W., Blau, O., Yang, H., Ogawa, S., Koeffler, H.P., 2015. Profiling of somatic mutations in acute myeloid leukemia with FLT3-ITD at diagnosis and relapse. Blood 126, 2491–

2501.
Jin, B., Ernst, J., Tiedemann, R.L., Xu, H., Sureshchandra, S., Kellis, M., Dalton, S., Liu, C., Choi, J.-H., Robertson, K.D., 2012. Linking DNA methyltransferases to epigenetic marks and nucleosome structure genome-wide in human tumor cells. Cell Rep 2, 1411–1424.
Kim, M.S., Kim, Y.R., Yoo, N.J., Lee, S.H., 2013. Mutational analysis of DNMT3A gene in acute leukemias and common solid cancers. APMIS 121, 85–94.
Lei, H., Oh, S.P., Okano, M., Juttermann, R., Goss, K.A., Jaenisch, R., Li, E., 1996. De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development 122, 3195–3205.
Leppert, S., Matarazzo, M.R., 2014. De novo DNMTs and DNA methylation: novel insights into disease pathogenesis and therapy from epigenomics. Curr Pharm Des 20, 1812–1818.
Ley, T.J., Ding, L., Walter, M.J., McLellan, M.D., Lamprecht, T., Larson, D.E., Kandoth, C., Payton, J.E., Baty, J., Welch, J., Harris, C.C., Lichti, C.F., Townsend, R.R., Fulton, R.S., Dooling, D.J., Koboldt, D.C., Schmidt, H., Zhang, Q., Osborne, J.R., Lin, L., O'Laughlin, M., McMichael, J.F., Delehaunty, K.D., McGrath, S.D., Fulton, L.A., Magrini, V.J., Vickery, T.L., Hundal, J., Cook, L.L., Conyers, J.J., Swift, G.W., Reed, J.P., Alldredge, P.A., Wylie, T., Walker, J., Kalicki, J., Watson, M.A., Heath, S., Shannon, W.D., Varghese, N., Nagarajan, R., Westervelt, P., Tomasson, M.H., Link, D.C., Graubert, T.A., Dipersio, J.F., Mardis, E.R., Wilson, R.K., 2010. DNMT3A mutations in acute myeloid leukemia. N Engl J Med 363, 2424–2433.
Li, E., Bestor, T.H., Jaenisch, R., 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69, 915–926.
Li, J., Jiang, T.-X., Hughes, M.W., Wu, P., Yu, J., Widelitz, R.B., Fan, G., Chuong, C.-M., 2012. Progressive alopecia reveals decreasing stem cell activation probability during aging of mice with epidermal deletion of DNA methyltransferase 1. J Invest Dermatol 132, 2681–2690.
Lian, C.G., Xu, Y., Ceol, C., Wu, F., Larson, A., Dresser, K., Xu, W., Tan, L., Hu, Y., Zhan, Q., Lee, C.-W., Hu, D., Lian, B.Q., Kleffel, S., Yang, Y., Neiswender, J., Khorasani, A.J., Fang, R., Lezcano, C., Duncan, L.M., Scolyer, R.A., Thompson, J.F.,

Kakavand, H., Houvras, Y., Zon, L.I., Mihm, M.C.J., Kaiser, U.B., Schatton, T., Woda, B.A., Murphy, G.F., Shi, Y.G., 2012. Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. Cell 150, 1135–1146.

Liao, Y., Gu, J., Wu, Y., Long, X., Ge, D.I., Xu, J., Ding, J., 2016. Low level of 5-Hydroxymethylcytosine predicts poor prognosis in non-small cell lung cancer. Oncol Lett 11, 3753–3760.

Lin, H., Yamada, Y., Nguyen, S., Linhart, H., Jackson-Grusby, L., Meissner, A., Meletis, K., Lo, G., Jaenisch, R., 2006. Suppression of intestinal neoplasia by deletion of Dnmt3b. Mol Cell Biol 26, 2976–2983.

Luo, C., Puigserver, P., 2016. Stem cells: Dietary fat promotes intestinal dysregulation. Nature 531, 42–43.

Ma, C., Kesarwala, A.H., Eggert, T., Medina-Echeverz, J., Kleiner, D.E., Jin, P., Stroncek, D.F., Terabe, M., Kapoor, V., ElGindi, M., Han, M., Thornton, A.M., Zhang, H., Egger, M., Luo, J., Felsher, D.W., McVicar, D.W., Weber, A., Heikenwalder, M., Greten, T.F., 2016. NAFLD causes selective CD4(+) T lymphocyte loss and promotes hepatocarcinogenesis. Nature 531, 253–257.

Mayle, A., Yang, L., Rodriguez, B., Zhou, T., Chang, E., Curry, C.V., Challen, G.A., Li, W., Wheeler, D., Rebel, V.I., Goodell, M.A., 2015. Dnmt3a loss predisposes murine hematopoietic stem cells to malignant transformation. Blood 125, 629–638.

Morita, R., Hirohashi, Y., Suzuki, H., Takahashi, A., Tamura, Y., Kanaseki, T., Asanuma, H., Inoda, S., Kondo, T., Hashino, S., Hasegawa, T., Tokino, T., Toyota, M., Asaka, M., Torigoe, T., Sato, N., 2013. DNA methyltransferase 1 is essential for initiation of the colon cancers. Exp Mol Pathol 94, 322–329.

Morselli, M., Pastor, W.A., Montanini, B., Nee, K., Ferrari, R., Fu, K., Bonora, G., Rubbi, L., Clark, A.T., Ottonello, S., Jacobsen, S.E., Pellegrini, M., 2015. In vivo targeting of de novo DNA methylation by histone modifications in yeast and mouse. Elife 4, e06205.

Nuhrenberg, T.G., Hammann, N., Schnick, T., Preissl, S., Witten, A., Stoll, M., Gilsbach, R., Neumann, F.-J., Hein, L., 2015. Cardiac Myocyte De Novo DNA Methyltransferases 3a/3b Are Dispensable for Cardiac Function and Remodeling after Chronic Pressure Overload in Mice. PLoS One 10, e0131019.

Okano, M., Bell, D.W., Haber, D.A., Li, E., 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de

novo methylation and mammalian development. Cell 99, 247–257.
Rasmussen, K.D., Jia, G., Johansen, J.V., Pedersen, M.T., Rapin, N., Bagger, F.O., Porse, B.T., Bernard, O.A., Christensen, J., Helin, K., 2015. Loss of TET2 in hematopoietic cells leads to DNA hypermethylation of active enhancers and induction of leukemogenesis. Genes Dev 29, 910–922.
Rinaldi, L., Benitah, S.A., 2014. Epigenetic regulation of adult stem cell function. FEBS J.
Rinaldi, L., Datta, D., Serrat, J., Morey, L., Solanas, G., Avgustinova, A., Blanco, E., Pons, J.I., Matallanas, D., Kriegsheim, Von, A., Di Croce, L., Benitah, S.A., 2016. Dnmt3a and Dnmt3b Associate with Enhancers to Regulate Human Epidermal Stem Cell Homeostasis. Cell Stem Cell.
Sheaffer, K.L., Elliott, E.N., Kaestner, K.H., 2016. DNA Hypomethylation Contributes to Genomic Instability and Intestinal Cancer Initiation. Cancer Prev Res (Phila) 9, 534–546.
Shen, H., Laird, P.W., 2013. Interplay between the cancer genome and epigenome. Cell 153, 38–55.
Shi, X., Yu, Y., Luo, M., Zhang, Z., Shi, S., Feng, X., Chen, Z., He, J., 2016. Loss of 5-Hydroxymethylcytosine Is an Independent Unfavorable Prognostic Factor for Esophageal Squamous Cell Carcinoma. PLoS One 11, e0153100.
Shlush, L.I., Zandi, S., Mitchell, A., Chen, W.C., Brandwein, J.M., Gupta, V., Kennedy, J.A., Schimmer, A.D., Schuh, A.C., Yee, K.W., McLeod, J.L., Doedens, M., Medeiros, J.J.F., Marke, R., Kim, H.J., Lee, K., McPherson, J.D., Hudson, T.J., Brown, A.M.K., Yousif, F., Trinh, Q.M., Stein, L.D., Minden, M.D., Wang, J.C.Y., Dick, J.E., 2014. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature 506, 328–333.
Steine, E.J., Ehrich, M., Bell, G.W., Raj, A., Reddy, S., van Oudenaarden, A., Jaenisch, R., Linhart, H.G., 2011. Genes methylated by DNA methyltransferase 3b are similar in mouse intestine and human colon cancer. J Clin Invest 121, 1748–1752.
Subramaniam, D., Thombre, R., Dhar, A., Anant, S., 2014. DNA methyltransferases: a novel target for prevention and therapy. Front Oncol 4, 80.
Sundberg, J.P., Sundberg, B.A., Beamer, W.G., 1997. Comparison

of chemical carcinogen skin tumor induction efficacy in inbred, mutant, and hybrid strains of mice: morphologic variations of induced tumors and absence of a papillomavirus cocarcinogen. Mol Carcinog 20, 19–32.

Ueda, Y., Okano, M., Williams, C., Chen, T., Georgopoulos, K., Li, E., 2006. Roles for Dnmt3b in mammalian development: a mouse model for the ICF syndrome. Development 133, 1183–1192.

Wang, L., Xu, S., Lee, J.-E., Baldridge, A., Grullon, S., Peng, W., Ge, K., 2013. Histone H3K9 methyltransferase G9a represses PPARgamma expression and adipogenesis. EMBO J 32, 45–59.

Witte, T., Plass, C., Gerhauser, C., 2014. Pan-cancer patterns of DNA methylation. Genome Med 6, 66.

Wu, H., Coskun, V., Tao, J., Xie, W., Ge, W., Yoshikawa, K., Li, E., Zhang, Y., Sun, Y.E., 2010. Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science 329, 444–448.

Yamada, Y., Jackson-Grusby, L., Linhart, H., Meissner, A., Eden, A., Lin, H., Jaenisch, R., 2005. Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Proc Natl Acad Sci U S A 102, 13580–13585.

Yang, L., Rodriguez, B., Mayle, A., Park, H.J., Lin, X., Luo, M., Jeong, M., Curry, C.V., Kim, S.-B., Ruau, D., Zhang, X., Zhou, T., Zhou, M., Rebel, V.I., Challen, G.A., Gottgens, B., Lee, J.-S., Rau, R., Li, W., Goodell, M.A., 2016. DNMT3A Loss Drives Enhancer Hypomethylation in FLT3-ITD-Associated Leukemias. Cancer Cell 29, 922–934.

Ye, H., Adane, B., Khan, N., Sullivan, T., Minhajuddin, M., Gasparetto, M., Stevens, B., Pei, S., Balys, M., Ashton, J.M., Klemm, D.J., Woolthuis, C.M., Stranahan, A.W., Park, C.Y., Jordan, C.T., 2016. Leukemic Stem Cells Evade Chemotherapy by Metabolic Adaptation to an Adipose Tissue Niche. Cell Stem Cell 19, 23–37.

Zhang, F., Liu, Y., Zhang, Z., Li, J., Wan, Y., Zhang, L., Wang, Y., Li, X., Xu, Y., Fu, X., Zhang, X., Zhang, M., Zhang, Z., Zhang, J., Yan, Q., Ye, J., Wang, Z., Chen, C.D., Lin, W., Li, Q., 2016. 5-hydroxymethylcytosine loss is associated with poor prognosis for patients with WHO grade II diffuse astrocytomas. Sci Rep 6, 20882.

Zhao, Q., Fan, Y.-C., Zhao, J., Gao, S., Zhao, Z.-H., Wang, K., 2013. DNA methylation patterns of peroxisome proliferator-

activated receptor gamma gene associated with liver fibrosis and inflammation in chronic hepatitis B. J Viral Hepat 20, 430–437.

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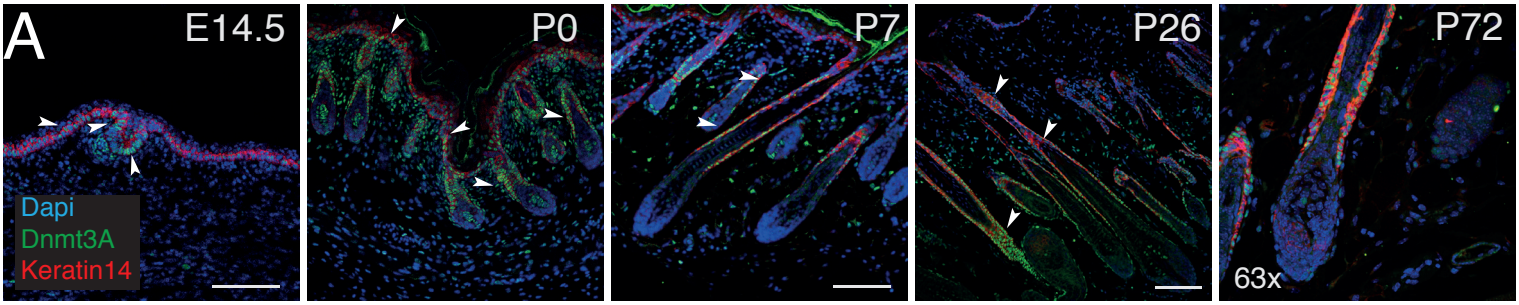
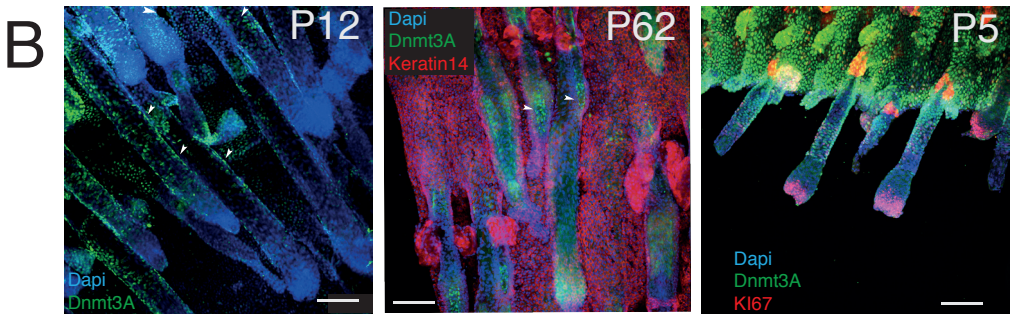
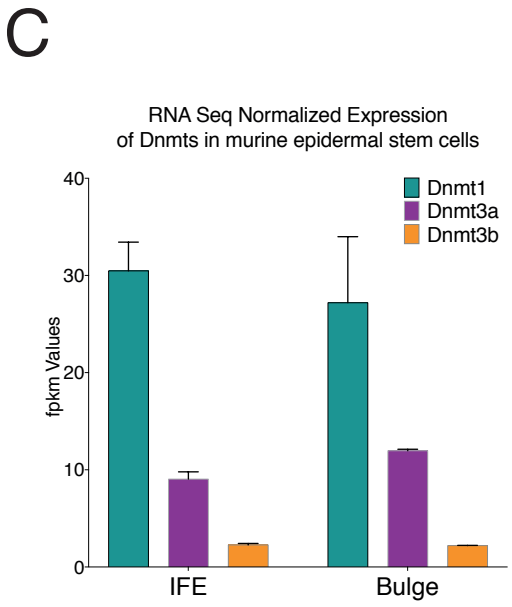
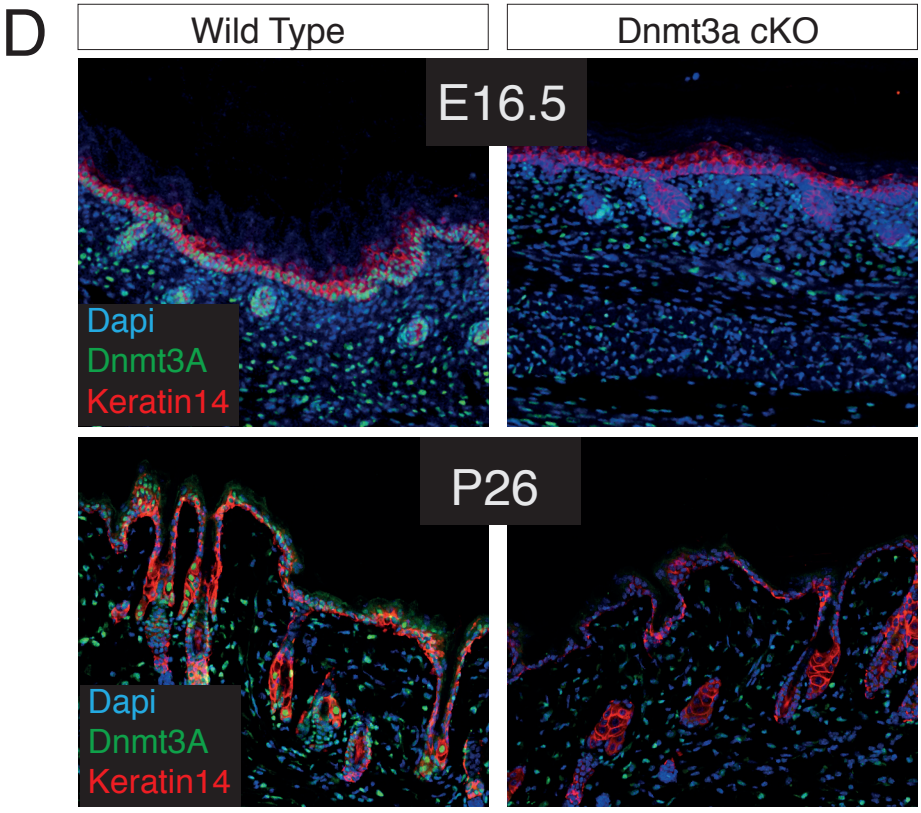
Figure S1









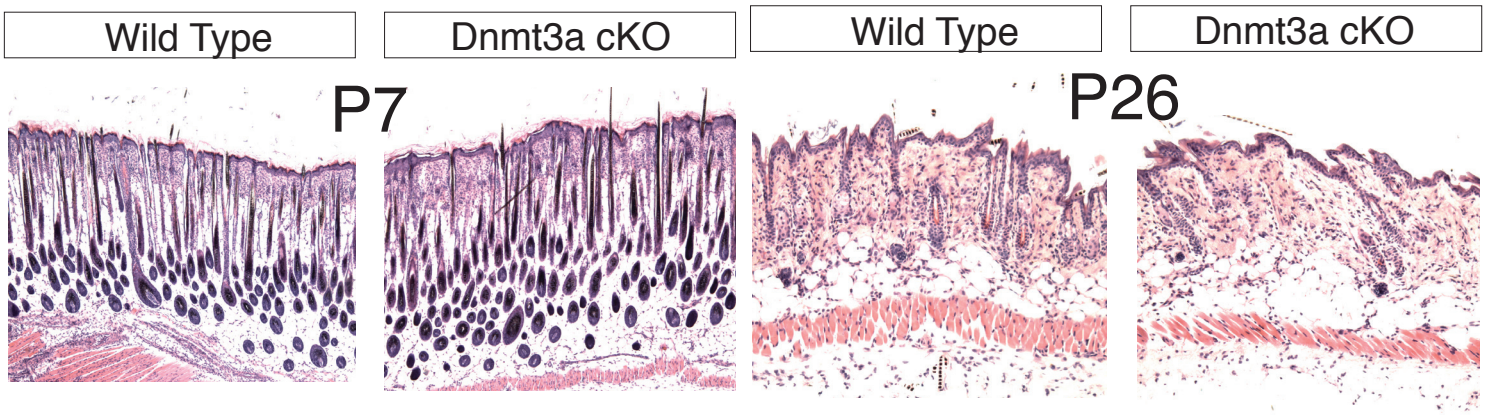


Figure S1: Dnmt3a is highly expressed in the interfollicular epidermis (IFE) and in the bulge of hair follicles in young mice.
(A-B) Immunofluorescence staining for Dnmt3a, Keratin 14 and Nuclei of back skin (A) and in tail skin
(B) isolated from wild-type animals at different ages. (C) Fpkm values of the Dnmt1,3a,3b from RNA-seq data performed
in interfollicular epidermal stem cells (IFE, n=4) and hair follicle stem cells (Bulge, n=3) FACS sorted after six weeks of DMBA/TPA treatment
(D) Immunofluorescence staining for Dnmt3a and keratin 14 of the back skin from wild-type or Dnmt3a cKO animals.
(E) Representative images (hematoxylin/eosin staining) of the back skin from wild-type and Dnmt3a cKO littermates at different ages.

Figure S2

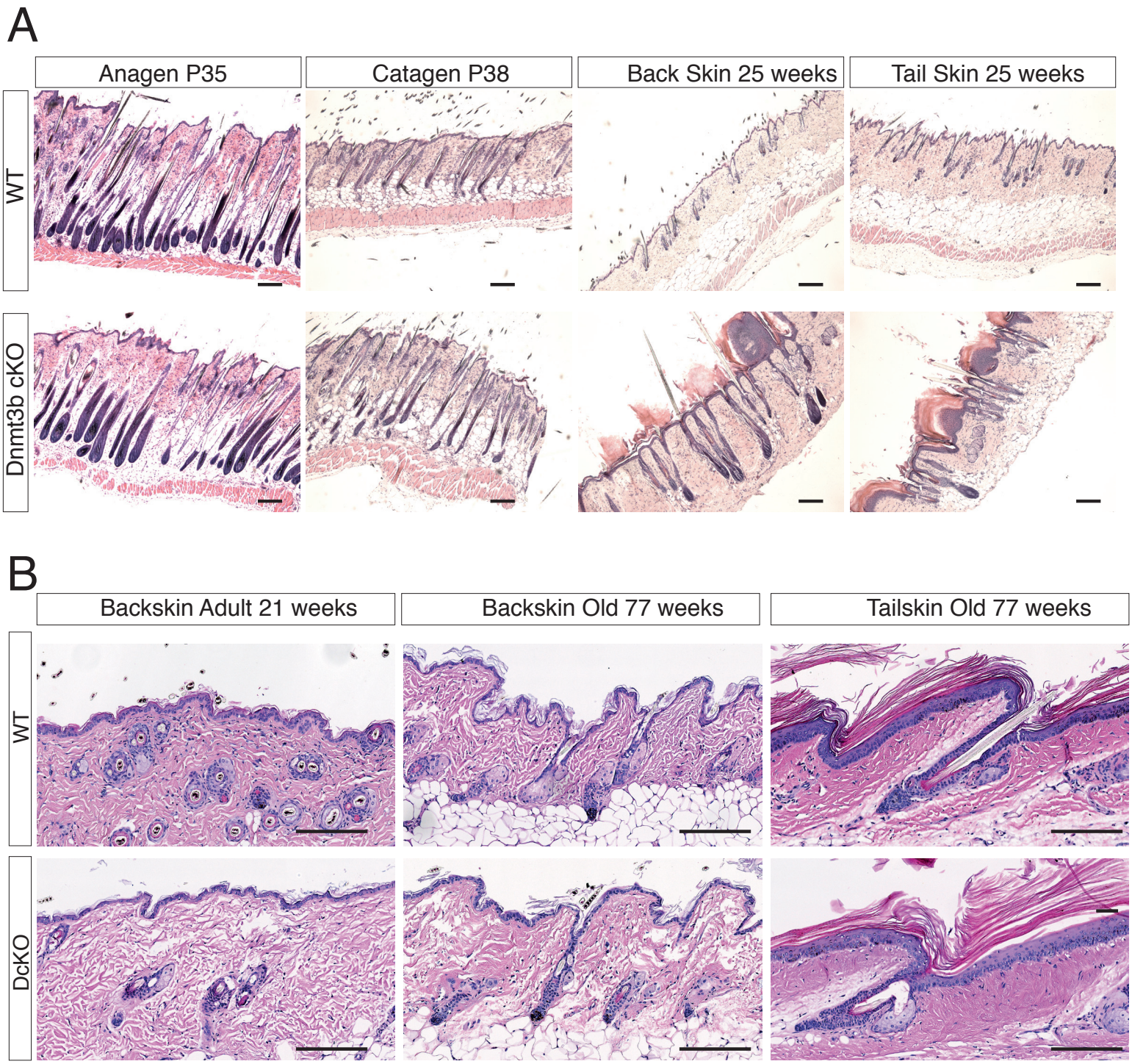


Figure S2: Dnmt3a and Dnmt3b double depletion does not affect epidermal tissue homeostasis.
(A) Representative images (hematoxylin/eosin staining) of back skin and tail skin from wild type and Dnmt3b cKO littermates at different ages.
(B) Representative images (hematoxylin/eosin staining) of back skin and tail skin from adult and aged wild type and cKO littermates.
Scale bars=100um

Figure S3

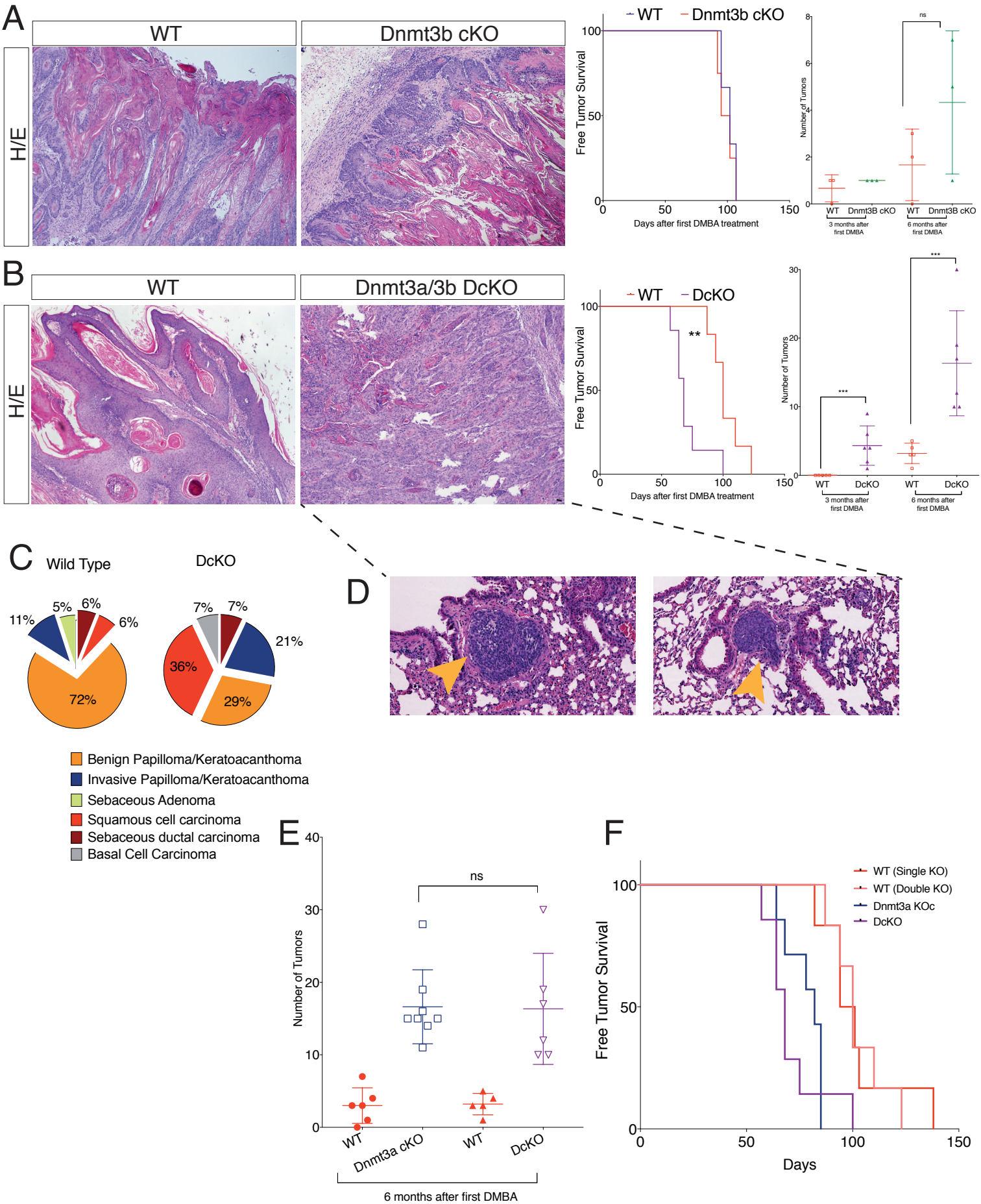


Figure S3: Dnmt3a and Dnmt3b double cKO animals developed severe and aggressive tumors, but single Dnmt3b cKO did not increase tumor initiation. (A) Left, representative images (hematoxylin/eosin staining) of skin tumors isolated from wild-type and Dnmt3b cKO littermates after 6 months of DMBA/TPA treatment. Right, time appearance expressed in percentages and number of skin tumors scored on wild-type and Dnmt3b cKO animals. (B) Left, representative images of skin tumors isolated from wild-type and Dnmt3a/Dnmt3b DcKO littermates after 6 months of DMBA/TPA treatment. Right, time appearance expressed in percentages and number of skin tumors on wild-type and Dnmt3a/Dnmt3b DcKO animals. (C) Histopathological analysis of the different subsets of skin tumors that appeared after DMBA/TPA treatment of wild-type or DcKO animals. (D) Representative images of metastatic nodules identified only in a percentage (33%) of the lungs of DcKO animals. (E-F) Time appearance expressed in percentages and number of skin tumors compared between WT, Dnmt3a cKO and DcKO.

Figure S4

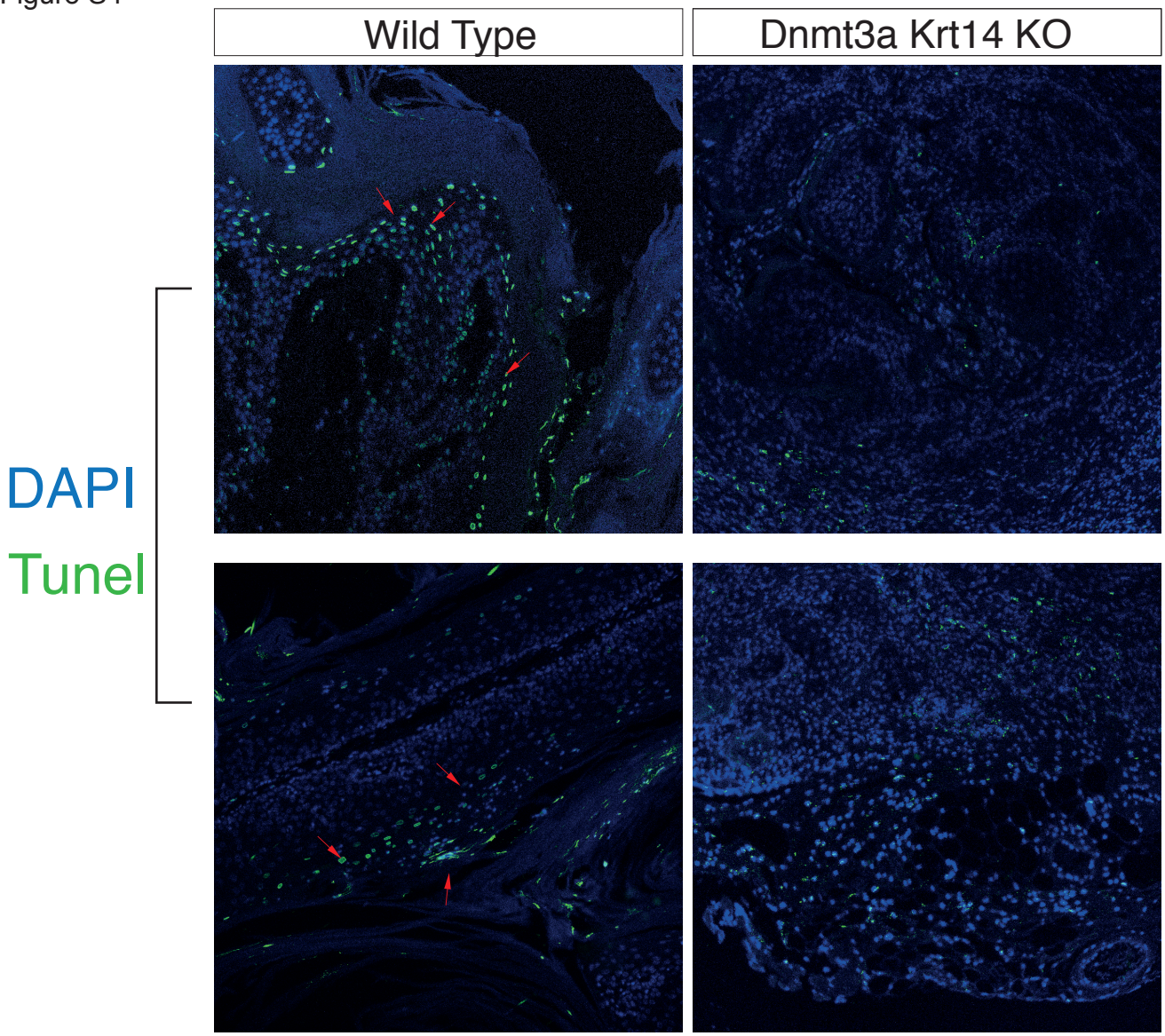
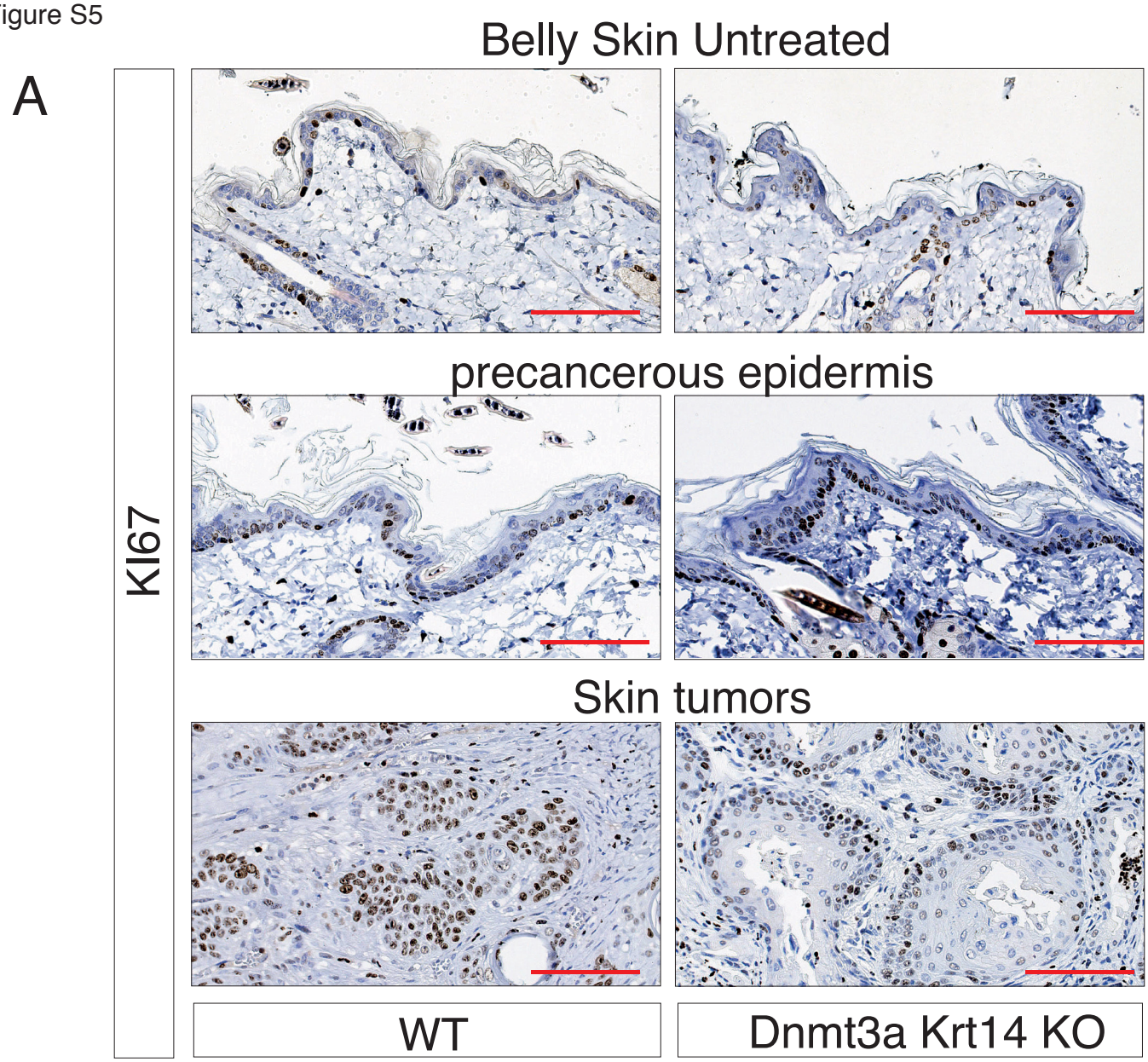


Figure S4: Loss of Dnmt3a results in a reduction of apoptosis in skin tumors.
TUNEL staining for apoptotic cells in skin tumors isolated from wild-type and Dnmt3a cKO animals.

Figure S5



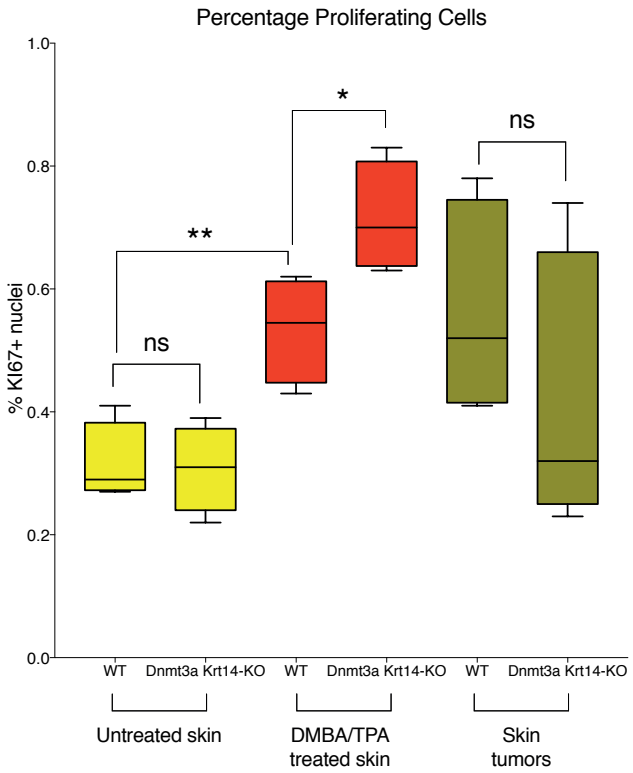
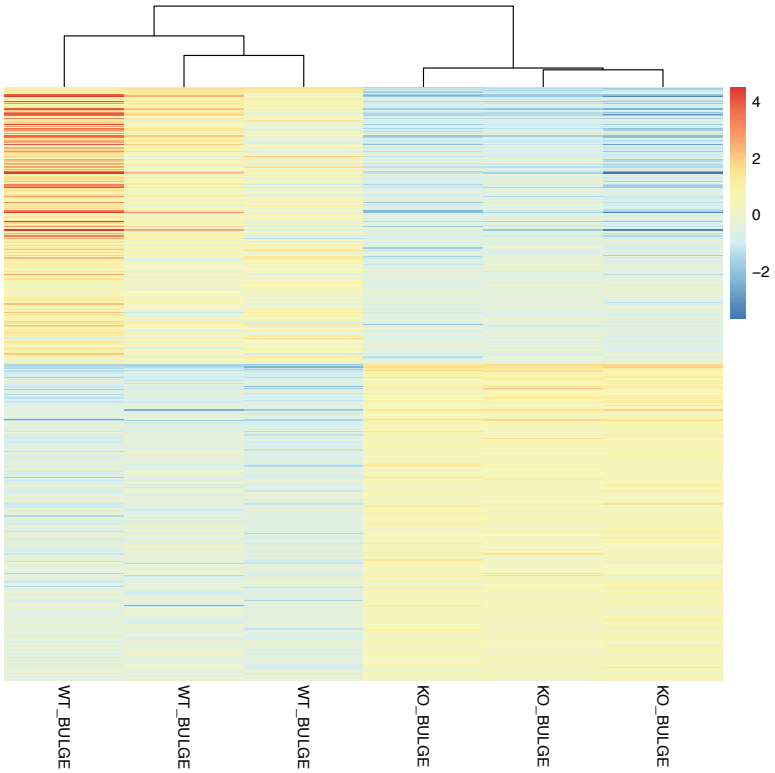


Figure S5: DMBA/TPA treatment induces an increase in cellular turnover in Dnmt3a cKO animals.
(A) Representative images of KI67 staining in treated or untreated back skin, and on skin tumors, for Dnmt3a cKO and wild-type littermates. (Scale Bars =100um)
(B) Quantification of KI67 staining using TMarker software, calculating percentages of KI67-positive cells as compared to all the interfollicular epidermal cells.

Figure S6

A



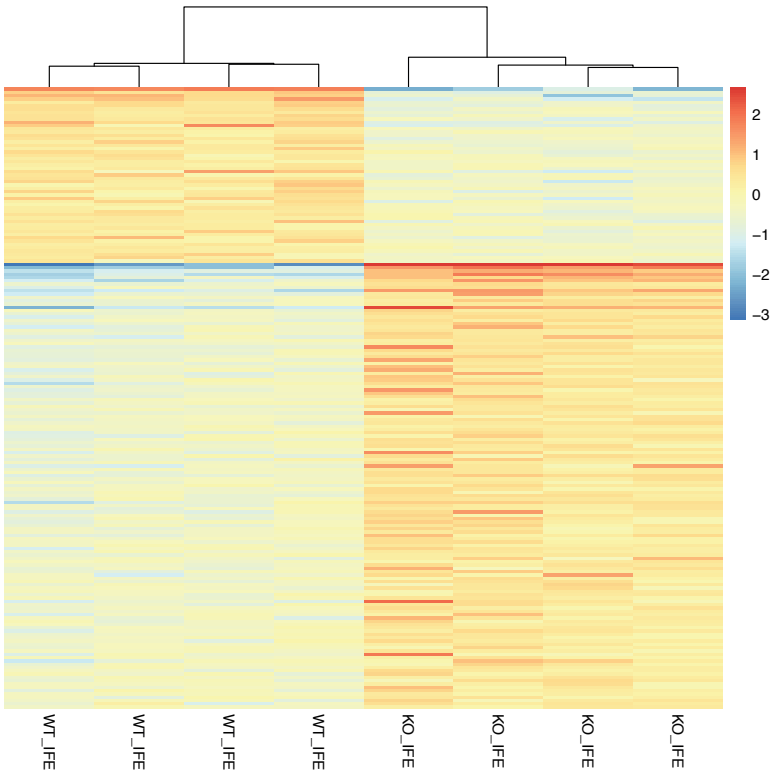
Downregulated in Dnmt3a KO bulge cells (233 genes)

GO Terms	P-value
cell migration	1.08e-15
Cell differentiation	1.52e-12
Signal transduction	8.77e-8
Response to chemical	3.78e-7

Upregulated in Dnmt3a KO bulge (265 genes)

GO Terms	P-value
Cell proliferation	1.29e-6
Regulation of cell adhesion	6.95e-5
Hair follicle maturation	1.46e-3
Wnt signaling pathway	1.84e-3

B



Downregulated in Dnmt3a KO IFE cells (53 genes)

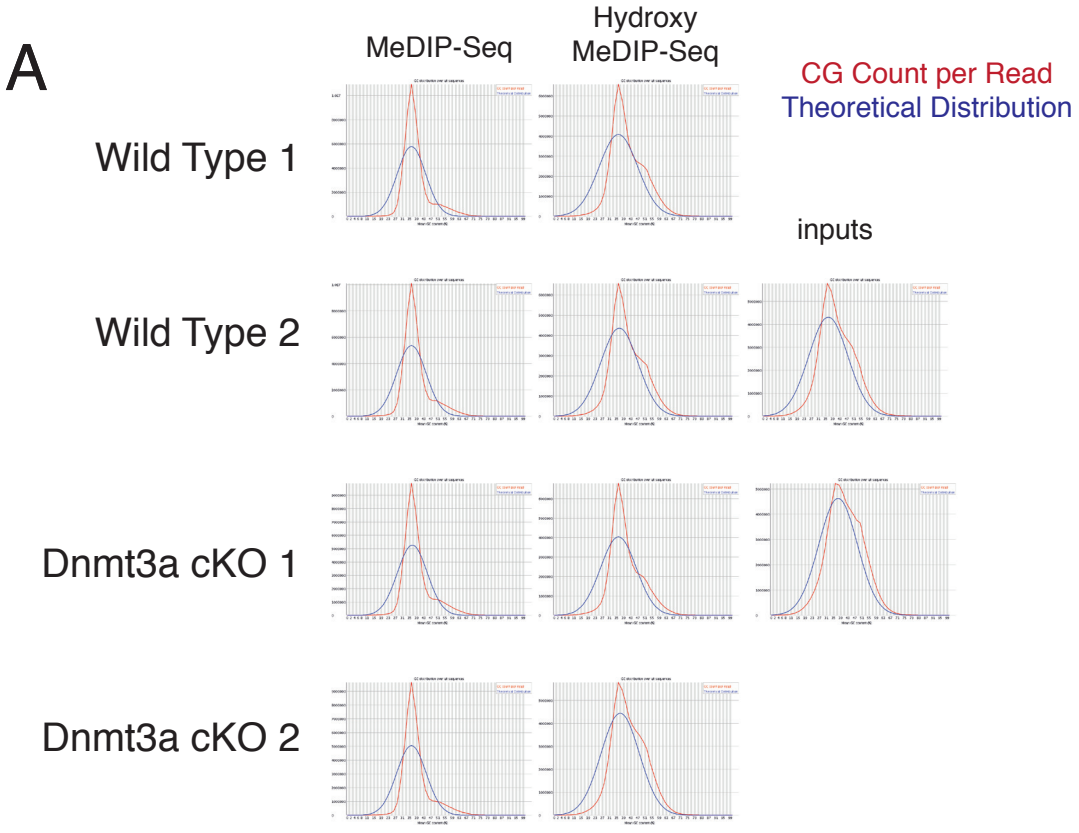
GO Terms	P-value
Cell differentiation	3.08e-6
Metabolic process	7.76e-5
Primary Metabolic Processes	1.34e-4
Notch pathway	3.01e-3

Upregulated in Dnmt3a KO IFE (135 genes)

GO Terms	P-value
Long chain fatty acid metabolic process	2.85e-4
Lipid biosynthetic process	1.10e-3
Fatty acid homeostasis	1.84e-3
cell growth	1.46E-03

Figure S6: Dnmt3a loss alters gene expression after six weeks of DMBA/TPA treatment.
(A) Left panel, heatmaps representing gene expression (rlog transformed values) of the 498 genes in sorted bulge hair follicle stem cells (Bulge) differentially expressed between wild-type (n = 3) and Dnmt3a cKO (n = 3). Right panel, gene ontology analysis of up and down-regulated genes. (B) Left panel, heatmaps representing gene expression (rlog transformed values) of the 188 differential expressed genes between wild-type (n = 4) and Dnmt3a cKO (n = 4) sorted interfollicular epidermal (IFE) stem cells. Right panel, gene ontology analysis of of up and down-regulated genes.

Figure S7



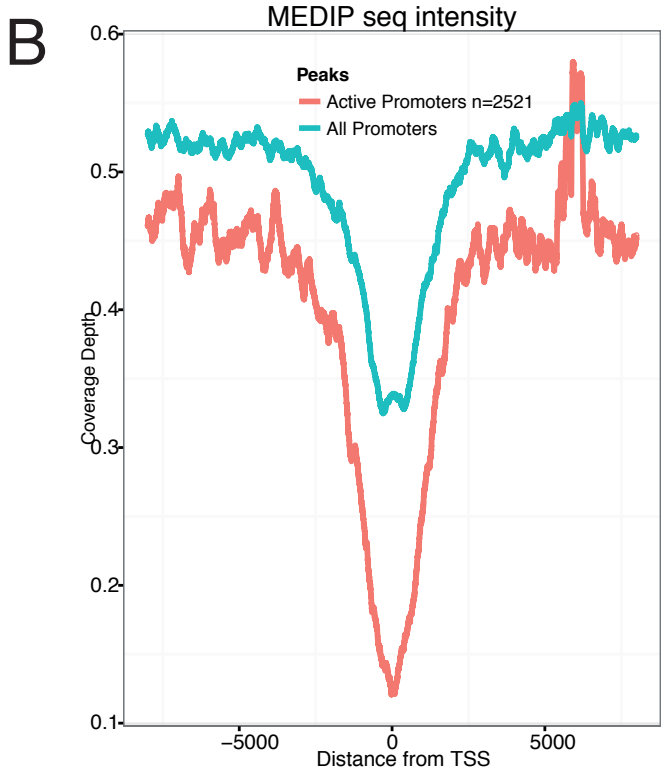


Figure S7: MeDIP-seq and hMeDIP-seq from sorted tumor cells.
(A) CpG count reads versus theoretical distribution in MeDIP and hMeDIP samples from wild-type and Dnmt3a cKO tumors.
(B) MeDIP-seq signals around active and non-active TSSs in wild-type tumor cells.

Materials and Methods

Chemical skin carcinogenesis

Inbred male or female Dnmt3a flox/flox (C57/Bl6) backcrossed to Krt14-CRE-YFP (C57/Bl6) for 6 generations were used for all animal experiments. Chemically-induced skin carcinogenesis was performed as previously described (Abel et al., 2009; Nassar et al., 2015), with a slight modification to yield high-frequency SCCs in the C57/Bl6 genetic background. Briefly, the back skin of 8 week-old mice—at which time hair follicles are in their resting phase (telogen)—was shaved and treated with the mutagen 7,12-dimethylbenz[a]anthracene (DMBA; 200 µl of 0.25 mg/ml solution in acetone) and the pro-inflammatory and pro-proliferation agent 12-O-tetradecanoyl phorbol-13-acetate (TPA; 200 µl of 0.02 mg/ml solution in acetone) once weekly for 6 weeks. For short DMBA experiments, animals were sacrificed and back skins were processed three days after the sixth TPA application. For tumor formation studies, treatment continued twice weekly with TPA (200 µl of 20 µg/ml solution in acetone) for up to 20 weeks, or until the largest tumor of each mouse reached 1.5 mm diameter, at which point animals were sacrificed. In total, 12 wild-type and 15 Dnmt3a cKO tumors from 6 and 8 mice, respectively, were included for tumor analyses.

Single-cell preparation and FACS analysis

To isolate pre-cancerous epidermal cells following short DMBA/TPA treatment, back skins were dissected and processed to single-cell suspensions as previously described (Jensen et al., 2010). To purify tumor cells, DMBA/TPA-induced SCCs were mechanically dissociated using a McIlwain Tissue Chopper (The Mickle Laboratory Engineering Co. LTD). Minced tumor tissue was digested under agitation in serum-free EMEM medium without calcium containing 2.5 mg/ml Collagenase I (Sigma Aldrich) and 0.75 mg/ml trypsin (Life Technologies) for 90 min at 37°C. Cells were pelleted, resuspended in 1–2 ml of 0.25% pre-warmed trypsin/EDTA (Life Technologies) containing 100 µg/ml DNase (Sigma Aldrich) per tumor, and incubated at 37°C for 2 min. Trypsin was inactivated by adding EMEM without calcium containing 10% chelated FBS. Cells were washed twice in PBS and filtered sequentially through 100 µm and 40 µm cell strainers.

For ChIP-seq, single-cell suspensions were cross-linked for 10 min at room temperature with 1% formaldehyde (methanol-free; Thermofisher, 28906) and quenched for 5 min to a final concentration of 0.125M glycine. Cells were washed 2× with cold PBS and frozen at –80°C.

For flow cytometry, epidermal or tumor cells were re-suspended at 1×10^7 cells/ml in PBS and labeled with CD49f-PE (clone NKI-GoH3, 1:200, AbD Serotec) and CD34-biotin (clone RAM34, 1:50, eBioscience) followed by streptavidin-APC (1:400, BD Biosciences). Tumor cell suspensions were additionally labeled

with lineage-BV605 (CD31, clone 390; CD45, clone 30-F11; TER119, clone TER119; all 1:100) (Biolegend) to exclude stromal cell contamination. Both epidermal and tumor cells were positive for YFP due to the presence of the Rosa26-YFP allele in the mice. Tumor cells (YFP+/lineage– cells), pre-cancerous epidermis of interfollicular epidermis (YFP+/CD49f^{high}/CD34– cells), and bulge

hair follicle stem cells (YFP+/CD49f^{high}/CD34+ cells) were FACS-sorted using a BD FACSAria Fusion flow cytometer (BD Biosciences). Between 3–20 × 10^4 cells were sorted and lysed in 1 ml of TRIzol for RNA and DNA isolation. After adding 200 µl chloroform, samples were vortexed for 30 second and then centrifuged at 12,000 g to separate the RNA-containing supernatant from the organic phase. RNA was precipitated with 1× volume of isopropanol, washed twice with 70% ethanol, and then used for library preparation. The interphase of the TRIzol solution (after removal of the supernatant) was precipitated adding 1× volume of isopropanol, centrifuged for 1 h at 4°C at 13,000 g, washed twice with ethanol, and digested overnight at 55°C with proteinase K (10 mg/ml) in TE 1× buffer. The following day, digested material was incubated 1 h at 37°C with RNase A and purified using a conventional phenol/chloroform separation. The DNA pellet was quantified, and DNA was used for library preparation for MeDIP-seq and hMeDIP-seq experiments.

MeDIP and hMeDIP sequencing

Purified genomic DNA (250 ng) from tumor cells was sonicated to

obtain fragments of 300–700 bp. Adaptors from the NEBNext Ultra DNA Library Prep Kit for Illumina were added to the fragmented DNA. DNA was denatured for 10 min at 99°C and cooled to avoid re-annealing. Fragmented DNA was incubated overnight with 1 μg of antibodies (5-methylcytosine, Abcam cat. # ab10805; 5-hydroxymethylcytosine, Active Motif, cat. # 39769) previously cross-linked with 15 μl of Dynabeads Protein A (Life Technologies). Immunocomplexes were recovered using 8 μl for 2 h. The following morning, DNA was washed three times for 10 min each, and purified DNA was extracted using QIAquick MinElute (Qiagen). Amplified libraries were prepared using NEBNext Ultra DNA Library Prep Kit for Illumina (E7370L) following the manufacturer's instructions.

RNA library preparation and sequencing

The libraries of total RNA from wild-type and Dnmt3a cKO tumors was prepared using the TruSeq®Stranded Total Sample Preparation kit (Illumina Inc.) according to the manufacturer's protocol. Each library was sequenced using TruSeq SBS Kit v3-HS, in paired end-mode with the read length 2 × 76 bp. A minimal of 137 million paired-end reads were generated for each sample run in one sequencing lane on HiSeq2000 (Illumina, Inc) following the manufacturer's protocol. Images analysis, base calling, and quality scoring of the run were processed using the manufacturer's software Real-Time Analysis (RTA 1.13.48) and followed by generation of FASTQ sequence files by CASAVA.

RNA-seq data processing

RNA-seq datasets were pre-processed by removing both low quality bases from the 3′- ends of the reads and adapter sequences using Trimmomatic (version 0.33) (Bolger et al., 2014). The trimmed reads were aligned to the mouse genome (UCSC mm10) using TopHat (version 2.0.13) (Trapnell et al., 2009), with default parameters and –g 5. Gene and transcript expression levels were quantified with HTSeq (version 0.6.1p1) (Anders et al., 2015). From the raw counts, counts per million (cpm) and fragments per kilobase of transcript per million mapped reads (fpkm) values were calculated. Differential expression analysis was performed using DESeq2 (Love et al., 2014) using a q-value cutoff of 0.05 and a fold-change cutoff of 1.5 to identify differentially expressed genes.

Chromatin immunoprecipitation–sequencing (ChIP-seq)

ChIP was performed as previously described (Morey et al., 2012). Briefly, frozen pelleted were lysed in 1 ml ChIP buffer (150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1% SDS, 0.5 mM DTT, and 1% Triton X-100) and sonicated for 30 min in a Bioruptor Pico (Diagenode). DNA fragments were de-crosslinked overnight at 65°C and checked with a bioanalyzer. After a DNA check, chromatin was diluted 1:5 with ChIP buffer with no SDS (150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 0.5 mM DTT, and 1% Triton X-100). Immunoprecipitation experiments for transcription factors used 30 μg of chromatin, and those for H3K27ac, 3 μg of chromatin. Antibodies (10 μg for Dnmt3a and 3 μg for H3K27ac)

were incubated overnight with the chromatin in ChIP buffer. Immunocomplexes were recovered with 40 μl of protein A bead slurry (Healthcare, cat. # 17-5280-01). Immunoprecipitated material was washed three times with low salt buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1% Triton) and 1× with high salt buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 1% Triton). DNA complexes were de-crosslinked at 65°C overnight, and DNA was then eluted in 50 μl of water using the PCR purification kit (QIAGEN). Antibodies used for ChIP were Dnmt3a (SantaCruz H-295) and H3K27ac (Merck Millipore, cat. # 07-360). Libraries for sequencing were prepared using NEBNext Ultra DNA Library Prep Kit from Illumina (E7370L) following the manufacturer's instructions.

ChIP-seq data processing

ChIP-seq datasets were aligned to the mouse genome build mm10 using BowTie (version 1.0.1) (Langmead et al., 2009); the parameters used were –k 1, –m 1, and –n 2. UCSC browser tracks (Kent et al., 2002) were created from the mapped bam file after converting it to bedGraph (normalized to 10 million reads) and subsequently bigWig format. Peak calling of Dnmt3A to determine regions of ChIP-seq enrichment over the background was done with the MACS version 1.4.1. Peaks of the methylation and hydroxymethylation datasets were determined similarly. For histone marks, MACS version 2 was used with parameters –broad, -q 0.01, and –g mm. ChIP-seq peaks were annotated using the annotatePeaks.pl script of the HOMER suite (version 4.6) (Heinz et al., 2010) using the UCSC mm10 annotation. The coverage depths

of different ChIP-seq experiments at specified regions were also calculated using the annotatePeaks.pl script. This generated a normalized coverage value of different sequencing experiments at equally-spaced bins spanning the region of interest. Bin size was set to 1 bp.

For the differential regulation analysis of MeDIP-seq data with replicates, common peaks were first determined among the replicates of the wild-type and KO samples separately. A consensus peakset was then created from the two common peaksets, and the read counts were calculated for all the peaks of the consensus peakset. DESeq2 (Love et al., 2014) was applied to calculate the differentially bound peaks using a padj < 0.05.

Immunofluorescence

Skin and tumors were isolated from mice, fixed in formalin 10% for 2 h at room temperature, and embed in paraffin. Sections were cut and stained on glass coverslips. Sections after deparaffinization were permeabilized with 0.5% Triton/PBS for 10 min, blocked with 10% goat serum, and stained overnight at 4°C with primary antibodies diluted in 1% goat serum. The morning after, sections were washed three times with PBS 1× with 10 min for each wash and stained with secondary antibody (1/1000). Nuclei were counterstained with DAPI (Roche). Primary antibodies were anti-Dnmt3a (1:100, SantaCruz H-295), anti-PPAR-γ (1:100 SantCruz), and anti-keratin 14 (1:500, Invitrogen); secondary antibodies were

anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 647 (1:500, Molecular Probes). Pictures were acquired using a Leica TCS SP5 confocal microscope

Statistical Analysis

To compare tumor burden between genotypes, we used a T-Test with 95% confidence. To compare free tumor survival differences and anagen entry differences we used a Chi-Square test. To compare Relative Methylation Score (RMS) levels and to compare normalized 5-hmC levels between wild type and Dnmt3a cKO sorted tumor cells we used a paired Wilcoxon Test. The same paired Wilcoxon test was used to measure differences in RNA expression.

KI67 staining and quantification

Skin and tumor sections were stained after deparaffinization with KI67 (Abcam ab15580) for 60 min. After two washes, section were incubated with Power Vision Rabbit (InmunoLogic) for 45 min. Positive staining was revealed using a chromogen DAB for 5 min (Dako). Counterstain for hematoxylin was incubated for 3 min (Dako).

Stained sections were scanned using a high resolution NanoZoomer 2.0 HT (Hamamatsu). KI67-positive nuclei in the interfollicular epidermis were measured using the TMarker software (Schuffler et al., 2013). Positive and negative nuclei for the staining were trained using the color deconvolution plugin and quantified using the

cancer nucleus classification plugin. Total number of positive nuclei were normalized to the total number of nuclei in the area considered. T-test was used to measure statistical difference among groups and genotypes.

Supplementary References

Abel, E.L., Angel, J.M., Kiguchi, K., DiGiovanni, J., 2009. Multi-stage chemical carcinogenesis in mouse skin: fundamentals and applications. Nat Protoc 4, 1350–1362.

Anders, S., Pyl, P.T., Huber, W., 2015. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120.

Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y.C., Laslo, P., Cheng, J.X., Murre, C., Singh, H., Glass, C.K., 2010. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell 38, 576–589.

Jensen, K.B., Driskell, R.R., Watt, F.M., 2010. Assaying proliferation and differentiation capacity of stem cells using disaggregated adult mouse epidermis. Nat Protoc 5, 898–911.

Kent, W.J., Sugnet, C.W., Furey, T.S., Roskin, K.M., Pringle, T.H., Zahler, A.M., Haussler, D., 2002. The human genome browser at UCSC. Genome Res 12, 996–1006.

Langmead, B., Trapnell, C., Pop, M., Salzberg, S.L., 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the

human genome. Genome Biol 10, R25.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15, 550.

Morey, L., Pascual, G., Cozzuto, L., Roma, G., Wutz, A., Benitah, S.A., Di Croce, L., 2012. Nonoverlapping functions of the Polycomb group Cbx family of proteins in embryonic stem cells. Cell Stem Cell 10, 47–62.

Nassar, D., Latil, M., Boeckx, B., Lambrechts, D., Blanpain, C., 2015. Genomic landscape of carcinogen-induced and genetically induced mouse skin squamous cell carcinoma. Nat Med 21, 946–954.

Schuffler, P.J., Fuchs, T.J., Ong, C.S., Wild, P.J., Rupp, N.J., Buhmann, J.M., 2013. TMARKER: A free software toolkit for histopathological cell counting and staining estimation. J Pathol Inform 4, S2.

Trapnell, C., Pachter, L., Salzberg, S.L., 2009. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25, 1105–1111.

Discussion

Discussion

Although 5-mC was reported to play a role in gene regulation more than 40 years ago (Holliday and Pugh, 1975; Riggs, 1975), we still have an incomplete understanding about the mechanisms behind DNA methylation, how it is regulated, and its roles during different cellular stages. What is clear is that 5-mC inhibits transcriptional initiation by inhibiting transcription factors binding to chromatin. This great discovery was established like a dogma into the DNA methylation scientific community, and for a long time 5-mC was suggested to be exclusively a static epigenetic modification of DNA that directly represses gene transcription. The advances of Next Generation Sequencing techniques started to challenge the dogma, with many different methods established to assess 5-mC genome-wide levels, including bisulphite whole-genome sequencing, reduced bisulphite sequencing, MeDIP sequencing, and MBD sequencing. These new sequencing techniques have provided tremendous amounts of information regarding the occupancy and the role of the 5-mC modification. In addition, the discovery of 5-hmC in 2009 opened an entire new epigenetic field, challenging the idea that 5-mC was the only stable modification in mammalian genomes.

The bioinformatic integration of DNA methylation data with other epigenetic modifications, such as histone modifications and transcription factor occupancy, gave new insights to the importance of 5-mC and 5-hmC. Interestingly, different work from many

laboratories pointed to the same direction: that differentially methylated regions through the transition of cell type A into cell type B were mostly found at distal regulatory regions (Colquitt et al., 2014; Hon et al., 2013; Plank and Dean, 2014; Rasmussen et al., 2015; Rinaldi and Benitah, 2014; Rinaldi et al., 2016; H. Wu et al., 2010). When I started my PhD, the first NGS experiment that we performed was the ChIP-seq of Dnmt3a and Dnmt3b in human epidermal stem cells and in differentiated keratinocytes (Rinaldi et al., 2016). We were at first quite intrigued by the results obtained: we could spot clearly Dnmt3a and Dnmt3b at a high number of active enhancers, but considering the accepted role of Dnmts in transcriptional repression at that time, the possible mechanism behind this observation was a complete mystery for us.

We later determined that Dnmt3a and Dnmt3b positively regulate gene expression through non-overlapping functions at these regulatory elements via distinct mechanisms. Our results showed that Dnmt3b promotes not only gene body methylation, as previously shown in other cell types (Baubec et al., 2015; Morselli et al., 2015), but also enhancer body DNA methylation, both of which are required to sustain high transcriptional activity of mRNAs and eRNAs (enhancer RNAs). For this, Dnmt3a, together with Tet2, was essential to maintain the high levels of 5-hmC at the regulatory regions (Figure 7).

Importantly, Dnmt3a and Dnmt3b regulate epidermal stem cells in two distinct ways: Dnmt3b is highly expressed in the stem cell state, and its depletion leads to a spontaneous differentiation; in contrast, Dnmt3a increases its expression during differentiation, and epidermal stem cells lacking Dnmt3a are incapable of properly undergoing differentiation. The function of Dnmt3a during differentiation relies on its ability to methylate DNA, and overexpression of a wild-type form, but not a catalytically inactive mutant, leads to spontaneous differentiation of epidermal stem cells. Nevertheless, Dnmt3a was also important also for the stem cell state in our system, suggesting a dual role for Dnmt3a in adult stem cells.

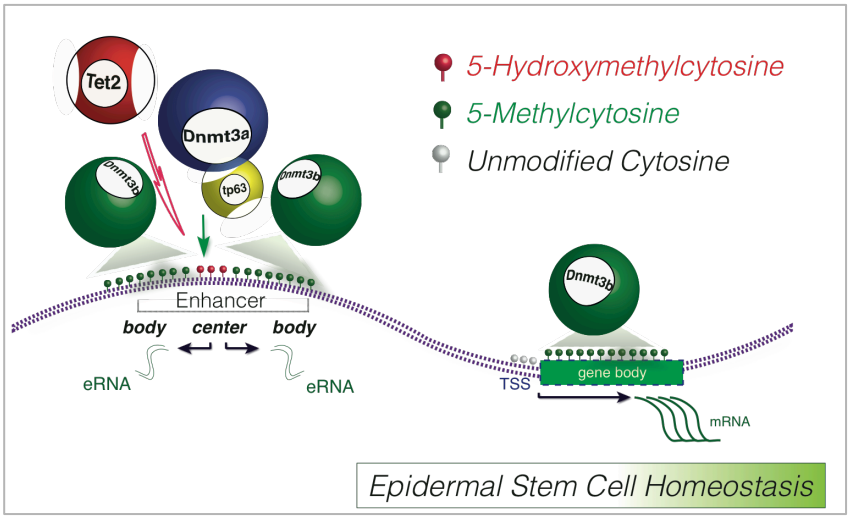


Figure 7: Model of Enhancer Regulation by Dnmt3a and Dnmt3b

To study the role of Dnmt3a and Dnmt3b in an *in vivo* setting, we established and analyzed conditional knockouts for Dnmt3a and/or Dnmt3b in epidermal tissue. We were surprised to notice no phenotypical difference of the cKOs to the wild-type animals, specially considering the numerous reports that have shown clearly the importance of several epigenetic regulators in maintaining the homeostasis of adult epidermis through direct regulation of epidermal stem cells functions.

The main role of skin is to protect the internal organs from water loss, parasites infection, and changes in temperatures. Facing the continuous challenges given by external stimuli makes skin a very robust tissue. At the genetic level, the deletion of specific genes can resolve in minor phenotypes as compared to the phenotypes observed in other organs. A clear example of this is the deletion of EED, the core subunit of the Polycomb Repressive Complex 2, a heterogeneous complex of proteins essential for establishing trimethylation of lysine 27 on the histone H3 tail (H3K27me3) and for repressing gene activation. EED conditional knockout in the epidermis leads to a minor phenotype (Dauber et al., 2016), but the deletion of the same gene in hematopoietic stem cells results in a complete exhaustion of adult hematopoietic stem cell pool and to the premature death of the animals (Xie et al., 2014). This extreme difference is just an example of how epidermal tissue is robust and resilient as compared to other organs. Nonetheless, Dnmt3a and Dnmt3b epidermal conditional KO did not lead to any strong

differences to the wild-type. Therefore, we decided to study the roles of Dnmt3a and Dnmt3b during skin tumorigenesis.

Interestingly, the Dnmt3a-deficient epidermis showed a tremendous increase in tumor initiation, while the Dnmt3b cKO mouse model did not show differences in tumor burden. We found that Dnmt3a is important to repress tumor initiation rather than tumor progression, a conclusion reached also by another group studying the role of Dnmt3a during leukemia formation (L. Yang et al., 2016).
Furthermore, the deletion of both Dnmt3a/b revealed a complementary relationship between Dnmt3a and Dnmt3b; in fact, the double cKO showed not only an increased tumor initiation but also the formation of aggressive skin tumors, which in some cases could also metastasize to the lungs of the animals.

Importantly, we further confirmed that the enhancers bound by Dnmt3a are highly enriched in 5-hmC. The loss of DNA modifications, caused by the loss of Dnmt3a, could thus be directly linked to the activity alteration of these enhancers. The intriguing link between enhancer activity–DNA modifications–tumorigenesis deserves further studies to elucidate how this is regulated.

Several reports have shown Dnmt3a to be frequently mutated in human tumors (M. S. Kim et al., 2013). Importantly, when looking at the timing of inactivation, Dnmt3a is frequently found to be one of the first mutations to occur during tumorigenesis (Shlush et al., 2014), highlighting the potent tumor suppressor role of this enzyme.

On the other hand, the impact of Dnmt1 or Dnmt3b in tumorigenesis is somewhat more controversial. Dnmt3b is often overexpressed in human tumors (Lin et al., 2006; Linhart et al., 2007) and is mutated in immunodeficiency syndrome (ICF) (Ehrlich, 2003; Jiang et al., 2005). The importance of Dnmt3b during tumorigenesis is not known but seems to be related to Dnmt3b-specificity as a genic methyltransferase (Baubec et al., 2015; Duymich et al., 2016; Linhart et al., 2007; Morselli et al., 2015; X. Yang et al., 2014). Also, the role of Dnmt1 is still poorly understood. Dnmt1 deletion slightly promotes colon tumor initiation but strongly suppresses tumor growth (Morita et al., 2013; Sheaffer et al., 2016; Yamada et al., 2005). It was therefore unexpected to observe the strong impact on tumor initiation and tumor progression of the Dnmt3a/Dnmt3b double knockout (Figure 8).

One of the possible links between Dnmt3a and tumor initiation is that Dnmt3a at enhancers is important for maintaining high levels of 5-hmC. The global reduction of 5-hmC is a hallmark of several cancer types, including squamous cell carcinoma, and is often correlated with poor prognosis (Ficz and Gribben, 2014; Lian et al., 2012; Liao et al., 2016; Shi et al., 2016; F. Zhang et al., 2016). However, it is unclear if the loss of 5-hmC is cause or consequence of tumor progression. Currently, we cannot exclude that precisely the loss of DNA modifications at regulatory elements could play a role in cancer initiation. We (and others) have demonstrated a clear relationship between Dnmt3a–Tet–5-hmC levels: the inactivation of this axis in adult stem cells predisposes the stem cell pool to

amplification, thereby leading to higher chances of tumor initiation. It will be imperative to follow-up with these results to determine driving forces behind tumor initiation.

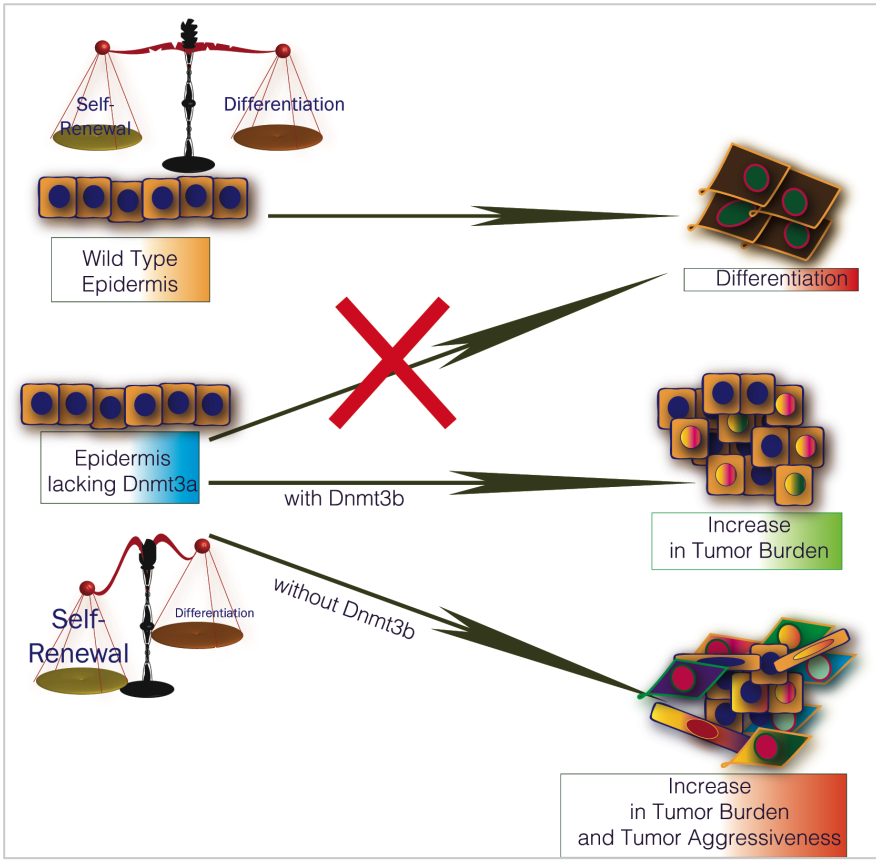


Figure 8: Scheme of tumor suppressor function of Dnmt3a and Dnmt3b in adult epidermis

Dnmt enzymes have complex, synergistic, redundant, and exclusive roles in mammalian genomes. It seems evident that Dnmt1 is important for tissue homeostasis throughout the entire animal lifespan, but its deletion does not lead to a predisposition for

acquiring strong oncogenic transformation (Fan et al., 2005; Li et al., 2012; Sen et al., 2010; Shaffer et al., 2014; Sheaffer et al., 2016). Dnmt3b is essential for the early stages of mammalian embryo development, and its deletion does not affect the homeostasis of adult tissues (Challen et al., 2014; Nuhrenberg et al., 2015; Okano et al., 1999). Dnmt3a in turn has only a minor impact on the homeostasis of adult tissues but strongly predisposes to tumor initiation (Challen et al., 2014; 2012; Mayle et al., 2015; Shlush et al., 2014; H. Wu et al., 2010; L. Yang et al., 2016). It is important, however, to appreciate the distinct roles of Dnmt3a during tumor initiation and within the tumor once formed. In fact, restoring Dnmt3a in leukemia samples did not block tumor progression, clearly indicating that Dnmt3a represses the initial steps of oncogenic transformation rather than tumor progression (L. Yang et al., 2016).

Taken together, we can conclude that Dnmt3a and Dnmt3b are essential for regulating the homeostasis of human epidermal stem cells. The methylation of promoters, gene bodies, and enhancers by the two Dnmt3a/b enzymes is essential not only for the self-renewal of epidermal stem cells but also for their proper differentiation. In fact, deletion of Dnmt3a alone or in combination with Dnmt3b results in acquiring an oncogenic identity. At this point, incorporating Dnmt3a into pre-tumoral mutational screening of human samples is to be strongly recommended.

Conclusions

Conclusions

1. The expression of Dnmt3a increases during epidermal stem cell differentiation while Dnmt3b diminishes

2. Dnmt3a and Dnmt3b associate with the most active enhancers in human epidermal stem cells in an H3K36me3-dependent manner

3. Dnmt3a and Tet2 establish the high levels of 5-hmC at active enhancers

4. Dnmt3b maintains high levels of 5-mC surrounding the center of enhancers

5. Dnmt3a is essential for proper differentiation of human epidermal stem cells while Dnmt3b is important for their self-renewal

6. Dnmt3a and Dnmt3b are dispensable for the formation of a functional murine epidermis

7. Dnmt3a strongly suppresses skin tumorigenesis

8. Dnmt3b deletion does not impact tumor initiation in skin cancer

9. Deletion of both Dnmt3a and Dnmt3b results in the increase of both tumor burden and tumor aggressiveness.

10. During skin tumorigenesis, Dnmt3a represses the PPAR genes and lipid metabolism pathway in a DNA methylation-dependent manner

References

References

Avgustinova, A., Benitah, S.A., 2016. Epigenetic control of adult stem cell function. Nat Rev Mol Cell Biol.

Bachman, M., Uribe-Lewis, S., Yang, X., Williams, M., Murrell, A., Balasubramanian, S., 2014. 5-Hydroxymethylcytosine is a predominantly stable DNA modification. Nat Chem 6, 1049–1055.

Ballare, C., Castellano, G., Gaveglia, L., Althammer, S., Gonzalez-Vallinas, J., Eyras, E., Le Dily, F., Zaurin, R., Soronellas, D., Vicent, G.P., Beato, M., 2013. Nucleosome-driven transcription factor binding and gene regulation. Mol Cell 49, 67–79.

Baubec, T., Colombo, D.F., Wirbelauer, C., Schmidt, J., Burger, L., Krebs, A.R., Akalin, A., Schubeler, D., 2015. Genomic profiling of DNA methyltransferases reveals a role for DNMT3B in genic methylation. Nature.

Beato, M., Eisfeld, K., 1997. Transcription factor access to chromatin. Nucleic Acids Res 25, 3559–3563.

Blanpain, C., Simons, B.D., 2013. Unravelling stem cell dynamics by lineage tracing. Nat Rev Mol Cell Biol 14, 489–502.

Bock, C., Beerman, I., Lien, W.-H., Smith, Z.D., Gu, H., Boyle, P., Gnirke, A., Fuchs, E., Rossi, D.J., Meissner, A., 2012. DNA methylation dynamics during in vivo differentiation of blood and skin stem cells. Mol Cell 47, 633–647.

Bostick, M., Kim, J.K., Esteve, P.-O., Clark, A., Pradhan, S., Jacobsen, S.E., 2007. UHRF1 plays a role in maintaining DNA methylation in mammalian cells. Science 317, 1760–1764.

Ceballos-Chavez, M., Subtil-Rodriguez, A., Giannopoulou, E.G., Soronellas, D., Vazquez-Chavez, E., Vicent, G.P., Elemento, O., Beato, M., Reyes, J.C., 2015. The chromatin Remodeler CHD8 is required for activation of progesterone receptor-dependent enhancers. PLoS Genet 11, e1005174.

Challen, G.A., Sun, D., Jeong, M., Luo, M., Jelinek, J., Berg, J.S., Bock, C., Vasanthakumar, A., Gu, H., Xi, Y., Liang, S., Lu, Y., Darlington, G.J., Meissner, A., Issa, J.-P.J., Godley, L.A., Li, W., Goodell, M.A., 2012. Dnmt3a is essential for hematopoietic stem cell differentiation. Nat Genet 44, 23–31.

Challen, G.A., Sun, D., Mayle, A., Jeong, M., Luo, M., Rodriguez, B., Mallaney, C., Celik, H., Yang, L., Xia, Z., Cullen, S., Berg,

J., Zheng, Y., Darlington, G.J., Li, W., Goodell, M.A., 2014. Dnmt3a and Dnmt3b have overlapping and distinct functions in hematopoietic stem cells. Cell Stem Cell 15, 350–364.

Charlet, J., Duymich, C.E., Lay, F.D., Mundbjerg, K., Dalsgaard Sorensen, K., Liang, G., Jones, P.A., 2016. Bivalent Regions of Cytosine Methylation and H3K27 Acetylation Suggest an Active Role for DNA Methylation at Enhancers. Mol Cell 62, 422–431.

Clayton, E., Doupe, D.P., Klein, A.M., Winton, D.J., Simons, B.D., Jones, P.H., 2007. A single type of progenitor cell maintains normal epidermis. Nature 446, 185–189.

Colquitt, B.M., Markenscoff-Papadimitriou, E., Duffie, R., Lomvardas, S., 2014. Dnmt3a regulates global gene expression in olfactory sensory neurons and enables odorant-induced transcription. Neuron 83, 823–838.

Dauber, K.L., Perdigoto, C.N., Valdes, V.J., Santoriello, F.J., Cohen, I., Ezhkova, E., 2016. Dissecting the Roles of Polycomb Repressive Complex 2 Subunits in the Control of Skin Development. J Invest Dermatol 136, 1647–1655.

Di Croce, L., Koop, R., Venditti, P., Westphal, H.M., Nightingale, K.P., Corona, D.F., Becker, P.B., Beato, M., 1999. Two-step synergism between the progesterone receptor and the DNA-binding domain of nuclear factor 1 on MMTV minichromosomes. Mol Cell 4, 45–54.

Duymich, C.E., Charlet, J., Yang, X., Jones, P.A., Liang, G., 2016. DNMT3B isoforms without catalytic activity stimulate gene body methylation as accessory proteins in somatic cells. Nat Commun 7, 11453.

Ehrlich, M., 2003. The ICF syndrome, a DNA methyltransferase 3B deficiency and immunodeficiency disease. Clin Immunol 109, 17–28.

Fan, G., Martinowich, K., Chin, M.H., He, F., Fouse, S.D., Hutnick, L., Hattori, D., Ge, W., Shen, Y., Wu, H., Hoeve, ten, J., Shuai, K., Sun, Y.E., 2005. DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. Development 132, 3345–3356.

Ficz, G., Branco, M.R., Seisenberger, S., Santos, F., Krueger, F., Hore, T.A., Marques, C.J., Andrews, S., Reik, W., 2011. Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473, 398–402.

Ficz, G., Gribben, J.G., 2014. Loss of 5-hydroxymethylcytosine in

cancer: cause or consequence? Genomics 104, 352–357.
Filion, G.J., Beato, M., 2015. 3D genome structure. Organization of the nucleus in space and time. FEBS Lett 589, 2867–2868.
Garcia-Prat, L., Martinez-Vicente, M., Perdiguero, E., Ortet, L., Rodriguez-Ubreva, J., Rebollo, E., Ruiz-Bonilla, V., Gutarra, S., Ballestar, E., Serrano, A.L., Sandri, M., Munoz-Canoves, P., 2016. Autophagy maintains stemness by preventing senescence. Nature 529, 37–42.
Goll, M.G., Kirpekar, F., Maggert, K.A., Yoder, J.A., Hsieh, C.-L., Zhang, X., Golic, K.G., Jacobsen, S.E., Bestor, T.H., 2006. Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science 311, 395–398.
Heintzman, N.D., Stuart, R.K., Hon, G., Fu, Y., Ching, C.W., Hawkins, R.D., Barrera, L.O., Van Calcar, S., Qu, C., Ching, K.A., Wang, W., Weng, Z., Green, R.D., Crawford, G.E., Ren, B., 2007. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat Genet 39, 311–318.
Hnisz, D., Abraham, B.J., Lee, T.I., Lau, A., Saint-Andre, V., Sigova, A.A., Hoke, H.A., Young, R.A., 2013. Super-enhancers in the control of cell identity and disease. Cell 155, 934–947.
Holliday, R., Pugh, J.E., 1975. DNA modification mechanisms and gene activity during development. Science 187, 226–232.
Hon, G.C., Rajagopal, N., Shen, Y., McCleary, D.F., Yue, F., Dang, M.D., Ren, B., 2013. Epigenetic memory at embryonic enhancers identified in DNA methylation maps from adult mouse tissues. Nat Genet 45, 1198–1206.
Jiang, Y.L., Rigolet, M., Bourc'his, D., Nigon, F., Bokesoy, I., Fryns, J.P., Hulten, M., Jonveaux, P., Maraschio, P., Megarbane, A., Moncla, A., Viegas-Pequignot, E., 2005. DNMT3B mutations and DNA methylation defect define two types of ICF syndrome. Hum Mutat 25, 56–63.
Jones, P.H., Watt, F.M., 1993. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 73, 713–724.
Kim, M.S., Kim, Y.R., Yoo, N.J., Lee, S.H., 2013. Mutational analysis of DNMT3A gene in acute leukemias and common solid cancers. APMIS 121, 85–94.
Kim, T.-H., Li, F., Ferreiro-Neira, I., Ho, L.-L., Luyten, A., Nalapareddy, K., Long, H., Verzi, M., Shivdasani, R.A., 2014. Broadly permissive intestinal chromatin underlies lateral

inhibition and cell plasticity. Nature 506, 511–515.
Kim, T.-K., Shiekhattar, R., 2015. Architectural and Functional Commonalities between Enhancers and Promoters. Cell 162, 948–959.
Li, J., Jiang, T.-X., Hughes, M.W., Wu, P., Yu, J., Widelitz, R.B., Fan, G., Chuong, C.-M., 2012. Progressive alopecia reveals decreasing stem cell activation probability during aging of mice with epidermal deletion of DNA methyltransferase 1. J Invest Dermatol 132, 2681–2690.
Lian, C.G., Xu, Y., Ceol, C., Wu, F., Larson, A., Dresser, K., Xu, W., Tan, L., Hu, Y., Zhan, Q., Lee, C.-W., Hu, D., Lian, B.Q., Kleffel, S., Yang, Y., Neiswender, J., Khorasani, A.J., Fang, R., Lezcano, C., Duncan, L.M., Scolyer, R.A., Thompson, J.F., Kakavand, H., Houvras, Y., Zon, L.I., Mihm, M.C.J., Kaiser, U.B., Schatton, T., Woda, B.A., Murphy, G.F., Shi, Y.G., 2012. Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. Cell 150, 1135–1146.
Liao, Y., Gu, J., Wu, Y., Long, X., Ge, D.I., Xu, J., Ding, J., 2016. Low level of 5-Hydroxymethylcytosine predicts poor prognosis in non-small cell lung cancer. Oncol Lett 11, 3753–3760.
Lin, H., Yamada, Y., Nguyen, S., Linhart, H., Jackson-Grusby, L., Meissner, A., Meletis, K., Lo, G., Jaenisch, R., 2006. Suppression of intestinal neoplasia by deletion of Dnmt3b. Mol Cell Biol 26, 2976–2983.
Linhart, H.G., Lin, H., Yamada, Y., Moran, E., Steine, E.J., Gokhale, S., Lo, G., Cantu, E., Ehrich, M., He, T., Meissner, A., Jaenisch, R., 2007. Dnmt3b promotes tumorigenesis in vivo by gene-specific de novo methylation and transcriptional silencing. Genes Dev 21, 3110–3122.
Luis, N.M., Morey, L., Mejetta, S., Pascual, G., Janich, P., Kuebler, B., Cozutto, L., Roma, G., Nascimento, E., Frye, M., Di Croce, L., Benitah, S.A., 2011. Regulation of human epidermal stem cell proliferation and senescence requires polycomb- dependent and -independent functions of Cbx4. Cell Stem Cell 9, 233–246.
Mascre, G., Dekoninck, S., Drogat, B., Youssef, K.K., Brohee, S., Sotiropoulou, P.A., Simons, B.D., Blanpain, C., 2012. Distinct contribution of stem and progenitor cells to epidermal maintenance. Nature 489, 257–262.
Mayle, A., Yang, L., Rodriguez, B., Zhou, T., Chang, E., Curry, C.V., Challen, G.A., Li, W., Wheeler, D., Rebel, V.I., Goodell,

M.A., 2015. Dnmt3a loss predisposes murine hematopoietic stem cells to malignant transformation. Blood 125, 629–638.

Meyenn, von, F., Iurlaro, M., Habibi, E., Liu, N.Q., Salehzadeh-Yazdi, A., Santos, F., Petrini, E., Milagre, I., Yu, M., Xie, Z., Kroeze, L.I., Nesterova, T.B., Jansen, J.H., Xie, H., He, C., Reik, W., Stunnenberg, H.G., 2016. Impairment of DNA Methylation Maintenance Is the Main Cause of Global Demethylation in Naive Embryonic Stem Cells. Mol Cell 62, 848–861.

Morita, R., Hirohashi, Y., Suzuki, H., Takahashi, A., Tamura, Y., Kanaseki, T., Asanuma, H., Inoda, S., Kondo, T., Hashino, S., Hasegawa, T., Tokino, T., Toyota, M., Asaka, M., Torigoe, T., Sato, N., 2013. DNA methyltransferase 1 is essential for initiation of the colon cancers. Exp Mol Pathol 94, 322–329.

Morselli, M., Pastor, W.A., Montanini, B., Nee, K., Ferrari, R., Fu, K., Bonora, G., Rubbi, L., Clark, A.T., Ottonello, S., Jacobsen, S.E., Pellegrini, M., 2015. In vivo targeting of de novo DNA methylation by histone modifications in yeast and mouse. Elife 4, e06205.

Nuhrenberg, T.G., Hammann, N., Schnick, T., Preissl, S., Witten, A., Stoll, M., Gilsbach, R., Neumann, F.-J., Hein, L., 2015. Cardiac Myocyte De Novo DNA Methyltransferases 3a/3b Are Dispensable for Cardiac Function and Remodeling after Chronic Pressure Overload in Mice. PLoS One 10, e0131019.

Okano, M., Bell, D.W., Haber, D.A., Li, E., 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247–257.

Pastor, W.A., Pape, U.J., Huang, Y., Henderson, H.R., Lister, R., Ko, M., McLoughlin, E.M., Brudno, Y., Mahapatra, S., Kapranov, P., Tahiliani, M., Daley, G.Q., Liu, X.S., Ecker, J.R., Milos, P.M., Agarwal, S., Rao, A., 2011. Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473, 394–397.

Plank, J.L., Dean, A., 2014. Enhancer Function: Mechanistic and Genome-Wide Insights Come Together. Mol Cell 55, 5–14.

Rada-Iglesias, A., Bajpai, R., Swigut, T., Brugmann, S.A., Flynn, R.A., Wysocka, J., 2011. A unique chromatin signature uncovers early developmental enhancers in humans. Nature 470, 279–283.

Rasmussen, K.D., Jia, G., Johansen, J.V., Pedersen, M.T., Rapin,

N., Bagger, F.O., Porse, B.T., Bernard, O.A., Christensen, J., Helin, K., 2015. Loss of TET2 in hematopoietic cells leads to DNA hypermethylation of active enhancers and induction of leukemogenesis. Genes Dev 29, 910–922.

Riggs, A.D., 1975. X inactivation, differentiation, and DNA methylation. Cytogenet Cell Genet 14, 9–25.

Rinaldi, L., Benitah, S.A., 2014. Epigenetic regulation of adult stem cell function. FEBS J.

Rinaldi, L., Datta, D., Serrat, J., Morey, L., Solanas, G., Avgustinova, A., Blanco, E., Pons, J.I., Matallanas, D., Kriegsheim, Von, A., Di Croce, L., Benitah, S.A., 2016. Dnmt3a and Dnmt3b Associate with Enhancers to Regulate Human Epidermal Stem Cell Homeostasis. Cell Stem Cell.

Seisenberger, S., Andrews, S., Krueger, F., Arand, J., Walter, J., Santos, F., Popp, C., Thienpont, B., Dean, W., Reik, W., 2012. The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. Mol Cell 48, 849–862.

Sen, G.L., Reuter, J.A., Webster, D.E., Zhu, L., Khavari, P.A., 2010. DNMT1 maintains progenitor function in self-renewing somatic tissue. Nature 463, 563–567.

Shaffer, B., McGraw, S., Xiao, S.C., Chan, D., Trasler, J., Chaillet, J.R., 2014. The Dnmt1 Intrinsically Disordered Domain Regulates Genomic Methylation During Development. Genetics.

Sharif, J., Muto, M., Takebayashi, S.-I., Suetake, I., Iwamatsu, A., Endo, T.A., Shinga, J., Mizutani-Koseki, Y., Toyoda, T., Okamura, K., Tajima, S., Mitsuya, K., Okano, M., Koseki, H., 2007. The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. Nature 450, 908–912.

Sheaffer, K.L., Elliott, E.N., Kaestner, K.H., 2016. DNA Hypomethylation Contributes to Genomic Instability and Intestinal Cancer Initiation. Cancer Prev Res (Phila) 9, 534–546.

Sheaffer, K.L., Kim, R., Aoki, R., Elliott, E.N., Schug, J., Burger, L., Schubeler, D., Kaestner, K.H., 2014. DNA methylation is required for the control of stem cell differentiation in the small intestine. Genes Dev 28, 652–664.

Shi, X., Yu, Y., Luo, M., Zhang, Z., Shi, S., Feng, X., Chen, Z., He, J., 2016. Loss of 5-Hydroxymethylcytosine Is an Independent Unfavorable Prognostic Factor for Esophageal Squamous Cell

Carcinoma. PLoS One 11, e0153100.
Shlush, L.I., Zandi, S., Mitchell, A., Chen, W.C., Brandwein, J.M., Gupta, V., Kennedy, J.A., Schimmer, A.D., Schuh, A.C., Yee, K.W., McLeod, J.L., Doedens, M., Medeiros, J.J.F., Marke, R., Kim, H.J., Lee, K., McPherson, J.D., Hudson, T.J., Brown, A.M.K., Yousif, F., Trinh, Q.M., Stein, L.D., Minden, M.D., Wang, J.C.Y., Dick, J.E., 2014. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature 506, 328–333.
Smith, Z.D., Meissner, A., 2013. DNA methylation: roles in mammalian development. Nat Rev Genet 14, 204–220.
Solanas, G., Benitah, S.A., 2013. Regenerating the skin: a task for the heterogeneous stem cell pool and surrounding niche. Nat Rev Mol Cell Biol 14, 737–748.
Sousa-Victor, P., Garcia-Prat, L., Serrano, A.L., Perdiguero, E., Munoz-Canoves, P., 2015. Muscle stem cell aging: regulation and rejuvenation. Trends Endocrinol Metab 26, 287–296.
Sousa-Victor, P., Gutarra, S., Garcia-Prat, L., Rodriguez-Ubreva, J., Ortet, L., Ruiz-Bonilla, V., Jardi, M., Ballestar, E., Gonzalez, S., Serrano, A.L., Perdiguero, E., Munoz-Canoves, P., 2014. Geriatric muscle stem cells switch reversible quiescence into senescence. Nature 506, 316–321.
Suetake, I., Shinozaki, F., Miyagawa, J., Takeshima, H., Tajima, S., 2004. DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. J Biol Chem 279, 27816–27823.
Sun, Z., Dai, N., Borgaro, J.G., Quimby, A., Sun, D., Correa, I.R.J., Zheng, Y., Zhu, Z., Guan, S., 2015. A sensitive approach to map genome-wide 5-hydroxymethylcytosine and 5-formylcytosine at single-base resolution. Mol Cell 57, 750–761.
Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L.M., Liu, D.R., Aravind, L., Rao, A., 2009. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324, 930–935.
Tuorto, F., Liebers, R., Musch, T., Schaefer, M., Hofmann, S., Kellner, S., Frye, M., Helm, M., Stoecklin, G., Lyko, F., 2012. RNA cytosine methylation by Dnmt2 and NSun2 promotes tRNA stability and protein synthesis. Nat Struct Mol Biol 19, 900–905.
van Oevelen, C., Collombet, S., Vicent, G., Hoogenkamp, M.,

Lepoivre, C., Badeaux, A., Bussmann, L., Sardina, J.L., Thieffry, D., Beato, M., Shi, Y., Bonifer, C., Graf, T., 2015. C/EBPalpha Activates Pre-existing and De Novo Macrophage Enhancers during Induced Pre-B Cell Transdifferentiation and Myelopoiesis. Stem Cell Reports 5, 232–247.

Watanabe, D., Suetake, I., Tada, T., Tajima, S., 2002. Stage- and cell-specific expression of Dnmt3a and Dnmt3b during embryogenesis. Mech Dev 118, 187–190.

Whyte, W.A., Orlando, D.A., Hnisz, D., Abraham, B.J., Lin, C.Y., Kagey, M.H., Rahl, P.B., Lee, T.I., Young, R.A., 2013. Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell 153, 307–319.

Wienholz, B.L., Kareta, M.S., Moarefi, A.H., Gordon, C.A., Ginno, P.A., Chedin, F., 2010. DNMT3L modulates significant and distinct flanking sequence preference for DNA methylation by DNMT3A and DNMT3B in vivo. PLoS Genet 6, e1001106.

Wu, H., Coskun, V., Tao, J., Xie, W., Ge, W., Yoshikawa, K., Li, E., Zhang, Y., Sun, Y.E., 2010. Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. Science 329, 444–448.

Wu, H., Zhang, Y., 2014. Reversing DNA methylation: mechanisms, genomics, and biological functions. Cell 156, 45–68.

Wu, S.C., Zhang, Y., 2010. Active DNA demethylation: many roads lead to Rome. Nat Rev Mol Cell Biol 11, 607–620.

Xie, H., Xu, J., Hsu, J.H., Nguyen, M., Fujiwara, Y., Peng, C., Orkin, S.H., 2014. Polycomb repressive complex 2 regulates normal hematopoietic stem cell function in a developmental-stage-specific manner. Cell Stem Cell 14, 68–80.

Yamada, Y., Jackson-Grusby, L., Linhart, H., Meissner, A., Eden, A., Lin, H., Jaenisch, R., 2005. Opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis. Proc Natl Acad Sci U S A 102, 13580–13585.

Yang, L., Rodriguez, B., Mayle, A., Park, H.J., Lin, X., Luo, M., Jeong, M., Curry, C.V., Kim, S.-B., Ruau, D., Zhang, X., Zhou, T., Zhou, M., Rebel, V.I., Challen, G.A., Gottgens, B., Lee, J.-S., Rau, R., Li, W., Goodell, M.A., 2016. DNMT3A Loss Drives Enhancer Hypomethylation in FLT3-ITD-Associated Leukemias. Cancer Cell 29, 922–934.

Yang, X., Han, H., De Carvalho, D.D., Lay, F.D., Jones, P.A., Liang, G., 2014. Gene body methylation can alter gene

expression and is a therapeutic target in cancer. Cancer Cell 26, 577–590.

Yu, M., Hon, G.C., Szulwach, K.E., Song, C.-X., Zhang, L., Kim, A., Li, X., Dai, Q., Shen, Y., Park, B., Min, J.-H., Jin, P., Ren, B., He, C., 2012. Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. Cell 149, 1368–1380.

Zhang, F., Liu, Y., Zhang, Z., Li, J., Wan, Y., Zhang, L., Wang, Y., Li, X., Xu, Y., Fu, X., Zhang, X., Zhang, M., Zhang, Z., Zhang, J., Yan, Q., Ye, J., Wang, Z., Chen, C.D., Lin, W., Li, Q., 2016. 5-hydroxymethylcytosine loss is associated with poor prognosis for patients with WHO grade II diffuse astrocytomas. Sci Rep 6, 20882.