Figures and figure supplements

Loss of Dnmt3a and Dnmt3b does not affect epidermal homeostasis but promotes squamous transformation through PPAR-γ

Lorenzo Rinaldi et al.
Figure 1. Dnmt3a loss shortens the onset of carcinogen-induced skin neoplasia, and increases tumor burden. (A) Representative pictures of wild-type and Dnmt3a-cKO animals after 5 months of treatment with DMBA/TPA. Graph in panel A represents the percentage of animals WT (n = 6) or Dnmt3a-cKO. (B) Number of tumors at 3 and 6 months after first DMBA/TPA treatment. (C) Survival analysis of animals at 3 and 6 months after first DMBA treatment. (D) Histological samples of papilloma, sebaceous adenoma, invasive papilloma, and SCC from wild-type and Dnmt3a-cKO mice. (E) Pie charts showing the percentage of different tumor types in wild-type and Dnmt3a-cKO mice.
cKO (n = 6) that entered into anagen after 2 weeks of treatment of DMBA/TPA, p=0.02, Chi-Square test. (B) Time of appearance, expressed in percentages of skin tumors on wild-type or Dnmt3a-cKO animals, p=0.005. (C) Number of skin tumors after 3 or 6 months of DMBA/TPA treatment, p=0.001 and p=0.0007. (D) Representative images (hematoxylin/eosin staining) of different subtypes of skin tumors. (E) Histopathological analysis of the different subsets of skin tumors that appeared after DMBA/TPA treatment of wild-type or Dnmt3a-cKO animals.

DOI: 10.7554/eLife.21697.003

The following source data is available for figure 1:

Source data 1. Data related to Figure 1B and Figure 1C.

DOI: 10.7554/eLife.21697.004
Figure 1—figure supplement 1. Dnmt3a is highly expressed in the basal cells of 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 wild type back skin (A) and tail skin (B) isolated at different ages.
Figure 1—figure supplement 1 continued on next page
Figure 1—figure supplement 1 continued

(C) Fpkm values of the Dnmts from RNA-seq data performed in wild type interfollicular epidermal stem cells (IFE, n = 4) and hair follicle stem cells (Bulge, n = 3) FACS sorted after 6 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. (F) Representative Images of one wild type and one Dnmt3a-cKO littermate (both females) age of five weeks.

DOI: 10.7554/eLife.21697.005
Dnmt3a and Dnmt3b double cKO animals develop more aggressive tumors than wild-type, Dnmt3a-cKO and Dnmt3b-cKO mice. (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.

Figure 2 continued on next page
treatment. Right, time of appearance of tumors shown as percentages in wild-type and Dnmt3b-cKO animals, and number of skin tumors after 3 or 6 months of DMBA/TPA treatment. (B) Left, representative images (hematoxylin/eosin staining) of skin tumors isolated from wild type and Dnmt3a/Dnmt3b DcKO littermates after 6 months of DMBA/TPA treatment. Right, time of appearance of tumors represented as percentages in wild-type and Dnmt3a/Dnmt3b DcKO animals, and number of skin tumors after 3 or 6 months of treatment with DMBA/TPA. (C–D) Number of tumors (left) and time of appearance (right) expressed as percentages, in wild type, Dnmt3a-cKO and DcKO animals after 6 months of DMBA/TPA treatment. (E) Histopathological analysis of the different subsets of skin tumors that appeared after DMBA/TPA treatment of wild type or DcKO animals. (F) Representative images of metastatic nodules identified only in a percentage (33%) of the lungs of DcKO animals, scale bar = 100 μm.
Figure 2—figure supplement 1. Deletion of Dnmt3b does not affect epidermal and hair follicle homeostasis. Representative images (hematoxylin/eosin staining) of back skin and tail skin from wild type and Dnmt3b-cKO littermates at different ages.

DOI: 10.7554/eLife.21697.007
Figure 2—figure supplement 2. Dnmt3b-KO and wild-type skin tumors are histologically indistinguishable. Representative images (hematoxylin/eosin staining) of different skin tumors isolated from wild type and Dnmt3b-cKO after 6 months of DMBA/TPA treatment.

Figure 2—figure supplement 2 continued on next page
Figure 2—figure supplement 3. Dnmt3b-KO tumors do not show changes in proliferation or apoptosis compared to their wild-type counterparts. (A) Left: Representative images of immunohistochemistry staining against the cell proliferation marker Ki67 in skin tumors isolated from wild type and Dnmt3b cKO mice. (B) Representative images of Tunel/DAPI double staining showing the percentage of apoptotic cells. The graphs show the percentage of proliferating cells and apoptotic cells in WT and Dnmt3b cKO tumors. The results indicate no significant difference (ns) in proliferation or apoptosis between WT and Dnmt3b cKO tumors.
Figure 2—figure supplement 3 continued

Dnmt3b-cKO after 6 months of DMBA/TPA treatment. Right- Quantification of Ki67 staining in wild type (n = 5) and Dnmt3b-KO (n = 5) skin tumors using the TMarker software. (B) Left-Representative images of TUNEL staining to detect apoptosis in skin tumors isolated from wild type and Dnmt3b-cKO after 6 months of DMBA/TPA treatment. Right-Quantification of Tunel staining in wild type (n = 5) and Dnmt3b-KO (n = 5) skin tumors using the TMarker software. Unpaired T-Test was used for statistics.

DOI: 10.7554/eLife.21697.009
Figure 2—figure supplement 4. The combined deletion of Dnmt3a and Dnmt3b does not affect epidermal homeostasis. (A) Representative images (hematoxylin/eosin staining) of back skin and tail skin from adult and aged wild type and DcKO littermates. (B) Immunofluorescence staining for 5-methylcytosine and Keratin 14 in aged (over 70 weeks old) wild type and DcKO littermates.

DOI: 10.7554/eLife.21697.010
Figure 2—figure supplement 5. Squamous cell carcinomas in Dnmt3a/Dnmt3b double KO mice express lower levels of epithelial markers compared to wild-type tumors. (A) Representative confocal images for E-Cadherin, Keratin 14 and DAPI in wild type, single Dnmt3a KO and double Dnmt3a/Dnmt3b KO squamous cell carcinomas. (B) Representative confocal images for Vimentin, Keratin 14 and DAPI in wild type, single Dnmt3a-cKO and double Dnmt3a/Dnmt3b cKO squamous cell carcinomas.

DOI: 10.7554/eLife.21697.011
Figure 2—figure supplement 6. The combined deletion of Dnmt3a and Dnmt3b favors the development of skin tumors with features of spindle cell carcinomas. (A–B) Hematoxylin/eosin staining and confocal images of two different spindle cell carcinomas developed in two DcKO animals.
Representative images of immunofluorescence staining to detect the expression of Vimentin, YFP and Keratin14. (C) Hematoxylin/eosin staining and confocal images of the stroma of a squamous cell carcinoma developed by a wild-type animal showing the absence of expression of Vimentin in the epithelial compartment of the tumor. Immunofluorescence staining shown correspond to Vimentin (green), YFP (grey), and Keratin14 (red). DOI: 10.7554/eLife.21697.012
**Figure 3.** Deletion of Dnmt3a results in increased tumor heterogeneity, and upregulation of genes related to lipid metabolism. (A) Schematic representation of FACS sorting strategy to isolate both RNA and DNA from Itga6pos cells within the tumors. (B) Heatmaps representing gene expression patterns. (C) PCA analysis showing the variance in gene expression. (D) Downregulated genes in Dnmt3a cKO tumors: 114 genes, and biological processes. (E) Upregulated genes in Dnmt3a cKO tumors: 277 genes, and signal transduction pathways.

**Table:**

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**Figure 3 continued on next page**
Figure 3 continued

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 RNA-seq samples from wild-type (n = 4) and Dnmt3a-cKO (n = 8) Itga6bright sorted tumor 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 wildtype and Dnmt3a-cKO animals.

DOI: 10.7554/eLife.21697.013
Figure 3—figure supplement 1. RNA samples submitted for sequencing were obtained from tumors scored predominantly as squamous cell carcinomas in wild-type and Dnmt3a-cKO mice. (A) Hematoxylin/eosin staining from the four wild-type tumors used for RNA-seq. (B) Hematoxylin/eosin staining from the four Dnmt3a-cKO tumors used for RNA-seq.
staining from the eight Dnmt3a-cKO tumors analyzed for RNA-seq. In A and B, immunofluorescence staining shown correspond to DAPI, ADFP (to ensure that no sebaceous adenomas were collected), and Krt14. (C) Representative hematoxylin/eosin staining and immunofluorescence of DAPI, ADFP and Krt14, of sebaceous adenomas eliminated from the RNA-seq study. Scale bar is 100 μm.

DOI: 10.7554/eLife.21697.014
Figure 3—figure supplement 2. Loss of Dnmt3a results in a reduction of apoptosis in skin tumors. (A) Representative images for TUNEL staining to detect apoptotic cells in skin tumors isolated from wild type and Dnmt3a-cKO animals. The right graph shows the quantification of the Tunel staining in Dnmt3a-cKO animals.

B

Percentage of Caspase Positive Cells Staining

WT

Dnmt3a cKO

*** p=0.0003

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Figure 3—figure supplement 2 continued

wild type (n = 12) and Dnmt3a-cKO (n = 17) tumors. (B) Representative images for active Caspase-3 staining to visualize apoptotic cells in skin tumors isolated from wild-type and Dnmt3a-cKO animals. The right panel shows the quantification of the staining in wild-type (n = 6) and Dnmt3a-cKO (n = 6) tumors. Scale bar is 100 μm.
DOI: 10.7554/eLife.21697.015

The following source data is available for figure 3:

Figure supplement 2—Source Data 1. Data related to Figure 3—figure supplement 2A–B.
DOI: 10.7554/eLife.21697.016
Figure 3—figure supplement 3. DMBA/TPA treatment induces an increase in cellular cell proliferation in Dnmt3a-cKO animals. (A) Representative images of Ki67 staining in treated or untreated back skin, and in skin tumors, of Dnmt3a-cKO and wild-type littermates. (B) Quantification of Ki67.
staining using the TMarker software, showing the percentages of Ki67-positive cells in the different conditions studied and normalized to the proliferation in the interfollicular epidermis of wild-type mice. Scale bar is 100 μm.

DOI: 10.7554/eLife.21697.017
**A**

Belly Skin Untreated

DMBA/TPA treated skin

Ki67

SCCs

WT

Dnmt3a cKO

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**B**

Percentage Proliferating Cells

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**C**

Days between appearance of first skin tumor and tumor collection

Free Tumor survival

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Rinaldi et al. eLife 2017;6:e21697. DOI: 10.7554/eLife.21697

Research article
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Figure 3—figure supplement 4. Dnmt3a-KO tumors express high levels of PPAR-γ. (A) CPM (Count per Million Read) values of the mRNA encoding for PPAR-γ obtained from the RNA-sequencing of the 12 tumors studied. (B) Representative immunofluorescence staining for DAPI, PPAR-γ and Krt14 in all the 12 tumors used for the RNA-sequencing experiment. Scale bar is 100 μm.
DOI: 10.7554/eLife.21697.018
The following source data is available for figure 3:

Figure supplement 4—Source Data 1. Data related to Figure 3—figure supplement 4B.
DOI: 10.7554/eLife.21697.019
Figure 4. Dnmt3a binds a subset of enhancers in tumor cells. (A) Schematic representation of a short treatment of DMBA/TPA in wild-type and Dnmt3a-cKO animals. (B) Genomic localizations of Dnmt3a determined by ChIP-seq of Dnmt3a in epidermal cells isolated from wild-type animals after 8 weeks old animals.

Enhancers bound by Dnmt3a (n=363)

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Figure 4 continued on next page.
Figure 4 continued

6 weeks of DMBA/TPA treatment. (C) Gene ontology analysis of the 363 H3K27ac-enriched regions (located at least 4 kb away from the TSS) also bound by Dnmt3a in isolated epidermis from wild-type animals after 6 weeks of DMBA/TPA. (D) Screenshot of enhancers bound by Dnmt3a in DMBA/TPA-treated skin in the FOS locus. All tracks are normalized to the number of mapped reads.

DOI: 10.7554/eLife.21697.020
Deletion of Dnmt3a alters the expression of genes involved in proliferation, lipid metabolism, epidermal differentiation, and Wnt signaling, after 6 weeks of DMBA/TPA treatment. (A) Left panel, heatmaps representing gene expression (rlog transformed.

**Figure 4—figure supplement 1 continued on next page**
values) of the 498 genes in sorted bulge hair follicle stem cells (Bulge) (Itga6\textsuperscript{bright}/CD34\textsuperscript{pos}) that were differentially expressed between wild-type (n = 3) and Dnmt3a-cKO (n = 3). Right panel, gene ontology analysis of the 498 differentially expressed genes up- or downregulated in Dnmt3a-cKO mice as compared to their wild-type littermates. (B) Left panel, heatmaps representing gene expression (log transformed values) of the 188 differentially expressed genes between wild type (n = 4) and Dnmt3a-cKO (n = 4) sorted interfollicular epidermal (IFE) basal cells (Itga6\textsuperscript{bright}/CD34\textsuperscript{neg}). Right panel, gene ontology analysis of the 188 differentially expressed genes that were up- or downregulated in Dnmt3a-cKO mice as compared to their wild-type littermates.

DOI: 10.7554/eLife.21697.021
Figure 5. Depletion of Dnmt3a leads to loss of DNA methylation and hydroxymethylation around its target enhancers. (A) Relative methylation score (CpG count) measured around 363 enhancers bound by Dnmt3a (−5 kb, +5 kb) from independent biological replicates of FACS sorted tumor cells from Rinaldi et al. eLife 2017;6:e21697. DOI: 10.7554/eLife.21697.

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Figure 5 continued

wild type (n = 2) and Dnmt3a-cKO (n = 2) (p<2.2 × 10^-16). (B) Relative methylation score (CpG count) measured around 2734 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, + 2 Kb) were quantified using HOMER software in independent biological replicates of FACS sorted tumor cells from wild-type (n = 2) and Dnmt3a-cKO (n = 2) mice 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 tumor cells.

DOI: 10.7554/eLife.21697.022
**A**

MEDIP seq intensity

Peaks
- Active Promoters n=2521
- All Promoters

Coverage Depth

Distance from TSS

**B**

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CG Count per Read
Theoretical Distribution

inputs

Rinaldi et al. eLife 2017;6:e21697. DOI: 10.7554/eLife.21697

Cancer Biology | Developmental Biology and Stem Cells
Figure 5—figure supplement 1. MeDIP-seq and hMeDIP-seq analysis 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 Itga6\textsuperscript{bright} tumor cells.

DOI: 10.7554/eLife.21697.023
Figure 6. Dnmt3a binds and methylates a subset of promoters of genes involved in lipid metabolism in DMBA/TPA-treated epidermal cells. (A) Relative methylation score (CpG count) measured around active and silenced promoters bound by Dnmt3a (−5 kb, +5 kb) from independent biological
Figure 6 continued

replicates of FACS-sorted tumor cells from wild type (n = 2) and Dnmt3a-cKO (n = 2) animals. (B) Relative methylation score (CpG count) measured around promoters 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=0.104). (C) CPM (Counts per Million) values of genes bound at the TSS by Dnmt3a in DMBA skin tumors from wild-type or Dnmt3a-cKO animals. (D) Gene ontology analysis, using Enrichr online software, of the 3521 genes bound at their promoter by Dnmt3a. (E) Screenshot of PPAR-γ gene, with all tracks normalized. (F) Normalized methylation score measured around TSS of Ppar-γ (~1 kb to +1 kb) bound by Dnmt3a. (G) CPM (Counts per Million) values of PPAR-γ measured by RNA-seq in sorted Itgad<sup>bright</sup> cells from DMBA/TPA-treated IFE and from DMBA skin tumors in wild-type and Dnmt3a-cKO mice. (H) Immunofluorescence staining for Krt14 and PPAR-γ of DMBA/TPA-treated skin and skin tumors from wild-type and Dnmt3a-cKO animals.

DOI: 10.7554/eLife.21697.024

The following source data is available for figure 6:

**Source data 1.** Data related to Figure 6G.

DOI: 10.7554/eLife.21697.025
Figure 7. PPAR-γ inhibition revert the tumor initiation phenotype of the Dnmt3a-cKO. (A) Schematic representation of the DMBA/TPA orthotopic treatment together PPAR-γ inhibitor (Sigma GW9662) treatment onto wild-type and Dnmt3a-cKO animals. (B) Time of appearance, expressed in days after first DMBA treatment. (C) Number of tumors per mouse after 120 days from first DMBA. (D) Tumor size comparison.
percentages of skin tumors on wild-type or Dnmt3a-cKO animals (vehicle and GW9662 treated): p=0.008, Chi-Square Test. (C) Number of skin tumors after 3 months of DMBA/TPA treatment plus GW9662 treatment, p=0.007 (Unpaired T-Test). (D) Tumors sizes expressed in millimeters (mm) after 3 months of DMBA/TPA plus GW9662 treatment.

DOI: 10.7554/eLife.21697.026

The following source data is available for figure 7:

Source data 1. Data related to Figure 7C.
DOI: 10.7554/eLife.21697.027
Cutaneous Skin Cancer data from GSE2503

Cutaneous Skin Cancer data from GSE45164

Cutaneous Skin Cancer data from GSE42677

Cutaneous Skin Cancer data from GSE53462

Rinaldi et al. eLife 2017;6:e21697. DOI: 10.7554/eLife.21697
The mRNA of Dnmt3a is downregulated in human cutaneous squamous cell carcinomas compared to normal human epidermis. mRNA expression of Dnmt3a in human healthy epidermis compared to actinic keratoses and Squamous Cell Carcinomas (SCC) quantified using GEO2R platform of the published databases (GSE2503, GSE42677, GSE45164, and GSE53462). Unpaired parametric T-Test was used for statistics.

DOI: 10.7554/eLife.21697.028

The following source data is available for figure 7:

Figure supplement 1—Source data 1. Data related to Figure 7—figure supplement 1.
DOI: 10.7554/eLife.21697.029