UNIVERSITY OF CALIFORNIA, SAN DIEGO

Epigenetic Control of Skin Inflammation by Commensal Microbes

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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2017

The Dissertation of James Asbury Sanford is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2017

DEDICATION

To the two most important women in my life—

my mother, who instilled in me at an early age a passion for science and a dedication to never quit learning;

and my wife, who has never wavered in her support of me throughout my time spent as a graduate student.

Without both of you, I would not be the person I am today.

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LIST OF ABBREVIATIONS

AD—Atopic dermatitis

AMP—Antimicrobial peptide

ATAC—Assay for transposase-accessible chromatin

CAMP—Cathelicidin antimicrobial peptide

ChIP—Chromatin immunoprecipitation

DAMP—Danger-associated molecular pattern

FFA—Free fatty acid

FFAR—Free fatty acid receptor

HAT—Histone acetyltransferase

hBD—Human beta-defensin

HDAC—Histone deacetylase

HDACi—Histone deacetylase inhibitor

IL—Interleukin

MALP-2—Macrophage activating lipopeptide 2

NES—Nuclear export signal

NHEK—Normal human epidermal keratinocyte

NLR—NOD-like receptor

NLS—Nuclear localization signal

NOD—Nucleotide-binding oligimerization domain

P. acnes—*Propionibacterium acnes*

PAMP—Pathogen-associated molecular pattern

Poly(I:C)—Polyinosinic:polycytidylic acid

PRR—Pattern recognition receptor

PTM—Post-translational modification

SC—Stratum corneum

SCFA—Short-chain fatty acid

SPF—Specific pathogen-free (mice)

TLR—Toll-like receptor

TNFα—Tumor necrosis factor α

TSA—Trichostatin A

TSLP—Thymic stromal lymphopoietin

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ABSTRACT OF THE DISSERTATION

Epigenetic Control of Skin Inflammation by Commensal Microbes

by

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Doctor of Philosophy in Biomedical Sciences

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The skin serves a vital role as our body's first line of defense against the external environment and the potentially pathogenic microbes we encounter. However, abundant, diverse communities of microbial species colonize the skin shortly after birth and evolve with us over time. Thus, the skin exists in a unique immunological state, where it must tolerate and avoid reacting to these resident microbes under normal conditions while remaining poised to respond rapidly and effectively at the first sign of danger. To accomplish this feat, tight regulation of the inflammatory and immune processes present in the skin is crucial.

This dissertation begins with a description of the processes relevant to the maintenance of immunological homeostasis in the skin. In particular, the

complex network of immune functions present in the skin is discussed, with a focus on the innate immune processes active in the epidermis and the role of keratinocytes in host defense. Additionally, we introduce the concept of epigenetic regulation of gene expression and discuss the roles of histone modifications, chromatin structure, and regulatory nuclear factors. The skin microbiome is described in detail, as are the impacts of these skin-resident microbes on cutaneous immunological processes and the consequences that can arise when their composition is altered.

Next, the hypothesis that the microbiome can influence epigenetic regulation of inflammatory gene expression in keratinocytes is explored. This work describes how a particular member of the skin microbiome can generate molecules that inhibit histone deacetylase (HDAC) activity, which has distinct effects in different cell types of the cutaneous innate immune system—specifically, an enhancement of TLR-mediated proinflammatory gene expression in keratinocytes. Furthermore, HDAC8 and HDAC9 are identified as key regulators of inflammatory gene expression in keratinocytes, as depletion of either of these enzymes greatly enhances the response to TLR activation.

Taken together, these results enhance our understanding of the impact of resident microbes on the immunological functions of the skin, and identify a mechanism by which tolerance to these microbes can be broken. This work advances our knowledge of the host-microbe interactions occurring at epithelial surfaces, and introduces a novel way of thinking about the mechanisms underlying the initiation and pathogenesis of inflammatory skin conditions.

Chapter I:

Introduction

Skin is the human body's most exposed organ, responsible for providing a barrier against the external environment that can resist a wide variety of insults and effectively respond to penetrating dangers (*1*). Not surprisingly, the skin is home to a robust and potent collection of immunological processes that serve to keep deeper tissues free from the adverse effects of the invasion of microbial and environmental challenges (*2*). However, the skin also hosts dense, diverse communities of microorganisms that reside on and within the skin and its appendages—a collection of inhabitants commonly referred to as the skin microbiome. Under normal conditions, these resident microbial communities exist without eliciting an inflammatory response from the host. As such, cells that compose the skin are tasked with the unique challenge of maintaining an intimate relationship with resident microbes without inducing unwarranted inflammation, while remaining poised to quickly and efficiently respond in times of danger. Thus, great importance is placed on the mechanisms that regulate expression of inflammatory genes in the skin, as failure to properly control and temporally induce these processes can result in immune-mediated inflammatory skin conditions or a skin environment that is prone to infection.

Within each human cell, the entire genomic content—approximately 3 billion nucleotide base pairs, which would reach roughly 2 meters in length when fully elongated—must be packaged into the nucleus, a small space with a diameter of a few microns. In order to achieve such a feat, the DNA must be compacted into higher order chromatin structures, ultimately resulting in the dense chromosomes visible during cell division. The simplest form of this

chromatin involves the formation of nucleosomes—small lengths of DNA, approximately 147 base pairs, wrapped around octamers of histone proteins, consisting of two copies each of the four core histone proteins (H2A, H2B, H3 and H4) (*3, 4*). These nucleosomes are often referred to as the "beads on a string" that are then further compacted and folded into dense fibers and loops. One can begin to imagine how the wrapping, folding, and compacting of DNA can greatly affect the accessibility of individual regions to transcriptional machinery necessary for gene expression to occur. Indeed, an entire field of study, termed "epigenetics," has emerged to investigate how changes to the chromatin structure can result in heritable, cell-type specific effects on gene expression without changing the underlying genetic sequence. More recently, the definition of epigenetics has expanded to include mechanisms that are not necessarily heritable, but are still functionally linked to transcriptional activity and distinct from the DNA sequence itself (*5*).

Among the most well studied aspects of epigenetic control of gene transcription are covalent modifications added to the DNA nucleotides or histone proteins around which DNA is wrapped (*6*). DNA can be modified by the addition of methyl groups to cytosine residues (DNA methylation), while histone proteins can have a variety of chemical groups added to amino acid residues, such as methylation, acetylation, phosphorylation, or ubiquitination (collectively termed histone post-translational modifications, or PTMs). In general, these modifications affect gene expression through two major mechanisms: first, they are important in influencing the interactions between the DNA and histone

proteins, as well as neighboring nucleosomes, ultimately affecting the higher levels of chromatin structure; second, these modifications can be sensed by distinct protein domains and affect the recruitment of transcriptional and regulatory machinery to specific regions of the genome. Modification of histones and DNA are highly dynamic processes, controlled by distinct sets of enzymes that catalyze their addition and removal, which are constantly being influenced by, and integrating signals from, the extracellular environment.

The project around which this dissertation is based began with a simple question: how might microbes residing on the skin affect epigenetic processes that regulate cutaneous immune functions? Our interest in this question began with an earlier observation that showed that expression of cathelicidin, a prominent antimicrobial peptide (AMP) in the skin and a key component of the cutaneous innate immune system, could be regulated in keratinocytes by DNA methylation in its promoter region (*7, 8*). This finding led us to wonder what other epigenetic mechanisms may be active in regulating the expression of AMPs, cytokines, chemokines, and other proinflammatory genes in keratinocytes, and how signals or products from the microbiome might affect these regulatory processes.

While a number of studies have previously identified key roles for epigenetic regulation of gene expression in keratinocytes, we were surprised to see that very few of these findings pertained to the role of keratinocytes in immunity—rather, the majority of this work was focused around the control of epidermal differentiation. However, within the field of immunology, the concept of

epigenetic regulation of inflammatory processes has recently become the focus of more in-depth study. In particular, the roles of histone acetylation and histone deacetylase enzymes in the control of immunity and inflammation have come to light as potentially important immune regulatory mechanisms. Histone acetylation, a modification first described in 1964 (*9*), occurs on the side chains of lysine residues found in the core, globular domains of histone proteins as well as their amino terminal tails. The balance of histone acetylation is controlled by the opposing activities of two classes of enzymes: histone acetyltransferases (HATs), which catalyze the addition of acetyl groups using acetyl-CoA as a substrate, and histone deacetylases (HDACs), which remove these chemical modifications. Acetylation of lysine residues masks the positive charge on these amino acids, which can reduce the electrostatic interaction between histones and negatively charged DNA; in general, histone acetylation is associated with the more loosely packed euchromatin, while deacetylated histones promote the more condensed heterochromatin. Additionally, acetylated lysine residues can be recognized by specific protein motifs, termed bromodomains, which are found in a wide variety of effector proteins. In this way, acetylation of histones can serve as a molecular docking point for other proteins to be recruited to regions of the genome.

Histone acetylation is generally accepted to be associated with a looser, more accessible chromatin structure and active gene transcription. Fittingly, inhibition of the enzymes that catalyze the removal of this modification from histone protein lysine residues has been shown to lead to a hyperacetylated

chromatin state (*10*), which would presumably result in overall higher levels of gene expression. Thus, it is somewhat surprising to note that a majority of the literature around the effects of HDAC inhibition on immune cells demonstrates a suppressive effect on the expression of proinflammatory genes, resulting in an overall anti-inflammatory phenotype (*11-18*). Importantly, a potent class of HDAC inhibitors is short-chain fatty acids (SCFAs)—small molecules generated by microbes through anaerobic fermentation. While commensal microbes in the intestinal tract have been shown to generate high levels of SCFAs through the breakdown of dietary fibers, members of the skin microbiome have also been shown to generate these molecules. However, the effect of molecules like SCFAs, and HDAC inhibition in general, on immune function of the skin has not been investigated previously.

With this information in mind, we began our studies with the hypothesis that inhibition of HDAC activity in keratinocytes would decrease the expression of proinflammatory genes in response to TLR activation. Much to our surprise, our initial experiments showed an opposite response in keratinocytes versus monocytes, as SCFAs and other HDAC inhibitors resulted in an increased response to TLR ligands. In this dissertation, we will describe our findings on the molecular effects of HDAC inhibition in keratinocytes, the relevant molecules generated by a member of the skin microbiome, and the individual HDAC enzymes responsible for controlling inflammatory gene expression in keratinocytes. We will also go on to describe our findings pertaining to the mechanism behind this HDAC-mediated control of inflammatory gene

expression. While these findings uncover a novel regulatory function of HDACs in keratinocyte-mediated inflammation, future studies will undoubtedly help to clarify the role of these and other epigenetic processes in the control of cutaneous immunity and inflammation.

Chapter II:

Skin-resident microbial communities and their interactions with the cutaneous innate immune system

Abstract

The skin is home to abundant, diverse communities of microbes that reside on the surface and within the various appendages present in the skin. Collectively referred to as the "skin microbiome," characterization of these microbial communities has enhanced our knowledge of the ecology of organisms present in normal skin. Surprisingly, the composition of these communities from different body sites exhibit high levels of both intra- and interpersonal variation, likely influenced by the vastly different environments of the skin across the body. Furthermore, studies have begun to bring to light the intimate relationships shared between host and resident microbes. In particular, it is apparent that just as host immunological factors and behaviors shape the composition of these communities, microbes present on the skin greatly impact the functions of human immunity. Thus, the skin immune system should be viewed as a collective mixture of both host- and microbe-derived elements acting in a mutualistic relationship. In this chapter, we will discuss the interactions of the skin microbiome and host immunity, as well as the role that dysbiosis of these communities plays in the development of various diseases of the skin.

Introduction

The skin is our most exposed organ, responsible for providing a barrier to the external environment that can resist a wide range of challenges and respond appropriately to penetrating dangers. However, despite a potent cutaneous immune system, many different microbial communities thrive on the surface. The challenge for the skin's immune system is that it is charged with resisting infections, but must do so under normal conditions in the absence of cell recruitment and inflammation. Failure to properly control and tolerate resident microbes results in skin disease. More recently, it has been hypothesized that the skin commensal microbial communities not only co-exist despite our immune defense network but actually modify immunity, therefore influencing normal skin health as well as participating in various dermatological conditions (*19*). The microbiota of the skin have therefore become the subject of much recent interest from the perspective of better understanding cutaneous disease and as a source for developing novel therapies for skin disease.

Historically, detection and characterization of the skin microbes depended on their cultivation from swabs of the skin surface (*20*). With the advent of DNA-based technologies for the detection and identification of microbial genes, it is now clear that the culturable microbes represent only a small fraction of the total organisms that interact at the surface. DNA sequencing techniques have sought to describe the diversity of microbes residing on and within our bodies, and as a shorthand for describing the ecology of the human body as a "biome," the microbial communities inhabiting us have collectively been called the "human

microbiome." This field received a great boost in 2007 when the National Institutes of Health initiated the Human Microbiome Project (HMP) with the intent of surveying and characterizing the microbes that reside at different body sites (*21*). The seminal HMP analysis of microbes from 18 body sites in over 240 healthy volunteers, completed in 2012, has begun to reveal the complex nature of the human microbial inhabitants and the incredible amount of both intra- and interpersonal variation in the communities residing throughout our bodies (*22*). Today, with the groundwork prepared by descriptive studies, we are now poised to uncover the intimate relationships that microbes share with their hosts and the influences they have on human health.

In this chapter, we will review recent findings of the role skin microbial communities play in host immunity, as well as explore the topic of dysbiosis as a participant in various pathologies of the skin. One finding made clear through the Human Microbiome Project is that the communities residing at different body sites are not at all uniform—the gastrointestinal, oral, nasal, vaginal, and skin-associated microbes vary greatly in their compositions (*22*). Adding to this complexity is the observation that different areas of the skin, which vary in physiologic aspects such as moisture, oiliness, and exposure to the external environment, harbor distinct groups of microbes. While this review will briefly address the nature of this diversity, we point the reader to several excellent publications for a more comprehensive characterization of the communities residing across the surfaces of our skin (*23-27*). Additionally, thorough discussions of the technologies currently used to analyze microbiome

composition and how these methods have revolutionized the way the human microbiome is viewed can be found elsewhere (*28, 29*). Our goal in this chapter is to begin to show how our existing information can be translated into a deeper functional understanding of how these may act to influence health.

The physical and cellular immune barrier of the skin

To understand the ecology of the skin surface and the factors influencing the skin microbiome it is first necessary to understand the elements contributing to the cutaneous environment. The skin barrier is most frequently thought of in terms of the outermost layer of the epidermis. For the purpose of this discussion of skin microbial communities, the epidermis will be discussed in greatest detail. However, it is important to recognize that the skin barrier consists of several layers below the epidermis that profoundly affect function and also harbor microbes (*30*). Additionally, it is important to recognize that an aqueous and lipid layer exists above the epidermis, also contributing to the ecology of the surface. Combined, all layers of the skin must prevent infection and the entry of harmful substances while controlling the loss of water and nutrients. At the forefront of this process to maintain homeostasis is the highly keratinized epidermis, the result of a specialized differentiation process of keratinocytes, the main cell type in the epidermal barrier (*31*).

Epidermal structure and composition contribute to physical immune barrier

The surface of the skin is formed by a network of cross-linked cornified cell envelopes and specialized lipid molecules creating the “bricks and mortar” structure of the epidermis (*32-34*). The ultrastructure of the skin surface is also riddled with invaginations, including sweat glands, hair follicles, and sebaceous glands. Two types of sweat glands exist: eccrine and apocrine. Eccrine sweat glands, which are distributed across nearly the entire skin surface, play a crucial role in thermoregulation. They secrete sweat that is almost entirely water directly onto the skin surface, the evaporation of which allows the body to cool (*35*). Eccrine sweat also contains salt and electrolytes, which work to acidify the skin. On the whole, the result of this process is a barrier that is cool, dry, and slightly acidic. This environment plays a major role in limiting the composition of microbes that can survive and proliferate. Furthermore, eccrine sweat glands constitutively express several antimicrobial peptides (AMPs), including cathelicidin and β-defensins (*36-38*). Thus, the density of eccrine sweat glands impacts microbial colonization of the skin. Apocrine sweat glands, which exist at birth but do not become active until puberty, have a more limited distribution, found primarily in sites such as the axilla, genitalia and perianal regions. These glands secrete their contents—an oily, odorless mixture of proteins, lipids, and steroids—into the hair canal (*35*). It is the degradation of these apocrine-derived compounds by resident bacteria that produces the characteristic odor of sweat (*39, 40*). Sebaceous glands are connected to hair follicles, forming the pilosebaceous unit. Sebaceous glands secrete the lipid-rich substance called

sebum, which works to lubricate the hair and skin. Indeed, it has long been realized that the pilosebaceous unit harbors distinct microbial communities, dominated by bacteria capable of thriving in the anoxic, lipid-rich environment, such as *Propionibacterium acnes* (*41, 42*). The breakdown of sebum generates free fatty acids, which work to control microbial colonization along with sebocyte-derived cathelicidin, β-defensins, and antimicrobial histones (*43-45*).

The differences in the physical characteristics of skin from different body sites are easily observed macroscopically. In some locations, such as the palms of the hands or soles of the feet, the skin is thick and hairless; other sites are thin and delicate, such as the eyelids. Sites like the scalp or axilla may support dense hair growth, and other locations produce more oil, such as the face, back, and chest. Importantly, these anatomic differences can strongly impact the microbial communities that reside on the skin. Figure 1 illustrates some of the fundamental differences in skin anatomy from various body sites. However, these innate, genetically defined differences in the anatomy of the skin at different sites are only a partial explanation of skin microbial diversity. An additional major variable to consider are individual behavioral factors that alter surface conditions. For example, the amount of exposure versus occlusion of body sites, the degree of detergent use, the application of lotions or cosmetic products, occupation, and where one lives all dramatically alter skin surface environments. Thus, the microbiome will be influenced by the structure and composition of the epidermis as well as individual behaviors that dictate the total nature of this environment. The skin's location at the interface with the outside

world therefore makes is most subject to environmental influences that will affect the microbiota.

Skin immunocytes comprise the cellular immune barrier

Within the skin, both innate and adaptive mechanisms contribute to immune function (*46-48*). Keratinocytes are the first active participant in the skin immune response. These epithelial cells express a number of pattern recognition receptors (PRRs) that sense microbes through recognition of conserved molecular entities such as lipoproteins, nucleic acids, cell wall components, and flagella. While keratinocytes express a number of antimicrobial peptides, cytokines, and chemokines at steady state, activation of PRRs can rapidly increase the expression these molecules, resulting in direct antimicrobial effects as well as recruitment and education of additional immune cells (*1*). Also found in the epidermis are Langerhans cells (LCs), a specific subset of dendritic cells. Historically viewed as constitutive immune-activating cells through their antigen-presenting roles, recent evidence supports the notion that LCs participate in promoting tolerance to self-antigens and commensal microbes through the induction of regulatory T cells at steady state (*49*). The history and currently changing views of the role of LCs is reviewed in depth elsewhere (*50, 51*). Furthermore, within and below the epidermis reside many more cells types with functional roles in cutaneous immunity. Cells involved in both innate and adaptive immunity can be found here: dendritic cells, macrophages, mast cells, natural killer cells, and a variety of T cells including $CD8^+$ memory T cells, $CD4^+$

T_H1, T_H2, and T_H17 cells, γδ T cells, NKT cells, and regulatory T cells (T_{reg}) (*46, 47*). Combined, there is considerable specialized capacity in the skin cellular immune system to react and change in response to microbes.

Therefore, to understand the skin microbial flora it is essential to recognize that unlike all other commonly studied areas of the microbiome such as gut and oral mucosa, the skin has the greatest diversity of variables that influence its surface characteristics and a wide variety of cell types that are positioned to interact with microbes. One can imagine the skin as a melting pot of different microenvironments, constantly shifting with influences from the outside world and the host immune system. As we shall describe, this creates a potential problem in describing the skin microbiome. Given the vast diversity of skin environments with different physical and chemical features it is logical to predict that each will support vastly different populations of microorganisms.

Keratinocytes in cutaneous innate immunity

Keratinocytes in particular play an extremely important role in cutaneous innate immunity, as they participate in formation of both the physical barrier and immunological barrier of the skin. First, the carefully controlled program of keratinocyte differentiation and cell death is crucial to the development of the stratum corneum (SC), the outermost layer of the epidermis that serves as the major hydrophobic barrier preventing the loss of body fluids and invasion of microbes (*32-34, 52*). In addition to its role as a physical barrier, the SC also serves as an antimicrobial barrier through its inclusion of various antimicrobial

lipids and peptides, as keratinocyte-mediated production of AMPs such as cathelicidin and defensins has been show to be critical for host defense against bacterial and viral infection (*53-57*). Thus, it is evident that the role of keratinocytes in forming the SC is crucial to skin immunity.

However, keratinocytes are not limited to barrier functions in terms of their participation in immune surveillance in the skin. Beginning in the 1970s and 1980s, researchers began to recognize keratinocytes for their ability to produce a variety of inflammatory mediators, either constitutively or following insult. As described above, keratinocytes possess the molecular machinery necessary to detect and respond to infection and injury in the form of numerous PRRs that respond to pathogen- and danger-associated molecular patterns (PAMPS and DAMPS, respectively). In particular, keratinocytes express a variety of members of the Toll-like receptor (TLR) family, including TLR1, TLR2, TLR3, TLR6, TLR7, and TLR9 (*58, 59*), as well as members of other classes of PRRs, such as nucleotide-binding oligimerization domain (NOD)-like receptors (NLRs), RIG-like helicase receptors (RLRs), and functional components of the inflammasome complex (*60, 61*). This array of receptors allows keratinocytes to sense numerous, diverse PAMPS and DAMPS and initiate immune responses through the production of cytokines, chemokines, and additional AMPs.

Indeed, keratinocytes are a major source of cytokine and chemokine production in the epidermis, and the generation of these molecules can initiate inflammatory processes through the recruitment and education of other immune cells to the skin (*62, 63*). Keratinocytes constitutively express some cytokines in

normal skin and can induce high levels of a variety of other cytokines and chemokines following PRR activation, including IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-18, TNFα, and TSLP (*64-68*). In addition, immune cells residing in or recruited to the skin can produce additional cytokines and molecules that further activate keratinocytes, establishing a cytokine network within the epidermis (*69*). However, it is important to note that keratinocyte cytokine production has been documented to occur before the infiltration of these cell types, demonstrating that keratinocytes can function as initiators of cutaneous inflammation. As such, it has been shown that the development of numerous inflammatory skin conditions, such as atopic dermatitis, psoriasis, and contact hypersensitivity disorders is controlled by keratinocyte expression of inflammatory mediators.

Characterization of the skin microbiome

Several recent studies have set out to investigate the composition of microbial communities on the skin at various anatomical locations. One of the key take-home points arising from these studies is that just as the microbiome of the skin differs greatly from that of the gastrointestinal tract or oral cavity, so too do the populations from different areas of the skin. This finding makes sense when one considers the vast differences in features of the skin from different body sites as described above. In light of these findings, the term “skin microbiome” must be used carefully: such a broad term, while distinguishing microbes on the skin from those in the gut or oral cavity, fails to address the site-based variation in microbes residing on the skin. One must be especially critical

when analyzing and comparing investigations of the “skin microbiome,” as the site studied and the environment the individual is exposed to can drastically impact the types of microbes identified. The definition of “biome” as being of a similar condition harboring a distinct community cannot be applied to the skin as a whole. However, as limited information has been assembled to accurately describe the composition and function of a “palm microbiome” or “axillary microbiome,” for now we must frequently lapse into the inherently incorrect term of the “skin microbiome.”

What is normal?

Based on 16S ribosomal RNA gene sequencing, the majority of “normal” bacterial inhabitants of the skin fall into four phyla: Actinobacteria, Bacteroidetes, Firmicutes, or Proteobacteria. While these are indeed the same four phyla that compose the majority of the bacteria present in the GI tract and oral cavity, these inhabitants are present at different ratios, and Actinobacteria are the most abundant inhabitants of many sites on the skin (*22, 70, 71*). Commensal microbes on the skin have also been classified as resident of transient depending on their ability to populate the skin—resident microbes survive on the skin for long periods of time, whereas transient microbes may be detected during one sampling event and absent the next. However, as we have seen earlier, the most influential factor in regards to the microbes inhabiting the skin is the body site being studied, and as a result skin has the most diverse bacterial communities of any of the major epithelial surfaces studied to date (Figure 2).

Current information on these sites can be divided into three categories: moist, sebaceous, and dry (*72*). Moist sites include areas such as the navel, the axilla, the inguinal crease (groin), the sole of the foot, antecubital fossa (inner elbow) and the popliteal fossa (back of the knee). The most abundant microbes colonizing these moist areas are *Staphylococcus* and *Corynebacterium* species, of the phyla Firmicutes and Actinobacteria, respectively (*72, 73*). Sebaceous sites, such as the forehead, the alar crease (side of the nostril), the retroauricular crease (behind the ear), and the back seem to harbor the least diverse populations of microbes. *Propionibacterium* species of the phyla Actinobacteria are the most common isolates from sebaceous areas, probably due to their ability to survive in these anaerobic, lipid-rich environments. Dry areas of the skin, including the forearm, various locations on the hand, and the buttocks, have been shown to have the most diversity in microbial inhabitants, with varying representations of the main four phyla (Figure 2). At present is still remains unclear what proportion of these organisms can actually survive or replicate on the skin and which are simply frequent environmental encounters.

Currently, the vast majority of microbiome-focused studies have concentrated on bacterial species. However, one must not forget additional microorganisms that are residents of the skin, including fungi, parasites, and viruses. While the methods for classifying and differentiating these microbes based on sequencing data are not as well developed as those for bacteria, some progress has been made towards characterizing these members of the microbiome. In regards to the fungi present on normal human skin, the most

commonly identified organisms are species of *Malassezia* (*74-76*). In fact, one study estimated *Malassezia* to account for up to 80% of fungi present, depending on the anatomical location sampled (*77*). Further investigation is warranted for identifying additional fungi that are components of the skin microbiome. *Demodex* mites, which are small, parasitic arthropods residing in pilosebaceous units, have been isolated and implicated in the pathogenesis of rosacea (*78, 79*). However, as these mites can also be found in normal skin, more investigation into their role as commensal microbes is needed (*80*). Viruses are perhaps the least studied of all members of the skin microbiota, though recent studies have begun to probe this subject. With the exception of viral pathogens that cause over disease (such as herpesviruses, papillomaviruses, and polyomaviruses, among others), study of this class of resident microbes has been largely neglected. Due to the great diversity already identified in the viral component of the microbiota, it is likely that these microbes play an important role in cutaneous immunity (*81, 82*). Part of the difficulty in conducting metagenomic studies of viral populations comes from the diversity in nucleic acid composition of different viral classes, which can possess either single- or double-strand DNA or RNA. Recent studies have worked to catalog the double-stranded DNA virome of the skin (*83, 84*). One interesting finding of these studies, as well as other characterizations of the skin microbiome as a whole, is that compared to bacteria and fungi, dsDNA viral communities were significantly less stable over time and more variable between individuals (*84, 85*). However, in contrast to the diversity seen in the dsDNA eukaryotic virome, there does appear to be more stability and

commonality in the population of bacteriophages that infect skin-resident bacteria like *P. acnes* and *S. epidermidis (85, 86)*. Bacteriophage populations are particularly interesting to consider, as the presence of these viruses can have dramatic effects on the composition of bacterial communities. In conclusion, while efforts to define the commensal non-bacterial microbes of the skin have advanced in recent years, at the present time few conclusions can be drawn regarding the functions of these populations in skin health and disease.

Hyper-diversity of bacterial communities of the skin

The most common theme to emerge from in-depth studies of the bacterial communities residing on the skin is the large amount of temporal and spatial diversity seen both between different individuals and within the same person. As mentioned, the body site sampled is a key determinant in the makeup of these communities, as different microbes have evolved to thrive in different ecological niches present across our bodies (*72, 73*). While some body sites that share similar characteristics do show significant similarities across individuals, a tremendous amount of interpersonal variation has been identified.

The skin is colonized by bacteria starting at birth. This initial skin microbiome has a very low diversity across the body, and is largely shaped by the delivery mode of the child—babies born through the vaginal canal will be colonized by microbes present in the mother's vaginal microbiome, while babies born through caesarian section will acquire a skin flora more similar to the mother's skin (*87*). In the first years of one's life, the microbiota at various body

sites develop more diversity as children explore their environment, change their diets, and are exposed to more people and animals (*88*). Studies have indicated that by 2.5 years of age, the intestinal microbiome of infants has developed to resemble that of adults (*89*); similarly, age has been shown to impact the composition of microbial communities, as great diversity was observed when comparing children at different stages of development with adults (*90*).

In healthy adults, the amount of diversity seen in skin commensal bacteria is staggering. In analyzing the bacteria present on the forearms of six individuals, Gao *et al* observed that less than 10% of identified genera were present across all individuals (*91*). Similarly, in surveying the palms of 51 healthy volunteers, Fierer *et al* report even greater diversity, with the average palm harboring over 150 species-level bacterial phylotypes (*92*). Furthermore, individuals displayed on average only 17% similarity between their two hands, and between individuals, the common members dropped to 13% (*92*). Much of this diversity correlates with gender, dominant hand, and time since last washing their hands. Additional studies have investigated the diversity between individuals, and in all cases, diversity was a unifying theme (*72, 93, 94*). Interestingly, a recent study showed that family members residing in the same home show much more similarity in their skin commensal bacteria than strangers; furthermore, pet owners showed a striking similarity with the bacterial communities present on their pets (*95*). Such observations further challenge our concept of the human biome and beg the question if we should actually include all the residents of our home and place of work in these discussions.

Functional associations of skin commensals with cutaneous immunity

The most interesting and important question to arise from studies of the human microbiome is to what extent these microbes impact our health. While a number of discoveries have been made regarding the importance of microbial communities in the gastrointestinal tract (*96-98*), much less is currently known about the role of microbes on the skin in the development and maintenance of our immune system. Conclusions drawn regarding the function of the gut microbiome in immunity are always complicated by the important role these microbes play in processing and absorption of nutrients. Thus, germ-free mice used in immunological studies will have dramatic, and often overlooked differences in nutrition compared to the control population. Studies of the skin microbiome are therefore somewhat more straightforward as their role in nutritional homeostasis is less likely to be a confounding variable.

Figure 3 summarizes the ways in which the commensal microbes of the skin are hypothesized to influence cutaneous immunity in the host. In the following sections, we will discuss recent experimental findings on the role of commensal microbes in cutaneous immunity.

<u>Healthy competition from our commensal microbes</u>

One method by which commensal microorganisms contribute to host immunity is through the inhibition of growth of pathogenic microbes. Indirectly, the presence of commensal microbes on the skin results in competition for

nutrients and space, thus greatly impacting the potential for growth when pathogens are introduced on the skin surface. Additionally, a large number of bacteria are known to directly restrict the growth of competitors through the production of antimicrobial compounds (*99*). These proteinaceous factors, called bacteriocins, are capable of inhibiting the growth of closely related species of bacteria while having no effect on the organisms which produce them. Recently, several lines of evidence have indicated that commensal bacteria from the skin produce molecules with antimicrobial properties that can function *in vivo* to restrict the growth of cutaneous pathogens (*100*).

One of the more abundant and frequently cultured members of the skin bacterial community is *Staphylococcus epidermidis*, a cousin of the frequent pathogen *Staphylococcus aureus*. While *S. epidermidis* does have the potential to cause serious infections, it has also been shown to produce several molecules that interfere with pathogen growth. Iwase *et al* illustrated that clinical isolates of *S. epidermidis* are able to inhibit *S. aureus* biofilm formation through the production of a serine protease, Esp, which also boosts the antimicrobial effects of hBD2 (*101*). Furthermore, the introduction of Esp-producing *S. epidermidis* into the nasal cavity of volunteers who were *S. aureus* carriers resulted in the clearance of *S. aureus* colonization, illustrating the clinical relevance of this commensal-produced protease (*101*). The mechanism behind the biofilm-disrupting capabilities of Esp have been recently elucidated: it seems that Esp specifically degrades several *S. aureus* proteins involved in biofilm formation and many human receptor proteins important for *S. aureus* colonization and infection

of host cells (*102*). *S. epidermidis* produces a variety of additional molecules that influence the growth of pathogenic microbes. In particular, phenol-soluble modulins (PSMs) have potent antimicrobial functions, possessing the ability to strongly interact with and cause leakage of microbial lipid membranes (*103*). These *S. epidermidis*-derived molecules selectively kill the skin pathogens *Streptococcus pyogenes* and *S. aureus*, cooperate with host-derived AMPs to increase bacterial killing, and can be incorporated into neutrophil extracellular traps (NETs), another innate host defense against infection (*103, 104*). Thus, *Staphylococcus epidermidis* possesses several weapons that contribute to the innate immune defense arsenal present in human skin.

Another common skin commensal bacteria is *Propionibacterium acnes* (*P. acnes*), and recent work indicates that this bacteria is capable of inhibiting the growth of MRSA (*105*). Briefly, *P. acnes* ferments glycerol, a metabolite that naturally occurs in human skin, into a number of short-chain fatty acids that result in a decreased intracellular pH within *S. aureus* to inhibit its growth. These findings were recapitulated *in vivo*, as the application of *P. acnes* and glycerol to wounds on mouse skin greatly decreased the bacterial burden when challenged with CA-MRSA isolates (*105*). These findings suggest that *P. acnes* may function to prevent pathogen growth in human skin, and could also be used to develop novel probiotic treatments for MRSA infections.

Commensal microbes control behavior of the host

Skin microbes also contribute to cutaneous immunity through their influence on the function of host cells. There is currently evidence for multiple mechanisms by which this can occur. Our group has shown that *Staphylococcus epidermidis,* sensed by keratinocytes via Toll-like receptor 2, boosts host immunity to *S. aureus* infection through increased expression of antimicrobial peptides such as β-defensins 2 and 3 (*106*). These results suggest a symbiotic relationship between hosts and commensal microbes that leaves the host more prepared to combat pathogenic infection. This is supported by findings from Wanke *et al* which demonstrate that signaling from the commensal microbes through TLR2 results in increased antimicrobial peptide expression in keratinocytes, and blocks NF-KB inhibition induced by pathogenic *S. aureus* (*107*). Indeed, the influence of commensal microbes extends to other cells types as well. For example, the role of commensals has been investigated in the setting of mast cell-mediated antiviral immunity. Wang *et al* demonstrated that TLR2 activation, mediated by LTA from *S. epidermidis*, results in greater numbers of mast cells being recruited to sites of viral challenge in the skin (*108*). Furthermore, the release of the AMP cathelicidin by these recruited mast cells was amplified by this TLR2 stimulus, resulting in increased antiviral immunity. Thus, signals from commensal microbes appear to be relevant to multiple cell types responding to a variety of microbial challenges in the skin. Together, these findings illustrate an important role for commensal bacteria in amplifying host immune defense against pathogens.

Commensal microbes like *S. epidermidis* also contribute to host immunity through maintenance of the epidermal barrier. TLR2-mediated recognition of LTA from *S. epidermidis* inhibited TLR3-driven inflammatory cytokine production in cultured keratinocytes and reduced levels of inflammation *in vivo* following wounding, a situation in which excessive inflammation would be detrimental to the host (*109*). Furthermore, activation of TLR2 has been shown to increase the tight junction barrier in cultured keratinocytes, illustrating another role for commensal microbes in maintaining barrier homeostasis, a crucial aspect of host defense (*110*).

Other evidence that commensal skin microbes are necessary and sufficient for the generation of optimal skin immunity have come from germ-free mice that failed to mount an adequate immune response to *Leishmania* (*111*). Recolonization of the gut was unable to restore cutaneous immune function, but exposure of the skin of these mice to *S. epidermidis* alone was sufficient to restore effector T cell levels and rescue the immune deficiency. These observations were linked to IL-1 signaling, as germ-free mice showed significant decreases in cutaneous IL-1α production. This evidence suggests that communication between commensal microbes and skin-resident cells is important for proper tuning of the local inflammatory milieu. Future studies should continue to probe the effects of commensal microbiota on the development of an effective immune environment.

Consequences of dysbiosis in the microbiome

As it is becoming increasingly clear that the microbiota make important contributions to normal immune development and function, it is logical that disease can be correlated with alterations in microbial communities. Several descriptive studies have identified differences in the microbes present in diseased skin versus those present in healthy skin. While studies indicate that an imbalance of microorganisms, termed dysbiosis, exists in numerous pathologies, these results have presented a sort of "chicken-or-the-egg" type conundrum, as it is not entirely clear whether alterations in the microbiome lead to disease, or whether underlying conditions result in an imbalance in microbial communities. These models are displayed in Figure 4.

Many microbes that are considered to be relatively harmless commensals can indeed cause serious infection in situations of immune suppression. This is supported by studies investigating the microbes present in chronic, non-healing ulcers that plague diabetic patients or the elderly, as well as the rates of coagulase-negative *Staphylococcal* infections seen in hospitals. Thus, one must keep in mind that even seemingly beneficial microorganisms can take on a pathogenic role when presented with the right opportunity.

<u>Dysbiosis in atopic dermatitis</u>

Atopic dermatitis (AD) is a chronic, relapsing, pruritic inflammatory skin condition that is non-contagious in nature, affecting approximately 15% of children in the United States. It has long been noted that AD flares are

associated with colonization and infection by *S. aureus*, and that antibiotic treatments targeting *S. aureus* are sometimes successful in temporary improvement of disease (*112*). However, it was not until very recently that a thorough comparison of the microbial communities present in lesional, non-lesional, and healthy skin was conducted (*113*). In this study, disease flares were associated with a decrease in the overall diversity of microbial communities on the skin, due to an expansion of *Staphylococcus* species up to 90% of the microbes detected. Interestingly, both *S. aureus* and *S. epidermidis* were noted to increase in untreated lesions—given previous data indicating the ability of *S. epidermidis* to inhibit *S. aureus*, the relationship between these two microorganisms in lesional skin remains unclear. The observation that resolution of disease flares is preceded by a restoration of microbial diversity strengthens the case for a link between skin resident microbes and AD; however, more investigation is needed to definitively assign a causative role for the microbiota in AD.

Adding to the complexity of the AD-microbiome interaction is the number of host factors that have been implicated in the onset of AD (*55*). To date, defects in several aspects of epidermal function have been implicated in AD: mutations in the filaggrin protein, an essential component of epidermal barrier formation (*114*); mutations in receptors and signaling molecules that sense microbes, such as TLR2, CARD4, and CD14; and diminished expression or function of antimicrobial peptides such as defensins, cathelicidin, and dermicidin (*115, 116*). With so many compounding factors, it remains unclear whether

changes in skin biology trigger alterations in microbial diversity or if overgrowth of *Staphylococcus* species occurs first and subsequently drives disease progression.

Dysbiosis in psoriasis

Psoriasis is an inflammatory skin condition, highlighted by erythematous, scaly plaques commonly occurring on the elbows, knees, scalp, and trunk (*117*). Unlike AD, psoriasis is seldom complicated by infection and is usually not pruritic. In contrast, this disorder is complicated by auto-immune phenomena including arthritis and co-morbidities including coronary vascular disease. Detailed analysis of the microbes present in lesional and non-lesional skin of psoriasis has focused on both bacterial (*118, 119*) and fungal (*74, 75*) communities. While these studies have identified potential differences in the composition of the microbiota between psoriatic and normal skin, no consensus microorganisms have been directly identified and linked to disease pathogenesis. Additionally, the number of innate immune-related genetic factors identified in patients with psoriasis further complicates the issue, again leaving researchers without a definitive cause-and-effect relationship between disease and microbial diversity (*117, 120*).

Opportunistic infections

Though commensal microorganisms typically inhabit our bodies peacefully, many do possess the ability to cause infection in the right setting.

Staphylococcus epidermidis, despite its many beneficial roles described previously, is a frequent cause of infections, particularly nosocomial deep tissue infections involving indwelling medical devices such as catheters (*121, 122*). Furthermore, numerous bacteria that are found in the normal skin microbiome frequently cause infection in chronic, non-healing wounds, which commonly occur in diabetic patients and the elderly (*123, 124*). While many factors of host biology contribute to the impaired healing of these wounds, it is noteworthy that the immune response to these commensal microbes in previously sterile tissues, resulting in prolonged inflammation, exacerbates the problem and creates a vicious cycle (*124, 125*).

Dysbiosis in acne vulgaris

Acne vulgaris is the most common skin condition, affecting upwards of 80% of the population to some extent at various periods of their life. While acne is a multi-factorial disease that involves numerous aspects of host biology, dermatologists have long recognized a relationship between the focal inflammation seen around hair follicles and the skin-resident bacterium *Propionibacterium acnes*, which thrives in the low oxygen, lipid-rich environment of the follicle (*126*). However, the exact relationship between this bacterium and inflammatory skin condition remains unclear and somewhat controversial. While studies investigating the microbial ecology of acne lesions consistently isolate *P. acnes* from the skin or follicles of acne patients, this should come as no surprise—in-depth characterization of the skin microbiome, as previously

described, identifies *P. acnes* as the major bacterial species isolated from sebaceous regions of the skin (*22, 72*).

Through *in vitro* studies, *P. acnes* has been shown to induce an innate immune response from various cell types, including keratinocytes, sebocytes, monocytes and macrophages (*45, 127-131*). Thus, it is understandable to contribute aspects of the inflammatory response seen in the skin acne patients to the presence of high levels of this bacterium in the follicles. The question then becomes, how can this bacterium be present seemingly ubiquitously across sebaceous skin sites, yet only trigger inflammatory immune reactions in select settings? Several possibilities exist to explain this phenomenon. First, it is possible that the development of inflammatory acne represents an alteration in the follicular microbiota. Some studies have shown that while healthy follicles are predominantly colonized with solely *P. acnes*, follicles from acne patients also contained other microbial species such as *Staphylococcus epidermidis* (*132*). Another possibility is that differences in the strains of *P. acnes* present in healthy versus acne-affected skin may account for the selective development of acne. A recent study showed that while the levels of *P. acnes* isolated from healthy versus diseased skin showed little difference, distinct strains were present in healthy versus diseased skin (*133*). Furthermore, it could be that differences in the follicular environment and metabolic state of the resident *P. acnes* can affect the host response to this bacterium, as we will discuss in more detail in the next chapter. Thus, it remains somewhat unclear the exact nature of the relationship between commensal *P. acnes* and the development of

inflammatory acne vulgaris, though the innate immune response to the presence of this microbe is widely accepted as an aspect of disease pathogenesis.

Conclusions

Recent and ongoing efforts have greatly enhanced our knowledge of the composition of microbial communities that inhabit the human body. With this descriptive information readily at hand, researchers are now poised to translate these findings into a deeper understanding of the complex relationships existing between commensals and their host. While several interesting discoveries have already been achieved, a number of important questions still remain to be answered. In particular, mechanistic details of the contributions of microbes to cutaneous immunity and the role of these commensal organisms in the initiation and progression of skin disease should be further pursued, as a deeper understanding of these processes will provide valuable information for the development of novel therapeutic strategies.

In Chapter III, we hypothesize that metabolites generated by members of the skin microbiota may influence host inflammatory and immune functions by interfering with epigenetic processes, in particular histone acetylation and HDAC function. These studies address the question regarding the role of commensal microbes in the pathogenesis of inflammatory skin conditions, in particular the inflammation seen around hair follicles in acne vulgaris.

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Chapter III:

Inhibition of HDAC8 and HDAC9 in keratinocytes by microbial short-chain fatty acids breaks immune tolerance of the epidermis to TLR ligands

Abstract

Epidermal keratinocytes participate in immune defense through their capacity to recognize danger, trigger inflammation, and resist infection. However, normal skin immune function must tolerate contact with an abundant community of commensal microbes without inflammation. We hypothesized that microbial environmental conditions dictate the production of molecules that influence epigenetic events and cause keratinocytes to break innate immune tolerance. *Propionibacterium acnes*, a commensal skin bacterium, produced the SCFAs propionate and valerate when provided a lipid source in hypoxic growth conditions, and these SCFAs inhibited HDAC activity. Inhibition of HDAC activity in keratinocytes promoted cytokine expression in response to ligands for TLR2 or TLR3. This response was opposite to the action of HDAC inhibition on production of cytokines from monocytes and involved HDAC8 and HDAC9 because siRNA silencing of these HDACs recapitulated the activity of SCFAs. Analysis of cytokine expression in mice confirmed the response of the epidermis as application of SCFA on the skin surface promoted cytokine expression, while subcutaneous administration was inhibitory. These findings show that the products of commensal microbes made under specific conditions will inhibit HDAC activity and break tolerance of the epidermis to inflammatory stimuli.

Introduction

Epithelial surfaces are the body's first line of defense against pathogenic microbes in the environment, and its physical and immunological integrity is crucial to health. Within human skin, a variety of cell types participate in immunological surveillance, including many classical immune cell subtypes acting in concert with keratinocytes that compose the epidermis (*47*). Keratinocytes directly participate in immunity as they are a major source of antimicrobial molecules and cytokines, and serve as a trigger of inflammation when the epithelium is exposed to danger- or pathogen-associated molecular patterns (DAMPs or PAMPs) including Toll-like Receptor 2 (TLR2) ligands present in bacteria that normally live on the skin such as *Propionibacterium acnes* (*P. acnes*) (*127, 128, 134*). Under normal conditions keratinocytes are in constant contact with a robust community of microbes that produces TLR2 ligands yet these abundant microbes only trigger inflammation in certain disease states. It is unknown how keratinocytes in some cases can tolerate normal microbial colonization yet at other times switch to the inflammatory response necessary when these microbes threaten infection.

Recent work has shed light onto the important role of epigenetic factors in the regulation of inflammatory gene expression in various cell types. These factors include DNA methylation, histone modifications, chromatin remodeling, and regulatory nuclear complexes that control transcriptional activity. Histone acetylation and histone deacetylase (HDAC) activity have been extensively studied in myeloid-derived cells such as monocytes, macrophages, and dendritic

cells. Overall, inhibitors of HDAC activity have been shown to decrease the expression of proinflammatory molecules in these cell types following TLR activation (*135-138*). This process has been hypothesized to be relevant to epithelial inflammatory homeostasis in the gut, as the microbes that populate the intestinal tract produce high levels of short-chain fatty acids that inhibit HDAC activity (*16, 139, 140*). These findings support beneficial clinical associations between the microbial metabolome of the gut and normal immune function (*141, 142*), and provide one mechanistic explanation for how bacteria can regulate inflammation.

While epigenetic processes have been described in keratinocytes in the setting of cellular differentiation (*8, 143-145*), little work has been dedicated to investigating the role of HDAC activity in keratinocyte innate immune and inflammatory functions. Recently, *Propionibacterium acnes* (*P. acnes*) was observed to generate short-chain fatty acids (SCFAs) when grown in anaerobic conditions with a lipid substrate for fermentation (*105*). This culture condition is physiologically relevant as it mimics the lipid-rich, low oxygen environment that exists in an occluded hair follicle. As some SCFAs are well-established inhibitors of HDAC activity (*146, 147*), we sought to investigate if these molecules influence the response of keratinocytes to TLR stimulation and determine whether HDAC enzymes regulate inflammatory and immune-related gene expression in the skin. Our results demonstrate that metabolites produced by members of the skin microbiome are a critical factor that turns off epithelial tolerance to resident bacteria.

Results

HDAC inhibition increases proinflammatory gene expression in keratinocytes

Butyrate is a SCFA that is produced by anaerobic fermentation and is known to be a potent inhibitor of HDAC activity (*10*). Through the production of butyrate, the microbiome has been suggested to exert epigenetic control of immune behaviors by modulating histone acetylation, acting to suppress cytokine production by cells like macrophages and dendritic cells (*135-138*). Consistent with this notion, we observed that the expression of mRNA for IL-6, TNFa, IL-8 and CCL2 induced by the TLR2/6 ligand MALP-2 was significantly decreased in human monocytes when exposed to sodium butyrate (Figure 5A). However, in contrast to the response of monocytes and other bone marrow-derived immunocytes previously studied, primary cultures of normal human epidermal keratinocytes (NHEK) significantly increased expression of these same cytokines and chemokines when exposed to equivalent concentrations of sodium butyrate and MALP-2 (Figure 5B).

The large magnitude of this unexpected proinflammatory response brought on by HDAC inhibition in keratinocytes prompted us to further characterize how HDAC inhibitors affect TLR responses in NHEK. Poly(I:C) is a ligand of TLR3 and is a potent inflammatory stimulus that models cutaneous immune responses to tissue necrosis and viral infections (*148*). Butyrate also increased NHEK IL-6 and IL-8 mRNA expression when exposed to this TLR3 ligand (Figure 5C). The abundance of IL-6 and IL-8 protein secreted into the culture media was also increased by butyrate exposure combined with either

TLR ligand (Figure 5D-E). Multiplex analysis of several other cytokines and chemokines demonstrated that RANTES was also increased when keratinocytes were co-treated with butyrate and MALP-2, but MCP-1, IL-1α, IL-1β, IP-10 and GM-CSF were not (Figure 5F). The effect of sodium butyrate on secretion of IL-6 and IL-8 was dose-dependent, with significant increases observed across micromolar to millimolar concentration ranges (Figure 5G-H). Similar stimulatory effects were also seen when cells were treated with other HDAC inhibitors, including trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) (Figure 6A-D). These results demonstrated that multiple different chemical inhibitors of HDAC activity increased inflammatory cytokine responses by cutaneous epithelial cells and supported the conclusion that the effect observed with sodium butyrate was due to the capacity of this SCFA to inhibit HDACs and not due to another response to this lipid.

To understand why inhibition of HDAC activity had an opposite effect on cytokine production in keratinocytes compared to bone marrow-derived immunocytes, we examined the role of the nucleosome remodeling and deacetylase (NuRD) complex. In macrophages, the NuRD complex acts as a negative regulator of inflammatory gene expression (*149, 150*). It was previously reported that in these cells, HDAC inhibitors increase both the expression and the chromatin-binding activity of Mi-2β/CHD4, the catalytic subunit of the NuRD complex (*136*). However, in contrast to monocytes, butyrate treatment did not increase Mi-2β transcript levels in keratinocytes (Figure 7A). Furthermore, contrary to the reported increases in Mi-2β/NuRD binding to the IL-6 promoter in

macrophages after HDAC inhibition, ChIP-qPCR analysis revealed no increases in Mi-2β binding to the IL-6 promoter in keratinocytes treated with sodium butyrate (Figure 7B). These results demonstrated that unlike monocytes or macrophages, keratinocytes lack the negative regulatory action that accompanies Mi-2β/NuRD activation by HDAC inhibitors.

HDAC8 and HDAC9 control inflammatory gene expression in keratinocytes

The HDAC inhibitors used in these experiments are broad acting and inhibit the activity of many of the 11 classical HDAC enzymes. We next sought to gain a better understanding of which particular HDACs are responsible for controlling proinflammatory gene expression in keratinocytes. First, we measured the transcript abundance of 11 HDACs in keratinocytes grown under conditions that mimic their state of differentiation in the basal layer of the epidermis, and then again after switching to a high calcium medium to promote differentiation to a state more reflective of keratinocytes in the superficial layers of the skin. We observed that HDAC2, HDAC3, HDAC8, HDAC9 and HDAC10 were the most highly expressed overall, but that HDACs 8-10 showed differential expression during differentiation. (Figure 8A). Expression of HDAC8 and HDAC9 in basal and differentiated epidermal keratinocytes was further validated by Western Blot (Figure 9A). Additionally, expression levels of the 11 HDAC enzymes was measured in a sebocyte cell line and compared to epidermal keratinocytes (Figure 9B).

We next utilized siRNA-mediated gene knockdown to systematically deplete these highly abundant HDACs in cultured keratinocytes, resulting in 90% or greater reduction in transcript levels (Figure 8B, Figure 10A). Similar to the response to broad-acting chemical inhibitors of HDAC activity, silencing of either HDAC8 or HDAC9 led to a robust increase in the TLR2/6-mediated induction of IL-6, IL-8, and CCL5 (Figure 8C-E). However, silencing of HDAC1, HDAC2, and HDAC3 did not consistently lead to increases in the responsiveness of keratinocytes to MALP-2 (Figure 10B-D). These observations suggested that the pro-inflammatory actions of SCFA were mediated by inhibition of HDAC8 and HDAC9. These HDACs have not been well studied in the skin, and were not previously known to influence immune function. Therefore, to better understand the potential role of HDAC8 and 9 in keratinocytes, the expression of these enzymes was studied by immunostaining of normal human skin. Both HDAC8 and HDAC9 were abundantly detected in the epidermis, with HDAC8 staining throughout the epidermis while HDAC9 was primarily expressed in basal layers (Figure 8F-G). Importantly, HDAC9 was highly expressed by keratinocytes lining hair follicles (Figure 8H), a site that is abundantly colonized by *P. acnes* and can provide the lipid-rich and hypoxic environment necessary for production of SCFAs (*30*). These results therefore suggested that HDAC8 and HDAC9 have relevant functions as negative regulators of inflammatory gene expression in the skin, and that inhibition of this tolerogenic activity can promote epithelial inflammation.

A commensal skin microbe inhibits HDAC activity

Having observed that the SCFA butyrate can enhance keratinocyte cytokine expression, we next directly tested if the SCFAs made by *P. acnes* could influence HDAC activity. To measure HDAC inhibition by the SCFAs made by *P. acnes,* two laboratory strains of *P. acnes* (ATCC6919 and ATCC29399) were grown anaerobically with and without 2% glycerol as a lipid substrate for fermentation. Bacterial supernatants were then collected, filtered, and used to stimulate keratinocytes in culture. The supernatants from bacteria grown in the presence of glycerol elicited increased production of the cytokines IL-6, IL-8, TSLP, and TNFα when compared to supernatants from bacteria grown without the glycerol substrate (Figure 11A-D). Furthermore, an assessment of acetylation of histone residues H3K9 and H3K27 by Western blot demonstrated that supernatants from *P. acnes* cultured with glycerol led to higher increases in levels of acetylation of these histone marks in keratinocytes (Figure 11E). Supernatants of *P. acnes* ATCC6919 cultured with glycerol increased H3K9 and H3K27 acetylation (9.2-fold and 2.7-fold over control, respectively) more than when cultured without glycerol (4.3-fold and 1.7-fold over control, respectively); similarly, ATCC29399 cultured with glycerol led to larger increases in H3K9 and H3K27 acetylation (9.4-fold and 2.3-fold over control, respectively) than when cultured without glycerol (5.4-fold and 1.3-fold over control, respectively) (Figure 12A-B).

Gas chromatography-mass spectrometry (GC-MS) analysis of the SCFAs produced by *P. acnes* cultured with glycerol revealed that these strains of *P.*

acnes most abundantly produced the SCFAs propionate and isovalerate (Table 1). These SCFAs were next directly tested for their capacity to alter histone acetylation. As seen with crude culture supernatants, increased acetylation of histone H3 lysine residues H3K9 and H3K27 was observed in keratinocytes following treatment with propionate (3.6-fold and 5.2-fold, respectively), butyrate (9.7-fold and 13.2-fold, respectively), or valerate (5.0-fold and 6.0-fold, respectively), but not acetate (1.6-fold and 2.0-fold, respectively) (Figure 13A, Figure 12C). Furthermore, as seen with butyrate, addition of propionate or valerate (but not acetate) also increased TLR2/6-mediated induction of IL-6 and IL-8 transcript and protein levels (Figure 13B-E). Consistent with this observation, chromatin immunoprecipitation analysis of H3K9ac also confirmed increased association with the promoter region of IL-6 in keratinocytes following butyrate treatment (Figure 13F). These results demonstrated that changes in levels of histone acetylation correlated with increased transcriptional activity of proinflammatory cytokines and chemokines in keratinocytes.

To explore potential effects of SCFAs not related to histone acetylation, we examined activation of alternate proinflammatory signaling pathways in keratinocytes. Previous reports have identified various membrane-bound G Protein-coupled receptors (GPCRs) as physiological receptors for SCFAs, and have attributed some of the effects of SCFAs on inflammatory gene expression to activation of these GPCRs (*151-153*). Blocking the signaling of membrane-bound free fatty acid receptors with pertussis toxin (PTX) failed to abrogate the SCFA-mediated increase of TLR-mediated cytokine expression in keratinocytes

(Figure 14A-C). Additionally, in cultured epidermal keratinocytes, expression levels of GPR41, GPR43, and GPR109a were extremely low, suggesting that these molecules do not increase cytokine response through GPCR activation (Figure 14D). Furthermore, propionate and butyrate had no effect on basal or TLR2/6-induced phosphorylation of JNK, p38, or NF-κB p65 and p105 subunits (Figure 14E). Taken in combination with our early data showing that SCFA treatment alone does not induce inflammatory gene expression, we concluded that these SCFAs were not directly affecting proinflammatory signaling pathways in keratinocytes. Together, these results show that when SCFAs are made under environmental conditions favoring fermentation they will increase histone acetylation and enhance cytokine production.

Inhibition of HDAC activity enhances the host defense response of the skin

RNA-sequencing analysis was next employed to gain a clearer understanding of the transcriptome-wide effects of HDAC inhibition on keratinocytes. First, we analyzed the effects of TLR2/6 stimulation in keratinocytes. Using a fold-change cutoff of 1.5 compared to control conditions, 387 genes were identified that were up-regulated and 68 genes that were down-regulated after 4 hours of exposure to MALP-2 (Figure 15A). Simultaneous treatment with butyrate led to further induction of 29% of the genes induced by TLR activation, and also significantly enhanced the expression of nearly half of the genes down-regulated by MALP-2 (Figure 15B). Gene ontology analysis of the genes that were most significantly enriched by butyrate compared to

stimulation with TLR2/6 ligand alone highlighted an increased response to wounding, immune response, inflammatory response and defense response as the biological processes most enriched (Figure 15C).

In order to determine if enhanced host defense in epithelial cells could also occur *in vivo*, we measured the skin cytokine response of mice after cutaneous exposure to propionate or butyrate. In the setting of a normal cutaneous microbiome, topical application of propionate or butyrate led to the induction of IL-1β, IL-6, CXCL1, CXCL2, and TSLP expression (Figure 16A). This enhanced cytokine response *in vivo* was consistent with the observed effects of HDAC inhibitors on cultured keratinocytes but opposite to that previously reported for macrophages. Therefore, to evaluate if the response was determined by the route of exposure, mice were instead exposed subcutaneously to propionate and *Propionibacterium acnes.* Under these conditions, propionate led to a modest decrease in the *P. acnes*-mediated induction of these same inflammatory cytokines and chemokines (Figure 16B). Together, these results support our *in vitro* data that demonstrate a cell type-specific effect of SCFAs and other HDAC inhibitors: namely, that epidermal keratinocytes display an increased responsiveness to Toll-like receptor activation when HDAC activity is inhibited, contrary to the anti-inflammatory effect of HDAC inhibition in monocytes.

Discussion

In these studies, we demonstrate that inhibitors of histone deacetylase (HDAC) enzymes will enhance the inflammatory response of epidermal keratinocytes to TLR ligands. While short-chain fatty acid HDAC inhibitors have been shown to block the TLR-mediated induction of inflammatory gene expression in cells of myeloid origin (*135-138*), a result that was validated in this work, our findings show that these molecules exert an opposite effect in these epithelial cells. Together with our observations that the skin commensal bacterium *P. acnes* produces SCFAs, these observations show how a shift in the microenvironment of a microbe will drive the loss of innate immune tolerance in the skin epithelium. The consequence of this response will be promotion of inflammation under specific environmental settings. A common example of this environment is the occluded hair follicle, a lipid-rich and anaerobic site; therefore, these observations may explain the inflammation observed in diseases such as acne and folliculitis.

Our initial experiments found that the addition of the SCFA butyrate to primary human keratinocyte cultures significantly increased the TLR2/6-mediated expression of pro-inflammatory cytokines such as IL-6, IL-8, CCL2 and TNFα. These results are in stark contrast to the effects of butyrate on inflammatory cytokine induction in monocytes, where an anti-inflammatory effect was observed. As similar increases in cytokine production were seen when keratinocytes were stimulated with other HDAC-inhibitory molecules, we hypothesized that the observed effects were due to changes in histone

acetylation in keratinocytes. Importantly, *P. acnes* produced SCFAs through anaerobic fermentation and supernatants from cultures grown with glycerol substrates induced higher levels of cytokine expression and inhibited HDAC activity more than supernatants from non-fermented bacterial cultures. These data directly demonstrated that products generated by metabolic changes in resident skin microbes can influence the responsiveness of epithelial cells.

As SCFAs and other chemical HDAC inhibitors are broad acting and inhibit most of the classical HDAC enzymes, we aimed to determine which particular HDAC family members are important in controlling inflammatory gene expression in keratinocytes. Through measurements of mRNA transcript levels, we identified HDAC2, HDAC3, HDAC8, and HDAC9 as the most highly expressed in NHEK cultures. We initially targeted HDAC1, HDAC2, and HDAC3 for depletion with siRNA techniques as these enzymes have previously been implicated in the regulation of cytokine and chemokine expression in other cell types (*11, 154, 155*). Surprisingly, loss of HDACs 1-3 did not consistently promote increased sensitivity to TLR2/6 ligands in keratinocytes. However, depletion of HDAC8 or HDAC9 led to a robust increase in the response to TLR2/6 ligands in keratinocytes, and was similar to the effects seen when cells were treated with chemical HDAC inhibitors. These results reveal that HDAC8 and HDAC9 in keratinocytes play an important role in repressing inflammatory gene expression, as depleting these enzymes or inhibiting their function increases the TLR-mediated induction of inflammatory cytokine and chemokine expression.

We next sought to characterize the short-chain fatty acids generated by *P. acnes.* In contrast to previous reports, we were unable to detect butyric acid production in our system, though high levels of propionic and isovaleric acid were present. As butyrate was not a dominant byproduct of *P. acnes* fermentation, we examined the effects of propionate and valerate on human keratinocytes. Propionate and valerate increased global levels of histone H3K9 and H3K27 acetylation, marks associated with actively transcribed regions of the genome, and promoted increased cytokine release comparable to butyrate. These results, in combination with experiments showing that culture supernatants from *P. acnes* grown with glycerol elicit higher cytokine response from keratinocytes than cultures grown without glycerol, suggest that skin-resident commensal microbes can generate biologically active short-chain fatty acids that act to increase histone acetylation and de-repress the expression of inflammatory genes in keratinocytes. However, further studies, including isolation and characterization of SCFAs from human skin or sebaceous material, and characterization of the capacity of different strains and species of commensal bacteria to generate SCFAs, should be done to link these molecules to *in vivo* effects. Additionally, the cytokine response by keratinocytes is only one element in the overall skin inflammatory response. For example, in our system, keratinocytes did not generate high levels of IL-1a and IL-1b, inflammatory mediators important to the pathogenesis of acne(*129, 156*). It is likely that production of additional cytokines by other cells in the skin / hair follicle is also influenced by SCFAs and contributes to the overall skin inflammatory phenotype. Future studies of the

physiological significance of HDAC inhibition in acne pathogenesis will depend on studies of human tissue as no acceptable model for acne currently exists in mice.

Prior studies have linked the immunomodulatory effects of SCFAs to their activation of the membrane-associated GPCRs, GPR41, GPR43, and GPR109a (*140, 151-153, 157*). In our experimental models, epidermal keratinocytes expressed very low levels of these receptors as measured by qPCR (Figure 14D). Furthermore, inhibiting the activation of these GPCRs with pertussis toxin failed to abrogate the increased cytokine response to the TLR2/6 ligand elicited by SCFA treatment. These data, combined with the observation that other non-SCFA HDAC inhibitors also enhance cytokine production, suggest that free fatty acid receptor activation is not an underlying mechanism of this response in keratinocytes. Additionally, some studies have demonstrated that in other innate immune cell types, SCFAs and other HDAC inhibitors influence cytokine production by affecting signaling events downstream of TLRs (*14, 157, 158*). However, we did not detect any differences in the activation of p38, JNK, or NF-κB subunits in keratinocytes treated with propionate or butyrate, either alone or in combination with TLR2/6 ligands. While it remains possible that HDAC inhibition may affect negative regulators of these pathways or molecules that impact transcription factor binding, we can conclude that SCFAs do not activate proinflammatory signaling events in keratinocytes, and instead likely mediate their effects thru modulation of histone acetylation. Indeed, treatment of human monocytes by SCFA resulted in increased expression of the repressive NuRD

nuclear complex, a phenomenon not seen in keratinocytes. This may explain why keratinocytes do not exhibit the same anti-inflammatory effects to SCFAs as seen in monocytes or macrophages.

We observed *in vivo* that topical application of short-chain fatty acids to the dorsal skin of mice led to induction of many of the same pro-inflammatory genes that were increased in our *in vitro* experiments. Interestingly, the induction of these cytokines and chemokines did not require the addition of any TLR ligands, suggesting that they function to increase the responsiveness of murine skin to the resident microbiota. However, when these short-chain fatty acids were injected intradermally along with *P. acnes*, inflammatory gene induction was abrogated. These results support our early observations of the opposing effects of short-chain fatty acids on different cell types: when epidermal keratinocytes are exposed to SCFAs and environmental triggers through topical application, inflammatory gene expression was induced; however, when the same stimuli were applied to the myeloid-derived immunocytes residing in the dermis, the effects were largely anti-inflammatory.

In summary, these results illustrate how short-chain fatty acids act through an epigenetic mechanism to enhance cytokine production from epidermal keratinocytes. Together with the observation that skin-resident bacterial species can generate these molecules through anaerobic fermentation, our findings help to explain the skin's shift towards mounting an inflammatory reaction to normally commensal microbes. While further work, including studies of clinical correlation, should be conducted to definitively link the generation of short-chain fatty acids to

the development of skin inflammation, our studies are consistent with accepted models that propose a shift to anaerobic conditions and *P. acnes* hyperproliferation within follicles as contributing factors to the disease progression of acne. Additional studies to elucidate the mechanisms behind the divergent effects of HDAC inhibition on inflammatory gene expression in keratinocytes and myeloid-derived immunocytes are also merited, as these processes could provide a new avenue for therapeutic intervention of failures of epithelial tolerance. In conclusion, these findings advance the understanding of the skin immune system by illustrating a molecular mechanism through which the environment dictates how skin commensal microbes regulate inflammation.

Methods

Study Design. This study was designed to biochemically and molecularly characterize the response of human epidermal keratinocytes in culture to products of commensal microbes. Pilot experiments were performed to determine the magnitude of cytokine responses; following this experimental replicates of at least 3 (indicated in figure legends) were performed and analyzed to determine statistical significance as defined by $P < 0.05$. Sample analysis was performed quantitatively in an unblinded manner and confirmed by at last 3 independent experiments as indicated in the figure legends.

Cell Culture and Reagents. Human epidermal keratinocytes (Life Technologies, #C-001-5C) were cultured in EpiLife medium supplemented with 60 uM $CaCl_2$, 1x EpiLife Defined Growth Supplement, and 1x antibiotic/antimycotic (all reagents from Life Technologies). Cells were maintained in culture for 5-6 passages; treatments were performed on cells at approximately 70% confluency. Peripheral blood mononuclear cells were isolated from freshly drawn human blood separated with Ficoll-paque reagent (GE Life Sciences). Leukocytes were incubated in tissue culture-treated dishes for three hours at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 uM β-mercaptoethanol, and 1x antibiotic/ antimycotic (Life Technologies); plates were washed and the adherent monocyte populations were used in subsequent assays. SEB-1 sebocytes were cultured in Sebomed basal medium (Millipore) supplemented with 5 ng/mL recombinant human epidermal growth factor (Sigma). Sodium

butyrate, sodium propionate, and valeric acid were purchased from Sigma-Aldrich. MALP-2 was purchased from Enzo Life Sciences. Poly(I:C) HMW was purchased from Invivogen.

RNA Isolation, cDNA synthesis, RT-qPCR. Cultured cells were lysed in TRIzol reagent (Life Technologies) or PureLink RNA Lysis Buffer (Ambion/ Life Technologies). RNA was isolated according to the TRIzol RNA isolation protocol or using the PureLink RNA Isolation Kit according to manufacturer's instructions. RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific), and up to 1 µg of total RNA was reverse-transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-RAD). 50 ng of cDNA was used in RT-qPCR reactions using Taqman Universal PCR Master Mix and Taqman Gene Expression Assays (Life Technologies). RT-qPCR reactions were run on the CFX96 Real-Time System (Bio-RAD) and gene expression was determined by Δ(Δ(Ct)), normalized to GAPDH or β-Actin.

ELISA and Luminex Assays. Cell culture supernatants were isolated and cellular debris was removed by centrifugation at 600 x *g* for 5 minutes. Cytokine protein concentrations were determined using BD OptEIA Elisa Kits (BD Biosciences) or Milliplex MAP Immunoassay kits (EMD Millipore) according to manufacturer's instructions. Milliplex assays were analyzed on a MAGPIX instrument (Luminex Corporation).

Chromatin Immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed as previously described (*159*) with slight modifications. Briefly, cultured cells were crosslinked in 1% Formaldehyde in PBS for 10 minutes at room temperature, and the reaction was quenched by the addition of 125 mM glycine for 5 minutes. Cells were washed twice with cold PBS, scraped into 15 mL conical tubes in 10 mL PBS, pelleted by centrifugation at 600 x *g* for 5 minutes, and cell pellets were flash-frozen in liquid nitrogen and stored at -80°C. Cell pellets were resuspended in 1 mL lysis buffer (5 mM PIPES pH 8.0/ 85 mM KCl/ 0.5% NP-40 +fresh 1X Roche Protease Inhibitor Cocktail) and incubated on ice for 5 minutes. Nuclei were pelleted by centrifuging at 400 x *g* for 5 minutes at 4°C, and resuspended in 1 mL RIPA buffer (1X PBS/ 1% NP-40/ 0.5% sodium deoxycholate/ 0.1% SDS + fresh 1X Roche Protease Inhibitor Cocktail). Nuclear preps were transferred to 15 mL tubes and chromatin was sheared using a Diagenode Bioruptor Sonicator (4 x 7.5 minute cycles of 30 seconds on, 60 seconds off). Samples were then centrifuged at 16,000 x *g* for 15 minutes at 4°C, and supernatants were removed for IP. 900 µL of sonicated chromatin was incubated at 4°C overnight with 5 µg of primary antibody coupled to Dynabeads (Invitrogen). Beads were washed 5 times with 1 mL LiCl wash buffer (100 mM Tris pH 7.5/ 500mM LiCl/ 1% NP-40/ 1% sodium deoxycholate) and once in TE, then resuspended in 200 µL IP elution buffer (1% SDS/ 0.1M $NaHCO_3$). Crosslinked chromatin was eluted from the beads by incubation at 65°C for 1 hour and centrifugation at 16,000 x *g* for 3 minutes; supernatants were collected and crosslink reversal was completed by incubating at 65°C overnight.

Immunoprecipitated DNA was isolated using a Qiagen PCR Cleanup Kit and used in qPCR reactions along with input DNA (45 µL (5%) of sheared chromatin, reverse-crosslinked but not subjected to immunoprecipitation).

RNA Sequencing. Purified RNA was submitted to the UCSD Institute for Genomic Medicine (IGM) core facility for library preparation and high-throughput next-generation sequencing. Libraries were constructed using TruSeq Stranded mRNA Library Prep Kits (Illumina) and run on the HiSeq 2500 instrument (Illumina). Raw data was analyzed using Partek Flow and Partek Genomics Suite software (Partek) to determine transcript abundance and differentially expressed genes between samples. Gene Ontology analysis was performed using the DAVID Bioinformatics Database (*160, 161*).

Western Blots. Cells were collected in RIPA buffer containing 1X protease inhibitor cocktail (Roche) and lysed by incubation in ice water in a sonic bath for 30 minutes. Cellular debris was removed by centrifugation at 12,000 rpm for 10 minutes at 4°C, and the supernatant was collected and analyzed by BCA assay (Pierce). Protein concentrations were normalized, and indicated amounts of protein were diluted 1:4 in 4x Laemmli Sample Buffer containing β-mercaptoethanol (Bio-RAD) and heated at 100°C for 8 minutes. Samples were cooled, run on Mini-PROTEAN TGX gels (Bio-RAD), and transferred to PVDF membranes using TransBlot Turbo Transfer Packs (Bio-RAD) according to manufacturer's instructions. Membranes were blocked with Odyssey blocking

buffer (Licor), incubated overnight at 4°C with primary antibodies against indicated targets, washed, and incubated with IRDye-labeled species-specific secondary antibodies (Licor) for 1 hour at room temperature. Membranes were subsequently washed and visualized using the Odyssey Infrared Imager (Licor). Odyssey program software was used to perform densitometry analysis.

Bacterial cultures and short-chain fatty acid analysis. *Propionibacterium acnes* (ATCC6919 and ATCC29399) were maintained in Difco Reinforced Clostridial Medium (BD Biosciences) at 37°C in anaerobic conditions using BD GasPak EZ pouches (BD Biosciences). For fermentation assays, *P. acnes* ($1x10^7$ CFU) was inoculated into 5 mL rich medium cultures with and without 2% glycerol. Cultures were grown anaerobically at 37°C for 10 days to allow for anaerobic fermentation. For determination of short-chain fatty acid concentrations, bacterial cultures were extracted using ethyl acetate as described previously, with modifications (*162*). Briefly, 1 mL of bacterial supernatant was acidified with 0.5% ortho-phosphoric acid (Fisher), spiked with ^{13}C-labeled butyric acid to 50 µg/mL, and extracted twice with 0.5 mL ethyl acetate (Omnisolv, EMD Millipore) by vortexing for 5 minutes, followed by centrifugation at 14,000 x *g* for 10 minutes. Supernatants were combined and analyzed by gas chromatography-mass spectrometry (GC-MS; EI (70eV)) using an Agilent 5890 Series II GC coupled with 5971 MS detector. (Agilent) following temperature gradient and conditions as in the original paper. Acetic, propionic, isobutyric, butyric, isovaleric and valeric acids were quantified against a six (6)

non-zero levels calibration curve using the Free Fatty Acids Test Standard Mix (Restek).

For experiments performed with keratinocytes, *P. acnes* was cultured in antibiotic-free keratinocyte growth medium (EpiLife, Life Technologies) with and without 2% glycerol in anaerobic pouches at 37°C for 10 days. Bacteria were pelleted and supernatants were filtered through 0.2 micron filters prior to addition to keratinocyte cultures.

Mice. SKH-1 Elite hairless mice were originally purchased from Charles River Laboratories, and experimental mice were born, bred, and maintained at UCSD. All animals were housed in the University of California, San Diego (UCSD) SPF facilities in accordance with the guidelines of the Institutional Animal Care and Use Committee of UCSD. Animals in experimental models were matched for gender and age.

Topical and subcutaneous application of short-chain fatty acids and Propionibacterium acnes. 2% agar plates were made using UltraPure Agarose (Invitrogen) containing sodium butyrate or sodium propionate (0.2M) and poured 5 mm thick the day before experiments. Biopsy punches were used to prepare agar discs 8 mm in diameter, and 10 µL of dH2O or propionate or butyrate (2M) was applied to the discs 30 minutes before application to mice. Agar discs were applied to the dorsal skin of mice and secured in place using Tegaderm transparent wound dressing (3M Company). For intradermal injections, *P. acnes*

($1x10^7$ CFU in 20 µL dH2O or 1M propionate) or vehicle control was injected into the dermis using a 31-gauge insulin syringe. 8 hours after agar disc application or injections, mice were sacrificed, dorsal skin dissected, and 6 mm punch biopsies were taken at the disc or injection sites for mRNA extraction and RT-qPCR analysis.

siRNA-mediated gene silencing. Genes of interest were knocked down in keratinocytes using Silencer Select siRNA (Thermo Fisher Scientific) according to manufacturers instructions. Briefly, siRNA was prepared at 100 nM in OPTI-MEM medium containing 2.5% Lipofectamine RNAiMAX reagent (Thermo Fisher) and incubated at room temperature for 5 minutes. The siRNA-Lipofectamine solution was then diluted 1:10 in EpiLife media, yielding a final siRNA concentration of 10 nM and final Lipofectamine concentration of 0.25%. siRNA was applied to keratinocyte cultures for 16 hours, after which fresh media was added to cells. siRNA-treated Keratinocytes were allowed to recover for 48-72 hours prior to further stimulation.

Immunohistochemistry. Healthy human skin was obtained from excess skin tissue taken from mole removal surgery. Tissue biopsies were first fixed with 4% PFA then cryoprotected with sucrose before being embedded in OCT compound. Frozen skin sections were then subjected to immunofluorescence staining as described before (*163*). In brief, fixed and permeabilized skin sections were blocked with buffer containing 0.2M Glycine, 30 mg/ml BSA and 0.1 mg/ml

Saponin in PBS before incubating with primary antibodies followed by appropriate 488- or Cy3-coupled secondary antibodies. Nuclei were counter-stained with DAPI. All images were taken with an Olympus BX41 microscope. All images shown are representative image of n=3~5 human samples.

Statistical Analysis. GraphPad Prism 6.0 software was used for data analysis. Statistical significance was determined by one-way or two-way ANOVA as indicated in figure legends. $P<0.05$ was considered statistically significant, where $*P<0.05$, $**P<0.01$, $***P<0.001$ and $****P<0.0001$; exact P-values are indicated in figure legends.

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Chapter IV:

Mechanistic insight into HDAC8 and HDAC9 control of inflammatory gene expression in keratinocytes

Abstract

We have shown that inhibition of histone deacetylase activity in epidermal keratinocytes—specifically, inhibition of HDAC8 or HDAC9—results in an increased response to Toll-like receptor activation. This response is phenotypically distinct from the established role of short-chain fatty acids and other HDAC inhibitors in repressing the expression of proinflammatory cytokines and chemokines from myeloid-derived cells such as macrophages and dendritic cells. Here, we examine the transcriptomic effects of HDAC8 and HDAC9 silencing in keratinocytes through RNA-sequencing, demonstrating that like SCFA treatment, depletion of these HDACs increases the expression of sets of genes related to host defense and immune responses. Furthermore, we explore the consequences of SCFA treatment or HDAC silencing in sebocytes, identifying another cell type in the skin in which cytokine expression is influenced by HDAC activity. Next, we performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) to evaluate the genomic targets of HDAC8 and HDAC9. Finally, we use a recently developed technique, assay for transposase-accessible chromatin with sequencing (ATAC-seq) to evaluate the accessibility of chromatin in keratinocytes following depletion of HDAC8 or HDAC9. Together, these experiments lay a foundation for gaining deeper molecular insight into the unique abilities of HDAC8 and HDAC9 to control the epigenetic landscape of keratinocyte chromatin and influence the expression of TLR-responsive genes.

Introduction

Our results from Chapter III indicate that in keratinocytes, the histone deacetylase enzymes HDAC8 and HDAC9 function to limit the expression of numerous proinflammatory genes following TLR activation, as depletion of either of these HDACs resulted in an enhanced response to the lipopeptide MALP-2. These findings were surprising, as the majority of studies investigating the role of HDAC inhibition in immune and inflammatory processes have demonstrated an anti-inflammatory effect. Furthermore, it is interesting to note that two distinct HDAC enzymes, which are categorized in separate HDAC classes based on structure, appear to have redundant effects in repressing the transcription of various cytokines and chemokines.

In human cells, there are 18 enzymes that have been identified as possessing deacetylase function. Eleven of these molecules are considered "classical" HDACs, which require zinc for enzymatic activity, and are conveniently named HDAC1 through HDAC11. Seven additional proteins, the sirtuin family, have deacetylase activity, but do not share sequence similarity with the HDAC family and have a distinct NAD^+- dependent mechanism of deacetylation. The 11 classical HDACs are further divided into 3 classes: Class I (HDAC1, HDAC2, HDAC3, and HDAC8); Class II, which is broken into Class IIa (HDAC4, HDAC5, HDAC7, and HDAC9) and Class IIb (HDAC6 and HDAC10); and Class IV, containing only HDAC11 (illustrated in Table 2). Though named for the initial discovery that these proteins can remove acetyl groups from histone proteins, HDACs in fact regulate the acetylation status of numerous cellular proteins

besides histones, with studies identifying several hundred proteins with lysine residues modified by acetylation (*164*). Lysine acetylation is now recognized as a major regulatory mechanism in a wide range of cellular functions.

Class I HDACs are broadly expressed throughout mammalian tissues, and are the most well studied of the classes in regards to modification of histone proteins and regulation of transcriptional activity (*165*). All 4 members of the Class I HDAC family (HDAC1, HDAC2, HDAC3, and HDAC8) contain nuclear localization signals (NLS), and HDAC3 is the only member that also contains a nuclear export signal (NES). Thus, these HDACs are typically located in the nucleus and have been well studied for the role in controlling histone acetylation. These HDACS are typically found as part of larger multi-protein complexes that dictate DNA- and chromatin-binding, substrate specificity, and deacetylase activity. Many major nuclear regulatory complexes contain class I HDACs: HDAC1 and HDAC2 are found in the Sin3, NuRD, and CoREST co-repressor complexes, and HDAC3 is the catalytic subunit of the N-CoR/SMRT complex. Interestingly, HDAC8 can form homodimers that are capable of deacetylating certain substrates in the absence of any other proteins (*166*), a seemingly unique function of this class I HDAC.

Class IIa HDACs (HDAC4, HDAC5, HDAC7, and HDAC9) have several unique features that distinguish them from other classes (*167*). First, all Class IIa members possess both a NLS and NES, and can shuttle between the nucleus and the cytoplasm. Indeed, subcellular localization appears to be a major mechanism of regulating the capacity of these enzymes to function as

transcriptional regulators. Besides the NES, Class IIa HDACs all share conserved N-terminal domains that harbor serine residues that are targets for phosphorylation, which has been shown to regulate their subcellular localization. Furthermore, this N-terminal domain allows for interaction with other tissue-specific transcription factors such as myocyte enhancer factor 2 (MEF2), and these interactions can be regulated by serine phosphorylation. Compared to Class I, Class IIa HDAC enzymes have markedly reduced deacetylase activity, with one study finding them to be 1000 times less efficient at removing acetyl groups from standard substrates (*168*)—thus, it has been suggested that the non-enzymatic functions of these enzymes may be of greater importance than other classes. Class IIa HDACs are known to exist in larger co-repressor complexes containing regulatory factors such as SMRT, N-CoR, and other HDACs such as HDAC3.

Given the structural and functional diversity of HDAC8 and HDAC9, yet the common effect of acting to limit TLR-mediated cytokine induction in keratinocytes, we set out to explore these molecules in greater detail. First, we evaluate the role of HDAC inhibitors, and specific depletion of HDAC8 and HDAC9, in another epithelial cell type of the skin, the sebocyte. Next, we employ mouse models in an attempt to understand the contributions of keratinocyte-specific deletion of HDAC9 or depletion of commensal skin microbiota to the cutaneous inflammatory response. Finally, we utilize multiple genome-wide techniques coupled with next-generation sequencing, including ChIP-seq, ATAC-seq, and RNA-seq, to evaluate the chromatin interactions and transcriptomic

effects of these HDACs. Together, these data bring us closer to understanding the mechanistic functions of histone deacetylase activity in keratinocyte inflammatory gene expression.

Results

Depletion of HDAC8 and HDAC9 increases proinflammatory gene expression in keratinocytes.

To gain a more complete picture of the effects of inhibition of HDAC8 and HDAC9 in keratinocytes, we knocked down these enzymes with siRNA and performed RNA-sequencing analysis following treatment with the TLR2/6 ligand MALP-2. To begin, we looked at the sets of genes that were up-regulated and down-regulated in keratinocytes treated with siRNA specific for HDAC8 or HDAC9 (siHDAC8 and siHDAC9) compared to a scrambled, negative control (siCtrl). Using a fold-change cutoff of 1.5, we identified 152 genes up-regulated and 268 down-regulated when HDAC8 was depleted, and 275 genes up-regulated and 252 genes down-regulated when HDAC9 was depleted (Table 3-Table 6). Validation of the efficiency of our siRNA approach was provided by the fact that HDAC8 and HDAC9 were the most highly down-regulated genes in their respective datasets (Table 4 and Table 6). Importantly, there were very few immune or inflammatory-related genes of which the expression was significantly altered by depletion of either HDAC8 or HDAC in this setting without TLR activation, indicating that depletion of these enzymes does not directly affect the baseline levels of cytokine transcription in keratinocytes.

Next, we looked at the response to MALP-2 stimulation in cells treated with negative control siRNA. Comparable to MALP-2 stimulation in our previous RNA-seq experiment involving cells without siRNA intervention (discussed in Chapter III), treatment with MALP-2 led to the induction of 393 genes by a fold-

change of 1.5 or greater (Figure 17A). Analyzing this gene list with gene ontology programs gave predictable results, as the most highly represented biological processes were inflammatory response, response to wounding, defense response, and immune response (Figure 17B). Interestingly, stimulation with MALP-2 resulted in a larger set of genes of which expression decreased by 1.5 fold or more: in this case, 1511 genes were down-regulated (Figure 17A). We then wanted to determine the overall effect of HDAC depletion on the set of MALP-2-responsive genes in keratinocytes. To this end, we investigated the response of the set of 393 genes up-regulated in our control cells by MALP-2 in our cells depleted of HDAC8 or HDAC9. In cells where HDAC8 was silenced, 6.4% of the MALP-2-induced genes were further induced by 1.5-fold or more, while 33.3% of the MALP-2-induced genes were inhibited by 1.5-fold or more and 60.3% were unchanged. In cells with HDAC9 silenced, a larger proportion of MALP-2-induced genes were further up-regulated (15.5%), while 30% were inhibited and 54.5% were left unchanged. These results indicate that depletion of HDAC8 or HDAC9 in keratinocytes has a range of effects on TLR-mediated proinflammatory gene expression. Compared to the pan-inhibitory SCFA application shown in Figure 15, depletion of individual HDACs results in a smaller set of genes whose expression is enhanced. Future studies will be aimed at validating the increased transcriptional response seen through RNA-seq analysis by RT-qPCR and examining the levels of protein production by ELISA and Western blot, with a focus on the MALP-2 mediated genes that are further increased by knockdown of both HDAC8 and HDAC9.

HDAC inhibition increases inflammatory gene expression in sebocytes.

As a parallel to our studies in keratinocytes, we decided to investigate the effects of HDAC inhibition in sebocytes. Sebocytes are the cell type that make up the sebaceuous gland, a lipid-secreting gland connected to the hair follicle—together, these structures form what it called the pilosebaceous unit. The generation of sebum by the sebaceous gland helps to lubricate the hair and skin surface; however, sebum is also utilized by *Propionibacterium acnes* as a nutrient source. As *P. acnes* resides in the follicle, this bacterium and its metabolic products such as short-chain fatty acids are likely to come in contact with sebocytes. Similar to the effect seen in keratinocytes, application of the SCFAs propionate, butyrate, and valerate led to increases in the production of various proinflammatory cytokines following stimulation with MALP-2, including IL-1β, IL-8, TNFα, CXCL2, CXCL10, TSLP and CAMP (Figure 18A-G). However, other cytokines that were enhanced by SCFAs in keratinocytes were not altered by SCFA treatment in sebocytes, including IL-6 and CCL5 (Figure 18H-I).

Interestingly, the expression of some proinflammatory genes (such as TSLP and CAMP) was induced in sebocytes by treatment with SCFAs alone, in the absence of any TLR stimulation (Figure 18F-G). This is consistent with previous reports demonstrating that in other cell types, such as cells of the colonic epithelium, butyrate alone is sufficient to induce the expression of cathelicidin (*169*), but contrasts our data from keratinocytes, in which SCFA treatment enhanced TLR-mediated cytokine induction, but did not have major

effects in the absence of this signal. This could be due to the expression of certain free fatty acid receptors (FFARs) in these different cell types. As described in Figure 14D, keratinocytes express extremely low levels of any of the 3 known receptors for SCFAs. However, as illustrated in Figure 19A, sebocytes show strong expression of the mRNA transcripts for all three of these receptors. Thus, it is possible that some of the immunomodulatory effects of SCFAs on sebocytes cytokine expression are due to the activation of these membrane-bound FFARs, in contrast to keratinocytes where these receptors are not sufficiently expressed.

We next sought to characterize the effects of HDAC silencing in sebocytes as we previously explored in keratinocytes. To this end, we first measured the expression of the 11 classical HDACs in SEB-1 sebocytes (Figure 19B). In general, the pattern of HDAC expression in sebocytes looked similar to differentiated keratinocytes, with HDAC1, HDAC2 and HDAC3 being the most highly expressed, and HDAC8 and HDAC9 showing modest levels of expression. Having identified HDAC8 and HDAC9 as key regulators of cytokine expression in keratinocytes, we utilized the siRNA-mediated gene silencing techniques performed in keratinocytes to knock down these enzymes first (Figure 20A-B). Similar to keratinocytes, depletion of either of these HDACs led to significantly higher TLR-mediated induction of numerous proinflammatory cytokines, including IL-1β, IL-6, IL-8, and CXCL10 (Figure 20C-F). Thus, our data demonstrate that in sebocytes, inhibition of HDAC function by SCFAs, and specifically the depletion of HDAC8 and HDAC9, leads to higher levels of proinflammatory

cytokine expression following TLR activation. In contrast to keratinocytes, however, exposure to SCFAs alone may be sufficient to induce the expression of some of these cytokines and chemokines.

Mice lacking HDAC9 in epidermal keratinocyte show increased inflammatory response to topical bacterial application.

As we have observed increased cytokine responses in keratinocytes and sebocytes depleted of HDAC9 *in vivo*, we sought to characterize the *in vivo* effects of deficiency of this enzyme. We obtained a genetically modified mouse line where the sequence encoding HDAC9 was flanked by *loxP* sites ($HDAC9^{flox/flox}$). We then crossed this mouse with our own genetically modified mice expressing the Cre recombinase enzyme under the control of the keratin 14 (KRT14) promoter (KRT14-Cre+/-), a gene which is highly specific for epidermal keratinocytes. The results of multiple generations of crossing these mice were transgenic animals lacking both copies of HDAC9 in KRT14-positive cells ($HDAC9^{flox/flox}$ KRT14-$Cre^{+/-}$) and littermate controls ($HDAC9^{flox/flox}$ KRT14-$Cre^{-/-}$) expressing HDAC9 in all cells.

We then challenged these transgenic mice with topically applied *Staphylococcus aureus* (USA300 strain) using a method established previously in our lab (*170*). Bacteria were applied to the skin for 8 hours, after which skin was collected and analyzed for the induction of proinflammatory cytokines and chemokines. Figure 21 shows mRNA expression levels of various inflammatory markers in mouse skin following bacterial challenge. In general, mice lacking

HDAC9 in epidermal keratinocytes induced modestly higher levels of genes such as IL-1β, CXCL2, TNFα, and TSLP following *S. aureus* application compared to littermate controls (Figure 21A-E), though the data from this pilot experiment do not result in statistical significance. Importantly, levels of HDAC9 in skin samples from transgenic mice were reduced compared to controls (Figure 21F)—however, as these samples were full-thickness skin biopsies, the expression of HDAC9 was not fully eliminated, as cells from the dermis that do not express KRT14 and thus do not have active expression of the Cre recombinase were included in the samples.

Our previous data from Chapter III regarding the induction of proinflammatory gene expression in mouse skin by topical application of short-chain fatty acids led us to hypothesize that these SCFAs, through their mechanism of inhibition of HDAC activity, caused epidermal keratinocytes to break tolerance to resident skin microbes. To further examine this hypothesis, we utilized two different methods to deplete commensal skin microbial populations. First, we treated mice with an antibiotic cocktail in drinking water for 4 weeks, resulting in a reduction in the number of bacteria detected in swabs of the skin (Figure 23H). Where mice containing an intact skin microbiota mounted a modest inflammatory response to topically applied propionate, antibiotic-treated mice largely failed to respond to this treatment (Figure 23A-G), illustrating that epidermal keratinocytes require both signals—i.e. TLR activation by commensal microbes and HDAC inhibition by SCFAs—to induce proinflammatory gene expression.

Next, we aimed to further elucidate the impact of commensal microbes in our model using a more precise experimental approach. To this end, we utilized gnotobiotic mice, which are bred and raised in a sterile environment and lack resident microbial communities at all body sites. We subjected these gnotobiotic mice and specific pathogen-free (SPF) control mice to the same topical propionate application and again evaluated the inflammatory response. Similar to our antibiotic-treated mice, gnotobiotic mice induced lower levels of numerous proinflammatory mediators following propionate application compared to SPF controls (Figure 24A-D). Thus, it appears that without a resident microbiome to supply ligands for TLRs and other PRRs in keratinocytes, SCFA-mediated inhibition of HDAC activity alone is not sufficient to induce the expression of proinflammatory cytokines and chemokines, a result that is consistent with our *in vitro* studies from Chapter III.

HDAC8 and HDAC9 chromatin immunoprecipitation.

One mechanism by which histone deacetylase enzymes exert their regulatory effects on transcriptional activity is through the alteration of chromatin structure brought on by the removal of lysine acetylation on histone proteins. HDACs are often recruited to regions of chromatin as part of larger multi-protein complexes, targeting their deacetylase function to specific areas of the genome. Thus, we sought to determine where in the genome of keratinocytes HDAC8 and HDAC9 might localize, as this could provide clues into the genes whose transcription may be modulated by these enzymes. Chromatin

immunoprecipitation (ChIP) is an established methodology for the profiling of interactions between proteins of interest and a cell's genetic content. Briefly, this method relies on the fixing of cells with formaldehyde, which works to covalently crosslink proteins, nucleic acids, and other macromolecular complexes with chemical constituents within approximately 2 angstroms. Fixed cells can then by lysed, chromatin sheared via sonication, and antibodies can be used to precipitate specific proteins along with their crosslinked partners. One can use this method to pull down transcription factors and other nuclear regulatory proteins along with their associated chromatin, which can then be analyzed using general PCR or next-generation sequencing technologies to identify genomic regions.

We set out to use ChIP to profile the areas where HDAC8 and HDAC9 associate with the genome to determine whether direct chromatin association is responsible for the regulation of any of genes where we saw increases in the TLR-mediated induction of expression following depletion of these HDACs. Using antibodies specific for HDAC8 and HDAC9, as well as isotype-matched IgG controls, we pulled down HDAC-associated regions of chromatin and obtained raw sequencing data from Illumina HiSeq 4000 analysis via the UCSD IGM core. Sequencing resulted in approximately 30 million reads per sample, of which 96-97% uniquely aligned to the human genome using the Bowtie2 aligner and the hg19 reference genome in Partek Flow software. These aligned reads were then processed using Partek Genomics Suite software to detect peaks that

were significantly enriched in each sample, providing approximately 2500 peaks per sample.

The genomic coordinates of these peaks were then used to identify the nearest gene-coding sequence to the peak—this analysis provides insight into whether HDACs are binding in a gene's promoter region, around the transcriptional start site, or within the coding sequence itself. After performing this analysis, and filtering out genes where our IgG control was also detected, we were left with a surprisingly small list of genes around which binding was detected—81 for HDAC8 and 75 for HDAC9, 38 of which were common between the two. The lists of genes that are nearest to regions where peaks were detected by ChIP-seq are displayed in Table 7. Surprisingly, this initial analysis did result in any correlation with the sets of genes further induced by MALP-2 following HDAC depletion identified through RNA-seq. These results could be indicative of indirect effects of HDAC8 and HDAC9 on inflammatory gene expression—rather than regulating transcriptional activity by binding and altering chromatin near these genes, these HDACs may control the expression or activity of upstream signaling molecules or transcription factors through acetylation—either acetylation of the factors themselves or of regions of chromatin regulating their expression. Furthermore, these HDACs could be regulating the acetylation status of distant regulatory regions such as enhancers or other cis-regulatory elements, sites that our current analysis fails to identify.

Chromatin accessibility following HDAC8 and HDAC9 depletion.

Packaging of a cell's genetic content into the nucleus involves the formation of chromatin, a compact and dynamic structure resulting from the association of DNA with histone proteins to form nucleosomes. This compaction can dictate transcription, as inactive regions of the genome are sequestered while biologically active regions, including promoter and enhancer regions, are left in a more open state, accessible to transcriptional machinery. Working to dictate chromatin structure are a variety of factors that come together to form and epigenetic code, including histone modifications, DNA methylation, nucleosome positioning, transcription factors and chromatin remodeling complexes. Recent technological advances have birthed a variety of extremely useful techniques that provide genome-wide information on this epigenetic structure, as methods such as ChIP-seq and DNase-seq can provide insight into transcription factor binding, nucleosome positioning, and chromatin accessibility. Recently, another technique was introduced to aid in the probing of chromatin structure, termed "Assay of Transposase-Accessible Chromatin with sequencing" or ATAC-seq (*171*). Briefly, ATAC-seq takes advantage of a hyperactive Tn5 transposase enzyme, which can insert adapters with known sequences at regions of open, accessible chromatin. These regions can then be amplified and analyzed by next-generation sequencing technology to gain a genome-wide picture of where chromatin is accessible. ATAC-seq is advantageous as it can be performed on small sample sizes compared to other techniques (typical experiments require

only 50,000 cells of starting material) with a protocol that only takes a few hours to complete.

With these considerations in mind, we set out to use ATAC-seq to determine the differences in chromatin accessibility brought on by depletion of HDAC8 or HDAC9 in keratinocytes. As an additional condition, we treated another group of keratinocytes with propionate, which is a strong inhibitor of HDAC function and presumably results in a looser, more open chromatin structure. We performed ATAC reactions using published protocols (*171, 172*) and obtained raw sequencing data from Illumina HiSeq 4000 analysis via the UCSD IGM core. Sequencing resulted in approximately 35 million reads per sample, of which roughly 55% aligned to the human genome using the Bowtie2 aligner and the hg19 reference genome in Partek Flow software. These aligned reads were then processed using Partek Genomics Suite software to detect peaks that were significantly enriched in each sample. Table 8 contains the information regarding reads, alignments, and peaks detected for each sample.

We then sought to identify peaks detected in samples lacking HDAC8 or HDAC9 or treated with propionate that were not detected in control samples, indicating that these regions were more accessible following HDAC inhibition. As illustrated in Table 8, we identified several thousand peaks in each of our three comparison samples that were not detected in the control sample. The genomic coordinates of these peaks can then be used to identify the nearest gene-coding sequence to the peak—this analysis can provide insight into whether HDAC inhibition results in increased chromatin accessibility in a gene's promoter region,

around the transcriptional start site, or within the coding sequence itself. Performing this analysis resulted in the identification of 2000 to 3000 genes per sample that exhibited increased chromatin accessibility in or near the coding sequence. Figure 24 is a Venn diagram showing the comparison of the identified genes from each sample, illustrating that each of the conditions—knockdown of HDAC8, HDAC9, or treatment with propionate—had a set of genes (roughly one-third) that were unique to that condition, while the remaining two-thirds were shared with one or more of the other conditions. Interestingly, 25% of the genes associated with peaks identified were common between all three conditions.

Discussion

In this chapter, we set out to attempt to uncover details that will assist in understanding the molecular mechanisms behind the control of inflammatory gene expression in keratinocytes by HDAC8 and HDAC9 and the increased cytokine response we observed in keratinocytes following TLR activation when these enzymes were inhibited. First, we evaluated the transcriptomic effects of siRNA-mediated knockdown of HDAC8 and HDAC9 by RNA-seq: initially, we looked at what genes exhibited altered expression in resting keratinocytes when HDAC8 or HDAC9 were depleted, and then we investigated how depletion of these enzymes changed the response to stimulation with the TLR2/6 ligand MALP-2. We next conducted experiments on another cell type in the skin, the sebocyte, to determine how treatment with short-chain fatty acids or depletion of HDAC8 or HDAC9 affected the response to MALP-2. We performed *in vivo* experiments using mice lacking HDAC9 in epidermal keratinocytes to evaluate how loss of this enzyme affected cytokine induction in response to short-term topical bacterial challenge. Additionally, we repeated *in vivo* experiments to measure the response to topical SCFA application when skin commensal microbes had been depleted, either via antibiotics supplied in drinking water or through the use of gnotobiotic mice. Finally, we attempted to evaluate chromatin-wide interactions of HDAC8 and HDAC9 using ChIP-seq, to determine where in the genome these molecules are localized, as well as ATAC-seq, to evaluate how chromatin accessibility is altered in keratinocytes when HDAC8 or HDAC9 are depleted.

Our RNA-seq data demonstrated that in untreated cells, depletion of HDAC8 or HDAC9 resulted in altered expression of 420 and 527 transcripts, respectively, of which approximately half were up-regulated and half were down-regulated. Importantly, HDAC8 and HDAC9 were the most highly down-regulated genes in their respective datasets, indicating that our siRNA methodology was effective in depleting the target genes and did not have major off-target effects. In regards to TLR-mediated gene induction, we saw that knocking down HDAC8 and HDAC9 enhanced 6.4% and 15.5% of the 393 genes induced by MALP-2. These numbers were somewhat lower than we expected based on our initial qPCR screening of candidate genes (illustrated in Figure 8), but it is important to note the differences in methodology between qPCR and RNA-seq that may lead to discrepancies in quantitative analysis.

Furthermore, the number of TLR-mediated genes further up-regulated by knockdown of specific HDACs was dramatically lower than our earlier RNA-seq experiments evaluating the effects of SCFA treatment (Figure 15B demonstrates that nearly 30% of genes induced by MALP-2 were further enhanced by propionate treatment). However, this makes sense when one considers that SCFAs are broad-acting, pan-inhibitory molecules that affect the activity of most of the 11 HDAC enzymes, in addition to the possible non-HDAC effects of SCFAs. Thus, the effects we see from SCFA treatment are likely to be the additive result of inhibiting all HDACs, whereas our siRNA experiments show the specific effects of loss of activity of HDAC8 or HDAC9. While relatively modest, we did observe an increased response to topical bacterial challenge in mice

lacking HDAC9 in epidermal keratinocytes, suggesting that the role of HDAC9 is consistent in keratinocytes *in vivo*. Future experiments with these mice, or with isolated epidermal keratinocytes, will be beneficial to more fully understand the role of HDAC9 in regulating inflammatory gene expression from keratinocytes. Additionally, the generation of a mouse line where HDAC8 can be conditionally deleted from keratinocytes and other cell types would be of great value in examining the *in vivo* effects of this enzyme.

We were intrigued by the similarity of our results from experiments in a sebocyte cell line stimulated with MALP-2 and SCFAs to those previously observed in keratinocytes. Sebocytes are an important cell type in the pilosebaceous unit, have been shown to be a prominent source of inflammatory cytokines in response to *P. acnes* stimulation, and are likely an active contributor to the inflammatory processes in the development of acne vulgaris (*45, 173*). As *P. acnes* resides within the follicle, sebocytes would be readily exposed to any SCFAs that this bacterium may generate. Like keratinocytes, these cells exhibit hyper-responsiveness to TLR activation when exposed to HDAC inhibitors, providing more support to the hypothesis that SCFA generation by *P. acnes* contributes to the development of inflammatory acne. Interestingly, and in contrast to our keratinocyte data, some proinflammatory cytokines and the antimicrobial peptide cathelicidin are induced in sebocytes by SCFA exposure alone, in the absence of any TLR activation. However, it has been noted that in other epithelial cell types—such as colonic epithelial cells and airway epithelial cells—treatment with butyrate results in the induction of CAMP expression (*169,*

174-176). In colonic epithelial cells in particular, this butyrate-mediated induction of CAMP expression was dependent on butyrate inducing cell differentiation. In addition to butyrate, other HDAC inhibitors (such as other SCFAs, TSA, or analogs of SCFAs such as phenylbutyrate) can induce CAMP expression in these cell types, with a variety of mechanistic explanations offered, ranging from induction of differentiation to activation of the vitamin D receptor to changes in histone acetylation (*177-179*). We hypothesize that the TLR-independent effects of SCFAs in sebocytes could be due to the high levels of known free fatty acid receptors expressed in these cells, which are only weakly expressed by keratinocytes. Future studies can be aimed at addressing this hypothesis, either through the depletion of these FFARs or blocking their activation using inhibitors such as pertussis toxin, and evaluating the effects of SCFA stimulation.

We also observed that consistent with keratinocytes, specific depletion of HDAC8 or HDAC9 in sebocytes recapitulated the effects of SCFA stimulation. While other HDACs have not yet been evaluated in this sebocytes model, this data provides more evidence for a previously unrecognized role of HDAC8 and HDAC9 in controlling the inflammatory response of epithelial cells. As sebocytes have a variety of additional functions in the skin not related to acne pathogenesis, such as sebum production and adipokine secretion (*180*), the effects of SCFAs and the role of HDAC8 and HDAC9 should be further explored in this cell type.

We next sought to further examine our hypothesis that in our *in vivo* mouse model of topical SCFA application, the cytokine response we observe is

due to epidermal keratinocytes losing tolerance to resident skin microbial communities. We utilized two models of skin commensal depletion—antibiotic treatment via drinking water and gnotobiotic mice. Our antibiotic model, though not completely effective, did result in a reduction in bacteria cultured from the skin that correlated with a decreased response to topically applied propionate. Future replication of this experiment should seek to more definitively evaluate the reduction in skin commensal bacteria by isolating and measuring microbial DNA rather than relying on culture-based methods; additionally, the response to propionate observed in control mice was weak compared to our prior experiments, making statistical significance difficult to achieve in these data.

Similar to the results in our antibiotic-treated mice, gnotobiotic mice lacking all commensal microbes also exhibited a markedly reduced cytokine response to topical propionate application. Experiments with gnotobiotic mice are somewhat harder to interpret, as the total lack of resident microbial communities can have wide-ranging effects on various developmental processes, including effects on the immune system. While we didn't detect major differences in baseline levels of cytokine expression in the skin of these mice compared to SPF controls, further evaluation of these mice should be performed to fully characterize the presence of different populations of cutaneous immunocytes. In all cases—whether mice are treated with antibiotics to deplete the skin microbiome or gnotobiotic mice are used—additional methods such as immunohistochemistry should be conducted to definitively identify keratinocytes as the source of the observed cytokine expression in response to SCFAs. As our

previous data demonstrates, there could be a contribution from sebocytes, even in the absence of microbial populations to provide TLR activation.

Finally, we have collected data from preliminary experiments aiming to evaluate the genome-wide effects of HDAC8 and HDAC9 that will presumably be of great value towards gaining mechanistic insight into the role of these enzymes. Chromatin immunoprecipitation (ChIP) studies allowed us to isolate and sequence regions of the genome with which HDAC8 and HDAC9 associate in untreated keratinocytes. Assay for transposase-accessible chromatin (ATAC) experiments identified regions of the genome that were accessible in untreated cells, and also identified regions where accessibility was increased following depletion of HDAC8, HDAC9, or treatment with propionate. Ultimately, these datasets should be cross-referenced with each other, as well as with datasets generated from our RNA-seq experiments of keratinocytes treated with MALP-2 following SCFA treatment or HDAC inhibition. Combining the data from these related experiments will allow us to address several key questions regarding the regulation of chromatin by HDAC8 and HDAC9 in keratinocytes. For example, are regions where these HDACs are shown to bind also regions where accessibility increases following HDAC inhibition or depletion? Furthermore, are these regions of HDAC binding or chromatin accessibility functionally relevant to the genes that show increased TLR-mediated expression when HDAC activity is compromised? If true, this data would suggest a previously unknown mechanism for direct control of certain inflammatory genes in keratinocytes by specific HDACs. Alternatively, it is possible that HDACs act to control master regulators

of cytokine induction—i.e. signaling molecules or transcription factors acting downstream of TLR2/6—or distant cis-regulatory chromatin elements such as enhancers that can modulate transcriptional activity. While these datasets are preliminary and need to be more comprehensively analyzed, we are hopeful that they will prove useful in developing a functional understanding of the mechanisms behind our observation of increased inflammatory response of keratinocytes following HDAC inhibition.

Together, these experiments support the hypothesis that inflammatory gene expression in keratinocytes is regulated by epigenetic mechanisms including histone acetylation, histone deacetylase activity, and chromatin accessibility. Furthermore, this data provides evidence that through the generation of HDAC-inhibitory molecules such as short-chain fatty acids, microbes that reside on the skin and other body sites can affect these epigenetic processes in host cells and have impacts on inflammatory and immunological processes. While the effects of HDAC inhibition have been largely anti-inflammatory in classical immune cells and models of intestinal inflammation, our *in vitro* and *in vivo* effects demonstrate that in cutaneous epithelium, SCFA-mediated inhibition of HDACs results in a breakdown of innate immune tolerance to resident microbial populations.

Methods

Cell Culture and Reagents. Human epidermal keratinocytes (Life Technologies, #C-001-5C) were cultured in EpiLife medium supplemented with 60 uM $CaCl_2$, 1x EpiLife Defined Growth Supplement, and 1x antibiotic/antimycotic (all reagents from Life Technologies). Cells were maintained in culture for 5-6 passages; treatments were performed on cells at approximately 70% confluency. SEB-1 sebocytes were cultured in Sebomed basal medium (Millipore) supplemented with 5 ng/mL recombinant human epidermal growth factor (Sigma). Sodium butyrate, sodium propionate, and valeric acid were purchased from Sigma-Aldrich. MALP-2 was purchased from Enzo Life Sciences. Poly(I:C) HMW was purchased from Invivogen.

siRNA-mediated gene silencing. Genes of interest were knocked down in keratinocytes and sebocytes using Silencer Select siRNA (Thermo Fisher Scientific) according to manufacturers instructions. Briefly, siRNA was prepared at 100 nM in OPTI-MEM medium containing 2.5% Lipofectamine RNAiMAX reagent (Thermo Fisher) and incubated at room temperature for 5 minutes. The siRNA-Lipofectamine solution was then diluted 1:10 in EpiLife media, yielding a final siRNA concentration of 10 nM and final Lipofectamine concentration of 0.25%. siRNA was applied to keratinocyte cultures for 16 hours, after which fresh media was added to cells. siRNA-treated Keratinocytes were allowed to recover for 48-72 hours prior to further stimulation.

Mice. All animals were housed in the University of California, San Diego (UCSD) facilities in accordance with the guidelines of the Institutional Animal Care and Use Committee of UCSD. All animal protocols were reviewed and approved by UCSD IACUC (approval number S09074). HDAC9$^{flox/flox}$ mice were generously provided from the laboratory of Dr. Neil Weintraub at Augusta University, and were bred with mice expressing Cre recombinase under control of the KRT14 promoter (KRT14-Cre) in our facilities to delete HDAC9 from epidermal keratinocytes. Mice were challenged topically with USA300 strain of *Staphylococcus aureus* as described previously (*170*) and RNA was isolated from skin samples and analyzed for expression of proinflammatory cytokines and chemokines by RT-qPCR. Epidermal keratinocytes were isolated from tail skin of these mice and cultured as described previously (*181, 182*), and treated with MALP-2 (400 ng/mL) for 4 hours, after which RNA was isolated and analyzed by RT-qPCR.

SKH-1 Elite hairless mice were originally purchased from Charles River Laboratories, and experimental mice were born, bred, and maintained at UCSD. To deplete commensal microbes, mice were supplied antibiotics in drinking water (50 units/ml benzylpenicillin potassium, 2.0 mg/ml neomycin sulfate and 0.5mg/ml cefoperazone sodium salt) for 4 weeks. Gnotobiotic mice and SPF control mice (Taconic C57BL/6) were bred and housed at the UCSD Gnotobiotic Facility. Antibiotic-treated, gnotobiotic and control mice were challenged topically with propionate solution or water as described previously (*183*).

Chromatin Immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed as previously described (*159*) with slight modifications. Briefly, cultured cells were crosslinked in 1% Formaldehyde in PBS for 10 minutes at room temperature, and the reaction was quenched by the addition of 125 mM glycine for 5 minutes. Cells were washed twice with cold PBS, scraped into 15 mL conical tubes in 10 mL PBS, pelleted by centrifugation at 600 x *g* for 5 minutes, and cell pellets were flash-frozen in liquid nitrogen and stored at -80°C. Cell pellets were resuspended in 1 mL lysis buffer (5 mM PIPES pH 8.0/ 85 mM KCl/ 0.5% NP-40 +fresh 1X Roche Protease Inhibitor Cocktail) and incubated on ice for 5 minutes. Nuclei were pelleted by centrifuging at 400 x *g* for 5 minutes at 4°C, and resuspended in 1 mL RIPA buffer (1X PBS/ 1% NP-40/ 0.5% sodium deoxycholate/ 0.1% SDS + fresh 1X Roche Protease Inhibitor Cocktail). Nuclear preps were transferred to 15 mL tubes and chromatin was sheared using a Diagenode Bioruptor Sonicator (4 x 7.5 minute cycles of 30 seconds on, 60 seconds off). Samples were then centrifuged at 16,000 x *g* for 15 minutes at 4°C, and supernatants were removed for IP. 900 µL of sonicated chromatin was incubated at 4°C overnight with 5 µg of primary antibody coupled to Dynabeads (Invitrogen). Beads were washed 5 times with 1 mL LiCl wash buffer (100 mM Tris pH 7.5/ 500mM LiCl/ 1% NP-40/ 1% sodium deoxycholate) and once in TE, then resuspended in 200 µL IP elution buffer (1% SDS/ 0.1M $NaHCO_3$). Crosslinked chromatin was eluted from the beads by incubation at 65°C for 1 hour and centrifugation at 16,000 x *g* for 3 minutes; supernatants were collected and crosslink reversal was completed by incubating at 65°C overnight.

Immunoprecipitated DNA was isolated using a Qiagen PCR Cleanup Kit and used in qPCR reactions along with input DNA (45 µL (5%) of sheared chromatin, reverse-crosslinked but not subjected to immunoprecipitation).

RNA Sequencing. Purified RNA was submitted to the UCSD Institute for Genomic Medicine (IGM) core facility for library preparation and high-throughput next-generation sequencing. Libraries were constructed using TruSeq Stranded mRNA Library Prep Kits (Illumina) and run on the HiSeq 2500 instrument (Illumina). Raw data was analyzed using Partek Flow and Partek Genomics Suite software (Partek) to determine transcript abundance and differentially expressed genes between samples. Gene Ontology analysis was performed using the DAVID Bioinformatics Database (*160, 161*).

Assay for Transposase-Accessible Chromatin with Sequencing. ATAC-seq was performed according to a protocol adapted from elsewhere (*171, 172*), with slight modifications. Briefly, nuclei from $2x10^5$ primary keratinocytes were isolated by lysing cells on ice in 200 uL ice-cold lysis buffer (10 mM Tris-HCl, pH=7.5, 10 mM NaCl, 3 mM MgCl2, 0.1% IGEPAL CA-630); 50 uL was then transferred to a new tube and nuclei were pelleted at 1000 *x g* for 10 minutes at 4°C. Supernatants were removed and nuclear pellets were resuspended in transposase reaction mix (25 uL TD buffer, 2.5 uL Tn5 transposase, 22.5 uL nuclease-free H_2O; all components from the Nextera DNA Library Prep Kit, Illumina) and incubated at 37°C for 30 minutes. Following transposition reaction,

DNA was cleaned up using MinElute PCR Purification Kit (QIAGEN) according to manufacturer's instructions and eluted in 10 uL H_2O. Libraries were then amplified using a KAPA Library Amplification Kit and customized Nextera PCR primers using the following PCR conditions: 72°C for 5 minutes, 98°C for 30 seconds, and then thermocycling (98°C for 10 seconds, 63°C for 30 seconds, and 72°C for 1 minute) for 11-13 cycles, monitoring the amplifications against standards provided in the KAPA kit to prevent oversaturation. Reactions were stopped and products cleaned up using MinElute PCR Purification Kit (QIAGEN), quantified using a Qubit dsDNA Assay (Thermo Fisher), visualized on a gel, and submitted to the UCSD IGM core for sequencing on an Illumina Hiseq 2500 instrument. Raw data was then processed and analyzed using Partek Flow and Partek Genomics Suite software (Partek) to determine genomic locations (using hg19 reference genome) of the amplified chromatin.

Statistical Analysis. GraphPad Prism 6.0 software was used for data analysis. Statistical significance was determined by one-way or two-way ANOVA as indicated in figure legends. $P<0.05$ was considered statistically significant, where *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$.

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Chapter V:

Conclusions and perspectives

Summary

In this dissertation, we began with a hypothesis that signals from skin-resident commensal microbes could influence epigenetic processes that control inflammatory gene expression in keratinocytes. Indeed, we confirmed that *Propionibacterium acnes* generates short-chain fatty acids that inhibit HDAC activity in keratinocytes. Based on previous reports of the effects of HDAC inhibitors on cytokine expression from cells of the innate immune system, we suspected that inhibition of HDAC activity would result in decreased expression of pro-inflammatory cytokines and antimicrobial molecules from keratinocytes following TLR activation. Surprisingly, however, these SCFAs exerted an opposite effect in keratinocytes than in myeloid-derived cells, and resulted in increased responsiveness to TLR ligands. These effects were not specific to SCFAs alone, as other, structurally distinct chemical HDAC inhibitors recapitulated this response, suggesting that these effects are linked to alteration of the epigenetic effects of histone deacetylase enzymes.

Additionally, we identified HDAC8 and HDAC9 as likely targets of these bacterial SCFAs, as depletion of either of these HDACs in keratinocytes also resulted in increased levels of cytokine expression following stimulation with TLR ligands. Together, these results shed light on a previously unrecognized mechanism of regulation of inflammatory gene expression in epithelial cells. Furthermore, this work provides a novel hypothesis for the development and pathogenesis of inflammatory acne vulgaris, and illustrates how the local microenvironment of follicles in the skin, host factors, and the microbiome can

combine to initiate focal inflammation. As summarized in Figure 25, we hypothesize that changes in the follicular microenvironment (including plugging of the follicle by hyperproliferative keratinocytes, excess production of sebum by the sebaceous gland, and a decrease in oxygen levels in the follicle) lead to an expansion of the resident *Propionibacterium acnes* population and a metabolic shift towards anaerobic fermentation of sebum lipids that results in the generation of short-chain fatty acids. These molecules then act on keratinocytes and sebocytes that line the follicle, inhibiting HDAC function—in particular, HDAC8 and HDAC9—and increasing the cytokine output in response to *P. acnes* and other commensal bacteria, ultimately leading to the recruitment and activation of immunocytes and the inflammation seen clinically around acne lesions.

Future studies will allow us to gain more insight into the mechanisms behind the regulation of inflammatory gene expression in keratinocytes by HDAC8 and HDAC9. Specific experiments designed to identify the genomic targets of these HDACs, as well as proteomic exploration into protein-protein interactions between these HDACs and other regulatory factors will shed light where and how HDAC8 and HDAC9 act to control gene expression. Furthermore, experiments with mice harboring targeted deletion of HDAC9 in epidermal keratinocytes or other cell types will allow for further understanding of the importance of HDAC9 in inflammatory processes as well as other specific functions of the skin. Additionally, future work will expand on our studies to more conclusively elucidate the role of *Propionibacterium acnes* on the development of

inflammatory acne, to determine whether our hypothesis of SCFA generation is indeed an underlying factor in disease progression.

Further characterization of HDAC8 and HDAC9 in regulation of keratinocyte cytokine expression

In Chapter III, we identified HDAC8 and HDAC9 as key regulators of cytokine expression in keratinocytes, as depleting either of these enzymes results in increased responsiveness to TLR activation comparable to when cells are treated with broad-acting HDAC inhibitors. In Chapter IV, we describe some of the steps taken to gain a better understanding of the mechanism by which these HDACs function to control inflammatory gene expression. ChIP-seq experiments provide a dataset towards an understanding of the genomic interactions of HDAC8 and HDAC9, and ATAC-seq allows us to investigate how depletion of HDAC8 or HDAC9 alters the overall accessibility of chromatin in the nucleus. While these genome-wide studies are helpful preliminary information, there is still much work to be done to gain a clearer understanding of how these histone deacetylase enzymes function in keratinocytes.

First, studies should be continued to investigate the chromatin interactions of HDAC8 and HDAC9. While our initial ChIP-seq experiment has provided some preliminary results for genomic locations of HDAC8 and HDAC9 localization, these studies should be replicated and validated. Furthermore, these studies were conducted only in resting, untreated cells. It would be of great interest to investigate how TLR activation or SCFA treatment may alter the

genomic binding of these enzymes. For example, it may be possible that following stimulation with a TLR ligand, one of these HDACs are recruited to regulatory regions such as promoters or enhancers that control inflammatory gene expression as a cellular mechanism to dampen or prevent prolonged gene expression. Thus, our initial experiments may not have identified all of the possible regions HDAC8 or HDAC9 bind to control gene expression. Furthermore, the effects of SCFA inhibition of HDAC activity on chromatin binding should be looked into to determine whether these molecules actually impair the ability of HDACs to localize to the necessary regions, or simply inhibit their ability to repress gene transcription through deacetylation.

Once regions of the genome where HDAC8 and HDAC9 bind have been definitively mapped, it would be informative to evaluate how the chromatin landscape changes around these regions when HDACs are inhibited or depleted from the cells. Our ATAC-seq data can be used to identify regions that are more open and accessible following knockdown of HDAC8 or HDAC9 or inhibition via propionate treatment, and it would be interesting to determine whether these are sites of direct HDAC binding. One could also conduct additional ChIP-seq experiments to look for histone modifications associated with active gene transcription or binding of key transcription factors or RNA polymerase when HDAC function has been reduced. Together, these studies could help to gain a more thorough understanding of how HDAC8 and HDAC9 regulate the epigenetic state of the keratinocyte genome and control transcriptional activity.

Finally, studies should be conducted to elucidate the protein-protein interactions of HDAC8 and HDAC9 in keratinocytes. HDACs are often found as part of larger protein complexes both within and outside of the nucleus, and their deacetylase activity is not limited to histone substrates (*166, 184, 185*). As such, HDACs can have impacts on the function of a wide variety of proteins within the cell that may help explain the increased responsiveness to TLR activation we see in keratinocytes. Co-immunoprecipitation experiments should be executed and paired with mass spectrometry analysis to identify proteins with which HDAC8 and HDAC9 interact in keratinocytes, and these studies could prove valuable in multiple ways. First, one might use these studies to identify multi-protein complexes that contain HDAC8 or HDAC9. These findings could help to focus the study of HDAC genomic interactions described above, and could be used to determine whether the deacetylase activity of HDAC8 or HDAC9 are integral to their effects on gene transcription. Additionally, this type of analysis could identify targets of HDAC8 and HDAC9-mediated deacetylation, potentially uncovering novel regulatory mechanisms. For example, it has been illustrated that reversible acetylation of the p65 subunit of NF-κB can control functions such as subcellular localization, DNA binding affinity, interaction with IκBα, and transcriptional activation (*186*). While our studies did not identify any alterations in the acetylation status of p65 following depletion of HDAC8 or HDAC9, it is possible that other key proteins involved in the regulation or induction of inflammatory gene expression are targets of these HDACs. As mentioned in regards to additional ChIP experiments, these co-IP experiments should be

conducted in keratinocytes not only in a resting state, but also following stimulation with TLR ligands or with HDAC inhibitors to fully elucidate where and how HDAC8 and HDAC9 exert their regulatory functions.

Propionibacterium acnes in acne pathogenesis

In regard to potential clinical implications, this work introduces a new hypothesis for the role of the commensal bacterium *Propionibacterium acnes* in the pathogenesis of inflammatory acne vulgaris. We show that under specific environmental conditions—namely, a low oxygen environment rich in lipid substrates, which are characteristics consistent with an occluded follicle in the skin—*P. acnes* can undergo anaerobic fermentation to generate short-chain fatty acids that inhibit HDAC activity in host cells and increase the cytokine response of epidermal keratinocytes to TLR activation. Thus, our work suggests that metabolic processes in *P. acnes,* dictated by the local microenvironment, may be a trigger for the initiation of keratinocyte inflammatory gene expression and subsequent immune infiltration seen in acne. However, these findings bring to light several additional questions relating to other recent studies regarding *P. acnes* in acne pathogenesis and the clinical implications of this disease.

First, it is interesting to consider the possibility that other skin commensal microbes may also have the ability to generate these biologically active SCFAs and initiate acne development. Recent work has suggested that in similar hypoxic, lipid-rich environments, *Staphylococcus epidermidis* can generate SCFAs such as butyrate (*187, 188*). Considering the fact that the microbiota of

the follicle may not be exclusively composed of *P. acnes*, other species of resident bacteria generating SCFAs could be a possible trigger for follicular inflammation. Strong support for our hypothesis of SCFAs being an initiator of keratinocyte inflammatory gene expression within the follicle could come from the detection of these SCFAs in clinical samples of affected follicles. While our *in vitro* work shows that bacteria can generate these molecules in an artificial environment, and our *in vivo* work demonstrates the effect of SCFAs on inflammation through different routes of exposure in the skin, we have yet to definitively prove that these processes are active and important in clinical acne. If true, the generation of these SCFAs within the follicle could be an advantageous target for therapeutic intervention.

Furthermore, it would be worthwhile to evaluate the capacity of different strains of *P. acnes* to generate SCFAs. As described by Fitz-Gibbon and colleagues in a recent characterization of *P. acnes* on healthy and diseased skin, several strains of this bacterium were identified that are found predominantly on the skin of acne patients but not in healthy individuals (*133*). Additional studies have demonstrated that distinct *P. acnes* strains differ in their effects on inducing proinflammatory cytokines and cell toxicity in keratinocytes and sebocytes (*45, 189*). Thus, it is interesting to wonder if the metabolic capabilities of these strains are different from those more commonly found in healthy skin. Obtaining a library of distinct *P. acnes* strains would allow for comparison of SCFA production in an *in vitro* setting, as we have previously performed. As the two different strains utilized in our experiments yielded different levels of SCFA generation, it

would not be surprising to see differences in healthy versus acne-associated ribotypes of *P. acnes*. Furthermore, one could utilize the full genomic sequences of the various strains to search for differences in genetic sequences coding for enzymes involved in the biosynthesis of SCFAs. However, genomic analysis performed in the original paper only identified a small number of differences between healthy and acne-associated strains, none of which were reported to be involved in metabolic processes. Furthermore, the presence or absence of a gene identified through sequencing does not definitively correlate with expression; thus, a more precise approach would involve analyzing the metatranscriptome of the follicular contents from healthy and acne-affected skin, allowing one to determine whether there are differences in expression levels of biological pathways controlling SCFA generation. This type of analysis, in combination with experiments aimed at detecting SCFAs from clinical samples as described above, could strongly support the notion that *P. acnes* fermentation and generation of SCFAs is linked to the development or pathogenesis of acne vulgaris.

Our hypothesis regarding environmental changes promoting the production of SCFAs by *P. acnes* and these molecules driving the cutaneous inflammatory response behind acne pathogenesis are consistent with host factors that impact skin biology and the development of acne. Acne most commonly occurs during puberty—affecting an estimated 95% of teenage boys and 85% of teenage girls (*190*)—when levels of androgen and estrogen hormones produced by the body are drastically changing. These hormones exert

various effects on the skin, including on cells of the pilosebaceous unit, such as keratinocytes, sebocytes, and dermal papilla cells, that are thought to contribute to the development of acne and fit well with our *P. acnes*-derived SCFA-mediated model. In particular, increased levels of the androgen hormones testosterone, dihydrotestosterone (DHT), and dehydroepiandrosterone sulfate (DHEA-S), which occur during puberty, especially in males, and have been linked to acne (*191*). Additionally, acne is a cutaneous manifestation in cases of hyperandrogenism in women, congenital adrenal hyperplasia (*192*), as part of the SAHA (seborrhea, acne, hirsutism and alopecia) syndrome (*193*), and often following topical or systemic anabolic steroid application (*194*)—all cases in which levels of androgen hormones are increased systemically and in the skin. Furthermore, positive correlations have been observed between serum levels of DHT and DHEA-S and acne lesion counts in adult men and women (*195*).

Mechanistically, androgen hormones are known to increase the proliferation, size, and levels of sebum lipogenesis in sebocytes (*196-198*). Furthermore, androgens and other hormones have been suggested to increase the proliferation of follicular keratinocytes, leading to follicular hyperkeratiniztion and comedogenesis (*199*). Taken together, this means that during times of increased androgen production—such as puberty—follicles of the skin are frequently plugged and hyper-sebaceous, consistent with clinical observations. These conditions would thus provide the low oxygen, lipid-rich environment for *P. acnes* to proliferate and generate SCFAs. As we model in Figure 25, the SCFAs generated by *P. acnes* could then act to inhibit HDACs in keratinocytes and

sebocytes, resulting in cytokine production and the recruitment of immune cells to further perpetuate the inflammatory condition.

Final Conclusions

In conclusion, this dissertation describes a novel effect of inhibition of histone deacetylase activity on the induction of proinflammatory gene expression in keratinocytes following Toll-like receptor activation. Contrary to the well-established role that HDAC inhibitors play in suppressing cytokine expression from cells such as monocytes and macrophages, we demonstrate that HDAC inhibition, and particularly inhibition of HDAC8 or HDAC9, results in keratinocytes that are hyper-responsive to TLR ligands. We also show that certain commensal skin microbes, such as *Propionibacterium acnes*, can generate short-chain fatty acids that inhibit keratinocyte HDAC activity and increase cytokine output. While continued studies should be conducted to more definitively determine the mechanisms by which HDAC enzymes control inflammatory gene expression in keratinocytes, our results have illustrated a crucial role for these epigenetic modifiers in the regulation of cutaneous immunity and inflammation, and identified a means through which members of the microbiome can affect this process. This work advances our understanding of the interactions between the skin and its resident microbial inhabitants, and has the opportunity to be relevant to the development of novel therapeutic strategies for managing inflammatory skin conditions.

APPENDIX

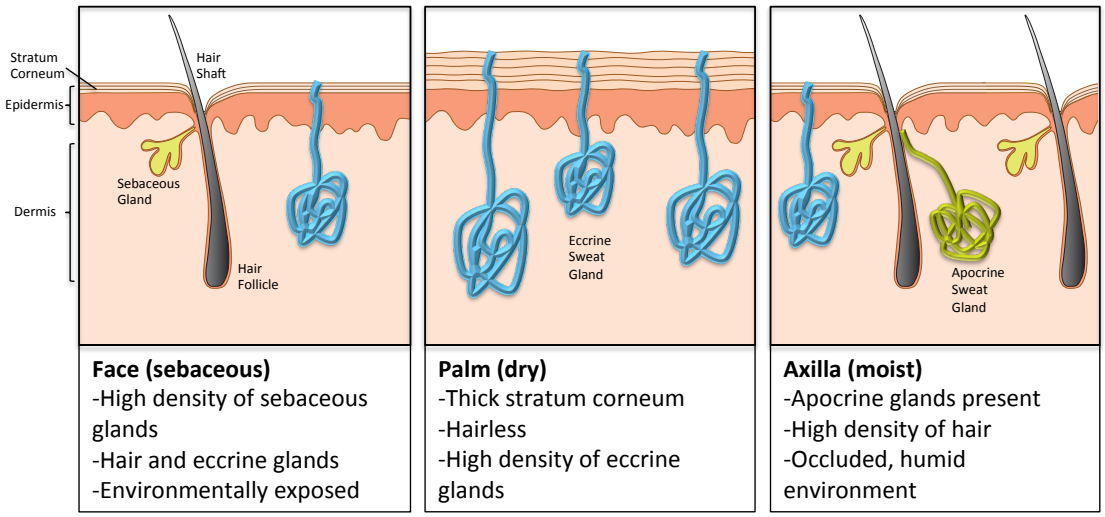

Figure 1. Diversity of the skin at different body sites.
The physical and chemical features of the skin are not uniform across the body; rather, different anatomical locations show vast diversity in organization and the distribution of appendages and glandular structures. Shown are representative illustrations of the three major categories of skin environments.

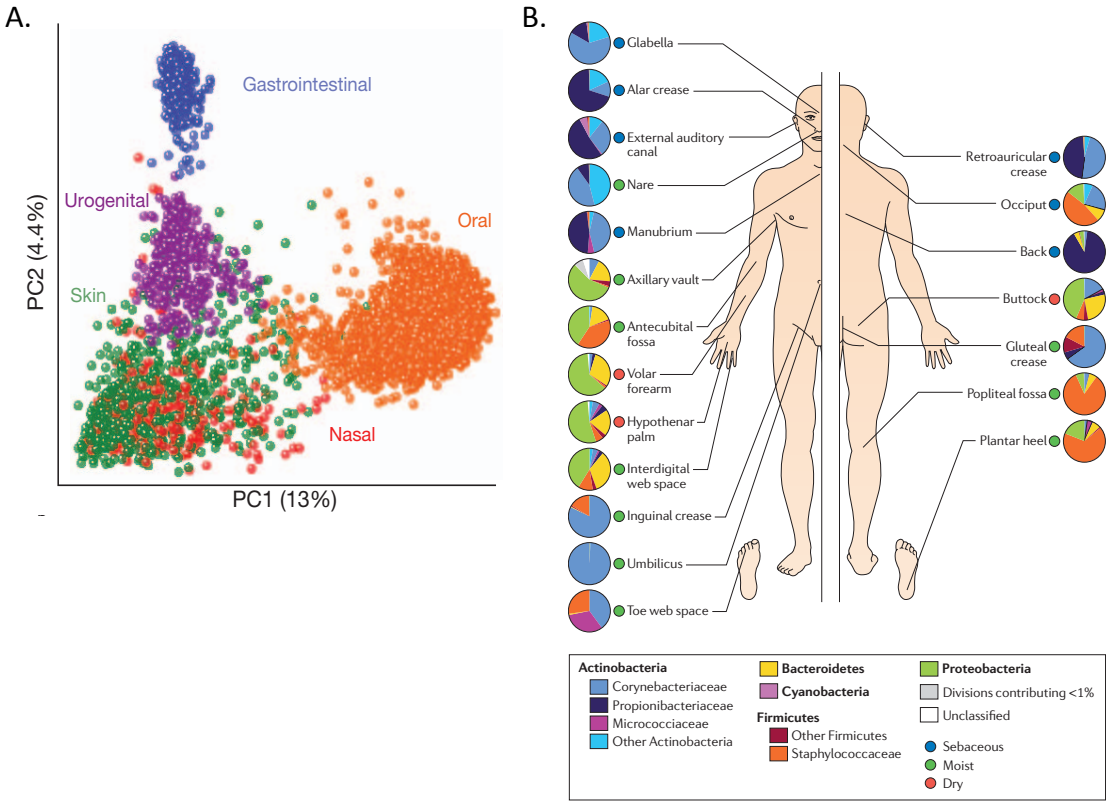

Figure 2. Diversity of the microbial communities populating the skin. **A)** Microbial communities inhabiting the skin are extremely diverse, more so than those residing at other epithelial surfaces of the body. **B)** In addition, communities residing on the skin at different anatomical locations differ greatly in their compositions. Reprinted from references (*22*) and (*25*) with permission from Nature Publishing Group.

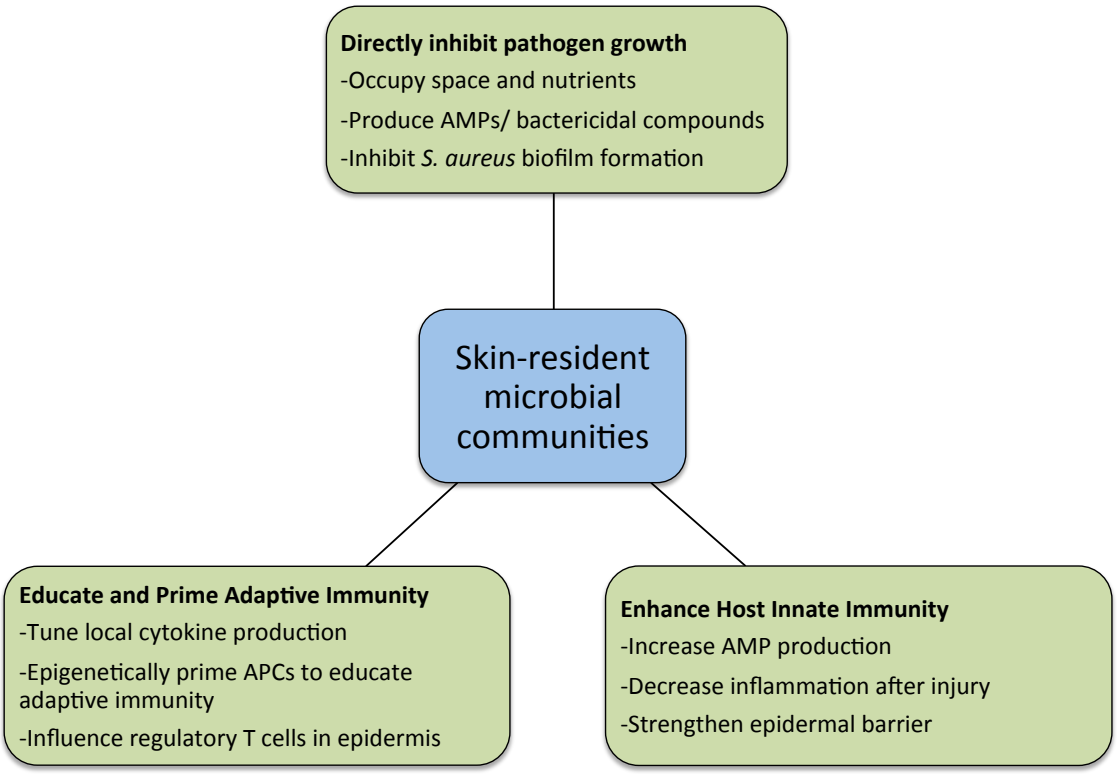

Figure 3. Functional associations with microbial communities on the skin. Microbial communities on the skin contribute to host immune defense through a variety of mechanisms, as illustrated here.

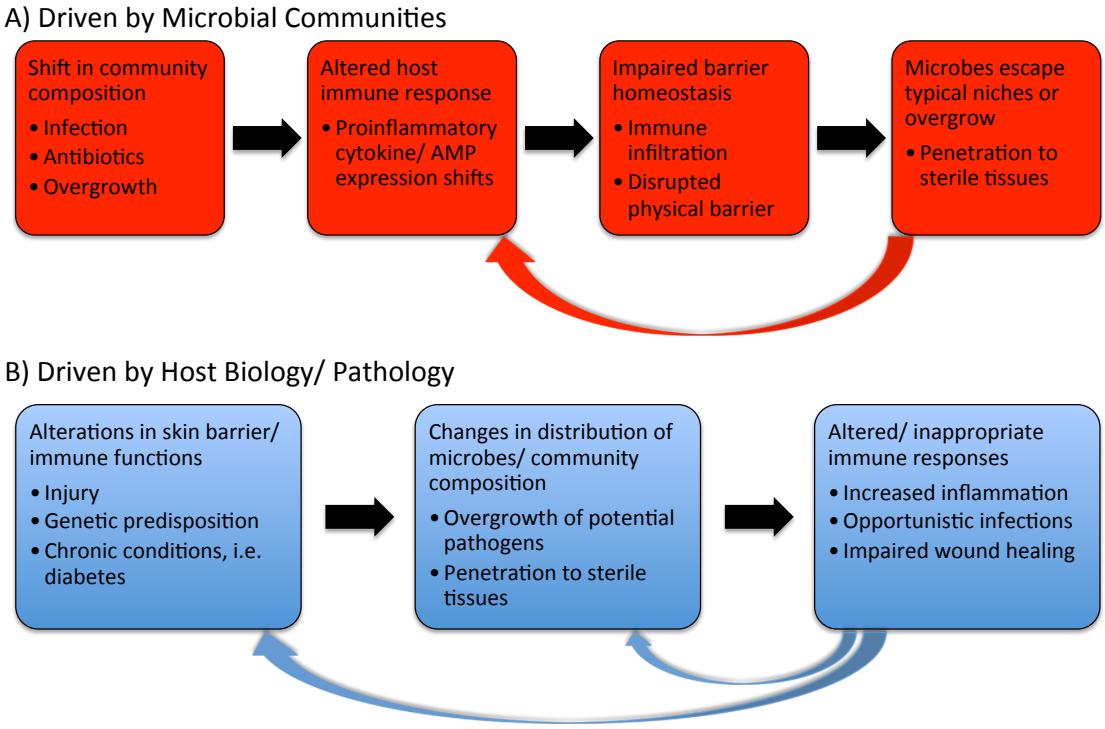

Figure 4. Models of dysbiosis of skin-resident microbes.
Dysbiosis of skin-resident microbes has been associated with a variety of dermatological pathologies; however, it remains unclear whether alterations in microbial communities or intrinsic features of the host initiate these processes.

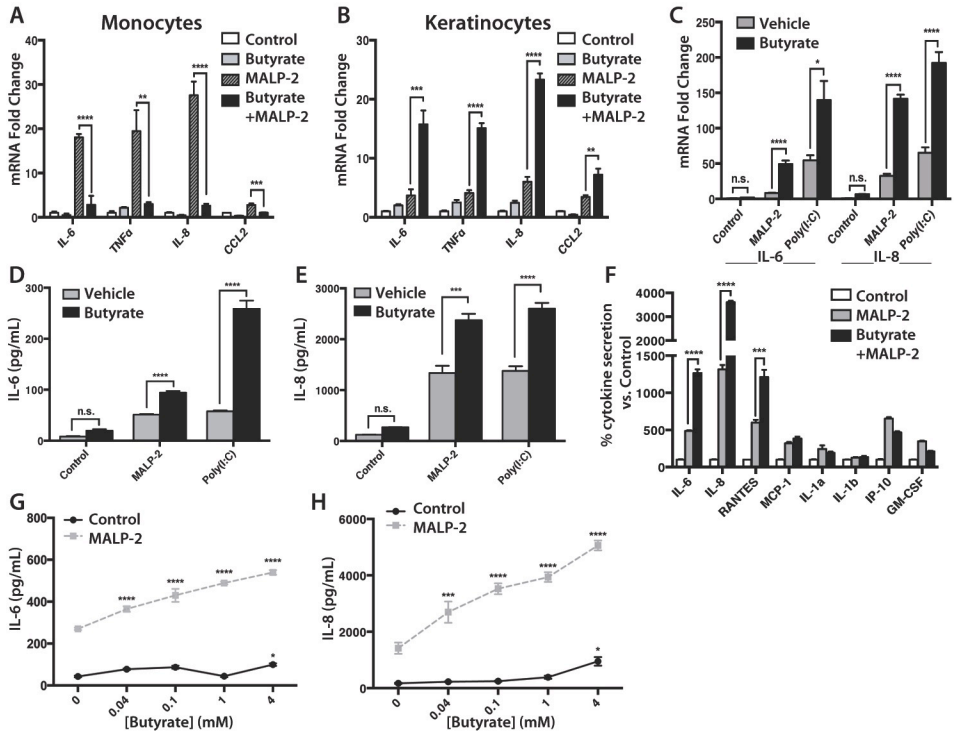

Figure 5. Butyrate inhibits TLR-mediated cytokine production from monocytes while increasing cytokine expression in keratinocytes.
Human monocytes or keratinocytes were incubated in culture medium +/- sodium butyrate (2mM) for 1 hour and subsequently stimulated with Toll-like Receptor ligands. (**A**) Cytokine mRNA expression in monocytes stimulated with MALP-2 (200 ng/mL) for 4 hours (n=3; *P*<0.0001 (IL6), *P*=0.0052 (TNF), *P*<0.0001 (IL8), *P*=0.0004 (CCL2); one-way ANOVA). (**B**) Cytokine mRNA expression in keratinocytes stimulated with MALP-2 for 4 hours (n=3; *P*=0.0008 (IL6), *P*<0.0001 (TNF), *P*<0.0001 (IL8), *P*=0.005 (CCL2); one-way ANOVA). (**C**) IL-6 and IL-8 mRNA expression in keratinocytes stimulated with MALP-2 or Poly(I:C) (1 ug/mL) for 4 hours (n=3; *P*=0.9929, *P*<0.0001, and *P*=0.0117 (IL6); *P*=0.6314, *P*<0.0001, and *P*<0.0001 (IL8); one-way ANOVA). (**D-E**) IL-6 and IL-8 levels in culture supernatants of keratinocytes stimulated with MALP-2 or Poly (I:C) for 24 hours (n=4; *P*=0.7438, *P*<0.0001, and *P*<0.0001 (IL6); *P*=0.7028, *P*=0.0003, and *P*<0.0001 (IL8); one-way ANOVA). (**F**) Secreted protein concentration from keratinocytes stimulated with MALP-2 (100 ng/mL) for 24 hours, normalized to untreated cells (n=3; *P*<0.0001 (IL6), *P*<0.0001 (IL8), and *P*=0.0009 (RANTES); one-way ANOVA). (**G-H**) IL-6 and IL-8 levels in culture supernatants of keratinocytes treated with MALP-2 (200 ng/mL) with increasing concentrations of sodium butyrate for 24 hours (n=3; *P*=0.019 (IL6 control); *P*<0.0001 (IL6 MALP-2); *P*=0.0262 (IL8 control); *P*=0.0004 and *P*<0.0001 (IL8 MALP2); tow-way ANOVA). All data are mean +/- SEM and representative of at least 3 experiments.

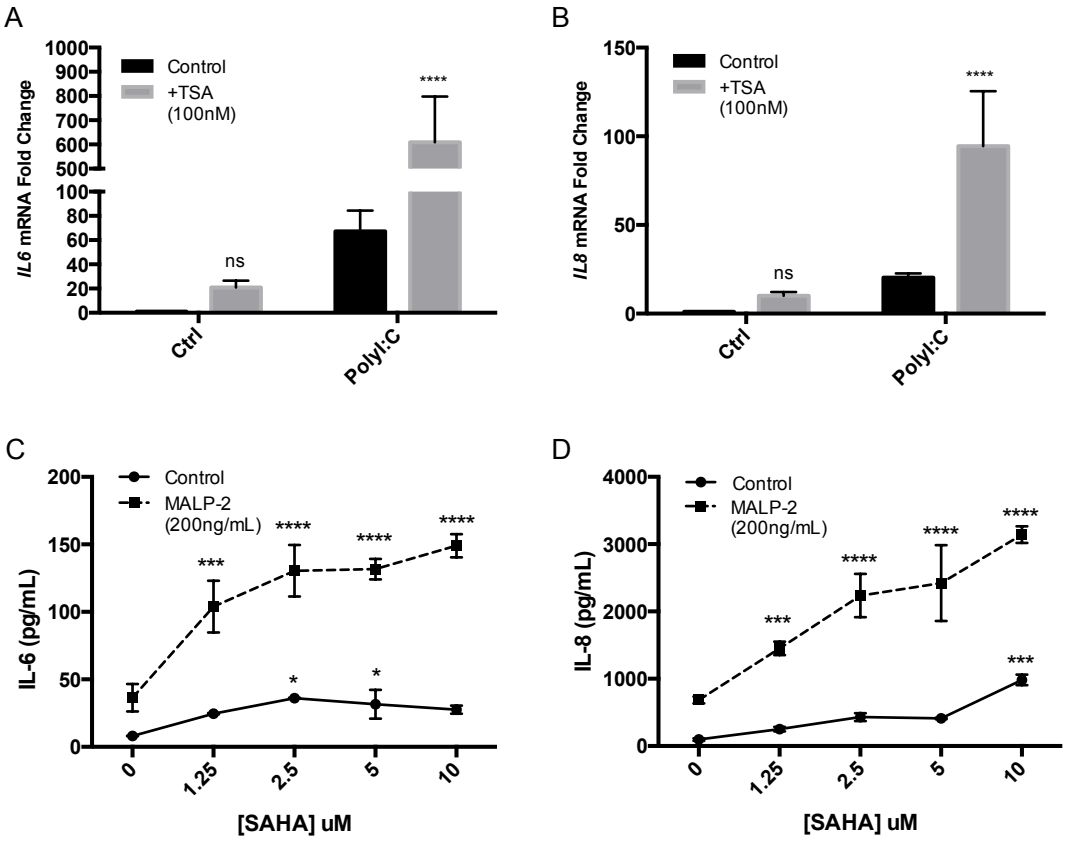

Figure 6. Chemical HDAC inhibition increases keratinocytes cytokine response to TLR activation.

(A-B) Human keratinocytes were stimulated with the TLR3 ligand Poly(I:C) (1 ug/mL) for 4 hours in the presence or absence of Trichostatin A (100nM) and induction of IL-6 and IL-8 mRNA was evaluated by RT-qPCR (n=4; *P*=0.9475 and *P*<0.0001 (IL6); *P*=0.6685 and *P*<0.0001 (IL8); two-way ANOVA). (**C-D**) Keratinocytes were stimulated with the TLR2/6 ligand MALP-2 (200 ng/mL) for 24 hours in the presence or absence of increasing concentrations of suberoylanilide hydroxamic acid (SAHA). Secreted levels of IL-6 and IL-8 in culture supernatants were evaluated by ELISA (n=3; *P*=0.3328, *P*=0.0270, *P*=0.0800 and *P*=0.1919 (IL6 Control); *P*<0.0001 (IL6 MALP-2); *P*=0.7925, *P*=0.1575, *P*=0.1999 and *P*<0.0001 (IL8 control); *P*=0.0003 and *P*<0.0001 (IL8 MALP-2); two-way ANOVA). Data shown as mean +/- SEM and representative of 3 independent experiments.

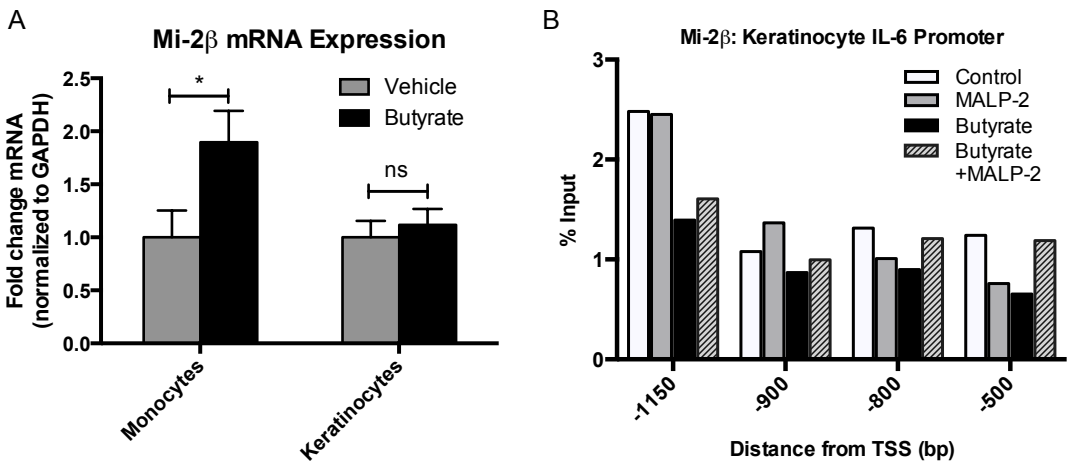

Figure 7. Negative regulatory NuRD complex is not induced in keratinocytes by butyrate.

(A) Monocytes or NHEK were treated with sodium butyrate (2mM) for 2 hours, after which the expression of CHD4 (Mi-2β) was analyzed by RT-qPCR and normalized against GAPDH (n=3; *P*=0.0164 (monocytes) and *P*=0.3918 (keratinocytes); 2-tailed student's unpaired T-test). Data are mean +/- SEM and representative of 2 independent experiments. (**B**) Chromatin immunoprecipitation (ChIP) was performed using an antibody against Mi-2 β in keratinocytes treated with MALP-2 (200ng/mL) or sodium butyrate (2mM) for 2 hours. Precipitated DNA was analyzed by RT-qPCR for regions of the IL-6 promoter (n=1; data representative of 2 independent experiments).

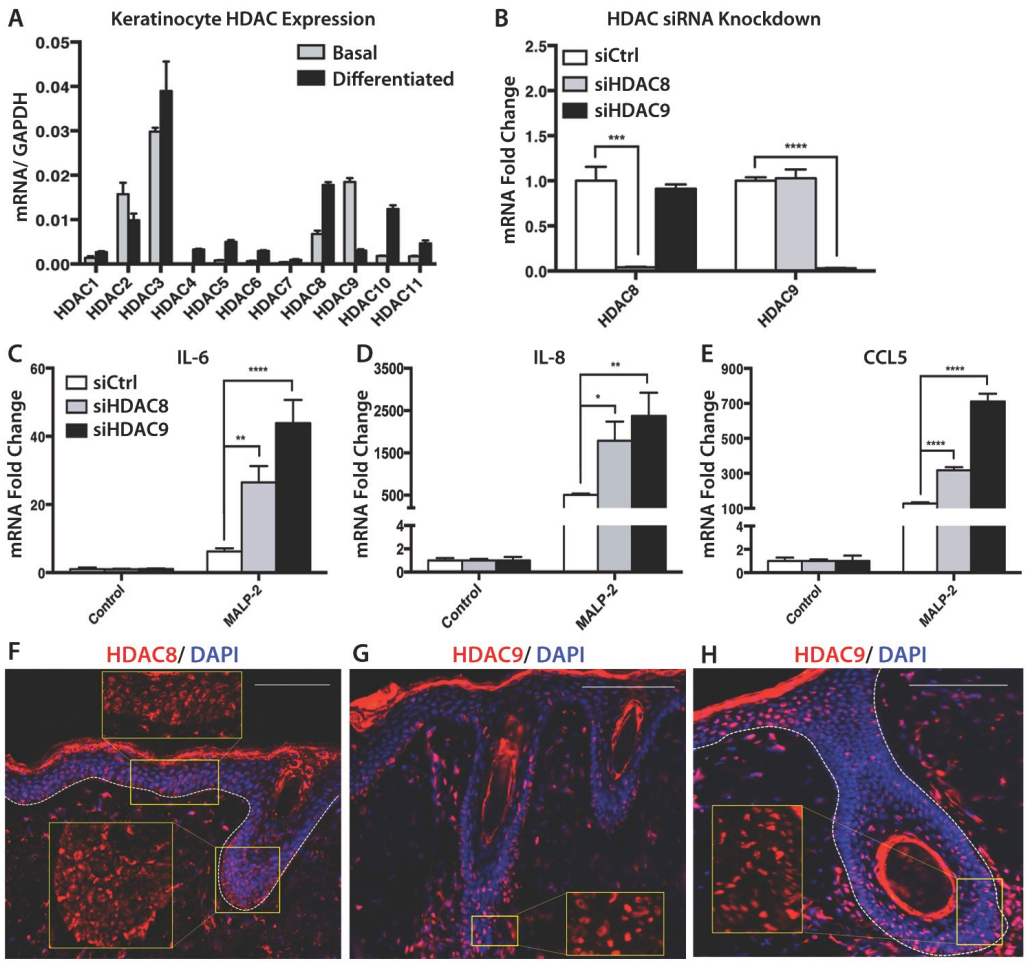

Figure 8. HDAC8 and HDAC9 control inflammatory gene expression in keratinocytes.

(A) Expression levels of 11 HDAC enzymes in basal or calcium-differentiated keratinocytes, normalized to GAPDH (n=3). **(B)** Expression of HDAC8 and HDAC9 in keratinocytes following siRNA-mediated gene knockdown (n=3; *P*=0.0008 (HDAC8); *P*<0.0001 (HDAC9); one-way ANOVA) (**C-E**) Levels of IL6, IL8, and CCL5 expression in keratinocytes treated with siRNA specific for HDAC8 or HDAC9, stimulated with MALP-2 for 4 hours (n=3; *P*=0.0034 and *P*<0.0001 (IL6); *P*=0.0237 and *P*=0.0019 (IL8); *P*<0.0001 (CCL5); two-way ANOVA). (**F-H**) Normal human skin sections were stained with anti-HDAC8 (F) or anti-HDAC9 (G-H) antibodies (red) as indicated and nuclei were counter-stained with DAPI (blue). Scale bar = 100 μm. Zoom-in pictures of HDAC8 or HDAC9 staining in epidermal keratinocytes or in the follicular keratinocytes surrounding the hair follicle are highlighted in yellow boxes. White dashed line indicates the epidermal/dermal junction. All data are mean +/- SEM and representative of at least 3 experiments.

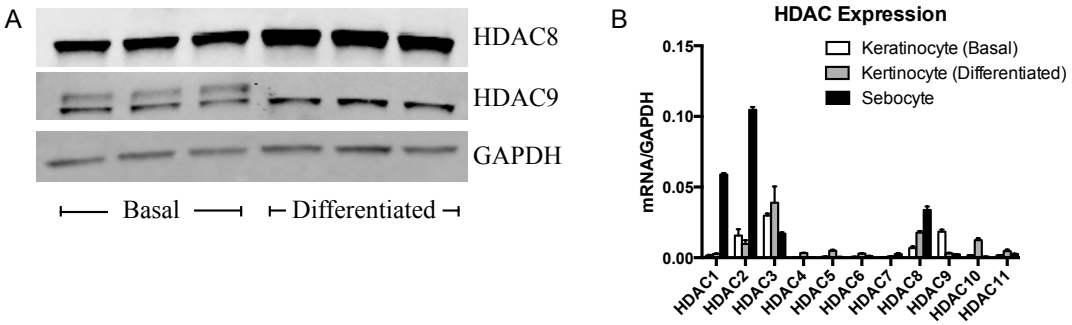

Figure 9. HDAC expression in human epidermal keratinocytes and sebocytes.
(**A**) Cell lysates were collected from human keratinocytes grown in sub-confluent conditions with low calcium (basal) or confluent conditions with high calcium (1.5 mM for 72 hours, differentiated) and analyzed by Western Blot for HDAC8, HDAC9, and GAPDH (n=3; representative of 3 independent experiments). (**B**) Expression levels of HDAC1- HDAC11 in SEB-1 human sebocytes and human keratinocytes (basal and differentiated) were measured by RT-qPCR and normalized against GAPDH. Data are mean +/- SEM of n=3 samples, representative of at least 3 independent experiments.

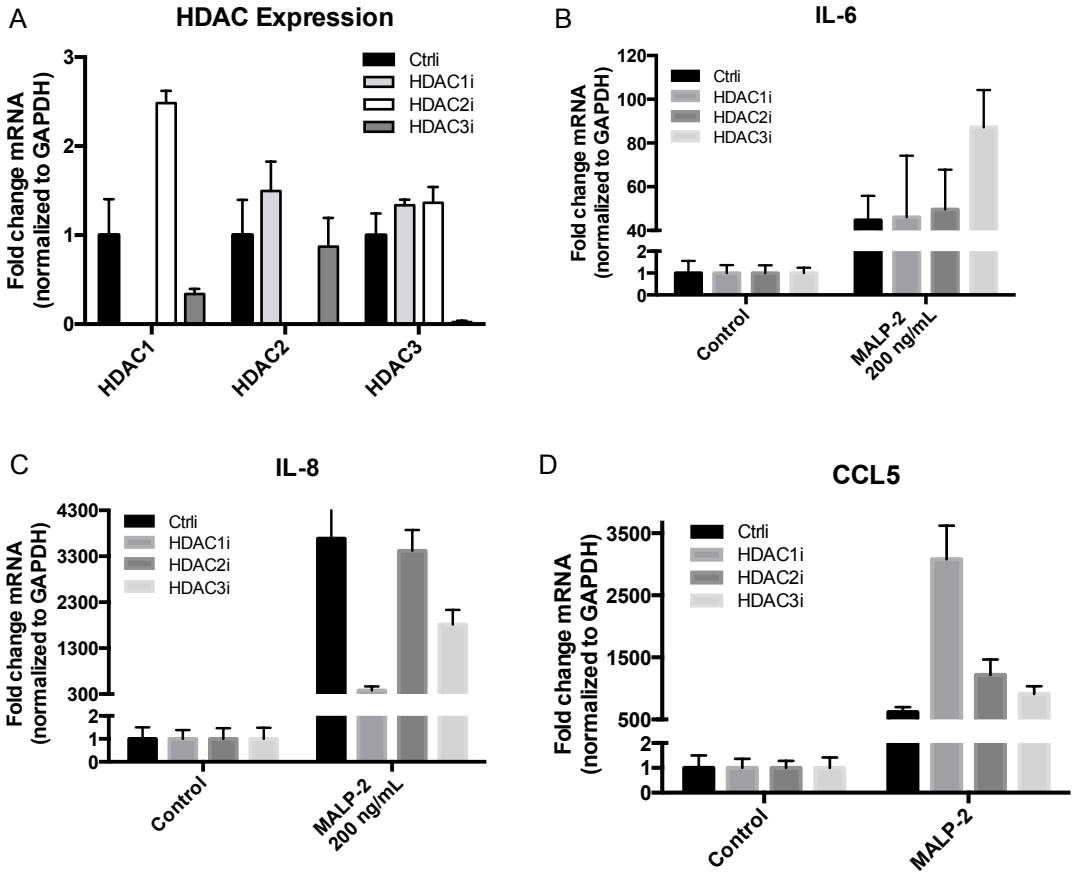

Figure 10. Knockdown of HDAC1, HDAC2, and HDAC3 does not increase keratinocyte cytokine response to TLR2/6 activation.
(**A**) Expression of HDAC1, HDAC2, and HDAC3 in keratinocytes following siRNA-mediated gene knockdown. (**B-D**) Levels of cytokine mRNA expression in keratinocytes treated with siRNA specific for HDAC1, HDAC2, and HDAC3, stimulated with MALP-2 for 4 hours (n=3; *P*=0.9993, *P*=0.9715, and *P*=0.0085 (IL6); *P*<0.0001, *P*=0.8129, and *P*<0.0001 (IL8); *P*<0.0001, *P*=0.0452, and *P*=0.4078 (CCL5); two-way ANOVA). Data are mean +/- SEM, n=3 for each group, and representative of 3 independent experiments.

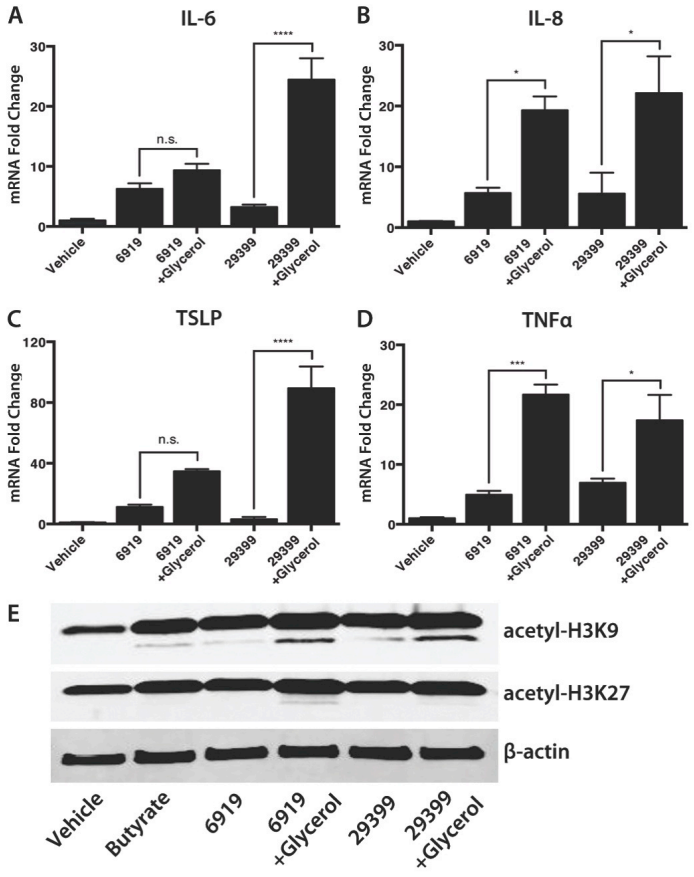

Figure 11. *Propionibacterium acnes* enhances cytokine expression from keratinocytes when cultured in fermentation-favoring conditions.
Culture supernatants of *Propionibacterium acnes* (ATCC6919 and ATCC29399, grown in anaerobic conditions with or without 2% glycerol for 14 days) were added to EpiLife media at 25% and used to stimulate keratinocytes for 4 hours. (**A-D**) mRNA expression levels of IL-6, IL-8, TSLP and TNFα, normalized to GAPDH (n=3; *P*=0.7146 and *P*<0.0001 (IL6); *P*=0.0305 and *P*=0.0106 (IL8); *P*=0.0558 and *P*<0.0001 (TSLP); *P*=0.0004 and *P*=0.0109 (TNF); one-way ANOVA). (**E**) Western blot analysis of histone acetylation in keratinocytes following stimulation with *P. acnes* supernatants. All data are mean +/- SEM and representative of at least 2 experiments.

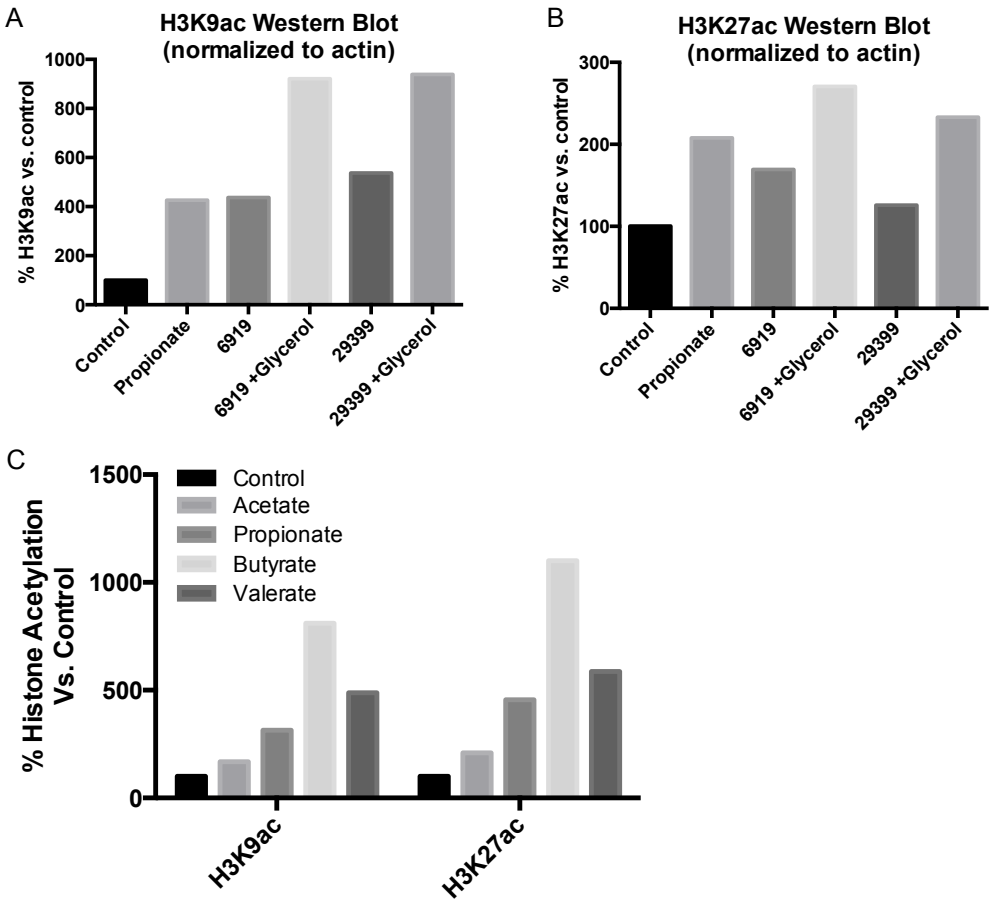

Figure 12. Quantification of histone acetylation in human epidermal keratinocytes.
(**A-B**) Quantification of H3K9 and H3K27 acetylation, normalized against β–actin, as measured by Western Blots from human keratinocytes treated with *P. acnes*-conditioned supernatants as illustrated in Figure 11E. (**C**) Quantification of H3K9 and H3K27 acetylation, normalized against β–actin, as measured by Western Blots of human keratinocytes cultured with the indicated short-chain fatty acids as illustrated in Figure 13A.

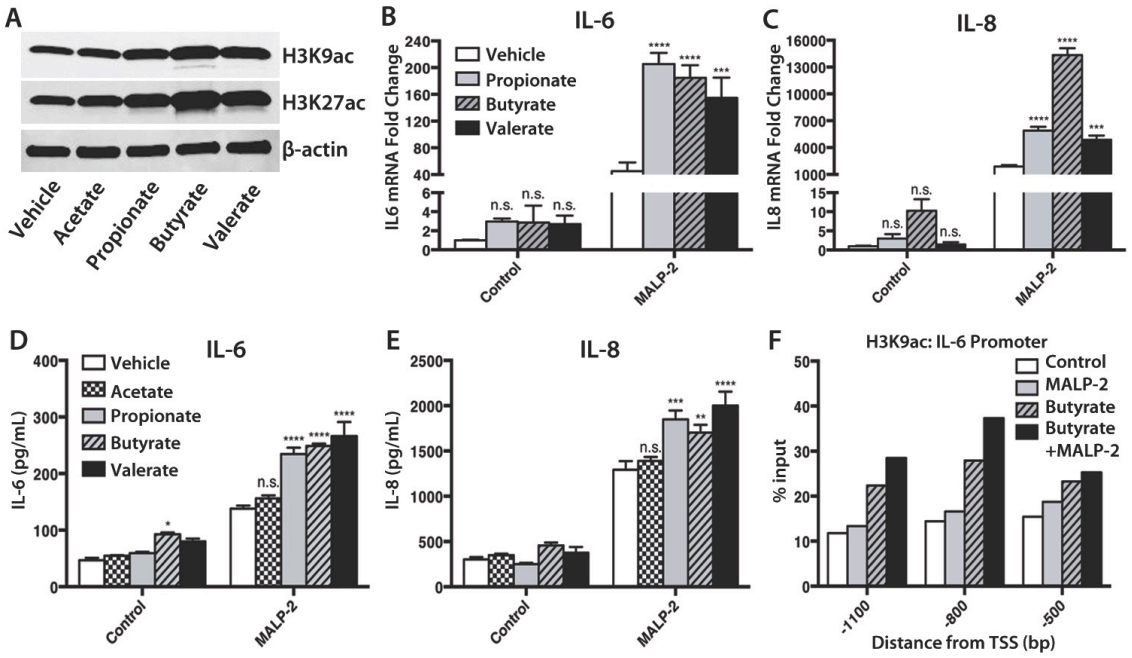

Figure 13. SCFA-induced increases in cytokine expression in keratinocytes are mediated through histone deacetylase inhibition.
Keratinocytes were treated with sodium acetate, sodium propionate, sodium butyrate, or valeric acid (2mM) +/- MALP-2 (200 ng/mL) for 4-24 hours. (**A**) Western Blot analysis of H3K9 and H3K27 acetylation following 4 hour SCFA treatment. (**B-C**) IL-6 and IL-8 mRNA expression in keratinocytes cultured with short-chain fatty acids and MALP-2 for 4 hours (n=3; *P*=0.9996, *P*=0.9996, and *P*=0.9997 (IL6 control); *P*<0.0001, *P*<0.0001, and *P*=0.0004 (IL6 MALP-2); *P*>0.9999 (IL8 control); *P*<0.0001, *P*<0.0001, and *P*=0.0002 (IL8 MALP-2); two-way ANOVA). (**D-E**) IL-6 and IL-8 levels in culture medium from keratinocytes cultured with short-chain fatty acids and MALP-2 for 24 hours (n=4; *P*=0.9724, *P*=0.8748, *P*=0.014, and *P*=0.1161 (IL6 control); *P*=0.6648 and *P*<0.0001 (IL6 MALP-2); *P*=0.9929, *P*=0.9884, *P*=0.6105, and *P*=0.9582 (IL8 control); *P*=0.892, *P*=0.0001, *P*=0.0052 and *P*<0.0001 (IL8 MALP-2); two-way ANOVA). All data are mean +/- SEM and representative of at least 3 experiments. (**F**) Chromatin immunoprecipitation (ChIP) was performed using antibody against acetylated H3K9 in keratinocytes treated with MALP-2 and sodium butyrate for 2 hours. Precipitated DNA was analyzed by RT-qPCR for regions of the IL-6 promoter (n=1, representative of 2 experiments).

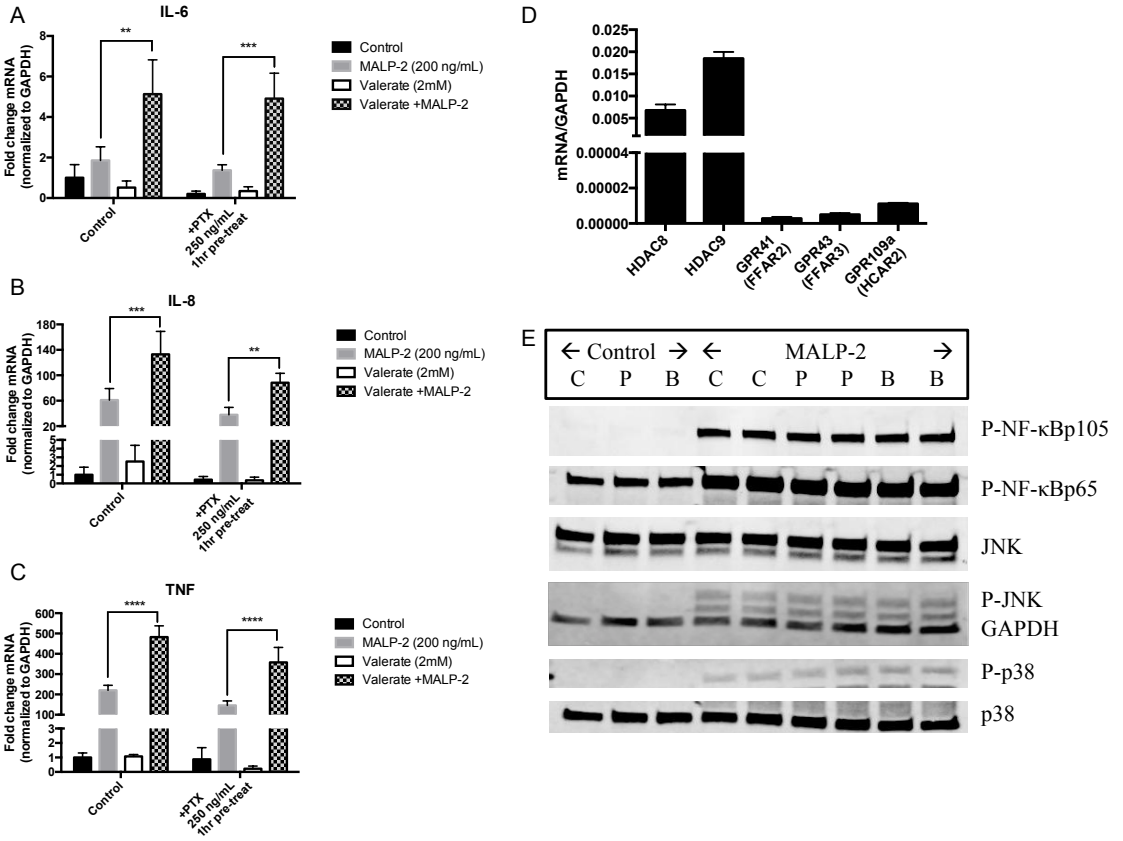

Figure 14. SCFAs do not increase TLR-mediated cytokine expression in keratinocytes by activation of free fatty acid receptors or NF-kB, p38, or JNK signaling.

(**A-C**) Free fatty acid receptor activation in keratinocytes was blocked by pertussis toxin (PTX) pre-treatment (250 ng/mL for 1 hour). Cells were then stimulated with MALP-2 (200 ng/mL) and valerate (2mM) for 4 hours and analyzed by RT-qPCR for cytokine mRNA expression (n=3; *P*=0.0013 and *P*=0.0006 (IL6); *P*=0.0004 and *P*=0.0084 (IL8); *P*<0.0001 (TNF); two-way ANOVA). Data are mean +/- SEM and representative of 3 independent experiments. (**D**) mRNA expression levels of free fatty acid receptors in keratinocytes was measured by RT-qPCR, normalized against GAPDH, and compared to expression of HDAC8 and HDAC9. Data are mean +/- SEM of n=3 samples, representative of 3 independent experiments. (**E**) Western Blot analysis of signaling pathway activation in keratinocytes treated with MALP-2 (400 ng/mL) for 30 minutes with propionate or butyrate (2mM) added 1 hour prior. Data are representative of 3 independent experiments.

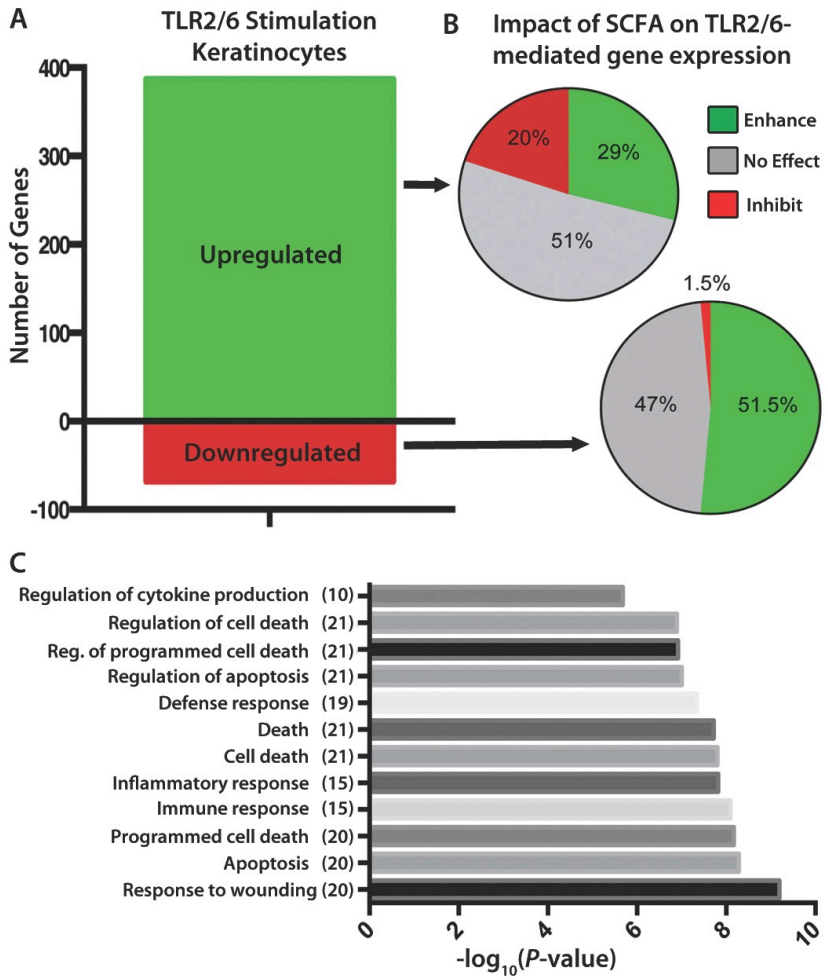

Figure 15. Up-regulation of inflammatory and immune response-related genes in keratinocytes by butyrate.
RNA-sequencing was performed on keratinocytes stimulated with MALP-2 (200 ng/mL) with or without butyrate (2mM) for 4 hours. (**A**) Identification of genes up- and down-regulated (fold change >1.5 vs. control) by MALP-2 stimulation in keratinocytes. (**B**) Analysis of the effects of butyrate treatment on gene sets modulated by MALP-2 (enhancement= fold-change >1.5 in butyrate +MALP-2 vs. vehicle +MALP-2; inhibition = fold change <-1.5 in butyrate +MALP-2 vs. vehicle +MALP-2; no effect = -1.5< fold-change <1.5). (**C**) Gene Ontology analysis of the MALP-2 induced genes of which expression was further increased by butyrate treatment in keratinocytes. The number of genes related to each biological process is indicated in parentheses.

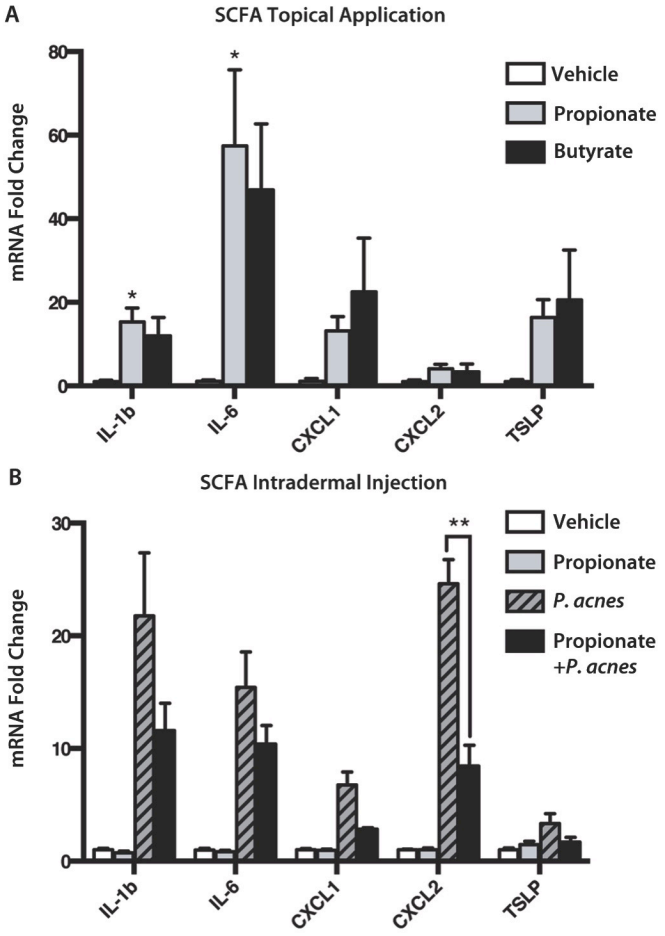

Figure 16. Route of exposure of short-chain fatty acids influences effects on inflammatory gene expression in mouse skin.
(A) Cytokine mRNA expression levels in mouse dorsal skin treated topically with agar discs containing propionate or butyrate (200 mM) for 8 hours (n=4; *P*=0.0205 and *P*=0.0669 (IL-1b); *P*=0.0338 and *P*=0.0792 (IL-6); *P*=0.4579 and *P*=0.1366 (CXCL1); *P*=0.1938 and *P*=0.3467 (CXCL2); *P*=0.2835 and *P*=0.1555 (TSLP); one-way ANOVA). (**B**) Cytokine mRNA expression levels in mouse dorsal skin 8 hours following intradermal injection of *Propionibacterium acnes* ($1x10^7$ CFU) with or without propionate (1M) (n=4; *P*=0.0708 (IL-1b); *P*=0.6874 (IL-6); *P*=0.3136 (CXCL1); *P*=0.0007 (CXCL2); *P*=0.0897 (TSLP); two-way ANOVA). All data are mean +/- SEM and representative of at least 2 experiments.

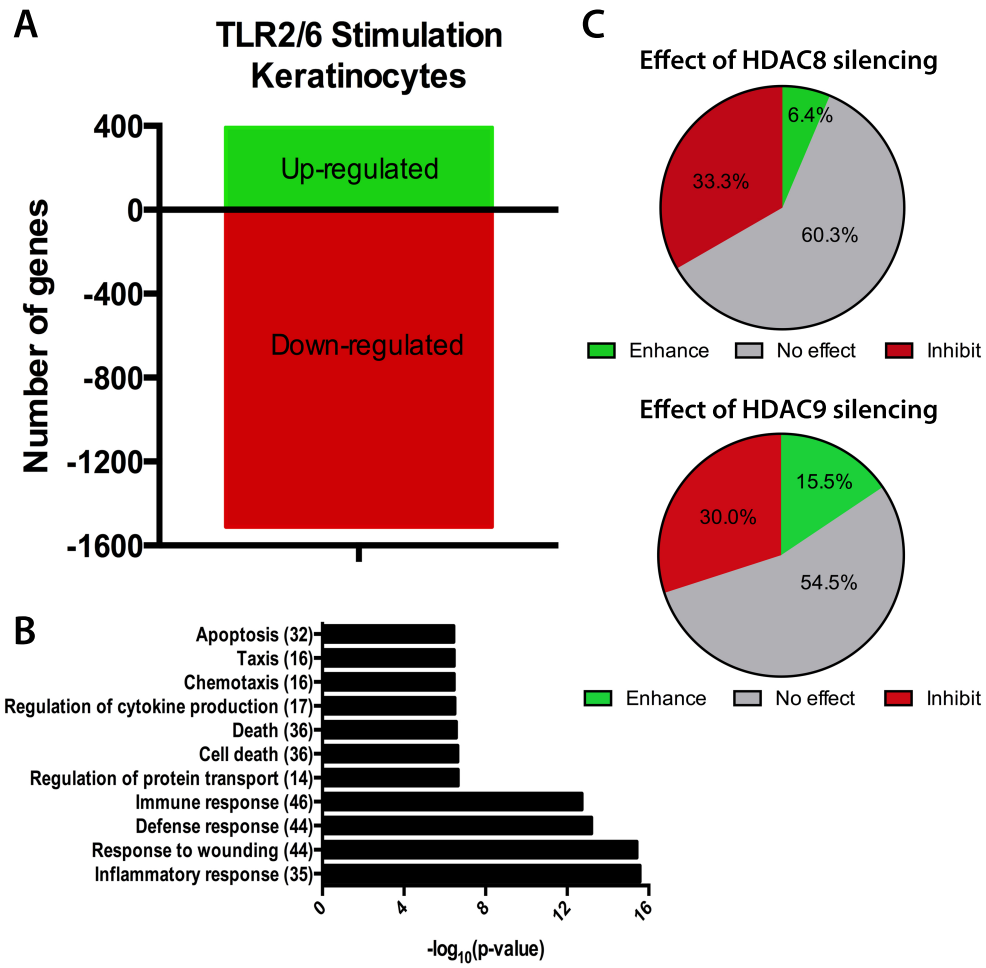

Figure 17. RNA-seq of keratinocytes treated with siRNA for HDAC8 or HDAC9 and stimulated with MALP-2.
Primary human epidermal keratinocytes were treated with negative control siRNA for 18 hours, then allowed to recover for 72 hours. Cells were then treated with MALP-2 (200 ng/mL) for 4 hours, and RNA was isolated and analyzed by next-generation sequencing. (**A**) Identification of genes up- and down-regulated (fold change >1.5 vs. control) by MALP-2 stimulation in keratinocytes. (**B**) Gene Ontology analysis of the list of genes up-regulated by MALP-2. (**C**) Analysis of the effects of silencing HDAC8 or HDAC9 on the genes up-regulated by MALP-2 in keratinocytes.

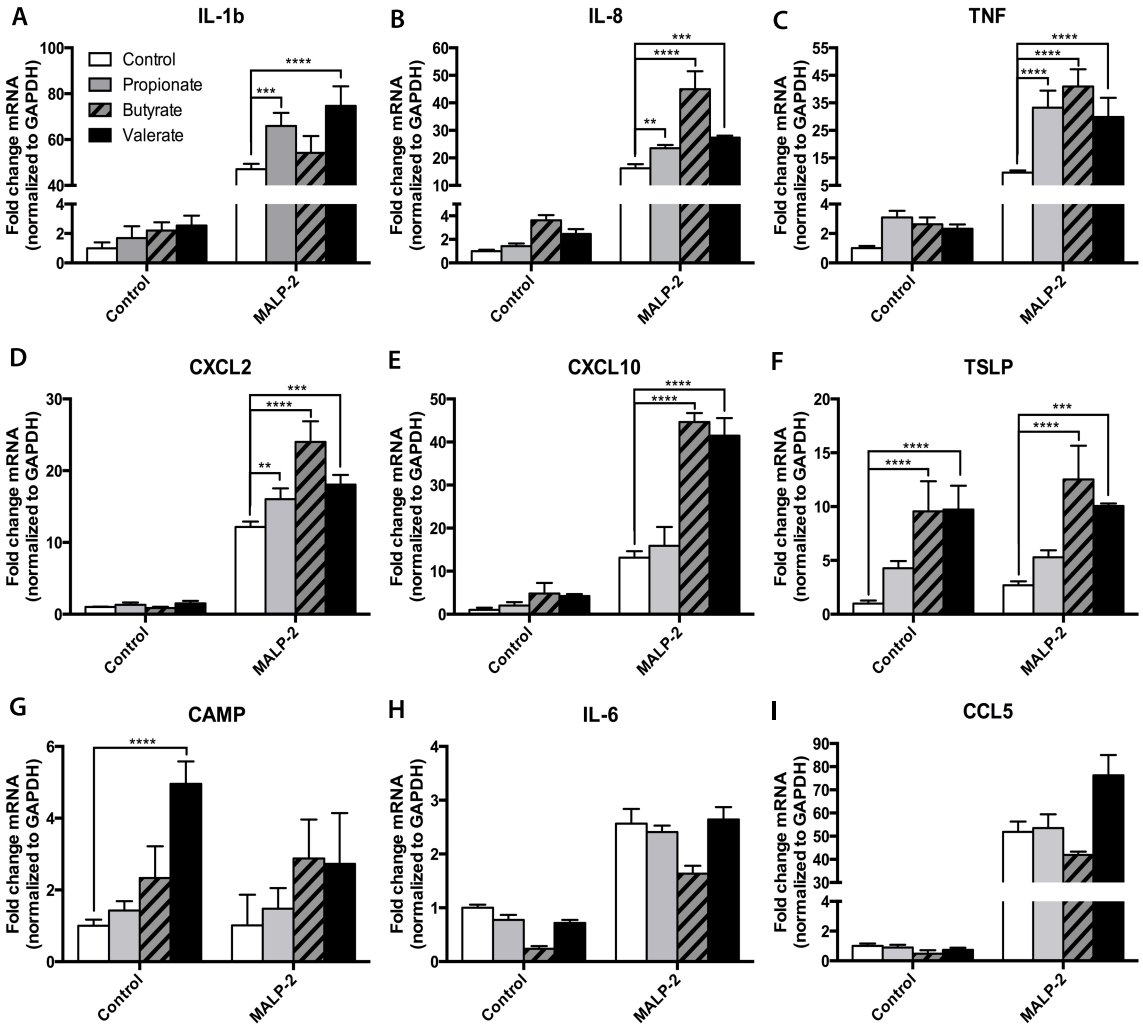

Figure 18. SCFAs increase proinflammatory cytokine expression from sebocytes.

(A-I) SEB-1 sebocytes were treated with the indicated short-chain fatty acids at 2 mM for 1 hour, followed by stimulation with MALP-2 (200 ng/mL) for 4 hours. Expression levels of the indicated cytokines were then analyzed by RT-qPCR and normalized to GAPDH. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ as indicated by two-way ANOVA. All data are mean +/- SEM, n=3.

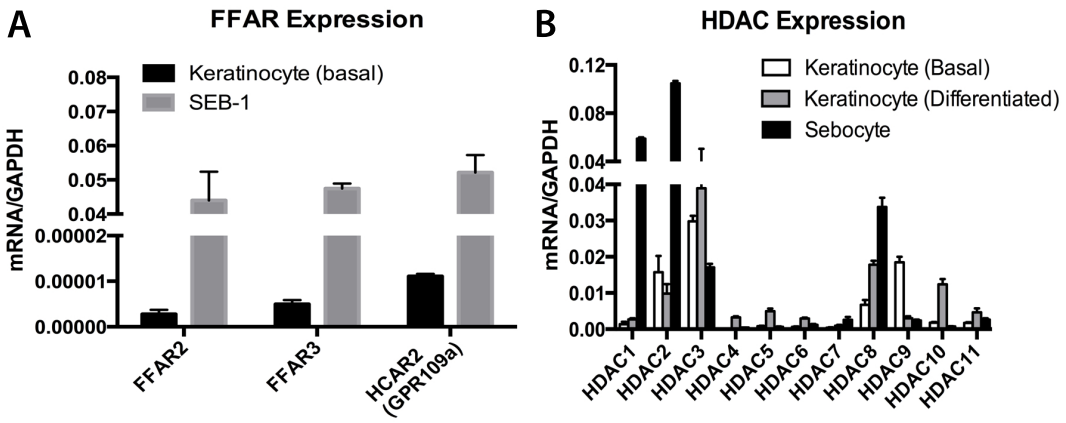

Figure 19. Expression levels of free fatty acid receptors and HDACs in sebocytes compared to keratinocytes.
(**A**) Expression levels of free fatty acid receptors in cultured basal keratinocytes and cultured SEB-1 sebocytes, normalized to GAPDH. (**B**) Expression levels of the 11 classical HDAC enzymes in cultured basal and differentiated keratinocytes and cultured SEB-1 sebocytes, normalized to GAPDH.

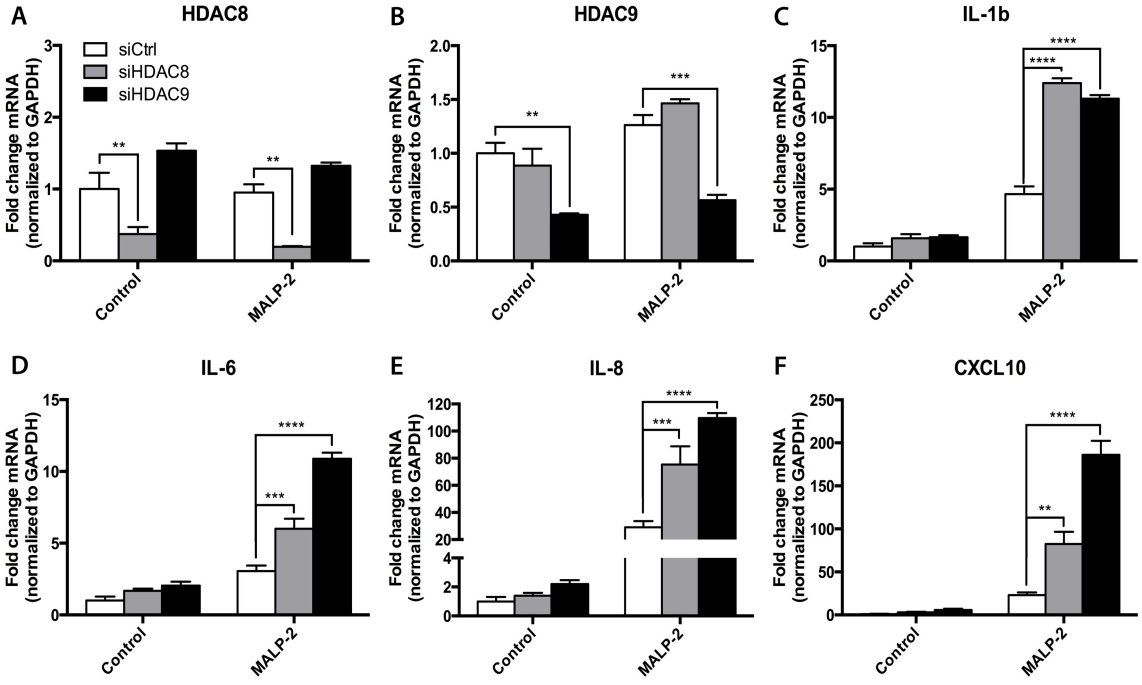

Figure 20. Depletion of HDAC8 or HDAC9 in sebocytes results in increased TLR-mediated cytokine expression.

(A-F) SEB-1 sebocytes were treated with siRNA for HDAC8 or HDAC9, or negative control, and allowed to recover for 72 hours. Cells were then treated with MALP-2 (200 ng/mL) for 4 hours, and expression levels of the indicated cytokines were analyzed by RT-qPCR and normalized to GAPDH. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ as indicated by two-way ANOVA. All data are mean +/- SEM, n=3.

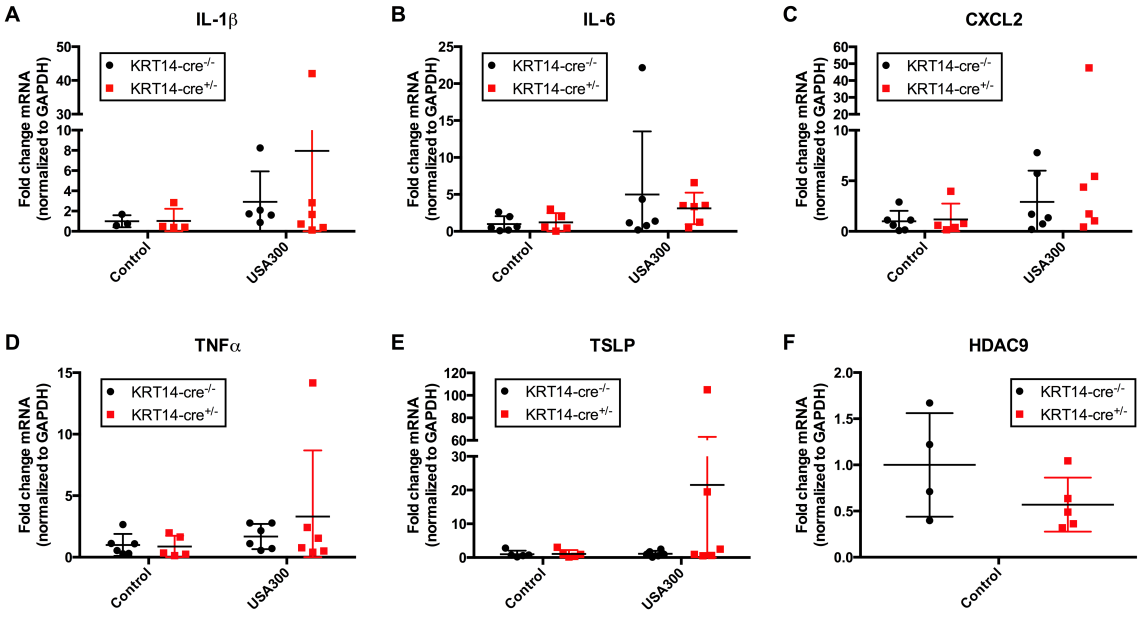

Figure 21. Mice lacking HDAC9 in epidermal keratinocytes exhibit higher cytokine response to topical bacterial challenge.

Mice (HDAC9$^{flox/flox}$) lacking HDAC9 in epidermal keratinocytes (KRT14-cre$^{+/-}$) or littermate controls with intact HDAC9 expression in epidermal keratinocytes (Krt14-cre$^{-/-}$) were challenged topically with *Staphylococcus aureus* (USA300) for 8 hours. Skin was then harvested and expression levels of various cytokines and chemokines (**A-E**) and HDAC9 (**F**) were measured by RT-qPCR.

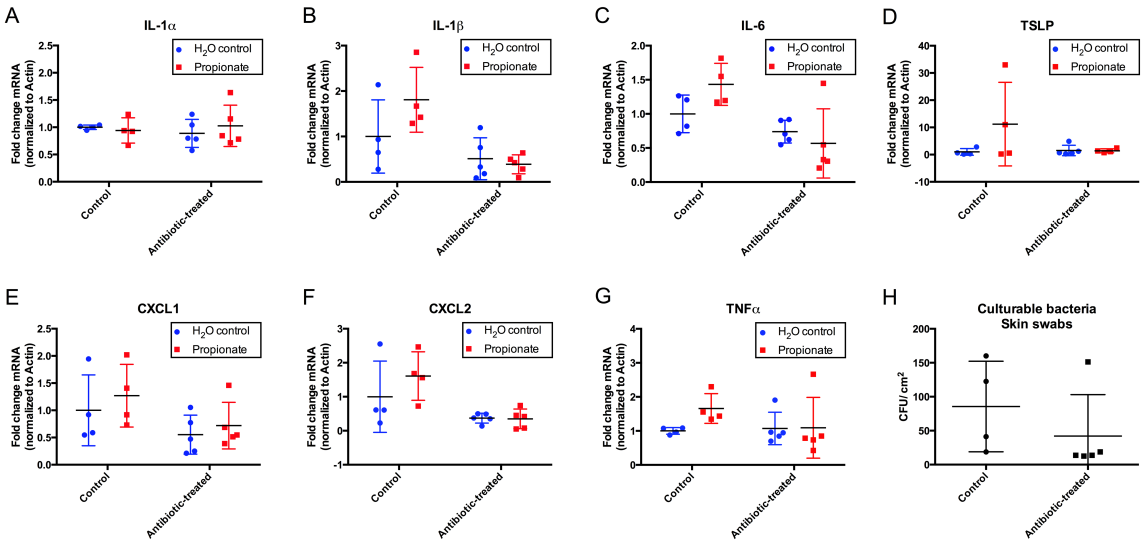

Figure 22. Mice with depleted commensal skin microbiota show decreased response to topical SCFA application.
SKH-1 elite mice were supplied antibiotics (50 units/ml benzylpenicillin potassium, 2.0 mg/ml neomycin sulfate and 0.5mg/ml cefoperazone sodium salt) in drinking water for 4 weeks to reduce cutaneous microbial communities. Following antibiotic treatment, mice were challenged topically with agar discs containing sodium propionate (200 mM) or water for 8 hours, after which mice were sacrificed and skin samples from application sites were collected and analyzed via RT-qPCR. (**A-G**) Expression levels of inflammatory cytokines in mouse skin. (**H**) Number of resident bacteria on the skin as measured by skin swabs and overnight culture.

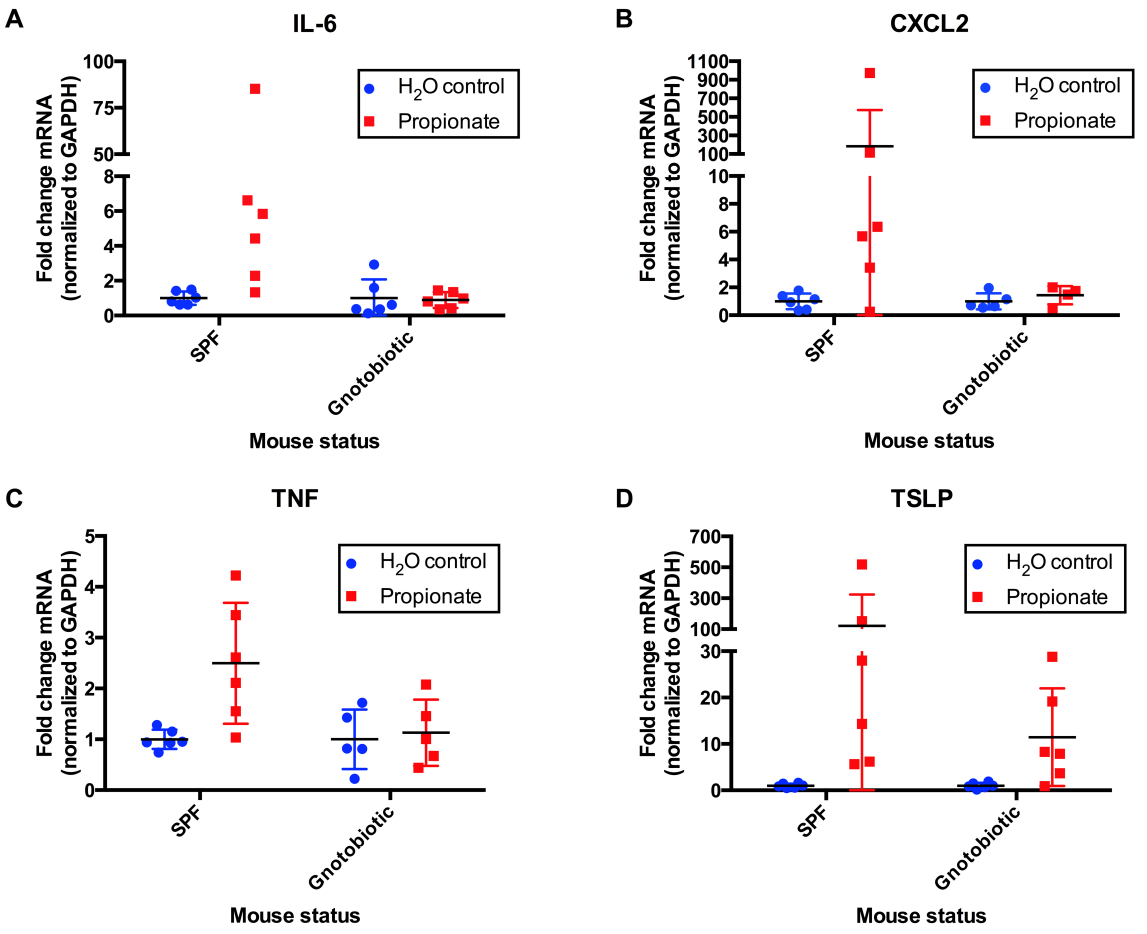

Figure 23. Mice lacking commensal skin microbiota show a decreased response to topical SCFA application.
Gnotobiotic mice lackin commensal skin microbes and specific-pathogen free (SPF) control mice were challenged topically by the application of 100 uL of sodium propionate (2 M) or water on 1 cm^2 gauze pads secured with adherent wound dressing for 16 hours, after which mice were sacrificed and skin samples were collected from sites of application and gene expression was analyzed via RT-qPCR. (**A-D**) Expression levels of inflammatory cytokines in mouse skin.

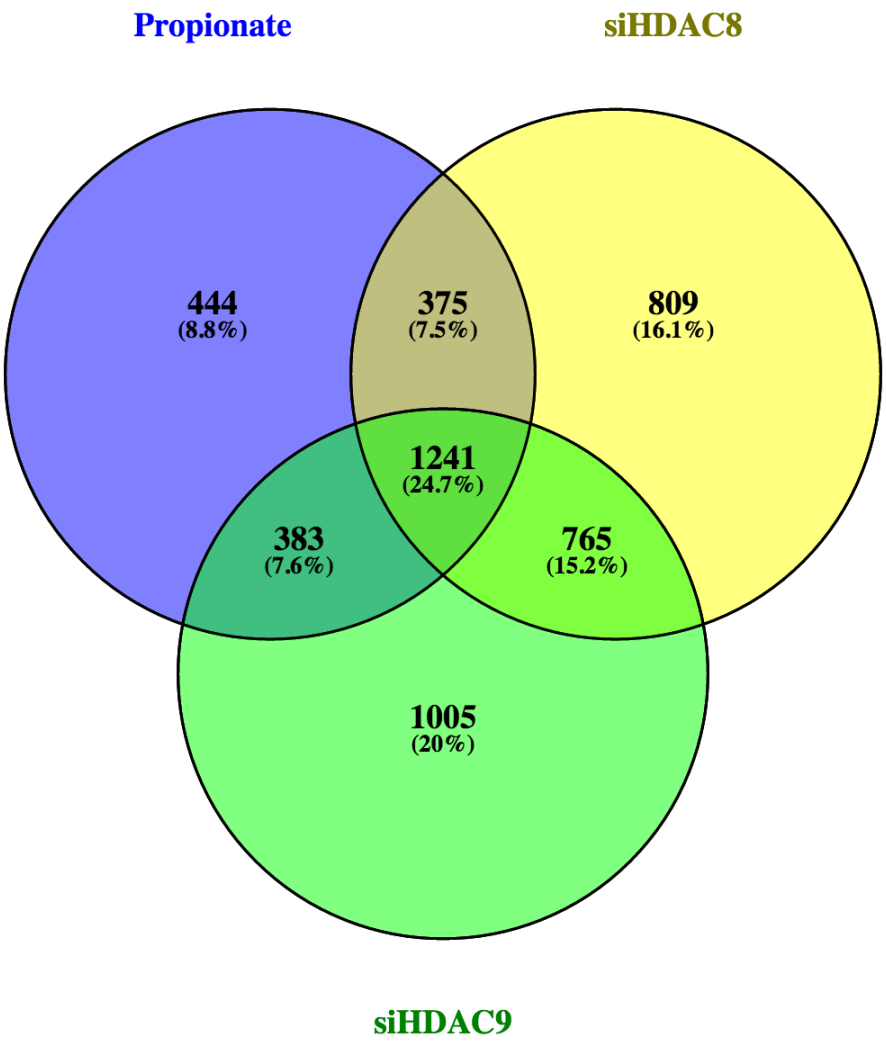

Figure 24. Comparison of lists of genes with increased chromatin accessibility as detected by ATAC-seq.
Analysis of ATAC-seq detected genomic regions where chromatin was detected as more accessible following depletion of HDAC8 (siHDAC8), HDAC9 (siHDAC9), or addition of propionate (propionate) than in control cells. These peaks were then used to identify the nearest genes; lists of genes with more accessible chromatin from the three conditions were compared and displayed here.

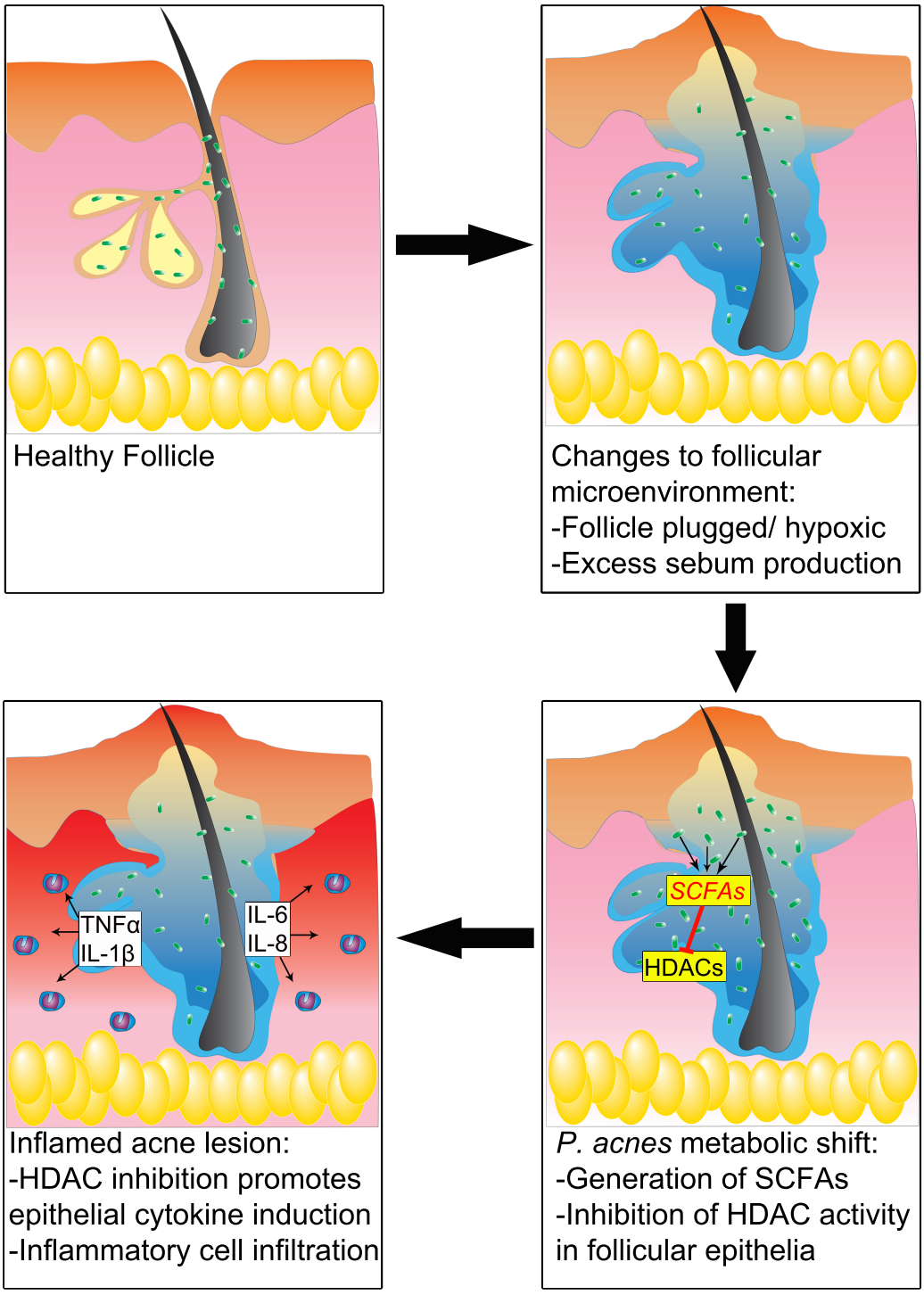

Figure 25. Model of *P. acnes*-derived, SCFA-mediated acne pathogenesis.

Table 1. Propionibacterium acnes produces propionate and valerate via anaerobic fermentation.
Concentrations (mM) of short-chain fatty acids produced by laboratory strains of *Propionibacterium acnes* (ATCC6919 and ATCC29399). Bacteria were cultured under anaerobic conditions in rich medium with or without 2% glycerol for 14 days. SCFA concentrations in culture supernatants were determined by GC-MS following ethyl acetate extraction.

Concentration SCFA (mM)	ATCC6919	ATCC6919 +Glycerol	ATCC29399	ATCC29399 +Glycerol	Media
Acetic	2.33	7.17	3.17	6.00	1.33
Propionic	1.62	75.14	4.59	175.81	0.14
Butyric	0.00	0.11	0.00	0.11	0.00
Isobutyric	0.11	0.11	0.11	0.11	0.11
Isovaleric	0.20	4.11	2.06	24.58	0.00

Table 2. Human histone deacetylase (HDAC) classifications.
The 11 classical histone deacetylase enzymes expressed in human cells are divided into several classes based on sequence homology and conserved domains. Here we illustrate the categorization of these proteins.

Class	Enzyme	Nuclear localization signal?	Nuclear export signal?	Expression in keratinocytes
I	HDAC1	Yes	No	Moderate
	HDAC2	Yes	No	High
	HDAC3	Yes	Yes	High
	HDAC8	Yes	No	High
IIa	HDAC4	Yes	Yes	Low
	HDAC5	Yes	Yes	Low
	HDAC7	Yes	Yes	Low
	HDAC9	Yes	Yes	High
IIb	HDAC6	Yes	Yes	Low
	HDAC10	No	Yes	Moderate
IV	HDAC11	No	No	Moderate

Table 3. List of genes up-regulated by loss of HDAC8 in keratinocytes.

Gene ID	Fold change (siHDAC8 vs. siCtrl)	Gene ID	Fold change (siHDAC8 vs. siCtrl)	Gene ID	Fold change (siHDAC8 vs. siCtrl)
HNRNPA1P10	4.48	IFI44L	1.84	COL1A1	1.67
FAM101A	4.47	DLG4	1.82	DDIT4L	1.67
SCHIP1	3.01	LIMD2	1.81	TMEM156	1.67
DNMT3B	2.87	NPHP1	1.79	APLN	1.66
IGFBP5	2.64	TP73	1.79	PYROXD2	1.66
TAGLN	2.47	AKAP12	1.79	CD74	1.66
TTLL3	2.23	LFNG	1.78	CNTNAP4	1.65
DERL3	2.23	MICU3	1.77	TMED7-TICAM2	1.65
FAT3	2.20	STMN3	1.74	JAG1	1.65
ADAMTSL1	2.19	PPP1R1B	1.74	DISP2	1.65
COL8A1	2.15	SYNDIG1	1.74	SCUBE2	1.65
SV2A	2.14	FES	1.74	PCYOX1L	1.65
TUB	2.08	CA10	1.74	LRRN1	1.64
MRGPRX3	2.07	SLIT3	1.73	MMP2	1.64
HNRNPH2	2.06	NRK	1.73	PCDHGA12	1.64
CD163L1	2.02	MYZAP	1.73	PABPC4L	1.63
GTF2H2C	2.02	NHSL2	1.72	DLK2	1.63
SLC13A5	2.02	EFNB3	1.71	CNPY4	1.63
DCLK1	2.00	HSP90B2P	1.71	ETV1	1.62
MGAM	1.98	LRRC23	1.71	C3orf18	1.62
COL5A2	1.97	IL33	1.71	SNCA	1.62
NGFR	1.97	CT62	1.71	KCNK2	1.62
CFH	1.96	LINC00623	1.70	SLC22A3	1.61
FAM156B	1.95	MYL9	1.70	THBS2	1.61
MMP19	1.95	ROR2	1.70	ARHGAP40	1.61
JMJD7-PLA2G4B	1.91	AGR2	1.70	SPC24	1.60
PDIA4	1.91	HSP90B1	1.69	PODXL	1.60
CFAP57	1.90	AZIN2	1.69	ZNF204P	1.60
CLDN11	1.88	MAP3K14	1.69	PAMR1	1.60
ADAMTS12	1.87	MAP6	1.69	CPVL	1.59
LSP1	1.87	PLEKHO1	1.68	TENM2	1.59
SULT1E1	1.86	MGC16275	1.68	CXCL14	1.59
CORO2B	1.85	BDKRB2	1.67	SLC4A4	1.59
HTR1B	1.85	NTN1	1.67	PLEKHG2	1.59

Table 3. List of genes up-regulated by loss of HDAC8 (continued).

Gene ID	Fold change (siHDAC8 vs. siCtrl)	Gene ID	Fold change (siHDAC8 vs. siCtrl)
CDH26	1.58	TGIF2	1.51
CCNJL	1.58	CD1D	1.51
TMEM8B	1.58	SLC7A2	1.51
HOTS	1.58	ERCC5	1.51
LINC01521	1.58	FLI1	1.51
CDK20	1.58	ARL10	1.51
SLC16A4	1.58	NLGN4X	1.51
PRICKLE1	1.58	BBS1	1.51
PTPRZ1	1.58	HSPE1-MOB4	1.51
TGFB2	1.58	NID1	1.50
MIR503HG	1.58	CD164L2	1.50
FARP2	1.57	HFE	1.50
MORN1	1.57	LIX1L	1.50
MIR31HG	1.57	PPP1R3C	1.50
TGIF2-C20orf24	1.56	MET	1.50
CRELD1	1.56	SHISA6	1.50
RGAG4	1.56	RAMP1	1.50
FLNC	1.56		
LIMCH1	1.56		
KDM4D	1.56		
ZNF284	1.56		
TCF7L1	1.55		
IFI44	1.55		
TAPT1	1.54		
SPRY1	1.53		
CLDN6	1.53		
CRELD2	1.53		
FGFR4	1.53		
BAIAP2-AS1	1.53		
TNFSF15	1.52		
AEBP1	1.52		
ACTA2	1.52		
ZNF503-AS2	1.51		

Table 4. List of genes down-regulated by loss of HDAC8 in keratinocytes.

Gene ID	Fold change (siHDAC8 vs. siCtrl)	Gene ID	Fold change (siHDAC8 vs. siCtrl)	Gene ID	Fold change (siHDAC8 vs. siCtrl)
HDAC8	-5.98	NOTCH3	-1.94	ANAPC10	-1.76
KRT1	-5.74	HIST3H2A	-1.93	CREBRF	-1.76
CDKN1C	-3.45	TPGS1	-1.92	HAP1	-1.76
FLG2	-3.42	CTU1	-1.90	SMG1P5	-1.76
PGBD3	-2.86	HINT3	-1.90	USP2	-1.76
TMEM160	-2.55	RHPN2	-1.89	EPPK1	-1.76
IGFL1	-2.54	KRT81	-1.87	CYBA	-1.75
RGS16	-2.43	SPRR2A	-1.87	SGK3	-1.74
WFDC21P	-2.37	PLEKHG6	-1.87	RHCG	-1.74
CST6	-2.33	ARFGEF3	-1.86	TLL2	-1.74
ELOVL7	-2.30	LINC00520	-1.86	CEBPA	-1.74
CALML5	-2.26	PVRL4	-1.85	GGT6	-1.74
CCDC64B	-2.23	CHADL	-1.85	CCNA1	-1.74
CRCT1	-2.19	UFSP1	-1.85	HSPB1	-1.73
CSF2	-2.15	NRARP	-1.85	VIM-AS1	-1.73
ELF3	-2.14	MT1E	-1.85	SAPCD2	-1.73
TMEM125	-2.13	CYSRT1	-1.84	LY6D	-1.73
KIF26A	-2.13	TMEM191A	-1.84	NDRG4	-1.73
SLC6A20	-2.13	ABHD17A	-1.83	MT1X	-1.73
IFIT2	-2.10	PVRIG2P	-1.83	SLC2A12	-1.73
GNAO1	-2.09	STEAP4	-1.83	ASPRV1	-1.72
C10orf10	-2.09	DYX1C1-CCPG1	-1.82	SPON2	-1.72
CCL20	-2.08	S100A8	-1.81	OASL	-1.72
KRTDAP	-2.06	GOLPH3L	-1.81	BMI1	-1.72
C4orf48	-2.06	ZNF750	-1.81	MDP1	-1.72
EIF4EBP3	-2.03	UCA1	-1.80	OCLN	-1.72
RPTN	-2.01	IPMK	-1.79	KCNQ1OT1	-1.72
SOWAHB	-2.01	FOXN1	-1.79	AGK	-1.71
LOC441455	-2.00	IRF5	-1.78	TNFAIP2	-1.71
HIST1H2BG	-1.99	LCE3D	-1.78	UPK2	-1.71
TEN1-CDK3	-1.98	MIF	-1.78	GPR180	-1.71
SLC39A10	-1.97	OVOL1	-1.78	CLDN7	-1.71
LY6G6C	-1.97	PDF	-1.77	CHST7	-1.70
EEPD1	-1.96	SERPINB1	-1.77	SLX1A	-1.70
NUDT10	-1.95	CXCL16	-1.77	SLX1B	-1.70

Table 4. List of genes down-regulated by loss of HDAC8 (continued).

Gene ID	Fold change (siHDAC8 vs. siCtrl)	Gene ID	Fold change (siHDAC8 vs. siCtrl)	Gene ID	Fold change (siHDAC8 vs. siCtrl)
LOC101927178	-1.70	SLC30A1	-1.64	PRKCDBP	-1.59
EVA1B	-1.70	WNT9A	-1.64	IL1R2	-1.59
APOBEC3B	-1.70	SRMS	-1.64	CCDC85B	-1.59
PRSS22	-1.70	IER5L	-1.63	SLC16A1	-1.59
15-Sep	-1.70	SYN1	-1.63	SPRR1A	-1.58
VNN1	-1.69	GPNMB	-1.63	SPRR2D	-1.58
PLA2G4E	-1.69	SPRR2B	-1.63	HCAR2	-1.58
RAB11FIP1	-1.69	IL32	-1.63	ALDH3B2	-1.58
ZNF460	-1.69	MIF-AS1	-1.63	SLC28A3	-1.58
LTBP3	-1.69	GPR63	-1.63	NID2	-1.58
KRT75	-1.69	LCE3E	-1.63	DENND2C	-1.58
HIST1H3D	-1.69	ANKRD9	-1.62	TMPRSS4	-1.58
SIGIRR	-1.68	LOC391322	-1.62	LOC100130476	-1.58
LRRC2	-1.68	ANKRD52	-1.62	NPAT	-1.57
PLA2G4D	-1.67	IRX3	-1.62	CLDN12	-1.57
P2RX5-TAX1BP3	-1.67	BMF	-1.62	RPP25	-1.57
GCH1	-1.67	SPRR2G	-1.62	COL9A2	-1.57
KRT78	-1.66	UBL7-AS1	-1.62	C20orf24	-1.57
LBX2-AS1	-1.66	GRHL3	-1.62	GJD3	-1.57
TRIM36	-1.66	BMP2	-1.62	CNFN	-1.57
SPRR2E	-1.66	UNC13D	-1.62	IL36G	-1.57
LCE1B	-1.66	TMEM255B	-1.61	MIB2	-1.57
C11orf52	-1.66	SPNS2	-1.61	RORA	-1.57
TMPRSS13	-1.66	ZDHHC21	-1.61	GRAP	-1.57
ADAT3	-1.65	PGAM1P5	-1.60	MAFB	-1.57
PLA2G3	-1.65	SPRR1B	-1.60	TRAPPC5	-1.56
FUT3	-1.64	FAM71E1	-1.60	FTH1	-1.56
PITX1	-1.64	CHST6	-1.60	GRHL1	-1.56
METRNL	-1.64	HSD11B1	-1.60	BMS1P6	-1.56
GAL	-1.64	CLDN4	-1.60	GRB7	-1.56
CEBPB	-1.64	CTAGE8	-1.59	SAA2	-1.56
IL36RN	-1.64	GPRASP2	-1.59	KLK8	-1.56
GFPT1	-1.64	PKN1	-1.59	DGUOK-AS1	-1.55
C10orf95	-1.64	SCAND1	-1.59	C19orf60	-1.55

Table 4. List of genes down-regulated by loss of HDAC8 (continued).

Gene ID	Fold change (siHDAC8 vs. siCtrl)	Gene ID	Fold change (siHDAC8 vs. siCtrl)
LYRM9	-1.55	C11orf54	-1.52
TMPRSS11E	-1.55	PPP4R4	-1.52
INA	-1.55	G0S2	-1.51
KRT73-AS1	-1.55	GULP1	-1.51
NIPSNAP3A	-1.55	TXNDC16	-1.51
SPRR3	-1.55	SULT2B1	-1.51
IDUA	-1.54	ADAM9	-1.51
CTXN1	-1.54	CES3	-1.51
S100A4	-1.54	FOXL2	-1.51
ARC	-1.54	SLC25A43	-1.51
PNRC1	-1.54	TPT1-AS1	-1.51
SPRR2F	-1.54	BLNK	-1.51
HAPLN3	-1.54	RHOV	-1.51
PTGS2	-1.53	FBXO32	-1.51
C4orf26	-1.53	PPM1L	-1.50
RND1	-1.53	SCML2	-1.50
GCNT2	-1.53	CYB5A	-1.50
IGFBP3	-1.53	TMCC2	-1.50
SAA1	-1.53	MFSD6	-1.50
TRHDE	-1.53	YOD1	-1.50
OGFRL1	-1.53	DBNDD1	-1.50
ANKRD13C	-1.52	PARD3B	-1.50
KRT80	-1.52	TGDS	-1.50
RFX5	-1.52	RTN4R	-1.50
RHPN1	-1.52	GPRC5C	-1.50
ISPD	-1.52	TRIM22	-1.50
IMPA1	-1.52	SOCS2	-1.50
GBP6	-1.52	HEPHL1	-1.50
DPF2	-1.52		
AIM1L	-1.52		
IQCJ-SCHIP1	-1.52		
SFT2D3	-1.52		
CCDC69	-1.52		

Table 5. List of genes up-regulated by loss of HDAC9 in keratinocytes.

Gene ID	Fold change (siHDAC9 vs. siCtrl)	Gene ID	Fold change (siHDAC9 vs. siCtrl)	Gene ID	Fold change (siHDAC9 vs. siCtrl)
MRGPRX3	4.77	TAGLN	1.95	P4HTM	1.78
AKAP12	3.16	CXCL14	1.95	HOXC5	1.78
SPOCD1	3.03	DNMT3B	1.95	C6orf223	1.78
C1R	2.99	PGBD5	1.91	PRDM8	1.78
IL33	2.98	UBA7	1.91	CD164L2	1.77
IFI44L	2.84	SLC36A1	1.91	DNAJB5	1.77
FAM101A	2.69	BAIAP2-AS1	1.90	PMEPA1	1.77
CRIP2	2.50	FUT4	1.89	ADM5	1.77
HNRNPH2	2.40	TUBB3	1.89	CCND3	1.76
CORO2B	2.39	TM4SF19	1.88	PLEKHA4	1.76
TMED7-TICAM2	2.34	LINC01521	1.88	TMEM8B	1.76
SCG5	2.34	VPS9D1	1.87	SARM1	1.76
CSF3	2.30	CCDC64	1.87	C7orf55	1.75
TAGLN3	2.29	TFPI2	1.86	COL1A1	1.75
MIR31HG	2.25	MMP19	1.86	RGAG4	1.74
TTLL3	2.24	DGCR5	1.86	RUNDC3A	1.74
MX2	2.22	PIGZ	1.85	CMPK2	1.73
DCLK1	2.21	GTF2H2C	1.85	FAM110C	1.73
DLG4	2.20	JAG1	1.85	CTC-338M12.4	1.73
RAB4B	2.19	COL5A2	1.84	SAMD14	1.73
ADAMTS15	2.19	SNCA	1.83	IL11	1.72
C11orf71	2.18	IL20	1.82	ISG15	1.72
IFI44	2.15	KCNJ15	1.82	OAS1	1.72
MYL9	2.09	JAK3	1.82	FANK1	1.72
CLDN15	2.08	NRXN3	1.82	MAGEE1	1.71
SULT1E1	2.07	MC1R	1.82	FGFR4	1.71
MX1	2.06	FGF1	1.81	MMP9	1.71
LBH	2.05	XYLT1	1.81	MAPK11	1.71
ZFHX2	2.03	GAMT	1.81	NEIL1	1.70
IFIT1	2.01	XAF1	1.81	TUB	1.70
SERPINI1	2.00	C10orf35	1.80	PPP1R3C	1.70
LRRC56	2.00	HCN2	1.80	PAOX	1.70
LIMD2	1.99	LINC00623	1.79	ENTPD2	1.70
IFI6	1.98	TYMP	1.79	PPP1R3B	1.69
RHOV	1.96	DISP2	1.79	NTSR1	1.69

Table 5. List of genes up-regulated by loss of HDAC9 (continued).

Gene ID	Fold change (siHDAC9 vs. siCtrl)	Gene ID	Fold change (siHDAC9 vs. siCtrl)	Gene ID	Fold change (siHDAC9 vs. siCtrl)
IFITM1	1.68	SLC27A6	1.61	DLX2	1.57
GLYCTK	1.68	IFI27	1.61	SAMD9L	1.57
CAPN5	1.68	TMEM217	1.61	LOC729737	1.57
CDK20	1.68	NAIP	1.61	DDX11L2	1.57
CFB	1.68	ZNF284	1.61	LINC01137	1.57
B3GNT4	1.68	GLB1L3	1.60	MVB12A	1.57
USP18	1.68	COL6A1	1.60	SEMA4F	1.57
TFEB	1.67	IFNK	1.60	LBHD1	1.57
CCDC92	1.67	GYLTL1B	1.60	TMEM92	1.57
MMP3	1.67	DHX58	1.60	IFI35	1.57
KLHL3	1.67	LINC00467	1.60	ST20-MTHFS	1.56
TRIM46	1.67	CLSTN3	1.60	GALNT4	1.56
SERPINE1	1.67	GLB1L	1.60	RHOF	1.56
ZNF717	1.67	SUSD1	1.60	AEBP1	1.56
TNFSF10	1.66	MAPRE3	1.59	ZDHHC14	1.56
HERC6	1.66	TDRD7	1.59	TMEM158	1.56
CERS6-AS1	1.66	EFEMP2	1.59	THAP7-AS1	1.56
LRRC4	1.66	PIK3C2G	1.59	NKAPL	1.56
PLEKHG2	1.65	ERCC5	1.58	ADAMTSL1	1.56
OAS2	1.65	IRF9	1.58	MAPK8IP1	1.56
TNC	1.65	ARHGAP40	1.58	AGPAT4	1.56
EFNB3	1.65	REEP2	1.58	ST20	1.56
BDKRB2	1.65	LURAP1	1.58	CTF1	1.56
ADGRL1	1.65	WTIP	1.58	HIST1H2AC	1.56
PYROXD2	1.64	LIMCH1	1.58	MOSPD3	1.56
MYZAP	1.64	FES	1.58	LAMA1	1.56
C6orf141	1.64	FAM189A2	1.58	MAN1B1-AS1	1.55
TMEM143	1.64	PARP9	1.58	PARP3	1.55
FKBP1B	1.63	RGS14	1.58	TMC4	1.55
LOC100506123	1.63	RHPN1	1.58	HTR1B	1.55
IGSF8	1.63	CLUHP3	1.58	PCDHGB5	1.55
YBEY	1.63	LTB4R	1.57	CSMD3	1.55
MMP10	1.62	SEZ6L2	1.57	STX1A	1.55

Table 5. List of genes up-regulated by loss of HDAC9 (continued).

Gene ID	Fold change (siHDAC9 vs. siCtrl)	Gene ID	Fold change (siHDAC9 vs. siCtrl)
GGT7	1.55	PRKACG	1.51
P3H4	1.55	LIX1L	1.51
MYADM	1.55	CSRNP1	1.51
ANO8	1.54	DYNC2LI1	1.51
OLFM2	1.54	SULF1	1.51
SLC16A5	1.54	FRS3	1.51
RTN2	1.54	WSB1	1.51
ARNT2	1.54	HSD11B1L	1.51
PXN-AS1	1.54	GOLGA7B	1.51
FAM63A	1.53	RFXAP	1.51
ABHD15	1.53	PCDHGB4	1.51
C19orf66	1.53	SLC46A3	1.51
EDNRA	1.53	RTN4RL1	1.51
TLCD2	1.53	COL8A1	1.51
ENG	1.52	CXCL8	1.51
FARP2	1.52	ULK4	1.50
DMTN	1.52	SLC52A3	1.50
MLLT11	1.52	ARL10	1.50
NUTM2D	1.52	WNK3	1.50
ISOC1	1.52	ANTXR2	1.50
LOC105374952	1.52	MRC2	1.50
EPHB2	1.52	ST3GAL4-AS1	1.50
NEURL1B	1.52	LOC728613	1.50
IFFO1	1.52	CNPY4	1.50
SLC9A5	1.52	ATP6V0E2	1.50
SPIRE2	1.52	ZNF461	1.50
NYAP1	1.52	RSG1	1.50
KLC4	1.52	OBSCN	1.50
MGAM	1.52	TIAF1	1.50
FBXO6	1.51	GSDMC	1.50
PDE4D	1.51	TNFSF12	1.50
SPTBN5	1.51	TSLP	1.50
NOTCH3	1.51	SHISA2	1.50

Table 6. List of genes down-regulated by loss of HDAC9 in keratinocytes.

Gene ID	Fold change (siHDAC9 vs. siCtrl)	Gene ID	Fold change (siHDAC9 vs. siCtrl)	Gene ID	Fold change (siHDAC9 vs. siCtrl)
HDAC9	-5.88	ELF3	-2.15	RPTN	-1.87
LCE3E	-3.09	CYP1A1	-2.11	FAM3C	-1.86
PLEKHA3	-2.99	CDSN	-2.10	SLC6A20	-1.86
LCE1A	-2.98	PADI2	-2.09	CCDC117	-1.86
KRT1	-2.97	KLK6	-2.07	ABLIM2	-1.86
NID2	-2.92	ASPRV1	-2.03	NANOS1	-1.86
SGMS2	-2.75	GNAO1	-2.03	CSF2	-1.86
LRAT	-2.69	C10orf10	-2.02	OLR1	-1.85
PADI1	-2.64	BLOC1S5	-2.01	IL1R2	-1.85
SPRR3	-2.64	RBM24	-2.00	PROS1	-1.84
STEAP4	-2.63	SLC2A12	-2.00	EPHX3	-1.84
CRCT1	-2.59	KRT80	-1.99	LOC101927267	-1.83
FAM72D	-2.58	GULP1	-1.97	SSPN	-1.82
MAP3K8	-2.58	CLDN11	-1.96	KRT8	-1.81
FLG2	-2.50	GRHL3	-1.96	KRTDAP	-1.81
CNKSR2	-2.49	ZNF704	-1.95	CNFN	-1.81
LCE3D	-2.41	LOX	-1.95	MRVI1	-1.81
IGFL1	-2.40	GPR1	-1.94	WWC2	-1.80
KPRP	-2.38	KLK10	-1.93	A2ML1	-1.80
NLRP10	-2.36	NEURL1	-1.93	USP32P1	-1.79
SPNS2	-2.35	NCCRP1	-1.92	CAMK2B	-1.79
UPK2	-2.35	SMPD3	-1.92	NKX3-1	-1.79
CHST6	-2.34	CCL20	-1.92	ICAM5	-1.79
MIA-RAB4B	-2.34	LINC00520	-1.92	CRISPLD2	-1.78
WBP1L	-2.30	KRT78	-1.92	CST6	-1.78
IGFBP3	-2.30	CALML5	-1.91	TPRG1	-1.78
ATP12A	-2.29	LRRC2	-1.91	KLK5	-1.77
QKI	-2.28	C6orf15	-1.91	FAM63B	-1.76
SLC4A4	-2.28	CDKN1C	-1.89	PRR9	-1.76
KRT15	-2.25	ZNF460	-1.88	ZFHX4	-1.75
IFIT2	-2.24	FBXO32	-1.88	HSD17B2	-1.75
PPP1R14C	-2.24	SBSN	-1.88	MAL2	-1.75
APOBEC3B	-2.21	H19	-1.88	AMMECR1	-1.75
LCE1E	-2.19	ENDOU	-1.87	IL36RN	-1.74
INHBA	-2.16	HSD11B1	-1.87	KRT18P55	-1.74

Table 6. List of genes down-regulated by loss of HDAC9 (continued).

Gene ID	Fold change (siHDAC9 vs. siCtrl)	Gene ID	Fold change (siHDAC9 vs. siCtrl)	Gene ID	Fold change (siHDAC9 vs. siCtrl)
ZNF488	-1.73	HOTS	-1.66	HOPX	-1.59
SPRR1A	-1.73	DSG1	-1.66	APLF	-1.59
CCSAP	-1.73	PPP4R4	-1.65	BIRC3	-1.58
AKR1C2	-1.73	VASN	-1.65	LRRN1	-1.58
SPATA6	-1.73	FUZ	-1.65	KLK11	-1.58
DENND2A	-1.72	CHSY1	-1.64	ROR1	-1.58
OLFML2A	-1.72	PALD1	-1.64	G2E3	-1.58
TTC7B	-1.71	RGS16	-1.64	LCN2	-1.58
GPNMB	-1.71	SPRR2G	-1.64	GAB1	-1.58
UCA1	-1.71	TXNRD1	-1.64	ANLN	-1.58
SMG1P5	-1.71	APCDD1	-1.64	DOLPP1	-1.58
SAA1	-1.70	PWAR5	-1.64	YOD1	-1.58
GGT6	-1.70	SCNN1A	-1.64	MTUS1	-1.58
CYP1B1	-1.70	RIMS2	-1.64	TMEM86A	-1.58
USP46	-1.69	SOX6	-1.63	OVOL2	-1.57
DCUN1D4	-1.69	CCDC138	-1.63	IFITM10	-1.57
LCE1B	-1.69	EXPH5	-1.63	CBX6	-1.57
PLBD1	-1.68	POC1B	-1.63	KLK8	-1.57
PIGL	-1.68	HMGB3	-1.63	TBC1D3P1-DHX40P1	-1.56
LMCD1	-1.68	SPRR2A	-1.63	GDA	-1.56
ALDH3A1	-1.68	ANKRD18A	-1.62	TMPRSS11E	-1.56
TYMS	-1.68	GCNT2	-1.62	EPGN	-1.56
AKR1C4	-1.68	RASSF5	-1.61	DNAJC16	-1.56
HAP1	-1.67	NXT2	-1.61	OVOL1	-1.56
ARHGAP42	-1.67	PALM2-AKAP2	-1.61	TMEM133	-1.56
BVES	-1.67	TNFAIP2	-1.61	TNF	-1.56
LINC00630	-1.67	PARD3B	-1.60	WBP1	-1.56
EMC3-AS1	-1.67	TMPRSS13	-1.60	CXCL5	-1.56
SLPI	-1.67	SORBS1	-1.60	SYT8	-1.56
LCE1F	-1.66	EFCAB1	-1.60	USP2	-1.55
ADGRE2	-1.66	DIP2C	-1.60	PHACTR3	-1.55
HEPHL1	-1.66	OXTR	-1.60	AKR1C3	-1.55
SCEL	-1.66	KRT81	-1.59	LOC100129940	-1.55

Table 6. List of genes down-regulated by loss of HDAC9 (continued).

Gene ID	Fold change (siHDAC9 vs. siCtrl)	Gene ID	Fold change (siHDAC9 vs. siCtrl)
LMO7	-1.55	FOLR3	-1.51
CADM1	-1.55	NIN	-1.51
DPYSL3	-1.54	ARFGEF3	-1.50
C3	-1.54	NDRG4	-1.50
TTC9	-1.54	CTAGE8	-1.50
HOXC4	-1.54	ZNF789	-1.50
SCUBE2	-1.54	KRT7	-1.50
GREB1L	-1.54	ARHGAP11B	-1.50
TSC22D2	-1.54	BRCA2	-1.50
ANKRD18B	-1.54	POLQ	-1.50
EPPK1	-1.53	LOC100128361	-1.50
CSDE1	-1.53	ZIK1	-1.50
L3HYPDH	-1.53	KCTD10	-1.50
TMEM55A	-1.53	FLG	-1.50
BCL2	-1.53		
CPS1	-1.53		
GCSH	-1.53		
LCA5	-1.53		
KLK7	-1.53		
OSER1-AS1	-1.52		
AKR1C1	-1.52		
PTPRM	-1.52		
ALDH3B2	-1.52		
CLIC3	-1.52		
LOC100288798	-1.52		
EME1	-1.52		
MYO3B	-1.52		
OCLN	-1.52		
NUDT10	-1.51		
GAL	-1.51		
RLF	-1.51		
GAREM1	-1.51		
SAA2	-1.51		
IDNK	-1.51		

Table 7. List of nearest genes to peaks identified through ChIP-seq of HDAC8 and HDAC9.

HDAC8		HDAC8 and HDAC9		HDAC9	
Gene ID	Distance to TSS (bp)	Gene ID	Distance to TSS (bp)	Gene ID	Distance to TSS (bp)
ABCB1	9394	ADAP1	49705	AGBL2	-6636
ADAMTS16	164751	AFAP1	75893	AK5	201020
AFG3L1P	14796	C16orf46	-8907	ANKLE2	2854
C5orf64	22796	CNTNAP2	2215266	ATP2C1	18891
CALN1	47094	DANT2	80710	BCL11A	85571
CNTLN	203232	DGCR6	-9633	CACNB2	-7590
CSGALNACT1	174894	FGF14	104648	CDKAL1	216481
DBET	3111	LINC00629	6367	CECR2	70264
EFHC2	92029	LOC100507412	6395	CELA2A	2182
ELL	30842	LOC105375545	-5597	COL6A2	-7105
FAM135B	289919	LOC143666	-9544	DOCK8	185288
FRG1	-8801	MIR424	-9679	DTX2P1-UPK3BP1-PMS2P11	18070
GLB1L3	32001	MIR503	-9992	EHBP1	370061
HHIPL2	-4128	MIR503HG	-9760	FAM120B	108175
HPGDS	2526	MIR548T	402016	FAM178B	-620
LDLRAD4	43473	MIR5692B	-6500	GALK2	121370
LINC01297	18339	MIR6724-1	-1058	GRAMD4	14712
LMF1-AS1	-6360	MIR6724-2	-1058	HIPK3	-4956
LOC101928539	-594	MIR6724-3	-1058	LOC105379477	5591
LOC101928880	-1215	MIR6724-4	-1058	LOC442028	52477
LOC63930	59170	MROH1	85396	MBNL3	5439
LOC730100	772758	MRPL23	-7348	MEGF6	27633
MCPH1	170083	OSGIN1	-2389	MIR32	-4869
MIPEPP3	79460	PAX3	89246	NTN5	3670
MIR3156-2	-7169	PDCD6	-4742	OTX1	-6097
MIR4768	-304	PPFIA2	322361	PRKAR1B	139024
MIR99AHG	971	RAPGEF6	119143	RAB3C	45289
NHS	50109	RBFOX1	331313	RAP2C-AS1	189580
PLCXD1	-7155	RGS19	-8665	RGPD5	56796
PPA2	24801	RNA45S5	-1750	RGPD6	58427

Table 7. List of nearest genes to peaks identified through ChIP-seq of HDAC8 and HDAC9 (continued).

HDAC8		HDAC8 and HDAC9		HDAC9	
Gene ID	Distance to TSS (bp)	Gene ID	Distance to TSS (bp)	Gene ID	Distance to TSS (bp)
RGPD4	18269	RNA5-8S5	-8351	SBNO2	-9879
RUNDC3B	75469	RRBP1	23088	SEC1P	31320
SART1	9702	SNORD131	-9407	TMEM128	10829
SLBP	5441	SNX16	-147	TMEM245	68727
SLC10A7	235510	TLK1	87750	URI1	61486
SLC25A13	28760	TOMM40	-8229	ZCWPW2	99643
SORBS2	129420	VPS53	68104	ZNF229	-8660
SPON1	150924	ZNF285	-9838		
STPG2	60757				
TMPRSS11A	1991				
TTC37	62315				
ZDHHC20	81738				
ZNF620	-4797				
ZPBP	122755				

Table 8. Sample statistics from ATAC-seq.

Sample	Number of Reads	Number of Aligned Reads (% of total)	Number of Peaks Detected	Unique Peaks (not in siCtrl)
siCtrl	33,899,014	18,389,608 (54.13%)	22,476	N/A
siHDAC8	47,128,892	25,268,950 (53.46%)	29,236	9,122
siHDAC9	35,965,748	20,589,282 (57.11%)	29,827	9,484
Propionate	41,173,534	23,057,622 (55.85%)	26,661	7,534

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