Curriculum Vitae

CHRISTINA A. YOUNG

Educational Background

2011	B.A.
	McDaniel College
	Westminster, Maryland
	Biochemistry
2011-Spring 2017	Ph.D.
	University of Maryland School of Medicine
	Baltimore, Maryland
	Biochemistry and Molecular Biology
	Advisor - Dr. Richard Eckert

Research Experience

2006-2007	Werner H. Kirsten Student Intern
	National Cancer Institute
	Frederick, Maryland
	Laboratory of Genomic Diversity
	Advisor – Dr. Warren Johnson
2007-2011	Research Assistant (full and part time)
	National Cancer Institute
	Frederick, Maryland
	Laboratory of Genomic Diversity
	Advisor – Dr. Jill Pecon-Slattery
Jun 2011-Aug 2011	Graduate Student
	University of Maryland School of Medicine
	Baltimore, Maryland

Anesthesiology

Advisor – Dr. Gary Fiskum

Dec 2011-Feb 2012 Graduate Student

University of Maryland School of Medicine

Baltimore, Maryland

Pediatrics

Advisor – Dr. Curt Civin

Feb 2012-May 2017 Graduate Student and Ph.D. Candidate

University of Maryland School of Medicine

Baltimore, Maryland

Biochemistry and Molecular Biology

Thesis Advisor - Dr. Richard Eckert

Honors and Awards

2007	McDaniel College Academic Scholarship ($100,000)
2007-2011	Summer Cancer Research Training Award (SCRTA), National Cancer Institute, Frederick, Maryland
2007-2011	McDaniel College Honors Program, College Scholar
2007-2011	Dean's List, McDaniel College: Highest Honors
2009-pres	Beta Beta Beta, The Biology National Honors Society Member
2010-pres	Gamma Sigma Epsilon, The Chemistry National Honors Society
	Member & Vice President
2011	Chemistry Departmental Honors, McDaniel College
2014	Graduate Program in Life Sciences Ph.D. Scholar of the Year Nominee

Publications

1. Perelman P, Johnson WE, Roos C, Seuanez HN, Horvath JE, et al. (2011) A Molecular Phylogeny of Living Primates. PLoS Genetics 7: e1001342. doi:10.1371/journal.phen.1001342. PMID: 21436896; PMCID: PMC3060065 (**Young CA** - *contributions acknowledged*)

2. Eckert RL, Adhikary G, **Young CA**, Jans R, Crish JF, Xu W, Rorke EA (2013) AP1 Transcription Factors in Epidermal Differentiation and Skin Cancer. J Skin Cancer 537028. PMID: 23762562; PMCID: PMC3676924

3. Clerc P, **Young CA**, Grigore AM, Fiskum G, Polster BM (2013) Magnesium Sulfate Protects Against the Bioenergetic Consequences of Chronic Glutamate Receptor Stimulation. PLoS ONE 8: e79982. PMID: 24236167; PMCID: PMC3827425

4. Rorke EA, Adhikary G, **Young CA**, Xu W, Eckert RL (2015) Suppressing AP1 Factor Signaling in Suprabasal Epidermis Produces a Keratoderma Phenotype. Journal of Investigative Dermatology 135(1), 170-80.

5. Rorke EA, Adhikary G, **Young CA**, Rice RH, Elias PM, Crumrine D, Meyer J, Blumenberg M, Eckert RL (2015) Structural and biochemical changes underlying a keratogerma-like phenotype in mice lacking suprabasal AP1 transcription factor function. Cell Death and Disease 6, e1647.

6. **Young CA**, Eckert RL, Crumrine D, Elias PM, Rorke EA (2017) Embryonic AP1 transcription factor deficiency causes an ichthyosis-related collodion baby-like phenotype. Journal of Investigative Dermatology. In press.

7. **Young CA**, Rorke EA, Xu W, Eckert RL (2017) Loss of epidermal AP1 transcription factor function reduces filaggrin level, alters chemokine expression and produces an ichthyosis-related phenotype. Cell Death and Disease. In press.

Conferences

Jul 2012 Transglutaminases in Human Disease Processes

Gordon Research Conference

Davidson, North Carolina

Jan

2013-2017 Biochemistry and Molecular Biology Departmental Retreat

University of Maryland School of Medicine

Baltimore, Maryland

April

2013-2017 Graduate Research Conference

University of Maryland School of Medicine

Baltimore, Maryland

May 2013 The 4th Annual Cancer Biology Research Retreat

University of Maryland School of Medicine

Baltimore, Maryland

Oct 2013 The 64th Annual American Association for Laboratory Animal Science National Meeting

Baltimore, Maryland

May 2014 Society for Investigative Dermatology Annual Meeting

Albuquerque, New Mexico

May 2015 Society for Investigative Dermatology Annual Meeting

Atlanta, Georgia

Invited Lecture

2014 McDaniel College, Gamma Sigma Epsilon Chemical Honor Society Lecture (Triet Bui, Vice-President), September 26, 2014 "Creation of a mouse model of human keratoderma"

Selected Poster Presentations

1. **Young CA** (2011) Multiplex Polymerase Chain Reaction Screen for Feline Immunodeficiency Virus. McDaniel College Chemistry Department Capstone Project, Westminster, Maryland (Oral)

2. **Young CA**, Rorke EA, Babus J, Eckert RL (2013) Inactivating AP1 Transcription Factor Function in Suprabasal Epidermis Produces a Loricrin Keratoderma Phenotype Associated with Enhanced Th1 Chemokine Production. Biochemistry and Molecular Biology Departmental Retreat, University of Maryland School of Medicine, Baltimore, Maryland (Poster)

3. **Young CA**, Rorke EA, Babus J, Eckert RL (2013) Inactivating AP1 An Enhanced Th1 Chemokine response in a mouse model of keratoderma. International Investigative Dermatology, Edinburgh, Scotland (Poster)

4. **Young CA**, Rorke EA, Babus J, Eckert RL (2013) A role of AP1 transcription factors in keratoderma. American Association for Laboratory Animal Science, Baltimore, Maryland (Poster)

5. **Young CA**, Rorke EA, Babus J, Eckert RL (2014) Inactivating AP1 Transcription Factor Function in Suprabasal Epidermis Produces a Loricrin Keratoderma Phenotype Associated with Enhanced Th1 Chemokine Production. Society for Investigative Dermatology Annual National Meeting, Albuquerque, New Mexico (Poster)

6. **Young CA**, Rorke EA, Babus J, Eckert RL (2015) Chemokine changes in suprabasal epidermis of TAM67 mice suggests a role for Th1 lymphocytes in keratoderma. Society for Investigative Dermatology Annual National Meeting, Atlanta, Georgia (Poster)

Summary of Skills

Technical Skills:

- Proficient in laboratory techniques such as fluorescent microscopy, cell culture, mouse handling, QPCR, ELISA, western blotting, and immunohistochemistry
- Protocol development and troubleshooting
- Microsoft Office, Sigma Plot, Adobe Photoshop and Corel Draw

Graduate Level Course Work:

- Mechanisms in Biomedical Sciences
- Advanced Biochemistry
- Advanced Molecular Biology
- Advanced Cancer Biology
- Cancer Biology: Research to Clinic

Communication Skills:

- Excellent oral and written communication skills
- Ability to effectively communicate research findings to technical and non-technical audiences
- Experienced in team work and mentoring

ABSTRACT

Title of Dissertation: A Possible Role for AP-1 Transcription Factors in Development of Epidermal Ichthyoses

Christina A. Young, Doctor of Philosophy, 2017

Dissertation Directed By: Richard L. Eckert, Ph.D., John F.B. Weaver Distinguished Professor and Chair of Biochemistry and Molecular Biology Department

Ichthyoses are highly debilitating and painful disorders characterized by thickening of the skin with marked hyperkeratosis and hyperplasia. Some ichthyosis-related skin disorders are caused by genetic mutation while other forms are acquired. AP-1 transcription factors are known regulators proliferation and differentiation in the epidermis and their activity is necessary in achieving homeostasis of the epidermal barrier. We have previously described a mouse model where inactivation of AP-1 transcription factor function in the suprabasal epidermis, via targeted expression of a dominant negative c-jun (TAM67), produces an ichthyosis-related phenotype. The observed phenotype includes keratinocyte hyperproliferation, delayed differentiation, hyperkeratosis, parakeratosis, extensive vasodilation/erythroderma, tail and digit pseudoainhum, reduced barrier integrity, reduced filaggrin level, and nuclear accumulation of loricrin. To further characterize this mouse model, we sought out to follow phenotype development during embryogenesis. TAM67 expression was turned on during embryogenesis at embryonic day (E) E13.5, E15.5, and E17.5. The E13.5 and E15.5 treated embryos had severely decreased filaggrin levels, nuclear loricrin, and were born with a collodion baby-like phenotype as seen in many human ichthyosis patients. These data suggest a role for AP-1 transcription factors in epidermal development. Next, we focused on the downstream signaling effects of suprabasal AP-

1 transcription factor inhibition in the adult mouse by assessing the role of specific chemokine mediators in phenotype development. We monitored impact on chemokine production and used knockout methods to study the role of the most highly induced chemokines in this process. Suprabasal AP-1 factor inactivation results in increased levels of T_h1 (IFNγ, CCL3, CCL5, CXCL9, CXCL10, and CXCL11) and T_h2 (CCL1, CCL2, CCL5, and CCL11) chemokines in epidermis and serum. S100A8 and S100A9 levels are also elevated. Interestingly, we observe no attenuation of phenotype when TAM67 is expressed in the epidermis of CXCR3 (CXCL9, CXCL10, and CXCL11 receptor) or S100A8/A9 knockout mice indicating lack of a role of these regulators in phenotype development. We propose that loss of AP-1 transcription factor function leads to impaired barrier function and enhanced epidermal chemokine production. These findings suggest that reduced AP-1 transcription factor activity may play a role in the pathogenesis of ichthyosis related skin disorders.

A Possible Role for AP-1 Transcription Factors in Development of Epidermal Ichthyoses

by
Christina A. Young

Dissertation submitted to the faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2017

DEDICATION

To Drs. Warren Johnson and Jill Pecon-Slattery, my first scientific mentors

ACKNOWLEDGEMENTS

First, I would like to thank my mentor, Dr. Richard Eckert, for giving me the opportunity to join his laboratory. I appreciate the insight and guidance from him and my committee members: Drs. Michal Zalzman, Achsah Keegan, Suzanne Ostrand-Rosenberg, David Kaetzel, and Qun Zhou. I also would like to thank members of the Eckert laboratory, in particular Dr. Ellen Rorke for being my unofficial mentor, Jan Babus for her care and attention to the mouse colony, and Wen Xu for her excellent organization and management of the laboratory. I must extend a special thanks to members of the Department of Biochemistry and Molecular Biology, in particular Dr. Gerald Wilson, Kathleen Reinecke, and Koula Kozmo – thank you for answering all of my questions and providing encouragement. I would like to acknowledge an exceptional scientist and professor from my alma mater, Dr. Susan Parrish, who helped shape my career pathway. Last but not least, I am grateful for the support and love of my husband Ricky, family, and friends, especially my UMB friends, Stephanie Gourley, Da Shi, and Dr. Kamalika Saha.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

A. THE EPIDERMIS:

The skin is the largest organ which creates an essential barrier between the organism and its environment. The barrier is responsible for preventing dehydration and UV radiation, as well as blocking entry of pathogens such as parasites, viruses, bacteria, and fungi. The skin is divided into the epidermis and dermis, lying atop the hypodermis, with a layer of fat and muscle underneath. The human epidermis is further subdivided into four layers: the basal, spinous, granular, and cornified layers (Figure 1-1). These layers are constantly being replaced via cell division and differentiation.

The basal layer, connected to the basement membrane, has important reservoirs of stem cells which give rise to the upper layers. These cells are responsible for cell division which replenishes all layers of the epidermis. Here keratin 5 (K5) and keratin 14 (K14) are expressed at high levels; keratin monomers assemble to form important intermediate filaments. Our research will be centered on the importance of a family of transcription factors above this layer referred to as suprabasal, i.e. more differentiated cells.

As cells divide in the basal layer, the cells detach from the basal lamina and move up into the next layer of the epidermis, the spinous layer. This layer is thick and comprised of multiple cell layers. Here cells turn on gene expression of various proteins essential for upper layers of the epidermis, including K1, K10, loricrin, filaggrin, involucrin, lipids, and caspase-14. Also, necessary cell-to-cell connections between keratinocytes called desmosomes are formed (Fuchs & Green, 1980). Desmosomes are connection complexes containing cell adhesion proteins and linking proteins which

attach two cells together through interaction with keratin cytoskeletal filaments. These connections are necessary for normal stratified squamous epithelium and are lost upon epithelial to mesenchymal transition (EMT), a very serious consequence in cancer progression.

The next layer, the granular layer, is so named due to the keratohyalin granules found there. Keratohyalin granules function to bind keratin intermediate filaments (KIF) together with the help of histidine- and cysteine-rich proteins and store the filaggrin precursor, profilaggrin. This is the last layer of viable keratinocytes. The stratum corneum is the outermost layer consisting of essential lipids (ceramides, cholesterol, and fatty acids), natural moisturizing factors (Feingold, 2007; Lopez, Cocera, Wertz, Lopez-Iglesias, & de la Maza, 2007; Nemes, Marekov, Fesus, & Steinert, 1999) and the dead keratinocytes from the lower layers, called corneocytes. The lipids create a water repellant barrier, while the cross-linked structural proteins provide physical strength. These cells are shed off constantly and the basal layer is responsible for replacing the upper layers with new cells. The terminal differentiation process will be discussed in the next section.

Keratinocytes may be the major cell population of the epidermis, but there are more cell types present to keep the skin functional. Immune cells are extremely important in responding to infection; epidermal specific cells include Langerhans cells and leukocytes, while dendritic cells, macrophages, and mast cells reside in the dermis. Melanocytes provide pigmentation and protection from UV-B rays via production of melanin. All of these cell types must communicate with one another and other organs of the body, which will be elaborated upon shortly.

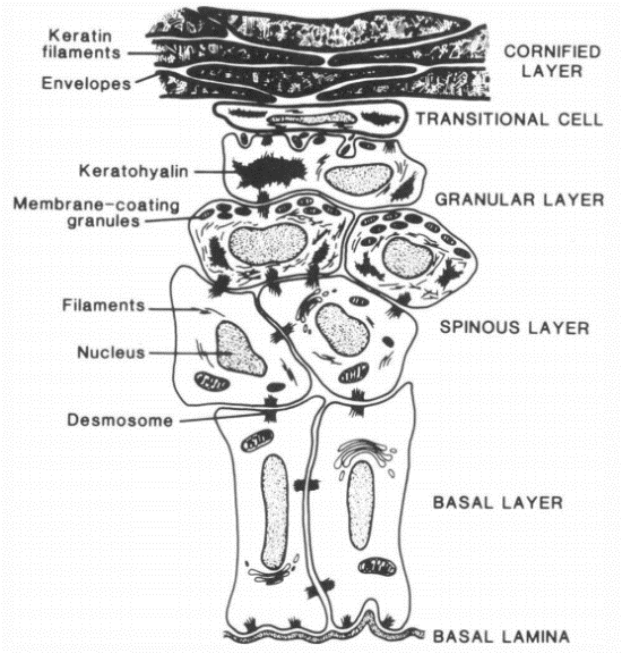

Figure 1-1: Layers of the epidermis The basal, proliferative layer is attached to the basal lamina separating the dermis from the epidermis. Suprabasal layers include the spinous layer and granular layer. The transitional cell represents the final steps in the terminal differentiation process where the cells die and flatten creating the cornified layer. Desmosomes are junctional complexes between keratinocytes. In the granular layer, keratohyalin granules contain profilaggrin where processing occurs to prepare for aggregating keratin filament bundles. These bundles of keratin filaments provide structure. (Eckert & Rorke, 1989)

B. BARRIER AND CORNIFICATION:

The epidermis has a very important process of terminal differentiation wherein the upper layers begin to differentiate, lose their nuclei and structural proteins become heavily crosslinked creating the barrier. If the terminal differentiation of keratinocytes is incomplete, the barrier is altered and consequences can be severe. Therefore this process is tightly regulated (Eckert, Crish, Banks, & Welter, 1997). One important form of regulation is control of gene expression via transcription factors. The work discussed here focuses on activator protein 1 (AP-1) transcription factor family activity in the suprabasal layer of the epidermis.

Keratinocytes begin the cornification process by turning on differentiation genes; many of which are located in the epidermal differentiation complex (EDC) (Mischke, Korge, Marenholz, Volz, & Ziegler, 1996). An increasing calcium gradient exists in the epidermis, with low concentrations in the basal layer and high levels in the stratum granulosum (Menon, Grayson, & Elias, 1985); this gradient triggers expression of some genes (Hohl, Lichti, Breitkreutz, Steinert, & Roop, 1991; Yuspa, Kilkenny, Steinert, & Roop, 1989). These gene products encode structural proteins including loricrin, involucrin, profilaggrin/filaggrin, various keratins, small proline rich proteins (SPRs), and the major cross-linking enzyme, transglutaminase (TG).

When these genes are transcribed and then translated, the keratinocytes are ready to make keratohyalin granules which store the precursor of filaggrin (filament aggregating protein), profilaggrin. As profilaggrin is broken down into smaller products via proteolysis, filaggrin aggregates the keratin intermediate filaments (KIF) and the cells begin to flatten. These filaments are composed of keratin binding partners which are bundled as cells undergo terminal differentiation. They are protective and provide structural integrity for the epidermis. Next, transglutaminases begin their cross-linking function by covalently attaching lipids and structural proteins as well as SPRs and loricrin (Eckert, Sturniolo, Broome, Ruse, & Rorke, 2005). Corneodesmosomes, modified desmosomes, are responsible for crosslinking the corneocytes and these biologically dead, but functional cells, along with the extracellular lipid matrix, provide a physically strong, water-tight barrier for the organism.

C. DERMATOSES:

Dermatoses can be uncomfortable, disfiguring, debilitating, painful, and even deadly. They range from minor dermatitis (i.e. rashes, red skin, and itchiness) to serious autoimmune dysfunction. Here we will discuss symptoms, biomolecular mechanisms, and treatments of ichthyoses. We will focus on ichthyosis, as our AP-1 transcription factor inactivated mouse model produces a phenotype that mimics the human disease.

When discussing ichthyosis, terminology can be a confusing and polarizing topic among dermatologists since many symptoms are found to be overlapping with other skin disorders. Recently, at the first ichthyosis consensus conference, revised nomenclature was agreed upon along with a reclassification of inherited ichthyoses (Oji et al., 2010). Ichthyosis is a broad term used to describe a family of skin disorders characterized by thick, dry, rough, scaly, or flaky skin. Inherited forms of ichthyosis are further classified as Mendelian disorders of cornification (MEDOC). These disorders, involving all or most of the integument, exhibit abnormal epidermal differentiation and desquamation evidenced by accelerated proliferation and/or impaired corneocyte shedding.

Some of these inherited ichthyoses include autosomal recessive congenital ichthyosis (ARCI- harlequin ichthyosis, lamellar ichthyosis, and congenital ichthyosiform erythroderma), Netherton syndrome, keratinopathic ichthyosis (bullous ichthyosis and epidermolytic hyperkeratosis), and ichthyosis vulgaris. The most common types of inherited ichthyosis are recessive X-linked ichthyosis and ichthyosis vulgaris with mutations in the steroid sulfatase and filaggrin genes, respectively (Oji et al., 2010).

Lastly, some types of ichthyosis can present with thickening of the skin, hyperkeratosis, and scaling; previously classified as keratodermas. These types of ichthyosis can be acquired (due to drug/chemical exposure, cancer, allergy, malnutrition, infection, etc.) (Patel, Zirwas, & English, 2007) or inherited (gene mutation passed down through families). For example, inherited ichthyoses have reported mutations in the following genes: keratins, loricrin, transglutaminase, desmoglein1, plakophilin, desmoplakin, connexins, and cathepsin C (Amagai & Stanley, 2012; Christiano, 1997; Kimyai-Asadi, Kotcher, & Jih, 2002). Palmoplantar keratoderma is a common type of keratoderma which affects the palms and soles of the feet. Some types of keratodermas are associated with other affected organs and can exhibit non-skin related symptoms such as hearing loss. Our aim is to understand the biomolecular mechanisms underlying ichthyoses using a unique mouse model.

D. ICHTHYOSES ASSOCIATED WITH FILAGGRIN AND LORICRIN PERTURBATION

Filaggrin is a small (37 kDa), cleaved product of profilaggrin. Profilaggrin, a large (~500 kDa) complex protein, is initially highly phosphorylated and stored in keratohyalin granules of the granular layer. During cornification, profilaggrin is dephosphorylated and proteolytically cleaved releasing filaggrin molecules into the cytoplasm (Resing, Dale, & Walsh, 1985; Resing, Johnson, & Walsh, 1993). Filaggrin acts as an aggregator of keratin filaments, creating tight bundles. Filaggrin is crosslinked to the cornified envelope along with the bundled keratin filaments which provides structure to the cell and is responsible for the flattened shape of the terminally differentiated keratinocytes, called corneocytes (Steinert & Marekov, 1995). Filaggrin is also further

degraded into amino acids which act as natural moisturizing factors (Rawlings & Harding, 2004) and contribute to an effective moisture barrier.

Ichthyosis vulgaris is a debilitating form of ichthyosis that involves filaggrin mutations. Filaggrin is essential for proper barrier development, as a keratin filament aggregator, as an envelope structural component and as a natural moisturizing factor; therefore it is unsurprising that mutations leading to filaggrin deficiency cause major perturbations to the skin. Filaggrin mutations are well documented in ichthyosis vulgaris. Ichthyosis vulgaris (OMIM 146700) is characterized by decreased filaggrin expression, resulting in small, rudimental keratohyalin granules, reduced or absent stratum granulosum, and clinically dry, scaly skin. Furthermore, ichthyosis vulgaris presents in a dose-dependent manner with homozygous and compound heterozygous mutations identified in the filaggrin gene leading to a spectrum of disease severity, moderate to severe (Gruber et al., 2011; McLean, 2016; Smith et al., 2006).

Another form of ichthyosis is loricrin keratoderma. Loricrin is the major protein of the cornified cell envelope, constituting 70-85% of the total protein mass of the cornified layer (Kalinin, Marekov, & Steinert, 2001; Steinert & Marekov, 1995; Steinert, Kartasova, & Marekov, 1998). Expression of loricrin occurs in the granular layer during terminal differentiation. Loricrin is heavily crosslinked to itself as well as small proline rich proteins (SPRs). It interacts with the keratin filaments and the involucrin-scaffolding at the cell membrane of corneocytes.

Loricrin keratoderma (a variant of the Vohwinkel Syndrome (OMIM 604117)) (Camisa & Rossana, 1984; Schmuth et al., 2004)) is an autosomal-dominant type of ichthyosis caused by a 1-bp insertion (730insG) in the loricrin gene (Korge et al., 1997).

Symptoms include hyperkeratosis (thickening of the stratum corneum) accentuated over joints, parakeratosis (retention of the nuclei in the stratum corneum), pseudoainhum (auto amputation of digits), and nuclear loricrin accumulation (Figure 1-2). Skin ultrastructure reveals electron dense intranuclear granules in granular cells, thin cornified envelopes in the lower regions of the stratum corneum, and abnormal extracellular lamellae (Oji et al., 2010). Noninflammatory diffuse palmoplantar keratoderma with a honeycomb pattern is also observed in these patients.

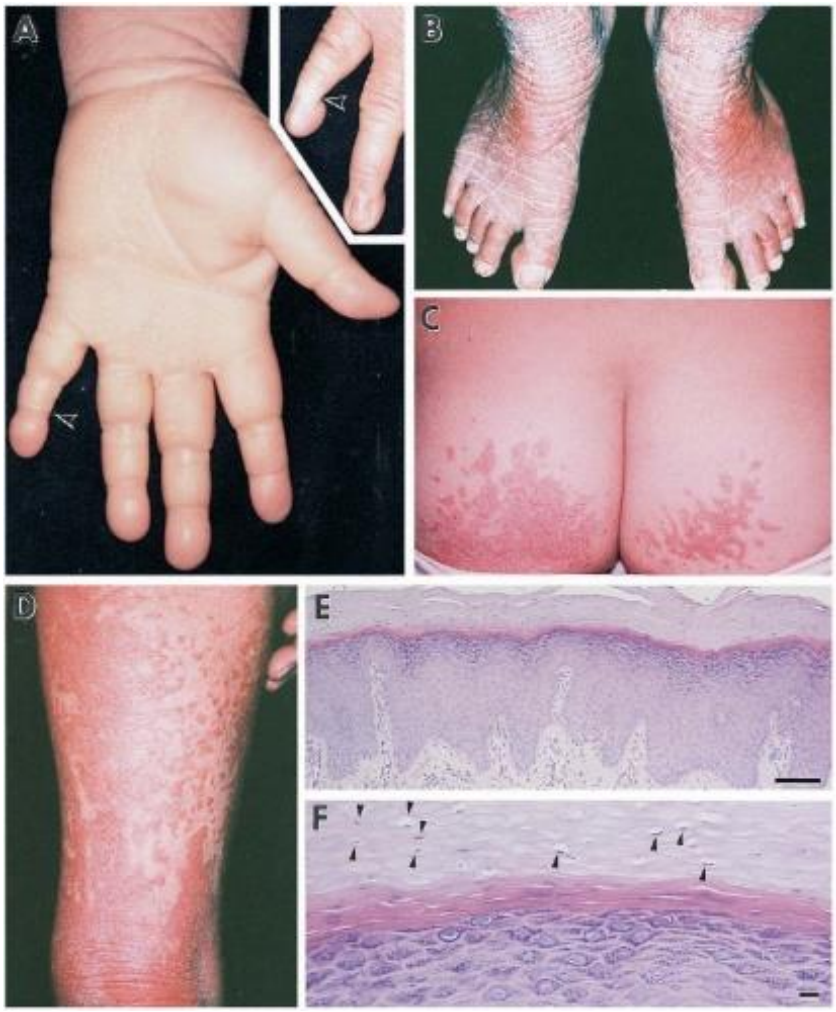

Figure 1-2: Loricrin keratoderma Human patients are photographed here exhibiting major symptoms of this skin disorder. A) Pseudoainum (autoamputation) of the digits. B/C/D) Dry, scaly skin in various regions of the body. E/F) H&E images of hyperproliferative basal layer and parakeratosis in the stratum corneum. Reprinted with permission (License # 4086650459814). (Ishida-Yamamoto et al., 1997)

E. ACTIVATOR PROTEIN 1 (AP-1) TRANSCRIPTION FACTORS:

Since transcriptional control over differentiation and proliferation genes maintains the crucial balance of the epidermis, we ventured to study the role of AP-1 transcription factors in this skin disorder. AP-1 transcription factors are a family of basic leucine zipper proteins comprised of c-jun, junB, junD, c-fos, FosB, Fra-1, and Fra-2 (Angel, Szabowski, & Schorpp-Kistner, 2001; Karin, Liu, & Zandi, 1997; Shaulian & Karin, 2001; Shaulian & Karin, 2002). They dimerize, bind specific sites within promoters of target genes, and affect transcription (Angel et al., 2001; Karin et al., 1997; Shaulian & Karin, 2001; Shaulian & Karin, 2002). AP-1 transcription factors most notably affect transcription of genes that control proliferation (Mizuno, Cho, Ma, Bode, & Dong, 2006; Shi & Isseroff, 2005), differentiation (Adhikary, Crish, Lass, & Eckert, 2004; Eckert et al., 2003; Eckert et al., 2004), and apoptosis (Efimova, Broome, & Eckert, 2004; Raj, Brash, & Grossman, 2006) and therefore play a critical role in epidermal maintenance. Homo- and heterodimers form via leucine zipper domains which are also responsible for dimer specificity and stability. Jun-Jun and Jun-Fos dimers preferentially bind to the phorbol 12-O-tetradecanoate-13-acetate (TPA)-responsive element (TRE) with a base sequence of TGACTCA (Hai & Curran, 1991). Jun-ATF dimers and ATF homodimers preferentially bind to the cAMP-responsive element (CRE) with a base sequence of TGACGTCA (Hai & Curran, 1991). The consensus sequence of AP-1 sites is TGAG/CTCA (Angel & Karin, 1991).

Upstream signaling cascades leading to AP-1 transcription factor activity are well studied. A differentiation stimulus, such as the calcium gradient discussed previously, activates novel protein kinase c (nPKC), Ras small GTPase, the mitogen activated

protein kinase (MAPK) phosphorylation cascade ending with p38δ driven increase in AP-1 transcription factor levels and control over AP-1 target genes (Adhikary, Chew, Reece, & Eckert, 2010; Eckert et al., 2003; Eckert et al., 2004; Efimova, LaCelle, Welter, & Eckert, 1998; Efimova & Eckert, 2000; Efimova, Deucher, Kuroki, Ohba, & Eckert, 2002; Efimova et al., 2004) (Figure 1-3). The MAPK phosphorylation cascade also flows into ERK, JNK, and other p38 isoform kinase signaling pathways, but the emphasis here is on p38δ and its role in regulating differentiation associated gene expression since we are studying the differentiating suprabasal layers of the epidermis.

Stimulated AP-1 transcription factors turn on transcription of early differentiation genes, growth factors, and signaling molecules. AP-1 controls gene expression of cornified envelope proteins such as transglutaminase (Liew & Yamanishi, 1992; Yamada et al., 1994), loricrin (DiSepio et al., 1995), and involucrin (Takahashi & Iizuka, 1993; Welter & Eckert, 1995). AP-1 factors also control expression of epidermal growth factor receptor (EGFR) which is essential for proliferation (Zenz et al., 2003). Keratinocytes can control their growth via a paracrine loop in communication with fibroblasts. IL-1 secreted by keratinocytes leads to c-Jun activity in the fibroblasts which in turn produce keratinocyte growth factor (KGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Maas-Szabowski et al., 2001; Szabowski et al., 2000).

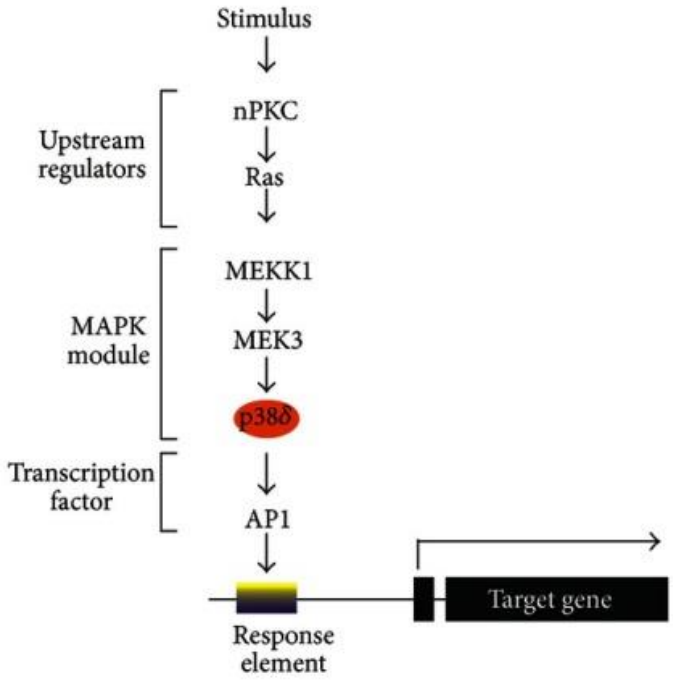

Figure 1-3: AP-1 stimulus Upstream regulators (nPKC and Ras) turn on the MAPK signaling cascade leading to activated p38δ and AP-1 transcription factor activity at AP-1 specific response elements in the promoters of target genes. (Eckert et al., 2013)

F. CREATING AN ANIMAL MODEL OF AP-1 FACTOR DEFICIENCY IN EPIDERMIS:

Scientific methods for studying a protein's role *in vitro* or *in vivo* include targeted and conditional knockdown, overexpression, or expression of a dominant negative form. A dominant negative form of the protein of interest acts antagonistically to affect all wild type forms, thus preventing normal activity. We utilized a dominant negative form of c-jun (TAM67), expressed in epidermis, to evaluate AP-1 transcription factor family inhibition on epidermal phenotype. Wild type c-jun has three domains: transactivation, DNA binding, and leucine zipper. To create the TAM67 construct, the C-terminus transactivation domain was deleted and a FLAG tag was added to the N-terminus for transgene detection (Figure 1-4). TAM67 protein can prevent transcriptional activity by homo dimerizing with other TAM67 proteins and blocking AP-1 binding sites or hetero

dimerizing with functional AP-1 transcription factors and quenching wild type activity by preventing transactivation (Brown, Chen, & Birrer, 1994; Han et al., 2012). Dominant negative c-jun has been used for the past 25 years in various scenarios including keratinocyte research (Cooper et al., 2003; Dong et al., 1997; Efimova et al., 1998; J. J. Li, Rhim, Schlegel, Vousden, & Colburn, 1998; Rorke, Adhikary, Jans, Crish, & Eckert, 2010; Rorke et al., 2015; Rorke, Adhikary, Young, Roop, & Eckert, 2015; Shen et al., 2006; Thompson, MacGowan, Young, Colburn, & Bowden, 2002; Young et al., 1999; Young, Farrell, Lambert, Awasthi, & Colburn, 2002).

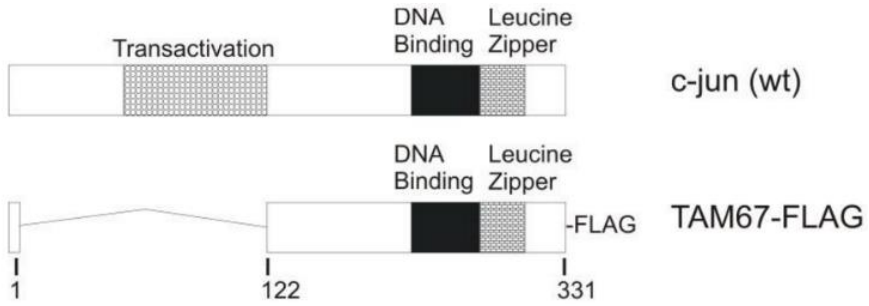

Figure 1-4: TAM67 construct The TAM67 construct (bottom) is pictured compared to wild type c-jun (top). The C-terminus transactivation domain has been deleted and a FLAG tag has been added to the N-terminus. The TAM67 protein can prevent transcriptional activity by homo dimerizing with other TAM67 proteins and blocking AP-1 binding sites or hetero dimerizing with functional AP-1 transcription factors and quenching wild type activity. (Han et al., 2012)

Important *in vivo* TAM67 experiments were completed by the Colburn laboratory using a basal targeted approach with the K14 promoter driving TAM67 expression in the mouse basal epidermis. While the basal targeted TAM67 mouse model does display interesting changes upon carcinogen challenge which further expands our knowledge of skin carcinogenesis at the molecular level (Cooper et al., 2003; Thompson, MacGowan et al., 2002; Tichelaar et al., 2010; Young et al., 1999; Young et al., 2002), there are no changes in epidermal appearance, nor any changes in keratinocyte proliferation. These data suggest that AP-1 transcription factors are not as important in the proliferative

basal layer as previously thought. Therefore, the Eckert laboratory endeavored to create a suprabasal targeted TAM67 mouse model to evaluate the role of suprabasal AP-1 transcription factor activities.

Suprabasal-specific expression of TAM67 was achieved by using the human involucrin promoter to drive expression of the Tet-on system (Tet0-TAM67-FLAG mouse). Involucrin is a structural protein that is expressed only in the suprabasal epidermis. The tetracycline responsive element (TRE) drives expression of the transgene, TAM67 (hINV-rTA mouse). When these two mice are crossed twenty-five percent of the pups are bitransgenic, herein called TAM67-rTA. A major advantage of this system is that transgene expression is inducible; it can be turned on and off through doxycycline treatment in the drinking water.

The resultant TAM67-rTA mouse model did attenuate tumorigenesis (Rorke et al., 2010), as previously seen in the basal driven system, but also had a surprising and unique phenotype, i.e. a visible change to the skin without stimulus. Characterization of the suprabasal TAM67 mouse phenotype was done by Rorke et al (Rorke, Adhikary, Young, Roop et al., 2015) (Figure 1-5).

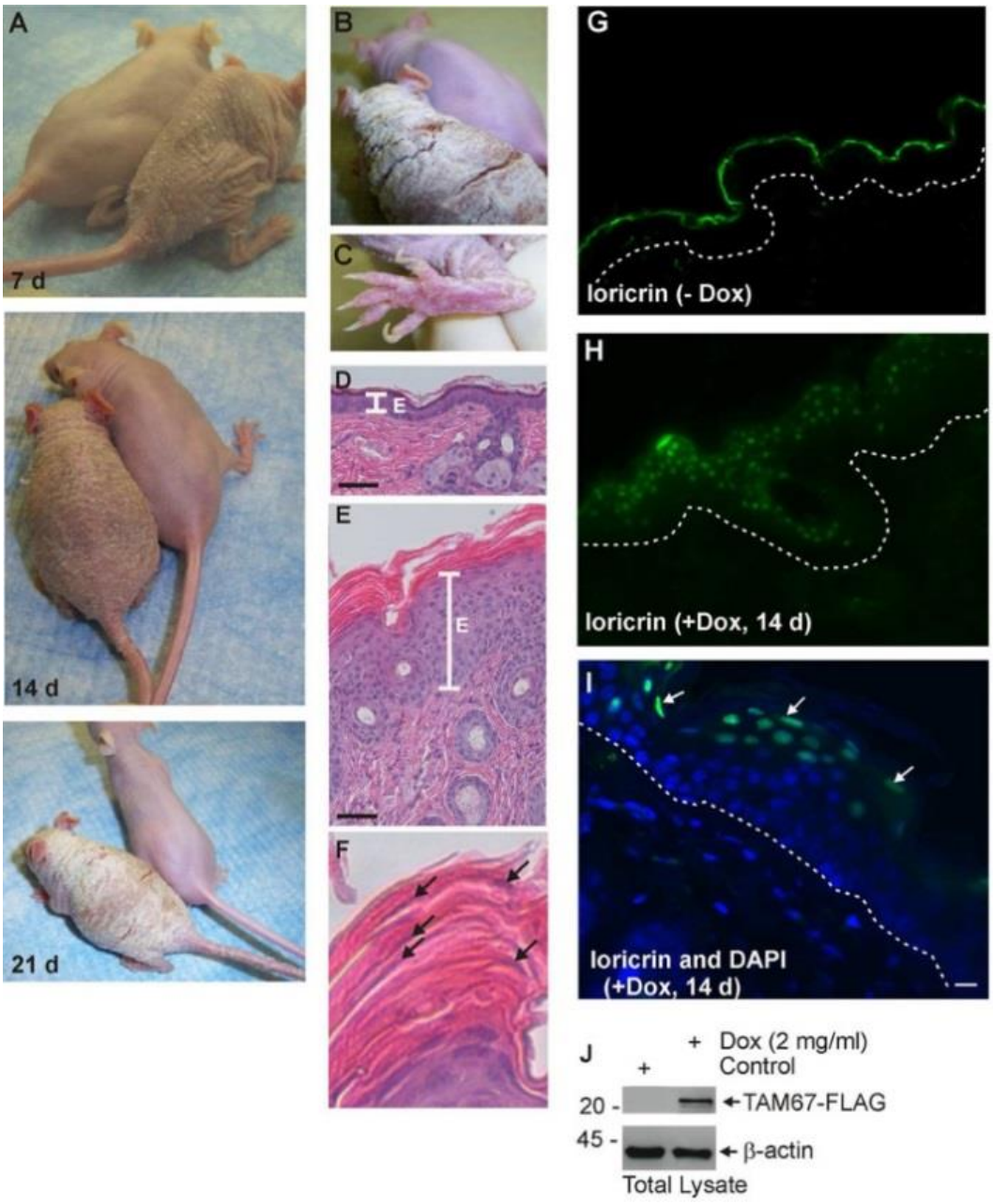

Figure 1-5: Suprabasal TAM67 mouse phenotype A) Hairless TAM67-rTA mice are photographed alongside their wild type littermates and show the flaky, dry phenotype which progresses to a thick scale after 21 days of transgene expression. B) This thick scale can have deep fissures. C) Scaling is also present on the bottom of the feet. D/E) H&E images of wild type skin (D) compared to TAM67-rTA skin (E). F) Nuclei are abnormally retained within the stratum corneum in TAM67-rTA epidermis. G/H/I) Loricrin is located cytoplasmically in wild type epidermis (G) compared to nuclear localization in TAM67-rTA expressing epidermis (H/I). J) TAM67-FLAG protein is detected in doxycycline treated TAM67-rTA mice, but not control mice. Reprinted with permission (License # 4086641071980). (Rorke, Adhikary, Young, Roop et al., 2015)

The phenotype of these mice begins with dry, flaky skin after one week of TAM67 expression. This continues over time to develop thick, dry, scaly skin by two to three weeks. This dry scaly skin is a symptom of human keratoderma called hyperkeratosis which develops deep fissures over time. The bottom of the feet also have extensive scale just like in palmoplantar keratoderma seen on the palms and soles in human patients.

Histological analysis of the skin shows the huge increase in epidermal thickness or hyperproliferation as early as 48 hours. This observed change in basal activity when suprabasal AP-1 transcription factors are inhibited suggests a communication pathway from the upper layers of the epidermis to the basal/proliferative layer and is the basis for the project outlined in chapter three. Also, parakeratosis or retention of nuclei in the stratum corneum is observed in the TAM67-rTA mice. All of these data suggest incomplete differentiation, thus affecting the mouse skin barrier and compromising the major function of the skin.

Another phenotypic hallmark of the suprabasal TAM67 epidermis is nuclear loricrin accumulation beginning around 48 hours after doxycycline treatment and transgene induction. Without doxycycline treatment, the loricrin protein can be found cytoplasmically in the upper layers of the epidermis. However upon doxycycline treatment, loricrin translocates into the nucleus and can no longer perform its structural function. Lastly these mice display a very unusual symptom, auto amputation of the tail or pseudoainhum. Constriction rings form around the tail, blocking blood flow and eventually amputating portions of the tail which do not regrow. Surprisingly, all of these

phenotypic and biochemical changes in the TAM67-rTA mice mimic the human ichthyosis previously discussed, loricrin keratoderma.

Other mouse models have been used to study this skin disorder by knocking out loricrin or expressing mutant loricrin. Loricrin knock out mice display a mild phenotype (Jarnik et al., 2002; Koch et al., 2000), while transgenic mice expressing a mutant form of loricrin have the loricrin keratoderma phenotype observed in the suprabasal TAM67 mouse (Suga et al., 2000), but the authors failed to describe any biomolecular events downstream of the presence of loricrin in the nucleus. It has been proposed that nuclear loricrin may be responsible for altering transcription (Yoneda et al., 2010; Yoneda et al., 2012). Interestingly, we found no effect on phenotype development of the suprabasal TAM67 mouse when crossed with a loricrin null mouse line (TAM67-rTA/LOR -/-) (Rorke et al., 2015); therefore if nuclear loricrin does play a role in transcription it is upstream of the AP-1 factors. All the work summarized heretofore pertains to the adult phenotype, but we must not forget the critical development of the epidermis in utero and resultant congenital skin disorders.

G. EMBRYONIC SKIN DEVELOPMENT:

Another interesting “symptom” associated with loricrin keratoderma is a neonatal syndrome called collodion baby. This phenotypic hallmark is observed in some forms of ichthyosis (Oji et al., 2010); thirty-six percent of loricrin keratoderma patients were born with this phenotype (Yeh, Yang, & Chao, 2013). The newborn appears to have an extra layer of skin which is red, taut, and shiny; this membrane is shed a couple weeks after birth (Gedicke, Traupe, Fischer, Tinschert, & Hennies, 2006; Matsumoto et al., 2001;

Takeichi & Akiyama, 2016; Vahlquist, 2010; Yeh et al., 2013) (Figure 1-6). Babies receive special attention in the neonatal intensive care unit with humidified incubators to prevent hypothermia, infection, and dehydration.

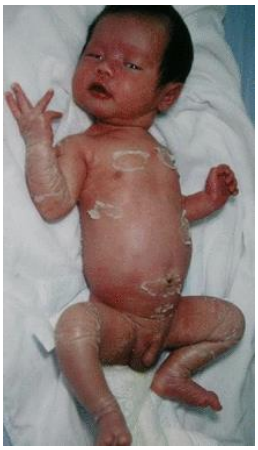

Figure 1-6: Collodion baby phenotype Patient exhibiting the collodion baby phenotype at birth photographed at three weeks of age. After treatment in the intensive care unit controlling dehydration and infection, small portions of this membrane remain and will continue to slough off over time. This patient may suffer from loricrin keratoderma later in life. Reprinted with permission (License # 4086640185522). (Yeh et al., 2013)

The observation of this neonatal phenotype and its connection to loricrin keratoderma sparked the study described in chapter two. But first, to better appreciate the role of AP-1 transcription factors we must understand the process of embryonic skin development and the formation of the first barrier.

Embryonic proliferation, differentiation, and cornification of keratinocytes proceeds in a very similar way compared to adult skin; however this is all happening for the very first time as the mouse develops and prepares for the air environment outside of the womb. The mouse epidermis develops from the surface ectoderm which begins as one proliferative layer. Cells in this layer commit to stratification at E9.5 with expression of K5 and K14 (Byrne, Tainsky, & Fuchs, 1994) and begin to stratify at E10.5 (M'Boneko & Merker, 1988). Next, suprabasal keratins, K1 and K10, are

expressed by E15.5 (Byrne et al., 1994), loricrin expression is turned on at E16.5 (Byrne et al., 1994; Mehrel et al., 1990; Yoneda & Steinert, 1993), and filaggrin appears at E17.5 (Rothnagel, Mehrel, Idler, Roop, & Steinert, 1987). Therefore under normal conditions, the mouse has a fully functional epidermis with a competent cornified layer by E18.5, approximately 2 days before birth.

Extensive research has been done on regulators of skin development including p-63 (Yang et al., 1998), notch signaling (Nickoloff et al., 2002; Okuyama et al., 2004; Rangarajan et al., 2001), NF-κB (J. Z. Qin et al., 1999; J. Z. Qin, Bacon, Chaturvedi, & Nickoloff, 2001; Seitz, Lin, Deng, & Khavari, 1998), bone morphogenetic protein (BMP) (Botchkarev, 2003; Botchkarev & Sharov, 2004; A. G. Li, Koster, & Wang, 2003; Mishina, 2003), Homeobox (Hox) genes (Chang, Kozono, Chida, Kuroki, & Huh, 1998; Detmer, Lawrence, & Largman, 1993; Mack et al., 2003; Magli, Largman, & Lawrence, 1997; Stelnicki et al., 1998), AP-2 (Byrne et al., 1994; Mitchell, Timmons, Hebert, Rigby, & Tjian, 1991), and CCAAT/enhancer binding proteins (C/EBP) (Maytin & Habener, 1998; Oh & Smart, 1998). We will focus on the role of AP-1 transcription factor signaling.

H. BIOCHEMICAL EFFECTS OF SUPRABASAL AP-1 TRANSCRIPTION FACTOR INHIBITION:

Recent work done in the Eckert laboratory explored the myriad of structural and biochemical changes which occur in the suprabasal TAM67 expressing mouse epidermis (Rorke et al., 2015). Three major techniques were employed: electron microscopy, mass spectrometry, and RNA array analysis.

Electron microscopy provided detailed images of wild type compared to suprabasal TAM67 expressing epidermis. Cornified envelope formation is defective with a reduction in the number of mature cornified envelopes as well as their thickness. Necessary cell-to-cell connections and structural components of the cells are abnormal including desmosomes and keratin filaments. Also the lipid matrix, in which the corneocytes reside, is severely altered with abnormal lamellar body contents, premature lamellar lipid secretion, and incomplete processing of secreted lipids. Discovery of all these changes at the molecular level make it unsurprising that the barrier of the TAM67-rTA mouse is dysfunctional and the phenotype worsens over time.

Mass spectrometry analysis of the cross-linked proteome revealed significant changes in many structural proteins, enzymes, and inflammation associated proteins and most of these changes were mirrored in the RNA array. TAM67 expressing epidermis had reduced levels of the following keratins: K10, K15, K23, K27 (type I) and K1, K2, K3, K5, K71, K77, K78 (type II) and increased levels of the hyperproliferation associated keratins: K6a, K6b, and K16. Changes in hair keratins and hair associated proteins were also observed. Early cornified envelope precursors were found to be increased (small proline-rich protein (Sprr) 1a, Sprr1b, Sprr2h, Sprr2i and involucrin) while late cornified envelope precursors were decreased (keratinocyte proline-rich protein (Kprp), loricrin, late cornified envelope (Lce) 1a1). Furthermore, filaggrin and filaggrin related proteins were dramatically reduced. This is important because the structural components of the cornified layer are dysregulated and therefore unable to construct functional corneocytes.

Proteins associated with inflammation and envelope defense were highly upregulated such as S100A8, S100A9, annexin (Anxa) 7, Anxa8, and fatty-acid-binding protein (Fabp) 5. This suggests the epidermis is responding to a real or perceived infection.

RNA array analysis of the entire epidermis revealed an increase in core desmosome proteins desmocollin 2 (DSC2) and desmoglein 3 (DSG3), while mass spectrometry picked up increased levels of desmosome tethering proteins, periplakin (Ppl), envoplakin (Evpl), plakophilin (Pkp) 1 and Pkp3. The proteins needed to create demosomal connections between cells are present, but the epidermis is unable to properly form functional desmosomes. This means that the cells are not linked and therefore fail to create a functional barrier.

These data confirm that the structure and function of TAM67-rTA epidermis is dramatically altered due to the inhibition of the AP-1 transcription factor family in the suprabasal layers. This work is crucial in better characterizing the biochemical and structural changes in this unique mouse model, but does not touch upon short-lived signaling molecules which provide communication to achieve epidermal homeostasis and barrier integrity.

I. CROSSTALK BETWEEN THE EPIDERMIS AND IMMUNE SYSTEM:

Signaling molecules (cytokines, chemokines, and interleukins) are essential in multicellular organisms for communication between organs and sensing the environment. Epidermal immune cells play an important role in the innate immune system by surveilling the outermost layers of the skin to detect pathogens or problems.

Some immune cells are resident to the skin while others infiltrate into the skin upon infection (Figure 1-7). Extensive cytokine research has been done in the fields of psoriasis and atopic dermatitis and therefore the changes in ligands, receptors, and activated signaling cascades are well documented. However, cytokine research is scant in the fields of ichthyosis and keratoderma. To understand the cytokines involved we must first define the predominant T helper (T_h) cell types: T_h1, T_h2, and T_h17. They arise from the differentiation of CD4+ effector T cells and coordinate immune responses. In general, T_h1 cells respond to infection while T_h2 cells direct asthma and allergic responses. The T_h17-mediated response is thought to control autoimmune pathways. However, differential expression of cytokines between these subtypes means that the helper T cell categories are not as distinct as once believed to be.

Along with nonspecific markers of inflammation, the cytokine profile of psoriasis is historically defined as increased levels of IFN-γ, TGF-α, IL-2, IL-6, IL-8, IL-12, IL-18, IL-23, IL-23R, LIF-1, and CXCL10 with decreased levels of IL-1, IL-4, IL-5 and IL-10, (Ferrari et al., 2015; Villanova, Di Meglio, & Nestle, 2013). This is predominantly a T_h1 response (Perera, Di Meglio, & Nestle, 2012), although recent studies have proposed a role for T_h17 cells as well (Gaffen, Jain, Garg, & Cua, 2014). On the other hand, atopic dermatitis is associated with a T_h2 immune response (McLean, 2016) leading to allergic inflammation and itchiness. Increased secretion of IL-4, IL-5, IL-13, IL-25, IL-31, IL-33, TSLP, elevated serum levels of immunoglobulin E (IgE) and decreased filaggrin levels define this disorder (Irvine, McLean, & Leung, 2011; Seguchi et al., 1996).

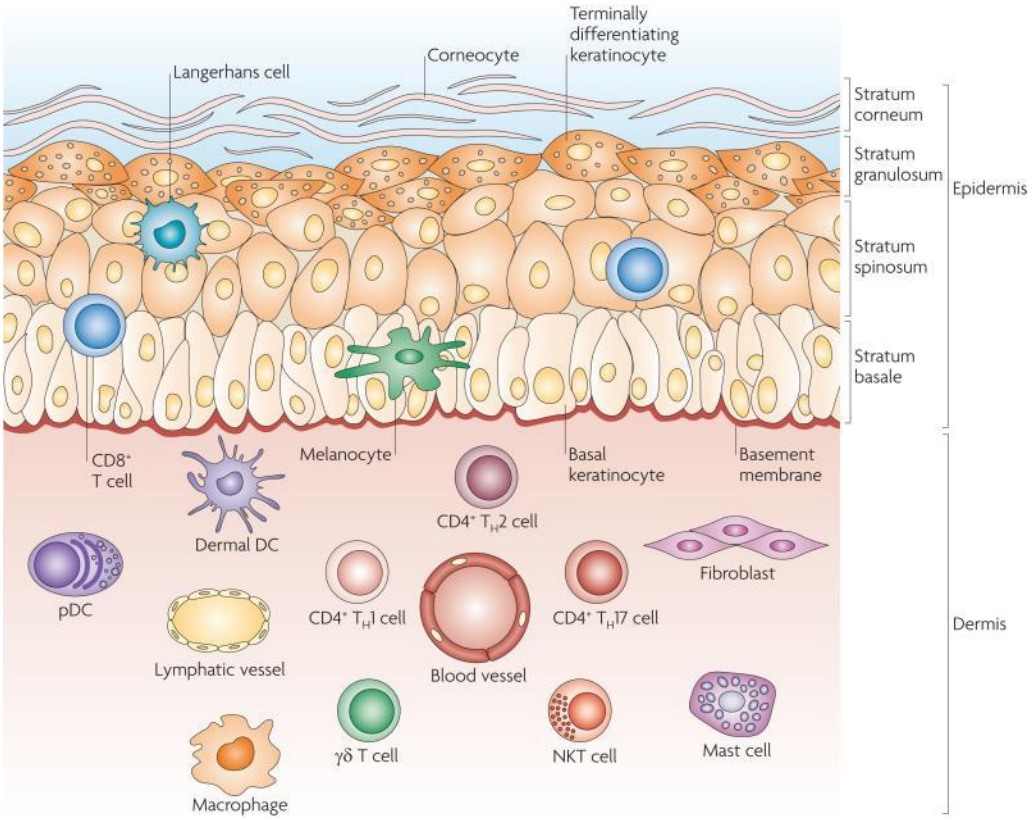

Figure 1-7: Immune cells of the skin Various immune cells reside within the epidermis such as Langerhans cells, while others migrate into the epidermis upon insult (CD8+ and CD4+ T cells). In the dermis, mast cells and macrophages, among others, surveil the area while the blood and lymphatic vessels transport cells throughout the body. Reprinted with permission (License # 4086990954012). (Nestle, Di Meglio, Qin, & Nickoloff, 2009)

AP-1 transcription factors can be involved with communication in multiple ways Examples include controlling gene expression of differentiation genes and thereby barrier formation and controlling gene expression of homeostatic cytokines. We have already provided evidence for the extensive AP-1 dependent-regulation of differentiation genes (involucrin, loricrin, filaggrin, keratin, etc...) and how inhibition of suprabasal AP-1 transcription factors disrupts the cornification process (Rorke et al., 2015; Rorke, Adhikary, Young, Roop et al., 2015). This leads to a dysfunctional barrier, but how do cells in the skin respond? Dendritic epidermal T cells (DETCs - sometimes also

referred to as γδT cells) are strategically positioned throughout the epidermis (Heath & Carbone, 2013; Macleod & Havran, 2011) to respond to a breach in the barrier and constitute 90% of mouse epidermal T cells (Nestle et al., 2009). Keratinocytes proliferate in response to growth factors produced by γδT cells (Di Meglio, Perera, & Nestle, 2011; Heath & Carbone, 2013), perhaps in an effort to regrow in the event of a wound. To achieve homeostasis and balance the cycles of proliferation and differentiation in the epidermis, cutaneous T cell-attracting chemokine (CTACK, now known as CCL27) is constitutively expressed by keratinocytes in normal skin and signals to immune surveillance T cells that the tissue is normal (Homey et al., 2002; Morales et al., 1999). In the absence of this regulatory signal the immune system can become activated.

The promoters of many inflammatory response genes, especially those encoding cytokines and chemokines, have functional AP-1 binding sites (Rahman & MacNee, 1998). CCL27 and IL1-α have putative AP-1 binding sites in their promoter region and we have found mRNA expression of CCL27 (-1.05) and IL1-α (-1.7) to be downregulated in the suprabasal TAM67-rTA mouse epidermis (Rorke et al., unpublished data). Perhaps the resultant TAM67 driven downregulation of CCL27 transcription triggers a disruption in skin homeostasis and initiates a response leading to the observed keratoderma-related phenotype. CCR10+ memory T cells (activated by CCL27) secrete TNFa and IFNγ (Albanesi, Scarponi, Giustizieri, & Girolomoni, 2005; Hudak et al., 2002). Keratinocytes constitutively bear the IFNγ receptor and are a primary target of IFNy (Albanesi et al., 2005; Barker, Sarma, Mitra, Dixit, & Nickoloff, 1990). Activated keratinocytes release predominantly T_h1 responders: CXCL9

(monokine induced by gamma interferon (MIG)), CXCL10 (interferon gamma-induced protein 10 (IP-10)), CXCL11 (interferon-inducible T-cell alpha chemoattractant (I-TAC)), CCL2, CCL27, CCL20, CCL5 (regulated on activation, normal T cell expressed and secreted (RANTES)), and IL-8 (Albanesi et al., 2001; Nestle et al., 2009). CXCL9, CXCL10, CXCL11, and CCL20 attract effector T cells (Nestle et al., 2009). A shift from T_h 2 (initiation of inflammation) to T_h 1 (chronic disease) is well documented in immune responses (Albanesi et al., 2001; Cavani et al., 1998; Cavani et al., 2000; Gittler et al., 2012; Grewe et al., 1998; Tuzun, Antonov, Dolar, & Wolf, 2007). However, T cells are not the only responders; dendritic cells also participate in the skin. For example, thymic stromal lymphopoeitin (TSLP) is a cytokine produced by a variety or cell types (including keratinocytes) which activates CD11b+ dermal dendritic cells (Malissen, Tamoutounour, & Henri, 2014). Similarly, langerhans cells, macrophages, mast cells, and natural killer cells are present and active in the skin. The suprabasal TAM67 mouse model provides an opportunity to examine the crosstalk between the epidermis and the immune system and will advance the study of ichthyosis/keratoderma.

J. GOALS OF THE STUDY:

1) Our first goal was to characterize the effects of suprabasal TAM67 expression in neonatal skin development. By inhibiting activity of suprabasal AP-1 transcription factors during embryogenesis, we can evaluate the effects upon gene expression of differentiation proteins during formation of the barrier. We hypothesized that the collodion baby phenotype, seen in a great proportion of human loricrin keratoderma patients, may be recapitulated in this mouse model. We treated pregnant dams with

doxycycline on embryonic (E) days E13.5, E15.5, and E17.5 to induce expression of TAM67 and then examined the embryos at E20. The phenotypes were also examined in newborn mice. Tissue from embryos on E20, the day before birth, was collected for immunostaining, ultrastructural, and cornified envelope analysis. If this neonatal phenotype is recapitulated, it would be significant as the first linking of a transcription factor to this ichthyosis phenotype.

2) The second goal is to investigate the biomolecular mechanisms behind the observed phenotype. This will be achieved through analysis of changes in signaling molecules, both at the RNA and protein level, to better understand how the epidermis communicates to the basal layer as well as the immune system. We hypothesize that a collection of cytokines, chemokines, and interleukins play a role in identifying the disruption in the balance between proliferation and differentiation caused by suprabasal AP-1 factor inhibition, and subsequently turn on signaling cascades that drive the disease phenotype. These studies have the potential to expand our understanding of human ichthyoses.

CHAPTER 2: EMBRYONIC AP-1 TRANSCRIPTION FACTOR DEFICIENCY CAUSES AN ICHTHYOSIS-RELATED COLLODION BABY-LIKE PHENOTYPE

A. ABSTRACT:

AP-1 transcription factors are important controllers of gene expression in the epidermis, and altered AP-1 factor function can perturb keratinocyte proliferation and differentiation. However, our understanding of how AP-1 signaling changes may underlie or exacerbate skin disease is limited. We have shown that inhibiting AP-1 factor function in adult epidermis leads to an ichthyosis/keratoderma-like phenotype. We now show that inhibiting AP-1 factor function in embryonic epidermis produces marked phenotypic changes including compromised barrier function, enhanced dehydration susceptibility and a collodion baby-like phenotype. This is coupled with reduced cornified envelope thickness and major ultrastructural changes. A major biochemical change is a reduction in filaggrin gene expression leading to reduced filaggrin and profilaggrin content. We propose that reduced AP-1 transcription factor signaling results in reduced filaggrin gene expression and filaggrin protein content which leads to reduced cornified envelope formation, reduced barrier integrity and a collodion baby-like phenotype. The presence of a collodion baby phenotype in the filaggrin-deficient epidermis is reminiscent of the collodion baby phenotype observed in newborns that are later diagnosed with ichthyosis.

B. INTRODUCTION:

AP-1 transcription factors comprise a family of proteins, including c-jun, junB, junD, c-fos and Fra-1 (Angel et al., 2001; Eckert et al., 1997), which form homo- and heterodimers that bind to specific DNA response elements in regulatory regions of target genes to drive transcription (Angel et al., 2001; Eckert et al., 1997). AP-1 factors in the epidermis are expressed in a differentiation-dependent manner (Welter & Eckert, 1995) and control expression of differentiation associated genes (Crish, Zaim, & Eckert, 1998; Crish, Gopalakrishnan, Bone, Gilliam, & Eckert, 2006), including filaggrin (Jang, Steinert, & Markova, 1996). We recently described a phenotype in adult mice that includes epidermal hyperproliferation, parakeratosis, hyperkeratosis, pseudoainhum, impaired barrier function, nuclear loricrin accumulation and reduced filaggrin level in response to suprabasal epidermis-specific inactivation of activator protein 1 (AP-1) transcription factor signaling (Rorke et al., 2010; Rorke et al., 2015; Rorke, Adhikary, Young, Roop et al., 2015). The skin is scaled, loricrin accumulates in the nucleus and filaggrin levels are reduced (Rorke, Adhikary, Young, Roop et al., 2015). In addition, constricting bands encircle the tail and digits in some animals (Rorke, Adhikary, Young, Roop et al., 2015). These novel findings suggest that loss of AP-1 factor signaling in the suprabasal epidermis is sufficient to cause a mouse phenotype that shares properties with keratoderma/ichthyosis. Ichthyosis is often associated with a collodion baby phenotype where babies are born encased in a translucent membrane (Gedicke et al., 2006; Matsumoto et al., 2001; Oji & Traupe, 2006; Oji & Traupe, 2009; Yeh et al., 2013). Thirty six percent of loricrin keratoderma patients, for example, are born as collodion babies (Yeh et al., 2013). In the present study we show that inactivation of

AP-1 factor function, during epidermal embryonic development, causes a collodion baby phenotype associated with reduced filaggrin mRNA and protein, nuclear loricrin localization, compromised barrier function and abnormal epidermal ultrastructure.

Ichthyosis is caused by inherited mutations in various genes involved in barrier formation, but can also be acquired in response to cancer, infection or treatment with medication (Oji et al., 2010). Filaggrin loss of function and reduced expression is a cause of ichthyosis vulgaris (Elias, Williams, Crumrine, & Schmuth, 2010; McLean, 2016; Oji et al., 2010; Richard, 2004; Smith et al., 2006; Takeichi & Akiyama, 2016). Consistent with these studies, our data suggest that loss of AP-1 transcription factor function results in loss of filaggrin gene expression and a reduction in filaggrin protein level, and that these events produce an ichthyosis-like phenotype.

C. MATERIALS AND METHODS:

Embryo Studies Construction of the TAM67-rTA transgenic mouse was previously described (Rorke et al., 2010). These mice encode a doxycycline-inducible cassette encoding TAM67, a dominant-negative form of c-jun that inhibits function of all AP-1 factors (Rorke et al., 2010). Induction of TAM67 expression results in an ichthyosis/keratoderma-like phenotype (Rorke et al., 2010). To study the role of AP-1 factor inactivation in embryonic epidermis, we mated heterozygous (TAM67+/-) and heterozygous (rTA+/-) mice and treated the pregnant dams with doxycycline (2 mg/ml) in drinking water beginning on embryonic days E13.5, E15.5, and E17.5. At E20, prior to birth, the pups were euthanized and harvested. We use the SKH1 hairless mouse genetic background so that epidermal phenotypes can be readily visualized (Rorke et

al., 2010; Rorke, Adhikary, Young, Roop et al., 2015). The SKH1 hairless mice are immune competent (Benavides, Oberyszyn, VanBuskirk, Reeve, & Kusewitt, 2009). Dorsal total skin was removed, sections were prepared for histology and the remaining skin was flash frozen in liquid nitrogen, powdered and stored at -80 C. These studies were approved by the Institutional Animal Care and Use Committee and followed the NIH Guidelines for the Care and Use of Laboratory Animals. Statistical analysis was performed using the student's t-test.

Skin permeability assay Pups were removed from the womb at E20, euthanized, rinsed in phosphate-buffered saline (PBS), immersed sequentially in 25%, 50%, 75% and 100% methanol (1 min each), PBS, 0.1% toluidine blue for 6 - 8 min, and then rinsed in PBS and photographed.

Cornified envelope images and quantification An 8 mm punch biopsy of dorsal embryonic skin was collected and the whole tissue (epidermis and dermis) was boiled for 20 min in 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM DTT, and 2% sodium dodecyl sulfate. Cornified envelopes were collected by centrifugation and counted using a hemocytometer. Data is graphed as average cornified envelope number/5 µl volume.

Filaggrin mRNA Filaggrin and filaggrin 2 mRNA level was measured by treating pregnant dams with 2 mg/ml doxycycline in drinking water beginning at E13.5, E15.5 and E17.5. The pups were excised at E20 and whole skin was flash frozen, pulverized and RNA was converted to cDNA followed by qRT-PCR detection of filaggrin and filaggrin 2 mRNA (Chew, Adhikary, Wilson, Reece, & Eckert, 2011). Filaggrin primer set 1 (P1): 5'-GAA TCC ATA TTT ACA GCA AAG CAC CTT G and 5'-GGT ATG TCC

AAT GTG ATT GCA CGA TTG. Filaggrin primer set 2 (P2): 5'-GAA GGA ACT TCT GGA AGG ACA AC and 5'-TCC ATC AGT TCC ACC ATG CCT C (Hansmann et al., 2012; Presland et al., 2000). Filaggrin 2 primers set: 5'-GAG CAA GGA TGA GCT AAA GGA AC and 5'-GCC ACG CCT ATG CTT CTT TGA C (Hansmann et al., 2012; Presland et al., 2000).

Immunological methods Immunofluorescence was performed using paraffin-embedded formalin-fixed sections (Rorke et al., 2010). Anti-loricrin (PRB-145P), anti-K14 (PRB-155P), anti-K10 (PRB-159P) and anti-Filaggrin (PRB-417P) were form Covance (Emeryville, CA). Anti-FLAG (M2) and anti-β-actin (A-5441) were from Sigma (St. Louis, MO), and anti-PCNA (sc-56) was from Santa Cruz (Dallas, TX). Anti-involucrin was prepared in our laboratory. Primary antibody binding was detected using Cy3-conjugated goat anti-rat IgG (A10522) or Alexafluor 488-conjugated goat anti-rabbit IgG (A11034) from Invitrogen (Waltham, MA).

Immunoblot extracts were prepared from flash frozen/powdered skin (epidermis + dermis) by dissolving in lysis buffer containing 0.625 M Tris-HCl, pH 7.5, 10% glycerol, 5% SDS, 5% β-mercaptoethanol. Equal amounts of protein were electrophoresed on 12% denaturing polyacrylamide gels.

Cell culture Total skin was harvested from newborn TAM67-negative and -positive pups and incubated overnight in 5 mg/ml dispase at 4°C. Epidermis was separated from the dermis and incubated in 0.25% trypsin at 37°C for 15 min. Incubation was stopped by adding soybean trypsin inhibitor to 0.5 mg/ml, and the cells were strained through a 100 μm nylon strainer and centrifuged at 2,000 rpm for 5 min. Cells were resuspended in low calcium (0.05 mM) Keratinocyte serum-free medium and plated at

79,000 cells per cm^2 in type I rat collagen-coated 100 mm culture dishes (Cultrex type I rat tail collagen, R&D Systems, 3440-100-01, Minneapolis, MN). The cells were fed every other day, and doxycycline was added at 1 µg/ml to induce TAM67-FLAG expression.

Electron microscopy Embryonic skin biopsies were taken for electron microscopy (Hou et al., 1991). Briefly, samples were minced to < 0.5 mm^3, fixed in modified Karnovsky's fixative overnight, postfixed in 1% aqueous osmium tetroxide containing 1.5% potassium ferrocyanide or in 0.2% ruthenium tetroxide (the latter only where indicated in figure legends). The samples were dehydrated in a graded ethanol series and embedded in an Epon-epoxy mixture. Ultrathin sections were examined, with or without further contrasting with lead citrate, in a JEOL electron microscope (JEOL USA, Inc, Peabody, MA, USA).

Gene array analysis For gene array analysis, we mated heterozygous (TAM67+/-) and heterozygous (rTA+/-) mice and treated the pregnant dams with doxycycline (2 mg/ml) in drinking water beginning on embryonic days E15.5. At E20 the pups were euthanized and RNA was collected from whole skin. RNA samples were pooled using epidermis from three separate mice. Approximately 5 to 8 µg of total RNA was reverse transcribed, amplified and labeled as described (D. Li et al., 2001) and labeled cRNAs were hybridized to Affymetrix Mouse Gene 1.0 ST Arrays (#901168) and 0.5 fold or greater change in expression was considered significant. For annotation we used the DAVID program [http://david.abcc.ncifcrf.gov] (Dennis et al., 2003).

D. RESULTS:

Role of AP-1 transcription factors in the embryonic epidermis AP-1 transcription factor inactivation alters epidermal differentiation in adult mice to produce a phenotype associated with nuclear loricrin accumulation and reduced filaggrin function, which are changes associated with ichthyosis (Rorke et al., 2015; Rorke, Adhikary, Young, Roop et al., 2015). Since a significant percentage of ichthyosis patients are born as collodion babies (Vahlquist, 2010; Yeh et al., 2013), we sought to determine whether inactivating AP-1 factor function in utero causes a collodion-like phenotype. We mated heterozygous (TAM67+/-) and heterozygous (rTA+/-) mice to generate control (TAM67-/rTA-, TAM67+/rTA-, TAM67-/rTA+) and TAM67-rTA (TAM67+/rTA+) embryos. We then treated the pregnant dams with 2 mg/ml doxycycline beginning at E13.5, E15.5 and E17.5 and harvested embryos at E20 (Fig. 2-1A). As anticipated, doxycycline treatment induces TAM67-FLAG expression in TAM67+/rTA+ embryos, but not in non-transgenic (control) embryos (Fig. 2-1B). To examine the impact on collodion phenotype, we initiated doxycycline treatment on E13.5, E15.5 and E17.5, and permitted the mice to be born. Figure 2-1C shows mouse images at 12 h post-birth. E13.5 and E15.5 TAM67-rTA mice display a collodion baby-like phenotype, which is not evident in the E17.5 mice. The phenotype was present in one hundred percent of E13.5 and E15.5 TAM67-positive mice, all of which died within 24 h after birth.

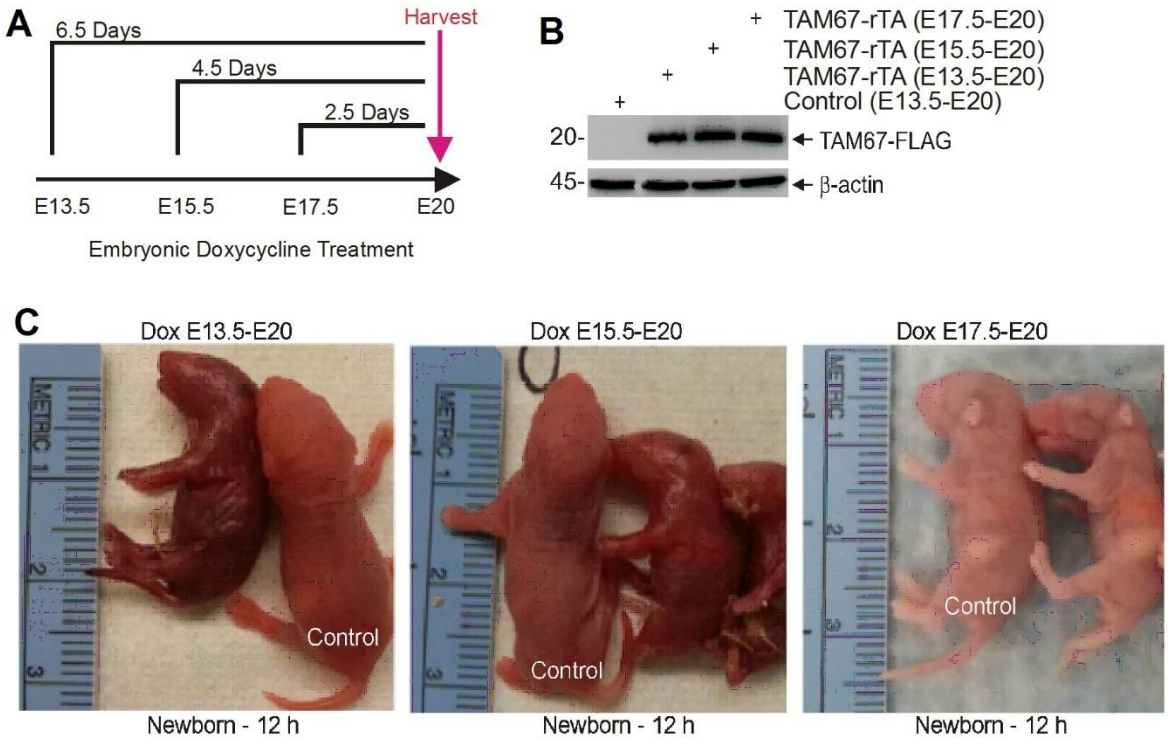

Figure 2-1: AP-1 factor inactivation in epidermis produces a collodion baby-like phenotype A) We mated heterozygous (TAM67+/-) and heterozygous (rTA+/-) mice and the pregnant dams were treated with 2 mg/ml doxycycline in drinking water beginning on E13.5, E15.5 and E17.5. The embryos were collected on E20 or as newborns. B) Skin extracts were prepared from individual embryos following treatment as indicated in panel A and TAM67-FLAG levels were assayed in extracts from E20 embryos by anti-FLAG immunoblot. C) Pregnant dams were treated with doxycycline as indicated in each panel and the phenotype of each newborn mouse was photographed at 12 h after birth. The mice are TAM67-positive except for those labeled control.

We next characterized the effect of AP-1 factor inactivation on epidermal histology. Fig. 2-2A shows that doxycycline-treated control mice display normal epidermal development which includes formation of a well-structured barrier (arrows). In contrast, in doxycycline-treated TAM67-rTA mice, the epidermis is thicker and the epidermal surface is less-structured. These changes are particularly apparent when treatment is initiated on E13.5 or E15.5. Initiating treatment on E17.5 produces a less perturbed epidermis with a more normal appearing surface. To assess the impact of TAM67 expression on barrier function, E13.5, E15.5 and E17.5 embryos were harvested on

E20 and challenged with toluidine blue. Figure 2-2B shows that the E13.5 and E15.5 mice have reduced barrier function, but that the E17.5 mice retain barrier function. We next boiled equal-sized samples of epidermis under reducing and denaturing conditions to prepare cornified envelopes. Control mice yield many envelopes, while very few are detected in E13.5 or E15.5 mice, and intermediate numbers in E17.5 mice (Fig. 2-2C/D). It is likely that the reduction in envelope number is due to formation of thin envelopes that are not resistant to boiling in detergent and reduction agent (Robinson, LaCelle, & Eckert, 1996; Robinson, Lapic, Welter, & Eckert, 1997). As will be discussed later, this is consistent with ultrastructure findings showing a reduction in envelope thickness.

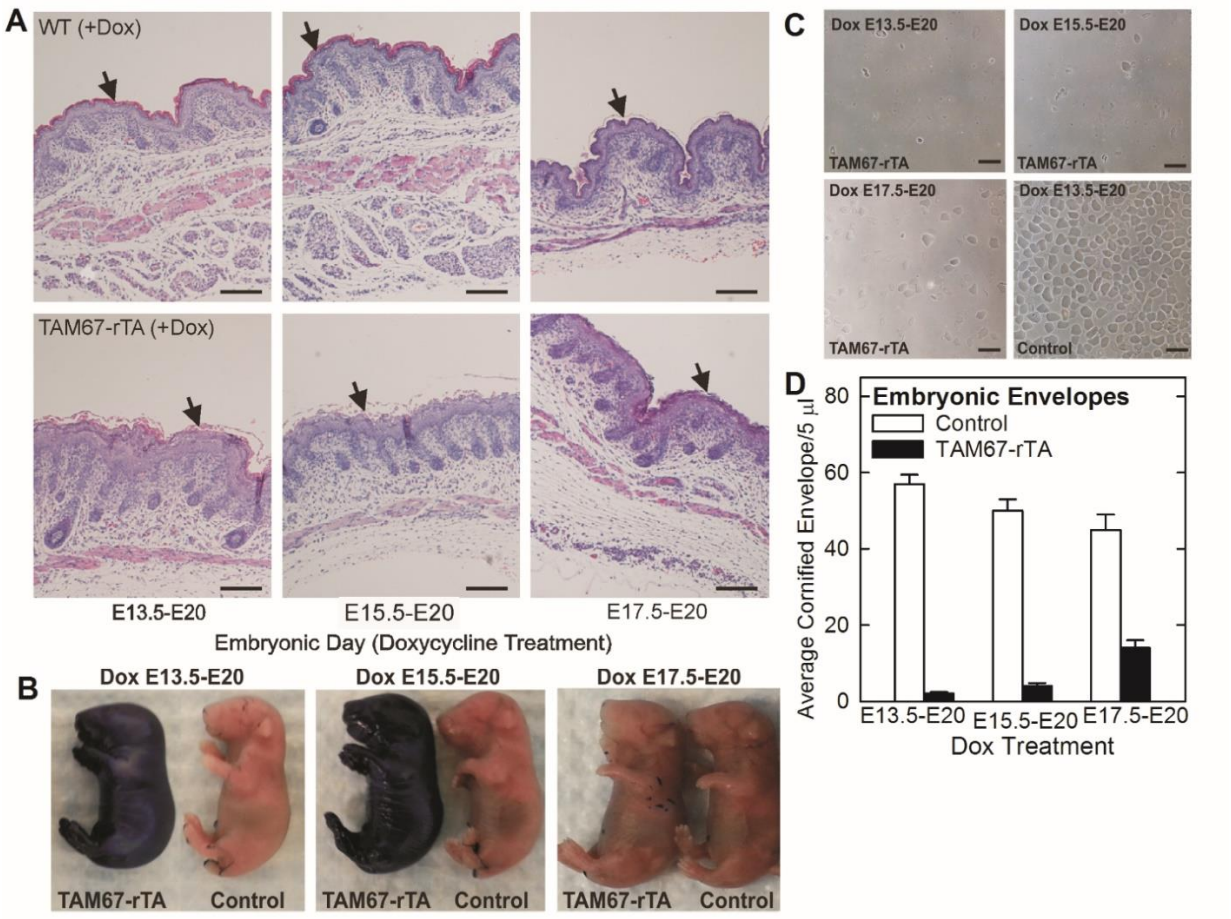

Figure 2-2: Impact of AP-1 factor inactivation on epidermal structure and barrier function A) H&E stained sections of embryonic epidermis were prepared from individual E20 embryos following treatment as indicated in Figure 2-1A. The arrows denote cornified layer. The bar = 100 microns. B) Embryos were collected at E20 and stained with 0.1% toluidine blue to assess skin permeability. C/D) Epidermis was harvested from embryos at E20 and boiled in denaturing/reducing buffer prior to cornified envelope counting. Bars = 100 microns. The values are mean ± SEM, n = 4. TAM67-rTA values are significantly reduced as compared to control ($p < 0.001$).

Expression of epidermal markers Similar to adult epidermis (Rorke et al., 2015; Rorke, Adhikary, Young, Roop et al., 2015), TAM67-FLAG localizes in the nucleus of suprabasal epidermal keratinocytes in doxycycline-treated TAM67-rTA embryos (Fig. 2-3A). Figure 2-3B shows that suprabasal TAM67 expression does not alter involucrin distribution (green stain). PCNA is present in proliferating cells in the basal epidermis in all samples (red stain) (Fig. 2-3B), although staining is generally less in control epidermis. Keratin 14 is a basal epidermal layer marker which displays little change in

tissue distribution (Fig. 2-3C). Loricrin redistributes from a membrane location in control epidermis to inside the nucleus in TAM67-FLAG expressing epidermis (Fig. 2-3D). This is also observed in TAM67-positive adult mouse epidermis (Rorke et al., 2015; Rorke, Adhikary, Young, Roop et al., 2015). Figure 2-3E shows that the overall levels of K10, K14, involucrin and loricrin are not markedly altered in TAM67-expressing epidermis.

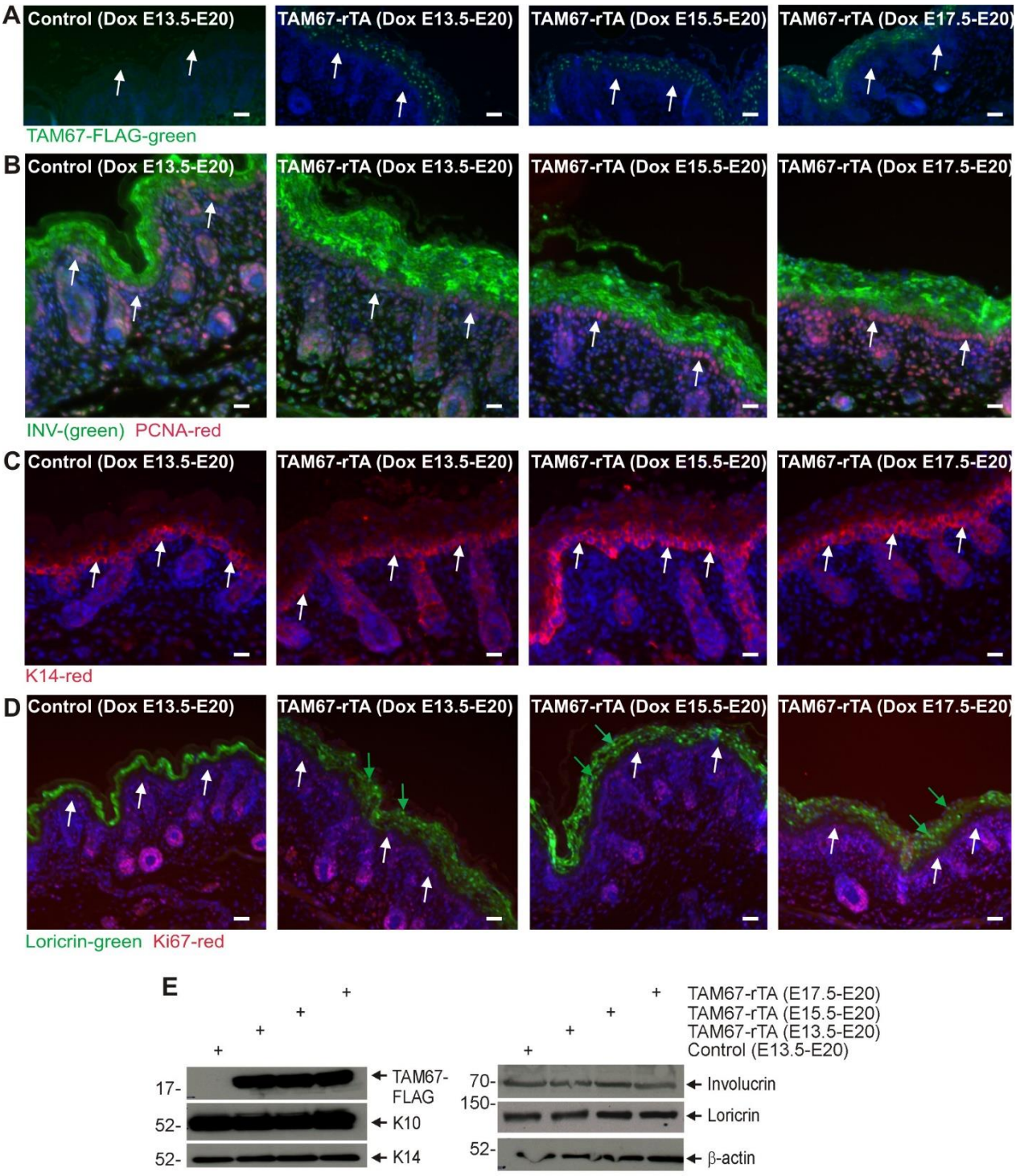

Figure 2-3: Distribution of differentiation markers in TAM67-positive embryonic epidermis
A/B/C/D) Pregnant dams were treated with 2 mg/ml doxycycline on E13.5-E20, E15.5-E20 or E17.5-E20 and the embryos were harvested on E20 and the epidermis sectioned. Sections were stained to detect the indicated epitopes and also DAPI stained to detect the nuclei. The white arrows indicate the epidermal basal layer and green arrows indicate nuclear loricrin. E) Following treatment of the dams with doxycycline as indicated, whole skin (dermis/epidermis) extracts were prepared from E20 embryos for immunoblot. Similar findings were observed in each of three replicate experiments.

To confirm loricrin nuclear translocation, we cultured murine keratinocytes derived from wild-type and TAM67-rTA mice in doxycycline-containing medium. In wild-type keratinocytes loricrin localizes in punctate foci throughout the cell and surrounding the nucleus (Fig. 2-4A). In contrast, loricrin localizes in the nucleus in TAM67-FLAG expressing cells (Fig. 2-4A). Figure 2-4B shows that TAM67 expression does not markedly alter K10, K6, loricrin or involucrin level in cultured murine keratinocytes.

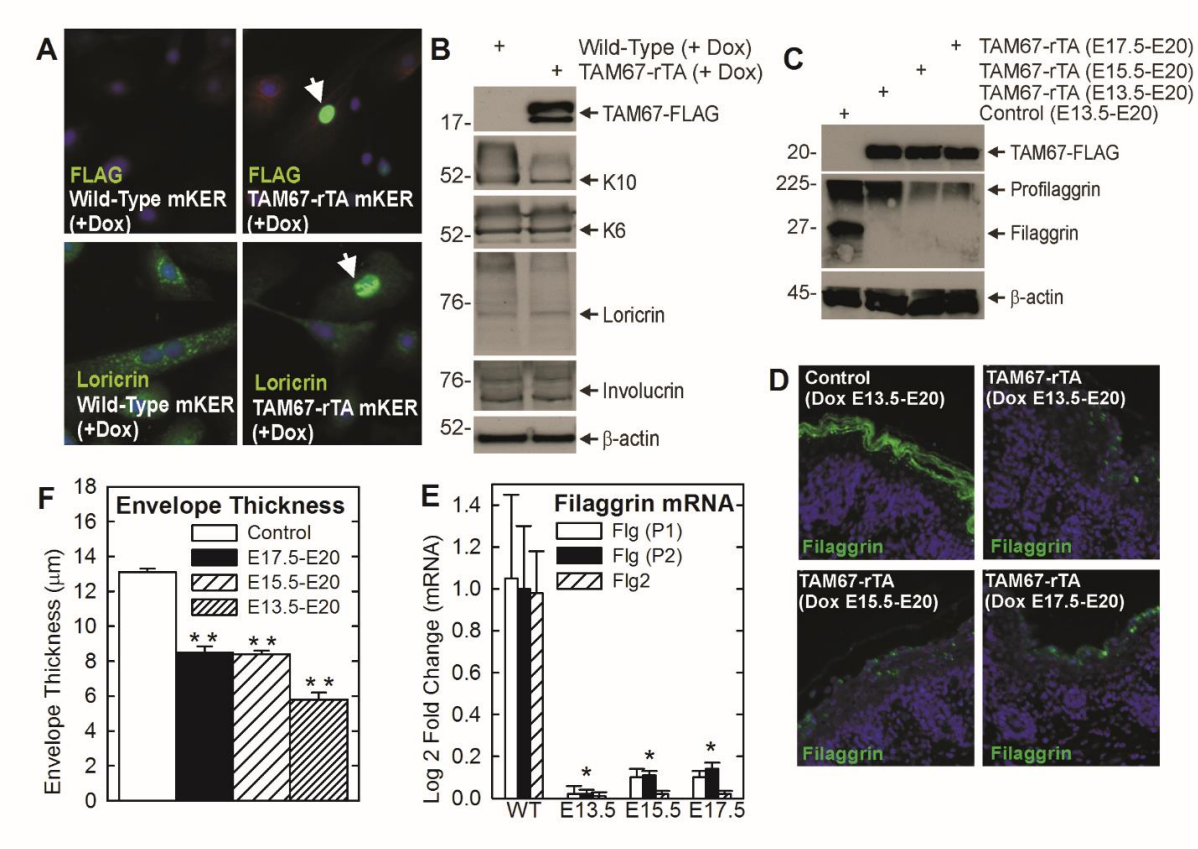

Figure 2-4: AP-1 factor loss regulates loricrin distribution and filaggrin level A/B) Murine keratinocytes were harvested from newborn mice and wild-type and TAM67-positive cells were grown as monolayer cultures and treated with 1 μg/ml doxycycline for 8 d. The cells were then fixed and stained to detect TAM67-FLAG (anti-FLAG) and loricrin. The arrows indicate nuclear localization of the TAM67-FLAG (top panels) and loricrin (bottom panels). Extracts were prepared from the above cells after doxycycline treatment for 8 d followed by immunoblot detection of the indicated proteins. Similar results were observed in each of three replicate experiments. C/D/E) Suprabasal epidermal TAM67 expression is associated with reduced profilaggrin and filaggrin levels. Pregnant dams were treated with 2 mg/ml doxycycline in drinking water on E13.5, E15.5 and E17.5 of embryo development and embryos were harvested at E20. Sections were collected and stained with anti-filaggrin to detect filaggrin distribution in the embryonic epidermis. Similar results were observed in each of four independent experiments. Whole skin extracts were collected for immunoblot detection of TAM67-FLAG (anti-FLAG) and profilaggrin/filaggrin (anti-filaggrin), and qRT-PCR detection of filaggrin and filaggrin 2 mRNA. The graph values are mean ± SEM, n = 3. The asterisks indicate a significant reduction in mRNA (compared to WT) as assessed using the student's t-test ($p < 0.001$). F) Cornified envelope thickness is reduced in TAM67-positive epidermis. EM images were measured for cornified envelope thickness. The values (μm) are mean ± SEM, n = 20, $p < 0.0001$.

Filaggrin expression Filaggrin is a key protein involved in cornified envelope assembly and barrier formation (Dale, Resing, & Lonsdale-Eccles, 1985; Irvine et al., 2011; Ishida-Yamamoto et al., 1999) and filaggrin loss-of-function and reduced level is observed in human ichthyosis vulgaris (McLean, 2016; Oji et al., 2010; Smith et al., 2006). Figure 2-4C shows that filaggrin and profilaggrin levels are markedly reduced in TAM67-positive embryonic epidermis. Immunostaining (Fig. 2-4D) confirms the reduction and reveals residual staining confined to punctate locations in the epidermis. RT-PCR analysis shows that the loss of filaggrin protein is associated with loss of filaggrin mRNA (Fig. 2-4E) and that filaggrin 2 encoding mRNA is also reduced (Fig. 2-4E).

Ultrastructure of TAM67-expressing embryonic epidermis Induction of TAM67 expression in embryonic epidermis at E13.5 or E15.5 results in loss of epidermal barrier function, but this is not observed in E17.5 epidermis (Fig. 2-2). This suggests that 2.5 d of exposure to TAM67 is not sufficient to induce changes in epidermal structure sufficient to compromise the barrier. However, we were concerned that this tissue may display ultrastructural changes, since we observe biochemical and cellular changes in the E17.5 epidermis (Fig. 2-3D and Fig. 2-4C). To examine epidermal ultrastructure, pregnant dams were treated with doxycycline beginning at the indicated embryonic day and epidermal tissue was processed at E20 for ultrastructural analysis. Analysis shows that cornified envelope thickness is significantly reduced in TAM67-positive E13.5, E15.5 and E17.5 epidermis (Fig. 2-4F). As shown in Figure 2-5, control epidermis displays a well-structured stratum corneum with normal secretion of granule contents at the stratum granulosum (SG)/stratum corneum (SC) junction (white arrows, upper

panel), and the presence of well-formed and abundant keratohyalin granules (K, lower panel). The extracellular lamellar bilayers are regular (upper insert), the cornified envelopes are thick and dense (middle insert) and abundant lamellar bodies are present (lower insert). In contrast, reduced-thickness cornified envelopes are observed in E13.5, E15.5 and E17.5 epidermis (black double arrows). In addition, keratohyalin granule (K) number is reduced, and morphology of cornified layer (e.g., E17.5, left insert) is abnormal. In general, the most severe ultrastructure changes are observed in epidermis from E13.5 and E15.5 mice.

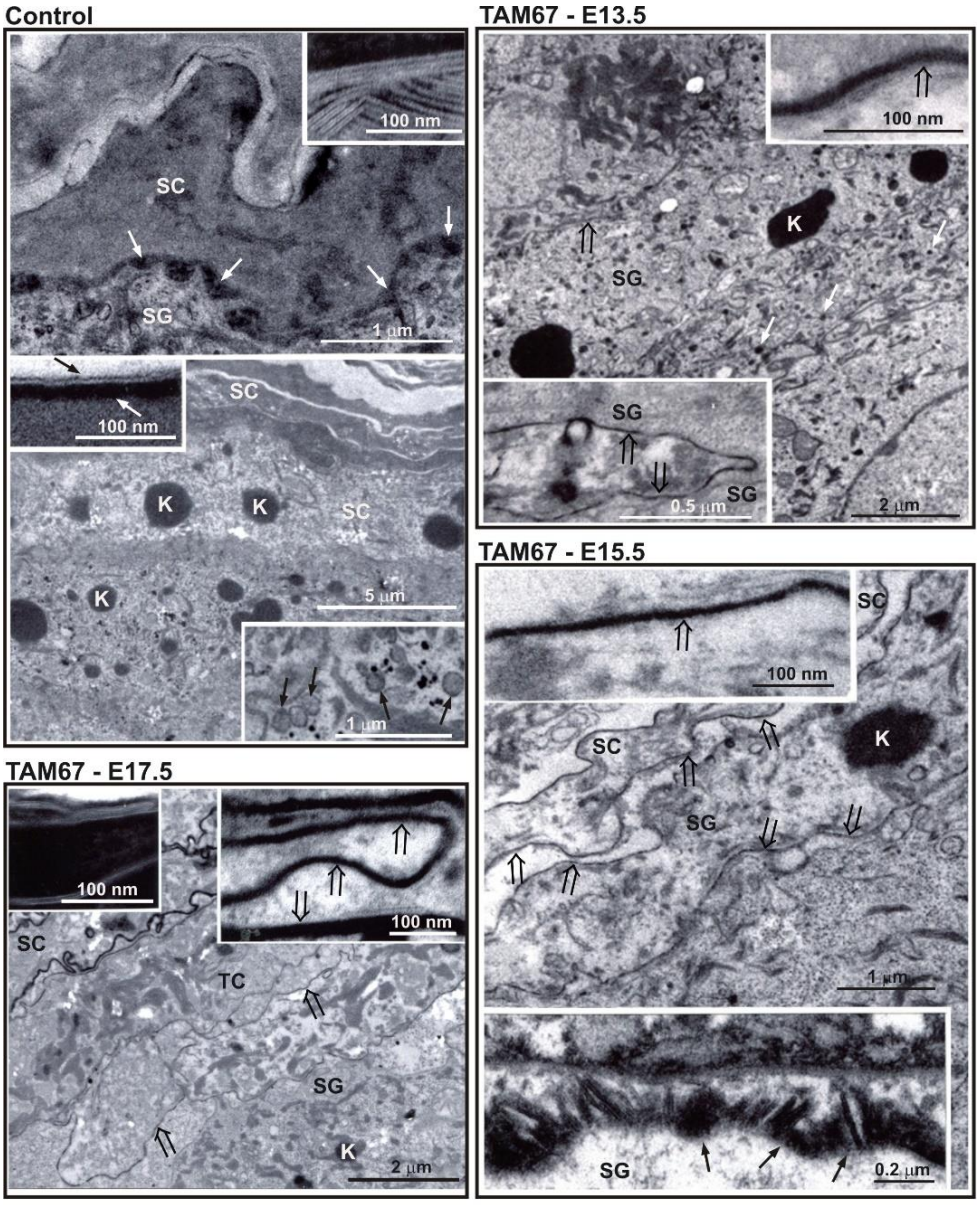

Figure 2-5: Ultrastructure of TAM67-positive embryonic epidermis Pregnant dams were treated with 2 mg/ml doxycycline beginning at E13.5, E15.5 and E17.5 and at E20 embryonic whole skin punch biopsies were harvested for ultrastructural characterization. Wild-type control embryos include TAM67-/rTA-, TAM67+/rTA- and TAM67-/rTA+ genotypes. Images from control epidermis show robust secretion of granular contents at the SG-SC junction (white arrows, upper panel), abundant keratohyalin granules (K, lower panel), extensive membrane arrays formed from processing of secreted lipids (top insert), formation of thick, well-formed cornified envelopes (middle insert, flanked by black and white arrows), and the presence of normal and abundant lamellar bodies in the SG (black arrows, bottom insert). In contrast, envelope thickness is reduced in E13.5, E15.5 and E17.5 mouse epidermis (black double arrows) and keratohyalin granule size and number are reduced (K). The solid white arrows in the E13.5 panel indicate lamellar bodies which appear normal and are present at normal numbers. In the E15.5 panel, the solid black arrows show defective secretion of granular contents at the SG-SC interface (lower insert). The left insert in the E17.5 panel indicates an enlarged view of the cornified layer. Abbreviations: SC, stratum corneum; TC, transition cells; SG, stratum granulosum; K, keratohyalin granules. The upper right insert in control, and the upper left insert in E17.5 were stained with ruthenium tetroxide.

Gene array analysis We next used gene array to compare mRNA expression in TAM67-negative and positive embryonic skin. Table 1 lists a collection of genes that are differentially expressed. TAM67-negative embryonic epidermis, which differentiates normally, is enriched in mRNA encoding cutaneous and hair keratins, hair keratin-associated proteins and late cornified envelope precursors. Filaggrin related genes, including filaggrin family member 2 and hornerin, are also enriched. In contrast, TAM67-positive embryonic skin is enriched in the proliferation associated keratins (Krt6a, Krt6b, Krt16), early envelope precursors, including the small proline-rich proteins, various proteases and proteasome inhibitors, and defensin family members.

E. DISCUSSION:

The AP-1 factor-deficiency phenotype We have previously reported that suprabasal inactivation of AP-1 transcription factor function in adult epidermis results in a phenotype including epidermal hyperproliferation, hyperkeratosis, parakeratosis, thickening of the cornified layer and excessive scale formation (Rorke et al., 2010; Rorke et al., 2015; Rorke, Adhikary, Young, Roop et al., 2015). However, we wanted to examine the impact in embryos, since keratoderma/ichthyosis patients are frequently born encased in a transparent collodion membrane (collodion baby) (Gedicke et al., 2006; Matsumoto et al., 2001; Oji et al., 2010; Takeichi & Akiyama, 2016; Vahlquist, 2010; Yeh et al., 2013). We therefore determined whether AP-1 factor inactivation in embryos could produce a collodion phenotype. We inactivated AP-1 factor function at E13.5, E15.5 and E17.5 and monitored for changes at E20. When TAM67 expression was induced at E13.5 and E15.5, all TAM67-expressing mice were born encased in a

collodion membrane. Histology of TAM67-positive E20 embryos revealed a thickened epidermis and a malformed epidermal cornified layer. The cornified layer appeared fragile and not tightly tethered to the underlying tissue. This phenotype is associated with a 95% reduction in cornified envelope formation and loss of barrier function. Envelope formation was measured by boiling equivalent skin samples in detergent and reducing agent. Since only well-formed envelopes survive this process (Robinson et al., 1996; Robinson et al., 1997), our finding suggest that the envelopes formed in TAM67-positive epidermis are fragile. In addition, the TAM67-positive embryos are born bloated, presumably due to abnormal fluid uptake in utero, and rapidly and visibly dehydrate following air exposure. This phenotype was associated with prominent ultrastructural changes. The most prominent include a reduction in keratohyalin granule number and the presence of thinner cornified envelopes.

In contrast, initiation of TAM67 expression at E17.5 produces mice that appear normal at birth and have normal epidermal barrier function. However, there are signs of abnormalities, as cornified envelope number is reduced by 65%, filaggrin protein and mRNA levels are reduced, and there are clear changes in epidermal ultrastructure including reduced keratohyalin granule number and thinner cornified envelopes. However, the ultrastructural changes are not as severe as those observed in E13.5 and E15.5 epidermis. Thus, it appears that disease severity progresses with the length of time that AP-1 factor function is inactivated in utero. The 2.5 day period of AP-1 factor inactivation in the E17.5 mice provides adequate time to initiate phenotype development but is not enough time to induce the enhanced dye permeability or collodion phenotype.

Immunostaining suggests minimal alteration in expression of some differentiation

marker proteins (Fig. 2-3). However, array analysis identified differentiation-associated changes in gene expression consistent with a shift towards enhanced proliferation and reduced differentiation. Messenger RNA encoding cutaneous and hair keratins, hair keratin-associated proteins, late cornified envelope precursors and filaggrin gene family members are enriched in TAM67-negative (control) epidermis. TAM67-positive embryonic skin is enriched in the proliferation associated keratins (Krt6a, Krt6b, Krt16), early envelope precursors, various proteases and proteasome inhibitors, defensin family members, and repetin. Thus, the array data suggests that TAM67 expression reduces differentiation and enhances proliferation.

Causing the phenotype We show that inactivation of AP-1 transcription factor function in the suprabasal epidermis creates a collodion-like phenotype similar to that observed in some newborn ichthyosis patients. However, there is previously no evidence of AP-1 transcription factors being involved in pathogenesis of these diseases. Thus, it is important to understand how perturbing epidermal AP-1 factor function may cause this phenotype. An important place to start is the impact of AP-1 factor inactivation on expression/function of proteins that are known to be involved in ichthyosis pathogenesis. Loricrin and filaggrin function are known to be perturbed, respectively, in loricrin keratoderma and ichthyosis vulgaris (Elias et al., 2010; Gruber et al., 2011; Oji et al., 2010; Richard, 2004; Sandilands et al., 2007; Takeichi & Akiyama, 2016), and we have described an altered subcellular distribution of loricrin and reduction in filaggrin level in TAM67-expressing adult mouse epidermis (Rorke et al., 2010; Rorke et al., 2015; Rorke, Adhikary, Young, Roop et al., 2015).

Loricrin is an abundant epidermal cornified envelope precursor protein (Candi,

Schmidt, & Melino, 2005; Hohl et al., 1991; Mehrel et al., 1990). In loricrin keratoderma, a frameshift mutation adds a nuclear localization signal to the loricrin c-terminus (Drera et al., 2008; Gedicke et al., 2006; Korge et al., 1997; Maestrini et al., 1996; Matsumoto et al., 2001; O'Driscoll et al., 2002; Takahashi, Ishida-Yamamoto, Kishi, Ohara, & Iizuka, 1999) which leads to its nuclear accumulation (Ishida-Yamamoto, Takahashi, & Iizuka, 1998; Ishida-Yamamoto, 2003). Although the mechanism is not well understood, there are several ideas about how nuclear loricrin may impact the epidermis. It is possible that nuclear accumulation of mutant loricrin reduces loricrin content at the cell periphery and thereby impairs cornified envelope assembly and that this leads to compromised barrier function. It is also possible that nuclear accumulation of loricrin alters keratinocyte gene expression to impair cell viability or alter epidermal differentiation or apoptosis. Consistent with such a role, we observe a marked shift of loricrin from the cytoplasmic to nuclear compartment in AP-1 factor inactivated epidermis of E13.5, E15.5 and E17.5 mice and in cultured cells derived from these mice. This fits with our previous findings showing nuclear loricrin localization in the epidermis of adult TAM67-expressing epidermis (Rorke et al., 2015; Rorke, Adhikary, Young, Roop et al., 2015). It is interesting that wild-type loricrin moves to the nucleus in the epidermal cells of these AP-1 factor perturbed mice (Rorke et al., 2015; Rorke, Adhikary, Young, Roop et al., 2015). This is in contrast to the human disease, where a frame-shift mutation creates a nuclear localization signal that moves loricrin to the nucleus (Drera et al., 2008; Gedicke et al., 2006; Korge et al., 1997; Maestrini et al., 1996; Matsumoto et al., 2001; O'Driscoll et al., 2002; Takahashi et al., 1999).

An alternate mechanism of phenotype development is filaggrin loss. Filaggrin is a

structural protein that is produced as a large precursor called profilaggrin that is the constituent of keratohyalin granules (Sandilands, Sutherland, Irvine, & McLean, 2009). Profilaggrin is dephosphorylated and proteolyzed into filaggrin monomers and these monomers bind to and bundle keratin intermediate filaments (Irvine, 2007). As the differentiation continues, the filaggrin monomers are processed to hygroscopic amino acids and these breakdown products function as moisturizing factors (Palmer et al., 2006). We show that TAM67-expressing embryonic epidermis displays markedly reduced filaggrin and profilaggrin content, and reduced keratohyalin granule number. An interesting protein profiling study revealed increased filaggrin in a limited number of patients with simultaneous mutations in filaggrin and other ichthyosis-relevant (steroid sulfatase, transglutaminase 1, gap junction protein beta 2) genes (Rice et al., 2013). However, some patients displayed no change or a reduction in filaggrin level. A complication of this study is the wide variation in filaggrin level observed even in epidermis from control patients (Rice et al., 2013). However, most studies report a reduction in filaggrin level in ichthyosis vulgaris (Gruber et al., 2011; Oji & Traupe, 2006; Oji & Traupe, 2009; Smith et al., 2006). For example, immunoblot/immunostaining studies show reduced keratohyalin granule number, and filaggrin and profilaggrin levels, in the epidermis of ichthyosis vulgaris patients (Smith et al., 2006). Filaggrin reduction is also observed in the epidermis of the flaky mouse, a model of ichthyosis vulgaris (Presland et al., 2000), and Amagai and colleagues described an ichthyosis phenotype that includes features (tail lesions) characteristic of keratoderma in filaggrin knockout mice (Kawasaki et al., 2012). As AP-1 factors function to maintain filaggrin gene expression (Jang et al., 1996), inhibiting AP-1 factor function may reduce filaggrin via a

transcriptional mechanism. Consistent with this, we observe a reduction in filaggrin and filaggrin 2 mRNA in TAM67-expressing embryonic epidermis. This is consistent with our report in adult epidermis showing that AP-1 factor inactivation reduces the level of mRNA encoding several filaggrin-related proteins (Rorke et al., 2015).

AP-1 factors and the epidermal phenotype Perturbing transcription factor expression in mice is known to produce a host of phenotypes and it can be difficult to assign a mechanism. However, our studies suggest that AP-1 factor inactivation reduces filaggrin gene expression to reduce profilaggrin and filaggrin content, keratohyalin granule formation, and barrier integrity. In this context, it is possible that compromised AP-1 factor function may play a role in the pathogenesis of ichthyotic disease via a mechanism that involves reduced filaggrin expression. This could be particularly important in acquired ichthyosis where disease onset is associated with altered nutritional or metabolic states, infection or use of medication (Oji et al., 2010). We suggest that it will be useful to assess whether AP-1 transcription factor protein level and/or function is compromised in these disease states. As an initial screen, useful information could be obtained by qRT-PCR analysis to measure the level of mRNA encoding the AP-1 transcription factors along with immunoblot to detect the corresponding proteins in epidermal samples derived from ichthyosis patients.

Table 1: Gene array analysis

Ratio: (log2)[a] ***TAM67+/TAM67-***	**Gene Name**	**Entrez Gene ID**	**Description**	**Category**
Gene expression elevated in TAM67-negative epidermis				
-1.98	*Krt23*	94179	Keratin 23	Cutaneous keratins
-0.62	*Krt7*	110310	Keratin 7	
-0.73	*Krt13*	16663	Keratin 13	
-0.87	*Krt4*	794486	Keratin 4	
-1.10	*Krt78*	332131	Keratin 78	
-0.60	*Krt84*	16680	Keratin 84	Hair keratins
-0.74	*Krt36*	16673	Keratin 36	
-0.66	*Krtap5-1*	50774	Keratin associated protein 5-1	
-0.97	*Gm10272*	16697	Keratin associated protein 12-4	
-2.66	*Kprp*	433619	Keratinocyte expressed proline-rich	
-3.66	*Lce1m*	66203	Late cornified envelope 1m	Late envelope
-3.17	*Lce1b*	68720	Late cornified envelope 1b	
-2.94	*Lce1c*	73719	Late cornified envelope 1c	
-2.22	*Lce1j*	545547	Late cornified envelope 1j	
-1.42	*Lce1f*	67828	Late cornified envelope 1f	
-1.07	*Lce1d*	69611	Late cornified envelope 1d	
-1.46	*Lor*	16939	Loricrin	
-3.39	*Flg2*	229574	Filaggrin family member 2	Filaggrin related

Table 1 Continued

-1.64	*Hrnr*	68723	Hornerin	
Gene expression elevated in TAM67-positive epidermis				
+4.46	*Krt16*	16666	Keratin 16 (proliferation-associated)	Cutaneous keratins
+2.10	*Krt6b*	16688	Keratin 6b (proliferation-associated)	
+1.40	*Krt6a*	16687	Keratin 6a (proliferation-associated)	
+2.89	*Sprr1b*	20754	Small proline rich protein 1b	Early envelope
+0.74	*Sprr1a*	20753	Small protein rich protein 1a	
+0.74	*Prr29*	75573	Proline rich 29	
+0.71	*Prrg4*	228413	Proline rich G-carboxyglutamic acid 4	
+0.99	*Serpin b11*	66957	Serine (or cysteine) peptidase inhibitor, clade B (ovalbumin) member 11	Proteases and protease inhibitors
+0.93	*Lyz2*	17105	Lysozyme 2	
+0.81	*Klk1b4*	18048	Kallikrein 1-related peptidase b4	
+0.71	*Cela3a*	242711	Chymotrypsin-like elastase family member 3A	
+0.70	*Cts3*	117066	Cathepsin 3	
+0.68	*Serpinb6c*	97848	Serine (or cysteine) peptidase inhibitor, clade B, member 6c	
+1.31	*Defa21*	66298	Defensin α21	Envelope defense
+0.94	*Defa-rs2*	13222	Defensin alpha-related sequence 2	

Table 1 Continued

+0.73	*Defa29*	13218	Defensin alpha 29	
+0.69	*Defb7*	246080	Defensin beta 7	
+1.31	*Defa21*	66298	Defensin α21	
+2.35	*Rptn*	20129	Repetin	Filaggrin related

[a]Negative log2 values indicate relative enrichment in TAM67-negative skin and positive numbers indicate relative enrichment in TAM67-positive skin.

CHAPTER 3: LOSS OF EPIDERMAL AP-1 TRANSCRIPTION FACTOR FUNCTION REDUCES FILAGGRIN LEVEL, ALTERS CHEMOKINE EXPRESSION AND PRODUCES AN ICHTHYOSIS-RELATED PHENOTYPE

A. ABSTRACT:

AP-1 transcription factors are important controllers of epidermal differentiation. Multiple family members are expressed in the epidermis in a differentiation-dependent manner where they function to regulate gene expression. To study the role of AP-1 factor signaling, TAM67 (dominant-negative c-jun) was inducibly expressed in the suprabasal epidermis. The TAM67-positive epidermis displays keratinocyte hyperproliferation, hyperkeratosis and parakeratosis, delayed differentiation, extensive subdermal vasodilation, nuclear loricrin localization, tail and digit pseudoainhum and reduced filaggrin level. These changes are associated with increased chemokine levels of IFNγ, CCL3, CCL5, CXCL9, CXCL10 and CXCL11 (Th1 chemokines), and CCL1, CCL2, CCL5 and CCL11 (Th2 chemokines) in the serum and epidermis. S100A8 and S100A9 protein levels are also markedly elevated. These changes in epidermal chemokine level are associated with increased levels of the corresponding chemokine mRNA. The largest increases were observed for CXCL9, CXCL10, CXCL11, and S100A8 and S100A9. To assess the role of CXCL9, CXCL10, CXCL11, which bind to CXCR3, on phenotype development, we expressed TAM67 in CXCR3 knockout mice. Using a similar strategy, we examine the role of S100A8 and S100A9. Surprisingly, loss of CXCR3 or S100A8/A9 did not attenuate phenotype development. These studies suggest that interfering with epidermal AP-1 factor signaling initiates a loss of barrier function leading to enhanced epidermal chemokine production and that these events influence epidermal differentiation.

B. INTRODUCTION:

The epidermis produces a barrier that functions to prevent loss of body fluids, fights infection, and is essential for life. Epidermal keratinocytes differentiate to form the barrier via a process that involves systematic destruction of intracellular structures leaving a network of covalently-crosslinked proteins, lipids and keratin bundles that form the barrier (Eckert, Crish, & Robinson, 1997). AP-1 transcription factors are essential regulators of this process (Crish et al., 1998; Crish, Bone, Banks, & Eckert, 2002; Crish et al., 2006; Eckert et al., 2003; Efimova et al., 1998; Efimova, Broome, & Eckert, 2003). They form homo- and heterodimers that bind DNA response elements to regulate gene expression (Angel et al., 2001). An example is the AP-1 site in the involucrin gene enhancer that is required for expression of involucrin in epidermis (Crish et al., 1998; Crish et al., 2002; Crish et al., 2006; Eckert et al., 2003; Efimova et al., 1998; Efimova et al., 2003). Disruption of AP-1 factor function can produce mouse phenotypes that mimic human epidermal diseases (Rorke et al., 2010; Zenz et al., 2005; Zenz & Wagner, 2006; Zenz et al., 2008). To investigate the role of AP-1 factors in epidermis, we inactivated AP-1 transcription factor function in the suprabasal epidermis using TAM67, a dominant-negative form of c-jun (Brown et al., 1994; Brown, Kim, Wise, Sabichi, & Birrer, 1996; Thompson, Gupta, Stratton, & Bowden, 2002). TAM67 dimerizes with AP-1 factor family members, but the resulting complex is not able to activate transcription. These animals display an epidermal phenotype including increased cell proliferation and delayed differentiation, extensive epidermal hyperkeratosis and parakeratosis, nuclear loricrin accumulation and digit and tail autoamputation (Rorke et al., 2010).

Biochemical characterization of the cross-linked proteome of the cornified envelope reveals a reduction in cutaneous keratins (Krt1, Krt5, Krt10), late cornified envelope precursor proteins, hair keratins and hair keratin-associated proteins, but increased incorporation of hyperproliferation-associated epidermal keratins (Krt6a, Krt6b and Krt16). We also observe increased incorporation of small proline-rich, S100, transglutaminase and inflammation-associated proteins (Rorke et al., 2015). In most cases, these changes are reflected by a parallel change in mRNA level. Ultrastructural characterization of the epidermis demonstrates reduced cornified envelope thickness, abnormal desmosomes and keratin filaments, abnormal lamellar body contents, premature lamellar lipid secretion, incomplete processing of secreted lipid and disrupted barrier formation (Rorke et al., 2015). Moreover, suprabasal AP-1 factor inactivation reduces expression of AP-1 factor-responsive late differentiation genes and increases expression of early differentiation genes. An important change is a reduction in expression of filaggrin family-related genes (Rorke et al., 2015), a change that is frequently observed in ichthyosis vulgaris and atopic dermatitis (Kawasaki et al., 2012; McLean, 2016; Smith et al., 2006). In these diseases, compromised barrier function is associated with migration of immune cells into the epidermis (Nedoszytko, Sokolowska-Wojdylo, Ruckemann-Dziurdzinska, Roszkiewicz, & Nowicki, 2014). These immune cells are thought to contribute to the development of disease phenotype due to the stimulatory impact of chemokines and cytokines on keratinocyte proliferation (Nedoszytko et al., 2014). In the present study, we characterize biochemical, structural and immune changes following inhibition of AP-1 factor function in the suprabasal epidermis. We show that structural proteins are altered in level, barrier function is

compromised and IFNγ, CCL3, CCL5, CXCL9, CXCL10 and CXCL11 (Th1), and CCL1, CCL2, CCL5 and CCL11 (Th2) chemokines are elevated. S100A8 and S100A9 levels are also increased. To begin to understand the role of selected chemokines in phenotype development, we show that the CXCR3 receptor and it ligands (CXCL9, CXCL10, CXCL11) and S100A8 and S100A9, although markedly overexpressed in TAM67-positive epidermis, are not required for phenotype development.

C. MATERIALS AND METHODS:

SKH1-TAM67-rTA mice TAM67 is a dominant-negative form of c-jun which lacks the c-jun amino terminus (Brown et al., 1994). TAM67 forms complexes with all AP-1 transcription factors but fails to activate transcription (Brown et al., 1994). This creates an AP-1 factor function-deficient environment. We cloned TAM67-FLAG into pTRE-Tight to produce pTRE-Tight-TAM67-FLAG. The Tet*O*-TAM67-FLAG-SV40 transcription cassette from this plasmid was microinjected into B6SJL embryos (Rorke et al., 2010) to produce TAM67-FLAG transgenic (strain 44) mice and the construct was then bred into an SKH-1 genetic background (Rorke et al., 2010). A FLAG epitope is included at the carboxyl terminus of TAM67 to facilitate detection (Rorke et al., 2010). We also utilize hINV-rTA transgenic mice, which harbor an expression cassette encoding the hINV promoter linked to rTA, maintained in an SKH-1 genetic background (Jaubert, Patel, Cheng, & Segre, 2004). TAM67-FLAG and hINV-rTA mice are bred to produce SKH1-TAM67-rTA mice. These mice express rTA in the suprabasal epidermal layers and addition of doxycycline converts rTA to a form that binds to the Tet*O* to turn on TAM67-FLAG expression in the suprabasal epidermis (Rorke et al., 2010). The mice

are genotyped using DNA-dependent PCR and primers that detect each transgene (Rorke et al., 2010). The SKH-1 genetic background was utilized in these studies because the mice are hairless and this facilitates visualization of the epidermis. SKH-1 mice are immune-competent. Mice were maintained in the University Of Maryland School Of Medicine animal facility. The protocols were approved by the Institutional Animal Care and Use Committee and comply with all NIH regulations. Induction of TAM67 expression was achieved by treating animals with 2 mg/ml doxycycline in drinking water. Statistical analysis used the student's t-test.

CXCR3-KO and S100A9-KO mice CXCR3 knockout (CXCR3-KO) mice were kindly provided by Dr. Bao Lu from Children's Hospital, Boston, MA (Hancock et al., 2000; Yates et al., 2007; Yates et al., 2010). S100A9 knockout (S100A9-KO) mice, which lack both S100A8 and S100A9 (Manitz et al., 2003), were kindly provided by Dr. Donna Kusewitt from MD Anderson Cancer Center, Houston, TX. Both CXCR3-KO and S100A9-KO mice were bred into an SKH-1 hairless genetic background for five generations then bred to the TAM67-FLAG and hINV-rTA mice separately. Then TAM67-FLAG/CXCR3-KO mice were crossed with hINV-rTA/CXCR3-KO mice to yield TAM67-FLAG-rTA/CXCR3-KO mice for experiments. Likewise, TAM67-FLAG/S100A9-KO mice were crossed with hINV-rTA/S100A9-KO mice to yield TAM67-FLAG-rTA/S100A9-KO mice for experiments.

Antibodies and immunological methods Immunofluorescence was performed using paraffin-embedded formalin-fixed sections (Rorke et al., 2010). K1 (PRB-165P), K6 (PRB-169P), K10 (PRB-159P), K14 (PRB-155P), filaggrin (PRB-417P) and loricrin (PRB-145P) antibodies were from Covance (Emeryville, CA, USA), and β-actin (A5441)

and FITC conjugated-anti-FLAG (M2) (F4049) antibodies were from (Sigma). CD3 (ab5690) was purchased from Abcam (Cambridge, MA, USA). Antibodies specific for proliferating cell nuclear antigen (PCNA, sc-56) CXCR3 (sc-6226), S100A8 (sc-8113) and S100A9 (sc-8115) were purchased from Santa Cruz (Dallas, TX). Anti-involucrin was prepared in our laboratory. All fluorophore-conjugated secondary antibodies were from Invitrogen (Waltham, MA). These included Cy3-conjugated goat anti-rat IGG (A10522) and Alexafluor 488-conjugated goat anti-rabbit IgG (A11031). For immunoblot analysis, epidermis was separated from dermis, frozen in liquid nitrogen, pulverized and suspended in dye-free Laemmli sample buffer (Rorke et al., 2010). The suspension was sonicated, centrifuged at 14,000 g, and the soluble extract electrophoresed on a polyacrylamide gel, transferred to nitrocellulose and immunoblotted (Crish et al., 2002; Crish et al., 2006). Unless otherwise indicated, immunohistological and immunoblot results were repeated in three separate experiments and sections and extracts were monitored from epidermis of three mice per treatment group. Evans Blue dye (E2129) and toluidine blue (89640) were obtained from Sigma-Aldrich (St. Louis, MO,).

Cytokine mRNA Array Total RNA was extracted using Illustra RNAspin Mini Isolation Kit (25-0500-70, GE Healthcare), and 0.5 μg of total RNA was reverse transcribed to cDNA using RT^2 First Strand Kit (330401, QIAGEN) according to manufacturers' protocol. RT^2 SYBR Green qPCR Mastermix (330500, QIAGEN) was prepared and cDNA added to the mix. PCR component mix (25 μl) was dispensed into each well of the RT^2 Profiler PCR array (Mouse Cytokines & Chemokines, PAMM-150ZF-6, QIAGEN), sealed, spun down and gene expression was measured by quantitative PCR using Roche LightCycler 480 System. Relative mRNA level was

analyzed by the comparative C_T method.

Cytokine Protein Array The Mouse Cytokine Array Panel A from R&D Systems (ARY006, Minneapolis, MN) was used to assess epidermal cytokine protein levels. Briefly, tissue lysates were homogenized in PBS with protease inhibitors and Triton X-100. Sample protein concentrations were quantified and 300 μg protein or 200 μl of serum was added to the prepared membranes. Next the membranes were incubated with the detection antibody cocktail and washed. Streptavidin-HRP and chemiluminescent detection reagents were added sequentially and the membranes were exposed to X-ray film. Pixel densities were quantified using Image J. Each signal doublet was normalized to the reference signal and then to the signal from TAM67-rTA mice not treated with doxycycline.

AMG487 Formulation AMG487, a CXCR3 inhibitor kindly provided by Amgen (San Francisco, CA) was prepared as previously described (Walser et al., 2006) with modifications as follows. AMG487 was dissolved in dimethyl sulfoxide at 20 mg/ml and then diluted with a 20% solution of 2-hydroxypropyl-β-cyclodextrin in water to a final concentration of 1.75 mg/ml. Mice were injected subcutaneously with 0.1 ml to achieve a final AMG487 concentration of 5 mg/kg body weight. Mice received 0.1 ml of either the inhibitor or vehicle injected subcutaneously. The drug and vehicle were stored at 4°C for the duration of the experiment.

Flow Cytometry Six to eight week old mice were treated for 2 - 8 d with 0 or 2 mg/ml doxycycline in drinking water and then back skin was harvested and incubated in 1% trypsin for 1 h at 37°C. The trypsin was inactivated by addition of 1 volume of 0.5 mg/ml soy bean trypsin inhibitor and the solution was gently pipetted to produce a single

cell suspension. The cells were pelleted and resuspended in calcium-free KSFM which was supplemented to a final concentration of 0.05 mM calcium chloride and chunks were removed by passage through a sterile nylon strainer (70 μm pore size, BD Falcon 352350). The cells were then resuspended in phosphate-buffered saline and 5 - 10 million cells were incubated (per sample) with the appropriate antibody for 30 min on ice. The cells were washed twice with PBS and then sorted using a FACSCanto II Sorter. Antibodies for cell sorting include rat anti-mouse CD11b APC (17-0112-81), rat anti-mouse F4/80 APC (17-4801-80) which detects mature macrophages, CD3 APC (17-0032-80), and hamster anti-mouse-CD183 (CXCR3) FITC (11-1831-80), which detects CXCR3, were purchased from Ebioscience (San Diego, CA).

Blood vessel permeability A 0.5% sterile solution of Evans Blue was prepared in PBS and filter-sterilized to remove any particulates and 200 μl of the Evans Blue solution was injected into the tail vein. After 30 min the mice were sacrificed by cervical dislocation and photographed. Whole skin biopsies were collected, weighted and placed into a tube containing 500 μl formamide and incubated for 24 - 48 h at 55°C to extract the Evans Blue dye. The tubes were then centrifuged and absorbance of the formamide/Evans Blue mixture was measured at 610 nm using formamide as the blank. Evans Blue uptake was monitored in five mice at each time point.

D. RESULTS:

We recently showed that inhibition of AP-1 factor function in the suprabasal epidermis marked changes the epidermal phenotype leading to an ichthyosis/keratoderma-like phenotype (Rorke et al., 2010; Rorke et al., 2015). The

present study expands upon these observations. Figure 3-1 shows the TAM67-FLAG time-dependent change in epidermal phenotype. The skin turns red at 12 - 24 h after induction of TAM67 expression and extended TAM67 expression leads to formation of a thick scaly cornified coat at 21 d (Fig. 3-1A). The phenotype is associated with increased blood flow to the skin, first observed at 12 and 24 h (Fig. 3-1B). Histologic sections reveal an increase in epidermal thickness which is also apparent at 12 - 24 h, with a massive increase in epidermis and stratum corneum thickness observed at 21 d (Fig. 3-1C). Ear thickness is also increased progressively and is 4-fold thicker at 21 d (Fig. 3-1D). The images in Figure 3-1C suggest a rapid change in blood flow to the skin that is evident at 24 h. To assess whether this is associated with increased vascular permeability, we injected Evans Blue dye and monitored its leakage from vessels into the surrounding tissue (Radu & Chernoff, 2013). Increased tissue Evans Blue dye accumulation is evident at 0.5 d and increases with time (Fig. 3-1E). Epidermal dye accumulation is readily visible in the skin at 2 d after induction of TAM67 expression (Fig. 3-1F), which indicates enhanced vessel permeability.

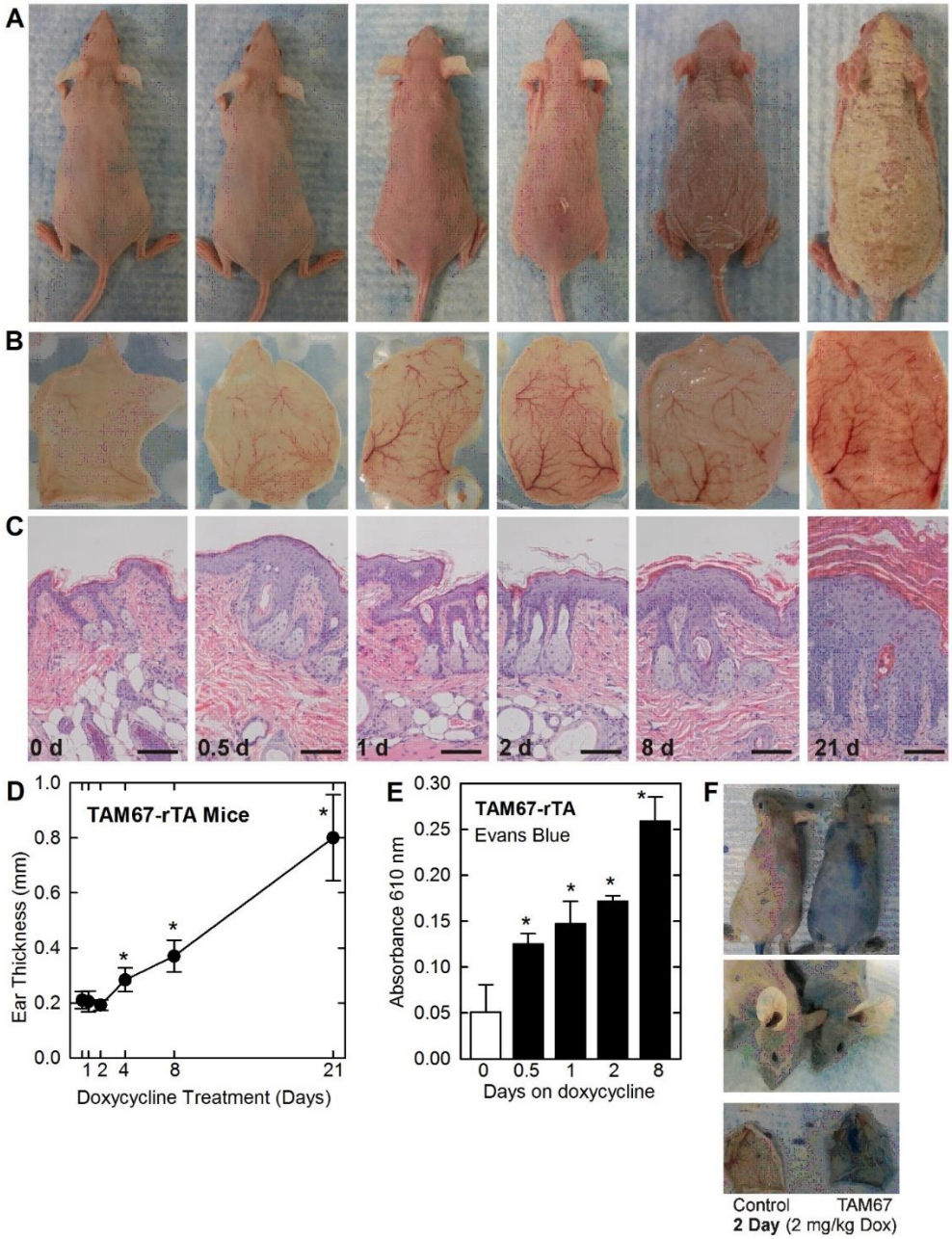

Figure 3-1: Development of TAM67 phenotype TAM67-rTA mice treated with 2 mg/ml doxycycline in drinking water for 0 - 21 d. A) TAM67-rTA mice were photographed at 0, 0.5, 1, 2, 8, and 21 d following initiation of doxycycline treatment. B) The back skin of TAM67-rTA mice was removed and flipped to reveal hypodermis and vascular network changes during phenotype development. C) H&E stained sections of TAM67-rTA mouse skin. The bars = 100 microns. D) Measurement of TAM67-rTA mouse ear thickness at indicated times after initiation of doxycycline treatment. The values are mean $\pm$ SEM, n = 6, $p < 0.001$. E/F) The Evans Blue dye assay reveals enhanced vessel permeability. TAM67-rTA mice were treated with 2 mg/ml doxycycline for 0.5 - 8 d (three mice/group, black bars) and then injected with Evans Blue dye solution. The open bar indicates dye level in control (no doxycycline) mice. The values are mean $\pm$ SEM. The treatment groups were significantly elevated compared to control (n = 3, $p < 0.05$). Photographs of day two mice indicate enhance epidermal dye uptake. The bottom panels indicate full thickness skin samples that were harvested following Evans Blue assay and is pictured inside up.

Chemokine levels in serum and epidermis To identify events that contribute to this phenotype, we monitored serum chemokine content and observed elevated levels of a host of chemokines including CXCL1, CXCL10, CCL1, CCL2, CCL12, CXCL9, IL-16 and IL-1F3 (Fig. 3-2A). Plotting chemokine level versus time reveals an early increase in CCL1 at 0.5 d with peak levels observed at days 2 to 21 (Fig. 3-2B). CXCL1 level is elevated at 4 and 8 d, and at 21 d other chemokines, including CXCL10, CCL1, CCL2, CCL12, CXCL9, IL-16 and IL-IF3 are increased (Fig. 3-2B). These findings indicate a strong early increase in CCL1 (0.5 d) (Wang et al., 2007), followed by an increase in CCL2 and CXCL10, CXCL1, CXCL9 and CXCL11 chemokines at 8 and 21 days.

Chemokine levels are also altered in the epidermis. TAM67-rTA mice were treated for 8 d with doxycycline and then epidermal chemokine levels were measured. Figure 3-2C/D shows that CCL1 and CCL2, CCL3, CCL5, CXCL9, CXCL10, CXCL11 and IFNγ chemokines are elevated. CXCL1, CXCL2, GM-CSF, TREM-1, TIMP-1 and compliment 5A levels are also increased (Fig. 3-2C/D). To understand the mechanism of this regulation, we measured mRNA level for selected chemokines. Figure 3-2E shows an increase in mRNA encoding CCL1 (7.7-fold), CCL2 (39-fold), CCL5 (33-fold), CCL7 (79.3-fold), CCL11 (16.8-fold), CCL3 (11.5-fold), CCL4 (12.4-fold), CXCL9 (16.8-fold), CXCL10 (625-fold), CXCL11 (13.2-fold) and IFNγ (13.1-fold) at 8 d after TAM67 induction. CCL17 (16-fold) and CCL20 (4-fold) were also increased at 8 d. An expanded plot of chemokine mRNA changes at 2 d after doxycycline addition shows increased levels of CCL1 (11.4-fold), CCL2 (2.2-fold), CCL7 (3.6-fold), CXCL1 (2.5-fold), CCL17 (2.1-fold), CCL20 (6.5-fold) and CXCL10 (3.3-fold) (Fig. 3-2E). These findings suggest that Th1 and Th2 chemokines are elevated in epidermis, and that

these increases are associated with increased mRNA level.

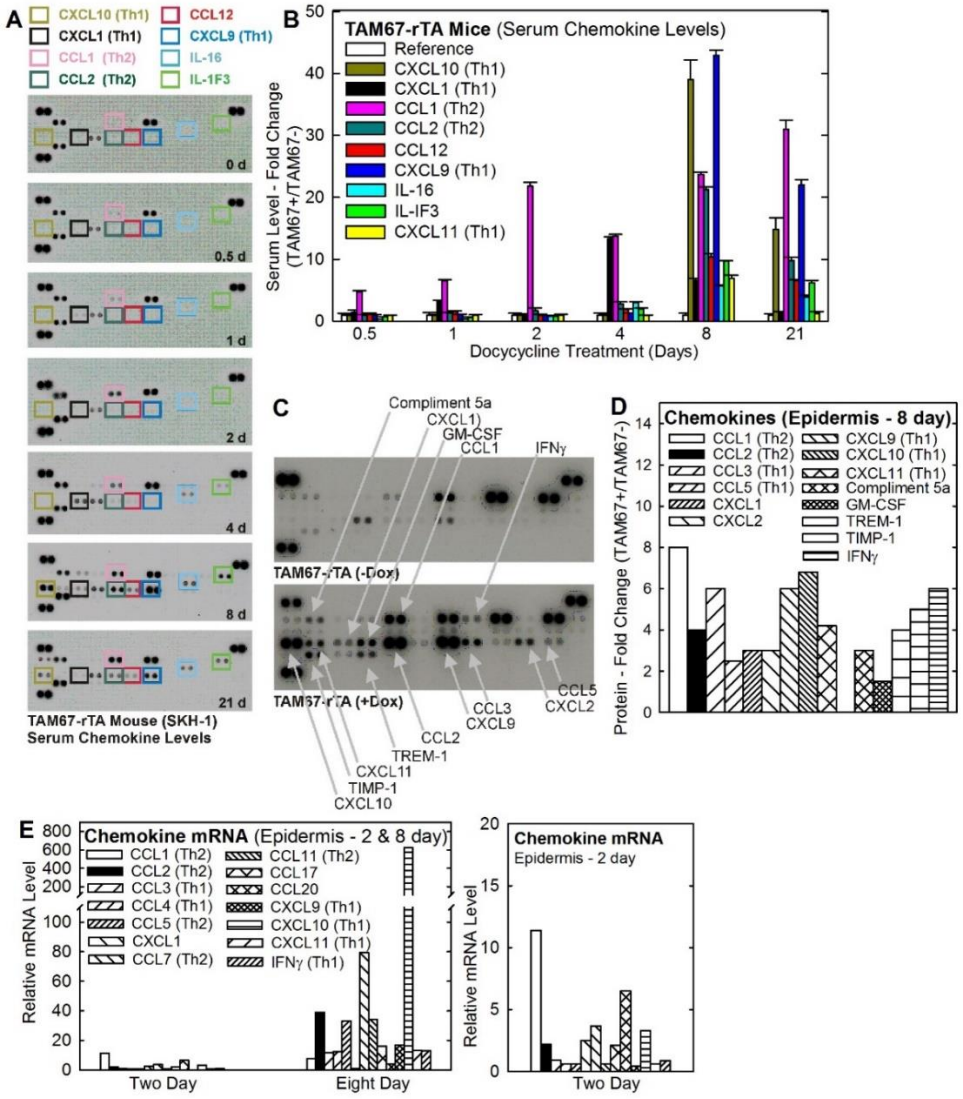

Figure 3-2: Chemokine levels in epidermis and serum A) TAM67-rTA mice were treated with 2 mg/ml doxycycline in drinking water for 0 - 21 days and serum was analyzed for chemokine content. The boxes indicate chemokines that change in level. Similar changes were observed in each of three independent experimental replicates. B) Pixel density graph depicts the levels of selected cytokines at 0.5 - 21 d after initiation of doxycycline treatment. The values are normalized to the day 0 time point (not shown) and show fold change. The values are mean $\pm$ SEM, n = 3, $p < 0.05$. C) TAM67-rTA mice were treated with or without doxycycline for 8 d and epidermal extracts were prepared and analyzed for chemokine content using the chemokine protein array. Upregulated chemokines are indicated. D) The graph presents a pixel density plot of data derived from panel C. Similar findings were observed in each of three replicate experiments. E) RNA, extracted from epidermis on 0, 2, and 8 d after initiation of doxycycline treatment of TAM67-rTA mice, was reverse transcribed to cDNA and applied to the cytokine and chemokine RNA array. Data was analyzed by the comparative C_T method and plotted as relative mRNA level. The right panel shows the two day data on an expanded scale. Similar trends were observed in three replicate experiments. The data indicates fold change as compared to the control (day zero) sample.

Immune cell invasion We next monitored the types of immune cells present in the dermis/epidermis in response to epidermal TAM67 expression. Histologic examination of H&E-stained sections revealed the presence of parakeratosis beginning at 2 d, mild to moderate dyskeratosis and hyperkeratosis at 8 days and severe dyskeratosis/hyperkeratosis at 21 d (Fig. 3-3A). Inflammation was detected in hair follicles, epidermis and stroma, and increased in severity with time. Consistent with these changes, cell sorting analysis reveals a marked increase in T-lymphocytes (CD3), neutrophils (CD11b) and macrophages (F4/80) (Fig. 3-3B). We also assessed the number of CXCR3-positive cells (receptor for CXCL9, 10, 11) and found no change (Fig. 3-3B). To identify the tissue localization of invading leukocytes, we stained epidermis from 8 d doxycycline-treated mice with anti-CD3 and found a 4-fold enrichment of CD3-positive lymphocytes in the epidermal suprabasal layers (Fig. 3-3C/D). We also observed a substantial increase in mast cells (Fig. 3-3E) and CD11b-positive neutrophils (Fig. 3-3F) at 21 d.

A

Histological Phenotype							
Day	0	0.5	1	2	4	8	21
Dyskeratosis	-	-	-	-	-	1+	3+
Parakeratosis	-	-	-	+	+	+	+
Hyperkeratosis	-	-	-	-	1+	2+	3+
Acute Inflammation (neutrophils) in Epithelium	-	-	-	-	-	-	2+
Acute Inflammation (neutrophils) in Hair Follicles	-	-	-	1+	1+	1+	3+
Acute Inflammation (neutrophils) in Stroma	-	-	-	-	-	-	3+
Chronic Inflammation (lymphocytes) in Epithelium	-	-	-	-	-	0+	0+
Chronic Inflammation (lymphocytes) in Hair Follicles	-	-	-	-	1+	1+	2+
Chronic Inflammation (lymphocytes) in Stroma	-	-	-	-	1+	1+	3+

Key: 0 = minimal/none, 0+ = focal, 1+ = mild, 2+ = moderate, 3+ = severe, + = present, - = absent

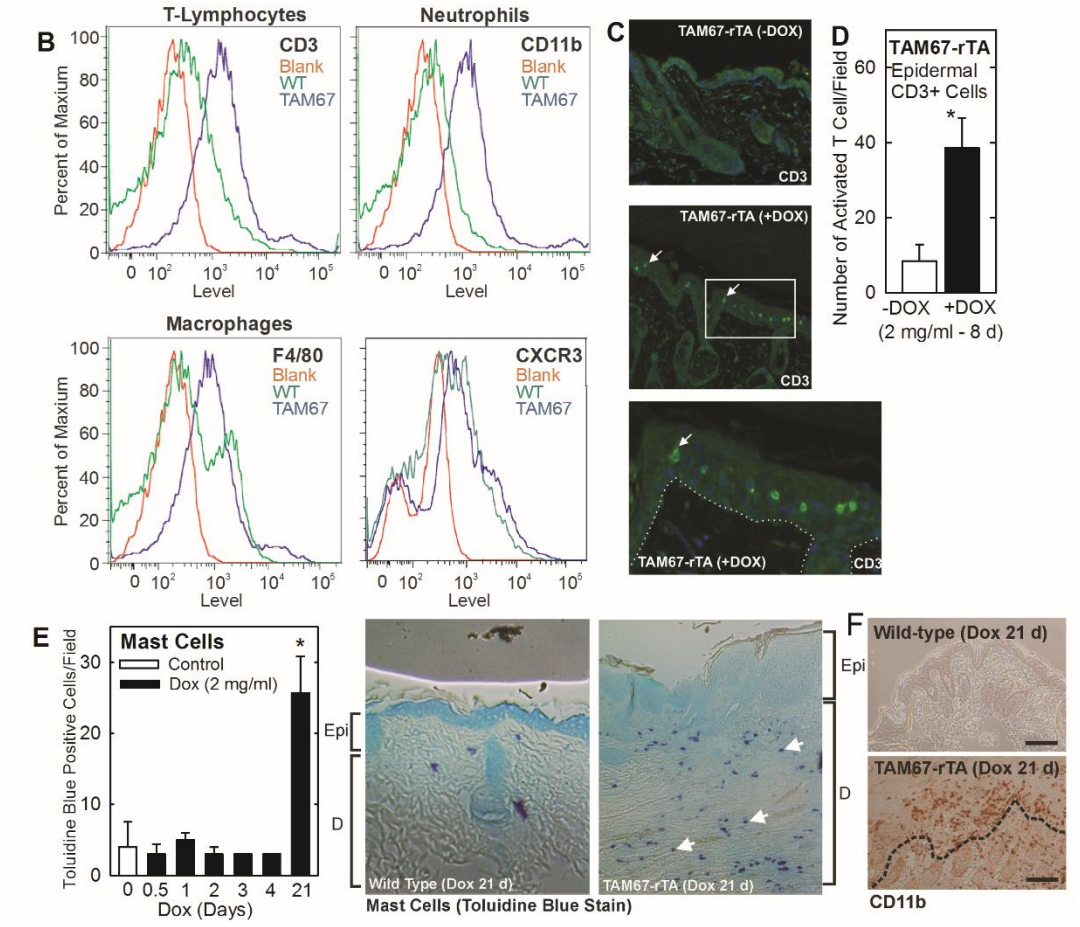

Figure 3-3: Immune cell involvement in TAM67-expressing skin A) Epidermal/dermal sections were prepared from mice at the indicated time points and prevalence of the indicated parameters was monitored by counting. The findings were scored by an expert pathologist. B) Flow cytometry data analysis of epidermal single cell suspensions from 8 d doxycycline treated WT and TAM67-rTA mice designed to detect CD3-, CD11b-, F4/80- and CXCR3-positive cells. (Red-unstained/blank, green-WT, blue-TAM67-positive). C/D) Immunofluorescent staining for CD3 in TAM67-rTA mouse skin treated with or without doxycycline for 8 d. Bottom image is magnification of white box from middle image. The number of interfollicular CD3+ (activated T cells) cells per field in 11 separate images was counted and graphed (control - open box, doxycycline-treated - closed boxes). The values are mean $\pm$ SEM, n = 11, $p < 0.001$. E) Toluidine blue stain to detect dermal mast cells in TAM67-rTA mice treated with doxycycline for 0 - 21 d. (Epi, epidermis; D, dermis). F) Detection of CD11b-positive cells in wild-type and TAM67-positive mice at 21 d after initiation of doxycycline treatment. The bar = 100 μm and the dashed line separates the epidermis and dermis.

Role of CXCL9, CXCL10, CXCL11 in phenotype development CXCL9, CXCL10 and CXCL11 are among the most increased chemokines, as assessed by mRNA and protein level, in TAM67-expressing epidermis (Fig. 3-2). Considering that CXCR3, the receptor for CXCL9, CXCL10 and CXCL11, is expressed by keratinocytes where it can stimulate proliferation (Winkler et al., 2011; Yates et al., 2007) and in activated T-lymphocytes as a chemotaxis mediator (S. Qin et al., 1998), we tested whether CXCR3 receptor function is required for generation of the TAM67-rTA mouse phenotype. Although CXCR3 ligand (CXCL9, CXCL10, CXCL11) levels are elevated, Figure 3-4A shows that CXCR3 receptor level is not changed in 8 d wild-type versus TAM67-positive epidermis (in the CXCR3-WT background), indicating that altered receptor level is not involved in generating the TAM67-associated epidermal phenotype. To examine the role of these chemokines and receptor, we produced TAM67-rTA/CXCR3-WT and TAM67-rTA/CXCR3-KO mice and monitored the impact on the TAM67-dependent phenotype. We treated WT/CXCR3-WT, TAM67-rTA/CXCR3-WT and TAM67-rTA/CXCR3-KO mice with doxycycline. Immunostaining confirms that CXCR3 is present in WT/CXCR3-WT and TAM67-rTA/CXCR3-WT mice but not in TAM67-rTA/CXCR3-KO mice (Fig. 3-4A). We next monitored the effect of CXCR3 knockout on the TAM67-associated phenotype. Figure 3-4B shows that presence or absence of CXCR3 does not alter phenotype severity in male or female mice. A severe flaking/scaly epidermal phenotype develops in the TAM67-expressing mice independent of whether CXCR3 is absent or present.

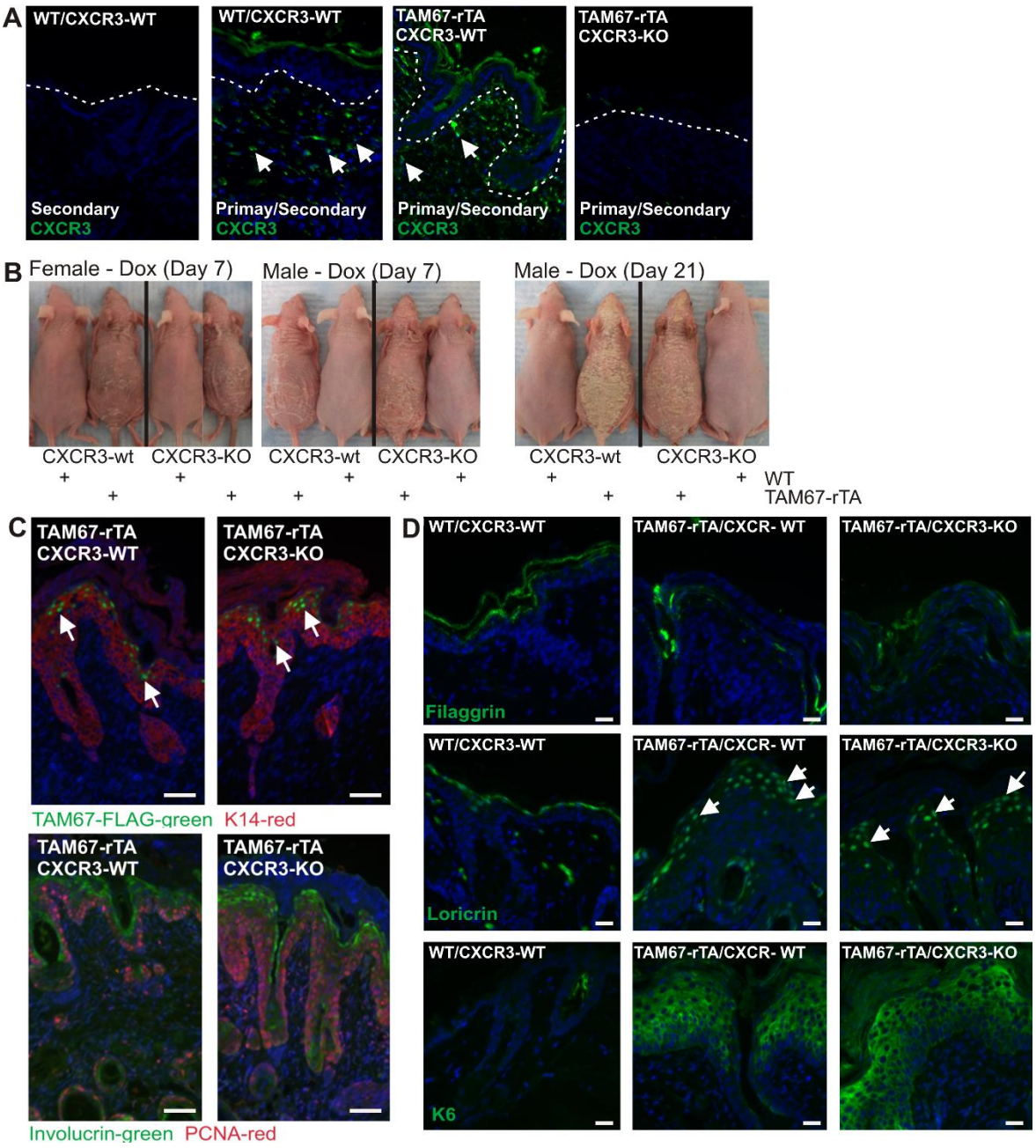

Figure 3-4: CXCR3 knockout does not prevent development of the keratoderma-like phenotype A) Immunofluorescent staining of CXCR3 in the indicated mouse genotypes. The dotted white line indicates dermal-epidermal junction. Arrows indicated positive CXCR3 staining which is absent in CXCR3-KO mice. B) Mice were treated for 7 and 21 d with 2 mg/ml doxycycline and the TAM67-rTA/CXCR3-WT and TAM67-rTA/CXCR3-KO mice were photographed alongside WT littermates. C) Mice were treated for 8 d with doxycycline and then epidermal sections were stained with the indicated antibodies and images were generated by fluorescence microscopy. The arrows indicate TAM67-FLAG-positive nuclei (green). The bar = 200 μm. D) Distribution of filaggrin, loricrin and K6 in epidermal sections prepared from WT/CXCR3-WT, TAM67-rTA/CXCR3-WT and TAM67-rTA/CXCR3-KO mice at 8 d after initiation of doxycycline treatment. The arrows indicate nuclear localization of loricrin in sections from TAM67-expressing mice. The bar = 200 μm.

To get a more detailed view of the impact of CXCR3 knockout on phenotype, we monitored epidermal marker expression as a measure of differentiation status. Figure 3-4C confirms that TAM67-FLAG is expressed in the nuclei of suprabasal keratinocytes (arrows) (Rorke et al., 2010; Rorke et al., 2015; Rorke, Adhikary, Young, Roop et al., 2015). K14, which in normal epidermis marks the epidermal basal layer (Eckert et al., 1997), is expressed in all epidermal layers in TAM67-expressing epidermis confirming expansion of the proliferative layer and delayed differentiation (Rorke et al., 2010). Multilayer staining of K14 is observed in the TAM67-expressing epidermis in presence or absence of CXCR3. Involucrin, a suprabasal marker (Eckert & Green, 1986; Eckert et al., 2004; Rice & Green, 1979), is confined to the suprabasal layer in all mice. PCNA staining, an indicator of proliferation, which is confined to the basal layer in normal epidermis, is observed in the basal and suprabasal layers which is indicative of hyperproliferation (Rorke et al., 2010; Rorke, Adhikary, Young, Roop et al., 2015), and CXCR3 status does not influence this distribution (Fig. 3-4C). Figure 3-4D shows reduced filaggrin level, abnormal nuclear accumulation of loricrin (arrows) and induction of expression of hyperproliferation-associated K6 in TAM67-expressing epidermis. These changes are identical in the CXCR3-WT and CXCR3-KO background. Finally, we used a pharmacologic approach to monitor the role of CXCR3, and showed that treatment with a CXCR3-selective inhibitor, AMG487, does not alter phenotype development (not shown). Based on this we conclude that CXCR3 (CXCL9/10/11) signaling is not required for phenotype development.

Role of S100A8 and S100A9 in phenotype development Our previous study showed that S100A8 and S100A9 are among the most upregulated genes in TAM67-

positive epidermis at both the mRNA and protein level (Rorke et al., 2015). We therefore sought to identify a potential role for these calcium-binding, proliferation and inflammation-associated proteins, in phenotype development in the TAM67-expressing epidermis. We bred the TAM67-rTA mice into a S100A9-KO background. S100A9-KO mice are interesting in that S100A9 knockout also results in loss of S100A8. Thus, these mice are deficient in both proteins (Manitz et al., 2003). We then characterized various features of phenotype development. Consistent with our previous report (Rorke et al., 2015), TAM67-expression is associated with increased expression of K6, S100A8 and S100A9 and reduced levels of loricrin and filaggrin (Fig. 3-5A). As expected, the level of K1 and K10, which are suprabasal keratin differentiation markers, remain relatively unchanged (Fig. 3-5A). Figure 3-5B/C shows the epidermal morphology and histology of control, TAM67-rTA/S100A9-WT and TAM67-rTA/S100A9-KO mice, and shows that phenotype development, as measured by gross appearance and by histology, is not significantly altered in the S100A9-KO environment. The TAM67-positive epidermis displays a scaly appearance with hyperplasia and hyperkeratosis regardless of S100 protein status. Figure 3-5D confirms nuclear loricrin localization in TAM67 expressing epidermis. Involucrin expression is detected in the suprabasal layers in all mice, and the PCNA proliferation marker, is distributed in both basal and suprabasal epidermal compartment in TAM67-positive mice independent of the presence or absence of S100A8/A9 (Fig. 3-5D).

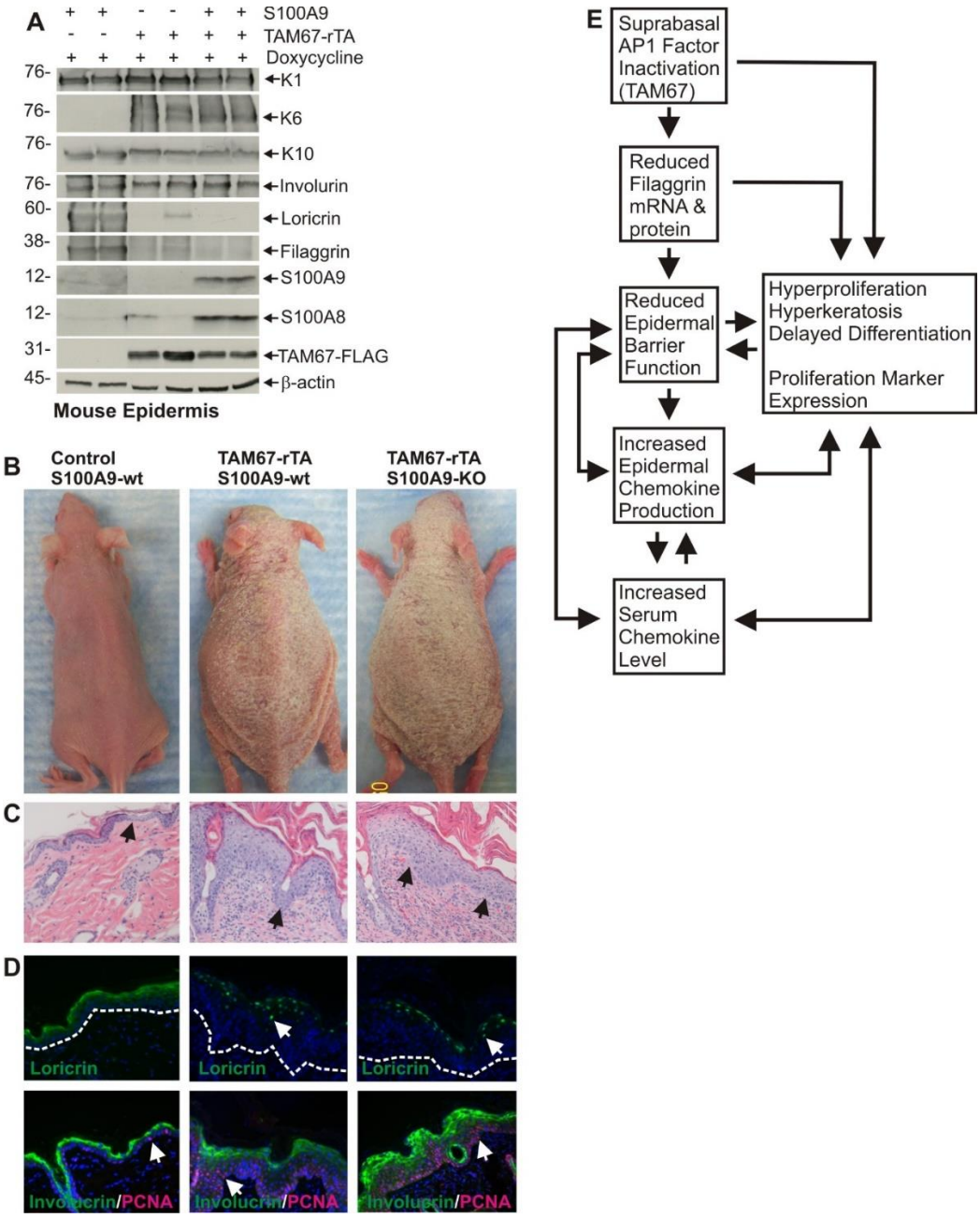

Figure 3-5: S100A8/A9 knockout does not prevent the keratoderma-like phenotype A) Mice of the indicated TAM67-rTA and S100A9 genotypes were treated for 10 d with doxycycline and epidermal extracts were prepared for immunoblot. B) WT/S100A9-WT, TAM67-rTA/S100A9-WT and TAM67-rTA/S100A9-KO mice were treated for 10 d with doxycycline before image collection. C) H&E stained sections of mouse skin corresponding to mice from panel B. Black arrows indicate the dermal-epidermal junction. D) Loricrin, involucrin and PCNA staining of epidermis from 10 d doxycycline-treated mice of the indicated genotypes. Dotted white lines indicate the dermal-epidermal junction and the arrows indicate either nuclear loricrin (upper panels) or nuclear PCNA staining (bottom panels). E) Model of phenotype development: AP-1 factor inaction leads to a reduction in filaggrin level and also has a direct impact on expression of other genes that control proliferation and differentiation. Loss of filaggrin leads to reduced barrier function (McLean, 2016; Smith et al., 2006) which exposes the mice to antigens that stimulate epidermal chemokine production and accumulation in the serum which further stimulate phenotype development. The arrows indicate the complex interactive feedback which is likely to exist.

E. DISCUSSION:

Morphological and biochemical response to AP-1 factor inactivation Inhibition of AP-1 transcription factor function in murine suprabasal epidermis initiates a poorly-understood cascade of changes in epidermal structure and function that produce a profound change in phenotype including nuclear loricrin accumulation, tail and digit autoamputation, hyperproliferation, hyperkeratosis, parakeratosis, delayed differentiation and reduced barrier integrity (Rorke et al., 2015; Rorke, Adhikary, Young, Roop et al., 2015). This is coupled with suprabasal expression of hyperproliferation-associated epidermal markers (K6, K14, K16) and reduced expression of filaggrin family members (Rorke et al., 2015; Rorke, Adhikary, Young, Roop et al., 2015). Our present findings show that this phenotype includes increased erythroderma and vascular permeabilization. Many of these features are observed in human epidermis in ichthyosis/keratoderma (Kawasaki et al., 2012; McLean, 2016; Smith et al., 2006).

These changes may be due to direct effects due to AP-1 factor loss on gene expression (e.g., filaggrin) and/or indirect effects associated with altered differentiation and reduced barrier function. A key example is the reduction in filaggrin mRNA and protein levels (Fig. 3-5A) (Rorke et al., 2015). As reviewed by Gutowska and Ogg, a number of chemokines (IL-4, IL-13, TNFα, IL-17, IL-22 and IL-24) are reported to suppress filaggrin mRNA and protein level (Gutowska-Owsiak & Ogg, 2013); however, we do not observe a major increase in these chemokines suggesting this is not a likely explanation for the reduction in filaggrin expression. Alternatively, AP-1 transcription factors bind directly to the filaggrin gene promoter to increase gene expression (Jang et al., 1996). Thus, loss of AP-1 factor function may directly lead to reduced filaggrin

mRNA and protein production.

Early chemokine response We also monitored the impact on AP-1 factor inaction on chemokine production. We observe increased CCL1 and CXCL1 levels in serum at early times (12 - 24 h) after TAM67 induction. We propose that these chemokines are produced in the epidermis and released to the serum, as we find elevated levels of CCL1 (11-fold) and CXCL1 (2.5-fold) mRNA at 2 d in epidermis. This is accompanied by a further increase in CCL1 (8-fold) and CXCL1 (2.5-fold) chemokines in 8 d epidermis. Keratinocyte-produced CCL1 targets CCR8 expressing T-lymphocytes and Langerhans cell precursors, and CXCL1 targets the CXCR2 receptor on keratinocytes (Kulke et al., 1998) and neutrophils (Cataisson et al., 2006) to promote proliferation and migration. Messenger RNA encoding other chemokines are elevated in epidermis at 2 d including CCL2, CCL3, CCL4, CCL5 (RANTES), CCL7, CCL11, CCL17, CCL20, CXCL9, CXCL10, CXCL11 and IFNγ. CCL1 (11-fold) and CCL20 (6-fold) levels are markedly increased. CCL20 is a keratinocyte-produced chemokine that has a role in recruiting CCR6-positive immature dendritic cells and T-lymphocytes from blood (Le Borgne et al., 2006; Paradis, Cole, Nelson, & Gladue, 2008; Varona, Cadenas, Gomez, Martinez-A, & Marquez, 2005). It is likely that these chemokines play a role in initiating and maintaining erythroderma.

Intermediate chemokine response Additional chemokine changes are observed at 8 days after TAM67-induction. These are associated with keratinocyte hyperproliferation and hyperkeratosis, and increased thickening of the epidermis. Analysis of mRNA isolated from 8 d epidermis reveals a marked increase in CCL2 (39-fold), CCL5 (RANTES) (33-fold), CCL7 (80-fold), CCL11 (33-fold) and CXCL10 (over

600-fold) which is associated with accumulation of CCL2, CCL12, CXCL9, CXCL10, IL-16 and IL-IF3 in epidermis and serum (Fig. 3-2). CXCL9 and CXCL10 are the most increased in serum (approximately 40-fold). Many of these chemokines are elevated in states of chronic inflammation and are involved in leukocyte recruitment. For example, CCL2 recruits dendritic and Langerhans cells, CCL5 recruits neutrophils, and CXCL9 and CXCL10 recruit T-lymphocytes. We observed increased accumulation of CD3-positive T-lymphocytes, CD11b-positive leukocytes and F4/80-positive macrophages in the epidermis at 8 d. It is interesting that the CD3-positive T-lymphocytes accumulate in the suprabasal epidermis.

Late chemokine response At 21 d the mice manifest an extensive scaling phenotype coupled with hyperproliferation and hyperkeratosis. This is associated with additional changes in the immune response including dermal accumulation of mast cells. Mast cell invasion of the epidermis is associated with response to allergens or pathogens (Sehra, Serezani, Ocana, Travers, & Kaplan, 2016) which is consistent with the finding that TAM67 expression in embryonic epidermis compromises the barrier (manuscript in preparation). The chemokine profiles remain generally very similar in 21 d mice as compared to 8 d, and the most highly elevated chemokines in the serum include CCL1, CXCL9 and CXCL10. It is interesting that the increase in chemokine level is nearly always associated with a parallel increase in the corresponding mRNA. We are not sure of the mechanism responsible for the changes in chemokine gene expression. This could be due to gene activation associated with loss of AP-1 factor-dependent repression, infiltration of cell types that encode the RNA, or indirect secondary effects (altered differentiation, reduced barrier function).

Impact of CXCR3 and S100A9 knockout on TAM67-rTA phenotype The above studies document wide ranging changes in chemokine expression in response to AP-1 inactivation. To investigate the role of individual chemokine/chemokine receptors, we focused on CXCL9/10/11 and the CXCR3 receptor, and S100A8 and S100A9. CXCL9, 10 and 11 interact specifically with the CXCR3 receptor (Groom & Luster, 2011; Lacotte, Brun, Muller, & Dumortier, 2009), and are among the most highly elevated chemokines at days 8 and 21 after TAM67 induction. To study their role in phenotype development, we bred the TAM67-rTA mice with CXCR3 knockout mice (Hancock et al., 2000; Yates et al., 2007; Yates et al., 2010). We anticipated that eliminating CXCR3-related signaling may attenuate development of the phenotype associated with AP-1 factor deficiency. However, elimination of CXCR3 signaling did not impact phenotype development. The mice develop the same morphological and histological changes including hyperproliferation and hyperkeratosis, and abnormal expression and subcellular distribution of K14, K6, filaggrin and loricrin, suggesting that CXCR3 related ligands are marginally important in phenotype development.

S100A8 and S100A9 are key inflammatory and anti-bacterial proteins in epidermis (Broome, Ryan, & Eckert, 2003; Eckert et al., 2004; Lee & Eckert, 2007; Robinson et al., 1997) that are highly elevated in some epidermal diseases (Eckert et al., 2004; Nakajima et al., 2013; Schonthaler et al., 2013). Recent studies indicate that S100A9 genetic deletion reduces phenotype severity in mouse inflammatory disease models (Schonthaler et al., 2013); however, we did not observe an appreciable change in disease onset or severity in a S100A9-null/S100A8-null environment. Likewise, S100A8 and S100A9 have been reported to stimulate keratinocyte proliferation which often

controls epidermal thickness (Iotzova-Weiss et al., 2015), but we did not detect any change in epidermal thickness in the S100A8/A9 null environment.

AP-1 factors in epidermis AP-1 factors are key controllers of differentiation-associated gene expression in epidermis (Eckert & Welter, 1996; Eckert et al., 1997; Eckert et al., 2004; Rossi, Jang, Ceci, Steinert, & Markova, 1998; Welter & Eckert, 1995) and loss of AP-1 function is associated with striking changes in epidermal differentiation. In many cases these changes are associated with disease (Mehic, Bakiri, Ghannadan, Wagner, & Tschachler, 2005; Rorke et al., 2010; Rorke, Adhikary, Young, Roop et al., 2015; Zenz & Wagner, 2006; Zenz et al., 2008). Inactivation of AP-1 factor function in the suprabasal epidermis of TAM67-rTA mice results in a phenotype that includes features that resemble ichthyosis and/or keratoderma. This includes reduced filaggrin level, keratinocyte hyperproliferation, hyperkeratosis and parakeratosis, impaired epidermal barrier function, a scaly epidermis and accumulation of chemokines in the skin and blood. A possible model of phenotype development is present in Figure 3-5E. In this model, AP-1 factor inaction leads to a reduction in filaggrin level and also has a direct impact on expression of other genes that control proliferation and differentiation. Filaggrin is an essential barrier component that is functionally inactivated in ichthyosis leading to reduced barrier function (McLean, 2016; Smith et al., 2006). We propose that loss of filaggrin leads to reduced barrier integrity and exposes the mice to antigens that stimulate epidermal chemokine production and accumulation in the serum. Increased chemokine accumulation would also be expected to further influence keratinocyte differentiation status and barrier function. Our present studies describe many chemokine changed, but clearly show, using genetic methods,

that S100A8/S100A9 and CXCR3 signaling (CXCL9/10/11) are not required for phenotype development.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

A. CONCLUSIONS:

In the experiments discussed in chapter two, we were able to further characterize our ichthyosis mouse model by extending the study from adult mice to studying the collodion baby phenotype in embryos/neonatal mice. Since a significant portion of human ichthyosis patients display a neonatal collodion phenotype (Yeh et al., 2013), we wanted to know if this phenotype would be recapitulated at birth in our ichthyosis mouse model. We targeted specific time windows during embryogenesis (chosen based on time-sensitive expression of differentiation genes) and evaluated the effects of AP-1 transcription factor inhibition on skin development. Long-term doxycycline treatment (E13.5 and E15.5) of TAM67 mouse neonates resulted in a collodion baby-like phenotype, evidenced by red neonates encased in a dry, taut membrane. These "collodion babies" quickly dehydrated and died approximately 12 hours after birth. Therefore we concluded that suprabasal AP-1 transcription factor activity is essential for normal epidermal development during this timeframe. Further support for this conclusion comes from the abolition of filaggrin as well as a drastic reduction in the number and thickness of cornified envelopes.

While short-term doxycycline treatment (E17.5) resulted in normal looking TAM67 neonates with a functional barrier at birth, they had significantly fewer normal cornified envelopes, moderate ultrastructural changes, and were as severely filaggrin-deficient as their E13.5 and E15.5 doxycycline treated TAM67 counterparts. This suggests that a functional barrier was formed prior to the effects of suprabasal AP-1

transcription factor inhibition, but the next cycle of cells will be defective and the sensitive neonates will not survive. These studies are the first of their kind to link a family of transcription factors (AP-1) to the collodion baby neonatal phenotype associated with ichthyosis.

The experiments laid out in chapter three were designed to identify changes in signaling molecules and therefore provide an important first step in better understanding the biomolecular mechanisms behind loricrin keratoderma and ichthyoses in general. This is the first characterization of the cytokine response in a mouse model of ichthyosis. Many changes (up/down) in cytokines, chemokines, and interleukins occurred and while all are not covered by the array used here, it focused the search to a reasonable list of targets to study. We further confirmed changes in protein levels identified on the array by evaluating changes in mRNA via qPCR.

After learning more about up and downregulated chemokines associated with T helper cell responses in the suprabasal TAM67-expressing epidermis, we wanted to identify the source of these signaling molecules. Were they secreted by keratinocytes or resident/infiltrating immune cells? We honed in our work to focus on immune changes in the skin to answer this question. We found epidermal T cells to be involved during early (8 days) phenotype development with infiltration of mast and dendritic cells occurring later (21 days). Since very early (0.5 day-1 day) changes were observed, such as erythroderma and basal hyperproliferation, we concluded that keratinocyte-related changes drive the phenotype while immune cells have a later role.

Furthermore, by breeding the suprabasal TAM67 mouse with CXCR3 and S100A9 knockout mice we were able to study the ichthyosis phenotype in the absence

of CXCL9/CXCL10/CXCL11 and S100A8/A9 signaling, respectively. While these knockout models both had no impact on phenotype development, they provide important information. Perhaps the dramatic upregulation of CXCL9/CXCL10/CXCL11 chemokines signal through a receptor other than CXCR3. Likewise, maybe the huge increase in S100A8/A9 proteins does not drive phenotype development, but rather is an attendant change associated with reduced barrier integrity. S100A8/A9 proteins may be responding as if there is a microbe invasion simply because the barrier is dysfunctional and an infection may be imminent.

The work described herein can be broadly summarized by the flow chart in Figure 4-1. AP-1 transcription factors were artificially inactivated in the suprabasal layers of the mouse epidermis using the TAM67 (dominant-negative c-jun) transgenic construct. This resulted in reduced filaggrin mRNA and protein levels and reduced epidermal barrier function. Further changes included basal hyperproliferation, dry, scaly skin, delayed differentiation, pseudoainhum, and nuclear loricrin accumulation. We saw a dramatic increase in epidermal chemokine production (CXCL9/10/11) as well as S100A8/A9 calcium-dependent signaling proteins. The epidermis is known to secrete signaling molecules into the serum to communicate both locally and systemically. This resulted in immune activation and movement of immune cells to the epidermis and dermis. Prolonged inactivation of suprabasal AP-1 transcription factors resulted in an ichthyosis phenotype. The arrows indicate the complex interactive feedback which is likely to exist. Disruption of the epidermal barrier initiates its own series of events coalescing with suprabasal AP-1 transcription factor inhibition to exacerbate phenotype development.

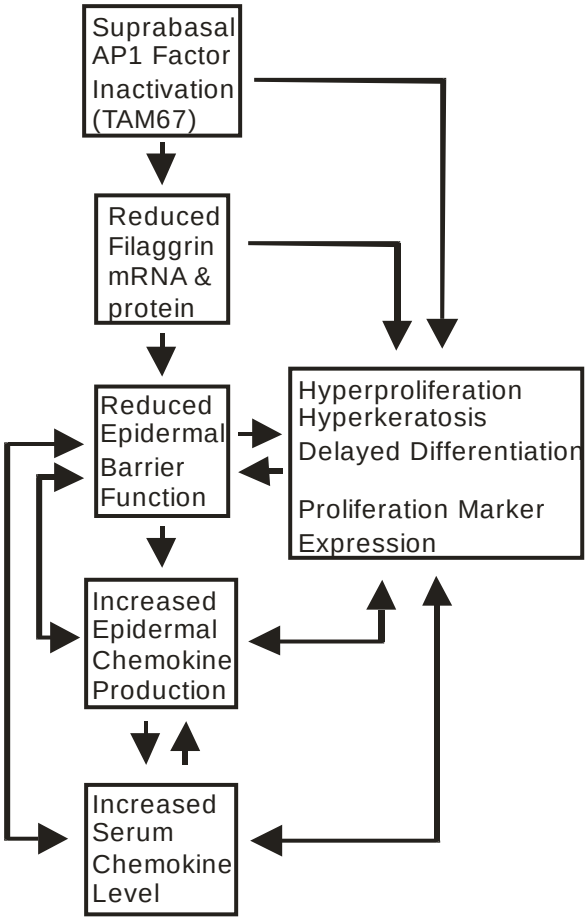

Figure 4-1: Working Model

B. FUTURE DIRECTIONS:

We have shown that inactivation of AP-1 transcription factor function in the suprabasal epidermal layer produced an ichthyosis/keratoderma-like phenotype in embryos and adult mice. This suggests that loss of AP-1 transcription factor function may have a role in genesis of the human diseases. An important future goal is to determine whether AP-1 factor loss or inactivation is associated with development of ichthyosis in human patients. Unfortunately, this specific type of keratoderma is rare and in general, skin biopsies are painful for patients suffering from dermatoses. We will

make efforts to obtain this tissue and examine AP-1 factor levels using protein blot, mRNA level analysis and immunohistology.

Next, we will explore the role of CCL1. CCL1 binds to CCR8 to control T cell-mediated immune protection in normal, healthy skin (McCully et al., 2012; Schaerli et al., 2004). Activated CCR8 receptor releases its own ligand, CCL1, in a positive feedback loop (Schaerli et al., 2004) and a change in this loop could signal epidermal perturbation. We find early upregulation (12 hours) of CCL1 mRNA in doxycycline-treated TAM67 mice (Figure 2-2) as well as CCR8+ cells in the follicular epidermis of both wild type and TAM67-expressing mice (Figure 4-2). Also, the gene encoding CCL1 has a putative AP-1 binding site; this suggests the possibility of TAM67-dependent control over the CCL1 promoter. Preliminary studies could use the novel CCR8 inhibitor, R243 (Aobious, Catalog number AOB2014), to block CCL1 binding in vivo. If the CCL1-CCR8 interaction and subsequent CCR8 activation is necessary for phenotype development, the phenotype of R243 treated TAM67-rTA mice would be diminished in severity/time of onset or absent.

CCR8+ T cells are known to secrete cytokines that may be involved in the initiation of T_h1 recall responses (TNFα and IFNγ) (Schaerli et al., 2004). TNFα may be responsible for inflammation and the observed erythroderma in the suprabasal TAM67 mouse phenotype. The role of TNFα in this phenotype could be assessed by injecting the mice with a TNFα inhibitor, such as etanercept (Enbrel), commonly used to treat psoriasis. Once injected with the monoclonal antibody, we could follow progression of erythroderma compared to non-treated TAM67-rTA mice. If the erythroderma is not present in the etanercept treated TAM67-rTA mice, we could conclude that TNFα plays

a role in the observed increase in blood flow to the epidermis. We also see a modest increase in IFNγ and a dramatic increase in IFNγ-inducible ligands, CXCL9, CXCL10, and CXCL11 (Figure 2-2). While the TAM67 keratoderma-like phenotype occurs regardless of CXCR3 status (Figure 3-4) those CXCR3 knockout experiments did not rule out noncanonical CXCL9, CXCL10, and CXCL11 signaling through other receptors. We could identify CXCL9, CXCL10, and CXCL11 receptor binding using ligand-based receptor capture (LRC) technology, TRICEPS (Frei, Moest, Novy, & Wollscheid, 2013). We could use a specific chemoproteomic reagent coupled to CXCL9, CXCL10, or CXCL11. This reagent allows for ligand-based capture and subsequent purification for quantitative mass spectrometry. Mass spectrometry could identify noncanonical binding of CXCL9, CXCL10, and CXCL11 to a known or unknown receptor which could play a role in development of the TAM67 keratoderma-like phenotype.

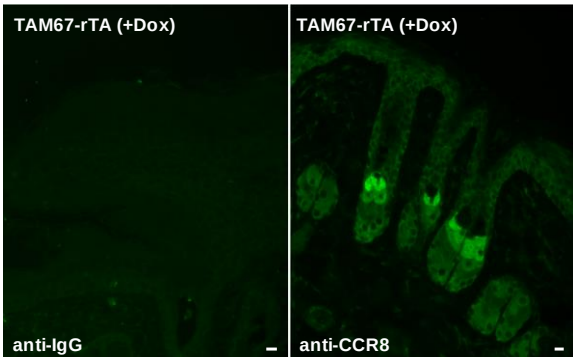

Figure 4-2: CCR8 immunohistochemistry CCL1 receptor, CCR8, expression was found to be localized to an unknown region of the follicular epidermis (hair follicle).

Lastly, a major query regarding this work is the role of loricrin in the nucleus. Does nuclear loricrin inhibit suprabasal AP-1 transcription factor activity, either directly or indirectly? In human loricrin keratoderma patients, the mutation in the loricrin gene creates a nuclear localization signal resulting in movement of loricrin from the cytoplasm to the nucleus where it accumulates. To assay the relationship between nuclear loricrin

and AP-1 transcription factor function, we could generate a mutant (nuclear) loricrin/AP-1 luciferase reporter transgenic mouse model. The luciferase reporter gene would be under the control of a promoter containing functional AP-1 binding sites. If those AP-1 sites were active, luciferase mRNA would be transcribed, then translated and measured. This would allow us to compare AP-1 activity between wild type and mutant loricrin-expressing epidermis *in vivo*. If mutant (nuclear) loricrin is inhibiting AP-1 transcription factor activity we would expect to see a decrease in luciferase levels. If true, we could then develop further experiments to identify specific protein-protein interactions between loricrin and either AP-1 transcription factors, or a mediator/co-factor.

REFERENCES

Adhikary, G., Chew, Y. C., Reece, E. A., & Eckert, R. L. (2010). PKC-delta and -eta, MEKK-1, MEK-6, MEK-3, and p38-delta are essential mediators of the response of normal human epidermal keratinocytes to differentiating agents. *The Journal of Investigative Dermatology, 130*(8), 2017-2030. doi:10.1038/jid.2010.108 [doi]

Adhikary, G., Crish, J., Lass, J., & Eckert, R. L. (2004). Regulation of involucrin expression in normal human corneal epithelial cells: A role for activator protein one. *Investigative Ophthalmology & Visual Science, 45*(4), 1080-1087.

Albanesi, C., Scarponi, C., Giustizieri, M. L., & Girolomoni, G. (2005). Keratinocytes in inflammatory skin diseases. *Current Drug Targets.Inflammation and Allergy, 4*(3), 329-334.

Albanesi, C., Scarponi, C., Sebastiani, S., Cavani, A., Federici, M., Sozzani, S., et al. (2001). A cytokine-to-chemokine axis between T lymphocytes and keratinocytes can favor Th1 cell accumulation in chronic inflammatory skin diseases. *Journal of Leukocyte Biology, 70*(4), 617-623.

Amagai, M., & Stanley, J. R. (2012). Desmoglein as a target in skin disease and beyond. *The Journal of Investigative Dermatology, 132*(3 Pt 2), 776-784. doi:10.1038/jid.2011.390 [doi]

Angel, P., & Karin, M. (1991). The role of jun, fos and the AP-1 complex in cell-proliferation and transformation. *Biochimica Et Biophysica Acta, 1072*(2-3), 129-157. doi:0304-419X(91)90011-9 [pii]

Angel, P., Szabowski, A., & Schorpp-Kistner, M. (2001). Function and regulation of AP-1 subunits in skin physiology and pathology. *Oncogene, 20*(19), 2413-2423. doi:10.1038/sj.onc.1204380 [doi]

Barker, J. N., Sarma, V., Mitra, R. S., Dixit, V. M., & Nickoloff, B. J. (1990). Marked synergism between tumor necrosis factor-alpha and interferon-gamma in regulation of keratinocyte-derived adhesion molecules and chemotactic factors. *The Journal of Clinical Investigation, 85*(2), 605-608. doi:10.1172/JCI114481 [doi]

Benavides, F., Oberyszyn, T. M., VanBuskirk, A. M., Reeve, V. E., & Kusewitt, D. F. (2009). The hairless mouse in skin research. *Journal of Dermatological Science, 53*(1), 10-18. doi:10.1016/j.jdermsci.2008.08.012 [doi]

Botchkarev, V. A. (2003). Bone morphogenetic proteins and their antagonists in skin and hair follicle biology. *The Journal of Investigative Dermatology, 120*(1), 36-47. doi:S0022-202X(15)30143-3 [pii]

Botchkarev, V. A., & Sharov, A. A. (2004). BMP signaling in the control of skin development and hair follicle growth. *Differentiation; Research in Biological Diversity, 72*(9-10), 512-526. doi:S0301-4681(09)60477-1 [pii]

Broome, A. M., Ryan, D., & Eckert, R. L. (2003). S100 protein subcellular localization during epidermal differentiation and psoriasis. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society, 51*(5), 675-685. doi:10.1177/002215540305100513 [doi]

Brown, P. H., Chen, T. K., & Birrer, M. J. (1994). Mechanism of action of a dominant-negative mutant of c-jun. *Oncogene, 9*(3), 791-799.

Brown, P. H., Kim, S. H., Wise, S. C., Sabichi, A. L., & Birrer, M. J. (1996). Dominant-negative mutants of cJun inhibit AP-1 activity through multiple mechanisms and with different potencies. *Cell Growth & Differentiation : The Molecular Biology Journal of the American Association for Cancer Research, 7*(8), 1013-1021.

Byrne, C., Tainsky, M., & Fuchs, E. (1994). Programming gene expression in developing epidermis. *Development (Cambridge, England), 120*(9), 2369-2383.

Camisa, C., & Rossana, C. (1984). Variant of keratoderma hereditaria mutilans (vohwinkel's syndrome). treatment with orally administered isotretinoin. *Archives of Dermatology, 120*(10), 1323-1328.

Candi, E., Schmidt, R., & Melino, G. (2005). The cornified envelope: A model of cell death in the skin. *Nature Reviews.Molecular Cell Biology, 6*(4), 328-340. doi:nrm1619 [pii]

Cataisson, C., Pearson, A. J., Tsien, M. Z., Mascia, F., Gao, J. L., Pastore, S., et al. (2006). CXCR2 ligands and G-CSF mediate PKCalpha-induced intraepidermal inflammation. *The Journal of Clinical Investigation, 116*(10), 2757-2766. doi:10.1172/JCI27514 [doi]

Cavani, A., Mei, D., Guerra, E., Corinti, S., Giani, M., Pirrotta, L., et al. (1998). Patients with allergic contact dermatitis to nickel and nonallergic individuals display different nickel-specific T cell responses. evidence for the presence of effector CD8+ and regulatory CD4+ T cells. *The Journal of Investigative Dermatology, 111*(4), 621-628. doi:10.1046/j.1523-1747.1998.00334.x [doi]

Cavani, A., Nasorri, F., Prezzi, C., Sebastiani, S., Albanesi, C., & Girolomoni, G. (2000). Human CD4+ T lymphocytes with remarkable regulatory functions on dendritic cells and nickel-specific Th1 immune responses. *The Journal of Investigative Dermatology, 114*(2), 295-302. doi:S0022-202X(15)40767-5 [pii]

Chang, P. Y., Kozono, T., Chida, K., Kuroki, T., & Huh, N. (1998). Differential expression of hox genes in multistage carcinogenesis of mouse skin. *Biochemical and Biophysical Research Communications, 248*(3), 749-752. doi:S0006291X98990763 [pii]

Chew, Y. C., Adhikary, G., Wilson, G. M., Reece, E. A., & Eckert, R. L. (2011). Protein kinase C (PKC) delta suppresses keratinocyte proliferation by increasing p21(Cip1) level by a KLF4 transcription factor-dependent mechanism. *The Journal of Biological Chemistry, 286*(33), 28772-28782. doi:10.1074/jbc.M110.205245 [doi]

Christiano, A. M. (1997). Frontiers in keratodermas: Pushing the envelope. *Trends in Genetics : TIG, 13*(6), 227-233. doi:S0168-9525(97)01104-9 [pii]

Cooper, S. J., MacGowan, J., Ranger-Moore, J., Young, M. R., Colburn, N. H., & Bowden, G. T. (2003). Expression of dominant negative c-jun inhibits ultraviolet B-induced squamous cell carcinoma number and size in an SKH-1 hairless mouse model. *Molecular Cancer Research : MCR, 1*(11), 848-854.

Crish, J. F., Bone, F., Banks, E. B., & Eckert, R. L. (2002). The human involucrin gene contains spatially distinct regulatory elements that regulate expression during early versus late epidermal differentiation. *Oncogene, 21*(5), 738-747. doi:10.1038/sj.onc.1205038 [doi]

Crish, J. F., Gopalakrishnan, R., Bone, F., Gilliam, A. C., & Eckert, R. L. (2006). The distal and proximal regulatory regions of the involucrin gene promoter have distinct functions and are required for in vivo involucrin expression. *The Journal of Investigative Dermatology, 126*(2), 305-314. doi:S0022-202X(15)32775-5 [pii]

Crish, J. F., Zaim, T. M., & Eckert, R. L. (1998). The distal regulatory region of the human involucrin promoter is required for expression in epidermis. *The Journal of Biological Chemistry, 273*(46), 30460-30465.

Dale, B. A., Resing, K. A., & Lonsdale-Eccles, J. D. (1985). Filaggrin: A keratin filament associated protein. *Annals of the New York Academy of Sciences, 455*, 330-342.

Dennis, G.,Jr, Sherman, B. T., Hosack, D. A., Yang, J., Gao, W., Lane, H. C., et al. (2003). DAVID: Database for annotation, visualization, and integrated discovery. *Genome Biology, 4*(5), P3.

Detmer, K., Lawrence, H. J., & Largman, C. (1993). Expression of class I homeobox genes in fetal and adult murine skin. *The Journal of Investigative Dermatology, 101*(4), 517-522. doi:S0022-202X(93)90399-3 [pii]

Di Meglio, P., Perera, G. K., & Nestle, F. O. (2011). The multitasking organ: Recent insights into skin immune function. *Immunity, 35*(6), 857-869. doi:10.1016/j.immuni.2011.12.003 [doi]

DiSepio, D., Jones, A., Longley, M. A., Bundman, D., Rothnagel, J. A., & Roop, D. R. (1995). The proximal promoter of the mouse loricrin gene contains a functional AP-1 element and directs keratinocyte-specific but not differentiation-specific expression. *The Journal of Biological Chemistry, 270*(18), 10792-10799.

Dong, Z., Crawford, H. C., Lavrovsky, V., Taub, D., Watts, R., Matrisian, L. M., et al. (1997). A dominant negative mutant of jun blocking 12-O-tetradecanoylphorbol-13-acetate-induced invasion in mouse keratinocytes. *Molecular Carcinogenesis, 19*(3), 204-212. doi:10.1002/(SICI)1098-2744(199707)19:3<204::AID-MC8>3.0.CO;2-D [pii]

Drera, B., Tadini, G., Balbo, F., Marchese, L., Barlati, S., & Colombi, M. (2008). De novo occurrence of the 730insG recurrent mutation in an italian family with the ichthyotic variant of vohwinkel syndrome, loricrin keratoderma. *Clinical Genetics, 73*(1), 85-88. doi:CGE914 [pii]

Eckert, R. L., Adhikary, G., Young, C. A., Jans, R., Crish, J. F., Xu, W., et al. (2013). AP1 transcription factors in epidermal differentiation and skin cancer. *Journal of Skin Cancer, 2013*, 537028. doi:10.1155/2013/537028 [doi]

Eckert, R. L., Broome, A. M., Ruse, M., Robinson, N., Ryan, D., & Lee, K. (2004). S100 proteins in the epidermis. *The Journal of Investigative Dermatology, 123*(1), 23-33. doi:10.1111/j.0022-202X.2004.22719.x [doi]

Eckert, R. L., Crish, J. F., Banks, E. B., & Welter, J. F. (1997). The epidermis: Genes on - genes off. *The Journal of Investigative Dermatology, 109*(4), 501-509. doi:S0022-202X(15)43015-5 [pii]

Eckert, R. L., Crish, J. F., Efimova, T., Dashti, S. R., Deucher, A., Bone, F., et al. (2004). Regulation of involucrin gene expression. *The Journal of Investigative Dermatology, 123*(1), 13-22. doi:10.1111/j.0022-202X.2004.22723.x [doi]

Eckert, R. L., Crish, J. F., & Robinson, N. A. (1997). The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. *Physiological Reviews, 77*(2), 397-424.

Eckert, R. L., Efimova, T., Balasubramanian, S., Crish, J. F., Bone, F., & Dashti, S. (2003). p38 mitogen-activated protein kinases on the body surface--a function for p38 delta. *The Journal of Investigative Dermatology, 120*(5), 823-828. doi:S0022-202X(15)30255-4 [pii]

Eckert, R. L., & Green, H. (1986). Structure and evolution of the human involucrin gene. *Cell, 46*(4), 583-589. doi:0092-8674(86)90884-6 [pii]

Eckert, R. L., & Rorke, E. A. (1989). Molecular biology of keratinocyte differentiation. *Environmental Health Perspectives, 80*, 109-116.

Eckert, R. L., Sturniolo, M. T., Broome, A. M., Ruse, M., & Rorke, E. A. (2005). Transglutaminase function in epidermis. *The Journal of Investigative Dermatology, 124*(3), 481-492. doi:S0022-202X(15)32208-9 [pii]

Eckert, R. L., & Welter, J. F. (1996). Epidermal keratinocytes - genes and their regulation. *Cell Death and Differentiation, 3*(4), 373-383.

Efimova, T., Broome, A. M., & Eckert, R. L. (2003). A regulatory role for p38 delta MAPK in keratinocyte differentiation. evidence for p38 delta-ERK1/2 complex formation. *The Journal of Biological Chemistry, 278*(36), 34277-34285. doi:10.1074/jbc.M302759200 [doi]

Efimova, T., Broome, A. M., & Eckert, R. L. (2004). Protein kinase cdelta regulates keratinocyte death and survival by regulating activity and subcellular localization of

a p38delta-extracellular signal-regulated kinase 1/2 complex. *Molecular and Cellular Biology, 24*(18), 8167-8183. doi:10.1128/MCB.24.18.8167-8183.2004 [doi]

Efimova, T., Deucher, A., Kuroki, T., Ohba, M., & Eckert, R. L. (2002). Novel protein kinase C isoforms regulate human keratinocyte differentiation by activating a p38 delta mitogen-activated protein kinase cascade that targets CCAAT/enhancer-binding protein alpha. *The Journal of Biological Chemistry, 277*(35), 31753-31760. doi:10.1074/jbc.M205098200 [doi]

Efimova, T., & Eckert, R. L. (2000). Regulation of human involucrin promoter activity by novel protein kinase C isoforms. *The Journal of Biological Chemistry, 275*(3), 1601-1607.

Efimova, T., LaCelle, P., Welter, J. F., & Eckert, R. L. (1998). Regulation of human involucrin promoter activity by a protein kinase C, ras, MEKK1, MEK3, p38/RK, AP1 signal transduction pathway. *The Journal of Biological Chemistry, 273*(38), 24387-24395.

Elias, P. M., Williams, M. L., Crumrine, D., & Schmuth, M. (2010). Inherited clinical disorders of lipid metabolism. *Current Problems in Dermatology, 39*, 30-88. doi:10.1159/000321084 [doi]

Feingold, K. R. (2007). The importance of lipids in cutaneous function. *Journal of Lipid Research, 48*(12), 2529-2530. doi:48/12/2529 [pii]

Ferrari, S. M., Ruffilli, I., Colaci, M., Antonelli, A., Ferri, C., & Fallahi, P. (2015). CXCL10 in psoriasis. *Advances in Medical Sciences, 60*(2), 349-354. doi:10.1016/j.advms.2015.07.011 [doi]

Frei, A. P., Moest, H., Novy, K., & Wollscheid, B. (2013). Ligand-based receptor identification on living cells and tissues using TRICEPS. *Nature Protocols, 8*(7), 1321-1336. doi:10.1038/nprot.2013.072 [doi]

Fuchs, E., & Green, H. (1980). Changes in keratin gene expression during terminal differentiation of the keratinocyte. *Cell, 19*(4), 1033-1042. doi:0092-8674(80)90094-X [pii]

Gaffen, S. L., Jain, R., Garg, A. V., & Cua, D. J. (2014). The IL-23-IL-17 immune axis: From mechanisms to therapeutic testing. *Nature Reviews.Immunology, 14*(9), 585-600. doi:10.1038/nri3707 [doi]

Gedicke, M. M., Traupe, H., Fischer, B., Tinschert, S., & Hennies, H. C. (2006). Towards characterization of palmoplantar keratoderma caused by gain-of-function mutation in loricrin: Analysis of a family and review of the literature. *The British Journal of Dermatology, 154*(1), 167-171. doi:BJD6995 [pii]

Gittler, J. K., Shemer, A., Suarez-Farinas, M., Fuentes-Duculan, J., Gulewicz, K. J., Wang, C. Q., et al. (2012). Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *The*

Journal of Allergy and Clinical Immunology, 130(6), 1344-1354. doi:10.1016/j.jaci.2012.07.012 [doi]

Grewe, M., Bruijnzeel-Koomen, C. A., Schopf, E., Thepen, T., Langeveld-Wildschut, A. G., Ruzicka, T., et al. (1998). A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunology Today, 19*(8), 359-361. doi:S0167-5699(98)01285-7 [pii]

Groom, J. R., & Luster, A. D. (2011). CXCR3 ligands: Redundant, collaborative and antagonistic functions. *Immunology and Cell Biology, 89*(2), 207-215. doi:10.1038/icb.2010.158 [doi]

Gruber, R., Elias, P. M., Crumrine, D., Lin, T. K., Brandner, J. M., Hachem, J. P., et al. (2011). Filaggrin genotype in ichthyosis vulgaris predicts abnormalities in epidermal structure and function. *The American Journal of Pathology, 178*(5), 2252-2263. doi:10.1016/j.ajpath.2011.01.053 [doi]

Gutowska-Owsiak, D., & Ogg, G. S. (2013). Cytokine regulation of the epidermal barrier. *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology, 43*(6), 586-598. doi:10.1111/cea.12023 [doi]

Hai, T., & Curran, T. (1991). Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proceedings of the National Academy of Sciences of the United States of America, 88*(9), 3720-3724.

Han, B., Rorke, E. A., Adhikary, G., Chew, Y. C., Xu, W., & Eckert, R. L. (2012). Suppression of AP1 transcription factor function in keratinocyte suppresses differentiation. *PloS One, 7*(5), e36941. doi:10.1371/journal.pone.0036941 [doi]

Hancock, W. W., Lu, B., Gao, W., Csizmadia, V., Faia, K., King, J. A., et al. (2000). Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *The Journal of Experimental Medicine, 192*(10), 1515-1520.

Hansmann, B., Ahrens, K., Wu, Z., Proksch, E., Meyer-Hoffert, U., & Schroder, J. M. (2012). Murine filaggrin-2 is involved in epithelial barrier function and down-regulated in metabolically induced skin barrier dysfunction. *Experimental Dermatology, 21*(4), 271-276. doi:10.1111/j.1600-0625.2012.01449.x [doi]

Heath, W. R., & Carbone, F. R. (2013). The skin-resident and migratory immune system in steady state and memory: Innate lymphocytes, dendritic cells and T cells. *Nature Immunology, 14*(10), 978-985. doi:10.1038/ni.2680 [doi]

Hohl, D., Lichti, U., Breitkreutz, D., Steinert, P. M., & Roop, D. R. (1991). Transcription of the human loricrin gene in vitro is induced by calcium and cell density and suppressed by retinoic acid. *The Journal of Investigative Dermatology, 96*(4), 414-418. doi:S0022-202X(91)90441-R [pii]

Hohl, D., Mehrel, T., Lichti, U., Turner, M. L., Roop, D. R., & Steinert, P. M. (1991). Characterization of human loricrin. structure and function of a new class of

epidermal cell envelope proteins. *The Journal of Biological Chemistry, 266*(10), 6626-6636.

Homey, B., Alenius, H., Muller, A., Soto, H., Bowman, E. P., Yuan, W., et al. (2002). CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nature Medicine, 8*(2), 157-165. doi:10.1038/nm0202-157 [doi]

Hou, S. Y., Mitra, A. K., White, S. H., Menon, G. K., Ghadially, R., & Elias, P. M. (1991). Membrane structures in normal and essential fatty acid-deficient stratum corneum: Characterization by ruthenium tetroxide staining and x-ray diffraction. *The Journal of Investigative Dermatology, 96*(2), 215-223. doi:S0022-202X(91)90385-4 [pii]

Hudak, S., Hagen, M., Liu, Y., Catron, D., Oldham, E., McEvoy, L. M., et al. (2002). Immune surveillance and effector functions of CCR10(+) skin homing T cells. *Journal of Immunology (Baltimore, Md.: 1950), 169*(3), 1189-1196.

Iotzova-Weiss, G., Dziunycz, P. J., Freiberger, S. N., Lauchli, S., Hafner, J., Vogl, T., et al. (2015). S100A8/A9 stimulates keratinocyte proliferation in the development of squamous cell carcinoma of the skin via the receptor for advanced glycation-end products. *PloS One, 10*(3), e0120971. doi:10.1371/journal.pone.0120971 [doi]

Irvine, A. D. (2007). Fleshing out filaggrin phenotypes. *The Journal of Investigative Dermatology, 127*(3), 504-507. doi:S0022-202X(15)33299-1 [pii]

Irvine, A. D., McLean, W. H., & Leung, D. Y. (2011). Filaggrin mutations associated with skin and allergic diseases. *The New England Journal of Medicine, 365*(14), 1315-1327. doi:10.1056/NEJMra1011040 [doi]

Ishida-Yamamoto, A. (2003). Loricrin keratoderma: A novel disease entity characterized by nuclear accumulation of mutant loricrin. *Journal of Dermatological Science, 31*(1), 3-8. doi:S0923181102001433 [pii]

Ishida-Yamamoto, A., McGrath, J. A., Lam, H., Iizuka, H., Friedman, R. A., & Christiano, A. M. (1997). The molecular pathology of progressive symmetric erythrokeratoderma: A frameshift mutation in the loricrin gene and perturbations in the cornified cell envelope. *American Journal of Human Genetics, 61*(3), 581-589. doi:S0002-9297(07)64321-3 [pii]

Ishida-Yamamoto, A., Takahashi, H., & Iizuka, H. (1998). Loricrin and human skin diseases: Molecular basis of loricrin keratodermas. *Histology and Histopathology, 13*(3), 819-826.

Ishida-Yamamoto, A., Tanaka, H., Nakane, H., Takahashi, H., Hashimoto, Y., & Iizuka, H. (1999). Programmed cell death in normal epidermis and loricrin keratoderma. multiple functions of profilaggrin in keratinization. *The Journal of Investigative Dermatology.Symposium Proceedings, 4*(2), 145-149. doi:S1087-0024(15)30251-3 [pii]

Jang, S. I., Steinert, P. M., & Markova, N. G. (1996). Activator protein 1 activity is involved in the regulation of the cell type-specific expression from the proximal promoter of the human profilaggrin gene. *The Journal of Biological Chemistry, 271*(39), 24105-24114.

Jarnik, M., de Viragh, P. A., Scharer, E., Bundman, D., Simon, M. N., Roop, D. R., et al. (2002). Quasi-normal cornified cell envelopes in loricrin knockout mice imply the existence of a loricrin backup system. *The Journal of Investigative Dermatology, 118*(1), 102-109. doi:S0022-202X(15)41536-2 [pii]

Jaubert, J., Patel, S., Cheng, J., & Segre, J. A. (2004). Tetracycline-regulated transactivators driven by the involucrin promoter to achieve epidermal conditional gene expression. *The Journal of Investigative Dermatology, 123*(2), 313-318. doi:10.1111/j.0022-202X.2004.23203.x [doi]

Kalinin, A., Marekov, L. N., & Steinert, P. M. (2001). Assembly of the epidermal cornified cell envelope. *Journal of Cell Science, 114*(Pt 17), 3069-3070.

Karin, M., Liu, Z., & Zandi, E. (1997). AP-1 function and regulation. *Current Opinion in Cell Biology, 9*(2), 240-246.

Kawasaki, H., Nagao, K., Kubo, A., Hata, T., Shimizu, A., Mizuno, H., et al. (2012). Altered stratum corneum barrier and enhanced percutaneous immune responses in filaggrin-null mice. *The Journal of Allergy and Clinical Immunology, 129*(6), 1538-46.e6. doi:10.1016/j.jaci.2012.01.068 [doi]

Kimyai-Asadi, A., Kotcher, L. B., & Jih, M. H. (2002). The molecular basis of hereditary palmoplantar keratodermas. *Journal of the American Academy of Dermatology, 47*(3), 327-43; quiz 344-6. doi:S0190962202000804 [pii]

Koch, P. J., de Viragh, P. A., Scharer, E., Bundman, D., Longley, M. A., Bickenbach, J., et al. (2000). Lessons from loricrin-deficient mice: Compensatory mechanisms maintaining skin barrier function in the absence of a major cornified envelope protein. *The Journal of Cell Biology, 151*(2), 389-400.

Korge, B. P., Ishida-Yamamoto, A., Punter, C., Dopping-Hepenstal, P. J., Iizuka, H., Stephenson, A., et al. (1997). Loricrin mutation in vohwinkel's keratoderma is unique to the variant with ichthyosis. *The Journal of Investigative Dermatology, 109*(4), 604-610. doi:S0022-202X(15)43032-5 [pii]

Kulke, R., Bornscheuer, E., Schluter, C., Bartels, J., Rowert, J., Sticherling, M., et al. (1998). The CXC receptor 2 is overexpressed in psoriatic epidermis. *The Journal of Investigative Dermatology, 110*(1), 90-94. doi:S0022-202X(15)37373-5 [pii]

Lacotte, S., Brun, S., Muller, S., & Dumortier, H. (2009). CXCR3, inflammation, and autoimmune diseases. *Annals of the New York Academy of Sciences, 1173*, 310-317. doi:10.1111/j.1749-6632.2009.04813.x [doi]

Le Borgne, M., Etchart, N., Goubier, A., Lira, S. A., Sirard, J. C., van Rooijen, N., et al. (2006). Dendritic cells rapidly recruited into epithelial tissues via CCR6/CCL20 are responsible for CD8+ T cell crosspriming in vivo. *Immunity, 24*(2), 191-201. doi:S1074-7613(06)00104-X [pii]

Lee, K. C., & Eckert, R. L. (2007). S100A7 (psoriasin)--mechanism of antibacterial action in wounds. *The Journal of Investigative Dermatology, 127*(4), 945-957. doi:S0022-202X(15)33314-5 [pii]

Li, A. G., Koster, M. I., & Wang, X. J. (2003). Roles of TGFbeta signaling in epidermal/appendage development. *Cytokine & Growth Factor Reviews, 14*(2), 99-111. doi:S1359610103000054 [pii]

Li, D., Turi, T. G., Schuck, A., Freedberg, I. M., Khitrov, G., & Blumenberg, M. (2001). Rays and arrays: The transcriptional program in the response of human epidermal keratinocytes to UVB illumination. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 15*(13), 2533-2535. doi:10.1096/fj.01-0172fje [doi]

Li, J. J., Rhim, J. S., Schlegel, R., Vousden, K. H., & Colburn, N. H. (1998). Expression of dominant negative jun inhibits elevated AP-1 and NF-kappaB transactivation and suppresses anchorage independent growth of HPV immortalized human keratinocytes. *Oncogene, 16*(21), 2711-2721. doi:10.1038/sj.onc.1201798 [doi]

Liew, F. M., & Yamanishi, K. (1992). Regulation of transglutaminase 1 gene expression by 12-O-tetradecanoylphorbol-13-acetate, dexamethasone, and retinoic acid in cultured human keratinocytes. *Experimental Cell Research, 202*(2), 310-315. doi:0014-4827(92)90080-R [pii]

Lopez, O., Cocera, M., Wertz, P. W., Lopez-Iglesias, C., & de la Maza, A. (2007). New arrangement of proteins and lipids in the stratum corneum cornified envelope. *Biochimica Et Biophysica Acta, 1768*(3), 521-529. doi:S0005-2736(06)00429-9 [pii]

Maas-Szabowski, N., Szabowski, A., Stark, H. J., Andrecht, S., Kolbus, A., Schorpp-Kistner, M., et al. (2001). Organotypic cocultures with genetically modified mouse fibroblasts as a tool to dissect molecular mechanisms regulating keratinocyte growth and differentiation. *The Journal of Investigative Dermatology, 116*(5), 816-820. doi:S0022-202X(15)41246-1 [pii]

Mack, J. A., Abramson, S. R., Ben, Y., Coffin, J. C., Rothrock, J. K., Maytin, E. V., et al. (2003). Hoxb13 knockout adult skin exhibits high levels of hyaluronan and enhanced wound healing. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 17*(10), 1352-1354. doi:10.1096/fj.02-0959fje [doi]

Macleod, A. S., & Havran, W. L. (2011). Functions of skin-resident gammadelta T cells. *Cellular and Molecular Life Sciences : CMLS, 68*(14), 2399-2408. doi:10.1007/s00018-011-0702-x [doi]

Maestrini, E., Monaco, A. P., McGrath, J. A., Ishida-Yamamoto, A., Camisa, C., Hovnanian, A., et al. (1996). A molecular defect in loricrin, the major component of the cornified cell envelope, underlies vohwinkel's syndrome. *Nature Genetics, 13*(1), 70-77. doi:10.1038/ng0596-70 [doi]

Magli, M. C., Largman, C., & Lawrence, H. J. (1997). Effects of HOX homeobox genes in blood cell differentiation. *Journal of Cellular Physiology, 173*(2), 168-177. doi:10.1002/(SICI)1097-4652(199711)173:2<168::AID-JCP16>3.0.CO;2-C [pii]

Malissen, B., Tamoutounour, S., & Henri, S. (2014). The origins and functions of dendritic cells and macrophages in the skin. *Nature Reviews.Immunology, 14*(6), 417-428. doi:10.1038/nri3683 [doi]

Manitz, M. P., Horst, B., Seeliger, S., Strey, A., Skryabin, B. V., Gunzer, M., et al. (2003). Loss of S100A9 (MRP14) results in reduced interleukin-8-induced CD11b surface expression, a polarized microfilament system, and diminished responsiveness to chemoattractants in vitro. *Molecular and Cellular Biology, 23*(3), 1034-1043.

Matsumoto, K., Muto, M., Seki, S., Saida, T., Horiuchi, N., Takahashi, H., et al. (2001). Loricrin keratoderma: A cause of congenital ichthyosiform erythroderma and collodion baby. *The British Journal of Dermatology, 145*(4), 657-660. doi:4412 [pii]

Maytin, E. V., & Habener, J. F. (1998). Transcription factors C/EBP alpha, C/EBP beta, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes in vitro and in vivo. *The Journal of Investigative Dermatology, 110*(3), 238-246. doi:S0022-202X(15)40025-9 [pii]

M'Boneko, V., & Merker, H. J. (1988). Development and morphology of the periderm of mouse embryos (days 9-12 of gestation). *Acta Anatomica, 133*(4), 325-336.

McCully, M. L., Ladell, K., Hakobyan, S., Mansel, R. E., Price, D. A., & Moser, B. (2012). Epidermis instructs skin homing receptor expression in human T cells. *Blood, 120*(23), 4591-4598. doi:10.1182/blood-2012-05-433037 [doi]

McLean, W. H. (2016). Filaggrin failure - from ichthyosis vulgaris to atopic eczema and beyond. *The British Journal of Dermatology, 175 Suppl 2*, 4-7. doi:10.1111/bjd.14997 [doi]

Mehic, D., Bakiri, L., Ghannadan, M., Wagner, E. F., & Tschachler, E. (2005). Fos and jun proteins are specifically expressed during differentiation of human keratinocytes. *The Journal of Investigative Dermatology, 124*(1), 212-220. doi:S0022-202X(15)32133-3 [pii]

Mehrel, T., Hohl, D., Rothnagel, J. A., Longley, M. A., Bundman, D., Cheng, C., et al. (1990). Identification of a major keratinocyte cell envelope protein, loricrin. *Cell, 61*(6), 1103-1112. doi:0092-8674(90)90073-N [pii]

Menon, G. K., Grayson, S., & Elias, P. M. (1985). Ionic calcium reservoirs in mammalian epidermis: Ultrastructural localization by ion-capture cytochemistry. *The Journal of Investigative Dermatology, 84*(6), 508-512. doi:S0022-202X(15)43678-4 [pii]

Mischke, D., Korge, B. P., Marenholz, I., Volz, A., & Ziegler, A. (1996). Genes encoding structural proteins of epidermal cornification and S100 calcium-binding proteins form a gene complex ("epidermal differentiation complex") on human chromosome 1q21. *The Journal of Investigative Dermatology, 106*(5), 989-992. doi:S0022-202X(15)42545-X [pii]

Mishina, Y. (2003). Function of bone morphogenetic protein signaling during mouse development. *Frontiers in Bioscience : A Journal and Virtual Library, 8*, d855-69.

Mitchell, P. J., Timmons, P. M., Hebert, J. M., Rigby, P. W., & Tjian, R. (1991). Transcription factor AP-2 is expressed in neural crest cell lineages during mouse embryogenesis. *Genes & Development, 5*(1), 105-119.

Mizuno, H., Cho, Y. Y., Ma, W. Y., Bode, A. M., & Dong, Z. (2006). Effects of MAP kinase inhibitors on epidermal growth factor-induced neoplastic transformation of human keratinocytes. *Molecular Carcinogenesis, 45*(1), 1-9. doi:10.1002/mc.20160 [doi]

Morales, J., Homey, B., Vicari, A. P., Hudak, S., Oldham, E., Hedrick, J., et al. (1999). CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proceedings of the National Academy of Sciences of the United States of America, 96*(25), 14470-14475.

Nakajima, K., Terao, M., Takaishi, M., Kataoka, S., Goto-Inoue, N., Setou, M., et al. (2013). Barrier abnormality due to ceramide deficiency leads to psoriasiform inflammation in a mouse model. *The Journal of Investigative Dermatology, 133*(11), 2555-2565. doi:10.1038/jid.2013.199 [doi]

Nedoszytko, B., Sokolowska-Wojdylo, M., Ruckemann-Dziurdzinska, K., Roszkiewicz, J., & Nowicki, R. J. (2014). Chemokines and cytokines network in the pathogenesis of the inflammatory skin diseases: Atopic dermatitis, psoriasis and skin mastocytosis. *Postepy Dermatologii i Alergologii, 31*(2), 84-91. doi:10.5114/pdia.2014.40920 [doi]

Nemes, Z., Marekov, L. N., Fesus, L., & Steinert, P. M. (1999). A novel function for transglutaminase 1: Attachment of long-chain omega-hydroxyceramides to involucrin by ester bond formation. *Proceedings of the National Academy of Sciences of the United States of America, 96*(15), 8402-8407.

Nestle, F. O., Di Meglio, P., Qin, J. Z., & Nickoloff, B. J. (2009). Skin immune sentinels in health and disease. *Nature Reviews.Immunology, 9*(10), 679-691. doi:10.1038/nri2622 [doi]

Nickoloff, B. J., Qin, J. Z., Chaturvedi, V., Denning, M. F., Bonish, B., & Miele, L. (2002). Jagged-1 mediated activation of notch signaling induces complete maturation of

human keratinocytes through NF-kappaB and PPARgamma. *Cell Death and Differentiation, 9*(8), 842-855. doi:10.1038/sj.cdd.4401036 [doi]

O'Driscoll, J., Muston, G. C., McGrath, J. A., Lam, H. M., Ashworth, J., & Christiano, A. M. (2002). A recurrent mutation in the loricrin gene underlies the ichthyotic variant of vohwinkel syndrome. *Clinical and Experimental Dermatology, 27*(3), 243-246. doi:1031 [pii]

Oh, H. S., & Smart, R. C. (1998). Expression of CCAAT/enhancer binding proteins (C/EBP) is associated with squamous differentiation in epidermis and isolated primary keratinocytes and is altered in skin neoplasms. *The Journal of Investigative Dermatology, 110*(6), 939-945. doi:10.1046/j.1523-1747.1998.00199.x [doi]

Oji, V., Tadini, G., Akiyama, M., Blanchet Bardon, C., Bodemer, C., Bourrat, E., et al. (2010). Revised nomenclature and classification of inherited ichthyoses: Results of the first ichthyosis consensus conference in soreze 2009. *Journal of the American Academy of Dermatology, 63*(4), 607-641. doi:10.1016/j.jaad.2009.11.020 [doi]

Oji, V., & Traupe, H. (2006). Ichthyoses: Differential diagnosis and molecular genetics. *European Journal of Dermatology : EJD, 16*(4), 349-359.

Oji, V., & Traupe, H. (2009). Ichthyosis: Clinical manifestations and practical treatment options. *American Journal of Clinical Dermatology, 10*(6), 351-364. doi:10.2165/11311070-000000000-00000 [doi]

Okuyama, R., Nguyen, B. C., Talora, C., Ogawa, E., Tommasi di Vignano, A., Lioumi, M., et al. (2004). High commitment of embryonic keratinocytes to terminal differentiation through a Notch1-caspase 3 regulatory mechanism. *Developmental Cell, 6*(4), 551-562. doi:S153458070400098X [pii]

Palmer, C. N., Irvine, A. D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S. P., et al. (2006). Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nature Genetics, 38*(4), 441-446. doi:ng1767 [pii]

Paradis, T. J., Cole, S. H., Nelson, R. T., & Gladue, R. P. (2008). Essential role of CCR6 in directing activated T cells to the skin during contact hypersensitivity. *The Journal of Investigative Dermatology, 128*(3), 628-633. doi:S0022-202X(15)33761-1 [pii]

Patel, S., Zirwas, M., & English, J. C.,3rd. (2007). Acquired palmoplantar keratoderma. *American Journal of Clinical Dermatology, 8*(1), 1-11. doi:811 [pii]

Perera, G. K., Di Meglio, P., & Nestle, F. O. (2012). Psoriasis. *Annual Review of Pathology, 7*, 385-422. doi:10.1146/annurev-pathol-011811-132448 [doi]

Presland, R. B., Boggess, D., Lewis, S. P., Hull, C., Fleckman, P., & Sundberg, J. P. (2000). Loss of normal profilaggrin and filaggrin in flaky tail (ft/ft) mice: An animal

model for the filaggrin-deficient skin disease ichthyosis vulgaris. *The Journal of Investigative Dermatology, 115*(6), 1072-1081. doi:S0022-202X(15)41085-1 [pii]

Qin, J. Z., Bacon, P., Chaturvedi, V., & Nickoloff, B. J. (2001). Role of NF-kappaB activity in apoptotic response of keratinocytes mediated by interferon-gamma, tumor necrosis factor-alpha, and tumor-necrosis-factor-related apoptosis-inducing ligand. *The Journal of Investigative Dermatology, 117*(4), 898-907. doi:S0022-202X(15)41394-6 [pii]

Qin, J. Z., Chaturvedi, V., Denning, M. F., Choubey, D., Diaz, M. O., & Nickoloff, B. J. (1999). Role of NF-kappaB in the apoptotic-resistant phenotype of keratinocytes. *The Journal of Biological Chemistry, 274*(53), 37957-37964.

Qin, S., Rottman, J. B., Myers, P., Kassam, N., Weinblatt, M., Loetscher, M., et al. (1998). The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *The Journal of Clinical Investigation, 101*(4), 746-754. doi:10.1172/JCI1422 [doi]

Radu, M., & Chernoff, J. (2013). An in vivo assay to test blood vessel permeability. *Journal of Visualized Experiments : JoVE, (73):e50062. doi*(73), e50062. doi:10.3791/50062 [doi]

Rahman, I., & MacNee, W. (1998). Role of transcription factors in inflammatory lung diseases. *Thorax, 53*(7), 601-612.

Raj, D., Brash, D. E., & Grossman, D. (2006). Keratinocyte apoptosis in epidermal development and disease. *The Journal of Investigative Dermatology, 126*(2), 243-257. doi:S0022-202X(15)32773-1 [pii]

Rangarajan, A., Talora, C., Okuyama, R., Nicolas, M., Mammucari, C., Oh, H., et al. (2001). Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *The EMBO Journal, 20*(13), 3427-3436. doi:10.1093/emboj/20.13.3427 [doi]

Rawlings, A. V., & Harding, C. R. (2004). Moisturization and skin barrier function. *Dermatologic Therapy, 17 Suppl 1*, 43-48. doi:04S1005 [pii]

Resing, K. A., Dale, B. A., & Walsh, K. A. (1985). Multiple copies of phosphorylated filaggrin in epidermal profilaggrin demonstrated by analysis of tryptic peptides. *Biochemistry, 24*(15), 4167-4175.

Resing, K. A., Johnson, R. S., & Walsh, K. A. (1993). Characterization of protease processing sites during conversion of rat profilaggrin to filaggrin. *Biochemistry, 32*(38), 10036-10045.

Rice, R. H., Bradshaw, K. M., Durbin-Johnson, B. P., Rocke, D. M., Eigenheer, R. A., Phinney, B. S., et al. (2013). Distinguishing ichthyoses by protein profiling. *PloS One, 8*(10), e75355. doi:10.1371/journal.pone.0075355 [doi]

Rice, R. H., & Green, H. (1979). Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: Activation of the cross-linking by calcium ions. *Cell, 18*(3), 681-694. doi:0092-8674(79)90123-5 [pii]

Richard, G. (2004). Molecular genetics of the ichthyoses. *American Journal of Medical Genetics.Part C, Seminars in Medical Genetics, 131C*(1), 32-44. doi:10.1002/ajmg.c.30032 [doi]

Robinson, N. A., LaCelle, P. T., & Eckert, R. L. (1996). Involucrin is a covalently crosslinked constituent of highly purified epidermal corneocytes: Evidence for a common pattern of involucrin crosslinking in vivo and in vitro. *The Journal of Investigative Dermatology, 107*(1), 101-107. doi:S0022-202X(15)42632-6 [pii]

Robinson, N. A., Lapic, S., Welter, J. F., & Eckert, R. L. (1997). S100A11, S100A10, annexin I, desmosomal proteins, small proline-rich proteins, plasminogen activator inhibitor-2, and involucrin are components of the cornified envelope of cultured human epidermal keratinocytes. *The Journal of Biological Chemistry, 272*(18), 12035-12046.

Rorke, E. A., Adhikary, G., Jans, R., Crish, J. F., & Eckert, R. L. (2010). AP1 factor inactivation in the suprabasal epidermis causes increased epidermal hyperproliferation and hyperkeratosis but reduced carcinogen-dependent tumor formation. *Oncogene, 29*(44), 5873-5882. doi:10.1038/onc.2010.315 [doi]

Rorke, E. A., Adhikary, G., Young, C. A., Rice, R. H., Elias, P. M., Crumrine, D., et al. (2015). Structural and biochemical changes underlying a keratoderma-like phenotype in mice lacking suprabasal AP1 transcription factor function. *Cell Death & Disease, 6*, e1647. doi:10.1038/cddis.2015.21 [doi]

Rorke, E. A., Adhikary, G., Young, C. A., Roop, D. R., & Eckert, R. L. (2015). Suppressing AP1 factor signaling in the suprabasal epidermis produces a keratoderma phenotype. *The Journal of Investigative Dermatology, 135*(1), 170-180. doi:10.1038/jid.2014.310 [doi]

Rossi, A., Jang, S. I., Ceci, R., Steinert, P. M., & Markova, N. G. (1998). Effect of AP1 transcription factors on the regulation of transcription in normal human epidermal keratinocytes. *The Journal of Investigative Dermatology, 110*(1), 34-40. doi:S0022-202X(15)37362-0 [pii]

Rothnagel, J. A., Mehrel, T., Idler, W. W., Roop, D. R., & Steinert, P. M. (1987). The gene for mouse epidermal filaggrin precursor. its partial characterization, expression, and sequence of a repeating filaggrin unit. *The Journal of Biological Chemistry, 262*(32), 15643-15648.

Sandilands, A., Sutherland, C., Irvine, A. D., & McLean, W. H. (2009). Filaggrin in the frontline: Role in skin barrier function and disease. *Journal of Cell Science, 122*(Pt 9), 1285-1294. doi:10.1242/jcs.033969 [doi]

Sandilands, A., Terron-Kwiatkowski, A., Hull, P. R., O'Regan, G. M., Clayton, T. H., Watson, R. M., et al. (2007). Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. *Nature Genetics, 39*(5), 650-654. doi:ng2020 [pii]

Schaerli, P., Ebert, L., Willimann, K., Blaser, A., Roos, R. S., Loetscher, P., et al. (2004). A skin-selective homing mechanism for human immune surveillance T cells. *The Journal of Experimental Medicine, 199*(9), 1265-1275. doi:10.1084/jem.20032177 [doi]

Schmuth, M., Fluhr, J. W., Crumrine, D. C., Uchida, Y., Hachem, J. P., Behne, M., et al. (2004). Structural and functional consequences of loricrin mutations in human loricrin keratoderma (vohwinkel syndrome with ichthyosis). *The Journal of Investigative Dermatology, 122*(4), 909-922. doi:10.1111/j.0022-202X.2004.22431.x [doi]

Schonthaler, H. B., Guinea-Viniegra, J., Wculek, S. K., Ruppen, I., Ximenez-Embun, P., Guio-Carrion, A., et al. (2013). S100A8-S100A9 protein complex mediates psoriasis by regulating the expression of complement factor C3. *Immunity, 39*(6), 1171-1181. doi:10.1016/j.immuni.2013.11.011 [doi]

Seguchi, T., Cui, C. Y., Kusuda, S., Takahashi, M., Aisu, K., & Tezuka, T. (1996). Decreased expression of filaggrin in atopic skin. *Archives of Dermatological Research, 288*(8), 442-446.

Sehra, S., Serezani, A. P., Ocana, J. A., Travers, J. B., & Kaplan, M. H. (2016). Mast cells regulate epidermal barrier function and the development of allergic skin inflammation. *The Journal of Investigative Dermatology, 136*(7), 1429-1437. doi:10.1016/j.jid.2016.03.019 [doi]

Seitz, C. S., Lin, Q., Deng, H., & Khavari, P. A. (1998). Alterations in NF-kappaB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-kappaB. *Proceedings of the National Academy of Sciences of the United States of America, 95*(5), 2307-2312.

Shaulian, E., & Karin, M. (2001). AP-1 in cell proliferation and survival. *Oncogene, 20*(19), 2390-2400. doi:10.1038/sj.onc.1204383 [doi]

Shaulian, E., & Karin, M. (2002). AP-1 as a regulator of cell life and death. *Nature Cell Biology, 4*(5), E131-6. doi:10.1038/ncb0502-e131 [doi]

Shen, Q., Zhang, Y., Uray, I. P., Hill, J. L., Kim, H. T., Lu, C., et al. (2006). The AP-1 transcription factor regulates postnatal mammary gland development. *Developmental Biology, 295*(2), 589-603. doi:S0012-1606(06)00245-4 [pii]

Shi, B., & Isseroff, R. R. (2005). Epidermal growth factor (EGF)-mediated DNA-binding activity of AP-1 is attenuated in senescent human epidermal keratinocytes. *Experimental Dermatology, 14*(7), 519-527. doi:EXD317 [pii]

Smith, F. J., Irvine, A. D., Terron-Kwiatkowski, A., Sandilands, A., Campbell, L. E., Zhao, Y., et al. (2006). Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. *Nature Genetics, 38*(3), 337-342. doi:ng1743 [pii]

Steinert, P. M., Kartasova, T., & Marekov, L. N. (1998). Biochemical evidence that small proline-rich proteins and trichohyalin function in epithelia by modulation of the biomechanical properties of their cornified cell envelopes. *The Journal of Biological Chemistry, 273*(19), 11758-11769.

Steinert, P. M., & Marekov, L. N. (1995). The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross-linked components of the human epidermal cornified cell envelope. *The Journal of Biological Chemistry, 270*(30), 17702-17711.

Stelnicki, E. J., Komuves, L. G., Kwong, A. O., Holmes, D., Klein, P., Rozenfeld, S., et al. (1998). HOX homeobox genes exhibit spatial and temporal changes in expression during human skin development. *The Journal of Investigative Dermatology, 110*(2), 110-115. doi:S0022-202X(15)39998-X [pii]

Suga, Y., Jarnik, M., Attar, P. S., Longley, M. A., Bundman, D., Steven, A. C., et al. (2000). Transgenic mice expressing a mutant form of loricrin reveal the molecular basis of the skin diseases, vohwinkel syndrome and progressive symmetric erythrokeratoderma. *The Journal of Cell Biology, 151*(2), 401-412.

Szabowski, A., Maas-Szabowski, N., Andrecht, S., Kolbus, A., Schorpp-Kistner, M., Fusenig, N. E., et al. (2000). c-jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. *Cell, 103*(5), 745-755. doi:S0092-8674(00)00178-1 [pii]

Takahashi, H., & Iizuka, H. (1993). Analysis of the 5'-upstream promoter region of human involucrin gene: Activation by 12-O-tetradecanoylphorbol-13-acetate. *The Journal of Investigative Dermatology, 100*(1), 10-15. doi:S0022-202X(93)92178-W [pii]

Takahashi, H., Ishida-Yamamoto, A., Kishi, A., Ohara, K., & Iizuka, H. (1999). Loricrin gene mutation in a japanese patient of vohwinkel's syndrome. *Journal of Dermatological Science, 19*(1), 44-47. doi:S0923-1811(98)00049-8 [pii]

Takeichi, T., & Akiyama, M. (2016). Inherited ichthyosis: Non-syndromic forms. *The Journal of Dermatology, 43*(3), 242-251. doi:10.1111/1346-8138.13243 [doi]

Thompson, E. J., Gupta, A., Stratton, M. S., & Bowden, G. T. (2002). Mechanism of action of a dominant negative c-jun mutant in inhibiting activator protein-1 activation. *Molecular Carcinogenesis, 35*(4), 157-162. doi:10.1002/mc.10090 [doi]

Thompson, E. J., MacGowan, J., Young, M. R., Colburn, N., & Bowden, G. T. (2002). A dominant negative c-jun specifically blocks okadaic acid-induced skin tumor promotion. *Cancer Research, 62*(11), 3044-3047.

Tichelaar, J. W., Yan, Y., Tan, Q., Wang, Y., Estensen, R. D., Young, M. R., et al. (2010). A dominant-negative c-jun mutant inhibits lung carcinogenesis in mice. *Cancer Prevention Research (Philadelphia, Pa.), 3*(9), 1148-1156. doi:10.1158/1940-6207.CAPR-10-0023 [doi]

Tuzun, Y., Antonov, M., Dolar, N., & Wolf, R. (2007). Keratinocyte cytokine and chemokine receptors. *Dermatologic Clinics, 25*(4), 467-76, vii. doi:S0733-8635(07)00061-7 [pii]

Vahlquist, A. (2010). Pleomorphic ichthyosis: Proposed name for a heterogeneous group of congenital ichthyoses with phenotypic shifting and mild residual scaling. *Acta Dermato-Venereologica, 90*(5), 454-460. doi:10.2340/00015555-0937 [doi]

Varona, R., Cadenas, V., Gomez, L., Martinez-A, C., & Marquez, G. (2005). CCR6 regulates CD4+ T-cell-mediated acute graft-versus-host disease responses. *Blood, 106*(1), 18-26. doi:2004-08-2996 [pii]

Villanova, F., Di Meglio, P., & Nestle, F. O. (2013). Biomarkers in psoriasis and psoriatic arthritis. *Annals of the Rheumatic Diseases, 72 Suppl 2*, ii104-10. doi:10.1136/annrheumdis-2012-203037 [doi]

Walser, T. C., Rifat, S., Ma, X., Kundu, N., Ward, C., Goloubeva, O., et al. (2006). Antagonism of CXCR3 inhibits lung metastasis in a murine model of metastatic breast cancer. *Cancer Research, 66*(15), 7701-7707. doi:66/15/7701 [pii]

Wang, G., Savinko, T., Wolff, H., Dieu-Nosjean, M. C., Kemeny, L., Homey, B., et al. (2007). Repeated epicutaneous exposures to ovalbumin progressively induce atopic dermatitis-like skin lesions in mice. *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology, 37*(1), 151-161. doi:CEA2621 [pii]

Welter, J. F., & Eckert, R. L. (1995). Differential expression of the fos and jun family members c-fos, fosB, fra-1, fra-2, c-jun, junB and junD during human epidermal keratinocyte differentiation. *Oncogene, 11*(12), 2681-2687.

Winkler, A. E., Brotman, J. J., Pittman, M. E., Judd, N. P., Lewis, J. S.,Jr, Schreiber, R. D., et al. (2011). CXCR3 enhances a T-cell-dependent epidermal proliferative response and promotes skin tumorigenesis. *Cancer Research, 71*(17), 5707-5716. doi:10.1158/0008-5472.CAN-11-0907 [doi]

Yamada, K., Yamanishi, K., Kakizuka, A., Kibe, Y., Doi, H., & Yasuno, H. (1994). Transcriptional regulation of human transglutaminase1 gene by signaling systems of protein kinase C, RAR/RXR and Jun/Fos in keratinocytes. *Biochemistry and Molecular Biology International, 34*(4), 827-836.

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., et al. (1998). P63, a P53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Molecular Cell, 2*(3), 305-316. doi:S1097-2765(00)80275-0 [pii]

Yates, C. C., Krishna, P., Whaley, D., Bodnar, R., Turner, T., & Wells, A. (2010). Lack of CXC chemokine receptor 3 signaling leads to hypertrophic and hypercellular scarring. *The American Journal of Pathology, 176*(4), 1743-1755. doi:10.2353/ajpath.2010.090564 [doi]

Yates, C. C., Whaley, D., Kulasekeran, P., Hancock, W. W., Lu, B., Bodnar, R., et al. (2007). Delayed and deficient dermal maturation in mice lacking the CXCR3 ELR-negative CXC chemokine receptor. *The American Journal of Pathology, 171*(2), 484-495. doi:S0002-9440(10)61982-7 [pii]

Yeh, J. M., Yang, M. H., & Chao, S. C. (2013). Collodion baby and loricrin keratoderma: A case report and mutation analysis. *Clinical and Experimental Dermatology, 38*(2), 147-150. doi:10.1111/j.1365-2230.2012.04426.x [doi]

Yoneda, K., Demitsu, T., Manabe, M., Igarashi, J., Kosaka, H., Inagaki, N., et al. (2010). Expression of wild-type, but not mutant, loricrin causes programmed cell death in HaCaT keratinocytes. *The Journal of Dermatology, 37*(11), 956-964. doi:10.1111/j.1346-8138.2010.00932.x [doi]

Yoneda, K., Nakagawa, T., Lawrence, O. T., Huard, J., Demitsu, T., Kubota, Y., et al. (2012). Interaction of the profilaggrin N-terminal domain with loricrin in human cultured keratinocytes and epidermis. *The Journal of Investigative Dermatology, 132*(4), 1206-1214. doi:10.1038/jid.2011.460 [doi]

Yoneda, K., & Steinert, P. M. (1993). Overexpression of human loricrin in transgenic mice produces a normal phenotype. *Proceedings of the National Academy of Sciences of the United States of America, 90*(22), 10754-10758.

Young, M. R., Farrell, L., Lambert, P., Awasthi, P., & Colburn, N. H. (2002). Protection against human papillomavirus type 16-E7 oncogene-induced tumorigenesis by in vivo expression of dominant-negative c-jun. *Molecular Carcinogenesis, 34*(2), 72-77. doi:10.1002/mc.10050 [doi]

Young, M. R., Li, J. J., Rincon, M., Flavell, R. A., Sathyanarayana, B. K., Hunziker, R., et al. (1999). Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. *Proceedings of the National Academy of Sciences of the United States of America, 96*(17), 9827-9832.

Yuspa, S. H., Kilkenny, A. E., Steinert, P. M., & Roop, D. R. (1989). Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations in vitro. *The Journal of Cell Biology, 109*(3), 1207-1217.

Zenz, R., Eferl, R., Kenner, L., Florin, L., Hummerich, L., Mehic, D., et al. (2005). Psoriasis-like skin disease and arthritis caused by inducible epidermal deletion of jun proteins. *Nature, 437*(7057), 369-375. doi:nature03963 [pii]

Zenz, R., Eferl, R., Scheinecker, C., Redlich, K., Smolen, J., Schonthaler, H. B., et al. (2008). Activator protein 1 (Fos/Jun) functions in inflammatory bone and skin disease. *Arthritis Research & Therapy, 10*(1), 201. doi:10.1186/ar2338 [doi]

Zenz, R., Scheuch, H., Martin, P., Frank, C., Eferl, R., Kenner, L., et al. (2003). c-jun regulates eyelid closure and skin tumor development through EGFR signaling. *Developmental Cell, 4*(6), 879-889. doi:S1534580703001618 [pii]

Zenz, R., & Wagner, E. F. (2006). Jun signalling in the epidermis: From developmental defects to psoriasis and skin tumors. *The International Journal of Biochemistry & Cell Biology, 38*(7), 1043-1049. doi:S1357-2725(05)00391-2 [pii]