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The rhythms of histones in regeneration: The epigenetic modifications determined by clock genes

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Abstract

The evolutionary establishment of an internal biological clock is a primordial event tightly associated with a 24-h period. Changes in the circadian rhythm can affect cellular functions, including proliferation, DNA repair and redox state. Even isolated organs, tissues and cells can maintain an autonomous circadian rhythm. These cell-autonomous molecular mechanisms are driven by intracellular clock genes, such as BMAL1. Little is known about the role of core clock genes and epigenetic modifications in the skin. Our focus was to identify BMAL1-driven epigenetic modifications associated with gene transcription by mapping the acetylation landscape of histones in epithelial cells responding to injury. We explored the role of BMAL1 in epidermal wound and tissue regeneration using a loss-of-function approach in vivo. We worked with BMAL1 knockout mice and a contraction-resistance wound healing protocol, determining the histone modifications using specific antibodies to detect the acetylation levels of histones H3 and H4. We found significant differences in the acetylation levels of histones in both homeostatic and injured skin with deregulated BMAL1. The intact skin displayed varied acetylation levels of histones H3 and H4, including hyperacetylation of H3 Lys 9 (H3K9). The most pronounced changes were observed at the repair site, with notable alterations in the acetylation pattern of histone H4. These

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AUTHOR CONTRIBUTIONS

RMC and CHS were involved in study design and securing funding. EJDS and CCSB were involved in experiments. CCSB, RMC and CHS were involved in manuscript draft and figures. CCSB, EJDS, MCB, RMC and CHS were involved in reviewing and editing.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest with the subject matter or materials discussed in the manuscript.

findings reveal the importance of histone modifications in response to injury and indicate that modulation of BMAL1 and its associated epigenetic events could be therapeutically harnessed to improve skin regeneration.

Keywords

epidermis; histone; repair; wound

1 | INTRODUCTION

The circadian clock genes drive the cyclic rhythm, maintaining an internal clock with strong ties to the overall health of an organism. Clock genes determine behavioural, physiological and biochemical functions. Clock genes were initially identified in the 90s. Later on, brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1) and CLOCK were shown to be main partners, becoming the master genes of the circadian clock. BMAL1 and CLOCK are members of the activatory feedback loop of the molecular clock and promote the expression of the negative arm of the molecular clock genes, such as period (PER1, PER2 and PER3) and cryptochrome (CRY1 and CRY2).¹

BMAL1 (also known as ARNTL or MOP3) and other clock genes are active in most cells of the body, and in many instances, BMAL1's function is independent of light.² The same set of clock genes maintains the cell-autonomous cyclic rhythms found in individual cells, tissues and organs, as these genes control genome-wide expression.^{3–5} The circadian genes, BMAL1 and CLOCK, are key regulators that promote chromatin modifications, leading to remodelling and transcriptional outputs.^{5–8}

A variety of functional studies on BMAL1 have shown that it plays a crucial role as a key player in tissue homeostasis and premature aging. Indeed, dysfunctional BMAL1 expression is associated with age-related pathologies, including cataracts, skeletal dysplasia, sarcopenia, poor reproduction and infertility.^{9–11} A lack of BMAL1 in the epidermis also results in dysfunctional stem cells, delayed healing, disrupted hair growth and an increased predisposition to cancer.^{11–16} Although there are reports showing that BMAL1 affects epithelial homeostasis and regeneration, little is known about the impact of BMAL1 dysfunction on epigenetic modifications, particularly in the epidermis.

Here, we determined the histone modifications present during homeostasis and revealed changes in the acetylation profile of histones in response to injury upon *Bmal1* deletion. We demonstrated that epigenetic modifications of histones H3 and H4 were evident in the skin of *Bmal1* mutant mice during homeostasis and that further changes in the acetylation status of histones H3 and H4 occurred in response to injury.

2 | MATERIALS AND METHODS

2.1 | Experimental mice and wound healing assay

Paraffin-embedded tissue specimens were obtained from *Bmal1* Knockout mice ($n = 5$, male, *Bmal1*^{-/-}, B6.129-Arntltm[1]Bra/J, Jackson Laboratory, Bar Harbor, ME, USA) and

wild-type (WT) mice ($n = 4$, male, control mice, colony 000664 C57BL/6J, Jackson Laboratory, Bar Harbor, ME, USA). The tissues were from full-thickness wounds created using a 5-mm-circular punch biopsy tool on the dorsal skin of 4–6-week-old mice. Silicone rings were affixed around the wound using interrupted sutures to prevent wound contraction. The wound, along with the wound bed, margins and adjacent skin tissue, was excised on post-operative Day 5.¹² Standard water and rodent chow were provided ad libitum in compliance with the American Association for Accreditation of Laboratory Animal Care (AAALAC) guidelines. Mice were housed in 12-h light/dark cycles and were observed daily by the investigators and animal husbandry staff. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.

2.2 | Immunofluorescence staining

Histological sections (5–8 μm) were prepared for immunofluorescence staining. The skin samples were collected at a standardized Zeitgeber time 2 (ZT2), when *Bmal1* is expressed in the WT mice and absent (due to the genetic deletion of the gene) in the test group *Bmal1*^{-/-} mice.^{2,17,18} Sections were deparaffinized using a xylene substitute solution (Safe Clear II) and then rehydrated through a descending ethanol series. Antigen retrieval was achieved using a citric acid buffer (pH 6). Unspecific binding was blocked using 3% bovine serum albumin (BSA) in 0.1% Triton phosphate-buffered saline (PBS). The tissue sections were incubated overnight with primary antibodies against Acetyl-Histone H3 (Lys 9; C5B11, Cell Signaling Tech, Danvers, MA); Acetyl-Histone H3 (Lys 27; 81735, Cell Signaling Tech, Danvers, MA); Acetyl-Histone H4 (Lys 5; PA5–40085, Invitrogen, Carlsbad, CA, USA); Acetyl-Histone H4 (Lys 8; 9HCLC, ThermoFisher Scientific); and Acetyl-Histone H4 (Lys 16; 135345, Cell Signaling Tech, Danvers, MA). Alexa Fluor 488 (A11034, Invitrogen, Carlsbad, CA, USA) was utilized as the secondary antibody, while Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) was employed for nuclear staining. Images were captured from at least three independent fields or regions of interest (ROIs) using a $\times 40$ objective on a Nikon Eclipse 80i microscope equipped with a QImaging ExiAqua camera (Nikon, Melville, NY, USA).

2.3 | Morphological analysis and histone assessment

Histological analysis was conducted on slides stained with Haematoxylin and Eosin (H&E). A morphological quantitative analysis was performed to investigate the hair follicles' growth cycle by an experienced pathologist. Tissue zones were identified as the epidermis, dermis, wound margin, migratory tongue and wound bed.¹⁹ Photomicrographs of the tissue samples were used to quantify cells positive for histone acetylation sites. The results are represented as the histone labeling index, defined as the percentage of histone-positive cells.²⁰ This is calculated by dividing the number of positive cells (in green) by the total number of cells (in blue), then multiplying by 100. Cell counting was conducted in up to nine independent fields within the epidermis using the ImageJ software.²¹ Radial and sunburst graphics displaying changes in histone acetylation levels were generated with the Flourish studio software (Kiln Enterprises Ltd, London, UK).

2.4 | Statistical analysis

The statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). *t*-Test was performed to determine the significant difference between the mean values from each dataset and the two experimental groups. The differences were considered statistically significant for *p*-value of $p \leq 0.05$. Asterisks denote statistical significance (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Data are expressed as mean $\pm$ SEM.

3 | RESULTS

3.1 | Bmal1 influences epigenetic modifications of histones H3 and H4 in the skin

The circadian clock genes play a key role in physiological processes in the skin. It can regulate skin homeostasis, hair follicle cycles and skin repair. As an example, we have reported that *Bmal1*^{-/-} mice displayed the delayed progression of the hair cycle (Figure S1A,B) as well as delayed skin wound healing.^{12,17} The circadian machinery has been also implicated in the modulation of transcription, a fundamental biological process that requires chromatin remodelling. To determine whether *Bmal1* modulates epigenetic changes associated with chromatin remodelling, we analysed the histone acetylation profile of *Bmal1* knockout animals. Tissues from the *Bmal1* knockout mice were compared with control mice (with *Bmal1*). We first examined the histone acetylation profile of the epidermal cells of the skin under homeostasis (Figure 1A). We found that *Bmal1* ablation displayed significant changes in the acetylation profile of the histones (Figure 1A–F). The quantitative analysis indicated a significant hyperacetylation of histone H3 Lysine 9 (Lys9 or H3K9) on *Bmal1*^{-/-} samples (43.11 ± 3.11) compared with the controls (14.76 ± 1.73 ; Figure 1B, $p \leq 0.0001$). Acetylation of H4 Lysine 8 (Lys8 or H4K8) followed a similar increasing pattern in the *Bmal1*^{-/-} mice (29.63 ± 4.14 ; controls 13.45 ± 1.29 ; Figure 1E, $p = 0.0002$). Conversely, deacetylation of H4 Lysine 16 (Lys16 or H4K16) occurred in the *Bmal1*^{-/-} cells (8.99 ± 1.41), compared with the controls (15.16 ± 1.15 ; Figure 1F, $p = 0.005$). No significant changes were found in the acetylation of histones H3 Lysine 27 (Lys27 or H3K27) and H4 Lysine 5 (Lys5 or H4K5; Figure 1C,D). Together, these results indicated that deregulation of the *Bmal1* gene exerts control over the histone acetylation in skin homeostasis, mainly on histones H3K9, H4K8 and H4K16 (Figure 1G).

3.2 | Acetylation of histone 4 is a significant event in injured skin lacking Bmal1

We and others determined that *Bmal1* is a key regulator of tissue regeneration^{12,22–29}; consequently, we asked whether *Bmal1* modulates the histone changes during skin repair (Figure 2A). We found that *Bmal1* ablation mostly affected histone H4 modifications (Figure 2B,C). Notably, we showed hyperacetylation of histone H4 at Lys 5 (45.07 ± 3.57), Lys 8 (15.92 ± 2.64) and Lys 16 (19.70 ± 1.34) on the injured skin of *Bmal1*^{-/-} mice, compared with the controls under the same conditions (Figure 2B, * $p \leq 0.05$ and **** $p \leq 0.0001$). We also showed that the acetylated histones (green stain) were present in the nucleus (Figure 2C). Since we found that *Bmal1* ablation mainly affected histone H4 modifications, we asked whether *Bmal1* modulates the histone changes in the dermis and wound bed. We showed that *Bmal1*^{-/-} mice presented a significant decrease in the acetylation levels at histone H3 at Lys 27 (42.79 ± 2.83) and histone H4 at Lys 8 (38.23 ± 3.06), and hyperacetylation of histone H4 at Lys 5 (51.88 ± 1.28) in the dermis (Figure

S2A). In contrast, we found hyperacetylation of histone H4 at Lys 5 (26.48 ± 1.54) and Lys 16 (35.59 ± 2.02) in the wound bed of *Bmal1*^{-/-} mice (Figure S2B, $**p \leq 0.01$ and $****p \leq 0.0001$).

3.3 | Hyperactivation of H4 is present in the *Bmal1*^{-/-} wound margins and newly formed epithelia

The reepithelization of the wound starts at the wound margins, which are sources of keratinocytes that will recover the wound (Figure 3A). Interestingly, we found that modulation of histones in the wound margin is highly affected by Bmal1. The cells at the wound margin displayed hypoacetylation of histone H3 Lys 9 ($*p \leq 0.05$), while histone H3 Lys 27 remains unaffected by *Bmal1* deletion (Figure 3B,C). Notably, acetylation of histone H4 increased in the injured skin depleted of *Bmal1* (Figure 3D–F), particularly the significant changes seen in H4 Lys 8 and 16 ($***p \leq 0.001$; Figure 3G). Next, we analysed the migratory epithelial tongue (Figure 3H). In the skin wound healing process, the epithelial cells from the wound margin proliferate and undergo a migration stage morphologically characterized as an epithelial projection, known as a migratory tongue, over the wound bed. The newly recovering epithelia present in controls and *Bmal1*^{-/-} mice displayed no differences for acetylation of Histones H3 Lys 9 and 27 and H4 Lys 8 and H4 Lys 16 (Figure 3I–M). Significant changes were also found at H4 Lys 5 (Figure 3K), which was hyperacetylated in the migratory tongue recovering the wound of *Bmal1*^{-/-} mice ($****p \leq 0.0001$; Figure 3N).

Overall, we found that modulation of histones H3 and H4 is dependent on Bmal1. The injured skin without Bmal1 exhibited hyperacetylation at histone H3 Lys 9. Moreover, the repair process in skin lacking Bmal1 significantly increased acetylation at histones H4 Lys 5 and 16 while decreasing it at histone H3 Lys 9. More specifically, the proliferative wound margin showed changes in acetylation of H3 Lys 9 and H4 Lys 16. In contrast, the migratory epithelia displayed modulation at histone H4 Lys 5 (Figure 4A,B).

4 | DISCUSSION

The genetic landscape of a cell is constantly responding to the environment and internal cues such as circadian oscillations. The circadian oscillation occurs in rhythm intervals along with the 24-h cycle, and self-sustained biological oscillators maintain it. The molecular mechanism maintaining the oscillations is an endogenous interlocking feedback loop among the clock genes BMAL1/CLOCK complex and clock repressors (*PER*, *CRY1* and *REV-ERB*).³⁰

Research on circadian clock genes has uncovered cell-autonomous circadian cycling in various peripheral tissues.^{3,4,5,31} The oscillations of these molecular and cellular clocks persist in peripheral tissue and dissociated cells, even when removed from the body. These rhythms are maintained by transcriptional feedback loops driven by the core clock components, BMAL1 and CLOCK.^{32–36} Moreover, when Bmal1 is deleted in specific tissues, the time-of-day-dependent synchronicity is lost, leading to increased cell proliferation and ROS production. This also affects skin sensitivity, particularly to UV-induced DNA damage.¹⁸ Beyond its role in managing peripheral tissue physiology,

Bmal1 governs skin cell differentiation, hair growth, stem cell quiescence and premature tissue ageing.^{1,2,14,16,37–41} Notably, skin-specific expression of BMAL1 influences several gene families tied to epidermal differentiation, along with alterations in the expression of CDKN1A, NR1D1 and c-Myc.^{2,41}

Follicular melanocytes synthesize melatonin, which may also play a role in this process. Previous studies have shown that BMAL1 binds to and regulates the promoter region of MITE, consequently modulating melanin synthesis.⁴² Melanin synthesis is linked to the anagen phase of the hair cycle, ceases during the catagen phase and is absent throughout the telogen stage.⁴³ Furthermore, there is evidence that melatonin assists in regulating skin pigmentation and the hair cycle due to its effects on melanin production. Indeed, melatonin has been demonstrated to be involved in skin regeneration, regulating ROS production, hair follicle growth and pigmentation.^{44–46} Additionally, while the serotonergic and melatoninergic systems are defective in C57BL/6 mice, this model remains relevant for investigating hair growth and pigmentation. Considering the many relevant studies that show changes in melanin and its partners in the skin correlate with the hair follicle cycle.^{17,43,47–50} and given that the BMAL1 gene plays a role in hair cycle progression, its function within the melatonin, serotonergic and melatoninergic systems presents avenues for future investigations.

While many studies have demonstrated that gene outputs largely adhere to circadian rhythms, the specific expression of coding genes often varies depending on the organ. This organ-specific expression has been observed in the skin, liver, kidney and lungs. Some of these organs exhibit more pronounced amplitude and levels of gene expression compared to tissues with less regenerative potential, such as muscles. Additionally, specific brain compartments such as the cerebellum and hypothalamus also have lower expression amplitude.^{31,51} Interestingly, these oscillations in transcriptomics are linked with epigenetic modifications that influence permissive chromatin states.

Acetylation of lysine residues in histone tails is a major post-translational modification that induces a transcription-permissive nucleosome conformation. This alters chromatin condensation and the availability of genes for transcription. These pivotal epigenetic events are often associated with modifications to histones 3 and 4.^{36,52–54} In this context, we present key findings from the realm of epithelial biology and regeneration. Using a model organism, we demonstrated that changes in histones 3 and 4 are influenced by the *Bmal1* clock gene during homeostasis, specifically affecting acetylation at histones H3 Lys9 and H4 Lys8, but not H3 Lys27. In *Bmal1* mutant mice, a more extensive set of changes manifests in response to injury. We found that the skin of *Bmal1* mutant mice exhibits impaired wound healing, marked by increased acetylation of H4 Lys 5 in the recovering epidermis and H4 Lys 16 in the wound margin. Additionally, the wound margin showed deacetylation of histone H3 Lys9. In line with our results highlighting the slow skin repair process, other researchers have reported a delayed impact of *Bmal1* on the regeneration of muscle, post-stroke brain recovery, intestines, and other tissues and organs.^{12,55–58} On the other hand, these findings indicate the potential therapeutic application of HDAC inhibitors to enhance wound treatment.

HDAC inhibitors lead to histone deacetylation; therefore, HDACi affecting histones H3 and H4 is particularly interesting. For instance, the HDACi valproic acid has been shown to increase the acetylation of histones H3 and H4, which is also known to play a role in promoting recovery from spinal cord and traumatic brain injuries.^{59,60} This idea is aligned with previous studies from our laboratory demonstrating that the healing and regeneration of skin are associated with a gradual deacetylation of histone H4 at Lysines 5, 8 and 16.^{61,62} Furthermore, therapies such as phototherapy applied to the skin and mucosa have been found to augment epithelial repair and induce histone H3 Lys 9 hypermethylation.^{63,64}

The small number of animals in each group can be considered a limitation in this study, which may reduce the statistical power. A larger sample size might have yielded smaller margins of error and more pronounced statistical differences between the two groups. Despite these limitations, our findings indicate the importance of histone modifications in response to injury and how modulation of BMAL1 and epigenetics could be therapeutically harnessed with HDACi to improve the regeneration of the skin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article.

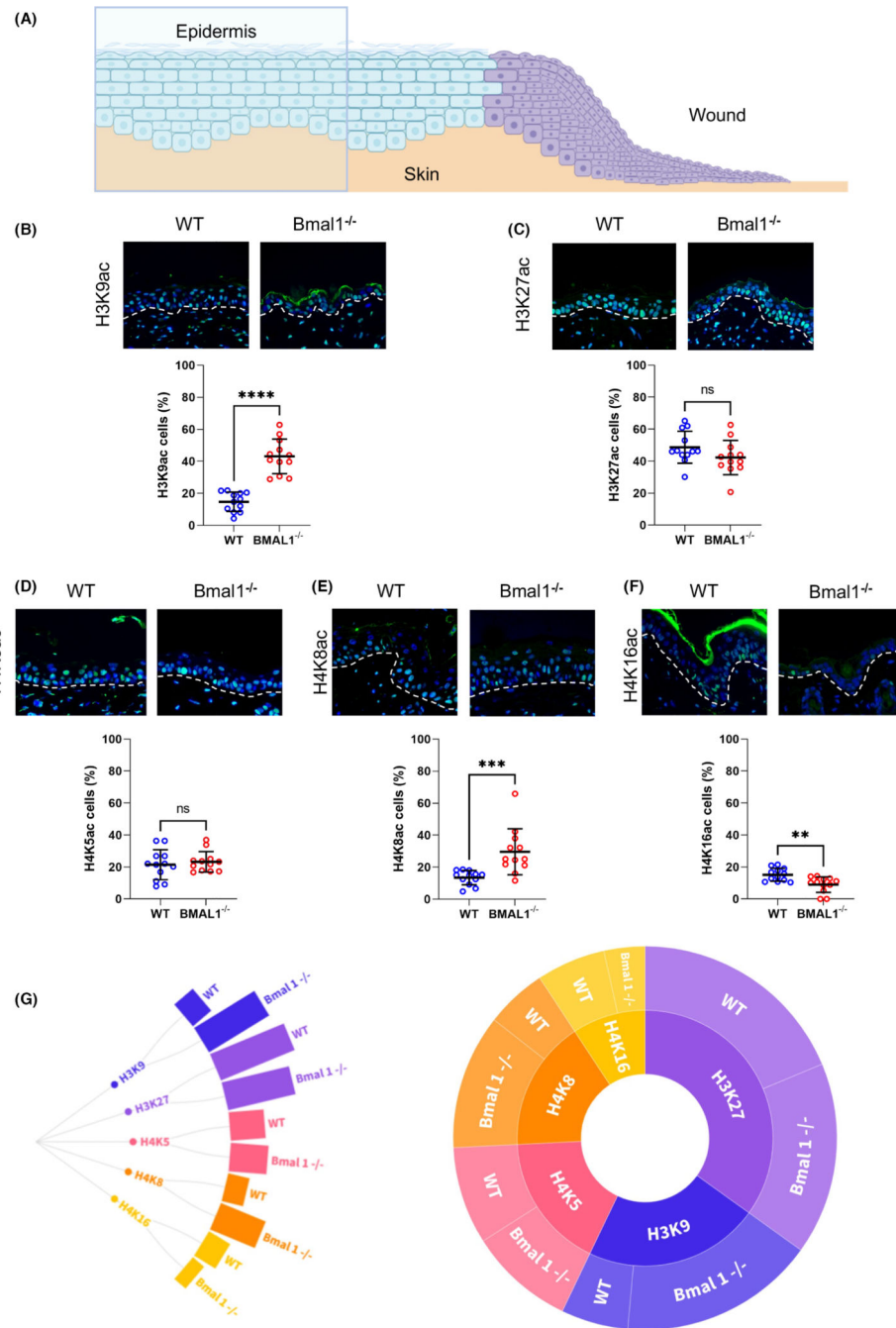
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**FIGURE 1.**

Bmal1 affects Histones H3 and H4 acetylation in the Epidermis. (A) Schematic representation of epidermis. The blue box highlights the uninjured epidermis, which displayed histone modifications. (B) The absence of the *Bmal1* gene induces significant hyperacetylation of histone H3 Lys 9 (H3K9). (C, D) *Bmal1*^{-/-} and wild-type mice show similar acetylation levels in the histones H3 Lys27 (H3K27) and H4 Lys5 (H4K5), and (E, F) significant hyperacetylation of H4 Lys8 (H4K8) and Lys 16 (H4K16) in the *Bmal1*^{-/-} mice (ns >0.05, **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001, *****p* ≤ 0.0001; Data showed

as percentage mean $\pm$ SEM, $n = 12$ ROI per group). Representative immunofluorescence images of acetylation levels of histones H3 and H4 are placed above each graph – Histone acetylation is found in the nucleus-green stain [Alexa Fluor 488]. Hoechst 33342 is used for nuclear counterstain. The dashed line delineates the epithelium. (G) Radial (left) and sunburst (right) representation of histone acetylation levels throughout the normal epidermis of control and *Bmal1*^{-/-} mice.

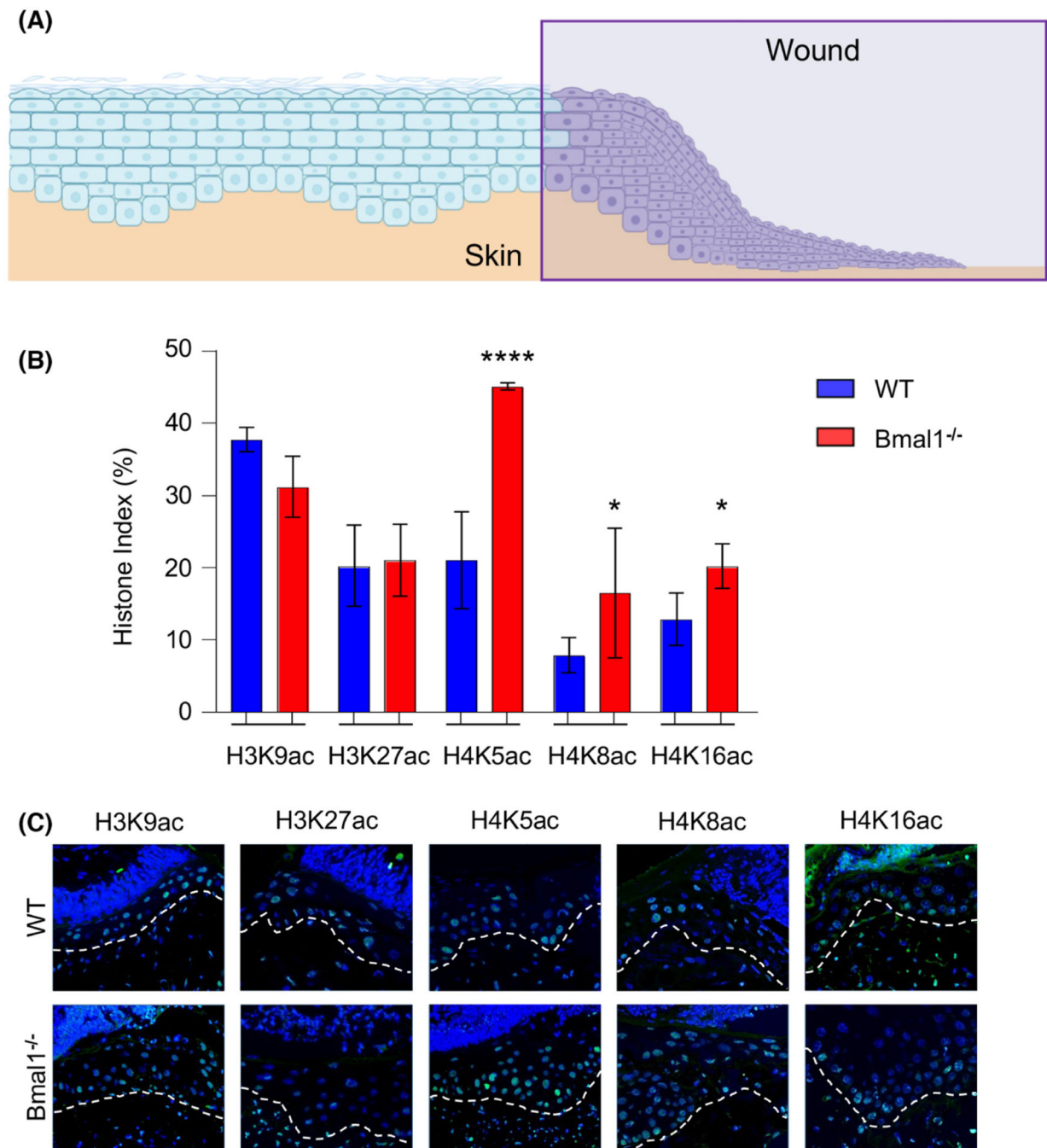


FIGURE 2.

Acetylation profile of histones H3 and H4 in the wound. (A) Schematic representation of the wound area is shown in the box. (B) Acetylation levels of the histone H3 at Lys 9 (H3K9) and Lys 27 (H3K27) are similar in the wild-type and *Bmal1*^{-/-} groups. Histone H4 also shows a significant increase of acetylation at Lys 8 (H4K8) and Lys 16 (H4K16) in the *Bmal1*^{-/-} group (ns > 0.05, **p* ≤ 0.05, *****p* ≤ 0.0001; Data showed as percentage mean ± SEM, *n* = 19–25 ROI per group). (C) Microphotographs show representative immunofluorescence images of histone acetylation profiles. Histone acetylation is in green

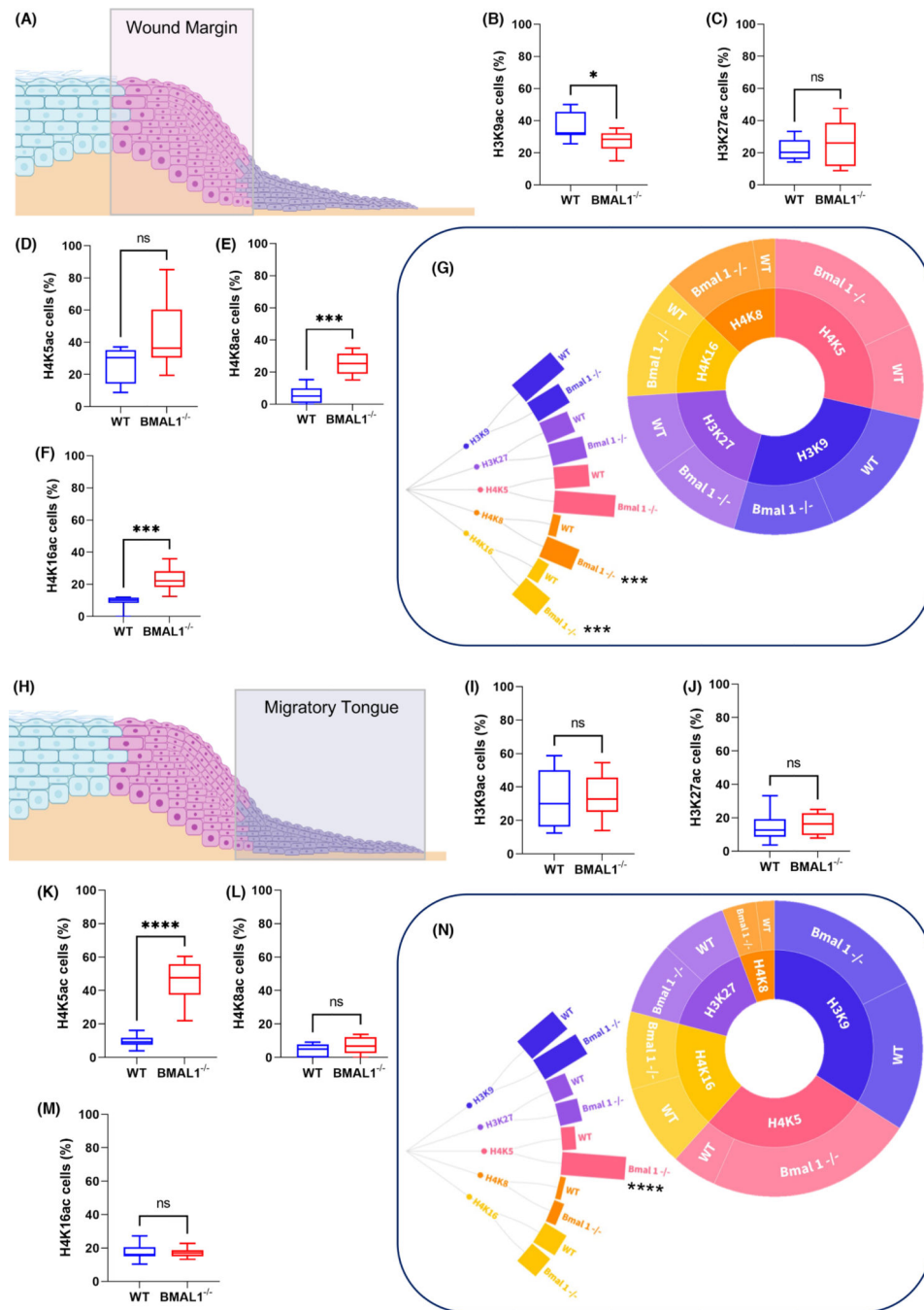
[Alexa Fluor 488], and counterstain is in blue [Hoechst 33342]. The dashed line delineates the epithelium.

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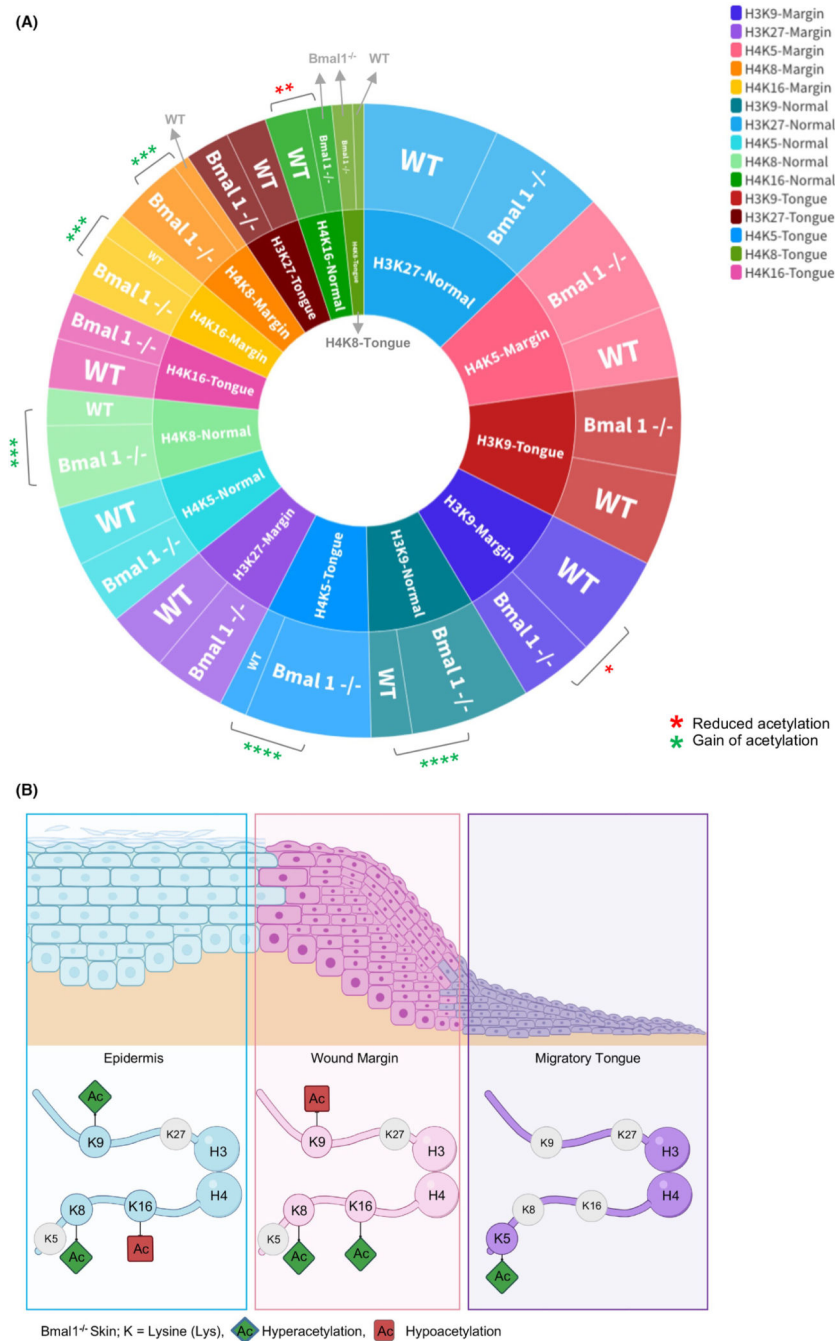
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**FIGURE 3.**

Histones H3 and H4 acetylation levels in the proliferative wound margin and (re)epithelialization. (A) Schematic representation of the wound margin in the wound area is highlighted in the box. (B) Histone H3 shows a significant decrease in Lys 9 acetylation (H3K9). (C, D) H3 Lys 27 (H3K27) and H4 Lys 5 (H4K5) presented similar acetylation levels in the wild-type and *Bmal1*^{-/-} mice; (E, F) H4 Lys 8 (H4K8) and Lys 16 (H4K16) are significant hyperacetylated in the *Bmal1*^{-/-} mice (ns > 0.05, **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001, *****p* ≤ 0.0001; Data shows the percentage mean ± SEM, *n* = 8–11 ROI

per group). (G) Radial (left) and sunburst (right) representation of histone acetylation levels throughout the wound margin of control and *Bmal1*^{-/-} mice. (H) Schematic representation of the migratory epithelial tongue forming the new wound recovering epithelia. (I, J) Histone H3 Lys 9 (H3K 9) and Lys 27 (H3K27) showed similar acetylation levels between the wild-type and *Bmal1*^{-/-} mice. (K) Histone H4 at Lys 5 (H4K5) is the only one to show a significant difference in the acetylation through the *Bmal1* gene modulation. (L, M) Histone H4 Lys 8 (H4K8) and Lys 16 (H4K16) express the same level of acetylation in both groups (ns >0.05, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; Data shows the percentage mean $\pm$ SEM $n = 9-12$ ROI per group). (N) Radial (left) and sunburst (right) representation of histone acetylation levels throughout the migratory epithelial cells (tongue) of control and *Bmal1*^{-/-} mice.

**FIGURE 4.**

BMAL1 modulates the acetylation landscape of epithelial cells responding to injury. (A) Global sunburst representation of all histone acetylation levels distributed throughout the normal epidermis as well as the wound margin and migratory epithelial cells sorted by the highest to the lowest acetylation values. Each cell size is determined by the mean percentage of positive cells according to each anatomical location of control and *Bmal1*^{-/-} mice ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$; Data shows the percentage mean $\pm$ SEM $n = 9-12$ ROI per group). (B) The absence of *Bmal1* leads to hyperacetylation of histone H3 at

Lys 9 (H3K9) and H4 at Lys 8 (H4K8), as well as hypoacetylation of histone H4 at Lys 16 (H4K16) in the epidermis under homeostasis. Upon injury, there is deacetylation of H3K9 and hyperacetylation of H4K8 and H4K16 in the proliferative wound margin. The histone H4K5 acetylation is a significant modification in the slower migratory tongue. In this way, the acetylation pattern may reduce proliferation and increase migration of the epithelial cells in this area.